Synthesis of mechanistic probes and mutational analysis of the sugar C4/C6 interactions in cytidine 5’-diphosphate-tyvelose 2-epimerase from Thermodesulfatator atlanticus

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SYNTHESIS OF MECHANISTIC PROBES AND MUTATIONAL ANALYSIS OF THE SUGAR C4/C6 INTERACTIONS IN CYTIDINE 5'-DIPHOSPHATE-TYVELOSE 2-EPIMERASE FROM *Thermodesulfatator atlanticus*

Experimental work of this Graduate Thesis was done at the Institute of Biotechnology and Biochemical Engineering at Graz University of Technology. The Thesis was made under the guidance of Univ.-Prof. Dipl.-Ing. Dr.techn. Bernd Nidetzky and with the help of Dipl.-Ing. Bakk.phil. BSc. Christian Rapp.

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SYNTHESIS OF MECHANISTIC PROBES AND MUTATIONAL ANALYSIS OF THE SUGAR C4/C6 INTERACTIONS IN CYTIDINE 5'-DIPHOSPHATE-TYVELOSE 2-EPIMERASE FROM *Thermodesulfatator atlanticus*

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Abstract: Mechanistic probes for investigation of CDP-Tyvelose 2-Epimerase - like enzyme from *Thermodesulfatator atlanticus* (*Ta*CPa2E) activity were synthesized. CDP-Lyxose, the mechanistic probe for characterization of C6 interaction with *Ta*CPa2E, was obtained by using chemical approach while CDP-4-Fluoro-4-deoxy-D-Glucose, the mechanistic probe for studying C4 interaction with the enzyme, was yielded by using enzymatic anomeric approach. The later substrate was produced after galactokinase from *Streptococcus pneumoniae* (GalKSpe4) and UDP-glucose pyrophosporylase from *Bifidobacterium longum* (UGPase), were produced in *E. coli*, purified, and therefrom used in the manufacturing of the substrate. Active mutants of *Ta*CPa2E - G206A and D204E were created and used in characterization of the C4/C6 interactions. The highest specific activity towards CDP-Lyxose was determined for wild-type *Ta*CPa2E (0.27 U/ml) and for the two mutants - G206A (0.50 U/ml) and D204E (0.41 U/ml), while CDP-Mannose seems to be better accepted by all three variants (0.02 U/ml for all three forms of the enzyme) than CDP-Glucose. *Ta*CPa2E showed more affinity towards C5 CDP-activated substrate than towards CDP-activated hexoses. Further research might shed more light on CDP-Glucose - and CDP-4-Fluoro-4-deoxy-D-Glucose - enzyme interactions.

Keywords: CDP-Tyvelose 2-Epimerase-like enzyme, mechanistic probes, mutational analysis

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SINTEZA MEHANISTIČKIH PROBA I MUTACIJSKA ANALIZA C4/C6 INTERAKCIJA UGLJIKOHIDRATA I CITIDIN 5'-DIFOSFAT TIVELOZE 2-EPIMERAZE IZ *Thermodesulfatator atlanticus*

Magdalena Merkaš, 1337/MB

Sažetak: Sintetizirane su mehnističke probe za istraživanje aktivnosti enzima sličnog CDP-Tiveloza 2-Epimerazi iz *Thermodesulfatator atlanticus* (*Ta*CPa2E). CDP-liksoza, mehanistička proba za karakterizaciju C6 interakcije supstrata s *Ta*CPa2E je dobivena kemijskom sintezom, dok je CDP-4-Fluoro-4-deoksi-D-glukoza, mehanistička proba za istraživanje C4 interakcije supstrata s enzimom, dobivena enzimskom sintezom. Ovaj drugi supstrat je proizveden nakon što su galaktokinaza iz *Streptococcus pneumoniae* (GalKSpe4) i UDP-glukoza pirofosforilaza iz *Bifidobacterium longum* (UGPase) sintetizirane u *E. coli*, pročišćene i onda korištene u proizvodnji ovoga supstrata. Aktivni mutanti *Ta*CPa2E - G206A i D204E također su sintetizirani i korišteni za karakterizaciju C4/C6 interakcija. Najveću specifičnu aktivnost prema CDP-liksozi pokazali su divlji tip *Ta*CPa2E (0.27 U/ml) i dva mutanta - G206A (0.50 U/ml) i D204E (0.41 U/ml), dok je CDP-manoza bolje prihvaćena kao susptrat kod sva tri enzima (0.02 U/ml za sve tri forme ovoga enzima) od CDP-glukoze. Ustanovljeno je da *Ta*CPa2E pokazuje bolju aktivnost prema C5 CDP-aktiviranom supstratu nego prema CDP-aktiviranim heksozama. Potrebna su daljnja istraživanja kako bi se bolje pojasnile interakcije CDP-glukoze i CDP-4-Fluoro-4-deoksi-D-glukoze s ovim enzimom.

Ključne riječi: CDP-Tiveloza 2-Epimerazi-sličan enzim, mehanističke probe, mutacijska analiza

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List of abbreviations

4-F-4-deoxy-D-Glc	4-Fluoro-4-deoxy-D-Glucose
4-F-4-deoxy-D-Glc-1-P	4-Fluoro-4-deoxy-D-Glucose-1-Phosphate
ATP	Adenosine 5'-Triphosphate
B-PER	Bacterial Protein Extraction Reagent
CDP	Cytidine 5'-Diphosphate
CDP-4-F-4-deoxy-D-Man- 1-P	CDP-4-Fluoro-4-deoxy-D-Mannose-1-Phosphate
CDP-4-F-4-deoxy-D-Man	CDP-4-Fluoro-4-deoxy-D-Mannose
CDP-Ara	CDP-L-Arabinose
CDP-Ara-1-P	CDP-L-Arabinose-1-Phosphate
CDP-Glc	CDP-D-Glucose
CDP-Lyx	CDP-D-Lyxose
CDP-Lyx-1-P	CDP-D-Lyxose-1-Phosphate
CDP-Man	CDP-D-Mannose
GDP-4-keto-6-deoxy-Man	GDP-4-keto-6-deoxy-Mannose
CDP-Par	CDP-D-Paratose
CDP-Rib	CDP-L-Ribose
CDP-Tyv	CDP-D-Tyvelose
CDP-Xyl	CDP-D-Xylose
CDP-Xyl-1-P	CDP-D-Xylose-1-Phosphate
CE	Capillary Electrophoresis
CMP	Cytidine 5'-Monophosphate
CTP	Cytidine 5'-Triphosphate
ddH_2O	Double-distilled water
dH_2O	Distilled water
D-Lyx	D-Lyxose
DMC	2-chloro-1,3-dimethylimidazolinium chloride
dNTP	Deoxynucleotides Triphosphate Solution Mix
DTT	Dithiothreitol

D-Xyl	D-Xylose
EDTA	2,2',2'',2''-(Ethane-1,2-diyldinitrilo)tetraacetic acid
FPLC	Fast Protein Liquid Chromatography
GalKSpe4	Galactokinase from *Streptococcus pneumoniae*
GDP-Man	GDP-Mannose
HPLC	High-Performance Liquid Chromatography
iPPase	Inorganic pyrophosphatase from *Thermococcus litoralis*
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
L-Ara	L-Arabinose
MOPS	3-morpholinopropane-1-sulfonic acid
NDP-sugars	Nucleoside diphosphate activated sugars
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PEP	2-Phosphoenolpyruvate
SDR	Short-chain dehydrogenases/reductases
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
***Ta*CPa2E**	CDP-Tyvelose 2-Epimerase from *Thermodesulfatator atlanticus*
TBAB	Tetra-n-butylammonium bromide
TyvE	CDP-Tyvelose 2-Epimerase
UDP-Gal	UDP-Galactose
UDP-Glc	UDP-Glucose
UDP-Xyl	UDP-Xylose
UGPase	UDP-Glucose pyrophosphorylase from *Bifidobacterium longum*

1. INTRODUCTION

Epimerization can be described as a reaction in which a change in the configuration of one chiral center of chiral molecule is catalyzed. Besides mono- and disaccharides, also nucleotide sugars undergo epimerization reactions. It was shown that this type of reaction plays a major role in metabolism, cell signaling, cell-cell recognition, energy delivery and more.

In this thesis, the focus is put on the investigation of the active site of a Cytidine-5'-Diphosphate (CDP)-Tyvelose 2-Epimerase-like enzyme from *Thermodesulfatator atlanticus* (*Ta*CPa2E). This enzyme belongs to the family of short-chain dehydrogenases/reductases (SDRs). *Ta*CPa2E catalyzes C2 epimerization in all nucleotide activated forms of D-Glucose (Glc) and derivatives thereof. In depth investigation of the enzyme's active site offers new possibilities for modifications of enzyme activity and substrate promiscuity. Changes in the active site by site-directed mutagenesis may alter the enzyme ability to accept other substrates in the sugar binding pocket and thus enable the synthesis of interesting "unusual" products.

Two approaches were designed to investigate the enzyme's active site: substrate synthesis and mutational analysis of *Ta*CPa2E. The first approach was to synthesize 4 CDP-sugars: CDP-Xylose (CDP-Xyl), CDP-Lyxose (CDP-Lyx), CDP-Arabinose (CDP-Ara) and CDP-4-Fluoro-4-deoxy-D-Glucose (CDP-4-F-4-deoxy-D-Glc), while CDP-Mannose (CDP-Man) and CDP-Glucose (CDP-Glc) were already available at the Institute for comparative analysis.

The second approach was production of two *Ta*CPa2E mutants by site-directed substitution of corresponding amino acid in the active site - G206A and D204E.

Finally, when the desired CDP-sugars and mutants were obtained, wild-type enzyme and mutants (G206A and D204E) activities were tested using the synthesized and already available CDP-sugars, by High-Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE).

2. THEORETICAL PART

2.1. SHORT-CHAIN DEHYDROGENASE SUPERFAMILY

The short-chain dehydrogenases/reductases (SDR) superfamily is a large family of NAD(P)H-dependent oxidoreductases, consisting of at least 140 enzymes (Kavanagh et al., 2008). It is one of the most common enzyme superfamily with members in all kingdoms. SDRs show activity for a wide range of substrates and play important roles in amino acid, hormone, cofactor, xenobiotic, and carbohydrate metabolism. It was shown that 25 % of all known dehydrogenases are from the SDR superfamily, and 75 % of all known dehydrogenases have a bacterial origin (Kavanagh et al., 2008)

Based on chain length, SDRs can be classified into two main categories, "classical" and "extended" (Kavanagh et al., 2008). The "classical" type chain is approximately 250 amino acid long, while in the C-terminal region of the "extended" type, additional 100 residues are added. More specific classification based upon sequence motifs includes three more types of SDRs: "intermediate", "complex" and "divergent". "Classical" and "intermediate" SDRs have similar one-domain structures, catalyzing mostly NAD(P)H-dependent oxidoreduction reactions. "Divergent" SDRs have an irregular pattern in the active site. Lysine residues usually replace methionine or hydrophobic residues in the active site. Enzymes in this group are mostly enoyl-thioester reductases. "Complex" SDRs make a large group of multidomain enzymes, like polyketide synthases (Kavanagh et al., 2008).

As shown in Figure 1, enzymes in this superfamily catalyze numerous reactions, like sulfotransferation, isomerization, decarboxylation, reduction of C = C or C = N bonds but also accept hydroxyl/carbonyl groups as substrates (Kavanagh et al., 2008).

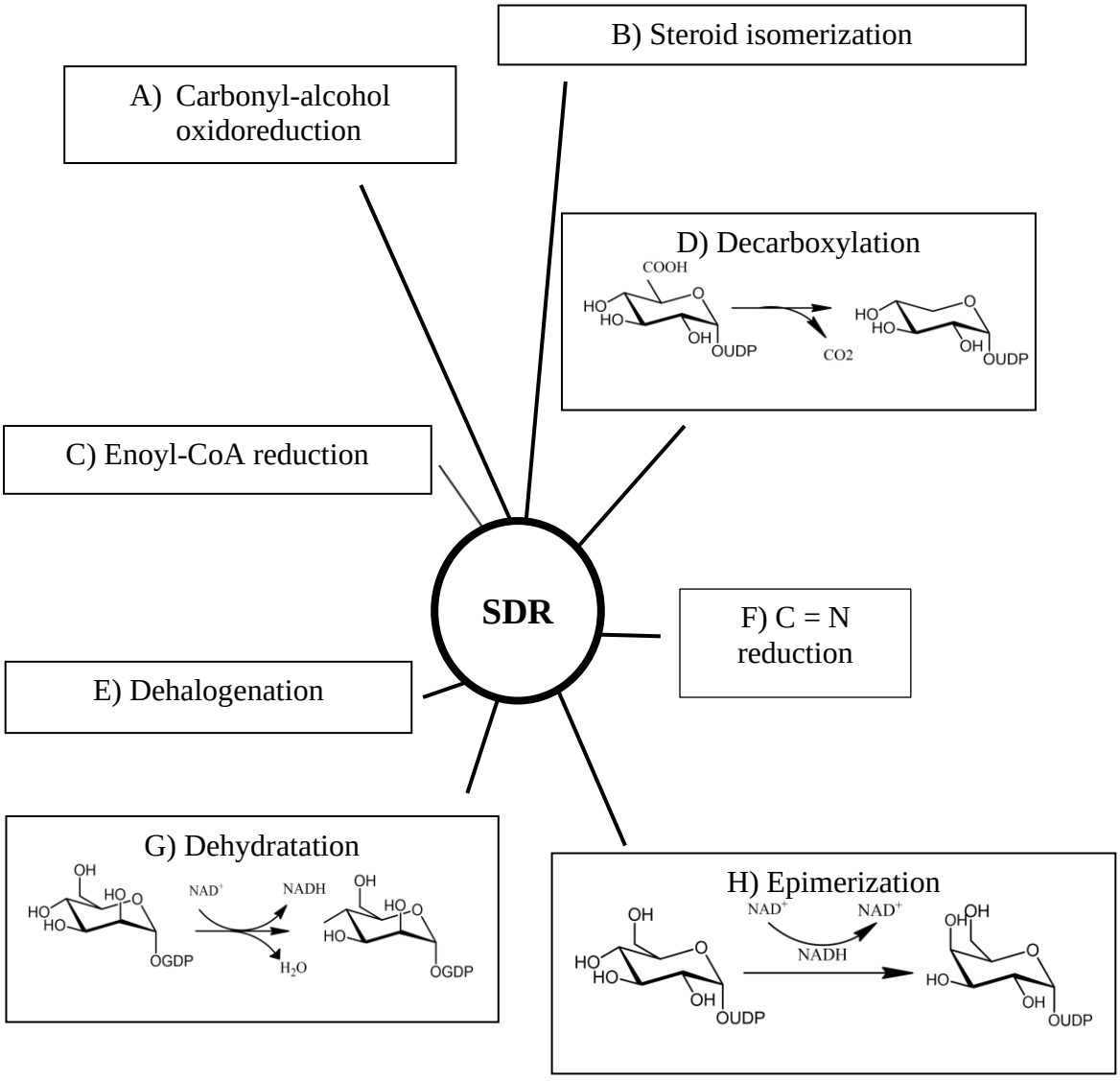


Figure 1. Reactions catalyzed by SDRs (modified from Kavanagh et al., 2008): A) carbonyl-alcohol oxidoreduction; B) steroid isomerization; C) enoyil-CoA reduction; D) decarboxylation of UDP-glucuronic acid to UDP-Xyl by UDP-xylose synthase; E) dehalogenation; F) C = N reduction; G) dehydration of GDP-Man to GDP-4-keto-6-deoxy-Man by GDP-4-keto-6-deoxy-mannose dehydratase; H) epimerization of UDP-Glc to UDP-Gal by UDP-glucose 4-epimerase.

According to pairwise comparisons, members of SDR superfamily share 20-30 % sequence similarity (Persson and Kallberg, 2013). All SDR enzymes share a specific domain at the N-terminus, a structural motif crucial for NAD(P) binding called Rossman-fold (Kavanagh et al., 2008).

The Rossman-fold is one of the most common structural motifs known in the protein world. This domain shows affinity for FAD and NAD(P) and is organized in *βαβαβ* structures, a series of alternating hydrogen bonded β-strands and α-helical segments. β-strands form the central β-sheet surrounded by α-helices (Hanukoglu, 2015) The order of the β-strands is 123456 from N- to the C-terminus (Figure 2). A tight loop connecting the first β-strand and α-helix shows a conserved GXGXXG sequence. In addition, this Gly-rich sequence domain interacts with negatively charged oxygens of pyrophosphate groups of the nucleotide cofactor (Shin and Kihara, 2019).

