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Valorisation du lactosérum par fermentation en une étape en utilisant un coculture des levures pour produire des biomolécules d'arôme et de saveur

Présenté par

Mariana Valdez Castillo
(M. Sc., Environnemental sciences)

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Jury d'évaluation

Président du jury et examinateur interne	Dr. Yann Le Bihan *Chercheur, CRIQ, Québec, Canada*
Examinateur externe	Dr. Mohammed Aider *Professeur, Université Laval, Québec, Canada*
Examinateur externe	Dr. Anaberta Cardador Martínez *Professeure, Tecnológico de Monterrey, Querétaro, Mexique*
Directeur de recherche	Dr. Patrice Couture *Professeur, INRS- ETE*
Codirectrice interne de recherche	Dr. Satinder Kaur Brar *Professeure, York University, Ontario, Canada*
Codirecteur interne de recherche	Dr. Jean François Blais *Professeur, INRS ETE*
Codirectrice externe de recherche	Dr. Sonia Lorena Arriaga Garcia *Professeure, IPICYT-Mexique*

Dédicace

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RÉSUMÉ

Le présent travail de recherche porte sur la valorisation de lactosérum par voie de fermentation en utilisant *Kluyveromyces marxianus* et *Debaryomyces hansenii*, deux levures connues par leur capacité de produire des composés ayant des propriétés d'arôme et de saveur. Le projet de recherche a été divisé en deux parties principales : la première section correspond à la détermination de l'état-de-art concernant la valorisation du lactosérum par des méthodes physiques, thermiques et biologiques. Parmi ces dernières, la fermentation du lactosérum en utilisant des levures a été identifiée parmi les options les plus appropriées. Ceci est attribuable au fait que les levures peuvent produire une grande variété de sous-produits d'intérêt industriel, tels que des alcools à haut masse molaire comme le 2-phényléthanol (2PE). Le 2PE est très apprécié dans les secteurs alimentaire, cosmétique et pharmaceutique et présente un prix de marché élevé. Cependant, sa production par fermentation est affectée principalement par la composition du milieu de culture (type de substrat et présence de la L-phénylalanine (L-Phe) en tant que précurseur), les conditions d'opération de la fermentation (aération, température et pH), l'accumulation des métabolites (éthanol) et les conditions cellulaires (âge et densité).

La deuxième section du projet a été planifiée selon l'état de l'art définie préalablement. La recherche expérimentale a été constituée de trois principaux sujets ayant comme objectif ultime le développement d'un bioprocédé pour produire du 2PE par fermentation de déchets agroalimentaires. Le recherche expérimentale a été développée comme suit : 1) une première étape afin d'identifier les principales biomolécules d'arôme et de saveur qui sont produites lors de la fermentation du lactosérum avec les levures *K. marxianus* et *D. hansenii* utilisées soit sous mode de monoculture, soit de co-culture; dans cette étape ont été déterminées les meilleurs conditions d'opération pour produire du 2PE à l'échelle laboratoire, 2) l'étude de l'effet de la densité cellulaire

de levures sur la production du 2PE; dans cette étape les modes de culture en suspension et de cellules immobilisées sur des supports ont été analysées et comparées réalisant la fermentation dans des fioles et en bioréacteur, et 3) l'étude de faisabilité technique de l'utilisation des biomasses résiduelles comme source renouvelable des précurseurs d'alcools de haute masse molaire en les ajoutant comme supplément pour augmenter la production des alcools lors de la fermentation du lactosérum; dans cette étape a été étudiée l'utilisation de sources renouvelables et peu dispendieuses riches en protéines afin d'avoir une incidence sur la rentabilité de la production de 2PE et de diminuer l'empreinte environnementale du procédé.

Les résultats ont montré que la fermentation aérobie du lactosérum à l'aide de levures co-cultivées améliore les paramètres cinétiques du 2PE par rapport à l'utilisation de monocultures. Ensuite, l'étude de l'effet de la concentration initiale de lactose et de L-Phe sur la production de 2PE a été réalisée. Un rendement de 0,81 $g_{2PE}/g_{L\text{-}Phe}$ a résulté de l'utilisation de 15,9 $g_{Lactose}$/L et 3 $g_{L\text{-}Phe}$/L ce qui représente une valeur compatible aux valeurs les plus élevées rapportées dans la littérature (0,78 $g_{2PE}/g_{L\text{-}Phe}$).

L'augmentation de la densité cellulaire par l'immobilisation des levures sur des supports inertes a conduit à une augmentation de la productivité de 2PE jusqu'à 9,45 ± 0,17 mg/L*h par rapport aux cellules en suspension de 6,64 ± 0,00 mg/L*h. Ceci a été attribué à la formation d'un biofilm de levure sur le support inerte, qui pourrait offrir une protection contre les alcools accumulés dans le bouillon de culture et permettre une meilleure adaptation des cellules de levure au milieu. À une échelle de fioles, l'ajout de sources naturelles de L-Phe a permis d'obtenir un faible rendement de 2PE avec les résidus de soja de 0,46 ± 0,01 $g_{2PE}/g_{L\text{-}Phe}$. En revanche, le rendement a été élevé avec levure résiduelle de bière (LUB) jusqu'à 2,44 ± 0,01 $g_{2PE}/g_{L\text{-}Phe}$ parce que les souches de levure étaient capables de dégrader les protéines de la LUB et de libérer la L-Phe dans le milieu. Ensuite,

le procédé a été mis à l'échelle dans des bioréacteurs d'un volume total de 2 L dans des conditions d'opération contrôlées et complètement aérobie. La productivité maximale de 2PE observée dans le bioréacteur a été de 38,4 mg/L*h, correspondant à une concentration de 2PE de 1843 mg/L au bout de 48 h de fermentation. Ces résultats montrent que l'utilisation de différents résidus comme matières premières biosourcées est une option prometteuse pour produire des biomolécules selon un mode de production durable. Le bioprocédé développé avec la levure en coculture et LUB dans cette étude a permis de multiplier par 4,7 la production de 2PE selon les données de la littérature (8,1 mg/L*h).

Mots-clés: valorisation du lactosérum, coculture de levures, immobilisation de levure, fermentation de cosubstrats, arômes, saveurs, 2-phényléthanol

ABSTRACT

The objectives of this project are related to the whey valorization *via* fermentation using *Kluyveromyces marxianus* and *Debaryomyces hansenii* to produce compounds with aroma and flavor properties. This project is performed in two steps: the first one is to define the state of the art of whey valorization by exploring both physical-thermal and biological methods. In this context, the yeast fermentation was identified as a suitable option to produce a large variety of byproducts of industrial interest, such as alcohols of high molecular weight like 2-phenylethanol (2PE). The 2PE is highly appreciated in the food, cosmetic and pharmaceutical sectors and presents an elevated market price. However, it was observed that its production by fermentation is mainly affected by the composition of the culture media (substrate and presence of the precursor L-Phe), the operating conditions of fermentation (aeration, temperature and pH), the production of other metabolites (ethanol) and the cellular conditions (age and density).

The second step of the project was planned according to the aforementioned state of art. The experimental research consisted of three main phases with the main objective of developing a bioprocess to produce 2PE by fermentation of agri-food residues: 1) the exploration of whey fermentation using *K. marxianus* and *D. hansenii* yeasts under monoculture and co-culture mode to produce aroma and flavor biomolecules; in this phase, the best conditions for the production of 2PE were determined at laboratory scale, 2) the effect of the yeasts cells immobilization onto inert supports on the production of 2PE was studied; in this phase, the culture in suspension and immobilized on supports developed on flasks were analyzed and compared, 3) the study of the feasibility of use biomass residual such as source of renewable precursors of alcohols with high molecular weight and supplements of whey fermentation to increase the production of alcohols

was performed; in this phase, the use of renewable and low-cost sources of proteins for the 2PE production was performed with the aim to decrease the environmental footprint of the bioprocess.

Results showed that the aerobic whey fermentation using the co-cultured yeasts improved the kinetic parameters of L-Phe rate consumption and production of 2PE in comparison to the use of monocultures. A subsequent optimization of initial concentration of lactose and L-Phe for the 2PE production was performed by response surface methodology. A yield production of 0.81 g_{2PE}/g_{Lphe} at 48 h resulted from the use of 15.9 $g_{Lactose}$/L and 3 $g_{L\text{-}Phe}$/L, showing to be a value compatible with the highest values reported in the literature (0.76 g_{2PE}/g_{Lphe}).

The increment on cellular density by means of the yeasts immobilization on inert supports led to the increment of 2PE productivity from 6.6±0.0 mg/L*h for cells in suspension to 9.5±0.2 mg/L*h for the immobilized yeast cells. This was attributed to the formation of yeast biofilm on the inert support, which could offer protection against the alcohols accumulated on the culture broth and allowed a better yeasts cells adaptation to the media. At flask scale, the use of natural sources of L-Phe let to a low 2PE yield with the soy residues (0.46 ± 0.01 $g_{2PE}/g_{L\text{-}Phe}$). Whereas, the yield was increased up to 2.44 ± 0.01 $g_{2PE}/g_{L\text{-}Phe}$ when brewer's spent yeasts (BSY) were used. This yield is attributed to the degradation of BSY proteins due to the metabolism of yeasts. Then, the process was scaled up to bioreactors of a total volume of 2 L under completely aerobic controlled operating conditions. The maximum 2PE productivity observed in bioreactor was 38.4 mg/L*h, corresponding to a 2PE concentration of 1843.2 mg/L at 48 h of fermentation.

These results demonstrated that the use of different residues as bio-based raw materials is a promising option to produce biomolecules in a sustainable production way. The bioprocess developed with the BSY increased up to 4.7-fold the 2PE production according to the results in the literature (8.1 mg/L*h).

Keywords: whey valorization, yeast co-culture, yeast immobilization, co-substrate fermentation, aromas, flavors, 2-phenylethanol

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TABLE DES MATIÈRES

Dédicace ii

REMERCEMENTS iii

RÉSUMÉ v

ABSTRACT viii

PUBLICATIONS DE CETTE THÈSE xi

CONFERENCES xii

LIST OF TABLES xix

LIST OF FIGURES xxi

LIST OF EQUATIONS xxv

LIST OF ABREVIATIONS AND SYMBOLS xxvi

CHAPPITRE UN: 1

CHAPITRE UN – PARTIE 1. INTRODUCTION 2

CHAPITRE UN – PARTIE 2 : REVUE DE LA LITTÉRATURE 7

Bioraffinage du lactosérum à l'aide de levures pour produire des arômes, des saveurs et des composés antioxydants à valeur ajoutée 7

1.1 Risques environnementaux 9

1.2 Valorisation du lactosérum 10

1.3 Valorisation biologique du lactosérum 12

1.4 Arômes, saveurs et antioxydants 13

1.4.1 Production de composés ASA par des procédés biologiques 17

1.4.2 Fermentation par des bactéries 18

1.4.3 Fermentation fongique 22

1.4.4 Fermentation par des levures 26

1.5 Technologies d'extraction et de purification du 2-phényléthanol 32

1.6 Applications sur le marché 33

CHAPITRE UN – PARTIE 3: PROBLÉMATIQUE 35

3.1 Les procédés actuels de valorisation du lactosérum sont coûteux 35

3.2 La fermentation du lactosérum nécessite des étapes de conditionnement pour permettre la croissance des microorganismes et de production des métabolites 35

3.3 La production de 2-phényléthanol dépend fortement de la composition du milieu 36

3.4 L'accumulation d'alcools dans le bouillon de culture peut diminuer la productivité du 2-phényléthanol par les levures 36

3.5 La production de 2-phényléthanol nécessite la présence de l'acide aminé précurseur (L-Phe) dans le milieu de culture ..... **Error! Bookmark not defined.**

3.6 La production de 2-phényléthanol dépend fortement des conditions de fermentation et du type de métabolisme des levures 37

CHAPITRE UN – PARTIE 4. HYPOTHÈSES 38

CHAPITRE UN – PARTIE 5. OBJECTIFS 41

CHAPITRE UN – PARTIE 6. ORIGINALITÉ 42

CHAPITRE UN – PARTIE 7. SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE .....**Error! Bookmark not defined.**

7.1 Production of aroma and flavor-rich fusel alcohols by cheese whey fermentation using the *Kluyveromyces marxianus* and *Debaryomyces hansenii* yeasts in monoculture and co-culture modes.....**Error! Bookmark not defined.**

7.2 Effect of the concetration of L-Phenylalanine and lactose on 2-phenylethanol production by whey fermentation using under *Kluyveromyces marxianus* and *Debaryomyces hansenii* co-culture mode .....**Error! Bookmark not defined.**

7.3 Effect of passive cell immobilization of co-cultures yeasts on the whey fermentation and alcohols production.....**Error! Bookmark not defined.**

7.4 Production of 2-phenylethanol by co-substrate fermentation of agri-food resdidues using *Kluyveromyces marxianus* and *Debaryomyces hansenii* co-culture mode**Error! Bookmark not defined.**

CHAPTER TWO: 44

CHAPTER TWO – PART 1. 45

Production of aroma and flavor-rich fusel alcohols by cheese whey fermentation using the *Kluyveromyces marxianus* and *Debaryomyces hansenii* yeasts in monoculture and co-culture modes.....45

Résumé 46

Abstract....47

Introduction....48

Material and methodology....50

Results and discussion....55

Conclusions....81

Acknowledgements....82

References....82

CHAPTER TWO – PART 2....88

Effect of the concentration of L-Phenylalanine and lactose on 2-Phenylethanol production by whey fermentation using the yeasts *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode....88

Résumé....89

Abstract....90

Introduction....91

Material and methods....95

Results and discussion....101

Conclusions....123

Acknowledgements....123

References....124

CHAPTER THREE – PART 1....128

Effect of passive cell immobilization of co-cultured yeasts on the whey fermentation and alcohols production....128

Résumé....129

Abstract....130

Introduction....131

Material and methodology....134

Results and discussion....141

Conclusions....164

Acknowledgements....166

References....166

CHAPTER FOUR - PART 1....173

Production of 2-Phenylethanol by co-substrate fermentation of agri-food residues using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode....173

Résumé....174

Abstract....175

Introduction....176

Material and methodology....178

Results and discussion....185

Conclusions....210

Acknowledgements....211

References 211

CONCLUSIONS ET RECOMMANDATIONS 216

CONCLUSIONS 216

RECOMMENDATIONS 217

APPENDIX 219

APPENDIX A (Supplementary Data – Chapter 2, Part 1) 220

APPRENDIX B (Supplementary Data – Chapter 2, Part 2) 227

APPRENDIX C (Supplementary Data – Chapter 3, Part 1) 229

APPRENDIX D (Supplementary Data – Chapter 4, Part 1) 231

LIST OF TABLES

Tableau 1 Types de lactosérum et leur composition 8

Tableau 2 Molécules asa produits par le bioraffinage du lactosérum avec des bactéries 21

Tableau 3 Molécules asa produits par le bioraffinage du lactosérum avec des champignons .. 25

Tableau 4 Molécules asa produits par le bioraffinage du lactosérum avec des levures 29

Tableau 5 Acides aminés essentiels impliqués dans la voie d'ehrlich et produits finaux 31

Table 2.1 Compounds with aroma and flavor properties identified in controls (sterilized and incubated) and metabolites found in culture broth under aerobic and anaerobic conditions 58

Table 2.2 Overall performance of yeasts during the LM, W and WY media fermentations assays using the monocultures. 66

Table 2.3 Two-way ANOVA table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under monoculture mode. Dependent variable: 2-Phenylethanol concentration 72

Table 2.4 Overall performance of yeasts during the fermentations assays for wy media using the monocultures and co-cultures. 74

Table 2.5 Ancova table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. Dependent variable: 2-Phenylethanol concentration 79

Table 2.6 Fermentation of whey using yeasts to produce 2-Phenylethanol 80

Table 2.7 Physicochemical characterization and composition of raw whey 102

Table 2.8 Biomolecules with aroma and flavor properties identified in the culture broth according to the sterilization method used: filtration, pasteurization and autoclave. These are the biomolecules identified at 72 h of fermentation of raw whey using *K. marxianus* and *D. hansenii* under co-culture mode. 107

Table 2.9 Analysis by ANCOVA of the effect of sterilization method on 2-Phenylethanol concentration during the whey fermentation using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. 110

Table 2.10 Levels of lactose and L-Phenylalanine as variables for the central composite design of k^2 model 116

Table 2.11 Levels of lactose and L-Phenylalanine as variables for the central composite design of k^2 model at 48 and 96 h of fermentation. 118

Table 2.12 Values of coefficients for the mathematical model parameters obtained by central composite design for each by-product.. 120

Table 3.1 Physical characteristics of inert supports used for the assays of whey fermentation to immobilize the yeast cells under co-culture mode……142

Table 3.2 Effect of supports on ph and zeta potential of aqueous and culture broth. For pH, it is shown the value before and after adding the support material. 154

Table 3.3 Overall performance of co-cultured yeasts during the fermentation assays of WM medium for suspended and immobilized yeast cells. The table shows the main kinetics parameters of yeast growth rate, consumption of L-Phe and production of main by-products. 160

Table 3.4 Statistical analysis of effect of the support used to immobilize yeast cells on 2-Phenylethanol production. The statistical analysis was done by method of anova of one way.. 163

Table 4.1 Chemical characterization of agri-food residues 186

Table 4.2 Overall performance of yeasts during the fermentation assays for media at flask level 196

Table 4.3 One-way ANOVA of the effect of agro-food residues co-fermentation using a co-culture of yeasts on 2-Phenylethanol production 199

Table 4.4 Overall performance of yeasts during the fermentation assays for WY media in the 2 L bioreactors 201

Table 4.5 One-way ANOVA of the effect of agro-food residues co-fermentation in bioreactors using a co-culture of yeasts on 2-Phenylethanol production.. 210

LIST OF FIGURES

Figure 1.1 Représentation graphique du projet : problèmes de récupération du lactosérum, étapes du projet et principaux points saillants.....6

Figure 1.2 Produits obtenus par le bioraffinage du lactosérum et leurs applications. AA: acides aminés; 2PE: 2-phényléthanol; GRAS: généralement reconnus inoffensifs.....13

Figure 2.1 Biomass production in culture broth of lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY) using *Kluyveromyces marxianus* (a) and *Debaryomyces hansenii* (b) yeasts. Open symbols indicate the aerobic □-LM, ○-W AND △-WY media fermentations. solid symbols indicate the anaerobic ■-LM, ●-W AND▲- WY media fermentations.....63

Figure 2.2 Production of alcohols by *Kluyveromyces marxianus* monocultures during the fermentations of lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W AND △-WY media fermentations. solid symbols indicate the anaerobic ■-LM, ●-W AND▲- WY media fermentations 68

Figure 2.3 Production of alcohols by *Debaryomyces hansenii* monocultures mode during the fermentation of lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W AND △-WY media fermentations. solid symbols indicate the anaerobic ■-LM, ●-W AND▲- WY media fermentations 71

Figure 2.4 Kinetics of biomass production of co-cultures for the cheese whey supplemented with yeast extract and peptone medium (WY) fermentation under aerobic conditions. Symbols indicate the performance of □-1KM:5DH, ■-1KM:2DH, ●-1KM:1DH, ▲-2KM:1DH, AND △-5KM:1DH.....73

Figure 2.5 Growth of (a) *Kluyveromyces marxianus* and (b) *Debaryomyces hansenii* as monoculture and co-culture mode for cheese whey supplemented with yeast extract and peptone (WY) medium-aerobic. Symbols indicate the performance of ▼-KM or DH monoculture, ▽-DH monoculture, □– 1KM:5DH, ■–1KM:2DH, ●– 1KM:1DH, ▲–2KM:1DH, △–5KM:1DH.....75

Figure 2. 6 Production of alcohols during the aerobic cheese whey supplemented with yeast extract and peptone (WY) medium fermentation using the co-cultures. Symbols indicate the performance of □– 1KM:5DH, ■–1KM:2DH, ●–1KM:1DH, ▲–2KM:1DH, △–5KM:1DH. 77

Figure 2.7 Growth of yeasts and alcohols production as co-culture mode and aerobic conditions for the fermentation of raw whey medium. growth is expresses as cell density (CD) as a function of time for (a) *Kluyveromyces marxianus* and (b) *Debaryomyces hansenii*. The cellular concentration is expressed as the logarithm of the ratio of cd at any time by the initial cellular density (CD_0). concentration of alcohols: c) 2-Phenylethanol (close symbols) and Isopentanol (open symbols), and d) Ethanol. The sterilization methods are identified as follows: ■–filtration, ●–pasteurization and ▲–autoclave. 105

Figure 2.8 Effect of initial concentration of lactose and L-Phenylalanine on the concentration of fusel alcohols in the culture broth for the fermentation of raw whey: a) 2-Phenylethanol at 48 h, b) 2-Phenylethanol at 96 h, and c) Isopentanol at 48 h of fermentation. 117

Figure 3.1 Photographies of biochar support showing: a) whole particle, b) zoom of clean surface by sem micrography, and c) zoom of support with immobilized cells by sem micrography. 143

Figure 3.2 Photographies of perlite support showing: a) whole particle, b) zoom of clean surface by sem micrography, and c) zoom of support with immobilized cells by sem micrography. 145

Figure 3.3 Photographies of filter Kaldnes plastic rings showing: a) whole particle, b) zoom of clean surface by sem micrography, and c) zoom of support with immobilized cells by sem micrography. 147

Figure 3.4 XRD spectra of clean supports: a) biochar, b) perlite and c) filter Kaldnes plastic rings. 148

figure 3. 5 FTIR spectra of clean supports: a) biochar, b) perlite and c) filter kaldnes plastic rings; and supports after being in contact with culture broth: d) biochar, e) perlite and f) filter Kaldnes plastic rings 151

Figure 3.6 Biomass concentration as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture. 156

Figure 3.7 Lactose concentration (continued lines) and ethanol concentration (dashed lines) as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture.....158

Figure 3.8 L-Phenylalanine concentration (a) and 2-Phenylethanol concentration (b) as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture. 162

Figure 4.1 Growth of a) *Kluyveromyces marxianus* and b) *Debaryomyces hansenii* as co-culture mode for whey medium-aerobic. Symbols indicate the co-culture growth for the ■-whey medium-control, ▼-whey medium supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●-whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts. 189

Figure 4.2 Production of proteins during the aerobic whey medium fermentation using the co-culture of yeasts. Symbols indicate the fermentation of ■-whey medium-control, ▼-whey medium supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●-whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts. 191

Figure 4.3 a) Lactose consumption; b) Ethanol production; c) L-Phenylalanine consumption; and d) 2-Phenylethanol production during the aerobic whey fermentation using the co-culture of yeasts. Symbols indicate the fermentation of ■-whey medium-control, ▼-whey medium supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●-whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts. 193

Figure 4.4 Kinetics of WM and WBSY fermentation. Growth of a) *Kluyveromyces marxianus* and b) *Debaryomyces hansenii* as co-culture mode for whey medium-aerobic in bioreactors. c) Lactose consumption and ethanol production during the whey medium-aerobic in bioreactors, and d) Soluble nitrogen consumption. Closed symbols indicate the consumption of total organic carbon and lactose consumption for the fermentation of ■-WM-control, and ▲-WBSY. Open symbols indicate the ethanol production for the fermentation of □-WM-control, and △- WBSY. 203

Figure 4.5 a) Total organic consumption, and b) Production of proteins during the whey medium-aerobic in bioreactors. Symbols indicate the fermentation for the ■-WM-control, and ▲-WBSY. 205
Figure 4.6 a) L-Phenylalanine concentration change, and b) 2-Phenylethanol production during the whey medium-aerobic in bioreactors. Symbols indicate the co-culture performance for the fermentation of ■-WM-control, and ▲-WBSY.....207

LIST OF EQUATIONS

$Y = B0 + B1X1 + B2X2 + B12X1X2 + B11X12 + B22X22$ ...**EQUATION 1**..... 100

$Y48H = 2.098 - 0.033 \cdot X1 + 0.317 \cdot X2 + 0.110 \cdot X1X2 - 0.069 \cdot X12 + 0.061 \cdot X22$ … **EQUATION 2**..... 121

$Y96H = 2.246 + 0.136 \cdot X1 - 0.041 \cdot X2 + 0.221 \cdot X1X2 - 0.058 \cdot X12 + 0.136 \cdot X22$ …**EQUATION 3**..... 121

$M = 1XA \cdot DXADT$ ... **EQUATION 4**..... 180

$DSDT = CINITIAL - CFINALELAPSED\ TIME\ OF\ FERMENTATION$ ... **EQUATION 5**..... 225

$DPDT = CMAXIMUMELAPSED\ TIME\ OF\ FERMENTATION$ ... **EQUATION 6** 226

$YPRODUCT = CMAXIMUMCINITIAL - CFINAL$ ... **EQUATION 7**..... 226

$L - PHE = ((C1 \cdot (500\ ML/1E - 6\ MLML))/2/3) \cdot 50 \cdot 35 \cdot MWL - PHE \cdot 1000\ MG/G \cdot 2$ ... **EQUATION 8** .. 230

LIST OF ABREVIATIONS AND SYMBOLS

1KM:5DH	Co-culture of KM and DH yeasts at a of ratio 1:5
1KM:2DH	Co-culture of KM and DH yeasts at a of ratio 1:2
1KM:1DH	Co-culture of KM and DH yeasts at a of ratio 1:1
2Hfuranone :	2(3H)-furanone,dihydro-4-hydroxy
2KM:1DH	Co-culture of KM and DH yeasts at a of ratio 2:1
2PE :	2-Phenylethanol
3D6Mpyranone :	3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one
4HDfuranone :	4-hydroxy-2,5-dimethyl-3(2H)-furanone
5Hfurfural :	5-hydroxymethylfurfural
5KM:1DH :	Co-culture of KM and DH yeasts at a of ratio 5:1
AA :	Amino acid
BA :	Butanoic acid
BSY :	Brewer's spent yeasts
CD :	Cellular density
COD :	Chemical oxygen demand
WP :	Cheese whey powder
CFU :	Colony Forming Units
DH :	*Debaryomyces hansenii* yeast strain Y-1408
DHA :	Dihydroxyacetone
GLY :	Glycerol
GC :	Gas chromatography
HPLC :	High-Performance Liquid Chromatography
KM :	*Kluyveromyces marxianus* yeast strain Y-1109
LC-MS/MS :	Liquid chromatography-tandem mass spectrometry
LC-HRMS :	Liquid chromatography – high resolution mass spectrometric
LM :	Lactose medium
L-Phe :	L-Phenylalanine
OD_{600} :	Optical Density at a wavelength of 600 nm
MRP :	Maillard reaction products
PA :	Propanoic acid

W : Cheese whey powder diluted in water to reach a lactose concentration of 45 g/L

WY : Cheese whey powder diluted in water to reach a lactose concentration of 45 g/L enriched with yeast extract and peptone

WHC : Culture medium composed of diluted cheese whey 20 $g_{Lactose}$/L, yeast extract 10 g/L, peptone 20 g/L, and hydrolyzed and crab headshells 0.9 g/L

WHS : Culture medium composed of of diluted cheese whey 20 $g_{Lactose}$/L, yeast extract 10 g/L, peptone 20 g/L, and hydrolyzed soy residue 2 g/L

WHBSY : Culture medium composed of of diluted cheese whey 20 $g_{Lactose}$/L, yeast extract 10 g/L, peptone 20 g/L, hydrolyzed brewer's spent yeasts 0.9 g/L

WM : Cheese whey diluted in water to reach a lactose concentration of 20 g/L. It is enriched with, L-Phenylalanine 3 g/L, yeast extract 10 g/L, and peptone 20 g/L

WMB : Whey medium fermentation using the co-culture immobilized on biochar (20 $g_{Lactose}$/L, 10 $g_{yeast\ extract}$/L, 20 $g_{peptone}$/L, and 3 $g_{L\text{-}Phenylalanine}$/L)

WMC : Whey medium fermentation using the co-culture in suspension (20 $g_{Lactose}$/L, 10 $g_{yeast\ extract}$/L, 20 $g_{peptone}$/L, and 3 $g_{L\text{-}Phenylalanine}$/L)

WMF : Whey medium fermentation using the co-culture immobilized on filter Kaldnes plastic rings (20 $g_{Lactose}$/L, 10 $g_{yeast\ extract}$/L, 20 $g_{peptone}$/L, and 3 $g_{L\text{-}Phenylalanine}$/L)

WMP : Whey medium fermentation using the co-culture immobilized on perlite (20 $g_{Lactose}$/L, 10 $g_{yeast\ extract}$/L, 20 $g_{peptone}$/L, and 3 $g_{L\text{-}Phenylalanine}$/L)

WNC : Culture medium composed of diluted cheese whey 20 $g_{Lactose}$/L, L-Phenylalanine 3 g/L, yeast extract 10 g/L, peptone 20 g/L, and crab headshells 0.9 g/L

WNS : Culture medium composed of diluted cheese whey 20 $g_{Lactose}$/L, L-Phenylalanine 3 g/L, yeast extract 10 g/L, peptone 20 g/L, and and soy residue 2 g/L

WNBSY : Culture medium composed of diluted cheese whey 20 $g_{Lactose}$/L, L-Phenylalanine 3 g/L, yeast extract 10 g/L, peptone 20 g/L, and and brewer's spent yeasts 0.9 g/L

WBSY : Culture medium composed of diluted cheese whey 20 $g_{Lactose}$/L, brewer's spent yeasts 61.11 g/L and L-Phenylalanine

μ : Growth rate

CHAPITRE UN:

Synthèse

CHAPITRE UN – PARTIE 1. INTRODUCTION

Ce travail présente les résultats d'une recherche bibliographique et expérimentale sur la valorisation du lactosérum pour produire du 2-phényléthanol (2PE) en utilisant les levures *Kluyveromyces marxianus* et *Debaryomyces hansenii*. La première partie de la recherche est la revue de la littérature et détermine l'état de l'art de la production et de la valorisation du lactosérum. Les types de lactosérum, la génération globale de lactosérum et les méthodes physico-thermiques et biologiques de valorisation sont discutés.

Le lactosérum est l'un des principaux sous-produits agro-industriels générés par la production de fromage et de yogourt. En 2021, le Canada a produit environ $4{,}4 \times 10^6$ tonnes de lactosérum, ce qui correspond à 2,23 % de la production mondiale de lactosérum. De même, la province du Québec, avec $2{,}24 \times 10^6$ tonnes de lactosérum par an, représente 51% de la production canadienne (Les Producteurs de lait du Québec, 2021). Le lactosérum est un résidu liquide obtenu par l'élimination des protéines de masse molaire élevé contenues dans le lait (comme la caséine) (Panesar et Kennedy, 2012). Environ 9 kg de lactosérum sont produits comme sous-produit pour chaque kg de fromage, et 2 kg de lactosérum pour chaque kilo de yaourt grec (Anand et al., 2013; Kyle et Amamcharla, 2016). Le type de lactosérum est défini par le procédé de transformation du lait duquel il est généré. Sur le plan de la matière sèche totale, le lactosérum est principalement composé de lactose (33 - 52 g/L), de protéines (5 - 15 g/L) et d'acide lactique (1,4 - 4,7 g/L), qui contribuent à sa demande chimique en oxygène (DCO) de 60 à 80 g O_2/L et à son bas pH de 3,9 à 5,6. Ainsi, le volume de lactosérum généré, son pH et sa DCO représentent un problème de gestion pour les industries laitières qui doivent traiter le lactosérum avant son rejet dans l'environnement ou dans les réseaux d'égouts.

CHAPITRE UN: SYNTHESE

Un domaine de recherche émergent est la valorisation du lactosérum. Par exemple, l'optimisation des méthodes physiques et thermiques telles que la filtration par membrane et le séchage par pulvérisation sont toujours d'actualité comme des domaines de recherche en lien avec la transformation et la valorisation du lactosérum. Ces méthodes sont utilisées pour concentrer les nutriments du lactosérum afin de produire des formules commerciales pour les athlètes, les enfants ou les produits alimentaires supplémentaires (Kosikowski, 1979; Panesar et Kennedy, 2012). Cependant, elles sont énergivores et par conséquent coûteuses. Comme alternative, la fermentation du lactosérum en utilisant des bactéries, des champignons et des levures sont des options de valorisation économiques et prometteuses pour obtenir des biocarburants, des probiotiques, des détergents, etc. Parmi celles-ci, la fermentation de levure produit des biomolécules à valeur ajoutée telles que des arômes, des saveurs et des antioxydants sans nécessiter de conditionnement préalable du lactosérum, tel que l'ajustement du pH, l'hydrolyse du lactose ou l'enrichissement avec d'autres sources de carbone (Dragone et al., 2009; Leclercq-Perlat et al., 2004; Löser et al., 2015a). La fermentation du petit-lait, la voie d'Ehrlich pour transformer les acides aminés en alcools de fusel et la production de 2PE sont abordées dans ce projet. Les alcools de fusel ont des propriétés aromatiques et gustatives, comme le 2PE, qui est très apprécié dans les secteurs alimentaire, cosmétique et pharmaceutique en raison de ses propriétés antimicrobiennes, de son arôme de rose et de sa saveur de miel (The Flavor and Extract Manufacturers Association of the United States (FEMA), 2005).

La revue de la littérature montre que les principaux facteurs qui affectent la bioproduction de 2PE sont la présence de L-Phe (précurseur du 2PE), le ratio entre la source de carbone et le L-Phe, la concentration de la biomasse et l'accumulation des métabolites. En conséquence, la bioproduction

de 2PE doit relever plusieurs défis pour être compétitive par rapport aux méthodes chimiques actuelles. Par exemple, le prix du marché du 2PE d'origine chimique est de 3,50 $/kg, tandis que celui du 2PE d'origine naturelle est de 100 $/kg (Etschmann et al., 2002; Fuzhou Farwell company, 2020; Jiangsu Juming Chemical Process Technology Co., 2020). L'utilisation de L-Phe renouvelable biosourcé permet d'augmenter la pertinence de la bioproduction de 2PE comme réponse au développement d'un bioprocédé compétitif. La L-Phe biosourcée devrait permettre d'obtenir du 2PE comme principal alcool de fusel. De plus, l'extraction et la purification du 2PE à partir de la culture doivent être revues pour développer de nouvelles méthodes qui répondent au défi de l'énergie, de la consommation et du nombre d'opérations unitaires. Cela permettra d'accroître la faisabilité technico-économique de l'ensemble du bioprocédé.

En fonction des défis mentionnés pour développer un bioprocédé faisable du point de vue technico-économique pour la valorisation du lactosérum, cette thèse a été divisée en quatre chapitres comme suit : le **chapitre 1** correspond à l'introduction, où l'état de l'art est défini, et les principaux problèmes pour la valorisation du lactosérum et sa fermentation son discutés. La revue de littérature a permis d'identifier les points faibles pour la fermentation du lactosérum utilisant des levures et des essais expérimentaux décrits dans les chapitres 2 à 4 ont été proposés afin de remédier ces lacunes du savoir scientifique et technologique. Le **chapitre 2** décrit l'étude de la fermentation du lactosérum en utilisant les levures non conventionnelles en mode monoculture et co-culture. La plage des conditions d'opération pour produire de 2PE a déterminée, spécialement pour la concentration initiale de lactose et de L-Phe. Le **chapitre 3** analyse l'effet de l'immobilisation des cellules de levure sur la production de 2PE. Finalement, dans le but d'améliorer le potentiel de valorisation du lactosérum, le **chapitre 4** montre que la fermentation peut être réalisée en utilisant une source d'azote biosourcée et renouvelable pour enrichir le lactosérum. Dans ce dernier chapitre

est discuté aussi l'effet de l'aération sur la production de 2PE et la fermentation de cosubstrats (résidus agroalimentaires) dans des bioréacteurs de 2 L. Le fil conducteur entre les chapitres est illustré de façon schématique dans la **Figure 1.1**. Les étapes expérimentales que permettent de surmonter les principaux problèmes techniques sont montrées, indiquant les faits saillants.

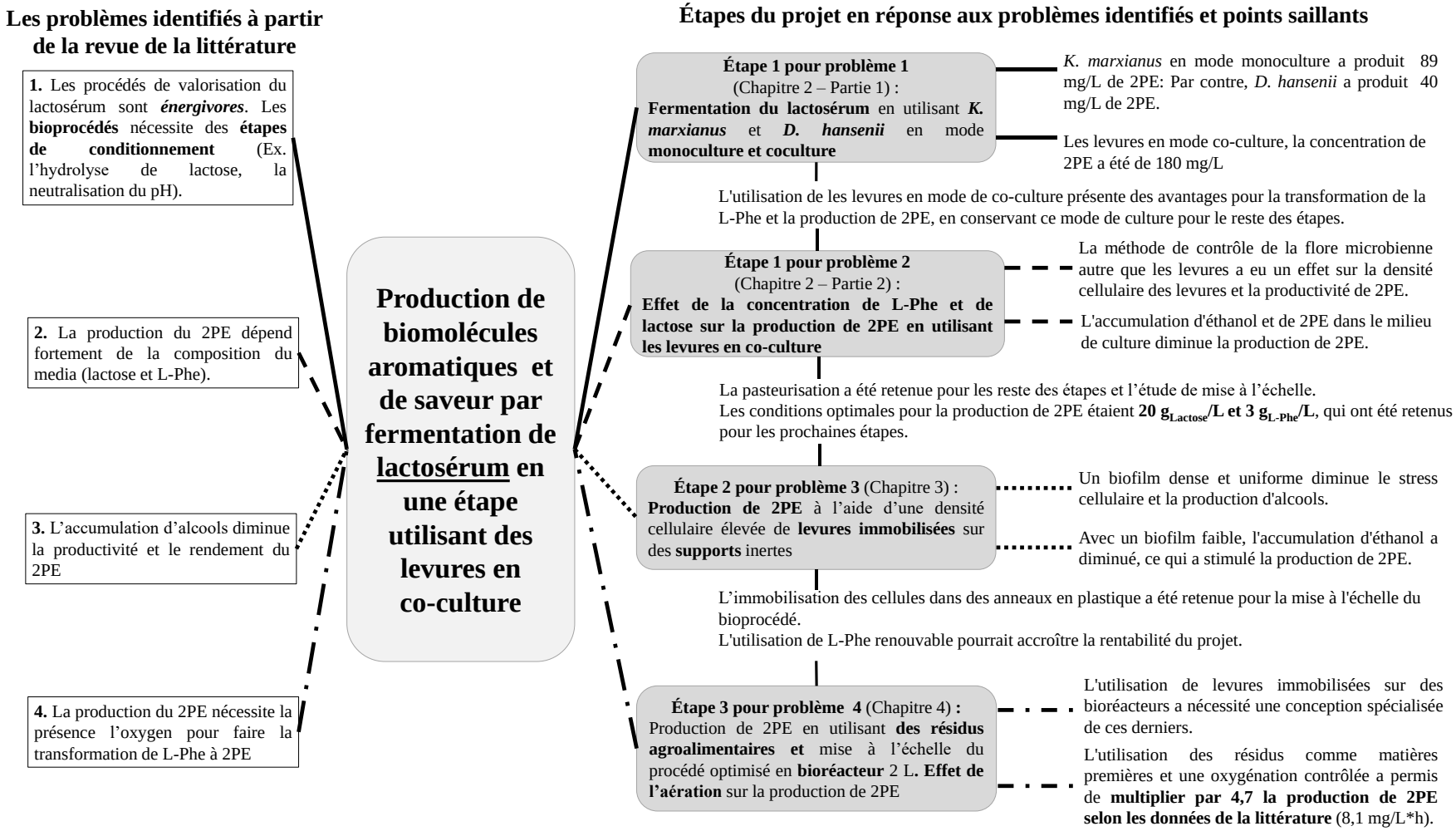

Figure 1. 1 Représentation graphique du projet : problèmes de récupération du lactosérum, étapes du projet et principaux points saillants.

CHAPITRE UN – PARTIE 2 : REVUE DE LA LITTÉRATURE

Bioraffinage du lactosérum à l'aide de levures pour produire des arômes, des saveurs et des composés antioxydants à valeur ajoutée

Ce chapitre est une mise à jour de l'état de l'art publié dans un article de synthèse au journal «***Critical Reviews in Biotechnology***» (Vol. 40, Issue 7, Pages 1–21. doi.org/10.1080/07388551.2020.1792407).

Le lactosérum est l'un des principaux sous-produits agro-industriels générés par l'industrie laitière, en particulier par la production de fromage et de yaourt. Le lactosérum est un résidu liquide obtenu par l'élimination des protéines de haute masse molaire contenues dans le lait (comme la caséine) (Panesar et Kennedy, 2012). Il représente 85 à 90% du volume initial du lait, y compris les protéines solubles de plus faible masse molaire (Kao et al., 2003).

Cela représente une perte importante de nutriments puisque le lactosérum conserve environ 55% des nutriments du lait (Jesus et al., 2015; Panesar et Kennedy, 2012). Il est principalement composé de lactose (45 – 50 g/L), d'acide lactique (1,4 – 5,0 g/L) et de protéines de lactosérum (5 – 15 g/L) (Anand et al., 2013; Tavares et al., 2012). Le type de lactosérum (acide ou doux) et sa composition varient en fonction de la technique de transformation utilisée dans la production de fromage et de yaourt. La présence d'acides organiques détermine son niveau de pH acide et contribue à sa faible alcalinité (inférieure à 0,22 g en $CaCO_3$/L) (Slavov, 2017; Smithers, 2015). Le Tableau 1 montre la composition de trois types de lactosérum.

Lactosérum acide

Le lactosérum acide résulte des procédés de coagulation du lait par fermentation ou addition d'acides organiques (Panesar et Kennedy, 2012). Lorsque de l'acide sulfurique ou tout autre acide

minéral est utilisé pour coaguler le lait, on parle de lactosérum industriel, qui possède toutes les propriétés du lactosérum acide (Panesar et Kennedy, 2012). Le lactosérum acide est également produit par l'acide lactique libéré par les bactéries lactiques (BAL), qui détermine le pH acide final du lactosérum et sa valeur de DCO résiduelle. Avec la diminution du pH, les protéines du lait précipitent et le caillé se forme. Les BAL métabolisent également les protéines du lait, ce qui entraîne une concentration plus faible de protéines dans le lactosérum acide obtenu par des méthodes biologiques. La solubilité des minéraux du lait augmentant avec l'acidité, la teneur en minéraux est plus élevée dans le lactosérum acide que dans les autres types de lactosérum (Tableau 1) (Villarreal, 2017).

Tableau 1 Types de lactosérum et leur composition

Paramètres	Lactosérum		
	Acide (Anand et al., 2013; Panesar et Kennedy, 2012; Smithers, 2015)	**Yaourt grec** (Kyle et Amamcharla, 2016; Smith et Grove, 2018)	**Doux** (Anand et al., 2013; Panesar et Kennedy, 2012; Smithers, 2015)
Eau (%)	95,5	93	93,3
pH	3,9 - 4,5	4,45	>5,6
Solides totaux (g/L)	64,2 - 70,0	50,0 – 60,0	63,0 - 70,0
DCO (g O_2/L)	~80	60	>60
Lactose (g/L)	44,0 - 46,0	33,6 - 41,1	46,0 - 52,0
Acide lactique (g/L)	4,7	4,0 - 5,0	1,4
Protéines totales (g/L)	2,42 - 5,3	1,77 - 4,6	6,0 - 10
Caséine (g/L)	0,42 - 0,78	NR	0,36 - 0,54
Azote inorganique (mg/g)	1,19	NR	1,3
Phosphate (g/L)	2,0 - 4,5	0,07 ± 0,005	1,0 - 3,0
Calcium (mg/g)	0,93 - 1,6	1,2-1,5	0,36 - 0,6
Zinc (µg/kg)	2 340	NR	110
Gras (g/L)	1	0,3-15,6	1
Cendres (g/L)	6	7,2	5,2

NR = non rapporté

CHAPITRE UN: SYNTHESE

Lactosérum de yaourt grec

La production de yaourt grec diffère de la production de fromage en ce qui concerne la préparation du lait. Pour fabriquer le yaourt grec, le lait est chauffé à des températures de 90 à 95°C pendant 5 – 10 min (International Dairy Federation, 2022). La chaleur précipite les protéines du lait, après quoi les deux phases sont homogénéisées et refroidies avant d'ajouter la culture de départ. Le yaourt est fabriqué par fermentation du mélange homogène avec une bactérie telle que *Lactobacillus sp*. ou *Streptococcus sp*. (Anand et al., 2013). Le mélange fermenté est épaissi et l'on obtient le lactosérum de yaourt grec, qui contient les 2/3 des composés du lait (Kyle et Amamcharla, 2016).

Lactosérum doux

Ce lactosérum doux est produit par l'ajout d'enzymes de type chymosine, également appelées complexe présure. Les micelles du lait ont une charge électrique négative externe et la chymosine est une protéase capable de couper les protéines des micelles. Par conséquent, les protéines de caséine floculent pour former le fromage blanc. Dans ce cas, le lactose n'est pas transformé en acide lactique (Anand et al., 2013).

Les micelles de caséine, qui sont chimiquement déstabilisées par la chymosine, entraînent une concentration de glycomacropeptides dans le lactosérum restant. Cependant, cela diminue la teneur des protéines β-lactoglobuline et α-lactalbumine. La teneur et le profil des acides aminés (AA) changent également en fonction du type de lactosérum (Anand et al., 2013). Le lactosérum doux est limité en AA essentiels par rapport au lactosérum acide.

1.1 Risques environnementaux

Le lactosérum représente un problème environnemental en raison de sa teneur élevée en DCO, de 60 à 85 g/L (Anand et al., 2013; Panesar et Kennedy, 2012; Smithers, 2015). Lorsqu'il est rejeté

dans l'environnement, il peut causer de graves problèmes de pollution. Lorsque le lactosérum est répandu sur le sol, il affecte la structure physique et chimique du sol, augmentant la teneur en azote, en phosphate et en minéraux (Slavov, 2017; Smithers, 2015). Le lactosérum peut induire la lixiviation des métaux lourds et augmenter leur biodisponibilité, ainsi que la bioaugmentation dans la chaîne trophique, en raison de son pH acide (Smithers, 2015). Le lactosérum rejeté dans les masses d'eau induit un changement dans les cycles biogéochimiques du phosphore, du fer, de l'azote et du carbone en raison de la modification de l'équilibre redox. Cela peut appauvrir l'oxygène dissous et déclencher l'eutrophisation (Panesar et Kennedy, 2012).

Environ 9 kg de lactosérum (Anand et al., 2013) sont produits comme déchets par chaque kilo de fromage, et 2 kg de lactosérum (Kyle et Amamcharla, 2016) par 1 kg de yaourt grec. Parallèlement, la production volumétrique de lactosérum a augmenté au cours des dernières années à un taux moyen de 3,8% de 2014 à 2018 (FAO, 2019; Food and Agriculture Organization of the United Nations, 2017) en raison de l'augmentation de la demande de produits laitiers (Smithers, 2015). Par conséquent, une quantité totale de $2{,}31 \times 10^7$ tonnes de lactosérum a été produite par la production mondiale de fromage en 2018.

1.2 Valorisation du lactosérum

La valorisation est définie comme un processus qui transforme les déchets par des méthodes physiques, thermiques, chimiques ou biologiques pour créer des produits qui peuvent être incorporés dans les chaînes de production, y compris dans le cadre de l'économie circulaire. L'utilisation de procédés de séchage par atomisation ou évaporation et de filtration sur membrane (ultrafiltration et osmose inverse) sont les méthodes les plus courantes pour accomplir la valorisation du lactosérum. Ces méthodes visent à concentrer le lactose, les protéines ou les nutriments présents dans le lactosérum, ce qui conduit à la production de poudre de lactosérum, de

lactose et de préparations pour nourrissons et sportifs (Aguirre-villegas et al., 2011; Das et al., 2016; Domínguez-Puerto et al., 2018; Khaire et Gogate, 2018; Singh et Banerjee, 2013). En conséquence, 13,5% du lactosérum produit au Canada est exporté sous forme de produits laitiers et 11,2% est consommé sous forme de poudre de lactosérum, de lactose, de préparations pour nourrissons, de préparations pour sportifs ou d'additifs alimentaires, le reste étant détourné vers d'autres usages, tels que l'alimentation animale (Food and Agriculture Organization of the United Nations, 2017).

Cependant, l'un des principaux problèmes qui entravent la valorisation du lactosérum acide et du lactosérum de yaourt grec est sa forte teneur en acide lactique qui augmentent la solubilité du lactose dans le lactosérum. Ceci empêche l'élimination de l'acide lactique par filtration ou cristallisation du lactose (Chandrapala et al., 2017). De plus, en raison de sa faible teneur en lactose et de son pH peu élevé, le lactosérum de yaourt grec n'est pas un lactosérum approprié pour être utilisé comme engrais agricole, aliments transformés et aliments pour animaux comme le lactosérum généré par la production de fromage (Smith et Grove, 2018). Un autre inconvénient de la concentration physique du lactosérum est le colmatage des membranes de filtration, et la nécessité d'un traitement supplémentaire pour détacher les protéines et les nutriments, ce qui augmente les coûts de production (Anand et al., 2013; Tejayadi et Cheryan, 1995). L'évaporation sous vide de l'eau du lactosérum à 60 - 95°C entraîne une forte consommation d'énergie, ainsi que la perte et la dégradation des composés du lactosérum (Tejayadi et Cheryan, 1995). Par exemple, les protéines sont dénaturées, ce qui diminue la valeur nutritionnelle du lactosérum et la qualité du produit final (Sánchez-Oliver et al., 2018). Il est donc nécessaire d'ajouter des AA essentiels (acide glutamique, leucine, arginine et acide aspartique), ce qui rend le procédé non rentable (Amorim et

al., 2018). Ces inconvénients demandent le développement de méthodes alternatives pour accomplir la rentable valorisation du lactosérum.

1.3 Valorisation biologique du lactosérum

Les traitements biologiques du lactosérum présentent plusieurs avantages par rapport aux méthodes physiques et thermiques. Ils sont réalisés dans des conditions d'opération ambiantes, ils utilisent des microorganismes pour produire des composés à valeur ajoutée et pour diminuer la teneur en DCO du lactosérum, ce qui les rend potentiellement réalisables, économiques et respectueux de l'environnement. La réduction du coût de transformation du lactosérum en fait une alternative intéressante pour valoriser le lactosérum, qui peut également être converti en produits de marché émergents. Les groupes de microorganismes utilisés pour réaliser la fermentation du lactosérum, les sous-produits et leurs applications sont présentés à la Figure 1.2. Les procédés biologiques sont prometteurs pour la valorisation du lactosérum en raison de leur capacité à métaboliser le lactose et les nutriments du lactosérum, en les transformant en produits d'intérêt industriel.

