Université du Québec
Institut National de la Recherche Scientifique
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# LA PRODUCTION DE PROTÉINES UNICELLULAIRES, CARACTÉRISATION ET RÉCUPÉRATION DES PROTÉINES DE LACTOSÉRUM DU LACTOSÉRUM FERMENTÉ

Par

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Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph.D.)
en sciences de l'eau

**Jury d'évaluation**

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# DÉDICACE

**This thesis is dedicated to my parents, my wife Sunita and my loving twins (Anish and Ankit).**

# REMERCIEMENTS

First and foremost, I feel immense pleasure to express my sincere sense of gratitude to my research supervisor **Prof. R.D. Tyagi** for his inspiring, valuable suggestions and regular encouragement during the period of my PhD research. Sir, you have been a steady influence throughout my PhD pursuit. You have oriented and supported me with promptness and care, and have always been patient and encouraging in times of new ideas and difficulties. Your ability to select and to approach convincing research problems, your high scientific standards, and your hard work set an example, which was transmissible and motivational for me. It was a great experience, and I am grateful to you for providing me the opportunity to pursue my research.

I take the opportunity to express my gratitude to **Prof. José R. Valéro** for providing me guidance and encouragement during the course of my travail dirigé. I express my sincere thanks to **Dr. R.Y. Surmapalli** for providing me guidance and inspiration during the research period.

I would like to acknowledge **Dr. Song Yan** for her appreciable suggestion, constructive discussions and support during the whole period of my research. I am grateful to **Dr. Jyothi Bezawada** and **Dr. C.M. Ajila**, for insightful comments during the research work. I would like to thank **Madam Nirmala Tyagi** for her suggestions and support throughout my stay in Quebec.

I would like to thank all the laboratory personnels, especially **Sébastien Duval, Stéfane Premont, René Rodrigue, Lise Rancourt and Pauline Fournier** for their technical support and maintaining the lab facility throughout the research work. I would like to express sincere gratitude to the student's secretary, **Suzanne Dussault**, **Johanne Desrosiers, Linda Aubert** and **Magos Sophie** for being always prompt in any kind of administrative help. I am grateful to the informatics support at INRS-ETE. I am also thankful to all the staff in INRS-ETE for proving the first class study and working environment.

I would also like to express my thanks to my colleagues **Tanaji More**, **Sridhar Pilli**, **Lalit Kumar** and **Indrani Bhattacharya** for their help during research work. I would like to acknowledge **Tarek**, **Gnepe Jean Robert**, **Douha**, **Nouha**, **Louis-Philippe Theroux, Adma**, **Brahima, Yassine Ouarda, Khalid Oubennaceur** and **Imen** for the French translation of synthèse part of

my thesis. At this point, I would like to thank all my colleagues who have made my stay in INRS-ETE enjoyable.

In particular, I would like to express my heartfelt gratitude to my parents, my wife and my brothers for everything they have done for me.

# RÉSUMÉ

Le lactosérum est un coproduit de la fabrication du fromage qui contient environ 55% des nutriments résiduels du lait. La présence de ces nutriments mène à une demande chimique en oxygène élevée (DCO), allant de 60 à 80 g/L, ce qui restreint le rejet direct du lactosérum dans les cours d'eau. D'un autre côté, la présence des nutriments comme le lactose, les protéines, les lipides et minéraux font du lactosérum une ressource pour la transformation et la production d'une grande variété de produits à valeurs ajoutées. Ces produits sont la poudre de lactosérum, les protéines de lactosérum, le bioéthanol, les biopolymères, les enzymes, le méthane, les protéines unicellulaires (PU) et les probiotiques. Parmi ces produits à valeurs ajoutées, la transformation du lactosérum et de son perméat en produits protéinés comme les PU est attrayante et de grande valeur puisqu'elle demande un simple procédé de biotransformation en réduisant simultanément la charge polluante. Cependant, la tendance pour la production de PU est d'utiliser uniquement le perméat. La séparation des protéines du lactosérum s'effectue par précipitation ou par filtration membranaire. La présence du lactose dans le lactosérum est un obstacle dans les deux procédés de séparation. Par ailleurs, le coût de récupération élevé et les faibles volumes disponibles font en sorte qu'il est difficile pour certaines industries d'appliquer les deux procédés séparément (c.a.d. la séparation des protéines de lactosérum et le traitement du perméat). Pour ces raisons, la biotransformation du lactosérum non-traité en biomasse pourrait être un meilleur choix pour l'utilisation du lactosérum que la biotransformation du perméat. Par la suite, les protéines résiduelles du lactosérum fermenté pourraient être récupérées facilement par précipitation ou par filtration membranaire. Les protéines récupérées pourraient être mélangées avec de la biomasse ou utilisées séparément. Donc, le but de cette étude était la production de PU de grade alimentaire, la caractérisation des protéines résiduelles et leur récupération en maintenant un équilibre entre le rendement et l'efficacité de l'enlèvement de la DCO.

La fermentation du lactosérum par *Kluyveromyces marxianus* s'est déroulée à 40 °C et pH 3,5 afin d'examiner simultanément la production de PU et l'enlèvement de la DCO, ainsi que pour déterminer les caractéristiques et le sort de la fraction des protéines solubles du lactosérum de même que caractériser les métabolites intermédiaires. Lors de la fermentation de type « batch », le rendement de biomasse obtenu ($Y_{x/s}$) était de 0,12 g de biomasse/g de lactose avec une réduction de la DCO de 55% (protéines incluses), tandis que la concentration de protéines

solubles du lactosérum a diminué de 5,6 à 4,1 g/L. L'électrophorèse a révélé que les protéines fermentées étaient différentes des protéines présentes au départ. Une analyse chromatographique a démontré un changement dans la composition des composés organiques après fermentation. Une fermentation continue avec un système de recyclage de cellules a résulté en un rendement de 0,19 g de biomasse/g lactose et une productivité de 0,26 g/L/h. L'efficacité de l'enlèvement de la DCO était de 78-79% avec une concentration de protéines résiduelles de 3,8-4,2 g/L.

Les levures ont été séparées du bouillon fermenté de la fermentation continue par centrifugation. Les protéines solubles résiduelles du surnageant de lactosérum fermenté (SLF) ont été précipitées par traitement à la chaleur (100 °C, pH 3,5 et 10 minutes de temps d'incubation) sous agitation. Le résultat de ce traitement a donné une récupération de 68% des protéines solubles résiduelles et un enlèvement simultané de la DCO résiduelle de 62%. Plus tard, le procédé en deux étapes de précipitation/coagulation des protéines solubles en combinaison avec le carbométhylcellulose (CMC) (traitement thermique suivi de la précipitation par CMC) a permis d'augmenter à 81% la récupération des protéines du total des protéines solubles du SLF.

La fermentation discontinue d'une monoculture (*K. marxianus*) et d'une culture mixte (*K. marxianus* et *C. krusei*) à des conditions extrêmes (40 °C et pH 3,5) a démontré de meilleurs résultats pour la culture mixte. En effet, une efficacité d'enlèvement de la DCO de 9% supérieure a été obtenue avec un rendement de biomasse supérieur de 19% et une productivité augmentée de 33%. L'enlèvement maximal de la DCO, 80%, (incluant les protéines résiduelles) a été obtenu à 24 heures de temps de rétention hydraulique (TRH) avec une productivité en biomasse de 0,17 g/L/h. Cependant, la productivité maximale de biomasse, soit 0,38 g/L/h et un enlèvement de 34% de la DCO ont été obtenus pour un TRH de 6 heures. Lors de la comparaison des cultures, le contenu en lysine de la biomasse de la culture mixte était plus élevé que celui de la biomasse de la monoculture.

Les cellules de levure *K. marxianus* ont été perméabilisées avec un détergent anionique, non toxique et biodégradable, le N-lauroyl sarcosine (N-LS), pour déterminer l'activité enzymatique intracellulaire de la β-galactosidase. Les paramètres du procédé de perméabilisation (la concentration de N-LS, le volume de solvant, la température et le temps d'incubation) ont été optimisés. L'activité enzymatique maximale de la β-galactosidase, 1220 IU/g de biomasse sèche, a été obtenue sous des conditions optimales. Les cellules perméabilisées ont par la suite

été évaluées pour leur capacité à hydrolyser le lactose du lactosérum. Le meilleur résultat pour l'hydrolyse du lactose, 91%, a été observe à 600 mg (poids sec de cellules/100 mL) dans une solution de poudre de lactosérum (5% p/v) à pH 6,5 et 30 °C pendant un temps d'incubation de 180 minutes. L'hydrolyse simultanée du lactose du lactosérum pour l'amélioration de la croissance de la levure *Saccharomyces cerevisiae*, une levure qui ne consume pas le lactose, a été évaluée avec les cellules perméabilisées de *K. marxianus.* La levure *S. cerevisiae* peut croître dans le lactosérum hydrolysé par *K. marxianus* en culture mixte.

La biomasse de levure a été traitée en deux étapes avec une combinaison de produits chimiques (N-LS et $NH_4OH$) pour réduire la teneur en acide nucléique afin d'obtenir des PU de qualité alimentaire. La combinaison de produits chimiques a réduit efficacement le contenu en acide nucléique sous 2% avec un relâchement externe simultané de nucléotides. Finalement, les caractéristiques et la récupération des protéines solubles résiduelles de lactosérum dans leur état natif ont été évaluées. La fraction des protéines de lactosérum fermenté est différente des protéines natives du lactosérum et sont partiellement hydrolysées lors de la fermentation. Les paramètres opérationnels d'ultrafiltration (flux de perméat et pression transmembranaire-PTM) ont été optimisés pour des membranes de 1 et 10 kDa. Un flux de perméat de 413 $L/h/m^2$ et une PTM de 80 kPa donne le meilleur résultat pour la membrane de 10 kDa, alors que pour la membrane de 1 kDa, le meilleur résultat a été obtenu avec un flux de perméat de 2760 $L/h/m^2$ et une PTM de 230 kPa. Les conditions optimisées combinées ont permis, respectivement, un recouvrement de 84% et 92% des protéines résiduelles solubles totales du surnagent de lactosérum fermenté d'une monoculture (*K. marxianus*) et d'une culture mixte (*K. marxianus* et *S. cerevisiae*).

Les PU ont été produites sous des conditions opératoires extrêmes à partir de lactosérum non traité suivi d'un recouvrement des protéines résiduelles par précipitation. La biomasse et les protéines solides récupérées pourraient être mélangées et pulvérisées à sec, ce qui pourra augmenter le contenu global en protéine et balancer le profil des acides aminés essentiels des PU. Les PU de la culture mixte (*K. marxianus* et *C. krusei*) ont été produites sous conditions non-aseptiques avec une efficacité d'enlèvement de la DCO supérieure, un rendement amélioré et une qualité du produit augmentée. Les deux microorganismes ont démontré une interaction physiologique. Le détergent a été efficace pour perméabiliser les cellules afin de permettre au lactose de pénétrer et de subir l'action des enzymes intracellulaires. Les cellules perméabilisées ont efficacement hydrolysé le lactose du lactosérum. Les PU de qualité alimentaire ont été

obtenues par prétraitement de la biomasse avec une combinaison de produits chimiques. Les protéines résiduelles après la fermentation ont été récupérées par ultrafiltration dans leur état natif. Donc, en se basant sur la présente étude, trois différentes approches pourraient potentiellement être appliquées pour la biotransformation du lactosérum non traité en produits protéinés.

# ABSTRACT

Cheese whey, a byproduct, is produced during manufacturing of cheese and contains around 55% of the milk residual nutrients. The presence of milk nutrients leads to a high chemical oxygen demand (COD) of 60-80 g/L, it restricts direct whey disposal into natural environmental streams. On the other hand, the presence of nutrients such as lactose, protein, lipids and minerals makes cheese whey a potential resource for the processing and production of a wide range of value-added products, such as whey powder, whey protein, bioethanol, biopolymers, enzymes, methane, single-cell protein (SCP) and probiotics. Among these, the transformation of whey and whey permeate into proteinaceus products such as SCP is attractive and demands since it involves a simple biotransformation process and simultaneous pollution load removal. However, the general trend for production of SCP is using whey permeate. The recovery of whey proteins is carried out by precipitation or membrane filtration. The presence of lactose in whey is an hindrance in both separation processes. Moreover, high recovery cost and low volume availability of whey make it hard for some companies to apply two processes separately (i.e. whey protein separation and whey permeate treatment). Therefore, biotransformation of raw cheese whey to biomass could be a better choice for whey utilization. Thereafter, the residual fermented whey proteins could be recovered via precipitation or membrane filtration with ease. The recovered proteins could be either mixed with biomass or used separately. Thus, the aim of the study was the production of feed- and food-grade SCP, the characterization of the residual proteins and their recovery by maintaining a balance between product yield and COD removal efficiency.

Cheese whey fermentation with *Kluyveromyces marxianus* was carried out at 40 $^{o}$C and pH 3.5 to examine simultaneous SCP production and COD removal, to determine the fate and characteristics of the soluble whey protein and to characterize the intermediate metabolites. During batch fermentation, the biomass yield ($Y_{x/s}$) obtained was 0.12 g biomass/g lactose with 55% COD reduction (including proteins), whereas soluble whey protein concentration decreased from 5.6 to 4.1 g/L. Electrophoresis revealed that the fermented whey protein characteristic was different from the native whey protein. Chromatographic analysis showed a change in the composition of organic compounds post-fermentation. Continuous fermentation with a cell recycle system resulted in a biomass yield of 0.19 g biomass/g lactose and a productivity of 0.26

g/L/h. The COD removal efficiency was 78-79% with a residual protein concentration of 3.8-4.2 g/L.

The yeast biomass was separated from the fermented broth from continuous fermentation by centrifugation. The residual soluble proteins from fermented whey supernatant (FWS) were precipitated by heat treatment (at 100 $^{o}$C, pH 3.5 and 10 min incubation) under agitation, which resulted in 68% of protein recovery and a simultaneous residual COD removal of 62%. Further, precipitation/coagulation of the soluble proteins in presence of carboxymethylcellulose (CMC) in two-step process (thermal treatment followed by CMC precipitation) enhanced protein recovery from FWS up to 81%.

Batch fermentation of mono (*K. marxianus*) and mixed (*K. marxianus* and *C. krusei*) cultures at extreme conditions (temperature 40 $^{o}$C and pH 3.5) showed that the mixed culture resulted in 9% higher COD removal efficacy, 19% higher biomass yield and 33% increased productivity. Maximal COD removal of 80% (including residual proteins) was obtained at 24 h HRT with a biomass productivity of 0.17 g/L/h; however, maximal biomass productivity of 0.38 g/L/h and 34% COD removal were obtained at 6 h HRT. The mixed culture biomass showed a higher content of lysine compared to monoculture biomass.

Yeast *K. marxianus* cells were permeabilized with the non-toxic, biodegradable, anionic detergent N-lauroyl sarcosine (N-LS) for accessing the intracellular β-galactosidase activity. The permeabilization process parameters (N-LS concentration, solvent volume, temperature and incubation time) were optimized. The maximal β-galactosidase activity of 1220 IU/g dry weight was obtained under the optimized conditions. Further, the permeabilized cells were evaluated for whey lactose hydrolysis. The maximal lactose hydrolysis of 91% was observed with 600 mg (dry cell weight/100 mL) in whey powder (5% w/v) solution with a 180-min incubation, pH 6.5 and 30 °C. Simultaneous whey lactose hydrolysis for increasing the growth of the non-lactose-consuming yeast *Saccharomyces cerevisiae* was evaluated with permeabilized *K. marxianus* cells. *S. cerevisiae* was able to grow in hydrolyzed whey with *K. marxianus* in a mixed culture mode.

The yeast biomass was treated in two steps with a combination of chemicals (N-LS and $NH_4OH$) to reduce the nucleic acid content in order to obtain food-grade SCP. The combination of chemical was effective to reduce the nucleic acid content below 2% with simultaneous release of

nucleotides. Finally, the characteristics and the recovery of the residual soluble whey proteins in native state was evaluated. The fermented whey protein fraction was different from the native whey protein fraction and it was partially hydrolyzed during fermentation. The ultrafiltration operational parameters (permeate flux and transmembrane pressure-TMP) were optimized for 1 and 10 kDa membranes. The permeate flux of 413 L/h/$m^2$ and TMP of 80 kPa resulted in highest recovery with the 10 kDa membrane followed by a permeate flux of 2760 L/h/$m^2$ and TMP of 230 kPa for the 1 kDa membrane. The optimized conditions in combination recovered 84% and 92% of the total residual soluble proteins from mono- (*K. marxianus*) and mixed cultures (*K. marxianus* and *S. cerevisiae*) fermented whey supernatants, respectively.

SCP was produced under extreme operating conditions from raw cheese whey followed by residual protein recovery through precipitation. The biomass and the recovered proteinaceous solids could be mixed and spray-dried, which will increase the overall protein content and balance the essential amino acids profile of SCP. The mixed culture (*K. marxianus* and *C. krusei*) SCP was produced under non-aseptic conditions with higher COD removal efficiency, enhanced yield and improved product quality. The two microorganisms have demonstrated physiological interactions. Permeabilization of yeast cells with a detergent was effective for accessing intracellular enzyme activity. Permeabilized cells were effective in hydrolyzing whey lactose. Food-grade SCP was obtained by pre-treatment of biomass with a combination of chemicals. The residual proteins after fermentation were recovered by ultrafiltration in their native state. Thus, based on the present study, three different approaches could be potentially applied to biotransform raw cheese whey into proteinaceous products.

# AVANT-PROPOS

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# LISTE DES ABRÉVIATIONS

| | |
|---|---|
| APHA | American Public Health Association |
| AAFCO | Association of American Feed Control Officials |
| α-LA | α-lactalbumin |
| β-LG | β-lactoglobulin |
| BOD | Biochemical oxygen demand |
| BSA | Bovine serum albumin |
| CMC | Carboxymethyl cellulose |
| COD | Chemical oxygen demand |
| CTAB | Cetyltrimethyl ammonium bromide |
| DNS | 3,5-Dinitrosalicylic acid |
| DO | Dissolve oxygen |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| ELISA | Enzyme-linked immunosorbent assay |
| EDTA | Ethylenediaminetetraacetic acid |
| ICP-AES | Inductively coupled plasma atomic emission spectroscopy |
| Igs | Immunoglobulins |
| GC-MS | Gas chromatography–mass spectrometry |
| GMP | Glycomacropeptide |
| GOS | Galactooligosaccharide |
| GRAS | Generally recognized as safe |
| HPLC | High-performance liquid chromatography |
| HRT | Hydraulic retention time |
| FAO | Food and Agriculture Organization |
| FPLC | Fast protein liquid chromatography |
| FWS | Fermented whey supernatant |
| LAB | Lactic acid bacteria |
| Lf | Lactoferrin |
| LP | Lactoperoxidase |
| MIC | Minimal inhibitory concentration |
| MRS | de Man, Rogosa and Sharpe |
| MW | Molecular weight |

| | |
|---|---|
| MWCO | Molecular weight cut-off |
| N-LS | N-Lauroylsarcosine |
| NTU | Nephelometric turbity units |
| PER | Protein efficiency ratio |
| PMSF | Phenyl methyl sulphonyl fluoride |
| RP-HPLC | Reversd-phase high-performance liquid chromatography |
| SCP | Single-cell protein |
| SS | Suspended solids |
| TFF | Tangential flow filtration |
| TS | Total solids |
| TSB | Tryptic Soya Broth |
| UF | Ultrafiltration |
| USDEC | US Dairy Export Council |
| US-FDA | U.S. Food and Drug Administration |
| VOCs | Volatile organic compounds |
| WHO | World Health Organization |
| WPC | Whey protein concentrate |
| WPI | Whey protein isolate |
| YM | Yeast Malt |

# CHAPITRE I

# SYNTHÈSE

# 1. REVUE DE LITTÉRATURE

## 1.1 INTRODUCTION

Le lactosérum est la fraction liquide issue de la coagulation de la caséine du lait durant la fabrication du fromage ou aussi durant l'extraction de la caséine en tant que sous-produit. La production mondiale en lactosérum a été estimée à 180.000.000-190.000.000 tonnes/an (Mollea *et al.,* 2013), dans laquelle l'Union européenne et les États-Unis contribuent avec près de 70%. Cette production de lactosérum augmente graduellement de 2% chaque année, suivant ainsi le taux de production de lait (Smithers, 2008). Bien que certaines de ces quantités de lactosérum sont utilisées en alimentation ou en industries alimentaires comme additifs, les grands volumes générés sont en train de créer un grand problème environnemental et sanitaire en raison des volumes importants de production d'une part, et en raison de la charge organique élevée d'autre part. La demande biochimique en oxygène (DBO) et la demande chimique en oxygène (DCO) du lactosérum sont aux environs de 30-50 g/L et de 60-80 g/L, respectivement (Guimarães *et al.,* 2010). L'élimination du lactosérum dans les égouts municipaux a été interdite par de nombreuses municipalités vu qu'il (le lactosérum) affecte le processus biologique de traitement des eaux usées dans les stations d'épuration. L'élimination du lactosérum par les méthodes conventionnelles pourrait être à l'origine de plusieurs problèmes de pollution, en particulier au niveau du sol, ce qui affecte sa structure physique et chimique ainsi que le rendement des cultures. Quand il est libéré dans les cours d'eau, le lactosérum réduit l'oxygène dissous, constituant ainsi une menace pour la vie aquatique, pour la santé humaine et pour l'environnement (Ghaly *et al.,* 2007).

La présence de nutriments résiduels (du lait) est à l'origine de la charge organique élevée dans le lactosérum. Ces éléments nutritifs peuvent être valorisés et exploités pour la production de divers produits à valeur ajoutée. Le progrès réalisé en biotechnologie les dernières années a été mis en service pour améliorer la gestion des effluents de lactosérum. Actuellement, une bonne fraction de lactosérum est transformée en produits à valeur ajoutée. Cependant, malgré tous ces efforts, d'importantes quantités de lactosérum (le petit-lait), qui représentent la moitié de la production totale, restent inutilisées. Ainsi, de nouvelles issues doivent être développées pour la valorisation du lactosérum pour la limitation de ses incidences sur l'environnement (Panesar *et al.,* 2007).

Deux méthodes sont utilisées actuellement pour la valorisation-transformation du lactosérum en produits à valeur ajoutée. La première est le traitement direct (transformation physique); cette méthode permet l'obtention de la poudre de lactosérum, de concentrés de protéines sériques, de l'isolats de protéines sériques, du perméat de lactosérum, du lactose et d'autres fractions. La deuxième méthode est basée sur des procédés biotechnologiques, le lactosérum etant utilisé comme substrat pour les enzymatiques/microorganisms, ce qui permet l'obtention de produits à valeur ajoutée. Ces produits sont utilisés principalement pour l'alimentation animale; il s'agit principalement de protéines unicellulaires (PU), de probiotiques, d'acides organiques, d'enzymes, de caroténoïdes, de bio-conservateurs, des gommes biologiques, d'exopolysaccharides et de bioplastiques (Kosseva *et al.,* 2009; Mollea *et al.,* 2013; Panesar *et al.,* 2013; Siso, 1996).

En raison de leurs valeurs fonctionnelles, les protéines de lactosérum (concentrés de protéines sériques, isolats de protéines sériques et protéines de lactosérum individuelles) sont très demandées sur le marché. Plusieurs applications ont été récemment développées pour les protéines de lactosérum en industrie pharmaceutique, par exemple, pour le développement de formulations pour le contrôle de la pression artérielle ou pour induire le sommeil (Korhonen, 2009; Modler, 2009). Les protéines de lactosérum ont la faculté d'être facilement transformables en peptides bioactifs par voie enzymatique ou fermentaire. Ainsi, l'étape la plus importante dans le traitement du petit lait de fromage est la récupération de la fraction protéique du lactosérum ou du perméat (fraction lactose) et sa conversion en produits à valeur ajoutée. La biotransformation du perméat de lactosérum pour la production des protéines (PU) et des galacto-oligosaccharides (GOS), par accumulation dans la biomasse, représente l'option la plus prometteuse (Gänzle *et al.,* 2008). La production de PU à partir de lactosérum est effectuée en utilisant les microorganismes qui sont capables de dégrader le lactose comme les levures *Kluyveromyces* spp. et *Candida* spp. et les bactéries *Lactobacillus* spp. (Panesar *et al.,* 2013).

## 1.2 LES CARACTÉRISTIQUES DU LACTOSÉRUM ET SA TRANSFORMATION EN PRODUITS À VALEUR AJOUTÉE

### 1.2.1 Les caractéristiques physiques

En général, le lactosérum est défini comme la partie du liquide ou du sérum de lait résiduel qui reste après la séparation du caillé à partir de la coagulation du lait par des enzymes protéolytiques ou des acides. Le lactosérum a une couleur jaune/vert, ou parfois même teinte bleuâtre, mais la couleur peut être changée avec le type de lait utilisé. Il peut être produit à partir de différents types de lait, mais le lait de vache est le plus populaire dans les pays occidentaux, alors que, dans d'autres régions du monde, le lait de chèvre, de brebis et même de chameau est utilisé dans la production de fromages et de lactosérum (Bordenave-Juchereau *et al.,* 2005; Smithers, 2008). En effet, le lactosérum représente environ 85-95% du volume de lait et conserve environ 55% des éléments nutritifs du lait (Varnam et Sutherland, 1994). La figure 1.1 représente l'analyse comparative du lait et de composés du lactosérum.

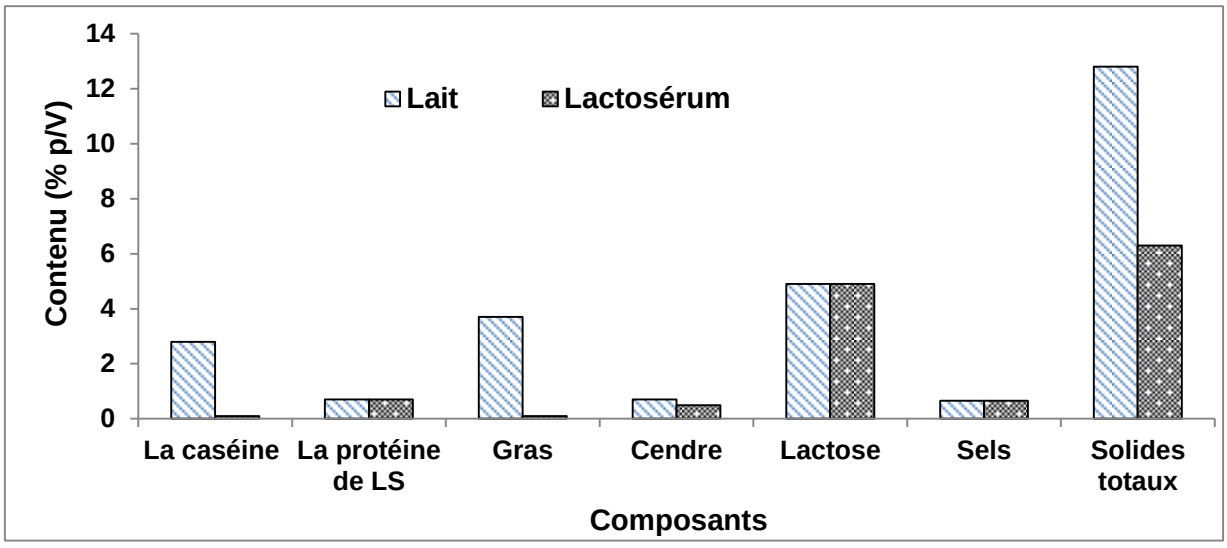


**Figure 1.1 La comparaison des composantes du lait de vache et du lactosérum (LS)**

## 1.2.2 Les caractéristiques chimiques

Le type et la composition du lactosérum dépend principalement des techniques de traitement utilisées dans l'élimination de la caséine du lait. Le type le plus souvent connu de lactosérum provient de la fabrication de fromages ou de certains produits de fromage à base de caséine, où la transformation est basée sur la coagulation de la caséine par la présure. La présure induite par la coagulation de la caséine se produit à un pH d'environ 6,5, donc, le lait produit pendant le traitement enzymatique est désigné comme lactosérum doux. Le second type de lactosérum est du lactosérum acide (pH inférieur à 5), qui est généré grâce à des acides ou des acides organiques afin de coaguler la caséine. La principale différence entre les deux types de lactosérum est son contenu minéral, son acidité et sa composition en protéines, comme indiqué dans le tableau 1.1 (Jelen *et al.,* 2003;. Kosseva *et al.,* 2009).

**Table 1.1 Les caractéristiques de la composition du lactosérum doux et du lactosérum acide**

| Composants | Lactosérum doux (g/L) | Le lactosérum acide (g/L) |
|---|---|---|
| Total des solides | 63,0 à 70,0 | 63,0 à 70,0 |
| Lactose | 46,0 à 52,0 | 44,0 à 46,0 |
| Protéines | 6,0-10,0 | 6,0-8,0 |
| Calcium | 0,4-0,6 | 1,2-1,6 |
| Phosphate | 1,0-3,0 | 2,0-4,5 |
| Lactate | 2.0 | 6.4 |
| Chlorure | 1.1 | 1.1 |

## 1.2.3 Utilisation du lactosérum pour la production de produits à valeur ajoutée

Environ 50% du total de lactosérum produit dans le monde est traité et transformé en différents produits alimentaires, dont environ 45% sont utilisés directement sous forme liquide, 30% sous forme lactosérum en poudre, 15% comme lactose et divers sous-produits, et le reste sous forme des concentrés de protéines de lactosérum (Kosseva *et al.,* 2009;. Panesar *et al.,* 2013).

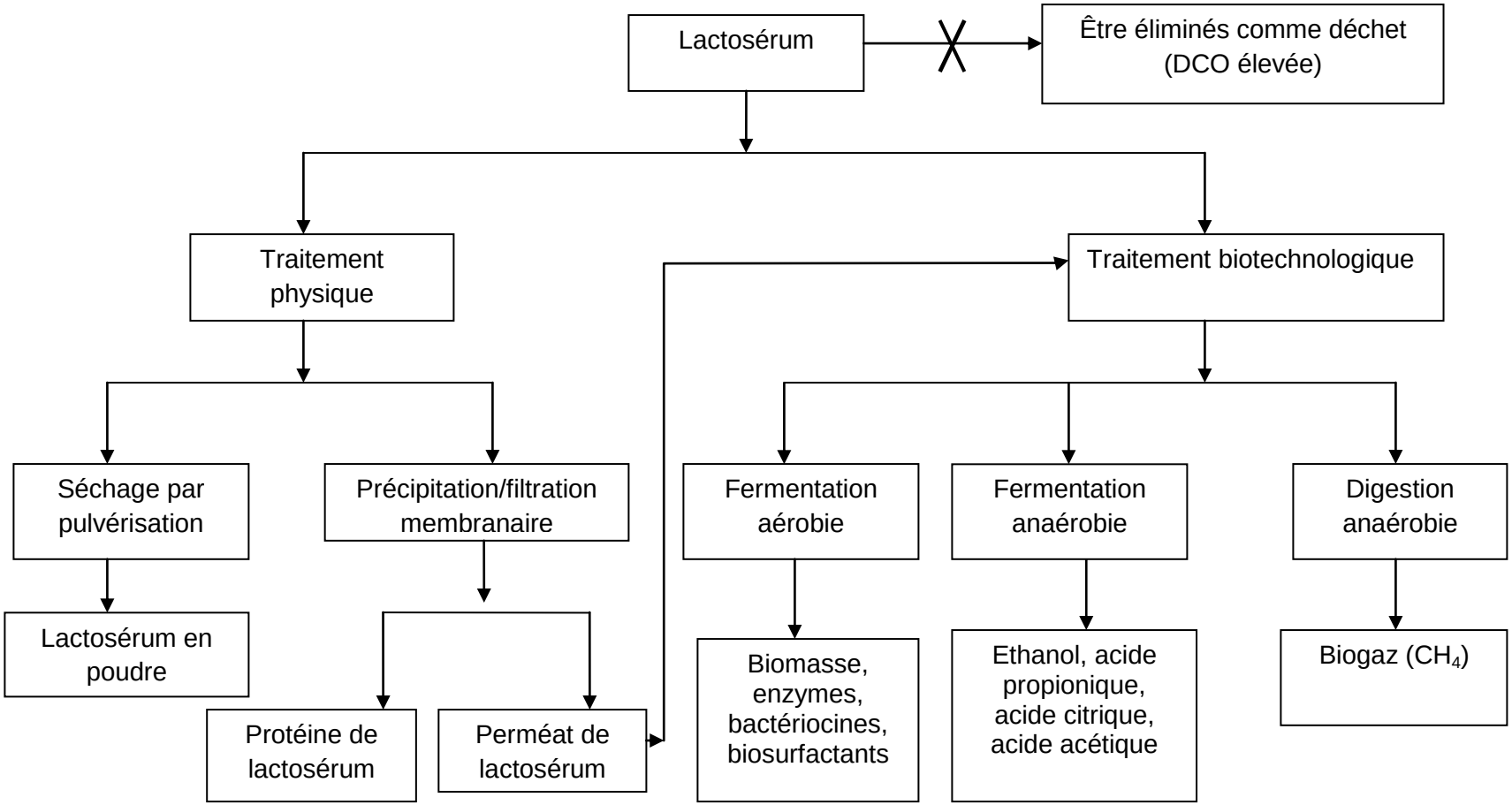


**Figure 1.2 Schéma du traitement du lactosérum par des méthodes physiques et biotechnologiques**

Étant donné que le lactose est le principal composant des matières solides du lactosérum, ainsi que les protéines solubles, les vitamines et des minéraux, divers procédés biotechnologiques ont été mis au point pour utiliser le lactosérum comme substrat pour produire des produits industriels importants tels que des enzymes, des protéines riches en biomasse et de l'éthanol. Par contre, d'autres procédés physiques sont utilisés pour la conversion directe de lactosérum en des produits importants (Morales *et al.,* 2006; Panesar *et al.,* 2013; Siso, 1996). Divers traitements biotechnologiques et physiques sont illustrés à la figure 1.2. Les traitements physiques et chimiques ont joué un rôle important dans la transformation du lactosérum en produits à valeur ajoutée. Microorganismes qui consomment le lactose sont utilisés pour la bioconversion directe de lactosérum.

La large gamme de produits à valeur ajoutée, tel que présenté dans le tableau 1.2, est obtenue à partir de lactosérum ou à partir du perméat de lactosérum par fermentation (Carlotti *et al.,* 1991; De León Rodríguez *et al.,* 2006; Guimarães *et al.,* 2010; Panesar *et al.,* 2013; Pham *et al.,* 2000; Wan *et al.,* 2008). Cependant, dans certains cas, l'hydrolyse du lactose est une première étape avant la fermentation par les microorganismes, ceux-ci étant incapables d'hydrolyser le lactose afin d'améliorer l'utilité de lactosérum. L'hydrolyse de la liaison β-glycosidique du lactose en glucose et galactose peut être réalisée soit avec des enzymes telles que la β-galactosidase (lactase), soit par hydrolyse acide (Siso, 1996; Zadow, 1992). Par exemple, l'éthanol a été produit à partir de lactosérum en tant que substrat par des souches la levure *Kluyveromyces marxianus* utilisant du lactose (Guimarães *et al.,* 2010). Des acides organiques tels que l'acide acétique, l'acide propionique, l'acide lactique et l'acide citrique, peuvent être produits à partir de lactosérum/le perméat de lactosérum par fermentation (El Aasar, 2006; Morales *et al.,* 2006; Mostafa, 2001; Mukhopadhyay *et al.,* 2005; Panesar *et al.,* 2007; Wan *et al.,* 2008). Le perméat de lactosérum est utilisé au cours de la fermentation comme substrat pour la production de polysaccharides, mais le lactose doit être tout d'abord hydrolysé (exemple: la gomme xanthane est produite par *Xanthomonas campestris* (Mesomo *et al.,* 2009).

**Table 1.2 Bioutilisation du lactosérum pour la production de produits à valeur ajoutée**

| Catégorie de produit | Microorganismes | Produit | Références |
|---|---|---|---|
| **Probiotiques et protéines unicellulaires** | *Le kefir* microflore | Protéine unicellulaire | Paraskevopoulou *et al.,* 2003 |
| | *Lactobacillus casei* | Probiotiques | Aguirre-Ezkauriatza *et al.,* 2010 |
| | *Candida kefyr* & *Candida valida* | Biomasse (levure) | Carlotti *et al.,* 1991 |
| **Enzymes** | *Bacillus* spp. | α-amylase | Bajpai, 1991 |
| | *Kluyveromyces marxianus,* | β-galactosidase | Fonseca *et al.,* 2008 |
| | *Candida rugosa* | Lipase | Tommaso *et al.,* 2011 |
| | *Escherichia coli* Recombinant | Pénicilline acylase | De León-Rodríguez *et al.,* 2006 |
| **Peptides et protéines** | *Lactococcus lactis* | Nisine (bacteriocine) | Liu *et al.,* 2005 |
| | | Peptides bioactifs | Hernández-Ledesma *et al.,* 2008 |
| | *Kluyveromyces marxianus* et *Lactobacillus rhamnosus* | Inhibiteur de l'ACE (antihypertenseur) | Hammé *et al.,* 2009 |
| **Oligosaccharides et biopolymères** | *Kluyveromyces marxianus* | Galacto-oligosaccharides | Petrova et Kujumdzieva, 2010 |
| | *Lactobacillus rhamnosus* | Expolysaccharides (EPS) | Pham *et al.,* 2000 |
| | *Xanthomonas campestris* | Gomme xanthane | Mesomo *et al.,* 2009 |
| | *Leuconostoc mesenteroides* | Dextrane & Fructose | Santos *et al.,* 2005 |
| | *Azotobacter chrooccum* | Poly-β-hydroxybutyrate (PHB) | Khanafari *et al.,* 2006 |
| | *Kluyveromyces lactis* | D-arabitol | Toyoda et Ohtaguchi, 2011 |
| | | Xylitol | Toyoda et Ohtaguchi, 2009 |
| | | Lactulose | Gänzle *et al.,* 2008 |
| | *Kluyveromyces marxianus* | Oligonucléotides | Belem et Lee, 1999a |
| | *Rizopus oryzae* | Chitosane | Chatterjee *et al.,* 2008 |

| | | | |
|---|---|---|---|
| **Acides organiques et produits biochimiques** | *Bactéries lactique* | Acide lactique | Panesar *et al.,* 2007 |
| | *Aspergillus niger* | Acide citrique | El Aasar, 2006 |
| | *Actinobacillus succinogenes* | Acide succinique | Wan *et al.,* 2008 |
| | *Kluyveromyces fragilis* | Acide acétique & glycérol | Mostafa, 2001 |
| | *Aspergillus niger* | Acide gluconique, lactones | Mukhopadhyay *et al.,* 2005 |
| | *Propionibacterium acidipropionici* | Acide propionique | Morales *et al.,* 2006 |
| | *Bacillus licheniformis* K51 | Biosurfactant | Joshi *et al.,* 2008 |
| | *Anaerobiospirillum succiniciproducens* | Succinate pour alimentation animale | Samuelov *et al.,* 1999 |
| **Agent de biocontrôle** | | *B. thuringiensis, Beauveria bassiana* et *Metarhizium anisopliae* | Kassa *et al.,* 2008 |
| | | *Steinernema carpocapsae* | Chavarría-Hernández *et al.,* 2006 |
| | | *Bacillus sphaericus* | El-Bendary *et al.,* 2008 |
| **Carburants et énergie** | | Biogaz | Gelegenis *et al.,* 2007 |
| | *K. marxianus* | Ethanol | Guimarães *et al.,* 2010 |
| | | Hydrogène | Davila-Vazquez *et al.,* 2009 |

Ainsi, le lactose, constituant du lactosérum, a été largement utilisé pour la production d'une large gamme d'exopolysaccharides, tels que les dextranes (par *Leuconostoc mesenteroides*), et les biopolymères comme le poly-β-hydroxy butyrate (PHB) (*Azotobacter chrooccum*) (Khanafari *et al.,* 2006; Pham *et al.,* 2000; Santos *et al.,* 2005). La production de méthane ou de biogaz par fermentation de lactosérum est un processus composé de trois étapes successives; l'hydrolyse de lactose/protéine, la fermentation (acidogénèse, acétogénèse) et la méthanisation. La production de méthane devrait donc représenter une source importante d'énergie utilisée comme carburant ou pour produire de l'électricité (Gelegenis *et al.,* 2007). La liste des produits (Tableau 1.2) montre le rôles de la biotechnologique dans la transformation des déchets ou des sous-produits dans différentes catégories (probiotiques, enzymes, protéines et peptides, biopolymères, produits biochimiques, agents de lutte biologique et carburants).

## 1.3 LA PRODUCTION DE PROTÉINES UNICELLULAIRES À PARTIR DE LACTOSÉRUM

### 1.3.1 Les protéines unicellulaires

Une protéine unicellulaire (PU) peut être définie comme la cellule biomasse des microorganismes. Les PU peuvent être produites en utilisant la matière carbonée des déchets disponibles en abondance. Les algues, les champignons/levure et les bactéries sont les principales sources de biomasse microbienne qui peuvent être employées comme source de PU (Paraskevopoulou *et al.,* 2003). Parmi les importantes caractéristiques des microorganismes unicellulairés, il y à la grande teneur en protéines, allant d'environ 40 à 80% de leur poids en cellules sèches, sur la base de protéines brutes. En outre, les protéines tendent à être de haute qualité, ressemblant plus à une protéine animale qu'à une protéine végétale et, par conséquent, aisément disponible de façon nutritive (Anvari et Khayati, 2011; Schultz *et al.,* 2006). Parmi les algues, champignons/levures et les bactéries, les levures sont préférées pour la production de PU en raison du etre fait que la levure peut facilement être propagée sur une source de carbone bon marché et facilement récoltée en raison de sa plus grande taille compareé à celle des bactéries. En outre, la levure contient moins d'acides nucléiques que les bactéries (Bekatorou *et al.,* 2006). Il existe divers microorganismes de GRAS (GRAS-generally recognized as safe) de catégorie (levures et bactéries), qui sont généralement utilisés pour produire les PU. Les plus couramment utiliseés parmi les espèces de levure sont *Saccharomyces*, *Kluyveromyces*

*marxianus*, *Candida, Pichia* et *Torulopsis*. Parmi les espèces bactériennes, *Lactobacillus*, *Cellulomonas* et *la bactérie Alcaligenes* sont les plus courantes (Bekatorou *et al.,* 2006).

La biotransformation du lactosérum ou du perméat de lactosérum en biomasse protéinique est l'une des meilleures utilisations du lactosérum (Grba *et al.,* 2002; Mansour *et al.,* 1993; Mawson, 1994). Les microorganismes utilisant le lactose (p. ex., *Kluyveromyces marxianus* spp.) sont les plus couramment utilisés pour la production de biomasse à partir du lactosérum. Cependant, l'accent a également été accordé pour la production de non-lactose consommant universellement la levure de boulanger acceptée (*S. cerevisiae*). Le procede industriel pour produire *S. cerevisiae* à partir du lactosérum a été adopté par la société Nutrisearch en 1983 au Kentucky. Le processus se compose de l'hydrolyse du lactose du lactosérum par une β-galactosidase immobilisée, suivie par une fermentation des sucres résultants (Siso, 1996). Plusieurs procédés ont été rapportés visant la production de biomasse à partir du lactosérum; parmi ces procédés, celui de Vienne et de Bel sont les plus importants. La production industrielle de biomasse microbienne à partir du lactosérum, pour ingrédients alimentaire, a commencé en France dans les Fromageries Bel autour de 1958. Les procédé impliquée trois espèces de levure (*Kluyveromyces lactis, K. Fragilis* et *Torulopsis bovina*), que ont été cultivées en équilibre sur du lactosérum en mode continue sur une période de plus d'un an, à pH 3,5 et à 38 $^{o}$C. Une haute température et un faible pH sont suggérés car ils réduisent le risque de contamination (Siso, 1996).

## 1.3.2 Facteurs qui affectent la production de protéines unicellulaires

Les facteurs qui influent sur la production de biomasse par une levure (PU), à partir du lactosérum, sont les microorganismes, la supplémentation en nutriments, les conditions de culture et le type du procedes de fermentation. Le microorganisme préféré est la levure.

### Microorganismes

La sélection des microorganismes pour la production de PU dépend de leur capacité à métaboliser le lactose du lactosérum. La production de biomasse est essentiellement limitée aux microorganismes consommant le lactose (Ghaly *et al.,* 2003), cependant, certains microorganismes qui ne consomment pas le lactose et qui prolifèrent sur les métabolites intermédiaires peuvent également être utilisés (Cristiani-Urbina *et al.,* 2000). Différentes espèces de levure, telles que *Kluyveromyces marxianus* (p. ex., *Kluyveromyces marxianus et K.*

*lactis*), *Candida* (p. ex., *Candida utilis*) et *Trichosporon* spp., sont employées, car elles sont capables d'assimiler le lactose (Mansour *et al.,* 1993; Mawson, 1994). Parmi ces espèces, les espèces de *Kluyveromyces marxianus*. ont été le plus largement étudiées (Grba *et al.,* 2002). La levure *K. marxianus* est la plus dominante et la plus couramment utilisée jusqu'à une échelle commerciale (Bekatorou *et al.,* 2006; Fonseca *et al.,* 2008). Les espèces bactériennes (comme *Lactobacillus brevis*, *Lactobacillus plantarum* et *Lactobacillus fermentum*), qui sont classées comme «GRAS» ou ont une histoire sûre dans les denrées alimentaires d'aliments peuvent être utilisées (AAFCO, 2010; Panesar *et al.,* 2013).

Un problème principal qui survient généralement au cours de la culture de *K. marxianus* à une concentration de lactose initiale eleveé est la production de produits (p ex., éthanol) au détriment de la formation de biomasse due à l'effet Crabtree. La classification de levures Crabtree-positive ou Crabtree-négative est fondée sur leur système d'absorption du glucose. Les microorganismes Crabtree-négatifs minimisent la perte de lactose en convertissant le lactose en éthanol. *K. marxianus* est classée comme Crabtree-négatif et est un microorganisme de pouvoir fermentaire facultatif (van Dijken *et al.,* 1993; Fonseca *et al.,* 2008). Il ne peut pas croître sous des conditions strictement anaérobiques et l'apparition de la formation de l'éthanol est presque exclusivement liée à la limitation d'oxygène (Bellaver *et al.,* 2004). La capacité de l'organisme modèle Crabtree-négatif *K. lactis* à métaboliser le lactose est principalement due à la présence de lactose perméase (codée par le gène *LAC12*) et de β-galactosidase (gène *LAC4*) (Rubio-Texeira, 2006).

En général, l'objectif des espèces de *Kluyveromyces marxianus* (p. ex., *K. marxianus*) est de produire de la biomasse et, simultanément, d'enlever la charge organique du lactosérum (Ghaly et Kamal, 2004; Schultz *et al.,* 2006). Cependant, la formation d'éthanol et d'autres métabolites a un effet négatif sur la biomasse et la réduction de DCO pendant la culture de *Kluyveromyces marxianus*. D'autre part, l'efficacité d'élimination de la DCO était supérieure avec la culture mixte (*K. marxianus* et *S. cerevisiae*) par rapport à la monoculture de *K. marxianus* (Domingues *et al.,* 2010). L'élimination de la DCO la plus élevée pendant la culture mixte de *K.marxianus* et *S. cerevisiae* (ne consommant pas de lactose) était le résultat de la consommation de certains des métabolites extracellulaires produits pendant la croissance de *Kluyveromyces marxianus* (Domingues *et al.,* 2010). En outre, l'augmentation de biomasse a été signalée lors de l'utilisation des cultures mixtes de *Torulopsis cremoris* et C. *utilis*. L'amélioration du rendement en biomasse peut être due au fait que les sous-produits métaboliques consommés par *C. utilis*

générés par *T. cremoris* entrainent une augmentation du rendement en biomasse avec l'élimination de la DCO la plus élevée (Cristiani-Urbina *et al.,* 2000).

### Nutriments

La croissance microbienne nécessite du carbone et de l'azote comme une source majeure de substrats. En outre, d'autres nutriments essentiels sont le soufre, le phosphore et des éléments en quantité mineure. La principale source de carbone dans le lactosérum est le lactose tandis que de l'azote organique est présent sous forme complexe (comme les protéines, les peptides et les acides aminés) (Ghaly et Kamal, 2004). En raison de la nature complexe de l'azote dans le lactosérum, les microorganismes ont besoin facilement assimilés la forme inorganique ou la forme organique simple d'azote (p. ex., l'urée) pour une croissance optimale. L'azote inorganique peut être fourni sous la forme d'ammoniac ou d'ammonium et de nitrates. L'assimilation de l'urée implique soit une dégradation extracellulaire par l'uréase, conduisant à la production d'ammoniac ou son transport et son assimilation par la voie de l'urée amydolyase (Roon et Levenberg, 1972). Des sources d'azote inorganiques influencent le pH du milieu de culture; l'assimilation d'un ion ammonium génère un proton, tandis que l'assimilation d'un ion nitrate consomme un proton. Cependant, l'assimilation de l'urée est neutre par rapport à l'équilibre de protons (Castrillo *et al.,* 1995). L'extrait de levure, qui est une bonne source d'acides aminés libres, de peptides et de vitamines du complexe B, a également été utilisé (Lukondeh *et al.,* 2005).

### pH

En général, le pH neutre est la valeur optimale pour la croissance des microorganismes. En outre, les levures sont généralement tolérantes au conditions acides et peuvent se développer dans un intervalle de pH de 4 à 4,5 (Battcock, 1998). En maintenant le pH en dessous de 4,5 durant la culture, on élimine la possibilité de contamination par des bactéries pathogènes (Ghaly et Kamal, 2004). Les levures sont capables de maintenir un pH cytosolique plus ou moins constant sur une plage extracellulaire du pH de 3,5 à 9 (Viegas et Sá-Correia, 1991). Cependant, Ghaly et Kamal (2004) ont rapporté que la stabilité du pH entre 4 et 5 est essentielle pour la croissance optimale et la survie de *K. fragilis*. La levure est capable de soutenir la croissance à un pH plus faible, mais une augmentation du rendement en biomasse de levure a été observée avec une augmentation du pH de 4 à 7 (Munawar *et al.,* 2010; Rajoka *et al.,* 2006).

## Température

Une analyse détaillée des effets de la température sur la croissance de la levure a été analysée par van Uden (1984). Les températures élevées donnent généralement lieu à des petits mutants qui peuvent affecter la perméabilité de la membrane cytoplamique. La variation de la perméabilité de la membrane cytoplamique peut conduire à une diminution de l'efficacité de génération d'énergie et a une augmentation des coût de la maintenance. L'augmentation du coût de la maintenance aboutit finalement à une diminution du rendement en biomasse. En outre, il est rapporté qu'une diminution du rendement à des températures élevées peut être due à la consommation de glucose par une fraction non viable de la culture (Van Uden et Madeira Lopes, 1976). L'intervalle de température optimale pour la levure *C. utilis* pour la production de biomasse est signalé à être entre 30 et 35 °C (Munawar *et al.,* 2010; Rajoka *et al.,* 2006). La plage de température comprise entre 25 et 35 °C a été rapportée comme étant optimale pour la croissance de la levure et la production de PU (Tableau 1.3).

**Table 1.3 Les conditions de fermentation, le mode de fermentation, les rendements et l'efficacité d'élimination pendant la production de protéines unicellulaires à partir du lactosérum**

| Fermentation | Substrat | Microorganismes | Source d'azote | pH | Temp. ($^oC$) | μ (1/h) | $Y_{x/s}$ (g/g) | Productivité (g/L/h) | Taux d'élimination de DCO (%) | Références |
|---|---|---|---|---|---|---|---|---|---|---|
| Batch | CW | *Candida kefyr* et *C. valida* | $(NH_4)_2SO_4$ + extrait de levure | 4,5 | 35 | - | 0,38 | - | - | Carlotti *et al.,* 1991 |
| Batch | CW | *Kluyveromyces fragilis* | - | 4,4 | 31 | 0,150 | 0,44 | NA | 90,6 (PE) | Ghaly et Kamal, 2004 |
| Batch | WP | *K. lactis* et *S. cerevisiae* | $(NH_4)_2$ $SO_4$ | 4,5 | 25 | - | - | 0,47 | 88,5 (DBO) | Moeini *et al.,* 2004 |
| Batch | DWC | *K. marxianus* | - | 5,8 | 30 | NA | 0,52 | NA | 90,0 (PE) | Schultz *et al.,* 2006 |
| Batch | DSWC | *K. marxianus* | - | 4,8 | 30 | NA | 0,48 | NA | 83,0 (PE) | Schultz *et al.,* 2006 |
| Batch | WP | *K. marxianus* | $(NH_4)_2$ $SO_4$ | 5,0 | 34 | 0,230 | 0,26 | 0,45 | 88,5 (PE) | Anvari et Khayati, 2011 |
| Fed-batch | WP | *Torulopsis cremoris* et *C. utilis* | $NH_4)_2$ $SO_4$ | 4,8 | 29 | - | 0,75 | - | 95,8 | Cristiani-Urbina *et al.,* 2000 |
| Fed-batch | DPP | *K. marxianus* | $(NH_4)_2SO_4$ + extrait de levure | 5,0 | 30 | - | 0,38 | 2,90 | NA | Lukondeh *et al.,* 2005 |
| Continu | CW | *K. fragilis* | - | 4,5 | 33 | 0,105 | 0,26 | 0,40 | NA | Ghaly *et al.,* 2005 |
| Continu | CW | *K. fragilis* | - | 4,5 | 35 | 0,041 | NA | 0,21 | NA | Ben-Hassan et Ghaly, 1995 |

***Remarque-** CW: lactosérum; WP: le perméat de lactosérum; DWC: le concentré de perméat de lactosérum doux; DSWC: le concentré de lactosérum acidifié; DPP: poudre de lactose déprotéiné; NA: non disponible; PE: Hors proteins; DBO: Demande biochimique en oxygène.

## Oxygène dissous

L'oxygène dissous (DO) est généralement présenté en tant que pourcentage de saturation au cours du procedé de fermentation. Différents pourcentages de la valeur de saturation en oxygène au cours de la fermentation de lactosérum ont été soulignés par différents chercheurs. Carlotti *et al.* (1991) ont rapporté le besoin de maintenir DO-dessus de 30% de saturation lors de la production de PU par la levure à partir de lactosérum. Cristiani-Urbina et al. (2000) ont également rapporté le besoin de maintenir DO dessus de 25% lors de la production. En outre, il a été conclu que la DO critique pour le lactose consommant par la levure est supérieure à 25% de saturation, autrement la culture des levures change son métabolisme de l'oxydation de type oxydation-fermentation mixte qui conduit à la formation des métabolites intermédiaires (p. ex., l'alcool, les aldehydes et les esters).

Le changement dans le métabolisme due à DO affecte le rendement de production de biomasse et l'efficacité de l'élimination de la charge organique (Cristiani-Urbina *et al.,* 2000). La formation des métabolites (p. ex., l'éthanol) a même été observée dans des conditions totalement aérobies (> DO saturation de 20%) lors de la production de biomasse de *K. marxianus* à partir du milieu à base de lactose (Lukondeh *et al.,* 2005).

## Mode de fermentation

Le fermenteur est un instrument, qui est mis en place pour réaliser un procédé de fermentation principalement pour la culture en masse de la plante, de cellules animales ou de microorganismes et que peut varier en taille de l'échelle du laboratoire, 1-10 L, à des modèles industriéls plusieurs centaines de litres en capacité. Les fermenteurs sont équipés d'un aérateur, qui fournit de l'oxygène à des procédés aérobies, et d'un agitateur pour obtenir un mélange homogène des ingredients. Un thermostat est utilisé pour réguler la température et un capteur de pH pour contrôler le pH (Nasseri *et al.,* 2011). Le procédé de fermentation peut être effectué en mode immergé ou en mode semisolide. Cependant, le mode submergé est le principal mode de production la production impliquer dans de biomasse à partir le lactosérum.

Le choix du mode de fonctionnement des différents procédés de fermentation submergés dépend du but du procédé. Cependant, trois modes principalement de fermentation sont utilisés (batch, fed-batch et continu) pour la production de biomasse à partir de lactosérum utilisant une levure. Un désirait but de cette fermentation est l'elimination de la DCO du lactosérum (Ghaly

etKamal, 2004;. Moeini *et al.,* 2004). Le premier but de la fermentation par le (batch) est d'identifier les conditions optimales à utilisant plus tard en mode continu ou discontinu. Il existe diverses études à l'échelle du laboratoire sur la fermentation discontinue (Anvari et Khayati, 2011; Cristiani-Urbina *et al.,* 2000;. Ghaly *et al.,* 2003).

En particulier, lorsque l'étude est sur le traitement des eaux usées, le mode préféré du procédé de fermentation est le mode continu. La fermentation continue est favorisée pour diverses en raison d'une l'opération sans interruption pendant une période de temps longue (Shuler et Kargi, 2002). De plus, un autre avantage de la fermentation en continu sur un lot est une réduction de la durée de fonctionnement avec une uniformité de produit attendu. Le processus aérobie continu a été utilisé avec succès pour la production de biomasse à partir de lactosérum en utilisant la levure *Kluyveromyces fragilis* (Ben-Hassan et Ghaly, 1995; Ghaly *et al.,* 2005).

Type fed-batch est un mode entre la fermentation en discontinu et la fermentation en continu. Le mode fed-batch offre certains avantages par rapport aux cultures en discontinu et aux cultures en mode continu. Les principaux avantages sont de donner des cellules de densité élevées, de contrôler les conditions de concentration du substrat, de contrôler la production des sous-produits ou les effets de la répression catabolique en raison de l'approvisionnement limité en substrat, et aussi de permettre le remplacement des pertes d'eau par évaporation (Shuler et Kargi, 2002). Étant donné que les fermentations fed-batch impliquent le contrôle de l'alimentation en source de carbone, le mode fed-batch dominance plus adapté pour la production de biomasse. Une concentration de biomasse trés élevée (105 g/L) a été signalée dans une culture fed-batch utilisant *K. marxianus* (Lukondeh *et al.,* 2005). Un résumé du rendement en biomasse ($Y_{x/s}$), du taux de croissance spécifique ($\mu_m$), de la productivité, des conditions de culture (pH, température et source d'azote), et du mode de fonctionnement du procédé de fermentation à partir de lactosérum est presenté dans le tableau 1.3.

### 1.3.3 Qualité des protéines unicellulaires produites à partir du lactosérum

La valeur alimentaire et l'utilité du PU sont fondeés sur sa teneur en éléments nutritifs et sa composition. Les principaux composants du PU sont des protéines, des glucides, des acides gras, des constituants de la paroi cellulaire, des acides nucléiques et des vitamines (Anupama et Ravindra, 2000). Parmi ces derniers, l'élément important, qui déterminent la qualité des PU est

la teneur en protéines et leur composition. Comme par American Feed Control Officials-AAFCO (2010) la teneur minimale en protéines necessaire pour à l'alimentation animale ne doit pas être inférieure à 40% p/p.

En outre, le principal facteur déterminant des PU est le profil en acide aminés des PU. La norme fixée par la FAO/WHO pour les acides aminés essentiels est présentée dans le tableau 1.4. Le principal inconvénient des protéines microbiennes est leur faible contenu en certains acides aminés essentiels comme contenant du soufre (cystéine et méthionine) et l'acide aminé lysine. La basse teneur en acides aminés essentiels des PU de lactose fermentating levure *Kluyveromyces marxianus* spp. est également indiqué (Paul *et al.,* 2002; Schultz *et al.,* 2006). La composition en acides aminés des PU dépend principalement des caractéristiques du microorganisme. Comme présenté dans le tableau 1.4, aucun des microorganismes ne donne la composition requise en acides aminés. Pour atteindre l'équilibre dans le profil des acides aminés, différentes stratégies ont été proposées telles que l'utilisation de levures enrichies en méthionine ou l'utilisation de cultures mixtes. Des mutants de *K. lactis* enrichies en methionine, et qui utilisant le perméat du lactosérum, ont été isolée pour améliorer les caractéristiques nutritionnelles de biomasse de la levure (Kitamoto et Nakahara, 1994).

**Table 1.4 Les profils en acides aminés essentiels de protéines unicellulaires de différents microorganismes et leur comparaison avec le profile standatd de la FAO**

| | g/100 g de protéines | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Microorganismes (substrat)** | **Leucine** | **Lysine** | **Thréonine** | **Valine** | **Isoleucine** | **Tyrosine** | **Méthionine** | **Methinine + cystine** | **Références** |
| *K. marxianus,* CBS 6556 (perméat de lactosérum) | 7,7 | - | 6,94 | 7,5 | 5,48 | 2,5 | 0,77 | - | Schultz *et al.,* 2006 |
| *Kluyveromyces marxianus,* ATCC 8554 (lactosérum) | 10,6 | 5,5 | 15,2 | ND * | 6,7 | - | 1,73 | 1,73 | Paez *et al.,* 2008 |
| *K. fragilis* | 3,8 | 1,8 | 2,5 | 3,0 | 5,2 | 1,7 | 0,5 | 1,4 | Martini *et al.,* 1979 |
| *Saccharomyces cerevisiae* (mélasse) | 7,9 | 8,2 | 4,8 | 5,5 | 5,5 | 1,2 | - | 4,1 | Paez *et al.,* 2008 |
| *Candida krusei* SO1 (Sorgho) | 5,6 | 6,5 | 4,1 | 4,0 | 4,18 | - | 0,91 | - | Konlani et al., 1996 |
| *Candida utilis* | 3,5 | 1,7 | 2,5 | 2,7 | 5,2 | 1,8 | 0,3 | 1,2 | Martini *et al.,* 1979 |
| *Candida utilis* | 4,4 | 7,6 | 3,4 | 4,0 | 3,2 | 2,60 | 1,5 | 4,2 | Adedayo *et al.,* 2011 |
| *Lactobacillus fermentum* 3957 (Bouillon de Trypticase Soja) | 6,3 | 7,1 | 3,6 | 4,9 | 4,4 | - | 2,0 | - | Erdman *et al.,* 1977 |
| La FAO/WHO | 7,0 | 5,5 | 4,0 | 5,0 | 4,0 | 2,80 | 2,20 | 3,5 | Joint FAO/WHO, 2007 |

ND: Non déterminé

Des protéines unicellulaires de cultures mixtes de *Trichoderma reesei* et *K. Marxianus,* cultivées sur des pulpes de betterav, sont censées contenir des acides aminés essentiels, ce qui se compare favorablement aux directives de la FAO (Anupama et Ravindra, 2000). L'autre facteur important qui détermine la valeur nutritionnelle des PU est leur performance réelle, qui est déterminée par leur digestibilité et le coefficient d'efficacité protéique (PER). La digestibilité des protéines est présentée en pourcentage et varie de 65 à 96% pour différentes protéines microbiennes. La valeur de PER varie de 0,6 à 2,6 (Anupama et Ravindra, 2000).

### 1.3.4 Les considérations de sécurité et de pré-traitement des protéines unicellulaires

L'examen de la sécurité est nécessaire pour l'application des PU soit comme aliments pour animaux ou ingrédients alimentaires. Le niveau de toxicité permis pour l'alimentation animale est généralement plus élevé par rapport. Les essais sur la toxicité du produit final doit inclure les tests de toxicité aigüe à court terme, suivie des études à long terme (Anupama et Ravindra, 2000). Le substrat utilisé pour la culture de PU représente le principal danger pour la sécurité. Le substrat utilisé pour la culture PU représente le principal danger pour la sécurité. Les PU produite à partir de différentes sources peut être un support de composants cancérigènes. Les PU peuvent contenir également des métaux lourds ou des composés métalliques, ce qui peut provoquer des mutations, même en très faibles quantités. En outre, la présence de mycotoxines est également un facteur important qui influe sur l'utilisation des PU (Anupama et Ravindra, 2000). Cependant, les PU produites à partir de lactosérum devraient être exemptes de contamination, parce le lactosérum est classé comme GRAS pour l'alimentation animale et pour les denrées alimentaires par la Food and Drug Administration des Etats Unis (FDA américaine).

Les PU nécessitent un assainissement et des procédés de purification avant que le produit final soit distribué pour la consommation par les normes de contrôle de qualité en particulier pour les applications alimentaires. En outre, l'utilisation de protéines microbiennes pour la consommation humaine nécessite les prétraitements: i) une désintégration non digestibles de parois cellulaires et ii) une réduction de contenu des acides nucléiques (Anupama et Ravindra, 2000; Nasseri *et al.,* 2011). Le but partiel ou complèt de la digestion de la paroi cellulaire est d'améliorer la digestibilité des PU de la levure. La désintégration ou la digestion de la paroi cellulaire microbienne est généralement obtenue par des méthodes mécaniques (p. ex., homogénéisateurs sous haute pression, broyage humide, sonication, l'extraction par pression et

le traitement de particules de meulage) et par d'autres méthodes (comme le traitement chimique, le traitement enzymatique et le traitement physique) (Nasseri *et al.,* 2011).

Le facteur inhibiteur important des PU admis comme aliments, est le contenu en acides nucléiques, qui varie de 8 à 25 g/100 g de protéines unicellulaires (Larsen et Joergensen, 1996). Un régime alimentaire ayant un contenu élevé en acides nucléiques aboutit à la production d'acide urique suite à la dégradation des acides nucléiques. L'acide urique s'accumule dans l'organisme en raison de l'absence de l'enzyme uricase chez l'homme. Une consommation humaine supérieure à 2 g d'acides nucléiques équivalent par jour peut conduire à la goutte et à une insuffisance rénale, et donc à la formation de calculs rénaux (Anupama et Ravindra, 2000; Larsen et Joergensen, 1996). Différents traitements, tels que le traitement chimique avec $NH_4OH$, NaOH ou NaCl, ou l'activation de nucléases endogènes (ribonuclease-RNase et désoxyribonucléase-DNase) pendant la phase finale de production de biomasse microbienne. Aussi le choc thermique a été utilisé pour réduire les acides nucléiques (Alvarez et Enriquez, 1988; Larsen et Joergensen, 1996; Nasseri *et al.,* 2011). Alvarez et Enriquez (1988) ont montré une réduction des acides nucléiques des levures *S. cerevisiae et K. fragilis* avec traitement au $NH_4OH$ (4.5%), avec un contenu acides nucléiques de moins de 2%. Larsen et Joergensen (1996) ont appliqué un choc thermique pour activer les RNase et DNase endogènes dans la bactérie *Methylococcus capsulatus,* ce qui a abouti à une réduction de 80% de la teneur en acides nucléiques, avec une perte de moins de 10% de protéines.

## 1.4 LES PROTÉINES FONCTIONNELLES ET NUTRITIONNELLES DU LACTOSÉRUM

L'intensification des activités de recherche, l'avancement des techniques de séparation et la disponibilité des instruments analytiques sophistiqués ont renforcé l'intérêt de séparer (ou concentrer) les différentes protéines du lactosérum. Les protéines obtenues à partir du lactosérum sont reconnues pour leurs nombreuses applications, notamment, dans les industries alimentaires et pharmaceutiques.

### 1.4.1 Les produits protéinés issus du lactosérum

Il existe plusieurs produits utiles, obtenus à partir d'un simple traitement physique du lactosérum liquide contenant différentes teneurs en protéines. Ces produits sont notamment; la poudre de

lactosérum, le lactosérum à teneur réduite en lactose, les concentrés de protéines sériques («WPC- whey protein concentrate») et l'isolats de protéines sériques («WPI- whey protein isolate») (Modler, 2009; USDEC, 2006). La poudre de lactosérum est obtenue par élimination de l'eau par séchage. Le lactosérum à teneur réduite en lactose est du lactosérum dont la teneur en lactose est inférieure à 60% p/p. L'WPI résulte de l'élimination des composés non protéiques présents dans le lactosérum afin d'atteindre différentes teneurs en protéines. Les WPC les plus connus sont WPC34, WPC50, WPC60, WPC75 et WPC80. Les valeurs numériques indiquent la teneur en protéines. Par exemple, WPC34 doit contenir pas moins de 34% de protéines. WPI résulte de l'élimination des constituants non protéiques du lactosérum afin d'atteindre des teneurs en protéines de plus de 90% (USDEC, 2006). Les compositions typiques de différent products, dérivés du lactosérum sont présentées au tableau 1.5 (USDEC, 2006). La valeur sur le marché d'un produit protéiné dépend de sa teneur en protéines. Par exemple, la valeur sur le marché du WPC est de 3 à 40 fois plus élevée que celle de la poudre de lactosérum (Baldasso *et al.,* 2011).

**Table 1.5 Les caractéristiques des différents produits protéiques issus du lactosérum**

| Produits | Protéines (%) | Lactose (%) | Matières grasses (% ) | Cendres (% ) | Humidité (%) |
|---|---|---|---|---|---|
| Poudre de lactosérum doux | 11,0-14,5 | 63,0-75,0 | 1,0-1,5 | 8,2-8,8 | 3,5-5,0 |
| Poudre de lactosérum acide | 11,0-13,5 | 61,0-70,0 | 0,5-1,5 | 9,8-12,3 | 3,5-5,0 |
| Lactosérum réduit en lactose | 18,0-24,0 | 52,0-58,0 | 1,0-4,0 | 11,0-22,0 | 3,0-4,0 |
| Lactosérum dématérialisé | 11,0-15,0 | 70,0-80,0 | 0,5-1,8 | 1,0-7,0 | 3,0-4,0 |
| WPC34 | 34,0-36,0 | 48,0-52,0 | 3,0-4,5 | 6,5-8,0 | 3,0-4,5 |
| WPC50 | 50,0-52,0 | 33,0-37,0 | 5,0-6,0 | 4,5-5,5 | 3,5-4,5 |
| WPC60 | 60,0-62,0 | 25,0-30,0 | 1,0-7,0 | 4,0-6,0 | 3,0-5,0 |
| WPC75 | 75,0-78,0 | 10,0-15,0 | 4,0-9,0 | 4,0-6,0 | 3,0-5,0 |
| WPC80 | 80,0-82,0 | 4,0-8,0 | 4,0-8,0 | 3,0-4,0 | 3,5-4,5 |
| WPI | 90,0-92,0 | 0,5-1,0 | 0,5-1,0 | 2,0-3,0 | 4,5 |

## 1.4.2 Les protéines de lactosérum individuelle composants

Les protéines de lactosérum sont un mélange de différentes protéines. Ce sont notamment la β-lactoglobuline (β-LG), la α-lactalbumine (α-LA), l'immunoglobuline (Ig), l'albumine de sérum de

boeuf (ASB), la lactoferrine (Lf), la lactoperoxydase (LP), le protéose-peptone et glycomacropeptide (GMP). À l'exception de la glycomacropeptide, toutes les autres protéines sont naturellement présentes dans le lactosérum, tandis que la glycomacropeptide est produite à partir de la caséine, lors de la première étape du traitement enzymatique du lactosérum (Madureira *et al.,* 2007). Selon le tableau 1.6, la composition et les caractéristiques des protéines sont différentes (Amiri et Valsaraj, 2004; Kim *et al.,* 2007; Krissansen, 2007). La composition des protéines dans le lactosérum indique qu'il s'agit d'un mélange hétérogène de différentes fractions protéiques qui ont leurs propres propriétés fonctionnelles, physiologiques et nutraceutiques.

**Table 1.6 La composition protéique et les caractéristiques des protéines du lactosérum**

| **Protéines** | **Composition (% m/m)** | **Masse moléculaire (kDa)** | **pH isoelectrique (pl)** |
|---|---|---|---|
| Immunoglobulines (Igs) | 8,0 | 150-1000 | 5,5-8,3 |
| Albumine de sérum de boeuf (ASB) | 5,0 | 66,0 | 5,13 |
| Lactoferrine (Lf) | 1,0 | 76,5 | 9,5-10,0 |
| β-Lactoglobuline (β-LG) | 40-50 | 18,3 | 5,35-5,49 |
| α-Lactalbuline (α-LA) | 12-15 | 14,0 | 4,2-4,5 |
| Glycomacropeptide (GMP) | 12,0 | 6,8 | 4,3-4,6 |
| Proteose-peptone | 0,19 | 4-22 | - |
| Lactoperoxidase (LP) | 0,5 | 78,0 | 9,5 |

## β-Lactoglobuline

La β-lactoglobuline est une importante et essentielle protéine du lactosérum. Elle représente environ 64% des protéines coagulables sous l'effet de la chaleur ou 51% des protéines totales du lactosérum. Elle est généralement présente sous la forme d'un dimère constitué de deux sous-unités identiques. Chaque sous-unité (monomère) est constituée d'un groupement sulfhydryle et deux liaisons disulfure. Le nombre d'acides aminés dans la chaîne peptidique simple de la β-lactoglobuline est 162. La solubilité de cette protéine dépend principalement du pH et de la force ionique (Hernandez-Ledesma *et al.,* 2011a; Walstra *et al.,* 2010). La dénaturation thermique de la β-lactglobuline se produit entre 70 et 75 °C tandis qu'elle forme des agrégats entre 78 et 82 $^{o}$C. La précipitation de la β-lactglobuline se produit avant celle de

l’α-lactalbumine. La dénaturation de l’α-lactalbumine est réversible, comparée à celles d'autres protéines de lactosérum. Par conséquent, elle est plus résistante à la chaleur par rapport à la β-lactoglobuline (Save *et al.,* 2005). Des produits à forte teneur en protéines telles que les WPI, sont enrichis en β-lactoglobuline, ce qui améliore leurs propriétés fonctionnelles, tels que la stabilité à l'émulsion, l'activité de l'émulsion, la viscosité et les propriétés de gélification, comparé aux WPC. La séquence d'acides aminés de la β-lactoglobuline a une similarité avec celle de la protéine de liaison du rétinol (provitamine A). C’est grâce à cette similarité que la β-lactoglobuline se lie à des molécules hydrophobes, telles que les vitamines liposolubles et les lipides (Modler, 2009).

## α-Lactalbumine

L’α-lactalbumine (α-LA) représente environ 11% des protéines totales du lactosérum. C’est une petite protéine globulaire à chaîne polypeptidique simple. La masse moléculaire de l’α-LA est 14 kDa, avec une chaine polypeptidique de 123 acides aminés et huit résidus de cystéine (Hernández-Ledesma *et al.,* 2011a; Markus *et al.,* 2002). Le calcium se lie fortement à l’α-LA et assure la stabilisation des conformations moléculaires. L’α-LA a plusieurs utilisations bénéfiques, notamment comme composante alimentaire. L’α-LA, comme composante alimentaire, augmente le ratio plasma/tryptophane relativement à la somme des autres acides aminés neutres (valine, isoleucine, leucine tyrosine et phénylalanine) qui ont des effets négatifs, tels que le déséquilibre du cerveau en sérotonine (5-hydroxytryptamine). L'amélioration de ce déséquilibre conduit à de meilleures performances cognitives, de l'humeur et du sommeil (Markus *et al.,* 2002). Elle aide à préserver l'oxydation des lipides dans le cycle métabolique et augmente la masse corporelle maigre chez le rat (Bouthegourd *et al.,* 2002). L’α-LA contribue également à l'absorption des minéraux, possède une activité antibactérienne, montre des effets immunomodulateurs et une activité anti-tumorale (Modler, 2009).

## L'albumine de sérum bovin

La protéine de lactosérum contient environ 5-6% d’albumine de sérum de boeuf, qui est pratiquement similaire à la concentration dans le lait humain. La masse moléculaire de l’ASB est 66,26 kDa avec 35 résidus de cystéine et 17 liaisons disulfure par molécule (Walstra *et al.,* 2010). Le nombre d'acides aminés dans la chaîne polypeptidique de l’ASB est 582 (Hernandez-Ledesma *et al.,* 2011a). L’ASB a une température de dénaturation de 64 °C, qui est presque similaire à celle de l’α-LA (62 $^{o}$C). Bien que la température de dénaturation de l’ASB est un peu élevée que celle de l’α-LA, elle précipite avant l’α-LA à cause de la nature réversible du précipité

α-LA (Modler, 2009). La caractéristique la plus importante de l'ASB est sa capacité à se lier de manière réversible à plusieurs ligands, grâce auxquels elle peut être utilisée en tant que la transporteur d'acides gras (Mollea *et al.,* 2013).

## Immunoglobulines

Les immunoglobulines (Igs) sont composées de trois classes principales: IgG, IgA et IgM. En outre, l'IgG est divisée en sous-classes: IgG1 et IgG2. La concentration totale des immunoglobulines dans le lactosérum est de l'ordre de 0,7 g/L et les concentrations d'IgG représentent jusqu'à 80% (p/p). Les IgG sont présentes sous forme de monomères, tandis que les IgA et IgM sont présents dans des formes de polymères (Mollea *et al.,* 2013). Le rôle principal des immunoglobulines est d'agglutiner les bactéries, de neutraliser les toxines et d'inactiver les virus. Les IgA protègent contre la protéolyse et peuvent également neutraliser des virus et des toxines bactériennes. L'IgM se révèlent être plus efficaces que les autres immunoglobulines, en termes du fixation de complément, de neutralisation des virus et d'agglutination des bactéries. Les IgG peuvent également résister à la digestion gastrique. Les autres rôles métaboliques sont la baisse de la pression sanguine, en réduisant de taux de cholesterol, et les utilisation comme substituts de lait pour nourrissons (Modler, 2000; Modler, 2009;. Mollea *et al.,* 2013).

## Glycomacropeptide

Le glymacromacropeptide (GMP) est aussi connu sous le nom de caséinomacropeptide (CMP). Le GMP est dérivé des composantes de la k-caséine du lait durant la fabrication du fromage par l'action d'enzymes de coagulation du lait (p. ex., la chymosine). Les résidus d'acides aminés du GMP sont de 64 et la masse moléculaire est 6,8 kDa. Le degré de glycosylation des molécules varie de 0 à 5 unités d'acide N-acétyl-neuraminique (acide sialique). Cependant, 25 à 50% des GMP peuvent être dépourvus de glycosylation de glucides. Le GMP sont thermostables et semblent être une partie de la fraction protéose-peptone (Modler, 2009). Les molécules de GMP ont une caractéristique simple en raison de l'absence de phénylalanine, de tryptophane, de tyrosine, d'histidine, d'arginine ou de cystéine. L'absence de phénylalanine dans le résidu rend cette protéine un ingrédient alimentaire utile pour les patients qui souffrent de phénylcétonurie (Modler, 2009). En outre, le GMP sont également riches en acides aminés ramifiés (Krissansen, 2007; Marshall, 2004).

## Lactoferrine

La glycoprotéine de fixation du fer lactoferrine (Lf) appartient à la famille des protéines de transfert. La lactoferrine est présente généralement dans les sécrétions exocrines du lait de mammifère, les larmes, le mucus et la salive. La présence de lactoferrine a également été rapportée dans le blanc d'oeuf, la conalbumine et ovatransferrine. La masse moléculaire de la lactoferrine est de 76,50 kDa et elle se compose d'une chaîne polypeptide simple de 700 résidus d'acides aminés (González-Chavez *et al.,* 2009; Modler, 2009). Les molécules de lactoferrine sont stables à la chaleur et résistantes à l'acide à pH 4,0 (Modler, 2009). Elles sont également résistantes à l'action de la trypsine et de la chymotrypsine, mais peuvent être hydrolysées par la pepsine (Modler, 2000). Traditionnellement, la lactoferrine est connue pour son activité antimicrobienne et antifongique contre une vaste gamme de bactéries et de levures (Hernández-Ledesma *et al.,* 2011a). La lactoferrine possède également une activité immunomodulatrice. Elle a des effets sur la croissance cellulaire et la différenciation, le développement embryonnaire, la myelopoesis, l'adhérence des cellules endothéliales, cytokines et la production de chimiokines (Cornish *et al.,* 2004; Ulber *et al.,* 2001).

## Proteose-peptone composant

La protéose-peptone est la fraction de protéine de lait qui reste en solution après que le lait ait été chauffé à 95 °C pendant 20 min, puis acidifié à pH 4,7. La protéose-peptone a quatre formes (forme 3, forme 5, forme 8-lente et forme 8-rapide). La forme 3 est la forme majeure avec 25% m/m (Krissansen, 2007). Les trois autres formes (forme 5, forme 8-lente et forme 8-rapide) proviennent de la β-caséine par action de la plasmine native, tandis que la forme-3 semble dériver des lipides de la membrane des globules. La protéose-peptone a été reconnue pour ses applications très limitées dans le domaine alimentaire. La forme 3 peut être utilisée comme un facteur bifidogène en raison de sa teneur élevée en glucides. Toutefois, peu de recherches ont été effectuées sur cet aspect (Sørensen et Petersen, 1993).

## Lactopéroxydase

La lactoperoxydase (LP) est une enzyme naturelle du système de défense des mammifères (Hernández-Ledesma *et al.,* 2011a). La masse moléculaire de la lactoperoxydase de 78 kDa constitue une chaîne polypeptidique simple de 612 résidus d'acides aminés. Elle contient 15 résidus de demi-cystine, un groupement hémique et environ 10% (m/m) de groupements glucidiques. L'activité enzymatique est affectée par la température et le temps de traitement. L'activité enzymatique est perdue à 62,5 °C pendant 30 min, à 70 °C pendant 15 min ou à 85 °C

pendant 15 s (Modler, 2009; Mollea *et al.,* 2013). La principale utilisation de la lactoperoxydase est la protection contre les microbes infectieux (Shin *et al.,* 2005).

### 1.4.3 Le traitement des produits protéinés du lactosérum

Le lactosérum est traité par différentes opérations unitaires pour obtenir le produit final. La première étape est la clarification, durant lequel le lactosérum est tamisé et/ou centrifugé pour éliminer l'excès de lait caillé issu de la production du fromage. La lipides sont également enlevés pour faciliter la filtration sur membrane (Zadow, 1992). Après la clarification et l'élimination des lipides, le lactosérum doit être pasteurisé immédiatement pour éliminer les pathogènes et inactiver les cultures starter du fromage. De plus, le prétraitement peut être appliqué pour décolorer le lactosérum en enlevant le rocou (Modler, 2009). L'étape du prétraitement est suivie de la concentration des protéines du lactosérum. Les méthodes de concentration couramment utilisées comprennent l'évaporation, l'osmose inverse et la filtration sur membrane (Akpinar-Bayazit *et al.,* 2009). L'évaporation est souvent réalisée sous vide à des températures inférieures à 100 °C afin de protéger les protéine du lactosérum de la dénaturation. Les inconvénients des procédés thermiques classiques d'évaporation sont l'exigence d'une grande quantité d'énergie, la haute teneur en cendres, et da presence de lactose dans le concentré. En outre, les caractéristiques des protéines thermolabiles peuvent changer, ce qui conduit à une modification des propriétés fonctionnelles et nutritionnelles du produit final (Baldasso *et al.,* 2011). La concentration du lactosérum par osmose inverse est réalisée par passage du lactosérum sous haute pression à travers une membrane de porosité d'environ 0,001 µm. Le lactosérum peut être concentré à 20-22% base séche, par osmose inverse. Parfois, l'osmose inverse est utilisée comme une étape de pré-concentration avant évaporation (Zadow, 1992).