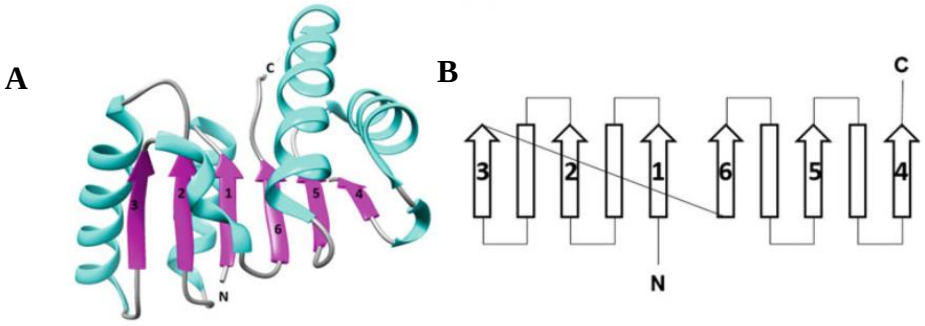


Figure 2. Rossman-fold structure (modified from Shin and Kihara, 2019)a). A) An example of the Rossman-fold. β-strands are colored in purple and α-helices in blue. B) The Rossman-fold shown as two-dimensional model. Arrows are β-strands and α-helices are squares. The strands are numbered from N-terminus.

Functional SDRs are often found to be monomeric, dimeric or tetrameric. Their active site is typically composed of a catalytic tetrad: Tyr, Lys, Ser and Asn. Members of the SDR superfamily do not require metal ions in the active site, which is characteristic for other dehydrogenase superfamilies (Persson and Kallberg, 2013).

2.2. CARBOHYDRATE EPIMERIZATION AND CDP-TYVELOSE 2-EPIMERASE-LIKE ENZYMES

Carbohydrates are the most various biomolecules on the Earth (Nelson and Cox, 2013). They play an important part in energy metabolism, cell-cell recognition and signaling. They also serve as structural molecules of plant and fungi cells, insects exoskeleton and more. Based on the size, carbohydrates can be divided into three groups: monosaccharides (single polyhydroxy aldehyde or ketone unit), oligosaccharides (short chains of monosaccharide units linked to glycosidic bonds) and polysaccharides (more than 20 monosaccharide units) (Nelson and Cox, 2013).

2.2.1. Nucleoside diphosphate activated sugars (NDP-sugars)

A special group of carbohydrates are nucleoside diphosphate activated sugars (NDP-sugars) which are widely spread in nature. The NDP-sugars play important roles in all living organisms. Their analogues can potentially serve as inhibitors in processes for drug synthesis and NDP-sugars serve as building blocks in biosynthesis of carbohydrates and glycoconjugates. Structurally, NDP-sugars are built of monosaccharide and a nucleoside mono- or diphosphate part (Mikkola, 2020).

All NDP-sugars share structure similarities, but also its structure vary in different species. For example, eukaryotes use a lower variety of nucleotides in the activation of sugars than bacteria. Plant cells contain NDP-sugar combinations that are not typical in human carbohydrates (Mikkola, 2020).

The synthesis of NDP-sugars involves a huge number of enzymatic processes that can build and modify sugar nucleotides, but two main biosynthetic pathways for NDP-sugars are *de novo* synthesis and salvage pathways (Mikkola, 2020). The first one uses dietary monosaccharides as the starting material while the salvage pathways recycle monosaccharides from *de novo* reactions. Synthesis of the same NDP-sugar can be carried through different pathways. Some of the enzymes involved in biosynthetic pathways of NDP-sugars are glycosyl transferases, polyisoprenol-

phosphate glycosyltranferases and phoshoglycosyl transferases. New chemical and enzymatic methods for NDP-sugar synthesis have also been proposed (Mikkola, 2020).

2.2.2. Epimerases

Epimerases catalyze the stereochemical inversion at a chiral center in a molecule with n > 1 asymmetric center. First, the enzyme breaks bond and then rearranges it in a non-stereospecific way. This process can involve three different bonds: a carbon-hydrogen bond, a carbon-heteroatom (N or O) bond, or a carbon-carbon bond (Tanner, 2002).

2.2.2.1. Epimerases acting on NDP-sugars

Epimerases can act on "activated" or "unactivated" stereogenic centers in NDP-sugars. Epimerization at the "activated" stereogenic centers proceeds *via* a deprotonation/reprotonation mechanism enabled by an "activated" stereogenic center next to an ester, carbonyl, or carboxyl acid group lowering the *pKa* of the proton in this center. In the enzyme's active site, a catalytic base facilitates proton abstraction from the substrate. Epimerization at "unactivated" stereocenter requires the use of a cofactor, NAD^+ or $NADP^+$. The proton in this case has a higher pKa impeding deprotonation/reprotonation, so the inversion of the stereochemistry is achieved by an oxidation/reduction strategy (Samuel and Tanner, 2002).

CDP-Tyvelose 2-Epimerase (TyvE) is a tetrameric bacterial enzyme and a member of the SDR superfamily. It operates at "unactivated" stereocentres interconverting CDP-Par and CDP-Tyv (Samuel and Tanner, 2002).

Initially, there were four mechanisms proposed for the reaction catalyzed by TyvE (Figure 3). Mechanisms A and B share the same NAD^+-mediated oxidation at the C2 position of the substrate. In the mechanism A, non-stereospecific oxidation of the substrate and reduction of the cofactor happen directly at the epimerization site resulting in respective epimer and NADH. In the mechanism B, a base-catalyzed enolization is followed by the return of the hydrate to C3 and C2 protonation on the opposite side of the pyranose (Samuel and Tanner, 2002).

The first step in mechanisms C and D is oxidation at position C4 resulting in a 4-ketopyranose intermediate. According to the mechanism C, base-catalyzed deprotonation at C3 and additional loss of a hydroxy group of the intermediate leads to the formation of an α, β-ketone. This α, β-ketone is unsaturated and the re-addition of H_2O occurs on the opposite side of the double bond with stereospecific reduction. This leads to the formation of corresponding epimer. With the mechanism D, the intermediate undergoes retro aldol cleavage of the C2-C3 bond. The epimer is formed after aldol addition to the opposite side of the C2 carbonyl with stereospecific reduction (Samuel and Tanner, 2002).

It was shown that no hydride replacement happens between C2 and C3, which ruled out the mechanism B (Samuel and Tanner, 2002). Further experiments have shown that a 4-fluoro analogues of CDP-Par can be used as substrates for the TyvE, so the mechanism D was rejected because it requires oxidation at the C4 position (Samuel and Tanner, 2002). Additional experiments were carried out and it was found that when substrates undergo extended incubation with the enzyme, accumulation of an abortive NADH-enzyme complex occurs. This was attributed to the release of an oxidized keto intermediate. The most important experiment showed that when C2 and C4 deuterated substrates were used and incubated for an extended time, only the C2 marked substrate led to marked NADH. The conclusion was, that the mechanism A is most likely happening (Samuel and Tanner, 2002).

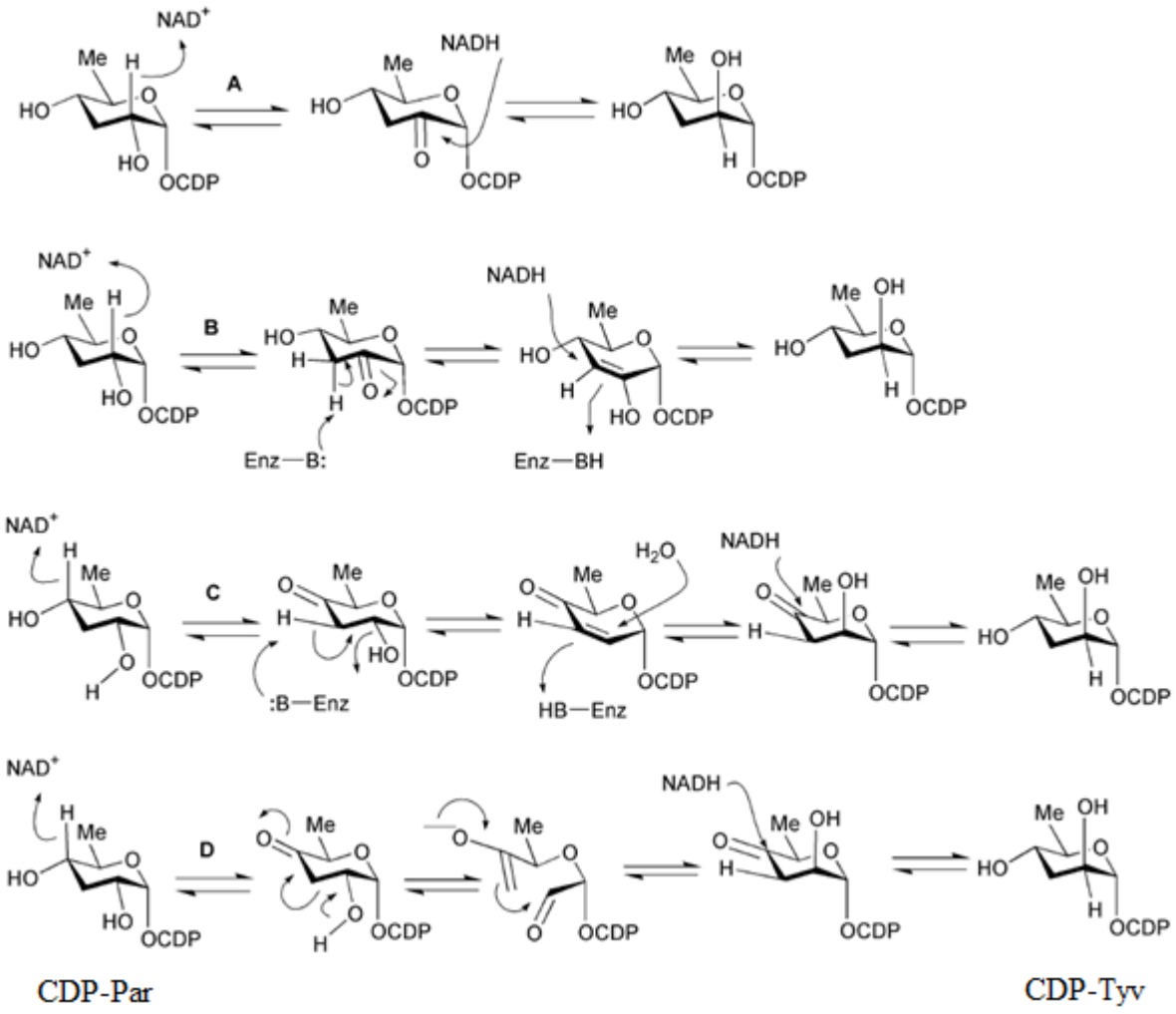


Figure 3. Initially proposed reaction mechanisms for TyvE (modified from Samuel and Tanner, 2002). Mechanism A) and B) share the same NAD^+-mediated oxidation at the C2 position. Mechanisms C) and D) share oxidation at position C4 resulting in a 4-ketopyranose intermediate. Me, Methyl group ($-CH_3$); OCDP, Cytidine 5'-Diphosphate linked to sugar; Enz-B, Enzyme with deprotonated active-site base; Enz-BH, Enzyme with protonated active-site base.

2.2.2.1.1. CDP-Tyvelose 2-Epimerase from Thermodesulfatator atlanticus

CDP-Tyvelose 2-Epimerase from *Thermodesulfatator atlanticus* (*Ta*CPa2E) is a promiscuous CDP-Tyvelose 2-Epimerase (TyvE)-like enzyme that catalyzes C2 epimerization in all nucleotide activated forms of D-Glc as well as its derivates. It was isolated from *Thermodesulfatator atlanticus*, a thermophile bacterium found in the walls of an active deep-sea hydrothermal vent on the Mid-Atlantic Ridge. *Ta*CPa2E is a functional homodimer with one tightly bound NAD^+/subunit with conserved residues in the catalytic center and characteristic residues for binding NAD^+ and CTP as in other TyvE-like epimerases (Rapp et al., 2021).

Rapp et al. (2021) confirmed C2 epimerization activity of *Ta*CPa2E by offering CDP-Glc to the purified enzyme. It was shown that the substrate was converted into a single detectable product, confirmed to be CDP-Man (Figure 4). An equilibrium ratio of CDP-Man/CDP-Glc of 0.7 was found (Rapp et al., 2021).

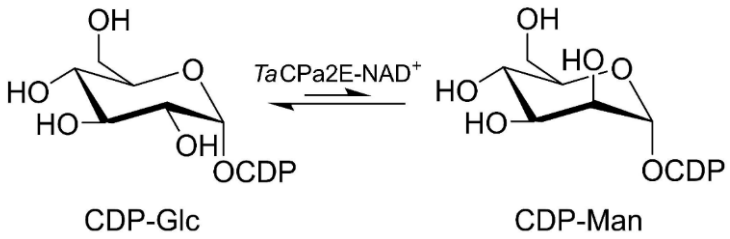


Figure 4. Interconversion of CDP-Glc and CDP-Man by *Ta*CPa2E (modified from Rapp et al., 2021)

In order to get insights into potential binding modes of CDP-Glc (C2-*gluco*-configuration) and CDP-Man (C2-*manno*-configuration) to *Ta*CPa2E, ligand dockings experiments were performed (Figure 5). Based on that, key residues in the enzyme's active site interacting with the before mentioned CDP-sugars were identified. Besides the well conserved catalytic triad (Tyr164, Thr124, Lys168), Ser193 and Asp204 are assumed to interact with C4-OH in the *gluco*- and *manno*-configuration of the substrates, respectively (Rapp et al., 2021). Mutational analysis revealed that site directed substitution of aspartate to alanine (Asp204Ala) leads to complete activity loss, while substitution of Asp204 to Ser reduces the activity of the enzyme by half. Key residues interacting with C6-OH comprise Gly206 (*gluco*-configuration) and Lys126 (*manno*-

configuration; (Rapp et al., 2021). Site-directed substitution of Lys126 or Gly206 performed previously (Rapp et al., 2021) leads to complete loss of enzymatic activity in Lys126Ala and Gly206Ser variants of *Ta*CPa2E. (Rapp et al., 2021). In addition, the epimerization reaction with wild-type *Ta*CPa2E using CDP-6-deoxy-D-Glucose as substrate shows a 5-fold increase in the reaction rate compared to the epimerization of CDP-Glc.

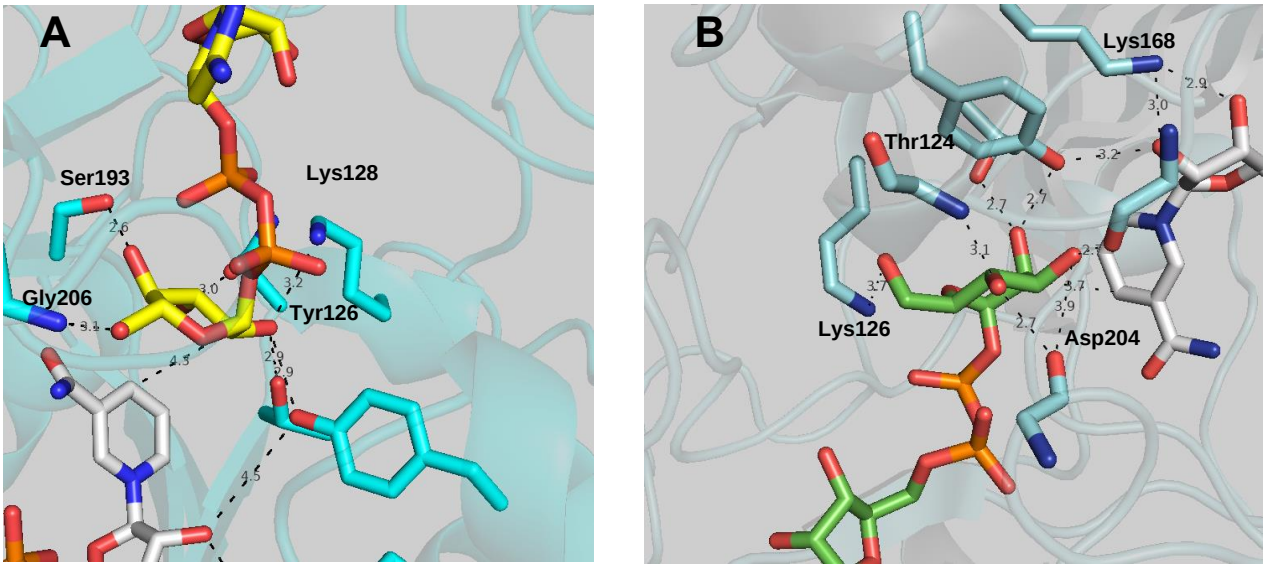


Figure 5. Proposed docked binding modes of CDP-Glc (*gluco*-configuration) (A) and CDP-Man (*manno*-configuration) (B) (modified from Rapp et al., 2021). Proposed key residues in the sugar binding pocket of the enzyme interacting with C4-OH are Ser193 (*gluco*-configuration) and Asp204 (*manno*-configuration). Proposed key residues interacting with C6-OH are: Gly206 (*gluco*-configuration) and Lys126 (*manno*-configuration). Representation of the enzyme's active site was performed using Pymol (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC).

