Immobilization of a whole cell catalyst for cellodextrin production

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IMMOBILIZATION OF A WHOLE CELL CATALYST FOR CELLODEXTRIN PRODUCTION

This study was carried out at the Institute of Biotechnology and Biochemical Engineering (BIOTE) at the Graz University of Technology under supervision of Bernd Nidetzky, Univ. Prof. Dipl. Ing. Dr. techn., with the assistance of Katharina Schwaiger, PhD Student. The Thesis was accomplished under supervision of Anita Slavica, Full Professor, at Faculty of Food Technology and Biotechnology University of Zagreb.

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IMMOBILIZATION OF A WHOLE CELL CATALYST FOR CELLODEXTRIN PRODUCTION

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Abstract: Enzymes sucrose phosphorylase from *Bifidobacterium adolescentis* (BaSP), cellobiose phosphorylase from *Cellulomonas uda* (CuCBP) and cellodextrin phosphorylase from *Clostridium cellulosi* (CcCDP) were co-expressed in *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new cells and immobilized in polyacrylamide matrix (PAM) with the aim of repeated use of immobilized cells, *i.e.* three enzymes in the production of soluble cellodextrins (COS). Three repetitive batch experiments were performed at three different temperatures (35, 40 and 45 °C), differing in number of cycles (9, 6 and 5, respectively), duration of each cycle (8, 12 and 6 h), cell loading (63, 40, 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$) and cell catalyst concentration (4, 3, 4 g $_{CDW}$/L). The impact of these variables has been investigated and a new experimental setup for the industrial production of COS has been proposed; six repetitive batch cycles should be performed at 40 °C, with a duration of each cycle 8-10 h, with cell loading higher than 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$, and concentration of the cell catalyst (higher than 4 g $_{CDW}$/L).

Keywords: soluble cellodextrins, immobilization, *Bifidobacterium adolescentis* sucrose phosphorylase (BaSP), *Cellulomonas uda* cellobiose phosphorylase (CuCBP), *Clostridium cellulosi* cellodextrin phosphorylase (CcCDP)
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PROIZVODNJA CELODEXTRINA POMOĆU IMOBILIZIRANIH STANICA

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Sažetak: Enzimi saharoza fosforilaza iz *Bifidobacterium adolescentis* (BaSP), celobioza fosforilaza iz *Cellulomonas uda* (CuCBP) i celodekstrin fosforilaza iz *Clostridium cellulosi* (CcCDP) su ko-eksprimirani u stanicama *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new. Stanice su zatim imobilizirane u poliakrilamidnom matriksu (PAM) s ciljem ponavljajućeg korištenja imobiliziranih stanica, odnosno ova tri enzima u proizvodnji topljivih celodekstrina (COS). Proizvodnja COS pomoću PAM imobiliziranih stanica tijekom ponavljajućih šaržnih eksperimenata provedena je na tri različite temperature (35, 40 i 45 °C) s različitim brojem ciklusa (9, 6 i 5), vremenom trajanja ciklusa (8, 12 i 6 h), udjelom stanica (63, 40 , 40 mg $_{s.t.}$/g $_{immobilizata}$) i koncentracijom stanica (4, 3 , 4 g $_{s.t.}$/L). Utjecaj ovih parametara je ispitan i predloženi su sljedeći uvjeti za provođenje ponavljajućih šarži za industrijsku proizvodnju COS-a; šest ponavljajućih šaržnih ciklusa treba provesti na 40 °C, s trajanjem ciklusa 8-10 h, s većim udjelom stanica u poliakrilamidnom matriksu (≥ 4 g $_{s.t.}$/L), i koncentracijom stanica (≥ 63 $_{s.t.}$/g $_{immobilizata}$).

Ključne riječi: topljivi celodekstrini, imobilizacija, celobioza fosforilaza iz *Cellulomonas uda* (CuCBP), celodekstrin fosforilaza iz *Clostridium cellulosi* (CcCDP), saharoza fosforilaza iz *Bifidobacterium adolescentis*
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1. INTRODUCTION

Interest in the production of soluble cello-oligosaccharides (COS) began in the late 20th century due to their beneficial effects on human health. Their application is intended as a sweetener that acts as a dietary fiber, which means that in addition to other positive characteristics, it has a positive effect on digestion as they cannot be digested by the human arsenal of digestive glycoside hydrolases (Zhong et al., 2020b). Despite wide usage forecasts, there are still no plants for their mass production, so their price is still extremely high. Cellodextrins, also called cello-oligosaccharides, containing between two and six β-(1,4) linked D-glucose units are defined as soluble (COS) and only such could be potentially used in human and animal nutrition.

This Thesis presents the small–scale production of cello-oligosaccharides, through repeated batch reactions. The *E. coli* catalyst chosen for production, carries the plasmid pPOLY_new which expresses three specific enzymes: sucrose phosphorylase isolated from *Bifidobacterium adolescentis* (BaSP), cellobiose phosphorylase isolated from *Cellulomonas uda* (CuCBP) and cellodextrin phosphorylase isolated from *Clostridium cellulosi* (CcCDP). These enzymes act in a cascade, starting with BaSP which converts the affordable substrate, sucrose, to fructose and αGlc1-P in the presence of inorganic phosphate. The role of CcCDP is to elongate cellobiose (G2), which is formed by CuCBP from glucose and αGlc1-P. Production of only COS with a strictly defined degree of polymerization (DP, the number of D-glucose units) less than or equal to six, depends greatly on the mentioned enzymes activity ratio, which is defined by measured enzymes activities and their corelation. Zhong and Nidetzky (2020) suggested that the optimal activity (U/mL) ratio of BaSP, CuCBP and CcCDP is 10/3/2 for COS production.

In order to preserve the BaSP, CuCBP and CcCDP activity for as long as possible with the possibility of their reuse, the production of COS was performed by immobilization of *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new, which carries the genes for BaSP, CuCBP and CcCDP expression, in a polyacrylamide matrix (PAM). PAM provides high mechanical stability, has the ability to protect cells and is easily separated from the reaction suspension, which allows reuse of expressed enzymes. Although whole cell immobilization is superior to enzyme immobilization, negative aspects of whole cell immobilization are changes in temperature and osmotic conditions that can affect membrane fluidity, and reproducibility of enzyme expression power on which is difficult to influence. However, usually for mass production in large quantities, whole cells immobilization techniques are superior to enzyme immobilization due to lower sensitivity to environmental conditions and easier handling.

Consequently, immobilized *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new cells were used as a whole cell catalyst. After adding the catalyst to a substrate solution, now reaction suspension, as it contains substrates and catalyst, the production of cellodextrins was started and it lasted specific time (reaction time). The reaction was ended by sieving the reaction suspension, which separated the immobilized catalyst from the reaction suspension, resulting in reactant/product mixture (abbreviated product mixture) as it contains produced COS, insoluble cellodextrins, unused substrates, and reaction intermediates. Afterward, immobilized catalyst was emerged again into new substrate solution, where the number of times emerging catalyst into new substrate solution defines the number of cycles conducted in each repetitive batch. Again, the catalyst was left in the reaction suspension until the reaction time ended, and the sieving step occurred again. To determine the effectiveness of using repetitive batch production of cellodextrins, specifically COS, with whole cells entrapped in PAM, experiments were performed at three different temperatures (35, 40 and 45 °C) with different number of reaction cycles (9, 6, and 5, respectively), each lasting differently (8, 12, and 6 h, respectively). The relationship between named variables and their influence on the composition of the product mixture, and the total amount of produced COS and insoluble cellodextrins was investigated.

2. LITERATURE REVIEW

2.1 SOLUBLE CELLO-OLIGOSACCHARIDES (COS)

So far cellulose is the most familiar dietary fiber, as it is the major component of every plant cell wall. The properties of cellulose are strength, its resistance to both biological and chemical hydrolysis, and water holding capacity. Cellodextrins are linear β-(1,4)-D-glucose oligosaccharides, which are comprised in cellulose polysaccharides chain. The oligosaccharides of cellulose make various forms, from short-chain soluble dimers, trimers, tetramers, pentamers and hexamers which are denoted as cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (C5), and cellohexaose (C6) to insoluble seven or longer-chain oligomers (Phirom-on and Apiraksakorn, 2021). Cellodextrins, that have a low DP, typically lower than 10 are easier to process and characterize, compared to the insoluble native cellulose. Cellulose, thanks to negatively charged hydroxyl groups attract water molecules and make cellulose absorb water well. This high-water holding capacity enables retention of many water molecules in the stomach, so swelled cellulose can improve satiety in humans and animals, which effectively manages bodyweight (Phirom-on and Apiraksakorn, 2021). Cellodextrins also have water holding capacity characteristic, and in addition, both cellulose and cellodextrins are not digested by the human organism so they can be used as prebiotic, dietary fiber or sweetener (Billès et al., 2017). The conclusion of the research conducted by Phirom-on and Apiraksakorn (2021) showed that the water-soluble cellulose from banana peel did not support pathogenic bacteria's growth, while it significantly enhanced the growth of *L. plantarum* TISTR 2075 (isolated from fermented vegetables). The authors also imply that *L. plantarum* TISTR 2075 and *L. casei* TISTR 1463 are able to produce extra-/intracellular β-glucosidase that directly releases glucose from G2, so potentially COS could be used as the alternative carbon source for probiotics in the fermentation of some food products, such as yogurt and cheese. Furthermore, mentioned research concludes that the water-soluble cellulose raises the prospect of a potential prebiotic fiber. A prebiotic has been defined as a selectively fermented ingredient that stimulates the immune system of the host, regulates its gastrointestinal function, so potential prebiotic component must fulfil the following criteria: nondigestible by the host, fermentation by the intestinal microbiota, and selective stimulation of growth and activity of beneficial intestinal bacteria (Pokusaeva et al., 2011). COS meet this requirement, and they seem promising as ingredients for human and animal nutrition (Zhong et al., 2019). Another study conducted by Zhong et al. (2020b), that compared COS with inulin,

trans-galacto-oligosaccharides, and G2, showed that COS had up to 4.1-fold higher stimulation of cell density for *Clostridium butyricum*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus paracasei* subsp. *paracasei*, which characterizes COS as selectively functional carbohydrates with prebiotic potential. In addition to the already mentioned benefits, COS may enhance human health by also reducing cholesterol, stabilizing blood glucose, and enhancing weight loss (Phirom-on and Apiraksakorn, 2021). To ensure a completely water-soluble product, cellodextrins must have a DP equal to or lower than six, to be called COS, which is a challenge in the production. Cellodextrins with a DP of about 8 - 10, are hardly soluble in water. DP of sugar is one of the crucial factors that affects the availability of carbohydrates for consumption by bacteria. The findings of Montagne et al. (2003) claim that water-soluble dietary fiber favoured the growth of beneficial microbiota and optimized pig gut health because, unlike insoluble fractions, as they are fermented faster, producing higher amounts of volatile fatty acids. Moreover, COS could be potentially used as a bulking agent, in cosmetics as additives applied as such or in chemically derivatized form (Zhong et al., 2019), also when combined with a fatty acid they can create a new totally bio-based amphiphilic compound (Billès et al., 2017).

2.2. COS PRODUCTION

Despite the wide promising use of COS, they are not widely available, mainly due to the lack of industrial processes for controlled production of COS with specific DPs, resulting in limited use due to their cost and limited availability (Zhong et al., 2019). Choice of suitable substrate(s) is critical for the COS production to become viable, as chosen substrate(s) must be available in suitable purity, whereas industrial sugars (*e.g.* sucrose and glucose) fulfil the demand, yet cellulose-based feedstocks raise concern due to their chemical and physical heterogeneity (Zhong and Nidetzky, 2020). Reported routes for COS production are based upon depolymerization of cellulose or bottom-up synthesis, while chemical and enzymatic methods are known for both types of routes (Billès et al., 2017).

2.2.1. Cellulose depolymerization

One way to produce cellodextrins is through chemical hydrolysis (*e.g.* acid-catalysed), with a moderate yield of cellulose oligomers (≤ 68 %) or by enzymatic hydrolysis of cellulose, which gives a mixture of cellodextrins (Billès et al., 2017).

2.2.1.1. Chemical and mechanical depolymerization

Chemical hydrolysis by fuming HCl has been widely used to hydrolyse cellulose to cellodextrins, with a required HCl concentration greater than 40 % (Zhang and Lynd, 2003). As the use of fuming HCl has safety problems and is quite challenging in terms of controlling conditions of cellulose dissolution and achieving reproducible results, the authors investigated the effect of mixed acids on microcrystalline cellulose. Their results seemed sufficient, taking into account readily available reagents and less risky conditions such as accidental release, or exposure to acid fumes. An interesting production of COS by mechanical depolymerization was introduced by Shrotri et al. (2013), by milling sulphuric acidified cellulose. Soluble fractions containing less than 10 monomer units were primarily linked via β-(1,4) glycosidic bonds together, while formed α-(1,6) bonds increased solubility of larger oligosaccharides and decreased depolymerization to glucose (Shrotri et al., 2013). Similar study was conducted by Dornath et al. (2015), concluding that co-impregnation of crystalline cellulose with sulfuric acid and glucose greatly improves bead milling efficiency of crystalline cellulose, resulting in production of cellodextrins with average DP of about seven and a yield of 92.3 ± 4.3 % by weight. Another example of mechanical depolymerization of cellulose is pyrolysis with the downside of low yield, which happens at high temperatures (100 to 1200 °C) where two decomposition mechanisms compete: the "unzipping" model, resulting in levoglucosan and random chain cleavage, resulting in cellulose oligomers (Billès et al., 2017). The next example of cellulose mechanical depolymerization is by using hot compressed water, introduced by Zhao et al. (2009), where supercritical water acts as a solvent as well as a reactant. In the mentioned study, temperature and reaction time were two key parameters that determined the products of cellulose hydrolysis. The highest yield of COS, where 40 % of produced cellodextrins had a DP up to five, was achieved under optimal conditions at 380 °C and a reaction time of 16 s. A recent study conducted by Tolonen et al. (2015), showed the production of cellodextrin with DP from two to nine, with a yield of 42 % achieved by treatment of cellulose with supercritical water at 380 °C, and 250 bar for 0.4 seconds. However, structural analysis of oligomeric and monomeric degradation products, formation of the cellodextrins with a levoglucosan end group, and dehydration and fractionation reactions happening in the reducing end may limit their use, especially in human food. Disadvantages of chemical decomposition of cellulose are its dependence on the nature of a particular cellulose substrate, and parameters such as temperature and time of hydrolysis process, which have a great impact

on the hydrolysis efficiency and require significant efforts in cellodextrin extraction (Zhang and Lynd, 2003).

2.2.1.2. Enzymatic depolymerization

COS can be obtained by enzymatic hydrolysis of cellulose, but naturally-occurring highly crystalline cellulose is hardly soluble in water and therefore less approachable for cellulase enzymatic decomposition (Yamasaki et al., 2012). In order to prevent complete depolymerization of cellulose to glucose, β-glucosidase should be inactivated as it hydrolyses G2 into glucose, which ultimately causes a decrease in COS production yield. Previously mentioned flaws and requirement to purify endoglucanases (E.C. 3.2.1.4) and cellobiohydrolyses (E.C. 3.2.1.91) from β-glucosidases (EC 3.2.1.21) presents big technological hurdle in large scale production (Zhong et al., 2019). However, results of Phirom-on and Apiraksakorn (2021) revealed that enzymatic hydrolysis by Celluclast®, cellulase from *Trichoderma reesei* obtained from Sigma Aldrich, provides higher contents of cellodextrins than did diluted-acid hydrolysis.

