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Cellodextrin Phosphorylase: Characterization and application in oligosaccharide synthesis

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Affidavit

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"I hear and I forget.

I see and I remember.

I do and I understand."

- Confucius -

Konfuzius, der chinesische Philosoph, beschreibt viele meiner Lernphasen während des Studiums ziemlich zutreffend, jedoch wäre es mir ohne die Unterstützung vieler toller Menschen wesentlich schwerer gefallen.

Ich möchte mich herzlichst beim gesamten Team des Institutes von Biotechnologie und Bioprozesstechnik bedanken, die mir während meiner praktischen Arbeit tatkräftig zur Seite gestanden haben. Besonderer Dank gilt meinen Betreuern Prof. Bernd Nidetzky und Dr. Christiane Luley, die auch in ihren stressigen Phasen immer eine offene Tür und ein besonderes Lächeln für mich übrig hatten.

Nun wird es Zeit auch meiner Familie großen Dank zu übermitteln. "Über Stock und Stein" ist hier nicht nur ein einfaches Sprichwort, es zeigt neben der Entfernung des Heimatortes, auch den Zusammenhalt in turbulenten Zeiten.

Was wäre ein Studium ohne Menschen, die einem mit der Zeit ans Herz gewachsen sind und auf die man sich immer verlassen kann. Danke an meine liebgewonnen Freunde Ana, Bianca, Katrin, Jasmin, Stefanie, Domenic und Max, ohne euch wäre ich um einige tolle Erinnerungen ärmer!

Abstract

Cellodextrin phosphorylase is a β-1,4-glucan phosphorylase. According to sequence similarity it is classified into glycoside hydrolase family GH94. The enzyme is most commonly known for the synthesis of synthetic crystalline cellulose while biocatalytic application for the synthesis of different oligosaccharide derivatives is rare due to lack of catalytic data.

This thesis focuses on characterization and application in oligosaccharide synthesis of cellodextrin phosphorylase from *Clostridium cellulosi*. Cloning experiments were performed in order to attach an N-terminal His-tag to the synthetic genetic construct. Protein expression was performed in *E. coli* BL21, a licence free strain. Different methods for the characterization and quantification were developed, e.g. TLC, HPAE-PAD and generally HPLC with different columns. The efficiency of this enzyme was compared with its homologue cellodextrin phosphorylase from *Clostridium stercorarium*.

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1. Introduction

Cellulose is an important component of plant cell walls and therefore the most abundant bio-polymer in the world. It is a β-1,4-linked glucose polymer with a degree of polymerization (DP) of >100. Many fungi and bacteria produce cellulases, which hydrolyse cellulose into β-D-glucose moieties [1].

1,4-β-D-glucooligosaccharides, known as cellodextrins, have a lower DP (n = 2-6) and are classified as non-digestible oligosaccharides [2]. They pass the human stomach and small intestine and reach the large intestine where they are assimilated by enteric bacteria. Enteric bacteria (probiotics) and cellodextrins (prebiotics) work together in a symbiotic manner and can improve human gut health [3] by e.g. increased production of short chain fatty acids like acetic acid, propionic acid, butyric acid and lactic acid. The produced short chain fatty acids are important for many physiological activities. Famous examples are the increase of mucosal blood amounts in gastrointestinal blood flow, the growth of gastrointestinal mucosal epithelial cells as well as the inhibition of cholesterol synthesis [2]. Cellodextrins are usually obtained as mixtures of oligosaccharides with different degrees of polymerization.

Some anaerobic bacteria have an intracellular phosphorolytic enzyme system to convert cellodextrins and thereby store energy in form of glucose-1-phosphate. The first phosphorolytic cleavage of cellodextrins was shown in *Clostridium thermocellum* [4]. The enzymes involved in this reaction are named β-D-glycoside phosphorylases. They are mainly classified into glycoside hydrolase family GH94 and act on β-linked structures such as cellobiose, cellodextrin, laminaribiose, N,N'-diacetylchitobiose and cellobionic acid [5]. The best characterized member among the GH94 enzymes is cellobiose phosphorylase (CBP, EC 2.4.1.20). This enzyme catalyses the reversible phosphorolysis of cellobiose into α-D-glucose-1-phosphate (Glc1-P) and D-glucose (Figure 1).

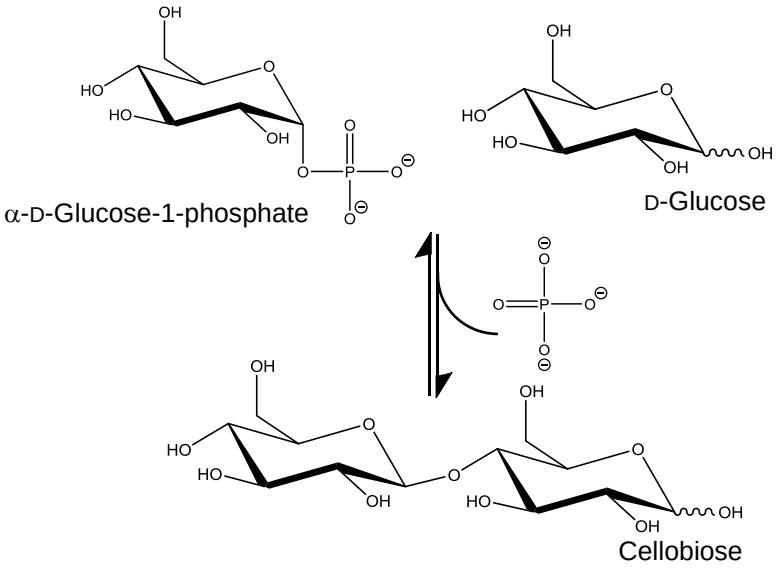

Figure 1: Reaction scheme of cellobiose phosphorylase.

Cellodextrin phosphorylase (CdexP, EC 2.4.1.49) is another enzyme belonging to family GH94. It catalyses the phosphorolysis of long-chain cellodextrins with a DP of more than 2 and the synthesis of short cellodextrins with a DP of > 2 [6]. The enzymatic reaction scheme is shown in Figure 2.

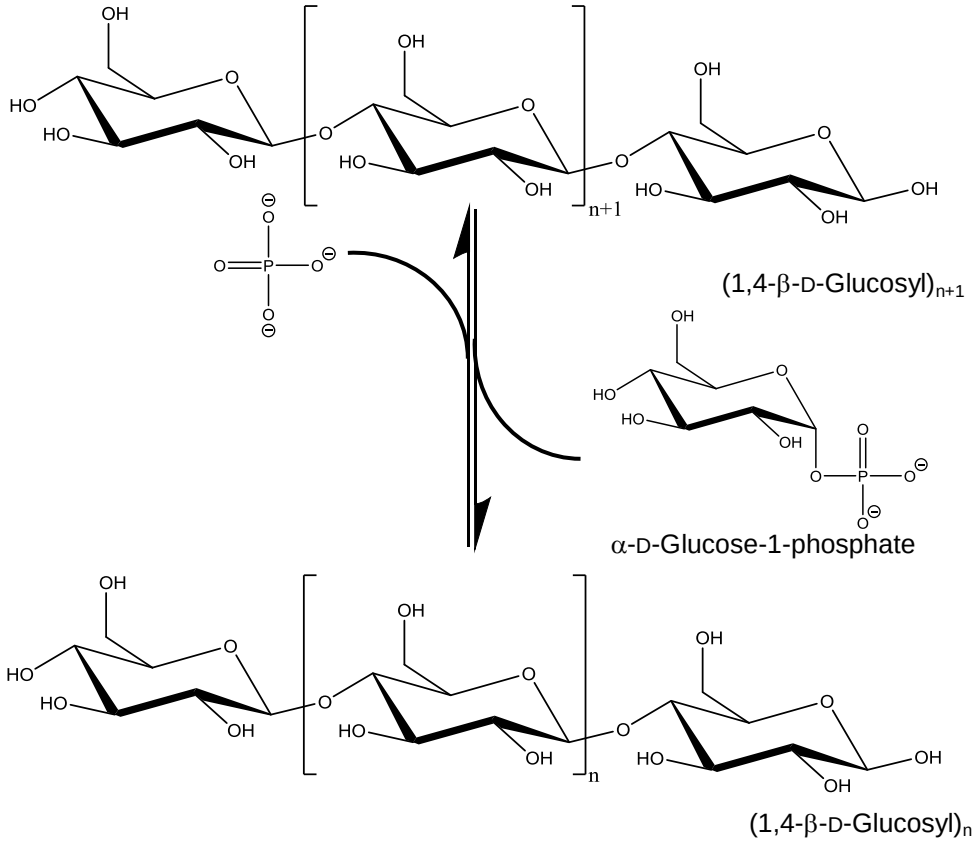

Figure 2: Reaction scheme of cellodextrin phosphorylase.

Although CBP and CdexP belong to the same GH family, they have different substrate specificities [4, 7, 8]. While both enzymes are mainly restricted to Glc1-P as glucosyl donor, CBP has a more restricted acceptor specificity than CdexP. CdexP is active on disaccharides and oligomeric acceptors while CBP only can convert monosaccharidic acceptor molecules.

The CBP crystal structure from *Clostridium thermocellum* reveals which amino acids correspond to the binding sites of phosphate and cellobiose. Significant differences of the glucose binding subsites -1 and +1 are observed. Structural changes in the enzyme, due to interactions between the substrate and a side chain at the subsite -1, allow the movement of said side chain to the active site pocket. Residues near the glucose binding subsite +1 are structurally conserved (PDB 2CQT). They determine in combination with the N-terminus the sugar binding characteristics of CBP. Similar enzymes show the same behaviour [9].

Next to the glucose binding subsites -1 and +1, CdexP possesses additional subsites called +2 and +3 (Figure 3) [10]. This additional subsites leads to the broader substrate specificity of the enzyme. In *Clostridium stercorarium,* an indole ring of W168 from CdexP is positioned parallel to the sugar ring, resulting in a hydrophobic stacking interaction. This type of interaction is a typical feature of subsites in carbohydrate-active enzymes and is probably involved in the binding of alkyl and aryl chains of acceptor molecules [8].

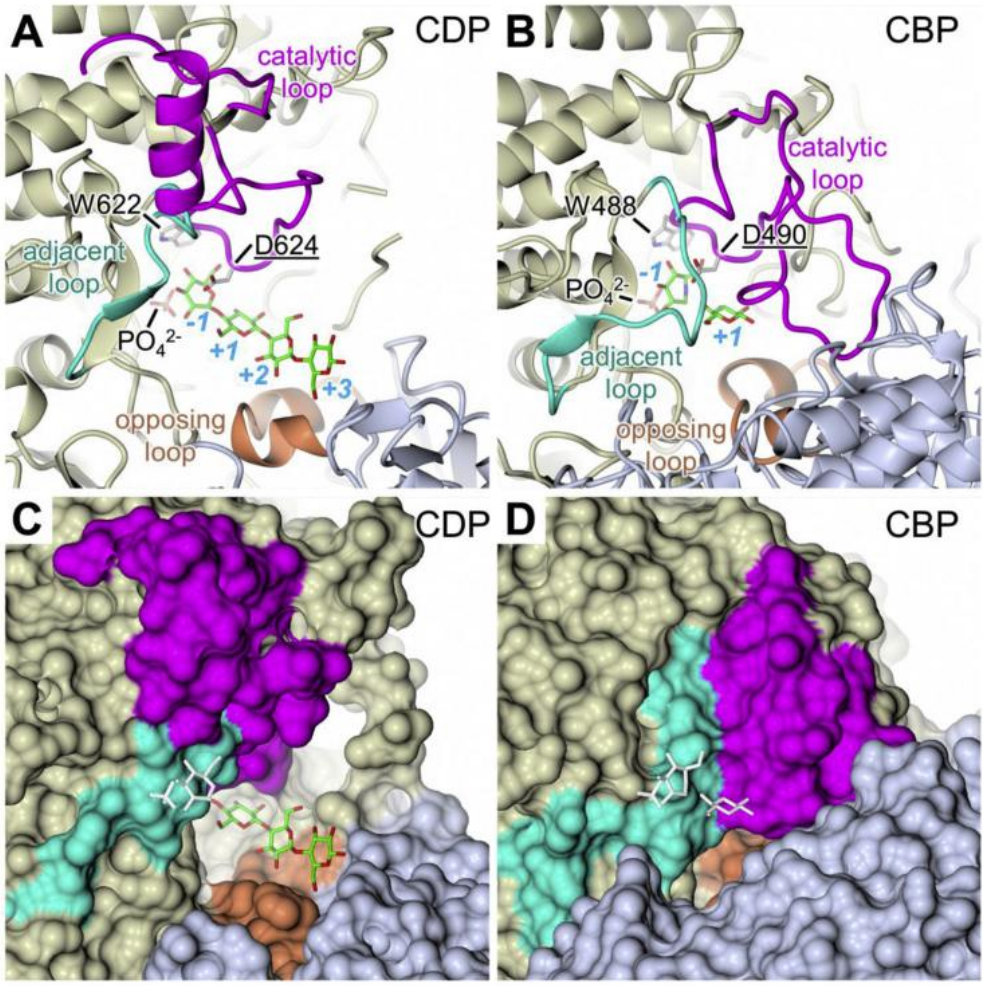

Figure 3: Comparison of the subsites in the two different GH94 enzymes CdexP and CBP. CdexP=CDP. A and B: cartoon representations with the ligands in stick representation and with the side-chains of the "platform", Trp and the catalytic Asp. C and D: molecular surface of the protein. Ligand: white. [10]

There isn't a similar residue present in CBP, which can partly explain its lack of activity on disaccharide acceptors. Another contributing aspect might be the different shape of the active site entrance. The entrance is "closed" at CBP due to a loop consisting of 19 residues, but in CdexP the loop is relatively "open" (4 residues). This could result in an easier access for longer acceptors and more flexibility for finding the optimal positioning in the active site [8]. The structure of CdexP from *Ruminiclostridium thermocellum* (formerly *Clostridium thermocellum* [10]) is shown in Figure 4.

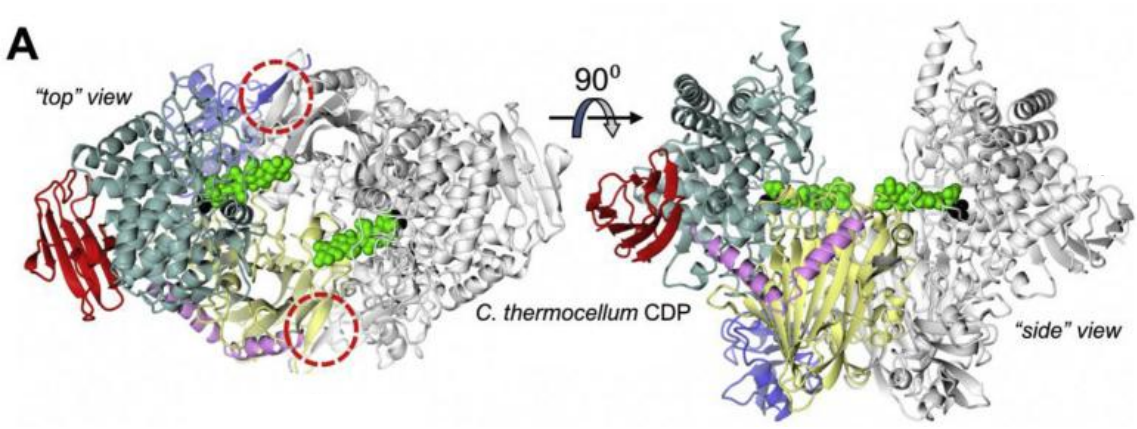

Figure 4: Structure of cellodextrin phosphorylase from *Ruminiclostridium thermocellum* (formerly *Clostridium thermocellum*). Green: two copies of cellotetraose. Black: phosphate. Blue-violet: N-terminal domain. Yellow: β-sandwich domain. Purple: two α-helix linker. Grey: catalytic-barrel domain. Red: C-terminal domain. Dashed red circles: indicate β-sheet interactions between N-terminal arms and β-sandwich domain. [10]

The combination of both enzymes with their reverse synthetic abilities is interesting for the biosynthesis of diverse disaccharides, oligosaccharides and glycolipids [11–13]. CBP acting on D-galactosyl L-rhamnose, D-mannosyl D-glucose, D-mannobiose, D-chitinbiose, D-cellobionic acid and D-cellobiose. CdexP has been used for the synthesis of crystalline cellulose and cellooligosaccharide derivatives [14]. The great industrial potential of CBP and CdexP motivated us to discover new variants with even more desirable properties, e.g. increased thermostability, increased activity, broader substrate specificity and so on.

In industrial applications, the commercial availability and the prices of educts strongly influence the economic viability of the overall production process. Table 1 shows current prices of commonly used substrates for the production of disaccharides and oligosaccharides. An enzyme-cascade reaction using CBP and CdexP can already reduce production cost. However the main cost factor is still Glc1-P, which is the donor substrate for both reactions.

Table 1: Substrate prices.

substance	price* per kg [€/kg]
α-D-Glucose-1-phosphate disodium tetrahydrate	~ 30,000
Cellobiose	~ 1,850
Phosphate	~ 30
D-Glucose	~ 30
Sucrose	~ 20

*prices taken from "www.carlroth.com" at 25th April 2018

Therefore, designing a reaction cascade with the three enzymes (sucrose phosphorylase (SP, EC 2.4.1.7), CBP and CdexP) may be the best choice for the industrial production of oligosaccharides. SP catalyses the reversible phosphorolysis of sucrose into Glc1-P and D-fructose (Figure 5). The enzyme belongs to the GH13 family, which is often referred to as the α-amylase family [15].

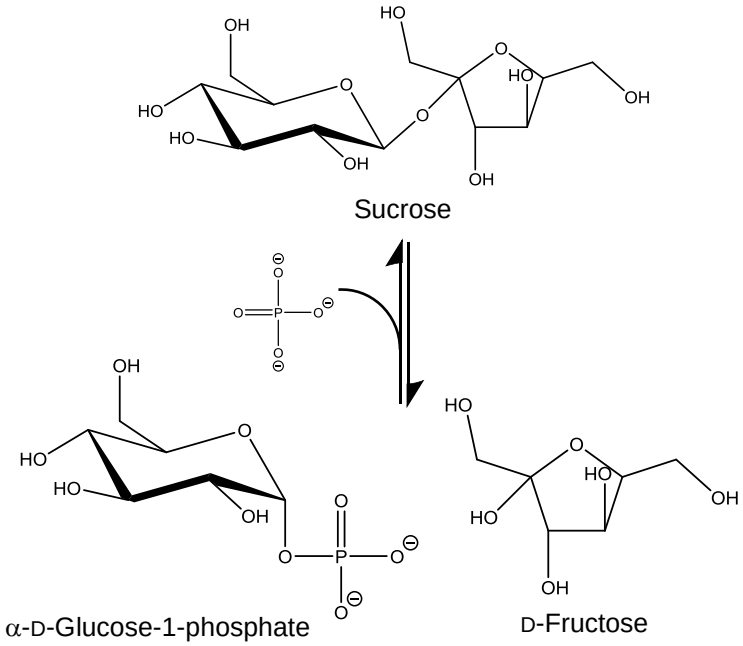

Figure 5: Reaction scheme of sucrose phosphorylase.

CdexP can also be used for the production of novel glycolipids. In recent years, glycolipids have gotten increasing attention as natural surfactants with a beneficial environmental profile. The enzyme from *Clostridium stercorarium* was found to display a broad donor and acceptor specificity. Sophorolipids and glucolipids could be efficiently glycosylated using Glc1-P or α-galactose-1-phosphate as glycosyl-donor. The glycolipids could also be used in applications for the pharmaceutical and nanomaterial industry [13].

This work focuses on the biochemical characterization of a putative cellodextrin phosphorylase from *Clostridium cellulosi.* This enzyme was evaluated as biocatalyst in oligosaccharide synthesis with a defined DP. The efficiency of this enzyme was compared with its homologue CdexP from *Clostridium stercorarium*.

2. Materials and Methods

2.1. Chemicals and strains

Yeast extract (ROTH), Peptone ex casein (ROTH), NaCl (≥99.5% ROTH), Ampicillin sodium salt (≥97% ROTH), Glycerol (≥98% ROTH), Isopropyl-β-D-thiogalactopyranoside (IPTG) (>99% ROTH), MOPS sodium salt (≥99% ROTH), ETDA disodium dihydrate (≥99% ROTH), Dithiothreitol (DTT, ≥99% ROTH), Imidazole (≥99% ROTH), NuPAGE® MOPS SDS Running Buffer (Invitrogen™; 20x Thermo Fisher Scientific), Albumin fraction V (≥98% ROTH), Bradford reagent (Roti®-Nanoquant 5x ROTH), Glucose-1-phosphate dipotassium (>80% Pfeifer & Langen), Cellobiose (95% Pfeifer & Langen), Na_2HPO_4 $2H_2O$ (≥98% ROTH), Ascorbic acid sodium salt (≥99% ROTH), HCl (37% ROTH), $Zn(CH_3COO)_2$ (≥99% Sigma-Aldrich), $(NH_4)_6Mo_7O_{24}$ $4H_2O$ (≥81.0-83.0% MoO_3 basis, Sigma-Aldrich), Acetonitrile (≥99.5% ROTH), Thymol (99% ROTH), Ethanol (96% ROTH), H_2SO_4 (96% ROTH), Cellotriose (95% Carbosynth), Cellotetraose (95% Carbosynth), Fructose (>99.5% ROTH), K_2HPO_4 (>98% ROTH), HEPES (≥99.5 ROTH), TRIS-HCl (99% ROTH), MES (≥99% ROTH), Citric acid (99.5% ROTH).

2.2. Cultivation and protein isolation

2.2.1. Protein Expression

Two overnight cultures (50 mL LB-media consisting of 10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl supplemented with 100 mg/L ampicillin) were inoculated with 10 µL of a glycerol stock of pC21e_1_His-CcCdexP in *E. coli* BL21 (Clone 7). The cultures were incubated at 30°C and 120 rpm over night. The main cultures (200 mL LB-media supplemented with 100 mg/L ampicillin) were inoculated to an OD_{600} of 0.01. Cultivation parameters were 37°C and 120 rpm until OD_{600} reached 0.8 to 1.0. Then protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in a final concentration of 1 mM followed by a second ampicillin dosage. The induction phase was carried out at 25°C and 120 rpm for about 18 hours.

2.2.2. Cell Harvest

The cells were harvested at 5,000 rpm and 4°C for 20 min. After a washing step with desalting buffer (20 mM MOPS, 300 mM NaCl, pH 7.0) the cell pellets were stored at -21°C.

2.2.3. Cell Lysis

5 mL lysis buffer (100 mM MOPS, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, pH 7.0) were added per gram of cell pellet. Defrosting and homogenization was performed on ice. For cell disruption two French Press cycles were carried out between 10,000 and 15,000 psi. The obtained slurry was centrifuged at 5,000 rpm and 4°C for 15 min. In a second step, the supernatant was split into 1.5 mL reaction tubes and centrifuged at 13,000 rpm and 7°C for 15 min. The cell-free extract was collected in a new 50-mL falcon tube. The remaining pellet was dissolved in the same volume of 6 M urea. Aliquots of the cell-free extract were stored at 4°C for protein quantification and activity measurements.

2.2.4. Protein Purification

A two-step chromatographic protocol consisting of affinity and size exclusion chromatography was employed for protein purification. In the first step, two 5-mL His-Trap SP columns (GE Healthcare) were equilibrated with binding buffer (20 mM MOPS, 300 mM NaCl, 20 mM imidazole, pH 7.5). The cell-free extract was then filtered through a 1.2 µm filter (Sartorius) and 10 mL were loaded onto the His-Trap SP columns. Protein elution was performed at a constant flow rate of 5 mL/min using a linear gradient from 0-100% of elution buffer (20 mM MOPS, 300 mM NaCl, 500 mM imidazole, pH 7.5). After pooling, 150 µL of each fraction were kept for further analysis (protein and activity measurement). The His-CcCdexP containing fractions were concentrated via ultrafiltration using Vivaspin Turbo 15 tubes with a cut-off of 10 kDa. 2.0 mL of the concentrated enzyme fraction were loaded on a HI-Trap Desalting column (GE Healthcare), which was previously equilibrated with storage buffer (20 mM MOPS, 300 mM NaCl, pH 7.0). After the desalting step, the purified His-CcCdexP preparation was stored at 4°C for further analysis.

2.2.5. SDS Page

For the SDS-PAGE 10 µL of sample (with a concentration of 1.0 mg/mL) were mixed with 10 µL of NUPAGE®, 4 µL of 50 mM DTT and 16 µL of ddH_2O. After a short centrifugation step, the mixture was incubated at 90°C for 10 min. 10 µL of this mixture were loaded onto the SDS-gel. The SDS-gel was run in NuPAGE® MOPS SDS Running Buffer (Invitrogen™), with a pH of 7.7 and at 200 V for around 55 min. The SDS-gel was stained with Coomassie Brilliant Blue using a microwave protocol. The gel, covered with staining solution (50 mL), was incubated for 30 s in the microwave (the solution should not boil), followed by incubation on an orbital shaker for 30 min. Before adding the destaining solution (50 mL), the gel was washed with ddH_2O. The gel, covered with destaining solution, was incubated for 30 s in the microwave (the solution should not boil), followed by an incubation step on an orbital shaker for 15 min. The destaining step was repeated twice.

2.3. Determination of protein concentration - Bradford-Assay

Bovine serum albumin fraction V was used as a standard. For this purpose, a stock of 2 mg/mL was diluted according to Table 2. The reaction mixture consisted of 10 µL sample, standard or water and 500 µL Bradford reagent. After vortexing, the reaction mixture was incubated at 25°C for 15 min before the absorbance was measured at 595 nm. Each sample was analysed in duplicates. The calibration curve was fitted in a non-linear mode using the Beckman software. The Bradford reagent (stored at 4°C) and calibration curve were used for two months.

Table 2: Dilutions for the Bradford calibration curve.

Albumin V [mg/mL]	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
Albumin V stock 2 mg/mL [µL]	50	100	150	200	250	300	350	400	450	500
ddH2O [µL]	950	900	850	800	750	700	650	600	550	500

2.4. Enzyme activity measurement

2.4.1. Activity assay

The reaction mixture for the activity assay contained glucose-1-phosphate (Glc1-P) as glucosyl donor, cellobiose (C) as glucosyl acceptor in 50 mM MOPS buffer pH 7.0 and the purified His-CcCdexP or the cell-free extract preparation as biocatalyst. The substrate mixture was pre-incubated at 55°C for 5 min before enzyme was added. Aliquots of 100 µL were taken at different points in time. The enzymatic reaction was stopped by incubation at 99°C for 5 min. The samples were centrifuged at 13,000 rpm and 7°C for 10 min. The supernatants were transferred to a new reaction tube and stored at 4°C before further analysis. The release of phosphate was determined according to Saheki.