Les technologies membranaires ont gagné de l'importance en raison de leur advantage côte économie d'énergie. Le WPC, obtenu par ultrafiltration du lactosérum, est disponible en haute qualité, en termes de teneur en protéines, état natif et propriétés fonctionnelles (Atra *et al.,* 2005; Limsawat et Pruksasri, 2010). L'ultrafiltration et la nanofiltration peuvent être définies comme de procédés à membrane sous pression pour la séparation et la concentration des substances ayant un poids moléculaire compris entre 1 kDa et 1000 kDa pour l'ultrafiltration et entre 100 et 500 Da pour la nanofiltration (Baker, 2002). En fonction des caractéristiques de rétention des membranes, il peut y avoir une différence significative dans la qualité nutritive du rétentat et du perméat. L'ultrafiltration, pour obtenir le WPC du liquide clarifié, peut concentrer jusqu'à 65% sur

une base de matière sèche. En outre, en diluant le rétentat avec de l'eau et en utilisant la diafiltration, la protéine peut être concentrée jusqu'à 80% (Varnam et Sutherland, 1994). Le WPC peut être produit soit en tant que liquide concentré soit en tant que poudre séchée aprés pulvérisation. L'organigramme général décrivant la production industrielle le WPC est présentée dans la figure 1.3. (USDEC, 2006).

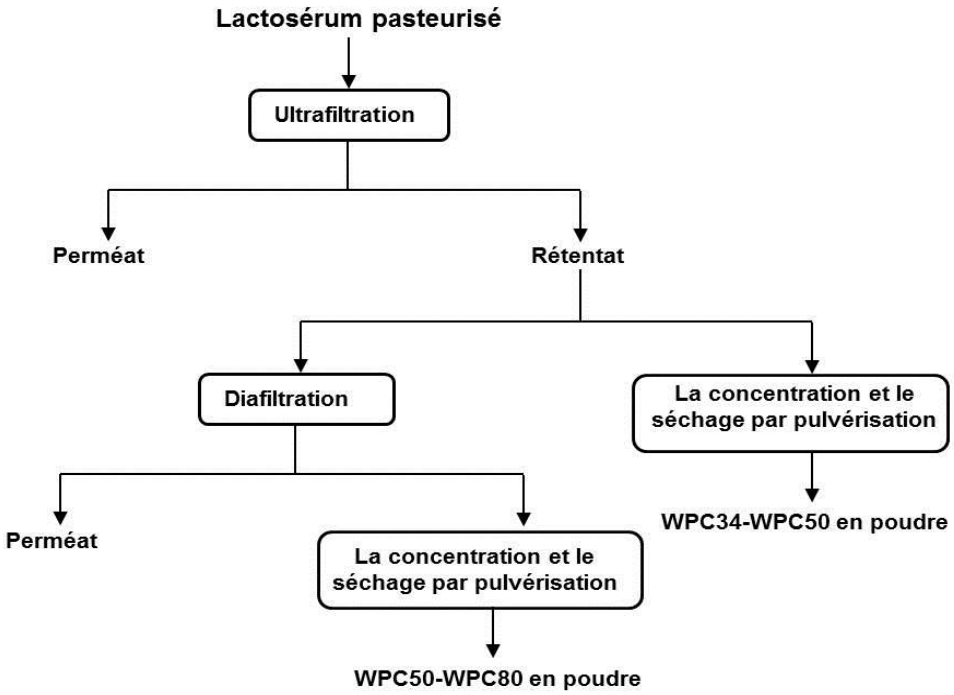


**Figure 1.3** Schéma du traitement de concentrés de protéines sériques (WPC)

Pour obtenir l'isolats de protéines sériques (WPI) ayant une plus grande (au dessus de 90%), une microfiltration supplémentaire ou un traitement par échange d'ions est nécessaire. Comme les autres protéines, les protéines du lactosérum sont également de nature amphotère et elles agissent comme un acide ou une base en fonction du pH du milieu. Les protéines de lactosérum ont une charge nette positive à des valeurs de pH en dessous du point isoélectrique et une charge nette négative au-dessus du point isoélectrique. De cette manière, le pH du milieu peut être ajusté, et les protéines sont donc des adsorbées sur un échangeur d'ions qui a une taille de pores et une surface appropriée pour la récupération de protéines à partir de solutions diluées (Varnam et Sutherland, 1994). Les protéines du lactosérum sont récupérées de l'échangeur

d'ions à un pH approximatifde 9 puis la solution peut être concentrée par ultrafiltration (Morr et Foegeding, 1990).

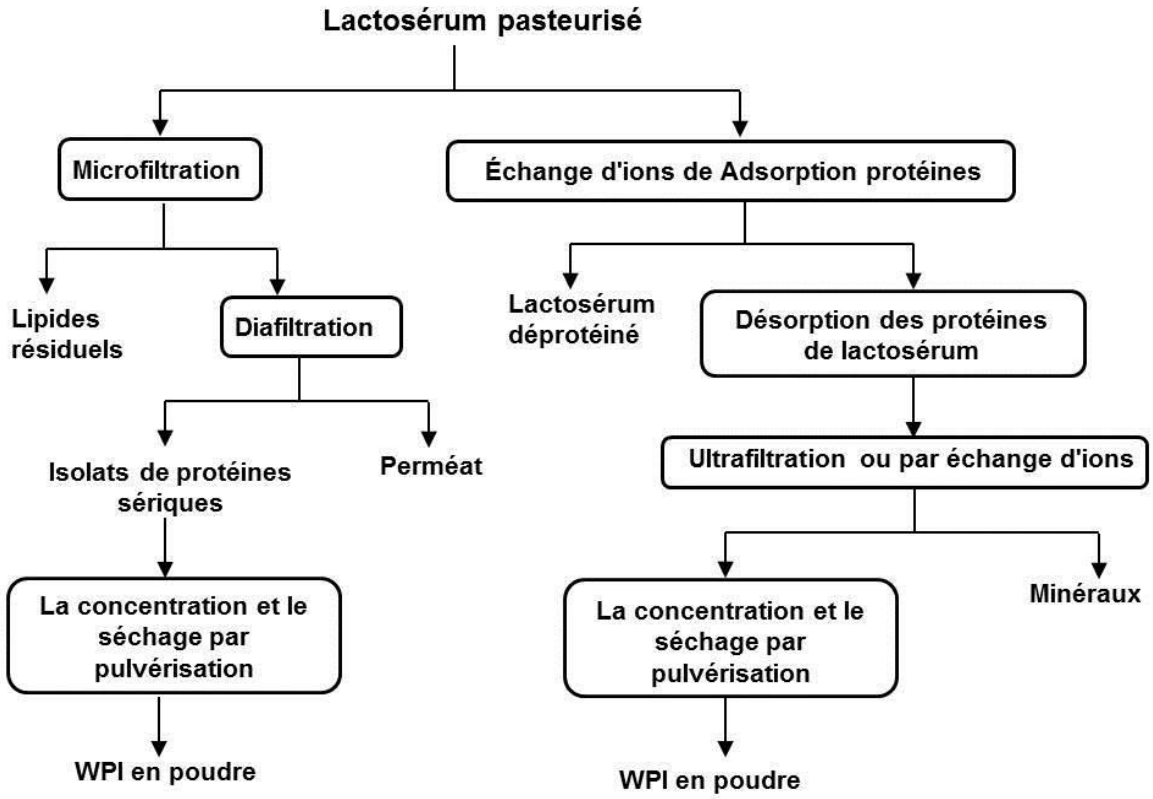


**Figure 1.4 Schéma de traitement d'isolat protéique de lactosérum (WPI)**

Généralement, le séchage par pulvérisation est le procédé utilisé pour le séchage à sec pour obtenir la poudre de lactosérum et des protéines de lactosérum, que sont souhaitables pour de nombreuses applications (Modler, 2009; Zadow, 1992). La poudre est obtenue à partir du séchoir par pulvérisation avec une teneur en humidité d'environ 5-7% (Henning *et al.,* 2006). Le lactosérum devient très collante durant le processus de séchage, donc, de l'équipement spécialisé est utilisé pour atteindre plus solides les concentrations (de 50% à 80% de solides). En outre, un deuxième ensemble de l'équipement est utilisé à cristalliser et à refroidir le produit rapidement. Il est ensuite introduit dans un tambour interne et séché à 95-96% de matières solides à 54 à 60 °C. Le produit du lactosérum séché, en outre, est traité dans un broyeur à marteaux pour réduire la taille des particules. Les différentes étapes impliquées dans le traitement de l'isolat de protéines du lactosérum sont représentées dans la figure 1.4 (USDEC, 2006).

## 1.4.4 Le fractionnement des protéines du lactosérum

Il y a un intérêt accru pour le développement des technologies rentables. L'isolement des différentes fractions de protéines du lactosérum permet l'acquisition de nouvelles parts de marché. En général, trois approches sont utilisées pour séparer ou fractionner les protéines de lactosérum: i) la précipitation/floculation par un traitement thermique ou en utilisant des polymères; ii) la technologie membranaire (l'ultrafiltration et la nanofiltration) et iii) la séparation par chromatographie.

### Séparation par précipitation

La séparation des protéines par précipitation consiste à ajuster les propriétés physiques de la solution afin de promouvoir le caractère insoluble. Des protéines sont généralement moins solubles à un pH proche du point isoélectrique et dans des solutions faibles en force ionique. Les conditions énoncées ci-dessus permettent de former des agrégats de protéines. Les traitements thermiques ont une incidence sur la structure et la solubilité des protéines de lactosérum. Les protéines de lactosérum précipite et lorsqu elles sont chauffées à plus de 90 °C pendant 10 min dans des conditions acides (pH 3,5 à 5,5). Il existe de nombreuses études sur la separations des protéines de lactosérum influencée par le pH, la température, et la concentration en protéines (Antoine et Souza, 2007; de la Fuente *et al.,* 2002.). À un pH isoélectrique, les protéines dénaturées par la chaleur interagissent via des réactions irréversibles du thiol-disulfure. Ensuite, elles forment des agrégats, qui se rejoignent ensuite

pour dimer de grosses particules sédimentables. C'est le cas du lactosérum ajusté à un pH 4,2 et chauffé à 65 °C afin de dénaturer et précipiter les agrégats α-lactalbumine (Pearce, 1983). Le surnageant a été appauvri en α-lactalbumine et enrichi en la β-lactoglobuline, qui est la protéine restante (Pearce, 1983). La tendance à l'agrégation augmente avec la concentration en protéines. Dans ces conditions opératoires, la β-lactoglobuline est restée soluble (plus de 98%), alors que l'ASB et les Igs co-précipitent. Les agrégats ont été partiellement solubilisés de nouveau par un réajustement du pH et de la température.

La précipitation ou le fractionnement est également effectué en utilisant des agents floculants tels que la bentonite, la carboxyméthylcellulose et le chitosane. Le fractionnement via des polymères (p. ex., des polysaccharides) sur la base de l'interaction électrostatique (les deux semaines ou fortes polyélectrolytes de charges opposées) entre les protéines et les polymères (Koupantsis et Kiosseoglou 2009; Vikelouda et Kiosseoglou, 2004; Xu *et al.,* 2001). Le chitosane permet une précipitation optimale de la β-lactoglobin avec un pH de 6,2 tandis que 80% des outres protéines du lactosérum demeurent soluble (Casal *et al.,* 2006).

### Séparation sur membranes

Il y a eu plusieurs études faites sur le fractionnement individuel des protéines de lactosérum à l'aide de technologies membranaires (ultrafiltration et nanofiltration). Deux étapes du procédé à membranes sont utilisées pour l'obtention l'α-lactalbumine enrichie à partir du lactosérum. Par exemple, une membrane XM50 (poids moléculaire limite 50 kDa) a été utilisée pour produire un perméat contenant principalement de l'α-lactalbumine, de la β-lactoglobuline et d'autres petites protéines/peptides. Une seconde membrane (poids moléculaire limite plus faible) a été utilisée pour enlever le caésinomacropeptide. Cependant, le produit final est composé uniquement d'α-lactalbumine et de β-lactoglobuline à un ratio de 2:1 (Zydney, 1998). De même, Mehra et Kelly (2004) ont proposé trois étapes séquentielles de fractionnement individuel des protéines de lactosérum, par filtration et ultrafiltration, avec différentes membranes de poids moléculaire limite (MWCO), et la modification de charge, sur la base d'un pH, tel que présenté dans la figure 1.5.

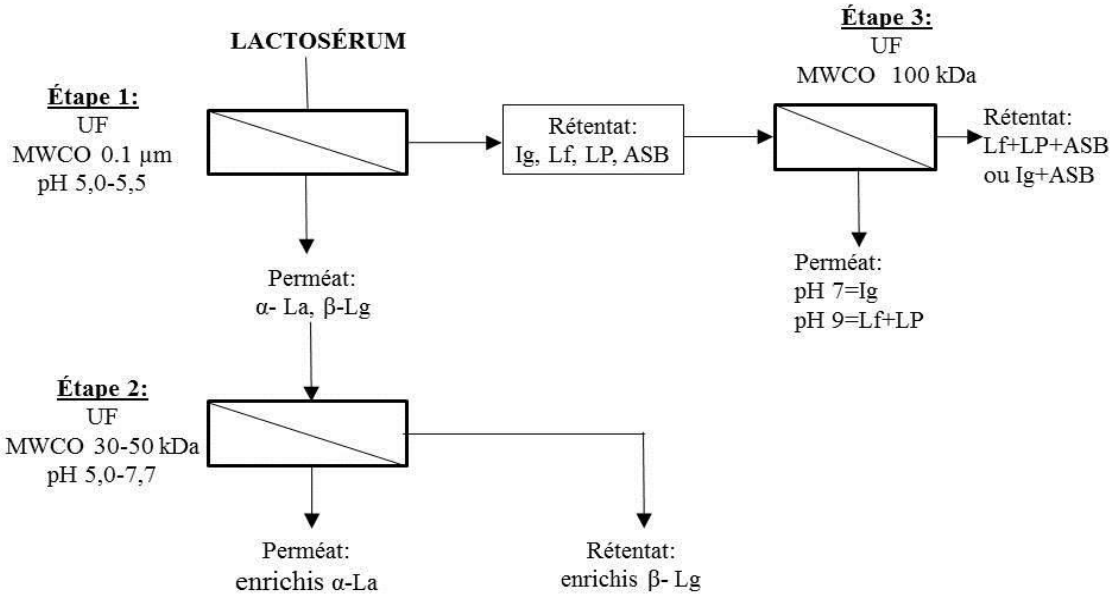


**Figure 1.5 Fractionnement séquentiel des protéines de lactosérum par le système de filtration membranaire à trois étapes**

## La Séparation par chromatographie

Les méthodes de séparation par chromatographie sont aussi très populaires dans le fractionnement individuel des protéines de lactosérum. Un schéma de fractionnement a ete proposé pour les IgG, la lactoferrine et la lactoperoxydase. Il est basé sur l'échange de cations sur S-HyperD-F, S Sepharose FF et Fractogel EMD-S 650 (S) (Hahn *et al.,* 1998). Naqvi *et al.* (2010) ont purifié la β-lactoglobuline par chromatographie sur gel à partir du lait de bovin. Ils ont utilisé la colonne Bio-Gel P10 (BIORED) à pH 3,0, laquelle fut éluée avec du chlorure de sodium à 18 mM. La récupération et le fractionnement des protéines de lactosérum, à partir de WPC (80% p/p), par chromatographie à interaction hydrophobe, a également été rapporté par Santos *et al.* (2011). Ils ont utilisé des protéines de référence et de WPC80 dissoutes dans un tampon phosphate avec du sulfate d'ammonium (1M) et utilisé une colonne HiPrep Octyl Sepharose FF couplée à une chromatographie liquide rapide des proteins (FPLC) système. L'élution était réalisée par diminution de la force ionique du tampon en utilisant un gradient de sel. La récupération obtenue fut de 45,2% de β-lactoglobuline avec 99,6% de pureté (Santos *et al.,* 2011).

### 1.4.5 Les application des produits enrichis en protéines et des protéines individuelles du lactosérum

Les protéines de lactosérum ont été reconnues pour différentes propriétés fonctionnelles et leur valeur nutritive. Les propriétés fonctionnelles sont principalement dues à des caractéristiques physiques, chimiques et structurelles (Jovanovic *et al.,* 2005). La valeur nutritionnelle est due à une forte teneur en acides aminés essentiels (en particulier ceux contenant du soufre) et ceux à chaîne ramifiée (isoleucine, leucine, et valine) (Baldasso *et al.,* 2011). De plus, les protéines de lactosérum sont classées comme GRAS pour des applications alimentaires et elles peuvent être utilisées dans de nombreux aliments transformés tels que les aliments sains, les produits laitiers, carnés, surgelés et les préparations pour nourrissons (Jayaprakasha et Brueckner, 1999; Morr et Foegeding, 1990). WPC80 et WPI sont fréquemment utilisés pour leurs propriétés fonctionnelles dans les produits de boulangerie pour améliorer la qualité de la cuisson, ou comme un remplacement de l'œuf, pour augmenter le rendement en fromage, dans le yogourt comme stabilisant, dans les trempettes et tartinades comme texturant, dans les confiseries, en remplacement de blanc d'œuf, et dans les produits à base de viande comme diluant (Varnam et Sutherland, 1994). Les protéines de lactosérum sont utilisées en boulangerie, pâtisserie, viandes et fruits de mer pour obtenir un gel à haute résistance avec une bonne capacité de rétention d'eau (Jovanovic *et al.,* 2005; Veith et Reynolds, 2004). L'interaction de la protéine se traduit par la formation de gel et d'un réseau élastique (Foegeding *et al.,* 2002). Les protéines de lactosérum peuvent également être utilisées comme agent épaississant ou émulsifiants en raison de leur capacité à stabiliser les émulsions (Onwulata et Huth, 2009). En outre, la formation de mousse est une propriété attrayante importante pour de nombreux produits alimentaires, tels que les garnitures fouettées, gâteaux, milk-shakes et desserts glacés (Jayaprakasha et Brueckner, 1999; Jovanovic *et al.,* 2005). La formulation de boissons enrichies en protéines de lactosérum appelées boissons prêtes-à-boire, lancée par USDEC, fournissent des protéines nutritionnelles particulièrement destinées aux athlètes (Rittmanic, 2006).

Les chercheurs en alimentation sont régulièrement à la recherche de nouvelles façons d'utiliser ou de transformer des protéines de lactosérum en ingrédients fonctionnels. Mishra *et al.* (2001) ont produit un complexe de protéines-polysaccharides avec WPC71 et de la pectine, une même proportion de deux biopolymères. Le complexe produit a montré une solubilité accrue, une émulsification, une gélification, la formation de mousse et la stabilité de la mousse par rapport aux protéines de lactosérum concentrées seule. La couche encapsulée a retardé la dégradation

oxydative de cacahouètes grillées à sec. Une autre étude a montré que l'utilisation de la transglutaminase en tant qu'agent de réticulation dans les films a amélioré la barrière à l'oxygène. Des recherches approfondies ont disparu pour faire des protéines de lactosérum en tant qu'agents de revêtement contre l'oxydation (Di Pierro *et al.,* 2006; Lin et Krochta, 2005). En outre, le rôle fonctionnel et nutritionnel de protéines de lactosérum individuelles est résumé dans la figure 1.6 (Hernández-Ledesma *et al.,* 2011a; Madureira *et al.,* 2007).

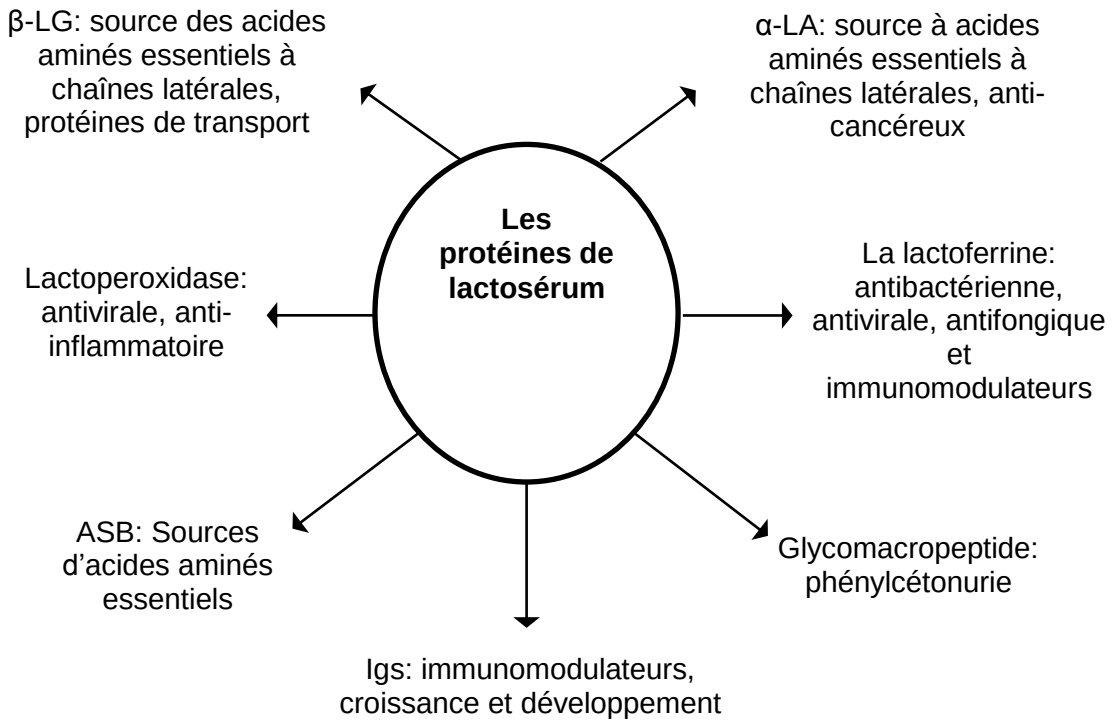


**Figure 1.6 Rôles individual fonctionnels et nutritionnels importants de protéines de lactosérum**

# 1.5 LES PEPTIDES BIOACTIFS ET LEUR TRANSFORMATION À PARTIR DU LACTOSÉRUM

## 1.5.1 Les peptides bioactifs

Les fragments de protéines spécifiques qui ont une influence positive sur les fonctions du corps et finalement, un impact sur la santé sont définis comme des peptides bioactifs. L'activité du peptide bioactif est fonction de sa séquence spécifique acides aminés et leur taille, qui varier de 2 à 20 résidus d'acides aminés (Korhonen, 2009). Le lactosérum peut être converti en divers peptides bioactifs suivant différents types de traitement.

### Peptide inhibiteur de l'ACE

Actuellement, l'inhibiteur de l'ACE (antihypertenseur) est utilisé de préférence avec le captopril, l'énalapril, le lisinopril et alecepril synthétisés chimiquement ou avec des peptides bioactifs produits par hydrolyse enzymatique du lait et des protéines de lactosérum, en utilisant des enzymes comme la trypsine, la pepsine et l'alcalase (Mota *et al.,* 2004). L'α-Lactorphine [YGLF, l'α-LA (f 50-53)], un tétrapeptide provenant de la protéolyse de bovins α-LA; la β-lactorphine [YLLF, β-LG (f 102-105)]; β-lactotensin [HIRL, β-LG (f 146-149)], un tétrapeptide issus de la protéolyse de bovins β-LG ont été rapportés pour montrer l'activité inhibitrice de l'enzyme angiotensine-I-conversion (ACE) (Belem *et al.,* 1999b; Sieber *et al.,* 2009). Le rôle principal de l'inhibiteur de l'ACE de peptides est dans la régulation de l'hypertension. L'inhibition de l'ACE conduit à une diminution du niveau du peptide angiotensine II vasoconstrictrice et de l'augmentation correspondante au niveau du peptide vasodilatoire, le bradykinine qui se traduit par une diminution globale de la pression artérielle (Fitzgerald et Murray, 2006; Saito, 2008). Actuellement, les fragments de β-LG dérivés de l'hydrolyse de l'isolat protéique de lactosérum sont commercialisés par BioZate (Danisco Foods International, Le Sueur, MN), pour réduire la pression artérielle (Madureira *et al.,* 2010).

### Peptides bioactifs avec activité morphinique

Les peptides opioïdes sont des peptides qui ont une affinité pour un récepteur aux opiacés ainsi que des opiacés qui ont un effet inhibiteur au naloxone (Pihlanto-Leppälä, 2000). Les peptides opioïdes présentent une activité pharmacologique similaire à celle de l'opium (morphine, alcaloïde) (Sharma *et al.,* 2011). Les peptides opioïdes typiques sont originaires de trois protéines précurseurs; proopiomélanocortine (endorphines), proencéphaline (enképhaline) et

prodynorphine (dynorphine). La caractéristique importante de peptides opioïdes typiques est d'avoir la même séquence N-terminale, Try-Gly-Gly-Phe (Pihlanto-Leppälä, 2000; Sharma *et al.,* 2011). Les séquences d'opioïdes présent en la structure native de la proteine α-LA, f (50-53) et β-LG, f (102-105) de lactosérum natif. Les peptides de l'α-LA et β-LG sont appelés α- et β-lactorphins (Pihlanto-Leppälä, 2000).

## Peptides bioactifs liant le fer

Protéines liant le fer est un peptide bioactif qui est produit par hydrolyse enzymatique de WPC. La lactoferricine est un exemple de liaison de fer à un peptide provenant de l'hydrolyse peptidique de WPC (Vegarud *et al.,* 2000; Wakabayashi *et al.,* 2003). De tels peptides du peuvent être utiles dans la prévention de l'anémie, d'une grande carence en fer, en particulier chez les enfants et les femmes (WHO, 2001). De plus, les peptides se liant au fer peuvent offrir un avantage sur l'administration prophylactique ou thérapeutique de la formulation orale, contenant des composés du fer non hémique, tel que le sulfate de fer, en atténuant le problème de la faible biodisponibilité et les effets secondaires dans le cas de l'anémie (Kim *et al.,* 2007).

## Les protéines de lactosérum hydrolysées

Généralement utilisées de façon standard dans la formule de lait infantile produit à partir de lait de vache ou de ses dérivés. Bien qu'il y ait beaucoup de similitude entre le lait humain et le lait de vache en ce qui concerne le profil des protéines, la différence est due à la présence de β-lactoglobuline dans le lait de vache. La β-lactoglobuline est absente dans le lait humain, et si elle est administrée à partir de sources extérieures, elle provoque l'allergie chez les nourrissons due à l'absence de la sécrétion de pepsines sous-développés. Par conséquent, le WPC est généralement allergique pour certains nourrissons en raison de la présence de β-lactoglobuline, qui est le composant majeur de la protéine de lactosérum. Il existe deux approches possibles pour éviter l'allergie aux protéines de lactosérum. La première est l'élimination de β-lactoglobuline à partir des produits de protéines de lactosérum par précipitation/ou filtration sur membrane (ultrafiltration). La seconde est la protéolyse des protéines de lactosérum par une protéase différente pour produire des protéines et des peptides de poids moléculaires inférieurs. Les protéines peuvent être largement ou partiellement hydrolysées pour les préparations des nourrissons. Cependant, certains chercheurs ont suggéré que seulement un hydrolysat très étendu doit être utilisé pour éviter toute réaction chez les nourrissons hautement sensibles (Chan *et al.,* 2002).

## 1.5.2 Méthodes de production de peptides bioactifs

Il existe un certain nombre de méthodes pour produire des peptides bioactifs. Les méthodes les plus couramment utilisées dépendent de l'agro-alimentaire: (i) le chauffage dans des conditions acides/alcalines (ii) l'hydrolyse enzymatique de protéines et (iii) l'activité microbienne (hydrolyse microbienne) dans les aliments fermentés. L'hydrolyse limitée de protéines connues libère des peptides bioactifs actifs. Cependant jusqu'à ce jour, la façon la plus courante pour produire des peptides bioactifs est la digestion enzymatique, en utilisant différentes échelles et techniques (Pihlanto-Leppälä, 2000). La trypsine a été utilisée de préférence pour l'hydrolyse de la protéine connue, qui clive à l'extrémité C-terminale de l'arginine (R) et la lysine (K) du résidu. Une autre importante action de l'enzyme chymotrypsine sur la chaîne latérale aromatique du C-terminal. Les protéases bactériennes et fongiques ont également été utilisées pour générer des peptides bioactifs tels que des peptides bioactifs inhibiteurs de l'ACE. L'hydrolyse enzymatique de la protéine ou du peptide précurseur peut être effectuée soit en mode discontinu soit en mode continu (dans des réacteurs à membrane) pour obtenir des peptides bioactifs (Madureira *et al.,* 2010).

A côté de l'hydrolyse enzymatique, le procédé de fermentation a également été identifié comme étant l'une des voies pour produire des peptides bioactifs. Les bactéries lactiques (LAB) sont la dominante parmi toutes, pour hydrolyser les protéines du lait, en particulier la caséine, en peptides bioactifs (Hernández-Ledesma *et al.,* 2011b). La protéine de lactosérum a été hydrolysée en des peptides inhibant l'ACE par l'action de levures et de bactéries protéolytiques. Les bactéries utilisées pour la fermentation est des espèces *Lactobacillus* et la levure *K. marxianus*. Les espèces *Lactobacillus* comme *L. rhamnosus* et *L. helveticus* CNP4 leur activité de protéolitique et leur production de peptide inhibant l'ACE. Les levures comme, *K*. *marxianus* et *S. cerevisiae* sont reconnues pour avoir une activité protéolytique sur les protéines de lactosérum pour produire des oligopeptides (Didelot *et al.,* 2006; Hamme *et al.,* 2009). Cependant, l'hydrolyse microbienne est encore à l'étape du laboratoire.

# 2. PROBLIÉMATIQUE

L'examen de la littérature montre que le lactosérum est un sous-produit valorisable des industries fromagères qui, s'il est directement déversé dans les eaux usées, pose des problèmes pour l'environnement. Cependant, s'il est traité par des procédés biotechnologique et physiques, ce sous-produit peut potentiellement être transformé en produits à valeur ajoutée, ce qui contribue à minimiser le problème de gestion environnementale y étant associé. Néanmoins, un problème demeure: l'excès de volume de production du lactosérum. Le procédé principal de biotransformation du lactosérum est la production de protéines unicellulaires (PU). Cependant, il existe de nombreux défis qui entravent l'adaptation commerciale de la production de PU et du traitement du lactosérum.

## 2.1 Problème 1

La biomasse est habituellement produite à l'aide du perméat de lactosérum, ce qui nécessite un traitement préalable pour séparer les protéines du lactosérum avant la fermentation. Les étapes du prétraitement, telle l'ultrafiltration, sont dispendieuses, surtout pour parvenir à séparer les protéines du lactosérum et obtenir un grand volume de perméat de lactosérum liquide. En outre, la séparation des protéines de lactosérum avant la fermentation n'est généralement pas dans l'intérêt des petites et moyennes industries fromagères en raison du faible volume de lactosérum pour en séparer les protéines, suivi par le traitement et la biotransformation du perméat de lactosérum.

## 2.2 Problème 2

Des cultures pures de la levure (*Kluyveromyces marxianus*) utilisant le lactose sont principalement utilisées pour la production de biomasse (PU), qui est utilisée comme aliments pour animaux. Cependant, le principal problème lors de la culture de la levure à haute concentration de lactose est la création de métabolites secondaires, qui conduit à une augmentation de la DCO résiduelle et à la réduction du rendement en levure. De plus, les cultures pures de *K. marxianus* ne contiennent pas certains acides aminés essentiels, ce qui réduit la valeur des PU comme nourriture animale ou pour leur application en alimentaition humaines.

## 2.3 Problème 3

Le sort et les méthodes de récupération des résidus solubles des protéines de lactosérum après fermentation sont inconnus.

## 2.4 Problème 4

La monoculture est moins efficace dans la dégradation de la DCO, contrairement aux cultures mixtes. Cependant, ceci nécessite des souches de levures acido-résistantes et thermo-tolérantes pour effectuer la fermentation en conditions non-aseptiques.

## 2.5 Problème 5

La production de biomasse de la levure plus accommodante *Saccharomyces cerevisiae* est effectuée après l'hydrolyse du lactose grâce à l'utilisation de β-galactosidase (libre ou par système enzymatique immobilisé). Toutefois, l'utilisation d'enzymes purs ou en systèmes immobilisés rend le processus coûteux. En outre, le prétraitement de la biomasse pour réduire son contenu en acides nucléiques pour une PU de qualité alimentaire nécessite également une méthode efficace.

## 2.6 Problème 6

Les protéines de lactosérum ont diverses propriétés fonctionnelles avec haute valeur nutritionnelle. Cependant, le pourcentage élevé de β-lactoglobuline rend les protéines du lactosérum allergènes chez les enfants en bas âge. Les protéines de lactosérum ont donc été hydrolysées par traitement enzymatique ou par un procédé de fermentation. Le traitement enzymatique est coûteux et le processus de fermentation nécessite diverses avancées technologiques, telles que la récupération de protéines de lactosérum à l'état natif après la fermentation et l'évaluation des activités biologiques in-vitro.

# 3. HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ

## 3.1 HYPOTHÈSES

Les souches de *K. marxianum* ont la capacité de métaboliser le lactose pour leur croissance, et sont utilisées comme source de PU pour la création de nourriture. L'hypothèse générale de cette étude est qu'il pourrait être possible de produire de la biomasse de levure en utilisant le lactosérum comme substrat, en éliminant simultanément la DCO. La production de métabolites peut être minimisée en jouant sur la concentration de lactose ou en employant des cultures mixtes. Les cultures mixtes de levures pour de la PU de grade alimentaire peuvent être produites par hydrolyse du lactose avec des cellules de levure perméabilisées. De plus, les protéines de lactosérum résiduelles peuvent être récupérées à l'état primaire par une filtration sur membrane.

### 3.1.1 Hypothèse 1

La fermentation du perméat de lactosérum avec la levure acido-résistante et thermo-tolérante *K. marxianus* est bien connue pour la production de biomasse avec réduction de la DCO simultanée. Ainsi, il pourrait être possible de produire la biomasse en utilisant le lactosérum sans retirer les protéines du lactosérum dans des conditions extrêmes (pH faible et haute température) afin de minimiser les risques de contamination. La caractérisation des métabolites intermédiaires et la détermination du sort des protéines de lactosérum pendant la fermentation fournira une information pour jouer sur l'efficacité de la dégradation de la DCO et le rendement en biomasse durant la fermentation.

### 3.1.2 Hypothèse 2

Le lactose contenu dans les milieux sera consommé durant la fermentation, laissant la plupart des protéines solubles dans le bouillon fermenté. La présence de lactose a un impact négatif sur la précipitation des protéines de lactosérum, donc une fois que le lactose est consommé (lors de la fermentation), il pourrait être possible de précipiter et récupérer les protéines solubles résiduelles avec facilité par l'optimisation de la méthode de précipitation. Les protéines

précipitées peuvent être mélangées avec la biomasse pour améliorer leur contenu en protéines et équilibrer les profils d'acides aminés essentiels des PU ainsi produites.

### 3.1.3 Hypothèse 3

Les cultures mixtes pourraient être produites en employant des levures consommatrices de lactose (*K. marxianus*) et des levures non consommatrices de lactose pour améliorer l'efficacité d'enlèvement de la DCO, la qualité des produits et opérer le procédè de fermentation sous conditions non-aseptiques employant des levures acido-résistantes et thermo-tolérantes.

### 3.1.4 Hypothèse 4

La production de cultures mixtes (surtout *Saccharomyces cerevisiae* avec *K. marxianus*), pour équilibrer les profils d'acides aminés essentiels, nécessite du lactose hydrolysé. L'hydrolyse est effectuée à l'aide d'enzymes β-galactosidase pures ou immobilisés, ce qui rend le processus coûteux. Cependant, il est possible de perméabiliser les cellules de *K. marxianus* (qui possèdent une activité β-galactosidase) avec des agents perméabilisants non-toxiques et ensuite d'utiliser ces cellules perméabilisées comme source d'enzymes pour hydrolyser le lactosérum lactose.

### 3.1.5 Hypothèse 5

Le prétraitement est effectué par traitement chimique ou enzymatique pour réduire le contenu en acides nucléiques des PU utilisées comme denrées alimentaires. Néanmoins, il est possible d'utiliser des détergents pour améliorer l'efficacité du traitement chimique et la libération des nucléotides, séparément. La séparation par membrane est la technologie privilégiée pour récupérer les protéines et peptides indigènes, lorsque le poids moléculaire des protéines et des peptides sont connus. Ainsi, il est possible d'utiliser la technologie des membranes pour séparer les protéines de lactosérum fermentées à l'état primaire après évaluation leur poids moléculaire.

## 3.2 OBJECTIFS

L'objectif global de ce project est de produire des PU de qualité alimentaire et de caractériser et récupérer les protéines de lactosérum résiduelles et solubles suite à la fermentation du lactosérum. Les objectifs spécifiques de recherche sont décrits ici.

### 3.2.1 Objectif 1

La production de PU, la caractérisation des métabolites intermédiaires de *K. marxianus* et la détermination du sort des protéines de lactosérum à partir du lactosérum.

### 3.2.2 Objectif 2

Le développement d'une méthode de récupération des protéines de lactosérum résiduelles solubles par précipitation suite à récuperation de la biomasse produite.

### 3.2.3 Objectif 3

L'évaluation des cultures mixtes de *K. marxianus* et *Candida krusei* pour la production de PU et le retrait de la charge organique du lactosérum sous conditions non-aseptiques.

### 3.2.4 Objectif 4

La perméabilisation de *K. marxianus* pour accés à la β-galactosidase et l'augmentation des cultures mixtes pour la production de biomasse.

*Objectif 4.1*

La perméabilisation de *K. marxianus* pour accés à la de la β-galactosidase et l'hydrolyse du lactose avec les cellules perméabilisées de *K. marxianus*.

*Objectif 4.2*

L'augmentation des cultures mixtes de *Saccharomyces cerevisiae* et *K. marxianus* sur le lactosérum hydrolysée.

### 3.2.5 Objectif 5

Le prétraitement de la biomasse de levure pour réduire le contenu en acides nucléiques et l'optimisation de l'ultrafiltration pour la récupération des protéines du lactosérum résiduelles solubles fermentées.

## 3.3 ORIGINALITÉ

La production de PU directement du lactosérum est une approche nouvelle pour éviter la séparation des protéines du lactosérum avant la fermentation. La production de biomasse à partir du lactosérum, en plus de la détermination du sort des protéines de lactosérum, leur caractérisation et leur récupération ainsi que la caractérisation des métabolites intermédiaires n'ont pas été effectués auparavant. En outre, la production de cultures mixtes sous conditions non-aseptiques n'a également jamais été explorée. De plus, la production de biomasse de qualité alimentaire provenant de cultures mixtes de levures utilisant des cellules perméabilisées de *K. marxianus* pour augmenter la population de *S. cerevisiae* est une approche novatrice. La récupération des protéines de lactosérum résiduelles fermentées après fermentation à l'état primaire n'a pas été rapportée à ce jour. Ainsi, l'étude proposée prévoit fournir une technologie viable pour la transformation du lactosérum en produits protéiniques.

# 4. RÉSULTATS ET DISCUSSION

Les résultats obtenus dans cette thèse sont présentés en 3 parties. La première partie présente la transformation du lactosérum, la production de protéines unicellulaires (PU) et la valorisation des résidus de protéines solubles après la fermentation (deux articles publiés, un est soumis). La deuxième partie démontre le potentiel biotechnologique de *Candida krusei* et son rôle en tant que membre de culture mixte pour la production de PU (deux articles publiés). La troisième partie est consacrée à la présentation de la perméabilisation des cellules de levure pour l'activité enzymatique, l'hydrolyse du lactose du lactosérum avec des cellules perméabilisées, l'augmentation de la culture mixte pour la production de PU de qualité alimentaire et la récupération de la protéine soluble résiduelle par ultrafiltration (un article publié, un est soumis).

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## 4.1 TRANSFORMATION DU LACTOSÉRUM, PRODUCTION DE PROTÉINES UNICELLULAIRES ET RÉCUPÉRATION DES PROTÉINES RÉSIDUELLES POST-FERMENTATION

### 4.1.1 Lactosérum: une ressource potentiellement transformable en bio-protéines, protéines fonctionnelles/nutritionnelles et peptides bioactifs (Chapitre II, partie 1)

Dans ce chapitre, les différents procédés de transformation du lactosérum en produits à valeur ajoutée sont présentés. En effet, l'objectif est de mettre en évidence les facteurs qui peuvent affecter la biotransformation du lactosérum en bioprotéine (PU). De plus, les procédés de séparation et de transformation des protéines du lactosérum en diverses protéines fonctionnelles et nutritionnelles ont été également évalués. Le lactosérum peut être transformé en produits à valeur ajoutée tels que la poudre de lactosérum, les protéines de lactosérum, le bioéthanol, les biopolymères, le méthane, les bioprotéines (PU) et les probiotiques. D'une part, le lactosérum et le perméat de lactosérum sont biotransformés en aliments riches en protéines et en PU de qualité alimentaire par une simple fermentation. D'autre part, le lactosérum est directement transformé en du concentré de protéines de lactosérum, en isolat de protéines de lactosérum et en des protéines de lactosérum individuelles. De plus, les protéines de lactosérum peuvent être transformées en peptides bioactifs par des procédés enzymatiques ou par fermentation.

### 4.1.2 La production de protéines unicellulaires et l'élimination simultanée de la DCO avec caractérisation des protéines résiduelles et des métabolites intermédiaires au cours de la fermentation du lactosérum par *K. marxianus* (Chapitre II, partie 2)

La fermentation du lactosérum utilisant *Kluyveromyces marxianus* a été effectuée à 40 °C et à un pH de 3,5. Elle a permis d'évaluer la production des PU et l'élimination simultanée de la DCO, de déterminer le sort des protéines du lactosérum et de caractériser les métabolites intermédiaires. De plus, le rendement en biomasse ($Y_{x/s}$) de 0,12 g biomasse/g de lactose et une réduction de la DCO (y compris des protéines) de 55% a été observée au cours de la fermentation en mode discontinu avec un inoculum de densité cellulaire normale. Le faible rendement de la biomasse pourrait être dû à des conditions extrêmes de fermentation, c'est-à-dire un pH faible et une haute température, qui ont été utilisés pour minimiser les risques de contamination. Par contre, la faible dégradation de la DCO est plutôt due à la concentration initialement élevée de lactose. Aussi, la concentration de protéines solubles du lactosérum a diminué de 5,6 à 4,1 g/L, ce qui indique la très faible quantité de protéines du lactosérum utilisée au cours de la fermentation. En outre, les résultats d'électrophorèse ont démontré que les protéines du lactosérum fermenté sont différentes des protéines du lactosérum initial. Ces résultats ont été confirmés par des analyses HPLC et GC-MS qui ont révélé une modification de la composition des composés organiques au cours de la fermentation. Certains des composés volatiles du lactosérum naturel ont été consommés alors que d'autres ont été générés lors de la fermentation. La fermentation discontinue avec un inoculum à concentration élevée a entraîné une augmentation du rendement en biomasse jusqu'à 0,19 g biomasse/g de lactose et une réduction de la DCO (incluant les protéines) de 80%, avec une concentration en protéines résiduelle de 4,5 g/L. Ainsi, une réduction importante de la DCO a été remarquée en raison d'un rapport de nourriture sur microorganismes diminuant la production des métabolites intermédiaires. Une troisième fermentation en mode discontinu a été réalisée avec un inoculum de densité cellulaire moyenne et a été remplacée par une fermentation continue avec un système de recyclage des cellules pour maintenir la concentration de la biomasse élevée. Dans ce cas, un rendement en biomasse de 0,19 g de biomasse/g de lactose et une productivité de 0,26 g/L/h ont été obtenus. Le taux d'élimination de la DCO a été de 78-79% avec une concentration en protéines résiduelles de 3.8 à 4.2 g/L. La forte DCO résiduelle rapportée en comparaison de celle obtenue dans des exemples de la littérature est due à la présence de protéines résiduelles solubles. Auparavant, la plupart des études ont été menées avec du perméat de lactosérum où la DCO résiduelle due aux protéines est négligeable.

### 4.1.3 Récupération des protéines solubles résiduelle par un procédé de précipitation en deux étapes avec réduction concomitante de la DCO du lactosérum de levure cultivées (Chapitre II, partie 3)

Le procédé de précipitation pour la récupération des protéines résiduelles solubles de lactosérum fermentés, suite à la culture de la levure (*K. marxianus*) par fermentation en mode continu, a été évalué. La biomasse a également été récupéré par centrifugation, et les protéines résiduelles solubles dans le surnageant du lactosérum fermenté a été précipitées par un traitement thermique. Une récupération des protéines solubles jusqu'à 53% et une élimination de la DCO jusqu'à 54% ont été obtenues dans les conditions optimales de température (100 °C) et de pH (4,5) pendant 10 minutes. Toutefois, une sédimentation des précipités due à la gravité a été obtenue à un pH de 3,5 avec 47% de récupération des protéines. De plus, l'étude a été effectuée à un pH de 3,5 avec agitation, ce qui a conduit à 68% de la récupération des protéines et à une DCO résiduelle de 62%. En effet, l'augmentation de la vitesse d'agitation (vitesse de cisaillement) induit l'agrégation des protéines précipitées par la chaleur. De plus, la précipitation et la coagulation des protéines solubles ont été aussi évaluées en présence de carboxyméthylcellulose (CMC). Par la suite, deux procédés de précipitations (CMC thermique suivi par précipitation) ont été combinés, ce qui a entraîné 81% de récupération des protéines totales. En outre, ce procédés n'a pas besoin de la centrifugation pour récupérer les protéines. Par conséquent, le précipité qui sédimente par gravité peut être mélangé avec de la biomasse centrifugée et séchée par pulvérisation pour obtenir un produit enrichi en protéines.

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## 4.2 POTENTIEL BIOTECHNOLOGIQUE DE LA LEVURE CANDIDA KRUSEI ET SON RÔLE EN TANT QUE MEMBRE DE CULTURE MIXTE POUR LA PRODUCTION DE PROTÉINES UNICELLULAIRES

### 4.2.1 *Candida krusei*: potentiel biotechnologiques et préoccupations quant à sa sécurité (Chapitre III, partie 1)

Une revue complète sur *Candida krusei* (le potentiel biotechnologique et les préoccupations sur sa sécurité) est présentée. L'espèce *Candida krusei* (un pathogène opportuniste) a été observée dans différents habitats. Elle attire de plus en plus l'attention avec son rôle biotechnologique diversifié. En effet, on la trouve dans de nombreux produits alimentaires fermentés ainsi que dans des produits laitiers. Elle a également été exploitée pour la production de divers enzymes

et produits biochimiques. En outre, elle est acido-résistante et thermo-tolérante. Elle pourrait être exploitée pour la production de PU à partir du lactosérum en culture mixte.

### 4.2.2 Culture mixte de *Kluyveromyces marxianus* et *Candida krusei* pour la production de protéines unicellulaires et l'élimination de la charge organique à partir du lactosérum (Chapitre III, partie 2)

L'étude a été menée pour évaluer le potentiel de la culture mixte de *K. marxianus* et *Candida krusei* par rapport à la culture pure de *K. marxianus* pour: améliorer le taux de réduction de la DCO, minimiser les risques de contamination en utilisant des conditions de fermentation extrêmes (c.-à-d. haute température et faible pH) et obtenir une meilleure qualité de PU au cours de la fermentation en continu et en discontinu. La fermentation aérobie en mode discontinu utilisant sont la culture pure (*K. marxianus*) sont la culture mixte (*K. marxianus et de C. krusei)* a été effectuée à 40 °C et à pH de 3,5. Également, la fermentation en mode continu a été réalisée à différents temps de rétention hydraulique (TRH) (6, 12, 18 et 24 h) pour éliminer au maximum la DCO et pour une production importante de biomasse. La fermentation en discontinu par la culture mixte a obtenu plus de 8,8% d'élimination de DCO avec 19% de rendement de la biomasse et 33% d'augmentation de productivité par rapport à la fermentation par la culture pure. Une forte dégradation de la DCO utilisant la culture mixte a été obtenue puisque la levure lactose-négative (*C. krusei*) consomme des métabolites intermédiaires au cours de la fermentation. Ce dernier phénomène a également conduit à une meilleure productivité et un meilleur rendement en biomasse. Le retrait maximal de DCO de 80,2% (y compris les protéines résiduelles) a été obtenu avec un TRH de 24 h, pour une productivité en biomasse de 0,17 g/L.h. Néanmoins, la productivité maximale en biomasse de 0,38 g/L/h, avec 34% de DCO éliminée, a été obtenue avec un TRH de 6 h. La croissance de *C. krusei* dans la culture mixte dans les deux cas de fermentation continue ou discontinue a montré que les deux types de levures ont des interactions physiologiques commensalistes. Ainsi, elle a montré que les PU de la culture mixte ont une forte teneur en protéines et sont enrichies en lysine, un acide aminé essentiel.

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## 4.3 PERMÉABILISATION DES CELLULES DE LEVURE, HYDROLYSE DU LACTOSE, AUGMENTATION DE LA CULTURE MIXTE POUR LA PRODUCTION DE PU ET RÉCUPÉRATION DES PROTÉINES RÉSIDUELLE APRÉS FERMENTATION

### 4.3.1 La perméabilisation de *Kluyveromyces marxianus* avec un détergent doux pour l'hydrolyse du lactose lactosérum et l'augmentation de la culture mixte (Chapitre IV, partie 1)

Les cellules *de K. marxianus* ont été perméabilisées avec le détergent anionique N-lauroyl sarcosine (N-LS), qui est non-toxique et biodégradable, pour améliorer accéder à enzymatique intracellulaire et l'hydrolyse du lactose. Dans ce contexte, des paramètres du procédé de perméabilisation comme la concentration de N-LS, le volume de solvant, la température et le temps d'incubation ont été optimisés. Ainsi, une activité maximale de la β-galactosidase de 1220 UI/g poids sec a été obtenue avec des cellules perméabilisées et les conditions optimisées suivantes : une concentration de N-LS de 1,5% (p/v), un volume d'eau de 1 ml (ou 1,0 g de poids humide/mL d'une solution de N-LS), la température de 25 °C et une dureé de 20 minutes d'incubation. De plus, la viabilité des cellules perméabilisées a également été évaluée. Une réduction de la viabilité des cellules de deux unites logarithmique après perméabilisation a été observée. Par la suite, une hydrolyse du lactose de lactosérum a été réalisée avec des cellules perméabilisées. Un maximum d'hydrolyse de 91% est atteint avec 600 mg (poids sec de cellules/100 mL) dans la poudre de lactosérum (5% p/v) pendant 180 minutes d'incubation à un pH de 6,5 et une température de 30 °C. En outre, le lactosérum hydrolysé a été évalué pour son améliorer la croissance de la levure *Saccharomyces cerevisiae* qui ne consomme pas le lactose. Il a été démontré que *S. cerevisiae* est également capable de croître dans le lactosérum hydrolysé en culture mixte avec *K. marxianus*.

### 4.3.2 Production de qualité alimentaire protéines unicellulaires, la caractérisation et la récupération des protéines résiduelles fermentés par ultrafiltration du lactosérum (Chapitre IV, partie 2).

L'étude a été effectuée afin de caractériser et récupérer les protéines solubles résiduelle après la production de la culture pure (*K. marxianus*) ou de la culture mixte (*K. marxianus et S. cerevisiae*), utilisant la biomasse de la levure en tant que PU de qualité alimentaire présent dans le lactosérum. La fermentation en mode discontinu a été effectuée à 35 °C et à un pH de 5,5

pour la culture pure et à 30 °C et un pH de 6,5 pour la culture mixte. Dans ces conditions, les rendements en biomasse obtenus étaient de 0,27 et 0,31 g de biomasse/g de lactose consommé pour la culture pure et de la culture mixte, respectivement. La biomasse et le lactosérum ont été obtenus dans le surnageant suite à une centrifugation du bouillon fermenté. Par la suite, la biomasse de levure a été traitée en deux étapes avec la N-LS et le $NH_4OH$, respectivement. Le traitement a entraîné une réduction de la teneur en acides nucléiques de moins de 2% (p/p) à partir de 11,4% (p/p). De plus, une caractérisation des protéines solubles a été évaluée par SDS-PAGE. Une fraction des protéines a été consommée au cours de la fermentation. Les résultats obtenus à partir de SDS-PAGE ont révélé que les protéines du lactosérum fermenté sont différentes des protéines de lactosérum original, et qu'elles sont partiellement hydrolysés durant la fermentation. Les paramètres de fonctionnement de l'ultrafiltration (flux de perméat et pression transmembranaire, PTM) ont été optimisés pour des membranes de 1 kDa et de 10 kDa afin de récupérer les protéines résiduelles du lactosérum fermenté. Un flux de perméat de 413 $L/h/m^2$ (LMH) avec une PTM de 80 kPa a montré un meilleur taux de récupération avec la membrane de 10 kDa suivis par un flux de perméat de 2760 $L/h/m^2$ et une pression transmembranaire de 210 kPa pour la membrane de 1 kDa. Avec ces conditions optimisées, une récupération de 84% et de 92% des protéines solubles résiduelles totales a été obtenue avec les surnageants de la culture pure et de la culture mixte du lactosérum fermenté, respectivement.

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# 5. CONCLUSIONS ET RECOMMANDATIONS

Sur la base de la présente étude, trois processus différents ont été développés, ce qui pourrait potentiellement être appliquées à biotransformer le lactosérum cru en produits protéiques. Le premier processus est la fermentation en continu (à haute température et un pH bas) avec recyclage des cellules, suivie de la récupération des protéines soluble résiduelle par précipitation. Le processus intégré (p. ex., production de PU, suivie de la récupération de protéines résiduelles) a donné lieu à une productivité plus élevée et plus de DCO d'élimination. Le produit (alimentation animale) a une teneur en protéines élevée et équilibrée en acides aminés essentiels. Le second processus est la fermentation en mode continu dans des conditions non aseptiques employant la culture mixte (*K. marxianus* et *C. krusei*), qui a abouti à plus efficacité d'élimination de DCO avec un rendement amélioré de la biomasse. Le PU produit (de l'alimentation animale) a une teneur en lysine plus élevé, qui est fortement preferée pour l'alimentation animale. Le troisième procédé est de produire une qualité alimentaire des PU de culture mixte (*K. marxianus* et *S. cerevisiae*) utilisant les cellules de *K. marxianus* perméabilisées comme source d'enzyme pour hydrolyser le lactose du lactosérum, suivie de la récupération de la protéine résiduelle par ultrafiltration. PU de qualité alimentaire a été obtenues par pré-traitement de la biomasse avec une combinaison de produits chimiques en deux étapes. Les protéines solubles résiduelles à l'état natif ont été récupérées par ultrafiltration.

Une analyse économique comparative est de mise pour les trois cas de processus de production de PU et de leur récupération afin de trouver le meilleur procédé conditionnel pour la valorisation du lactosérum.

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# CHAPITRE II

# TRANSFORMATION DU LACTOSÉRUM, PRODUCTION DE PROTÉINES UNICELLULAIRES ET LA RÉCUPÉRATION DES PROTÉINES RÉSIDUELLES POST-FERMENTATION

# PARTIE 1

# CHEESE WHEY: A POTENTIAL RESOURCE TO TRANSFORM INTO BIOPROTEIN, FUNCTIONAL/NUTRITIONAL PROTEINS AND BIOACTIVE PEPTIDES

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**Submitted to Biotechnology Advances**

# RÉSUMÉ

Les sous-produits (de lactosérum) de l'industrie de la production du fromage sont considérés comme des matières polluantes en raison de leur forte DBO et des concentrations résultantes en DCO. La forte charge organique du lactosérum provient en fait de la présence d'éléments nutritifs résiduels dans le lait. Comme la demande pour les produits dérivés du lait augmente, elle conduit à la production de grandes quantités de lactosérum, ce qui occasionne un grave problème de gestion. Dans le but de surmonter les problèmes de gestion liés à cette production de lactosérum, différentes approches technologiques ont été utilisées pour convertir le lactosérum en produits à valeur ajoutée. Concrètement, l'avancement technologique a permis de favoriser l'utilisation du lactosérum et de la moitié de la totalité produite en lactosérum en les transformant en produits à valeur ajoutée, tels que la poudre de lactosérum, les protéines du petit-lait, le perméat de lactosérum, le bioéthanol, les biopolymères, le méthane, les bioprotéines et les probiotiques. Parmi les différents produits à valeur ajoutée, la transformation du lactosérum en produits protéiques est avantageuse et exigeante. Le principal facteur qui importe dans la transformation du lactosérum en produit protéique, en ce qu'il rend cette transformation avantageuse, est sa nature généralement reconnue comme inoffensive (GRAS- *generally recognized as safe*). Le lactosérum et le perméat de lactosérum sont biotransformés, grâce à un procédé de fermentation, en aliments protéiques et en bioprotéines/protéines d'origine unicellulaire de qualité alimentaire. D'autre part, le lactosérum est traité directement pour obtenir des concentrés de protéines du lactosérum, l'isolat de protéines du lactosérum et des protéines individuelles du lactosérum. En outre, les protéines du lactosérum sont également transformées en peptides bioactifs via des procédés enzymatiques ou de fermentation. Les produits protéiques ont aussi des applications fonctionnelles, nutritionnelles et thérapeutiques. Ainsi, la disponibilité, les caractéristiques et les procédés de transformation/biotransformation du lactosérum en divers produits protéiques à valeur ajoutée sont présentés et discutés dans cet article.

**Mots-clés:** Lactosérum; Fermentation; Transformation; Biotransformation; Bioprotéine; Protéine fonctionnelle/nutritionnelle; Les peptides bioactifs.

## ABSTRACT

The byproduct of cheese-producing industries, cheese whey, is considered as an environmental pollutant due to its high BOD and COD concentrations. The high organic load of whey arises from the presence of residual milk nutrients. As demand for milk-derived products is increasing, it leads to increased production of whey, which poses a serious management problem. To overcome this problem, various technological approaches have been employed to convert whey into value-added products. These technological advancements have enhanced whey utilization and about 50% of the total produced whey is now transformed into value-added products such as whey powder, whey protein, whey permeate, bioethanol, biopolymers, methane, bioprotein (single cell protein) and probiotics. Among various value-added products, the transformation of whey into proteinaceous products is attractive and demanding. The main important factor which is attractive for transformation of whey into proteinaceous products is the generally recognized as safe (GRAS) regulatory status of whey. Whey and whey permeate are biotransformed into proteinaceous feed and food-grade bioprotein/single cell protein through fermentation. On the other hand, whey can be directly processed to obtain whey protein concentrate, whey protein isolate, and individual whey proteins. Further, whey proteins are also transformed into bioactive peptides via enzymatic or fermentation processes. The proteinaceous products have applications as functional, nutritional and therapeutic commodities. Whey characteristics, and its transformation processes for proteinaceous products such as bioproteins, functional/nutritional protein and bioactive peptides are covered in this review.



**Keywords:** Cheese whey; Fermentation; Transformation; Biotransformation; Bioprotein; Functional/Nutritional protein; Bioactive peptides.

# 1. INTRODUCTION

The food processing industries, such as dairy and cheese processing plants, generate large volumes of liquid waste including “cheese whey”. Whey is liquid portion produced during cheese-making or during coagulation of the milk casein process as a byproduct. The current total worldwide production of whey is estimated at about 180 to 190 million tons/year (Mollea et al., 2013). The major share of whey production comes from the European Union and the USA (approximately 70% of the total world whey). Moreover, the whey production rate is increasing with similar rates of milk production. The global growth rate of whey generation is around 2% per annum (Smithers, 2008). A portion of cheese whey is used for animal feed and in food applications. However, whey creates significant environmental and health issues due to its large volume production and high organic content. The biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values of whey are about 30-50 g/L and 60-80 g/L, respectively (Guimarães et al., 2010). Disposal of whey into municipal sewers has been banned by many local authorities as it interrupts the biological process of wastewater treatment plants. Whey dumping on land creates severe pollution concerns for the surrounding environment by affecting the physicochemical characteristics of soil that results in decreased crop yields. Furthermore, when poured into water bodies, it reduces the dissolved oxygen, hampers biodegradability, and poses a major risk to aquatic life, and to environment and human health (Ghaly et al., 2007). Biodegradability of dairy effluents was studied by Janczukowicz et al. (2008) and they concluded that the effluents generated from all dairy factories could be treated together without any problem, except for whey, because whey contains complex components. Whey components are difficult to degrade and create a major problem to any wastewater treatment plant that treats other effluents. Therefore, proper management (treatment or re-use) of cheese whey, which would be economical and ecofriendly, is required before its disposal.

Responsible factors for the high organic load of whey is the presence of residual milk nutrients (lactose, proteins, lipids and vitamins). On the other hand, the presence of whey nutrients is considered as a potential resource for the production of various value-added products. Various advanced technologies are in use to tackle this whey management issue, and major fraction of whey is utilized and transformed today into valuable products. However, still a significant amount of whey remains unutilized. So, whey must be further processed to attain maximal benefits and to limit its environmental pollution impact (Panesar et al., 2007).

Processing of whey is carried out by 2 procedures to transform whey into value-added products. The first one is direct processing (physical or thermal treatment) of whey to obtain whey powder, whey protein concentrate, whey protein isolate, whey permeate, lactose, and other fractions. The second one involves biotechnological processing, where whey is used as substrate for various microbial/enzymatic processes to obtain valuable end products. Such products are animal feed, bioprotein (single cell protein-SCP), probiotics, organic acids, enzymes, carotenoids, bio-preservatives, biological gums, exopolysaccharides, and bioplastics (Kosseva et al., 2009; Mollea et al., 2013; Panesar et al., 2013; Siso, 1996). These products and methods used have been demonstrated to be technically achievable. However, research is continuing to make value-added products even more economically viable.

Demand for whey is increasing to manufacture whey proteins due to their high functional and nutritional values. Whey proteins have also several applications in the pharmaceutical industry. Specific protein fractions are used to control blood pressure and to induce sleep (Korhonen, 2009; Modler, 2009). Peters (2005) has evaluated the techno-economic feasibility of whey processing alternatives and concluded that the transformation of whey into whey protein concentrate generates a large stream of whey permeate (lactose fraction), which needs further processing. Therefore, a whey protein concentrate as the only product is not very profitable. Additionally, the value of whey protein isolate can be further enhanced by enzymatic hydrolysis of the proteins to non-allergenic peptide fractions. Further, whey proteins have the potential to be transformed into bioactive peptides via enzymatic or fermentation processes. So, an important step in processing of cheese whey is protein fraction recovery and whey permeate (lactose fraction) conversion to different valuable products. Biotransformation of whey permeate into protein-rich biomass (SCP) and into galacto-oligosaccharides (GOS) are most feasible options, among others at the present time. Bioprotein/SCP production from whey is carried out by employing specific lactose-consuming microorganisms, especially yeasts (*Kluyveromyces* spp. and *Candida* spp.) and bacteria such as *Lactobacillus* spp. (Panesar et al., 2013). Moreover, non-lactose consuming microorganisms are used for production of SCP through hydrolysis of lactose into monosaccharides (glucose and galactose). The bioprotein could be used as animal feed or as a protein source for humans and GOS could be used as a replacement for antibiotics in animal feeding. This makes cheese whey promising to influence the whey market (Gänzle et al., 2008). The main emphasis of this paper is on cheese whey utilization for the production of the bioprotein, on factors affecting the production, quality, acceptability of bioprotein, and on processing and production of functional/nutritional and bioactive peptides.

# 2. WHEY CHARACTERISTICS AND TRANSFORMATION INTO VALUE-ADDED PRODUCTS

## 2.1 Physical characteristics

Whey is residue that remains after recovery of the curd from the clotting of milk with proteolytic enzymes or acid. Colour of whey varies from a dominantly yellow/green colour to rarely a bluish tinge, but colour can vary with type of milk used. Whey is produced from all types of milk, but cow's milk is most common in the western countries. In some regions of the world, goat's, sheep's and camel's milk are also used in the production of cheese and cheese whey (Bordenave-Juchereau et al., 2005; Smithers, 2008). Whey shares about 85-95% of milk volume and contains around 55% of the milk nutrients (Varnam and Sutherland, 1994). Fig. 1 shows a comparative analysis of milk and of whey.

## 2.2 Chemical characteristics

The type and composition of whey depend mainly upon the processing techniques used in casein removal from milk. The most frequently encountered type of cheese whey results from the coagulation of casein by rennet (an industrial casein-clotting enzymatic complex containing chymosin or other casein-coagulating enzymes). Coagulation of casein by rennet occurs at around pH 6.5 thus, whey produced during the enzymatic treatment is called sweet whey. Acid whey (pH less than 5) is generated when coagulation of casein is achieved through the addition of mineral or organic acids. The main difference between the two types of whey are mineral content, acidity and content of the whey protein fractions (Table 1) (Jelen et al., 2003; Kosseva et al., 2009).

## 2.3 Whey utilization for production of value-added products

The presence of residual milk nutrients has drawn an interest to transform whey into value-added products. Around 50% of the total produced whey worldwide is transformed into different food stuffs. These food items include about 45% in liquid form, 30% as dried whey powder, 15% as lactose and various byproducts from its elimination and remaining as whey protein concentrates (Kosseva et al., 2009; Panesar et al., 2013). Since the major fraction of whey solids is lactose along with soluble proteins, vitamins and minerals, various biotechnological

processes have been applied to utilize whey as substrate to produce industrially important and valuable products such as enzymes, protein rich biomass and ethanol. Alternatively, physical/thermal processes are used for direct conversion of whey into important products (Morales et al., 2006; Panesar et al., 2013; Siso, 1996). The outline of various biotechnological and other processes is illustrated in Fig. 2. Both routes of processing have played an important role in the transformation of whey into value-added products. Whey is directly used for fermentation using lactose-consuming microorganism for its bioconversion. The broad range of value-added products, as presented in Table 2, is obtained from whey or whey permeate via fermentation (Carlotti et al., 1991; De León-Rodríguez et al., 2006; Guimarães et al., 2010; Panesar et al., 2013; Pham et al., 2000; Wan et al., 2008). However, in some cases, lactose hydrolysis is performed before fermentation, when microorganisms are unable to hydrolyze lactose, to enhance whey usefulness. Hydrolysis of lactose into monosaccharides (glucose and galactose) is performed either with the enzyme β-galactosidase or by acid hydrolysis (Siso, 1996; Zadow, 1992). For example, ethanol is produced using whey as substrate by lactose-utilizing yeast *Kluyveromyces marxianus* strains. Produced ethanol can be used as an energy source (as fuel) or further used as substrate for vinegar or acetic acid production (Guimarães et al., 2010). Different organic acids like acetic acid, propionic acid, lactic acid and citric acid can be produced from whey/whey permeate by fermentation. Most of the organic acids are used as speciality chemicals. For example, lactic acid is used to make propylene oxide, the polylactic acid polymers and propylene glycol or acrylic fibers. Acetic acid is used to produce calcium magnesium acetate (CMA) which is used in large amounts as road or airport de-icer. Acetic acid is also used as a raw material (as substrate) for bioplastic (polyhydroxyalkanoates) production. Similarly, propionic acid is used as preservative, herbicides and chemical intermediates (Dias et al., 2006; El Aasar, 2006; Morales et al., 2006; Mostafa, 2001; Mukhopadhyay et al., 2005; Panesar et al., 2007; Wan et al., 2008). Hydrolyzed lactose from whey permeate is used as a substrate for the production of polysaccharides by fermentation such as xanthan gum (a heteropolysaccharide) produced by *Xanthomonas campestris* (Mesomo et al., 2009). Whey lactose is also used for the production of other exopolysaccharides such as dextrans (*Leuconostoc mesenteroides*), and biopolymers such as poly-β-hydroxy butyrate (PHB) (*Azotobacter chrooccum*) (Khanafari et al., 2006; Pham et al., 2000; Santos et al., 2005). Anaerobic digestion of whey results in the production of methane or biogas and is a process comprised of three successive steps, lactose (and protein) hydrolysis, fermentation (acidogenesis, acetogenesis) and methanogenesis. The produced methane can be used as a source of energy (as fuel) or to generate electricity (Gelegenis et al., 2007). The list of products

in Table 2 shows biotechnological role in the transformation of whey into different product categories (probiotics, enzymes, proteins and peptides, biopolymers, biochemicals, biocontrol agents and fuel).

# 3. BIOTRANSFORMATION OF WHEY/WHEY PERMEATE INTO BIOPROTEIN

## 3.1 Bioprotein

Bioprotein or single cell protein (SCP) may be defined as microbial biomass. SCP may be produced by cultivation on various and abundant carbon sources. Bacteria, yeast, fungi and algae are the main sources of microbial biomass, which can be employed as sources of bioprotein/SCP (Paraskevopoulou et al., 2003). The trademark name Bioprotein® is given for single cell protein, based on the transformation of methane, by the addition of ammonia and oxygen, into biomass (protein source) (VKM, 2006). The important characteristics of single cell microorganisms are their substantial content of crude protein, which varies from 40 to 80% w/w of dry cell weight. In addition, protein quality is high and more closely resembles to that of animal protein compared to plant proteins. Therefore, readily available nutritionally (Anvari and Khayati, 2011; Schultz et al., 2006). Additionally, there are a number of other advantages in the production of microbial proteinaceous biomass compared to conventional source crops protein used as food and feed. These are: (i) rapid succession of generations (algae, 2-6 h; yeast, 1-3 h; bacteria, 0.5-2 h); (ii) high protein content on a dry mass basis; (iii) broad spectrum of substrate (carbon sources) utilization; (iv) production in continuous cultures of consistent quality; (v) independence of environmental conditions, low land requirements and ecologically beneficial and (vi) potential for cellular, molecular and genetic alterations (Anupama and Ravindra, 2000; Bekatorou et al., 2006; Nasseri et al., 2011; Srividya et al., 2014). Among the algae, fungi/yeast and bacteria, yeasts are preferred for the production of bioprotein due to the fact that yeast can easily be propagated on cheap carbon sources and are easily harvested due to their bigger size. Additionally, yeasts contain less nucleic acids than bacteria (Bekatorou et al., 2006). There are various GRAS microorganisms (yeast and bacteria), that are often used to produce SCP. The most commonly used yeast genera for SCP/bioprotein production are *Saccharomyces*, *Kluyveromyces*, *Candida, Pichia* and *Torulopsis*. Among the bacterial genera, *Lactobacillus*, *Cellulomonas* and *Alcaligenes* are the most common. The yeast *Saccharomyces cerevisiae* has

been commonly used due to its availability as a byproduct from the beer industry (Bekatorou et al., 2006).

Biotransformation of whey or of whey permeate into proteinaceous biomass (bioprotein) is one of the best options for whey utilization (Grba et al., 2002; Mansour et al., 1993; Mawson, 1994). The lactose-utilizing microorganisms (e.g. *Kluyveromyces* spp.) are more commonly used for biomass production from whey. However, attention has also been given to production of non-lactose consuming universally accepted baker's yeast (*S. cerevisiae*) for food and feed applications. The industrial process to produce *S. cerevisiae* using whey was implemented by the Nutrisearch Company in 1983 in Kentucky (USA). The production method was comprised of two steps: (a) lactose hydrolysis in whey with an immobilized β-galactosidase enzyme, followed by (b) monosaccharides fermentation (Siso, 1996). Numerous processes have been reported for biomass cultivation from whey, and among these the Vienna process and the Bel process are prominent. Production of microbial biomass at industrial scale from whey for food applications started in France at the Fromageries Le Bel around 1958, where a consortium of 3 yeasts (*Kluyveromyces lactis, K. fragilis* and *Torulopsis bovina*) was grown in equilibrium on whey permeate in a continuous fermentation system for a period of over 1 year, at pH 3.5 and 38 °C. The extreme culture conditions (high temperature and low pH) were applied to reduce the probability of contamination (Siso, 1996). During the fermentation oxygen supply was kept high to diminish ethanol production. However, ethanol formation by *Kluyveromyces* spp. has been reported in a fully aerated system. In order to avoid ethanol production, a mixed culture including the non-lactose consuming yeast *T. bovina* was employed. *T. bovina* utilized ethanol produced during the fermentation (Siso, 1996).

## 3.2 Factors affecting and production process of bioprotein

The factors that affect the production of yeast biomass (bioprotein/SCP) using whey as substrate are microorganisms, nutrients supplementation, culture conditions and type of fermentation process. The preferred microorganism is yeast.

### 3.2.1 Microorganisms

Selection of microorganism for SCP production depends on ability to metabolize whey lactose. The production of biomass is mostly limited to lactose-consuming microorganisms (Ghaly et al.,

2003), however, some non-lactose consuming microorganisms (e.g. *Candida utilis* and *Candida valida*), which grow on intermediate metabolites can also be used (Cristiani-Urbina et al., 2000). Different yeast species such as *Kluyveromyces* (e.g. *Kluyveromyces lactis* and *K. marxianus*), *Candida* (e.g. *Candida pseudotropicalis*) and *Trichosporon*, are employed since they are capable of metabolizing lactose (Mansour et al., 1993; Mawson, 1994). Among these, *Kluyveromyces* species have been most widely studied (Grba et al., 2002). The yeast *K. marxianus* is the dominant and most commonly used one, up to commercial scale (Bekatorou et al., 2006; Fonseca et al., 2008). The bacterial species (like *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fermentum* etc.), which are categorized as GRAS or have a safe history in food and feed applications, can also be used (AAFCO, 2010; Panesar et al., 2013).

One main problem that generally occurs during cultivation of *K. marxianus* at higher initial lactose concentration is the production of byproducts (intermediate metabolites) at the expense of the biomass yield due to the Crabtree effect. The classification of Crabtree-positive and -negative yeasts is based on their glucose-uptake mechanism. Crabtree negative microorganisms optimize their use of lactose by converting lactose into ethanol. *K. marxianus* is classified as a Crabtree-negative yeast and is a facultative fermentative microorganism (Fonseca et al., 2008; van Dijken et al., 1993). *Kluyveromyces* spp. cannot grow in severely anaerobic environments and, thus the formation of ethanol is entirely associated with oxygen supply (Bellaver et al., 2004). The ability of the Crabtree-negative model organism *K. lactis* to metabolise lactose is mainly due to the existence of a lactose permease, which is encoded by the *LAC12* gene, and of a β-galactosidase which is encoded by the *LAC4* gene (Rubio-Texeira, 2006). Glucose and galactose are the products of lactose hydrolysis due to action of the β-galactosidase. Intracellular glucose is metabolized through glycolysis whereas galactose is metabolised by the Leloir pathway (Leloir pathway enzymes encoded by the *GAL* genes and activated in the presence of galactose) (Sellick et al., 2008). *K. fragilis* (now known as *K. marxianus*) has been also reported to change its cellular metabolic pathway, from oxidative to a mixed oxidative-fermentative type that resulted in other metabolic end products such as aldehydes and esters. The formation of byproducts (intermediate metabolites) led to a reduction of the biomass yield on lactose, which affects the economics of the process and also affects the efficacy of organic matter elimination (Cristiani-Urbina et al., 2000). Additionally, biomass yield has been reported to decrease with an increase in lactose concentration and this may be due to increased production of metabolic products. The production of metabolites has also been

reported during aerated cultures of *K. lactis*, *C. pseudotropicalis, Torulopsis cremoris* and *C. kefyr* (Cristiani-Urbina et al., 2000).

The general purpose of cultivation of *Kluyveromyces* spp. (e.g. *K. marxianus*) is to produce biomass, and simultaneously, to remove the organic load of cheese whey (Ghaly and Kamal, 2004; Schultz et al., 2006). However, the formation of ethanol and of other metabolites has a detrimental effect on biomass yield and COD reduction during the cultivation of *Kluyveromyces* species. In contrast, COD degradation efficiency was higher with a mixed culture of *K. marxianus* and *S. cerevisiae* compared to the monoculture of *K. marxianus* (Domingues et al., 2010). The higher COD degradation with the mixed culture of *K. marxianus* and *S. cerevisiae* (non-lactose consuming) was due to consumption of part of the extracellular intermediate metabolic products of *Kluyveromyces* spp. (Domingues et al., 2010). Therefore, to minimize the generation of metabolic byproducts and enhance productivity, mixed yeast cultures can be used. A higher biomass yield has been reported employing mixed cultures of *Torulopsis cremoris* and *C. utilis*. The improvement in biomass yield may be due to the fact that *C. utilis* consumed the metabolic byproducts of *T. cremoris*, which resulted in increased biomass production with higher COD removal efficiency (Cristiani-Urbina et al., 2000). Yadav et al. (2014a) also reported a higher biomass yield and COD removal efficiency during mixed-culture cultivation of *K. marxianus* and *Candida krusei* (non-lactose consuming yeast). However, the main challenging issue during cultivation of mixed cultures is the stability of the microbial consortium, which must be studied before implementation of any mixed culture at industrial scale.

### 3.2.2 Nutrients

Biomass production or microbial growth requires carbon and nitrogen as a major source of nutrients. Additionally, other essential nutrients are sulfur, phosphorus and some elements in minor quantity. The major source of carbon in whey is lactose whereas organic nitrogen is present in the complex form (as protein, peptides, and amino acids) (Ghaly and Kamal, 2004). Due to the complex nature of nitrogen in whey, microorganisms require readily assimilated inorganic or simple organic forms of nitrogen for optimal growth. Inorganic nitrogen can be provided as ammonia/ammonium salts and nitrates. Most of the yeasts and fungi assimilate ammonia and ammonium salts. The simplest form of organic nitrogen source is urea, which is used either by a extracellular urease that leads to ammonia generation or transported and used through the urea amydolyase pathway (Roon and Levenberg, 1972). Supplementation of a

inorganic nitrogen source (ammonium sulphate) has been reported to improve biomass yield during SCP production from cheese whey (Cristiani-Urbina et al., 2000; Moeini et al., 2004). Inorganic nitrogen sources influence the pH of the culture medium such as assimilation of one ammonium ion produces one proton, whereas assimilation of one nitrate ion utilises one proton. However, assimilation of urea is neutral regarding proton equilibrium (Castrillo et al., 1995). Yeast extract, which is rich in free amino acids, peptides and B-vitamins has been also used (Lukondeh et al., 2005). Cheese whey is rich in minerals, however; during biotransformation into biomass, supplementation of trace elements, ammonium and calcium has been recommended with sweet whey concentrates while, supplementation of ammonium, trace elements and vitamins was required for sour whey concentrates (Schultz et al., 2006).

### 3.2.3 pH

In general, neutral pH is the optimum for the growth of microorganisms (bacteria and yeasts). In addition, yeasts are usually acid-tolerant and can grow in the pH range of 4 to 4.5 (Battcock, 1998). Keeping the pH below 4.5 during cultivation eliminates possibility of contamination by pathogenic bacteria (Ghaly and Kamal, 2004). The most advisable pH range for the prevention of contamination from pathogenic bacteria is pH 3-4 (Yadav et al., 2014a). Generally, yeasts have the capacity to maintain a constant cytosolic pH over an extracellular pH range from 3.5 to 9.0 (Viegas and Sa-Correia, 1991). However, Ghaly and Kamal (2004) suggested maintaining the pH the of culture medium between pH 4 and 5 to achieve optimal growth and survival of *K. fragilis*. The yeast is able to sustain growth at lower pH, but an increase in the yield of yeast biomass was observed with an increase of pH from 4 to 7 (Munawar et al., 2010; Rajoka et al., 2006). However, Adoki (2008) reported that a *Candida* species was capable to grow at pH values between 3.0 and 5.8. A pH is a key factor for the growth and proliferation of microorganisms. Alteration in pH stresses the cells, which increases the requirement for maintenance energy that results in lower yields and lower productivity of the process. Thus, optimal pH and its maintenance are essential during cultivation.

### 3.2.4 Temperature

The effect of temperature on yeasts growth has been comprehensively analyzed by van Uden (1984). An increase in temperature generally promotes the generation of petite mutants that may affect the permeability of the plasma membrane. The change in permeability of plasma the

membrane may lead to a reduction of energy generation efficiency and to an increasing in energy mainaintance. The increase in maintenance energy will finally end up in a decrease of biomass yield. Additionally, a decrease in yield at high temperatures has been reported and this may have been due to glucose dissimilation by a non-viable fraction of the culture (Van-Uden and Madeira-Lopes, 1976). The optimal temperature range of the yeast *C. utilis* for biomass yield is reported to be between 30 and 35 $^{o}$C (Munawar et al., 2010; Rajoka et al., 2006). The temperature range between 25 and 35 $^{o}$C has been reported to be optimal for yeast growth and SCP cultivation (Table 3).

### 3.2.5 Dissolved oxygen

Dissolved oxygen (DO) is generally presented as percentage of saturation during the fermentation process. Various percentages of oxygen saturation during whey fermentation have been reported by different researchers. Carlotti et al. (1991) maintained DO above 30% saturation during production of yeast SCP from whey. Cristiani-Urbina et al. (2000) also reported to maintaining DO above 25% during production. Moreover, it has been concluded that the critical DO value for lactose-consuming yeasts is above 25% saturation, otherwise yeasts cultures switch their metabolic pathways from oxidative to the mixed oxidative-fermentative type, which results in the formation of intermediate metabolites (e.g. alcohol, aldehyde, esters, etc.). The change in metabolism due to DO affects the economics of the process and the efficacy of organic load removal (Cristiani-Urbina et al., 2000). Metabolites (e.g. ethanol) formation was even observed with fully aerobic conditions (DO > 20% saturation) during production of *K. marxianus* biomass in a lactose-based medium (Lukondeh et al., 2005). Thus, it is essential to maintain the DO above 20% saturation during the production process, as a minimum.

### 3.2.6 Mode of fermentation process

A fermenter is an apparatus, which is used for biomass cultivation of plant, animal or microbial cells and it can vary in size, from laboratory scale (1 to 10 L) to industrial models of several hundreds of litres in capacity. A fermentation process may be conducted as submerged or as solid state fermentation (Nasseri et al., 2011). However, submerged type of fermentation is the dominant mode of fermentation process applied to the production of biomass from whey.

