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INHIBITION DE LA CROISSANCE DE *CANDIDA KRUSEI* ET ÉVALUATION DU PROFIL NUTRITIONNEL DE LA FERMENTATION DE *SACCHAROMYCES UNISPORA* À PARTIR DU LACTOSERUM

Par

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DÉDICACE

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RÉSUMÉ

Le lactosérum provenant de la fabrication du fromage est considéré comme une importante source de pollution de l'environnement, car il cause une demande chimique en oxygène (DCO) supérieure à 60 000 mg⁄L et une demande biochimique en oxygène (DBO) supérieure à 30 000 mg⁄L. Environ 35 % du lactosérum pourrait servir dans l'alimentation humaine et animale puisque celui-ci contient la moitié des nutriments présents dans le lait. Les autres utilisations du lactosérum sont la production de boissons riches en protéines, par exemple les boissons protéinées, le lait fermenté et les suppléments de protéines. Le lactosérum peut même être utilisé comme engrais en agriculture. Le lactose dans le lactosérum est la source principale de DCO et le retrait des protéines du lactosérum réduit la DCO de 10 000 mg⁄L. Le lactosérum contient également quelques métaux lourds (Al, Cd et Pb) en faibles concentrations. Des techniques de filtration et d'ultrafiltration, par exemple, sont utilisées pour convertir les protéines du lactosérum en une forme plus concentrée permettant de récupérer jusqu'à 50 % des protéines (sous la forme de perméat de lactosérum). Ces concentrés protéiques sont une option économique pour la production de protéines d'organismes unicellulaires (POU). Cela permet de réduire la pollution et résout en grande partie le problème de gestion des déchets. Les POU pourraient permettre de nourrir à bon marché une population mondiale en forte croissance. La production de protéines microbiennes n'est pas contrainte par les conditions environnementales. Cependant, le choix des microorganismes est important pour la production de POU, car les microbes doivent pouvoir obtenir du lactosérum les nutriments nécessaires au maintien des fonctions de leurs cellules. Le choix de la communauté microbienne est aussi basé sur certains critères de sécurité, la communauté microbienne ne doit pas altérer les propriétés fonctionnelles de la nourriture à laquelle on l'ajoute, les microbes ne doivent pas être pathogènes, etc. Ainsi, le but de cette étude était de produire des aliments destinés aux humains et aux animaux avec des microbes autorisés, en l'absence de microbes non désirés, et d'en examiner le profil nutritif dans des monocultures et des cultures mixtes.

La fermentation du lactosérum a été effectuée à pH 3,5 et à 40 °C avec *Kluyveromyces marxianus*, *Candida krusei* est alors ressorti comme un contaminant biologique. Les méthodes utilisées pour éliminer *C. krusei* comprenaient des approches chimiques, biochimiques et à l'aide de bionanoparticules. Les approches chimiques et biochimiques étaient efficaces pour inhiber *C. krusei*, mais *K. marxianus* était également affecté. De plus, les approches chimiques et biochimiques n'étaient pas très économiques pour la fermentation à grande échelle. Des approches d'inhibition sélective ont été comparées et il a été observé que 350 μM d'une protéine tueuse conjuguée à de l'argent (Ag-KT4561) d'une taille de 200 nm pourrait complètement éliminer *C. krusei* en affectant seulement partiellement *K. marxianus* et *Saccharomyces unispora* à pH 5,5 et à 30 °C. On a même observé que *K. marxianus* avait un meilleur profil de croissance à pH 5,5 plutôt que 3,5. La concentration de l'ion métallique d'argent a été calculée à 3,96 mM et la stabilité du complexe protéine-argent a été vérifiée sur plus de 20 semaines. Le complexe protéine-argent n'a pas formé de complexes métalliques avec les minéraux présents dans le lactosérum et, par conséquent, il est considéré comme un composé stable. Puisque l'argent est fortement antimicrobien, l'utilisation d'Ag-KT4561 peut éliminer d'autres contaminants biologiques et contribuer à ce que la fermentation s'effectue en conditions aseptiques. Cependant, les ions d'argent affectaient partiellement *K. marxianus*, mais dans une faible mesure.

Le profil nutritif de *S. unisporus* a été étudié en faisant varier la source de carbone (sucrose, glycérol), d'azote (urée, sulfate d'ammonium) et le pH. *S. unisporus* est considéré comme une levure probiotique parce qu'il produit des intermédiaires nutritifs. *S. unisporus* pouvait consommer une vaste gamme de sources de carbone excluant le lactose et il pouvait consommer 0,4 % (poids/volume) de sulfate d'ammonium comme source d'azote. *S. unisporus* s'est avéré strictement urée négatif. La monoculture de *S. unisporus* était enrichie en lysine (84,0 mg/g), valine (74,4 mg/g), acide aspartique (83,3 mg/g) et glycine (77,0 mg/g). La biomasse de *S. unisporus* contenait les minéraux essentiels, incluant Ca, S, Na, Mg, P et K. La teneur en acides gras observée pour *S. unisporus* était de 0,86 % (poids/poids) et en protéines de 4.2 (g/L). Le pH idéal pour la production de POU avec *S. unisporus* en monoculture était de 5,5.

L'hydrolyse acide du lactosérum et du perméat a été réalisée et *S. unisporus* a été cultivé en monoculture et en culture mixte avec *K. marxianus*. Il a été observé que 0,2 % (p/p) de H_2SO_4 pouvait hydrolyser le perméat de lactosérum et 90 % du lactose était converti en glucose et en galactose en 70 min. Alors que 0,4 % (p/p) de H_2SO_4 permettait de convertir le lactose en glucose et en galactose en 60 min. *S. unisporus* et *K. marxianus* ont été cultivés dans du lactosérum hydrolysé avec 0,4 % (p/v) de sulfate d'ammonium et une meilleure croissance a été observée pour *K. marxianus* ($7,8 \times 10^8$ CFU/mL) que pour *S. unisporus* ($5,6 \times 10^7$ CFU/mL), parce que ce dernier peut consommer partiellement le galactose tandis que *K. marxianus* peut consommer autant les monosaccharides que le surplus de lactose.

La fermentation en culture mixte (*S. unisporus* et *K. marxianus*) montrait une légère augmentation de la teneur en acides aminés essentiels, en particulier la leucine et l'isoleucine (79,9 mg/g), tandis que pour la biomasse de *S. unisporus* en monoculture, une concentration de seulement 59 mg/g a été mesurée. Il a même été observé que la production de biomasse en culture mixte générait des intermédiaires (furanméthanol, formate furfurylique, acides organiques faibles) importants en biotechnologies. Cependant, aucune quantification n'a été effectuée.

En vue de la production d'aliments pour les humains et les animaux, le contaminant biologique a été éliminé et la levure autorisée a été ajoutée. *C. krusei* a été tué grâce à cette approche de synthèse verte rentable en maintenant les conditions de fermentation à pH 5,5 avec une température de 30 °C et à l'aide de l'agent biologique de conservation Ag-KT4561. Après l'élimination de *C. krusei*, la levure autorisée *S. unisporus* a été ajoutée dans la fermentation en monoculture et en culture mixte. La qualité des POU en termes de valeur nutritive était augmentée lorsque *S. unisporus* était cultivé avec *K. marxianus* dans du lactosérum et du perméat. Par conséquent, sur la base de ces deux études, il est possible de conclure que le contaminant biologique (*C. krusei*) peut être éliminé de façon aseptique dans des fermentations en culture mixte par une approche rentable de chimie verte.

ABSTRACT

Cheese whey is considered as a significant source of environmental pollution as the chemical oxygen demand (COD) is greater than 60, 000 ppm and biological oxygen demand (BOD) is greater than 30, 000 ppm. Approximately 35% of the cheese whey is considered for food and feed because cheese whey consists of half of the nutrients, which are present in milk. The other uses of cheese whey are by the production of beverages, which are rich in proteins for example protein drinks, fermented milk, and sports protein. Cheese whey is also utilized in agriculture as a fertilizer. The major source of high COD is the presence of lactose in cheese whey and it has been observed that the removal of proteins from cheese whey reduces the COD by 10, 000 ppm. Cheese whey contains some heavy metals (Al, Cd, and Pb) in lower quantities. Techniques such as filtration and ultra-filtration are utilized for the conversion of whey protein into more concentrated form leading up to 50% of protein recovery (as whey permeate). These protein concentrates are used in single cell protein production (SCP) as an economic alternative. This helps in controlling the pollution and solves the waste disposal problem to a larger extent. SCP can feed the ever-increasing world population at much cheaper rates. Microbial protein can be an alternative source, which can be grown in a any surface area irrespective of the environmental conditions. However for the production of SCP, right choice of microorganisms is important, as microbe must be able to derive nutrients from cheese whey for maintaining the cell functions. Additionally, the choice of the microbial community is based on certain criteria such as general safety from the microbe, the microbial community must not alter the functional properties of food to which it is added, the microbe must not be pathogenic in nature etc. Based on this, the aim of the study was the production of food and feed by eliminating the unwanted microbe, addition of the acceptable microbe, study of the nutritive profile in a mono and mixed culture.

Cheese whey fermentation was carried out at pH 3.5 and 40 °C with *K. marxianus* but *C. krusei* was emerging as a biological contaminant. The methods applied for the removal of *C. krusei* were chemical, biochemical and bio-nanoparticle approaches. Chemical and biochemical approaches were effective in inhibiting *C. krusei* but *K. marxianus* was also getting affected. However chemical and biochemical approaches were not very

economical for large-scale fermentation. Selective inhibition approaches were compared and it was observed that 350 uM of silver-killer protein conjugate (Ag-KT4561) of size 200 nm could eliminate *C. krusei* completely with partial effect on *K. marxianus* and *S. unisporus* at pH 5.5 and 30 °C. It was also observed that *K. marxianus* has a better growth profile at pH 5.5 rather than in pH 3.5. The concentration of the metallic silver ion is calculated as 3.96 mM and stability of the silver-protein complex were verified for more than 20 weeks. The silver-protein complex did not form any metallo-complex with minerals present in cheese whey and hence it is a stable compound. As silver is highly antimicrobial, usage of Ag-KT4561 can eliminate other biological contaminants and help run the fermentation under aseptic conditions. However silver ions had partial effect on *K. marxianus* but not to a larger extent.

A study was conducted for the nutritive profile of *S. unisporus* by varying the carbon (sucrose, glycerol), nitrogen source (urea and ammonium sulphate) and pH. *S. unisporus* is coined as a probiotic yeast because it generates nutritive intermediates. *S. unisporus* could consume vast range of carbon sources excluding lactose and 0.4% (w/v) of ammonium sulphate as nitrogen source. *S. unisporus* was found to be strictly urea-negative. The mono-culture of *S. unisporus* was found enriched in lysine (84.0 mg/g), valine (74.4 mg/g), asparatic acid (83.3 mg/g) and glycine (77.0 mg/g). The *S. unisporus* biomass had the essential minerals including Ca, S, Na, Mg, P and K. The fatty acid content observed for *S. unisporus* were 0.86% (w/w) and protein content of 4.2 (g/L). The ideal pH of *S. unisporus* for SCP production as a mono-culture was observed at 5.5.

Further acid hydrolysis of cheese whey and permeate were performed and *S. unisporus* was grown as mono-culture and mixed culture with *K. marxianus*. It was observed that 0.2% (w/w) of H_2SO_4 can perform hydrolysis of whey permeate with 90% lactose converted to glucose and galactose in 70 min. However 0.4% (w/w) of H_2SO_4 converted lactose from cheese whey into glucose and galactose in 60 min. *S. unisporus* and *K. marxianus* were grown in hydrolyzed whey along with 0.4% (w/v) of ammonium sulphate and it was observed that *K. marxianus* (7.8×10^8 CFU/mL) has better growth than *S. unisporus* (5.6×10^7 CFU/mL) because *S. unisporus* can partially consume galactose whereas *K. marxianus* can consume both the monosaccharides and the left over lactose.

Eventually, fermentation with the mixed culture (*S. unisporus* and *K. marxianus*) has shown slight increment in the essential amino acid content especially with leucine and isoleucine (79.9 mg/g), whereas in the case of mono-culture *S. unisporus* biomass of only 59 mg/g was observed. It was also observed that mixed culture biomass production generated intermediates (furanmethanol, furfuryl formate, weak organic acids), which have biotechnological importance. However no quantification has been performed.

Therefore for the production of food and feed, elimination of the biological contaminant was performed followed by addition of the acceptable yeast. *C. krusei* was killed in an economic and green synthesis approach by maintaining the fermentation conditions at pH 5.5 and temperature 30 °C with the help of bio preservative Ag-KT4561. After the removal of *C. krusei*, *S. unisporus* was added as acceptable yeast in mono-culture fermentation and in mixed-culture fermentation. *S. unisporus* has enhanced SCP quality in terms of nutritive value when grown along with *K. marxianus* in cheese whey and permeate. Therefore based on these two set of studies it is concluded that biological contaminant (*C. krusei*) can be removed aseptically in mixed culture fermentations in an economical green chemistry approach.

AVANT -PROPOS

PUBLICATIONS/MANUSCRIPTS DANS CETTE THÈSE

1. **Bhattacharya, I.,** Bezawada, J., Chandran, A., Yan, S., and Tyagi R.D. (2015). *Candida krusei*: factors influencing its growth and inhibition by chemical, biochemical and nanoparticle approaches. *Journal of Microbiology, Biotechnology and Food science* (under review).
2. **Bhattacharya, I.,** Bezawada, J., Yan, S., and Tyagi R.D. (2015). Optimization and production of silver-protein conjugate as growth inhibitor. *Journal of Bionanoscience,* 9(4), 261-269.
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4. **Bhattacharya, I.,** Yan, S., Yadav, J. S. S., Tyagi, R. D., & Surampalli, R. Y. (2013). *Saccharomyces unisporus*: Biotechnological Potential and Present Status. *Comprehensive Reviews in Food Science and Food Safety*, *12*(4), 353-363.
5. **Bhattacharya, I.,** Bezawada, J., Yan, S., and Tyagi R.D. (2015). Nutritional profile of *Saccharomyces unisporus* for single cell protein production: from classical to genomic approach. *Journal of Microbiology, Biotechnology and Food science* (under review).
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2. **Bhattacharya, I.**, Yan, S., Tyagi, R.D., and Surampalli, R.Y. (2012). Biomass production and simultaneous bioconversion of toxic furfural from cheese industry waste water (cheese whey) into furanmethanol and furancarboxyaldehyde (Poster). Dans: 27e Congres de L'Est Du Canada sur Canadian Association on water Quality (CAWQ), Sherbrooke, Canada.

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1. **Indrani Bhattacharya**, R.D. Tyagi, Jose Valero. (2014). Recovery of PHA using different detergents and optimization of different process parameters. Report No : 1354, INRS-ETE, Centre de Eau Terre Environnement, Quebec Canada, submitted on : 1st July, 2014.

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LISTE DES ABRÉVIATIONS

Ag-KT4561	Silver-killer protein
AgNPs	Silver Nanoparticles
AOX	Alternative Oxidase
AT	Adenine-Tyrosine
ATCC	Ameican Type Culture Collection
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
CW	Cheese Whey
EDTA	Ethylenediaminetetraacetic Acid
EDS	Energy Dispersive X Ray Spectrometry
EFFCA	European Food and Feed Cultures Association
EPA	US Environmental Protection Agency
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FAME	Fatty Acid Methyl Ester
FDA	Food and Drug Administration
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Regarded as Safe
GT	Gliotoxin
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectrometry
IDF	International Dairy Federation
IFO	Institute for Fermentation, Japan
LAB	Lactic Acid Bacteria

LC-MS/MS	Liquid Chromatography-Mass Spectrometry
MMP	Mitochondrial Membrane Potential
MUFA	MonoUnsaturated Fatty Acids
NPs	Nanoparticles
PCA	Principal Component Analysis
PMSF	PhenylMethylSulfonyl Fluoride
PUFA	PolyUnsaturated Fatty Acids
ROX	Reactive Oxygen Species
SAP	Secreted Asparatic Proteinases
SCP	Single Cell Protein
SEM	Scanning Electron Microscopy
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV-Vis	UV-Visual Spectrometry
WP	Whey Permeate
YEPD	Yeast Extract Peptone Dextrose
YT	Yeast Extract Tryptone

CHAPTIER I

SYNTHÈSE

REVUE DE LITTÉRATURE

1. INTRODUCTION

Dans les industries de transformation des aliments, le fromage est l'un des principaux produits laitiers qui joue un rôle important dans la nutrition humaine. Le type de fromage produit dépend fortement des conditions appliquées au cours de la production ainsi que de la technologie envisagée pour le produire. Étant donné que le lait est périssable, le fromage est considéré comme étant une meilleure alternative car il a une plus longue durée de vie. Au cours de la production du fromage, une grande quantité de déchets liquides, sous forme de lactosérum, est générée. Selon la littérature, le lactosérum est formé suite à la coagulation de la caséine du lait (Hinrichs 2001). Malgré son importance, le lactosérum représente une source de contamination des eaux (Chegini *et* Taheri 2013). Les recherches effectuées par Chatzipaschali *et* Stamatis (2012) ont montré que des valeurs de DCO et de DBO supérieures à 60 000 mg⁄L et 30 000 mg⁄L respectivement ont été enregistrées. En effet, les plus importants producteurs de lactosérum au monde sont les États-Unis (4,4 Mt), suivis par le Brésil (0,5 Mt), l'Argentine (0,4 Mt) et l'Australie (0,3 Mt) produisant au total 190 Mt par an dans le monde (Juliano *et* Clarke 2013, Mollea *et al.,* 2013). Environ 35% du lactosérum de fromage est utilisé comme nourriture pour les animaux. Le reste du lactosérum de fromage est utilisé en tant que boissons riches en protéines tels que les boissons protéinées, le lait fermenté et les protéines de sport. De plus, le lactosérum de fromage peut être directement utilisé dans l'irrigation (comme engrais agricole) et en tant que support d'herbicides (Lambou *et al.,* 1975).

Le principal constituant du lactosérum est le lactose dont la contribution dans la demande en oxygène est assez importante. La récupération de certaines protéines présentes dans le lactosérum permet de produire environ 10 000 mg⁄L de la DCO. Cependant, la présence de certains métaux lourds tels que le Cd et le Pb en faibles concentrations contribue à l'effet polluant de ce liquide. En effet, la présence de tels composants dans le lactosérum provoquent des effets toxiques sur les microorganismes aquatiques. Sa disposition dans le sol peut aussi contribuer à son effet toxique (Juliano *et* Clarke 2013). Selon l'union européenne, 45% du lactosérum de fromage peut être transformé en produits alimentaires

utilisés directement sous forme de poudre (comme concentrés de protéines) et en tant qu'additifs dans les boissons suite à la présence du lactose présent dans le lactosérum de fromage (Chatzipaschali *et* Stamatis 2012). Il est possible d'extraire le lactose par cristallisation. Ce phénomène consiste à fragmenter le lactosérum à une température allant de 50 à 75 °C et en présence d'un pH variant entre 5,5 et 7 en utilisant une colonne de résine de polystyrène sulfonée. Par conséquent, le lactosérum sera concentré et une partie du lactose sera sous forme cristallisée. Les cristaux ainsi formés seront par la suite séchés (Harju *et al.,* 1990). Le lactose est principalement utilisé dans les préparations pour nourrissons ou comme agent de revêtement des comprimés dans les industries pharmaceutiques. Cependant, l'utilisation du lactose en tant que produit alimentaire est limitée étant donné sa faible digestibilité et sa faible solubilité due à sa forme cristalline. D' autre part, le lactose venant remplacer le sirop d'amidon peut être hydrolysé et utilisé comme un édulcorant dans la confiserie. Ce sucre peut également être utilisé lors de la fermentation en tant que substrat pour la production de produits à valeur ajoutée tels que les protéines à cellule individuelle (PU) et d'autres produits biologiques (éthanol, butanol, des acides organiques et des enzymes) (Chatzipaschali *et* Stamatis 2012).

D'autres études ont montré que les molécules de lactose peuvent être extraites par ultrafiltration. En effet, une fois le lactosérum introduit dans la membrane, seules les petites molécules du lactose seront capables d'y passer. Le perméat, dans ce cas, présentera le reste du lactosérum. Les molécules de lactose ainsi éluées seront utilisées dans de nombreuses industries alimentaires (Barile *et al.,* 2009). Il est bien connu que la protéine de lactosérum est une protéine de haute qualité et qui est très riche en acides aminés (Chegini *et* Taheri 2013). L'élimination de ce composé peut être réalisée par l'intermédiaire des microorganismes aquatiques. Ces derniers diminuent considérablement le taux de pollution dans les eaux usées. Les PU produites suites à l'utilisation du lactose sont capables de nourrir toute une population à un coût minimum. Les protéines sont les essences de la vie et doivent être abordables pour l'homme et les animaux. Les protéines microbiennes peuvent être une autre source cultivée indépendamment des conditions environnementales (Gomashe *et al.,* 2014). Les levures telles que *Kluyveromyces* et *Candida* sont cultivées dans le lactosérum de fromage pour produire le PU. Elles peuvent facilement métaboliser le lactose présent dans le

lactosérum de fromage; c'est pourquoi *Kluyveromyces* est l'organisme le plus largement utilisé en monoculture. Les cultures mixtes sont principalement utilisées pour la production de PU. Les combinaisons largement utilisées pour la production de PU sont des souches de *Kluyveromyces* et de *Saccharomyces cerevisiae* (Moeini *et al.,* 2004). Le microbe produisant le PU doit tirer les nutriments du lactosérum de fromage pour le maintien de la substance cellulaire (Ghaly *et al.,* 1993).

1.1 CARACTÉRISTIQUES DU LACTOSÉRUM DE FROMAGE

1.1.1 Caractéristiques physiques

Le lactosérum de fromage est un liquide jaune-vert qui est évacué à partir du fromage lors de sa fabrication en grains. Le lactosérum a un goût fade mais s'oxyde rapidement en formant des arômes rassis. Le lactosérum se compose de la moitié de protéines trouvées dans le lait (Chegini et Taheri 2013). Dans certaines parties du monde, les animaux tels que les chameaux, les chèvres et les moutons sont même utilisés pour la production du fromage et du lactosérum de fromage, car ce dernier peut être produit à partir de n'importe quel type de lait (Ghaly *et* Singh 2007). Une étude comparative du lait et du lactosérum est présentée à la figure 1.1.

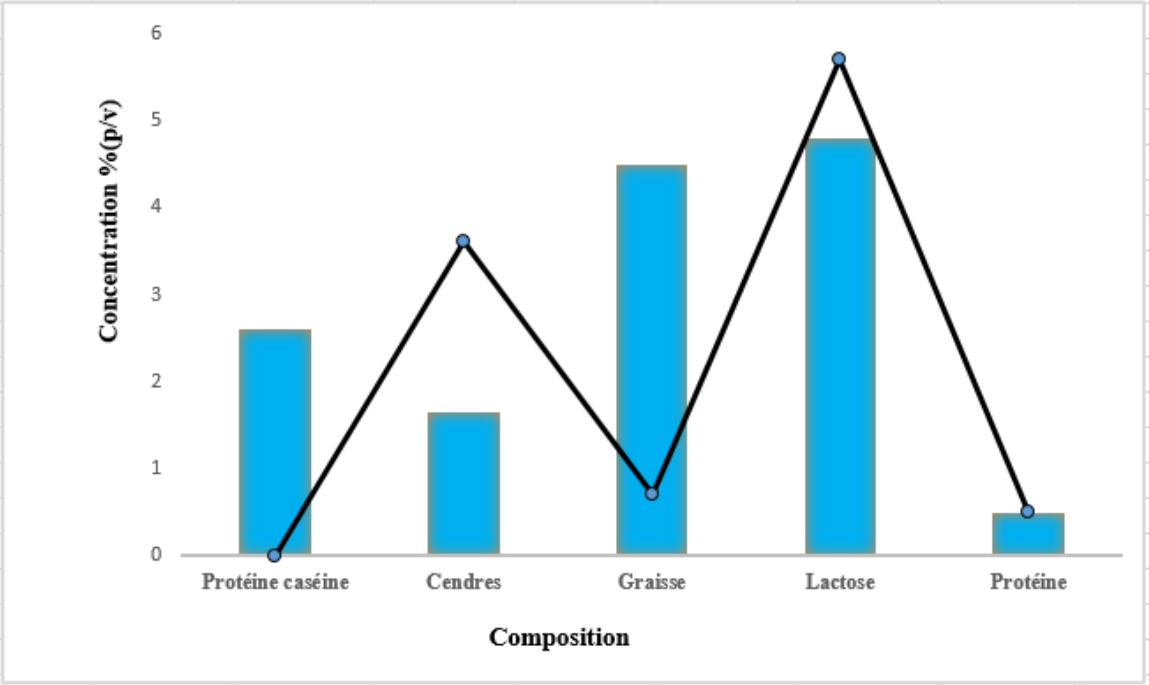

Figure 1.1 Composition du lactosérum et du lait de vache

1.1.2 Caractéristiques chimiques

Le lactosérum se présente sous forme de quatre catégories différentes : le lactosérum sucré, le lactosérum acide, le lactosérum de caséine et le lactosérum salé (Awad *et al.*, 2013). La composition du lactosérum varie en fonction de la source de lait ou de la méthode de production. Bien que les composants principaux du lactosérum soient les glucides (lactose), d'autres minéraux sont présents en faibles quantités tels que les vitamines A, D, le fer, le cuivre et l'iode. Le lactosérum ne peut pas être utilisé sous forme liquide, il est donc transformé en divers produits tels que la poudre de lactosérum séchée, le concentré protéique de lactosérum et le lactosérum condensé (Chegini *et* Taheri 2013). La caséine et les protéines du lactosérum sont les principaux constituants des protéines de lait. La caséine constitue 80% de la teneur en protéines présentes dans le lait et est isolée soit par précipitation ou par l'ajout d'un acide. Les 20% restants sont les protéines de lactosérum qui ont des protéines globulaires. Ces protéines globulaires sont

solubles dans une large gamme de pH (Sindayikengera *et* Xia 2006). Une composition comparative de lactosérum acide, salé et sucré est présentée à la figure 1.2. Le lactosérum acide présente un pH inférieur à 5 et le lactosérum sucré présente une valeur de pH entre 6 et 7. Le lactosérum salé a une concentration maximale en sel de 15% (p/v) (Vardhanabhuti *et al.*, 2001). Le lactosérum salé est difficile à traiter en raison des niveaux élevés de salinité (Awad *et al.*, 2013). Le lactosérum acide contient moins de protéines en raison de la nature acide élevée (Chatzipaschali *et* Stamatis 2012).

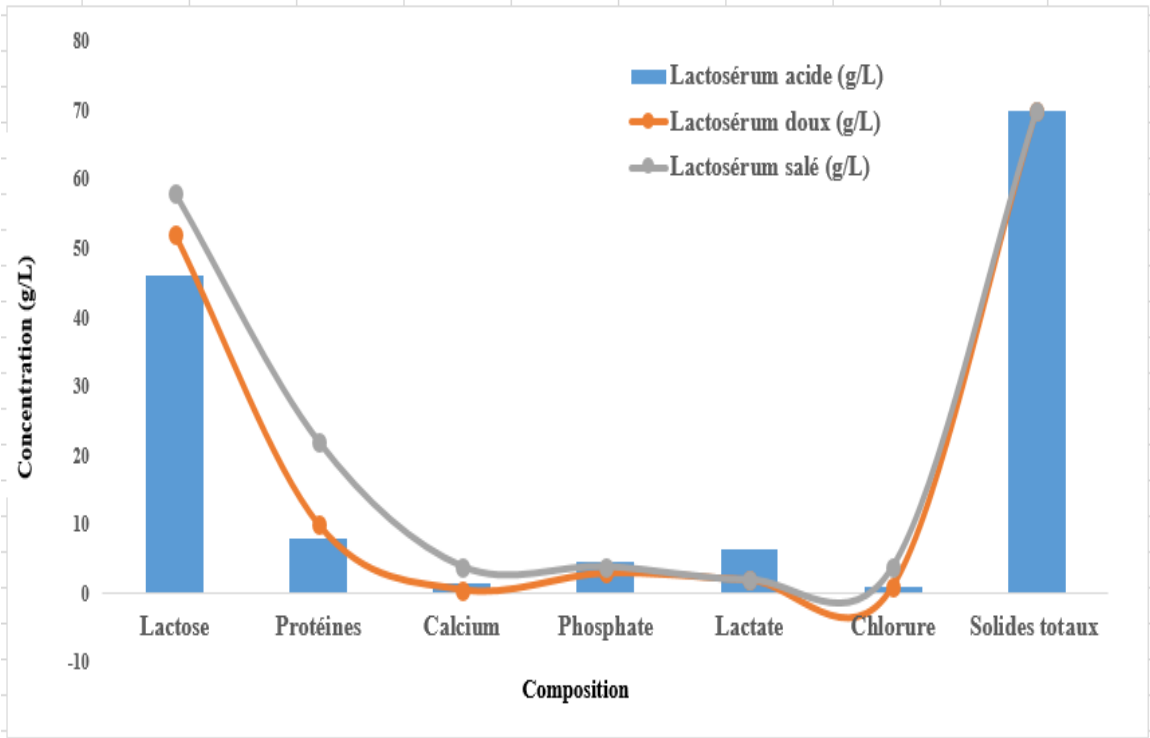

Figure 1.2 Comparaison de lactosérum acide, sucré et salé

1.2 UTILISATION DU LACTOSÉRUM DE FROMAGE POUR LA SYNTHÈSE DE PRODUITS À VALEUR AJOUTÉE

1.2.1 Produits à valeur ajoutée

La protéine de lactosérum a une valeur biologique similaire à d'autres sources de protéines comme le soja ou les protéines d'œuf. La valeur biologique peut être définie comme le rapport des nutriments requis et utilisés par le corps. Il est démontré qu'au cours des années, suite à la transformation enzymatique du lactose en galacto-oligosaccharides et en fructo-oligosaccharides, la protéine du lactosérum peut être utilisée comme probiotique (Cruz *et al.,* 2010). Des techniques telles que la filtration et l'ultrafiltration sont utilisées pour concentrer la protéine de lactosérum jusqu'à 50% (Jauregi *et* Welderufael 2010). De plus, environ 50% du lactosérum produit est transformé en alimentation humaine et animale. Il s'agit notamment de 30% (poudre de lactosérum séchée) et le reste est converti en produits industriels importants comme la biomasse riche en protéines unicellulaires (PU), les substances polymériques extracellulaires (SPEs), les enzymes, les produits pharmaceutiques, le bioéthanol (Briczinski et Roberts 2002, Mollea *et al.,* 2013). Dans certains cas, l'hydrolyse du lactose est effectuée lors de la fermentation de sorte qu'il peut être consommé par les levures. Certains micro-organismes tel que *Saccharomyces cerevisiae* qui sont impliqués dans la fermentation du lactosérum de fromage ou du perméat de lactosérum ne sont pas en mesure d'utiliser le lactose directement, donc le lactose est hydrolysé en glucose et galactose dans le but de faciliter son assimilation par les microbes et produire des PU (Khan *et al.,* 2011). Autres que les PU, d'autres produits à valeur ajoutée sont mentionnés dans la figure 1.3 et le tableau 1.1. Par exemple, le bioéthanol est produit par des souches de *Kluyveromyces* qui est en outre utilisé pour la production d'acide acétique (Nasseri *et* al., 2011).

Suite à la fermentation du lactose, divers autres acides organiques sont produits tels que l'acide lactique, l'acide citrique, l'acide malique et l'acide propionique (Mollea *et al.,* 2013). Les principales protéines du lactosérum (β-lactoglobuline, α-lactalbumine, la lactoferrine, le lysozyme, l'albumine de sérum bovin, des immunoglobulines (Ig), la

lactoperoxydase) sont des protéines qui présentent des acides aminés essentiels et ramifiés. Ceux-ci peuvent renforcer le système immunitaire des êtres humains et apporter des avantages pour la santé. Un autre fractionnement de protéines peut établir plus de valeur complémentaire pour les formules infantiles et boissons sportives (Jauregi *et* Welderufael 2010). Les produits de protéines de lactosérum peuvent être présentés sous trois formes principales : a) hydrolysats b) isolés et c) concentrés. Celles-ci ont une acceptation limitée par les industries agro-alimentaires; indépendamment du fait que toutes les protéines de lactosérum ont des qualités diverses, y compris des applications nutritionnelles et biologiques (Mollea *et al.*, 2013).

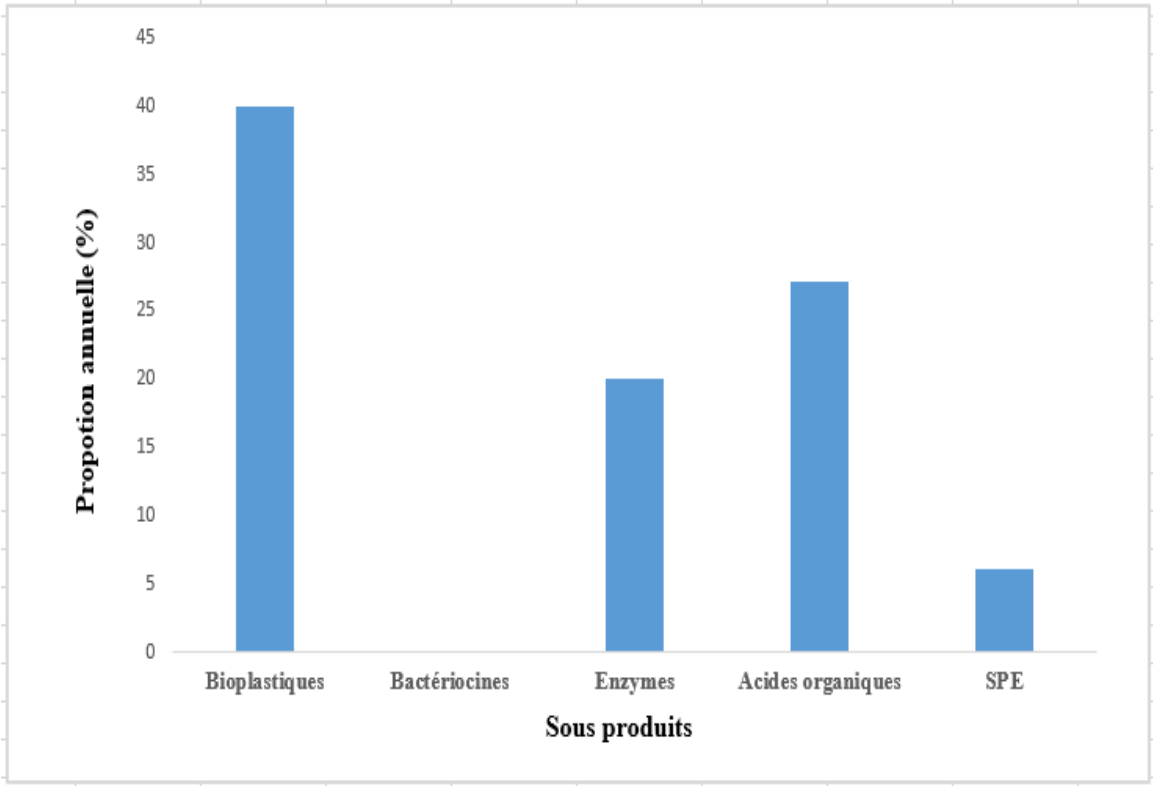

Figure 1.3 Proportion annuelle (%) de produits à valeur ajoutée fabriqués à partir du lactosérum de fromage

Tableau 1.1 Utilisation de lactosérum pour la valeur ajoutée

Catégories des produits	Microorganismes	Produits	Réferences
Probiotiques et Prébiotiques	*Candida valida*, *Candida kefir*	biomasse (levure), protéines unicellulaires	Paraskevopoulou *et al.,* 2003; Aguirre-Ezkauriatza *et al.,* 2010
Enzymes	*Kluyveromyces marxianus*	β-galactosidase	Fonseca *et al.,* 2008
	Bacillus spp.	α-amylase	Bajpai 1991
	Candida rugosa	lipase	Tommaso *et al.,* 2010
Protéines	*Lactobacillus lactis*	nisin (Bacteriocin)	Liu *et al.,* 2005
	Kluyveromyces marxianus	ACE-inhibitory peptides	Hamme *et al.,* 2009
Biopolymères	*Kluyveromyces marxianus*	oligonucleotides, galacto-oligosaccharides	Petrova *et* Kujumdzieva 2014
	Rhizopus oryzae	chitosane	Chatterjee *et al.,* 2008
	Azotobacter chrooccum	PHB	Khanafari *et al.,* 2006
	Leuconostoc mesenteroides	dextrane et fructose	Santos *et al.,* 2005
Agent de biocontrôle	*Beauveria bassiana, Bacillus sphaericus, Metarhizium anisopliae*		El-Bendary *et al.,* 2008, Kassa *et al.,* 2008

Acides organiques	*Aspergillus niger*	acide citrique, acide gluconique	Mukhopadhyay *et al.*, 2005, El Aasar 2006
	Anaerobiospirillum succiniciproducens	Alimentation animale riche en succinate	Samuelov *et al.*, 1999
	Kluyveromyces fragilis	Acide Acétique et glycérol	Mostafa 2001
	Bactéries lactiques (LAB)	Acide lactique	Panesar *et al.*, 2007
	Bacillus licheniformis K51	Biosurfactant	Joshi *et al.*, 2008

1.2.2 Protéines unicellulaires (PU)

Le secteur agricole est très important dans des pays comme les États-Unis. Généralement, les déchets agricoles générés au cours de ces activités sont utilisés pour la production des PU (Ware *et* Clarence 1977). La pénurie mondiale de protéines est un problème majeur. Des efforts sont déployés afin d'opter pour des sources de protéines alternatives et non conventionnelles. De nouvelles protéines sont produites par des microbes tels que les levures, les bactéries, les algues et les champignons. Ces composés biologiques sont connus sous le nom de protéines unicellulaires (PU). Peu importe les conditions saisonnières, ces microbes sont capables de produire des quantités élevées en PU utilisées par la suite dans les denrées alimentaires. Cependant, la valeur nutritionnelle de ces protéines est limitée par le code génétique de ces microbes (figure 1.4). Contrairement aux levures, les cellules bactériennes présentent une faible taille ce qui réduit leur capacité à produire des PU. D'autre part, les levures contiennent de faibles teneurs en méthionine (Israelidis, 2013). La biomasse de microalgues est utilisée dans la fabrication de produits pharmaceutiques. Ainsi, les PU seront par la suite utilisées dans l'alimentation animale et marine (Nasseri *et al.,* 2011). En se basant sur la quantité des acides nucléiques, les levures ont moins d'acides nucléiques en comparaison avec d'autres microorganismes (bactéries, champignons, algues) (Bekatorou *et al.*, 2006).