We compared the cellodextrin phosphorylase from *Clostridium cellulosi* to the homologue enzyme from *Clostridium stercorarium.* We were provided with *C. stercorarium* enzyme from the Department of biochemical and microbial technology at the Ghent University. This enzyme has a temperature optimum of 65°C and a pH optimum of 6.5 [8].

2.4.2. Saheki Assay

Na_2HPO_4 $2H_2O$ was used as standard. A 5 mM stock solution was diluted according to Table 3. The test solution consisted of 1/5 of a 10% ascorbic acid solution (pH adjusted to 5.0 with 1/100 (vol%) of 4 M HCl; prepared fresh daily) and 4/5 of a molybdate reagent (100 mM $Zn(CH_3COO)_2$ and 15 mM $(NH_4)_6Mo_7O_{24}$ $4H_2O$, stored in 50-mL falcon tubes protected from light). The reaction mixture, consisting of 470 µL of test solution and 30 µL of sample or standard, was vortexed and incubated at 25°C for 15 min. The formed phosphomolybdate complex was reduced by ascorbic acid at pH 5.0 in the presence of Zn^{2+} and the produced chromophore was measured at 850 nm [16]. The calibration curve was fitted in a linear mode using the Beckman software. The test solution was only used for measurements within 5 h.

Table 3: Dilutions for the Saheki calibration curve

P_i [mM]	**0.0**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
P_i stock 5 mM [µL]	0	4	8	12	16	20
ddH_2O [µL]	100	96	92	88	84	80

2.5. Determination of kinetic parameters - v_{max} and K_m

2.5.1. Kinetic parameters in synthesis direction

v_{max} for the synthesis reaction and K_m for glucose-1-phosphat were determined using different concentrations of Glc1-P and a fixed concentration of cellobiose. To ensure no pH changes, a 50 mM MOPS-buffer was used to maintain a pH of 7.0. The used concentrations are shown in Table 4. The substrate mixture was pre-incubated at 55°C for 5 min before the enzyme was added. The enzymatic reaction was stopped after 5 min by incubation at 99°C. The samples were then centrifuged at 13,000 rpm and 7°C for 10 min. The supernatants were transferred to new reaction tubes and stored at 4°C before phosphate concentration was determined using the Saheki assay. The results were fitted according to a Michaelis-Menten kinetic (Sigma Plot, see Equation 1) and v_{max} and K_m were calculated.

Equation 1: $$f = \frac{y_0 \cdot x}{K_m + x}$$

Table 4: Substrate concentrations for the determination of kinetic parameters in synthesis direction.

Glc1-P [mM]	Cellobiose [mM]	Enzyme [mg/mL]	MOPS [mM]
0.0	50.0	0.066	50.0
2.0			
3.0			
3.5			
4.0			
4.5			
5.0			
8.0			
10.0			
15.0			
30.0			
60.0			
90.0			

2.5.2. Kinetic parameters in phosphorolysis direction

v_{max} for the phosphorolysis reaction and K_m for cellobiose were determined using different concentrations cellobiose and a fixed concentration of Glc1-P. A 50 mM MOPS-buffer was used to maintain a pH of 7.0. The used concentrations are shown in Table 5. The substrate mixture was pre-incubated at 55°C for 5 min before the enzyme was added. The enzymatic reaction was stopped after 5 min by incubation at 99°C. The samples were centrifuged at 13,000 rpm and 7°C for 10 min. The supernatants were transferred to new reaction tubes and stored at 4°C before phosphate concentration was determined using the Saheki assay. The results were fitted according to a Michaelis-Menten kinetic (Sigma Plot, see Equation 1) and v_{max} and K_m were calculated.

Table 5: Substrate concentrations for the determination of kinetic parameters in phosphorolysis direction.

Cellobiose [mM]	Glc1-P [mM]	Enzyme [mg/mL]	MOPS [mM]
0.0	20.0	0.066	50.0
4.0			
6.0			
8.0			
10.0			
11.0			
12.0			
13.0			
25.0			
50.0			
75.0			
100.0			

2.6. Product Characterization

2.6.1. Thin layer chromatography (TLC)

A thin layer chromatography was carried out. For this purpose, a piece of the TLC aluminium sheets was cut out for a quick determination of the oligomeric product state. TLC aluminium sheets of the type silica gel 60 with fluorescent indicator (MERCK, pre-coated, 20x20 sheet, layer thickness 0.2mm) were used. We used acetonitrile:water (75:25; 85:15; final setting 75:25 (v/v)) as mobile phase [17] and thymol:ethanol:H_2SO_4 (0.5:95:5; w:v:v) as colouring reagent [18]. This solution was prepared on site. The acetonitrile:water mobile phase was mixed fresh for every use. The colouring reagent was stored at 4 °C protected from light (aluminium foil was wrapped around the bottle) for more than 3 months. After spotting of 1 µL (finally 0.5 µL) of sample, Glc1-P, cellobiose and glucose (different concentrations) the TLC plate was transferred into the saturated developing chamber. The plate was developed twice. The TLC plate was dried before the colouring reagent was sprayed on the surface. After heat development the carbohydrates were identified as pink spots.

2.6.2. High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

For HPAE-PAD analysis CarboPac PA10 Column (Thermo Scientific) was used on a Dionex device. The Dionex device was equipped with a "CHROMELEON 6.50" software for monitoring and control, an electrochemical detector ED50 with an pH-Ag/AgCl electrode and an refractive index detector "Shodex RI-101". A new method had to be developed for product quantification (Figure 6). 400 µM fructose was used as internal standard. Oligosaccharides standards had a maximum concentration of 500 µM.

```
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        Pressure.UpperLimit =   2950
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        %B.Equate =     "wasser"
        %C.Equate =     "500 mM NaOAc"
        %D.Equate =     "%D"
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        Wait    FlushState
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        pH.UpperLimit =         13.0
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Integration = End
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        Waveform  Time = 0.44, Potential = -0.10
        Waveform  Time = 0.50, Potential = -0.10
        WaitForTemperature =    False
        Wait    SampleReady

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        %D =    0.0
        Curve = 5
        Load
        Wait    CycleTimeState
        Inject
        Wait    InjectState
        ECD_1.AcqOn
```



```
        ECD_Total.AcqOn
        Flow =  0.85
        %B =    72.5
        %C =    2.5
        %D =    0.0
        Curve = 5

20.100  Flow =  0.85
        %B =    65.0
        %C =    10.0
        %D =    0.0
        Curve = 5

40.100  Flow =  0.85
        %B =    60.0
        %C =    15.0
        %D =    0.0
        Curve = 5

50.100  Flow =  0.85
        %B =    35.0
        %C =    40.0
        %D =    0.0
        Curve = 5

60.100  ECD_1.AcqOff
        ECD_Total.AcqOff
        Flow =  0.85
        %B =    72.5
        %C =    2.5
        %D =    0.0
        Curve = 5

        End
```


Figure 6: Method for the quantification of oligosaccharides with HPAE-PAD.

2.6.3. High performance liquid chromatography (HPLC)

All HPLC analyses were carried out on a Merck-Hitachi LaChrom HPLC instrument equipped with a Merck L-7490 RI detector.

2.6.3.1. HPLC analysis on Aminex HPX-87H column with RI detection

Aminex HPX-87H column was performed at 65°C and a flow rate of 0.3 mL/min using 5 mM H_2SO_4 as mobile phase.

2.6.3.2. HPLC analysis on Lichrospher 100 C18 RP column with RI detection

Analysis on a Lichrospher 100 C18 RP column was performed at 30°C and a flow rate of 0.2 mL/min using 10 mM K_2HPO_4 with a pH of 2.0 as mobile phase. Samples and standards were filtrated via 96-well plate for centrifuges before measurement on the HPLC instrument.

2.6.3.3. HPLC analysis on Aminex HPX-87C column with RI detection

Aminex HPX-87C column was performed at 85°C and a flow rate of 0.2 mL/min using water as mobile phase. Standard concentrations ranged from 1 - 20 mM.

2.6.3.4. HPLC analysis on Phenomenex Luna NH_2 column with RI detection

Analysis on an Phenomenex Luna NH_2, 5 µm, 100 Å was performed at 60°C was and a flow rate of 1.5 mL/min using 37.5% acetonitrile in water as mobile phase. Standard concentrations ranged from 2 - 25 mM. Standard concentrations were correlated with the peak areas at the corresponding retention times. For rough characterization of the higher oligomeric products, where no standards are available, all areas were divided by the molecular weight of the corresponding product. This generated data was further used to calculate the approximate concentration via the standard curve of cellotetraose.

3. Results and Discussion

3.1. Determination of protein concentration

3.1.1. Cellodextrin phosphorylase from *C. cellulosi*

Table 6: Protein and activity measurements during protein purification.

Sample	Volume [mL]	Total protein [mg]	Total protein [%]	Activity [U/mL]	Total activity [%]	Activity [U/mg]
E. coli cell-free extract	10	514	100	202	100	3.9
His-Trap Loading, fraction 1-4	40	390	76			
His-Trap Elution, fraction 10-13	40	150	29			
Concentrated His-CcCdexP	3	116	23			
HI-Trap Purified His-CcCdexP	6	99	19	230	68	13.9

After the two-step chromatographic purification, approximately 70% of the total enzyme activity was found in the purified enzyme fraction. The specific activity of this fraction was increased 3.6-fold compared to the *E. coli* cell-free extract (Table 6).

3.1.2. Cellodextrin phosphorylase from *C. stercorarium*

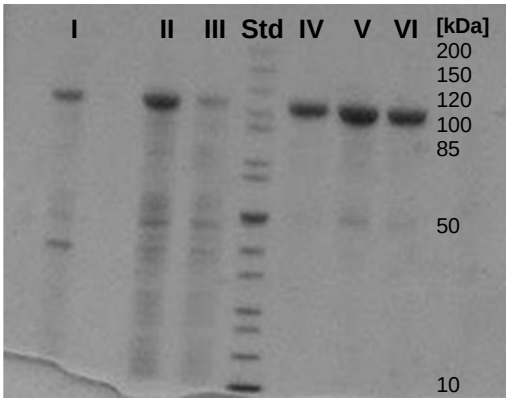
Table 7: Protein and activity measurements during protein purification.

Sample	Volume [mL]	Total protein [mg]	Total protein [%]	Activity [U/mL]	Total activity [%]	Activity [U/mg]
E. coli cell-free extract	10.0	325.5	100	56.6	100	1.7
His-Trap Loading, fraction 1-7	35.0	197.4	60.6			
His-Trap Elution, fraction 23-27	25.0	3.6	1.2			
Concentrated Purified His-CsCdexP	2.8	3.4	1	29.6	15	24.7

Approximatley 15% of the total enzyme activity was found in the purified enzyme fraction after the chromatographic purification. Compared to the *E. coli* cell-free extract, the specific activity of this fraction was increased 14-fold (Table 7).

3.2. SDS-PAGE - Cellodextrin phosphorylase from *C. cellulosi*

Figure 7 shows the purity of the different His-CcCdexP fractions during protein purification. Overexpression of His-CcCdexP could be detected by a strong band at >100 kDa which corresponds well with the expected molecular weight for His-CcCdexP of 112.8 kDa (calculated using the "Compute pI/Mw tool" on web.expasy.org).

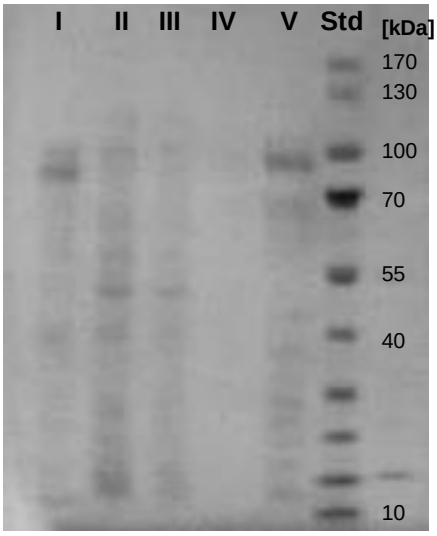


I: Inclusion bodies fraction
II: Cell-free extract
III: His-Trap Loading, fraction 1-4
IV: His-Trap Elution, fraction 10-13
V: Concentrated His-CcCdexP
VI: HI-Trap Purified His-CcCdexP
Std: PageRuler™ Protein Ladder

Figure 7: SDS-gel of the different fractions (I-VI) during protein purification.

3.3. SDS-PAGE - Cellodextrin phosphorylase from *C. stercorarium*

Figure 8 shows the purity of the different His-CsCdexP fractions during protein purification. Overexpression of His-CsCdexP could be detected by a band at <100 kDa which corresponds well with the expected molecular weight for His-CsCdexP of 91.5 kDa [8].



I: Inclusion bodies fraction
II: Cell-free extract
III: His-Trap Loading, fraction 1-7
IV: His-Trap Elution, fraction 23-27
V: Concentrated Purified His-CsCdexP
Std: PageRuler™ Prestained Protein Ladder

Figure 8: SDS-gel of the different fractions (I-V) during protein purification.

3.4. Activity assay using cell-free extract or purified His-CcCdexP

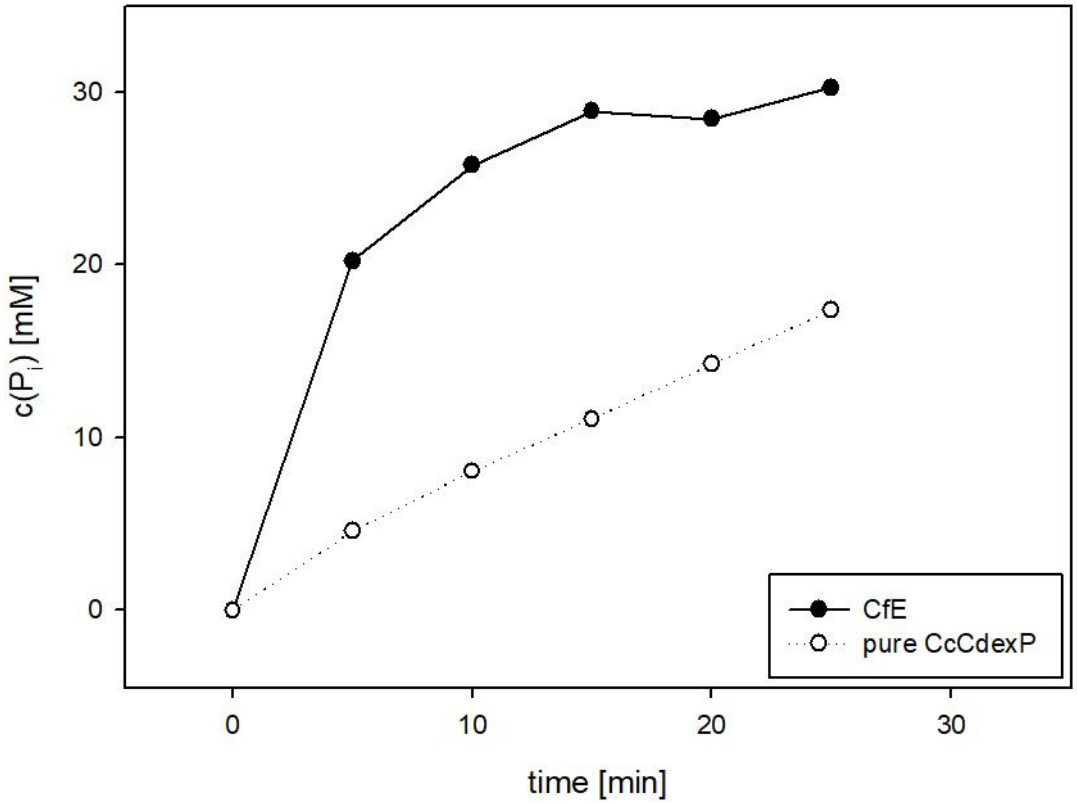


Figure 9: Determination of glucosyl transfer activity of His-CcCdexP. The release of phosphate was recorded over time and determined according to Saheki. The reaction mixture contained 40 mM Glc1-P, 50 mM cellobiose and purified cellodextrin phosphorylase (CcCdexP, empty dots, 0.066 mg/mL enzyme concentration) or cell free extract (CfE, filled dots, 1.03 mg/mL enzyme concentration). The reaction took place in 50 mM MOPS with a pH of 7.0 and a temperature of 55°C. The enzyme reaction was stopped at different time points by incubation at 99°C for 5 min.

Using the cell-free extract (1.03 mg/mL total protein) the release of phosphate was faster within the first 5 min compared to the purified enzyme fraction (0.066 mg/mL purified His-CcCdexP). As expected, the His-CcCdexP concentration was higher in the cell-free extract compared to the purified enzyme fraction. The purified His-CcCdexP showed a linear increase over time (Figure 9).

3.5. Activity assay using cell-free extract or purified His-CsCdexP

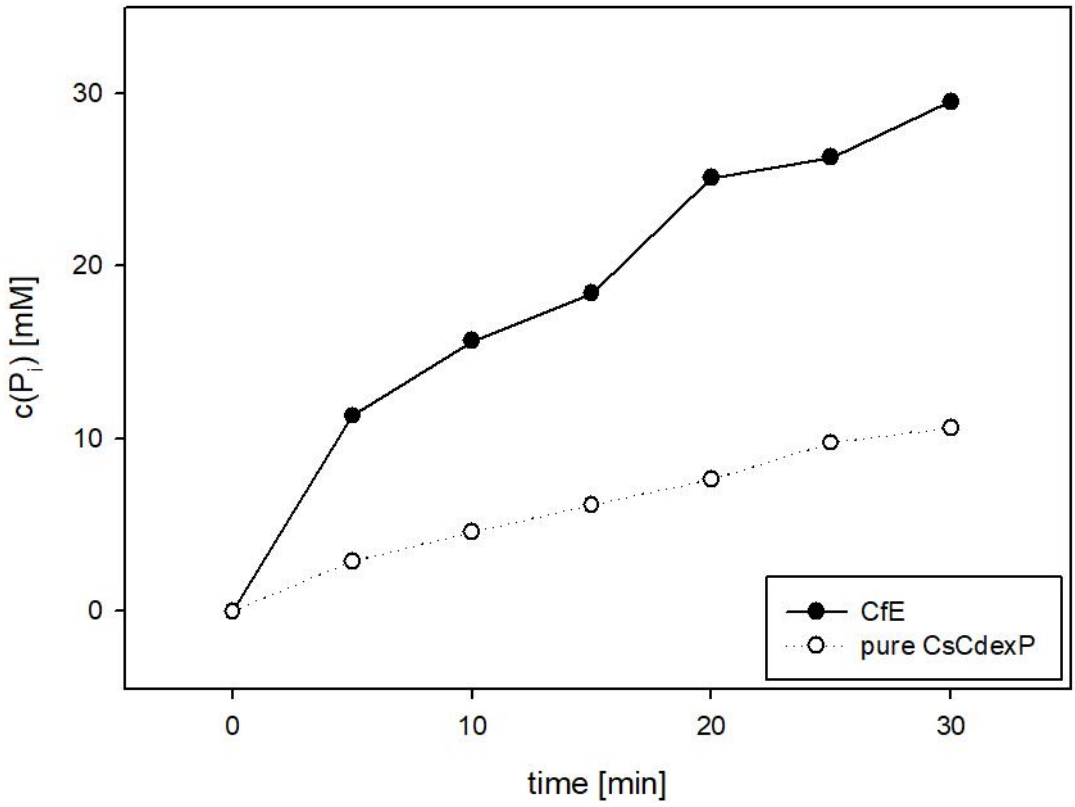


Figure 10: Determination of glucosyl transfer activity of His-CsCdexP. The release of phosphate was recorded over time and determined according to Saheki. The reaction mixture contained 50 mM Glc1-P, 50 mM cellobiose and purified cellodextrin phosphorylase (CsCdexP, empty dots, 0.023 mg/mL enzyme concentration) or cell free extract (CfE, filled dots, 1.30 mg/mL enzyme concentration). The reaction took place in 50 mM MOPS with a pH of 6.5 and a temperature of 65°C. The enzyme reaction was stopped at different time points by incubation at 99°C for 5 min.

The release of phosphate was faster within the first 5 min using the cell-free extract (1.30 mg/mL total protein) compared to the purified enzyme fraction (0.023 mg/mL purified His-CsCdexP). The purified His-CsCdexP reactions showed a linear increase over time (Figure 10).

3.6. *Clostridium cellulosi*: Determination of kinetic parameters - v_{max} and K_m

3.6.1. Synthesis direction

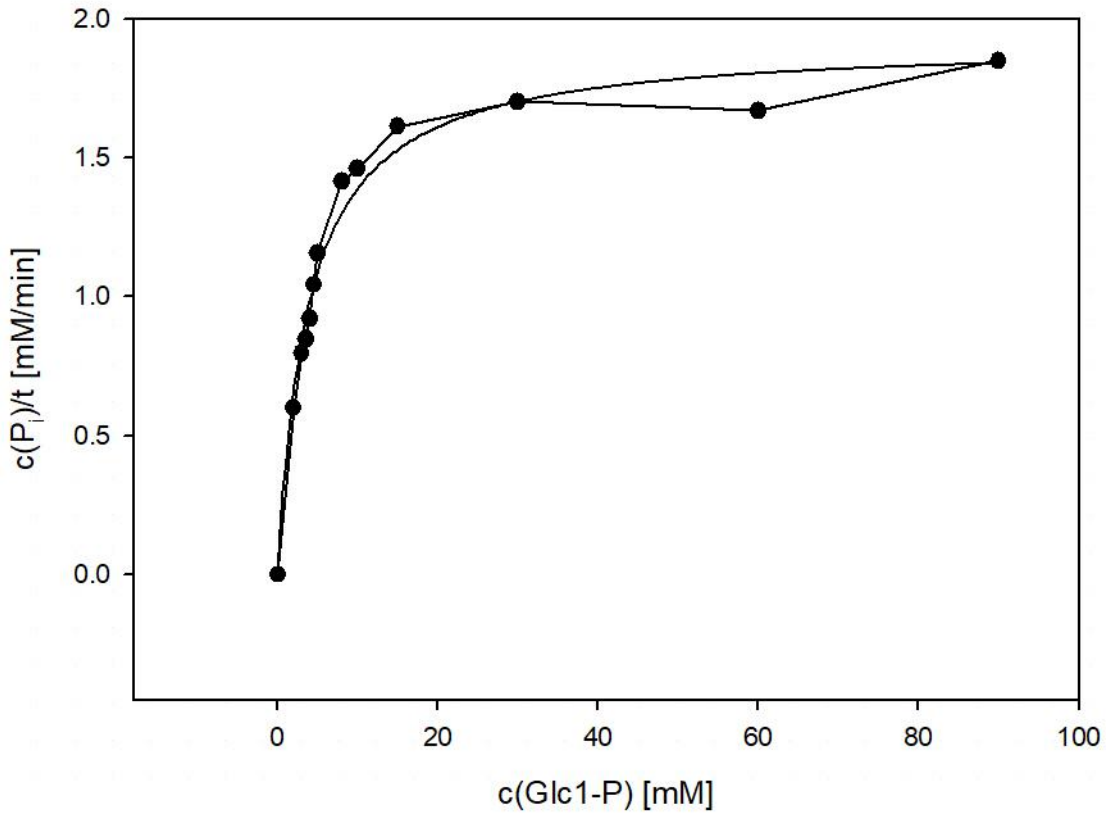


Figure 11: Determination of apparent kinetic parameters in synthesis direction. The reaction mixture contained 0-90 mM Glc1-P, 50 mM cellobiose and 0.066 mg/mL purified His-CcCdexP. The reaction took place in 50 mM MOPS with a pH of 7.0 and a temperature of 55°C. Enzymatic activity was stopped after 5 min by incubation at 99°C for 5 min. Release of phosphate was determined according to Saheki.

The results from Figure 11 were fitted according to a Michaelis-Menten kinetic equation (Sigma Plot, see Equation 1) and revealed a K_m value of 3.80 ± 0.35 mM for Glc1-P and v_{max} value of 1.92 ± 0.05 mM/min in synthesis direction.

3.6.2. Phosphorolysis direction

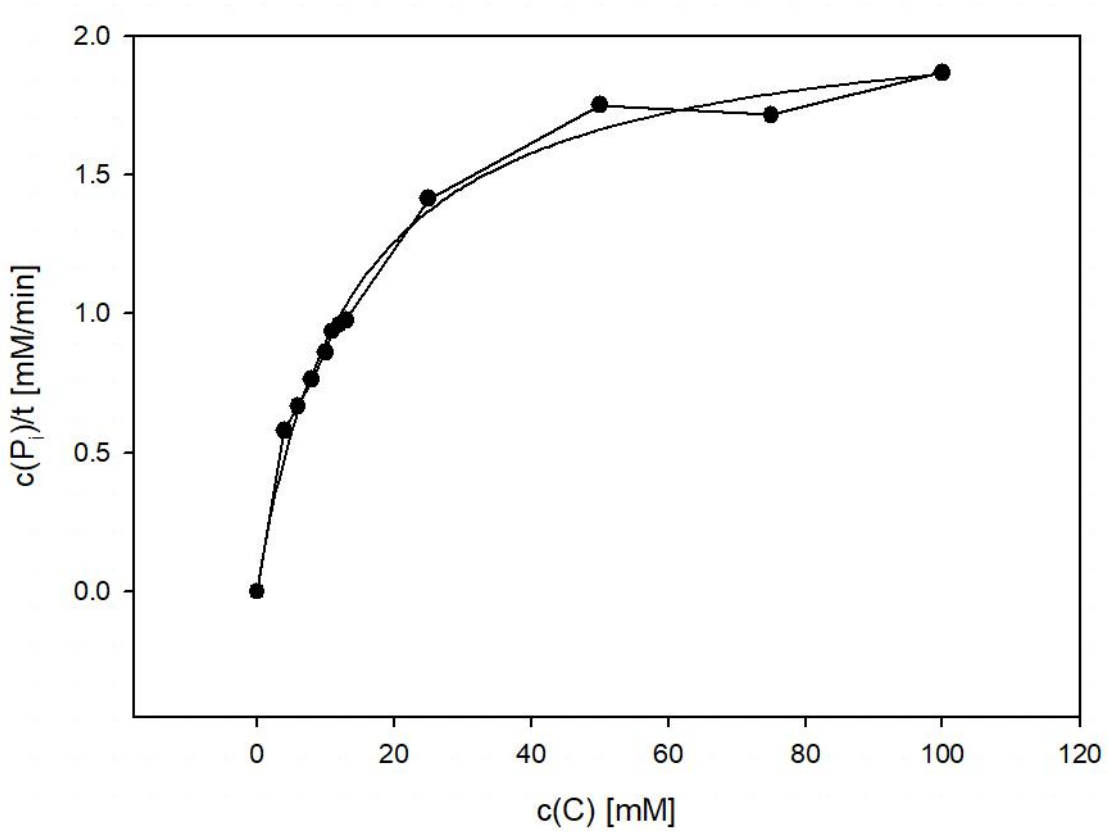


Figure 12: Determination of apparent kinetic parameters in phosphorolysis direction. The reaction mixture contained 20 mM Glc1-P, 0-100 mM cellobiose and 0.066 mg/mL purified His-CcCdexP. The reaction took place in 50 mM MOPS with a pH of 7.0 and a temperature of 55°C. Enzymatic activity was stopped after 5 min by incubation at 99°C for 5 min. Release of phosphate was determined according to Saheki.

