Faculty of Bioscience Engineering
Academic Year 2014 – 2015

Demonstrating the necessity of enzyme engineering: towards a highly efficient process for the synthesis of kojibiose

Anoek Van Canneyt

Promotor: Prof. dr. Tom Desmet

Tutor: ir. Karel De Winter

Master's dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioscience Engineering: Chemistry and Bioprocess Technology

Faculty of Bioscience Engineering
Academic Year 2014 – 2015

Demonstrating the necessity of enzyme engineering: towards a highly efficient process for the synthesis of kojibiose

Anoek Van Canneyt

Promotor: Prof. dr. Tom Desmet

Tutor: ir. Karel De Winter

Master's dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioscience Engineering: Chemistry and Bioprocess Technology

IMPORTANT – PLEASE READ THIS FIRST

Anoek Van Canneyt
Ghent, June 2015

Promotor:
Prof. dr. Tom Desmet

Tutor:
ir. Karel De Winter

Contact person Ghent University:
Prof. dr. Tom Desmet
tom.desmet@ugent.be
Center for Industrial Biotechnology and Biocatalysis
Coupure Links 653, 9000 Gent

PREFACE

"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mahatma Gandhi

The completion of this thesis has been a challenging and interesting endeavor, which would not be possible without the support of many.

At first, I would like to thank my tutor ir. Karel De Winter. You and your never ending enthusiasm motivated me day after day to get the maximum out of this research. I can't tell how much I have learned from you the past year, going from practical techniques in the lab to the problem solving way of thinking. You taught me how to become a good research scientist and you were always there answering my questions, throwing new ideas, helping me understand the world of enzymes by numerous discussions. I am really glad we've got to know each other. Thank you, once again.

Secondly, I would like to thank my promotor Prof. dr. Tom Desmet for giving me the opportunity to complete this master thesis within Inbio.be. Your course in the last bachelor year evoked my interest in the world of biocatalysis and lead to the choice of this subject as thesis topic.

Off course I can not forget the other thesis students at Inbio.be, Paul, Dana and Sylvie in particular. Paul, you have been a really good friend for me the past five years. After so many group works, parties and good conversations, I deeply value our friendship. Also this year, you were always there for a pep-talk, a joke or just to complain about my problems in the lab. The same is true for Sylvie and Dana. Girls, I'm really glad we've got to know each other this year and I'm sure graduating will not be the end of our friendship. I would also like to thank everyone working at Inbio.be. Thank you for the great atmosphere in the lab. The famous *Inbio Party Committee* guaranteed having a lot of good memories of my thesis year.

But at the end, none of this would have been possible without the support of my family and friends. My parents in particular, Mia and Wim, for believing in me but also for giving me the financial support to complete this study. My sister, Manon, who has been my *study buddy* for five years. Thank you for supporting me and calming me down in moments of stress. My brother, Aaron, who was always there to cheer me up and who helped me to relativize things. My friends from the *Chiro,* to clear my mind in the weekends by having a lot of fun. My friends I have met these past five years, relieving our stress with a nice party or drink, but also listening to each other in difficult times. Thank you for making my student life so unforgettable. Finally I want to thank Emiel. Throughout these five years you have been my listening ear. You were always capable of motivating me in hard times. I am deeply grateful.

Anoek Van Canneyt May 24, 2015

ABSTRACT

Over the past decade, prebiotica have gained increasing attention due to their ability to improve the microbial balance in the human gut, thereby inducing favorable effects for human health. In this area, non-digestible carbohydrates are an interesting group of compounds. The disaccharide kojibiose (2-O-α-D-glucopyranosyl D-glucopyranose) displays promising prebiotic properties. The bottleneck for further research and application of this molecule is however its limited availability. No viable production processes for kojibiose exist to date, since isolation is not economically profitable and chemical methods suffer from low yields, the use of solvents and the need for multiple (de)protection steps. Therefore, this thesis aimed to develop a highly efficient biotechnological process for the synthesis of kojibiose with the use of enzyme engineered sucrose phosphorylase from *Bifidobacterium adolescentis* as biocatalyst.

First, one of the mutant enzymes - created in previous research - was selected, displaying the most promising properties for the synthesis of kojibiose and further downstream process steps. The average selectivity of BaSP_L341I_Q345S was found to be 97.1 % while an average kojibiose yield of 51.1 % was obtained after 8 h of reaction at 55 °C. To provide sufficient amounts of this enzyme, a large scale fermentation was performed. Subsequently its thermostability was compared to that of the wild type. The half-life was found to be 35.1 h at 60 °C, which is 70 % of the wild type's half-life (49.0 h). Further research revealed that the decrease in thermostability was caused by the Q345S mutation. However, at a temperature of 55 °C, no decrease in activity was observed after 96 h.

Reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S at 55 °C resulted in a kojibiose concentration of 350 mM after approximately 24 h. Subsequently a yeast treatment was performed to remove the contaminating carbohydrates. The addition of 30 g/L spray dried baker's yeast allowed the removal of all carbohydrates in the reaction mixture after 6-8 h at 30 °C. Next, the solution was evaporated to Brix 48, followed by cooling crystallization. As a result, after washing with ethanol, kojibiose crystals with a purity higher than 99.5 % on HPLC and HPAEC-PAD were obtained.

Further research focused on the intensification of the process, whereby the aim was to increase the kojibiose concentration. Therefore, on the one hand, substrate concentrations were increased while on the other hand glucose isomerase was added. Indeed, the latter enzyme is capable of converting fructose, released in the reaction step, to the desirable acceptor glucose. This strategy allowed higher kojibiose concentrations, while also the Carbohydrate Efficiency could be significantly improved. Reacting 1.8 M sucrose with 0.2 M glucose in the presence of 2 mg/mL BaSP_L341I_Q345S and 20 g/L glucose isomerase led to the formation of approximately 1.5 M kojibiose after 70 h at 55 °C. As a proof of concept, this process, coupled with the previous developed downstream process procedure, was performed on a 10 L scale, resulting in the isolation of 3 kg kojibiose with a purity exceeding 99.8 % and 1 kg kojibiose with a purity of 99.0 %. Moreover, elimination of the yeast treatment purification step was realized by increasing the sucrose concentration further to 2.0 M. This resulted in the formation of kojibiose crystals displaying a purity of 97.5 %.

SAMENVATTING

Het afgelopen decennium hebben prebiotica steeds meer aandacht gekregen vanwege hun vermogen om de microbiële balans in het darmkanaal van de mens te verbeteren, wat voordelige effecten voor de gezondheid kan teweegbrengen. In dat gebied, zijn niet verteerbare koolhydraten een interessante groep componenten. De disacharide kojibiose (2-O-α-D-glucopyranosyl D-glucopyranose) vertoont veelbelovende prebiotische eigenschappen. Het grootste probleem voor verder onderzoek en toepassing van deze molecule is echter zijn gelimiteerde beschikbaarheid. Tot op de dag van vandaag bestaan er geen voor de hand liggende processen voor de synthese van kojibiose, aangezien isolatie niet economisch rendabel is en chemische methoden nadelen - zoals lage rendementen, het gebruik van solventen en de nood aan verschillende de(protectie) stappen - met zich meebrengen. Om deze reden werd in deze thesis een uiterst efficiënt biotechnologisch proces voor de synthese van kojibiose ontwikkeld gebruik makende van *enzyme engineered* sucrose phosphorylase van *Bifidobacterium adolescentis* als biokatalysator.

Eerst en vooral werd één van de mutanten – gecreëerd in eerder onderzoek – geselecteerd die de meeste belovende eigenschappen voor de synthese van kojibiose en verdere downstream processing, vertoonde. De gemiddelde selectiviteit van de L341I_Q345S mutant bedroeg 97.1 %, terwijl een gemiddeld kojibiose rendement van 51.1 % werd bereikt na 8 h reactie bij 55 °C. Om voldoende hoeveelheden van dit enzym te voorzien, werd vervolgens een grootschalige fermentatie uitgevoerd. Daarna werd de thermostabiliteit van de mutant vergeleken met die van het wild type. Bij 60 °C werd een t_{50} waarde van 35.1 h vastgesteld, welke 70 % van de t_{50} waarde van het wild type bedraagt (49.0 h). Verder onderzoek onthulde dat de daling in thermostabiliteit te wijten was aan de Q345S mutatie. Bij een temperatuur van 55 °C werd echter geen daling in activiteit vastgesteld na 96 h.

500 mM sucrose laten reageren met 500 mM glucose in de aanwezigheid van 2 mg/mL BaSP_L341I_Q345S bij 55 °C resulteerde in een kojibiose concentratie van 350 mM na ongeveer 24 h. Vervolgens werd een gistbehandeling uitgevoerd om de contamineerde koolhydraten te verwijderen. De toevoeging van 30 g/L gesproeidroogde gist resulteerde in de verwijdering van alle koolhydraten in het reactiemengsel na 6-8 h bij 30 °C. Daarna werd de oplossing ingedampt tot Brix 48, gevolgd door koelingskristallisatie. Als resultaat werden, na wassen met ethanol, kojibiose kristallen met een zuiverheid groter dan 99.5 % op HPLC en HPAEC-PAD bekomen.

Verder onderzoek focuste op de intensifiëring van het proces, waarbij men poogde de concentratie kojibiose te verhogen. Hiervoor werden enerzijds substraat concentraties verhoogd. Anderzijds werd glucose isomerase toegevoegd. Dit enzym is immers in staat om fructose, dat vrijkomt tijdens de reactie, te converteren naar de gewenste acceptor glucose. Deze strategie liet niet alleen hogere kojibiose concentraties toe, ook de *Carbohydrate Efficicency* kon significant verbeterd worden. 1.8 M sucrose laten reageren met 0.2 M glucose in de aanwezigheid van 2 mg/mL BaSP_L341I_Q345S en 20 g/L glucose isomerase leidde tot de vorming van ongeveer 1.5 M kojibiose na 70 h bij 55 °C. Als *proof of concept*, werd dit proces, gekoppeld met de eerder ontwikkelde downstream proces

procedure, op 10 L schaal uitgevoerd, wat resulteerde in de isolatie van 3 kg kojibiose met een zuiverheid hoger dan 99.8 % en 1 kg kojibiose met een zuiverheid van 99.0 %. Bovendien was eliminatie van de gistbehandeling als opzuiveringsstap mogelijk door de sucrose concentratie verder te verhogen naar 2.0 M. Dit resulteerde in de vorming van kojibiose kristallen met een zuiverheid van 97.5 %.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMMOENG 101	5-tetra alkyl ammonium chloride
BaSP	Sucrose phosphorylase from *Bifidobacterium adolescentis*
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAZymes	Carbohydrate active enzymes
CE	Carbohydrate Efficiency
CP	Cellulose phosphorylase
DSP	Downstream processing
E. coli	*Escherichia coli*
EC	Enzyme commission
FOS	Fructo-oligosaccharides
G1P	Glucose 1-phosphate
Gal1P	Galactose 1-phosphate
GE	Glucose Equivalents
GH	Glycosyl hydrolase
GI	Glucose isomerase
GP	Glycosyl phosphorylase
GT	Glycosyl transferase
HIV	Human immunodeficiency virus
HPAEC	High performance anionic exchange chromatography
HPLC	High performance liquid chromatography
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
AEC	Anion exchange chromatography
KP	Kojibiose phosphorylase
LB	Luria broth
LmSP	Sucrose phosphorylase from *Leuconostoc mesenteroides*
MOPS	3-(N-morpholino)propanesulfonic acid
OD	Optical density
PAD	Pulsed amperometric detection
PAGE	Polyamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SP	Sucrose phosphorylase
TG	Transglycosidase
UDP	Uridine diphosphate
ZnCl	Zinkchloride

CHAPTER I

INTRODUCTION

Prebiotics have recently gained increasing attention since these components stimulate the growth of beneficial bacterial populations in the human gut (Ziemer & Gibson, 1998; Gibson & Roberfroid, 1995). In this area, non-digestible carbohydrates are an interesting group of components. The disaccharide kojibiose (2-O-α-D-glucopyranosyl D-glucopyranose) displays promising prebiotic properties (Sanz *et al.*, 2005). The α-1,2 bond is largely resistant to the action of enzymes in the digestive tract, but can indeed be handled by specific micro-organisms such as lactobacilli (Chaen *et al.*, 2001). Besides its prebiotic properties, kojibiose has the ability to inhibit α-glucosidase I (Ogawa *et al.*, 1998). These inhibitors are suggested to be interesting candidate drugs for the treatment of HIV-1 infections (Borges de Melo *et al.*, 2006). Moreover this inhibitory action results in limiting the digestion of dietary carbohydrates. Kojibiose could therefore be useful to counter diabetes, obesity and cardiovascular diseases (Phung *et al.*, 2014).

The main bottleneck for the interesting applications of kojibiose is however its limited availability. Kojibiose is naturally present in honey, beer, sake and koji but the amounts are too low for extraction on large scale (Matsuda, 1959; Hough *et al.*, 1982; Watanabe & Aso, 1959). Chemical synthesis has tried to circumvent this problem via modified Koenigs-Knorr reactions but due to the need of multiple (de)protection steps, this method suffers from low yields (Igarashi *et al.*, 1975). Isolation from the partial acetolysis of dextran from *Leuconostoc mesenteroides* is currently the best method, but unfortunately this involves a multi-step process using several chemical reagents (Duke *et al.*, 1973).

Alternatively, biocatalytic approaches can be addressed for the synthesis of kojibiose. Enzymes are known to be very efficient biocatalysts in a broad range of carbohydrate related reactions, since they are active under mild reactions conditions and can be highly specific (Desmet & Soetaert, 2011). Indeed, the enzyme sucrose phosphorylase from *Bifidobacterium adolescentis* is capable of synthesizing kojibiose, starting from sucrose and D-glucose, both renewable, cheap and readily available substrate sources (Kitao *et al.*, 1994). The overall kojibiose yield is however lowered by the side-formation of maltose, which accounts for 65 % of the formed glucobioses. Enzyme engineering was proved to be an efficient tool to enhance the selectivity of sucrose phosphorylase towards the synthesis of kojibiose (Verhaeghe, 2014; Van Lul *et al.*, 2014).

The aim of this master thesis was to develop a highly efficient process for the synthesis of kojibiose with the use of enzyme engineered sucrose phosphorylase. Several research strategies were addressed to achieve this goal. First, sufficient amounts of purified BaSP had to be produced (Section IV.1). Next, it was investigated which mutant possessed the most desirable properties in terms of activity and selectivity (Section IV.2). The search for an efficient downstream processing method was the focus of the following section (Section IV.3). Hereby yeast treatment and crystallization were the main applied tools. The necessity of enzyme engineering was demonstrated in Section IV.4. Subsequently, efforts were made to further increase the concentration of kojibiose in the enzymatic reaction by increasing substrate concentrations and using the ability of glucose isomerase to convert fructose - released by conversion - into glucose, which is the acceptor for the synthesis of kojibiose (Section IV.5). Finally the feasibility of the developed process was demonstrated through production of kojibiose on 10 L scale (Section IV.6).

CHAPTER II
LITERATURE REVIEW

1 Kojibiose

1.1. Introduction

The disaccharide kojibiose consists of two glucose units bound to each other via an α-1,2 linkage, resulting in the systematic name 2-O-α-D-glucopyranosyl D-glucopyranose (Figure II.1). Kojibiose is widespread in nature, either as a constituent of polysaccharides and the carbohydrate chain of glycoproteins (Wang *et al.*, 1999; Ogawa *et al.*, 1998), or in the free form in honey (Watanabe & Aso, 1959), beer (Hough *et al.*, 1982), sake and koji (Matsuda, 1959). The name kojibiose is derived from the latter, which is a crucial ingredient in sake brewing. Although kojibiose is thus present in a variety of natural occurring products, its concentration in these products is typically only in the range of 1 % (m/m) (Mateo & Bosch-Reig, 1997), which explains the current high cost of about € 156/mg (Sigma-Aldrich[1]).

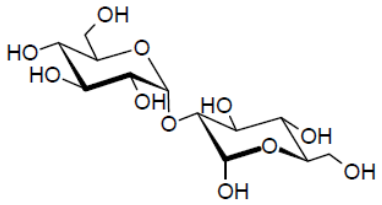


Figure II.1: Chemical structure of kojibiose.

1.2. Applications of kojibiose

Although studies on kojibiose are scarce due to its limited availability, some health-promoting properties have been assigned to this disaccharide.

1.2.1. Prebiotic

Kojibiose is known for its prebiotic properties (Sanz *et al.*, 2005). Prebiotics are non-digestible food components that stimulate the growth of beneficial bacterial populations in the human gut, such as bifidobacteria and lactobacilli. In this manner, the microbial balance in the gut is improved, which can induce favorable effects to human health (Ziemer & Gibson, 1998).

[1] 03/05/2015

The term prebiotica must not be confused with probiotica. A probiotic is a living microbial food supplement that directly improves the microbial balance in the human colon (Ziemer & Gibson, 1998). The most commonly used probiotics, lactobacilli and bifidobacteria, are usually added to fermented milk products. Alternatively, they can also be found as lyophilized powders. From this definition, it is clear that probiotics are living microbial supplements. Although there is some debate as to whether the concept should include unviable micro-organisms, or even bacterial fragments. Synbiotics on the other hand, are a mixture of pre- and probiotics. These mixtures stimulate the survival and implantation of the selected microbial strains (Ziemer & Gibson, 1998).

Although the use of probiotics seems an interesting concept, concerns about the survivability and colonization in the human colon have been described (Gibson & Roberfroid, 1995). Therefore, prebiotics have recently gained the most attention. In this area, non-digestible carbohydrates are an interesting group. The health effects, as described in the definition of a prebiotic, of these carbohydrates include delayed gastric emptying, modulation of the gastrointestinal tract transit times, improved glucose tolerance, reduced fat and cholesterol absorption via binding of bile acids, increased volume and water carrying capacity of intestinal contents, and modulation of microbial fermentation with increased short chain fatty acids production and decreased pH and ammonia production (Roberfroid, 1996; Cummings & Macfarlane, 2002). The combination of these effects could potentially result in improved host health by reducing intestinal disturbances (constipation and diarrhea), cardiovascular disease and intestinal cancer (Ziemer & Gibson, 1998; Roberfroid, 1993).

In that respect, fructo-oligosaccharides (FOS) have received increasing attention over the past decade (Gibson & Roberfroid, 1995). FOS are present in relative high concentrations in chicory, garlic and onion. Next to that, FOS can also be produced enzymatically, allowing commercial large scale production (Crittenden & Playne, 1996). The digestive enzymes of human and animals lack the ability to cleave the β-glycosidic linkages that combine the fructose residues in FOS. As a result, FOS arrive intact in the human colon (Mikkelsen *et al.*, 2004). There, specific microbial enzymes, including β-fructosidase, are able to act on these bonds resulting in the degradation of FOS (Barrangou *et al.*, 2003). Only probiotic microorganisms possess these appropriate enzymes. As a result, selective growth of probiotic microorganisms is stimulated (Ziemer & Gibson, 1998).

The same elucidation can be applied to explain the prebiotic properties of kojibiose. Microorganisms such as lactobacilli and bifidobacteria possess cell-associated glycosidases, which allow degradation of kojibiose (Sanz *et al.*, 2005). The liberated monosaccharides can then be taken up by these specific organisms, rather than other microorganisms present in a mixed culture community like the human colon. Kojibiose derivatives, such as kojioligosaccharides, are utilized by fewer strains but more selectively than kojibiose itself (Nakada *et al.*, 2003). Sanz *et al.* screened about 20 disaccharides for their prebiotic properties, pointing out kojibiose as the best prebiotic compound. Next to kojibiose, sophorose, 4-β-galactobiose, isomaltose, 6-β-galactobiose, 6-β-mannobiose and nigerose also showed strong prebiotic potency. Remarkably, all these disaccharides score better

than the previously described FOS (van den Broek *et al.*, 2008). Moreover, this study revealed that α-disaccharides resulted in higher prebiotic potency compared to β-disaccharides (Sanz *et al.*, 2005).

1.2.2. α-glucosidase I inhibitor

Kojibiose and derivatives have the ability to inhibit α-glucosidase I (Ogawa *et al.*, 1998), as demonstrated in plants (Zeng & Elbein, 1998) and rat liver microsomes (Ugalde *et al.*, 2005). It is supposed that kojibiose acts as a transition state analogue of this enzyme, thereby inhibiting its biological activity (Ogawa *et al.*, 1998). This characteristic is extremely captivating, since inhibitors that act upon α-glucosidase I are suggested to be interesting candidate drugs for the treatment of HIV type 1 infections (Borges de Melo *et al.*, 2006). HIV contains an envelope which is composed of viral glycoproteins. These glycoproteins are essential in the viral life cycle. Processing of these glycoproteins is performed by the human cellular machinery. Infection of HIV occurs by binding of such a glycoprotein to the CD4 surface antigen. CD4-T-cells are lymphocytes who coordinate the immune system in case of infection. By attacking these cells, HIV is not only capable of reproducing itself, but it also shuts down the mechanism by which the human body protects itself against all sorts of pathogens. The bond between the HIV glycoprotein and the CD4 surface antigen allows fusion with the cellular membrane, thus viral entry into the cell. Treatment of HIV-1 infected cells with α-glucosidase inhibitors, hampers the formation of the glycoprotein, thereby inhibiting the fusion of HIV and thus virus spread (Mehta *et al.*, 1998).

Next to HIV-1 treatment, the α-glucosidase I inhibitory activity of kojibiose gives rise to another interesting application. Normally, α-glucosidases are used to degrade diet polysaccharides to monosaccharide units, which are then able to be metabolically absorbed and used by the organism (Borges de Melo *et al.*, 2006). α-glucosidase inhibitors limit thus the digestion of dietary carbohydrates and could therefore be useful to counter diabetes, obesity and cardiovascular diseases (Phung *et al.*, 2014; Standl & Schnell, 2012).

1.3. Production of kojibiose

1.3.1. Isolation

As previously described, kojibiose is naturally present in honey, beer, sake and koji. It was first isolated from sake during studies on unfermentable sugars (Aso & Shibasaki, 1953). A few years later, it was also discovered in honey as its crystalline α- and β-octa-acetate form (Watanabe & Aso, 1959; Aso & Fumio, 1961). However, the concentrations are extremely low in these sources, rendering isolation on an industrial scale rather pointless. In honey for example, only 3.67 mg of kojibiose was eventually obtained from 100 g honey after numerous isolation steps including extraction, evaporation and chromatography (Watanabe & Aso, 1959). Another route to obtain kojibiose is by processing the acid hydrolysate of sweet potato starch (Sato & Aso, 1957). Furthermore, when incubating several *Aspergillus* species with glucose and maltose, kojibiose is produced (Aso & Shibasaki, 1953; Nikolov *et al.*, 1989). However, the isolation of kojibiose from this complex mixture has not been reported to date.

1.3.2. Partial acetolysis of dextran

Currently, the best known method for the production of kojibiose is through partial acetolysis of dextran from *Leuconostoc mesenteroides* NRRL B-1299-S (Chaen *et al.*, 2001; Matsuda *et al.*, 1961; Suzuki & Hehre, 1964). Both immunochemical observations (Suzuki & Hehre, 1964) and optical rotational shifts, caused by the formation of cuprammonium complexes (Scott *et al.*, 1957), suggest that the derived dextran from this strain contains a high proportion of α-1,2-glycosidic bonds. The isolation of kojibiose from this matrix is however quite intensive. First, an ice-cold mixture of acetic anhydride and sulfuric acid was added to a suspension of dry, finely pulverized dextran. The reaction mixture was stirred magnetically for 3-4 days at 25 °C. Afterwards, this mixture was neutralized with sodium hydrogen carbonate, extracted with chloroform and evaporated. The resulting solution was then dissolved in hot methanol, cooled and centrifuged at low speed. The supernatant was decanted and seeded with α-kojibiose octa-acetate crystals, after which the mixture was allowed to crystallize during 3-4 days at room temperature. The crystals were filtered off and finally washed with a small amount of cold methanol, resulting in a yield of approximately 17 % (w/w) (Duke *et al.*, 1973). In conclusion, the latter multi-step process involves the use of many chemical reagents such as acetic anhydride, sulfuric acid, chloroform and methanol, while only low yields are obtained.

1.3.3. Chemical synthesis

Kojibiose can also be obtained through chemical synthesis. This is performed via modified Koenigs-Knorr reactions. Starting from 1,3,4,6-tetra-O-acetyl-α-D-glucopyranose and zinc chloride as reagents, a mixture of glucose, sophorose and kojibiose can be obtained (Igarashi *et al.*, 1975; Wolfrom *et al.*, 1963; McGrath *et al.*, 1969). Hereby, McGrath *et al.* determined a ratio of sophorose over kojibiose of circa 2:1, amongst some trisaccharides and tetrasaccharides. After purification of the reaction mixture, typically very low overall yields in the range of only 0.48 % are reported (McGrath *et al.*, 1969). Other disadvantages of the latter method are the need of multiple protection and deprotection steps, the use of silver or mercury catalysts, the use of heavy metals (ZnCl) and the necessity of solvents such as acetonitrile. In conclusion, although chemical synthesis allows the isolation of kojibiose, this method proved to be rather inefficient.

1.3.4. Enzymatic synthesis

Because of the many drawbacks concerning the previous described methods, the quest for more efficient ways for the production of kojibiose was ongoing. Enzymes are known to be efficient biocatalysts in a broad range of carbohydrate related reactions. Accordingly, several enzymatic procedures were developed for the synthesis of kojibiose. An overview of the currently existing methods will be given in this section.

1.3.4.1. α-glucosidases and α-glucoamylases

At first, enzymes with a typically broad specificity such as α-glucosidases and α-glucoamylases were considered (Fujimoto *et al.*, 1988; Cantarella *et al.*, 1994). An α-glucosidase (e.g. from *Saccharomyces*

sp.) or an α-glucoamylase (e.g. from *Aspergillus niger*) was incubated with glucose or starch, respectively. At the end of the reaction, a mixture of α-linked disaccharides was obtained: kojibiose, nigerose, maltose and isomaltose. Although the use of toxic catalysts and solvents can be avoided, time consuming downstream process methods would be required to isolate kojibiose from these mixtures. Therefore enzymes with a broad specificity should be avoided for the synthesis of kojibiose.

1.3.4.2. Glucansucrase

Glucansucrase naturally catalyzes the polymerization of glucosyl residues obtained through the hydrolysis of sucrose, with release of fructose (Monsan *et al.*, 2010). Depending on the specificity of the enzyme, a wide range of glucans can be synthesized, varying in type of glycosidic bonds (α -1,2; α-1,3; α-1,4; α-1,6), size, structure, degree of branching and spatial arrangement. These enzymes are also able to transfer the glucosyl unit from sucrose onto hydroxylated acceptor molecules. Recently, enzyme engineering has been successfully applied on glucansucrase DSR-E from *Leuconostoc mesenteroides* NRRL B-1299 to increase the specificity for α-1,2-glycosidic bonds. Unfortunately, next to the synthesis of kojibiose from sucrose and glucose, the enzyme is also capable of glycosylating fructose, resulting in the formation of leucrose (Glc α(1→5) Fruc) and maltulose (Glc α(1→4) Fruc) as byproducts (Brison *et al.*, 2012).