2.2.2. Bottom-up synthesis of COS

2.2.2.1. Chemical bottom-up synthesis

In 1996 Nakastubo et al. successfully synthesized cellulose oligomers with very low DP (< 4) by an eight-step chemical reaction pathway, starting from monomer 3-*O*-benzyl-6-*O*-pivaloyl-α-glucopyranose (Billès et al., 2017). Authors also imply that the main advantage of the chemical route is its ability to target a specific DP, which provides good purity but only after a multi-step chemical reaction pathway, the higher the DP, the more numerous are the chemical steps.

2.2.2.2. Enzymatic bottom-up synthesis

Production of cellodextrins with a specific DP through bottom-up chemical synthesis involves multistep procedures, which is not suitable for bulk production, so enzymatic bottom-up synthesis seems more promising as it is conducted in very mild reaction conditions, with fewer by-products (Billès et al., 2017). Cellodextrins production can be started using two step syntheses beginning with cellobiose phosphorylase (CBP, EC 2.4.1.20) that catalyses the

synthesis of G2 from glucose, or with cellodextrin phosphorylase (CDP, EC 2.4.1.49) in one step that elongates G2 to form cellodextrins with wide DP range. Depolymerization of starch to G2 by glucan and cellobiose phosphorylase (EC 2.4.1.1 and EC 2.4.1.20, respectively) was demonstrated by Suzuki et al. (2009), with a rather low conversion yield because starch is a compound of high molecular weight (difficult to dissolve) with a complex (branched) structure. The first complete enzymatic production of COS was presented by Zhong et. al. (2019) in a linear-cascade reaction using a CBP from *Cellulomonas uda* (CuCBP; GenBank identifier AAQ20920.1) and the CDP from *Clostridium cellulosi* (CcCDP; GenBank identifier CDZ24361.1). Their goal was to produce cellodextrin products with a DP ≤ six, which is a limiting solubility factor. The obtained results imply that the product DP can be controlled kinetically, by adjusting the time or enzyme activity, and more importantly by the molar ratio of substrates (glucose/αGlc1-P). In particular, when 200 mmol/L of αGlc1-P was used for the production of COS, the ratio of glucose/αGlc1-P should be 0.25 or higher. Zhong et. al. (2019) optimized the reaction conditions that resulted in producing only COS, with DP from three to six [~96 expressed in product yield percentage by weight (wt %)] by using 200 mmol/L αGlc1-P, 200 mmol/L $MgCl_2$, 50 mmol/L glucose as substrates, and catalysts CuCBP, CcCDP with activity ratio 3:2 (U/mL). Also, they imply that *in situ* phosphate removal by precipitation with Mg^{2+} from reaction suspension is extremely important for overcoming kinetic and thermodynamic restrictions of the overall conversion, since the CuCBP is phosphate inhibited. Zhong et. al. (2019) also examined the possibility of generating COS by G2 and αGlc1-P as substrates, by using only one enzyme, CcCDP which elongates G2 to cellodextrins with different DP. They concluded that glucose as a substrate and two-enzyme (CuCBP and CcCDP) reaction cascade has more potential in the COS production, than one-enzyme (CcCDP) reaction in which G2 is elongated. Although, the product composition was similar in both experiments, substrate affordability and better glucose solubility (glucose solubility is 909 g/L, while G2 solubility is 120 g/L) seemed to be the deciding factors for choosing the two-enzyme approach. In the next study conducted by Zhong and Nidetzky (2020) a new approach with a more affordable substrate (sucrose) was introduced, since the aforementioned experiments had the need for addition of expensive αGlc1-P into reaction suspension, which is not suitable for bulk production. In the mentioned study production of COS was suggested in two separate steps and in one step by continuous production. The continuous production in one step experiment is the base of this Thesis, where three enzymes BaSP, CuCBP and CcCDP act in a cascade and produce wanted COS and unwanted insoluble cellodextrins, according to the reaction scheme shown in Figure 1. In the reaction conducted by BaSP the inexpensive sucrose substrate is

converted to fructose and αGlc 1-P, which eliminated the need of adding expensive αGlc 1-P into reaction suspension, which is needed for both - G2 and cellodextrin synthesis. The conditions selected for the overall conversion from sucrose to COS were pH 7.0 and 45 °C, as optimal pH profiles for CcCDP and CuCBP is from pH 7.0 to 6.5, while temperature optima is from 55 °C to 40 °C, respectively (Zhong et al., 2019).

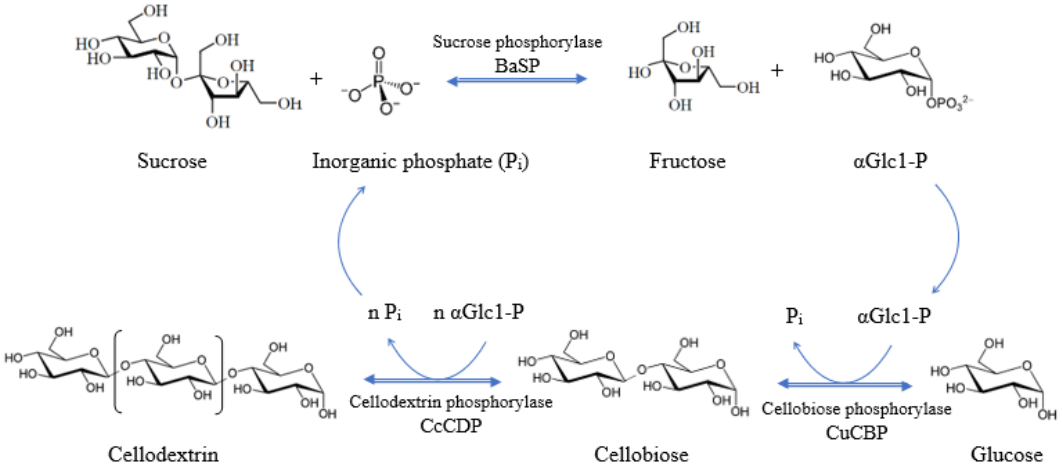

Figure 1. Reaction scheme for the production of cellodextrin, where three enzymes: BaSP, CuCBP, CcCDP act in a cascade. n symbolizes the number of reactions that CcCDP conducts, if n = 1, CcCDP binds D-glucose to G2 by β-(1,4) linkage, and formed cellodextrin is denoted as G3. For n = 2 or more, CcCDP binds D-glucose to G3 or longer-chain cellodextrin. For COS production n can be from one to four [G3 to G6] (*according to* Zhong and Nidetzky, 2019)

Zhong and Nidetzky (2020) conducted the first experiment with all three enzymes for the COS production in two separate steps, wherein in the first 2 h step the BaSP converted sucrose and phosphate (200 mmol/L each) to ~180 mmol/L αGlc1-P. Whereas, in the following 1 h step CuCBP formed G2, from glucose and previously produced αGlc1-P (molar ratio of glucose/αGlc1-P was 0.25), which was then elongated to cellodextrins with different DP range by the CcCDP. Based on their previous findings from 2019, 180 mmol/L $MgCl_2$ was added to the reaction suspension because the magnesium salt of phosphate, released from αGlc1-P, induces a CuCBP-CcCDP reaction toward the COS production. The calculated productivity of the COS production after the two-step reaction was 9 g/(L h) with the highest product yield of G3 of 31 wt % and G4 of 37 wt %. To present conversion efficiency, the molar yield of reaction was defined as the mole ratio (mol%), where the overall conversion of sucrose (200 mmol/L) was 58 mol% after 3 h of reaction. This yield is considerably lower than expected from

estimates, but could be increased by the higher initial concentration of phosphate, however it would consequently increase the requirements for the separation of magnesium phosphate precipitate from insoluble cellodextrins. The magnesium phosphate precipitate can be removed from insoluble cellodextrins by dissolving it at slightly acidic conditions (pH 4.5; Zhong et al., 2019).

Therefore, Zhong and Nidetzky (2020) introduced the continuous COS production, with all three enzymes (BaSP, CuCBP, CcCDP) in one reaction pot under the same conditions (pH 7.0, 45 °C), with a reduced phosphate concentration (50 mmol/L). Their results show that the COS production was at its maximum after 1.5 h of reaction time with a concentration of 32 g/L of the COS, and afterward the concentration of COS decreased due to the progressing formation of insoluble cellodextrins, 28 mol % at 3 h reaction time. However, the molar yield of the COS was considerably lower than theoretical, which was explained by the unused αGlc1-P (released from sucrose) for G2 or cellodextrin formation. In this continuous COS production experiment from 1.5 to 3 h the composition of the produced COS completely changed from 29 to 19 wt % for G3, from 34 to 26 wt % for G4, from 26 to 37 wt % for G5, and from 12 to 18 wt % for G6, as in this period accumulated shorter-chain cellodextrins (*i.e.* G3 and G4) were elongated. Zhong and Nidetzky (2020) suggested that the continuous one step COS production, compared to separate mode - two step COS production, has a higher conversion efficiency, which resulted in a lower proportion of insoluble cellodextrins.

2.3. IMMOBILIZATION TECHNIQUES

Free enzymes are not always ideal catalysts for industrial applications, as they are generally unstable and cannot be used in organic solvents or at elevated temperatures. Furthermore, enzymes used in batch process after only one usage are generally discarded without recovery. The need to find easily producible, environmentally friendly and inexpensive catalysts leads to intensive research and development of enzymes and whole cells immobilization techniques (Zajkoska et al., 2013). In addition, in order to use enzymes more economically and efficiently, and to eliminate the process of enzyme extraction from microbial cells, direct immobilization of whole microbial cells into polymer lattice has been attempted since the early 1970s (Guisan et al., 2020). Since then, immobilized enzymes and cells are used in organic synthesis, clinical and chemical analysis, the food industry, medicine, etc. (Chibata and Tosa, 1981). Depending on the type and nature of the interactions between the catalyst and the supporting material, either enzymes or whole cells, a variety of immobilization techniques have been developed.

These interactions can be referred to as adsorption and entrapment in which there are no covalent interactions between catalyst and support carriers, while covalent attachment and cross-linking belong to chemical methods (Liu et al., 2018). There is no unique immobilization method that is suitable for all reactions because the catalyst and material used depend largely on the type and conditions of the catalytic process. In addition, it is necessary to take into account the different properties of substrates, products and ultimately their end use (Homaei et al., 2013). All of the methods have merits and drawbacks, so new are constantly being developed to improve the effectiveness of immobilization.

Homaei et al. (2013) characterized every immobilization method, for example adsorption is simple, fast, inexpensive and effective but oftentimes reversible as the binding forces are sensible to changes in pH, temperature and ionic strength, resulting in poor catalyst stability. Interaction between enzyme/cell and support material with charged surfaces relies only on naturally present functional groups and is based on different intermolecular chemical bonds, such as a combination of electrostatic or hydrophobic interactions, van der Waals forces, and hydrogen bonds (Liu et al., 2018).

Covalent bonding among enzyme/cell and support material is a more stable form of immobilization as bonds are formed by chemical reactions between supporting material and catalysts side chain amino acids, such as lysine, cysteine, or aspartic and glutamic acid residues. In addition, functional groups of amino, carboxylic, imidazole, indolyl and phenolic hydroxyl groups are favourable for the formation of covalent bonds (Liu et. al., 2018) However, the active sites of the enzyme can be inactivated due to conformational changes, which may result in reduced activity.

Cross-linking is based on intermolecular reactions, where catalysts whole cells or enzymes are cross-linked to the support matrices using bifunctional reagents such as isocyanate, *N*, *N*′-ethylene bismaleimide and glutaraldehyde, which is the most adapted in practice (Liu et al., 2018). This type of chemical interaction involves covalent bonds that improve catalyst reuse and stability, but on the other hand, may decrease catalytic activities during the cross-linking process. A new approach to enzyme immobilization has emerged, where the catalytic activities of the enzyme are preserved as active sites are protected by first cross-linking enzymes to each other to synthesize the cross-linked enzyme aggregates (CLEAs) and afterward by bifunctional reagents used to cross-link the aggregated (Zhang et al., 2011). The authors characterize this Combi-CLEA method as a combination purification and immobilization in one step, and predict the use of two or more enzymes in multi-step syntheses providing numerous potential benefits: less reactor volume, higher volumetric and space-time yield, shorter cycle time.

Entrapment and micro-encapsulation techniques ensure high catalytic activities as there are no covalent bonds between enzymes and support matrices so enzyme conformations are maintained but often have characteristic diffusional problems.

Enzyme immobilization provides pure products, oftentimes without the need for their separation, but the use of whole cells prevents conformational changes of enzymes and thus allows the use of enzymes in severe reaction conditions or in unconventional (non-aqueous) reaction media. Whole cell immobilization eliminates the need for sometimes expensive enzyme extraction and purification, while undoubtedly providing the most familiar environment for enzymes. In addition, whole cells are being the cheapest form of catalyst formulation as they supply the needed cofactors for biotransformation's and simplify their regeneration, while making an external and expensive addition of cofactors needless.

2.3.1. Whole cell immobilization

Some of the desired properties and performances of supporting materials for whole cell immobilization are high biocatalysts loading efficiency, nontoxicity, favourable chemical and mechanical stability, wide applicability, high biocompatibility, as well as affordability. The most frequently used technique for whole cell immobilization is entrapment, which is based on the enclosing cells within a rigid network to prevent cells release into the surrounding medium. On the other hand, used matrix with entrapped cells should be porous enough to enable the diffusion of substrates and products (Suzana et al., 2013). The matrices that use entrapment technique could be divided into hydrogels (alginate, κ-carrageenan, chitosan), thermogels (agar, agarose, cellulose), and synthetic polymers [PAM, polyvinyl alcohol (PVA), polyurethane] (Guisan et al., 2020). Synthetic polymers, such as PAM or PVA overcome the poor mechanical stability and the high biodegradability of natural polymers (Carballeira et al., 2009). Schlieker and Vorlop presented many features of new PVA-based hydrogel trapping technique called LentiKats®, with intended use for living cells and cross-linked enzymes. Some of its many outstanding advantages are excellent physical and mechanical stability (elasticity, low abrasion), non-biodegradability, high inner porosity, and high immobilization capacity. The authors present some successful immobilization projects by using LentiKats® (Lentikats Biotechnology), *e.g*. bioconversion of raw glycerol to 1,3-propanediol, bioethanol production from nonsterile molasses, reduction of energy consumption for sewage treatment, and production of (*R*)-cyanohydrins. Unlike polyacrylamide monomer, LentiKats® doesn't show any toxic properties towards microorganisms, enzymes. Furthermore, entrapment of the catalyst

occurs in mild environmental conditions, allowing very high survival of cells in the biocatalyst matrix. The particles are thin in structure (diameter 200–400 μm), which helps minimizing the diffusion limitations, while on the other hand are easy to separate from the liquid media in repeated or continuous experiments by sieving. To potentially overcome the diffusional problems that may occur, cells can be permeabilized by using organic solvents (*e.g.* ethanol) or surfactants (*e.g.* cetrimonium bromide) (Cárdenas-Fernández et al., 2012).