The results from Figure 12 were fitted according to a Michaelis-Menten kinetic equation (Sigma Plot, see Equation 1) and revealed a K_m value of 13.59 ± 0.98 mM for cellobiose and v_{max} value of 2.11 ± 0.06 mM/min in phosphorolysis direction.

3.7. Characterization of oligosaccharide products

3.7.1. Thin layer chromatography (TLC)

To get a first estimation of the oligomeric product state a thin layer chromatography was performed. The next few figures show experimental challenges encountered due to unfit mobile phase composition and substrate concentration.

The same reaction mixture was analysed using different mobile phase concentrations. The acetonitrile:water concentration of 85:25 (v/v) (Figure 13) was too hydrophobic and all spots got smirked. With an acetonitrile:water concentration of 75:25 (v/v) (Figure 14) smirked spots were visible above 50 mM of cellobiose, where nearly no other products could be detected. These results indicate that the cellobiose concentration has to be below 50 mM for TLC analysis.

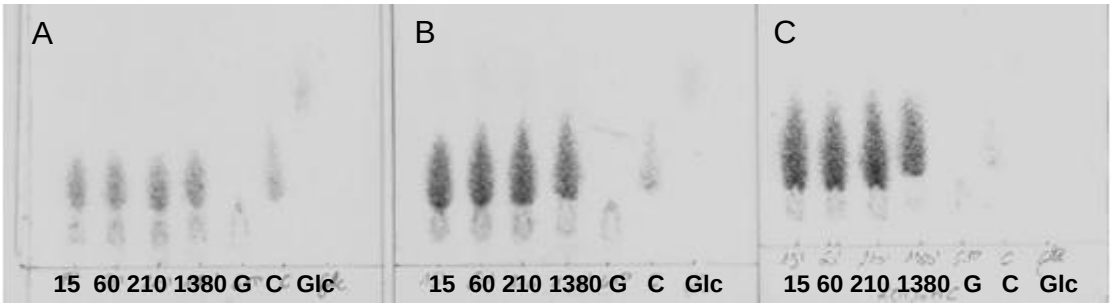


Figure 13: TLC analysis with acetonitrile:water (85:15; v/v), 1 run, 1 µL sample. Numbers = reaction time in minutes, G = Glc1-P, C = Cellobiose, Glc = Glucose. Standard concentration: 20 mM. Reaction parameters: 50 mM MOPS at pH 6.5, 55°C, 15 mM Glc1-P, 0.066 mg/mL purified His-CcCdexP. A: 50 mM C, B: 100 mM C, C: 200 mM C.

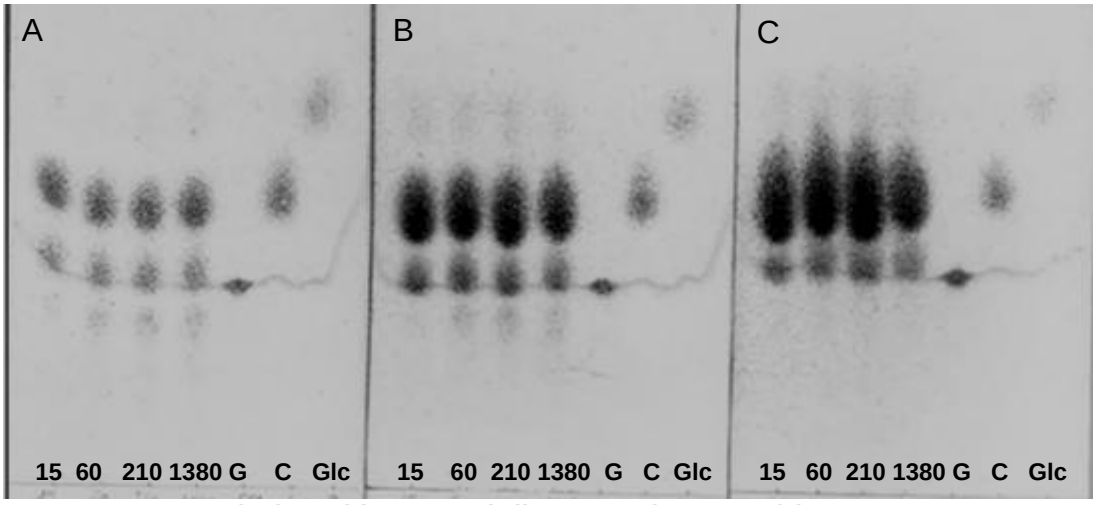


Figure 14: TLC analysis with acetonitrile:water (75:25; v/v), 1 run, 1 µL sample. Numbers = reaction time in minutes, G = Glc1-P, C = Cellobiose, Glc = Glucose. Standard concentration: 20 mM. Reaction parameters: 50 mM MOPS at pH 6.5, 55°C, 15 mM Glc1-P, 0.066 mg/mL purified His-CcCdexP. A: 50 mM C, B: 100 mM C, C: 200 mM C.

Testing of different Glc1-P concentrations with a mobile phase of acetonitrile:water of 75:25 (v/v) showed that also that substrate was problematic for TLC analysis at higher concentration. At 15 mM Glc1-P clear spots could be identified (Figure 15), but at 50 mM all spots were smirked (Figure 16). For further TLC analysis we decided to use Glc1-P concentrations not higher than 30 mM.

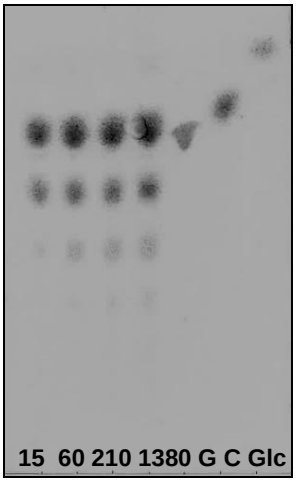


Figure 15: TLC analysis with acetonitrile:water (75:25; v/v), 2nd run, 1 µL sample. Numbers = reaction time in minutes, G = Glc1-P, C = Cellobiose, Glc = Glucose. Standard concentration: 20 mM. Reaction parameters: 50 mM MOPS at pH 6.5, 55°C, 15 mM Glc1-P, 50 mM C, 0.066 mg/mL purified His-CcCdexP.

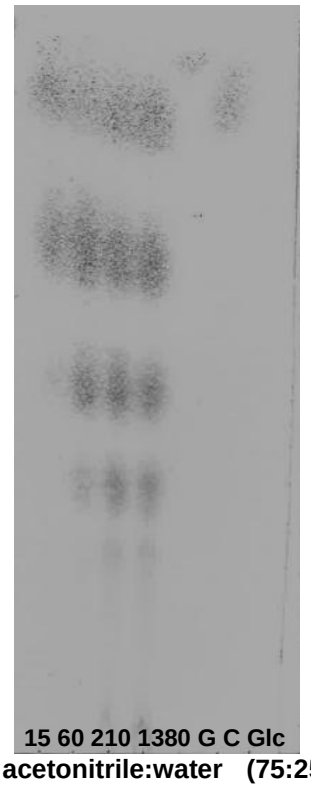


Figure 16: TLC analysis with acetonitrile:water (75:25; v/v), 2nd run, 1 µL sample. Numbers = reaction time in minutes, G = Glc1-P, C = Cellobiose, Glc = Glucose. Standard concentration: 20 mM. Reaciton parameters: 50 mM MOPS pH 6.5, 55°C, 50 mM Glc1-P, 50 mM C, 0.066 mg/mL purified His-CcCdexP.

Figure 17 shows a ideal TLC analysis with clearly defined spots using optimized conditions. Glc1-P appears as a defined line below cellotriose. At prolonged reaction time product spots of higher oligomeric state were obtained.

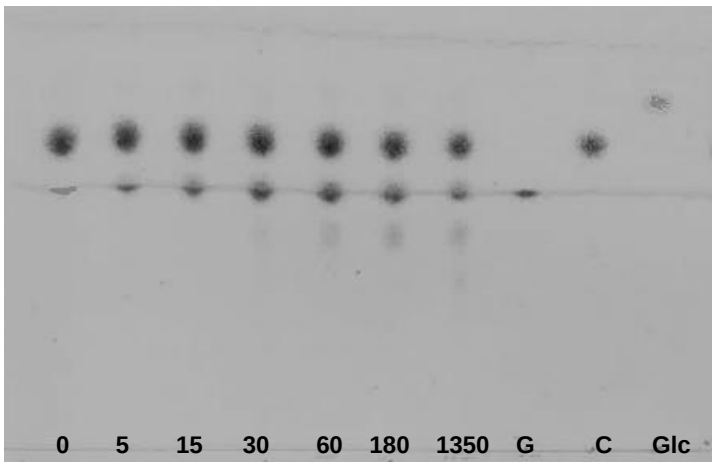


Figure 17: TLC analysis with acetonitrile:water (75:25; v/v), 2nd run, 0.5 µL sample. Numbers = reaction time in minutes, G = Glc1-P, C = Cellobiose, Glc = Glucose. Standard concentration: 20 mM. Reaction parameters: 50 mM TRIS-HCl at pH 7.0, 55°C, 15 mM Glc1-P, 50 mM C, 0.066 mg/mL purified His-CcCdexP.

3.7.2. High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

With the developed method it was possible to identify products of higher oligomeric state than by TLC. A general chromatogram is shown in Figure 18. The huge disadvantages of this method were on the one hand the long measuring time of 60 minutes and on the other hand the interaction of Glc1-P and inorganic phosphate with the CarboPac PA10 column. Fructose was used as internal standard. However, product quantification was still influenced by interaction of the phosphorylated compounds with the column which makes comparison of different reaction conditions difficult. The following results (shown as area) were all correlated with the internal fructose standard.

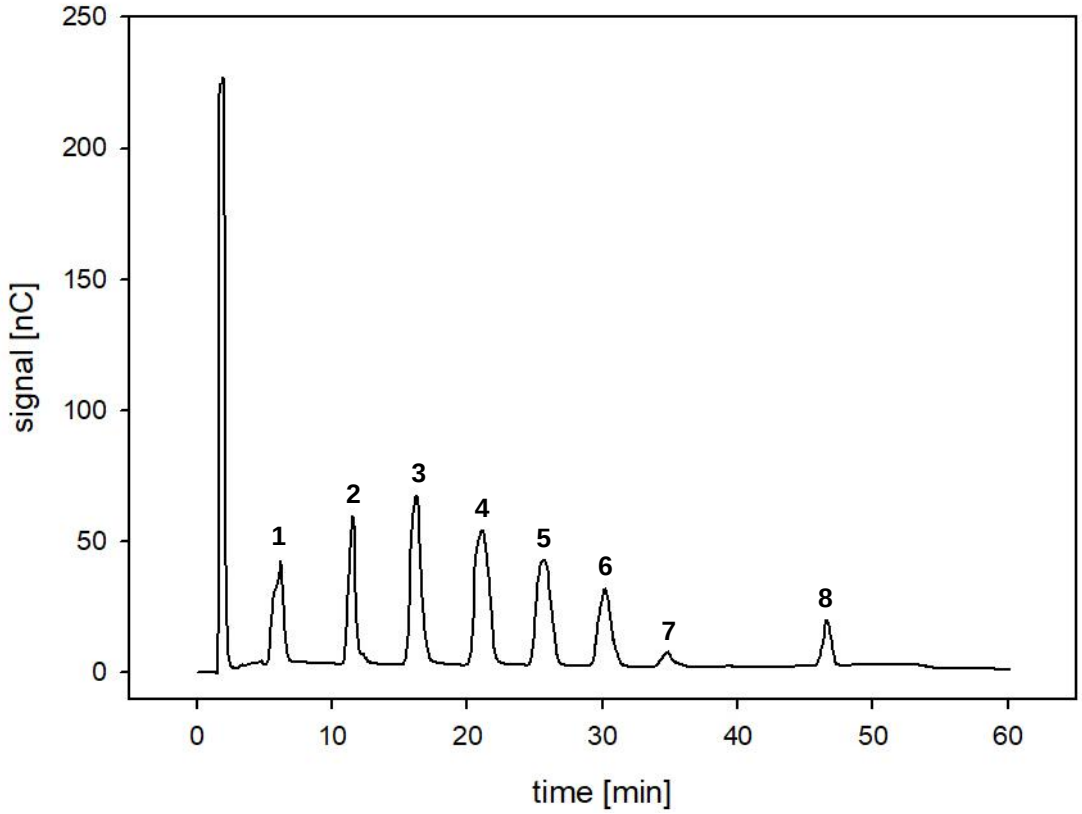


Figure 18: Product spectra obtained with HPAE-PAD. 1 = internal standard fructose, 2 = cellobiose, 3 = cellotriose, 4 = cellotetraose, 5 = cellopentaose, 6 = cellohexaose, 7 = celloheptaose, 8 = Glc1-P.

First the influence of donor and acceptor substrate ratio on product yield and product spectra was investigated. Three different concentrations of cellobiose were tested, at the same concentration of Glc1-P and the other way around. Also equimolar concentrations were considered.

To got a number how high this influence is, the measured areas (Crude Data, Table 21 - Table 24) were calculated within a product. The area of 1440 min were divided by the area of 360 min to saw the influence over time. For the ratio change one time point of the high excess was divided by the lower one. 1.0 represent no changes, numbers >1 an increasing product and numbers <1 a decrease. Results are shown in Table 8 - Table 11.

Using equimolar concentration of cellobiose and Glc1-P (Figure 19) the timeline shows that after 60 min only cellotriose and cellotetraose were produced. After 360 min the amount of cellotriose and cellotetraose was increased about 1.4-fold for cellotriose and 5.0-fold for cellotetraose. Also two other oligosaccharide products appeared (cellopentaose and cellohexaose). The reaction overnight (1440 min) revealed a lower amount of cellotriose, a similar amount of cellotetraose and a increase of cellopentaose and cellohexaose compared to the 360 min time point. These results are linked with the fact, that cellodextrin phosphorylase also accepts the produced oligosaccharides as glycosyl acceptor.

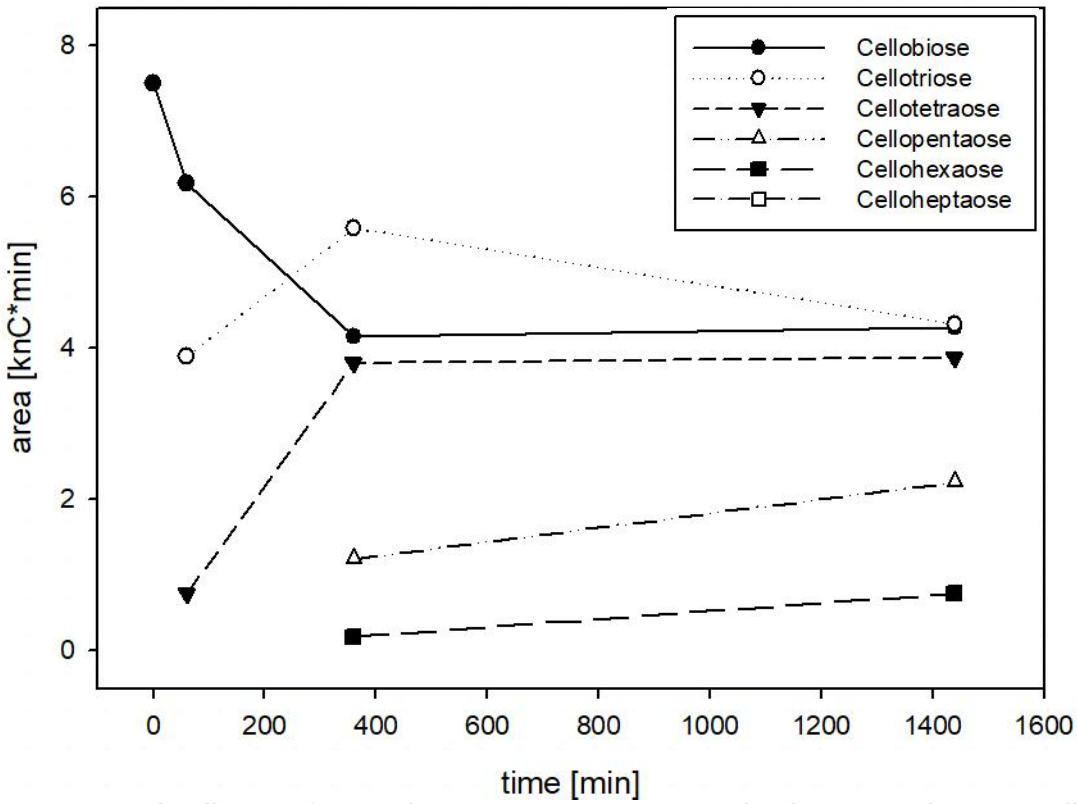
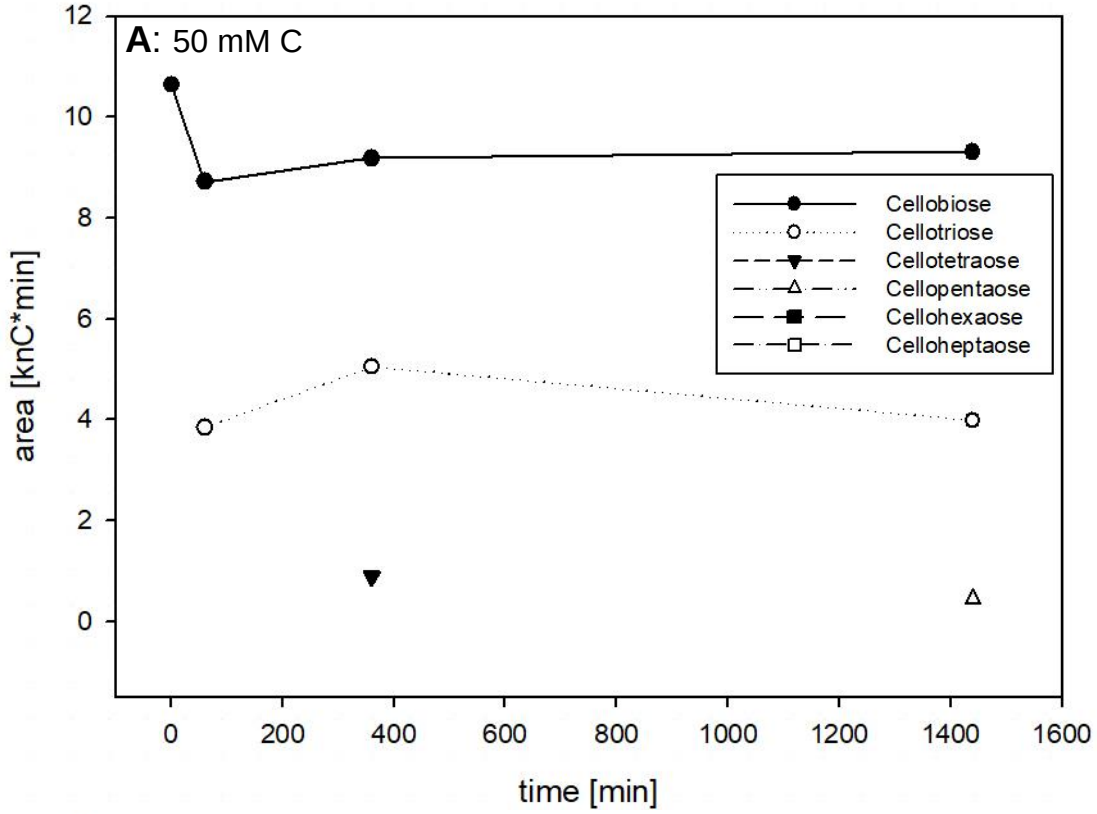


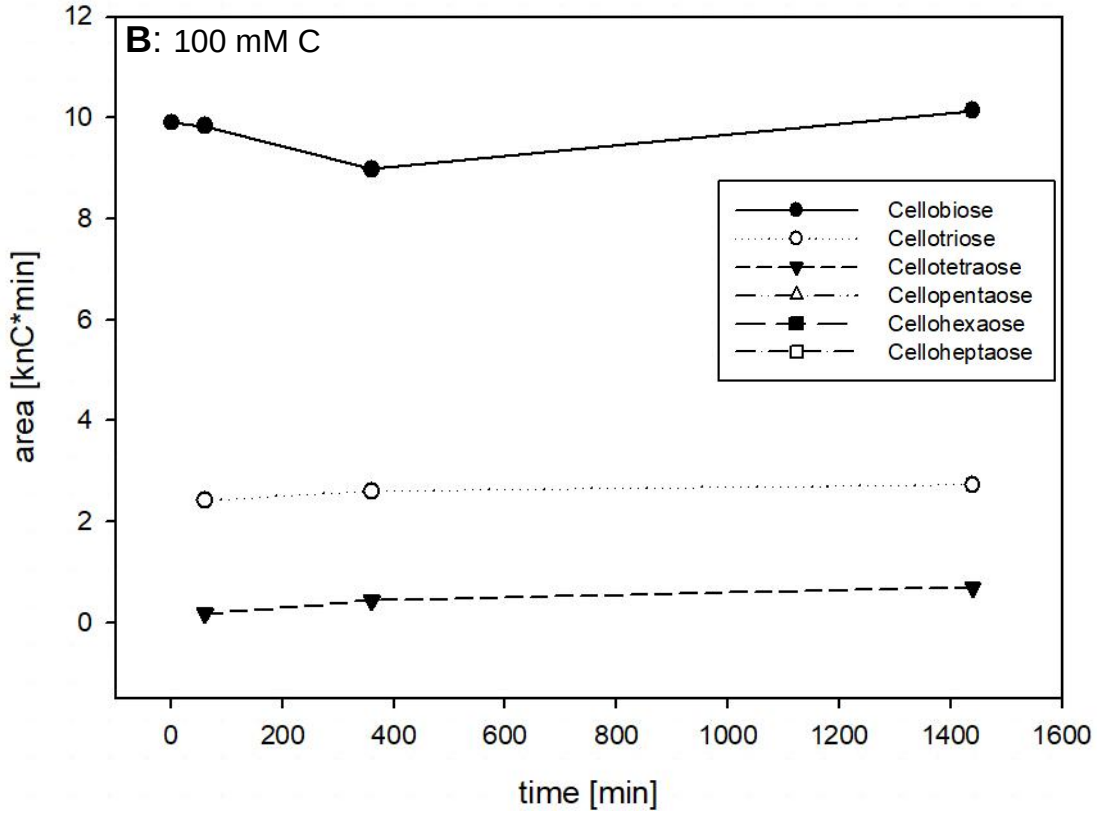
Figure 19: Timeline of product spectra at equimolar reaction conditions. 50 mM Glc1-P, 50 mM C in 50 mM Tris-HCl pH 7.0 and 0.066 mg/mL purified His-CcCdexP. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose.

Table 8: Product spectra changes for the equimolar reaction conditions. C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose. 1.0 = no change, >1.0 = increase, <1.0 = decrease.

Glc1-P/C/pH [mM/mM/pH]	time [min]	C	C3	C4	C5	C6	C7
50/50/7.0	60	0.7	1.4	5.0			
	360						
	360	1.0	0.8	1.0	1.8	4.0	
	1440						

The analysis of different concentrations of glycosyl donor (Glc1-P) and glycosyl acceptor (cellobiose) revealed that the ratio has a strong impact on the oligomeric product state. A 3.3-fold excess of cellobiose over Glc1-P showed that cellotriose, cellotetraose and cellopentaose were the main products (Figure 20, A), while a 13.3-fold excess revealed only cellotriose production (Figure 20, C) and a 6.7-fold excess produced mainly cellotriose and cellotetraose (Figure 20, B).


A: 50 mM C
area [knC*min]
time [min]
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose


B: 100 mM C
area [knC*min]
time [min]
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose

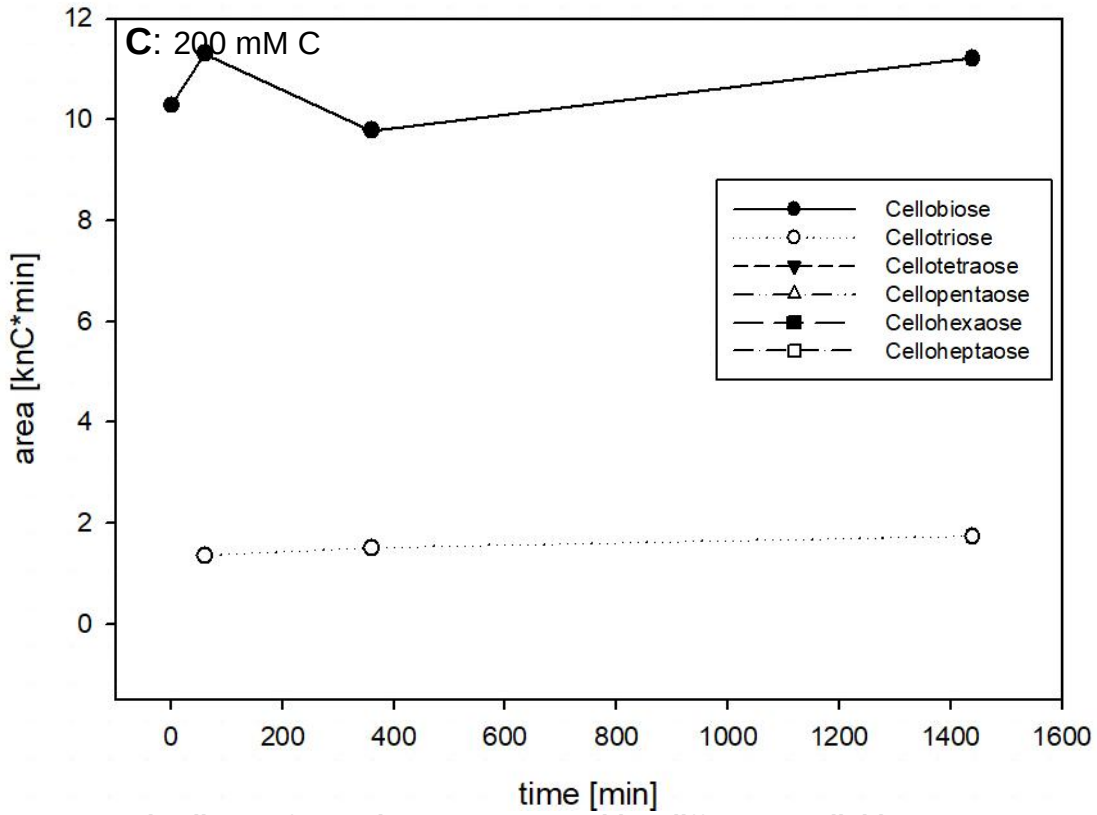


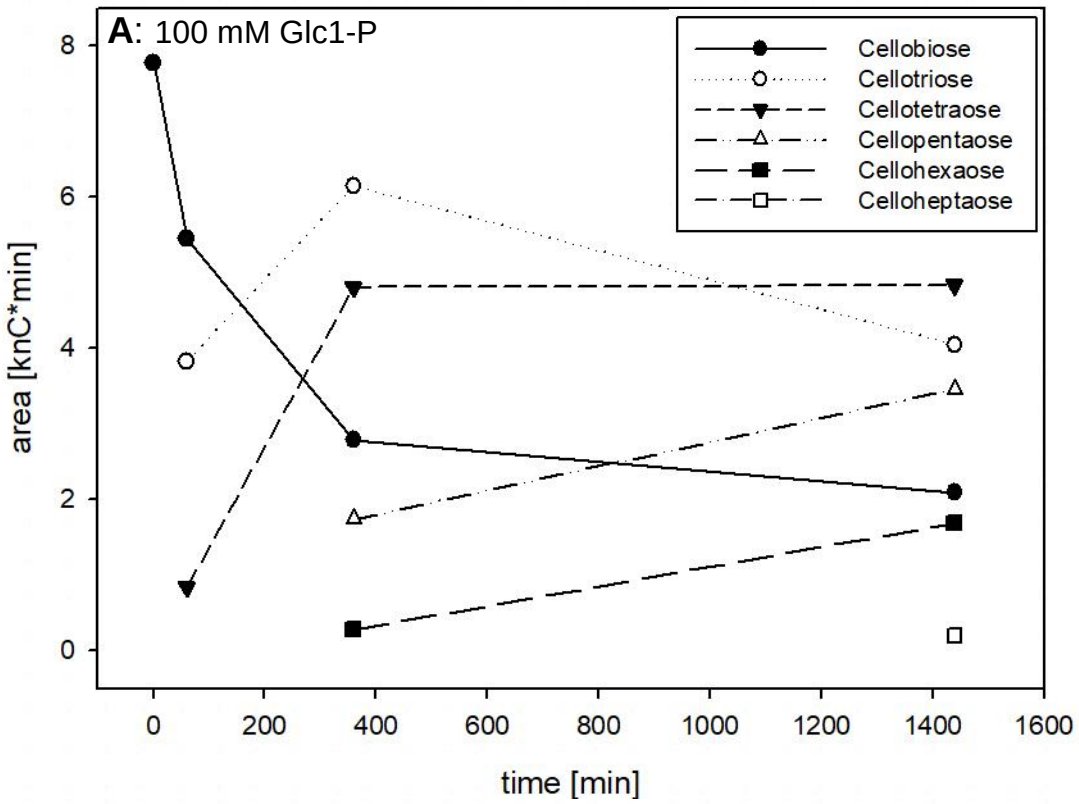
Figure 20: Timeline of product spectra with different cellobiose concentrations. 15 mM Glc1-P in 50 mM Tris-HCl pH 7.0 and 0.066 mg/mL purified His-CcCdexP. A: 50 mM cellobiose, B: 100 mM cellobiose, C: 200 mM cellobiose. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose.