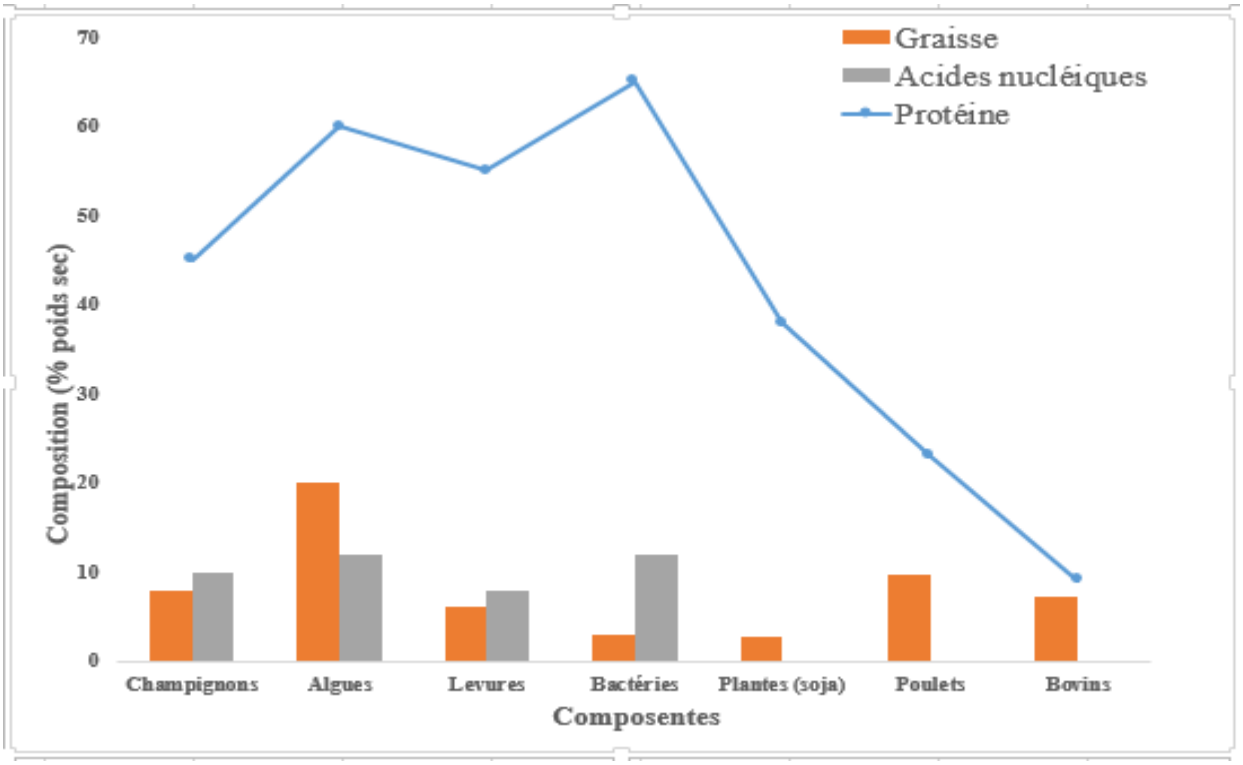

Figure 1.4 Comparaison de différents microorganismes basée sur les protéines, les graisses et les acides nucléiques

La bioconversion des protéines de lactosérum en biomasse protéique est la meilleure option pour l'utilisation du lactosérum (Grba *et al.*, 2002). Bien que les microbes assimilant le lactose soient la meilleure option utilisée pour la production de la biomasse, *Saccharomyces cerevisiae*, même si elle est une levure non-consommatrice de lactose, est considérée comme l'organisme modèle pour la production des PU (Khan *et al.*, 2011).

1.3 Choix du microorganisme

Le choix d'un microorganisme est basé sur certains prérequis. Le microorganisme ne doit ni être pathogène, ni produire de toxines. Si le PU produit par le microorganisme est incorporé dans l'alimentation, celui-ci doit être sans arrière-goût tout en assurant les propriétés fonctionnelles du met dans lequel il est ajouté. Les conditions de fermentation pour la production de PU devraient permettre une bonne croissance cellulaire maximale, un bon rendement, pH, tolérance à la température et des coûts économiques avantageux. Les conditions de la fermentation ne devraient pas permettre une contamination biologique ou chimique. Un autre facteur significatif

portant sur le choix du microorganisme produisant le PU réside dans l'efficacité de celui-ci à convertir les nutriments du milieu de culture en protéine (Moon 1977, Ware *et* Clarence 1977). La stabilité de la culture durant la fermentation est également importante. D'autres facteurs qui affectent la production des PU sont le substrat et la source de carbone présent dans le substrat. Le coût du substrat et la nature de celui-ci sont également des facteurs à prendre en compte (Detroy *et* Hesseltine 1978). Le facteur final dans la production de PU est la facilité à récolter celui-ci. La production de PU entre dans la ligne des technologies qui ont pour objectif de réduire la pénurie de protéines. Ceux-ci évoluent en même temps que les procédés de bioconversion permettant de valoriser des milieux résiduels en milieu nutritionnel. Les microorganismes sélectionnés permettraient une production importante de biomasse, riche en source de protéines. Le microorganisme le plus utilisé afin de produire du PU est le *S. cerevisiae*. Khan *et al.* (2011) ont produit différents volumes de protéines en utilisant cette même souche dans différents déchets provenant de fruits. Ces microorganismes sont capables de croître dans des milieux résiduels à faible coût.

En général, les champignons ont la capacité de croître dans un spectre important de glucide. Ils peuvent également résister à des pH très bas, ce qui est intéressant dans la mesure où les contaminants tolèrent moins les pH acides. Toutefois, comparativement aux bactéries et aux levures, leur taux de croissance est plus faible. De plus, il est noté dans la littérature que certains types de champignons produisent des toxines et que leur mycélium est déficient en méthionine (Ware *et* Clarence 1977). En général, les bactéries ont un taux de croissance important et supportent des températures élevées. L'aération est facilitée dans les fermentations bactériennes. Les bactéries produisent des protéines intéressantes, comparativement à celles des levures. Les protéines contenues dans les bactéries avoisinent 60 à 66% tandis que cette concentration tourne autour de 45% chez les levures. La stabilité génétique est un facteur vital chez les bactéries (Vardhanabhuti *et al.,* 2001). Cependant, des endotoxines peuvent se retrouver chez certaines cellules bactériennes alors que les algues entraînent une pauvre digestibilité chez l'homme et les non-ruminants. Les algues entraînent parfois une saveur âpre qui n'est pas acceptable dans l'alimentation humaine et animale (Nizet *et* Esko 2009). Parmi les levures, la production de PU est limitée majoritairement par l'assimilation du lactose par les levures (Ghaly *et al.,* 2003). Toutefois, les levures qui ont de la difficulté à assimiler le lactose (*candida* spp et

saccharomyces spp) peuvent croître grâce à des produits intermédiaires. Les microorganismes assimilant le lactose pour la production de PU sont usuellement les espèces *Kluyveromyces* telles que *Kluyveromyces marxianus*, *Kluyveromyces fragilis*, *Kluyveromyces bulgaricus*, et *Kluyveromyces lactis* (Mahmoud *et* Kosikowski 1982). L'objectif principal pour la culture de *Kluyveromyces* est de produire une biomasse et de réduire la charge organique (Schultz *et al.*, 2006). Toutefois, la production de composés intermédiaires a un impact négatif sur la production de la biomasse. La dégradation du COD est efficace avec une concentration élevée de *K. marxianus* (Yadav *et al.*, 2014)

1.3.1 Microorganisme non accepté

Les microorganismes non souhaitables dans l'alimentation humaine et animale sont mentionnés ci-dessous:

1.3.1.1 *Candida krusei*

C. krusei est un agent pathogène, parmi les espèces non-*albicans* de *Candida*, qui est généralement isolé à partir de patients ayant une déficience immunitaire et de ceux qui ont des hépatologies malignes (Fleischmann *et* Sripuntanagoon 2011). L'infection causée par *C. krusei* est la fongémie, qui est associée à une mortalité importante (Abbas *et al.*, 2000). Les échantillons cliniques de *C. krusei* sont collectés et traités grâce à des médicaments capables d'éliminer les infections telles que le fluconazole, qui réprime *C. krusei* pour une durée limitée (Wingard *et al.*, 1991, Samaranayake, 1997, Dassanayake *et al.*, 2000, Arévalo *et al.*, 2003)

C. krusei est considérée comme un champignon pathogène résistant aux médicaments (Taylor *et* Webster 2011). Les azoles qui ont été utilisés pour la suppression du cytochrome P-450, qui est l'isoenzyme de *C. krusei*, ont échoué. *C. krusei* diffère des autres espèces de *Candida*. Elle est capable de croître à haute température (42 °C), en aérobie et en anaérobie. *C. krusei* est une levure formant une pellicule (Fleischmann *et* Sripuntanagoon 2011). C. krusei se classe chez les levures ascoporogènes de la classe des saccharomycetoidées (Kreger-van Rij 1984). Beaucoup d'inhibiteurs biologiques ont été utilisés afin d'inhiber la croissance de *C. krusei*. Ces inhibiteurs sont des protéines ou d'autres composés produits par des microorganismes. Apparemment, *C. krusei* développe une résistance à certains inhibiteurs qui possèdent des effets contre *Candida*. Ceci dépend du volume d'inhibiteurs produits, lorsqu'ils sont consommés par *C. krusei*, ce qui

affecte sa croissance. À cause de cette raison, les analyses des voies métaboliques sont obligatoires, étant donné que certains composés (inibiteurs) sont produits à certaines températures. Afin de bien comprendre l'effet pathogène engendré par *C. krusei*, il est important de comprendre la physiologie cellulaire de *C. krusei*. Il est également important de déterminer les protéines, les lipides et les autres métabolites/toxines qui sont responsables des effets pathogènes de *C. krusei*. Toutefois, *C. krusei* est utilisé comme un déclencheur d'inoculum dans une culture mixe associée à l'espèce *Saccharomyces* dans certains produits alimentaires.

1.3.1.1.1 Toxicité de *C. krusei*

La gliotoxine (GT) est un métabolite secondaire des champignons, qui a des propriétés immunosuppressives pléiotropiques (mutation des gènes). Celui-ci fait partie de la classe des épipolythiodioxopiperazines (Gardiner *et al.,* 2005). Les gliotoxines sont divisées en gliotoxines extracellulaire et intracellulaire. La gliotoxine inhibe le farnesyl transférase (impliqué dans la régulation cellulaire) (Reid *et al.,* 2004, Vigushin *et al.,* 2004). Elle est également impliquée dans la virulence chez les espèces de *Candida*. La structure de GT comprend des ponts disulfures qui peuvent inactiver les protéines par des réactions avec le groupement thiol dans la membrane cellulaire (Chamilos *et al.,* 2008). La gliotoxine interfère avec le métabolisme du glutathionne situé à l'intérieur de la cellule (Beaver et Waring, 1996). La candidiase causée par la famille des *Candida* est associée à des concentrations élevées en gliotoxine (Shah *et al.,* 1995, Khan *et al.,* 2010). La gliotoxine est considérée comme une forme d'endotoxine (molécule structurale de la bactérie reconnue par le système immunitaire) (Birdsall 2011). L'identification de la mycotoxine ou de la gliotoxine dans un tissu ou chez le fluide corporel d'un patient indique la présence de *C. krusei* ou d'une autre espèce de *Candida*.

Les protéinases aspartiques (PAS) sécrétées chez les *Candida* qui sont capables de détruire les tissus sont les PAS 2, 5 et 9 (Naglik *et al.,* 2003, Khan *et al.,* 2010). Les cellules de *Candida* contiennent des liaisons entre les protéinases et les cellules (Pärnänen 2010) à pH 4,5 (Hamal *et al.,* 2004). Naglik *et al.,* (2008) ont prouvé que *C. krusei* est le plus faible producteur de PAS et ce ne sont pas toutes les souches de *C. krusei* qui sont protéolytiques (Dosta´l *et al.,* 2003). Ozkan *et al.,* (2005) ont déterminé que la production de protéases extracellulaires par *C. krusei* peut causer des mycoses chez les êtres humains.

Hwpl est la première protéine de surface connue chez les cellules de *Candida*, ce qui fait d'elle une excellente source thérapeutique (Nobile *et al.,* 2006). L'adhésion des protéines/hwp1 (mannoprotéine) est réticulée à l'épithélium cellulaire humain par transglutaminase, causant la candidièse (Daniels *et al.,* 2003, Laín *et al.,* 2007). Les protéines Hwpl sont considérées comme des tueuses silencieuses (D'Enfert 2009) étant donné qu'elles sont similaires au gluten du blé et qu'elles interfèrent dans le mécanisme de la coagulation sanguine. Ainsi, elles sont considérées comme des tueuses silencieuses, une fois à l'intérieur du système sanguin (Nieuwenhuizen *et al.,* 2005, Panteris *et* Karamanolis 2005, Stepniak *et* Koning 2006).

Les autres protéines produites par *C. krusei* sont l'hyaluronidase et le sulfatase chondroitine qui sont considérés comme étant des facteurs virulents. D'autres protéines virulentes sont les phospholipases qui sont capables d'envahir les tissus (Khan *et al.,* 2010). Certains des hydrolases synthétisés par *C. krusei* sont des hydrolats de caséines et des hydrolats d'hémoglobine (Okumura *et al.,* 2007). De plus, quelques protéines à faible poids moléculaire sont produites par *C. krusei* (Vigushin *et al.,* 2004)

La production de mannoprotéines se déroule dans le cas du *C. krusei* lorsque le sucre utilisé est du glucose (Henrique *et al.,* 2006). Toutefois, la présence de mannoprotéines (protéines avec un grand nombre de groupe de mannose qui sont hautement antigéniques) n'est pas détectée lorsqu'il y a présence d'autres formes de glucides. *C. krusei* ne consomme pas le lactose, donc sa présence n'est pas relevée dans des produits contenant du lactose (Bekatorou *et al.,* 2006). Les composants de PU provenant de *C. krusei* sont: protéines (47-50% (p/p)), gras/lipides (5-13% (p/p)), et acides nucléiques (9,70%) (FAO) (Konlani *et al.,* 1996).

1.3.1.1.2 Inhibiteurs connus de *C. krusei*

Les inhibiteurs de *C. krusei* sont répertoriés en termes chimiques, biochimiques et nanoparticulaires (NPs). Ces derniers sont utilisés afin d'éradiquer les *C. krusei*.

1.3.1.1.2.1 Inhibiteurs chimiques comme agents antimicrobiens

Candida krusei est devenu au fil du temps un agent pathogène envahissant de plus en plus important, en particulier chez les patients immunodéprimés (Venkateswarlu *et al.,* 1997,

Macreadie *et al.,* 2006). Pour éradiquer *I. orientalis* (*C. krusei*), la plupart des thérapies sont conçues pour viser : 1) un composant de la paroi cellulaire unique aux champignons (Georgopapadakou 2001, Gozalbo *et al.,* 2004); 2) la voie de biosynthèse de l'ergostérol ou son produit final, l'ergostérol (un stérol). Des médicaments antifongiques sont utilisés de manière efficace pour des conditions spécifiques telles que des infections aux levures *Candida*. Les études sur l'utilisation des médicaments antifongiques sont soutenues par le National Institute of Allergy and Infectious Diseases et le Mycology Research Unit de l'Université Duke aux États-Unis. Des cas ont été rapportés où ces médicaments antifongiques ont causé de rares anomalies foetales chez les animaux, cependant leur utilisation a été approuvée par la FDA (Food and Drug Administration des États-Unis).

Candida spp causent des maladies graves chez les patients immunodéprimés. L'introduction d'inhibiteurs de la calcineurine comme agents antifongiques a causé une augmentation de la sensibilité de *C. krusei* aux antifongiques qui s'attaquent à l'intégrité de la membrane cellulaire. *In vitro,* le fenpropimorphe et la terbinafine montrent une activité fongicide synergique avec les inhibiteurs de calcineurine contre *C. krusei.* Ces classes de médicaments antifongiques sont restreintes à des utilisations agricoles ou à des applications médicales pour traiter des infections systématiques en raison d'une meilleure efficacité en thérapie combinée, ce qui constitue une approche thérapeutique viable. Parmi les médicaments antifongiques, les inhibiteurs de la calcineurine, spécifiquement FK506, inhibent fortement Erg2 et Erg24 de la voie métabolique de *C. krusei* (Onyewu *et al.,* 2003, Reedy *et al.,* 2006, Steinbach *et al.,* 2007, Walker *et al.,* 2008, Kelly *et* Kavanagh 2011). Les azoles sont plus fongistatiques que fongicides.

Le miconazole inhibe la biosynthèse de l'ergostérol (un composant essentiel des membranes cellulaires fongiques) conduisant à des changements de perméabilité. Des intermédiaires méthylés de stérol qui sont toxiques s'accumulent suite à l'inhibition par le miconazole, celui-ci modifie le cytosquelette d'actine et produit des dérivés réactifs de l'oxygène (DRO) causant la mort de cellules. Ces DRO sont des avenues potentielles pour des applications thérapeutiques dans le traitement de la candidose buccale (Wei *et al.,* 2011). La métergoline favorise la mort des cellules de *C. krusei* par la dépolarisation du potentiel de la membrane mitochondriale et l'accumulation de DRO, suivie de la translocation de la membrane plasmique de la

phosphatidylsérine vers la surface externe et des dommages à l'ADN. En résumé, les cellules de *Candida* spp meurent par des processus chimiques (Kang *et al.,* 2011).

Les échinocandines sont spécifiques aux cellules fongiques. La 1, 3-D-glucane synthase est absente des cellules humaines et son mode d'action unique est spécifique aux parois cellulaires fongiques, ce qui élimine potentiellement la toxicité pour l'être humain (McGee *et* Tereso 2003). Il a été rapporté que des composés de plantes médicinales chinoises et coréennes (berbérines) possèdent une activité antifongique et une faible toxicité pour l'hôte (Park *et al.,* 1999, Enriz *et* Freile 2006, Wei *et al.,* 2011). L'acide sorbique et l'acide benzoïque sont efficaces à des températures légèrement plus élevées que la température maximale pour la croissance des microorganismes (Maneesri *et* Maneesri 2009). Les alcaloïdes de *L. angustifolius* et *Genista* spp. inhibent spécifiquement la souche ATCC 14243 de *C. krusei*, le mécanisme d'inhibition n'est cependant pas connu (Erdemoglu *et al.,* 2007).

L'inhibition par l'acide salicylhydroxamique est basée sur l'hypothèse que l'activité des halogènes organiques adsorbables (AOX) augmente lorsque la capacité de la voie qui dépend du cytochrome est dépassée puisque (i) la constante de Michaelis (*K*m) pour l'ubiquinol du complexe du cytochrome *bc*1 est beaucoup plus faible que pour les AOX et (ii) l'activité maximale des AOX est élevée. Normalement, le réservoir de quinone est oxydé par le cytochrome *bc*1, mais les électrons sont acceptés par les AOX lorsque le réservoir de quinone est largement réduit (Pang *et al.,* 2010). L'inhibition de *C. krusei* par la lactoferrine diminue fortement en présence de cations monovalents (Na^+, K^+) et bivalents (Ca^{2+}, Mg^{2+}) (Viejo - Díaz *et al.,* 2004).

L'utilisation de divers composés chimiques et médicaments pour inhiber *C. krusei* est limitée, car ces médicaments ne sont pas efficaces contre d'autres espèces de la même famille. Peu de médicaments antifongiques ont montré une activité *in vitro* contre *Kluyveromyces marxianus.* Ils n'ont pas été rapportés comme étant nocifs pour d'autres espèces de *Candida*.

1.3.1.1.2.2 Inhibiteurs biochimiques comme agents antimicrobiens

Une autre méthode d'inhibition est de nature biologique et se produit lorsque la présence d'un organisme inhibe celle d'un autre. *Hanseniaspora. uvarum* produit une protéine (poids moléculaire 18 kDa) qui est létale pour la plupart des levures incluant *C. krusei* (Schmitt *et al.,* 1997). *Williopsis mrakii* et *Aspergillus niger* produisent la mycocine HMK qui est très stable aux variations de pH et de température. Elle est utilisée comme agent de conservation polyvalent dans les aliments pour les humains et les animaux. La mycocine brute ou purifiée est utilisée comme additif et est efficace contre une vaste gamme de pathogènes, dont *C. krusei* D1241 (Lowes *et al.,* 2000).

Le mécanisme exact par lequel la protéine tueuse KT4561 de *W. saturnus* exerce son activité létale contre *C. krusei* reste à déterminer (Hodgson *et al.,* 1995). La protéine KT4561 agit de façon similaire à la protéine K1 de *S. cerevisiae* qui semble causer des dommages à la membrane cellulaire par la formation d'un lien qui ne dépend pas de l'énergie entre la toxine et un récepteur de la paroi cellulaire du complexe (1→6)-β-D-glucane. Elle crée un lien additionnel, dépendant de l'énergie, entre la toxine et un récepteur présent dans la membrane cytoplasmique (Breinig *et al.,* 2002). Ceci est suivi d'une plus grande perméabilité de la membrane, de la perte de H^+, K^+ et d'ATP, et par la suite de la mort des cellules (Marquina *et al.,* 2002). Une étude de Kerr (1994) a montré que *Pseudomonas aeruginosa* possède un mécanisme inhibiteur contre *C. krusei* ATCC 6258.

Le mécanisme d'action des protéines tueuses dépend du pH, de la température et de la souche. Ces toxines/protéines tueuses pourraient avoir des applications biotechnologiques dans l'industrie de la fermentation et en médecine. Lorsque des souches sensibles sont présentes dans un milieu, la levure tueuse croît abondamment en produisant des protéines qui sont aussi tueuses. Dans l'industrie de la fermentation, les levures commerciales sont moins compétitives que les levures causant des détériorations. Ces dernières sont en général sensibles aux souches tueuses de levures.

1.3.1.1.2.3 Nanoparticules comme agents antimicrobiens

C. krusei étant résistant à plusieurs médicaments, de nouvelles options ont émergé pour le combattre de façon efficace. Il s'agit des nanoparticules (NP) qui sont considérées comme une source potentielle de nouveaux agents antimicrobiens (Nozari *et al.,* 2012). Les avantages de ces nouveaux agents antimicrobiens sont qu'ils possèdent une activité à large spectre et ont moins tendance à induire de la résistance. En raison de leurs propriétés physiques et chimiques uniques et de leur rapport surface-volume élevé, ces NP présentent une vaste gamme d'activités biologiques (Taylor *et* Webster 2011). Les diverses formes de NP d'usage courant utilisent l'or, l'argent, le cuivre ou l'oxyde de zinc (Rai *et* Ingle 2014) et d'autres NP biodégradables courants utilisent du chitosane, de l'acide polylactique, de la gélatine et du poly-D-L-lactide-co-glycolide (Singh *et* Mishra 2013). Par exemple, les nanoparticules d'argent sont commercialisées et utilisées comme agents antimicrobiens dans le traitement des plaies (antiseptique), dans les implants osseux, dans les matériaux dentaires, et également comme agents antibactériens, antiprotozoaires, anticancéreux et antifongiques. Le premier médicament comprenant des NP approuvé par la FDA a été le Doxil composé de doxorubicine. Ce nanomédicament vise spécifiquement les cellules tumorales via la phase aqueuse intraliposomale (Barenholz, 2012). L'activité antibactérienne du nanoargent a d'abord été décrite dans le cas d'*Escherichia coli* dans lequel les NP créent des perturbations membranaires qui conduisent à leur intériorisation et par la suite à des effets intracellulaires comprenant la production de dérivés réactifs de l'oxygène (DRO), une interaction avec les groupes –SH, l'inhibition de la synthèse de protéines et une interaction avec des molécules contenant du phosphore (ADN) (Taylor *et* Webster 2011, Rai *et al.,* 2014). Les NP de grandes tailles (environ 800 nm) démontrent un niveau élevé de cytotoxicité dans les tests de culture cellulaire, tandis que les NP métalliques de plus petites tailles ont une moins grande toxicité. Les agents de coiffage conjugués à des NP d'argent sont compatibles biologiquement et réduisent les infections à des niveaux contrôlés. Les NP d'argent sont efficaces pour détecter des cibles pathogènes potentielles parmi les microorganismes et ces NP peuvent être combinées à des agents antimicrobiens conventionnels pour combattre les infections (Rai *et al.,* 2014). À ce jour, la production mondiale annuelle de NP d'argent est d'environ 55 tonnes (Bondarenko *et al.,* 2013).

1.3.1.1.2.4 Exposition aux NP et leur toxicité

Globalement, l'utilisation des NP comme agents antimicrobiens, désodorisants, filtres dans des polymères, dans les bacs d'entreposage de nourriture, etc. a augmenté de 0,15 milliard $ US (2002) à 740 milliards $ US (2014). L'exposition des humains et des animaux aux NP est connue (Food Safety Authority of Ireland 2008, Wagner 2013). L'utilisation des NP dans l'industrie des aliments et des boissons a cru de façon exponentielle depuis 20 ans, il est donc difficile d'estimer la toxicité des NP dans les matériaux en contact avec la nourriture (Shekhon 2014). Au niveau cellulaire, une toxicité est observée au niveau des canaux ioniques et des pores lorsque les NP sont plus petits que 0,7 nm. Aux États-Unis, les utilisations de NP sont réglementées par la FDA, tandis que dans l'Union européenne, c'est par le règlement EC 1935/2004 (Wagner 2013). La Food Safety Authority of Ireland (2008) a autorisé l'utilisation de NP d'argent comme suppléments alimentaires (250 mL de MesoSilver se vend 29 $ US). Les NP les plus couramment fabriqués sont les nanoargents qui sont listés dans la base de données des produits de consommation d'Environnement Canada (Green *et* Ndegwa 2011).

1.3.2 Microorganisme autorisé

Le microorganisme autorisé pour la production d'aliments destinés aux humains et aux animaux est décrit ci-dessous :

1.3.2.1 *Saccharomyces unisporus*

Le taux de production d'éthanol par *S. unisporus* est plus lent que celui de *K. marxianus*. *S. unisporus* effectue une fermentation propre dans le lait et le lactosérum (Montanari *et al.*, 1996). Très peu d'oxygène est nécessaire pour que les levures *Saccharomyces* synthétisent des stérols et des acides gras insaturés. Une particularité des cellules de *Saccharomyces* est qu'elles ne peuvent pas faire fermenter les sucres, en particulier lorsque les conditions anaérobies durent longtemps, car elles requièrent des précurseurs préformés des lipides pour croître qui ne sont synthétisés qu'en conditions aérobies. En laboratoire, de l'ergostérol et du Tween 80 (une source d'acide oléique qui est un acide gras insaturé) sont ajoutés au milieu pour étudier les espèces de *Saccharomyces* en conditions anaérobies (Ingledew 1999). Les levures extraites des fromages

bleus de Cabrales d'Espagne jouent un rôle dans l'affinage du fromage (Callon *et al.,* 2006). La plupart des études ont été faites sur des grains de kéfir et ses sous-produits et ont rapporté que les métabolites des levures, comme *S. unisporus,* donnent du goût aux produits et fournissent un milieu de croissance pour les bactéries (Maalouf *et al.,* 2011). Cependant, les effets potentiels de la consommation de ces métabolites ne sont pas connus. *S. unisporus* se développe efficacement dans du fromage; cependant on ne connait pas quels sont les enzymes intracellulaires et extracellulaires produits par cette levure. Parmi les produits laitiers fermentés, le koumis (2 % d'alcool à pH 4,0) est obtenu à partir de lait de jument; sa flore microbienne est dominée par des bactéries thermophiles d'acide lactique ainsi que par *K. marxianus* et des espèces de *Saccharomyces*, spécifiquement *S. unisporus* (Hansen *et* Jakobsen, 2004). Les microorganismes communément présents dans le kéfir sont *S. florentinus*, *S. globosus* et *S. unisporus.* (Coppola *et al.,* 2008). Ce dernier est étroitement apparenté sur le plan phylogénétique à *S. florentinus* et les deux sont classées parmi les espèces autorisées par la Fédération internationale du lait (FIL-IDF) et l'European Food and Feed Cultures Association (EFFCA) pour la consommation humaine et animale (Bourdichon *et al.,* 2012).

1.3.2.2 Présence dans les aliments et utilisations potentielles

S. unisporus présent dans le jus d'orange fermenté a été identifié et classifié par différentes techniques moléculaires (Heras-Vazquez *et al.,* 2003, Canibe *et al.,* 2010). La présence de *S. unisporus* a aussi été observée dans le jus de canne à sucre (Qureshi *et al.,* 2007). Le jus de légumes favorise également la croissance de *S. unisporus* (Savard *et al.,* 2002a, b). *S. unisporus* est important dans la production de vinaigre de petit-lait (Rainieri *et* Zambonelli 2009). Cependant, davantage de recherches sont nécessaires pour comprendre la présence et le rôle exact de *S. unisporus* dans tous ces aliments. Eijk *et* Johannes (1995) ont inventé la méthode de préparation de la pâte limitée en substrat (un mélange de farine et d'eau) pour laquelle *S. unisporus* était une des levures proposées pour la préparation du pain à l'aide de levure (Gatto *et* Torriani 2004). Dans de telles préparations de pâte, *S. unisporus,* ou une autre des levures proposées, consomme les sucres fermentescibles, tandis que des sucres non fermentescibles sont ajoutés à la pâte à pain pour ajuster son goût sucré. Un exemple d'un tel additif agissant comme

supplément artificiel de sucre est le perméat de lactosérum; la quantité ajoutée à la pâte à pain varie de 0,1 à 10 % (poids/poids).

La souche de *S. unisporus* IFO (Institute for Fermentation, Japon) 0298 a la capacité de convertir le cétoisophorone en lévodione (un composé organique volatil). La lévodione est impliquée dans l'activité de l'oxydoréductase et joue également un rôle important dans la synthèse commerciale de caroténoïdes. En présence d'autres levures, *S. unisporus* convertit 2, 6, 6-triméthyle-2-cyclohexane-1, 4-dione en (6R)-2, 2, 6-triméthylecyclohexane-1, 4-dione (un isomère de lévodione) (Fukuoka *et al.,* 2002). Le temps moyen requis pour la production de lévodione par *S. unisporus* a été mesuré à 16 h, avec un taux final de production de 16,7 g de lévodione/kg de cellules de levure/h; alors que le taux de production mesuré n'était que de 8,8 g de lévodione/kg de cellules de levure/h avec *S. cerevisiae* (Fukuoka *et al.,* 2002). L'alcool prénylique est le principal composé odorant du café torréfié et des huiles essentielles. Les cellules de *S. unisporus* produisent une importante quantité d'alcool prénylique en milieu nutritif. *S. unisporus* a également le potentiel de produire du géranylegéraniol (un intermédiaire dans la biosynthèse des vitamines E et K) et du farnesol, qui font tous deux partie de la famille de l'alcool prénylique (Obata *et al.,* 2005). Le farnesol a été rapporté comme étant un agent antitumoral (Obata *et al.,* 2005). Si une souche sauvage de levure produit du géranylegéraniol ou du farnesol de façon naturelle, la croissance de cette levure peut être stimulée pour augmenter la production de géranylegéraniol ou de farnesol dans un milieu peu coûteux et le procédé rentable pourrait mener à une production de masse des intermédiaires. En plus de *S. unisporus*, *S. cerevisiae* et *S. dairensis*, certaines espèces de *Saccharomyces* produisent naturellement du farnesol (Muramatsu *et al.,* 2002). Une forte production de farnesol contrôle la filamentation des champignons qui sont polymorphes et pathogènes (Albuquerque *et* Casadevall 2012).

S. unisporus ne produit aucune odeur phénolique pendant toute la période d'incubation. La présence d'un arôme phénolique dans un aliment fermenté indique une détérioration de la nourriture. Cependant, *S. unisporus* produit un arôme phénolique lorsqu'il consomme efficacement de l'acide p-coumarique (un dérivé du 4-éthylphénol) et il produit du 4-éthylphénol comme intermédiaire (Loureiro *et* Querol 1999, Rodriques *et al.,* 2001). La quantité de 4-éthylphénol produite est proportionnelle à la concentration de l'acide p-coumarique dans le

milieu. Le 4-éthylphénol est un phénol volatil responsable de la détérioration du vin (Rodriques et al, 2001). *S. unisporus* avec d'autres levures produisent entre 0,7 et 2,5 % (poids/volume) d'éthanol (Zajsek *et* Gorsek 2010). *S. unisporus* démontre également l'effet Crabtree indiquant qu'il produit de l'éthanol, du pyruvate, de l'acétate, du succinate et des glycérols par le cycle de Krebs (Merico *et al.*, 2006). La production de composés aromatiques volatils et non volatils par *S. unisporus* dans les produits alimentaires fermentés a un impact positif dans tous les aliments destinés aux humains ou aux animaux.

1.3.3 Conditions de fermentation (pH et température)

1.3.3.1 pH

Un pH neutre est considéré comme optimal pour la croissance des levures et des bactéries. Toutefois, les levures peuvent tolérer un milieu acide et peuvent croître à des pH de 4 à 4,5 (Narendranath *et* Power 2005). Durant la fermentation, un pH inférieur à 4 élimine les bactéries pathogéniques (Ghaly *et* Kamal 2004). Les levures ont la capacité de réguler leur pH cytosolique dans des gammes de pH de l'ordre de 3 à 9 (Viegas *et* Sa-Correia 1991). Dans le cas de la levure *Kluyveromyces fragilis*, le pH optimal pour sa culture est entre 4 et 5, en vue d'obtenir une croissance optimale et une survie importante (Ghaly *et* Kamal 2004). Toutefois, les levures sont capables de croître à des pH faibles, alors qu'une croissance importante est observée en augmentant le pH de 4 à 7 (Rajoka *et al.*, 2006, Munawar *et al.*, 2010). Ainsi, il est essentiel d'optimiser le pH du milieu pour la culture des levures et des bactéries.

1.3.3.2 Température

L'effet de la température sur les microorganismes a été analysé par Van Uden (1984). Il a observé que l'augmentation de la température entraîne des mutations qui peuvent affecter la perméabilité de la membrane plasmique. Lorsque que celle-ci change, il y a réduction de l'efficacité de la production énergétique et augmentation de l'énergie nécessaire pour la maintenance cellulaire. Si cette dernière augmente, la production de la biomasse diminue. Par ailleurs, à température élevée, la dissimulation du glucose grâce à la fraction non viable de la

culture est observée (Rajoka *et al.*, 2006, Munawar *et al.*, 2010). Pour la production de PU, la température optimale suggérée est de 25 à 35 °C en ce qui concerne l'inoculum des levures.

2.1 PROBLÉMATIQUE

La revue de littérature a montré que le lactosérum est un contaminant autant en milieu terrestre qu'aquatique. Le lactosérum est massivement produit par l'industrie fromagère mondiale. Le lactosérum a le potentiel d'être converti en produits à valeur ajoutée tels que les protéines d'organismes unicellulaires (POU). Celles-ci peuvent être produites efficacement à l'aide de microorganismes autorisés pour les POU. La production de POU requiert cependant que la présence de toute forme de contamination biologique ou chimique soit vérifiée minutieusement et que l'efficacité des microorganismes pouvant être utilisés pour la production de POU soit acceptable pour des applications alimentaires destinées aux humains ou aux animaux sur le plan commercial ou industriel. Les différentes problématiques sont mentionnées ci-dessous.

2.1 Première problématique

Lors de la production de POU à l'aide des microorganismes, *K. marxianus, Lactobacillus fermentum,* et *S. unisporus*, *C. krusei* est ressorti comme un contaminant biologique. Même si *C. krusei* est un microorganisme qui ne consomme pas de lactose, il consommait les nutriments essentiels du lactosérum en poudre et sa croissance était plus efficace que celle de *K. marxianus* et des autres microorganismes. *C. krusei* n'étant pas autorisé par la FDA, il ne doit donc pas se retrouver dans les aliments pour les humains ou les animaux. Des conditions de faible pH (3,5) et de basse température (40 °C) ont été utilisées pour la fermentation afin de contrôler la contamination par *C. krusei,* mais ces mesures se sont révélées inefficaces à cette fin. Il est donc nécessaire d'évaluer diverses méthodes d'inhibition pour éliminer *C. krusei* qui seraient efficaces, économiques et non toxiques.

2.2 Seconde problématique

Les cultures mixtes produisent des biomasses plus nutritives que les monocultures. Peu de microbes consomment du lactose et *K. marxianus* en est un. Lors de la fermentation comprenant *K. marxianus, S. unisporus* et *L. fermentum*, ces deux derniers microbes n'ont pas montré une bonne croissance par rapport à *K. marxianus,* car ils ne consomment pas de lactose. *S. unisporus* est une levure autorisée pour la production d'aliments destinés aux humains et aux animaux par l'Union européenne, mais son profil nutritif n'est pas connu.

