IMMOBILIZATION OF RECOMBINANT LEVANASE AND ITS APPLICATION IN BI-ENZYMATIC SYSTEMS AIMING AT FRUCTOOLIGOSACCHARIDE AND OLIGOLEVAN SYNTHESIS

By

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Suggested Short Title

SYNTHESIS OF FRUCTOOLIGOSACCHARIDES AND OLIGOLEVANS BY IMMOBILIZED LEVANASE AND ITS BI-ENZYMATIC SYSTEM

Abstract

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The high interest in the health benefits of prebiotics, especially their ability to modulate and improve gut microbiota balance, has fueled the development of efficient and novel approaches. In particular, β-(2→6) fructooligosaccharides (FOSs) and fructooligomers exhibit higher prebiotic potential than commercial β-(2→1)-FOSs, owing to increased colonic persistence and selective fermentation. However, the FOSs and their corresponding polysaccharides levans have been found and extracted from limited plant sources in low concentration, so enzymatic approaches have emerged to bridge the gap. Two approaches were adopted including 1) applying levansucrases (LS) (LS, EC 2.4.1), which are fructofuranosidases and able to catalyze transfructosylation reaction from sucrose to produce FOSs and levans; 2) taking advantage of levanases (E.C.3.2.1.65), which are glycosyl-hydrolytic enzymes that catalyze the hydrolysis of levans into β-(2→6) FOSs and fructooligomers. The novel recombinant endo-levanases from *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), *Dyadobacter fermentans* (LEV-D.F.), previously expressed and purified, have shown promising endo-levanase activity. The present research work aimed at the immobilization of the novel recombinant enzymes on functionalized agarose derivatives. Screening the enzyme immobilization efficacy on positively, negatively, chelating, and partially hydrophobic agarose derivatives was preformed. LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA, and LEV-D.F./Gly-Ag-IDA/Cu were chosen for the further studies due to possessing the best compromise between the immobilized enzyme activity yield, retention of specific levanase activity, and retained levanase activity after incubation at 50 ^{o}C. The kinetic parameters, thermal stability, product profile, and the immobilized enzyme reusability were investigated for each selected immobilized enzyme. The results revealed that the use of a temperature of 15 ^{o}C and the high molecular weight levans can maximize the release of FOSs and limit that of fructose. Indeed, increasing the temperature shifted the end-product profile towards lower molecular weight FOSs with an exception in the case of the LMW levan-immobilized LEV-D.F./Gly-Ag-IDA/Cu reaction system. Compared to LMW levan, the use of HMW levan favored the GF_7 formation over the shorter chain FOSs. Immobilized LEV-C.O. on Gly-Ag-IDA showed a high product selectivity towards GF_7 production with no release of shorter oligosaccharides; however, its half-life decreased from 202.4 min to 78.8 min upon immobilization. On the other hand, LEV-D.F. exhibited the lowest selectivity but a substantial thermal stability improvement

by around 9-time increase of the enzyme half-life after immobilization. In addition, immobilised LEV-C.O. on Gly-Ag-IDA and immobilised LEV-B.B. on Gly-Ag showed the highest and the lowest reusability upon four successive enzymatic reactions, respectively. Based on the product profile and enzyme reusability results, the immobilized LEV-C.O. was chosen as the most promising option to initiate an immobilized bi-enzymatic system from the combination of the immobilized levanase and the immobilized levansucrase from *Bacillus amyloliquefaciens* (LS-B.A.) on Gly-Ag-IDA/Cu, previously reported to exhibit superior ability in levan synthesis. The potential interference of the selected enzymes was investigated, and the results showed an interference between the LS and LEV towards sucrose. Based on the interference, the ratios of the LS and LEV were adjusted in the bi-enzymatic systems. The two-step, one-step, and co-immobilized bi-enzymatic systems were assessed for FOSs synthesis from sucrose. The two-step bi-enzymatic reaction (1:1 LS/LEV) resulted in the highest oligo yield and enzyme selectivity by producing 45.7% (w/w) of GF_7 after 48 h incubation at 15 ^{o}C, while the co-immobilized system (with the initial ratio of 1 U:0.67 U LS/LEV) ended up the lowest yield and highest relative proportion of GF_2 (10.8% and 23.2% respectively). The two-step bi-enzymatic system produced a detectable level of levans during the bi-enzymatic reaction which indicated the importance of the primary incubation time for levan formation by the LS to achieve higher oligo yields. Also, the product profile study showed the synthesis of levan by LS continued to happen even after the addition of LEV to reach a maximum yield of 24.8% (w/w) after 5 h followed by a decrease in the levan yield and a significant increase in the oligo yield (45.7%, w/w) at 48 h. The use of immobilized LS-B.A. favored the synthesis of HMW levans (>10000 kDa) by producing the highest ratio of the levans at the beginning of the bi-enzymatic reaction. The two-step immobilized bi-enzymatic system was used for conducting an RSM optimization by applying a five-level, two variable central composite rotatable design (CCRD). According to the statistical calculation, applying 15 h and 50 % LS proportion would result in the maximum oligo yield (63%, w/w) indicating the credibility of the effects of the incubation time and the LS proportion. Indeed, the most important factor in oligo yield improvement was the primary incubation time at which the LS carried out the levan synthesis. In the case of short first step incubation time, the presence of short-chain levans would promote excessive hydrolyzing activity of the levanase that decreased the oligo yield; also, long first step incubation time would result in oligo yield decrease but due to suppressed levanase activity.

RÉSUMÉ

L'intérêt élevé par les bénéfices pour la santé des prébiotiques, en particulier leur capacité à moduler et à améliorer l'équilibre du microbiote intestinal, a alimenté le besoin de développement d'approches efficaces et innovantes. En particulier, les β-(2→6) fructooligosaccharides (FOS) et les fructooligomères présentent un potentiel prébiotique plus élevé que les β-(2→1)-FOS commerciaux, en raison de leur persistance colonique accrue et de leur fermentation sélective. Cependant, les FOS et leurs levanes polysaccharides correspondants ne peuvent etre extraits de sources végétales limitées qu'à des faibles concentrations. A cet égard, des approches enzymatiques ont émergé pour combler cette limitation. Deux approches ont été adoptées, dont 1) l'application de lévansucrases (LS, EC 2.4.1) qui sont des fructofuranosidases et capables de catalyser la réaction de transfructosylation à partir du saccharose pour produire des FOS et des levanes; 2) l'utilisation des lévanases (E.C.3.2.1.65), qui sont des enzymes glycosyl-hydrolytiques et capables de catalyser l'hydrolyse des lévanes en β-(2→6) FOS et fructooligomères. Les nouvelles endo-lévanases recombinantes de *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), *Dyadobacter fermentans* (LEV-D.F.), précédemment exprimées et purifiées, ont montré une activité endo-lévanase prometteuse. Le présent travail de recherche visait à immobiliser les nouvelles enzymes recombinantes sur des dérivés d'agarose fonctionnalisés. Le criblage de l'efficacité d'immobilisation des enzymes sur les dérivés d'agarose ayant des charges positifs, ceuxnégatifs, des chélateurs et partiellement hydrophobes a été préformé. LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA et LEV-D.F./Gly-Ag-IDA/Cu ont été choisis pour les études ultérieures car ils ont conduit au meilleur compromis entre le rendement de l'activité enzymatique immobilisée, la rétention d'activité lévanase spécifique, et activité lévanase conservée après incubation à 50 ^{o}C. Les paramètres cinétiques, la stabilité thermique, le profil du produit et la réutilisabilité des enzymes immobilisées ont été étudiés pour chaque enzyme immobilisée sélectionnée. Le rendement total, le rendement en oligo et le profil du produit final, ont révélé que l'utilisation d'une température de 15 ^{o}C et des levans de haut poids moléculaire peuvent maximiser la libération de FOS et limiter celle du fructose; en effet, l'augmentation de la température a déplacé le profil du produit final vers des FOS de poids moléculaire plus faible, à l'exception du système de réaction LEV-DF/Gly-Ag-IDA / Cu immobilisé par levan LMW, et par rapport au levan LMW, l'utilisation du levan HMW a favorisé la formation de GF_7 par rapport aux FOS à chaîne plus courte. LEV-C.O. immobilisé sur Gly-Ag-IDA a montré une sélectivité élevée du produit envers

la production de GF_7 sans libération d'oligosaccharides plus courts, bien que sa demi-vie ait diminué de 202,4 min à 78,8 min par immobilisation. D'un autre côté, LEV-D.F. présentait la sélectivité la plus faible mais une amélioration substantielle de la stabilité thermique par une augmentation d'environ 9 fois de la demi-vie de l'enzyme après immobilisation. En outre, LEV-C.O./Gly-Ag-IDA et LEV-B.B./Gly-Ag ont montré la réutilisation la plus élevée et la plus faible sur quatre réactions enzymatiques successives, respectivement. Sur la base du profil du produit et des résultats de réutilisation des enzymes, le LEV-C.O immobilisé a été choisi comme l'option la plus prometteuse pour initier un système bi-enzymatique immobilisé à partir de la combinaison de la lévanase immobilisée et de la lévansucrase immobilisée de *Bacillus amyloliquefaciens* (LS-B.A.) sur Gly-Ag-IDA/Cu, qui avait précédemment démontré une capacité supérieure en synthèse de levan. L'interférence potentielle des enzymes sélectionnées a été étudiée et les résultats ont montré une interférence entre le LS et le LEV envers le saccharose. Sur la base de l'interférence, les rapports LS et LEV ont été ajustés dans les systèmes bi-enzymatiques. Les systèmes bi-enzymatiques en deux étapes, en une étape et co-immobilisés ont été évalués pour la synthèse des FOS à partir du saccharose. La réaction bi-enzymatique en deux étapes (1: 1 LS/LEV) a conduit au rendement en oligo et la sélectivité enzymatique les plus élevés en produisant 45,7% (w/w) de GF_7 après 48 h d'incubation à 15 ^{o}C, tandis que le système co-immobilisé (avec le rapport initial de 1 U: 0.67 U LS/LEV) a obtenu le rendement le plus bas et la proportion relative la plus élevée de GF2 (10,8% et 23,2%, respectivement). Le système bi-enzymatique en deux étapes a produit un niveau détectable de levans ; alors que celui en deux étapes a démontré un niveau détectable de levanes pendant la réaction bi-enzymatique qui a indiqué l'importance du temps d'incubation primaire pour la formation de levane par le LS pour obtenir des rendements oligo plus élevés. De plus, l'étude du profil de produit a montré que la synthèse de levane par LS continuait de se produire même après l'ajout de LEV pour atteindre un rendement maximal de 24,8% (w/w) après 5 h, suivie d'une diminution du rendement en levane et d'une augmentation significative du rendement en oligo (45,7%, w/w) à 48 h. L'utilisation de LS-B.A immobilisé A favorisé la synthèse des levanes HMW (> 10000 kDa) en produisant le ratio le plus élevé de levanes au début de la réaction bi-enzymatique. Le système bi-enzymatique immobilisé en deux étapes a été utilisé pour effectuer une optimisation par RSM en appliquant une conception rotative composite centrale (CCRD) à cinq niveaux et deux variables. Selon le calcul statistique, le temps de réaction de 15 h et de 50% de LS entraînerait un rendement d'oligo maximal (63%) indiquant la crédibilité des

effets du temps d'incubation et de la proportion de LS. En effet, le facteur le plus important dans l'amélioration du rendement en oligo était le temps d'incubation primaire auquel le LS a effectué la synthèse du levane. Dans le cas d'une courte durée d'incubation de la première étape, la présence de levanes à chaîne courte favoriserait une activité d'hydrolyse excessive de la lévanase qui diminuait le rendement en oligo; en outre, un long temps d'incubation de la première étape entraînerait une diminution du rendement en oligo mais en raison de la suppression de l'activité lévanase.

STATEMENT FROM THE THESIS OFFICE

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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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As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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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 prebiotics especially fructooligosaccharides (FOSs), the challenges facing levan-type FOS synthesis, the strategies applied to accomplish enzymatic levan-type FOS synthesis, and outlines the research objectives of the current study.

Chapter II comprises a literature review of the studies relevant to prebiotic classification, current methods of prebiotic production, especially enzymatic approaches, enzyme immobilization and co-immobilization strategies, the advantages and potential risks related to such, and bi-enzymatic systems developed for levan-type FOS production. Finally, the analytical techniques for quantitative and qualitative FOS analysis are reviewed.

Chapter III presents the results of the immobilization of three selected recombinant levanases on glyoxyl agarose derivatives. The main enzyme immobilization features, including enzyme activity yield, retained specific enzyme activity, and retained enzyme activity after incubation at 50 ^{o}C, were discussed as they are related to the support type and the levanase properties. The best support that compromises between the immobilisation parameters were selected for each levanase. The product profile and the reusability of immobilized levanases were also examined to identify the best-immobilized levanase for the bi-enzymatic system design.

In Chapter IV, bi-enzymatic systems based on the combined use of selected immobilized levanase and immobilized levansucrase were investigated. The bi-enzymatic systems were set in different modes, two-step, one-step, and co-immobilized. Oligo yield, levan yield, total yield, and the product profile of the bi-enzymatic systems were determined. Finally, an RSM design of the most promising immobilized bi-enzymatic system was carried out in order to study the effect of reaction parameters and to optimize the oligo yield.

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

Ag	Agarose
AMU	Atomic mass unit
ATCC	American type culture collection
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
EC	number Enzyme classification number
EDA	Ethylenediamine
ETOH	Ethanol
FOSs	Fructooligosaccharides
Fru	Fructose
Gal	Galactose
Gly-Ag	Glyoxyl Agarose
HPAEC-PAD	High-pressure anion exchange chromatography with a pulsed amperiometric detector
HPLC	High Pressure Liquid Chromatography
HPSEC	High Pressure Size Exclusion Chromatography
IDA	Iminodiacetic acid
Kcat	Turnover number
Km	Michaelis-Menton constant
LEV	Levanase
LS	Levansucrase
Man	Mannose
MANEA	Monoaminoethyl-N-ethyl agarose
MeOH	Methanol
MW	Molecular weight
n	Hill coefficient
PEG	Polyethylene glycool
PEI	Polyethylenimine
PST	Potassium sodium tartrate
RPM	Rotations per minute
SCFA	Short chain fatty acid
scFOS	Short chain fructooligosaccharides (2-5 saccharide units)
SDS-PAGE	Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
Suc	Sucrose
TEA	Triethylamine
Vmax	Maximum velocity

CHAPTER I

General Introduction

Non-digestible oligosaccharides are important food ingredients and crucial to the health of the digestive system as they serve as prebiotic components of the foods. The requirements of an effective prebiotic are as follows: 1) being resistant to the gastric acidity and the hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) being fermentable by the intestinal microflora; and 3) being selective stimulant of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (Gibson et al., 2004; Roberfroid, 2007). Fructooligosaccharides (FOSs) are an important group of non-digestible oligosaccharides and have been applied as low-calorie and alternative sweeteners (Petal, 2011). As an effective prebiotic food ingredient, fructooligosaccharides (FOSs) are resistant to the digestion in the small intestine and can be fermented to short-chain fatty acids in the large intestine (Roberfroid, 2007). The fermentation of FOSs results in the proliferation of beneficial colonic bacteria through decreasing the colonic and intracellular pH. FOSs have also been used as a sweetener to lower the calorie content of traditional foods suitable for a diabetic diet (Alles et al., 1999).

Inulin- and levan-type FOSs are two main sub-groups of these non-digestible oligosaccharides. Recently, there has been an increased interest in levan-type FOSs, which are characterized by β-(2,6)-glycosidic bonds, because of their higher prebiotic capacity and their increased colonic persistence compared to the inulin-type FOSs (W. Zhang et al., 2019). However, there are only fewer reports on levan-type FOSs, and no abundant natural sources of these prebiotics. Levanase has been identified as a potential biocatalyst for the production of levan-type FOSs from the hydrolysis of levan polysaccharides. However, the use of levanase is still limited by their low thermal stability and availability. In the present study, immobilisation of levanase was investigated in order to enhance their catalytic action, to improve their thermal stability, to modulate their end-product specificity and to allow their reusability.

Synergistic effect of a combination of levansucrase from *Bacillus amyloliquefaciens* (LS-B.A.) and endo-inulinase in the production of FOSs has been investigated by Tian and Karboune (2013). The synthesis has been carried out either in one-step or two-step bi-enzymatic system. In this bi-enzymatic system, levansucrase catalyzes the synthesis of levan or oligolevans from sucrose as the starting material, while the endo-inulinase hydrolyzes the levan products to oligolevans and FOSs. The levansucrase/endo-inulinase reaction system led mainly to the production of short-chain FOSs. In addition, the hydrolytic action of endo-inulinase was hindered by the high molecular

weight (HMW) of levans. It can be hypothesized that the use of highly specific endo-levanases (instead of endo-inulinase) for the hydrolysis of β-(2,6)-glycosidic bonds may enhance the product specificity of the bi-enzymatic system. In the present study, the application of the immobilized levanases in combination with the immobilized levansucrase in the bi-enzymatic system was investigated. The immobilized bi-enzymatic systems have been evaluated in terms of yield and end-product profile.

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

1. Study the immobilization of selected levanases and assess their catalytic efficiency

    1.1. Investigate different strategies for the immobilization of levanases from *Belliella baltica*, *Capnocytophaga ochracea*, *Dyadobacter fermentans*.

    1.2. Determine the immobilization efficiency in terms of protein immobilization yield, activity immobilization yield, retention of activity, and end-product profile.

    1.3. Study the thermal stability and the kinetic parameters of the immobilized levanases (Km, kcat, Vmax)

    1.4. Characterize the end-profile of immobilized levanases using well-defined reaction parameters.

2. Study the combined use of immobilized levanase and levansucrase in the bi-enzymatic system to produce levan-type FOSs from sucrose as an abundant substrate.

    2.1. Investigate different strategies for conducting the bi-enzymatic system: one step mode; two-step mode; the use of levansucrase and levanase immobilized on different supports; or co-immobilized on the same support.

    2.2. Determine the efficiency of the bi-enzymatic systems in terms of total yield, oligo yield and end-product profile (MW distribution of FOSs).

    2.3. Optimize the two-step bi-enzymatic system and determine the predictive models that help better to understand the effects of reaction parameters.

CHAPTER II

Literature Review

2.1 Introduction

Functional food and nutraceutical industry are growing fast and the market is demanding for the novel products that address the emerging health issues and improve the quality of human life. The future of the industry is bright and according to Statista, a well-known online portal for global statistics, the revenue of the industry is going to boom from about 174.75 billion in 2019 to over 275.77 billion U.S. dollars in 2025. Amongst the functional foods, oligosaccharides have attracted a high interest as food ingredients and pharmacological supplements. The non-digestible oligosaccharides have been applied as dietary fibre, sweetener, weight controlling agent and humectant in confectioneries, bakeries and breweries (Patel, 2011).

2.2 Oligosaccharide Classification upon their Bioactivity

Oligosaccharides can be categorized as digestible and non-digestible (Mussatto et al., 2007), with the main difference being the resistance of non-digestible oligosaccharides to the hydrolytic activities of human digestive enzymes (e.g., α-glucosidase, maltase-isomaltase, and sucrase) due to the presence of β-glycosidic bonds (Roberfroid et al., 2000). Non-digestible carbohydrates are undigested or minimally digested in the stomach or small intestine but are able to migrate to the large intestine where they may be utilized by gut microbiota as substrates resulting in a number of metabolites (Esawy et al., 2013).

Indigestible carbohydrates have been classified based on their type of monomer, their glycosidic linkages, and their source. According to Paeschke and Aimutis, indigestible carbohydrates can be categorized into ten groups including fructans, galactans, synthetic non-digestible carbohydrates (SNC), grain, fruits and vegetable oligo and polysaccharides (GFVOP), resistant starches, galactomannan polysaccharides, microbial polysaccharides, seaweed polysaccharides, glucomannan polysaccharides, and tree exudate polysaccharides (Paeschke & Aimutis, 2011). The list of known and approved non-digestible carbohydrates has updated regularly by FDA and Health Canada due to emerging new synthetic or natural forms. A group of well-known industrialized prebiotics involves fructans which are produced by polymerization or oligomerization of fructose, and are divided into three sub-groups: inulin, fructooligosaccharide (FOS) and oligofructose (OFS), and levan. They are classified based on the type of glycosidic bonds and the degree of polymerization (DP); that is, inulins have β (2→1) glycosidic bond often with a terminal glucose,

while levans have β (2→6) linkages, and FOSs could have both glycosidic linkages (Paeschke & Aimutis, 2011).

FOSs have been reported to be more selectively fermented by *lactobacilli* and *bifidobacteria* than other non-digestible oligosaccharides (NDOs) (Olano-Martin et al., 2002) which may be attributed to the bacterial membrane structure, for example membrane-bound β-fructofuranosidase is capable of hydrolyzing FOSs (Perrin et al., 2001). In addition, FOSs have been proven to promote the population of beneficial bacteria in the intestinal tract (Videla et al., 2001; Butel et al., 2002; Guigoz et al., 2002; Hoentjen et al., 2005; Osman et al., 2006; Vos et al., 2006). In the case of bifidobacteria, improved resistance against bile and consequently better survival rate and adherence in the colon can be achieved by the virtue of FOSs (Perrin et al., 2000). β-fructofuranosidase from *B. adolescentis* has been reported to hydrolyze both the levan-type FOSs (ex. neokestose) and the inulin-type FOSs (ex. as 1-kestose) although they have shown higher substrate specificity towards neokestose (Omori et al., 2010). Most importantly, levan-type FOSs have proven to be significantly more effective prebiotics than inulin-type FOS (Marín-Navarro et al., 2015; Omori et al., 2010) and been of great commercial interest because only inulin-type FOS are currently available (Kilian et al., 2002) which mainly contain 1-kestose (Glc-Fru2), nystose (Glc-Fru3) as well as fructofuranosylnystose (Glc-Fru4) (Plou et al., 2007b; Rastall, 2010). The commercial FOSs are rapidly fermented by anaerobic bacteria in the proximal colon which results in the formation of short-chain fatty acids (SCFAs) that promote a healthy digestive system although it does so only in this restricted part of the intestinal tract. Indeed, carbohydrate depletion causes proteolytic fermentation to dominate the activity of anaerobes in the distal colon resulting in the formation of phenolic compounds, amines and ammonium, which are considered toxic metabolites (Manning & Gibson, 2004). Furthermore, long-chain levan-type FOSs have been shown to function as NDOs more effectively than the short-chain type (Lovegrove et al., 2017) and can promote prebiotic activity in the distal colon, the region most susceptible to colon cancer (Rastall & Maitin, 2002). In addition, longer chain FOSs as prebiotics in infant formula are less likely to induce intestinal discomfort (Fanaro et al., 2005).

2.3 Fructan Classification and Structure

2.3.1 Fructooligosaccharides (FOSs)

Fructooligosaccharies are made up of 3 to 10 monosaccharides including fructose monomers with β (2→1) or β (2→6) glycosidic bond and often contain a terminal D-glucose joined by a α (1→2) glycosidic linkage. They are usually found in fruits and vegetables such as banana, onion, chicory root, garlic, asparagus, jicama, and leeks. Although some grains and cereals such as wheat and barley contain FOS (Campbell et al., 1997), the Jerusalem artichoke and its relative yacón as well as the Blue Agave plant have been found to have the highest concentrations of FOS of cultured plants (Dumitriu, 2005).

FOSs can be classified as inulin-type, neoinulin-type, levan-type, neolevan-type and mixed levan-type. In inulin-type, D-fructosyl units are attached by β (2→1) glycosidic linkages and the simplest molecular structure in this class is 1-kestose, a trisaccharide with terminal glucose joined with an α (1→2) glycosidic bond (Figure 1). In levan-type, the glycosidic bond is β (2→6) joining the fructosyl moieties and the simplest compound is 6-kestose, a trisaccharide with a terminal glucosyl moiety attached through an α (1→2) glycosidic bond. In either neoinulin- or neolevan- types, the core structure is a glucoside moiety attached to the fructosyl moieties through β (1→2) or β (6→2) glycosides linkages. The most basic compound in this category is neokestose (Figure 2.1). Finally, the mixed levan-type has both β (1→2) and β (6→2) glycosides linkages between fructosyl moieties, but the glucoside moiety is the terminal group and not the core structure. The basic structure in this class is a tetrasaccharide called bifurcose in which the core structure, fructose, is joined by two other fructoses via β (2→1) and β (2→6) glycosidic bonds respectively and a terminal glucoside moiety is attached by an α (1→1) glycosidic linkage (Figure 2.1). It is noteworthy to add that galactooligosaccharides (GOS) are the animal-sourced version of FOS which are known as non-digestible oligosaccharides and naturally found in many animals' milk including cow and human; that is, FOS are oligomers of fructose with terminal glucose and GOS are oligomers of galactose with terminal glucose. However, the glycosidic linkages of GOS are more varied. Coulier et al. have studied Vivinal GOS (a commercial GOS) and found the predominance of the (1→4)-linked β-D-Gal residue in the oligosaccharides, but other linkages such as (1→6)-linked β-D-Gal and (1→3)-linked β-D-Gal were also observed (Hernández et al., 2012). In the case of shorter oligosaccharides (di- and trisaccharides), the reducing terminal

glucose might be attached through positions 2, 3, 4, or 6, while in a longer oligosaccharide chain, linkages at the position 4 predominate.

2.3.2 Polysaccharides: Inulin and Levan

Inulin is a polysaccharide of *D*-fructosides joined by *β* (2→1) glycosidic linkages with a terminal *D*-glucoside moiety. It is the second most abundant carbohydrate designed for energy storage after starch. The degree of polymerization depends on the origin of inulin; that is, bacterial inulin could contain up to 1000 monomers, while inulin of plant-origin does not usually have more than 70 monomers (Robyt, 1998). Paeschke & Aimutis reported that inulins are found in the roots and tubers of Compositae family, which includes aster, dahlias, cosmos, chicory, lettuce, and Jerusalem artichokes (Paeschke & Aimutis, 2011). They are also found in the Liliaceae family, comprising of lily bulbs, onion, and tulips. Although the substantially higher degree of polymerization (DP) of bacterial inulins would make them better options for being starting material in inulin-type FOS production, the plant sources would benefit from the high availability and less laborious production procedure. Levans have more complex structures than inulin as they have β (2→6) linked fructose, with branches of fructose linked by β (2→1) glycosidic bonds.

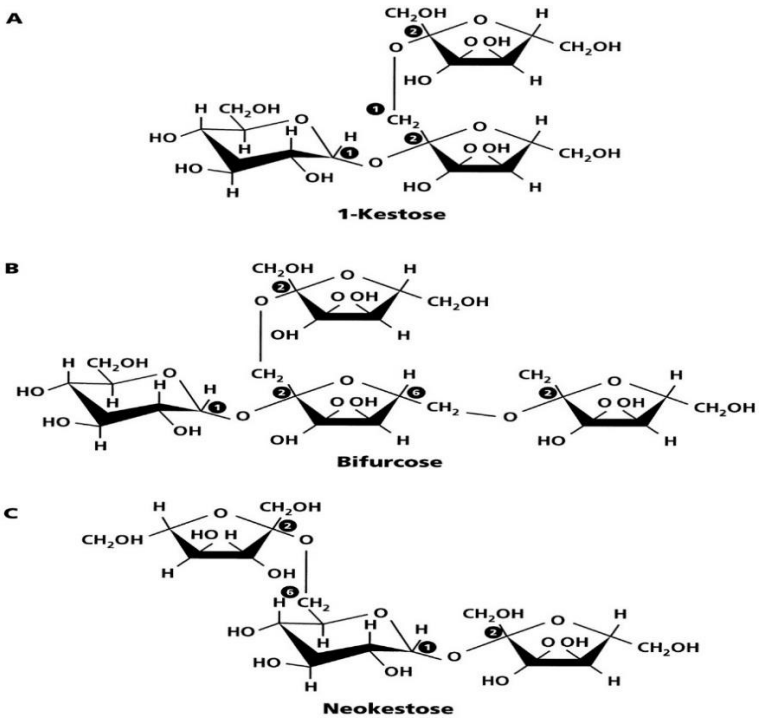

Figure 2. 1. The simplest FOS representative of the sub-groups

The long-chain levans in plant sources have much higher DP and consequently greater molecular weight (e.g., 1700–1850 kDa) in comparison with inulin (Hendry & Wallace, 1993). The DP in levans also depends on the origin; and bacterial levans generally have much higher DP than plant levans (Hendry & Wallace, 1993).

2.4 Production of Fructans

Fructans can be obtained using three methods: plant extraction, chemical synthesis, and enzymatic production. The first method utilizes plant sources as raw material, specifically chicory (Cichorium intybus) and is the most common technique to produce inulin in the food industry. A plant source that can commercially produce levans or levan-type FOSs has not been reported (Paeschke & Aimutis, 2011).