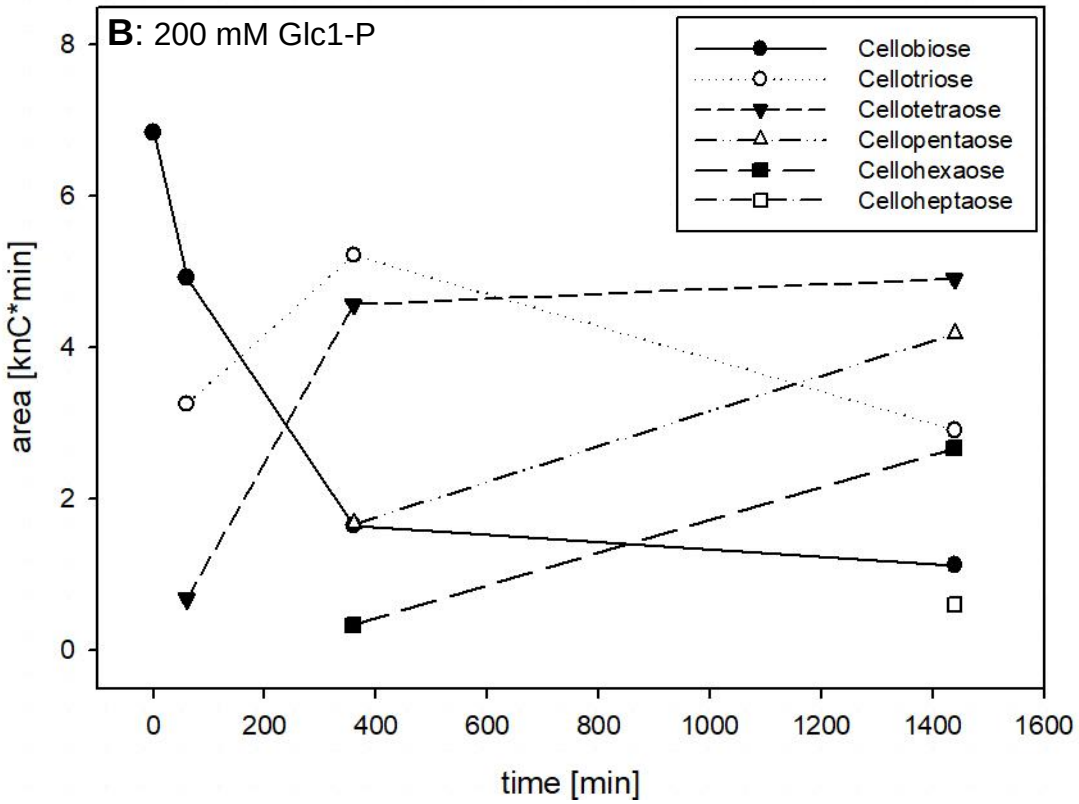
Table 9: Product spectra changes for reaction condtions with excess of the cellobiose. C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose. 1.0 = no change, >1.0 = increase, <1.0 = decrease.

Glc1-P/C/pH [mM/mM/pH]	time [min]	C	C3	C4	C5	C6	C7
A 15/50/7.0	360	0.5	0.8				
	1440						
B 15/100/7.0	360	1.1	1.0	1.6			
	1440						
C 15/200/7.0	360	1.1	1.2				
	1440						
B/A	360	0.5	0.5	0.5			
	1440	1.1	0.7				
C/B	360	1.1	0.6				
	1440	1.1	0.6				

Product spectra in the presence of excess of Glc1-P are shown in Figure 21. A 2-fold excess of Glc1-P over cellobiose (Figure 21, A) revealed mainly cellotetraose, cellotriose and cellopentaose formation, but also small amounts of cellohexaose and celloheptaose were detected. The 4-fold and 6-fold excess of Glc1-P over cellobiose revealed a similar product spectra (Figure 21, B and C). Cellotetraose and celloheptaose were the main products, while cellotriose concentration was reduced in favour of cellohexaose. The effect that cellohexaose increased over time are shown in all three reaction conditions. This product also increased with higher Glc1-P excess over cellobiose, where the range is between 6.1-fold to 8.3-fold (Table 10).

Generally speaking, the oligosaccharide spectrum is smaller at earlier reaction times than at later time points.


A: 100 mM Glc1-P
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
2
4
6
8
0
200
400
600
800
1000
1200
1400
1600


B: 200 mM Glc1-P
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
2
4
6
8
0
200
400
600
800
1000
1200
1400
1600

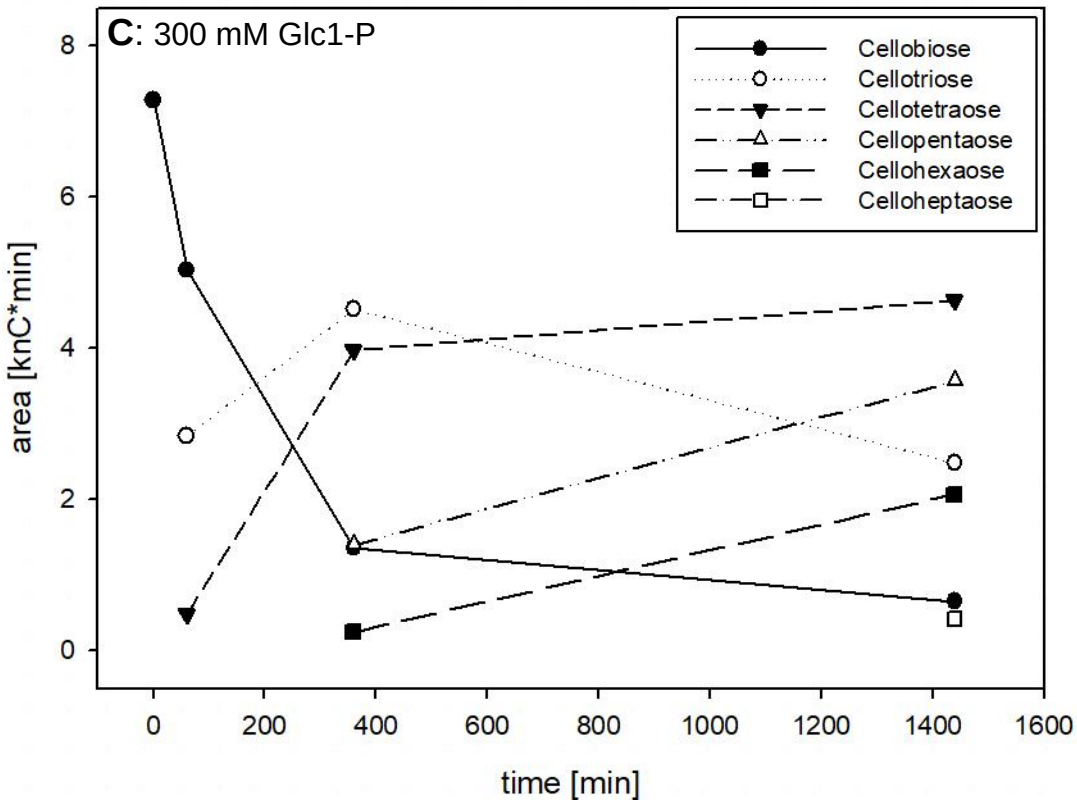


Figure 21: Timeline of product spectra with different Glc1-P concentrations. 50 mM cellobiose in 50 mM Tris-HCl pH 7.0 and 0.066 mg/mL purified His-CcCdexP. A: 100 mM Glc1-P, B: 200 mM Glc1-P, C: 300 mM Glc1-P. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose.

Table 10: Product spectra changes for reaction conditions with excess of Glc1-P. C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose. 1.0 = no change, >1.0 = increase, <1.0 = decrease.

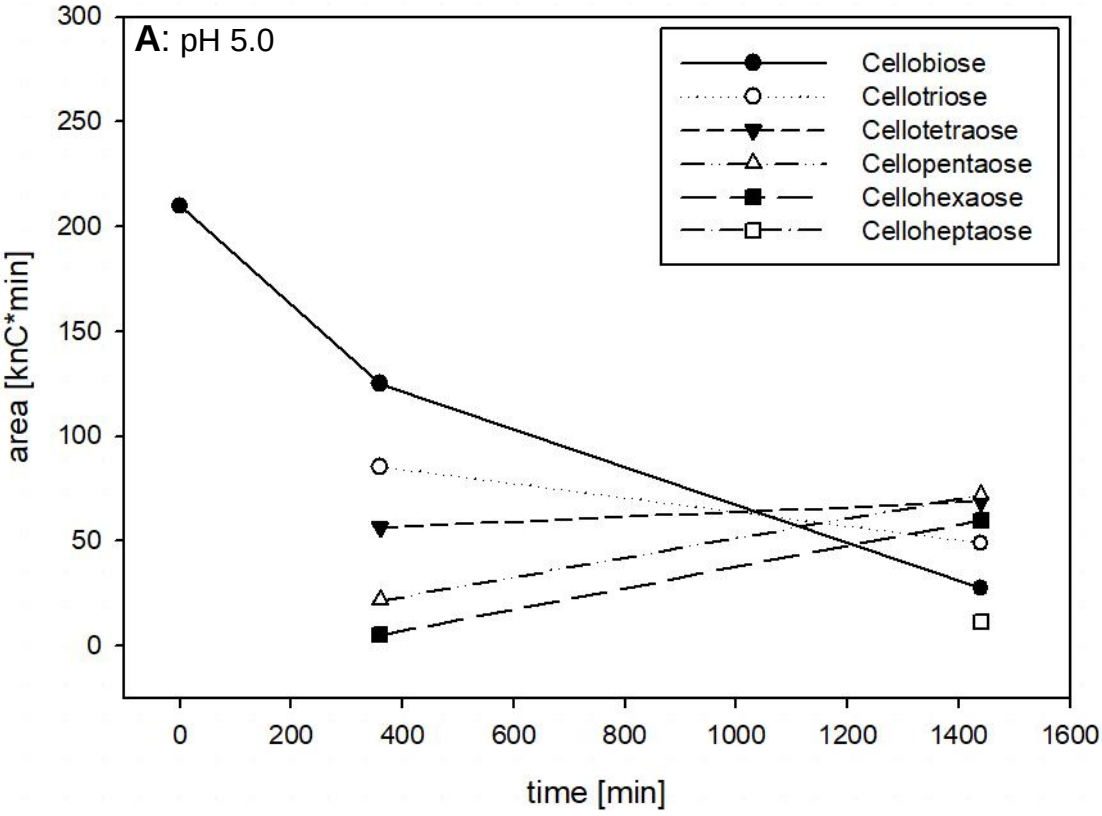
Glc1-P/C/pH [mM/mM/-]	time [min]	C	C3	C4	C5	C6	C7
A 100/50/7.0	360	0.8	0.7	1.0	2.0	6.1	
	1440						
B 200/50/7.0	360	0.7	0.6	1.1	2.5	7.8	
	1440						
C 300/50/7.0	360	0.5	0.5	1.2	2.5	8.3	
	1440						
B/A	360	0.6	0.8	1.0	1.0	1.2	
	1440	0.5	0.7	1.0	1.2	1.6	2.9
C/B	360	0.8	0.9	0.9	0.8	0.7	
	1440	0.6	0.9	0.9	0.9	0.8	0.7

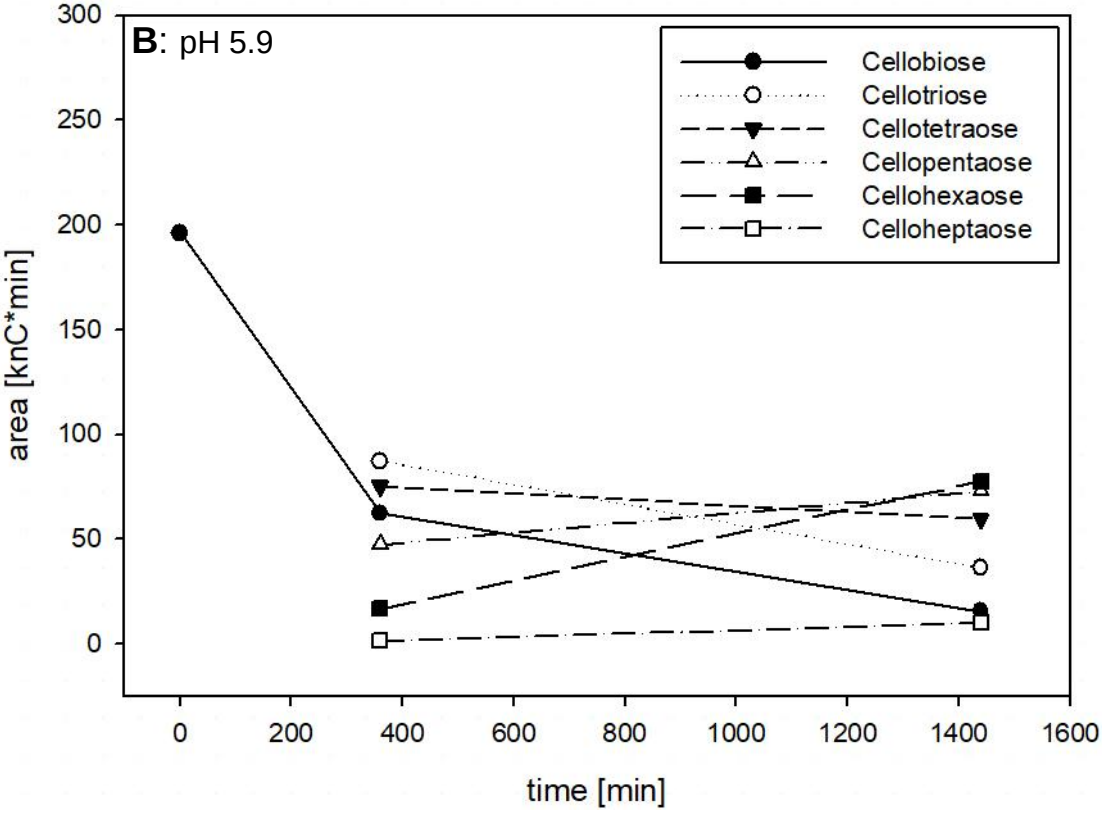
Another aspect to be considered was the influence of different pH values and the presence of dithiotreitol (DTT, 5 mM) on the product spectra. The influence of DTT was mentioned in literature [4]. Previous experiments showed that higher glucosyl donor concentrations are better to study the influence of different parameters on the product spectra. We therefore chose 300 mM of Glc1-P and 50 mM of cellobiose.

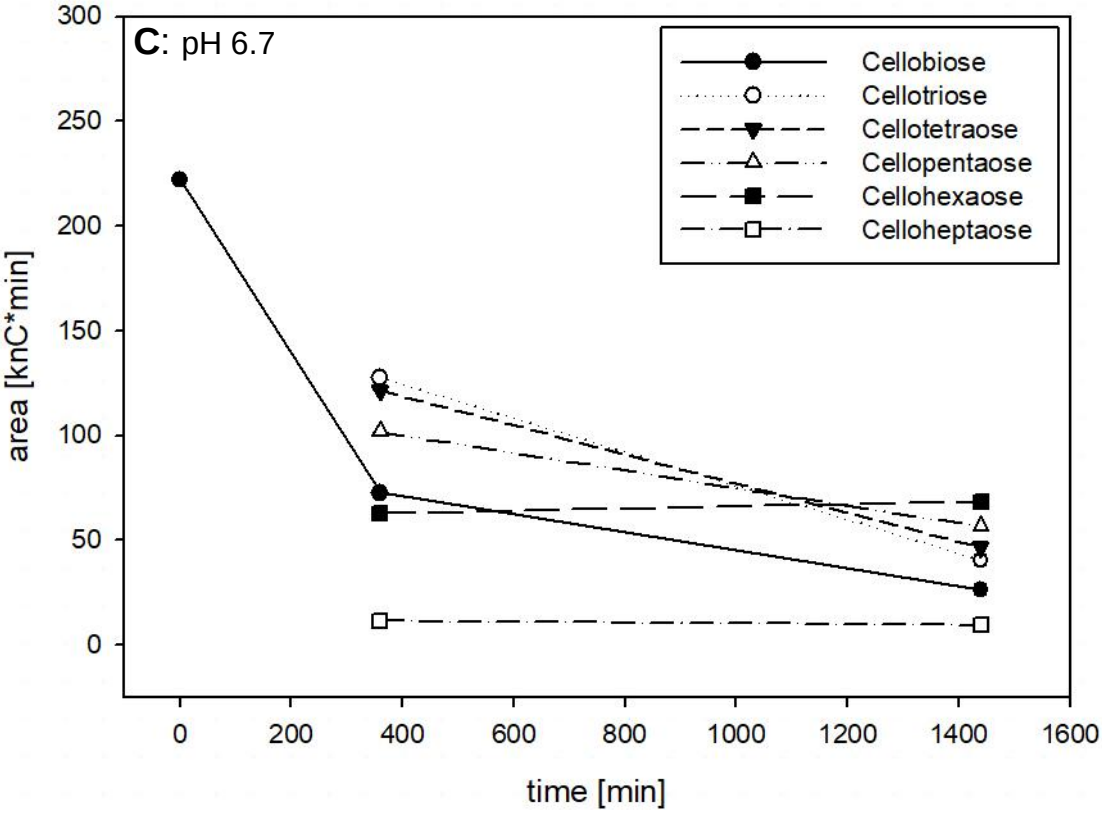
Figure 22 shows time courses of product formation record at different pH values in the absence and presence of DTT. After 1440 min, all the different assay conditions revealed similar product distribution and similar product amounts. While after 360 min a pH-dependence could be observed. pH 5.0 showed the lowest concentrations of oligomeric product formation. pH 5.9 and 6.7 (Figure 22, B and C) showed that the concentration of the higher cellodextrins increased by 1.1- to 12.5-fold, respectively (Table 11, B/A and C/A). At pH 6.7 and 7.1 (Figure 22, C and D) similar results were obtained, but the pH difference was only ΔpH 0.4. Figure 22 E shows the influence of DTT at pH 8.0 on product formation. After 360 min the oligomeric products with DP>5 were more increased than at the optimum condition (1.3- to 2.5-fold, Table 11, E/D). DTT stabilizes the disulfide-bounds of the enzyme. Due to the fact that *Clostridium cellulosi* is an anaerobe bacteria exposure to air affects the enzyme structure and thus the reaction reactivity. DTT could help to stabilize the enzyme towards oxidative denaturation.

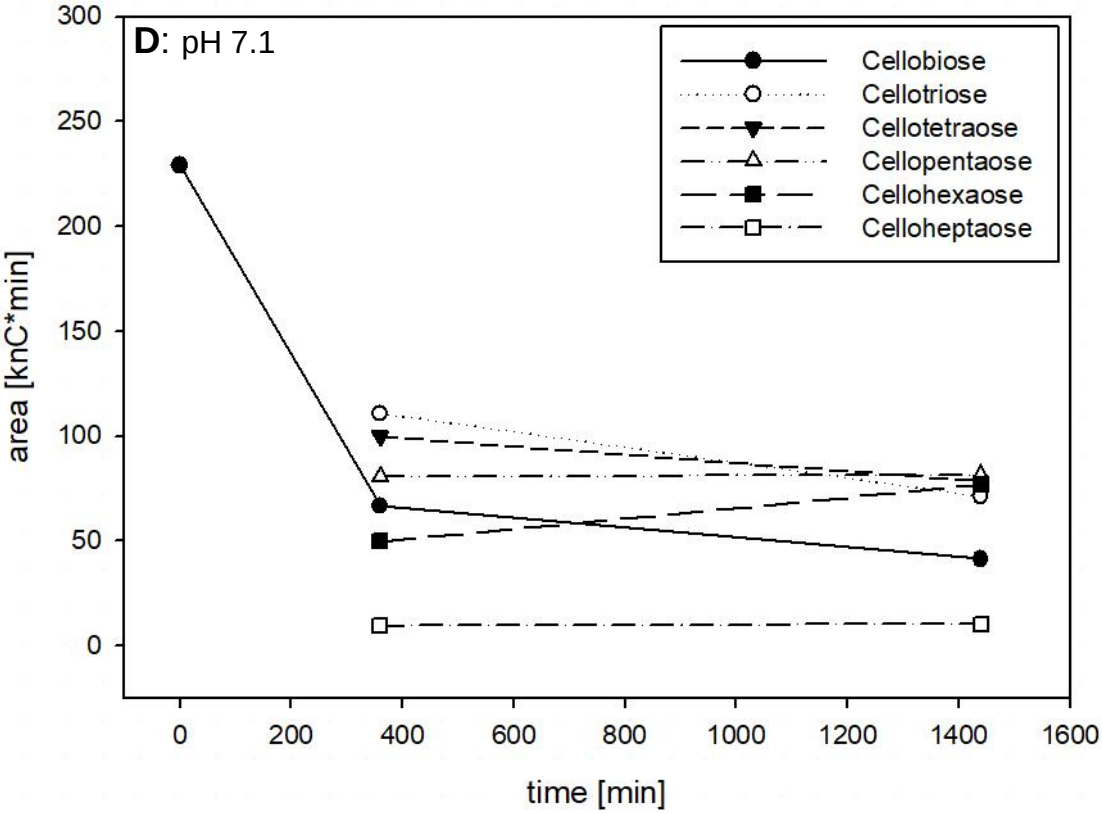
At a pH of 8.5 (Figure 22, F) mainly cellotriose was formed and the product spectrum did not really change over time. This could mean that the cellodextrin phosphorylase does not remain active for long under this condition, due to enzyme inactivation.

Which correlates well with the pH optimum. The enzyme shows only 77.4% activity at pH 8.5 correlated to the optimum pH 7.1 at 360 min. Nethertheless this could be a good regulation point, in order to get mainly cellotriose for further product isolation.


A: pH 5.0
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200
1400
1600


B: pH 5.9
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200
1400
1600


C: pH 6.7
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200
1400
1600


D: pH 7.1
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
area [knC*min]
time [min]
0
50
100
150
200
250
300
0
200
400
600
800
1000
1200
1400
1600

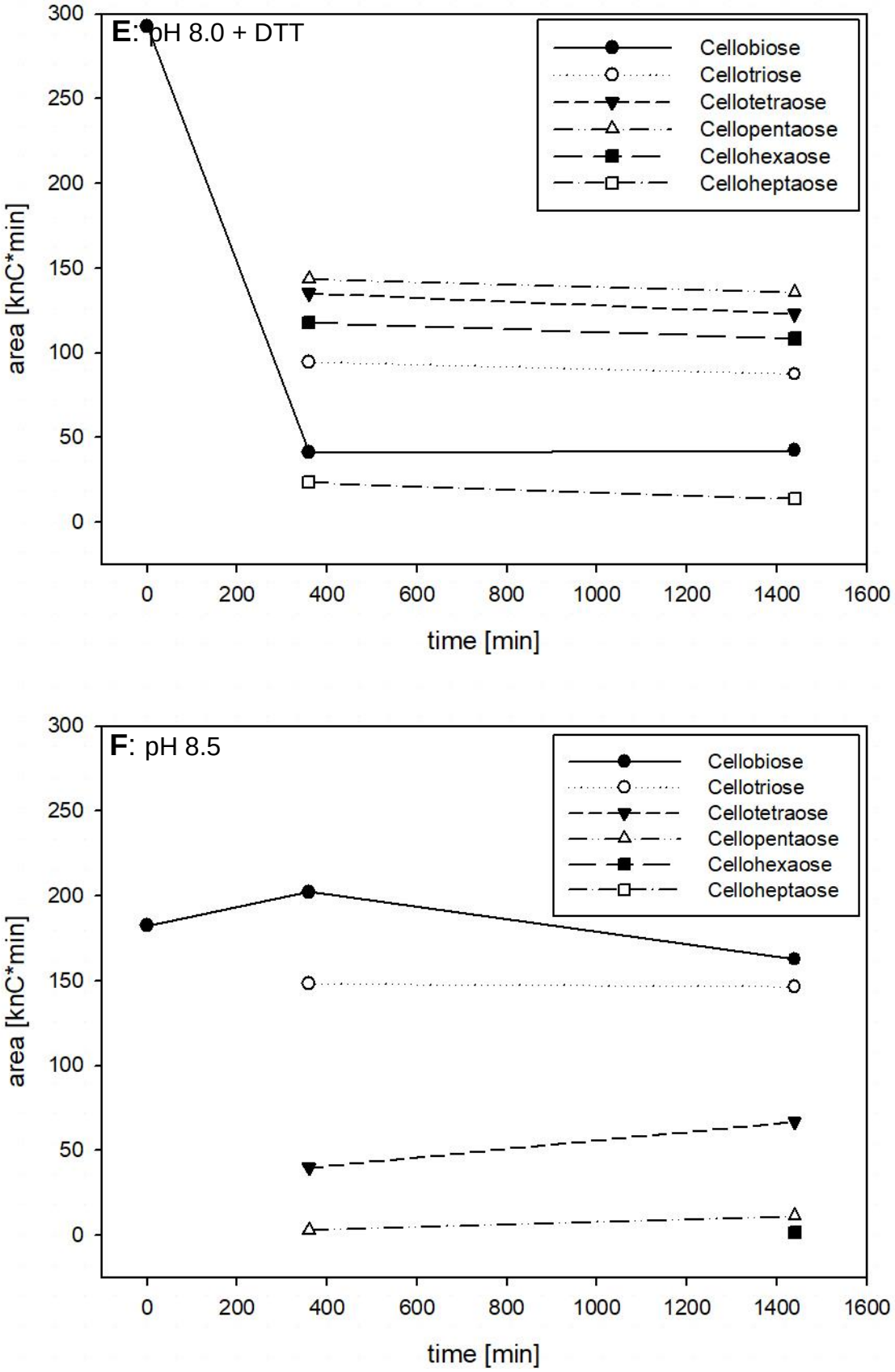


Figure 22: Timeline of product spectra at different pH values and addition of DTT.
300 mM Glc1-P, 50 mM cellobiose in 50 mM buffer (letters A-F) and 0.066 mg/mL purified His-CcCdexP. A: Citric acid pH 5.04, B: MES pH 5.94, C: Citric acid pH 6.68, D: MES pH 7.13, E: TRIS-HCl pH 7.97 + 5 mM DTT, F: HEPES pH 8.49. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose.