2.3 Troisième problématique

La présence de lactose dans le lactosérum provenant du fromage cause une augmentation de la demande chimique en oxygène (DCO) lorsque le lactosérum est rejeté en milieu terrestre ou aquatique. Le lactosérum est un additif autorisé dans la nourriture humaine et animale, mais la présence de lactose limite son utilisation comme supplément alimentaire, car une grande partie de la population mondiale est intolérante au lactose. De plus, ne consommant pas de lactose, *S. unisporus* ne peut pas être cultivée dans du lactosérum pour produire des POU.

3. HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ

3.1 HYPOTHÈSES

Les hypothèses suivantes sont émises à partir des problématiques susmentionnées :

3.1.1 Hypothèse 1

Les méthodes utilisées pour éliminer *C. krusei* sont des approches chimiques, biologiques ou à l'aide de nanoparticules. Ces différentes approches ont été prouvées efficaces pour inhiber *C. krusei*, mais la meilleure méthode pour éliminer *C. krusei* de cultures mixtes n'est pas rapportée dans la littérature. Ainsi, une étude des différentes approches (chimiques, biologiques et à l'aide de nanoparticules) pourrait permettre de déterminer la méthode d'inhibition la plus efficace pour éliminer complètement *C. krusei* sans affecter la croissance de *K. marxianus* et *S. unisporus* pour la production de POU.

3.1.2 Hypothèse 2

Les cultures mixtes offrent une meilleure production de POU que les monocultures. Il est donc envisagé de cultiver *S. unisporus* en culture mixte avec *K. marxianus*. Le profil nutritif de *S. unisporus* n'est cependant pas connu, mais il s'agit d'une levure autorisée pour l'alimentation humaine et animale. Ainsi, l'étude du profil métabolique de *S. unisporus* en utilisant diverses sources de carbone et d'azote pourrait permettre de déterminer le profil de croissance et le profil nutritionnel complet de *S. unisporus*. Cette information pourra aider à choisir les meilleures sources de carbone et d'azote pour produire des POU en culture mixte avec *K. marxianus*.

3.1.3 Hypothèse 3

Puisque le lactosérum possède des concentrations élevées en lactose, celui-ci doit être dégradé en monosaccharides par hydrolyse. L'hydrolyse acide du lactosérum est privilégiée par rapport à l'hydrolyse enzymatique, car elle est plus économique, et un acide comme l'acide sulfurique peut hydrolyser efficacement le lactose du lactosérum en glucose et en galactose. *S. unisporus* peut consommer le glucose et le galactose hydrolysés pour croître efficacement en culture mixte avec *K. marxianus* et ainsi produire une biomasse à forte teneur en protéines.

3.2 OBJECTIFS

L'objectif général de ces travaux de recherche est l'élimination du contaminant biologique *C. krusei* d'une fermentation en culture mixte et l'amélioration de la croissance de *S. unisporus* en culture mixte lors de la production de POU.

3.2.1 Objectif 1

Évaluation des approches chimiques et biochimiques pour inhiber *C. krusei* sans affecter la croissance des levures autorisées (*K. marxianus* et *S. unisporus*).

3.2.2 Objectif 2

Production et optimisation d'une nanoparticule de protéine conjuguée à de l'argent pour inhiber *C. krusei*.

3.2.3 Objectif 3

Étude du profil métabolique de *S. unisporus* en variant les sources de carbone et d'azote en vue de l'utiliser pour produire des POU en culture mixte.

3.2.4 Objectif 4

Hydrolyse acide du lactosérum et production de POU en culture mixte (*S. unisporus* et *K. marxianus*) à partir du lactosérum hydrolysé.

3.3 ORIGINALITÉ

1. L'inhibition de *C. krusei* dans une culture mixte durant la production de POU en utilisant des nanoparticules biologiques (protéine tueuse de *W. saturnus*) est une approche totalement originale.

2. Le profil alimentaire de *S. unisporus* n'a pas été précédemment rapporté dans la littérature.

3. La production de POU en culture mixte avec *K. marxianus* et *S. unisporus* est également une approche nouvelle qui n'a jamais été réalisée auparavant.

4. MÉTHODOLOGIE

4.1 Optimisation et production de protéine d'argent conjugué

4.1.1 Production de la biomasse de *Williopsis saturnus*

La préculture de *W. saturnus* a été préparée dans des fioles de 500 ml en utilisant 100 ml du milieu YEPD stérilisé. Le milieu de production contenant 5% (p / v) de mélasse, 4,5% (p / v) de petit lait de fromage et 0,22% (p / v) d'urée a été pasteurisé à 80 °C pendant 20 min. Une boucle complète de la culture *W. saturnus* a alors été transférée dans le milieu pasteurisé et la fiole a été maintenue à 28 °C dans un agitateur à 150 rotations par minute pendant 24 h. Des échantillons ont été prélevés à intervalles réguliers pour mesurer la concentration de cellules et de protéines. La concentration en protéines a été mesurée en utilisant la méthode de Folin-Lowry (Lowry *et al.,* 1951).

4.1.2 Optimisation des paramètres du procédé

- Différentes concentrations de nitrate d'argent ($AgNO_3$) allant de 0,01 M à 5 M ont été préparées en double dans de l'eau déionisée dans des bouteilles ambrées stériles et conservées à température ambiante.
- De plus, différentes concentrations en $AgNO_3$ ont été ajoutées à 72 µL du surnageant de *W. saturnus* et à 5 ml d'H_2O dans des tubes en verre de 15 ml ont été incubées à la température (20 - 40 °C) sous agitation continue en utilisant un agitateur magnétique à 150 rotations par minute. Un changement de couleur a été observé du jaune clair au brun jaunâtre foncé qui indique la formation d'Ag-NPS. L'intensité de la couleur a été mesurée à l'aide d'un spectrophotomètre UV-Vis (Carry 100 Bio®, Varian USA) de 300-700 nm.
- Après l'optimisation de la concentration de 0,1 M d'$AgNO_3$, différents volumes (25-200 µL) de 0,1 M $AgNO_3$ ont été testés avec 72 µL du surnageant de *W. saturnus* et 5 ml d'H_2O.
- Différents volumes du surnageant de *W. saturnus* (96-220 µL / 5 ml d'H_2O) ont été testés en même temps que la solution optimale d'$AgNO_3$ pour la réduction complète des ions d'argent (Ag^+).
- Des temps de réaction différents ont été testés à des concentrations optimales d'$AgNO_3$ et du surnageant de *W. saturnus* à une température optimale de 25 °C. Le mélange réactionnel dans les tubes de verre de 15 ml a été incubé pendant 24 h. Des échantillons

ont été prélevés à chaque 2 h pour mesurer l'intensité de couleur du mélange réactionnel en utilisant un spectrophotomètre UV-Vis.

4.1.3 Caractérisation des conjugués de protéine-argent synthétisés

- Les conjugués d'Ag-NPS synthétisés ont été centrifugées à 10 000 g pendant 2 minutes à une température ambiante de 20 °C suivie d'une filtration du surnageant au moyen d'un filtre de 0,45 micromètre.
- La solution de nanoparticules d'argent filtré a été caractérisée en utilisant l'infrarouge transformé de Fourier (FTIR), le zetasizer, l'analyse au microscope électronique à balayage (MEB) et l'analyse de la dispersion d'énergie au rayon X (EDS).

4.1.4 Bonne méthode de diffusion

L'argent conjugué à une protéine synthétisée (Ag-KT4561) a été testé contre *C. krusei, K. marxianus* et *S. unisporus* en utilisant la méthode de diffusion de Nathan *et al.* (1978). Les plaques de gélose ont été inoculées avec *C. krusei, K. marxianus* et *S. unisporus* séparément et des puits de 6 mm de diamètre ont été faits sur ces plaques. De plus, 40 µL d'Ag-KT4561 ont été chargés dans les puits et maintenus à 30 °C et les plaques ont été évaluées après 24 h en zone d'inhibition.

4.2 Évaluation des méthodes chimiques, biochimiques et liées aux nanoparticules sur l'inhibition de *C. krusei*

4.2.1 Microorganismes

Les micro-organismes *K. marxianus* et *C. krusei* ont été cultivés sur le milieu YEPD et l'ensemencement a été fait sur des plaques de gélose YEPD.

4.2.2 Lyophilisation

La lyophilisation du surnageant *W. saturnus* a été réalisée avec l'aide d'un lyophilisateur à 40°C et à 15 °C (SP Scientific, États-Unis).

4.2.3 Expériences d'inhibition

Les expériences ont été effectuées en agitant les fioles et en utilisant 500 ml de fromage de petit lait dans deux fioles d'Erlenmeyer. La pasteurisation a été effectuée à 80°C pendant 20 min. Après la pasteurisation, le petit lait de fromage a été inoculé avec *C. krusei* et *K. marxianus* et

incubée dans l'incubateur agitateur à 30 °C et 150 rotations par minute. Les différentes études d'inhibition suivantes ont été effectuées :

4.2.3.1 Approches chimiques

- L'effet de différentes températures (28 °C, 40 °C et 45 °C) et différents pH (2, 3.5, 4, 4.5, 5, 5.5, 6 et 9) a été testé sur l'inhibition de *C. krusei* dans une culture mixte (*C. krusei, K. marxianus* et *W. saturnus*).
- L'effet de la concentration de H_2O_2 (100, 200, 300, 400, 600, 800, 2400, 3200 et 4000 mg⁄L) avec différentes températures (28 °C, 40 °C et 45 °C) a été évalué sur l'inhibition de *C. krusei* dans un milieu mixte (*C. krusei, K. marxianus*, et *W. saturnus*).
- L'effet de la concentration de NaCl (1,5 M, 2 M) sur l'inhibition de *C. krusei* dans une culture mixte (*C. krusei* et *K. marxianus*) a été étudié.

4.2.3.2 Approches biochimiques

- L'effet de l'huile de girofle (0,4% et 0,5%) sur l'inhibition de *C. krusei* en culture mixte (*C. krusei* et *K. marxianus*) a été étudié.
- L'effet du surnageant lyophilisé de *W. saturnus* et de l'ensemble du bouillon fermenté de *W. saturnus* lyophilisé (200, 300 et 400 mg/ml) sur l'inhibition de *C. krusei* a été évalué dans une culture mixte (*C. krusei* et *K. marxinaus).*
- L'effet de différentes concentrations en H_2O_2 (2400, 3200 et 4000 mg/L), ainsi que du surnageant lyophilisé de *W. saturnus* et l'ensemble de bouillon fermenté de *W. saturnus* lyophilisé (200, 300 et 400 mg/ml) sur l'inhibition de *C. krusei* a été testé dans une culture mixte (*C. krusei* et *K. marxinaus*).

4.2.3.3 Approche liée aux nanoparticules

L'effet de synthèse Ag-KT4561 (10 uM - 1 mM) sur l'inhibition de *C. krusei* a été réalisé dans une culture mixte (*C. krusei* et *K. marxianus*).

4.3 Profil nutritionnel de *S. unisporus*

4.3.1 Analyse du profil d'utilisation du substrat en utilisant le système Biolog

S. unisporus NRRL Y-1556 a été cultivé sur une plaque de YEPD à 30 °C pendant 24 h. L'inoculum a été préparé par mise en suspension des cellules provenant de la plaque de YEPD

dans de l'eau distillée stérile pour obtenir 45-47% de transmittance (%T) avec le turbidimètre biolog. 100 µL d'inoculum de chaque échantillon ont été distribués dans chaque puits de la microplaque pour levure du système Biolog (Biolog Inc., Hayward, CA, USA). La microplaque inoculée a été incubée à 30 °C pendant 12, 24, 36 et 48 h, respectivement. La microplaque a été lue par le lecteur de microplaque et traitée pour déterminer le profil d'utilisation des substrats.

4.3.2 Optimisation des conditions de croissance

4.3.2.1 Variation des sources de carbone

Différentes sources de carbone ont été choisies telles que les mélasses (3%, 5% ou 7% en p/v, des mélasses basées sur des cannes à sucre typiques contiennent 35 à 42% (p/v) de saccharose) (Doelle *et* Doelle, 1990) et du glycérol pur (5%, 7% ou 9% p/ v), ainsi que 4,5% (p/v) du petit lait de fromage et 0,22% (p/v) d'urée). La pré-culture de *S. unisporus* a été préparée en utilisant (100 mL) d'un milieu YEPD avec une boucle complète de la culture dans des fioles d'Erlenmeyer de 500 ml. L'inoculum de 2% (v/v) du milieu de pré-culture est utilisé pour inoculer les milieux de production pasteurisée (80 °C pendant 20 min) contenant différentes concentrations de mélasse et de glycérol pur ainsi que 4,5% (p/v) de poudre de petit lait et 0,22% (p/v) d'urée. Après l'inoculation, les fioles ont été gardées à 150 rotations par minute et à 28 °C pendant 48 h dans un incubateur agitateur. Des échantillons ont été prélevés à intervalles réguliers pour mesurer la concentration cellulaire.

4.3.2.2 Variation des sources d'azote

Différentes sources d'azote telles que l'urée (0,22% p/v) et le sulfate d'ammonium à différentes concentrations (0,2%, 0,4% et 0,6% (p/v)) ont été ajoutées au milieu contenant de la mélasse et du petit lait de fromage. La fermentation avec *S. unisporus* a été effectuée selon la procédure décrite dans la section de variation de la source de carbone.

4.3.2.3 Variation du pH

La variation du pH dans le milieu au cours de la fermentation du petit lait de fromage avec *S. unisporus* dans des fioles d'Erlenmeyer a été étudiée. Le milieu de production a été préparé avec divers pH (3,5, 4,5 et 5,5) en utilisant 1 N NaOH et 1 N H_2SO_4.

4.3.3 Méthode statistique

L'analyse des composantes principales (PCA 1 et 2) a été réalisée en utilisant l'outil statistique R à l'aide du progiciel FactoMine.R sur les profils d'acides aminés obtenus à différentes températures. L'analyse statistique des différents profils d'acides aminés a été réalisée par PCA 1 et plus tard, PCA 2 a été effectuée sur la base du codon d'acides aminés identifiés par PCA 1. Cette méthode est similaire à la méthode de Dom *et al.* (2003).

4.4 Hydrolyse acide du petit lait et du perméat de fromage pour la croissance de *S. unisporus*

4.4.1 Hydrolyse acide du petit lait de fromage et du perméat du petit lait

L'hydrolyse acide du petit lait de fromage et du perméat du petit lait a été effectuée à l'aide de H_2SO_4. 200 ml de petit-lait de fromage (4,5% (p/ v)) et le perméat du petit lait (5% (p/v)) ont été pris dans différentes bouteilles de 1 L et différentes concentrations de H_2SO_4 telles que 0,2, 0,3 et 0,4% (p/p) ont été ajoutées à chaque bouteille et traités thermiquement à 121 °C pendant différentes périodes de temps (15, 30, 60 et 120 min).

4.5 Méthodes analytiques

4.5.1 Analyse des carbohydrates

Le petit lait de fromage hydrolysé et le perméat du petit lait ont été centrifugés à 10 000 g pendant 10 min à 4°C et le surnageant a été recueilli. Le surnageant a été encore filtré à l'aide d'un microfiltre de 0,45 µm (Thermo Scientific, USA). L'analyse des carbohydrates a été effectuée en utilisant la chromatographie en phase liquide et la méthode de spectroscopie de masse (LC-MS / MS) (Thermo TSQ Quantum) équipée d'une ionisation par électropulvérisation (ESI) en mode d'ions négatifs. La colonne analytique utilisée était celle de Carbohydrate Zorbax avec des spécifications de 4,6 mm x 150 mm (Agilent sciences de la vie). Le volume d'injection était de 10 µL et le standard interne utilisé était le Glucose-D2. La phase mobile utilisée pour l'analyse des carbohydrates était composée de 75% d'acétonitrile mélangé avec 0,1% de NH_4OH et 25% d'eau mélangée avec 0,1% de NH_4OH. Cette méthode est modifiée pour l'analyse (McRae *et* Monreal 2011).

4.5.2 Composés volatils

Les composés volatils présents dans le bouillon de fermentation de S. ont été déterminés par chromatographie en phase gazeuse et spectrométrie de masse (GC-MS). Le bouillon fermenté a été centrifugé à 10 000 g à 4 °C et pendant 15 min et le surnageant a été recueilli. La colonne capillaire utilisée était HP - INNOWax (30 m ; 0,25 mm, en utilisant de l'azote comme gaz porteur), et un rapport de division de 1/100. Les échantillons de surnageant sont dilués avec du méthanol (1: 1) et 10 µL d'acide acétique (étalon interne). L'échantillon blanc a été préparé avec 2 ml de méthanol et 10 µL d'acide acétique. Les échantillons dilués et celui blanc ont été mis dans des flacons de GC et analysés par GC-MS (Agilent Chrompack HP-6820).

4.5.3 Acides gras

Les profils d'acides gras ont été effectués par chromatographie en phase gazeuse et spectrométrie de masse (GC-MS) après la conversion en lipides dérivés d'esters méthyliques (Mooney *et al.*, 2007). Les échantillons ont été centrifugés à basse vitesse pour faciliter la séparation des phases. La phase organique est ensuite prise pour le GC, l'analyse des esters méthyliques d'acides gras est réalisée dans le chromatographe Varian 3900 équipé d'un détecteur à ionisation de flamme (FID) et d'un échantillonneur automatique. La colonne était le facteur Varian VF-23ms quatre dont les spécifications sont 260°C 3PA (30 mm, 0,25 mm, 0,25 mm). Les acides gras ont été identifiés par comparaison avec des standards d'esters méthyliques (FAME 32 FAME mélange C16-C18, C4-C24 FAME, Supelco, France; C16: 1 n-9, Cayman, France) et quantifiés en utilisant un standard interne, l'acide heptadécanoïque (C17 : 0, Sigma) (Milinski *et al.*, 2008).

4.5.4 Acides aminés

Les compositions essentielles d'acides aminés du bouillon de fermentation de S. lyophilisé ont été analysées par hydrolyse de la poudre avec 6 N de HCl à 110 °C pendant 24 h. Les échantillons digérés contenant du HCl ont été remises en suspension dans une solution tampon d'acétate et passées à l'intérieur d'un microfiltre de 0,45 µm (Thermo Scientific, USA). Cette méthode a été légèrement modifiée par Lourenço *et al.* (2002). Les acides aminés ont été libérés par hydrolyse et ont été analysés par chromatographie en phase liquide et spectrométrie de masse (LC-MS / MS) (TSQ Quantum Access, Thermo Scientific).

4.5.5 Production d'éthanol

Le surnageant de bouillon de fermentation a été prélevé pour l'analyse de l'éthanol. La solution standard interne de propanol à une concentration de 0,01% (v/v) a été ajoutée aux échantillons d'essai et aux standards (1,00 à 0,005 g / dL) (Tiscione *et al.*, 2011). L'éthanol produit a été analysé par FID (Varian Chrompack, CP-3800).

4.5.6 Analyse des métaux

Les concentrations en métaux présents dans les échantillons lyophilisés de *S. unisporus* ont été analysées par ICP-AES (Varian Vista AX) après une digestion partielle de l'échantillon liquide par la méthode de digestion à l'acide nitrique et au peroxyde d'hydrogène (HNO_3 et H_2O_2) (US EPA Procédé 1982).

4.5.7 Estimation des protéines

La poudre lyophilisée de la biomasse de *S. unisporus* a été soumise aux ultrasons (Lopez *et al.*, 2010) et la protéine a été estimée par la méthode de Folin-Lowry (Lowry *et al.*, 1951).

5. RÉSULTATS ET DISCUSSION

Les résultats obtenus sont présentés dans le manuscrit de thèse en 2 parties. La 1ère partie présente l'optimisation et la production de « protéine tueuse » conjuguée avec des nanoparticules d'argent. L'application du produit chimique, biologique et la protéine conjuguée avec des nanoparticules d'argent dans le fromage de lactosérum pour l'élimination de *C. krusei* en mono-culture et en culture mixte (deux articles acceptés et un soumis). La 2ième partie présente le profil nutritif de *S. unisporus* dans le fromage de lactosérum, l'hydrolyse de l'acide a été effectuée sur le fromage de lactosérum et *S. unisporus* et *K. marxianus* étaient cultivés en culture mixte pour des fins alimentaires (nourritures pour animaux) (un article est publié et deux articles sont soumis).

5.1 Inhibition de *C. krusei* par une approche économique lors de la production de PU

5.1.1 Facteurs influant la croissance et l'inhibition des *C. krusei* par des approches chimiques, biochimiques et nanotechnologiques

C. krusei est décrit comme l'un des microorganismes pathogènes d'origine alimentaire qui relève du genre *Candida*. Il a été majoritairement isolé à partir de patients immunodéprimés ayant une importance clinique minime. Les mesures possibles d'inhibitions de *C. krusei* se composent de méthodes chimiques, biochimiques et l'utilisation de nanoparticules. Les inhibiteurs chimiques de *C. krusei* sont limités à cause de la non-efficacité de nombreux médicaments à base d'azole contre le pathogène. D'autres moyens d'inhibition de *C. krusei* consistent en l'utilisation de plantes médicinales, le stress osmotique en utilisant le chlorure de sodium (NaCl) ou le glucose et les huiles essentielles comme l'huile de clou de girofle (*Syzygium aromaticum*). En industrie alimentaire et de boissons, l'utilisation d'inhibiteurs biologiques constitués de protéines tueuses soit *Hanseniaspora uvarum*, *Williopsis mrakii* ou *Pleurotus ostreatus* est observée. D'autres moyens d'inhibition de *C. krusei* évoquent l'ingénierie de nanoparticules (NP). Bien que *C. krusei* est moins virulente que *C. albicans*, elle a cependant un potentiel protéolytique et produit des phospholipases. Malgré que *C. krusei* n'ait pas été acceptée en tant que producteur de

PUs, elle est cependant utilisée dans la fabrication du vin, de la bouillie et dans la fermentation boulangère. *C. krusei* est significativement différente des autres souches du genre *Candida spp*. aussi bien en structure et métabolisme qu'à l'égard du comportement de défense suite à son exposition à l'hôte.

5.1.2 Optimisation et production de nanoparticules d'argent-protéine conjuguées comme bioconservateur

Williopsis saturnus est connue comme une «levure tueuse» efficace contre *Candida albicans.* Cette leuvre qui fait partie de la microflore naturelle du fromage est délibérément utilisée dans les industries alimentaires et de boissons. L'efficacité de la protéine conjuguée produite par *W. saturnus* est amplifiée si elle est sous forme nanométrique. La capacité de la protéine de *W. saturnus* tueuse (AC 4561) complexée à des nanoparticules d'argent n'a jamais été explorée. Différents paramètres ont été optimisés et caractérisés pour la conjugaison des KT4561 et des ions d'argent. On a constaté que les nanoparticules Ag-IP ont été entièrement synthétisées en utilisant la protéine *W. saturnus* (4,3 g / L) et 200 µL de 0,1 M $AgNO_3$ (3,96 mM) à un temps de réaction de 4 h. La caractérisation a été réalisée en utilisant les techniques de spectrophotométrie UV-VIS, IRTF et EDS ainsi que la microscopie électronique MEB. La spectroscopie IRTF a confirmé que seules les protéines sont liées à des ions d'argent tandis que la réduction EDS a confirmé que l'argent est la seule particule métallique présente dans le conjugué protéine-argent de 200 nm. Le MEB confirme que les NPs synthétisées présentent une structure cristalline. La méthode de diffusion dosée du conjugué protéine-argent a été effectuée sur *C. krusei, K. marxianus* et *S. unisporus*. C. *krusei* a été inhibée avec succès tandis qu'une inhibition partielle a été observée pour *K. marxianus* et *S. unisporus*, sous l'effet des ions d'argent. Il a été observé que l'activité inhibitrice de l'Ag-KT4561 (les nanoparticules d'argent) a augmenté de l'ordre de 3 par rapport à la protéine pure. Une étude plus approfondie a été menée par la production en masse des NPs Ag-KT4561 étant donné qu'elles sont économiques, qu'elles ont un temps de réaction court et qu'elles soutiennent la notion de chimie verte.

5.1.3 Évaluation du pouvoir inhibiteur de la levure *Candida krusei* affectant les produits alimentaires pendant le processus de fermentation via des approches chimiques, biochimiques et nanotechnologiques

Divers procédés chimiques, biochimiques et nanotechnologiques ont été évalués pour inhiber *C. krusei* sans nuire à la croissance de la levure *K. marxianus*. Cependant, on a observé une inhibition efficace à l'aide de *W. saturnus* et d'H_2O_2, avec une combinaison spécifique de pH 5,0 et de température 40 °C, mais l'approche la plus efficace a été l'utilisation des nanoparticules Ag-KT 4561. Parmi les méthodes chimiques, l'utilisation de H_2O_2 fonctionne très bien dans une plage de pH comprise entre 4,0 et 10,0 et à une température de 30 °C ou plus. À une très forte concentration en H_2O_2 (4000 mg/L), à 45 °C et à un pH de 5,5 l'inhibition de *C. krusei* a été réussie sans affecter *K. marxianus*. Le surnageant de la culture de *W. saturnus* a été lyophilisé et utilisé pour l'inhibition de *C. krusei* avec 2400 mg/L de H_2O_2. Néanmoins, la dose de H_2O_2 s'est avérée concentrée, par conséquent, des nanoparticules d'argent ont été synthétisées pour réduire la quantité de protéines utilisées et pour renforcer l'efficacité de son activité. Il a été observé que 350 µM (contenant 1 ppm d'ions Ag réduits) de nanoparticules d'argent synthétisées (NP-Ag) de la protéine tueuse de *W. saturnus* peuvent inhiber *C. krusei* tout en ayant un effet partiel sur *K. marxianus*. La méthode NP-Ag s'est avérée être une approche efficace pour sa fonction inhibitrice par rapport à des moyens chimiques et biochimiques. La méthode NP-Ag est également très économique car la production de 20 000 L d'Ag-KT4561 ne coûterait que 863 $CAD. En outre, le test de stabilité a confirmé l'efficacité des nanoparticules d'argent sur la protéine *C. krusei* pendant plus de 20 semaines. En conclusion, l'approche de nanoparticules peut être potentiellement utilisée pour l'inhibition de *C. krusei* avec ou sans effet partiel sur d'autres microbes et le processus peut être exécuté en mode de culture non aseptique.

5.2 Étude du profil nutritionnel de *S. unisporus* et son utilisation en culture mixte pour la production de PUs

5.2.1 *Saccharomyces unisporus*: potentiel biotechnologique et contexte actuel

Saccharomyces spp. a été associée à la nourriture et l'alimentation du bétail et ainsi reconnu comme probiotique. Parmi les espèces du genre *Saccharomyces* spp., on retrouve *S. unisporus* qui a été observée dans différents produits laitiers. Un certain intérêt a été dédié à cette espèce d'importance industrielle, grâce à sa présence dans les produits laitiers et à sa grande ressemblance avec *S. florentinus*. Ces deux espèces sont acceptées par la Fédération internationale de produits laitiers et l'Association Européenne d'alimentation et de la culture alimentaire, pour des procédés alimentaires et pour une alimentation animale. Comme *S. unisporus* se trouve dans différents produits alimentaires, il est considéré comme le candidat potentiel au statut sécuritaire (GRAS). Dans des études antérieures, il a été confirmé que *S. unisporus* a la capacité de convertir le cétoisophorone en lévodione, une molécule essentiellement utilisée dans les industries pharmaceutiques. *S. unisporus* produit certains acides gras insaturés de type Oméga ayant un effet curatif. Cependant, le profil nutritionnel de *S. unisporus* reste entièrement méconnu par rapport à sa capacité de produire des Protéines Unicellulaires (PU). De cette constatation, l'exploration des métabolites secondaires et intermédiaires de l'espèce pourrait être prometteur (protéines, acides aminés, acides gras, teneur en minéraux et autres métabolites). Cette revue de littérature est une tentative de décrire l'ubiquité de *S. unisporus* pour la production des PUs.

5.2.2 Profil nutritionnel de *Saccharomyces unisporus* pour la production de Protéines Unicellulaires: de l'approche classique à l'approche génomique

S. unisporus a été cultivée dans un milieu de lactosérum avec diverses sources de carbone telles que les mélasses et le glycérol, différentes sources d'azote telles que l'urée et le sulfate d'ammonium dans un objectif d'optimiser la production des PUs. Nous avons observé que le milieu de lactosérum enrichi de 7% (p/v) de mélasses contenant l'équivalent de 10 g/L de sucrose et 0,4% de sulfate d'ammonium permet de produire une concentration de PUs notamment plus élevée à 4,2 g/L pour un pH 5,5. La croissance de *S. unisporus* s'est avérée négative à l'urée. L'analyse des A.As a été réalisée et nous avons constaté que la souche *S. unisporus* est capable de générer les A.As essentiels comme la lysine (84,0 mg/g poids sec), la valine (74,4 mg/g), l'acide aspartique (83,3 mg/g), la glycine (84,0 mg/g) et la cystéine (77,0 mg/g) respectivement confirmés par le profil LC-MS/MS. Selon les standards de la FAO, la concentration en acides gras des PUs ne devrait pas dépasser 3% (p/p), celle de *S. unisporus*

étudiée étant de 0,86% (p/p). Nous avons également observé que *S. unisporus* secrète 2 g/L d'éthanol à 36 h de la durée de fermentation en conditions de milieu contenant les mélasses comme source de carbone. Consécutivement, nous concluons que *S. unisporus* obéit au phénomène de « l'effet Crabtree » et pourrait même être cultivé pour la production du bioéthanol. Les études des fréquences codon ont montré que *S. unisporus* est riche en éléments A et T (Adénosine et Thionine) à environ 65% dans la composition de leurs PUs, ce qui se concorde avec *S. cerevisiae*. À la base des analyses du profil nutritionnel, *S. unisporus* pourrait bien être maintenue aussi bien en mono-culture qu'en culture mixte à condition de fournir les sources nutritives nécessaires en carbone et azote.

5.2.3 Hydrolyse acide du lactosérum pour la croissance de *Saccharomyces unisporus*

L'hydrolyse acide du lactosérum du fromage et du perméat avait un effet positif sur la croissance de *S. unisporus* en mono-culture et en culture mixte. Différentes concentrations de H_2SO_4 (0,2 - 0,3 - 0,4 p/p %) ont été utilisées pour l'hydrolyse et nous avons constaté que la concentration de 0,2% (p/p) de H_2SO_4 peut efficacement hydrolyser le perméat de fromage en environ 70 min. Une hydrolyse complète du lactosérum a été obtenue avec 0,4% (p/p) de H_2SO_4 en 60 min. Il a été conclu que la monoculture de *S. unisporus* dans le lactosérum ou le perméat est prometteuse respectivement dans les deux cas obtenus lors des analyses par ($4,0 \times 10^8$) UFC/mL et ($2,0 \times 10^8$) UFC/mL comparativement aux substrats non hydrolysés respectivement ($2,1 \times 10^5$) UFC/mL et ($2,0 \times 10^5$) UFC/mL. Dans les substrats hydrolysés, *S. unisporus* et *K. marxianus* ont été fermentés ensemble (culture mixte) et il a été observé que les deux souches étaient bien maintenues avec une meilleure croissance distinguée chez *K. marxianus.* En conclusion, le perméat du fromage ainsi que le lactosérum pourraient être utilisés pour la fermentation microbienne en vue de la production des PUs dans une culture de type mixte. Nous avons également observé que *S. unisporus* produit certains métabolites essentiels tels que le phényl-éthyl-alcool et le furan méthanol, quand il s'agit d'un milieu hydrolysé, ceci étant confirmé par les résultats GC-MS.

6. CONCLUSION ET RECOMMANDATIONS

PARTIE 1 : CONCLUSION

On peut tirer de cette étude les deux conclusions présentées ci-dessous. Seuls les microorganismes approuvés par la FDA peuvent être utilisés pour produire des POU. *K. marxianus* est une levure approuvée pour la production de POU, mais *C. krusei* n'est pas approuvé pour les aliments destinés aux humains et aux animaux; il s'agit d'un contaminant biologique. Certaines étapes doivent donc être effectuées afin d'éliminer la présence de *C. krusei* sans affecter la croissance de *K. marxianus*.

Des approches chimiques (NaCl, H_2O_2) et biochimiques (essence de girofle, surnageant de *W. saturnus* et surnageant de *W. saturnus* avec H_2O_2) sont efficaces pour éliminer *C. krusei*, mais la quantité requise d'agent de conservation est très élevée ce qui augmente les coûts d'opération. Par conséquent, la méthode envisagée pour éliminer *C. krusei* d'une culture mixte avec *K. marxianus* consiste à utiliser 350 µM d'une protéine tueuse conjuguée à des ions d'argent Ag-KT4561 (avec une concentration en argent réduite à 1 mg⁄L efficace à 30 °C et pH 5,5). *K. marxianus* présente un meilleur profil de croissance à pH 5,5 que 3,5. L'agent biologique de conservation Ag-KT4561 est un composé stable qui n'a pas formé de complexes métalliques avec les minéraux présents dans le lactosérum. L'utilisation d'Ag-KT4561 dans le lactosérum pourrait potentiellement éliminer d'autres contaminants biologiques en plus de *C. krusei* durant la fermentation.

S. unisporus peut consommer une vaste gamme de sources de carbone excluant le lactose et il peut consommer 0,4 % (p/v) de sulfate d'ammonium. *S. unisporus* est strictement urée négatif. La monoculture de *S. unisporus* était enrichie en lysine (84,0 mg/g), en valine (74,4 mg/g), en acide aspartique (83,3 mg/g) et en glycine (77,0 mg/g). La biomasse de *S. unisporus* contenait les minéraux essentiels (Ca, S, Na, Mg, P et K). Sa teneur en acides gras était de 0,86 % (p/p) et en protéines de 4,2 g/L et était riche en bases A et T comme *S. cerevisiae*. Le profil nutritionnel complet de *S. unisporus* en fait un producteur efficace de POU.

S. unisporus a été cultivé en culture mixte avec *K. marxianus*. Ce dernier avait une meilleure croissance (7,8 x 10^8 UFC/mL) que *S. unisporus* (5,6 x 10^7 UFC/mL) parce qu'il peut consommer autant les monosaccharides que le surplus de lactose tandis que *S. unisporus* ne peut

que consommer le galactose partiellement. La fermentation en culture mixte (*S. unisporus* et *K. marxianus*) a montré une légère augmentation des acides aminés essentiels, en particulier la leucine et l'isoleucine (79,9 mg/g), et a généré des intermédiaires comme le furanméthanol, le formate furfurylique et des acides organiques faibles, qui sont importants en biotechnologies. Cependant, aucune quantification des intermédiaires produits n'a été effectuée.

Cette étude nous permet donc de conclure que le contaminant biologique peut être éliminé par une approche verte et rentable et que l'ajout du microorganisme autorisé (*S. unisporus*) pour la production de POU peut générer une biomasse de meilleure qualité.

PARTIE 2 : RECOMMANDATIONS

En se basant sur les résultats obtenus et afin d'étudier plus profondément l'utilisation de lactosérum dans les applications alimentaires destinées aux humains ou aux animaux, les recommandations suivantes ont été proposées :

1. La méthode de bioconservation peut être modifiée en microfiltrant la protéine tueuse *W. saturnus* et la préparation des protéines filtrées conjuguées à des ions d'argent peut permettre de réduire la quantité d'agent de conservation requise et donc les coûts.
2. Pour la croissance de *W. saturnus*, les matières premières utilisées sont le lactosérum, la mélasse et l'urée. Il est recommandé de remplacer le lactosérum par du perméat, car il n'y a aucune protéine soluble présente dans le perméat de lactosérum pouvant interférer avec la protéine tueuse *W. saturnus*.
3. Il est fortement recommandé d'étudier davantage l'effet à long terme du conjugué d'argent-protéine sur le contaminant biologique. De plus, il est recommandé que l'Agence canadienne d'inspection des aliments approuve ce conjugué comme agent de conservation d'origine naturelle. Le conjugué argent-protéine pourrait même remplacer certains antibiotiques dans le domaine médical, car *C. krusei* est un microbe pathogène important chez les personnes immunodéprimées.
4. Il est recommandé d'étudier la production d'agents biologiques de conservation utilisant l'oxyde de fer et une protéine tueuse, car l'oxyde de fer n'est pas antimicrobien comme les ions d'argent et il n'inhibera pas les microorganismes utiles.
5. Il est recommandé d'étudier la thermostabilité d'Ag-KT4561 (conjugué argent-protéine). Ceci permettra d'évaluer la stabilité du conjugué aux températures élevées.
6. Il est recommandé d'étudier les protéines et les enzymes intracellulaires et extracellulaires de *S. unisporus*, puisque cela n'a jamais été fait. D'autre part, l'élimination des acides nucléiques de la biomasse de *S. unisporus* doit être effectuée en vue d'utilisations alimentaires.
7. Il est recommandé d'étudier quantitativement les autres intermédiaires, par exemple les acides organiques, produits par *S. unisporus* puisque ce dernier génère des intermédiaires bénéfiques importants en biotechnologies.

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CHAPTIRE II

ARTICLES

PARTIE I
L'INHIBITION DE *C. KRUSEI* PAR UNE APPROCHE ÉCONOMIQUE LORS DE LA PRODUCTION DE PU

ARTICLE 1

Candida krusei: Factors influencing its growth and inhibition by chemical, biochemical and nanoparticle approaches

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ARTICLE 2

Optimization and Production of Silver-Protein Conjugate as Bio preservative

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ARTICLE 3

Evaluation of inhibitory measures for food spoiler yeast *Candida krusei* during fermentation process by chemical, biochemical and nanoparticle approaches

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PARTIE II

ÉTUDE DU PROFIL NUTRITIONNEL DE *S. UNISPORUS* ET SON UTILISATION EN CULTURE MIXTE POUR LA PRODUCTION DE PUS

ARTICLE 1

Saccharomyces unisporus: Biotechnological Potential and Present Status

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RECONNAISSANCE

Dans cet article de revue, l'idée originale a été proposée par le professeur R.D. Tyagi. Les données ont été recueillies par Indrani Bhattacharya. Le manuscrit a été écrit par Indrani Bhattacharya. Les corrections et la mise en forme du manuscrit ont été faites par le Dr. Song Yan et le Dr. Jay Shankar Yadav. Le Professeur R.D. Tyagi et le Dr. Rao Surampalli ont finalisé l'article.