1.3.4.3. Kojibiose phosphorylase

Kojibiose phosphorylase (KP) from *Thermoanaerobacter brockii* ATCC 35407 is a more specific enzyme that catalyzes the α-1,2 transglucosylation from β-D-glucose 1-phosphate (β-D-G1P) as the glucosyl donor to an appropriate acceptor sugar (Nakai *et al.*, 2013). If glucose is used as the acceptor, kojibiose can be synthesized. The greatest drawback however is the requirement of β-D-G1P, which is only available in limited amounts and is therefore an expensive substrate (€ 22 730/g, Carbosynth). A solution that could overcome this problem was developed by Chaen *et al.* and involves a coupled enzymatic process starting from maltose using 2 enzymes: kojibiose phosphorylase and maltose phosphorylase. The latter, originating from *Enterococcus hirae* IFO 3181, produces the expensive donor substrate β-D-G1P in situ through phosphorolysis of maltose. This is followed by the action of KP resulting eventually in the production of kojibiose. Due to the side production of considerable amounts of glucose, maltose and different kojioligosaccharides, the overall yield of kojibiose in this process is limited to only 19.1 % (Chaen *et al.*, 2001).

1.3.4.4. Dextransucrase and β-galactosidase

Recently, a sustainable biotechnological process for the efficient synthesis of kojibiose was published in the Green Chemistry journal (Díez-Municio *et al.*, 2014). The latter process starts from sucrose as donor substrate and lactose as acceptor substrate, both readily available and cheap carbohydrates (Figure II.2). Frist, a transglucosylation reaction was performed with dextransucrase from *Leuconostic mesenteroides* B-512F, whereby the glucose unit from sucrose was transferred to lactose, resulting in 4'-galactosyl-kojibiose. Subsequently, a hydrolysis step was performed with β-galactosidase from *Kluyveromyces lactis* to obtain kojibiose. Since monosaccharides (e.g. fructose)

inhibit the activity of β-galactosidase, removal of residual monosaccharides was necessary after the first transglucosylation step. This was achieved by yeast treatment with *Saccharomyces cerevisiae*.

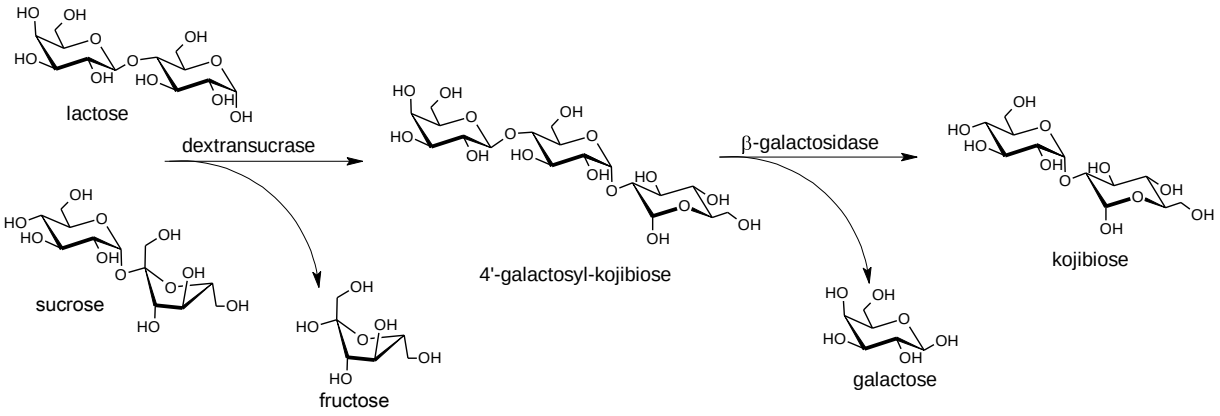


Figure II.2: Mechanism of the synthesis of kojibiose by transglucosylation of lactose catalyzed by dextransucrase from *Leuconostoc mesenteroides* B-512F, yeast treatment with *Saccharomyces cerevisiae* and hydrolysis with β-galactosidase from *Kluyveromyces lactis* (adapted from Díez-Municio *et al.,* 2014).

Afterwards, a purification stage was required to obtain the synthesized kojibiose out of the mixture of carbohydrates: fructose, glucose, galactose, leucrose and unidentified trisaccharides, all byproducts from the enzymatic reaction steps. Two different strategies were studied: yeast treatment purification and chromatographic purification. Yeast treatment seems a very attractive method, since it provides an easy en cheap way to purify the product. Unfortunately, a purity of only 68 % was obtained, due to the presence of leucrose and unidentified trisaccharides, products which the yeast can not metabolize. Moreover this purity was obtained only after 42 h of yeast treatment. To increase the purity of kojibiose and to reduce the total process time, a preparative liquid chromatography purification step was performed, using silica as the stationary phase and a mixture of acetonitrile/water as the mobile phase. In this way, kojibiose with a purity higher than 99 % was obtained. However, the yield of kojibiose synthesized with the described process amounted only to 38 % (in weight with respect to the initial amount of lactose). Moreover the sustainability of this process decreases dramatically using liquid chromatography. In conclusion, efforts need to be made to obtain higher yields and purities in order to make this process economically feasible at industrial scale.

Although these enzymatic methods provide more efficient ways for the synthesis of kojibiose than the isolation and chemical synthesis, they suffer from low yields due to considerable amounts of byproducts. In summary, the currently existing processes are far from optimal and are not attractive for production on large scale.

2 Enzymatic synthesis of di- and oligosaccharides

2.1. Introduction

The synthesis of di- and oligosaccharides – such as kojibiose – can be achieved through the attachment of a glycosyl moiety on a carbohydrate (van den Broek *et al.*, 2008). Glycosylation can also be performed on non-carbohydrates, thereby altering the physiological properties of these compounds (Desmet *et al.*, 2012; De Roode *et al.*, 2003). Although much work has been done on the chemical synthesis of carbohydrates, the selective formation of a glycosidic bond remains a difficult task to date (Nicolaou & Mitchell, 2001). It requires multiple protection and deprotection steps, resulting in time consuming procedures with low overall yields. Moreover, chemical glycosylation generates large amounts of waste and often toxic catalysts are involved (Desmet & Soetaert, 2011). To counter these disadvantages, enzymes could be used, since they are active under mild reaction conditions and can be highly specific, which eliminates the need for protection steps. Moreover, it has been calculated that reactions via enzymatic glycosylation generate 5-fold less waste and have a 15-fold higher space-time yield (De Roode *et al.*, 2003). Therefore, enzymes are interesting tools for the synthesis of carbohydrates and are nowadays the focus of attention.

2.2. CAZymes

Enzymes that show activity towards glycosidic bonds are known as carbohydrate-active enzymes, or CAZymes (Desmet & Soetaert, 2011). Four types of CAZymes can be distinguished (Figure II.3). Glycosyl transferases (GT) typically use nucleotide activated sugars as donor, from which a glycosyl group is transferred to an acceptor. Transglycosidases (TG) are capable of synthesizing carbohydrates by transferring a glycosyl group from one carbohydrate chain to another. Glycoside hydrolases (GH) and glycoside phosphorylases (GP) catalyze the degradation of glycosidic bonds. In case of GP, inorganic phosphate is the acceptor substrate, while with GH, the glycosyl group is transferred to water. According to the Enzyme Commission (EC) classification, GH belong to the EC.3.2 class. GT, TG and GP are all part of one major class, the glycosyl transferases (EC.2.4) The 'true' glycosyl transferases are also called Leloir transferases, to discriminate them from the other enzymes in subclass 2.4 (Desmet & Soetaert, 2011).

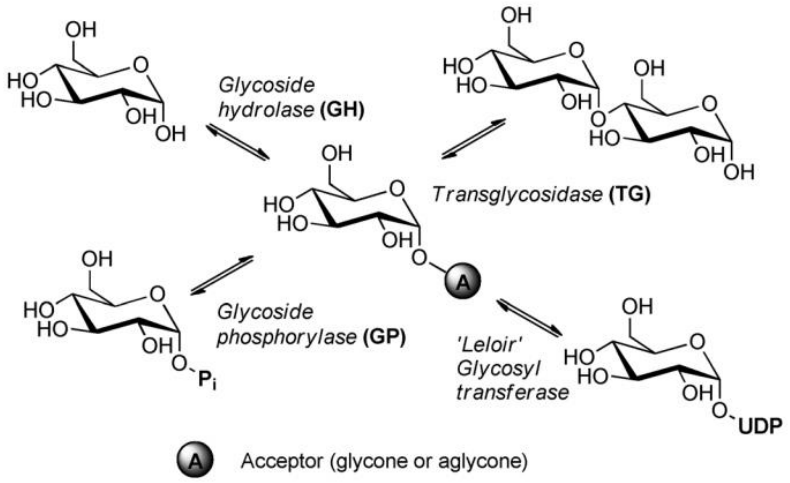


Figure II.3: Overview of the four different types of CAZymes (Desmet *et al.*, 2012).

2.2.1.Glycosyl transferases

GT's or Leloir transferases usually catalyze *in vivo* glycosylation (Kitaoka & Hayashi, 2002). Due to the use of high energy donors (e.g. UDP-glucose), high yields can be obtained with these enzymes. Interestingly, Leloir transferases have a strict substrate specificity, avoiding the formation of side products (Kittl & Withers, 2010; Desmet *et al.*, 2012). From this, GT's seem attractive tools for glycosylation. Two main issues hinder however the large scale industrial application of GT's. On the one hand, the nucleotide activated sugar donors are quite expensive (e.g. € 181.80/g UDP-glucose, Carbosynth). On the other hand, GT's are associated with the cell membrane and thus difficult to extract. Moreover, once extracted, they show a low stability (Kitaoka & Hayashi, 2002). Consequently, the industrial applications of these enzymes are limited to the synthesis of expensive glycosides with therapeutic properties such as N-acetyllactosamine, a key component of the glycan epitopes present on glycoproteins, produced by galactosyl transferase (Palcic, 1999). Improving the stability of GT's and the development of alternative ways to produce the expensive donors could greatly increase the potential of these enzymes.

2.2.2.Transglycosidases

TG's catalyze the transfer of carbohydrate moieties from one chain to another. They are basically glycosidases that are able to avoid water as acceptor substrate, resulting in a high degree of kinetic control (Seibel *et al.*, 2006). Interestingly, some TG's employ sucrose as donor substrate. This is not only a very cheap and readily available substrate, it is also a very reactive molecule that allows yields comparable with those achieved with the expensive nucleotide activated sugar donors of the Leloir transferases (Monsan & Ouarné 2009). Examples include the synthesis of the low-glycemic sweetener isomaltulose by sucrose mutase (Monsan *et al.*, 2010) and the production of FOS by fructansucrase (Kralj *et al.*, 2008). Unfortunately, their application in industry has been hampered due to the small number of available specificities. Enzyme engineering could offer a solution to this problem (Desmet *et al.*, 2012).

2.2.3.Glycoside hydrolases

Although GH's naturally catalyze the degradation of glycosidic bonds, these enzymes can be forced to create glycosidic bonds via either thermodynamic control or kinetic control. Thermodynamic control or reverse hydrolysis is based on the principle of Le Châtelier. The equilibrium can be shifted towards synthesis instead of hydrolysis by using high substrate concentrations or media with a low water content (Desmet & Soetaert, 2011). Kinetic control is based on transglycosylation, which occurs through interception of the glycosyl enzyme intermediate by an acceptor other than water. Activated donor substrates and optimization of the reaction conditions can be applied to maximize the ratio of transglycosylation over hydrolysis (Desmet & Soetaert, 2011). Advantages of these type of enzymes are their readily, cheap availability and their wide range of donor specificities. Examples include the synthesis of rutinose from L-rhamnose and D-glucose with the highly specific α-L-rhamnosidase from *Aspergillus niger* (Martearena *et al.*, 2008), while β-mannosides were successfully produced with endo-β-mannosidase from *Lilium longiflorum* (Bojarová & Kren, 2009; Sasaki *et al.*, 2005). Nevertheless, GH's typically suffer from poor yields, low regioselectivity - resulting in a mixture of products in case of multiple hydroxyl group containing acceptors - and the possibility of formed products being hydrolyzed again, called secondary hydrolysis (Desmet *et al.*, 2012).

A major breakthrough was realized with the introduction of glycosynthases. These are glycosidases mutated in their active site, resulting in elimination of their hydrolytic activity, though still capable of performing transglycosylation, when a suitable activated donor is used. Glycosynthase variants of α-L-fucosidase and β-glucuronidase have, for example, been created to synthesize oligosaccharide structures of considerable biological significance (Wada *et al.*, 2008; Müllegger *et al.*, 2006). Nonetheless, the applications of glycosynthases still face some challenges with substrate specificity, regioselectivity and optimal yields (Osanjo *et al.*, 2013).

2.2.4.Glycoside phosphorylases

In vivo, GPs catalyze the degradation of the glycosidic linkage in oligosaccharides with the use of inorganic phosphate, resulting in the production of a glycosyl phosphate and a carbohydrate of reduced chain length (Kitaoka & Hayashi, 2002). A list of known phosphorylases with their characteristics is presented in Table II.1.

The physiological role of GP's lays in providing an efficient energy metabolism *in vivo* (Desmet & Soetaert, 2012). The phosphate group of the glycosyl phosphate can simply be transferred from the C1- to the C6-position by a mutase and is than capable of entering the glycolysis pathway. This amounts to the saving of one molecule ATP compared to hydrolytic degradation of saccharides, where activation with a kinase is necessary (de Kok *et al.*, 2011). Phosphorolytic reactions are reversible due to the high energy content of the glycosyl phosphate, allowing their application for synthetic purposes *in vitro* (Kitaoka & Hayashi, 2002). Moreover, the glycosyl donor is more easily produced and thus cheaper, compared to the expensive nucleotide activated sugars required with GT's. This is a crucial benefit for industrial applications. α-glucose 1-phosphate (α-G1P), for example,

can be produced from the cheap and readily available substrate sucrose (De Winter *et al.*, 2011; Goedl *et al.*, 2011). Next to that, the acceptor specificity of phosphorylases comprises a wide range of mono-, di- and oligosaccharides.

Table II.1: Overview of known phosphorylases to date (Adapted from Nakai *et al.*, 2013).

CAZy	Mechanism	EC	Name	Substrate	Product
GH13	Retention	2.4.1.7	Sucrose phosphorylase	Glc-α(1,2)β-Fru	α-Glc-1P
		2.4.99.16	α(1,4)-Glucan:maltose-1P maltosyltransferase	Glc-[α(1,4)-Glc]$_n$	α-Mal-1P
GH65	Inversion	2.4.1.64	Trehalose phosphorylase	Glc-α(1,1)α-Glc	β-Glc-1P
		2.4.1.230	Kojibiose phosphorylase	Glc-α(1,2)α-Glc	β-Glc-1P
		2.4.1.279	Nigerose phosphorylase	Glc-α(1,3)α-Glc	β-Glc-1P
		2.4.1.8	Maltose phosphorylase	Glc-α(1,4)α-Glc	β-Glc-1P
		2.4.1.216	Trehalose-6P phosphorylase	Glc-α(1,1)α-Glc-6P	β-Glc-1P
		2.4.1.282	3-O-α-Glucosyl-L-rhamnose phosphorylase	Glc-α(1,3)-L-Rha	β-Glc-1P
GH94	Inversion	2.4.1.20	Cellobiose phosphorylase	Glc-β(1,4)-Glc	α-Glc-1P
		2.4.1.31	Laminaribiose phosphorylase	Glc-β(1,3)-Glc	α-Glc-1P
		2.4.1.49	Cellodextrin phosphorylase	Glc-[β(1,4)-Glc]$_n$	α-Glc-1P
		2.4.1.280	N,N'-Diacetylchitobiose phosphorylase	GlcNAc-β(1,4)-GlcNAc	α-GlcNAc-1P
GH112	Inversion	2.4.1.211	(Ga)Lacto-N-biose phosphorylase	Gal-β(1,3)-GlcNAc	α-Gal-1P
		2.4.1.247	Galactosylrhamnose phosphorylase	Gal-β(1,4)-L-Rha	α-Gal-1P
GH130	Inversion	2.4.1.281	Mannosylglucose phosphorylase	Man-β(1,4)-Glc	α-Man-1P
		2.4.1.-	Mannooligosaccharide phosphorylase	Man-[β(1,4)-Man]$_n$	α-Man-1P
—	Inversion	2.4.1.30	Laminaridextrin phosphorylase	Glc-[β(1,3)-Glc]$_n$	α-Glc-1P
		2.4.1.97	β(1,3)-Glucan phosphorylase	Glc-[β(1,3)-Glc]$_n$	α-Glc-1P
GT4	Retention	2.4.1.231	Trehalose phosphorylase	Glc-α(1,1)α-Glc	α-Glc-1P
GT35	Retention	2.4.1.1	Glycogen phosphorylase	Glc-[α(1,4)-Glc]$_n$	α-Glc-1P

As can be derived from Table II.1, a significant number of phosphorylases are known to date. Considering their reversible reaction potential, phosphorylases are powerful catalysts for efficient synthesis of particular di- and oligosaccharides from a donor sugar 1-phosphate and suitable carbohydrate acceptors with strict regioselectivity (Nakai *et al.*, 2013). A profound example hereby is the synthesis of kojibiose from β-G1P using kojibiose phosphorylase (Chaen *et al.*, 2001). Unfortunately β-G1P is an expensive substrate, although solutions to overcome this problem were developed, as mentioned in Section II.1.3.4.3.

GP's however show the disadvantage of having lower product yields than GT's (<65 %) (Desmet & Soetaert, 2012). In addition, they suffer from a narrow donor specificity: mostly their activity is limited to G1P. Over the past decade, enzyme engineering has proven an interesting tool for expanding the specificity of phosphorylases. In this field, most work has been performed on cellobiose phosphorylase (CP). A CP enzyme variant from *Cellulomonas uda*, lactose phosphorylase 3 (LP3), obtained through directed evolution and protein engineering, is able to use α-galactose 1-phosphate (Gal1P) as donor (De Groeve *et al.*, 2010). As a result, lactose could be produced with LP3 using Gal1P as glycosyl donor and glucose as acceptor (De Groeve, 2009). This synthesis route for lactose is not only simpler than the route mammals use in nature, but also provides a completely new way of synthesizing lactose using natural substrates. The produced lactose, which is not directly from animal origin, can be of interest in certain pharmaceutical applications (De Groeve *et al.*, 2010).

The LP3 mutant also shows activity towards cellobiose as acceptor, making the synthesis of lactocellobiose possible. The latter may have prebiotic properties (De Groeve *et al.*, 2010).

Another interesting enzyme is sucrose phosphorylase, which uses a double displacement mechanism (see Section II.3.3). This enzyme can also act as a TG without the participation of phosphate through glycosyl transfer to suitable acceptor molecules. Sucrose phosphorylase seems therefore a very attractive biocatalyst. Indeed, the enzyme displays a remarkable broad acceptor specificity (Luley-Goedl & Nidetzky, 2010) and uses a reactive substrate, allowing high yields (Desmet & Soetaert, 2012).

3 Sucrose phosphorylase

3.1. General properties and classification

Sucrose phosphorylase (SP) is a bacterial GP that catalyzes the reversible conversion of sucrose and phosphate into α-D-glucose 1-phosphate and β-D-fructose (Goedl *et al.*, 2010). SP was first discovered in 1942 (Kagan *et al.*, 1942) in *Leuconostoc mesenteroides,* but it has so far been identified in different species such as *Bifidobacterium adolescentis, Pseudomonas saccharophila* and *Streptococcus mutans* (van den Broek *et al.*, 2004; Lee *et al.*, 2006). The SP originating from *Bifidobacterium adolescentis* (BaSP) is the most stable one. Moreover, the crystal structure of this enzyme was characterized by Sprogoe *et al.* in 2004. BaSP consists of 504 amino acids and has a molecular mass of 56 189 g/mol (Sprogoe *et al.*, 2004). Some important physico-chemical properties are listed in Table II.2.

Table II.2: Physico-chemical properties of sucrose phosphorylase from *Bifidobacterium adolescentis*.

Parameter	SP value	Reference
Specific activity towards sucrose	213 U/mg	Cerdobbel *et al.*, 2011
Deduced molecular mass	56 189 Da	van den Broeck *et al.*, 2004
Molecular mass (SDS-PAGE)	58 kDa	van den Broeck *et al.*, 2004
Native molecular mass (gel permeation)	129 kDa	van den Broeck *et al.*, 2004
Optimum pH	pH 6.5	Cerdobbel *et al.*, 2010a
Optimum temperature	58 °C	Cerdobbel *et al.*, 2010a

The native molecular mass is determined via gel permeation, a technique in which the quaternary structure of the protein is conserved. With SDS-PAGE, the enzyme is denatured, resulting in the detection of the molecular mass of the monomer. Comparison of the values from these 2 techniques indicates clearly a dimeric structure for BaSP (van den Broek *et al.*, 2004). SP is classified as EC.2.4.1.7 (Schwarz & Nidetzky, 2006). The number refers to a subdivision of the hexosyltransferases (EC.2.4.1), which is in turn a subset of the GT's (EC.2.4). However, according to the CAZy database, SP is a member of the glycoside hydrolase 13 (GH13) family. This classification system, in contrast to EC classification, is based on similarities in amino acid sequences rather than the reaction catalyzed,

thereby considering resemblances in reaction mechanism and evolutionary relationships (Cantarel *et al.*, 2009). The GH13 family is also known as the α-amylase family and despite its EC classification as GT, SP is structurally more related to enzymes of the GH's (EC.3.2) (Goedl *et al.*, 2010)

3.2. Structural properties

In Figure II.4, the 3D-crystal structure of the monomeric subunit (left) and the dimeric enzyme (right) of BaSP is shown. The polypeptide chain is folded into a structure with four domains, named A, B, B' and C (Sprogoe *et al.*, 2004). Domain A refers to the $(\beta/\alpha)_8$-barrel, typical for members of the GH-13 family. It contains eight alternating parallel β-sheets and α-helices. The catalytic domain including the active site is situated in this barrel. The active site incorporates a triad of two aspartic acids (Asp192, Asp290) and one glutamic acid (Glu232). These active side residues are highly conserved in members of the GH-13 family (Luley-Goedl & Nidetzky, 2010). Domains B and B', both situated close to the active site, are considered to be involved in the enzyme's specificity (Sprogoe *et al.*, 2004). The folding of domain C is unique among the GH-13 family, while its function has not yet been discovered (Mirza *et al.*, 2006).

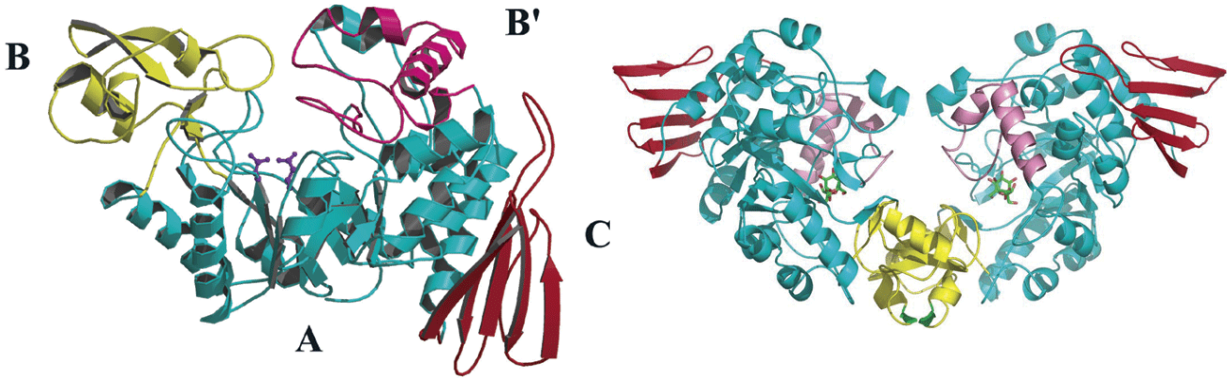


Figure II.4: *(Left)* Representation of the monomer structure of BaSP. Domain A is displayed in blue, domain B in yellow, domain B' in magenta, and domain C in red. The catalytic active residues (Asp192 and Glu232) are shown in purple. *(Right)* Representation of the dimer structure of BaSP (Sprogoe *et al.*, 2004).

3.3. Reaction mechanism

SP uses an α-retaining double displacement mechanism to convert sucrose and inorganic phosphate into α-D-G1P and D-fructose (Luley-Goedl & Nidetzky, 2010). The mechanism can be explained in detail using Figure II.5. Three acidic residues contribute to the catalytic subside: Asp192, Asp290 and Glu232. In the first step, Asp192 acts as a nucleophile by attacking the anomeric carbon of the glucose unit of the donor substrate sucrose. Meanwhile Glu232, acting here as an acid catalyst, loses its carboxylic proton to the glycosidic bond oxygen. This results in the release of D-fructose and the formation of a covalent glucosyl-enzyme intermediate, which has an inverted β configuration. Subsequently the deprotonated Glu232-residue, now acting as a base catalyst, deprotonates the inorganic phosphate which on its turn is capable of attacking the C1-carbon of the intermediate. As a result, α-D-G1P is released from the enzyme, causing a second anomeric inversion and thus a net anomeric retention (Aerts, 2011b; Desmet & Soetaert, 2011).

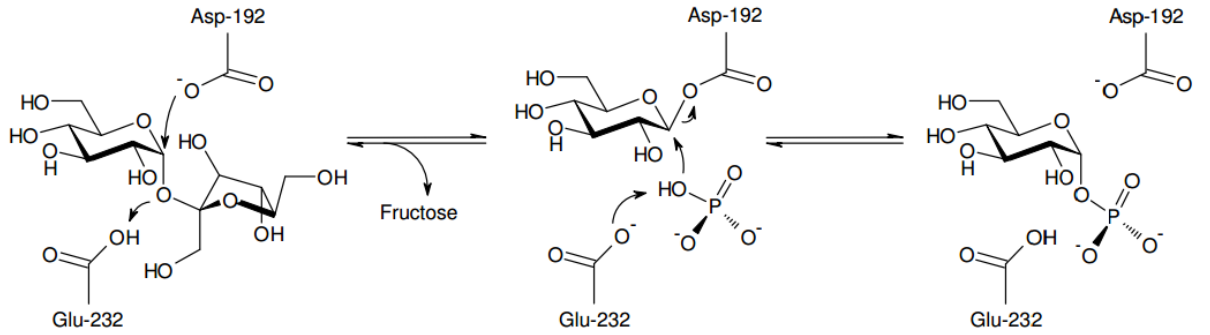


Figure II.5: Catalytic mechanism of SP. Catalytically important residues from BaSP are indicated: Asp192 (catalytic nucleophile) and Glu232 (catalytic acid/base) (Luley-Goedl & Nidetzky, 2010).