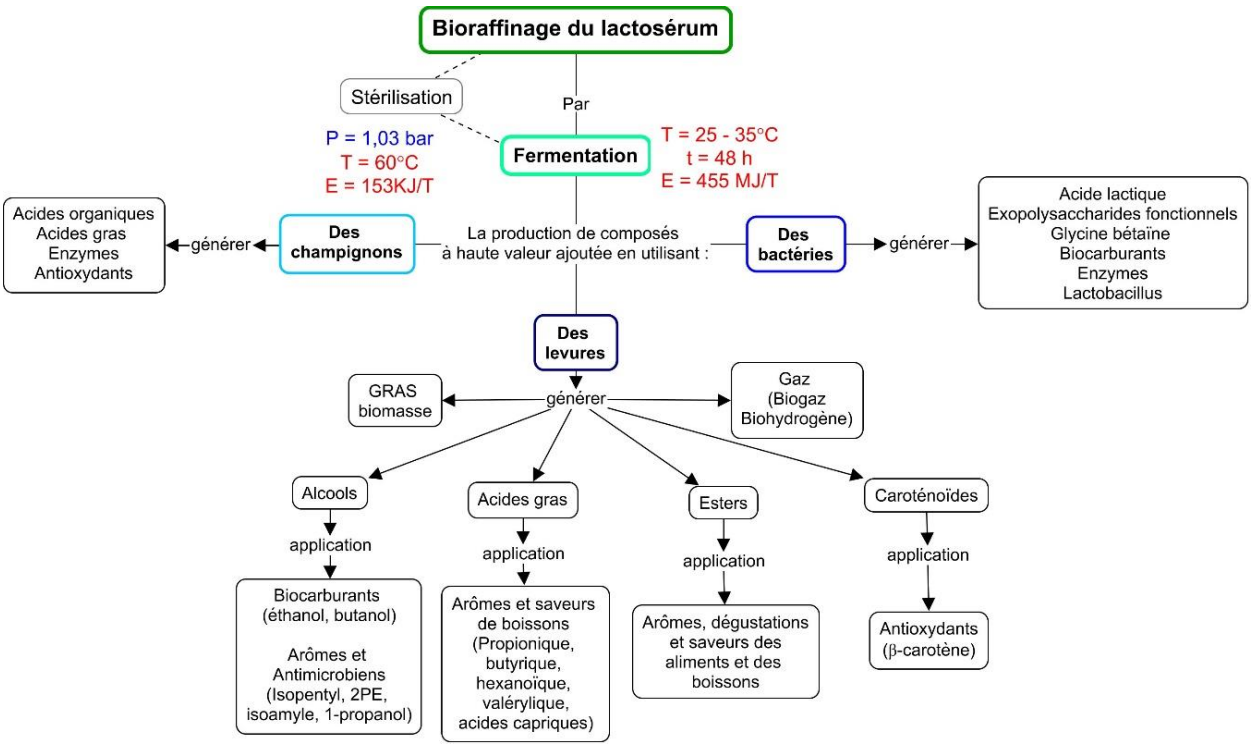

Figure 1. 2 Produits obtenus par le bioraffinage du lactosérum et leurs applications. AA: acides aminés; 2PE: 2-phényléthanol; GRAS: Généralement reconnus inoffensifs

1.4 Arômes, saveurs et antioxydants

Les arômes, saveurs et antioxydants (ASA) sont des molécules à haute valeur ajoutée, consommés en grande quantité par les industries alimentaire, cosmétique et pharmaceutique. Ils sont utilisés pour améliorer les aspects organoleptiques des produits finis. Ils sont considérés comme des composés GRAS (Généralement reconnus inoffensifs, pour son acronyme en anglais), ce qui signifie qu'ils ne sont pas toxiques et peuvent être utilisés dans toutes sortes de produits ingérés ou appliqués sur la peau. Les molécules ASA possèdent des fonctionnalités chimiques spécifiques, qui sont considérées en les regroupant dans des classes bien définies selon le système de Beilstein, comme les alcools acycliques, isocycliques et hétérocycliques (Bertuzzi et al., 2018; Reuss et al., 2012).

CHAPITRE UN: SYNTHESE

Les groupes fonctionnels d'ASA les plus courants sont les alcools, les lactones, les aldéhydes, les cétones, les acides gras, les esters, les phénols et les composés organosulfurés, et la plupart d'entre eux sont des composés organiques volatils (COV) (Bertuzzi et al., 2018; Hazelwood et al., 2008). Les interactions et la réactivité des COV et des autres composés présents dans leur proximité sont les principaux facteurs qui affectent la stabilité de l'arôme et de la saveur dans les produits finis (Weerawatanakorn et al., 2015). Plusieurs de ces COV et autres biomolécules valorisées sont susceptibles de s'oxyder. Cette réaction peut être limitée par l'utilisation d'antioxydants. Leur utilisation préserve les arômes et les saveurs des produits finis, tels que les cosmétiques ou les produits pharmaceutiques (Santos et al., 2012; Siddaiah et al., 2006)(Santos et al., 2012; Siddaiah et al., 2006).

Les antioxydants ont également un effet bénéfique sur la santé animale et humaine ; ils peuvent prévenir le vieillissement prématuré, la modification des protéines, les cataractes ou le cancer, entre autres affections (Blanco et Blanco, 2017). Les microorganismes présentent un rendement plus élevé que les plantes ou les animaux, et leur utilisation évite la génération de sous-produits toxiques (Prazeres et al., 2012). Plusieurs microorganismes peuvent être utilisés pour produire des ASA, car ils ne sont pas pathogènes et produisent une grande quantité d'ASA sans nécessiter de modification génétique [33,39].

La liste des molécules ASA utilisés dans l'industrie est longue. L'un des plus couramment utilisés est l'acide lactique (AL). Sa production par fermentation du lactosérum à l'aide de bactéries a été largement étudiée, comme indiqué ci-dessous (Tableau 2) (Panesar et Kennedy, 2012). L'AL est utilisé comme agent de conservation dans les produits alimentaires, et également comme composé de base pour les produits pharmaceutiques. Il s'agit d'un important composé à valeur ajoutée dont le prix actuel sur le marché est d'environ 10 à 12 $US/kg (Archine pharmaceutical Co., 2020).

CHAPITRE UN: SYNTHESE

Les groupements hydroxyles sont l'un des principaux groupes fonctionnels chimiques qui contribuent à l'arôme et à la saveur des produits alimentaires. Certains d'entre eux sont obtenus par la fermentation du lactosérum. Par exemple, le 2,3-butanediol (2,3-BD) est un alcool produit naturellement par les bactéries de l'acide lactique (BAL). Le 2,3-BD peut être utilisé seul ou comme précurseur d'autres molécules ASA. Il peut être oxydé en acétoïne, puis en diacétyle. Ces trois composés (acétoïne, diacétyle et 2,3-BD) sont des additifs alimentaires. Ils sont des agents aromatisants pour les produits laitiers, tels que la margarine, le pop-corn beurré, la crème ou le fromage (Huchede et al., 2019). Ce sont des produits à haute valeur ajoutée dans l'industrie alimentaire, où l'acétoïne peut atteindre un prix de vente de 50 à 100 $US/kg (Farwell, 2020), le diacétyle environ 100 $US/kg (Shandong Zhi Shang Chemical Co., 2020), et le 2,3-BD 20 $US/kg (Jinan Finer Chemical Co., 2020). Dans le cas du 2,3-BD, la valeur du marché mondial est d'environ 43 milliards de dollars (Mihalcea et al., 2011).

L'éthanol et les alcools de fusel sont d'autres molécules ASA très consommés et ils sont généralement produits par la fermentation du lactosérum par des levures. Le rendement en éthanol par des méthodes biologiques est plus élevé que celui des alcools de fusel parce que sa production ne nécessite pas d'électrons et d'énergie. Au contraire, les levures produisent des molécules énergétiques (adénosine triphosphate), qui sont consommées pendant la croissance cellulaire. Dans le cas des alcools de fusel, la privation de la source d'azote inorganique induit leur production. Pendant la phase de croissance stationnaire des levures, celles-ci peuvent transformer l'AA en alcools de fusel par la voie d'Ehrlich (Hazelwood et al., 2008). Les alcools de fusel peuvent également être produits par les levures par la voie "*de-novo*" (également appelée voie "*shikimate*"), qui consiste à consommer des AA synthétisés par elles-mêmes. Ces voies ont été largement étudiées, montrant les principales étapes des mécanismes réactionnels, tels que les réactions de

transamination, de décarboxylation et de déshydrogénation pour convertir des AA en leurs alcools de fusel respectifs (Chua et al., 2017; de Matos et al., 2017; Qian et al., 2019; Varela, 2016). L'un des alcools de fusel les plus importants est le 2-phényléthanol (2PE) en raison de sa stabilité, de son arôme de rose et de sa saveur de miel, ainsi que de ses propriétés antiseptiques (Etschmann et al., 2002; Qian et al., 2019). Grâce à ces caractéristiques, le 2PE est un composé très demandé dans les secteurs alimentaire, pharmaceutique et cosmétique. Le 2PE biosourcé a un prix sur le marché international de 100 $/kg (Ambeed USA, 2022; Chem-Impex International, 2022).

Les microorganismes peuvent également produire des acides de fusel homologues en utilisant les mêmes voies que pour les alcools de fusel. Dans le cas des acides homologues, les microorganismes réduisent le composé décarboxylé, qui est un aldéhyde, au lieu de le déshydrogéner pour produire l'alcool de fusel (Hazelwood et al., 2008).

Les esters des acides gras à chaîne courte donnent des saveurs et des arômes fruités aux aliments et aux boissons (Izawa et al., 2015). Ils peuvent être perçus à des faibles concentrations : le benzoate d'éthyle, par exemple, peut être détecté à partir de 1 mg/L. Au contraire, les esters peuvent provoquer un goût de sensation rance à des concentrations élevées. Les lactones sont des esters cycliques, qui donnent des arômes de fruits, de noix de coco ou de noix (Longo et Sanromán, 2016). Les esters peuvent être biosynthétisés par estérification et alcoolyse dans les voies liées à la production d'acides gras chez les bactéries et les levures (Liu et al., 2004). Les esters sont également utilisés dans l'alimentation et les cosmétiques, mais contrairement aux autres molécules d'ASA, leur prix de marché est faible, environ 1,5 à 3 $/kg (Shandong Zhi Shang Chemical Co., 2020)

Un antioxydant est un composé qui s'oppose à l'effet négatif des espèces réactives de l’oxygène (ERO) telles que l'oxygène et les radicaux libres (Blanco et Blanco, 2017). Les antioxydants sont produits naturellement lorsque l'oxygène est le dernier accepteur d'électrons dans la chaîne

respiratoire et comme défense en réponse aux agents toxiques abiotiques et biotiques. Les tocophérols (phénols méthylés), les acides organiques (ascorbique, citrique, caféique, férulique, gallique) et les flavonoïdes (catéchines, quercétine) sont les antioxydants les plus utilisés (Abbas, 2006; Siddaiah et al., 2006). Certains pigments, comme les caroténoïdes (β-carotène, γ-carotène, astaxanthine et lycopène), ont également des propriétés antioxydantes. Les caroténoïdes sont produits par les microorganismes de manière intracellulaire, et ils sont utilisés comme antioxydants pour prévenir les dommages cellulaires. Les caroténoïdes sont des additifs alimentaires et cosmétiques qui remplissent à la fois les fonctions d'antioxydant et de colorant (Azmi et al., 2011). Un autre antioxydant bien connu est l'acide citrique qui peut agir comme agent chélateur ou donneur d'hydrogène aux radicaux libres. La chélation des ions métalliques empêche l'oxydation des acides gras polyinsaturés. Le second mécanisme consiste à piéger le peroxyde d'hydrogène en donnant un atome d'hydrogène au radical libre, créant ainsi un produit moins réactif et moins toxique. Grâce à ces propriétés, l'acide citrique est largement utilisé dans les industries alimentaire et pharmaceutique, comme conservateur (Gervasi et al., 2018; Sawant, 2018; Schneider et al., 2013). Le prix des antioxydants oscille entre 13 et 180 $/kg (Guangzhou ZIO Chemical Co., 2020; Xi'an Imaherb Biotech Co., 2020).

1.4.1 Production de molécules ASA par des procédés biologiques

Cette revue de la littérature se concentre sur les métabolites produits par des microorganismes sauvage ou par des microorganismes sélectionnés par voie de l'adaptation. Ceci est dû aux restrictions sur l'utilisation des métabolites produits par des microorganismes génétiquement modifiés dans les produits alimentaires et les boissons. De même, la gestion des déchets produits par ces microorganismes doit être effectuée selon des règles strictes de manipulation et d'élimination. La production d'ASA par des procédés biologiques utilisant les trois principaux

groupes microbiens industriels (bactéries, levures et champignons) est abordée ci-dessous. Le Tableau 2 présente une grande variété de composés produits par la fermentation du lactosérum à l'aide de bactéries, de champignons et de levures. Il indique le type de lactosérum utilisé et s'il a été complété par une source de carbone externe ou s'il a été prétraité avant la fermentation.

1.4.2 Fermentation par des bactéries

La fermentation du lactosérum à l'aide de bactéries peut produire des acides organiques, des aldéhydes et des cétones. Plusieurs microorganismes peuvent consommer le lactose du lactosérum comme principale source de carbone. Dans certains cas, lorsque la biomasse microbienne est constituée de microorganismes bénéfiques pour la santé humaine, comme les lactobacilles et les lactocoques, ils peuvent également être valorisés comme probiotiques, encapsulés dans des formulations solides et stables (Teijeiro et al., 2018). Le Tableau 2 indique les conditions de fermentation avec des bactéries et quelques remarques concernant ces études.

L'AL est le composé le plus couramment produit par la fermentation du lactosérum à l'aide de bactéries. Les bactéries les plus souvent utilisées pour produire l'AL sont des souches de *Lactobacillus*, qui présentent une sélectivité et une affinité pour la consommation de sucre, le glucose étant le sucre le plus apprécié, suivi du fructose, galactose et lactose (Singhvi et al., 2018). Juodeikiene et al. (2016) ont observé que le rendement en LA pour les *Lactobacillus* est compris entre 0,06 et 0,09 g AL/g substrat pour une concentration initiale de lactose de 20 g/L à 40 g/L. Cependant, à des concentrations de lactose supérieures à cette plage, les lactobacilles peuvent être inhibés. Par exemple, Juodeikiene et al. (2016) ont également observé que pour une concentration initiale de lactose de 60 g/L, le rendement en AL diminuait à 0,035 $g_{AL}/g_{substrat}$. Si les bactéries présentent des taux de production d'AL élevés, ces derniers peuvent s'accumuler dans le bouillon de culture et l'acidifier. Certaines études ont indiqué que pour une concentration d'AL supérieure

à 20 g/L, le pH du bouillon de culture passait de 5,5 à 3,5, et que cette acidification était suffisante pour inhiber la croissance bactérienne (Cheirsilp et al., 2018; Juodeikiene et al., 2016). L'augmentation de la concentration de protons dans le bouillon de culture due à l'acidification peut affecter le potentiel membranaire des bactéries lactiques et le transport du substrat dans la cellule. Par conséquent, il est recommandé d'éliminer l'AL du bouillon de fermentation pour améliorer le rendement global de la fermentation (Ghafouri et al., 2018).

La production d'aldéhydes et de cétones est directement associée à une bio-oxydation partielle de l'alcool. La stabilité des produits obtenus par oxydation de l'alcool dépend fortement du pH, en particulier dans la marge de 3,5 à 4,0 (Gallardo-Escamilla et al., 2005). Par exemple, lorsque le pH du bouillon de culture pour les fermentations utilisant du lactosérum diminue à 4,5, l'oxydation du précurseur 2,3-BD est induite et du diacétyle est formé chez *Lactobacillus* sp., *Streptococcus* sp. et *Enterococcus* sp. (Mauriello et al., 2001).

Gutierrez et Debarr (1996) ont étudié la fermentation du perméat de lactosérum à un pH de 5,8 en utilisant des BL. Ils ont observé la présence de diacétyle (aldéhyde) et d'acétoïne (cétone) à une concentration de 13 mg/L et 120 mg/L, respectivement. La production de diacétyle et d'acétoïne est causée par la bio-oxydation partielle du 2,3-BD. Le 2,3-BD est produit par le cycle de l'acide tricarboxylique et il est oxydé en acétoïne par l'enzyme α-acétolactase. Cette oxydation se produit à un pH d'environ 4,5. Dans ces conditions, l'acétoïne peut être transformée en diacétyle par une réaction réversible (Mauriello et al., 2001). Par exemple, la réduction du diacétyle dans les mêmes conditions a été observée (Gutierrez et Debarr, 1996; Reuss et al., 2012). La présence de cofacteurs enzymatiques tels que Cu^{2+} pour l'α-acétolactase (une enzyme liée au cycle de l'acide tricarboxylique), est un facteur clé pour augmenter la production de cétones et d'aldéhydes. Gutierrez et Debarr (1996) ont observé que pour un milieu de culture enrichi avec 63 mg/L de Cu^{2+}

et 5 g/L d'extrait de levure, la concentration finale de diacétyle et d'acétoïne était de 28 mg/L et 360 mg/L, respectivement. Cependant, une inhibition du substrat a été observée lorsque la concentration en extrait de levure a été augmentée à 10 g/L.

Tableau 2 Molécules ASA produits par le bioraffinage du lactosérum avec des bactéries

Substrat	Microorganisme	Conditions de fermentation				Produit	Observations	Ref.
		T (°C)	pH	Temps (h)	Agitation (rpm)			
Lactosérum doux	*Lactobacillus bulgaricus* *Pediococcus acidilactici* *P. pentosaceus*	37	6.5	72	0	Acide lactique	Utilisation de la co-culture de bactéries. Hydrolyse enzymatique préalable du lactose. Lavage des cellules à des taux de dilution allant jusqu'à 0,04 h^{-1}. Concentration finale de 6 100 mg/L.	(Juodeikiene et al., 2016)
Lactosérum doux	*L. kefiranofaciends*	30	6.5	24	100	Acide lactique	Conditions anaérobies. Inhibition des bactéries due à l'accumulation d'acide lactique et au faible pH. Production d'exopolysacharides par le stress du pH. Fonctionnement des réacteurs en mode batch et feed-batch avec une concentration finale de 33 000 et 135 000 mg/L, respectivement.	(Cheirsilp et al., 2018)
Lactosérum	*Lactococcal lactis*	30	4.5	18	-	Acétoïne	Concentration finale 4,814 mg/L	(Mauriello et al., 2001)
	Enterococcus feacalis	40	4.5	18	-	Acétoïne	Concentration finale 0,818 mg/L	(Mauriello et al., 2001)
	Streptococcus thermophilus	40	4.5	18	-	Acétoïne et 2-méthyl-1-butanol	Concentration finale de 0,55 mg/L pour l'acétoine et de 1,661 mg/L pour 2-méthyl-1-butanol.	(Mauriello et al., 2001)

La fermentation du lactosérum par des souches d'entérocoques et de lactocoques a été étudiée. Mauriello et al. (2001) ont observé que certaines de ces bactéries ont produit plusieurs composés, tels que le furfural, l'éthylbenzène, le butanoate, le phénol, l'hexadécane et le tétradécane (Mauriello et al., 2001). Cependant, la fermentation du lactosérum n'a pas été optimisée et les bactéries ont donné des rendements inférieurs à 0,002 g produit/g lactose (Mauriello et al., 2001). Le mécanisme par lequel ces bactéries produisent les molécules ASA ligneux et fruitiers mentionnés précédemment est une activité de dégradation du citrate au cours du cycle de l'acide tricarboxylique.

1.4.3 Fermentation fongique

Les champignons ont un avantage naturel sur les bactéries dans la production d'antioxydants. Cependant, la production d'antioxydants tels que l'acide citrique à partir de la fermentation du lactosérum par les champignons est limitée par la formation de galactose lors de l'hydrolyse du lactose (Sawant, 2018). Le galactose est un sucre qui provoque la répression du glucose, interférant avec la glycolyse et la respiration de l'oxygène. Le galactose est également un inhibiteur de l'oxoglutarate déshydrogénase et de la pyruvate déshydrogénase, enzymes clés du cycle de l'acide tricarboxylique et de la production d'acide citrique (El-Holi et Al-Delaimy, 2003; Sawant, 2018). Le Tableau 3 indique les conditions de fermentation avec des champignons et quelques remarques concernant ces études.

El-Holi et Al-Delaimy (2003) ont rapporté la production d'acide citrique par *Aspergillus niger*. En utilisant une concentration initiale de lactose de 49 g/L, ils ont corroboré que la fermentation du lactosérum ne convient pas à la production d'acide citrique. Le rendement maximal rapporté était de 0,049 g d'acide citrique/g lactose en 8 jours avec une concentration d'acide citrique de 2,43 g/L. Les auteurs ont constaté que l'ajout de saccharose à 15% (p/v) au lactosérum pendant la même

période de 8 jours a permis d'augmenter le rendement à 0,28 g d'acide citrique/g lactose et la concentration d'acide citrique à 18,25 g/L, ce qui représente une hausse du rendement de 5 fois.

La production d'acide citrique par les champignons pourrait être induite en soumettant les cellules à un stress osmotique en les mettant en contact avec des substances exogènes qui compromettent la membrane cellulaire (El-Samragy et al., 1996; Sawant, 2018). Par exemple, certaines études ont montré que la concentration et le rendement en acide citrique étaient légèrement augmentés, passant respectivement de 1,06 g/L à 1,30 g/L et de 0,056 à 0,074 $g_{d'acide\ citrique}/g_{lactose}$, lorsque 4 g/L de méthanol étaient ajoutés au milieu de culture avec lactosérum (El-Samragy et al., 1996). L'ajout de 10 g/L de sels a augmenté le stress osmotique et a réduit la concentration d'acide citrique de 1,06 g/L à 0,76 g/L et la consommation de lactose de 19 g/L à 3,1 g/L (El-Samragy et al., 1996).

Les zygomycètes ont été utilisés pour valoriser du lactosérum en caroténoïdes après une pré-hydrolyse du lactose. *Blakeslea trispora*, qui a des formes sexués et asexuées, a été utilisé pour fermenter du lactosérum hydrolysé (49 g/L) (Varzakakou et al., 2010). Les résultats ont montré que la morphologie sexuelle des champignons était corrélée à la production de caroténoïdes. La population de *B. trispora* avec un ratio de formes sexuelles:asexuelles de 1:10 était capable de produire 3 caroténoïdes : β-carotène, γ-carotène et lycopène. Les champignons ont consommé jusqu'à 90% des sucres après 8 jours, et à ce moment-là, la production de caroténoïdes et la biomasse ont atteint un rendement et une concentration maximaux, 175 mg caroténoïdes/g biomasse et 8 g/L, respectivement (Varzakakou et al., 2010). De même, le γ-carotène et le lycopène étaient des intermédiaires dans la voie métabolique pour produire le β-carotène. De ce fait, la concentration en β-carotène était 5 fois plus élevée que pour les deux autres, 112 et 168 mg/L, respectivement (Varzakakou et al., 2010; Varzakakou et Roukas, 2010). Cependant, après 10 jours de fermentation, le rendement a chuté à 160 mg caroténoïdes/g biomasse en raison de la

dégradation des caroténoïdes par les enzymes fongiques (Varzakakou et al., 2010; Varzakakou et Roukas, 2010).

L'un des principaux problèmes de l'utilisation de souches fongiques telles que *Blakeslea trispora*, *Mucor circinelloides*, *F. lusitanicus* ou *Mortierella isabellina* est qu'elles ne peuvent pas facilement hydrolyser le lactose du lactosérum (Demir et al., 2013), et les étapes supplémentaires de prétraitement pour l'hydrolyser augmentent les coûts de production. De plus, la longue durée de fermentation entraîne des coûts élevés et des temps de fermentation allant de 7 à 12 jours sont nécessaires (Wu et Hansen, 2008).

Tableau 3 Molécules ASA produits par le bioraffinage du lactosérum avec des champignons

Substrat	Microorganisme	Conditions de fermentation				Produit	Observations	Ref.
		T (°C)	pH	Temps (h)	Agitation (rpm)			
Lactosérum acide + saccharose	*Aspergillus niger*	30	3	20	-	Acide citrique	Fermentation discontinue réalisée avec une culture liquide de surface. Lorsque le lactose a été utilisé comme source de carbone, la concentration finale d'acide citrique était de 2 430 mg/L. Amélioration de 750% du rendement en acide citrique après l'ajout de 15% p/v de saccharose au lactosérum (18 250 mg/L).	(El-Holi et Al-Delaimy, 2003)
Lactosérum doux déprotéiné	*Aspergillus niger*	30	3.5	15	150	Acide citrique	Fermentation discontinue La concentration maximale d'acide citrique à 9 jours était de 1 060 mg/L.	(El-Samragy et al., 1996)
Lactosérum doux, filtré et déprotéiné	*Mucur azygosporus*	30	5.5 - 6.6	10	150	β-Carotène	Lactosérum complété par une fermentation discontinue à l'amidon. Désacidification du lactosérum. Inhibition causée par la production de β-Carotène (0,4 mg/L). Corrélation positive entre la biomasse des champignons et la production de carotène	(Azmi et al., 2011)
Lactosérum doux, hydrolysés et déprotéiné	*Blakeslea trispora*	26	7.0 - 7.5	12	200	β-Carotène γ-Carotène Lycopène	Fermentation discontinue Le pH doit être de 7-7.5, sinon la production de pigments diminue. Des concentrations de 1 120 mg/L de β-Carotène, 168 mg/L de γ-Carotène et 112 mg/L de Lycopène ont été atteintes à 8 jours.	(Varzakakou et al., 2010)

1.4.4 Fermentation par des levures

Les levures sont des biocatalyseurs dotés d'une machinerie enzymatique polyvalente ; par exemple, elles peuvent hydrolyser le lactose et métaboliser les sels d'acides organiques présents dans le lactosérum, comme le lactate et l'acétate (Löser et al., 2015b). Elles peuvent transformer des biomolécules en molécules ASA avec des exigences nutritionnelles minimales. Les levures peuvent se développer même dans des conditions de faible pH, dans lesquelles les bactéries et les champignons échouent pendant la fermentation du lactosérum. Le Tableau 4 indique les conditions de fermentation avec des levures et certaines remarques en lien avec ces études.

La biosynthèse des esters par les levures fait intervenir deux mécanismes enzymatiques: l'estérification et l'alcoolyse (Löser et al., 2015b). L'estérification englobe la formation d'esters à partir d'alcools et d'acides carboxyliques ; l'alcoolyse produit des esters à partir d'alcools et d'acylglycérols ou d'alcools et d'acides gras (Dragone et al., 2009; Izawa et al., 2015; Löser et al., 2015a).

Löser et al. (2015) ont étudié la production d'acétate d'éthyle (AE) par estérification, en fermentant du lactosérum doux partiellement déminéralisé avec *K. marxianus*. La concentration initiale de lactose était de 3,9 g/L et le lactosérum a été supplémenté avec 10 g/L de $(NH_4)_2SO_4$. L'effet des oligo-éléments a également été analysé. Ils ont observé que l'absence de fer et de cuivre limite le transfert d'électrons entre NADH et NAD et l'activité de l'aconitase et de la succinate déshydrogénase (enzymes agissant dans le cycle ATC). Le flux d'acétyl-SCoA est diminué ainsi que l'oxydation du pyruvate par le cycle ATC. Cela favorise la formation d'éthanol et aussi sa réaction avec le pyruvate pour former de l'acétate d'éthyle par estérification, avec un rendement final de 0,198 g AE/g lactose. Lorsque la fermentation du lactosérum est réalisée dans des

conditions anaérobies et supplémentée en Fe et Cu, un rendement de 0,008 g AE/g lactose peut être obtenu (Löser et al., 2015b).

Pour la production d'alcools de fusel par la voie d'Ehrlich, les levures doivent être cultivées dans un milieu présentant une carence en azote inorganique et avec des sources riches en AA, pour induire les levures à consommer l'azote organique (Hazelwood et al., 2008). Peu d'études ont rapporté la production d'alcools de fusel et, plus particulièrement, de 2PE par fermentation du lactosérum à l'aide de levures. Leclercq-Perlat et al. (2004) ont rapporté la fermentation du lactosérum dans des conditions limitées en oxygène et en utilisant *D. hansenii* (Leclercq-Perlat et al., 2004). Une concentration de 191 mg/L de 2PE a été obtenue à 96 h. On suppose que la levure a suivi la voie d'Ehrlich avec un rendement calculé de 0,064 $g_{2PE}/g_{L\text{-}Phe}$.

Dragone et al. (2009) ont rapporté la fermentation de lactosérum doux déprotéiné en utilisant *K. marxianus* (Dragone et al., 2009). La fermentation a été réalisée dans un bioréacteur à air pulsé fonctionnant en mode continu avec un volume opérationnel effectif de 700 L et un débit de 140 L/h. Les auteurs ont identifié 40 composés volatils, parmi lesquels les alcools supérieurs étaient les plus abondants, ainsi que l'acétate d'éthyle. Ils ont produit 3,9 mg 2PE/L*h pendant la fermentation continue du lactosérum. Cette faible productivité peut être liée à l'élimination des protéines du lactosérum avant la fermentation. Dans ce cas, la concentration de la L-Phe a diminué, ce qui a un impact sur la production de 2PE (Dragone et al., 2009).

Conde-Baéz et al. (2019) ont demontré que *K. marxianus* est capable d'utiliser du lactose de lactosérum doux enrichi avec de la L-Phe. La concentration initiale dans le bouillon de culture pour les sources de carbone et d'azote était de 54 g de lactose/L, 1 g de L-Phe/L et 0,45 g de $(NH_4)_2SO_4$/L (Conde-Báez et al., 2019). Le sulfate d'ammonium stimule la croissance de la levure, mais il ne maintient pas la croissance cellulaire tout au long du procédé de fermentation. Par contre, la

présence d'AA dans le bouillon de culture stimule l'activation des gènes pour la production d'aminotransférases, qui sont essentielles pour commencer la transformation des AA et leur utilisation comme source d'azote. (Fonseca et al., 2008; Hazelwood et al., 2008; Qian et al., 2019). Ceci favorise la consommation de L-Phe sans minéralisation complète. Le 2PE est un sous-produit de l'utilisation de L-Phe comme source d'azote. L'hydrolyse de l'acide aminé libère ainsi un groupe fonctionnel phénolique qui est transformé en alcool. Après 96 h de fermentation, le 2PE a été produit avec un rendement de 0,78 g 2PE/g L-Phe (Conde-Báez et al., 2019).

Wittmann et al. (2002) ont réalisé la fermentation de milieux synthétiques contenant du glucose (77 g/L), du L-Phe (7 g/L) et des sels de phosphate (Wittmann et al., 2002). Le rendement maximal qu'ils ont obtenu a été de 0,68 g 2PE/g L-Phe. Ceci indique que le rendement en 2PE se situe dans une plage de 0,7-0,8 g 2PE/g L-Phe (Conde-Báez et al., 2019; Wittmann et al., 2002).

Tableau 4 Molécules ASA produits par le bioraffinage du lactosérum avec des levures

Substrat	Microorganisme	Conditions de fermentation				Produit	Observations	Ref.
		T (°C)	pH	Temps (h)	Agitation (rpm)			
Lactosérum doux **+ $(NH_4)_2SO_4$** **+ L-Phe**	*Kluyveromyces marxianus*	30	4.8	96	180	2-phényléthanol (2PE)	Fermentation discontinue. Promotion de la privation d'azote. La production de 2PE a été induite par L-Phe. La concentration finale de 2PE était de 750 mg/L de produit.	(Conde-Báez et al., 2019)
Lactosérum **+ Saccharose de betterave** **+ L-Phe**	*Saccharomyces cerevisiae*	30	5.4	72	240	2PE	Fermentation discontinue. Limitation de l'utilisation du lactose. Augmentation du rendement de production jusqu'à 21% pour la consommation de saccharose. La concentration finale de 2PE était de 3 280 mg/L de produit.	(Chreptowicz et al., 2018)
Lactosérum acide déprotéiné	*Kluyveromyces marxianus*	30	4	2	-	Alcools (isoamyl, isobutyl 1-propanol et isopentyle) Acétate d'éthyle 2PE	Fermentation continue dans un bioréacteur air-lift (volume effectif = 700 L). 37% (v/v) d'éthanol ont été produits. Faible concentration des produits. Production multiple de composés aromatiques. La concentration finale d'isoamyle, d'isobutyle, de 1-propanol, d'isopentyle, d'acétate d'éthyle et de 2PE était respectivement de 887 mg/L, 542 mg/L, 266 mg/L, 176 mg/L, 138 mg/L et 7,8 mg/L.	(Dragone et al., 2009)
Lactosérum synthétique de fromage	*Kluyveromyces marxianus*	25	4.8 - 5.8	96	150	Acétate d'éthyle (AE)	Fermentation discontinue. Désacidification des milieux avec une concentration finale de 108,9 mg/L.	(Leclercq-Perlat et al., 2004)
Lactosérum synthétique de fromage	*Debaryomyces hansenii*	25	4.8 - 5.8	96	150	2PE	Fermentation discontinue avec désacidification du milieu de culture. La concentration finale était de 191 mg/L.	(Leclercq-Perlat et al., 2004)

CHAPITRE UN: SYNTHESE

Selon la littérature, la production d'arômes et de saveurs par les levures dépend fortement du type et de la concentration d'AA. Il existe des AA à chaîne ramifiée, des AA aromatiques et des AA contenant du soufre qui peuvent déclencher l'activation séquentielle de plusieurs gènes pour produire des alcools de fusel ou des acides de fusel. Les concentrations courantes d'AA dans le lactosérum acide et le lactosérum de yaourt sont détaillées au Tableau 5, ainsi que les alcools de fusel et les acides de fusel homologues que les levures peuvent produire (Hazelwood et al., 2008).

Tableau 5 Acides aminés essentiels impliqués dans la voie d'Ehrlich et produits finaux (modifié à partir de Hazelwood et al., 2008)

Acide aminé	**Lactosérum**		**Alcools de fusel**	**Acides de fusel**
	Acide (mg/L) (Anand et al., 2013; Huffman et Ferreira, 2011)	**Yaourt grec (mg/L)** (Kawase et Furuse, 2019)		
Leucine	119,0	12,2	3-Méthylbutanol (alcool isoamylique)	3-Méthylbutanoate
Isoleucine	54,0	5,93	2-Méthylbutanol	2-Méthylbutanoate
Valine	51,0	31,3	2-Méthylpropanol (alcool isobutylique)	Propanoate de 2-méthyl
Phénylalanine	0,0 - 68,0	2,53	2-Phényléthanol	2-Phényléthanoate
Tyrosine	0,0 - 68,0	1,22	Tyrosol	p-hydroxyphénylacétate
Tryptophan	20,0	38,5	Tryptophol	Acide 3-Indoleacetique
Méthionine	48,9 - 84,75	-	Méthionol	3-méthylthiopropionate

1.5 Technologies d'extraction et de purification de 2-phényléthanol

En plus de la production de 2PE, il existe des problèmes liés à la séparation, l'extraction et la purification de 2PE. Les cellules de levures sont plus grandes que celles de bactéries, cela représente un avantage lors de la fermentation du lactosérum (Gao et al., 2012), car jusqu'à 99% des cellules de levure peuvent être retirées du bouillon fermenté par centrifugation (Vieira et al., 2019). La microfiltration peut également être utilisée pour l'élimination des levures. L'utilisation d'un flux transversal au lieu du flux frontal est préférée en raison de la réduction de l'encrassement et de l'augmentation de la récupération des levures. Le perméat du surnageant peut-être directement envoyé vers des unités d'extraction du 2PE (Etschmann et al., 2002; Qian et al., 2019).

Une technologie d'extraction de 2PE est celle des membranes de polystyrène pour l'échange d'ions (Amberlite) (Qian et al., 2019; Savina, 1999). Son efficacité globale de récupération est de 70 à 85% (Savina, 1999). Il s'agit déjà d'une technologie commerciale brevetée, mais elle nécessite des étapes de nettoyage en raison de l'encrassement ; cela use les membranes, qui doivent être éliminées à la fin de leur vie utile (Savina, 1999).

L'extraction liquide-liquide peut être respectueuse de l'environnement et consiste à utiliser l'acétate d'éthyle comme solvant (phase organique) (Chreptowicz et al., 2016). L'efficacité finale de la récupération de 2PE est de 78%. Une étape ultérieure de séchage de 12 h est nécessaire, en utilisant le $MgSO_4$ comme agent de séchage. Finalement, l'évaporation de l'acétate d'éthyle doit être réalisée pour obtenir du 2PE à 99% de pureté, atteignant le standard requis pour une utilisation dans des produits alimentaires, cosmétiques et pharmaceutiques (Chreptowicz et al., 2016). L'efficacité de cette méthode d'extraction est comparable à celles testées avec des résines, des solvants fixés dans des supports et la pervaporation. Bien que l'extraction avec des liquides ioniques ou du CO_2

supercritique puisse fortement augmenter l'efficacité d'extraction et de récupération, ces technologies sont encore en développement et leur utilisation industrielle reste coûteuse.

1.6 Applications de 2PE sur le marché

À l'échelle mondiale, 4 536 t de 2PE ont été produites en 2016. Sur cette quantité, 10% a été utilisé comme parfum et 3% comme additif alimentaire (National Center for Biotechnology Information, 2020; U.S. Environmental Protection Agency, 2020). Le 87% restant a été détourné vers la fabrication de pesticides, d'antifongiques ou d'antiseptiques (Etschmann et al., 2002). Le 2PE a été inclus comme additif dans plusieurs produits, tels que les peintures, les composés de traitement de l'eau (adoucisseurs, anticalcaires), les cires et les purificateurs d'air (National Center for Biotechnology Information, 2020). Ces produits de base et ces produits industriels peuvent être couverts par la production de 2PE provenant de procédés chimiques et de microorganismes génétiquement modifiés. *Saccharomyces cerevisiae* et *Escherichia coli* sont les microorganismes les plus souvent modifiés génétiquement pour produire du 2PE. Des données rapportées ont montré que ces microorganismes étaient capables de produire jusqu'à 12,5 g/L de 2PE avec du glucose comme substrat (Wang et al., 2019). Néanmoins, la voie chimique utilisant le mécanisme de réaction de Friedel-Crafts est le principal procédé de fabrication industrielle (U.S. Environmental Protection Agency, 2020).

Le 2PE est obtenu par la réaction entre l'oxyde d'éthylène et le benzène, catalysée par le chlorure d'aluminium pour produire de l'oxyde de propylène. Ce dernier est hydrolysé avec un acide inorganique fort, tel que HCl, pour donner du 2PE. Cette réaction est réalisée à basse pression, inférieure à la pression atmosphérique, et à des températures entre 150 et 250°C. Dans ces conditions, le rendement de la réaction est d'environ 0,65 $g_{2PE}/g_{réactif}$ (Etschmann et al., 2002). Malgré le rendement élevé, cette réaction génère des sous-produits neurotoxiques tels que le

styrène et l'$Al(OH)Cl_2$ (National Center for Biotechnology Information, 2020). Pour cette raison, la production de 2PE au moyen de la valorisation du lactosérum en utilisant des levures est une alternative émergente. Cette alternative évite la production de composés toxiques et n'est pas un procédé énergivore.

D'ailleurs, la quantité de 2PE utilisée pour la consommation humaine sous forme de produits de soins personnels tels que les savons, les médicaments, les cosmétiques et les gels douche n'est pas bien documentée. Ces produits et aliments entrent en contact direct avec le corps humain, et l'utilisation de 2PE dérivés de microorganismes génétiquement modifiés représenterait un risque et serait interdit complètement pour des aliments (Goverment of Canada, 2020). Par conséquent, avec les données actuellement disponibles, au moins 13% du marché du 2PE représente une opportunité potentielle pour la production de 2PE par des moyens biologiques utilisant des microorganismes sauvages et non pathogènes, pour éviter la production des composés toxiques qui sont générés par les procédés chimiques.

CHAPITRE UN – PARTIE 3: PROBLÉMATIQUE

À partir de l'état de l'art de la valorisation du lactosérum, les principaux problèmes pour la production biologique de 2PE ont été identifiés comme suit :

3.1 Les procédés actuels de valorisation du lactosérum sont coûteux

La biovalorisation du lactosérum en molécules industrielles est un domaine de recherche émergent car les méthodes physiques et thermiques actuelles sont énergivores et coûteuses. Elles concentrent les nutriments du lactosérum et pour obtenir des formulations sèches il faut évaporer l'eau, par des procédés thermiques. Ce qui représente une consommation d'énergie très élevée et la dégradation des composés thermolabiles, tels que les protéines, diminuant la valeur nutritionnelle des produits finaux. Par ailleurs, le prix de ces produits est inférieur à celui des spécialités chimiques telles que les biomolécules d'arôme et de saveur qui peuvent être obtenues par transformation biologique du lactosérum.

3.2 La fermentation bactérienne ou fongique du lactosérum nécessite des étapes de conditionnement pour permettre la croissance des microorganismes et de production des métabolites

Le lactosérum contient du lactose (33 - 52 g/L), des protéines (5 - 15 g/L) et de l'acide lactique (1,4 - 4,7 g/L), avec un pH de 3,9 à 5,6. Malgré cette composition riche en carbone et en azote organique, il est nécessaire de conditionner le lactosérum pour le fermenter à l'aide de bactéries ou de champignons. Plusieurs des microorganismes ne sont pas capables d'hydrolyser le lactose ou ne tolèrent pas un pH acide. Dans le cas de la source de carbone, il est nécessaire d'hydrolyser le lactose en glucose et galactose, qui sont des substrats carbonés presque universels. Pour le pH du

lactosérum, il est nécessaire d'augmenter celui-ci en ajoutant une base (ex. NaOH) ou un tampon (ex. K_2HPO_4). Les réactifs et étapes additionnelles augmentent les coûts de production.

Dans le cas des levures, qui peuvent métaboliser le lactose et tolérer un pH acide, leur métabolisme peut être affecté par des concentrations de lactose et de minéraux supérieures à 8 et 2% (p/p) respectivement. Sous ces conditions, les voies métaboliques des levures peuvent produire préférentiellement du glycérol au lieu des biomolécules souhaitées.

3.3 La production de 2-phényléthanol dépend fortement de la composition du milieu

La principale source de carbone et d'azote du lactosérum est le lactose et les acides aminés, respectivement. Les levures ont besoin de consommer des précurseurs des alcools de fusel, qui sont des acides aminés. Plus précisément, pour la production de 2PE, de la L-Phe est nécessaire. Cependant, la concentration élevée de lactose et la faible concentration de L-Phe dans le lactosérum (0 – 68 mg/L) limitent la production de 2PE. Afin d'obtenir une productivité élevée de 2PE, il est nécessaire d'enrichir le milieu de culture avec une source supplémentaire de L-Phe. En général, les sources de L-Phe sont la poudre d'extrait de levure, la peptone ou la caséine, qui sont coûteuses

3.4 L'accumulation d'alcools dans le bouillon de culture peut diminuer la productivité du 2-phényléthanol par les levures

Les levures peuvent produire une diversité de composés pendant la fermentation. Dans le cas de la fermentation des sucres, l'un des principaux sous-produits est l'éthanol. Si sa concentration augment beaucoup par son accumulation, il induit la dénaturalisation des protéines de la membrane cellulaire, inhibe la croissance et provoque la mort cellulaire. Un effet similaire est observé lorsque le 2PE s’accumule. L'élimination des alcools pour éviter ce phénomène augmentera également les coûts de production.

3.5 La production de 2-phényléthanol dépend fortement de l'aération

La production de 2-phényléthanol par fermentation submergée peut être réalisées sous conditions hypoxiques ou aérobies. Dans le cas d'une fermentation aérobie, la productivité et la concentration de 2-phényléthanol sont plus élevées que dans une fermentation anaérobie, étant respectivement 8,12 mg/L*h et 780 mg/L pour l'aérobie et 2,04 mg/L*h et de 196 mg/L pour l'anaérobie. Cependant, les deux études existantes ont été réalisées dans des fioles, ce qui montre la nécessité d'étudier l'effet d'une aération contrôlée sur la fermentation du lactosérum et la production de 2PE.

CHAPITRE UN – PARTIE 4. HYPOTHÈSES

Les hypothèses suivantes énoncent les principales suppositions qui seront vérifiées dans la présente recherche et qui constituent la base des activités expérimentales :

Hypothèse 1 pour le problème 3.1

1) Selon la littérature, *Kluyveromyces marxianus* et *Debaryomyces hansenii* peuvent consommer les nutriments présents dans le lactosérum et produire des composés d'arômes et de saveurs, notamment des alcools de fusel. En fonction de cette observation, l'hypothèse suivante a été formulée : « **le lactosérum de fromage serait un substrat adéquat et peu coûteux pour produire des alcools de fusel, en particulier le 2PE par fermentation par des levures, ce qui se traduirait par un procédé rentable »**.

Hypothèse 2 pour le problème 3.2

K. marxianus et *D. hansenii* peuvent consommer le lactose comme source de carbone sans nécessiter d'hydrolyse préalable. En conséquence, il est supposé que les deux levures, seules ou en mode de co-culture, seront capables de consommer et d'épuiser le lactose dans un processus de fermentation. Ainsi, « **les levures pourraient fermenter le lactose et transformer les acides aminés contenus dans le lactosérum en développant un processus compétitif** ».

Hypothèse 3 pour le problème 3.2/3.3

Le lactose du petit-lait est la source de carbone des levures, mais en même temps, une concentration élevée de lactose pourrait avoir un effet négatif sur les levures. En fonction de la tolérance métabolique de chaque levure au lactose, l'hypothèse suivante a été formulée : « **la concentration initiale des nutriments et du précurseur de 2PE, en particulier le lactose et la L-Phe, peut être optimisée en termes d'obtention du taux de croissance maximal et de la production de 2PE »**.

Hypothèse 4 pour le problème 3.4

La co-culture de levures crée des interactions synergiques pour la consommation de substrat et la production de métabolites. En fonction des différents besoins en nutriments de chaque levure et de leurs réponses métaboliques, l'hypothèse suivante a été formulée : « **l'utilisation d'une co-culture de KM et DH pourrait diminuer l'accumulation de métabolites, tels que l'éthanol et le glycérol, qui seraient consommés lorsque le lactose est épuisé ».**

Hypothèse 5 pour le problème 3.4

La production de 2PE est fonction de la concentration de la biomasse car la consommation de sources de carbone et d'azote augmente avec le nombre de cellules actives. Selon cette observation, l'hypothèse suivante a été émise : « **la concentration cellulaire peut être augmentée** a) **en utilisant une concentration initiale élevée de nutriments, et** b) **en produisant des biofilms par immobilisation des levures sur des supports inertes, ce qui est plus résistant aux variations des facteurs environnementaux »**.

Hypothèse 6 pour le problème 3.5

La concentration de L-Phe peut être augmentée en ajoutant des nutriments ou du L-Phe pure, ce qui augmente les coûts de fermentation et affecte négativement la durabilité du processus. En conséquence, l'hypothèse suivante a été formulée : « **La production de 2PE par fermentation de lactosérum enrichi d'une source de protéines renouvelables d'origine biologique sera similaire à la production utilisant de la L-Phe pure. De plus, la L-Phe biosourcée pourrait être économique, diminuant les coûts du bioprocédé et l'empreinte environnementale »**.

Hypothèse 7 pour le problème 3.6

Un milieu un niveau contrôlé d'oxygène permet une bio-oxydation efficace du lactose et donc une transformation efficace du L-Phe via la voie d'Ehrlich. D'après cette observation, l'hypothèse suivante a été émise : « **le passage à l'échelle du procédé de bioproduction de 2PE dans des bioréacteurs de 2L maintiendra le rendement ainsi que la productivité du 2PE grâce à l'aération constante et au contrôle du pH et de la température »**.

CHAPITRE UN – PARTIE 5. OBJECTIFS

L'objectif général de la présente étude est le développement d'un procédé économique de fermentation de lactosérum enrichi d'une source d'azote organique en utilisant des levures pour produire du 2-phényléthanol. Ce bioprocédé permettra l'utilisation de ressources renouvelables telles que les résidus agro-industriels comme co-substrats pour la fermentation. De cette manière, la valorisation des résidus agroalimentaires peut contribuer à l'économie circulaire de l'industrie laitière. Les objectifs spécifiques suivants ont été dérivés de la revue de la littérature et ils permettront de prouver les hypothèses énoncées ci-dessus :

1) Comparer la fermentation du lactosérum en utilisant *Kluyveromyces marxianus* NRRL-Y-1109 et *Debaryomyces hansenii* NRRL-Y-1448 en mode monoculture pour produire des biomolécules d'arôme et de saveur. L'identification et la quantification des biomolécules d'arôme et de saveur permettront de déterminer les meilleures conditions de fermentation.

2) Déterminer la meilleure composition du milieu de culture pour la croissance de *Kluyveromyces marxianus* (Y-1109) et *Debaryomyces hansenii* (Y-1448) en mode monoculture. La synthèse de la biomasse et la quantification de la densité cellulaire permettront d'identifier les meilleures conditions pour la croissance cellulaire et la production de 2PE.

3) Développer la fermentation du lactosérum en utilisant les meilleures conditions obtenues dans l'objectif (2) et les levures *Kluyveromyces marxianus* (NRRL-Y-1109) et *Debaryomyces hansenii* (NRRL-Y-1448) en mode de co-culture pour produire du 2PE. Ceci permettra de déterminer la meilleure condition de fermentation du lactosérum pour produire du 2PE parmi l'utilisation de levures en mode monoculture ou en mode de co-culture.

4) Maximiser la bioproduction de 2PE en variant la concentration initiale de lactose et de L-Phe pour la fermentation du lactosérum en utilisant le co-culture des levures *Kluyveromyces marxianus* (Y-1109) et *Debaryomyces hansenii* (Y-1448) obtenu dans l'objectif (3). Le rendement maximal en 2PE et l'accumulation d'éthanol la plus faible détermineront la meilleure condition pour la production de 2PE. 5) Étudier l'effet de l'immobilisation des cellules de levures sur la bioproduction de 2PE en utilisant des supports inertes (perlite, kaldnes media et biochar) . Cela a comme but d'augmenter la densité cellulaire et en conséquence la performance du bioréacteur par une consommation rapide du substrat et une production élevée du produit.

6) Identifier une source biologique de L-Phe pour compléter la fermentation du lactosérum en utilisant le meilleur mode de culture de *Kluyveromyces marxianus* (Y-1109) et *Debaryomyces hansenii* (Y-1448) obtenu dans l'objectif (2). La co-fermentation du lactosérum avec la source biologique de L-Phe augmentera la viabilité de la bioproduction de 2PE.

7) Déterminer l'effet du L-Phe biosourcé sur la production de 2PE pendant la co-fermentation du lactosérum enrichi avec la source biologique de L-Phe la plus approprié identifié dans l'objectif (6).

8) Valider les conditions optimisées pour la bioproduction de 2PE dans un bioréacteur de 2 L. La quantification de la concentration de 2PE, de la consommation de L-Phe et de lactose permettra de déterminer la productivité et le rendement après la mise à l'échelle du procédé.

CHAPITRE UN – PARTIE 6. ORIGINALITÉ

Selon les hypothèses et les objectifs mentionnés ci-dessus, l'originalité de ce projet de recherche peut être définie ainsi : La valorisation du lactosérum a été largement étudiée par des méthodes physiques, thermiques et biologiques. Les méthodes physiques et thermiques se concentrent sur la

concentration des nutriments du lactosérum en utilisant des unités énergivores qui sont des procédés coûteux. En général, la valorisation biologique du lactosérum a été développée avec des microorganismes qui ne peuvent pas consommer de lactose. Certaines levures en monoculture ont également été utilisées en raison de leur capacité à consommer le lactose sans qu'il soit nécessaire de l'hydrolyser. Cependant, la plupart d'entre elles présentent des rendements et une productivité faibles en matière de composés aromatiques et gustatifs. Dans le cas de la production de 2PE par fermentation du lactosérum à l'aide de levures, les informations sont quasi inexistantes. La production d'alcools de fusel par des levures à partir d'un résidu agroalimentaire important (le lactosérum) fait défaut, même si les levures sont parmi les principaux producteurs de ces composés. Dans ce contexte, **l'originalité** du présent projet réside dans les nouvelles approches pour fermenter du **lactosérum pour produire du 2PE**. **L'utilisation pour première fois de deux levures** bien étudiées et robustes, ***Kluyveromyces marxianus*** et ***Debaryomyces hansenii,*** permettra de trouver les meilleures conditions sous lesquelles les levures agissent synergétiquement pour diminuer le temps de fermentation et augmenter la productivité de 2PE. Par ailleurs, **la co-fermentation du lactosérum et d'une source d'azote biosourcée renouvelable combinée avec l'immobilisation de levures comblera plusieurs lacunes au niveau de connaissances scientifiques et techniques**. Dans son ensemble, le projet de recherche produira des informations très importantes pour **développer un bioprocédé compétitif pour produire du 2PE, en termes d'aspects techniques et économiques ayant comme but ultime un transfert technologique accéléré**.