2.3.2. Polyacrylamide matrix (PAM) immobilization

Polyacrylamide gel has been used extensively to immobilize microbial cells, but its first application was introduced by Bernfeld and Wan in 1963 (Powell, 1984). Japanese company Tanabe Seyiaku Co. first performed industrial application with immobilized cells in 1973, by producing L-aspartic acid via *E. coli* cells immobilized in PAM (Zajkoska et al., 2013). The immobilization process begins by emerging a cell suspension in a solution of the selected matrix, and the biocatalyst formation begins with a temperature change or using a chemical reagent, which accelerates polymerization. The main advantages of this immobilization technique are a significant increase in productivity, due to easy separation that allows reuse, which consequently reduces operational costs, high process stability and the protection of labile cells (Guisan et al., 2020) Furthermore, reuse is possible, because PAM is a polymer resistant to bacterial contamination and insensitive towards organic solvents (Carballeira et al., 2009). However, this method has some drawbacks, for example, decrease of the activity or even inactivation of the enzymes during the immobilization procedure by the action of toxic acrylamide monomer, dimitylamino-propionitril, potassium persulfate, or by the heat of the polymerization reaction (Chibata and Tosa, 1981). Other limitations are additional costs, diffusion limitations that can cause a decrease in reaction kinetics, and abrasion of the immobilization matrix during use in bioreactors (Schlieker and Vorlop, 2006). The rate-limiting step in the reaction with this immobilized system is the mass transfer of substrates and reaction products, especially a high molecular weight compounds, which in some cases can cause inhibition (Carballeira et al., 2009). Using an immobilized cell system is not recommended when cells contain interfering enzymes that cannot be inactivated or removed (Chibata and Tosa, 1981). The cost of the immobilization matrix, as well as its storage stability and disposal upon exhaustion of the biocatalyst should be considered when choosing the immobilization technique. Additionally, every immobilization technique should be adjusted for every microorganism.

3. EXPERIMENTAL PART

3.1. MATERIALS

Table 1 shows all of the chemicals used for the experimental work in this Graduate Thesis. In Table 2 *E. coli* strains with used plasmids are listed. Medium used for cultivation of *E. coli* strains is Lysogeny Broth (LB) medium (10 g/L peptone, 10 g/L NaCl and 5 g/L yeast extract). Prepared media was sterilized in autoclave at 121 °C and 1 bar for 20 min. After sterilization and cooling, 100 mg/L of ampicillin sodium salt (Amp) was added to the medium. Gel, protein ladder, dye, solution and stains used for SDS-PAGE are shown in Table 3. In Table 4 enzymes used in the research are listed. Buffers prepared for the enzyme activity analysis are shown in Table 5. In Table 6 equipment used in this research is listed. To adjust pH of all buffers HCl, H_3PO_4, NaOH or KOH were used. All buffers were filtered using a MF-Millipore® Membrane Filter, 0.45 µm pore size (Sigma-Aldrich, St. Louis, USA).

Table 1. Chemicals used in the research

Chemicals	Company/Institution
distilled water (dH_2O)	BIOTE, TU Graz
double distilled water (ddH_2O, nanopure water)	BIOTE, TU Graz
2,2',2",2'"-(ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA, >99 %)	Sigma-Aldrich (St. Louis, USA)
3-dimethylaminoproprionitrile solution	Sigma Aldrich (St. Louis, MO, USA)
p-nitrophenyl β-D-cellobioside (PNP-C)	Carbosynth Ltd. (Berkshire, UK)
Acrylamide	Sigma Aldrich (St. Louis, MO, USA)
Amoniumheptamolybdat-Tetrahydrat	Carl Roth GmbH (Karlsruhe, DE)
Ampicillin sodium salt (Amp, >97 %)	Carl Roth GmbH (Karlsruhe, DE)
L(+)-Ascorbic acid sodium salt	Carl Roth GmbH (Karlsruhe, DE)
Bovine serum albumin (BSA) (pH 7.0, >98%)	Sigma-Aldrich (St. Louis, USA)
Calcium chloride ($CaCl_2$ >93 %)	Sigma-Aldrich (St. Louis, USA)
Cellobiose (G2)	Pfeifer and Langen Gmbh and Co. KG (Elsdorf, DE)
Cellohexaose (G6)	Megazyme Ltd. (Wicklow, IE)
Cellopentaose (G5)	Megazyme Ltd. (Wicklow, IE)

Table 1. Chemicals used in the research (continuation)

Chemicals	Company/Institution
Cellotetraose (G4)	Megazyme Ltd. (Wicklow, IE)
Cellotriose (G3)	Megazyme Ltd. (Wicklow, IE)
D (+)-Glucose (>99 %)	Carl Roth GmbH (Karlsruhe, DE)
Ethanol (EtOH, 96 %)	Carl Roth GmbH (Karlsruhe, DE)
Hydrogen chloride (HCl > 99 %)	Sigma-Aldrich (St. Louis, USA)
Kalium peroxydisulfate ($K_2S_2O_8$)	E. Merck, Darmstadt
Magnesium chloride ($MgCl_2$, > 98.5 %)	Carl Roth GmbH (Karlsruhe, DE)
MES buffer	Carl Roth GmbH (Karlsruhe, DE)
Nicotinamide adenine dinucleotide, oxidized form (NAD^+ > 98 %)	Carl Roth GmbH (Karlsruhe, DE)
Peptone from casein	Carl Roth GmbH (Karlsruhe, DE)
Potassium dihydrogen phosphate (KH_2PO_4, > 99.5 %)	E. Merck KG (Darmstadt, DE)
Potassium persulfate solution	E. Merck KG (Darmstadt, DE)
Zinc acetate dihydrate [$Zn(CH_3COO)_2$ $2H_2O$, > 99 %]	Sigma-Aldrich (St. Louis, USA)
Yeast extract	Carl Roth GmbH (Karlsruhe, Germany)

Table 2. *E. coli* strains with two different plasmids which

Strain with plasmid	Company/Institution	Enzymes expressed
E. coli BL21 (DE3) -*agp*$^-$ pPOLY_new	BIOTE, TU Graz	BaSP, CuCBP, CcCDP
E. coli BL21 (DE3) -*agp*$^-$ pBICI_strong_2	BIOTE, TU Graz	BaSP, CuCBP

Cell suspensions of the listed strains were prepared by resuspending harvested (*E. coli* BL21 (DE3) -*agp*$^-$ pBICI_strong_2 or *E.coli* BL21 (DE3) -*agp*$^-$ pPOLY_new) cells in 50 mmol/L MES buffer, pH 7.0. These two strains differ by the plasmid which carries the genes for specific enzymes expression.

Table 3. Gel, gel markers, stains, solutions, protein ladder used for SDS-PAGE

Gel, gel markers and stains	Company/Institution
Coomassie staining solution (50 % methanol, 10 % CH_3COOH, 40 % H_2O)	BIOTE, TU Graz
Destaining solution (40 % CH_3OH, 10 % CH_3COOH)	BIOTE, TU Graz
InstantBlue™ Coomassie Protein Stain	Expedeon (Cambridge, UK)
NuPAGE™ Bis-Tris Mini Protein Gels 10 or 15-well	Thermo Fisher Scientific (Waltham, USA)
PageRuler™Prestained Protein Ladder	Thermo Fisher Scientific (Waltham, USA)
NuPAGE™ LDS Sample buffer 4x (SDS-PAGE)	Thermo Fisher Scientific (Waltham, USA)
NuPAGE™ MOPS Running buffer (SDS-PAGE)	Thermo Fisher Scientific (Waltham, USA)

Table 4. Enzymes used in the research

Enzyme	Company/Institution
Phosphoglucomutase (PGM) from rabbit muscle	Sigma-Aldrich/Merck (Darmstadt, DE)
glucose-6-phosphate dehydrogenase (G6P-DH) from *Leuconostoc mesenteroides*	Sigma-Aldrich/Merck (Darmstadt, DE)
N-terminal $Strep_{II}$-tag sucrose phosphorylase from *Bifidobacterium adolescentis* (BaSP)	BIOTE, TU Graz
N-terminal His_6-tag cellobiose phosphorylase from *Cellulomonas uda* (CuCBP)	BIOTE, TU Graz
N-terminal His_6-tag cellodextrin phosphorylase from *Clostridium cellulosi* (CuCDP)	BIOTE, TU Graz

Table 5. Buffers prepared for the enzyme activity analysis

Assay	Assay mixture	Assay buffer
α-D-glucose 1-phosphate assay	400 µL of assay buffer 10 µL NAD^+ 2 µL G6P-DH [350 U/mL] 40 µL sample 2 µL PGM [400 U/mL]	50 mmol/L Tris/HCl 10 mmol/L $MgCl_2$ 10 mmol/L EDTA 10 µmol/L glucose glucose 1,6 bis-phosphate pH 7.7 (adjusted with 5 mol/L HCl)
Continuous coupled activity assay	380 µL of assay buffer 40 µL NAD^+ 2 µL G6P-DH [958.5 U/mL] 2 µL PGM [846 U/mL] 10 µL sample 130 µL sucrose	50 mmol/L KH_2PO_4, 10 mmol/L $MgCl_2$ 10 mmol/L EDTA glucose 1,6 bis-phosphate pH 7.0
Phosphate assay	9 µL sample 141 µL assay buffer	15 mL molybdate reagent (15 mmol/L ammonium molybdate, 100 mmol/L $Zn(CH_3COO)_2 \cdot 2H_2O$) 5 mL ascorbic reagent (100 g/L ascorbic acid dissolved in distilled (nanopure) water)

BaSP activity was measured by the continuous coupled assay, while activity of this enzyme in the PAM immobilized and free *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new cells was measured by using the G1P assay.

The G1P assay was used to determine the activity of the purified CuCBP in the direction of αGlc1-P formation, while the phosphate assay was used to determine the activity in the direction of G2 synthesis. CuCBP activity in the free cells and in the PAM immobilized *E. coli* BL21 (DE3) -*agp*⁻ pBICI_strong_2 cells was measured by using the phosphate assay.

The activity of the purified CcCDP and its activity in immobilized *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new cells was measured by using phosphate assay.

Substrates for the G1P assay were dissolved in 50 mmol/L MES buffer, pH 7.0, while substrates for the phosphate assay were dissolved in 50 mmol/L MES buffer, pH 7.0, prepared with double distilled water (nanopure water). The phosphate assay is very sensitive to phosphate, so use of nanopure water is required.

Table 6. Equipment used in the research

Instrument	**Company/ Institution**
5424R Centrifuge	Eppendorf AG (Hamburg, DE)
5810R Centrifuge A-4-62-MTP	Eppendorf AG (Hamburg, DE)
691 pH Meter	Metrohm (Herisau, CH)
Analytical balance Sartorius LE244S	DWS (IL, USA)
Laminar BioAir AURA-2000 M.A.C.	EuroClone S.p.A. (Milan, IT)
Certomat BS-1 Shaking Incubator	Sartorius (Göttingen, DE)
DU®800 Spectrophotometer	Beckman Coulter (Brea, USA)
Nanodrop (DS-11 Spectrophotometer)	DeNovix Inc. (Wilmington, USA)
Entris® Laboratory Balance	Sartorius (Göttingen, DE)
Luna 5 µm NH_2 column (100 Å, 250 × 4.6 mm)	Phenomenex (Aschaffenburg, DE)
Merck Hitachi L-7100	Merck (Darmstadt, DE)
HPLC YMC-Pack Polyamine II, 12 nm, 5µm, 250 × 4.6 mm	YMC Co., Ltd. (Shimogyo-ku, Kyoto, JP)
Oven universal	Memmert GmbH + Co. KG (Schwabach, DE)
YMC-Pack Guard Cartridge, Polyamine, 5 µm, 20 mm × 4.0 mm	YMC Co., Ltd (Shimogyo-ku, Kyoto, JP)
Variomag® Multi-Magnetic Stirrer	Thermo Fisher Scientific Inc. (Walthman, MA, USA)

3.2. METHODS

3.2.1. A whole cell catalyst preparation for COS production

3.2.1.1. Cultivation of E. coli strains, enzymes expression and cell suspension preparation

Catalyst used for the cellodextrin production *E.coli* BL21 (DE3) -*agp*⁻ was previously transformed with pPOLY_new plasmid, that co-expresses enzymes needed for COS production: BaSP ($Strep_{II}$-tag, GenBank identifier AF543301.1), CuCbP (His_6-tag; GenBank identifier AAQ20920.1), and CcCDP (His_6-tag; GenBank identifier CDZ24361.1).

To avoid degradation of the produced intermediate αG1-P, the glucose 1-phosphatase gene (*agp*) was previously knocked-out of the strain. The plasmid pBICI_strong (Schwaiger et al., 2020), was chosen for co-expression of BaSP, and CuCBP (cellobiose production), as CuCBP activity cannot be determined from *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new catalyst, as produced G2 is a substrate for the cellodextrin synthesis that the third enzyme in the enzymatic cascade, CcCDP, elongates to G3 (or longer chain cellodextrins).

The inoculum for the main cultures [*E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new, and *E. coli* BL21 (DE3) -*agp*⁻ pBICI_strong_2] were obtained from 25 % glycerol stocks, 50 µL of each glycerol stock was transferred into 50 mL of LB medium. These precultures, supplemented with 100 mg/mL Amp, were grown at 30 °C (130 rpm) overnight. Each main culture medium (250 mL LB medium supplemented with 100 mg/L Amp) was inoculated with preculture to an optical density (OD) of 0.03. All cell densities were determined at 600 nm, where the LB medium was used as a blank. Biomass production of both strains was obtained at 37 °C and 135 rpm to an OD of 0.8 to 1.0. Afterward, the enzymes expression in the main cultures was induced by the addition of 500 µL of 0.5 mol/L isopropyl-β-D-thiogalactopyranosid (IPTG), and cells were incubated overnight at 25 °C and 110 rpm. Cultivation of both *E. coli* strains followed the same procedure, and both strains were harvested by centrifugation at 44000 rcf (relative centrifugal force) at 4 °C for 20 min (Sorvall® Evolution™ RC Superspeed Centrifuge, Thermo Fisher Scientific, Waltham, USA), which separated supernatant from the cells (precipitate). The precipitate was weighed, and the volume (50 mmol/L MES buffer pH 7.0) in which cells needed to be resuspended was determined, as follows. Volume of 50 mmol/L MES buffer pH 7.0 was calculated by multiplying the weight of the precipitate by 6, *e. g.* if the weight of the harvested cells was 26.4 g, so the added volume was 158.4 mL [6:1 precipitate weight, volume per weight (v/w)]. By resuspending the cells in the determined volume, the cell

suspension was prepared, which cell wet weight and cell dry weight had to be determined (see Chapter *3.2.1.2.* below). In the final step of the cell suspension preparation, aliquots of ~10 -15 mL portions were stored at -80 °C, and thawed when used again. Mentioned freeze-thawing step, permeabilizes the cell membrane, which reduces the mass-transfer barrier tremendously (Schwaiger et al., 2021).