2.2.2.1.1.1. Active site of CDP-Tyvelose 2-Epimerase from Thermodesulfatator atlanticus

This thesis focuses on investigating enzyme-substrate interactions at positions C4 and C6 of the sugar moiety *via* synthesis of substrates analogues and mutational analysis.

As previously described, the *Ta*CPa2E catalyzed epimerization rate acceleration when CDP-6-deoxy-D-Glucose was used, prompted the question whether the reaction rate would be further enhanced by creating an even less bulky substrate than the CDP-6-deoxy substrate. In order to investigate the influence of C6-OH and the surrounding residues on the enzyme catalyzed epimerization rate, the idea was to synthesize CDP-Xyl or CDP-Lyx, both pentose sugars without C6-OH (Figure 6), and to offer them as substrates to wild-type *Ta*CPa2E and selected mutants thereof.

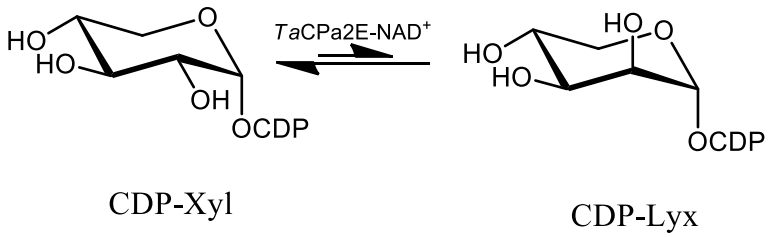


Figure 6. Proposed interconversion of CDP-Xyl to CDP-Lyx and *vice versa* by *Ta*CPa2E.

Additionally, the aim of this thesis was to elucidate interactions at the position C4 of sugar moiety by involving CDP-Ara (Figure 7).

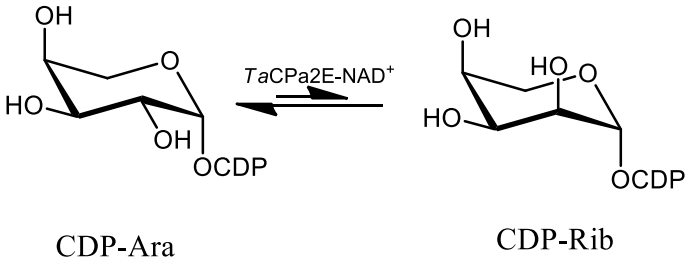
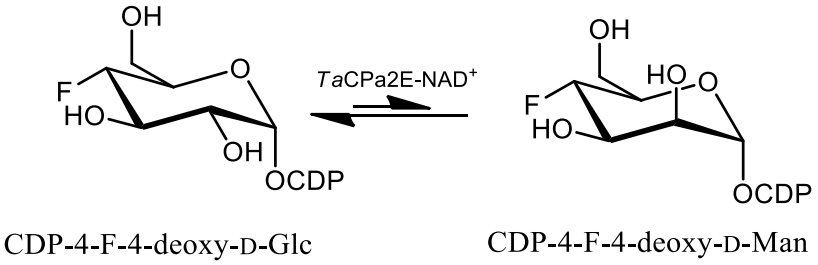


Figure 7. Proposed interconversion of CDP-Ara to CDP-Rib by *Ta*CPa2E.

The combined findings of removing the C6-OH from substrate leading to an accelerated epimerization rate in *gluco*-configurated probes and the lack of activity with CDP-Gal using wild-

type *Ta*CPa2E were raising the question whether a deoxy-*galacto*-configurated probe would enables an epimerization reaction (Rapp et al., 2021).

To further expand the insights into C4-OH interactions with *Ta*CPa2E enzyme active site, the synthesis of CDP-4-F-4-deoxy-D-Glc was targeted as a substrate (Figure 8). The underlying hypothesis was that if hydrogen bonding interaction at C4-OH with *Ta*CPa2E enzyme active site is substantial for proper positioning of the substrate in *Ta*CPa2E enzyme active site, fluorine could partially compensate the role of C4-OH.



CDP-4-F-4-deoxy-D-Glc CDP-4-F-4-deoxy-D-Man

Figure 8. Proposed interconversion of CDP-4-F-4-deoxy-D-Glc to CDP-4-F-4-deoxy-D-Man by *Ta*CPa2E.

So, site-directed mutagenesis of essential amino acid residues in the active site of *Ta*CPa2E should be applied as another tool to obtain more clarity in enzyme-substrate interactions affecting positions C4 and C6 of the sugar moiety. Point mutations should be introduced at Gly206 and Asp204 to investigate interactions in the active site. As described before, Gly206 interacts with the C6-OH of Glc and mutation of Gly206 to Ser results in complete inactivity (Rapp et al., 2021). This theory put forward that an exchange of Gly to Ala, creating G206A mutant, would lead to decreased activity of *Ta*CPa2E, but not complete loss of enzymatic activity. The second desired mutant was D204E. Exchange of Asp at position 204 to Glu would lead to a longer side chain in the enzyme active site and could increase intramolecular interactions and preserve the activity.

3. EXPERIMENTAL PART

3.1. MATERIALS

All chemicals; media; competent *E. coli* strains; plasmids; primers for PCR; kits for plasmid isolation and purification; buffers for SDS-PAGE, PCR, purification and HPLC analysis; gels and gel stains; enzymes and filters are listed in Tables 1-13. All instruments used for experiments are reported in Table 14.

Table 1: The chemicals used in this research.

Chemicals	**Company**
(Double) Distilled Water [(d)dH_2O]	The Institute of Biotechnology and Biochemical Engineering, TU Graz
1,4- Dithiothreitol (DTT, >99 %)	Carl Roth GmbH (Karlsruhe, Germany)
2,2',2'',2''-(Ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA, > 99 %)	Sigma-Aldrich (St. Louis, USA)
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris, > 99.9 %)	Carl Roth GmbH (Karlsruhe, Germany)
2-chloro-1,3-dimethylimidazolinium chloride (DMC, 95 %)	Sigma-Aldrich (St. Louis, USA)
4-F-4-deoxy-D-Glc	Carbosynth Ltd (Compton UK)
Acetic acid (HAc, 96 %)	Sigma-Aldrich (St. Louis, USA)
Acetonitrile (ACN, > 99.9 %)	Chem-Lab (Zedelgem, Belgium)
Adenosine 5'-triphosphate disodium salt hydrate, 99 %	Sigma-Aldrich (St. Louis, USA)
Albumin Fraction V ≥ 98 %	Carl Roth GmbH (Karlsruhe, Germany)
Ampicillin sodium salt (Amp, > 97 %)	Carl Roth GmbH (Karlsruhe, Germany)
B-PER™ Bacterial Protein Extraction Reagent	Thermo Fisher Scientific (Waltham, USA)
CDP-Glc	The Institute of Biotechnology and Biochemical Engineering, TU Graz

Table 1: The chemicals used for the research (continuation).

Chemicals	**Company**
CDP-Man	The Institute of Biotechnology and Biochemical Engineering, TU Graz
Copper (II) chloride (99 %)	Sigma-Aldrich (St. Louis, USA)
Cytidine 5'-monophosphate disodium salt	Carbosynth Ltd (Compton UK)
Cytidine 5'-triphosphate disodium salt	Carbosynth Ltd (Compton UK)
D(+)-Xylose	E. Merck KG (Darmstadt, Germany)
Deoxynucleotides Triphosphate Solution Mix (dNTP) (10 mM)	Thermo Fisher Scientific (Waltham, USA)
Desthiobiotin (>98 %)	Sigma-Aldrich (St. Louis, USA)
Deuterium oxide (D_2O, 99.8%)	Euristop (Saint-Aubin, France)
Dipotassium phosphate (K_2HPO_4, >99 %)	E. Merck KG (Darmstadt, Germany)
D-Lyx	Sigma-Aldrich (St. Louis, USA)
Ethanol (96 %)	Carl Roth GmbH (Karlsruhe, Germany)
Glycerol (> 98 %)	Carl Roth GmbH (Karlsruhe, Germany)
Hydrogen chloride (HCl, > 37 %)	Sigma-Aldrich (St. Louis, USA)
Imidazole (≥ 99 %)	Carl Roth GmbH (Karlsruhe, Germany)
Hydrogen chloride (HCl, > 37 %)	Sigma-Aldrich (St. Louis, USA)
Isopropyl β-D-1-thiogalactopyranoside (IPTG, > 99%)	Sigma-Aldrich (St. Louis, USA)
Kanamycin sulphate (≥ 50 IU/mg)	Carl Roth GmbH (Karlsruhe, Germany)
L-Ara	LOBA Feinchemie (Wien, Austria)
Magnesium chloride ($MgCl_2$, > 98.5 %)	Carl Roth GmbH (Karlsruhe, Germany)
Methanol (> 99.9 %)	Carl Roth GmbH (Karlsruhe, Germany)
2-Methyl-2-oxazoline (98 %)	Sigma-Aldrich (St. Louis, USA)
Diethyl ether (> 99.5 %)	Carl Roth GmbH (Karlsruhe, Germany)
Dichloromethane (≥ 99.5 %)	Sigma-Aldrich (St. Louis, USA)
Dimethylformamide (DMF)	Sigma-Aldrich (St. Louis, USA)
Phosphoric acid (H_3PO_4, 85 %)	Sigma-Aldrich (St. Louis, USA)

Table 1: The chemicals used for the research (continuation).

Chemicals	**Company**
Potassium chloride (KCl, > 99.5 %)	Carl Roth GmbH (Karlsruhe, Germany)
Potassium dihydrogen phosphate (KH_2PO_4, > 99.5 %)	E. Merck KG (Darmstadt, Germany)
Potassium hydroxide (KOH, 85 %)	Carl Roth GmbH (Karlsruhe, Germany)
p-Toluensulfonyl hydrazide (97 %)	Sigma-Aldrich (St. Louis, USA)
Sodium chloride (NaCl, > 99.5 %)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium dihydrogen phosphate (NaH_2PO_4, > 98 %)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium hydroxide (NaOH, 99 %)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium tetraborate (99 %)	Sigma-Aldrich (St. Louis, USA)
Soy peptone	Carl Roth GmbH (Karlsruhe, Germany)
Tetrabutylammonium bromide (TBAB, > 99 %)	Carl Roth GmbH (Karlsruhe, Germany)
Thymol (≥ 99 %)	Carl Roth GmbH (Karlsruhe, Germany)
Triethylamine (≥ 99.5 %)	E. Merck KG (Darmstadt, Germany)

Table 2. Media used for cultivation of *E. coli* strains.

Medium	**Composition**
Lysogeny Broth (LB) medium	10 g/L (1 % w/v) peptone 10 g/L (1 % w/v) NaCl 5 g/L (0.5 % w/v) yeast extract
S.O.C. Medium (Thermo Fisher Scientific (Waltham, USA))	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glc

Table 2. Media used for cultivation of *E. coli* strains (continuation).

Medium	**Composition**
Terrific Broth (TB) medium	10 g/L (1 % w/v) peptone 10 g/L (1 % w/v) NaCl 5 g/L (0.5 % w/v) yeast extract 20% v/v Phosphate buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4)

Sterilization of media was done in an autoclave at 121 °C and 1 bar for 15 min.

Table 3. *E. coli* strains used for expression of wild-type and mutants of *Ta*CPa2E.

Bacterial strains	**Company**
E. coli BL21(DE3)	The Institute of Biotechnology and Biochemical Engineering, TU Graz
E. coli BL21(DE3) LEMO21	New England Biolabs (Ipswich, USA)
E. coli NEB5α	New England Biolabs (Ipswich, USA)

Table 4. Plasmids used for transformation of *E. coli* strains.

Plasmids	**Company**
pET21a_*Ta*CPa2E	The Institute of Biotechnology and Biochemical Engineering, TU Graz
pET28a_GalKSpe4	The Institute of Biotechnology and Biochemical Engineering, TU Graz
pET30_UGPase	The Institute of Biotechnology and Biochemical Engineering, TU Graz
pET-STRP3_iPPase	The Institute of Biotechnology and Biochemical Engineering, TU Graz

Table 5. Primers used for PCR for G206A and D204E *Ta*CPa2E mutants.

Primer name	**Sequence**
G206A forward primer	5‘-GCAACCTATGATCAGGCGTGGATTGGTTGG-3’
G206A reverse primer	5‘-CCAACCAATCCACGCCTGATCATAGGTTGC-3’
D204E forward primer	5‘-CAGTTTGCAACCTATGAACAGGGTTGGATTGG-3’
D204E reverse primer	5‘-CCAATCCAACCCTGTTCATAGGTTGCAAACTG-3’

Table 6. Kits used for isolation and purification of PCR products.

Kits	**Company**
Wizard® Plus SV Minipreps DNA Purification System	Promega Corporation (Madison, USA)
Wizard® SV Gel and PCR Clean-up System	Promega Corporation (Madison, USA)

Table 7. Buffers for SDS-PAGE and PCR.

Buffers	**Company**
NuPAGE™ LDS Sample buffer 4x	Thermo Fisher Scientific (Waltham, USA)
NuPAGE™ MOPS Running buffer	Thermo Fisher Scientific (Waltham, USA)
Phusion 5x High Fidelity (HF) buffer	Thermo Fisher Scientific (Waltham, USA)
Q5® 5x reaction buffer	New England Biolabs (Ipswich, USA)
Tango *Dpn*I buffer	Thermo Fisher Scientific (Waltham, USA)

Table 8. Buffers prepared for purification and storage of *Ta*CPa2E enzymes, for CDP-sugars purification, and for TLC and HPLC analysis.

Buffer	**Composition**
Acetate buffer A for anion exchange chromatography	20 mM Acetic acid, pH 4.3
Acetate buffer B for anion exchange chromatography	1 M Acetic acid, pH 4.3
Borate buffer for CE	20 mM Sodium tetraborate, pH 9.3
His-stripping buffer	20 mM Na_2HPO_4 500 mM NaCl 50 mM Ethylenediaminetetraacetic acid pH 7.5 (adjusted with with HCl)
His-trap binding buffer (A)	10 mM Ethylenediaminetetraacetic acid 500 mM NaCl 5 % glycerol 5 mM imidazole pH 7.5 (adjusted with with HCl)
His-trap elution buffer (B)	500 mM NaCl 5 % glycerol 300 mM imidazole pH 7.5 (adjusted with with HCl)
Protein storage buffer for GalKspe4 and UGPase	50 mM Tris Hydrogen chloride 10 % glycerol pH 8.0 (adjusted with with HCl)
Protein storage buffer for *Ta*CPa2E	100 mM Tris 500 mM NaCl pH 7.5 (adjusted with with HCl)

Table 8. Buffers prepared for purification and storage of *Ta*CPa2E enzymes, for CDP-sugars purification, and for TLC and HPLC analysis (continuation).

Buffer	**Composition**
Strep-trap elution buffer (E)	100 mM Tris/HCl 150 mM NaCl 2.5 mM desthbiotin pH 8 (adjusted with with HCl)
Strep-trap regeneration buffer (R)	100 mM Tris 150 mM NaCl pH 8.0 (adjusted with with HCl)
Strep-trap washing buffer (W)	100 mM Tris 150 mM NaCl pH 8 (adjusted with with HCl)
TBAB buffer	20 mM K_2HPO_4/KH_2PO_4 40 mM TBAB pH 5.9 (adjusted with with HCl and KOH)
Thymol staining solution	0.5 % w/v Thymol 95 % v/v Ethanol 5 % v/v H_2SO_4
TLC eluent solution	50 % Buthanol 25 % Acetic acid 25 % H_2O

Buffers pH was adjusted using HCl, H_3PO_4, NaOH, or KOH. To filtrate buffers, 0.45 μm cellulose acetate filter (GE Healthcare Life Sciences, Chicago, USA) was used.

Table 9. Columns used for protein purification and HPLC analysis.