The choice of the production mode of submerged fermentation depends on the purpose of the process. However, all three modes of fermentation process, i.e. batch, fed-batch and continuous process, have been reported for the production of biomass (bioprotein) from whey using yeasts. However, a secondary but significant purpose is to reduce the COD of the whey (Ghaly and Kamal, 2004; Moeini et al., 2004). An initial study using batch fermentation is a prerequisite to find the optimal condition for continuous or fed-batch fermentation. Various lab-scale studies have been reported on batch fermentation of whey (Anvari and Khayati, 2011; Cristiani-Urbina et al., 2000; Ghaly et al., 2003).

When the purpose is specifically wastewater treatment, the preferred mode of the fermentation process is continuous fermentation. Continuous fermentation is favoured for various obvious reasons, such as the economics of the process due to uninterrupted operations for long periods of time (Shuler and Kargi, 2002). Moreover, another advantage of continuous fermentation over a batch one is the reduction in operational time (e.g. inoculum preparation, harvesting, cleaning, and recharging the bioreactor) with expected product uniformity. High sugar contents during the continuous process may ultimately result in pyruvate accumulation, thus, stimulating higher glycolytic flux that may result in a decrease in final biomass yields. Growth is an oxidative process under subcritical substrate flux and limited carbon source with an adequate oxygen supply. However, growth will be oxido-reductive, under conditions of critical or super-critical glucose flux and both oxidative and fermentative glucose metabolism will occur concurrently (Castrillo and Ugalde, 1993). Continuous fermentation has been applied effectively for biomass production from cheese whey employing the yeast *Kluyveromyces fragilis* (Ben-Hassan and Ghaly, 1995; Ghaly et al., 2005).

Fed-batch, which is a mode of fermentation between batch and continuous fermentation, offers some advantages over batch and continuous processes. The major advantages are higher cell densities, controlled conditions of substrate concentration, control on the generation of byproducts, or catabolite suppression, and also allow maintenance of cell culture volume (Shuler and Kargi, 2002). Furthermore, an increase in lactose content can enhance pyruvate accumulation due to a higher glycolytic flux in the yeasts that finally results in a reduction of the biomass yield (Belem and Lee, 1999). Since fed-batch fermentations allow for facile control of carbon source supply by adjustment of the feeding rate, they are the most suited for biomass production. Comparatively, a high cell concentration of 105 g/L was reported in fed-batch cultures using *K. marxianus* (Lukondeh et al., 2005). A summary of biomass yields ($Y_{x/s}$), specific

growth rates ($\mu_m$), productivities, cultivation conditions (pH, temperature, and nitrogen source), and modes of the fermentation process employing cheese whey is presented in Table 3.

## 3.3 Quality of cultivated bioprotein from whey

The food value and the usefulness of bioprotein (SCP) depend on the nutrient content and composition of the bioprotein. The main components of bioprotein are proteins, carbohydrates, fatty acids, cell wall components, nucleic acids and vitamins. The composition of bioprotein for animal feed or food applications should always be analyzed (Anupama and Ravindra, 2000). Among these, the most important component, which determines the quality of the bioprotein, is the content in proteins and its composition. As per American Feed Control Officials-AAFCO (2010) guidelines, the protein content for animal feed applications should not be less than 40% w/w. Estimation of crude protein is generally based on total nitrogen, which is multiplied by the factor 6.25. Furthermore, the main decisive factor is the amino acids profile of bioprotein. The standard fixed by the FAO/WHO for the essential amino acids profile given in Table 4. The main drawback of microbial protein is the low content in some essential amino acids such as sulfur containing amino acids (cysteine and methionine) and lysine. The low cocentration in essential amino acids in biopreotein (or SCP) of *Kluyveromyces* yeast has been reported (Paul et al., 2002; Schultz et al., 2006). The amino acid composition of bioprotein depends mainly on the characteristic of the microorganism used. As presented in Table 4 none of the single microorganism studied fulfils the required amino acid composition. To achieve the balanced amino acid profile, different strategies have been suggested such as the use of methionine-enriched yeast strains or the use of mixed cultures. *K. lactis* mutants, which was methionine-enriched isolate and grown on whey permeate to improve the nutritional value of yeast biomass (Kitamoto and Nakahara, 1994).

Mixed culture SCP of *Trichoderma reesei* and *K. marxianus* cultivated on beet pulp, was reported to show the desired amino acid composition according to the FAO guidelines (Anupama and Ravindra, 2000). The cultivation of mixed culture of *K. marxianus* with yeasts like *Candida* and *Saccharomyces* may also fulfill the requirement for an essential amino acids profile as well as enhances COD removal efficiency. Furthermore, biotransformation of whey (which contains whey protein) in place of whey permeate (without whey proteins) may also be an option to produce higher value bioprotein. Whey protein are rich in lysine and in sulfur containing amino acids and mixing them with biomass protein will balance the composition of essential amino

acids (Yadav et al., 2014d). Additionally, the approach will be more attractive for small and medium scale industries because they do not have a sufficient volume of whey to recover the whey proteins and treat whey permeate separately (Yadav et al., 2014d). Others important factor that determines the nutritional value of bioprotein is its actual performance, which is determined by protein digestibility and the protein efficiency ratio (PER). Protein digestibility is presented in percentage and varies from 65 to 96% for different microbial proteins. The PER value varies from 0.6 to 2.6 (Anupama and Ravindra, 2000). Besides the nutritional value of the proteins, the other impacts of bioprotein on consumers are palatability, allergies and gastrointestinal effects, toxicological effects and carcinogenesis (Adedayo et al., 2011). Therefore, assessment of these factors is essential to determine the value of bioprotein.

## 3.4 Safety considerations and pre-treatment of bioprotein

Safety considerations arise from the bioprotein applications either as animal feed or as food ingredients. Acceptable toxicity levels are higher for animals than for humans. Toxicity evaluation of the end product must comprise short-term acute toxicity evaluation followed by extensive and long term-studies (Anupama and Ravindra, 2000). The substrate used for SCP cultivation represents the main safety hazard. The bioprotein produced/cultivated from different sources of substrate may be a carrier of carcinogenic components. Bioprotein may also be contaminated with heavy metals or metallic components that may increase the probability of mutations, even at low concentrations. Furthermore, the presence of mycotoxins is an important factor, which affects the use of bioprotein (SCP). Therefore, purification of the end consumer product is essential before using bioprotein as animal feed or as food ingredients (Anupama and Ravindra, 2000). However, the bioprotein produced from cheese whey should be considered as safe as cheese whey has GRAS status for animal feed and food ingredients by the United State Food and Drug Administration (US-FDA).

A rigorous hygiene and purification process is required for food-grade SCP as an end-product as per standard quality control norms. Additionally, the use of microbial protein (bioprotein) for human consumption requires pretreatments for: (i) disintegration of the non-digestible cell walls and (ii) reduction of the nucleic acid content (Anupama and Ravindra, 2000; Nasseri et al., 2011). The purpose of partial or complete digestion of microbial cell walls is to enhance digestibility of the yeast bioprotein. The disintegration or digestion of microbial cell walls is generally achieved by mechanical methods (e.g. high pressure homogenizers, wet milling,

sonication, pressure extraction and treatment with grinding particles) and non-mechanical methods (e.g. chemical and enzymatic treatment) (Nasseri et al., 2011).

The second and significant inhibitory factor associated with bioprotein intake as food is its content of nucleic acids, which varies from 8 to 25 g/100 g cell protein (Larsen and Joergensen, 1996). Foods with a higher nucleic acid content increase the level of uric acid in the body due to nucleic acid degradation. The accumulation of uric acid in the body occurs due to the lack of the uricase enzyme in humans. Ingestion of nucleic acids superior to 2 g equivalent per day by humans may lead to kidney stone development and gout disease. Therefore, the nucleic acid content of the bioprotein must be reduced to an acceptable range (< 2.0 g nucleic acid/day) (Anupama and Ravindra, 2000; Larsen and Joergensen, 1996). Different treatments such chemical treatment with ammonium hydroxide, sodium chloride or activation of endogenous nucleases (ribonuclease-RNase and deoxyribonuclease-DNase) during the final stage of microbial biomass production as well as thermal shocks has been applied to decrease nucleic acids (Alvarez and Enriquez, 1988; Larsen and Joergensen, 1996; Nasseri et al., 2011). Alvarez and Enriquez (1988) reported nucleic acid reduction of the yeast *S. cerevisiae* and *K. fragilis* after treatment with ammonium hydroxide (4.5%) to give a nucleic acid content less than 2%. Larsen and Joergensen (1996) applied a heat shock method to activate endogenous RNase and DNase in the bacterium *Methylococcus capsulatus* which resulted in a 80% reduction in nucleic acid content with loss of less than 10% in protein. Exogenous nucleases have also been applied to hydrolyse the nucleic acids. The derivatives of pancreatic RNase and endonuclease of *Staphylococcus aureus* as insolubilized thermostable form have been employed to decrease the nucleic acids content in bioprotein/SCP of yeast. The exogenous enzymatic treatment decreased the nucleic acid content from 5-15% to 0.5% with a simultaneous protein loss of only 6% (Martinez et al., 1990). Both enzymatic and chemical treatments have their disadvantages i.e. loss of protein during treatment. Moreover, alkaline treatment at high temperature generates toxic compounds such as lysinoalanine (Nasseri et al., 2011). Therefore, the method of choice for pretreatment should be considered based on the factors (protein loss and toxicity).

## 4. FUNCTIONAL/NUTRITIONAL WHEY PROTEINS FROM WHEY

Increased research activities, advances in separation technology and availability of sophisticated analytical instruments have increased the interest to separate out (concentrate) various

proteinaceous products from whey. The processed proteins from whey are applied in the food industry due to their excellent functional and nutritional properties.

## 4.1 Whey-based proteinaceous products

There are various useful products, which can be obtained through simple physical processing from liquid whey with different protein contents. These products are whey powder, reduced lactose whey, whey protein concentrate (WPC) and whey protein isolate (WPI) (Modler, 2009; USDEC, 2006). The simplest dried whey product is whey powder, which is obtained by removal of water via spray drying. Reduced lactose whey is a product that contains a lactose concentration less than 60%. WPC results from the removal of non-protein fractions to attain a defined protein content. The commonly available WPC products are WPC34, WPC50, WPC60, WPC75 and WPC80. The numerical value defines the percentage of protein in the product such as WPC34 must contain not less than 34% protein. Similarly, WPI is obtained through removal of the non-protein fraction to achieve protein concentrations higher than 90% in the final product (USDEC, 2006). The US-FDA (2013) have fixed specifications according to, which WPC protein content should be not less than 25%. The other components, such as fat should be between 1 and 10% and moisture should be between 2 and 6%. The ash value is fixed between 2 and 15% whereas the lactose content should not be more than 60% and the limit of heavy metals is 10 ppm. Furthermore, whey or WPC should be produced from pasteurized milk or the WPC must be decontaminated or its equivalent before application as food (US-FDA, 2013). The typical whey protein content of various whey derived proteinaceous products is summarized in Table 5 (USDEC, 2006). The market value of proteinaceous product depends on the protein content of the product; the WPC value is 3 to 40 times higher than that of whey powder (Baldasso et al., 2011).

## 4.2 Individual whey protein components

Whey proteins are a mixture of different individual whey proteins, which are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin (Lf), lactoperoxidase (LP), proteose-peptone and glycomacropeptide (GMP). Excluding glycomacropeptide, all other protein components are naturally present in whey, while glycomacropeptide is produced from casein during the first step of enzymatic cheese processing (Madureira et al., 2007). The content and characteristics of each individual protein is different as

presented in Table 6 (Amiri and Valsaraj, 2004; Kim et al., 2007; Krissansen, 2007). Whey protein composition indicates that it is a heterogeneous mixture of different individual proteins, with its specific distinctive functional, physiological and neutraceutical characteristics.

### 4.2.1 β-Lactoglobulin

β-lactglobulin (β-LG) is an important and major component of whey proteins. It accounts for around 64% of heat-coagulable proteins or 51% of the total whey proteins. The molecular weight (MW) of this protein ranges from 18.20 to 18.36 kDa. It is generally present as a dimer of 2 identical subunits with each monomer consisting of one sulfhydryl group and two disulphide bonds. The number of amino acids in the single peptide chain of β-LG is 162. Solubility of this protein is mainly dependent on pH and ionic strength (Hernández-Ledesma et al., 2011a; Walstra et al., 2010). The heat denaturation of β-LG occurs between 70 to 75 $^{o}$C and forms aggregates at 78 to 82 $^{o}$C. The precipitation of β-LG occurs before that of α-LA. Denaturation of α-LA is reversible compared to other whey proteins; therefore, it is more resistant to heat compared to β-LG (Sava et al., 2005). High protein content products, such as WPI, are enriched with β-LG. The high content of β-LG in WPI leads to enhanced functional properties like emulsion stability, activity, viscosity and gelling properties compared to WPC. The amino acid sequence of β-LG shows similarity with that of the retinol-binding protein (provitamins A) and consequently it binds to hydrophobic molecules such as fat-soluble vitamins as well as lipids (Modler, 2009).

### 4.2.2 α-Lactalbumin

α-lactalbumin (α-LA) comprises around 11% of the total whey proteins and is a small globular protein with a single polypeptide chain. The molecular mass of α-LA is 14 kDa, a 123 amino acids polypeptide containing eight cysteine residues (Hernández-Ledesma et al., 2011a; Markus et al., 2002). Calcium strongly binds to α-LA and provides stabilization of the molecular conformation. α-LA has several beneficial uses as a dietary component. α-LA with dietary ingredients, increases the level of plasma tryptophan compared to dominant neutral amino acids (valine, isoleucine, leucine tyrosine and phenylalanine), which has the negative effects of an imbalance of brain serotonin (5-hydroxytryptamine). Improvement in this imbalance leads to better cognitive performance, mood and sleep (Markus et al., 2002). It helps to maintain lipid oxidation and increases lean body mass in rat models (Bouthegourd et al., 2002). α-LA helps in

the absorption of minerals, possesses antibacterial activity, shows immunomodulatory effects and antitumor activity (Modler, 2009).

### 4.2.3 Bovine serum albumin

Whey protein consists of around 5-6% in bovine serum albumin (BSA), which is almost similar to its human milk concentration. The nature of BSA is very heterogeneous and its concentration in milk is approximately identical to that present in bovine blood. The molecular mass of BSA is 66.26 kDa, with 35 cysteine residues and 17 disulphide linkages per molecule (Walstra et al., 2010). The number of amino acids in the BSA polypeptide chain is 582 (Hernández-Ledesma et al., 2011a). BSA has a denaturation temperature of 64 $^{o}$C, which is almost similar to that of α-LA (62 $^{o}$C). Though the temperature at which BSA gets denatured is a little higher compared to that of α-LA, BSA precipitates before α-LA because of the reversible nature of α-LA precipitate (Modler, 2009). Important characteristic of BSA is its capacity to bind reversibly to several ligands and because of this it can be used as carrier of fatty acids (Mollea et al., 2013). Moreover, it can also be used as a source of essential amino acids, but due to availability of other sources, there is no real demand for it (Modler, 2009).

### 4.2.4 Immunoglobulins

Bovine immunoglobulins (Igs) are comprised of three main classes: IgG, IgA and IgM. Further, IgG has two subclasses, IgG1 and IgG2. The total content of immunoglobulins in whey is around 0.7 g/L and IgG shares up to 80% (w/w) of the total. IgGs are present in a monomeric form, whereas IgA and IgM are present in polymeric forms (Mollea et al., 2013). The main role of immunoglobulins is to agglutinate bacteria, neutralize toxins and inactivate viruses. IgA protects from proteolysis and it can also neutralize viruses and bacterial toxins. IgM is more efficient than other immunoglobulins in terms of complement fixation, neutralizing viruses and agglutination of bacteria. IgG can also withstand gastric digestion. The other reported metabolic roles of IgG are lowering of the blood pressure by reducing the cholesterol level and use of immunoglobulin preparation in infant formula as milk replacers (Modler, 2000; Modler, 2009; Mollea et al., 2013).

### 4.2.5 Glycomacropeptide

Glymacromacropeptide (GMP) also known as caseinomacropeptide (CMP). GMP is derived from the k-casein component of milk during cheese processing by the action of the milk-clotting enzyme (e.g. chymosin). Amino acid residue of GMP is 64 and has a MW of 6.8 kDa with an isoelectric pH of 4.3 to 4.6. The glycosylation degree of the molecules varies from 0 to 5 trisaccharide units of N-acetyl-neuraminic acid (sialic acid). However, 25 to 50% of the GMP can be devoid of glycosylation. GMP is thermo-stable and are part of the proteose-peptone fraction (Modler, 2009). The GMP molecules have unique characteristics due to the absence of phenylalanine, tryptophan, tyrosine, histidine, arginine or cysteine residues. Due to absence of aromatic amino acids in the molecule, GMP does not show absorption at 280 nm (Rajput et al. 2013) The absence of phenylalanine makes this protein a valuable dietary ingredient for patients who are suffering from phenylketonuria. Phenylketonuria is a genetic disorder and around 15 thousand people in the United States sufferg from this defect (Modler, 2009). Thus, whey can be used as a potential source of GMP to fulfill the nutritional profile of such patients. Moreover, GMP is rich in branched chain amino acids (Krissansen, 2007; Marshall, 2004).

### 4.2.6 Lactoferrin

The iron-binding glycoprotein lactoferrin (Lf) belongs to the transferring family of proteins. Lactoferrin generally is found in exocrine secretions of mammal's milk, tears, mucus and saliva. Presence of lactoferrin has also been reported in egg white, conalbumin and ovatransferrin. The MW of lactoferrin is 76.50 kDa and lactoferrin consists of a single polypeptide chain of 700 amino acid (Gonzalez-Chavez et al., 2009; Modler, 2009). The polypeptide chain may contain one or two carbohydrate chains. It has a very high isoelectric point of 9.5 (for the lactoferrin 'a' variant) and 10.0 (for the lactoferrin 'b' variant). The lactoferrin molecules are heat stable and acid resistant at pH 4.0 (Modler, 2009). It is also resistant to the action of trypsin and chymotrypsin but it can be hydrolysed with pepsin (Modler, 2000). Traditionally, lactoferrin is known for its antimicrobial and antifungal activity against a wide range of bacteria and yeasts (Hernández-Ledesma et al., 2011a). Lactoferrin also shows immunomodulatory activity. It has influence on cell growth and differentiation, embryonic development, myelopoesis, endothelial cell adhesion, cytokine and chemokine generation (Cornish et al., 2004; Ulber et al., 2001).

### 4.2.7 Proteose-peptone component

Proteose-peptone is the fraction of milk protein which remains soluble in milk when milk is heated at 95 $^{o}$C for 20 min under acidic condition (pH 4.7). Proteose-peptone has 4 components (component 3, component 5, component 8-slow and component 8-fast). Component 3 is the major one and makes with 25% by weight (Krissansen, 2007). The other three peptides components originate from β-casein due to action of the native plasmin, while component-3 appears to derive from the fat globule membrane. Proteose-peptone has been recognized for very limited consideration in terms of food application. Component 3 may be used as a bifidogenic factor due to its higher carbohydrate content, however, research is lacking on this aspect (Sorensen and Petersen, 1993).

### 4.2.8 Lactoperoxidase

Lactoperoxidase (LP) is a natural enzyme of the mammals' host defense system (Hernández-Ledesma et al., 2011a). The molecular mass of lactoperoxidase is 78 kDa. This enzyme is a single polypeptide chain of 612 amino acid residues. It contains 15 half-cystine residues, one heam group and about 10% w/w of carbohydrate moieties. The enzymatic activity is influenced by temperature and time, i.e. enzymatic activity is lost at 62.5 $^{o}$C after 30 min, 70 $^{o}$C after 15 min or 85 $^{o}$C after 15 s (Modler, 2009; Mollea et al., 2013). The main use of lactoperoxidase is as a protective factor against infectious microbes. Mice infected by the influenza virus can be attenuated by oral administration of lactoperoxidase (Shin et al., 2005). Increases in superoxide production have been reported in sheep neutrophils after dose dependent application of lactoperoxidase (Wong et al., 1997).

## 4.3 Processing of proteinaceous whey products

Liquid whey is processed through different unit operations to obtain the desired final product. The first step is clarification, which eliminates residual curd. Fat is also removed to facilitate membrane filtration (Zadow, 1992). After that, whey is pasteurized to eradicate pathogens and to deactivate the starter cultures. Further, pretreatment can be applied to decolourize whey by removing annatto (Modler, 2009). The pretreatment step is followed by concentration of the whey protein. The commonly used concentration methods include evaporation, reverse osmosis and membrane filtration (microfiltration, ultrafiltration, pervaporation, nanofiltration and

electrodialysis) (Akpinar-Bayizit et al., 2009). Evaporation is often achieved under vacuum at temperatures lower than 100 $^{o}$C to protect whey proteins from denaturation. The disadvantage of conventional thermal evaporation methods is high energy requirement, high ash values and presence of lactose in the final product. Moreover, the thermolabile protein characteristics may change, which leads to change in the functional and nutritional properties of the final product (Baldasso et al., 2011). Concentration of whey via reverse osmosis is carried out by passing whey under pressure via membranes with a pore size of around 0.001 µm and the product can be concentrated up to 20-22% solids (Zadow, 1992). However, the most preferred method to concentrate whey proteins in their native state is membrane filtration (ultrafiltration and nanofiltration) (Atra et al., 2005).

Membrane technology has gained great interest because of its energy saving advantage. WPCs, which are obtained by whey ultrafiltration, are available in high quality in terms of protein content and functional properties (Limsawat and Pruksasri, 2010). Ultrafiltration (UF) and nanofiltration are defined as pressure-driven membrane separation and concentration processes for target components in the MW ranges from 1 to 1000 kDa by ultrafiltration and from 100 to 500 Da through nanofiltration (Baker, 2002). Generally, higher MW whey components (e.g. proteins and fat) are retained on the retentate side whereas lower MW components (e.g. lactose, minerals and vitamins) are partitioned on both sides of the membrane (i.e. retentate and permeate sides) (Kessler, 2002). Ultrafiltration to obtain WPC from a clarified liquid can concentrate up to 65% solids. Further, a more concentrated proteins fraction (up to 80% solids) is obtained by diafiltration, which involves dilution the retentate with water (Varnam and Sutherland, 1994). However, diafiltration cannot remove all of the lactose from the retentate. Further, to enhance the purity of whey proteins, a combination of ultrafiltration and microfiltration is used, which can yield a WPC containing up to 99% in whey proteins (Henning et al., 2006). WPC can be produced either as concentrated liquid or dried powder after spray drying. The general flow chart describing the industrial production of WPC is presented in Fig. 3 (USDEC, 2006).

Whey protein isolate-WPI (protein content above 90%) is obtained by performing a additional microfiltration or an ion exchange chromatography. Like other proteins, whey proteins are also amphoteric in nature; they may behave either as an acid or base with respect to medium pH. Generally, any protein possesses a net positive charge at pH values below its isoelectric point and a net negative charge above the isoelectric point. In this manner, the pH of the medium can be adjusted to adsorb proteins onto an ion exchange and therefore, concentrate them from dilute

solutions (Varnam and Sutherland, 1994). Further, proteins are eluated from the ion exchanger at ~ pH 9, and then concentrated by ultrafiltration (Morr and Foegeding, 1990).

Generally, spray drying is the method used for drying to obtain dry whey powder and whey proteins, which are desired for many applications (Zadow, 1992). The powder is obtained from the spray dryer with a moisture content around 5-7% (Henning et al., 2006). Whey becomes very sticky during the drying process, therefore, specialized equipment is used to achieve higher solids concentrations (from 50% to 80% solids). Further, a second set of equipment is used to crystallise and to cool down the product rapidly. The cold product is sent to an internal drum and dried up to 95 to 96% solids at 54-60 $^{o}$C. Then the dried whey product is milled by a hammer mill to decrease particle size. The various steps involved in WPI processing are represented in Fig. 4 (USDEC, 2006).

## 4.4 Methods for fractionation of individual whey proteins

There is increasing interest to develop cost-efficient technologies for the isolation of individual whey protein fractions in order to capture growing market opportunities. Generally three approaches are used to separate or fractionate individual whey proteins: (i) precipitation/flocculation by heat treatment or using polymers; (ii) membrane technology (ultrafiltration and nanofiltration) and (iii) chromatographic separation.

### 4.4.1 Separation by precipitation

The separation of protein by precipitation involves adjustment of solution physical properties to decrease protein solubility. Proteins generally have low solubility at a pH near the isoelectric point and in low ionic concentrated solutions. Therefore, by applying the above conditions (i.e. adjusting pH near isoelectric point) proteins will start to aggregate. Heat treatments, when applied, modify the whey protein structure and solubility. Protein precipitate when whey is heated above 90 $^{o}$C for 10 min in acidic conditions (pH 3.5 to 5.5). There are various fundamental studies on whey protein aggregation, which is as affected by pH, temperature and protein concentration (Antoine and Souza, 2007; de la Fuente et al., 2002). At isoelectric pH, heat denatured proteins interact via irreversible thiol-disulphide bonding and form aggregates. Further, the aggregated molecules will coalesce resulting finally in sedimentable precipitates. For example, a precipitate of α-LA from liquid whey was obtained by adjustement of pH at 4.2 and

heat treatment at 65 $^{o}$C (Pearce, 1983). The supernatant was exhausted in α-LA and enriched with the remaining proteins like β-LG. Pearce (1983) has reported that α-LA has more of a tendency to aggregate at a pH between pH 4.2 and 4.6 and at a temperature between 50 and 65 °C. The aggregation tendency increases with an increase of protein content. Moreover, in this environment, β-LG completely remains soluble (more than 98%), while other major proteins (BSA and Ig) co-precipitate. Re-solubilization of the aggregates was partially obtained by re-adjustment of pH and temperature. An optimized set of conditions has also been reported by Bramaud et al. (1997) for whey protein fractionation based on selective aggregation of α-LA. Precipitation or fractionations were also carried out by using a flocculating agent such as bentonite, carboxymethyl cellulose and chitosan. The fractionation with polymers (e.g. polysaccharides) based on electrostatic interactions (the two-week or strong opposites charged polyelectrolytes) between proteins and polymers (Yadav et al., 2014d). Chitosan was found to be more effective for β-LG precipitation from whey at pH 6.2 and around 80% of the other whey protein fractions was left in the solution (Casal et al., 2006).

### 4.4.2 Separation by membrane technology

There have been several reports on the fractionation of individual whey proteins using membrane technology (ultrafiltration and nanofiltration). For example, a two-step membrane separation process for an α-LA-enriched product has been reported. In the first stage, an XM50 (50 kDa molecular weight cutoff-MWCO) membrane was applied, which retained higher MW proteins on the retentate side while the permeate contained mainly α-LA, β-LG and smaller proteins. Then, a second membrane (lower MWCO) was used to remove caesinomacropeptide. The end product had an α-LA- to -β-LG ratio of 2:1 (Zydney, 1998). Similarly, Mehra and Kelly (2004), proposed three-stage sequential fractionation of individual whey proteins via ultrafiltration filtration using different MWCO membranes and charge modifications based on pH (Fig. 5).

### 4.4.3 Chromatographic separation

Chromatographic methods of separation are also dominant for the fractionation and purification of individual whey proteins. Especially, preparative chromatographic techniques are more attractive. Though chromatographic process development is easy, its optimization is a complicated process as it involves various process parameters like selection of the column matrix, salts, buffer, organic solvent, temperature and gradients. A fractionation scheme has

been reported for IgG, lactoferrin and lactoperoxidase employing cation exchang matrices such as S-HyperD-F, S Sepharose FF and Fractogel EMD-S 650 (S) (Hahn et al., 1998). Naqvi et al. (2010) purified the β-LG using gel chromatography from bovine milk whey. They have used the column Bio-Gel P10 (Biored) at pH 3.0 and eluated the protein with 18 mM sodium chloride. Separation and purification of whey proteins from WPC80 using hydrophobic interaction chromatography has also been reported by Santos et al. (2011). They have used standard proteins and a WPC80 solution in phosphate buffer with ammonium sulfate (1M). The solubilized sample was loaded onto a HiPrep Octyl Sepharose FF column using a fast protein liquid chromatography (FPLC) system. A lower ionic strength buffer with a salt gradient was used to eluate the proteins. The recovery obtained was 45.2% for β-LG with 99.6% purity (Santos et al., 2011). Among the three separation techniques, more emphasis has been placed on membrane filtration and chromatographic separation. Furthermore, the use of supercritical carbon dioxide ($SCO_2$) has also been applied to manipulate pH of solution at different temperatures to precipitate some components of whey proteins and leaving some in soluble form. Thereafter, the soluble form could be concentrated by membrane filtration (ultrafiltration) (Bonnaillie et al., 2014). Casein glycomacropeptide of 94% purity from WPI has been reported by employing $SCO_2$ and ultrafiltration. Use of $SCO_2$ can avoid the use of buffer solutions.

## 4.5 Application of protein enrich products and individuals whey proteins

Whey proteins have been recognized for their various functional properties and nutritional value. The functional properties are mainly due to their physical, chemical and structural characteristics (Jovanović et al., 2005), and the nutritional value is directly linked to the concentration of essential amino acids (especially sulfur-containing ones) and branched-chain amino acids (isoleucine, leucine and valine) (Baldasso et al., 2011). Moreover, whey proteins have GRAS status for food applications and can be used in various food products such as healthy foods, dairy foods, meat products, frozen foods and baby food formulations (Jayaprakasha and Brueckner, 1999; Morr and Foegeding, 1990). WPC80 and WPI are frequently used to enhance functional properties like baking quality, egg replacement, in cheese to enhance yield, as a stabilizer in yogurt, in dips and spreads as a texturizer and as an extender in meat products (Varnam and Sutherland, 1994). Whey proteins are used in bakery, confectionary, meats and seafood to obtain a strong gel with high water-holding capacity (Jovanović et al., 2005; Veith and Reynolds; 2004). Protein interactions result in gel formation and form an elastic network

(Foegeding et al., 2002). Whey proteins can also be used as thickeners or emulsifiers due to their ability to stabilize emulsions such as in processed meats and desserts. Whey proteins emulsification properties mainly depend on pH. The emulsification is lowest near the isoelectric point (Onwulata and Huth, 2009). Foaming is an important attractive property for many food products, such as whipped toppings, cakes, milkshake and frozen desserts. Whey proteins induce foam formation by lowering the interfacial tension and exhibit their best properties near the isoelectric point (Jayaprakasha and Brueckner, 1999; Jovanović et al., 2005). The whey protein-fortified beverage formulation known as ready-to-drink beverages has been initiated by the US Dairy Export Council-USDEC, which provides nutritional proteins that target especially athletes (Rittmanic, 2006).

The researchers in food science are regularly looking for new methods to develop or to transform whey proteins into more functional ingredients. Mishra et al. (2001) have developed a protein-polysaccharides compound made up of WPC71 and pectin, in a 1:1 ratio. The produced complex showed enhanced solubility, emulsification, gelation, foam formation and foam stability over WPC alone. Moreover, an encapsulated layer of whey protein delayed the oxidative degradation of dry-roasted peanuts. Furthermore, use of transglutaminase as a cross-linking agent with whey protein in the film enhanced oxygen barrier. Extensive research has been conducted to use whey proteins as coating agents against oxidation (Di Pierro et al., 2006; Lin and Krochta, 2005). Additionally, the functional and nutritional role of individual whey proteins are summarized in Fig. 6 (Hernández-Ledesma et al., 2011a; Modler, 2009; Mollea et al., 2013).

# 5. BIOACTIVE PEPTIDES AND THEIR PROCESSING FROM WHEY

## 5.1 Bioactive peptides

A defined protein fragment, which shows a positive influence on body functions and finally impact on health, is defined as bioactive peptide. The activity of a bioactive peptide depends on its intrinsic amino acid sequence and size, which varies between 2 and 20 amino acid residue (Korhonen, 2009). Whey proteins can be converted into various bioactive peptides following different types of treatment.

### 5.1.1 ACE-inhibitory peptide

Presently, the ACE-inhibitors (antihypertensive agents) such as captopril, enalapril, alecepril and lisinopril are synthesized chemically or alternatively bioactive peptides are generated by enzymatic hydrolysis of milk and whey proteins, using enzymes like trypsin, pepsin and alcalase. After hydrolysis, peptides are fractionated and identified through reverse phase HPLC employing a UV detector (Mota et al., 2004). α-Lactorphin [YGLF, α-LA(f 50-53)], a tetra-peptide originating from the proteolysis of bovine α-LA; β-lactorphin [YLLF, β-LG(f 102-105)]; β-lactotensin [HIRL, β-LG (f 146-149)], tetra-peptides originating from the proteolysis of bovine β-LG, etc., have been reported to show angiotensin-I-converting enzyme (ACE) inhibitory activity (Belem et al., 1999; Sieber et al., 2009). The ACE-inhibitory peptides are used for the control of hypertension (high blood pressure). The physical approach for blood pressure management includes dietary modification and exercise while drug management includes calcium-channel agonists, angiotensin-II receptor blockers, diuretics and angiotensin-converting enzyme (ACE) inhibitors. ACE inhibition controls the concentration of the vasoconstrictory peptide, angiotensin-II, and with an enhancement in the concentration of the vasodialatory peptide, bradykinin, this finally leads to a decrease in blood pressure (Fitzgerald and Murray, 2006). The ACE-inhibitory peptides inactivate the angiotensin-converting enzyme, which is responsible for developing hypertension inducing factors in the body as mechanism presented in Fig. 7 (Saito, 2008).

Presently, fragments of β-LG derived from the hydrolysis of whey protein isolate are marketed as BioZate (Danisco Foods International, Le Sueur, MN), which are claimed to lower blood pressure. A study has been conducted with the BioZate 1 product on 30 borderline hypertensive subjects for 6 weeks with a placebo-control, in which the placebo was un-hydrolysed WPI. A decrease in blood pressure of 8 mmHg was documented compared with to placebo group (Madureira et al., 2010).

### 5.1.2 Bioactive peptides with opioid activity

Opioid peptides are those bioactive peptides that shows an affinity with opiate receptors along with opiate like effects, and are inhibited by naloxone (an opioid antagonist) (Pihlanto-Leppälä, 2000). Moreover, the pharmacological activity of opioid peptides is similar to that of opium (morphine, an alkaloid) (Sharma et al., 2011). Three precursor proteins, proopiomelanocortin (endorphins), proenkephalin (enkephalin) and prodynorphin (dynorphin) usually generate opioid

peptides. The important characteristic of typical opioid peptides is having an identical N-terminal sequence, Try-Gly-Gly-Phe (Pihlanto-Leppälä, 2000; Sharma et al., 2011). Opioid-like sequences present in the native bovine whey protein structure of α-LA f (50-53) and β-LG f (102-105). The peptides from α-LA and β-LG have been termed α- and β-lactorphins, respectively (Pihlanto-Leppälä, 2000). The enzymatic treatment required to liberate lactorphins involve pepsin for α-LA hydrolysis and trypsin for β-LG hydrolysis. α-lactorphins are reported to exert low but stable opioid activity while β-lactorphins exert a clear, non-stimulatory opioid activity in the guinea pig ileum when bound to opioid receptors. The binding affinity of these peptides with receptors is low, and is more specific towards the μ-type receptor. Both α and β-lactorphins were found to displace naloxone from its binding sites at micromolar concentrations. Moreover, these two peptides have also reported to possess antihypertensive activity (Madureira et al., 2010; Pihlanto-Leppälä, 2000).

### 5.1.3 Iron-binding bioactive peptide

Iron-binding bioactive peptides are produced by enzymatic hydrolysis of whey protein concentrate (WPC). Lactoferricin is an example of an iron-binding peptide derived from peptic hydrolysis of WPC (Vegarud et al., 2000; Wakabayashi et al. , 2003). Iron-binding peptides can be helpful in the management of anemia (a wide spread iron deficiency), especially in kids and women (WHO, 2001). In addition, it may be more useful compared to oral formulations of prophylactic or therapeutic doses of non-heme iron components like iron sulphate, by mitigating the low bioavailability and side effect problems in anemic subject. These iron-binding peptides make iron supplementation safe and more efficient (Kim et al., 2007).

### 5.1.4 Hydrolysed whey protein

Commonly used standard base in infant milk formula is developed from the cow's milk or its derived products. Though there is much similarity between human and cow's milk proteins, however, differences arise due to the presence of β-LG in cow's milk. β-LG is absent in human milk and if it is administered it might cause allergy in infants due to the lack of underdeveloped pepsin secretion. Therefore, WPC is generally allergenic to some infants due to the presence of β-LG, which is a major component of whey protein. There are two approaches available to avoid allerginicity of whey proteins. The first one is elimination of β-LG from the whey protein products by precipitation/or membrane filtration (ultrafiltration). The second one is proteolysis of the whey

proteins by different proteases to produce lower MW proteins and peptides. Infant formulations may be prepared either from extensively or from partially hydrolyzed whey proteins. However, some researchers have suggested that only the extensive hydrolys is giving peptides with MW less than 3 kDa is acceptable for more sensitive infants to avoid any reactions (Chan et al., 2002). Alexander et al. (2010) have extensively reviewed the benefits of partially hydrolyzed 100% whey protein for infant formula and suggested that it was effective to reduce the risk of incidence of atopic dermatitis.

## 5.2 Process for production of bioactive peptides

There are various methods available to produce bioactive peptides. The most commonly used methods depend on the food processing: (i) heating under alkali/acid conditions; (ii) enzymatic hydrolysis and (iii) microbial proteolysis of the fermented foods. Limited hydrolysis of well-known proteins releases bioactive peptides. However, till date, the dominant procedure to generate bioactive peptides is enzymatic breakdown, utilizing different techniques and scales (Pihlanto-Leppälä, 2000). Trypsin (a pancreatic enzyme) has been preferably used for the hydrolysis of known proteins, which cleaves at the C-terminal end of arginine (R) and lysine (K) residues. Another important enzyme, chymotrypsin, acts on aromatic side chain of the C-terminal. Bacterial and fungal proteases have also been employed to produce bioactive peptides like ACE-inhibitory peptides. The enzymatic hydrolysis of precursor proteins or peptides may be conducted either in a batch or in a continuous (in membrane reactors) mode to obtain bioactive peptides (Madureira et al., 2010). The development of membrane reactors (or continuous process) for hydrolysis of proteins is increasing to overcome the high cost of batch processes. The higher cost in batch processes arise due to the need for large quantities of enzymes, energy and labor costs. To avoid the higher cost of batch process, a membrane bioreactor with an ultrafiltration membrane of a MWCO 3 kDa has been reported by Guadix et al. (2006) for the manufacturing of whey protein hydrolysates.

Beside enzymatic hydrolysis, fermentation has also been identified as one of the routes to produce bioactive peptides. Lactic acid bacteria (LAB) are the dominant ones to hydrolyze milk proteins, especially casein, to bioactive peptides (Hernandez-Ledesma et al., 2011b). Whey proteins have been hydrolyzed to ACE-Inhibitory peptides by the action of yeast and bacteria. The bacteria used in these fermentation processes are *Lactobacillus* spp. and the yeast *K. marxianus. Lactobacillus* spp. like *L. rhamnosus* and *L. helveticus* CNP4 are well established for

producing ACE-Inhibitory peptides. Yeast species like *K. marxianus* and *S. cerevisiae* have been reported to have proteolytic activity on whey proteins for producing oligopeptides (Didelot et al., 2006; Hamme et al., 2009). However, such microbial hydrolysis is still carried out only at laboratory scales. The produced bioactive peptides during these hydrolysis processes are purified and characterized through analytical techniques. The commonly used technique for peptides purification is reversed phase chromatography. Further, the purified peptides are generally characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Butylina et al., 2006). The in-vitro peptide activity is evaluated using chemical or biochemical assay methods. Antigenicity of the hydrolyzed whey product is evaluated using an enzyme-linked immunosorbent assay (ELISA) (Guadix et al., 2006). Finally, the bioactive peptides are separated or concentrated from hydrolysate product by membrane separation. The preferred methods of membrane separation for bioactive peptides are ultrafiltration and nanofiltration (Bazinet and Firdaous, 2009; 2013).

## 6. CONCLUSIONS

The present surpluses amount of cheese whey may be potentially transformed into various value-added products to tackle the environmental problems. In addition, the GRAS status of cheese whey allow to produce proteinaceous products such as yeast bioprotein (SCP), and functional and/or nutritional proteins and bioactive peptides. The biotechnological transformation of whey or whey permeate into bioprotein is advantageous in two ways i.e. whey management and production of proteinaceous animal feed or food ingredients. On the other hand, physical processing has potential to transform whey into functional and nutritional proteins (WPC, WPI and individual whey proteins). Furthermore, combined processing i.e. enzymatic treatments or fermentation processes lead to generation of bioactive peptides (antihypertensive, opioid, iron-binding and hydrolysed whey proteins) from whey. Thus, combined physical and biotechnological processing has potential for achieving a complete transformation of whey into various proteinaceous products.

## 7. FUTURE PROSPECTIVE

The quality of bioprotein, which mainly depends on the essential amino acid profile, may be enhanced by: (i) employing yeast stains rich in sulfur-containing amino acids; or (ii) employing mixed cultures to achieve the desired amino acid profile. Additionally, mixed cultures have

shown more potential for higher COD removal. Mixed culture cultivation of *K. marxianus* and *C. krusei* at higher temperature and low pH could be a good option for animal feed production. The extreme cultivation conditions will provide an opportunity to run the fermentation process under non-aseptic conditions. This potential consortium should be applied on an industrial scale. Another option to improve bioprotein quality is by replacing whey permeate with whey following fermentation thereafter, the residual unutilized whey protein would be mixed with biomass protein to enhance protein content and to balance the essential amino acids profile. This, approach will be highly beneficial for small- and -medium scale cheese producing companies, thus, permetting to utilize crude whey for the production of protein-rich products and to enhance value of their waste product economically.

Food-grade bioprotein can easily be produced from whey by processing of crude biomass. Moreover, good quality, food-grade SCP can also be produced from cheese whey employing a mixed culture of *K. marxianus* and *S. cerevisiae.* Partial or complete hydrolysis of lactose could be achieved using permeabilized yeast cells (Yadav et al., 2014c). Moreover, processing of crude bioprotein (SCP) could be achieved by employing a combined method of treatment (detergent in combination with chemicals), such as a mild detergent to release the nucleotides and to make the yeast cells fragile. Further, addition of a chemical treatment will yield a food-grade product. Emphasis should also be given to fractionate the yeast cell wall components for value addition. Another opportunity using permeabilized yeast cells is the simultaneous generation of galactooligosaccharide during whey fermentation (Yadav et al., 2014c). The galactooligosaccharides could be recovered with the residual whey proteins that might give partially hydrolysed whey protein and GOS. Such a type of product is currently being marketed by company like Nestle. Thus, fermentation and membrane technology may play an important role in the production such products. However, these all aspects need a systematic study to translate them into real products processes.

Another opportunity employing fermentation is production of bioactive peptides. *K. marxinaus* was reported to produce bioactive peptides during fermentation, which needs to be developed into a pilot scale process. Further, proteolysis of whey proteins can be improved by employing a protease-producing *Lactobacillus* species. Till date, most of the bioactive peptide characterization and recovery studies have used WPC or WPI. Therefore, development of analytical methods to characterize the bioactive peptides from fermentation and recovery processes (membrane filtration) are still needed. Additionally, a recent trend is to get two

products at the same time to enhance the economics of any fermentation process. Like production of nisin (antibacterial peptide) employing *Lactococcus lactis* and *K. marxinaus*, has GRAS status, and after separation of extracellular nisin, the biomass can be used as animal feed. Some aroma compounds like phenyl ethyl alcohol can also be produced during biomass production. The byproduct can be recovered after fermentation. Another opportunity with the fermentation of cheese whey, instead that of whey permeate is that it will be easier to apply a precipitation or an ultrafiltration method to recover the residual proteins because lactose has been depleted. This strategy also needs to be explored further.

## ACKNOWLEDGEMENTS

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support**.**

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**Table 1. Compositional characteristic of sweet whey and acid whey**

| Constituents | Sweet whey (g/L) | Acid whey (g/L) |
|---|---|---|
| Total solids | 63.0-70.0 | 63.0-70.0 |
| Lactose | 46.0-52.0 | 44.0-46.0 |
| Protein | 6.0-10.0 | 6.0-8.0 |
| Fat | 5.0 | 0.4 |
| Lactate | 2.0 | 6.4 |
| Ash | 5.0 | 8.0 |
| Calcium | 0.4-0.6 | 1.2-1.6 |
| Phosphate | 1.0-3.0 | 2.0-4.5 |
| Chloride | 1.1 | 1.1 |

**Table 2. Bioutilization of cheese whey for production of value-added products**

| Product category | Microorganism | Product | References |
|---|---|---|---|
| **Probiotics and biomass (single cell protein)** | *Kefir* microflora | Single-cell protein | Paraskevopoulou et al., 2003 |
| | *Lactobacillus casei* | Probiotics | Aguirre-Ezkauriatza et al., 2010 |
| | *Candida kefyr* & *Candida valida* | Biomass (Yeast) | Carlotti et al., 1991 |
| **Enzymes** | *Bacillus* spp. | α-amylase | Bajpai, 1991 |
| | *Kluyveromyces marxianus* | β-galactosidase | Fonseca et al., 2008 |
| | *Candida rugosa* | Lipase | Tommaso et al., 2010 |
| | Recombinant *Escherichia coli* | Penicillin acylase | De León-Rodríguez et al., 2006 |
| **Peptides and proteins** | *Lactococcus lactis* | Nisin (Bacteriocin) | Liu et al., 2005 |
| | | Bioactive peptides | Hernandez-Ledesma et al., 2008 |
| | *Kluyveromyces marxianus* & *Lactobacillus rhamnosus* | ACE-Inhibitory peptides (Antihypertensive) | Hamme et al., 2009 |
| **Oligosaccharides and biopolymers** | *Kluyveromyces marxianus* | Galacto-oligosaccharides | Petrova & Kujumdzieva, 2014 |
| | *Lactobacillus rhamnosus* | Expolysaccharides (EPS) | Pham et al., 2000 |
| | *Xanthomonas campestris* | Xanthan gum | Mesomo et al., 2009 |
| | *Leuconostoc mesenteroides* | Dextran & Fructose | (Santos et al., 2005) |
| | *Azotobacter chrooccum* | Poly-β-hydroxy butyrate (PHB) | Khanafari et al., 2006 |
| | *Kluyveromyces lactis* | D-arabitol | Toyoda & Ohtaguchi, 2011 |
| | | Xylitol | Toyoda & Ohtaguchi, 2009 |
| | | Lactulose | Gänzle et al., 2008 |
| | *Kluyveromyces marxianus* | Oligonucleotides | Belem & Lee, 1999 |
| | *Rhizopus oryzae* | Chitosan | Chatterjee et al., 2008 |

| | | | |
|---|---|---|---|
| **Organic acids and biochemicals** | Lactic acid bacteria (LAB) | Lactic acid | Panesar et al., 2007 |
| | *Aspergillus niger* | Citric acid | El Aasar, 2006 |
| | *Actinobacillus succinogenes* | Succinic acid | Wan et al., 2008b |
| | *Kluyveromyces fragilis* | Acetic acid & glycerol | Mostafa, 2001 |
| | *Aspergillus niger* | Gluconic acid | Mukhopadhyay et al., 2005 |
| | *Propionibacterium acidipropionici* | Propionic acid | Morales et al., 2006 |
| | *Bacillus licheniformis* K51 | Biosurfactant | Joshi et al., 2008 |
| | *Anaerobiospirillum succiniciproducens* | Succinate-rich animal feed supplement | Samuelov et al., 1999 |
| **Biocontrol agent** | | *Beauveria bassiana* & *Metarhizium anisopliae* | Kassa et al., 2008 |
| | | *Steinernema carpocapsae* | Chavarria-Hernandez et al., 2006 |
| | | *Bacillus sphaericus* | El-Bendary et al., 2008 |
| **Fuel/Energy** | | Biogas | Gelegenis et al., 2007 |
| | *K. marxianus* | Ethanol | Zafar & Owais, 2006; Guimarães et al., 2010 |
| | | Hydrogen | Davila-Vazquez et al., 2009 |

**Table 3. Cultivation conditions and process yields during different mode of fermentation for bioprotein production from whey**

| Fermentation | Substrate | Microorganism | Nitrogen source | pH | Temp. ($^oC$) | μ (1/h) | $Y_{x/s}$ (g/g) | Productivity (g/L/h) | COD removal efficiency (%) | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Batch | CW | *Candida kefyr* & *C. valida* | $(NH_4)_2$ $SO_4$ +yeast extract | 4.5 | 35 | - | 0.38 | - | - | Carlotti et al., 1991 |
| Batch | CW | *Kluyveromyces fragilis* | - | 4.4 | 31 | 0.150 | 0.44 | ND | 90.6 | Ghaly & Kamal, 2004 |
| Batch | WP | *K. lactis* & *Saccharomyces cerevisiae* | $(NH_4)_2$ $SO_4$ | 4.5 | 25 | - | - | 0.47 | 88.5 | Moeini et al., 2004 |
| Batch | DWC | *K. marxianus* | - | 5.8 | 30 | ND | 0.52 | ND | 90.0 | Schultz et al., 2006 |
| Batch | DSWC | *K. marxianus* | - | 4.8 | 30 | ND | 0.48 | ND | 83.0 | Schultz et al., 2006 |
| Batch | WP | *K. marxianus* | $(NH_4)_2$ $SO_4$ | 5.0 | 34 | 0.230 | 0.26 | 0.45 | 88.5 | Anvari & Khayati, 2011 |
| Batch | CW | *K. marxianus* | - | 3.5 | 40 | 0.200 | 0.12 | 0.16 | 55.0 | Yadav et al., 2014a |
| Batch | CW | *K. marxianus* | Urea | 3.5 | 40 | 0.210 | 0.26 | 0.15 | 78.0 | Yadav et al., 2014c |
| Batch | CW | *K. marxianus+C. krusei* | Urea | 3.5 | 40 | 0.200 | 0.31 | 0.20 | 86.8 | Yadav et al., 2014c |
| Fed-batch | WP | *Torulopsis cremoris* & *C. utilis* | $(NH_4)_2$ $SO_4$ | 4.8 | 29 | - | 0.75 | - | 95.8 | Cristiani-Urbina et al., 2000 |
| Fed-batch | DPP | *K. marxianus* | $(NH_4)_2SO_4$ + yeast extract | 5.0 | 30 | - | 0.38 | 2.90 | ND | Lukondeh et al., 2005 |
| Continuous | CW | *K. fragilis* | - | 4.5 | 33 | 0.105 | 0.26 | 0.40 | ND | Ghaly et al., 2005 |
| Continuous | CW | *K. fragilis* | - | 4.5 | 35 | 0.041 | NA | 0.21 | ND | Ben-Hassan & Ghaly, 1995 |
| Continuous | CW | *K. marxianus* | Urea | 3.5 | 40 | 0.026 | 0.19 | 0.26 | 78.5 | Yadav et al., 2014a |

***Note**- CW: Cheese whey; WP: Whey permeate; DWC: De-proteinized sweet whey concentrate; DSWC: De-proteinized sour whey concentrate; DPP: Deproteinated lactose powder; ND: Not defined.

**Table 4. Essential amino acid profile of SCPs of different microorganisms and their comparison with FAO standard**

| | g/100 g Protein | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Microorganisms (substrate)** | **Leucine** | **Lysine** | **Threonine** | **Valine** | **Isoleucine** | **Tyrosine** | **Methionine** | **Methinine + Cystine** | **References** |
| *Kluyveromyces marxianus* CBS 6556 (whey permeate) | 7.7 | - | 6.94 | 7.5 | 5.48 | 2.5 | 0.77 | - | Schultz et al., 2006 |
| *Kluyveromyces marxianus* ATCC 8554 (whey) | 10.6 | 5.5 | 15.2 | ND* | 6.7 | - | 1.73 | 1.73 | Paez et al., 2008 |
| *K. fragilis* | 3.8 | 1.8 | 2.5 | 3.0 | 5.2 | 1.7 | 0.5 | 1.4 | Martini et al., 1979 |
| *Saccharomyces cerevisiae* (molasses) | 7.9 | 8.2 | 4.8 | 5.5 | 5.5 | 1.2 | - | 4.1 | Paez et al., 2008 |
| *Candida krusei* SO1 (sorghum) | 5.6 | 6.5 | 4.1 | 4.0 | 4.18 | - | 0.91 | - | Konlani et al., 1996 |
| *Candida utilis* | 3.5 | 1.7 | 2.5 | 2.7 | 5.2 | 1.8 | 0.3 | 1.2 | Martini et al., 1979 |
| *Candida utilis* | 4.4 | 7.6 | 3.4 | 4.0 | 3.2 | 2.60 | 1.5 | 4.2 | Adedayo et al., 2011 |
| *Lactobacillus fermentum* 3957 (trypticase soy broth) | 6.3 | 7.1 | 3.6 | 4.9 | 4.4 | - | 2.0 | - | Erdman et al., 1977 |
| FAO/WHO | 7.0 | 5.5 | 4.0 | 5.0 | 4.0 | 2.80 | 2.20 | 3.5 | FAO/WHO, 2007 |

ND: Not determined

**Table 5. Typical components of various whey-derived proteinaceous products**

| Products | Protein (%) | Lactose (%) | Fat (% ) | Ash (% ) | Moisture (%) |
|---|---|---|---|---|---|
| Sweet whey powder | 11.0-14.5 | 63.0-75.0 | 1.0-1.5 | 8.2-8.8 | 3.5-5.0 |
| Acid whey powder | 11.0-13.5 | 61.0-70.0 | 0.5-1.5 | 9.8-12.3 | 3.5-5.0 |
| Reduced lactose whey | 18.0-24.0 | 52.0-58.0 | 1.0-4.0 | 11.0-22.0 | 3.0-4.0 |
| Dematerialized whey | 11.0-15.0 | 70.0-80.0 | 0.5-1.8 | 1.0-7.0 | 3.0-4.0 |
| WPC34 | 34.0-36.0 | 48.0-52.0 | 3.0-4.5 | 6.5-8.0 | 3.0-4.5 |
| WPC50 | 50.0-52.0 | 33.0-37.0 | 5.0-6.0 | 4.5-5.5 | 3.5-4.5 |
| WPC60 | 60.0-62.0 | 25.0-30.0 | 1.0-7.0 | 4.0-6.0 | 3.0-5.0 |
| WPC75 | 75.0-78.0 | 10.0-15.0 | 4.0-9.0 | 4.0-6.0 | 3.0-5.0 |
| WPC80 | 80.0-82.0 | 4.0-8.0 | 4.0-8.0 | 3.0-4.0 | 3.5-4.5 |
| WPI | 90.0-92.0 | 0.5-1.0 | 0.5-1.0 | 2.0-3.0 | 4.5 |

**Table 6. Protein composition and basic characteristics of the whey proteins**

| Protein | Content (% w/w) | Molecular mass (kDa) | Isoelectric pH (pI) |
|---|---|---|---|
| Immunoglobulin's(Igs) | 8.0 | 150-1000 | 5.5-8.3 |
| Bovine serum albumin (BSA) | 5.0 | 66.0 | 5.13 |
| Lactoferrin (Lf) | 1.0 | 76.5 | 9.5-10.0 |
| β-Lactoglobulin (β-LG) | 40-50 | 18.3 | 5.35-5.49 |
| α-Lactalbulin (α-LA) | 12-15 | 14.0 | 4.2-4.5 |
| Glycomacropeptide (GMP) | 12.0 | 6.8 | 4.3-4.6 |
| Proteose- peptone | 0.19 | 4-22 | - |
| Lactoperoxidase(LP) | 0.5 | 78.0 | 9.5 |

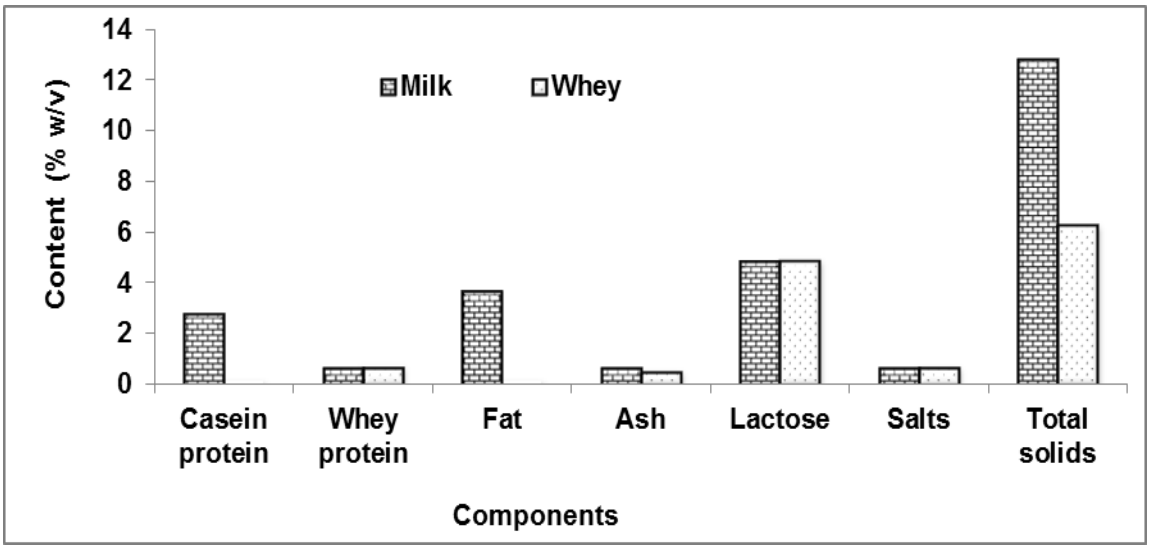


**Figure 1. Comparison of the proximate analysis of bovine milk and its whey**

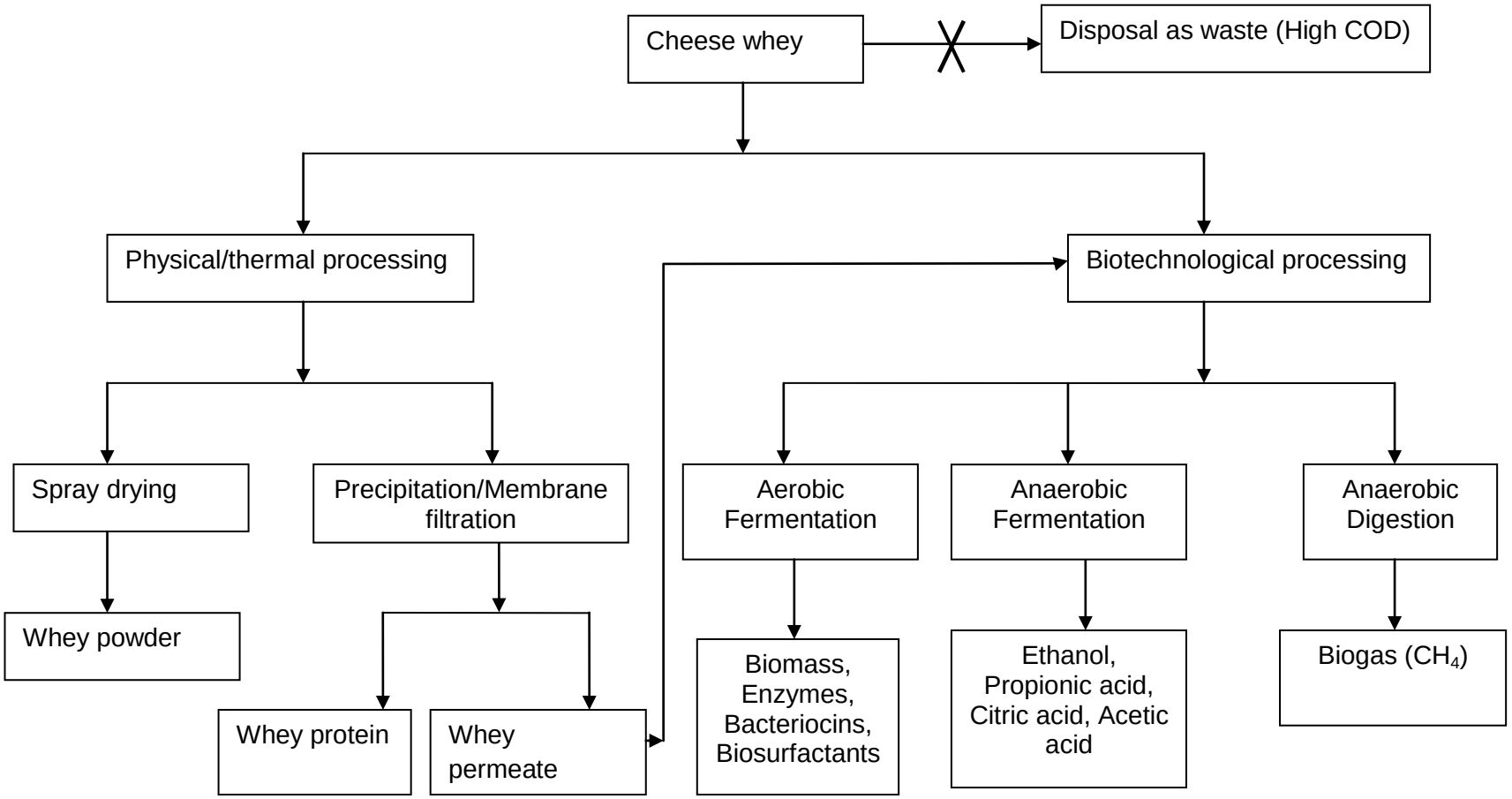


**Figure 2. Flow diagram of the processing of whey by physical/thermal and biotechnological method**

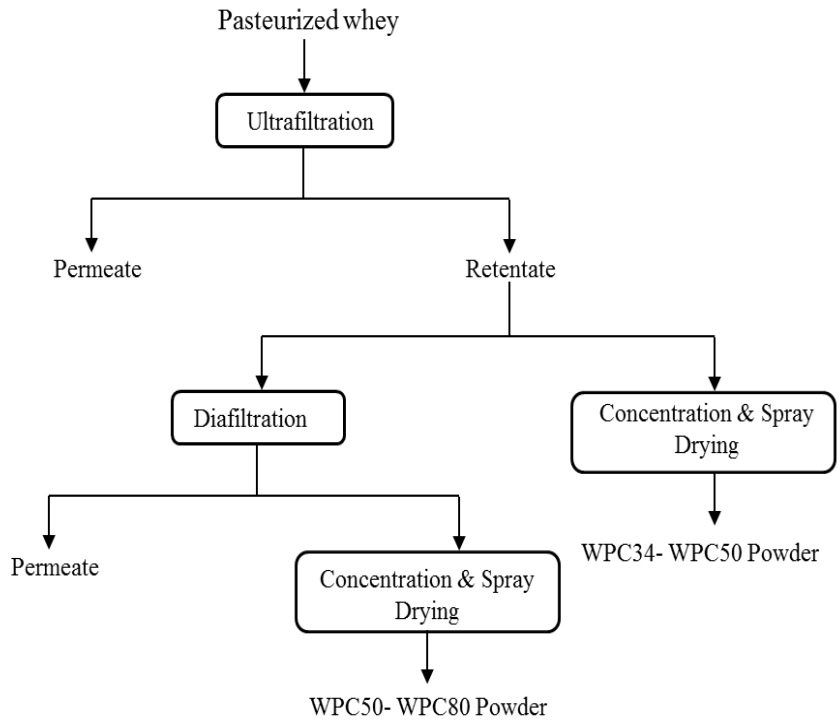


**Figure 3. Processing for the production of various whey protein concentrates (WPCs)**

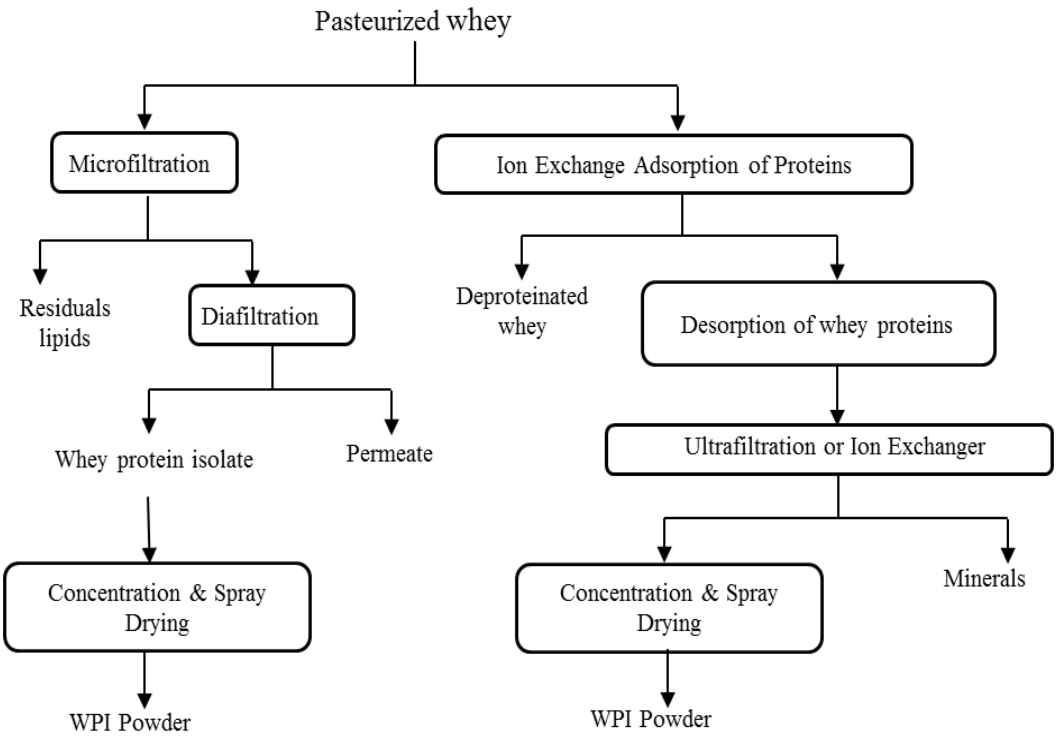


**Figure 4. Processing for the production of whey protein isolate (WPI)**

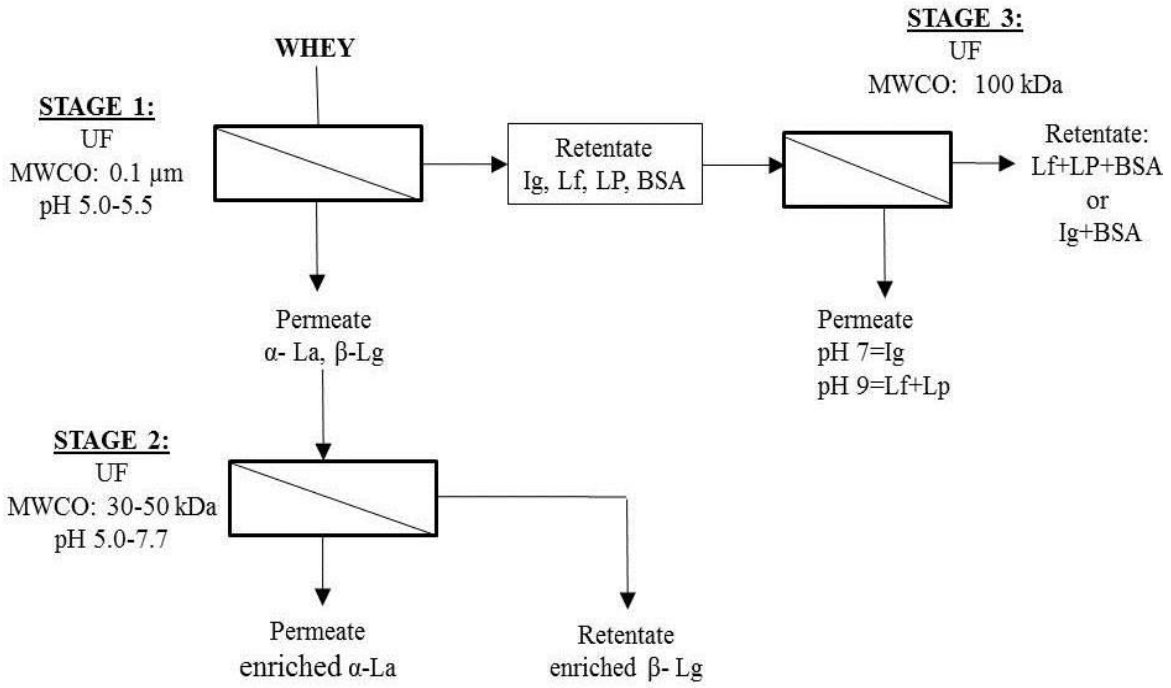


**Figure 5. Three-stage membrane filtration system for sequential fractionation of whey proteins from whey**

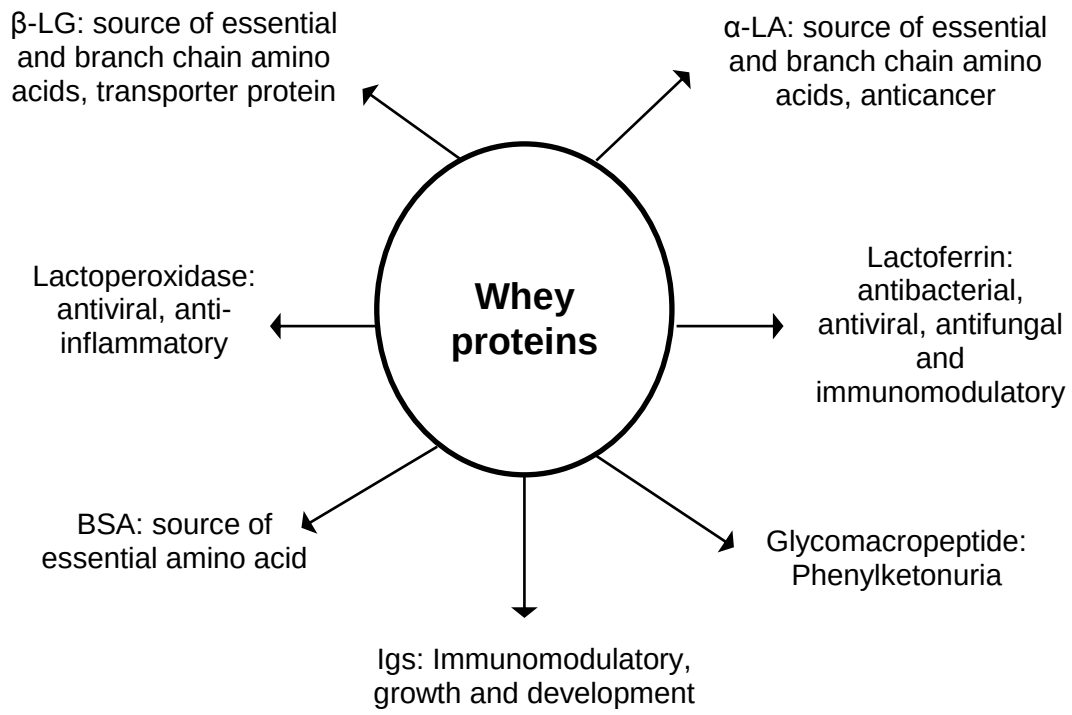


**Figure 6. Important functional and nutritional roles of individual whey proteins**

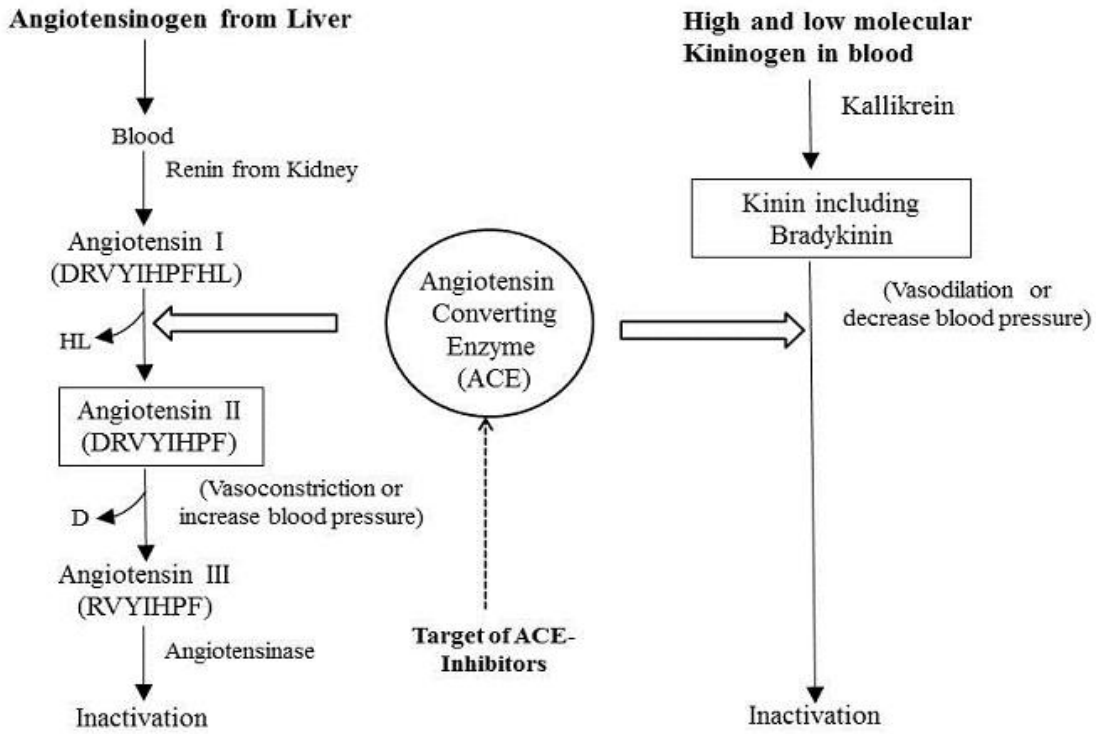


**Figure 7. The regulatory system of human blood pressure by the rennin-angiotensin system and the Kallikrein-Kinin system**

# PARTIE 2

# SIMULTANEOUS SINGLE CELL PROTEIN PRODUCTION AND COD REMOVAL WITH CHARACTERIZATION OF THE RESIDUAL PROTEINS AND INTERMEDIATE METABOLITES DURING WHEY FERMENTATION BY K. MARXIANUS

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**Bioprocess and Biosystems Engineering**

**37(2014):1017-29**

# RÉSUMÉ

La fermentation du lactosérum avec *K. marxianus* a été effectuée à 40 $^{o}$C et à pH 3,5 dans le but d'examiner la production simultanée des protéines unicellulaires (PU) avec l'élimination de la DCO, mais aussi afin de déterminer le sort des protéines solubles du lactosérum et de caractériser les métabolites intermédiaires. Après 36 heures de fermentation discontinue, la concentration de la biomasse a augmenté de 2,0 à 6,0 g/L avec une réduction de 55% en DCO (incluant les protéines), tandis que la concentration en proteins solubles du lactosérum a diminué de 5,6 g/L à 4,1 g/L. La nature différente des protéines du lactosérum fermenté, par rapport aux protéines naturelles du lactosérum, a été confirmée via électrophorèse. Les analyses HPLC et GC-MS ont révélé une modification de la composition en composés organiques pendant la fermentation. Une forte concentration de l'inoculum a donné lieu, après 36 heures de fermentation discontinue, à une augmentation de la concentration de la biomasse de 10,3 à 15,9 g/L, à une réduction en DCO de 80% (incluant les protéines), ainsi qu'à une concentration de 4,5 g/L en protéines résiduelles. Après 22 heures de fermentation discontinue, la concentration de la biomasse a augmenté de 7,3 à 12,4 g/L avec une réduction de 71% de la DCO et 4,3 g/L de concentration en protéines résiduelles. À la suite de ce troisième procédé, le mode discontinu de fermentation a été remplacé par un mode continu avec recyclage cellulaire, et l'état à l'équilibre a été atteint après 60 heures supplémentaires dont le rendement en biomasse était de 0,19 g de biomasse/g de lactose avec 0,26 g/L.h de productivité. L'efficacité d'élimination de la DCO était de l'ordre de 78-79% avec une teneur en protéine résiduelle de 3,8 à 4,2 g/L. Le procédé continu de fermentation aérobie avec recyclage cellulaire a ainsi pu être appliqué à la production de protéines d'origine unicellulaire provenant du lactosérum de fromage, avec une importante élimination de la DCO à pH faible et à température élevée.

**Mots-clés:** Protéines unicellulaire; Lactosérum; DCO; Protéines du lactosérum; Métabolites intermédiaires; Recyclage cellulaire.

## ABSTRACT

Cheese whey fermentation with *K. marxianus* was carried out at 40 $^{o}$C, pH 3.5, to examine simultaneously single-cell protein (SCP) production and COD removal, to determine the fate of soluble whey protein and to characterize the intermediate metabolites. After 36 h of batch fermentation, biomass concentration increased from 2.0 to 6.0 g/L with 55% COD reduction (including proteins), whereas the soluble whey protein concentration decreased from 5.6 g/L to 4.1 g/L. It was confirmed through electrophoresis (SDS-PAGE) that the fermented whey proteins were different from the native whey proteins. HPLC and GC-MS analyses revealed a change in composition of organic compounds post-fermentation. High inoculum concentration in batch fermentation resulted in an increase in biomass concentration from 10.3 to 15.9 g/L with 80% COD reduction (including proteins) within 36 h with a residual protein concentration of 4.5 g/L. In a third batch fermentation, the biomass concentration increased from 7.3 to 12.4 g/L with 71% of COD removal and a residual protein concentration of 4.3 g/L after 22 h. After 22 h, the batch process was shifted to a continuous process with cell recycle and the steady state was achieved after another 60 h with a biomass yield of 0.19 g biomass/g lactose and a productivity of 0.26 g/L.h. COD removal efficiency was 78-79% with residual protein content of 3.8-4.2 g/L. The aerobic continuous fermentation process with cell recycle could be applied to single-cell protein production from cheese whey with substantial COD removal at low pH and high temperature from cheese whey.



**Keywords:** Single-cell protein; Cheese whey; COD; Whey protein; Intermediate metabolites; Cell recycle.

# INTRODUCTION

Cheese whey is a byproduct of the cheese-producing industry, which is considered as an environmental pollutant due to its high biological oxygen demand (BOD) of 40-60 g/L and its chemical oxygen demand (COD) of 60-80 g/L. The main components of cheese whey are lactose (4.5-5.0% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (0.8-1.0% w/v) [1, 2]. Whey proteins are the major second dominant component in cheese whey. Whey protein is a mixture of different proteins which are composed of β-lactoglobulin (40-50% w/w), α-lactalbumin (12-15% w/w), immunoglobulin's (8% w/w), bovine serum albumin (5% w/w), lactoferrin (1% w/w), lactoperoxidase (0.5% w/w), proteose-peptone and glycomacropeptide (12% w/w) [3].

Production of cheese whey is on the rise due to increase in demand for milk and milk-derived products. As per a Food and Agriculture Organization (FAO) 2005 report, the worldwide annual production of cheese whey was around 139 billion kilograms of which 35.2 and 3.1 billion kilogram were generated in the USA and Canada, respectively, with a global annual growth rate of 2% [4-6]. Around half of the whey is either dumped into sewers or spread on land [4]. The disposal and land applications have detrimental impacts on health and the environment [4, 7]. The availability of surplus amounts of cheese whey provides an opportunity to apply various biotechnological processes to convert it into value-added products such as ethanol, lactic acid, enzymes, biopolymers, biogas and single-cell protein (SCP) [1, 2]. Bioconversion of whey into SCP has become one of the best solutions and is carried out in many countries [1, 2, 8]. The use of whey for the production of proteinaceous yeast biomass is advantageous because it is a simple process and final discharge of the whey is facilitated due to reduced organic content by utilization of lactose during yeast biomass production.

SCP is generally produced using yeasts such as *Kluyveromyces*, *Candida* and *Trichosporon* species as they can naturally metabolize whey lactose [9]. *Kluyveromyces marxianus* species have been the most widely studied for SCP production from cheese whey and categorised as generally regarded as safe (GRAS) microorganisms for food and feed applications [9-11]. SCP production is a good alternative for whey treatment; however, there are some challenges: i) economics of the process and contamination problems; ii) low efficiency of COD removal; iii) fate and recovery of the soluble whey proteins after fermentation (when raw whey is fermented without protein removal). On-site production of SCP as animal feed using cheese whey is not commercialised due to contamination problems and poor economic returns [12-15]. The fermentation at extreme conditions such

as low pH (3 to 4) and high temperature (40 to 45 $^{o}$C) has been suggested to prevent the contamination problem [1, 16]. A few large scale companies are able to remove the whey proteins before fermentation through membrane separation technology (ultrafiltration and nanofiltration); however, application of membrane technology is not possible for small- and medium-scale companies for economic reasons [15, 17]. Till date, most of the studies and trends for SCP production use whey permeate (after removal of whey proteins by ultrafiltration) [2, 18-20]. Therefore, there is a need for an alternative process, one which could efficiently tackle the problem with on-site utilization of cheese whey.

SCP production and COD removal can be carried out employing basic bioprocess engineering principles through the running of different modes of fermentation, such as a batch, fed-batch or continuous process. However, each process has its own advantages and disadvantages. The choice of the mode depends on the objective i.e. production or treatment or both. However, batch process information is always essential before developping a continuous or fed-batch process. Moreover, a continuous process is usually preferred at industrial scales for economic reasons [21].

Thus, the aim of the present research was to study a fermentation strategy for yeast SCP production, with high COD removal, using raw cheese whey (with proteins) as substrate, at low pH and high temperature to minimise contamination. Further, the study was also undertaken to characterise the residual soluble whey proteins and the intermediate compounds produced during the fermentation.

# MATERIALS AND METHODS

## Cheese whey

Fresh cheese whey was obtained from the cheese producing industry in Quebec, Canada, and stored at -20 $^{o}$C until used. The major composition of the cheese whey is presented in Table 1. A higher lactose concentration in the whey increases the chances of ethanol formation [22]. Therefore, cheese whey with a COD of 68.0±2.0 g/L was diluted to a COD of approximately 50.0 g/L, and the diluted whey was used in batch or continuous fermentation processes.

