# Bioconversion Process Assisted by Non-Conventional Techniques for the Production of Lactobionic Acid from Lactose and Dairy-by-Products

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Suggested Short Title

# BIOCONVERSION OF LACTOSE AND DAIRY-BY-PRODUCTS INTO LACTOBIONIC ACID

**ABSTRACT**

Lactose as an important by-product of the dairy industry can be converted to a variety of value-added derivatives, including lactobionic acid (LBA, 4-O-b-D-galactopyranosyl-D-gluconic acid). LBA provides diverse functional properties, including chelating, antioxidant and humectant, making it a potent ingredient for food, pharmaceutical and cosmetics applications. In the present research, selected biocatalytic systems (lactoYIELD-LY/ Catazyme®-Cataz; LY/Cataz/Laccase; LY/Cataz /Laccase/mediator) were investigated for bioconversion of lactose and by-products of the dairy industry (including milk and whey permeates) into LBA. The end-product profile was analyzed using mass spectrometry, HPLC/Q- TOF MS. Controlling pH (5.6-5.8) during the time-course of the bioconversion showed a significant effect on the yield and productivity of LBA production, when LY/Cataz was used as a biocatalytic system. Ultrasonication (amplitude of 15 and pulse duration 3 s, off time 7 s) and microwave (100 W/g) assistance were investigated. Compared to the conventional bioconversion, no significant improvement in the LBA production was obtained with ultrasound-assisted bioconversion reactions, whereas the microwave-assisted one shortened efficiently the reaction time. The *in-situ* generation of oxygen was usefully achieved by the supplementation of reaction media with hydrogen peroxide, which was decomposed into oxygen by Cataz. Similar bioconversion yield results were achieved when the reaction media was supplemented by oxygen (air at 690 mL/min) or hydrogen peroxide (0.5%, v/v). However, LY/Cataz/Laccase/mediator system supplemented with oxygen showed a higher yield and productivity at a high lactose concentration of 200 mg/ml compared to LY/Cataz system. On the other hand, LY/Cataz system performed better in the presence of hydrogen peroxide added to generate in-situ oxygen by catalase. Lactose present in milk permeate was completely bioconverted to LBA in all investigated biocatalytic systems. While LY/Cataz system was identified as the most appropriate biocatalytic system for the bioconversion of whey permeate into LBA. Using the conventional bioconversion, the highest productivity of 17.5 and 21.1 g/l/h was obtained with whey permeate and lactose, respectively, using LY/Cataz system at a constant pH and in the presence of 0.5% hydrogen peroxide (v/v).

Mechano-milling activation was investigated for the bioconversion of lactose and whey permeate into LBA. The LY/Cataz was used as the selected biocatalytic system, which was supplemented by hydrogen peroxide to generate *in situ* oxygen. After ball-milling the reaction mixture

(substrate/LY/Cataz) at 20 Hz for 30 min in the absence of bulk media, the aging step was initiated by adding buffer and hydrogen peroxide (0.5% v/v) and run for 8 h under controlled pH (5.6-5.8) and temperature (38 °C). As expected, the highest bioconversion yields were obtained at low substrate concentrations. When lactose or whey permeate concentration was increased from 100 to 800 mg/ml, the bioconversion yields decreased, but the productivity increased to reach 30.1-38.9 and 8.4-12.7g/l.h, respectively. The other components of whey permeate were found to have an inhibitory effect on the efficiency of the biocatalytic system. In addition, polyethylene glycol exhibited a protective effect, which was significant in the presence of lactose than whey permeate as a substrate. A five-level, three variables central composite rotatable design (CCRD) was conducted to optimize the mechano-milling assisted bioconversion of lactose and whey permeate. The significant variables that affected the bioconversion yield and productivity of lactose bioconversion were substrate concentration and hydrogen peroxide amount. For the bioconversion of whey permeate, substrate concentration and LY/Cataz amount displayed the major contribution to the bioconversion yield and the productivity. Among interactive effects, the interaction between substrate concentration and enzyme amount exhibited an antagonistic effect on the bioconversion yield of lactose into LBA, while the interaction between substrate concentration and hydrogen peroxide amount exhibited a synergistic effect on the productivity. None of the interactions between variables was significant in the predictive models of both whey permeate-bioconversion yield and productivity. For the bioconversion of whey permeate into LBA, the highest productivity of 24 g/l.h was obtained at lactose concentration of 799.94 mg/ml, LY/Cataz amount of 0.079% (w/w) and hydrogen peroxide concentration 0.621%(v/v). As far as the authors are aware, this productivity is among the highest reported so far.

**RÉSUMÉ**

Le lactose en tant qu'un co-produit important de l'industrie laitière peut être converti en une variété de dérivés à haute valeur ajoutée, y compris l'acide lactobionique (ALB, acide 4-O-b-D-galactopyranosyl-D-gluconique). Le ALB offre diverses propriétés fonctionnelles, notamment chélatantes, antioxydantes et humectantes, ce qui en fait un ingrédient d'intérêt pour les applications alimentaires, pharmaceutiques et cosmétiques. Dans la présente etude, des systèmes biocatalytiques sélectionnés (lactoYIELD-LY/Catazyme®-Cataz; LY/Cataz/Laccase; LY/Cataz/Laccase/mediator) ont été étudiés pour la bioconversion du lactose et des co-produits de l'industrie laitière (à savoir, les perméats du lait et du lactosérum) en ALB. Le profil du produit final a été analysé à l'aide de la spectrométrie de masse, HPLC/Q- TOF MS. Le control du pH (5.6-5.8) tout en long de la cinétique de la bioconversion a montré un effet significatif sur le rendement et la productivité de la production de ALB, lorsque LY/Cataz était utilisé comme système biocatalytique. L'assistance par ultrasons (amplitude de 15 et durée de l'impulsion de 3 s, temps d'arrêt de 7 s) et micro-ondes (100 W / g) a été étudiée. Par rapport à la bioconversion conventionnelle, aucune amélioration significative de la production du ALB a été obtenue suite à des bioconversion assistées par ultrasons, tandis que la bioconversion assistée par micro-ondes a raccourci efficacement le temps de réaction. La génération *in situ* d'oxygène a été utilement réalisée par la supplémentation des milieux réactionnels avec du peroxyde d'hydrogène, qui a été décomposé en oxygène par Cataz. Des rendements de bioconversion similaires ont été obtenus lorsque le milieu réactionnel a été supplémenté par de l'oxygène (air à 690 mL/min) ou du peroxyde d'hydrogène (0.5 %, v/v). Cependant, le système LY/Cataz/Laccase/médiateur supplémenté par de l'oxygène a montré un rendement et une productivité plus élevé à une concentration élevée de lactose de 200 mg/ml par rapport au système LY/Cataz. D'autre part, le système LY/Cataz a mieux fonctionné en présence de peroxyde d'hydrogène ajouté pour générer de l'oxygène *in situ* par catalase. Le lactose présent dans le perméat de lait a été complètement bioconverti en ALB dans tous les systèmes biocatalytiques étudiés. Alors que le système LY/Cataz a été identifié comme le système biocatalytique le plus approprié pour la bioconversion du perméat de lactosérum en ALB. En utilisant la bioconversion conventionnelle, la productivité la plus élevée de 17,5 et 21,1 g/l/h a été obtenue avec du perméat de lactosérum et du lactose, respectivement, en utilisant le système LY/Cataz à pH constant et en présence de peroxyde d'hydrogène à 0,5% (v/v).

L'activation par mechano-broyage a été étudiée pour la bioconversion du lactose et du perméat de lactosérum en ALB. Le LY/Cataz a été utilisé comme système biocatalytique sélectionné, qui a été supplémenté par du peroxyde d'hydrogène pour générer de l'oxygène *in situ.* Après broyage à billes du mélange réactionnel (substrat/LY/Cataz) à 20 Hz pendant 30 min en l'absence du tampon du milieu, l'étape de vieillissement a été initiée par l'ajout de tampon et du peroxyde d'hydrogène (0,5% v/v) qui a duré 8 h sous pH contrôlé (5,6-5,8) et température constante (38 °C). Comme prévu, les rendements de bioconversion les plus élevés ont été obtenus à des faibles concentrations de substrat. Lorsque la concentration de lactose ou de perméat de lactosérum a été augmentée de 100 à 800 mg/ml, les rendements de bioconversion ont diminué, mais la productivité a augmenté pour atteindre 30,1-38,9 et 8,4-12,7g/l.h, respectivement. Les autres composants du perméat de lactosérum se sont avérés avoir un effet inhibiteur sur l'efficacité du système biocatalytique. De plus, le polyéthylène glycol exerçait un effet protecteur, qui était significatif en présence de lactose que le perméat de lactosérum comme substrat. Une conception rotative composite centrale (CCRD) à cinq niveaux et trois variables a été réalisée pour optimiser la bioconversion du lactose et du perméat de lactosérum assistée par mécano-broyage. Les variables significatives qui ont affecté le rendement et la productivité de la bioconversion au lactose étaient la concentration du substrat et la quantité de peroxyde d'hydrogène. Pour la bioconversion du perméat de lactosérum, la concentration du substrat et la quantité de LY/Cataz ont montré la contribution majeure au rendement de bioconversion et à la productivité. Parmi les effets interactifs, l'interaction entre la concentration du substrat et la quantité d'enzymes a montré un effet antagoniste sur le rendement de bioconversion du lactose en ALB, tandis que l'interaction entre la concentration du substrat et la quantité de peroxyde d'hydrogène a montré un effet synergique sur la productivité. Aucune des interactions entre les variables n'était significative dans les modèles prédictifs du rendement et de la productivité de la bioconversion du perméat du lactosérum. Pour la bioconversion du perméat de lactosérum en ALB, la productivité la plus élevée de 24 g/l.h a été obtenue à une concentration de lactose de 799,94 mg/ml, une quantité de LY/Cataz de 0,079 % (p/p) et une concentration de peroxyde d'hydrogène de 0,621 % (v/v). Pour autant que les auteurs le sachent, cette productivité est parmi les plus élevées rapportées jusqu'à présent.

**STATEMENT FROM THE THESIS OFFICE**

According to the regulations of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

*Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.*

*The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.*

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*When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.*

## PREFACE AND CONTRIBUTION OF AUTHORS

This thesis includes five chapters. Chapter I provides a general introduction to lactobionic acid production, the challenges facing ultrasound-assisted, microwave-assisted and mechano-milling methods in lactose bioconversion, the strategies applied to accomplish enzymatic bioconversion of lactose, and outlines the research objectives of the current study.

Chapter II comprises a literature review of the studies relevant to lactobionic acid (LBA) and the current methods of production, especially enzymatic approaches. Finally, the analytical techniques for quantitative and qualitative LBA analysis are reviewed.

Chapter III presents the results of the conventional method for production of LBA with different enzyme's combination either in presence or absence of oxygen and the results of microwave-assisted technique has been used on the production. The effect of the activation of substrate with ultrasonication in production yield and productivity in comparison with conventional method was studied. The study on the presence of hydrogen peroxide by catalase as a source of required oxygen in different concentration was investigated.

In Chapter IV, describes the employing of the mechano-milling process for LBA production. The effect of the presence of the polyethylene glycol in mechano-milling reaction was considered. Finally, an RSM design has been used in mechano-milling bioconversion technique in order to study the effect of reaction parameters and their interaction to optimize the lactobionic acid yield and productivity.

Finally, Chapter V covers an overall summary of the current research results.

The author was responsible for the experimental work and the preparation of the first draft of the thesis.

Dr. Salwa Karboune, the supervisor of the current M.Sc. research project, guided the entire research framework and reviewed all the presented chapters in this thesis prior to the submission.

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## NOMENCLATURE/LIST OF ABBREVIATIONS

| | |
|---|---|
| Catazyme | Catazyme® 25 L |
| CDH | cellobiose dehydrogenase |
| GFOR | Glucose–fructose oxidoreductase |
| $H_2O_2$ | Hydrogen peroxide |
| Lac | Laccases from *Trametes versicolor* |
| ABTS | 2,2’azino-bis (3-ethylbenzothiazoline-6-sulfonic acid |
| RP-HPLC | Reverse phase-high performance liquid chromatography |
| LBA | Lactobionic Acid |
| LC-MS | Liquid chromatography–mass spectrometry |
| LY | LactoYIELD |
| ESI | Electrospray ionization |
| $NH_4OH$ | Ammonium hydroxide |
| QTOF | Quadruple-Time-of-Flight |
| PEG | Polyethylene glycool |
| RPM | Rotations per minute |
| RSM | Response surface methodology |
| CCRD | Central composite rotatable design |

# CHAPTER I. GENERAL INTRODUCTION

The use of agri-food by products as biomasses to produce value-added ingredients has drawn a particular attention because of their abundance and their renewability. Valorizing these by-products is also part of the efforts to increase sustainability of the agri-food industry and to achieve the circular economy concept. Carbohydrates are the most abundant renewable resources available among the biomass categories (wood, energy crops, agricultural residues, food waste and industrial waste) (Lichtenthaler & Peters, 2004). For instance, the major component of whey, which is the main by-product of the dairy industry, is lactose (5-7%), followed by proteins (0.8-1.2%), lipids (0.06-3%) and ash (Pesta, Meyer-Pittroff, & Russ, 2007; Rocha & Guerra, 2020). Whey is abundantly generated (1 kg of cheese generated 9 kg of whey) and is being mainly valorized as a source of proteins. Upon this valorization, whey permeate, the by-product of whey protein production, is generated and contains between 65-85% lactose, 8-20% minerals and 3-8% proteins. However, the use of lactose is limited by its low solubility and sweetness. There is a high interest in the development of innovative biotransformations to produce value-added lactose derivatives. Indeed, lactose can be used as the starting materials for the production of epilactose, galacto-oligosaccharides, lactitol, lactobionic acid, lactosucrose, lactulose, sialyllactose and tagatose (Bramhankar et al., 2018). Among these lactose derivatives, lactobionic acid (LBA, 4-O-β-D-galactopyranosyl-D-gluconic acid), obtained from the oxidation of free aldehyde group of glucose on the lactose, has diverse functional properties, including chelating, antioxidant and humectant. These properties make LBA of great potential for food and pharmaceutical applications (Cardoso, Marques, Dagostin, & Masson, 2019).

Although there are different methods for producing LBA (chemical, electrochemical, heterogeneous catalytic oxidations), the green biocatalytic approaches have received a high attention because of their high selectivity, specificity, and mild reaction conditions. In addition, the ingredients produced by the environmentally friendly bioprocesses such as enzyme or using microorganisms as biocatalysts can be labelled as natural ingredients. Selected enzymatic processes have been investigated for oxidizing lactose to lactobionic acid. For instance, the combined of use of lactose oxidase and catalase has shown an ability to catalyze the formation of LBA from lactose. Indeed, lactose oxidase oxidizes lactose and transfers their electrons to molecular oxygen, which results in the hydrogen peroxide formation; while catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen. However, the productivity and the efficiency of this biocatalytic system is still limited. Emerging technologies, such ultrasound and

microwave, can assist the biotransformations and improve the yields and productivity (Delgado-Povedano & De Castro, 2015). It can also be hypothesized that the collapse of acoustic bubbles upon applying ultrasounds can enhance the amount of dissolved oxygen in the reaction media and promote the oxidation reaction (D. Wang et al., 2018). Mechano-milling is another technology that has the potential of providing some benefits, including alternative selectivity, shorter reaction times, reduced waste and the ability to access to the reaction pathways not accessible in solution (Q. Cao, Nicholson, Jones, & Browne, 2019). Mechano-milling process can alter both thermodynamic and kinetics of covalent bond forming reactions (Pang, Ishiyama, Kubota, & Ito, 2019). The assistance of mechano-milling has been explored in a range of synthetic transformations, but not for the valorization of whey lactose (Nicholson et al., 2021).

The main objective of the present research was to investigate selected approaches for the biotranformation of dairy-by-products, including milk and whey permeates, into LBA. The assistance of ultrasound, microwave and mechano-milling was studied, and their ability to increase the yield and the productivity as well as to shorten the reaction time was assessed. The effects of selected reaction parameters on the efficiency of this biotransformation were also studied.

The specific objectives which have been set to achieve the aforementioned objectives are as follows:

- Investigate the efficiency of selected biocatalytic systems that explore the complementarity and the synergistic actions between biocatalysts
- Study the effects of the assistance of microwave and ultrasound on the biotransformation yield and productivity.
- Assess the efficiency of the mechano-milling assistance in the lactose and dairy by-product biotransformation into LBA.
- Study the effects of reaction parameters and optimize the yield and the productivity of the biotransformation of whey permeate and lactose into LBA.

# CHAPTER II. LITERATURE REVIEW

## 2.1. Introduction

The dairy industry is undeniably one of the deep-rooted and most industrialized organization for over 3000 years, which represents all the activities connected to milk production, transformation, and distribution. Milk has a complex composition containing thousands of constituents from which is produced hundreds of different ingredients. Traditionally milk has been consumed in well known dairy commodities such as whole milk, cheese, yogurt, and butter (United States, Nutrition, & Team, 2014). Global demand for dairy products has continuously increased in consequence of the increasing population growth rate, urbanization, and the development of healthier dietary habits. On the other hand, this increase in demand has resulted in the generation of waste streams and by-products. A large quantity of bio-based waste, primarily whey is generated by dairy industry. Novel processing techniques are required as it is associated with a large polluting capacity due to the presence of dissolved sugars, proteins, fats, and residues of processing additives. In turn, dairy wastewater treatment processing is challenging due to the enormous quantities; however, the conversion of lactose to valuable ingredients is promising. Valorization of the dairy by-product is undoubtedly profitable for industry because it minimizes the environmental impact and cost of waste management as well as produces added-value ingredients (Rasmussen, Suwal, van den Berg, Yazdi, & Ahrné, 2020).

## 2.2. Valorization of dairy industry by-products

Whey is a liquid resulting from the coagulation and removal of milk casein during cheese-making which is a value-added by product that is widely used in the enrichment of food products (Brandelli, Daroit, & Corrêa, 2015). Cheese whey is primarily used for the whey protein production through ultrafiltration process; however, another stream, known as whey permeate (WP), with composition of minerals (6–20%), proteins (0.5–3%), lactose (70–90%), and lipids (< 2%) on a dry mass basis is produced through this process (Chan et al., 2020). Differences in raw material as well as differences in the procedure can result in variations in the functional and nutritional properties of whey products, acid (pH <5) and sweet (pH 6-7) whey are the main types of them.

Sweet whey powder, with a pH of at least 5.6, is primarily achieved using enzymatic coagulation (rennet-coagulation) of Cheddar, Swiss, Mozzarella, Monterey Jack, and similar cheeses (Council, 2004). Acid whey powder, with a pH less than 5.1, is obtained from the manufacture of acid-

coagulated cheeses such as cottage, cream cheese, and ricotta. Acid whey is higher in ash and lower in protein contents when compared to sweet whey and its application is limited in alimentation because of their acidic flavour content.

In order to produce about 1 kg of cheese, 9 kg of whey is created being traditionally considered as a waste product (Kosikowski, 1979). Annually close to 200 million tons of cheese whey are generated worldwide (Addai et al., 2020), and about half of this volume has been considered as a waste product. Due to the high level of organic and inorganic nutritional compounds in the whey, it can be considered of a major environmental threat (Farkas et al., 2019). Whey contains approximately biochemical oxygen demand (BOD) of 30000-50000 ppm and chemical oxygen demand (COD) of 60000-80000ppm (Risner, Shayevitz, Haapala, Meunier-Goddik, & Hughes, 2018). Since lactose being largely responsible for the high BOD and COD, bioconversion of whey lactose into added value component can reduce more than 75% of the BOD (Mawson, 1994). The results of the study that has been done by Ghaly et al. (2007) revealed that, the physical and chemical structure of soil get affected by discarded whey and as a result crop yield is decreased (Ghaly, Mahmoud, Rushton, & Arab, 2007).

Whey valorization technology is predominately applied to lactose taking advantage of its functional characteristics. The biotechnological applications of lactose as a source of carbon include the production of bioethanol, vinegar, environmentally safe disinfectant (S Santos et al., 2019), surface active compounds, single-cell protein, green plastics, food additive, pharmaceutical matrices, artificial sweeteners and soil bio-stimulants (Y. Wang, 2017). Lactose can be used as a sweetener in food applications or can be converted to valuable derivatives (R. P. Kumar et al., 2020). The key ingredients that can be produced from lactose are galactooligosaccharides, lactosucrose and lactulose with prebiotic functionality, lactobionic acid with antioxidant properties and lactitol as sweetener with prebiotic functions (Panghal et al., 2018; Rocha & Guerra, 2020).

### 2.3. Milk sugar

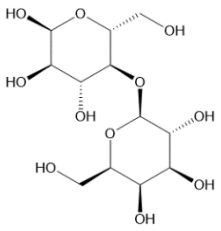
Milk homogeneous sugar, named lactose is present in all mammal's milk, which made up of the monosaccharides, glucose and galactose, which are linked by a β1-4 glycosidic bond named as O-β-D-galactopyranosyl-(1-4)-β-D-glucose, $C_{12}H_{22}O_{11}$.(Rawn & Ouellette, 2018) (scheme 2.1). The configuration of glucose around the C1 is not stable. The glucose moiety may exist in two isomers, the α and the β forms. In α-lactose (monohydrate), the hydroxyl group on the C1 of glucose is

orientated downwards and its upwards in the β-lactose (anhydride) form. Lactose is a major constituent in whey, permeate and skim milk powder. Lactose is mostly recovered from whey by the crystallization process (Gänzle, Haase, & Jelen, 2008). The crystallization behaviour of lactose is an important factor in the production of various lactose ingredients.