RÉSUMÉ

Les levures du genre *Saccharomyces* ont une longue histoire d'usages traditionnels et d'effets bénéfiques. Davantage, les levures *Saccharomyces* ont été associées à l'alimentation du bétail et sont également considérés comme probiotiques. La présence de *Saccharomyces unisporus* a été enregistrée dans divers produits laitiers et est devenu un sujet d'intérêt et de grande importance. *S. unisporus* a démontré un rôle important dans la maturation du fromage et la production de produits laitiers fermentés tels que le kéfir et le koumis. L'absence de pseudohyphes pendant le cycle de vie de *S. unisporus* est une indication de non pathogénicité. La recherche a été mise au point sur la présence de *S. unisporus* dans les produits de qualité alimentaire et la ressemblance entre *S. unisporus* et *S. florentinus*. Ces deux espèces ont été acceptées par la Fédération Internationale de Produits Laitiers et l'Association Européenne d'aliments et de culture alimentaire pour des applications alimentaires et l'alimentation animale. D'autant plus qu'au fil des années, *S. unisporus* est déjà devenu une partie intégrante de divers produits laitiers, il est généralement considéré comme un candidat potentiel au statut sécuritaire (GRAS). *S. unisporus* a la capacité de convertir la cétoisophorone en lévodione, un précurseur pharmaceutique important. *S. unisporus* est considérée comme un producteur potentiel de farnesol pouvant contrôler la filamentation de micro-organismes pathogènes. Outre celà, *S. unisporus* produit certains acides gras non-saturés oméga qui combattent les maladies. Désormais, les possibilités d'exploitation de *S. unisporus* pour ses intermédiaires utiles sont les enzymes et les acides gras qu'elle produit. Dans ce contexte, cette revue de la littérature vise à décrire et à discuter de l'omniprésence de *S. unisporus* dans les produits

alimentaires, sa composition cellulaire, les voies de régulation, et sa synthèse des acides gras et des enzymes.

ABSTRACT

Yeast species *Saccharomyces* has a long history of traditional applications and beneficial effects. Along with it, *Saccharomyces* has been associated with livestock food and feed and has emerged as a probioticum. Among these presence of the *Saccharomyces unisporus* has been documented in various dairy products and has become a subject of interest and great importance. *S. unisporus* has shown a significant role in the ripening of cheese and production of fermented milk products such as kefir and koumiss. The absence of pseudohyphae during the life cycle of *S. unisporus* is an indication of non pathogenicity. Significance has been laid on the presence of *S. unisporus* in food-grade products and a close proximity of *S. unisporus* to *S. florentinus* and both of these species are accepted by the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA) for food and feed applications. Since over the years, *S. unisporus* has already become a part of various dairy products, *S. unisporus* can be considered as a potential candidate for generally regarded as safe (GRAS) status. *S. unisporus* has the capacity to convert ketoisophorone to levodione, which is an important pharmaceutical precursor. *S. unisporus* are considered as the potential producers of farnesol which eventually controls filamentation of pathogenic microorganism. Apart from that, *S. unisporus* produces certain omega unsaturated fatty acids which combat diseases. Henceforth, the areas which *S. unisporus* can be possibly exploited for its useful intermediates are the enzymes and fatty acids it produces. In this context, this review attempts to describe and discuss the ubiquity of *S. unisporus* in food products, cellular composition, regulatory pathways, and its synthesis of fatty acids and enzymes.

Keywords: *Saccharomyces unisporus*; Fatty acids; Regulatory pathways; Carbon sources; Volatile compounds; Toxin; Intracellular proteins.

1.INTRODUCTION

Yeasts are known for their biotechnological applications and therefore interest in searching and characterizing new yeast species has increased over the years. Yeasts are mainly used for the production of beverages, cereal-based food, enzymes, fine chemicals, single-cell protein, and flavoring compounds (Eijk and Johannes 1995; Gatto and Torriani 2004; Wang 2008). Yeasts are also used for research in genetics, molecular biology, and cell biology, and most of the focuses are on the 2 species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. However, some nonconventional yeast species like *S. unisporus* draw interest because of their biotechnological potentials due to ubiquitous presence, non-pathogenicity, and probiotic nature (Sinnott 2010). For the possible benefits of human and animal health, organisms which are termed as probiotic can be incorporated into dietary adjuncts for the maintenance of a healthy gastrointestional balance (Lourens-Hattingh and Viljoen 2001). Mostly incorporated organisms are *Lactobacillus* along with *Saccharomyces*. *S. unisporus* yeast was discovered by Holm (Jorgensen 1920). Under the genus *Saccharomyces*, 10 species are accepted of which *S. unisporus*, which is also called as *Kazachstania unispora* (Lu and others 2004) is one of them. *S. unisporus* is an auxotrophic organism (Vogl and others 2008) and it is found to be the dominant yeast in traditional dairy products (Montanari and Grazia 1997; Coppola and others 2008; Rahman and others 2009; Yildiz 2010; Bourdichon and others 2012). A unique synchronization of *Kluyveromyces marxianus* and *S. unisporus* (*Kazachstania*) species have been found in milk-fermented products, such as dahi, suusac, gariss, kefir, shubat and koumiss (Narvhus and Gadaga 2003; Lore and others 2005; Abdelgadir and others 2008; Rizk and others 2008)**.**

S. unisporus is the slowest producer of ethanol and performs a clean fermentation in milk and whey (Montanari and others 1996). Oxygen is required in very minute amounts for the yeasts *Saccharomyces* to synthesize sterols and unsaturated fatty acids. A specialty about *Saccharomyces* cells is that they cannot ferment sugars, especially under anaerobic conditions over a long period because they require pre-formed lipid precursors for growth which are synthesized under aerobic condition. In laboratory practices, ergosterol and Tween 80 (defined source of oleic acid which is an unsaturated fatty acid)

are added to the medium to study *Saccharomyces* species anaerobically (Ingledew 1999). Yeast organisms extracted from Spanish blue-veined cabrales cheese have been found to contribute towards the maturation and ripening of the cheese (Callon and others 2006).

Most of the studies have been done on kefir grains and by-products, where it is mentioned that metabolites from yeast, such as *S. unisporus* give taste to the products and also provide a milieu for the growth of bacteria (Maalouf and others 2011). However, the significance of consumption of these metabolites is not yet known. *S. unisporus* grows efficiently in cheese; however the enzymes and extracellular enzymes produced by this yeast still remain a subject of study. Furthermore, there is a debate whether this organism is generally regarded as safe (GRAS) in food products and animal feed. Therefore, this review intends to summarize the research available on the existence of *S. unisporus* in natural food and feed, as well as other pertinent information regarding its safe use with other potential applications.

2.Taxonomy and Biology

2.1 Taxonomy

S. unisporus can be classified as belonging to the fungi, which are the largest eukaryotic group consisting of yeasts and molds, of which one of the phylum is the sac fungi known as Ascomycota. A subphylum of Ascomycota is *Saccharomycotina* and one class there is *Saccharomycetes*; *Saccharomycetales* is an order which falls under the class *Saccharomycetes,* since they reproduce by budding. Family is *Saccharomycetaceae*, and genus is *Kazachstania*. However, *S. unisporus* has also been placed under species *Zygosaccharomyces* (Alvarez-Martin and others 2007). Scientific classification has been presented in Figure 1.

Species of the genus *Saccharomyces* are divided into a group of petite-positive and petite-negative. A typical specialty of a *Saccharomyces* species is that, when it is allowed to grow in the absence of oxygen, it generates respiratory-deficient mitochondrial mutants which are called petites. Similar observations have been recorded for *S. unisporus*. This is a unique trait of *Saccharomyces* clade. A petite-positive

organism can efficiently grow in the absence of oxygen and its survival is not dependent on the loss of mtDNA. A petite-negative organism cannot grow on fermented carbon sources and it cannot grow after the loss of mtDNA (Wang 2008). Petite-positive species are further divided into *sensu stricto* (species with narrow circumscription) and *sensu lato* species (species with wider circumscription). *S. unisporus* falls into the category of *Saccharomyces sensu lato* species (Broker and Harthus 1997; Piskur and others 1998; Loureiro and Querol 1999; Marinoni and others 1999**;** Groth and others 2000; Cliften and others 2005) along with other *sensu lato* species which are *Saccharomyces turicensis*, *Saccharomyces bulderi*, *Saccharomyces zonatus* and *Saccharomyces spencerorum* (Mikata and others 2001). While *Saccharomyces bayanus* and S*accharomyces eubayanus* falls in the *sensu stricto* group (Libkind and others 2011). Other *Saccharomyces* spp. which is *Saccharomyces chevalieri* and *Saccharomyces ellipsoideus* have close associations with *S. cerevisiae* (Soltesova and others 2000; Hoff 2012). Hence a phylogenetic tree (Figure 2) has been drawn to identify the *Saccharomyces sensu stricto* and *sensu lato* spp. in clusters. Apparently, Table 1 has been presented based on the distance matrix and evolutionary distance of one *Saccharomyces* spp. from another *Saccharomyces* spp.

2.2 Biology

2.2.1 Morphology and reproduction

S. unisporus ascospores are refractive and round with an average diameter of 4.5 μm and length of 3 μm. Vegetative cells are transformed directly into asci containing one and occasionally two globose to subglobose ascospores (Yarrow 1984). The yeast actively grows in presence of yeast malt agar and sporulation is observed in the absence of acetate. The spores appear at the end of 40 h at 25 °C and after 72 h at 15 °C (Yarrow 1984). This yeast does not form a scum but shows simply a ring in old cultures. The external morphology consists of a cream, flat layer. It is usually glossy and smooth, sometimes with light striations (Yarrow 1984). Morphology of *S. unisporus* is shown in Figure 3 obtained through scanning electron microscopy (SEM).

The cell components are coenzyme Q6 (Kurtzman 2003) and %Mol G+C (Guanine + Cytosine) range is 32.4 (Vaughan-Martini and Kurtzman 1988). However, in

the genus *Saccharomyces* there is less G+C content. The size of the mitochondrial DNA is detected as 29 kilobase pairs, with an identification of sites for *MspI* gene/ type-2 restriction enzyme after 11 kilobase pairs and after subsequent 8 kilobase pairs *HaeIII* gene/ type-2 restriction enzyme. The other important gene site (*Ori*/*rep* gene) has not been detected. Composition of *S. unisporus* can be identified based on the most popular organism *S. cerevisiae* with which *S. unisporus* has a 59.6% identity match. In all genetic comparisons, *S. cerevisiae* is taken as the base organism. Out of 59.6%, the percentage of noncoded region is 69.5 and the percentage of coded region is 40.4 (when compared with *S. cerevisiae*). The predicted protein types are hypothetical protein, polyprotein, and homolog of co-factor B. *S. unisporus* has chromosomes [Chr2:363049(start)-362771(end), Chr3:100839 (start)-101343(end), and Chr5:551117(start)-550863(end)] and they match with those of *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Piskur and others 1998). The total genomic size up to the year 1999 was 11560 kb (Piskur and others 1998; Cliften and others 2001).

The external morphology of *S. unisporus* is entire or undulating; pseudohyphae are not formed. Pseudohyphae are morphologically different from hyphae. The pseudohyphae or hyphae are virulent factors, and hence an organism having pseudohyphae or hyphae is considered as pathogenic (Sudbery and others 2004). However, hyphae are far more pathogenic than pseudohyphae. During yeast growth, first stage is the formation of pseudohyphae, which may or may not convert into hyphae. Since *S. unisporus* does not even form pseudohyphae, therefore it cannot be considered pathogenic. Also, pseudohyphae are formed when yeast cells do not fully separate after every cell cycle completion (Sudbery and others 2004). On the other hand, *S. cerevisiae* can form pseudohyphae but hyphae are not formed (Bastidas and Heitman 2009). However, all strains of *S. cerevisiae* do not form pseudohyphae (Llanos and others 2006; Perez-Torrado and others 2012); only diploid strains of *S. cerevisiae* develop pseudohyphae under nitrogen starvation condition (Strudwick and others 2010). Single copies of genes responsible for the development of pseudohyphae in haploid cells of yeasts are a1/α2 repressor. These repressors, which are transcriptional repressors stimulate the pseudophyphal pathways in yeasts when environmental condition is devoid

of nitrogen. In case of diploid cells, when *MAT* locus as *MAT*a/*MAT*α, *MAT*α/*MAT*α, and *MAT*a/*MAT*a are present in the yeast cells, pseudohyphae is likely to form (Lo and others 1997; Wolfe 2006). Thus, based on pseudohyphae/hyphae formation characteristics, a phylogenetic tree was drawn (Figure 4) to correlate the pathogenicity, where maximum parsimony method has been used for aligning species of *Saccharomyces* complex and *Candida albicans* ATCC 18804.

Studies have been carried out to know whether *S. unisporus* could sustain extreme conditions and whether the organism needs any extra chemicals for sustaining the drastic conditions. Merico and others (2006) tried to grow *S. unisporus* in synthetic medium with fortification of antimycin A (concentration range of 0.5-25 µM). *S. unisporus* grew efficiently at all dosages of antimycin A up to the 7^{th} day. However, other strains of *Saccharomyces* species could grow only when the medium was supplemented with lysine (Mikata and others 2001), glutamic acid, and acetoin (Loureiro and Querol 1999; Merico and others 2006). Antimycin A (secondary metabolite) is produced by *Streptomyces*, a genus of Actinobacteria. This chemical compound binds to the cytochrome c reductase and thus inhibits the oxidation of ubiquinol (Neft and Farley 1972).

2.2.2 Growth conditions and substrate (carbon, nitrogen, and vitamin requirements)

S. unisporus is a nonlactose fermenting yeast (Montanari and others 2000; Merenstein and others 2009). The compounds which *S. unisporus* can efficiently consume are listed in Table 2. *S. unisporus* ISA 1097 where ISA stands for Instituto Superior de Agronomia grew efficiently when the carbon sources were glucose, sucrose, maltose, cycloheximide, and sorbic acid (Rodriques and others 2001). It does not grow on sucrose, maltose or lactose (Wang and others 2008) and produces succinic acid (Sahasrabudhe and Sankpal 2001). Considering this organism's sugars metabolism, it could be related to *Saccharomyces mali duclauxi.* Further, *S. unisporus* is capable of utilizing nitrogenous compounds that are found in the environment (except nitrates and nitrites) as the sole nitrogen source. *S. unisporus* assimilates ethylamine (Middelhoven 2002), cadaverine, and lysine (Andorra and others 2010), which are not usually consumed by all *Saccharomyces* **(**James and others 1997**).**

Thiamine is the only vitamin required as exogenous source for growth of *S. unisporus*. *S. unisporus* grows faster in the presence of thiamine in culture media rather than in the absence of thiamine. It is classified auxotrophic because it fails to synthesize thiamine as an essential compound (Wightman and Meacock 2003). It is co-related with *S. servazzi* and *S. castellii* because of the lack of gene *THI5*/enzyme involved in the synthesis of thiamine precursor. It follows HMP (4-amino-5-hydroxylmethyl-2-methylpyrimidine) and HET (5-(2-hydroxy-ethyl)-4-methylthiazole) pathways. These pathways undergo phosphorylation and are condensed to thiamine monophosphate (Vogl and others 2008). However, *S. unisporus* cannot use thiamine monophosphate for its growth.

THI9 gene is strongly up-regulated (activated) by a supply of thiamine and that implies thiamine transport. This also specifies the absence of another significant gene, *BSU1*, which is up-regulated when pyridoxine and thiamine are present endogenously. The minimum concentration of thiamine required for growth of *S. unisporus* is 1.2 µM (Vogl and others 2008), however *THI9* starts expressing even when a lower concentration of thiamine is present in the medium. Lower glucose concentration or absence of glucose in the medium enables a strong reduction in thiamine uptake. Thiamine-dependent *S. unisporus* also requires efficient plasma membrane transport proteins for the acquisition of thiamine. However, in most of the organisms, thiamine is not produced in higher quantities than are being required for TDP (thiamine diphosphate) biosynthesis.

Thiamine plays a major role in carbohydrate metabolism in living cells. In *S. unisporus*, thiamine represses 60 or more genes and most of them are involved in thiamine metabolism. Vogl and others (2008) demonstrated a current model of thiamine-sensing, where TDP works as the intracellular thiamine signal. It is an important cofactor of transketolase enzyme complexes in the oxidative decarboxylation of oxo-acids (Vogl and others 2008).

3.Potential applications

3.1 Presence and role in milk-derived products

Graziella and Grazia (1997) studied the yeast microflora of 94 samples of Central Asian koumiss and found *S. unisporus* as a dominant species (in 68% of the samples examined). The species was tested for sugar fermentation and it was found that *S. unisporus* is comparatively less capable of producing alcohol from grape must. *S. unisporus* has been found on the surface of different types of cheese and has been shown to be important for cheese quality as it performs cheese ripening and maturation (Corsetti and others 2001; Callon and others 2006; Jacques and Casaregola 2008). It was also stated that *S. unisporus* was the only isolated species of the *Saccharomyces* genus and constituted 5% of all the identified yeast from cheese (Tornadijo and others 1998). Furthermore, *S. unisporus* was identified by Nunez and others (1981) from Cabrales cheese, and its population was 3% of total isolates obtained from this cheese.

Kefir production is started using kefir grains which consists of a rubbery matrix embedded with different types of microorganisms. Kefir grains contain lactic-acid bacteria, acetic acid bacteria, lactose-fermenting yeasts of *Kluyveromyces* spp. which are *Kluyveromyces marxianus* and *Kluyveromyces lactis*, and nonlactose fermenting yeasts are *Saccharomyces unisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus* (Angulo and others 1993; Pintado and others 1996; Wyder and Puhan 1997; Wyder and others 1997; Marquina and others 2002; Heras-Vazquez and others 2003; Farnworth and Mainville 2003; Farnworth 2005; Mainville and others 2006; Latorre-Garcıa and others 2007, Păucean and Socaciu 2008; CodexAlimentarius 2011). A broader spectrum of yeast species was found among samples of home-made kefir samples, of which *Issatchenkia orientalis*, *S. unisporus*, *S. exiguous*, and *Saccharomyces humaticus* were the most representative species (Latorre-Garcıa and others 2007). *S. unisporus* is also involved in a unique process patented in United States for the production of kefir-like fermented milk (Saita and others 1991).

Viili is a category of fermented milk which originated in Scandinavia, it is claimed to possess functional benefits and health-improving potential. *K. marxianus*, *S.*

unisporus, and *Pichia fermentans* were identified in viili starters corresponding to 58, 11, and 31% of total cell counts, respectively (Wang and others 2008). In a study by Canibe and others (2010) fermented liquid feed samples for feeding pigs from 40 Danish farms were prepared and further microbial and biochemical variations were determined. The feed group was divided into 2 groups, a high- intake group and low intake group. In both groups, compositions of yeasts constituted 85-91% of the total isolates of microorganisms. Variable isolated yeasts were *S. unisporus* along with other *Saccharomyces* species.

Among the fermented milk products, koumiss (2% alcohol at pH 4.0) is prepared from mare's milk; its microflora is dominated by thermophilic lactic acid bacteria (LAB) along with *K. marxianus* and *Saccharomyces* species, specifically *S. unisporus* (Hansen and Jakobsen 2004). In kefir, the microorganisms often found are *S. florentinus*, *S. globosus,* along with *S. unisporus* (Coppola and others 2008). *S. unisporus* has shown close relation with *S. florentinus*, and both of these organisms come under the category of species which are accepted by the International Dairy Federation (IDF) and European Food and Feed Cultures Association (EFFCA) as suitable organisms for consumption by human beings or animals (Bourdichon and others 2012).

Even for edible biomass production, *K. marxianus* and *S. unisporus* are the common yeast species exploited (Lewandowski 2011). Apart from that, *S. unisporus* has shown presence in dahi (a milk fermented product from India), gariss (which is a fermented camel milk product), shubat (which is similar to gariss), and suusac along with other beneficial yeasts and bacteria (Rizk and others 2008).

3.2 Interaction with lactic acid bacteria

S. unisporus is present in dairy products, but usually at a lower concentration than other lactose-fermenting yeast species, such as *Kluyveromyces* species (Wouters and others 2002). *S. unisporus* has a marked presence in koumiss, a product produced in Central Asia and the former Soviet Union derived from the lactic and alcoholic fermentation of mare's milk (Curadi and others 2001). The microflora of koumiss

consists mainly of thermophilic LAB and species of *Saccharomyces*. The yeasts consist of lactose-fermenting and lactose-non-fermenting species. The yeast producing mare milk fermentation (alcoholic) is a lactose-fermenting species from the *Kluyveromyces* genus and, more often, nonlactose-fermenting strains belonging to *Saccharomyces* genus (Montanari and Grazia 1997; Frölich-Wyder 2003; Cagno and others 2004).

In *Saccharomyces*, a frequently found species is *S. unisporus*, which vigorously ferments galactose (Montanari and Grazia 1997; Wouters and others 2002). Currently, koumiss is manufactured at an industrial level in many countries (Tamime and others 1999; Litopoulou-Tzanetaki and Tzanetakis 2000). The interaction between yeast and LAB may be stimulation or inhibition of each other in a co-culture. They compete for growth nutrients or they will produce certain metabolic products that stimulate growth of the other organism (Neviani and others 2001; Lopitz-Otsoa and others 2006). *S. unisporus* is one of the isolates from different batches of kefir grains. Apparently, other *Lactobacillus* spp. are also isolated from the same. The unique interaction between yeasts and LAB for example *Lactobacillus kefiranofaciens* demonstrates stability in a mixed culture of kefir or any other milk fermentation product (Lopitz-Otsoa and others 2006).

Certain yeasts in kefir are lactose-positive and most of them consume galactose, including *S. unisporus* (Yarrow 1984**)**. On the contrary, yeasts may produce vitamins which may enhance the growth of LAB (Lopitz-Otsoa and others 2006). Commensalism between yeast and LAB is taken into consideration for the production of single-cell protein (SCP) in cheese whey (a by-product of the cheese industry). LAB produces DL-pyro-glutamic acid and lactic acid; however, none of the yeasts produces glutamic acid (Gadaga and others 2001). In kefir grains, when complex microfloras were considered, often they were classified as homofermentative lactic acid bacteria, heterrofermentative lactic acid bacteria, lactose-assimilating yeast and nonlactose-assimilating yeast. During the production of kefirs, homofermentative lactic acid bacteria were simulated by the intermediates produced from the other 3 groups. For the kefir production and enhancement, presence of yeasts for example *S. unisporus* is a must as excessive accumulation of lactic acid can inhibit *L. kefiranofaciens*, while gradual removal of lactic

acid enhances the production of kefir. Yeasts otherwise provide vitamins, amino acids and other growth factors which influence the bacterial growth (Cheirsilp and others 2003).

3.3 Presence in other fermented food

S. unisporus was found in fermented orange fruit and juice and was identified and classified by different molecular techniques (Heras-Vazquez and others 2003; Canibe and others 2010). The presence of *S. unisporus* has also been observed in sugarcane juice (Qureshi and others 2007). Apart from those, vegetable juice favors growth of *S. unisporus* (Savard and others 2002a, b). The role of *S. unisporus* was found important in whey vinegar production (Rainieri and Zambonelli 2009). However, the exact role of *S. unisporus* for its existence in these items is not identified and further research is required to exactly define its function.

Eijk and Johannes (1995) invented the process of preparing substrate-limited dough (a mixture of flour and water), where *S. unisporus* was one of the proposed yeast for the preparation of yeast-leavened bread dough (Gatto and Torriani 2004). In such dough preparations, the proposed yeasts or *S. unisporus* consumes the fermentable sugars, whereas the nonfermentable sugars are added additionally to the bread dough for adjusting the sweetness of the bread dough. An example of such additive, which acts as artificial sugar supplement, is whey permeate; it is added in the range of 0.1-10% (w/w) to the bread dough.

3.4 Production of volatile compounds

S. unisporus IFO (Institute for Fermentation, Japan) 0298 strain has the capacity to convert ketoisophorone into levodione (a volatile organic compound). Levodione is involved in oxidoreductase activity and also plays a significant role in the commercial synthesis of carotenoids. In the presence of other yeasts, *S. unisporus* converts 2,6,6-trimethyl-2-cyclohexane-1,4-dione into (6R)-2,2,6-trimethylcyclohexane-1, 4-dione (an isomer of levodione) (Fukuoka and others 2002) as shown in Figure 5. The average time needed for levodione production by *S. unisporus* was 16 h, with a final production rate of

16.7 g levodione/kg yeast cells/h; whereas the production rate was only 8.8 g levodione/kg yeast cells/h when *S. cerevisiae* was used (Fukuoka and others 2002).

Prenyl alcohol is the major component of odorants in roasted coffee and essential oils. *S. unisporus* cells produce a significant amount of prenyl alcohol in a nutrient medium. It also has the potential to produce geranylgeraniol (which is an intermediate in the biosynthesis of vitamins E and K) and farnesol, which are typical members of the prenyl alcohol family (Obata and others 2005). Farnesol (one of the major natural alcohols) is produced by *S. unisporus*. Farnesol has been considered as an anti-tumor agent (Obata and others 2005). If a wild-type strain of yeast which produces geranylgeraniol or farnesol naturally, yeast growth can be stimulated and geranylgeraniol or farnesol can be produced at a much higher rate in a medium which will be inexpensive and economic, leading to mass production of the intermediates. Along with *S. unisporus*, *S. cerevisiae* and *S. dairensis* are certain species of *Saccharomyces* which are the natural producers of farnesol (Muramatsu and others 2002). Extensive production of farnesol controls filamentation in fungi which are polymorphic and pathogenic for example *C. albicans*, henceforth destroying the harshest of opportunistic organisms (Albuquerque and Casadevall 2012).

The entire *Saccharomyces* complex shows a well-balanced fermentative metabolism. *S. unisporus* produces mutants when allowed to grow under drastic conditions with rearranged mtDNA molecules. This condition is called petite phenotype and *S. unisporus* is petite-positive (Merico and others 2006, Fekete and others 2007). Petite-positives can survive well anaerobically. Under anaerobic conditions, respiratory biochemical pathways are shut down and the only way the yeast cells generate energy is through substrate-level phosphorylation. Under such conditions, cells maintain their redox balance by the production of glycerol through glycerol 3-phosphate dehydrogenase and production of succinate by fumarate reductase. During such conditions, the mitochondrion usually does not play a significant role in energy metabolism (Merico and others 2006).

Savard and others (2007) stated that food products containing a minimum concentration of ethanol at pH 3-11 were more prone to microbial spoilage. *S. unisporus* can efficiently grow in this range of pH (3-9) (Loureiro and Querol 1999) and produce low ethanol concentration and, therefore, is mainly considered as a food spoiler (Savard and others 2007). The strains of *S. unisporus* do not perform clean alcohol fermentation as it produces some other minor byproducts, such as glycerol, succinic acid and acetic acid (Montanari and Grazia 1997). Two strains of *S. unisporus*, BR174 and BR180 isolated from mangrove bromeliads and tested for alcohol fermentation were found to be less tolerant to high ethanol concentration (5 g/L) (Morais and others 1996).

S. unisporus does not produce any phenolic odor during the entire incubation period as presence of phenolic aroma in a fermented food product can be considered as a food spoiler. However, it generates a phenolic aroma whenever it efficiently consumes *p*-coumaric acid (derivative of 4-ethylphenol) and produces 4-ethylphenol as an intermediate (Loureiro and Querol 1999; Rodriques and others 2001). The amount of 4-ethylphenol produced is proportional to the concentration of *p*-coumaric acid in the medium. The 4-ethylphenol is a volatile phenol responsible for wine spoilage (Rodriques and others 2001). *S. unisporus* along with other yeasts produces 0.7-2.5% (w/v) of ethanol (Zajsek and Gorsek 2010). *S. unisporus* also demonstrates the "crabtree" effect, which means it produces ethanol, pyruvate, acetate, succinate, and glycerols following the TCA cycle (Merico and others 2006). Production of volatile and nonvolatile flavoring compounds by *S. unisporus* in fermented food products has a positive impact on any food and feed.

3.5 Fatty acids profile of *S. unisporus*

Over the years, single cell proteins (SCP) are best known for their high nutritive value as they have higher protein, vitamin, and fatty acid contents; and they are used as a supplement in food and feed (Lewandowski 2011). Fatty acids constitute 5% of the overall cell weight of *S. unisporus* (Kock and others 1986). *S. unisporus* produces palmitic acid and oleic acid. Middle chain fatty acids (saturated or unsaturated) are present in *S. unisporus* ranging from carbon 14:0 to 18:1. When compared to other

Saccharomyces spp., *S. unisporus* cells contain a high percentage of palmitoleate, followed by palmitic acid, and oleic acid. Other fatty acids like myristic (Kock and others 1986) and linolenic acids are absent in this species of *Saccharomyces* (Grillitsch and others 2011). Palmitoleic acid is an omega-7 monounsaturated fatty acid, which is also a major constituent of human adipose tissues. Current-day research shows that palmitoleic acid (which *S. unisporus* is known to produce) acts as the signaling molecule to help fight weight gain (Yarrow 1984). Palmitate is the first product of lipogenesis (fatty acid synthesis) and is considered as an antioxidant and a source of vitamin A (Beare-Rogers and others 2001).

Furthermore, no unusual fatty acid chain has been reported in *S. unisporus* as yet. *S. unisporus* produces high concentration of fatty acids in the presence of glucose, sucrose, and low concentrations of lower-chain fatty acids have been recorded in the presence of maltose and galactose, whereas it does not produce any fatty acid in the presence of lactose at temperatures of 25 °C and 30 °C (Obata and others 2005).

S. unisporus did not produce linoleic acid (C18:2) and linolenic acid (C18:3) **(**Kock and others 1986; Loureiro and Querol 1999). However, it is not known if *S. unisporus* produces other C18 fatty acids. The absence of C18 polyunsaturated fatty acids is considered as a chemical marker of a strain being a pathogen or a food spoiler in the food industry. Therefore, production of C18 fatty acids by *S. unisporus* must be investigated.

3.6 Biochemical sensitivity of *S. unisporus*

Unlike other *Saccharomyces* species, *S. unisporus* is resistant to organic acids. The strain *S. unisporus* Y-42 was tested against a set of organic acids including acetic acid, lactic acid, and propionic acid, individually and in mixture. A mixture of lactic acid 0.7% (w/v), acetic acid 0.3% (w/v), and propionic acid at 0.2% (w/v), when added to a vegetable juice medium, completely inhibited growth of *S. unisporus* (Savard and others 2002a, b). Another effective inhibitory effect of *S. unisporus* has been noticed by an easily hydrolyzable chitosan-acid complex comprised of a chitosan oligomer complexed

with an acid radical or an acid with a molecular weight of 0.5 kDa to 1.2 kDa. *S.unisporus* Y-42 also exhibited sensitivity to chitosan hydroxylates at pH 3.8. *S. unisporus* is resistant when the pH is low. A microscopic view of the inhibited *S. unisporus* by chitosan-acid complex showed an irregular coating around its surface, which generalizes the phenomenon of cell suffocation. The mechanism proposed is the reactive amino groups in chitosan which interacts with various anionic groups on the yeast cell surface and thus creating a layer (Savard and others 2007).

S. unisporus has been found to partially inhibit a food pathogen *Listeria monocytogenes.* Goerges and others (2006) showed in an agar-membrane assay that *S. unisporus* produces a toxin responsible for eradicating this common food pathogen, which has not been biochemically characterized. Haruji and others (2008) claimed the use of yeast (*Saccharomyces* and non-*Saccharomyces*) to produce an inhibitor for the production of secondary bile acid. *S. unisporus* was also found to be a significant producer of an inhibitor which inhibits secondary bile acid production. Deoxycholic acid is a bile acid and *S. unisporus* absorbs up to 65.5% of this acid. Another bile acid, chenodeoxycholic acid, is also absorbed by *S. unisporus* up to 58.8% (Haruji and others 2008).

3.7 Biochemistry and Molecular Biology

3.7.1 Intracellular proteins and intergenic sequences

mtDNA (mitochondrial DNA) of *Saccharomyces sensu lato* species, for example, *S. unisporus,* contains fewer guanine-cytosine (GC) clusters and length is usually smaller than 50 kb. Some researchers have also reported GC clusters of less than 100 bps (Groth and others 2000; Spirek and others 2003). The size of mtDNA for *S. unisporus* is 29 kb and GC cluster content is 23% in mtDNA (Piskur and others 1998). Groth and others (2000) studied 2 mitochondrial genes (*SSU* and *ATP9*) from various *Saccharomyces* species and phylogenetic trees were drawn based on mtDNA molecules, of which *S. unisporus* was found to have close relationship with *S. exiguus* species in *SSU* gene; whereas *S. unisporus* exhibited close relatedness with *S. servazzii* in *ATP9* gene (Groth and others 2000).

The gene order in *Saccharomyces* spp. differs among each other (Keogh and others 1998). Gene order of the same species is karyotype but is more heterogeneous. The chromosome numbers in *Saccharomyces* spp. vary from 8-16, whereas *Saccahromyces sensu* is a strict group which is interfertile and *S. unisporus* chromosome numbers are usually 11-13 (Petersen and others 1999). In the *Saccharomyces sensu lato* species, small chromosomes (less than 0.5 Mb) are present; very different from the *Saccharomyces sensu stricto* species which has larger mtDNA molecules and highly extensive intergenic regions (Marinoni and others 1999; Spirek and others 2003).

Ho protein is an intracellular protein, highly conserved in all *Saccharomyces* species including *S. unisporus*. The latter has two domains of nuclear localization sequences (NLS1 and NLS2), where NLS1 is inside the endonuclease domain, and NLS2 is inside the zinc finger domain. Ho proteins are induced by domestication of *VMA1* interin (Bakhrat and others 2006). *S. unisporus* produces *VMA1*-derived endonucleases known as VDE (Okuda and others 2003; Posey and others 2004). Okuda and others (2003) studied endonuclease activity of *S. unisporus* IFO0316 and showed that VDE proteins were expressed; however, no endonuclease activity was recorded. *S. unisporus* contains 45.6% hydrophobic, 25.9% neutral, and 28.0% hydrophilic amino acids in *VMA1* inteins (Okuda and others 2003).

Bakhrat and others (2006) studied common sequential regions of Ho and *VMA1* strains. Ho and *VMA1* are intracellular proteins and fall into 2 different phylogenetic groups. Ho is marked as the proteosome substrate within the nucleus. In a bootstrap alignment, *VMA1* interim sequence present in *S. unisporus* is closely related to *S. cariocanus, S. castellii*, and *S. dairenensis* species (Bakhrat and others 2006). Posey and others (2004) speculated duplication of endonuclease gene followed by their fusion which furnished monomeric proteins (like PI-SceI) for *S. cerevisiae.* In the case of *S. unisporus* this process gave an enzyme known as PI-SunIP. Percentage identity for this monomeric protein (PI-SceI) is 33% and the region conserved at position 301st is lysine,

341st position is threonine, and 403rd position it is lysine again. PI-SunIP is an active homing enzyme (Posey and others 2004).

Presence or absence of *ori-rep-tra* sequences has divided the *Saccharomyces* species into 2 different categories of *sensu stricto* and *sensu lato* (Piskur and others 1995). In mtDNA of *S. cerevisiae*, a unique class of intergenic sequences is formed by the *ori-rep-tra* sequences, which are available in multiple copies. These sequences are supposed to be conserved in the same genus or species. However, these set of sequences are absent in *S. unisporus* and other yeasts of the same species. The sequence of *ori-rep-tra* is specific only for a limited number of closely related species.

In a process, where levodione is converted to actinol with the help of ketoisophorone reductase gene, which are derived from the group of organisms, one of them is *S. unisporus* IFO0298. Further ahead, actinol is extracted from the reaction mixture, where a recombinant microorganism is derived by direct transformation of the host microorganisms, for example *Saccharomyces* and *Candida* (Hoshino and others 2005). Actinol is a useful compound as it prevents osteoporosis.

A phylogenetic tree (Figure 6) has been drawn for *Saccharomyces sensu lato* species based on the D1/D2 domain sequences of 26S rDNA *S. unisporus* NRRL Y-1556 was found closely related to *S. servazzii* and *S. martiniae*, while *S. cariocanus*, *S. dairenesis*, and *S. castelli* are distantly related. Most of the species from *Saccharomyces sensu lato* are nonsporulating, for that reason interfertility tests were not performed on the organisms falling into this group (Spirek and others 2003). Such closeness with other important organisms of the *Saccharomyces* spp. makes *S. unisporus* a potential candidate for recombinant DNA technology. Apart from that, the presence of *S. unisporus* in various fermented milk products and fermented fruit products, along with other species, reveals that *S. unisporus* was the most conspicuous species of yeast found in kefir (Latorre-Garcia and others 2007) of which rRNA genes has been sequenced.

3.7.2 Regulatory pathways

When 18S rDNA of *S. unisporus* is aligned along with 18S rDNA of other yeast**,** the nearest 2 organisms which were highlighted were *K. sinensis* and *S. transvaalensis* which have a 97% sequence match (Blast N) (James and others 1997)**.** Another nearest organism is *S. servazzii* with which *S. unisporus* exhibits 99% sequence similarity (Blast N) (James and others 1997). Some strains of *Saccharomyces* genus have 97% coverage with *S. cerevisiae*, and the metabolic pathways that *S. cerevisiae* follows are phenylalanine, tyrosine, and tryptophan biosynthesis; histidine and lysine biosynthesis; fatty acid biosynthesis; fatty acid elongation; triglyceride biosynthesis; sphingolipid biosynthesis; and phospholipid biosynthesis. These are a few of the major pathways which *S. unisporus* might be following, but they are not exactly known and, therefore, further research is required on this topic.