L'originalité du présent projet peut être résumée comme le développement d'un nouveau bioprocédé à haute rendement, économique et techniquement faisable pour produire de 2PE en combinant pour toute première fois deux levures non traditionnelles (*K. marxianus* et *D. hansenii*) immobilisées et en co-culture avec la fermentation de deux résidus agroalimentaires.

CHAPTER TWO:

Cheese whey fermentation using two yeast strains to produce aroma and flavor biomolecules

CHAPTER TWO – PART 1.

Production of aroma and flavor-rich fusel alcohols by whey fermentation using the *Kluyveromyces marxianus* and *Debaryomyces hansenii* yeasts in monoculture and co-culture modes

Mariana Valdez Castillo[1], Hamed Tamasbi[2], Vinayak Laxman Pachapur[1], Satinder Kaur Brar[1,3], Dajana Vuckovic[2], Dimitri Sitnikov[2], Sonia Arriaga[4], Jean-François Blais[1], Antonio Ávalos Ramirez[1,5]

[1] Institut national de la recherche scientifique, Centre-Eau Terre Environnement, 490, Rue de la Couronne, Québec, Québec G1K9A9, Canada.

[2] Department of Chemistry and Biochemistry Loyola Campus, Concordia University, 7141 Sherbrooke Street West, Montreal, Québec, H4B 1R6, Canada.

[3] Department of Civil Engineering, Lassonde School of Engineering, York University, Toronto, Ontario M3J 1P3, Canada.

[4] Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4a Sección 78216, México.

[5] Centre National en Électrochimie et en Technologies Environnementales Inc. 2263, avenue du Collège, C.P. 610, Shawinigan (Québec) G9N 6V8, Canada.

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Résumé

Le lactosérum est l'un des principaux sous-produits agro-industriels générés par la production de fromage. Il s'agit d'un résidu à bas pH et à haute teneur en composés organiques et inorganiques, tels que le lactose, les protéines et les minéraux qui peuvent être utilisés comme nutriments dans la fermentation. L'objectif de la présente étude était de produire des biomolécules ayant des propriétés aromatiques et de saveur par la fermentation du lactosérum en utilisant *Kluyveromyces marxianus* et *Debaryomyces hansenii*. Ces levures ont produit et accumulé dans le bouillon de culture des composés aromatiques et de saveur dont les principaux sont l'éthanol, le glycérol, l'acide propanoïque, la dihydroxyacétone, le méthionol, l'isopentanol et le 2-phényléthanol (2PE). Ce dernier a été retenu comme biomolécule cible, en raison de son potentiel de commercialisation industrielle.

Les deux levures ont été capables de métaboliser la L-Phénylalanine (L-Phe) pour produire du 2PE, en monoculture et en co-culture. Lorsque les levures ont été utilisées seules, *K. marxianus* a atteint la plus haute concentration de 2PE, 82±28 mg/L, par fermentation aérobie, soit un rendement en 2PE de 0,16±0,08 $g_{2PE}/g_{L\text{-}Phe}$ et une productivité de 0,86±0,18 mg_{2PE}/L*h pour une durée de fermentation de 96 h. Cependant, sous un mode de co-culture, le rendement en 2PE a été de 0,38 g_{2PE}/gL-Phe, soit deux fois plus que le rendement maximal en monoculture. Ce rendement correspondait à une productivité de 1,93±0,02 mg_{2PE}/L*h. Les résultats montrent que l'utilisation des levures en co-culture peut améliorer la production de composés à valeur ajoutée.

Mots clés : Fermentation aérobie du lactosérum, levures, biotransformation, L-phénylananine, 2-phényléthanol

Chapter two: Cheese whey fermentation using two yeast strains

Abstract

Whey is one of the main agro-industrial by-products generated in the production of cheese. It is a residue with low pH and high content of organic and inorganic compounds, such as lactose, proteins and minerals that can be used as nutrients in fermentation. The aim of the present study was to produce biomolecules with aroma and flavor properties by whey fermentation using *Kluyveromyces marxianus* and *Debaryomyces hansenii* . These yeasts produced and accumulated in the culture broth aroma and flavor compounds, the main ones being ethanol, glycerol, propanoic acid, dihydroxyacetone, methionol, isopentanol and 2-phenylethanol (2PE). This last one was retained as the target biomolecule, because of its potential to be commercialized industrially.

Both yeasts were able to metabolize L-Phenylalanine (L-Phe) to produce 2PE, in monoculture and co-culture modes. When yeasts were used by themselves, *K. marxianus* produced the highest 2PE concentration 82±28 mg/L under aerobic fermentation, i.e. a 2PE yield of 0.16±0.08 $g_{2PE}/g_{L\text{-}Phe}$ and a productivity of 0.86±0.18 $mg_{2PE}/L*h$ at a fermentation time of 96 h. Whereas in co-culture mode the 2PE yield was 0.38 $g_{2PE}/g_{L\text{-}Phe}$, twice as high as the maximum yield for monocultures. This yield corresponded to a productivity of 1.93±0.02 $mg_{2PE}/L*h$. Results show that the use of the yeasts in co-culture may improve the production of value-added compounds, because the biotransformation of substrates increased.

Keywords: aerobic whey fermentation, yeasts, biotransformation, L-Phenylananine, 2-phenylethanol

Chapter two: Cheese whey fermentation using two yeast strains

Introduction

Canada is one of the ten main cheese producer countries, reporting $5.2x10^5$ tonnes of cheese in 2019 (Goverment of Canada, 2019). Cheddar and mozzarella are the most common types of cheese in Canada. For example, 164 million kg of cheddar cheese was produced in 2019, representing 31% of the dairy cheese market. The consumption of cheese increases, causing an increase in by-products generation.

Whey is a liquid residue derived from the milk proteins coagulation which is promoted by the use of acids, starter microorganisms-cultures or an enzymatic action (Anand et al., 2013; Panesar and Kennedy, 2012; Smithers, 2015). In general, the production of 1 kg of cheese generates 9 kg of whey (Anand et al., 2013); therefore, in Canada in 2019 we can estimate the production of whey at approximately $4.7x10^6$ tonnes (FAO, 2019; Index mundi, 2020), representing 2.04 % of global whey generation (FAO, 2019; Index mundi, 2020). Whey contains 55% milk nutrients, which could be considered as economical waste for the dairy industry. Whey contains lactose 45 g/L, proteins from 2.42 to 10 g/L, inorganic nitrogen from 1.3 to 1.19 mg/g and phosphate from 1 to 4.5 g/L, representing a chemical oxygen demand (COD) of 60 – 80 g/L (Anand et al., 2013; Panesar and Kennedy, 2012; Smithers, 2015). It has a pH of 3.8 to 5.6, with low alkalinity (lower than 0.22 g as $CaCO_3$/L) (Slavov, 2017; Smithers, 2015). Whey is an effluent with a high organic and inorganic load, representing an environmental risk when released without treatment. It would contribute to the eutrophication of water bodies, the agglomeration of soil structures and the increase of salts in environmental matrixes (Panesar and Kennedy, 2012).

Chapter two: Cheese whey fermentation using two yeast strains

Biotechnological processes have been developed to valorize the whey. Among them, fermentation can transform the whey into high value-added compounds, such as aromas and flavors. Whey fermentation has been carried out using lactic acid bacteria, fungi and yeasts (Cheirsilp et al., 2018; Dragone et al., 2009; El-Holi and Al-Delaimy, 2003; Mauriello et al., 2001). Of those, yeasts present advantages in fermentation because they tolerate low pH and they can directly consume whey lactose as a source of carbon. They can also transform amino acids into alcohols with high molecular weight (fusel alcohols), by the Ehrlich pathway (Conde-Báez et al., 2019; Wittmann et al., 2002). The fusel alcohols compounds are in high demand as aroma and flavor additives in the food, cosmetic and pharmaceutical sectors. Among the fusel alcohols, the 2PE is a compound of interest because of its aroma (rose-honey) and antimicrobial properties. It is currently used as an additive in pesticides, preservative in foods and aroma in personal care products. It is a high valued compound, with a current market price around $100/kg (Fuzhou Farwell company, 2020; Jiangsu Juming Chemical Process Technology Co., 2020).

The biological production of 2PE is limited by the use of L-Phenylalanine (L-Phe) as a precursor and the 2PE yield ranging from 0.08 to 0.78 $g_{2PE}/g_{L\text{-}Phe}$ depending on the operating conditions (Conde-Báez et al., 2019; Leclercq-Perlat et al., 2004; Wittmann et al., 2002). Thus, the objective of the present research was to study whey fermentation using *K. marxianus* and *D. hansenii* yeasts to produce aromas and flavors. The study was performed in two stages. First, the use of *K. marxianus* and *D. hansenii* to produce aromas and flavors was validated, analyzing the main biomolecules that can be obtained. Second, the effect of the yeast culture mode (mono- and co-culture) and media composition on 2PE production

was investigated. To our knowledge, there are no previous studies about the co-culture of these two yeasts using whey to produce 2PE.

Material and methodology

Culture media

Whey powder (WP) was used as a substrate (CRINO/Agropur, Canada). The WP composition (% w/w) was: crude proteins 2, moisture 5, lactose 80, and ash 9. Yeast extract (Fisher Scientific, Canada) and peptone (Organotechnie S.A.S, Canada) were used as a source of nitrogen supplements. Lactose (Sigma-Aldrich, Canada) was used as a reference carbon source. Yeast malt extract medium (YME) containing glucose 10 g/L, malt extract 3 g/L, peptone 5 g/L, and yeast extract 3 g/L was prepared to revive the yeasts.

Phenylalanine and mobile phase additives were purchased from Millipore Sigma (Oakville, ON, Canada) and phenylalanine(d5) from CDN Isotopes (Pointe-Claire, QC, Canada). LC-MS grade solvents were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada).

Lactose medium (LM) was used as a reference; it contained lactose 20 g/L, yeast extract 10 g/L, and peptone 20 g/L. To study the effect of an external nitrogen source, two media were prepared as follows: one was prepared with 54 g/L of WP in water which contained lactose at a concentration of 45 g/L (medium “W”), the other was similar to W, but supplemented with 10 g/L of yeast extract and 20 g/L of peptone (medium “WY”). The pH was adjusted to 4.5 with a 6M HCl solution.

Chapter two: Cheese whey fermentation using two yeast strains

Yeasts and inoculum

Kluyveromyces marxianus NRRL Y-1109 and *Debaryomyces hansenii* NRRL Y-1448 were selected for their capacity to metabolize lactose and proteins and to produce aroma and flavor biomolecules. The yeasts were conserved in Petri plates with YME solidified with 20 g/L of agar and following the instructions of collection cultures where they were acquired (United States Department of Agriculture; USDA). One loop of grown-up yeast colonies was transferred to 30 mL of LM medium contained in 125 mL flask and incubated at 25 °C and 200 rpm during 20 h to prepare the inoculum.

Fermentation conditions

In the first stage, anaerobic and aerobic fermentations were carried out in 125 mL serum bottles or flasks, respectively. The LM, W, WY were added to containers and autoclave sterilized at 121 °C for 20 min. To study the *K. marxianus* or *D. hansenii* monoculture effect, the inoculum was added to the containers using a concentration of $1.5x10^7$ CFU/mL of each yeast strain, which corresponded to an optical density (OD_{600}) of 0.1. The OD_{600} was measured using a UV-VIS spectrophotometer (Epoch microplate spectrophotometer 2666795, BioTek, Vermont, U.S.A.). Assays were performed in duplicate and the fermentation lasted 96 h. Temperature and agitation were set at 25 °C and 200 rpm respectively. Samples of 1 mL were taken at 0, 4, 8, 24, 32, 48, 54, 72 and 96 hours for biomass analysis. Additionally, the samples taken at 0, 24, 48 and 96 h were used for yeast growth and metabolite analysis and those taken at 0, 14, 32, 54, 72 and 108 h for L-Phe analysis. Aerobic and anaerobic controls of media incubated under the same conditions were

carried out to corroborate sterility and determine the molecules naturally present in each medium.

In the second stage, five co-culture ratios of *K. marxianus* and *D. hansenii* were tested. For the co-culture assays, the inoculum of each yeast was added to have five mixture ratios of *K. marxianus* and *D. hansenii* in the culture broth at time 0 h according to the OD_{600}. The *K. marxianus*: *D. hansenii* mixture ratios were: 0.1:0.5, 0.1:0.2, 0.1:0.1, 0.2:0.1 and 0.5:0.1 and were identified as 1KM:5DH, 1KM:2DH, 1KM:1DH, 2KM:1DH and 5KM:1DH respectively. The co-culture assays were performed under aerobic fermentation of WY during 96 h at 25 °C and 200 rpm. The samples were taken at the same fermentation time as monoculture assays.

Kinetics of yeast growth and metabolite production

The growth rate was calculated for biomass concentration (dry basis) and cell density by counting plates (Lawford and Rousseau, 1993). Biomass concentration was used for the monocultures and cell density for the co-cultures. To determine the L-Phe consumption rate, data were fitted using zero-order reaction kinetics (Lawford and Rousseau, 1993; Villadsen et al., 2011).

The 2PE productivity was calculated by dividing the concentration of 2PE at 96 h by the elapsed time of the fermentation, in this case 96 h. Finally, the production yield was calculated as the ratio of the mass of 2PE to the mass of L-Phe consumed.

Sample handling

Samples of monoculture and co-culture assays were handled as follows: sample volumes of 1 mL were taken at the aforementioned times. Each sample was transferred to microtubes

and centrifuged (Minispin plus, Eppendorf) at 9660*x g* for 2 min. The supernatant was stored at -20 °C until analysis. Pellets were dried at 60 °C for 24 h to quantify biomass.

To determine the cellular density during co-culture assays, serial dilutions (from 10^{-1} to 10^{-8}) from samples of 1 mL were prepared and cultured in plates containing solid LM with 20 g/L of agar.

2.6 Preliminary identification of aroma and flavor metabolites using GC-MS

In the first stages, the identification of metabolites was performed at 72 h of fermentation using gas chromatography (Trace 1310, Thermo Scientific), coupled with a mass spectrometry detector (ISQ Thermo Scientific) (GCMS). The identification method was adapted from Leclercq-Perlat et al. (2004). The GCMS was equipped with a CP-Wax 57 CB (Agilent Technologies Inc.) column with a length of 25 m, an internal diameter of 0.25 mm, and a film thickness of 0.20 µm. The GC temperature for the injector and detector were both set to 240 °C. 0.7 µl of the undiluted sample was injected in the splitless mode. The oven temperature was held at 70 °C for 3 min, then it increased to 200 °C, at 5 °C/min, and finally held at 200 °C for 6 min. Helium at 103 kPa was used as a gas carrier, at a flow of 1 mL/min. To identify the biomolecules, the match factor, reverse match factor and probability in the library of the mass spectral database were calculated (Agilent Technologies, Inc).

Quantification of lactose, L-Phe and metabolites

Lactose was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, using a Surveyor Instrument) using the method reported earlier (Osorio-González et al., 2019). The LC-MS was equipped with a DF column (4.6×150 mm, 5 µm). A solution of acetonitrile:water (89:11) was used as mobile phase. The injection volume was 20 µL and

analytes were eluted at a flow rate of 0.3 mL/min. D6-glucose (20 µL/mL) was used as the internal standard and lactose concentration was calculated based on the analyte areas normalized to the areas of D6-glucose.

To determine the L-Phe concentration, the samples of culture broth were analyzed by Liquid chromatography – high resolution mass spectrometric (LC-HRMS). LC-HRMS analysis was executed on an Agilent 1290 Infinity II liquid chromatograph connected to QTOF 6545 (Agilent Technologies, ON, Canada). 2 x 150 mm, mixed-mode Scherzo SM-C18 column with 2 x 5 mm guard packed with 3 µm particles, (Imtakt, Portland, USA) at 35 °C and a flow rate of 0.2 mL/min were used for the analysis. The injection volume was 10 µL for all analyses. The gradient was generated using mobile phase A consisting of 5 mM ammonium formate in water and mobile phase B, consisting of 0.5% (v/v) formic acid in methanol. The run began with 2 min isocratic 100% A, followed by a linear increase to 24% B in 6 min and a further increase to 90% B in 0.1 min. 90%B was held for 3.9 min and the column was then re-equilibrated for 8 min to starting conditions of 100% A. Positive electrospray ionization was used for the analysis, with the capillary voltage set to 3500 V, nozzle voltage to 800 V, fragmentor voltage to 175 V, drying gas temperature to 250 °C and sheath gas temperature to 275 °C. Data were acquired in the 50-1000 m/z range at an acquisition rate of 3 spectra/s. The column output was directed into waste during two segments: (i) between 0 and 2 min and (ii) between 8.5 and 14.5 min to reduce contamination of the source. Phenylalanine quantitation was executed using Mass Hunter Quantitative Analysis (v. 10) software and weighted (1/x) calibration curves were built using the peak areas extracted with a mass accuracy window of 20 mg/L. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed throughout the sample analysis using signals

at m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (HP-921)).

The quantification of metabolites was determined using gas chromatography (Thermo Scientific), equipped with flame ionization detection (GC-FID). The quantification method was adapted from Dragon et al. 2009. The GC-FID was equipped with a Split/Splitless injector capillary column CP-Wax 57 CB (25 m, 0.25 mm and film thickness of 0.20 µm; Agilent Technologies Inc.). The temperature of the injector and detector was set to 250 °C. The oven temperature was held at 50 °C for 5 min, then it increased to 220 °C at 3 °C/min, and held at 220 °C for 10 min. Helium at 125 kPa was used as the gas carrier. 2 µl of the sample was injected in the splitless mode (vent time, 15 s); isobutanol (internal standard) was added to the sample at a concentration of 1 mg/L (Dragone et al., 2009). The volatile compounds were identified by comparing the retention times with those of standard compounds, and the quantification was performed using GC Agilent software.

Statistical analysis

Two-way ANOVA analysis was performed to determine if the 2PE production was a function of media composition and fermentation conditions (aerobic or anaerobic). Whereas for the co-cultures, an ANCOVA of one-factor analysis was performed to determine if the 2PE production was a function of co-culture ratios. Both ANOVA and ANCOVA analyses were run in R software using the concentration of 2PE as the response variable.

Results and discussion

The first stage of the study was the screening and identification of compounds with aroma and flavor properties produced for the whey fermentation.

Effect of the media composition on biomolecules production under monoculture fermentation mode

The analysis of aroma and flavor molecules of control helped distinguish the molecules produced by yeasts from those that were initially present in the culture media. The GC-MS method allowed semi-quantitative determination of each compound, using the relative area obtained by chromatogram integration and expressed with arbitrary units of counts*min. The spectrum that presented a probability higher than 80% for a specific compound was retained as the most likely compound.

Compounds present in the culture medium

The compounds identified in controls and culture broth for both aerobic and anaerobic fermentation are shown in Table 2.1. The controls for all culture media contained acetic acid, 5-3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one (3D6pyranone) and 5-hydroxymethylfurfural (5Hfurfural). The last one was the most abundant compound in LM and W media controls, presenting the highest relative area of $39.5x10^7$ counts*min in the W-aerobic control. Maltol was only found in W and WY media with relative areas of $0.2\pm0.03x10^7$ and $4.1\pm1.3x10^7$ counts*min, respectively; and 3D6pyranone was the most abundant in WY media with a relative area of $10.7\pm0.8x10^7$ counts*min. In general, acetic acid, 3D6pyranone, maltol and 5Hfurfural were produced under aerobic fermentation.

All the compounds shown in Table 2.1 have aroma and/or flavor properties. Specifically, the compounds identified in the controls are commonly associated with dairy products and they confer buttery, nutty or caramel flavors (Singla et al., 2018). These compounds are commonly

produced by the Maillard reaction, which occurs at temperatures from 100 °C to 149 °C. The generation of Maillard reaction products (MRP) occurs when lactose reacts with the amino groups of glycine, proline, lysine, arginine or histidine to form deoxy-ketoses. Subsequently, reduction reactions formed enol groups (alkenes with a hydroxyl group) in the carbonyl structure, resulting in furfural and pyranosyl type compounds (Singla et al., 2018). Huffman and Ferreira (2011) reported that the cheese whey naturally contains lactose and amino acids, such as lysine, arginine and histidine. In the present study, the concentration of the amino acids in the medium was increased by adding yeast extract and peptone. The enrichment in amino acids could increase the production of MRP, which was a function of media composition and sterilization conditions. According to Table 2.1, the relative area of acetic acid, 3D6pyranone, maltol and 5Hfurfural is higher under aerobic than under anaerobic sterilization conditions using in hermetic serologic bottles. This suggested that the content of oxygen increased the production of MRP. For example, Munanairi et al. (2007) observed that the production of MRP from ribose 5-phosphate was higher when it was transformed through aerobically thermochemical process (Munanairi et al., 2007).

Table 2. 1 Compounds with aroma and flavor properties identified in controls (sterilized and incubated) and metabolites found in culture broth under aerobic and anaerobic conditions at 72 h of fermentation.

Compounds	Relative area (counts*min) x $1x10^7$																	
	Aerobic									Anaerobic								
	Lactose medium			Cheese whey medium			Cheese whey with yeast extract and peptone medium			Lactose medium			Cheese whey medium			Cheese whey with yeast extract and peptone medium		
	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH
Maillard reaction products																		
Acetic acid	0.26	0.18	0.18	3.2	0.26	6.77	3.69	1.90	-	0.20	1.36	2.31	0.21	0.96	7.11	1.95	0.41	4.47
Maltol	-	-	-	0.22	0.11	1.36	5.41	-	-	-	-	-	0.15	0.05	9.75	2.73	-	5.86
3D6Mpyranone	2.47	1.35	3.40	9.47	0.52	10.60	11.60	0.60	0.98	0.32	1.47	3.32	1.99	0.21	9.53	9.90	0.57	11.70
5Hfurfural	19.60	-	-	39.50	5.24	1.42	4.92	-	-	105	-	-	16.70	1.25	1.65	4.41	-	4.18
4HDfuranone	0.21	-	-	-	-	-	0.59	-	-	-	-	-	-	-	-	0.30	-	-
2Hfuranone	0.87	-	-	4.91	0.15	4.91	3.93	-	0.14	1.20	-	-	-	0.76	-	1.71	4.16	1.09
1-hydroxy-2-propanone	-	-	-	-	-	-	0.46	-	-	-	-	-	-	-	-	0.16	-	0.56
Metabolites																		
Propanoic acid	-	0.69	0.33	-	0.76	-	-	4.55	0.19	-	-	-	-	-	-	-	-	-
Butanoic acid	-	0.14	-	-	-	0.85	-	-	-	-	-	-	-	-	1.09	-	0.34	-
Glycerol	-	-	-	-	12.20	-	-	10.90	-	-	-	-	-	27.70	-	-	21.20	-
Dihydroxyacetone	-	-	-	-	-	0.57	-	-	0.71	-	-	-	-	-	0.93	-	-	0.62
Methionol	-	-	-	-	-	-	-	0.19	-	-	-	-	-	-	-	-	-	-
Isopentanol	-	0.63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.22	-
2-phenylethanol	-	2.10	0.52	-	1.21		-	5.91	1.11	-	0.39	0.10	-	0.98	-	-	0.72	0.12

CT = control; KM = *Kluyveromyces marxianus*; DH = *Debaryomyces hansenii*; 3D6Mpyranone = 3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one; 5Hfurfural = 5-Hydroxymethylfurfural; 2Hfuranone = 2(3H)-Furanone,dihydro-4-hydroxy; 4HDfuranone = 4-Hydroxy-2,5-dimethyl-3(2H)-furanone

3.1.2. Compounds produced by fermentation

At 72 h of aerobic and anaerobic fermentation, the culture broth was analyzed by GC-MS to determine which compounds had been produced or consumed by the yeasts. At this point, the microbial growth for all the assays was at the beginning of the stationary phase, meaning that both primary and secondary metabolites should be present. According to Table 2.1, the most important compounds identified in the culture broth at 72 h were as follows:

Acetic acid. After anaerobic fermentation, the relative amount of acetic acid was higher than using aerobic fermentation for all media, except for WY with *K. marxianus*. In the pathway to produce acetic acid, yeasts consume lactose and transform it into glyceraldehyde-3-phosphate, then to pyruvate, and finally to ethanol and acetic acid. Under stressful conditions, such as limited oxygen, low pH and/or the presence of inhibitors (for example ethanol and MRP), the acetic acid is produced and accumulated in the culture media. Christensen et al. (2011) observed an accumulation of ethanol up to 24 g/L when *K. marxianus* was used to ferment whey at 32 °C for 50 h. This would cause the production of acetic acid, which accumulated at a concentration of 1 g/L (Christensen et al., 2011).

5Hfurfural. For LM and WY fermentations, 5Hfurfural was detected only for WY anaerobic fermentation with *D. hansenii*. The relative area of 5Hfurfural was similar to the respective control. For the W medium fermentation assays, it was smaller than in the controls. It is known that furfurals are inhibitors for both *K. marxianus* and *D. hansenii* yeasts (Duarte et al., 2005; Flores-Cosio et al., 2018). Nevertheless, as a stress-response, these yeasts produce enzymes able to attack pentoses present in the structure of furfurals (Duarte et al., 2005; Flores-Cosio et al., 2018). According to

Table 2.1, both yeasts could remove 5Hfurfural, which disappeared or decreased in concentration at 72 h for all fermentations.

Pyranosyl type compounds. 3D6Mpyranone is a pyranosyl type compound which was found in all assays. Its relative amount after fermentation was higher than in the controls for all assays using *D. hansenii*, except for WY media. Similarly, the relative amount of maltol, another pyranosyl type compound, was higher for W and WY anaerobic fermentation than in controls also using *D. hansenii*. *D. hansenii* could produce the homodimeric type III polyketide synthase. This enzyme is produced by means of the polyketide pathway and triggered by the presence of the lactose. The enzyme catalyzes the formation of multiple ketene groups and cyclization reactions of organic carbon structures. The polyketide synthase carboxylates the acetyl-coenzyme A to transform it into malonyl coenzyme A. Then, condensation chain reactions are initiated to cycle the malonyl coenzyme A. The final step involves enolization reactions to form structures such as 3D6Mpyranone and maltol (Saunders et al., 2015; Talapatra et al., 2015).

Propionic (PA) and Butanoic (BA) acids. Both yeasts produced PA, and BA under aerobic conditions, with PA being more abundant than BA in WY fermented by *K. marxianus* ($4.5x10^7$ counts*min). They are produced by the degradation of pyruvate by the acetyl CoA carboxylase (Chaturvedi et al., 2018; Hirst and Richter, 2016). Both PA and BA are flavor and aromatic compounds commonly found on the cheese surface during ripening. To the best of our knowledge, this is the first study reporting that *D. hansenii* and *K. marxianus* can specifically produce these acids.

Glycerol (GLY). Glycerol was only identified in W and WY fermented by *K. marxianus*. Glycerol was the most abundant metabolite, with relative areas in a range of $11.5\pm0.6x10^7$ to $24.4\pm3.2x10^7$

counts*min under aerobic and anaerobic conditions respectively. Its production is caused by an osmotic stress-response of *K. marxianus*. Glycerol is a cell-protector compound produced during the transformation of glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase (Saini et al., 2017). In the present study, the osmotic stress could be caused by the high initial lactose concentration in W and WY media (45 g/L), which was 2.25 fold times than the lactose concentration of LM (20 g/L). Also, the salts naturally present in the WP contributed to increasing the osmotic pressure. Some studies showed that increasing the initial lactose concentration from 50 g/L to 100 g/L induced the production of GLY by *K. marxianus*, which accumulated at a concentration of 2.2 g/L and 2.7 g/L, respectively (Beniwal et al., 2017; Saini et al., 2017).

Dihydroxyacetone (DHA). This compound was only produced by *D. hansenii* at an average relative area of $0.70\pm0.1x10^7$ counts*min for the assays with W and WY media. Some studies have reported that DHA is a by-product of GLY transformation. Similarly to *K. marxianus*, *D. hansenii* can produce and accumulate GLY to equilibrate the osmotic pressure of the environment. In the case of whey fermentation, *D. hansenii* could accumulate the GLY intracellularly as a response to the osmotic pressure produced by lactose and salts (Adler et al., 1985). In contrast with *K. marxianus*, *D. hansenii* can transform GLY when it is over accumulated, stimulating the transcription of four key enzymes: glycerol-3-phosphate dehydrogenase, dihydroxyacetone kinase, glycerol dehydrogenase and a phosphatase. The first enzyme catalyzes the production of glycerol-3-phosphate from dihydroxyacetone phosphate, which can be transformed into dihydroxyacetone by the dihydroxyacetone kinase. Then, the glycerol-3-phosphate is transformed into GLY by the phosphatase, and simultaneously the glycerol-dehydrogenase along with the kinase transform the intracellular accumulated GLY into DHA and dihydroacetone phosphate (Adler et al., 1985; Gori et al., 2007). This allows the production of dihydroxyacetone and prevents the intracellular

accumulation or the excretion of GLY. This phenomenon was observed for the first time by Adler et al. (1985) and later confirmed by Gori et al. (2007) when the NaCl at 8 % (wt/v) promoted the accumulation of GLY and DHA in *D. hansenii* cells (Adler et al., 1985; Gori et al., 2007).

Fusel alcohols. The production of 2PE, isopentanol and methionol was stimulated under aerobic conditions. 2PE was identified in all the fermentation assays performed with *K. marxianus*, whereas for *D. hansenii* the 2PE was only produced when LM and WY media were fermented. Isopentanol and methionol were produced by *K. marxianus* under aerobic conditions. The production of fusel alcohols is limited by the availability of amino acids, which are transformed into their respective fusel alcohol by the Ehrlich pathway (Hazelwood et al., 2008). All the media tested (LM, W and WY) contained L-Phe, leucine and methionine, which were provided by yeast extract, peptone and the WP itself (Huffman and Ferreira, 2011). These amino acids can be transformed into 2PE, isopentanol and methionol, respectively. In the fusel alcohols pathway, the pyruvate is transformed into α-ketoglutarate during the Krebs cycle. In this way, the production of fusel alcohols is stimulated by the presence of oxygen. The α-ketoglutarate acts as an electron acceptor from the transamination of amino acids, and subsequent decarboxylation and final dehydrogenation are carried out to produce the respective fusel alcohols (Hazelwood et al., 2008; Wittmann et al., 2002). Wittman et al. (2002) shown that the presence of α-ketoglutarate is necessary to produce 2PE from L-Phe during glucose fermentation (Wittmann et al., 2002).

The first stage determined the compounds that can be produced by whey fermentation with *K. marxianus* and *D. hansenii*. For the next stages of the present study, L-Phe consumption and the production of 2PE were monitored. Also, the isopentanol concentration through the kinetics was determined by GC method. Whereas, the production of methionol was not studied further because

it was only identified in WY aerobic fermentation by *K. marxianus*. GLY is not considered either an aromatic compound or a flavor, thus, in this study, its production was not followed further.

Whey fermentation under monoculture mode

Yeast growth

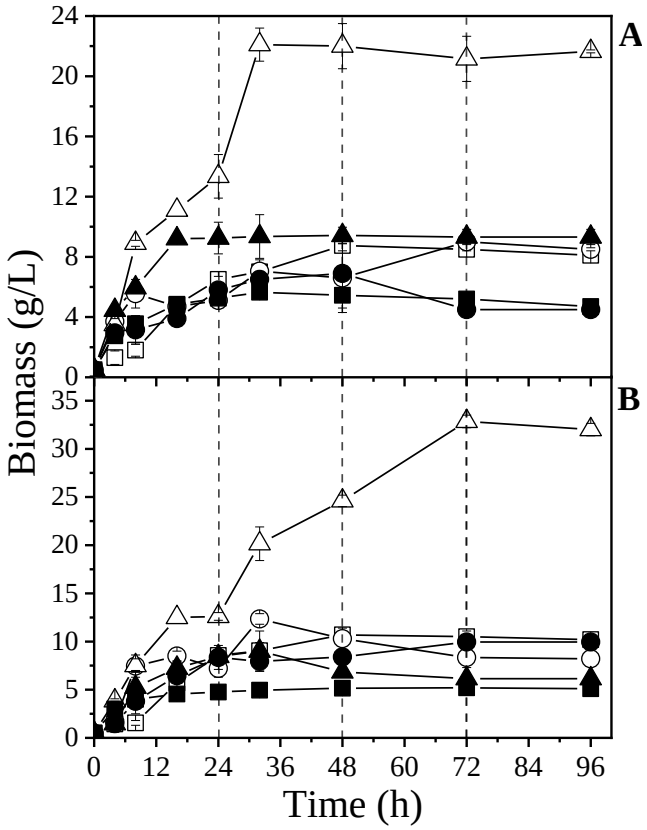

Figure 2. 1 Biomass production in culture broth of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY) using *Kluyveromyces marxianus* (A) and *Debaryomyces hansenii* (B) yeasts. Open symbols indicate the aerobic □-LM, ○-W and △-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and ▲-WY media fermentations.

Figure 2.1 shows the biomass concentration (dry basis) of *K. marxianus* and *D. hansenii* for aerobic and anaerobic fermentations under monoculture mode. For the aerobic assays, the exponential phase occurred from 8 h to 32 h, except for *D. hansenii* in WY, where the exponential phase ended

at 72 h. The anaerobic fermentation did not present a lag phase and the exponential phase finished within 24 h. For both yeasts, Table 2.2 shows the kinetics parameters of growth rate (μ), L-Phe consumption, maximum yield and maximum productivity of 2PE under the fermentation conditions. The μ for *D. hansenii* was always higher than for *K. marxianus*, except for LM-anaerobic, where it was nearly similar. Among all assays, the μ of *D. hansenii* (0.16 h^{-1}) calculated for WY medium fermentation was the maximum value observed.

The μ is a kinetics parameter defined by the strain and fermentation conditions. For WY-aerobic, the μ of both *K. marxianus* and *D. hansenii* strains was higher than for the rest of the assays. This shows that the aerobic fermentation of a culture medium enriched with organic nitrogen was favorable for *K. marxianus* and *D. hansenii* growth. The μ for *D. hansenii* fermenting glucose (20 g/L) was 0.21 h^{-1} (Breuer and Harms, 2006; Neves et al., 1997), and for *K. marxianus* fermenting lactose (50 g/L) at 35 °C, was 0.55 h^{-1}. (Saini et al., 2017).

The μ obtained in this study was smaller than the μ of *D. hansenii* and *K. marxianus* of other studies from 0.21 to 0.55 h^{-1} (Breuer and Harms, 2006; Neves et al., 1997; Saini et al., 2017). However, the μ of the present study was not obtained under optimal conditions. The highest biomass concentration was observed for WY-aerobic, which can be explained by the enrichment of the medium with yeast extract and peptone as an organic nitrogen source.

For almost all fermentations, the biomass concentration after reaching the stationary phase was in the range of 5.25 to 12.35 g/L. However, for WY-aerobic using either *K. marxianus* or *D. hansenii* the biomass concentration was higher, 22 g/L and 33 g/L respectively. This can be explained by the combination of a rich media and oxygen availability. The electrons donated by the oxidation of lactose are used to produce energy which is mainly used for cell synthesis (Christensen et al., 2011;

Rittman and McCarty, 2001), or store it under chemical bonds (example ATP). This suggests that under aerobic fermentation *K. marxianus* and *D. hansenii* used energy for cell synthesis.

In the case of WY-aerobic using *D. hansenii*, the highest biomass concentration of 33 g/L can be explained by the effect of a high concentration of lactose and salts in media. Generally, *K. marxianus* excretes the glycerol, while *D. hansenii* accumulates it inside the cells. For example, some studies show that *D. hansenii* could accumulate up to 0.34 $g_{GLY}/g_{biomass}$ when the concentration of NaCl was 16 % wt/v (Adler et al., 1985).

The DHA is also formed using the glycerol pathway, where DHA can be transformed into glycerol utilizing the glycerol dehydrogenase and the coenzyme NADP/NADPH. In the present study, the DHA concentration was lower than 82 mg/L except for the W fermentation using *D. hansenii*, where it was 1719±328 and 1876±114 mg/L under aerobic and anaerobic conditions respectively. The W medium was the only one not enriched with peptone and yeast extract. This suggests that the production of energetic molecules was limited, and as a consequence, the DHA was not converted into glycerol, and was excreted (Adler et al., 1985; Gori et al., 2007).

Table 2. 2 Overall performance of yeasts during the LM, W and WY media fermentations assays using the monocultures.

Medium Fermentation	Initial lactose concentration (g/L)	Lphe concentration (mg/L)			Growth rate - μ (h^{-1})		L-Phe consumption rate ($mg_{L\text{-}Phe}/L*h$)		Maximum yield production ($g_{2PE}/g_{L\text{-}Phe}$)		Maximum productivity ($mg_{2PE}/L*h$)	
		Initial	At 72 h for KM	At 72 h for DH	KM	DH	KM	DH	KM	DH	KM	DH
LM-aerobic	20	491	237.5±43.1	350.9±15.50	0.06	0.09	5.41±0.71	3.54±0.26	0.20±0.01	0.11±0.01	0.52 ± 0.05	0.16±0.01
LM-anaerobic	20	491	316.7±27.3	386.4±1.20	0.08	0.07	3.35±0.11	2.26±0.25	0.05±0.01	0.01±0.00	0.09 ± 0.05	0.02±0.00
W-aerobic	45	6.7	0	0	0.06	0.09	0.21±0.00	0.21±0.00	0.76±0.01	0.38±0.03	0.05± 0.00	0.03±0.01
W-anaerobic	45	6.7	0	0	0.08	0.11	0.21±0.00	0.21±0.00	0.55±0.1	0.30±0.07	0.04±0.01	0.02±0.00
WY-aerobic	45	494	0	132.4±75.8	0.13	0.16	7.04±0.00	5.27±1.16	0.16±0.02	0.11±0.04	0.86±0.18	0.42±0.06
WY-anaerobic	45	494	352.9±5.7	410.6±6.6	0.10	0.11	1.20±0.04	1.58±0.62	0.11±0.01	0.04±0.01	0.16±0.01	0.06±0.01

LM = Lactose medium; W = cheese whey medium; WY = cheese whey medium enriched with yeast extract and peptone

L-Phe consumption and alcohols production of 2PE and ethanol by *Kluyveromyces marxianus*

Figure 2.2 shows the production of 2PE and ethanol for aerobic and anaerobic fermentation using *K. marxianus*. The aerobic condition stimulated the production of 2PE by *K. marxianus*, especially for WY fermentations. For this case, the highest production of 2PE was observed, 2.46±0.70 mg_{2PE} at 96 h (corresponding to a concentration of 82.12±16.88 mg/L). At this point, *K. marxianus* also presented a maximum 2PE yield of 0.16±0.03 $mg_{2PE}/mg_{L\text{-}Phe}$ and the highest L-Phe consumption rate of 7.04 $mg_{L\text{-}Phe}$/L*h (Table 2.2). In contrast to kinetics parameters such as productivity, the production yield is not a good indicator of metabolite production for 2PE. For example, for W media, the yields for aerobic and anaerobic fermentations were 0.75 and 0.55 $g_{2PE}/g_{L\text{-}Phe}$, respectively. However, the L-Phe consumption rate for W-aerobic was the lowest value. Additionally, for W and WY aerobic and W-anaerobic fermentation, the L-Phe was completely consumed, whereas, for the LM-aerobic, LM-anaerobic and WY-anaerobic, the L-Phe was partially consumed, corresponding to 51.6, 35.5 and 29.3% of the initial concentration of L-Phe 492.3±1.3 $mg_{L\text{-}Phe}$ (equivalent to a mass of 14.8±0.0 $mg_{L\text{-}Phe}$), as expressed in Table 2.2.

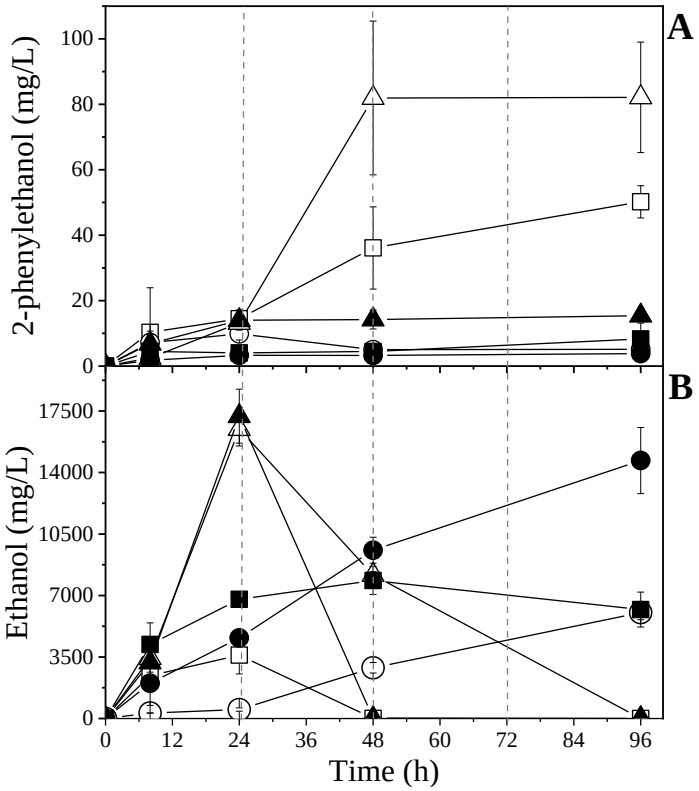

Figure 2. 2 Production of alcohols by *Kluyveromyces marxianus* monocultures during the fermentations of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W and △-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and▲- WY media fermentations

The ethanol concentration was higher for the anaerobic assays than for the aerobic ones. The highest ethanol concentration was observed around the end of the exponential growth phase in all cases. Maximum ethanol concentrations corresponded to WY-aerobic and anaerobic with 16.5±2.3 and 17.2±1.5 $g_{ethanol}$/L respectively. After the maximum accumulation, the ethanol was quickly consumed decreasing nearly to 0 $g_{ethanol}$/L at 48 h. The opposite pattern was observed for the W medium, where ethanol concentration continued increasing.

Similar to biomass production, the L-Phe consumption was affected by the dissolved oxygen and the media composition. For example, under aerobic conditions, the consumption of L-Phe was

complete for WY and W media. This consumption can occur as follows: lactose is oxidized to pyruvate which is further oxidized by the Krebs cycle to produce α-ketoglutarate which enters the Ehrlich pathway where the L-Phe is transformed to form phenylpyruvate. Then, it is decarboxylated producing phenylacetaldehyde, and reduced on 2PE, ending the reaction. This last step involves the use of a reductase or an alcohol dehydrogenase to catalyze the aldehyde reduction coupled with the oxidation of NAD(P)H to $NAD(P)^+$. Hence, the production of 2PE is an energy consumption step without being essential for cell growth. 2PE is a secondary metabolite, which accumulates at the end of the exponential growth phase; for this reason, in the present study, it appeared after 24 h. Since aerobic metabolism produces more energetic molecules than the anaerobic one (Hazelwood et al., 2008; Wittmann et al., 2002), the aerobic consumption rate of L-Phe was faster than the anaerobic one.

The low 2PE yield for the LM and WY assays shown in Table 2.2 can be attributed mainly to the presence in the culture broth of amino acids other than L-Phe. These amino acids will also be transformed through the Ehrlich pathway into their respective fusel alcohols. For example, the isopentanol which is produced from leucine was accumulated in higher concentration than 2PE under aerobic conditions, from 120.0±13.5 to 200.1±23.8 mg/L (data not shown), which corresponds to a mass from 3.6±0.4 to 6.0±0.7 $mg_{isopentanol}$, respectively. Additionally, under aerobic conditions the 2PE can be transesterified into 2-phenylethylacetate, decreasing its concentration in the media. The ester is formed in the presence of Acetyl CoA and an alcohol dehydrogenase which uses 2PE as a precursor (Etschmann et al., 2005; Wittmann et al., 2002). Wittman et al. (2002) studied the 2PE and 2-phenylethylacetate production during the fermentation of glucose (77 g/L) and of L-Phe (7 g/L) using *K. marxianus*, with resulting yields of 0.65 $g_{2PE}/g_{L\text{-}Phe}$ and 0.08 $g_{2\text{-}phenylethylacetate}/g_{L\text{-}Phe}$ (Wittmann et al., 2002).

In the present study, the WY-aerobic presented the highest content of 2PE. This medium contained 45 g/L of lactose and it was supplemented with an external nitrogen source. The combination of both lactose content and high nitrogen contributed to obtaining the highest production of 2PE (0.86 mg_{2PE}/L*h).

Lactose concentration was monitored for this WY medium only (data not shown), and at 24 h it was nearly 0 g/L. Then, *K. marxianus* should begin to consume ethanol as a carbon source, and as shown in Figure 2.2A this time corresponding to the end of *K. marxianus* exponential growth phase. This observation confirms that 2PE is associated with the stationary phase, where secondary metabolites are produced. Wittman et. al. (2002) studied glucose fermentation using *K. marxianus*. They observed that *K. marxianus* produced ethanol, glycerol and pyruvate. When glucose was depleted at 28 h of fermentation, ethanol, glycerol and pyruvate were used as a carbon source (Wittmann et al., 2002). Assuming that ethanol was consumed because lactose was depleted, the continued accumulation of ethanol for W may indicate that the lactose was not depleted during the 96 h of fermentation.

L-Phe consumption and alcohols production of 2PE and ethanol by *Debaryomyces hansenii*

Figure 2.3 shows the 2PE and ethanol production by *D. hansenii* during the aerobic and anaerobic assays. For the aerobic assays of W and WY and W-anaerobic, the L-Phe was totally consumed at 96 h and the faster consumption (5.73 $mg_{L\text{-}Phe}$/L*h) was observed for WY-aerobic. For the LM-aerobic and LM-anaerobic and WY-anaerobic, the initial concentration of L-Phe (492.3±1.3 $mg_{L\text{-}Phe}$/L) decreased by 28, 21.3 and 33.9% respectively.

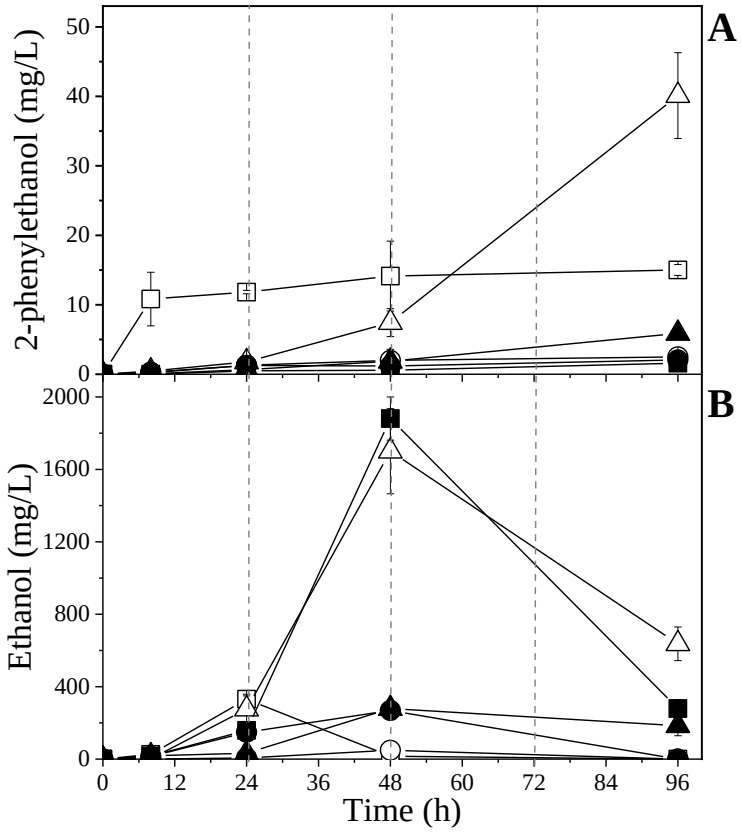

Figure 2. 3 Production of alcohols by *Debaryomyces hansenii* monocultures mode during the fermentation of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W and △-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and ▲- WY media fermentations

The highest 2PE concentration (40.1±6.17 mg/L) occurred at 96 h of WY-aerobic (Figure 1.3). For the ethanol concentration, it was lower than 300 mg/L for all assays except for LM-anaerobic and WY-aerobic, which presented 1880.89±0.12 and 1700.38±0.23 mg/L at 48 h respectively.

Figures 2.2 and 2.3 show that the metabolism of *D. hansenii* behaved differently from *K. marxianus* for the same fermentation conditions. The L-Phe was consumed similarly by both yeasts, but the consumption rate was slower for *D. hansenii*. Apparently, in this yeast the lactose carbon was mainly derived to biomass synthesis (yield of 0.73 $g_{biomass}/g_{lactose}$), instead of ethanol (0.04 $g_{ethanol}/g_{lactose}$), which was observed at a lower concentration than in *K. marxianus* assays. As

mentioned before, the 2PE is a secondary metabolite, and it could be detected after 24 h or 48 h of fermentation. Its accumulation was more pronounced when ethanol was consumed, maybe because the availability of lactose decreased, which could be associated with inducing 2PE formation.

The statistical analysis (two-way ANOVA, $\alpha = 0.95$, indicated in Table 2.3) confirmed that the *K. marxianus* strain presented the highest 2PE production when grown in WY under aerobic conditions. For this reason, WY-aerobic was selected to perform the next step of the co-culture of *K. marxianus* and *D. hansenii*.

Table 2. 3 Two-Way ANOVA table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under monoculture mode. Dependent variable: 2-phenylethanol concentration

Source of variation: *Medium and fermentation conditions*	*Yeast strain*	*Pr(>|t|)*	Residual standard error	Degrees of freedom	Multiple R-squared	Adjusted R-squared	*P*-value
LM-Aerobic	KM	$2.25x10^{-5}$*	9.216	96	0.79	0.74	$2.2 x10^{-16}$
	DH	0.089					
LM-Anaerobic	KM	0.676					
	DH	0.891					
W-aerobic	KM	0.784					
	DH	0.938					
W-anaerobic	KM	0.682					
	DH	0.936					
WY-aerobic	KM	0.001*					
	DH	$3.33x10^{-12}$*					
WY-anaerobic	KM	0.209					
	DH	0.979					

**Significant at 5% level ($P \leq 0.05$). Pr(>|t|); proportion of the t distribution at that degree of freedom which is greater than the absolute value of t sta- tistic. KM; Kluyveromyces marxianus, DH; Debaryomyces hansenii.*

Whey fermentation under co-culture mode

Growth performance and ecological behavior

Figure 2.4 shows the biomass concentration (dry basis) of the co-cultures assays as a function of time under the WY-aerobic. The growth pattern was similar for the five ratios studied, with

exponential growth phase up to 72 h. The 1KM:1DH ratio presented the highest biomass concentration of 25.25±0.75 g/L.

Table 2.4 shows the μ, cell density, L-Phe consumption rate, 2PE productivity and 2PE and ethanol yield under monoculture and co-culture modes. The μ was calculated by cellular counting, and the μ of co-cultures was always lower than in monocultures, except for 1KM:1DH, where it was higher. The yeast colonies were easy to differentiate. Figure 2.5 shows the normalized concentration of *K. marxianus* and *D. hansenii* as $\log_{10}$ of cell density at any time/cell density at an initial time for monoculture and co-culture.