3.2.1.2. Determination of the cell wet weight and cell dry weight

Cell wet weight (CWW) was determined in triplicates by centrifugation of 1 mL cell suspension for 10 min at 21100 rcf. After the supernatant was carefully removed, the CWW was determined by an analytical balance [Sartorius LE244S analytical balance DWS (IL, United States)]. Afterward, the tubes were left overnight in the oven [Memmert GmbH + Co. KG (Schwabach, DE] at 70 °C for dry cell weight (CDW) determination also by the analytical balance. Measured CDW and CWW were used to express concentration of the prepared cell suspension (mg/mL).

3.2.1.3. Cell free extract preparation

An aliquot of the cell suspension (see Chapter *3.2.1.1.*) was thawed and ultra-sonicated (Branson Ultrasonics^{TM} Microtips Probe 1/8'' dia 418-A, Thermo Fisher Scientific Inc., Waltham, MA, USA) using three times a 6-min run (2 s pulse on, 4 s pulse off, where 30 % amplitude is the distance between horn's vibrating surface position in the horn's fully extended and fully contracted states, measured in microns). Sonication was performed in a plastic beaker with a stirring-bar inserted. The beaker was kept on ice during the sonication and stirred at 200 rpm on a Variomag® Multi-Magnetic Stirrer (Thermo Fisher Scientific Inc. Walthman, MA, USA). The insoluble and soluble fractions of the cell lysate were separated by centrifuging at 21100 rcf at 4 °C for 1 h (5424R Centrifuge, Eppendorf AG, Hamburg, DE).

3.2.1.4. PAM immobilization

Immobilization of both *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new, and *E. coli* BL21 (DE3) -*agp*$^{-}$ pBICI_strong_2 cells in PAM was performed according to the following steps.

In the first step, an aliquot of a 1 g CDW of frozen cell suspension (see Section *3.2.1.1*) was thawed at room temperature (RT, 25 °C). Obtained defrosted cell suspension was centrifuged and resuspended in 100 mmol/L MES buffer, pH 7.0, to a final volume of 20 mL. The suspension was thoroughly stirred on ice. Then, 37.5 g of acrylamide (Sigma Aldrich, St. Louis,

MO, USA) and 0.2 g of *N, N'*-methylenebisacrylamide (Carl Roth, Karlsruhe, DE) were dissolved in the suspension, and 2.5 mL of a 5% 3-dimethylaminoproprionitrile solution [Sigma Aldrich, (St. Louis, MO, USA)] (diluted with nanopure water) was added as a polymerization accelerator. Polymerization itself was started by adding 2.5 mL of a 2.5% potassium persulfate solution (Merck, Darmstadt, DE) (diluted with distilled water). The total volume of the polymerization solution was thus 25 mL, and the process of polymerization was performed on ice at 4 °C for 30 min. The obtained PAM with entrapped whole *E. coli* cells was weighed by an analytical balance and the measured weight was denoted as the weight of the PAM (with immobilized cells). PAM with immobilized cells was then cut by either a hand blender or by hand with a scalpel to particles differing in size. Particles were sieved and resulted in a size of PAM 0.25-2 mm (small particles) or 3-5 mm (big particles), as shown in Figure 2. Cell loading of the PAM particles was determined by dividing the CDW weight (described in Chapter *3.2.1.2.*) with the weight of the PAM (with immobilized cells).

The cell loading of 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$ was used in repetitive batch experiments conducted at 45 °C and 30 °C (see Chapter 3.2.2.), while in the repetitive batch experiment performed at 35 °C an aliquot of 2 g CDW was taken for polymerization in PAM, which resulted in higher cell loading of 63 mg $_{CDW}$/g $_{PAM\text{-}particles}$.

a b

Figure 2. The small (a) and the big PAM particles (b)

3.2.2. Repetitive batch experiments for cellodextrins production

Bioconversion of sucrose and glucose to cellodextrins with the free *E. coli* cells and the PAM particles (see Chapter *3.2.1.4.*) as catalysts were performed in 500 mL borosilicate glass bottles

equipped with magnetic stirrer bars (Economy Rotilabo®, 25 × 8 mm, Carl Roth GmbH, Karlsruhe, DE).

When choosing the free cells as the catalyst, the prepared cell suspension (see Chapter *3.2.1.1.*) was thawed, centrifuged and supernatant was discarded. Volume of cell suspension that was centrifuged and separated from the cells, and to which substrate solution was added was determined by the following equation:

$$V_1 = \frac{V_2 \cdot c_2}{c_1} \quad [1]$$

Where V_1 is the volume of cell suspension which was centrifuged, V_2 is 250 mL (end volume of the reaction suspension), c_1 was the wanted concentration of cell catalyst (3 or 4 g $_{CDW}$/L), c_2 is concentration of the prepared cell suspension (mg $_{CDW}$/mL; see Chapter *3.2.1.2.*).

The precipitate (the *E. coli* cells) was resuspended in 250 mL substrate solution in the final concentration of 4 g $_{CDW}$/L of the reaction volume for repetitive batch experiments conducted at 35 °C, and in the concentration of 3 g $_{CDW}$/L of the reaction volume in the repetitive batch experiments conducted at 40 °C (for the CDW determination see Chapter *3.2.1.2.)*.

In the case when the PAM particles was the catalyst (see Chapter *3.2.1.4.*), the specific mass of the PAM-particles, calculated by using equation [2] (below), which depended on the cell loading, was added to substrate solution resulting in the catalyst concentration of 4 and 3 g $_{CDW}$/L of the reaction volume for repetitive batch experiments conducted at 45 °C and 35 °C or at 40 °C, respectively.

$$m_{PAM-particles} = \frac{c_1}{\text{cell loading}} \cdot V_2 \quad [2]$$

Where c_1 is the catalyst concentration (4 or 3 g $_{CDW}$/L), V_2 is the end volume of reaction suspension (250 mL), where cell loading (mg $_{CDW}$/g $_{PAM\text{-}particles}$) depended on the temperature of the repetitive batch experiments (40 mg $_{CDW}$/g $_{PAM\text{-}particles\ e}$ at 40 and 45 °C, and 63 mg $_{CDW}$/g $_{PAM\text{-}particles}$ at 35 °C).

The reaction volume of the substrate solution was 250 mL containing 500 mmol/L sucrose, 200 mmol/L glucose, 50 mmol/L phosphate in 50 mmol/L MES buffer, pH 7.0. Repetitive batch experiments were conducted at 35, 40 and 45 °C (incubation shaker Certomat BS-1, Sartorius, Göttingen, DE) and at 300 rpm using Variomag® Multi-Magnetic Stirrer (Thermo Fisher Scientific Inc. Walthman, MA, USA). First cycle of the cellodextrins production in repetitive

batch experiments was started by adding either the free or the PAM immobilized cells in the substrate solution, now reaction suspension. After distinctive period of time (at 45 °C after 6 h, at 40 °C after 12 h, and at 35 °C after 8 h) the first cycle was stopped by separating the catalyst from the reaction suspension, now called reactant/product mixture, abbreviated product mixture, as it contains produced COS, insoluble cellodextrins and unused substrates. After separation, the samples of the gained product mixture (without catalyst) were stored at 4 °C until HPLC measurement was performed (see Chapter 3.3.3.).

If the PAM particles was used as a catalyst, the separation step was done by sieving the PAM particles from the reactant/product mixture by using sieve with 180 µm cut off. Now the separated PAM particles (catalyst) was washed with deionized water and afterward, applied directly to a new substrate solution (repetitive batch reaction at 40 °C) or stored overnight at 4 °C until the next cycle was started (repetitive batch reactions at 35 and 45 °C).

When the free cells were used as a catalyst, cells were separated from the reaction suspension by centrifugation, 4400 rcf at 4 °C for 20 min (Sorvall® Evolution^{TM} RC Superspeed Centrifuge, Thermo Fisher Scientific, Waltham, USA), which resulted in product mixture and separated whole cells, which were either resuspended in 50 mmol/L MES buffer, pH 7.0, and stored at 4 °C until next cycle of the repetitive batch experiment was conducted (repetitive batch experiments conducted at 35 and 45 °C), or directly applied to the fresh substrate solution (repetitive batch experiments at 40 °C).

To remove insoluble cellodextrins and enzymes from obtained product mixtures that may have leaked from the PAM particles the product mixture was centrifuged (20 min, 4 °C, 15000 rcf Ultracentrifuge Sorvall RC-5B Superspeed, Thermo Fisher Scientific Inc., Waltham, MA, USA), and supernatant was stored at 4 °C until further downstream processing was performed, *i.e.* removal of fermentable sugars (fructose, glucose, sucrose) from the product mixtures.

3.3. ANALYTICS

3.3.1.SDS-PAGE and staining

SDS-PAGE was performed to confirm the expression of three desirable enzymes. Preparation of *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new cell free extract is already described in Chapter *3.2.1.3.* Cell free extract was centrifuged at 15000 rcf for 2 min (5424R Centrifuge, Eppendorf AG, Hamburg, DE) and the supernatant was then diluted to a total soluble *E. coli* protein concentration of 2 mg/mL. The samples were prepared by taking 10 µL of the diluted

supernatant and mixing it with 10 µL of a previously prepared master mix, consisting of 5 µL NuPAGE™ LDS sample buffer (4×), 4 µL nanopure water and 1 µL DTT (1 mol/L) solution. The prepared samples were incubated at 95 °C for 5 min to denature the proteins before loading (10 µL) them onto NuPAGE™ 4 to 12 % Bis-Tris gel. The PageRuler™ Prestained Protein Ladder (10 to 180 kDa) was used as a standard (5 µL). The electrophoresis was performed at 170 V for 60 min in NuPAGE™ 1×MOPS buffer. After SDS-PAGE was done, the gel was stained by a staining solution for one hour or longer. Subsequently the gel was distained to visualize protein bands. Another SDS-PAGE was performed to identify the purity of BaSP, CUCBP, CcCDP obtained from BIOTE.

3.3.2. Determination of the protein concentration

The protein concentration was determined using the Roti®-Quant (Carl Roth GmbH, Karlsruhe, DE) according to Bradford (Kruger, 2009). The sample (10 µL) was mixed with 500 µL of the 1:5 diluted dye reagent and after 15 min of incubation at RT, the absorbance was measured at 595 nm. Calculation of the concentration was done using a bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) calibration curve in the range of 0.1 to 1.0 mg/mL.

3.3.3. HPLC measurements

Quantification of main components (sucrose, fructose, glucose, G2, G3, G4, G5, G6) of the product mixture was performed by HPLC method using a Merck Hitachi L-7100 system (Merck, Darmstadt, DE) equipped with an autosampler and refractive index (RI) detector.

Analysis of sucrose, fructose, glucose and G2 was performed with hydrophilic interaction chromatography column YMC-Pack Polyamine II/S-5 µm/12 nm column (250 mm × 4.6 mm, YMC Co., Ltd., Shimogyo-ku, Kyoto, JP) and an additionally installed pre-column (YMC-Pack Guard Cartridge, Polyamine 20 mm × 4.0 mm; YMC Co., Ltd., Shimogyo-ku, Kyoto, JP). The flow rate of an acetonitrile-water mixture (75:25, v/v) of 1 mL/min was performed isocratically. Separations were performed at RT, the injection volume per sample was set to 20 µL and 35 min run time.

COS of different DP were analysed using a hydrophilic interaction chromatography column Luna 5 µm NH_2 column (100 Å, 250 × 4.6 mm; Phenomenex, Aschaffenburg, DE) at 40 °C. Acetonitrile-water (67.5:32.5, v/v) was used as the mobile phase at a flow rate of 1.5 mL/min and a run time of 25 min with the injection volume per sample set to 20 µL.

In both cases (quantification of sucrose, fructose, glucose, G2, G3, G4, G5, G6) detected RI peaks were analysed using the software Chromeleon Chromatography Data System (Thermo Fischer Scientific Inc., Waltham, MA, USA). The calibration was done with authentic standards sucrose, fructose, glucose, and G2, G3, G4, G5, G6 (Megazyme Ltd., Wicklow, IE).

3.3.4. Activity assay

3.3.4.1. Continuous coupled assay

This assay is based on the conversion of αGlc1-P, which is being released during the assay procedure. The continuous coupled assay mixture (see Table 5) consisted of the assay buffer (50 mmol/L KH_2PO_4, 10 mmol/L $MgCl_2$, 10 mmol/L EDTA, pH 7.0, and solid crumbles of glucose-1,6-bisphophate), 40 µL of 20 mg/mL NAD^+ dissolved in distilled water, 2 µL of 958.5 U/mL G6P-DH [21 µL of G6P-DH (Sigma-Aldrich/Merck (Darmstadt, DE) dissolved in 79 µL 50 mmol/L MES buffer, pH 7.0], 2 µL of 846 U/mL PGM (32 µL of the enzyme was diluted with 68 µL 50 mmol/L MES buffer, pH 7.0), and 10 µL of properly diluted purified enzyme. The reaction is started by adding the substrate. αGlc1-P is converted by phosphoglucomutase (PGM) from rabbit muscle (Sigma-Aldrich/Merck, Darmstadt, DE) into D-glucose-6-phosphate, which can be assayed by using NAD^+-dependent D-glucose-6-phosphate dehydrogenase (G6P-DH) from *Leuconostoc mesenteroides* (Sigma-Aldrich/Merck, Darmstadt, DE). Conversion of NAD^+ to NADH in the G6P-DH catalysed reaction is then monitored spectrophotometrically at 340 nm (DU® 800 UV/Vis Spectrophotometer, Beckman Coulter, Brea, CA, USA). The assay is performed at 30 °C for 15 min by measuring absorbance of the mixture every 16.8 s. One unit (U) of the enzyme activity (a) is defined as the enzyme amount producing 1µmol of αGlc1-P per min under stated conditions. The absorbance per minute ($\frac{\Delta E}{\Delta t}$) was obtained and used in the activity (a) estimation, as calculated by using the following equation:

$$a = \frac{\Delta E}{\Delta t} \cdot \frac{Vtotal}{Vsample \times \varepsilon \times d} \quad [3]$$

V_{total} is the total volume of the assay mixture (564 µL) with V_{sample} as a sample volume (10 µL), the molar absorption coefficient for NADH (ε) is 6.22 L/(mmol cm) and d (1 cm) is the light path length through the cuvette.

3.3.4.2. G1P assay

The G1P assay is actually based on the same principle as the continuous coupled enzymatic assay, as previously described (see Chapter *3.3.4.1*.). There is one alteration in the G1P assay compared to the continuous coupled assay in which αGlc1-P production is measured during the assay, while in the G1P assay previously produced αGlc1-P is measured. In both assays, αGlc1-P concentration is measured by the reduction of NAD^+ to NADH in the PGM/G6P-DH catalysed reaction.

Before determining the concentration of the produced αGlc1-P by the G1P assay, the reaction mixture that produces αGlc1-P consists of a substrate solution (depending on the enzyme or type of catalyst) and a properly diluted catalyst (purified enzyme, free or immobilized cell catalyst), details in Chapters *3.3.5.2, 3.3.6.1.* Every two min, up to 12 min, a sample is taken out of the reaction mixture, heated to 99 °C for five min, and further analysed by the G1P assay to determine the concentration of released αGlc1-P.