Table 11: Product spectra changes for reaction conditions with different pH values and addition of DTT. C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose. 1.0 = no change, >1.0 = increase, <1.0 = decrease.

Glc1-P/C/pH [mM/mM/-]	time [min]	C	C3	C4	C5	C6	C7
E 300/50/8.0 + 5mM DTT	360	1.0	0.9	0.9	0.9	0.9	0.6
	1440						
F 300/50/8.5	360	0.8	1.0	1.7	3.8		
	1440						
C 300/50/6.7	360	0.4	0.3	0.4	0.6	1.1	0.8
	1440						
D 300/50/7.1	360	0.6	0.6	0.8	1.0	1.5	1.1
	1440						
A 300/50/5.0	360	0.2	0.6	1.2	3.4	11.9	
	1440						
B 300/50/5.9	360	0.2	0.4	0.8	1.5	4.7	7.1
	1440						
E/D	360	0.6	0.9	1.4	1.8	2.4	2.5
	1440	1.0	1.2	1.6	1.7	1.4	1.3
F/D	360	3.0	1.3	0.4	0.0		
	1440	3.9	2.1	0.9	0.1	0.0	
C/D	360	1.1	1.2	1.2	1.3	1.3	1.2
	1440	0.6	0.6	0.6	0.7	0.9	0.9
A/D	360	1.9	0.8	0.6	0.3	0.1	
	1440	0.7	0.7	0.9	0.9	0.8	1.1
B/D	360	0.9	0.8	0.8	0.6	0.3	0.1
	1440	0.4	0.5	0.8	0.9	1.0	1.0
B/A	360	0.5	1.0	1.3	2.2	3.3	
	1440	0.6	0.7	0.9	1.0	1.3	0.9
C/A	360	0.6	1.5	2.2	4.7	12.5	
	1440	1.0	0.8	0.7	0.8	1.1	0.8

3.7.3. High performance liquid chromatography (HPLC)

3.7.3.1. HPLC analysis on Aminex HPX-87H column with RI detection

The Aminex HPX-87H column was tested for analysis of the product mixtures. Figure 23 shows an overlay of the chromatograms of the standards cellobiose and Glc1-P (dashed line) and the product mixtures (solid line). Overlapping peaks of substrate and product revealed that the column cannot be used for product quantification.

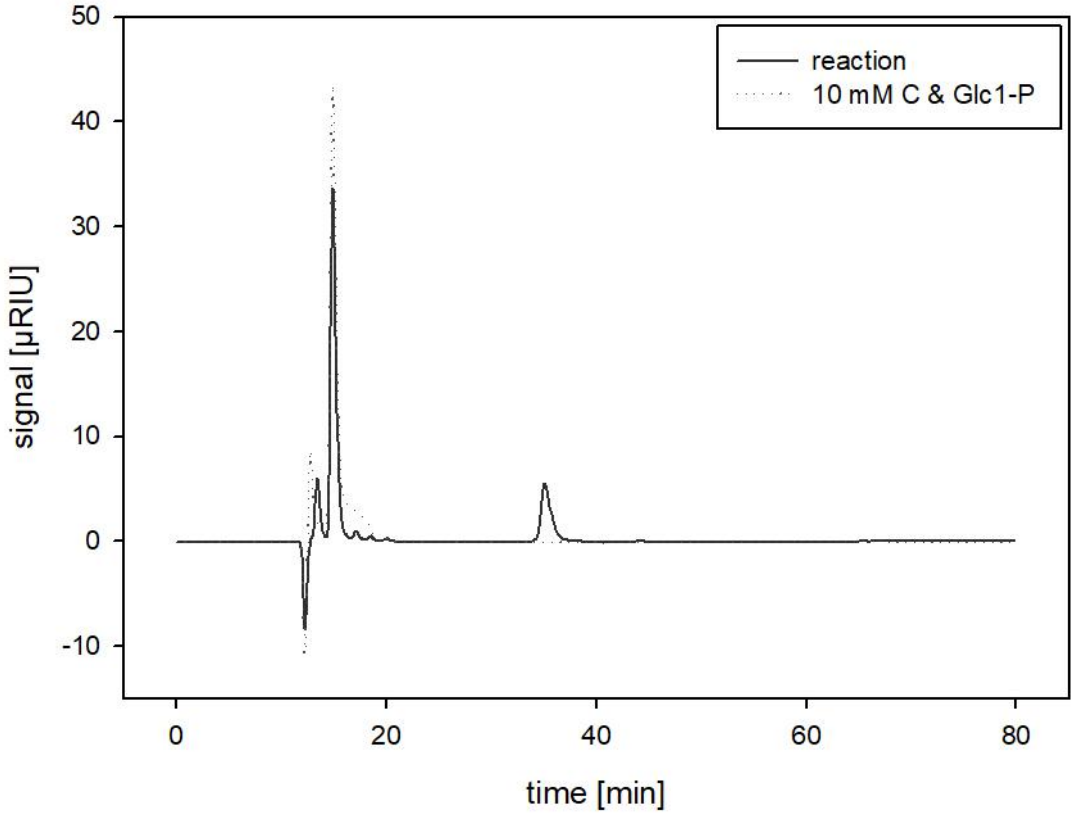


Figure 23: HPLC chromatograms of product mixture and standard on an Aminex HPX-87H column. Operating conditions: 65°C, flow rate: 0.3 mL/min, mobile phase: 5 mM H_2SO_4. Solid line = 1380 min reaction assay with 100 mM cellobiose, 50 mM Glc1-P, 50 mM TRIS-HCl pH 7.0 and 0.066 mg/mL His-CcCdexP. Dashed line = standard mixture with 10 mM cellobiose (C) and Glc1-P in ddH_2O.

3.7.3.2. HPLC analysis on a Lichrospher 100 C18 RP column with RI detection

The use of the Lichrospher 100 C18 RP column brought upon some difficulties. Figure 24 shows that Glc1-P (first peak of dashed line) and the buffer-system (dotted line) were co-eluting. Interestingly, the standard mixture (Glc1-P and cellobiose, dashed line) showed two peaks for cellobiose, while the single standards had only one peak. Moreover peak resolution of the reaction sample (solid line) could not be achieved. Therefore the use of Lichrospher 100 C18 RP column was not further pursued.

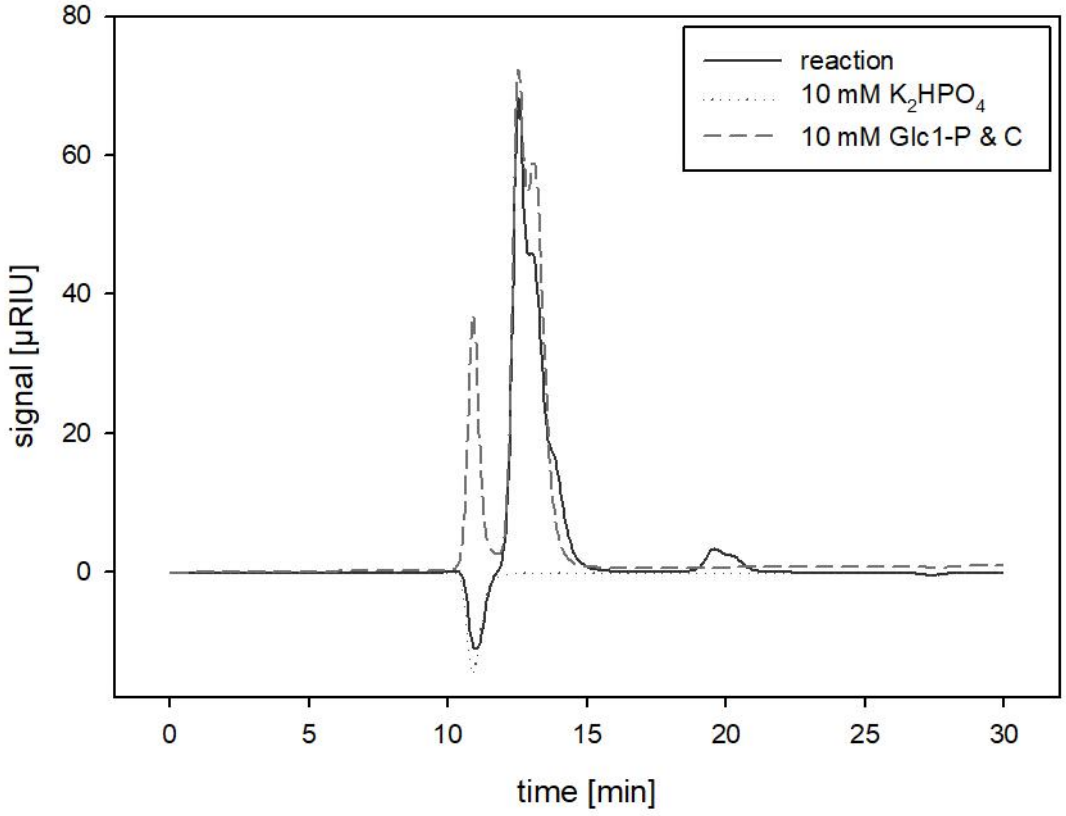


Figure 24: HPLC chromatograms of product mixture, buffer system and standard on a Lichrospher 100 C18 RP column. Operating conditions: 30°C, flow rate: 0.2 mL/min, mobile phase: 10 mM K_2HPO_4 at pH 2.0. Solid line = 1380 min reaction assay with 50 mM cellobiose, 30 mM Glc1-P, 50 mM MOPS pH 6.5 and 0.066 mg/mL His-CcCdexP. Dotted line = 10 mM K_2HPO_4 buffer system at pH 2.0. Dashed line = standard mixture with 10 mM cellobiose (C) and Glc1-P in ddH_2O.

3.7.3.3. Aminex HPX-87C column

The HPLC chromatograms of product mixture and standards on an Aminex HPX-87C column are shown in Figure 25. The chromatograms were recorded under optimized conditions where the best possible resolution of the reaction mixture was obtained. Product quantification was also difficult, due to overlapping peaks. Peak 1 corresponds to the fraction containing Glc1-P, oligomeric products with DP>5 and part of the buffer signal. Peak 5 corresponds to the fraction containing glucose (standard chromatogram, dotted line) and the second buffer signal. Note, that the buffer system (MOPS) eluted with two peaks from the column.

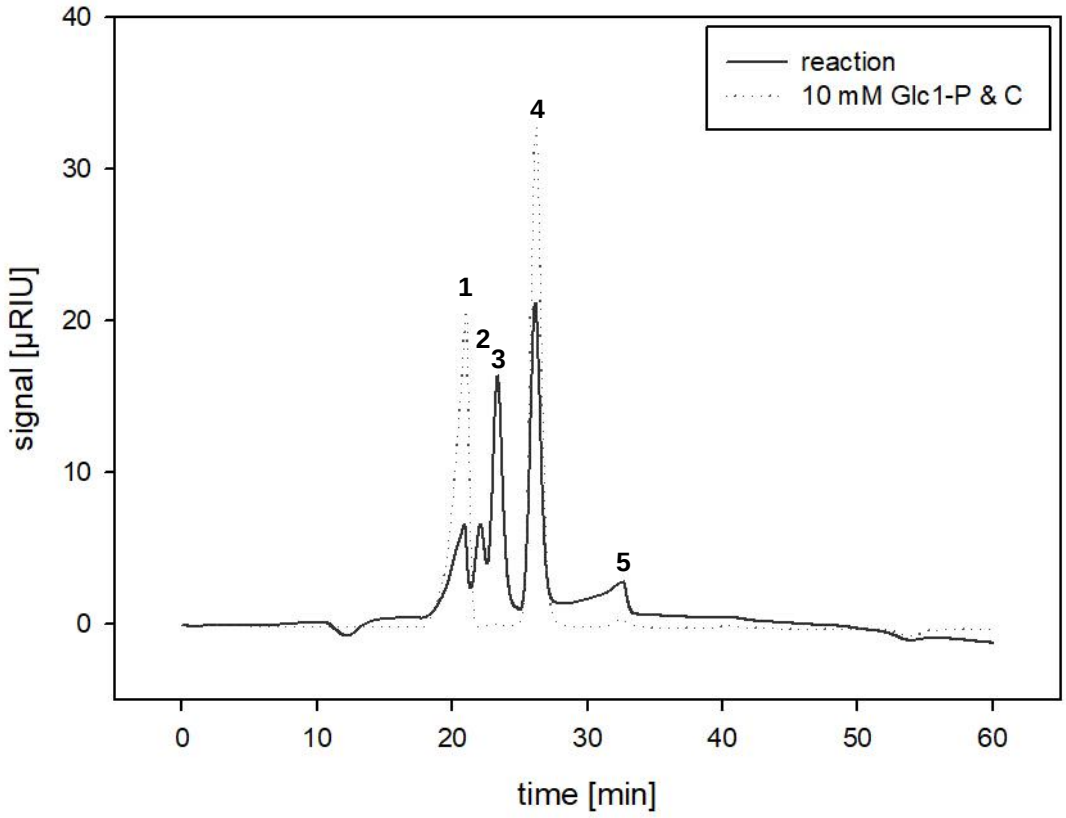
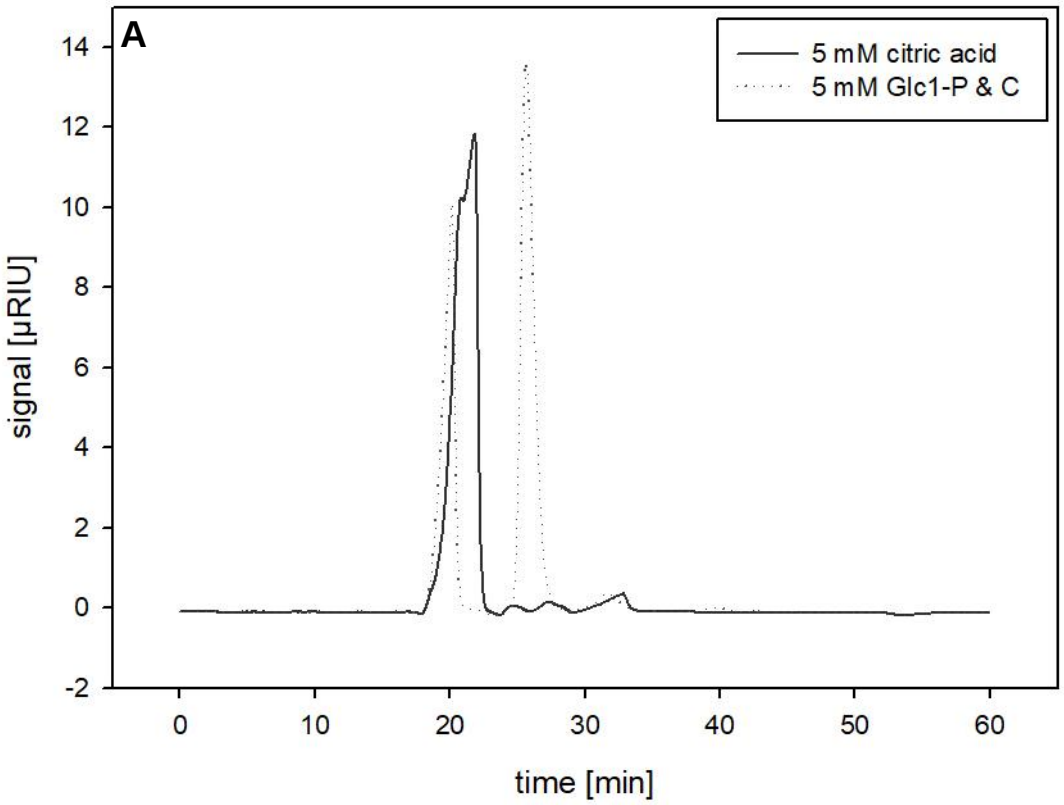
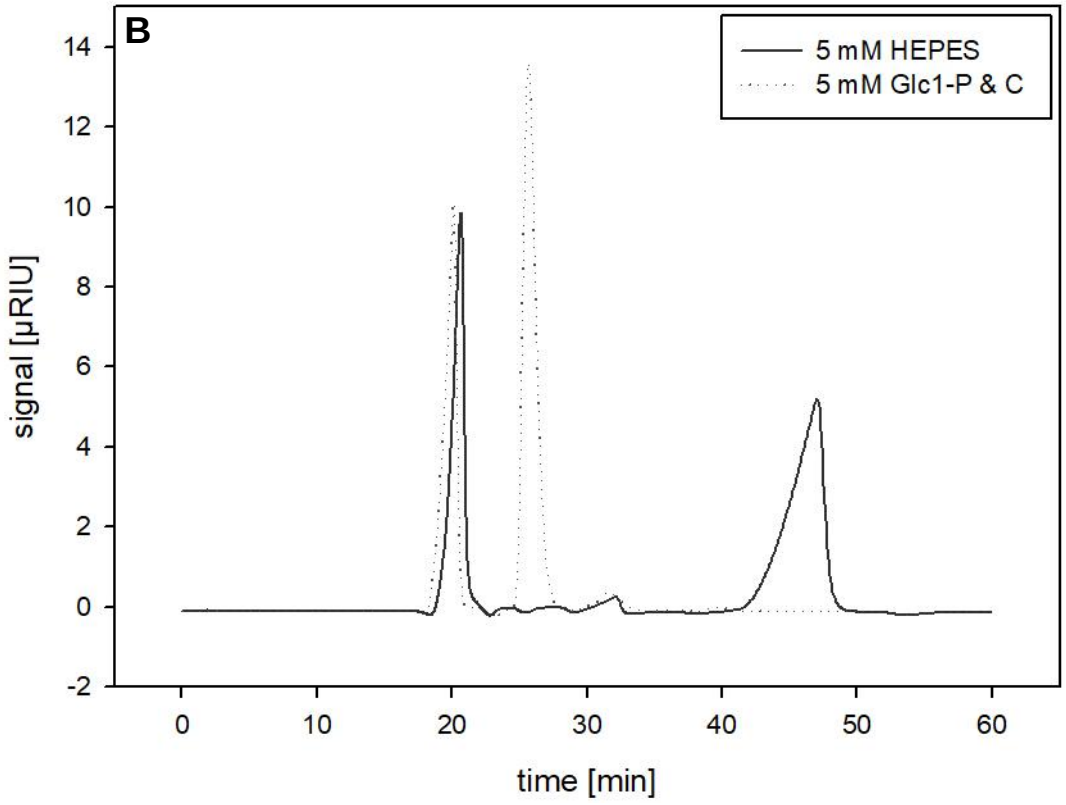


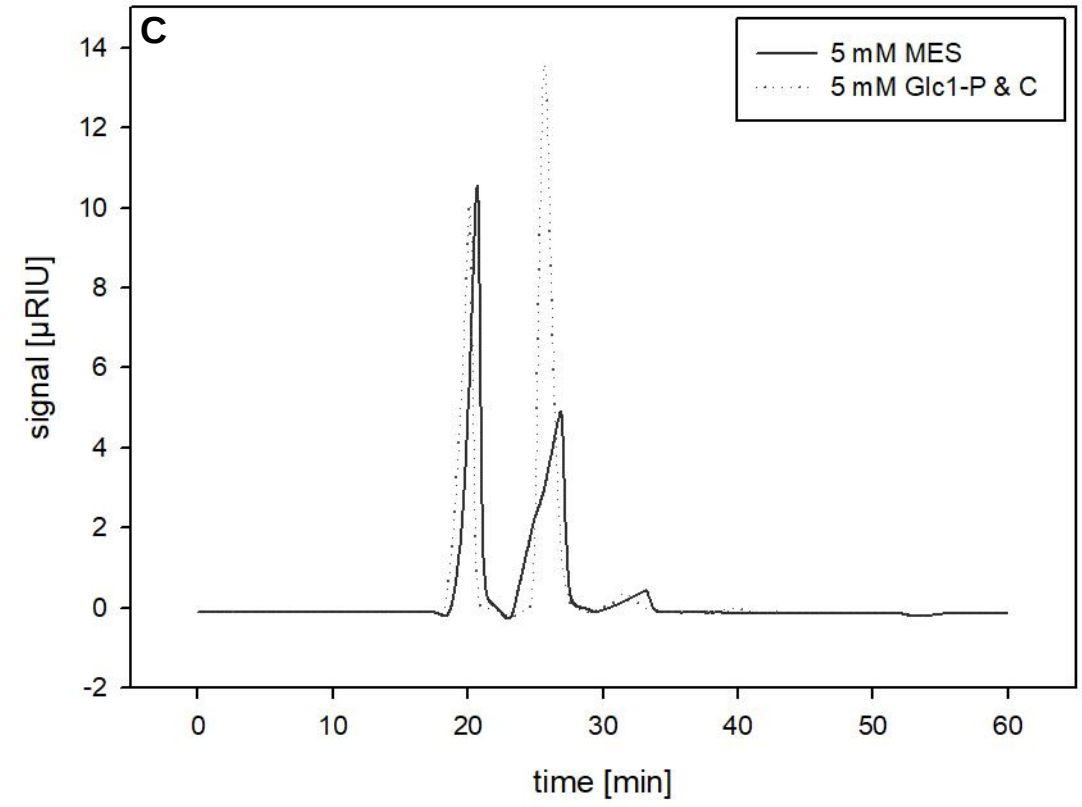
Figure 25: HPLC chromatograms of product mixture and standards on an Aminex HPX-87C column. Operating conditions: 85°C, flow rate: 0.2 mL/min, mobile phase: ddH_2O. Solid line = 1440 min reaction assay with 50 mM cellobiose, 30 mM Glc1-P, 50 mM MOPS pH 6.5 and 0.066 mg/mL His-CcCdexP. Dotted line = standard mixture with 10 mM cellobiose (C) and Glc1-P in ddH_2O. 1 = Glc1-P + buffer + oligomeric products with DP>5, 2 = cellotetraose, 3 = cellotriose, 4 = cellobiose, 5 = glucose + buffer.

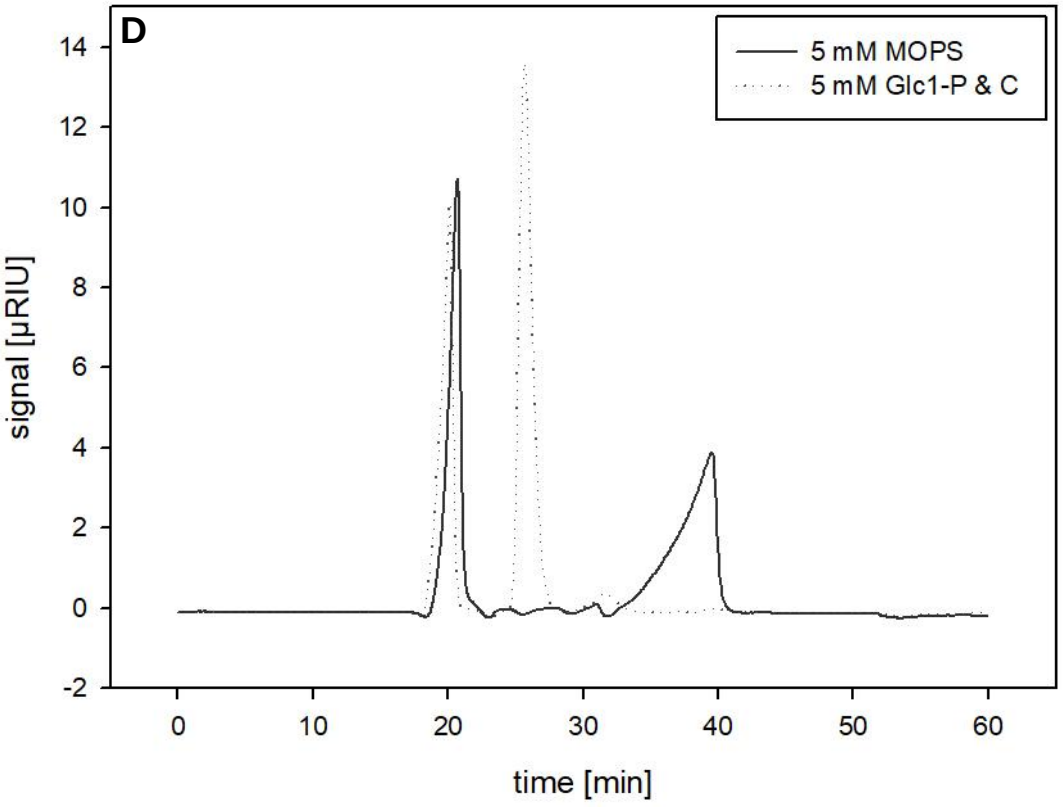
In order to get rid of co-eluting buffer signals, other commonly used buffers were tested. Unfortunately, all the different buffer-systems were at some points overlapping with the expected produced peaks (Figure 26, A-E). HEPES, MES and MOPS were even giving two peaks. Tris-HCl seemed to be the most suitable buffer-system due to early elution and small signal peak high. We also tested the enzymatic reaction only in water and obtained similar results as with the different buffer-

systems. However a pH change during the reaction could change the efficiency and may influence the product spectra. We observed a slight shift in retention times with higher substrate concentrations and with higher oligomeric product formation.