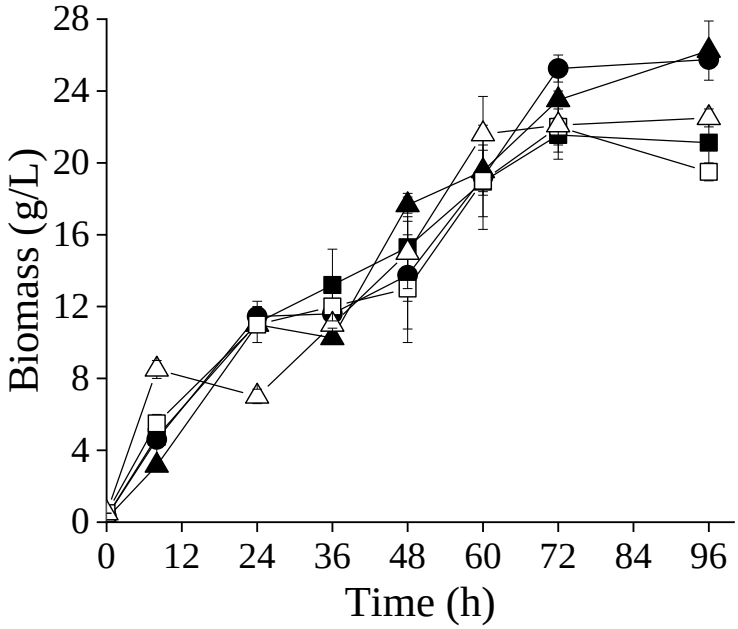

Figure 2. 4 Kinetics of biomass production of co-cultures for the cheese whey supplemented with yeast extract and peptone medium (WY) fermentation under aerobic conditions. Symbols indicate the performance of □-1KM:5DH, ■-1KM:2DH, ●-1KM:1DH, ▲-2KM:1DH, and △-5KM:1DH

Table 2. 4 Overall performance of yeasts during the fermentations assays for WY media using the monocultures and co-cultures.

Parameter	Culture						
	KM	**DH**	**1KM:5DH**	**1KM:2DH**	**1KM:1DH**	**2KM:1DH**	**5KM:1DH**
Growth rate - μ (h^{-1})	0.13	0.16	KM=0.01 DH=0.06	KM=0.14 DH=0.06	KM=0.21 DH=0.13	KM=0.08 DH=0.12	KM=0.10 DH=0.00
CD ($1x10^7 CFU_{KM}/mL)_{0h}$	1.60±0.10	NA	1.50±0.04	1.20±0.10	1.20±0.00	2.41±0.20	3.90±0.20
CD ($1x10^7 CFU_{KM}/mL)_{8h}$	11.80±1.20	NA	20.00±0.10	31.00±3.00	10.00±0.13	5.00±0.01	41.00±2.30
CD ($1x10^7 CFU_{KM}/mL)_{24h}$	111.00±3.00	NA	30.00±0.00	49.50±3.00	221±0.1	27.00±6.00	45.00±5.00
CD ($1x10^7 CFU_{KM}/mL)_{48h}$	880.00±70.00	NA	65.00±5.00	49.20±3.00	83.00±2.00	116.00±6.00	920.00±0.50
CD ($1x10^7 CFU_{KM}/mL)_{96h}$	56.50	NA	94.00±6.00	43.50±4.5	72.5±0.1	11.90±0.50	61.00±13.00
CD ($1x10^7 CFU_{DH}/mL)_{0h}$	NA	1.40±0.10	6.00±0.05	1.97±0.10	1.51±0.00	1.51±0.00	1.60±0.01
CD ($1x10^7 CFU_{DH}/mL)_{8h}$	NA	20.00±4.20	21.00±2.00	11.00±2.00	35.00±0.13	7.00±0.01	0.10±0.01
CD ($1x10^7 CFU_{DH}/mL)_{24h}$	NA	95.00±3.00	35.00±5.00	15.00±0.00	49.50±2.50	32.00±1.00	0.00±0.00
CD ($1x10^7 CFU_{DH}/mL)_{48h}$	NA	265.00±6.50	115.00±5.00	13.00±3.00	20.00±2.00	23.00±3.00	0.00±0.00
CD ($1x10^7 CFU_{DH}/mL)_{96h}$	NA	99.00±8.00	0.00±0.00	6.50±0.50	2.00±0.01	1.00±0.50	0.00±0.00
L-Phenylalanie consumption rate ($mg_{Lphe}/L*h$)	7.04±0.00	5.27±1.16	4.54±0.00	9.58±0.00	9.54±0.00	9.48±0.00	10.38±0.00
2-phenylethanol productivity ($mg_{2PE}/L*h$)	0.86±0.18	0.42±0.06	1.46±0.22	1.63±0.03	1.30±0.10	1.41±0.18	1.93±0.02
2-phenylethanol yield production (g_{2PE}/g_{Lphe})	0.16±0.02	0.11±0.04	0.27±0.03	0.31±0.08	0.26±0.02	0.28±0.04	0.38±0.00
Ethanol productivity ($mg_{ethanol}/L*h$)	0.69±0.04	0.04±0.00	0.56±0.01	0.62±0.01	0.16±0.01	0.55±0.02	0.64±0.02
Ethanol yield production ($g_{ethanol}/g_{lactose})_{24h}$	0.36±0.00	0.04±0.00[α]	0.30±0.00	0.33±0.00	0.17±0.00[α]	0.29±0.00	0.34±0.00

CD= Cell density; NA= Not applicable.
[α]The value was obtained at 48 h of fermentation

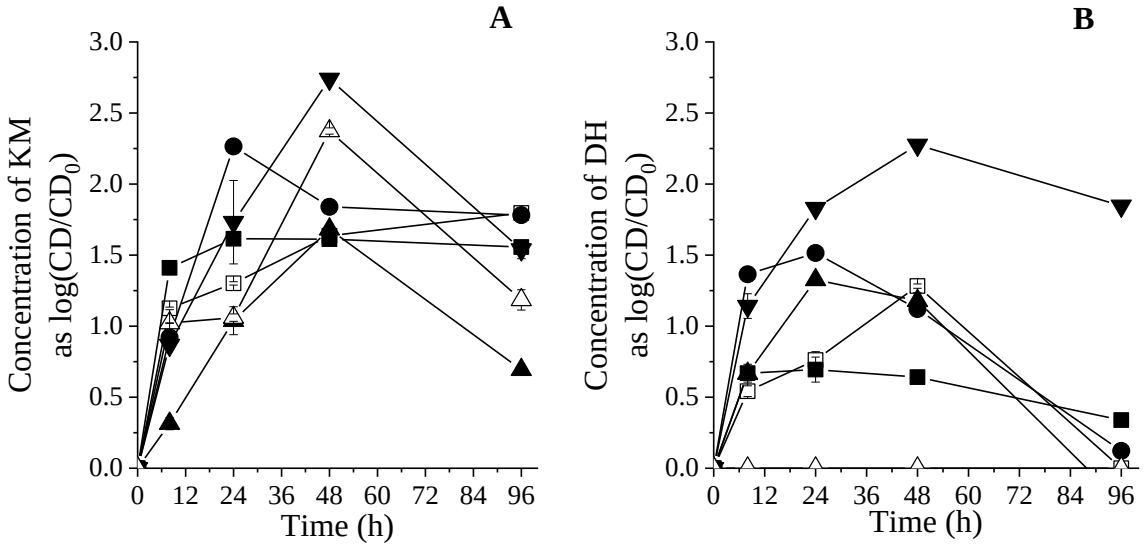

Figure 2. 5 Growth of (A) *Kluyveromyces marxianus* and (B) *Debaryomyces hansenii* as monoculture and co-culture mode for cheese whey supplemented with yeast extract and peptone (WY) medium-aerobic. Symbols indicate the performance of ▼-KM or DH monoculture, ▽-DH monoculture, □– 1KM:5DH, ■–1KM:2DH, ●– 1KM:1DH, ▲– 2KM:1DH, △–5KM:1DH.

In general, *K. marxianus* showed a higher growth rate than *D. hansenii*, even for 1KM:5DH, where DH was inoculated with a concentration 5 times higher than *K. marxianus*. For 1KM:5DH, the final cell density of *D. hansenii* was higher than *K. marxianus*, but *K. marxianus* increased its initial cell density ($1.50\pm0.04x10^7$ CFU/mL) up to 1.63 times, whereas, *D. hansenii* increased its initial cell density ($6.00\pm0.05x10^7$ CFU/mL) by only 1.28 times. Moreover, when the inoculum of *K. marxianus* was 5 times higher than *D. hansenii* (5KM:1DH), the *K. marxianus* yeast dominated from the beginning of the fermentation, and no colonies of *D. hansenii* were observed on the plates.

These results suggest that the interactions between the two yeasts are a competitive relationship. Several reports indicate the production of mycocins by both yeasts. Mycocins are extracellular proteins that can inhibit the β-glucan synthesis, a key compound in the

structure of the cell wall. Also, mycocins interfere with the synthesis of genetic material and consequently with cell division, producing the known killer phenomenon (Banjara et al., 2016; Nascimento et al., 2020). Banjara et al. (2016) observed the production of mycocins by *D. hansenii* strains which were able to inhibit *Candida albicans* and *C. tropicalis* yeasts, especially under pH conditions from 4.5 to 5.5 and temperatures from 25 °C to 30 °C (Banjara et al., 2016). In addition, Chen et al. (2015) studied the production of mycocins by *K. marxianus* at pH 2 and 8, and the inhibitory effect on *Escherichia coli* was mainly observed in a temperature range from 25 °C to 45 °C (Chen et al., 2015). This suggests that *K. marxianus* and *D. hansenii* could also produce mycocins as a survival mechanism and predominate in the environment.

2PE and ethanol production under co-culture mode.

Figure 2.6 shows the production of 2PE and ethanol by the five co-culture ratios. For all assays, L-Phe was depleted and the L-Phe consumption rate for 5KM:1DH was the fastest (10.38 $mg_{L\text{-}Phe}$/L*h), followed by 1KM:2DH (9.58 $mg_{L\text{-}Phe}$/L*h).

2PE production was higher for co-cultures than for monocultures. The highest productivity was observed for 5KM:1DH (1.93±0.02 mg_{2PE}/L*h), which corresponded to the highest mass of 2PE accumulated in the culture broth of 5.57±0.07 mg_{2PE} (corresponding to a concentration of 185.72±2.28 mg/L). Figure 5A shows that at 48 h *K. marxianus* and *D. hansenii* yeasts in co-culture mode produced a 2PE mass of 2.39±0.07 mg_{2PE} or higher, in comparison to the monoculture mode using *K. marxianus*, which took 96 h to produce 2.46±0.70 mg_{2PE} (Figure 2).

For all co-culture assays, an ethanol accumulation of 396.5±17.9 to 458.1±11.1 $mg_{ethanol}$ (corresponding to a concentration of 13217.7±599.9 to 15276±367.9 mg/L) was observed at 24 h, except for 1KM:1DH, where an ethanol mass of 226.8±14.7 mg was observed at 48 h (concentration of 7560±490 mg/L). The highest ethanol mass (458.1±11.1 mg) was observed for 5KM:1DH, corresponding to a yield of 0.34 $g_{ethanol}/g_{lactose}$. Moreover, for all co-culture assays, the lactose was depleted at 24 h, except for 1KM:1DH, where it occurred at 48 h (data not shown).

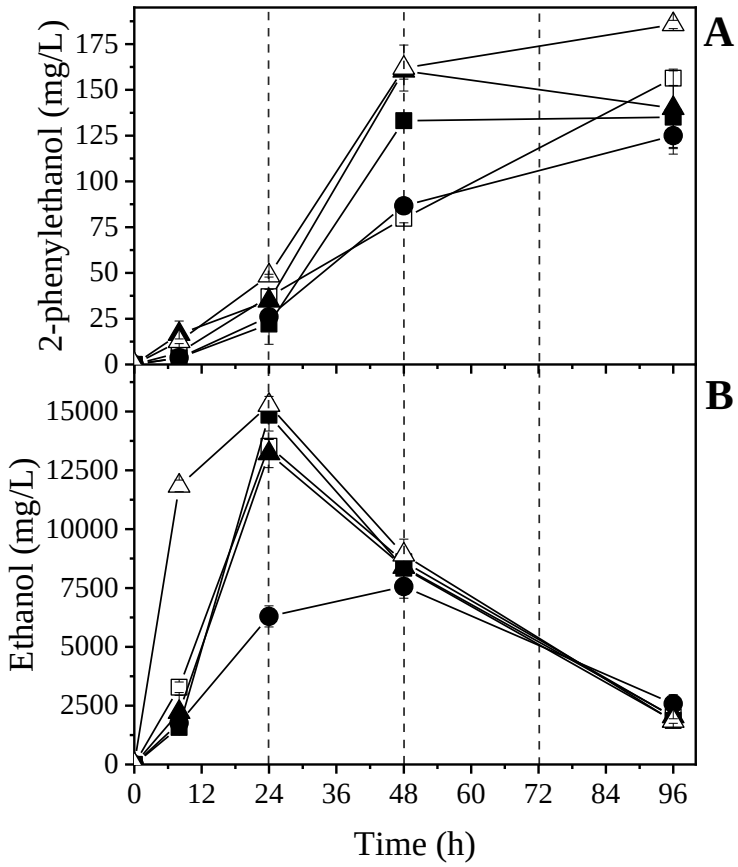

Figure 2. 6 Production of alcohols during the aerobic cheese whey supplemented with yeast extract and peptone (WY) medium fermentation using the co-cultures. Symbols indicate the performance of □– 1KM:5DH, ■–1KM:2DH, ●–1KM:1DH, ▲–2KM:1DH, △–5KM:1DH.

The use of co-cultures increased the L-Phe consumption rate. Both yeasts consumed nutrients, such as lactose and organic nitrogen, and *K. marxianus* had an advantage over *D. hansenii* because of its faster growth (defined by μ) in comparison to *D. hansenii* during the co-cultures assays. Also, *K. marxianus* has a higher lactose oxidation capacity than *D. hansenii*, allowing *K. marxianus* to produce more energy to transform L-Phe into 2PE. Rodrigues et al. (2016) studied a co-culture using *Sacharomyces cerevisae* and *Kluyveromyces lactis* for a whey and carob sugars mix fermentation. They observed a faster and complete depletion of sugars when they used the co-culture instead of monoculture fermentation (Rodrigues et al., 2016).

Since 2PE is a secondary metabolite induced by the presence of L-Phe, the presence of L-Phe in the culture broth is necessary. When this amino acid is consumed, its amine is incorporated into the essential metabolic pathways of yeasts, such as for protein formation, biomass synthesis or energy production (Krebs cycle). In addition, in co-culture mode both yeasts compete for lactose consumption, and this could quickly energize the cells. All this led to higher 2PE production than the sum of individual production with *K. marxianus* and *D. hansenii* in monoculture mode.

The statistical analysis (ANCOVA of one factor, $\alpha = 0.95$, indicated in Table 2.5) showed that the yeasts growing under co-culture mode and WY-aerobic were the best conditions for the production of 2PE, especially the 5KM:1DH. According to the statistical analysis, the cumulative production of 2PE was a function of biomass content.

Table 2. 5 ANCOVA table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. Dependent variable: 2-Phenylethanol concentration

Source of variation: *Medium and fermentation conditions*	*Yeast strain*	*Pr(>|t|)*	Residual standard error	Degrees of freedom	Multiple R-squared	Adjusted R-squared	*P*-value
WY-aerobic	KM	2.74 × 10-4*	19.42	102	0.879	0.84	2.2 x10^{-16}
	DH	0.125					
	1KM:5DH	1.15 × 10-5*					
	1KM:2DH	2.85 × 10-7*					
	1KM:1DH	4.58 × 10-6*					
	2KM:1DH	1.43 × 10-7*					
	5KM:1DH	7.78 × 10-10*					

*Significant at 5% level ($P \leq 0.05$). Pr(>|t|); proportion of the *t* distribution at that degree of freedom which is greater than the absolute value of *t* statistic. KM; *Kluyveromyces marxianus*, DH; *Debaryomyces hansenii*.

In contrast, ethanol production was not affected by either biomass or cell density. Table 2.6 shows the studies where whey was fermented by yeast with the aim to produce 2PE. It is expressed by the 2PE yield obtained in other studies compared to the present study. The present study can be a competitive process since the whey was not supplemented directly with L-Phe and both yeasts produced 2PE. Additionally, the use of the co-culture mode improved the transformation of L-Phe into 2PE in comparison with the monocultures used in this study and in Leclerq-Perlat et al. (2004).

This behavior has been observed previously, showing that the ethanol yield varies slightly when single yeasts are grown under co-culture mode. Rodrigues et al. (2016) observed an ethanol yield of 0.40 $g_{ethanol}/g_{sugars}$ for *S. cerevisae* and *K. lactis* under both monoculture and co-culture conditions (Rodrigues et al., 2016). Once lactose is consumed, the yeasts will use

other carbon sources present in the culture broth, such as ethanol and glycerol. Both yeasts will interact to consume these molecules and survive.

Table 2. 6 Fermentation of whey using yeasts to produce 2-Phenylethanol

Culture media	Microorganisms	T (°C)	pH	Time (h)	Mode operation	Yield ($g_{2PE}/g_{L\text{-}Phe}$)	References
Whey + $(NH_4)_2SO_4$ + Lphe	*Kluyveromyces marxianus*	30	4.8	96	Batch	0.75	Conde-Báez et al., 2019;
Whey +Beet sucrose +$(NH_4)_2SO_4$ +Lphe	*Saccharomyces cerevisae*	30	5.4	72	Batch	0.65	Chreptowicz et al., 2018;
Whey	*Debaryomyces hansenii*	25	4.8	96	Batch	0.06	Leclerq-Perlat et al., 2004;
Whey	*Kluyveromyces marxianus*	30	4	NR	Continuous	Not reported	Dragone et al., 2009
Whey + Yeast extract and Peptone	*Kluyveromyces marxianus*	25	4.5	96	Batch/aerobic Batch/anaerobic	0.16 0.11	This study
Whey + Yeast extract and Peptone	*Debaryomyces hansenii*	25	4.5	96	Batch	0.11 0.04	This study
Whey + Yeast extract and Peptone	Co-culture of *Kluyveromyces marxianus* and *Debaryomyces hansenii*	25	4.5	96	Batch	0.32	This study

NR = Not reported

For example, *K. marxianus* released glycerol which could be consumed by *D. hansenii*. Then, *D. hansenii* produced and released, ethanol, which could be used by *K. marxianus* as an alternative carbon source. This could cause a decrease in ethanol in the culture media after 24 h, and even its complete depletion. The ethanol consumption can occur by alcohol dehydrogenase which reduces a NAD^+ enzyme to NADH, where the ethanol is transformed into an aldehyde. This reaction releases energy which is used for cell preservation and biomass synthesis (Hazelwood et al., 2008; Rittman and McCarty, 2001). For this reason, the

use of co-cultures could improve the lactose and L-Phe consumption, and their transformation into 2PE.

Conclusions

Whey fermentation using yeasts to produce compounds with aromatic and flavoring properties was studied. The production of Maillard reaction products (MRP) was observed in sterilized media, probably due to the thermal reactions. Their relative abundance varied based on the content of oxygen, lactose and amino acids on the medium. The MRP identified were 5-hydroxymethylfurfural (5Hfurfural), pyranosyl type compounds and acetic acid. The content of the MRP was modified by yeast fermentation. For example, 5Hfurfural was consumed. The enriched fermentation broth matrix produced aromatic and flavoring compounds, such as organic acids, dihydroxyacetone, isopentanol and 2-phenylethanol which were produced and excreted by both yeasts. The biomass synthesis and metabolite production were affected by the media composition, oxygen availability, yeast strain and culture mode. Thus, in the present study, the aerobic fermentation of whey powder enriched with yeast extract and peptone (as organic nitrogen sources) using *Kluyveromyces marxianus* and *Debaryomyces hansenii* at a mixture ratio of 5:1 resulted in the best conditions to produce 2PE. Under these operating conditions, the 2PE yield was 0.16±0.03 $g_{2PE}/g_{L\text{-}Phe}$, corresponding to a productivity of 1.93±0.02 $mg_{2PE}/L*h$, which is competitive compared with studies reported in the literature using glucose as a substrate. The present study shows that the valorization of whey into value-added biomolecules has great potential.

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CHAPTER TWO – PART 2.

Effect of the concentration of L-Phenylalanine and lactose on 2-Phenylethanol production by whey fermentation using the yeasts *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode

Mariana Valdez Castillo[1], Vinayak Laxman Pachapur[1], Satinder Kaur Brar[1,2], Sonia Arriaga[3], Jean-François Blais[1], Antonio Avalos Ramirez[1,4]

[1] Institut national de la recherche scientifique, Centre-Eau Terre Environnement, 490, Rue de la Couronne, Québec, Québec G1K9A9, Canada.

[2] Department of Civil Engineering, Lassonde School of Engineering, York University, Toronto, Ontario M3J 1P3, Canada.

[3] Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4a Sección 78216, México.

[4] Centre National en Électrochimie et en Technologies Environnementales Inc. 2263, avenue du Collège, C.P. 610, Shawinigan (Québec) G9N 6V8, Canada.

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Résumé

Le lactosérum est un sous-produit généré par l'industrie laitière qui contient du lactose et des protéines. En tant que méthode de valorisation, la fermentation est une pratique biotechnologique permettant d'obtenir des biomolécules à haute valeur ajoutée à partir du lactosérum, comme le 2-phényléthanol (2PE). Trois méthodes de stérilisation ont été évaluées afin de déterminer laquelle était la plus appropriée pour la fermentation du lactosérum en utilisant *Kluyveromyces marxianus* et *Debaryomyces hansenii* en mode de co-culture. Ensuite, l'effet des concentrations initiales de lactose et de L-phénylalanine (L-Phe) sur la production de 2PE a été étudié en utilisant un plan composite central. La concentration initiale de lactose et de L-Phe de 40 g/L et 4 g/L respectivement, a permis d'obtenir la plus forte concentration de 2PE (2.55 ± 0.12 g/L). Le principal effet du lactose sur la production de 2PE a été l'induction vigoureuse de la croissance de la levure en 24 h de fermentation. La présence de L-Phe dans le milieu de culture a favorisé la production de 2PE pendant le métabolisme secondaire de la levure, correspondant à la phase de croissance stationnaire.

Mots clés : Valorisation du lactosérum doux, fermentation, co-culture de levures, alcools de fusel, 2-phényléthanol

Abstract

Whey is a by-product generated by the dairy industry which contains lactose and proteins. As a valorization method, fermentation is a biotechnological practice to obtain high value-added biomolecules from whey, such as 2-phenylethanol (2PE). Sterilization methods were assessed to determine which was more appropriate for whey fermentation using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. Then, the effect of lactose and L-Phenylalanine (L-Phe) initial concentrations on the production of 2PE was studied using a central composite design. The initial concentration of lactose and L-Phe of 40 g/L and 4 g/L respectively, allowed to obtain the highest concentration of 2PE (2.55 ± 0.12 g/L). The main effect of lactose on the production of 2PE was the vigorous induction of yeast growth in 24 h of fermentation. The L-Phe presence in the culture medium promoted the production of 2PE during the yeast's secondary metabolism, corresponding to the stationary growth phase.

Keywords

Whey valorization, fermentation, co-culture of yeasts, fusel alcohols, 2-phenylethanol

Introduction

Global milk transformation into cheese is ranked as the third-largest product of the dairy industry. In 2021, the global cheese market was valued as 72.26$ billion and it is expected an annual growth rate of 6.81% by 2026 (Laura Wood, 2021). Specifically in Canada, milk transformation is the second largest industry in the agro-industrial sector, with the Province of Quebec being the largest cheese producer in the country. In 2021, the Province of Quebec contributed 51% of the Canadian cheese production (Gouvernement du Québec, 2021). The transformation of milk into cheese or yogurt generates a liquid by-product called whey. This by-product is derived from the coagulation of proteins and fat of the milk, at a rate of around 9 kg of whey per kilogram of cheese (Anand et al., 2013; Kyle and Amamcharla, 2016). According to an economic analysis of cheese production, the Province of Quebec generates around 2.0 $x10^6$ t of whey per year, and Canada 6.0 $x10^6$ t per year (Munger et al., 2017). Whey presents high content of organic compounds, mainly lactose (33 – 52 g/L), proteins (5 – 15 g/L) and lactic acid (1.4 – 4.7 g/L), producing a chemical oxygen demand (COD) from 60 to 80 g O_2/L and low pH, from 3.9 to 5.6 (Valdez Castillo et al., 2020). The volume, pH and COD of whey represent a management problem for the dairy industries, which have to remove the organic load and/or neutralize it before releasing the whey into the environment or sewage.

Whey fermentation using bacteria, fungi or yeast strains is an economical and promising valorization option to obtain biomolecules such as biofuels, probiotics, surfactants, etc. Among others, whey fermentation using yeasts produces value-added biomolecules such as aromas, flavors, and antioxidants with no need for prior treatment of the whey, such as

adjustment of pH or hydrolysis of lactose (Dragone et al., 2009; Leclercq-Perlat et al., 2004; Löser et al., 2015b). Yeasts can transform amino acids into their respective fusel alcohols by the Ehrlich pathway. The amino acids are submitted to transamination, followed up by decarboxylation and dehydrogenation to form the corresponding alcohol (Hazelwood et al., 2008).

One of the most promising microorganisms in whey fermentation is the yeast *Kluyveromyces marxianus*. This yeast can hydrolyze the lactose using the β-galactosidase that it produces. The lactose is then hydrolyzed to glucose and galactose, which are transformed into pyruvate by the glycolysis pathway (Pires et al., 2014). Then, pyruvate can be oxidized into ethanol and acetic acid by anaerobic fermentation, or transformed into organic acids by the metabolic pathway of Krebs cycle (Hazelwood et al., 2008; Pires et al., 2014). In addition, *K. marxianus* can transform amino acids such as leucine, methionine and L-Phenylalanine (L-Phe) into their respective fusel alcohols: 3-methylbutan-1-ol (isoamyl alcohol), 3-(methylthio)-1-propanol (methionol) and 2-phenylethanol (2PE) (Valdez Castillo et al., 2021). The yeast *Debaryomyces hansenii* can also hydrolyze the lactose and produce compounds with aroma and flavor properties, for example, 2PE, isopentanol, and dihydroxyacetone (Leclercq-Perlat et al., 2004; Valdez Castillo et al., 2021).

The majority of studies of whey fermentation using yeasts have been oriented to the monoculture mode. Leclercq-Perlat et al. (2004) studied whey fermentation under batch and oxygen-limited conditions using either *K. marxianus* or *D. hansenii* under monoculture mode. They aimed to transform the amino acids present in a synthetic whey medium. *D.*

hansenii was able to produce 191 mg/L of 2PE, whereas *K. marxianus* produced 5.54 mg/L of isoamyl alcohol (Leclercq-Perlat et al., 2004).

Conde-Báez et al. (2019) studied aerobic whey fermentation using *K. marxianus*. The whey was enriched with pure L-Phe and the maximum 2PE accumulation was 780 mg/L for a yield of 0.75 $mg_{2PE}/mg_{L\text{-}Phe}$. The $(NH_4)_2SO_4$ was used as a nitrogen source showing that a suitable nitrogen source combined with aerobic conditions could increase the 2PE yield in comparison with the anaerobic fermentation or oxygen-limited conditions (Conde-Báez et al., 2019).

Valdez Castillo et al. (2021) studied both aerobic and anaerobic whey fermentation with *K. marxianus* and *D. hansenii*. The whey was enriched with yeast extract and peptone as organic nitrogen sources. The fermentation assays were carried out under monoculture and co-culture mode using these two yeast strains. The highest concentration of 2PE was obtained for the co-culture, corresponding to 185.72 mg/L and a yield of 0.38 $mg_{2PE}/mg_{L\text{-}Phe}$. This yield was 2.37 and 3.45-fold higher than the highest yield obtained with *K. marxianus* and *D. hansenii* under monoculture mode, respectively (Valdez Castillo et al., 2021). The use of co-cultured yeasts fermentation can improve the yeasts cells adaptation to the media, decreasing the bioprocess time, and increasing the productivity of the target product. This is possible due to either synergetic or competitive relationships between the yeasts (Rodrigues et al., 2016; Valdez Castillo et al., 2021).

Whereas, the interest in 2PE is for its rose-like aroma, flavor (fruit, honey, lilac, rose, wine) and antimicrobial properties. These properties, combined with the biosourced production of 2PE, have increased the demand for the biomolecule in the food, cosmetic and

pharmaceutical sectors (Conde-Báez et al., 2019; Valdez Castillo et al., 2021). The current 2PE market size surpassed 240$ million in 2020 and it is estimated a constant annual growth rate of 5.5% by 2027 (Kunal Ahuja and Kritika Mamtani, 2021). However, chemical methods such as Friedel craft reactions are the common production *via*. These methods are energy-consuming and generate toxic residues (Qian et al., 2019).

The type of fermentation, the fermentation conditions and the type of whey sterilization method can affect the production of biomolecules with aroma and flavor properties. Previous studies showed that the sterilization of whey at 120°C for 20 min can lead to the formation of Maillard reaction products such as furans, furfurals and pyranone compounds (Valdez Castillo et al., 2021). These compounds can inhibit the growth of yeast strains and the production of secondary metabolites such as 2PE.

The aim of the present study is to analyse the effect of lactose and L-Phe initial concentrations (main operating parameters) on whey valorization through fermentation using a co-culture of *K. marxianus* and *D. hansenii* in order to produce 2PE. The ultimate objective of the present research is the scaling up of the knowledge to an industrial level. For this reason, the effect of the method of whey sterilization on yeast growth and 2PE production was analyzed. To the best of our knowledge, this is the first study reporting the effect of the initial concentration of lactose in whey and L-Phenylalanine on the production of fusel alcohols using *K. marxianus* and *D. hansenii* under co-culture conditions.

Material and methods

The experiments were divided in two steps. The first step aimed to determine the most favorable whey sterilization method for the production of biomolecules with aroma and flavor properties. The second step studied the effect of L-Phe and lactose initial concentration on the production of 2PE and other biomolecules with aroma and flavor properties.

Materials

Yeast extract (Fisher Scientific, Canada) and peptone (Organotechnie S.A.S, Canada) were used for organic nitrogen supplementation. Lactose (Sigma-Aldrich, Canada) and L-Phenylalanine (Fisher Scientific, Canada, > 99%) were used as internal standards for liquid chromatography-mass spectrometric analysis. L-Phe (BioShop life science products, Canada, > 98%) was used to enrich the whey.

Kluyveromyces marxianus NRRL Y-1109 and *Debaryomyces hansenii* NRRL Y-1448 were selected by their capacity to transform L-Phe into 2PE (collection culture of United States Department of Agriculture-USDA). The revival, conservation and inoculum preparation of yeasts was done as described by Valdez-Castillo et. al. (2021) (Valdez Castillo et al., 2021). Brief, the yeasts were revived in yeast malt extract medium (YEM) and conserved in petri plates with YEM solidified. The inoculum was prepared in a medium containing lactose (45 g/L), yeast extract (10 g/L), and peptone (20 g/L), identified as LYP. To prepare the inoculum of each yeast, one loop of grown-up colonies of *K. marxianus* and *D. hansenii* was transferred to a flask of 125 mL filled with 30 mL of sterile LYP. Flasks were incubated at 25°C and 200 rpm for 20 h.

Raw whey characterization

Raw whey was used as the base of the substrate for yeast fermentation. It was donated by "Centre de Recherche Industrielle du Québec-CRIQ (Quebec, Canada)" and conserved at -20°C until used for fermentation. The raw whey was characterized for pH (Teknokroma pH meter digital, pH-2005), chemical oxygen demand (COD), total solids (TS), dissolved organic carbon (TOC-L analyzer, Shimadzu VCPH), and total nitrogen (Kjeldahl analyzer, Shimadzu VCPH) according to the standards methods described in APHA, 1999. The concentration of cations was determined by inductively coupled plasma optical emission spectrometry (5110 ICP-OES analyzer, Agilent Technologies). Whey was also characterized in lactose and L-Phe content using liquid chromatography-tandem mass spectrophotometry described below.

Heat treatment and Sterilization methods and biomolecules production

For the first step of the study, three methods of sterilization were analyzed: 1) filtration with membrane (pore size of 0.22 μm); 2) pasteurization (68°C for 30 min); and 3) autoclave (121°C for 20 min).

Whey fermentation

A co-culture ratio of *K. marxianus* and *D. hansenii* was used to ferment the whey sterilized by membrane, pasteurization, and autoclave. For the fermentation assays, the raw whey was used as culture medium and it was inoculated adjusting the initial cellular density (CD) of *K. marxianus* and *D. hansenii* at $5.0x10^7$ and $1.0x10^7$ CFU/mL. The flasks were incubated at 25 °C and 200 rpm for 96 h. Samples were taken at fermentation time of 0, 8, 24, 48, 72 and

96 h to determine biomass (dry basis), CD, and biomolecules with flavor and aroma properties (identification and quantification), and they were handled as indicated in previous study (Valdez Castillo et al., 2021). Briefly, 1 mL of culture broth was taken and centrifuged at 9,660 x g for 2 min (Mini spin plus, Eppendorf). The supernatant was stored at -20 °C until analysis. Pellets were dried at 60 °C for 24 h to quantify biomass. CD was determined by plate counting in solid LYP with 20 g/L of agar using serial dilutions (from 10^{-1} to 10^{-7}). In addition, in order to determine if the bioprocess can be used as a strategy for the disposal of dairy wastes, the COD was determined at 96 h of fermentation.

Identification of aroma and flavor molecules produced during sterilization and fermentation

The identification of secondary metabolites with aroma and flavour properties was performed at 72 h of fermentation using gas chromatography (Trace 1310, Thermo Scientific), coupled with a mass spectrometry detector (ISQ, Thermo Scientific) (GCMS), following the protocole developed by Valdez-Castillo et. al. (2021) (Valdez Castillo et al., 2021).

Lactose quantification

Lactose concentration was analyzed by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS). The system included a Finnigan surveyor LC pump linked to a TSQ Quantum access triple-quadruple mass spectrometer (Thermo Scientific) and an electrospray ionization interface. The mass spectrometer was operated in SRM negative ionization mode. It was equipped with an autosampler set at a temperature of 20 °C. Volumes of 20 µL of the samples were injected into the column Shodex HILICpak VG-50 2D (150 × 2 mm, 5 µm; Canadian Life Science). The column oven was set at 40 °C and the flow rate at

0.2 mL/min from 0 to 10 min. Then, the flow rate changed from 0.4 mL/min to 10.1 mL/min for 26 min. After this, the flow rate decreased to 0.2 mL/min for 28 min. Glucose-D2 98% purity (CDN Isotope, Pointe-Claire, Québec) and Lactose 99% purity (Sigma Aldrich, Canada) were used as internal standards for the detection and construction of a calibration curve, respectively. Samples were eluted with a mobile phase which included a mix of 89% acetonitrile and 11% water buffered with 0.2% (w/v) NH_4OH. The lactose was characterized by using signals like the mass of precursor and product ions (m/z: 341), and Glucose-D2 precursor m/z 181.1 and product m/z 91.1. The concentration was calculated based on the analyte areas and the calibration curve.

L-Phenylalanine quantification

The L-Phe concentration was determined by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS, Thermo Scientific). The system included a Finnigan surveyor LC pump linked to TSQ Quantum access triple-quadruple mass spectrometer (Thermo Scientific) and an electrospray ionization interface. The mass spectrometer was operated in SRM positive ionization mode. It was equipped with an autosampler set at a temperature of 10 °C and configured for a 96-well microtiter plate. Volumes of 10 μL of the samples were injected into the column HALO AQ-C18 (2.1×50 mm, 2.7 μm; Crawford Scientific). The column oven was set at 35 °C and the flow rate at 0.2 mL/min. L-Phe was used as the internal standard for the detection and construction of the calibration curve. Samples were eluted with a mobile phase which included a mix A of 95% water with 0.1% formic acid and mix B of 5% acetonitrile with 0.1% formic acid. The gradient elution started with 90% of mix A and 10% of mix B. It was constant till 0.5 min and then the mobile phase

A started to gradually decrease (30%). Subsequently, the gradient was kept constant until 8 min and then it came back to the initial setup. The L-Phe was characterized by a specific mass of precursor and product ions (m/z: 166.03/120.07), and the concentration was calculated based on the analyte areas and the calibration curve.

Quantification of metabolites

The biomolecules 2PE, ethanol, isopentanol, propanoic acid, butyric acid and dihydroxyacetone were identified as fermentation metabolites among the compounds detected previously and the method of Dragon et al., 2009 (Dragone et al., 2009) was adapted to quantify them. Briefly, a gas chromatograph (Trace 1310, Thermo Scientific) equipped with flame ionization detector (GC-FID), and capillary column CP-Wax 57 CB (Agilent Technologies Inc.) was used according to the conditions specified by Valdez-Castillo et al. (2021).

Experimental plan for the effect of culture medium composition on biomolecules production.

For the second step of the study, a central composite design (CCD) was used to study the effect of the initial concentration of L-Phe and lactose on the production of 2PE, ethanol, isopentanol, propanoic acid, butyric acid and dihydroxyacetone. The production of biomolecules was analyzed using the response surface methodology (RSM). The model was constructed using the Design-Expert®-7 software (Stat-Ease Inc., Minneapolis, MN) and Rstudio software. The maximum lactose concentration in raw whey of 45 g/L was considered the highest value for the experimental design. According to the literature, the maximum concentration of L-Phe which does not inhibit the growth of *K. marxianus* is 4 g/L (Azevedo

de Lima et al., 2018). This was set as the highest value for the experimental design. Thus, the CCD was a model of two factors and three levels, high, midpoint and low, which are depicted by (+1), (0) and (-1). The axial points were determined using α of $\pm$ 1.4114. Three replicates of midpoint were included to fit a model of second-order response surface to experimental data. Equation 1 shows the mathematical equation expression for the second-order response surface model:

$$\boldsymbol{Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2}$$ **...Equation 1**

Where β_0 is the intercept value, β_1 and β_2 the first-order coefficient values for lactose and L-Phe, respectively, β_{12} the two-way interaction coefficient for both lactose and L-Phe variables, β_{11} and β_{22} the second-order coefficient values for lactose and L-Phe, respectively, and x_1 and x_2 the lactose and L-Phe concentrations, respectively.

Aerobic fermentation assays were carried out in 250 mL flasks. The empty flasks with cotton caps were sterilized by autoclave at 121 °C for 20 min. The whey media was prepared as follows: the whey was sterilized by pasteurization at 68 °C for 30 min to avoid the precipitation of whey proteins. Autoclaved yeast extract and peptone solutions were added to the pasteurized whey until obtaining a concentration of 10 g/L and 20 g/L. They were used as an organic nitrogen source. Under sterile conditions, the necessary volume of pasteurized whey was added to the sterile flasks according to the lactose concentrations of the experimental design. Then, the total volume in each flask was topped to 30 mL with sterile water. Finally, L-Phe was added at the concentration specified in the experimental design.

Statistical analysis

For the first step, an analysis of one-way ANCOVA (p-value < 0.05) with the Tukey test as a post hoc test was performed using the R core team 2020 software. The analysis was used to study the effect of the sterilization method on the production of 2PE.

For the second step, the relation between the parameters and the response variable was determined by design matrix evaluation using the response surface quadratic model for interactions. The effect of the lactose and L-Phe initial concentration on the production of biomolecules was analyzed by the response surface model for the two factors at a 95% of confidence interval (p-value < 0.05).

Results and discussion

Characterization of raw whey

The physicochemical characteristics and the composition of the raw whey were determined in terms of pH, lactose, COD, carbon, nitrogen, L-Phe and cations content. Whey had a pH of 6.50 ± 1.00 and contained lactose 45.0 ± 2.1 g/L, COD 60.33 ± 0.86 g O_2/L, total nitrogen 0.79 ± 0.01 g/L, and L-Phe 13.08 ± 0.90 mg/L. The K^+ was the most abundant cation, at 1,094 ± 1 mg/L (data shown in Table 2.7).

The raw whey used in this study was generated by the addition of chymosin enzymes to the milk, producing a raw whey with a nearly neutral pH (Anand et al., 2013; Risner et al., 2019). This parameter is important for the growth of microorganisms during fermentation. In the case of *K. marxianus* and *D. hansenii* yeasts, the pH of 6.5 was appropriate for their growth.

In addition, yeast growth is affected by the lactose (main carbon source) and nitrogen content of the raw whey. In this study, the raw sweet whey presented a C/N ratio of 55.69 ± 1.26. The total nitrogen content of the sweet whey is a result of both protein nitrogen and non-protein nitrogen. The β-lactoglobulin and α-lactalbumin are the main source or protein nitrogen in sweet whey. Non-protein nitrogen compounds are mainly free amino acids and ammonium nitrogen compounds (Anand et al., 2013; Rodrigues et al., 2016).

Table 2. 7 Physicochemical characterization and composition of raw whey

Parameter	**Value**
pH	6.50 ± 1.00
Total solid (g/L)	50.34 ± 1.44
Lactose (g/L)	45.00 ± 2.10
Chemical oxygen demand (g O_2/L)	60.33 ± 0.86
Total nitrogen (g/L)	0.79 ± 0.01
Organic Carbon (g/L)	15.11 ± 0.08
Inorganic Carbon (mg/L)	4.00 ± 0.00
L-Phe (mg/L)	13.08 ± 0.90
Ca (mg/L)	247 ± 1.00
Cu (mg/L)	0.013 ± 0.002
Fe (mg/L)	0.033 ± 0.003
K (mg/L)	1 094.00 ± 1.00
Mg (mg/L)	57.00 ± 0.52
Zn (mg/L)	0.05 ± 0.00

The concentration of total nitrogen of 0.79 ± 0.01 g/L in the raw sweet whey is in agreement with other studies, which indicate total nitrogen in sweet whey varying from 0.5 to 1.4 g/L due to the low concentration of proteins in whey (Anand et al., 2013; Rodrigues et al., 2016). For whey fermentation, studies in the literature report that it is necessary to enrich the whey with either organic or inorganic nitrogen sources. A nitrogen concentration range from 3 to 6 g/L is required to promote the consumption of lactose and the growth of the yeasts (Löser et al., 2015a; Rodrigues et al., 2016; Valdez Castillo et al., 2021). For this reason, the raw sweet whey used in this study was enriched with yeast extract and peptone as organic nitrogen sources. The nitrogen concentration in the whey medium was 3.7 g/L for the experimental design.

The concentration of L-Phe of 13.08 ± 0.90 mg/L can be considered low, as it can be present in whey in a range going from 0 to 68 mg/L (Huffman and Ferreira, 2011). The low concentration of L-Phe could be due to the specificity of chymosin. This enzyme cuts the casein into peptides of different length, releasing mainly glycomacropeptides, β-lactoglobulin and α-lactalbumin in the liquid phase (whey) (Anand et al., 2013). The glycomacropetide is a protein fraction of sweet whey, it is rich in branched amino acids but deficient in aromatic amino acids, such as L-Phe, tryptophan and tyrosine. This means that the solid fraction (proteins and lipids coagulated) would contain more L-Phe than released peptides (Anand et al., 2013; Kassem, 2015).

The content of K^+ was in agreement with previous reports, since potassium is one of the most abundant minerals found in whey (Anand et al., 2013). K^+ is a key parameter for the activity of the β-galactosidase enzyme produced by both yeast strains used in this study. K^+ activates

the enzyme and induces conformational changes in its structure. These conformational changes improve the binding with the substrate, in this case the lactose (Rajakala and Karthigai Selvi, 2006).

Effect of the method of control of microorganisms during the whey fermentation

Cell density

The effect of the sterilization method on normalized cell density (with respect to initial cell density of each yeast) is shown in Figure 2.7A for *K. marxianus* and Figure 2.7B for *D. hansenii*. Since the initial cell density and the growth were different for each yeast, the analysis was done using normalized cell densities. For example, the initial cell density of *K. marxianus* and *D. hansenii* was $5.0x10^7$ CFU/mL $1.0x10^7$ CFU/mL, respectively. For *K. marxianus*, the maximum normalized cell density was observed at 24 h of fermentation for the raw whey sterilized by pasteurization, followed by the raw whey sterilized by autoclave. After 24 h of fermentation, the normalized cell density decreased from 2.1 ± 0.4 to 0.3 ± 0.0 log of cell density/cell $density_{initial}$ which corresponded to $5.2x10^9 \pm 1.1x10^8$ and $8.5x10^7 \pm 1.1x10^7$ $CFU_{K.marxianus}$/mL, respectively. Similar findings were made at 48 h for the other two fermentation assays. For *D. hansenii* the maximum normalized cell density was also observed at 24 h of fermentation for the raw whey sterilized by pasteurization. The lowest growth of either *K. marxianus* or *D. hansenii* was observed when whey was sterilized by filtration. In this case, contamination was observed after 24 h of fermentation. This was confirmed by the presence of clear colonies with circular form, flat elevation and entire margin different from the morphology of *K. marxianus* and *D. hansenii* in the plate counting. The staining of these colonies showed gram-positive bacillus which could be lactic acid

bacteria or ripening bacteria such as microbacteriaceae (Mauriello et al., 2001; Montel et al., 2014) .

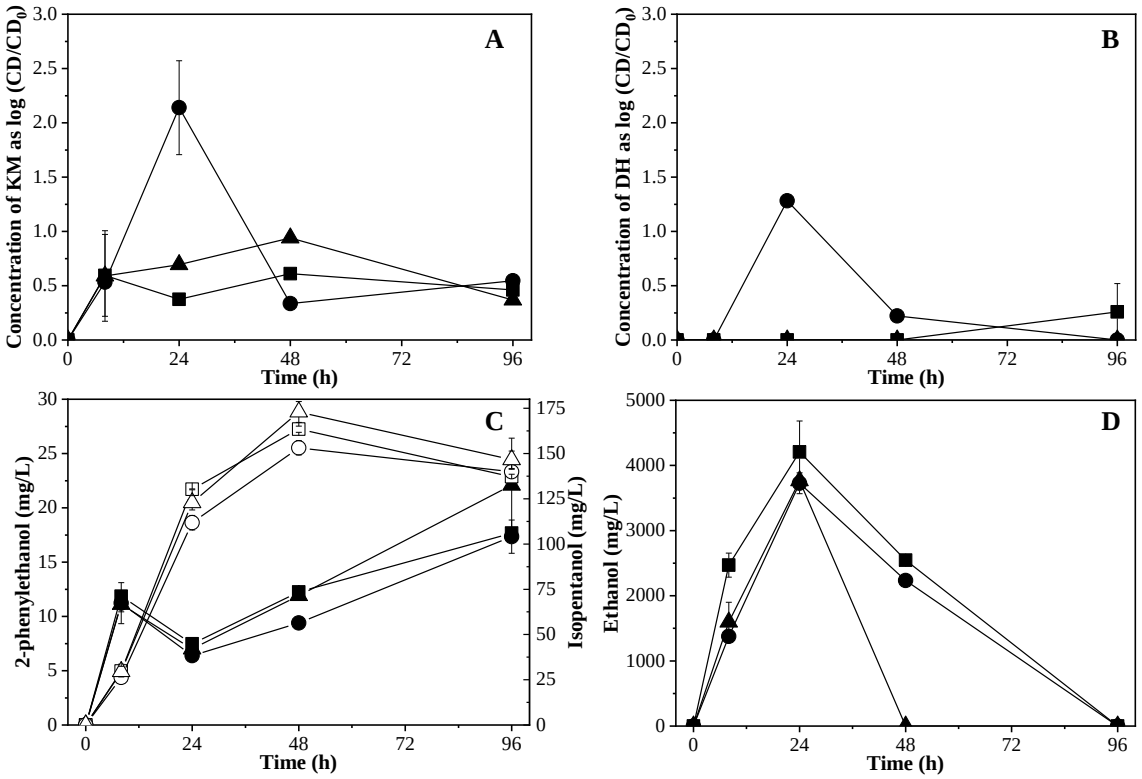

Figure 2. 7 Growth of yeasts and alcohols production as co-culture mode and aerobic conditions for the fermentation of raw whey medium. Growth is expresses as cell density (CD) as a function of time for (A) *Kluyveromyces marxianus* and (B) *Debaryomyces hansenii*. The cellular concentration is expressed as the logarithm of the ratio of CD at any time by the initial cellular density (CD_0). Concentration of alcohols: C) 2-phenylethanol (closed symbols) and isopentanol (open symbols), and D) ethanol. The sterilization methods are identified as follows: ■─filtration, ●─pasteurization and ▲–autoclave.

These bacteria have a common size of 1 – 5 µm long and 0.2 – 1 µm diameter, which could lead to the bacteria going through the membrane filter (Gaveau et al., 2017; Morandi et al., 2019). This explains the lowest growth for filtration as a sterilization method, as yeast growth is affected by the presence of other microorganisms in the culture medium.

In this study, *K. marxianus* and *D. hansenii* colonies were dominant over the other microorganism colonies through the fermentation time. However, the presence of the other microorganisms could contribute to the consumption of lactose in the whey and the production of lactic acid. This could decrease the cell density of the yeast strains because of faster starvation and probable inhibition by some bacterial by-products. Christensen et al. (2011) studied the fermentation of a non-sterile whey using a strain of *K. marxianus*. They observed the presence of lactic acid bacteria and lactic acid in the raw whey. The presence of other microorganisms and lactic acid affected the lactose consumption and limited the growth of *K. marxianus* during whey fermentation (Christensen et al., 2011).

Figure 2.7B shows that *D. hansenii* was more sensitive to whey sterilization by autoclave. In comparison to *K. marxianus*, there was no presence of *D. hansenii* colonies on the agar plates when whey sterilized by autoclave was fermented. This can be attributed to the presence of inhibitory growth compounds such as furfurals and pyronones compounds, as was reported in previous studies (Valdez Castillo et al., 2021). The low cell density of *D. hansenii* can also be explained by the dominance of whey fermentation by *K. marxianus* since this yeast had a higher cell density at the beginning of the fermentation.

Similar observations were made in previous studies by Valdez et al. (2021), who studied whey fermentation enriched with organic nitrogen source using a co-culture of *K. marxianus* and *D. hansenii*. The cell density of *K. marxianus* was $1.1x10^9 \pm 3.0x10^7$ $CFU_{K.\ marxianus}$/mL at 24 h of fermentation. It was also observed that *K. marxianus* was always the dominant yeast even if its initial cell density was lower than the cell density of *D. hansenii* (Valdez Castillo et al., 2021). Conde-Báez et al. (2019) observed a *K. marxianus* cell density of

$2.2x10^9 \pm 9.9x10^7$ $CFU_{K.\ marxianu}$/mL at 72 h of fermentation for acid whey fermentation using *K. marxianus* under monoculture mode (Conde-Báez et al., 2019).

Production of biomolecules

The effect of the whey sterilization method on the production of biomolecules with aroma and flavor properties during fermentation was studied. Table 2.8 shows the aroma and flavor molecules identified in the culture broth by GC-MS analysis at 72 h of fermentation. Only alcohols (2PE, isopentanol and 2,3-butanediol) were identified for all sterilization methods. Other aromas and flavor molecules were identified in some cases, for example, paraldehyde and organic acids in the filtration and pasteurization methods of whey sterilization.

Table 2. 8 Biomolecules with aroma and flavor properties identified in the culture broth according to the sterilization method used: filtration, pasteurization and autoclave. These are the biomolecules identified at 72 h of fermentation of raw whey using *K. marxianus* and *D. hansenii* under co-culture mode.

Biomolecule	**Relative area (counts*min) *x* $1x10^7$**		
	Filtration	**Pasteurization**	**Autoclave**
2-Phenylethanol	0.249 ± 0.008	0.208 ± 0.007	0.235 ± 0.008
Isopentanol	1.177 ± 0.086	0.972 ± 0.068	1.126 ± 0.026
2,3-Butanediol	0.439 ± 0.158	0.260 ± 0.062	0.033 ± 0.002
Propanoic acid	0.122 ± 0.023	0.193 ± 0.016	0.000 ± 0.000
Butyric acid	0.930 ± 0.095	0.930 ± 0.079	0.000 ± 0.000

Among the aroma and flavor molecules, isopentanol was the most abundant compound, followed by 2PE. In the absence of a quantitative method, the relative area (counts*min) calculated similarly for all compounds was used to determine which are more abundant. The

highest relative area of both isopentanol and 2PE observed in the raw whey sterilized by filtration was 1.117 ± 0.086 and 0.249 ± 0.008 counts*min (x 10^7), respectively.