The G1P assay mixture (see Table 5) for determination of concentration of produced αGlc1-P consists of: 400 µL of assay buffer (50 mmol/L Tris-HCl buffer, pH 7.7; 10 mmol/L $MgSO_4$, 10 mmol/L EDTA, pH 7.0, and solid crumbles of glucose-1,6-bisphophate), 2 mmol/L NAD^+ (dissolved in distilled water).

Volume of 40 µL of the sample, which was taken every 2 min until 12 min reaction time from the reaction mixture, was then added to the assay mixture. After adding 2 µL of 350 U/mL G6P-DH to the assay mixture, ~5 min later the absorbance of already present NAD^+ was measured (A1) at 340 nm. When the A1 value no longer increased, 400 U/mL PGM was added to the test mixture and the absorbance (A2) was read at 340 nm until the reaction was complete, *i.e* until there was no change in absorbance. The enzyme activity (a), was calculated by using the following equation:

$$a = \frac{\Delta A \times Vtotal}{Vsample \times \varepsilon \times d} \quad [4]$$

ΔA=A2-A1, V_{total} = 454 µL, ε = 6.22 L/(mmol cm) and d = 1 cm.

3.3.4.3. The phosphate assay

This colorimetric assay is based on measuring released inorganic phosphate (Pi), which is released from αGlc1-P (Saheki et al., 1985). Prior to performing the phosphate assay, a reaction where αGlc1-P is formed needs to be conducted. The reaction where αGlc1-P is formed starts

by adding a properly diluted catalyst (volume of purified enzyme or free / immobilized cells) to a substrate solution (volume and composition depending on the type of enzyme or catalyst), more details are available in Chapters *3.3.5.2., 3.3.5.3., 3.3.6.2., 3.3.6.3*. The substrates are dissolved in 50 mmol/L MES buffer, pH 7.0 (MES dissolved in nanopure water). Every two min, up to 12 min, a sample (volume depends on the catalyst – purified enzyme, the immobilized or the free cells) is taken out of the reaction mixture, heated to 99 °C for five min, and further analysed by the assay colorimetric phosphate assay (Saheki et al., 1985).

Pi forms a complex with ammonium molybdate in the presence of Zn^{2+}, which strongly absorbs ultraviolet light (Saheki et al., 1985). Namely, the phosphomolybdate complex is reduced by ascorbic acid (Carl Roth GmbH, Karlsruhe, DE) (Table 1) and the chromophore produced shows maximum absorption at 850 nm. In the absence of phosphate, the chromophore is not produced, while the presence of Zn^{2+} the phosphomolybdate complex achieves a maximum absorption at 350 nm.

Measurements were done in 96 well-plate by adding 9 µL of sample (taken every 2 min until 12 min reaction time), 141 µL of the assay buffer (see the phosphate assay in Table 5) and after 15 min incubation at 25 °C the absorbance was read at 850 nm against blank (nanopure water). Calculation of the inorganic phosphate concentration was done using a calibration curve in the range 0.0 to 1.0 mmol/L phosphate, where K_2HPO_4 was used for preparation of standards. One unit (1 U) of the enzyme activity is the amount of the enzyme producing 1 µmol phosphate per min under the stated conditions.

3.3.5. Determination of the enzymes activities

The three purified enzymes were already available at the BIOTE, and their activities were measured by using activity assays described in Chapter 3.3.4. BaSP activity was measured at 30 °C by using continuous coupled assay. CuCBP activity was measured at two temperatures, 30 and 45 °C by using G1P and phosphate assay, depending on the reaction direction. CcCDP activity was also measured at two temperatures (30 and 45 °C) by using phosphate assay.

3.3.5.1. BaSP activity

The continuous coupled assay (see Chapter *3.3.4.1.*) was used to determine the activity of BaSP in phosphorolysis direction (N = 3), where properly diluted purified BaSP enzyme (obtained from BIOTE) was added as a sample (10 µL). The continuous coupled assay measurement

started by adding substrate (130 µL of sucrose, 250 mmol/L). The activity of BaSP was followed according reaction shown in Figure 3.

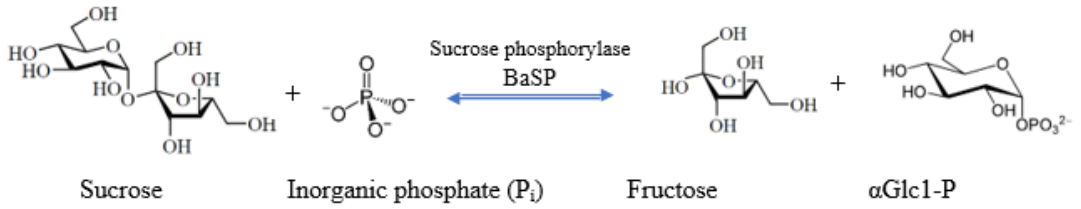

Figure 3. The reaction scheme of BaSP: the conversion of sucrose to D-fructose and αGlc1-P in the presence of inorganic phosphate (Pi)

3.3.5.2. CuCBP activity

Since CuCBP is performing a reversible reaction, as shown in Figure 4, the enzyme activity had to be measured both ways (Hidaka et al., 2006). αGlc1-P formation (phosphorolysis) was measured using the αGlc1-P assay (the G1P assay, Chapter *3.3.4.2*), while G2 synthesis was measured by using the phosphate assay (Chapter *3.3.4.3*.). Both - G2 phosphorolysis (the αGlc1-P formation) and G2 synthesis (the phosphate formation) were measured at two different temperatures - 30 and 45 °C.

To determine activity towards αGlc1-P formation (phosphorolysis) the total volume of the reaction mixture (V_{total}= 600 µL) consisted of 540 µL reaction solution (50 mmol/L G2 and 50 mmol/L K_2HPO_4 dissolved in 50 mmol/L MES buffer, pH 7.0) and 60 µL of properly diluted purified CuCBP (the BIOTE). Every two min, up to 12 min, a 100 µL sample was taken out of the reaction mixture, heated to 99 °C for five min, and further analysed by the G1P assay (see Chapter *3.3.4.2*.).

The phosphate assay (see Chapter *3.3.4.3*.) was used to determine CuCBP activity towards G2 synthesis, where reaction mixture (V_{total} = 600 µL) consisted of 540 µL of the reaction solution (50 mmol/L αGlc1-P and 50 mmol/L glucose dissolved in 50 mmol/L MES buffer (dissolved in nanopure water), pH 7.0) and 60 µL of properly diluted purified CuCBP (the BIOTE). Every two min, up to 12 min, a 100 µL sample was taken, heated to 99 °C for five min, and further analysed by the colorimetric assay (Saheki et al., 1985) in which the phosphate (Pi) is released from αGlc1-P.

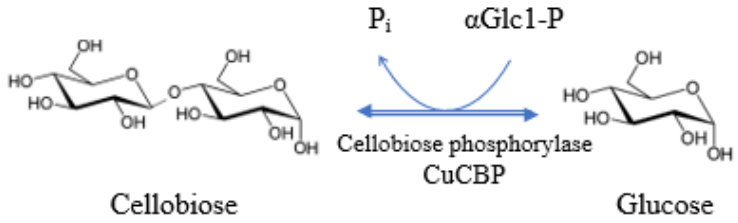

Figure 4. The reaction scheme of the reversible reaction catalysed by CuCBP

3.3.5.3. CcCDP activity

The CcCDP conducts a reaction shown in Figure 5, and its activity was measured only towards cellodextrin production by using two types of substrate - G2 and its analogue, *p*-nitrophenyl β-D-cellobioside (PNP-C). The measurements were performed at 30 and at 45 °C by using the phosphate assay (see Chapter *3.3.4.3.*).

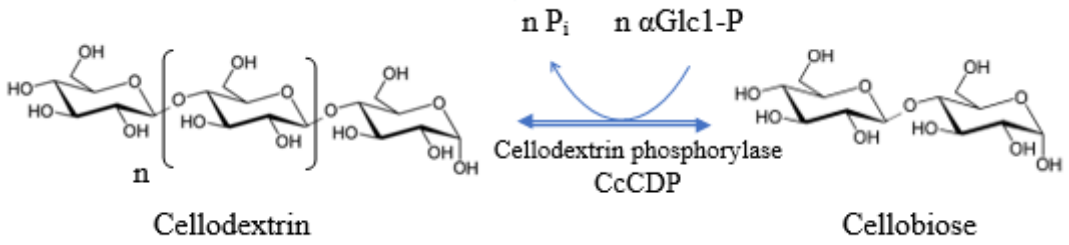

Figure 5. CcCDP reaction scheme: the enzyme uses short cellodextrins (DP ≤ two) and αGlc1-P as substrates. n symbolizes the number of reactions that CcCDP conducts. If n = 1, CcCDP binds D-glucose to G2 by β-(1,4) linkage, and formed cellodextrin is denoted as G3. For n=2 or more, CcCDP binds D-glucose to G3 or longer-chain cellodextrin. For COS production n can be from one to four [G3 to G6], including also G2

The substrate solution consisted of 50 mmol/L of G2 or PNP-C and 50 mmol/L αGlc1-P as substrates in 50 mmol/L MES buffer (dissolved in nanopure water), pH 7.0. Reactions were started by adding 60 µL of purified CcCDP (the BIOTE), to a preheated 540 µL of the substrate solution. Every two min, up to 12 min, a 100 µL sample was taken, heated to 99 °C for five min, and further analysed by measuring released phosphate from αGlc1-P by the colorimetric assay, as described previously (see Chapter *3.3.4.2.*).

3.3.6. Determination of the activity of the three enzymes (BaSP, CuCBP, and CcCDP) in free *E. coli* cells and in the immobilized cells (PAM-particles)

Before estimation of the activities of the three enzymes (BaSP, CuCBP, and CcCDP) that react in a cascade and produce the COS, the specific volume of the cell suspension (see Chapter *3.2.1.1*) or the specific weight of the PAM particles (see Chapter *3.2.1.4*) were determined. The specific volume of the cell suspension or the specific weight of the PAM particles was added to the substrate solutions, corresponding the enzyme which activity was determined. In table 7 are listed strains and types of catalyst, and activity assays used for determination of each enzyme activity. The activities of CuCBP and CcCDP were determined by using the substrate solutions described in details in Chapters *3.3.6.2.* and *3.3.6.3.*, respectively, and also in Table 7. If the cell suspension was used as a catalyst, the specific volume added to the substrate solution was calculated by the equation [1], where V_1 is the volume of the cell suspension added to the substrate solution, V_2 = 2.0 mL (end volume of the reaction mixture), c_2 is the desired concentration of the cell catalyst (1.0, 0.5 or 0.25 mg $_{CDW}$/mL), these concentrations were determined by preliminary tests which ensured higher measurement accuracy, and c_1 is concentration of cell suspension (mg $_{CDW}$/mL; see Chapter *3.2.1.2.*).
In the case of determining the enzymes activities in the PAM immobilized cells, the weight of the PAM particles (mg) added to a 2.0 mL preheated substrate mixture solutions was calculated by the equation [2], where concentrations of the cell catalyst were 1.0, 1.5 or 3.0 mg $_{CDW}$/mL, V is the end volume of the reaction mixture (2.0 mL), where cell loading (mg $_{CDW}$/g $_{PAM\text{-}particles}$) depended on the temperature of the repetitive batch experiments.

Table 7. Enzymes, catalysts, catalyst type and methods for determination of the enzymes activities (see Chapter *3.3.4.3.*)

Enzyme	A whole cell catalyst	Catalyst type	activity assay
BaSP	*E.coli* BL21 (DE3) -*agp*$^-$ pPOLY_new	Immobilized cells / Free cells	the G1P assay
CuCBP	*E.coli* BL21 (DE3) -*agp*$^-$ pBICI_strong_2	Immobilized cells / Free cells	the phosphate assay
CcCDP	*E.coli* BL21 (DE3) -*agp*$^-$ pPOLY_new	Immobilized cells / Free cells	the phosphate assay

3.3.6.1. BaSP activity

Sucrose phosphorylase activity was measured in the free *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new cells and in *E.coli* BL21 (DE3) -*agp*⁻ pPOLY_new PAM immobilized cells by using the G1P assay (see Chapter *3.3.4.2.)*. The reaction mixture for determining the BaSP activity consisted of substrate solution (250 mmol/L sucrose and 50 mmol/L K_2HPO_4; E. Merck KG, Darmstadt, DE) dissolved in 50 mmol/L MES buffer, pH 7.0, and the catalyst. In the case of determining BaSP activity in the free cells, specific volume of *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new cell suspension added to the substrate solution was calculated by using equation [1]. In the case of determining BaSP activity in the PAM immobilized cells, the specific weight of the PAM particles was calculated by equation [2], which was added to the substrate solution.

The reaction was started by adding the catalyst to the substrate solution with an end volume of 2.0 mL. Every two min, up to 12 min, a 200 µL sample was taken out, heated to 99 °C for five min, and further analysed to measure released αGlc1-P. The procedure for determining the released αGlc1-P is already described in Chapter *3.3.4.2.*

3.3.6.2. CuCBP activity

The CuCBP activity was measured in the free and in the PAM immobilized *E. coli* BL21 (DE3) -*agp*⁻ pBICI_strong_2 cells, which only expressed two (BaSP and CuCBP) out of the three enzymes (no CcCDP). Determination of CuCBP activity in the PAM immobilized cells in absence of the CcCDP activity was crucial to avoid interference deriving from CcCDP activity. The measurements of CuCBP activity were performed by adding the catalyst, *i.e.* specific volume of the free or the *E.coli* BL21 (DE3) -*agp*⁻ pPOLY_new PAM immobilized cells - small particles, to the substrate solution (50 mmol/L αGlc1-P and 50 mmol/L glucose dissolved in 50 mmol/L MES buffer - dissolved in nanopure water, pH 7.0).

Specific volume of the free cells was calculated by using equation [1], while the specific weight of the PAM particles was calculated by using equation [2]. Every two min, up to 12 min, a 200 µL sample was taken, heated to 99 °C for five min, and further analysed by measuring released phosphate from αGlc1-P by the colorimetric assay (see Chapter *3.3.4.3.*).

3.3.6.3. CcCDP activity

The CcCDP activity was measured by the phosphate assay (see Chapter *3.3.4.3.*) in the direction of cellodextrin synthesis, where the substrate solution consisted of 50 mmol/L PNP-C and

50 mmol/L αGlc1-P, dissolved in 50 mmol/L MES buffer (dissolved in nanopure water), pH 7.0. The substrate analogue (PNP-C) was used to prevent possible interference from ultimately present CuCBP which has a too small active site to accommodate the analogue (Zhong et al., 2020a). In this way, it is ensured that only CcCDP activity is being measured, as PNP-C is not a suitable substrate for CuCBP catalysed phosphorolysis reaction. Reactions were started by adding the catalyst - the free *E.coli* BL21 (DE3) -*agp*⁻ pPOLY_new cells or the *E.coli* BL21 (DE3) -*agp*⁻ pPOLY_new immobilized cells to a substrate solution to an end volume of 2 mL. Every two min, up to 12 min, a 200 µL sample was taken, heated to 99 °C for five min, and further analysed by measuring released phosphate from αGlc1-P (see Chapter *3.3.4.3.*).