Columns	**Company**
HisTrap™ HP 5 mL	GE Healthcare Life Sciences (Chicago, USA)
StrepTrap™ HP 5 mL	GE Healthcare Life Sciences (Chicago, USA)
Superdex G-10 size-exclusion column	GE Healthcare Life Sciences (Chicago, USA)
TOYOPEARL Super Q-650M anion exchange column 125 mL	GE Healthcare Life Sciences (Chicago, USA)
Kinetex® 5 µm EVO C18 100 Å, 150 x 4.6 mm	Phenomenex (Torrance, USA)

Table 10. Gels used for SDS-page.

Gels	**Company**
NuPAGE™ Bis-Tris Mini Protein Gels, 10 or 15-well	Thermo Fisher Scientific (Waltham, USA)

Table 11. Gel markers and stain used for SDS-PAGE.

Gel stains and markers	**Company**
1 kb DN Ladder	New England Biolabs (Ipswich, USA)
Gel Loading Dye, Purple 6x, no SDS	New England Biolabs (Ipswich, USA)
HDGreen™ Plus DNA Stain	INTAS (Göttingen, Germany)
InstantBlue™ Coomassie Protein Stain	Expedeon (Cambridge, UK)
PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific (Waltham, USA)
Coomasie staining solution (50 % Methanol 10 % Acetic acid 40 % H_2O)	The Institute of Biotechnology and Biochemical Engineering, TU Graz
Destaining solution (40 % Methanol 10 % Acetic acid)	The Institute of Biotechnology and Biochemical Engineering, TU Graz

Table 12. Enzymes used in the research.

Enzymes	**Company**
DpnI Restriction Enzyme	Thermo Fisher Scientific (Waltham, USA)
Galactokinase from *Streptococcus pneumoniae* (GalKSpe4)	The Institute of Biotechnology and Biochemical Engineering, TU Graz
iPPase (Inorganic pyrophosphatase) from *Thermococcus litoralis*	The Institute of Biotechnology and Biochemical Engineering, TU Graz
Q5® High-Fidelity DNA Polymerase	New England Biolabs (Ipswich, USA)
*Ta*CPa2E wild-type enzyme	The Institute of Biotechnology and Biochemical Engineering, TU Graz
UGPase wild-type enzyme from *Bifidobacterium longum*	The Institute of Biotechnology and Biochemical Engineering, TU Graz
Pyruvate kinase from rabbit muscle	Sigma-Aldrich (St. Louis, USA)

Table 13. Filters used for buffers filtration and protein ultracentrifugation.

Filters	**Company**
Vivaspin 20, 30 kDa cut-off	Sartorius (Göttingen, Germany)
Vivaspin 500, 10 kDa cut-off	Sartorius (Göttingen, Germany)
Whatman™ Cellulose Acetate Membrane 0.45 μm	GE Healthcare Life Sciences (Chicago, USA)
Whatman™ Syringe Filters 0.45 μm	GE Healthcare Life Sciences (Chicago, USA)

Table 14. Equipment used for the experiments.

Instruments	**Company**
5424R Centrifuge	Eppendorf AG (Hamburg, Germany)
5810R Centrifuge A-4-62-MTP Rotor	Eppendorf AG (Hamburg, Germany)
691 pH Meter	Metrohm (Herisau, Switzerland)
ÄKTA FPLC system	GE Healthcare Life Sciences (Chicago, USA)
ÄKTAprime Plus	GE Healthcare Life Sciences (Chicago, USA)
Laminar BioAir AURA-2000 M.A.C.	EuroClone S.p.A. (Milan, Italy)
Certomat BS-1 Shaking Incubator	Sartorius (Göttingen, Germany)
CO8000 Cell Density Meter	Biochrom WPA (Cambridge, UK)
DS-11 Spectrophotometer	DeNovix Inc. (Wilmington, USA)
Entris® Laboratory Balance	Sartorius (Göttingen, Germany)
G1600AX 3D Capillary Electophoresis	Agilent Technologies (Santa Clara, USA)
Laborota 4000 rotary evaporator	Heidolph Instruments (Schwabach, Germany)
Mini-Sub Cell GT System for SDS-PAGE	Bio-Rad Laboratories Inc. (Hercules, USA)
MP-300V Power Supply for SDS-PAGE	Major Science Co. LTD. (Saratoga, USA)
Shimadzu® HPLC-20	Shimadzu Corporation (Kiyamachi-Nijo, Japan)
Sorvall® Evolution™ RC Superspeed Centrifuge	Thermo Fisher Scientific (Waltham, USA)
ThermoMixer® Comfort	Eppendorf AG (Hamburg, Germany)
TM 01 Vortex Mixer	Retsch GmbH (Haan, Germany)
Varian INOVA 500-MHz NMR spectrometer	Agilent Technologies (Santa Clara, USA)
Varioklav® Laboratory Autoclave	Thermo Fisher Scientific (Waltham, USA)
Vibra-Cells Processor VCX130	Sonics & Materials Inc. (Newtown, USA)
ZWY-B3222 Orbital Floor Shaker	Labwit Scientific (Victoria, Australia)

3.2. METHODS

3.2.1. Enzyme production

3.2.1.1. Media preparation

LB medium and TB medium were prepared in 1000 mL baffled shake flasks. Each flask contained 250 mL of the medium and was autoclaved. Adequate volume of antibiotic solution was added to the medium to obtain final concentration of 50 µg/mL of antibiotic in medium (see Table 15).

3.2.1.2. Plasmids

E. coli BL21 (DE3) LEMO21 expression strain was transformed by plasmids with genes encoding wild-type enzymes, as follows: *Ta*CPa2E (pET21a_*Ta*CPa2E) and GalKSpe4 (pET28a_GalKSpe4). *E. coli* BL21 (DE3) was transformed with pET30_UGPase. All those transformants were already available at the Institute of Biotechnology and Biochemical Engineering. As a part of this thesis, *E. coli* BL21 (DE3) LEMO21 was transformed by pET21a_*Ta*CPa2E_G206A and pET21a_*Ta*CPa2E_D204E (QuikChange^{TM} site-directed mutagenesis; see chapter 3.2.1.2.1.) in order to obtain the two *Ta*CPa2E mutants. iPPase (pET-STRP3_iPPase) was already available as purified enzyme at the Institute. Details of the transformation and the expression are organized in Table 15.

Table 15. Enzymes, *E. coli* strains, plasmids, media, antibiotic resistance, induction by IPTG and tags used in this research.

Enzyme	***E. coli* strain**	**Plasmid**	**Medium**	**Antibiotic**	**IPTG (mM)**	**Tag**
wild-type *Ta*CPa2E	BL21 (DE3) LEMO21	pET21a_TaCPa2E	TB	ampicillin	0.1	His
*Ta*CPa2E G206A	BL21 (DE3) LEMO21	pET21a_*Ta*CPa2E_G206A	TB	ampicillin	0.1	His
*Ta*CPa2E D204E	BL21 (DE3) LEMO21	pET21a_*Ta*CPa2E_D204E	TB	ampicillin	0.1	His
GalKSpe4	BL21 (DE3) LEMO21	pET28_GalKSpe4	LB	kanamycin	0.2	Strep
UGPase	BL21 (DE3)	pET30_UGPase	LB	kanamycin	0.1	His

3.2.1.2.1. Site-directed mutagenesis

Both primers for *Ta*CPa2E mutants (G206A and D204E) are shown in chapter 3.1, Table 5. They were ordered from Sigma-Aldrich (Darmstadt, Germany). As a template, the pET21a_*Ta*CPa2E plasmid was used to create mutational variants.

For each of the two mutants (G206A and D204E), three PCR reaction mixtures were set in separate Eppendorf tubes (Table 16). The first reaction served as a control reaction and the pET21a_*Ta*CPa2E plasmid was used as template.

Table 16. Composition of PCR reaction mixtures for site-directed mutagenesis.

Volume (μL)	**Composition**		
Total – 50	1. reaction	2. reaction	3. reaction
0.8	Template plasmid (25 ng/μl stock)	Template plasmid (25 ng/μl stock)	Template plasmid (25 ng/μl stock)
10.0	Q5® 5x reaction buffer	Q5® 5x reaction buffer	Q5® 5x reaction buffer
1.0	Deoxynucleotide Solution Mix (10 mM)	Deoxynucleotide Solution dMix (10 mM)	Deoxynucleotide Solution Mix (10 mM)
36.5	Autoclaved ddH_2O	Autoclaved dd H_2O	Autoclaved dd H_2O
1.0	Autoclaved ddH_2O	Forward primer (10 μM stock)	Reverse primer (10 μM stock)
0.5	Q5® High-Fidelity DNA Polymerase	Q5® High-Fidelity DNA Polymerase	Q5® High-Fidelity DNA Polymerase

The temperature and time profile for the PCR reactions are shown in Table 17. The initial phase of linear PCR amplification was carried out separately for both primers in order to avoid the complementary binding of primers and their dimerization. The initial phase consisted of three cycles of denaturation, annealing and extension. After the initial phase, 2nd and 3rd PCR reaction mixtures were mixed and again separated in two Eppendorf tubes and PCR amplification was carried out for additional 15 cycles.

Table 17. Temperature and time profile for the PCR reactions.

PCR step	Temperature (°C)	Time
Initial denaturation	98	30 s
Denaturation	98	10 s
Annealing	60	15 s
Extension	72	6 min
Final extension	72	5 min
Hold	10	∞

After the PCR reaction, digestion of template plasmid was performed by adding 6 µL of Tango *Dpn*I buffer (Thermo Fisher Scientific Waltham, USA) and 1 µL of DpnI Restriction Enzyme (Thermo Fisher Scientific, Waltham, USA) in each Eppendorf tube and mixtures were incubated overnight at 37 °C. Afterwards, denaturation of DpnI was performed by incubating the reaction mixtures at 80 °C for 20 min. Resulting mixture was centrifuged for 1.5 min at high speed (10000 rpm) to remove denatured DpnI. Supernatants were used for isolation of corresponding plasmids (see below).

3.2.1.2.2. Isolation and sequencing of plasmids after PCR reaction

Plasmid DNA isolation and purification were done by using the Wizard® SV Gel (Promega Corporation, Madison, USA) and PCR Clean-up system (Promega Corporation, Madison, USA). 5 µL of isolated and purified plasmid DNA was used to heat-shock transform 20 µL of chemically competent *E. coli* NEB5α cells (42 °C for 30 s). First, the cells with plasmid DNA were incubated for 30 min on ice and subsequently subjected to the heat-shock transformation. For recovery of the cells, suspension was immediately after the heat-shock placed on ice for 2 min and then 400 µL of S.O.C. medium was added in each transformation mixture. Afterwards, cell growth was carried for 60 min at 37 °C and 135 rpm. Resulting cell suspension was centrifuged (2 min at 10000 rpm; 5424R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant was partially discarded in order to have approximately 100 µL of supernatant left in the tube. The remaining 100 µL of supernatant was resuspended with cell precipitate and plated on LB plates containing ampicillin (50 µg/mL), and incubated overnight at 37 °C without shaking. A single colony of transformants

was taken and resuspended in 10 mL of LB medium with ampicillin (50 µg/mL) and incubated overnight at 37 °C and 135 rpm. The overnight cultures were used for plasmid isolation by using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, USA). Protocol for isolation of plasmid DNA was followed almost in all, except one step. For the elution of plasmid DNA part, 30 µL of ddH_2O heated to 70 °C was used instead of Nuclease-Free Water. Concentration of purified plasmids was determined by using Nanodrop at 260 nm (DS-11 Spectrophotometer, DeNovix Inc. Wilmington, USA). 20 µL samples (10 µL of isolated plasmid solution + 10 µL of ddH_2O) were taken and sent for sequencing to LGC Genomics GmbH (Berlin, Germany).

3.2.1.2.3. Glycerol stocks preparation

2 µL of purified plasmid containing mutant plasmid DNA was used to transform 50 µL of *E. coli* BL21 (DE3) LEMO21 cells. The protocol was comparable to the procedure used to transform *E. coli* NEB5α cells with the only one difference that heat shock was carried for 10 sec instead of 30 sec (see chapter 3.2.1.2.2.). A single colony was taken from the LB plates and 10 mL LB medium with ampicillin was inoculated and cultivated overnight (37 °C and 135 rpm) to obtain the pre-culture. Glycerol stocks for each mutant were prepared from 800 µL of freshly prepared pre-culture and 200 µL of glycerol. Glycerol stocks were stored at -70 °C until further usage.

3.2.1.3. Cultivation of transformed E. coli strains (for production of wild-type Ta*CPa2E, GalKspe4, UGPase and* Ta*CPa2E mutants)*

Fresh pre-cultures of transformed *E. coli* strains were prepared from their glycerol stocks in order to express wild-type *Ta*CPa2E, the two mutants (G206A and D204E), then GalKSpe4 and UGPase. Glycerol stocks for production of wild-type *Ta*CPa2E, GalKSpe4 and UGPase were already available at the Institute. 30 µL from each glycerol stock was resuspended in 10 mL sterile LB or TB medium with ampicillin (50 µg/ml) or kanamycin (50 µg/mL) (Table 15) and cells were incubated in 10 mL of the media overnight at 37 °C and 135 rpm. Volume of 2.5 mL of freshly pre-cultures were used as inocula (1.0 % v/v) for main cultures (see chapter 3.2.1.1.) and obtained

suspensions were incubated at 37 °C and 135 rpm. Cell growth was monitored by measuring optical density (OD) at 600 nm (OD_{600}). When the OD_{600} reached a value between 0.8 and 1.0 AU, the gene expression was induced by the addition of adequate concentration of IPTG (Table 15) and then the suspensions were incubated overnight (18 °C and 110 rpm). Cell harvesting was conducted by centrifugation for 25 minutes at 5000 rpm and 4 °C (Sorvall® Evolution™ RC Superspeed Centrifuge, Thermo Fisher Scientific, Waltham, USA). The cell pellet was collected and resuspended in 5-10 mL of His-trap binding buffer for wild-type *Ta*CPa2E, then for the two mutants (G206A and D204E) and UGPase or 5-10 mL of Strep-trap washing buffer (W) for GalKSpe4. Resulting suspensions were used for preparation of cell extracts (see below).

3.2.1.4. Preparation of cell extracts

Sonication (Vibra-Cells Processor VCX130, Sonics & Materials Inc. (Newtown, USA)) was used for cell lysis (Table 18). Amplitude was given as % (the distance between horn's vibrating surface position in the horns's fully extended and fully contracted states, measured in microns). Obtained cell extracts (approximately 50 ml) were divided into aliquots of 2 mL in Eppendorf tubes and centrifuged for 1 h at 4 °C and 15000 rpm (5423 Centrifuge, Eppendorf AG, Hamburg, Germany). The pellet was discharged, and the supernatant was collected and filtered through a filter (Whatman™ Syringe Filters 0.45 μm, GE Healthcare Life Sciences Chicago, USA) and used for the purification of the enzymes (see chapter 3.2.1.5) and also for SDS-PAGE (see chapter 3.2.1.7.).

Table 18: Sonication settings used for each enzyme.

Enzyme	**Program**	**Amplitude**	**Time/min**
GalKSpe4	2 sec on 5 sec off	65 %	7
UGPase	2 sec on 5 sec off	60 %	7
Wild-type *Ta*CPa2E	2 sec on 5 sec off	70 %	7
*Ta*CPa2E D204E	2 sec on 5 sec off	70 %	7
*Ta*CPa2E G206A	2 sec on 5 sec off	70 %	7

3.2.1.5. Enzymes purification by chromatography

Recombinant proteins are often expressed with a fused affinity tag on the N- or C-terminus. Genes encoding UGPase and wild-type and mutants of *Ta*CPa2E were fused with a sequence of six histidines (the His-tag) on the C-terminal end. Histidine residues have affinity for metal ions (Ni^{2+} or Cu^{2+}) bound to the column. This enables interaction between only the protein of interest and the His-tag column (Spriestersbach et al., 2015). In this thesis proteins were bound to the column [HisTrap™ HP 5 ml, GE Healthcare Life Science, Chicago, USA connected to a ÄKTAprime Plus (GE Healthcare Life Sciences, Chicago, USA) liquid chromatography device] using His-Trap biding buffer (A). A gradient with His-trap elution buffer (B) was increasing from 0 to 100 % with 2 ml/min flow and a total volume of 120 mL was used to elute the His-tagged proteins (UGPase, wild-type and mutants of *Ta*CPa2E). The imidazole (300 mM) competes with the His-tagged proteins bound to metal ions on the column and enables elution of the His-tagged proteins (Spriestersbach et al., 2015). At approximately 30 % of His-trap elution buffer (B) unspecific proteins from cell extracts were eluted, while proteins of interest were eluted between 60-100 % of His-trap elution buffer (B). Flowthrough fractions, washing fractions and elution fractions were kept and used for loading on SDS-PAGE gel (see chapter 3.2.1.7.).