Amorphous lactose, non-crystallized form, is the most common form of lactose, which results from the rapid evaporation/drying leading to the same ratio of α- and β-lactose. The presence of amorphous lactose in whey powder promotes caking due to its extremely hygroscopic property. The most common form of lactose with more applications is α-lactose monohydrate ($C_{12}H_{22}O_{11}\cdot H_2O$). However, each crystal variant consists of small amounts of the other isomer. Indeed, β-lactose contains almost 20% of anhydrous α-lactose, and α- lactose monohydrate includes about 5% β-lactose anhydride.

Lactose solubility is lower compared to other types of carbohydrates. According to the solubility curve (Fig. 2.2), the solubility of β-lactose is considerably greater than that of α-lactose. The solubility chart reveals that the solution is saturated with the α-form below 93.5 °C and with the β-form above 93.5 °C (Shendurse & Khedkar, 2016). Consequently, the values of the final solubility are the same because of the α/β equilibrium. Interestingly, the super solubility curve of the solution highlights that below the line and above the final solubility line, the solution will not crystallize unless lactose seed crystals are introduced. This region is known as the metastable area.

Currently, the largest application of lactose includes its incorporation into infant formulas as a nutritional supplement (Jelen & Tossavainen, 2003). It has also been used in pharmaceutical products as a filler or coating agent for tablets due to its light flavour and mild sweetness. The other application for lactose is as a substrate for the synthesis of added-value lactose derivatives such as lactobionic acid, lactulose, lactitol, lactosucrose, galacto-oligosaccharides, and lactosyl urea (Guimarães, Teixeira, & Domingues, 2010).




**Scheme. 2.1**. Lactose chemical structure

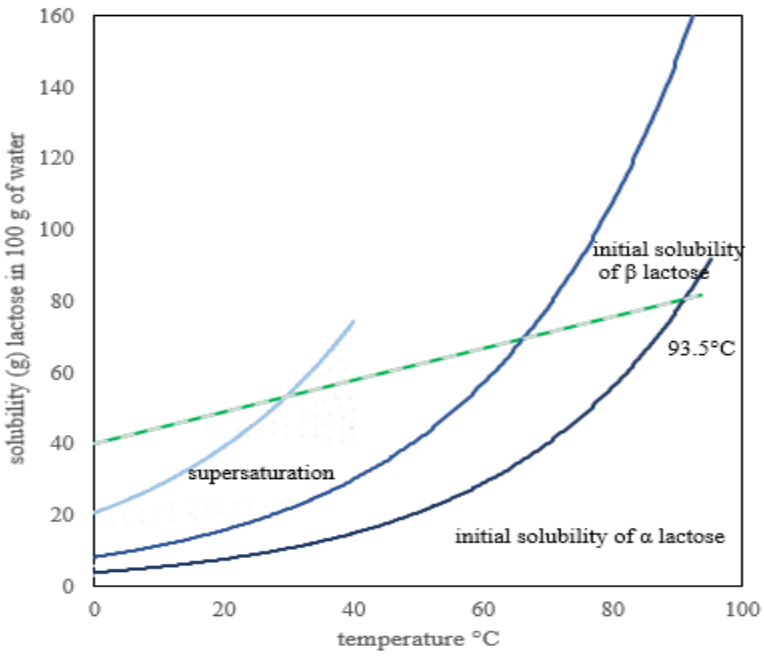



**Figure 2.2**. Lactose solubility curve (adapted from Caballero et al., 2015)

## 2.4. Lactose derivatives

In the last few decades, diet has shifted from principal role of food as a source of energy to applying functional food ingredients for additional health effects. The latest trend in the food industry, is focusing on the diets ability to modify the body's physiological functionality to prevent some diseases (Koletzko et al., 1998; Liu, 2020).Carbohydrates play a key role in the human diet as they are responsible for 50–60% of total required energy. Conversely, high carbohydrate diets are associated with increased risks of chronic diseases. To this end, research on production of functional carbohydrates has drawn increasing interest. Lactose as an inexpensive disaccharide can be converted to value-added functional carbohydrate products such as lactulose, epilactose, and galactooligosaccharides by isomerization in alkaline solution, epimerization, or transgalactosylation reactions, respectively (Guevara-Alvarado, Gutiérrez-Méndez, Carrillo-Pérez, Vargas-Bello-Pérez, & Rodríguez-Figueroa, 2020). Table 2.1. summarises the lactose conversions to produce value-added functional carbohydrate products

Transglycosidases including β-galactosidase (EC 3.2.1.23) and β-glucosidases (EC 3.2.1.21), are well-known for their hydrolytic and transglycosylation activities (Mu & Chen, 2021). The bond between glucose and galactose in lactose can be broken down by a specific enzyme named β-

galactosidase (Ansari & Satar, 2012; Wallenfels, Lehmann, & Malhotra, 1960). The hydrolytic activity of β- galactosidase is used in the production of low-lactose or lactose-free milk powders. However, under controlled reaction conditions, β-galactosidase can express a transgalactosylation activity by catalyzing the formation of glycosidic bond between saccharides. This ability allows β-galactosidase to catalyse the synthesis of galactooligosaccharide from lactose by adding galactose unit to lactose (Park, Oh et al. 2010, Torres, Gonçalves et al. 2010). Indeed, galactooligosaccharide consists of a chain of galactose units with a glucose unit at the reducing terminus. At the beginning of the reaction, transgalactosylation activity prevails when the concentration of lactose is high; as the reaction proceeds, a shift toward the hydrolytic reaction can occur (Prenosil, Stuker, Bourne, & bioengineering, 1987). Transgalactosylation performs through two steps: the first step is the formation of the galactosyl–enzyme complex with releasing of glucose; in the second step, enzyme-galactosyl complex is transferred to an accepter that contains a hydroxyl group. If the solution has a low concentration of lactose, water can be a competitor to act as an acceptor, rather than glucose and lactose (Albayrak, Yang, & Bioengineering, 2002).

On the other hand, sorbitol from glucose and lactitol from lactose can be produced by catalytic hydrogenation (W. Zhang, Chen, Chen, Wu, & Mu, 2020). Lactobionic acid (LBA, lactose oxidation) and gluconic acid (glucose moieties oxidation) can be obtained by oxidation reaction. Lactulose is a synthetic oligosaccharide possessing numerous physiological functions and applications in food and pharmaceutical industries. Lactulose is a ketose isomer of lactose that it is neither naturally occurring or hydrolyze by the small-intestinal enzyme, lactase (Q. Chen, Xiao, & Wu, 2021).

Another, less known lactose derivative is the bioactive trisaccharide lactosucrose, which is scarcely hydrolyzed by human digestive system and can be selectively used by *Bifidobacterium* (Kawase, Pilgrim, Araki, & Hashimoto, 2001). Lactosucrose is not found in the nature, and it is hard to produce chemically. Lactosucrose is 30% as sweet as sucrose and consists of glucose, galactose and fructose. It can be synthesized from lactose and sucrose using a transfructosylation reaction catalyzed by either β-fructofuranosidase or levansucrase (Park, Choi, & Oh, 2005). This specific sugar. Currently lactosucrose is commercially produced in Japan by Ensuiko Sugar Refining Co. and Hayashibara Shoji, Inc (Xu, Zhang, & Wu, 2021).

**Table 2.1**. Summary of lactose conversion to added value components.

| Reactions | Main products | By- products | Functionalities | References |
|---|---|---|---|---|
| Oxidation | Lactobionic acid | Hydrogen peroxide or 2-keto- lactobionic acid | Antioxidant, chelating agent, acidulent, humectant, bio-detergent | (Cardoso et al., 2019) |
| Polymerization | Galactooligosaccharides | Glucose, Lactose | Dietary fiber and prebiotic, enfant furmula | (Renfei Zhao, Duan, Yang, Xiao, & Lu, 2021) |
| Hydrogenation | Lactilol | Sorbitol, Galactitol, Lactuitol, Lactobionic acid | Sweetener, Laxative effect, prebiotic, mineral adsorption | (Martínez-Monteagudo, Enteshari, & Metzger, 2019) |
| Isomerization | Lactulose | Galactose, Tagatose, Epilactose, formic acid | Laxative effect, prebiotic, mineral adsorption | (Pazourek, 2019) |
| Hydrolysis | Glucose and galactose | Hydroxymethylfurfural | Sweetener, lactose-free formulation | (Rocha & Guerra, 2020) |

## 2.5. Enzyme-catalyzed biotransformations

Enzymes are biological molecules that accelerate chemical reactions. In food applications, they have been used for the food improvement from ancient era. Enzymes are selective for their substrates, as a result only a few reactions can be catalyzed in presence of them among many possibilities (Smith et al., 1998). The use of enzymes as powerful processing aids helped to overcome many limitations in the beer and cheese production (Tucker & Woods, 1995). Enzymes have also been employed in the production of functional ingredients, such as fructooligosaccharides (Hill et al., 2019; Sirouspour, 2020). The combination of catalytic function, specificity and mild reaction conditions makes the enzymes the favoured catalysts in the biotransformation reactions. The application of commercial enzymes in the food industry are either for hydrolysis of biological polysaccharide or for improving the food texture.

### *2.5.1. Cellobiose oxidase*

Cellobiose oxidase (EC 1.1.99.18.) belongs to oxidoreductases family and catalyzes the oxidation of cellobiose by transferring the reducing equivalents to the molecular oxygen (Younus, 2019). In particular, lactose oxidase (LactoYIELD; LY) is a flavoprotein that exhibits a high specificity toward the oxidation of lactose into lactobiono-δ-lactone, which is spontaneously converted into LBA (Ahmad, Brinch, Friis, & Pedersen, 2004). The electrons taken during lactose oxidation are transferred to the FAD domain, and are further used in the reduction of $O_2$, with the production of hydrogen peroxide ($H_2O_2$) as secondary product. The lactose oxidase activity of LY is favored at pH 5.5 to 7.0 and at 38°C, and it is completely inactivated above 80°C (Lund, Nikolajsen, & van den Brink, 2020). Ahmad et al. (2004) reported that the use of lactose oxidase in food is harmless for human consumption. Another application of lactose oxidase includes it use as a tissue preservation in the medical industry (Ahmad et al., 2004).

### *2.5.2. Catalase*

Catalase (EC 1.11.1.6) is a common enzyme found in all living organisms, and depending on its source, it exhibits different properties. However, one important function of catalase is to catalyse the decomposition of hydrogen peroxide into molecular oxygen and water (Y. Zhang, He, & Simpson, 2018). This enzyme plays a vital role in quenching the reactive oxygen species (Beer, 1952). The activity of catalase can be measured by spectrophotometry, oxygen electrode method

as well as electrochemistry. Catalase is produced by fungi, bacterial plants, animals and humans. Catalase from *Aspergillus niger* has many commercial applications as it offers high stability in severe conditions. Some applications of catalase include food and textile bleaching, corrosion, medicine (treatment of H1N1 flu and wound healing), biosensors, polymer and pharmaceutical synthesis and genetic bioengineering (AYDEMİR & KURU, 2003; Kadam, Tamboli, Sambhare, Jeon, & Govindwar, 2018; Kaushal, Mehandia, Singh, Raina, & Arya, 2018). In the biotransformation applications, catalase is used to decompose hydrogen peroxide produced in the reaction in the presence of oxidases.

#### *2.5.3. Laccase*

Laccase (EC 1.10.3.2) is a ligninolytic enzyme with ability to oxidize phenolic substrates with an affiliated reduction of oxygen to water (Giardina et al., 2010). Laccases from numerous bacteria and fungi have been used for detoxification of pretreated and un-pretreated feedstock either with or without mediator (Fillat et al., 2017). The application of laccase on protein modification has been reported (Pradyawong, Qi, Sun, & Wang, 2019). Inn the recent study reported by Li et al. (2021), the effect of laccase modifications on the potato protein functionality was investigated. The assessment was done through direct or indirect cross-linking in the presence of ferulic acid as a mediator and conjugation with polysaccharide (M. Li, Blecker, & Karboune, 2021). In the dairy application, the effect of laccase mediator system on the yield, quality, the colorimetric and sensory properties of the "Giuncata" cheese was studied. The results revealed there is similarity in milk coagulation properties with and without laccase mediator system; however, the yield, the solid recovery, the dry matter and protein contents were higher in the presence of laccase mediator system (Loi et al., 2020). In the LBA production, cellobiose dehydrogenase (CDH) from *Aspergillus fumigatus* /laccase (Lac) system were applied in presence of redox mediator (J. Yang, Xu, Long, & Ding, 2021).

### 2.6. Lactobionic acid production

LBA (4-0-β-galactopyranosyl-D-gluconic acid) with molecular weight of 358.3 Da with pKa 3.6 belongs to the aldobionic acid family, which comprises a galactose moiety linked with a gluconic acid via an ether-like linkage (H. Chen & Zhong, 2017). LBA, with a melting point range of 128-130 °C, is completely water soluble and slightly soluble in anhydrous ethanol and methanol. It is not normally found in nature, but it may be formed by oxidation of the free aldehyde group of

lactose. LBA is a chelating agent, and different salts of LBA have been produced like calcium, cupric and ferric salts. Calcium salt of LBA was used as a good source of calcium in pharmaceutical preparations and as a food additive (Minal, Balakrishnan, Chaudhary, & Jain, 2017). LBA can also be used as a liver preservation for organ transplants as it suppresses cell swelling during hypothermic storage (Rusciano et al., 2018). LBA can be produced catalytically, electrochemically, chemically, enzymatically or by fermentation.

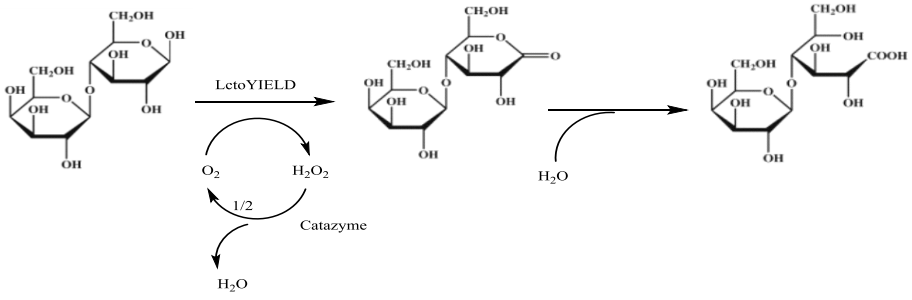
### *2.6.1. Biocatalytic oxidation*

Conversion of lactose to LBA using specific enzymes or microorganisms as biocatalysts is called biocatalytic oxidation. The mechanism of this reaction comprises the formation of an intermediate of lactobiono-O̊-lactone, which is subsequently hydrolyzed to LBA (Minal et al., 2017). Biocatalytic oxidation of lactose is favourably efficient and selective under specific conditions. The yield of LBA produced by biocatalytic oxidation is higher compared to microbial fermentation, but enzymes can be unstable under process conditions (Nordkvist, Nielsen, & Villadsen, 2007). The enzymes, known to catalyze the oxidation of lactose, are glucose-fructose oxidoreductase/ glucono-α-lactonase (KIRYU, KISO, & MURAKAMI, 2016), cellobiose dehydrogenase (Qiaopeng Tian et al., 2018), and carbohydrate oxidase (S.-F. Lin, C.-K. Li, & Y.-P. Chung, 2019). Table 2.2 summarizes the efficiency of the biocatalytic oxidation systems. Cellobiose dehydrogenase is the most used enzyme to produce LBA. This extracellular enzyme is produced by many fungi, such as *Sclerotiumrolfsii, Phanerochaete chrysosporium,* and *Trametes versicolor,* and needs a redox mediator like 2, 2-azinobis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2, 6-dichloroindophenol (DGIP) and *ortho*- and *para*-quinones to act as electron acceptors. The mediator can be regenerated during the reaction by adding an additional enzyme like laccase (Nyanhongo, Thallinger, & Guebitz, 2017). The challenges in the biocatalytic oxidation are the need of the regeneration of coenzyme or the formation of hydrogen peroxide, which causes enzyme inactivation (Nath, Verasztó, Basak, Koris, Kovacs, et al., 2016). LactoYIELD (LY) produced by Chr. Hansen and Novozymes contains a carbohydrate acceptor oxido-reductase from *Microdochium nivale* and catalase (scheme 2.3). LY uses dissolved oxygen as an electron acceptor, which makes it cost friendly for industrial application. The pH must be kept constant by adequate addition of sodium, calcium, or potassium ions to the reaction.

Conversion of lactobionate salts to acid form is completed by passing the solution through cation exchange resins.

**Table 2.2.** Production of lactobionic acid (LBA) by biocatalytic oxidation of lactose.

| Biocatalysts | Redox mediator | Productivity (g/L h) | Yield | Reference |
|---|---|---|---|---|
| Cellobiose dehydrogenase from *Aspergillus fumigatus* /Laccase | ABTS | 7.14 | 100 | (J. Yang et al., 2021) |
| Cellobiose dehydrogenase from *Sclerotium rolfsii*/Laccase | ABTS | 3 | 100 | (Van Hecke et al., 2009) |
| Cellobiose dehydrogenase mutants from *Phanerochaete chrysosporium* in *Pichia pastoris* KM71H strain | | | 100 | (Balaž et al., 2020) |
| Cellobiose dehydrogenase from *Termitomyces clypeatus* | | 0.01 | 100 | (Saha, Ghosh, Mukherjee, Bose, & Mukherjee, 2008) |
| Cellobiose dehydrogenase from *Volvariella volvacea* expressed in *Pichia pastoris* | | | | (K. Chen, Liu, Long, & Ding, 2017) |
| Lactose oxidase from *Myrmecridium flexuosum* NUK- 21 | | | | (S. F. Lin, C. K. Li, & Y. P. Chung, 2019) |
| Carbohydrate oxidase from *Microdochium nivale* / catalase | | 4 | 98 | (Hua, Nordkvist, Nielsen, & Villadsen, 2007) |
| Glucose–fructose oxidoreductase and glucono-δ-lactonase from *Zymomonas mobilis* ATCC 29191 | | 5.6 | 85 | (da Silva, Pedruzzi, & Rodrigues, 2011) |




**Scheme 2.3**. LactoYIELD catalyzed conversion of lactose to lactobionic acid.

### *2.6.2. Microbial production of lactobionic acid*

*Filamentous fungi, red algae, Acetobacter orientalis* and *acetic acid* bacteria have been used in lactose oxidation (Alonso, Rendueles, & Díaz, 2013c; Kiryu et al., 2012; Malvessi et al., 2013; Oe & Kimura, 2008).The first bacterium reported with ability to convert lactose to lactobionic acid was *Pseudomonas* and it was investigated by Stodola and Lockwood (1947). Among 15 *Pseudomonas* species, only *Pseudomonas graveolens* species (known as *teatrolens*) has shown the ability to convert lactose (Stodola & Lockwood, 1947). Other strains that are able to produce LBA include *Zymomonas mobilis, Burkholderia cepacian,* and *Acetobacter orientalis* (Alonso, Rendueles, & Díaz, 2013b; Kiryu et al., 2012; Murakami et al., 2003). Some of the strains used in lactobionic acid production with high corresponding yields resulted in mutant strains, which are associated with safety concerns. In addition, a purification step is needed to separate LBA from the culture media.

### *2.6.3. Electrochemical oxidation*

Electrolytic oxidation process is another method to produce LBA. In this method, lactose is converted to LBA on noble metal electrodes such as Ni, Au, Pt and Cu (Danaee, Jafarian, Forouzandeh, Gobal, & Mahjani, 2008; Karim-Nezhad et al., 2009; X. Zhao, Liu, Chen, Lin, & Lu, 2007). Oxide electrodes are suitable for the oxidation of carbohydrate because of the formation of hydrogen bonds via hydroxyl groups and adsorption on the oxide layer.

### *2.6.4. Heterogeneous catalytic oxidation*

Heterogeneous chemical oxidation of lactose occurs in presence of air or oxygen using catalysts such as gold or palladium (Regenhardt et al., 2018; Tomar, Sharma, Nishimura, & Ebitani, 2016). The easily integration of this method with other lactose conversion processes make it a very favourable process. In this method, the tension of oxygen plays a critical role where emphasis for its control is necessary(C. I. Meyer et al., 2016). Pd (Palladium) and Au (Gold) with different supports are the most common catalysts that were used in LBA production. Indeed. Pd on different base such as Pd/MCM-22 zeolites, Pd-V/C and Pd/Carbon were used as catalyst in lactose oxidation (Mäki-Arvela et al., 2010; Wojciechowska, Klewicki, Sójka, & Klewicka, 2017). The Pd catalysts are limited by over-oxidation and self-poisoning. However, this drastically decreased when using Au since it has high stability against overoxidation. Using supported Au catalysts

under mild conditions has shown high selectivity in production of LBA (C. I. Meyer et al., 2016). Bimetallic Ag/Au (Silver/Gold) on $Al_2O_3$ support was also studied as catalyst for the production of LBA and was shown its efficiency to be dependent on the Ag/Au ratio (C. Meyer et al., 2018). During this reaction, LBA is synthesised as the main product; however, lactulose and 2-keto LBA are produced as undesirable co-products depending on the catalyst activity and selectivity, and the reaction conditions. Ideal reaction operating conditions include atmospheric pressure, alkaline media (pH 8.0 to 9.0), temperature between 50-70 ºC, using air or oxygen as eco-friendly oxidizing agents. Increasing pH values promotes the isomerization of lactose into lactulose. In contrast, decreasing the pH values cause deactivation of the catalyst because of the strong adsorption of the reaction products that occupy the active sites (Gutierrez, Hamoudi, & Belkacemi, 2011).