## Microorganism

A strain of yeast *K. marxianus* was isolated from cheese whey and identified by biochemical and molecular methods in our laboratory (accession number GQ 506972). The culture was subcultured on de Man, Rogosa and Sharpe (MRS) agar plates and incubated at 35 $^{o}$C for 24 h and preserved at 4 $^{o}$C for subsequent use.

## Inoculum preparation

MRS broth was prepared and sterilized at 121 $^{o}$C for 15 min. A loopful of *K. marxianus* grown on MRS agar plates was used to inoculate 100 mL Erlenmeyer flasks containing 20 mL of MRS medium. The flasks were incubated in an incubator shaker at 150 rpm and 35 $^{o}$C for 12 h. The actively grown cells from the flasks were used as a pre-culture to inoculate 2 L Erlenmeyer flasks containing 500 mL of pasteurized cheese whey and the flasks incubated under the conditions described above. The actively grown cells from the flask were used as inoculum to inoculate 10 L of medium placed in a bioreactor.

## Fermentation

Fermentation was carried out in a stirred tank 15 L bioreactor (working volume: 10 L, Biogenie, Quebec, Canada) equipped with accessories and a programmable logic control (PLC) system for dissolved oxygen (DO), pH, anti-foam, impeller speed, aeration rate and temperature. The software (iFix 3.5, Intellution, USA) allowed for automatic set-point control and integration of all parameters via PLC. Before each sterilization cycle, the polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR, Canada). The oxygen probe was calibrated to zero (using $N_2$ gas) and to 100% (air saturated water). Fresh cheese whey (10 L) with initial COD 50.0 g/L was transferred to the 15 L bioreactor followed by pasteurization for 15 min at 80 $^{o}$C and pH 3.5 (original pH of the whey). After pasteurization, the temperature was reduced to 40 $^{o}$C and the DO probe was re-calibrated. The fermentation was conducted at low pH (3.5) and high temperature (40 $^{o}$C) to minimize contamination, which is one of the most common problems in industry [1, 16]. The bioreactor was inoculated described below. Three fermentation runs were conducted with normal, high and medium-cell-density inoculums as described below.

### Fermentation with normal cell density inoculum

The first batch bioreactor was inoculated with 5% (v/v) inoculum (initial suspended solids concentration of 2.0 g/L) of *K. marxianus* which was prepared as described earlier. To keep the DO above 25% saturation (critical DO for *K. marxianus)*, air flow and agitation rates were appropriately adjusted. pH 3.5 was maintained using 4 N $H_2SO_4$ or 4 N NaOH and a polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was used to control foaming during fermentation. The fermentation was carried out for 36 h.

### Fermentation with high-cell-density inoculum

The second batch fermentation was carried out with a high-cell-density inoculum (initial suspended solids concentration of 10.3 g/L). The inoculum was prepared by growing *K. marxianus* in a 10 L (working volume) bioreactor for 24 h. To obtain rapid growth of *K. marxianus*, the fermentation (inoculum production) was conducted at pH 5.5 and at 35 $^{o}$C using diluted cheese whey (initial COD 50.0 g/L) as substrate. The fermented broth was centrifuged aseptically to obtain a concentrated biomass, which was used as the inoculum.

Bioreactor preparation and feed pasteurization for the second batch was done similarly to the first batch fermentation, except that feed (cheese whey) was supplemented with urea (0.22% w/v, following optimization studies in shake flasks) as extra nitrogen source to minimize nitrogen limitation. Urea as nitrogen supplement was chosen because it is cheaper compared to other nitrogen sources and its consumption, unlike that of ammonium sulfate, does not change the pH of the medium [23]. The fermentation was conducted at pH 3.5 and 40 $^{o}$C for 36 h.

### Batch fermentation with medium-cell-density inoculum followed a switch to continuous fermentation

The third batch fermentation was conducted using a medium cell density inoculum (initial suspended solids concentration of 7.3 g/L). The inoculum was prepared similarly to the one for the second batch. The batch fermentation process in this case was switched to a continuous process with an hydraulic retention time (HRT) of 24 h, after 22 h of batch operation. Bioreactor preparation and feed (fortified with 0.22% w/v of urea) pasteurization were conducted as described for the second batch fermentation.

In batch fermentation, the specific growth rate (μ) is calculated from the slope of the line of a semi log plot of cell concentration versus time. For continuous fermentation with cell recycle system, at steady state the following equations were derived using mass balance [21] in the bioreactor (Fig. 1) to calculate the specific growth rate (μ):

$$\mu = D[1 + r(1 - \alpha)] \qquad (1)$$

Where,

$$\text{Factor of cell concentration } (\alpha) = \frac{X_r}{X}$$

$$D = \frac{1}{HRT}$$

$$r = \frac{F_r}{F}$$

In which X and $X_r$ are the biomass concentration in the bioreactor and in the centrifuged biomass (recycle stream) respectively; *F*, $F_r$, $F_1$, *r*, *V* and *D* represent the feed flow rate (L/h), recycle flow stream (L/h), total flow in the reactor (L/h), recycle ratio, working volume of the reactor (L) and the dilution rate (1/h). $X_0$ and $X_2$ represent the biomass concentration in feed and supernatant, respectively (Fig. 1). The high-cell-density biomass (between 12.0-13.0 g/L) during the continuous fermentation was maintained by recycling centrifuged biomass. Raw cheese whey feeding (fortified with 0.22% w/v urea), sampling, and biomass recycling feed flow were controlled using an external peristaltic pump (Masterflex, Cole-Palmer, USA). The recycled biomass was prepared by centrifuging the fermented effluent of the bioreactor and the volume was maintained as desired. When the system reached a steady state, it was operated continuously for 8 days (192 h) at 24 h HRT.

## Analytical methods

Samples were withdrawn at regular intervals (in all three runs) and analyzed for cell concentration (colony forming unit-CFU), yeast biomass (suspended solids), reducing sugar (lactose), COD and soluble proteins. Total cell count (CFU/mL) was determined by a standard agar plate technique. The appropriately diluted samples were plated on MRS agar and the plates incubated at 35 $^{o}$C for 24 h to get isolated colonies. Suspended solids were measured according to the APHA Standard Methods [24]. Reducing sugar (lactose) concentration was measured by using the dinitro salicylic acid (DNS) method [25]. 1 mL of appropriately diluted sample, 2 mL of distilled water and 2 mL of DNS solution were

transferred to a test tube, followed by incubation in a water bath at 100 $^{o}$C for 5 min. After that, 15 mL of distilled water (to cool down the reaction mixture, and thus, to stop the reaction and to dilute the colour) was added to the reaction mixture. Absorbance of the solution was read in a spectrophotometer at 540 nm. COD was measured according to APHA Standard Methods [24] using a closed reflex, colorimetric method. Soluble proteins concentration was determined according to the Lowry et al. [26] method using 1 mg/mL bovine serum albumin (BSA) as standard.

The protein content in the biomass at the end of the first batch fermentation was determined using the method described by Lopez et al. [27] after some modifications. The biomass was treated with lysis buffer, which contained 5 mL/L of Triton X-100, 0.372 g/L of ethylenediaminetetraacetic acid (EDTA) and 0.035 g/L of phenylmethylsulfonyl fluoride (PMSF). Then, the biomass suspension was incubated for 20 min under shaking conditions at room temperature. After incubation, the sample was sonicated for 5 min with an ultrasonic homogenizer Auto tune 750W (Cole-Parmer Instruments, Vernon Hills, Illinois, US) to increase lysis of the cells and release the proteins. Protein concentration was measured using the method of Lowry et al. [26]. All the analyses were carried out in triplicates and the average values are presented (with standard error less than 5% of the mean).

## Characterization of residual proteins and of the intermediate compounds

### Electrophoresis of native whey and fermented whey proteins

The nature of the residual proteins in the centrifuged supernatant of the first batch yeast fermented cheese whey was evaluated using electrophoresis and the results compared to those obtained with the native whey proteins. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the protocol of Laemmli [28]. Acrylamide/bis-acrylamide gel 10% was used to prepare the resolving gel, whereas 5% Acrylamide/bis-acrylamide gel was used as stacking gel. Samples were prepared with sample loading buffer and heated for 3 min at 100 $^{o}$C before loading the gel. One lane was also loaded with molecular weight (MW) markers (Sigma, USA) to evaluate the approximate MW of the proteins. After loading the samples, gel electrophoresis was conducted in a VWR electrophoresis unit and was run at a constant voltage of 100 mV for 4 h. Then gel was rinsed with water and stained with a Coomassie Blue solution for 45 min followed by de-staining overnight.

## Evaluation of sugar and intermediate compounds in whey and fermented whey

Cheese whey and centrifuged fermented whey (supernatant) were characterized for their sugars and intermediate compounds (produced during fermentation) to evaluate the components responsible for residual COD in the supernatant. The analyses was carried out by HPLC and GC-MS.

### HPLC analysis

Analysis of sugars and intermediate compounds produced during fermentation was performed using HPLC. The samples of cheese whey and fermented broth were centrifuged at 5000 rpm for 10 min to remove the suspended particles. The collected supernatants from the centrifuged samples were filtered to obtain a clear liquid using Nanosep MF Centrifugal Devices purchased from Pall Life Sciences, USA. The following conditions were used for sugar analysis by HPLC: mobile phase solvent, 480 mM sodium hydroxide solution, isocratic flow rate, 0.4 mL/min; analytical column 4x250 mm dimension (Carbopack$^{TM}$MA1) from Dionex, Dionex ED40 Electrochemical Detector (ED). The data were analyzed using Chromeleon software [29]. The retention time of standard sugars were determined by running standard glucose, galactose and lactose (Sigma Chemicals, USA). After determination of the retention time of the standards, the samples were run through the HPLC under optimal conditions to obtain the sugars and other intermediates profile.

### GC-MS analysis

Cheese whey and centrifuged fermented whey supernatant samples were extracted for analysis of intermediate compounds in two different ways. Firstly, the samples were directly extracted with solvent and, secondly, the samples were distilled and the distillates were extracted by solvent. The two extraction methods were used because precipitation occurred during direct solvent extraction, whereas there were chances of loss of some volatile compounds during distillation. Whey and the supernatant samples (original and distillate) were extracted with dichloromethane ($CH_2Cl_2$). 100 mL of sample (whey or supernatant) was extracted five times with 5 mL of $CH_2Cl_2$ each time. The extracted samples in $CH_2Cl_2$ were concentrated using liquid nitrogen, followed by filtration through a 0.4 µm filter. The filtered samples were used for analysis of the intermediate compounds through GC-MS. GC-MS analysis was carried out after some modifications of the Dragone et al. [30] protocol. A PerkinElmer Clarus-500 GC-MS system was used. Separation of the different compounds was performed on a fused silica capillary Innowax column (length-30 m, I.D 0.25 mm, film 0.25 µm, Agilent Technology) with 1 µL of injected sample. The injection was performed in the split mode (10:1). Helium was used as the career gas at a constant flow of 1 mL/min.

Oven temperature was programmed from 60 to 250 °C at a rate of 5 °C/min with an initial 5 min hold time and a final hold time of 20 min. The detector was set to the electronic impact mode (70 eV), with an acquisition range from m/z 29 to m/z 360. Identification of the volatile and semi-volatile compounds was performed using the Turbo Mass software and the National Institute of Standard and Technology (NIST) library.

# RESULT AND DISCUSSION

## Variation of different parameters during cheese whey fermentation with *K. marxianus*

The first batch fermentation was conducted without supplementation in nitrogen due to the fact that previous research reports did not use extra nitrogen when cheese whey was used as growth medium for *Kluyveromyces* species [7, 31]. Furthermore, this allowed us to evaluate the protein consumption profile and the fate of the proteins without addition of external nitrogen. The nitrogen source was supplemented only when whey permeate was used as growth medium [13, 20].

The results for cell count, biomass (suspended solids), lactose, COD and soluble protein concentration during the fermentation are presented in Fig. 2. The cell count (CFU/mL) of *K. marxianus* increased with time and reached a maximum of $4.60 \times 10^8$ CFU/mL in 30 h. Yeast biomass (suspended solids) concentration during fermentation was also increased and reached 6.0 g/L at 24 h of fermentation. The values for specific growth rate ($\mu_m$), productivity (g/L.h) and yield ($Y_{x/s}$) were found to be 0.20 $h^{-1}$, 0.16 g/L.h and 0.12 g biomass/g lactose consumed, respectively. The specific growth rate ($\mu_m$) of 0.37 $h^{-1}$, biomass yield ($Y_{x/s}$) of 0.37 g/g, biomass concentration 15.0 g/L and biomass productivity of 1.27 g/L.h of *K. marxianus* at pH 5.0 and at 30 $^oC$ have been reported by Lukondeh et al. [32] with 40.0 g/L in initial lactose concentration in a synthetic medium. A comparatively lower biomass yield and a lower productivity in the present work might be due to two reasons: a) extreme fermentation conditions (i.e. low pH 3.5 and high temperature (40 $^oC$)) as used in this work to minimise the contamination problem, and b) deficiency in easily assimilable nitrogen of the medium, because external nitrogen was not supplemented and only a small quantity of the proteins (26% of the initial protein) present in the medium was consumed by the yeast (as presented below). However, nitrogen deficiency may not have been very important as Ghaly and Kamal [7] reported a higher yield (0.44 g/g) but low specific growth rate (0.15 $h^{-1}$) (Table 3) during the production of SCP using undiluted cheese whey at 31 $^oC$ and pH 4.4, without external nitrogen supplementation. In order to eliminate this possibility, we conducted shake flasks

experiments (initial pH 3.5, but not controlled during the experiments) at different concentrations of urea and we found that a 0.22% w/v urea concentration was optimal leading to a 10% improvement in biomass yield. On the other hand, the maximal yield and productivity of *Kluyveromyces* species have been reported at pH 4.4 and 5.8 and temperature 31 and 30 $^{o}$C in batch processes, respectively while cultivating *K. marxianus* on cheese whey [7] and whey permeate [20] (Table 3). Thus, yeast cells cultivated at higher temperature and lower pH, as done in this study, show higher maintenance energy (diverting a greater amount of lactose towards catabolism), which leads to a decrease in biomass yield [33].

The protein content of yeast biomass (or suspended solids) at the end of fermentation was found to be 42% w/w which is higher than the required minimal protein content (40% w/w) for yeast biomass to serve as animal feed as per the guidelines of the Association of American Feed Control Officials (AAFCO) [34].

Most of the lactose (30.0 g/L) was consumed within 18 h and the final lactose concentration was less than 1.0 g/L (Fig. 2). The soluble whey protein concentration decreased up to 12 h (Fig. 2) and slightly increased from 12 to 18 h, which may be due to the excretion of cellular proteins and enzymes. After that, the soluble protein concentration remained almost constant until the end of the fermentation (36 h). A similar trend in protein consumption has been reported [22]. The decrease in soluble protein concentration during the first 12 h of fermentation could be due to their consumption by microorganisms, as nitrogen source. This hypothesis was supported by the fact that *K. marxianus* and *Lactobacillus* species are known to show proteolytic activity on whey proteins [35, 36]. *K. marxianus* is known to possess a serine carboxypeptidase and a lysine aminopeptidase [37]. The stable concentration of soluble proteins after 18 h might be due to partial consumption of the proteins and to simultaneous secretion of extracellular enzymes by *K. marxianus* (such as inulinase and lipase), as this yeast is known to produce various enzymes [11].

COD decreased rapidly up to 18 h (Fig. 2) of fermentation followed by a slower reduction. This could be due to the rapid increase in yeast cells, which consumed most of the lactose during the first 18 h. However, COD continued to decrease from 18 h until the end of fermentation (36 h), which could be due to the consumption of intermediate compounds that might have formed during the first 18 h or were already present in the cheese whey. A COD reduction of 55% was achieved at the end of the fermentation. The high residual COD (21.8 g/L) in the fermented whey supernatant was due to residual soluble proteins (4.1 g/L) and the other residual organic compounds (present in cheese whey as well as those produced during

fermentation) [38]. Intermediate metabolites such as organic acids (pyruvic acid, malic acid, acetic acid, citric acid, propionic acid, fumaric acid and others) were reported to be produced during whey fermentation by *K. marxianus* [30, 32]. Moreover, it has been reported that the production of intermediate metabolites increased with high initial lactose concentration during fermentation due to a change in the metabolic pathways of *K. marxianus* from oxidative to mixed oxidative [13].

## Characteristics of the native whey proteins and of the fermented whey proteins

SDS-PAGE was carried out on native whey proteins and on supernatants (centrifuged fermented broth) containing residual whey proteins (Fig. 3). Results clearly demonstrated that the nature of the whey proteins changed during fermentation. The native whey proteins (lanes 1 and 2) were in the higher molecular weight (MW) range (mostly above 29 kDa) while the supernatant proteins were largely in the lower MW range (below 29 kDa). Thus, the SDS-PAGE results confirmed that the whey proteins were partially hydrolyzed to gave a lower MW range of proteins during fermentation. Proteolysis of goat whey proteins has also been reported with *K. marxianus* and *Lactobacillus* species [35, 36].

## Sugars and intermediate compounds in whey and fermented whey

### HPLC analysis

The results of HPLC analysis of cheese whey and of supernatants from fermented whey are presented in Fig. 4. The retention times were determined for the three sugars: glucose (24.7 min), galactose (27.63 min) and lactose (29.32 min) (chromatogram not shown). This method was found to be good for analysis of the three sugars at a time with clear separated peaks. The main sugar component of cheese whey is lactose, which was also evident in the HPLC analysis (Fig. 4). The HPLC chromatogram of the supernatant (Fig. 4) indicates that there was no residual lactose after fermentation; however, the presence of many other peaks indicate the presence of secondary metabolites generated during fermentation. These intermediate compounds were responsible for the residual COD after fermentation. It was difficult to identify all of these unknown compounds in the supernatant by HPLC (because standard compounds are required to detect these unknown compounds). Therefore, further identification of these intermediate compounds was carried out using GC-MS.

### GC-MS analysis

The individual separated compounds were identified by GC-MS on the basis of the NIST library and the results are presented in Figs. 5 and 6 and in Table 2. The obtained GC-MS

chromatogram for cheese whey and that for fermented whey supernatant are presented. Both samples were directly extracted with $CH_2Cl_2$. Cheese whey as well as the supernatant contained different groups of volatile compounds such as carbonyls, alcohols, esters, acids, furans and phenols. A comparison of the organic compounds of cheese whey before and after fermentation reveals that some compounds (such as 2-methyl propanol, formic acid, isomaltol, etc.) were degraded and some new intermediate metabolites (such as 2,3-butanediol; 2-furanemethanol; butyrolactone, glycerin, etc.) were generated during fermentation. The degradation of original cheese whey compounds might be responsible for the COD reduction after lactose utilization. The volatile organic compounds (VOCs) and semi-VOCs identified during this study (fermentation conducted at a lower pH and at higher temperature) in cheese whey (Table 2) are similar to those identified (in cheese whey or whey powder) by other researchers [38, 39]. This study also confirmed that some valuable compounds (like phenylethyl alcohol, an aroma compound) are produced during fermentation. The production of aroma compounds (like fruit esters, carboxylic acids, ketones, furans, alcohols and isoamyl acetate) by *K. marxianus* has also been documented [11].

## Fermentation with high cell-density-inoculum

The first batch results revealed low COD reduction (55%) and low biomass yield. In order to obtain higher COD reduction, the second fermentation batch was conducted with a higher cell density inoculum (high initial biomass concentration) considering the fact that a high cell concentration can reduce the diversion of *K. marxianus,* metabolic pathway towards mixed oxidative metabolism, and thus, the less amounts of intermediates will be formed. Further, higher degradation of intermediate metabolites was expected at a higher cell concentration.

The variation in cell count, biomass, lactose, soluble proteins and COD during the fermentation with a high cell density inoculum (10.3 g/L initial suspended solids) is presented in Fig. 7. The cell count (CFU/mL) increased from an initial ($1.03x10^9$ CFU/mL) to a maximum of $1.70x10^9$ CFU/mL in the first 12 h. Initially, the cell count increased rapidly followed by a slow or no growth (Fig. 7). After 12 h, an increase in cell concentration was not observed, which could be due to deficiency in a growth-limiting substrate such as lactose. A similar profile for biomass (suspended solids) increase was also observed; biomass increased from an initial 10.3 g/L to a maximum of 17.1 g/L at 12 h (Fig. 7); thereafter, the biomass started decreasing. The overall increase in biomass (15.9 - 10.3 = 5.6 g/L) at the end of fermentation (36 h) was 39% higher compared to the first batch (biomass increase of 4.0 g/L) and the yield of biomass was also increased by 58% (Table 3). The increase in biomass concentration and

biomass yield could be attributed to two reasons: a) utilization of more whey lactose for oxidative metabolism and b) availability of easily assimilable nitrogen source as urea. However, the yield of biomass was still lower than those observed with processes operated at pH 4.4 and a temperature of 31 $^{o}$C (Table 3) using cheese whey as growth medium. This is due to the higher maintenance energy required to maintain cells at low pH and higher temperature. Additionally, maintenance energy requirement further increased due to higher cell concentrations used in this batch [31, 40]. The specific growth rate (μ), productivity (g/L.h) and yield ($Y_{x/s}$) are presented in Table 3.

Lactose consumption was rapid during the first 18 h (Fig. 7) and approximately more than 98% of the lactose was consumed during this period giving a residual lactose concentration less than 0.7 g/L. The soluble protein concentration decreased during the first 6 h followed by a slight increase at 12 h, which might be due to excretion of some extracellular proteins or enzymes, and it remained almost constant during the rest of the fermentation process with residual protein concentration 4.5 g/L. This again indicates that the proteins could not be utilized after a certain extent. Though urea was supplemented in this batch, however, the addition of urea did not show any effect on the protein consumption trend (i.e. first a decrease, followed by a slight increase and then, the protein content stayed constant as observed in the first batch).

A COD reduction of 80% was obtained at the end of 36 h of fermentation. COD and lactose decreased rapidly during the first 18 h of fermentation and this is attributed to the high cell density. After lactose consumption (i.e. after 18 h), the COD reduction continued slowly up to 36 h of fermentation and this was probably due to consumption of the intermediate metabolites produced, similarly to the first batch fermentation (Table 2). The residual COD at the end of fermentation was 9.6 g/L. The residual COD was due to residual soluble proteins (5.4 g/L) and to lactose (0.8 g/L). The rest 3.3 g/L COD could be due to the intermediate compounds, which *K. marxianus* has produced (as characterized in the first batch fermentation). The value of COD associated to the intermediate metabolites is much lower compared to the COD value observed in the first batch (15.8 g/L). This COD reduction of 80% at the end of fermentation was 25% higher than that of the first batch fermentation (which was operated with a 2.0 g/L initial biomass concentration as inoculum). The results revealed that the degradation of organic compounds (original whey and those substances produced during the fermentation with *K. marxianus*) was higher with a high-cell-density inoculum (initial suspended solids of 10.3 g/L), which resulted in low residual COD. The consumption of intermediate metabolites as carbon sources by *Kluyveromyces* species has been reported to be of diauxic metabolic nature [41]. The second reason for the low residual

COD might be the a low intermediates production due to lower lactose-to-cell (biomass) ratio that resulted in increased oxidative metabolism [13]. The finding of this batch study i.e. higher COD reduction, could be applied to the bioconversion of whey into SCP in a continuous process employing a similar initial cell concentration.

## Batch fermentation with medium-cell-density inoculum and the shifted to continuous fermentation

The second batch fermentation results revealed that a fermentation with a higher cell density at start increased COD degradation efficiency. However, it might be difficult to maintain high cell concentration (15.0-17.0 g/L) during a fermentation process. Therefore, the third batch fermentation was started with a lower cell concentration compared to the second batch fermentation. The variations in the value of the different parameters during this batch fermentation with a medium-cell-density inoculum (7.3 g/L initial suspended solids), such as cell count, biomass, lactose, soluble proteins, and COD are presented in Fig. 8. The cell count (CFU/mL) of *K. marxianus* increased from an initial value of $6.20 \times 10^8$ CFU/mL to a maximum of $1.44 \times 10^9$ CFU/mL at 22 h. Similarly to the second batch fermentation, the increase in cell count (CFU/mL) was rapid. A similar profile for biomass increase was also noticed, which increased from 7.3 g/L to 12.4 g/L at 22 h (Fig. 8). Lactose concentration decreased from 32.4 g/L to 0.7 g/L and a COD removal of 71% was obtained at 22 h. The soluble protein content decreased from 5.7 to 4.3 g/L and there was no further change or little change in the protein concentration with time.

After 22 h, the batch fermentation was switched to a continuous mode with a 24 h HRT. The cell recycle system was used to keep a high biomass concentration (12.0 to 13.0 g/L, almost close to the cell concentration in the second batch) so that the COD degradation would remain high. Steady state during the continuous process was obtained after 60 h of operation. The variations in lactose, COD and soluble protein concentration during the batch plus continuous fermentation are shown in Fig. 8. The lactose concentration increased from 0.7 to 4.3 g/L when the batch process was shifted to the continuous mode and then slowly decreased to 0.8 g/L. The lactose consumption at steady state was 97.5%, whereas the effluent COD concentration varied between 10.0 and 12.0 g/L (Fig. 8). The soluble protein concentration in the effluent at steady state ranged from 3.8 g/L to 4.2 g/L. The specific growth rate ($h^{-1}$), biomass yield ($Y_{x/s}$), productivity (g/L.h) and % of COD reduction during the continuous process are shown in Table 3.

The results of the continuous fermentation with cell recycle showed that a higher COD reduction (up to 78-79%) could be achieved during a fermentation conducted at low pH (3.5) and high temperature (40 $^{o}$C). The biomass productivity 0.26 g/L.h was comparable to that of the batch process (second run). However, the batch process productivity does not include the downtime required for inoculum preparation, harvesting, cleaning and recharging of the bioreactor. This will lower batch productivity of a real-world process. The other advantage of low pH and high temperature as operating conditions is that it provide an opportunity to run the continuous process with cell recycle for a long time without contamination, which is one of the main obstacles of a continuous process for large-scale operations [21].

Studies by others researchers [7, 20, 31, 42, 43] on SCP production and COD removal during batch and continuous processes have been presented in Table 3, which showed somewhat higher yields and higher COD removal efficiencies (in batch processes). These studies were conducted under favorable fermentation conditions (pH 4.5 to 6.0 and temperature 30 to 35 $^{o}$C) where the chances of contamination were higher. Thus, to run the process without contamination while using cheese whey without sterilization, an all loss in yield would be acceptable. Moreover, most of the studies were conducted using whey permeate (Table 3) and reported higher COD removal values. This was due to the absence of residual proteins in the whey permeate. In the present case, COD removal up to 95% could be achieved if the residual soluble proteins are to be recovered from the supernatant. The recovered protein could be mixed with the biomass to increase overall SCP production.

This study provides an alternative continuous process for the biotransformation of raw cheese whey into SCP without removing the whey proteins before fermentation, which will reduce the whey management problem. Also, the residual soluble proteins (3.9 g/L) could be recovered from the fermented whey supernatant and mixed with the biomass (SCP). This could increase the protein content of the final product and further reduce the effluent COD (4.8 g/L). However, this would require further study to optimize the protein recovery process.

## CONCLUSIONS

Biomass concentration increased from 2.0 to 6.0 g/L after 24 h in the case of the batch fermentation with a low-cell-density inoculum. The COD reduction after 36 h was 55% with a residual soluble protein concentration of 4.1 g/L. The SDS-PAGE results revealed that the residual proteins were different (low molecular weight) from the native whey proteins. HPLC and GC-MS analyses indicated that there was no residual lactose in the supernatant, but many other intermediate compounds present were responsible for the residual COD along

with residual proteins. The fermentation with a high-cell-density inoculum resulted in higher COD removal (up to 80%) with a biomass productivity 0.25 g/L.h. The protein consumption trend was similar with all batch fermentations, with residual concentrations ranged between 4.1 and 4.5 g/L. The finding of a higher COD removal with the batch process led us to a continuous process using a cell recycle system. Continuous fermentation with cell recycle gave a COD reduction of up to 78.5% with a biomass productivity of 0.26 g/L.h. Thus, this study established that continuous fermentation with cell recycle could be applied to produce SCP with simultaneous high COD removal. Further studies are required for recovering of the residual soluble proteins and to decrease COD even further.

## ACKNOWLEDGEMENTS

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support.

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**Table 1. Composition of the cheese whey**

| Characteristics | Concentration |
|---|---|
| Color | Yellowish |
| Total solids (g/L) | 65±2 |
| Suspended solids (g/L) | 0.7±0.1 |
| Protein (g/L) | 7±0.5 |
| Lactose (g/L) | 45±2 |
| COD (g/L) | 68±2 |
| pH | 3.5±0.3 |
| Calcium (g/L) | 0.45 |
| Phosphorus (g/L) | 0.45 |
| Potassium (g/L) | 1.50 |
| Sodium (g/L) | 0.77 |

**Table 2. Organic compounds identified in cheese whey and in supernatant from *K. marxianus* fermented cheese whey**

| Before fermentation | | After fermentation | |
|---|---|---|---|
| **VOC** | **RT(min)** | **VOC** | **RT (min)** |
| 2-Methylpropanol | 4.20 | 3-Methylbutanal | 7.25 |
| 3-Methylbutanal | 7.28 | 2-Butane,3-hydroxy | 9.40 |
| Acetic acid hydrazide | 9.82 | Acetic acid hydrazide | 9.80 |
| Propionic acid, 2-hydroxy, methyl ester | 11.33 | Furfural | 14.59 |
| Acetic acid | 13.94 | Acetic acid | 14.86 |
| Furfural | 14.64 | 2,3-Butanidiol | 16.78 |
| Ethanone,1-(2-furanyl) | 15.64 | Octanol-1 (internal standard) | 17.04 |
| Formic acid | 15.40 | Propionic acid,2-methyl | 17.29 |
| Octonol-1 (internal standard) | 16.39 | 2-Furanethanol | 17.61 |
| 2-Furancarboxyaldehyde,5-methyl | 17.37 | Butyrolactone | 18.61 |
| 2-Cyclopentane-1-4-dione | 17.58 | Butanoic acid | 18.82 |
| Isomaltol | 18.53 | Butanoic acid,3-methyl | 19.76 |
| Butanoic acid | 18.81 | Acetamide | 21.72 |
| 2-Furanmethanol | 18.91 | 1-Pentadecanamine,N,N-dimethyl | 22.82 |
| Butanoic acid,3-methyl | 19.80 | Hexanoic acid | 23.65 |
| 2(5H)-furan | 21.49 | Phenylethyl alcohol | 24.88 |
| 1,2-Cyclopentanedione | 21.85 | 2(3H) Furanone,dihydroxy-4,4-dimethyl | 27.24 |
| 2-Cyclohexen-1-ol | 22.10 | Octanoic acid | 27.95 |
| Formamide, N-(2-phenylethyl) | 22.90 | 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl | 31.56 |
| Hexanoic acid | 23.63 | n-Decanoic acid | 31.89 |
| Maltol | 25.33 | Glycerine | 32.44 |
| Octanoic acid | 27.95 | 2-Furancarboxyaldehyde,5-(hydroxymethyl) | 35.53 |
| 2-Propanone,1,3-dihydroxy | 27.72 | 2H-pyron-2-one,tetrahydro-4-hydroxy-4-methyl | 36.33 |
| Methyltartonic acid | 30.32 | 2(3H)-Furanone,dihydroxy-4-hydroxy | 37.06 |
| 4H-pyran-4-one,2,3-dihydro -3,5-dihydro-6-methyl | 31.09 | Not applicable (NA) | NA |
| 9-Decanoic acid | 32.94 | NA | NA |
| Butanedioic acid, monomethylester | 33.07 | NA | NA |
| 2-Furancarboxyaldehyd,5-(hydroxymethyl) | 35.08 | NA | NA |
| 2(3H) Furanone,dihyro-4-hydroxy | 36.60 | NA | NA |
| Tetradecanoic acid | 38.84 | NA | NA |
| n-Hexanoic acid | 42.00 | NA | NA |

**Table 3. Process parameter values and results obtained with different modes of fermentation**

| Fermentation | Substrate | Initial COD (g/L) | pH | Temp. ($^{o}$C) | μ (1/h) | $Y_{x/s}$ (g/g) | Productivity (g/L.h) | COD removal efficiency (%) | References |
|---|---|---|---|---|---|---|---|---|---|
| Batch -1, normal inoculum | CW | 48.9 | 3.5 | 40 | 0.200 | 0.12 | 0.16 | **55.0** (including protein) | This study |
| Batch -2, high cell density inoculum | CW | 49.3 | 3.5 | 40 | 0.027 | 0.19 | 0.25 | **80.0** (including protein) | This study |
| Batch -3, medium cell-density inoculum | CW | 49.9 | 3.5 | 40 | 0.017 | 0.16 | 0.21 | **71.0** (including protein) | This study |
| Continuous with cell recycle | CW | 49.9 | 3.5 | 40 | 0.026 | 0.19 | 0.26 | **78.5** (including protein) | This study |
| Batch | CW | 59.6 | 4.4 | 31 | 0.150 | 0.44 | NA | **90.6** (excluding protein) | [7] |
| Continuous | CW | NA | 4.5 | 33 | 0.105 | 0.26 | 0.40 | NA | [31] |
| Continuous | CW | NA | 4.5 | 35 | 0.041 | NA | 0.21 | NA | [42] |
| Batch | DWC | 150.0 | 5.8 | 30 | NA | 0.52 | NA | **90.0** (excluding protein) | [20] |
| Batch | DSWC | 193.0 | 4.8 | 30 | NA | 0.48 | NA | **83.0** (excluding protein) | [20] |
| Batch | WP | NA | 5.0 | 34 | 0.230 | 0.26 | 0.45 | **88.5** (excluding protein) | [43] |

***Note**- CW: Cheese whey; WP: Whey permeate; DWC: De-proteinized sweet whey concentrate; DSWC: De-proteinized sour whey concentrate; NA: Not available

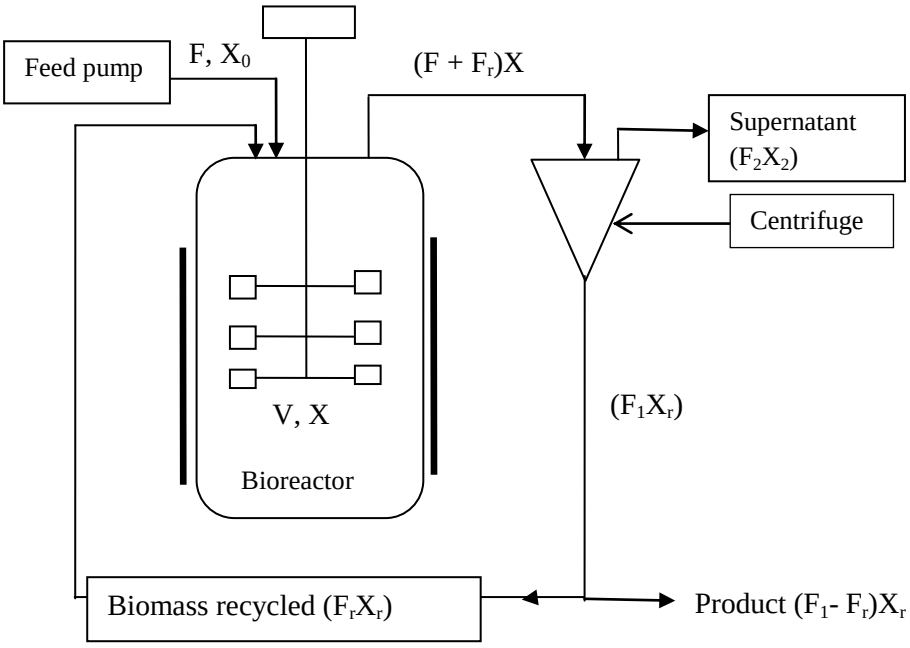



**Figure 1. Schematic diagram of continuous fermentation with a cell recycle system**

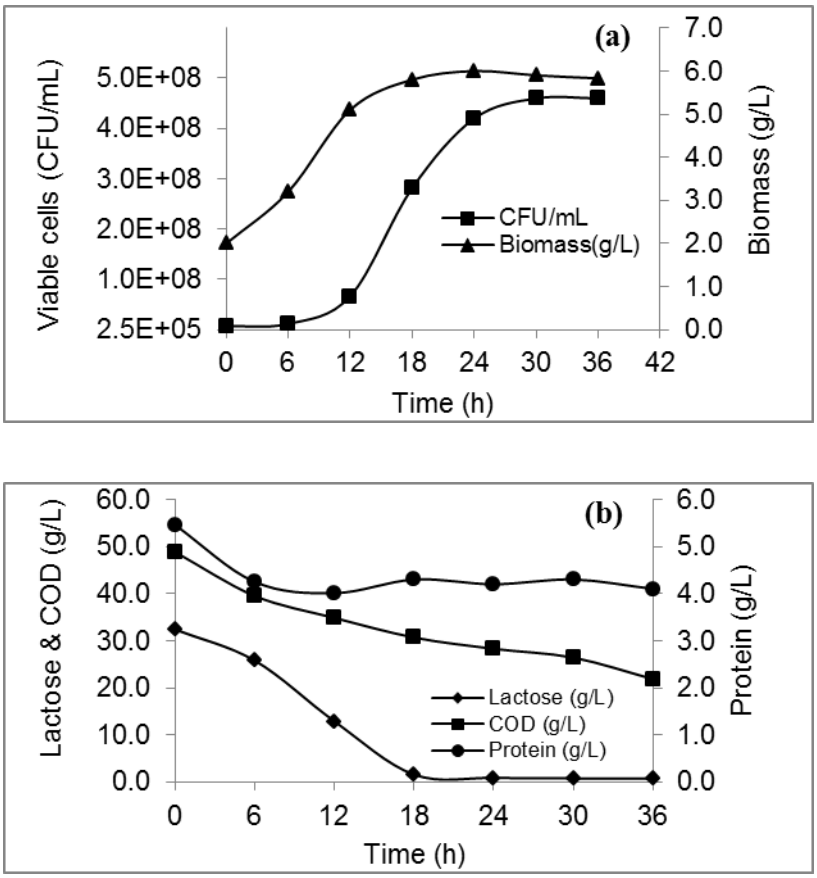



**Figure 2. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during fermentation; (b) Variation in lactose, COD and protein concentration during fermentation**

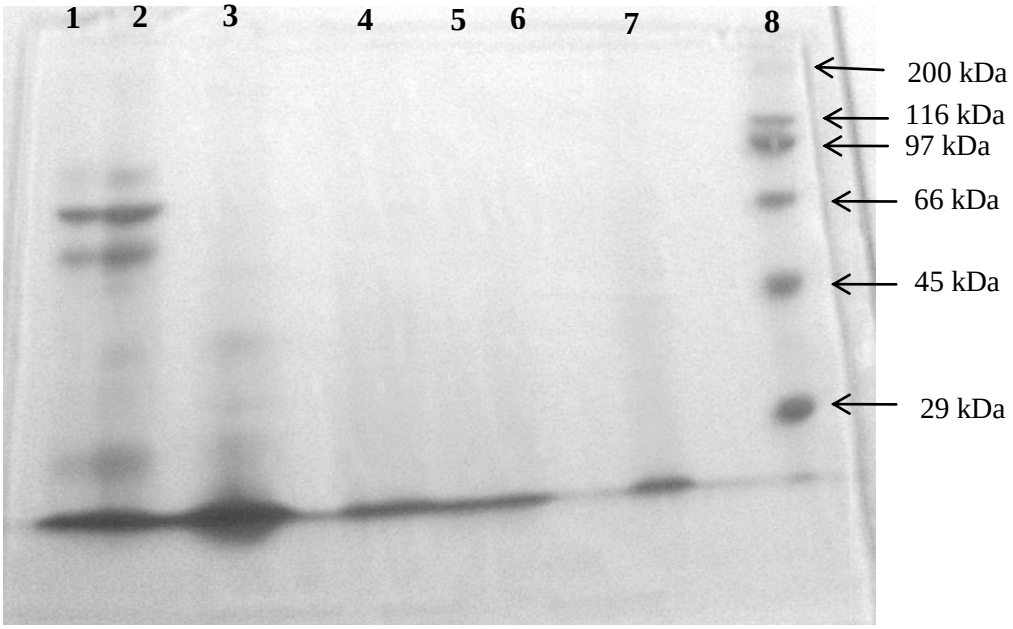



**Figure 3. SDS-PAGE with native whey and fermented whey supernatant proteins (lanes 1 and 2 whey proteins; lanes 3, 4, 5, 6 and 7 supernatant proteins; and lane 8 MW marker)**

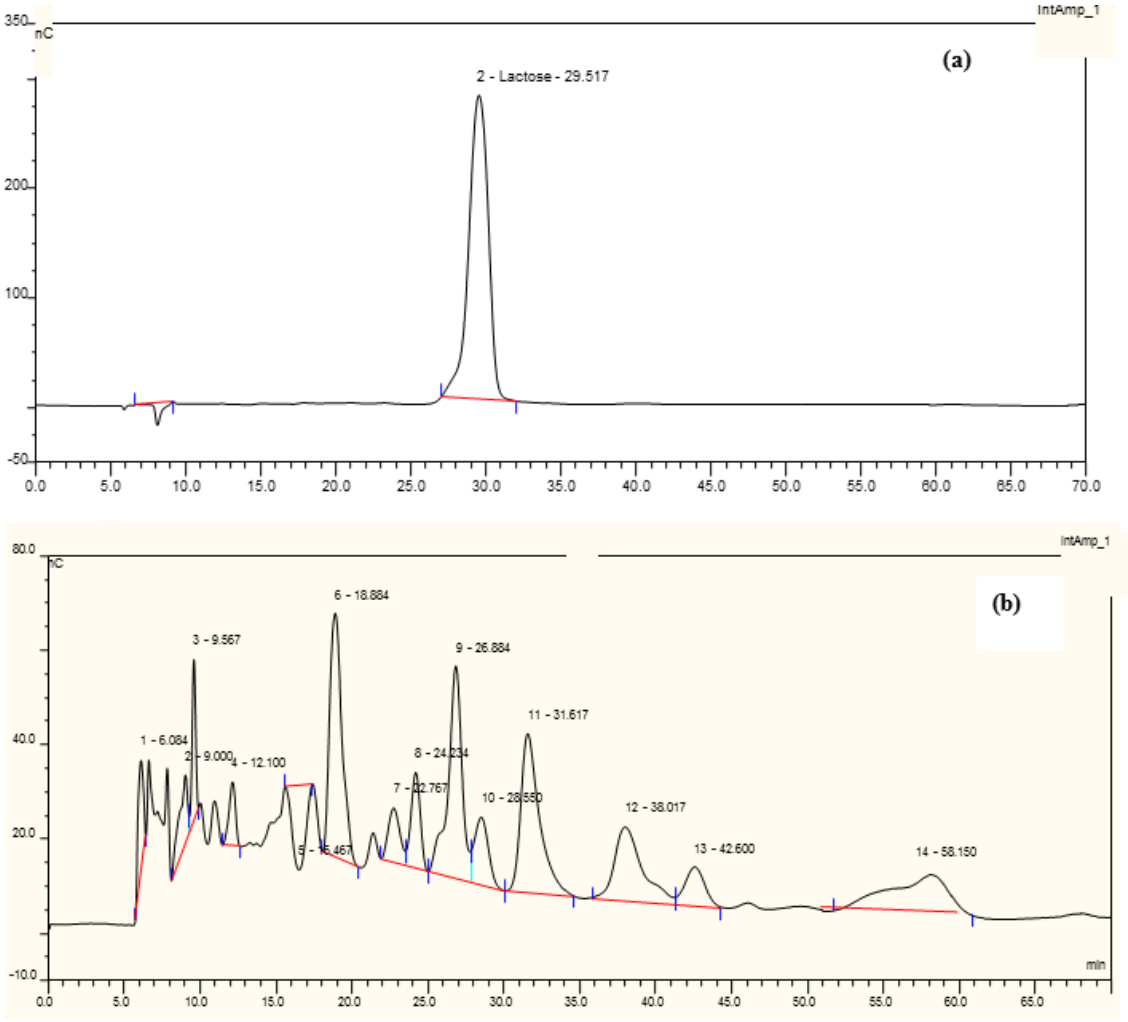



**Figure 4. (a) HPLC chromatogram of a cheese whey sample indicates the presence of lactose; (b) HPLC chromatogram of a supernatant of fermented whey indicates the presence of various intermediate compounds other than lactose**

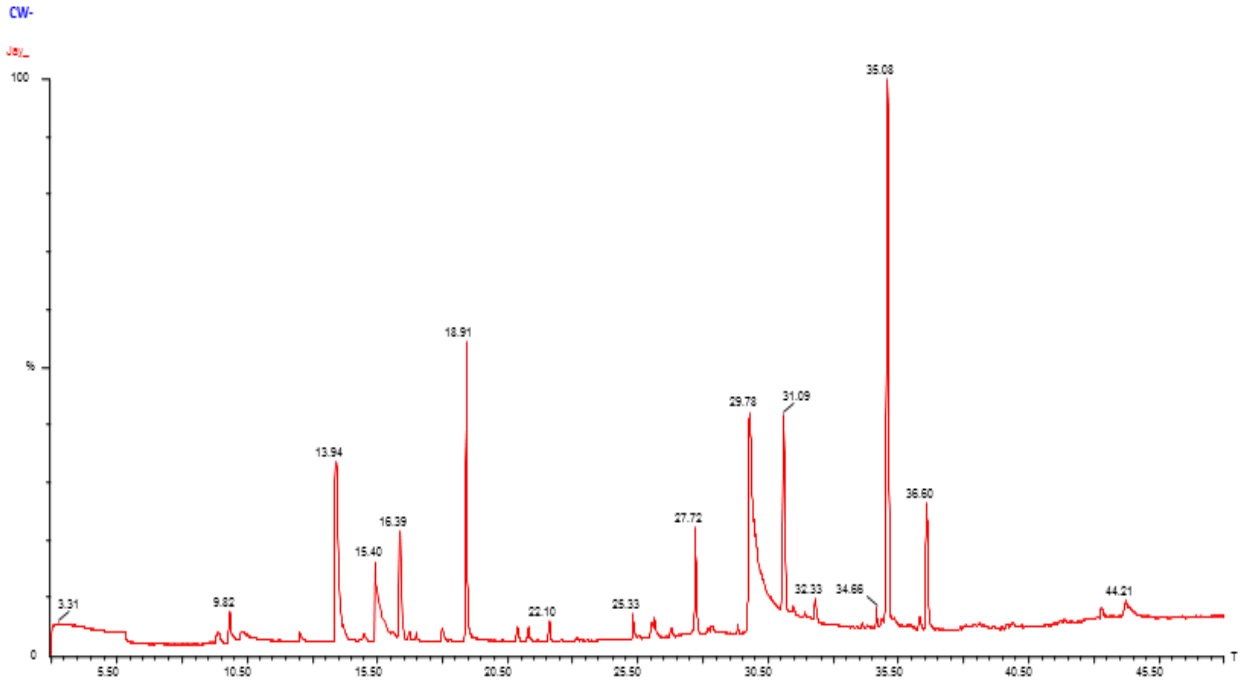
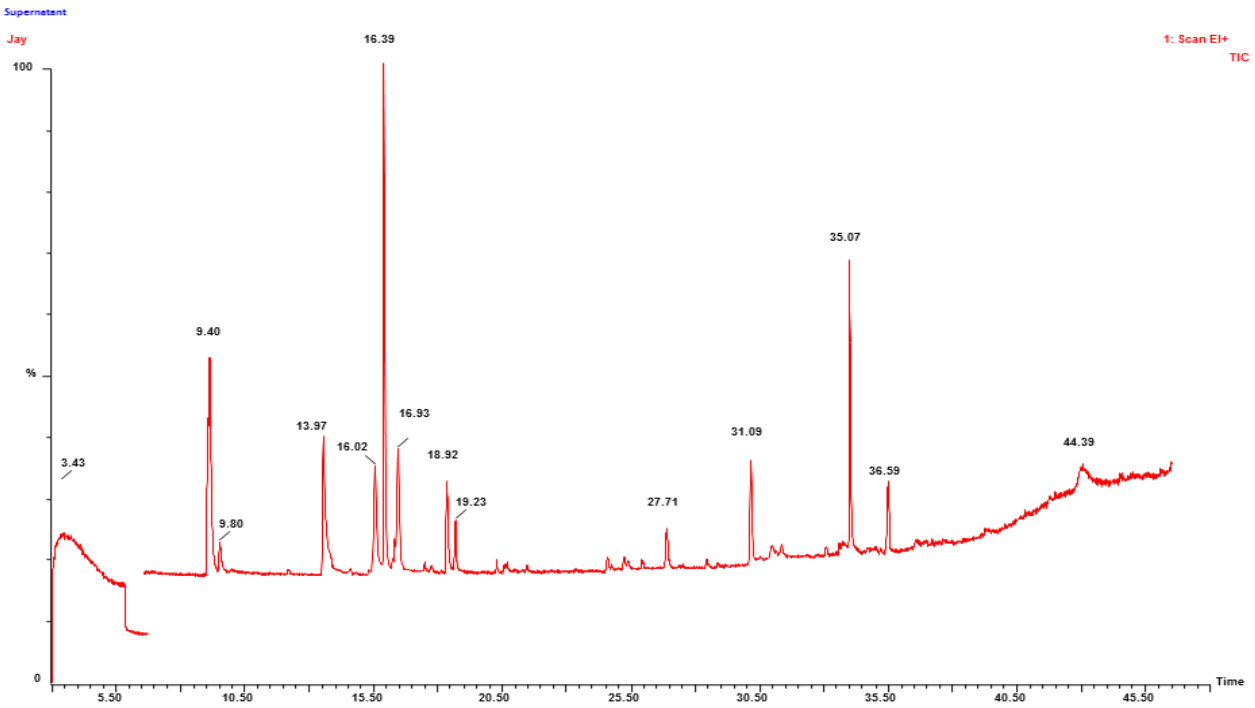



**Figure 5. GC-MS chromatogram of cheese whey***

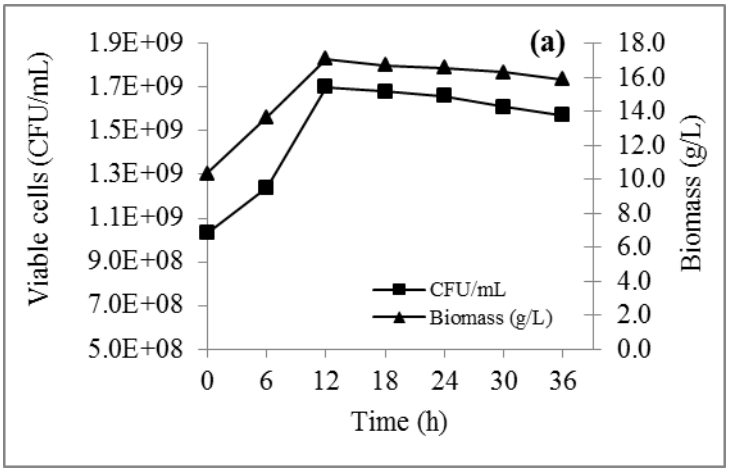
**From the left:*** Acetic acid hydrazide (9.82); Acetic acid (13.94); Formic acid (15.40); octanol-1, internal std. (16.39); 2-furan methanol (18.91); 2-cyclohexene-1-ol (22.10); Maltol (25.33); 2-propanone1,3-dihydroxy (27.72); 2-hydroxy,propionic acid (29.78); 4-H-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl (31.09); 2-furan,carboxaldehyde,5-hydroxy,methyl (35.08); 2(3H)-furanone,dihydro-4-hydroxy (36.60)




Retention Time (min)

**Figure 6. GC-MS chromatogram of supernatant***

**From the left:*** 2-butanone 3-hydroxy (9.40); Acetic acid hydrazide (9.80); Acetic acid (13.97); 2,3-butanediol (16.02); octanol-1 internal std. (16.39); 2-furan methanol (18.92); Pentanoic acid 3-methyl (19.23); Maltol (25.33); 2-propanone-1,3-dihydroxy (27.71); 4-H-pyran-4-one 2,3-dihydro-3,5-dihydroxy-6-methyl (31.09); 2-furan carboxaldehyde 5-hydroxy methyl (35.07); 2(3H)-furanone,dihydro-4-droxy (36.59)



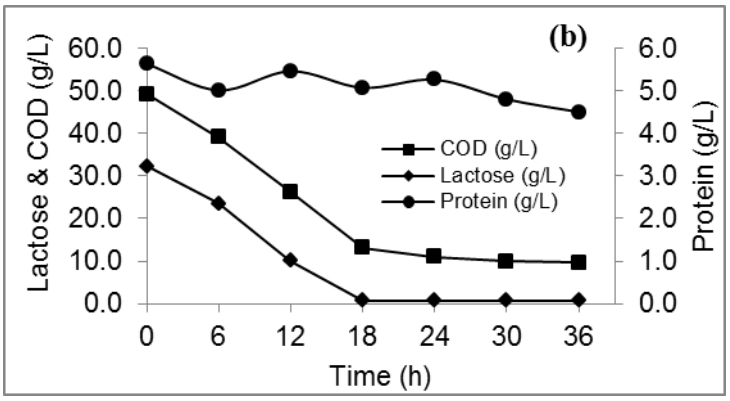




**Figure 7. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during fermentation with a high cell density inoculum; (b) Variation in lactose, COD and protein concentration during fermentation a with high cell density inoculum**

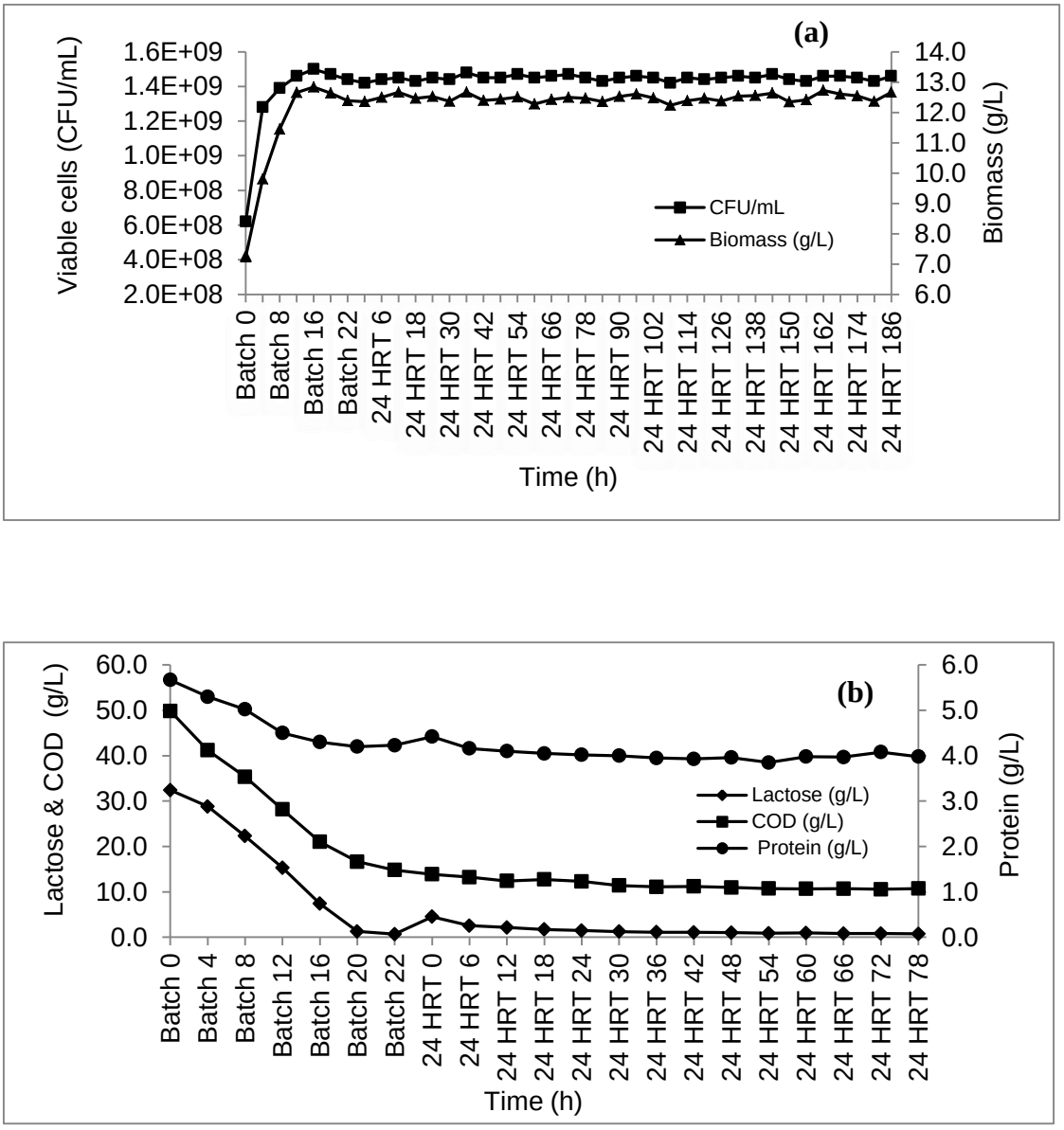



**Figure 8. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during batch and continuous fermentation; (b) Variation in lactose, COD and protein concentration during batch and continuous fermentation (with cell recycle)**

# PARTIE 3

# RECOVERY OF RESIDUAL SOLUBLE PROTEIN BY TWO-STEP PRECIPITATION PROCESS WITH CONCOMITANT COD REDUCTION FROM THE YEAST-CULTIVATED CHEESE WHEY

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**Bioprocess and Biosystems Engineering (2014)**

**37(2014):1825-1837**

# RÉSUMÉ

La présente étude a été menée dans le but de récupérer les protéines résiduelles solubles des cultures de levures (*K. marxianus*) sur lactosérum de fromage. La fermentation en continu du lactosérum a été effectuée à 40 $^{o}$C et à pH 3,5 avec un système de recyclage cellulaire. La biomasse de levures a été séparée du bouillon de fermentation par centrifugation, et les protéines résiduelles solubles contenues dans le surnageant du petit-lait fermenté ont été précipitées par traitement thermique (incubation pendant 10 minutes à 100 $^{o}$C et à pH 4,5). La récupération maximale en protéines solubles était de 53% et elle a été atteinte à pH 4,5 en combinaison avec 54% d'élimination de la DCO résiduelle. Les précipités sédimentables par gravité ont cependant été obtenus à pH 3,5 en combinaison avec 47% de récupération en protéines. Par conséquent, l'étude en réacteur (à grande échelle) a été réalisée à pH 3,5 avec agitation, ce qui a abouti à 68% de récupération en protéines résiduelles solubles simultanément avec 62% d'élimination de la DCO résiduelles. Également, la précipitation/coagulation des protéines solubles a par après été évaluée en utilisant la carboxyméthylcellulose, puis deux procédé de précipitation ont été combinés (un processus thermique suivi d'un processus via la carboxyméthylcellulose) afin d'accroître la précipitation des protéines du surnageant, laquelle a finalement permis d'atteindre jusqu'à 81% de récupération en protéines solubles. Ce procédé optimisé a donc pu être appliqué à la récupération des protéines résiduelles de la fermentation du lactosérum de fromage, et ce, sans centrifugation.

**Mots-clés:** Fermentation; Protéines du lactosérum; Précipitation thermique; Carboxyméthylcellulose; Récupération; DCO.

## ABSTRACT

The present study was conducted to recover the residual soluble proteins after cultivation of yeast (*K. marxianus*) in cheese whey. Continuous fermentation of cheese whey with cell recycle was carried out at 40 $^{o}$C and pH 3.5. The yeast biomass was separated from the fermented broth by centrifugation and the residual soluble proteins from the fermented whey supernatant were precipitated by heat treatment (at 100 $^{o}$C, pH 4.5 and 10 min incubation). A maximal soluble protein recovery up to 53% was achieved at pH 4.5 with 54% residual COD removal. However, sedimentable precipitates were obtained at pH 3.5 with 47% protein recovery. Therefore, the reactor (scale up) study was conducted at pH 3.5 with agitation, which resulted in 68% of residual soluble protein recovery and simultaneously a residual COD removal value of 62%. Further precipitation/coagulation of the soluble proteins was also evaluated using carboxymethyl cellulose and then two precipitation processes (thermal followed by carboxymethyl cellulose precipitation) were combined to increase protein precipitation, which finally yielded up to 81% of total soluble protein recovery from the supernatant. This optimized process could be applied to recover the residual proteins left after fermentation of cheese whey without use of centrifugation.



**Keywords:** Fermentation; Whey protein; Thermal precipitation; Carboxymethyl cellulose; Recovery; COD.

## INTRODUCTION

The protein content of cheese whey is around 0.6-0.8% w/v, which is the second dominant component after lactose. Whey proteins are generally recognized as safe (GRAS) for food applications and is used in many processed foods such as healthy foods, dairy foods, meat products, frozen foods and infant formulas [1, 2]. The main components of whey protein are β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, lactoferrin, lactoperoxidase, proteose-peptone and glycomacropeptide, with their different physical characteristics [3]. Yeast *Kluyveromyces* species have been widely studied for single-cell protein (SCP) production from cheese whey [4, 5]; however, cultivation of *K. marxianus* has not been efficient in utilizing whey proteins. During SCP production, lactose gets utilized completely but at the end of fermentation, a major fraction of the proteins remains unutilized [6]. The unutilized proteins are then washed due to lack of knowledge about their characteristics and recovery methods.

The recovery of whey proteins by membrane separation (ultrafiltration and nanofiltration), and by precipitation after thermal treatment has been well documented [7, 8]. However, for to economic reasons, membrane separation technology, in general, is not employed by medium- and small- scale cheese-producing companies [9]. Thermal precipitation is not commonly used for protein separation from cheese whey due to the presence of lactose, which has a negative impact on β-lactoglobulin and α-lactabumin precipitation (which constitute more than 50% of total whey proteins) [10-12].

Thermal precipitation and precipitation/coagulation using polymers are the two most commonly used methods for whey protein separation. Thermal precipitation is based on whey protein characteristics such as, denaturation temperature, isoelectric point/isoelectric pH (pI) and electrostatic interaction [7]. Protein precipitation/coagulation by coagulating agents is based on electrostatic attraction, and therefore, it depends on the target protein characteristics and on the nature of polymer used. There are many polymers (coagulating agents) which are generally used to coagulate proteins and fats from wastewaters from food processing plants. Examples of coagulating agents are carboxymethylcellulose (CMC), hydroxylethylcellulose (HEC), lignosulphonate, bentonite, chitosan, xanthan gum, alum and ferric chloride [9, 13, 14]. Bentonite and CMC have GRAS status and both can be used in animal feed as supplement [15]. The impact of HEC, which has been reported as whey protein precipitant and non-toxic in nature, has also been tested on liver and kidney in albino rats [16].

Most studies till date have reported on native whey protein recovery. However, following cultivation of the yeast *K. marxianus* in cheese whey, changes in the characteristics of the whey proteins have been reported [6]. The changes in characteristics may affect the recovery of residual soluble proteins while applying the same methods and conditions as used for the recovery of native whey proteins. Therefore, there is a need to find an economical and efficient method for residual soluble protein recovery after cultivation of yeast. The recovered residual proteins could be mixed with the yeast biomass (produced during whey fermentation and to be used as animal feed) to increase the protein content as well as to enhance the essential amino acids profile of the final product. A biomass with high protein content as animal feed is highly desirable.

Thus, the objective of this study was to investigate a method for residual protein recovery from the supernatant of *K. marxianus* fermented cheese whey. The specific objectives of the research included: i) Optimization of different parameters of thermal precipitation for residual protein recovery; ii) evaluation of coagulation/precipitation of residual protein using the non-toxic polymer CMC; iii) evaluation of a combination of the two methods for enhancement of protein recovery.

# MATERIALS AND METHODS

## Fermentation and downstream processing

Fermented whey broth [6], after cultivation of *K. marxianus,* was collected from the continuous fermentation process with cell recycle operated at 40 $^{o}$C and pH 3.5. The fermented whey broth was centrifuged to separate biomass and fermented whey supernatant (FWS). The centrifugation was conducted at 9682 x g for 15 min at 20±1 $^{o}$C in a Beckman Coulter centrifuge (model Avanti J-25 XP) using the JAL rotor (Beckman USA). The FWS was stored in cold room (4 $^{o}$C) until used. The initial protein content, chemical oxygen demand (COD), pH and turbidity (NTU) of the FWS was 3.9 g/L, 10.10 g/L, 3.5 and 580 NTU, respectively. CMC sodium salt, having a high viscosity 2440 mPas was purchased from Calbiochem (Canada). A stock solution of CMC (30.0 g/L) was prepared by dissolving CMC in hot distilled water (60 $^{o}$C) with stirring and left overnight for complete solubilisation. The CMC stock was kept at 4 $^{o}$C.

## Precipitation of proteins through thermal treatment

### Effect of temperature

Precipitation of the residual soluble whey proteins was carried out by treating the FWS (pH 3.5) at different temperatures (80, 90, 95 and 100 $^{o}$C). For the thermal treatments, the FWS 500.0 mL (in one litre glass bottles) was heat-treated in an autoclave for 15 min. Precipitates from thermally treated FWS were separated by centrifugation at 8000 rpm (10,808 x g) for 10 min using a Sorvall RC5C centrifuge. Supernatants of thermally treated FWS were used for protein analysis.

### Effect of pH

To determine the optimal pH for maximal protein precipitation through heat treatment, FWS samples were heated at 100 $^{o}$C for 15 min at different initial pH values (3.0, 3.5, 4.0, 4.5, 5.0 and 5.5) in an autoclave. The precipitates were separated by centrifugation. Supernatants of thermally treated FWS were used for protein analysis.

### Treatment time

To determine the effective incubation time of heat treatment, FWS were heat-treated for different times (5, 10, 15 and 20 min). Two different initial pH values (3.5 and 4.5) were also tested and kept the FWS in an autoclave at 100 $^{o}$C. After heat treatment, the precipitates were separated by centrifugation. Supernatants of thermally treated FWS were used for protein and COD analysis. Precipitates were also used for cations/anions analysis.

### Impact of agitation rate on precipitation and process scale up

Based on the optimized values identified above at small scale (1 L), the effect of agitation speed (100, 150, 200 and 250 rpm) on precipitation of proteins from FWS was evaluated at pH 3.5, 100 $^{o}$C, and a treatment time of 10 min using a 15 L reactor. The reactor was equipped with accessories for precise control of pH, temperature and agitation speed (Fig. 1). The reactor (15 L, total volume) used in the study was fitted with 4 baffles and an agitator, comprising three impellers. The geometry of the impeller was three six-flat blade turbines (Rushton turbine) with a diameter of 0.08 m. The average shear rate ($s^{-1}$) was calculated based on the geometry of the impeller using the formula (Equation 1) [17].

$\gamma = k_s N$ (1)

Where γ is the average shear rate, $k_s$ is a constant, which depends on the geometry of the impeller and N is the rotational impeller speed. After heat treatment in the reactor, FSW samples were centrifuged and the supernatants used for analysis.

## Coagulation/precipitation using CMC

### Precipitation at room temperature

Coagulation/precipitation of proteins from FWS with addition of CMC was conducted as reported in the literature at room temperature (20 $^{o}$C) [14]. Coagulation/precipitation of FWS samples was carried by first adding CMC (0.50% w/v) to FWS at different initial pH values (3.0, 3.5, 4.0, 4.5, 5.5 and 6.5) and at room temperature (20 $^{o}$C). The initial pH of FWS was adjusted using 1 N $H_2SO_4$ acid or 1 N NaOH. Then, the samples were mixed at 70 rpm for 10 min in a jar test apparatus using 1000 mL glass beakers. After mixing, the samples were kept at room temperature for 1 h (for settling). The precipitates were separated by centrifugation at 8000 rpm for 10 min. The residual protein concentration in the supernatant was measured. The treated samples at different pH value were also kept in 1 L graduated glass bottles overnight to determine gravity sedimentation of the precipitates.

### Precipitation at elevated temperature

Protein precipitation from FWS at elevated temperature was carried out by adding the 0.25% w/v concentration of CMC to FWS at different pH values (3.0, 3.5, 4.0, 4.5, 5.5 and 6.5) with good agitation. Then the samples were heated for 10 min at 100 $^{o}$C. Following this treatment, the samples were left to settle for sedimentation of the precipitate to occur.

## Combination of thermal precipitation followed by coagulation with CMC

### Effect of pH at which CMC was added after heat treatment

FWS was first heat-treated at pH 3.5, 100 $^{o}$C for 10 min. Then the solution was cooled to 70 $^{o}$C and pH was adjusted to the desired value (5.0, 5.5, 6.0 and 6.5). Then CMC of concentration 0.25% w/v was added to each sample. The samples were mixed in a jar test apparatus for 10 min and kept for 2 h to settle the precipitates. Protein content and COD were measured in the supernatant following sedimentation.

### Heat treatment followed by addition of different concentrations of CMC

The effective concentration of CMC for precipitation of the residual proteins was optimized by fortifying the heat-treated supernatants (pH 3.5, 100 $^{o}$C, 10 min) with different concentrations of CMC (0.0, 0.05, 0.075, 0.10, 0.20 and 0.25% w/v) followed by adjustment of pH to 5.5. The samples were mixed for 10 min in a jar test apparatus and kept in graduated cylinders (500 mL) overnight (12 h) to settle for sedimentation to occur.

### Addition of CMC at different temperatures

The optimal temperature for CMC addition was evaluated by adding the 0.05% w/v concentration of CMC, pH 5.5 at different temperatures (20, 35, 50 and 75 $^{o}$C). Prior to this, FWS was heat-treated at 100 $^{o}$C, pH 3.5, 10 min followed by cooling to different temperatures (20, 35, 50 and 75 $^{o}$C). After cooling to the desired temperature, pH was adjusted to 5.5 and the 0.05% w/v concentration of CMC was added. The treated solutions were mixed and kept settle for sedimentation to occur.

### Optimization of mixing time and determination of sediment volume

Mixing time was optimized, using the optimal values for CMC concentration, pH and temperature in a 15 L reactor (Fig. 1). Firstly fermented whey supernatant was heat-treated at 100 $^{o}$C, pH 3.5 for 10 min at an agitation rate of 250 rpm. After the heat-treatment, the temperature was brought down to 50 $^{o}$C and 0.05% w/v CMC was added followed by pH adjustment to 5.5. Mixing was done at 50 $^{o}$C at an agitation rate of 250 rpm for up to 60 min. Samples were withdrawn at 15 min intervals and left to settle for sedimentation to occur. Sediment volume was also measured in only heat treated solution and CMC added solution to compare the compactness of the sediments after overnight (12 h) gravity settling. Zone settling velocity of the FWS after CMC addition was evaluated as per Metcalf and Eddy [18]. After thermal and CMC treatment the FWS mixtures were transferred to 1 L graduated glass cylinders. The height of the settled zone with respect to time (each 15 min) was recorded and the slope of the straight line (settled zone height versus settling time) was determined to calculate the zone settling velocity.

## Analysis

Protein concentration of the samples was measured according to Lowry et al. [19]. The standard curve was drawn with different concentrations of bovine serum albumin (BSA)

obtained by diluting a 1 mg/mL BSA stock solution. Suspended solids (SS) and COD of the samples were measured by Standard Methods [20]. Turbidity of the samples was measured using the Micro 100 Turbidity meter, Hach Company.

Cations and anions contents of the precipitates obtained at pH 3.5 and 4.5 were analyzed by ICP-AES (Varian Vista AX, USA) after partial digestion of the samples (of the precipitated proteins) using an acid-peroxide reagent ($HNO_3$ and $H_2O_2$). To partially digest the samples, 0.5 g of dry precipitated sample was taken in a 50 mL Pyrex glass tube and 5 mL of 50% $HNO_3$ was added followed by heating for 10 min at 95 ± 2 $^oC$. Concentrated $HNO_3$ (2.5 mL) was added to the samples and digestion continued at 95 ± 2 $^oC$ for 1 h. After this 1 h digestion, 9 mL of 30% (v/v) $H_2O_2$ was added followed by digestion for 2 h at 95 ± 2 $^oC$. Finally, 2.5 mL of concentrated $HNO_3$ was added and the samples heated for 15 min. The final digestates were diluted up to 50 mL with Milli-Q water and used for metals analysis by ICP-AES. Quality control of elemental analysis by ICP-AES was performed with two certified liquid samples (multi-element standards, catalog number 900-Q30-100 (Lot SC-8305871) and 990-Q30-101 (Lot SC7256497), SCP Science, Lasalle, QC, Canada).

All bench-scale experiments were conducted in duplicates, whereas all of the reactor-scale experiments were carried out one time only. However, analysis of all samples was carried out in triplicates, irrespective of the experiment (lab- or reactor-scales) and the average value was reported (with standard error less than 5% of the mean).

# RESULT AND DISCUSSION

## Recovery of residual proteins by thermal precipitation

The change in the molecular weight (MW) of native proteins due to proteolysis would change the characteristics of these proteins, and therefore, this would require different thermal precipitation conditions to recover the residual fermented whey proteins. A second inhibitory factor, lactose is fortunately not present in fermented whey supernatants. The important factors, which affect precipitation of whey proteins, are pH, heating temperature, ionic strength and protein concentration. The major component of whey proteins is β-lactoglobulin and it shares around 50% of the total whey proteins. The pI of β-lactoglobulin is in the pH range of 5.2-5.4 [21]. Aging (treatment time) and agitation also affect protein precipitation and the particles size of precipitates. Increase in aging with agitation rate increases the precipitation of α-lactalbumin; however, particle size of the precipitates is inversely proportional to agitation [22]. During heat precipitation, protein molecules became unfolded

(denaturation), and then, aggregation occurs due to covalent binding between active sulphydryl (-SH) groups of the two intermolecular protein surfaces (i.e. thiol/disulphide exchange) [23, 24]. The second step of the aggregation mechanism is interaction (due to fluid motion and collision of particles within the fluid) between the different denatured molecules, which leads to formation of the aggregates. The second step of aggregation controlls the rate of aggregation at elevated temperatures, and thus, making stirring (agitation) an important parameters during heat induced denaturation process [23].

### Effect of temperature

Precipitation of proteins increased with an increase of temperature and maximal precipitation (46%) was observed at 100 $^{o}$C (Fig. 2a). A temperature above 100 $^{o}$C was not tested to prevent boiling of water and also denaturation of proteins at higher temperature. At lower temperatures only negligible protein precipitation (at 80 $^{o}$C only 2.5%) was observed. The temperature needed for unfolding the native whey protein has been reported to be higher than 70±2 $^{o}$C and precipitation was directly proportional to the temperature used [25]. Temperature is the critical factor for breaking the disulphide bonds and to create new thiol-disulphide bonds [26]. Thus, the residual soluble proteins in the yeast FWS followed almost the same trend in precipitation as that of the native proteins in cheese whey.

### Effect of pH

The results revealed that the optimal pH for thermal precipitation (at 100 $^{o}$C) was between 4.0 and 4.5 with 53% protein precipitation (Fig. 2b). There was a decrease in protein precipitation at pH above 4.5, which indicated that the pI of most of the residual proteins (formed during fermentation due to hydrolysis) was between pH 4-4.5. The proteins typically have the least solubility near their pI [27]. The maximal precipitation was obtained at pH 4.5. However, the precipitated solids (mostly protein) at pH 3.5 settled without centrifugation, but with comparatively less protein recovery (47% of the total). The observed optimal pH for maximal residual protein precipitation was different from that of the native whey proteins (pH of 5.3) [28]. This could be due to a change in the nature of the proteins after fermentation [6]. Due to the presence of new (hydrolysed) proteins with a much reduced MW and a different pI, different precipitation condition were needed. The majority of the studies on whey protein precipitation have been carried out using pure β-lactoglobulin as model protein and the dominant mechanism of heat-induced aggregation has been reported to be unfolding of the protein, which results in exposure and activation of free sulphydryl groups. The activated free sulphydryl groups form aggregates via a thiol/disulphide exchange reaction between an

activated β-lactoglobulin intermediate and a free (reactive) thiol group. β-lactoglobulin also unfolds and aggregates through a series of parallel and successive steps, which involves hydrophobically-driven association reactions. Unfolding reactions of protein would be the rate-limiting step at low treatment temperature, at pH values close to the pI (5.2) and at high ionic strength; whereas aggregation reactions would be reduced at high treatment temperature, pH values far from the pIs and at low ionic strength. At very acidic pH values (2-3), β-lactoglobulin bears only positive charge in contrast to its amphoteric behaviour at neutral pH. At low pH condition, electrostatic interactions between monomers are purely repulsive and two opposite effects on protein aggregation would be involved. First, an increase in ionic strength reduces the intermolecular repulsion (between molecules) because of the screening of charged groups, and thus, increasing the aggregation rate. Second, an increase in ionic strength increases the denaturation temperature of the protein because screening of the charged groups reduces intra-molecular repulsion, therefore, increasing conformational stability [7]. The mechanism discussed above should be applicable to the fermented whey proteins as they are mostly derived from native whey proteins.

## Treatment time

Effect of different treatment duration on precipitation of the residual proteins was studied at both pH 3.5 and 4.5 and the results are given in Figs. 3a and 3b. The results indicate that in both cases a thermal treatment time of 5 min (after reaching the temperature of 100 $^{o}$C) was sufficient for maximal soluble protein precipitation. There was no significant increase in precipitated proteins after 5 min of heat treatment. At the same time, residual COD after precipitation at pH 3.5 and 4.5 was 47.5% and 46%, respectively. Thus, during precipitation, COD reduction from FWS up to 52.5 and 54% was achieved in both cases (Figs. 3a and 3b). The protein precipitates obtained at pH 3.5 settled well (within ~8-10 h) without any need for centrifugation. The heat treatment time for maximal precipitation (5-10 min at 100 $^{o}$C) is comparatively less than that required (30 min at 92±2 $^{o}$C) for precipitation of the cheese whey protein in presence of lactose [28]. The maximum of protein precipitation was only 47% at pH 3.5, but COD reduction was comparatively high as compared to the protein precipitated. Generally, 1.0 g of protein equals to 1.5 g of COD [29] {total COD removed (5.4 g) - COD removed due to protein precipitation (2.70 g) = 2.70 g of extra COD was removed during the precipitation}. The higher COD reduction during precipitation than that equivalent to protein precipitated might be due to the removal of other organic (phenolic) compounds together with the precipitated proteins. The interaction between phenolic/polyphenolic compounds and proteins (plants, milk and whey proteins) during precipitation is well documented [30-32] and

could be one of the reasons for the removal of organics, which would explain the higher COD reduction.

Protein-polyphenolic complexes are formed due to various weak interactions such as hydrophobic, van der Waals and hydrogen bonding. However, hydrophobic interactions are reported to be dominant [33-35]. Moreover, interactions due to hydrophobic bonding between bovine serum albumin (type of milk or whey protein) and flavour compounds (esters, aldehydes and ketones) have also been reported [36]. The presence of such organic compounds (e.g. phenylethyl alcohol, aldehydes, ketones and acids) in FWS has been reported in our previous work [6]. Thus, these compounds are most likely to be removed along with proteins during precipitation. The concentration of these organic compounds in the precipitated proteins should not be an issue for safety and quality of the product (i.e. precipitated proteins) as the presence of such organic compounds has also been reported in the cheese whey-based fermented beer, and kefir-like beverages [37, 38].

The quality control issue might be raised due to the presence of synthetic hormones (such as recombinant bovine growth hormone-rBGH) in cheese whey, which is used by dairy farmers to increase milk production in cows. The hormones have been used in the USA since their approval by the Food and Drug Administration (FDA) in 1993, but their use is not permitted in the European Union, Canada, Australia, New Zealand, Japan and Israel, since 2000. The presence of such hormones in milk has been confirmed by various studies. However, there is no consensus on their harmful effects or not on consumers. These hormones are mostly degraded in the digestive system when a milk containing them is ingested by human/animal [39, 40]. On the other hand, the use of hormones leads to adverse effects on cows, such as higher rates of mastitis, foot problems, and reactions at the injection site. Thus, to avoid such infections the use of antibiotics has been in practice. The residues of those antibiotics in milk may lead to antibiotics resistance and to health risk for humans and therefore precautions are needed. The most commonly used antibiotics in lactating cows are penicillin. Other than penicillin, the most used antibiotics are oxytetracycline, sulfadiazine, metronidazole, chloramphanicol, cephalosporins, streptomycin, and rifampicin. Most commonly excreted antibiotics in milk are oxytetracycline, chloramphenicol and streptomycin [41-43]. In conclusion, hormones have no side-effect if present in milk. However, due to the adverse effects of hormones on cows, the use of antibiotics is higher that finally end up in the milk. This concern might be raised in the USA, however, countries like Canada, which banned the use of lactating hormones and therefore, at present safe from these concerns.

The other side of whey proteins is that it can decrease the absorption of antibiotics in the body due to interaction of antibiotics with calcium. A well-established example of such interaction is between tetracycline and calcium [44]. In one sense the interaction of antibiotics with protein will be beneficial in order to reduce absorption and the side-effects of antibiotics, if the antibiotics are present in recovered protein and these used by the consumer. It may be possible that antibiotics present in cheese whey are degraded during yeast fermentation, which needs to be verified. Degradation of some of the whey native organic compounds was observed as reported in our previous study [6].Other chemicals such as herbicides and pesticides are also of concern for the quality of milk and dairy products [43]. However, these can be divided in both the cheese and the whey fraction, thus, diluting their concentration, which still needs to be verified.

## Cations/anions content of the precipitates

The content of cations and anions was determined in both the precipitates obtained at pH 3.5 and 4.5. Generally speaking, content of anions and cations in the precipitates was significantly higher at low pH i.e. pH 3.5 (Table 1). Protein molecules at low pH usually exist as positively charged entities [7]. Therefore, interaction of anions with positively charged proteins (at pH 3.5) resulted in higher precipitation of anions ($SO_4^{2-}$ and $PO_4^{3-}$). Cross-bridging (between phosphate and sulfate attached to protein with cations) could be the dominant mechanism to explain the higher precipitation of monovalent and divalent cations at low pH (3.5). Another reason behind $Ca^{2+}$ precipitation might be the presence of phosphoserine in the protein molecules [45]. Phosphoserine molecules bind with calcium, and probably with other cations, leading to their precipitation. Also, the higher mineral contents of the precipitates at low pH (3.5) produced dense flocs, which resulted in fast settling and compact sediments, and thus, obviating the need of centrifugation (as discussed above). The high content of minerals (e.g. calcium, potassium, sodium, phosphorus and sulfur) could enhance the value of proteins in the precipitated solids as these are well known essential minerals present in protein rich animal feeds [46].