2.4.1 Fructan Chemical Synthesis

FOSs can be produced by chemical synthesis in two ways including polymerization of monosaccharides and hydrolyzation of polysaccharides. In the bottom-up strategy in which the synthesis begins from the monomers, considering they have various functional groups and chiral centers, selective protection-deprotection steps are necessary to control the stereochemical and regiochemical specificity of the desired glycosidic bonds. Moreover, the chemical synthesis of FOSs is a multi-step process with laborious and costly procedures and involves toxic reagents that are not safe to use based on food safety guidelines (Plante et al., 2003). In the top-down strategy in which the synthesis begins from polysaccharides, selective chemical hydrolysis is challenging to achieve, so a complex mixture of products can be produced containing brown contaminants resulting from the conventional heating procedure (Warrand & Janssen, 2007).

2.4.2 Enzyme-mediated Fructan Production: Fructosidase, Fructanase (Fructosyltransferase) and Inulinase

There are two strategies for the enzymatic synthesis of fructans. The first one is the bottom-up strategy; that is, the enzymes are used to produce fructans by transfructosylation from simple saccharides containing fructose such as sucrose to produce FOSs and corresponding polymers. β-fructofuranosidases (EC 3.2.1.26) and fructosyl-transferases (EC 2.4.1) are two groups of enzymes that follow the bottom-up strategy by cleaving fructosyl moieties from simple carbohydrates and coupling them to obtain a higher degree of polymerization (Anwar et al., 2010). The second

strategy is top-down in which high molecular fructans undergo controlled hydrolyzation by the use of fructanases (Sangeetha et al., 2005a).

2.4.2.1 Enzymatic Fructan Synthesis by Using the Top-Down Strategy

FOSs can be produced using the top-down strategy, in which the controlled hydrolysis of fructans can produce the desirable FOSs by using endo-fructanases. The top-down strategy offers the selective hydrolysis of inulins or levans to produce controlled molecular weight of inulin-type and levan-type FOSs. The prerequisite of the strategy is the availability of the fructans, which can be isolated from natural sources or produced by fermentation or by enzymes. Endo-inulinases can break down β (2→1) glycosidic bonds of inulin to produce inulin-type FOSs. The mixture of these inulin-type FOSs obtained by endo-inulinase is similar to that produced by enzymatic transfructosylation, but they may have a higher degree of polymerization (Sangeetha et al., 2005b) and do not always have the terminal glucosides (Rastall, 2010). On the other hand, endo-levanases can hydrolyse β (2→6) glycosidic bonds of levan to produce levan-type FOSs. Chen and Karboune (2017) have reported levanases from *Belliella baltica* (LEV-B.B.), *Dyadobacter fermentans* (LEV-D.F.), *Capnocytophaga ochracea* (LEV-C.O.) showing high levels of endo-hydrolytic activity on levans than inulin. Although the reaction mechanism of levanases has not been fully elucidated, the active residues involved in the hydrolysis activity were suggested to be from aspartate and glutamate acting as nucleophile and proton donor, respectively (Song & Kim, 2002). The hydrolysis of the glycosidic bond can take place with either inversion or retention of the configuration of the anomeric carbon. A levanase from *Microbacterium laevaniformans* was used in multiple sequence alignments with other fructosylhydrolases to analyze the conserved residues and to determine the crucial amino acids functioning in the active site. The amino acids were identified as Asp86 and Glu2707/Cys271/Pro272 (Song & Kim, 2002). The isoelectric point of levanases is low and ranges from 4.1 to 4.8 with the optimum pH between 5.0 and 7.0 (Kang et al., 1998; Lim & Kang, 1998).Their optimum reaction temperatures vary between 30 °C to 60 °C (Yokota et al., 1993), and their molecular weights are found to be in the range of 38-135 kDa (Murakami et al., 1990).

2.4.2.2 Enzymatic Fructan Synthesis by Using the Bottom-to-Up Strategy

β-fructofuranosidases (EC 3.2.1.26), which have been obtained from *Aureobasidium pullulans* (Yun, 1996) and *A. niger* (Hidaka et al., 1988), are powerful tools for the catalysis of the

transfructosylation reactions. They have been applied industrially to produce FOSs using sucrose as the starting material with a yield of 51.9% (w/w) (Kurakake et al., 2010). The downsides of this type of enzyme are their modest yield as well as their poor specificity and regioselectivity, as their product profile shows a quite complex mixture of the FOSs (Plou et al., 2007). The effectiveness of the FOS production method is dependent on the origin of the enzymes and the reaction conditions, such as sucrose concentration, *p*H, and reaction temperature. In fact, the transfructosylation reaction is favorable under neutral or basic conditions and low temperatures since the hydrolysis reaction competes significantly with the transfructosylation reaction (Plou et al., 2007). To make the transfructosylation reaction favorable, three strategies can be followed: i) removal of the transfructosylation reaction end product from the reaction mixture by crystallization, selective adsorption, or coupling through another enzymatic reaction (Plou et al., 2007); ii) application of high-affinity fructosyl acceptors to suppress hydrolyzation reaction; iii) genetic modifications of enzymes by site-directed mutagenesis, improving the intrinsic affinity of the original enzymes towards transfructosylation (Perugino et al., 2004). Fructansucrases are another group of enzymes applied for the synthesis of fructans. They are able to carry out the transfructosylation reaction on both levan- (with β (2→6) glycosidic bonds) and inulin-type fructans (with β (2→1) glycosidic bonds) (Andersone et al., 2004). Based on the type of glycosidic linkages that are being produced by fructansucrases, they are divided into two sub-groups: levansucrases (LSs) for β (2→6) and inulinsucrases for the synthesis of β (2→1) glycosidic bonds. These enzymes can be produced by both gram-positive and gram-negative bacteria. *Bacillus sp.* and *Zymomonas sp.* have been used for LS production (Kim, et al., 2005; Nakapong et al., 2013), while inulinsucrases are exclusively found in lactic acid bacteria (Hijum et al., 2002).

2.5 Levanase

Levanases (2,6-β-D-fructanohydrolase, EC 3.2.1.65) catalyze the hydrolyzation of β-(2,6)-linked fructans consisting of various FOSs and levanoligosaccharides (Mardo et al., 2017; Soon Lim & Kyung Kang, 1998). They belong to glycoside hydrolase (GH) family 32 which also includes invertases (EC 3.2.1.26), endo-inulinases (EC 3.2.1.7), exo-inulinases (EC 3.2.1.80), and other enzymes with homologous topology and sequence motifs (Mardo et al., 2017). Levanases are divided into two types: 1) exo-levanase (EC 3.2.1.64), and 2) endo-levanase (EC 3.2.1.65). However, some levanases have been reported to not be classified either as exo- or endo-levanases

as they produced fructose and FOSs (Miller & Somers, 1978, Igarashi et al., 1987, Chaudhary et al., 1996). Exo-levanases hydrolyze levans to produce mainly fructose (Zhang et al., 2019). In terms of microbial sources, exo-levanases have been identified in *Pseudomonas* species (Avigad & Zelikson, 1963), *S. salivarius* KTA- 19 (Tanaka et al., 1983), *Streptomyces* species No. 7-3 (Murakami et al., 1990), *Streptomyces exfoliates* F3-2 (Yokota et al., 1993), and *Streptomyces sp.* K52 (Kang et al., 1998). Endo- levanases catalyze the levan hydrolysis to produce FOSs and/or fructooligomers (Zhang et al., 2019). These enzymes have been reported in some microbial sources including *Arthrobacter* species (Avigad & Bauer, 1966), *Bacillus* species (Miasnikov, 1997), and *G. diazotrophicus* SRT4 (Menedez et al., 2004). Furthermore, levanases have been reported in yeasts and filamentous fungi (Dahech et al., 2013).

There has been limited data about the 3D structure of levanases due to their scarcity; that is, these enzymes have been identified in only fifteen microbial sources, and, in general, they are highly branched and heterogeneous in terms of molecular weight and the structural elements responsible for substrate binding and specificity (Mardo et al., 2017; Zhang et al., 2019). Therefore, the study of the 3D structures and mechanisms of their actions are still new and challenging research topics. Endo-levanases have been identified to have a 5-bladed β-propeller fold and their hydrolyzing activity is due to the action of a proton donor (i.e., glutamate), and a nucleophile (predominantly aspartate) (Mardo et al., 2017). Also, endo-levanases produce different FOSs in terms of degree of polymerization (DP) based on their origins (Zhang et al., 2019). For example, levanases from *Bacillus sp*. L7 (Miasnikov 1997) and *Bacillus licheniformis* (Zhang et al., 2019) break down levans into a variety of FOSs with a degree of polymerization of 2–10. However, levanases from *Bacillus sp*. 71 and *Pseudomonas* K-52 showed higher selectivity by producing levanheptaose (DP = 6) (Zhang et al., 2019) and levanoctaose (DP = 7) (Kang et al., 1998) as the main products of levan hydrolysis. Also, the levanase from *Pseudomonas sp.* 43 exhibited high selectivity towards levanbiose production by hydrolyzing levans (Kang et al. 1999).

In terms of substrate specificity, levanases generally have the ability to hydrolyze sucrose and inulin besides levans (Miasnikov, 1997). Also, levanases from *Rhodotorula sp.*, *Treponema zioleckii*, and *Gluconacetobacter diazotrophicus* proved to be able to hydrolyze inulin, phlein, and other short-chain FOSs as well as levans (Zhang et al., 2019).

2.6 Levansucrase

2.6.1 Properties and Functions

LSs are powerful catalysts that carry out transfructosylation reaction with β (2→6) glycosidic linkages by using different acceptors. The physicochemical properties of LSs, such as their primary structure (amino acid sequences), tertiary structure (their active site conformations), sizes and molecular weights, end-product profiles, regio-specificity, and donor/acceptor specificity are dependent on their origin. Both gram-positive (Belghith et al., 2012) and gram-negative bacteria (Vigants et al., 2003) can produce LSs. Microbial LSs have been shown to have molecular weights between 45 to 220 kDa (Hettwer et al., 1995). The optimal pH of bacterial LSs is between 5.0 to 6.6 (Homann et al., 2007), and the optimal temperature between 25 to 60 ^{o}C; for example, the optimum temperature of *B. megaterium* is quite high (45 to 50 ^{o}C) (Homann et al., 2007), while for others such as *laevaniformans,* it is 30 ^{o}C (Park et al., 2003).

2.6.2 Types of Levansucrase-catalysed Reactions

LS, an enzyme responsible for *β*-D-fructosylation, can carry out four different reactions based on the acceptor (Figure 2.2): i) transfructosylation which requires sucrose, its analogs, or other FOSs; ii) hydrolysis which becomes favorable in the presence of water as the main acceptor; iii) exchange which takes place in the presence of monosaccharides; and iv) polymerization which is the predominant reaction in the presence of levans which serve as the main acceptors (Martinez-Fleites et al., 2005).

The ratio of potential products is primarily dependent on the origin of the enzyme. For example, *B. subtilis* and *B. megaterium* mainly produce levans from sucrose (Homann et al., 2007), while *Gluconacetobacter diazotrophicus*' products are predominantly short FOSs (Hernandez et al., 1995). Moreover, *Z. mobilis* produces a mixture of FOSs and levans (Homann et al., 2007).

The mechanism of all aforementioned reactions begins with the same first step which is the formation of fructosyl-enzyme intermediate. However, the ability of the fructosyl-enzyme intermediate to transfer the fructosyl moiety to an acceptor is heavily dependent on the origin of the enzyme and consequently, on its structure (Hernandez et al., 1995). This diversity allows different species to meet their requirement of life by modifying the change in structure and reactivity of the enzyme (Song & Jacques, 1999).

2.6.3 Mechanism of Levansucrase functions

According to different types of studies on the mechanism of LS including sequence alignment, site-directed mutagenesis, and structural studies (Meng & Futterer, 2003), there are always three amino acids in the active site of any LSs which are directly involved in the transfructosylation reaction though their position in the protein chain and in the active site are dependent on the origin of the LS. For example, in *B. subtilis* LS, the three amino acids are Asp86, Glu352, and Asp 247 (Meng & Futterer, 2008), but in *B. megaterium LS*, they are Asp95, Asp257, Glu352 (Strube et al., 2011). In general, one of the Asp in each case (the lower number in both cases) acts as a nucleophile to attack the anomeric center, the second Asp creates strong hydrogen bonds with OH groups of C-3 and C-4 of the fructosyl unit to stabilize the transition state of the transfructosylation reaction, and finally, the Glu plays a role as an acid-base catalyst. The catalytic reaction follows a "ping-pong" mechanism, in which the enzyme is transformed into a fructosyl-enzyme intermediate, followed by the transfructosylation product release (Hijum et al., 2003; Meng & Futterer, 2003).

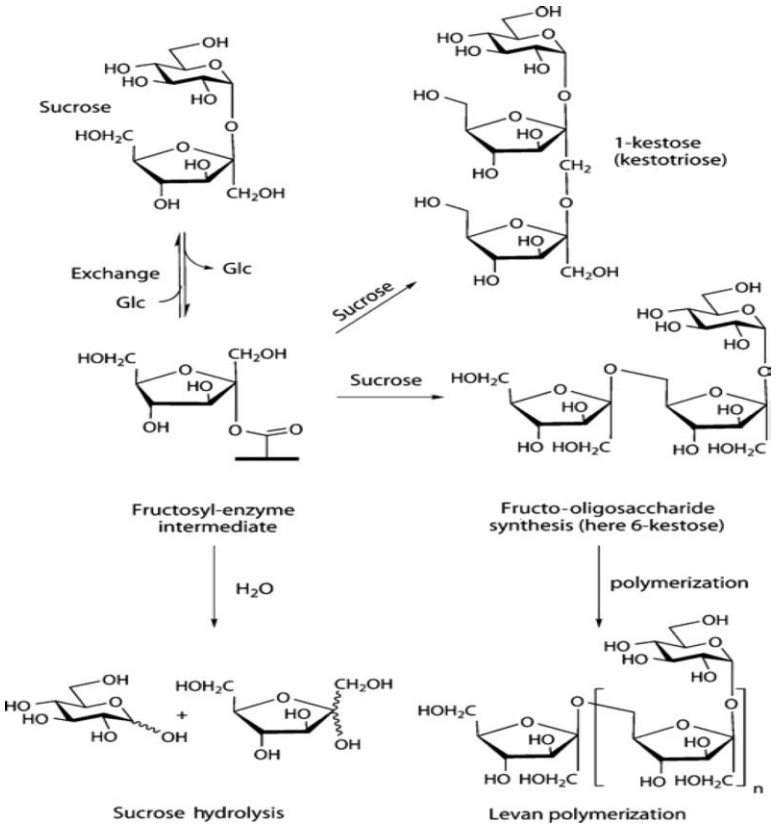

Figure 2. 2. Possible reactions catalyzed by LSs (Martinez-Fleites et al., 2005)

2.6.4 Levansucrase Substrates Specificity

The affinity of LSs towards carbohydrate donors primarily depends on the origin of the LSs. For example, LS of *Z. mobilis* can use both sucrose and raffinose as a donor, but has a higher affinity toward raffinose, especially at low substrate concentrations (Andersone et al., 2004). On the other hand, *M. laevaniformans* LS has almost the same affinity toward both sucrose and raffinose in comparison with stachyose (Kim et al., 2005). The more likely the residue connected to fructosyl can be stabilized by the subsites of LSs, the more is the affinity for the donor, so short-chain FOSs are not usually preferred donors as the position and orientation of their tails are not similar to sucrose. In fact, a trisaccharide or tetrasaccharide can serve as a fructosyl donor provided the LS has available and suitable +2 of +3 subsites to stabilize the tails (Chuankhayan et al., 2010). The ability of LSs' subsites to stabilize special types of FOSs allows the LSs able to use the FOSs as acceptors of the new fructosyl moiety coming from the fructosyl-enzyme intermediate. For example, maltose, cellobiose, melibiose, and lactose were found to be the best acceptors, while sugar alcohols like xylitol and arabitol had the lowest affinity toward being an acceptor for *B. subtilis* (Seibel et al., 2006). Reducing saccharides have been shown to have a greater ability as acceptors compared to non-reducing saccharides in the case of *B. subtilis*, *R. aquatilis*, and *M. laevaniformans* (Kim et al., 2005; Park et al., 2003; Seibel et al., 2006).

2.6.5 End-product Profile and Product Specificity

To explain each mode of action of LSs, "non-processive" *vs* "processive" mechanisms have been proposed (Ozimek et al., 2006). Each LS's active site has several subsites which are labeled as -1, +1, +2, +3, and so on. Transfructosylation occurs at subsites -1 and +1 by positioning the fructosyl moiety on subsite -1 and the glucosyl moiety on subsite +1; the subsequent formation of the fructosyl-enzyme intermediate occurs at subsite -1 and the departure of glucose at subsite +1. The ratio of potential products formed based on the intermediate depends on the capability of the vacant subsites to stabilize a special acceptor. Indeed, if they could stabilize water in the subsites, the dominant reaction would be hydrolysis, while in the case of stabilization of a monosaccharide, oligosaccharide or polysaccharide, the product would be a disaccharide, a longer chain of an oligosaccharide, or a longer chain of a polysaccharide, respectively (Figure 2.3). A study showed that not only is the active site composition of the LS crucial for enzymatic activity, but other parts of the enzyme could determine the ratio of the potential products because of their role in stabilizing or destabilizing oligosaccharide acceptors (Strube et al., 2011).

2.7 Enzyme Immobilization and Co-immobilization

Enzyme immobilization is a common strategy for the stabilization of enzymes by attaching them onto an inert material to increase their rigidity. As enzymes are intrinsically sensitive molecules and can undergo a reversible or irreversible conformational change due to temperature or environmental variations, immobilization has been used to avoid the undesirable folding which can change the tertiary structure and consequently the active site of the enzyme (Mozhaev & Melik-Nubarov, 1990). The support material is usually water-insoluble to produce an easy-to-recover enzyme although soluble supports have also been used to produce more stable soluble enzymes (Mozhaev & Melik-Nubarov, 1990)

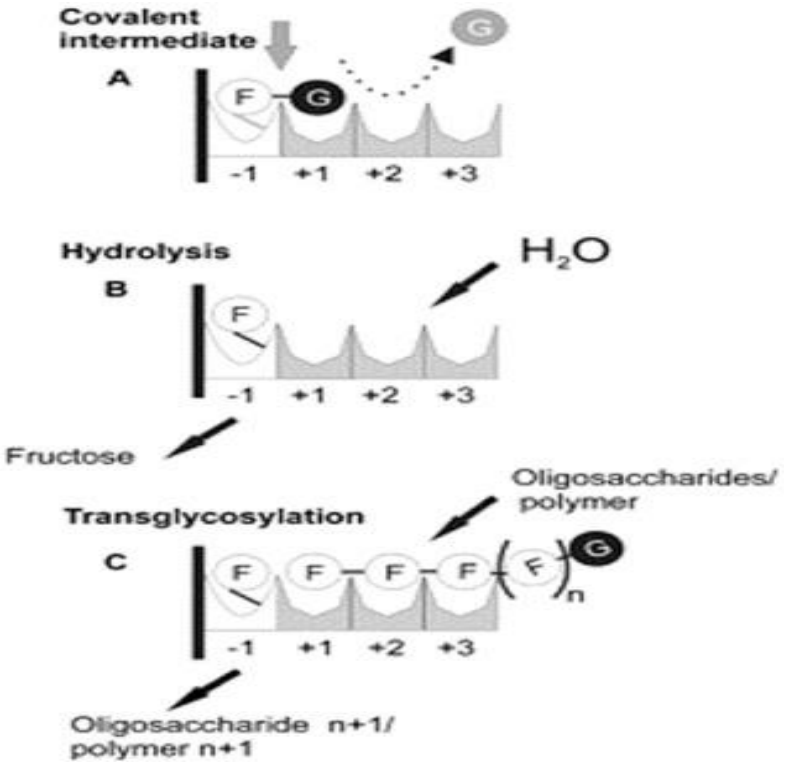

Figure 2. 3. Schematic presentation of LSs active site (Ozimek et al., 2006)

2.7.1 Enzyme Immobilization Advantages

To make an enzyme reusable and consequently economically viable for industrial applications, immobilization has proven to be a successful method (Tischer & Kasche, 1999). Furthermore, enzyme immobilization can result in improved enzyme stability and even modification of the enzyme's original activity in a targeted way (Tischer & Kasche, 1999).

2.7.2 Strategies of immobilization

Enzyme immobilization could be categorized as either reversible immobilization techniques, including adsorption, ionic binding, affinity binding, and metal binding, or irreversible ones, including covalent binding, entrapment, and aggregation (Figure 2.4) (Karav et al., 2017). The drawback of reversible techniques is the possibility of enzyme leaching and consequently enzyme loss although these techniques offer the possibility of reloading the support with fresh enzyme when the activity of the immobilized enzyme decreases. On the other hand, irreversible immobilization can minimize enzyme loss, but could result in dramatically decreasing enzyme activity due to the deformation of the enzyme's active site and intrinsic diffusion issues (Karav et al., 2017).

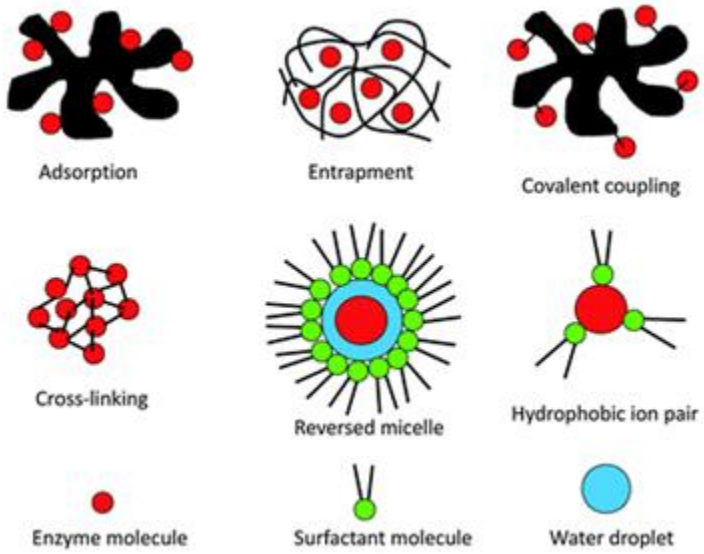

Figure 2. 4. Different techniques for enzyme immobilization (Karav et al., 2017)

2.8 Enzyme Co-immobilization and its Challenges

The co-immobilization of enzymes has been used to influence the combined catalytic activity of two or more enzymes. This strategy has used the same techniques as simple enzyme immobilization (Jia et al, 2014). However, co-immobilization presents challenges to obtaining successful immobilization as it needs to meet the spatial and proximity requirements of the enzymes to show an effective combination of their individual reactivity. This has been addressed by creating multi-enzyme complexes (MECs), where a series of enzymatic reactions take place without any departure of the intermediates from the MECs before the formation of the final

product. In fact, the intermediates are transferred between MECs' subunits very efficiently. Not only does the suitable conformation of each individually immobilized enzyme matter, but their relative position and proximity also needs to be considered as a vital factor for a successful co-localization of the enzymes (Jia et al., 2014).

2.8.1 Some Examples of Enzyme Co-immobilization

As mentioned in the previous paragraph, enzyme co-immobilization techniques use the same strategies as simple immobilization. However, the number of publications in this field is still not comparable with single enzyme immobilization. There are several publications about enzyme co-immobilization by physical adsorption which have mostly focused on co-immobilization of glucose oxidase and catalase. This combination is crucial for sustainable enzymatic oxidation of glucose. Due to the accumulation of hydrogen peroxide as the side product of glucose oxidation, denaturation of glucose oxidase occurs because of the oxidation of methionine and cysteine residues of the enzyme (Zhao et al., 2018). In 2014, Mahdizadeh and Eskandarian developed the co-immobilized system of glucose oxidase and catalase on biosynthesized nanoporous SiO_2 to remove dissolved oxygen in water to control corrosion of boilers (Mahdizadeh & Eskandarian, 2014). In this article nanoporous SiO_2 was dispersed in a solution of the enzymes in acetate buffer to adsorb the enzymes onto the nanoporous platform. In 2017, Christwardana *et al* reported an effective co-immobilization of the enzymes on polyethyleneimine (PEI) wrapped in carbon nanotubes (CNT) by manipulation of the positive charge on the surface of PEI, which is adsorbed on the negatively charged surface of CNT, and the negative charge of the surface of the enzymes in the co-immobilization conditions (Christwardana et al., 2017). In this method, the platform was produced first by loading the CNT with PEI at pH 7, then the mixture of the enzymes in a buffer solution at pH 7.4 was added to the platform. In another example of using an adsorption strategy for co-immobilization, glucose oxidase and horseradish peroxidase were immobilized with polyelectrolyte layers on the surface of silica microparticles. This co-immobilization has been carried out in both one layer and separated layers and it has been shown that the overall rate of hydrogen peroxide conversion was around 2.5 times in the case of one-layer co-immobilization than with the separated layer immobilization (Pescador et al., 2008).

Using the physical entrapment technique, malic and alanine dehydrogenase have been co-immobilized in hybrid gel fibers of cellulose acetate (CA) and zirconium (Zr) alkoxide; while reusability has been shown, the total activity of the immobilized enzymes is one-fifth of the free

enzymes (Nakane et al., 2010). Co-immobilization of glucose oxidase and catalase by entrapping them in silica inverse opal (IO-SiO2) templating by polyacrylamide microsphere (PAM) has been recently reported by Zhao et al. (2018). The system has been used for glucose removal from commercial isomaltooligosaccharide and maintained 79.2% removal efficiency after 6-time use (Zhao et al., 2018). In another study, α-amylase and glucoamylase, starch-converting enzymes, were immobilized on surface-modified carriers using co-immobilization and simple immobilization strategies (Park et al., 2005). The co-immobilized system on hydrophilic silica gel and DEAE-cellulose entrapped in alginate beads exhibited 92.3 and 88.9% of the immobilized enzymes' activity after 10-time use, respectively (Park et al., 2005).

Covalent binding has also been used for enzyme co-immobilization although it is not a recommended strategy for enzymes highly sensitive to new covalent bonds (Jia et al., 2014). A significant development in this field has been the co-immobilization of three cysteine-tagged cellulases including endo-glucanase (EGIVCBDII), exo-glucanase (CBHII), and β-glucosidase (BglB) on gold nanoparticles (AuNP) and gold-doped magnetic silica nanoparticles (Au-MSNP). This combination has proven to be an effective enzymatic system to produce cellobiose and glucose from cellulose (Cho et al., 2012). The covalent co-immobilization of commercial α-amylase, cellulase, protease, and lipase, the properties of the co-immobilized enzymes, and their application in stain removal have been reported (Pundir & Chauhan, 2012). In this method, the surface of PVC beakers and bristles have been treated by a mixture of concentrated nitric and sulfuric acids to oxidatively cleave the polymers and produce a shorter chain with a terminal methylidene group which can undergo a condensation reaction with glutaraldehyde. Finally, the modified surface has been used for the coupling of aldehyde groups of the modified surface and the amino groups on the enzyme surface. Commercial lipase, glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO) and peroxidase were co-immobilized covalently on arylamine glass beads supported on a plastic strip through diazotization with a conjugation yield of 89.1 mg/g and 64.1% retention of specific activity (Minakshi & Pundir, 2008; Singh et al., 2013). Amide bond formation between hexadecylamine-modified poly-aspartic acid (HPASP) as the support and superoxide dismutase and catalase, in which both were fused to an elastin-like polypeptide (ELP), have been manipulated for co-immobilization of the enzymes and greater stability and higher activity have been shown with this combination for scavenging superoxide anion (Mao et al., 2017). Recently, co-immobilization of α-amylase, protease, and pectinase by

glutaraldehyde-activated chitosan, as a platform for covalent co-immobilization, and sodium alginate, as a platform for entrapment, have been reported. According to the findings, co-immobilization using covalent bonds has shown higher specific activity in comparison with the entrapment technique (Gur et al., 2018).

As a combination of covalent bond attachment and entrapment techniques for enzyme co-immobilization, Dongen *et al* have reported immobilization of glucose oxidase, *Candida antarctica* lipase B (CaLB) as well as horseradish peroxidize (HRP) entrapped in a polymersome lumen, the bilayer membrane, and on the surface of the membrane, respectively (2009). HRP was anchored by the cycloaddition reaction of azide functional groups, attached to the enzyme's lysine residues, and acetylene groups on the platform. This combination was used to perform a three-step reaction using glucose acetate as the initial substrate which was subsequently deacetylated by lipase and oxidized by glucose oxidase yielding peroxide, and the peroxide was subsequently used by peroxidase to oxidize 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS (Dongen et al., 2009; Singh et al., 2013). In this method, the porous polymersome based on isocyanopeptide and styrene monomers were used to immobilize the three enzymes. Glucose oxidase is entrapped in in an aqueous compartment of the polymersome lumen, while CalB is entrapped in the bilayer membrane; finally, the covalent bond attachment is been modified to affix HRP to the outer side of the polymersome.