Both isopentanol and 2PE are produced by yeasts using the Ehrlich pathway in which amino acids are transformed into their respective fusel alcohol molecule. For isopentanol the precursor amino acid is leucine and for 2PE is L-Phe. Since the target compound in this study was 2PE, the only amino acid quantified was L-Phe. Despite the fact isoleucine was not quantified, concentrations of leucine in whey from 100 to 120 mg/L have been reported (Anand et al., 2013; Huffman and Ferreira, 2011).

Table 2.8 shows that 2,3-butanediol was produced, and this metabolite is associated with common metabolic pathways. For example, in other yeasts, it was observed that lactose is oxidized into pyruvate by the glycolysis pathway. Pyruvate is further oxidized into 2-acetolactate by acetolactate synthase. Then, 2-acetolactate is spontaneously oxidized by the presence of oxygen to form diacetyl. Diacetyl is reduced to acetoin by the action of dehydrogenase enzymes of NADH. Acetoin can be reduced to 2,3-butanediol by butanediol dehydrogenase (González et al., 2010).

The concentration of biomolecules produced during whey fermentation was determined at 0, 24, 48 and 96 h by GC-FID. The biomolecules traced were 2PE, ethanol, isopentanol, dihydroxyacetone, propanoic acid and butyric acid. The variation of concentration of 2PE and isopentanol is shown in Figure 2.7C. The highest concentration of 2PE was observed at 96 h for all sterilization methods, 17.7 ± 0.1, 17.3 ± 1.5 and 22.1 ± 4.3 mg/L for filtration, pasteurization and autoclave respectively. The highest concentration of isopentanol, on the

other hand, was observed at 48 h, 163.5 ± 1.7, 153.2 ± 4.0 and 173.0 ± 5.8 mg/L for filtration, pasteurization, and autoclave, respectively.

L-Phe was naturally present in the raw whey. In its free form (solubilized in the whey), its concentration was 13.1 ± 0.9 mg/L. The 2PE yield observed would correspond to 1.3 $mg_{2PE}/mg_{L\text{-}Phe\text{-}consumed}$, which was nearly twice as high as the maximum theoretical yield that can be obtained (0.74 $mg_{2PE}/mg_{L\text{-}Phe}$). The yield was calculated using the concentration of free L-Phe presented at 0 and 96 h of fermentation. But, this amino acid is also present in the structure of the whey proteins β-lactoglobulin and α-lactalbumin (Anand et al., 2013). If they are hydrolyzed, the L-Phe would be released, increasing its bioavailability and the production of 2PE. For this reason, the hydrolysis of these proteins modifies the free L-Phe content, and the observed yield for the whole fermentation is not an indicator in this case. In previous studies, Valdez-Castillo et al. (2021) reported the fermentation of whey powder containing only 6.7 mg/L of free L-Phe, and obtained a maximum 2PE concentration of 5.1 ± 0.0 mg/L, and a yield of 0.75 $mg_{2PE}/mg_{L\text{-}Phe\text{-}consumed}$, similar to the maximum theoretical yield.

The study of the effect of the sterilization method on the production of 2PE was performed using an ANCOVA analysis. The results indicated that only the whey sterilized by autoclave had a negative effect on the production of 2PE, whereas the whey sterilized by pasteurization was the optimal method to produce 2PE (data shown in Table 2.9).

Figure 2.7D shows that for ethanol, the highest concentration was observed at 24 h, 4207 ± 476, 3728 ± 160, and 3764 ± 34 mg/L for the fermentation of raw whey sterilized by filtration, pasteurization, and autoclave respectively.

Table 2. 9 Analysis by ANCOVA of the effect of sterilization method on 2-phenylethanol concentration during the whey fermentation using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode.

Sterilization method	*Pr(>|t|)*	Residual standard error	Degrees of freedom	Multiple R-squared	Adjusted R-squared	p-value
Autoclave	0.001*					
Filtration	0.747	3.913	26	0.783	0.746	$1.169x10^{-6}$
Pasteurization	0.382					

"" Significant at 5% level (P ≤ 0.05). Pr(>|t|); proportion of the t distribution at that degree of freedom which is greater than the absolute value of t statistic.*

In all cases, at 24 h of fermentation, the lactose in the culture broth had been completely depleted (data not shown). The corresponding ethanol yield was 0.09 ± 0.01, 0.08 ± 0.00 and 0.08 ± 0.00 $mg/mg_{lactose\text{-}consumed}$ for the whey sterilized by filtration, pasteurization and autoclave, respectively. In all cases, ethanol concentration decreased after 24 h of fermentation, especially for the raw whey sterilized by autoclave. Lactose from raw whey was transformed by the yeasts into ethanol. According to alcoholic fermentation, lactose is firstly hydrolyzed into galactose and glucose. These carbohydrates are transformed into glyceraldehyde-3-phosphate which is further oxidized into pyruvate by means of the glycolysis pathway, and finally, the pyruvate is oxidized into CO_2 and ethanol. The alcoholic fermentation is limited under aerobic conditions. When oxygen is present, the electrons donated from the lactose oxidation are used to store energy in bonds of biomolecules such as ATP or NAD^+ or in cell synthesis (Beniwal et al., 2017). In the present study, all the fermentation assays were carried on under aerobic conditions. For this reason, low ethanol concentration was observed. Christensen et al. (2011) studied anaerobic whey fermentation

with *K. marxianus* in cap flasks. The fermentation conditions were an initial lactose concentration of 48 ± 0.8 g/L, pH of 6.34 ± 0.5, 30°C and 100 rpm. They observed an ethanol concentration of 14.0 ± 0.8 g/L at 24 h, corresponding to a yield of 0.5 ± 0.0 $mg_{ethanol}/mg_{lactose}$ (Christensen et al., 2011). This corroborates that ethanol production is promoted when whey fermentation is carried on under anaerobic instead of aerobic conditions as it was observed in the present study. In previous studies, an ethanol yield of 0.34 $mg/mg_{lactose}$ at 24 h using *K. marxianus* and *D. hansenii* under co-culture mode to ferment cheese whey enriched with organic nitrogen was observed (Valdez Castillo et al., 2021).

As it was mentioned, lactose was completely depleted after the first 24 h of fermentation (show in supplementary material SF1). In the absence of primary carbon source (lactose), the yeasts can adapt their metabolism to consume a new source of carbon. In the case of *K. marxianus* and *D. hansenii*, they can consume other molecules in the absence of sugars, for example, ethanol is oxidized by means of alcohol dehydrogenase producing an aldehyde and after CO_2. This oxidation releases the energy for cell preservation. Figure 2.7D shows that after 24 h of fermentation, yeasts metabolism switched to consume and oxidize ethanol, corresponding to the depletion of lactose and the decrease of cell density, which was observed in Figure 2.7A and 2.7B for all the fermentation assays. However, the ethanol concentration in the culture broth and the energy that cells can obtain from the dehydrogenation of ethanol is not enough to keep the growth of yeast cells. Instead of the growth, it was promoted the death of cells until reach a cell concentration that was suitable to maintain with the available ethanol in the culture broth. The cell density observed at 48 h of fermentation of whey sterilized by pasteurization is similar to the cell density observed for the two other

fermentation assays with whey sterilized by filtration and autoclave. This was also observed in previous study by Valdez-Castillo et al. (2021). They observed that both *K. marxianus* and *D. hansenii* could consume ethanol under monoculture and co-culture mode after lactose depletion. Wittman et al., (2002) studied the metabolomics flux of glucose and production of ethanol using *K. marxianus*. They observed that at 30 h of fermentation glucose had been completely consumed corresponding to the maximum concentration of ethanol, then the yeast consumed the ethanol (Wittmann et al., 2002). These studies confirm the observation of ethanol as alternative carbon source for yeasts under co-culture mode.

The yeasts can present two kinds of metabolism: the primary metabolism associated with growth and division of cells and the secondary metabolism which is nonessential for growth, but where specialized biomolecules are produced. During the primary metabolism, the yeasts produce metabolites to stock carbon and energy for later use, such as ethanol, glycerol, and organic acids. Ethanol is produced as final product during alcoholic fermentation or organic respiration of lactose. Lactose is transformed to pyruvate by means of glycolysis pathway, and this last compound is oxidized into acetaldehyde which is further reduced into ethanol; however, its accumulation can affect the metabolism of the yeast. Ethanol could be toxic at a concentration equal to 20-25 g/L or higher, because it can damage the cell membrane by denaturalizing proteins and finally causing the cellular lysis of yeasts (Christensen et al., 2011; Rodrigues et al., 2016). In order to avoid accumulation of ethanol in the media, yeasts can activate the secondary metabolism stage. The secondary metabolism produces specialized biomolecules such as fusel alcohols, esters, and carbonyls. These biomolecules

are regulators and signaling molecules which promote the adaptation of yeast cells to environmental changes (Hazelwood et al., 2008).

For the following assays, the pasteurization method was selected to carry on the whey fermentation because it produced the highest cell density and the lowest accumulation of either isopentanol and ethanol. This corroborates that the sterilization method is a key parameter to promote or inhibit the yeast cells growth.

In addition, the final COD of whey sterilized by membrane, pasteurization, and autoclave was 20.0 ± 0.2, 20.4 ± 0.0, and 29.7 ± 0.2 gO_2/L, respectively. This corresponds to a COD decreasing of 66.7 ± 2.7% in comparison to the initial COD of whey (60.3 ± 0.9 gO_2/L). This shows that the whey fermentation using yeasts under co-culture mode can be used as strategy for dairy waste disposal.

Effect of the initial concentration of lactose and L-Phe on alcohols production

Effect of lactose and L-Phe on 2PE production.

For these assays, the raw whey was enriched with pure L-Phe to observe its effect on 2PE production. Organic nitrogen was added to avoid limiting growth and 2PE production.

The levels of lactose and L-Phenylalanine as variables for CCD experimental plan is shown in Table 2.10. The initial concentration of lactose ranged from 15.86 to 44.14 g/L, and that of L-Phe from 1.58 to 4.41 g/L. All experiments were performed in duplicate and the average concentration of 2PE, ethanol, isopentanol, propanoic acid, butyric acid and dihydroxyacetone was used as response variable for the statistical analysis. For all

biomolecules, the concentration was determined at 48 h of fermentation. Additionally, the concentration of ethanol and 2PE was also determined at 8 h and 96 h, respectively.

Table 2.10 shows that at 48 h of fermentation, lactose was depleted for almost all assays, except for the lactose concentration of 40 g/L or higher. In these cases, the lactose concentration at 48 h ranged from 3.05 to 6.46 g/L (data shown in Table 2.11). For an initial lactose and L-Phe concentration of 40 and 4 g/L respectively, the highest concentration of 2PE at 48 h and 96 h was 2.48 ± 0.00 and 2.55 ± 0.01 g/L. This led to a yield of 0.89 ± 0.01$mg_{2PE}/mg_{L\text{-}Phe\text{-}consumed}$. For a lactose and L-Phe initial concentration of 15.86 and 3 g/L respectively, the 2PE concentration was 2.12 ± 0.01 g/L at 96 h, corresponding to a yield of 0.80 ± 0.00 $mg_{2PE}/mg_{L\text{-}Phe\text{-}consumed}$, which was slightly higher than the maximum theoretical yield (0.74 $mg_{2PE}/mg_{L\text{-}Phe}$). Figure 2.8A shows the 2PE concentration as a function of the initial concentration of lactose and L-Phe at 48 h of fermentation. A constant increment in the production of 2PE was observed, even for the highest concentration of L-Phe (4.41 g/L).

The results indicated that the initial concentration of both lactose and L-Phe are key variables to improve the production of 2PE. Lactose is necessary for biomass synthesis and as the source of electrons used for the assimilation of amino acids such as L-Phe. Yeast cells prefer to assimilate amino acids with a simple carbon backbone such as asparagine, glutamine, glutamic acid and alanine. These amino acids trigger the formation of α-ketoglutarate and pyruvate and support primary and secondary metabolism in cells (Garavaglia et al., 2007; Mas et al., 2014). To assimilate complex amino acids such as L-Phe, on the other hand, the yeast cells need to reduce the α-ketoglutarate through the Ehrlich pathway (Garavaglia et al.,

2007). Yeast cells shift metabolism to the Ehrlich pathway when complex amino acids are present in the medium at high concentration.

Table 2. 10 Levels of lactose and L-Phenylalanine as variables for the central composite design of k^2 model to determine their effect on the production of biomolecules at 48 and 96 h of fermentation and to construct a statistical predictive model.

Run	Initial concentration (g/L)		Concentration of biomolecules (g/L)							Yield ($g_{2PE}/g_{L\text{-}Phe\text{-}consumed}$)
			48 h						96 h	48 h
	Lactose	L-Phe	ET	ISOP	PA	BA	DHA	2PE	2PE	2PE
1	20.00	2.00	0.00±0.00	0.05±0.00	0.08±0.01	0.06±0.00	0.25±0.01	1.94±0.00	2.06±0.00	0.95±0.00
2	40.00	2.00	4.15±0.01	0.09±0.00	0.09±0.01	0.04±0.01	0.25±0.00	1.65±0.00	1.70±0.01	0.82±0.01
3	20.00	4.00	0.00±0.00	0.05±0.00	0.09±0.01	0.08±0.00	0.25±0.00	2.33±0.00	2.34±0.03	0.75±0.01
4	40.00	4.00	4.14±0.00	0.07±0.01	0.07±0.01	0.23±0.00	0.25±0.00	2.48±0.00	2.55±0.01	0.89±0.01
5	30.00	3.00	1.29±0.00	0.08±0.01	0.07±0.01	0.04±0.01	0.26±0.01	1.90±0.00	2.21±0.00	0.64±0.02
6	30.00	3.00	1.36±0.00	0.06±0.00	0.07±0.00	0.05±0.00	0.25±0.00	2.20±0.01	2.27±0.02	0.73±0.03
7	30.00	3.00	1.99±0.01	0.07±0.00	0.08±0.00	0.05±0.00	0.26±0.00	2.19±0.00	2.24±0.00	0.73±0.01
8	15.86	3.00	0.00±0.00	0.04±0.01	0.07±0.00	0.08±0.00	0.25±0.00	2.28±0.00	2.12±0.01	0.80±0.00
9	44.14	3.00	4.81±0.01	0.07±0.00	0.06±0.00	0.11±0.01	0.24±0.00	2.19±0.00	2.21±0.00	0.78±0.01
10	30.00	1.59	1.50±0.01	0.09±0.00	0.10±0.02	0.04±0.00	0.25±0.00	1.51±0.00	1.99±0.00	0.65±0.00
11	30.00	4.41	1.92±0.01	0.07±0.00	0.08±0.00	0.08±0.01	0.24±0.00	2.44±0.00	1.75±0.01	0.67±0.01

2PE, 2-phenylethanol. ET, ethanol. ISOP, isopentanol. PA, propanoic acid. BA, butyric acid. DHA, Dihydroxyacetone.

The levels for x1: -α= 15.86 g/L; -1 = 20.00 g/L ; 0 = 30.00 g/L ; 1 = 40.00 g/L ; +α = 44.14 g/L

The levels for x2 : -α= 1.58 g/L; -1 = 2.00 g/L ; 0 = 3.00 g/L ; 1 = 4.00 g/L ; +α = 4.41 g/L

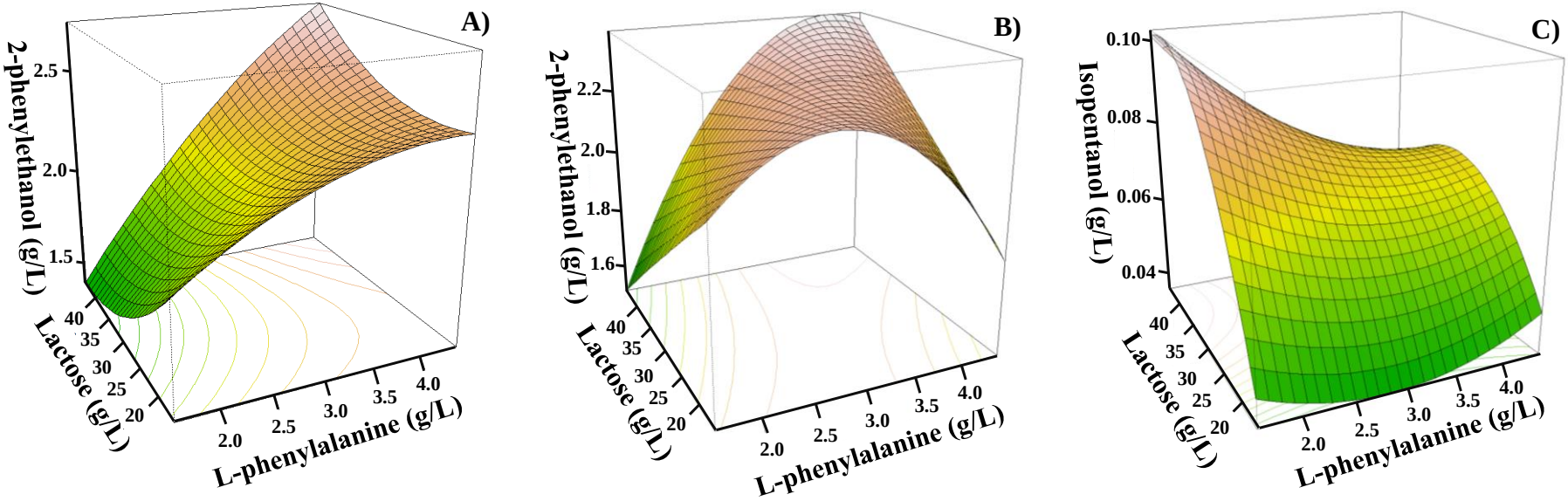

Figure 2. 8 Effect of initial concentration of lactose and L-Phenylalanine on the concentration of fusel alcohols in the culture broth for the fermentation of raw whey: A) 2-phenylethanol at 48 h, B) 2-phenylethanol at 96 h, and C) Isopentanol at 48 h of fermentation.

Table 2. 11 Levels of lactose and L-Phenylalanine as variables for the central composite design of k^2 model at 48 and 96 h of fermentation.

Run	Initial concentration (g/L)		Concentration (g/L)	
	Lactose	L-Phe	Lactose at 48 h of fermentation	L-Phe at 96 h of fermentation
1	20.00	2.00	0.01	0.00
2	40.00	2.00	0.01	0.00
3	20.00	4.00	0.23	0.30
4	40.00	4.00	3.06	1.20
5	30.00	3.00	0.01	0.03
6	30.00	3.00	0.00	0.00
7	30.00	3.00	0.00	0.00
8	15.86	3.00	0.07	0.28
9	44.14	3.00	6.47	0.24
10	30.00	1.59	0.00	0.95
11	30.00	4.41	0.75	0.67

The levels for x1: -α= 15.86 g/L; -1 = 20.00 g/L ; 0 = 30.00 g/L ; 1 = 40.00 g/L ; +α = 44.14 g/L

The levels for x2 : -α= 1.58 g/L; -1 = 2.00 g/L ; 0 = 3.00 g/L ; 1 = 4.00 g/L ; +α = 4.41 g/L

Garavaglia et al. (2007) studied the fermentation of grape must using *K. marxianus* at an initial reducing sugars concentration of 186.2 g/L. They observed a 2PE concentration of 0.59 g/L under pH, temperature and L-Phe concentration conditions of 6, 34 °C and 4.5 g/L respectively. They observed an inhibition due to the ethanol production, showing that not only the L-Phe concentration must be taken into account but also the initial concentration of the carbon source.

The effect of the initial concentration of lactose on 2PE production can be also related to the type of metabolism or growth phase of yeasts under co-culture mode. Since the stationary growth phase of both yeasts ended until 96 h of fermentation, the 2PE concentration at this time was included in the experimental design.

Figure 2.8B shows the 2PE concentration as a function of the initial concentration of lactose and L-Phe at 96 h of fermentation. For an L-Phe concentration of 3 g/L, the concentration of 2PE was 2.21 ± 0.05 g/L, or a yield of 0.74 ± 0.05 $g_{2PE}/g_{L\text{-}Phe}$, which is the maximum theoretical yield.

The results in Table 2.12 indicate that the model proposed for the production of 2PE as a function of the initial concentration of lactose and L-Phe is suitable. The statistical analysis of the model shown in Table 2.12, it indicates that both variables have an effect on the production of 2PE. The model was created in the form of a polynomial equation in order to predict the concentration of each biomolecule as a function of both variables. This polynomial equation was fitted to experimental data at 48 and 96 h of fermentation and the polynomial factors obtained are shown in equations 2 and 3 respectively:

Table 2. 12 Values of coefficients for the mathematical model parameters obtained by central composite design for each by-product. The coefficients correspond to the general mathematical model $\beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 = Y$ where Y is the predictive concentration of the respective by-product and the coefficients were obtained using the experimental design presented in Table 2.10.

By-product	Intercept	First order terms		Two-way interaction	Second-order terms		Adjustment of the CCD model		
		x_1	x_2	$x_1 x_2$	x_1^2	x_2^2	R^2	*P*-value	Lack of fit value
2-phenylethanol (48 h)	2.098*	-0.033	0.317*	0.110*	-0.069	0.061	0.941	0.004	0.997
2-phenylethanol (96 h)	2.246*	0.136*	-0.041*	0.221*	-0.058	-0.136*	0.841	0.046	0.075
Ethanol (48 h)	1.548*	1.886*	0.074	-0.001	0.431*	0.085	0.979	0.003	0.618
Ethanol (8 h)	3.766*	-0.346*	0.434*	-0.563*	-0.231*	-0.296*	0.949	0.003	0.145
Isopentanol	0.073*	0.012*	-0.007*	-0.006*	0.010*	0.004	0.949	0.003	0.882
Propanoic acid	0.074*	-9.743×10^{-3}*	6.242×10^{-3}	-2.115×10^{-5}	3.202×10^{-6}	1.844×10^{-3}	0.716	0.167	0.705
Butyric acid	0.045*	0.020	0.033*	0.042*	0.032*	0.013	0.811	0.068	0.022*
Dihidroxyacetone	0.259*	-0.002	-0.002	-0.0002	-0.006*	-0.004*	0.696	0.191	0.728

"" Significant at 5% level of confidence ($P \leq 0.05$).*

$$Y_{48h} = 2.098 - 0.033 \cdot x_1 + 0.317 \cdot x_2 + 0.110 \cdot x_1 x_2 - 0.069 \cdot x_1^2 + 0.061 \cdot x_2^2$$ **… Equation 2**

$$Y_{96h} = 2.246 + 0.136 \cdot x_1 - 0.041 \cdot x_2 + 0.221 \cdot x_1 x_2 - 0.058 \cdot x_1^2 + 0.136 \cdot x_2^2$$ **…Equation 3**

With each equation it is possible to determine the regions where the maximum concentration of 2PE occurs at these times of fermentation. For example, at 48 h the optimal theoretical value for lactose is 19.45 g/L and for L-Phe 4.62 g/L; this will lead to the maximum 2PE concentration of 2.37 g/L. Whereas, at 96 h the optimal theoretical value for lactose and L-Phe is 22.49 and 2.99 g/L respectively, to obtain a maximum 2PE concentration of 2.24 g/L. According to the experimental results, the maximum L-Phe concentration that yeast could consume was 3 g/L. When a L-Phe concentration higher than 4.0 g/L was used to supplement the raw whey, the L-Phe consumption range was from 70 to 75 %. In this case, it would be preferable to use 3 $g_{L\text{-}Phe}$/L as maximum initial concentration in order to avoid wasting the amino acid and thus increase the profitability of the 2PE production.

Effect of lactose and L-Phe on isopentanol production.

Figure 2.8C shows that isopentanol concentration had a positive correlation with the increment of lactose. The highest isopentanol concentration was 0.093 g/L when the initial concentration of lactose was in the range of 30.00 to 40.00 g/L and that of L-Phe of 1.58 to 2.00 g/L. Outside of this range, the isopentanol concentration decreased with the L-Phe concentration. For example, the lowest concentration of isopentanol was 0.039 at an L-Phe initial concentration of 3.00 g/L. The precursor amino acid for isopentanol is leucine, and the response surface of isopentanol concentration indicates that the increment of L-Phe can inhibit the production of isopentanol. This was corroborated by the isopentanol concentration observed in the first step of this study. In this

case, when L-Phe was not added, the concentration of isopentanol was 0.175 g/L at 48 h of fermentation, nearly twice as high as the highest concentration observed during the second step.

Effect of lactose and L-Phe on ethanol production.

Figure 2.9A and 2.9B shows ethanol concentration as a function of the initial concentration of lactose and L-Phe at 8 and 48 h of fermentation respectively. A positive correlation between the initial concentration of lactose and ethanol concentration was observed for both fermentation times. The highest concentration of ethanol at 8 h of fermentation was 4.79 ± 0.02 g/L, at an initial concentration of lactose and L-Phe of 40.00 and 2.00 g/L respectively. Whereas, when both initial concentrations were increased to 44.14 g/L of lactose and 3.00 g/L of L-Phe, the ethanol concentration decreased to 2.82 ± 0.01 g/L. Figure 2.9 shows that the increment of the initial concentration of L-Phe inhibited ethanol production despite the increment of the initial lactose concentration. This suggests that the concentration of L-Phe of 3.00 g/L is a threshold for the consumption of L-Phe as source of nitrogen and carbon. Below this concentration, the L-Phe is used to obtain energy and produce primary metabolites. Above this concentration, the L-Phe is preferentially transformed via the Ehrlich pathway. As a consequence, the electrons to produce pyruvate and further oxidation to ethanol are limited because the Ehrlich pathway is an energy-consuming metabolic route (Garavaglia et al., 2007; Mas et al., 2014). The results obtained in this study are comparable to those obtained by Garavaglia et al. (2007). They studied grape must fermentation using *K. marxianus* with an initial pH of 6.5 and at a temperature of 30 °C and observed an ethanol concentration of 5 g/L for the first 8 hours of fermentation (Garavaglia et al., 2007).

In addition, Figure 2.9B shows that the maximum concentration of ethanol was obtained at 48 h of fermentation. This supports the theory about the deviation of carbon and nitrogen to the Ehlrich

pathway when L-Phe is present at high concentration, causing a delay on ethanol production. For example, in the first step when L-Phe was not added, the maximum concentration of ethanol was observed at only 24 h of fermentation. This is comparable with the study of Azevedo de Lima et al. (2018). They fermented whey enriched with 1 g/L of L-Phe using *K. marxianus* and observed that the addition of L-Phe caused the inhibition of ethanol production (Azevedo de Lima et al., 2018).

Conclusions

The yeasts *Kluyveromyces marxianus* and *Debaryomyces hansenii* were used under co-culture mode to ferment sweet whey. They consumed the lactose and the amino acids from whey to support their growth and produce flavor and aroma biomolecules. The method to control microbial contamination affected the cell density of yeasts, being the pasteurization which promoted the yeast growth. The yeasts transformed the L-Phe into 2PE as secondary metabolite. The initial concentration of lactose and L-Phe was a determinative parameter for 2PE production, which was accumulated at a concentration of up to 2.55 g/L, showing the potential of the bioprocess for whey valorization.

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CHAPTER 3:

Production of 2-phenylethanol using yeasts immobilized systems onto inert supports

CHAPTER THREE – PART 1

Effect of passive cell immobilization of co-cultured yeasts on the whey fermentation and alcohols production

Mariana Valdez Castillo[1], Satinder Kaur Brar[1,2], Sonia Arriaga[3], Jean-François Blais[1], Antonio Avalos Ramirez[1,4]

[1] Institut national de la recherche scientifique, Centre-Eau Terre Environnement, 490, Rue de la Couronne, Québec, Québec G1K9A9, Canada.

[2] Department of Civil Engineering, Lassonde School of Engineering, York University, Toronto, Ontario M3J 1P3, Canada.

[3] Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4a Sección 78216, México.

[4] Centre National en Électrochimie et en Technologies Environnementales Inc. 2263, avenue du Collège, C.P. 610, Shawinigan (Québec) G9N 6V8, Canada.

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Résumé

Le lactosérum est l'un des principaux résidus de l'industrie laitière et sa valorisation par fermentation est une pratique émergente qui contribue à l'économie circulaire et au développement durable. La fermentation du lactosérum avec des souches de levures spécialisées produit des biomolécules à valeur ajoutée, comme les alcools de fusel de grand intérêt pour les industries pharmaceutiques, alimentaires et cosmétiques en raison de leurs propriétés aromatiques et gustatives. La présente étude visait à développer la fermentation du lactosérum avec les levures *Kluveromyces marxianus* et *Debaryomyces hansenii* immobilisées sur un support inerte, afin d'augmenter la densité cellulaire et la production de 2-phényléthanol (2PE). Du biochar produit à partir de matières premières ligneuses, de la perlite, et des anneaux plastiques Kaldnes ont été utilisés comme supports pour l'immobilisation des cellules. Ils ont été choisis parce que leur structure poreuse et leur surface rugueuse est differente. De plus, les groupes fonctionnels tels que l'hydroxyle, le carbonyle, le siloxane et les hydrocarbures aliphatiques étaient utiles pour le développement de liaisons covalentes et de forces électrostatiques entre les cellules et le support. L'immobilisation de la levure a augmenté la production de 2PE, en particulier sur les anneaux plastiques et la perlite, obtenant 0,64 ± 0,01 g/L de 2PE pour la culture en suspension et jusqu'à 0,90 ± 0,01 g/L pour la co-culture immobilisée sur les anneaux plastiques Kaldnes.

Mots clés : Fermentation du lactosérum, immobilisation des levures, biocharbon de bois, perlite, anneaux en plastique, production de 2-phényléthanol.

Abstract

Whey is one of the main residues of the dairy industry and its valorization by fermentation is an emergent practice that contributes to the circular economy and sustainable development. Whey fermentation with specialized yeast strains produces value-added biomolecules, such as fusel alcohols of high interest for the pharmaceutic, food and cosmetic industries due to their aromatic and flavor properties. The present study aimed to develop the whey fermentation with the yeasts *Kluveromyces marxianus* and *Debaryomyces hansenii* immobilized on inert support, to increase cell density and 2-phenylethanol (2PE) production. Biochar synthesized from wood feedstock, perlite and filter Kaldnes plastic rings were used as supports for the cell immobilization. They were selected based on their different porous structure and rough surface. Also, functional groups such as hydroxyl, carbonyl, siloxane, and aliphatic hydrocarbons were useful for the development of covalent bonds and electrostatic forces between cells-support. The yeast immobilization increased the 2PE production, especially on the plastic rings and perlite, obtaining 0.64 ± 0.01 g/L of 2PE for the suspended culture and up to 0.90 ± 0.01 g/L for the immobilized co-culture on filter Kaldnes plastic rings.

Keywords: Whey fermentation, yeast immobilization, wood biochar, perlite, plastic rings, 2-phenylethanol production.

Introduction

Canada is one of the ten major cheese-producing countries in the world, with the Province of Quebec contributing the largest share (Chrétien et al., 2019; Statistics Canada, 2017). Cheese production generates a liquid by-product called whey, which is the result of the coagulation of the milk proteins and lipids (Christensen et al., 2011; Díez-Antolínez et al., 2018). The coagulation is promoted due to the acidification of milk by the addition of organic or inorganic acids or of chymosin, an enzyme that cuts the casein into peptides, releasing glycomacropeptides, β-lactoglobulin and α-lactalbumin (Anand et al., 2013). Whey retains about 55% of the milk nutrients such as lactose (33.6 – 52.0 g/L), proteins of low molecular weight (1.8 – 10.0 g/L), and minerals (0.4 – 1.6 g/L), which contribute to the high chemical oxygen demand (COD), between 60 and 80 g O_2/L (Conde-Báez et al., 2019; Zotta et al., 2020). Whey is currently used as a fertilizer of crops lands or animal feedstock, but these activities can promote the acidification of soil and cause diarrhea to animals (Díez-Antolínez et al., 2018; Sebastián-Nicolás et al., 2020).

Globally, the United Nations Industrial Development Organization (UNIDO) and the European Union (EU) promote the implementation of a circular economy based-model in the agricultural sector. Strategies include the creation of policies on a well-functioning EU market for secondary raw materials, providing technical assistance for biomass transformation into energy and compost, and the application of biotechnology in the valorization of food wastes. UNIDO and EU also assist the development of Regional Industrial Clusters in providing technical and business support facilities based on zero-waste principles (European Commission, 2020; United Nations Industrial Development Organization, 2021). In the case of the particular case of the Province of Quebec, in 2020 the "Ministry of Sustainable Development, Environment, and Fight Against Climate Change (MELCC, for its French acronym)", published its strategy for the valorization of organic matter

such as agro-industrial residues (RECYC-QUÉBEC, 2018). In this context, the development of new bioprocesses to obtain active biomolecules from whey will contribute to its valorization and the circular economy of the dairy industry.

An emerging perspective for whey valorization is the production of biomolecules with aroma and flavor properties, such as 2-phenylethanol (2PE) (Conde-Báez et al., 2019; Dragone et al., 2009; Leclercq-Perlat et al., 2004; Valdez Castillo et al., 2021). The 2PE is a fusel alcohol highly valued by the pharmaceutical, food and cosmetic industries for its rose-like aroma, honey flavor and antimicrobial properties (Chreptowicz et al., 2016; Liu et al., 2014). The current market price of natural 2PE is from 70 – 100 $US/kg (Alibaba, 2022; Ambeed USA, 2022; Chem-Impex International, 2022). It can be produced by the specific transformation of L-Phenylalanine (L-Phe) carried out in yeast cells by the Ehrlich pathway. In general terms, this pathway consists of deamination, decarboxylation and aldehyde reduction to transform the amino acids into their respective fusel alcohols (Hazelwood et al., 2008; Pires et al., 2014).

Whey fermentation using yeasts to produce 2PE is a common practice (Conde-Báez et al., 2019; Dragone et al., 2009; Leclercq-Perlat et al., 2004). However, the accumulation of inhibitory compounds such as ethanol, organic acids and the 2PE is a bottleneck in whey fermentation (Conde-Báez et al., 2019; Dragone et al., 2009; Leclercq-Perlat et al., 2004; Valdez Castillo et al., 2021). To overcome these constraints, strategies such as co-culturing (Rodrigues et al., 2016; Valdez Castillo et al., 2021) and cell immobilization (Christensen et al., 2011; Díez-Antolínez et al., 2018) have been explored.

In previous studies, Valdez-Castillo et al. (2021) studied whey fermentation using a co-culture of *Kluyveromyces marxianus* and *Debaryomyces hansenii*, doubling the 2PE productivity and yield compared to suspended yeasts under monoculture mode. The increase in 2PE production was due

to the apparent synergic interaction of the yeasts and their high cellular density (Valdez Castillo et al., 2021). To obtain stable high cellular density and optimize the production of biomolecules, the active and passive immobilization of yeast cells is a promising alternative (Christensen et al., 2011; Díez-Antolínez et al., 2018; Wilkowska et al., 2014).

Immobilization is recommended when alcohols are produced because the biofilm that forms over the support protects the cells against toxic compounds such as ethanol. The biofilm also improves the transport of nutrients from the culture broth to the yeast cell (Christensen et al., 2011; Díez-Antolínez et al., 2018; Rittman and McCarty, 2001; Wilkowska et al., 2014). Suitable support must be easy to handle, mechanical and chemically stable, non-toxic for the cells and with high surface area (Rittman and McCarty, 2001). Díez-Antolínez et al. (2018) observed that the immobilization of *K. marxianus* increased the lactose consumption and prolonged the stationary growth phase during whey fermentation, when compared to the suspended culture (Díez-Antolínez et al., 2018). The stationary growth phase on yeasts is closely associated with the production of secondary metabolites such as 2PE (Hazelwood et al., 2008; Pires et al., 2014)

The present study aimed to analyze the effect of the immobilization of co-cultured yeasts on whey fermentation, using the production of 2PE and ethanol as response parameters. A passive immobilization of co-cultured *K. marxianus* and *D. hansenii* was performed using perlite, plastic rings and biochar as supports. This led to the comparison of 2PE production in suspended and immobilized cells. To the best of our knowledge, the effect of porosity, roughness and functional groups properties on the biofilm formation using co-cultured yeasts had not been reported. more so for 2PE production.

Material and methodology

Materials

Whey powder from bovine milk (Sigma-Aldrich, Canada, 1002641465) with a composition (% w/w) of lactose 75.5, and crude proteins 11.0 was used as substrate. Yeast extract (Fisher Scientific, Canada) and peptone (Organotechnie S.A.S, Canada) served as the source of organic nitrogen. Lactose (Sigma-Aldrich, Canada) and L-Phenylalanine powder (BioShop life science products, Canada, >98%) were the internal standards for the analytical method of liquid chromatography-mass spectrometric. L-Phenylalanine (L-Phe) powder was added to enrich the whey for the fermentation assays. Triethylamine (Fisher, Canada), methanol (Fisher, Canada, >98%), hydrochloric acid (Fisher, Canada), disodium hydrogen phosphate (Na_2HPO_4), and phenyl isothiocyanate (Sigma-Aldrich, Canada, >99%) were used for the determination of amino acids. Ethanol (Fisher, Canada, >98%) and 2-phenylethanol (Fisher, Canada, >98%) were the internal standards for the High-Performance Liquid Chromatography (HPLC).

The supports used to immobilize the yeast cells were biochar from wood torrefaction, perlite (Holland Basics Premiun Coarse, Canada), and filter Kaldnes plastic rings (K1 micro, Garden Supply, USA). The biochar was kindly donated by Innofibre – Centre d'innovation des produits cellulosiques (Trois-Rivières, Canada). These supports were selected for their easy handling, non-toxicity to the yeast cells, surface area and chemical stability.

Glutaraldehyde (Sigma-Aldrich, G7651-10ml, grade I, 50% aqueous solution) was used as the fixation agent prior to the analysis using the scanning electron microscope.

Culture media

Lactose medium (LM) was used to prepare the yeast inocula, containing lactose 45 g/L, yeast extract 10 g/L, and peptone 20 g/L. For the fermentation assays, whey powder from bovine milk was used as a culture broth basis. The powder was diluted in distilled water for a lactose concentration of 20 g/L, corresponding to 26.47 g/L of whey powder. Yeast extract 10 g/L, peptone 20 g/L, and L-Phe 3 g/L were added to the dissolved whey to form the whey medium (WM). The pH of WM was adjusted at 6.5 with NaOH 6M or HCl 6M.

Inoculum preparation

Kluyveromyces marxianus NRRL Y-1109 and *Debaryomyces hansenii* NRRL Y-1448 were acquired from the collection culture of the United States Department of Agriculture-USDA. Yeasts were conserved in glycerol stocks at -80°C. To prepare the inoculum for the fermentation assays, 100 µL of glycerol stock of either *K. marxianus* or *D. hansenii* and 50 mL of LM previously sterilized by autoclave (120°C, 20 min) were placed in a 250 mL flask. The inoculum of each yeast strain was incubated at 25°C and 200 rpm for 20 h in a rotary incubator (INNOVA 44 New Brunswick Scientific).

Methods

Fermentation using supports

Aerobic fermentations were carried out in 250 mL flasks with cotton caps filled with 30 mL of WM. Before carrying out the yeast fermentation, adsorption assays were performed to determine if supports could take molecules of lactose and L-Phe when they were not covered with biofilm at the beginning of fermentation. Flasks with the WM were filled with 1 g of either perlite, filter Kaldnes plastic rings or biochar, and sterilized at 121°C for 15 min. Physical characteristics of each support are indicated on Table 3.1. The flasks were incubated at 25°C, 200 rpm for 0.5 h to reach

the equilibrium between the supports and the WM. Samples were taken at 0 and 0.5 h to determine the lactose, amino acid, ethanol and 2PE content.

To study the effect of suspended and immobilized co-culture of yeasts on whey fermentation and alcohols production, four fermentation treatments were performed. For the first treatment, 12 flasks with WM were filled with 1 g of perlite and sterilized at 121°C for 15 min. The flasks were inoculated by adjusting the initial OD_{600} with a UV-VIS spectrophotometer (Eppendorf BioPhotometer plus). Based on previous a previous study, where the initial OD600 of *K. marxianus* and *D. hansenii* mixture ratio of 0.5:0.1 was optimal for the obtention of 2PE, the ratio for the co-cultures yeasts was selected (Valdez-Castillo et al., 2021). This corresponds to an initial cell density of $5x10^7$ and $1x10^7$ CFU/mL of *K. marxianus* and *D. hansenii*, respectively. For the treatments with 1 g of filter Kaldnes plastics rings or biochar, the process was repeated. The last treatment was the co-culture in suspension, which was used as a control. The flasks were incubated in a rotary incubator (INNOVA 44 New Brunswick Scientific) at temperature and agitation of 25°C and 200 rpm respectively.

Samples were taken at 0, 24, 48 and 96 h to determine the concentration of lactose, L-Phe, biomass (dry basis), ethanol and 2PE.

The kinetics parameters were calculated as explained in previous studies (Valdez Castillo et al., 2021). In brief, L-Phe consumption rate data were fitted using zero-order reaction kinetics. Whereas the biomass production was calculated considering the maximum yeast biomass concentration and it was divided by 24 h. Ethanol and 2PE productivity were calculated considering their concentration at 24 and 96 h of fermentation, respectively. The concentration was divided by either 24 or 96, which was the time yeast cells produced the maximum concentration of ethanol

and 2PE, respectively. The production yield of ethanol and 2PE was calculated as a ratio of the consumed mass of lactose and L-Phe, respectively.

Sample handling

Samples of adsorption assays and fermentation assays with and without support were handled as follows: for each sampling time, three flasks were taken, and the content was filtrated through a Whatman No. 1 filter with a pore size of 11 µm. The solids retained in the filter were dried at 105°C for 24 h. For fermentation with perlite, plastic rings or biochar, the support mass was subtracted from the dried sample weight to obtain the weight of the basis dry biomass only. The filtrate was centrifuged (Eppendorf 5804 R – Benchtop centrifuge) at 16 639 *x g*, 4°C for 10 min. The pellets were dried at 105°C for 24 h and the dry weight was added to the dried weight obtained with the aforementioned Whatman filter. The supernatant was used to determine the L-Phe concentration. The supernatant was filtrated (polyvinylidene difluoride-PVDF membrane, 0.45 µm, Fisher) to determine lactose, ethanol, and 2PE content. The filtrated and non-filtrated supernatants were stored at -20°C until analysis.

Characterization of the inert supports

Filter Kaldnes plastic rings were milled using an industrial miller (Fritsch 19.5720/021704) to determine the zeta potential and physical structure (crystallography) by X-ray diffraction analysis (XRD). Perlite and biochar were milled with pestle and mortar to a fine powder. The resulting milled powder and plastic particles were sifted through a 1 mm sieve.

pH and Zeta potential. 100 mg of sifted support (perlite, filter Kaldnes plastic rings and biochar) were suspended in 100 mL of deionized water. The mix was agitated at 200 rpm and 25°C for 24 h

to obtain a colloidal solution. This colloidal solution was also characterized in terms of pH (benchtop pH meter, Accumet AB250).

The resulting solution was used to determine the surface charge in a zeta potential analyzer (Malvern Instruments Nano-ZS ZEN 3600). Around 1 mL of the colloidal solution was used to fill a polycarbonate cell with a gold-plated electrode The plastic rings had the lowest with caps. Samples were analyzed using the phase analysis light scattering method with water as solvent at 20°C. The same process was repeated with particles suspended in WM.

X-ray crystallography. The structure, phase and crystal orientation of the supports were analyzed using a Bruker D2 PHASER Benchtop X-ray diffractometer. An incident wavelength radiation ($Cu_{K\alpha}$) of 1.5404 Å was used to sweep the samples in diffraction angles going from $5_{2\theta}$ to $80_{2\theta}$, using a 0.05 step size, at a scan rate of 1°/min and a temperature of 25°C. The voltage and current used were 40 kV and 40 mA respectively.

Fourier-transform infrared spectroscopy. The chemical structures of the sieved supports were characterized with a Thermo Scientific Nicolet IS5 total reflectance-Fourier transform infrared (FTIR) spectrometer (USA) within a frequency range of 500 – 4000 cm^{-1}.

Scanning electron microscope imaging. The surface morphology of the cleaned and dried supports was analyzed using a ZEISS EVO MA10 scanning electron microscope operated at 15 kV. After cultivation, supports particles were removed from the culture broth and immediately fixed in a 2.5% glutaraldehyde in phosphate-buffered saline solution for 2 h at 23°C. After fixation, the samples were washed twice in a phosphate-buffered saline solution (pH = 7) and dehydrated with a series of ethanol solutions (30, 50, 70, 95, and 100%). Each ethanol solution was used for 15 min, and the samples were allowed to dry overnight in a desiccator. The samples were gold coated and visualized using a ZEISS EVO MA10 scanning electron microscope operated at 15 kV.

Quantification of lactose and ethanol

The samples were thawed to 4°C for analysis. High performance liquid chromatography (HPLC Agilent Technologies 1260 infinity, USA) coupled with a diode array detector of 1260 RID and 1290 DAD were used to quantify lactose and ethanol respectively. The HPLC was equipped with an aminex HPX-87H column (Bio-Rad Laboratories, Inc., USA) with a length of 300 mm, an internal diameter of 7.8 mm and a film thickness of 5 µm. The column temperature was set at 50°C with a mobile phase of sulfuric acid 0.008 N diluted in water. The flow rate was set at 0.6 mL/min for 30 min. A volume of 5 µL of the sample was injected into the column.

Quantification of 2-Phenylethanol

The samples were thawed up at 4°C for analysis. High-performance liquid chromatography (HPLC Agilent Technologies 1230 infinity II, USA) coupled with a UV-visible forward optical scanning detector was used to determine the concentration of 2PE on culture broth samples. The HPLC was equipped with a ZORBAX Eclipse XDB-C18 (Agilent Technologies Inc., USA) column with a length of 250 mm, an internal diameter of 4.6 mm and a film thickness of 5 µm. A mobile phase of water: methanol (50:50) was used at a flow rate of 0.5 mL/min for 30 min, and the detection wavelength was 370 nm. The temperature of the column was set at 30°C and the volume of injection was 10 µL.

Derivatization and quantification of L-Phe

Culture broth samples of the four fermentation assays and amino acid standard (L-Phe) were submitted to derivatization for the analysis of amino acids adapting the method of Vilasoa-Martínez et al. (2007) as follows: 20 µL of the sample were placed in a vial and dried at 65°C for 2 h. 30 µL of solution methanol-water-triethylamine (4:4:1) were added and vigorously mixed with a vortex

for 10 s, and dried at 65°C for 10 min. 30 µL of methanol-water-triethylamine-phenyl isothiocyanate (7:1:1:1) were added and vortex-mixed for 30 s. The vial was left to stand at room temperature for 20 min. The resulting solution was dried for 15 min at 65°C. Prior to injection, 150 µl of 5 mM Na_2HPO_4 with 5% v/v acetonitrile were added to the dried sample. The pH was adjusted to 7 with phosphoric acid 1.07 M and was vortex-mixed for 30 – 60 s. Finally, 100 µL of the neutral derivatized amino acids solution were diluted with 400 µL of 5 mM Na_2HPO_4 with 5% acetonitrile.

A standard solution, containing 1 mM of the amino acid standard in HCl 0.1 M, was obtained and derivatized as aforementioned.

The derivatized samples and standard solutions were analyzed by HPLC (Agilent Technologies 1260 infinity, USA) with a UV-Visible forward optical scanning detector. The HPLC was equipped with a ZORBAX Eclipse XDB-C18 (Agilent Technologies Inc., USA) column with a length of 250 mm, an internal diameter of 4.6 mm and a film thickness of 5 µm. The temperature of the column was set at 27°C. Two mobile phases were used. Mobile phase A was 0.14 M sodium acetate buffer containing trimethylamine 0.05 % (v/v) at a pH of 6.2. The pH of mobile phase A was adjusted with acetic acid. Mobile phase B was a solution of acetonitrile and water (60:40). A gradient of the two mobiles phases was used as described by Vilasoa-Martínez et al. (2007). The flow rate was set at 0.9 mL/min and the detection wavelength was 254 nm (Vilasoa-Martínez et al., 2007).

Statistical analysis

An analysis of one-way ANOVA (p-value < 0.05) with the Tukey test as a post hoc test was performed in R core team 2020 software. The analysis was used to study the effect of the suspended and immobilized co-culture of yeasts on the production of 2PE. The independent variable was the

type of treatment (suspended yeasts cells, and immobilized yeast cells on biochar, perlite and plastic rings), and the dependent variable was the concentration of 2PE at the end of the fermentation.

Results and discussion

Physicochemical characteristics of supports and their effect on yeast biofilm formation

Characterization of the supports in terms of physical parameters

The type of immobilization promoted in this study was the adhesion of cells to the surface of the supports. The immobilization grade was determined by physical and chemical parameters, such as the surface area, porosity, roughness, surface charge, media pH and chemical composition of the supports. Table 3.1 shows the physical characteristics of three supports: perlite, filter Kaldnes plastic rings and biochar. Perlite presented the highest porosity, 65%, followed by biochar and negligible for the plastic rings. Whereas biochar presented the highest surface area, 3.00 m^2/g, this could be due to the roughness of the biochar and its particle size (3.10 ± 0.20 mm), which was smaller than perlite (5.60 ± 1.10 mm) and plastic rings (9.37 ± 0.10 mm).

Specific surface area, porosity and roughness are physical parameters that affect the adhesion of cells because they will affect the sensation of shear force on the surface. When the shear force is low, the cells are in contact with the material longer and they can establish bonds like charge attraction, covalent bonds between functional groups and physical retention. As an example, high roughness like perlite and small pores like biochar will produce zones with low shear forces.

Table 3. 1 Physical characteristics of inert supports used for the assays of whey fermentation to immobilize the yeast cells under co-culture mode.

Properties	Inert supports		
	Perlite	**Filter Kaldnes plastic rings**	**Biochar**
Density (g/cm^3)	0.078 ± 0.01	0.96 ± 0.20	0.33 ± 0.01
Porosity (%)	<65[A]	n.a.	50 – 70[C]
BET surface area (m^2/g)	1.20 ± 0.20[A]	0.931± 0.01[B]	3.00± 0.01[C]
article size (mm)	5.60 ± 1.10	9.37 ± 0.10	3.10 ± 0.20

Information from producers: A (Holland Basics Premiun Coarse, Canada), B (K1 micro, Garden Supply, USA), and C (Innofibre – Centre d'innovation des produits cellulosiques, Quebec, Canada).

n.a., Not apply.

An electron microscopy analysis was performed to observe the surface of each support before and after fermentation. Figure 3.1a shows photography of one average particle of biochar and Figure 3.1b and 3.1c show the SEM micrography (500x zoom) of the surface of biochar. Figure 3.1b shows clean biochar and Figure 3.1c biochar after whey fermentation with yeast cells immobilized over the surface. The biochar pores (Figure 3.1b) had thin channels where yeasts cells could enter, avoiding their removal by agitation, protecting the cells to start the formation of a biofilm. In addition, the roughness of the surface derived from the partial degradation of lignin and cellulose decreased the effect of shear force. This contributes to the development of a uniform thin layer of biofilm over the particles as shown in Figure 1c. Kyriakou et al. (2019) used biochar to immobilize *K. marxianus.* Their biochar was obtained by pyrolysis at 500^{o}C, producing mainly macropores due to the complete degradation of hemicellulose, lignin and cellulose. They observed the entrapment and agglomeration of *K. marxianus* cells inside the biochar pores in comparison to the uniform layer observed in Figure 1c of the present study (Kyriakou et al., 2019). This confirms

that the porosity and roughness of the surface are important parameters to create favorable conditions for cell adhesion.