## 2.7. Applications of lactobionic acid

### *2.7.1. Food applications*

Increasing business profitability and maintaining the quality of products are two main pillars for food companies that motivate the processors to seek innovative methods in the development of new products and processes. LBA has many applications within the food industry based on its unique characteristics including acidulant, antioxidant, stabilizer, antimicrobial, and moisturizing properties (Cardoso et al., 2019; Illanes et al., 2016). According to the Food and Drug Administration, LBA is allowed to use in its salt form as stabilizer, gelling agent and antioxidant in dessert and its application as a food additive has increased tremendously (FDA, 2011; FDA, Food, & Administration, 2018). This polyhydroxy acid can serve as an acidifying agent in fermented milk products (J. Cao, Fu, Gao, & Zheng, 2019). In dairy desserts, it can be used as a mineral absorption enhancer (Oe & Kimura, 2008), and plays an aging inhibitor role when it comes to bread manufacturing (Goderska, 2019; Minal et al., 2017). The presence of LBA in dairy- and other product manufacturing processes provides not only stabilizing effect and acidulant properties but also improves the sensory attributes (Merrill & Singh, 2011; Minal et al., 2017; Nelles, Nesheim, & Nesheim, 2019). LBA can be added to the food either directly mixing (up to 10%) or produced *in situ* (by enzyme) (Koka et al., 2008).

### *2.7.2. Drug-delivery systems*

Controlled drug delivery is defined as a system which releases and distributes the therapeutic agents at a determined rate and time (Lesmes & McClements, 2009; Unagolla & Jayasuriya, 2018). Targeted delivery strategy has drawn the attention not only because of its boosted efficacy, but also for its reduced side effects. LBA was applied as a ligand in a targeted delivery for liver cells. Biocompatibility, ion-chelating ability biodegradability and its synergistic combination are unique characteristics of LBA, which makes it a potential drug-vehicle for the treatment of various diseases (W. Yang et al., 2010). Samui et al. (2019) have developed a multifunctional nanoscale LBA modified by $NH_2$-MIL-53(Al) NMOFs, metal organic framework for targeted cell imaging and liver cancer therapy by loading the anticancer drug doxorubicin (DOX) in hepatocellular carcinoma cell. According to their study greater cell death achieved by DOX-loaded LA-targeted NMOFs compared bare NMOFs. The results revealed that the fluorescent property of the $NH_2$-MIL-53 NMOFs remains unaltered after conjugation with LBA, which makes it advantageous therapeutic for cell imaging and targeted drug delivery (Samui, Pal, Karmakar, & Sahu, 2019).

### *2.7.3. Nanoparticle diagnosis*

LBA recently has been applied in the nanoparticles surface modification for biomedicine application. LBA as a bio- functionalized agent can be used as surface coating materials to enhance their properties such as biodegradability, biocompatibility, detection sensitivity, water-solubility, longer stability, and lower cytotoxicity (Knopp, Tang, & Niessner, 2009; Limo et al., 2018). The bio-functionalized nanoparticles have a high capability to be used in biomedical applications including cancer therapies, MRI, bio-detection and labelling (Iacobazzi et al., 2017; Kekkonen et al., 2009; Samui et al., 2019; Ruirui Zhao et al., 2017).

### *2.7.4. Tissue engineering*

Tissue engineering as a novel field aims at substituting, rejuvenating and regenerating injured tissues. As an example, for tissue engineering can mention to the development of using hepatocytes of liver tissue engineering for liver malfunction treatment. There is no report of direct contribution of LBA on tissue repair, however, the mechanism is accelerated in presence of this organic acid (Jain, Damania, & Kumar, 2014); (Alonso, 2018).

### *2.7.5. Cosmetic industry*

The skin is the main protector of internal organs in human that covers an area of approximately 2 $m^2$ (V. Cheng & Li, 2007). The skin is frequently exposed to free radicals, which cause skin aging. Polyhydroxy acids, which is a new generation of α-Has, is recently used in a number of cosmetic and therapeutic formulations for obtaining the beneficial effects for the skin (Kornhauser, Coelho, & Hearing, 2010). Due to therapeutic efficacy of LBA, the cosmetics industry is employing it as the active constituent in anti-aging and regenerative skin-care products. As a cosmetic ingredient, LBA offers multiple benefits for the therapeutic treatment of dermatological pathologies such as atopic dermatitis, rosacea and it can be used in anti-acne products. LBA's antioxidant role exhibits strong moisturizing effect, reduce wrinkles, exfoliative, and humectant properties.

### *2.7.6. Chemical industry*

Sugar-based surfactants drawn the attention in the industry field. Lactobionic acid as a candidate can be used as sugar-based surfactant as well as a co-builder in biodegradable detergents. Its unique properties such as iron-chelating and emulsifying characteristics can make it a potential agent for many industrial purposes (Gerling & Wilke, 1991). All applied improvement and development on LBA-based surfactants have been recently done resulting on elaboration of its functionality, enhancement of its surface properties and more importantly a reduced amount of environmental impact (Bize, Blanzat, & Rico-Lattes, 2010). Lactobionic acid with amide compositions is previously presented by Gerling et al. (1991) because of its specific characteristics such as higher foam stabilizing, cleaning, emulsifying, and softening properties in aqueous systems. Recently, it is being produced as an active ingredient with antibacterial component and it delivers superb preservation stability on goods (Araki, Fujii, Ueno, Fujii, & Takahane, 2006). It is also suggested that it be used as a building block for the bioconversion of novel polymers (Gumel, Annuar, & Chisti, 2013; Kakasi-Zsurka et al., 2011). Its role on functionalization of agents for the industrial systems is investigated such as functionalized carbon nanotubes with LBA amide amphiphile molecules to adsorb proteins is investigated (Feng et al., 2009).

## 2.8. Microwave-assisted the synthesis of organic compounds

The microwave radiation is a form of electromagnetic spectrum that falls at the lower frequency 0.3 to 300GHz (Rajak & Mishra, 2004). Organic synthesis has been carried out by conventional

approach that needs an external heat source. Transferring energy into the system can be considered as inefficient in conventional methods, because of the thermal conductivity and materials penetration. Consequently, the temperature of the container being higher than the reaction media. In microwave method, the key role is the ability of a specific material to first absorb microwave radiation energy and then convert it into heat (A. Yadav, S. Mohite, & C. Magdum, 2020). Conventional method for synthesis necessitates longer time; while microwave radiation synthesis promotes faster reaction and higher conversion rate. Microwave radiation is influenced by two key factors: the pre-exponential or 'A' factor, which is the molecular mobility and the vibrations frequency (A. R. Yadav, S. K. Mohite, & C. S. Magdum, 2020a, 2020b).

### 2.9. Ultrasound-assisted the enzymatic synthesis

The ultrasound radiation with the frequency in the range of 20–100 kHz has emerged in biocatalytic processes and has shown improved bioconversion yields (Rico-Rodríguez, Villamiel, Ruiz-Aceituno, Serrato, & Montilla, 2020). Ultrasound irradiation can be used either in enzymatic systems such as hydrolysis and synthesis or in live biological systems such as cell cultures and microbial fermentations. Homogeneous or quasi- homogeneous, heterogeneous or biphasic systems can be assisted by ultrasound irradiation (Soria, Villamiel, & Montilla, 2017). The collapse of acoustic bubbles upon ultrasound irradiation can dissolve oxygen, and as a result the concentration of oxygen increased in the reaction mixture. This process has been proven in waste water treatment in which oxygen is needed as a reactant (D. Wang et al., 2018; Q.-Q. Zhang & Jin, 2015). The ultrasound can also generate energy that can modify the vibrational and rotational states of the molecules (Rico-Rodríguez et al., 2020; Y.-G. Zhang et al., 2020). This technique was investigated for the synthesis of galactooligosaccharides and many other organic compounds (de Carvalho Silvello, Martínez, & Goldbeck, 2020; Rico-Rodríguez, Serrato, Montilla, & Villamiel, 2018).

### 2.10. Mechano-milling assisted the conversion

The mechano-milling approach modified the traditional crushing and griding methods by conducting them under vibration at frequencies of 5 to 60 Hz (Toda, 1995). The mechano-milling method has been mainly used in the field of supramolecular chemistry, organic synthesis and nano-chemistry (José G. Hernández & Friščić, 2015; Margetić & Štrukil, 2016a, 2016b). In particular, it has been used to promote green chemistry in the synthesis of inorganic compounds, co-crystals,

metal-ligand complexes and polymers (Braga, Maini, & Grepioni, 2013; Groote, van Haandel, & Sijbesma, 2012; Haehnel, Sagara, Simon, & Weder, 2015; Šepelák, Düvel, Wilkening, Becker, & Heitjans, 2013). Decreasing in the use of hazardous and toxic solvents, minimizing the magnitude of catalyst and reagents and reducing in the chemical waste are the foundation of green chemistry (DeVierno Kreuder et al., 2017).

### 2.11. Structural analysis

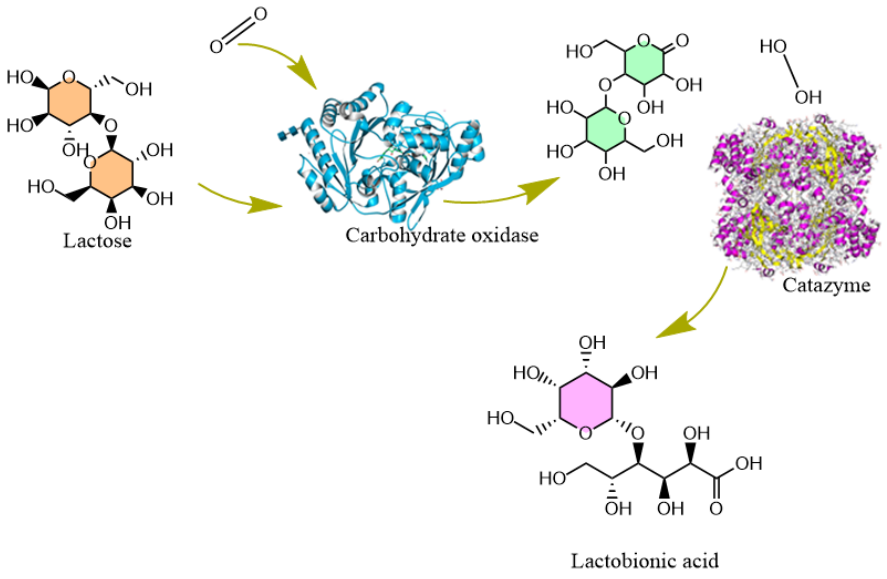
Due to the high sensitivity and specificity of mass spectroscopy (MS) compared to other chromatographic detectors, coupling of mass spectroscopy to chromatographic techniques has always been desirable. Mass spectroscopy can identify the molecule structure by determination of the mass-to-charge ratio of ionized molecule. LC-MS technique has been used in the analysis of lactose derivatives such as hetero-oligosaccharides from whey (İspirli & Dertli, 2021). Hydrophilic interaction chromatography (HILIC) is known for its ability to separate highly polar substances (Turcotte, Sakai, America, & KK, 2014). LBA was analyzed using HILIC column and MS/MS in negative ionization mode (Farouk & Azzazy, 2020). The method linearity was in the range of 5-60 ng/mL ($r^2>0.99$), with accuracy of >98 %.

### 2.12. Conclusion

LBA is a versatile ingredient that can be employed by food developers not only as a value-added component but also to assist food formulation as a humectant, antibacterial, anti-obesity chelating, stabilizer, acidulant, and moisturizing agent. It also helps to reduce dairy wastewaters to initiate a functional food products. This unique organic acid can be considered as a suitable molecule for the use as a carrier of a variety of drugs for enhanced bioavailability in drug delivery. Considerable research has been done so far using catalytic, electrochemical and biocatalytic oxidation to oxidize of the free aldehyde group of glucose on the lactose molecule to the carboxyl group for production of LBA in milk or whey. Generally, all methods are suitable for LBA synthesis, and yields of varied from 20 to 100% have been reported. However, catalyst or biocatalyst type has effect on method feasibility and the reaction yield.

# CHAPTER III

Investigate the efficiency of selected biocatalytic systems for the bioconversion of lactose and dairy by-products into lactobionic acid



## CONNECTING STATEMENT

In the literature review, presented in Chapter II, the valorization of dairy by-products, the properties of whey lactose, LBA production and LBA applications are discussed. The assessment of different biocatalytic systems to produce LBA from lactose, together with the investigation of the *in-situ* generation of oxygen, are reported in Chapter III. The application of the best identified biocatalytic systems for the conversion of lactose present in whey and milk permeates into LAB was performed. The results of the biotransformation were discussed in terms of yield and productivity.

### 3.1. Abstract

Selected biocatalytic systems (lactoYIELD-LY/ Catazyme®-Cataz; LY/Cataz/Laccase; LY/Cataz /Laccase/mediator) were investigated to bioconvert an abundant renewal resource produced by the dairy industry, lactose, into lactobionic acid. LY/Cataz/Laccase/mediator system was not affected by the decrease in the pH over the reaction time course, while the efficiency of LY/Cataz system was significantly dependent on the control of pH. The ultrasound-assisted biocatalytic systems showed similar bioconversion efficiency than the conventional systems, while the microwave-assisted systems helped to shorten the reaction time. At constant pH, the oxygen supplementation enhanced the bioconversion yield of LBA in both LY/Cataz/Laccase/mediator and LY/Cataz biocatalytic systems. LY/Cataz/Laccase/mediator system supplemented with oxygen showed a higher yield and productivity at a high lactose concentration of 200 mg/ml compared to LY/Cataz system. On the other hand, LY/Cataz system performed better in the presence of hydrogen peroxide added to generate *in-situ* oxygen by catalase. Lactose present in milk permeate was completely bioconverted to LBA in all investigated biocatalytic systems. While LY/Cataz system was identified as the most appropriate biocatalytic system for the bioconversion of whey permeate into LBA. A compromise between the yield and the productivity was achieved by using the appropriate biocatalytic system, lactose source and oxygen source. The highest productivity of 17.5 and 21.1 g/l/h was obtained with whey permeate and lactose, respectively, using LY/Cataz system at a constant pH and in the presence of 0.5% hydrogen peroxide (v/v).

## 3.2. Introduction

Carbohydrates are the amplest components that can be found in different forms. A number of hydroxyl groups is carried by carbohydrate in which each of them can be presented as a potential site for the chemical activity. Lactose, a major constituent of mammalian milk, is an important by-product of the dairy industry. For instance, whey, as a nutrient-rich by-product stream of cheese manufacturing, contains 63-80 % (w/w) lactose (Guo & Wang, 2019). However, the use of lactose is limited by its low solubility and sweetness. There is a high interest in innovative bioconversions to produce value-added lactose derivatives and achieve a circular economy (Hennig, Brosowski, & Majer, 2016; Mirabella, Castellani, & Sala, 2014; Nizami et al., 2017).

Among lactose derivatives, lactobionic acid (LBA, 4-O-b-D-galactopyranosyl-D-gluconic acid) can be obtained from the oxidation of free aldehyde group of glucose on the lactose. LBA has shown diverse functional properties, including chelating, antioxidant and humectant, making it a potent ingredient for food and pharmaceutical applications (Cardoso et al., 2019). Industrially, LBA has been mainly produced by a chemical oxidation method (Sarenkova & Ciprovica, 2018). Contrary to the chemical synthesis, the biocatalytic approaches offer additional benefits due to their high selectivity, specificity and mild reaction conditions (Alonso, Rendueles, & Díaz, 2013a; Sarenkova & Ciprovica, 2018). In addition, the ingredients produce by the environmentally-friendly biocatalytic processes can be labelled as natural ones.

Selected bi-enzymatic systems have been reported in the literature for the production of LBA, including carbohydrate oxidase/catalase (Nordkvist et al., 2007) and cellobiose dehydrogenase/laccase (J. Yang et al., 2021). Oxygen as an electron acceptor takes an important role in carbohydrate oxidase/catalase system. Laccase as a regenerating enzyme in combination with 2-2-azinobis-(3-ehtylbenzothiazoline-6-sulfonate) (ABTS) electron acceptor in cellobiose dehydrogenase/laccase system was studied by (Dhariwal, Mavrov, & Schroeder, 2006). Although the biocatalytic systems for the production of LBA reported so far led to high yields, their productivity for an efficient scale up of the production is still limited by the oxygen/substrate diffusion rate and the efficiency of the regeneration systems. Ultrasound and microwave-assisted biocatalytic reactions can generate cavitations and rapid heating of polar materials, enhancing the reaction rate and the productivity (Umego, He, Ren, Xu, & Ma, 2020; D. Wang et al., 2018). To the best of our knowledge, ultrasound and microwave-assisted biocatalytic reactions have not been

investigated to produce LAB. In addition, the continuous *in-situ* generation of oxygen may help to overcome the limited transfer and solubility of the oxygen in the reaction media. In the present study, different biocatalytic systems were compared in terms of their efficiency to produce LBA from lactose. The *in-situ* generation of oxygen by supplementing the reaction mixtures with hydrogen peroxide and catalase was also investigated. Indeed, catalase being an oxidoreductase enzyme catalyses the decomposition of hydrogen peroxide to water and oxygen. The application of the best identified biocatalytic systems for the conversion of lactose present in whey and milk permeates into LAB was performed.

## 3.3. Materials and Methods

### *3.3.1 Materials*

Lactose monohydrate powder, D- (+)-glucose, D- (+)-galactose, LBA and laccase were obtained from Sigma-Aldrich (Oakville, ON, Canada). LactoYIELD and catalase (Catazyme 25 L) were obtained from Novozymes. Whey permeate (11.55% protein, 96.02% solid, 7.57% ash, and a minimum of 76.09% lactose by weight) and milk permeate (0.25% protein, 5.65% solid, 0.6% ash, and a minimum of 4.80% lactose by weight) were obtained from Agropur cooperative. Hydrogen peroxide solution, 30 % (w/w) in $H_2O$, contains stabilizer was purchased from sigma. Formic acid, laccase from *Trametes versicolor* (LacTv), pH indicator strips and ABTS were purchased from Sigma Aldrich (St-Louis, MO). Acetonitrile and methanol HPLC grade and water LC/MS grade were purchased from Fisher Scientific (Mississauga, ON).

### *3.3.2. Bioconversion reaction*

Bioconversion of lactose to LBA was carried out in the presence and absence of oxygen over a reaction time course of 24 h. The reaction mixtures consisted of lactose at a concentration varying from 50 to 800 mg/ml and 0.02 % (w/w substrate) of each of LactoYIELD (LY) and Catazyme (Cataz) prepared in ammonium acetate buffer (25 mM, pH 5.8). For selected reaction mixtures, laccase was added at 0.01% (w/w substrate). The reactions were performed at 38°C under continuous agitation and controlled pH by adding ammonium hydroxide (2 M). In the presence of oxygen, the substrate was air aerated at flow rate of 690 mL/min. At selected reaction times, the enzymatic reactions were halted by immersing them in a boiling water-bath for 5 min. The enzymatic reactions were carried out in duplicates, along with blank trials containing all reaction components except the enzymes. LBA concentration was estimated by high pressure liquid

chromatography (HPLC) system coupled to the 6560 ion mobility (Q-TOF -MS). Bioconversion reactions were performed in triplicates. A two-way ANOVA followed by mean comparison was performed using the Tukey test. *p*-values ≤ 0.05 were considered to be statistically significant.

### *3.3.3. Ultrasound and microwave-assisted bioconversion reaction*

For ultrasonic-assisted reactions, lactose substrate was subjected to ultrasound pretreatment by immersing an ultrasound microtip probe (Kunshan Ultrasonic Instruments Co., Ltd., Suzhou, China) in its solution. The ultrasonication parameters were set as following: ultrasonic output (15 W/ml), ultrasonic intermittent ratio (3 s/7 s, working/waiting) and ultrasonication time (1 h). After ultrasonic pre-treatment, the enzymes were added to the mixture, and the reaction mixtures were incubated at 38°C under 250 rpm agitation and controlled pH at 5.8.

Microwave-assisted reactions were carried out using a Panasonic Inverter Microwave reactor. The reaction mixtures consisted of lactose solution (100 mg/ml) and selected multi-enzymatic systems in ammonium acetate buffer (25 mM, pH 5.8). The reaction mixtures were subjected to microwave irradiation power (110 and 330 W) radiation with an algorithm of 20 s on and 60 s off. During the microwave irradiation process, temperature was kept at the range from 20-35◦C for 2 h. The reaction mixtures were analysed by HPLC-MS-QTOF to quantify the produced LBA.

### *3.3.4. In-situ generation of oxygen for lactose bioconversion*

*In situ* oxygen generation was investigated by supplementing the reaction media with hydrogen peroxide and catalase. Indeed, the catalase catalysed the breaking of hydrogen peroxide into oxygen needed for the bioconversion reaction. The reaction media were prepared in 50 mM ammonium acetate buffer (pH 5.8) by mixing substrates (lactose or whey permeate) at a concentration of 100, 200 and 400 mg/ml and LZ/Cataz at 0.02% (w/w, substrate) and laccase at 0.1% (w/w, substrate) and ABTS at 2 mM. The reactions were performed by adding different concentrations of hydrogen peroxide (0.1, 0.5, 1%, v/w and 1 %gradually). The reaction mixtures were incubated for 8 h at 38 °C. Thereafter, the reactions were halted by immersing them in a boiling water-bath for 5 min, and the samples were then centrifuged (Eppendorf 5430R) at 14 000 x g for 10 min and analysed by HPLC-MS-QTOF to quantify the produced LBA.

### *3.3.5. Bioconversion of dairy by-products-based lactose into lactobionic Acid*

Whey and milk permeates were used as a lactose source. Whey permeate contains 11.5% (w/w) protein, 7.6% (w/w) ash and 76.1% (w/w) lactose. While milk permeate is composed of 5.7% (w/v) solid, 0.25% (w/v) protein, 0.6% (w/v) ash and 4.8% (w/v) lactose contents. The reaction mixtures consisted of whey permeate concentration varying from 100 to 400 mg/mL and 0.02 % (w/w substrate) of each of LY and Cataz prepared in ammonium acetate buffer (25 mM, pH 5.8). For selected reaction mixtures, laccase was added at 0.01% (w/w mg substrate). The reactions were performed at 38°C under continuous agitation and controlled pH by adding ammonium hydroxide (2 M).