2.9 Levanase and Levansucrase Immobilization

The immobilization of levanases has not been fully investigated so far most likely due to the intrinsic scarcity of the enzymes although there are several reports of immobilization of other glycosidases that the most relevant ones will be addressed in this part. The levanase immobilization on hydroxyapatite by the combination of adsorption and crosslinking strategies was reported. The immobilized levanase was designed as a toothpaste ingredient for degrading dental plaques. The immobilized levanase was reported to degrad between 0.44-0.49 g of levan/min. g support (Kuboki et al, 1989). β-glucosidase was immobilized on functionalized agarose derivatives containing reactive groups such as polyethylenimine (PEI), glyoxyl (linear aliphaticaldehydes) and amine-epoxy. Using reversible immobilization on agarose-PEI and irreversible attachment (covalent immobilization) on glyoxyl agarose resulted in preserving the enzyme activity but poor thermal stability. However, covalent immobilization on amine-epoxy agarose supports preserved 80% of the β-glucosidase activity and was around 200 times more stable than the soluble enzyme (Vieira

et al., 2011). Aminated commercial glucoamylase was immobilized on highly activated glyoxyl-agarose support and ended up preserving 50% of activity as well as enhancing the free enzyme stability by more than 500-fold (Tardioli et al., 2011). Amyloglucosidase (AMG) from *Aspergillus niger*, produced by solid state fermentation, was covalently linked to the magnetic nanoparticle (MNP) to form a monolayer of AMG (MNP–AMG) which subsequently used as a platform for immobilization of crosslinked aggregates of free AMG (MNP–AMGn). The immobilization resulted in high recovery (92.8%) of enzyme activity and improved the enzyme thermal stability (Gupta et al., 2013). The immobilization of a recombinant endo-1,5-arabinanase from *Aspergillus niveus*, was carried out by covalent binding onto agarose-modified supports, including glyoxyl iminodiacetic acid–Ni^{2+}, glyoxyl amine, glyoxyl (4% and 10%) and cyanogen bromide activated sepharose. The highest and the lowest yield of immobilization was reported to be obtained by immobilization on glyoxyl amine and glyoxyl (96%) glyoxyl iminodiacetic acid–Ni^{2+} (43%), respectively. However, the immobilized enzyme on glyoxyl 4 and 10% were shown the highest thermal stability improvement by 4.0 and 10.3-fold factor at 70 ˚C. Finally, the product profile study of the major hydrolysis product of debranched arabinan or arabinopentaose by the immobilized endo-1,5-arabinanase on glyoxyl agarose was reported to be arabinobiose (Damasio et al., 2012). The immobilization of β-Xylosidases from *Aspergillus niger* USP-67 on DEAE-Sepharose, Polyethyleneimine (PEI)-Sepharose, Q-Sepharose, CM-Sepharose, Sulphopropil-Sepharose and MANAE-agarose was reported showing the the best result with PEI-Sepharose (94% of immobilization yield). The immobilized enzyme exhibited higher thermal stability than the soluble enzyme and other immobilized enzymes (half-life of about 50 min, at 65 ˚C) (Benassi et al., 2013). Commercial xylanolytic enzyme cocktail known as Bioxilanase L PLUS (BIO) was immobilized by multipoint covalent attachment under alkaline conditions on agarose beads highly activated with aldehyde groups resulting in thermal stability improvement (half-life of approximately 50 h at pH 7.0 and 60 °C). Also covering the covalently immobilized enzymatic system by PEI 10 kDa showed enhancing the thermal stability by100-fold (de Oliveira et al., 2018). Microbial LSs which can be used to produce levans must be stabilized due to their intrinsic thermal sensitivity and high risk of hydrolyzation reaction. Therefore, different immobilization techniques have been used to overcome the limitations. In Table 2.1, a summary of published articles in this field is presented. According to the table, the effective immobilization techniques have been either physical adsorption or covalent binding. This could be due to the diffusion limitation which

Table 2.1. A list of publications describing immobilized levansucrase for levan synthesis[a].

Microorganism	Biocatalyst	Results
Zymomonas mobilis	Covalently immobilized on Vinyl sulfone-activatedsilica	The immobilized enzyme product profile shifted to FOSs and the enzyme retained activity was 25% (Santos-M et al., 2016)
Bacillus amyloliquefaciens	Covalently immobilized on functionalized glyoxyl agarose	Transfructosylation/hydrolysis ratio increased 120% by enzyme immobilization (Hill, et al., 2016))
Bacillus subtilis NRC33a	Immobilization by covalent binding on chitosan through glutaraldehyde	Highest immobilization yield (85.51 %) of the enzyme, and the immobiliz enzyme retained 51.13 % activity after 14 repeated uses (Esawy, Mahmou
Zymomonas mobilis	Purified recombinant enzyme immobilized on titanium-activated magnetite	The thermostability significantly improved with the maximal production yield of levan of 42 % from 100 g L^{-1} of sucrose (Jang et al., 2001)
Zymomonas mobilis	Purified recombinant enzyme immobilized on hydroxyapatite	Compared with the free enzyme, the immobilized enzyme produced a much greater proportion of low-molecular weight levan (Jang et al., 2000)
Zymomonas mobilis ATCC 10988	Crude recombinant enzyme immobilized on chitin beads	With 20 % (w/v) sucrose, the production of levan reached 83 g/L using the immobilized enzyme, and a total production of 480 g/L was obtained by recycling the immobilized enzyme seven times (Chiang, Wang, Chen, & Chao, 2009)

[a]: This information has been borrowed from Li's review about different applications of LSs (W. Li, S. Yu, T. Zhang, B. Jiang, & W. Mu, 2015).

adversely affects levanase activity as the enzyme needs FOSs to reach the active site as the main acceptor in the case of polymerization mode (Hill et al., 2016). In accordance with the diffusion limitation, covalent binding and multi-covalent binding are the most stable interactions between the support and the enzyme to have been used for LS immobilization (Hill et al., 2016). According to the findings of the article, the best compromise between the immobilization activity yield and retention of enzyme activiyt after immoblization was obtainted for Sepabeads® HA (52.7% and 98.8%, respectively) and glyoxyl agarose-IDA/Cu (80.1% and 67%, respectivley). Moreover, the thermal stability results showed that glyoxyl agarose-IDA/Cu and glyoxyl agarose presented the highest thermal stability with factors of around 14 and 106 times, respectively. However, immobilization through Sepabeads® HA did not significantly improve the stability of LS but did increased the ratio of transfructosylation/hydrolysis by 2.3 times. In fact, immobilization on glyoxyl agarose-IDA/Cu demonstrated the best compromise between three key properties including retention of activity (67.0%), transfructosylation/ hydrolysis ratio (120%) and thermal stability factor (13.6).

2.10 Enzyme Modification

To boost the enzyme affinity toward a support with special functional groups, enzyme modifications play a role. However, achieving selective modifications is often problematic because a special moiety must preferentially react in the presence of so many other side chains as competitors (Chalker & Davis, 2011). Enzyme modification can be divided into two sub-groups: pre-immobilized and post-immobilized enzyme modifications.

2.10.1 Pre-immobilization Modification

Cysteine is the most used residue as a tag in comparison with other alternatives like tyrosine or serine because of its high nucleophilicity and the feasibility of further modifications. Cysteine can be easily converted into a disulfide functional group to modify the spatial shape or surface chemistry of a protein. Simply mixing a thiol with cysteine-tagged protein in the presence of oxygen would result in a mixed disulfide, but undesirable symmetrical disulfides can also be produced under this condition (Chalker et al., 2011). Therefore, applying reagents that can react faster than the intramolecular reaction of cysteine residues is necessary. These reagents contain methanethiosulfonate (MTS) (Davis& Jone s, 1998), phenylthiosulfonate (PTS) (Gamblin et al., 2003), or phenylselenenylsulfide (SeS) (Figure 2.5) (Chalker et al., 2011; Gamblin et al., 2004).

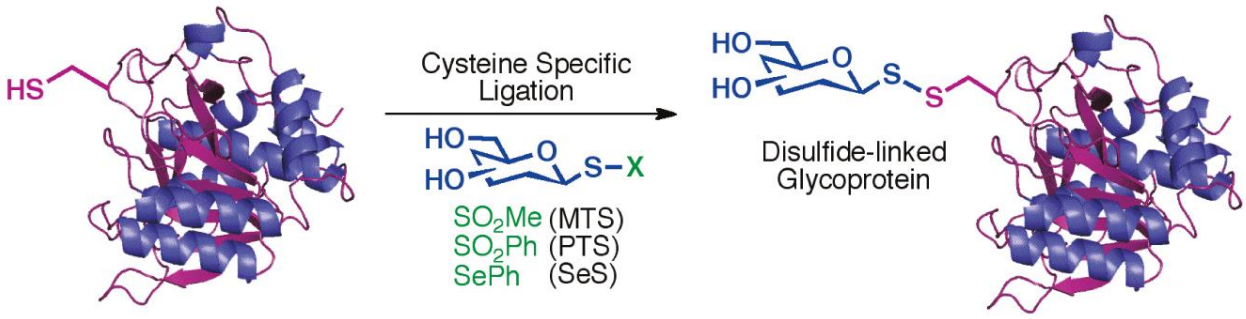

Figure 2. 5. Selective disulfide preparation from cysteine residues of an enzyme (Chalker et al., 2011

The ease of these procedures in preparing disulfides has resulted in emerging thiolation reagents like Lawesson's reagent which can directly convert alcohols to the corresponding thiols without the use of any protecting group (Figure 2.6) (Bernardes & Davis, 2006).

The susceptibility of the disulfide to reduction and its subsequent instability in a reducing medium has brought about two new techniques: desulfurization of disulfides to thioether and conjugate addition to dehydroalanine.

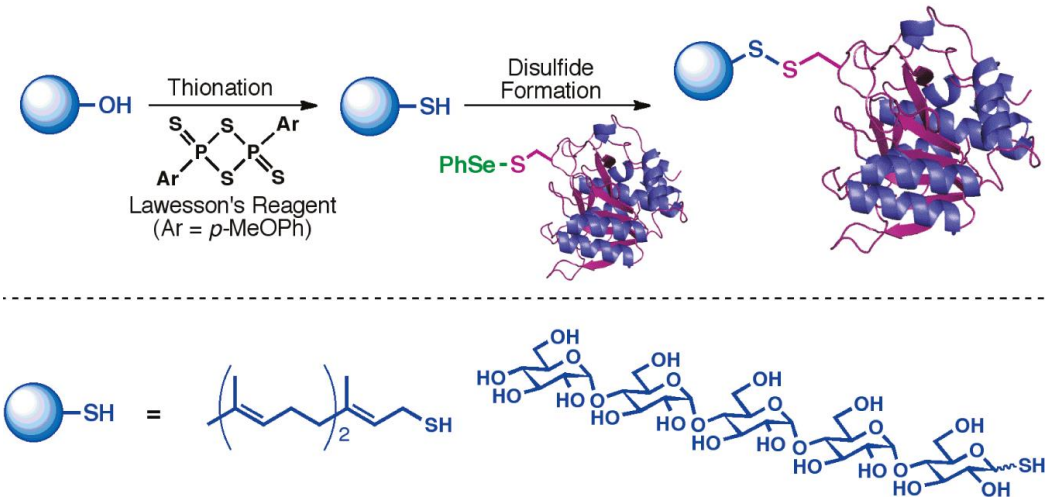

Figure 2.6. Selective disulfide functional group formation by Lawesson's reagent (Bernardes & Davis, 2006)

Desulfurization occurs by applying electron-rich phosphines such as hexamethylphosphorus-triamide (HMPT) which produces thioether from disulfide derivatives. According to Figure 2.6, HMPT acts as a nucleophile and supplements the cysteine's sulfur in the disulfide functional group. The nucleophilic addition results in the formation of dehydroalanine, HMPTS, whose formation is considered as the driving force of the reaction, and a nucleophilic-sulfide-containing

fragment. The final step is the addition of the new nucleophile to dehydroalanine to produce the thioether functional group. This desulfurization presents the first glycosylation of cysteine residues on proteins (Bernardes et al., 2008).

The conversion of cysteine to dehydroalanine is the second transformation of disulfides to a more stable functional group. Syn-elimination of sulfinylimide, produced by the reaction of cysteine's thiol group with *O*-mesitylsulfonylhydroxylamine (MSH), results in the dehydroalanine formation. It has proven a reliable method for incorporating dehydroalanine in a single-cysteine-tagged protein (Figure 2.7 and 2.8) (Bernardes & Davis, 2008). Site-directed immobilization is a recent advancement in the enzyme immobilization field, and it is crucial for situations that are dependent on a specific orientation of an enzyme on a support. To obtain site-directed immobilization, site-directed mutagenesis has been designed to insert desired amino acids called a tag to the specific sites of an enzyme (Hernandez & Fernandez-Lafuente, 2011). Although this strategy is more complex as it requires complete knowledge of the enzyme structure and determining a suitable microorganism for growing the modified enzyme, the production cost is similar to the wild enzyme. The goal of site-directed mutagenesis in this field is to modify the sequence of the native gene so that the newly expressed protein can be immobilized based on a

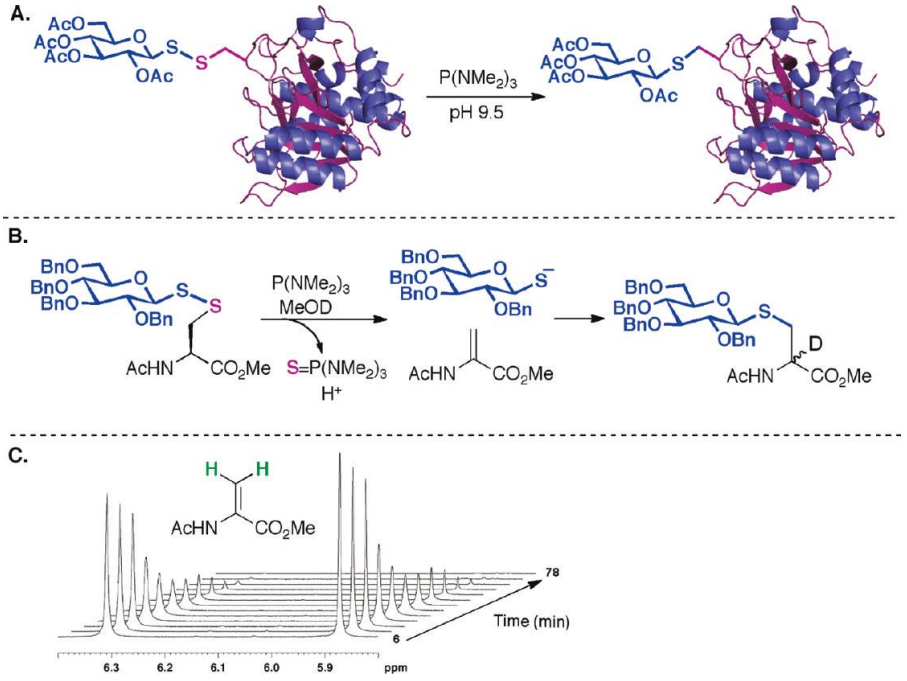

Figure 2.7. Disulfide functional group conversion to thioether by using HMPT (a), the mechanism of the reaction (b), and the crude HNMR of the reaction mixture (c) (Bernardes & Davis, 2008)

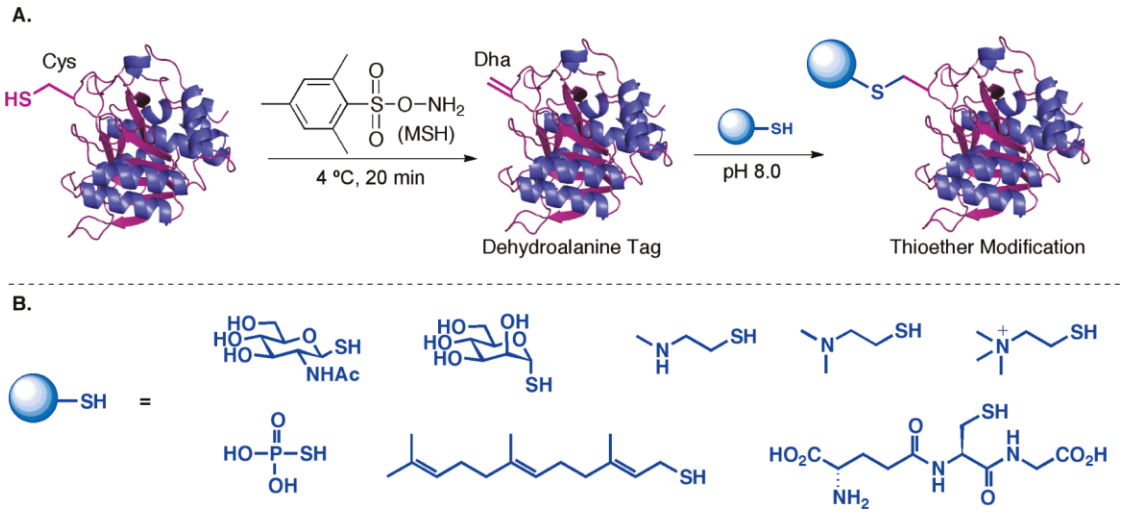

Figure 2.8. Dehydroalanine formation and its subsequent transformations (Bernardes & Davis, 2008)

specific immobilization protocol, while the native gene cannot. There are several strategies to manage tagged enzyme immobilization which will be presented in the next section. The first strategy is immobilization using tag-specific antibodies; that is, the antibody tags supported on the platform are used to attach to the tagged enzyme by its specific affinity to the antibody (Vishwanath et al., 1997). This strategy is useful when either the N- or C- terminus of the enzyme is available for the tag fusion and the desired orientation is aligned with one of the termini. In a similar strategy, a polypeptide tag containing active lysine has been used to attach the enzyme to a specific immobilized protein such as β-casein by applying transglutaminase (Tominaga et al., 2005). Another strategy has taken advantage of the strong interaction between biotin and avidin; that is, the biotinylated enzyme can be attached to a support which contains immobilized avidin. The drawback of this method is the difficulty of covalent bond formation between the platform and the enzyme after the primary immobilization due to the biotin/avidin interaction. In fact, avidin as a big base for immobilization blocks the enzyme from the active surface of the platform (Vishwanath & Huang, 1995). In the second version of tagged protein immobilization, simpler tags with a high affinity towards specific functional groups on the support have been used. Histidine tagged enzymes are the most common example of this strategy. In this case, the support containing transition metal chelates such as Ni^{+2}, Co^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2}, or Fe^{+3} undergo a reversible interaction with the His residues as electron-donating groups. Though there may be an interaction between histidine residues of the wild enzymes and the support, poly-His tagged proteins may be fixed onto the support by the interaction of two His groups of the tag with one

chelating metal on the support. This technique has been mainly used for developing biosensors. For example, immobilization of a hexa-His-tagged acetylcholine esterase by using Ni^{+2}-iminodiacetic acid modified magnetic beads has been reported to improve the limit of detection of the enzyme ten times compared to the enzyme immobilized by entrapment (Csöregi & Gorton, 1993). In another example, a hexa-His-tagged horseradish peroxidase and its native version immobilized on a gold electrode have been compared and only the recombinant enzyme produced a high and stable current response to H_2O_2, due to the enzyme's bioelectrocatalytic reduction by electron transfer between gold and HRP. The electrode was shown to improve H_2O_2 detection up to 10 nM (Ferapontova et al., 2001).

Cysteine tags installed by site-directed mutagenesis have presented the most simple and versatile way of controlling enzyme orientation. The tagged enzymes can be immobilized either on a support containing activated disulfide which can be produced by treatment of a thiol-containing platform with reagents containing one of methanethiosulfonate (MTS) (Davis et al., 1998), phenylthiosulfonate (PTS) (Gamblin et al., 2003), or phenylselenenylsulfide (SeS) (Chalker et al., 2011; Gamblin et al., 2004) resulting in a thiol-disulfide interchange or, on gold-containing platforms, by utilizing the strong affinity of thiol functional groups of the tags and the support's gold component (Kobatake & Aizawa, 1999). For example, site-directed mutagenesis has been used to replace a serine far from the active site of *subtilisin* BPN for an effective immobilization. The enzyme has been recognized as sensitive to immobilization due to the proximity of its active site to the likely area of immobilization, so replacing the serine moiety with a cysteine tag far from the active site could lead to a site directed immobilization without jeopardizing its activity (Viswanath & Bhattacharyya, 1998). Comparison between the recovered activity of the mutant enzyme and the natural enzyme immobilized on PVC-silica membrane containing thiol-reactive groups showed 35% improvement in specific activity. Immobilization of cysteine-tagged protein G, an antibody binding protein on bare gold, has been reported. In this study, *Streptococcus* protein G has been engineered for installing various types of cysteine tags at its N-terminus to achieve the well-oriented protein G film on the gold platform. The results have shown significant improvement in antigen detection of the immobilized protein and been recommended for oriented antibody immobilization in immunosensors (Lee et al., 2007).

Site-directed immobilization can be combined with subsequent convenient immobilization methods to bring about greater degrees of rigidity and stability. For example, lipase from

Geobacillus thermocatenulatus (BTL2), genetically engineered by site-directed mutagenesis, has been first immobilized on disulfide-aldehyde supports by oriented immobilization through its engineered cysteine moieties and then rigidified by subsequent reactions between amine residues of lysine groups around the engineered cysteines. This process has resulted in a fully enantioselective biocatalyst with *ee*>99% in reference reactions including kinetic resolution of *rac*-2-*O*-butyryl-2-phenylacetic acid and asymmetric hydrolysis of phenylglutaric acid dimethyl diester. However, oriented immobilization by the introduction of cysteines and activated disulfide groups on the support has given similar results in the case of the asymmetric hydrolysis reaction but much lower kinetic resolution yield (*ee* = 27%) (Godoy et al., 2013). The research group has improved the site-directed/multipoint covalent attachment (MCA) combination by modifying the residues around one engineered cysteine moiety to obtain a higher level of rigidification and better performance of the enzyme. This modification has carried out by genetic amination and/or chemical amination of the residues around the engineered cysteine. By this strategy, two highly stabilized derivatives of chemically aminated lipases have been immobilized by site-directed MCA and by exploiting the modification of the surrounding surface of Cys344 or Cys40 residues. The first one has shown 2.4-fold more productivity than the reference derivative and the second one was 40% more selective with the same activity as the reference in the production of polyunsaturated fatty acids (Godoy & Guisán, 2014). In 2016, Zhang *et al.* have reported the use of thiol-ene click chemistry for the immobilization of a cysteine-tagged dehydrogenase on modified carbon electrodes. The modification was completed using electrochemical reduction of the corresponding diazonium salts generated *in situ* from 4-vinylaniline (Figure 2.9) (Zhang et al., 2016). Besides cysteine, as a natural tag, incorporation of unnatural amino acids in the protein structure have been used to produce unnatural-amino acid-tagged proteins. For example, allyl selenide, homoallylglycine (Hag) (Hest & Tirrell, 2000), azidohomoalanine (Aha) (Kiick & Bertozzi, 2002), and homopropargylglycine (Hpg) tags (Hest et al., 2000) offer several chemical reactions such as olefin metathesis, palladium and copper-mediated cross-coupling, and copper catalyzed [3+2] cycloaddition which have introduced further potential enzyme modifications (Figure 2.10) (Chalker et al., 2011).

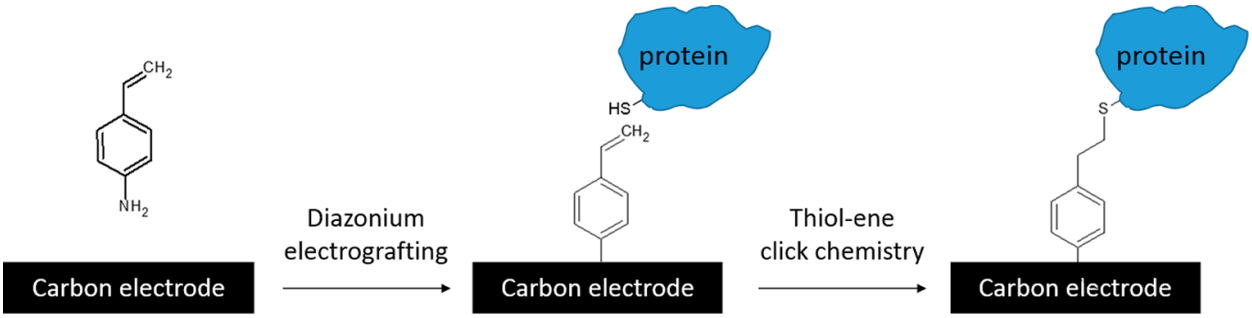

Figure 2.9. Schematic representation of vinylphenyl grafted carbon electrode preparation and site-directed immobilization by exploiting thiol-ene click chemistry (Zhang et al., 2016)

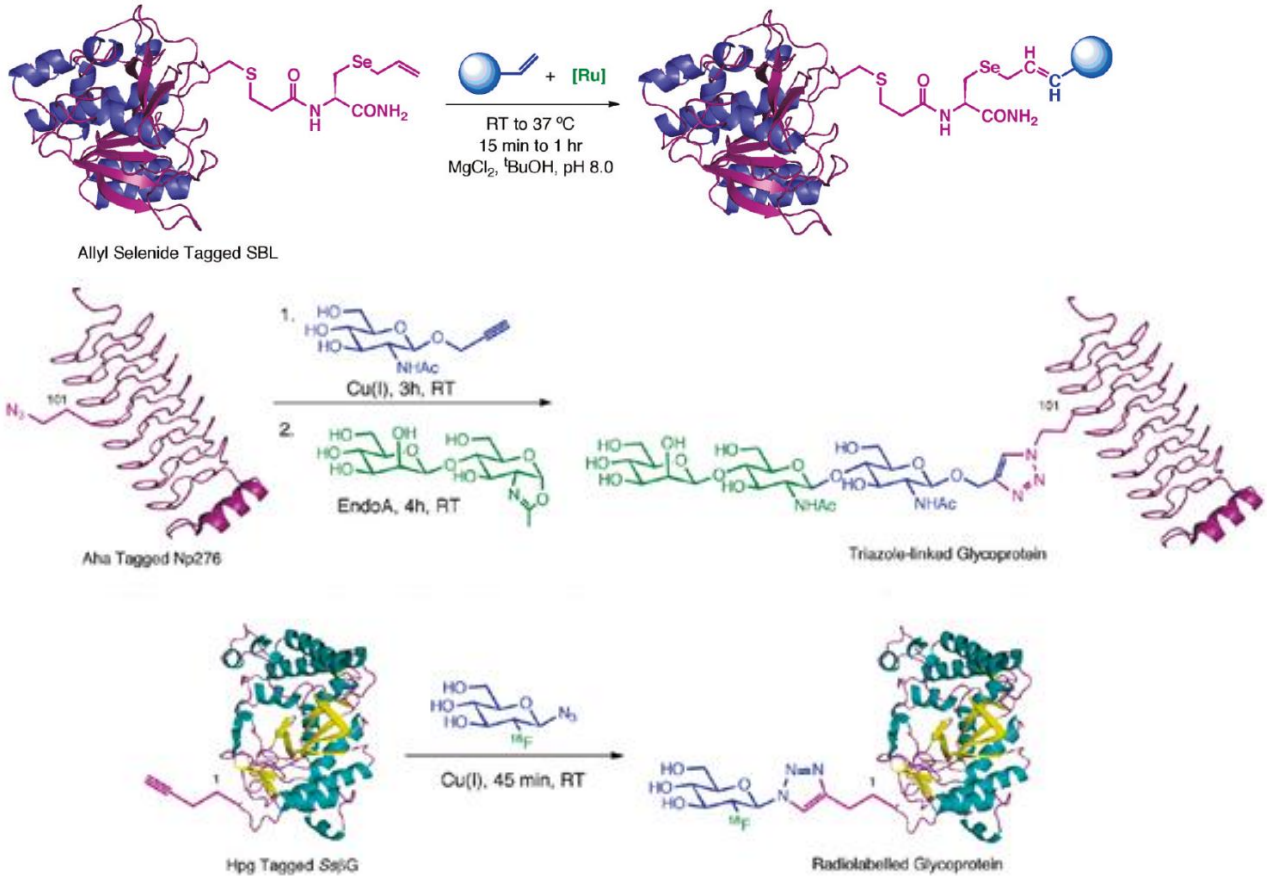

Figure 2.10. Metathesis and [3+2] cycloaddition reactions by unnatural tags (Chalker, 2011)

This field of research is still emerging and innovative strategies for obtaining the highest level of control on enzyme immobilization have been reported. For instance, formylglycine-generating enzymes with the ability of selective recognition and oxidation of cysteine residues within the sulfatase sub-motif at the terminus of proteins have been exploited to produce aldehyde-bearing formylglycine (FGly) at haloacid dehalogenase ST2570 from *Sulfolobus takodaii*. The C-terminal

aldehyde-tagged ST2570 has been selectively immobilized on SBA-15 functionalized by aminopropyltriethoxysilane to introduce amino groups on the support. The site-specific immobilized enzyme has shown 3 times higher thermal stability, 1.2 times higher catalytic ability and improved operational stability in comparison with the corresponding free enzyme; moreover, it has retained 60% of its original activity after seven cycles of batch operation (Jian, et al., 2016).