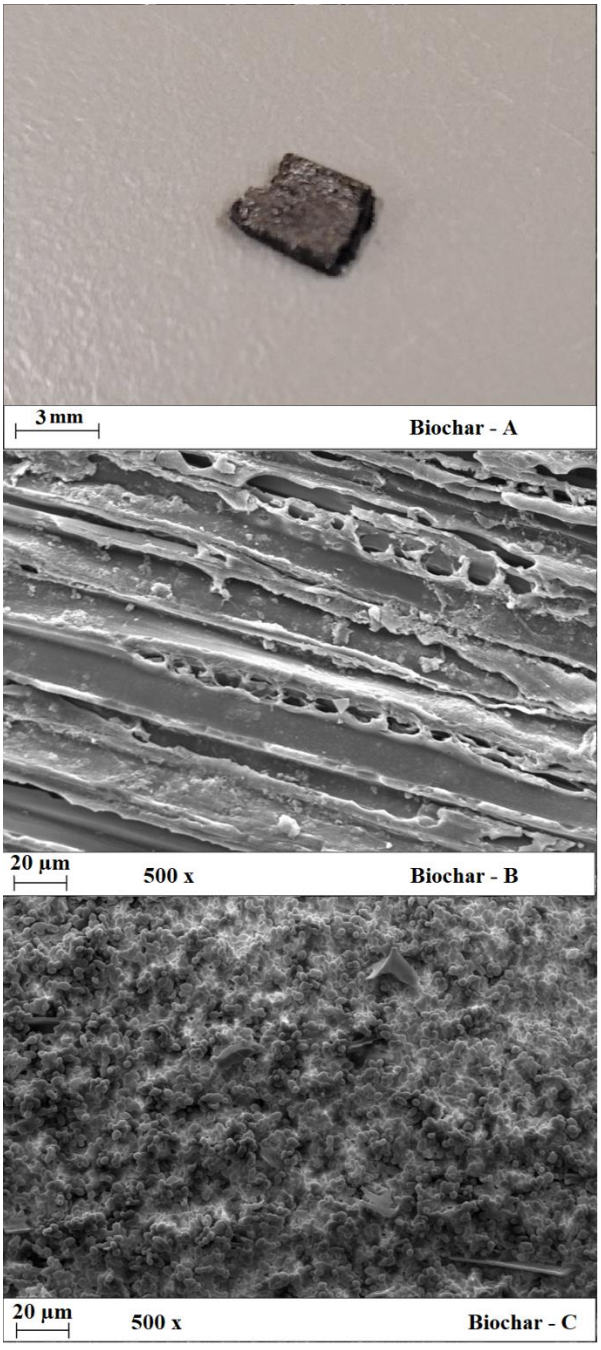

Figure 3. 1 Photographies of biochar support showing: a) Whole particle, b) Zoom of clean surface by SEM micrography, and c) Zoom of support with immobilized cells by SEM micrography.

Figure 3.2a shows photography of one average perlite particle, Figure 3.2b the 500x zoom of the surface of perlite particle before fermentation, and Figure 3.2c after fermentation. The latter shows the immobilization of yeasts cells, concentrated in low exposed areas. Perlite is a natural volcanic rock characterized by its large irregular pores and high roughness (Anicua Sánchez et al., 2009). Porous diameter size ranges from 100 to 700 nm, in the class of macropores (Anicua Sánchez et al., 2009; Leng et al., 2021).

These pores could allow the retention of cells. However, because of their large size there are large open areas (Figure 3.2b) that cannot prevent the formation of turbulence inside the pores, probably presenting more zones with high shear forces over the surface than the biochar particles. This can limit the adhesion of yeast cells on the surface. Also, the friction of the particles and the force of agitation over the perlite surface can lead to the detachment of some yeast cells weakly attached, decreasing the rate and uniformity of biofilm formation. Figure 3.2c shows that the biofilm of yeast cells over the perlite surface had a lower density and it was less uniform than the biofilm formed on biochar particles. Foroughi et al. (2018) studied the immobilization of *Sacharomyces cerevisae* cells on perlite with a particle size ranging from 3 to 5 mm. They observed the entrapment of the yeast cells into the perlite pores at the end of 48 h, forming pores spots rather than a uniform biofilm as observed in the present study (Figure 3.2c).

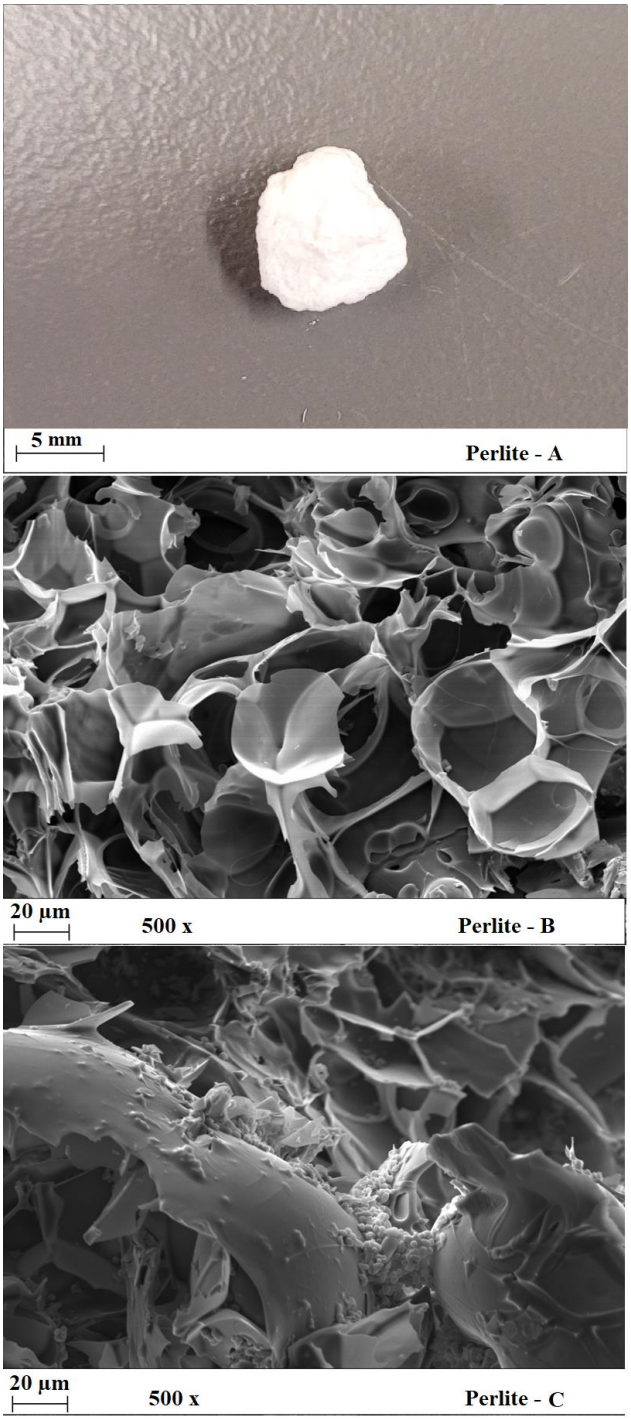

Figure 3. 2 Photographies of perlite support showing: a) Whole particle, b) Zoom of clean surface by SEM micrography, and c) Zoom of support with immobilized cells by SEM micrography.

Figure 3.3a shows the photography of one average plastic ring particle, Figure 3.3b the 505x zoom of the surface of plastic rings before fermentation and 3.3c after fermentation. The plastic rings had the lowest surface roughness, with negligible porosity, as discussed earlier. This represented low physical fixation points for developed biofilm. For this reason, some biofilm was lost during the manipulation of support to analyze the surface by SEM, especially during the fixation of cells with glutaraldehyde. For this reason, the micrography of Figure 3.3c showed a low density of yeast cells in comparison with biochar and perlite supports. Due to the geometry of the rings, with four internal macro cavities, the specific surface area could be comparable to perlite. Despite the absence of pores, the macrostructure of rings, with several "V" points, favors a lower shear force in these sections, which can help the adhesion of the yeast cells. However, the yeast cells are not protected against the fluid flow caused by the constant agitation and the absence of roughness and pores leads to the detachment of cells and the formation of a weak biofilm. This was observed during the film fixation with glutaraldehyde for microscopy analysis. Normally, plastic materials in contact with cells cause electrostatic interactions such as van der Waals forces, ionic and hydrogen bonding interactions. These forces allowed the binding of yeast cells and plastic materials but they were reversible surface interactions. This means that the binding was weak and could not resist the detachment of cells by abrasion caused by the friction of rings under constant agitation and the shear force of the medium over the ring surface (Bickerstaff, 2003; Thorne and Williams, 1999).

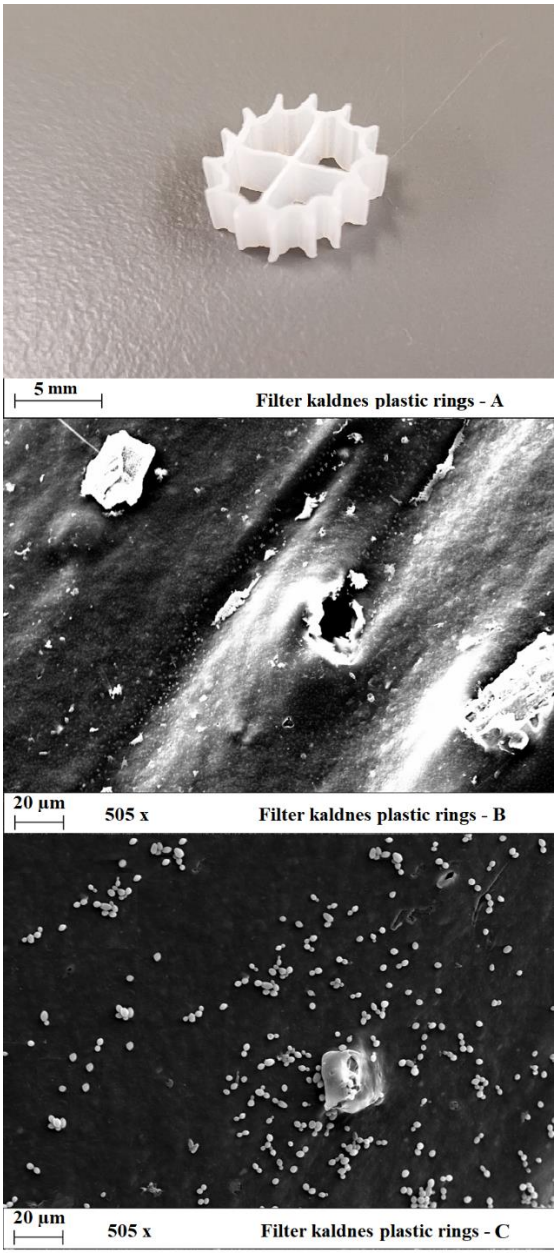

Figure 3. 3 Photographies of filter Kaldnes plastic rings showing: a) Whole particle, b) Zoom of clean surface by SEM micrography, and c) Zoom of support with immobilized cells by SEM micrography.

Analysis of crystal structures and chemical composition of supports

XR crystallography was performed for the three supports to determine their crystal structure and thereby the main components. Figures 3.4a, 3.4b and 3.4c show the XRD spectra for the biochar, perlite, and plastic rings, respectively.

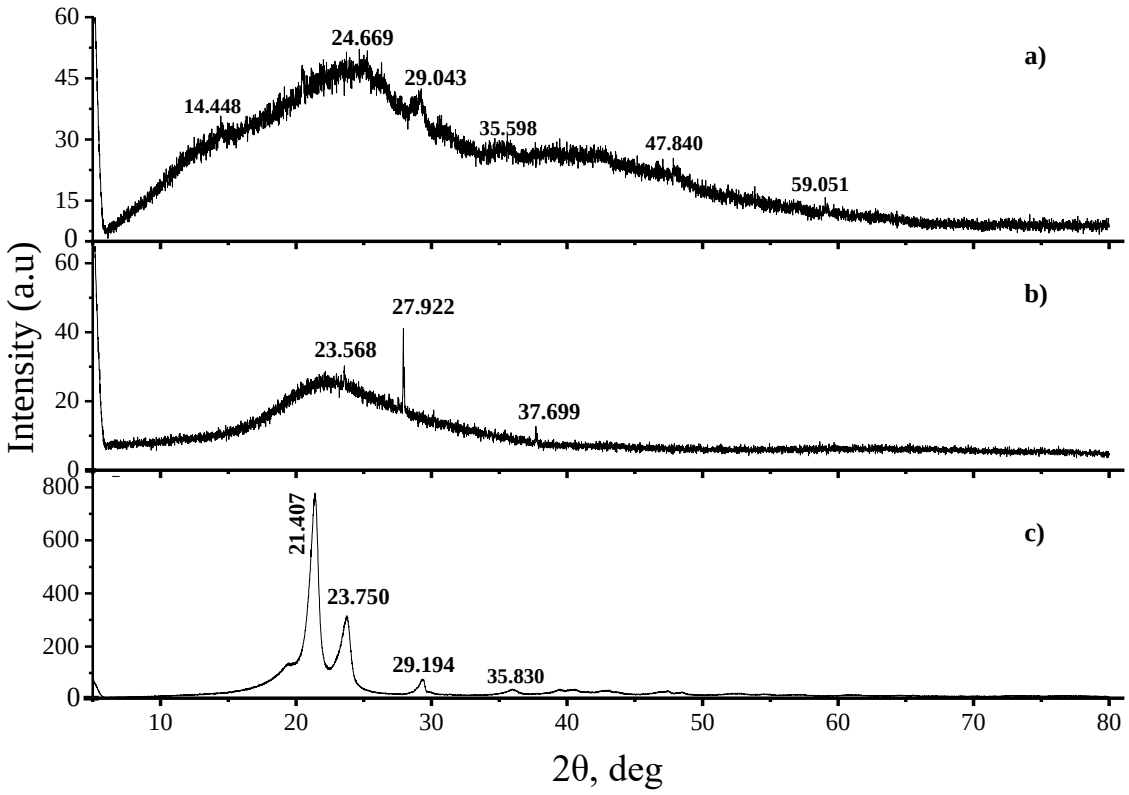

Figure 3. 4 XRD spectra of clean supports: a) Biochar, b) Perlite and c) Filter Kaldnes plastic rings.

Figure 3.4a shows 6 peaks with low intensity at 2θ = 14.44; 24.66; 29.04; 35.59; 47.84; and 59.05°. The first two peaks indicate the presence of cellulose and lignin in the biochar, which aligns with the roasting process used to produce the biochar at a temperature of around 250ºC, which produced an incomplete degradation of the cellulose and lignin. The peaks from 2θ = 29.04° to 2θ = 59.05° correspond to the crystalline structure of the calcite ($CaCO_3$). In addition to the natural accumulation of calcium in the wood structure, the presence of gypsum in the residual wood may have reacted with carbon dioxide and carbon monoxide during torrefaction to form calcium carbonate (Kyriakou et al., 2019; Leng et al., 2021).

The two polymers of biochar, cellulose, and lignin are considered complex carbon structures due to the glucose units or the crosslinked phenolic compounds respectively. Both polymers are not

degraded by *K. marxianus* and *D. hansenii*, as these do not produce the enzymes to hydrolyse the cellulose and lignin structures (Wei and Zhang, 2018; Williams et al., 2019). Instead, the biochar polymers confer mechanical resistance and chemical stability to biochar for biofilm formation. Pires et al. (2012) studied the use of cellulose-based materials for the biofilm yeast-like formation. They observed the formation of a thick biofilm without degradation of the support (Pires et al., 2012).

For perlite, three main peaks were observed at $2\theta = 23.56°$, $2\theta = 27.92°$ and $2\theta = 37.69°$ (see Figure 3.4b). This indicates the presence of the anorthite structure, present in perlite. It is a rock with 14.4% of calcium, 19.4% of aluminum, 20.2% silicon, and 46.0% oxygen (Erdogan, 2015). These elements were not toxic for the yeast cells, facilitating the adherence and formation of biofilm. Foroughi et al. (2018) studied the adhesion of *Saccharomyces cerevisiae* on perlite beads. They observed a strong adhesion of yeasts cells on the surface after 48 h of contact (Foroughi et al., 2018). This corroborated the use of perlite as support for the yeast cells' immobilization.

For the plastic rings, the two peaks at $2\theta = 21.41°$, $2\theta = 23.75°$ are distinctive of high-density polyethylene (see Figure 3.3c). In addition, the peaks at 29.19° and 35.83° corresponded to lattice planes, which indicated the packing of plane layers of high-density polyethylene to form a homogeneous and mechanically resistant structure. The XRD spectra indicated that the high-density polyethylene used in this study has an orthorhombic solid structure (Benabid et al., 2019).

High-density polyethylene is a support material widely used for bacteria-like biofilm formation in wastewater treatment (Al-Amshawee and Yunus, 2021). To the best of our knowledge, this material has not been studied for the specific immobilization of yeast cells. However, this support was selected due to its high mechanic resistance, lightweight, low polarity, - nearly neutral -, and complex hydrocarbon structure which can provide adhesion points for yeast cells. The

orthorhombic crystal structure indicated that particles have a rectangular prism arrangement with plane surfaces intersecting at 90° angles. Because of this, not all the surface area of plastic rings was exposed to the same abrasion forces from the culture medium fluid movement. The angles of contact between fluid and support may contribute to the easier detachment of cells in comparison to perlite (spherical structure) or biochar (cylindrical structure). In addition, the absence of pores in the plastic rings could limit the adhesion of cells.

Presence of functional groups on the support surface

For the identification of functional groups on the surface of the supports, FTIR analyses were performed. Figure 3.5 shows the FTIR spectra for the three supports. The FTIR spectra for biochar (Figure 3.5a) presented three stretching bands at 1696.56, 1588.57 and 1157.08 cm^{-1}, corresponding to the stretching vibrations of carbonyl groups (RC(=O)R'), aromatic C=C and polysaccharides from cellulose and lignin –C–O–C–, respectively. The peak at 1588.57 cm^{-1} could also be the stretching vibration of C=O stretching associated with the $CaCO_3$, identified previously in the XRD spectra of biochar (Behazin et al., 2016; Liu et al., 2012). The low intensity of a band in the region from 3423 to 2900 cm^{-1} in Figure 5a indicated the overlap of phenolic groups and aliphatic OH stretching groups. The phenolic groups can come from the lignin, associated with the C=C aromatic vibrations, whereas the aliphatic-OH groups could correspond to the partially degraded cellulose (Heidenreich et al., 2016; Liu et al., 2012).

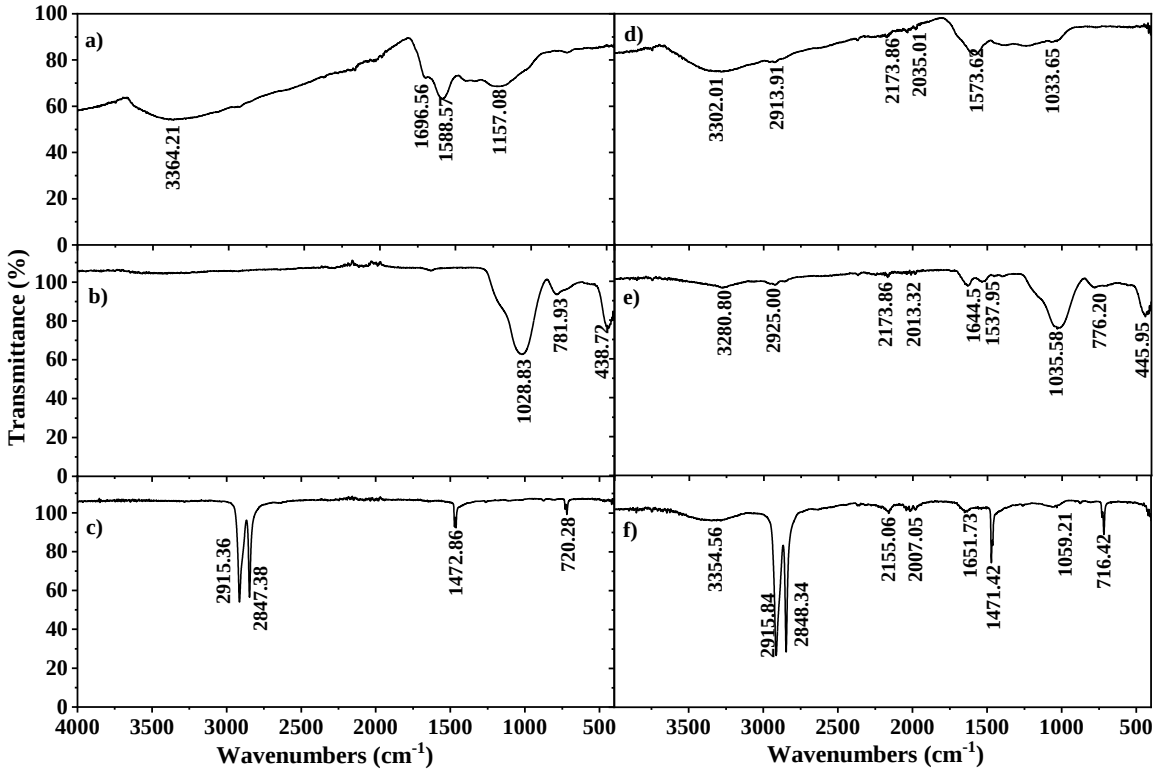

Figure 3. 5 FTIR spectra of clean supports: a) Biochar, b) Perlite and c) Filter Kaldnes plastic rings; and supports after being in contact with culture broth: d) Biochar, e) Perlite and f) Filter Kaldnes plastic rings

The functional groups of biochar have polar characteristics, meaning that they have positive and negative charges. For example, the hydrogen from –OH has a partial positive charge, but the oxygen is more electronegative and has a partial negative charge. On the other hand, the oxygen in the ether groups (–C–O–C–) and carbonyl groups (RC(=O)R') can share electrons to create hydrogen bonds with the hydroxyl functional groups. This leads to the formation of chemical bonds between the functional groups of biochar and the functional groups of yeast cell walls. The cell walls of *K. marxianus* and *D. hansenii* are mainly constituted of mannose, proteins and glycoproteins (Nguyen et al., 1998; Perpetuini et al., 2019). The main functional groups of these molecules are –OH, present in polysaccharides and proteins, and COOH and amino groups, present

in proteins and glycoproteins (Nguyen et al., 1998; Perpetuini et al., 2019). Due to the electrostatic forces between O and H, O and C, and H and N atoms, the functional groups of biochar and cell walls can interact and create covalent bonds which also contribute to the attachment of cells to the biochar surface. Kyriakou et al. (2019) observed that the immobilization of *K. marxianus* cells was more efficient on biochar produced from wood feedstock in comparison to biochar produced from biomass like sewage sludge (Kyriakou et al., 2019). This was attributed to the presence of lignin, which could increase the interactions of cells with the surface of the wood biochar (Kyriakou et al., 2019).

The Figure 3.5b shows the FTIR spectra of perlite. Three bands at 1028.83, 781.93, and 438.72 cm^{-1} were identified. They correspond to the Si-O-Si asymmetric stretching bonds (commonly identified as siloxane functional groups and specific for perlite), the symmetrical stretching of Si-O-Si and the symmetrical stretching of Si-O-Al (corroborating the aluminosilicate phase), respectively. The presence of Si-O and Si-O-Al are associated with the structure of the anorthite identified in the XRD spectra (Figure 3.4b). The band's pattern shown in Figure 3.5b is in agreement with other reports of perlite characterization (Erdogan, 2015).

Silicon is a highly reactive element due to its electronegativity and metalloid properties. As identified in Figure 3.5b, the presence of Si-O and Si-Si functional groups may contribute to the immobilization of yeast cells on the surface of perlite. Yeast cells contain proteins covalently linked to polysaccharides, called glycoproteins (Nguyen et al., 1998; Perpetuini et al., 2019). Glycoproteins have –OH, -COOH and $-NH_2$ groups that can interact with the functional groups on the perlite surface. In this context, Si-O and Si-Si can form covalent and hydrogen bonds with the H atoms of –OH, -COOH and $-NH_2$ functional groups present on the cell walls (Canham, 2014; Perpetuini et al., 2019). Also, the Si-H bond is more stable than Si-O, due to the lower

electronegativity of H in comparison to O (Canham, 2014). This contributed to the attachment of yeast cells to the surface of perlite. Foroughi et al. (2018) observed that both yeast cells and perlite have hydrophobic properties which contribute to the attachment and accumulation of yeast over the perlite surface (Foroughi et al., 2018). The hydrophobicity of yeast cells was attributed to the presence of proteins mainly constituted of hydrophobic amino acids such as valine, alanine, and leucine. The hydrophobicity of perlite was attributed to the presence of siloxane functional group (Canham, 2014; Perpetuini et al., 2019).

The FTIR spectra of filter Kaldnes plastic rings is shown in Figure 3.5c. Four intense bands were identified at 2915.36, 2847.38, 1472.86 and 720.28 cm^{-1} and corresponded to aliphatic hydrocarbon bonds. The peaks at 2915.36 and 2847.38 cm^{-1} correspond to an asymmetric C-H bond and a symmetric C-H bond respectively (Benabid et al., 2019). These aliphatic hydrocarbon bonds have nonpolar and hydrophobic properties. This leads to the direct interaction of the hydrophobic amino acids of proteins present on yeast cell walls. This interaction allows the development of electrostatic forces such as van der Waals forces and hydrogen bonding interactions (Benabid et al., 2019; Bickerstaff, 2003). Hydrogen bonding can be created between the amino groups and the carboxylic groups of the proteins, as well as the hydroxyl groups of polysaccharides of cell walls (Canham, 2014; Perpetuini et al., 2019). Díez-Antolínez et al. (2018) studied the effect of the immobilization of *K. marxianus* on plastic, glass and alumina supports on whey permeate fermentation to produce ethanol. They observed that the production of ethanol was more stable over time when yeast cells were immobilized on glass and alumina supports rather than on plastic support because the material may affect the cell activity (Díez-Antolínez et al., 2018). This agrees with the fragile and slim biofilm observed in the present study for plastic rings.

pH and surface charge of aqueous suspensions

The pH and surface charge of supports suspended in water and WM is shown in Table 3.2. The pH of water and WM were 6.81 ± 0.25 and 6.57 ± 0.15, respectively. When the particles were added to the liquids, their pH changed. The biochar slightly acidified both the water and the WM, whereas, the perlite increased their pH.

Table 3. 2 Effect of supports on pH and Zeta potential of aqueous and culture broth. For pH, it is shown the value before and after adding the support material.

Suspension medium	pH				Zeta potential (mV)		
	Without supports	With biochar	With perlite	With Kaldnes plastic rings	Biochar	Perlite	Filter Kaldnes plastic rings
Water	6.81 ± 0.25	6.50 ± 0.02	7.52 ± 0.01	6.50 ± 0.01	-23.87 ± 0.45	-60.47 ± 0.94	-10.46 ± 3.26
WM	6.57 ± 0.15	6.49 ± 0.01	7.20 ± 0.40	6.50 ± 0.00	-6.31 ± 0.21	-7.22 ± 0.27	-6.87 ± 0.07

WM, Whey medium.

The acidification of water and WM when the biochar particles were added is attributed to the presence of hydroxyl and phenol groups in the biochar. Both functional groups can donate hydrogen protons to aqueous media. The functional groups of biochar act as Lewis acid to form the hydronium ion acidifying the medium (Behazin et al., 2016). The silicon in perlite, on the other hand, can accept hydrogen protons from H_2O. This leads to the removal of hydrogen and the slight increment in pH once the perlite particles are suspended in the water and the WM (Canham, 2014). Despite the slight initial pH change, the supports can be used for the immobilization of yeast cells: after adjusting the pH to 6.5, it remained stable.

Table 3.2 also shows the zeta potential of the particles suspended in water and WM at a pH of 6.5. This matches the pH of the fermentation assays. For all materials, the negative net surface charge was higher in water than in WM. Perlite presented the highest negative net surface charge of -60.5 ± 0.9 mV in water, followed by biochar and plastic rings with -23.9 ± 0.5 and -10.5 ± 3.3 mV respectively. The negative zeta potential of the three supports is highly dependent on the functional groups previously identified. The Si-O can be responsible for the net negative charge of perlite. The carbonyl and ether groups of biochar have a negative net charge normally attributed to the oxygen. For the plastic rings, the aliphatic hydrocarbons are stable, and charges can be balanced to show a zeta potential near zero. When the supports were suspended in WM, their negative net surface charge was in a narrow range, from -7.2 ± 0.3 to -6.3 ± 0.2 mV. The decrease in net negative charge for the three supports when suspended in WM indicated that functional groups of biomolecules present in WM, such as proteins, can interact with the negative charges of the surface of supports and be adsorbed on the surface of supports. This can improve cell adhesion over the surface supports because the repulsion forces between the negative charges of cells and surface decreased.

To corroborate this, particles of the three supports suspended in WM were filtrated and dried at 60°C. Dried particles were analyzed by FTIR and spectra are shown in Figure 3.5. The new spectra showed the presence of amino and hydroxyl groups in the region from 3000 to 3500 cm^{-1} and amino and carboxyl groups from 1000 to 2000 cm^{-1} for all materials. To confirm this observation, particles of the three supports suspended in WM were filtrated, washed three times with deionized water, dried at 60°C and analyzed by FTIR. The spectra obtained were similar to those of the clean particles.

Lavaisse et al. (2019) analyzed the variation of the zeta potential of yeast cells under aerobic growth conditions. The cells had negative zeta potential in a range from -10 to -20 mV (Lavaisse et al., 2019), which is near the zeta potential observed for the three supports suspended in WM (Table 3.2). This suggested that electrostatic forces played an important role in the yeast adhesion, because the difference in net negative charge between yeast cell and support would have been low (Díez-Antolínez et al., 2018; Foroughi et al., 2018; Perpetuini et al., 2019).

Effect of yeast immobilization on whey fermentation

Biomass production

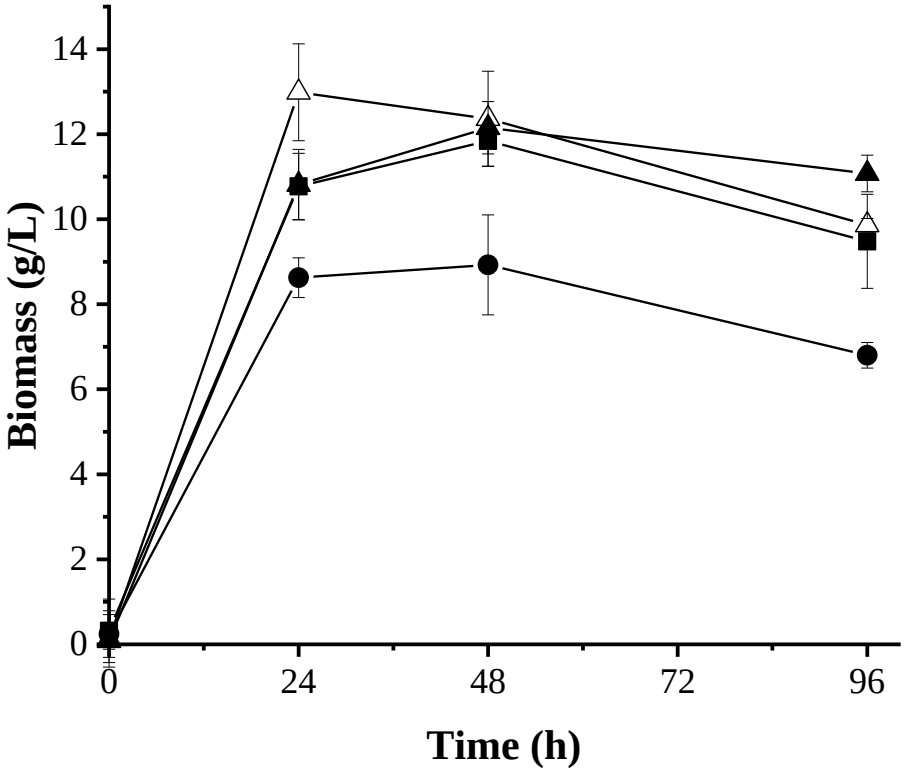

Figure 3. 6 Biomass concentration as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture.

Figure 3.6 shows the total biomass concentration (dry basis) of suspended cells and immobilized cells. For all assays, the exponential phase occurred within the first 24 h of fermentation. The co-culture immobilized on perlite presented the highest biomass concentration, with a maximum of 12.98 ± 1.14 g/L at 24 h, and the co-culture immobilized on biochar presented the lowest, with a maximum of 8.62 ± 0.46 g/L at 24 h. For the four fermentation assays, Table 3.3 showed the biomass production and kinetics parameters of alcohols yield and productivity, and L-Phe consumption rate. The co-culture immobilized on perlite had the highest biomass production, followed by the co-culture immobilized on plastic rings, at 0.54 ± 0.05 and 0.45 ± 0.03 $g_{biomass}$/L*h, respectively.

The production of biomass is directly related to the consumption of lactose and nitrogen. The fermentation with biochar produced a lower concentration of biomass (Figure 3.6), but the biofilm developed over biochar surface was uniform and apparently had high cell density, compacted as shown in Figure 3.1c. This biofilm can also form in multilayers, which limits nutrient transfer from the culture broth to the deepest layer of biofilm. This could be due to the formation of a better structured biofilm than with other supports. The biofilm of biochar did not need to produce a lot of adhesins, glycoproteins that have the function of selective cell-cell and cell-environment adhesion. Among the biofilm's functions is protection against hazardous environmental factors and increased nutrient uptake. Since the biofilm of biochar formed quickly and was well structured, as shown in Figure 1c, offering these functionalities to cell yeasts, the consumption rate of lactose and ethanol were slower than in the other cases, as shown in Figure 3.7.

For the other two supports, the need to produce a high amount of adhesins to form the biofilm can explain both higher biomass accumulation (yeasts and exopolymers) and substrate consumption. The adhesins attach to the membrane cell and they are produced in response to stress, nutrient

limitation or quorum-sensing biomolecules produced by the yeasts, such as ethanol and fusel alcohols (Chauhan and Mohan Karuppayil, 2021).

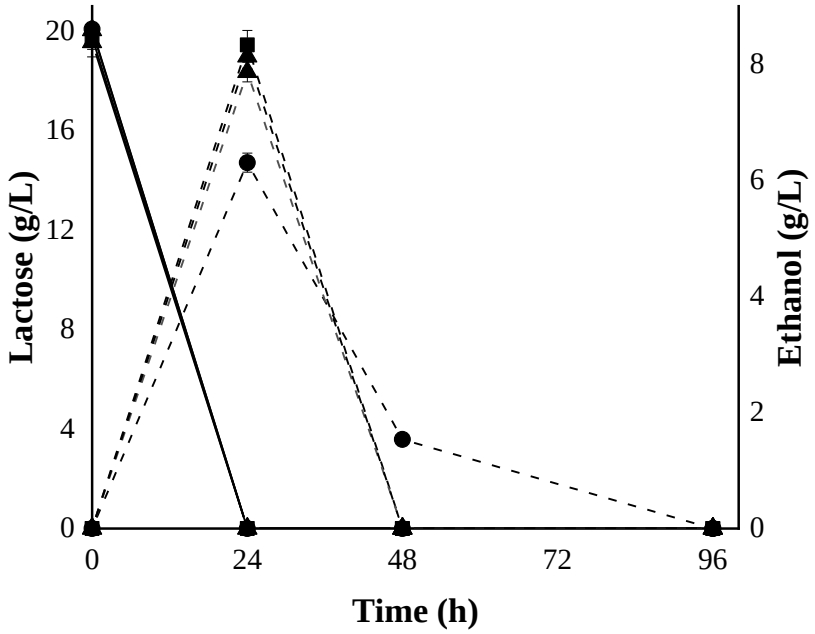

Figure 3. 7 Lactose concentration (continued lines) and ethanol concentration (dashed lines) as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture.

Ethanol and fusel alcohols are by-products of lactose fermentation and amino acids transformed by the Ehrlich pathway, respectively (Conde-Báez et al., 2019; Valdez Castillo et al., 2021). To produce adhesins, the MAP Kinase-dependent pathway and Ras-cAMP pathway are activated once the yeast cells detect lactose in the medium. Both pathways are overexpressed if there are ethanol and fusel alcohols in the medium. This is a defense mechanism, as alcohols can denaturalize the membrane cells (Chauhan and Mohan Karuppayil, 2021). Thus, for perlite and plastic rings the induction of adhesins to form the biofilm and protect cells against high ethanol concentration was higher than for biochar, which explains why plastic and perlite had higher biomass production than biochar. Li et al. (2017) studied the effect of 2PE, tyrosol and tryptophol on the growth of *S.*

cerevisae and observed that these fusel alcohols inhibited growth, and stimulated the morphological cell change from buds to hyphaes or pseudohyphaes to flocculate and protect the cells (Li et al., 2017).

Perlite produced the highest concentration of biomass. Figure 3.2c shows a thin film over the surface of perlite, which may be exopolymers such as adhesins, and not uniform agglomerates in the interstices of perlite walls where pores narrow.

For the fermentation system using the plastic rings and the co-culture in suspension, the biomass concentration was similar. This may be attributed to weak electrostatic forces developed between cells-support and cells-cells once they attach to the plastic rings. Figure 3c shows that the surface of the plastic ring is covered by a thin film and the yeast cells attach to this film, like in perlite. However, the absence of pores and roughness in plastic rings did not produce strong adhesion between the biofilm and the surface, causing some of the biofilm easily detached.

Lactose consumption and ethanol production

Figure 3.7 shows the consumption of lactose and the production of ethanol for all fermentation assays. Lactose was completely consumed within the first 24 h of fermentation, the exponential growth phase of the yeasts (Figure 3.6).

For all assays, the maximum concentration of ethanol occurred at 24 h. It was around 7.7 ± 0.8 g/L in all cases, except for biochar, with 6.3 ± 0.2 g/L. Similarly, for all fermentation assays, except for biochar, ethanol was depleted at 48 h of fermentation. Table 3.3 shows the productivity and yield of ethanol with respect to the lactose consumed. The co-culture in suspension and the yeast cells immobilized on plastic rings had the highest ethanol yield of 0.41 ± 0.01 $g_{ethanol}/g_{lactose}$.

Table 3. 3 Overall performance of co-cultured yeasts during the fermentation assays of WM medium for suspended and immobilized yeast cells. The table shows the main kinetics parameters of yeast growth rate, consumption of L-Phe and production of main by-products.

Parameter	Treatment			
	Suspended cells	**Immobilized cells on biochar**	**Immobilized cells on Kaldnes plastic rings**	**Immobilized cells on perlite**
Growth rate (1/h)	0.418 ± 0.019	0.337 ± 0.029	0.447 ± 0.028	0.531 ± 0.042
Ethanol productivity ($g_{ethanol}$/L*h)	0.347 ± 0.010	0.263 ± 0.007	0.339 ± 0.007	0.328 ± 0.007
Ethanol yield ($g_{ethanol}/g_{lactose}$)	0.423 ± 0.023	0.314 ± 0.009	0.416 ± 0.001	0.393 ± 0.007
L-Phenylalanine consumption rate (g/L*h)[a]	0.019 ± 0.001	0.021 ± 0.005	0.034 ± 0.002	0.021 ± 0.001
2-phenylethanol productivity (mg/L*h)	6.641 ± 0.000	6.606 ± 0.133	9.449 ± 0.167	9.365 ± 0.096
2-phenylethanol yield (g_{2PE}/g_{Lphe})[a]	0.659 ± 0.020	0.553 ± 0.070	0.856 ± 0.046	0.543 ± 0.007

[a] Calculated using the concentration of free L-Phenylalanine.

To satisfy their metabolic needs, the yeasts began consuming ethanol once lactose was depleted. Initially, the production of ethanol will increase the production of metabolites to protect yeast cells, such as adhesins, as discussed previously. By the time ethanol became the main carbon source and biofilm had been developed, yeasts could change their metabolism to assimilate ethanol, which was completely consumed at 48 h, except in the case of biochar. This suggests that the ethanol present in the culture broth is associated with the biomass produced, the three fermentations with higher biomass concentration than biochar having produced more ethanol and consumed it faster.

Díez-Antolínez studied the immobilization of *K. marxianus* on glass beads and plastic during the fermentation of whey. They observed that ethanol production was dependent of initial lactose concentration and biomass concentration instead of the immobilized cells (Díez-Antolínez et al., 2018). This confirms that ethanol production is highly dependent on the primary metabolism of the yeast cells as one of the main by-products derived from lactose oxidation.

L-Phe consumption and 2-phenylethanol production

Figure 3.8 shows the L-Phe consumption and the production of 2PE for all assays. Table 3.3 shows the L-Phe consumption rate and the productivity and yield of 2PE. In all cases, the maximum concentration of 2-phenylethanol (2PE) was observed at 96 h of fermentation, with yeasts immobilized on plastic rings showing the highest concentration, 907 ± 16 mg/L. This concentration corresponds to an observed yield of 0.85 ± 0.04 $g_{2PE}/g_{consumed\text{-}L\text{-}Phe}$, higher than the theoretical yield. The productivity was close to 9.4 mg/L*h. The final L-Phe concentration in the culture broth was 2.70 ± 0.04 $g_{L\text{-}Phe}$/L, with an apparent L-Phe consumption of only 1.024 $g_{L\text{-}Phe}$ of the L-Phe initially solubilized in the culture broth.

For the fermentation of yeasts immobilized on biochar and in suspension, 2PE productivity was around 6.0 and 6.6 mg/L*h, respectively. For biochar, the 2PE concentration at 48 h of fermentation was lowest in comparison with the other three assays (Figure 3.8b).

The conversion of L-Phe into 2PE is mainly observed during the stationary phase. 2PE is a secondary metabolite produced when the carbon source is consumed. Figures 3.8a and 3.8b show that in all cases at 24 h the L-Phe consumption was similar, and the production of 2PE did not present a difference. The variations in consumption and production trends were observed after 24 h. In the case of biochar, the ethanol was not completely consumed at 48 h, which can explain why the yeasts had not completely changed their metabolism to mainly produce secondary metabolites,

as ethanol as a carbon source was available for a longer period. On the contrary, the other three cases presented sustained production of 2PE because lactose first and then ethanol was completely consumed.

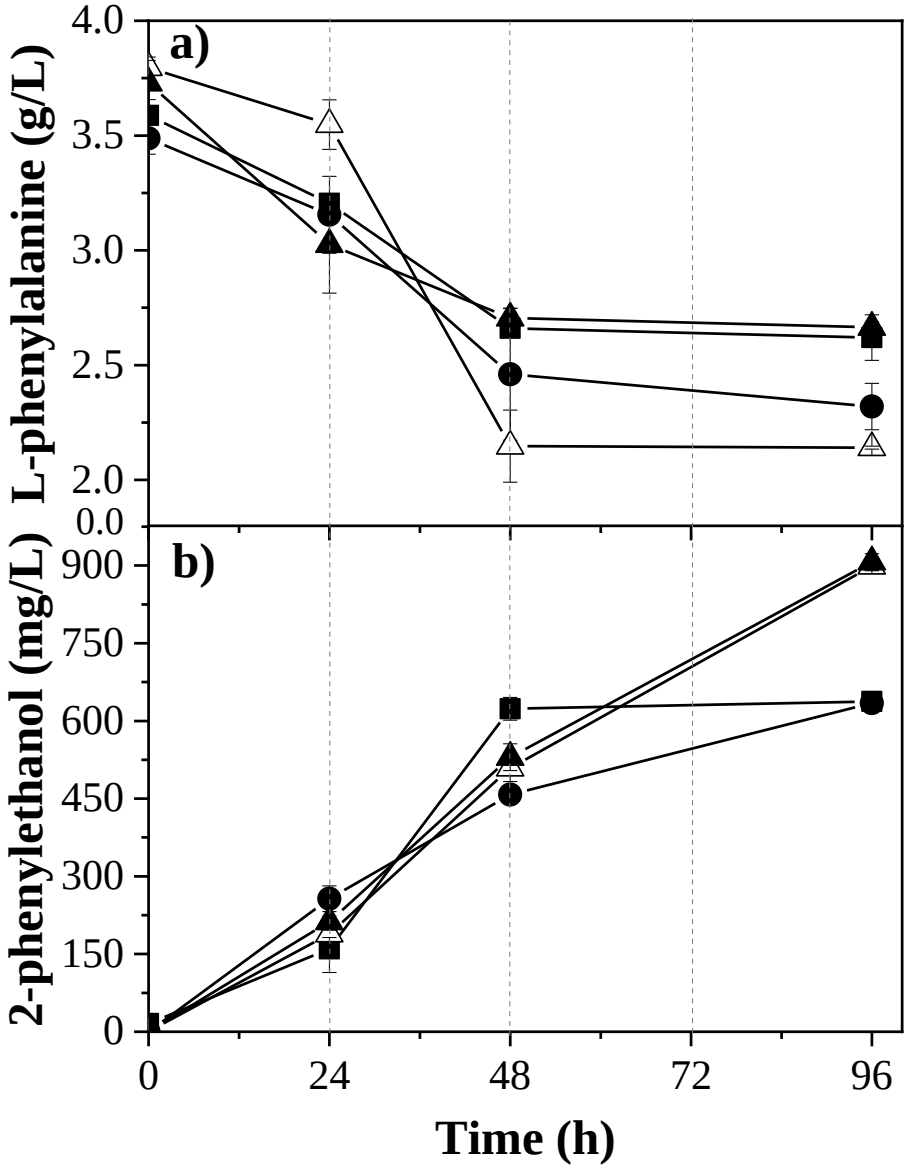

Figure 3. 8 L-Phenylalanine concentration (A) and 2-Phenylethanol concentration (B) as a function of time and kind of support for: ■-without support (suspended cells), ●- biochar, △- perlite, and ▲- filter Kaldnes plastic rings. The fermentation assays were done for whey medium and yeast co-culture.

The statistical analysis of ANOVA, pairwise comparison and Tukey test showed that the production of 2PE was higher for yeasts immobilized on plastic rings and perlite than on biochar or suspended cells (data shown in Table 3.4). The interactions of the yeasts with the supports determined the metabolic routes, inducing fast consumption of the substrate and quickly reaching the stationary growth phase with substrate starvation or slower consumption of the substrate and keeping nutrients for the stationary phase. This leads to select supports that will make it more difficult for yeasts to attach to them if the aim is to produce secondary metabolites.

Table 3. 4 Statistical analysis of effect of the support used to immobilize yeast cells on 2-phenylethanol production. The statistical analysis was done by method of ANOVA of one way. The treatments make reference to the comparison of the performance of immobilized cells against to suspended cells.

Source of variation	**Degree of freedom**	$\sum$ ***Squares***	***Mean squares***	***F* - value**	***p* – value**
Biochar	3	0.142	0.0475	188	$9.28x10^{-5}$*
Residuals	4	0.001	0.0002		
Treatments	**Diff**	**Lwr**	**Upr**	***p* – value adjusted**	
WMC-WMB	0.004	-0.061	0.068	0.993	
WMF-WMB	0.273	0.208	0.337	$2.42x10^{-3}$*	
WMP-WMB	0.265	0.200	0.329	$2.69x10^{-3}$*	
WMF-WMC	0.269	0.204	0.333	$2.55x10^{-3}$*	
WMP-WMC	0.261	0.196	0.325	$2.85x10^{-3}$*	
WMP-WMF	-0.008	-0.072	0.056	0.954	

*Significant at 5% level ($p \leq 0.05$). Treatments make reference to the comparison of the performance of immobilized cells against to suspended cells. **WMC**: whey medium fermentation using the co-culture in suspension; **WMB**: whey medium fermentation using the co-culture immobilized on biochar; **WMP**: whey medium fermentation using the co-culture immobilized on perlite; **WMF**: whey medium fermentation using the co-culture immobilized on filter Kaldnes plastic rings; **Diff**: difference between means of the two groups; **Lwr, Upr**: the lower and the upper end point of the confidence interval at 95% (default); and ***p* – value adjusted:** p-value after adjustment for the multiple comparisons.

For the production of 2PE, studies are focused in the use of pure carbon sources such as glucose (Pires et al., 2014; Valdez Castillo et al., 2020). In addition, whey fermentation using non-engineered yeast to produce fusel alcohols is an emergent bioprocess for whey valorization. For example, Conde-Baez et al. (2019) studied the sweet whey enriched with L-Phe fermentation using *K. marxianus* under monoculture mode. They observed a concentration of 780 ± 0.02 mg_{2PE}/L in 96 h of fermentation. These results are similar to the results observed with the co-cultured yeasts at 48 h of fermentation (Figure 3.8). This shows that the use of co-cultured yeasts increased the productivity of 2-phenylethanol. In addition, the use of immobilized co-cultured yeasts produced up to 907 ± 16 mg/L, which represents a yield of 0.85 ± 0.01 $g_{2PE}/g_{L\text{-}Phe}$. To the best of our knowledge, this high yield has not been reported before. These results contribute to elucidating the approaches to whey valorization and main variables for the production of either ethanol or fusel alcohols.

In light of these results, the plastic rings were the best inert supports in comparison with biochar and perlite, because of their homogeneity in physicochemical characteristics and constant availability on the market. In addition, the plastic rings allowed the development of a yeast biofilm which increased 2PE productivity. In this sense, future work scaling up the process to bioreactors must consider the filter Kaldnes plastic rings.

Conclusions

Whey fermentation using the *K. marxianus* and *D. hansenii* yeasts was performed under co-culture mode and with suspended and immobilized cells. Biochar, perlite, and filter Kaldnes plastic rings were used to immobilize the yeasts. The supports were characterized and their effect on the production of ethanol and 2PE was analyzed. Biochar had good characteristics as a support, with high porosity and roughness, as well as the presence of functional groups such as carbonyl,

hydroxyl, and ether which can interact with the functional groups of the cell wall of yeasts, such as carboxyl, amino and hydroxyl. Perlite presented high porosity, but it had macropores. The presence of siloxane functional groups on the perlite surface leads to the supposition of the formation of covalent bonds between cells and support. The plastic rings presented poor physical characteristics to start biofilm formation, such as negligible porosity and poor roughness. But their composition indicated that electrostatic forces between the cell walls and the aliphatic hydrocarbon groups of plastic rings could be an important parameter for biofilm development.

The functional groups on the surface of the supports conferred them a high net negative charge, up to -60.5 ± 1.0 mV, but when they were submerged into the culture media, they interacted with biomolecules present in the media, adjusting the zeta potential at a level nearly identical to that of the yeasts cells. The interactions of the cells with the support surfaces were determinants for yeast growth and metabolism. The support that presented the best characteristics for yeast immobilization led to developing a uniform and dense biofilm, but it was at a disadvantage when it came to 2PE production because yeasts were less stressed. The use of perlite and plastic rings induced the quick consumption of lactose and ethanol. The ethanol productivity was 0.4 ± 0.0 $g_{ethanol}$/L*h for yeast cells immobilized on plastic rings. The immobilization of yeast cells on perlite and plastic rings induced early production of 2PE and maintained the conversion of L-Phe to 2PE during the stationary phase because yeasts were under substrate starvation. Maximum 2PE productivity was observed with the yeast cells immobilized on plastic rings, a value of 9.5 ± 0.2 mg/L*h, whereas the 2PE productivity observed with the culture in suspension was 6.6 ± 0.0 mg/L*h.

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CHAPTER 4:

Production of 2-phenylethanol using biobased L-phenylalanine and scale-up of the process

CHAPTER FOUR - PART 1

Production of 2-phenylethanol by co-substrate fermentation of agri-food residues using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode

Mariana Valdez Castillo[1], Satinder Kaur Brar[1,2], Sonia Arriaga[3], Jean-François Blais[1], Antonio Avalos Ramirez[4]

[1] Institut national de la recherche scientifique, Centre-Eau Terre Environnement, 490, Rue de la Couronne, G1K9A9, Québec, Québec, Canada.