3.7.3 Potential of recombinant DNA technology

Advancement in recombinant DNA (rDNA) technology has become a potential molecular biology tool which is used to exploit industrially potential microorganism to increase the yield or productivity of a desired product. The application of rDNA technology sometimes becomes essential for an effective industrial process. *S. unisporus* possesses much higher active biotransformation of ketoisophorone to levodione, with the help of old yellow enzyme (OYE) / ketoisophorone reductase, as compared to *S. cerevisiae*. The gene responsible for the enzyme, OYE (EC 1.6.99.1), has been cloned from *S. cerevisiae* and expressed in *E. coli* (Wada and others 2003). However, *S. unisporus* OYE has not been cloned yet and expressed until now. The other possibility is the insertion of levodione reductase gene to make recombinant *S. unisporus*. The recombinant *S. unisporus*, in that case, could convert ketoisophorone directly to actinol and complete the entire process in a single step (Hoshino and others 2006).

Similar applications can be achieved when other potential genes of *S. unisporus* can be cloned for the purpose of producing useful and beneficial compounds, which otherwise *S. unisporus* is producing only in minimal quantities. The scope of rDNA technology has a wide perspective when certain genes are considered for cloning, which

otherwise might be producing significant compounds or proteins, and in the long run must satisfy the concept of genetically modified organism (GMO) for long-time-frame usage.

4.Conclusions

The ubiquitous presence of *S. unisporus* in fermented milk (viili), cheese, and certain fruits, (grapes and oranges) has been long observed. *S. unisporus* is also one of the important yeasts found in food products like koumiss and kefir-based milk products. *S. unisporus* grows efficiently in a medium where carbon sources are glucose, galactose, sucrose, maltose, sorbic acid, and cycloheximide, but the yeast does not consume lactose. Growth of *S. unisporus* takes a boost in the presence of thiamine. It also produces thiamine monophosphate but cannot consume it.

A unique interaction between LAB and *S. unisporus* has been observed in certain milk products like kefir and koumiss. *S. unisporus* produces metabolites which LAB consumes and, on the other hand, LAB produces glutamic acid and lactic acid which *S. unisporus* consumes. *S. unisporus* efficiently produces middle-chain fatty acids upto C18:1. Other important fatty acids produced by the organism are palmitoleic, palmitic, and oleic acids. It is not known if it produces polyunsaturated C18 fatty acid to class it as safe or unsafe. Absence of C18 polyunsaturated fatty acid chains are considered as chemical markers of an organism being a pathogen.

It is a significant producer of levodione and actinol, which shows oxidoreductase activity and synthesis of carotenoids. Other essential volatile compounds are prenyl alcohol and farnesol. Certain sets of organic acids, which are lactic acid, acetic acid, and propionic acid, can inhibit *S. unisporus*. An importance of *S. unisporus* is that it inhibits certain food pathogens like *L. monocytogenes* and degrade secondary bile acids effectively. Regulatory metabolic pathways of *S. unisporus* have not been well defined until now and further research is required. Whole ranges of intergenic and intragenic sequences have been studied in *S. unisporus* to get it placed at the right subspecies of *Saccharomyces* and classified as a *sensu lato* species. Closeness of *S. unisporus* with *S. florentinus* has been observed, and both the organisms are accepted under IDF and

EFFCA as food cultures to be present in food and feed; these aspects predict that *S. unisporus* can be considered as a GRAS organism.

5.Future perspective

Aspects of using *S. unisporus* commercially in food products should be in concordance with the benefits and its natural occurrence in food items. Certain patented compounds which are levodione, has health benefits for the human race, but many other compounds are still unknown which *S. unisporus* might be producing under different culture media, for example, in cheese whey. The study of proteins also remains a significant researchable aspect because so far only limited numbers of extracellular proteins have been reported. Certain toxins are also produced by *S. unisporus* and they kill pathogens; there could be more and more could be produced at a commercial scale and thus would require further research. By listing all the properties of *S. unisporus* it can be categorized as a useful organism. Production of middle-chain fatty acids and significant amounts of intermediates by *S. unisporus*, when grown along with LAB, good-quality SCP can be produced which can be used for animal feed or food. It is an abundant by occurring organism in certain milk-based products and fruits that are being produced efficiently now by the food industry.

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Table 1- Observed evolutionary (percentage) distance of one *Saccharomyces* spp. from the other displayed in matrix-technological relationships using standard distance matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		56	54	97	88	45	88	100	94	59	57	95	58	95	94	95
2	56		95	54	53	43	53	56	54	66	65	56	65	56	50	95
3	54	95		57	53	42	53	54	53	63	65	57	67	54	52	95
4	97	54	57		90	45	90	97	94	58	59	94	62	94	95	95
5	88	53	53	90		44	99	88	89	61	61	85	62	85	90	86
6	45	43	42	45	44		44	46	41	53	56	45	52	40	47	86
7	88	53	53	90	99	44		88	89	61	62	86	62	85	90	86
8	100	56	54	97	88	46	88		94	59	57	95	58	95	94	95
9	94	54	53	94	89	41	89	94		64	60	93	62	94	93	92
10	59	66	63	58	61	53	61	59	64		91	64	94	60	64	93
11	57	65	65	59	61	56	62	57	60	91		58	94	57	58	94
12	95	56	57	94	85	45	86	95	93	64	58		60	94	96	95
13	58	65	67	62	62	52	62	58	62	94	94	60		62	59	96
14	95	56	54	94	85	40	85	95	94	60	57	94	62		95	96
15	94	50	52	95	90	47	90	94	93	64	58	96	59	95		90
16	95	95	95	95	86	86	86	95	92	93	94	95	96	96	90	

Note: 1- *S. bayanus* MCYC 623, 2- *S. bulderi* 30-1, 3- *S. cariocanus* NCYC 2890T, 4- *S. cerevisiae* AG4, 5- *S. chevalieri* MUCL 27815, 6- *S. dairenensis* NRRL Y-12639, 7- *S. ellipsoideus* MUCL 38888, 8- *S. eubayanus* CBS 1538, 9- *S. exiguus* 2-3, 10- *S. florentinus* NRRL Y-1560, 11- *S. martiniae* NRRL Y-409, 12- *S. spencerorum* NRRL Y-17920, 13- *S. turicensis* NRRL Y-27345, 14- *S. unisporus* SA21S02, 15- *S. zonatus* NBRC 100504, 16- *S. castellii* NRRL Y-12630

Table 2- Consumption of different carbon, nitrogen, and others substrate by *S. unisporus*

Compound	Assimilation	Fermentation	Reference
Glucose	(+)	(+)	Rodriques and others 2001
Lactose	(+) by certain *S. unisporus*	(-)	Cheirsilp and others 2003
Galactose	(+)	(+)	Wyder 2001
Trehalose	(+)	(-)	Mikata and others 2001
Sorbic acid	(+)	(+)	Rodriques and others 2001
Sucrose	(+)	(+)	Rodriques and others 2001; Qureshi and other 2007
Maltose	(+)	(+)	Rodriques and others 2001; Qureshi and other 2007
Lactic acid	(-)	(-)	Wyder 2001
Succinate	(+)	(-)	Mikata and others 2001
Lysine	(+)	(-)	Mikata and others 2001
Cadaverine	(+)	(-)	Mikata and others 2001; Lu and others 2004
Cycloheximide	(+)	(+)	Rodriques and others 2001; Middelhoven 2002
Ethanol	(+)	(+)	Rodriques and others 2001
p-Coumaric acid	(+)	(+)	Rodriques and others 2001
Glycerol	(+)	(+)	Rodriques and others 2001
Ethylamide	(+)	(+)	Middelhoven 2002; Mikata and others 2001
Vitamins	(+)	(+)	Middelhoven 2002
Max. growth T (ºC)			Middelhoven 2002; Qureshi and

other 2007

Note: (+) Positive growth; (-) no growth.

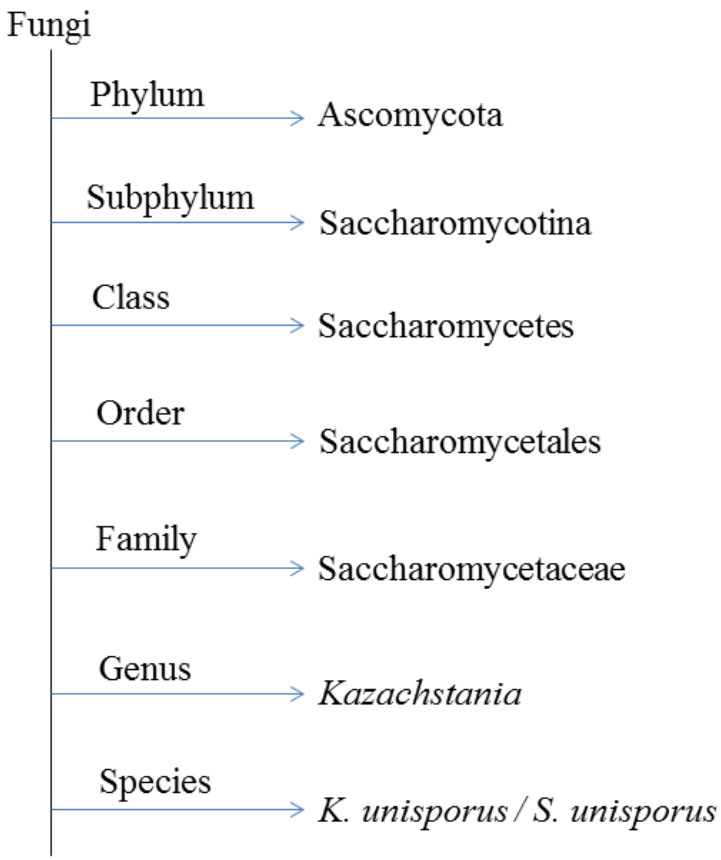


Figure 1- Scientific classification of *S. unisporus*

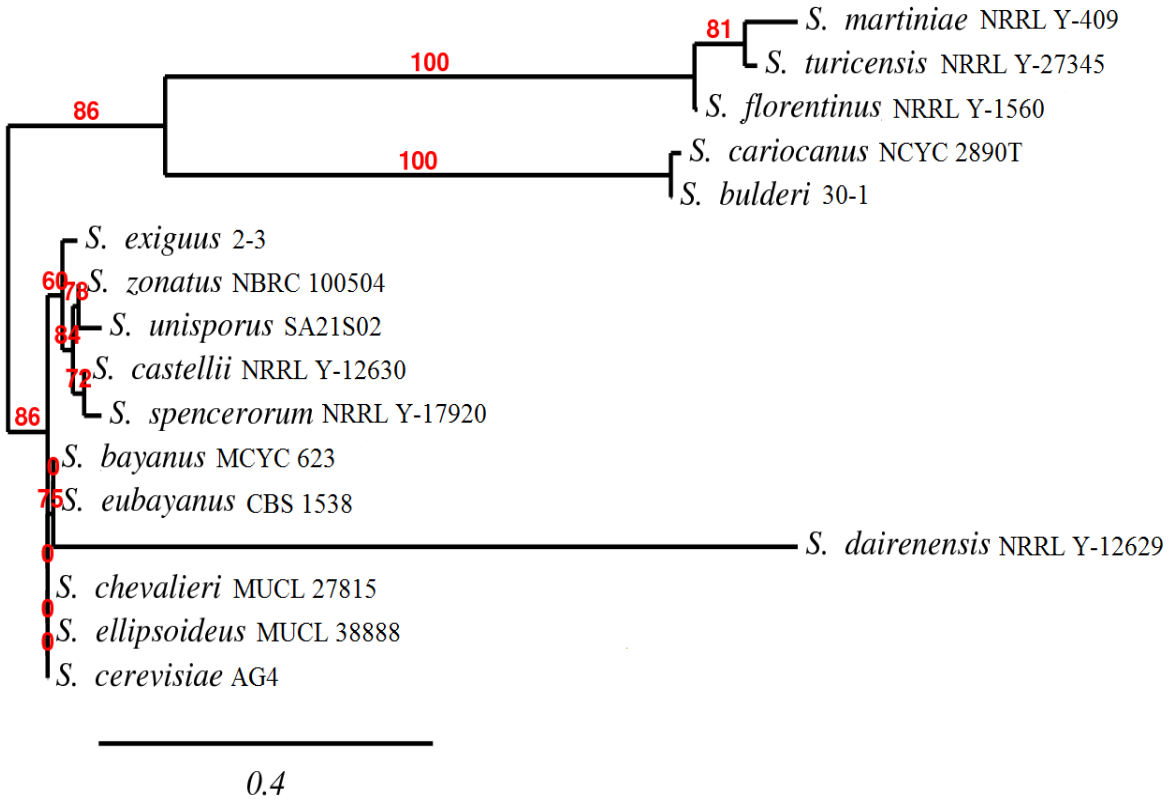
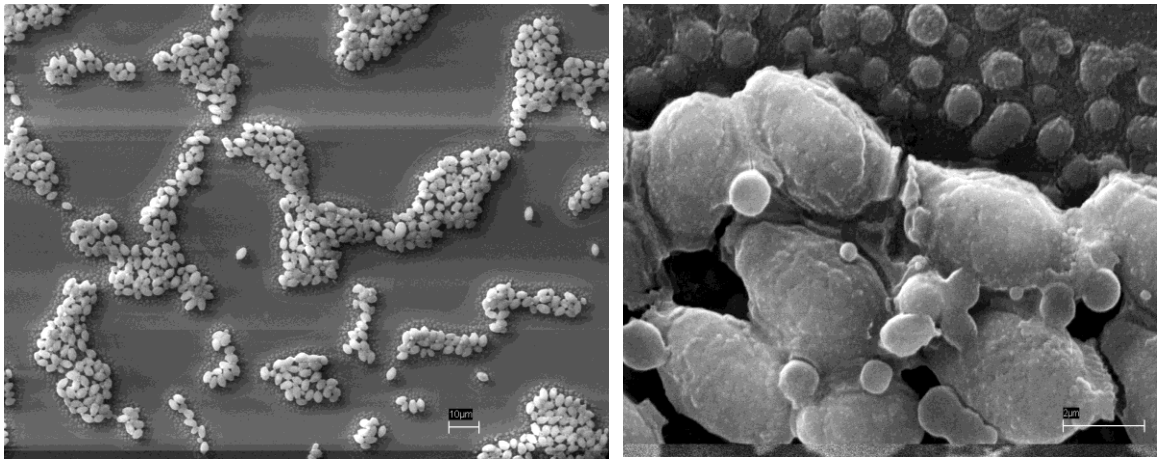


Figure 2- A phylogenetic tree based on Maximum parsimony method clustering *Saccharomyces sensu stricto* and *sensu lato* spp. Scale bar indicates 0.4 nucleotide substitution per site.



B

A

Figure 3- *S. unisporus* cells through SEM (scanning electron microscopy): A) Colony formation of *S. unisporus.* The *S. unisporus* cells, oval-shaped, are joined through small clusters. (SEM original magnification 2 000), **B)** *S. unisporus* cells conjoint with each other and budding simultaneously (SEM original magnification 30 000).

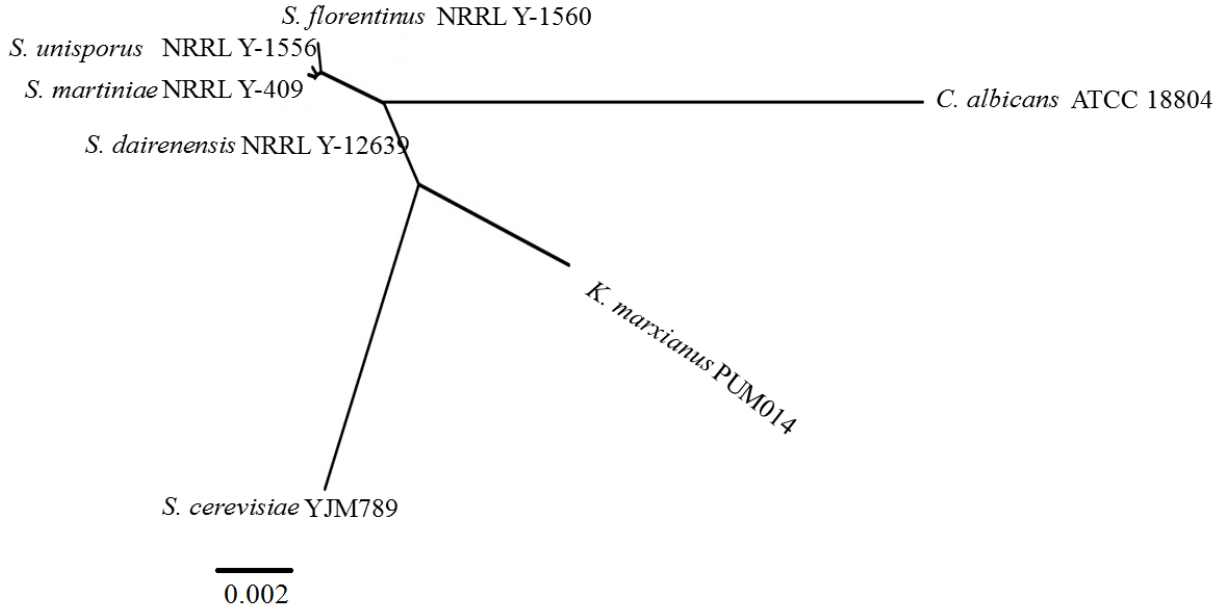
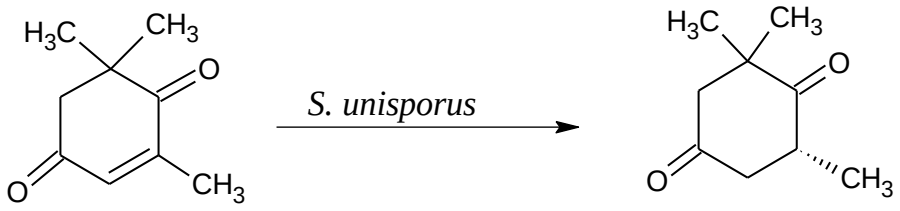


Figure 4- Maximum parsimony method for *Saccharomyces* complex and *K. marxianus* 26S rDNA partial sequences; where *S. unisporus* NRRL Y-1556, *S. martiniae* NRRL Y-409, *S. florentinus* NRRL Y-1560, and *S. dairenesis* NRRL Y-12639 fall into the same sub cluster. *S. unisporus* NRRL Y-1556 is relatively closer to *S. cerevisiae* YJM789 and distant from *C. albicans* ATCC 18804 and *K. marxianus* PUMY014; where *C. albicans* is a known pathogen and *S. cerevisiae* and *K. marxianus* fall under the generally regarded as safe (GRAS) yeasts. Scale bar indicates 0.002 nucleotide substitution per site.



2,6,6-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone)

(6R)-2,2,6-trimethyl-cyclohexane-1,4-dione ((6R)-levodione)

Figure 5- Biotransformation of ketoisophorone to levodione by *S. unisporus*

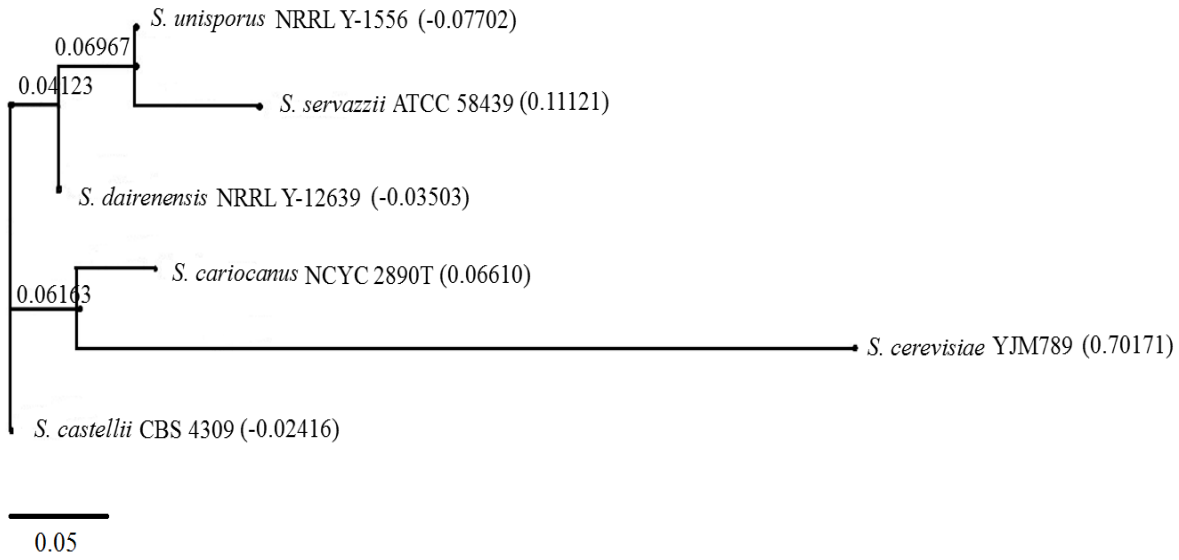


Figure 6- UPGMA method for the *Saccharomyces* complex, where *S. unisporus* are aligned against *S. cariocanus,* *S. castelli*, *S. servazzii*, *S. cerevisiae*, and *S. dairenesis*. ***S. unisporus* NRRL Y-1556** was closely related to *S. servazzii* ATCC 58439 though *S. dairenesis* NRRL Y-12639 fall into the same cluster as *S. unisporus* NRRL Y-1556 and *S. servazzii* ATCC 58439. *S. unisporus* is distantly related to *S. castellii* and *S. cariocanus*, when 26S rDNA is compared based on D1/D2 domains. Scale bar indicates 0.05 nucleotide substitution per site.

ARTICLE 2

Nutritional profile of *Saccharomyces unisporus* for single cell protein production: from classical to genomic approach

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ARTICLE 3

Acid hydrolysis of cheese whey for the growth of *Saccharomyces unisporus*

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Yet to be submitted

RECONNAISSANCE

L'originalité de cet article a été proposée par Indrani Bhattacharya. Les protocoles, les expériences et les analyses ont été approuvés par le Professeur R.D. Tyagi. L'article a été rédigé par Indrani Bhattacharya et corrigé par Dr. Jyothi Bezawada et Dr. Song Yan.

RÉSUMÉ

L'hydrolyse acide du lactosérum et du perméat de lactosérum a été effectuée pour induire la croissance de *S. unisporus* en culture mixte ou en mono-culture. Cette levure ne peut pas consommer le lactose. Différentes concentrations en H_2SO_4 (0,2, 0,3 0,4 p/p%) ont été utilisées pour l'hydrolyse. D'une part, on a constaté que 0,2% (p/p) de H_2SO_4 est suffisant pour l'hydrolyse complète du perméat de lactosérum avec près de 90% de lactose hydrolysé en glucose et galactose en 70 min seulement. D'autre part, l'hydrolyse complète de lactosérum a été obtenue avec 0,4% de H_2SO_4 (p/p) en 60 min. On a également montré que *S. unisporus* comme mono-culture a été cultivée de manière très efficace à la fois dans le lactosérum hydrolysé ($4,8 \times 10^8$) UFC⁄mL et dans le perméat ($2,0 \times 10^8$) UFC⁄mL par rapport au lactosérum non hydrolysé ($2,1 \times 10^5$) UFC⁄mL et le perméat ($2,0 \times 10^5$) UFC⁄mL. De plus, les levures *S. unisporus* et *K. marxianus* ont été cultivées ensemble dans le lactosérum hydrolysé et le perméat et il a été remarqué que les deux organismes ont été bien cultivés ainsi que dans la culture mixte. Par contre, la croissance de *K. marxianus* était plus élevée par rapport à *S. unisporus*. Par conséquent, l'hydrolyse du lactosérum et du perméat de lactosérum, peut être utilisée pour la croissance de la culture mixte produisant ainsi les PUs. De plus, lorsque que *S. unisporus* est cultivée dans des substrats ou des médias hydrolysés, il peut produire également certains métabolites intermédiaires essentiels tels que l'alcool phényl-éthyle; furane méthanol… etc.

ABSTRACT

Acid hydrolysis of lactose in cheese whey and whey permeate have been carried out for effective growth of *S. unisporus* (mixed culture or mono culture) as it cannot consume lactose. Different concentrations of H_2SO_4 (0.2, 0.3 0.4 w/w%) were used for the hydrolysis. It was observed that 0.2% (w/w) of H_2SO_4 were good for complete hydrolysis of whey permeate as 90% lactose hydrolyzed into glucose and galactose in 70 min. Complete hydrolysis of cheese whey was attained with 0.4% H_2SO_4 (w/w) in 60 min. It was also found that *S. unisporus* as monoculture was grown in very effectively in both hydrolyzed cheese whey (4.0×10^8) CFU⁄mL and permeate (2.0×10^8) CFU⁄mL compared to unhydrolyzed cheese whey (2.1×10^5) CFU⁄mL and permeate (2.0×10^5) CFU⁄mL. *S. unisporus* and *K. marxianus* were grown together in hydrolyzed whey and permeate and it was found that both organisms were growing well as mixed culture, but *K. marxianus* growth was higher compared to *S. unisporus*. Therefore hydrolyzed cheese whey and whey permeate can be used for the growth of mixed culture and produce SCPs. *S. unisporus* also produces some essential intermediate metabolites such as phenyl ethyl alcohol, furan methanol, etc while growing in hydrolyzed media.

KEYWORDS: Acid hydrolysis, Cheese whey, Whey permeate, Amino acids, Volatile intermediates

1.INTRODUCTION

Whey is the liquid remaining after the production of cheese or the removal of fat and casein (80% of the proteins) from milk. Whey still contains about 50% of the nutrients present in milk, comprising milk sugar (lactose), serum proteins (whey proteins), minerals, a small amount of fat, and most of the water soluble minor nutrients from milk such as vitamins. The low concentration of these components makes their recovery process un-economical. Whey is treated as a waste and if discharged without any treatment creating pollution problems (Carvalho et al., 2013). Lactose is the major component of the whey and can be economically utilized by its conversion to value-added products such as single cell proteins (SCPs), enzymes, ethanol, lactic acid etc (Tsakali et al., 2010). Biotransformation of cheese whey into nutritive and economical SCPs is one of the promising technologies (Fitzpatrick and O'Keeffe 2001). SCPs are preferred over animal and plant proteins as a supplement in food and feed because of their easy handling and compatibility to grow in a limited surface area irrespective of the environmental conditions by utilizing low cost carbon sources (Coughlin and Nickerson, 1974).

Whey protein is considered a high quality protein because of presence of all essential amino acids and physiochemical properties. Apart from the high nutritional quality, whey protein has been recognized for several physiological functions which are antimicrobials, growth promotional activity and immunoactivity, and other growth factors respectively (Ismail and Gu, 2009). The disposal of lactose waste is concern as of its high biological oxygen demand and chemical oxygen demands (BOD and COD) which leads to environmental problems. Apart from that lactose is disaccharides barely digestable by a varied human population (Lin and Nickerson, 1976; Szczodrak, 1999). The objective of this research is to minimize the lactose-disposal in wastewater by methods like hydrolysis of lactose. Conversion of lactose into monosaccharides reduces the lactose loss and rather conversion it into beneficial products at the same time (Coughlin and Nickerson, 1974; Abril and Stull, 1989).

The monosaccharides (glucose and galactose) are easily digestable by most of the single cell proteins (SCP) producing microorganisms. So far, lactose can be hydrolyzed

by basic two methods which are enzymatic treatment and other one is acid treatment. Mostly the enzymatic treatment is the most preferred one because of the milder treatments; whereas acid treatment for hydrolysis has a high variation of pH and temperatures (Namvar-Mahboub and Pakizeh, 2012). Apart from the enzymatic approach in the industrial scale, even solid acids were considered for lactose breakdown (Elliott et al., 2001). The basic disadvantage of opting for acid hydrolysis is that during acid hydrolysis of lactose at high acid concentrations and elevated temperatures leads to undesired products apart from the monosaccharaides, and have corrosive nature of the treatment is disabling its implementation in an industrial-scale (Hatzinikolaou et al., 2005; Harju et al., 2012). Acid hydrolysis is an economic approach than the pure/crude enzymatic approach in an industrial level fermentation which is expensive. Apart from that the enzymes for hydrolysis must be from GRAS microbes. Other than that the physiological characteristic of the microorganisms fermenting on the hydrolyzed product must be known in order to produce SCPs (Akpinar et al., 2010). These are certain challenges in whey lactose conversion and this study has been done on *S. unisporus* and *Kluyveromyces marxinaus* individually and in a mixed culture.

2. MATERIALS AND METHODS

2.1 MATERIALS

The chemicals used in the experiments were of analytical grade. Yeast Extract (Fisher Scientific, USA), Malt Extract (Oxoid Ltd., Basingstoke, England), Meat Peptone (Organotechnie SA., La Courneuve, France), Glucose (Fisher Scientific, USA), Cheese whey (Agropur, Canada), Whey permeate (Agropur, Canada), Ammonium sulfate (Sigma Aldrich, Canada), H_2SO_4 (Fisher Scientific, USA), NaOH (PELS, Quebec).

2.2 MICROOGANISMS

S unisporus NRRL Y-1556 was isolated from cheese whey in our laboratory for the production of SCP. Strains were sub-cultured on yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L (YEPD) agar slants and stored at 4 ^{o}C for further use. Similarly *K. marxianus* which was isolated from cheese whey was also sub-cultured.

2.3 ACID HYDROLYSIS OF CHEESE WHEY AND WHEY PERMEATE

Acid hydrolysis of cheese whey and whey permeate were carried out using H_2SO_4. 200 mL of cheese whey (4.5% (w/v)) and whey permeate (5% (w/v)) were taken in different 1 L bottles and different concentrations of H_2SO_4 such as 0.2, 0.3 and 0.4% (w/w) were added in each bottle, pH was adjusted to 2.0 and heat treated at 121 °C for different time periods (15 min, 30 min, 60 min and 120 min). After the treatment all the samples were analyzed for hydrolyzed sugars.

2.4 PRODUCTION OF MICROBIAL CULTURE

Pre-culture of *S. unisporus* and *K. marxianus* was prepared in YEPD medium (100 mL) using 500 mL Erlenmeyer flasks. 2% (v/v) of inoculum from *S. unisporus* and *K. marxianus* pre-cultures were used to inoculate the production media (cheese whey and ammonium sulphate) in two different flasks separately and incubated at 30 °C, pH 5.5 for 24 h. Samples were withdrawn at regular intervals and cell concentration was measured.

2.5 ANALYTICAL METHODS

2.5.1 Carbohydrate Analysis

Carbohydrate analysis was performed using Liquid Chromatography- Mass Spectroscopy (LC-MS/MS) method. Hydrolyzed cheese whey and whey permeate were centrifuged at 10 000 g for 10 min at 4 °C and supernatant was collected. The supernatant was further filtered using micro filter of 0.45 µ (Thermo Scientific, USA). Samples were diluted 10 times and collected in 2 mL vials and placed in the auto-sampler of the LC-MS/MS (Thermo TSQ Quantum) equipped with an Electrospray Ionization (ESI) in negative ion mode. The analytical column used was Zorbax Carbohydrate of the specifications 4.6 mm x 150 mm (Agilent Life Sciences). The injection volume was 10 µL and the internal standard used was Glucose-D2. The mobile phase used for carbohydrate analysis was 75% acetonitrile mixed with 0.1% NH_4OH and 25% Water mixed with 0.1% NH_4OH (McRae and Monread, 2011).

2.5.2 Protein Estimation

The lyophilized powder of *S. unisporus* biomass was ultra-sonicated by the method described in Lopez et al., 2010 with slight modifications. The biomass was

treated with lysis buffer comprising of 5 mL/L (Triton X-100), 0.372 g/L (ethylenediaminetetraacetic acid (EDTA)) and 0.035 g/L (phenylmethylsulfonyl fluoride (PMSF)). Further the biomass was incubated at 25 ºC for 20 min in incubator shaker. After incubation, the treated biomass was sonicated for 5 min in with ultrasonic homogenizer Autotune 750W (Cole-Parmer Instruments, Vernon Hills, Illinois, US). The sonication was performed in order to release the protein. Further the protein was estimated by Folin-Lowry method (Lowry et al., 1951).

2.5.3 Amino acids

The essential amino acid profile of *S. unisporus* and *K. marxianus* biomass was analysed by LC-MS/MS (TSQ Quantum Access, Thermo Scientific) using the slightly modified method of Lourenço et al., (2002). The mixed culture biomass samples were hydrolysed with 6 N HCl at 110 ºC for 24 h. Digested samples were re suspended in acetate buffer and filtered using 0.45 µ (Thermo Scientific., USA) micro-filter. The amino acids were released as a result of hydrolysis and were analysed by LC-MS/MS. The column used was C18 HyperSil-Gold 100 mm x 2.1 mm x 3 µm; the solvents ratio used was 40% water mixed with 0.1% formic acid further mixed with 60% acetonitrile. The flow rate was maintained at 0.5 mL/min.

2.5.4 Volatile compounds

The volatile compounds present in the *S. unisporus* fermented broth were determined using gas chromatography – mass spectrometry (GC-MS). Fermented broth was centrifuged 10 000 g, 4 °C and 15 min and the supernatant was collected. The capillary column used was HP - INNOWax (30 mm, 0.25 mm, using nitrogen as carrier gas) and a split ratio of 1/100. The supernatant samples are diluted with methanol (1:1) and 10 µL of acetic acid (internal standard). Blank was prepared with 2 mL methanol and 10 µL of acetic acid. Diluted samples and blank were taken in GC vials and analyzed using GC-MS (Agilent Chrompack HP-6820). The oven temperature used was maintained at 60 °C for 8 min, and then temperature was gradually raised at a rate of 3 °C per min to 180 °C and maintained for 5 min. The temperature of the injection port was 250 °C. The components of the sample were identified by comparing the spectra with

those of the known compounds stored in NIST library (2010). The chromatographic effluent was used as a sample inlet for the mass spectrometer (Annan et al., 2003).

3. RESULTS AND DISCUSSION

3.1 Acid hydrolysis of cheese whey and permeate

Hydrolysis of cheese whey and whey permeate was studied using different concentrations of H_2SO_4 and results were presented in Figure 1, 2 and 3. Fig 1 shows that lactose (50 g/L) in permeate was almost hydrolyzed into glucose (10 g/L) and galactose (11.3 g/L) with 0.2% w/w H_2SO_4 in 70 min, whereas in cheese whey only partial lactose hydrolysis was observed. However during hydrolysis of whey permeate, brown coloration, precipitation and oligosaccharides occurred. It was measured by loss of free glucose (Fig 1b). These effect the physical and chemical characteristics of the H_2SO_4-hydrolysed whey. Similar trends were observed when whey was treated with HCl by Coughlin and Nickerson, 1974. The discoloration of the whey might be due to the formation of browning pigments followed by precipitation which occurs by denaturation of the whey proteins and simultaneous production of insoluble browning products. I another case, cheese whey (30 g/L) lactose was completely hydrolyzed in 90 min with 0.3% H_2SO_4 and only in 60 min when 0.4% H_2SO_4 was used (Fig 2 and 3). Unlike whey permeate, lesser formation of oligosaccharides occurred in cheese whey hydrolysates. The hydrolysis time for lactose hydrolysis is bit high compared to the study by Elliot et al., 2001. From the results it is also clear that further increase in H_2SO_4 did not show any improvement in lactose hydrolysis of whey permeate (Fig 4). Different hydrolysis pattern of cheese whey and whey permeate could be due to their different composition as cheese whey contains protein which may hinder the hydrolysis process. In the literature it was reported that H_2SO_4 is less effective than HCl for the hydrolysis of lactose (Vujicic et al., 1977). On Contrary to the literature, in the current study H_2SO_4 was found to be very efficient in hydrolyzing lactose of cheese whey and whey permeate. These characteristics indicate that whey after being hydrolysed must undergo further treatment before being suitable for food and feed.

3.2 Growth of *S. unisporus* (mono-culture) in hydrolyzed whey and permeate

Hydrolyzed cheese whey (with 0.4% H_2SO_4) and whey permeate (with 0.2% H_2SO_4) were evaluated for the growth of *S. unisporus* and results are presented in Figure 5. Results indicate that *S. unisporus* utilized glucose more efficiently than galactose. Maximum cell concentrations were obtained at 18 h of fermentation for both cheese whey (4.0×10^8) and permeate (2.0×10^8). *S. unisporus* has shown better growth in cheese whey compared to whey permeate. Comparatively good growth of *S. unisporus* in cheese whey after hydrolysis could be due to the presence of free amino acids in cheese whey which is otherwise absent in whey permeate Other than that sweet whey has more free amino acid concentration than salt whey (Božanic et al., 2014).

3.3 Growth of *S. unisporus* and *K. marxianus* as a mixed-culture

S. unisporus and *K. marxianus* were grown together in hydrolyzed cheese whey and whey permeates and results are presented in (Figure 6). *K. marxianus* can consume both glucose and galactose efficiently. *S. unisporus* can consume only glucose efficiently and galactose partially. Maximum cell concentrations of *S. unisporus* was observed at 18 h in whey (5.6×10^7) and permeate (6.8×10^7); for *K. marxianus* maximum growth was observed at 12 h in cheese whey (7.8×10^8) and permeate (8.1×10^8). High cell concentration of *K. marxianus* than *S. unisporus* is due to the fact that it can consume left over lactose along with hydrolyzed glucose and galactose.

3.4 Volatile compounds

The volatile compounds generated by *S. unisporus* when inoculated in acid hydrolyzed whey and permeate were analyzed by GC-MS and the results are shown in Table 1. Certain weak organic acids such as butanoic acid, propionic acid, citric acid and pentadieonic acid are produced during growth of *S. unisporus* in cheese whey and in mixed culture of *S. unisporus* and *K. marxianus*. Production of certain compounds depends heavily on the substrate present in medium. *S. unisporus* produced aromatic compounds such as furfuryl formate, furanmethanol (volatile compounds). Another useful compound produced is phenyl ethyl alcohol which has antimicrobial effects (Bhattacharya et al., 2011). Another compound detected by GC-MS was pentadienoic

acid. This compound may be produced within the *S. unisporus* cell by the interconversion of 2-n-propyl-4-pentenoic acid into 2-propyl-2, 4-pentadienoic acid. Pentadienoic acid is responsible for the Ca signaling pathway, which is entirely intracellular (Umezawa et al., 2010).