C
5 mM MES
5 mM Glc1-P & C
signal [µRIU]
time [min]


D
5 mM MOPS
5 mM Glc1-P & C
signal [µRIU]
time [min]

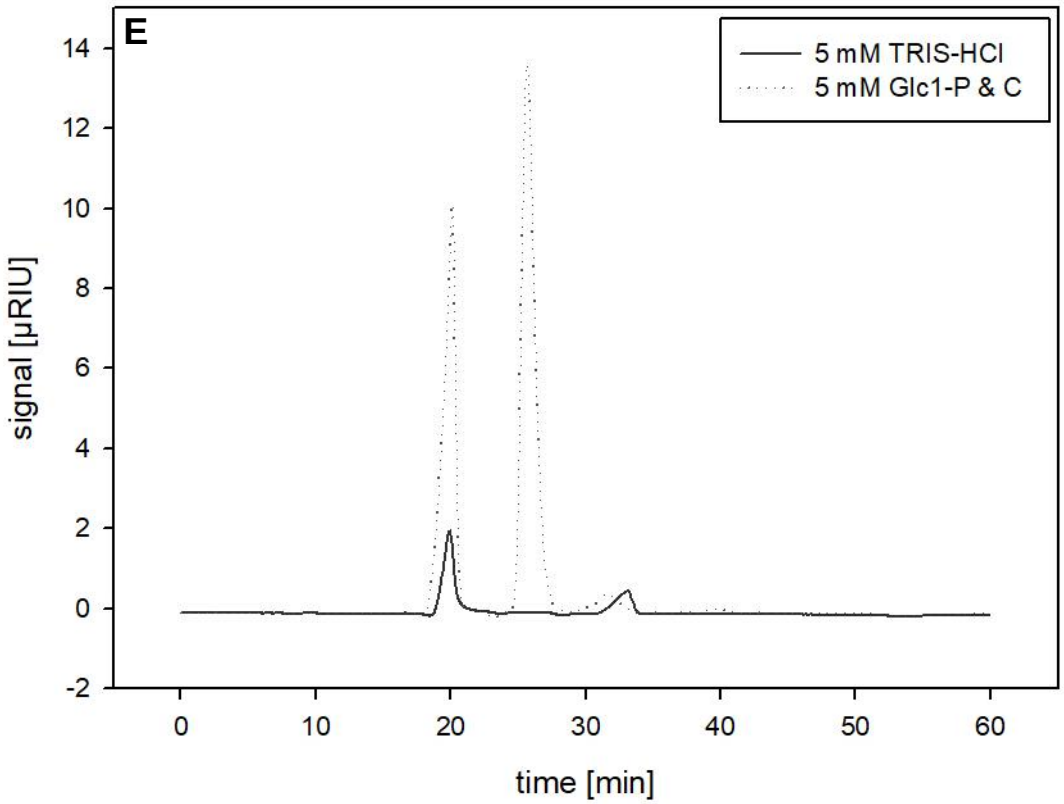


Figure 26: HPLC chromatograms of different buffer-systems on an Aminex HPX-87C column. Operating conditions: 85°C, flow rate: 0.2 mL/min, mobile phase: ddH_2O. Dotted line = 5 mM standard mixture with cellobiose (C) and Glc1-P in ddH_2O. Solid line: 5 mM buffer, A = citric acid, B = HEPES, C = MES, D = MOPS. E = TRIS-HCl.

3.7.3.4. HPLC analysis on a Phenomenex Luna NH_2 column with RI detection

With the developed method, described in Material and Methods, it was possible to detect and also quantify the different oligomeric products. A general chromatogram is shown in Figure 27. The concentration of cellobiose, cellotriose and cellotetraose was calculated with the corresponding areas using a measured standard curve. For the other products the individual peak area was divided by the molecular weight of the corresponding product to obtain a correlated area value. These values were used to estimate product concentrations via the cellotetraose standard curve. For the standards cellobiose, cellotriose and cellotetraose the measured peak areas and the correlated ones are shown in Table 12. For better visualization Figure 28 was created. Correlated area data was similar but not identical for each standard substrate at the different concentrations (standard curves for cellobiose, cellotriose and cellotetraose are shown in Figure 29). The observed differences could be due to the different integration of the product peaks. We assume that this correlation is also true for higher oligomeric products, where no standards are available. Therefore we calculated the concentration of cellopentaose, cellohexaose and celloheptaose using the calibration curve of cellotetraose. Figure 30 - Figure 35 show the concentration for cellobiose, cellotriose and cellotetraose, calculated via corresponding standards curves and the concentration for cellopentaose, cellohexaose and celloheptaose, calculated via correlated areas.

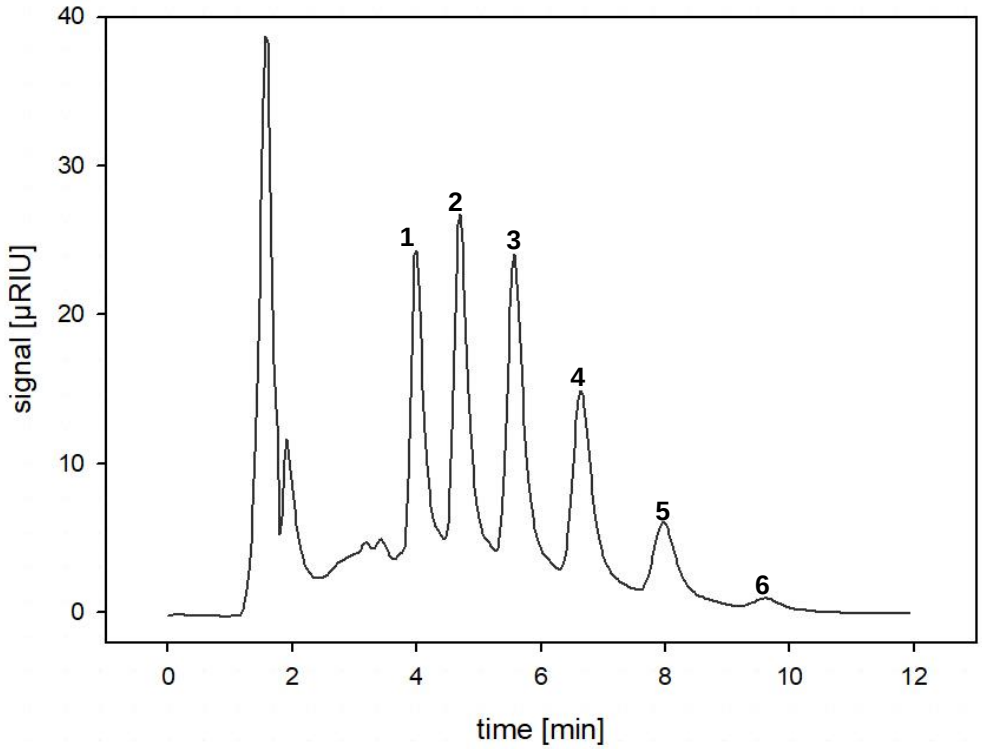


Figure 27: HPLC chromatogram of product mixture on a Phenomenex Luna NH_2 column. Operating conditions: 60°C, flow rate: 1.5 mL/min , mobile phase:37.5% acetonitrile in water . 1 = cellobiose, 2 = cellotriose,3 = cellotetraose, 4 = cellopentaose, 5 = cellohexaose, 6 = celloheptaose.

Table 12: Peak area and correlated peak area for cellobiose, cellotriose and cellotetraose.

Standard concentration c [mM]	Cellobiose	Cellotriose	Cellotetraose
	Peak area [µRIU*min]		
2	52849	87854	112038
5	120410	195972	268264
10	223739	380423	498265
15	342306	579843	754567
20	455186	775919	1007184
25	557471	966751	1303406
50	1333183		
100	2679522		
	Correlated peak area [µRIU*min*mol/g]		
	Molecular Weight [g/mol]		
	242.3	**504.4**	**666.6**
2	218.1	174.2	168.1
5	496.9	388.5	402.4
10	923.4	754.2	747.5
15	1412.7	1149.6	1132.0
20	1878.6	1538.3	1510.9
25	2300.7	1916.6	1955.3
50	5502.2		
100	11058.7		

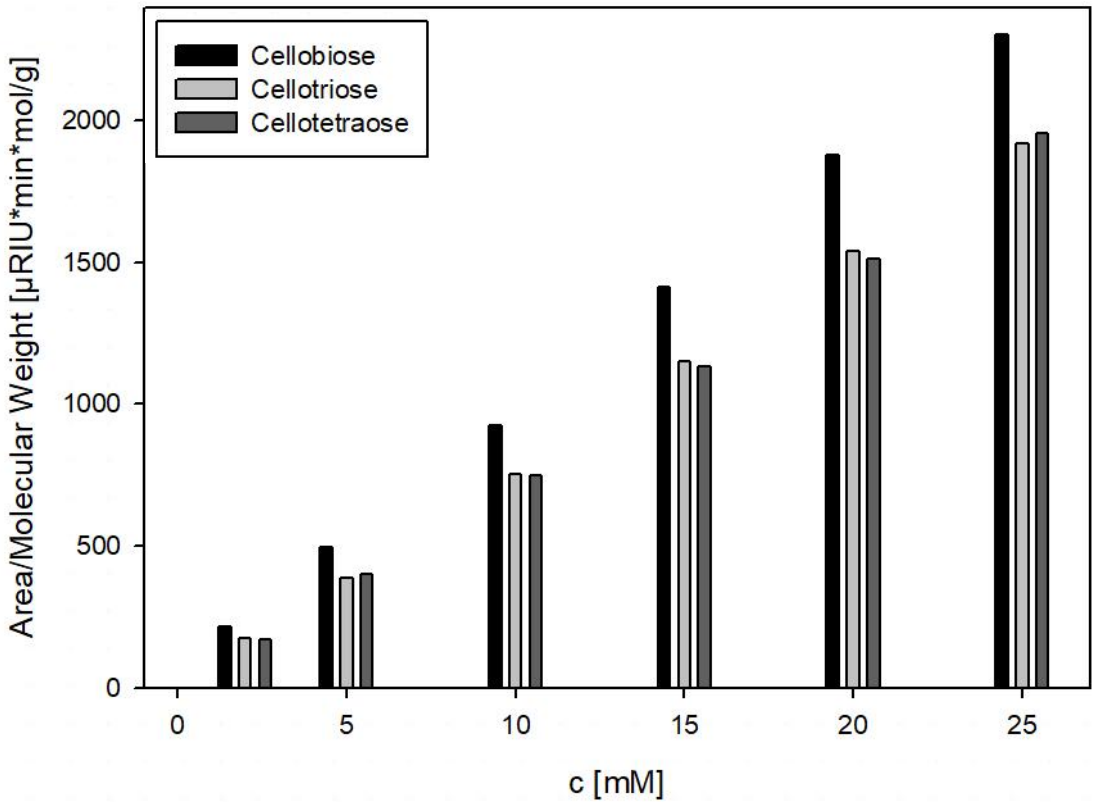


Figure 28: Visualisation of peak areas correlated with the molecular weight for the standards cellobiose, cellotriose and cellotetraose.

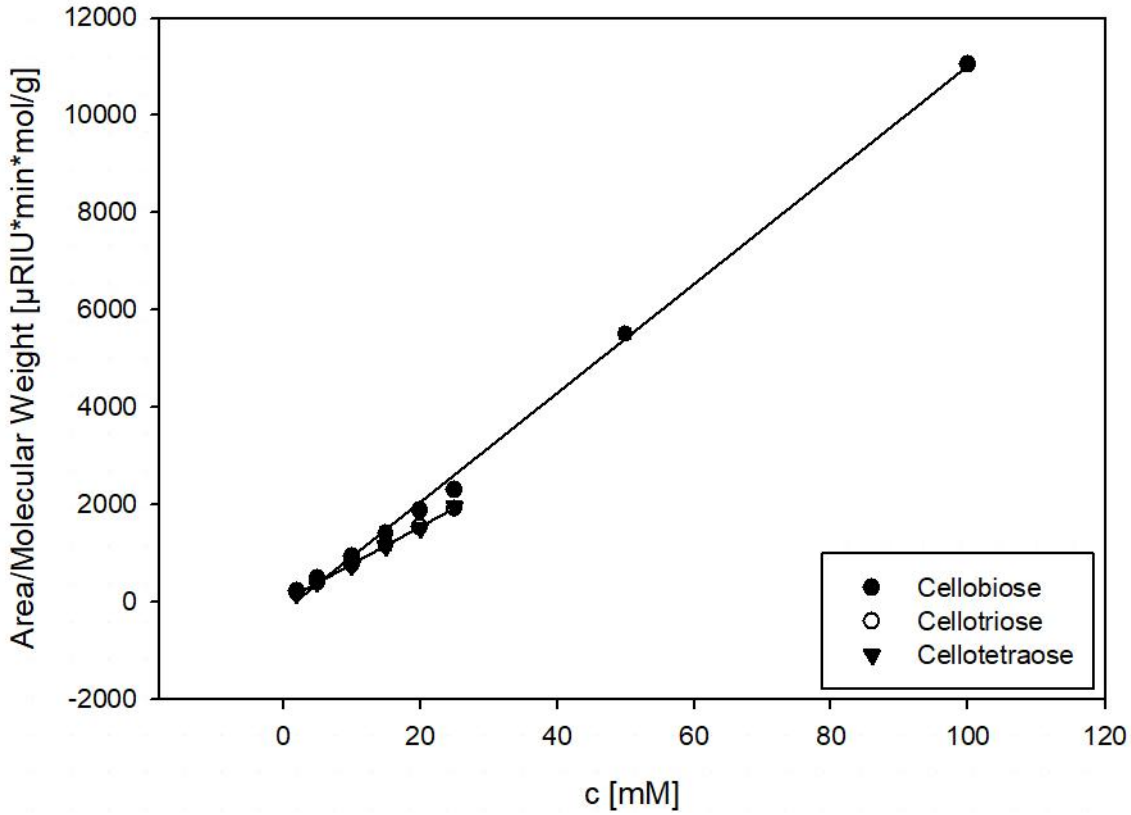


Figure 29: Standard curves using correlated area peaks for cellobiose, cellotriose and cellotetraose. The points were fitted using a linear fit equation with Sigma Plot. Cellobiose: y = 112.2x-209.0, Cellotriose: y = 76.2x+9.5, Cellotetraose: y=76.7x+1.6.

The influence of the acceptor and donor ratio on the product spectra was tested again at 360 min and 1440 min (Figure 30). In accordance with previous from HPAE-PAD analysis it could again be demonstrated that the glycosyl donor concentration has a huge impact on the oligomeric product state.

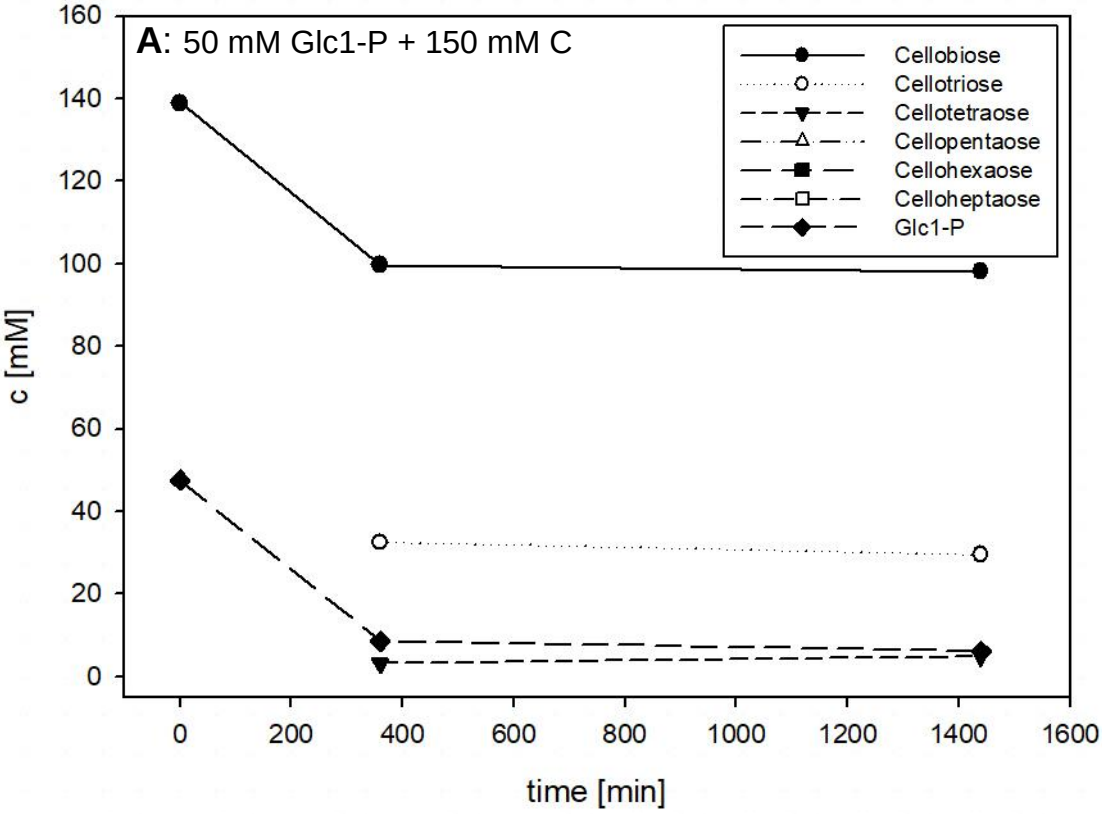
However this time we were also able to quantify the oligomeric products.

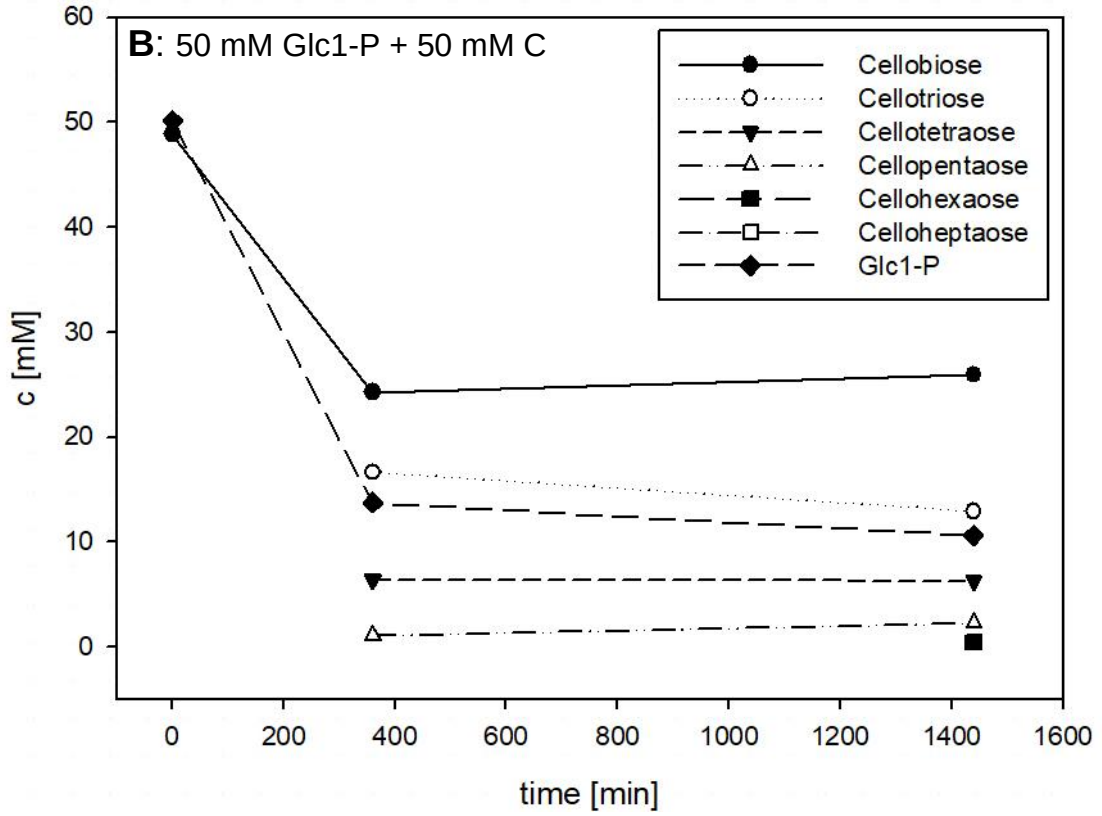
Having a 3-fold excess of acceptor cellobiose in the reaction mixture, approximately 30 mM cellotriose and 4 mM cellotetraose were produced, while no other higher oligomeric products were formed (Figure 30, A). 82% of Glc1-P were already converted after 360 min and the conversion yield did only increase marginally up to 87% after 1440 min.

An equimolar ratio of acceptor and donor revealed similar cellotriose but lower cellotetraose concentrations after 360 min compared to the reaction conditions using 2-fold and 4-fold excess of Glc1-P (Figure 31). After 1440 min the cellotriose concentration was higher under equimolar conditions compared to excess of Glc1-P (Figure 31). While the concentration of higher oligomeric products like cellotetraose, cellopentaose, cellohexaose and celloheptaose was increased in general the dependency of donor/acceptor ratio was still the same as described after 360 min.

The difference between the 2-fold and the 4-fold excess of Glc1-P can be clearly seen at reaction time point 1440 min. Using a 2-fold excess of Glc1-P over cellobiose (Figure 30, C) the main products were 10.8 mM cellotriose followed by 8.5 mM cellotetraose and 4.5 mM cellopentaose. While using a 4-fold excess of Glc1-P over cellobiose (Figure 30, D) the main product was 9.5 mM cellotetraose, followed by 6.4 mM cellotriose and 4.0 mM cellohexaose. Interesting the cellopentaose concentration was lowest which might reflect varying selectivity of the enzyme towards the different acceptor substrates. According to our results cellopentaose might be a better acceptor substrate than cellotetraose.

To summarize the influence of donor/acceptor ratio on the product spectra, two additional bar charts were created. One shows the product spectra after 360 min (Figure 31, A), the other one after 1440 min (Figure 31, B).


A: 50 mM Glc1-P + 150 mM C
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
Glc1-P
c [mM]
time [min]
0
20
40
60
80
100
120
140
160
0
200
400
600
800
1000
1200
1400
1600


B: 50 mM Glc1-P + 50 mM C
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
Glc1-P
c [mM]
time [min]
0
10
20
30
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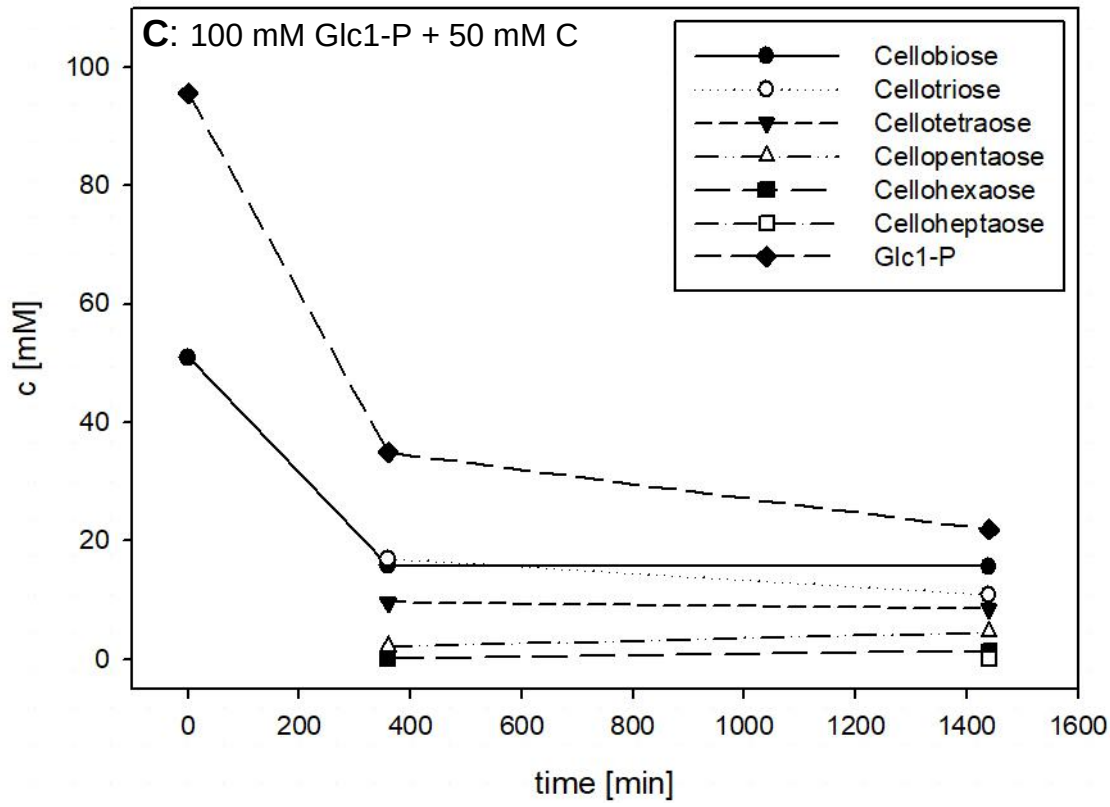
50
60
0
200
400
600
800
1000
1200
1400
1600



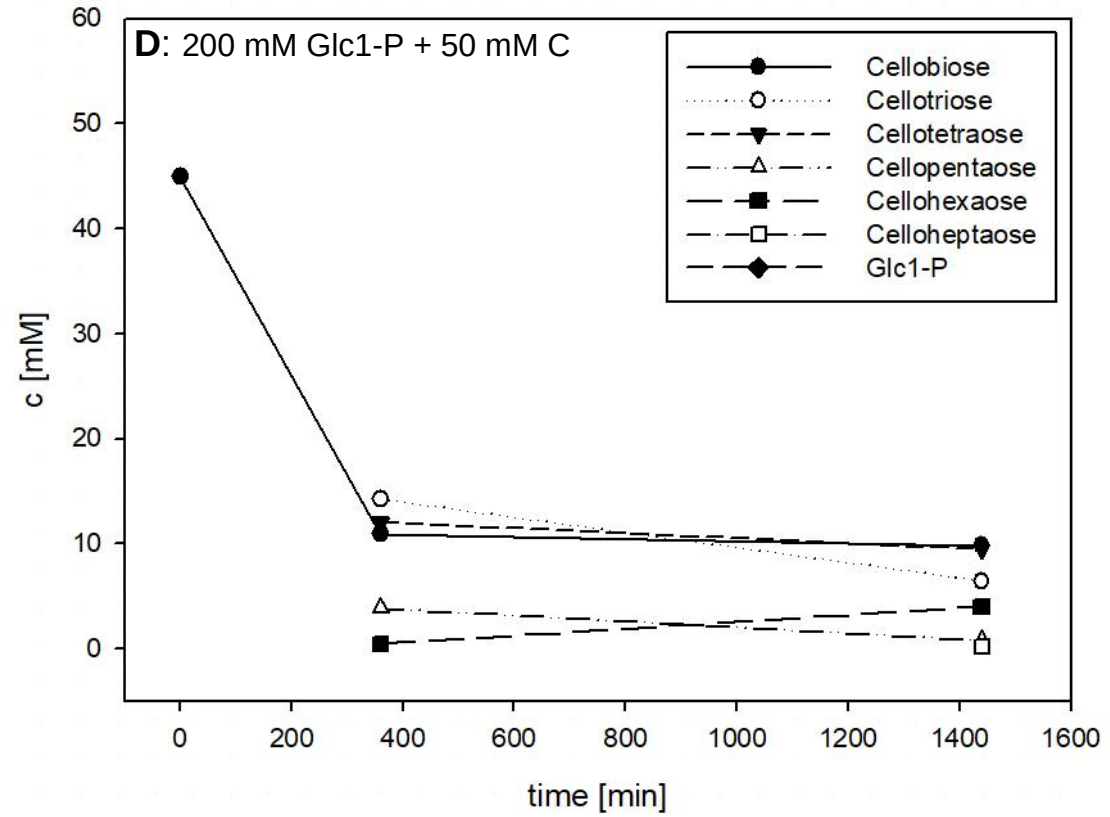


Figure 30: Timeline of product spectra at different substrate concentrations. All reactions were performed in 50 mM TRIS-HCl pH 7.3 in the presence of 0.066 mg/mL purified His-CcCdexP. A: 50 mM Glc1-P and 150 mM cellobiose, B: 50 mM Glc1-P and 50 mM cellobiose, C: 100 mM Glc1-P and 50 mM cellobiose, D: 200 mM Glc1-P and 50 mM cellobiose. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose, filled diamond: Glc1-P.