2.10.2 Post-immobilisation Modification

Crosslinking enzyme aggregates (CLEAs) was presented for the first time in the 1960s as a promising strategy for enzyme immobilization. In this approach, enzymes are precipitated out of solution by the addition of salts such as ammonium sulfate, application of water-miscible organic solvents, or by the use of non-ionic polymers such as polyethyleneglycol to form a physical aggregate. Subsequently, a bifunctional reagent such as glutaraldehyde is used perform the cross-linking reaction. This method causes minimum deformation of the tertiary structure of enzymes and circumvents the pre-purification of enzymes as this can be done during or after immobilization (Karav et al., 2017). Recently, new polyfunctional high-molecular weight cross-linkers have attracted more interest as they can decrease the risk of deactivation of crosslinked enzymes which may happen due to their active sites being blocked during the cross-linking process (Karav et al., 2017; Mateo et al., 2004).

2.10.3 Isosteric vs Non-isosteric Modification

Enzyme modification is an effective way to manipulate natural catalysts to carry out a desired reaction. Although chemical synthesis of large catalysts (mass ~ 10 kDa) from scratch is challenging, it provides very helpful tools to modify the natural enzyme structure and its reactivity. In other words, ribosomal synthesis of proteins can produce natural enzymes because of its stereo- and regio-specificity. Tor modify the enzyme structure, this strategy imposes strict limitations such as its specific capability to introduce L-amino acids, which are also canonical. Therefore, chemical synthesis offers diverse possibilities to achieve desired modifications to circumventing the limitations. Enzyme modifications can be classified into two major groups: isosteric and non-isosteric replacement (Kohrer & Bhandary, 2009).

Chemical isosteric modifications are simple but are not regio-specific nor reproducible. A successful example of this type of modification shows that the active serine residue of the protease active site from *subtilisin* can be converted into a cysteine residue which loses protease activity

and becomes an active enzyme for ester hydrolyzation (Kohrer & Bhandary, 2009). Non-isosteric modifications include all changes in the enzyme structure done by adding a non-amino acid moiety, a non-canonical amino acid, or a new oligopeptide to the original enzyme. There are several strategies to achieve this kind of modification such as C-C cross-coupling, azide-mediated cycloaddition reaction, and backbone modification (Chalker et al., 2011).

2.11 Methods for the Analysis of Fructooligosaccharides, Fructo-oligomers and Levans

FOS and fructan analysis can be preformed by chromatographic techniques, NMR spectroscopy, and Mass spectrometry

2.11.1 Chromatographic Techniques

Chromatography is the method of choice in carbohydrate qualitative and quantitative analyses due to high resolution, fast analysis, direct injection of the sample without or with little pre-treatment and the feasibility of automation (Corradini et al., 2012). There are two major challenges which must be addressed for successful chromatographic analysis of carbohydrates: the selection of a suitable column able to effectively separate the carbohydrates, and the selection of a suitable detector which is sensitive enough to offer an acceptable limit of detection (LOD) but is also compatible with the eluent used for the separation (Corradini et al., 2012).

Although hydrophilic interaction chromatography, which is based on bonded-phase packaging-based silica columns, and reversed-phase chromatography have been used for carbohydrate analysis, these methods have shown some major downsides such as instability, short lifespan of the bonded phases and poor column performance in selectivity and efficiency (Corradini et al., 2012). To circumvent the difficulties, anion exchange columns have proven highly effective. As carbohydrates are weak acids with pK_a of 12-14, their hydroxyl groups are partially or completely deprotonated at high pH values. Therefore, the oxyanions can be selectively eluted in a high-performance anion exchange chromatography (HPAEC) in a single run (Corradini et al., 2012). Anion exchange columns are based on quaternary-ammonium-bonded pellicular resins on which the order of elution time is correlated with decreasing pK_a value knowing the acidity of glucose's hydroxyl groups in the following order: 1-OH>2-OH>6-OH>3-OH>4-OH as reference values. Moreover, aldoses show higher retention time than reduced alditols and the retention time usually increases with increasing the number of monosaccharides carbon atoms, as well as increasing chain length of oligosaccharide homologous series. Monosaccharides and disaccharides are

usually analyzed by CarboPack PA1 and CarboPack PA10 using isocratic elution with 10-20 mM sodium hydroxide solutions. In the case of oligo- and polysaccharide (until DP of 85) separation, sodium acetate is usually used as a stronger eluent, although sodium hydroxide is still used due to the requirement of a strongly basic environment (Corradini et al., 2012).

The subsequent challenge of chromatographic carbohydrate analysis is the type of detector compatible with anion exchange chromatography conditions and eluents. First, neutral carbohydrates do not have chromophores and fluorophores, so their UV-absorbances are in the range of 190-210 nm, where organic mobile phase modifiers such as acetonitrile have strong absorbance. Second, some detection methods are sensitive to changes in mobile phase, so gradient elution is not compatible with this kind of detector. For example, refractometric detectors can be compatible with basic eluent of anionic exchange chromatography, but they are sensitive to eluent changes and cannot support gradient elution (Corradini et al., 2012). Considering the aforementioned issues of carbohydrate detection, electrochemical detection has been found effective to address all necessities of HPAEC analysis of carbohydrates. In constant potential amperometric detection usually done with a glassy carbon amperometric electrode, aromatic compounds, especially phenols, aminophenols, catecholamines, and other metabolic amines can easily get oxidized and produce a strong response in the detector; however, carbohydrates, lacking functional groups to stabilize the intermediates of anodic oxidation, have no response in the detector. Hence, gold and platinum electrodes have been used to offer a catalytic electrode surface able to oxidize aldehyde and terminal alcohol moieties in carbohydrates. The drawback of this method is the deterioration of the electrode surface due to the adsorption of the oxidation products, resulting in a reduced response overtime. To address the issue, pulsed electrochemical detectors (PED) have been used. PED is based on applying a simple three-step potential waveform to achieve a stable electrode response (Corradini et al., 2012). Analyte oxidation current is measured by the integration of the response for the duration of time t_{det}. Moreover, the electrode function is recovered during the reduction period. It is noteworthy to mention that the sensitivity of PED in the detection of alditols, monosaccharides, oligosaccharides, and polysaccharides with a gold electrode is optimal under basic conditions (pH > 12), which is also the ideal condition of the eluent for HPAEC.

High-pressure size-exclusion chromatography (HPSEC) equipped with a refractometer detector could be also applied for product profile studies. Although HPAEC offers a better resolution and

LOD, HPSEC could be a faster and easier method if the difference in the retention time of the components and their concentrations are satisfactory. To perform a product profile study by HPSEC two different columns are necessary, including an aligned-in-sequence of (7.8 mm x 30 cm) TSKgel G3000PWXL-CP and TSKgel G5000PWXL-CP for polysaccharide measurements, and (7.8 mm x 30 cm) TSKgel G-Oligo-PW for oligosaccharide analysis.

2.11.2 NMR spectroscopy

To elucidate the structure of an oligosaccharide, the following information must be addressed: (a) the monosaccharide composition (b) the anomeric configurations of each glycosidically-linked monosaccharide unit (c) the linkage between monosaccharide units and (d) the attached groups. NMR spectroscopy has offered the most complete picture in carbohydrate structural elucidation regarding not only the primary structure of carbohydrates but also information on the conformation and carbohydrate molecular dynamics (Table 2.2) (Agrawal, 1992).

2.11.3 Tandem Mass Spectrometry (MS/MS)

Mass spectrometry (MS) is a powerful method to analyze the structure of organic compounds, but has three major limitations: 1) compounds having complex matrix cannot be characterized without pre-treatment and matrix removal; b) this technique is not able to provide sensitive and selective analysis of complex mixtures; c) for large molecules such as peptides and polysaccharides, mass spectra are very complex and difficult to interpret (Zaia, 2004). To circumvent the difficulties, tandem mass spectrometry (MS/MS) has emerged. This technique provides two benefits lacking from simple MS: a) structural elucidation of unknown and complex molecules; b) analysis of complex mixtures with minimal sample clean-up. Although complex oligosaccharide analysis in a biological matrix by MS is still challenging, new MS/MS techniques can effectively analyze them if sample preparation producing equal ionization response is undertaken. The MS/MS sample preparation steps are as follows: chromatographic separation, peralkylation, and methyl esterification. Peralkylated ions are highly effective in producing the most informative MS/MS spectra, particularly for branched oligo- and polysaccharides. However, this method is not recommended for samples less than 5 mg due to the risk of sample loss. It must be highlighted that the use of native or minimally modified derivatives is not considered as a reliable source of mass

Table 2.2. List of useful NMR techniques in carbohydrate structural elucidation

Structural information	**NMR**
Number of sugar residues	a. Integrated ^{1}D ^{1}H NMR spectrum b. ^{13}C NMR Spectrum c. 2D ^{1}H-^{1}H correlation spectroscopy for connectivity analysis d. 2D ^{1}H-^{13}C correlation spectroscopy
Constituent monosaccharides	a. ^{1}H NMR chemical shifts b. ^{1}H NMR vicinal coupling constant ($^{3}J_{H-H}$) c. 2D homonuclear correlation spectroscopy (COSY[a], HOHAHA[b]) d. ^{13}C NMR chemical shifts e. ^{1}H-^{13}C correlation spectroscopy
Anomeric configuration	a. ^{1}H NMR chemical shifts and vicinal coupling constants b. ^{13}C NMR chemical shifts and ^{13}C-^{l}H coupling constants c. Intraresidue NOE[c]
Linkage sites and sequence	a. ^{1}H and ^{13}C NMR chemical shifts b. Interresidue NOE c. Long-range homo- and heteronuclear correlation
Position of appended groups	a. ^{1}H and 13C NMR chemical shifts b. Interresidue NOE c. Long-range homo- and heteronuclear correlation

[a]: Correlation Spectroscopy
[b]: Homonuclear Hartmann Hahn which is equal to Total Correlated Spectroscop (TCOSY)
[c]: Nuclear Overhauser effect. This table has been borrowed from a review about NMR spectroscopy applications in carbohydrate structural elucidation published by Agrawal (Agrawal, 1992)

data as protonated native ions can undergo internal-residue rearrangements (Zaia, 2004). Because of metastable fragmentation in MALDI and TOF/TOF MS, the most reliable mass spectrometry technique is a low-energy collision-induced dissociation (CID) in which the precursor ion is selected and fragmented in a collision chamber before a mass spectrum of fragments is acquired. CID is the most common method of fragmentation in MS/Ms. The precursor ion enters the collision cell containing He, as an energized-chemically inert gas, to promote fragmentation of the ion (Zaia, 2004). Conventional low-pressure MALDI-TOF has been used to profile oligosaccharide mixtures by adopting necessary measures to avoid metastable ions, but it has not been recommended for sulfated oligosaccharides because of the high level of fragmentation in the source. Moreover, electrospray ionization (ESI) has been found as a suitable ionization method for

fragile oligosaccharides such as sialylated or sulfated oligosaccharides (Zaia, 2004). Finally, MALDI-QoTOF offers enough sensitivity to analyze oligosaccharides released from SDS-PAGE gels (Zaia, 2004).

2.12 Conclusion

Novel prebiotic levan-type FOSs have been identified as a great candidates. This type of FOSs has proven higher resistance and selectivity in their fermentation in the human gastrointestinal tract and hence better performance as prebiotics, than the commercially available inulin-type FOSs. However, their production is still challenging as there are limited plant sources of these FOSs. Although levanase-catalysed the hydrolysis of levans is a potential approach to produce levan-type FOSs, it is still limited by the low thermal stability and scarcity of levanases Enzyme immobilization can improve levanase thermal stability and modulate its product profile in order to produce levan-type FOSs in a commercially feasible way. Furthermore, bi-enzymatic systems composed of immobilized levansucrase and levanase would have the ability to produce the desired FOSs from abundant and inexpensive starting material sucrose.

CHAPTER III

Selected Recombinant Levanase Immobilization

Abstract

The immobilization of levanases from *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), *Dyadobacter fermentans* (LEV-D.F.) on functionalized agarose, including glyoxyl agarose, glyoxyl agorose-TEA, glyoxyl agarose-IDA, and glyoxyl agarose-IDA/Cu were investigated. The enzyme activity yield, retention of enzyme activity, and retained activity after incubation at 50 ^{o}C were measured, and the best immobilization conditions and support were identified. Glyoxyl-Ag, glyoxyl-Ag-IDA, glyoxyl-Ag-IDA/Cu supports led to the best compromises for the immobilization of LEV-B.B., LEV-C.O., and LEV-D.F., respectively. Immobilized LEV-D.F. on glyoxyl-Ag-IDA/Cu showed the highest thermal stability improvement by recording almost 9-time higher half-life than that of the free enzyme. Immobilized LEV-B.B. on Gly-Ag, also, showed thermal stability improvement by increasing the enzyme half-life from 109.5 to 133.3 min after immobilization. However, LEV-C.O. exhibited a decline in the half-life from 202.4 to 78.8 min upon immobilization. Total yield, oligo yield and end-product profile, revealed the use of temperature of 15 ^{o}C and high molecular weight levans can maximize the release of FOSs and limit that of fructose; indeed, increasing the temperature shifted the end-product profile towards lower molecular weight FOSs with an exception in the case of the LMW levan-immobilized LEV-D.F./Gly-Ag-IDA/Cu reaction system, and compared to LMW levan, the use of HMW levan favored the GF_7 formation over the shorter chain FOSs. Immobilized LEV-C.O. on glyoxyl-Ag-IDA demonstrated superior end-product specificity towards GF_7 (23.1%), with no release of other FOSs. The highest oligo yield (27.8%) was obtained by the immobilized LEV-B.B. on Glyoxyl-Ag .The reusability of the three immobilized enzymes was investigated by using the same enzymes for four consecutive batches. The immobilized LEV-C.O. exhibited the highest retained activity (37.7%), while the LEV-B.B. exhibited the lowest retained activity (12.6%) after four hydrolysis reactions.

3.1 Introduction

Levanases (2,6-β-D-fructanohydrolase, EC 3.2.1.65), which belong to the glycoside hydrolase family 68 (GH68), catalyse the hydrolysis of *β*-(2,6)-linked fructans to produce various levan-type fructooligosaccharides (FOSs) (Lim & Kang, 1998). Levanases include two sub-groups: 1) exo-levanase (EC 3.2.1.64) releases mainly fructose from levans, and 2) endo-levanase (EC 3.2.1.65) produces fructooligosaccharides (FOSs). Endo-levanases have been identified and produced in *Bacillus* species (Porras-Domínguez et al., 2014), *Arthrobacter* species (Avigad & Bauer, 1966), *G. diazotrophicus* SRT4 (Menéndez et al., 2004) and *Vibrio natriegens* and *Arthrobacter aurescens* (Chen et al., 2020). The main end-products of endo-levanases catalysing the hydrolysis of levan were reported to be mainly levanbiose (DP 2); however, levanases from *Streptomyces sp.* and *Pseudomonas K-52* exhibited a high end-product specificity towards levanheptaose and levanoctaose (Kang et al., 1998; Zhang et al., 2019). The active residues of levanase responsible for levan hydrolysis have been identified to be proton donor (i.e., glutamate), and nucleophile (predominantly aspartate) ones (Mardo et al., 2014; Mardo et al., 2017). To date, there is still no clear understanding what structural elements of levanase and intermolecular interactions at its active site that determine the levanase specificity and end-product profile.

The levanase-catalyzed hydrolysis reaction has been recognized as a relevant synthetic route for the synthesis of novel *β*-(2-6)-FOSs from levan. However, this attractive approach is still limited by the poor availability of levanases, their low stability and the lack of understanding of their mechanism. Only few studies have carried out gene mining, high throughput screening, and gene expression to discover and produce new levanases from selected microbial sources (Chen et al., 2020; Porras-Domínguez et al., 2014). In the previous study of our research group, Chen et al. (2020) have reported a vast gene mining and high throughput screening, which started from 1902 genetic sequence study followed by the selection of 123 bacterial source candidates of levanases for screening their hydrolytic activity, their substrate specificity towards high and low molecular weight levans and their thermal stability. Amongst the screened genes, 10 top candidates were expressed in *Eschericia coli* BL21(DE3) and characterized. Levanases from *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), and *Dyadobacter fermentans* (LEV-D.F.) exhibited the highest level of endo-hydrolytic activity. Further immobilization of these levanases can help to modulate their specificity and their end-product profiles, while enhancing their thermal stability and allowing for their easy reuse. As far as the authors are aware, only one study has

investigated the immobilization of levanases on functionalized hydroxy apatite by glutaraldehyde aiming at its application in toothpaste (Kuboki et al., 1989). As part of our on-going research, the present research was aimed at the investigation of the immobilization of levanases from *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), and *Dyadobacter fermentans* (LEV-D.F.) through multi-point attachments on selected modified and unmodified agarose beads as supports. Indeed, through pre-immobilization treatments, different linkers, such as carboxyl groups, thiols groups and metallic chelating groups, can be incorporated into the agarose supports and promote controlled adsorption on specific regions of the enzyme. For instance, immobilization of LS from *Bacillus amyloliquefaciens* on glyoxyl agarose containing iminodiacetic acid moieties as powerful ligands loaded by cupric ions as chelating agents (glyoxyl agarose-IDA/Cu) resulted in superior transfructosylation/hydrolysis ratio (120%), retention of enzyme activity (67.0%), and thermal stability (stability factor of 13.6) (Hill et al., 2016). However, the same LS immobilized on Gly-Ag-TEA, positively charged support, or Gly-Ag, possessing a partially hydrophobic surface, resulted in almost one-third and two-third retention of enzyme activity and two-third enzyme activity yield (Hill et al., 2016). Understanding of the effect of pre-immobilisation treatments on the immobilization efficiency of levanases still needs to be elucidated. The first part of our current research was aimed at the investigation of different pre-immobilisation treatments for the immobilization of the selected levanases. The immobilization efficiency was assessed by determining the activity immobilization yield, the retention of specific activity, and the end-product profile. The thermal stability and the kinetic parameters (v_{max}, Km, K_{cat}) as well as the enzyme reusability were also studied.

3.2 Materials and Methods

3.2.1 Materials

Lysogeny Broth (LB) ingredients as growth media tryptone, NaCl, and yeast extract, Terrific Broth (TB), carbenicillin antibiotic, and isopropyl β- D-1-thiogalactopyranoside (IPTG) were purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Mono- and disaccharide standards (i.e., D-(-)-fructose, D-(+)-glucose and sucrose) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Fructooligosaccharide standards (i.e., 1-kestose, nystose and 1^F-fructofuranosylnystose) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals and reagents, including 3,5-dinitrosalicylic acid (DNS), potassium sodium (+)-

tartrate, Sodium dodecyl sulfate (SDS), K_2HPO_4, KH_2PO_4, NaOH, NaOAc, imidazole, and PIPES were also purchased from Sigma-Aldrich (Oakville, ON, Canada). GE Healthcare Life Sciences HisTrap^{TM} FF 1 mL columns (Mississauga, ON, Canada) were used for purification of levanases. Agarose 10BCL was purchased from Agarose Bead Technologies. Finally, Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON, Canada).

3.2.2 Levanase Production, Recovery and Purification

LB media made of 40% (w/v) tryptone, 20% (w/v) yeast extract, 40% (w/v) NaCl and carbenicillin (0.1 mg/mL) was used for aerobically preculturing *E. coli* BL21(DE3)-pLysS over-expressing levanase-encoding genes for *B. baltica* (LEV-B.B.), *C. ochracea* (LEV-C.O.), and *D. fermentans* (LEV-D.F.). Preculturing took overnight incubation at 37 ℃ and 250 rpm with continuous agitation (New Brunswick Scientific) to reach the optical density (OD) of 1.2-1.4 at 600 nm. Culturing step was carried out in commercial TB media (47.6 g/L) containing carbenicillin (0.1 mg/mL final concentration) using 50-times dilution of the precultured samples; upon achieving a final OD of 1.2-1.4. IPTG was added to the culture media to reach a final concentration of 1 mM. Incubation proceeded at 25 ℃ for 20 h before cell mass separation by centrifugation at 4 ℃ (8000 rpm for 20 min). Pellets containing cells were re-suspended in sonication buffer [10% (w/v) glycerol, 30 mM PIPES, 30 mM NaCl, pH 7.2] prior to being treated by lysozyme (4 mg/g cell mass) and DNase (2000 U, 4 μL/g cell mass), followed by a 1-hour incubation period at 18 ℃ and 50 rpm in an orbital shaker. The cell suspension was ultrasonicated and centrifuged at 4 ℃ (10000 rpm for 1 h) to obtain crude enzyme extract, which was subsequently dialyzed against 5 mM potassium phosphate buffer (pH 6.0) with a membrane cut-off of 6-8 kDa at 4 ℃, and freeze dried at -40°C. Purification by affinity chromatography (IMAC) on HisTrap^{TM} FF 1 mL column were carried out using imidazole solutions at 5, 10, 50, 100, and 200 mM as the eluent, prepared in 30 mM PIPES buffer containing 30 mM NaCl and 10 % (v/v) glycerol(pH 6.4). The purity of the recovered fractions upon affinity chromatography were subjected to electrophoretic analysis using the 15% SDS polyacrylamide gels (Bio-Rad, Qc, Canada). SDS-polyacrylamide gel electrophoresis was conducted at 120 V in 10-time diluted Tris/Glycine/SDS buffer containing 25 mM, 192 mM, 0.1% of each, respectively. Pure enzyme fractions were obtained when the imidazole gradient reached concentrations of 100 mM-200 mM.

3.2.3 Levanase Activity Assay

One unit of levanase activity was defined as the amount of the biocatalyst that produces 1 μmol of reducing sugar per min by hydrolyzing low (LMW, 9±0.6 kD) or high-molecular weight (HMW, 55±1.5 kDa) levans as the substrates. The glycosyl-hydrolytic activity of each levanase was assayed by measuring the release of reducing sugars by the 3,5- DNS method. 125 μL of levanase suspension (15-45 μg protein) in 50 mM potassium phosphate buffer (pH 6.0) was added to 125 μL of levan substrate (1% w/v) in order to initiate the reaction. In tandem with the reaction, one blank containing only substrate solution was carried out. After 20 min of reaction at 37 °C, 375 μL of DNS reagent [1% (w/v) DNS, 1.6% (w/v) NaOH] was added, and the reaction mixture was boiled for 5 minutes for complete enzyme inactivation. Finally, 125 μL of potassium sodium tartrate (50%, w/v) was added. All measurements were done at duplicate, and the absorbance was measured at 540 nm (Spectrophotometer, Beckman, DU 800 UV/Visible). The amount of released reducing sugars was determined from a standard curve constructed with glucose (0.0-12.0 mM). The protein content of purified levanase suspension was determined using Bradford protein assay and bovine serum albumin as standard (1-20 μg/mL). Specific enzyme activity was defined as the enzyme units in μmol of reducing sugar per min per mg protein.

3.2.4 Preparation and Functionalization of Glyoxyl Agarose-based Supports

The functionalized glyoxyl-agaroses were prepared based on of the methods reported by Mateo *et al*. (2010) and Hill *et al*. (2015).

Epoxy-Activated Agarose. $NaBH_4$ solution (0.45%, w/v) was prepared in NaOH solution (0.656 M). Agarose 10-BCL (14%, w/v) was suspended in the mixture made of $NaBH_4$ solution, acetone, and epichlorohydrin at a ratio of 4:1.45: 1 (v:v:v). The mixture was stirred overnight at 25°C at 150 rpm. The recovered modified support was washed with distilled water until the filtrate pH value reached 7.

Glyoxyl Agarose (Gly-Ag). Epoxy activated agarose was hydrolyzed by adding 1000 ml 0.5 M H_2SO_4 and agitating the mixture on a shaker with 150 rpm for 4 h at 25°C. The support was filtered on a sintered glass filter and washed with 1000 ml distilled water to reach pH 6. The hydroxyl groups were oxidized by 1000 ml $NaIO_4$ (0.02 M) agitating for 90 min at 25 ^oC and then washed with 1000 ml distilled water.

Glyoxy agarose-IDA/Cu (Gly-Ag-IDA/Cu). 1000 ml iminodiacetic acid (IDA) solution (0.5 M) at pH 11 was mixed with wet, epoxy-activated agarose produced in the first step. The reaction was

mixed by a shaker with 150 rpm for 36 h at 25 °C. Then, the support was filtered and washed by 1000 ml distilled water. The remaining diols were oxidized by treating with 1000 ml NaIO4 (0.02 M) for 90 min followed by washing the support by 1000 ml distilled water. Afterwards, the support was mixed with 1000 ml $CuSO_4$ solution containing 30 mg/ml for 1 h at 25°C. Finally, the support was filtered and washed with 500 ml distilled water. The wet support was stored at 4 °C for further use.

Glyoxyl agarose-TEA (Gly-Ag-TEA). Epoxy activated agarose 10-BCL prepared in the first step was added to 1000 ml solution of acetone and water in the ratio of 1:1 (v:v) containing 0.1 M triethylamine adjusted at pH 12.5. The suspension was mixed by a shaker at 150 rpm for 48 h; then, the support was washed with 500 ml distilled water to reach pH 7. Remaining hydroxyl groups were oxidized by $NaIO_4$ with the same procedure used for glyoxyl agarose preparation.

3.2.5 Selected Levanase Immobilization

The immobilization of levanases on selected supports was carried out in phosphate buffer (600 mM, pH 6), at 4 ^oC and using a protein loading concentration of 1-5mg/g wet support. The immobilization was initiated by adding the enzyme suspension to the wet support and was carried under a gently agitation for a period varying from 8 to 24 h. The free enzyme solution was incubated at the same immobilization conditions in order to estimate the enzyme activity loss that may have occurred during the immobilization procedure. The support, containing immobilized enzyme, was recovered by centrifugation (8000 rpm, 2 min) and washed twice by the 50 mM of potassium buffer (pH 6) to remove any unbound enzyme on support. The immobilized levanase was resuspended in potassium phosphate buffer (50 mM, pH 6) and the activity was measured. Levanase immobilization activity yield and retention of specific levanase activity were determined over the immobilization time course in order to identify the optimal immobilization time. Levanase immobilization activity yield (%) was calculated as the difference between the total units of the free enzyme solution and the supernatant solution divided by the total units of the free enzyme multiplied by 100. Retention of levanase activity was calculated as the ratio of the specific activity of the immobilized enzyme divided by the specific activity of the free enzyme solution multiplied by 100.

3.2.6 Assessment of Thermal Stability

The thermal stability was investigated by incubating the free and immobilized levanases in potassium phosphate buffer (50 mM, pH 6) at 50 °C under orbital shaking at 50 rpm. Every 30 min over 4 h incubation time, an aliquot was taken, and the residual levanase hydrolytic activity was measured as described above. The half-life of the immobilized enzymes was estimated based on the levanase activity decrease over the incubation time.

3.2.7 Kinetic Parameter Measurement

Kinetic parameters (i.e., v_{max}, Km, and Hill coefficient) were measured using low- and high-MW levans as substrates at concentration range varying from 0.05 to 1.0% (w/v). The enzyme activity of each levanase was plotted against the substrate concentration, and the kinetic parameters were computed using SigmaPlot 14.0 software based on Michaelis-Menten and Hill models to identify the model that better fitted the data. Michaelis-Menten (a) and Hill model (b) are described by the following equations:

$$\text{a) } V = Vmax * \frac{[S]}{Km + [S]}$$

$$\text{b) } V = Vmax * \frac{[S]^n}{Km + [S]^n}$$

3.2.8 Product Profile Study

The hydrolysis reaction was initiated by adding the immobilized levanase (0.2 U/mg substrate) to the levan substrate solution at 0.5% (w/v) in 50 mM phosphate buffer (pH 6). The hydrolysis reaction was carried out at 15 and 35 ^{o}C using LMW or HMW levans as substrates at 50 rpm. Over the reaction time course, aliquots were taken from the reaction mixtures. The product profile of the end-products was determined by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector and Breeze™ 2 software. The samples were eluted with isocratic elution at a flow rate of 0.4 ml/min. The polysaccharide analysis was carried out on TSKgel G3000PWXL-CP and TSKgel G5000PWXL-CP, aligned in sequence using 0.1 M NaCl solution as the eluent. While the oligosaccharide analysis was performed on TSKgel G-Oligo-PW column using the HPLC grade water as the eluent. Carbohydrate calibration curves were constructed using dextran and oligosaccharide standards. Total yield was calculated as the percentage of the hydrolyzed levan substrate concentration over the initial one. The oligo yield was measured as the percentage of the released

fructooligosaccharides concentration over the initial levan concentration. Product profile was evaluated based on the standard curves of *D*-fructose, *D*-glucose, sucrose, 1-kestose, nystose and 1^F-fructofuranosylnystose (GF4) for oligosaccharide analysis and the standard curves of dextrans (12 to 640 kD) for polysaccharide analysis as the reported procedure for FOS product profile study (Tian, et al., 2014).