[2] Department of Civil Engineering, Lassonde School of Engineering, York University, M3J 1P3, Toronto, Ontario, Canada.

[3] Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4a Sección 78216, México.

[4] Centre National en Électrochimie et en Technologies Environnementales Inc. 2263, avenue du Collège, G9N 6V8, Shawinigan (Québec), Canada.

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Résumé

Le lactosérum est un résidu laitier riche en nutriments généré lors de la production de fromage et de yaourt. Le lactose et les protéines du lactosérum peuvent être transformés par des levures en éthanol et en biomolécules possédant des propriétés aromatiques et gustatives. Par exemple, le 2-phényéthanol (2PE) est produit par la voie d'Ehrlich en utilisant la L-phénylalanine (L-Phe) comme précurseur. Le 2PE est très apprécié dans l'industrie en raison de ses propriétés organoleptiques et biocides. La présente étude visait à valoriser le lactosérum de fromage et des résidus agroalimentaires riches en L-Phe pour développer un procédé économique et durable de production de 2PE. *Kluyveromyces marxianus* et *Debaryomyces hansenii* ont été utilisés pour fermenter le lactose du lactosérum et spécialement la source de L-Phe. Des résidus de crabe, de soja et de la production de bière ont été utilisés comme source d'azote pour les levures. La fermentation du lactosérum a été réalisée dans des bioréacteurs de 2 L. Une aération constante de 0,5 volume de cuve par minute (vvm) et l'ajout de L-Phe et de levures résiduelles de production de bière comme source d'azote ont conduit à une productivité de 0,04 g_{2PE}/L*h. Alors que le blanc contenant de l'extrait de levure et de la peptone a conduit à une productivité de 2PE de 0,01 g_{2PE}/L*h. Le présent bioprocédé a montré une productivité de 2PE 4 fois supérieure aux meilleurs résultats rapportés dans la littérature. Cela montre que la fermentation de résidus agroalimentaires comme co-substrats a un fort potentiel pour leur valorisation et peut contribuer à l'économie circulaire de l'industrie laitière et au recyclage du carbone.

Mots clés : Lactosérum de fromage, résidus agroalimentaires, fermentation de levure, 2-phényléthanol, bioréacteur à cuve agitée.

Abstract

Whey is a nutrient-rich dairy residue generated in the production of cheese and yogurt. Lactose and proteins in whey can be transformed by yeast into ethanol and biomolecules with aroma and flavor properties. For example, 2-Phenyethanol (2PE) is produced by the Ehrlich pathway using L-Phenylalanine (L-Phe) as precursor. 2PE is highly appreciated in the industry due to its organoleptic and biocidal properties. The present study aimed to valorize cheese whey and agri-food residues rich in L-Phe to develop an economic and sustainable process to produce 2PE. *Kluyveromyces marxianus* and *Debaryomyces hansenii* were used to ferment the lactose in whey and specially L-Phe source. Crab headshells, soy residue, and brewer's spent yeasts were used as a nitrogen source for yeasts. The whey fermentation was performed in 2 L bioreactors. Constant aeration of 0.5 vessel volume per minute (vvm) and the addition of L-Phe and brewer's spent yeasts as nitrogen source led to 2PE productivity of 0.04 g_{2PE}/L*h. Whereas, the blank containing yeast extract and peptone led to 2PE productivity of 0.01 g_{2PE}/L*h. The present bioprocess showed a 2PE productivity 4 times higher than the best results reported in the literature. This shows that the fermentation of agri-food residues as co-substrates has high potential for their valorization and can contribute to the circular economy of the dairy industry and carbon recycling.

Keywords: Cheese whey, agro-food residues, yeast fermentation, 2-phenylethanol, stirred-tank bioreactor

Introduction

The agri-food industry is an important social economical sector of Canada and Quebec generating 2.5 billion dollars per year. For example, Canada is one of the top ten major cheese producers in the world. The Province of Quebec with $2.49x10^5$ tons of cheese per year, represents 51% of the Canadian production, generating 83,000 direct and indirect jobs. This shows the importance of the dairy industry in Quebec's economy, especially in local regions (Les Producteurs de lait du Québec, 2021; Les Producteurs de lait du Quebec, 2017). The agri-food sector in Quebec is recognized for its production of seafood, cereals, legumes, and brewery (Bedford, 2021; BeerCanada, 2020). However, the residues of the agri-food sector can be a big problem, because they are issued in great volume and represent an important pollutant load for environmental systems. For example, cheese production generates a liquid by-product called whey, which contains about 55% of milk solids, such as lactose (in a concentration range from 33.6 to 52.0 g/L), proteins (from 1.8 to 10.0 g/L), and minerals (from 0.4 to 1.6 g/L). The content of solids in whey is equivalent to a chemical oxygen demand (COD) in the range from 60 to 80 g O_2/L (Valdez Castillo et al., 2021). The management of whey needs considerable human and material resources. Among the current whey managing practices is land spreading, but this can cause soil and water pollution due to the high pollutant load (Valdez Castillo et al., 2021).

In this context, whey valorization is an emerging practice for its management. The biological way to valorize whey is an interesting option that decreases the pollutant load by obtaining high value-added biomolecules The new environmental rules oriented to deviate organic residues from landfills promote the research and development of new valorization processes (Agropur, 2021;

Mordor Intelligence, 2021). The fermentation processes using non-traditional yeasts, such as *K. marxianus* and *D. hansenii*, offer several advantages to produce value-added biomolecules.

These yeasts can hydrolyze the lactose to produce ethanol, and under starvation conditions, they can transform amino acids into fusel alcohols through the Ehrlich pathway, such as the 2-phenylethanol (2PE) (Karim et al., 2020; Valdez Castillo et al., 2021). This alcohol is highly appreciated by the pharmaceutic, food, and cosmetic industries because of its aroma, flavor and antiseptic properties with a market price of around 100 $US/kg (Ambeed USA, 2022). However, the accumulation of alcohols and organic acids during fermentation can inhibit yeasts growth and the fermentation is a time-consuming process.

In order to overcome the limitations for whey fermentation, strategies such as cell immobilization (Christensen et al., 2011; Díez-Antolínez et al., 2018) or co-culturing (Rodrigues et al., 2016; Valdez Castillo et al., 2021) have been explored. For example, Valdez-Castillo et al. (2021) showed that the whey fermentation using *K. marxianus* and *D. hansenii* under co-culture mode let to double the 2PE productivity in comparison to the monoculture. In addition, the incorporation of other agri-food residues to replace high costly additives such as nitrogen and amino acid sources, improve the cost-benefit of the process.

The main aim of this study was to a fermentation process to valorize agri-food residues into 2PE. The project studied the effect of several agri-food residues as nitrogen sources on yeast growth and 2PE production. In order to develop a sustainable bioprocess to valorize agri-food residues under the principles of the circular economy.

Material and methodology

The study was performed in two steps. The first step aimed to test the cheese whey as the main carbon source and three agri-food residues (crab headshells, soy residue, and brewer's spent yeasts) as a biobased L-Phe source to produce 2PE. The second step aimed to scale up the co-fermentation of residues in bioreactors of 2 L to study the effect of aeration on the transformation of L-Phe into 2PE.

Agri-food residues handling and characterization

Whey, crab headshells, soy residue, and brewer's spent yeasts (BSY) were the substrates for the fermentation assays. Residues were obtained from Quebec enterprises (Canada). Whey was conserved at -20°C until its use. The crab headshells were rinsed with distilled water, dried at 60°C for 48 h (Thermo Herathern Fisher oven, OMS180), and milled to obtain particles of 1 – 2 mm (Fritsch 19.5720/021704). Then, the crab headshells particles were milled with pestle and mortar to obtain a fine powder. The soy residue was dried at 60°C for 48 h (Thermo Herathern Fisher oven, OMS180) and milled with pestle and mortar to obtain a fine powder. Whereas the BSY was conserved at -20°C until be used for fermentation assays.

The four residues were characterized in terms of total nitrogen (Kjeldahl analyzer, Shimadzu VCPH), total solids, and ashes (APHA, 1999), total proteins by Lowry's method. Concentration of cations by inductively coupled plasma optical emission spectrometry (ICP-OES OPTIMA 4300 DV). For whey and BSY were also determined the pH (benchtop pH meter, Accument AB250), chemical oxygen demand (HACH kits and spectrophotometer), and dissolved organic carbon (TOC-L analyzer, Shimadzu) according to APHA et al. (1999).

Culture media

The medium for inoculum contained lactose 45 g/L (Sigma-Aldrich, Canada, >98%), yeast extract 10 g/L (Organotechnie S.A.S, Canada), and peptone 20 g/L (Organotechnie S.A.S, Canada) (LYP medium). For fermentation, the whey was diluted with distilled water to obtain a lactose concentration of 20 g/L. Yeast extract 10 g/L and peptone 20 g/L were added (whey-based medium).

For the first step of the study, the whey-based medium was enriched with L-Phe 3 g/L (BioShop life science products, Canada, >98%) (WM). Hydrolyzed and non-hydrolyzed crab headshells 0.90 g, soy residue 2.00 g, and BSY 0.9 g were added to enrich the whey-based medium to have a final concentration of 3 g/L of L-Phe. Whey-based medium with hydrolyzed crab residues, soy residue and BSY was identified as WHC, WHS and WHBSY, respectively. Whereas the whey-based medium with non-hydrolyzed crab residues, soy residue and BSY was identified as WNC, WNS and WNBSY, respectively. The pH of each medium was adjusted at 6.5 with KOH 6M or HCl 6M. Then, 30 mL of media was placed in Erlenmeyer flasks of 250 mL and sterilized by autoclave at 120°C for 15 min.

Inoculum

Kluyveromyces marxianus NRRL Y-1109 and *Debaryomyces hansenii* NRRL Y-1448 were seed in 50 mL of LYP in a 250 mL flask. The inoculum was incubated at 25°C and 200 rpm for 20 h in a rotary incubator (INNOVA 44 New Brunswick Scientific).

Fermentation conditions

For the fermentation assays, WM, WNC, WNS, WNBSY, WHC, WHS and WHBSY media were inoculated with both *K. marxianus* and *D. hansenii* for an initial OD_{600} (UV-VIS spectrophotometer, Eppendorf BioPhotometer plus) of 0.5 and 0.1, corresponding to an initial cellular density of $5.0x10^7$ and $1.0x10^7$ CFU/mL, respectively. This co-culture ratio was selected based on a previous study by Valdez Castillo et. al. (2021). Fermentation was carried out at 25°C and 200 rpm for 120 h. Samples of culture broth were taken at 0, 24, 48, and 120 h to determine cellular density, and concentration of lactose, L-Phe, ethanol and 2PE.

Samples were handled as indicated by Valdez-Castillo et. al. (2021). Briefly, 1 mL sample was centrifuged at 16 639 *x g*, 4°C for 2 min (Eppendorf 5804 R- Benchtop centrifuge). The supernatant was used to determine total proteins, amino acids, lactose, ethanol, and 2PE concentration. 100 µL of non centrifuged sample was used to determine the cellular density by counting in plates by serial dilutions (10^{-3} – 10^{-8}) plated on solid LYP with 15% (w/v) of agar, and incubated at 25°C for 24 – 48 h.

The specific growth rate due to biomass synthesis was determined according to the Monod equation modified from Rittman and McCarty (2001), as follows:

$$\boldsymbol{\mu = \left(\frac{1}{X_a} \cdot \frac{dX_a}{dt}\right)} \text{ ... } \textbf{Equation 4}$$

In which "µ" (h^{-1}) is the growth rate due to synthesis during the exponential phase of either *K. marxianus* or *D. hansenii*. "Xa" stands for the initial cell density of either *K. marxianus* or *D. hansenii* (CFU_{yeast}/mL), and "dXa" is the difference between the initial and highest cellular density

in the region of the exponential growth phase. Whereas, "dt" is the specific elapsed time of the exponential growth phase.

Lactose and L-Phe consumption were determined by considering the difference between the initial and their lowest concentration divided by the elapsed time of fermentation. Whereas for ethanol and 2PE productivity, the highest concentration of each alcohol was divided by the elapsed time of fermentation where it was identified.

Batch fermentation in 2 L bioreactor

Diluted whey was enriched with 61.11 g/L of BSY and 3 g/L of L-Phe (WBSY medium). The BSY was used as the nitrogen source instead of yeast extract and peptone. WM was used as control.

Fermentations were carried out in 2-L stirred tank bioreactors (New Brunswick scientific BioFlo/Celligen 115, USA). 1.8 L of either WM or WBSY medium was added to bioreactor and sterilized at 121°C for 30min by autoclave (Steris Amsco Lab 250). The inoculum was added to bioreactors for an initial cellular density of $5.0x10^7$ and $1.0x10^7$ CFU/mL of *K. marxianus* and *D. hansenii*, respectively. The agitation cascade system was from 300 to 600 rpm in order to keep the oxygen level in the media. The pH was maintained at 6.5 ± 0.5 using HCl and KOH solutions 4M. The temperature was maintained at 25°C and controlled with a chilling system and heating jacket. Aeration was set at 30% of oxygen level in media with 0.5 of vessel volume per minute (vvm). Samples of culture broth were taken at 0, 7, 24, 31, 48, 53 and 72 h to determine the concentration of lactose, L-Phe, ethanol, 2PE, TOC. Total proteins and amino acids concentration were determined following the Lowry method and derivatization of amino acids described below. Samples were handled as indicated in "section 2.4".

Determination of protein content by Lowry's method

The extraction of proteins from solid residues (crab, soy, and BSY) was performed by basic hydrolysis adapting the method reported by Alabaraoye et al. (2018). Briefly, 1 g of each residue was placed in Erlenmeyer flasks of 250 mL and 20 mL of NaOH (1 M) was added and agitated at 250 rpm, 60°C for 30 min. Bovine serum albumin (Sigma-Aldrich, Canada, >98%) standard solution in distilled water was subjected to the protein extraction method and used to make the calibration curve.

Protein content was determined by adapting the colorimetric Lowry's method reported by Lucarini and Kilikian (1999). Briefly, 250 µL of the sample was taken and mixed with 1.25 mL of freshly prepared solution of $CuSO_4*5H_2O$ (2% w/v), sodium potassium tartrate (4% w/v), and Na_2CO_3 (3 w/v). This mix was homogenized by vortex-mixed for 30 sec, and it reacted for 20 min. Then 250 µL of Folin-Ciocalteu reagent (Fisher, Canada, 2M) diluted with distilled water in a ratio of 1:1 was added and homogenized by vortex-mixed for 30 sec, and it was let react for 40 min. The absorbance was determined at a wavelength of 750 nm (DR 2700 BenchPlus spectrophotometer, HACH).

Extraction, derivatization and quantification of amino acids

The extraction of amino acids of residues was performed by acid hydrolysis. 100 mg of each residue were placed in screw-cap polypropylene tubes (17 mm x 120 mm) and 10 mL of 6M hydrochloric acid (Fisher, Canada, 37% v/v) was added. The suspension was homogenized by vortexing for 30 sec. Then, the air of tubes was replaced with nitrogen, and tubes were placed in an electric oven (Thermo Herathern Fisher oven, OMS180) at 105°C for 24 h.

Culture broth samples of fermentation assays and amino acid standard (L-Phe, BioShop life science products, Canada, >98%) were derivatized for the analysis of amino acids adapting the method of Vilasoa-Martínez et al. (2007) as follows. Methanol, triethylamine, and disodium hydrogen phosphate were purchased from Fisher, Canada (purity > 98%). Whereas phenyl isothiocyanate was purchased from Sigma-Aldrich, Canada (purity > 99%). 20 µL of the sample were placed in a vial and dried at 65°C for 2 h. 30 µL of solution methanol-water-triethylamine (4:4:1) were added and vigorously mixed with a vortex for 10 s, and dried at 65°C for 10 min. 30 µL of methanol-water-triethylamine-phenyl isothiocyanate (7:1:1:1) were added and vortex-mixed for 30 s. The vial was left at room temperature for 20 min, and then it was dried for 15 min at 65°C. Then, 150 µL of 5 mM Na_2HPO_4 with 5% v/v acetonitrile solution was added to the dried sample. The pH was adjusted to 7 with 10 µL of phosphoric acid 1.07 M. Tubes were vortex-mixed for 60 s. Finally, 100 µL of the neutral derivatized amino acids solution were diluted with 400 µL of 5 mM Na_2HPO_4 with 5% acetonitrile. A standard solution, containing 1 mM of L-Phe in HCl 0.1 M, was obtained and derivatized as aforementioned.

The derivatized samples were analyzed by High-performance liquid chromatography (HPLC, Agilent Technologies 1260 infinity, USA) with a UV-Visible forward optical scanning detector. The HPLC was equipped with a ZORBAX Eclipse XDB-C18 (Agilent Technologies Inc., USA) column with a length of 250 mm, an internal diameter of 4.6 mm and a film thickness of 5 µm. The temperature of the column was set at 27°C. Two mobile phases were used. Mobile phase A was 0.14 M sodium acetate buffer containing trimethylamine 0.05% (v/v) at a pH of 6.2 adjusted with acetic acid (Fisher, Canada, HPLC grade). Mobile phase B was a solution of acetonitrile (Fisher, Canada, HPLC grade) and water (60:40). A gradient of the two mobile phases was used as

described by Vilasoa-Martínez et al. (2007). The flow rate was set at 0.9 mL/min and the detection wavelength was 254 nm.

Quantification of lactose and ethanol

The samples were thawed to 4°C for analysis. HPLC (Agilent Technologies 1260 infinity, USA) coupled with a diode array detector of 1260 RID and 1290 DAD were used to quantify lactose and ethanol respectively. Lactose (Sigma-Aldrich, Canada, >98%) and ethanol (Fisher, Canada, >98%) were used as internal standard and for the calibration curve. The HPLC was equipped with an aminex HPX-87H (Agilent technologies, USA) column with a length of 300 mm, an internal diameter of 7.8 mm and a film thickness of 5 µm. The column temperature was set at 50°C with a mobile phase of sulfuric acid 0.008 N diluted in water. The flow rate was set at 0.6 mL/min for 30 min. A volume of 5 µL of the sample was injected into the column.

Quantification of 2-Phenylethanol

The samples were thawed up at 4°C for analysis. HPLC (Agilent Technologies 1230 infinity II, USA) coupled with a UV-visible forward optical scanning detector was used to determine the concentration of 2PE on culture broth samples. Pure 2PE (Fisher, Canada, >98%) was used as internal standard and for the calibration curve. The HPLC was equipped with a ZORBAX Eclipse XDB-C18 (Agilent Technologies Inc., USA) column with a length of 250 mm, an internal diameter of 4.6 mm and a film thickness of 5 µm. A mobile phase of water:methanol (50:50) was used at a flow rate of 0.5 mL/min for 30 min, and the detection wavelength was 370 nm. The temperature of the column was set at 30°C and the volume of injection was 10 µL.

Statistical analysis

For the first step of the study, an analysis of one-way ANOVA (p-value < 0.05) with the Tukey test as a post hoc test was performed in R core team 2020 software. The analysis was used to study the effect of the addition of hydrolyzed and non-hydrolyzed biosources of L-Phe to the base whey medium on the 2PE production. The independent variable was the type of culture media (WNC, WNS, WNBSY, WHC, WHS, and WHBSY) compared with the control fermentation of WM, and the dependent variable was the concentration of 2PE at the end of the fermentation.

For the second step of the study, an analysis of one-way ANOVA (p-value < 0.05) with the Tukey test as a post hoc test was performed in R core team 2020 software. The analysis was used to study the effect of WM and WBSY fermentation medium on 2PE production. The independent variable was the medium type, whereas the dependent variable was the concentration of 2PE at the end of the fermentation.

Results and discussion

Characterization of agri-food residues

The characterization of whey, crab shells, soy residue and brewer's spent yeasts (BSY) are shown in Table 4.1. Whey had a pH of 4.9 ± 0.0, lactose concentration of 36.5 ± 0.0 g/L, being the methionine and leucine the most abundant amino acids with 0.1 ± 0.0 and 0.05 ± 0.0 g/L, respectively. Additionally, the most abundant cation identified by ICP was potassium (K^+) with a concentration of 1.1 ± 0.0 mg/L_{whey}. All the aforementioned components contributed to the COD concentration of 63.1 ± 2.8 g O_2/L_{whey}.

Table 4. 1 Chemical characterization of agri-food residues

Parameters	Agrí-food residues			
	Whey	**Crab heads**	**Soy residues**	**Brewer's spent yeasts**
pH	4.9 ± 0.1	N.A.	N.A.	5.4 ± 0.0
Total solids ($g/kg_{residue}$)	48.4 ± 0.1	592 ± 1	201 ± 1	208 ± 13
Ashes ($g/kg_{residue}$)	3.6 ± 0.9	408 ± 1	6.5 ± 0.1	4.2 ± 0.1
Lactose ($g/L_{residue}$)	36.5 ± 0.1	N.A.	N.A.	N.A.
Chemical oxygen demand ($g\ O_2/L_{residue}$)	63.1 ± 2.9	N.A.	N.A.	321 ± 10
Total nitrogen-Kjeldahl ($g_{Total\text{-}N}/L_{residue}$)	0.9 ± 0.1	52.2 ± 0.1	9.6 ± 0.0	11.7 ± 1.4
Total proteins ($g/L_{residue}$)	0.6 ± 0.0	13.4 ± 0.4	2.98 ± 0.04	1.56 ± 0.01
L-Phenylalanine ($mg/L_{residue}$)	31.3 ± 0.3	146 ± 12	75.0 ± 4.0	126 ± 21
Valine (mg/L)	4.13 ± 0.65	6.42 ± 4.21	29.5 ± 0.6	21.3 ± 2.6
Methionine (mg/L)	121 ± 3	29.4 ± 2.2	0.00 ± 0.00	19.4 ± 1.7
Isoleucine (mg/L)	3.53 ± 0.97	5.71 ± 1.92	64.7 ± 3.2	69.4 ± 8.0
Leucine (mg/L)	55.5 ± 8.8	1.33 ± 0.15	12.5 ± 6.2	6.16 ± 0.99
Tryptophan (mg/L)	44.6 ± 1.3	59.9 ± 10.9	71.7 ± 5.2	70.3 ± 21.4
Calcium-Ca^{2+} ($mg/L_{residue}$)	274 ± 5	129 000 ± 1 000	544 ± 13	319 ± 19
Potassium-K^+ ($mg/L_{residue}$)	1090 ± 10	3 470 ± 10	1 770 ± 20	3 090 ± 6
Magnesium-Mg^+ ($mg/L_{residue}$)	58.0 ± 0.1	8 020 ± 10	245 ± 4	578 ± 7
Sodium-Na^+ ($mg/L_{residue}$)	372 ± 1	9 060 ± 30	51.0 ± 1.3	15.0 ± 0.5

N.A.= Not applicable.

For the generation of cheese whey, the proteins of milk were coagulated using a starter culture and a proteolytic enzyme (rennet). These acidified the milk and affected the concentration of lactose

on cheese whey (Powell et al., 2011). The lactose content was in the range of studies reported in the literature, from 36 to 52 g/L for acid whey (Chandrapala et al., 2015; Risner et al., 2019).

Whereas, the rennet is a protease assisting the break of milk proteins and curd formation, affecting the final concentration of amino acids of cheese whey (Anand et al., 2013; Powell et al., 2011; Risner et al., 2019). Anand et al. (2013) reported that the major protein fraction in acid whey is the α-lactalbumin, β-lactoglobulin, and bovine serum albumin. These proteins are rich in methionine, leucine, and isoleucine amino acids (Anand et al., 2013). This is in accordance with the abundance of methionine and leucine observed in the whey used on the present study.

In addition, the acid pH of whey increases the solubility of cations such as calcium and potassium. Potassium is essential for the activity and structural changes of β-galactosidase enzyme, which is produced by the yeasts to consume lactose as the key carbon source (Rajakala and Karthigai Selvi, 2006; Valdez Castillo et al., 2021).

The residue with the highest total nitrogen and protein content was the crab headshells, with 52.2 ± 0.0 $g_{Total\text{-}N}/L_{residue}$ and 13.4 ± 0.4 $g_{proteins}/L_{residue}$, respectively. The proteins concentration corresponds to 0.3 ± 0.0 $g_{proteins}/g_{dry\text{-}residue}$. For L-Phe, crab heads, soy residue, and BSY contained 146.4 ± 12.0, 75.0 ± 4.0, and 126.0 ± 20.5 mg/L, respectively.

The high content of total nitrogen in residual crab heads can be contained by its high content of proteins (34.2 % w/w) and the chitin, which is a long chain polymer of N-acetylglucosamine. Vilasoa-Martínez et al. (2007) observed 0.35 ± 0.03 $g_{proteins}/g_{dry\text{-}residue}$ of proteins in the shells of *Chionoecetes opilio* (crab specie). The total protein content was similar to that observed by Vilasoa-Martínez et al. (2007). This corroborates that the crab headshells could be a potential source of nitrogen for fermentation.

The L-Phe content in crab headshells, soy residue, and BSY was in the range from 75 to 146 mg/L. These values are close to the values reported in the literature for crab shells and BSY (Stanojevic et al., 2013; Vieira et al., 2019; Vilasoa-Martínez et al., 2007). For the crab residues, the main L-Phe source is the shell (Vilasoa-Martínez et al., 2007). Proteins of soy are conglycinin and glycinin, rich in cysteine and glycine, and as consequence with low L-Phe (Dranginis et al., 2007; Stanojevic et al., 2013).

Yeasts growth and proteins production

Figure 4.1a and 4.1b show the cell density of *K. marxianus* and *D. hansenii*, respectively. The initial cell density of *K. marxianus* and *D. hansenii* was $5.0x10^7$ and $1.0x10^7$ CFU/mL, respectively. Because of this, the normalized cell density (CD) with respect to the initial cell density was used to show the growth. For *K. marxianus*, the maximum CD was observed at 48 h of WM fermentation, followed by WNHB fermentation at 24 h. This corresponds to 3.4 ± 0.0 and 2.8 ± 0.0 log $CD_{\textit{K. marxianus}}/CD_{initial}$, respectively.

The yeast growth depends on the available carbon source in the culture broth, in this case, lactose in whey. However, yeast growth was inhibited by the presence of the non-hydrolyzed and hydrolyzed residues. For example, crab headshells presented the highest concentration of cations (Ca^{2+}, K^+, Mg^{2+} and Na^+). Whereas, a high concentration of K^+ was observed for the soy residue and BSY. As was aforementioned, the K^+ is a key cation for the activation and performance of the β-galactosidase enzyme for the hydrolysis of lactose. In addition, the K^+ and Na^+ are key cations for the transport and redox reactions of energetic molecules such as adenosine triphosphate, which occurs in the yeast's organelles and the yeast membrane. In this sense, a balance between cations

concentrations is needed to regulate the growth of yeasts and the production of by-products (Illarionov et al., 2021).

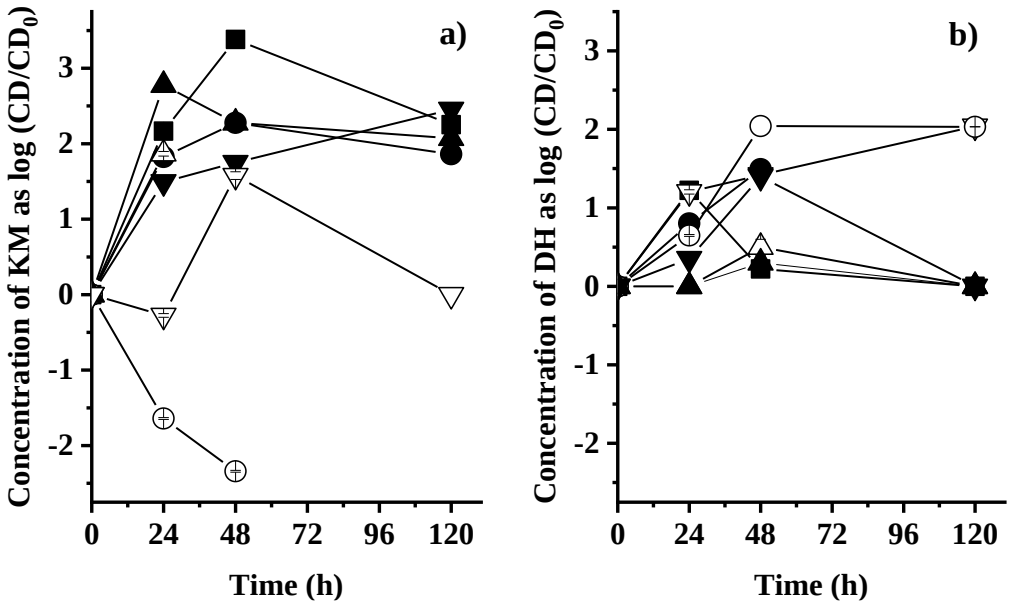

Figure 4. 1 Growth of a) *Kluyveromyces marxianus* and b) *Debaryomyces hansenii* as co-culture mode for whey medium-aerobic. Symbols indicate the co-culture growth for the ■-whey medium-control, ▼-whey medium supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●- whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts.

However, the effect of the four cations in each medium could promote an osmotic stress-response on yeast cells. Because of this, the concentration of both Cl^- and K^+in the hydrolyzed media was calculated considering the used volume and the molarity of either HCl or KOH. For WHC, WHS, and WHBSY media, 10 mL of HCl (6M) was used to hydrolyze the corresponding residues. The resulting hydrolyzed solution was neutralized with 7.5 mL of KOH (6M). This contributed with $6x10^3$ mg/L of Cl^- and $2.3x10^5$ mg/L of K^+, respectively. The high concentration of either Cl^- or K^+ could inhibit the growth observed in the present study for the WNC and media with hydrolyzed

residues. Illarionov et al. (2021) studied the salt stress of KCl, NaCl, and their combination on *K. marxianus* during glucose fermentation. The growth rate of *K. marxianus* was 0.6 and 0.3 h^{-1} at KCl concentrations of 978 mg/L and $5.8x10^4$ mg/L, respectively. Whereas, when the yeast was cultivated at a KCl concentration of $7.82x10^4$ mg/L, its growth was completely inhibited. The same growth inhibition effect was observed when $2.3x10^4$ mg/L of KCl and 1,149 mg/L of NaCl were used. This effect was attributed to salt stress caused for the high concentration of cations and Cl^- which decreased the volume of the vacuoles and the yeast capacity to respond to osmotic pressure (Illarionov et al., 2021). This is in agreement with the results observed in Figure 4.1, corresponding to the high concentration of K^+, Na^+, and Cl^- that had a negative synergetic effect on the *K. marxianus* growth.

Figure 4.1b shows that *D. hansenii* had a better adaptation for the fermentation of hydrolyzed residues than *K. marxianus*. This allowed the dominance of *D. hansenii* over *K. marxianus* in terms of growth. The maximum CD of *D. hansenii* was observed at 48 h of WHS fermentation and 120 h of WHC fermentation. For both fermentation assays, the CD was 2.04 ± 0.00 log $CD_{D. hansenii}/CD_{initial.}$.

D. hansenii is a halotolerant yeast that allows the resistance to stressful osmotic environments. Under salt stress, *D. hansenii* can produce and accumulate glycerol to create an osmotic pressure balance between the environment and the intracellular space (Calahorra et al., 2009; Valdez Castillo et al., 2021). This could lead the better adaptation to the high concentration of cations in the media with hydrolyzed residues. Calahorra et al. (2009) studied the effect of the KCl and NaCl on glucose fermentation by *D. hansenii*. Results showed a positive correlation between the salts concentration and glycerol production.

Figure 4.2 shows the protein concentration in culture broth for all the fermentation assays. The maximum concentration of proteins (36.87 ± 0.13 $g_{protein}$/L) was observed at 24 h for WHS medium fermentation. The other fermentation assays presented constant increase of protein concentration.

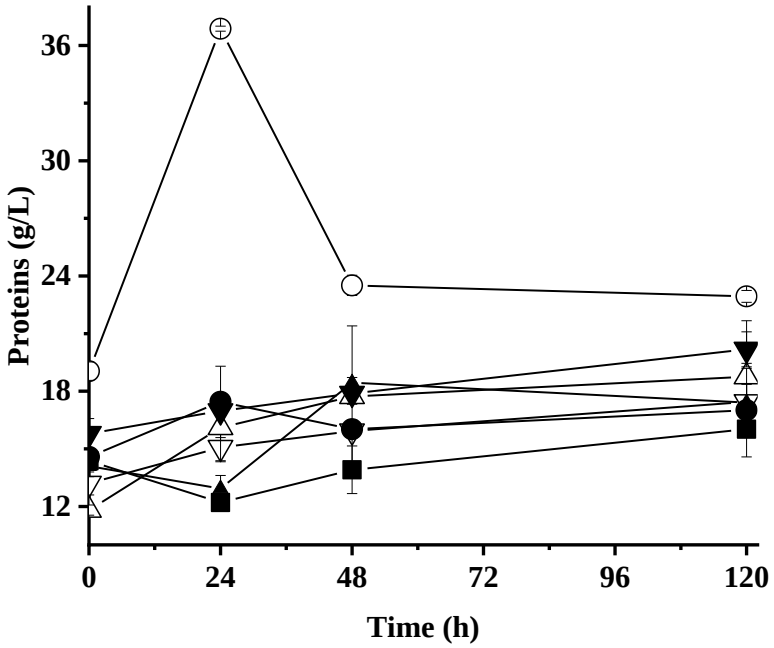

Figure 4. 2 Production of proteins during the aerobic whey medium fermentation using the co-culture of yeasts. Symbols indicate the fermentation of ■-whey medium-control, ▼-whey medium supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●-whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts.

These results can be related to the type and availability of organic nitrogen sources in the medium. For WM the organic nitrogen source was the yeast extract and peptone, which can be easily metabolized. Whereas for the rest of the fermentation assays, the organic nitrogen source was the yeast extract, peptone, and agri-food residues. The agri-food residues contain complex protein matrices, which could promote the production of yeasts' proteases (Jacob et al., 2019). In this context, the continuous increment of protein concentration suggests that yeasts produced proteases

to break the protein matrix of non-hydrolyzed residues to use them as nitrogen sources. Whereas for the hydrolyzed residues, the continuous increment in protein concentration can be derived from biomass growth. The production of proteases in *K. marxianus* and *D. hansenii* is a process related to the fermentation conditions and the nitrogen source in the growth medium (Easwaran et al., 2022; Kumura et al., 2002). *K. marxianus* can produce and excrete pectinases, inulinases, and carboxypeptidases. The production and release of proteases in *K. marxianus* are promoted when the yeast is cultivated at a pH range from 5 to 6.5, and in presence of complex proteins as nitrogen source. The acid pH facilitates the mass transfer by increasing the plasma membrane H^+-ATPase activity. Whereas the complex proteins stimulate the enzyme production to cleave the proteins and release the amino acids, which can be further catabolized to use the amino group in them as nitrogen source (Easwaran et al., 2022; Foukis et al., 2012). Foukis et al., (2012) cultivated *K. marxianus* in a medium containing glucose 10 g/L, yeast nitrogen base without amino acids 5 g/L, and bovine serum albumin 0.4 g/L. They isolated and purified an exoprotease that was able to cleave the amino acids from the carboxyl end of proteins. Kumura et al. (2002) studied the fermentation of synthetic milk ultrafiltrate medium with a pH of 6.2 using *D. hansenii*. They observed an enzyme attached to the cell wall that can degrade β-casein. In addition, they isolated and characterized endoproteases from the cytosol of *D. hansenii* that were able to degradate α and β-casein. This suggested that the proteolytic activity is a cell lysis depending-phenomena in D. hansenii. These results are comparable with the results observed in Figure 2 and the increment of protein content in the medium, especially after the stationary growth phase of yeast (Figure 1), which can be related to the lysis of cells and the release of peptidases.

Effect of cheese whey enriched with L-Phe biosource fermentation on lactose consumption and ethanol production

Figure 4.3 shows the lactose, ethanol, L-Phe and 2PE concentration change through fermentation time. In addition, Table 4.2 shows the consumption of lactose and L-Phe rates, and alcohols production for the co-fermentation assays. The lactose consumption was slowed for WHC, WHS, WHBSY, and WNC media with an average consumption rate of 0.1 ± 0.0 $g_{lactose}$/L*h. Whereas for the fermentation WNBSY, the lactose consumption rate was 1.0 ± 0.0 $g_{lactose}$/L*h, being 8.25-fold higher than the lactose consumption for WHBSY fermentation.

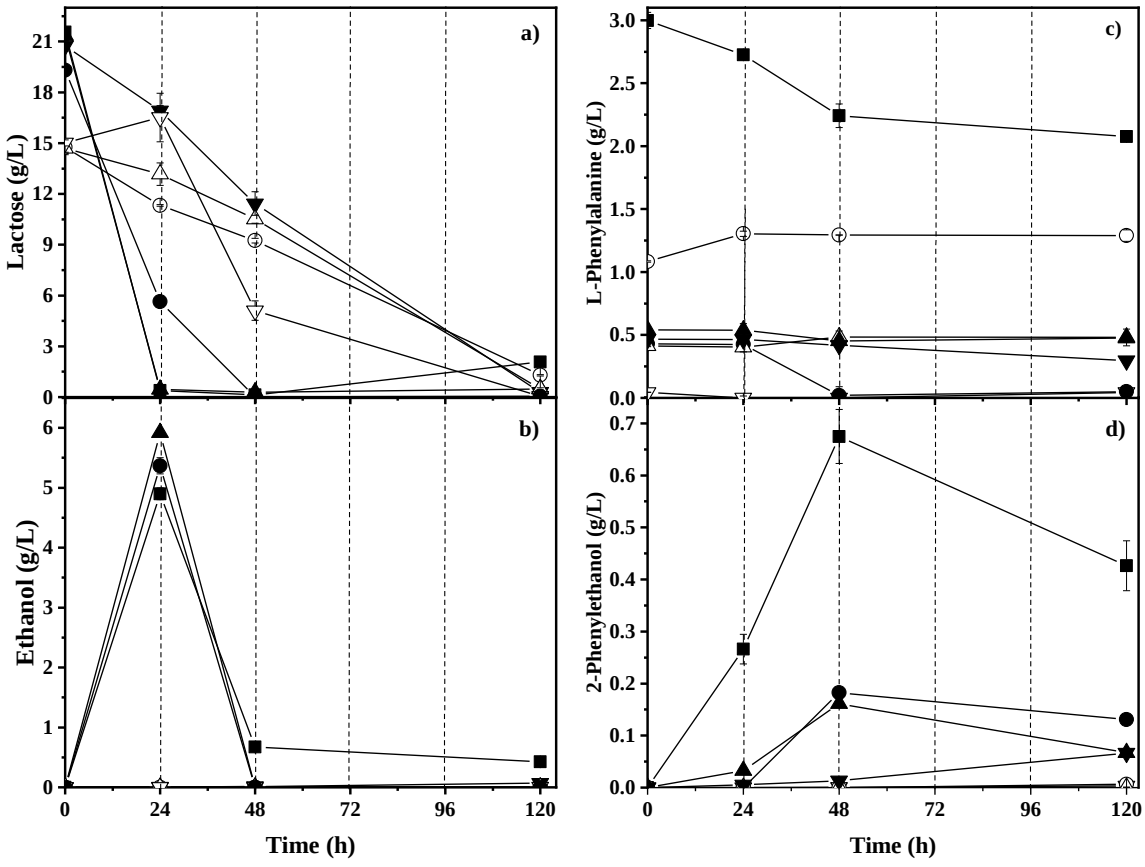

Figure 4. 3 a) Lactose consumption; b) ethanol production; c) L-Phenylalanine consumption; and d) 2-Phenylethanol production during the aerobic whey fermentation using the co-culture of yeasts. Symbols indicate the fermentation of ■-whey medium-control, ▼-whey medium

supplemented with non-hydrolyzed crab, ▽-whey medium supplemented with hydrolyzed crab, ●- whey medium supplemented with non-hydrolyzed soy, ○-whey medium supplemented with hydrolyzed soy, ▲-whey medium supplemented with non-hydrolyzed brewer's spent yeasts, and △-whey medium supplemented with hydrolyzed brewer's spent yeasts.

As it was discussed before, lactose is hydrolyzed by the yeasts and its oxidation through the glycolysis pathway leads to the formation of pyruvate and energetic molecules such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide hydrogen (NADH). Then pyruvate is further oxidized, leading to its use in biomass and biomolecules synthesis (Beniwal et al., 2017; Christensen et al., 2011). However, the fermentation of hydrolyzed residues and WNC delayed the lactose consumption due to the high concentration of cations in media. The synergetic effect of cations on yeasts cells observed in media with hydrolyzed residues could affect the permeability of the cell membrane by increasing the plasma membrane H^+-ATPase activity. This facilitates the transport of lactose from the culture broth to the intracellular space, generating an osmotic shock in the yeast cells (Sánchez et al., 2018). This led to slow the yeasts growth and the lactose consumption rate (Illarionov et al., 2021; Saini et al., 2017; Sánchez et al., 2018).

In this context, Figure 4.3b shows that ethanol production was completely inhibited during the fermentation of WHS, WHC, WHBSY and WNC. Whereas for WNBSY, the highest productivity, yield, and concentration of ethanol of 0.3 ± 0.1 $g_{ethanol}/L*h$, 0.25 ± 0.0 $g_{ethanol}/g_{lactose}$ and 5.9 ± 0.1 $g_{ethanol}/L$ were respectively observed. After 48 h of fermentation, the ethanol concentration decreased for all the fermentation assays.

Ethanol production is related to the production and oxidation of pyruvate. Pyruvate can be oxidized into acetaldehyde by a dehydrogenase enzyme. This aldehyde is further oxidized to produce ethanol

as end-product. However, ethanol production is limited by the presence of oxygen (Das et al., 2016a). In this study, all the fermentation assays were carried out under aerobic conditions, limiting ethanol production.

When lactose was depleted at 24 h of fermentation for WM, WNBSY and WNS the yeast can start to consume ethanol as a new carbon source. The ethanol consumption is carried out by alcohol dehydrogenase, releasing energy that is used for the preservation of cells. This allowed the stationary growth phase of yeasts observed from 24 to 120 h of fermentation.

Table 4. 2 Overall performance of yeasts during the fermentation assays for media at flask level

Parameter	Fermentation conditions						
	WM-Control	**WHC**	**WNC**	**WHS**	**WNS**	**WHBSY**	**WNBSY**
Specific growth rate (h^{-1})	KM = 0.02 ± 0.00 DH = 0.04 ± 0.00	KM = 0.02 ± 0.00 DH = 0.01 ± 0.00	KM = 0.01 ± 0.00 DH = 0.02 ± 0.000	KM = 0.00 ± 0.00 DH = 0.02 ± 0.00	KM = 0.02 ± 0.00 DH = 0.02 ± 0.00	KM = 0.04 ± 0.00 DH = 0.00 ± 0.00	KM = 0.04 ± 0.00 DH = 0.00 ± 0.00
Lactose consumption rate ($g_{lacrose}$/L*h)	0.88 ± 0.09	0.12 ± 0.01	0.12 ± 0.00	0.11 ± 0.00	0.61 ± 0.00	0.12 ± 0.01	0.99 ± 0.01
Ethanol productivity ($g_{ethanol}$/L*h)	0.20 ± 0.01	$2.83x10^{-5}$± 0.00	$1.00x10^{-3}$ ± 0.00	$5.25x10^{-5}$ ± 0.00	$4.00x10^{-3}$ ± 0.00	$2.11x10^{-3}$ ± 0.00	0.25 ± 0.00
Ethanol yield ($g_{ethanol}/g_{lactose})_{24h}$	0.23 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.01	0.00 ± 0.00	0.25 ± 0.00
L-Phenylalanine consumption rate ($mg_{L\text{-}Phe}$/L*h)	15.8 ± 0.7	5.65 ± 0.55	1.70 ± 0.10	4.40 ± 0.10	8.55 ± 0.10	2.80 ± 0.20	3.65 ± 0.15
2-Phenylethanol productivity (mg_{2PE}/L*h)	17.0 ± 1.0	0.04 ± 0.00	0.65 ± 0.04	0.08 ± 0.00	7.60 ± 0.00	0.00 ± 0.00	3.35 ± 0.05
2-Phenylethanol yield production ($g_{2PE}/g_{L\text{-}Phe}$)	0.89 ± 0.01	0.34 ± 0.00	0.40 ± 0.01	0.00 ± 0.00	0.46 ± 0.01	0.00 ± 0.00	2.44 ± 0.10

WM: whey medium fermentation which is used as control and comparative reference of the other media; **WHBSY**: whey enriched with hydrolyzed brewer's spent yeast; **WHC:** whey enriched with hydrolyzed crab residues; **WHS**: whey enriched with hydrolyzed soy residues; **WNBSY**: whey enriched with non-hydrolyzed brewer's spent yeast; **WNC:** whey enriched with non-hydrolyzed crab residues; **WNS**: whey enriched with non-hydrolyzed soy residues

Effect of cheese whey enriched with L-Phe biosource fermentation on L-Phe consumption and 2PE production

Figure 4.3c shows the L-Phe concentration during the fermentation time of media. The initial concentration of L-Phe was different for each media. The highest initial L-Phe concentration of 2.99 ± 0.06 g/L was observed for the WM medium, and decreased to 0.5 ± 0.1 g/L for the other media.

The low initial L-Phe concentration in the medium with the hydrolyzed residues is attributed to degradation of amino acids during the neutralization of pH with KOH. As the residues were treated with a strong acid solution to break the proteins, they had to be neutralized before fermentation. This increased the temperature and could lead to the degradation of peptides and amino acids (Singla et al., 2018).

For WNC, WNS, and WNBSY, the initial L-Phe concentration was 0.5 ± 0.1 $g_{L\text{-}Phe}$/L. This is attributed to the yeast extract and peptone that were added to the media. The presence of L-Phe is limited by the complex proteins matrices of residues (Vieira et al., 2019).

Figure 4.3c and Table 4.2 show that the consumption of L-Phe was almost negligible for all the media except for WM and WNS. The L-Phe transformation into 2PE is an energetic-consuming step. In the present study, when the lactose and ethanol were depleted after 48 h of fermentation, the cells need to divert the energy mostly for the preservation of the cell. This could limit the transformation of L-Phe through the Ehrlich pathway during the WM and WHS fermentation.

Figure 4.3d shows the 2PE concentration for all fermentation assays. The highest 2PE concentration was 674.9 ± 52.1 mg/L at 48 h for WM. WNS and WNBSY had 182.3 ± 0.0 and

161.3 ± 1.2 mg_{2PE}/L, respectively, at 48 h of fermentation. However, for WM fermentation, it was observed that 2PE concentration decreased from 674.85 ± 5.20 to 426.20 ± 4.80 mg/L at 120 h.

The high yield of 2PE for the WNHB (2.44 ± 0.10 $mg_{2PE}/mg_{L\text{-}Phe}$) shown in Table 4.2 could be attributed to the continuously biological hydrolysis of proteins of BSY. This allowed keeping a constant L-Phe concentration in the medium despite its consumption and transformation. Conde-Baez et al. (2019) studied the fermentation of acid whey using *K. marxianus* at pH of 4.8. The initial concentration of lactose and L-Phe was 45 and 1 g/L. They observed a 2PE productivity of 9.29 mg_{2PE}/L*h at 72 h of fermentation. In contrast, the 2PE productivity in this study using a co-culture for the whey fermentation was 14.05 mg_{2PE}/L*h.

The statistical analysis shows that the use of the hydrolyzed and non-hydrolyzed residues negatively affected the 2PE production (data shown in Table 4.3). However, the non-hydrolyzed BSY was selected as a nitrogen source for the second step of the study because it promoted the growth of the yeasts and the continuous production of proteins.

Table 4. 3 One-way ANOVA of the effect of agro-food residues co-fermentation using a co-culture of yeasts on 2-phenylethanol production. The treatments comprise fermentation of hydrolyzed and non-hydrolyzed agri-food residues.

Source of variation	**Degree of freedom**	$\sum$ ***Squares***	***Mean squares***	***F* - value**	***p* – value**
Type of medium	6	0.36	0.06	4.47	0.00458*
Residuals	21	0.28	0.01		
Treatments	**Diff**	**Lwr**	**Upr**	***p* – value adjusted**	
WHB-WM	-0.34163	-0.60885	-0.0744	0.006914*	
WHC-WM	-0.34086	-0.60809	-0.07363	0.007061*	
WHS-WM	-0.34018	-0.6074	-0.07295	0.007195*	
WNHB-WM	-0.27664	-0.54387	-0.00941	0.039353*	
WNHC-WM	-0.32059	-0.58782	-0.05336	0.012288*	
WNHS-WM	-0.26359	-0.53082	0.003641	0.054791	
WHC-WHB	0.000763	-0.26647	0.267991	1	
WHS-WHB	0.00145	-0.26578	0.268679	1	
WNHB-WHB	0.064988	-0.20224	0.332216	0.983412	
WNHC-WHB	0.021038	-0.24619	0.288266	0.99997	
WNHS-WHB	0.078038	-0.18919	0.345266	0.959445	
WHS-WHC	0.000688	-0.26654	0.267916	1	
WNHB-WHC	0.064225	-0.203	0.331454	0.984375	
WNHC-WHC	0.020275	-0.24695	0.287504	0.999976	
WNHS-WHC	0.077275	-0.18995	0.344504	0.961276	
WNHB-WHS	0.063538	-0.20369	0.330766	0.985207	
WNHC-WHS	0.019588	-0.24764	0.286816	0.999981	
WNHS-WHS	0.076588	-0.19064	0.343816	0.962878	
WNHC-WNHB	-0.04395	-0.31118	0.223279	0.997938	
WNHS-WNHB	0.01305	-0.25418	0.280279	0.999998	
WNHS-WNHC	0.057	-0.21023	0.324229	0.991572	

*Significant at 5% level ($p \leq 0.05$). **WM**: whey medium fermentation which is used as control and comparative reference of the other media; **WHBSY**: whey enriched with hydrolyzed brewer's spent yeast; **WHC:** whey enriched with hydrolyzed crab residues; **WHS**: whey enriched with hydrolyzed soy residues; **WNBSY**: whey enriched with non-hydrolyzed brewer's spent yeast; **WNC:** whey enriched with non-hydrolyzed crab residues; **WNS**: whey enriched with non-hydrolyzed soy residues; **Diff**: the difference between means of the two groups; **Lwr, Upr**: the lower and the upper endpoint of the confidence interval at 95% (default); and ***p*-value adjusted:** p-value after adjustment for the multiple comparisons.

Co-fermentation of whey and brewer's spent yeast in aerated bioreactor

Yeast growth response under controlled aeration

The fermentation of WM and WBSY were performed in 2 L bioreactors. Table 4.4 shows the initial conditions of fermentation in terms of lactose, nitrogen, pH, COD, and cations content. Table 4.4 also shows the determined consumption rates, alcohols yield, and alcohols productivity. Whereas Figure 4.4 shows the kinetics of fermentation in terms of yeast growth, lactose and nitrogen consumption, and ethanol production.