### *3.3.6. Characterization of reaction mixture by LC-MS*

The reaction mixtures were analyzed by LC-MS using an Agilent 1290 Infinity II LC system coupled to the 6560 ion mobility Q-TOF -MS (Agilent Technologies, Santa Clara, USA). The LC separation was conducted on a Poreshell120 EC-C18 analytical column (Agilent Technologies; 2.7 µm × 3 mm × 100 mm) connected with a Poreshell120 EC-C18 guard column (Agilent Technologies; 2.7 µm × 3 mm × 5 mm). The mobile phase A was HPLC water with 25 mM ammonium acetate and 0.1% formic acid and the mobile phase B was methanol with 25 mM ammonium acetate and 0.1% formic acid. HPLC parameters were as follows: injection volume was 1 µL, the flow rate was 0.4 ml/min and the column temperature was set to 35°C. The mobile phase profile used for the run in negative ion mode was 97% A (0 to 2.0 min), 50% A (2.0 to 4.0 min), linear decrease to 0% A and 100% B (4.0 to 5.0 min), hold at 100% B (5.0-7.0 min), decrease to 3% B (7.0 to 7.5 min) and hold 3% B (7.5 to 9.5 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in positive ionization modes. MS conditions were as follows: for ESI-, the drying gas temperature was 150 °C, drying gas flow rate was 11 l/min, sheath gas temperature was 375°C, sheath gas flow rate was 12 L/min, the pressure on the nebulizer was 30 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 30 V and the nozzle voltage was 1000 V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 100 and 1100 at a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. Reference ions (*m/z* at 121.0508 and 922.0098 for ESI-) were used for automatic mass recalibration of each acquired spectrum. Data treatment was conducted using Quantitative Analysis B.07.01 from Agilent MassHunter Workstation Software.

#### *3.3.7. Statistical analysis*

Statistical analyses were performed using XLSTAT software (Addinsoft, New York, NY, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$).

### 3.4. Results and discussion

#### *3.4.1. Effect of type of biocatalytic systems on lactobionic acid production*

Selected biocatalytic systems (LY/Cataz; LY/Cataz/Laccase; LY/Cataz/Laccase/mediator) were investigated for the bioconversion of lactose into LBA. LactoYIELD® (LY) expresses a cellobiose oxidase (EC 1.1.99.18) activity that catalyzes the oxidation of lactose into LBA; while Catazyme® (Cataz) is a catalase (EC 1.11.1.6) that catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen. The addition of laccase mediated by ABTS to the combined LY/Cataz bienzymatic system was also investigated. Laccase is a multi-copper oxido-reductase enzyme (EC 1.10.3.2) that catalyzes the oxidation of a wide range of inorganic and organic substances by using oxygen as an electron acceptor. The use of redox mediators can promote the formation of stable radicals that oxidize other molecules not directly oxidized by the enzyme itself (M. Li, Liu, Kermasha, & Karboune, 2021).

Figure 1 shows that the MS-MS spectrum of the main product of the bioconversion reaction corresponds to LBA with molecular ions appearing at m/z 358.11112613. The fragmented ions of LBA appeared at m/z 183.6979,177.0408, 159.0301, 130.0212, 113.4926 and 99.0089. Similarly, Farouk and Azzazy (2020) reported similar fragmented pattern of LBA. In addition, molecular feature extraction was performed on the end-product profile of reaction mixtures using the "Find by Molecular Feature" function for the nontargeted approach. For the bioconversion reactions that were carried out in the absence of oxygen, additional minor peaks were identified and corresponded to residual lactose, gluconic acid, sorbitol and lactic acid. However, lactobiono-δ-lactone, which is an intermediate compound in LBA production was observed, when the bioconversion reactions were run in the presence of oxygen or hydrogen peroxide.

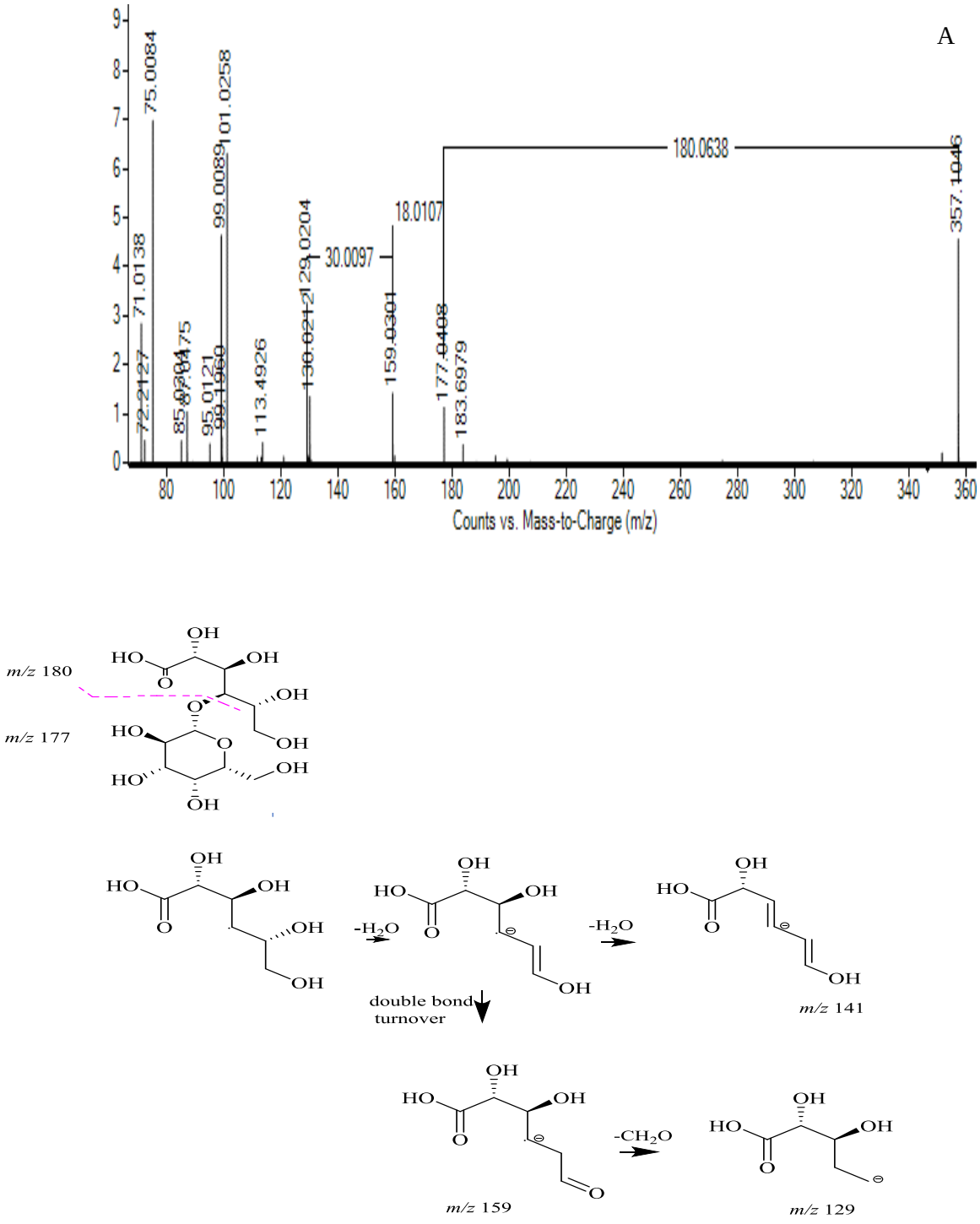



**Figure 3.1.** A. MS/MS fragmentation mass spectrum for lactobionic acid (LBA, $C_{12}H_{22}O_{12}$), eluted at retention time of 3.0 min, in a negative ion mode and with a collision energy of 20 V. B. a proposed fragmentation pattern of LBA

Table 3.1 shows the bioconversion yields at selected lactose concentrations and reaction times, with/without pH control. The highest bioconversion yields of 94-98% were achieved using the LY/Cataz/Laccase/mediator biocatalytic system at lactose concentrations of 50 and 100 mg/ml. No production of LBA was obtained in the laccase/mediator biocatalytic system, revealing the contribution of laccase/mediator in the propagation phase of the oxidation reaction and not the initiation phase. In addition, the results also reveal that the use of LY/Cataz/laccase biocatalytic system without mediator led to a similar bioconversion yield than that of LY/Cataz/Laccase/mediator biocatalytic system after 8 h reaction at a low lactose concentration of 50 mg/ml; however, at higher lactose concentrations of 100 and 150 mg/ml, lower bioconversion yields were obtained in the LY/Cataz/laccase biocatalytic system without mediator. Increasing the reaction time to 24h seems to be more beneficial for LY/Cataz/Laccase/mediator biocatalytic system (up to 60 % increases) than LY/Cataz/laccase one (up to 34% increases).

The results (Table 3.1) also indicate that increasing the lactose concentration to 150 mg/ml led to a significant decrease in the bioconversion yield in all biocatalytic systems. At this highest lactose concentration of 150 mg/ml, the maximum bioconversion yield of 64% was achieved in the LY/Cataz biocatalytic system with pH control after 24 h reaction. Such effect may be attributed to substrate/product inhibition and/or to the limited availability of oxygen. Similar results were reported by Kim et al. (2020) in which the LBA yield decreased from 80 to 48%, when the initial lactose concentration was increased from 50 to 95 g/l. As the reaction was proceeded, the production of LBA contributed to a decrease in the pH of the reaction medium, affecting the catalytic efficiency of the enzymes. Indeed, LactoYIELD (LY) was reported to exhibit a maximum activity in the pH range of 4 to 9, while Catazyme® (Cataz) showed a broad optimum pH range of 4 to 8.3 (Röcker, Schmitt, Pasch, Ebert, & Grossmann, 2016). In addition, the limited stability of biocatalysts under acidic conditions can also affect their catalytic efficiency in the production of LAB. Contrary to other biocatalytic systems, LY/Cataz/Laccase/mediator system was not significantly affected by the pH decrease (Table 3.1). Indeed, bioconversion yields of 29 to 80% and 28 to 98% were obtained in LY/Cataz/Laccase/mediator system, upon 24h of reaction, without and with pH control, respectively. These results may reveal the dominance of the non-enzymatic oxidation reaction in the LY/Cataz/Laccase/mediator system, which can be attributed to the generation of stable radicals in the presence of laccase and ABTS mediator.

**Table 3.1.** Effect of type of biocatalytic systems on lactobionic acid (LBA) production yield and productivity

| | Initial lactose concentration | | | | | |
|---|---|---|---|---|---|---|
| | 50 mg/ml | | 100 mg/ml | | 150 mg/ml | |
| Biocatalyst Systems | yield (%) | productivity (g/l.h) | yield (%) | productivity (g/l.h) | yield (%) | productivity (g/l.h) |
| LY/Cataz[1a] | 42.0±8.2 $^{B}$ | 2.6 | 37.7±4.3$^{BC}$ | 4.7 | 12.0±3.0 $^{DE}$ | 2.3 |
| LY/Cataz/Laccase [1a] | 17.8±0.4 $^{EF}$ | 1.1 | 8.9±0.4 $^{DE}$ | 1.1 | 5.1±0.9 $^{EF}$ | 1.0 |
| LY/Cataz/Laccase/mediator [1a] | 18.2±1.2 $^{EF}$ | 1.1 | 24.1±2.8 $^{CD}$ | 3.0 | 11.1±2.9 $^{EF}$ | 2.0 |
| Laccase/mediator [1a] | 0.1±0.0 $^{F}$ | 0.0 | 0.1±0.0 $^{E}$ | 0.4 | 0.1±0.0 $^{F}$ | 0.4 |
| LY/Cataz/Laccase/mediator [1b] | 76.2±4.7 $^{B}$ | 5.9 | 79.9± 7.2 $^{A}$ | 8.0 | 29.4±15.7 $^{C}$ | 5.4 |
| LY/Cataz [2a] | 53.9±3.3 $^{CD}$ | 3.5 | 50.1±3.8 $^{B}$ | 6.3 | 45.7±6.3 $^{B}$ | 7.8 |
| LY/Cataz [2b] | 73.2±4.7 $^{BC}$ | 5.0 | 84.3±14.1 $^{A}$ | 72.8 | 63.7±4.7 $^{A}$ | 7.2 |
| LY/Cataz/Laccase/mediator [2a] | 30.0±8.5 $^{DE}$ | 1.9 | 38.5±1.5 $^{B}$ | 4.8 | 23.3±3.5 $^{CD}$ | 4.4 |
| LY/Cataz/Laccase/mediator [2b] | 98.0±2.7 $^{A}$ | 6.1 | 97.6±1.6 $^{A}$ | 12.1 | 27.8±1.1 $^{C}$ | 4.1 |

[1a] no O2/no pH control/8 h

[1b] no O2/no pH control/24 h

[2a] no O2/ pH control/8 h

[2b] no O2/ pH control/24 h

[A,F] Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

The results also show that in the LY/Cataz biocatalytic system, the effect of the pH decrease on the bioconversion yield increased with increasing substrate concentration and reaction time; indeed, higher bioconversion yields of 41 to 56% were obtained under a controlled pH at 8h reaction as compared to the non-controlled pH conditions (12 to 45%). The pH control improved the efficiency of the LY/Cataz biocatalytic system.

### *3.4.2. Ultrasound and microwave-assisted bioconversion reaction*

Ultrasound and microwave-assisted approaches have been studied as alternatives to the conventional methods as they can allow substrate activation, mass transfer acceleration during bioconversion, heat generation, high purity of products, less side-products and shortening of reaction time (Rico-Rodríguez et al., 2020; Zarei, Golmakani, Keramat, Majdinasab, & Karami, 2021). Indeed, poor solubility of lactose and the non-homogenous distribution of oxygen can limit the bioconversion of lactose into LBA.

It is known that ultrasonic waves at frequencies of 20 kHz to 3 MHz can generate cavitation (e.g' ultrasonic waves) known for their ultrasound's physical and chemical effects (W. Li & Ashokkumar, 2018). The effect of ultrasound pre-treatment of lactose on the bioconversion yield is shown in Figure 3.2. The results (Table 3.1; Fig. 3.2) indicate no significant difference ($p<0.05$) in the bioconversion yields between the conventional method and ultrasound-assisted biocatalytic systems. Rico-Rodriguez et al. (2020) reported that the ultrasound assistance had no effect on the galactooligosaccharide production by β-galactosidase, but it had a positive effect on the oxidation of glucose to gluconic acid by glucose oxidase in the bienzymatic β-galactosidase/glucose oxidase system (Rico-Rodríguez et al., 2020). On the other hand, Demirhan and Özbek (2009) have found that the ultrasound assistance enhanced the hydrolysis of lactose by β-galactosidase at an acoustic power of 20 W, while increasing the acoustic power from 20 to 100 W resulted in a decrease in the lactose hydrolysis extent (Demirhan & Özbek, 2009).

Figure 3.1 also shows that the supplementation of the reaction mixture with oxygen significantly increased the bioconversion yield of LBA in both biocatalytic systems (LY/Cataz/Laccase/mediator; LY/Cataz). Higher bioconversion yield of 52.3 (%) and productivity 13.2 (g/l.h) was achieved at higher concentration of lactose (200 mg/ml), when LY/Cataz/Laccase/mediator biocatalytic system was used.

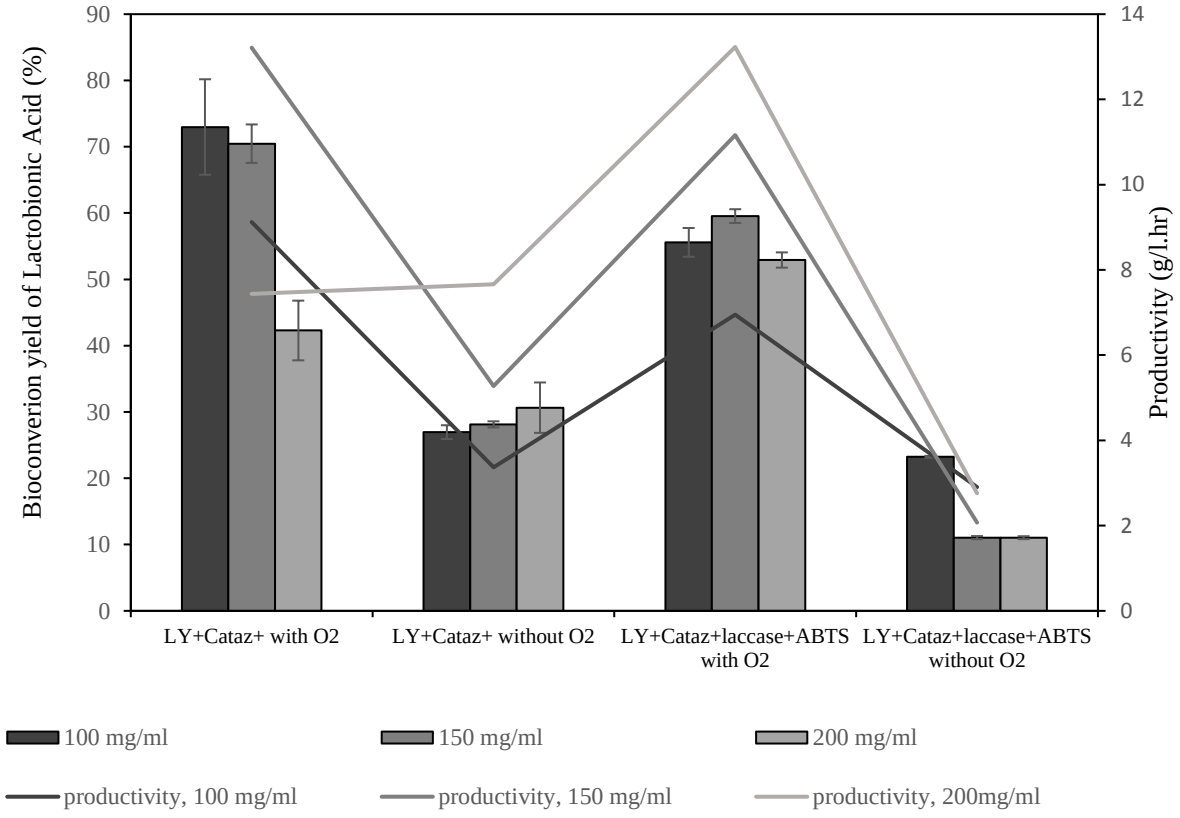



**Figure 3.2.** Effect of ultrasound pre-treatment of lactose on the bioconversion yield and the productivity of selected biocatalytic systems after 8h reaction time.

On the other hand, in the LY/Cataz biocatalytic system, higher bioconversion yield of 70.5 % and productivity of 13.2 g/l.h were obtained at the lactose concentration of 150 mg/ml; increasing lactose concentration to 200 mg/ml led to a decrease in the bioconversion yield to 43% and productivity to 7.4 g/l.h. At high substrate concentrations, the interactions between substrate-enzyme may be limited by the availability of substrates at the enzyme's microenvironment, by the competing behavior of substrates/products at the enzyme active site and by the presence of intermolecular interactions between the reaction components (Couto, Karboune, & Mathew, 2010; Karam, Karboune, St-Louis, & Kermasha, 2009; Karboune, Neufeld, & Kermasha, 2005). In addition, the use of multiple enzymes in the reaction biocatalytic system can affect the catalytic properties and the interactive behaviors of nanocomponent enzymes toward substrates, resulting in different reaction outcomes (Liu, 2017). As compared to LY/Cataz biocatalytic system, the high efficiency of LY/Cataz/Laccase/mediator system at a high lactose concentration of 200 mg/ml can be attributed to the generation of high number of stable radicals, enhancing the extension of the oxidation reaction.

The microwave-assisted bioconversion was evaluated for its ability to shorten the reaction time and accelerate the reaction rate. Table 3.2 shows that the yields of LBA were low in the absence of oxygen. Increasing the microwave power from 110 to 330 W affected the bioconversion yield. This result may be attributed to the enzyme inactivation by the thermal effect of microwaves. Such effect was limited by the use of non-aqueous media in which enzyme may exhibit a high thermal stability (Réjasse, Besson, Legoy, & Lamare, 2006; H. Zhao, 2010). In the present study, the use of acetonitrile/buffer (20/80, v/v) as a reaction medium didn't enhance the yield and the productivity of LBA produced in the LY/Cataz biocatalytic system. The results also show that the microwave-assisted bioconversion of lactose at 100 mg/ml in the presence of oxygen led to a yield of 44% with productivity of 11 g/l.h after 2hr of reaction in the LY/Cataz biocatalytic system. The use of conventional method at the same conditions resulted in a yield of 73% with the productivity of 3.4 g/l.h (Fig. 3.1). High productivity can be achieved using microwave-assisted bioconversion compared to the conventional method. As far as the authors are aware, the microwave-assisted bioconversion of lactose into LBA was not reported so far. Microwave-assisted approach was investigated for the isomerization of lactose to lactulose in alkaline solution, resulting in 11% of conversion upon 50 or 60 s of treatment (Nooshkam & Madadlou, 2016).