GalKSpe4 was fused with Strep-tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys; (Schmidt and Skerra, 2007)) and purified by using Strep-Tactin bound to the column [StrepTrap™ HP 5 ml, GE Healthcare Life Science, Chicago, USA connected to a ÄKTAprime Plus (GE Healthcare Life Sciences, Chicago, USA) liquid chromatography device]. The Strep-tagged GalKSpe4 bound to the column [Strep-Trap washing buffer (W)] and then eluted with 100 % of Strep-Trap elution buffer (E). The elution was performed without buffer gradient, because Strep-Trap contains desthiobiotin (2.5 mM) which shows a higher affinity to Strep-Tactin than Strep-tagged protein (Junttila et al., 2005). Again, flowthrough fractions, washing fractions and elution fractions were taken for loading on SDS-PAGE gel (see chapter 3.2.1.7.).

3.2.1.6. Determination of protein concentration

Protein concentration was measured with Nanodrop (DS-11 Spectrophotometer, DeNovix Inc. Wilmington, USA) at 280 nm. When successful purification was confirmed by SDS-PAGE (see

chapter 3.2.1.7), elution fractions of wild-type *Ta*CPa2E and mutants (G206A and D204E) GalKSpe4 and UGPase were concentrated with Vivaspin tubes (MWCO 30 kDa and 50 kDa). Also, proteins were rebuffered with protein storage buffers, as described in Table 8 (see chapter 3.1.). GalKSpe and UGPase were rebuffered with the same storage buffer (50 mM Tris/Hcl, 10 % glycerol and pH 8.0) while *Ta*CPa2E wild-type and mutants were rebuffered with another buffer (100 mM Tris, 500 mM NaCl and pH 7.5).

Concentrated proteins were aliquoted and marked (E_1-E_n) and stored in tubes at -70 °C.

3.2.1.7. SDS-PAGE and gel staining

To check the purity of the expressed enzymes, samples were collected from different purification steps. The pellet (insoluble fraction) separated by the centrifugation after sonication of the main *E. coli* cultures was resuspended in 1 mL of Bacterial Protein Extraction Reagent (B-PER™) and centrifuged for 2 min at 15000 rpm (5424R Centrifuge, Eppendorf AG, Hamburg, Germany). Extract obtained in this way was also analyzed by SDS-PAGE.

The samples were prepared by adding 10 µL NuPAGE™ sample buffer, 16 µL ddH_2O and 4 µL DTT solution to the 10 µL of protein sample. Mixtures were incubated for 10 min at 95 °C to denature the proteins. 5 µL of PageRuler™ Prestained Protein Ladder and 10 µL of each prepared protein sample were loaded to NuPAGE™ Bis-Tris Mini Protein Gels (Thermo Fisher Scientific, Waltham, USA). The electrophoresis run for 60 min at 170 V in NuPAGE™ MOPS Running buffer (Thermo Fisher Scientific, Waltham, USA). The gels were stained with Coomassie blue staining solution and destained with the destaining solution (40 % Methanol, 10 % Acetic acid) (see Table 11, chapter 3.1.)

3.2.2. Synthesis of substrates (CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc) for wild-type *Ta*CPa2E and its mutants

3.2.2.1. Anomeric approach

Anomeric approach in synthesis of CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc consisted of two steps - anomeric phosphorylation of L-Ara, D-Xyl, D-Lyx and 4-F-4-deoxy-D-Glc to L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P and 4-F-4-deoxy-D-Glc-1-P by GalKSpe4, and then CMP coupling of L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P and 4-F-4-deoxy-D-Glc-1-P to CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc by UGPase. All required enzymes (GalKSpe4 and UGPase) were previously produced and prepared, as described in chapter 3.2.1.

3.2.2.1.1. Anomeric phosphorylation of L-Ara, D-Xyl, D-Lyx and 4-F-4-deoxy-D-Glc

3.2.2.1.1.1. Enzymatical anomeric phosphorylation of L-Ara, D-Xyl, D-Lyx and 4-F-4-deoxy-D-Glc

For anomeric phosphorylation of L-Ara, D-Xyl, D-Lyx and 4-F-4-deoxy-D-Glc, the activity of GalKSpe4 kinase was tested using a recycling system. The recycling system uses a pyruvate kinase, an enzyme that catalyzes reversible phosphorylation of pyruvate using ATP forming PEP and ADP. This enables ATP regeneration in reaction mixture when applied in the reverse direction (Munoz and Ponce, 2003). Series of reactions were set. Reaction mixture for production of L-Ara-1-P, D-Xyl-1-P and D-Lyx-1-P contained 25 mM (33.8 mg) of L-Ara, D-Xyl or D-Lyx, 1 mM ATP (5.0 mg), 30 mM PEP (45.37 mg) and 6 mM $MgCl_2$ (11.07 mg) dissolved in 9 mL of 50 mM Tris/HCl buffer at pH 7.5. Reaction mixture for production of 4-F-4-deoxy-D-Glc-1-P contained 50 mM of 4-F-4-deoxy-D-Glc (81.9 mg), while remaining components were the same: 1 mM ATP (5.0 mg), 30 mM PEP (45.37 mg) and 6 mM $MgCl_2$ (11.07 mg) dissolved in 9 mL of 50 mM Tris/HCl buffer at pH 7.5. 10 U/mL of pyruvate kinase (2.5 µL, 4000 U/mL) and 4 mg/mL of GalKSpe4 (0.95 mL, 38 mg/mL) were then added to the reaction mixtures to start the phosphorylation.

The reaction mixture was incubated at 37 °C for 20 h. The phosphorylation progress was monitored by TLC (see chapter 3.2.2.3.) at different points of time (0 h and/or 1 h, 2 h and/or 20 h), respectively.

3.2.2.1.1.2. Chemical anomeric phosphorylation of D-Xyl

To obtain D-Xyl-1-P, another approach was tried - chemical anomeric phosphorylation. Modified protocol (Edgar et al., 2012) for production of D-Xyl-1-P was used. D-Xyl (150 mg) and *p*-toluenesulfonyl hydrazide (206.4 mg) were suspended in dimethylformamide (DMF) (300 µL) and dH_2O (150 µL) in 1.5 mL Eppendorf tube. Acetic acid (99.5%, 30 µL) was added to the mixture and incubated at 37 °C (without stirring) for three days. Afterwards, the undissolved precipitate was left in Eppendorf tube while the supernatant was collected and poured into diethyl ether (24 ml, 99.5 %), and the resulting mixture was strongly shaken until a white precipitate was formed. The mixture was left for 5 minute, the supernatant (diethyl ether) was discharged and precipitate was collected by using spatula.

110 mg of collected precipitate was added to the first scintillation vial with dry DMF (4.8 mL) and the mixture was stirred with magnetic stirrer for 15 minutes at 23 °C. In the second scintillation vial anhydrous cupric chloride (195 mg) was placed and dissolved in dry DMF (1.2 mL). Next, 2-methyl-2-oxazoline (0.12 mL) was added to the solution and the mixture was shaken by magnetic stirrer for few minutes. In third scintillation vial crystalline phosphoric acid (525 mg) was dissolved in dry DMF (1.2 mL), shaken, and added to the second scintillation vial. The mixture was shaken for 30 seconds and then added to the first scintillation vial via syringe. The resulting mixture was stirred for 18 h at 23 °C. The reaction mixture was poured into dichloromethane (36.5 mL) and left for 20 min. After 20 min, the formed precipitate was collected *via* centrifugation. The collected precipitate was diluted with ethanol (18.25 mL) and dissolved in DMF (3.65 mL). In the meanwhile, an aqueous saturated solution of barium hydroxide was prepared and slowly added to the previously diluted precipitate until the pH was approximately 8-9. The resulting precipitate was filtered off and washed with ~18.5 mL of hot distilled H_2O (~ 70 °C). The filtrate was concentrated via rotary evaporation and analyzed on TLC. After the formation of D-Xyl-1-p was

indicated on TLC, the concentrated filtrate was freeze-dried, dissolved in D_2O and analyzed on NMR (see chapter 3.2.2.6.).

3.2.2.1.2. CMP coupling of 4-F-4-deoxy-D-Glc-1-P

CMP coupling is transfer of CMP group from CTP to sugar-1-Phosphate to obtain CDP-sugar, catalyzed by pyrophosphorylase.

When successful anomeric phosphorylation was confirmed using TLC and/or NMR (chapter 3.2.2.3.) the second step was enzymatic CMP coupling of successfully obtained phosphorylated product (4-F-4-deoxy-D-Glc-1-P) to CDP-sugar. Only 4-F-4-deoxy-D-Glc-1-P was used as substrate for enzymatic coupling to produce CDP-4-F-4-deoxy-D-Glc.

The reaction mixture containing 4-F-4-deoxy-D-Glc-1-P (see chapter 3.2.2.1.1.1.) was centrifuged at 15000 rpm for 20 min (5424R Centrifuge, Eppendorf AG, Hamburg, Germany) in order to remove previously used enzymes (GalKSpe4 and pyruvate kinase). The supernatant was collected and enzymatic nucleotidyl transfer to CDP-4-F-4-deoxy-D-Glc was initiated by addition of 50 mM CTP (245.7 mg), 1.3 % Albumin Fraction V (0.117.0 mg, 1 % v/v), 2 mg/mL of UGPase (0.28 mL, 64.0 mg/mL) and 0.2 mg/mL iPPase (15 µL, 119.0 mg/mL) to reaction mixture. Reaction mixture was incubated at 30 °C for 20 h.

10 µL sample was taken after 20 h and mixed in 1:100 in a volume of 60 µL with a mixture of methanol and ddH_2O, centrifuged for 20 min at 15000 rpm and analyzed on CE (see chapter 3.2.2.4.).

3.2.2.2. Chemical approach for CDP-sugar synthesis (CDP-Ara, CDP-Lyx and CDP-Xyl)

Three reaction mixtures were set to obtain each of the three sugar nucleotides - CDP-Ara, CDP-Xyl and CDP-Lyx according to the protocol, as described below:

The starting material comprised L-Ara, D-Xyl or D-Lyx. Each sugar (27 mg, 150 µmol) was dissolved in D_2O/CH_3CN (deuterium oxide/acetonitrile) (2:1, 400 µL), while in parallel DMC (76 mg, 450 µmol) was dissolved in D_2O/CH_3CN (2:1, 200 µL). Dissolved DMC was added to the

sugar solution. Then, triethylamine (251 µL, 1.80 mmol) and CDP (104.72 mg, 152 µmol) were added to the sugar solution. The resulting mixture was stirred at 4 °C for 3 days.

After 3 days, samples were taken and mixed in 1:100 in a volume of 60 µL with a mixture of methanol and ddH_2O and centrifuged for 20 min at 15000 rpm. The supernatant was analyzed using HPLC and compounds were detected at 262 nm (see chapter 3.2.2.4.).

3.2.2.3. Thin layer chromatography (TLC)

To confirm formation of phosphorylated products (L-Ara-1-P, D-Xyl-1-P, D-Lyx-1-P, 4-F-4-deoxy-D-Glc-1-P), TLC was used. Reaction samples were taken and mixed 1:1 with methanol and centrifuged (5424R Centrifuge, Eppendorf AG, Hamburg, Germany) at 15000 rpm for 20 minutes. 2 µL of supernatant was spotted on the plates and a mixture of butanol, acetic acid and H_2O (2:1:1) was used as mobile phase.

After running, the silica plate was dried and stained with staining mixture prepared from thymol (0.5 w/v), ethanol (95 w/v) and concentrated H_2SO_4. The stained silica plate was dried and heated to visualize the phosphorylated product.

3.2.2.4. Product analysis using Capillary Electrophoresis or High-Performance Liquid Chromatography

To confirm production of CDP-sugars, Capillary Electrophoresis (CE) or High-Performance Liquid Chromatography (HPLC) was used. The samples for HPLC analysis were taken from the reaction mixture and prepared by mixing 25 µL of solution with 25 µL of methanol (99 %). The samples were centrifuged at 15000 rpm for 15 min (5424R Centrifuge, Eppendorf AG, Hamburg, Germany). Kinetex® column (5 µm EVO C18 100 Å, 150 x 4.6 mm; Shimadzu Corporation, Kiyamachi-Nijo, Japan) and HPLC system (Shimadzu® HPLC-20, Shimadzu Corporation, Kiyamachi-Nijo, Japan) with UV/Vis detector (262 nm) were used. The mobile phase was 100 % TBAB buffer, with flow rate 2 mL/min, for 8 min at 42 °C. Products were identified by retention times shown on HPLC chromatograph.

The samples for CE were prepared on the same way as samples for the HPLC. G1600AX 3D Capillary Electophoresis (Agilent Technologies, Santa Clara, USA) was used with Borate buffer as mobile phase, at 50 °C and 11 min separation time. Products were identified by retention times shown on CE chromatograph.

3.2.2.5. Purification of CDP-sugars

Obtained CDP-sugars (CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc) were purified in two chromatographic steps each. CDP-Ara was not further purified. The first chromatographic step was anion exchange chromatography using a 125 ml TOYOPEARL SuperQ-650M anion exchange column GE (Healthcare Life Sciences, Chicago, USA), connected to an ÄKTA FPLC system (GE Healthcare Life Sciences,Chicago, USA). The sugar reaction mixture was dissolved in a total volume of 5 mL Acetate buffer A for anion exchange chromatography, pH 4.3, and loaded onto the column. Elution of compounds bound to column was achieved with a stepwise gradient of the Acetate buffer B for anion exchange chromatography, pH 4.3.

Fractions obtained by anion exchange were analyzed on HPLC (see chapter 3.2.2.4.) and fractions with confirmed pure CDP-sugar on HPLC were combined and concentrated on a rotary evaporator (Laborota 4000 rotary evaporator, Heidolph Instruments, Schwabach, Germany). The concentration of fractions with pure CDP-sugars was performed at 42 °C and reduced pressure (20-30 mbar) until final volume of 10-20 mL was reached. The second chromatographic step needed was size exclusion to separate CDP-sugars from acetic acid. Superdex G-10 size-exclusion column (GE Healthcare Life Sciences, Chicago, USA) connected to the ÄKTA FPLC (GE Healthcare Life Sciences,Chicago, USA) system was used with ddH_2O as eluent. Fractions obtained by size exclusion were analyzed on HPLC (see chapter 3.2.2.4.) and fractions with confirmed pure CDP-sugar were combined and concentrated on a rotary evaporator (Laborota 4000 rotary evaporator, Heidolph Instruments, Schwabach, Germany). Product purity was checked on HPLC (see chapter 3.2.2.4.) and/or ^{1}H-NMR (see chapter 3.2.2.6.).

3.2.2.6. Identification of products (CDP-Lyxose, CDP-Xylose and Xyl-1-P) using ^{1}H-NMR

To confirm the identity of the products (CDP-Lyx, CDP-Xyl and Xyl-1-P), further assays were performed using ^{1}H-NMR (Varian INOVA 500-MHz NMR, Agilent Technologies Santa Clara, USA). Products were previously freeze dried, dissolved in 700 µL D_2O and taken at the Institute for Organic Chemistry at TU Graz to analyze.

3.3. MUTATIONAL ANALYSIS OF *Ta*CPa2E

After obtaining the desired CDP-sugars (CDP-Lyx and CDP-4-F-4-deoxy-D-Glc) (chapter 3.2.2.) and *Ta*CPa2E mutants (G206A and D204E) (chapter 3.2.1.), a series of reactions were performed to test the enzyme activity and compare the activity of the mutants and the wild-type *Ta*CPa2E enzyme against different CDP-sugars. As substrates, prepared CDP-Lyx and CDP-4-F-4-deoxy-D-Glc were used. CDP-Man and CDP-Glc were already available at the Institute, and also used as substrates. Different concentrations of enzymes were used (0.2 or 1.0 mg/mL of *Ta*CPa2E wild-type and 0.2 mg/mL, 1.0 mg/mL or 5.0 mg/mL of *Ta*CP2aE mutants). Substrate concentrations in reaction mixture (3 mM or 4 mM) were used, depending on the reaction rate and the concentration of the obtained substrates. Reactions were performed in 100 mM MOPS buffer at pH 7.5 and incubated at 60 °C for 1 h. 3 µL of samples were taken after 0, 2, 5, 10, 30 and 60 min and dissolved in 27 µL of ddH_20 and 30 µL of methanol. Samples were centrifuged for 20 min at 15000 rpm, supernatant was taken and formation of product was analyzed on CE (see chapter 3.2.2.4.).