Table 4. 4 Overall performance of yeasts during the fermentation assays for WY media in the 2 L bioreactors

Parameter	Fermentation conditions	
	WM	**WBSY**
Initial lactose concentration (g/L)	19.7 ± 0.3	19.6 ± 0.1
Initial L-Phenylalanine concentration (g/L)	3.42 ± 0.21	3.21 ± 0.45
Initial brewer's spent yeast mass (g)	0.00 ± 0.00	110 ± 0
Initial total organic carbon (g/L)	17.6 ± 0.5	12.6 ± 0.2
Initial total nitrogen (g/L)	3.58 ± 0.31	2.17 ± 0.12
Initial soluble nitrogen (g/L)	2.92 ± 0.00	0.99 ± 0.00
Initial C/N ratio	4.94 ± 0.28	5.52 ± 0.10
Initial pH	6.50 ± 0.02	6.50 ± 0.02
Initial total chemical oxygen demand (gO_2/L)	39.30 ± 0.15	36.60 ± 0.51
Initial K^+ (mg/L)	1289.00 ± 64.46	1084.00 ± 54.17
Initial Na^+ (mg/L)	2526.00 ± 126.29	167 ± 8.34
Initial Mg^{2+} (mg/L)	30.00 ± 0.12	75 ± 0.02
Growth rate at 24 h of fermentation- μ (h^{-1})	KM= 0.04 ± $0.00_{24\ h}$ DH= 0.01 ± $0.00_{24\ h}$	KM= 0.04 ± $0.00_{24\ h}$ DH =0.01 ± $0.00_{72\ h}$
Lactose consumption rate ($g_{lactose}$/L*h) $_{24\ h}$	0.83 ± 0.01	0.81 ± 0.01
Ethanol productivity ($g_{ethanol}$/L*h)$_{8h}$	0.10 ± 0.02	0.28 ± 0.01
Ethanol yield production ($g_{ethanol}$/$g_{lactose}$)$_{8h}$	0.30 ± 0.00	0.73 ± 0.02
L-Phenylalanie consumption rate ($mg_{L\text{-}Phe}$/L*h)	59.5 ± $0.1_{31\ h}$	132 ± $23_{24\ h}$
2-Phenylethanol productivity (mg_{2PE}/L*h)	29.2 ± $0.2_{31\ h}$	38.4 ± $0.6_{48\ h}$
2-Phenylethanol yield production (g_{2PE}/$g_{L\text{-}Phe}$)	0.36 ± $0.02_{31\ h}$	0.61 ± $0.01_{48\ h}$
Final total chemical oxygen demand (gO_2/L)	20.80 ± 0.76	7.00 ± 0.20

Sub-indices near to the values of each kinetics parameter corresponds to the time of fermentation at which they were calculated. **WM**: whey medium fermentation which is used as control and comparative reference of the other media. **WBSY:** whey medium enriched with brewer's spents yeasts.

Figure 4.4a and 4.4b show the CD of *K. marxianus* and *D. hansenii*, respectively. For *K. marxianus,* the cell density was 2.18 ± 0.02 and 1.99 ± 0.04 log $CD_{K.\ marxianus}/CD_{initial}$ for WM and WBSY, respectively at 24 h of fermentation. This means that cell density increased from $5x10^7$ to $1.09x10^8$ ± $1.17x10^6$ and $9.98x10^7$ ± $1.90x10^6$ $CFU_{K.\ marxianus}$/mL for WM and WBSY, respectively. The stationary growth phase was identified from 24 to 48 h of fermentation in both media. For WBSY fermentation, the cell density incremented up to 2.43 ± 0.04 log $CD_{K.\ marxianus}$/cell $density_{initial}$ ($1.22x10^8$ ± $2.09x10^6$ $CFU_{K.\ marxianus}$/mL) at 72 h of fermentation. Whereas for *D. hansenii* in WM, it was observed a cell density of 1.52 ± 0.01 log $CD_{D.\ hansenii}/CD_{initial}$ ($7.61x10^7$ ± $4.75x10^5$ $CFU_{D.\ hansenii}$/mL). After this, the death phase was observed. For WBSY, a lag phase was observed during the 48 h of fermentation, and then a cell density of 2.62 ± 0.00 log $CD_{D.\ hansenii}/CD_{initial}$. ($1.31x10^8$ ± $6.40x10^4$ $CFU_{D.\ hansenii}$/mL) was observed.

Figure 4.4c shows the consumption of lactose and production of ethanol. For both fermentation assays, lactose was completely depleted at 24 h. A maximum concentration of ethanol of 2.0 ± 0.1 g/L was observed at 8 h in WBSY, corresponding to a productivity of 0.28 ± 0.01 $g_{ethanol}$/L*h.

The airflow rate of 1 L/min allowed a level of 30% of oxygen in both culture media. This promoted the synthesis of biomass for both yeasts rather than ethanol production, in comparison with the flask level experiments of the first part of the study. Oxygen is the last electron acceptor of the electron chain under aerobic fermentation of lactose. This means that the oxidation of lactose is releasing carbon and energetic biomolecules used mainly for biomass synthesis. This accelerated the ethanol production but decreased its accumulation in the bioreactors in comparison with the flask. Similar results were observed by Beniwal et al. (2017). They studied the aerobic fermentation

of whey using *K. marxianus* in a 3 L bioreactor, and they observed an ethanol concentration of 2 g/L at 8 h of fermentation.

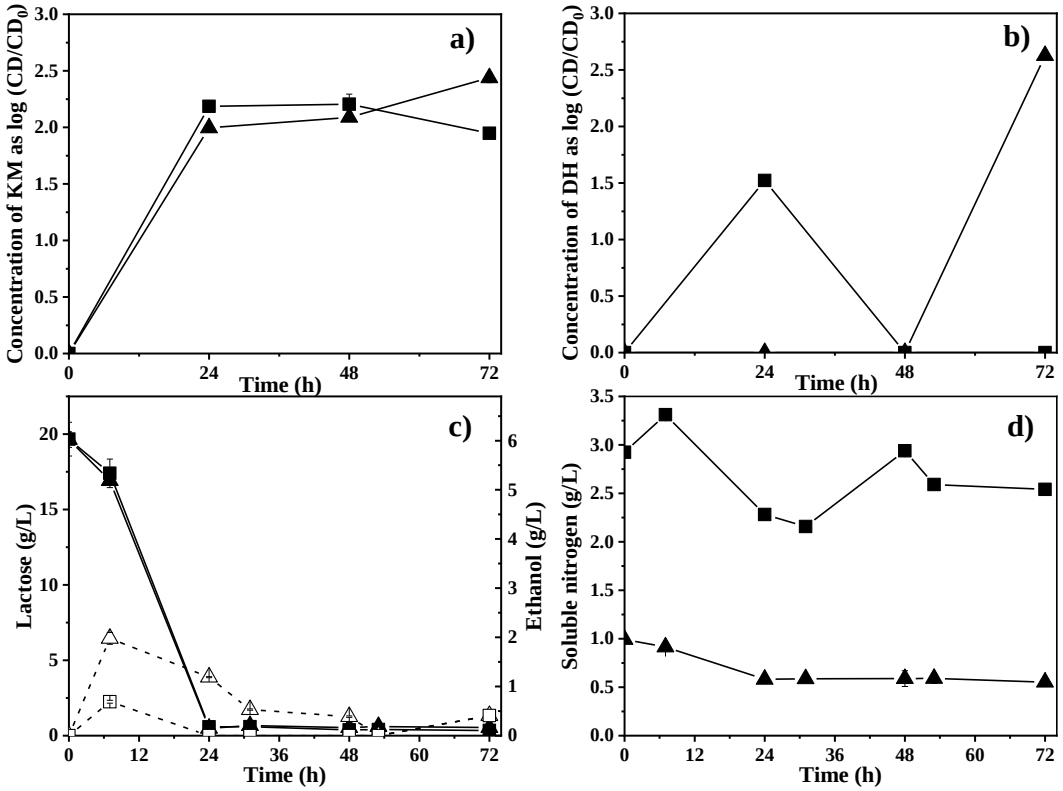

Figure 4. 4 Kinetics of WM and WBSY fermentation. Growth of a) *Kluyveromyces marxianus* and b) *Debaryomyces hansenii* as co-culture mode for whey medium-aerobic in bioreactors. c) lactose consumption and ethanol production during the whey medium-aerobic in bioreactors, and d) soluble nitrogen consumption. Closed symbols indicate the consumption of total organic carbon and lactose consumption for the fermentation of ■-WM-control, and ▲-WBSY. Open symbols indicate the ethanol production for the fermentation of □-WM-control, and △- WBSY.

Figure 4.4d shows the initial concentration of soluble nitrogen for both assays, which corresponds to 2.92 ± 0.00 and 0.99 ± 0.00 g/L for WM and WBSY, respectively. For both assays, a decrease of nitrogen concentration was observed until 24 h. The BSY is a complex matrix mainly composed of glycoproteins. These glycoproteins contain saccharides such as glucose, and proteins (Jacob et al., 2019; Vieira et al., 2019). Thus, BSY could be used as both nitrogen and carbon source

contributing to the higher ethanol production in comparison to the ethanol production in WM. In addition, for the WBSY fermentation, it was observed that ethanol was consumed slower than the ethanol accumulated in WM. This could allow a slight increment of the cellular density for both yeasts after 48 h of fermentation as it was observed in Figure 4.4a and 4.4b. Deesuth et al. (2015) studied the fermentation of sweet sorghum juice (200 g/L of total sugars) using *Saccharomyces cerevisiae* for the production of ethanol. The medium was fermented with and without dried BSY (13.5 g/L), and the ethanol productivity was increased from 1.9 to 3.9 g/L*h, respectively. This was attributed to the content of Mg^{2+} (concentration not mentioned) and assimilable nitrogen (373 mg/L) in BSY, which induced a protective effect on the yeast membrane and promoted the growth of yeast cells in the first 12 h, respectively. According to the concentration of the cations shown in Table 4.4, WBSY contained 2.5 fold higher Mg^{2+} concentration than WM, which could promote a protective effect on yeasts cells against the ethanol produced, improving both yeast growth and ethanol production.

Consumption of total organic carbon and proteins

Figure 4.5a shows the total organic carbon (TOC). The TOC decreased from 17.61 ± 0.57 to 7.92 ± 0.32 g/L for WM, and from 12.60 ± 0.20 to 4.66 ± 0.12 g/L for WBSY. The decrease of TOC in the culture media is related to the consumption of some compounds. For example, the lactose was completely consumed before 24 h of fermentation. Ethanol and biomass were produced as by-products of yeast primary metabolism within the first 8 h of fermentation, contributing to the constant TOC for WM and WBSY.

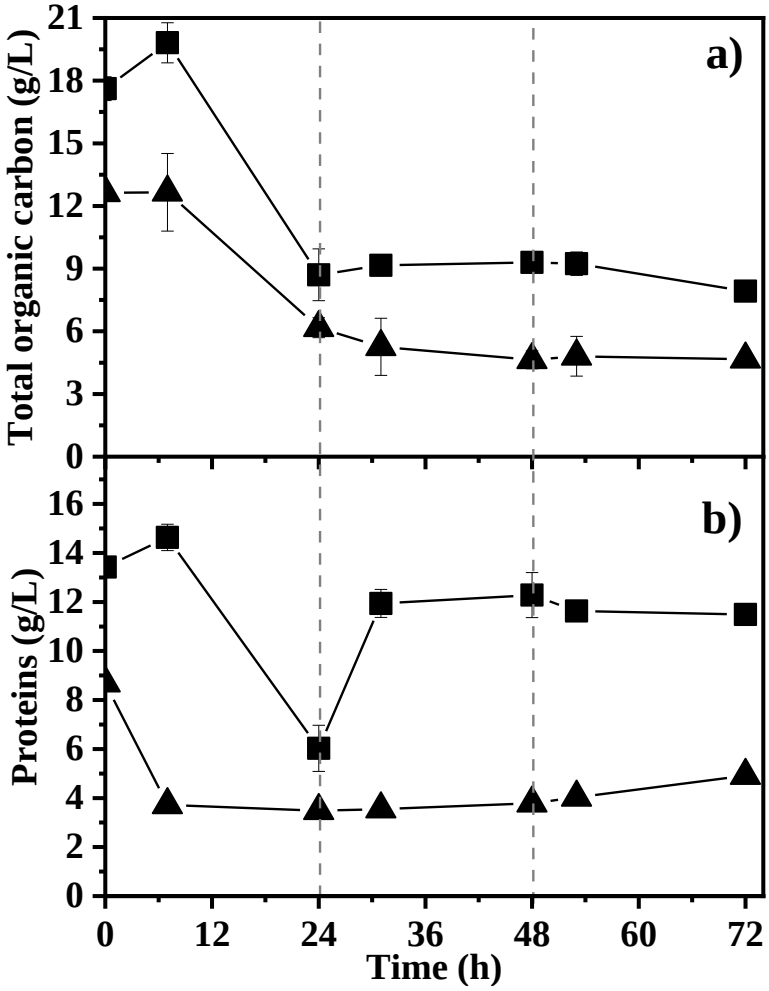

Figure 4. 5 a) Total organic consumption, and b) production of proteins during the whey medium-aerobic in bioreactors. Symbols indicate the fermentation for the ■-WM-control, and ▲-WBSY.

These results can be compared to the COD content in both media shown in Table 4.4. The initial total COD of WM and WBSY was 39.3 ± 0.1 and 36.6 ± 0.5 g O_2/L, respectively. The final total COD of WM and WBSY was 20.8 ± 0.8 and 7.0 ± 0.2 g O_2/L, respectively. Whereas the final soluble COD was under the limits of detection for both culture broth, showing the potential of the bioprocess to treat agri-food residues. According to the Canadian regulatory limits of discharge of

wastewater systems effluents regulation SOR/2012-139, the final soluble COD content in media fits this limit of discharge ($\leq$ 0.02 g O_2/L).

Figure 4.5b shows the initial protein concentration of 13.42 ± 0.15 and 8.68 ± 0.12 g/L for WM and WBSY, respectively. For WM, a decrease of protein concentration was observed within 24 h. Then, an increase of proteins up to 11.94 ± 0.57 g/L was observed at 31 h. Whereas for WBSY, a decrease of 57.3 ± 0.6% (5.0 ± 0.1 g/L) of proteins was observed.

The variation of proteins in WM and WBSY could be related to the source of nitrogen. For example, WM medium contained yeast extract and peptone which are assimilable nitrogen sources. The increment of proteins after 24 h could be related to the biomass growth. Whereas in the WBSY medium, the source of nitrogen was the whey and the BSY, which limit the available nitrogen in the culture broth. This could lead to nitrogen starvation, showing that this starvation is not a limiting parameter for either consumption of lactose or growth of yeasts.

Consumption of L-Phenylalanine and 2PE production

Figure 4.6a shows the L-Phe concentration during the fermentation assays. For WM, the highest L-Phe consumption was observed within the 31 h, corresponding to a concentration of 1.2 ± 0.0 $g_{L\text{-}Phe}$/L and a consumption rate of 0.1 ± 0.0 $g_{L\text{-}Phe}$/L*h. After this, the L-Phe concentration constantly increased. Whereas for WBSY, 89.1% of L-Phe (2.71 g/L) was consumed at 24 h, corresponding to a consumption rate of 0.13 ± 0.02 $g_{L\text{-}Phe}$/L*h. This suggests that the consumption of L-Phe is attributed only during the exponential phase of the yeasts.

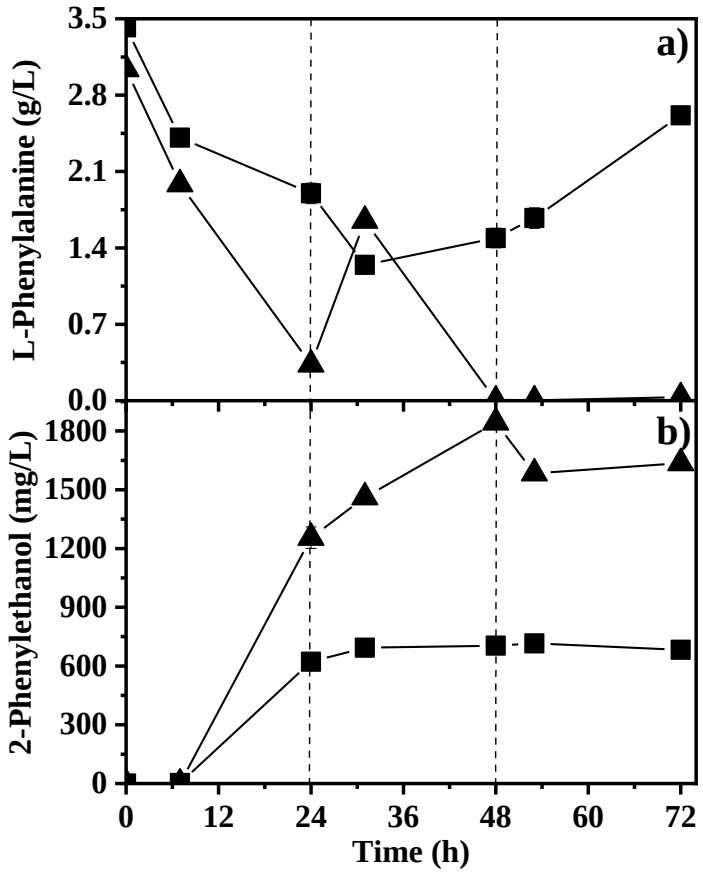

Figure 4. 6 a) L-Phenylalanine concentration change, and b) 2-Phenylethanol production during the whey medium-aerobic in bioreactors. Symbols indicate the co-culture performance for the fermentation of ■-WM-control, and ▲-WBSY.

The consumption of L-Phe in WM can be related to the primary metabolism of the yeasts. The amino group of the L-Phe can be used as nitrogen by means of its transamination into phenylpyruvate. The transamination reduces the α-ketoglutarate to glutamate by oxidizing the L-Phe and realizing energy. Yeast cells switch to this alternative pathway under nitrogen starvation conditions for the regeneration of energetic biomolecules such as NADH. This is possible under aerobic conditions, and due to the constant level of oxygen at 30% in the culture media, the yeasts were able to transform 2.18 ± 0.01 $g_{L\text{-}Phe}$/L. This is 2.9 times higher than the concentration

transformed at the flask level shown in Figure 4.3c. For WM, the increase of L-Phe concentration could be attributed to the lysis of *D. hansenii* cells after their death phase, realizing L-Phe to the medium. In addition, the activity of *K. marxianus* and the production of proteases could contribute to the increase of L-Phe concentration by releasing the amino acids from the proteins of residues.

For WM, the increase of L-Phe concentration could be attributed to the lysis of *D. hansenii* cells after their death phase, realizing L-Phe to the medium. In addition, the activity of *K. marxianus* and the production of proteases could contribute to the increase of L-Phe concentration by releasing the amino acids from the proteins of residues.

For WBSY, the increment of L-Phe at 31 h could be related to the action of the proteases degrading the complex protein matrix of BSY and whey. After this, both *K. marxianus* and *D. hansenii* increased their cell density, consuming 1.6 ± 0.0 $g_{L\text{-}Phe}$/L in the medium. Shu et al. (2021) studied the fermentation of glucose 60 g/L, and L-Phe 4 g/L using *S. cerevisiae* to produce 2PE. They studied the effect of aeration and temperature on the L-Phe consumption rate. They observed the highest consumption rate of 0.02 $g_{L\text{-}Phe}$/L*h at 1.3 vvm of aeration and 25°C. The L-Phe consumption rate obtained for WM and WBSY fermentation in the present study is 2.38 and 5.28 fold higher than the consumption rate obtained by Shu et al. (2021).

Figure 4.6b shows the 2PE during the fermentation assays. For WM, the highest 2PE concentration of 0.7 ± 0.1 g/L was observed at 31 h, corresponding to a productivity and a yield of 0.03 ± 0.01 g_{2PE}/L*h and 0.4 ± 0.1 $g_{2PE}/g_{L\text{-}Phe}$ (Table 4.4), respectively. In contrast for the WBSY, the highest 2PE concentration of 1.84 ± 0.03 g/L was observed at 48 h. This corresponds to a productivity of 0.04 ± 0.01 g_{2PE}/L*h and a yield of 0.6 ± 0.0 $g_{2PE}/g_{L\text{-}Phe}$ (Table 4.4).

The high productivity and yield of 2PE determined for WBSY were related to the quantity and nitrogen source in the medium. In this media, the quantity of available nitrogen (shown in Figure 4.4d) could promote a nitrogen starvation, forcing the yeasts to increase the transamination of L-Phe to use it as nitrogen source. In addition, the 30% level of oxygen in media could facilitate the reduction of L-Phe and its transformation into 2PE, resulting in the highest concentration of 2PE observed at 48 h of WBSY fermentation. The oxygen level could also promote the production of sterols and unsaturated fatty acids in the yeasts (Cao et al., 2016). Sterols and fatty acids act as protective compounds of the cell wall membrane, which could help the yeast to increase their tolerance to the presence of alcohols in the culture broth. For example, 2PE is a natural antimicrobial that denaturalizes the proteins in the membrane of microorganism cells. However, further studies need to be done to detect and quantify the sterols and fatty acids produced by the yeast and how they act as protective agents. The statistical analysis showed that the use of BSY as nitrogen source improved the 2PE production in comparison to the use of yeast extract and peptone (data shown in Table 4.5). Conde-Baez et al. (2019) studied the fermentation of whey using *K. marxianus*. They enriched the whey with 1 g/L of L-Phe and 0.4 mg/L of $(NH_4)_2SO_4$. They observed a 2PE concentration of 0.8 g/L at 96 h (Conde-Báez et al., 2019). The 2PE concentration observed in the present study using the co-cultured yeast to ferment whey and BSY is at least 2.25 fold higher than those values reported in the literature valorizing the whey. This shows the potential of the bioprocess implemented in this study to complete the valorization of agro-food residues and produce a high value-added compound.

Table 4. 5 One-way ANOVA of the effect of agro-food residues co-fermentation in bioreactors using a co-culture of yeasts on 2-phenylethanol production. The treatments are the fermentation of whey medium and whey enriched with brewer's spent yeast as co-substrate.

Source of variation	Degree of freedom	$\sum Squares$	*Mean squares*	*F* - value	*p* – value
Type of medium	1	2.72	2.72	8.16	0.0083*
Residuals	26	8.65	0.33		
treatments	**Diff**	**Lwr**	**Upr**	***p*-value adjusted**	
WM-WBSY	-0.6229	-1.071052	-0.1747482	0.0083031*	

*Significant at 5% level ($p \leq 0.05$). **WM**: whey medium fermentation which is used as control and comparative reference of the other media; **WBSY**: whey enriched with brewer's spent yeast and L-Phenylalanine. **Diff**: difference between means of the two groups; **Lwr, Upr**: the lower and the upper-end point of the confidence interval at 95% (default); and ***p*-value adjusted:** p-value after adjustment for the multiple comparisons.

Conclusions

The valorization of whey and agri-food residues was studied by means of fermentation using co-cultured yeasts, *Kluyveromyces marxianus* and *Debaryomyces hansenii*. Non-hydrolyzed agro-food residues were used as nitrogen sources for the growth of the yeasts. The synergetic effect of cations present in hydrolyzed residues promoted the growth inhibition on *K. marxianus*. Whereas, *D.hansenii* showed a better adaptation to the osmotic stress caused for the cations. In addition, it was observed that the consumption of L-Phe is a primary-metabolism process, and the 2PE production is a secondary-metabolism process. This corresponds to the exponential growth phase and the stationary growth phase of the yeasts, respectively. The controlled aeration and nitrogen starvation promoted in WBSY led to the highest productivity, yield, and accumulation of 2PE, which corresponds to 38.4 ± 0.6 $mg_{2PE}/L*h$, 0.6 ± 0.0 $g_{2PE}/g_{L\text{-}Phe}$ and 1.8 ± 0.1 g_{2PE}/L at 48 h.

Results are competitive with 2PE production studies for whey valorization and synthetic media fermentation. This shows the potential of using agro-food residues as carbon and nitrogen for their co-fermentation by a co-culture of yeasts.

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CHAPITRE CINQ - CONCLUSIONS ET RECOMMANDATIONS

CONCLUSIONS

Les principales conclusions de ce projet de recherche sont :

1. Les levures *Kluyveromyces marxianus* et *Debaryomyces hansenii* utilisées en monoculture ou en co-culture peuvent fermenter le lactosérum. Ces levures permettent de produire des composés aromatiques et de saveur, comme le 2-phényléthanol (2PE) et cette production est affectée par la composition du milieu de culture, la présence d'oxygène, la souche de levure et le mode de culture. Étant la fermentation aérobie de poudre de lactosérum en utilisant *K. marxianus* et *D. hansenii* avec un ratio 5:1 la meilleure condition pour la production de 2PE.

2. La méthode de contrôle de la flore microbienne autre que *K. marxianus* et *D. hansenii* a eu un effet sur la densité cellulaire des levures. Parmi trois méthodes testées, la pasteurisation a été retenue parce qu'elle a eu comme effet une croissance plus élevée des levures pendant la fermentation, ce qui a stimulé la production de 2PE.

3. Les levures peuvent consommer le lactose et produire de l'éthanol. L'ajout d'acides aminés a permis d'augmenter la production d'alcools de fusel. Les levures ont transformé spécifiquement la L-Phe en 2PE comme métabolite secondaire. La concentration initiale de lactose et de L-Phe a été un paramètre déterminant pour la production de 2PE étant les conditions optimales 20 $g_{Lactose}$/L et 3 $g_{L\text{-}Phe}$/L.

4. Les levures ont été immobilisées sur de biochar, de perlite et d'anneaux en plastique (Kaldness) afin d'augmenter la densité cellulaire et la production de 2PE. Le biochar a présenté les meilleures caractéristiques pour l'immobilisation des levures, permettant le développement d'un biofilm uniforme et dense, mais avec une faible production de 2PE. Cependant, les levures immobilisées

sur perlite et anneaux en plastique ont consommé rapidement le lactose et l'éthanol (qui a agi comme sous-produit de réserve). Ce qui a induit une production rapide et soutenue de 2PE.

5. Des résidus agroalimentaires peuvent être utilisés comme sources d'azote par les levures. Les résidus riches en protéines, comme la levure résiduelle de bière, sont une source renouvelable d'acides aminés et permettent d'obtenir des rendements élevés en 2PE. Le projet de recherche a montré que la production de biomolécules à haute valeur ajoutée sous une modalité de production durable est possible grâce à la valorisation de résidus agroalimentaires par voie fermentative.

RECOMMANDATIONS

En fonction des limitations technologiques et scientifiques identifiées pendant la définition de l'état de l'art et des difficultés observées pendant les essais expérimentaux, les recommandations suivantes sont proposées :

1. L'étude des monocultures et des co-cultures de levures suggère qu'il y a des interactions synergiques. Afin d'identifier les causes et les effets de ces interactions sur la production des biomolécules, l'étude des changements morphologiques, des flux métabolomiques et de la production de protéines spécialisées telles que les mycocines et les glycoprotéines est recommandée. Ceci contribuerait à trouver les facteurs qui stimulent la production de 2PE ou d'autres sous-produits d'intérêt industriel.

2. La fermentation de lactosérum peut être réalisée sous le concept de bioraffinerie et d'économie circulaire grâce à la composition des cellules de levure qui sont riches en protéines, saccharides et vitamines. L'extraction, séparation et purification des composés prisés par les secteurs alimentaire, pharmaceutique et cosmétique, augmenterait le potentiel de développer un bioprocédé

économiquement faisable et de cibler le mode de production zéro déchets. L'approfondissement dans la caractérisation de la biomasse résiduelle et l'identification de voies de valorisation sont fortement recommandés.

3. Afin de réaliser un transfert technologique efficace du bioprocédé à une échelle pilote ou industrielle, il est fortement recommandé d'optimiser les paramètres d'agitation et d'aération. Ces paramètres définissent le transfert de masse de l'oxygène affectant la transformation de la L-Phe en 2PE. L'optimisation en bioréacteur de la production de 2PE permettra de diminuer le coût global du bioprocédé et d'augmenter la rentabilité.

4. Dans le but d'augmenter la réussite de la mise à l'échelle pilote ou industrielle du bioprocédé, le développement des méthodes de récupération in situ des métabolites est recommandé. Ce qui aura comme effet le contrôle de la concentration d'alcools évitant d'atteindre leurs seuils de toxicité. Par exemple, pour récupérer et purifier l'éthanol et le 2PE des systèmes comme la pervaporation, l'adsorption, le stripping de gaz, la fermentation sous vide ou les bioréacteurs biphasiques (pour une extraction liquide-liquide) peuvent être appliqués.

APPENDIX

APPENDIX A (Supplementary Data – Chapter 2, Part 1)

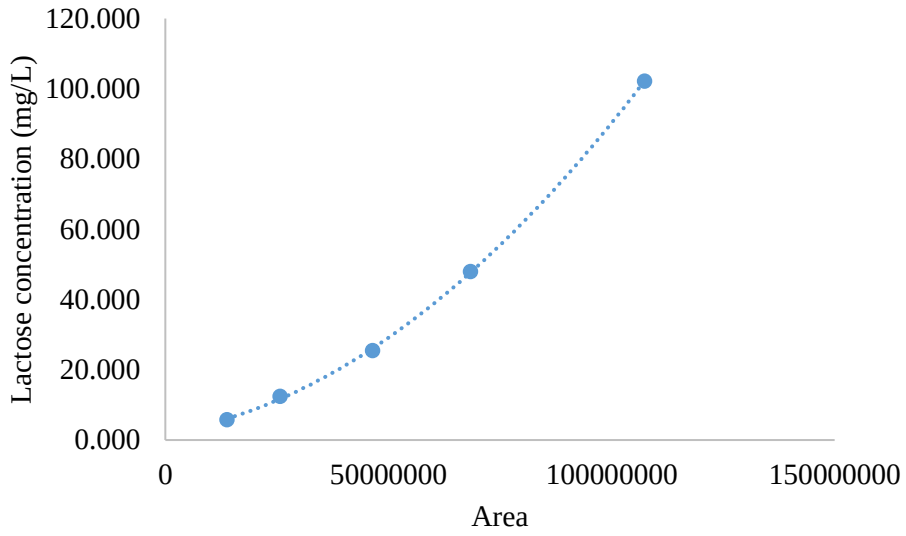

Figure A1. 1 Lactose calibration curve – LC-MS/MS

Component Name	Curve Index	Weighting Index	Origin Index	Equation
Lactose	Quadratic	Equal	Include	Y = 1.68069+0.729777*X-0.00318959*X^2 R^2 = 0.9949

Filename	Sample Type	Sample Name	Integ. Type	Area	ISTD Area	Area Ratio	Specified Amount	Calculated Amount	%Diff	%RSD-AMT	Peak Status	Level	Units	RT	Sample ID
std1	Std Bracket	Sample	Method Settings	13801585	2398174	5.755	5.000	5.726	15 %	0.0%		std4	mg/L	15.92	1
std2	Std Bracket	Sample	Method Settings	25741676	2509436	10.258	10.000	12.428	24 %	0.0%		std5	mg/L	16.06	1
std3	Std Bracket	Sample	Method Settings	46408132	2550940	18.193	25.000	25.459	2%	0.0%		std6	mg/L	16.20	1
std4	Std Bracket	Sample	Method Settings	68379024	2331493	29.328	50.000	47.923	-4%	0.0%		std7	mg/L	16.25	1
std5	Std Bracket	Sample	Method Settings	107398929	2500805	42.946	100.000	102.156	2%	0.0%	Response High	std8	mg/L	16.20	1

Example of identification of aroma and flavor metabolites using GC-MS

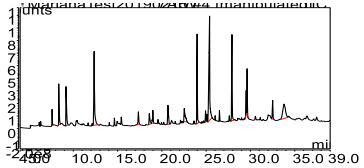

Figure A1. 2 Identification of aroma and flavor metabolites produced by yeasts during the whey fermentation

Sample: Aerobic fermentation of whey non-supplemented with nitrogen source

Fermentation time: 72 h

Peak No. TIC	Ret.Time min TIC	1st Hit SI TIC	Library Compound TIC	CAS Number TIC	Area counts*min TIC	% Prob
1	7.60858667	779	Butane, 1,2:3,4-diepoxy-, (±)-	298-18-0	6.98E+06	46
2	8.357415	950	Acetic acid	64-19-7	2.57E+07	83
3	9.172925	900	Oxalic acid	144-62-7	3.27E+07	45
4	12.35747	924	2-Furanmethanol	98-00-0	7.26E+07	52.5
5	17.349655	840	Phenylethyl Alcohol	60-12-8	1.21E+07	78.17
6	18.5832833	859	Maltol	118-71-8	1.13E+07	82.3
7	18.98338	815	Furyl hydroxymethyl ketone	17678-19-2	8.53E+06	40.91
8	19.5655	651	d-Glycero-d-ido-heptose	0	2.33E+06	8.47
9	20.6882917	802	2-Propanone, 1,3-dihydroxy-	96-26-4	1.33E+07	75.97
10	20.75948	699	2-Buten-1-ol	6117-91-5	5.26E+06	10.14
11	22.5283717	782	(S)-2-Hydroxypropanoic acid	79-33-4	1.93E+07	19.39
12	23.9809717	891	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	28564-83-2	5.25E+07	94.67
13	24.9307483	735	Propanoic acid, 3-hydroxy-	503-66-2	7.65E+06	18.21
14	25.3867133	910	Glycerin	56-81-5	1.22E+08	92.38
15	26.4986917	679	Nonanediamide, N,N'-di-benzoyloxy-	0	6.51E+06	9.68
16	27.9125433	881	5-Hydroxymethylfurfural	67-47-0	5.24E+07	89.03
17	29.51653	791	2(3H)-Furanone, dihydro-4-hydroxy-	5469-16-9	1.54E+07	69.08
18	29.6228617	744	1,2,6-Hexanetriol	106-69-4	2.47E+07	23.86
19	32.5055333	707	a-D-Glucopyranoside, O-a-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fructofuranosyl	597-12-6	1.43E+07	33.39
20	33.7887233	889	trans-13-Octadecenoic acid	693-71-0	5.02E+07	15.65

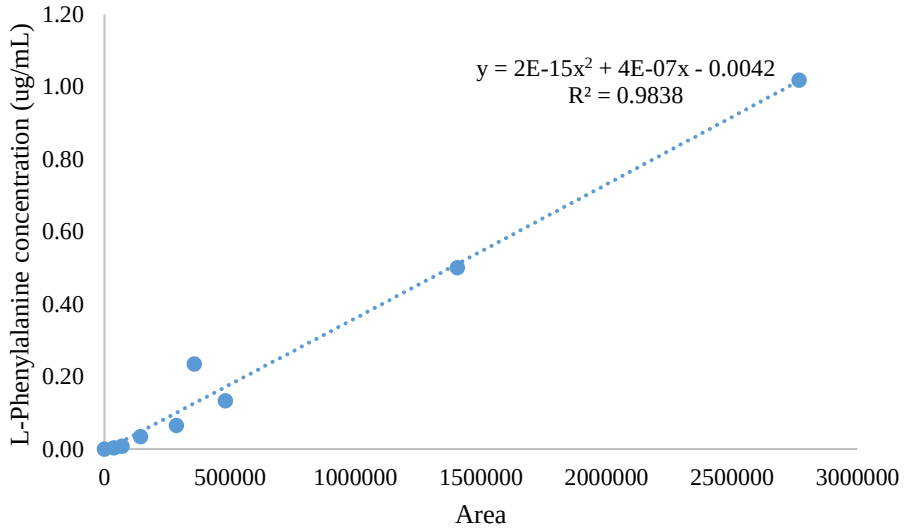

Figure A1. 3 L-Phenylalanine calibration curve using LC-MS/MS

Calibration curve and QC runs					
Name	Notes	Area	Conc ug/mL	Accuracy	Area
AA CC 0		0	0.00		398505
AA CC p008	0.0078 ug/mL	37719	0.004	88.8	341752
AA CC p016	0.0156 ug/mL	70051	0.008	100.9	315838
AA CC p032	0.0313 ug/mL	144626	0.035	91.5	376746
AA CC p064	0.0625 ug/mL	287336	0.066	112.5	315296
AA CC p125	0.125 ug/mL	481482	0.134	105.4	285143
AA CC p250	0.25 ug/mL	358144	0.235	106.8	105437
AA CC p500	0.5 ug/mL	1406226	0.501	93.9	235944
AA CC 1000	1 ug/mL	2767425	1.019	100.2	218078

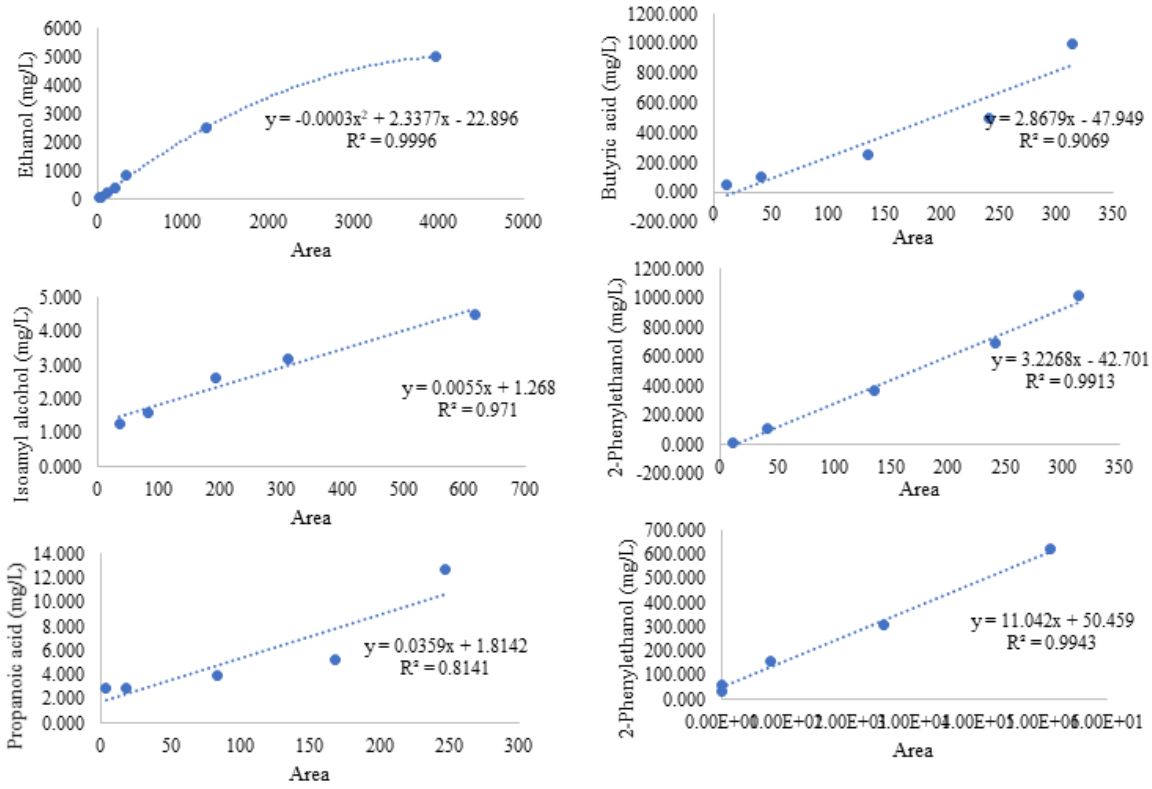

Figure A1. 4 Calibration curves of metabolites with aroma and flavor properties quantified with GC-FID

Area	RT	Signal	Compound	Lvl	Amount [mg/L]	Area	RT	Signal	Compound	Lvl	Amount [mg/L]
22.5				1	41	11.5	8.423	FID1A	Butyric acid	1	50.000
51.1				2	82	42.3				2	100.000
111				3	205	135.8				3	250.000
202.9	2.301	FID1A	Ethanol	4	410	242.1				4	500.000
341.3				5	820	314.4				5	1000.000
1274				6	2500	11.5	9.984	FID1A	2-Phenlethanol	1	13.000
3964				7	5000	42.3				2	106.000
35.8				1	1.286	135.8				3	365.000
81.8				2	1.580	242.1				4	690.000
194.8	5.633	FID1A	Isoamyl alcohol	3	2.600	314.4				5	1020.000
310.9				4	3.200	0.00E+00	10.881	FID1A	Dihydroxyacetone	1	31.000
617.5				5	4.500	0.00E+00				2	62.000
3.9				1	2.934	7.7				3	156.000
18.7				2	2.928	25.3				4	311.000
84.2	7.899	FID1A	Propanoic acid	3	3.994	51.2				5	622.000
168.7				4	5.290	11.5	8.423	FID1A	Butyric acid	1	50.000
247.4				5	12.683	42.3				2	100.000

Equation for determine the substrate consumption rate

$$\frac{dS}{dt} = \frac{C_{initial} - C_{final}}{Elapsed\ time\ of\ fermentation}$$ ... **Equation 5**

In which lactose or L-Phenylalanine are considered the substrates for ethanol or 2-Phenylethanol, respectively. Thus, dS/dt indicate the substrate consumption rate ($M \cdot L^{-3} \cdot T^{-1}$). Whereas the $C_{initial}$ and C_{final} are the initial concentration and the final concentration of substrate ($M \cdot L^{-3}$). The *elapsed time of fermentation* is the time when the final concentration of substrate is considered (T).

Equation for determine the productivity of by-products

$$\frac{dP}{dt} = \frac{C_{maximum}}{Elapsed\ time\ of\ fermentation} \text{ ... Equation 6}$$

In which the by-products are considered either ethanol or the 2-Phenylethanol. Thus, dP/dt indicates the productivity of the target by-product ($M \cdot L^{-3} \cdot T^{-1}$). Whereas the $C_{maximum}$ is the maximum concentration of by-product observed ($M \cdot L^{-3}$). The *elapsed time of fermentation* is the time when the maximum concentration is considered (T).

Equation for determine the yield of by-products

$$Y_{product} = \frac{C_{maximum}}{C_{initial} - C_{final}} \text{ ... Equation 7}$$

In which the by-products are considered either ethanol or the 2-Phenylethanol. Thus, $Y_{product}$ indicates the yield of the target by-product ($M_{by\text{-}product} \cdot M^{-1}_{substrate}$). Whereas the $C_{maximum}$ is the maximum concentration of by-product observed ($M \cdot L^{-3}$). $C_{initial}$ and C_{final} are the initial concentration and the final concentration of substrate ($M \cdot L^{-3}$)

APPENDIX B (Supplementary Data – Chapter 2, Part 2)

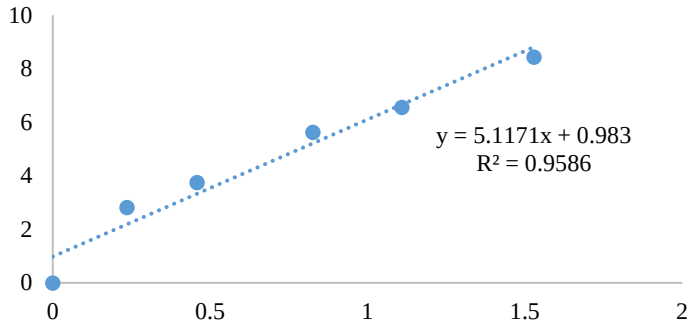

Figure A2. 1 Calibration curve for chemical oxygen demand

Glucose Concentration g/L	COD	Abs (600 nm) 1	Abs (600 nm) 2	Average Abs (600 nm)	COD
0	0	0	0	0	0
3	2.811621	0.2374	0.2337	0.23555	2.811621
4	3.748828	0.4594	0.4577	0.45855	3.748828
6	5.623243	0.8301	0.8223	0.8262	5.623243
7	6.56045	1.1082	1.1109	1.10955	6.56045
9	8.434864	1.5237	1.5342	1.52895	8.434864

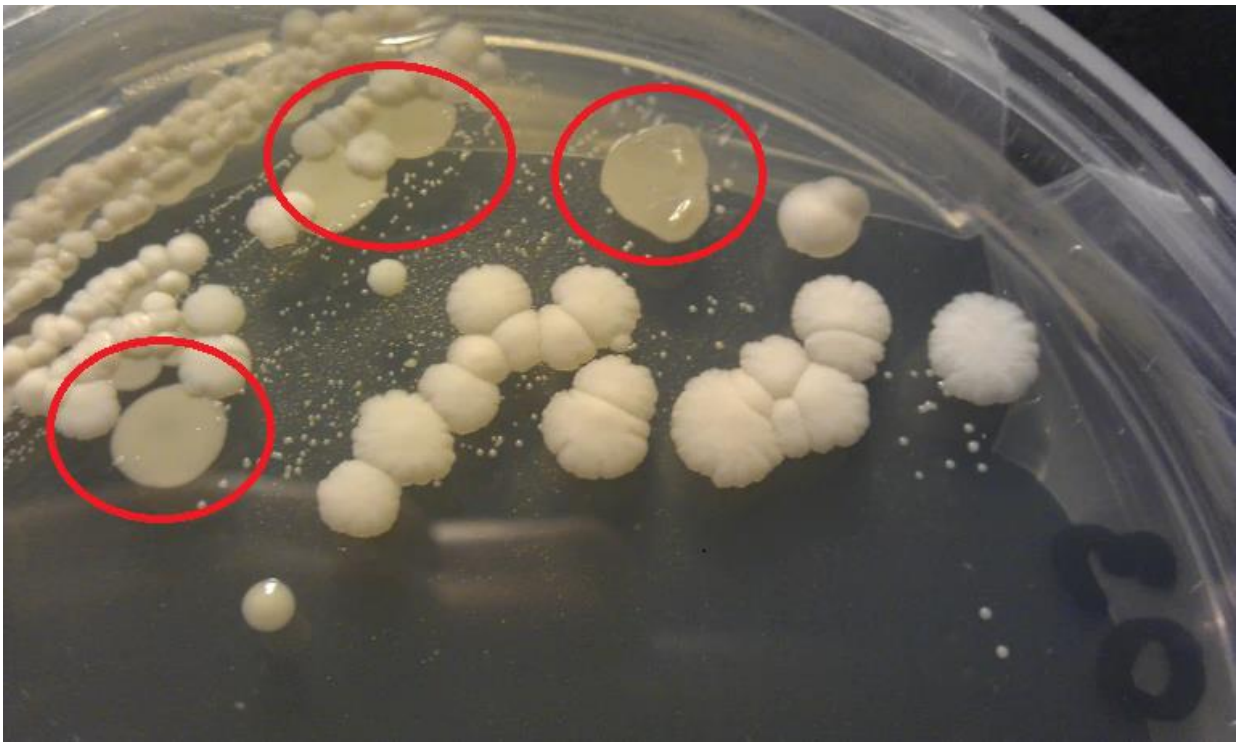

Figure A2. 2 Contamination of co-cultures yeast. The colonies signalled in a red circle are the gram-positive bacteria discussed in Chapter 2 – part 2.

APPENDIX C (Supplementary Data – Chapter 3, Part 1)

Concentration (mg/L)	Area 2-Phenylethanol
0	0
46.7	3238.947
93.4	6584.804
233.5	15916.73
467.0	32108.962

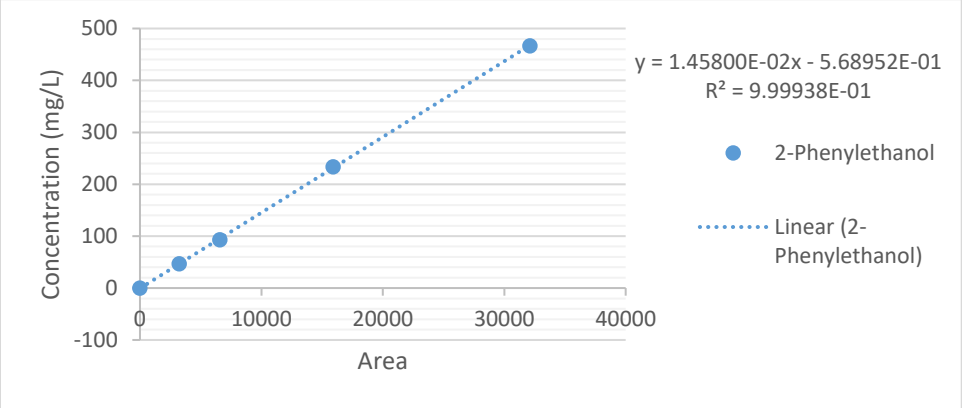

Figure A3. 1 Calibration curve of 2-Phenylethanol with HPLC

Area phenylalanine	Concentration (M)
2.29400	0.00000
204.15300	0.00005
834.17900	0.00020
1666.92100	0.00050
3436.80800	0.00110

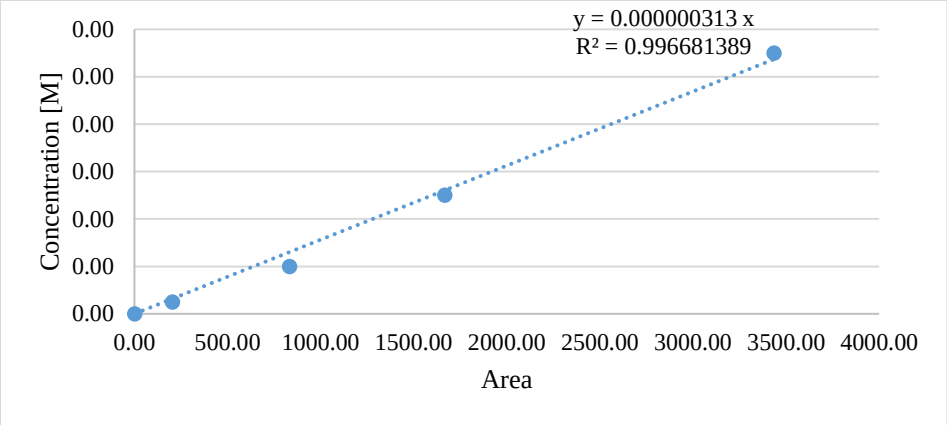

Figure A3. 2 Calibration curve of 2-Phenylethanol with HPLC

Equation to calculate the concentration (mg/L) of L-Phenylalanine in derivatized samples:

$$\boldsymbol{L-Phe = \left(\frac{C_1 \cdot \left(\frac{500\,\mu L}{1x10^{-6}\frac{mL}{\mu L}} \right)}{\frac{2}{3}} \right) \cdot 50 \cdot 35 \cdot MW_{L-Phe} \cdot 1000\ \frac{mg}{g} \cdot 2} \ldots \textbf{Equation 8}$$

In which C_1 is the molar concentration calculated with the calibration curve in Figure A3.2, followed by the factor of conversion of the total volume of the derivatized and neutralized sample (0.001), "2/3" is the proportion of the sample used for the neutralization, "50 and 35" are the volumes taken in each step of the derivation, MW is the molecular weight of L-Phe. Finally, 1000 mg/g is a factor of conversion, and the whole number is multiplied by 2 because of the dilution made during the HPLC analysis.

APPENDIX D (Supplementary Data – Chapter 4, Part 1)

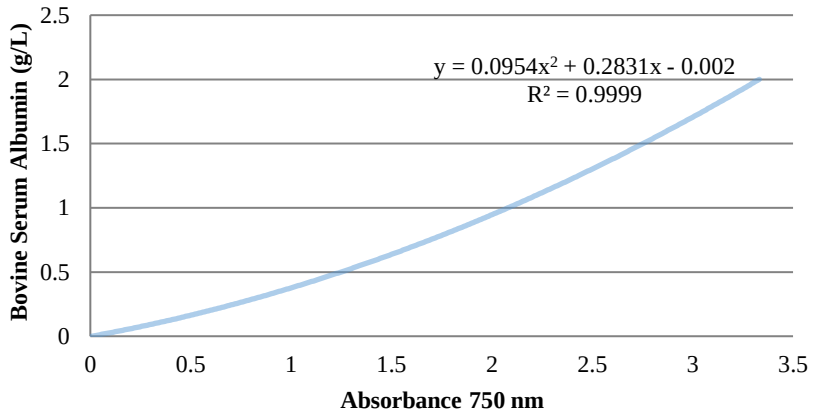

Figure 4A. 1 Calibration curve for proteins determination by Lowry method

Abs 750 nm	Concentration of Bovine serum albumin (g/L)	
0	0	
0.601	0.2	
1.43	0.6	
1.947	0.9	
2.476	1.3	
3	1.7	
3.332	2	
1.271	0.5	QC1
1.225	0.5	QC2
	0.511933	CORRECT
	0.487957	CORRECT