**Table 3.2.** Effect of microwave irradiation on lactobionic acid (LBA) production yield and productivity

| | Initial lactose concentration | | | | | |
|---|---|---|---|---|---|---|
| | 50 mg/ml | | 100 mg/ml | | 150 mg/ml | |
| Biocatalyst System | yield (%) | productivity (g/l.h) | yield (%) | productivity (g/l.h) | yield (%) | productivity (g/l.h) |
| LY/Cataz [3a] | 7.4±0.1 [C] | 1.9 | 3.9±0.1 [B] | 2.0 | 3.4±0.1 [B] | 2.6 |
| LY/Cataz/Laccase/mediator[3a] | 6.4±0.4 [C] | 2.1 | 2.2±0.2 [BC] | 1.8 | 0.1±0.0 [D] | 1.8 |
| LY/Cataz/Laccase/mediator[3b] | 0.8±0.0 [D] | 0.2 | 0.3±0.1 [D] | 0.2 | 0.2±0.0 [D] | 0.2 |
| LY/Cataz[3b] | 0.9±0.0 [D] | 0.2 | 0.3±0.0 [D] | 0.2 | 0.1±0.1 [D] | 0.1 |
| LY/Cataz[3c] | 5.6±0.1 [C] | 1.4 | 2.4±0.1[B] | 1.2 | 1.6±0.1 [BC] | 1.2 |
| LY/Cataz[3d] | 44±0.4 [A] | 11.0 | 21.1±0.1 [A] | 10.5 | 13.4±0.1 [A] | 10.1 |

[3a] no O2/ pH control/2 h/microwave/p:110 W, time on/off: 20s/60s

[3b] no O2/ pH control/2 h/microwave/p:330 W, time on/off: 20s/60s

[3c] no O2/ pH control/20 minutes/microwave/ACN:water (20:80(v/v))/p:110 W

[3d] supplementation with O2/ pH control/2 h/microwave/p:110 W, time on/off: 60s/5min

A,D Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

Microwave assistance has been used in different enzymatic reactions, such as protease- (subtilisin Carlsberg and a-chymotrypsin) and lipase-catalyzed esterification and transesterification (Roy & Gupta, 2003; Yadav & Lathi, 2005) reactions. Recently, it has been reported that the microwave heating could affect the amino acid fluctuations in the aglycone subsites of xylanase, reducing the formation of xylose and increasing the yield of xylooligosaccharides in the microwave-assisted reactions compared to conventional heating (Mobarec, Villagomez, Karlsson, & Linares-Pastén, 2021).

### *3.4.3. In-situ generation of oxygen for the bioconversion of lactose into lactobionic acid*

LactoYIELD contains cellobiose oxidase that oxidizes the anomeric carbon of β-D-glucose using molecular oxygen as an electron acceptor producing hydrogen peroxide and D-glucono-δ-lactone, which in the presence of water spontaneously hydrolyzes to LBA. Contrary to cellobiose oxidase, cellobiose dehydrogenase requires a cofactor as an electron acceptor and regenerative enzyme. In the present study, the reaction-catalyzed by cellobiose oxidase was continuously supplemented by the oxygen through the injection of the air. Since oxygen solubility is poor in water (approx. 8.3 mg/l at 25 °C), the mass transfer of oxygen from gas phase to liquid phase can limit the reaction rate. In addition, the area of the gas−liquid interface is equivalent to the inner surface area of the tube in contact with the reaction media (J. F. Chen, Chen, Wang, Shao, & Li, 2011). Furthermore, it has been reported that carbohydrate oxidase can be deactivated by direct exposure to gas-liquid interfaces (Bhagia, Dhir, Kumar, & Wyman, 2018; Lou et al., 2018). Therefore, the *in situ* generation of oxygen by the supplementation of reaction mixtures with hydrogen peroxide that can decompose exothermically into oxygen and water in the presence of catalysts (manganese dioxide, potassium permanganate, and silver) or enzyme such as catalase can be a potential strategy (Nooshkam & Madadlou, 2016). The chemical decomposition of hydrogen peroxide requires alkaline conditions and suitably elevated temperatures of about 50°C or more. These conditions are not appropriate for the enzymatic oxidation reaction catalysed by cellobiose hydrolase. In the present study, catalase enzyme was used to decompose the hydrogen peroxide into molecular oxygen and water in the fed-batch procedure (Equation 3.1, 3.2) (García-García, Rocha-Martin, Fernandez-Lorente, & Guisan, 2018). The heme-containing catalase, first, bonds with a molecule of hydrogen peroxide and break it up into water and oxygen atom, which subsequently joined to

the iron atom present in the heme. At the next step, the second oxygen atom is attached to the iron, the molecules of water and oxygen gas are released.

$$H_2O_2 + Fe^{III}\text{-enzyme} \longrightarrow H_2O + O{=}Fe^{IV}\text{-enzyme} \quad (3.1)$$

$$H_2O_2 + O{=}Fe^{IV}\text{-enzyme} \longrightarrow H_2O + O_2 + Fe^{III}\text{-enzyme} \quad (3.2)$$

In addition, it has been shown that the rate of conversion of lactose to LBA by carbohydrate oxidase increased in the presence of catalase (Zappi, White, Hwang, Bajpai, & Qasim, 2000). Figure 3.2 shows the bioconversion yield and the productivity in the presence of oxygen (air supplementation at 690 ml/min) and hydrogen peroxide (from 0.1 to 1%) as well as in the absence of oxygen. Generally, substrate oxidation by natura enzymes is occurred by two ways: *(i)* oxygenation oxidation, that oxygen serves as an oxidant, and *(ii)* dehydrogenation oxidation that protons and electrons are eliminated from the substrate and transferred to an electron acceptor (Huang & Groves, 2017). Glucose oxidase has shown an ability to transfer electrons not only from glucose to oxygen but also to other electron receptors, such as ferrocene (Fc) and ABTS+• (Trifonov et al., 2013). As far as the authors are aware, it is unknown if lactose oxidase can transfer the electron from lactose to other electron receptors than oxygen (Lin, Li, & Chung, 2020). The results (Fig. 3.3) show that the presence of oxygen significantly enhanced the bioconversion yield and the productivity of LBA production in the LY/Cataz/Laccase/mediator biocatalytic system when laccase and ABTS mediator were added to the reaction mixtures after 3 h. It has been reported that the substrate specificity of laccase can be extended to include nonphenolic compounds in the presence of ABTS that acts as a co-oxidant (Collins, Dobson, & Field, 1998). Indeed, ABTS is oxidized by laccase to form a cation radical ($ABTS^+$), which can be efficiently reduced back to ABTS in the presence of organic acids, resulting in the formation of $O_2^-$ as an intermediate of hydrogen peroxide formation. It can be hypothesized that adding laccase and ABTS after the initiation of the oxidation reaction of lactose by cellobiose oxidase (LactoYIELD) may have favored the redox cycle of $ABTS^+$/ABTS, resulting in the formation of a pool of $O_2^-$, which may have promoted the oxidation of lactose in LBA. On the other hand, Potthast et al. (1996) have proposed another co-oxidant role of ABTS in which ABTS activates the laccase by transferring one electron to the enzyme, hence initiating the ability of enzyme to proceed with an electron transfer from the substrate to dioxygen in a two-electron transfer process (Potthast, Rosenau, Chen, & Gratzl, 1996).

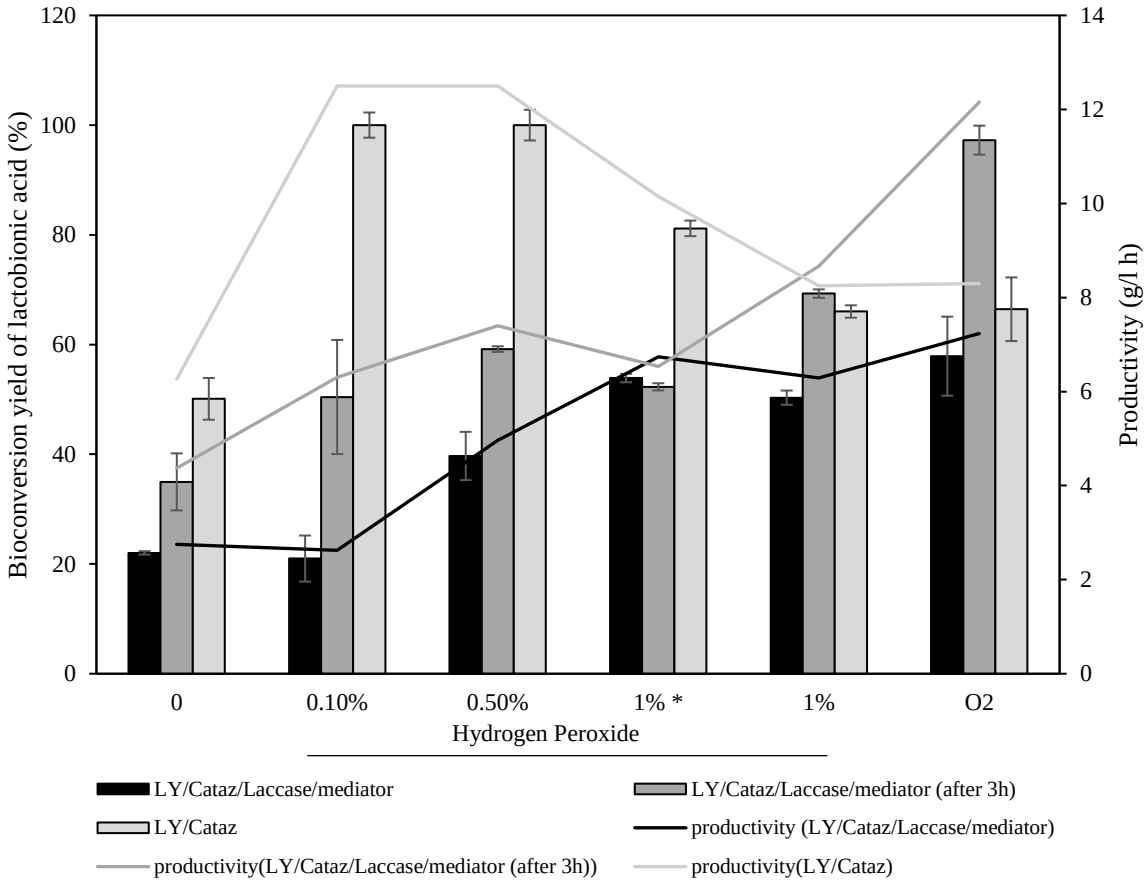



**Figure. 3.3.** The effects of different sources of oxygen on the yield and the productivity of bioconversion using selected biocatalytic systems *(i)* LY/Cataz/ laccase/ABTS *(ii)* LY/Cataz/ when laccase and mediator added after 3 h, *(iii)* LY/Cataz. Supplementation of the media with air was done at 690 ml/min. The *in-situ* generation of oxygen by catazyme was assessed at selected hydrogen peroxide concentrations of 0.1%, 0.5,1% (v/v).

1%*: Hydrogen peroxide was sequentially added to the reaction media (5 times) over the reaction time course.

The addition of hydrogen peroxide to generate *in-situ* oxygen by catalase was investigated over a broad range of 0.1 to 1% (v/w). The complete bioconversion of lactose into LBA was achieved when 0.1 and 0.5% (v/w) of hydrogen peroxide were added to the reaction mixture of the LY/Cataz biocatalytic system (Fig. 3.2). Similarly, Chapman et al. (2018) reported an increase in the productivity of galactose oxidase up to 5-fold in a tubular microreactor upon the addition of hydrogen peroxide (Chapman, Cosgrove, Turner, Kapur, & Blacker, 2018). Increasing the hydrogen peroxide to 1% in this LY/Cataz biocatalytic system resulted in a decrease in the bioconversion yield to 66%; this can be attributed to the inhibitory effect of hydrogen peroxide on the enzyme activity. Such inhibitory effect was reduced when 1% hydrogen peroxide was gradually added in the LY/Cataz biocatalytic system (yield of 81%). It has been reported that hydrogen peroxide can modify the peptide core of an enzyme and adversely affect its catalytic activity or stability (Hernandez, Berenguer-Murcia, C Rodrigues, & Fernandez-Lafuente, 2012).

Contrary to LY/Cataz system, LY/Cataz/Laccase/mediator system led to a higher bioconversion yield of 58% and productivity of 7.24 g/l.h, when the reaction mixture was supplemented with oxygen than with hydrogen peroxide. In addition, the bioconversion yield and the productivity of LBA in the LY/Cataz/Laccase/mediator system increased with the increase in the hydrogen peroxide concentration to achieve 54% and 6.74 g/l.h, respectively at 1% (v/v). These results reveal the low catalytic efficiency of Cataz in converting hydrogen peroxide into oxygen in the presence of laccase and ABTS mediator. Indeed, an increase in the bioconversion yield to 66% and the productivity of LBA to 8.66 g/l.h was observed when hydrogen peroxide (1%, v/v) was added to the LY/Cataz/Laccase/mediator system after 3 h of reaction. These results reveal the non-synergistic interactions between the $O_2^-$ generation upon $ABTS^+$/ABTS redox cycle and the $O_2$ release upon the breakdown of hydrogen peroxide by catalase in the LY/Cataz/Laccase/mediator system.

### *3.4.4. Bioconversion of dairy by-products into lactobionic acid*

Whey and milk permeate were investigated as the starting materials for the bioconversion of dairy by-products into LBA. Whey permeate contains 76.1% (w/w) lactose, 11.5% (w/w) protein and 7.6% (w/w) ash and, whereas milk permeate is composed of 4.8% (w/v) lactose, 0.25% (w/v) protein and 0.6% (w/v) ash. LY/Cataz and LY/Cataz/Laccase/mediator systems were used as a biocatalytic system. For LY/Cataz/Laccase/mediator system, the effect of the addition of

laccase/mediator after the initial stage of reaction (3h) was also assessed. Figure 3.3. shows the yield and the productivity of the bioconversion of whey (100 mg/ml lactose) and milk (48 mg/ml) permeate into LBA. It is important to note that the milk permeate was used as reaction medium and substrate source. The biocatalytic systems were supplemented by air at 690 ml/min.

The results show that lactose present in the milk permeate was completely bioconverted into LBA in all investigated biocatalytic systems. The productivity of the bioconversion of milk permeate into LBA was estimated at 5.93 g/l.h. Contrary to milk permeate, the yield and the productivity of the bioconversion of whey permeate into LBA were dependent on the type of biocatalytic system. The addition of laccase/mediator in the LY/Cataz system led to a decrease in the yield from 100% to 77.8% and in the productivity from 12.5 to 9.7g/l.h. The results (Fig 3.4) also indicate that the addition of laccase/mediator after the initial stage of the reaction didn't improve the yield and the productivity of the bioconversion of whey permeate into LBA. Contrary to whey permeate, the highest yield and the productivity of the bioconversion of lactose into LBA in the presence of oxygen were achieved upon the use of LY/Cataz/Laccase/mediator (after 3 h) system. The different behaviors of lactose and whey permeate may be attributed to the presence of protein and ash in the latter starting material. Indeed, laccase can oxidize selected amino acid (cysteine, tyrosine) residues of proteins by removal of one electron resulting in the formation of free radicals, which can undergo further nonenzymatic reactions including disproportionation, polymerization, and fragmentation. Such reaction may have occurred in the presence of whey permeate containing proteins; and it may have affected the catalytic behavior of laccase and the co-oxidant role of mediator in the oxidation reaction of lactose into LBA. The aggregation of enzymes in the presence of laccase and whey permeate may also explain the lower yield in the LY/Cataz/Laccase/mediator system. Indeed, it has been reported that the irreversible aggregation of the proteins at the air–liquid interface may limit the production of LBA in the cellobiose dehydrogenase/laccase biocatalytic system ((Ludwig et al., 2004).

The productivity of the bioconversion process is highly dependent on the initial concentration of substrate. The effect of substrate concentration on the bioconversion yield and productivity was investigated using lactose and whey permeate as a starting material (Fig. 3.4B). The reaction mixtures were supplemented with the hydrogen peroxide (0.5%, v/v) to generate *in-situ* the oxygen by catalase.

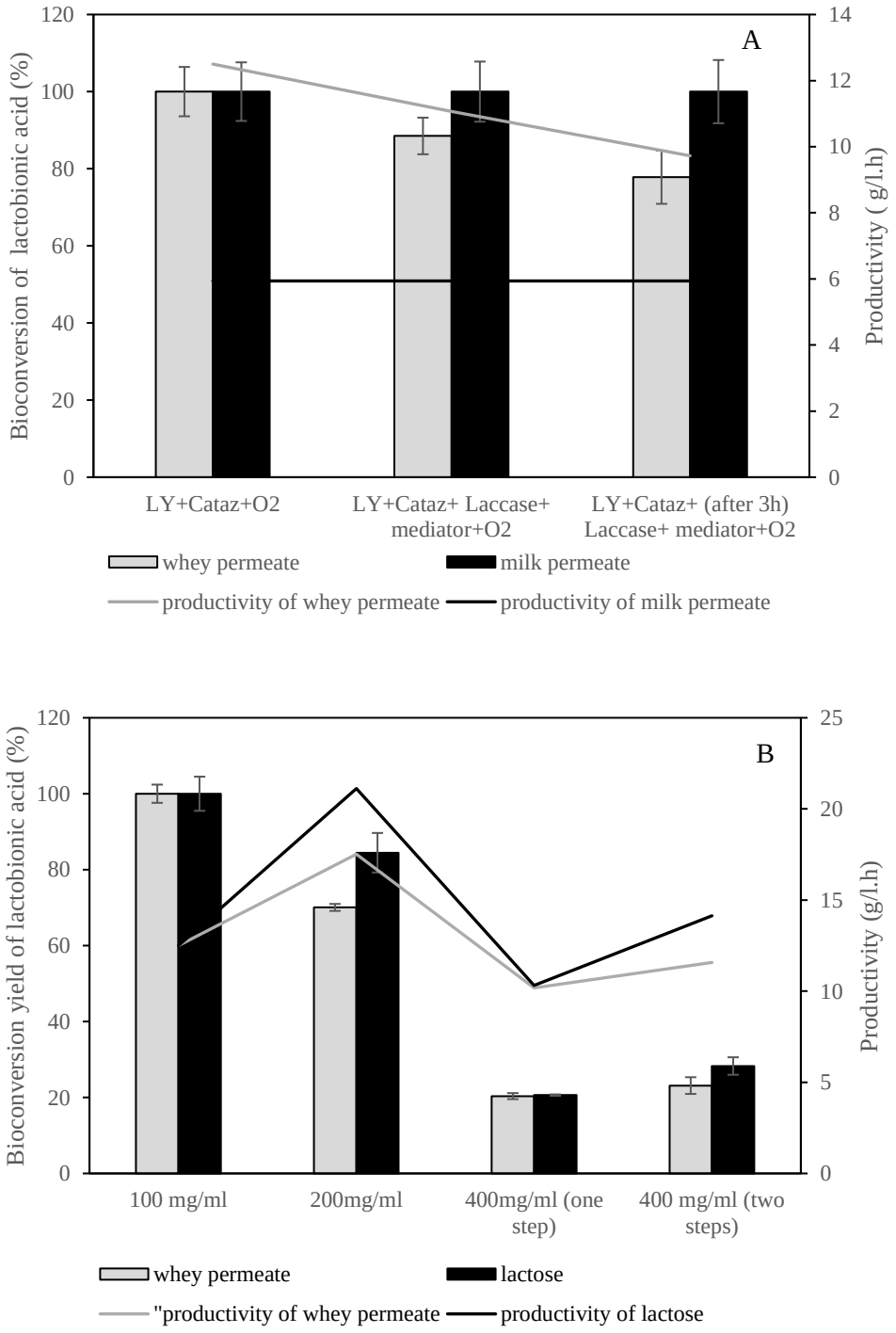



**Figure. 3.4**. **A**. Yield and productivity of the bioconversion of whey and milk permeate into LBA in the selected biocatalytic systems supplemented with air (690 ml/min) at constant pH (5.8) and temperature (38°C). **B.** Yield and productivity of the bioconversion of lactose and whey permeate into LBA in the LY/Cataz system supplemented with hydrogen peroxide (0.55, v/v) at constant pH (5.8) and temperature (38°C).

Figure 3.4B shows that increasing whey permeate and lactose concentration from 100 to 200 mg/ml led to a decrease in the bioconversion yield from 100% to 70 and 84%, respectively; while the productivity increased from 12.5 g/l.h to 17.5 and 21.1 g/l.h. Further increase of the substrate concentration to 400 mg/ml resulted in a significant decrease in the yield to 20% and no increase in the productivity was observed. The low bioconversion yield at high substrate concentration may be attributed to the high viscosity of reaction mixture and/or to the inhibitory substrate effect ((M. Wang et al., 2015). Nordkvist et al. (2007) have found that the inhomogeneities in pH caused by non-efficient mixing resulted in carbohydrate oxidase deactivation, and the high lactose concentration up to 200mg/ml provided a protective effect against deactivation by base.

To minimize the substrate/product inhibition, fed-batch system, in which the substrate was used in two steps, was investigated. It has been reported that the fed batch can also avoid unwanted parallel reactions and favor the desired reactions (Guajardo, Schrebler, & de María, 2019; Kasmi, 2018). The enzymatic production of lactulose was found to be 10 times higher using repeated fed-batch process (Ramírez et al., 2021). Figure 3.3B. shows that the bioconversion yields (23.4-28.3%) were slightly higher compared to the conventional batch process.

A summary of the efficiency of biocatalytic processes for the LBA production is given in Table 3.3. Comparison of the results reported in literature with the present study show that adding hydrogen peroxide as a source for oxygen was beneficial for the bioconversion of lactose into LBA. Our study has shown the highest yield and productivity for the bioconversion of whey permeate into LBA.