3.3.6.4. Activity ratio of the three enzymes

Activity ratio of the three enzymes (BaSP, CuCBP and CcCDP) was calculated after determining their activities (Chapters *3.3.6.1-3.3.6.3*). The activities are expressed in U/mg, based on the measured activities in the free cells or in the PAM immobilized cells (mg $_{CDW}$). Production of COS greatly depends on the activity ratio of the enzyme cascade.

3.3.7. Total turnover number and space time yield

To describe production efficiency, values were expressed as total turnover number (TTN; equation [5]) and space time yield (STY; equation [6]).

$$TTN = \frac{c_t}{c_1} \quad [5]$$

$$STY = \frac{c_t}{t} \quad [6]$$

where c_t is the total concentration of COS (g/L) referring to cellodextrins with $2 \leq DP \leq 6$; c_1 is the catalyst concentration (g $_{CDW}$/L), and t is the reaction time (h).

4.RESULTS AND DISCUSSION

As the main goal of this Thesis was to produce COS, *E. coli* BL21(DE3) *agp*⁻ pPOLY_new was used as a catalyst, since it expresses enzymes BaSP, CuCBP, CcCDP that react in the enzymatic cascade producing COS and insoluble cellodextrins. *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cells were immobilized in the PAM (Chapter *3.2.1.4.*), and in its immobilized form used in repetitive batch reaction experiments as a catalyst. In these experiments, it was important to ensure that the activity of the three enzymes (BaSP, CuCBP and CcCDP), as well as their stability during the batch cycles, maintained sufficient value so that most of the produced cellodextrins remains soluble.

4.1 EXPRESSION OF BaSP, CuCBP, and CcCDP IN *E. coli* BL21(DE3) *agp*⁻AND ACTIVITY OF FREE AND PAM IMMOBILIZED CELLS

As the main goal of this Thesis was to produce the COS, the used cell catalyst had to express three specific enzymes: BaSP, CuCBP, and CcCDP, that act efficiently in a highly dynamic enzymatic cascade. A whole cell catalyst *E. coli* BL21(DE3) *agp*⁻ pPOLY_new carries the plasmid for their expression, and therefore efficiency of BaSP, CuCBP, CcCDP expression was examined (Figure 6).

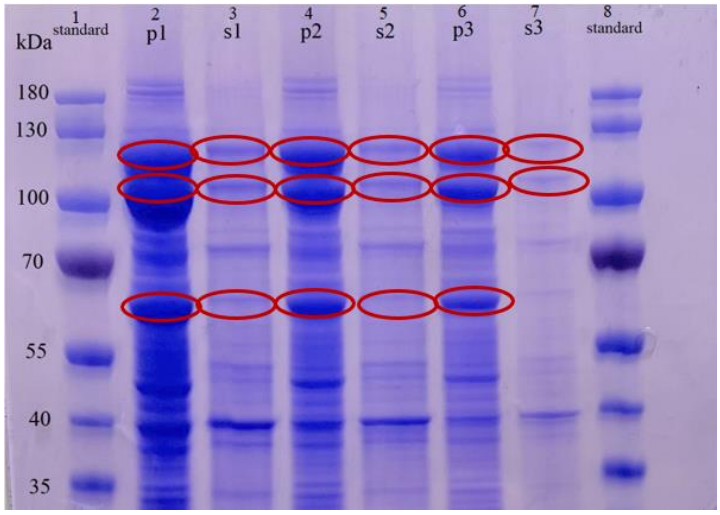

Figure 6. SDS-PAGE of pellets (p) and supernatants (s) obtained after centrifugation of *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cell extract (see chapters 3.3.1, *3.2.1.3*) Lines 1 and 8, standard PageRuler™ Prestained Protein Ladder; lines 2, 4 and 6, pellets diluted in the 50 mmol/L MES buffer, pH 7.0, with different ratios of pellets and the MES buffer (p2, 1 : 2; p2, 1 : 5; p3, 1 : 10); lines 3, 5 and 7, supernatants (s) also diluted in the MES buffer in the same ratios. Red circles indicate bands of tagged proteins: CcCDP (112.8 kDa), CuCBP (92.7 kDa) and BaSP (57.7 kDa)

Figure 6 shows that *E. coli* BL21(DE3) *agp*⁻ pPOLY_new successfully co-expressed the three desired enzymes - BaSP, CuCBP, and CcCDP based on their molecular mass. Further, stability of the PAM immobilized *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cells are compared to the stability of the free *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cells (Figure 7). Experiment was carried out as described in Chapter 3.3.6., where *E. coli* BL21(DE3) *agp*⁻ pPOLY_new catalyst was used for estimation of stability of BaSP and CcCDP, while CuCBP activity was determined in *E. coli* BL21(DE3) -*agp*⁻ pBICI_strong_2.

The optimal temperature for the phosphorylase activity of the BaSP is 58 °C, which is surprisingly high for an enzyme from a mesophilic source - *Bifidobacterium adolescentis* (Cerdobbel et al., 2010), while temperature optima of the remaining two enzymes (CuCBP and CcCDP) is between 45 °C and 55 °C (Zhong and Nidetzky, 2020). Based on these data, first experiment was conducted at 45 °C to establish if immobilization of the catalyst in PAM is sufficient enough for maintaining enzymes activities. This activity experiment was performed by leaving the immobilized *E. coli* cell catalyst in the reaction suspension for 48 h at 45 °C, and the enzymes activities were measured after 0, 24 and 48 h.

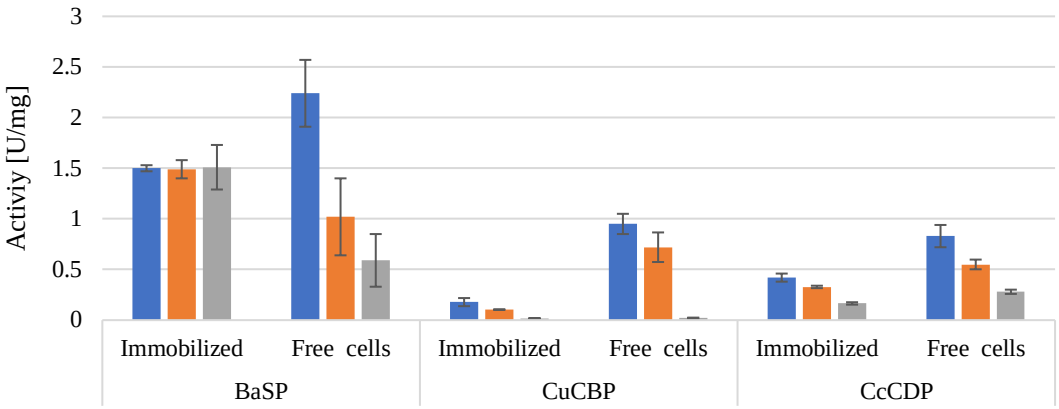

Figure 7. Comparison of the enzymes activities in the free cells and the PAM immobilized cells. The activities were determined at 45 °C, as described in Chapter 3.3.6. at different time points [*t* = 0 h (■); 24 h (■); 48 h (■)]

At the reaction temperature of 45 °C BaSP loses 64 % of its activity over 48 h in the free *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cells, while there is no decrease of BaSP activity in the PAM *E. coli* BL21(DE3) *agp*⁻ pPOLY_new immobilized cells over the same period of time. The decrease of the CcCDP activity in free *E. coli* BL21(DE3) *agp*⁻ pPOLY_new cells (66 %) is similar to the decrease in the PAM *E. coli* BL21(DE3) *agp*⁻ pPOLY_new immobilized cells (60 %).

CuCBP activity couldn't be measured in the *E. coli* BL21(DE3) *agp*⁻ pPOLY_new catalyst as G2, which is produced by CuCBP, is further converted by CcCDP to COS with different DPs. So, CuCBP activity was measured in free *E. coli* BL21 (DE3) -*agp*⁻ pBICI_strong_2 cells and in the PAM *E. coli* BL21 (DE3) -*agp*⁻ pBICI_strong_2 immobilized cells, and obtained results show that CuCBP loses almost completely its activity after 48 h at 45 °C.

Based on obtained data it was concluded that the immobilization of the *E. coli* in the PAM is sufficient for maintaining at least one enzyme activity (BaSP) and it is important to further optimize use of the whole cell immobilized catalyst. Therefore, the activities of the three purified enzymes were determined (see below).

4.2.THE ACTIVITIES OF PURIFIED ENZYMES - BaSP, CuCBP, AND CcCDP AT TWO DIFFERENT TEMPERATURES

Preliminary determinations of the activities of the three purified enzymes (BaSP, CuCBP and CcCDP) have been performed to determine the optimal temperature range for their activity. The

purified proteins (BaSP, CuCBP and CcCDP) have been already available (the BIOTE). First, purity of the proteins was checked by SDS-PAGE (Figure 8).

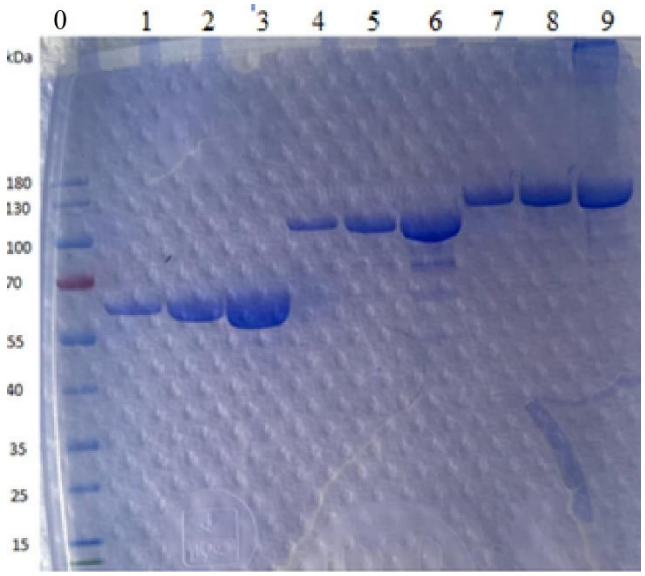

Figure 8. SDS-PAGE of the three purified proteins - BaSP, CuCBP and CcCDP. Three different concentrations of each protein were used for the SDS-PAGE (0.5, 1.0 and 2.5 mg/mL). Line 0, standard PageRuler^{TM}Prestained Protein Ladder; lines 1 - 3, BaSP; lines 4 - 6, CuCBP; lines 7 - 9, CcCDP

After confirming that obtained enzymes were successfully purified, the activities of the three purified enzymes were determined, as described previously (Chapter 3.3.5.). The BaSP activity was measured at 30 °C, in the phosphorolysis direction by the continuous coupled assay (Chapter *3.3.5.1.*), 117.3 ± 10.3 U/mg, which corresponds to the literature data (van den Broek et al., 2004). Because of relatively high activity compared to the other two enzymes (CuCBP, CcCDP), there was no concern regarding reverse reaction catalysed by this enzyme (the synthesis). Also, results presented in Figure 7 clearly show that this enzyme should not be the weakest in the three-enzyme reaction cascade. Therefore, it remained to determine the activity of the two remaining enzymes (CuCBP and CcCDP), as described before (Chapters *3.3.5.2.* - *3.3.5.3.*), and obtained results are presented in Tables 8 and 9.

Table 8. The activity of the purified CuCBP (U/mg)

temperature (°C) /direction	phosphorolysis	synthesis	factor (-)
30	4.86 ± 1.01	11.2 + 0.21	2.3
45	16.04 ± 1.78	21.88 ± 0.76	1.3

The CuCBP activity in the phosphorolysis direction at both temperatures is considerably lower than its activity in the synthesis direction. Having in mind that the concentrations of G2 and, on the other side of the reaction, glucose and αGlc1-P, were the same (50 mmol/L, see Chapter *3.3.5.2.*) the CuCBP dynamic equilibrium leans towards G2 synthesis, which is the desired direction for the COS production.

Table 9. The activity of the purified CcCDP (U/mg) - the synthesis direction

temperature (°C)	Substrate		factor (-)
	PNP-C	Cellobiose (G2)	
30	8.1 ± 0.1	4.4 ± 0.3	1.8
45	17.7 ± 2.1	14.0 ± 1.4	1.2

Here, the affinity of the purified CcCDP towards PNP-C, the substrate analogue, and its natural substrate – G2 at two different temperatures can be compared. The PNP-C cannot be used as a substrate by CuCBP (the active site of CuCBP cannot accommodate the PNP-C molecule) and, therefore, by using this substrate only CcCDP activity was reached. The affinity of the CcCDP for PNP-C is higher than for G2, as clearly proven by the results (Table 9). All further determinations of the CcCDP activities reported in this Thesis were accomplished by using the PNP-C. This implies that actual CcCDP activity should be corrected for the factor from Table 9, because the reactions catalysed by CcCDP, which occurs within *E. coli* BL21(DE3) *agp*⁻ pPOLY_new, requires their natural substrate - G2, and not its analogue which is used for activity determinations.

Obtained activities of the purified enzymes (Tables 8 and 9) provided initial information about the temperature range (35, 40 and 45 °C) in which further experiments with *E. coli* BL21(DE3) *agp*⁻ pPOLY_new in its free form and the PAM immobilized form should be performed (details in Chapter 3.3.6.).

4.3.PRODUCTION OF COS BY REPETITIVE BATCHES

4.3.1. Loss of proteins from the PAM during the repetitive batch experiments

The production of the COS depends largely on the immobilization technique required for enzymes and cell-protection. While PAM provides high mechanical strength, the acrylamide monomer is highly toxic, hence, entrapped cells or enzymes can be very easily harmed during polymerization (Li, 2017) and further use of immobilized catalyst. As clearly shown in Figure 7, where enzymes activities in the free cells are much higher than in the PAM immobilized cells, toxicity of the acrylamide monomer might be the main reason for this activities decrease. So, when compared with the free cells, the BaSP, the CcCDP and the CuCBP activities decrease due to the PAM immobilization by 32, 54 and 81 %, respectively. Another reason for the activities decrease can be the cell leakage from the PAM matrix, which is unavoidable due to differences in osmolarity between the PAM matrix and the storing 50 mmol/L MES buffer, pH 7.0. The two forms of catalyst (the free cells and the PAM immobilized cells) were used for the production of COS by the enzymatic cascade in repetitive batches experiments (see Chapter 3.2.2.). Additionally constant stirring causes PAM particles degradation, and possibly leads to cell leakage from the PAM matrix and also protein leakage from the cells. The inevitable swelling and shrinking properties of PAM occur when reaction conditions change, and its intensity depends on the gel composition and temperature difference, *e.g.* during the storage (4 °C) and the batch reactions (35, 40 and 45 °C), as already reported by Neamtu et al. (2006). Therefore, it was necessary to define the protein leakage from the PAM immobilized cells (Figure 9).