Even though there was no direct production of furfural compound was observed, metabolites of furfural such as furancarboxaldehyde, furfurylformate and furanmethanol were detected. Furfural can be coined as toxic if present in higher quantities. But *S. unisporus* can transform this toxic furfural into its isomeric forms like carboxaldehyde, formate and methanol, which has lesser toxic effects. This indicates that if certain pathogen is producing furfural, *S. unisporus* can convert it into much acceptable and less toxic compound (carboxylate and methanol forms).

3.4 Amino acids

The total protein content of the mixed culture biomass (*S. unisporus* and *K. marxianus*) was found to be 5.2 g/L and the amino acid composition of the mixed culture is mentioned in Table 2. From the amino acids analysis it was observed that there is definite increase in the specific amino acids production specifically leucine and isoleucine (79.7 mg/g) from an initial 59.87 mg/g *S. unisporus* biomass when mixed culture was grown in hydrolyzed cheese whey. Other amino acids where increment in concentration was observed was arginine (77.3 mg/g) from an initial concentration of *S. unisporus* arginine 42.05 mg/g, similarly aspartic acid (91.6 mg/g) from an initial *S. unisporus* aspartic acid 83.33 mg/g, serine (44.5 mg/g), alanine (56.3 mg/g) and glycine (87.5 mg/g) respectively (Unpublished data).

4. CONCLUSION

0.2% (w/w) of H_2SO_4 is sufficient to completely hydrolyze the lactose in whey permeate in 120 min. However complete hydrolysis of cheese whey lactose was obtained with 0.3% (w/w) of H_2SO_4. It can break down lactose to glucose and galactose in whey powder in 90 min. *S. unisporus* and *K. marxianus* can together grow well in hydrolyzed cheese whey and permeate, but *S. unisporus* growth was high when it was used as mono-culture. High cell concentration was observed with mixed culture (*S. unisporus* and *K.*

marxianus) compared to mono-culture (*S. unisporus*). An efficient increase was observed in essential amino acids (leucine and isoleucine) and other partially essential amino acids (arginine and glycine). Beneficial metabolites (phenylethyl alcohol, furanmethanol and organic acids) were produced by *S. unisporus* as a mono- and mixed culture but no quantifications was performed.

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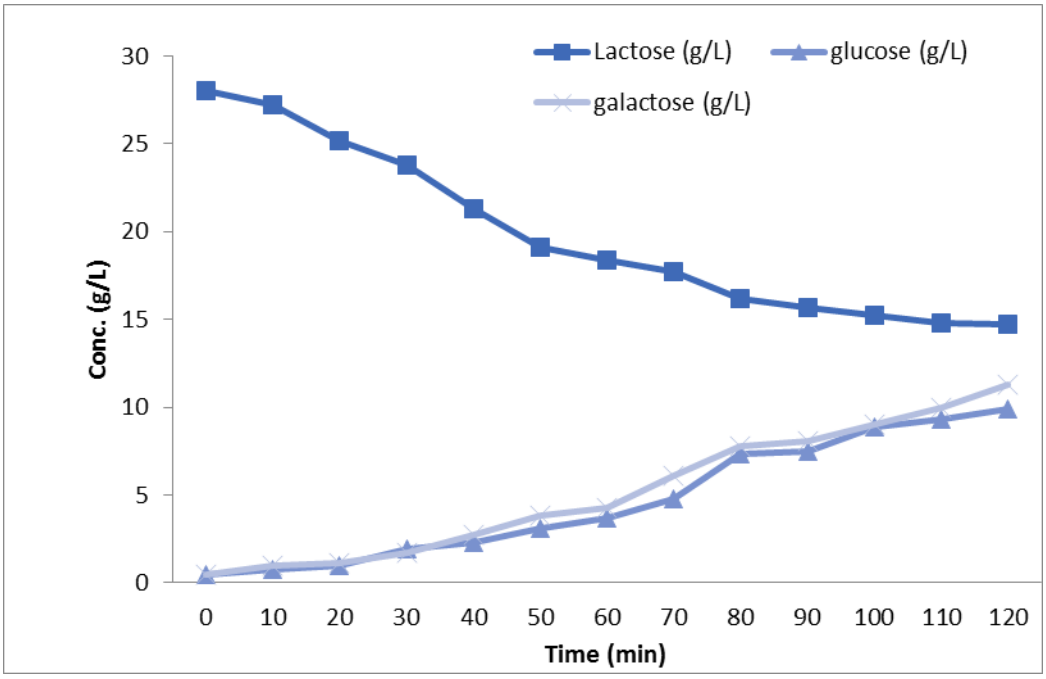
Table 1: Analysis of volatile compounds by GC-MS produced by *S. unisporus* (mono culture) and *S. unisporus* and *K. marxianus* (mixed culture) where values are presented in form of Retention time (RT)

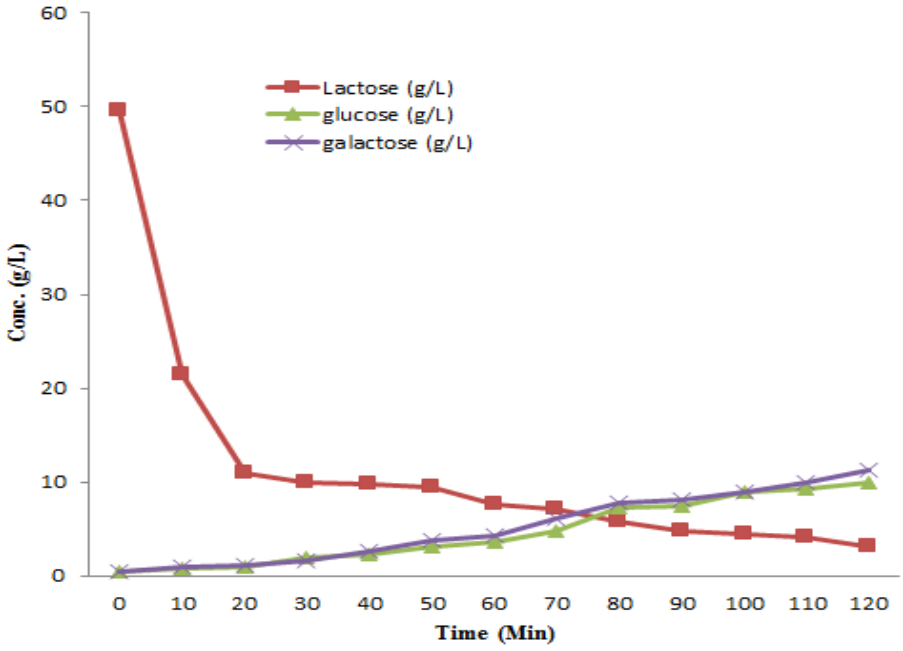
Volatile Compound	Cheese whey	*S. unisporus*	*S. unisporus + K. marxianus*
Alcohols			
Butanol	-	23.70	23.70
3-Pentanol	-	14.95	14.95
2-Pentanol	-	-	11.1
1-Octanol	-	33.39	33.39
Heptanol	-	-	15.63
4-Nonanol	-	-	4.9
2-cyclopentanol	-	12.89	12.89
furfuryl alcohol	-	20.33	-
2-Hexanol	14.88		
2-Methyl-3-butanol	13.42		
Phenylethyl alcohol	-	14.18	14.18
Carbonyls			
2-Pentanone	-	5.07	5.07
Hexanal	-	9.03	9.03
2-Heptanal	-	15.63	15.63
2-Decanal	-	11.3	11.3
Benzeneacetaldehyde	-	17.69	17.69
Acids			
Acetic acid (Internal standard)	14.97	14.97	14.97
Butyric acid	7.149	7.149	7.149
Propanoic acid	12.08	12.08	12.08
Pentadienoic acid	-	18.53	18.53
Butanoic acid	-	28.63	28.63
Citric acid	-	29.09	29.09
3,3-Dimethyl-propanoic acid	-	17.25	17.25
Pentanoic acid	-	18.02	18.02
Succinic acid	7.01	-	-
Malic acid	8.64	-	-
Other Intermediates			
Furfuryl formate	-	15.672	-
2-Furanmethanol	-	18.56	-
Methyl-2-0-benzyl-d-arabinofuranoside	-	22.41	22.41
6-Oxabicyclo/ hexanone furfuryl alcohol	-	20.33	20.33

Table 2: Analysis of amino acids of *S. unisporus* and *K. marxianus* as a mixed culture for SCP production

Amino Acid	***S. unisporus* + *K. marxianus* (mg/g)**
ARG	77.3
LYS	85.1
HIS	46.2
LEU+ILE	79.7
MET	8.1
PHE	34.7
THR	39.1
TRP	8.1
VAL	78.7
Trans-PRO	59.2
CYS	70.6
PRO	59.5
ASP	91.6
GLN	8.1
SER	44.5
GLU	76.4
ALA	56.3
GLY	87.5
TYR	42.2
ASN	26.5

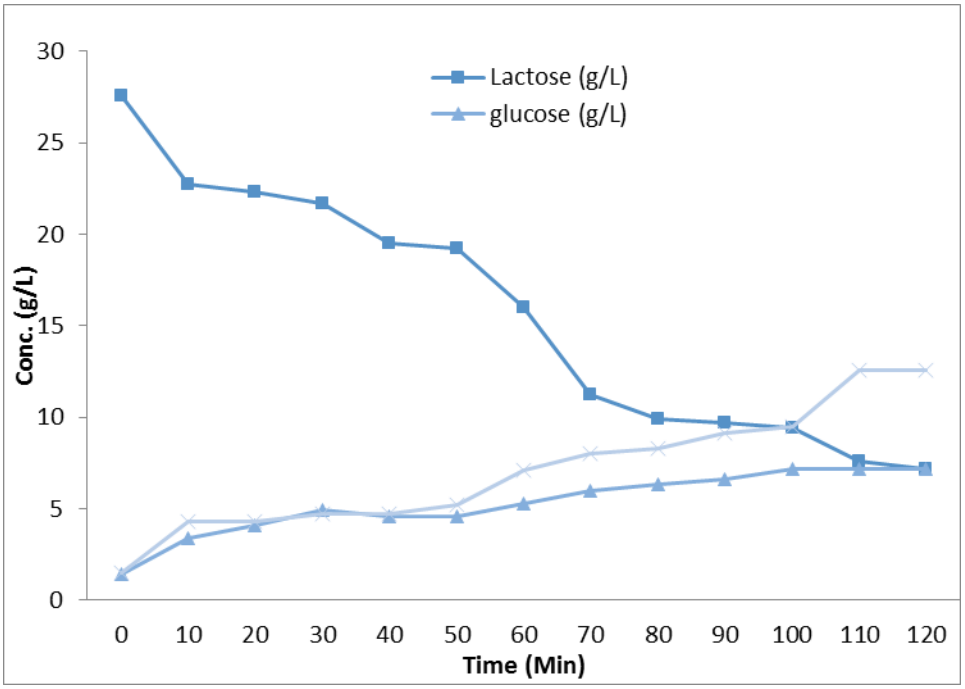


a

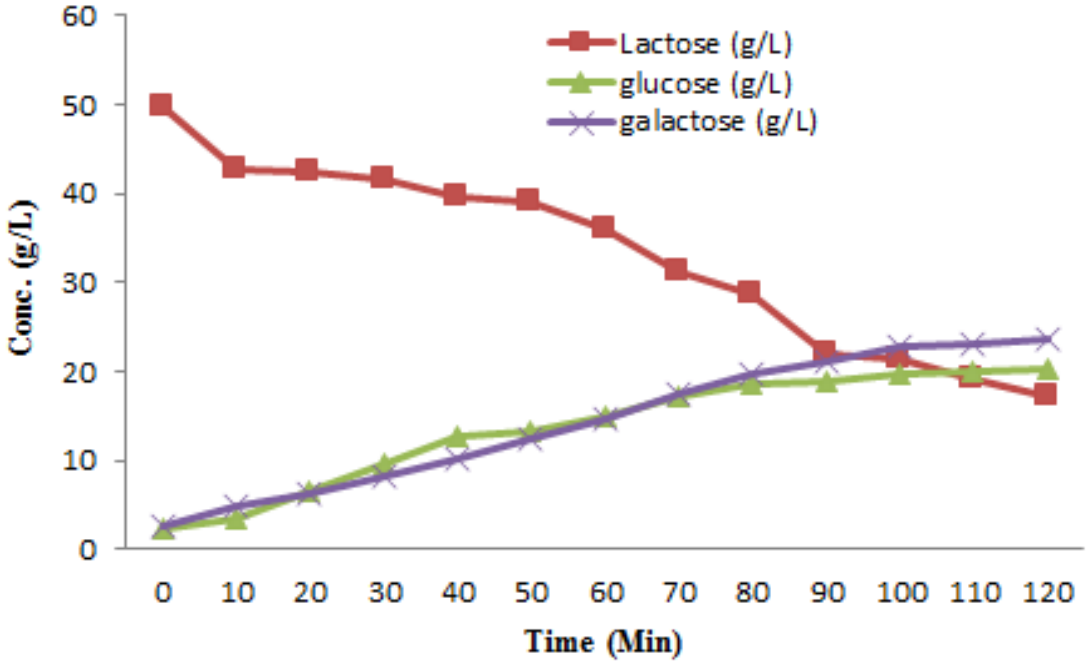


b

Figure 1. Acid hydrolysis at 0.2wt% H_2SO_4 **a)** whey **b)** permeate



a



b

Figure 2. Acid hydrolysis at 0.3wt% H_2SO_4 **a)** whey **b)** permeate

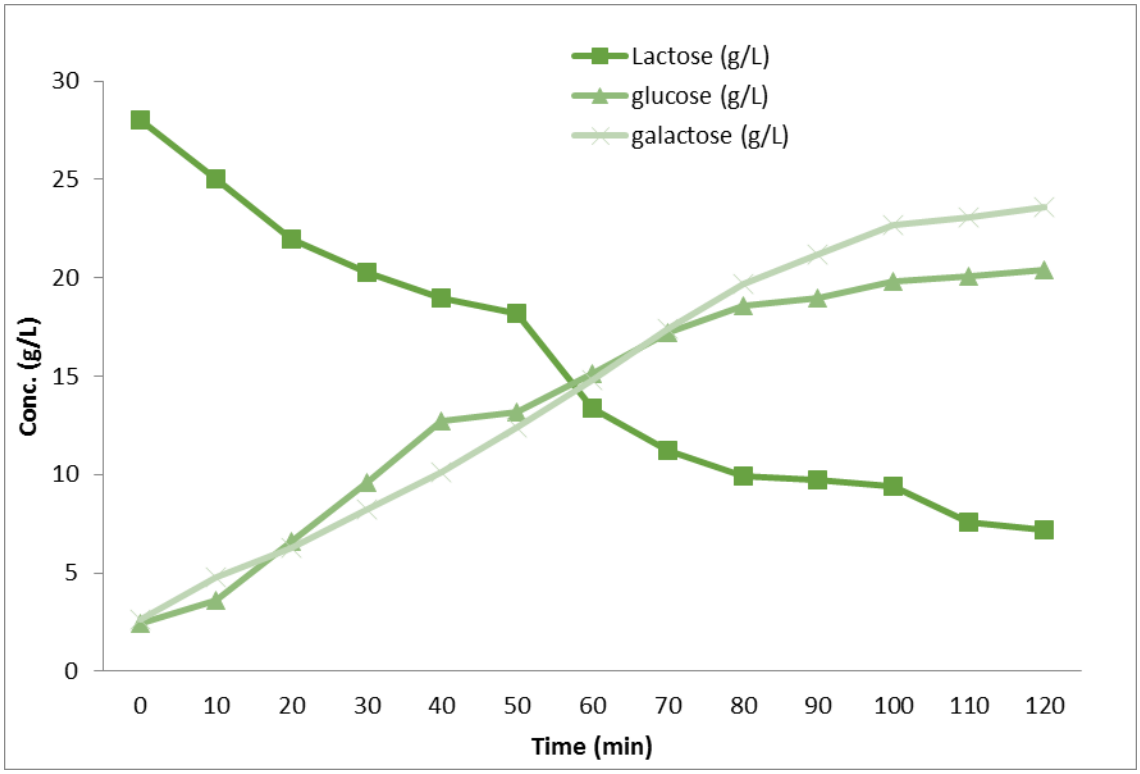


Figure 3. Acid hydrolysis at 0.4wt% H_2SO_4 in whey

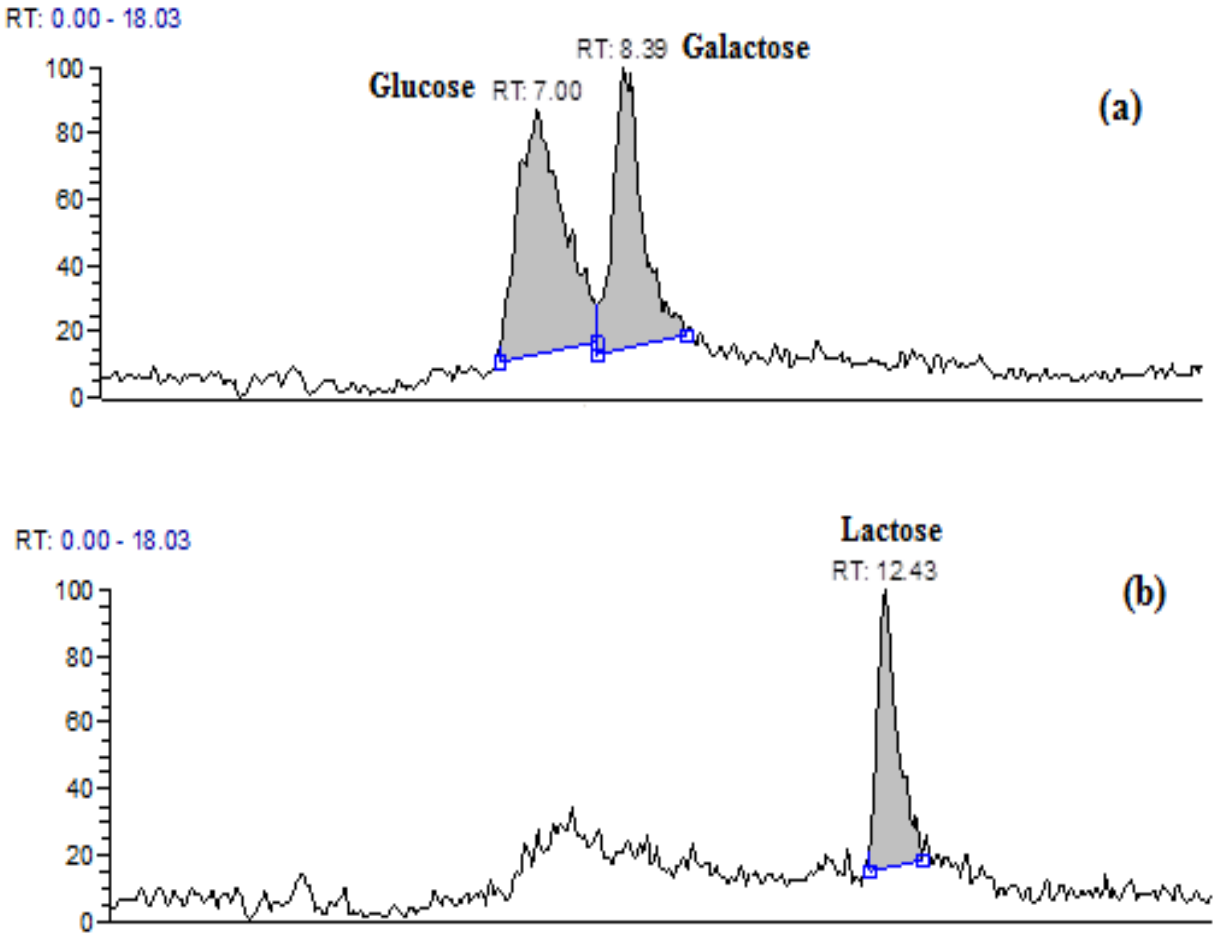


Figure 4. a) LC-MS/MS chromatogram of the hydrolyzed whey lactose by acid hydrolysis, **b)** LC-MS/MS chromatogram of the whey lactose before acid hydrolysis

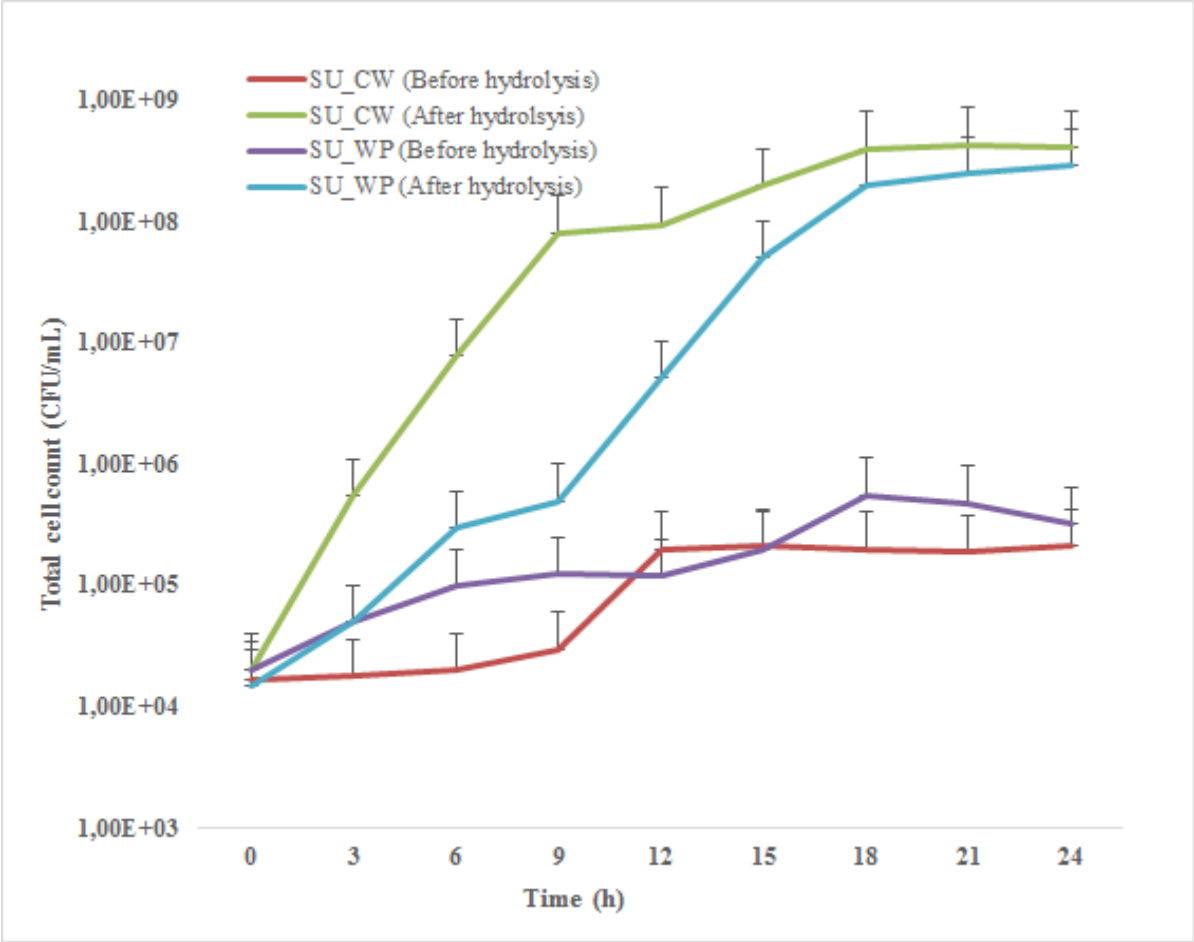


Figure 5. Growth of *S. unisporus* as a mono-culture in cheese whey (CW), hydrolyzed cheese whey, Hydrolyzed permeate and whey permeate (WP)

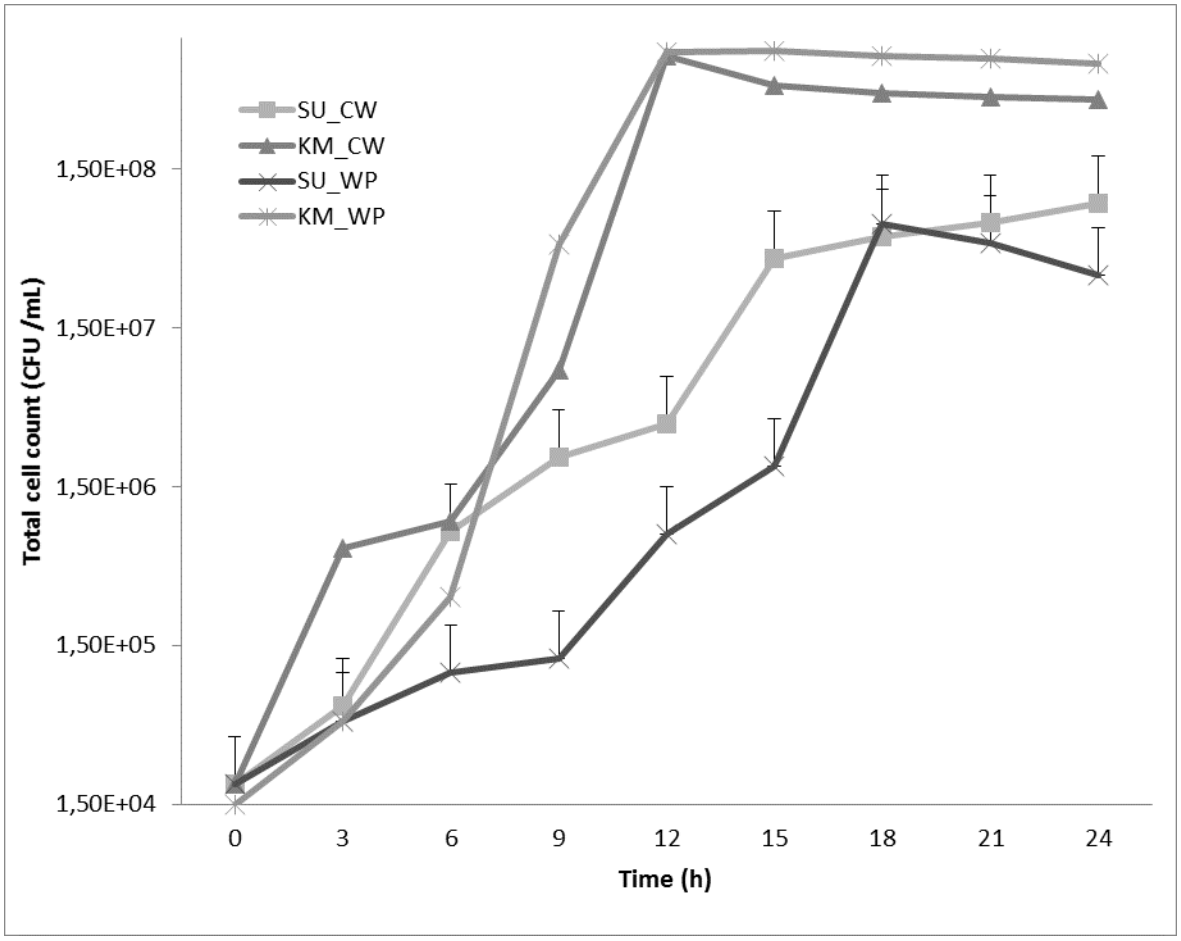


Figure 6. Growth of *K. marxianus* (KM) and *S. unisporus* (SU) as a mixed culture in hydrolyzed cheese whey (CW) and permeate (WP)

CHAPTIER III

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ANNEXES

ANNEXE 1
Raw Data

Optimization and Production of silver-protein conjugate as bio preservative

Figure 1. Profiles of cell and protein concentrations of *W. saturnus* while grown in cheese whey, molasses and urea

Time (h)	CFU/mL	Protein (g/L)
0	7,70E+05	2,14
3	2,10E+06	2,24
6	9,00E+06	3,56
9	1,56E+07	5,54
12	4,00E+07	5,16
15	5,47E+07	7,48
18	6,14E+07	8,91
21	5,94E+07	10,56
24	4,83E+07	9,88

Figure 3. Synthesis of NPs from *W. saturnus* protein using various concentrations of $AgNO_3$

Wavelength	0.1 M	0.2 M	0.5 M	1 M	.01 M	.05 M
300	1,027	1,064	0,973	0,769	0,898	0,797
305	0,924	0,97	0,876	0,629	0,798	0,115
310	0,839	0,108	0,105	0,576	0,156	0,11
315	0,77	0,106	0,105	0,531	0,148	0,106
320	0,717	0,105	0,105774194	0,378	0,14	0,102
325	0,676	0,105	0,103531342	0,367	0,133	0,098
330	0,647	0,105	0,10128849	0,139	0,127	0,095
335	0,624	0,105	0,099045638	0,133	0,122	0,092
340	0,603	0,105	0,105774194	0,128	0,117	0,089
345	0,589	0,105	0,103531342	0,122	0,112	0,086
350	0,578	0,105	0,10128849	0,117	0,108	0,084

355	0,57	0,105	0,09	0,113	0,104	0,081
360	0,566	0,105	0,087	0,108	0,1	0,079
365	0,566	0,105	0,085	0,104	0,097	0,077
370	0,567	0,105	0,083	0,079	0,094	0,075
375	0,57	0,105	0,081	0,077	0,091	0,074
380	0,574	0,105	0,079	0,075	0,088	0,072
385	0,578	0,105	0,077	0,074	0,086	0,07
390	0,581	0,105	0,075	0,139	0,083	0,069
395	0,584	0,105	0,074	0,133	0,081	0,068
400	0,585	0,105	0,072	0,128	0,079	0,066
405	0,586	0,105	0,09	0,122	0,077	0,065
410	0,585	0,129	0,087	0,117	0,076	0,064
415	0,583	0,126	0,085	0,113	0,074	0,063
420	0,579	0,123	0,083	0,108	0,072	0,115
425	0,575	0,121	0,081	0,104	0,071	0,11
430	0,568	0,119	0,079	0,101	0,07	0,106
435	0,56	0,116	0,077	0,097	0,068	0,102
440	0,55	0,114	0,075	0,094	0,108	0,098
445	0,538	0,112	0,074	0,091	0,104	0,095
450	0,527	0,11	0,072	0,088	0,1	0,092
455	0,513	0,108	0,09	0,086	0,097	0,089
460	0,499	0,106	0,087	0,083	0,094	0,086
465	0,483	0,105	0,085	0,081	0,091	0,084
470	0,468	0,105774194	0,083	0,079	0,088	0,081
475	0,45	0,103531342	0,081	0,077	0,086	0,079
480	0,433	0,10128849	0,079	0,075	0,083	0,077
485	0,416	0,099045638	0,077	0,074	0,081	0,075

490	0,398	0,135	0,075	0,072	0,079	0,074
495	0,382	0,132	0,09	0,071	0,077	0,072
500	0,365	0,129	0,087	0,069	0,076	0,07
505	0,348	0,126	0,085	0,068	0,074	0,069
510	0,33	0,123	0,083	0,067	0,072	0,068
515	0,314	0,121	0,081	0,066	0,071	0,066
520	0,299	0,119	0,079	0,065	0,07	0,065
525	0,284	0,116	0,077	0,064	0,068	0,064
530	0,271	0,114	0,075	0,063	0,108	0,063
535	0,257	0,112	0,074	0,062	0,104	0,115
540	0,244	0,11	0,09	0,061	0,1	0,11
545	0,233	0,11	0,087	0,074	0,097	0,106
550	0,222	0,11	0,085	0,139	0,094	0,102
555	0,212	0,11	0,083	0,133	0,091	0,098
560	0,202	0,11	0,081	0,128	0,088	0,095
565	0,193	0,11	0,079	0,122	0,086	0,092
570	0,185	0,11	0,077	0,117	0,083	0,089
575	0,177	0,11	0,075	0,113	0,081	0,086
580	0,169	0,11	0,074	0,108	0,079	0,084
585	0,162	0,11	0,072	0,104	0,077	0,081
590	0,156	0,11	0,131	0,101	0,076	0,079
595	0,15	0,11	0,126	0,097	0,074	0,077
600	0,145	0,164	0,121	0,094	0,072	0,075
605	0,14	0,159	0,117	0,091	0,071	0,074
610	0,136	0,155	0,112	0,088	0,07	0,072
615	0,131	0,15	0,109	0,086	0,068	0,07
620	0,127	0,146	0,105	0,083	0,1	0,069

625	0,123	0,142	0,101	0,081	0,097	0,068
630	0,119	0,138	0,098	0,079	0,094	0,066
635	0,116	0,135	0,095	0,077	0,091	0,065
640	0,113	0,132	0,092	0,075	0,088	0,064
645	0,11	0,129	0,09	0,074	0,086	0,063
650	0,107	0,126	0,087	0,072	0,083	0,077
655	0,104	0,123	0,085	0,071	0,081	0,075
660	0,102	0,121	0,083	0,069	0,079	0,074
665	0,1	0,119	0,081	0,068	0,077	0,072
670	0,097	0,116	0,079	0,067	0,076	0,07
675	0,095	0,114	0,077	0,066	0,074	0,069
680	0,093	0,112	0,075	0,065	0,072	0,068
685	0,091	0,11	0,074	0,064	0,071	0,066
690	0,09	0,108	0,072	0,063	0,07	0,065
695	0,088	0,106	0,071	0,062	0,068	0,064
700	0,087	0,105	0,069	0,061	0,067	0,063

Figure 4. a) Optimization of 0.1 M $AgNO_3$ volume (25 – 200 µL) for synthesis of NPs

Wavelength	50 uL (0.1 M AgNO3)	100 uL (0.1 M AgNO3)	25 uL (0.1 M AgNO3)	200 uL (0.1 M AgNO3)
300	0,425	0,466	0,412	0,702
305	0,378	0,416	0,365	0,622
310	0,341	0,375	0,329	0,558
315	0,313	0,344	0,302	0,511
320	0,294	0,323	0,282	0,477
325	0,279	0,307	0,267	0,454
330	0,27	0,299	0,256	0,441

335	0,262	0,295	0,247	0,433
340	0,255	0,293	0,238	0,429
345	0,251	0,294	0,232	0,429
350	0,248	0,297	0,227	0,431
355	0,246	0,302	0,223	0,437
360	0,246	0,308	0,221	0,446
365	0,247	0,316	0,22	0,457
370	0,249	0,325	0,22	0,47
375	0,252	0,335	0,221	0,483
380	0,255	0,345	0,223	0,497
385	0,259	0,355	0,224	0,511
390	0,262	0,364	0,226	0,524
395	0,265	0,373	0,227	0,536
400	0,267	0,38	0,227	0,546
405	0,268	0,387	0,227	0,555
410	0,269	0,392	0,226	0,561
415	0,269	0,396	0,225	0,566
420	0,267	0,399	0,222	0,568
425	0,264	0,399	0,219	0,568
430	0,261	0,398	0,215	0,565
435	0,256	0,395	0,21	0,56
440	0,25	0,39	0,205	0,551
445	0,244	0,383	0,199	0,54
450	0,237	0,375	0,193	0,528
455	0,229	0,365	0,186	0,512
460	0,221	0,354	0,18	0,495
465	0,213	0,341	0,173	0,477
470	0,204	0,328	0,166	0,458
475	0,195	0,313	0,159	0,436

480	0,186	0,299	0,152	0,415
485	0,177	0,284	0,146	0,395
490	0,169	0,269	0,139	0,372
495	0,161	0,255	0,133	0,352
500	0,153	0,241	0,127	0,331
505	0,145	0,227	0,122	0,311
510	0,137	0,212	0,116	0,29
515	0,13	0,2	0,111	0,271
520	0,123	0,188	0,106	0,254
525	0,117	0,176	0,101	0,237
530	0,112	0,167	0,097	0,222
535	0,106	0,157	0,092	0,208
540	0,101	0,147	0,089	0,194
545	0,097	0,14	0,085	0,182
550	0,092	0,132	0,082	0,171
555	0,088	0,125	0,079	0,161
560	0,085	0,118	0,076	0,151
565	0,081	0,112	0,073	0,143
570	0,078	0,107	0,071	0,135
575	0,075	0,102	0,069	0,127
580	0,073	0,098	0,067	0,121
585	0,07	0,093	0,065	0,115
590	0,068	0,089	0,063	0,109
595	0,066	0,086	0,061	0,104
600	0,065	0,083	0,06	0,1
605	0,063	0,08	0,058	0,096
610	0,061	0,078	0,057	0,092
615	0,059	0,075	0,056	0,088
620	0,058	0,073	0,054	0,085

625	0,057	0,071	0,053	0,082
630	0,056	0,069	0,052	0,08
635	0,054	0,067	0,051	0,077
640	0,053	0,065	0,05	0,075
645	0,052	0,064	0,05	0,073
650	0,052	0,063	0,049	0,071
655	0,051	0,061	0,048	0,069
660	0,05	0,06	0,048	0,067
665	0,049	0,059	0,047	0,066
670	0,048	0,058	0,046	0,064
675	0,048	0,057	0,046	0,063
680	0,047	0,056	0,045	0,061
685	0,047	0,055	0,045	0,06
690	0,046	0,054	0,044	0,059
695	0,046	0,054	0,044	0,058
700	0,045	0,053	0,044	0,057
705	0,045	0,053	0,044	0,057
710	0,045	0,053	0,044	0,056
715	0,045	0,052	0,044	0,056
720	0,045	0,052	0,044	0,055
725	0,045	0,052	0,044	0,055
730	0,046	0,053	0,045	0,056
735	0,047	0,053	0,046	0,056
740	0,047	0,054	0,046	0,056
745	0,047	0,053	0,046	0,056
750	0,047	0,053	0,046	0,055
755	0,046	0,052	0,046	0,055
760	0,046	0,052	0,045	0,054
765	0,045	0,052	0,045	0,054