Because SP displays a double displacement mechanism, it can catalyze three types of overall reactions, as shown in Figure II.6. Once the covalent glucosyl-enzyme intermediate is formed, the glucosyl moiety can be transferred to phosphate (phosphorolysis), water (hydrolysis), or an alternative acceptor (glycosylation) (Desmet *et al.*, 2012; Goedl *et al.*, 2010). Interestingly, the range of acceptor substrates is broad, due to the crystallographically observed conformational flexibility of the acceptor biding site in BaSP (Goedl *et al.*, 2010). A very diverse group of compounds can thus be glycosylated with this enzyme, making SP a very powerful biocatalyst for glycosylation. Examples include the synthesis of 3-O-α-D-glucopyranosyl-(E)-resveratrol in presence of the ionic liquid AMMOENG 101 (De Winter *et al.*, 2013) and the synthesis of 2-O-α-D-glucopyranosyl pyrogallol in a biphasic solvent system containing MOPS buffer and ethyl acetate in a ratio of 5:3 (De Winter *et al.*, 2014).

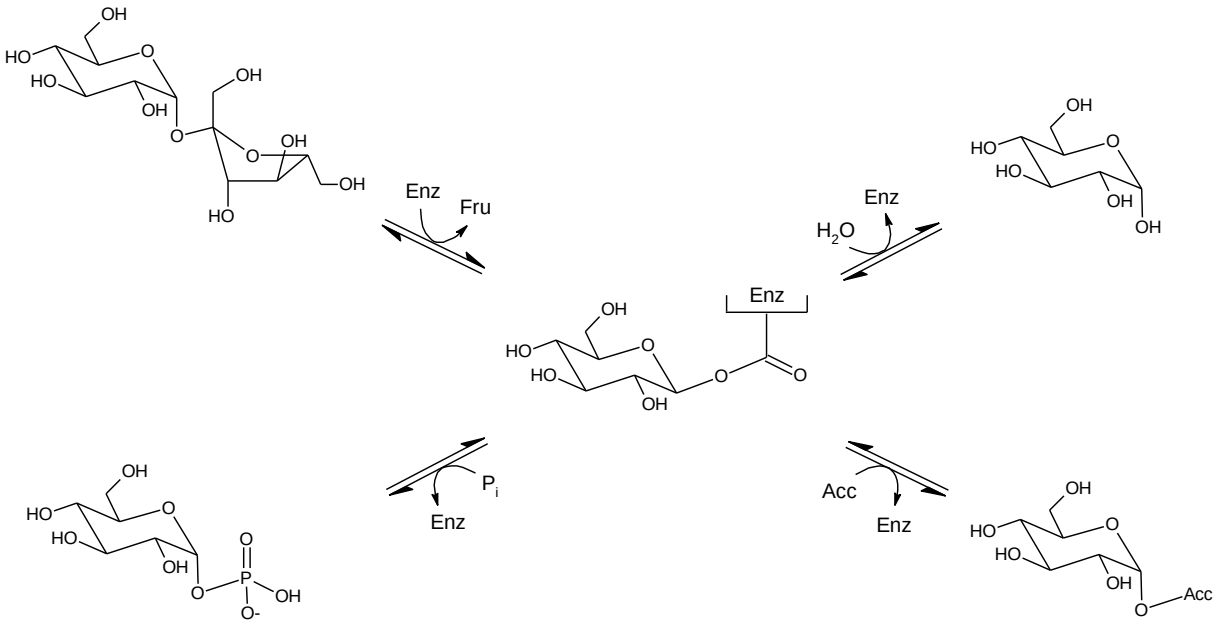


Figure II.6: The different reactions catalyzed by SP: phosphorolysis, hydrolysis and glycosylation.

3.4. Engineering of SP for the synthesis of kojibiose

The broad acceptor specificity of SP has proved useful for the synthesis of glycosides and also shows potential towards carbohydrate synthesis (Desmet *et al.*, 2012). Indeed, the synthesis of glucobioses could be achieved starting from sucrose as donor substrate and glucose as acceptor substrate. Kitao *et al.* reported in 1994 the synthesis of kojibiose using SP from *Leuconostoc mesenteroides* (LmSP) (Kitao *et al.*, 1994). The reaction scheme is shown in Figure II.7.

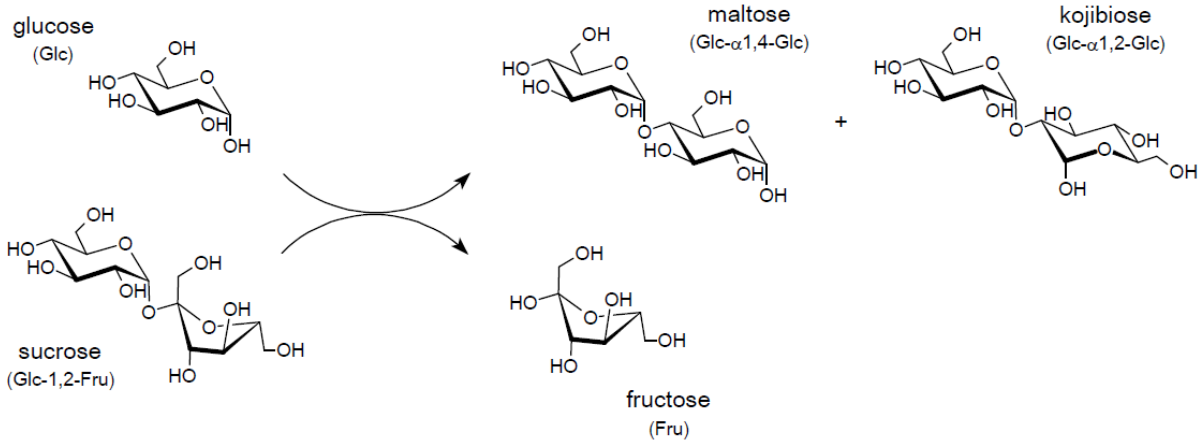


Figure II.7: Reaction of SP with sucrose as donor substrate and glucose as acceptor substrate, resulting in the formation of kojibiose and maltose.

Besides glucose, water can also act as acceptor leading to the formation of glucose and fructose (not depicted on the figure). The overall kojibiose yield is however lowered by the side formation of maltose. The selectivity can be defined as the amount of kojibiose formed divided by the total glucobiose formation (kojibiose + maltose). The selectivity of the wild type SP from *Leuconostoc mestenteroides* amounts to 50 % (Goedl *et al.*, 2010), while the selectivity of the enzyme from *Bifidobacterium adolescentis* was found the be only 35 % (Verhaeghe, 2014). Applications on industrial scale require however a selectivity of at least 95 %. Consequently enzyme engineering is required to overcome this problem. Using the most stable variant as a starting point is hereby preferable, making BaSP the variant of choice, rather than LmSP (Cerdobbel *et al.*, 2010a).

Using BaSP for the production of kojibiose is thus advantageous, since it displays the highest temperature optimum of all known SP's (58 °C). However temperatures of 60 °C or higher are preferred for carbohydrate conversions, mainly to avoid microbial contamination (Haki & Rakshit, 2003). Therefore the thermostability of BaSP was recently improved by means of mutagenesis and immobilization. Multipoint covalent immobilization of BaSP on Sepabeads EC-HFA has been found to shift the optimum temperature from 58 to 65 °C. Moreover, both thermal and pH stability were considerably increased (Cerdobbel *et al.*, 2010a). Further improvement of the thermostability of BaSP was achieved by producing cross-linked enzyme aggregates (CLEAs) using glutaraldehyde, whereby the optimum temperature was shifted to 75 °C (Cerdobbel *et al.*, 2010b). This research also lead to the development of imprinted CLEAs (iCLEAs) of BaSP, where improved stability and altered specificity were combined (De Winter *et al.*, 2012).

In order to enhance the selectivity of BaSP towards the production of kojibiose, enzyme engineering was applied by Verhaeghe *et al.* using semi-rational mutagenesis. It was shown that all residues in the acceptor site of BaSP contribute to the binding of the wild type substrates and that the enzyme is highly optimized to perform its wild type reaction. As a consequence, it was stated that the acceptor site would need drastic changes to completely shift the specificity. Since in this case, the goal was only to alter the preference for one or two existing but competing reactions, the hypothesis was made that small modifications in the acceptor site could lead to the desired goal. To that end, all positions in the acceptor site were randomized one by one by creating single site-saturation libraries. From this, it became clear that the selectivity could indeed be altered by small changes. In some cases the preference of maltose formation was enhanced, while for other mutants the opposite was observed. Moreover, not only the selectivity, but also the activity was affected, although it comprised most often a decrease. The best mutant was found to be L341I, having a selectivity (79 %) and activity (0.37 U/mg) more than twice as high compared to the wild type enzyme (35 % and 0.15 U/mg, respectively). High selectivities (62-73 %) were also observed for P134R, P134W, Y344I, Q345N, Q345S and P134V.

The next step was to combine the improved point mutated variants to further enhance the selectivity of BaSP towards kojibiose. This was done either rationally or based on a statistical model. The former refers to combination of the best single mutant L341I with the next best mutations in terms of selectivity, i.e. P134V, Q345N and Q345S. Combination of Q345N or Q345S with L341I gave rise to an increase in selectivity from 79 % to 93-94 % for both double mutants. The activity was unfortunately decreased to a value below that of the wild type enzyme. P134V_L341I in contrast had an improved activity, albeit at the cost of selectivity. The selectivity of L341I_Q345N was further optimized by introducing an alanine at position 344. In that way, a selectivity of 95.7 % could be achieved for L341I_Y344A_Q345N, together with a slight increase in activity compared to the double mutant lacking the Y344A mutation (Table II.3). Although no production process has been established to date, these mutants display promising characteristics for the synthesis of kojibiose.

Table II.3: Values for selectivity and specific activity of the wild type enzyme of BaSP and four created mutants. Data were obtained from the research of Tom Verhaeghe and calculated from initial reaction rates obtained from reactions at 58°C, pH 7 in the presence of 100 mM sucrose and 200 mM glucose (Van Lul *et al.*, 2014).

Enzyme	Selectivity (%)	Specific activity (U/mg)
Wild Type	35	0.15
L341I	79	0.37
L341I_Q345S	93-94	0.10
L341I_Q345N	93-94	0.05
L341I_Y344A_Q345N	95.7	0.06

4 Downstream processing

After biocatalytic reactions, downstream processing (DSP) methods are often required to recover the product of interest through separation from for example byproducts and substrates. In industry, DSP has the primary aim to recover the product as efficiently and reproducibly as possible, while maximizing the yield of recovery and minimizing the costs (Soetaert, 2012). The amount of steps are ideally kept to a minimum, since multistep purification procedures go hand in hand with significant product losses and higher process costs. Depending on the application area, different purities are required: bulk products in contrast to pharmaceutical agents, for example. For bulk chemicals, DSP accounts typically for 30 % of the total productions costs and this number can even increase up to 70 – 90 % for specialty small-scale products found in food and pharmaceutical industries (Strube *et al.*, 2011). An overview of the most important methods concerning the purification of carbohydrates is given below.

4.1. Chromatography

Chromatographic techniques are generally applied when a high purity of the product is required. The technique involves the use of a stationary and mobile phase and the separation is based on the difference in interaction of the components in a mixture with the stationary phase: the stronger the interaction, the later the component will elute with the mobile phase (Snyder *et al.*, 2011). A distinction needs to be made between analytic chromatography and preparative chromatography (Scott, 2003). The primary goal of analytic chromatography is peak profile monitoring for quantitative estimation and elution time measurement, while preparative chromatography involves the actual collection of an eluted component making the latter a purification technique instead of an analytical technique.

For the separation of carbohydrate mixtures on preparative scale, hydrophilic interaction chromatographic (HIC) systems using silica as the stationary phase and acetonitrile-water mixtures as the mobile phase are typically used (Corradini *et al.*, 2012). The main drawback is however the significant loss of mass during this purification step. The enzymatic production of kojibiose for example, with a purity higher than 99 % was only possible with the use of HIC, whereby the yield of the process amounted only to 38 %, as described in Section II.1.3.4.4. (Díez-Municio *et al.*, 2014).

Another preparative chromatographic technique frequently used for the fractionation of carbohydrates is size exclusion chromatography (SEC) or gel filtration. In SEC, carbohydrates are separated according to their molecular size (Sanz & Martínez-Castro, 2007). The technique is mainly used for the fractionation of oligo- and polysaccharides. Oligosaccharides from caprine milk whey for example, were fractionated to yield 28 fractions of 0.125 L using a BPG 100/950 column with a Superdex 30™ gel bed volume of 4.2 L. The target fractions were the first to be eluted, thereby separating them from the lower molecular mass carbohydrates, such as lactose, which eluted later (Oliveira *et al.*, 2012).

Ion exchange chromatography has been widely used by the sugar industry to fractionate mono- and oligosaccharides (Sanz & Martínez-Castro, 2007). Many carbohydrates are weak acids with pK_a values in the range of 12 to 14. Consequently under alkaline conditions, their hydroxyl groups are partially or totally transformed into anions. Using a positively charged stationary phase, this class of compounds can be selectively eluted (Corradini *et al.*, 2012). The latter technique is called anion exchange chromatography (AEC) and the stationary phase involves usually a resin. α-Gal1P, synthesized with LP3 from lactose and phosphate, was purified using AEC on Dowex 1-X8 and proved to be very efficient since 9.5 g of purified product was obtained from a 1 L reaction volume (De Groeve *et al.*, 2010).

Using chromatography as a DSP method results in a high purity of the product, but unfortunately this technique is expensive. Next to that, the use of organic solvents makes chromatography in many cases a very unsustainable purification method, which is both economically and ecologically detrimental. Moreover, these solvents are not compatible with many intended applications of carbohydrate products (De Winter *et al.*, 2013).

4.2. Yeast treatment

4.2.1. Introduction

Yeast treatment provides a cheap and efficient way of purification that can be applied to remove carbohydrate byproducts. The common baker's yeast *Saccharomyces cerevisiae* can be used to remove mono- and disaccharides from a carbohydrate mixture (Yoon *et al.*, 2003). Although this seems a very attractive method, it is crucial that the yeast only metabolizes the unwanted carbohydrates whereby the desired products remain unaffected. In the next section an overview of the specificity of *S. cerevisiae* towards the removal of saccharides is given.

4.2.2. Specificity

Under anaerobic conditions, monosaccharides are metabolized into ethanol and carbon dioxide, whereby two ATP molecules are generated (Figure II.8). The first step involves the degradation of the carbohydrates into two pyruvate molecules via the glycolysis or Embden-Meyerhof-Parnas pathway. Subsequently pyruvate is decarboxylated with pyruvate decarboxylase to CO_2 and acetaldehyde. By means of an alcohol dehydrogenase and NADH, the acetaldehyde is reduced to ethanol, regenerating NAD^+, necessary for the glycolysis reactions (Yoon *et al.*, 2003; Rodrigues *et al.*, 2006; Faria-oliveira *et al.*, 2013; van Maris *et al.*, 2006; van Dijken *et al.*, 1993; Flikweert, 1999).

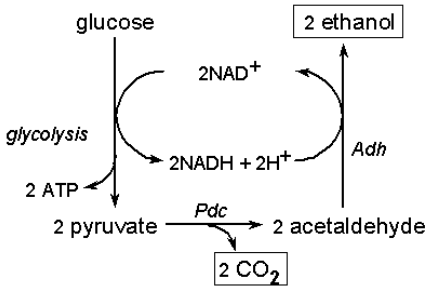


Figure II.8: Schematic representation of glucose dissimilation in *Saccharomyces cerevisiae* under anaerobic conditions (Flikweert, 1999). Pdc, pyruvate decarboxylase; Adh, alcohol dyhdrogenase

Yoon *et al.* studied the specificity and preference of *Saccharomyces cerevisiae* in removing carbohydrates by fermentation. Beads of immobilized yeast were added to different carbohydrate solutions, followed by reaction at 37°C for 24 h. After the yeast treatment, the carbohydrate solutions were analyzed via thin layer chromatography (Yoon *et al.*, 2003).

The common monosaccharides D-glucose, D-fructose, D-mannose and D-galactose were readily metabolized by the yeast, glucose being the preferable substrate (Faria-oliveira *et al.*, 2013). The α-linked disaccharides maltose and sucrose were also completely removed. Maltose was hydrolyzed into two glucose units by the yeast's α-glucosidase, while sucrose was found to be converted into fructose and glucose by the extracellular invertase enzyme. Isomaltose and α,α-trehalose were also found to be hydrolyzed by yeast's α-glucosidase and trehalase, respectively, but only partial removal after 24 h was observed. The β-linked disaccharides cellobiose and lactose were not removed, most likely due to the lack of β-glucosidase activity under the conditions of fermentation (Rosi *et al.*, 1994).

4.2.3. Examples of yeast purification

A continuous production process for α-G1P was developed by De Winter *et al.* with immobilized BaSP starting from sucrose. A packed-bed reactor was constructed with a space-time yield of 179 g/L/h at 60 °C, the temperature at which carbohydrate conversions are operated at industrial scale. The produced α-G1P could be purified by yeast treatment with baker's yeast (20 g/L) and could be recovered in crystalline form with a yield of 86 % (De Winter *et al.*, 2011).

Diez-Municio *et al.* reported a scalable process for the enzymatic synthesis of kojibiose from sucrose and lactose. Monosaccharide byproducts were removed using a *Saccharomyces cerevisiae* yeast treatment, resulting in a kojibiose purity of 68 % (Díez-Municio *et al.*, 2014).

Two salicin analogues, α-salicin and α-isosalicin, were enzymatically synthesized by transglycosylation reactions. α-salicin was produced with cyclomaltodextrin glucanyltransferase starting from cyclomaltohexaose and salicyl alcohol, followed by reactions with α-amylase and glucoamlase. α-isosalicin was synthesized with dextransucrase from sucrose and salicyl alcohol, followed by reactions with dextranase and glucoamylase. In both procedures, immobilized yeast was used to remove fermentable saccharides, which were produced as byproducts from the enzyme

reactions (Yoon *et al.*, 2004). The α-salicin analogues are expected to be better absorbed after oral administration than the β-salicin prodrug in aspirine (Gray *et al.*, 1979).

4.3. Crystallization

Crystallization of complex molecules on production scale is increasingly investigated because it perfectly integrates purification, concentration and sometimes formulation (Strube *et al.*, 2011). Transfer from laboratory scale into batch reactors for pilot and production scale is however not straightforward. In this section, some fundamental principles of crystallization are discussed.

Crystallization is the transformation of one or more substances from the amorphous solid, liquid, or gaseous phase to the crystalline phase (Mersmann *et al.*, 2011). For crystallization to occur, a solution has to be supersaturated. In a saturated solution, two states exist in equilibrium, the solid phase and one consisting of molecules free in solution. At saturation, no net increase in the proportion of solid phase can occur since it would be counterbalanced by an equivalent dissolution. Thus, crystals do not grow from a saturated solution. The system must be in a non-equilibrium, or supersaturated state to provide the thermodynamic driving force for crystallization (McPherson, 1990). Supersaturation can be achieved by cooling the solution or by evaporating the solvent. These techniques are called cooling and evaporative crystallization, respectively. Other techniques, such as varying the pH or using precipitating agents, to achieve supersaturation exist, but the latter are most commonly used in crystallization of carbohydrates (Linhardt & Bazin, 2001).

Cooling crystallization can be used if the solubility of the substance which is to be crystallized, considerably increases with increasing temperature. A typical mode of batch operation is cooling the undersaturated solution in a crystallizer, whereby to cooling rate is adjusted such that the supersaturation is roughly constant during the cooling process (Mersmann *et al.*, 2011). When the solubility of the component hardly increases with temperature, evaporative crystallization can be applied. The undersaturated solution is fed into the crystallizer and heated upon the boiling point, so that the solvent evaporates. Since the boiling point is function of pressure, also low pressure or even vacuum can be applied to evaporate the solvent. Here again it is advantageous to adjust the evaporation rate so that the supersaturation remains constant with time.

Crystallization from a supersaturated solution can start either through the formation of new nuclei followed by crystal growth or through the further growth onto an existing crystal surface (Mersmann *et al.*, 2011). The latter is called seeding. A seed provides a template of the desired component on which further molecules can assemble. Nucleation requires in general a higher degree of supersaturation than seeding because it is energetically more favorable to add to an already existing crystal plane than to form a new nucleus (Stura & Wilson, 1991). Therefore the seeding technique can be applied to facilitate the crystallization process.

From partial acetolysis of dextran, kojibiose octa-acetate crystals were obtained via cooling crystallization with a yield of 17 % (w/w) (Duke *et al.*, 1973). Crystalline β-sophorose octa-acetate

was isolated from glucose via the Koenigs-Knorr reaction with an overall yield of 28.2 % (Koeppen, 1968). α-G1P could be obtained in a crystallized form using both evaporation and cooling crystallization with a yield of 86 % (De Winter *et al.*, 2011).

A technique that is closely related to crystallization is precipitation. The driving force for the latter is also supersaturation, but amorphous solids are formed as the end product, in contrast to crystals having a well ordered 3D structure (Strube *et al.*, 2011). Five α-linked diglucosyl disaccharides (isomaltose, nigerose, kojibiose, maltose, and α,β-trehalose) and two trisaccharides (isomaltotriose and panose) were produced in 90 % aqueous solutions of 2-methoxyethyl ether, 2-ethoxyethyl ether or triethylene glycol dimethyl ether by glucoamylase-catalyzed glucose condensation. The use of these solvents led to the precipitation of the products (Cantarella *et al.*, 1994).

CHAPTER III

MATERIALS AND METHODS

1 Production and purification of recombinant SP

1.1. Expression in Erlenmeyer flasks

E. coli BL21 cells were used for transformation with the constitutive expression plasmids pCXshP34_BaSP, pCXshP34_BaSP_L341I, pCXshP34_BaSP_Q345S, pCXshP34_BaSP_L341I_Q345S, pCXshP34_BaSP_L341I_Q345N, pCXshP34_BaSP_L341I_Y344A_Q345N (Aerts, 2011a). The resulting strains were routinely grown at 37 °C on 5 mL autoclaved LB medium (10 g/L tryptone, 5 g yeast extract/L, 5 g NaCl/L, pH 7.0) supplemented with 100 mg/L ampicillin. After approximately 9 h of incubation with continuous shaking at 200 rpm, one percent (v/v) of inoculum was added to a 2 L Erlenmeyer flask containing 500 mL autoclaved LB medium, supplemented with ampicillin to a final concentration of 100 mg/L. After overnight growth, the cells were harvested by centrifugation (10 000 rpm, 4 °C, 15 min) with a Sorvall RC-6+ centrifuge (Thermo Scientific). The obtained cell pellets were frozen and stored at -20 °C until further processing.
Strains were stored in 2 mL cryovials containing inoculum at -20 °C (months) or at -80 °C (years) in the presence of sterilized 70 % glycerol, which is used as a cryoprotectant (1:1 (v/v)).

1.2. Fermentation

For the production of large amounts of SP, a fermentation at 30 L scale was performed. To that end, an inoculum volume of 1 L was prepared, using the previous described method. Subsequently the culture was inoculated onto 16 L of double LB medium (20 g/L tryptone, 10 g yeast extract/L, 5 g NaCl/L, pH 7.0) and sterile supplemented with 1 L glucose solution containing ampicillin, in such a way that a concentration of 30 g/L glucose and 100 mg/L ampicillin was obtained in a 30 L Biostat C reactor (B. Braun Biotech). The temperature, stirring speed and aeration rate were set at 37 °C, 800 rpm and 1 vvm, respectively. The dissolved oxygen concentration was maintained above 40 %, by increasing the agitation rate from 800 to 1000 rpm after 3 h of fermentation. When necessary, anti-foam was added manually to prevent foaming. The pH was controlled automatically at 7.0 with 25 % NaOH and 1 M H_2SO_4. Optical density measurements were performed using 1 mL samples in discardable cuvettes in an UVIKON 922 spectrophotometer at 600 nm (OD_{600}). When a

sufficiently high OD_{600} value was obtained, cells were harvested by centrifugation as described in the previous section.

1.3. Enzyme purification

Since sucrose phosphorylase is produced intracellular by *E. coli* BL21 cells, several steps are required to obtain a purified enzyme solution.

1.3.1. Cell lysis

First of all, a pellet originating from a 500 mL culture was dissolved in 20 mL lysis buffer in order to break the cell walls and free the enzymes from the pellet. Lysis buffer contained 5 mM MOPS pH 7.0, 1 mg/mL lysozyme and 0.1 mM phenylmethylsulfonylfluoride. After 30 minutes of incubation on ice, the cells were exposed to 2 times 3 minutes of sonification at level 3 with a 50 % cycle time (Branson 250 Sonifier). Next, the lysate was centrifuged with a Heraeus Biofuge stratus centrifuge (15 000 rpm, 4 °C, 15 min) to remove cell debris, resulting in crude cell extract.

1.3.2. Heat purification

To further purify the enzyme, a heat treatment was applied since the target enzyme is highly thermostable (Cerdobbel *et al.*, 2010a). The crude cell extract was placed in a heat bath at 58 °C for 15 minutes. Afterwards, the denatured proteins were removed by centrifugation with a Heraeus Biofuge stratus centrifuge (15 000 rpm, 4 °C, 15 min). Eventually, the purified BaSP solution was stored at 4 °C until further use.

1.3.3. Ni-NTA affinity chromatography

If a high purity of the enzyme was required, purification by means of nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography was applied, since enzymes were constructed to have a 6xHis-tag. Following solutions were to be prepared in advance (Table III.1).

Table III.1: Composition of buffers used during Ni-NTA affinity chromatography.

Buffer	Composition
Lysis buffer	50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4 100 µM PMSF, 1mg/mL lysozyme
Equilibration buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4
Wash buffer	50 mM NaH_2PO_4, 300 mM NaCl, 35 mM imidazole, pH 7.4
Elution buffer	50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole, pH 7.4
MOPS buffer	50 mM MOPS, pH 7.0

Cell lysis was performed as described in Section III.1.3.1, whereby lysis buffer was in this case composed as depicted in Table III.1. For the purification of crude cell extract originating from 500 mL culture, a column containing 1 mL bed volume HisPurTM Ni-NTA resin (Thermo Scientific) was equilibrated with 4 mL equilibration buffer. The crude cell extract was applied and the column was subsequently washed with 3 x 4 mL wash buffer. Next, the enzyme was eluted using 3 x 1 mL elution buffer. To eliminate the denaturing effect of imidazole on the enzyme, the buffer was exchanged for a 50 mM MOPS buffer pH 7.0 by iterative centrifugation in a Centricon YM-30 centrifugal filter unit (Millipore) (MWCO 30 kDa) at 4500 rpm for 9 to 15 min. The Centricon YM-30 tube reduced the solutions volume from 4 mL to 250-1000 µL four or five times. In the end the final imidazole concentration was lowered about 4000x. The enzymes were then transferred to an Eppendorf tube and stored at 4 °C until further use.

2 Enzyme characterization

2.1. Protein concentration: BCA assay

The Pierce™ BCA Protein Assay Kit was used to determine the protein concentration. This assay is based on the reduction of Cu^{2+} to Cu^{1+} by proteins brought in an alkaline medium. The cuprous cation (Cu^{1+}) can be detected colorimetrically at 540 nm using a reagent containing bicinchoninic acid (BCA). A standard dilution series of bovine serum albumin (BSA) was prepared and the protocol as described in the kit was followed.