4. RESULTS AND DISCUSSION

4.1. ENZYME PRODUCTION

4.1.1. Production of wild-type GalKSpe4 and UGPase, and wild-type and mutants of *Ta*CPa2E

4.1.1.1. Production of GalKSpe4 and UGPase required for substrates synthesis (CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc)

The aim of this master thesis was to investigate the active site of *Ta*CPa2E by using substrate analogues and mutational analysis.

For that purpose, one kinase (GalKSpe4) was produced for phosphorylation of L-Ara, D-Lyx, D-Xyl and 4-F-4-deoxy-D-Glc to L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P and 4-F-4-deoxy-D-Glc-1-P, and one pyrophosphorylase (UGPase) to perform CMP coupling of L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P and 4-F-4-deoxy-D-Glc-1-P to CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc.

GalKSpe4 and UGPase were expressed and purified, as described in chapters 3.2.1. Purity of produced proteins is shown in Figure 9. The desired enzymes were obtained with molecular masses of ~58 kDa (UGPase) and ~45 kDa (GalKspe4) which corresponds to literature data for UGPase (Li et al., 2016) and GalKspe4 (Zou et al., 2011).

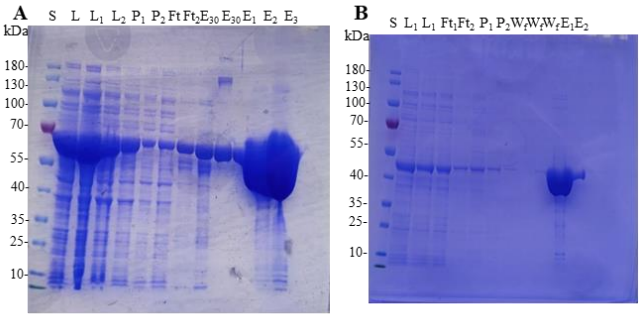


Figure 9. SDS-PAGE gels of GalKSpe4 and UGPase. The molecular mass ladder (S), the lysate (L_n), the insoluble fraction (P_n), washing fractions (W_n), flow through fractions (Ft_n), elution fractions at 30 % elution buffer (E_{30}). Pure protein samples are labelled as E_1-E_4. A) UGPase, B) GalKSpe4.

4.1.1.2. Production of wild-type TaCPa2E

The wild-type *Ta*CPa2E was expressed in *E. coli* BL21 (DE3) LEMO21 (chapter 3.2.1.2.3) and purified by using His-tag purification (chapter 3.2.1.5.). SDS-PAGE gel of the purified protein is shown on Fig. 10.

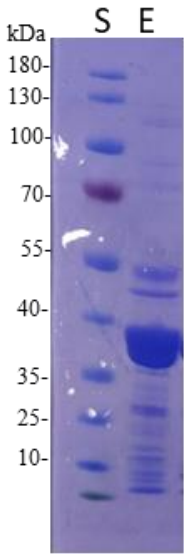


Figure 10. SDS-PAGE gels of wild-type *Ta*CPa2E. The molecular mass ladder is labelled as S, and pure *Ta*CPa2E protein is labelled as E.

Expression and purification of the wild-type *Ta*CPa2E was successful (Figure 10) and the desired enzyme was obtained with molecular mass of ~39 which corresponds to literature data (Rapp et al., 2021).

4.1.1.3. Production of mutants of Ta*CPa2E (G206A and D204E)*

Two *Ta*CPa2E mutants (G206A and D204E) were constructed to test the influence of site-directed substitution of amino acids in active site on the enzymatic activity. As previously explained (see chapter .2.2.1.1.1.), G206A was prepared to investigate interactions at substrate position C6 with *Ta*CPa2E active site, and D204E to investigate interactions at substrate position C4 with *Ta*CPa2E active site. Site-directed mutagenesis (see chapter 3.2.1.2.1.) was successful and confirmed by sequencing for both mutants expressed in *E. coli* BL21 (DE3) LEMO21. The *E.*

coli BL21 (DE3) LEMO21 cells for mutated proteins (G206A and D204E) production were grown and mutated proteins were purified under the same conditions as the wild-type *Ta*CPa2E (see chapter 3.2.1.). The mutated enzymes were purified using His-tag affinity chromatography (see chapter 3.2.1.5.), and corresponding chromatograms are shown in Figures 11 and 12.

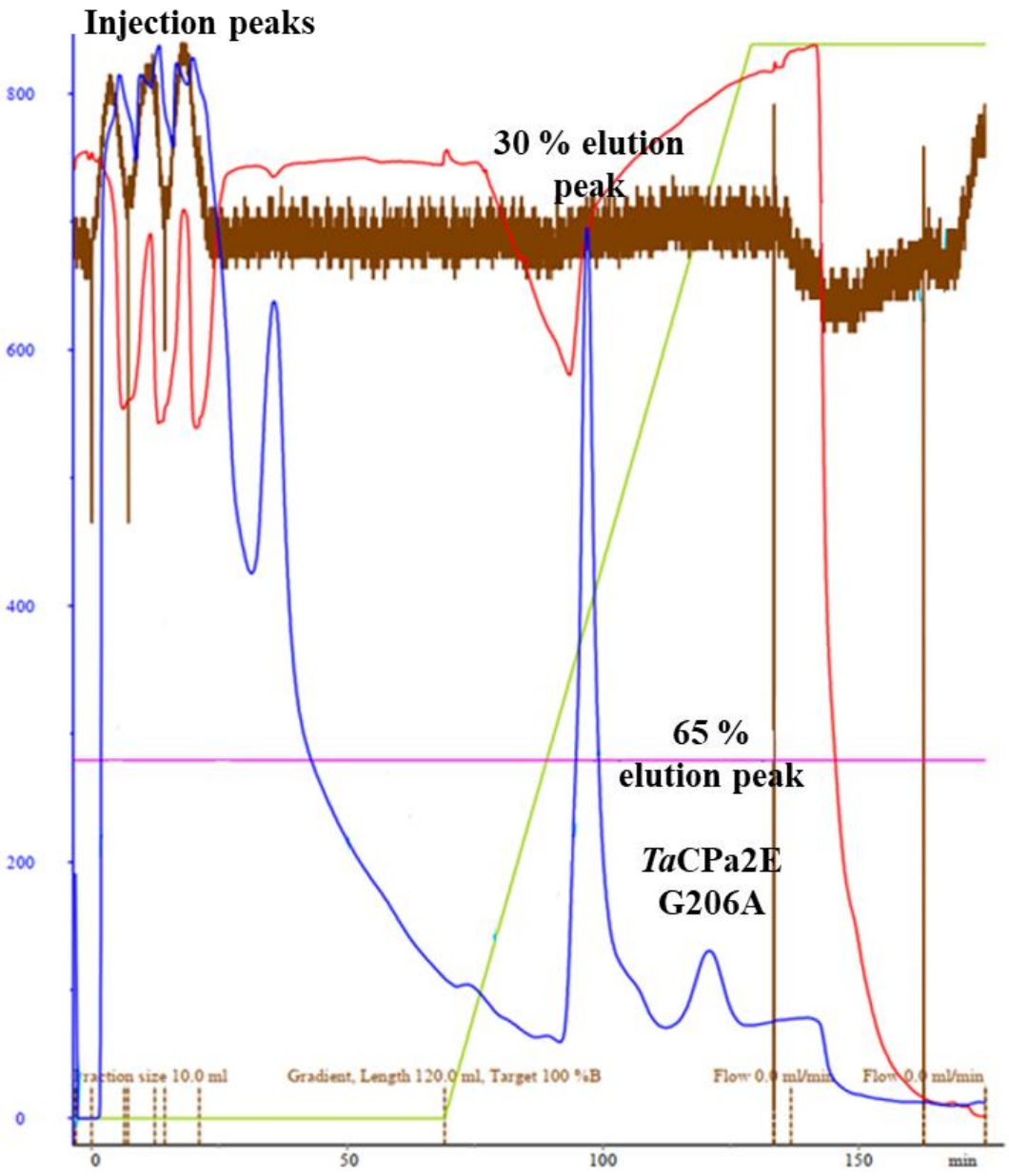


Figure 11. Chromatogram of His-tag purification of *Ta*CPa2E G206A mutant. The blue line shows UV-absorbance, the red line shows conductivity, the brown line pressure. Green line shows % of His-trap elution buffer (B). Injection peaks are labelled. G206A is eluted at 65 % of His-trap elution buffer (B), while untargeted *E. coli* proteins are eluted at 30 % of His-trap elution buffer (B).

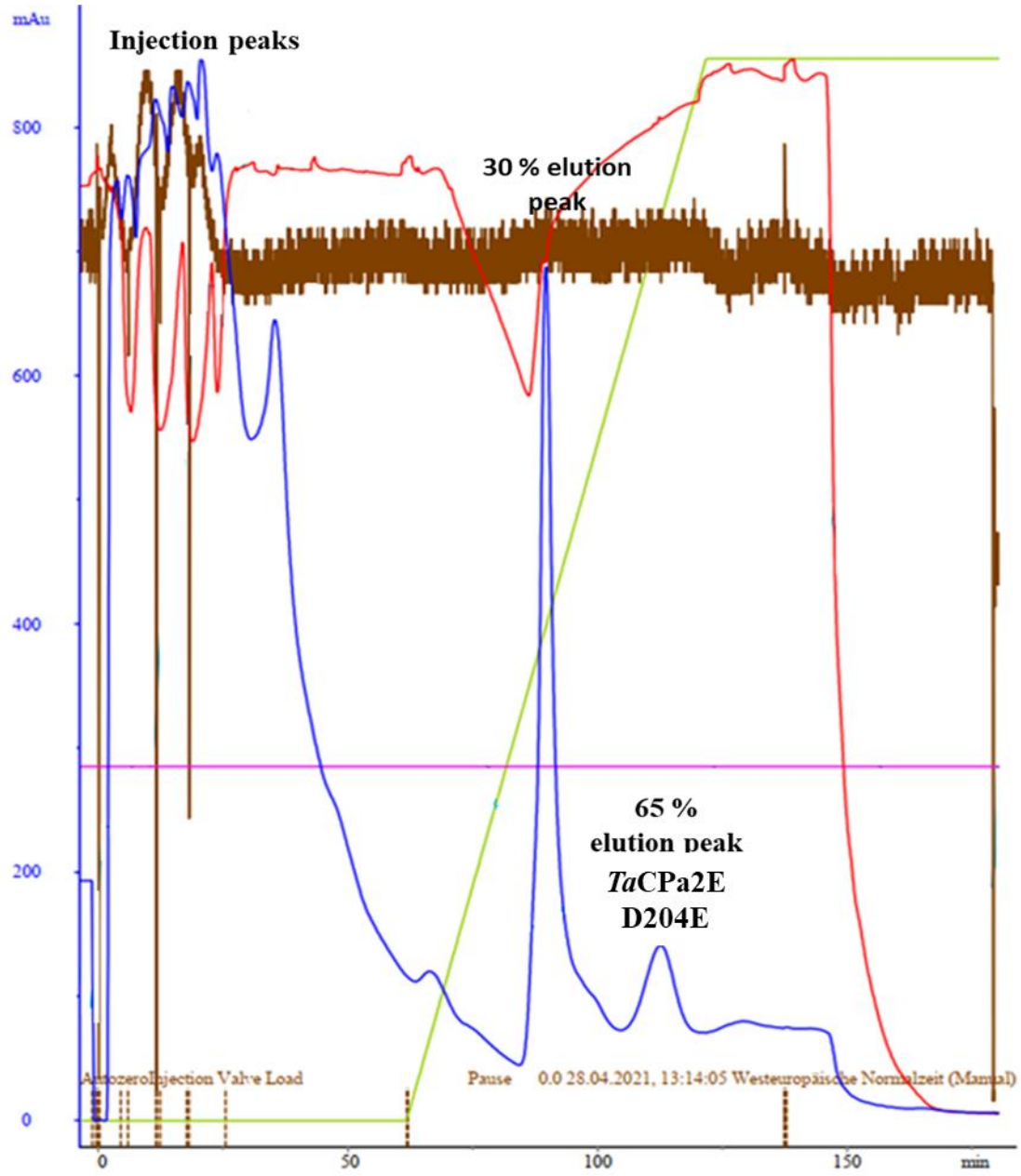


Figure 12. Chromatogram of His-tag purification of *Ta*CPa2E D204E. The blue line shows UV-absorbance, the red line shows conductivity, the brown line pressure. Injection peaks are labelled. Green line shows % of buffer His-trap elution buffer (B). D204E is eluted at 65 % of His-trap elution buffer (B) while untargeted *E. coli* proteins are eluted at 30 % of His-trap elution buffer (B).

After concentrating eluted fractions that formed protein peak (see chapter 3.2.1.6.), mutated enzyme sizes and purity was compared to wild-type enzyme on SDS-PAGE gel (Figure 13).

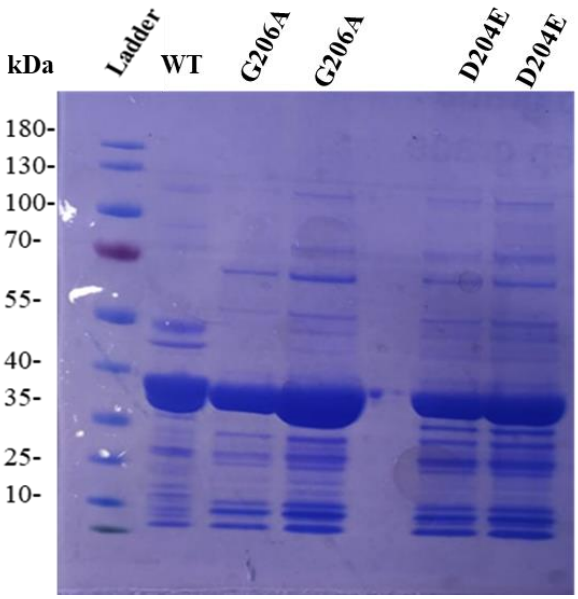


Figure 13. SDS-PAGE of *Ta*CPa2E mutants compared to wild-type (WT) *Ta*CPa2E. Wild-type *Ta*CPa2E (WT) and mutants are labelled G206A and D204E. Molecular mass ladder (Ladder) is used as standard for molecular mass determination.

According to the results showed in Figure 13. purification was successful and both mutant enzymes were obtained with good purity and molecular weight of ~39 kDa, comparable to the molecular weight of the wild-type enzyme.

4.2. SYNTHESIS OF SUBSTRATES FOR WILD-TYPE *Ta*CPa2E AND MUTANTS OF *Ta*CPa2E

4.2.1. CDP-sugars (CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc) synthesis

The initial idea was to synthesize four substrates (CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc) for activity testing of *Ta*CPa2E. Two main approaches were tried – anomeric and chemical. Synthesis of CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc was initially tried using anomeric approach (see chapter 3.2.2.1.). However, full anomeric synthesis was only successful for production of CDP-4-F-4-deoxy-D-Glc, so production of CDP-Ara, CDP-Lyx, CDP-Xyl was additionally tried by using chemical approach (see chapter 3.2.2.2.).

4.2.1.1. Anomeric approach

The first step of anomeric approach was anomeric enzymatical phosphorylation of L-Ara, D-Lyx, D-Xyl and CDP-4-F-4-deoxy-D-Glc using kinase GalKSpe4 or a chemical phosphorylation (only for D-Xyl), whereas the second step involved CMP coupling of obtained phosphorylated sugars (L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P or 4-F-4-deoxy-D-Glc-1-P) *via* pyrophosphorylase UGPase (Figure 14).

The general idea behind the anomeric approach was to synthesize α-anomers of the CDP-Ara, CDP-Lyx, CDP-Xyl and CDP-4-F-deoxy-D-Glc. As mentioned above, kinases generally show specificity for α-anomers. Anomeric phosphorylation of L-Ara resulted in low phosphorylation rate to L-Ara-1-P. Anomeric phosphorylation of 4-F-4-deoxy-D-Glc-1-P was obtained using GalKSpe4, while D-Xyl-1-P was obtained using chemical phosphorylation of D-Xyl. D-Lyx-1-P was not obtained by anomeric phosphorylation of D-Lyx. CMP coupling was only successful for 4-F-4-deoxy-D-Glc.