770	0,045	0,051	0,045	0,053
775	0,045	0,051	0,044	0,052
780	0,044	0,05	0,044	0,052
785	0,044	0,05	0,043	0,051
790	0,044	0,049	0,043	0,05
795	0,043	0,048	0,043	0,05
800	0,043	0,048	0,042	0,049

Figure 4. b) Optimization of *W. saturnus* supernatant (WS_sn) for synthesis of NPs

Wavelength	220 uL WS_sn	180 uL WS_sn	208 uL WS_sn	154 uL WS_sn	130 uL WS_sn	96 uL WS_sn	72 uL WS_sn
300	0,358	0,697	0,425	0,363	0,466	0,501	0,412
305	0,319	0,616	0,378	0,324	0,416	0,448	0,365
310	0,288	0,553	0,341	0,292	0,375	0,405	0,329
315	0,265	0,507	0,313	0,27	0,344	0,373	0,302
320	0,248	0,472	0,294	0,253	0,323	0,35	0,282
325	0,236	0,449	0,279	0,24	0,307	0,334	0,267
330	0,228	0,432	0,27	0,232	0,299	0,324	0,256
335	0,223	0,421	0,262	0,226	0,295	0,319	0,247
340	0,219	0,411	0,255	0,22	0,293	0,316	0,238
345	0,217	0,405	0,251	0,217	0,294	0,316	0,232
350	0,216	0,401	0,248	0,214	0,297	0,319	0,227
355	0,217	0,4	0,246	0,213	0,302	0,323	0,223
360	0,219	0,402	0,246	0,214	0,308	0,329	0,221
365	0,222	0,406	0,247	0,215	0,316	0,336	0,22
370	0,226	0,411	0,249	0,218	0,325	0,345	0,22
375	0,23	0,418	0,252	0,221	0,335	0,354	0,221
380	0,235	0,426	0,255	0,224	0,345	0,363	0,223

385	0,24	0,433	0,259	0,227	0,355	0,372	0,224
390	0,244	0,44	0,262	0,23	0,364	0,381	0,226
395	0,248	0,446	0,265	0,233	0,373	0,389	0,227
400	0,252	0,451	0,267	0,235	0,38	0,396	0,227
405	0,255	0,454	0,268	0,236	0,387	0,403	0,227
410	0,257	0,456	0,269	0,237	0,392	0,407	0,226
415	0,259	0,456	0,269	0,237	0,396	0,411	0,225
420	0,259	0,454	0,267	0,235	0,399	0,413	0,222
425	0,259	0,451	0,264	0,233	0,399	0,414	0,219
430	0,257	0,446	0,261	0,23	0,398	0,413	0,215
435	0,254	0,438	0,256	0,226	0,395	0,41	0,21
440	0,251	0,429	0,25	0,221	0,39	0,405	0,205
445	0,246	0,418	0,244	0,216	0,383	0,398	0,199
450	0,241	0,407	0,237	0,21	0,375	0,391	0,193
455	0,234	0,394	0,229	0,203	0,365	0,381	0,186
460	0,227	0,38	0,221	0,196	0,354	0,37	0,18
465	0,22	0,365	0,213	0,189	0,341	0,358	0,173
470	0,212	0,35	0,204	0,182	0,328	0,345	0,166
475	0,203	0,333	0,195	0,174	0,313	0,33	0,159
480	0,195	0,317	0,186	0,166	0,299	0,315	0,152
485	0,187	0,302	0,177	0,159	0,284	0,301	0,146
490	0,178	0,285	0,169	0,151	0,269	0,285	0,139
495	0,17	0,27	0,161	0,145	0,255	0,271	0,133
500	0,162	0,255	0,153	0,138	0,241	0,257	0,127
505	0,154	0,24	0,145	0,131	0,227	0,242	0,122
510	0,145	0,226	0,137	0,124	0,212	0,227	0,116
515	0,138	0,213	0,13	0,118	0,2	0,214	0,111
520	0,131	0,201	0,123	0,113	0,188	0,202	0,106
525	0,124	0,188	0,117	0,107	0,176	0,189	0,101

530	0,118	0,178	0,112	0,102	0,167	0,179	0,097
535	0,113	0,168	0,106	0,098	0,157	0,169	0,092
540	0,107	0,158	0,101	0,093	0,147	0,159	0,089
545	0,103	0,15	0,097	0,089	0,14	0,15	0,085
550	0,098	0,142	0,092	0,086	0,132	0,143	0,082
555	0,094	0,135	0,088	0,082	0,125	0,135	0,079
560	0,09	0,128	0,085	0,079	0,118	0,128	0,076
565	0,087	0,122	0,081	0,076	0,112	0,122	0,073
570	0,083	0,116	0,078	0,073	0,107	0,116	0,071
575	0,08	0,111	0,075	0,071	0,102	0,111	0,069
580	0,078	0,106	0,073	0,069	0,098	0,107	0,067
585	0,075	0,102	0,07	0,067	0,093	0,102	0,065
590	0,073	0,097	0,068	0,064	0,089	0,098	0,063
595	0,071	0,094	0,066	0,063	0,086	0,094	0,061
600	0,07	0,09	0,065	0,061	0,083	0,092	0,06
605	0,068	0,087	0,063	0,06	0,08	0,088	0,058
610	0,066	0,084	0,061	0,059	0,078	0,086	0,057
615	0,065	0,082	0,059	0,057	0,075	0,083	0,056
620	0,063	0,079	0,058	0,056	0,073	0,081	0,054
625	0,062	0,077	0,057	0,055	0,071	0,078	0,053
630	0,061	0,075	0,056	0,054	0,069	0,076	0,052
635	0,06	0,073	0,054	0,053	0,067	0,074	0,051
640	0,058	0,071	0,053	0,052	0,065	0,073	0,05
645	0,058	0,07	0,052	0,051	0,064	0,071	0,05
650	0,057	0,068	0,052	0,05	0,063	0,07	0,049
655	0,056	0,067	0,051	0,049	0,061	0,068	0,048
660	0,055	0,065	0,05	0,049	0,06	0,067	0,048
665	0,055	0,064	0,049	0,048	0,059	0,066	0,047
670	0,054	0,063	0,048	0,048	0,058	0,065	0,046

675	0,053	0,062	0,048	0,047	0,057	0,064	0,046
680	0,053	0,061	0,047	0,047	0,056	0,063	0,045
685	0,052	0,06	0,047	0,046	0,055	0,062	0,045
690	0,052	0,059	0,046	0,046	0,054	0,061	0,044
695	0,051	0,058	0,046	0,045	0,054	0,06	0,044
700	0,051	0,058	0,045	0,045	0,053	0,059	0,044
705	0,051	0,057	0,045	0,045	0,053	0,059	0,044
710	0,052	0,056	0,045	0,045	0,053	0,058	0,044
715	0,052	0,055	0,045	0,045	0,052	0,058	0,044
720	0,051	0,056	0,045	0,045	0,052	0,058	0,044
725	0,051	0,056	0,045	0,045	0,052	0,058	0,044
730	0,052	0,057	0,046	0,046	0,053	0,058	0,045
735	0,053	0,057	0,047	0,047	0,053	0,059	0,046
740	0,053	0,057	0,047	0,047	0,054	0,059	0,046
745	0,053	0,057	0,047	0,047	0,053	0,059	0,046
750	0,053	0,056	0,047	0,047	0,053	0,058	0,046
755	0,052	0,056	0,046	0,046	0,052	0,058	0,046
760	0,052	0,055	0,046	0,046	0,052	0,057	0,045
765	0,052	0,055	0,045	0,046	0,052	0,057	0,045
770	0,052	0,054	0,045	0,046	0,051	0,056	0,045
775	0,051	0,054	0,045	0,045	0,051	0,056	0,044
780	0,051	0,053	0,044	0,045	0,05	0,055	0,044
785	0,051	0,053	0,044	0,044	0,05	0,054	0,043
790	0,05	0,052	0,044	0,044	0,049	0,054	0,043
795	0,05	0,051	0,043	0,044	0,048	0,053	0,043
800	0,05	0,051	0,043	0,043	0,048	0,053	0,042

Figure 5. Utilization profile of protein during synthesis of NPs

Time (h)	*W. saturnus* Protein g/L
0	4,8775
2	0,8075
4	0,7225
6	0,6275
12	0,53
18	0,465

Figure 6. a) Profile of NPs synthesis using 180 µL of *W. saturnus* supernatant at different reaction time

Wavelength	0 h	2 h	4 h	6 h	8 h	10 h	12 h	14 h	16 h	18 h	20 h	22 h	24 h
300	1,064	0,629	0,798	0,115	0,501	0,412	0,401	0,702	1,027	0,973	0,629	0,798	0,115
305	0,97	0,576	0,156	0,11	0,448	0,365	0,359	0,622	0,924	0,876	0,576	0,156	0,11
310	0,548	0,531	0,148	0,106	0,405	0,329	0,328	0,558	0,839	0,105	0,531	0,148	0,106
315	0,336	0,378	0,14	0,102	0,373	0,302	0,303	0,511	0,77	0,105	0,378	0,14	0,102
320	0,205	0,367	0,133	0,098	0,35	0,282	0,284	0,477	0,717	0,105774194	0,367	0,133	0,098
325	0,105	0,139	0,127	0,095	0,334	0,267	0,269	0,454	0,676	0,103531342	0,139	0,127	0,095
330	0,105	0,133	0,122	0,092	0,324	0,256	0,258	0,441	0,647	0,10128849	0,133	0,122	0,092
335	0,105	0,128	0,117	0,089	0,319	0,247	0,248	0,433	0,624	0,099045638	0,128	0,117	0,089
340	0,105	0,122	0,112	0,086	0,316	0,238	0,239	0,429	0,603	0,105774194	0,122	0,112	0,086
345	0,105	0,117	0,108	0,084	0,316	0,232	0,231	0,429	0,589	0,103531342	0,117	0,108	0,084
350	0,105	0,113	0,104	0,081	0,319	0,227	0,224	0,431	0,578	0,10128849	0,113	0,104	0,081
355	0,105	0,108	0,1	0,079	0,323	0,223	0,219	0,437	0,57	0,09	0,108	0,1	0,079
360	0,105	0,104	0,097	0,077	0,329	0,221	0,214	0,446	0,566	0,087	0,104	0,097	0,077
365	0,105	0,079	0,094	0,075	0,336	0,22	0,211	0,457	0,566	0,085	0,079	0,094	0,075
370	0,105	0,077	0,091	0,074	0,345	0,22	0,21	0,47	0,567	0,083	0,077	0,091	0,074
375	0,105	0,075	0,088	0,072	0,354	0,221	0,209	0,483	0,57	0,081	0,075	0,088	0,072
380	0,105	0,074	0,086	0,07	0,363	0,223	0,208	0,497	0,574	0,079	0,074	0,086	0,07
385	0,105	0,139	0,083	0,069	0,372	0,224	0,208	0,511	0,578	0,077	0,139	0,083	0,069
390	0,105	0,133	0,081	0,068	0,381	0,226	0,208	0,524	0,581	0,075	0,133	0,081	0,068

395	0,105	0,128	0,079	0,066	0,389	0,227	0,208	0,536	0,584	0,074	0,128	0,079	0,066
400	0,105	0,122	0,077	0,065	0,396	0,227	0,207	0,546	0,585	0,072	0,122	0,077	0,065
405	0,105	0,117	0,076	0,064	0,403	0,227	0,207	0,555	0,586	0,09	0,117	0,076	0,064
410	0,129	0,113	0,074	0,063	0,407	0,226	0,206	0,561	0,585	0,087	0,113	0,074	0,063
415	0,126	0,108	0,072	0,115	0,411	0,225	0,205	0,566	0,583	0,085	0,108	0,072	0,115
420	0,123	0,104	0,071	0,11	0,413	0,222	0,203	0,568	0,579	0,083	0,104	0,071	0,11
425	0,121	0,101	0,07	0,106	0,414	0,219	0,201	0,568	0,575	0,081	0,101	0,07	0,106
430	0,119	0,097	0,068	0,102	0,413	0,215	0,198	0,565	0,568	0,079	0,097	0,068	0,102
435	0,116	0,094	0,108	0,098	0,41	0,21	0,195	0,56	0,56	0,077	0,094	0,108	0,098
440	0,114	0,091	0,104	0,095	0,405	0,205	0,191	0,551	0,55	0,075	0,091	0,104	0,095
445	0,112	0,088	0,1	0,092	0,398	0,199	0,187	0,54	0,538	0,074	0,088	0,1	0,092
450	0,11	0,086	0,097	0,089	0,391	0,193	0,183	0,528	0,527	0,072	0,086	0,097	0,089
455	0,108	0,083	0,094	0,086	0,381	0,186	0,178	0,512	0,513	0,09	0,083	0,094	0,086
460	0,106	0,081	0,091	0,084	0,37	0,18	0,173	0,495	0,499	0,087	0,081	0,091	0,084
465	0,105	0,079	0,088	0,081	0,358	0,173	0,168	0,477	0,483	0,085	0,079	0,088	0,081
470	0,105774194	0,077	0,086	0,079	0,345	0,166	0,163	0,458	0,468	0,083	0,077	0,086	0,079
475	0,103531342	0,075	0,083	0,077	0,33	0,159	0,158	0,436	0,45	0,081	0,075	0,083	0,077
480	0,10128849	0,074	0,081	0,075	0,315	0,152	0,152	0,415	0,433	0,079	0,074	0,081	0,075
485	0,099045638	0,072	0,079	0,074	0,301	0,146	0,147	0,395	0,416	0,077	0,072	0,079	0,074
490	0,135	0,071	0,077	0,072	0,285	0,139	0,142	0,372	0,398	0,075	0,071	0,077	0,072

495	0,132	0,069	0,076	0,07	0,271	0,133	0,138	0,352	0,382	0,09	0,069	0,076	0,07
500	0,129	0,068	0,074	0,069	0,257	0,127	0,133	0,331	0,365	0,087	0,068	0,074	0,069
505	0,126	0,067	0,072	0,068	0,242	0,122	0,129	0,311	0,348	0,085	0,067	0,072	0,068
510	0,123	0,066	0,071	0,066	0,227	0,116	0,124	0,29	0,33	0,083	0,066	0,071	0,066
515	0,121	0,065	0,07	0,065	0,214	0,111	0,12	0,271	0,314	0,081	0,065	0,07	0,065
520	0,119	0,064	0,068	0,064	0,202	0,106	0,115	0,254	0,299	0,079	0,064	0,068	0,064
525	0,116	0,063	0,108	0,063	0,189	0,101	0,111	0,237	0,284	0,077	0,063	0,108	0,063
530	0,114	0,062	0,104	0,115	0,179	0,097	0,108	0,222	0,271	0,075	0,062	0,104	0,115
535	0,112	0,061	0,1	0,11	0,169	0,092	0,104	0,208	0,257	0,074	0,061	0,1	0,11
540	0,11	0,074	0,097	0,106	0,159	0,089	0,101	0,194	0,244	0,09	0,074	0,097	0,106
545	0,11	0,139	0,094	0,102	0,15	0,085	0,098	0,182	0,233	0,087	0,139	0,094	0,102
550	0,11	0,133	0,091	0,098	0,143	0,082	0,095	0,171	0,222	0,085	0,133	0,091	0,098
555	0,11	0,128	0,088	0,095	0,135	0,079	0,092	0,161	0,212	0,083	0,128	0,088	0,095
560	0,11	0,122	0,086	0,092	0,128	0,076	0,089	0,151	0,202	0,081	0,122	0,086	0,092
565	0,11	0,117	0,083	0,089	0,122	0,073	0,086	0,143	0,193	0,079	0,117	0,083	0,089
570	0,11	0,113	0,081	0,086	0,116	0,071	0,084	0,135	0,185	0,077	0,113	0,081	0,086
575	0,11	0,108	0,079	0,084	0,111	0,069	0,082	0,127	0,177	0,075	0,108	0,079	0,084
580	0,11	0,104	0,077	0,081	0,107	0,067	0,08	0,121	0,169	0,074	0,104	0,077	0,081
585	0,11	0,101	0,076	0,079	0,102	0,065	0,077	0,115	0,162	0,072	0,101	0,076	0,079
590	0,11	0,097	0,074	0,077	0,098	0,063	0,075	0,109	0,156	0,131	0,097	0,074	0,077

595	0,11	0,094	0,072	0,075	0,094	0,061	0,074	0,104	0,15	0,126	0,094	0,072	0,075
600	0,164	0,091	0,071	0,074	0,092	0,06	0,072	0,1	0,145	0,121	0,091	0,071	0,074
605	0,159	0,088	0,07	0,072	0,088	0,058	0,071	0,096	0,14	0,117	0,088	0,07	0,072
610	0,155	0,086	0,068	0,07	0,086	0,057	0,07	0,092	0,136	0,112	0,086	0,068	0,07
615	0,15	0,083	0,1	0,069	0,083	0,056	0,068	0,088	0,131	0,109	0,083	0,1	0,069
620	0,146	0,081	0,097	0,068	0,081	0,054	0,067	0,085	0,127	0,105	0,081	0,097	0,068
625	0,142	0,079	0,094	0,066	0,078	0,053	0,066	0,082	0,123	0,101	0,079	0,094	0,066
630	0,138	0,077	0,091	0,065	0,076	0,052	0,065	0,08	0,119	0,098	0,077	0,091	0,065
635	0,135	0,075	0,088	0,064	0,074	0,051	0,063	0,077	0,116	0,095	0,075	0,088	0,064
640	0,132	0,074	0,086	0,063	0,073	0,05	0,062	0,075	0,113	0,092	0,074	0,086	0,063
645	0,129	0,072	0,083	0,077	0,071	0,05	0,062	0,073	0,11	0,09	0,072	0,083	0,077
650	0,126	0,071	0,081	0,075	0,07	0,049	0,061	0,071	0,107	0,087	0,071	0,081	0,075
655	0,123	0,069	0,079	0,074	0,068	0,048	0,06	0,069	0,104	0,085	0,069	0,079	0,074
660	0,121	0,068	0,077	0,072	0,067	0,048	0,059	0,067	0,102	0,083	0,068	0,077	0,072
665	0,119	0,067	0,076	0,07	0,066	0,047	0,059	0,066	0,1	0,081	0,067	0,076	0,07
670	0,116	0,066	0,074	0,069	0,065	0,046	0,058	0,064	0,097	0,079	0,066	0,074	0,069
675	0,114	0,065	0,072	0,068	0,064	0,046	0,057	0,063	0,095	0,077	0,065	0,072	0,068
680	0,112	0,064	0,071	0,066	0,063	0,045	0,057	0,061	0,093	0,075	0,064	0,071	0,066
685	0,11	0,063	0,07	0,065	0,062	0,045	0,056	0,06	0,091	0,074	0,063	0,07	0,065
690	0,108	0,062	0,068	0,064	0,061	0,044	0,056	0,059	0,09	0,072	0,062	0,068	0,064

695	0,106	0,062	0,068	0,064	0,06	0,044	0,056	0,058	0,088	0,071	0,062	0,068	0,064
700	0,105	0,061	0,067	0,063	0,059	0,044	0,055	0,057	0,087	0,069	0,061	0,067	0,063

Figure 6. b) Profile of NPs synthesis using different volumes of *W. saturnus* supernatant

Wavelength (nm)	180 uL WS supernatant	154 uL WS supernatant	168 uL WS supernatant
280	3,924	3,872	3,953
285	3,738	3,691	3,735
290	3,695	3,679	3,734
295	3,567	3,61	3,759
300	3,302	3,422	3,831
305	2,977	3,158	3,691
310	2,708	2,918	3,516
315	2,502	2,725	3,349
320	2,35	2,579	3,224
325	2,241	2,474	3,123
330	2,167	2,402	3,055
335	2,11	2,341	2,996
340	2,074	2,302	2,949
345	2,047	2,272	2,931
350	2,035	2,252	2,914
355	2,027	2,242	2,906
360	2,031	2,243	2,899
365	2,046	2,236	2,89
370	2,058	2,244	2,9
375	2,068	2,254	2,902
380	2,094	2,257	2,906
385	2,1	2,262	2,908
390	2,103	2,267	2,906
395	2,114	2,256	2,902
400	2,112	2,257	2,903
405	2,117	2,257	2,895
410	2,104	2,252	2,885
415	2,091	2,239	2,867
420	2,065	2,213	2,845
425	2,047	2,19	2,822
430	2,004	2,156	2,788
435	1,97	2,116	2,763
440	1,94	2,085	2,731
445	1,906	2,058	2,699
450	1,883	2,028	2,665
455	1,833	1,981	2,63

460	1,792	1,93	2,596
465	1,75	1,894	2,559
470	1,709	1,853	2,52
475	1,662	1,806	2,48
480	1,616	1,77	2,441
485	1,601	1,743	2,411
490	1,552	1,701	2,366
495	1,514	1,657	2,328
500	1,487	1,632	2,286
505	1,438	1,582	2,251
510	1,401	1,539	2,212
515	1,357	1,509	2,172
520	1,333	1,482	2,131
525	1,284	1,436	2,084
530	1,278	1,42	2,052
535	1,233	1,373	2,017
540	1,188	1,319	1,981
545	1,182	1,315	1,956
550	1,143	1,289	1,923
555	1,114	1,261	1,884
560	1,081	1,232	1,846
565	1,062	1,198	1,811
570	1,03	1,172	1,778
575	1,018	1,139	1,757
580	0,993	1,123	1,733
585	0,962	1,103	1,709
590	0,95	1,088	1,686
595	0,933	1,076	1,655
600	0,917	1,065	1,63
605	0,911	1,044	1,605
610	0,895	1,033	1,581
615	0,879	1,01	1,564
620	0,865	0,99	1,556
625	0,846	0,976	1,538
630	0,841	0,968	1,523
635	0,831	0,959	1,51
640	0,83	0,95	1,497
645	0,819	0,944	1,484
650	0,806	0,933	1,473
655	0,801	0,93	1,462
660	0,805	0,918	1,454

665	0,793	0,906	1,443
670	0,788	0,905	1,436
675	0,782	0,893	1,428
680	0,779	0,886	1,421
685	0,77	0,887	1,416
690	0,761	0,875	1,408
695	0,763	0,869	1,401
700	0,764	0,861	1,394

ANNEXE 2
Raw Data

Evaluation of inhibitory measures for food spoiler yeast *Candida krusei* during fermentation process by chemical, biochemical and nanoparticle approaches

Figure 1. a) Impact of 1.5 M NaCl b) 2.0 M NaCl on *C. krusei* and *K. marxianus* in YEPD medium at pH 3.5 and 40 ºC (shake flask experiments)

Time (h)	Km	Km + 1.5 M NaCl	Ck	Ck + 1.5 M NaCl
0	2,00E+04	3,00E+03	5,00E+03	1,00E+03
2	4,00E+04	5,00E+03	7,00E+03	4,00E+03
4	5,00E+04	9,00E+04	4,00E+04	7,00E+04
6	2,00E+05	7,00E+05	5,00E+04	6,00E+04
8	4,00E+06	2,00E+06	2,00E+05	5,00E+05
10	5,00E+07	3,00E+07	1,00E+06	6,00E+06
12	1,00E+08	4,00E+08	6,00E+07	5,00E+07
14	3,00E+08	6,00E+08	8,00E+07	7,00E+07
16	8,00E+07	1,00E+08	3,00E+08	7,00E+08
18	5,00E+07	3,00E+07	5,00E+08	8,00E+08
20	3,00E+06	7,00E+06	4,00E+07	2,00E+07
22	5,00E+06	5,00E+06	3,00E+06	7,00E+06
24	4,00E+06	9,00E+06	7,00E+05	5,00E+06

Time (h)	Km	Km + 2.0 M NaCl	Ck	Ck + 2.0 M NaCl
0	2,00E+04	3,00E+03	1,00E+03	0,00E+00
2	5,00E+04	3,70E+03	2,00E+03	0,00E+00
4	3,00E+05	4,00E+03	5,00E+03	0,00E+00
6	5,00E+05	5,00E+04	8,00E+04	0,00E+00
8	8,00E+06	6,00E+06	6,00E+05	0,00E+00
10	5,00E+07	4,00E+07	5,00E+06	0,00E+00
12	3,00E+08	5,00E+08	8,00E+06	0,00E+00
14	4,00E+08	7,00E+08	4,00E+07	0,00E+00
16	8,00E+07	3,00E+07	4,00E+07	0,00E+00
18	5,00E+07	6,00E+06	7,00E+07	0,00E+00
20	9,00E+06	4,00E+06	6,00E+07	0,00E+00
22	6,00E+06	5,00E+05	8,00E+06	0,00E+00
24	5,00E+06	4,00E+05	3,00E+06	0,00E+00

Figure 2. Impact of H_2O_2 on *C. krusei, K. marxianus* in cheese whey at pH 6.0, 28 ºC with 300 and 400 ppm H_2O_2

Time (h)	Ck with 300 ppm H_2O_2	Km with 300 ppm H_2O_2	Ck with 400 ppm H_2O_2	Km with 400 ppm H_2O_2
0	3,14E+05	2,80E+05	2,40E+05	3,17E+05
6	2,60E+06	4,30E+05	2,45E+06	2,10E+06
12	2,20E+07	3,10E+06	2,70E+07	1,14E+07
18	2,44E+07	2,31E+07	3,20E+07	1,00E+07
24	2,00E+07	4,52E+07	5,00E+07	9,70E+06

Figure 3. Impact of H_2O_2 and W. saturnus on the mixed culture (*C. krusei* and *K. marxianus)* in cheese whey at pH (3.5, 4.5) and 28 ºC (shake flask experiments) a) with 300 ppm H_2O_2 only b) with 300 ppm H_2O_2 and 1% (v/v) *W. saturnus*

Time (h)	Ck (3.5)	Km (3.5)	Ck (4.5)	Km (3.5)
0	3,70E+05	5,70E+05	1,70E+05	3,80E+04
6	2,70E+05	3,40E+05	1,70E+05	8,70E+04
12	2,70E+05	3,70E+05	6,70E+02	2,10E+05
18	5,34E+04	2,00E+05	5,90E+02	1,60E+06
24	5,00E+04	1,00E+05	5,00E+02	8,40E+05

Time (h)	Ck (3.5)	Km (3.5)	Ws (3.5)	Ck (4.5)	Km (4.5)	Ws (4.5)
0	5,00E+04	3,40E+04	2,70E+04	2,00E+05	9,70E+05	5,70E+05
6	5,40E+04	2,70E+05	1,70E+05	1,30E+04	1,83E+05	1,13E+05
12	6,00E+04	3,00E+04	2,34E+04	3,40E+03	2,20E+05	1,30E+05
18	2,70E+05	5,34E+04	6,67E+04	3,00E+03	2,80E+04	9,00E+04
24	4,34E+05	6,34E+05	5,70E+05	2,30E+03	4,13E+04	1,70E+04

Figure 4. Impact of 2400 ppm H_2O_2 with 200- 400 mg/mL (156-200 µg/mL killer protein) of lyophilized *W. saturnus* (Ws) powder on the mixed culture (*C. krusei* (ck) and *K. marxianus* (Km)) in the fermenter broth at pH 5.0, 40 ºC (shake flask experiments)

Time (h)	Ck + 200mg/ml (Ws_su)	Km + 200mg/ml(Ws_su)	Ck + 300mg/ml(W s_su)	Km + 300mg/ml(Ws_s u)	Ck + 400mg/ml(Ws_s u)	Km + 400mg/ml(Ws_s u)
0	1,80E+06	3,00E+06	1,50E+06	5,20E+06	1,68E+06	3,00E+06
3	1,27E+06	3,90E+06	1,19E+06	5,80E+06	1,27E+06	3,35E+06
6	2,30E+05	5,00E+06	2,98E+05	6,23E+06	1,80E+05	5,12E+06
12	1,10E+05	6,10E+06	1,00E+05	6,36E+06	2,40E+04	6,00E+06
18	1,60E+04	2,00E+07	1,27E+04	3,20E+07	1,00E+04	2,87E+07
24	3,76E+03	1,87E+07	2,00E+03	3,00E+07	2,00E+02	1,50E+07

Figure 5. a) UV-Vis spectrometry showed peak at 410 nm at 12 h and maximum at 48 during bulk preparation of Ag-KT4561

Wavelength	Ag-KT4561 12 h	Ag-KT4561 48 h
300	0,769	1,064
305	0,694	0,97
310	0,629	0,889
315	0,576	0,823
320	0,531	0,766
325	0,495	0,721
330	0,468	0,686
335	0,445	0,657
340	0,424	0,631
345	0,406	0,611
350	0,391	0,595
355	0,378	0,582
360	0,367	0,572
365	0,36	0,567
370	0,354	0,563
375	0,35	0,562
380	0,347	0,562
385	0,344	0,562
390	0,342	0,563
395	0,339	0,563
400	0,337	0,563

405	0,335	0,562
410	0,332	0,561
415	0,329	0,558
420	0,326	0,555
425	0,322	0,551
430	0,318	0,545
435	0,313	0,539
440	0,308	0,53
445	0,302	0,52
450	0,295	0,511
455	0,288	0,499
460	0,281	0,487
465	0,274	0,474
470	0,266	0,46
475	0,257	0,445
480	0,249	0,43
485	0,241	0,416
490	0,232	0,4
495	0,224	0,386
500	0,215	0,371
505	0,206	0,355
510	0,198	0,339
515	0,19	0,325
520	0,182	0,311
525	0,173	0,297
530	0,166	0,285
535	0,159	0,272
540	0,152	0,26
545	0,146	0,249
550	0,139	0,239
555	0,133	0,229
560	0,128	0,219
565	0,122	0,211
570	0,117	0,203
575	0,113	0,195
580	0,108	0,188
585	0,104	0,181
590	0,101	0,174
595	0,097	0,169
600	0,094	0,164
605	0,091	0,159
610	0,088	0,155
615	0,086	0,15
620	0,083	0,146
625	0,081	0,142

630	0,079	0,138
635	0,077	0,135
640	0,075	0,132
645	0,074	0,129
650	0,072	0,126
655	0,071	0,123
660	0,069	0,121
665	0,068	0,119
670	0,067	0,116
675	0,066	0,114
680	0,065	0,112
685	0,064	0,11
690	0,063	0,108
695	0,062	0,106
700	0,061	0,105

Figure 5. b) A 12 h study of *C. krusei* when various concentration of Ag-KT4561 was mixed with cheese whey

Conc. Ag-KT4561 (uM)	0 h	3 h	6 h	9 h	12 h
10	7,50E+06	1,80E+07	2,50E+07	7,90E+07	1,10E+08
20	3,00E+06	5,00E+06	2,20E+07	3,40E+07	8,00E+07
40	1,00E+06	7,00E+06	9,00E+07	1,50E+08	1,50E+08
50	6,00E+06	1,85E+07	1,30E+07	6,00E+07	6,50E+07
100	5,00E+06	2,30E+07	6,00E+07	6,80E+07	9,70E+07
200	2,46E+06	2,75E+07	6,15E+07	8,70E+07	1,20E+08
250	6,00E+06	1,13E+07	4,01E+07	5,12E+07	1,12E+08
350	7,00E+06	0,00E+00	0,00E+00	0,00E+00	0,00E+00
500	7,12E+06	0	0	0	0

Figure 6. a) A 12 h study of *K. marxianus* when various concentration of Ag-KT4561 was mixed with cheese whey

Conc. Ag-KT4561 (uM)	0 h	3 h	6 h	9 h	12 h
10	1,00E+07	1,40E+08	4,90E+08	5,00E+08	9,00E+08
20	1,20E+06	7,50E+06	5,25E+08	5,90E+08	7,70E+08
40	1,30E+06	1,00E+07	1,13E+08	5,00E+08	9,40E+08
50	1,90E+07	2,00E+07	5,20E+08	6,10E+08	6,25E+08
100	8,00E+06	1,10E+08	1,30E+08	1,50E+08	4,80E+08
200	3,50E+07	1,10E+08	1,19E+08	1,39E+08	5,60E+08
250	2,10E+07	6,80E+07	8,16E+07	9,00E+08	1,00E+09
350	2,00E+07	4,10E+05	5,01E+06	5,10E+07	2,60E+08
500	1,30E+07	2,80E+05	4,51E+05	7,80E+06	9,00E+06
1000	1,00E+07	0	0	0	0

Figure 6. b) A stability test done for 20 weeks representing growth of *K. marxianus* (KM) at a minimum of 2.1 x 10^8 and no traces of *C. krusei*

Weeks	KM + Conc. Ag-kt4561 350 uM	CK
1	2,20E+08	0
2	2,10E+08	0
3	1,91E+08	0
4	2,01E+08	0
5	2,10E+08	0
6	1,90E+08	0
7	1,95E+08	0
8	2,00E+08	0
9	1,93E+08	0
10	1,89E+08	0
11	2,01E+08	0
12	1,87E+08	0
13	1,89E+08	0
14	1,98E+08	0
15	2,00E+08	0
16	2,01E+08	0
17	2,15E+08	0

18	2,00E+08	0
19	2,20E+08	0
20	2,15E+08	0

ANNEXE 3
Raw Data

Nutritional profile of *Saccharomyces unisporus* for single cell protein production: from classical to genomic approach

Figure 1. a) *S. unisporus* growth in synthetic medium (YEPD), cheese whey (CW) 4.5% (w/v) and cheese whey (CW+ U) (4.5% (w/v)) supplemented with urea (0.22% (w/v))

Time (h)	SU_YEPD (control)	SU_CW	SU_CW+UREA
0	1,50E+03	1,70E+03	1,75E+03
6	5,00E+04	1,83E+03	1,90E+03
12	3,00E+05	2,00E+04	2,34E+04
18	5,00E+05	3,00E+04	4,78E+04
24	5,20E+06	2,00E+05	4,34E+05
30	5,00E+07	3,80E+05	4,20E+05
36	5,60E+07	5,00E+05	6,00E+05
42	4,80E+06	2,30E+05	2,30E+05
48	3,20E+06	3,00E+04	3,50E+04

Figure 1. b) *S. unisporus* growth at various concentrations of molasses along with cheese whey

Time (h)	SU_MOLASSES	SU_CW+MOLASSES (3%)+UREA (0.22%)	SU_CW+MOLASSES (5%)+UREA (0.22%)	SU_CW+MOLASSES (7%)+UREA (0.22%)	SU_CW+MOLASSES (7%)
0	1,75E+03	1,50E+03	1,70E+03	1,62E+03	1,75E+03
6	1,90E+03	5,00E+03	1,83E+04	1,93E+04	2,20E+03
12	2,34E+03	3,00E+04	2,50E+04	2,20E+05	2,56E+04
18	4,78E+03	4,30E+04	5,00E+05	3,70E+06	9,88E+04
24	9,54E+03	5,20E+05	3,60E+06	1,07E+07	3,34E+05
30	4,20E+04	5,00E+06	4,30E+06	4,76E+07	4,20E+06
36	6,00E+04	5,60E+06	5,00E+07	6,00E+07	6,00E+06
42	2,30E+04	4,80E+05	5,60E+07	5,00E+07	5,30E+06
48	2,75E+03	3,20E+05	3,00E+06	3,30E+07	3,10E+06

Figure 2. *S. unisporus* growth at various concentrations of glycerol and cheese whey

Time(h)	SU_CW+MOLASSES (5%)+UREA	SU_CW+Glycerol (3%) + urea (0,22%)	SU_CW+Glycerol (5%) + urea (0.22%)	SU_CW+Glycerol (7%) + urea (0.22%)
0	1,70E+03	1,75E+03	1,50E+03	1,70E+03
6	1,83E+04	1,90E+03	3,20E+03	1,83E+03
12	2,50E+04	2,34E+03	3,00E+03	2,50E+03
18	5,00E+05	4,78E+03	4,30E+03	5,00E+03
24	3,60E+06	1,34E+04	2,54E+04	1,34E+04
30	4,30E+06	4,20E+04	5,00E+04	4,30E+04
36	5,00E+07	6,00E+04	5,60E+04	5,00E+04
42	5,60E+07	2,30E+04	4,80E+04	5,60E+04
48	3,00E+06	2,00E+04	2,35E+04	3,05E+04

Figure 3. *S. unisporus* growth at various concentrations of ammonium sulphate with cheese whey and 7% (w/v) molasses

Time (h)	SU_CW+MOLASSES (7%)+UREA	SU_CW+MOLASSES (7%)	SU_CW+ AS (0,2%)	SU_CW+ AS (0,4%)	SU_CW+ AS (0,6%)	SU_MOLASSES (7%)+ AS(0,2%)	SU_MOLASSES (7%)+ AS(0,4%)	SU_MOLASSES (7%)+ AS(0,6%)
0	1,70E+03	1,75E+03	1,50E+03	1,32E+03	1,21E+03	1,23E+03	1,64E+03	1,72E+03
6	1,83E+04	2,20E+03	3,20E+03	3,13E+03	2,10E+03	1,50E+03	1,90E+03	2,30E+03
12	2,50E+04	2,56E+04	3,00E+04	3,33E+04	2,90E+04	1,90E+04	2,61E+04	1,34E+04
18	5,00E+05	9,88E+04	2,50E+05	4,12E+05	3,90E+05	2,38E+05	6,58E+05	4,78E+04
24	3,60E+06	3,34E+05	4,34E+05	2,54E+06	2,78E+06	3,24E+06	8,75E+06	4,54E+05
30	4,30E+06	4,20E+06	5,00E+06	4,91E+06	5,10E+06	3,20E+07	4,20E+07	6,50E+06
36	5,00E+07	6,00E+06	5,60E+06	5,23E+06	5,56E+06	1,20E+08	9,80E+07	4,30E+07
42	5,60E+07	5,30E+06	4,80E+06	4,51E+06	4,50E+06	1,00E+08	1,20E+08	2,31E+07
48	3,00E+06	3,10E+06	2,35E+06	3,10E+06	8,15E+05	9,18E+07	1,05E+08	2,15E+07