3.2.9 Enzyme Reusability

Enzyme reusability was evaluated by performing 4 successive hydrolysis reactions using the same immobilized levanase. The reaction conditions consisted of 0.5% (w/v) HMW levan, 0.2 levanase U/mg substrate, potassium phosphate buffer (50 mM, pH 6) and 15^{o}C. After each 2 h of reaction, the immobilized levanase was recovered and washed with potassium phosphate buffer (50 mM, pH 6) before reusing it. At each reaction, the total yield and oligo yield were determined.

3.3 Result and Discussion

3.3.1 Immobilization of Levanase on Selected Supports

The recombinant levanases LEV-B.B., LEV-C.O., and LEV-D.F. were immobilized on selected functionalized agarose-10 BCL supports. Pre-immobilization treatments for the modification of agarose support with glyoxyl groups, IDA, IDA-Cu (copper), and TEA possessing hydrophobic, anionic, chelating, and cationic surface, respectively, were carried out. These treatments can promote the multi-covalent attachments of levanases on agarose support. Gly-Ag support with relatively a high hydrophobic surface can result in the immobilization of levanases by hydrophobic interactions and reversible covalent linkages as the result of the formation of imine groups upon the reaction of aldehyde moieties of Gly-AG with the active amine residues of levanase (Mateo et al., 2010). Gly-Ag-IDA can primarily promote the physical adsorption step, by ionic interactions between the negatively charged IDA groups and positively charged residues (e.g. lysine, histidine, and arginine) on the enzyme's surface. Amongst the positively charged amino acid residues, lysine groups were identified as the most likely residues that can make reversible imine covalent linkages (Mateo et al., 2010). Contrary to Gly-Ag-IDA, Gly-Ag-IDA/Cu can act as a chelating support and bind to the enzyme through the chelation of sulfhydryl or amine groups on the enzyme's surface and cupric ions on the support. However, the positively charged Gly-Ag-TEA is expected to promote mainly the ionic interactions between negatively charged aspartate and glutamate residues on the enzyme, and triethyl ammonium groups on the support. Indeed, 3D structure of an enzyme

can help the identification of some available binding sites for immobilisation. Only one crystal structure of endo-levanase from a human gut commensal *Bacteroides thetaiotaomicron* has been recently reported (Ernits et al., 2019). It was still considered worthwhile to analyze the amino acid sequences of the selected levanases and identify potential binding immobilization sites. Table 3.1S shows the primary structures of the levanases and the abundance of the negatively and positively charged amino acids in the enzymes and their active sites. Table 3.1 summarizes the results for the immobilization of levanases on selected modified agarose supports. The results show that the highest immobilization yields of 100, 82.2, and 88.3% were achieved upon the use of Gly-Ag-IDA/Cu for the immobilization of LEV-B.B, LEV-C.O., and LEV-D.F., respectively. These results could be attributed to the strength of the primary interactions between the support and the enzymes, which were mainly of chelation type instead of ionic or hydrophobic interactions. Indeed, according to the amino acid sequences, a number of chelating amino acids such as arginine, lysine, glutamate, and aspartate on the enzyme surface as well as the cysteine tag of the enzymes can be involved in the chelating interactions with Gly-Ag-IDA/Cu. For the other investigated supports, the immobilization yield was dependent on the microbial source of levanase and the type of support. Contrary to other levanases (46.3-64%), LEV-B.B. (85.1%) showed higher immobilization yield upon immobilization of Gly-Ag, which is in agreement with the low abundance of charged residues as well as the high abundance of non-polar residues especially phenylalanine outside the active site. Although LEV-D.F has similar low abundance charged residues outside its active site as LEV-B.B., its immobilization on Gly-Ag led to 64% of immobilization yield; this can be attributed to the fact that the number of non-polar residues of LEV-D.F., especially phenylalanine, is significantly lower than LEV-B.B. The results also show that LEV-C.O. with the highest charged residues outside its active site exhibited the lowest enzyme immobilization yield of 46.3% on the Gly-Ag support. LEV-B.B. showed significant higher affinity toward the positively charged support, leading to substantially higher yield upon immobilization on Gly-Ag-TEA (80.8%) than on Gly- Ag-IDA (51.3%). LEV-D.F behaved similarly in terms of the immobilization yield upon its immobilization on the positively-charged Gly-Ag-TEA and the negatively-charged Gly-Ag-IDA supports (63.4% and 56.9%, respectively). These results are supported by the higher number of negatively charged residues in

Table 3.1S. The amino acid sequences and the number of the negatively charged and positively charged amino acids in the primary structures and active sites of LEV-B.B., LEV-C.O., and LEV-D.F. (the data were borrowed from Uniport database)

LEV-B.B.

MNTRISTLFL FISITFFQAC SQKETVVEEN REFDEQFRPQ YHFSPPANWM NDPNGMVYFE GEYHLFYQYY PDGNVWGPMH WGHAISTDLI HWEHLPIAIY 100
PDDLGWIFSGSAVVDWENTS GLGTGNQPPM IAIYTYHLDS GEKAGRDDYQ TQGIAYSNDK GRTWTKYENN PVLANPGIKD FRDPKVTWHE ESESWIMSLA 200
VKDKISFYTS SNLLEWTYQS DFNPDWAAYG GVWECPDLFP ITTDSGEEKW ILLVSINPGG PNGGSATQYF VGDFDGRVFT TETTEVKWLD YGADNYAGVT 300
WSDVPKEDGR RLFLGWMSNW LYANEVPTEV WRSAMTVPRS LELMKNGDDY SIASRPVEEL EKLRESTKEQ EGDLISLTSD VLEIEMKSLG GDFKMTFSND 400
QGDKLVIDKT DDLVLFDRSQ AGLKDFSDVF ATVHNVPLKG VEVKDIRIFL DRSSIEIFFN DGESVITELI FPTSAYTELS LQGMDSKVEI HLLKSIWGN 490

Asp and Glu in the primary structure	**78**	**Asp and Glu in active site**	**64**
Arg and Lys in the primary structure	**38**	**Arg and Lys in active site**	**30**

LEV-C.O.

MNNKLIAGLG VLTLTACQQN TDNLIIEDFE SGTYANWTVE GDAFGATPAT GSYTGQQPVI DFEGKFLANS FNNGDDSRGT LTSKEFTIKR DYINFLIGGG 100
THPDTYIELL VEGKSVLQTR SLFETETLQW LTWDVKPYKN KKATIRIVDN QRGGWGHILI DQIEQGNKQK SVFMTDYTRT FEAKDKYLLI PIEDQAVENK 200
VQLSVDGTLV GEPMTIRIAQ NKIDYWMPIA IEAYKGKKVT LTFAVAKTTD MGLAEIKQSA EYNFNYNEKY RPLYHFTPQY GWMNDPNGMV YLDGVFHLFY 300
QYNPYGARWG NMHWGHTVSK DLVNWEYKPY VLVPDKLGAI FSGSAVIDHE NTAGFGKGAM VAIFTSAGER QTQSIAYSLD GGKTFTKYEG NPVLTDANII 400
DFRDPKVFWH APSKQWVMSL ATTQTITFYG SKNLKEWTRL SEFGEGLGGH GGVWECPDLF PLTYEGKTKW VLFVSINPGG PNGGSATQYF IGNFDGKTFT 500
PDTMSYPLWLDYGRDNYAGVTWSNVPATDG RRLFIGWMSN WDYANETPTQ NFRSAMTVAR VLRLVHNGEH LVVASEPVKE LESLRREAVL LGDKTRTNTS 600
DAITFENFLP NNQGAYELTF TVTPNETDSF SFALENAKGE TIKYLFDGAN KTLSVDRSKS SVAFNANFAE TLIKAPMVAK KSYTVRLLVD KSSTELFVNN 700
GEVVQTNAVF PSEVYNTLRF NTSKGTLTLN NVTVYKLK 730

Asp and Glu in the primary structure	**83**	**Asp and Glu in active site**	**45**
Arg and Lys in the primary structure	**70**	**Arg and Lys in active site**	**40**

LEV-D.F.

MIDKFINMKK LTILAALLTTNFAHAQETPEKYRPQFHFSPKANWMNDPNGMVFHNGTYHLFYQYYPDAKV WGPMHWGHAT SKDMLHWKEQ TIALYPDSLG 100
YIFSGSAVVD VNNTSGFGKD GKAPLVAIFT HHNPVIEKQK TGLHEYQSIA YSLDDGKSWT KYSGNPVLPN PGITDFRDPK VCWYEPQKKW VMTLATKDRI 200
TFYSSPDLKK WSKESEFGAN AGAHGGVWECPDLFPLMHEGKQVWVLIVNI NPGGPNKGSAGQYFLGDFDG KTFTANSSKT KWLDWGTDNYAAVTFSNTGN 300
RRLLMGWMSN WQYANQVPTD PWRSANTISR ELALTAVDKE LYLTSVPARE LDAIEEGGYS KQNMAAKAPV NLAPKSGNPS GLFRLDFETA SVADFELVLS 400
NKAGNELLIG YDQASNQYYI DRSKSGKTDF EAGFAQKHFA PRLSKNGKID FTLVADVASV EVFADGGLTV MTDIFFPETP LSELSIKSVK GIQVKDLQYS 500
TLKPSME 507

Asp and Glu in the primary structure	**54**	**Asp and Glu in active site**	**46**
Arg and Lys in the primary structure	**51**	**Arg and Lys in active site**	**44**

Table 3. 1. Immobilization results of the selected levanases on functionalized glyoxyl agarose supports

Enzyme	Support	Immobilization Yield (%)[a]	Retention of Specific Activity (%)[b]	Activity of Immobilized Levanase (µmol /min.g support)[c]
Levanase from *B. Baltica*	Gly-Ag	85.1 (±6.7)	45.2 (±1.1)	2.1 (±0.2)
	Gly-Ag-IDA	51.3 (±4.2)	22.3 (±3.0)	0.9 (±0.1)
	Gly-Ag-IDA/Cu	100 (±2.1)	100 (±1.3)	5.1 (±0.4)
	Gly-Ag-TEA	80.8 (±0.2)	31.4 (1.4)	1.4 (±0.2)
Levanase from *C. Ochracea*	Gly-Ag	46.3 (±2.7)	34.0 (±5.6)	1.9 (±0.1)
	Gly-Ag-IDA	46.4 (±1.5)	44.4 (±1.4)	2.2 (±0.3)
	Gly-Ag-IDA/Cu	82.2 (±4.6)	65.6 (±5.2)	6.3 (0.5)
	Gly-Ag-TEA	30.7 (±2.6)	31.4 (±7.6)	1.8 (±0.2)
Levanase from *D. fermentans*	Gly-Ag	64.0 (±2.9)	13.5 (±1.8)	1.3 (0.1)
	Gly-Ag-IDA	56.9 (±1.4)	21.1 (2.2)	1.9 (0.3)
	Gly-Ag-IDA/Cu	88.3 (±0.9)	28.9 (±4.0)	6.4 (0.7)
	Gly-Ag-TEA	63.4 (±0.6)	14.6 (±1.9)	1.6 (±0.5)

[a] Immobilization yield was calculated as the difference between the total units of the free enzyme solution and the supernatant solution divided by the total units of the free enzyme multiple by 100.

[b] Retention of specific activity was calculated as the ratio of the specific activity of the immobilized enzyme divided by the specific activity of the free enzyme solution multiplied by 100.

[c] Activity of the immobilized levanase per gram of support.

the composition of the enzyme primary structures presented in Table 3.1S. However, compared to Gly-Ag-TEA (30.7%), the higher immobilization yield of LEV-C.O on Gly-Ag-IDA (46.4%) indicates its higher affinity towards negatively charged support, which may be attributed to the better accessibility of the positively charged residues, such as protonated histidine, arginine, and lysine, on the LEV-CO considering this enzyme exhibits the highest charged residues than the other investigated levanase candidates. At the best of the authors knowledge, the immobilization of levanase on the selected heterofunctional supports has not been yet reported. Only a patent on the levanase immobilization on hydroxyapatite by the combination of adsorption and crosslinking strategies was reported (Kuboki et al., 1989). The immobilization efficacy was evaluated based on substrate decomposition rate per gram of the immobilized levanase being reported to be between 0.44-0.49 gram of levan/min. g support; however, the immobilization yield, retention of specific enzyme activity, and activity of immobilized levanase were not reported (Kuboki et al., 1989). Hill, Karboune and Mateo (2016) reported the immobilization of LS from *Bacillus amyloliquefaciens* on the functionalized agarose including Gly-Ag, Gly-Ag-IDA/Cu, and Gly-Ag-TEA. Comparison between the immobilization yields of LS on the functionalized agarose including Gly-Ag, Gly-Ag-IDA/Cu, and Gly-Ag-TEA, Eupergit®C-IDA showed the similar trends as the obtained ones for LEV-B.B., and LEV-D.F. immobilization; indeed, the immobilization yields were reported to be high for Gly-Ag-IDA/Cu and Gly-Ag (80.1%, and 81.0%, respectively) and moderate to low for Gly Ag-TEA, and Eupergit®C-IDA, as negatively charged support, (47.9% and 21.7%, respectively) (Hill et al., 2016).

In terms of retention of specific levanase activity, the highest values were achieved, when Gly-Ag-IDA-Cu support was used for the immobilisation of LEV-B.B. (100%), LEV-C.O. (65.6%), and LEV-D.F. (28.9%) as compared to other modified supports. The low retention of specific activity of LEV-D.F. can be explained by the significant changes in its tridimensional structure upon immobilization, the steric hindrance affecting the substrate binding at the enzyme active site, and the presence of substrate diffusional limitations. Levanases with low thermal stability would be more susceptible to tridimensional structure change and to the subsequent denaturation. Indeed, the residual enzyme activity of free levanases after 1-hour incubation at 50 ^{o}C were reported to be 77.97%, 84.62%, and only 8.98% for LEV-B.B., LEV-C.O., and LEV-D.F. respectively (Chen et al., 2020). These results could suggest the enzyme denaturation as the main reason for the observed low retention of the specific activity of LEV-D.F upon immobilization. Contrary to Gly-Ag-

IDA/Cu (100%), the use of other functionalized agarose supports, Gly-Ag-IDA and Gly-Ag, for the immobilization of LEV-B.B., resulted in moderate to low retention of specific activity of 22.3% and 45.2%, respectively. These significant losses of specific activity are most likely due to steric hindrance and/or changes in the tridimensional structure considering the high thermal stability of the free LEV-B.B. (Chen et al., 2020). Chen and Karboune reported that there were numerous conserved glutamate (Glu) groups in the selected levanases. Furthermore, the recent X-ray study on levanase from *B. thetaiotaomicron* showed that the active site was composed of a nucleophile (Asp41), a transition state stabilizer (Asp169) and an acid/base catalyst (Glu221) (Ernits et al., 2019). Considering the high abundance of aspartate (Asp) and glutamate (Glu) residues in the active site of selected levanases, particularly LEV-C.O., the involvement of these residues in the immobilization on Gly-Ag-IDA/Cu. may have resulted in the steric hindrance of the active site of the immobilized enzyme, limiting its accessibility to the substrate

The overall results show that the binding affinity of support-enzyme interactions depicted by the immobilization yield follows more or less the same trend as the specific activity retention. As a result, the activity of immobilized levanase per gram of support showed similar trends as the retention of specific activity. Gly-Ag-IDA/Cu support led to the highest activity of immobilized levanase of 5.1, 6.3, and 6.4 µmol /min. g support for LEV-B.B., LEV-C.O., and LEV-D.F. respectively. For LEV-B.B., the second highest value was achieved upon the use of Gly-Ag (2.1 µmol /min. g support), while for LEV.C.O. and LEV-D.F., Gly-Ag-IDA showed the second highest values (2.2 and 1.9 µmol /min. g support respectively). The reported retention of specific enzyme activity of the LS from *Bacillus amyloliquefaciens* showed the highest value for Gly-Ag-IDA/Cu (67.0%), which is in agreement with our findings and comparable with the record for LEV-C.O. (65.6%) but significantly lower than LEV-B.B.'s value (100%).

As part of the assessment of the efficiency of the supports for the immobilization of selected levanases, the retained levanase activity after incubation at 50 ^{o}C for 20 min to 4 h was investigated. Figure 3.1a-c indicates a comparison between the retained enzyme activity of the free enzymes and their corresponding immobilized forms for LEV-B.B., LEV-C.O., and LEV-D.F. For LEV-B.B, the addition of the IDA functional groups to Gly-Ag supports to create Gly-Ag-IDA (48.1%), did significantly enhance the retained activity upon incubation at 50 ^{o}C in comparison to the free enzyme (20.4%). Gly-Ag and Gly-Ag-TEA led to similar retained activity (35.4%, 35.6%

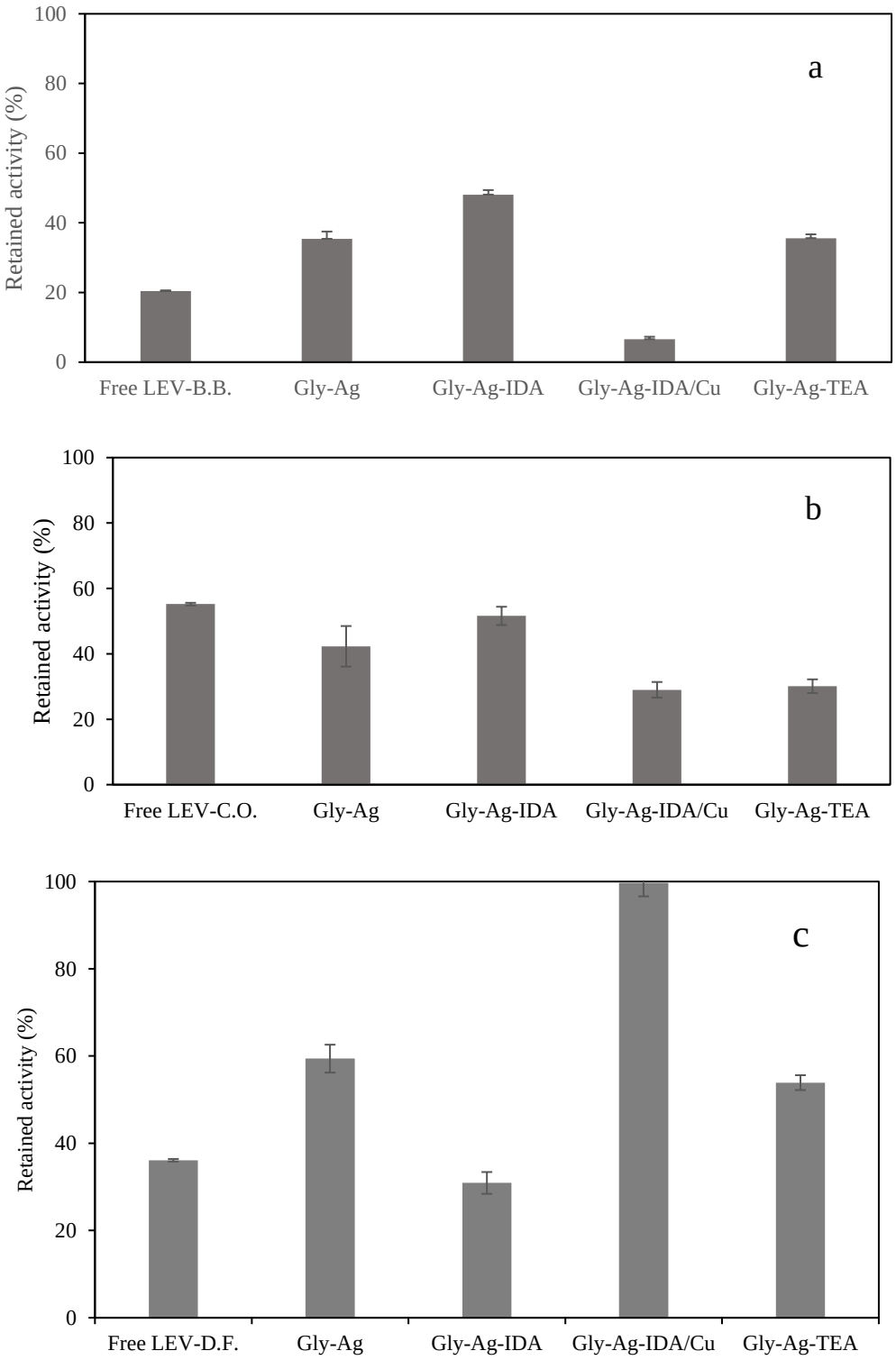

Figure 3. 1 a-c. Comparison between retained levanase activity after incubation at 50 ºC for free and immobilized LEV-B.B. (a), LEV-C.O. (b), and LEV-D.F. (c) after 4h, 1h, and 20 min incubation, respectively.

respectively). However, the incorporation of Cu into Gly-Ag-IDA to yield Gly-Ag-IDA-Cu decreased significantly the level of the retained activity of LEV-B.B, upon incubation at 50 ^{o}C, to 6.6% although it resulted in the highest retention of the specific activity.

These results reveal that support-enzyme interactions involved in the immobilization of LEV-B.B on Gly-Ag-IDA-Cu maintain a good molecular flexibility (high catalysis of immobilized enzyme), but affect the structural stability of the levanase (a tridimensional structure more prone to denaturation). The finding may be attributed to the fact that LEV-B.B. possesses the lowest abundance of Asp and Glu far from the active site, which may have increased the probability of having the chelation of Asp and Glu at the active site with copper ions on the support at the elevated temperature. For LEV-C.O., the immobilization on Gly-Ag and Gly-Ag-IDA resulted in a moderately lower retained enzyme activity (42.3% and 51.6%, respectively) than the free enzyme (55.2%); while a substantial decrease in the retained activity of LEV-C.O was observed upon immobilization on Gly-Ag-IDA/Cu and Gly-Ag-TEA (29.0% and 30.1%, respectively). On the other hand, LEV-D.F. exhibited substantial retained enzyme activity after incubation. An almost complete retained enzyme activity (99.7%) was achieved for immobilization on Gly-Ag-IDA/Cu. This result indicates that Gly-Ag-IDA/Cu had not only the ability to effectively immobilized LEV-D.F. but also to stabilize the enzyme. These effects could be attributed to the intrinsic instability of free LEV-D.F., which is due to its high flexibility; the immobilization can impose higher rigidity on the enzyme to make it more stable. The immobilization of LEV-D.F on Gly-Ag and Gly-Ag-TEA resulted in a moderate improvement in the retained enzyme activity (59.4% and 53.9%) in comparison the the free enzyme (36.1%), whereas the immobilization on Gly-Ag-IDA resulted in a slight decrease in the retained enzyme activity (30.9%).

Comparing the enzyme activity yield, retention of specific enzyme activity, and retained enzyme activity after incubation of the three levanases was preformed to select the most appropriate immobilization support for each levanase.

3.3.2 Thermal stability study of the selected immobilized levanases

The effect of immobilization on the thermal stability of selected levanases was evaluated at 50°C using the most appropriate supports: LEV-B.B. immobilized on Gly-Ag (LEV-B.B./Gly-Ag), LEV-C.O. immobilized on Gly-Ag-IDA (LEV-C.O./Gly-Ag-IDA) and LEV-D.F. immobilized on Gly-Ag-IDA/Cu (LEV-D.F./Gly-Ag-IDA/Cu). Figure 3.2a-c shows the thermal inactivation kinetics of the free and immobilized levanases. The immobilized LEV-B.B. on Gly-Ag showed higher thermal stability than the free enzyme. Indeed, the high thermal stability of immobilized LEV-B.B. on Gly-Ag was exhibited up to 100 min of incubation, where the retained activity reached a plateau at 83%.While the thermal inactivation kinetic of the free LEV-B.B. followed more or less a linear deactivation rate to retain 55% of its initial activity after 100 min incubation. Above 100 min, the retained activity of immobilized LEV-B.B. decreased significantly to reach the same level as the free enzyme. Both free and immobilized Lev-B.B reached a plateau with 17% and 28% retained activity at 280 min, respectively. The presence of two plateaux in the thermal inactivation of immobilized LEV-B-B may be attributed to the multi-covalent attachment, which may have led to two stabilization effects. The half-life of the free and immobilized LEV-B.B. was estimated at 109.5 and 133.3 min, respectively. On the other hand, immobilized LEV-C.O. on Gly-Ag-IDA showed lower thermal stability with half-life of 78.8 min in comparison to the free enzyme (202.4 min). The lower thermal stability for the immobilized LEV-C.O. may reveal the intrinsic sensitivity of LEV-C.O. to tridimensional structure change nearby the active site; this may have happened as the result of the high abundance of charged residues nearby the active site of LEV-C.O. that can be involved in the multi-attachment of LEV-C.O. on Gly-Ag-IDA (Table 3.1S). Interestingly, LEV-D.F. showed the highest thermal stability improvement with a 9-time increase in the half-life (from 3.3 min to 27.8 min) upon immobilization on Gly-Ag-IA/Cu. The most significant difference between the thermal inactivation kinetics of free and immobilized LEV-DF was observed during the first stage of 15 min- incubation; at which the free enzyme showed a sharp decrease in the retained activity from 100 to 7%, while the immobilized enzyme retained over 90% of its initial activity. The high thermal stability of the immobilized LEV-DF can be attributed to the rigidification of its structure by multi-covalent attachments on the support.

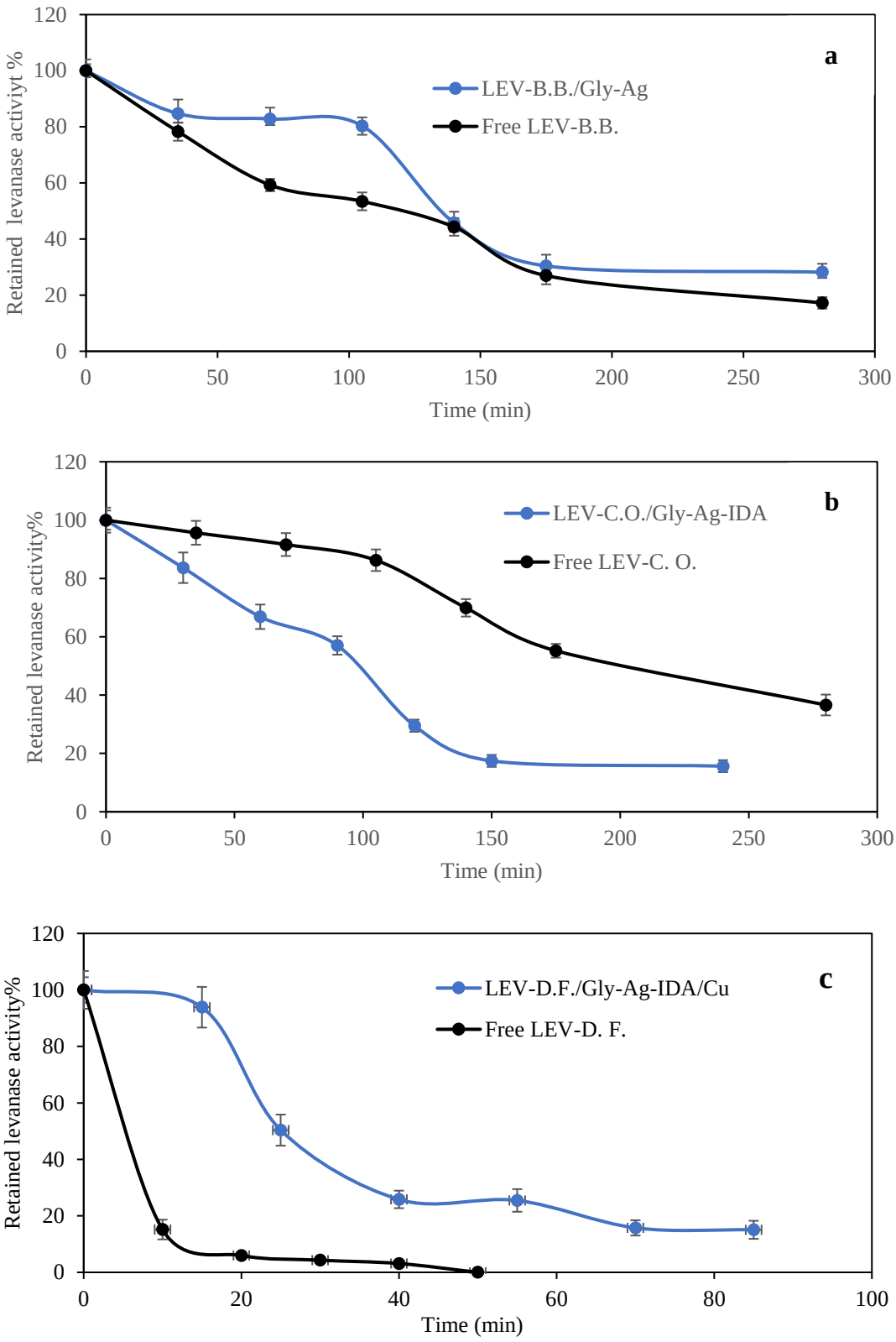

Figure 3. 2 a-c. Thermal stability kinetics of the selected immobilized and free levanases

3.3.3 Kinetic parameter study of the selected immobilized levanases

The kinetic parameters of immobilized LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA, and LEV-D.F./Gly-Ag-IDA/Cu were determined using LMW- and HMW levans as substrates at concentrations ranging between 0.05 to 1%. The kinetics of the hydrolytic activities of three selected immobilized levanases were best fitted to the Hill model ($R^2 > 0.9$) (Table 3.2) than Michaelis-Menten model ($R^2 < 0.8$). The hill model fits sigmoidal, saturated non-Michaelis–Menten kinetics, in which the enzyme cooperativity is significant. Similarly, free LEV-B.B., LEV-C.O., and LEV-D.F. kinetic studies were reported to follow Hill model (Chen et al., 2020). The results also show that the K_m values of LMW levan for all immobilized levanases are substantially higher than those of HMW one. Indeed, the K_m values for the immobilized LEV-B.B., LEV-C.O., LEV-D.F. towards LMW levan were around 9, 5, 6 higher, respectively, than the corresponding values of HMW levan. These experimental findings reveal the higher affinity of all three immobilized levanases towards HMW levan than LMW one. These results can be attributed to the micro-environment effect, which may have favored the substrate binding of HMW levan. Contrary to the immobilized LEV-B.B, and LEV-D.F, it has been reported that the free corresponding levanases have more or less the same affinity towards LMW- and HMW levans (Chen et al., 2020). On the other hand, the free LEV-C.O. was found to exhibit two-time higher affinity towards LMW levan than HMW one.

k_{cat} or turnover number, which is a measure of the number of substrates converted into the product by one molecule of enzyme per unit of time, was more of less similar for LMW and HMW levans for the immobilized LEV-B.B. and LEV-D.F. but significantly different in the case of the immobilized LEV-C.O. (33.2 and 46.3 S^{-1} for LMW and HMW levan, respectively). The immobilized LEV-B.B. and LEV-D.F. showed the lowest (18.6-20.9 S^{-1}) and the highest (74.9-78.0 S^{-1}) k_{cat}, respectively. Compared to the reported k_{cat} values for the corresponding free levanases (98.02-193.20 S^{-1}) (Chen et al., 2020), the immobilization led to the one-fifth to one-sixth of the k_{cat} for LEV-B.B. and LEV-C.O., while LEV.D.F. showed significantly closer values for the free and immobilized enzyme (99.21-101.10 for the free enzyme and 74.91-78.05 S^{-1} for the immobilized one). k_{cat}/K_m can be used to compare the catalytic efficiency of immobilized levanases. Comparing the k_{cat}/K_m showed substantially higher values for HMW levan than LMW one for all immobilized levanases. The higher values for HMW levan revealed the higher efficiency of the immobilized levanases for acting on this substrate than LMW levan, which was in agreement with K_m values.