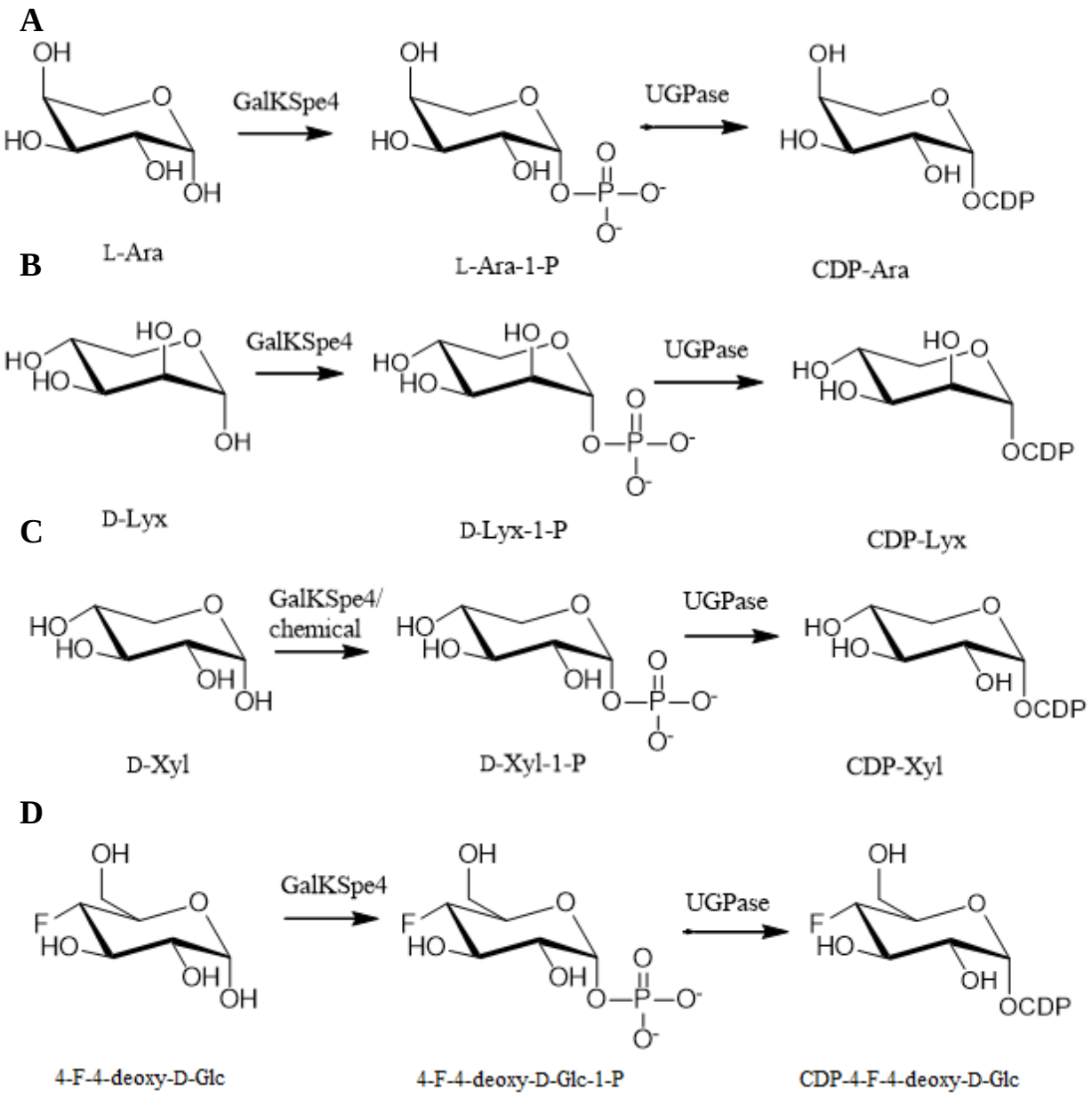


Figure 14. Proposed anomeric approach for substrate synthesis with two steps. The first step is anomeric phosphorylation of L-Ara, D-Lyx, D-Xyl and CDP-4-F-4-deoxy-D-Glc using GalKSpe4 (and chemical phosphorylation for D-Xyl). The second step is CMP coupling of L-Ara-1-P, D-Lyx-1-P, D-Xyl-1-P or 4-F-4-deoxy-D-Glc-1-P. Synthesis: A) CDP-Ara, B) CDP-Lyx, C) CDP-Xyl, D) CDP-4-F-4-deoxy-D-Glc.

4.2.1.1.1. Synthesis of L-Ara-1-P as a substrate for synthesis of CDP-Ara by using UGPase

Anomeric phosphorylation of L-Ara to L-Ara-1-P was tried with GalKSpe4. Although product formation was visible using TLC (Figure 15), phosphorylation rate was low. Due to low phosphorylation rate, L-Ara-1-P was not used for CMP coupling using UGPase.

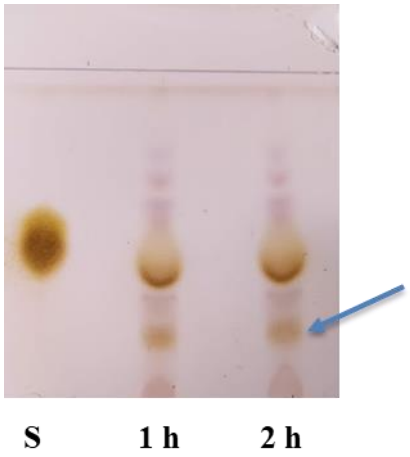


Figure 15. Thin layer chromatography (TLC) plate of phosphorylation of L-Ara to L-Ara-1-P using GalKSpe4. L-Ara standard is labelled (S). Reaction progress was checked after 1 h and 2 h. Reaction progress after 0 h is not shown. The product (L-Ara 1-P) is indicated with an arrow.

4.2.1.1.2. Synthesis of D-Lyx-1-P as a substrate for synthesis of CDP-Lyx by using UGPase

Anomeric phosphorylation of D-Lyx was tried using enzymatical phosphorylation using GalKSpe4. According to the TLC (Figure 16) anomeric enzymatical phosphorylation was not successful. No phosphorylation of D-Lyx to D-Lyx-1-P was obtained.

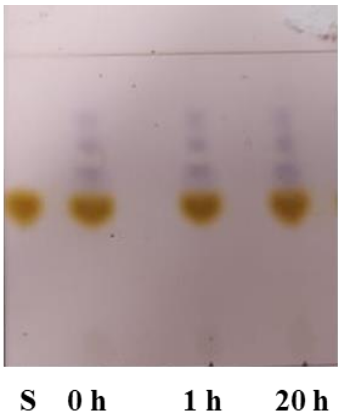


Figure 16. TLC plate of anomeric enzymatical phosphorylation of D-Lyx to D-Lyx-1-P using GalKSpe4. Reaction progress was checked after 0 h, 1 h and 20 h. D-Lyx standard (S) is labelled.

4.2.1.1.3. Synthesis of D-Xyl-P as a substrate for synthesis of CDP-Xyl by using UGPase

Anomeric phosphorylation of D-Xyl was tried using chemical and enzymatical phosphorylation. According to the TLC (Figure 17) anomeric enzymatical phosphorylation was not successful because low phosphorylation rate of D-Xyl to D-Xyl-1-P was obtained.

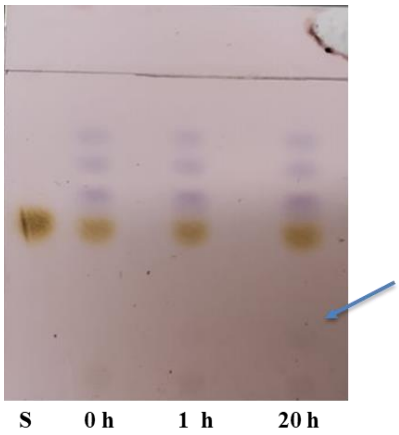


Figure 17. TLC plate of anomeric enzymatical phosphorylation of D-Xyl to D-Xyl-1-P. Reaction progress was checked after 0 h, 1 h and 20 h. D-Xyl standard (S) is labelled. D-Xyl-1-P is indicated with an arrow.

On the other hand, chemical anomeric phosphorylation of D-Xyl to D-Xyl-1-P was successful. Reaction progress was checked using TLC (Figure 18) ending up in a conversion of roughly 90 %. Using ^{1}H-NMR (see chapter 3.2.2.6), a 6.6:1 mixture of α:β anomers could be confirmed (Figure 19), which approximately corresponds to the results reported in the literature (5:1 mixture of α:β anomers) (Edgar et al., 2012).

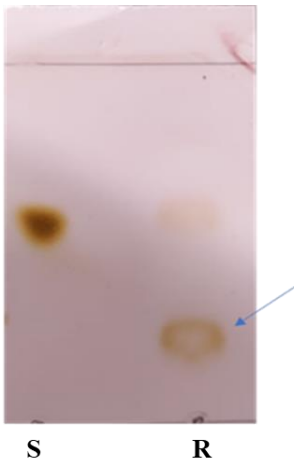


Figure 18. TLC plate of anomeric chemical phosphorylation of D-Xyl to D-Xyl-1-P. D-Xyl standard is labelled (S) and reaction mixture (R). D-Xyl-1-P is highlighted with an arrow.

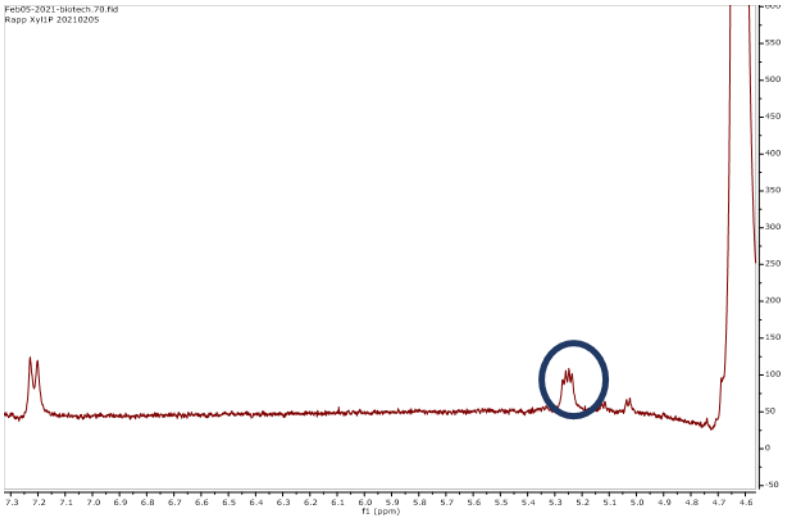


Figure 19. **^{1}H-NMR**: Xyl-1-P. The α -anomeric signal is circled.

However, obtained D-Xyl-1-P was not used for CMP coupling due to the fact that lots of chemicals were used in production of this product and no additional purifications were used at the moment.

4.2.1.1.4. Synthesis of 4-F-4-deoxy-D-Glc-1-P as a substrate for synthesis CDP-4-F-4-deoxy-D-Glc by using UGPase

Phosphorylation of 4-F-4-deoxy-D-Glc to 4-F-4-deoxy-D-Glc-1-P was obtained using conditions as explained in chapter 3.2.2.1.1. After 20 h of reaction time, approximately 50 % phosphorylation yield was achieved (Figure 20). The obtained results are in accordance with the results reported in the literature using a similar compound (Iacovino et al., 2020).

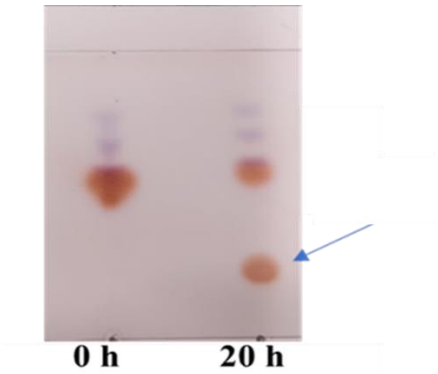


Figure 20. TLC plate of phosphorylation of 4-F-4-deoxy-D-Glc to 4-F-4-deoxy-D-Glc-1-P with GalKSpe4. Reaction progress was checked after 0 h and 20 h. The phosphorylated product is marked with an arrow.

4.2.1.5. CMP coupling of 4-F-4-deoxy-D-Glc-1-P to CDP-4-F-4-deoxy-D-Glc by using UGPase

As previously explained, L-Ara-1-P, D-Lyx-1-P and D-Xyl-1-P were not used for CMP coupling (chapters 4.2.1.1.1, 4.2.1.1.2. and 4.2.1.1.3.).

The second step of anomeric enzymatical approach was enzymatic-nucleotidyl transfer as described in chapter 3.2.2.1.1.2. The progress was analyzed on CE (Figure 21) and product peak

was observed. An important difference introduced in this synthesis compared to the protocol described in the literature (Iacovino et al., 2020) is the usage of a recycling system which allows ATP recycling without additional need for ATP, ADP and AMP purification. Obtained product was used for further purification.

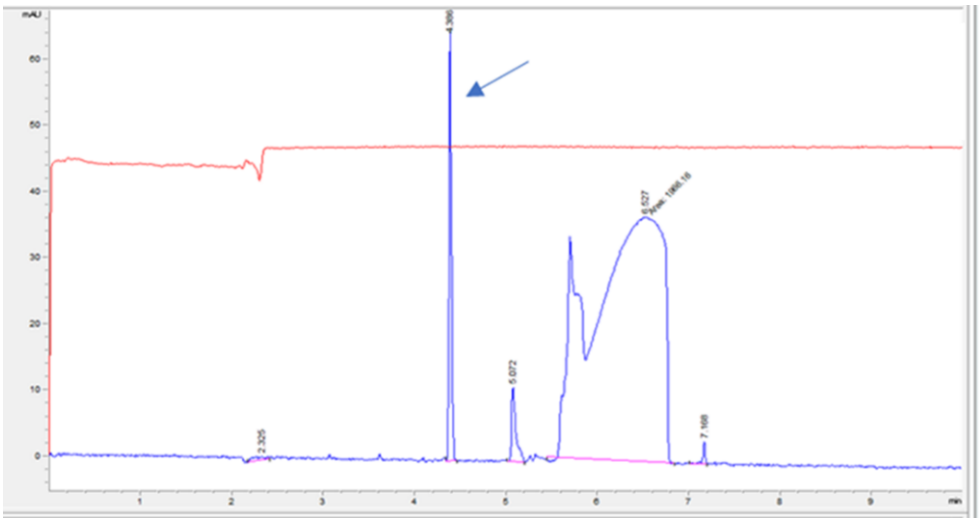


Figure 21. CE chromatogram of nucleotidyl transfer of 4-F-4-deoxy-D-Glc-1-P to CDP-4-F-4-deoxy-D-Glc after 20 h. CDP-4-F-4-deoxy-D-Glc is indicated by an arrow.

4.2.1.2. Chemical approach - Synthesis of CDP-Ara, CDP-Lyx and CDP-Xyl

Due to successful anomeric approach in the synthesis of CDP-4-F-4-deoxy-D-Glc, there was no attempt to produce CDP-4-F-4-deoxy-D-Glc by using chemical approach.

An alternative approach for the synthesis of CDP-Ara, CDP-Lyx, and CDP-Xyl and was tried as described in chapter 3.2.2.1.3. One step synthesis protocol for the synthesis of sugar nucleotides was tested to obtain these 3 sugar nucleotides (CDP-Ara, CDP-Lyx, and CDP-Xyl). (Miyagawa et al., 2020). Reaction conditions were the same for each sugar. In addition, each reaction mixture went through the same purification treatment (see chapter 3.2.2.5.) and reaction mixtures were analyzed on HPLC (Figure 22) (see chapter 3.2.2.4.). CDP-Lyx and CDP-Xyl obtained by chemical approach were used for further purification, while CDP-Ara was not due to low HPLC peak obtained.