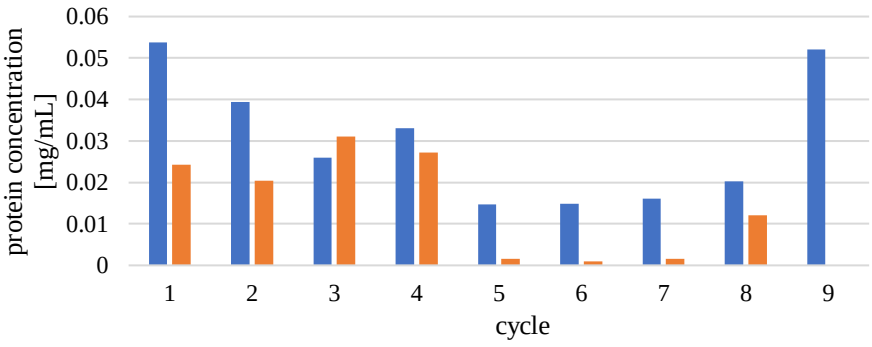

Figure 9. Leakage of proteins from the small PAM immobilized *E. coli* BL21(DE3) *agp*⁻ pPOLY_new particles in the reactant/product mixture (blue) or in the storage MES buffer (orange) over nine repetitive batches at 35 °C . The measurements were performed as described in Chapters *3.2.1.4.* and 3.3.2.

The concentrations of total proteins in the reactant/product mixture or in the storage MES buffer were determined by the Bradford method (see Chapter 3.3.2.). The total protein concentrations shown in Figure 9 are quite low and at the photometer accuracy limit, so the obtained data give only an approximate value of the leaked proteins, but the detection itself proves that the leak has occurred. Certainly, the temperature difference affects the PAM properties: at the storage MES buffer temperature (4 °C) the particles shrink, while in the repetitive batch experiments (*e.g.* 35 °C) the particles swell. In addition, constant stirring promotes cell leakage of the poorly entrapped cells, and consequently also protein leakage.

The weight of the small PAM particles after the first few cycles of each batch at all three temperatures (35, 40 and 45 °C) was measured by an analytical balance and there were no significant differences between cycles, while the volume of the particles increased and their colour got brighter, from brownish to yellow-white. The increase in the volume and the change of the particle colour can be explained by the accumulation of insoluble cellodextrins in the PAM pores. It can also be assumed that over the same time whole cells leaked from the PAM matrix. The weight of the PAM with immobilized cells was also decreasing and *e.g.* at the beginning of the experiment performed at 35 °C the mass of the immobilized catalyst was 15.77 g and after nine batch cycles was only 9.5 g, which is a 40 % loss in weight. This weight loss can be explained by PAM degradation which was most likely caused by constant stirring of the reactant/product mixture and MES storage buffer.

Further, the storage time of the catalysts (the free cells and the PAM immobilized cells) affects COS production because it prolongs the total time during which the catalysts are submitted to the mixing. Over time, the cell and the protein leakage inevitably occurs, and it is greater at the beginning of the repetitive batches experiments, and, fortunately, decreases over time.

Thus, an alternative immobilization technique - microencapsulation technology can be used to overcome the disadvantages encountered with the PAM cell entrapment, such as limited cell loading due to the small void space of the polymer matrix and cell leakage (Zhu, 2007). The LentiKats capture technique also seems promising, with the advantages listed before in this Thesis in Chapter 2.3.1.

4.3.2. Activity of the three enzymes in the free cells and the small PAM particles

Stability and activity tests of the three enzymes in the free cells and the small PAM particles (diameter of 0.25 - 2.0 mm) were performed. Preparation of the PAM immobilized cells is described in detail in Chapter *3.2.1.4.* Briefly, the G1P assay for BaSP activity was performed

(Chapter *3.3.5.2.*), while the activity of CcCDP and CuCBP were obtained by using the phosphate assay, as described elsewhere (Saheki et al., 1985; Chapters *3.3.5.2.*, *3.3.5.3.*). Obtained results are presented in Figures 7 and 10.

Determined activities of BaSP, CuCBP, and CcCDP in the free cells and in the small PAM particles are shown in Figure 7. Since the CcCDP activity was always measured with PNP-C as a substrate, this activity should be corrected for the factor of 1.2 (see Table 9). The activity ratios at 45 °C were calculated (see Chapter *3.3.6.4.*) for the three enzymes (BaSP/CuCBP/CcCDP) in the free cells and in the small PAM particles, 2.3/1.0/0.8 and 1.5/0.2/0.4 U/mg, respectively. The results from Zhong and Nidetzky (2020) suggest that the optimal activity ratio for the conversion performed by the three enzymes at 45 °C is 10.0/3.0/2.0 (U/mL).

CuCBP and CcCDP activity was also measured in the small PAM particles at 40 °C. Surprisingly, obtained results showed higher activity of CuCBP at 40 °C (Figure 7) compared to its activity measured at 45 °C,.

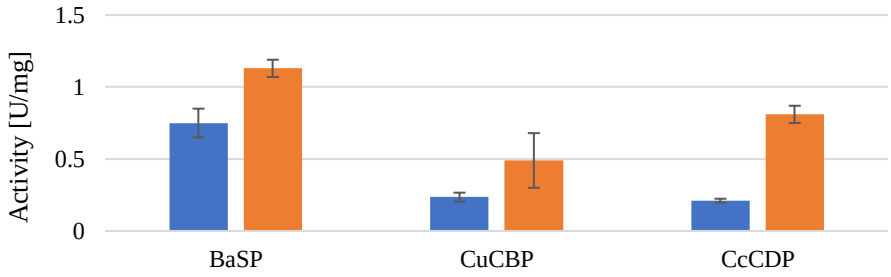

Figure 10. The activity of BaSP, CuCBP, and CcCDP in the free cells (orange) and the small PAM particles (blue) at 35 °C (*N* = 3)

At 35 °C the activity ratio of enzymes (BaSP/CuCBP/CcCDP) is 1.2/0.5/0.8 in the free cells and 0.8/0.2/0.2 U/mg in the small PAM particles. However, with respect to the results in Table 9, the activity of CcCDP is lower so this ratio is approximate, as the exact factor was not measured at this temperature, but the assumed activity could be about 1.6 times lower. Also, Figure 10 shows that all enzymes lose activity right after immobilization. So, at 35 °C BaSP activity decreases by 34 %, CuCBP 52 % and CcCDP 75 % after immobilization of the *E.coli* in the PAM.

From data presented in this chapter it can be seen that with a decrease of temperature, the activity of CuCBP increases, and the loss of activity after the PAM immobilization is less affected (at 45 °C the decrease of activity is 81 %, and at 40 °C decrease is 76 %). However,

the opposite effect can be seen with CcCDP activity, where activity decreases with decreasing temperature. The loss of BaSP activity caused by the PAM immobilization is almost the same at 35 °C (34 %) and 45 °C (32 %), indicating that the PAM immobilization has different effects on different enzymes.

4.3.3. Production of COS at different temperatures

The experimental set-up for the production of the COS by using the free cells and the PAM immobilized cells is described in Chapter 3.2.1. In short, the reaction suspension consists of the substrate solution (sucrose, glucose, and phosphate in the 50 mmol/L MES buffer, pH 7.0) and specific concentration of the catalyst, which differs depending on the temperature at which the COS production was performed. A sucrose/glucose ratio of 2.5/1.0, selected according to the results of Zhong and Nidetzky (2020), successfully eliminates the precipitation of insoluble cellodextrins and enables efficient utilization of sucrose. Additionally, the authors showed that a sucrose/phosphate ratio lower or equal to 8.0 increases the tendency of the insoluble product formation, therefore a 10/1 ratio of sucrose/glucose was applied in this Thesis.

4.3.3.1. Production of COS at 45 °C

The results of the repeated batch experiments performed at 45 °C with two types of catalysts - the free cells and the PAM immobilized cells divided into two groups (the small particles with a diameter of 0.25 - 2.0 mm, and the large particles with diameter of > 2.0 mm, Figure 2) are shown in Figure 11. As described previously (Chapter 3.2.2.) this repetitive batch experiment consisted of five cycles and each cycle lasted 6.0 h. The period of time between two consecutive cycles was 18.0 h, during which the catalysts was stored in the storage 50 mmol/L MES buffer, pH 7.0, at 4 °C.

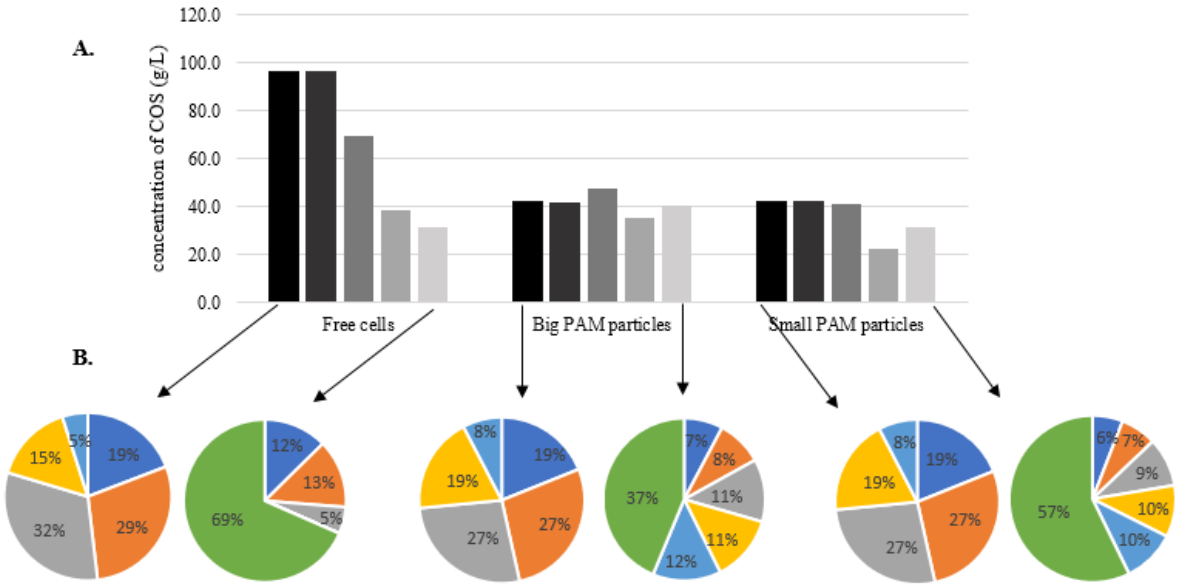

Figure 11. A. The concentration of COS after five cycles of the repetitive batch experiments catalysed by the free cells, the big PAM particles, and the small PAM particles, carried out at 45 °C. Cycles: first (■); second (■); third (■); fourth (■); fifth (■); and **B.** the composition of produced cellodextrins after the first and the fifth cycle. G2, cellobiose (■); G3, cellotriose (■); G4, cellotetraose (■); G5, cellopentaose (■); G6, cellohexaose (■); insoluble cellodextrins (■)

Production of the COS (102.6 - 96.2 g/L) by the free cells (3 g $_{CDW}$/L) during the first two cycles was superior to all other experiments (PAM particles with catalyst concentration 4 g $_{CDW}$/L). The rest of the three cycles were comparable to the performance of the big and small PAM immobilized cells/particles with 69.5 - 31.6 g/L of COS. Regarding the PAM particles, their different sizes impact differently the production of COS. The big PAM particles seem to be more stable and easier to recycle. However, the bigger PAM particles the smaller is the specific surface area and resulting in a smaller concentration of produced COS (*e.g.* after the first cycle 75 g/L of the COS was produced by the small PAM particles and only 60 g/L of the COS by the big PAM particles). As the main goal of this Thesis is to maximize the COS production, the small PAM particles were selected for further experiments.

The calculated TTN (Chapter 3.3.7.) of the catalysts after the first cycle for the free cells and the small PAM particles were 34 and 18 g/g $_{CDW}$, respectively. After five cycles the TTN for the free cells was drastically lower, yet still higher than for the small PAM particles (11 versus 8 g/g $_{CDW}$, respectively). However, for the production of the COS with the free cells the time period of 30 h (after five cycles) seemed too long because then the product mixture consisted of 69 % insoluble cellodextrins. Looking at the COS composition after the first and fifth cycle

(Figure 11B), it might be concluded that all catalyst preparations follow the same trend in which an increase of longer-chain cellodextrins can be observed in the last batch. This can be explained by looking at the data shown in Figure 7, where CcCDP activity measured after each time point is always higher than CuCBP activity, which resulted in a more pronounced elongation process which led to the accumulation of insoluble cellodextrins in immobilized (45 °C repetitive batch experiment). Additionally, altered PAM properties (depending on the time and temperature of the experiment) cause extreme cell leakage, which consequently deteriorates the COS production.

4.3.3.2. Production of COS at 40 °C

The catalyst concentration selected for this repetitive batch experiment was 3 g $_{CDW}$/L, while the cell loading was 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$. The repetitive batch experiments conducted at 40 °C had seven cycles and each cycle lasted 12 h to compensate lower catalyst concentration. In this repetitive batch experiment, the recycled small particles were immediately applied to a fresh substrate solution so that there was no storage between cycles. The composition of the product mixture is shown in Figure 12.

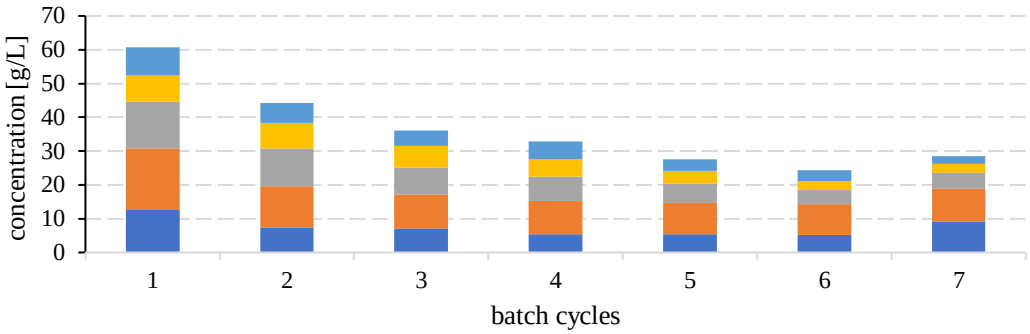

Figure 12. Concentration of COS produced by the small PAM particles during seven cycles of the repetitive batch experiments performed at 40 °C. G2, cellobiose (■); G3, cellotriose (■); G4, cellotetraose (■); G5, cellopentaose (■); G6, cellohexaose (■)

Figure 12 shows a linear decrease in COS concentration after each cycle, except in the last cycle, where an increase in COS concentration can be observed. The results for the last cycle are most likely a failure in the measurement, and therefore this last cycle was not taken into consideration. Since this experiment was performed at the lower temperature (40 °C), with lower catalyst concentration (3 g $_{CDW}$/L) the duration of each cycle was doubled to 12 h and one (two) more cycle(s) was/were performed then in the previous experiment (45 °C, five cycles, 6 h each cycle, immobilized catalyst concentration 4 g $_{CDW}$/L).

So, after six cycles at 40 °C and a time period of 72 h, the production of the COS decreased by 61 % when compared to the first cycle (after 12 h). Additionally, after 72 h production of COS dropped to 27.6 g/L, while the similar drop in the COS production conducted at 45 °C occurred already after 30 h reaction time. This indicates that the temperature of 40 °C is more suitable for the COS production under described conditions than the temperature of 45 °C.