The lower k_{cat} and higher K_m for the immobilized levanases resulted in substantially lower k_{cat}/K_m values compared to the corresponding free enzymes (Chen et al., 2020). However, the low k_{cat}/K_m cannot only be interpreted as a lower catalytic efficiency of each levanase due to immobilization (Eisenthal et al., 2007), but it reflected the immobilization effects on k_{cat} and K_m and can be attributed to the mass diffusional limitation or altering the 3D structure of the enzymes due to immobilization.

Hill coefficient provides information regarding the cooperativity of enzyme binding sites towards a substrate. Hill coefficient higher than one reveals a positive cooperativity between the binding sites of an enzyme, while values lower than 1 means negative cooperativity between enzyme binding sites revealing an apparent inhibiting effect of the substrate. Hill coefficient equal to one indicates independent binding affinity between the binding sites of an enzyme or a single substrate binding site; this corresponds to the Michaelis-Menten model. Immobilized LEV.B.B./Gly-Ag showed more and less similar Hill coefficients for both LMW and HMW levans (1.58 and 0.94, respectively) than the free enzyme (1.34 and 1, respectively). Similarly, immobilized LEV-D.F./Gly-Ag-IDA/Cu exhibited the same Hill coefficients for both LMW and HMW levans (0.98) as the free enzyme. However, immobilized LEV-C.O. showed a significant change in the kinetic properties as their Hill coefficients for both LMW and HMW levans (1.70 and 1.58, respectively) were different from those reported for the free LEV-C.O. (2.09 and 0.82, respectively) (Chen et al., 2020).

Table 3. 2. Kinetic parameters of LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA, LEV-D.F./Gly-Ag-IDA/Cu using 1% LMW- and HMW levan

Enzyme	Substrate	Vmax[c] (µmol/mg*min)	Km[d] (µM)	k_{cat} (S^{-1})	Catalytic Efficiency k_{cat}/Km	Hill Coefficient	R^2
LEV-B.B/Gly-Ag	LMW Levan[a]	19.54 (±1.65)	259.65	18.56	0.07	1.58	0.99
	HMW Levan[b]	22.04 (±2.58)	30.52	20.94	0.69	0.94	0.91
LEV-C.O/Gly-Ag-IDA	LMW Levan	24.02 (±0.91)	472.71	33.15	0.07	1.70	0.95
	HMW Levan	33.55 (±4.36)	88.35	46.31	0.52	1.58	0.92
LEV-D.F/Gly-Ag-IDA/Cu	LMW Levan	79.27 (± 1.23)	221.47	74.91	0.34	0.98	0.93
	HMW Levan	82.59 (± 5.34)	39.35	78.05	1.98	0.98	0.95

[a] LMW levan, low molecular weight levan

[b] HMW levan, High molecular weight levan

[c] v_{max}, measured as the release of reducing sugars from low- or high-molecular weight levan in µmol per mg enzyme per minute

[d] Km, the concentration (µM) of low- or high-molecular weight levan reached at ½ vmax

3.3.4 End-product profile study of the selected immobilized levanases

The end-product profile of the hydrolysis reactions of LMW and HMW levans, catalyzed by the selected immobilized levanases, was studied. Figure 3.3a-c shows the total yield, the oligo yield and the MW distribution of the FOSs. The difference between total yield and oligo yield provides an indication about the monosaccharide (fructose) yield. The results show that the immobilized LEV-B.B. acting on LMW and HMW levans led to an increase in the total yield from 56.8-60.2 to 67.6-76.6% and from 60.8-60.9 to 76.6-72.9%, at 35 ^{o}C and 15 ^{o}C, respectively, when the reaction time was increased from 2 to 6 h. In contrast, the oligo yield of the hydrolysis reactions-catalyzed by immobilized LEV-B.B. remained more or less constant at 15 ^{o}C (23.4- 25.2%, LWM; 27.8-27.9%, HMW) and decreased at 35 ^{o}C (29.1 to 26.3%, LMW; 30.1 to 24.4%, HMW) when the reaction time increased from 2 to 6 h. These results reveal that longer reaction time promoted exo-hydrolysis by immobilized LEV-B.B. than endo-hydrolysis, releasing monosaccharides. The immobilized LEV-C.O. and LEV-D.F. showed the same total yield pattern as the immobilized LEV-B.B over the time course. However, contrary to immobilized LEV-B.B, immobilized LEV-C.O. led to higher yields at 35 ^{o}C (51.6- 64.9%, LMW; 55.5 to 65.6%, HWM) than at 15 ^{o}C (48.9 to 53.5%, LMW; 44.6 to 50.9%, HMW). The HMW levan hydrolysis by the immobilized LEV-C.O. led to higher oligo-yields (23.3-24.8%, 2hr) than the LMW levan hydrolysis (12.3-13.4%, 6 hr). The results also indicate that the immobilized LEV-D.F. led to more or less similar total yields at 15 ^{o}C (49.1-56.0%, LWM; 48.2-57.3%, HMW) and at 35 ^{o}C (46.5- 54.9%, LMW; 45.3-59.8%, HWM). However, the oligo yield of the hydrolysis reaction-catalyzed by LEV-D.F. was dependent not only on the type of substrate (LMW, HMW), but also on the temperature and reaction time; the highest oligo yield at 15 ^{o}C and 35 ^{o}C was achieved upon the hydrolysis of LMW levan for 2h (17.3%) and HMW levan for 6 h (27.2%).

As an overall, LEV-B.B./Gly-Ag showed the highest oligo yields of 29.1 and 30.9% for the hydrolysis of LMW and HMW levan, respectively, at 35 ^{o}C after 2h reaction. A better compromise was obtained for the hydrolysis of HMW levan with LEV-C.O./Gly-Ag-IDA at 15 ^{o}C with a 23.1% oligo yield and a 44.6% total yield. According to Figure 3.3a-c, for HMW and LMW levans, LEV-B.B./Gly-Ag reaction system resulted in the release of GF_7 as the main oligosaccharide (82.9-84.4% at 15 ^{o}C; 45.6-74.4% at 35 ^{o}C, relative proportion of oligo yield).

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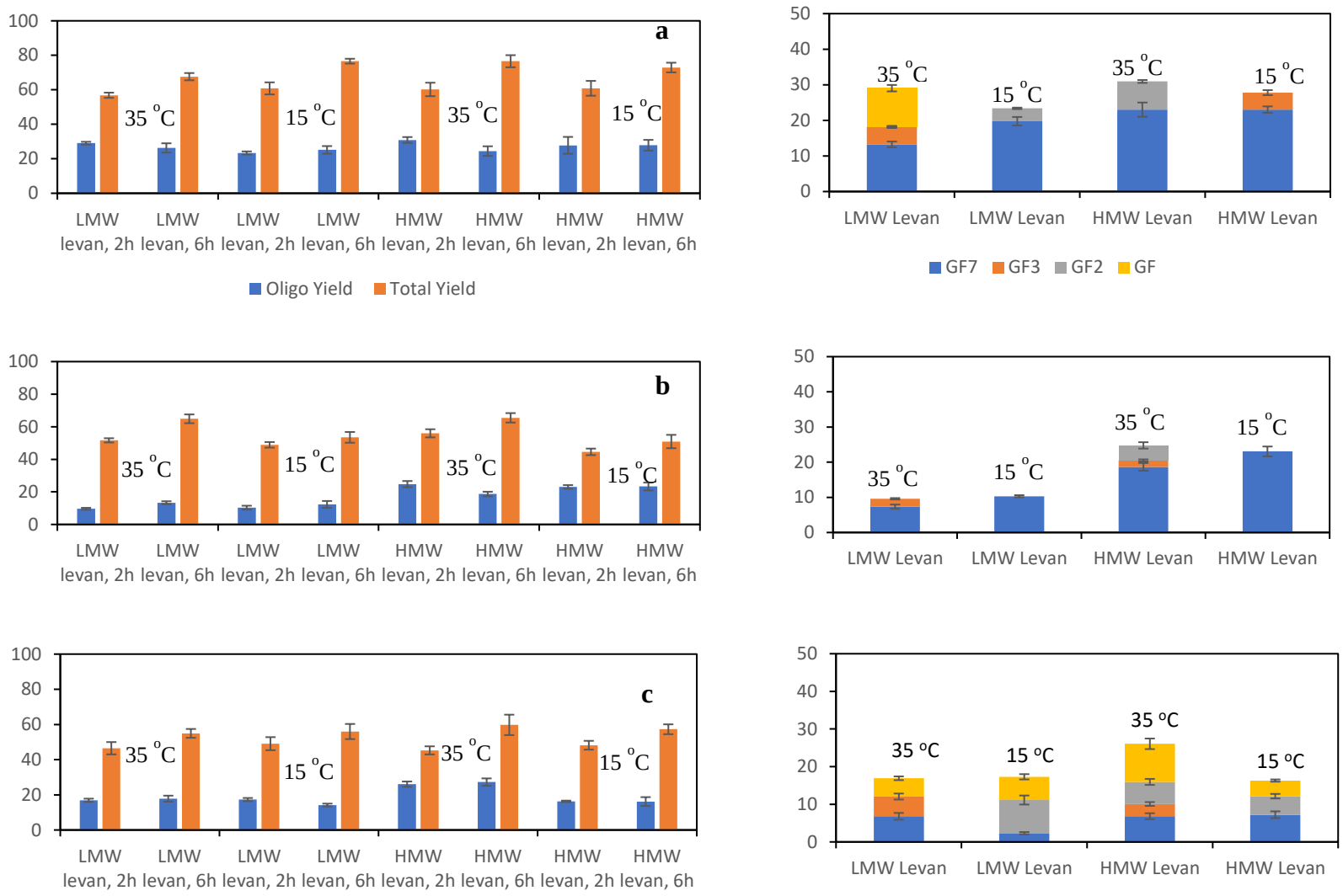


Figure 3. 3 a-c. Total and oligo yields (left graphs) for 2 and 6 h incubation and product profile (right graphs) for 2 h incubation of a) LEV-B.B./Gly-Ag, b) LEV-C.O./Gly-Ag-IDA, c) LEV-D.F./Gly-Ag-IDA/Cu with 0.5% (w/v) LMW and HMW levan at the ratio of 0.2 U of enzyme activity per 1 mg substrate at 35 and 15 ºC and in phosphate buffer (50 mM, pH 6)

Increasing the temperature to 35 °C shifted the end-product profile toward lower MW FOSs (GF3, GF2, GF) in LMW levan- LEV-B.B./Gly-Ag reaction system; while it didn't affect significantly the end-product profile of HMW- LEV-B.B./Gly-Ag reaction system. Similarly to LEV-B.B./Gly-Ag reaction system, LEV-C.O./Gly-Ag-IDA ones with both LMW and HMW levans led to the release of GF_7 as the main oligosaccharide (100 % at 15 °C; 64.8-7.6.6% at 35 °C, relative proportion of oligo yield). The higher temperature of reaction (35°C) shifted the end-product profile toward low MW oligosaccharides (GF_3, GF_2) in both LMW- and HMW- LEV-C.O./Gly-Ag-IDA reaction systems. While the HMW levan- LEV-C.O./Gly-Ag-IDA reaction system at 15 °C showed a high reaction selectivity toward GF_7 (100% relative proportion of oligo yield) with no release of a detectable amount of the shorter FOSs. The LEV-D.F./Gly-Ag-IDA/Cu reaction systems with LMW and HMW levans produced the lowest GF_7 yield compared to the other immobilized levanases reaction systems. In a different pattern, increasing the temperature from 15 to 35 °C increased the GF_7 yield (13.4 to 40.2%, relative proportion of oligo yield) of the LMW levan- LEV-D.F./Gly-Ag-IDA/Cu reaction system, while it maintained it constant for the HMW levan one (44.3 to 26.1%, relative proportion of oligo yield). The HMW levan- LEV-D.F./Gly-Ag-IDA/Cu reaction system led also to an increase in the yield of low MW oligosaccharide (GF_2, GF) when the temperature was increased from 15 to 35 °C. As an overall, increasing the temperature shifted the end-product profile towards lower molecular weight FOSs with an exception in the case of the LMW levan-immobilized LEV-D.F./Gly-Ag-IDA/Cu reaction system. Compared to LMW levan, the use of HMW levan favored the GF_7 formation over the shorter chain FOSs.

As far as the authors are aware, no study has investigated the end-product profile of immobilised levanases. However, the reported end-product profiles of the endo-levanases were dependent on the origin the enzymes (W. Zhang et al., 2019). For instance, the levanase form *Bacillus sp. L7* and *Bacillus licheniformis* hydrolysed levans into scFOSs with a degree of polymerization between 2 and 10 (Zhang et al., 2019). However, the levanase from *Pseudomonas K-52* demonstrated the ability to hydrolyse levans into a mixture of scFOSs enriched by levanoctaose, with DP = 7 (Kang et al., 1998). Although free LEV-B.B., LEV-C.O., and LEV-D.F. were reported to led to a high oligo-yield (50-62%) compared to the immobilized ones, they were found to exhibit less reaction selectivity, resulting in the release of a mixture of a short chain FOSs, GF_5, GF_4, GF_3, GF2 and GF (Chen et al., 2020). Therefore, the immobilization of LEV-B.B., LEV-C.O., and LEV-D.F. could

modulate the enzyme specificity and help to control the molecular size of the hydrolysis products by producing GF_7 as the main FOS.

3.3.5 Reusability study of the selected immobilized levanases

One of the advantageous features of immobilized enzyme is its reusability. The reusability of an immobilized enzyme depends not only on its thermal stability but also on their stability against leaching from the solid immobilization support. To assess the enzyme reusability, the total conversion yield was determined for four consecutive batches of hydrolysis of HMW levan. Figure 3.4a-c shows the total conversion yields upon four consecutive reuses of the same immobilized levanases. As expected, the total conversion yield decreased upon the reuse of immobilized levanase. LEV-B.B./Gly-Ag showed the most significant decrease in the conversion yield from 57.7 to 7.25% after four reuses. While the reusability of LEV-C.O./Gly-Ag-IDA and LEV-D.F./Gly-Ag-IDA/Cu resulted in a decrease in the total conversion yield from 49.4 to 18.6 and from 50.0% to 18.09%, respectively. The presence of IDA on the Gly-Ag seems to limit the loss of the enzyme activity. Indeed, although LEV-B.B./Gly-Ag showed the highest half-life (133 min), it resulted in the most limited reusability, which can be attributed to the leaching of levanase from the support and/or to the substrate/product inhibition. The immobilized LEV-C.O./Gly-Ag-IDA and LEV-D.F./Gly-Ag-IDA/Cu, on the other hand, showed the highest total yield after the fourth batch, conserving 37.7 and 36.1% of the maximum yield, respectively, although they exhibit a shorter half-life of 78.8 and 27.8 min, than the immobilized LEV-B.B. The superior reusability of the immobilized LEV-C.O. and LEV.D.F. in comparison with the immobilized LEV-B.B. could indicate the importance of the type of enzyme-support interaction in preventing enzyme leaching and accordingly in immobilized enzyme reusability.

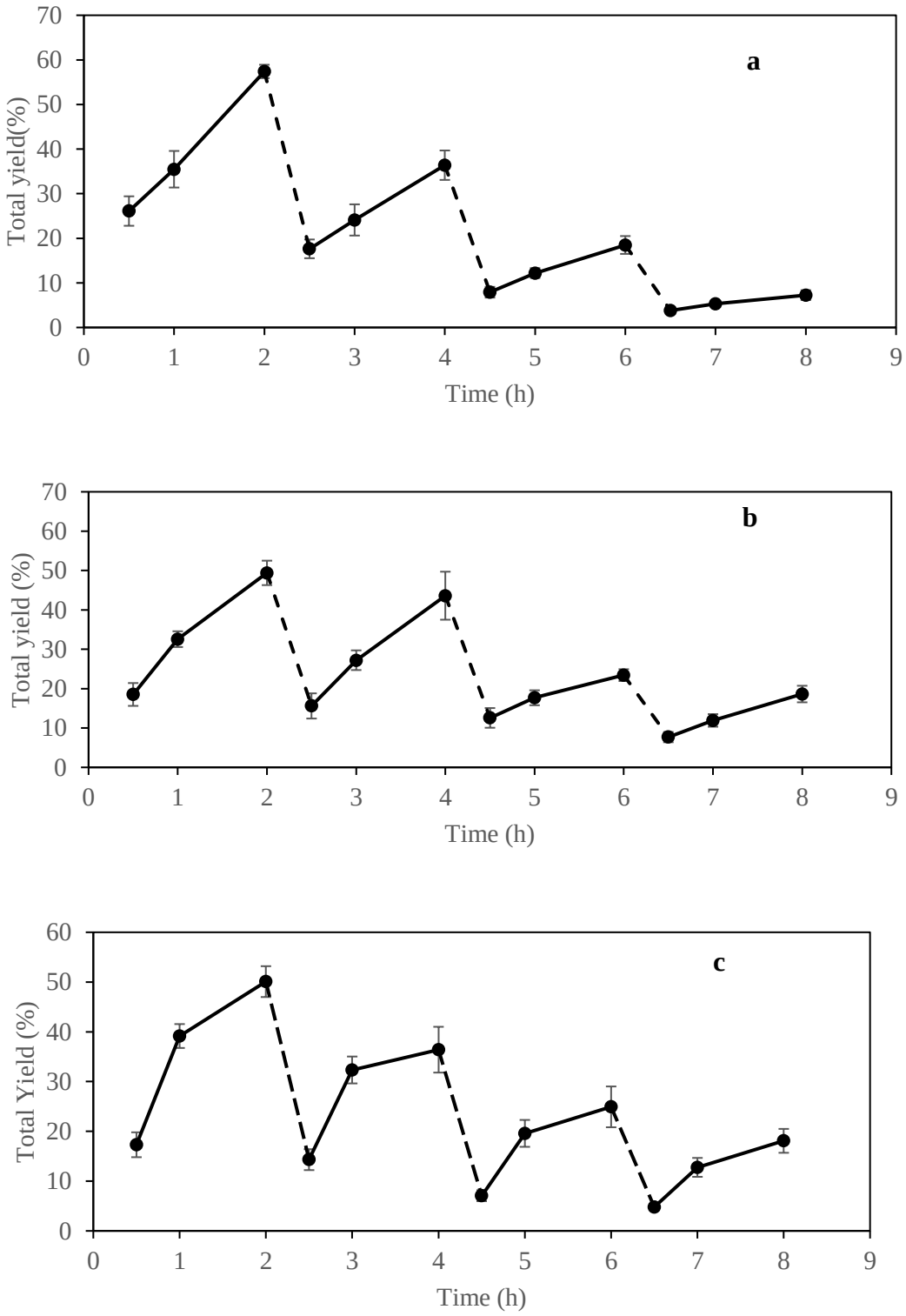


Figure 3. 4 a-c. The enzyme reusability graphs of a) LEV-B.B./Gly-Ag, b) LEV-C.O./Gly-Ag-IDA, c) LEV-D.F./Gly-Ag-IDA/Cu by applying 2 h incubation for each batch at 15 ºC in phosphate buffer (50 mM, pH 6)

3.4 Conclusion

Recombinant levanases form *B. baltica* (LEV-B.B.), *C. ochracea* (LEV-C.O.), *D. fermentans* (LEV-D.F.) were immobilized on modified glyoxyl agarose supports. Modified glyoxyl agarose supports possessing the ability to make muti-attachment immobilization, including Gly-Ag, Gly-Ag-TEA and Gly-Ag-IDA, and Gly-Ag-IDA-Cu were tested. LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA, and LEV-D.F./Gly-Ag-IDA/Cu were identified as the most appropriate immobilized levanases as they do allow the best compromise between the immobilized enzyme activity yield, retention of specific levanase activiy, and retained levanase activity after incubation at 50 ^{o}C. The results showed that the improvement in the thermal stability and the product selectivity upon immobilization on functionalized glyoxyl agarose was dependent on the type of the levanase and the immobilization support. All the immobilized levanases followed Hill Model. The immobilized LEV-B.B. and LEV-D.F. showed the lowest and the highest k_{cat}s, respectively. Comparing the k_{cat}/K_m showed substantially higher values for HMW levan than LMW one for all immobilized levanases showing the higher efficiency of the immobilized levanases for acting on HMW levan. Immobilized LEV-C.O. on Gly-Ag-IDA showed a high product selectivity towards GF_7 production (100% of the relative oligo yield) with no release of shorter oligosaccharides, while a decrease in the thermal stability was observed upon immobilisation; this decrease in the thermal stability was compensate by it high reuasibility in a batch reactor. On the other hand, immobilized LEV-D.F. on Gly-Ag-IDA/Cu exhibited lower reaction selectivity but a significant thermal stability improvement upon immobilization (9 times). Immobilized LEV-B.B. on Gly-Ag exhibited a moderate enzyme selectivity and a half-life improvement. In addition, LEV-C.O./Gly-Ag-IDA and LEV-B.B./Gly-Ag showed the highest and the lowest reusability upon four consecutive enzymatic reactions, respectively.

CHAPTER IV

Bi-enzymatic Immobilized LS-B.A./LEV-C.O. Systems

Abstract

In this chapter, the bi-enzymatic systems based on the combined use of immobilized levansucrase from *Bacillus amyloliquefaciens* (LS-B.A.) on Gly-Ag-IDA/Cu and immobilized levanase from *Capnocytophaga ochracea* (LEV-C.O.) on Gly-Ag-IDA were investigated. Two-step (1:1 LS/LEV), and one-step bi-enzymatic systems (1:1, 1:0.67, 1:0.1, 1:07, LS/LEV) were investigated. In the one-step bi-enzymatic system, LS and LEV were either co-immobilized or immobilized separately on selected supports. The effect of the enrichment of the one-step immobilized LS/LEV bienzymatic system with levan (0.5%; 55±1.5 kDa) was also studied. The FOSs, levan and total product yields as well as the product profile were characterised in order to assess the efficiency of each bi-enzymatic system. The highest oligo yield was achieved in the two-step bi-enzymatic system (45.7%), while the lowest one was obtained upon the use of the one-step co-immobilized bi-enzymatic system (10.8%). Adding 0.5% levan to the one-step immobilized LS/LEV bienzymatic system led to an increase in the oligo yield to 28.0%. The presence of HMW levans (1000-10000 kDa) was found to limit the hydrolytic action of levanase, while it does suppress the sucrose hydrolysis. Two-step bi-enzymatic system resulted in a superior product specificity by producing 45.7% GF_7 and only 3.5% GF, while the other bi-enzymatic systems led to a mixture of GF_3, GF_2 and GF_7, with the last one being the most abundant one. The optimization of the two-step bi-enzymatic system by response surface methodology led to the development of predictive models of oligo, levan and total yields. The interaction between the reaction time of the first LS step and the LS:LEV ratio occurred mainly at longer time; no significant effect of LS:LEV ratio was observed at the lower range of reaction time. The optimum yield of 63% can be achieved upon the use of LS/LEV ratio of 1:1 (U:U) and reaction time of 15h for the first LS step, followed by 48 h for the second LEV step.

4.1 Introduction

β-2-6, levan-type fructooligosaccharides (FOSs) have attracted a great interest as prebiotics. The levan-type FOSs can be produced upon the hydrolytic action of endo-levanases on levan polysaccharides (Zhang et al., 2019). However, this top-to-bottom strategy is limited by the low availability of levans and their low concentrations. Contrary to inulin (Paeschke & Aimutis, 2011), only limited sources of levans have been identified including timothy grass (*Phleum pratense*) and agave (Matsuhira et al., 2014). The levan-type FOSs can be produced through LS-catalyzed the transfructosylation of the abundant sucrose substrate (Hill et al., 2017). LSs (EC 2.4.1.10), which are belong to the glycoside hydrolase family 68 (GH68) can catalyze four reactions: the hydrolysis of sucrose, the exchange reaction, the fructosylation and the polymerisation reaction. LSs from Gram-negative bacteria synthesize mostly FOSs and low amounts of levan; while LSs from Gram-positive bacteria synthesize dominantly high-molecular weight levan (González-Garcinuño et al., 2017). Recently, some hypotheses and structural features have been put forward to describe the hydrolysis/transfructosylation ratio (Xu et al., 2018) and the polymerization/oligomerization ratio (Raga-Carbajal et al., 2015). However, LS end-product profile depends not on the microbial origin of the enzyme, but also on the reaction conditions and the predominant acceptor (Li et al., 2015). For instance, LS from *Bacillus amyloliquefaciens* (LEV-BA) was reported to produce 47% (w/w) levan and only 3% (w/w) levan-type FOSs in the reaction system at sucrose concentration of 0.6 M in 100 mM phosphate buffer, pH 6, (Tian & Karboune, 2012). Recently, gene cloning and the over-expression of 32 LSs in *E. coli* have resulted in finding the LS with high thermal stability and HMW levan production capacity (Hill et al., 2019).

As part of our effort to improve the production of controlled molecular size FOSs, a bienzymatic system based on the synergistic actions of LS from *B. amyloliquefaciens* (LEV-B.A.) and endo-inulinase from *Aspergillus niger,* was investigated for the synthesis of FOSs and oligolevans using sucrose as an abundant substrate (Tian et al., 2014). LS catalyzes the synthesis of levan from sucrose, whilst the endo-inulinase hydrolyses levan into FOSs and oligolevans. The end-product profile study of the bi-enzymatic LS/endo-inulinase system revealed that levan formation by LS was the prerequisite of FOSs synthesis by the endo-inulinase action (Tian, et al., 2014). Endo-inulinase does not exhibit high specificity towards β-(2-6) levan and was shown to be not able to hydrolyze efficiently HMW levan (Tian et al, 2014). In the present study, immobilized levanase

from *Capnocytophaga ochracea* (LEV-C.O.) on Gly-Ag-IDA was used in combination with immobilized LS from *B. amyloliquefaciens* (LEV-B.A.) on Gly-Ag-IDA/Cu to study the bi-enzymatic system. In our previous study, the immobilization of LEV-B.A. on Gly-Ag-IDA/Cu was reported to result in superior transfructosylation/hydrolysis ratio (120%), retention of enzyme activity (67.0%), and thermal stability (stability factor of 13.6) (Hill et al., 2016). While the immobilization of LEV-C.O. on Gly-Ag-IDA led to the immobilization yield of 46.4%, retention of specific activity of 44.4%, and 2.2 μmol /min.g of activity of immobilized levanase per gram of support (chapter III). To assess the effect of micro-environment on the catalytic efficiency of bi-enzymatic system, co-immobilization of LS (LEV-B.A.) and levanase (LEV-C.O.) on the same Gly-Ag-IDA/Cu support was also studied. The efficiency and the end-product profile of one and two-step bi-enzymatic systems were determined and discussed. The bi-enzymatic system was optimized using response surface methodology, and the predictive models were developed and used to better understand the effects of reaction parameters.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

Sucrose, *D*-(-)-fructose, *D*-(+)-glucose, 3,5-dinitrosalicylic acid (DNS), NaOH, polyethylene glycol (PEG) 200, potassium sodium tartrate ($KNaC_4H_4O_6$), $NaIO_4$, $NaBH_4$, iminodiacetic acid $HN(CH_2CO_2H)_2$, and $CuSO_4$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $CaHPO_4$, $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $Na_2HPO_4.2H_2O$, $NaMoO_4.2H_2O$, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, Bovine Serum Albumin, and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Agarose 10BCL was purchased from Agarose Bead Technologies. *B. amyloliquefaciens* (ATCC 23350) was obtained from American Type Culture Collection (Manassas,VA, USA). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON, Canada).