Figure 4. *S. unisporus* growth at various pH in cheese whey with molasses and ammonium sulphate

Time (h)	SU_CW+MOLASSES (7%)+AS(0.4%) (pH 3.5)	SU_CW+MOLASSES (7%)+AS (0.4%) (pH 5.5)	SU_CW+MOLASSES (7%)+AS (0.4%) (pH 4.5)
0	1,75E+03	1,23E+03	1,50E+03
6	1,90E+03	1,50E+04	4,10E+03
12	2,34E+04	1,90E+04	3,00E+05
18	4,78E+04	2,38E+05	4,30E+05
24	1,34E+05	3,24E+06	2,54E+06
30	4,20E+05	1,60E+07	5,20E+07
36	5,90E+06	3,00E+08	1,00E+08
42	4,30E+06	2,80E+08	9,00E+07
48	3,54E+06	1,25E+08	8,15E+07

Figure 5. Fatty acid profile of *S. unisporus* and *S.cerevisiae* b) Variation in mono-unsaturated and polyunsaturated fatty acids profile of *S. unisporus* and *S.cerevisiae*

Saturated FA			
Fatty Acids (%wt)	*S. cerevisiae*	*S. unisporus* NRRL Y-1556	FAO (2009)
C10:0	1,1	1,2	1
C12:0	4,8	5,2	1
C14:0	8,8	7,3	11
C16:0	26,8	24	28
C18:0	6,1	5	2
C20:0	1,194	2	4
C22:0	5,7	1,4	2
MUFA			
Fatty Acids (%wt)	*S. cerevisiae*	*S. unisporus* NRRL Y-1556	FAO (2009)
C16:1	16,6	12,1	1 to 36
C18:1	25,7	22,3	2 to 25
PUFA n=6			
Fatty Acids (%wt)	*S. cerevisiae*	*S. unisporus* NRRL Y-1556	FAO (2009)
C18:2	10,1	7,3	1 to 3
Total Fatty acids	0,962	0,8612	

Fatty Acids (%wt)	*S. cerevisiae*	*S. unisporus* NRRL Y-1556	FAO (2009)
C16:1	16,6	12,1	36
C18:1	25,7	22,3	25
C18:2	10,1	7,3	3
Total Fatty acids	0,962	0,8612	2,5

Figure 6. Amino acid profile of *S. unisporus*

FAO (mg/g)	*S. unisporus* mg/g	Composés
0	42,05128205	ARG
42	83,97435897	LYS
14	46,15384615	HIS
42	59,87179487	LEU+ILE
22	8,974358974	MET
28	38,07692308	PHE
40	36,15384615	THR
8,5	8,974358974	TRP
42	74,35897436	VAL
0	59,29487179	Trans-PRO
20	76,92307692	CYS
0	50,70512821	PRO
0	83,33333333	ASP
0	8,974358974	GLN
0	37,05128205	SER
0	71,15384615	GLU
0	15,12820513	ALA
0	83,97435897	GLY
0	40	TYR
0	26,79487179	ASN

ANNEXE 4
Raw Data

Acid hydrolysis of cheese whey for the growth of *Saccharomyces unisporus*

Figure 1. Acid hydrolysis at 0.2wt% H_2SO_4 a) whey b) permeate

Time (min)	Lactose (g/L)	glucose (g/L)	galactose (g/L)
0	28	0,49	0,48
10	27,2	1	1
20	25,2	1	1,1
30	23,8	2	1,7
40	21,3	2	3
50	19,1	3	3,8
60	18,4	4	4,3
70	17,7	5	6,1
80	16,2	7	7,8
90	15,7	8	8,1
100	15,2	9	9
110	14,8	9	10
120	14,7	10	11,3

Time (min)	Lactose (g/L)	glucose (g/L)	galactose (g/L)
0	49,5	0,49	0,48
10	21,5	1	1
20	11	1	1,1
30	9,9	2	1,7
40	9,7	2	3
50	9,4	3	3,8
60	7,6	4	4,3
70	7,2	5	6,1
80	5,8	7	7,8
90	4,8	8	8,1
100	4,5	9	9
110	4,2	9	10
120	3,2	10	11,3

Figure 2. Acid hydrolysis at 0.3wt% H_2SO_4 a) whey b) permeate

Time (min)	Lactose (g/L)	glucose (g/L)	galactose (g/L)
0	27,6	1,4	1,5
10	22,7	3	4
20	22,3	4	4,3
30	21,7	5	4,7
40	19,5	5	5
50	19,2	5	5,2
60	16	5,3	7,1
70	11,2	6	8
80	9,9	6,3	8,3
90	9,7	6,6	9,1
100	9,4	7,2	9,5
110	7,6	7,2	12,6
120	7,2	7,2	12,6

Time (min)	Lactose (g/L)	glucose (g/L)	galactose (g/L)
0	49,6	2,4	2,6
10	42,7	4	5
20	42,3	7	6,3
30	41,7	10	8,2
40	39,5	13	10
50	39,2	13	12,4
60	36	15	14,8
70	31,2	17	17,4
80	28,8	19	19,7
90	22	19	21,2
100	21,4	20	22,7
110	19,3	20	23,1
120	17,3	20	23,6

Figure 3. Acid hydrolysis at 0.4wt% H_2SO_4 in whey

Time (min)	Lactose (g/L)	glucose (g/L)	galactose (g/L)
0	28	2,4	2,6
10	25	4	5
20	22	7	6,3
30	20,3	10	8,2
40	19	13	10
50	18,2	13	12,4
60	13,4	15	14,8
70	11,2	17	17,4
80	9,9	19	19,7
90	9,7	19	21,2
100	9,4	20	22,7
110	7,6	20	23,1
120	7,2	20	23,6

Figure 5. Growth of *S. unisporus* (mono-culture in cheese whey (CW), hydrolyzed CW, hydrolyzed permeate and whey permeate (WP)

Time (h)	SU_CW (Before hydrolysis)	SU_CW (After hydrolysis)	SU_WP (Before hydrolysis)	SU_WP (After hydrolysis)
0	1,70E+04	2,01E+04	2,00E+04	1,50E+04
3	1,80E+04	5,44E+05	5,00E+04	5,00E+04
6	2,00E+04	7,80E+06	1,00E+05	3,00E+05
9	3,00E+04	8,10E+07	1,23E+05	5,00E+05
12	2,00E+05	9,40E+07	1,20E+05	5,20E+06
15	2,10E+05	2,00E+08	2,00E+05	5,00E+07
18	2,00E+05	4,00E+08	5,60E+05	2,00E+08
21	1,90E+05	4,30E+08	4,80E+05	2,50E+08
24	2,10E+05	4,10E+08	3,20E+05	2,90E+08

Figure 6. Growth of *S. unisporus* and *K. marxianus* in hydrolyzed cheese whey (CW), hydrolyzed permeate (WP)

Time (h)	SU_CW	KM_CW	SU_WP	KM_WP
0	2,01E+04	2,00E+04	2,00E+04	1,50E+04
3	6,20E+04	6,14E+05	5,00E+04	5,00E+04
6	7,80E+05	9,00E+05	1,00E+05	3,00E+05
9	2,30E+06	8,10E+06	1,23E+05	5,00E+07
12	3,70E+06	7,80E+08	7,50E+05	8,10E+08
15	4,10E+07	5,00E+08	2,00E+06	8,30E+08
18	5,60E+07	4,50E+08	6,80E+07	7,80E+08
21	6,90E+07	4,30E+08	5,10E+07	7,50E+08
24	9,10E+07	4,10E+08	3,20E+07	6,90E+08

ANNEXE 5
Synthesis in English

2. PROBLEMS

The literature review has shown that cheese whey is an environmental contaminant both in land and in water. Cheese whey is produced from cheese industries globally at a massive scale. But cheese whey has the potential to be converted to value-added products such as SCP. SCP can be produced efficiently with the help of microorganisms which are acceptable in the production of SCP. However for the production of SCPs, factors such as the presence of any form of biological or chemical contaminant must be thoroughly verified or efficiency of microorganisms which can be utilized for SCP production must be acceptable for food and feed application in a commercial or industrial level. The problem areas are mentioned below.

2.1 Problem 1

During the production of SCP using the microorganisms (*K. marxianus*, *L. fermentum*, and *S. unisporus*), C. *krusei* was emerging as a biological contaminant. In spite of *C. krusei* being a non-lactose consuming microorganism, it was consuming the essential nutrients out of the cheese whey powder and the growth of *C. krusei* is more effective than *K. marxianus* and other microorganisms. *C. krusei* cannot be considered for food and feed application as it is not acceptable by FDA. During fermentation, low pH of 3.5 and high temperature of 40 °C were used to control *C. krusei* contamination, however they were not effective. Therefore it is necessary to evaluate various inhibition methods to remove *C. krusei*, which are effective, economical, and non-toxic

2.2 Problem 2

Mixed culture provides better nutritive biomass than mono-culture. There are few lactose-consuming microbes and *K. marxianus* is one of them. While conducting fermentation with *K. marxianus*, *S. unisporus* and *L. fermentum*, it was observed that *S. unisporus* and *L. fermentum* were not growing along with *K. marxianus* as they are non-lactose consuming microbes. But *S. unisporus* is an acceptable yeast for the production of

food and feed in the European Union, however the nutritive profile of *S. unisporus* is not known yet.

2.3 Problem 3

Presence of lactose in cheese whey enables the rise of COD when disposed in land or in water. Cheese whey can be acceptable as a value addition in food and feed, but the lactose present in the cheese whey is restricting its use as food and feed supplement as large section of the world population cannot consume lactose due to lactose intolerance. Also non-lactose consuming yeast cannot be grown in cheese whey for SCP production.

3. HYPOTHESIS, OBJECTIVES AND ORIGINALITY

3.1 Hypothesis

Based on problems mentioned, the following hypotheses are drawn:

3.1.1 Hypothesis 1

For the removal of *C. krusei*, the methods applied are either chemical, biological or nanoparticle approaches. These chemical, biological and nanoparticles are proven to be effective in inhibiting *C. krusei*, however the better inhibitory method to remove *C. krusei* from mixed culture is not known from the literature. Therefore, investigation of various chemical, biological and nanoparticle approaches could provide the better inhibitory method to remove *C. krusei* completely without affecting the growth of *K. marxianus* and *S. unisporus* during SCP production.

3.1.2 Hypothesis 2

For SCP production, mixed culture is better than mono culture, so *S. unisporus* is considered for mixed culture along with *K. marxianus*. However nutritive profile of *S. unisporus* is not defined yet but it is an acceptable yeast for food and feed. So studying the metabolic profile of *S. unisporus* using various carbon and nitrogen sources could provide the complete growth and nutritional profile of *S. unisporus*. This information could help to choose the best carbon and nitrogen source to grow along with *K. marxianus* for mixed culture SCP.

3.1.3 Hypothesis 3

As cheese whey has high concentrations of lactose, it has to be degraded to monosaccharides with the help of hydrolysis. Acid hydrolysis of cheese whey is preferred over enzymatic hydrolysis as it is economical than enzymatic hydrolysis and sulfuric acid can efficiently hydrolyze cheese whey lactose to glucose and galactose. *S. unisporus* can consume hydrolyzed glucose and galactose to grow efficiently along with *K. marxianus* in mixed culture and could produce biomass with high protein content.

3.2 OBJECTIFS

The global objective of this research work is removal of biological contaminant *C. krusei* from the mixed culture fermentation and improved growth of *S. unisporus* in mixed culture during SCP production.

3.2.1 Objectif 1

Evaluation of chemical and biochemical approaches towards inhibition of *C. krusei* without affecting the growth of acceptable yeasts (*K. marxianus* and *S. unisporus*).

3.2.2 Objectif 2

Production and optimization of protein conjugated silver nanoparticle for inhibition of *C. krusei*.

3.2.3 Objectif 3

Study of metabolic profile of *S. unisporus* by varying carbon and nitrogen sources to use it for SCP production in mixed culture.

3.2.4 Objectif 4

Acid hydrolysis of cheese whey and production of mixed culture SCP (*S. unisporus* and *K. marxianus*) using hydrolyzed cheese whey.

3.3 ORIGINALITY

1. Inhibition of *C. krusei* from mixed culture during SCP production using biological nanoparticles (killer protein from *W. saturnus*) is completely novel approach.

2. Nutritional profile of *S. unisporus* is not studied before in the literature.

3. Mixed culture SCP production using *K. marxianus* and *S. unisporus* is also a new approach, as it not considered before.

4.METHODOLOGY

4.1 Optimization and production of silver-protein conjugate

4.1.1 Production of *Williopsis saturnus* biomass

Pre-culture of *W. saturnus* was prepared in 500 mL flasks using 100 mL of sterilized YEPD medium. The production medium containing 5% (w/v) of molasses and 4.5% (w/v) of cheese whey, 0.22% (w/v) of urea was pasteurized at 80 ^{o}C for 20 min. A loop full of the *W. saturnus* culture was then transferred to pasteurized medium and the flask was kept at 28 ^{o}C in an agitating shaker at 150 rpm for 24 h. Samples were withdrawn at regular intervals to measure cell and protein concentration. Protein concentration was measured using Folin-Lowry method (Lowry et al., 1951).

4.1.2 Optimization of the process parameters

- Different concentrations of silver nitrate ($AgNO_3$) concentration ranging from 0.01M - 5 M were prepared in double deionized water in sterile amber bottles and kept at room temperature.
- Further, different concentrations of $AgNO_3$ were added to 72 µL of *W. saturnus* supernatant and 5mL of dH_2O in 15 mL glass tubes were incubated at temperatures (20 - 40 °C) under continuous stirring using magnetic stirrer at 150 rpm. A change in color was observed from light yellow to dark yellowish-brown which indicates the formation of Ag-NPs. The color intensity was measured using UV-Vis spectrophotometer (Carry 100 Bio®, Varian USA) at 300-700 nm.
- After optimization of 0.1M $AgNO_3$ concentration, different volumes (25-200 µL) of 0.1 M $AgNO_3$ were tested with 72 µL of *W. saturnus* supernatant and 5mL of dH_2O.
- Different volumes of *W. saturnus* supernatant (96 - 220 µL/5 mL dH_2O) were tested along with optimum $AgNO_3$ solution for the complete reduction of silver ions (Ag^{+}).
- Different reaction times were tested with optimum concentrations of *W. saturnus* supernatant and $AgNO_3$ at optimum temperature of 25 °C. The reaction mixture in

the 15 mL glass tubes were incubated for 24 h. Samples were withdrawn at every 2 h to measure the color intensity of the reaction mixture using UV-Vis spectrophotometer.

4.1.3 Characterization of synthesized silver-protein conjugate

- Synthesized Ag-NPs were centrifuged at 10,000g for 2 min at room temperature (20 °C) followed by filtration of supernatant using 0.45 micron filter.
- The filtered silver nanoparticles solution was characterized using Fourier transform infrared (FTIR), zetasizer, Scanning Electron Microscope analysis (SEM) and Energy Dispersive X-ray Analyzer (EDS).

4.1.4 Well-diffusion method

The synthesized silver-protein conjugate (Ag-KT4561) was tested against *C. krusei*, *K. marxianus* and *S. unisporus* using Nathan et al., 1978 well diffusion method. Agar plates were inoculated with *C. krusei*, *K. marxianus* and *S. unisporus* separately and wells of 6 mm diameter were made on agar plates. Further 40 µL of Ag-KT4561 were loaded in the wells and kept at 30 °C and plates were evaluated after 24 h zone of inhibition.

4.2 Evaluation of chemical, biochemical and nanoparticle methods on *C. krusei* inhibition

4.2.1 Microorganisms

The microorganisms *K. marxianus* and *C. krusei* were grown on YEPD media and plating was done one YEPD agar plates.

4.2.2 Lyophilization

Lyophilization of the *W. saturnus* supernatant was carried out with the help of Lyophilizer at -40 °C to 15 °C (SP Scientific, USA).

4.2.3 Inhibition experiments

Shake flask experiments were carried using 500 mL of cheese whey in 2 L ErlynMayer flasks. Pasteurization was done at 80 °C for 20 min. After pasteurization, cheese whey was inoculated with *C. krusei* and *K. marxianus* and incubated in the shaker incubator at 30 °C and 150 rpm. The following inhibition studies were performed:

4.2.3.1 Chemical approaches

- Effect of different temperatures (28, 40 and 45 °C) and different pH (2.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 9.0) were tested on the inhibition of *C. krusei* in a mixed culture (*C. krusei*, *K. marxianus*, and *W. saturnus*).
- Effect of H_2O_2 concentration (100, 200, 300, 400, 600, 800, 2400, 3200 and 4000 ppm) along with different temperatures (28 40 and 45 °C) were tested on the inhibition of *C. krusei* in a mixed culture (*C. krusei*, *K. marxianus*, and *W. saturnus*).
- Effect of NaCl concentration (1.5 and 2 M) on the inhibition of *C. krusei* in a mixed culture (*C. krusei* and *K. marxianus*) was tested.

4.2.3.2 Biochemical approaches

- Effect of clove oil (0.4 and 0.5%) on the inhibition of *C. krusei* in a mixed culture (*C. krusei* and *K. marxianus*) was carried out.
- Effect of lyophilized *W. saturnus* supernatant and lyophilized whole fermented broth of *W. saturnus* (200, 300 and 400 mg/mL) for inhibition of *C. krusei* were carried out in a mixed culture (*C. krusei* and *K.marxinaus*).
- Effect of different H_2O_2 concentration (2400, 3200, 4000 ppm) along with *W. saturnus* lyophilized supernatant and *W. saturnus* whole fermented broth lyophilized (200, 300 and 400 mg/mL) on the inhibition of *C. krusei* were tested in a mixed culture (*C. krusei* and *K. marxinaus*).

4.2.3.3 Nanoparticle approach

- Effect of synthesized Ag-KT4561 (10 µM – 1 mM) on the inhibition of *C. krusei* was carried out in a mixed culture (*C. krusei* and *K. marxianus*).

4.3 Nutritional profile of *S. unisporus*

4.3.1 Analysis of substrate utilization profile using Biolog system

S. unisporus NRRL Y-1556 was grown in the YEPD plate at 33 °C for 24 h. The inoculum was prepared by suspending cells from the YEPD plate in the sterile distilled water to give 45-47% transmittance (%T) with Biolog Turbidimeter. 100 µL of inoculum samples were dispensed into each well of the Biolog Yeast (YT) microplate of the Biolog system (Biolog Inc., Hayward, CA, USA). The inoculated microplate was incubated at 30 °C for 12, 24, 36 and 48 h, respectively. Microplate was read by the microplate reader and processed to determine the substrate utilization profile.

4.3.2 Optimization of growth conditions

4.3.2.1 Variation in carbon sources

Different carbon sources were chosen such as molasses (3%, 5%, or 7% w/v, typical sugarcane-based molasses contains 35-42% (w/v) of sucrose) (Doelle and Doelle, 1990) and pure glycerol (5%, 7%, or 9% w/v) along with cheese whey 4.5% (w/v) and urea (0.22% w/v). Pre-culture of *S. unisporus* was prepared using YEPD medium (100 mL) with loop full of culture in 500 mL Erlenmeyer flasks. Inoculum of 2% (v/v) from pre-culture was used to inoculate the pasteurized production (80 ^{o}C for 20 min) media, which contains different concentrations of molasses and pure glycerol along with 4.5% (w/v) of whey powder and 0.22% (w/v) of urea. After inoculation, flasks were kept at 150 rpm and 28 ^{o}C for 48 h in an incubator shaker. Samples were withdrawn at regular intervals to measure the cell concentration.

4.3.2.2 Variation in nitrogen sources

Different nitrogen sources such as urea (0.22 % w/v) and ammonium sulphate at various concentrations (0.2%, 0.4% and 0.6% (w/v)) were added to medium containing molasses and cheese whey. Fermentation with *S. unisporus* was carried out same as described in the section of variation in carbon source.

4.3.2.3 Variation in pH

Variation in pH of media during the fermentation of cheese whey with *S. unisporus* in Erlenmeyer flasks was studied. Production media was prepared with various pH (3.5, 4.5, and 5.5) using 1 N NaOH and 1 N H_2SO_4.

4.3.3 Statistical method

Principal component analysis (PCA 1 and 2) was performed using statistical tool R using the package FactoMine.R on amino acid profiles obtained at various temperatures. Statistical analysis of various amino acid profiles were carried out by PCA 1, further PCA 2 was performed based on the codon of the amino acids identified through PCA 1. This method is similar to the method of Dom et al., (2003).

4.4 Acid hydrolysis of cheese whey and permeate for the growth of *S. unisporus*

4.4.1 Acid hydrolysis of cheese whey and whey permeate

Acid hydrolysis of cheese whey and whey permeate were carried out using H_2SO_4. 200 mL of cheese whey (4.5% (w/v)) and whey permeate (5% (w/v)) were taken in different 1 L bottles and different concentrations of H_2SO_4 such as 0.2, 0.3 and 0.4% (w/w) were added in each bottle and heat treated at 121 °C for different time periods (15, 30, 60 and 120 min).

4.5 Analytical methods

4.5.1 Carbohydrate Analysis

Hydrolyzed cheese whey and whey permeate were centrifuged at 10 000 g for 10 min at 4 °C and supernatant was collected. The supernatant was further filtered using micro filter of 0.45 µ (Thermo Scientific, USA). Carbohydrate analysis was performed using Liquid Chromatography- Mass Spectroscopy (LC-MS/MS) method (Thermo TSQ Quantum) equipped with an Electrospray Ionization (ESI) in negative ion mode. The analytical column used was Zorbax Carbohydrate of the specifications 4.6 mm x 150 mm (Agilent Life Sciences). The injection volume was 10µL and the internal standard used was

Glucose-D2. The mobile phase used for carbohydrate analysis was 75% acetonitrile mixed with 0.1% NH_4OH and 25% water mixed with 0.1% NH_4OH. This method is modified for the analysis (McRae and Monreal, 2011).

4.5.2 Volatile compounds

The volatile compounds present in the *S. unisporus* fermented broth were determined using gas chromatography – mass spectrometry (GC-MS). Fermented broth was centrifuged 10 000 g, 4 °C and 15 min and the supernatant was collected. The capillary column used was HP - INNOWax (30 mm, 0.25 mm, using nitrogen as carrier gas) and a split ratio of 1/100. The supernatant samples are diluted with methanol (1:1) and 10 µL of acetic acid (internal standard). Blank was prepared with 2 mL methanol and 10 µL of acetic acid. Diluted samples and blank were taken in GC vials and analyzed using GC-MS (Agilent Chrompack HP-6820).

4.5.3 Fatty acids

Fatty acids profiling was done by gas chromatography-mass spectrometry (GC-MS) after converting lipid into methyl ester derivatives (Mooney et al., 2007). The samples were centrifuged at low speed to facilitate phase separation. The organic phase was then taken for the GC, the analysis of the methyl esters of fatty acids performed in the chromatograph Varian 3900 equipped with a flame ionization detector (FID) and auto sampler. The column was Varian VF-23ms factor four whose specification is 260 ºC 3PA (30, 0.2 and 0.25 mm). Fatty acids were identified by comparison with standards of methyl esters (FAME 32 FAME mix C16-C18, C4-C24 FAME, Supelco, France; C16: 1 n-9, Cayman, France) and quantified using the internal standard heptadecanoic acid (C17: 0, Sigma) (Milinsk et al., 2008).

4.5.4 Amino acids

The essential amino acid compositions of the *S. unisporus* lyophilized fermentation broth was analyzed by hydrolyzing the powder with 6 N HCl at 110 ºC for 24 h. Digested samples containing HCl were re-suspended in acetate buffer and passed through 0.45 µ micro-filter (Thermo Scientific., USA), which was slightly modified method of

Lourenço et al., (2002). The amino acids were released as a result of hydrolysis and were analysed by Liquid chromatography-mass spectrometry (LC-MS/MS) (TSQ Quantum Access, Thermo Scientific).

4.5.5 Ethanol production

The supernatant of fermenter broth was taken for ethanol analysis. The internal standard propanol at a concentration of 0.01% (v/v) was added to the test samples and standards (1.00 - 0.005 g/dL) (Tiscione et al., 2011). The ethanol produced was analyzed by FID (Varian Chrompack, CP-3800).

4.5.6 Metal analysis

Metal concentrations of *S. unisporus* lyophilized samples were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Varian Vista AX, US) after partial digestion of liquid sample with acid-peroxide (HNO_3 and H_2O_2) digestion method (US EPA Method, 1982).

4.5.7 Protein Estimation

The lyophilized powder of *S. unisporus* biomass was ultra-sonicated (Lopez et al., 2010) and protein was estimated by Folin-Lowry method (Lowry et al., 1951).

2. RESULTS AND DISCUSSION

The results obtained are presented in this thesis in 2 parts. The 1st part presents the optimization and production of killer protein conjugated with silver nanoparticles. The application of chemical, biological and protein conjugated with silver nanoparticles in cheese whey for the elimination of *C. krusei* in mono-culture and mixed culture (three articles submitted). The 2nd part presents the nutritive profiling of *S. unisporus* in cheese whey, further acid hydrolysis was performed on cheese whey and *S. unisporus* and *K. marxianus* were grown as a mixed culture for food and feed production (one article is published and two articles submitted).

5.1 Inhibition of *C. krusei* by an economical approach during SCP production

5.1.1 *Candida krusei*: Factors influencing its growth and inhibition by chemical, biochemical and nanoparticle approaches

C. krusei is described as one of the food pathogens which falls under the genus *Candida*. It has been mostly isolated from patients who are immune-deficient and are of minimal clinical significance. The possible ways of inhibitions of *C. krusei* are through chemical, biochemical and nanoparticle methods. Chemical inhibitors for the inhibition of *C. krusei* are getting restricted, as many azole drugs are not effective against *C. krusei*. Other ways of inhibiting *C. krusei* is by the use of medicinal plants, osmotic stress using NaCl or glucose and essential oils such as clove oil (*Syzygium aromaticum*). In food and beverages industry, use of biological inhibitors consisting of killer proteins either from *Hanseniaspora uvarum*, *Williopsis mrakii*, *or Pleurotus ostreatus* are observed. Other ways of inhibiting C. *krusei* is by the use of engineered nanoparticle (NP). Although *C. krusei* is less virulent than C. *albicans*, but still it has proteolytic potential and produces phospholipases. In spite of *C. krusei* not being acceptable as a SCP producer, it is used in wine making, porridge, and dough fermentation. C. *krusei* is significantly different from

other medically important *Candida* spp. in its structural and metabolic features and exhibits different behavioral patterns towards host defenses.

5.1.2 Optimization and Production of Silver-Protein Conjugate as Bio preservative

Williopsis saturnus is known as the effective killer yeast against non-*candida albicans* spp. *W. saturnus* comes under natural microflora of cheese and is deliberately used in food and beverages industries. Efficiency of the killer protein produced by *W. saturnus* is increased if it is in nanometer size. The capability of *W. saturnus* killer protein (KT4561) conjugating it with silver nanoparticles has never been explored. Various parameters were optimized and characterized for the conjugation of KT4561 and silver ions. It was observed that Ag-NPs were completely synthesized by utilizing *W. saturnus* protein (4.3 g/L) and 200 µL of 0.1 M $AgNO_3$ (3.96 mM) at reaction time of 4 h. Characterization was performed using UV-VIS, FTIR, EDS, and SEM techniques. FTIR confirms that only proteins are bonded with the reduced silver ions and EDS confirms that silver is the only metal particle present in the protein-silver conjugate of 200 nm. SEM confirms the synthesized NPs as crystalline structures. Well-assay diffusion method of the protein-silver conjugate was performed on *C. krusei*, *K. marxianus* and *S. unisporus. C. krusei* was successfully inhibited while partial inhibition was observed for *K. marxianus* and *S. unisporus* which caused by silver ions. It was observed that the killer activity of the Ag-KT4561 (silver nanoparticles) has increased three-folds compared to pure protein. Further, study was conducted by preparation of the bulk Ag-KT456. Because it is economical, consumes less time and supports green chemistry.

5.1.3 Evaluation of inhibitory measures for food spoiler yeast *Candida krusei* during fermentation process by chemical, biochemical and nanoparticle approaches

Various chemical, biochemical and biomolecule-nanoparticle methods were evaluated for the inhibition of *C. krusei* without hampering the growth of dairy yeast *K. marxianus*. Effective inhibition was observed with the help of H_2O_2, *W. saturnus* at specific combination of pH and temperature (pH 5.0 and 40 °C) but the efficient approach was the use of Ag-KT4561 nanoparticles. Among the chemical methods H_2O_2 works best at pH

range 4.0 to 10.0 and at temperature 30 °C or above. However very high concentration of H_2O_2 (4000 ppm) at 45 °C and pH 5.5 did completely inhibit *C. krusei* without effecting *K. marxianus*. *W. saturnus* supernatant was lyophilized and utilized for *C. krusei* inhibition along with 2400 ppm of H_2O_2. But 2400 ppm of H_2O_2 is very high concentration, therefore nanoparticle with silver was synthesized to reduce the quantity of killer protein used and enhance the efficiency of protein. It was observed that 350 µM (containing 1 ppm of reduced Ag ions) of synthesized silver nano-particle (AgNPs) of the killer protein from *W. saturnus* can completely inhibit *C. krusei* with partial effect on *K. marxianus*. The AgNPs method was found to be an efficient inhibitory approach in comparison to chemical and biochemical ways. AgNP method is also very economical, as production of 20,000 L of Ag-KT4561 would cost only 863 CAD. Further the stability test has confirmed the effectiveness of protein silver nanoparticles on *C. krusei* for more than 20 weeks. Therefore nanoparticle approach can be potentially utilized for *C. krusei* inhibition with partial or no-effect on other microbes and the process can be run non-aseptically.

5.2 Study of the nutritional profile of *S. unisporus* and utilizing it for mixed culture SCP production

5.2.1 *Saccharomyces unisporus*: Biotechnological Potential and Present Status

Saccharomyces spp has been associated with livestock food and feed and is a probioticum. Among these *Saccharomyces* spp presence of the *S. unisporus* was observed in different dairy products. Significance has been laid on the presence of *S. unisporus* in food-grade products and a close proximity of *S. unisporus* to *S. florentinus* and both of these species are accepted by the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA) for food and feed applications. As *S. unisporus* is found in different food-grade products, it is considered as the potential candidate because it is generally regarded as safe (GRAS) status. In previous studies, it

was confirmed that *S. unisporus* has the capacity to convert ketoisophorone to levodione, which is basically used in pharmaceutical industries. *S. unisporus* produces certain omega unsaturated fatty acids which combat diseases. As entire nutritional profile of *S. unisporus* is not known yet for SCP production the possible areas where *S. unisporus* can be exploited are for intermediates (proteins, amino acids, fatty acids, mineral content and other intermediates). This review is an attempt to describe the ubiquity of *S. unisporus* in SCP production.

5.2.2 Nutritional profile of *Saccharomyces unisporus* for single cell protein production: from classical to genomic approach

S. unisporus was grown in cheese whey with various types of carbon sources such as molasses and glycerol and nitrogen sources such as urea and ammonium sulphate for the single cell protein production (SCP). It was observed that cheese whey along with 7% (w/v) of molasses with sucrose content of 10 g/L and 0.4% (w/v) of ammonium sulphate produced higher SCP with total protein content of 4.2 (g/L) at pH 5.5. *S. unisporus* was found to be urea-negative. Amino acid analysis was performed and it was observed that *S. unisporus* has the capacity to produce essential amino acids, which are lysine (84.0 mg/g dry cell), valine (74.4 mg/g), asparatic acid (83.3 mg/g), glycine (84.0 mg/g) and cysteine (77.0 mg/g) respectively as confirmed by LC-MS/MS. According to FAO standards the concentration of fatty acid in SCP must be lower than 3.0 % (w/w) and fatty acids concentration produced by *S. unisporus* is 0.86 % (w/w). It was even observed that *S. unisporus* produced 2 g/L of ethanol at 36 h of fermentation with 7% (w/v) molasses in the medium as *S. unisporus* is crab-tree positive. *S. unisporus* can be even exploited for bioethanol production. Study of the codon frequencies showed that *S. unisporus* is AT rich (65%) in SCP similar to *S. cerevisiae*. Based on the nutritional profile of *S. unisporus*, it can be grown as a mono-culture as well as mixed culture when provided with optimum carbon and nitrogen source.

5.2.3 Acid hydrolysis of cheese whey for the growth of *Saccharomyces unisporus*

Acid hydrolysis of cheese whey and whey permeate has been carried out for the effective growth of *S. unisporus* as mono-culture and mixed-culture. Different concentrations of

H_2SO_4 (0.2, 0.3 0.4 w/w %) were utilized for the hydrolysis and it was observed that 0.2% (w/w) of H_2SO_4 can efficiently hydrolyze whey permeate in 70 min. Complete hydrolysis of cheese whey was obtained with 0.4% H_2SO_4 (w/w) in 60 min. It was also found that *S. unisporus* as mono culture was grown very effectively in both hydrolyzed cheese whey (4.0×10^8) and permeate (2.0×10^8) compared to unhydrolyzed cheese whey (2.1×10^5) and permeate (2.0×10^5). *S. unisporus* and *K. marxianus* were cultivated in hydrolyzed whey and permeate and it was observed that both the yeasts are growing well but growth of *K. marxianus* is higher than *S. unisporus*. Henceforth cheese whey and whey permeate can be used for the growth of mixed culture and produce SCP. It was also observed that *S. unisporus* produced certain essential intermediates such as phenyl ethyl alcohol, furan methanol etc while grown in hydrolyzed media which has been confirmed by GC-MS.

PART 1: CONCLUSION

The conclusions that are presented in this study are mentioned in two different parts. For SCP production only microorganisms which are acceptable by Food and Drug Administration must be utilized. *K. marxianus* is a reported approved yeast meant for SCP production, but *C. krusei* is not reported as an approved yeast for food and feed but is a biological contaminant. Steps are ensured for the elimination of *C. krusei* without having any effect on *K. marxianus*.

Approaches such as chemical (NaCl, H_2O_2), and biochemical (clove oil, *W. saturnus* supernatant and *W. saturnus* supernatant along with H_2O_2) are effective in eliminating *C. krusei* but the preservative quantity required for eliminating *C. krusei* is very high which in turn is increasing the economic cost of the preservative used. Therefore the steps considered for the removal of *C. krusei* from the mixed culture of *K. marxianus* and *C. krusei* is by the use of silver ion-killer protein conjugate (Ag-KT4561 of 350 uM (with reduced silver concentration of 1 ppm effective at pH 5.5, 30 °C). Apart from that *K. marxianus* has a better growth profile at pH 5.5 rather than 3.5. Ag-KT4561 bio preservative is a stable compound and did not form any metallo-complex with minerals present in cheese whey. Usage of Ag-KT4561 in cheese whey can eliminate other biological contaminants along with *C. krusei* during cheese whey fermentation.

S. unisporus can consume wide range of carbon sources except lactose and can consume nitrogen source (0.4% (w/v) of ammonium sulphate). *S. unisporus* is strictly urea-negative. The mono-culture of *S. unisporus* was found to be enriched in lysine (84.0 mg/g), valine (74.4 mg/g), asparatic acid (83.3 mg/g) and glycine (77.0 mg/g). The *S. unisporus* biomass has the essential minerals which are Ca, S, Na, Mg, P and K. The *S. unisporus* biomass also has fatty acid content of 0.86% (w/w) and protein content of 4.2

(g/L) and is found to be AT-rich similar to *S. cerevisiae*. Entire nutritional profile makes it as an efficient SCP producer.

Further *S. unisporus* is exploited as mixed culture with *K. marxianus*. *K. marxianus* (7.8 x 10^8 CFU/mL) has a better growth than *S. unisporus* (5.6 x 10^7 CFU/mL) because *S. unisporus* can partially consume galactose whereas *K. marxianus* can consume both the monosaccharides and even the left over lactose. Fermentation with the mixed culture (*S. unisporus* and *K. marxianus*) has shown slight increment in the essential amino acids especially leucine and isoleucine (79.9 mg/g), also production of intermediates such as furanmethanol, furfuryl formate, weak organic acids that has biotechnological importance were observed. However, no quantification of the intermediates produced was done.

From these two sets of studies it can be concluded that the biological contaminant has to be eliminated in an economic and green way whereas the addition of the acceptable microorganism (*S. unisporus*) for the SCP production can lead to a better biomass.

PART 2: RECOMMANDATIONS

Based on the results as above utilization of cheese whey for food and feed applications can further be explored with the recommendations mentioned below:

1. The bio-preservation method can be modified by micro-filtration of the killer protein from *W. saturnus* and further preparation of the conjugation of filtered protein and silver ions can bring down the bio-preservative requirement and the cost of the bio-preservative.

2. For the growth of *W. saturnus* the raw materials used are cheese whey, molasses and urea. It is recommended to replace cheese whey with whey permeate as there is no soluble protein present in whey permeate which will interfere with *W. saturnus* killer protein.

3. A strong recommendation is to have further study on the effect of silver-protein conjugate on biological contaminant in the long run and it is even recommended for the Canadian Food Inspection Agency to accept it as food preservative of natural origin. Silver-protein conjugate can even replace antibiotics in the medical sector as *C. krusei* is a notable pathogen for immune-compromised people.

4. Study on the production of bio preservatives using iron oxide and killer protein because iron oxide is not antimicrobial like silver ions and it will not inhibit the useful microorganisms.

5. The study on temperature stability of Ag-KT4561 (silver-protein conjugate) must be carried out. This will enable us to understand the stability of the conjugate at higher temperatures.

6. A brief study of the intracellular and extracellular proteins and enzymes has to be conducted for *S. unisporus* as no study has been conducted so far on it. Apart from

that removal of nucleic acids from *S. unisporus* biomass has to be performed for food applications.

7. Quantitative study of the other intermediates for example organic acids produced from *S. unisporus* are recommended as *S. unisporus* produces beneficial intermediates of biotechnological importance.