2.2. Enzyme activity

The activity of the wild type BaSP could be quantified by measuring the release of D-fructose with the discontinuous bicinchoninic acid (BCA) assay for reducing sugars using sucrose as the donor substrate (Waffenschmidt & Jaenicke, 1987). The latter can only be applied for purified enzyme solutions as other enzymatic reactions could also set free fructose (for example hydrolysis of sucrose), which could falsify the results.

First of all, the BCA working solution was prepared. This solution was only prepared when needed, since it could not be stored for longer than one week in the dark at room temperature. Solution A en B were to be prepared in advance according to the following:

Solution A

Solution A was prepared by adding 150 mg 4-4'-dicarboxy-2,2'-biquinoline to 100 mL MilliQ water. Next, 7.16 g of anhydrous Na_2CO_3 was added and diluted to 115 mL MilliQ water.

Solution B

Solution B was prepared by dissolving 3.5 g aspartic acid and 5 g anhydrous Na_2CO_3 in 100 mL MilliQ water. Next, 1.704 g $CuSO_4 \cdot 5H_2O$ was dissolved in 40 mL MilliQ water. Both solutions were mixed and MilliQ water is added to 150 mL.

Solutions A and B were stored at 4 °C. Finally, the working solution was prepared by adding 23 mL solution A, 1 mL solution B and 6 mL EtOH in a tinfoil wrapped scott flask.

The activity of the wild type BaSP was determined by adding 50 µL enzyme solution to 450 µL preheated substrate solution (0.1 M phosphate buffer, 0.1 M sucrose, pH 7.0) in a 1.7 mL Eppendorf tube, placed in a heating block at 37 °C. After homogenization, eight samples were taken at fixed intervals. For this purpose, 50 µL was added to a microtiterplate filled with 150 µL BCA solution. Due to the high pH of the BCA solution (pH 11.25), the enzyme denatures immediately, without the requirement for heat inactivation. A standard series of fructose was added and the plate was incubated for 30 min at 70 °C. During this time, the color formation reaction takes place. Afterwards, the absorbance was measured at 540 nm using a 680XR microplate reader spectrophotometer (Bio-Rad).

This enzyme test allowed plotting the absorbance of the samples at 540 nm in function of time (min). Also, the absorbance of the standard series was plotted in function of the concentration of fructose (µM). The ratio of the sample slope (mOD/min) and the reference slope (mOD/µM) resulted in the rate of fructose formed (µM/min). This value was then converted to enzyme units. One unit of SP activity corresponded to the release of 1 µmole fructose from 0.1 M sucrose in 0.1 M phosphate buffer at pH 7.0 and 37 °C.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was applied to measure enzyme activity of the mutants and determine parameters such as selectivity, conversion, yield, hydrolysis, efficiency and productivity.

2.3. Enzyme stability

The thermostability of the wild type and the mutants L341I, Q345S and L341I_Q345N was evaluated by incubating 1.7 mL Eppendorf tubes containing 0.5 mg/mL of Ni-NTA purified enzyme (diluted in 50 mM MOPS pH 7.0) in a water bath at 60 °C. Their residual activity was measured using the following assay: Reactions starting from 500 mM sucrose and 500 mM glucose were performed in the presence of the specific enzyme at a certain concentration, whereby a sample was taken at a certain time point and analyzed with HPAEC-PAD (Table III.2). The peak area of kojibiose was determined and the activity after 0 h incubation was set as 100 % relative activity.

Table III.2: Assay for the determination of the residual activity.

Enzyme	Concentration (mg/mL)	Time sample (h)
Wild type	0.250	6
L341I	0.125	1
Q345S	0.250	6
L341I_Q345S	0.250	3

Their half-life at 60 °C was determined by sampling at regular intervals until the residual activity had dropped to 50 %. The corresponding t_{50}-values were calculated from a first-order fit of the stability curve.

3 Production of kojibiose

3.1. Reaction setup

Reactions with glucose as acceptor and sucrose as donor were performed in MilliQ water at 55 °C. Small screenings were performed in 1.7 mL Eppendorf tubes placed in a thermoshaker at 1400 rpm, whereby both substrate and enzyme concentrations varied. Subsequently, reactions on medium scale were performed in 50 mL falcons with continuous shaking at 750 rpm. Upscaling of this reaction to liter scale was performed in a 2 L glass flask with magnetic stirring. The flask was embedded in silicone oil, providing temperature control. Finally, the reaction was scaled up to a volume of 10 L. This was performed in a 30 L Biostat C reactor (B. Braun Biotech). The temperature and stirring speed were set at 55 °C and 35 rpm, respectively. To further improve the efficiency of the reaction, glucose isomerase was added. This was kindly provided by Novozymes™ under the immobilized form, named Sweetzyme® IT Extra. Before inactivating the reaction for 10 min at 95 °C, glucose isomerase was removed using Seitz® K Series Depth Filter Sheets.

3.2. Downstream processing

3.2.1. Yeast treatment

In order to remove the contaminating carbohydrates (glucose, fructose, sucrose and maltose) in the reaction mixture, a yeast treatment was performed. Unless stated otherwise, 30 g/L spray dried baker's yeast *Saccharomyces Cerevisiae* from Algist Bruggeman was added to the reaction. The yeast treatment was performed at 30 °C for 6 to 12 h. Further, an industrial strain used for ethanol production (Ethanol Red®, Leaf Technologies) was tested on its ability to remove the contaminating carbohydrates. To that end, analogue experiments were performed with the use of 30 g/L spray dried Ethanol Red® at 30 °C.

The yeast treatment was monitored by HPAEC-PAD analysis. Therefore, 80 µL samples were spun down for 5 min at 14 000 rpm. 50 µL of the supernatant was inactivated with 450 µL 7.5 mM NaOH and subsequently placed on ice for 10 minutes. Next, particulates were removed by centrifugation (5 min, 14 000 rpm) and supernatant was adequately diluted in MilliQ water.

When all contaminating carbohydrates were metabolized, the yeast was removed by centrifugation (5000 rpm, 4 °C, 15 min) with a Sorvall RC-6+ centrifuge (Thermo Scientific). To remove any suspended solids, microfiltration was performed using a sterile vacuum filtration system (Corning Incorporated) with a pore size of 0.22 µm.

3.2.1.1. Ethanol collection

To quantify the amount of ethanol produced by the baker's yeast during the treatment, a 500 mL mixture obtained by reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S, was treated anaerobically with 30 g/L spray dried baker's yeast at 30 °C and low stirring speed (50 rpm). Samples were taken every 2 h and analyzed with HPLC.

3.2.2.Crystallization

After yeast treatment and microfiltration, the solution was evaporated by means of a rotavapor (Büchi R-200). The temperature and pressure were set at 50 °C and 50 mbar, respectively. The evaporation was allowed to proceed until the desired Brix value was obtained. The Brix value was determined by measuring the refractive index of the solution with an ATAGO handrefractometer. Subsequently the solution was cooled to room temperature. During this step, crystallization occurred. After overnight incubation at room temperature, the solution was placed on ice to further promote crystal growth. Finally, the crystals were washed with ethanol and dried to the air.

3.2.2.1. Microscopic appearance of the crystals

To map the microscopic appearance of the kojibiose crystals, a small amount of crystals was placed on a substrate glass, moistened with a drop of ethanol and covered with a microscopic cover glass. This was placed under a microscope (Olympus CH30) and pictures were taken at constant diaphragm light and zoom stand of the camera. For each Brix value a 100x and 400x magnification picture was taken.

3.2.2.2. ICUMSA score

The International Commission for Uniform Methods of Sugar Analysis (ICUMSA) has developed a method for characterizing the whiteness of carbohydrate crystals. It is based on the absorbance at 420 nm of a carbohydrate solution. This solution is prepared as a mixture of carbohydrates in MilliQ water. The carbohydrate score is then calculated as follows:

$$ICUMSA\ 420\ Carbohydrate\ Score = \frac{(Absorbance_{420nm}\ of\ carbohydrate\ solution * 1000)}{c * b}$$

Where

c = concentration of carbohydrate solution in g/mL

b = cell path length expressed in cm

By measuring its absorbance, the yellowness of the carbohydrate is calculated. The lower the ICUMSA score, the whither the carbohydrate.

To evaluate the whiteness of kojibiose crystals, 100 mg crystals were dissolved in 200 µL mQ water, resulting in a concentration of 0.5 g/mL. The absorbance was measured at 415 nm using a 680R microplate reader spectrophotometer (Bio-Rad). The measurement was performed in Thermo Scientific Nunc Edge 96-well Plates, whereof the cell path length was calculated as 0.57 cm.

4 Separation and quantification

4.1. HPAEC-PAD analysis

Reactions were monitored by a Dionex ICS-3000 Ion Chromatography system (Thermo Scientific) with a CarboPac PA20 pH-stable anion exchange column for carbohydrate separation, coupled to a Pulsed Amperometric Detection (PAD) system. Chromeleon software was used for data collection and analysis. All samples were analyzed at 30 °C with a fixed flow rate of 0.5 mL/min. The composition of the eluens was varied in time. Separation of all substrate and (potential) product peaks (trehalose, glucose, fructose, sucrose, isomaltose, kojibiose and maltose) was achieved with a 30 min method (Table III.3). After 13 min of isocratic elution with 30 mM NaOH, the concentration was gradually increased to 100 mM over 5 min, kept constant for 3 min and decreased again to 30 mM within 1 min, followed by an equilibration period of 8 min.

Table III.3: HPAEC-PAD method for the separation of samples originating from reactions for the production of kojibiose with BaSP and mutants. Variation of the ratio A:B is given as a function of time, where solution A is MilliQ water and solution B is 100 mM NaOH. The flow rate was constant at 0.5 mL/min.

Time (min)	A (%)	B (%)
0.0	70.0	30.0
13.0	70.0	30.0
18.0	0.0	100.0
21.0	0.0	100.0
22.0	70.0	30.0
30.0	70.0	30.0

Standards were prepared for trehalose, glucose, fructose, sucrose, isomaltose, kojibiose and maltose. Determination of the retention times allowed peak identification (Table III.4) while assembling standard curves allowed the quantification of the peaks.

Table III.4: Overview of the retention times of the carbohydrate separation with the 30 min method.

Peak	Retention time (min)
trehalose	2.0
glucose	6.1
fructose	7.2
sucrose	8.6
isomaltose	12.1
kojibiose	16.0
maltose	26.4

Reactions were monitored by taking samples at regular time intervals. After intensely mixing of the reaction mixture, 50 µL sample was inactivated in 450 µL of 7.5 mM NaOH and subsequently placed

on ice for 10 minutes. Next, particulates were removed by centrifugation (5 min, 14 000 rpm) and supernatant was adequately diluted in MilliQ water.

4.2. HPLC analysis

High Performance Liquid Chromatography (HPLC) analysis was used to quantify glycerol and ethanol peaks, since HPAEC-PAD analysis was not able to separate these peaks adequately. The HPLC system was composed of a Varian Prostar 410 autosampler, a Croco-cil oven (at 30 °C), a Varian Prostar 230 pump, a hydrogen based Aminex HPX-87H column from BIO-RAD Laboratories, and a Varian Prostar 320 UV/Vis detector. Elution was performed isocratically with MilliQ water containing 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Sampling was identical as described for HPAEC-PAD analysis.

CHAPTER IV
RESULTS AND DISCUSSION

1 Purification of recombinant SP

As indicated in the Literature Study, sucrose phosphorylase from *Bifidobacterium adolescentis* was the biocatalyst of interest in this research. Consequently *E. coli* BL21 cells, containing the constitutive expression plasmid pCXshP34_BaSP, were grown in Erlenmeyer flasks as described in Chapter III. Currently, two methods for purification of the enzyme are available: Ni-NTA affinity chromatography and heat purification. To evaluate these methods, the activity of the enzyme was measured before and after each purification method. The crude cell extract, obtained by treating 20 mL culture, yielded 3552 U of SP activity. After heat purification an activity of 3129 U was obtained, while Ni-NTA affinity chromatography resulted in only 428 U SP activity.

From these experiments, also the purification yield (Y_p) and purification level (L_p) could be calculated. The former is defined as the enzymatic activity to be found in the purified enzyme preparations compared to the activity present in the crude cell extract, while the latter expresses to what extent the purified enzyme preparations have a higher specific activity compared to the crude cell extract (Table IV.1).

Table IV.1: Heat and Ni-NTA purification of the wild type BaSP. Enzyme activity was calculated by measuring the release of D-fructose with the discontinuous bicinchoninic acid (BCA) assay for reducing sugars, while the Pierce™ BCA Protein Assay Kit was used to determine the protein concentration (Chapter III).

	Total activity (U)	Specific activity (U/mg)	Y_p (%)	L_p
Crude cell extract	3552	12.8	-	-
Heat purification	3129	17.5	88.1	1.4
Ni-NTA	428	98.9	12.1	7.7

Heat purification was found to retain roughly 88 % of the SP activity, although 5.5 times less pure enzyme preparations were obtained compared to Ni-NTA purification. Since the latter yields only 12.1 % of the total SP activity, heat purification is the purification method of choice. This technique yields a sufficiently pure enzyme preparation while avoiding the labour-intensive Ni-NTA chromatography.

To investigate the performance of the wild type BaSP towards the production of kojibiose, a reaction containing 500 mM sucrose, 500 mM glucose and 2 mg/mL enzyme diluted in 50 mM MOPS buffer pH 7.0 was performed at 55 °C. After 16 h, approximately 100 mM kojibiose and 200 mM maltose were formed. The fructose concentration was found to be 500 mM which indicates the occurrence of hydrolysis. Indeed, fructose is released during the formation of maltose and kojibiose in equimolar amounts, while hydrolysis of sucrose results in an additional release of fructose.

In accordance with the results of Verhaeghe (Verhaeghe, 2014), kojibiose accounted for only 33 % of the formed glucobioses. The overall kojibiose yield was thus strongly lowered by the formation of maltose, which amounted to 67 % of the total product formation. For this reason, enzyme engineering was previously applied to enhance the specificity of BaSP towards the production of kojibiose. The research of Verhaeghe resulted in the creation of four mutant enzymes with improved properties towards the production of kojibiose: L341I, L341I_Q345S, L341I_Q345N and L341I_Y344A_Q345N (Table II.3). The goal of the following section was to decide which enzyme to use for the development of the kojibiose production process. Furthermore, suitable enzyme and substrate concentrations were determined.

2 Enzyme selection

2.1. Turn over

The kojibiose synthesis potential of the previously described mutants was evaluated by reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL enzyme at 55 °C (Figure IV.1).

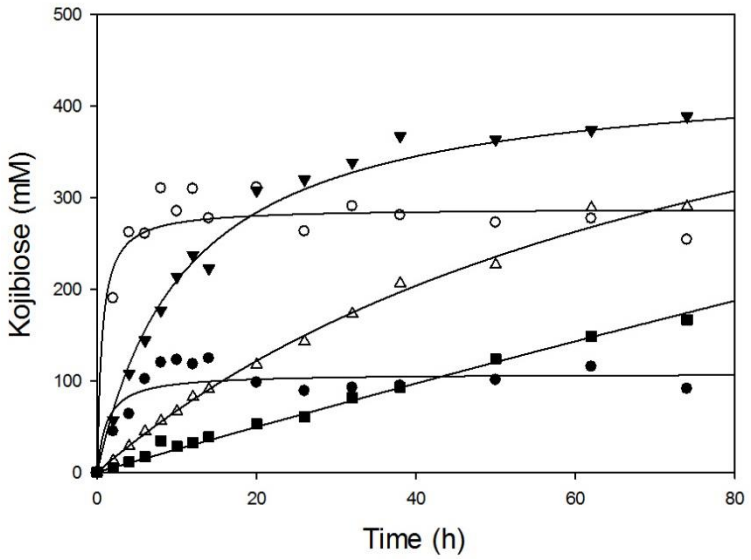


Figure IV.1: Synthesis of kojibiose with various mutants at 55 °C. The reactions were performed in a 50 mM MOPS buffer at pH 7.0 containing 500 mM sucrose, 500 mM glucose and 2 mg/mL wild type (●), L341I (○), L341I_Q345S (▼), L341I_Q345N (△), and L341I_Y344A_Q345N (■) SP.

The graph reveals that the L341I mutant is the fastest enzyme for the synthesis of kojibiose. The wild type enzyme has a velocity close to the L341I_Q345S mutant. These three reactions reached their equilibrium in the timeframe of sampling, while reactions with the L341I_Q345N and the L341I_Y344A_Q345N mutant did not. The latter is clearly the slowest enzyme, which is in accordance with the results of Verhaeghe (Verhaeghe, 2014). The wild type enzyme reached a kojibiose concentration of only 100 mM. Interestingly, the L341I mutant formed 250 mM kojibiose, while the L341I_Q345S mutant reached a kojibiose concentration of 350 mM. These mutual differences are the result of differences in selectivity and hydrolysis, which is discussed in the following section.

2.2. Other characteristics

To further evaluate the performance of the various mutants, an experiment was set up in which all mutants were analyzed under different substrate and enzyme concentrations. To this end, 35 reactions were prepared (Table IV.2). Samples were taken every 2 h and subsequently analyzed with HPAEC-PAD. Six parameters were calculated according to the formulas below: selectivity, conversion, yield, hydrolysis, efficiency and productivity.

$$Selectivity\ (\%) = \frac{[kojibiose]}{[kojibiose] + [maltose] + [isomaltose]} * 100$$

$$Conversion\ (\%) = \left(1 - \frac{[sucrose]}{[sucrose]_0}\right) * 100$$

$$Yield\ (\%) = \frac{[kojibiose]}{[sucrose]_0} * 100$$

$$Hydrolysis\ (\%) = \frac{[fructose] - [kojibiose] - [maltose] - [isomaltose]}{[fructose]} * 100$$

$$Efficiency\ (\%) = \frac{[kojibiose]}{[fructose]} * 100$$

$$Productivity\ (\frac{g\ kojibiose}{g\ protein * h}) = \frac{[kojibiose\ (M)] * 342.3\frac{g}{mol}}{\left[protein\ (\frac{g}{L})\right] * time\ (h)}$$

Hereby $sucrose_0$ represents the initial sucrose concentration. Values for each of these parameters after 8 h of reaction can be found in Table IV.2. Previously, substrate inhibition was reported for the L341I_Q345S and the L341I_Q345N mutant when reacting 200 mM sucrose with glucose concentrations above 500 mM (Verhaeghe, 2014). However, under production conditions no such effects were observed. The selectivity of all mutants was found to be higher than 75 %, implicating a major improvement compared to the wild type. Indeed, the other parameters showed strong mutual differences between the various mutants, while also the reaction conditions seemed to significantly influence the outcome of the reactions. For a good interpretation of the data, average values for the parameters were calculated from all conditions with the same enzyme (Table IV.3).

Table IV.2: Experimental design and determination of selectivity, conversion, yield, hydrolysis, efficiency and productivity for various mutant enzymes under different substrate and enzyme concentrations. Values for the parameters were calculated according to the previously described formulas after 8 h of reaction at 55 °C.

Reaction	Concentration glucose (mM)	Concentration sucrose (mM)	Enzyme concentration (mg/mL)	Enzyme	Selectivity (%)	Conversion (%)	Yield (%)	Hydrolysis (%)	Efficiency (%)	Productivity (g kojibiose/(g protein*h))
1	100	100	1	L341I	86.1	100.0	34.7	66.8	28.6	1.5
2	250	250	1	L341I	81.4	100.0	47.3	39.3	49.5	5.1
3	500	500	1	L341I	78.8	95.9	49.8	33.8	52.2	10.7
4	250	500	1	L341I	79.1	93.8	36.3	49.1	40.3	7.8
5	1000	1000	1	L341I	77.5	67.9	38.1	17.2	64.2	16.3
6	100	100	3	L341I	85.2	100.0	33.8	67.6	27.6	0.5
7	250	250	3	L341I	80.7	100.0	47.6	43.3	45.7	1.7
8	500	500	3	L341I	77.3	100.0	58.0	10.2	69.4	4.1
9	250	500	3	L341I	76.2	100.0	41.2	22.2	59.2	2.9
10	1000	1000	3	L341I	74.4	100.0	47.7	0.0	78.1	6.8
11	100	100	1	L341I_Q354S	98.7	99.7	63.1	64.0	35.6	2.7
12	250	250	1	L341I_Q354S	97.6	43.4	45.0	37.0	61.5	4.8
13	500	500	1	L341I_Q354S	96.8	31.4	36.5	24.7	72.9	7.8
14	250	500	1	L341I_Q354S	97.3	35.5	25.8	36.4	61.9	5.5
15	1000	1000	1	L341I_Q354S	96.2	47.1	29.0	13.5	83.2	12.4
16	100	100	3	L341I_Q354S	99.0	100.0	52.3	47.7	51.8	0.7
17	250	250	3	L341I_Q354S	97.6	99.9	68.0	31.7	66.7	2.4
18	500	500	3	L341I_Q354S	96.0	78.6	71.5	17.7	79.0	5.1
19	250	500	3	L341I_Q354S	96.1	69.3	63.0	29.1	68.1	4.5
20	1000	1000	3	L341I_Q354S	95.4	73.7	56.7	5.1	90.6	8.1
21	100	100	2	L341I_Q354N	100.0	61.5	25.4	43.4	56.6	0.5
22	250	250	2	L341I_Q354N	100.0	14.9	19.1	86.5	13.5	1.0
23	500	500	2	L341I_Q354N	97.5	19.2	17.2	0.0	100.0	1.8
24	250	500	2	L341I_Q354N	98.7	24.9	13.0	43.0	56.3	1.4
25	1000	1000	2	L341I_Q354N	96.6	42.7	16.5	22.4	75.0	3.5
26	100	100	4	L341I_Q354N	100.0	85.3	34.7	48.2	51.8	0.4
27	250	250	4	L341I_Q354N	99.4	37.0	28.5	40.3	59.3	0.8
28	500	500	4	L341I_Q354N	98.0	34.2	24.0	28.9	69.7	1.3
29	250	500	4	L341I_Q354N	97.7	29.8	21.7	39.2	59.4	1.2
30	1000	1000	4	L341I_Q354N	95.5	37.4	34.2	12.0	84.0	3.7
31	100	100	4	L341I_Y344A_Q354N	100.0	39.3	11.4	40.9	59.1	0.1
32	250	250	4	L341I_Y344A_Q354N	100.0	13.9	7.4	24.7	75.3	0.2
33	500	500	4	L341I_Y344A_Q354N	100.0	14.7	8.7	0.0	100.0	0.5
34	250	500	4	L341I_Y344A_Q354N	100.0	17.1	6.4	23.2	76.8	0.3
35	1000	1000	4	L341I_Y344A_Q354N	100.0	59.4	3.1	22.1	77.9	0.3

Table IV.3: Average values for selectivity, conversion, yield, hydrolysis, efficiency and productivity from all conditions depicted in Table IV.2 with the same enzyme.

Enzyme	Selectivity (%)	Conversion (%)	Yield (%)	Hydrolysis (%)	Efficiency (%)	Productivity (g kojibiose/(g protein*h))
L341I	79.7	95.7	43.5	34.5	51.5	5.7
L341I_Q345S	97.1	67.8	51.1	30.7	67.1	5.4
L341II_Q345N	98.3	38.7	23.4	36.0	62.6	1.6
L341I_Y344A_Q345N	100.0	28.9	7.4	19.5	77.8	0.3

The triple mutant, L341I_Y344A_Q345N, displayed an average selectivity of 100.0 %. This implies an exclusively formation of kojibiose as glucobiose. In addition, the percentage hydrolysis was found to be only 19.5 % - the lowest value of all mutants - indicating an efficient use of the substrate. This was reflected in an average efficiency of 77.8 %. Unfortunately, this mutant suffered from low production rates (Section IV.2.1). After 8 h, the conversion was found to be 28.9 % and a kojibiose yield of only 7.4 % was obtained. For this reason, it was decided to eliminate this mutant. In contrast, the single mutant, L341I, reached an average conversion of 95.7 %, but the selectivity was found to be only 79.7 %. To avoid time-consuming purification steps and the loss of efficiency due to the formation of side products, also this mutant was eliminated. The L341I_Q345S and the L341I_Q345N mutants displayed the best balance between selectivity and activity. However, considering the difference in yield and productivity, the choice for the L341I_Q345S mutant was obvious. After 8 h, 67.8 % of the donor substrate was converted, resulting in a kojibiose yield of 51.1 %. The percentage hydrolysis was found to be 30.7 %, while a productivity of 5.4 gram kojibiose per gram protein per hour was reached after 8 h. In conclusion, the L341I_Q345S mutant is able to produce satisfying amounts kojibiose in a reasonable timeframe, whereby on average a selectivity of 97.1 % is reached.

Next, the effect of the initial sucrose concentration on the parameters hydrolysis and selectivity for the L341I_Q345S and the L341I_Q345N mutant was scrutinized (Figure IV.2). A decrease in hydrolysis with increasing sucrose concentrations was observed, while the selectivity remained roughly stable.

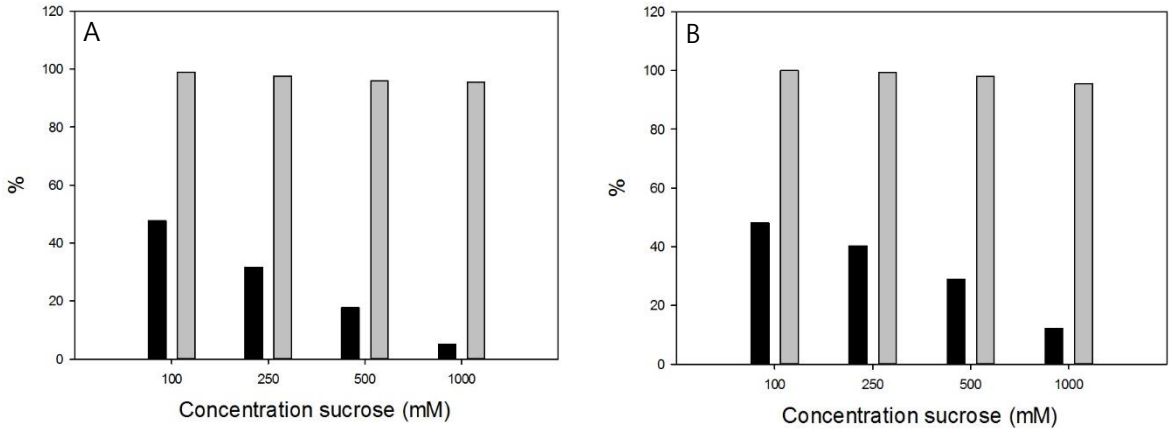


Figure IV.2: Percentage hydrolysis (black) and selectivity (grey) in function of the sucrose concentration from reactions performed in a 50 mM MOPS buffer at pH 7.0 supplemented with a certain concentration sucrose, the same amount glucose and 3 mg/mL BaSP_L341I_Q345S (A) or 4 mg/mL BaSP_L341I_Q345N (B) at 55 °C.

Although hydrolysis was lower upon using 1000 mM sucrose, further experiments were performed with 500 mM sucrose, which reduces hydrolysis while avoiding high viscosity related to elevated sucrose concentrations.

2.3. Production of recombinant BaSP_L341I_Q345S

In a next step, the enzyme BaSP_L341I_Q345S was produced at large scale in a 30 L fermentor, as described more detailed in Chapter III. After approximately 8 h of fermentation an OD_{600} of 35 was reached (Table IV.4) and the cells were harvested by centrifugation.