4.2.2 Production and Purification of LS from *B. amyloliquefaciens*

Culturing was carried out by adding 4 ml of the pre-cultured media of *Bacillus amyloliquefaciens* (ATCC23350) to 400 ml of modified mineral salt medium and incubating at 120 rpm on an orbital shaker at 35 ^oC for 24 h. The culturing medium was made of (in g/L) $NaHPO_4.H_2O$ (2. 67), KH_2PO_4 (1.36), $(NH_4)_2SO_4$ (0. 5), $FeSO_4$ (0.05), $MnSO_4.H_2O$ (0.0018), $Na_2MoO_4.H_2O$ (0.0025),

$CaPO_4.2H_2O$ (0.01), $MgSO_4.H_2O$ (0.02), and yeast extract 10.0 g/L. Afterward, the culture medium was centrifuged at 8000 rpm, and 4 °C for 20 min. The pellets were resuspended in potassium phosphate buffer (50 mM, pH 6) containing 1% Triton X-100. The bio-mass disruption was carried out by ultrasonication for 6 min and 25 s, set at 15 kHz with 25/50 s cycles. Then, the suspension was centrifuged (8000 rpm, 4°C) for 15 min followed by PEG-200 (30%) addition to pre-purify LS. The solution was stirred gently at 4 °C for 14 h. The recovered protein precipitate was recovered by centrifugation (4 °C, 12 000 rpm, 45 min), resuspended in potassium phosphate buffer (50 mM, pH 6) and then dialyzed against the same buffer with a molecular weight cut-off of 6-8 kDa. Finally, the dialyzed sample was freeze-dried and stored at -80 °C. The protein content of pre-purified LS-BA extract was determined by Bradford protein assay using the bovine serum albumin as a standard.

4.2.3 Production of Levanases

LB media, made of 40% (w/v) tryptone, 20% (w/v) yeast extract, 40% (w/v) NaCl and (0.1 mg/mL) carbenicillin, was used for aerobically preculturing *E. coli* BL21(DE3)-pLysS over-expressing levanase-encoding genes for *C. ochracea* (LEV-C.O.). Preculturing took overnight incubation at 37 °C and 250 rpm with continuous agitation (New Brunswick Scientific) to reach the optical density (OD) of 1.2-1.4 at 600 nm. Culturing step was carried out in commercial Terrific Broth (TB) media (47.6 g/L) containing carbenicillin (0.1 mg/mL final concentration) using 50-times dilution of the precultured samples; upon achieving a final optical density (OD) of 1.2-1.4, the IPTG was added to the culture media to reach a final concentration of 1 mM. Incubation proceeded at 25 °C for 20 h before cell mass separation by centrifugation at 4 °C (8000 rpm for 20 min). Pellets containing cells were re-suspended in sonication buffer [10% (w/v) glycerol, 30 mM PIPES, 30 mM NaCl, pH 7.2] prior to being treated by lysozyme (4 mg/g cell mass) and DNase (2000 U, 4 µL/g cell mass), followed by a 1-hour incubation period at 18 °C and 50 rpm in an orbital shaker. The cell suspension was ultrasonicated and centrifuged at 4 °C (10000 rpm for 1 h) to obtain crude enzyme extract, which was subsequently dialyzed against 5 mM potassium phosphate buffer (pH 6.0) with a membrane cut-off of 6-8 kDa at 4 °C, and freeze dried at -40 °C. Purification by affinity chromatography (IMAC) on HisTrap^{TM} FF 1 mL column were carried out using imidazole solutions at 5, 10, 50, 100, and 200 mM as the eluent, prepared in 30 mM PIPES buffer containing 30 mM NaCl and 10 % (v/v) glycerol (pH 6.4). The purity of the recovered

fractions upon affinity chromatography were subjected to electrophoretic analysis using the 15% SDS polyacrylamide gels (Bio-Rad, Qc, Canada). SDS-polyacrylamide gel electrophoresis was conducted at 120 V in 10-time diluted Tris/Glycine/SDS buffer containing 25 mM, 192 mM, 0.1% of each, respectively. Pure enzyme fractions were obtained when the imidazole gradient reached concentrations of 100 mM-200 mM.

4.2.4 Levansucrase and Levanase Activity Assay

A unit of total LS and LEV activity was defined as the amount the biocatalyst that released 1 μmol of reducing sugars from sucrose and levan, respectively, per min. To initiate the transfructosylation reaction,125 μL the LS (20-200 μg protein) diluted in 50 mM potassium phosphate buffer (pH 6.0) was incubated with sucrose solution (1.8 M, 125 μL) for 20 minutes at 30°C. While the hydrolysis reaction-catalyzed by LEV was initiated by mixing 125 μL the LEV (15-45 μg protein) diluted in 50 mM potassium phosphate buffer (pH 6.0) with the levan solution (1%, 125 μL) for 20 minutes at 37 °C. To quantify the reducing sugars, 375 μL DNS reagent (1% (w/v) DNS, 1.6% (w/v) NaOH) was added to the reaction mixtures, which were then boiled for 5 minutes for complete enzyme inactivation. Finally, 125 μL potassium sodium tartrate (50% w/v) was added to stabilize the colorimetric reaction. All measurements were done in duplicate, and absorbance was measured by DU 800 UV/Visible Spectrophotometer, Beckman at 540 nm. The enzyme and substrate blanks were carried out in parallel with the reaction. The amount of released reducing sugars was determined from a standard curve constructed with glucose (0.0-12.0 mM). Enzyme activity, expressed by μmol/min.ml, was calculated by plotting the reducing sugar concentration against the enzyme concentration. Specific enzyme activity was expressed as the enzymatic units (μmol/min) per mg proteins.

4.2.5 Preparation and Functionalization of Glyoxyl Agarose-based Supports

The functionalized glyoxyl-agaroses were prepared based on of the methods reported by Mateo et al. (2010) and Hill et al. (2015).

Epoxy-Activated Agarose. $NaBH_4$ solution (0.45%, w/v) was prepared in NaOH solution (0.656 M). Agarose 10-BCL (14%, w/v) was suspended in the mixture made of NaBH4 solution, acetone, and epichlorohydrin at a ratio of 4:1.45: 1 (v:v;v). The mixture was stirred overnight at 25 °C at 150 rpm. The recovered modified support was washed with distilled water until the filtrate pH value reached 7.

Glyoxyl Agarose (Gly-Ag). Epoxy activated agarose was hydrolyzed by adding 1000 ml 0.5 M H_2SO_4 and agitating the mixture on a shaker with 150 rpm for 4 h at 25 °C. The support was filtered on a sintered glass filter and washed with 1000 ml distilled water to reach pH 6. The hydroxyl groups were oxidized by 1000 ml $NaIO_4$ (0.02 M) agitating for 90 min at 25 ^oC and then washed with 1000 ml distilled water.

Glyoxy agarose-IDA/Cu (Gly-Ag-IDA/Cu). 1000 ml iminodiacetic acid (IDA) solution (0.5 M) at pH 11 was mixed with wet, epoxy-activated agarose produced in the first step. The reaction was mixed by a shaker with 150 rpm for 36 h at 25 °C. Then, the support was filtered and washed by 1000 ml distilled water. The remaining diols were oxidized by treating with 1000 ml $NaIO_4$ (0.02 M) for 90 min followed by washing the support by 1000 ml distilled water. Afterwards, the support was mixed with 1000 ml $CuSO_4$ solution containing 30 mg/ml for 1 h at 25 °C. Finally, the support was filtered and washed with 500 ml distilled water. The wet support was stored at 4 °C for further use.

4.2.6 Levansucrase and Levanase Immobilization

The immobilization of LEV-CO on selected supports was carried out in phosphate buffer (600 mM, pH 6) at 4 ^oC and using a protein loading concentrations of 1 mg/g wet support. The immobilization was initiated by adding the enzyme suspension to the wet support and was carried under a gently agitation for a period varying from 8 to 24 h. The immobilization of LS-BA on selected supports was preformed in phosphate buffer (608 mM, pH 6.8) at 4 ^oC and using a protein loading concentrations of 9.09 mg/g wet support. The immobilization was initiated by adding the enzyme suspension to the wet support and was carried out under a gently agitation for a period varying from 8 to 48 h. The support, containing immobilized enzyme, was recovered by centrifugation (8000 rpm, 2 min) and washed twice by the 50 mM of potassium buffer (pH 6.0) to remove any unbound enzyme on support. The immobilized enzymes were resuspended in potassium phosphate buffer (50 mM, pH 6) and their activities were measured. LS and LEV immobilization activity yield and retention of specific enzyme activity were determined over the immobilization time course in order to identify the optimal immobilization time. LS and LEV immobilization activity yield (%) was calculated as the difference between the total units of the free enzyme solution and the supernatant solution divided by the total units of the free enzyme multiplied by 100. Retention of LEV and LS specific activity was calculated as the ratio of the

specific activity of the immobilized enzyme divided by the specific activity of the free enzyme solution multiplied by 100.

4.2.7 Co-immobilization of LS-B.A. and LEV-C.O.

To co-immobilize LS-B.A and LEV-C.O. on Gly-Ag-IDA/Cu support, both enzymes were mixed at ratios of 1:0.67 and 1:0.07 (U:U) in 600 mM phosphate buffer (pH 6.4). The Gly-Ag-IDA/Cu support was added to the enzyme suspensions to achieve a protein loading of 1 mg of proteins/g support. The suspensions were gently mixed under shaking mode for 24 h. LS/LEV immobilization activity yields were measured during the immobilization procedure to monitor the enzyme immobilization progress. After 24h, when there was no detectable LS or LEV activity in the supernatant, the co-immobilized LS/LEV system was recovered by centrifugation (8000 rpm, 2 min) and washed twice by phosphate buffer (50 mM, pH 6) to remove any unbound protein.

4.2.8 Time Course and End-Product Profile of the Bi-enzymatic Reaction System

The bi-enzymatic reactions were initiated by adding the immobilized LS-B.A. and LEV-C.O., either in a one-step or two-step reaction at ratios of 0.6 U:0.6 U or 0.6 U:0.06 U to sucrose solution at 600 mM in 100 mM phosphate buffer (pH 6). The reactions were carried out at 15 ^{o}C and 50 rpm. Over the reaction time course, aliquots were taken from the reaction mixtures. The end-product profile was determined by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector and Breeze™ 2 software. The samples were eluted with isocratic elution at a flow rate of 0.4 ml/min. The polysaccharide analysis was carried out on TSKgel G3000PWXL-CP and TSKgel G5000PWXL-CP, aligned in sequence using 0.1 M NaCl solution as the eluent. While the oligosaccharide analysis was performed on TSKgel G-Oligo-PW column using the HPLC grade water as the eluent. Total yield was calculated as the percentage of the sucrose concentration consumed in a period of time over the initial one. The oligo yield was measured as the percentage of the released fructooligosaccharides concentration over the initial sucrose concentration. Product profile was determined based on the standard curves of D-fructose, D-glucose, sucrose, 1-kestose, nystose and 1F-fructofuranosylnystose (GF4) for the oligosaccharide analysis and the standard curves of dextrans (12 to 640 kD) for polysaccharide analysis as the reported procedure for FOS product profile study (Tian et al., 2014).

4.2.9 Optimization of LS-B.A. and LEV-C.O. Bi-enzymatic Reaction System

The effects of bi-enzymatic system parameters were studied using RSM and a central composite rotatable design (CCRD). Factors considered important for the bi-enzymatic system were the first step incubation time (6-24 h) and the LS proportion (50.00-83.33%), while other conditions such as incubation time for the second step (48 h), the ratio of the total enzyme unit per sucrose solution volume (1.2 U/1 ml), the sucrose solution concentration (600 mM), temperature (15 ^{o}C), and the buffer (100 mM PB with pH 6) were kept fixed. A five-level, two variable central composite rotatable design was created using Design Expert® Software (version 8.0.7) (Box & Behnken, 1960). The full designs consisted of 4 factorial points, 4 axial points, and 2 center points and the levels of the parameters were determined based on the preliminary trials. Total product yield (total, %), the fructooligosaccharide yield (oligo, %) and the levan yield (poly, %) were the quantified responses.

4.3 Results and Discussion

4.3.1 Two-step and one-step bi-enzymatic Systems of the Immobilized LS and LEV

The LS/LEV bi-enzymatic system was investigated in one-step and two-step reactions. In the two-step reaction, the transfructosylation reaction of sucrose was first conducted by immobilized LS-B.A on Gly-Ag-IDA/Cu; then after 12 h of reaction period, immobilized LEV-C.O on Gly-Ag-IDA was added to yield LS: LEV ratio of 1:1 (U:U). In the one-step bi-enzymatic system, the combined use of immobilized LS-B.A/Gly-Ag-IDA/Cu and LEV-C.O./Gly-Ag-IDA or co-immobilized LS/LEV on Gly-Ag-IDA/Cu was carried out.

The use of co-immobilization may favor the substrate channeling mode as the dominating way for transferring the reaction intermediates between the active sites of the co-immobilized LEV and LS instead of the intermediate's diffusion mode through the solution (Ji, Wang, Tan, Zhu, & Li, 2016). Contrary to the two-step reaction system, interference between the enzyme activities may occur in the one-step reaction system, when their combined use affects the availability of their substrates and their thermodynamic equilibrium. In this case, the ratio of the enzymes must be determined based upon the enzyme activities towards the starting material and the desired product (Ji et al., 2016). Our preliminary trials showed that immobilized LS-B.A./Gly-Ag-IDA/Cu didn't exhibit activity towards HMW and LMW levans, which were further used as substrates by LEV; while LEV-C.O./Gly-Ag showed a hydrolytic activity towards the initial sucrose substrate of LS-

B.A./Gly-Ag-IDA/Cu. The potential interference of enzyme activities was assessed by using LS/LEV ratios of 1:0.1 and 1:1 (U:U) in the one step enzymatic reaction.

To assess the efficiency of the immobilization of LEV and LS on selected supports, Gly-Ag-IDA and Gly-Ag-IDA/cu, respectively, the immobilization yield and the retention of activity were determined. LEV-C.O. immobilized on Gly-Ag-IDA resulted in 46.4% and 44.4% retention of activity, according to the results of Chapter III, while LS-B.A. immobilized on Gly-Ag-IDA/Cu led to 76.5% of immobilization yield and 81.6% of retention of activity after 24 h incubation (data not shown). In the case of co-immobilization of LEV and LS on Gly-Ag-IDA/cu, the immobilization couldn't be monitored at the same time for both biocatalysts. The immobilization time course showed that under the selected conditions (600 mM phosphate buffer, pH 6,), both LEV-C.O. and LS-B.A. are completely immobilized after 24 h incubation (Figure 4.1). Also, separately immobilized LEV-C.O. and LS-B.A. on Gly-Ag-IDA/Cu showed the enzymes retained 13.5% and 100% of their initial activity after immobilization, respectively. Considering these results, the amount of LEV and LS were adjusted to achieve the appropriate targeted ratio of co-immobilized LS:LEV of 1:1 and 1:0.1 (U:U).

4.3.2 Time Courses for Bi-enzymatic Systems

The time courses for the two-step, one-step, and co-immobilized bi-enzymatic systems were investigated over 48 h reaction period using the selected LS:LEV unit ratios (Figure 4.2). In the one-step immobilized LS:LEV reaction system, the 1:0.1 LS:LEV unit ratio was found to be the most appropriate, limiting the hydrolysis of sucrose and favoring the transfructosylation reaction-catalyzed by LS.

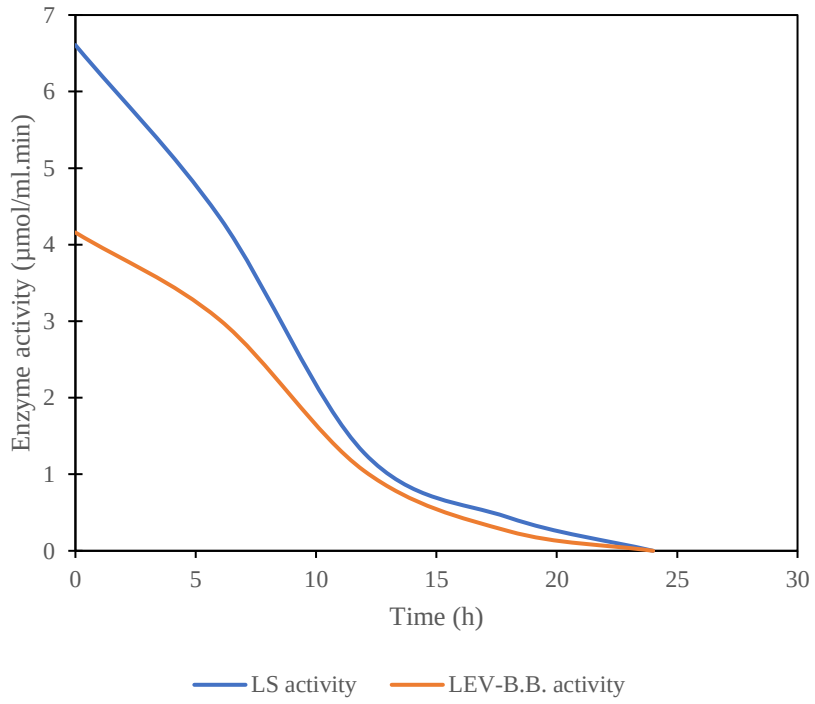


Figure 4.1. Enzyme activity in the immobilization supernatant of LS-B.A. and LEV-C.O. on Gly-Ag-IDA/Cu in 600 mM phosphate buffer, pH 6, and 1 g support per 1 mg of the enzymes at 4 ºC

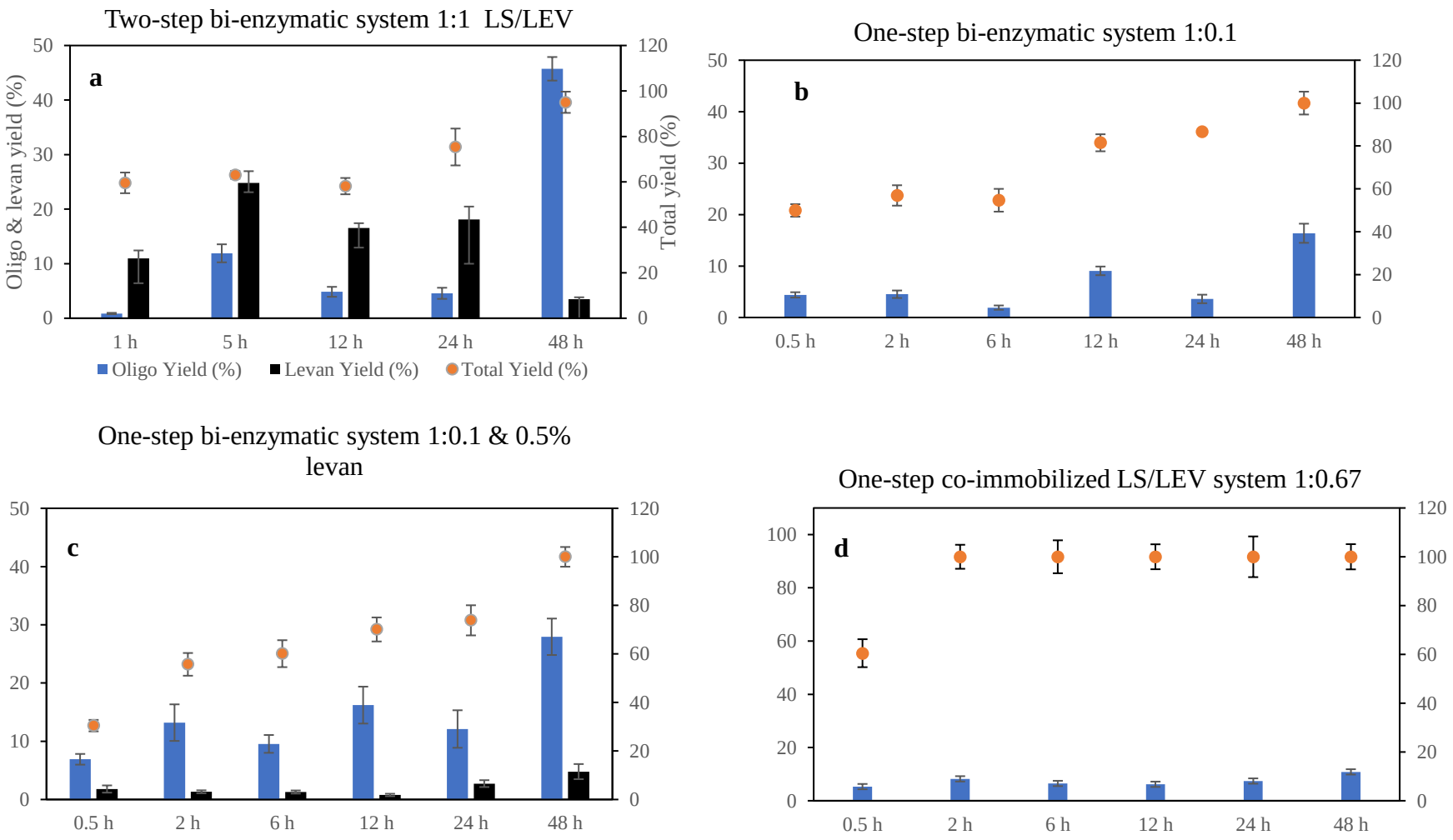


Figure 4.2a-d. Time courses for the bi-enzymatic reaction systems: the oligo, levan, and total yields

The effect of the addition of levan in the one-step immobilized LS:LEV reaction system was also investigated. For the one-step co-immobilized LS:LEV reaction system, the 1:0.67 initial ratio was identified as the best appropriate one. Contrary to the one-step reaction systems, a 1:1 ratio was used in the two-step bienzymatic system. The results (Figure 4.2) show that the overall yield of the sucrose conversion increased with the reaction time to reach a maximum value at 48 h in all investigated bi-enzymatic systems. Contrary to the two bienzymatic systems, the one-step co-immobilised LS:LEV reaction system converted completely the sucrose after 2h of reaction into mainly monosaccharides; indeed, this bi-enzymatic system resulted in the lowest oligo yield of 10.8%. This result can be attributed to the competitive interference of LEV activity, limiting the transfructosylation activity of LS and favoring the hydrolysis of sucrose. As both LS and LEV are not immobilized on the same support in the one-step immobilized LS:LEV reaction system, the microenvironment effect is expected to be less significant in this system as compared to the one-step co-immobilised LS:LEV one. The experimental findings (Figure 4.2b) show that the use of the one-step immobilized LS:LEV reaction system led to an oligo yield of 16.4% after 48 h of reaction. Such result reveals that the competitive interference between LEV and LS does occur in the macro and microenvironment of both biocatalysts. However, this interference seems to happen at a lower rate in the one-step immobilized LS:LEV reaction system compared to the co-immobilized one attributed to the lower impact of microenvironment effec. Furthermore, no detectable level of levan was detected in the one-step immobilized LS:LEV bi-enzymatic system during the reaction time course, confirming the inhibitory effect of LEV on the transfructosylation activity of LS in this system. The addition of levan in the one-step immobilized LS:LEV reaction system increased the oligo yield to 28.0% after 48 h of reaction (Figure 4.2c). The MW distribution of levans in this one-step bi-enzymatic system enriched with 0.5% levan (55±1.5 kDa) revealed the increase in the levan yield from 1.8 to 4.8% and the shift towards to high MW levans (1.5% of 5-100 kDa and 3.3% of 100-10000 kDa) over the time course up to 48 h (Figure 4.3b). The levan seems to limit the competitive interference of LEV with the transfructosylation activity of LS. This may have been achieved through the mass action effect or by limiting the LEV/Sucrose or FOSs binding. Amongst the bi-enzymatic systems, the highest oligo yield of 45.7% was obtained in the two-step bi-enzymatic one. The two-step bi-enzymatic system showed the production of levans during the reaction time course.

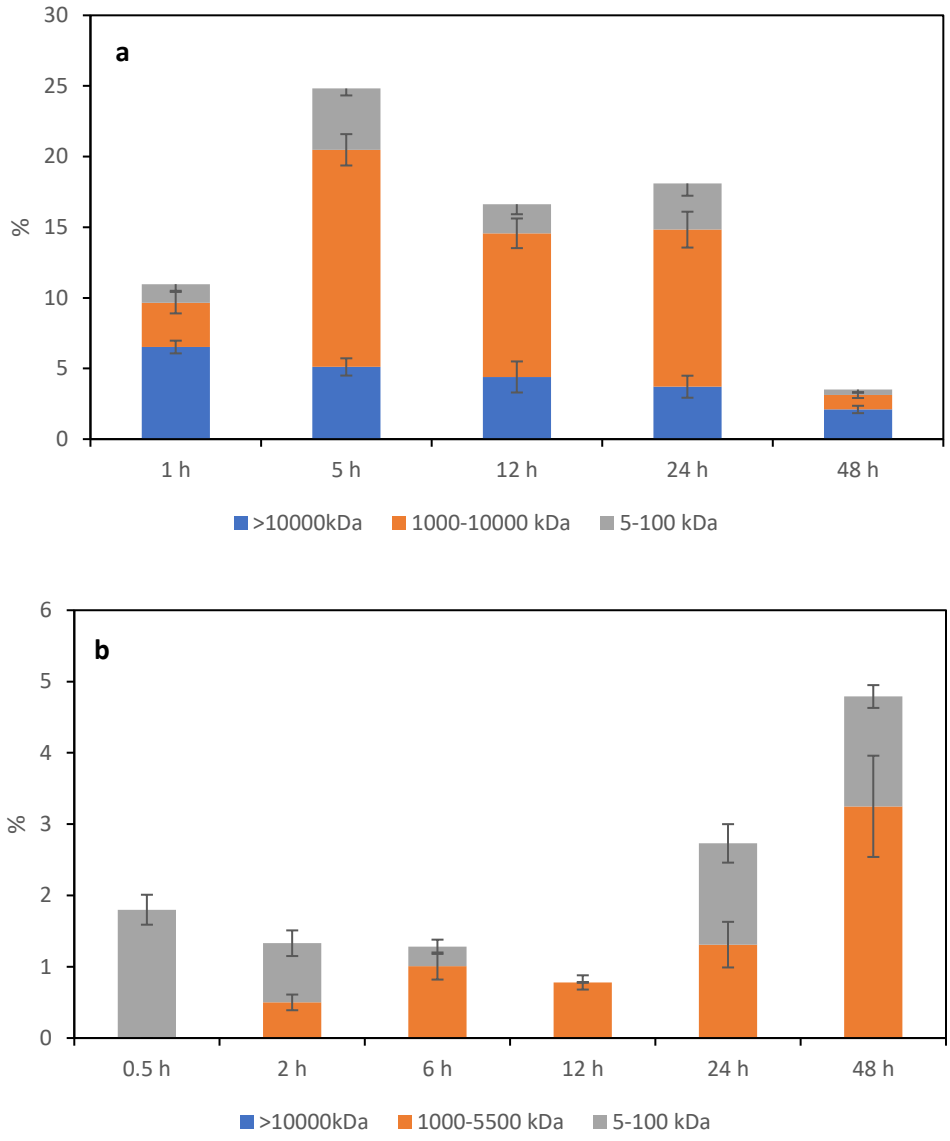


Figure 4.3a-b. Molecular mass distribution of the produced levans during the bi-enzymatic reactions in: a) the two-step and b) the one-step immobilized bi-enzymatic system with 0.5% levan

Interestingly, the synthesis of levan by LS continued to happen even after the addition of LEV to reach a maximum yield of 24.8% after 5 h; upon this reaction time, the commitment decrease in the levan yield was accompanied with a significant increase in the oligo yield at 48 h. The MW distribution of the produced levans during the time course of the two-step bi-enzymatic reaction system is presented in Figure 4.3.a. The use of immobilized LS-B.A. did favor the synthesis of HMW levans (>10,000 kDa). As the reaction of the bi-enzymatic system was proceeded to 5 hr, the MW of levans shifted towards the lower ranges of 1000-10000 kDa (15.4% yield), while the levan and oligo yields increased from 11.0 to 24.8% and from 0.9 to 11.6%, respectively. These changes were accompanied with almost the same total yield implying that the LS and the LEV had higher affinity towards levans than sucrose. The total conversion yield of the bi-enzymatic system during the first stage of the time course (0-12h) remained constant (~ 58%). This limited increase at the early stage of the bi-enzymatic reaction can be attributed to the substrate steric hindrance of LEV due to the HMW distribution of levans produced by LS. This result also reveals the importance of the intermediate levan substrates with the appropriate MW for their conversion into FOSs by LEV. Indeed, a significant increase in the oligo-yield to 45.7% was obtained upon the advanced reaction time of 48 h. The synergistic actions between the LS and LEV can be modulated by controlling the availability of the appropriate MW distribution of levans. The end-product profile of the FOSs produced after 48 h incubation is shown in Figure 4.4. The results indicate that the main FOS end-products in all the bi-enzymatic systems was GF_7. Similarly, the hydrolysis reaction of levan by immobilized LEV-C.O. on Gly-Ag-IDA resulted mainly in the release of GF_7 (see chapter III). The experimental findings confirm the high product specificity of LEV-CO towards GF7. Other minor FOSs (< 4%), including GF3, GF2 and GF, were also released. The co-immobilized LS:LEV reaction system produced the highest relative proportion of GF_2 (23.2%); this can be attributed to the limited production and accumulation of levans and oligolevans as intermediates, as a result of the co-immobilization of LV and LES. As an overall, the two-step bienzymatic system was identified as the best one in terms of oligo yield and product specificity (45.7% GF_7 and 3.5% GF); therefore, further optimization was preformed on this system.