## Role of agitation in precipitating proteins in a large-scale process

The role of the agitation rate (shear rate) on protein precipitation was evaluated and the results obtained are presented in Table 2. The results showed that there was an increase in protein precipitation with agitation. Heat treatment with agitation increased protein precipitation from 47% to 68%. The effect of agitation was considerable and demonstrated that the shear rate is an important factor. Initially, protein precipitation increased from 47% to

61% with only a small increment in agitation (Table 2), which indicates that agitation is required for aggregation of the heat-precipitated proteins. Further increments in the shear rate did not substantially increase precipitation. Agitation in a reactor increases the shear rate between particles, which leads to higher interactions between unfolded protein molecules, and thus, results in higher aggregation. Increased interactions of active molecules resulted in an increase of protein precipitation in dynamic state compared to the precipitation in static state. Later increase in shear rate with agitation did not significantly increase the precipitation rate, which indicated that the interaction of the active unfolded protein was almost completed. A similar trend for the shear rate effect on whey protein precipitation has been reported and revealed that the initial shear rate was important in whey protein aggregation. Shear rate increases aggregation and decreases the particle size of the aggregates leading to higher compactness of the precipitate [22, 24, 28].

Overall, thermal protein precipitation was increased by 13.3% at pH 4.5 compared to pH 3.5. Although thermal protein precipitation at pH 4.5 was significantly higher, pH 3.5 was selected for further studies due to the fact that, at this pH protein (precipitated) separation was possible without centrifugation. Treatment time had an insignificant effect (less than 0.8% variation) on protein precipitation. However, it was observed that the precipitate was more stable with the treatment time of 10 min. Agitation had the most significant impact (47.8% increment) on the thermal protein precipitation.

The residual COD removal from FWS was 62% (6.24 g/L). The overall COD reduction from cheese whey (79% during fermentation and 12.5% during protein precipitation) was approximately 91.5%, which is a substantial pollution load reduction. The precipitated solids settled by gravity without centrifugation. The scale-up (reactor) study indicated that the process could be used in cheese processing plants where yeast biomass (animal feed) production becomes the option of choice.

## Precipitation/coagulation with CMC

### Precipitation with CMC at room temperature

The attractive interaction between proteins and polysaccharides has been used in many processes to recover proteins or to stabilize food products. The attractive forces between proteins and polysaccharides arise from electrostatic interactions of the two -weak or -strong oppositely charged polyelectrolytes that forms a complex. The electrostatically-bound complexes may be either soluble or insoluble [47]. CMC, an anionic polysaccharide, is very

effective as a protein-precipitating agent. The strong interaction of anionic polysaccharides with proteins is assumed to take place at pH values between the pI of the protein and the pK value of the polysaccharides [48].

An initial experiment with CMC was conducted to identify the optimal pH value. The results for precipitation obtained with a 0.50% w/v concentration of CMC at different pH are presented in Fig. 4. The results established that the optimal pH for maximal protein precipitation with CMC was pH 5.5 (Fig. 4). Above and below the optimal pH, protein precipitation decreased. The reason for the change in the degree of precipitation at different pH values is probably due to changes in charge distribution; precipitation is known to occur due to electrostatic attraction between proteins and CMC. The charge on the protein molecules and CMC depends on the pH of the medium (solution) [49]. The optimal pH for maximal precipitation/coagulation of protein from cheese whey with CMC has been reported to be between 3 to 4 for native whey proteins [14, 50], values that one substantially different from that identified in the present study (pH 5.5). The difference in the optimal pH is due to a change in the nature of the residual soluble proteins during yeast fermentation. The maximum of protein precipitated at the optimal pH was 28.5% w/w of the initial protein level. The precipitates settled slowly (more than 15 h) with a high residual turbidity (150 NTU). Therefore, under these conditions, the proteins would not be separated only by gravity settling and centrifugation was required for effective separation of the precipitates.

## Precipitation/coagulation at elevated temperature

Protein precipitation with FWS samples at different initial pH values (3.0, 3.5, 4.0, 4.5, 5.5 and 6.5) fortified with 0.25% w/v CMC and concomitant heat treatment for 10 min at 100 $^{o}$C was studied. Surprisingly, the results observed no precipitation (visually no precipitation at any pH). Inhibition of protein aggregation was probably due to destabilization of the electrostatic forces between proteins and CMC or to interactions between the globular proteins and CMC preventing aggregation of the complexes. It has been reported that, at higher temperatures hydrophobic interactions between protein molecules may overcome the electrostatic interactions between proteins and anionic polysaccharides. The interaction of globular proteins with charged polysaccharides can inhibit thermal aggregation of proteins. On a similar line, the inhibition of aggregation of bovine serum albumin in presence of pectin or alginate has been reported [51].

## Combination of thermal precipitation followed by coagulation with CMC

The results from thermal protein precipitation or from polymer-assisted (CMC) precipitation were analysed and compared. Thermal precipitation was found to be more efficient to recover residual soluble proteins from (68% of total soluble protein recovered) FWS. Moreover, recovery through thermal precipitation at pH 3.5, as said before, did not require centrifugation and the precipitated proteins could be recovered by gravity settling alone. On the other, hand polymer-assisted precipitation alone gave only 28% of total protein recovery. Therefore, to enhance the further protein recovery, combinations of these two individual precipitation methods were evaluated as described below.

### Effect of pH at which CMC was added after heat treatment

Protein precipitation from FWS with heat treatment (at 100 $^{o}$C, pH 3.5 for 10 min) followed by adjustment of pH at different values (3.5, 5.0, 5.5, 6.0 and 6.5) and by addition of 0.25% w/v of CMC was tested (Fig. 5a). The results revealed that, after the heat treatment, the optimal pH for CMC addition was 5.5 for maximal protein precipitation/coagulation. This optimal pH was similar to the one found for precipitation at ambient temperature. The results showed that maximal interaction of CMC with proteins was at pH 5.5 and this might have been due to the nature of the residual proteins, following thermal precipitation, which were positively charged molecules able to forms complexs with CMC. Above and below pH 5.5, a decrease in precipitation occurred. As said before, a decrease in the efficiency of precipitation above and below pH 5.5 was due to changes in the charge on the interacting molecules. Furthermore, after heat treatment at pH 3.5, the pH of the solution needs to be increased to pH 5.5 for protein and CMC interactions to happen.

### Optimization of CMC concentration in heat-treated solution

The optimal concentration of CMC required after heat treatment (at 100 $^{o}$C, pH 3.5 for 10 min) followed by pH adjustment to 5.5 and addition of different concentrations of CMC was investigated (Fig 5b). Maximal precipitation of proteins was obtained with 0.05% w/v CMC. At CMC concentrations above 0.05% w/v, there was a decrease in protein precipitation and an apperent increase in the COD value of the residual FWS solutions. This increase in COD values indicates that CMC concentrations above the optimal value did not bind with the protein and remained in the solution, which resulted in increased COD value.

## Addition of CMC at different temperatures

Samples of FWS were heat-treated (at 100 $^{o}$C, pH 3.5 for 10 min) and cooled to different temperatures followed by addition of CMC (0.05% w/v) (Fig. 5c). The results revealed that the optimal temperature (for maximal precipitation of proteins and maximal COD reduction) for addition of CMC was 50 $^{o}$C. A temperature of 75 $^{o}$C, resulted in a decrease in precipitation. The reason might be exposure of the hydrophobic groups and that resulted to overcome the electrostatic attraction as explained before. A temperature below 50 $^{o}$C gave somewhat lower protein precipitation results. Thus, when temperature reaches 50 $^{o}$C (during cooling); CMC should then be added to attain maximal precipitation of proteins.

Overall, addition of CMC significantly increased (13.4%) protein precipitation at pH 5.5 as compared to pH 3.5. Addition of 0.05% CMC significantly increased (20.8%) protein precipitation compared to the control (no CMC addition). CMC addition was carried out during the process of cooling of the sample, once the temperature reached 50 $^{o}$C. Protein precipitation was relatively higher (20.2%) at 50 $^{o}$C.

## Optimization of mixing time and determination of sediment volume

The effect of mixing time after the heat treatment, cooling to 50 $^{o}$C, adjustment of pH and addition of CMC (0.05% w/v, pH 5.5 at 50 $^{o}$C) was evaluated and the results are presented in Table 3. The results indicate that a 15 min mixing time was sufficient for maximal protein precipitation. Mixing times of more than 15 min gave no significant increase in protein precipitation. This confirms that a 15 min mixing time at 250 rpm in the reactor is optimal for maximal protein precipitation. Mixing is an important factor, which is responsible for the interactions between proteins and CMC. The requirement of mixing or shear rate up to an optimal level is reported to be important to improve process efficiency using polymer precipitation [52]. Overall, thermal treatment followed by CMC addition increased protein precipitation by 18.1% as compared to thermal treatment alone.

The sediment volume after overnight (12 h) gravity settling of the various samples was measured after different mixing times. The results indicates that addition of CMC helped the precipitates to settle down more compactly (reduce sediment volume) compared to only heat treated samples (Table 3; Fig. 6). Compactness of the precipitate is an additional advantage besides increase protein recovery. Turbidity of the FWS during precipitation/coagulation process was also removed and it decreased from 580 NTU (FWS) to 10-20 NTU (supernatant after protein precipitation) (Table 3). The low turbidity indicated that FWS was

highly purified and that the purified FWS could be disposed or reused as dilution water for the cheese whey needed prior to fermentation. The calculated zone settling velocity of the precipitate was 0.48 cm/min. This value could be used to define the shape/size of a settler or clarifier for a real word process.

On the basis of the previously reported work by Yadav et al. [6] and from the present study, an integrated plant could be designed for SCP production with recovery of the residual soluble whey proteins. The results of the previous study (i.e. continuous fermentation at pH 3.5 and 40 $^{o}$C with cell recycle) followed by the present study on protein recovery, a process treating 1000 L/day of diluted whey could be implemented as shown in Fig. 7. The recovered proteins could be used to increase the protein content of the yeast biomass. The yeast biomass protein content obtained was 42% w/w (Fig 7, option 1), which could be mixed with the precipitated proteinaceous solids containing approximately 56% w/w protein. Addition of these proteinaceous solids could increase the protein content approximately up to 49% w/w (Fig 7, option 2). The settled proteinaceous solids could also be spray dried together with the yeast biomass slurry, and thus, substantial savings could be achieved. Mixing of the recovered proteinaceous solids will enhance product quality in two ways: i) the overall protein content of the produced SCP will be increased; ii) the amino acid profile of the SCP will also be balanced due to the higher content of sulfur-containing amino acids (cysteine and methionine) in the whey-derived proteins. Moreover, the digestibility of whey proteins is reported to be higher over other sources of proteins [53]. On the other hand, the SCP from the whey fermenting yeast *K. marxianus* generally lacks sulfur-containing amino acids [54]. Thus, the overall protein quality will be better compared to SCP alone and this will improve the value of the proposed proteinaceous product.

## CONCLUSIONS

The optimal conditions for maximal residual protein precipitation from fermented whey supernatant were pH 4.5, temperature of 100 $^{o}$C for 5 min. The gravity sedimentable precipitates were found at pH 3.5. Sedimentation of the precipitates obviates the need of centrifugation for protein recovery, and therefore, pH 3.5 was used for scale up. Precipitation at pH 3.5 also gave precipitates rich in mineral contents, which could be mixed with the yeast biomass to increase the final mineral content of the product. The combination of two precipitation processes (thermal treatment followed by CMC addition) increased protein recovery to approximately 81%. Residual COD removal was also increased during the combined process. The acidity of treated water reached toward neutrality (pH 5.5) from acidic (pH 3.5) and could be discarded to natural stream. The protein content of the final product

was increased from 42% w/w to 49% w/w by mixing precipitates with yeast biomass with an increase in quantity of product of approximately 48%. Overall COD removal increased from 79% (removed during fermentation) to 93% (including 14% removal during precipitation). This optimised method for residual protein recovery could be useful for industrial scale processes where raw cheese whey is directly used to produce SCP for animal feed purposes.

## ACKNOWLEDGEMENTS

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support**.**

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**Table 1. Cations and anions content of precipitates obtained at pH-3.5 and pH-4.5**

| Cations and anions in precipitates | Al | B | Ca | Fe | K | Mg | Mn | Na | P | S |
|---|---|---|---|---|---|---|---|---|---|---|
| pH 3.5 (g/kg precipitates) | 0.01 | 0.03 | 6.66 | 0.05 | 17.96 | 1.95 | 0.07 | 12.36 | 7.30 | 38.76 |
| pH 4.5 (g/kg precipitates) | 0.01 | 0.24 | 3.17 | 0.05 | 5.76 | 0.87 | 0.08 | 4.87 | 2.40 | 21.29 |

**Table 2. Effect of different agitation rates (average shear rate) on protein precipitation and COD removal at treatment conditions: time 10 min, pH 3.5 and temperature of 100 $^{o}$C**

| Agitation speed (rpm) | Average shear rate ($s^{-1}$) | % of protein precipitated | % of COD reduction during precipitation | Suspended solids (g/L) after precipitation | Protein content in the precipitated solids (% w/w) |
|---|---|---|---|---|---|
| 0 | 0 | 46.3±1.19 | 53.0±1.16 | 3.3 | 55.0 |
| 100 | 50 | 61.4±0.72 | 59.8±0.95 | 4.4 | 56.0 |
| 150 | 75 | 62.6±0.74 | 60.5±0.97 | 4.4 | 56.0 |
| 200 | 100 | 67.7±0.94 | 61.8±1.10 | 4.7 | 55.5 |
| 250 | 125 | 68.5±0.67 | 62.0±0.67 | 4.8 | 55.0 |

**Table 3. Effect of mixing time on precipitation with CMC at reactor scale***

| Mixing time (min) | % of protein precipitated | % of COD removal | Turbidity (NTU) after 12 h settling | Sediment volume (mL/500 mL) (after 12 h) | Suspended solids (S.S) in the precipitated sediment (g/L) | (% w/w) of protein content in the precipitated solids |
|---|---|---|---|---|---|---|
| 10 (only heating) | 68.6±0.82 | 62.0±1.64 | 16.6 | 130 | 18.8 | 55.8 |
| 15 | 80.3±1.85 | 67.6±2.57 | 16.3 | 110 | 25.3 | 56.3 |
| 30 | 80.8±1.54 | 67.6±1.96 | 11.3 | 110 | 25.4 | 56.5 |
| 45 | 81.8±1.88 | 67.3±2.62 | 9.6 | 100 | 28.3 | 56.4 |
| 60 | 81.3±2.20 | 67.4±2.35 | 8.6 | 100 | 28.1 | 56.4 |

*CMC concentration of 0.05% w/v, pH 5.5, 50 $^{o}$C and agitation rate of 250 rpm after heat treatment (pH 3.5, 100 $^{o}$C, 250 rpm, 10 min).

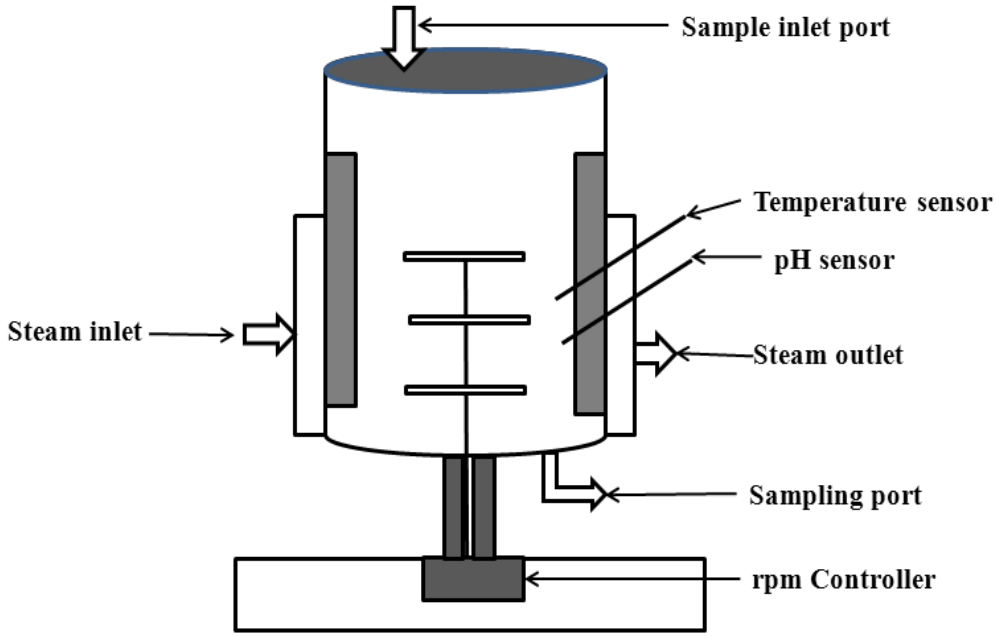


**Figure 1. Schematic diagram of the reactor tank used for protein precipitation**

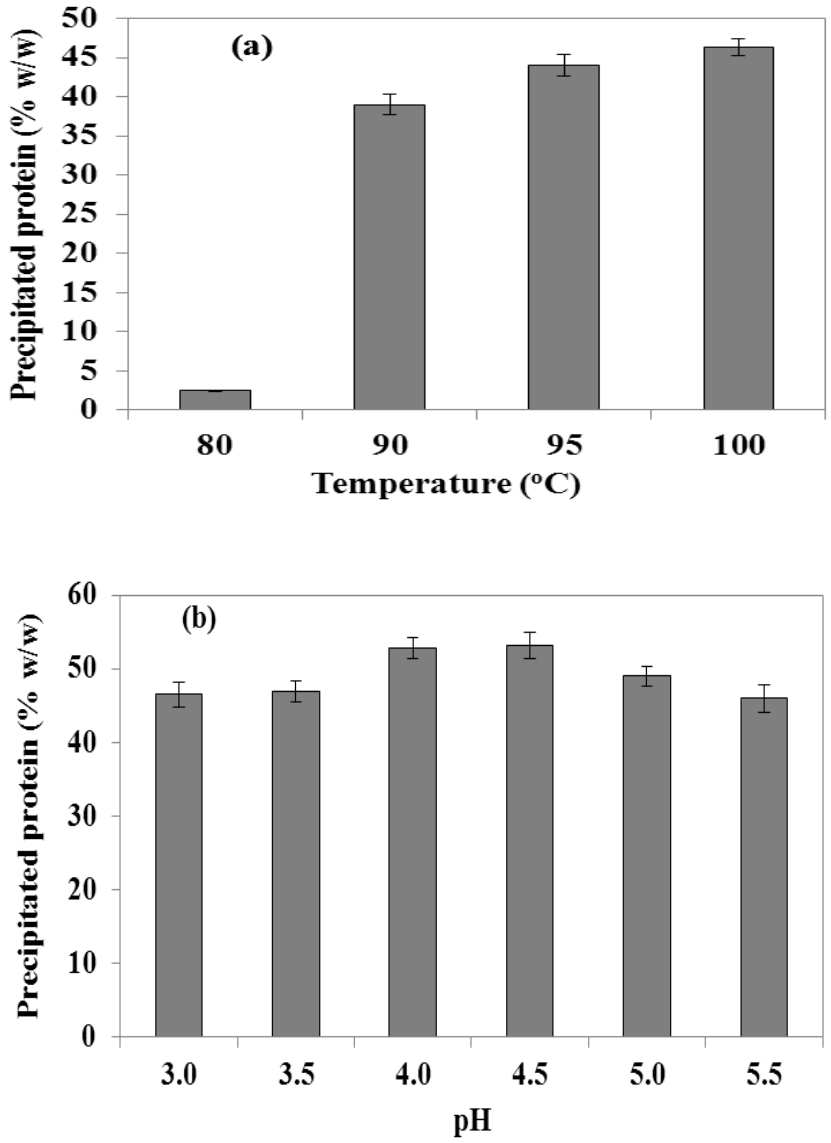


**Figure 2. (a) Effect of temperature on precipitation of residual proteins in FWS at pH 3.5 with a treatment time of 15 min; (b) Effect of pH on precipitation of proteins from FWS at 100 $^{o}$C for 15 min**

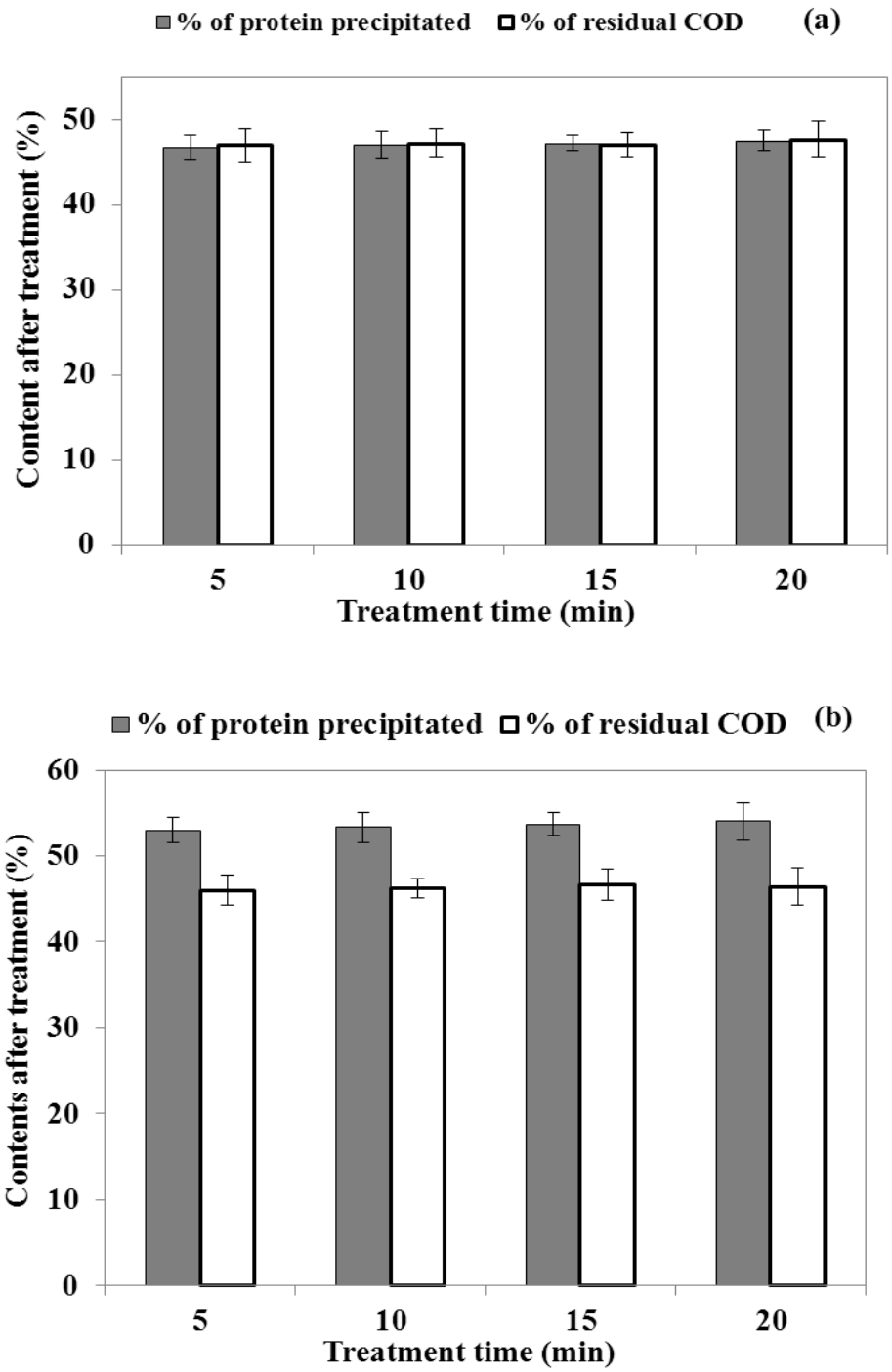


**Figure 3. (a) Effect of different incubation times on proteins precipitation and COD removal at 100 $^{o}$C and pH 3.5; (b) Effect of different incubation times on proteins precipitation and COD removal at pH 4.5 at 100 $^{o}$C**

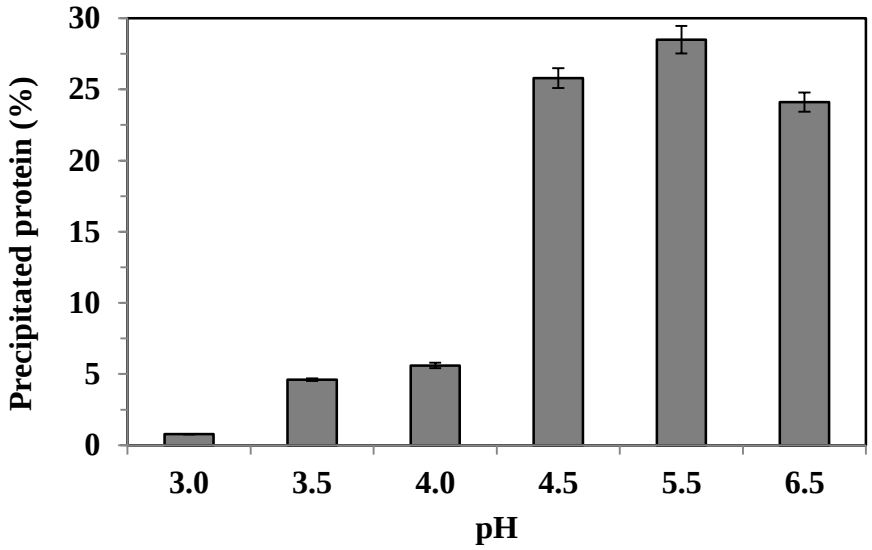


**Figure 4. Effect of different pH on precipitation of proteins with a 0.50% w/v concentration of CMC**

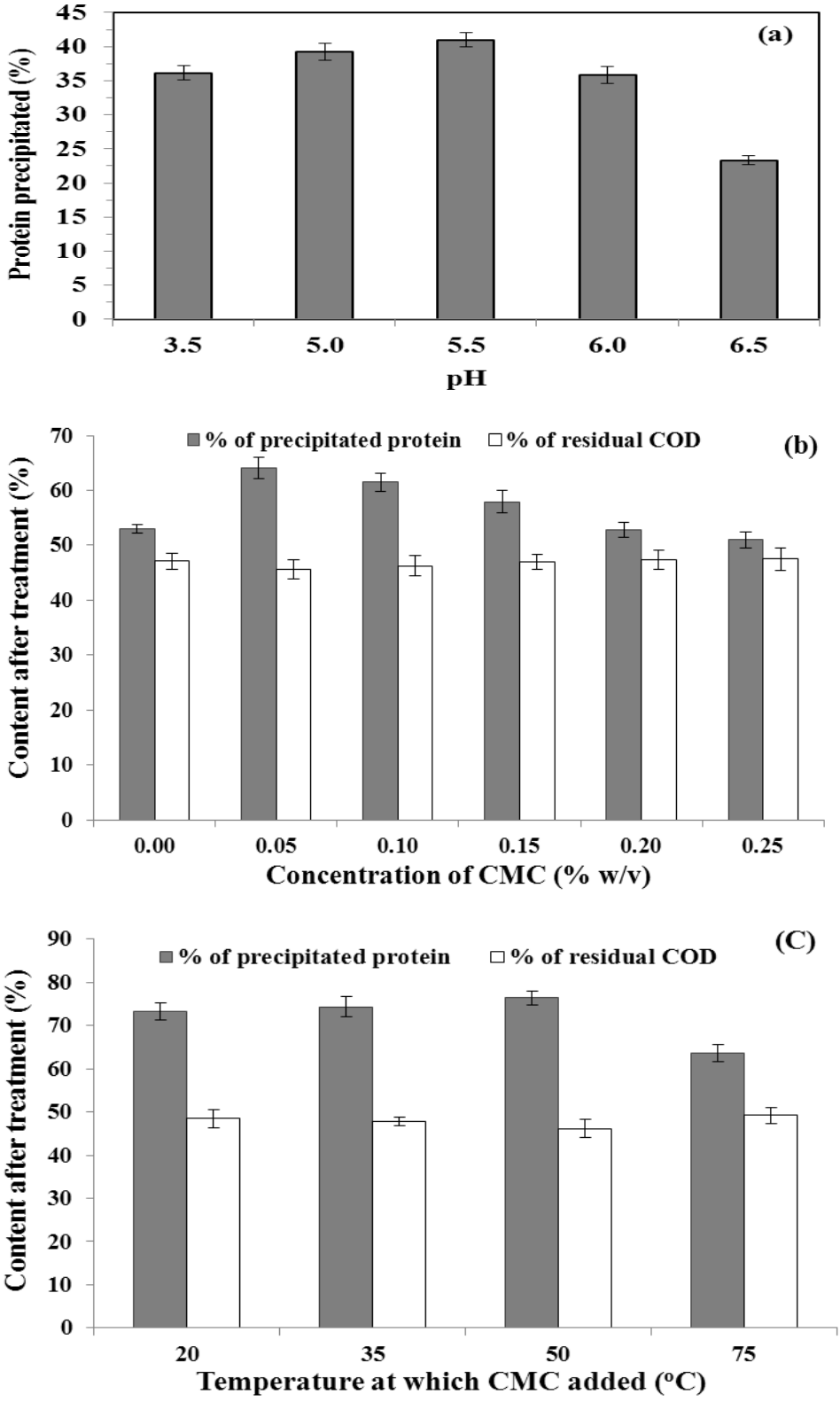


**Figure 5. (a) Heat treatment followed by change of pH and addition of CMC; (b) Heat treatment followed by addition of different conc. of CMC at pH 5.5; (c) Effect of temp. on precipitation with CMC at pH 5.5 after heat treatment**

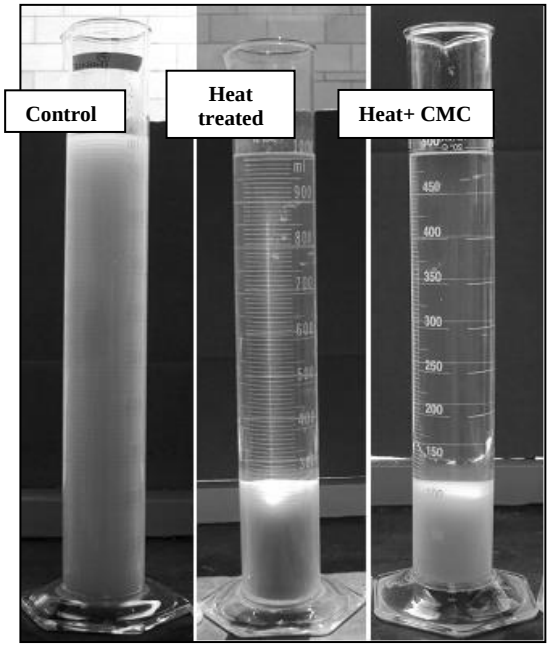


**Figure 6. Precipitate of proteins obtained after only heat treatment and after heat treatment followed by CMC addition**

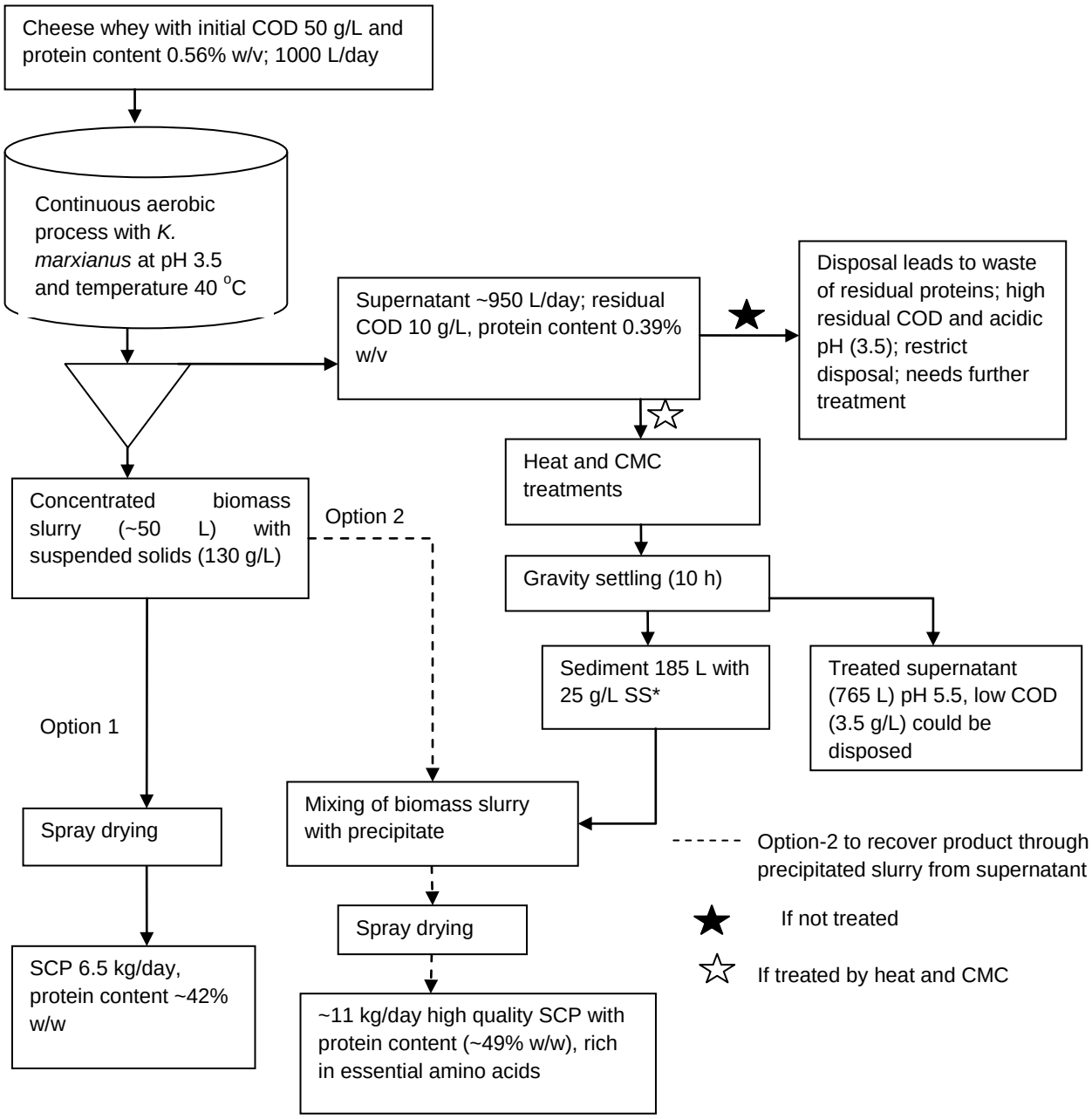


**Figure 7. Flow chart summarizing the results and future prospectives**

***SS**: suspended solids

# CHAPITRE III

# POTENTIEL BIOTECHNOLOGIQUE DE LA LEVURE CANDIDA KRUSEI ET SON RÔLE EN TANT QUE MEMBRE DE CULTURES MIXTES POUR LA PRODUCTION DE PROTÉINES UNICELLULAIRES

# PARTIE 1

# YEAST CANDIDA KRUSEI: BIOTECHNOLOGICAL POTENTIAL AND CONCERNS ABOUT ITS SAFETY

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**Canadian Journal of Microbiology**

**58(8) (2012): 937-952**

# RÉSUMÉ

Les levures sont traditionnellement utilisées à des fins d'applications biotechnologiques et les espèces *Saccharomyces* constituent les représentantes les plus fréquentes. Parmi les espèces de levures utilisées, *Candida krusei* isolée de différents habitats, a suscité au cours des dernières années un intérêt accru à cause de ses rôles variés en biotechnologie. On la retrouve dans plusieurs types d'aliments et de produits laitiers fermentés, et elle a aussi été exploitée pour produire des composés biochimiques et des enzymes. Cependant, à cause de sa nature pathogène opportuniste, elle est l'objet d'une attention scientifique particulière en regard de la sécurité de son exploitation industrielle. *Candida krusei* cause des infections chez les patients immunosupprimés comme ceux atteints du virus d'immunodéficience humaine (VIH) – syndrome de l'immunodéficience acquise (SIDA) et les patients cancéreux. L'augmentation récente de l'utilisation de médicaments immunosuppresseurs a aussi accru les risques d'infection à *C. krusei*. *Candida krusei* possède une résistance intrinsèque à plusieurs médicaments antifongiques de type triazole, notamment le fluconazole, qui est la principale drogue utilisée en thérapie antifongique; ainsi son utilisation industrielle sécuritaire soulève de sérieuses questions.

**Mots-clés**: *Candida krusei*; Biologie de la levure; Applications biotechnologiques; Pathogénicité.

## ABSTRACT

Yeasts have a tradition in biotechnological applications and *Saccharomyces* species are the most dominating representatives. Among the yeast species, *Candida krusei* has been isolated from different habitats and in recent years, it has gained increased interest because of its diverse biotechnological roles. It is found in many fermented food items and dairy products and it has also been exploited for production of biochemicals and enzymes. However, because of its opportunistic pathogenic nature, it draws scientific attention regarding the safety of its industrial exploitation. *Candida krusei* generally causes infections in immunocompromised patients, such as those suffering from the Human immunodeficiency virus-acquired immune deficiency syndrome, and also in cancer patients. The recent increase in the use of immunosuppressive drugs has increased the chances of *C. krusei* infections. *Candida krusei* possesses an intrinsic resistance to many triazole antifungal drugs, especially fluconazole, which is a main drug used in antifungal therapy; therefore, there is serious concern regarding its safe industrial use.



**Keywords:** *Candida krusei*; Yeast biology, Biotechnological applications; Pathogenicity.

# INTRODUCTION

Yeasts are well known for their biotechnological potential or industrial applications. Yeasts are usually used in both traditional and modern biotechnology for the production of food products, beverages, enzymes, fine chemicals, and pharmaceuticals. Yeasts are also used in the production of single-cell protein, flavouring compounds, and in various biotransformations (Satyanarayana and Kunze 2009). Modern research is also exploiting yeasts for research in genetics, molecular biololy and cell biology. Though it is true that the majority of the research has been focused on 2 species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, another medically important yeast, *Candida albicans*, is being exploited mainly in biomedical and clinical research. *Saccharomyces cerevisiae* is the most biotechnologically exploited yeast species because of its Generally Regarded as Safe (GRAS) status, as designated by the United States Food and Drug Administration. There is an increasing interest in the use of molecular biology tools and processes to develop some nonconventional yeast strains for the production of beneficial products. Among nonconventional yeasts, *Candida* species mainly draw interest for their biotechnological applications.

The important pathogenic species in the genus *Candida* are *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*. These species contribute to more than 80% of the clinical isolates, while species like *Candida krusei*, *Candida parapsilosis*, *Candida guilliermondii*, and *Candida kefyr* strains are isolated sporadically and are thought to be less virulent (Samaranayake and Samaranayake 1994). Recent clinical data also show similar trends regarding the *Candida* species, where *C. albicans* is the predominant yeast in blood infections, accounting for 55% of the total yeast isolates in the United States. It is also dominant in Canada, Latin America, and Europe. After *C. albicans*, the dominant species are *C. glabrata* (21%), *C. parapsilosis* (11%), *C. tropicalis* (9%), *C. krusei* (2%), and other *Candida* species (2%) (Pfaller et al. 2006, 2008; Fenn 2007). Samie and Mashao (2012) reported on the diversity of yeast infection from HIV-infected patients with tuberculosis from South Africa. The identified species were *Cryptococcus neoformans* (28%), *C. parapsilosis* (19%), *C. krusei* (16%), *C. albicans* (15%), *C. tropicalis* (13%), and others (4%).

*Candida krusei* draws the attention of many researchers for its potential biotechnological exploitation. *Candida krusei* shows various biotechnological potentials, but the literature available on its industrial applications is limited. The main reason for this is its opportunistic pathogenic nature. *Candida krusei* represents the imperfect form of *Issatchenkia orientalis*

(Barnett et al. 2000). *Candida krusei* presence has been documented in different natural food products like cheese (Prillinger et al. 1999), cassava fermentation (Oyewole 2001), tapai (Chiang et al. 2006), and togwa (Tanzanian fermented food) (Mugula et al. 2003). Though *C. krusei* has various beneficial roles, its use is limited because of its pathogenicity, drug resistance, and current scenario of medication where immunosuppressive drugs are commonly used (Hachem et al. 2008; Garnacho-Montero et al. 2010). This article reviews both the positive impacts of *C. krusei*, namely its role in fermented food production and potential use in modern biotechnology, and its negative impacts, such as causing infection and being drug-resistant.

## TAXONOMY AND BIOLOGY

Yeast species are found within 2 phyla of the Fungi kingdom: the Ascomycota and the Basidiomycota. Under certain conditions, ascomycetous yeasts, such as *Saccharomyces* and *Candida* species, form ascospores, while basidomycetous yeasts, such as *Trichosporon* and *Rhodotorula* species, are known to produce external spores (Suh et al. 2006; Morrow and Fraser 2009). Fungi have sexual and asexual forms during their life cycle. The sexual form (teleomorph) is considered as the perfect form, and the asexual form (anamorph) is considered as the imperfect form (Jacques and Casaregola 2008). Fungi or yeast identification by conventional methods is based on colony morphology, physiology, and biochemical tests (assimilation of specific carbon source and fermentation tests), but identification by these means has some disadvantages, such as the phenotypic characters may be variable, the methods take a long time (minimum 48 h), and the results may be misleading (Guarro et al. 1999). Therefore, the limitations of these methods has encouraged the application of molecular biology approaches. The variation in the D1–D2 region of the large subunit (26S) of ribosomal DNA was used as a basis to develop a standard test for identification of yeasts and for understanding interspecific phylogenetic relationships (Kurtzman and Robnett 1998). Polymerase chain reaction (PCR)-based methods such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) approaches are extensively used for identification of yeast species. The RAPD technique is based on application of a short (10 bases long) arbitrary primer to amplify the total genomic DNA at low annealing temperature by PCR. Amplification products are usually separated on agarose gels and stained with ethidium bromide. Thanos et al. (1996) used comparative analysis of band patterns generated by RAPD to identify different *Candida* species. RFLP analysis is also a widely used method for yeast species classification and is based on the internal transcribed sequences region of their ribosomal RNA (rRNA) genes. RFLP methodology involves fragmentation of DNA by a restriction enzyme (e.g., *Eco*RI and

*Msp*I) and separation of the DNA fragments through agarose gel electrophoresis, followed by hybridization of a labeled DNA probe to a Southern-blotted genomic DNA digested fragment. On the basis of the rRNA gene sequence, *C. krusei* has been identified from kefir (Fernandez-Espinar et al. 2006; Latorre-García et al. 2007). Another molecular method, nucleic acid sequence based amplification (NASBA), is used to identify the six medically important *Candida* species, including *C. krusei*. NASBA is a specific and very sensitive RNA amplification technique, which is based on the action of three enzymes, reverse transcriptase, RNase H, and T7 RNA polymerase, in isothermal conditions. In *Candida* species, the conserved regions of the 18S rRNA gene are targeted for amplification. Labelled oligonucleotide probes are then hybridized to an internal specific sequence of the *Candida* species (Trtkova and Raclavsky 2006).

In 1960, *Issatchenkia* was proposed as genus name by Kudryavtsev for the ascosporic state of *C. krusei*. This genus was characterized by formation of spherical, possibly roughened, ascospores formed in a persistent ascus. Kurtzman et al. (1980) assigned additional species to this genus as *Issatchenkia orientalis*. However, D1–D2 LSU rRNA gene sequence analysis suggested that *Issatchenkia* species are members of the *Pichia membranifaciens* clade (Kurtzman and Robnett 1998). Some *Issatchenkia* species were described initially as *Pichia* species, and valid names in this genus already exist for *Pichia scutulata* and *Pichia terricola*. The species name *Pichia orientalis* was earlier given to Guilliermond, a taxon of which a culture is no longer available. So, in 1998, Kurtzman transferred *I. orientalis* to *Pichia* under a new species, namely *Pichia kudriavzevii* (Kurtzman et al. 2008). Though the species name of *I. orientalis* was changed, most literature still presents *C. krusei* as *I. orientalis*. Studies of diverse molecular markers clearly validated that *C. krusei* belongs to the Candida clade species. Almost all *Candida* species translate CUG codons (called the CTG clade) as serine in place of leucine except *C. glabrata* and *C. krusei* (Butler et al. 2009). Evolutionary studies and phylogenetic analysis of *Candida* species based on small-subunit rRNA sequences reveals that *C. krusei* is relatively more closely related to *C. parapsilosis* than *C. albicans* (Barns et al. 1991). A mitochondrial gene is also used to evaluate phylogenetic relationships among *Candida* species, and based on this gene, Yokoyama et al. (2000) showed the high interspecies and low intraspecies divergence of the cytochrome b gene sequence. Using cytochrome b gene sequences from the GenBank database of *Candida* species and *S. cerevisiae*, a phylogenetic tree was made to compare the relationship among these yeast species. The phylogenetic tree was drawn using the neighbor-joining method. The statistical method used is maximum likelihood, after complete deletion of gaps in the cytochrome b gene using the nearest neighbor heuristic method (Fig. 1).

*Candida krusei* cells are generally elongated and look like “long grains of rice” with a dimensions of 2.2-5.6 µm × 4.3- 15.2 µm. Budding is the normal process of replication in *C. krusei* in which new cells are formed by the protrusion of smaller daughter cells from the mother cell. The maximal temperature at which *C. krusei* can grow is 43-45 °C. Most *Candida* species require biotin for optimal growth and some have extra requirements of vitamins, but *C. krusei* can grow in vitamin-free media (Samaranayake and Samaranayake 1994). All known isolates of *C. krusei* are able to utilize N-acetylglucosamine, DL-lactate, glycerol, and glucose. Some of the strains can even assimilate galactose, sorbose, and inositol (Hayford and Jakobsen 1999).

On Sabouraud’s dextrose agar, *C. krusei* colonies are white to cream in colour, smooth, glassy, and wrinkled, making them difficult to differentiate from other non-*C. albicans* species by classical methods; however, *C. krusei* colonies are identified as dry, flat, and rough-textured with a pale pink colour in the centre and white edges on CHROMagar Candida medium. CHROMagar Candida medium supplemented with Pal’s agar allows for the identification of mixed Candida culture of *Candida dubliniensis*, *C. tropicalis*, *C. albicans*, and *C. krusei* (Bernal et al. 1996; Sahand et al. 2005). The morphology of *C. krusei* isolated from Toddy palm in Thailand and then grown for 48 h on yeast malt agar media was described as oval to elongated cells under a scanning electron microscope (Tuntiwongwanich and Leenanon 2009).

Yeasts within the *Candida* clade have evolved an extremely divergent pattern of sexual development. *Candida albicans* undergoes a parasexual cycle wherein two diploid cells mate, resulting in cell fusion and a ploidy increase (2N to 4N), and the tetraploid cells then undergo mitosis and random chromosome loss to return to the diploid state with no recognized meiosis. No sexual cycle has yet been described for *C. tropicalis* or *C. parapsilosis* (Butler et al. 2009; Lee et al. 2010). *Candida krusei* is an asexual (anamorphic) form that does not produce ascospores, while *I. orientalis* represents the teleomorph (non-spore) form and persists in warmer regions. The transformation between the two forms is a complex process and it usually depends on the environmental conditions (Barnett et al. 2000; Jolly et al. 2006). Table 1 summarizes important characteristics of different *Candida* species and compares them with those a *S. cerevisiae*.

*Candida krusei* has dual effects for human beings, because traditionally it is considered as a nonpathogenic yeast owing to its presence in many fermented food products, but nowadays it is identified and classified as a non-albicans opportunistic pathogenic yeast.

# TRADITIONAL AND INDUSTRIAL BIOTECHNOLOGICAL APPLICATIONS

Fermentation is one of the oldest forms of food processing techniques. Presently, the products of modern yeast biotechnology form the backbone of many commercially important sectors such as foods, beverages, pharmaceuticals, industrial enzymes and other biochemicals through fermentation processes (Satyanarayana and Kunze 2009). *C. krusei* has shown its presence and importance in various traditional fermented food products, in the production of enzymes, bioproducts, biochemicals and bioremediation applications, which are discussed below.

## Presence and role of *C. krusei* in traditional fermented food products

### Milk products

*Candida krusei* is present in Armada cheese, which is produced from raw goat's milk in North Spain using farmstead procedures without adding a starter culture, i.e., the ripening process is completed by the natural flora of the milk. This implies a possible role of the yeast flora, including *C. krusei*, in the development of flavours in this cheese (Tornadijo et al. 1998). *Candida krusei* is also present in acid curd cheese produced from quarg and plays a role in flavour production of the cheese (Bockelmann et al. 2005). Suusac is a Kenyan traditional fermented camel's milk product, which is produced by spontaneous fermentation via a natural flora, including lactic acid bacteria and yeast such as *C. krusei* (Lore et al. 2005). Kefir is a fermented milk beverage produced from Kefir grains of bacteria and yeasts in the milk. The presence of *C. krusei* has been documented during mass cultivation of kefir (Witthuhn et al. 2005).

### Cocoa fermentation

Cocoa beans are the seeds of fruit pods from the tree *Theobroma cacao*, which is cultivated in tropical regions all over the world. Cocoa beans are embedded in a mucilaginous pulp inside the pods. The fermentation of cocoa is generally done by traditional methods. *Candida krusei*, which is naturally present on the cocoa pods, is a key microorganism in the fermentation process of cocoa. The fermentation helps to break down the mucilaginous pulp

surrounding the beans and causes death of the embryo. The fermentation of cocoa by *C. krusei* develops the chocolate aroma and eliminates the bitterness (Jespersen et al. 2005; Gálvez et al. 2007). The yeast diversity during Ghanaian cocoa bean heap fermentation has been characterized by Daniel et al. (2009) with the help of PCR fingerprinting. *Pichia kudriavzevii* (*I. orientalis*) (30%), *S. cerevisiae* (24%), and *Hanseniaspora opuntiae* (20%) were identified as dominant species of the total microbial community.

## Cassava fermentation

Cassava is a root crop of the tropics. Fufu, which is consumed in Africa, is produced by fermentation of cassava predominantly by lactic acid bacteria (LAB) (e.g., *Lactobacillus plantarum*) and yeasts, with *C. krusei* the dominant yeast species. The main role of *C. krusei* in the fermentation process is to influence the odour of fufu (Oyewole 2001). Gari is a solid-state naturally fermented product that is produced traditionally from cassava pulp in Nigeria. The microorganisms reported for gari production with the use of rudimentary equipment are mainly lactic acid bacteria and yeasts. The main yeast species involved in gari production and aroma development are *S. cerevisiae* and *C. krusei* (Oguntoyinbo 2008). Also, the use of *C. krusei* in ogi production (a traditional fermented food of Nigeria) has been reported by Omemu and Andeosun (2010). Agbelima is another fermented product famous in Ivory Coast and Ghana. It is used in the preparation of conventional meals, such as banku, akple and kenkey (Ellis et al. 1997). The microflora involved in the fermentation of cassava into agbelima shows the presence of LAB species, such as *Lactobacillus brevis*, *L. plantarum*, and *Lactobacillus mesenteroides* and of the yeasts, *C. krusei*, *C. tropicalis* and *Zygosaccharomyces bailii* (Amoa-Awua et al. 1997).

## Tapai and Boza

The indigenous fermented alcoholic beverage tapai is well known in the Kadazan–Dusan–Murut ethnic group of Sabah and is consumed during festive occasions and gatherings. Tapai is produced by fermentation of glutinous rice by yeast and LAB. The yeasts involved in the process have been identified as *S. cerevisiae*, *C. krusei*, *Candida pelliculosa*, *Candida guillermondii*, *Candida magnolia* and *Rhodotorula glutinis*, and the LAB are *L. brevis* and *L. plantarum* (Chiang et al. 2006). The main role of yeasts in these fermented food products is to improve the flavour. Tapai is a safe fermented food product to consume because of the absence of *Enterobacteriaceae* at the end of the fermentation process, probably because of the low pH and the increase in the content of alcohol (up to 12% v/v) (Chiang et al. 2006).

Boza is a rare beverage in the pious Muslim countries that has been also characterized by the presence of *C. krusei* (Teramoto et al. 2001).

### Koko

Koko is a traditional fermented product from Ghanaian maize dough. The microflora that is present naturally in koko has been identified as *Lactobacillus fermentum*, *S. cerevisiae* and *C. krusei* (Hayford and Jakobsen 1999). The detailed role of the microflora present during production of koko has been determined by Annan et al. (2003). The role of *C. krusei* in the fermentation process is the production of aroma compounds such as alcohols, carbonyls, esters, and acids. The combination of *C. krusei* and *L. fermentum* leads to a high sensory score compared to spontaneous fermentations only.

### Elubo

Yams (*Dioscorea rotundata*) constitute the most important food crop in West Africa. Elubo (yam flour) is produced by a fermentation process involving *Lactobacillus* spp. and yeasts, such as *Pichia burtonii* and *C. krusei* (Achi and Akubor 2000).

## Other indigenous fermented foods

There are various Asian indigenous fermented food products that are prepared in a natural fermentation processes. These fermented food products play an important role in household food preparation and in medium and large-scale industries. Kombucha is a beverage produced by fermentation of sweetened boiled tea by a mixed culture of yeast and acetic acid bacteria in which the presence of *C. krusei* has been reported (Aidoo et al. 2006). Papad is an important condiment or savoury food that is prepared from blackgram, lentil, redgram, or greengram flour by fermentation with *S. cerevisiae* and *C. krusei* (Shurpalekar 1986). Wadi, traditionally consumed in India, Bangladesh, and Pakistan is used as a spicy condiment. Wadi is prepared by inoculating the sterilized ingredients with a mixed culture of *C. krusei* and *L. mesenteroides*. The role of these microorganisms in wadi preparation is to increase acidification, leavening, and nutritional factors (B vitamins) (Sandhu and Soni 1989).

## Role of *C. krusei* in enzyme production

### Phytase

Phytases are enzymes that release soluble inorganic phosphate by hydrolyzing phytates. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) are classified as phosphohydrolases and are responsible for catalyzing the sequential release of phosphate from phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate), which is an abundant storage form of organic phosphorus in plant seeds and grains. The acceptance of this enzyme as animal feed supplement (Yi et al. 1996) and its potential in improving human nutrition are increasing worldwide (Da Silva et al. 2005; Kumar et al. 2010). The high-level intake of dietary phytate severely impedes the absorption of important trace elements, such as iron and zinc, in the digestive tract. The phytate content could be reduced through supplementing phytases in human food, and it is an effective way to reduce the negative effects of phytate on mineralization (Greiner and Konietzny 2006). Other applications in the area of aquaculture are also being extensively explored (Robinson et al. 1996). Moreover, some *myo*-inositol phosphates have shown important pharmacological effects, such as prevention of diabetes complications and antiinflammatory activity (Claxon et al. 1990; Bezprozvanny et al. 1991). The use of a specific *myo*-inositol triphosphate as pain killer has also been proposed (Siren 1995). Surprisingly, the esters of *myo*-inositol triphosphate have been shown to exert significant inhibitory effects against retroviral infections, including HIV (Siren 1998).

*Candida krusei* (*I. orientalis*) produces an intracellular phytase. Biodegradation of phytate by *C. krusei* has nutritional importance in that it increases bioavailability of divalent minerals such as iron, zinc, calcium, and magnesium (Quan et al. 2001, 2002). The detailed steps of dephosphorylation of myo-inositol hexakisphosphate ($IP_6$) by a phytase from *C. krusei* WZ-001 were determined by Quan et al. (2003) (Fig. 2). The significant increase in specific phytase activity under phosphate-depleted conditions has been observed in *C. krusei* compared to other *Candida* species (Tsang 2011). So, the enhanced capability of specific phytase activity by *C. krusei* could be useful for industrial applications. Togwa is a naturally fermented product generally prepared in villages in the countryside of Eastern Africa, and it is identified with higher phytase activity due to the presence of *I. orientalis* (Hellström et al. 2010).

### Carbonyl Reductase

The enzyme carbonyl reductase belongs to the class of oxidoreductases that are generally cytosolic enzymes of low molecular weight. Carbonyl reductase is capable of catalyzing the NAD(P)H-dependent reduction of various natural and unnatural carbonyl compounds (Kaluzna et al. 2005; Kira and Onishi 2009). Carbonyl reductase has been purified and characterized from *C. krusei* SW2026 (Li et al. 2010). Ethyl (R)-2-hydroxy-4-phenylbutyrate ((R)-HPBE), an important intermediate in the synthesis of angiotensin-converting enzyme inhibitors, is produced by enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate using *C. krusei* SW2026. The microbial biotransformations that are biocatalyzed by a NADPH-dependent carbonyl reductase are presented in Fig. 3 (Zhang et al. 2009). Angiotensin-converting enzyme inhibitors like enalapril, lisinopril, benazepril, and ramipril are drugs used for the treatment of hypertension (Iroyukifujita et al. 2000; Weinberg et al. 2000). The present approach for synthesis of (R)-HPBE is multistep chemical synthesis. Multistep chemical synthesis has various draw backs, such as multiple steps, consumption of large amounts of toxic chemical reagents, and low yield of the enantioselective final product (Oda et al. 1998; Herold et al. 2000). Therefore, use of a biotechnological process for enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate for production of (R)-HPBE employing *C. krusei* could be an alternative green chemistry approach.

## Role of *C. krusei* in bioproducts and biochemicals

### Single-cell protein

Single-cell protein (SCP) is produced by unicellular microorganisms, such as yeast, algae, and bacteria. It is a good source of protein and is useful for animal feed and human food in cases of protein malnutrition. *Candida krusei* SO1 co-cultured with *Saccharomyces* spp. strain LK3G has been used in the production of SCP from sorghum hydrolysate media. The crude protein content reported was 47%-50% for *C. krusei* SO1 and 45%-48% for *Saccharomyces* spp. strain LK3G. The amino acid profiles of the mixed-culture protein had a composition almost similar to that of the Food Agriculture Organization (FAO) references except for its content of methionine and cysteine (Konlani et al. 1996). A mixed culture of *Geotrichum candidum*, *Hansenula anomala*, and *C. krusei* from whiskey distillery spent has been stabilized for microbial protein production. The mixed-culture protein would be suitable as a dietary supplement for nonruminant animals. A true protein content of 38%-41.7% w/w in the mixed microbial biomass has been reported (Barker et al. 1982, 1983). Though the amino acid profile of SCP produced by *C. krusei* has been reported and is similar to the FAO

guidelines, toxicological data with reference to the United Nations FAO/WHO are scanty. Therefore, more information on the beneficial and toxicological effects are still needed before SCP commercialization.

## Folate biofortification

Folates (vitamins B9) are important cofactors for nucleotide biosynthesis, which is essential for cellular replication and growth. A folate biosynthesis pathway naturally exists in plants, yeast, and in some bacterial species. Hence, they are able to synthesize natural folates. Mammals lack the ability to synthesize folates, and therefore, they are dependent on an adequate dietary intake (Moslehi-Jenabian et al. 2010). A balanced folate intake prevents the risk of neural tube defects in the foetus, cardiovascular diseases, megaloblastic anaemia and in cancer patients, especially in colon cancer patients (Bailey et al. 2003; Jägerstad et al. 2004). Presently, chemically synthesized folic acid or pteroylglutamic acid (Fig. 4a) is mostly used, but it has the side effect of masking pernicious anemia. Because of this side effect, interest in the use of natural folates has been increasing. The natural folates differ from folic acid in the reduction state of the pteroyl group, the nature of the substituents on the pteridine ring, and the number of glutamyl residues attached to the pteroyl group (Fig. 4b). Naturally occurring folates comprise 5-methyltetrahydrofolate; 5-formyltetrahydrofolate; 10-formyltetrahydrofolate; 5,10-methylenetetrahydrofolate; 5,10-methenyltetrahydrofolate; 5-formiminotetrahydrofolate; 5,6,7,8-tetrahydrofolate; and dihydrofolate (LeBlanc et al. 2007).

Currently, yeast has been identified to enhance folate concentration in food products by fermentation (Jägerstad et al. 2005). The folate content of traditionally fermented maize-based porridge, called togwa, consumed in rural areas in Tanzania, has been investigated. The yeasts originally identified from togwa are *C. krusei* (*I. orientalis*), *Pichia anomala*, *S. cerevisiae*, *Kluyveromyces marxianus* and *C. glabrata* (Hjortmo et al. 2008). Kefir, a fermented milk beverage, has also been reported to have high folate content. Yeasts responsible for kefir fermentation are *K. marxianus*, *Saccharomyces exiguus*, *Candida lambica* and *C. krusei* (*I. orientalis*) (Witthuhn et al. 2005; Patring et al. 2006). Detailed studies are required to evaluate the role of *C. krusei* in folate biofortification to augment folate content in fermented foods.

## Glycerol

Glycerol is produced by chemical synthesis from petrochemicals, or by hydrolysis of oils and fats, and by microbial fermentation. In recent years, the supply of oils and fats has been

limited and the cost of propylene has increased, so, the production of glycerol by microbial fermentation process is emerging as an alternative method (Wang et al. 2001). *Candida krusei*, an osmophilic yeast, produces up to 179 g glycerol/L of water to balance the high extracellular osmotic pressure in media containing a high glucose concentration (Omori et al. 1995; Liu et al. 2002; Chen et al. 2006).

### Ethanol

*Candida krusei* IA-1 is identified as an ethanol-producing yeast that does not produce organic acids like succinic acid as by-product, as it is the case with the ethanol-producing yeast *S. cerevisiae*. The main advantage of the absence of succinic acid in the fermented broth is in the separation process. The separation of bioethanol is done by pervaporation using a silicon rubber-coated silicate membrane, which is prone to damage by succinic acid. Separation of ethanol by pervaporation is gaining interest due to its cost effectiveness over the traditional distillation method of separation (Nakayama et al. 2008; lkegami et al. 2009). The bacterial species *Zymomonas mobiliz* also produces ethanol without producing succinic acid, but its industrial application is limited because of its narrow spectrum of sugar (glucose, sucrose, and fructose) fermentation (Lin and Tanaka 2006). *Issatchenkia orientalis* has been isolated from a continuous ethanol production process in which the fermentation system was fed with sugar cane syrup and *S. cerevisiae* cells were recycled. The detailed role of the isolated yeast *I. orientalis* was determined to be its capacity to produce ethanol at higher temperatures (above 40 °C) (Gallardo et al. 2011). The capability of *I. orientalis* to produce ethanol at higher temperatures could be useful for industrial fermentations in tropical climates where the maintenance of an efficient refrigeration system is costly. The production of ethanol at higher temperatures has one more advantage: there is less chance of contamination (Gallardo et al. 2011). A thermotolerant strain of *P. kudriavzevii* (*I. orientalis*) has been documented by Dhaliwal et al. (2011). The isolated thermotolerant, galactose-adapted *P. kudriavzevii* showed ethanol production up to 71.9 g/L from sugarcane juice.

## Role of *C. krusei* in bioremediation

### Decolorization of synthetic dye

Synthetic dyes are xenobiotic chemicals and are difficult to degrade in nature. Currently, the textile industry is one of the major generators of liquid effluent pollutants due to the high quantities of water used in dyeing processes. Therefore, the removal of dyes from aqueous effluents of the textile industry has received considerable research attention (Borchert and

Libra 2001). Biodegradation is one of the promising approaches for the remediation of synthetic dyes in wastewaters because of its cost effectiveness, efficiency and environment-friendly nature (Verma and Madamwar 2003; Jirasripongpun et al. 2007). *Candida krusei* G-1 has been identified as an efficient colour remover from dyes like reactive azo dye and Reactive Brilliant Red K-2BP (Yu and Wen 2005). *Candida krusei* has been isolated from textile industry wastewaters and reported to degrade basic violet 3 (triphenylmethane dye). A maximal decolorization of basic violet 3 up to 74% and 100% has been reported in sucrose and sugarcane bagasse extract supplemented media, respectively. The degradation of basic violet 3 proceeds through stepwise reduction and demethylation processes (Deivasigamani and Das 2011). An increase in the activities of the enzymes nicotinamide adenine dinucleotide-dichlorophenol indophenol reductase and laccase has been observed after the decolorization process. Melanoidins are complex carbohydrate-based, nitrogen-containing high molecular weight polymers formed during the final stage of the Maillard reaction. Chemically, melanoidins are multicomponent polymers consisting of protein-polyphenol-oligosaccharide complexes (Brudzynski and Miotto 2011). The melanoidin pigment, which is present in molasses wastewater as a dark brown pigment, has been documented to be decolorized by yeast *I. orientalis* No. SF9-246 (Tondee et al. 2008). Though limited work has been carried out on bioremediation employing yeasts, yeasts have some advantages in bioremediation due to their fast growth and ability to resist unfavourable environmental conditions (Yang et al. 2003). These properties of yeasts could be useful in future for bioremediation of dyes to control environmental pollutants.

## POTENTIAL RECOMBINANT TECHNOLOGY

There are various important functions of *C. krusei* that could be exploited through genetic engineering manipulations. The genes for biotechnologically important enzymes such as carbonyl reductase and phytase from *C. krusei* could be cloned and overexpressed in bacteria, such as *E. coli*. Even though up to now no work has been carried out with *C. krusei*, *C. parapsilosis* genes closely related (more than 80%), based on cytochrome b gene sequences (Yokoyama et al. 2000), have been cloned. Kosa et al. (2007) constructed 4 cloning vectors pBP1, pBP2, pPK1 and pPK2 for *C. parapsilosis*. The plasmids were based on the *E. coli* vector pUC19, which possesses a ColE1 origin of replication and a β-lactamase gene conferring resistance to ampicillin. The expression vectors pBP3, pBP5 and pBP7 have been also constructed by Kosa et al. (2007). The constructed vectors are suitable for genetic studies of *C. parapsilosis* and functional analysis of its genes, such as expression of cloned genes, intracellular localization of protein products, and monitoring of promoter activity. Both *C. krusei* and *C. parapsilosis* contain a gene encoding for a carbonyl reductase

and the biocatalytic properties of the carbonyl reductase are suitable for diverse industrial applications. The gene that encodes (R)-specific carbonyl reductase (*rCR*) from *C. parapsilosis* CCTCCM203011 has been cloned and sequenced. The *rCR* gene expressed in *E. coli* JM109 produced (R)-1-phenyl-1,2-ethanediol from β-hydroxyactophenone without any additive to regenerate $NAD^+$ from NADH (Xu et al. 2008). Cloning and expression of *rCR* could be applied to *C. krusei* using suitable vectors. Another interesting physiological feature of *C. parapsilosis*, similar to *C. krusei*, is its ability to degrade various aromatic compounds via an operative gentisate pathway that includes flavoprotein monooxygenase catalyzing the regioselective hydroxylation of phenol derivatives. Interestingly, the *C. parapsilosis* genes CPAG-01781 and CPAG-03408, which encode homologs of salicylate 1-monooxygenase and gentisate 1,2-dioxygenase, respectively, are not found in the *C. albicans* genome. This indicates that degradation of phenolic compounds has particular physiological functions in *C. parapsilosis* species (Nosek et al. 2009).

# PATHOGENIC ROLE OF C. KRUSEI IN HUMAN DISEASE

## Epidemiology and pathogenicity

Opportunistic fungal or yeast infections are one of the major reasons of morbidity and mortality in immunocompromised patients. *Candida* species are usually found as commensal organisms in association with human and animal hosts. The most common *Candida* species causing infection is *C. albicans* and, of the non-*albicans* species, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* are the most common causes of infection (Fidel et al. 1999). The classification of fungal infections is dependent on the degree of tissue involvement and the mode of entry of the pathogen. Infection classifications are as follows: (i) superficial infections are localized to the skin, hair, and nails; (ii) subcutaneous infections are confined to the dermis, subcutaneous tissue, or adjacent structures; and (iii) systemic infections are confined to deep infection of the internal organs. With systemic infections, the infection spreads from the original site of infection and enters the blood stream. Systemic infections usually occur only in immunocompromised patients (Odom 1994; Hay 2006).

The severity of fungal or yeast infections depend upon the various host defence mechanisms. There are various defence mechanisms that play an important role in host defence against microbial invasion. The epidermal host defence mechanism involves desquamation of the epithelium, increased saliva production, fungistatic factors in saliva such as lactoferrin, lactoperoxidase, and lysozymes, interaction with the resident bacterial flora, and adherence inhibitors like mucins (glycoproteins). Activation of CD4 T-lymphocytes and of

neutrophil leucocytes and secretion of immunoglobulin A antibodies are also part of the host defence mechanism (Rogers and Balish 1980; Romani et al. 1996).

The pathogenesis caused by *Candida* species proceeds through different stages. The first step of pathogenesis is adherence of *Candida* to epithelial cells of the host. Adhesion is prerequisite to colonization and invasion of the *Candida* spp. virulence and it is initiated with different factors produced by virulence genes such as *Hwp1p*, *EAP1*, *CaMnt1p* and *Csf4*. Adhesion is followed by invasion into submucosa, angioinvasion adherence to endothelial cells and dissemination, resulting in systemic candidiasis (Odds 1994; Segal 2006).

*Candida krusei* has emerged in recent years as an opportunistic, nosocomial fungal pathogen responsible for 2% of candidemia cases (Kang et al. 2010), with a particular preference for neutropenic adult cancer patients (Wingard et al. 1991). The largest proportion of *C. krusei* infections has been seen in patients with leukaemia (13%-25%) and the lowest in neonates (Abi-Said et al. 1997). It colonizes and infects the vagina, urinary tract, or gastrointestinal tract of immunocompetent persons and may be fatal, particularly in immunocompromised cancer patients. The susceptibility of *C. krusei* infections is correlated with the use of broad spectrum antibiotics, corticosteroids, antitumoral agents, oral contraceptives and HIV protease inhibitors and with an increase in the number of immunocompromised patients (Eggimann et al. 2003).

*Candida krusei* mainly causes fungaemias (also known as candidemia or invasive candidiasis), which is the infection of blood with fungi or yeast; endophthalmitis, an inflammation of the internal coats of the eye; endocarditis; and osteomyelitis, an infection of bone marrow (Krcmery and Barnes 2002). For the past two decades, the increased incidence of nosocomial fungal infections has been reported owing to the invasive use of medical devices and wider use of broad spectrum antimicrobial agents (Hachem et al. 2008).

Similar to *C. albicans*, *C. krusei* has several virulence attributes. Some of these include adherence to host surfaces, production of phospholipases and proteinases, antigenic variability, dimorphic transition (yeast to hypha), phenotypic switching or capability of switching among different cell phenotypes, and modulation of the host's immune response (Samaranayake et al. 1998; Calderone and Fonzi 2001). Most of the virulence genes have been well identified and characterized in *C. albicans*, but they are not well characterized in *C. krusei* owing to the unavailability of its whole genome sequence (Pang et al. 2012). *Candida krusei* is usually found in two basic morphological forms (dimorphism): yeast and pseudohyphae (Samaranayake and Samaranayake 1994). It is generally accepted that

dimorphism is a virulence trait and is coregulated with other virulence factors associated with cellular morphology (Brown and Gow 1999). Normally, the yeast state is noninvasive, whereas the transition to pseudohyphae makes *C. krusei* invasive (Samaranayake et al. 1998). Though hyphae formation takes place in *C. krusei*, it is less effective compared to *C. albicans*. Studies on rat epithelial and mucosal cells have shown that *C. krusei* hyphae do not penetrate into deep tissue as effectively as *C. albicans* hyphae. Furthermore, colonization in the tissue by *C. krusei* is less effective than colonization by *C. albicans* (Samaranayake et al. 1998; Dorko et al. 2001).

Phenotypic switching is recognized as one of the important virulence factors of *Candida* species. The switched state helps *Candida* to rapidly adapt to different environmental conditions. Under the phenotypic switched condition, *C. krusei* also shows higher adherence ability and the lowest susceptibility towards antifungal agents. In addition, *C. krusei* exhibits high hydrophobicity, which enhances its adherence to the host (Samaranayake and Samaranayake 1994; Arzmi et al. 2012).

The hydrolytic enzymes (exoenzymes) secreted by pathogenic fungi are the major virulence factors for their invasiveness. The virulence property of *Candida* species depends on their ability to secrete exoenzymes (proteinases and phospholipases), and the activities of these enzymes are influenced by the medium. The exoenzyme secretions by *C. albicans* is more pronounced than that of *C. krusei*, therefore, *C. krusei* is less virulent (Costa et al. 2010). In a liver transplant recipient infected with *C. krusei*, two main hydrolytic enzymes were identified: esterase lipase C8 and valine arylamidase (Kawecki et al. 2006). Table 2 summarizes the diseases caused by *C. krusei* in general and in susceptible patients.

## Classification and mechanism of action of antifungal (anti-*Candida*) drugs

To understand antifungal drugs and their mechanism of action requires a brief look at the chemical nature of drugs. Antifungal drugs are generally classified into the following groups on the basis of their chemical structure, and the structure of representative antifungal agents of each group is shown in Fig. 5 (Ghannoum and Rice 1999; Odds et al. 2003; Cannon et al. 2007).

**1. Azoles**- examples include ketoconazole, fluconazole, itraconazole, and voriconazole. All azole antifungal agents work principally by inhibiting the cytochrome *P*-450 14a-demethylase. This enzyme is involved in the sterol biosynthesis pathway that leads to synthesis of ergosterol from lanosterol. Ergosterol serves as a bioregulator of membrane

fluidity and asymmetry, which is essential to maintaining membrane integrity in fungal cells (Fig. 6) (Georgopapadakou and Walsh 1996; Ghannoum and Rice 1999).

**2. Polyenes**- main examples are nystatin and amphotericin B. Antifungal polyenes bind to ergosterol, which is the main component of fungal cell membranes, forming a transmembrane channel that leads to monovalent ion leakage and finally to fungal cell death (Georgopapadakou and Walsh 1996; Ghannoum and Rice 1999).

**3. Allylamines/thiocarbamates-** terbinafine and naftifine are classified under this class. Terbinafine is an allylamine that inhibits ergosterol biosynthesis by inhibiting squalene epoxidase. Ergosterol biosynthesis is started by squalene epoxidation, which is catalyzed by squalene epoxidase from the precursor squelene (Fig. 6) (Ghannoum and Rice 1999; Odds et al. 2003).

**4. Others-** an example is 5-flurocytosine, a fluorinated pyrimidine, which acts by interfering with RNA, DNA and protein synthesis in fungal cell. One of the most recently developed classes of antifungal drugs is cyclic lipopeptides called echinocandines. This group is represented by caspofungin, micafungin and anidulafungin. These drugs act by retarding cell wall biosynthesis by inhibiting (1,3)-D-β-glucan synthase (Georgopapadakou and Walsh 1996; Ghannoum and Rice 1999; Odds et al. 2003).

## Drug resistance and management of *C. krusei* infection

*C. krusei* possesses an intrinsic resistance to many azole antifungal agents, especially fluconazole, and some strains have elevated minimal inhibitory concentrations (MIC) to itraconazole (MIC 0.5 to 1.0 mg/L). All *C. krusei* isolates have been reported as resistant to fluconazole and some isolates also exhibit decreased susptibility to amphotericine B and flucytosine (Pfaller and Diekema 2007; Pfaller et al. 2008). Table 3 summarises the MIC of regularly used antifungal drugs against *C. krusei* in comparison to *C. albicans*. Microbiological antifungal resistance may be defined as non-susceptibility of fungal infections to antifungal therapy. Resistance can be measured by *in vitro* susceptibility testing in which the MIC of the drug exceeds the susceptibility break point for that organism. Antifungal drug resistance has been classified as primary (intrinsic)- resistance found to be present inheritably among some fungi without any earlier exposure to antifungal drugs or as secondary (acquired) resistance, which develops after exposure to antifungal drugs owing to stable or transient genotype alteration (Kontoyiannis and Lewis 2002; Kanafani and Perfect 2008).

Three main mechanisms which are responsible for azole resistance have been described in *Candida* species. The first one is an alteration of the target enzyme, 14α-demethylase which is encoded by the *ERG11* gene, the key enzyme of ergosterol biosynthesis. Some microorganisms overexpress the 14α-demethylase gene and/or the enzyme is less susceptible to azoles inhibition. The second mechanism is lowered accumulation of the drug at the target site due to action of efflux pumps. Efflux pumps are encoded by the family of transporter genes *Cdr1-Cdr2* and *Mdr1*. The third mechanism is alteration in sterol biosynthesis caused by a deficiency in $C_{5(6)}$ desaturase (Anderson 2005; Kanafani and Perfect 2008; Perlin 2009).

Azole resistance (especially fluconazole) by strains of *C. krusei* is mainly the result of decreased affinity of 14a-demethylase encoded by the *ERG11* gene to fluconazole (Orozco et al. 1998; Anderson 2005; Kanafani and Perfect 2008). One recent study documented the presence of an alternative oxidase in *C. krusei* and its impact on generation of reactive oxygen species by fluconazole. The results showed there was a reduction of reactive oxygen species generation due to the alternative oxidase which resulted in a decrease of susceptibility of *C. krusei* to fluconazole (Costa-de-Oliveira et al. 2012). Some isolates of *C. krusei* also show reduced susceptibility to caspofungin, which is an echinocandin family antifungal drug. Mutation in the amino acid region of *Fks1p*, a component of the catalytic subunit of (1,3)-b-D-glucan synthase, has been associated with caspofungin resistance in *C. krusei* (Hakki et al. 2006; Kahn et al. 2007).

The mortality rate as a result of *C. krusei* infection is high (up to 60%) due to multidrug resistance, which makes its management problematic (Pfaller and Diekema 2007; Pfaller et al. 2008; Kang et al. 2010); therefore, early detection of the pathogen is prerequisite for therapy. The diagnosis can be done accurately with the help of modern molecular techniques. PCR and NASBA-based techniques have potential for early diagnosis of fungal infection (Perlin 2009). New data suggests that most recently developed azole agents like posaconazole, ravuconazole, and glucan inhibitors, like micafungin and anidulafungin, have activity against *C. krusei* and other more resistant *Candida* species (Pfaller et al. 2008). Among the systemically active antifungal agents, echinocandins, especially anidulafungin, appear to be the most active (Denning 2002).

## CONCLUSIONS AND FUTURE PERSPECTIVES

The traditional and industrial applications, epidemiology, pathogenicity, drug resistance, and brief history of *C. krusei* have been reviewed. The available literature indicates that *C. krusei*

is significantly different from the other nonpathogenic yeasts and medically important *Candida* species. *Candida krusei* has some distinct characteristics, which are as follows:

- It is naturally present in many traditional fermented food products.
- It has potential to produce valuable enzymes, bioproducts and biochemicals.
- It can be used in bioremediation of xenobiotic compounds.
- It is an opportunistic pathogen mainly in immunocompromised humans.
- Present scenario of medication makes patients more susceptible towards infection by *C. krusei*.
- *C. krusei* possesses multidrug resistance genes, which leads to high mortality rate.

Because of its dual nature (traditional and industrial potentialities as well as pathogenic role), the main concern regarding this yeast is how it could be exploited for its beneficial purposes while at the same time preventing its pathogenicity. Its presence in various traditional food products (i.e., milk products, cocoa fermentation, cassava fermentation, tapai, and koko) indicates the possibility that there is no secretion of mycotoxins in the final fermented products, interestingly *C. krusei* is documented as a mycotoxin decontaminating agent (Shetty and Jespersen 2006). Still, there is a need to take extra precautions during the production of SCP (i.e., there should not be any live yeast cells in the final product), as it is an opportunistic pathogen. Future research should place emphasis on determining the actual nature of all *C. krusei* strains with the help of molecular biology tools such as PCR-RFLP and RAPD-PCR. To date, no work has been done to exploit the nonpathogenic nature of *C. krusei* strains, but the traditional presence of *C. krusei* strains in several fermented food products indicates that some strains may be nonpathogenic. Biomedical science clearly considers it an opportunistic pathogen, so a clear line needs to be drawn for its industrial exploitation.

*Candida krusei*, in mixed culture with *K. marxianus*, has shown good growth and biomass production with efficient removal of chemical oxygen demand from cheese whey. The biomass could be used as SCP (J.S.S. Yadav, J. Bezawada, S. Yan, and R.D. Tyagi. unpublished result). Cheese whey, a by-product of the dairy industry, contains lactose (4.5%-5% w/v) and it is considered an environmental pollutant owing to its high chemical oxygen demand (Siso 1996). As reported, there is no toxic effect of the *C. krusei* SCP, thus, it could be considered for production of SCP. However, toxicity and allerginicity of SCP should be evaluated in long term clinical trials. The mixed culture of *K. marxianus* and *C. krusei* could also be utilized for ethanol production using cheese whey as fermentation substrate. Production of ethanol using *K. marxianus* and *C. krusei* will be the most exploited research

interest in the near future. *Candida krusei* can be more useful for ethanol production in tropical climates because it can grow at high temperatures. The other areas of research that should be pursued include the exploitation of *C. krusei* for biotechnological purposes (such as utilization for production of enzymes such as phytase and carbonyl reductase) and biodegradation (bioremediation). Until now, *C. krusei* genes, which have potential biotechnological application, have not been genetically cloned and expressed; hence, in the future, more studies are required to reveal the nature of the genes that can be expressed and exploited for biotechnological benefits.