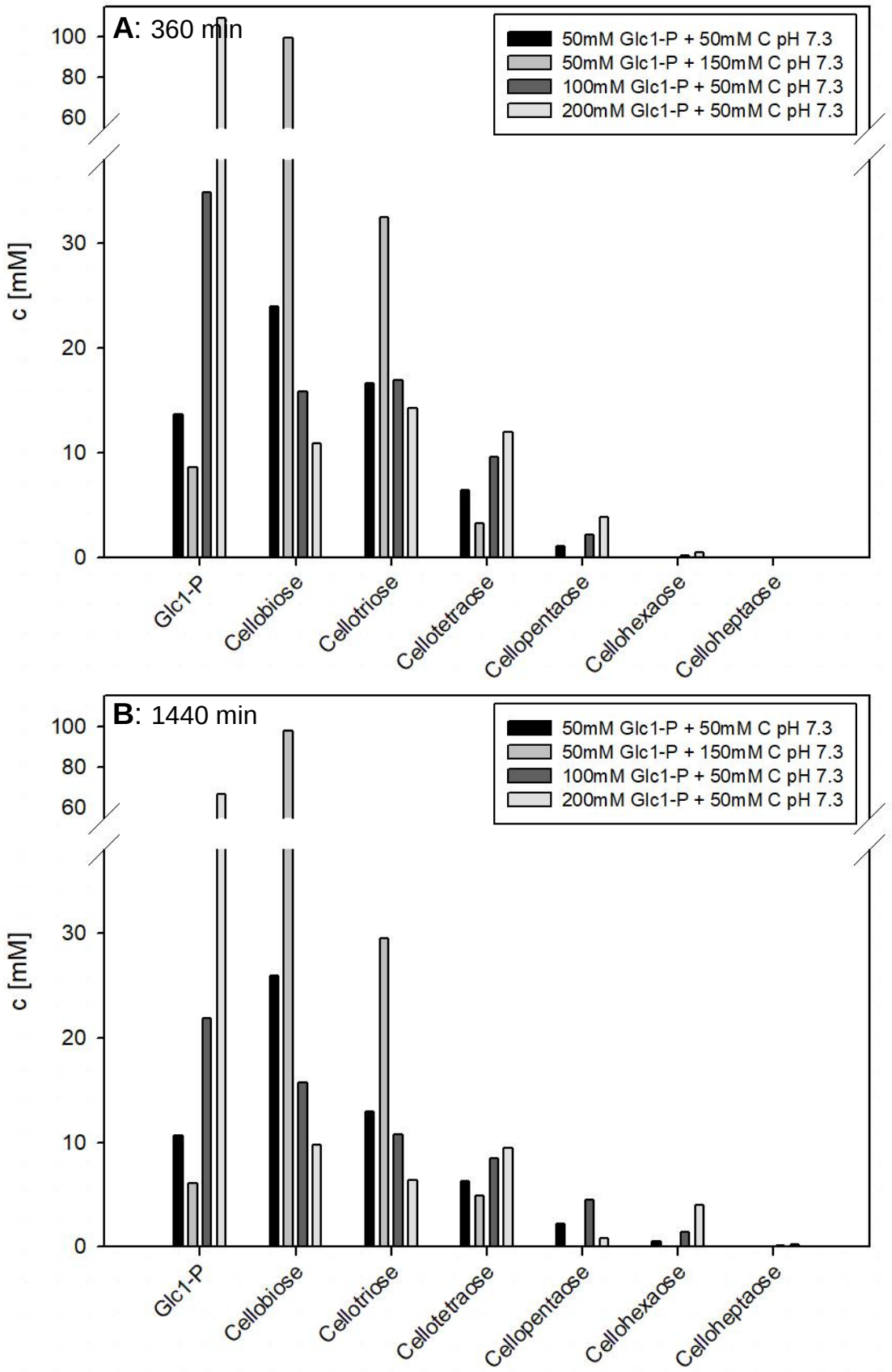
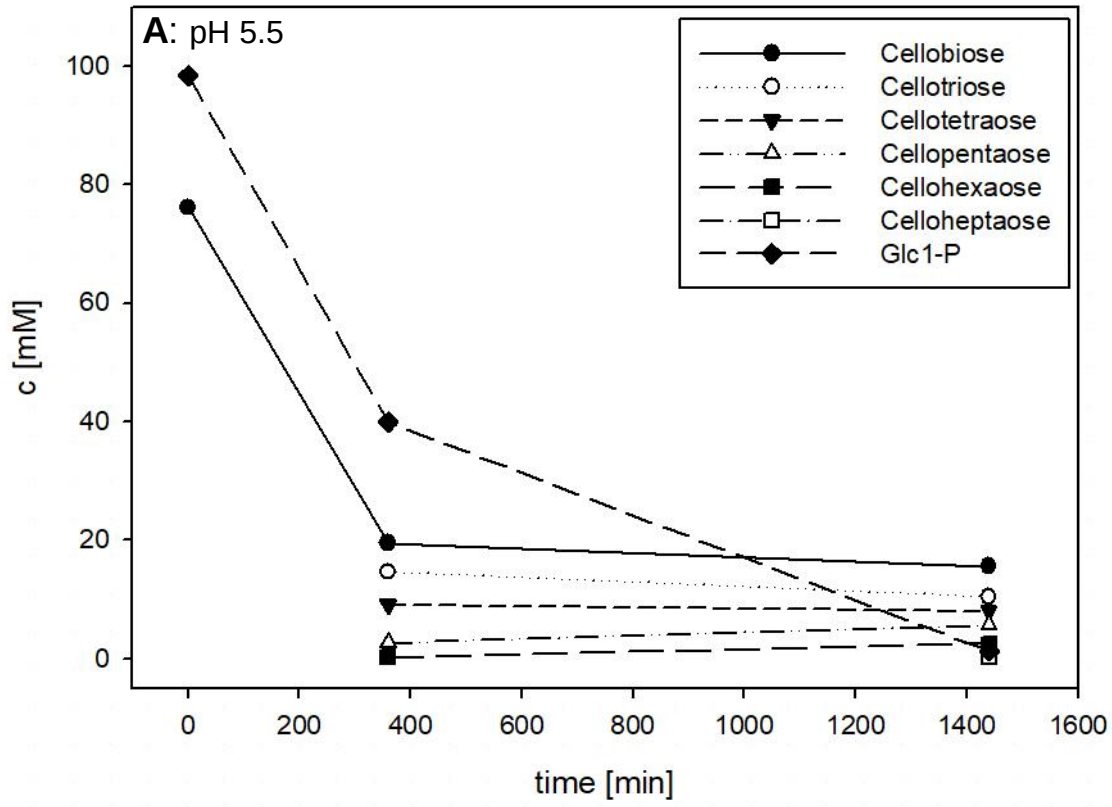


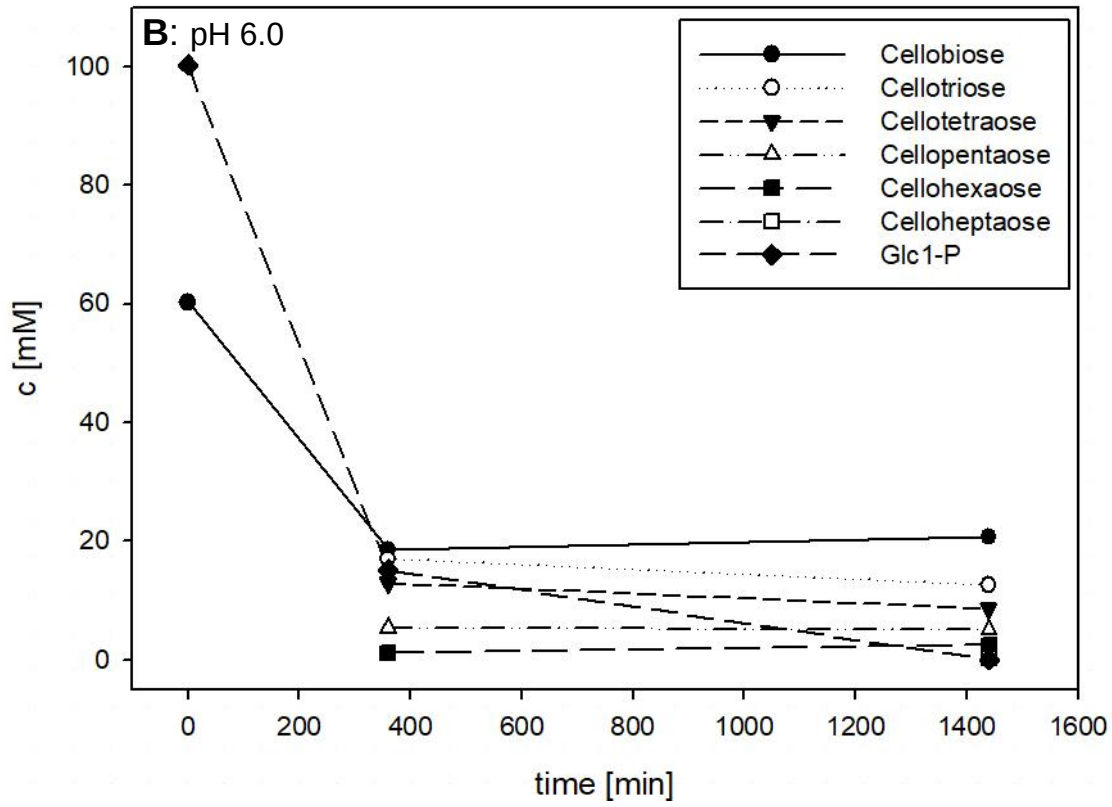
Figure 31: Bar diagrams of product spectra obtained at different donor/acceptor ratios at 360 min (A) and 1440 min (B). All reactions were performed in 50 mM TRIS-HCl pH 7.3 and in the presence of 0.066 mg/mL purified His-CcCdexP. Grey: 50 mM Glc1-P and 150 mM cellobiose, black: 50 mM Glc1-P and 50 mM cellobiose, dark grey: 100 mM Glc1-P and 50 mM cellobiose, light grey: 200 mM Glc1-P and 50 mM cellobiose.

The influence of different pH values on the product spectra were also analyzed after 360 min and 1440 min of reaction. A 2-fold excess of glycosyl donor over acceptor was used (substrate concentration: 100 mM Glc1-P and 50 mM C).

Figure 32 shows the timelines of product spectra recorded at different pH values. The lowest pH tested was pH 5.0 (Figure 32 A), where cellotriose was the main product (Crude Data Table 25: 360 min: 14.7 mM and 1440 min: 10.5 mM). The concentration of the higher oligomeric products decreased with oligomeric product state. The same was true for pH 6.0 (Figure 32 B, Crude Data Table 25: 360 min: 17.0 mM and 1440 min: 12.6 mM). After 1440 min >95% of Glc1-P was converted.

At the enzyme's optimum pH of 7.3 and at a pH of 8.5 lower concentrations of products with DP>5 were detected compared to pH 5.5 and 6.0. Cellotriose was the main product at both pH values with 16.9 mM (pH 7.3) and 28.0 mM (pH 8.5) after 360 min. After 1440 min, product concentration ratios changed. While at pH 8.5 cellotriose remained the main product with 31.0 mM (2.5-fold increase over cellotetraose), at pH 7.3 the concentration difference between cellotriose and cellotetraose was only 1.3-fold and also cellohexaose and celloheptaose were formed at a significant amount. This can be due to enzyme destabilisation that influences to formation of the enzyme`s active site.


A: pH 5.5
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
Glc1-P
c [mM]
time [min]
0
20
40
60
80
100
0
200
400
600
800
1000
1200
1400
1600


B: pH 6.0
Cellobiose
Cellotriose
Cellotetraose
Cellopentaose
Cellohexaose
Celloheptaose
Glc1-P
c [mM]
time [min]
0
20
40
60
80
100
0
200
400
600
800
1000
1200
1400
1600

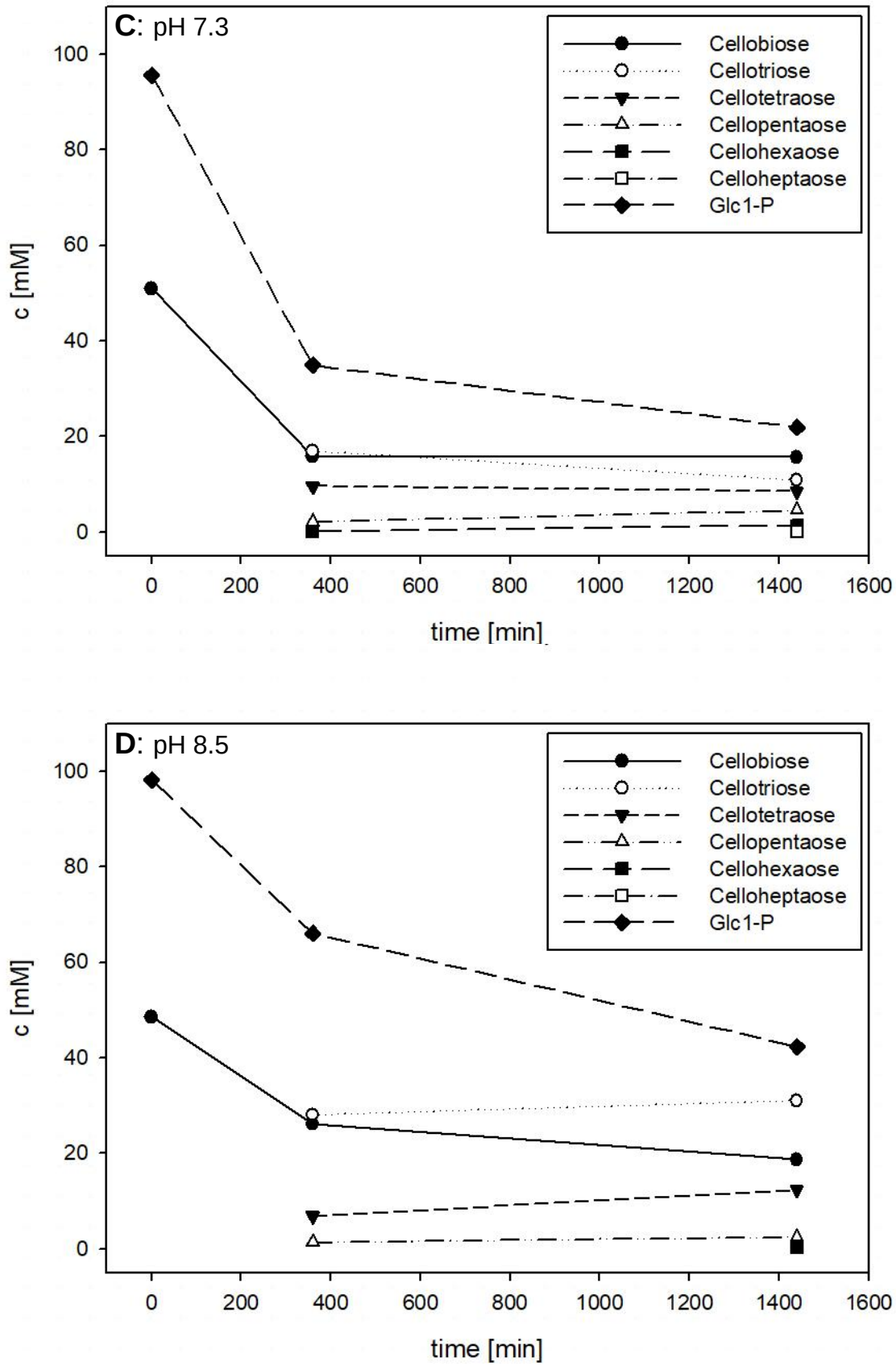


Figure 32: Timeline of product spectra at different pH values. Reaction assay: 100 mM Glc1-P, 50 mM cellobiose in 50 mM buffer (letters A-D) and 0.066 mg/mL purified His-CcCdexP. A: citric acid pH 5.5, B: MES pH 6.0, C: optimum pH 7.3 in TRIS-HCl, D: HEPES pH 8.5. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose, filled diamond: Glc1-P.

A summary of the influence of the different pH values on the product spectra is given in Figure 33. The figure at the top (Figure 33, A) shows the product spectra after 360 min of reaction, the bottom one (Figure 33, B) after 1440 min.

At both time points (Figure 33 A, B) cellotriose was always the main product under all conditions tested. Cellotriose accounts to approximately 57% of the total product mixtures at pH 8.5 after 360 min.

When looking at the product spectra after 1440 min (Figure 33, B), it becomes obvious that the cellotriose concentration of 31.0 mM for pH 8.5 is outstanding. Cellobiose concentration was decreased in the favour of cellotetraose. All other pH values showed a similar distribution and every oligosaccharide could be detected.

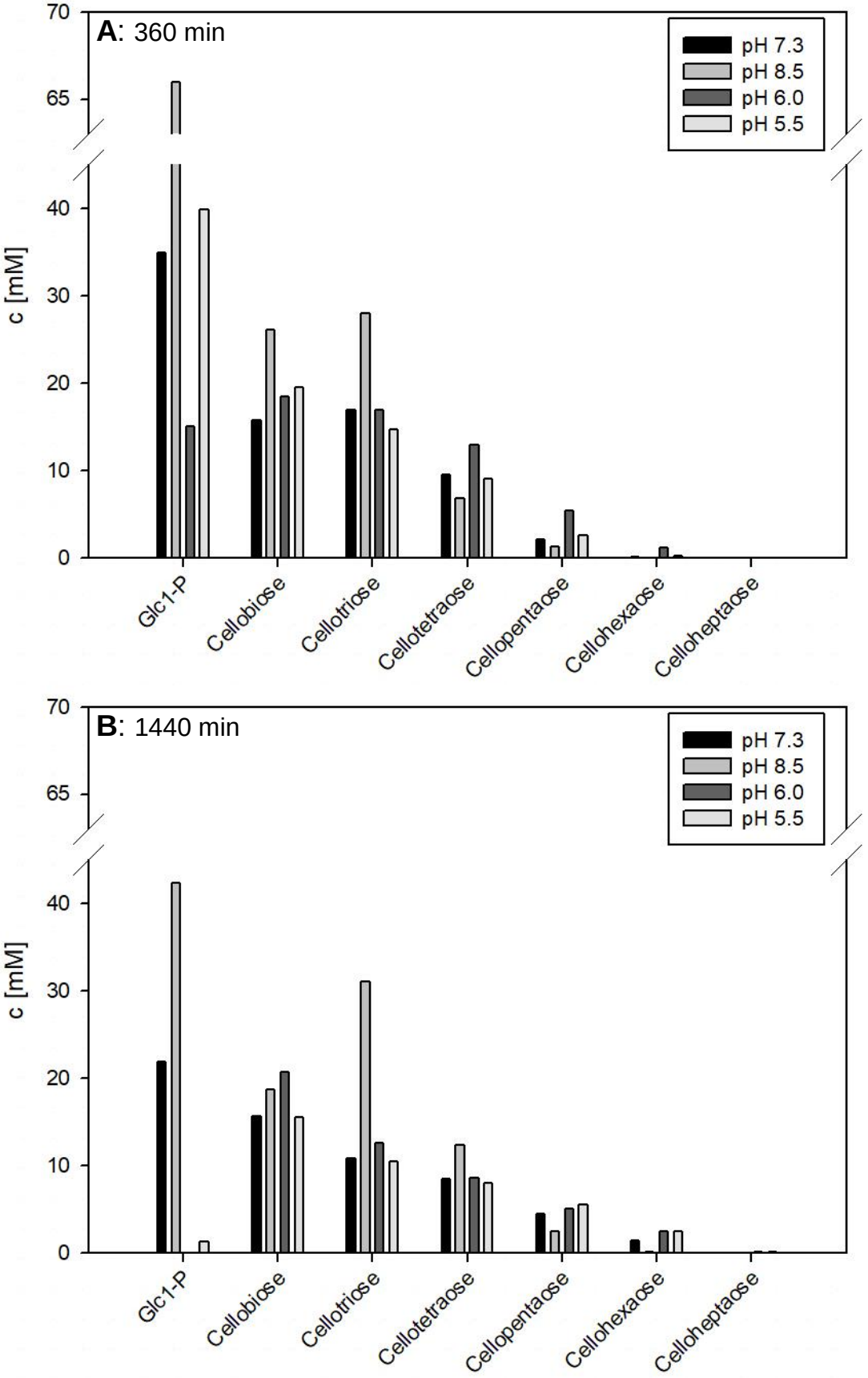


Figure 33: Bar diagrams of product spectra at different reaction time points and different pH values. Reaction assay: 100 mM Glc1-P, 50 mM cellobiose in 50 mM buffer and 0.066 mg/mL purified His-CcCdexP. Buffer: pH 7.3 = TRIS-HCl (black), pH 8.5 = HEPES (grey), pH 5.5 = citric acid (light grey), pH 6.0 = MES (dark grey). A: 360 min, B: 1440 min.

The concentrations of cellobiose, cellotriose and cellotetraose were calculated using an individual standard curve (Figure 29) and the results are shown in Table 13. Comparing the start concentration of cellobiose with the measured, calculated concentration, it is clear, that some integration errors at the individual chromatograms occur. However, the results fit in an acceptable manner.

Table 13: Quantification of the substrates cellobiose and Glc1-P and the products cellotriose and cellotetraose.

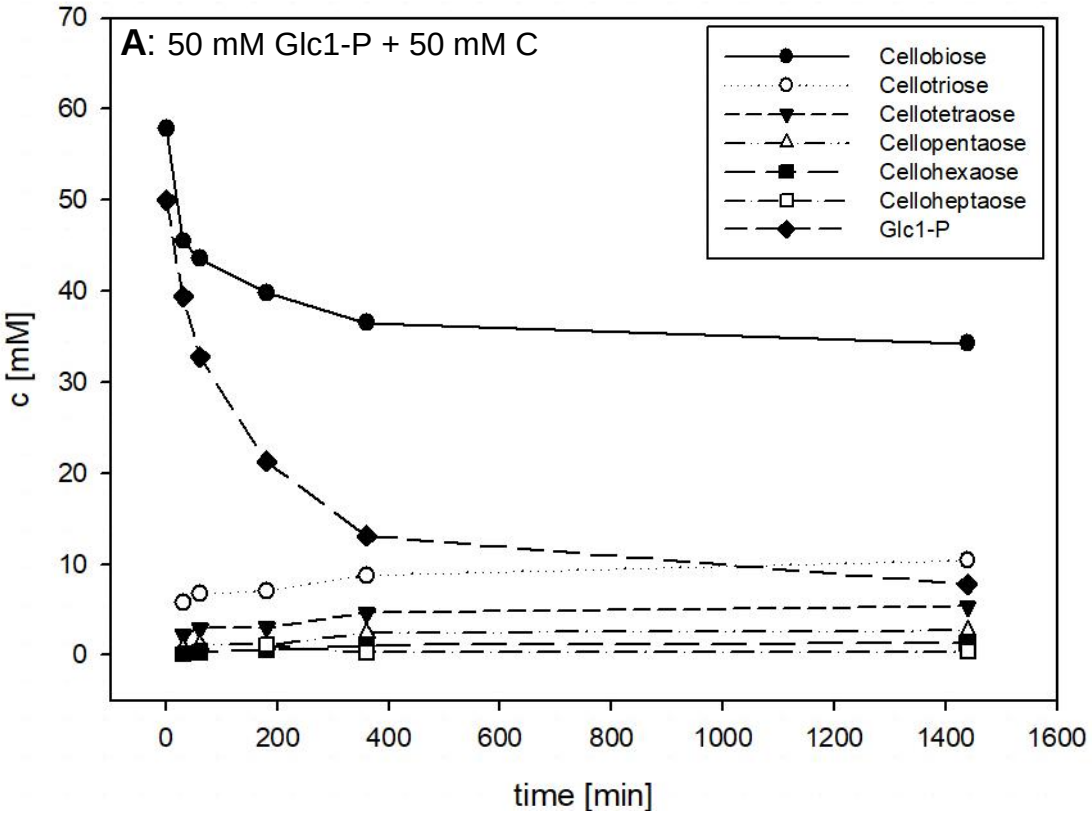
Glc1-P/C/pH [mM/mM/-]	time [min]	Cellobiose [mM]	Cellotriose [mM]	Cellotetraose [mM]	Glc1-P left [mM]
15/100/7.3	0	99.1			14.4
	360	80.0	14.0	1.0	1.9
	1440	80.1	13.0	1.4	2.0
50/50/7.3	0	48.8			50.1
	360	24.2	16.6	6.3	13.7
	1440	25.9	12.9	6.3	10.6
50/150/7.3	0	139.0			47.5
	360	99.7	32.5	3.2	8.6
	1440	98.1	29.5	4.9	6.1
100/50/7.3	0	51.0			95.6
	360	15.8	16.9	9.6	34.9
	1440	15.7	10.8	8.5	21.9
200/50/7.3	0	44.9			183.4
	360	10.9	14.2	12.1	109.2
	1440	9.8	6.4	9.5	67.0
100/50/8.5	0	48.6			98.2
	360	26.1	28.1	6.9	66.0
	1440	18.7	31.0	12.4	42.3
100/50/5.5	0	76.1			98.4
	360	19.5	14.7	9.1	39.9
	1440	15.6	10.5	8.0	1.3
100/50/6.0	0	60.2			100.2
	360	18.5	17.0	12.9	15.1
	1440	20.7	12.6	8.6	0.0

3.8. Cellodextrin phosphorylase from *C. cellulosi* vs. *C. stercorarium*

Influence of different donor and acceptor substrate ratios on product spectra were also tested for the homologue enzyme of *Clostridium stercorarium* and the results are shown in Figure 34. Figure 34 A shows the results from equimolar assay conditions, and Figure 34 B shows the results from a 2-fold excess of Glc1-P over cellobiose.