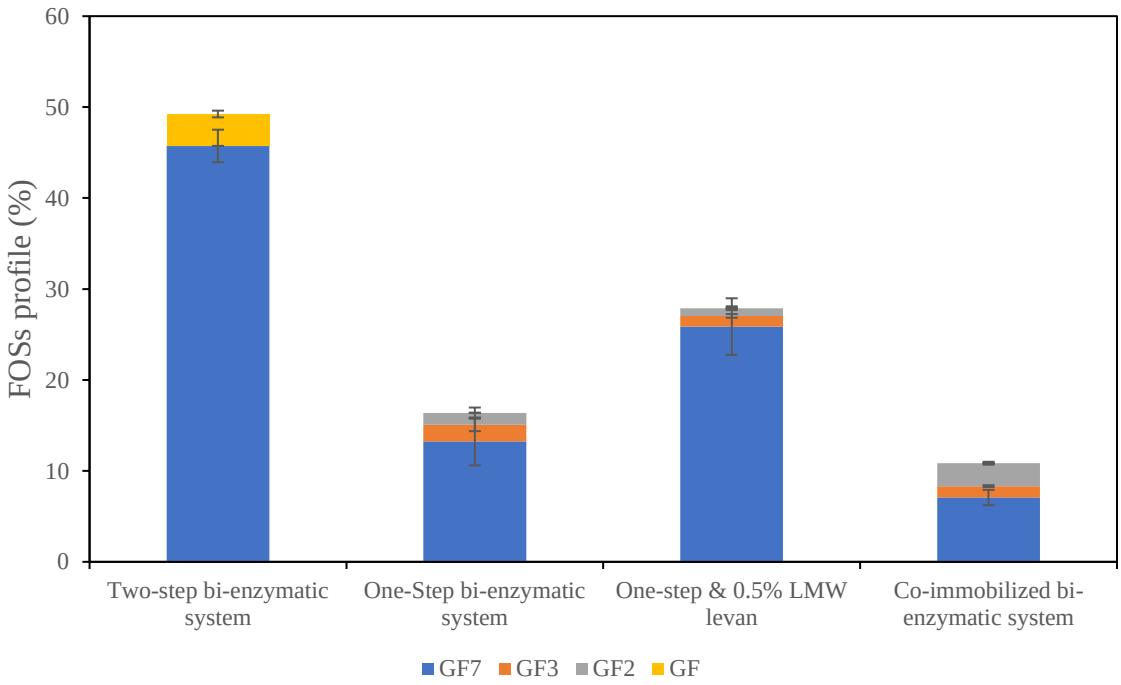


Figure 4.4. The product profile of the FOSs produced by the two-step, one-step with and without the levan additive, and co-immobilized bi-enzymatic systems after 48h incubation

4.3.3 Two-step Bi-enzymatic System Optimization by RSM

To enhance the efficiency of the two-step immobilized LS:LEV bi-enzymatic system, the LS:LEV ratio (LS relative proportion) and the reaction time were optimized using RSM methodology. The use of RSM can limit the labor-intensive process of stepwise optimization and allow the investigation of the potential combinatorial effects of the parameters besides their independent effects. A five-level, two variable central composite rotatable design (CCRD) was used to perform the optimization (Table 4.1). The full design consisted of 4 factorial points, 4 axial points, and 2 center points. The levels of the parameters were determined based on the preliminary trials. The first parameter was the immobilized LS proportion (A, 50.00-83.33 U%), which was defined as the percentage of the LS unit per that of the total LS/LEV units by keeping the total unit of the enzymes per volume of the sucrose solution constant. The second parameter was the reaction time of the primary step of the bi-enzymatic reaction system: LS/B.A.-catalyzed the transfructosylation reaction (6-24h). The quantified responses were the total product yield (total, %), the FOSs yield (oligo, %) and the levan yield (poly, %).

The best-fitting model was determined by multiple regression analyses of the experimental data and they were statistically checked by the coefficients of determination (R^2) and adjusted R-squared (Adj R^2) values, model lack of fit test, and *P*-value. In addition, Box-Cox plot was used to determine the appropriate power transformation needed to normalise the response data. The recommended rounded Lambda values by the Design Expert software for the transformation of the data were 1 (no transformation) for total and oligo yields as well as 0.50 (square root transformation) for poly yield. The analyses of variance (ANOVA) are summarized in Table 4.2. The results show that a reduced cubic model was statistically the most suitable for the description of the variations of total yield (*F*-value of 51.63 and *P*-value of <0.0001) and oligo yield (*F*-value of 316.68 and *P*-value of 0.0032) with the reaction parameters. While the linear model was well fitted for the poly (levan) yield (*F*-value of 24.91 and *P*-value of 0.0007). The lack of fit was not significant relative to pure error with *P*-values of 0.051 to 0.258. In addition, for total yield and oligo yield models, the predicted R^2 of 0.9232 and 0.9426 were in agreement with the adjusted R^2 of 0.9411 and 0.9959, respectively. Adeq Precision (e.g. signal to noise ratio) values of 19.458 and 49.304 indicate the adequate signals and the suitability of the models to navigate the design space. Although poly yield model showed a predicted R^2 of 0.7121, it was reasonably in accordance with

Table 4. 3. Factorial experimental design and experimental results of the two-step bi-enzymatic systems

LS Units (U/ml reaction volume)[a]	Lev Units (U/ml reaction volume)[b]	LS Proportion (Units %)[c]	Reaction Time for LS (h)	Total Product Yield (%)[d]	Levan Yield (%)[e]	Fructoligosaccharide Yield (%)[f]
0.94	0.26	78.33	21.36	97.19 (±3.57)	9.37 (±1.02)	54.48 (±2.71)
0.80	0.40	66.67	15.00	99.79 (±5.43)	3.98 (±0.47)	61.54 (±3.94)
0.66	0.54	55.00	21.36	99.55 (±6.11)	4.49 (±0.38)	61.29 (±2.55)
1.00	0.20	83.33	15.00	98.13 (±4.87)	10.76 (±1.26)	53.51 (±4.15)
0.66	0.54	55.00	8.64	99.75 (±7.38)	0.00	37.63 (±4.74)
0.94	0.26	78.33	8.64	99.11 (±8.32)	0.00	41.38 (±3.18)
0.80	0.40	66.67	24.00	97.46 (±7.22)	15.79 (±1.15)	48.56 (±5.66)
0.60	0.60	50.00	15.00	99.72 (±6.74)	1.83 (±0.19)	63.56 (±4.60)
0.80	0.40	66.67	6.00	100.00 (±8.59)	0.00	33.21 (±2.97)
0.80	0.40	66.67	15.00	99.02 (±4.05)	3.49 (±0.23)	61.43 (±3.88)

[a] LS unit was defined in $\mu mol.ml^{-1}.min^{-1}$ and the amount of the enzyme was applied in 1 ml of 600 mM sucrose solution in 100 mM phosphate buffer, pH 6

[b] LEV unit was defined in $\mu mol.ml^{-1}.min^{-1}$ and the amount of the enzyme was determined by abstracting the applied LS form the total unit of the bienzymatic system (0.6 U)

[c] The percentage of the applied LS per total unit of the bi-enzymatic system (0.6 U)

[d] The percentage of the sucrose converted to any product by the action of the bi-enzymatic system after 48 h of incubation in the second step of the two-step bi-enzymatic system

[e] The percentage of the sucrose converted to all LMW or HMW levans by the action of the bi-enzymatic system after 48 h of incubation in the second step of the two-step bi-enzymatic system

[f] The percentage of the sucrose converted to any FOSs by the action of the bi-enzymatic system after 48 h of incubation in the second step of the two-step bi-enzymatic system

the adjusted R^2 of 0.8416; i.e. the difference is less than 0.2.; the Adeq Precision of poly yield model of 12.202 indicates also an adequate signal.

The fitted models for total yield, oligo yield and poly yield in terms of coded factors are given by Equations 1, 2 and 3.

$$Total\ Yield = 99.41 - 0.6505A - 0.8970B - 0.4294AB - 0.2212A^2 - 0.3224B^2 + 0.3641A^2B$$ (Equation 1)

$$Oligosaccharide\ Yield = 61.48 - 3.51A + 5.43B - 2.27AB - 1.60A^2 - 10.47B^2 + 3.39A^2B + 2.38AB^2$$ (Equation 2)

$$Sqrt\ (Levan\ Yield) = 1.76 + 0.4566A + 1.35B$$ (Equation 3)

The significance of each coefficient was determined using the *F*-test and *p*-value. The variables are deemed more significant if the *F*-value is bigger and the *p*-value is smaller. As expected, the linear term with the largest effect on total, oligo and poly yields was the reaction time of the first LS step of bienzymatic system (B, *F*-value of 44.64-248.80, *p*-value of < 0.004).

In the total and oligo yield predictive models, the linear term of LS:LEV ratio (A, *F value* of 106.36-117.51, P<0.009), was also a significant parameter modulating the synthesis of FOSs and the total conversion of sucrose. As compared to the quadatic term of LS:LEV ratio (A^2, *F*-value of 7.94-25.70, *p*-value of 0.0368), that of the reaction time of the first LS step of bienzymatic system (B^2, *F*-value of 16.20-1052.77, *p*-value of 0.00014) exhibited more significant effect on the total and oligo yield. However, the poly yield seems to be less affected by the LS:LEV ratio (A, *F value* of 5.17, P<0.057) than the reaction time of the first LS step. In the total and oligo yield predictive models, the interaction between LS:LEV ratio and reaction time (AB, *F-value* of 25.31-43.64, *P<0.02*) exhibited significant effects on these responses; in addition, the negative sign of the cross-product coefficient (AB) reveals their negative antagonistic interaction.

The relationships between the reaction parameters and the yields can be better understood by studying the planned series of two-dimensional (2D) contour plots of fitted models. In the 2D contour plot, the curves of equal response values are drawn on a plane. In fact, each contour represents a specific value for the height of the surface. The 2D contour plots presented in Figure 4.5 illustrate the interaction effect of the reaction time of the first LS step of the bi-enzymatic systems and the LS:LEV ratio on the predicted total, oligo and poly yields.

Table 4. 4. Analysis of variance (ANOVA) for the two-step bi-enzymatic system of the immobilized LS/LEV

	Total Product Yield (%)			**Levan Yield (%)**			**Oligosaccharide Yield (%)**		
	Sum of Squares	**F-value**	**p-value**	**Sum of Squares**	**F-value**	**p-value**	**Sum of Squares**	**F-value**	**p-value**
Model	18.08	51.63	< 0.0001	16.27	24.91	0.0007	1050.50	316.68	0.0032
A-LS/LEV	6.86	117.51	< 0.0001	1.69	5.17	0.0571	50.41	106.36	0.0093
B-reaction Time for LS	6.44	110.27	< 0.0001	14.58	44.64	0.0003	117.90	248.80	0.0040
AB	1.48	25.31	0.0002				20.68	43.64	0.0222
A^2	0.4636	7.94	0.0145				12.18	25.70	0.0368
B^2	0.9454	16.20	0.0014				498.90	1052.77	0.0009
A^2B	0.5331	9.13	0.0098				23.07	48.67	0.0199
AB^2							11.45	24.16	0.0390
Lack of Fit	0.1656	1.54	0.2581	2.28	47.92	0.1101	0.9419	161.24	0.0500

According to the 2D contour plot (Figure 4.5a), the total yield varied between 93 and 100%, over the investigated reaction parameters range. A complete conversion of sucrose was achieved in the lower range of reaction time and LS:LEV ratio (corresponding of lower LS proportion). However, increasing the reaction time and LS:LEV ratio decreased to some minimal extent the total yield. The 2D oligo yield contour plot reveals that the interaction between the reaction time of the first LS step and the LS:LEV ratio (ellipsoidal lines) occurred mainly upon longer reaction time; no significant effect of LS:LEV ratio was observed at the lower range of reaction time. As an overall, the predictive oligo yield increased when the reaction time of the first LS step increased; further increase in the reaction time beyond 17 h led to a slight decrease in the oligo yield. These results reveal the significance of the LS-catalyzed transfructosylation and the modulation of the MW of levan. A compromise between the levan production and the levan MW should be achieved in order to maximize the oligo yield. At the lower range of reaction time, the oligo yield remained more or less constant, when the LS:LEV (LS proportion) ratio increased. Considering the fact that sucrose was more or less completely converted at the lower range of reaction time, it can be hypothesized that the accumulation of levan does not only contribute to the oligo-yield, but it does also limit the hydrolysis of sucrose in the bi-enzymatic system. While upon longer reaction time of the first LS step, increasing the LS:LEV (LS proportion from 50 to 83%) ratio resulted in a decrease in the oligo yield from 66.0 to 55%. An equal unit proportion of LS and LEV seems to be more appropriate for maximising the oligo-yield. According to the predictive plot of poly yield, a longer reaction time of the fist LS step and a higher unit of the LS can result in higher poly yield. In fact, the highest poly yield (10.8%) was produced upon 15 h of LS reaction using 83.33% of the LS.

Using the predictive models, the optimum parameters of the immobilized LS/B.A.-LEV/C.O. bi-enzymatic reaction system with the highest oligo yield were determined. It predicted oligo yield of 63% with a total product yield of 99% that can be achieved when the first step of bienzymatic system is performed with 50% immobilized LS units over a reaction time course of 15 h. Longer reaction time favored the levans production, but also the elongation of the MW distribution, limiting the hydrolytic action of LEV.

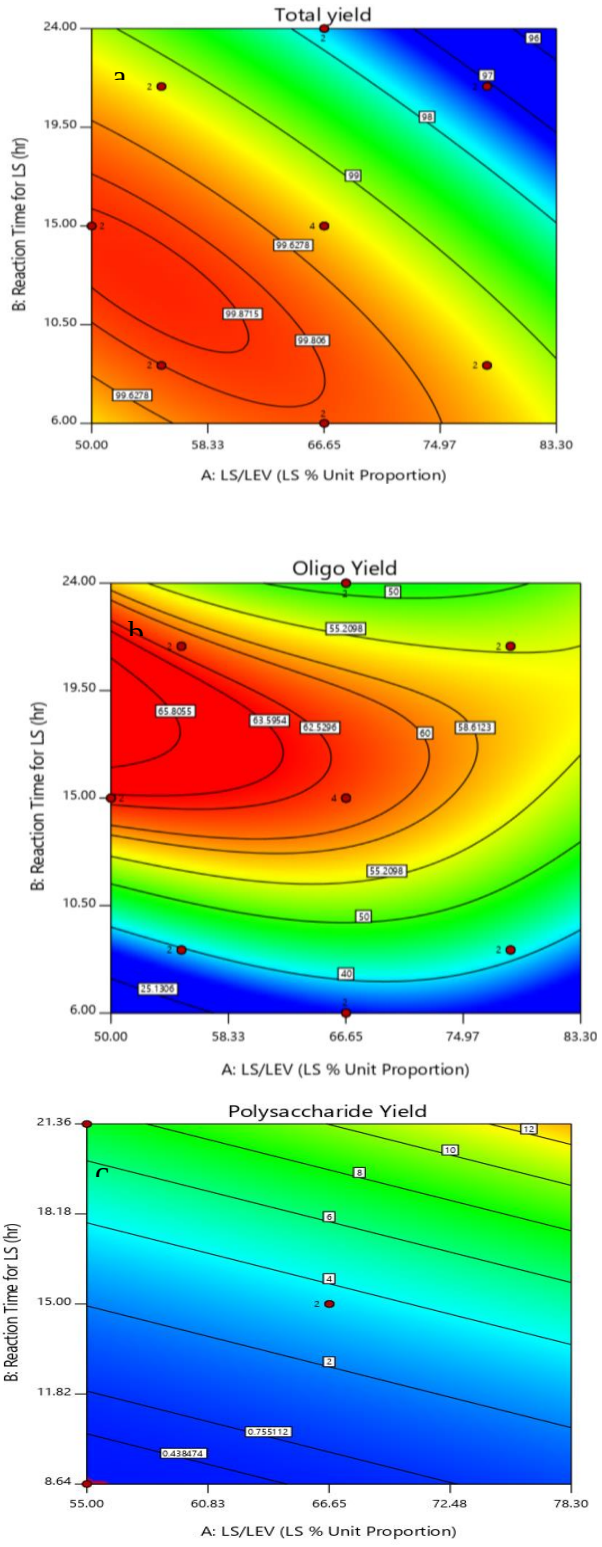


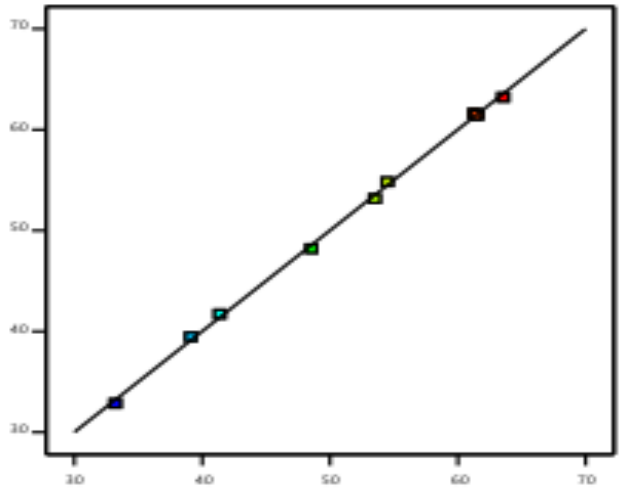
Figure 4.5a-c. Contour plots of the total yield, the poly yield, and the oligo yield as a function of the first step incubation time (6-24 h), and the LS proportion (50-83.33%). High Low

The agreement between the experimental and predicted oligo yield, total yield, and levan yield were presented by potting the experimental findings against the predicted values (Figure 4.6.a-c). The experimental and predicted values of oligosaccharide yields are in great agreement on the whole spectrum of values (33.2-63.6%). Levan yield showed the highest deviations between the experimental and predicted values. The deviations can be attributed to the intrinsically low levan yield (0-4.0%) which accordingly makes effects of random errors more highlighted.

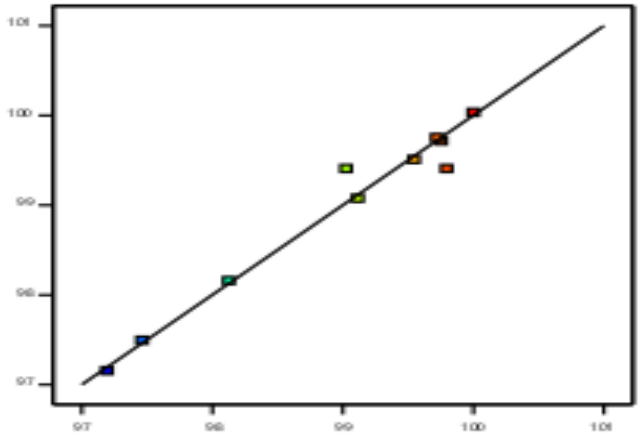
4.4 Conclusion

To wrap up, two-step immobilized bi-enzymatic system made up of LS-B.A./Gly-Ag-IDA/Cu and LEV-C.O./Gly-Ag-IDA (1U:1U ratio) was found as the most promising system for FOS synthesis by producing the highest oligosaccharide yield and selectivity (45.7% GF_7, and 3.5% GF), while the lowest oligo yield and the highest relative proportion of GF_2 were resulted by one-step co-immobilized bi-enzymatic system of LS/LEV on Gly-Ag-IDA/Cu with the initial ratio of 1U:0.67U (10.8% and 23.2% respectively). Monitoring the produced levans during the time course of the immobilized bi-enzymatic system reactions showed the importance of the presence of the levans as the prerequisite of FOS synthesis, that is, the two-step immobilized bi-enzymatic system showed the highest levan yield (24.8%) during the time course of the reaction which led the system to the highest oligo yield at the end of the reaction. On the contrary, neither on-step immobilized bi-enzymatic system nor one-step co-immobilized bi-enzymatic system produced a detectable level of levans during the time course their reactions and ended up substantially lower oligo yields (16.4 and 10.8%, respectively). Adding 0.5% levan to the one-step immobilized LS/LEV bienzymatic system could promote an increase in the oligo yield (28.0%). The presence of high molecular weight levans (1000-10000 kDa) was found to limit the hydrolytic action of LEV while it does suppress the sucrose hydrolysis. The optimization of the condition of the two-step immobilized bi-enzymatic system by RSM led to proposing first step LS incubation time as more important factor than LS proportion in oligosaccharide production. 15 h of the first step LS incubation time and 50% LS proportion were proposed as the optimum conditions to achieve the highest oligo yield 63%. Finally, plotting the experimental findings against predicted values of oligo yield, total yield, and levan yield showed the credibility of the optimization results.

a



b



c

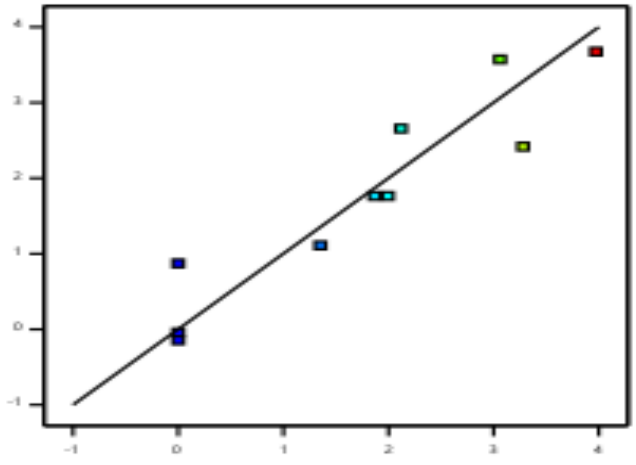


Figure 4.6a-c. Plotting the experimental values of oligo yield (a), total yield (b), and levan yield (c) against the corresponding predicted values. X-axis and y-axis are presenting the experimental and predicted values, respectively.

CHAPTER V

GENERAL CONCLUSION AND FUTURE WORK

Levan-type FOSs production by immobilized LEVs and by bi-enzymatic systems of immobilized LSs and LEVs has been the ultimate goal of the present work. Enzyme immobilization has been selected as the potential strategy to increase enzyme stability, to modulate enzyme selectivity and to allow the reusability of the biocatalysts. Previous work in our laboratory has shown the superior ability of the immobilized LS from *Bacillus amyloliquefaciens* (LS-B.A.) on Gly-Ag-IDA/Cu in levan synthesis, and the high endo-hydrolytic activity of the recombinant LEV form *Belliella baltica* (LEV-B.B.), *Capnocytophaga ochracea* (LEV-C.O.), *Dyadobacter fermentans* (LEV-D.F.). As part of our on-going efforts, the selected LEVs were immobilized on selected modified Glyoxyl agarose supports, and the combined use of immobilized LS-BA and LEVs in a bi-enzymatic system was investigated. Glyoxyl agarose derivatives possessing the ability to make multi-attachment, including Gly-Ag (hydrophobic), Gly-Ag-TEA (positively charged), Gly-Ag-IDA (negatively charged), and Gly-Ag-IDA-Cu (chelating), were used as immobilization supports. LEV-B.B./Gly-Ag, LEV-C.O./Gly-Ag-IDA, and LEV-D.F./Gly-Ag-IDA/Cu led to the most appropriate compromises between the immobilized enzyme activity yield, retention of specific enzyme activity, and retained levanase activity after incubation at 50 ^{o}C. Accordingly, the kinetic parameters, the thermal stability, the product profile, and the enzyme reusability of the selected immobilized LEVs were further investigated. The results showed that the improvement in the thermal stability and the product selectivity upon immobilization on functionalized glyoxyl agarose was dependent on the LEV properties, the type of immobilisation support, and the reaction conditions. In general, increasing the temperature shifted the end-product profile towards lower molecular weight FOSs with an exception in the case of the LMW levan-immobilized LEV-D.F./Gly-Ag-IDA/Cu reaction system; and compared to LMW levan, the use of HMW levan favored the GF_7 formation over the shorter chain FOSs. Immobilized LEV-C.O. on Gly-Ag-IDA showed the highest product selectivity towards GF_7 production (23.1%) with no release of shorter oligosaccharides although its half-life decreased from 202.4 min to 78.8 min by immobilization. On the other hand, LEV-D.F. exhibited the lowest selectivity but a significant thermal stability improvement by around 9-folds compared to the corresponding free enzyme. On the other hand, LEV-B.B./Gly-Ag exhibited moderate enzyme selectivity and slight half-life improvement. In addition, LEV-C.O./Gly-Ag-IDA and LEV-B.B./Gly-Ag showed the highest and the lowest reusability upon four successive enzymatic reactions, respectively. Based on the product profile and enzyme reusability results, the immobilized LEV-C.O. was chosen as the most promising

biocatalyst for the design of immobilized bi-enzymatic system by combining it with the immobilized LS from *Bacillus amyloliquefaciens* (LS-B.A.) on Gly-Ag-IDA/Cu.

The second part of the research was dedicated to combining the selected immobilized LEV and immobilized LS. The potential interference of the selected enzymes was investigated, and the results showed a significant interference between the LS and LEV towards sucrose. According to the interference, the ratios of the LS and LEV were adjusted in the bi-enzymatic systems. The two-step, one-step, and co-immobilized bi-enzymatic systems were assessed for FOSs synthesis from sucrose. The two-step bi-enzymatic reaction (LS/LEV ratio of 1U:1U) resulted in the highest oligo yield and enzyme selectivity by producing 45.7% of GF_7 after 48 h incubation at 15 ^{o}C. While the co-immobilized system (LS/LEV ratio of 1 U:0.67 U) led to the lowest oligo yield (10.8%) and selectivity, by producing the highest relative proportion of GF_2 (23.2%) and by resulting in the fastest reaction rate. The competitive interference of LEV activity, which have limited the transfructosylation activity of LS, was substantially higher in the co-immobilized system than in the one-step bi-enzymatic system, indicating the promotion of LS hydrolytic activity as a result of the microenvironment effect. However, the interference of enzyme activity in the one-step immobilized bi-enzymatic system was still so significant that the reaction did not produce a detectable level of levans during the time course of the enzymatic reaction and ended up by 16.4% of oligo yield. The two-step bi-enzymatic system and the one-step bi-enzymatic system enriched with 0.5% levan (55±1.5 kDa) resulted in 28.0% of oligo yield and the production of levans; this revealed the importance of the primary step of the bi-enzymatic system for levan formation by the LS and the limiting effect of the levan on hydrolyzing action of LEV on sucrose.

The two-step immobilized bi-enzymatic system was used for conducting an RSM optimization by applying a five-level, two variable central composite rotatable design (CCRD). According to the statistical calculation, applying 15 h and 50 % LS proportion resulted in the maximum oligo yield of 63%, confirming the significance of the effects of the reaction time and the LS proportion. Indeed, the most important factor in oligo yield improvement was the primary reaction time at which the LS carried out the levan synthesis. In the case of the use of short first step reaction time, the presence of short-chain levans promoted an excessive hydrolyzing activity of LEV that decreased the oligo yield. While the use of long first step reaction time suppressed LEV activity and resulted in a decrease in the oligo yield.

Considering the fact that combining LEV-C.O./Gly-Ag-IDA and LS-B.A./Gly-Ag-IDA/Cu in a two-step bi-enzymatic system could result in a superior oligo yield and enzyme selectivity, the system would have the high potential of industrializing levan-type FOS production. However, further research needs to be conducted to further stabilize the immobilized LEV-C.O./Gly-Ag-IDA by crosslinking or through polymer interactions. Also, the possibility of immobilization of LEV on novel supports such as magnetic nanoparticles, which have the ability of being separated by simple physical separation in magnetic field, can address the challenge of separating immobilized LS/LEV components after each reaction.

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