Table IV.4: OD_{600} values measured during the fermentation. OD was measured with an UVIKON 922 spectrophotometer at 600 nm. Every measurement was preceded by a blank measurement with MilliQ water.

Time (h)	OD_{600}
0	0.9
2	3.0
5.5	26.5
6.5	29.9
7.5	34.1

2.4. Stability

Although enzyme variants with increased selectivity were revealed, altering the structure of biocatalysts is known to influence their stability (Khan & Vihinen, 2010). Therefore, the thermostability of mutant L341I_Q345S was evaluated. When incubating the enzyme at 55 °C, no decrease in activity was observed after 96 h. However, at 60 °C a significant difference in thermostability was observed. The half-life (t_{50}) of the mutant, defined as the time at which the relative activity amounts to 50 % of the initial activity, was found to be only 70 % of the wild type's t_{50}. The double mutation resulted thus in a decrease in thermostability. In order to find out whether this decrease was caused by the L341I or the Q345S mutation, a similar experiment was performed with the L341I and the Q345S mutant (Figure IV.3).

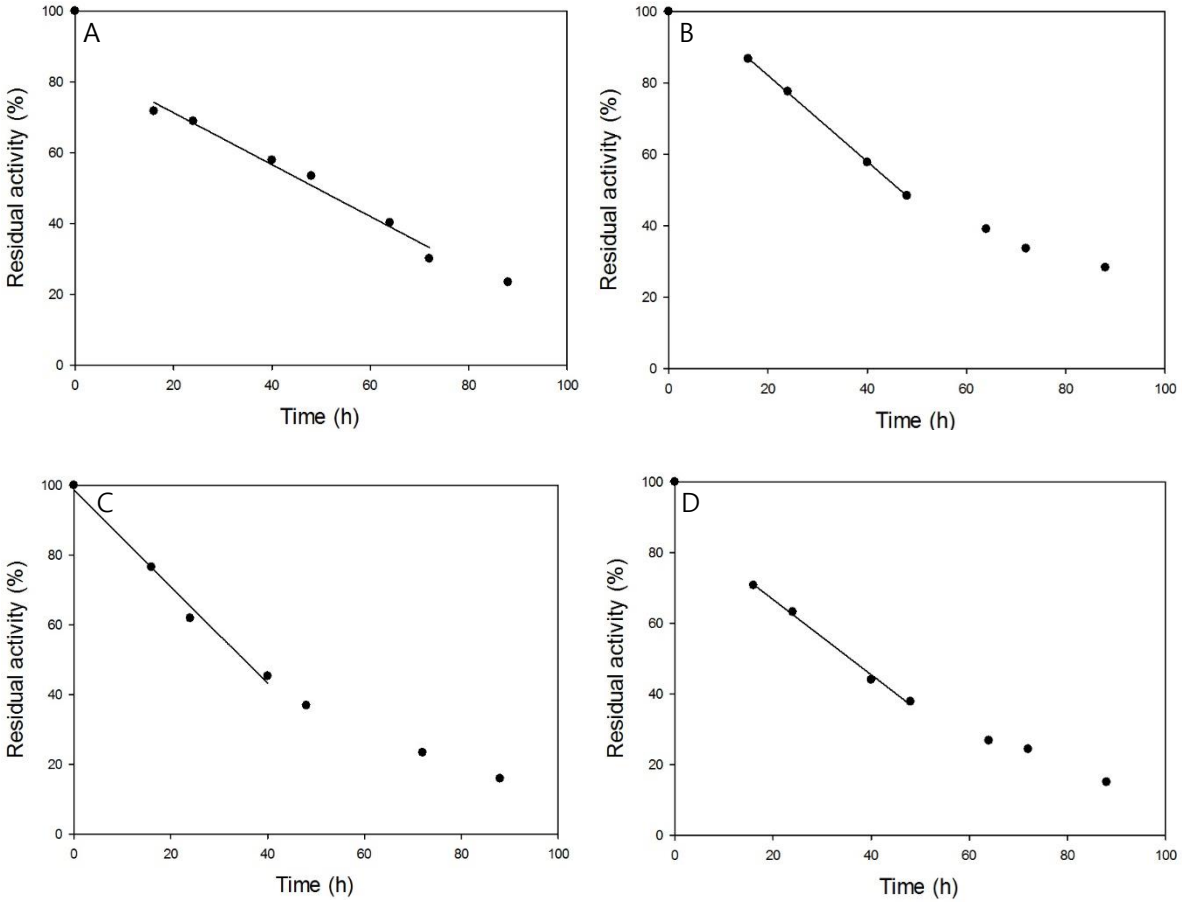


Figure IV.3: Stability curves at 60 °C accompanied by the linear part of the corresponding first-order fit for the wild type (A), L341I (B), Q345S (C) and L341I_Q345S (D). Residual activity was measured as described in Chapter III. The activity after 0 h incubation was set as 100 % relative activity.

In a next step, the t_{50}-value of each enzyme was calculated from the first-order fit of the linear part of the corresponding stability curve (Table IV.5).

Table IV.5: Half-life (t_{50}) values of wild type and mutants at 60 °C, calculated from a first-order fit of the linear part of the corresponding stability curve of which the R^2 value is given.

Enzyme	t_{50} (h)	R^2
Wild type	49.0	0.980
L341I	46.6	0.999
Q345S	35.1	0.989
L341I_Q345S	35.7	0.996

Table IV.5 reveals that the decrease in thermostability of the L341I_Q345S mutant is caused by the Q345S mutation. From these results, it was decided to set the optimal reaction temperature at 55 °C. Indeed, at this temperature, the mutant does not suffer from activity decrease, while the probability for microbial contamination is minimized.

2.5. Enzyme concentration

From Section IV.2.1, it is known that an enzyme concentration of 2 mg/mL BaSP_L341I_Q345S results in a kojibiose concentration of 350 mM after approximately 24 h upon reacting 500 mM sucrose with 500 mM glucose at 55 °C. In this section it was investigated if lower enzyme concentrations lead to satisfying amounts kojibiose in a realistic timeframe.

Four reactions were performed starting from 500 mM sucrose and 500 mM glucose. Enzyme was added in a concentration of 1 mg/mL, 0.5 mg/ml, 0.25 mg/mL and 0.1 mg/mL, respectively. The reactions were monitored during 9 days by taking samples every 24 h followed by HPAEC-PAD analysis (Figure IV.4).

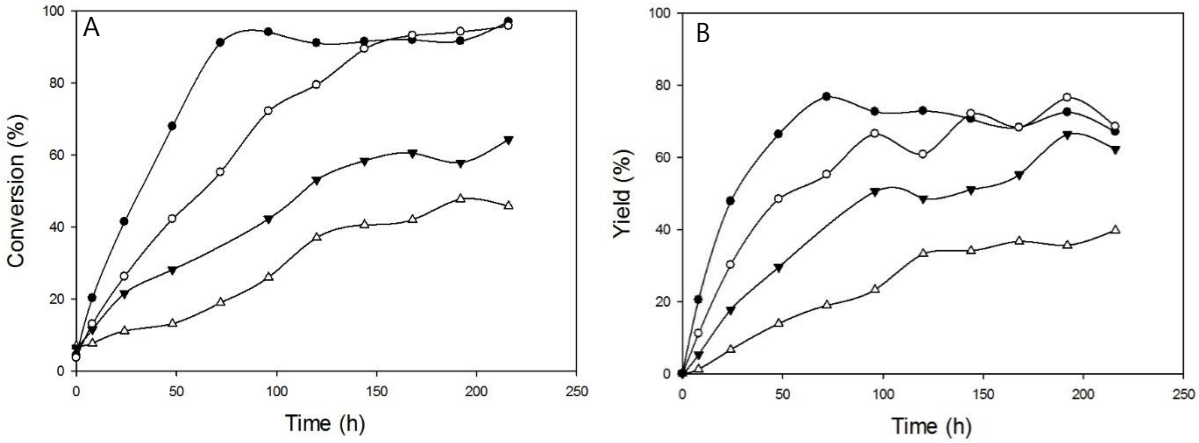


Figure IV.4: Conversion (A) and yield (B) in function of time. Reactions were performed at 55 °C in a 50 mM MOPS buffer at pH 7.0 containing 500 mM sucrose, 500 mM glucose and 1.0 mg/mL (●), 0.5 mg/mL (○), 0.25 mg/mL (▼) and 0.1 mg/mL (△) BaSP_L341I_Q345S.

An enzyme concentration of 1 mg/mL resulted in an acceptable conversion of 91 % after 72 h. In contrast, only 46 % and 64 % conversion was reached after 216 h in the case of 0.1 mg/mL and 0.25 mg/mL enzyme, respectively. The latter enzyme concentrations were eliminated since reactions could not be performed at a reasonable timescale. When using 0.5 mg/mL, the same level of conversion was obtained as with 1 mg/mL, although requiring a doubling in reaction time (144 h). Figure IV.4.B displays a yield of 77 % using 1 mg/mL after 72 h, while a yield of 72 % was obtained using 0.5 mg/mL after 144 h. This corresponds with a kojibiose concentration of 380 and 360 mM, respectively. The percentage hydrolysis was similar for all 4 enzyme concentrations, fluctuating around 20 %, while a selectivity of approximately 94 % was calculated for each enzyme concentration, barely varying in function of time.

In conclusion, comparable amounts of kojibiose were obtained using 1 and 0.5 mg/mL as when using 2 mg/mL enzyme, although requiring more time. Interestingly, higher productivities were reached when using lower enzyme concentrations (Figure IV.5). Therefore, a balance has to be found between prolonged reaction times, and lower productivities. Indeed, using 2 mg/mL enzyme required only 24 to 32 h to drive the reaction to completion, while with 1 mg/mL and 0.5 mg/mL,

72 and 144 h were necessary, respectively. From a practical point of view, it was decided to continue with 2 mg/mL enzyme, although it was shown that the concentration of enzyme could be reduced at the expense of increased reaction durations.

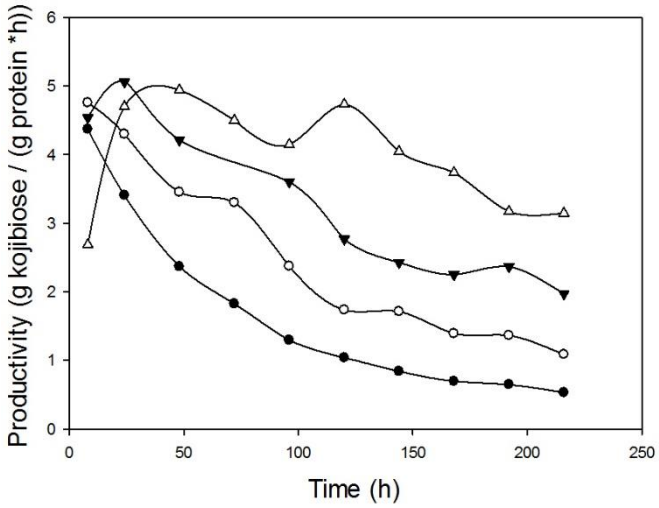


Figure IV.5: Productivity in function of time. The reactions were performed at 55 °C in a 50 mM MOPS buffer at pH 7.0 containing 500 mM sucrose, 500 mM glucose and 1.0 mg/mL (●), 0.5 mg/mL (○), 0.25 mg/mL (▼) and 0.1 mg/mL (△) BaSP_L341I_Q345S.

3 Downstream processing

3.1. Yeast treatment

The goal of this purification step was to remove the contaminating carbohydrates. As indicated in the Literature Study, the baker's yeast *Saccharomyces cerevisiae* is able to metabolize the latter with formation of CO_2 and ethanol. The aspect that is of utmost importance is whether the yeast also breaks down kojibiose or not.

3.1.1. Yeast formulation

A small test was performed to decide which formulation of baker's yeast to use: fresh or spray dried. To a reaction containing 50 mM kojibiose, 52 mM glucose and 86 mM fructose, yeast in a concentration of 20 g/L was added, once in fresh form, once as the spray dried formulation. The yeast originated in both cases from Algist Bruggeman. Samples were taken after 2 and 4 h and subsequently analyzed with HPAEC-PAD (Figure IV.6). Both treatments were performed at 30 °C.

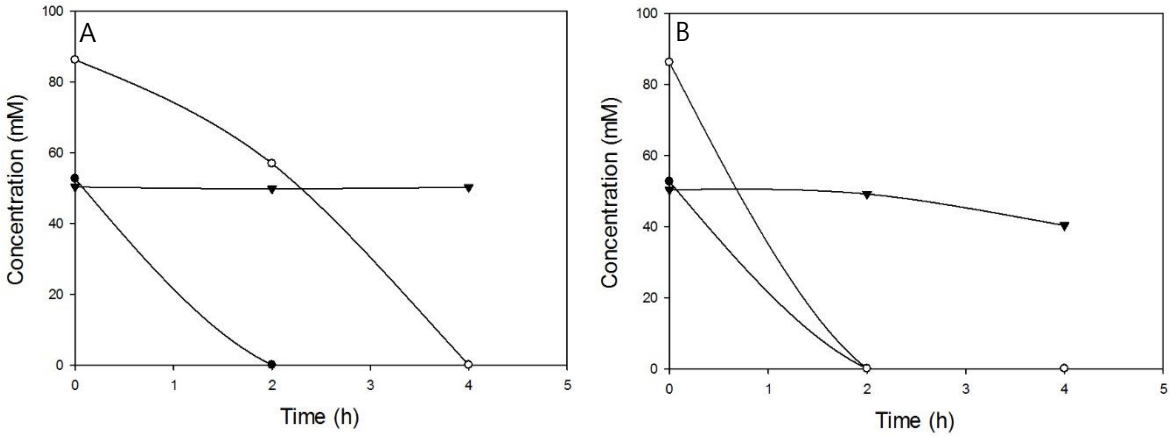


Figure IV.6: Concentration glucose (●), fructose (○) and kojibiose (▼) in function of time after adding 20 g/L fresh baker's yeast (A) or spray dried baker's yeast (B) to a reaction mixture containing 52 mM glucose, 86 mM fructose and 50 mM kojibiose at a temperature of 30 °C.

Using spray dried yeast resulted in a completely removal of glucose and fructose after 2 h. Interestingly, although relatively slow, breakdown of kojibiose was observed after depletion of glucose and fructose. Upon treatment with the fresh yeast, glucose was metabolized after 2 h. In contrast, fructose was only completely removed after 4 h. These results are logical, since the added concentration was based on mass, fresh yeast containing more water than spray dried yeast. Spray dried yeast is however more straightforward in practical use. For the rest of this research, the spray dried formulation of the baker's yeast *Saccharomyces cerevisiae* was used.

3.1.2.Optimization

Since it was shown that baker's yeast is able to remove the monosaccharides without metabolizing kojibiose, yeast treatment seems an attractive DSP technique, enabling a cheap and efficient purification. To dertermine the conditions resulting in an optimal carbohydrate removal, a reaction mixture obtained by reacting 1000 mM sucrose with 1000 mM glucose in the presence of 3 mg/mL BaSP_L341I_Q345S at 55 °C was prepared. The mixture contained 350 mM glucose, 850 mM fructose, 50 mM sucrose, 680 mM kojibiose and 40 mM maltose. Hereof a two and four times dilution was prepared. Since it was shown that pH influences the growth of baker's yeast (Al-Mudhaffer, 1978), the effect of pH was also investigated. To that end, each of these fractions was subsequently divided in two, whereby the pH was set at 7.0 and 5.0 by means of MilliQ water and KOAC buffer, respectively. To each of these six fractions 30 g/L spray dried yeast was added. Samples were taken at regular time intervals and analyzed with HPAEC-PAD (Figure IV.7).

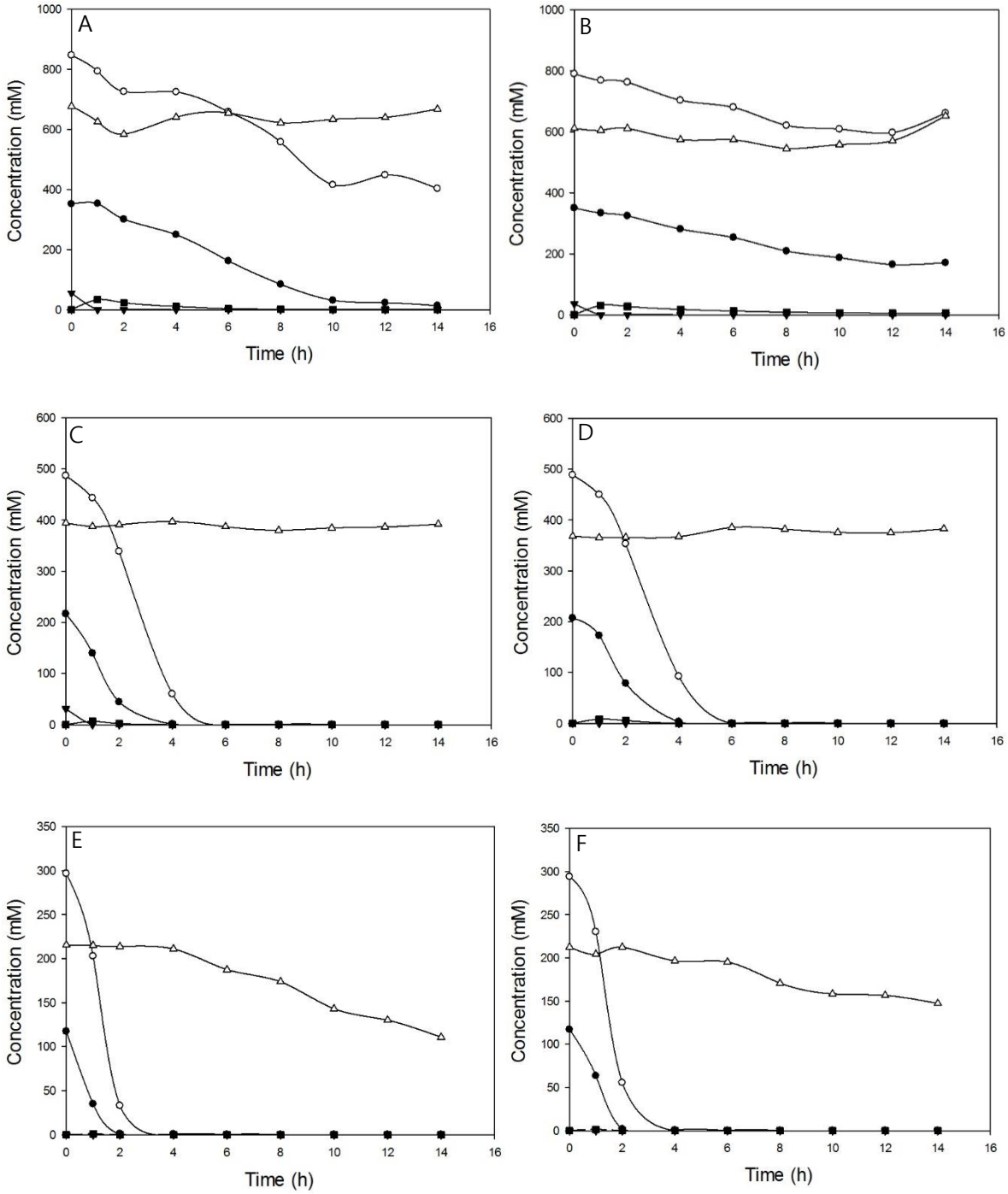


Figure IV.7: Concentration glucose (●), fructose (○), sucrose (▼), kojibiose (△) and maltose (■) in function of time after adding 30 g/L spray dried yeast to a reaction containing 350 mM glucose, 850 mM fructose, 50 mM sucrose, 680 mM kojibiose and 40 mM maltose. A: undiluted pH 7.0; B: undiluted pH 5.0; C: 2x dilution pH 7.0; D: 2x dilution pH 5.0; E: 4x dilution pH 7.0; F: 4x dilution pH 5.0. All treatments were performed at 30 °C.

Considering the graphs of the undiluted fraction (Figure IV.7 A and B), it is deduced that the yeast suffered from osmotic stress due to high carbohydrate concentrations. After 14 h glucose and fructose were still present in large amounts. In contrast, a two times dilution resulted in the removal of glucose and fructose after 4 and 6 h, respectively (Figure IV.7 C and D). Sucrose was immediately converted into its monosaccharides glucose and fructose by the yeast's extracellular invertase enzyme. Interestingly, maltose was also broken down, while kojibiose remained unaffected,

regardless their similar structure. The four times dilution showed a completed glucose removal after 2 h, while fructose was removed after 4 h (Figure IV.7 E and F). However, upon depletion of glucose and fructose, the yeast started to metabolize kojibiose. Considering the two and four times dilutions, it can be concluded that the effect of the pH was insignificant, since fructose and glucose were removed at exactly the same moment (Figure IV.7 C, D, E and F).

To predict the effect of the contaminating carbohydrates on the yeast treatment purification step, the concept of Glucose Equivalents (GE) was introduced. Hereby, 1 mM glucose or fructose accounts for 1 GE, while 1 mM sucrose or maltose accounts for 2 GE. The sum of the Glucose Equivalents was calculated for each condition at the beginning of the yeast treatment (Table IV.6).

Table IV.6: Sum of the Glucose Equivalents at the beginning of the yeast treatment under the conditions depicted in Figure IV.7. For the calculation of the GE, 1 mM glucose or fructose is considered as 1 GE, while 1 mM sucrose or maltose accounts for 2 GE.

	pH 5.0	pH 7.0
undiluted	1212	1308
2x dilution	696	766
4x dilution	411	414

Considering Figure IV.7 and Table IV.6, it can be concluded that the yeast suffers from significant osmotic stress when the sum of the GE amounts to 1212. However, at a total GE of 766, the yeast is capable of removing the contaminating carbohydrates in a timeframe of 6 h. Further experiments showed that the sum of the GE should not exceed 1000 in order to efficiently remove the contaminating carbohydrates by means of yeast treatment.

Another effect that could be noticed from Figure IV.7, relates to the breakdown of kojibiose. Remarkably, yeast metabolizes kojibiose faster under conditions of lower carbohydrate concentrations, comparing Figure IV.7 C and D to Figure IV.7 E and F. Even though this is in contrast with our expectations, this effect is beneficial for further research and production on large scale. Indeed, working at higher concentrations not only results in higher amounts of kojibiose, also a bigger safety margin can be incorporated. From these results, the condition of a two times dilution seems the most attractive. Since the starting point was 1000 mM substrate, a two times dilution corresponds approximately to a substrate concentration of 500 mM. In this case, high amounts of kojibiose are synthesized, while the contaminating carbohydrates are efficiently removed.

In conclusion, at the end of the yeast treatment a solution containing mainly kojibiose is obtained. A factor that however needs to be considered is the production of stress metabolites by the yeast during the purification step. On the HPAEC-PAD chromatogram (Figure IV.8), two unknown peaks were detected eluting after respectively 1.1 and 2.0 minutes. While the former peak overlaps with the injection signal, the peak at 2.00 corresponds to trehalose. Since the latter peak was already observed in the beginning of the yeast treatment, while no trehalose was detected at the end of

the reaction, it could be presumed that trehalose was present in the formulation of the spray dried yeast. Due to the overlap with the injection peak, samples were also subjected to HPLC analysis.

From these results, it was concluded that the unknown peak referred to the presence of glycerol, ethanol and MOPS. MOPS is present in the sample because the enzymatic reaction was diluted in a 50 mM MOPS buffer at pH 7.0. The latter could easily be replaced by MilliQ water. The presence of ethanol was predictable, considering the metabolic pathway of *Saccharomyces Cerevisiae* (Section II.4.2.2). An interesting option hereby, would be the distillation of ethanol out of the reaction mixture, as ethanol would be a valuable side product, rather than a waste product. Glycerol however, could hamper the final purification step - crystallization - and was therefore studied in detail (Section IV.3.2).

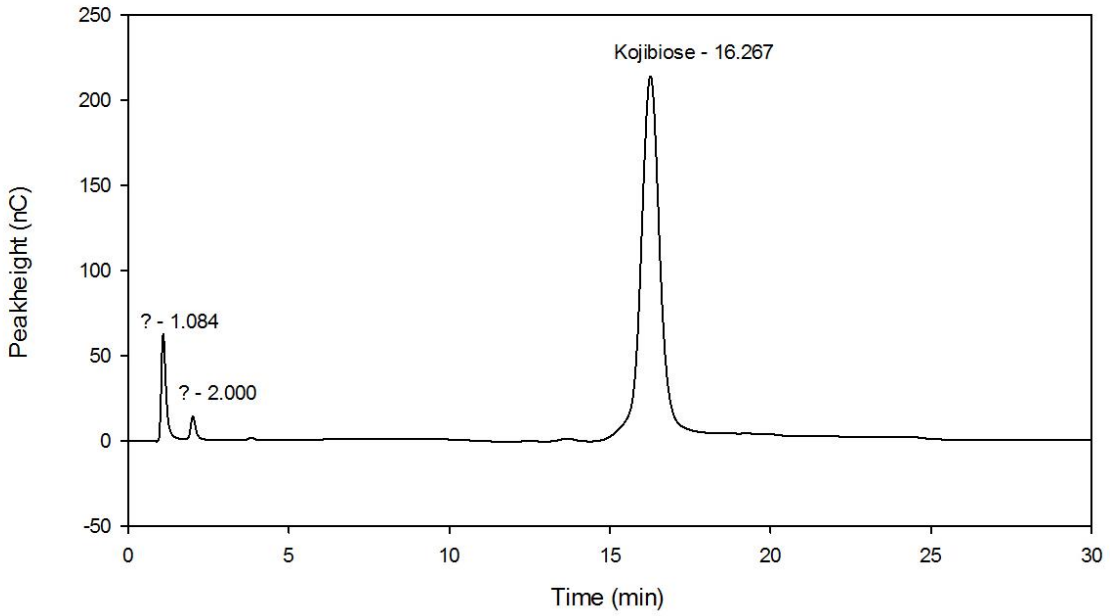


Figure IV.8: HPAEC-PAD chromatogram from a sample at the end of the yeast treatment of a reaction starting from 500 mM sucrose, 500 mM glucose and 2 mg/mL BaSP_L341I_Q345S. Spray dried baker's yeast in a concentration of 30 g/L was added at 30 °C after inactivation of the reaction mixture.

3.1.3.Ethanol production

Considering the collection of the ethanol formed during the yeast treatment via distillation, high concentrations of ethanol are desirable. Therefore, a comparison was made between the common baker's yeast and an industrial strain of *Saccharomyces cerevisiae* used for ethanol production (Ethanol Red®). A reaction mixture obtained by reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S for 24 h, was divided in two fractions. One fraction was treated with 30 g/L spray dried baker's yeast, while the other was treated with the same concentration of spray dried Ethanol Red®. Both treatments were performed at 30 °C. Samples were taken every 2 h and analyzed with HPAEC-PAD (Figure IV.9).