## ACKNOWLEDGEMENTS

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support**.**

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**Table 1. Comparison of some properties of pathogenic yeast *Candida* species and *S. cerevisiae***

| **Properties** | ***C. albicans*** | ***C. glabrata*** | ***C. tropicalis*** | ***C. parapsilosis*** | ***C. krusei*** | ***S. cerevisiae*** | **References** |
|---|---|---|---|---|---|---|---|
| Genome size (Mb) | 14.3 | 12.3 | 14.5 | 13.3 | Unknown | 12.5 | Butler et al. 2009; ten Cate et al. 2009; Inglis et al. 2011 |
| No. of genes | 6107 | 5212 | 6258 | 5733 | Unknown | 5770 | Butler et al. 2009; Inglis et al. 2011 |
| Ploidy | Diploid | Haploid | Diploid | Aneuploid/ diploid | Diploid | Haploid/ diploid | Butler et al. 2009; Lee et al. 2010; ten Cate et al. 2009 |
| CTG clade | Yes | No | Yes | Yes | No | No | Butler et al. 2009; ten Cate et al. 2009 |
| Pseudohyphae | Yes | Yes | Yes | Yes | Yes | Yes | ten Cate et al. 2009 |
| Hyphae formation | Yes | No | No | No | No | No | ten Cate et al. 2009 |
| Pathogenicity* | **+++** | **++** | **++** | **++** | **+** | **-** | Butler et al. 2009; Alby and Bennett 2010 |
| Reproduction | Asexual/ parasexual | Asexual | Asexual | Asexual | Asexual/ sexual | Sexual/asexual | Lee et al. 2010 |

*****Pathogenicity: +++, highest; ++, moderate; +, least; -, none.

**Table 2. Summary of diseases caused by *Candida krusei***

| Disease | Susceptible patient | References |
|---|---|---|
| Fungaemia | Neutropenic | Munoz et al. 2005; Abbas et al. 2000 |
| Soft tissue abscess | Osteoarthritis | Jiang et al. 2006 |
| Pneumonia | Post- transplantation | Petrocheilou-Paschou et al. 2002 |
| Oral Candidiasis | Leprosy | Reichart et al. 2002 |
| Candidemia | Allogeneic marrow transplant | Safdar et al. 2001 |
| Oncholysis | Healthy | Rao et al. 2004 |
| Vaginitis | Older age | Singh et al. 2002 |
| Spondylodiscitis | Acute myeloid leukemia | Peman 2006 |

**Table 3. Comparison to antifungal drugs susceptibility of *C. krusei* and *C. albicans***

| ***Candida* species** | **Range of minimal inhibitory concn. (µg/mL) of:** | | | | | **References** |
|---|---|---|---|---|---|---|
| | Fluconazole | Voriconazole | Amphotericin B | Caspofungin | Micafungin | |
| *C. krusei* | 32.0-64.0 | 0.25-1.0 | 0.5-4.0 | 0.06-2.0 | 0.03-0.25 | Hakki et al. 2006; Singh et al. 2002; Pfaller et al. 2006 |
| *C. albicans* | 0.13-8.0 | 0.006-0.13 | 0.25-0.50 | 0.05-1.0 | 0.06-0.25 | Fukuoka et al. 2003; Junqueira et al. 2011; Pfaller et al. 2006 |

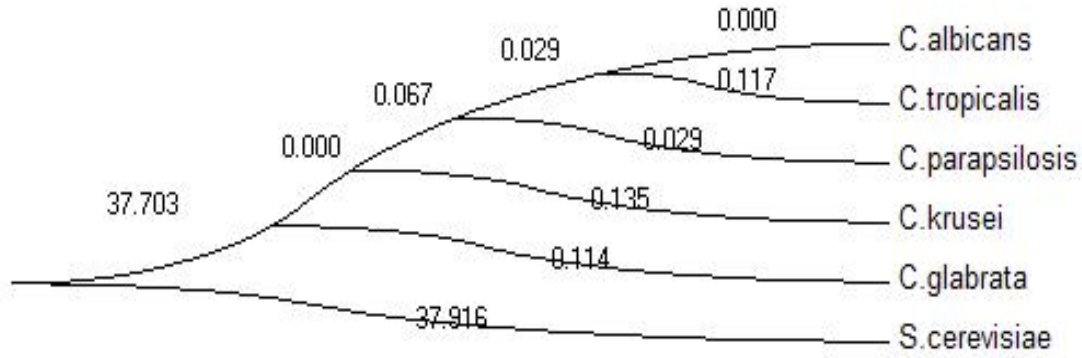



**Figure 1. Phylogenetic tree illustrating the relationship of *C. krusei* with *Cadida* species, based on the cytochrome b gene**

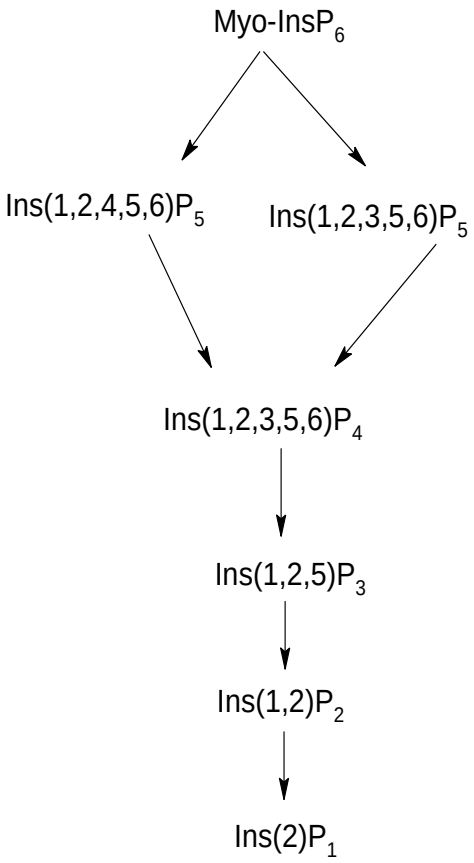



**Figure 2. Pathways of dephosphorylation of myo-inositol hexakisphosphate (*myo*-$IP_6$) catalyzed by the phytases of *Candida krusei* WZ-001**

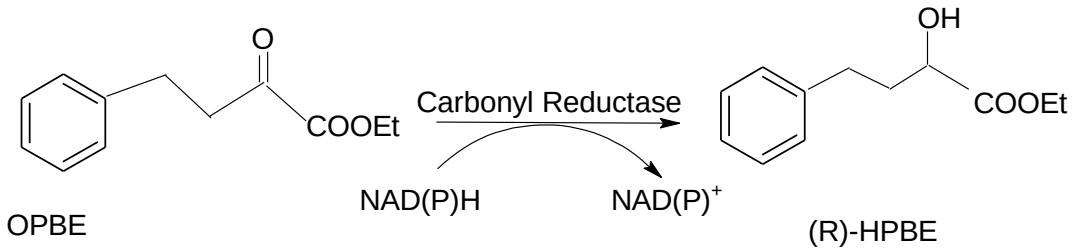
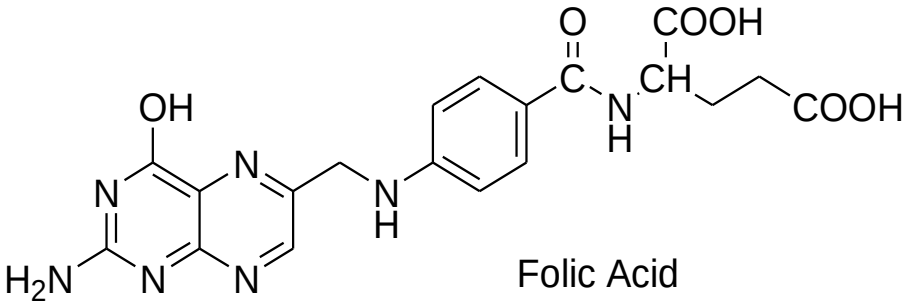



**Figure 3. *C. krusei* SW2026 mediated asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) to ethyl(R)-2-hydroxy-4-phenylbutyrate [(R)- HPBE]**

**a)**




**b)**

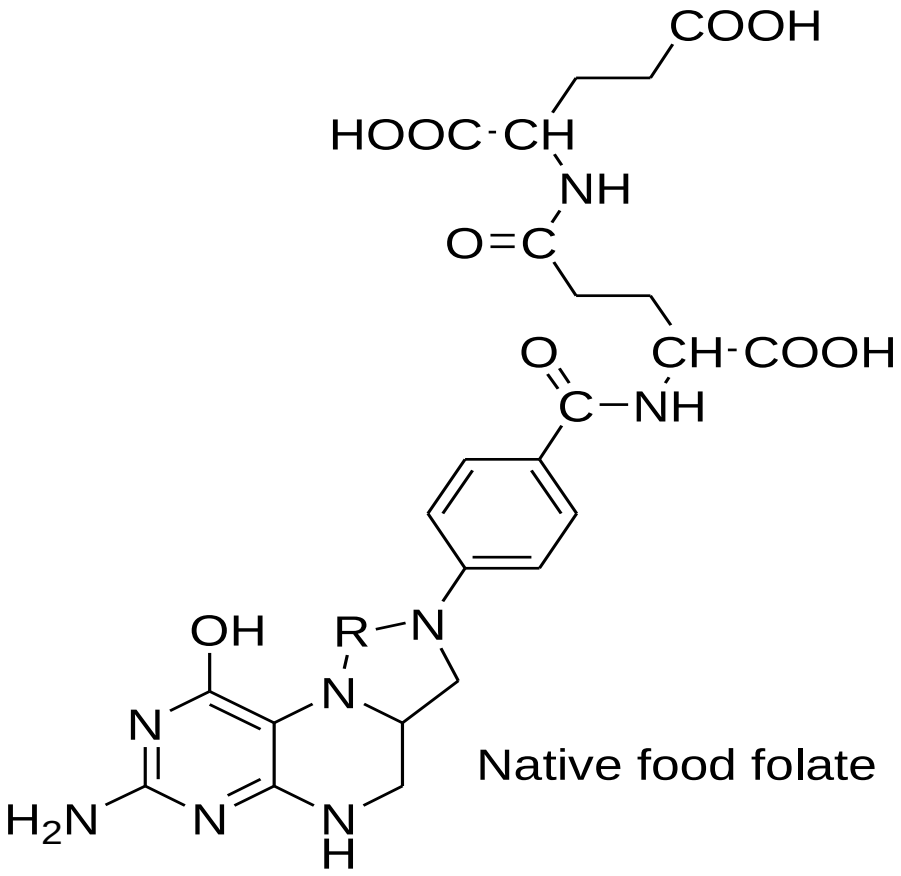
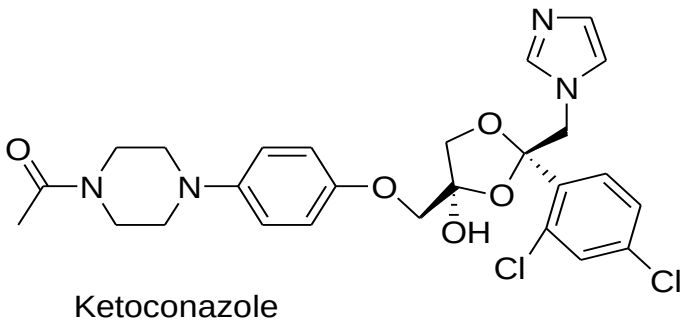
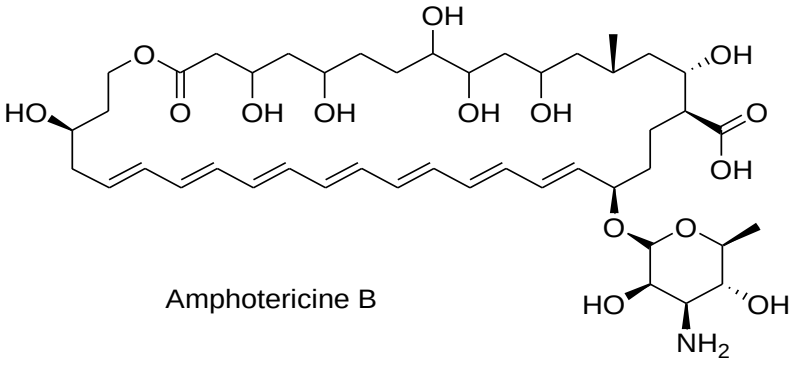


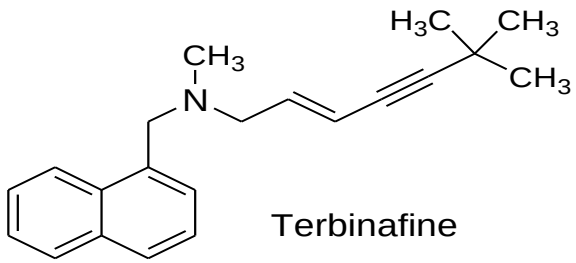

**Figure 4. Chemical difference between (a) folic acid (pteroly-L-glutamic acid) and (b) native food folates. Substitutens (R): -$CH_3$, methyl; -CHO, formyl; -CH=NH formimino; -$CH_2$, methylene; -CH= methenyl**



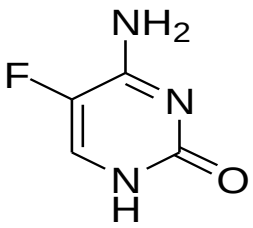

Ketoconazole




Amphotericine B




Terbinafine




5-Fluorocytosine

**Figure 5. Structure of representative antifungal agents**

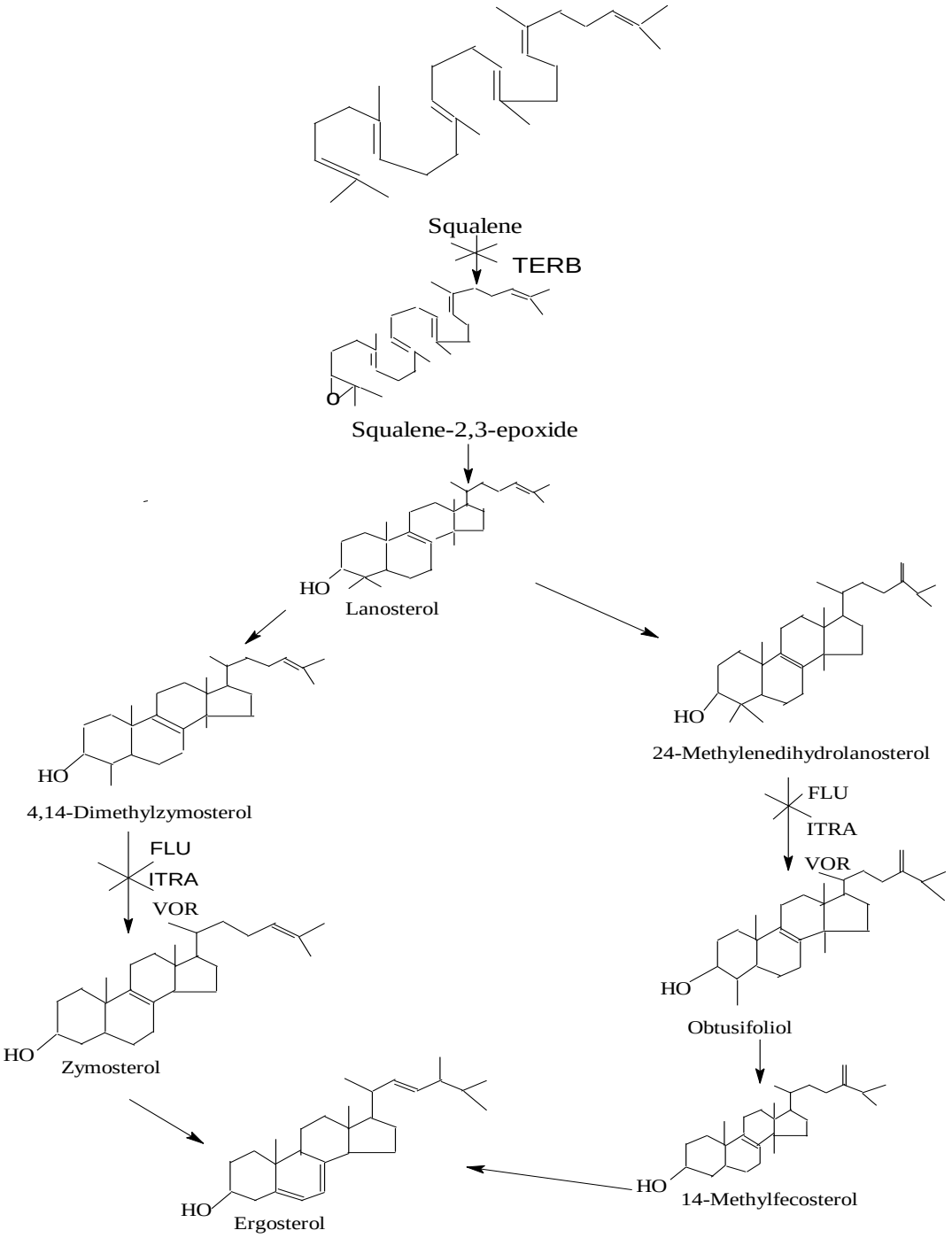



**Figure 6. Ergosterol biosynthesis pathways. Sites where antifungal agent acts and blocks ergosterol biosynthesis. Antifungal agents: terbinafine (TERB), fluconazole (FLU), itraconazol (ITRA) and voriconazole (VOR)**

# PARTIE 2

# MIXED CULTURE OF KLUYVEROMYCES MARXIANUS AND CANDIDA KRUSEI FOR SINGLE-CELL PROTEIN PRODUCTION AND ORGANIC LOAD REMOVAL FROM WHEY

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**Bioresource Technology**

**164 (2014) 119–127**

# RÉSUMÉ

L'étude réalisée visait à évaluer le potentiel des cultures mixtes de *Kluyveromyces marxianus* et *Candida krusei* afin d'améliorer l'efficacité d'élimination de la DCO, de minimiser la contamination lorsque des conditions extrêmes ont lieu pendant la fermentation aérobie continue et discontinue (une haute température fixée à 40 $^{o}$C et un faible pH fixé à 3,5), et d'améliorer la qualité des protéines unicellulaires (PU) obtenues lors de l'utilisation du lactosérum comme substrat. La fermentation discontinue des monocultures et les résultats obtenus avec les cultures mixtes ont montré que ces dernières ont engendré 8,8% de plus en efficacité d'élimination de la DCO, en combinaison avec un rendement de 19% de plus en biomasse et une productivité de 33% supérieure. L'élimination maximale de 80,2% de la DCO (incluant les protéines résiduelles) a été obtenue après 24 heures de temps de rétention hydraulique (TRH) en combinaison avec 0,17 g/L/h de productivité en biomasse. La productivité maximale de 0,38 g/L/h en biomasse a toutefois été atteinte après 6 heures de TRH en combinaison mais avec seulement 34% d'élimination en DCO. Les résultats ont montrés que les cultures mixtes de levures employées dans cette étude sont résistantes aux conditions acides et thermiques extrêmes, et représentent une avenue prometteuse dans la production des PU (alimentation animale), conjointement avec l'élimination de la DCO en ces conditions d'opération extrêmes.

**Mots-clés:** Lactosérum; *Candida krusei*; *Kluyveromyces marxianus*; Cultures mixtes; Fermentation continue.

## ABSTRACT

The study was conducted to evaluate the potential of mixed culture of *Kluyveromyces marxianus* and *Candida krusei* to enhance COD removal efficiency, minimize contamination at extreme conditions (high temperature 40 $^{o}$C and low pH 3.5) during batch and continuous aerobic fermentation and to obtain improved quality single-cell protein (SCP) using whey as substrate. Batch fermentations of a mono-culture and a mixed culture showed that the mixed culture resulted in 8.8% higher COD removal with a 19% higher biomass yield and 33% of increased productivity. The maximal COD removal of a 80.2% (including residual protein) was obtained at 24 h HRT with a biomass productivity of 0.17 g/L/h; however, a maximal biomass productivity of 0.38 g/L/h but only 34% COD removal were obtained at 6 h HRT. The results showed that the mixed culture of acid resistance and thermotolerant yeasts is a potential approach for producing SCP (animal feed) and simultaneous COD removal under extreme operating conditions.



**Keywords:** Cheese whey; *Candida krusei*; *Kluyveromyces marxianus*; Mixed culture; Continuous fermentation.

# 1. INTRODUCTION

Cheese whey, a by-product of cheese producing industries is produced during the coagulation of milk casein. The generation of large volumes of cheese whey poses an environmental problem due to its high organic matter content. The high organic content leads to chemical oxygen demand (COD) of 60-80 g/L and biological oxygen demand (BOD) of 40-60 g/L, respectively (Guimarães et al., 2010). Generally, cheese whey contains lactose (4.5-5.0% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (0.8-1.0% w/v) (Panesar and Kennedy, 2012). Production of cheese whey is increasing day by day as the demand for milk and milk-derived products increases. As per a Food and Agriculture Organization (FAO) 2005 report, the worldwide annual production of cheese whey is around 139 billion kilograms of which 35.2 and 3.1 billion kilogram are generated in the USA and Canada, respectively (Ghaly et al., 2007; Yadav et al., 2013). About 50% of the total whey produced is either dumped into sewers or applied on land (Ghaly et al., 2007). The disposal and land application have negative effect on health and environment (Ghaly and Kamal, 2004; Ghaly et al., 2007). The surplus availability of whey and its nutrient content provide an opportunity to use several biotechnological routes to transform it into value added products such as ethanol, lactic acid, enzymes, biopolymer, biogas, kefir-like whey drinks, probiotics and single-cell protein (SCP) (Koutinas et al., 2009; Mollea et al., 2013; Panesar and Kennedy, 2012). Among these, biotransformation of whey into SCP has arisen as one of the attractive options and is employed in several countries (Mollea et al., 2013; Panesar and Kennedy, 2012). The bioconversion of whey (mainly lactose) into yeast biomass has advantages such as it is a simple wastewater treatment process (i.e. COD removal), and final discharge of the whey has been facilitated since the pollutant load is significantly reduced.

The most widely yeast species used for SCP production from cheese whey is *Kluyveromyces marxianus*, which is categorised as generally regarded as safe (GRAS) microorganism for food and feed applications (Fonseca et al., 2008). Though SCP production using lactose-utilizing yeast (*K. marxianus*) is a good alternative for whey treatment (crude whey or whey permeate) and the most widely employed practice, however, there are some challenges during whey processing and the bioconversion process: i) contamination during fermentation; ii) low COD removal efficiency; iii) lower product quality of the produced SCP; iv) whey processing on site in small and medium scale industries; v) poor economic return (Chatzipaschali and Stamatis, 2012; Çinar et al., 2006; Cristiani-Urbina et al., 2000; Spălățelu, 2012). Fermentation under extreme conditions such as low pH and high temperature has been suggested to prevent the contamination problem especially with

pathogenic bacteria (Pacheco and Galindo, 2010). These conditions are unfavourable for most of the microorganisms used for whey bioconversion. Another important issue for whey processing to produce SCP is the fact that most of the results reported in the literature have employed whey permeate for the biotransformation process. The recovery of whey protein is carried out by protein precipitation or by membrane filtration before fermentation to produce SCP. The presence of lactose in whey poses a hindrance during the recovery process of protein by precipitation or by membrane filtration. Moreover, some companies do not want to apply two processes separately (i.e. whey protein separation and whey permeate treatment) for economic reasons. Therefore, recovery of protein before fermentation is not of interest especially for small and medium scale industries (Yadav et al., 2014). However, if the protein is recovered after fermentation, lactose interference will be eliminated due to its consumption the during fermentation process.

Further, the economics of SCP production and of simultaneous COD reduction could be improved using a continuous fermentation process; however, such a process is prone to contamination. A continuous fermentation process can help to reduce the cost of the process and it can be employed for bioconversion of large volumes of whey (i.e. simultaneous SCP production and wastewater treatment) provided the contamination problem is minimized. Moreover, product quality (amino acid profile of SCP) can be improved by employing a mixed culture.

Therefore, there is a need for a strategy and a process which could efficiently tackle the above problems with on-site utilization of cheese whey. The problem of contamination could be minimized by using extreme conditions like low pH (3-4) and high temperature (40-45 $^{o}$C) during the fermentation process; however, this required acid resistant and thermotolerant microorganisms. Furthermore, employing a mixed culture, especially a combination of lactose- and non-lactose consuming microorganisms, which could grow on the metabolites of the lactose-utilizing yeast (*K. marxianus*), could be highly efficient for COD removal. *K. marxianus* is used as the principal microorganism to produce SCP. This strain is poor in sulfur containing amino acids and in lysine. The mixed culture (*Candida krusei* & *K. marxianus*) will be useful to improve protein quality as *C. krusei* contains these amino acids (Konlani et al., 1996; Rajoka et al., 2012).

The yeast *C. krusei* (*Issatchenkia orientalis*), which is generally present in milk and in other dairy products (Lavoie et al., 2012) has not been exploited for its industrial potential. *C. krusei* could be applied for production of SCP as animal feed with simultaneous removal of COD from cheese whey because it does not excrete any extracellular toxin (Yadav et al.,

2012). However, *C. krusei* is regarded as an opportunistic pathogen (in immunocompromised hosts). *C. krusei* is acid resistant and thermotolerant, it can grow at low pH (3-4) and at high temperature (40-43 °C) (Shin et al., 2002; Yadav et al., 2012). The natural presence of *C. krusei* has been documented in many traditional fermented foods (Armada cheese, suusac, kefir, cocoa & cassava fermentation, tapai, koko, elubo, etc.). *C. krusei* can grow on cheese whey in a mixed culture mode and it could be used as SCP (animal feed) after spray drying. It was suggested that *C krusei* should be cultivated in a mixed culture with *K. marxianus* (Yadav et al., 2012).

Thus, the objective of this work was to evaluate *C. krusei* as a potential candidate in a mixed culture, with *K. marxianus,* for production of SCP with simultaneous removal of COD from cheese whey under extreme fermentation conditions. Furthermore, a continuous fermentation process employing the mixed culture was also studied at different hydraulic retention times (HRT). Additionally, the major composition of the mixed culture SCP produced was evaluated.

# 2. MATERIALS AND METHODS

## 2.1 Cheese whey

Fresh cheese whey was acquired from a cheese producing company in Quebec, Canada and stored at -20 $^{o}$C until used. A high initial lactose concentration is responsible for generation of higher concentrations of intermediate metabolites during the SCP production process that led to lower yields and lower COD removal (Cristiani-Urbina et al., 2000). Therefore, the cheese whey with an initial value of COD 68.0±2.0 g/L was diluted approximately 30.0 g/L, and the diluted cheese whey was used in batch and continuous fermentation experiments. In this study, a higher dilution of whey compared to that of dilution used in chapter two was used because in this study, the objective was to use whey without pasteurization. Thus, there is no cost factor related to pasteurization.

## 2.2 Microorganisms

Strains of the yeasts *K. marxianus* and *C. krusei* were isolated from cheese whey and identified by biochemical and molecular methods (*K. marxianus* strain CHY1612 and *C. krusei* strain NRRL Y-5396, respectively). The culture was sub-cultured on de Man, Rogosa and Sharpe (MRS) agar plates, and incubated at 35 $^{o}$C for 48 h and then preserved at 4 $^{o}$C for future use.

## 2.3 Inoculum preparation

Tryptic Soy Broth (TSB) was prepared and sterilized at 121 $^{o}$C for 15 min. A loopful of *K. marxianus* and *C. krusei* grown on MRS agar plates was used to inoculate separately 100 mL Erlenmeyer flasks containing 20 mL of TSB medium. The flasks were incubated in a rotatory incubator shaker at 150 rpm, 35 $^{o}$C for 12 h. The actively grown cells from the flasks were used as a pre-culture (2% v/v) to inoculate 2000 mL Erlenmeyer flasks containing 500 mL of pasteurized cheese whey supplemented with urea (0.15% w/v) as nitrogen source for *K. marxianus* and sterilized TSB medium for *C. krusei*, respectively. The inoculated flasks were incubated under the conditions described above. The actively grown cells from the flasks were used as inoculum to inoculate 10 L of pasteurized whey medium in the bioreactor for mono-culture (*K. marxianus*) and mixed culture (*K. marxianus* and *C. krusei*), respectively.

## 2.4 Fermentation

Fermentation was carried out in a stirred tank 15 L bioreactor (working volume: 10 L, Biogenie, Quebec, Canada) equipped with accessories and a programmable logic control (PLC) system for dissolved oxygen (DO), pH, anti-foam, impeller speed, aeration rate and temperature. The software (iFix 3.5, Intellution, USA) allowed for automatic set-point control and integration of all parameters via PLC. Before each sterilization cycle, the polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4.0 and 7.0 (VWR, Canada). The oxygen probe was calibrated to zero (using $N_2$ gas) and to 100% (air saturated water). Diluted cheese whey (10 L) of 30.0 g/L COD and supplemented with 0.15% w/v of urea was transferred to the 15 L bioreactor followed by pasteurization for 20 min at 80 $^{o}$C and pH 3.5. After pasteurization, the temperature was cooled to 40 $^{o}$C and the DO probe was re-calibrated. The fermentation was conducted at pH 3.5 and 40 $^{o}$C to minimize chances of contamination. Three fermentation runs were conducted using either the mono-culture (*K. marxianus*) approach or the mixed culture (*K. marxianus* and *C. krusei*) approach as described below.

### 2.4.1 Batch fermentation with mono-culture

The first batch bioreactor was inoculated with 5% (v/v) of *K. marxianus*, which was prepared as described earlier. In order to keep the DO above 25% saturation (critical DO for *K. marxianus*), air flow and agitation rates were appropriately adjusted. pH 3.5 was maintained using 4 N $H_2SO_4$ or 4 N NaOH and an polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v)

solution was used to control foaming during fermentation. The fermentation was carried out for 30 h (based on the previous study of batch fermentation and when the DO was increased the fermentation was stopped).

### 2.4.2 Batch fermentation with mixed culture

The second batch bioreactor was inoculated with a 6.5% (v/v) inoculum (4% v/v of *K. marxianus* and 2.5% v/v of *C. krusei*), which was prepared as described earlier (each culture was grown separately, *K. marxianus* in cheese whey and *C. krusei* in TSB medium). The DO was maintained above 25% saturation by controlling the air flow rate and/or the agitation rate. pH 3.5 was maintained using 4 N $H_2SO_4$ or 4 N NaOH and a polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was used to control foaming during fermentation.

### 2.4.3 Batch fermentation switched to continuous fermentation with mixed culture

The third batch fermentation was conducted using a mixed culture inoculum of 10% v/v (5% v/v of *K. marxianus* and 5% v/v of 10 times concentrated cells of *C. krusei* grown in TSB medium, centrifuged and re-suspended in diluted pasteurized cheese whey) to increase the initial cell concentration of *C. krusei*. Bioreactor preparation and feed pasteurization were similar to the first and second batch of the fermentation as mentioned above. The fermentation, after 22 h (when DO just started increasing due to depletion of main substarte lactose) of batch operation, in this case, was switched to a continuous process and run at different hydraulic retention times-HRT (HRT is reciprocal of dilution rate) of 6, 12, 18 and 24 h. The constant volume of the reactor was maintained by feeding (inflow) diluted unpasteurized cheese whey (unpasteurized cheese whey was used to evaluate contamination during the continuous process) supplemented with 0.15% w/v of urea at a predetermined flow rate with a peristaltic pump and pumping out (outflow) the fermented broth at an equal flow rate with second peristaltic pump (Masterflex, Cole-Palmer, USA). All samples were drawn from the fermenter outflow. With batch fermentation, the specific growth rate ($\mu_m$) was calculated from the slope of the line of semi-log plot of cell concentration versus time, while with the continuous fermentation mode the specific growth rate was equal to dilution rate (working volume/feed flow rate).

## 2.5 Analytical methods

Samples were withdrawn at regular intervals (during batch and continuous fermentation) and analyzed for viable cell count (colony forming unit-CFU), yeast biomass (suspended solids), lactose, COD and soluble proteins. The total cell count (CFU/mL) was determined by the standard agar plate technique. The appropriately diluted samples were plated on MRS-agar plates and incubated at 35 $^{o}$C for 48 h to obtain the isolated colonies. Suspended solids (dry weight) were measured using APHA Standard Methods (APHA, 2005). Lactose concentration was measured by using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). COD was measured according to APHA Standard Methods (APHA, 2005) using the closed reflex, colorimetric method. Soluble protein concentration was determined by the Lowry et al. (1951) method.

Sample used to determine the protein content of biomass were prepared using the procedure reported by Yadav et al. (2013). In brief, biomass was lyophilized and treated with lysis buffer (5.0 mL/L of Triton X-100, 0.372 g/L of ethylenediaminetetraacetic acid (EDTA) and 0.0345 g/L of phenylmethylsulfonyl fluoride (PMSF)). Then, the treated biomass suspension was incubated for 20 min under shaking conditions at room temperature to lyse the cells. After incubation, the samples were sonicated for 5 min with an ultrasonic homogenizer Auto tune 750W (Cole-Parmer Instruments, Vernon Hills, Illinois, US) to increase cells lysis and release of the proteins. The protein content was measured by the Lowry et al. (1951) method.

The carbohydrates content of biomass was measured the using standard phenol sulfuric acid method (DuBois et al., 1956). The lipids content of the biomass was determined by a Soxhlet extraction procedure using petroleum ether as solvent (Horwitz & International, 2011). Ash content of SCP was measured by the standard procedure described in AOAC (Horwitz & International, 2011). The individual minerals content was determined by ICP-AES (Varian Vista AX, USA) analysis after digestion of biomass.

The essential amino acid compositions of the SCP were analyzed through hydrolyzing the lyophilized SCP with 6 N HCl at 110 ºC for 22 h. Digested samples containing HCl were evaporated in vacuum lyophilizer and re-suspended in acetate buffer (Lourenço et al., 2002). The amino acids produced as a result of SCP hydrolysis were analyzed by HPLC. HPLC conditions used were: analytical column Aminopac PA10 of dimention 2x250 mm, Detector: Electrochemical Detector (ED), mobile phase solvent: 1 M sodium acetate, flow rate: 0.25 mL/min, gradient flow was used. The data were analysed using Chromeleon software. All

analyses were carried out in triplicate and average values of each parameter are presented (with standard error less than 5% of the mean).

# 3. RESULTS AND DISCUSSION

## 3.1 Batch fermentation with mono-culture

The data for the various parameters (cell count, biomass, lactose, COD and soluble proteins) during cheese whey fermentation with the mono-culture (*K. marxianus*) are illustrated in Figs. 1a and 1b. The cell count (CFU/mL) of *K. marxianus* (initial $7.0 \times 10^6$ CFU/mL) increased with time and reached a maximum of $5.10 \times 10^8$ CFU/mL at 20 h. From 20 to 30 h of fermentation, the cell concentration remained constant. Yeast biomass (suspended solids) concentration increased with time and reached maximum of 6.6 g/L at 20 h. The specific growth rate ($\mu_m$), productivity (g/L/h) and the biomass yield ($Y_{x/s,}$ based on lactose) were found to be 0.21 $h^{-1}$, 0.15 g/L/h and 0.26 g biomass/g lactose consumed, respectively. A specific growth rate ($\mu_m$) of 0.37 $h^{-1}$, a productivity of 1.27 g/L/h and a biomass yield ($Y_{x/s}$) of 0.37 g/g of *K. marxianus* at pH 5.0 and 30 $^oC$ have been reported by Lukondeh et al. (2005) from an initial lactose concentration of 40 g/L in a synthetic medium. A higher yield (0.44 g/g) but low specific growth rate (0.15 $h^{-1}$) have been reported by Ghaly and Kamal (2004) during SCP production from cheese whey at 31 $^oC$ and pH 4.4. Relatively, speaking the lower biomass yield and productivity in the current work might be due to the extreme process conditions (i.e. low pH 3.5 and high temperature of 40 $^oC$) used in this work to diminish contamination risk. The maximal yield and productivity of *Kluyveromyces* species have been reported at pH 4.4 and 5.8 and 31 and 30 $^oC$ in batch processes while cultivating *K. marxianus* on cheese whey (Ghaly and Kamal, 2004) and on whey permeate (Schultz et al., 2006), respectively (Table 1). Thus, yeast cells cultivated at higher temperature and low pH, as done in the present study, required higher maintenance energy (i.e. higher amount of lactose diverted towards catabolism), which led to a decrease in the biomass yield (Yadav et al., 2013).

Most of the lactose (initial concentration 17.2 g/L) was consumed within the first 16 h and reached zero at 20 h (Fig. 1b). The soluble whey protein concentration initially decreased very slightly up to 12 h (Fig. 1b) and slightly increased from 12 to 16 h, which may be due to excretion of extracellular proteins and enzymes. Then, the soluble protein concentration remained almost constant until the end of the fermentation process (30 h). A similar trend for the protein consumption profile has been observed by Yadav et al. (2013) during whey fermentation. Reduction in soluble protein concentration in the first 12 h of fermentation could be due to its utilization as nitrogen source by the microorganism. The constant concentration

of soluble protein after 16 h might be due to partial consumption of proteins and concurrent secretion of extracellular enzymes by *K. marxianus* (such as inulinase and lipase) as this yeast is known to excrete various enzymes (Fonseca et al., 2008).

COD (initial 30.6 g/L) decreased rapidly up to 16 h (Fig. 1b) followed by a slow reduction until 30 h. A rapid decrease of COD coincided with the exponential increase in cell count and a fast consumption of most of the lactose (during the first 16 h). However, the COD continued decreasing after 16 h until the end of fermentation (30 h), which could be due to consumption of the intermediates compounds that might already have been present in the cheese whey or formed during the first 16 h. A COD reduction of 78% was achieved with a residual COD value of 6.8 g/L at the end of fermentation. The residual COD was due to residual soluble proteins (3.4 g/L) and to other residual organic compounds (originally present in cheese and new intermediates produced during the fermentation). Organic intermediate metabolites such as organic acids and others were reported to be produced during whey fermentation by *K. marxianus* (Lukondeh et al., 2005; Yadav et al., 2013).

## 3.2 Batch fermentation with mixed culture

The variation in the values of different parameters (total cell count, individual cell count, biomass, lactose, residual COD and soluble protein concentrations) during cheese whey fermentation with a mixed culture (*K. marxianus* and *C. krusei*) are presented in Figs. 2a and 2b.

The initial population of *K. marxianus* (initial count: $5.0 \times 10^6$ CFU/mL) was comparatively just a bit higher than that of *C. krusei* (initial count: $3.50 \times 10^6$ CFU/mL). The growth of *K. marxianus* (specific growth rate of 0.20 $h^{-1}$) was also higher compared to *C. krusei* (specific growth rate of 0.14 $h^{-1}$) (Fig. 2a). This was due to the presence of lactose as main carbon source, which is not consumed by *C. krusei*. The overall (based on total cell count) specific growth rate was 0.20 $h^{-1}$. The biomass yield and productivity of the mixed culture have been summarised in Table 1. The growth of *C. krusei* was not too rapid, however *C. krusei* was able to grow in the medium (Fig. 2a) and increased from an initial cell count of $3.50 \times 10^6$ CFU/mL to $5.40 \times 10^7$ CFU/mL at the end of fermentation. Growth of *C. krusei* indicates that it is able to utilize other carbon sources (i.e. other than lactose). These carbon sources might be intermediate metabolites produced by *K. marxianus.* Because *C. krusei* was not able to grow in pure cheese whey, however, it was able to grow in the fermented broth supernatant of *K. marxianus* (an observation from flask culture results). The final concentration of *K. marxianus* after 30 h reached $4.70 \times 10^8$ CFU/mL. The overall increase in biomass was higher

with the mixed culture (6.1 g/L) compared to the mono-culture (4.7 g/L) as shown with the previous batch experiment (i.e. mono-culture). The increase in biomass concentration led to an increase in the biomass yield and productivity (Table 1). Thus, these results established that *C. krusei* has potential to grow in a mixed culture at higher temperature (40 $^{o}$C) and low pH (3.5). Similar results of an increase in biomass yield (due to consumption of extracellular intermediate metabolites by one yeast) during production of SCP using a mixed culture of *Torulopsis cremoris* and *Candida utilis* (Cristiani-Urbina et al., 2000) and for *C. utilis* and *Brevibacterium lactofermentus* (Rajoka et al., 2012) have been reported.

The lactose concentration decreased during fermentation from 19.2 g/L to 0.0 g/L in 20 h (Fig. 2b). The soluble whey protein concentration initially decreased (similar to the mono-culture case) up to 12 h (Fig. 2b) and slightly increased from 12 to 16 h. Then, the soluble protein concentration remained almost constant until the end of the fermentation process (30 h). COD also decreased with time as shown in Fig. 2b. COD decreased from 31.7 g/L to 4.2 g/L at the end of fermentation (30 h). The overall reduction of COD with the mixed culture was 86.8%, which was 8.8% higher than with the mono-culture during a same fermentation duration (30 h). The 8.8% higher reduction does not look statistically significant, however, when the residual COD was calculated separately for each component (i.e. lactose, proteins and intermediate metabolites), as shown in Table 1, the reduction of COD due to intermediate metabolites utilization was approximately 2.5 g/L. The residual COD associated with intermediate metabolites (0.3 g/L) with mixed culture is almost negligible compared to the mono-culture where COD associated with intermediate metabolites was 2.5 g/L. The difference indicates that intermediate metabolites were consumed during the mixed culture fermentation, which resulted in a lower residual COD. This proved that a mixed culture has potential to remove more COD compared to a mono-culture.

A similar trend in COD reduction has been reported with another mixed culture (yeast *Kluyveromyces fragilis* and bacterium *Serratia marcescens*) compared to a mono-culture (*K. fragilis*) during production of protease and COD removal from cheese whey (Ustáriz et al., 2007). A mixed culture of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* at pH 5 and 30 $^{o}$C has also been reported to enhance COD removal and biomass yield (Ghata et al., 2013). *S. cerevisiae* has been reported to grow in mixed culture; however, it cannot grow at high temperature and low pH, which is an essential condition for prevention of pathogenic bacterial contamination. Batch and fed-batch cultures have also been reported using a mixed culture of *Torulopsis cremoris* and *Candida utilis* for biomass production and COD removal from cheese whey at pH 4.8 and 29 $^{o}$C (Cristiani-Urbina et al., 2000). The batch fermentation results of this study showed that the mixed culture has higher potential in terms of COD

removal, biomass yield and productivity. Therefore, this mixed combination was subsequently used in a continuous fermentation process. A continuous process was employed for subsequent study because it is more practical when the purpose is both i.e. continuous wastewater treatment as well as biotransformation (Yadav et al., 2013). Additionally, previous reports have reported comparatively higher COD removal results (Table 1) but most of these studies were conducted using whey permeate and thus the residual COD due to proteins was negligible.

## 3.3 Batch fermentation followed by continuous fermentation with mixed culture

### 3.3.1 Variation of individual populations of *K. marxianus* and *C. krusei*

The variation of the individual populations of *C. krusei* and *K. marxianus* during batch and continuous fermentation are shown in Fig. 3a. An increase in more than one log in *C. krusei* (initial $2.60 \times 10^7$ CFU/mL) population (maximum up to $6.20 \times 10^8$ CFU/mL at 20 h) and *K. marxianus* (initial $2.30 \times 10^6$ CFU/mL) population (maximum up to $9.50 \times 10^7$ CFU/mL) during the batch fermentation was observed (Fig. 3a). An increase in the *K. marxianus*/*C. krusei* ratio during the batch fermentation from 0.08 to 0.15 was also observed.

The batch fermentation process, after 22 h, was changed to the continuous mode. At 24 h HRT, from beginning of the continuous process up to 48 h, the *C. krusei* population was higher than that of *K. marxianus*. At 54 h, the *K. marxianus* and *C. krusei* population were approximately equal (Fig. 3a). A reason for this might be the addition of fresh medium whey, which contains mainly lactose and less of other the organic components. The limitation in consumable carbon limited growth of *C. krusei* whereas *K. marxianus* grew at a faster rate compared to *C. krusei*. The *K. marxianus*/*C. krusei* ratio varied during the continuous fermentation (from 24 h HRT to 6 h HRT) and this ratio increased with HRT. A maximal *K. marxianus*/*C. krusei* ratio 3.5 was found at 6 h HRT while the minimal *K. marxianus*/*C. krusei* ratio of 0.05 was observed at 24 h HRT (at steady state). The increase in ratio at the lowest HRT (6 h HRT) value (or higher dilution rate) might be due to increase in availability of lactose. Due to the availability of lactose *K. marxianus* was able to grow continuously; however, at higher dilution rate (low HRT), the concentration of intermediate metabolites probably become diluted resulting in less availability of intermediate metabolites as carbon source for *C. krusei*. The ratio of *K. marxianus*/*C. krusei* varied with HRT during the continuous fermentation; however, *C. krusei* sustained the growth during whole fermentation period (22 h of batch followed by 168 h of continuous process).

The interaction among microorganisms in mixed cultures, especially with a lactose-positive and a lactose-negative exists due to mutualism or commensalism. Commensalism is a type of interaction in which one organism benefits from the interaction while the other strain is not affected (Smid and Lacroix, 2013). A commensalism interaction of *Candiada kefyr* (now known as *K. marxianus*) with the lactose-negative yeast *Candida valida* LY497 during SCP production using cheese whey has been reported (Carlotti et al., 1991). A 20% increase in biomass yield was reported with the mixed culture over the mono-culture. A specific physiological interaction has not been reported between *K. marxianus* and *C. krusei*, however, Pedersen et al. (2012) reported higher percentages of these two yeasts in the fermented food product fura (West African fermented cereal) from different sources, which supports the existence of physiological interaction-between these two yeast species as well as the present study supports this interaction.

### 3.3.2 Variation in lactose, COD and soluble protein concentration during batch and continuous fermentation

The variation in lactose, COD and soluble protein concentration during batch and continuous fermentation is presented in Fig. 3b and in Table 1. The lactose concentration decreased during the batch fermentation from 17.5 g/L to 1.1 g/L. The soluble protein concentration showed a similar trend as observed in the first two batch runs and its concentration decreased from 3.9 to 3.1 g/L. The COD concentration decreased from 29.7 g/L to 9.3 g/L from 0 to 22 h of batch operation (Fig. 3b).

At the start of the continuous process with 24 h HRT steady state was achieved after 54 hrs of continuous operation. The lactose concentration in the bioreactor effluent initially increased from 1.1 g/L to 3.3 g/L after switching from batch to continuous. This transient increase in lactose concentration was due to osmotic stress arising from feeding of new substrate. The microorganisms required time for homeostatic adjustment (Brauer et al., 2005). The residual lactose concentration at steady state was 1.8 g/L, and thus, consumption of lactose reached 91%. At 18 h HRT the lactose concentration in the bioreactor effluent decreased from 2.9 to 1.4 g/L and a lactose consumption value of 92% at steady state was observed. At 12 h HRT, the lactose concentration in the bioreactor effluent significantly increased (increased from 3.4 to 6.6 g/L) and lactose consumption was only 63.4% at steady state. At 6 h HRT, the lactose concentration in the bioreactor effluent increased from 8.8 to 11.8 g/L and lactose consumption was only 34.3% at steady state. The reason for these increases in residual lactose concentration at lower HRT values is that the microorganisms

were not getting sufficient time to utilize lactose supplied by the increased dilution rates. The soluble protein concentrations during continuous fermentation ranged from 2.5 to 3.9 g/L depending on HRT. However, the lowest concentrations of proteins were observed at 24 h HRT while the highest concentrations were observed at 12 h and 6 h HRT.

The variation in COD concentration, residual COD, COD due to each component and percentage of COD removal during continuous fermentation is shown in Fig. 3b and Table 1. At 24 h HRT, the effluent COD concentration varied between 8.9 g/L and 5.7 g/L. However, the residual COD at steady state (Table 1) associated with the intermediate metabolites was much lower and this indicates that consumption of these metabolites was similar to that in batch fermentation using the mixed culture. At 18 h HRT, the effluent COD first decreased from 7.6 g/L to 6.0 g/L up to 24 h. However after 24 h, the COD concentration increased to 9.2 g/L. The increase in COD concentration might be related to poor growth of the *K. marxianus* population (Fig. 3a) that resulting in higher accumulation or production of intermediate metabolites. At 12 h and 6 h HRT, the COD concentrations were 16.2 and 22.3 g/L, respectively. Continuous fermentation with *K. fragilis* using cheese whey for SCP production has been carried out by other researchers, however the fermentation conditions used were different (pH 4.5 and 33 $^{o}$C), which were reported to be optimal conditions for *Kluyveromyces* species (Ghaly et al., 2005). The optimal HRT reported for SCP production was 12 h, however, there is no report correlating COD removal efficiency and SCP production.

Biomass increased from 6.0 g/L to 10.1 g/L at 22 h growth during batch fermentation. The yield of biomass (0.16 g/g lactose) was comparatively lower compared to the previous batch results and this might be due to the high concentration of inoculum, which probably consumed more carbon source for maintenance energy (Yadav et al., 2013). Moreover, this yield was measured only after 22 h of batch fermentation when lactose and intermediate metabolites had not been fully consumed.

The results for biomass yield and productivity with respect to lactose consumed at steady state and their variation during the continuous process are presented in Table 1 and Fig. 3a, respectively. The higher variation in biomass yield was observed at 24 h HRT compared to 18 h HRT. At 12 h and 6 h HRT, the biomass yield increased during the process (Table 1). Higher biomass yield and productivity have also been reported at lower HRT values during continuous SCP production from cheese whey (Table 1) by other researchers (Ben-Hassan and Ghaly, 1995; Ghaly et al., 2005). Considering the fact that higher COD removal or lactose consumption was observed at 18 h and 24 h HRT (Table 1), therefore, these HRT

values could be employed for simultaneous COD removal and biomass production during continuous process. However, when the purpose is only biomass production then lower HRT could be employed. Samples were also examined for contamination by other microorganisms. No contamination was detected during 168 h of continuous cultivation and that was due to the extreme process conditions (i.e. low pH and high temperature) employed. Thus, the combination of these two yeast strains has the potential for concurrent cultivation to produce SCP with better quality of protein and COD removal (i.e. treatment of whey) at industrial scale under non-aseptic conditions in a continuous process.

## 3.4 Compositional analysis of biomass (SCP)

The produced biomass (SCP) from 24 h HRT was analyzed for its major chemical components. The composition of the mixed culture biomass showed that the major components were protein (43.4% w/w), carbohydrates (33.6% w/w), crude fiber (4.6% w/w), lipids (6.4% w/w) and ash (minerals) (8.4% w/w). Similar compositions of SCP produced from whey permeate have been reported by Anvari and Khayati (2011) and Paul et al. (2002) using pure *Kluyveromyces* species. The protein content of SCP from *C. krusei* strain SO1 has been reported to be 47.53 and 50.10% w/w, grown in synthetic medium and sorghum medium, respectively (Konlani et al., 1996). As per requirement of Association of American Feed Control Official Incorporated (AAFCO), the minimal protein content for SCP should not be less than 40% w/w (AAFCO, 2010). The details of the minerals profile also showed a balanced composition with the following values for individual minerals (mg/100 g dry cells weight): calcium (1700 mg), phosphorus (2120 mg), potassium (1700 mg), sulfur (610 mg), sodium (200), magnesium (170 mg), iron (0.15 mg), manganese (0.007 mg), copper (0.03), zinc (0.13), molybdenum (0.01 mg), and nickel (0.007). There were no heavy metals (toxic metals) detected in the SCP.

The essential amino acid profile of the mixed culture biomass was also evaluated and the results are presented in Table 2. The amino acid profile shows that the mixed culture SCP was balanced in the essential amino acid content. Moreover, it was expected that the lysine content should be high as *C. krusei* is reported to be rich in this amino acid. Lysine is one of the most essential amino acids along with sulfur-containing amino acid (Rajoka et al., 2012). The method used for amino acid analysis was not able to detect the presence of methionine and cysteine. Though the methionine content of *C. krusei* is reported to be less than that required by the Food Agriculture Organization (FAO) (Konlani et al., 1996), however, the methionine content of *C. krusei* is higher than the methionine content reported in *K. fragilis* (Paul et al., 2002). Thus, the mixed culture (*K. marxianus* and *C. krusei*) amino acid profile

should be better compared to that of the mono-culture *(K. marxianus)*. Important information obtained during the minerals analysis is the high sulfur content of the SCP (as given above). This high sulfur content might be due to sulfur-containing amino acids.

The biomass from the fermented broth could be separated by centrifugation or through membrane filtration (ultrafiltration). Separation of biomass via centrifugation will require one more step (either precipitation or membrane filtration) to recover the residual soluble whey proteins. Though, ultrafiltration could simultaneously recover the biomass as well as the proteins. The recovery of the residual soluble protein through ultrafiltration needs to be optimized. However, recovery of residual soluble protein by precipitation from the supernatant of yeast-grown (*K. marxianus*) on cheese whey has been optimized, with a residual protein recovery up to 81%, which further reduced the residual COD (Yadav et al., 2014). Protein precipitation does not require a centrifugation step because the precipitate can be separated by gravity settling. The optimal conditions for protein precipitation developed earlier could be applied on the supernatant of the mixed culture (*K. marxianus* and *C. krusei*) cultivated on cheese whey, with a similar recovery of the proteins. Thus, the present study of mixed culture SCP production from cheese whey could be integrated with our earlier developed technique (Yadav et al., 2014). The overall productivity and COD removal efficiency of the proposed process will be increased through recovery of the residual soluble protein (2.3 g/L) via precipitation. The recovered protein could be spray-dried (along with the biomass), and thus, this will help to increase the protein content of SCP (the final product). The biomass protein content obtained was 43.2% w/w, which will be further increased when mixed with the recovered precipitated proteinaceous solids. Thus, based on our calculations for the integrated process (fermentation and protein precipitation), the expected results as shown in Table 3. Mixing of the recovered proteinaceous solids with the biomass will enhance product quality in two ways: i) the overall protein content of the produced SCP will be increased; ii) the amino acid profile of the SCP will also be balanced due to a higher content of sulfur-containing amino acids (cysteine and methionine) and of lysine in the whey-derived proteins. Thus, the overall protein quality will be better compared to that of the pure culture SCP and this improve the value of the proteinaceous product (animal feed). COD removal is also enhanced, to 91-93% (Table 3). However, to implement this potential integrated process further studies are required i.e. scale-up and economic analysis.

## 4. CONCLUSIONS

A mixed culture is a better choice for SCP production and COD removal at higher temperature and lower pH. *C. krusei* and *K. marxianus* showed physiological interactions.

Continuous fermentation at 24 h HRT was optimal for higher COD removal with substantial yield and productivity. Additionally, mixed culture SCP is enriched with lysine and meet with the minimal required protein content. The extreme process conditions will minimize the contamination and thus, the continuous process can be operated under non-aseptic conditions. Residual protein recovery through precipitation increased the productivity from 0.17 to 0.31 g/L/h and COD removal efficiency, from 80% to 91-93%. Further, study is warranted to scale up the process in an integrated scenario. These results naturally lead to the need for scaling up the process using an intergrated scenario i.e. mixed culture SCP production + recovery of the residual proteins by precipitation.

## ACKNOWLEDGEMENT

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support.

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**Table 1. Summary of residual substrates, yield and COD removal efficiency during batch and continuous processes with mono-culture and mixed culture conducted at pH 3.5 and 40 $^{o}$C using cheese whey as substrate**

| Fermentation | pH | Temp. ($^{o}$C) | Total residual COD (g/L) | COD due to lactose (g/L) | COD due to proteins (g/L) | COD due to metabolites (g/L) | μ ($h^{-1}$) | $Y_{x/s}$ (g/g) | Productivity (g/L/h) | COD removal efficiency (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Batch-1 (*K. marxianus*) | 3.5 | 40 | 6.8 | 0.0 | 4.0 | 2.5 | 0.21 | 0.26 | 0.15 | 78.0 (IP) | This study |
| Batch-2 (*K. marxianus* + *C. krusei*) | 3.5 | 40 | 4.2 | 0.0 | 3.9 | 0.3 | 0.20 | 0.31 | 0.20 | 86.8 (IP) | This study |
| Batch (*K. fragilis*) | 4.4 | 31 | 5.4 | 0.0 | 0.0 | Na | 0.15 | 0.44 | Na | 90.6 (EP) | Ghaly and Kamal, 2004 |
| Batch (*K. marxianus*) | 5.0 | 34 | Na | Na | 0.0 | Na | 0.23 | 0.26 | 0.45 | 88.5 (EP) | Anvari and Khayati, 2011 |
| Batch (*K. marxianus*) | 5.8 | 30 | 15.0 | Na | 0.0 | Na | Na | 0.52 | Na | 90.0 (EP) | Schultz et al., 2006 |
| Batch (*T. cremoris* + *C. utilis*) | 4.8 | 29 | 10.2 | 0.0 | 0.0 | Na | 0.25 | 0.46 | Na | 92.7 (EP) | Cristiani-Urbina et al., 2000 |
| Continuous process | | | | | | | | | | | |
| 24 h HRT | 3.5 | 40 | 5.9 | 2.0 | 2.9 | 0.98 | 0.041 | 0.17 | 0.17 | 80.2 (IP) | This study |
| 18 h HRT | 3.5 | 40 | 8.4 | 1.7 | 3.5 | 3.2 | 0.055 | 0.21 | 0.21 | 72.2 (IP) | This study |
| 12 h HRT | 3.5 | 40 | 16.2 | 6.5 | 4.0 | 5.3 | 0.083 | 0.23 | 0.26 | 46.0 (IP) | This study |
| 6 h HRT | 3.5 | 40 | 22.2 | 13.4 | 3.5 | 5.4 | 0.167 | 0.27 | 0.38 | 26.0 (IP) | This study |
| 24 h HRT (*K. fragilis*) | 4.5 | 33 | Na | Na | Na | Na | 0.105 | 0.26 | 0.40 | Na | Ghaly et al., 2005 |
| 12 h HRT (*K. fragilis*) | 4.5 | 33 | Na | Na | Na | Na | 0.087 | 0.74 | 2.85 | Na | Ghaly et al., 2005 |
| 12 h HRT (*K. fragilis*) | 4.5 | 33 | Na | Na | Na | Na | 0.083 | 0.74 | 2.86 | Na | Ben-Hassan and Ghaly et al., 1995 |

***Na:** not available; IP: including protein; EP: excluding proteins

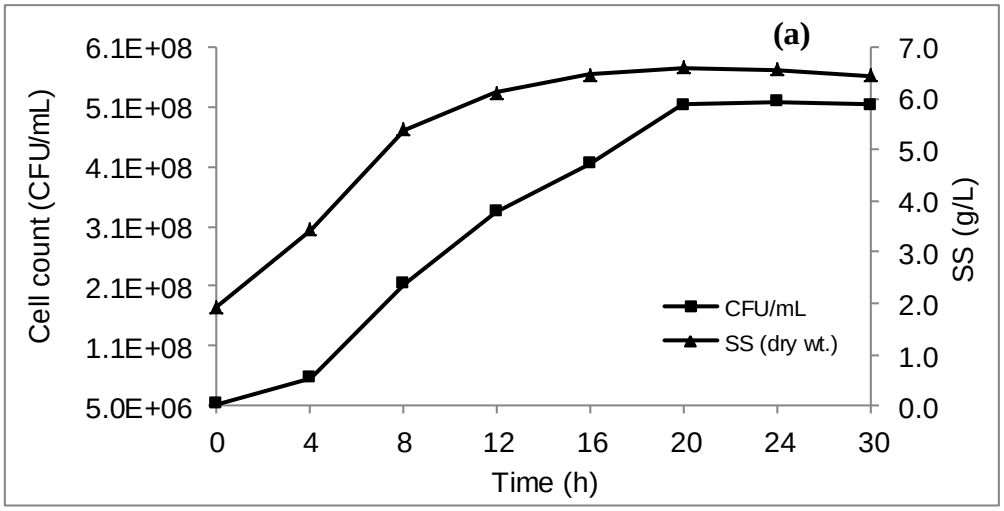
**Table 2. Amino acid profile of mono-culture and mixed culture SCP**

| | Amino acid (g/100 g protein) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Yeast** | **Leucine** | **Lysine** | **Threonine** | **Valine** | **Isoleucine** | **Tyrosine** | **Methionine** | **Methionine + Cystine** | **Phenylalanine** | **Tryptophan** |
| *K. marxianus* | 9.42 | 6.02 | ND | ND | 3.50 | 3.32 | ND | ND | ND | ND |
| *(K. marxianus +C. krusei*) | 7.40 | 9.8 | ND | ND | 3.80 | 1.8 | ND | ND | ND | ND |
| FAO/WHO | 7.0 | 5.5 | 4.0 | 5.0 | 4.20 | 2.80 | 2.20 | 3.5 | 2.8 | 1.4 |

***ND**: Not determined

**Table 3. Prediction for further improvement of the process efficiency by recovering the residual soluble proteins employing the Yadav et al. (2014) two-step precipitation method**

| Mixed culture product recovery conditions | Productivity (g/L/h) | Protein content of SCP (% w/w) | COD removal efficiency (%) |
|---|---|---|---|
| Recovery of SCP (biomass) after continuous fermentation by centrifugation (present status) | 0.17 | 43.4 | 80.2 |
| Predicted process efficiency after recovery of the residual soluble proteins using the two-step precipitation method | 0.31 | 47.4 | 91-93 |



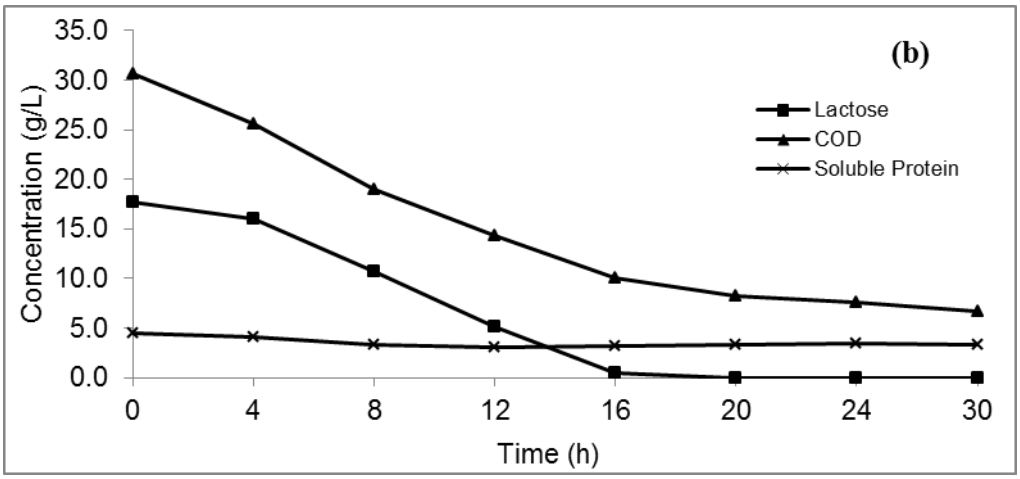
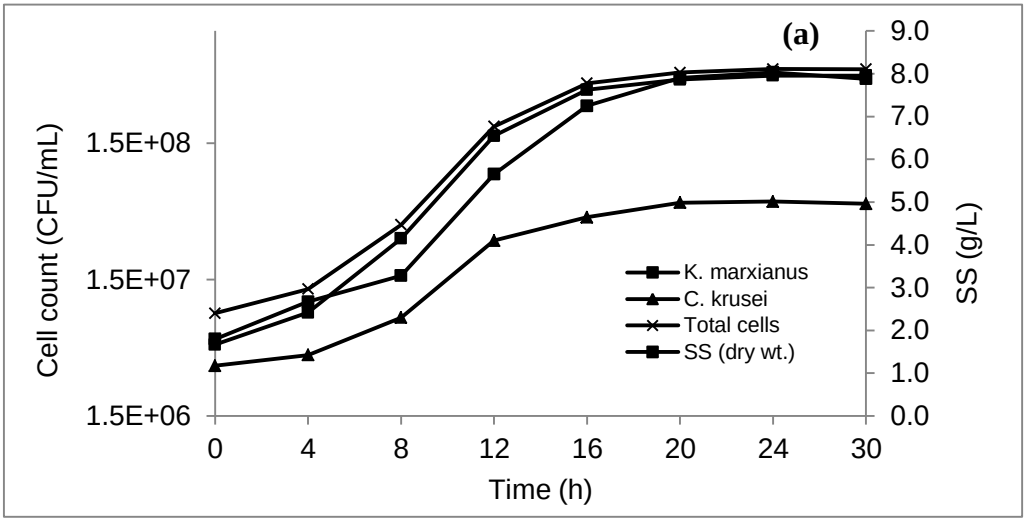




**Figure 1. (a) Growth profile of *K. marxianus* during batch fermentation in cheese whey; (b) Variation in substrates profiles during fermentation**



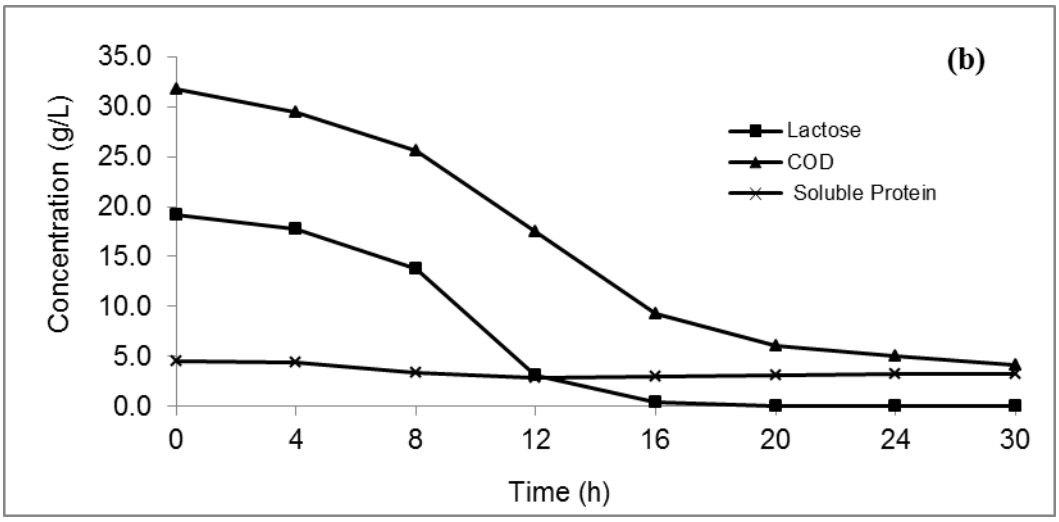




**Figure 2. (a) Growth profile during mixed culture (*K. marxianus* and *C. krusei*) fermentation in cheese whey; (b) Variation in substrates profiles during the mixed culture fermentation**

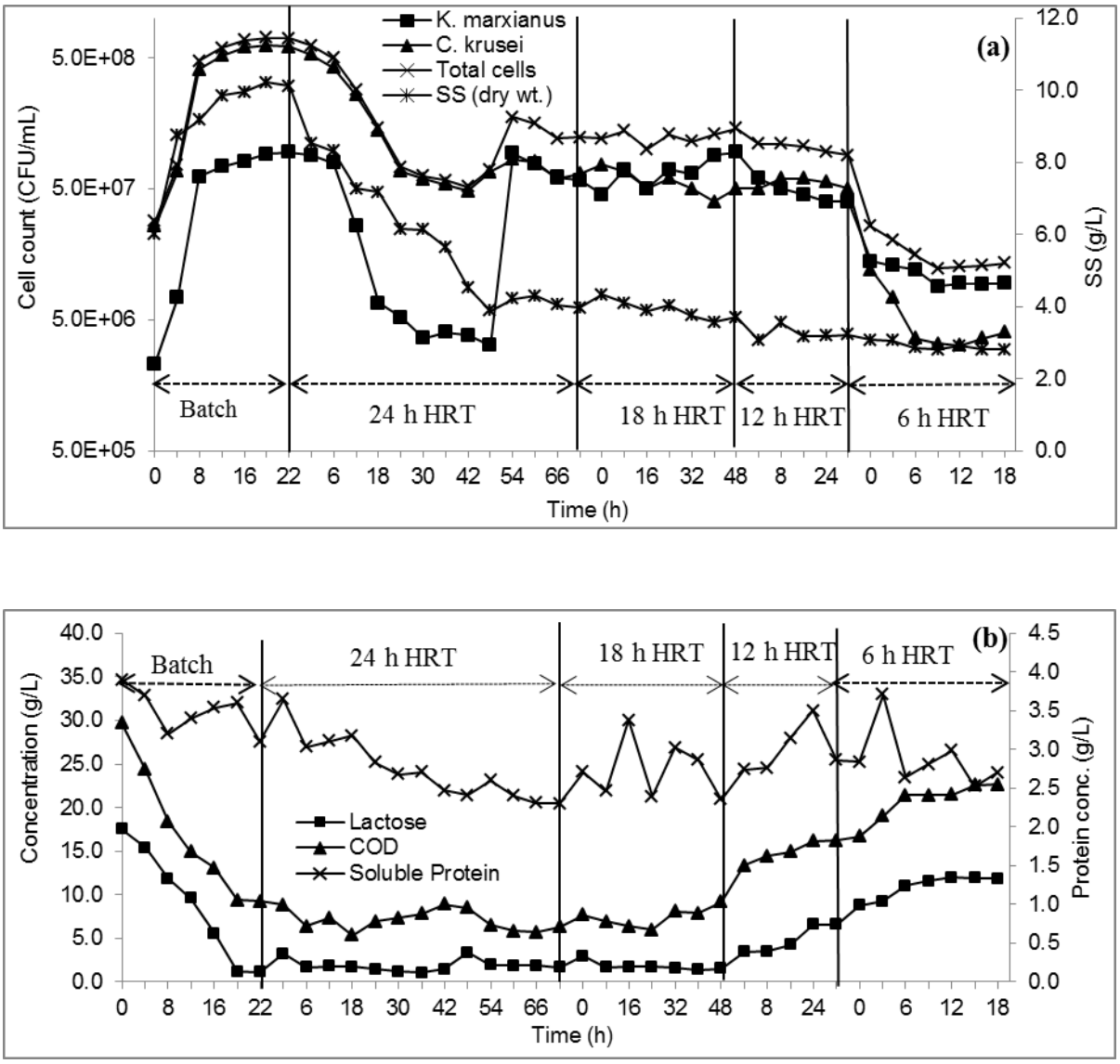



**Figure 3. (a) Growth profile of mixed culture (*K. marxianus* and *C. krusei*) during continuous fermentation at different HRT (6, 12, 18 and 24 h); (b) Variation in substrates profiles during continuous fermentation at different HRT**

# CHAPITRE IV

# PERMÉABILISATION DES CELLULES DE LEVURE, HYDROLYSE DU LACTOSE, AUGMENTATION DE LA CULTURE MIXTE POUR LA PRODUCTION DE PU ET RÉCUPÉRATION DES PROTÉINES RÉSIDUELLE APRÉS FERMENTATION

# PARTIE 1

# PERMEABILIZATION OF KLUYVEROMYCES MARXIANUS WITH MILD DETERGENT FOR WHEY LACTOSE HYDROLYSIS AND AUGMENTATION OF MIXED CULTURE

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**Applied Biochemistry and Biotechnology**

**172: 6 (2014), 3207-3222**

# RÉSUMÉ

Le lactosérum est un sous-produit issu de l'industrie de production du fromage. Son utilisation aussi bien que son élimination entraînent un défi de taille puisque très peu de microorganismes ont la capacité de métaboliser le lactosérum. Une des approches d'application biotechnologique visant à pallier à ce problème est basée sur l'hydrolyse enzymatique du lactosérum en glucose et en galactose à l'aide de l'enzyme de la β-galactosidase. À cette fin, les cellules de *Kluyveromyces marxianus* ont été perméabilisées avec du N-lauroyl sarcosine (N-LS), un détergent anionique, biodégradable et non toxique. Puis, les paramètres du procédé de perméabilisation ont été optimisés (concentration en N-LS, volume de solvant, température, et temps d'incubation). L'utilisation des cellules perméabilisées sous conditions optimales a ainsi permis d'atteindre l'activité maximale de la β-galactosidase, sont 1220 IU/g de masse sèche. Par ailleurs, la viabilité des cellules perméabilisées a été évaluée, ce qui a montré que les cellules étaient viables, mais que cette viabilité était réduite de deux cycles logarithmiques. Les cellules perméabilisées ont aussi été évaluées pour leur capacité à hydrolyser le lactose contenu dans le lactosérum. Une hydrolyse maximale de 91% du lactose a été observée en utilisant 600 mg de cellules (masse sèche/100 mL) dans une solution de poudre de lactosérum (5% m/v) incubée à 30 $^{o}$C et à pH 6,5 pendant 180 minutes. De plus, le lactosérum hydrolysé a été évalué pour son aptitude à améliorer la croissance de *Saccharomyces cerevisiae*, une levure qui ne possède pas l'enzyme hydrolysant le lactose. Il s'est avéré que *S. cerevisiae*, avec la présence simultanée de *K. marxianus,* était capable de croître dans ce milieu. La présente étude confirme que le N-LS a pu être employé afin de rendre les cellules de *K. marxianus* perméables, et donc de rendre disponible leur activité enzymatique pour l'hydrolyse du lactosérum.

**Mots-clés:** β-galactosidase; *Kluyveromyces marxianus*; N-lauroyl sarcosine; Perméabilisation; Hydrolyse du lactose; Cultures mixtes; Protéines unicellulaire.

## ABSTRACT

Cheese whey is a by-product of cheese manufacturing, and the utilization of whey is a challenging problem either to use it or to dispose of it, because only few microorganisms can metabolize the whey lactose. Enzymatic hydrolysis of whey lactose to glucose and galactose by β-galactosidase is one of the possible the approaches for biotechnological application. *Kluyveromyces marxianus* cells were permeabilized using the non-toxic, biodegradable, anionic detergent N-lauroyl sarcosine (N-LS) to access its enzyme activity. The permeabilization process parameters (N-LS concentration, solvent volume, temperature and incubation time) were optimized. A maximal β-galactosidase activity of 1220 IU/g dry cell weight was obtained using the optimized conditions. Moreover, viability of the permeabilized cells was also evaluated, which showed that cells were alive however, viability was reduced by two log unit. The permeabilized cells were evaluated for whey lactose hydrolysis. A maximal lactose hydrolysis of 91% was observed with 600 mg (dry cell weight/100 mL) in a whey powder (5% w/v) solution after 180 min of incubation, pH 6.5 and 30 $^{o}$C. Further, the hydrolyzed whey was evaluated for growth of the non-lactose consuming yeast *Saccharomyces cerevisae*. *S. cerevisiae* was able to grow in the hydrolyzed whey simultaneously with *K. marxianus*. The study confirmed that N-LS could be used to permeabilize *K. marxianus* cells in order to access its enzyme activity.



**Keywords:** β-galactosidase; *Kluyveromyces marxianus*; N-lauroyl sarcosine; Permeabilization; Lactose hydrolysis; Mixed culture; Single-cell protein.

## INTRODUCTION

Cheese whey is a by-product of cheese manufacturing and is considered as an environmental pollutant due to its high biological oxygen demand (BOD). The main components of liquid cheese whey are lactose (4.5-5.0% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried matter) [1, 2]. Lactose is primarily responsible for the high BOD. Increase in the production of cheese has resulted in a serious problem of whey management and disposal [3, 4]. However, lactose and protein components of whey also make it a nutritious resource for conversion into value-added products via various physical and biological processes. Physical methods involved are direct spray drying to convert liquid whey into whey powder or through separation of whey protein followed by spray drying of the permeate to obtain whey permeate powder. Whey proteins are used as a source of various functional and nutritional proteins, whereas whey permeate is used as a source of lactose in various applications such as fillers and diluent in the pharmaceutical industry [1, 2, 5]. Whey permeate (lactose) is also used to produce various products such as lactobionic acid, lactulose, lactitol, lactosucrose and galacto-oligosaccharides (GOS) [6]. These physical methods are effective for value addition to whey but due to surplus amounts of whey, still half of the produced whey remains unutilized. Therefore, to tackle this issue, various biotechnological processes have been applied to biotransform whey into value-added products like enzymes, single-cell protein (SCP), bioethanol, polyhydroxyalkaonates and GOS, etc. [1, 2, 5].

The major challenge in whey bio-utilization is lactose, which cannot be metabolized by the majority of microorganisms. Hence, alternative biotechnological approaches have been employed to use lactose. One approach is to hydrolyze the whey lactose by a free or an immobilized enzyme into a mixture of glucose and galactose, and subsequently, to use the hydrolyzed sugars either as a sweet syrup or as a substrate to grow non-lactose consuming microorganism to generate various products (such as baker's yeast SCP for feed and food) [7, 8]. Protein-rich SCP also has been produced using hydrolyzed whey lactose while employing a mixed culture of lactose-consuming and non-lactose consuming yeasts [9, 10]. Thus, lactose hydrolysis can make whey suitable for fermentation by lactose-negative microorganisms such as *Saccharomyces cerevisiae.*

β-galactosidase (β-D-galactoctohydrolase, EC 3.2.1.23) is an enzyme which is responsible for the release of D-galactosyl residues from polymers, oligosaccharides or secondary metabolites. This enzyme can be obtained from bacteria, yeasts (*Kluyveromyces* spp.) and

moulds (*Aspergillus* spp.). β-galactosidase is also used for the hydrolysis of lactose to solve the problem associated with lactose crystallization in frozen concentrated dessert and that of milk consumption by lactose intolerant individuals and tackle the disposal problem of whey [6, 7]. Pure or analytical grade β-galactosidase is expensive therefore, an alternative method is necessary to make the enzyme available at low cost.

The permeabilization technology is one of the effective tools, normally used to increase the accessibility to intracellular enzymes. Permeabilized cells could be used as a source of intracellular enzymes as biocatalysts for biotransformation of substrates to various products [11, 12]. There are many chemicals and detergents (ethanol, iso-propanol, n-butanol, n-propanol, toluene, benzene, chloroform, Triton X-100, sodium dodacyl sulphate (SDS), digitonin and cetyltrimethyl ammonium bromide (CTAB) available for the permeabilization of yeast cells [13-15]. However, most of them are toxic and non-biodegradable, which hinders their application, especially in feed and food processing industry.

N-lauroyl sacosine (N-LS), an amino acid derived detergent (an anionic detergent made up of amino acid sacosine and fatty acid) is non-toxic, biodegradable and completely metabolizable in the human body into sarcosine and fatty acids [16-18]. Thus, it can be safely used for permeabilizing yeast cells to exploit various intracellular enzyme activities. A trace residue of N-LS (if remained in the permeabilized cells after washing) will not be harmful due to its low toxicity and used in leave on products (cosmetics) at a concentration up to 5% w/v [19]. Thus, permeabilized cells could be safely applied for whey lactose hydrolysis.

Therefore, the objectives of the present work were: i) to study the pemeabilization efficiency of N-LS for the yeast *Kluyveromyces marxianus* to make the intracellular enzyme available ii) evaluation of the permeabilized cells for hydrolysis of whey lactose and iii) use the hydrolyzed whey to favor growth of a non-lactose consuming yeast for the production of SCP in a mixed culture.

## MATERIALS AND METHODS

N-lauroyl sarcosine, lactose, glucose and galactose standards and 2-nitrophenyl β-D-galactopyranoside (ONPG) were obtained from Sigma Chemicals, USA. Hydrogen peroxide was from Lab Mat Canada and whey powder was from a cheese factory (Fromage Saputo, St. Hyacinthe, Quebec, Canada).

## Microorganisms

Yeast strains (*Kluyveromyces marxianus* and *Saccharomyces cerevisiae*) were isolated from the cheese whey and identified by biochemical and molecular methods (accession number GQ 506972 and GQ 506975, respectively). The yeasts cultures were propagated on Yeast Malt (YM) plates (Quebact Laboratories Inc., Quebec, Canada) plates at 35 $^{o}$C for 48 h and preserved at 4 $^{o}$C for future use.

## Inoculum preparation

YM broth was prepared and sterilized at 121 $^{o}$C for 15 min. A loopful of *K. marxianus* grown on YM agar was used to inoculate a 100 mL Erlenmeyer flask containing 20 mL of sterilized YM broth medium. The flasks were incubated in a rotary incubator shaker at 150 rpm and at 35 $^{o}$C for 18 h. The actively grown cells from these flasks were used as a pre-culture to inoculate a 2000 mL flask containing 500 mL of pasteurized whey powder (4.5% w/v) supplemented with urea (0.22% w/v) as nitrogen source for *K. marxianus* or sterilized YM broth medium for *S. cerevisiae*.

## Production of yeast biomass

Production of yeast (*K. marxianus*) biomass was carried out in a stirred tank 15 L fermentor (working volume: 10 L, Biogenie, Quebec, Canada) equipped with accessories and a programmable logic control (PLC) system for dissolved oxygen (DO), pH, anti-foam, impeller speed, aeration rate and temperature. The software (iFix 3.5, Intellution, USA) allowed for automatic set-point control and integration of all parameters via PLC. Before each sterilization cycle, the polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4.0 and 7.0 (VWR Canada). The oxygen probe was calibrated to zero (using $N_2$ degassed water) and 100% (air saturated water). The fermentor was sterilized at 121 $^{o}$C for 15 min in-situ by water to maintain sterility. Subsequently, the fermentor was charged with reconstituted cheese whey (9.5 L) using whey powder (4.5% w/v) and urea (0.22% w/v). A polypropylene glycol solution (0.1% v/v) (PPG, Sigma-Canada) was used as antifoaming agent. The fermentor with medium was pasteurized in-situ at 80 °C for 20 min. When the fermentor was cooled to 35 °C, the pH was adjusted to 5.5 and the DO probe was recalibrated to zero.

The fermentor was then inoculated aseptically with 5% (v/v) of a *K. maxianus* pre-culture in the exponential phase (15 h-age). Air flow and agitation rates were adjusted during

fermentation in order to keep the DO above 25% saturation. The temperature was maintained at 35 $^{o}$C, and pH 5.5 (optimal pH for maximal production of β-galactosidase in whey medium) was maintained by adding 4 N NaOH or 4 N $H_2SO_4$ throughout the fermentation. The fermentation was carried out until 24 h and culture was then harvested.

## Permeabilization of yeast cells

*K. marxianus* biomass from the fermented broth was recovered by centrifugation at 5000 rpm for 10 min. One gram wet weight (0.17 g dry weight) of *K. marxianus* cells was suspended in a 1 mL solution of 1.5% (w/v) of freshly prepared N-lauroyl sacosine (N-LS) and incubated at 25 $^{o}$C for 20 min under shaking condition (150 rpm). After treatment with the detergent, the cells were separated by centrifugation at 5000 rpm for 10 min. N-LS-treated cells were washed twice with sterile distilled water to remove excess detergent and the permeabilized cells (permeabilized cells were used as a source of the crude enzymes β-galactosidase and catalase) were used to measure enzyme activity. Different permeabilization process parameters were optimized and the methodology used for each parameter is given below.

### Effect of N-LS concentration

1.0 g wet weight of yeast cells was treated with a 1 mL solution containing different concentrations (1.0 to 3.5% w/v) of N-LS prepared in sterilized distilled water. β-galactosidase activity of the permeabilized cells was determined after centrifugation.

### Effect of solvent volume

1.0 g wet weight of yeast cells was treated with 15 mg of N-LS dissolved in different volumes (1 to 6 mL) of solvent (water). The permeabilized cells were used to measure β-galactosidase activity.