**Table 3.3**. A summary of performance of LBA production process studied by several authors using dairy-by products

| Substrate | Biocatalytic systems | Substrate concentration/$O_2$ Source | Bioconversion Yield/Productivity | References |
|---|---|---|---|---|
| Lactose | CDH/lac/mediator [a] | 100 mg/ml | 100%, 7.14 g/l h | (J. Yang et al., 2021) |
| Lactose | carbohydrate oxidase/Catal | 50/100/200 mg/ml/ air | | |
| Whey permeate powder | carbohydrate oxidase/cataz | 6% | >90% | (Budtz, Vindelov, Nielsen, Ashie, & Nordkvist, 2007) |
| Lactose/Fructose | GFOR/GL [b] | 239 mg/ml, 108 mg/ml | 70%, 6.9 g/l h | (Carra et al., 2020) |
| Lactose/Fructose | GFOR/GL [b] | 65-0130 mg/ml | 85%, 5.6 g/l h | (da Silva et al., 2011) |
| Lactose | GDH [c] from *P. taetrolens*/ air | 200 g/l | 8.70 g/l h | (Oh, Jang, Lee, et al., 2020) |
| Lactose | CDH/lac | 10 mg/ml | 95-98%, 3.1 g/l h | (Van Hecke et al., 2011) |
| Lactose | MQO, EC 1.1.5.4 [d] | 200 mg/ml | 100%, 0.62 g/l h, | (Oh, Jang, Hong, & Eom, 2020) |
| Lactose | CDH-3-HAA-lac [e] | 205 mg/ml | 96%, 3.1 g/l h | (Q Tian et al., 2018) |
| Lactose | CDH/lac | 68 mg/ml | 100%, 3.0-21.0 g/l h | (Ludwig et al., 2004) |
| Lactose | CDH/lac | 17 mg/ml | 100%, 7.2-27 g/l h | (Baminger et al., 2001) |
| Lactose | Carbohydrate oxidase/Cataz | 50-100 mg/ml, $O_2$ | 98% | (Nordkvist et al., 2007) |
| Lactose and Whey permeate | LOX/ Catal [f] | 50 mg/ml, air | 98% | (Hua et al., 2007) |
| Lactose | LY/Cataz | 100/150/ 200 mg/ml / air | 42-79%, 7.6-13.2 g/l h | The present study |
| Lactose | LY/Cataz/lac/ABTS | 100/150/ 200 mg/ml / air | 59.4-55.6%, 6.9-13.2 g/l h | The present study |
| Whey permeate | LY/Cataz | 100/200/ 400 mg/ml / $H_2O_2$ | 23-100%, 11.6-17.5 g/l h | The present study |
| Lactose | LY/Cataz/lac/ABTS | 100/200/ 400 mg/ml / $H_2O_2$ | 28.3-100%, 14.4-21.1 g/l h | The present study |

[a] cellobiose dehydrogenase / laccase; [b] glucose–fructose oxidoreductase /glucono-δ-lactonase; [c] glucose dehydrogenase; [d] malate: quinone oxidoreductase; [e] cellobiose dehydrogenase/ 3-Hydroxyanthranilic acid/ laccase; [f] lactose oxidase/catalase.

### 3.5. Conclusion

Although develop and transforming process for producing high-value derivatives from dairy by-products has always been an exciting contribution in dairy industry, the design of new method for utilization of whey has always been a challenge. LBA and its salts as added-value food materials have drawn the attention because of the taste, solubility and the health promoting functions that can be used in the food, pharmaceutical and chemical industries. LBA was produced by selected biocatalytic systems in the presence or the absence of oxygen to better understand the oxygen limitation influences on LBA production. LY/Cataz/Laccase/mediator system was not significantly affected by the pH decrease. The *in-situ* generation of oxygen upon the supplementation of reaction mixtures by hydrogen peroxide was successfully achieved. LY/Cataz system showed a high bioconversion yield and productivity of LBA in the presence of hydrogen peroxide. While LY/Cataz/Laccase/mediator system performed better in the presence of oxygen and when laccase/mediator were added at the intermediate stage of the rection. The application of ultrasound did not affect the bioconversion yield and productivity; however, microwave assistance enhanced the productivity and shortened the reaction time. All investigated lactose sources (lactose, whey permeate, milk permeate) were shown to be suitable for LBA production. A compromise between the yield and the productivity was achieved upon the use of appropriate biocatalytic system, oxygen source and substrate concentration.

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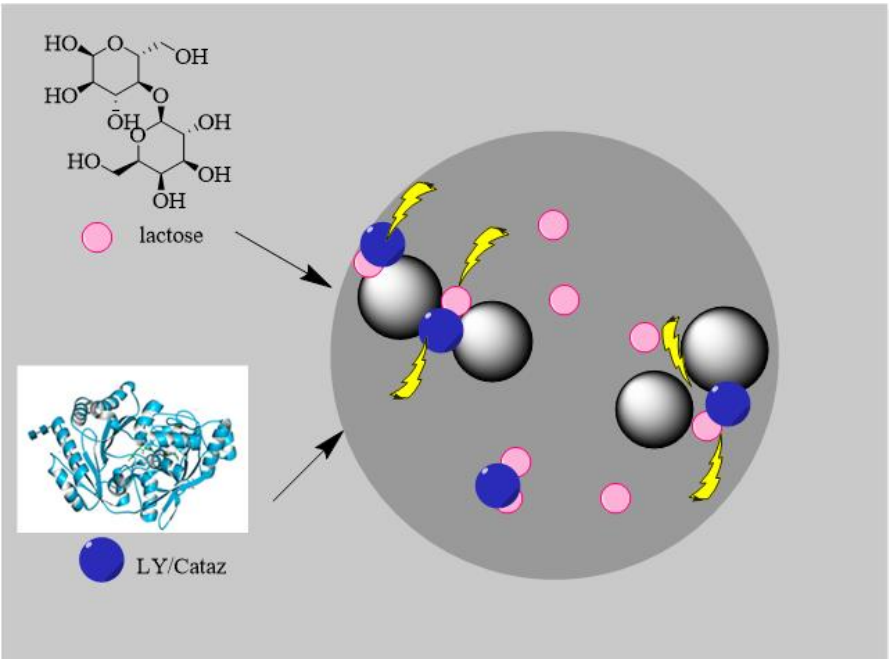
# CHAPTER IV

Investigation and Optimization of the Mechano-Assisted Bioconversion of Lactose and Whey Permeate into LBA



## CONNECTING STATEMENT

In Chapter III, different biocatalytic systems were assessed for their ability to produce LBA from lactose. The best identified biocatalytic systems were applied for the bioconversion of lactose present in whey and milk permeates into LAB. A compromise between the yield and the productivity was achieved by using the appropriate biocatalytic system, lactose source and oxygen source. In light of our findings, the study presented in chapter IV focused on the investigation of the enzymatic production of LBA, from lactose and whey permeate, assisted by mechanochemistry via mechano-ball milling at a broad range of substrate concentration. The addition of hydrogen peroxide for the *in-situ* generation of oxygen in the mechano- milling assisted biocatalytic system was also evaluated. The study of the effects of reaction parameters of the mechano-milling-assisted biocatalytic system provided an understanding of their interactions and their ability to maximize the productivity.

## 4.1 Abstract

The assistance of the mechanochemistry *via* mechano-ball milling for the enzymatic bioconversion of lactose and whey permeate into lactobionic acid (LBA) was investigated for the first time in the present study. The combined LactoYIELD-LY/ Catazyme®-Cataz was used as the selected biocatalytic system, which was supplemented by hydrogen peroxide to generate *in situ* oxygen. As expected, the highest bioconversion yields were obtained at low substrate concentrations. When lactose or whey permeate concentration was increased from 100 to 800 mg/ml, the productivity increased to reach 30.1-38.9 and 8.4-12.7g/l.h, respectively. The other components of whey permeate were found to have an inhibitory effect on the efficiency of the biocatalytic system. In addition, polyethylene glycol exhibited a protective effect, which was significant in the presence of lactose than whey permeate as a substrate. The significant variables that affected the bioconversion yield and productivity of lactose bioconversion were substrate concentration and hydrogen peroxide amount. For the bioconversion of whey permeate, substrate concentration and LY/Cataz amount displayed the major contribution to the bioconversion yield and the productivity. Among interactive effects, the interaction between substrate concentration and enzyme amount exhibited an antagonistic effect on the bioconversion yield of lactose into LBA. While the interaction between substrate concentration and hydrogen peroxide amount exhibited a synergistic effect on the productivity. None of the interactions between variables was significant in the predictive models of both whey permeate-bioconversion yield and productivity. For the bioconversion of whey permeate into LBA, the highest productivity of 24 g/l.h was obtained at lactose concentration of 799.94 mg/ml, LY/Cataz amount of 0.079% (w/w) and hydrogen peroxide concentration 0.621%(v/v). As far as the authors are aware, this productivity is among the highest reported so far.

## 4.2. Introduction

Whey permeate, which is the main by-product of dairy industry, is obtained upon the recovery of proteins from whey and contains a high amount of lactose (75–80% on dry basis)(Nath, Verasztó, Basak, Koris, Kovács, et al., 2016). The use of whey proteins as functional food ingredients has considerably increased over the last years (Livney, 2010). However, the lactose usage is still limited because of its technological challenges, including poor solubility, low sweetness index, malabsorption and intolerance by a certain population (S. Cheng & Martínez- Monteagudo, 2019). Therefore, there is an increasing interest in the bioconversion of lactose from whey permeate into added-value compounds such as lactobionic acid (LBA) (Martínez-Monteagudo et al., 2019). The various functionalities of LBA, including antioxidant, chelating and humectant, make it a unique compound for food applications (Gutiérrez, Hamoudi, & Belkacemi, 2012). In our previous study (chapter 3), selected biocatalytic systems were investigated for the bioconversion of lactose and whey/milk permeates into LBA. Increasing the productivity of these biocatalytic systems can be achieved by mechanochemistry assistance.

Mechanochemistry is defined as "a reaction caused by the mechanical energy" (Fernandez-Bertran, 1999). The use of mechanical energy instead of conventional stimulants (i.e. electricity, heat, light) to initiate the chemical processes has been utilised on insoluble inorganic compounds and recently on organic materials and nano-catalysts (Baláž et al., 2013; Traboni, Bedini, Vessella, & Iadonisi, 2020). Indeed, the combination of friction and shear energy through mechano-ball milling can create shape and crystal defects on the surface of reactants, resulting in changes in their physico-chemical properties and their reactivity; such changes can enhance the creation of diversified compounds (Mehta, Das, Kumar, Pandey, & Mehrotra, 2010). For instance to overcome the crystallinity and poor solubility of cellulose, the mechano-assisted method was used for the saccharification and derivatization of the cellulose (Achar, Bose, & Mal, 2017; Kuga & Wu, 2019). Mechano-milling assistance has also been investigated as a potential approach for controlling potato starch modification and increasing adsorption capacity of chitosan, biochar nanomaterials and cellulose nanofibers (Juarez-Arellano et al., 2021; M. Kumar et al., 2020; Piras, Fernández-Prieto, & De Borggraeve, 2019; Qiu, Vakili, Cagnetta, Huang, & Yu, 2020). In the biocatalytic processes, mechano-milling assistance has recently attracted attention as it can offer many benefits that include a decrease in the reaction volume, a high efficiency of solvent-free reaction and an enhanced bioconversion of poorly soluble substrates (Basile & Dalena, 2019; José

G Hernández, Ardila-Fierro, Crawford, James, & Bolm, 2017; Perona, Hoyos, Farrán, & Hernáiz, 2020). However, most of studied mechano-milling assisted bioconversions have been focused on the de-polymerisation of biomass components and the hydrolysis of feedstock (Basile & Dalena, 2019; José G Hernández et al., 2017; Perona et al., 2020). As far as the authors are aware, no study has so far investigated the use of mechano-milling assistance for the bioconversion of lactose into LBA. Mechano-milling assisted enzymatic reactions can be performed in the presence of minimal volume of a liquid, named liquid-assisted grinding, which can mimic the enzyme environment (Pérez-Venegas & Juaristi, 2020; Therien, Hammerer, Friscic, & Auclair, 2019). The efficiency of mechano-milling assisted enzymatic reactions has been enhanced by using consecutive cycles of milling activation and reactive aging; in this last cycle, the milled reaction mixture is incubated at a low ratio of water to reactants (v/w) (Friščić, Mottillo, & Titi, 2020).

The objective of the present research was to investigate the enzymatic production of LBA, from lactose and whey permeate, assisted by mechanochemistry *via* mechano-ball milling. The effects of mechano-milling process on the bioconversion yield and productivity of LBA were determined at a broad range of substrate concentration. The addition of hydrogen peroxide for *in-situ* generation of oxygen in the mechano- milling assisted biocatalytic system was also evaluated. To assess and understand the effects of reaction parameters of the mechano-ball milling assisted biocatalytic system, the response surface methodology was used.

## 4.3 Materials and methods

### *4.3.1. Materials*

Lactose monohydrate powder, D- (+)-glucose, D- (+)-galactose, LBA were obtained from Sigma-Aldrich (Oakville, ON, Canada). LactoYIELD and Catalase (Catazyme 25 L) from Chr. Hansen (Milwaukee, WI, USA). Whey permeate (11.55% protein, 96.02% solid, 7.57% ash, and a minimum of 76.09% lactose, w/w) was obtained from Agropur cooperative (Qc, Canada). Acetonitrile, methanol, formic acid and laccase from *Trametes versicolor* (LacTv) , pH indicator strips (2 to 9), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), hydrogen peroxide solution, 30 % (w/w) in $H_2O$, contains stabilizer and polyethylene glycol (PEG) 8000 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium hydroxide, acetonitrile and methanol HPLC grade and water LC/MS grade were purchased from Fisher Scientific (Hampton, NH).

### *4.3.2. Bioconversion reaction*

Bioconversion of lactose to LBA was carried out up to 8 h reaction time in the presence of hydrogen peroxide (0.5 %v/v) to generate *in -situ* oxygen. The reaction mixtures consisted of lactose substrate at a concentration of 100 to 800 mg/ml and LactoYIELD/Catazyme (LY/CataZ) biocatalysts at 0.02/0.02 to 0.2/0.2% (w/w substrate) concentration in ammonium acetate buffer (25 mM, pH 5.8). The reactions were performed at 38°C under controlled pH by adding ammonium hydroxide (0.2 M). At selected reaction times, the enzymatic reactions were halted by immersing them in a boiling water-bath for 10 min. The enzymatic reactions were carried out in triplicate, along with blank trial containing all reaction components except the biocatalysts. LBA concentration was estimated by High Performance Liquid Chromatography (HPLC) system coupled to the 6560 ion mobility Q-TOF mass spectrometer (Agilent).

### *4.3.3 Mechano-milling assisted bioconversion reaction*

The mechano-milling assisted reactions were conducted as described by Xing and Yaylayan (2021) in a stainless-steel grinding jar (10 mL) with one steel ball (3.2 mm diameter; ball/sample ratio ~32:1) for creating inner friction (Xing & Yaylayan, 2021). The jars were seated in the Retsch Mixer Mill (MM 400, Newtown, PA, US) that performs radial oscillations in a horizontal position without coolant (the external jar temperature was ~25°C) at a frequency of 20 Hz for 30 min. Consecutive cycles of milling activation and reactive aging were performed. For milling activation cycle, the reaction mixtures consisted of lactose solution and LY/CataZ biocatalyst suspension. After mechano-milling for 30 min at 20 Hz, the reaction mixtures were collected, and ammonium acetate buffer (50 mM, pH 5.8) was added to achieve a final lactose concentration of 100 to 800 mg/ml with 0.2 or 0.02 % (w/w substrate) of each biocatalyst. The bioconversion reaction mixtures were then incubated at 38 °C under 250 rpm for 8 h reactive aging cycle at constant pH 5.6-5.8 (adjusted with ammonium hydroxide). In addition to the use of pure lactose, whey permeate was used as a source of lactose. The mechano-milling assisted bioconversion reactions were also conducted in the presence of PEG 8000 (0.01 %, w/w substrate) to protect the enzymes for mechano/thermal-inactivation. LBA concentration was estimated by HPLC coupled to the 6560 ion mobility Q-TOF-MS.

### *4.3.4. Assessment of inhibitory effect of high substrate concentration*

To assess the inhibitory effect of high substrate concentration, the "fed-batch" strategy that consisted of a sequential loading of substrate during enzymatic bioconversion was investigated. LBA production was carried at high lactose concentration of 800 mg/ml using batch and fed-batch reactor. In the batch reactor, the bioconversion reaction was carried out as described above by adding the substrate and the biocatalysts in one step. For the fed-batch reactor, both substrate (2* 400 mg/ml) and LY/CataZ biocatalysts (2* 0.02/0.02% or 0.2/0.2%, w/w substrate) were added in two steps within 4 h interval. The batch and fed-batch reactors were carried out in parallel at 38°C under controlled pH of 5.6-5.8 by adding ammonium hydroxide (0.2 M). Hydrogen peroxide at 0.5 % v/v was added to the reaction to produce needed oxygen. Batch and fed-batch reactors were carried out in triplicate, along with blank trial containing all reaction components except the biocatalysts. The bioconversion reactions were also conducted in the presence of 0.01 % (w/w substrate) of PEG 8000. LBA concentration was estimated by HPLC coupled to the 6560 ion mobility Q-TOF-MS.

### *4.3.5 Optimization of mechano-milling assisted production of LBA*

The effects of mechano-milling assisted bioconversion parameters were studied using response surface methodology (RSM), a central composite rotatable design (CCRD). A five-level, three variable central composite rotatable design was created using Design Expert® Software (version 8.0.7) (Stat-Ease, inc. Minneapolis, MN, USA). The designs comprised of 6 factorial points, 8 axial points, and 6 center points. Three independent variables included $x_1$, lactose (100-800 mg/ml); $x_2$, enzyme amount (LY/CataZ, 0.015/0.015-0.15/0.15 % w/w substrate), and $x_3$, hydrogen peroxide concentration (0-1 % v/v). The milling cycle was carried at a frequency of 20 Hz for 30 min, while the aging cycle was run at 38 °C under controlled pH 5.6-5.8 and agitation at 250 rpm for 8 h. All reactions were carried out in the presence of PEG (0.01 %, w/w substrate). The quantified responses were the bioconversion yield (%, lactobionic w/substrate w) and the productivity (g/l.h). All reactions were run in triplicate.

### *4.3.6. Statistical analysis*

For approximating response surface, the obtained bioconversion yield (%) and productivity from the described design, was fitted to the general model Equation 4.1 using the software Design-Expert 8.0.2 (Stat-Ease, inc. Minneapolis, MN, USA):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i+1 \le j \le k}^{1 \le i \le k} \beta_{ij} X_i X_j \quad \text{(Eq. 4.1)}$$

$\beta_o$, $X_i$s (i=1-5), $\beta_{i,}$, $\beta_{ii}$, and $\beta_{ij}$ are the constant coefficient, coded independent variables, the coefficient for the linear effect the coefficient for the quadratic effect, and the coefficient for the interaction effect, respectively.

### *4.3.7. Characterization of reaction mixtures by LC-MS*

The reaction mixtures were analyzed by HPLC-MS using an Agilent 1290 Infinity II LC system coupled to the 6560 ion mobility Q-TOF -MS (Agilent Technologies, Santa Clara, USA). The LC separation was conducted on a Poreshell120 EC-C18 analytical column (Agilent Technologies; 2.7 μm × 3 mm × 100 mm) connected with a Poreshell120 EC-C18 guard column (Agilent Technologies; 2.7 μm × 3 mm × 5 mm). Two solvents A (water with 25 mM ammonium acetate and 0.1% formic acid) and B (methanol with 25 mM ammonium acetate and 0.1% formic acid) were used to construct the gradient for the elution of the reaction components: 97% A (0 to 2.0 min), 50% A (2.0 to 4.0 min), linear decrease to 0% A (4.0 to 5.0 min), hold at 100% B (5.0-7.0 min), decrease to 3% B (7.0 to 7.5 min) and hold 3% B (7.50 to 9.50 min). The injection volume, the flow rate and the column temperature were set at 1 μL, 0.15 mL/min and 35°C, respectively. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization modes. MS conditions for ESI- were as follow: the drying gas temperature was 150 °C, drying gas flow rate was 11 L/min, sheath gas temperature was 375°C, sheath gas flow rate was 12 L/min, the pressure on the nebulizer was 30 psi, the capillary voltage was 4000 V, the fragmentor voltage was 175 V, the skimmer voltage was 30 V and the nozzle voltage was 1000 V. Full scan MS data was recorded between mass-to-charge ratios (*m/z*) 100 and 1100 at a scan rate of 2 spectra/s, and was collected at both centroid and profile mode. Reference ions (*m/z* at 121.0508 and 922.0098 for ESI) were used for automatic mass recalibration of each acquired spectrum. Data was processed using Mass-Hunter Qualitative Analysis software (Agilent Technologies). This allows the identification and the discovery of unidentified minor compounds.

## 4.4. Results and discussion

### *4.4.1. Effect of mechano-milling assistance on LBA production*

The mechano-milling assisted bioconversion of lactose and whey permeate into LBA was investigated using consecutive cycles of milling activation and reactive aging. We hypothesize that the presence of heat and mechanical action under mechano-milling assistance can enhance the availability of lactose, promote substrate-enzyme interactions, and increase the mass transfer rate of reactants at enzymes' micro-environment. Indeed, the lactose substrate has a limited solubility of 24% (w/v) (Shendurse & Khedkar, 2016). In the mechano-milling assisted bioconversions, lactose and whey permeate were used at concentrations ranging from 100 to 800 mg/ml, corresponding to 10 to 80% (w/v).