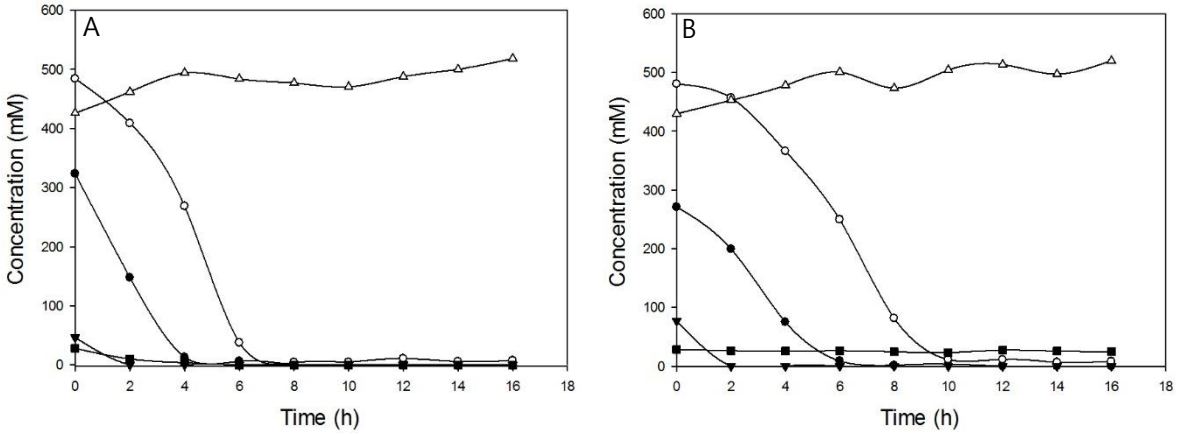


Figure IV.9: Concentration glucose (●), fructose (○), sucrose (▼), kojibiose (△) and maltose (■) in function of time after adding 30 g/L baker's yeast (A) or Ethanol Red® (B) to a reaction mixture obtained by reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S for 24 h.

Although both yeasts performed well, a slower removal of the contaminating carbohydrates was observed upon using Ethanol Red®. The addition of the latter resulted in the removal of glucose and fructose after 6 and 10 h, respectively, while with baker's yeast, glucose was already metabolized after 4 h, fructose after 6 h. Nevertheless, the main drawback of Ethanol Red® is the fact that the maltose concentration remained constant throughout the treatment (Figure IV.9.B). Apparently, this strain is not able to metabolize maltose. In contrast, baker's yeast consumed maltose completely after 6 h. This problem could be circumvented by the addition of maltase to the reaction, but since the cost of this enzyme would probably exceed the value of ethanol, it was decided not to work further with Ethanol Red®.

In a next step, the amount of ethanol produced by the baker's yeast during the treatment was quantified (Figure IV.10).

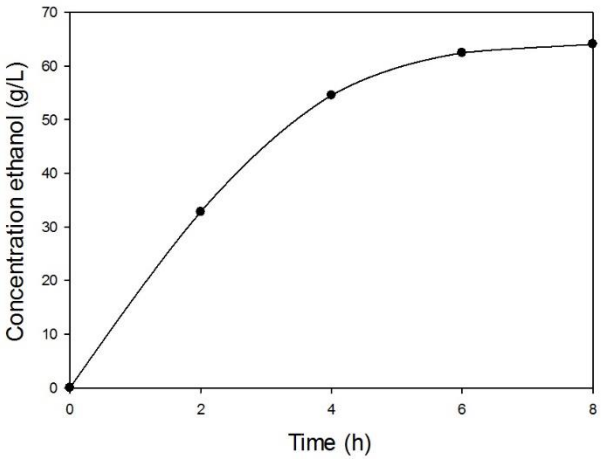


Figure IV.10: Production of ethanol during the yeast treatment of a reaction starting from 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S.

8 h of yeast treatment resulted in an ethanol concentration of 64 g/L. This result illustrates the usefulness of ethanol collection. Indeed reasonable amounts of ethanol are obtained - comparable with those produced by industrial strains for ethanol production (Chen *et al.*, 2008) – while the yeast was able to remove the contaminating carbohydrates adequately. In conclusion, yeast treatment provides an extremely efficient and cheap way to purify the reaction mixture while moreover, the collection of ethanol produced by the yeast, was proven to be useful.

3.2. Crystallization

Yeast treatment resulted in the removal of contaminating carbohydrates. Consequently a solution containing mainly kojibiose was obtained, despite some minor amounts of yeast metabolites - glycerol and ethanol - and trehalose. The yeast was removed by centrifugation and the solution was subjected to a microfiltration as described in Chapter III. As final purification step, this solution was crystallized. To that end, and evaporative cooling crystallization was designed: the solution was first evaporated to a certain Brix, whereafter the temperature was slowly decreased from 50 °C to 4 °C.

3.2.1.Relation between degrees Brix and concentration kojibiose

To assign the concentration kojibiose present in a solution from which the Brix value is known, a standard curve was composed. To that end, various solutions were evaporated to a certain Brix, whereof the concentration kojibiose was determined via HPAEC-PAD analysis (Figure IV.11).

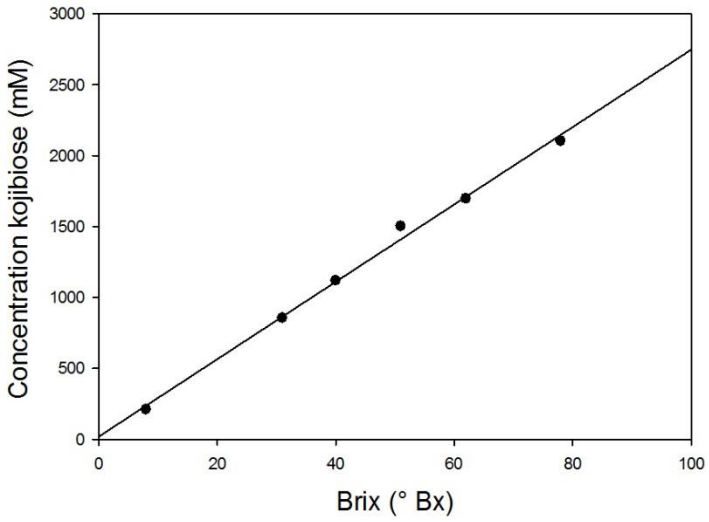


Figure IV.11: Concentration kojibiose in function of degrees Brix.

The first-order fit has the following equation:

$$y = 27.614x$$
$$with\ R^2 = 0.995$$

3.2.2.Evaluation of the crystallization process in function of the Brix

Next, the effect of the kojibiose concentration at the start of the cooling crystallization was evaluated. To that end, a reaction mixture obtained by reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S, was evaporated to various Brix values. The crystallization process was evaluated on the basis of five parameters: the appearance of the crystals, the microscopic appearance of the crystals, the ICUMSA score, the crystallization yield and the purity. Each parameter was determined for crystals originating from 8 different Brix solutions: 75 °Bx, 60 °Bx, 55 °Bx, 50 °Bx, 48 °Bx, 46 °Bx, 44 °Bx and 42 °Bx.

In a first step, pictures of the crystals originating from the various Brix solutions were taken (Figure IV.12).

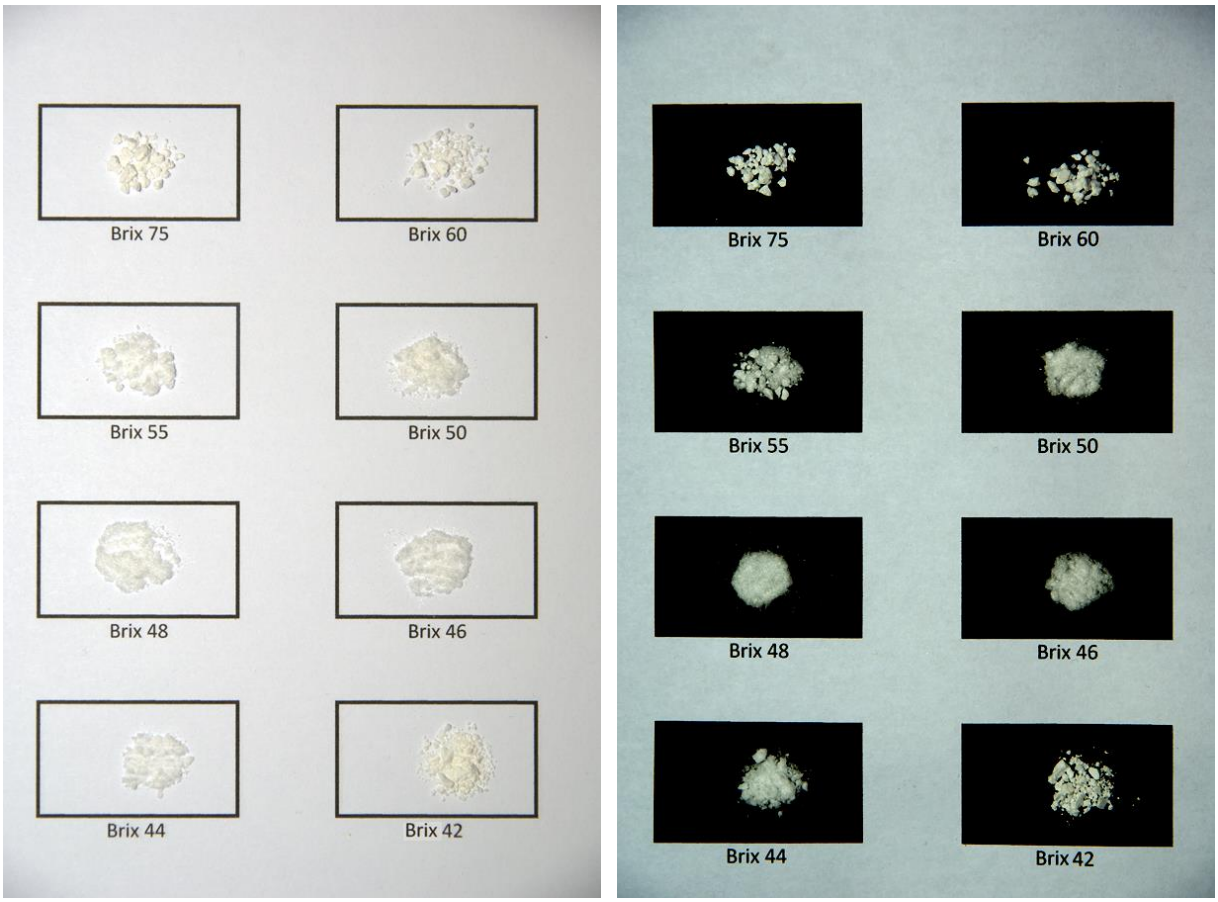
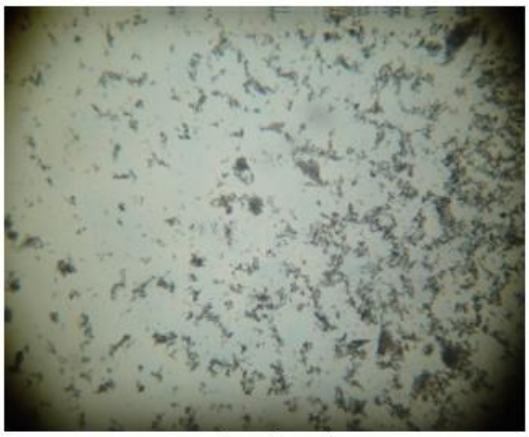
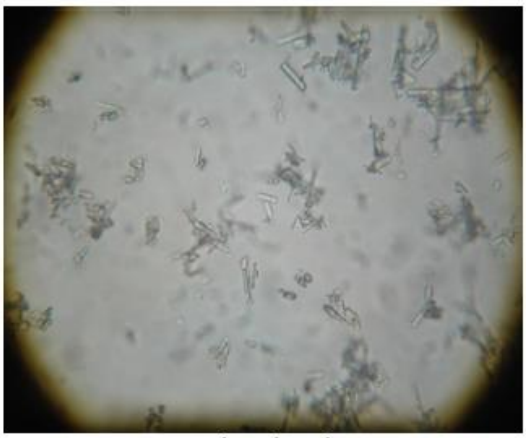


Figure IV.12: Pictures of crystals originating from various Brix solutions against a white (left) and black (right) background.

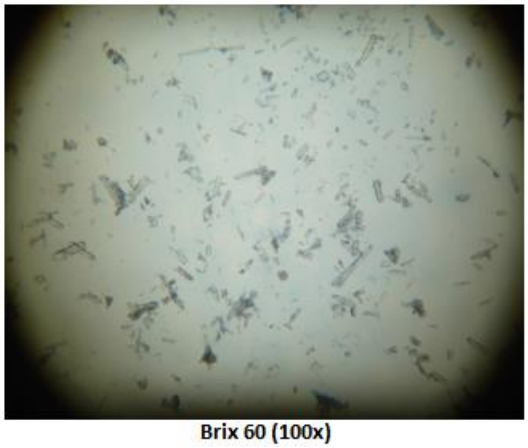
Next, the microscopic appearance of the crystals was mapped. Therefore microscope pictures were taken as described in Chapter III (Figure IV.13). Both 100x and 400x magnification are shown.



Brix 75 (100x)

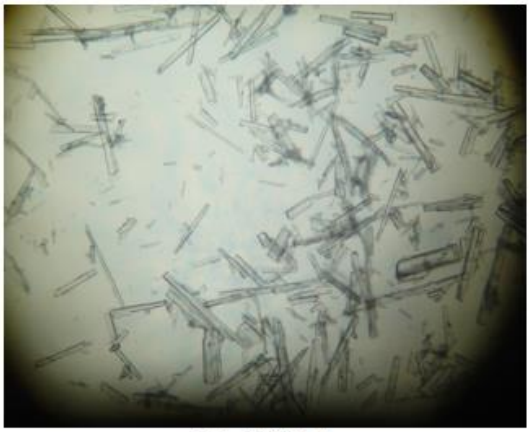


Brix 75 (400x)

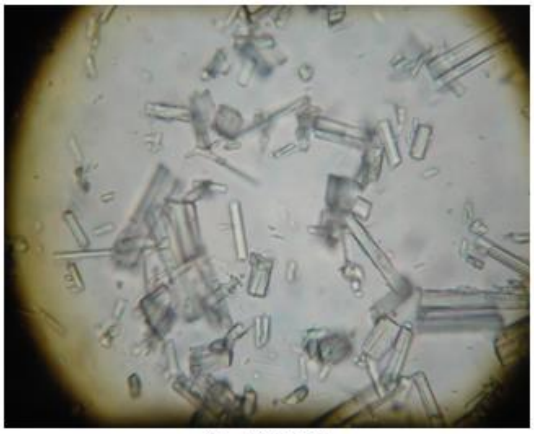


Brix 60 (100x)

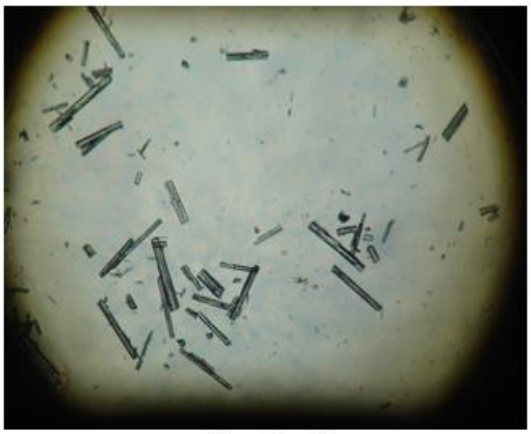
Brix 60 (400x)



Brix 55 (100x)



Brix 55 (400x)



Brix 50 (100x)

Brix 50 (400x)

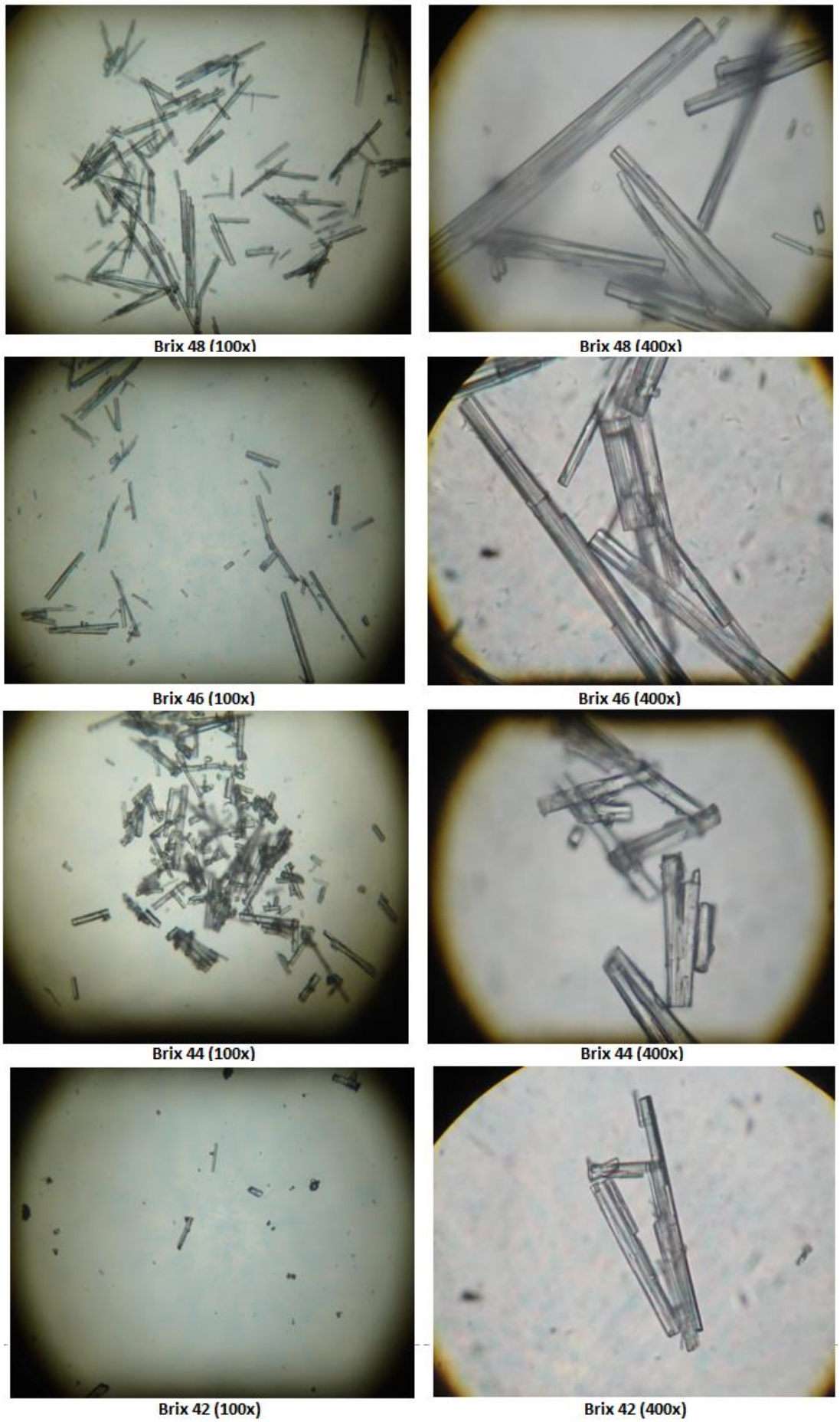


Figure IV.13: Microscopic appearance of crystals originating from various Brix solutions.

Subsequently, the ICUMSA score was calculated following the in Chapter III described protocol. In addition, the purity of the various crystals was determined via HPAEC-PAD and HPLC analysis and the crystallization yield was calculated in function of the Brix. A summary of all these results can be found in Table IV.7.

Table IV.7: Calculated ICUMSA score, purity and crystallization yield for crystals originating from various Brix solutions.

Brix	ICUMSA score	Purity HPAEC-PAD (%)	Purity HPLC (%)	Crystallization Yield (%)
75	543.9	96.40	98.41	85.5
60	522.8	96.66	94.16	72.5
55	361.4	98.08	99.87	74.9
50	340.4	98.47	99.90	74.2
48	273.7	99.79	99.98	72.3
46	312.3	98.08	99.95	58.2
44	308.8	98.17	99.86	51.7
42	933.3	96.12	89.45	46.2

The ICUMSA score displayed a minimum value of 273.7 for Brix 48. This implies that the whitest crystals are obtained originating from a Brix 48 solution. Since the whiteness of the crystals is an indication for their purity, these crystals should show the highest purity. Indeed, HPAEC-PAD analysis resulted in a maximum purity of 99.79 %, while HPLC analysis revealed a purity of 99.98 %. Investigating the appearance of the crystals favored the crystals obtained with Brix 50, Brix 48 and Brix 46 (Figure IV.12). Starting from a Brix of 48, the needle-like structure of the crystals was even visible to the naked eye. Crystals originating from the other Brix solutions had a more granulated structure. This observation was supported by the microscopic analysis whereby the biggest needle-like crystals were obtained from a Brix 48 solution (Figure IV.13). However, the yield of the crystallization process decreased with decreasing Brix values (Table IV.7). Evaluating these five parameters revealed an optimum Brix value at the start of the cooling crystallization of 48. Indeed, starting from this Brix allows the formation of crystals with a purity exceeding 99.5 %, while an acceptable crystallization yield of roughly 72 % is obtained. Interestingly, higher crystallization yields up to 85.5 % could be achieved at the expense of purity.

3.2.3. Influence of glycerol on the crystallization process

During the yeast treatment, glycerol and ethanol are formed, as described in Section IV.3.1. Because the former is known to hamper crystallization, the influence of this component on the crystallization process was investigated. To that end, known concentrations of glycerol were added to reaction mixtures before starting the crystallization protocol. Surprisingly, solutions with addition of glycerol up to 300 mM, could be conveniently crystallized. These results demonstrate the advantages of an integrated approach (i.e. exploring all parameters/possibilities of the entire DSP strategy at once; rather than applying a common sequential based optimization). Indeed, no optimization efforts

were required in order to minimize glycerol production by the yeast, since it had no influence on the crystallization process.

4 Necessity of enzyme engineering

Considering the previous results, an efficient process for the synthesis of kojibiose was developed, with the use of an enzyme engineered variant of sucrose phosphorylase as biocatalyst. In this section the necessity of enzyme engineering is demonstrated. To that end, the developed process was performed both with the wild type BaSP and with the L341I_Q345S mutant.

4.1. Reaction

Both enzymes were grown in Erlenmeyer flasks and purified by means of heat purification. Subsequently, two reactions were performed at 55 °C starting from 500 mM sucrose and 500 mM glucose in the presence of 2 mg/mL wild type BaSP or BaSP_L341I_Q345S during 24 h, followed by HPAEC-PAD analysis (Figure IV.14).

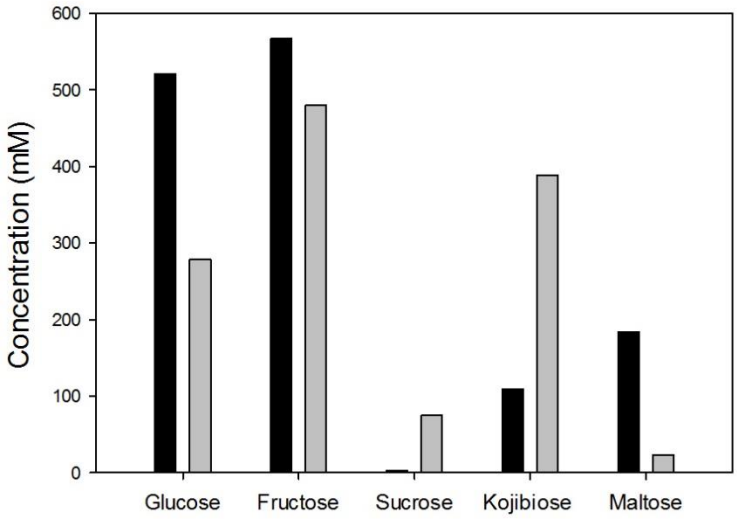


Figure IV.14: Concentrations glucose, fructose, sucrose, kojibiose and maltose after 24 h reaction at 55 °C starting from 500 mM sucrose and 500 mM glucose in the presence of 2 mg/mL wild type BaSP (black) or BaSP_L341I_Q345S (grey).

Considering the concentrations kojibiose and maltose, the difference in selectivity between the wild type and the mutant is immediately highlighted. The wild type produced more maltose than kojibiose, while with the mutant enzyme only a small fraction of the total product formation concerned maltose. To compare the performance of both enzymes, the parameters described in Section IV.2 were calculated (Table IV.8).

Table IV.8: Selectivity, conversion, yield, hydrolysis, efficiency and productivity calculated according to formulas given in Section IV.2 from reactions at 55 °C starting from 500 mM sucrose and 500 mM glucose in the presence of 2 mg/mL wild type BaSP or BaSP_L341I_Q345S after 24 h.

	Wild type	L341I_Q345S
Selectivity (%)	37.3	94.3
Conversion (%)	100.0	85.1
Yield (%)	21.8	77.8
Hydrolysis (%)	48.4	14.1
Efficiency (%)	19.2	81.0
Productivity (g kojibiose/ (g protein*h))	0.8	2.8

The selectivity of the wild type was found to be 37.3 %. In contrast, a selectivity of 94.3 % was calculated for the mutant. The significantly increase in selectivity resulted in a kojibiose yield of roughly 78 %, while with the wild type, the yield was found to be only 21.8 %. Important to notice is the difference in hydrolysis. Using the mutant resulted in a decrease of hydrolysis from 48.4 to 14.1 %, which indicates much more efficient use of the substrates. Further, the mutant obtained an efficiency of 81 %, while a productivity of 2.8 g kojibiose per g protein per hour was calculated. Comparing these values to those obtained with the wild type – 19.2 % efficiency and 0.8 g kojibiose per gram protein per h, respectively - proves once more the significant improvement of the enzyme engineered variant towards the production of kojibiose.

4.2. Yeast treatment

Subsequently, the reactions were inactivated and placed at 30 °C with addition of 30 g/L baker's yeast. After 7.5 h, the yeast treatment was finished for the mutant, while the wild type required 12.5 h. At the end of the treatment, samples were taken and analyzed with HPAEC-PAD. The resulting chromatograms can be found in Addendum (Figure A.2 and Figure A.3). Remarkably, the chromatogram of the wild type revealed two peaks, with retention times 3.6 and 12.7 min, which did not appear in the chromatogram of the mutant. Although several attempts were made, these peaks could not be elucidated. Moreover, the scale of the chromatograms is different, indicating a much higher peak area for kojibiose with the mutant compared to the wild type.

4.3. Crystallization

In a next step, the yeast was removed by centrifugation and the two reaction mixtures were subjected to a microfiltration. The solutions were subsequently evaporated to a Brix value of 48, whereafter cooling crystallization was performed. Interestingly, no crystallization occurred with the wild type enzyme, while crystals with a purity higher than 99.5 % (both on HPLC and HPAEC-PAD) were obtained with the L341I_Q345S mutant (Figure IV.15).

Figure IV.15: Demonstrating the necessity of enzyme engineering: with the wild type, no crystallization was observed (left), while crystals with a high purity (> 99.5 %) were obtained with the BaSP_L341I_Q345S mutant (right).

This experiment demonstrates the crucial role of enzyme engineering. Due to the development of a mutant with improved properties towards the synthesis of kojibiose, an efficient process could be developed resulting in kojibiose crystals having a purity higher than 99.5 %.