4.3.3.3. Production of COS at 35 °C

The repetitive batch experiments conducted at 35 °C had nine cycles and each cycle lasted 8 h, where in between cycles the small PAM particles spent 16 h in the storage MES buffer at 4 °C. The 10 °C temperature decrease in this experiment was compensated with almost doubled cell loading of 63 mg $_{CDW}$/g $_{PAM\text{-}particles}$. The composition of the product mixture is shown in Figure 13. The catalyst concentration in this experiment was 4 g $_{CDW}$ /L.

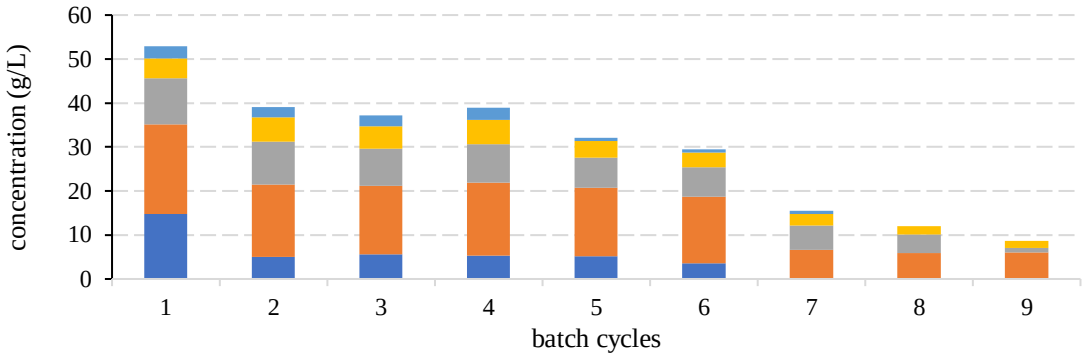

Figure 13. COS composition in the product mixture collected after every batch cycle conducted at 35 °C. G2, cellobiose (■); G3, cellotriose (■); G4, cellotetraose (■); G5, cellopentaose (■); G6, cellohexaose (■)

Figure 13 shows that a 10 °C temperature difference did not significantly slow down the production of COS. It seems that the production of COS at lower temperature was compensated with higher loading of *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new in the small PAM particles and the data obtained showed that the stability of CuCBP, which is the weakest member in the enzymatic cascade (Figure 7), at this temperature seemed sufficient for at least 48 h for the efficient COS production. From the first to the last cycle performed at 35 °C a drop in the COS production of 86 % was observed, indicating that over time, independent of the temperature, all enzymes lose their activities, or, another possibility is that they leak from the PAM. The reaction time was the same as in the repetitive batch experiments performed at 40 °C - in total 72 h, but

here the small PAM particles were stored between each cycle, and this obviously affected the overall COS production. The effect of the storage time and the low temperature on the small PAM particles properties can be seen when the COS productions at 40 and 35 °C are compared. The overall produced COS within the same time span of 72 h at 35 °C (66.5 g) was only 15 % higher compared to COS produced at 40 °C (56.5 g). Given that the catalyst concentration was 1.6 times higher at 35 °C with almost doubled cell loading (63 mg $_{CDW}$ /g $_{PAM\text{-}particles}$), the results show that the optimal temperature for COS production leans more towards 40 °C, with the optimal number of cycles in batch experiments around six, where each cycle should last about eight hours.

4.3.4. Product mixture composition

CuCBP activity cannot be directly measured in the immobilized *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new cell catalyst, which was used for the COS production, so its activity over time, and therefore stability, was measured by comparing product mixture compositions of the first and last batch cycles. If the composition of the product mixture in the first and last batch cycle differs by a large value (*i.e.* completely different ratio of G2/G3/G4/G5/G6 in the first and last cycle) it means that the CuCBP has lost its activity, and is no longer stable, which leads to increased production of insoluble cellodextrins. Comparing only two cycles from each repetitive batch experiment carried out at different temperatures (35, 40 and 45 °C) - the first cycle and the last cycle, the efficiency of the COS production was assessed (Figures 11, 12, 13). The variables examined in this Thesis are: the reaction time and the temperature, the number of repetitive batch cycles, the catalyst concentration, and the cell loading. Figures 11, 12, and 13 show the composition and concentration of the COS produced in the first and last batch at given temperatures (35, 40 and 45 °C), with the experiments differing in the number of cycles (9, 6, and 5, respectively) and different reaction times (8, 12 and 6 h per cycle, respectively), where *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new immobilized in the small PAM particles was used as the catalyst.

Figure 11A shows a 58 % reduction in the COS production by comparing the first and fifth batch, and Figure 11B shows the completely different ratio (G2/G3/G4/G5/G6) of the COS produced in the experiment conducted at 45 °C, (6 h and 30 h reaction time). This completely different composition of the product mixture, with an increased concentration of longer chain COS, which leads to accumulation of unwanted insoluble cellodextrins over time, indicates CuCBP instability (at 45 °C). Thus, 45 °C seems too high for the COS production over stated

period of time, so the COS production can be further optimized by lowering the reaction temperature or shortening the reaction time. Furthermore, these results are completely in line with the results shown in Figure 7, which show that CuCBP loses 45 % and 90 % of its activity after 24 and 48 h, respectively.

From the first to a sixth repetitive batch experiment conducted at 40 °C catalyst spent 72 h in the reaction suspension with a decrease in the COS production of 55 %, with a similar ratio (G2/G3/G4/G5/G6) of the produced COS, indicating that CuCBP is still stable after 72 h.

In the last repetitive batch experiment conducted at 35 °C the highest decrease in the COS production of 84 % (when comparing the COS production after 8 and 72 h) happened, suggesting that this reaction time is too long for the efficient COS production. But given the results obtained at 40 °C where CuCBP is stable after the same reaction time (72 h), this implies that the storage time between two consecutive cycles greatly affects the total COS production. In addition, the initial batch at the indicated temperature (35 °C) had the lowest COS concentration (53 g/L), suggesting that 35 °C is not suitable for rapid COS production.

However, the production of COS at 35 °C could be optimized by reducing the storage time between two consecutive cycles.

Most similar product mixtures in the initial and last batch can be seen in the batch carried out at 40 °C, so the optimal temperature for the COS production leans more to this temperature. To optimize the experiments carried out at 40 °C, it is necessary to introduce a higher catalyst concentration and cell loading, because cell leakage happens inevitably at any temperature due to mandatory stirring. This should be feasible because the production of the whole cells catalyst and its PAM immobilized version in this case is neither expensive nor difficult to achieve. If implemented on an industrial scale, time and overall productivity of the COS production are the most important factors. When choosing the optimal temperature, it should be taken into account that longer catalyst stability is achieved at lower bioprocess temperatures, but higher temperature means higher catalyst activity and thus higher productivity, so the optimal temperature should be less than 45 and higher than 35 °C, ideally around 40 °C.

4.3.4.1. Space time yield and total turnover number

STY and TNN are calculated, as described in Chapter *3.3.7.* Results are shown in Table 10, where values are calculated after each batch at the set temperatures, where the PAM immobilized *E. coli* BL21 (DE3) -*agp*$^{-}$ pPOLY_new small particles were used as the catalyst.

Table 10. The COS production catalysed by the PAM immobilized *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new (small particles) evaluated by STY calculated after each repetitive batch cycle at specific temperature

STY [g/(L h)]			
batch cycle	45 °C	40 °C	35 °C
1	12.2	5.1	6.6
2	7.1	3.7	4.9
3	6.8	3.0	4.6
4	4.4	2.7	4.9
5	5.1	2.3	4.0
6		2.0	3.7
7		2.4	1.9
8			1.5
9			1.1
mean	**7.1**	**3.0**	**3.7**

The highest STY of 12.2 g/(L h) was obtained at 45 °C. At this temperature all other values are considerably higher than the values at the two lower temperatures (40 °C and 35 °C).

Table 11. The COS production catalysed by the PAM immobilized *E. coli* BL21 (DE3) -*agp*⁻ pPOLY_new (small particles) evaluated by TTN calculated after each repetitive batch cycle at specific temperature

TTN [g $_{product}$/g $_{CDW}$]			
batch cycle	45 °C	40 °C	35 °C
1	18.2	20.2	13.2
2	10.6	14.8	9.8
3	10.2	12.0	9.3
4	6.6	11.0	9.7
5	7.7	9.2	8.0
6		8.1	7.4
7		9.5	3.9
8			3.0
9			2.2
total	**53.3**	**84.8**	**66.5**

TTN presented in Table 11 at 45 °C (6 h cycle) and 35 °C (8 h cycle) are comparable as they have the same cell concentration (4 g $_{CDW}$/L), but different cell loading of the PAM particles, 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$ and doubled at 35 °C 63 mg $_{CDW}$/g $_{PAM\text{-}particles}$. Surprisingly, they do not

differ much, not even in a single batch with respect to a difference of 10 degrees and 2 h more time per cycle. These results confirm the better stability of the enzymes at 35 °C, which then contributes to the better production of the COS. However, all enzymes lose their activity over time (Figure 13, last batch at 35 °C) or were leaking from the PAM, but the higher cell loading and lower stirring rate might contribute to the catalyst stability and diminish the PAM (both the small and the big PAM particles) shrinking and swelling effect.

Additionally, the results obtained from Zhong and Nidetzky (2020) show that the optimal activity ratio of BaSP/CuCBP/CcCDP should be 10/3/2 (U/mL) within the conversion time range (1– 1.5 h) at 45 °C, and under these conditions, all produced cellodextrins were soluble, with final productivity of 21.3 g/(L h). Comparing these results with here obtained STY (Table 10) calculated after 6 h at 45 °C of 12.2 g/(L h), it can be concluded that the enzyme activity ratio drastically impacts the COS production efficiency and the product composition, *e.g.* too high CcCDP activity will lead to the production of insoluble cellodextrins. The activity ratio of BaSP/CuCBP/CcCDP obtained at 45 °C in this thesis is 1.5/0.2/1.4 (U/mg).

STY (Table 10) at 35 °C is increased in comparison to values obtained at 40 °C due to shortened reaction time. A period of 12 h of cycle used at 40 °C would not be necessary at 40 °C if one would have a catalyst concentration of 4 g $_{CDW}$/L. At 40 °C more COS (226 g/L) is produced with less cell loading (40 g $_{CDW}$/g $_{PAM\text{-}particles}$), suggesting that the efficient COS production through repetitive batches can be carried out on indicated temperature - 40 °C. For further optimization and more efficient COS production, it is necessary to introduce a higher loading of PAM cells (higher than 40 g $_{CDW}$/g $_{PAM\text{-}particles}$) and a higher concentration of the catalyst in the reaction suspension ($\geq$ 4 g $_{CDW}$/L). Additionally, minimizing storage time between cycles would help maintain enzyme activity for a longer time, so the recommended optimal number of cycles (six) could be increased, while the optimal reaction time of 8 to 10 h may be extended.

5. CONCLUSIONS

Based on the results presented in this Graduate Thesis, the following conclusions can be drawn:

1. Expression of the three enzymes: sucrose phosphorylase from *Bifidobacterium adolescentis* (BaSP), cellobiose phosphorylase from *Cellulomonas uda* (CuCBP) and cellodextrin phosphorylase from *Clostridium cellulosi* (CcCDP) in *E. coli* BL21 (DE3) *agp*- pPOLY_new was successful, but a whole cell catalyst [*E. coli* BL21 (DE3) *agp*- pPOLY_new] immobilization in the polyacrylamide matrix (PAM) negatively affected activity of all three enzymes, BaSP activity decreased by 34 %, CuCBP by 52 % and CcCDP by 75 % (measured at 45 °C). However, the PAM immobilization was required in order to repetitively use entrapped *E. coli* cells *i.e.* the three enzymes for production of soluble cellodextrins (COS) in the enzymatic cascade reaction.
2. The three purified enzymes possess desirable activities under described conditions, especially at higher temperature, as follows: the BaSP favours phosphorolysis of sucrose (117.3 ± 10.3 U/mg at 30 °C; n.d. at 45 °C); the CuCBP prefers cellobiose synthesis (22.5 ± 0.4 U/mg, 45 °C); the CcCDP activity probed towards the substrate (cellobiose) analogue (PNP-C) is also promising (17.2 ± 2.4 U/mg; 45 °C).
3. During the employment of the whole cell catalyst - the PAM immobilized *E. coli* cells for the cellodextrin production *via* repetitive batch experiments, protein leakage from the immobilized cells occurred and consequently deteriorated the cellodextrin production. The activity of the three enzymes in the free *E. coli* cells was higher than their activity in the PAM particles, and in both cases the optimal temperature for the enzymatic cascade reaction created for the efficient COS production was chosen to be 35 - 45 °C.
4. The COS production by using the PAM immobilized particles (small particles) *via* repetitive batch experiments probed at three different temperatures (35, 40 and 45 °C) with different number of cycles (9, 6, 5, respectively) with distinct duration of each cycle (8, 12 and 6 h, respectively), cell loading (63 , 40 and 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$, respectively) and concentration of the cell catalyst (4, 3 and 4 g $_{CDW}$/L, respectively) showed that the optimal temperature is around 40 °C. At this temperature the weakest enzyme in the COS production enzymatic cascade - CuCBP (within small PAM particles) possess the highest activity. The CuCBP activity and stability are essential for efficient COS production.

5. Based on all obtained results a new experimental set up is proposed in order to reach higher space time yield and total turnover number, and make the COS production closer to industrial production: number of cycles within one repetitive batch experiments of six, duration of each cycle between 8 and 10 h, higher cell loading (higher than 40 mg $_{CDW}$/g $_{PAM\text{-}particles}$) and concentration of the cell catalyst (higher than 4 g $_{CDW}$/L).

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List of abbreviations

Amp	Ampicillin sodium salt
αGlc1-P	α-glucose-1-phosphate
ATP	Adenosine 5'-triphosphate
BaSP	Sucrose phosphorylase from *Bifidobacterium adolescentis*
CcCDP	Cellodextrin phosphorylase from *Clostridium cellulosi*
CDW	Cell dry weight
CWW	Cell wet weight
CuCBP	Cellobiose phosphorylase from *Cellulomonas uda*
COS	Soluble cello-oligosaccharides
Double distilled water (ddH_2O)	nanopure water
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
mol %	molar yield of reaction, defined as the mole ratio
n	the number of reactions that CcCDP conducts
N	Number of replicates in measurement
EDTA	Ethylenediaminetetraacetic acid
G1P assay	α-glucose-1-phosphate assay
G2	cellbiose
G3	cellotriose
G4	cellotetraose
G5	cellopentaose
G6	cellohexaose
IPTG	Isopropyl-β-D- thiogalactopyranoside
OD	optical density
PAM	Polyacrylamide matrix
Pi	inorganic phosphate
PVA	polyvinyl alcohol
PNP-C	*p*-nitrophenyl β-D-cellobioside
rcf	relative centrifugal force
RI	refractive index
RT	room temperature
v/v	volume per volume
v/w	volume per weight
wt %	product yield of reaction, defined as the weight ratio
w/w	weight per weight

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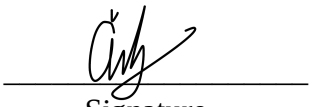

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