### Effect of incubation temperature

1.0 g wet weight of yeast cells was mixed with 1 mL of the N-LS solution (1.5% w/v) and incubated at different temperatures (20 to 45 $^{o}$C). β-galactosidase activity of the permeabilized cells was determined after centrifugation.

### Effect of incubation time

1.0 g wet weight of yeast cells was mixed with 1 mL of the solution of N-LS (1.5% w/v) and incubated at 25 $^{o}$C for different incubation times (10 to 60 min). The permeabilized cells after centrifugation were used to estimate enzyme activity.

## Hydrolysis of whey lactose using permeabilized *K. marxianus* cells

One hundred mL of a whey powder 5% (w/v) solution (pH 6.5) was prepared in Erlenmeyer flasks and inoculated with different amounts (150 mg, 300 mg, 450 mg, 600 mg and 675 mg dry weight) of permeabilized cells. Inoculated flasks were incubated at 30 $^{o}$C in an orbital shaker at 100 rpm for 3 h. Samples were withdrawn at regular intervals and at the end of hydrolysis. Enzyme activity (lactose hydrolysis) was stopped by heating the samples at 100 $^{o}$C for 10 min. The samples were centrifuged at 5000 rpm for 5 min and the supernatant of each sample was used to determine the hydrolysis products with high performance liquid chromatography (HPLC). The HPLC method of sugar analysis was chosen to measure hydrolysis process because the β-galactosidase enzyme may also show transgalactosylation activity and produce galacto-oligosaccharides (GOS) during the hydrolysis process.

## Production of mixed culture SCP in hydrolyzed whey

The *K. marxianus* cells were produced in a 15 L fermentor as described before (production of yeast biomass). The fermented broth was harvested after 24 h. The required volume of the fermented broth was centrifuged to obtain a biomass pellet and the pellet was permeabilized with the N-LS solution (1.5% w/v) at 25 $^{o}$C for 20 min at the ratio of 1.0 g wet weight/1 mL of N-LS solution. The permeabilized cells were centrifuged and washed with sterilized distilled water.

For the production of SCP, the prepared permeabilized *K. marxianus* cells (0.15% w/v dry weight) were added to the pasteurized reconstituted whey powder (4.5% w/v) solution supplemented with 0.22% w/v urea. Simultaneously, *S. cerevisiae* (10% v/v inoculum) was added to obtain a mixed culture. In this case, the permeabilized cells of *K. marxianus* not only supplied the needed enzyme, but also served as inoculum. The fermentation was carried out at pH 6.5 (optimal pH for hydrolysis of lactose by β-galactosidase) and 30 $^{o}$C. Samples were withdrawn at 6 h intervals and subsequently used for analysis.

## Analytical methods

### β-galactosidase assay

β-galactosidase activity was measured by the method of Miller [20]. The re-suspended permeabilized cells in distilled water served as source of crude enzyme. The reaction mixture contained 3 mL of Z-buffer, 1 mL of permeabilized cells (8 x concentrated compared to fermented broth) and 1 mL of ONPG (4 mg/mL). The reaction mixture was incubated at 30 $^{o}$C until the yellow colour developed (approximately 30 min). After incubation, the reaction was stopped by adding 3 mL of 0.4 M $Na_2CO_3$. The samples were centrifuged to separate the permeabilized cells at 5000 rpm for 5 min and the supernatant was collected. The produced colour in the supernatant was measured at 420 nm via spectrophotometer. One unit of enzyme activity is defined as one µmole of 2-nitrophenol liberated per min under the standard assay conditions.

### Catalase assay

Catalase activity was measured by the method of Aebi [21] in which $H_2O_2$ degradation was monitored by measuring a decrease in absorbance at 240 nm due to catalase reaction. Hydrogen peroxide (5-40 mM solutions) was used as standard to determine enzyme activity. The catalase activity of permeabilized cells was measured by incubating 0.5 mL of the suspension of permeabilized cells (8 x concentrated compared to fermented broth) in 10 mL $H_2O_2$ (40 mM solution), prepared in 50 mM phosphate buffer (pH 7.0), and incubated for 10 min with shaking. After incubation, the reaction was stopped by adding 1.5 mL $H_2SO_4$ (4 N). The samples were centrifuged (at 5000 rpm for 5 min) and absorbance was measured on the supernatant. One unit of enzyme activity is defined as the decomposition of one µmole of $H_2O_2$ per min at 25 $^{o}$C and pH 7.0.

### Viability of permeabilized cells

Viability of the permeabilized cells was determined as colony-forming units per milliliter (CFU/mL) by the serial dilution plating technique. The appropriately diluted samples of permeabilized cells or of non-permeabilized cells were plated on YM agar plates and incubated for 48 h at 35 °C to form fully developed colonies.

### Analysis of whey lactose hydrolysis

The HPLC conditions used for sugar analysis were; mobile phase 480 mM sodium hydroxide; isocratic flow rate, 0.4 mL/min; column, CarbopackTM MA1, analytical 4x250 mm dimension from Dionex; equipped with pump, auto sampler and Dionex ED40 Electrochemical Detector. The data were analyzed using the Chromeleon software [22].

The soluble protein concentration was measured by the Lowry [23] method. The standard curve was drawn with different concentrations of bovine serum albumin (BSA) obtained by diluting the 1 mg/mL BSA stock solution. The reducing sugar concentration was measured by the dinitro-salicylic acid (DNS) method of Miller [24]. The standard curve was drawn with different concentrations of lactose using a 2.0 g/L of lactose stock solution. All estimates were carried out in triplicate and the average values are presented (with standard error less than 5% of the mean).

## RESULTS AND DISCUSSION

## Permeabilization of yeast cells

### Effect of N-LS concentration

The impact of permeabilization of *K. maxianus* cells at different concentrations of N-LS is presented in Fig. 1. The maximal β-galactosidase activity was obtained at the N-LS concentration of 1.5% (w/v). Decreased enzyme activity at higher N-LS concentration could have been due to either cell lysis or interaction of membrane proteins with N-LS, which resulted in enzyme leakage, from the permeabilized cells to inactivation of the enzyme. At lower concentration of N-LS (<1.5% w/v) the lower enzyme activity was probably due to less effective permeabilization of the cells [25]. Thus, the concentration of a permeabilizing agent plays an important role in determining whether the cells are permeabilized or lysed during treatment. The affinity of the detergent towards the cell membrane could also affect the permeabilization process [17]. The mechanism of permeabilization (or alteration in membrane permeability) depends on the interaction of different detergents (ionic, zwitterion and non-ionic) with proteins and lipids of the cell membrane, which results in pore formation in the membrane or perturbation of the membrane structure [26, 27].

## Effect of solvent volume

The results revealed that the maximal enzyme activity was obtained when 1 mL of N-LS (containing total 15 mg of N-LS) was used to permeabilize 1.0 g wet weight of cells (Fig. 2). The enzyme activity decreased with the increase in volume of the solvent (water). The results indicated that volume of permeabilizing media (or cells to detergent ratio/unit volume of solvent) plays an important role in permeabilization of *K. marxianus* cells. This probably due to low interaction of N-LS molecules with yeast cells when the volume of solvent increases (or the cell concentration in detergent solution decreases). Moreover, the phenomenon of micelles formation must also play a key role above and below the critical micelle concentration (CMC), which in turn decreases the permeabilization effect and thus the enzyme activity.

It is known that process parameters (concentration of detergent, for example) for permeabilization differ for different types of microorganisms [11]. The permeabilization study on *S. cerevisiae* for catalase activity reported by Abraham and Bhat [17] revealed that the effective concentration of N-LS was 2% w/v. This might be due to the nature of the enzyme (catalase) or to a difference in the yeast species (*S. cerevisiae*). Moreover, one important information obtained during this N-LS volume optimization study was that the requirement in solvent (water) was very minor (1 mL with 15 mg N-LS/1.0 g cells wet weight). Due to this low requirement for the detergent solution, the released protein and nucleotides after permeabilization could be easily recovered because they were in a concentrated form. The recovered proteins and nucleotides could be used as flavouring agents and as nutrients in aquaculture [28].

## Effect of temperature

The optimal temperature to achieve maximal enzyme activity was 25 $^{o}$C (Fig. 3). Decreases in enzyme activity at higher temperatures may have been due to heat inactivation of the enzyme or increased deleterious enzyme-detergent interaction or activation of membrane bound endogenous proteases. Activation of these proteases may have degraded the enzyme β-galactosidase. A higher protease activity has been reported at temperatures above 25 $^{o}$C [29]. Low activity of the enzyme β-galactosidase at 20 $^{o}$C might have been caused by a reduction in the efficiency of the detergent.

## Effect of incubation time

An incubation time of 20 min was found to be the optimal to achieve maximal β-galactosidase activity (Fig. 4). Enzyme activity decreased with incubation time above and under 20 min. However, enzyme activity was stabilized after 30 min of incubation time. A decrease in enzyme activity when incubated for longer than 20 min could be attributed to inactivation or lysis of yeast cells by the permeabilizing agent or inactivation of β-galactosidase protein by proteases [17]. The lower enzyme activity with incubation times lower than 20 min could be due to insufficient permeabilization of the yeast cells [25]. A maximal activity of 1220 IU/g dry cell weight of β-galactosidase was observed with the optimal conditions (20 min and 25 $^{o}$C).

Use of permeabilized microbial cells as enzyme source has advantages over use of pure or free enzymes (such as low purification cost and enhanced enzyme stability). The permeabilization technique has been applied to *Kluyveromyces* spp. for β-galactosidase activity employing different permeabilizing agents. Various yeast strains of *K. marxianus* and of *K. fragilis* were permeabilized and different levels of β-galactosidase activity were reported [13, 15, 25, 30-32] by several researchers, as presented in Table 1. Though some chemicals such as organic solvents, CTAB and digitonin showed a slightly higher permeabilization efficiency (enzyme activity) compared to N-LS permeabilization, but due to their higher toxicity (lower LD50 value) these chemicals cannot be used especially in the context of food ingredients.

## Catalase activity in the permeabilized cells

A catalase activity of 38 000 IU/g dry cell weight was found under the conditions found optimal for the permeabilization of *K. marxianus* cells to achieve maximal β-galactosidase activity. The catalase activity in the permeabilized *K. marxianus* cells demonstrated that other intracellular enzyme activities could also be made available during permeabilization. Simultaneous presence of catalase along with β-galactosidase would be an added advantage because the premeabilized cells could also be used as an enzyme cocktail. Thus, permeabilized cells with catalase activity could be utilized simultaneously for degradation of hydrogen peroxide as well as hydrolysis of whey lactose. Hydrogen peroxide is used for cold pasteurization during the processing of cheese, and therefore, it is necessary to remove residual hydrogen peroxide before culturing microorganism in cheese whey [33].

### Viability of permeabilized cells

The viability of yeast cells was evaluated before and after permeabilization. The results revealed that the cells were viable after permeabilization but the total cells concentration decreased by 2 log units during permeabilization. Cell viability decreased from $4.30x10^8$ CFU/mL to $5.20x10^6$ CFU/mL. This might be due to inactivation or lysis of the cells brought about by the action of detergent. However, cell lysis seems improbable because β-galactosidase activity was not observed in the supernatant. A reduction in cell viability up to 15% together with a change in cell morphology has been reported after permeabilization of the yeast *Pichia anomala* with 5% v/v Triton X-100 [34]. Since viability of cells after permeabilization remained high, it is possible that some of the lactose might have been consumed by the yeast cells during hydrolysis. In order to verify this possibility, the concentration of glucose and lactose was monitored before and after hydrolysis (3 h). It was found that 15.5 g/L of glucose was released after maximal hydrolysis. The glucose concentration measured (15.5 g/L) was lower by 0.9 g/L than that theoretically expected from the hydrolysis of 32.7 g/L of lactose (initial concentration at the beginning of hydrolysis). The reduction in glucose concentration might have been due to consumption by the viable yeast cells within 3 h. Thus, the consumption of sugars during hydrolysis was not appreciable.

This is the first study to show the extent of viability after permeabilization of *K. marxianus* cells with a detergent. The lysis of cells should have been minimal otherwise the enzyme would have been released from the cells and consequently a loss of enzyme activity would have occured. This result is contrary to ethanol-permeabilized cells, where yeast cells lost viability after permeabilization (an observation evaluated from ethanol-permeabilized *K. marxianus* cells). On the other hand, viability of cells might be useful when the purpose is to produce galacto-oligosaccharides (GOS). During the enzymatic synthesis process, galactose (2 to 20 molecules) will be converted to GOS and residual glucose (generally 1 molecule used in synthesis of GOS) [35] will be consumed by the viable cells.

## Hydrolysis of whey lactose by permeabilized yeast cells

The permeabilized yeast cells were tested for whey lactose hydrolysis (Figs 5 & 6). Keeping in view that other reactions such as trangalactosylation could occur, the hydrolysis products of lactose by β-galactosidase were measured by HPLC. Same β-galactosidases from various *K. marxianus* strains possess a higher specificity towards lactose hydrolysis and some are more specific towards trangalactosylation activity [36]. The percentage of unhydrolyzed and hydrolyzed lactose in whey was calculated from the standard curve for lactose. The HPLC

chromatogram results (Fig. 5a - unhydrolyzed lactose, and Fig. 5b - hydrolyzed lactose) clearly indicate that whey lactose was hydrolyzed by β-galactosidase to glucose and galactose with minor trangalactosylation activity because the peak height of galactose was comparatively smaller to that of the glucose peak. The fourth peak appearing in the chromatogram was for GOS (Fig. 5b).

The amount of whey lactose hydrolyzed increased with time up to a permeabilized yeast cells concentration of 600 mg dry weight/100 mL of whey powder solution (Fig. 6). A maximal lactose hydrolysis of 91% was obtained with a permeabilized cell concentration of 600 mg dry cell weight per 100 mL of whey powder solution (5% w/v whey powder or lactose concentration of 3.6% w/v) after three hours of incubation. A permeabilized cell concentration above 600 mg dry cell weight/100 mL of whey powder solution did not show any further increase in the hydrolysis. At the same time, 51% hydrolysis was achieved with 150 mg biomass within 3 h. The increased lactose hydrolysis at higher permeabilized cell concentrations is due to increased availability of β-galactosidase enzyme. Lactose hydrolysis did not increase beyond 600 mg permeabilized cells/100 mL whey powder solution and this could be due to substrate (lactose) limitation or product (galactose) inhibition [37].

Based on the results obtained with different enzyme-to-substrate [permeabilized cells:substrate (lactose)] ratio, the kinetics of lactose hydrolysis was evaluated and first order kinetics showed the best fit with the data. The integral method of analysis has been used to evaluate the reaction kinetics of the hydrolysis. The relationship between substrate concentration and time of hydrolysis for a first order reaction was calculated using the following equation:

$$\ln(C_t/C_0) = -kt \quad (1)$$

Where $C_t$ is the substrate concentration at time t, $C_0$ is the initial substrate concentration and k is the apparent rate constant ($min^{-1}$). A plot of $\ln(C_t/C_0)$ versus time ($t$) gave a straight line with a slope of $-k$. Linear regression analysis of the plots gave the k-values with their respective coefficient of determination ($R^2$) value as presented in Table 2. Half-life ($t_{1/2}$) of the substrate was calculated by the first order kinetics half-life equation:

$$t_{1/2} = 0.693/k \quad (2)$$

The kinetics results obtained indicated that as the enzyme:substrate (E:S) ratio increased, the rate of hydrolysis increased from 51% to a maximum of 92% when the E:S ratio increased from 1:240 to 1:53. Similarly, the trend of half-life for substrate disappearance was also observed and decreased from 178 min to 50 min. However, with higher E:S ratios, there was not much variation in the half-life of the substrate. Thus, a E:S ratio could be chosen

based on the purpose of lactose hydrolysis (i.e. complete, partial or slow hydrolysis). The other parameter values related to enzyme kinetics are summarized in Table 2.

These results proved that N-LS permeabilized yeast cells could be used to hydrolyze whey lactose, and subsequently, the hydrolysate could be used to produce various products such as mixed culture SCP, ethanol and/or milk lactose hydrolysis. Moreover, lactose can also be used for biotransformation to GOS after selecting a specific β-galactosidase with transgalactosidase activity. Lactose hydrolysis up to 89 to 90.5% has been reported from skim milk and whey using *Kluyveromyces* permeabilized cells [31, 38]. Furthermore, whey lactose hydrolysis carried out by cetyltrimethyl ammonium bromide (CTAB)-permeabilized yeast cells and subsequent use of the hydrolyzed lactose for bioethanol production by non-lactose consuming yeast has been reported [39].

## Production of mixed culture SCP in hydrolyzed whey

The variations in reducing sugar, soluble protein concentration, cell count and suspended solids (yeast biomass) during hydrolyzed whey (via permeabilized *K. marxianus* cells) batch fermentation are shown in Fig. 7. The reducing sugar concentration during the batch fermentation decreased from 32.8 to 1.1 g/L. A sugar consumption of 96% was observed after 24 h of batch fermentation. The soluble protein content during the fermentation decreased from 8.2 to 7.8 g/L. A higher concentration of residual soluble protein in the fermented broth was observed compared to the fermentation without use of permeabilized *K. marxianus* (residual protein content was 5.2 g/L). The higher residual soluble protein concentration during fermentation with permeabilized cells might be due to release of soluble proteins from the permeabilized *K. marxianus* cells or excretion of some extracellular enzymes during the mixed culture cultivation (*K. marxianus* and *S. cerevisiae*). The biomass, in terms of suspended solids steadily increased from 2.3 to 9.2 g/L. Cell counts of *K. marxianus* and *S. cerevisiae* increased with time and active growth was observed after 6 hrs of fermentation (Fig. 7). The initial slow growth might have been due to the fact that *K. marxianus* cells were in a permeabilized state (almost inactivated cells) and it took some time before they start growing. Moreover, *S. cerevisiae* growth depends on the availability of glucose and galactose in the medium generated as a result of hydrolysis of lactose by the permeabilized cells and that may have delayed growth. Even though viable *K. marxianus* cells (or inoculum) were not added during the fermentation, the permeabilized cells of *K. marxianus* started growing. The *K. marxianus*/*S. cerevisiae* ratio during the batch fermentation (0-24 h) was increased from 0.6 to 1.3. An increase in the ratio of *K. marxianus*/*S. cerevisiae* might have been due to the fact that *K. marxianus* can utilize all

three sugars (glucose, galactose and lactose) while *S. cerevisiae* can consume only glucose and galactose. Moreover, the concentration of *S. cerevisiae* could be enhanced by using a higher concentration of permeabilized cells to increase hydrolysis, if necessary. The yields ($Y_{x/s}$), productivities (g/L.h) and sugar degradation rates (g/L.h) were compared between fermentation of whey without hydrolysis with *K. marxianus* (profile not presented) and fermentation with the mixed culture (*K. marxianus* and *S. cerevisiae*) in hydrolyzed whey. The yield, productivity and sugar degradation rate were found to be 0.26 g biomass/g lactose, 0.32 g/L.h and 1.25 g/L.h in the case of no lactose hydrolysis while they were 0.22 g biomass/g lactose, 0.29 g/L.h and 1.32 g/L.h in the case of hydrolysis, respectively. The biomass yield was a little higher with no hydrolysis, however, this could be compensated by the higher content of residual soluble proteins, which could be recovered and added to the biomass, or they could be used separately.

Growth of *S. cerevisiae* in the mixed culture confirmed that permeabilized cells could be applied for mixed culture biomass production (*K. marxianus* and *S. cerevisiae*), which is in higher demand for food grade yeast. The mixed culture biomass could be used as a source of protein with a balanced composition of amino acids [39]. *K. marxianus* usually lacks sulfur-containing amino acids [40] while *S. cerevisiae* possesses them. The hydrolyzed whey lactose can provide an alternate cheap medium for baker's yeast production without addition of other carbon sources, as done by some researchers who added molasses in cheese whey to improve growth of baker's yeast in a mixed culture [41]. Further, the observed yield and productivity during the fermentation process could be increased by applying a fed-batch process using one time permeabilized cells and thus reduce the production cost.

There is also the possibility of performing simultaneous galacto-oligosaccharides production in the same batch or fed-batch process due to transgalactosylation activity of the β-galactosidase. Moreover, as said above, the released nucleotides and proteins during permeabilization could be used as flavoring agents and as nutrients in aquaculture. This study shows that the permeabilization process can enhance the usefulness of whey for non-lactose consuming yeasts. Permeabilized *K. marxianus* cells can also be used for the production of lactose-free milk, as a cheap alternative, over the use of a pure enzyme for the hydrolysis.

## CONCLUSION

This study demonstrated that the non-toxic and biodegradable detergent (N-LS) can be successfully used to permeabilize *K. marxianus* cells. In order to obtain maximal β-

galactosidase activity, the process parameters for permeabilization of *K. marxianus* cells were optimized to gave the following values: N-LS concentration of 1.5% w/v, solvent (water) volume of 1 mL (or 1.0 g wet weight/mL of N-LS solution), temperature of 25 $^{o}$C and incubation time of 20 min. The permeabilized *K. marxianus* cells also exhibited catalase activity. This is an added advantage, which could be exploited for degradation of $H_2O_2$ present in cheese whey. More than 90% of the whey lactose hydrolysis (from 50.0 g/L of whey powder solution) was achieved within 3 h with 6.0 g dry weight of permeabilized cells. It was established that the hydrolyzed whey could be effectively used to grow the mixed culture (for SCP production) consisting of lactose-consuming (*K. marxianus*) yeast and of a non-lactose consuming yeast (*S. cerevisiae*). Additional studies are needed to test this new process at pilot scale as well as to optimize the recovery of the soluble proteins after fermentation. Moreover, it is also desirable to purify the released protein and nucleotides during permeabilization for its potential applications.

## ACKNOWLEDGEMENTS

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support.

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**Table 1. Comparison of β-galactosidase activity with different permeabilizing agents and their toxicity**

| Yeast strains | Permeabilizing agents | Conc. of permeabilizing agent | | Enzyme activity | | Toxic (LD50) | References |
|---|---|---|---|---|---|---|---|
| | | (mg/g wet wt.) | (mg/g dry wt.) | (IU/g wet wt.) | (IU/g dry wt.) | | |
| *K. marxianus* | N-LS | 15 | 90 | 203.3 | 1220 | No (>5000 mg/kg) | This study |
| *K. marxianus* | Toluene: ethanol (1:1) | N.a | N.a | N.a | 1610 | Yes | [32] |
| *K. marxianus* | CTAB | N. a | 136 | N.a | 1290 | Yes (410 mg/kg) | [38] |
| *K. marxianus* | CTAB | 10 | | 220.9 | N.a | Yes (410 mg/kg) | [13] |
| *K. marxianus* | Ethanol (50 % v/v) | N.a | N.a | N.a | 1540 | Yes (6200 mg/kg) | [15] |
| *K. marxianus* | Ethanol (50% v/v) | N.a | N.a | N.a | 1573 | Yes (6200 mg/kg) | [31] |
| *K. fragilis* | Digitonin | 10 | N.a | 212.0 | N.a | Yes (50 mg/kg) | [30] |
| *K. fragilis* | Triton X-100 | 107 | N.a | 51.7 | N.a | Yes (1800 mg/kg) | [30] |
| *K. marxianus* | Toluene (20%) | N.a | N.a | N.a | 1432 | Yes (13 mg/kg fish) | [32] |

* N.a: Not available; LD50: Lethal dose

**Table 2. Kinetics of whey lactose hydrolysis with permeabilized cells in relation to different E:S ratios during the 180 min-incubation**

| Hydrolysates | E:S | % of lactose hydrolyse | Rate equation | $R^2$ | Rate constant ($min^{-1}$) | Half-life of the substrate (min) |
|---|---|---|---|---|---|---|
| 1 | 1:240 | 51 | y= -0.0037x - 0.0828 | 0.9729 | 0.0037 | 178 |
| 2 | 1:120 | 68 | y = -0.0059x - 0.1405 | 0.9718 | 0.0059 | 108 |
| 3 | 1:80 | 84 | y = -0.0094x - 0.109 | 0.9746 | 0.0094 | 68 |
| 4 | 1:60 | 91 | y = -0.0135x - 0.1043 | 0.991 | 0.0135 | 51 |
| 5 | 1:53 | 92 | y = -0.014x - 0.1587 | 0.9816 | 0.0140 | 50 |

***Note** E:S [permeabilized cells:substrate (mg:mg)]

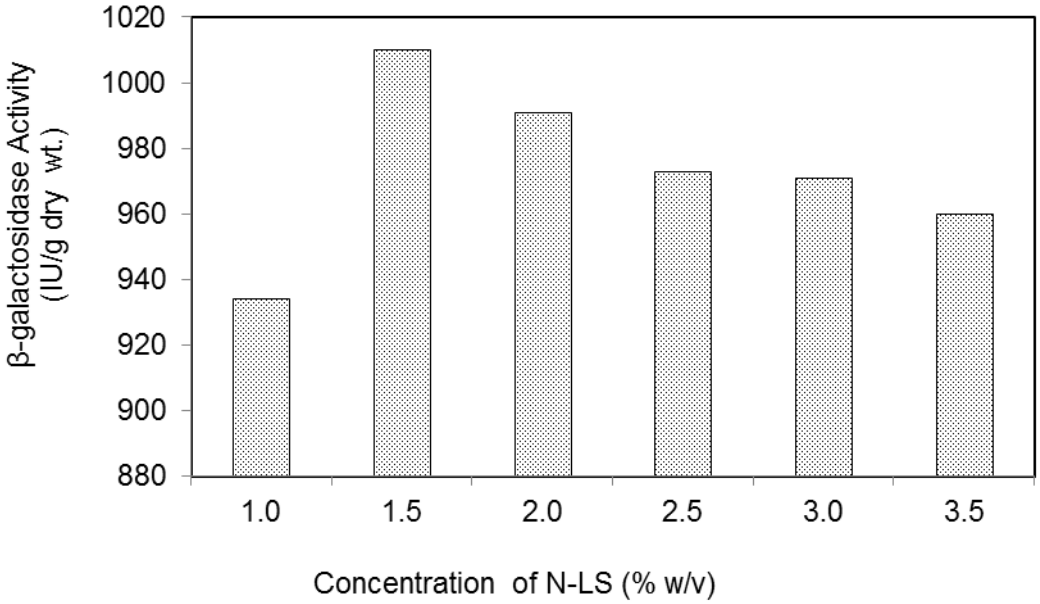


**Figure 1. Effect of N-LS concentration on permeabilization of *K. marxianus* cells**

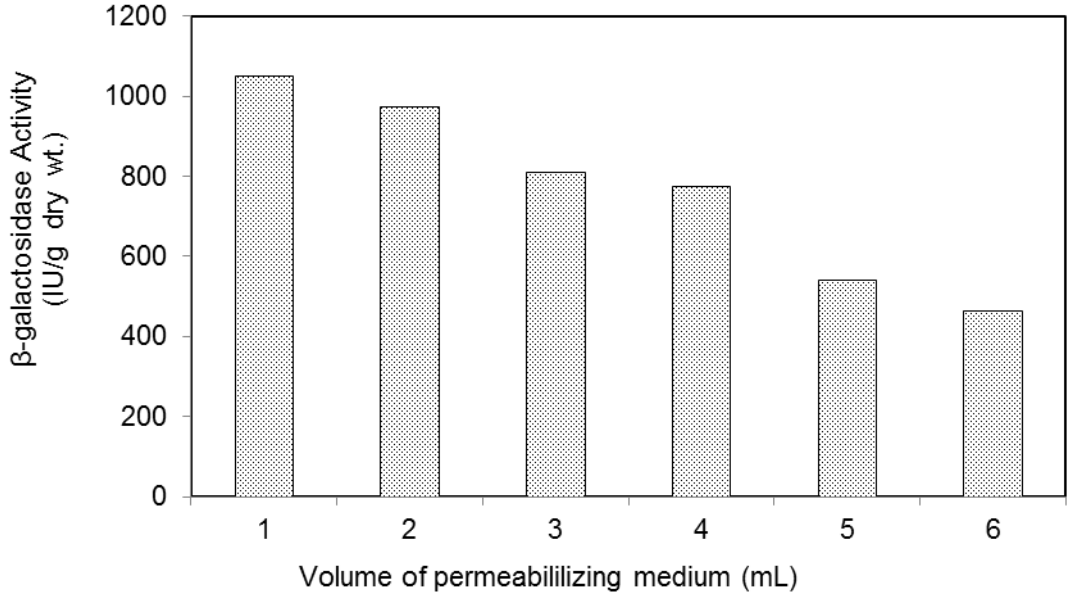


**Figure 2. Effect of solvent (water) volume with constant amount of N-LS on permeabilization of *K. marxianus* cells**

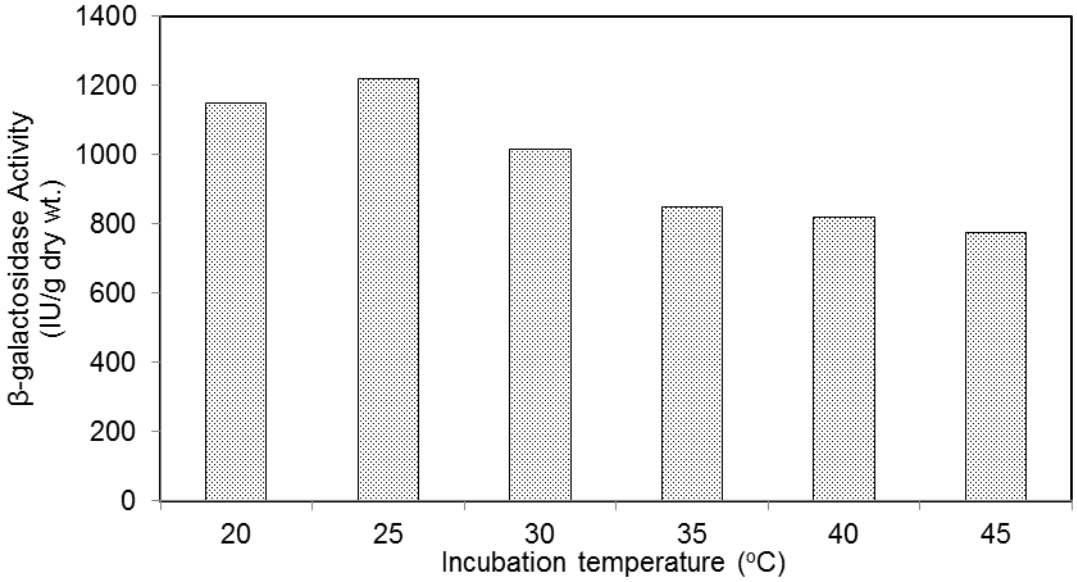


**Figure 3. Effect of incubation temperature on permeabilization of *K. marxianus* cells**

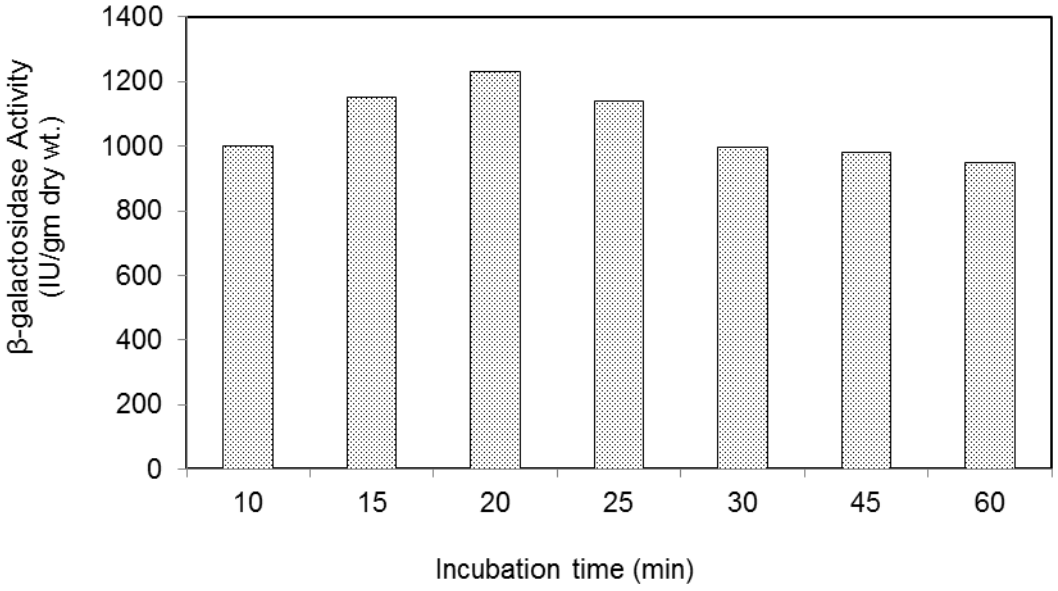


**Figure 4. Effect of incubation times at 25 $^{o}$C on permeabilization of *K. marxianus* cells**

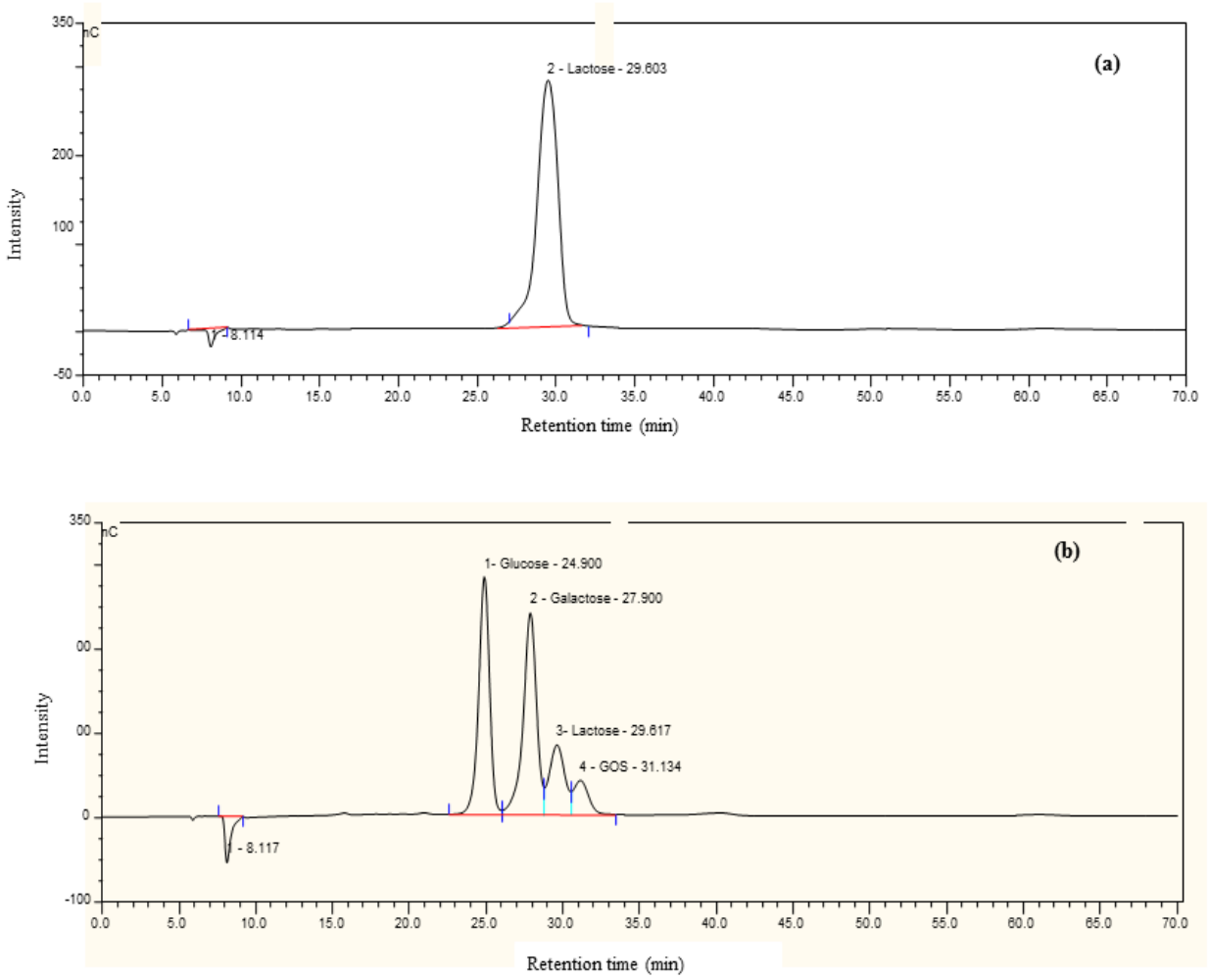


**Figure 5. (a) HPLC chromatogram of unhydrolyzed whey lactose; (b) HPLC chromatogram of hydrolyzed whey lactose to glucose and galactose by β-galactosidase**

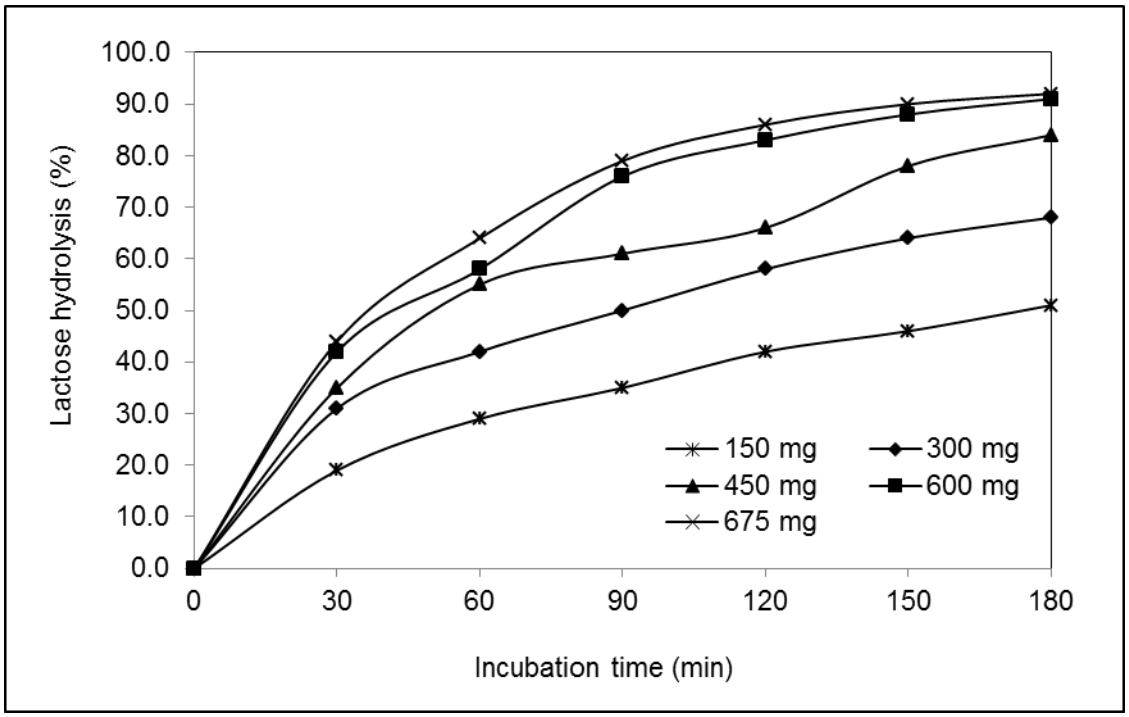


**Figure 6. Hydrolysis of whey lactose by permeabilized yeast cells with different dry cell weight loads and at different incubation times**

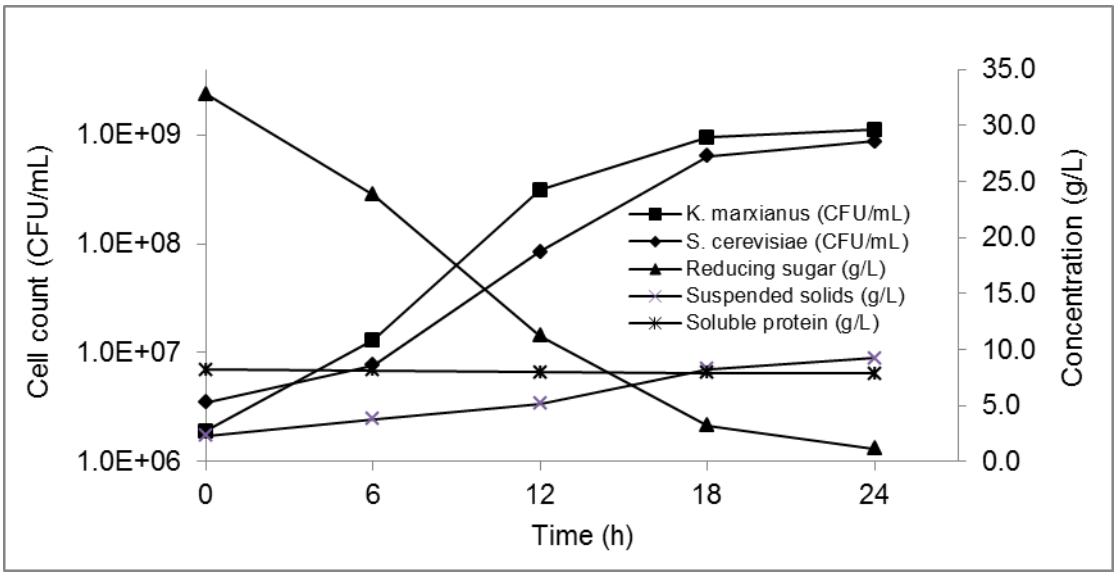


**Figure 7. Cells count, biomass and substrate profiles during the production of mixed culture SCP in hydrolyzed whey lactose**

# PARTIE 2

# FOOD-GRADE SINGLE-CELL PROTEIN PRODUCTION, CHARACTERIZATION AND ULTRAFILTRATION RECOVERY OF RESIDUAL FERMENTED WHEY PROTEINS FROM WHEY

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**Submitted to Food and Bioproducts Processing**

# RÉSUMÉ

Cette étude a été effectuée afin de caractériser et de récupérer les protéines résiduelles solubles engendrées par des monocultures (*Kluyveromyces marxianus*) et des cultures mixtes (*K. marxianus* et *Saccharomyces cerevisiae*) de levures sur protéines unicellulaires de qualité alimentaire, ces dernières étant issues du lactosérum. La fermentation discontinue a été réalisée à 35 $^{o}$C et à pH 5,5 pour les monocultures et à 30 $^{o}$C et à pH 6,5 pour les cultures mixtes. Les biomasses de levures ont été séparées du bouillon de fermentation par centrifugation, laissant les protéines résiduelles solubles dans le surnageant du lactosérum fermenté (SLF). Les biomasses de levures (monocultures et cultures mixtes) ont alors été traitées en deux étapes par une combinaison novatrice de réactifs (N-lauroyl sarcosine et $NH_4OH$) permettant la réduction de la teneur en acides nucléiques selon le niveau désiré (inférieur à 2% p/p) pour les PU de qualité alimentaire. De plus, les caractéristiques des protéines solubles issues de la fermentation ont été évaluées par électrophorèse. Pendant la fermentation, il s'est avéré qu'une fraction des protéines du lactosérum était consommée. Les résultats de l'électrophorèse ont ainsi révélé que les protéines du lactosérum fermenté sont différentes des protéines natives contenues dans le lactosérum, et que ces dernières sont partiellement hydrolysées pendant la fermentation. Les paramètres opérationnels de l'ultrafiltration (flux de perméat et pression transmembranaire- PTM) ont été optimisés pour des membranes de 1 et 10 kDa. Les valeurs maximales de récupération des protéines résiduelles solubles ont été atteintes selon les conditions suivantes 413 L/h/m$^2$ en flux de perméat et 80 kPa en PTM pour la membrane de 10 kDa, suivie de 2760 L/h/m$^2$ en flux de perméat et 230 kPa en PTM pour la membrane de 1 kDa. Finalement, la combinaison des deux conditions optimales a permis de récupérer 84% et 92% des protéines résiduelles solubles issues des monocultures et des cultures mixtes SLF, respectivement.

**Mots-clés**: Lactosérum; Fermentation; Protéines unicellulaire; Protéines du lactosérum; Électrophorèse; Ultrafiltration; Récupération.

## ABSTRACT

This study was carried out to characterize and recover residual soluble proteins after cultivation of mono- (*Kluyveromyces marxianus*) and mixed culture (*K. marxianus* and *Saccharomyces cerevisiae*) on whey, to serve as food-grade single-cell protein (SCP). Batch fermentation was carried out at 35 $^{o}$C and pH 5.5 for mono-culture and 30 $^{o}$C and pH 6.5 for mixed culture. The yeasts biomass was separated from the fermented broth by centrifugation leaving residual soluble proteins in the fermented whey supernatant (FWS). The yeasts biomass was treated in two-step with a novel combination of chemicals (N-lauroyl sarcosine and $NH_4OH$), which reduced the nucleic acid content in the biomass to the desired level (below 2% w/w) for food-grade SCP. The characteristics of the proteins were evaluated by electrophoresis. During fermentation, a fraction of the whey proteins was consumed. The electrophoresis results revealed that the fermented proteins were different from the native whey proteins and that they were partially hydrolyzed. The ultrafiltration operational parameters were optimized (in series) using 10 kDa and 1 kDa membranes. A permeate flux of 413 LMH (L/h/m$^2$) and transmembrane pressure-TMP of 80 kPa resulted in the highest recovery with the 10 kDa membrane followed by a permeate flux of 2760 LMH and a TMP of 210 kPa were determined for the 1 kDa membrane. Recovery of the total proteins under these optimized conditions was 84% and 92% from mono- and mixed culture FWS, respectively.



**Keywords:** Cheese whey; Fermentation; Single-cell protein, Whey protein; Electrophoresis; Ultrafiltration; Recovery.

# 1. INTRODUCTION

Cheese whey is produced after precipitation and removal of milk casein during cheese manufacturing. In terms of milk volume, cheese whey shares around 90-95% and contains around 55% of the milk nutrients (Spălăţelu, 2012). Due to the presence of milk components (nutrients), the chemical oxygen demand (COD) of whey is high (60-80 g/L) and considered as an environmental problem. The main components of cheese whey are lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (0.8-1.0% w/v) (Spălăţelu, 2012; Panesar and Kennedy, 2012). Whey proteins are the second dominant component in cheese whey, and is categorized as generally recognized as safe (GRAS) for food and pharmaceutical applications. Whey proteins are an heterogeneous mixture of different individual proteins, which are β-lactoglobulin (40-50% w/w), α-lactalbumin (12-15% w/w), immunoglobulins (8% w/w), bovine serum albumin (5% w/w), lactoferrin (1% w/w), lactoperoxidase (0.5% w/w), proteose-peptone and glycomacropeptide (12% w/w) (Madureira et al., 2007). Due to the presence of these nutrients, whey is utilized for value-added product such as various types of proteins (e.g. whey protein concentrate, whey protein isolate, individual whey proteins and hydrolyzed whey proteins) and lactose. Whey proteins are used in many processed foods such as healthy foods, dairy foods, meat products, frozen foods and infant formulas (Jayaprakasha and Brueckner, 1999; Modler, 2009). Furthermore, whey or whey permeate is biotransformed into value-added products such as single-cell protein (SCP) with simultaneous pollution load removal (Panesar and Kennedy, 2012; Yadav et al., 2014a).

SCP can be directly used for animal feed as a source of protein. However, direct use is restricted for food application because of higher content of nucleic acids in SCP. Too high a nucleic acid consumption in humans may lead to the development of gout disease due to accumulation of uric acid in the body (Anupama and Ravindra, 2000). Therefore, different techniques have been proposed for the reduction of nucleic acid from SCP to make SCP useful for food application. Chemical (e.g. sodium chloride, ammonium hydroxide and sodium hydroxide) and enzymatic (e.g. ribonuclease and deoxyribonuclease) treatments are generally employed to pre-treat the biomass in order to reduce the nucleic acid content to a desired level (below 2% w/w) (Alvarez and Enrquez, 1988; Parajó et al., 1995; Larsen and Joergensen, 1996; Anupama and Ravindra, 2000)

Yeast biomass production as SCP using cheese whey as raw material has been extensively studied employing *Kluyveromyces* species. However, *K. marxianus* has not been efficient in

consuming the whey proteins. During the fermentation process, lactose gets consumed entirely, but a major fraction of the protein remained unutilized. Furthermore, the characteristics of native whey proteins have been reported to change during fermentation (Yadav et al., 2014a). Moreover, Yadav et al. (2014b) reported the presence of residual soluble proteins after cultivation of mixed culture of *K. marxianus* and *Saccharomyces cerevisiae* on hydrolyzed lactose in cheese whey.

Whey protein recovery by thermal precipitation, chromatography and membrane separation (ultrafiltration and nanofiltration) are well established methods and used in commercial practice (De la Fuente et al., 2002; Chollangi and Hossain, 2007; Akpinar-Bayizit et al., 2009). The precipitation method involves the adjustment of physical properties (e.g. salt, pH and heat treatment) of the medium to enhance insolubility of proteins (Tovar-Jiménez et al., 2012). However, thermal precipitation has the disadvantage of changing the characteristics of native whey proteins, which can affect the functional and nutritional values of the proteins. Additionally, a change in precipitation method was required for residual soluble protein recovery after fermentation (Yadav et al., 2014c). Chromatographic methods (e.g. gel permeation, hydrophobic interaction and affinity chromatography) are also popular for separation and purification of whey proteins or of individual whey proteins. Especially, preparative chromatographic techniques are used for large scale protein separation (Almécija et al., 2007). A chromatographic method of separation is easy to develop, but it is a complex process to optimize because of the various process parameters such as choice of column matrix, salt, buffer, organic solvent, temperature and gradient (Bonnaillie et al., 2014). To circumvent thermal denaturation and complex chromatographic protocol method development, recovery of the whey proteins by membrane filtration (ultrafiltration) has been chosen as the best alternate approach (Akpinar-Bayizit et al., 2009; Tovar-Jiménez et al., 2012).

Membrane separation (ultrafiltration) of whey components is based on the retention of proteins and permeation of lactose, minerals and other lower molecular mass components. Membrane separation occurs due to the transmembrane pressure (TMP) difference at the two sides of the membrane, i.e. higher on the retentate side and lower on the permeate side. Furthermore, ultrafiltration does not increase the temperature and thus, does not involve the phase change of the product (protein) (Chollangi and Hossain, 2007; Baldasso et al., 2011). Ultrafiltration is an effective method for whey protein separation. However, additional steps of diafiltration and/or ion exchange adsorption are needed to obtain lactose free or highly purified whey proteins (e.g. whey protein isolate contains > 90% w/w of protein). Moreover, the presence of lactose has a negative impact on permeate/feed flux (i.e. reduction of flux)

(Yorgun et al., 2008). On the other hand, fermented cheese whey will not have residual lactose, therefore, the negative impact on permeate/feed flux due to lactose will be minimized during protein recovery. The absence of lactose in the supernatant will increase the efficiency of the ultrafiltration process and enhance the recovered proteins purity. However, ultrafiltration requires to know a suitable molecular weight cutoff membrane and optimum operating parameters to recover the protein. Therefore, there is a need to find suitable membrane cutoff and values of the optimal parameters for residual soluble protein recovery after cultivation of the yeast (mono or mixed culture).

Therefore, the objective of this study was to characterize the fermented whey proteins and to investigate a method for residual protein recovery from the fermented whey supernatant of either the mono- (*K. marxianus*) or mixed culture (*K. marxianus* and *S. cerevisiae*) to serve as food-grade SCP. The specific objectives were: i) production of food-grade SCP; ii) characterization of the mono and mixed-culture fermented whey proteins and iii) optimization of the recovery of residual soluble whey proteins by ultrafiltration.

# 2. MATERIALS AND METHODS

## 2.1 Microorganisms

Yeast strains (*Kluyveromyces marxianus* and *Saccharomyces cerevisiae*) were isolated from cheese whey and identified by biochemical and molecular methods (accession number GQ 506972 and GQ 506975, respectively). The yeast cultures were propagated on Yeast Malt (YM) agar plates at 35 $^{o}$C for 48 h and preserved at 4 $^{o}$C for future use.

## 2.2 Inoculum preparation

YM broth was prepared and sterilized at 121 $^{o}$C for 15 min. A loopful of each *K. marxianus* and *S. cerevisiae* culture grown separately on YM agar plates was used to inoculate a 100 mL Erlenmeyer flasks containing 20 mL of sterilized YM broth medium. The flasks were incubated in a rotary incubator shaker at 150 rpm and 35 $^{o}$C for 18 h. The actively grown cells from these flasks were used as a pre-culture to inoculate a 2.0 L flask containing 500 mL solution of pasteurized whey powder (4.5% w/v) (whey powder obtained from a cheese factory, Fromage Saputo, St. Hyacinthe, Quebec) supplemented with urea (0.22% w/v) as nitrogen source for *K. marxianus. S. cerevisiae* pre-culture was produced in YM broth.

## 2.3 Production of mono-culture (*K. marxianus*) yeast biomass

Production of mono-culture yeast (*K. marxianus*) biomass was carried out in a stirred tank 15 L fermentor (working volume: 10 L, Biogenie, Quebec) equipped with accessories and a programmable logic control (PLC) system for dissolved oxygen (DO), pH, antifoam, impeller speed, aeration rate and temperature. The software (iFix 3.5, Intellution, USA) allowed for automatic set-point control and integration of all parameters via PLC. The polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers (pH 4.0 and 7.0) before each sterilization cycle. The oxygen probe was calibrated to zero (by using nitrogen) and to 100% (air-saturated water). The fermentor was filled with water, and was pre-sterilized in-situ to maintain sterility. After that, water was drained and the fermentor was charged with the whey powder (4.5% w/v) solution and urea (0.22% w/v). A polypropylene glycol (PPG, Sigma-Canada) solution (0.1% v/v) was used as antifoam agent. Cheese whey medium in the fermentor was pasteurized in-situ at 80 °C for 20 min. The fermentor was cooled to 35 $^{o}$C, and the pH was adjusted to 5.5. The DO probe was recalibrated to zero (with nitrogen) and to 100% saturation (by using maximum aeration and agitation).

The fermentor was aspectically inoculated with a pre-culture of *K. marxianus* (5% v/v). After inoculation, the air flow and agitation rates were adjusted during fermentation in order to keep the DO above 25% saturation. The temperature was maintained at 35 $^{o}$C and pH 5.5 was maintained with the help of 4 N NaOH or 4 N $H_2SO_4$. The fermentation was carried out until 30 h and then harvested.

## 2.4 Production of mixed culture yeast biomass

The production of mixed culture biomass was carried out in hydrolyzed whey lactose. The lactose in cheese whey was hydrolysed employing permeabilized cells of *K. marxianus* as reported in our earlier work (Yadav et al., 2014b). The fermented broth of a *K. marxianus* mono-culture was centrifuged to obtain the biomass pellet. The pellet was permeabilized with a detergent N-Lauroylsarcosine (N-LS) solution (1.5% w/v) at 25 $^{o}$C for 20 min at a ratio of 1.0 g wet wt pellet/mL (1.0 g wet wt equal to 0.18 g dry wt) of N-LS solution. The permeabilized cells were centrifuged and washed with sterilized distilled water. The washed permeabilized *K. marxianus* cells (0.20% w/v dry wt) were added to the pasteurized whey powder (4.5% w/v) solution supplemented with 0.22% w/v urea. Simultaneously, an inoculum of *K. marxianus* (2.5% v/v) and *S. cerevisiae* (7.5% v/v) was also added to obtain a mixed culture. The permeabilized cells of *K. marxianus* not only provided the enzyme for hydrolysis

of whey lactose, but also served as partial inoculum due to their remains viability. The fermentation was carried out at pH 6.5 and 30 $^{o}$C for 30 h and then harvested.

## 2.5 Downstream processing

Fermented broths after cultivation of the mono- (*K. marxianus*) and the mixed culture (*K. marxianus* and *S. cerevisiae*) were collected. The broths were centrifuged to separate the biomass from the fermented whey supernatant (FWS). The centrifugation was conducted at 8 000 rpm (10 808 g) for 15 min using a Sorvall RC5C centrifuge at 4 $^{o}$C. The biomass and FWS were stored in a cold room (4 $^{o}$C) until used. The supernatant was used for the characterization and recovery of the residual soluble proteins.

### 2.5.1 Treatment of biomass to reduce the nucleic acid content

Yeast biomass of mono or mixed culture was chemically treated to reduce the nucleic acid content. A N-LS solution (1.5% w/v) at 25 $^{o}$C for 20 min at the ratio of one g biomass wet wt/mL of N-LS solution (the conditions were optimized in one of our previous study) followed by a treatment with 2.5 mL of different concentrations of $NH_4OH$ (1.25, 2.5, 3.75 and 5% v/v) at 65 $^{o}$C for 30 min were used. Firstly, biomass was treated with N-LS and centrifuged. The pellet and supernatant were collected. The pellet was again treated with $NH_4OH$ and the supernatant was collected. The level of released nucleic acids in the pooled supernatants were measured and compared with the level of total nucleic acids of untreated biomass (control).

### 2.5.2 Electrophoresis of native whey and fermented whey proteins

The nature of the residual soluble proteins in the yeast fermented whey supernatants (mono and mixed culture) were evaluated using electrophoresis in comparison with the native whey powder proteins. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the protocol of Laemmli (1970). Acrylamide/bis-acrylamide (10%) gel was used as resolving gel whereas acrylamide/bis-acrylamide (5%) gel was used as stacking gel. Samples were prepared with sample loading buffer and heated for 3 min at 100 $^{o}$C before loading. One lane was also loaded with molecular weight (MW) markers (Precision plus protein standard, Bio-Rad, USA) to evaluate the MW distribution of the proteins. After loading the samples, gel electrophoresis was conducted in a VWR electrophoresis unit and run at constant voltage (120 V) for 6 h. Then the gel was washed with water and stained with

Coomassie Blue solution for 45 min followed by destaining overnight in the destaining solution.

### 2.5.3 Ultrafiltration

#### Principle of operation and filter cleaning

A tangential flow filtration unit (PREP/SCALE-TFF, Cartridges Millipore) with a recirculation system was used for ultrafiltration. Pressure was applied by a pump (Casy Load, Master Flex, Millipore) to force a portion of the fluid through the membrane to the permeate side. The sample to be filtered was brought to room temperature (25 $^{o}$C) before start of the ultrafiltration study. The process consisted of feeding a volume of the sample (referred as "feed") through the membrane in order to concentrate the proteins (the concentrated volume is referred as "retentate"). The flow of the sample was controlled by means of a pump to obtain the desired flow and permeate flux. The flow of permeate, generally, depends on the transmembrane pressure (TMP) and the resistance of the membrane. After ultrafiltration, the permeate and retentate were collected and they were used for measurement of their respective volume and protein concentration.

After each ultrafiltration operation, the liquid in the membrane was completely drained off and the membrane was washed with water until complete cleaning before the next operation. Afterward, the membrane was first cleaned with water, followed by washing with an alkaline solution (0.1 N NaOH). The alkaline solution was passed through the membrane until the membrane was clean.

#### Membrane size

Ultrafiltration membranes for tangential flow filtration (TFF) or cross flow filtration (CFF) with molecular weight cutoffs (MWCO) of 1 kDa and 10 kDa were used in the present study (Millipore, prep/scale spiral wound TFF). The membranes were made up of regenerated cellulose and was of the spiral wound TFF module PLCC with a surface area of 0.093 $m^2$ (1 kDa) and 0.557 $m^2$ (10 kDa). The other details and characteristics of the membranes are presented in Table 1.

#### Optimization of ultrafiltration parameters

Permeate flux and transmembrane pressure (TMP) are the two important operational parameters to be controlled during ultrafiltration.

The permeate flux is determined with pure water at the beginning of the experiment using equation (1):

$$J = \frac{1}{A} \cdot \frac{dV}{dt} \qquad (1)$$

Where *J* is the permeate flux (L/h/m$^2$ or LMH), *A* the surface area of the membrane (m$^2$), *V* the permeate volume and *t* is the unit time (h). For optimization of the permeate flux, the experiment was carried out of various values of permeate flux of 267-505 LMH for the 10 kDa and permeate flux of 1345-3261 LMH for the 1 kDa membrane, respectively.

The transmembrane pressure, the pressure exerted on the membrane surface is calculated using equation (2):

$$\mathrm{TMP} = \frac{[P_{feed} + P_{ret}]}{2} - P_{perm} \qquad (2)$$

Where $P_{feed}$ and $P_{ret}$ are the feed and retentate pressure, respectively, and $P_{perm}$ is the permeate pressure, which is equal to atmospheric pressure. To optimize the TMP, TMP values between 60 and 90 kPa for the 10 kDa membrane and TMP values between 150 and 250 kPa for the 1 kDa membrane were tested. Samples were withdrawn to determine the soluble proteins and total solids in the retentate and permeate.

## 2.6 Analytical

The soluble protein concentration was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. The reducing sugar concentration was determined by the dinitro-salicylic acid (DNS) method of Miller (1959). Total nucleic acids content were estimated by a spectrophotometric method (Zachleder, 1984). COD, total solids and suspended solids were measured by standard APHA methods (2005). The total cell count (colony forming unit-CFU) was quantified by the standard agar plate method. All estimates were carried out in triplicate and the average value of each parameter was presented (with standard error less than 5% of the mean).

# 3. RESULTS AND DISCUSSION

## 3.1 Biomass production and nucleic acid removal

### 3.1.1 Fermentation profiles, yields and COD removal efficiency of mono and mixed culture

The online fermentation profiles during mono- (*K. marxianus*) and mixed cultures (*K. marxianus* and *S. cerevisiae*) are presented in Fig. 1 (a and b). The online profiles show the variations in air flow rate, agitation and DO during fermentation while pH and temperature remained constant. DO started decreasing from the beginning. It was then maintained above 25% (critical DO) by increasing the agitation and/or aeration. The high demand of oxygen between 7 and 18 h indicates that the yeast started growing in exponential phase. After 22 h, the demand for the oxygen had been decreased. The decrease in oxygen demand indicated that the main substrate (lactose) had been utilized and that the stationary growth phase had started.

The yield coefficient and important parameter values in both cases after 30 h of fermentation are presented in Table 2. After 30 h of fermentation, lactose had been consumed in both the fermentations. The residual components in the fermented whey supernatant were soluble proteins and intermediate metabolites, which were responsible for the residual COD. The fermentation yields were comparable to our previously reported study on mono- and mixed culture biomass (SCP) production from cheese whey (Yadav et al., 2014b).

### 3.1.2 Nucleic acid removal from mono and mixed culture biomass

A combined two-step treatment, i.e. treatment with a non-toxic detergent (N-LS) followed by a $NH_4OH$ treatment was carried out. The purpose of the using a non-toxic detergent before the $NH_4OH$ treatment was to release the cytoplasmic nucleotides into the N-LS solution, which could be recovered separately and used as flavouring agents. Moreover, the initial treatment with detergent makes the cells fragile, leading to enhanced $NH_4OH$ treatment. Different concentrations of $NH_4OH$ (1.25, 2.5, 3.75 and 5.0% v/v) was used and optimal concentration found was 3.75% v/v $NH_4OH$ (13.7 mL of diluted $NH_4OH$/g dry cell wt). In the first step, treatment with N-LS (1.5% w/v) resulted in a decrease of 1.8% (w/w) nucleic acids in the form of nucleotides from an initial nucleic acids content of 11.4% (w/w) in the biomass. Further, in the second step treatment with $NH_4OH$ resulted in a total decrease (N-LS + $NH_4OH$ treatment) of 9.6% of the initial. On the other hand, when the same concentration (3.75% v/v) of $NH_4OH$ was used alone, the total decrease of nucleic acid was only 6.0% of the initial. Thus, the combined treatment of detergent and $NH_4OH$ showed a synergistic effect

for nucleic acid release. During the two step treatment, the overall protein loss was approximately 18% w/w. The final protein content of the biomass after treatment was found to be approximately 49% w/w. Parajó et al. (1995) have used $NH_4OH$ to reduce nucleic acid content and reported an optimal concentration of 4.5% v/v (13.2 mL of diluted $NH_4OH$/g dry cell wt) in order to reduce the nucleic acid content below 2% w/w from *Candida utilis*. Thus, the present study shows better results due to use of N-LS as compared to those previously reported and attributed to a lower requirement for $NH_4OH$. In the present study the amount of $NH_4OH$ required to treat 1 kg dry biomass is approximately 513 mL of concentrated $NH_4OH$ (14.5 N) whereas the literature value (Parajó et al., 1995) will be 594 mL of concentrated $NH_4OH$. The lower requirement for $NH_4OH$ will be helpful to eliminate the formation of toxic compound (lysinoalanine), which depends on the concentration of alkali, temperature and time during the treatment process (Pomeranz, 1991). Thus, this is a novel approach to obtain a food-grade SCP. Moreover, an increase in the digestibility of *Candida utilis* biomass after treatment with $NH_4OH$ has been reported (Parajó et al., 1995).

## 3.2 Characterization of the fermented whey proteins

Electrophoresis was carried out to fractionate the proteins of whey powder (native whey proteins) and residual soluble fermented whey supernatant (FWS) proteins and the results are presented in Fig. 2. Results demonstrated that the native whey proteins showed a wider range of molecular mass (MW) distributions (lanes 5 and 6). Although, the whey proteins are not clearly differentiated, their molecular masses can be approximated based on the MW markers (lane 7). The dominant native whey proteins are immunoglobulins (MW 150-1000 kDa), lactoferrin (MW 76 kDa), bovine serum albumin (MW 66 kDa), β-lactoglobulin (monomer 18 kDa and dimer 36 kDa), α-lactalbumin (MW 14 kDa) and glycomacropeptide (MW 6.8 kDa) (Tovar Jiménez et al., 2012). The nature of the native whey proteins were modified during fermentation and mostly converted into lower MW proteins. Moreover, the light bands of the fermented whey protein indicate that the dominated whey proteins β-lactoglobulin and α-lactalbumin has also been partially hydrolyzed and consumed during fermentation. Thus, the electrophoresis results confirmed that the whey proteins were partially hydrolyzed during fermentation to gave lower MW proteins. Proteolysis of goat whey proteins has been reported by *K. marxianus* and *Lactobacillus* species (Hamme et al., 2009). Moreover, Yadav et al. (2014a) also reported on whey protein hydrolysis during liquid cheese whey fermentation by *K. marxianus*. This fermentation study was conducted with whey powder solution (i.e. reconstituted whey), which was obtained by spray drying. However, the proteolysis pattern observed in the previous study (Yadav et al., 2014a) is almost similar to the one in the present study. Thus, spray drying did not have any impact on the microbial

proteolysis. Additionally, proteolysis of the individual whey protein lactoferrin to produce an antihypertensive bioactive peptides by *K. marxianus* has also been reported by García-Tejedor et al. (2014). Lanes 1 and 2 representing mixed culture fermented whey proteins also revealed almost similar pattern in protein MW distribution. Thus, this study confirmed that both the cultures (i.e. mono and mixed culture) have the capacity to partially hydrolyze and consume the whey protein during fermentation. Didelot et al. (2006) also reported on the proteolysis of whey proteins by *S. cerevisiae* during whey fermentation. Therefore, the recovery of the residual soluble proteins post-fermentation will probably require different strategy of recovery through membrane filtration as the most of the residual fermented whey proteins lies below 25 kDa.

## 3.3 Selection of the ultrafiltration membrane

Based on the molecular mass distribution observed above, two ultrafiltration membranes (1 and 10 kDa MWCO) were chosen to recover the residual soluble proteins post-fermentation. The cellulose membranes were chosen because of their higher antifauling effect compared to polyether-sulphone membranes (Luo et al., 2011). However, the use of the 1 kDa MWCO membrane led to substantial foaming during direct filtration of FWS. Therefore, the first optimization study was conducted using 10 kDa membrane and the permeate from 10 kDa was filtered through 1 kDa membrane to further recover the protein.

### 3.3.1 Permeate flux for the 10 kDa membrane

The recovered protein value and total solids profiles in the retentate with respect to permeate flux at a constant TMP is presented in Fig. 3. Different permeate flux values between 267 LMH and 505 LMH were studied. For each permeate flux value, the retentate pressure was adjusted in order to maintain the same TMP of 70 kPa. The permeate flux of 413 LMH gave the highest values for protein and total solids in the retentate (Fig. 3). The retentate volume was concentrated 9 times volume of the initial value of FWS, which resulted in an increase in soluble protein concentration up to 27.7 g/L, from an initial 4.6 g/L value. The protein and total solids concentrations in the retentate were decreased at highest permeate flux value tested (505 LMH). The generation of turbulence effect might be the reason to decrease the protein and total solids concentration in the retentate at high flux. At higher permeate fluxes proteins might be degraded and products expelled to the permeate side (Millipore, 2003). Furthermore, turbulence can cause severe foaming in the retentate that may create a vacuum and decrease the permeate flux to below the optimum value. Foaming was more

dominant during vibration of the filtration unit at higher permeate fluxes because of the presence of soluble proteins in the medium (Abdel-Ghani, 2000).

### 3.3.2 Transmembrane pressure for the 10 kDa membrane

TMP values in the range of 60 to 90 kPa were tested to identify the optimal value for a fixed permeate flux (413 LMH). The soluble protein and total solids profiles in the retentate are presented in Fig. 4. The highest protein recovery was found at 80 kPa. The retentate was concentrated by 9 times with the protein concentration of 28.6 g/L in the retentate.

A lower or higher value of TMP than 80 kPa resulted in a decrease in the concentration of proteins in the retentate. The reason might be feed pressure, which may not have been sufficient enough to force the solution through the membrane. On the other hand, a loss of protein may have taken place in the tubes or on the surface of the membrane at the highest TMP value. Higher TMP also increases foam formation in the ultrafiltration membranes. Foaming resulted in the loss of protein on the membrane or some fractions were retained in tubes because foam cannot be retained either in the permeate or in the retentate. Clogging of some of the membrane pores by solutes may also be responsible for the generation of extra surfaces for adsorption and caking. Several other factors play a role in the losses through ultrafiltration membranes, such as the molecular mass of the solute components nearer to the MWCO of the membranes i.e. pore size of the membranes. In addition, formation of smaller fragments of solute molecules due to shear forces could also contribute to the losses (Powell and Timperman, 2005).

The optimized permeate flux and TMP resulted in a total protein recovery of 60.9%. Around 1.8 g/L of soluble protein was still found in the permeate. The presence of a high protein concentration in the permeate was linked to the presence of lower MW whey proteins (e.g. glycomacropeptide) and other small peptides (below 10 kDa) created during fermentation from the native whey protein. Most of the previous literature reported 95 to 100% recovery of whey protein employing 10 kDa regenerated cellulosic membranes (Chollangi and Hossain, 2007; Luo et al., 2011). Atra et al. (2005) also reported up to 98% of whey protein recovery employing a polyvinil-difluoride (Zoltek Rt MAVIBRAN) ultrafiltration membrane. This indicates that the different nature of the whey proteins post-fermentation (smaller MW, as it is also clear from electrophoresis result, Fig. 2) resulted in a higher concentration of permeate proteins during ultrafiltration with the 10 kDa membrane. To recover the residual proteins from the permeate, further ultrafiltration through a 1 kDa membrane was evaluated as discussed below.

### 3.2.3 Permeate flux for the 1 kDa membrane

The protein and total solids profiles in the retentate with respect to different permeate fluxes (of permeate from 10 kDa membrane, mono-culture FWS) at a constant TMP value (170 kPa) are presented in Fig. 5. The permeate flux of 2760 LMH gave the highest values of protein and total solids in the retentate (Fig. 5). A higher permeate flux was required to filter the protein through 1 kDa membrane as compared to that for the10 kDa membrane.

### 3.2.4 Transmembrane pressure for the 1 kDa membrane

TMP values in the range of 150 to 250 kPa were tested to find the optimal TMP value. The soluble proteins and total solids profiles in the retentate are presented in Fig. 6. The highest protein concentration was obtained with 210 kPa. The retentate was concentrated 9 times of the initial value of the permeate from the 10 kDa membrane of mono-culture FWS. The filtration at optimum values of permeate flux and TMP resulted in a recovery of 59.4% of the residual proteins from the 10 kDa permeate (mono-culture). The overall total protein recovery with the combination of two membranes in series reached 84% starting from the mono-culture FWS. A similar approach, i.e. combination of ultrafiltration (MWCO of 10 kDa) and nanofiltration (MWCO of 1 kDa) in series has been reported by Butylina et al. (2006) to recover the whey proteins and whey derived peptides from sweet whey. Thus, the two ultrafiltration membranes operated in series can be applied to recover lactose-free and partially hydrolyzed whey proteins.

The filtration process (with 10 and 1 kDa ultrafiltration membranes) at similar optimized conditions (those found for mono-culture FWS) was evaluated to recover protein from the mixed culture fermented whey supernatant. A similar trend of protein recovery was observed. The optimized conditions for the 10 kDa ultrafiltration membrane resulted in 76.4% protein recovery, while the1 kDa ultrafiltration membrane resulted in 67% protein (remaining after 10 kDa filtration) recovery from the permeate of 10 kDa ultrafiltration. The overall protein recovery from the mixed culture supernatant in the combined ultrafiltration membranes (1 and 10 kDa in series) was 92%. Thus, the optimized conditions can be used successfully to recover the protein from the supernatant (by mono or mixed culture) of the fermented whey.

### 3.3.5 COD reduction during the ultrafiltration

The ultrafiltration recovery of proteins resulted in further reduction of residual COD in the fermented whey supernatant. The COD decreased from 11.58 g/L to 3.78 g/L in case of

mono-culture FWS, while in case of mixed culture FWS, it was reduced from 13.92 g/L to 4.15 g/L. The overall COD removal during combined treatment, i.e. fermentation followed by ultrafiltration (the combined process) of FWS resulted in 92.2% and 92% from mono and mixed culture, respectively. In both cases the COD reduction was almost equal, however, the mixed culture SCP will be better in food quality because of the presence of balanced amino acids composition (especially sulfur containing amino acids) (Yadav et al., 2014b). Moreover, this approach of COD removal will be the best alternative to combined processes of ultrafiltration, nanofiltration and reverse osmosis applied for whey treatment, which reduced the COD down to 2.8 g/L (Yorgun et al., 2008). The present approach resulted in the proteinaceous value-added products (food-grade SCP and highly purified whey-derived protein) plus pollution load minimization.

The overall production of food-grade SCP (i.e. SCP with reduced nucleic acid content) and recovery of residual soluble proteins in the native state could be implemented as shown in figure 7. Higher biomass yield (compared to whey permeate fermentation) and highly purified partially hydrolyzed protein could be obtained from the cheese whey biotransformation through the present approach. The ultrafiltrated fermented proteins have purity more than 90%, which is equal to whey protein isolate. Moreover, partially hydrolyzed protein has been reported to possess higher value due to reduced allergenicity and increased digestibility (Bu et al., 2013). Further, studies are needed to evaluate the bioactivity of the recovered proteins. There is a higher probability of the presence of bioactive peptides (e.g. antihypertensive and opioid bioactive peptides) in the second fraction of ultrafiltered proteins. Additionally, economic analysis and a scale-up study is also required for large-scale food-grade SCP production and protein recovery from cheese whey.

## 4. CONCLUSIONS

The food-grade SCP could be obtained from the mono and mixed culture yeast biomass by the combined treatment of detergent and $NH_4OH$ in two steps. The fermentation of cheese whey with pure and mixed culture showed that the whey proteins were partially hydrolyzed and somewhat consumed during fermentation using two approaches (mono and mixed culture). Further, electrophoresis results revealed that the majority of the fermented whey proteins molecular mass was below 25 kDa. The protein recovery from the lactose free fermented whey supernatant through a series of ultrafiltration (in 10 kDa and 1 kDa) process recovered 84% and 92% of residual protein from mono and mixed culture FWS, respectively. The process operation under optimized conditions can recover the residual soluble protein (left after fermentation of cheese whey) with a purity of approximately 90% or higher. The

recovered protein could be used as an alternative to whey protein isolate. Additionally, the whole process potentially reduced the cheese whey COD.

## ACKNOWLEDGEMENTS

Authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grant A4984, RDCPJ379601-08, and Canada Research Chair) for their financial support**.**

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**Table 1. Specification of the membranes used**

| Description | Membrane-I | Membrane-II |
|---|---|---|
| Type | Prep/scale spiral wound TFF | Prep/scale spiral wound TFF |
| Filter type | Ultrafiltration | Ultrafiltration |
| Length (cm) | 15.2 | 39.9 |
| Diameter (cm) | 5.8 | 5.8 |
| Minimum working volume (mL) | 100 | 250 |
| pH range | 2.0- 10.0 | 2.0- 13.0 |
| Filtration area ($m^2$) | 0.093 | 0.557 |
| Maximum operating temp. (◦C) | 80 | 80 |
| Filter material | Regenerated cellulose | Regenerated cellulose |
| Maximum inlet pressure, bar (psi) | 0- 5.5 (0-80) | 0- 5.5 (0-80) |
| Molecular weight cutoff, (kDa) | 1 | 10 |
| Configuration | Spiral wound cartridge | Spiral wound cartridge |

**Table 2. Yields, productivities and process efficiencies during mono- and mixed culture after 30 h fermentation**

| S. No | Fermentation profile and yields | Mono-culture | Mixed culture |
|---|---|---|---|
| 1. | *K. marxianus*/ *S. cerevisiae* ratio | -- | 2.73 |
| 2. | Biomass yield (g biomass/g lactose consumed) | 0.27 | 0.31 |
| 3. | Productivity (g/L.h) | 0.29 | 0.33 |
| 4. | Initial protein concentration (g/L) | 6.40 | 8.53 |
| 5. | Residual protein concentration (g/L) | 4.60 | 7.20 |
| 6. | Residual COD (g/L) | 11.58 | 13.92 |
| 7. | % COD removal | 75.60 | 73.30 |

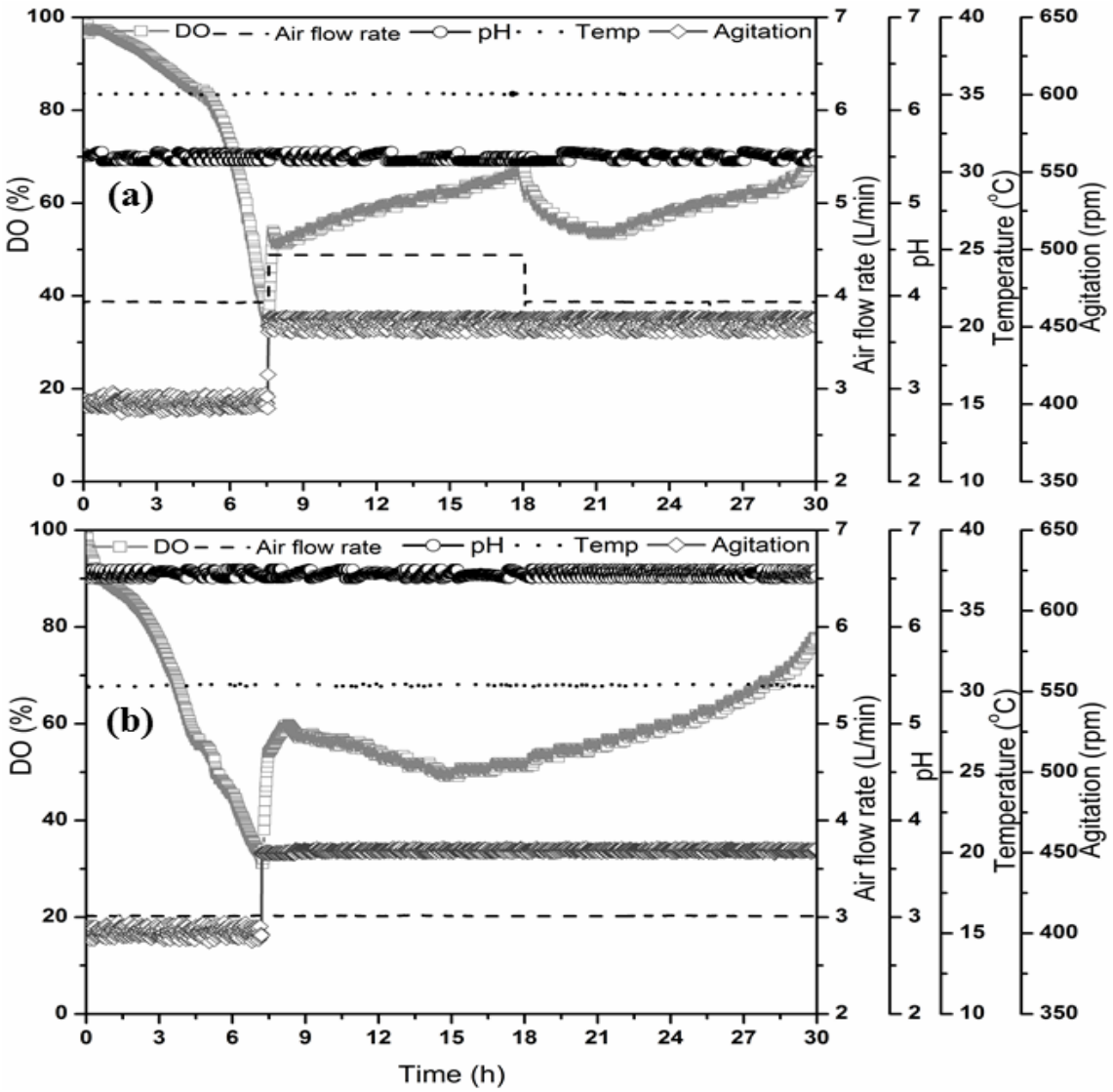


**Figure 1. (a) Online parameters profile during mono-culture batch fermentation (*K. marxianus*); (b) online parameters profile during mixed culture batch fermentation***

*White square (□) with gray solid line denotes dissolved oxygen (% saturation), black (- -) dash line denotes the air flow rate (L/min), white circle (○) black solid line denotes pH, black (…) dot line denotes temperature ($^{o}$C) and hollow star (◊) with a solid line denotes agitation (rpm).