5 Process intensification

5.1. Goal and strategy

Although a process for the biotechnological synthesis of kojibiose has been described earlier this year (Díez-Municio *et al.*, 2014), we developed an extremely convenient and efficient procedure, allowing simultaneously higher yields and purities, while using considerably cheaper substrates. Encouraged by these results, conditions enabling the efficient synthesis of kojibiose were further optimized. First, substrate concentrations were increased, allowing higher concentrations kojibiose. Indeed, higher substrate concentrations result in less hydrolysis, as described in Section IV.2.2. Secondly, attempts to increase the efficiency of the enzymatic reaction were the focus of attention. An interesting parameter hereby is the atom efficiency, defined as (Trost, 1991):

$$Atom\ Efficiency\ (\%) = \frac{mass\ of\ atoms_{desired\ product}}{mass\ of\ atoms_{reactants}} * 100$$

Since however, the molecules in our process involve exclusively carbohydrates, this definition could be simplified to the concept of Carbohydrate Efficiency (CE). The latter is defined as:

$$Carbohydrate\ Efficiency\ (\%) = \frac{number\ of\ Glucose\ Equivalents_{desired\ product}}{number\ of\ Glucose\ Equivalents_{reactants}} * 100$$

Hereby the desired product involves kojibiose, which accounts for 2 GE, while the reactants concern the substrates glucose and sucrose, accounting for 1 and 2 GE, respectively. Considering the reaction scheme of sucrose phosphorylase (Section II.3.4), the release of fructose engenders intrinsic a low Carbohydrate Efficiency, since this molecule is not incorporated in the desired product, kojibiose.

A solution to circumvent this loss was found in the addition of glucose isomerase (GI). This enzyme catalyzes the reversible isomerization of D-glucose to D-fructose. At equilibrium, the fructose content in a reaction mixture was determined to be 52 % of the initial glucose concentration (Demerdash & Attia, 1992). The optimum temperature of GI ranges from 60 to 80 °C while the optimum pH range of GI is generally between pH 7.0 and 9.0 (Bhosale *et al.*, 1996). Interestingly, these values fit well with the applied reaction conditions of 55 °C and pH 7.0. Indeed, GI could theoretically convert the fructose released by conversion into glucose, which is the acceptor for the synthesis of kojibiose. Moreover, the formed glucose would be incorporated in kojibiose, thereby pulling the conversion towards the synthesis of glucose. The latter strategy should not only allow higher kojibiose concentrations, also the CE should significantly increase. Indeed the reaction would exclusively require the addition of sucrose, since glucose is provided by the recycling of fructose.

5.2. Effect of kojibiose on yeast treatment

Increasing the substrate concentrations increases concurrently the stress for the yeast in the subsequent purification step, as described in Section IV.3.1.2. Therefore, the influence of the kojibiose concentration on the yeast treatment was investigated. A reaction starting from 500 mM sucrose and 500 mM glucose was performed for 24 h at 55 °C with 2 mg/mL BaSP_L341I_Q345S. The reaction mixture was inactivated and subdivided into six fractions. To each of these fractions an additional amount of kojibiose was added: 0, 25, 50, 100, 250 and 500 mM, respectively. Subsequently 30 g/L yeast was added to all six fractions and these were incubated at 30 °C (Figure IV.16).

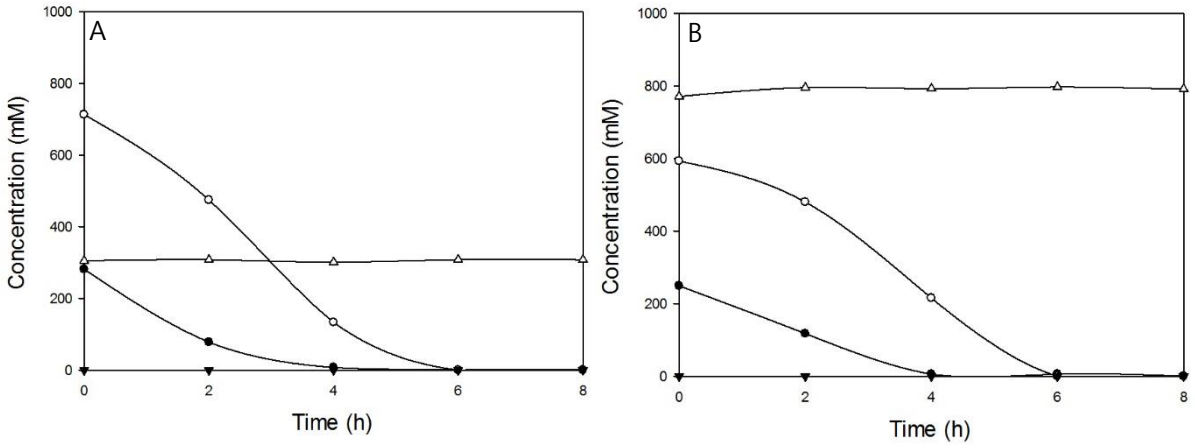


Figure IV.16 Concentration glucose (●), fructose (○), sucrose (▼) and kojibiose (△) in function of time after adding 30 g/L spray dried yeast to a reaction containing 280 mM glucose, 690 mM fructose, 30 mM sucrose, 300 mM (+0) kojibiose (A) or 800 mM (+500) kojibiose (B).

Concentrations up to 800 mM kojibiose did not influence the yeast treatment: glucose was removed after 4 h, fructose after 6 h, as was the case without the addition of kojibiose. Interestingly, kojibiose causes no significant stress to the yeast. This emphases once more the beneficial use of GI: converting fructose into glucose, serving on its turn as acceptor for the synthesis of kojibiose, decreases the stress for the yeast.

5.3. Process intensification

As previously described, reacting 500 mM sucrose with 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S results in a concentration kojibiose of approximately 380 mM. To increase this value, an experiment was set up in which five different substrate concentrations were evaluated. Reactions were performed at 55 °C with 2 mg/mL BaSP_L341I_Q345S for 24 h whereafter 5 mg/mL GI was added to half of the reaction volume (Figure IV.17.A).

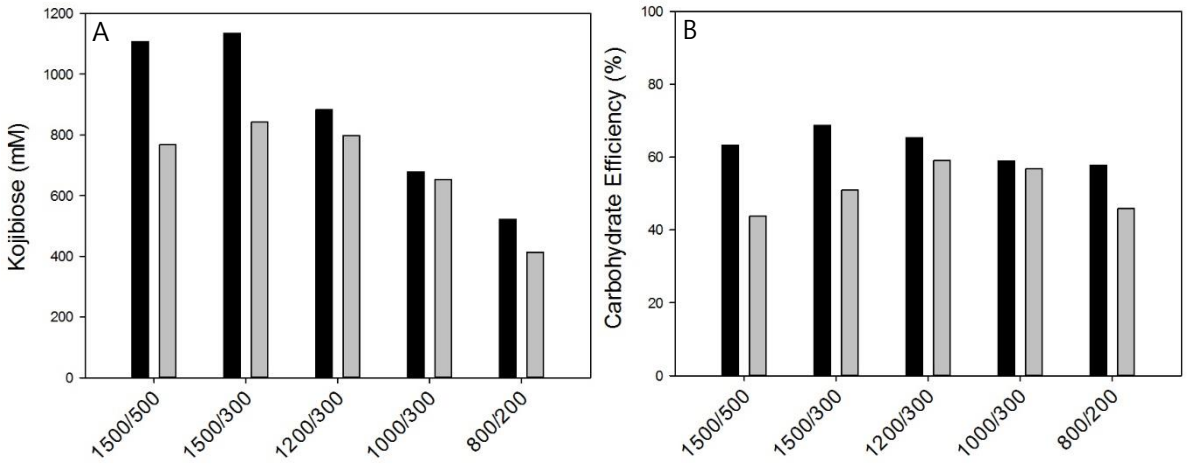


Figure IV.17: Concentration kojibiose (A) and Carbohydrate Efficiency (B) after 63 h reaction with 2 mg/mL BaSP_L341I_Q345S at 55 °C starting from five different substrate concentrations. After 24 h, 5 mg/mL GI was added to half of the reaction volume (black), the other half served as reference (grey). On the x-axis, the first number refers to the initial sucrose concentration, while the second number involves the initial glucose concentration.

The addition of GI resulted in a higher kojibiose concentration compared to the reference. Indeed, GI allows the recuperation of fructose by its conversion to the acceptor glucose. Moreover, this conversion is pulled by the synthesis of kojibiose, which incorporates the formed glucose. Reacting 1500 mM sucrose with 300 mM glucose resulted in a kojibiose concentration of 1135 mM, which is a threefold increase compared to the preliminary optimal conditions of 500 mM sucrose and 500 mM glucose (350 mM). Glucose Equivalents for the contaminating carbohydrates were found to be lower than 1000 for each reaction, indicating the feasibility of the yeast treatment (see Section 3.1.2). In addition, Carbohydrate Efficiencies were calculated (Figure IV.17.B).

The Carbohydrate Efficiency was found to be higher with the use of GI. Moreover, higher sucrose over glucose ratio's resulted in higher CE's. Therefore, it was decided to further increase this ratio. However, the glucose concentration should not be brought to zero, since this would significantly lower the velocity of the reaction. Subsequently 3 reactions starting from 1600, 1800 and 2000 mM sucrose, respectively and 200 mM glucose were performed at 55 °C in the presence of 2 mg/mL BaSP_L341I_Q345S. After 2 h of reaction, 20 mg/mL GI was added. Carbohydrate Efficiencies were calculated after 69 h (Table IV.9). This was compared to the CE of the process published in the Green Chemistry journal earlier this year (Díez-Municio *et al.*, 2014) and to the CE obtained with the preliminary optimal conditions described in Section IV.2.

Table IV.9: Comparison of the concentration kojibiose and the Carbohydrate Efficiency (CE) obtained with the process of Díez-Municio *et al.* (250 mM sucrose, 250 mM lactose), with the preliminary optimal conditions from Section IV.2 (500 mM sucrose, 500 mM glucose) and with reactions starting from 1600 mM, 1800 mM or 2000 mM sucrose and 200 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S at 55 °C during 69 h whereby 20g/L GI was added after 2 h of reaction.

Sucrose (mM)	Glucose (mM)	Kojibiose (mM)	CE (%)
250	250[a]	95	19.0
500	500	350	46.7
1600	200	1332	78.4
1800	200	1507	79.3
2000	200	1664	79.2

[a] lactose was used as acceptor substrate instead of glucose (Díez-Municio *et al.*, 2014)

Full conversion resulted in a final concentration kojibiose of 1332 mM, 1507 mM and 1664 mM starting from 1600 mM, 1800 mM and 2000 mM sucrose, respectively. The latter involves a more than fourfold increase compared to the preliminary optimal conditions (350 mM). It should be emphasized that the Carbohydrate Efficiency of our preliminary process (46.7 %) is already a significant improvement compared to the process described in the Green Chemistry journal (19.0 %). Moreover, due to process intensification, this value was further increased to nearly 80 %, which proves the role of GI in providing an efficient use of the substrate. After inactivating the reaction mixture and removal of GI, 30 g/L spray dried yeast was added and incubated at 30 °C. The reaction starting from 2000 mM sucrose showed however crystallization of kojibiose during the yeast treatment due to the high concentrations and the temperature drop from 55 °C to 30 °C. From this experiment, it was concluded that 1800 mM sucrose is the upper boundary in the process intensification story, whereby an increase from 350 mM to 1507 mM kojibiose was realized. This value corresponds to an impressive concentration of 516 g/L, which demonstrates the power of biocatalysis. 12 h of yeast treatment resulted in a complete removal of glucose and fructose (Figure IV.18). It should be noticed that during the filtration of GI some kojibiose was lost (due to the contact with the cold filtration unit), resulting in a lower concentration kojibiose at the beginning of the yeast treatment compared to the end of the reaction. After evaporation, crystallization and washing, kojibiose crystals with a purity higher than 99.5 % were obtained.

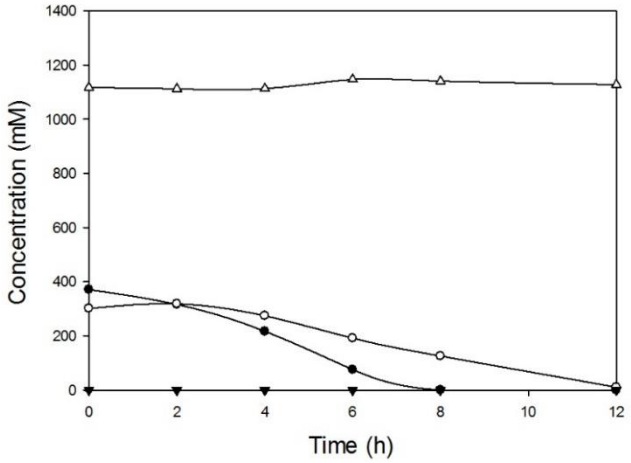


Figure IV.18: Concentration glucose (●), fructose (○), sucrose (▼) and kojibiose (△) in function of time after adding 30 g/L spray dried yeast to a reaction containing 410 mM glucose, 370 mM fructose, 30 mM sucrose and 1507 mM kojibiose.

Concerning the crystallization of kojibiose during the yeast treatment starting from 2000 mM sucrose, the idea was to eliminate the yeast treatment purification step. To that end, the reaction starting from 2000 mM sucrose and 200 mM glucose was repeated. After filtration of GI, a Brix value of 50 was measured. Consequently the reaction mixture was placed on ice for two days. Indeed, the solution crystallized and after several washing steps with ethanol, the crystals were analyzed with HPAEC-PAD. A purity of 97.5 % was calculated; spores of glucose and fructose were identified as impurities.

5.4. Inactivation of the reaction

Prior to performing a large scale process, the necessity of enzyme inactivation was investigated, since this energy consuming step involves a temperature increase to 95 °C for 10 minutes. Therefore 1800 mM sucrose was reacted with 200 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S at 55 °C for 48 h. Without inactivation of the reaction mixture, 30 g/L yeast was added. Samples were taken every 2 h and analyzed with HPAEC-PAD (Figure IV.19).

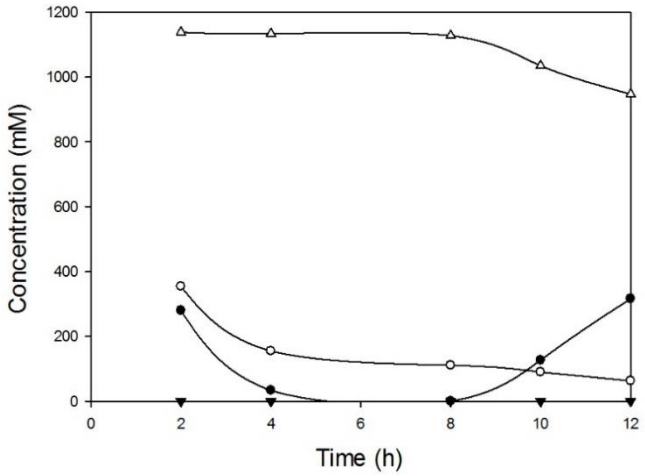


Figure IV.19: Concentration glucose (●), fructose (○), sucrose (▼) and kojibiose (△) in function of time after adding 30 g/L spray dried yeast to a reaction starting from 1.8 M sucrose and 0.2 M glucose in the presence of 2 mg/mL BaSP_L341I_Q345S at 55 °C.

During the first 8 h, fructose and glucose were metabolized by the yeast as previously described. Remarkably, hereafter, an increase in glucose concentration was observed. One could argue that the enzyme - still active since it was not inactivated – performed hydrolysis on kojibiose since water was the only possible acceptor present in the reaction mixture, resulting in the release of two glucose units. This hypothesis could declare the increase in glucose concentrations concurrently with a decrease in kojibiose concentration. To confirm this assumption, 1 M kojibiose was incubated at 55 °C in the presence of 2 mg/mL BaSP_L341I_Q345S. After 6 h, a sample was taken and analyzed with HPAEC-PAD. Indeed a peak of glucose was detected, corresponding to 220 mM glucose. This indicates that kojibiose is subject to secondary hydrolysis by BaSP_L341I_Q345S. From this, it can be concluded that inactivating the reaction mixture is necessary since otherwise the product degrades in further process steps due to hydrolysis.

5.5. Conclusion

In summary, depending on the desired purity, two highly efficient processes were developed. Crystals with a purity exceeding 99.5 % are obtained by reacting 1.8 M sucrose with 0.2 M glucose in the presence of 2 mg/mL BaSP_L341I_Q345S for approximately 70 h at 55 °C, whereby 20 mg/mL is added after 2 h. After removal of GI and inactivation of the reaction mixture, 30 g/L spray dried yeast provides the removal of the contaminating carbohydrates. Evaporation to a Brix value of 48 followed by temperature decrease to 4 °C leads to the crystallization of kojibiose with a purity exceeding 99.5 %. When a bulk purity of 97.5 % is sufficient, an even more efficient process can be performed. To that end, 2.0 M sucrose is reacted with 0.2 M glucose in the presence of 2 mg/mL BaSP_L341I_Q345S for approximately 70 h at 55 °C with addition of 20 g/L GI after 2 h. GI is removed by filtration and the reaction mixture is placed on ice until crystallization occurs, whereby the crystals display a purity of 97.5 %

6 Proof of concept: production at 10 L scale

As a proof of concept, the process starting from 1.8 M sucrose was performed at 10 L scale. It should be noticed that the enzyme concentration was decreased to 1 mg/mL. Indeed, it was shown in Section IV.2.5 that similar concentrations kojibiose were reached, provided that the reaction time was increased. Moreover, a higher productivity was reached using lower enzyme concentrations (Figure IV.5). Although upscaling was proven to be a labor-intensive challenge, it was concluded that the process is extremely robust and eventually resulted in the synthesis of roughly 3 kg kojibiose crystals with a purity exceeding 99.8 % (Figure IV.20). In addition, a second batch of approximately 1 kg kojibiose was obtained originating from the non-crystallized fraction of the first batch. These crystals displayed a purity of 99.0 %.

Figure IV.20: Reactor, purification and main result of the production at 10 L scale: 3 kg kojibiose with a purity higher than 99.8 %.

CHAPTER V

CONCLUSION AND FUTURE PERSPECTIVES

The aim of this master thesis was to develop an efficient process for the synthesis of kojibiose with the use of sucrose phosphorylase from *Bifidobacterium adolescentis*. This enzyme is able to synthesize kojibiose out of the cheap and readily available substrates D-glucose and sucrose. The yield is however strongly lowered by the formation of maltose, which accounts for 65 % of the total product formation. For this reason, enzyme engineering was applied prior to this thesis, which resulted in the creation of four mutant enzymes displaying a higher selectivity towards the synthesis of kojibiose.

For a comprehensive assessment of the synthesis potential, sufficient amounts of the wild type BaSP and mutants had to be available. Therefore cultures containing the proper plasmid were grown in Erlenmeyer flasks whereafter the enzyme was purified by means of a heat treatment, resulting in a purification yield of 88.1 % for the wild type.

In a next step, one of the mutants was selected for the further development of the production process. Therefore, multiple parameters such as the selectivity, conversion, yield, hydrolysis, efficiency and productivity, were calculated for each mutant from reactions under various substrate and enzyme concentrations. BaSP_L341I_Q345S displayed the optimal balance between activity and selectivity, whereby kojibiose accounted for 97.1 % of the total product formation. Preliminary optimal reaction conditions of 500 mM sucrose, 500 mM glucose, 2 mg/mL BaSP_L341I_Q345S and 55 °C, resulted in a kojibiose concentration of 350 mM after approximately 24 h.

Subsequently an efficient downstream process procedure was developed. Hereby the contaminating carbohydrates were removed by means of a yeast treatment. The spray dried baker's yeast *Saccharomyces cerevisiae* was able to remove the glucose, fructose, sucrose and maltose present in the reaction mixture in a timeframe of about 6 to 8 h, while kojibiose remained unaffected. After removal of the yeast by centrifugation, the solution was evaporated to an optimized Brix value of 48. Cooling crystallization resulted in kojibiose crystals with a purity higher than 99.5 % both on HPAEC-PAD and HPLC.

The last phase of the research incorporated the intensification of the process, whereby conditions enabling the efficient synthesis of kojibiose were further optimized. Higher substrate concentrations and the addition of glucose isomerase, capable of converting fructose to the acceptor substrate glucose, resulted in a major increase in kojibiose concentration. Moreover due to the use of glucose isomerase, the Carbohydrate Efficiency was significantly improved.

Depending on the desired purity two highly efficient processes were eventually developed. Crystals of high purity (> 99.5 %) were obtained by reacting 1.8 M sucrose with 0.2 M glucose in the presence of 2 mg/mL BaSP_L341I_Q345S for approximately 70 h at 55 °C, whereby 20 g/L glucose isomerase was added after 2 h. At the end of the reaction a kojibiose concentration of 1507 mM was obtained, which is a more than fourfold increase compared to the preliminary optimal conditions, where a concentration of 350 mM was reached. Moreover the Carbohydrate Efficiency increased from 46.7 % to nearly 80 %, which demonstrates strongly the beneficial effect of glucose isomerase. After removal of GI and inactivation of the reaction mixture, 30 g/L spray dried baker's yeast provided the removal of the contaminating carbohydrates. Evaporation to a Brix value of 48 followed by temperature decrease to 4 °C led to the crystallization of kojibiose. After washing the crystals with ethanol, a purity of 99.5 % was obtained. The feasibility of this process was demonstrated through production of kojibiose on 10 L scale. The final outcome was the production of 3 kg kojibiose with a purity exceeding 99.8 % and 1 kg kojibiose displaying a purity of 99.0 %.

Interestingly, when the initial sucrose concentration was further increased to 2.0 M, the yeast treatment purification step could be eliminated, although at the expense of purity. At the end of the reaction, glucose isomerase was removed by filtration and the reaction mixture was placed on ice until crystallization occurred. Washing with ethanol resulted in kojibiose crystals with a purity of 97.5 %.

Through enzyme and process engineering, a highly efficient process for the synthesis of kojibiose with the use of enzyme engineered sucrose phosphorylase from *Bifidobacterium adolescentis* was developed. Concentrations exceeding 500 g/L kojibiose were reached during the enzymatic reaction, which demonstrates clearly the power of biocatalysis. Previous limitations concerning the synthesis of kojibiose - such as the use of chemical reagents, the need for multiple (de)protection steps and low yields - were eliminated. The ultimate result of this master thesis is the availability of kojibiose at reasonable cost, which opens perspectives for its interesting applications as prebiotic and α-glucosidase I inhibitor. Moreover kojibiose could be the starting point for the enzymatic synthesis of kojibiose-analogues and kojioligosaccharides, which are proved to display even stronger prebiotic properties (Chaen *et al.*, 2001).

CHAPTER VI

BIBLIOGRAPHY

Aerts, D. *et al.*, 2011a. A constitutive expression system for high-throughput screening. *Engineering in Life Sciences*, 11(1), pp.10–19.

Aerts, D. *et al.*, 2011b. Transglucosylation potential of six sucrose phosphorylases toward different classes of acceptors. *Carbohydrate research*, 346(13), pp.1860–1867.

Al-Mudhaffar, S., 1978. Studies on the conditions of growth of baker's yeast, grown in solution of molasses or debis. *Food/Nahrung*, 22(4), pp.385–389.

Aso, K. & Fumio, Y., 1961. Crystalline α-Kojibiose. *Nature*, 189, pp.753.

Aso, K. & Shibasaki, K., 1953. Studies on the unfermentable sugars (I-III). *Tohoku Journal of Agricultural Research*, 3(2), pp.337–358.

Barrangou, R. *et al.*, 2003. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. *Proceedings of the National Academy of Sciences of the United States of America*, 100(15), pp.8957–89562.

Bhosale, S.H., Rao, M.B. & Deshpande, V.V., 1996. Molecular and industrial aspects of glucose isomerase. *Microbiological reviews*, 60(2), pp.280–300.

Bojarová, P. & Kren, V., 2009. Glycosidases: a key to tailored carbohydrates. *Trends in biotechnology*, 27(4), pp.199–209.

Borges de Melo, E., da Silveira Gomes, A. & Carvalho, I., 2006. α- and β-Glucosidase inhibitors: chemical structure and biological activity. *Tetrahedron*, 62(44), pp.10277–10302.

Brison, Y. *et al.*, 2012. Functional and structural characterization of α-(1->2) branching sucrase derived from DSR-E glucansucrase. *The Journal of biological chemistry*, 287(11), pp.7915–7924.

Van den Broek, L. *et al.*, 2004. Physico-chemical and transglucosylation properties of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis* DSM20083. *Applied microbiology and biotechnology*, 65(2), pp.219–227.

Van den Broek, L. *et al.*, 2008. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Molecular nutrition & food research*, 52(1), pp.146–163.

Cantarel, B.L. *et al.*, 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic acids research*, 37(1), pp.233–238.

Cantarella, L., Nikolov, Z.L. & Reilly, P.J., 1994. Disaccharide production by glucoamylase in aqueous ether mixtures. *Enzyme and Microbial Technology*, 16(5), pp.383–387.

Cerdobbel, A. *et al.*, 2010a. Increasing the thermostability of sucrose phosphorylase by multipoint covalent immobilization. *Journal of biotechnology*, 150(1), pp.125–130.

Cerdobbel, A. *et al.*, 2010b. Sucrose phosphorylase as cross-linked enzyme aggregate: Improved thermal stability for industrial applications. *Biotechnology journal*, 5(11), pp.1192–1197.

Chaen, H. *et al.*, 2001. Enzymatic synthesis of kojioligosaccharides using kojibiose phosphorylase. *Journal of Bioscience and Bioengineering*, 92(2), pp.177–182.

Chen, Y. *et al.*, 2008. Anaerobic yeast fermentation for the production of ethanol in a versatile lab fermentor. *Nature Methods*, 5(12), pp.4–5.

Corradini, C., Cavazza, A. & Bignardi, C., 2012. High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection as a Powerful Tool to Evaluate Carbohydrates of Food Interest: Principles and Applications. *International Journal of Carbohydrate Chemistry*, 2012, pp.1–13.

Crittenden, R.G. & Playne, M.J., 1996. Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science & Technology*, 7(11), pp.353–361.

Cummings, J.H. & Macfarlane, G.T., 2002. Gastrointestinal effects of prebiotics. *British Journal of Nutrition*, 87(2), pp.145–151.

Demerdash, M. & Attia, R.M., 1992. Equilibrium kinetics of D-glucose to D-fructose isomerization catalyzed by glucose isomerase enzyme from *Streptomyces phaeochromogenus*. *Zentralblatt fur Mikrobiologie*, 147(5), pp.297–303.

Desmet, T. *et al.*, 2012. Enzymatic glycosylation of small molecules: challenging substrates require tailored catalysts. *Chemistry-a European Journal*, 18(35), pp.10786–10801.

Desmet, T. & Soetaert, W., 2012. Broadening the synthetic potential of disaccharide phosphorylases through enzyme engineering. *Process Biochemistry*, 47(1), pp.11–17.

Desmet, T. & Soetaert, W., 2011. Enzymatic glycosyl transfer: mechanisms and applications. *Biocatalysis and Biotransformation*, 29(1), pp.1–18.