Figures 4.1 and 4.2 show the bioconversion yield and productivity of LBA produced by mechano-milling assisted bioconversion of lactose and whey permeate. Using 0.02/0.02% (w/w substrate) of LY/Cataz, the bioconversion yield decreased from 100 to 30.4% and from 61.7 to 7.5% when lactose and whey permeate concentration was increased from 100 to 800 mg/ml, respectively. These results can be due to enzyme denaturation and/or substrate/product inhibition. Substrate inhibition may occur when enzymes have a specialized site at which a second substrate molecule can bind and act as an allosteric inhibitor (Reed, Lieb, & Nijhout, 2010). The formation of enzyme-substrate complex that cannot react can also lead to substrate inhibition (Kasper, Andrews, & Park, 2014). While product inhibition can be the result of the formation of a stable enzyme-product complex, which sequesters free enzyme as product is formed (Kasper et al., 2014). At high LY/Cataz amount of 0.2/0.2% (w/w), the bioconversion yields obtained with lactose substrate were lower compared to those observed at LY/Cataz amount of 0.02/0.02% (w/w). These results may be attributed to the presence of enzyme-enzyme interactions and/or to the inhibitory effect of the degradation products that may have been highly generated at high enzyme amount. Contrary to lactose, similar bioconversion yields were achieved with whey permeate at 0.02/0.02 and 0.2/0.2% (w/w) LY/Cataz amount. The other components of whey permeate, composed of 11.5% protein, 7.6% ash and 76.1% lactose (w/w), may have limited the inhibitory effect at high enzyme amount.

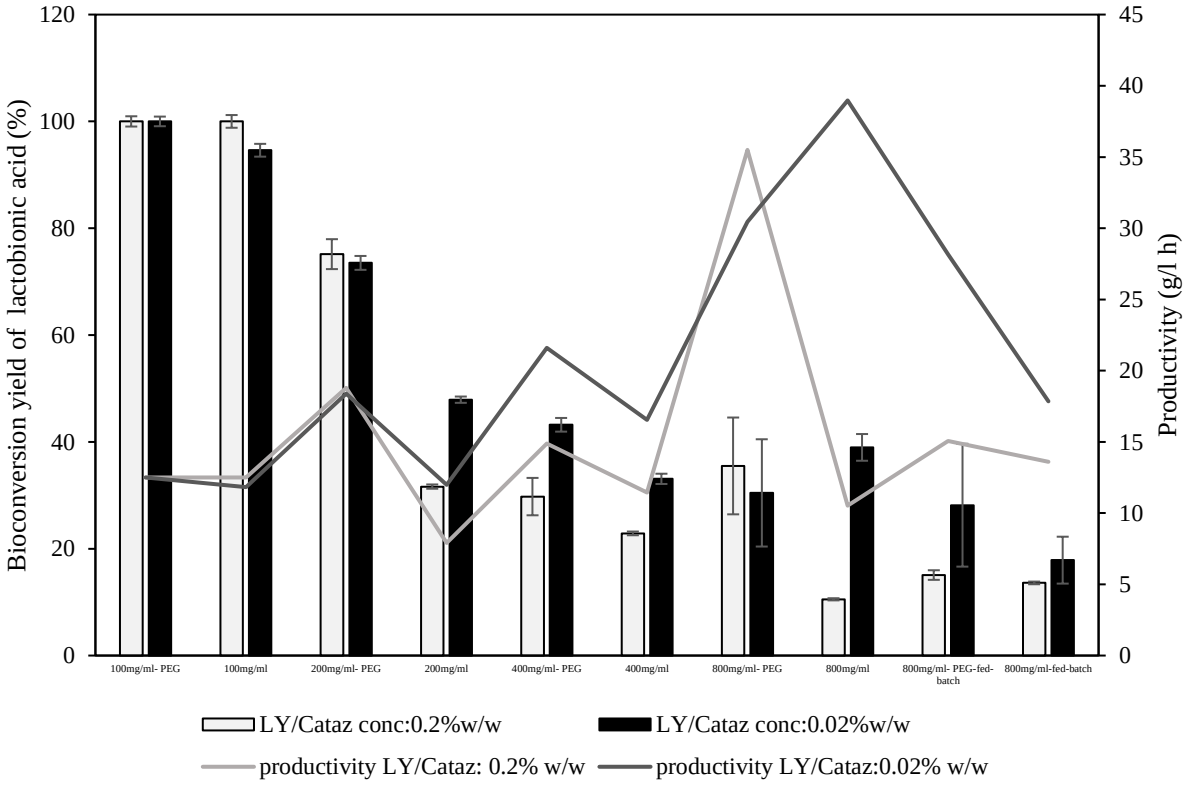



**Figure 4.1.** Mechano-milling assisted bioconversion of lactose into LBA by LactoYIELD/Catazyme (LY/CataZ) at a concentration of 0.02/0.02% and 0.2/0.2% (w/w substrate). The milling cycle was carried at a frequency of 20 Hz for 30 min, while the aging cycle was run at 38 °C under controlled pH (5.6-5.8) and agitation (250 rpm) for 8 h

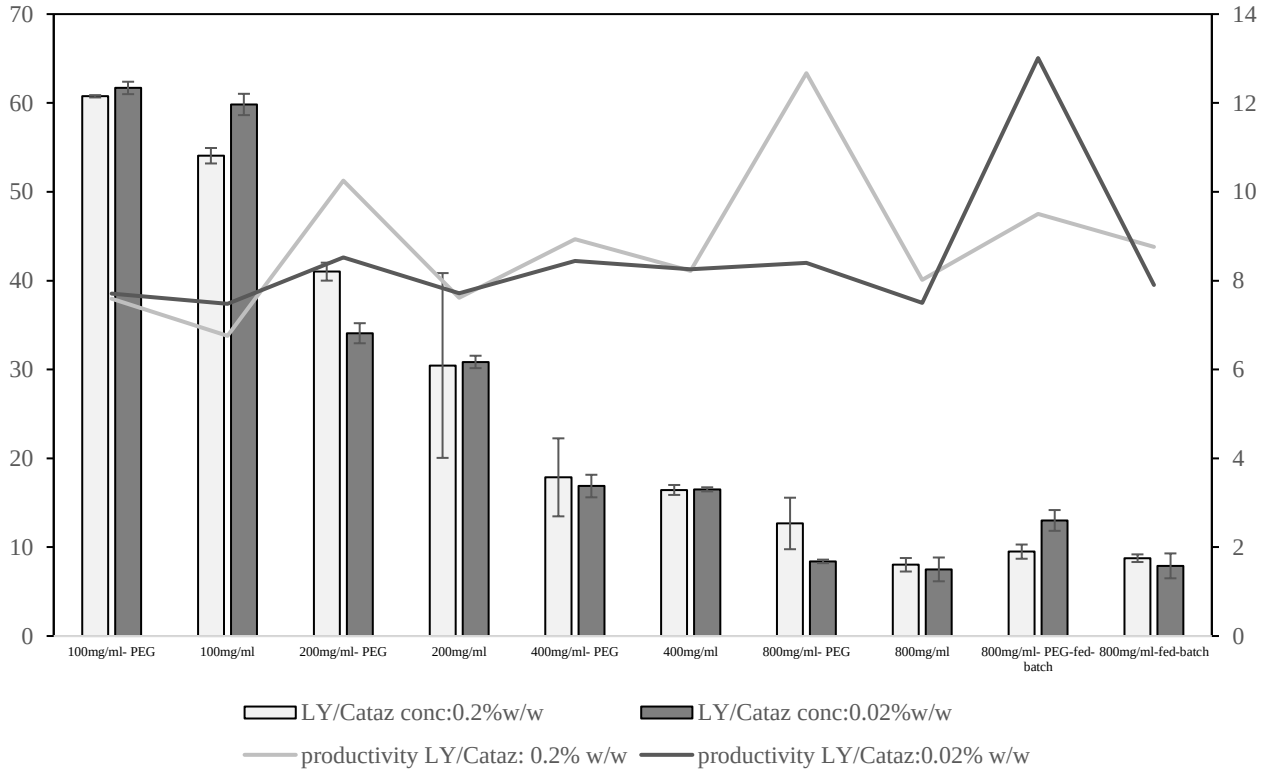



**Figure 4.2.** Mechano-milling assisted bioconversion of whey permeate into LBA by LactoYIELD/Catazyme (LY/CataZ) at a concentration of 0.02/0.02% and 0.2/0.2% (w/w susbtrate). The milling cycle was carried at a frequency of 20 Hz for 30 min, while the aging cycle was run at 38 °C under controlled pH (5.6-5.8) and agitation (250 rpm) for 8 h

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To prevent the thermal-denaturation of biocatalysts under mechano-milling forces, the mechano-milling assisted bioconversion reactions were conducted in the presence of PEG 8 000. Figures 4.1 and 4.2 show that PEG exhibited a protective effect, which was significant in the presence of lactose than whey permeate as a substrate. The use of PEG anionic polymers to form non-covalent bioconjugates with enzymes was proven to be a potential approach for enzyme stabilization (Pérez et al., 2018). Indeed. the PEG stabilizing effect was attributed to the ability of PEG to suppress the enzyme aggregation and to delay the unfolding at elevated temperatures (Wang et al., 2021). The PEG results agree with the previous hypothesis that whey permeate components may have protected the enzymes, limiting the formation of a shell of surfactants compared to the presence of only lactose. The number of hydrogen bonds between the cationized proteins and PEG chain was reported to correlate with enzyme thermostability (Perez et al., 2018).

Figures 4.1 and 4.2 also indicate that the use of fed-batch approach in which the substrate was sequentially added (200 mg/ml-2 steps) did not enhance the production of LBA from lactose. A decrease in the bioconversion yield from 38.9 to 17.9% and 30.4 to 28.1% was even observed at 0.02/0.02% (w/w) of LY/Cataz with and without PEG 8 000, respectively, using lactose substrate. Contrary to lactose substrate, the use of fed-batch approach with whey permeate substrate resulted in more or similar bioconversion yields as the batch system.

The determination of productivity is a key element towards assessing the feasibility of bioconversion. Figure 4.1 shows that when lactose concentration was increased from 100 to 800 mg/ml, the productivity increased significantly to reach a maximum value of 38.9 g/l.h at 800 mg/ml. The results also show that the batches with lower LY/Cataz amount of 0.02/0.02% (w/w) were more productive than the ones with 0.2/0.2% (w/w) enzymes at the same lactose substrate concentration. In addition, the presence of PEG 8 000 had a positive effect on the reaction productivity. These results reveal that a more productive process for the bioconversion of lactose into LBA can be achieved with the mechano-milling assistance and the use of high substrate concentration and PEG stabiliser.

In comparison to lactose substrate, increasing whey permeate concentration didn’t increase significantly the productivity of LBA (Figure 4.2). An increase in the productivity from 7.6 to 12.7 g/l.h was observed when the whey permeate concentration was increased from 100 to 800 mg/l at LY/Cataz amount of 0.2/0.2 (w/w) and in the presence of PEG 8 000. The use of

0.02/0.02% of LY/Cataz requires the use of fed-batch process in order to achieve a maximum productivity of 13.01 g/l.h. These results reveal that the other components of whey permeate hindered the complete conversion of lactose into LBA.

Using carbohydrate oxidase from *Microdochium nivale*/catalase (Catazyme 25 L), a productivity of 4 g/l.h was reported after 12h reaction using lactose concentration of 50 mg/ml (Hua et al., 2007). On the other hand, a productivity in the range of 7.2-27 g/l.h was obtained when cellobiose dehydrogenase (CDH) from *Athelia (Sclerotium) rolfsii* CBS 191.62/ laccase from *Trametes pubescens* MB 89 were employed (Baminger et al., 2001). Lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3/ catalase resulted in 7.4 to 11.5 g/l.h productivity at lactose concentration of 10% (w/v). Productivity of 7.9 to 11.5 was reported for CDH from Microdochium *nivale*/ catalase (Catazyme 25 L) system, while CDH from white mold/laccase from *Trametes versicolor* productivity was reported to be 1.8 g/l.h (Budtz et al., 2007; Dhariwal et al., 2006). Glucose/fructose oxidoreductase/ glucono-δ-lactonase enzymes from *Zymomonas mobilis* system resulted in 5.6 g/l.h productivity, whereas the use of glucose/fructose oxidoreductase from permeabilized cells of *Z. mobilis* led to a productivity of 0.8 to 2.3 g/l.h (da Silva et al., 2011; Satory et al., 1997). As an overall, the productivities achieved in our study are higher than most ones reported in the literature.

### *4.4.2. Study the effects of bioconversion parameters*

RSM contains of a collection of mathematical and statistical systems applied for the development of a functional relationship between a response of interest, *y*, and a number of associated control (or input) variables denoted by $x_1$, $x_2$,…,$x_k$ (Chelladurai et al., 2021). The principal advantage of using Design Expert technique is to minimize the number of experiments, which result in saving experimentation time and materials as well as predict a response by developing a mathematical model (Kalavathy, Regupathi, Pillai, & Miranda, 2009). In the present study, the effects of variables, including substrate concentration ($x_1$, 100-658.111, mg/ml), hydrogen peroxide concentration ($x_2$, 0-1 %, v/v) and enzymes concentration ($x_3$, 0.015-0.15 %, w/w substrate), on the bioconversion yield and productivity of LBA were investigated using RSM. Multiple linear regression technique was employed to predict the outcome of a response by using various explanatory variables by the coefficients of determination ($R^2$), adjusted R-squared (Adj $R^2$) values, *P*-value and model lack of fit test. The observed results are depicted in Table 4.1.

**Table 4.1**. Factorial experimental design and experimental results of mechano-milling assisted bioconversion of lactose and whey

| | | | | Lactose bioconversion | | Whey permeate bioconversion | |
|---|---|---|---|---|---|---|---|
| Run | $x_1$: Substrate concentration (mg/ml) | $x_2$: Hydrogen peroxide concentration (%, v/v) | $x_3$: Enzyme concentration (%, w/w) | Bioconversion yield (%) [a] | Productivity g/lh [b] | Bioconversion yield (%) [a] | Productivity g/lh [b] |
| 1 | 658.1 | 0.20 | 0.042 | 19.8±5.8 | 16.3 | 19.7±2.8 | 16.2 |
| 2 | 658.1 | 0.80 | 0.123 | 24.7±4.0 | 20.3 | 27.2±3.5 | 22.4 |
| 3 | 100.0 | 0.50 | 0.083 | 94.4±2.6 | 11.8 | 77.6±5.3 | 9.7 |
| 4 | 450.0 | 0.00 | 0.083 | 21.5±1.7 | 12.1 | 20.3±1.3 | 11.4 |
| 5 | 658.1 | 0.20 | 0.123 | 20.3±2.3 | 16.7 | 29.8±4.5 | 24.5 |
| 6 | 241.9 | 0.20 | 0.042 | 52.2±1.7 | 15.8 | 42.8±1.3 | 12.9 |
| 7 | 450.0 | 0.50 | 0.083 | 34.7±2.9 | 19.5 | 24.5±3.1 | 13.8 |
| 8 | 800.0 | 0.50 | 0.083 | 23.5±1.1 | 23.5 | 16.9±2.8 | 17.0 |
| 9 | 450.0 | 0.50 | 0.083 | 38.4±1.6 | 21.6 | 23.3±2.2 | 13.1 |
| 10 | 450.0 | 0.50 | 0.015 | 28.7±1.6 | 16.1 | 20.2±2.3 | 11.4 |
| 11 | 450.0 | 0.50 | 0.083 | 34.3±4.1 | 19.3 | 23.4±1.7 | 13.2 |
| 12 | 241.9 | 0.80 | 0.123 | 72.0±1.7 | 21.8 | 35.3±1.6 | 10.7 |
| 13 | 450.0 | 0.50 | 0.083 | 35.8±1.5 | 20.1 | 29.1±2.4 | 16.4 |
| 14 | 450.0 | 0.50 | 0.083 | 35.2±1.4 | 19.8 | 23.1±1.7 | 13.0 |
| 15 | 241.9 | 0.20 | 0.123 | 58.9±1.2 | 17.8 | 33.1±1.5 | 10.0 |
| 16 | 241.9 | 0.80 | 0.042 | 61.4±2.6 | 18.6 | 42.5±1.9 | 12.9 |
| 17 | 450.0 | 1.00 | 0.083 | 40.1±1.9 | 22.6 | 31.9±2.8 | 17.9 |
| 18 | 450.0 | 0.50 | 0.083 | 34.7±1.9 | 19.5 | 28.8±3.2 | 16.2 |
| 19 | 658.1 | 0.80 | 0.042 | 31.8±2.8 | 26.2 | 20.2±12.3 | 16.6 |
| 20 | 450.0 | 0.50 | 0.150 | 35.9±1.6 | 20.2 | 28.8±3.2 | 16.2 |

[a] Bioconversion yield was calculated as the amount of produced LBA per the amount of lactose substrate, multiplied per 100.

[b] Productivity was estimated as the amount of produced LBA per l of reactor volume per h of reaction.

Using whey permeate and lactose as a starting material, the highest bioconversion yield of LBA of 77.6 and 94.4 %, respectively, were obtained in the presence of 0.5% (v/v) hydrogen peroxide, 100 mg/ml of substarte and 0.0825% (w/w) LY/Cataz (run #3). However, the conditions of this run#3 led to the lowest productivity for the bioconversion of both lactose and whey permeate. While the highest productivity of 26.15 g/l.h was achieved at 658.1 mg/ml lactose in the presence of 0.8% (v/v) hydrogen peroxide and 0.04% (w/w) LY/Cataz (runs #19). The use of whey permeate at 658.1 mg/ml in the presence of 0.2 (v/v) hydrogen peroxide and 0.1% (w/w) LY/Cataz allowed a productivity of 24.57 g/l.h (run#5). On the other hand, the lowest bioconversion yields of 16.9 and 19.8% were obtained at 800 mg/ml whey permeate/0.5% hydrogen peroxide/0.08% LY/Cata (run #8) and at 658.1 mg/ml lactose/0.2% hydrogen peroxide/0.04% LY/Cataz (run #1), respectively.

4.4.2.1. Analysis of variance and model fitting

To find the best bioconversion yield and productivity models, Box-Cox plot was used to determine the appropriate power transformation needed to normalise the response data. The Lambda value indicates the power to which all data should be raised in order to transform the data into a normal distribution. The recommended rounded Lambda values by the Design Expert software for the transformation of the data were -1 (inverse transformation) for the bioconversion yield and the productivity predictive models with whey permeate as a source of lactose substrate.

Based on the analysis of variance (ANOVA) for the design (Table 4.2), a reduced quadratic model was statistically suitable for the description of LBA yield (*F*-value of 191.8 and *p*-value of 0.0001), whereas the reduced 2F model was well fitted for the description of the variations of the productivity of LBA when lactose used as substrate (*F*-value of 22.75 and *P*-value of 0.0001). However, when whey permeate was used as a substrate, linear model was fitted for both bioconversion yield (*F*-value of 13.14 and *p*-value of 0.0001) and productivity (*F*-value of 11.2 and *p*-value of 0.0003). For all models, the lack of fit was not significant relative to pure error with *F* values of 2.53 to 3.7 and *p*-values of 0.073 to 0.158. These results indicate a good quality of the fit and its ability to predict within a range of variables employed. In addition, the predicted coefficient of determination ($R^2$) values was in reasonable agreement with the adjusted $R^2$ ones.

**Table 4.2**. Analysis of variance (ANOVA) for the mechano-milling assisted bioconversion of lactose and whey permeate into LBA

| | Lactose bioconversion | | | | Whey permeate bioconversion | | | |
|---|---|---|---|---|---|---|---|---|
| | Bioconversion yield (%)[a] | | Productivity (g/l.h)[b] | | Bioconversion yield (%) | | Productivity (g/l.h) | |
| Source | F-value | p-value | F-value | p-value | F-value | p-value | F-value | p-value |
| Model | 191.80 | < 0.0001 | 22.75 | < 0.0001 | 13.14 | 0.0001 | 11.20 | 0.0003 |
| $x_1$-Substrate concentration | 876.73 | < 0.0001 | 17.87 | 0.0007 | 34.72 | < 0.0001 | 30.35 | < 0.0001 |
| $x_2$- Hydrogen peroxide | 60.08 | < 0.0001 | 62.08 | < 0.0001 | 1.52 | 0.2348 | 2.11 | 0.1660 |
| $x_3$-Enzyme concentration | 6.45 | 0.0247 | 4.01 | 0.0635 | 3.16 | 0.0943 | 1.14 | 0.3006 |
| $x_1x_2$ | | | 7.04 | 0.0181 | - | - | - | - |
| $x_1x_3$ | 12.07 | 0.0041 | - | - | - | - | - | - |
| $x_1^2$ | 185.34 | < 0.0001 | - | - | - | - | - | - |
| $x_2^2$ | 3.74 | 0.0750 | | | | | | |
| Lack of Fit | 3.7 | 0.0828 | 3.8 | 0.0738 | 3.14 | 0.1081 | 2.53 | 0.1582 |

[a] Reduced quadratic model; [b] reduced 2F model; [c] Transformation inverse and linear model.

The fitted models for the LBA yield and productivity in terms of actual factors are given by Equations 4.2 and 4.3 for lactose and 4.4 and 4.5 for whey permeate.

Lactose: Bioconversion yield (%) = $88.95 - 0.25\ x_1 + 31.24\ x_2 + 203.44\ x_3 - 0.359\ x_1\ x_3 + 2.01\ 10^{-4}\ x_1^{\ 2} - 14.01\ x_2^{\ 2}$ (Eq. 4.2)

Lactose: Productivity (g/l.h) = $19.37 + 1.64\ x_1 + 3.06\ x_2 + 0.7782\ x_3 + 1.35\ x_1\ x_2$ (Eq. 4.3)

Whey Permeate: 1/Bioconversion yield (%) = $0.025 + 5.01\ 10^{-5}\ x_1 - 0.0074\ x_2 - 0.078\ x_3$ (Eq. 4.4)

Whey Permeate: 1/Productivity (g/l.h) = $0.119 - 7.6\ 10^{-5}\ x_1 - 0.014\ x_2 - 0.076\ x_3$ (Eq. 4.5)

Where $x_1$ is substrate concentration (mg/ml); $x_2$ is hydrogen peroxide ($H_2O_2$, % v/v); $x_3$ is enzyme concentration (%w/w substrate).