In contrast to the reaction of His-CcCdexP (Figure 30) the excess of Glc1-P over cellobiose did not show a significant influence on the product spectra of His-CsCdexP. However, a definite conclusion cannot be drawn as different amounts of active enzyme were used. Due to problems during protein purification of His-CsCdexP, an enzyme preparation with a low overall yield of only 1% was obtained. The Glc1-P excess over cellobiose experiments should be repeated under identical conditions as described for His-CcCdexP.

In Table 14 the concentrations of the measured calculated cellobiose, cellotriose and cellotetraose are shown.



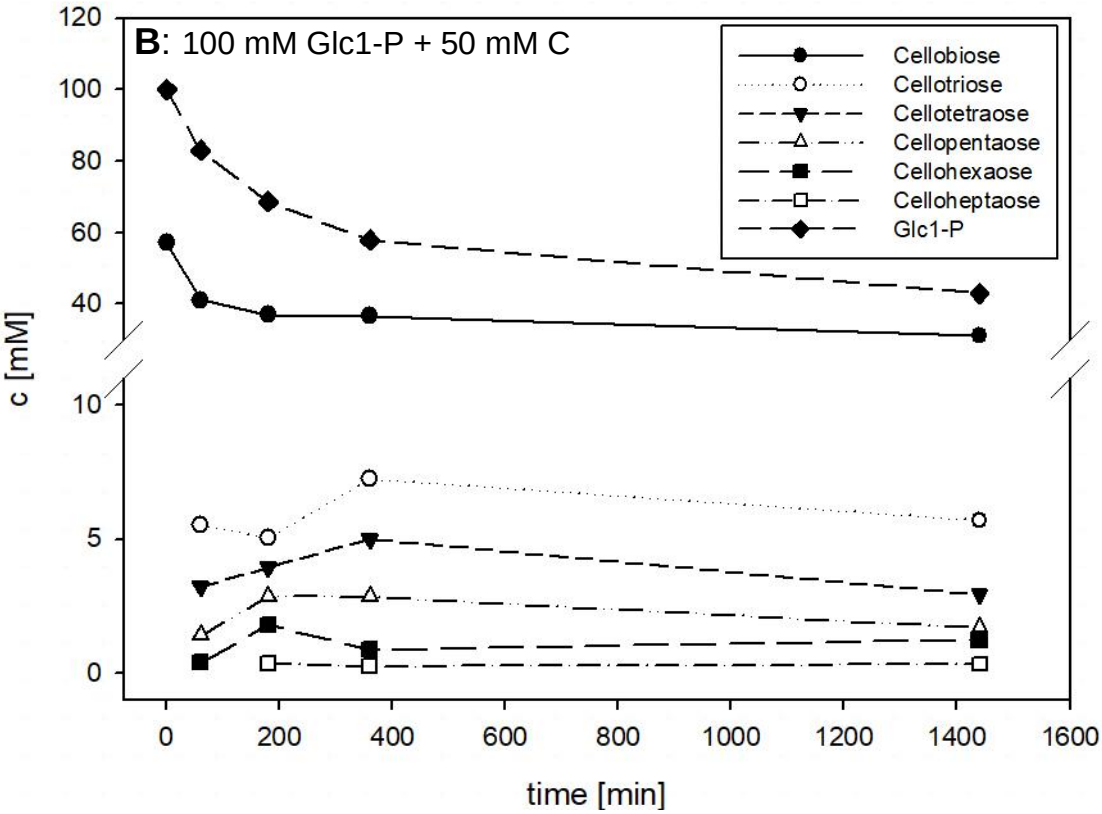


Figure 34: Timeline of product spectra at two different donor/acceptor ratios for cellodextrin phosphorylase from *Clostridium stercorarium*.
A: equimolar concentration: 50 mM Glc1-P and 50 mM cellobiose (C) in 50 mM MOPS at pH 6.5 and 0.023 mg/mL purified His-CsCdexP. B: 100 mM Glc1-P and 50 mM cellobiose (C) in 50 mM MOPS at pH 6.5 and 0.023 mg/mL purified His-CsCdexP. Filled circle: cellobiose, empty circle: cellotriose, filled tangial down: cellotetraose, empty tangial up: cellopentaose, filled square: cellohexaose, empty square: celloheptaose, , filled diamond: Glc1-P.

Table 14: Quantification of the substrate cellobiose and the products cellotriose and cellotetraose from transfer reaction with His-CsCdexP.

G1P/C/pH [mM/mM/-]	time [min]	Cellobiose [mM]	Cellotriose [mM]	Cellotetraose [mM]	Glc1-P left [mM]
50/50/6.5	0	57.9			50.0
	30	45.5	5.8	2.3	39.4
	60	43.6	6.7	3.0	32.8
	180	39.8	7.1	3.0	21.3
	360	36.6	8.8	4.7	13.1
	1440	34.3	10.4	5.4	7.8
100/50/6.5	0	57.1			100.0
	60	41.1	5.5	3.2	82.9
	180	37.0	5.0	3.9	68.6
	360	36.6	7.2	5.0	57.8
	1440	31.2	5.7	2.9	43.0

The major differences between the two enzyme variants occur at the kinetic parameter level for cellobiose (Table 15). The K_m value of cellobiose is 30 times higher for His-CcCdexP compared to His-CsCdexP (K_m = 0.45 mM). The HPLC analysis of the different product spectra on a Phenomenex Luna NH_2 column reveals influence on the higher oligomeric product spectra at lower time points (Figure 35) for the different enzyme variants. A lower catalytic amount of His-CsCdexP in the reaction mixture (0.15 U), compared to His-CcCdexP (0.23 U), yielded higher concentration of oligosaccharides with DP>5 (cellopentaose, cellohexaose, celloheptaose). Due to a similar Glc1-P consumption (His-CcCdexP: 74% at 360 min and 79% at 1440 min, His-CsCdexP: 74% at 360 min and 84% at 1440 min) at equimolar donor/acceptor ratio one can elucidate, that the lower K_m of cellobiose is the reason for the difference in the product spectra. However, to prove this statement, the experiment should be repeated using the same catalytic amount for each enzyme. The individual product concentrations of cellotriose and cellotetraose are shown in Table 13 for His-CcCdexP and in Table 14 for His-CsCdexP.

Table 15: Comparison of important parameters for cellodextrin phosphorylase from *C. cellulosi* and *C. stercorarium*.

		Cellodextrin Phosphorylase *Clostridium cellulosi*	**Cellodextrin Phosphorylase *Clostridium stercorarium****
Reaction optima			
pH		7.3	6.5
T		55	65
Cloning		6x His, N-terminal	
Sequence similarity		70%	
Kinetic parameter			
Glc1-P	K_m [mM]	2.8	2.4
	k_{cat} [s^{-1}]	51.5	27.4
	k_{cat}/K_m [s^{-1} mM^{-1}]	18.4	11.2
Cellobiose	K_m [mM]	13.6	0.45
	k_{cat} [s^{-1}]	56.6	18.1
	k_{cat}/K_m [s^{-1} mM^{-1}]	4.2	40.2

*[8]

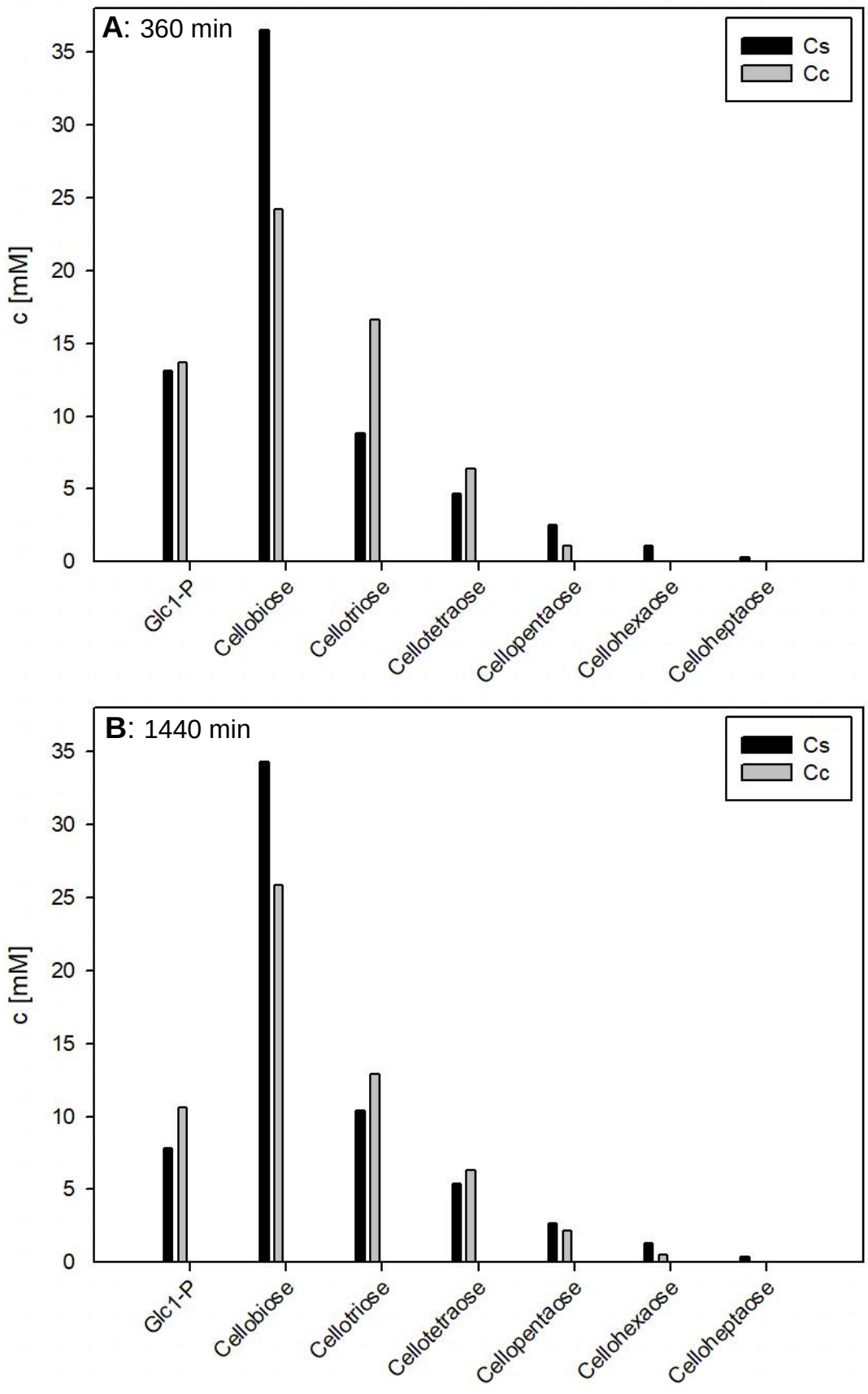


Figure 35: Bar diagram of product spectra at two reaction time points with the homologues cellodextrin phosphorylase from *Clostridium cellulosi* and *Clostridium stercorarium*. A: 360 min, B: 1440 min. *Clostridium cellulosi* (grey, Cc): 50 mM Glc1-P, 50 mM cellobiose (C) in 50 mM TRIS-HCl pH 7.3 and 0.066 mg/mL purified His-CcCdexP (0.23 U). Clostridium stercorarium (black, Cs): 50 mM Glc1-P, 50 mM cellobiose(C) in 50 mM MOPS pH 6.5 and 0.023 mg/mL purified His-CsCdexP (0.15 U).

We also compared the two enzyme variants by their chemical equilibrium. Both CdexPs catalyze the same reaction (Figure 2), but produce different concentrations of higher oligomeric products. K_{eq} was calculated using Equation 2 and 3 were used. The results are shown in Table 16.

Equation 2: $$K_{eq} = \frac{[P_i] \cdot [elongated\_glu\cos ylresidues]}{[Glc1-P] \cdot [C]}$$

Equation 3: $$[elongated\_glu\cos ylresidues] = [C3] + 2 \cdot [C4] + 3 \cdot [C5] + 4 \cdot [C6] + 5 \cdot [C7]$$
with C3, cellotriose; C4, cellotetraose; C5, cellopentaose; C6, cellohexaose and C7, celloheptaose.

Table 16: Determination of chemical equilibrium.

Glc1-P/C/pH [mM/mM/-]	time [min]	C3 [mM]	C4 [mM]	C5 [mM]	C6 [mM]	C7 [mM]	P_i = Glc1-P* [mM]	C left [mM]	elongated glucosyl-residues [mM]	K_{eq}
His-CcCdexP 50/50/7.3	0						0.0	48.8		
	360	16.6	6.4	1.1			36.4	24.2	32.7	3.6
	1440	12.9	6.3	2.2	0.5		39.5	25.9	34.1	5.0
His-CsCdexP 50/50/6.5	0						0.0	57.9		
	360	8.8	4.7	2.5	1.1	0.3	36.9	36.5	31.6	2.4
	1440	10.4	5.4	2.7	1.3	0.4	42.2	34.3	36.5	5.8

*consumed

The calculated chemical equilibrium constants of the two enzyme variants were similar at time point 1440 min, where the products and reactants had no further tendency to change with time. At time point 360 min the dynamic equilibrium had not been reached and therefore calculation of K_{eq} was not applicable. Glc1-P consumption was similar for both enzyme variants, while cellobiose consumption was approximately 1.3-fold decreased for His-CsCdexP. K_{eq} of His-CcCdexP was 0.8-fold decreased at 1440 min compared to His-CsCdexP. Overall, it can be concluded that the equilibrium coefficient K_{eq} is very similar for both enzyme variants at the state of dynamic equilibrium although the product spectra differ a lot in respect to higher oligomeric products (see Table 16).

4. Conclusions

202,000 Units recombinant CdexP from *Clostridium cellulosi* were produced per litre fermentation media during shake flask expression in *E. coli* BL21. Other recombinant expression studies of homologous enzyme variants mentioned in literature reported lower levels. The recombinant expression for the cellodextrin phosphorylase from *Clostridium thermocellum* (Y4 strain) generated 71,900 U/L fermentation media [4], whereas the expression of the enzyme from *Clostridium stercorarium (E. coli* BL21-RIC strain) reached only 2,200 U/L [8].

After a two-step chromatographic purification protocol, approximately 70% of the total enzyme activity was found in the purified His-CcCdexP fraction. Thereby, the specific activity was increased by 3.5-fold compared to the cell-free extract. For the purified His-CsCdexP fraction, only 14% of the total enzyme activity could be recovered after chromatographic purification, however with a 14-fold increase in specific activity. Both enzymes showed a stable linear glucosyl-transfer-activity under their optimal conditions.

The main challenge of this work was to develop a fast and reproducible method for quantification of oligomeric product mixture. This challenge could be successfully met by HPLC analysis on a Phenomenex Luna NH_2 column where it was possible to separate and quantify products up to celloheptaose within 12 min. Many other publications only characterized the products by TLC [4, 19]. There is one report where high performance anion-exchange chromatography with pulsed amperometric detection on a Carbopac PA200 column was used for product analysis (measurement time of 35 min). Within the same report quantitative HPLC on an TSKgel Amide-80 column was used for product isolation [12]. Hiraishi et. al obtained similar quantification data using HPLC analysis on an Asahipak NH2P-50 4E column, where one analysis run took 20 min and products up to cellohexaose could be separated [20].

Interestingly, product analysis of the homologous enzymes His-CcCdexP and His-CsCdexP revealed that their product spectra are different, which can be explained by the difference in K_m value for cellobiose. The K_m value of cellobiose is 30 times higher for His-CcCdexP than for His-CsCdexP, although both enzymes carry an N-terminal His-tag and the kinetic parameters were determined under optimal reaction conditions for each enzyme variant. Using equimolar concentration of the substrates Glc1-P and cellobiose a similar Glc1-P turnover was obtained after 24 h (His-CcCdexP: 11 mM, His-CsCdexP: 8 mM). While the Glc1-P turnover was similar one has to mention that different catalytic amounts of His-CcCdexP (0.23 U) and His-CsCdexP (0.15 U) were used. Interestingly, the reaction with His-CsCdexP produced oligomeric products with DP>5 in higher concentration. For an accurate determination, the experiment has to be repeated with the same catalytic amount of the enzymes. Moreover the Glc1-P turnover did only increase marginally (His-CcCdexP and His-CsCdexP: 1.1-fold) from 6 hours to 24 hours, which leads to the assumption that both enzymes suffer from product inhibition by the released phosphate.

For an industrial application, an enzyme that can produce mainly one product is the biocatalyst of choice. We therefore investigated different reaction conditions to control the oligomeric product formation. The change in product spectra was studied at different pH values and at different donor/acceptor substrate ratios.

A product yield of 57% of cellotriose was reached after 360 min when 100 mM Glc1-P, 50 mM cellobiose were used at a pH of 8.5. 57% of cellotriose correspond to 28.1 mM or 14.2 g/L with a space time yield of 2.4 g/(L*h) under the assay conditions. Other products came up to 43% and were composed of cellotetraose, cellopentaose and cellohexaose.

Changing to optimal enzyme environment to pH 7.3, the best product yield of 68% of cellotriose was reached after 360 min using 50 mM Glc1-P and 150 mM cellobiose. Here 68% of cellotriose correspond to 32.5 mM or 16.4 g/L with a space time yield of 2.7 g/(L*h) under the assay conditions used. The other product cellotetraose came to 32%.

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Crude Data

Table 17: P_i concentration for the activity assay of *C. cellulosi* (Figure 9).

time [min]	CfE	purified His-CcCdexP
	P_i [mM]	P_i [mM]
0	0.0	0.0
5	20.2	4.6
10	25.8	8.0
15	28.9	11.1
20	28.5	14.2
25	30.3	17.4

Table 18: P_i concentration for the activity assay of *C. stercorarium* (Figure 10).

time [min]	CfE	purified His-CsCdexP
	P_i [mM]	P_i [mM]
0	0.0	0.0
5	11.3	2.9
10	15.7	4.6
15	18.4	6.2
20	25.1	7.6
25	26.3	9.8
30	29.5	10.6

Table 19: P_i concentration and consumption for the kinetic parameters in synthesis direction (Figure 11).

c(Glc1-P) [mM]	time [min]	$c(P_i)$ [mM]	$c(P_i)/t$ [mM/min]
0.0	5	0.00	0.00
2.0		3.00	0.60
3.0		3.98	0.80
3.5		4.23	0.85
4.0		4.60	0.92
4.5		5.22	1.04
5.0		5.79	1.16
8.0		7.07	1.41
10.0		7.31	1.46
15.0		8.06	1.61
30.0		8.51	1.70
60.0		8.35	1.67
90.0		9.25	1.85

Table 20: P_i concentration and consumption for the kinetic parameters in phosphorolyis direction (Figure 12).

c(C) [mM]	time [min]	c(P_i) [mM]	c(P_i)/t [mM/min]
0.0	5	0.00	0.00
4.0		2.90	0.58
6.0		3.34	0.67
8.0		3.82	0.76
10.0		4.31	0.86
11.0		4.69	0.94
12.0		4.80	0.96
13.0		4.89	0.98
25.0		7.07	1.41
50.0		8.76	1.75
75.0		8.58	1.72
100.0		9.34	1.87

Table 21: Area-Data for the HPAE-PAD analysis - equimolar reaction condition (Figure 19).

time [min]	Cellobiose [knC*min]	Cellotriose [knC*min]	Cellotetraose [knC*min]	Cellopentaose [knC*min]	Cellohexaose [knC*min]	Celloheptaose [knC*min]
0	7.50					
60	6.18	3.89	0.76			
360	4.15	5.58	3.80	1.21	0.19	
1440	4.27	4.32	3.88	2.23	0.76	

Table 22: Area-Data for the HPAE-PAD analysis - different cellobiose concentrations (Figure 20).

time [min]	Cellobiose [knC*min]	Cellotriose [knC*min]	Cellotetraose [knC*min]	Cellopentaose [knC*min]	Cellohexaose [knC*min]	Celloheptaose [knC*min]	
0	10.64						A
60	8.72	3.85					
360	19.19	5.05	0.90				
1440	9.31	3.99		0.45			
0	9.90						B
60	9.84	2.42	0.18				
360	8.98	2.61	0.44				
1440	10.13	2.73	0.70				
0	10.28						C
60	11.31	1.36					
360	9.79	1.51					
1440	11.21	1.74					

Table 23: Area-Data for the HPAE-PAD analysis - different Glc1-P concentrations (Figure 21).

time [min]	Cellobiose [knC*min]	Cellotriose [knC*min]	Cellotetraose [knC*min]	Cellopentaose [knC*min]	Cellohexaose [knC*min]	Celloheptaose [knC*min]	
0	7.77						
60	5.45	3.83	0.84				
360	2.79	6.14	4.81	1.74	0.28		**A**
1440	2.09	4.05	4.83	3.46	1.68	0.20	
0	6.84						
60	4.92	3.26	0.68				
360	1.65	5.22	4.57	1.67	0.34		**B**
1440	1.13	2.91	4.91	4.19	2.67	0.60	
0	7.28						
60	5.03	2.84	0.48				
360	1.36	4.51	3.97	1.40	0.25		**C**
1440	0.66	2.48	4.63	3.57	2.07	0.42	

Table 24: Area-Data for the HPAE-PAD analysis - different pH values and addition of DTT (Figure 22).

time [min]	Cellbiose [knC*min]	Cellotriose [knC*min]	Cellotetraose [knC*min]	Cellopentaose [knC*min]	Cellohexaose [knC*min]	Celloheptaose [knC*min]	
0	292.47						
360	41.03	94.52	135.06	143.21	117.79	23.20	**E**
1440	42.26	87.42	122.90	135.59	108.20	13.62	
0	182.41						
360	202.19	148.05	39.78	2.94			**F**
1440	162.67	146.31	66.80	11.10	1.60		
0	221.94						
360	72.53	127.46	121.43	101.60	62.82	11.48	**C**
1440	26.25	40.37	46.38	56.30	68.35	9.39	
0	228.78						
360	66.61	110.68	99.73	80.62	49.67	9.46	**D**
1440	41.28	71.08	78.43	81.39	76.57	10.23	
0	209.75						
360	124.98	85.33	56.43	21.44	5.04		**A**
1440	27.56	48.86	68.69	71.84	59.89	11.50	
0	196.07						
360	62.32	87.29	75.26	47.30	16.59	1.39	**B**
1440	15.53	36.15	59.43	72.93	77.44	9.86	

Table 25: Correlated concentrations for the product spectra analysis of *C. cellulosi* at different substrate concentrations and pH values with Phenomenex Luna NH_2 column (Figure 30 and Figure 33). C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose.

Glc1-P/C/pH [mM/mM/-]	time [min]	C [mM]	C3 [mM]	C4 [mM]	C5 [mM]	C6 [mM]	C7 [mM]	Glc1-P left [mM]	Glc1-P conversion [%]
15/100/7.3	0	99.1						14.4	0
	360	80.0	14.0	1.0				1.9	86.8
	1440	80.1	13.1	1.4				2.0	86.1
50/50/7.3	0	48.8						50.1	0.0
	360	24.2	16.6	6.4	1.1			13.7	73.7
	1440	25.9	12.9	6.3	2.2	0.5		10.6	78.8
50/150/7.3	0	139.0						47.5	0.0
	360	99.7	32.5	3.2				8.6	81.9
	1440	98.1	29.5	4.9				6.1	87.2
100/50/7.3	0	51.0						95.6	0.0
	360	15.8	16.9	9.6	2.1	0.2		34.9	63.5
	1440	15.7	10.8	8.5	4.5	1.4	0.1	21.9	77.1
200/50/7.3	0	44.9						183.4	0.0
	360	10.9	14.2	12.0	3.8	0.5		109.2	40.5
	1440	9.8	6.4	9.5	0.8	4.0	0.2	67.0	63.5
100/50/8.5	0	48.6						98.2	0.0
	360	26.1	28.0	6.9	1.3			66.0	32.8
	1440	18.7	31.0	12.4	2.5	0.2		42.3	56.9
100/50/5.0	0	76.1						98.4	0.0
	360	19.5	14.7	9.1	2.6	0,3		39.9	59.5
	1440	15.6	10.5	8.0	5.6	2.5	0.2	1.3	98.7
100/50/6.0	0	60.2						100.2	0.0
	360	18.5	17.0	12.9	5.4	1.2		15.1	84.9
	1440	20.7	12.6	8.6	5.1	2.5	0.2	0.0	100.0

Table 26: Correlated concentrations for the product spectra analysis of *C. stercorarium* at different substrate concentrations with Phenomenex Luna NH_2 column (Figure 34). C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose.

Glc1-P/C/pH [mM/mM/-]	time [min]	C [mM]	C3 [mM]	C4 [mM]	C5 [mM]	C6 [mM]	C7 [mM]	Glc1-P left [mM]	Glc1-P conversion [%]
50/50/6.5	0	57.9						50.0	0.0
	30	45.5	5.8	2.3	0.7	0.2		39.4	21.2
	60	43.6	6.7	3.0	1.2	0.4		32.8	34.4
	180	39.8	7.1	3.0	1.1	0.6	1.1	21.3	57.4
	360	36.5	8.8	4.7	2.5	1.1	0.3	13.1	73.8
	1440	34.3	10.4	5.4	2.7	1.3	0.4	7.8	84.4
100/50/6.5	0	57.1						100.0	0.0
	60	41.1	5.5	3.2	1.4	0.4		82.9	17.1
	180	37.0	5.0	3.9	2.9	1.8	0.3	68.6	31.4
	360	36.6	7.2	5.0	2.8	0.9	0.3	57.8	42.2
	1440	31.2	5.7	2.9	1.7	1.2	0.3	43.0	57.0

Table 27: Correlated concentration for the product spectra analysis of *C. stercorarium* and *C. cellulosi* with Phenomenex Luna NH_2 column (Figure 35). C = cellobiose, C3 = cellotriose, C4 = cellotetraose, C5 = cellopentaose, C6 = cellohexaose, C7 = celloheptaose.

organism	Glc1-P/C/pH [mM/mM/-]	time [min]	C [mM]	C3 [mM]	C4 [mM]	C5 [mM]	C6 [mM]	C7 [mM]	Glc1-P left [mM]	Glc1-P conversion [%]
Clostridium cellulosi	50/50/7.3	0	48.8						50.1	0.0
		360	24.2	16.6	6.4	1.1			13.7	72.7
		1440	25.9	12.9	6.3	2.2	0.5		10.6	78.8
Clostridium stercorarium	50/50/6.5	0	57.9						50.0	0.0
		360	36.5	8.8	4.7	2.5	1.1	0.3	13.1	73.8
		1440	34.3	10.4	5.4	2.7	1.3	0.4	7.8	84.4