Díez-Municio, M. *et al.*, 2014. A sustainable biotechnological process for the efficient synthesis of kojibiose. *Green Chemistry*, 16(4), pp.2219–2226.

Van Dijken, J.P., Weusthuis, R.A. & Pronk, J.T., 1993. Kinetics of growth and sugar consumption in yeasts. *Antonie van Leeuwenhoek*, 63(3-4), pp.343–352.

Duke, J., Little, N. & Goldstein, I., 1973. Preparation of cyrstalline α-kojibiose octaacetate from dextran B-1299-S: Its conversion into p-nitrophenyl and p-isothiocyanatophenyl β-kojibioside. *Carbohydrate research*, 27(1), pp.193–198.

Faria-oliveira, F., Puga, S. & Ferreira, C., 2013. Yeast : World ' s Finest Chef. In *Food Industry*.

Flikweert, M.T., 1999. *Physiological roles of pyruvate decarboxylase in Saccharomyces cerevisiae*. PhD thesis, Delft University of Technology.

Fujimoto, H., Nishida, H. & Ajisaka, K., 1988. Enzymatic Syntheses of Glucobioses by a Condensation Reaction with alpha-glucosidase, beta-glucosdase and glucoamylase. *agricultural and biological chemistry*, 52(6), pp.1345–1351.

Gibson, G.R. & Roberfroid, M.B., 1995. Critical Review Dietary Modulation of the Human Colonie Microbiota : Introducing the Concept of Prebiotics. *Journal of nutrition*, 125, pp.1401–1412.

Goedl, C. *et al.*, 2011. Recombinant sucrose phosphorylase from *Leuconostoc mesenteroides*: characterization, kinetic studies of transglucosylation, and application of immobilised enzyme for production of alpha-D-glucose 1-phosphate. *Journal of biotechnology*, 129(1), pp.77–86.

Goedl, C. *et al.*, 2010. Sucrose phosphorylase: a powerful transglucosylation catalyst for synthesis of α-D-glucosides as industrial fine chemicals. *Biocatalysis and Biotransformation*, 28(1), pp.10–21.

Gray, G.M., Lally, B.C. & Conklin, K.A., 1979. Action of intestinal sucrase-isomaltase and its free monomers on an alpha-limit dextrin. *Journal of Biological Chemistry*, 254(13), pp.6038–6043.

De Groeve, M.R.M., 2009. *Engineering of cellobiose phosphorylase for glycoside synthesis*. PhD thesis, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

De Groeve, M.R.M., Desmet, T. & Soetaert, W., 2010. Engineering of cellobiose phosphorylase for glycoside synthesis. *Journal of Biotechnology*, 156(4), pp.253–260.

Haki, G. & Rakshit, S., 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technology*, 89(1), pp.17–34.

Hough, J.S., Stevens, R. & Young, T.W., 1982. : In *Malting and brewing science, Volume II Hopped word and Beer*. New York, USA: Chapman and Hall, pp. 776–838.

Igarashi, K., Irisawa, J. & Honma, T., 1975. Syntheses of a-D-linked disaccharides. *Carbohydrate Research*, 39, pp.341–343.

Kagan, B.O., Latker, S.N. & Zfasman, E.M., 1942. Phosphorolysis of saccharose by cultures of *Leuconostoc mesenteroides*. *Biokhimiya*, 7, pp.93–108.

Khan, S. & Vihinen, M.A., 2010. Performance of Protein Stability Predictors. *Human mutation*, 31(6), pp.675–684.

Kitao, S. *et al.*, 1994. Formation of kojibiose and nigerose by sucrose phosphorylase. *Bioscience, biotechnology and biochemistry*, 58(4), pp.790–791.

Kitaoka, M. & Hayashi, K., 2002. Carbohydrate-Processing Phosphorolytic Enzymes. *Trends in Glycoscience and Glycotechnology*, 14(75), pp.35–50.

Kittl, R. & Withers, S.G., 2010. New approaches to enzymatic glycoside synthesis through directed evolution. *Carbohydrate research*, 345(10), pp.1272–1279.

Koeppen, B., 1968. Some observations on the synthesis of sophorose. *Carbohydrate research*, 7(4), pp.410–413.

De Kok, S. *et al.*, 2011. Increasing free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression of a heterologous maltose phosphorylase. *Metabolic engineering*, 13(5), pp.518–526.

Kralj, S. *et al.*, 2008. Fructansucrase enzymes and sucrose analogues: A new approach for the synthesis of unique fructo-oligosaccharides. *Biocatalysis and Biotransformation*, 26(1-2), pp.32–41.

Lee, J.-H. *et al.*, 2006. Molecular cloning of a gene encoding the sucrose phosphorylase from *Leuconostoc mesenteroides* B-1149 and the expression in *Escherichia coli*. *Enzyme and Microbial Technology*, 39(4), pp.612–620.

Linhardt, R.J. & Bazin, H.G., 2001. Separation and Purification of Carbohydrates. In *Glycoscience: Chemistry and Chemical Biology I–III*. pp. 63–74.

Van Lul, J., Verhaeghe, T. & Desmet, T., 2014. *Improving sucrose phosphorylase for the production of a prebiotic sugar.* Master thesis, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

Luley-Goedl, C. & Nidetzky, B., 2010. Carbohydrate synthesis by disaccharide phosphorylases: reactions, catalytic mechanisms and application in the glycosciences. *Biotechnology journal*, 5(12), pp.1324–1338.

Van Maris, A. *et al.*, 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. , 90(4), pp.391–418.

Martearena, M.R., Mirta, D. & Ellenrieder, G., 2008. Synthesis of rutinosides and rutinose by reverse hydrolysis catalyzed by fungal α-L-rhamnosidases. *Biocatalysis and Biotransformation*, 26(3), pp.177–185.

Mateo, R. & Bosch-Reig, F., 1997. Sugar profiles of Spanish unifloral honeys. *Food Chemistry*, 60(1), pp.33–41.

Matsuda, K., 1959. Studies on the disaccharides in koji extract and sake. V. Isolation and identification of kojibiose. *Nippon Nogeikagaku Kaishi*, 33, pp.719–723.

Matsuda, K. *et al.*, 1961. Isolation of nigerose and kojibiose from dextrans. *Nature*, 191, pp.278.

McGrath, D., Lee, E.E. & O'Colla, P.S., 1969. The chemical synthesis of polysaccharides: Part II.(1→ 2)-,(1→ 3)-, and (1→ 4)-linked glucans. *Carbohydrate Research*, 11(4), pp.461–465.

McPherson, A., 1990. Current approaches to macromolecular crystallization. *European Journal of Biochemistry*, 189(1), pp.1–23.

Mehta, A. *et al.*, 1998. Alpha-glucosidase inhibitors as potential broad based anti-viral agents. *FEBS letters*, 430(1), pp.17–22.

Mersmann, A., Kind, M. & Stichlmair, J., 2011. Crystallization. In *Thermal Separation Technology*. pp. 413–481.

Mikkelsen, L.L., Knudsen, K.E.B. & Jensen, B.B., 2004. In vitro fermentation of fructo-oligosaccharides and transgalacto-oligosaccharides by adapted and unadapted bacterial populations from the gastrointestinal tract of piglets. *Animal Feed Science and Technology*, 116(3-4), pp.225–238.

Mirza, O. *et al.*, 2006. Structural rearrangements of sucrose phosphorylase from *Bifidobacterium adolescentis* during sucrose conversion. *The Journal of biological chemistry*, 281(46), pp.35576–35584.

Monsan, P. & Ouarné, F., 2009. Oligosaccharides derived from sucrose. *Prebiotics and probiotics science and technology*, pp.293–336.

Monsan, P., Remaud-Siméon, M. & André, I., 2010. Transglucosidases as efficient tools for oligosaccharide and glucoconjugate synthesis. *Current opinion in microbiology*, 13(3), pp.293–300.

Müllegger, J., Chen, H.M. & Chan, W.Y., 2006. Thermostable Glycosynthases and Thioglycoligases Derived from Thermotoga maritima β-Glucuronidase. *ChemBioChem*, 7(7), pp.1028–1030.

Nakada, T. *et al.*, 2003. Kojioligosaccharides: application of kojibiose phosphorylase on the formation of various kojioligosaccharides. *Oligosaccharides food Agric.*, 849, pp.104–117.

Nakai, H. *et al.*, 2013. Recent development of phosphorylases possessing large potential for oligosaccharide synthesis. *Current opinion in chemical biology*, 17(2), pp.301–309.

Nicolaou, K.C. & Mitchell, H.J., 2001. Adventures in Carbohydrate Chemistry : New Synthetic Technologies , Chemical Synthesis , Molecular Design , and Chemical Biology. *Angewandte Chemie International Edition*, 40(9), pp.1576–1624.

Nikolov, Z.L., Meagher, M.M. & Reilly, P.J., 1989. Kinetics, equilibria, and modeling of the formation of oligosaccharides from D-glucose with *Aspergillus niger* glucoamylases I and II. *Biotechnology and bioengineering*, 34(5), pp.694–704.

Ogawa, S., Ashiura, M. & Uchida, C., 1998. Synthesis of alpha-glucosidase inhibitors : kojibiose- type pseudodisaccharides and a related pseudotrisaccharide. *Carbohydrate research*, 370, pp.83–95.

Oliveira, D.L. *et al.*, 2012. Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. *International Dairy Journal*, 24(2), pp.102–106.

Osanjo, G.O., Bulimo, W.D. & Mulaa, F.J., 2013. Engineering the functional fitness of transglycosidases and glycosynthases by directed evolution. *African Journal of Biotechnology*, 10(10), pp.1727–1735.

Palcic, M.M., 1999. Biocatalytic synthesis of oligosaccharides. *Current Opinion in Biotechnology*, 10(6), pp.616–624.

Phung, O.J. *et al.*, 2014. Effect of Noninsulin Antidiabetic Drugs Added to Metformin Therapy on Glycemic Control , Weight Gain , and Hypoglycemia in Type 2 Diabetes. *Jama*, 303(14), pp.1410–1418.

Roberfroid, M., 1993. Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects. *Critical Reviews in Food Science & Nutrition*, 33(2), pp.103–148.

Roberfroid, M.B., 1996. Functional effects of food components and the gastrointestinal system : Chicory fructooligosaccharide. *Nutrition reviews*, 54(11), pp.38–42.

Rodrigues, F., Ludovico, P. & Leão, C., 2006. Sugar Metabolism in Yeasts : an Overview of Aerobic and Anaerobic Glucose Catabolism. In *Biodiversity and ecophysiology of yeasts*. pp. 101–121.

De Roode, B.M. *et al.*, 2003. Perspectives for the industrial enzymatic production of glycosides. *Biotechnology progress*, 19(5), pp.1391–1402.

Rosi, I., Vinella, M. & Domizio, P., 1994. Characterization of β-glucosidase activity in yeasts of oenological origin. *Journal of Applied Bacteriology*, 77(5), pp.519–527.

Sanz, M.L., Gibson, G.R. & Rastall, R.A., 2005. Influence of disaccharide structure on prebiotic selectivity in vitro. *Journal of agricultural and food chemistry*, 53(13), pp.5192–5199.

Sanz, M.L. & Martínez-Castro, I., 2007. Recent developments in sample preparation for chromatographic analysis of carbohydrates. *Journal of Chromatography A*, 1153(1-2), pp.74–89.

Sasaki, A. *et al.*, 2005. Synthesis of beta-mannosides using the transglycosylation activity of endo-beta-mannosidase from *Lilium longiflorum*. *The FEBS journal*, 272(7), pp.1660–1668.

Sato, A. & Aso, K., 1957. Kojibiose (2-O-alpha-D-glucopyranosyl-D-glucose) - isolation and structure - isolation for hydrol. *Nature*, 180, pp.984–985.

Schwarz, A. & Nidetzky, B., 2006. Asp-196-->Ala mutant of *Leuconostoc mesenteroides* sucrose phosphorylase exhibits altered stereochemical course and kinetic mechanism of glucosyl transfer to and from phosphate. *FEBS letters*, 580(16), pp.3905–3910.

Scott, R.P.W., 2003. *Preparative chromatography*, pp.71.

Scott, T.A., Hellman, N.N. & Senti, F.R., 1957. Characterization of dextrans by the optical rotation of their cuprammonium complexes. *Journal of the American Chemical Society*, 79(5), pp.1178–1182.

Seibel, J. *et al.*, 2006. Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase). *Carbohydrate research*, 341(14), pp.2335–2349.

Snyder, L.R., Kirkland, J.J. & Dolan., J.W., 2011. *Introduction to modern liquid chromatography* John Wiley & Sons, ed.,

Soetaert, W., 2012. *Industrial biotechnology*. Course, Ghent, Faculty Bio-engineering Sciences, pp.268.

Sprogoe, D. *et al.*, 2004. Crystal Structure of Sucrose Phosphorylase from *Bifidobacterium adolescentis*. *Biochemistry*, 43(5), pp.1156–1162.

Standl, E. & Schnell, O., 2012. Alpha-glucosidase inhibitors 2012 - cardiovascular considerations and trial evaluation. *Diabetes & vascular disease research*, 9(3), pp.163–169.

Strube, J. *et al.*, 2011. Process Development and Design of Downstream Processes. *Chemie Ingenieur Technik*, 83(7), pp.1044–1065.

Stura, E.A. & Wilson, I.A., 1991. Applications of the streak seeding technique in protein crystallization. *Journal of crystal growth*, 110, pp.270–282.

Suzuki, H. & Hehre, E.J., 1964. The differentiation of serotype A and B dextrans by means of partial acetolysis. *Archives of Biochemistry and Biophysics*, 104(2), pp.305–313.

Trost, B.M., 1991. The atom economy-a search for synthetic efficiency. *Science*, 254(5037), pp.1471–1477.

Ugalde, R.A., Staneloni, R.J. & Leloir, L.F., 2005. Microsomal Glucosidases of Rat Liver. Partial Purification and Inhibition by Disaccharides. *European Journal of Biochemistry*, 113(1), pp.97–103.

Verhaeghe, T.F., 2014. *Engineering of sucrose phosphorylase for the selective production of kojibiose*. PhD thesis, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

Wada, J. *et al.*, 2008. 1,2-alpha-l-Fucosynthase: a glycosynthase derived from an inverting alpha-glycosidase with an unusual reaction mechanism. *FEBS letters*, 582(27), pp.3739–3743.

Waffenschmidt, S. & Jaenicke, L., 1987. Assay of reducing sugars in the nanomole range with 2, 2'-bicinchoninate. *Analytical biochemistry*, 165(2), pp.337–340.

Wang, Y. *et al.*, 1999. Structure of an antigenic teichoic acid shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. *Carbohydrate Research*, 316(1-4), pp.155–160.

Watanabe, T. & Aso, K., 1959. Isolation of kojibiose from honey. *Nature*, 183, pp.1740.

De Winter, K. *et al.*, 2013. Ionic liquids as cosolvents for glycosylation by sucrose phosphorylase: balancing acceptor solubility and enzyme stability. *Green Chemistry*, 15(7), pp.1949–1955.

De Winter, K. *et al.*, 2011. Operational stability of immobilized sucrose phosphorylase: continuous production of alpha-glucose-1-phosphate at elevated temperatures. *Process Biochemistry*, 46, pp.2074–2078.

De Winter, K. *et al.*, 2014. Biphasic catalysis with disaccharide phosphorylases: Chemoenzymatic synthesis of α- D -glucosides using sucrose phosphorylase. *Organic Process Research and Development*, 18, pp.781–787.

De Winter, K., Soetaert, W. & Desmet, T., 2012. An Imprinted Cross-Linked Enzyme Aggregate (iCLEA) of Sucrose Phosphorylase: Combining Improved Stability with Altered Specificity. *International Journal of Molecular Sciences*, 13(9), pp.11332–11341.

Wolfrom, M.L., Thompson, A. & Lineback, D.R., 1963. Isopropyl Tetra-O-acetyl-α-D-glucopyranoside; A Synthesis of Kojibiose. *The Journal of Organic Chemistry*, 28(3), pp.860–861.

Yoon, S., Bruce, D. & Robyt, J.F., 2004. Enzymatic synthesis of two salicin analogues by reaction of salicyl alcohol with *Bacillus macerans* cyclomaltodextrin glucanyltransferase and *Leuconostoc mesenteroides* B-742CB dextransucrase. , 339(8), pp.1517–1529.

Yoon, S., Mukerjea, R. & Robyt, J.F., 2003. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydrate Research*, 338(23), pp.1127–1132.

Zeng, Y.C. & Elbein, a D., 1998. Purification to homogeneity and properties of plant glucosidase I. *Archives of biochemistry and biophysics*, 355(1), pp.26–34.

Ziemer, C.J. & Gibson, G.R., 1998. An Overview of Probiotics , Prebiotics and Synbiotics in the Functional Food Concept : Perspectives and Future Strategies. *International Dairy Journal*, 8(5-6), pp.473–479.

Addendum

1 List of products

Company: Acros organics
Sodium carbonate anhydrous
Company: Algist Bruggeman
Spraydried baker's yeast
Company: Baker analsed
Sodium hydroxide
Company: BD
Yeast extract
Company: Carbosynth
Kojibiose
Company: CMS
Trehalose
Company: Chem-lab
Disolol
Glycerol
Company: Fiers
Acetone
Company: Fluka
Ampcillin
4-4'-dicarboxy-2,2'-biquinoline
Lysozyme, from chicken egg white
Tryptone
Company: Leaf technologies
Ethanol Red
Company: Merck
Ethanol
Imidazole
Company: Novozymes
Glucose isomerase
Company: Pierce
Coomassie Briljant Blue R-250
Company: Purelab
MilliQ water

Company: Sigma-Aldrich
Copper(II) sulfate pentahydrate
D-Glucose
Phenylmethylsulfonyl fluoride
Maltose
D-Fructose
Isomaltose
Nigerose
MOPS
Sucrose
L-Aspartic acid sodium salt monohydrate
Sodium chloride
Sodium phosphate monobasic
Sodium phosphate dibasic
Company: Thermo Scientific
HisPur™ Ni-NTA Resin
Company: Veolia
Distilled water
Company: VWR
Antifoam silicone snapsil RE20
Inbio.be: Inhouse preparation
Kojibiose

2 Additional information

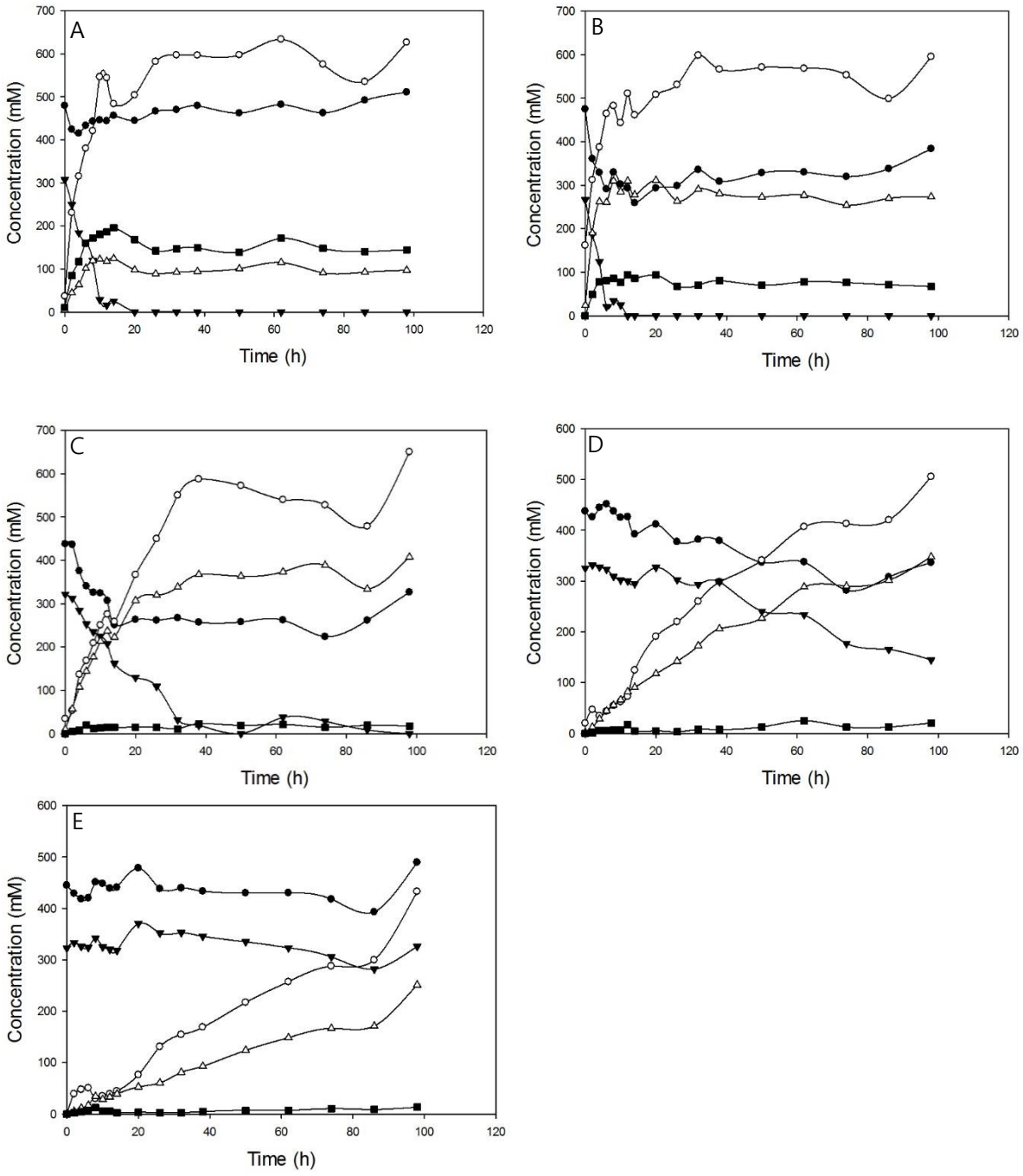


Figure A.1 : Concentration glucose (●), fructose (○), sucrose (▼), kojibiose (△) and maltose (■) in fucntion of time from reactions performed in a 50 mM MOPS buffer at pH 7.0 containing 500 mM sucrose, 500 mM glucose and 2 mg/mL wild type (A), L341I (B), L341I_Q345S (C), L341I_Q345N (D), and L341I_Y344A_Q345N (E).

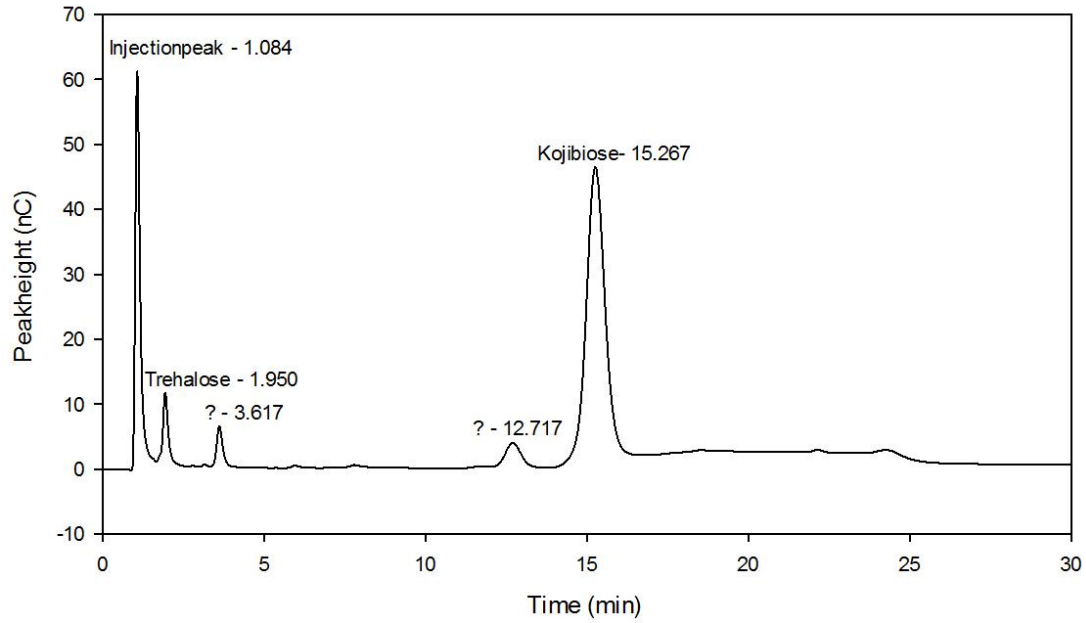


Figure A.2: HPAEC-PAD chromatogram from a sample after 12.5 h yeast treatment of a reaction starting from 500 mM sucrose and 500 mM glucose in the presence of 2 mg/mL wild type BaSP. Spray dried baker's yeast in a concentration of 30 g/L was added after inactivation of the reaction mixture.

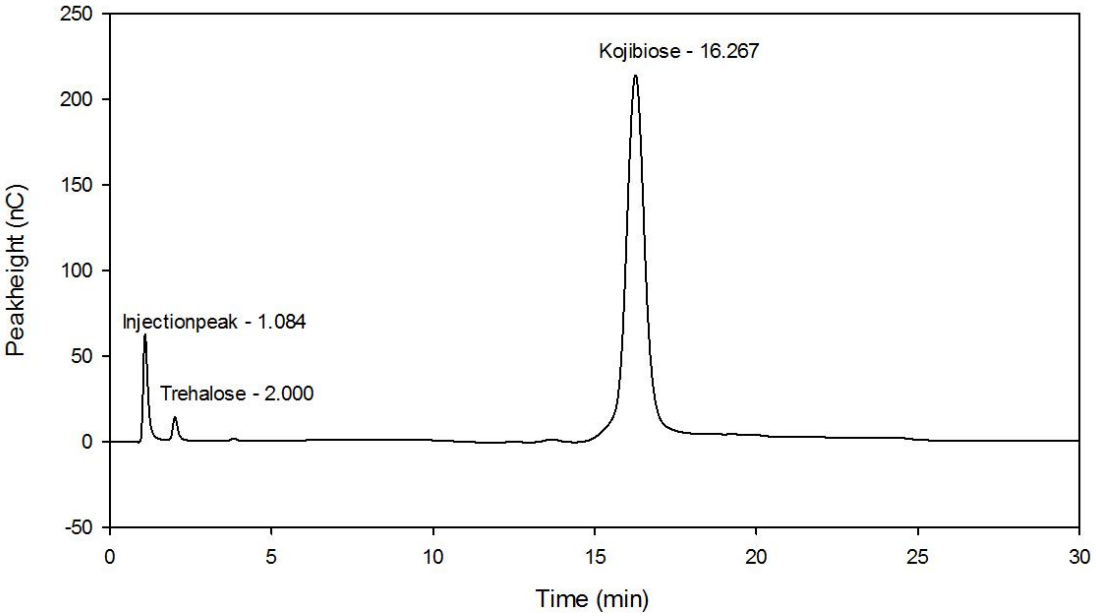


Figure A.3: HPAEC-PAD chromatogram from a sample after 7.5 h yeast treatment of a reaction starting from 500 mM sucrose and 500 mM glucose in the presence of 2 mg/mL BaSP_L341I_Q345S. Spray dried baker's yeast in a concentration of 30 g/L was added after inactivation of the reaction mixture.