Among the linear terms, the significant variables that affected the bioconversion yield and productivity models with lactose substrate were substrate concentration ($x_1$, *F of 17.9-876.3; P of 0.007-<0.0001* ) and hydrogen peroxide amount ($x_2$, *F of 60.1-62.1; P of <0.0001*). In addition, in the bioconversion yield model, the quadratic effects of these last parameters ($x_1^2$, $x_2^2$, *F of 3.7-185.3; P of 0.075-<0.0001*) were also significant. Among the interactive effects, the interaction between substrate concentration ($x_1$) and enzyme amount ($x_3$) was significant in the lactose-bioconversion yield predictive model (*F* of 12.1; *P* of 0.0041). While the interaction between substrate concentration ($x_1$) and hydrogen peroxide amount ($x_2$) exhibited more effect in the lactose-productivity predictive model (*F* of 7.04; *P* of 0.0181). As per the equation coefficients, $x_1x_3$ interaction has a negative coefficient, revealing its antagonistic effect in the lactose-bioconversion yield predictive model. On the other hand, the positive coefficient of $x_1x_2$ in the lactose- productivity predictive model reveals their synergistic interaction.

Table 4.4 also shows that the substrate concentration ($x_1$) displayed the major contribution to the models of both whey permeate-bioconversion yield and productivity (*F of 34.7-30.3; P of <0.0001*). Enzyme amount ($x_3$) was the variable with the second largest effect on the bioconversion yield (*F of 3.2-1.1; P of 0.0943-0.3006*), but not on the productivity with whey permeate substrate. None of the interactions between variables was significant in the predictive models of both whey permeate-bioconversion yield and productivity.

4.4.2.2. Investigation of the interactive effects of reaction parameters

Contour plots is a graphical technique with a two-dimensional input and a one-dimensional output. The two-dimensions (2D) contour plots from the fitted model is helpful to better understand the relationships between the reaction parameters. The 2D contour plots are illustrated in Figures 4.3 and 4.4, showing the interactive effects between hydrogen peroxide concentration/ lactose concentration ($x_1$), enzymes amount ($x_3$)/ lactose concentration ($x_1$), hydrogen peroxide concentration ($x_2$)/ whey lactose concentration ($x_1$), enzymes concentration ($x_3$)/ whey lactose concentration ($x_1$) on the predicted bioconversion yield and productivity. When lactose was used as substrate, higher amount of enzymes and lower concentration of lactose were found to have a more significant positive effect on the bioconversion yield of LBA (Figure. 4.3c). As the substrate concentration increased, the bioconversion efficiency decreased potentially due to the substrate diffusional limitations, the enzyme denaturation and/or to the product/substrate inhibition (Butre, Sforza, Gruppen, & Wierenga, 2014). With whey permeate substrate, the enzyme amount exhibited more effect on the bioconversion yield in which an increase was observed as the enzyme amount increased from 0.04 to 0.15 % (w/w, substrate) (Figure 4.4c). This contribution of the enzyme amount to the bioconversion yield of LBA was more pronounced at the lower range of whey permeate concentration. Indeed, the lactobionic-lactone is the primary product of lactose oxidation, which is further hydrolysed to LBA. Higher enzyme amount may have affected the reaction equilibrium resulting in its shift towards esterification.

The productivity predictive models reveal that higher productivity can be achieved by increasing substrate concentration and enzyme amount. However, the contribution of whey permeate concentration to the enhancement of productivity was more significant in the presence of high enzyme amount (Figure 4.4f). These results also show that in productivity model of whey permeate, there is some inhibitory activity on enzyme, and more enzyme is needed to obtain high productivity. While in the lactose-productivity model, the effect of substrate concentration was not dependent on the enzyme amount (Figure 4.3f). The results also confirm that the hydrogen peroxide concentration was more determinant for the productivity than the bioconversion yield. Indeed, in the productivity model of lactose, the positive contribution of increasing hydrogen peroxide concentration was not dependent on the enzyme concentration (Figure 4.3d,e). In contrast, the productivity model of whey permeate revealed the increasing contribution of hydrogen peroxide concentration to the productivity at higher enzyme amount (Figure 4.e).

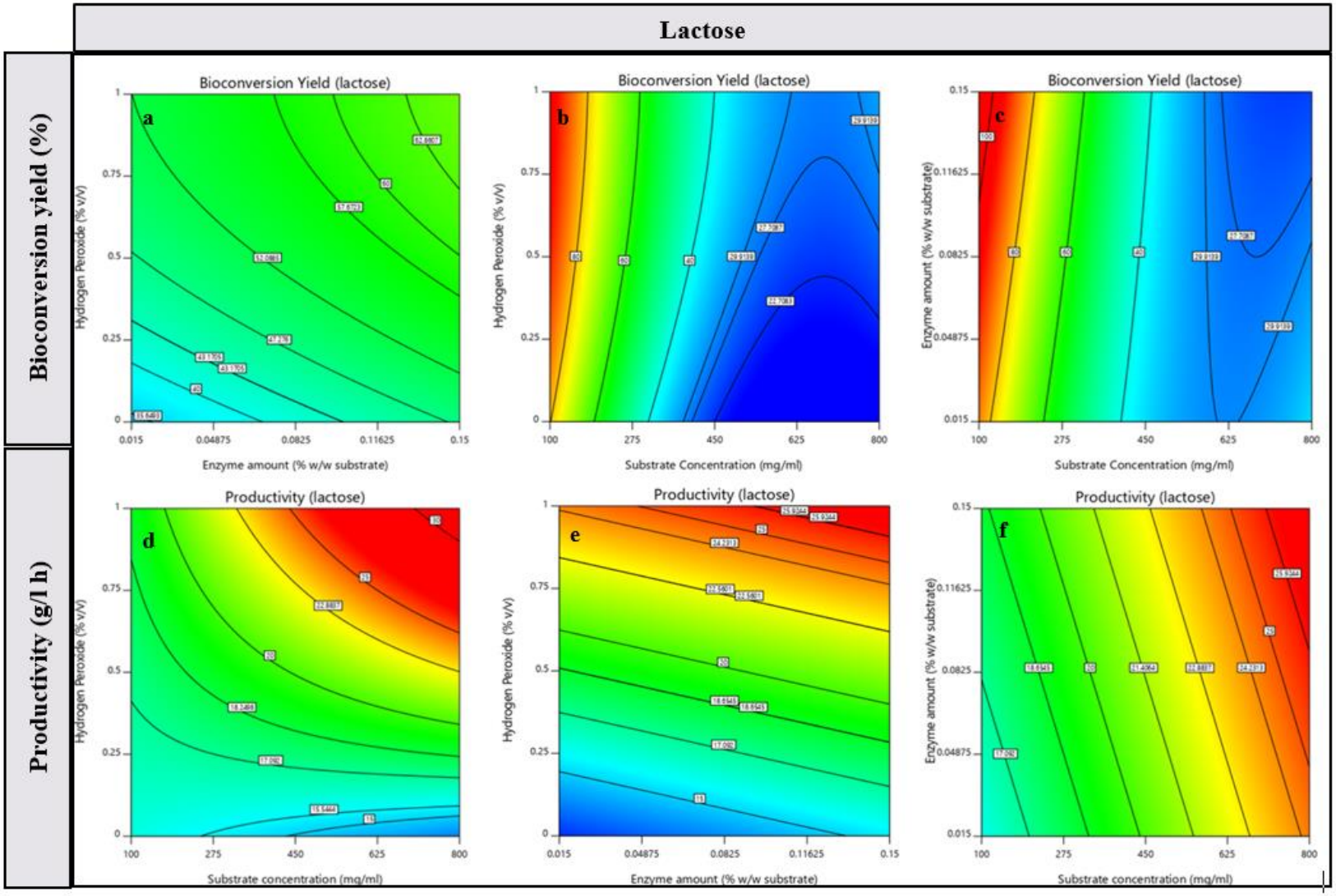


**Figure 4.3 a-f.** Contour plots. a-c are showing the bioconversion yield of lactose, d-f are for productivity of lactose.

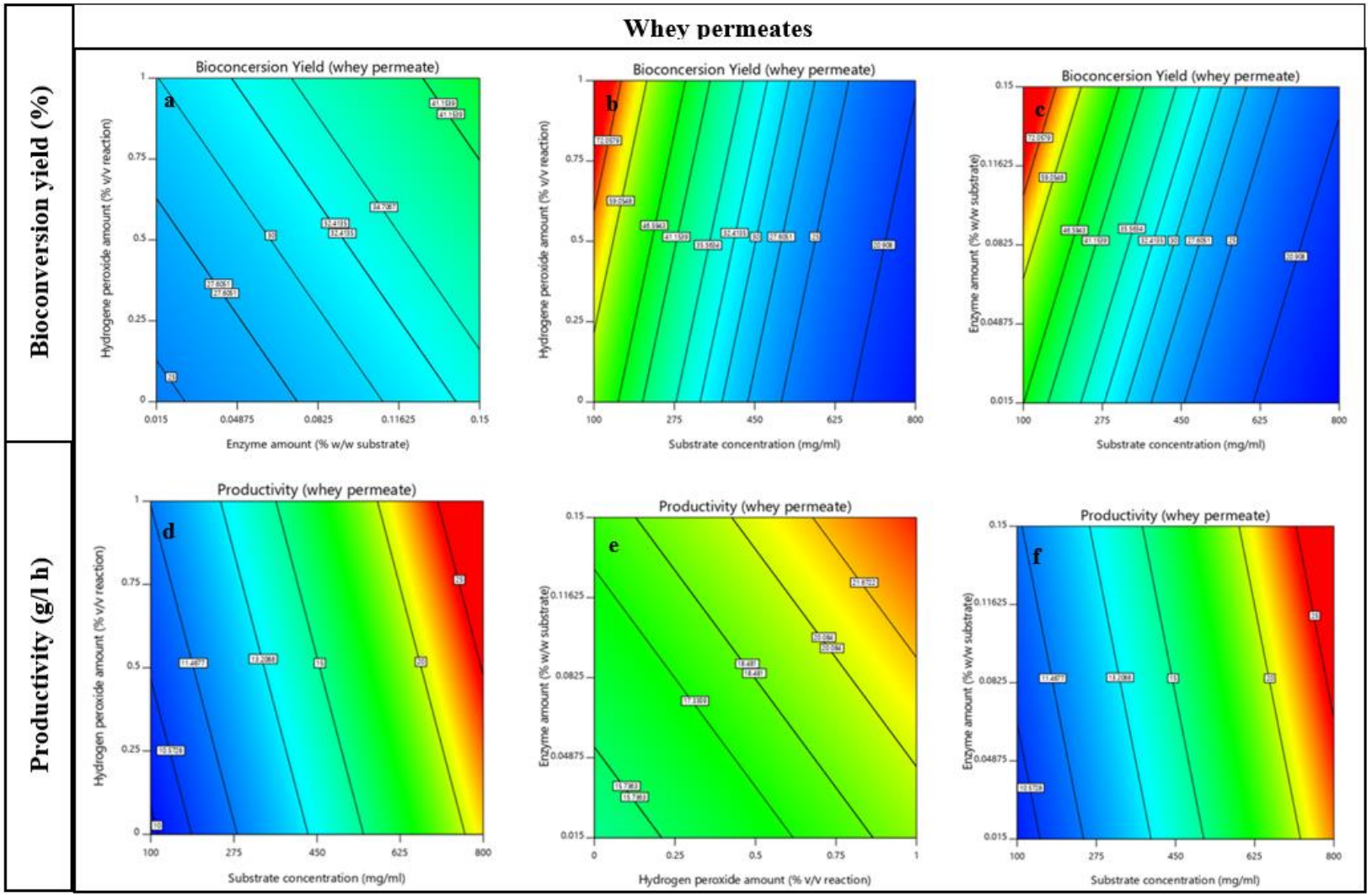


**Figure 4.4 a-f.** Contour plots a-c showing the bioconversion of whey permeate, d-f the contour plots of productivity when the substrate is whey permeate.

Hydrogen peroxide seemed to have more effect on the productivity of the lactose bioconversion than the whey permeate bioconversion (Figures 4.3d, 4.4.d). The hydrogen peroxide is converted by catalase into oxygen, which may favour the bioconversion (Kaushal et al., 2018). It has been reported that the presence of the dissolved oxygen affects kinetic of the biocatalytic oxidation reactions (Lindeque & Woodley, 2020).

### *4.4.4. Model validation and optimization of conditions*

To validate the predictive models, the optimum conditions for maximizing the bioconversion yield and the productivity of LBA were identified and carried out in duplicate. Table 4.3 shows the optimum conditions including initial lactose concentration, LY/Cataz amount and hydrogen peroxide concentration, and their corresponding responses. The experimental values were within the predicted intervals of 95% confidence. This confirms the validity of the established models for the production of LBA by mechano-milling assisted bioconversion of lactose and whey permeate by LY/Cataz biocatalysts. Using lactose substrate, the maximum bioconversion yield requires low lactose concentration of 118.73 mg/ml, high enzymes amount of 0.108 %(w/w) and hydrogen peroxide concentration of 0.75% (v/v); while the maximum productivity was achieved at higher lactose concentration of 782.556 mg/ml, moderate enzyme amount of 0.074 % (w/w) and higher hydrogen peroxide concentration of 0.977 % (v/v). For whey permeate, the maximum bioconversion yield was obtained at low substrate concentration of 100 mg/ml, enzymes amount of 0.0825% (w/w) and moderate hydrogen peroxide concentration of 0.5% (v/v), whereas the highest productivity was obtained at higher lactose concentration of 799.94 mg/ml, moderate enzyme amount of 0.079% (w/w) and moderate hydrogen peroxide concentration 0.621%(v/v).

A fed-batch process was carried out in order to assess the effects of the side-products on the bioconversion efficiency. After each 8 h of reaction, new LY/Cataz biocatalysts and hydrogen peroxide were added to the reaction mixture, and the reaction was run for three cycles of 8 h. The results (data not shown) show no significant increase in the bioconversion yield and the productivity after adding new biocatalysts and hydrogen peroxide. These revealed the effects of side-products on the biocatalysts' catalytic efficiency and on the reaction thermodynamic, limiting the further conversion of lactose and whey permeate into LBA.

The reaction mixtures after each cycle were analyzed by LC-MS and the reaction components were identified by 'Find by Formula' algorithm and 'Find by Molecular Feature'. Tables 4.4 summarizes the identified compounds of the reaction mixtures with lactose and solid whey substrates. Sorbitol and gluconic acid were identified in the reaction mixtures with lactose and whey permeate substrates. On the other hand, citric acid was only present in the whey permeate reaction mixture.

**Table 4.3**. Predicted and experimental bioconversion yield and productivity values for the mechano-milling assisted bioconversion of whey permeate and lactose into lactobionic acid (LBA).

| | | Lactose concentration (mg/ml) | Hydrogen peroxide concentration (%v/v) | Enzyme concentration (%w/w) | Predicted value | Experimental value | 95%CI low | 95%CI high |
|---|---|---|---|---|---|---|---|---|
| Whey permeate | Maximum productivity (g/l.h) | 799.9 | 0.62 | 0.079 | 24.23 | 25.08±2.8 | 24.651 | 32.77 |
| | Maximum yield (%) | 100.0 | 0.50 | 0.083 | 77.31 | 77.6±0.38 | 69.17 | 0.9426 |
| Lactose | Maximum Productivity (g/l.h) | 782.6 | 0.98 | 0.074 | 29.67 | 28.26±5.6 | 26.27 | 36.62 |
| | Maximum Yield (%) | 118.7 | 0.75 | 0.108 | 94.82 | 81.17±2.3 | 82.63 | 99.89 |

**Table 4.4.** Qualitative HPLC-MS QTOF analysis of the LBA when lactose used as substrate and clarification of empirical formula and putative identification

| Reaction Components | Empirical Formula | Molecular Mass (Da) | Peak Area | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Lactose (cycle 1) | Lactose (cycle 2) | Lactose (cycle 3) | Whey Permeate (cycle 1) | Whey Permeate (cycle 2) | Whey Permeate (cycle3) |
| Sorbitol | $C_6H_{14}O_6$ | 182.0792 | 148155 | 959638 | 1030908 | 280635 | 920938 | 985079 |
| Gluconic acid | $C_6H_{12}O_7$ | 196.0584 | 59228 | 157664 | 125912 | 243938 | 167835 | 137708 |
| Lactitol | $C_{12}H_{24}O_{11}$ | 344.1318 | - | - | | 4270 | - | 71405 |
| Lactone | $C_{12}H_{20}O_{11}$ | 340.1001 | 39683 | 4932 | | 18624 | - | - |
| Citric acid | $C_6H_8O_7$ | 192.0269 | - | - | | 56930 | | 73125 |

When lactose was used as substrate, sorbitol concentration increased continuously after each cycle, whereas gluconic acid concentration increased in the second cycle and then showed a little decline for the third cycle. Contrarily, when solid whey was used as substrate, sorbitol and gluconic acid peak areas decreased in the second cycle and remained relatively constant at the third one.

### 4.5. Conclusion

A mechano-milling-assisted biocatalytic system was successfully applied for the bioconversion of lactose and whey permeate into LBA using LY/Cataz biocatalysts and hydrogen peroxide supplementation. Mechano-milling assistance was proven to be a potential approach for enhancing the yield and productivity of bioconversion at high substrate concentrations. The other components of whey permeate were found to have an inhibitory effect on the efficiency of the biocatalytic system. PEG enhanced the bioconversion efficiency as a result of its stabilizing effect on biocatalysts upon mechano-frictions. The study of the effects of hydrogen peroxide (*in situ* oxygen source), lactose and enzymes concentration on the yield and productivity of LBA contributed to the understanding of their linear and interactive effects. Among interactive effects, the interaction between substrate concentration and enzyme amount exhibited an antagonistic effect on the bioconversion yield of lactose into LBA, While the interaction between substrate concentration and hydrogen peroxide amount exhibited a synergistic effect on the productivity. None of the interactions between variables was significant in the predictive models of both whey permeate-bioconversion yield and productivity. Higher substrate concentration and hydrogen peroxide amount were found to maximize the productivity.

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# CHAPTER V

GENERAL CONCLUSION AND FUTURE WORK

Bioconversion of lactose and dairy by-products into LBA using *in situ* generated oxygen and alternative approaches was the goal of the present work. Three alternative approaches investigated to produce LBA were: (***i***) activation of substrate by ultrasonication and microwave assistances; (***ii***) generation of *in situ* oxygen as an electron acceptor for carbohydrate oxidase by supplementing the reaction media with the hydrogen peroxide in the presence of catalase; ***(iii)*** applying mechano-milling assisted approach for enhancing the yield and productivity of the bioconversion at high substrate concentrations.

Enzymatic pathway has been selected as the strategy to convert lactose to LBA. The results revealed the efficiency of lactoYIELD/catazyme (LY/Cataz) in the presence of molecular oxygen to bioconvert lactose, whey permeate and milk permeate into LBA. LY/Cataz/Laccase/mediator system was not significantly affected by the pH decrease. The *in-situ* generation of oxygen through the supplementation of reaction mixtures by hydrogen peroxide in the presence of catalase was successfully achieved. An optimal hydrogen peroxide concentration that compromised between the efficient generation of oxygen and the limited enzyme inhibition by hydrogen peroxide was determined. In addition, the addition of hydrogen peroxide after the initial stage of reaction resulted in an increase in the bioconversion yield. It was also found that LY/Cataz biocatalytic system showed a high bioconversion yield and productivity of LBA in the presence of hydrogen peroxide; while LY/Cataz/Laccase/mediator system performed better in the presence of oxygen and when laccase/mediator were added at the intermediate stage of the rection. Ultrasound and microwave assistances were applied in order to generate hydro-mechanical shear force created by cavitation bubbles and rapid heating. The application of ultrasound resulted to more or less similiar bioconversion yield and productivity as the conventional method. However, microwave assistance enhanced the productivity and shortened the reaction time. All investigated lactose sources (lactose, whey permeate, milk permeate) were shown to be suitable for LBA production. A compromise between the yield and the productivity was achieved upon the use of appropriate biocatalytic system, oxygen source and substrate concentration.

The use of mechanical forces to induce the chemical reactivity has been applied in organic and inorganic synthesis. For the first time, the present study investigated the mechano-milling assistance for the bioconversion of lactose and whey permeate to LBA. Indeed, the mechano-milling assistance allowed the use of higher substrate concentrations, and the results showed a significant increase in the productivity of the bioconversion. Mechano-milling assistance was

proven to be a potential approach for enhancing the yield and productivity of bioconversion at high substrate concentrations. The hydrogen peroxide concentration was more determinant for the productivity than the bioconversion yield. Compared to lactose, the use of whey permeate led to lower yield and productivity, revealing the inhibitory effects of other components of whey permeate on the efficiency of the biocatalytic system. PEG enhanced the bioconversion efficiency as a result of its stabilizing effect on biocatalysts upon mechano-frictions. The study of the effects of hydrogen peroxide (*in situ* oxygen source), lactose and enzymes concentration on the yield and productivity of LBA contributed to the understanding of their linear and interactive effects. The parameters that significantly affected the bioconversion yield and productivity of lactose bioconversion were substrate concentration and hydrogen peroxide amount. For the bioconversion of whey permeate, substrate concentration and LY/Cataz amount displayed the major contribution to the bioconversion yield and the productivity. Among interactive effects, the interaction between substrate concentration and enzyme amount exhibited an antagonistic effect on the bioconversion yield of lactose into LBA; while the interaction between substrate concentration and hydrogen peroxide amount exhibited a synergistic effect on the productivity. None of the interactions between variables was significant in the predictive models of both whey permeate-bioconversion yield and productivity. The use of higher substrate concentration and hydrogen peroxide amount was found to maximize the productivity.

Assessment of the mechano-milling parameters (frequency, duration) effect on the bioconversion yield, using immobilized enzymes on this system and using other bioconversion by this technique can address the challenge of further work.

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