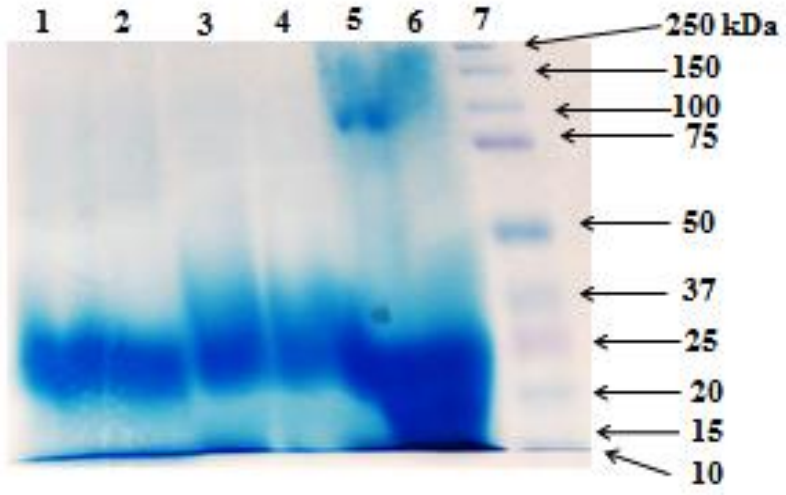


**Figure 2. SDS-PAGE of native whey proteins and of fermented whey supernatant proteins (lanes 1 and 2: mixed culture fermented proteins; lanes 3 and 4: mono-culture fermented whey proteins; lanes 5 and 6 native whey proteins, lane 7: MW marker)**

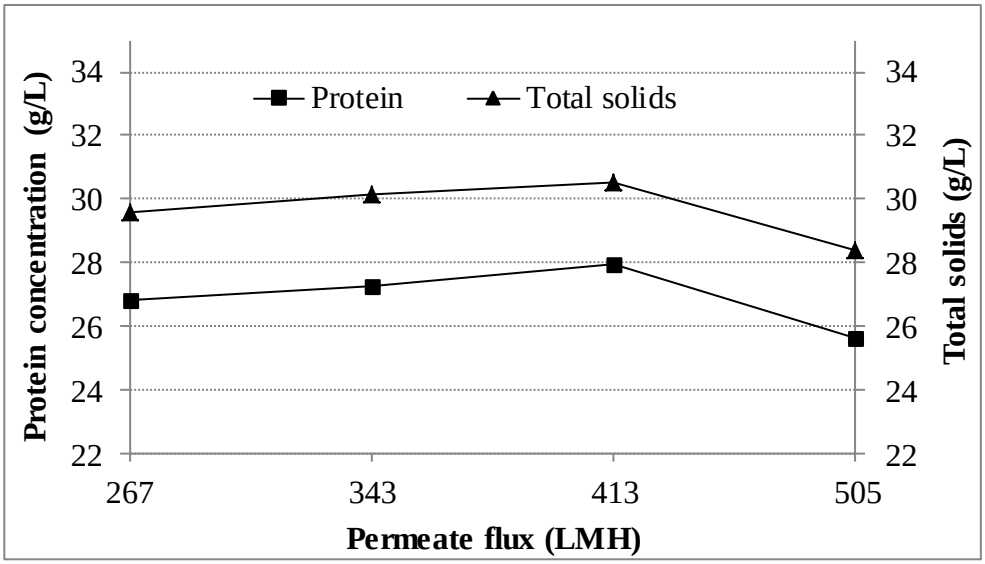


**Figure 3. Variation of protein content in the retentate with the 10 kDa membrane at different permeate flux using mono-culture fermented whey supernatant**

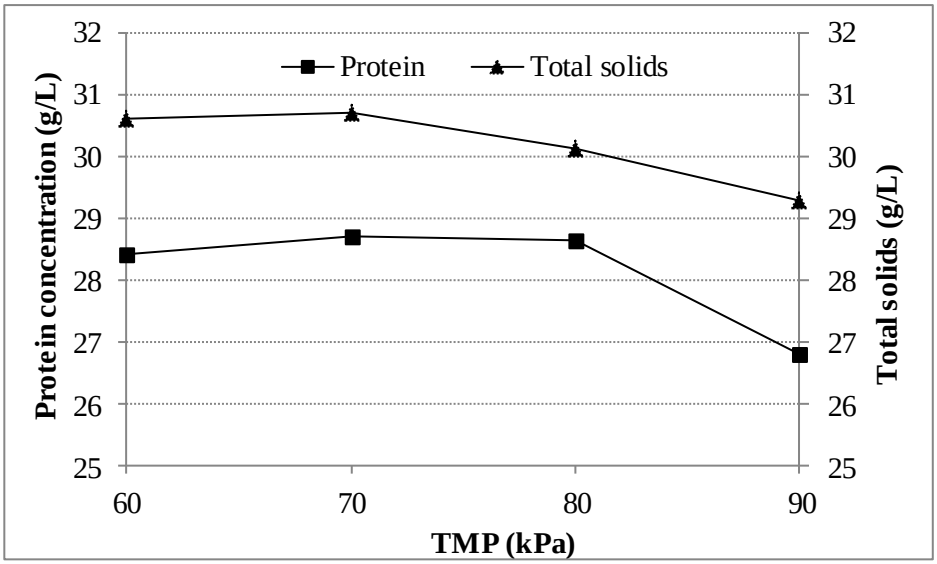


**Figure 4. Variation of protein content in the retentate with the 10 kDa membrane at different TMP values using mono-culture fermented whey supernatant**

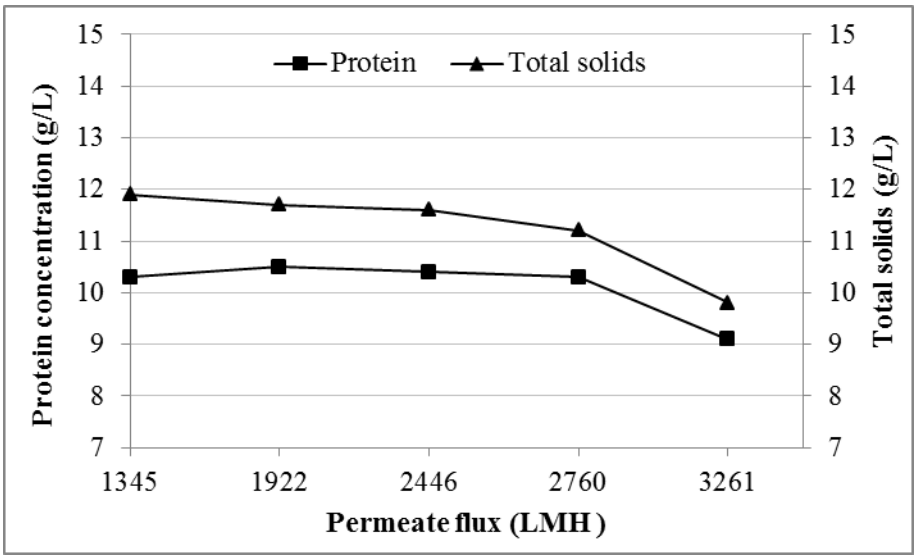


**Figure 5. Variation of protein content in the retentate of 1 kDa membrane at different permeate flux using 10 kDa permeate**

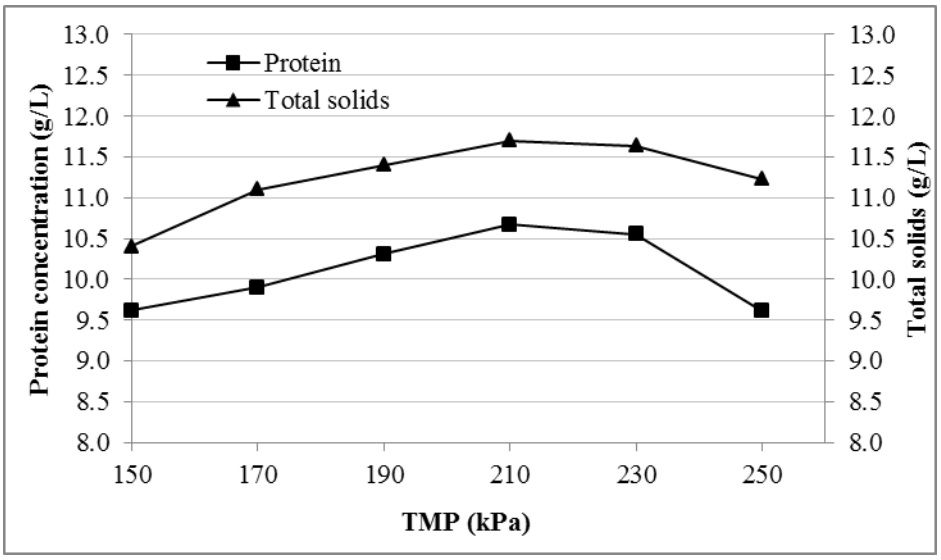


**Figure 6. Variation of protein content in the retentate with the 1 kDa membrane at different TMP values using 10 kDa permeate as feed**

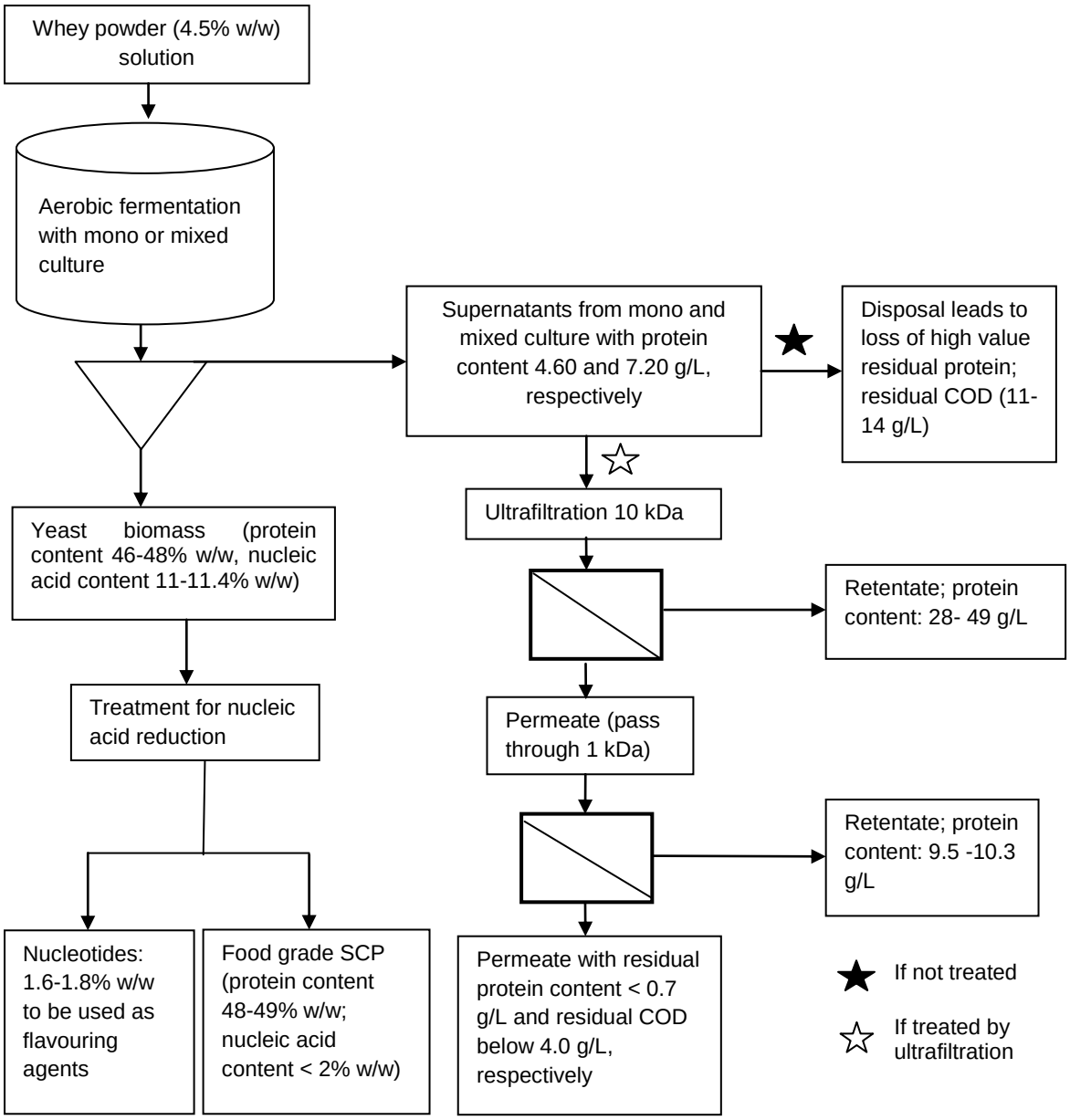


**Figure 7. Flow chart of the combined process for food-grade SCP production and recovery of the residual soluble proteins after cheese whey fermentation**

# CHAPITRE V

# CONCLUSIONS ET RECOMMANDATIONS

# PARTIE 1: CONCLUSIONS

En conclusion, la présente étude a démontré que *K. marxianus* comme PU peut être cultivée à haute température et faible pH, réduisant ainsi les chances de contamination, à partir du lactosérum. De plus, une faible quantité des protéines du lactosérum à été consommée au cours de la fermentation. Cependant, la nature des protéines solubles résiduelles était différente par rapport à celle des protéines naturelles du lactosérum. Les analyses de HPLC et GC-MS effectuées sur le surnageant de lactosérum fermenté ont montré que la présence de métabolites intermédiaires est le facteur responsable de la haute DCO résiduelle. Le taux d'élimination de la DCO élevé et la productivité améliorée de la biomasse ont été obtenus lors de la fermentation avec un inoculum à densité cellulaire élevée. Le meilleur taux de réduction de la DCO et la meilleure productivité de biomasse ont été obtenus au cours de la fermentation en continu avec recyclage des cellules.

La sédimentation du précipité de protéines de lactosérum fermenté due à la gravité a été obtenue à un pH de 3,5 et une température de 100 °C pendant 10 minutes. La sédimentation gravitaire évite la nécessité de la centrifugation pour récupérer les protéines. En outre, les précipités obtenus à un pH de 3,5 sont riches en minéraux et peuvent ainsi être mélangés à la biomasse de levure pour augmenter la teneur minérale finale du produit. La combinaison des deux étapes de précipitation (traitement thermique suivi par l'addition de CMC) a augmenté le taux de récupération de protéines jusqu'à 81%, ainsi que le taux d'élimination de la DCO résiduelle. La teneur en protéines du produit final a été augmentée de 42% (poids/poids) à 49% (poids/poids) en mélangeant le précipité avec la biomasse. En outre, une augmentation de la quantité de produit d'environ 48% a été observée. La DCO a été éliminée à 93% suite à une combinaison des deux types de fermentation suivie d'une précipitation. Par conséquent, la méthode développée et utilisée pour récupérer les protéines résiduelles pourrait être appliquée à l'échelle industrielle (en particulier dans les petites et les moyennes industries) pour produire la PU qui peut être utilisée par la suite dans l'alimentation animale.

La culture mixte de *C. krusei* et *K. marxianus* a donné un rendement plus élevé et un taux d'élimination important de la DCO en comparaison avec la culture pure de *K. marxianus*. Par conséquent, les deux levures ont montré une interaction physiologique entre elles. De plus, la fermentation en continu à 24 h TRH a montré un taux d'élimination élevé de la DCO avec un meilleur rendement et une bonne productivité. Aussi, la PU de la culture mixte est enrichie en lysine. En effet, le procédé de fermentation en continu peut être recommandé dans des conditions non-aseptiques. La récupération des protéines résiduelles à travers

deux étapes de précipitation a amélioré la productivité de 0,17 à 0,31 g/L.h et a augmenté le taux de réduction de la DCO de 80% à 91-93%.

Le détergent non-toxique et biodégradable N-PE peut être utilisé pour perméabiliser les cellules de *K. marxianus* afin d'avoir accés à l'enzyme β-galactosidase intracellulaire. Plus de 90% du lactose du lactosérum (à partir de 50,0 g/L de la solution de poudre de lactosérum) a été hydrolysé pendant seulement 3 heures avec 6,0 g de poids sec de cellules perméabilisées. Le lactosérum hydrolysé avec cellules perméabilisées peut être utilisé efficacement pour développer une culture mixte de levures qui consomme le lactose (*K. marxianus*) et d'autres qui ne consomment pas le lactose (*S. cerevisiae*).

Pour conclure, la PU de qualité alimentaire peut être obtenue par le traitement combiné de détergent et de $NH_4OH$ à partir de la biomasse de levure avec une libération de nucléotides (comme agent aromatisant). De plus, les protéines de lactosérum ont été partiellement hydrolysées et consommées au cours de la fermentation dans les deux cas (culture pure et culture mixte). Aussi, les résultats de SDS-PAGE ont révélé que la majorité de la masse moléculaire de la protéine de lactosérum fermenté est inférieure à 25 kDa. Les protéines de lactosérum fermenté peuvent être récupérées par l'intermédiaire d'un système d'ultrafiltration combiné (membranes de 10 kDa et 1 kDa) avec presque 90% de pureté des protéines.

Sur la base de la présente étude, trois processus différents ont été développés, ce qui pourrait potentiellement être appliquées à biotransformer le lactosérum cru en produits protéiques. Le premier processus est la fermentation en continu (à haute température et un pH bas) avec recyclage des cellules, suivie de la récupération des protéines soluble résiduelle par précipitation. Le processus intégré (p. ex., production de PU, suivie de la récupération de protéines résiduelles) a donné lieu à une productivité plus élevée et plus de DCO d'élimination. Le produit (alimentation animale) a une teneur en protéines élevée et équilibrée en acides aminés essentiels. Le second processus est la fermentation en mode continu dans des conditions non aseptiques employant la culture mixte (*K. marxianus* et *C. krusei*), qui a abouti à plus efficacité d'élimination de DCO avec un rendement amélioré de la biomasse. Le PU produit (de l'alimentation animale) a une teneur en lysine plus élevé, qui est fortement preferée pour l'alimentation animale. Le troisième procédé est de produire une qualité alimentaire des PU de culture mixte (*K. marxianus* et *S. cerevisiae*) utilisant les cellules de *K. marxianus* perméabilisées comme source d'enzyme pour hydrolyser le lactose du lactosérum, suivie de la récupération de la protéine résiduelle par ultrafiltration. PU de qualité alimentaire a été obtenues par pré-traitement de la biomasse avec une combinaison

de produits chimiques en deux étapes. Les protéines solubles résiduelles à l'état natif ont été récupérées par ultrafiltration.

## PARTIE 2: RECOMMANDATIONS

Les futures recherches sur la production de PU pour les applications alimentaire du lactosérum devraient tenir compte des recommandations suivantes:

**1.** L'étude à grande échelle est justifiée pour la production de la PU. La PU peut être utilisée dans l'alimentation animale avec une élimination importante de la DCO dans un procédé intégré, c'est-à-dire la production de la biomasse utilisant le système de recyclage des cellules et la récupération résiduelle des protéines solubles avec un procédé de précipitation en deux étapes.

**2.** L'étude à grande échelle est également recommandée pour la production de la PU dans la culture mixte (*K. marxianus et C. krusei*), cette PU pourrait par la suite etré utilisée comme aliments pour les animaux avec une meilleure réduction de la DCO dans des conditions non-aseptiques. En outre, l'accent devrait également être mis sur l'enregistrement de la PU produite de la culture mixte à l'Agence Canadienne d'Inspection des Aliments ou de tout autre organisme de réglementation afin d'approuver *Candida krusei* comme membre d'consortium de PU pouvant être utilisé dans l'alimentation animale.

**3.** La réutilisation de l'eau traitée pour la dilution du lactosérum doit être étudiée pour éviter la dépendance sur l'approvisionnement en eau municipalité.

**4.** Les PU de qualité alimentaire de la culture pure ou mixte ont été obtenues à partir de biomasse, en combinant un prétraitement par détergent + alcali. Cependant, une étude plus approfondie devrait être menée afin de purifier les nucléotides libérés et d'analyser les propriétés fonctionnelles des protéines de la biomasse traitée. Une ultrafiltration pourrait être appliquée pour retrouver la grande pureté de protéines de lactosérum fermenté à l'état natif. Cependant, l'étude à grande échelle pour l'ultrafiltration et l'évaluation de la bio-activité de la protéine de lactosérum fermenté doit d'abord être effectuée.

5. Une analyse économique comparative est de mise pour les trois cas de processus de production de PU et de leur récupération afin de trouver le meilleur procédé conditionnel pour la valorisation du lactosérum.

6. Possibilité de synthèse de prébiotiques (galacto-oligosaccharides) pendant la production de la culture mixte de cellules perméabilisées employant comme source d'enzyme (β-galactosidase). En outre,la possibilité de la production d'autres sous-produits tels que l'alcool phényle éthylique doit être étudiée pour rendre le procédé plus économique et attractif.

7. La précipitation des composés organiques en cours de procédés de précipitation de protéines doit être étudiée pour explorer les mécanismes de base.

8. Etude comparative doit être effectuée sur le profil d'acides aminés essentielles des protéine unicellulaires unique produites à partir de lactosérum et le perméat de lactosérum.

# ANNEXES

# ANNEXE 1

**Raw data**

**Simultaneous single-cell protein production and COD removal with characterization of the residual proteins and of the intermediate metabolites during whey fermentation by *K. marxianus* (Chapter 2, part 2)**

**Figure 2. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during fermentation**

| Time (h) | CFU/mL | Biomass (g/L) |
|---|---|---|
| 0 | 7.40E+06 | 2.00 |
| 6 | 1.21E+07 | 3.20 |
| 12 | 6.70E+07 | 5.10 |
| 18 | 2.82E+08 | 5.80 |
| 24 | 4.20E+08 | 6.00 |
| 30 | 4.60E+08 | 5.90 |
| 36 | 4.60E+08 | 5.83 |

**Figure 2. (b) Variation in lactose, COD and protein concentration during fermentation**

| Time (h) | Lactose (g/L) | COD (g/L) | Protein (g/L) |
|---|---|---|---|
| 0 | 32.40 | 48.85 | 5.46 |
| 6 | 25.80 | 39.52 | 4.25 |
| 12 | 12.90 | 34.85 | 4.01 |
| 18 | 1.57 | 30.68 | 4.30 |
| 24 | 0.87 | 28.28 | 4.20 |
| 30 | 0.78 | 26.41 | 4.30 |
| 36 | 0.75 | 21.84 | 4.10 |

**Figure 7. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during fermentation with high cell density inoculum**

| Time (h) | CFU/mL | Biomass (g/L) |
|---|---|---|
| 0 | 1.03E+09 | 10.34 |
| 6 | 1.24E+09 | 13.67 |
| 12 | 1.70E+09 | 17.08 |
| 18 | 1.68E+09 | 16.72 |
| 24 | 1.66E+09 | 16.55 |
| 30 | 1.61E+09 | 16.32 |
| 36 | 1.57E+09 | 15.90 |

**Figure 7. (b) Variation in lactose, COD and protein concentration during fermentation with high cell density inoculum**

| Time (h) | COD (g/L) | Lactose (g/L) | Protein (g/L) |
|---|---|---|---|
| 0 | 49.29 | 32.40 | 5.64 |
| 6 | 39.21 | 23.53 | 5.02 |
| 12 | 26.35 | 10.14 | 5.47 |
| 18 | 13.14 | 0.76 | 5.08 |
| 24 | 11.06 | 0.75 | 5.27 |
| 30 | 9.87 | 0.75 | 4.81 |
| 36 | 9.56 | 0.76 | 4.51 |

**Figure 8. (a) Variation in viable cell concentration (CFU/mL) and biomass with respect to time during batch and continuous fermentation**

| Fermentation time (h) | CFU/mL | Biomass (g/L) |
|---|---|---|
| Batch 0 | 6.20E+08 | 7.25 |
| Batch 4 | 1.28E+09 | 9.81 |
| Batch 8 | 1.39E+09 | 11.45 |
| Batch 12 | 1.46E+09 | 12.66 |
| Batch 16 | 1.50E+09 | 12.84 |
| Batch 20 | 1.47E+09 | 12.64 |
| Batch 22 | 1.44E+09 | 12.39 |
| 24 HRT 0 | 1.42E+09 | 12.36 |
| 24 HRT 6 | 1.44E+09 | 12.50 |
| 24 HRT 12 | 1.45E+09 | 12.67 |
| 24 HRT 18 | 1.43E+09 | 12.47 |
| 24 HRT 24 | 1.45E+09 | 12.53 |
| 24 HRT 30 | 1.44E+09 | 12.37 |
| 24 HRT 36 | 1.48E+09 | 12.67 |
| 24 HRT 42 | 1.45E+09 | 12.40 |
| 24 HRT 48 | 1.45E+09 | 12.44 |
| 24 HRT 54 | 1.47E+09 | 12.51 |
| 24 HRT 60 | 1.45E+09 | 12.28 |
| 24 HRT 66 | 1.46E+09 | 12.43 |
| 24 HRT 72 | 1.47E+09 | 12.50 |
| 24 HRT 78 | 1.45E+09 | 12.47 |
| 24 HRT 84 | 1.43E+09 | 12.36 |
| 24 HRT 90 | 1.45E+09 | 12.53 |
| 24 HRT 96 | 1.46E+09 | 12.61 |
| 24 HRT 102 | 1.45E+09 | 12.48 |

| | | |
|---|---|---|
| 24 HRT 108 | 1.42E+09 | 12.24 |
| 24 HRT 114 | 1.45E+09 | 12.39 |
| 24 HRT 120 | 1.44E+09 | 12.47 |
| 24 HRT 126 | 1.45E+09 | 12.38 |
| 24 HRT 132 | 1.46E+09 | 12.54 |
| 24 HRT 138 | 1.45E+09 | 12.56 |
| 24 HRT 144 | 1.47E+09 | 12.64 |
| 24 HRT 150 | 1.44E+09 | 12.35 |
| 24 HRT 156 | 1.43E+09 | 12.42 |
| 24 HRT 162 | 1.46E+09 | 12.73 |
| 24 HRT 168 | 1.46E+09 | 12.61 |
| 24 HRT 174 | 1.45E+09 | 12.55 |
| 24 HRT 180 | 1.43E+09 | 12.37 |
| 24 HRT 186 | 1.46E+09 | 12.67 |
| 24 HRT 192 | 1.42E+09 | 12.42 |
| 25 HRT 198 | 1.48E+09 | 12.77 |
| 26 HRT 204 | 1.49E+09 | 12.87 |
| 27 HRT 210 | 1.46E+09 | 12.67 |
| 28 HRT 216 | 1.42E+09 | 12.36 |
| 29 HRT 222 | 1.43E+09 | 12.53 |
| 30 HRT 228 | 1.42E+09 | 12.48 |
| 31 HRT 234 | 1.40E+09 | 12.25 |
| 32 HRT 240 | 1.44E+09 | 12.53 |
| 33 HRT 246 | 1.42E+09 | 12.34 |
| 34 HRT 252 | 1.47E+09 | 12.87 |
| 35 HRT 258 | 1.46E+09 | 12.77 |
| 36 HRT 264 | 1.49E+09 | 12.94 |
| 37 HRT 270 | 1.48E+09 | 12.87 |
| 38 HRT 276 | 1.45E+09 | 12.53 |
| 39 HRT 282 | 1.46E+09 | 12.77 |
| 40 HRT 288 | 1.47E+09 | 12.52 |
| 41 HRT 294 | 1.42E+09 | 12.34 |
| 42 HRT 200 | 1.42E+09 | 12.28 |

**Figure 8. (b) Variation in lactose, COD and protein concentration during batch and continuous fermentation (with cell recycle)**

| Fermentation time (h) | Lactose (g/L) | COD (g/L) | Protein (g/L) |
|---|---|---|---|
| Batch 0 | 32.40 | 49.86 | 5.67 |
| Batch 4 | 28.80 | 41.21 | 5.30 |
| Batch 8 | 22.30 | 35.35 | 5.02 |
| Batch 12 | 15.30 | 28.16 | 4.50 |
| Batch 16 | 7.40 | 21.01 | 4.30 |
| Batch 20 | 1.30 | 16.65 | 4.20 |
| Batch 22 | 0.68 | 14.80 | 4.23 |
| 24 HRT 0 | 4.52 | 13.87 | 4.42 |
| 24 HRT 6 | 2.57 | 13.23 | 4.16 |
| 24 HRT 12 | 2.15 | 12.42 | 4.10 |
| 24 HRT 18 | 1.72 | 12.73 | 4.05 |
| 24 HRT 24 | 1.49 | 12.30 | 4.02 |
| 24 HRT 30 | 1.25 | 11.40 | 4.00 |
| 24 HRT 36 | 1.12 | 11.12 | 3.95 |
| 24 HRT 42 | 1.08 | 11.20 | 3.93 |
| 24 HRT 48 | 1.02 | 10.98 | 3.96 |
| 24 HRT 54 | 0.89 | 10.74 | 3.85 |
| 24 HRT 60 | 0.96 | 10.68 | 3.98 |
| 24 HRT 66 | 0.82 | 10.70 | 3.97 |
| 24 HRT 72 | 0.82 | 10.58 | 4.08 |
| 24 HRT 78 | 0.75 | 10.75 | 3.98 |

# ANNEXE 2

**Raw data**

**Recovery of residual soluble proteins by a two-step precipitation process with concomitant COD reduction from the yeast cultivated cheese whey (Chapter 2, part 3)**

**Figure 2. (a) Effect of temperature on precipitation of residual proteins in the FWS at pH 3.5 and treatment time 15 min**

| Temperature ($^{o}$C) | % of protein precipitated |
|---|---|
| 80 | 2.5±0.11 |
| 90 | 39.0±1.30 |
| 95 | 44.0±1.41 |
| 100 | 46.3±1.04 |

**Figure 2. (b) Effect of different pH on precipitation of proteins from FWS at 100 $^{o}$C for 15 min**

| pH | % of protein precipitated |
|---|---|
| 3.0 | 46.5± 1.6 |
| 3.5 | 47.0±1.40 |
| 4.0 | 52.8±1.42 |
| 4.5 | 53.3±1.76 |
| 5.0 | 49.0±1.27 |
| 5.5 | 46.0± 1.87 |

**Figure 3. (a) Effect of different incubation times on proteins precipitation and COD removal at 100 $^{o}$C and pH 3.5**

| Treatment time (min) | % of protein precipitated | % of residual COD |
|---|---|---|
| 5 | 46.8±1.45 | 47.0±2.02 |
| 10 | 47.0±1.60 | 47.3±1.70 |
| 15 | 47.3±0.99 | 47.0±1.46 |
| 20 | 47.5±1.25 | 47.7±2.15 |

**Figure 3. (b) Effect of different incubation times on proteins precipitation and COD removal at pH 4.5**

| Treatment time (min) | % of protein precipitated | % of residual COD |
|---|---|---|
| 5 | 53.0±1.47 | 46.0±1.69 |
| 10 | 53.3±1.70 | 46.2±1.11 |
| 15 | 53.7±1.29 | 46.6±1.79 |
| 20 | 54.0±2.21 | 46.4±2.13 |

**Figure 4. Effect of different pH on precipitation of proteins with 0.50% w/v concentration of CMC**

| pH | % of protein precipitated |
|---|---|
| 3.0 | 0.77±0.02 |
| 3.5 | 4.60±0.09 |
| 4.0 | 5.60±0.19 |
| 4.5 | 25.80±0.70 |
| 5.5 | 28.50±0.97 |
| 6.5 | 24.10±0.67 |

**Figure 5. (a) Heat treatment (at 100 $^{o}$C, pH 3.5 for 10 min) followed by change of pH and addition of CMC (0.25% w/v)**

| pH | % of protein precipitated |
|---|---|
| 3.5 | 36.15±1.01 |
| 5.0 | 39.20±1.25 |
| 5.5 | 41.00±1.07 |
| 6.0 | 35.89±1.24 |
| 6.5 | 23.33±0.69 |

**Figure 5. (b) Heat treatment (at 100 $^{o}$C, pH 3.5 for 10 min) followed by addition of a different concentration of CMC at pH 5.5**

| Conc. of CMC (% w/v) | % of precipitated protein | % of residual COD |
|---|---|---|
| 0.00 | 53.08±0.80 | 47.13±1.46 |
| 0.05 | 64.10±1.99 | 45.54±1.78 |
| 0.10 | 61.54±1.66 | 46.29±1.90 |
| 0.15 | 57.95±2.09 | 46.90±1.36 |
| 0.20 | 52.82±1.43 | 47.37±1.75 |
| 0.25 | 51.03±1.44 | 47.52±2.04 |

**Figure 5. (c) Effect of temperature on precipitation with CMC (0.05% w/v) at pH 5.5 after heat treatment (at pH 3.5, 100 $^{o}$C, for 10 min)**

| Temperature ($^{o}$C) at CMC added | % of precipitated protein | % of residual COD |
|---|---|---|
| 20 | 73.33±1.98 | 48.43±1.99 |
| 35 | 74.36±2.31 | 47.82±1.00 |
| 50 | 76.41±1.60 | 46.14±2.12 |
| 75 | 63.59±2.03 | 49.23±1.87 |

# ANNEXE 3

**Raw data**

**Mixed culture of *Kluyveromyces marxianus* and *Candida krusei* for single-cell protein production and organic load removal from whey (Chapter 3, part 2)**

**Figure 1. (a) Growth profile of *K. marxianus* during batch fermentation in cheese whey**

| Time (h) | *K. marxianus* (CFU/mL) | SS (g/L) |
|---|---|---|
| 0 | 7.00E+06 | 1.94 |
| 4 | 5.20E+07 | 3.43 |
| 8 | 2.10E+08 | 5.39 |
| 12 | 3.30E+08 | 6.13 |
| 16 | 4.10E+08 | 6.48 |
| 20 | 5.10E+08 | 6.62 |
| 24 | 5.14E+08 | 6.57 |
| 30 | 5.10E+08 | 6.46 |

**Figure 1. (b) Variation in substrates profiles during fermentation**

| Time (h) | Lactose (g/L) | COD (g/L) | Soluble protein (g/L) |
|---|---|---|---|
| 0 | 17.70 | 30.64 | 4.45 |
| 4 | 16.04 | 25.56 | 4.13 |
| 8 | 10.71 | 18.92 | 3.37 |
| 12 | 5.20 | 14.28 | 3.04 |
| 16 | 0.53 | 10.01 | 3.18 |
| 20 | 0 | 8.28 | 3.38 |
| 24 | 0 | 7.57 | 3.52 |
| 30 | 0 | 6.75 | 3.35 |

**Figure 2. (a) Growth profile during mixed culture fermentation (*K. marxianus* and *C. krusei*) in cheese whey**

| Time (h) | *K. marxianus* (CFU/mL) | *C. krusei* (CFU/mL) | Total cells (CFU/mL) | SS (g/L) |
|---|---|---|---|---|
| 0 | 5.00E+06 | 3.50E+06 | 8.50E+06 | 1.80 |
| 4 | 8.60E+06 | 4.20E+06 | 1.28E+07 | 2.67 |
| 8 | 3.00E+07 | 7.90E+06 | 3.79E+07 | 3.28 |
| 12 | 1.70E+08 | 2.90E+07 | 1.99E+08 | 5.65 |
| 16 | 3.70E+08 | 4.30E+07 | 4.13E+08 | 7.25 |
| 20 | 4.40E+08 | 5.50E+07 | 4.95E+08 | 7.90 |
| 24 | 4.70E+08 | 5.60E+07 | 5.26E+08 | 8.03 |
| 30 | 4.70E+08 | 5.40E+07 | 5.24E+08 | 7.88 |

**Figure 2. (b) Variation in substrates profiles during mixed culture fermentation**

| Time (h) | Lactose (g/L) | COD (g/L) | Soluble protein (g/L) |
|---|---|---|---|
| 0 | 19.2 | 31.7 | 4.56 |
| 4 | 17.7 | 29.4 | 4.36 |
| 8 | 13.8 | 25.6 | 3.34 |
| 12 | 3.2 | 17.5 | 2.86 |
| 16 | 0.4 | 9.3 | 2.95 |
| 20 | 0 | 6.0 | 3.12 |
| 24 | 0 | 5.1 | 3.24 |
| 30 | 0 | 4.2 | 3.25 |

**Figure 3. (a) Growth profile of mixed culture (*K. marxianus* and *C. krusei*) during continuous fermentation at different HRT (6, 12, 18 and 24 h)**

| Fermentation time (h) | *K. marxianus* (CFU/mL) | *C. krusei* (CFU/mL) | Total cells (CFU/mL) | SS (g/L) |
|---|---|---|---|---|
| 0 | 2.30E+06 | 2.60E+07 | 2.83E+07 | 6.02 |
| 4 | 7.40E+06 | 6.80E+07 | 7.54E+07 | 8.75 |
| 8 | 6.20E+07 | 4.10E+08 | 4.72E+08 | 9.17 |
| 12 | 7.40E+07 | 5.20E+08 | 5.94E+08 | 9.85 |
| 16 | 8.10E+07 | 6.00E+08 | 6.81E+08 | 9.95 |
| 20 | 9.20E+07 | 6.20E+08 | 7.12E+08 | 10.23 |
| 22 | 9.50E+07 | 6.10E+08 | 7.05E+08 | 10.13 |
| 0 | 9.00E+07 | 5.30E+08 | 6.20E+08 | 8.53 |
| 6 | 7.90E+07 | 4.20E+08 | 4.99E+08 | 8.33 |
| 12 | 2.60E+07 | 2.60E+08 | 2.86E+08 | 7.28 |
| 18 | 6.70E+06 | 1.40E+08 | 1.47E+08 | 7.18 |
| 24 | 5.20E+06 | 6.80E+07 | 7.32E+07 | 6.15 |
| 30 | 3.60E+06 | 5.90E+07 | 6.26E+07 | 6.13 |
| 36 | 4.00E+06 | 5.40E+07 | 5.80E+07 | 5.65 |
| 42 | 3.80E+06 | 4.80E+07 | 5.18E+07 | 4.53 |
| 48 | 3.20E+06 | 6.70E+07 | 7.02E+07 | 3.89 |
| 54 | 9.30E+07 | 8.40E+07 | 1.77E+08 | 4.22 |
| 60 | 7.70E+07 | 8.00E+07 | 1.57E+08 | 4.30 |
| 66 | 6.10E+07 | 6.00E+07 | 1.21E+08 | 4.05 |
| 72 | 5.80E+07 | 6.50E+07 | 1.23E+08 | 3.96 |
| 0 | 4.50E+07 | 7.60E+07 | 1.21E+08 | 4.33 |
| 8 | 7.00E+07 | 6.80E+07 | 1.38E+08 | 4.10 |
| 16 | 5.00E+07 | 5.00E+07 | 1.00E+08 | 3.88 |
| 24 | 7.00E+07 | 6.00E+07 | 1.30E+08 | 4.03 |
| 32 | 6.50E+07 | 5.00E+07 | 1.15E+08 | 3.75 |
| 40 | 9.00E+07 | 4.00E+07 | 1.30E+08 | 3.58 |
| 48 | 9.50E+07 | 5.00E+07 | 1.45E+08 | 3.70 |
| 0 | 6.00E+07 | 5.00E+07 | 1.10E+08 | 3.05 |

| | | | | |
|---|---|---|---|---|
| 8 | 5.00E+07 | 6.00E+07 | 1.10E+08 | 3.55 |
| 16 | 4.50E+07 | 6.00E+07 | 1.05E+08 | 3.18 |
| 24 | 4.00E+07 | 5.60E+07 | 9.60E+07 | 3.18 |
| 30 | 4.00E+07 | 5.00E+07 | 9.00E+07 | 3.22 |
| 0 | 1.40E+07 | 1.20E+07 | 2.60E+07 | 3.08 |
| 3 | 1.30E+07 | 7.40E+06 | 2.04E+07 | 3.05 |
| 6 | 1.20E+07 | 3.60E+06 | 1.56E+07 | 2.85 |
| 9 | 9.00E+06 | 3.30E+06 | 1.23E+07 | 2.80 |
| 12 | 9.50E+06 | 3.20E+06 | 1.27E+07 | 2.90 |
| 15 | 9.40E+06 | 3.60E+06 | 1.30E+07 | 2.80 |
| 18 | 9.50E+06 | 4.00E+06 | 1.35E+07 | 2.80 |

**Figure 3. (b) Variation in substrates profiles during continuous fermentation at different HRT (6, 12, 18 and 24 h)**

| Fermentation time (h) | Lactose (g/L) | COD (g/L) | Soluble protein (g/L) |
|---|---|---|---|
| 0 | 17.50 | 29.69 | 3.90 |
| 4 | 15.43 | 24.36 | 3.69 |
| 8 | 11.85 | 18.39 | 3.20 |
| 12 | 9.55 | 14.91 | 3.40 |
| 16 | 5.56 | 13.08 | 3.54 |
| 20 | 1.15 | 9.35 | 3.60 |
| 22 | 1.10 | 9.25 | 3.10 |
| 0 | 3.25 | 8.81 | 3.65 |
| 6 | 1.65 | 6.35 | 3.03 |
| 12 | 1.80 | 7.29 | 3.11 |
| 18 | 1.70 | 5.31 | 3.18 |
| 24 | 1.42 | 6.93 | 2.83 |
| 30 | 1.15 | 7.34 | 2.68 |
| 36 | 1.05 | 7.80 | 2.71 |
| 42 | 1.47 | 8.91 | 2.47 |
| 48 | 3.35 | 8.48 | 2.41 |

| | | | |
|---|---|---|---|
| 54 | 1.92 | 6.47 | 2.60 |
| 60 | 1.86 | 5.80 | 2.40 |
| 66 | 1.84 | 5.69 | 2.31 |
| 72 | 1.68 | 6.31 | 2.30 |
| 0 | 2.93 | 7.64 | 2.71 |
| 8 | 1.68 | 6.94 | 2.46 |
| 16 | 1.72 | 6.35 | 3.38 |
| 24 | 1.70 | 5.98 | 2.40 |
| 32 | 1.60 | 8.07 | 3.02 |
| 40 | 1.40 | 7.85 | 2.87 |
| 48 | 1.50 | 9.15 | 2.35 |
| 0 | 3.40 | 13.35 | 2.74 |
| 8 | 3.47 | 14.42 | 2.76 |
| 16 | 4.25 | 14.93 | 3.15 |
| 24 | 6.60 | 16.17 | 3.50 |
| 30 | 6.60 | 16.24 | 2.86 |
| 0 | 8.77 | 16.69 | 2.84 |
| 3 | 9.27 | 19.02 | 3.71 |
| 6 | 11.02 | 21.40 | 2.64 |
| 9 | 11.56 | 21.40 | 2.81 |
| 12 | 11.97 | 21.48 | 2.99 |
| 15 | 11.92 | 22.58 | 2.53 |
| 18 | 11.83 | 22.62 | 2.70 |

# ANNEXE 4

**Raw data**

## Permeabilization of *Kluyveromyces marxianus* with mild detergent for whey lactose hydrolysis and augmentation of mixed culture (Chapter 4, part 1)

**Figure 1. Effect of N-LS concentration on permeabilization of *K. marxianus* cells**

| NLS concentration (% w/v) | Enzyme activity (IU)/g dry cell wt. |
|---|---|
| 1.0 | 934 |
| 1.5 | 1010 |
| 2.0 | 991 |
| 2.5 | 973 |
| 3.0 | 971 |
| 3.5 | 960 |

**Figure 2. Effect of solvent (water) volume on permeabilization of *K. marxianus* cells**

| Volume of solvent with 1 mL NLS | Enzyme activity (IU)/ g dry cell wt. |
|---|---|
| 1.0 | 1050 |
| 2.0 | 973 |
| 3.0 | 810 |
| 4.0 | 776 |
| 5.0 | 541 |
| 6.0 | 466 |

**Figure 3. Effect of incubation temperature on permeabilization of *K. marxianus* cells**

| Incubation temperature ($^{o}$C) | Enzyme activity (IU)/ g dry cell wt. |
|---|---|
| 20 | 1150 |
| 25 | 1220 |
| 30 | 1015 |
| 35 | 849 |
| 40 | 820 |
| 45 | 776 |

**Figure 4. Effect of incubation time on permeabilization of *K. marxianus* cells**

| Incubation time (min) | Enzyme activity (IU)/g dry cell wt. |
|---|---|
| 10 | 1000 |
| 15 | 1150 |
| 20 | 1230 |
| 25 | 1140 |
| 30 | 995 |
| 45 | 980 |
| 60 | 950 |

**Figure 6. Hydrolysis of whey lactose by permeabilized yeast cells with different dry cells wt loads and at different incubation times**

| Time (min) | Hydrolysis (%) with 150 mg | Hydrolysis (%) with 300 mg | Hydrolysis (%) with 450 mg | Hydrolysis (%) with 600 mg | Hydrolysis (%) with 675 mg |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 19 | 31 | 35 | 42 | 44 |
| 60 | 29 | 42 | 55 | 58 | 64 |
| 90 | 35 | 50 | 61 | 76 | 79 |
| 120 | 42 | 58 | 66 | 83 | 86 |
| 150 | 46 | 64 | 78 | 88 | 90 |
| 180 | 51 | 68 | 84 | 91 | 92 |

**Figure 7. Cells count, biomass and substrate profiles during the production of mixed culture SCP in hydrolyzed whey lactose**

| Time (h) | *K. marxianus* (CFU/mL) | *S. cerevisiae* (CFU/mL) | Reducing sugar (g/L) | SS (g/L) | Soluble protein (g/L) |
|---|---|---|---|---|---|
| 0 | 1.90E+06 | 3.50E+06 | 32.80 | 2.30 | 8.18 |
| 6 | 1.30E+07 | 7.60E+06 | 23.87 | 3.76 | 8.12 |
| 12 | 3.10E+08 | 8.50E+07 | 11.24 | 5.13 | 7.98 |
| 18 | 9.40E+08 | 6.40E+08 | 3.27 | 8.26 | 7.88 |
| 24 | 1.12E+09 | 8.70E+08 | 1.12 | 9.20 | 7.82 |

# ANNEXE 5

**Raw data**

## Food-grade single-cell protein production, characterization and ultrafiltration recovery of residual fermented whey proteins from whey (Chapter 4, part 2)

**Figure 1. (a) Online parameters profile during mono-culture (*K. marxianus*) batch fermentation**

| Time (h) | DO (%) | Agitation (rpm) | pH | Temp. ($^{o}$C) | Air flowrate (L/min) |
|---|---|---|---|---|---|
| 0.00 | 99.57 | 400.00 | 5.52 | 35.09 | 3.90 |
| 0.51 | 97.22 | 400.17 | 5.54 | 35.02 | 3.94 |
| 1.00 | 96.87 | 401.04 | 5.46 | 35.00 | 3.94 |
| 1.50 | 95.27 | 402.79 | 5.50 | 35.02 | 3.94 |
| 2.00 | 94.05 | 399.87 | 5.50 | 35.06 | 3.94 |
| 2.50 | 92.40 | 399.29 | 5.46 | 35.02 | 3.94 |
| 3.01 | 90.40 | 397.83 | 5.55 | 35.09 | 3.94 |
| 3.50 | 88.55 | 401.62 | 5.50 | 35.02 | 3.94 |
| 4.01 | 86.77 | 400.46 | 5.54 | 35.06 | 3.94 |
| 4.50 | 84.75 | 401.33 | 5.45 | 35.02 | 3.94 |
| 5.00 | 83.35 | 397.83 | 5.53 | 35.00 | 3.93 |
| 5.51 | 79.30 | 398.71 | 5.54 | 35.00 | 3.93 |
| 6.00 | 72.95 | 401.62 | 5.46 | 35.04 | 3.94 |
| 6.51 | 62.85 | 398.42 | 5.53 | 35.08 | 3.93 |
| 7.01 | 49.45 | 403.08 | 5.45 | 35.03 | 3.93 |
| 7.51 | 35.70 | 404.83 | 5.45 | 35.09 | 3.94 |
| 8.01 | 51.73 | 455.58 | 5.53 | 35.03 | 4.44 |
| 8.50 | 52.65 | 454.12 | 5.50 | 35.07 | 4.44 |
| 9.01 | 53.92 | 446.54 | 5.54 | 35.03 | 4.44 |
| 9.50 | 54.60 | 455.50 | 5.45 | 35.03 | 4.44 |
| 10.01 | 55.02 | 455.33 | 5.50 | 35.03 | 4.44 |
| 10.50 | 57.00 | 455.29 | 5.46 | 35.03 | 4.44 |
| 11.00 | 57.60 | 455.67 | 5.55 | 35.09 | 4.44 |
| 11.50 | 58.15 | 447.12 | 5.50 | 35.09 | 4.44 |
| 12.00 | 58.37 | 456.17 | 5.54 | 35.09 | 4.44 |
| 12.51 | 59.95 | 456.17 | 5.45 | 35.09 | 4.44 |
| 13.00 | 60.70 | 455.50 | 5.45 | 35.09 | 4.44 |
| 13.51 | 60.87 | 455.00 | 5.46 | 35.05 | 4.44 |
| 14.01 | 61.32 | 453.83 | 5.46 | 35.05 | 4.44 |
| 14.50 | 62.00 | 450.33 | 5.50 | 35.02 | 4.44 |
| 15.01 | 62.07 | 452.67 | 5.51 | 35.05 | 4.44 |
| 15.50 | 62.22 | 451.21 | 5.46 | 35.05 | 4.44 |
| 16.01 | 63.62 | 450.92 | 5.45 | 35.05 | 4.44 |
| 16.50 | 64.50 | 450.87 | 5.50 | 35.09 | 4.44 |
| 17.01 | 64.17 | 455.67 | 5.46 | 35.02 | 4.44 |
| 17.50 | 66.32 | 451.79 | 5.45 | 35.04 | 4.44 |
| 18.00 | 66.50 | 446.25 | 5.50 | 35.03 | 4.44 |
| 18.51 | 61.50 | 452.37 | 5.45 | 35.04 | 3.94 |

| | | | | | |
|---|---|---|---|---|---|
| 19.00 | 57.58 | 450.87 | 5.45 | 35.02 | 3.94 |
| 19.51 | 56.97 | 446.83 | 5.46 | 35.04 | 3.94 |
| 20.01 | 55.17 | 452.08 | 5.55 | 35.08 | 3.94 |
| 20.50 | 54.45 | 455.75 | 5.54 | 35.09 | 3.94 |
| 21.01 | 53.54 | 453.25 | 5.53 | 35.09 | 3.94 |
| 21.50 | 53.72 | 450.33 | 5.51 | 35.08 | 3.94 |
| 22.01 | 53.97 | 452.96 | 5.45 | 35.08 | 3.94 |
| 22.50 | 55.62 | 453.25 | 5.51 | 35.08 | 3.93 |
| 23.01 | 56.50 | 448.87 | 5.50 | 35.04 | 3.94 |
| 23.51 | 56.42 | 453.83 | 5.53 | 35.08 | 3.93 |
| 24.00 | 57.37 | 450.33 | 5.50 | 35.03 | 3.93 |
| 24.51 | 58.17 | 446.83 | 5.45 | 35.08 | 3.93 |
| 25.00 | 59.52 | 450.29 | 5.52 | 35.00 | 3.93 |
| 25.51 | 59.95 | 455.50 | 5.45 | 35.00 | 3.94 |
| 26.01 | 60.07 | 452.96 | 5.51 | 35.04 | 3.94 |
| 26.51 | 60.30 | 450.29 | 5.45 | 35.00 | 3.94 |
| 27.01 | 61.72 | 455.92 | 5.46 | 35.00 | 3.94 |
| 27.50 | 62.80 | 452.96 | 5.54 | 35.08 | 3.94 |
| 28.01 | 62.30 | 452.67 | 5.50 | 35.00 | 3.94 |
| 28.50 | 63.40 | 455.42 | 5.45 | 35.00 | 3.94 |
| 29.01 | 65.72 | 455.96 | 5.51 | 35.04 | 3.94 |
| 29.50 | 66.70 | 451.79 | 5.45 | 35.00 | 3.94 |
| 29.99 | 70.15 | 455.50 | 5.45 | 35.06 | 3.94 |

**Figure 1. (b) online parameters profile during mixed culture *(K. marxianus & S. cerevisiae)* batch fermentation**

| Time (h) | DO (%) | Agitation (rpm) | pH | Temp. ($^{o}$C) | Air flowrate (L/min) |
|---|---|---|---|---|---|
| 0.00 | 99.05 | 403.08 | 6.56 | 30.42 | 3.01 |
| 0.51 | 91.95 | 399.29 | 6.55 | 30.35 | 3.01 |
| 1.01 | 88.82 | 398.71 | 6.59 | 30.30 | 3.02 |
| 1.50 | 86.87 | 397.54 | 6.50 | 30.30 | 3.01 |
| 2.01 | 84.62 | 404.54 | 6.55 | 30.30 | 3.02 |
| 2.50 | 81.80 | 399.87 | 6.59 | 30.35 | 3.01 |
| 3.01 | 77.27 | 398.12 | 6.58 | 30.30 | 3.01 |
| 3.52 | 71.42 | 401.92 | 6.57 | 30.35 | 3.01 |
| 4.01 | 64.40 | 402.21 | 6.52 | 30.35 | 3.01 |
| 4.51 | 57.13 | 404.54 | 6.56 | 30.43 | 3.01 |
| 5.00 | 54.98 | 405.42 | 6.55 | 30.43 | 3.01 |
| 5.49 | 48.52 | 398.42 | 6.52 | 30.43 | 3.01 |
| 6.00 | 45.53 | 397.54 | 6.51 | 30.30 | 3.01 |
| 6.51 | 39.60 | 400.46 | 6.53 | 30.47 | 3.02 |
| 7.01 | 34.10 | 403.37 | 6.55 | 30.42 | 3.02 |

| | | | | | |
|---|---|---|---|---|---|
| 7.51 | 54.10 | 449.87 | 6.51 | 30.42 | 3.02 |
| 8.01 | 58.30 | 449.71 | 6.60 | 30.42 | 3.01 |
| 8.50 | 58.60 | 449.58 | 6.53 | 30.42 | 3.02 |
| 9.01 | 57.58 | 450.05 | 6.58 | 30.42 | 3.02 |
| 9.50 | 56.95 | 451.58 | 6.53 | 30.42 | 3.01 |
| 9.99 | 56.33 | 450.62 | 6.57 | 30.42 | 3.02 |
| 10.51 | 56.33 | 451.05 | 6.59 | 30.38 | 3.02 |
| 11.00 | 55.30 | 451.96 | 6.50 | 30.38 | 3.01 |
| 11.51 | 54.95 | 451.75 | 6.55 | 30.30 | 3.01 |
| 12.00 | 54.08 | 451.71 | 6.55 | 30.34 | 3.01 |
| 12.51 | 52.78 | 451.87 | 6.54 | 30.38 | 3.02 |
| 13.00 | 53.45 | 450.92 | 6.57 | 30.47 | 3.02 |
| 13.51 | 51.85 | 452.50 | 6.59 | 30.47 | 3.02 |
| 14.01 | 51.05 | 451.05 | 6.50 | 30.30 | 3.02 |
| 14.50 | 50.20 | 450.62 | 6.59 | 30.43 | 3.02 |
| 15.01 | 49.85 | 451.50 | 6.57 | 30.43 | 3.01 |
| 15.50 | 50.40 | 451.45 | 6.53 | 30.43 | 3.01 |
| 16.01 | 50.20 | 450.33 | 6.54 | 30.43 | 3.01 |
| 16.51 | 50.65 | 452.00 | 6.54 | 30.30 | 3.01 |
| 17.01 | 51.25 | 451.92 | 6.52 | 30.34 | 3.01 |
| 17.51 | 51.95 | 451.79 | 6.59 | 30.30 | 3.01 |
| 18.00 | 51.52 | 451.96 | 6.53 | 30.30 | 3.01 |
| 18.51 | 53.25 | 451.50 | 6.53 | 30.47 | 3.01 |
| 19.00 | 53.80 | 450.17 | 6.59 | 30.38 | 3.01 |
| 19.51 | 54.55 | 451.17 | 6.51 | 30.42 | 3.01 |
| 20.01 | 54.78 | 450.75 | 6.59 | 30.34 | 3.01 |
| 20.50 | 54.65 | 450.87 | 6.55 | 30.38 | 3.01 |
| 21.01 | 55.33 | 451.67 | 6.59 | 30.30 | 3.01 |
| 21.50 | 56.48 | 452.50 | 6.51 | 30.34 | 3.01 |
| 22.01 | 56.65 | 450.25 | 6.51 | 30.42 | 3.01 |
| 22.50 | 57.98 | 451.96 | 6.58 | 30.34 | 3.02 |
| 22.99 | 58.50 | 450.46 | 6.52 | 30.42 | 3.02 |
| 23.51 | 58.59 | 451.71 | 6.56 | 30.42 | 3.02 |
| 24.00 | 59.48 | 451.71 | 6.51 | 30.42 | 3.02 |
| 24.51 | 60.98 | 450.83 | 6.52 | 30.46 | 3.02 |
| 25.00 | 60.25 | 451.45 | 6.58 | 30.46 | 3.02 |
| 25.51 | 62.50 | 452.71 | 6.54 | 30.46 | 3.02 |
| 26.01 | 62.95 | 451.17 | 6.52 | 30.42 | 3.01 |
| 26.51 | 64.45 | 452.37 | 6.56 | 30.46 | 3.01 |
| 27.01 | 65.50 | 451.05 | 6.53 | 30.46 | 3.01 |
| 27.50 | 66.65 | 450.55 | 6.56 | 30.42 | 3.01 |
| 28.01 | 68.65 | 451.79 | 6.57 | 30.30 | 3.01 |
| 28.50 | 69.33 | 451.79 | 6.58 | 30.47 | 3.01 |

| | | | | | |
|---|---|---|---|---|---|
| 29.01 | 71.75 | 451.71 | 6.55 | 30.34 | 3.01 |
| 29.51 | 74.80 | 450.87 | 6.53 | 30.30 | 3.01 |
| 29.99 | 77.33 | 452.37 | 6.53 | 30.38 | 3.01 |

**Figure 3. Variation of protein content in the retentate with the 10 kDa membrane at different permeate flux using mono-culture fermented whey supernatant**

| Permeate flux (L/h/m$^2$) | Protein concentration (g/L) | Total solids (g/L) |
|---|---|---|
| 267 | 26.80 | 29.60 |
| 343 | 27.26 | 30.17 |
| 413 | 27.94 | 30.50 |
| 505 | 25.63 | 28.40 |

**Figure 4. Variation of protein content in the retentate with the 10 kDa membrane at different TMP values using mono-culture fermented whey supernatant**

| TMP (kPa) | Protein concentration (g/L) | Total solids (g/L) |
|---|---|---|
| 60 | 28.42 | 30.60 |
| 70 | 28.70 | 30.70 |
| 80 | 28.63 | 30.14 |
| 90 | 26.80 | 29.30 |

**Figure 5. Variation of protein content in the retentate of 1 kDa membrane at different permeate flux using 10 kDa permeate**

| Permeate flux (L/h/m$^2$) | Protein content (g/L) | Total solids (g/L) |
|---|---|---|
| 1345 | 10.3 | 11.9 |
| 1922 | 10.5 | 11.7 |
| 2446 | 10.4 | 11.6 |
| 2760 | 10.3 | 11.2 |
| 3261 | 9.1 | 9.8 |

**Figure 6. Variations of protein content in the retentate with 1 kDa membrane at different TMP values using 10 kDa permeate as feed**

| TMP (kPa) | Protein content (g/L) | Total solids (g/L) |
|---|---|---|
| 150 | 9.6 | 10.4 |
| 170 | 9.9 | 11.1 |
| 190 | 10.3 | 11.4 |
| 210 | 10.7 | 11.7 |
| 230 | 10.6 | 11.6 |
| 250 | 9.6 | 11.2 |

# ANNEXE 6

**Synthèse (Version anglaise)**

**Synopsis (English version)**

# 2. PROBLEMS

The literature review shows that the cheese whey is a valuable byproduct of cheese producing industries and if it is directly dumped into the sewage, creates environmental problems. However, if it is processed via biotechnological and physical processing it has potential to transform into value-added products and helps to minimize the whey management problem. However, problems still exist due to excess volume of whey production. The one dominant biotransformation process for whey utilization is the production of single-cell protein (SCP). However, there are many challenges that hamper the commercial adaptation of SCP production and whey treatment.

## 2.1 Problem 1

Biomass is usually produced using whey permeate, which requires pre-treatment to separate the whey protein before fermentation. The pre-treatment steps such as ultrafiltration is expensive to separate whey protein and leaving large volume of liquid whey permeate. Furthermore, the separation of whey protein before fermentation is generally not in the interest of small and medium scale cheese producing industry due to low volume of whey to separate the whey protein, followed by the treatment or biotransformation of whey permeate.

## 2.2 Problem 2

Pure culture of lactose utilizing yeast (*Kluyveromyces marxianus*) is mostly used for the production of biomass (SCP), which is used as animal feed or food. However, the main problem during cultivation of this yeast at high lactose concentration is the generation of secondary metabolites, which leads to the higher residual COD and reduction of biomass yield. Moreover, pure culture of *K. marxianus* lacks some essential amino acids that reduce the SCP value as animal feed or food applications.

## 2.3 Problem 3

The fate and the recovery methods of the residual soluble whey protein after fermentation are unknown.

## 2.4 Problem 4

Monoculture is less efficient in COD degradation, whereas mixed cultures were efficient for COD degradation. However, this requires acid resistance and thermotolerent yeast strains to operate the fermentation under non-aseptic condition.

## 2.5 Problem 5

The biomass production of more acceptable yeast *Saccharomyces cerevisiae* is carried out after hydrolysis of lactose using pure β-galactosidase (free or immobilized enzyme system). However, the use of pure or immobilized enzyme system makes the process expensive. Moreover, pre-treatment of biomass to reduce its nucleic acid content for food grade SCP also needs an efficient method.

## 2.6 Problem 6

Whey proteins have various functional properties with high nutritional value. However, higher percentage of β-lactoglobulin make the whey proteins allergic to infants. So whey proteins were hydrolyzed via enzymatic treatment or by fermentation. Enzymatic treatment is expensive and fermentation process needs various technological developments (i.e. recovery of whey protein in native state after fermentation and evaluation of in-vitro biological activity).

# 3. HYPOTHESIS, OBJECTIVES AND ORIGINALITY

## 3.1 HYPOTHESIS

The strains of *K. marxianum* has the capacity to metabolize the whey lactose for its growth, and used as a source of SCP for animal feed and food. The general hypothesis of this research is: it could be possible to produce yeast biomass using cheese whey as a substrate with simultaneous removal of COD. The metabolite generation can be minimised by manipulation of lactose concentration or employing mixed culture. The mixed culture yeast biomass for food grade SCP can be produced by hydrolyzing the whey lactose employing permeabilized yeast cells. Further, the residual whey protein can be recovered in native state by membrane filtration.

### 3.1.1 Hypothesis 1

Fermentation of whey permeate with acid tolerant and thermotolerant yeast *K. marxianus* is well known for the production of biomass and simultaneous COD reduction. Thus, it could be possible to produce the biomass using cheese whey without removing the whey protein under extreme conditions (low pH and high temperature) that minimize the chances of contamination. The characterization of intermediate metabolites and determination of whey protein fate during fermentation will provide an information to manipulate the COD degradation efficiency and biomass yield.

### 3.1.2 Hypothesis 2

Lactose in the medium will be consumed during fermentation, leaving most of the soluble protein in the fermented broth. The presence of lactose has negative impact on the precipitation of whey protein, therefore once the lactose is consumed (during fermentation), it would be possible to precipitate and recover the residual soluble protein with ease by optimizing the precipitation method. The precipitated protein can be mixed with the biomass to enhance its protein content and balance the essential amino acids profile of the produced SCP.

### 3.1.3 Hypothesis 3

The mixed culture could be produced employing lactose consuming yeast (*K. marxianus*) with non-lactose consuming yeast to enhance the COD removal efficiency, improve the product quality and operate the fermentation process under non-aseptic conditions employing acid resistance and thermotolerant yeast.

### 3.1.4 Hypothesis 4

Cultivation of mixed culture (especially *Saccharomyces cerevisiae* with *K. marxianus*), to balance the essential amino acids profile needs hydrolyzed whey lactose. Hydrolysis is carried out using pure β-galactosidase enzyme or using immobilized enzyme, which makes the process expensive. However, it is possible to permeabilize *K. marxianus* cells (which possess β-galactosidase activity) with non-toxic permeabilizing agents and subsequently use of the permeabilized cells as a source of enzyme to hydrolyze whey lactose.

### 3.1.5 Hypothesis 5

The pre-treatment is carried out by chemical or enzymatic treatment to reduce the nucleic acid content from the SCP to be used as food. However, it is possible to use detergent to enhance the efficiency of chemical treatment and release of nucleotides, separately. Membrane separation is a preferred technology to recover the native protein and peptides, when molecular weight of protein and peptides are known. Thus, it is possible to use membrane technology to separate the fermented whey protein in native state after evaluation of molecular weight.

## 3.2 Objectives

The global research objective is to produce feed and food grade single-cell protein, characterization and recovery of residual soluble whey protein from cheese whey fermentation. The specific research objectives are as follows:

### 3.2.1 Objective 1

Production of single-cell protein, characterization of intermediate metabolites of *K. marxianus* and determination of the fate of whey protein from cheese whey.

### 3.2.2 Objective 2

Development of recovery method for residual soluble fermented whey protein by precipitation after yeast cultivation.

### 3.2.3 Objective 3

Evaluation of mixed culture of *K. marxianus* and *Candida krusei* for single-cell protein production and organic load removal from whey under non-aseptic condition.

### 3.2.4 Objective 4

Permeabilization of *K. marxianus* for β-galactosidase activity and augmentation of mixed culture for biomass production.

#### *3.2.4.1 Objective 4.1*

Permeabilization of *K. marxianus* for β-galactosidase activity and hydrolysis of whey lactose with permeabilized *K. marxianus* cells.

#### *3.2.4.2 Objective 4.2*

Augmentation of mixed culture of *Saccharomyces cerevisiae* and *K. marxianus* on hydrolyzed whey.

### 3.2.5 Objective 5

Pre-treatment of yeast biomass to reduce the nucleic acid content and optimization of ultrafiltration for recovery of residual soluble fermented whey protein.

## 3.3 ORIGINALITY

The production of single-cell protein directly from cheese whey is a novel approach to avoid the separation of whey protein before fermentation. The biomass production from cheese whey with determination of the fate of whey protein, their characterization and recovery as well as characterization of intermediate metabolites has not been studied. Moreover, production of mixed culture under non-aseptic conditions has also been not explored. Further, production of food grade mixed culture yeast biomass using permeabilized *K. marxianus* cells to increase the population of *S. cerevisiae* is a novel approach. The recovery of residual fermented whey protein post fermentation in native state is not reported. Thus, the proposed research is expected to provide a feasible technology for the transformation of whey into proteinaceous products.

# 4. RESULTS AND DISCUSSION

The obtained results of this thesis are presented in the 3 parts. The first part is comprised of the transformation of whey, single-cell protein (SCP) production and recovery of residual soluble protein post-fermentation (2 articles published; one is submitted). The second part comprises of the biotechnological potential of *Candida krusei* and its role as a mixed culture consortium for the production of SCP (2 articles published). The third part is about the permibilization of yeast cells for enzyme activity, hydrolyze whey lactose with permeabilized cells, augmentation of mixed culture for food grade SCP production and recovery of residual soluble protein through ultrafiltration (1 article published; one is submitted). Results are presented accordingly.

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## 4.1 Transformation of whey, single cell-protein production and residual protein recovery post-fermentation

### 4.1.1 Cheese whey: a potential resource to transform into bioprotein, functional/nutritional protein and bioactive peptides (Chapter II, part 1)

An overview of cheese whey transformation processes for the value added products are presented. The main emphasis has been given to the processes and factors affecting the biotransformation of cheese whey into bioprotein/single-cell protein. Additionally, separation and transformation processes of whey protein into various functional and nutritional proteins have also been reviewed. Cheese whey can be transformed into the value added products such as whey powder, whey protein, bioethanol, biopolymers, methane, bioprotein (single-cell protein) and probiotics. Whey and whey permeate is biotransformed to proteinaceuous feed and food grade single-cell protein through fermentation. On the other hand, whey is directly processed into whey protein concentrate, whey protein isolate and individual whey proteins. Further, whey proteins have the potential to transform into bioactive peptides via enzymatic or fermentation processes.

### 4.1.2 Simultaneous single-cell protein production and COD removal with characterization of residual protein and intermediate metabolites during whey fermentation by *K. marxianus* (Chapter II, part 2)

The fermentation of cheese whey with *Kluyveromyces marxianus* was carried out at 40 $^{o}$C and pH 3.5 to examine the simultaneous single-cell protein (SCP) production and chemical oxygen demand (COD) removal, determine the fate of whey protein and to characterize

intermediate metabolites. The biomass yield ($Y_{x/s}$) of 0.12 g biomass/g lactose and 55% of COD reduction (including protein) was observed during batch fermentation with normal cell density inoculum. Comparatively lower biomass yield might be due to extreme fermentation conditions, i.e. low pH and high temperature, which were used to minimize the chance of contamination. The lower COD degradation was due to high initial lactose concentration. The soluble whey protein concentration decreased from 5.6 to 4.1 g/L, which indicated that the very minor quantity of whey protein was utilized during fermentation. Further, the electrophoresis result indicates that the fermented whey protein was different from native whey protein. HPLC and GC-MS analysis revealed a change in the composition of organic compounds post-fermentation. Some of the whey native volatile compounds were consumed while some new compounds were generated during fermentation. Batch fermentation with high initial inoculum concentration resulted in an increase in the biomass yield up to 0.19 g biomass/g lactose and 80% COD reduction (including protein) with residual protein concentration of 4.5 g/L. The higher COD reduction was due to the lower food to microorganism ratio that decrease the higher intermediate metabolite generation. Third batch fermentation, which was conducted with medium cell density inoculum and shifted to the continuous fermentation with cell recycle system to maintain the higher biomass concentration. The biomass yield of 0.19 g biomass/g lactose and productivity of 0.26 g/L/h was obtained during continuous fermentation. The COD removal efficiency was 78-79% with residual protein concentration of 3.8-4.2 g/L. The higher residual COD compared to the literature report was due to the presence of residual soluble protein. Previously, most of the studies were conducted with whey permeate where the residual COD due to protein was negligible.

### 4.1.3 Recovery of residual soluble protein by two-step precipitation process with concomitant COD reduction from the yeast-cultivated cheese whey (Chapter II, part 3)

The precipitation method for the recovery of residual soluble fermented whey protein remained after yeast (*K. marxianus*) cultivation by continuous fermentation was evaluated. The yeast biomass was separated from the fermented broth by centrifugation, and the residual soluble protein from fermented whey supernatant was precipitated by heat treatment. The maximum soluble protein recovery up to 53% and 54% of residual COD removal were obtained at the optimum temperature 100 $^{o}$C, pH 4.5 and time 10 min. However, gravity sedimentable precipitates were obtained at pH 3.5 with 47% of protein recovery. Further, the study was conducted at pH 3.5 with agitation, which resulted in 68% of protein recovery and simultaneously residual COD removal of 62%. The increase in agitation

rate (shear rate) enhanced the aggregation of heat precipitated protein. Further, precipitation/coagulation of soluble protein was evaluated with carboxymethyl cellulose (CMC) and then two precipitation (thermal followed by CMC precipitation) processes were combined. The combined precipitation process resulted in 81% of total protein recovery. The precipitation process did not require the centrifugation to separate the protein. The gravity sedimentable precipitate slurry could be mixed with centrifuged biomass and spray dried to obtain an enriched proteinaceous product.

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## 4.2 Biotechnological potential of yeast Candida krusei and its role as a mixed culture consortium for the production of SCP

### 4.2.1 *Candida krusei*: biotechnological potentials and concerns about its safety (Chapter III, part 1)

A comprehensive review about the two sides (i.e., biotechnological potentials and concerns about its safety) of *Candida krusei* has been done. The yeast species, *Candida krusei* (an opportunistic pathogen) has been reported from different habitats. It has gained increased attention because of its diverse biotechnological role. It is found in many fermented food items and dairy products. It has also been exploited for the production of various biochemical and enzymes. Moreover, it is an acid resistant and thermotolerant in nature, which could be exploited for the production of single-cell protein from cheese whey in mixed culture.

### 4.2.2 Mixed culture of *Kluyveromyces marxianus* and *Candida krusei* for single-cell protein production and organic load removal from whey (Chapter III, part 2)

The study was conducted to evaluate the potential of mixed culture of *K. marxianus* and *Candida krusei* over a mono-culture of *K. marxianus* to enhance COD removal efficiency, minimize the chances of contamination using extreme fermentation conditions (i.e. high temperature and low pH) and to obtain improved quality SCP during batch and continuous fermentation. The aerobic batch fermentation with mono-culture (*K. marxianus*) and mixed culture (*K. marxianus* and *C. krusei*) was carried out at 40 $^{o}$C and pH 3.5. The continuous fermentation at different hydraulic retention times (HRT) (6, 12, 18 and 24 h) was conducted for maximum COD removal and biomass production. The batch fermentation of mixed culture resulted in 8.8% higher COD removal efficiency with 19% higher biomass yield and 33% increased productivity than that of mono-culture. The higher COD degradation with mixed

culture was obtained due to the fact that lactose negative yeast (*C. krusei*) consumed intermediate metabolites during fermentation. Moreover, the intermediate metabolites consumption also resulted in enhanced biomass yield and productivity. The maximum COD removal 80.2% (including residual protein) was obtained at 24 h HRT with biomass productivity of 0.17 g/L/h, however, maximum biomass productivity 0.38 g/L/h with 34% COD removal were obtained at 6 h HRT. The growth of *C. krusei* in the mixed culture during batch and continuous fermentation showed that the two yeasts have commensalism type of physiological interaction. The mixed culture SCP is composed of desired protein content and enriched with lysine essential amino acid.

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## 4.3 Permibilization of yeast cells, hydrolysis of whey lactose, augmentation of mixed culture for SCP production and recovery of residual soluble fermented whey protein

### 4.3.1 Permeabilization of *Kluyveromyces marxianus* with mild detergent for whey lactose hydrolysis and augmentation of mixed culture (Chapter IV, part 1)

*K. marxianus* cells were permeabilized with non-toxic, biodegradable, anionic detergent N-lauroyl sarcosine (N-LS) for the intracellular enzyme activity and lactose hydrolysis. The permeabilization process parameters (N-LS concentration, solvent volume, temperature and incubation time) were optimized. The maximum β-galactosidase activity of 1,220 IU/g dry weight was obtained with permeabilized cells under the optimized conditions: N-LS concentration of 1.5% w/v, water volume of 1 mL (or 1.0 g wet weight/mL of N-LS solution), temperature 25 $^{o}$C and incubation time of 20 min. Moreover, the viability of the permeabilized cells was also evaluated, which showed that cells were alive; with viability reduction of two log cycles after permeabilization. The whey lactose hydrolysis was conducted with permeabilized cells. The maximum lactose hydrolysis of 91% was observed with 600 mg (dry cell weight/100 mL) in whey powder (5% w/v) solution at 180-min incubation, pH 6.5 and 30 °C. Further, the hydrolyzed whey was evaluated for amelioration of growth of non-lactose-consuming yeast *Saccharomyces cerevisiae*. *S. cerevisiae* was able to the grow in hydrolyzed whey with *K. marxianus* in mixed culture.

### 4.3.2 Characterization and ultrafiltration recovery of residual soluble protein from food grade yeast-fermented whey (Chapter IV, part 2).

The study was carried out to characterize and recovery of the residual soluble protein after cultivation of mono (*K. marxianus*) and mixed (*K. marxianus* and *S. cerevisiae*) culture yeast biomass as food grade SCP from cheese whey. The batch fermentation was carried out at 35 $^{o}$C and pH 5.5 for mono-culture and at 30 $^{o}$C and pH 6.5 for mixed culture. The obtained biomass yields were 0.27 and 0.31 g biomass/g lactose consumed from mono and mixed culture, respectively. The fermented broth was centrifuged to obtain biomass and fermented whey supernatant. The yeast biomass was treated in two steps with N-LS and $NH_4OH$, respectively. The treatment resulted in a reduction of nucleic acid content below 2% w/w from initial 11.4% w/w. The characteristics of soluble protein after fermentation were evaluated by SDS-PAGE. During fermentation a fraction of whey protein was consumed. The SDS-PAGE result revealed that the fermented whey protein is different from the native whey protein and partially hydrolyzed during fermentation. The ultrafiltration operational parameters (permeate flux and transmembrane pressure-TMP) were optimized for 1 and 10 kDa membranes to recover the residual fermented whey protein. The permeate flux of 413 L/h/m$^2$ and TMP of 80 kPa resulted in the highest recovery with 10 kDa membrane followed by a permeate flux of 2760 L/h/m$^2$ and TMP of 230 kPa for 1 kDa membrane. These optimized conditions in combination recovered 84% and 92% of total residual soluble protein from mono and mixed culture fermented whey supernatant, respectively.

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# 5. CONCLUSIONS AND RECOMMENDATIONS

## 5.1 CONCLUSIONS

Yeast *K. marxianus* as SCP could be cultivated with minimal chances of contamination at high temperature and low pH from cheese whey. A very minor quantity of whey protein consumption was observed during fermentation. However, the nature of residual soluble proteins were different from native whey proteins. HPLC and GC-MS analysis of fermented whey supernatant showed that the intermediate metabolites presence was the responsible factor for high residual COD along with residual protein. The higher COD removal and enhanced biomass productivity were obtained during fermentation with high cell density inoculum. The higher COD reduction and biomass productivity were achieved during continuous fermentation with a cell recycle system.

The gravity sedimentable precipitates of protein from fermented whey supernatant were obtained at pH 3.5, temperature 100 $^{o}$C and treatment time of 10 min. The gravity sedimentation obviates the need of centrifugation for protein recovery. Additionally, precipitates obtained at pH 3.5 was rich in mineral content, which could be mixed with the yeast biomass to increase the final mineral content of the product. The combination of two step precipitation process (thermal treatment followed by CMC addition) increased the protein recovery up to 81% with further residual COD removal. The protein content of the final product was increased from 42% w/w to 49% w/w by mixing the precipitates with yeast biomass, and an increase in the quantity of the product by approximately 48%. The overall COD removal in combination of fermentations, followed by precipitation was reaching up to 93%. The developed method for residual protein recovery could be applied at industrial scale (especially by small and medium scale industries) to produce the SCP as animal feed.

The mixed culture of *C. krusei* and *K. marxianus* gave higher biomass yield and COD removal efficiency over a mono-culture of *K. marxianus*. Moreover, they have shown the physiological interaction with each other. The continuous fermentation at 24 h HRT is optimum for higher COD removal with substantial yield and productivity. In addition, SCP from mixed culture was enriched with lysine amino acid. The continuous process can be operated in non-aseptic condition. Residual protein recovery through two steps precipitation will increase the productivity from 0.17 to 0.31 g/L/h and COD removal efficiency from 80% to 91-93%.

The non-toxic and biodegradable detergent (N-LS) can be used to permeabilize *K. marxianus* cells for intracellular β-galactosidase activity. More than 90% of whey lactose (from 50.0 g/L of whey powder solution) hydrolysis was achieved within 3 h with 6.0 g dry weight of permeabilized cells. The hydrolyzed whey with permeabilized cells could be effectively used to grow a mixed culture of lactose consuming (*K. marxianus*) and non-lactose consuming (*S. cerevisiae*) yeasts.

The food grade SCP could be obtained by the combined treatment of detergent and $NH_4OH$ from yeast biomass with simultaneous release of nucleotides (as flavoring agent). The whey proteins were partially hydrolyzed and consumed during the fermentation in two cases (mono and mixed culture). The SDS-PAGE result revealed that the majority of the fermented whey protein molecular mass was remained below 25 kDa. The fermented whey protein could be recovered through combined (10 and 1 kDa membrane) ultrafiltration with approximately 90% of protein purity.

Based on the present study, three different processes have been developed, which could be potentially applied to biotransform the raw cheese whey into proteinaceous products. The first process is the continuous fermentation (at high temperature and low pH) with cell recycle, followed by residual soluble protein recovery through precipitation. The integrated process (i.e. production of SCP, followed by residual protein recovery) resulted in higher productivity and higher COD removal. The product (SCP to be used animal feed) has high protein content and balanced with essential amino acids. The second process is the continuous fermentation under non-aseptic conditions employing mixed culture (*K. marxianus* and *C. krusei*), which resulted in higher COD removal efficiency and enhanced biomass yield. The produced SCP (animal feed) has higher lysine content, which is highly desired for animal feed. The third process is to produce food-grade mixed culture (*K. marxianus* and *S. cerevisiae*) SCP using permeabilized *K. marxianus* cells as a source of enzyme to hydrolyse whey lactose, followed by residual protein recovery through ultrafiltration. Food-grade SCP was obtained by pre-treatment of biomass with a combination of chemicals in two-step. The residual soluble proteins after fermentation were recovered by ultrafiltration in native state.

## 5.2 Recommendations

Future research of single-cell protein production for feed and food application from cheese whey should consider the following recommendations:

1. Scale up study is warranted for the production of SCP for use as animal feed with simultaneous COD removal in an integrated process, i.e., production of biomass employing cell recycle system and residual soluble protein recovery through two steps precipitation process.

2. Scale up study is also necessary for the production of mixed culture (*K. marxianus* and *C. krusei*) SCP to be used as animal feed and COD removal in an integrated scenario under non-aseptic conditions. Moreover, the emphasis should also be given for the registration of this mixed culture SCP at Canadian Food Inspection Agency or other regulatory body to accept the *Candida krusei* as a consortium of SCP to be used as animal feed.

3. Reuse of treated water for dilution of cheese whey should be investigated to avoid the dependency on municipal water supply.

4. The food grade mono and mixed culture SCP were obtained from biomass by combining pre-treatment of detergent and alkali. However, further study should be conducted to purify the released nucleotides and functional properties of treated biomass protein. Ultrafiltration could be applied to recover the high purity fermented whey protein in native state. However, scale-up study for ultrafiltration and the bioactivity evaluation of fermented whey protein should also be conducted.

5. A comparative economic analysis is required, considering all three cases of single-cell protein production and protein recovery processes to find the best conditional based process for whey valorization.

6. Possibility of synthesis of prebiotics (galacto-oligosaccharides) during production of mixed culture employing permeabilized of cells as a source of enzyme (β-galactosidase). Furthermore, the possibility of the production of other byproducts such as phenyl ethyl alcohol should also be investigated to make the process further economically attractive.

7. The precipitation of organic compounds during protein precipitation processes should be investigated to explore the basic mechanism.

8. Comparative study should be conducted on the essential amino acid profile of the single cell proteins produced from whey permeate and cheese whey.