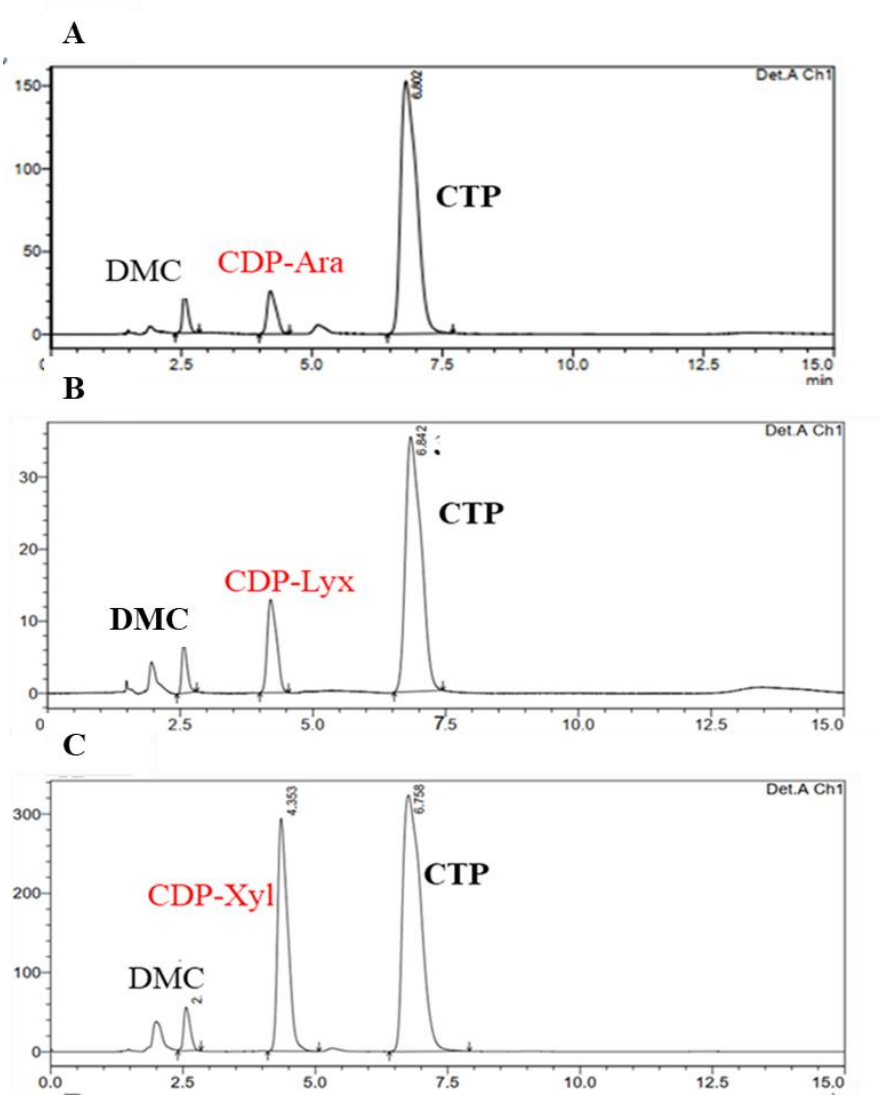


Figure 22. HPLC chromatogram for chemical approach: A) production of CDP-Ara; B) production of CDP-Lyx; C) production of CDP-Xyl. The peaks coming from CDP-sugars (CDP-Ara, CDP-Lyx and CDP-Xyl), DMC and CTP are shown.

4.2.2. Purification of CDP-sugars (CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc)

After the conversion of corresponding sugars (D-Lyx, D-Xyl and 4-F-4-deoxy-D-Glc) to the desired CDP-sugars (CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc) as final products to be used as substrates for *Ta*CPa2E, their identity was confirmed using HPLC (see chapter 3.2.2.4.) or CE (see chapter 3.2.2.4.). CDP-Ara was not used in purification, because HPLC peak which indicates CDP-Ara was low (see above). Their purification was done, as follows. For all CDP-sugars the same purification procedure was used (see chapter 3.2.2.5.). Two chromatographic steps were employed. The first chromatographic step was anion exchange chromatography (see chapter 3.2.2.5.) used to separate CDP-sugars from other reaction mixture components. The fractions with the expected product (Figure 23) were analyzed, combined, and concentrated using rotary evaporator.

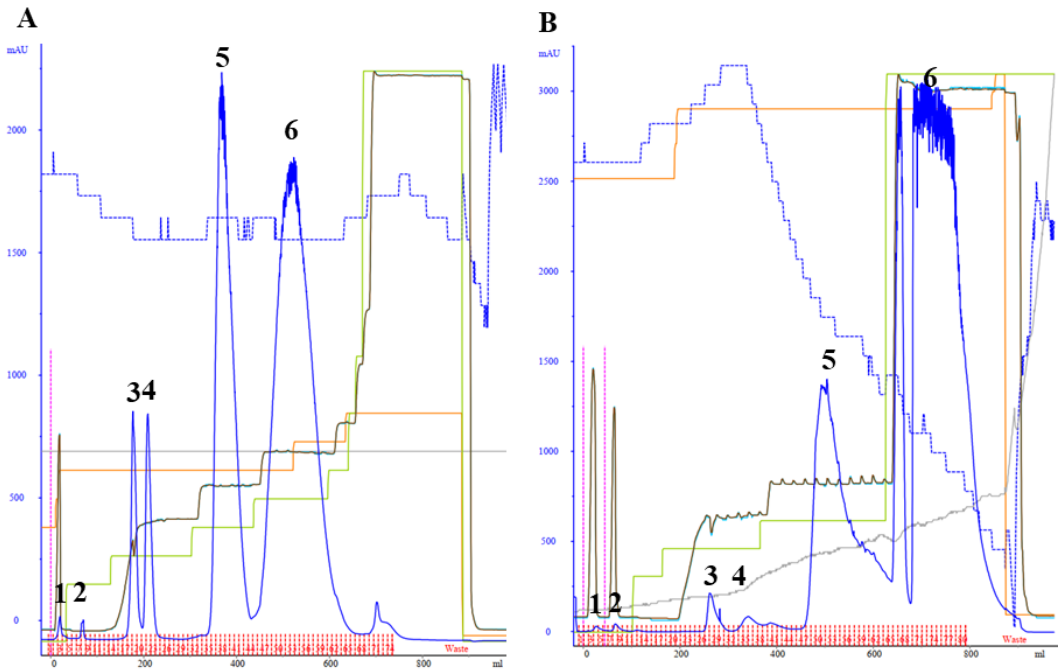


Figure 23. Chromatogram showing anion exchange chromatography performed on A) CDP-Xyl, B) CDP-4-F-4-deoxy-D-Glc, shown as example. The blue line is the UV-absorbance at 280 nm, the brown line is conductivity and green one is concentration of elution buffer. The pink lines indicate injection points. A) Fraction with peaks 1, 2, 3, 4, 6 were: CMP, CDP, DMC and CTP mix. Fractions with peak 5 contained pure product (CDP-Xyl) were taken and combined. B) Fractions with peaks 1,2,3,6 were CMP, CDP, ATP and CTP mix. Fractions within peaks 3 and 5 containing pure product (CDP-4-F-deoxy-D-Glc) were taken and combined.

The second step was size exclusion chromatography (see chapter 3.2.2.5.) to desalt the purified product. The mobile phase used was ddH_2O (Figure 24).

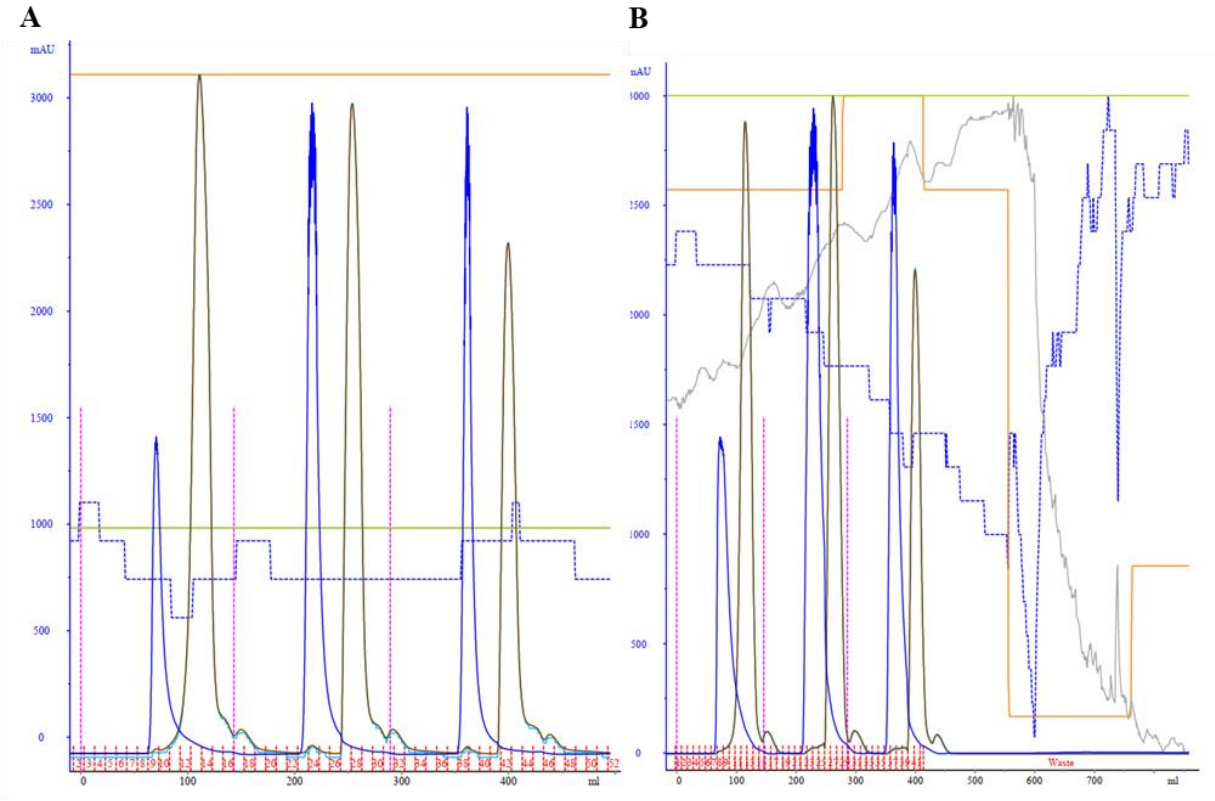


Figure 24. ÄKTA chromatogram showing size exclusion chromatography on A) CDP-Xyl, B) CDP-4-F-4-deoxy-D-Glc, shown as example. The blue line is UV-absorbance at 280 nm, the brown line is conductivity, and the pink lines indicate injection points. Blue peaks indicate products while brown peaks indicate acetate.

Fractions containing product were combined and concentrated using rotary evaporator and the purity of CDP-Lyx, CDP-Xyl and CDP-4-F-4-deoxy-D-Glc was analyzed on HPLC or CE. The rest was freeze dried and the mass was calculated. Sugar nucleotides obtained by chemical synthesis were further dissolved in D_2O and analyzed using ^{1}H-NMR (see chapter 3.2.2.6.).

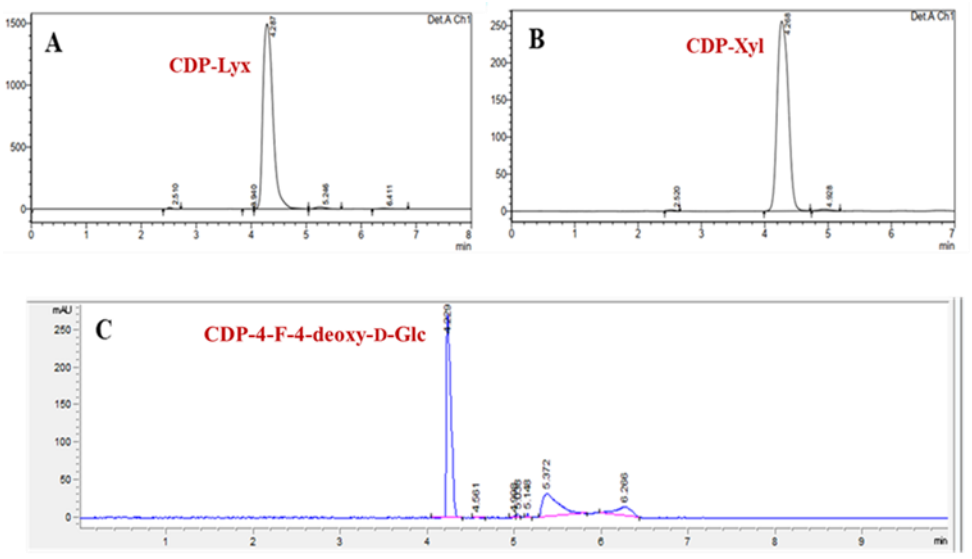


Figure 25. HPLC and CE chromatograms after CDP-sugars purification. A) HPLC chromatogram of CDP-Lyx. B) HPLC chromatogram of CDP-Xyl. C) CE chromatogram of CDP-4-F-4-deoxy-D-Glc.

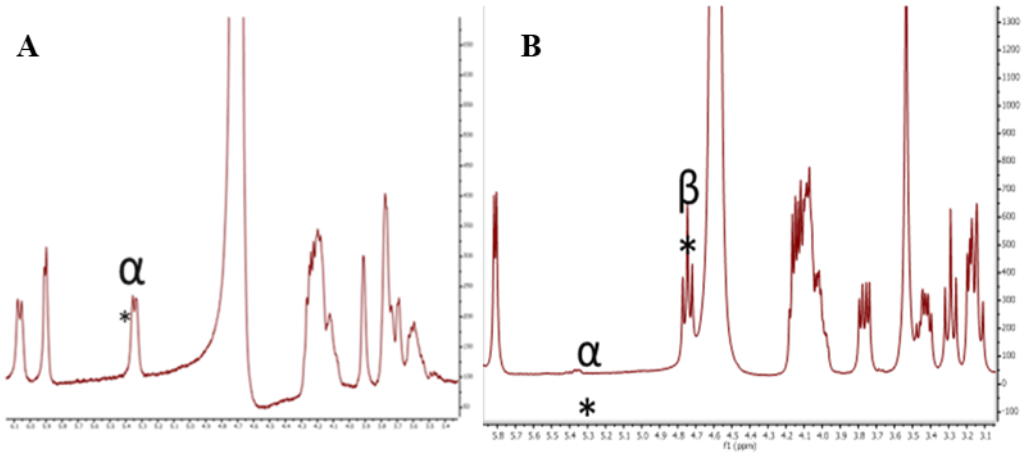


Figure 26. ^{1}H-NMR analysis. A) CDP-Lyx is obtained with only α anomeric signal. B) CDP Xyl is obtained with α:β ratio 1:10.

After purification and analysis, it was possible to draw conclusions about sugar nucleotide production. CDP-Xyl was obtained with good purity and mass (98.3 %, 6.11 mg) (Figure 25 B)

but with α:β ratio 1:10 (Figure 26 B). Although less amount of CDP-Lyx was produced (3.6 mg), it was most successful production. Purity was good (98.6 %, Figure 25 A) and only α anomeric signal was recorded (Figure 26 A). CDP-4-F-4-deoxy-D-Glc was produced by anomeric enzymatical approach so ^{1}H-NMR analysis was not performed. However, it requires further purification because according to the CE (Figure 25 C) purity is only 61.60 %.

4.3. DETERMINATION OF SPECIFIC ACTIVITY OF WILD-TYPE AND MUTANS OF *Ta*CPa2E

When wild-type and mutants of *Ta*CPa2E were expressed and purified, next step was activity testing. CDP-Lyx and CDP-4-F-4-deoxy-D-Glc were successfully synthesized and used in determination of activity of wild-type and mutants of *Ta*CPa2E.

Also, two additional substrates - CDP-Glc and CDP-Man were already available at the Institute and therefore used in activity experiments.

Adequate concentration of wild-type *Ta*CPa2E or mutants (G206A and D204E) was used. Reactions were carried out for 60 or 120 min at 60 °C. Samples were taken in different points of time, dissolved in methanol-ddH_20 mixture and analyzed on CE.

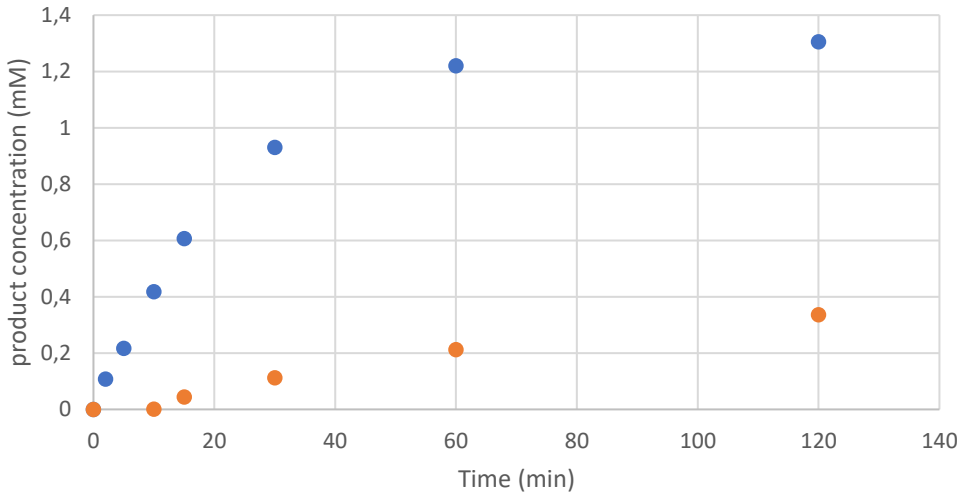


Figure 27. Time course conversion of CDP-Lyx to CDP-Xyl (blue), and conversion of CDP-Man to CDP-Glc (orange) using wild-type *Ta*CPa2E. The substrate concentration was 4 mM, and the enzyme concentration was 0.2 mg/mL.

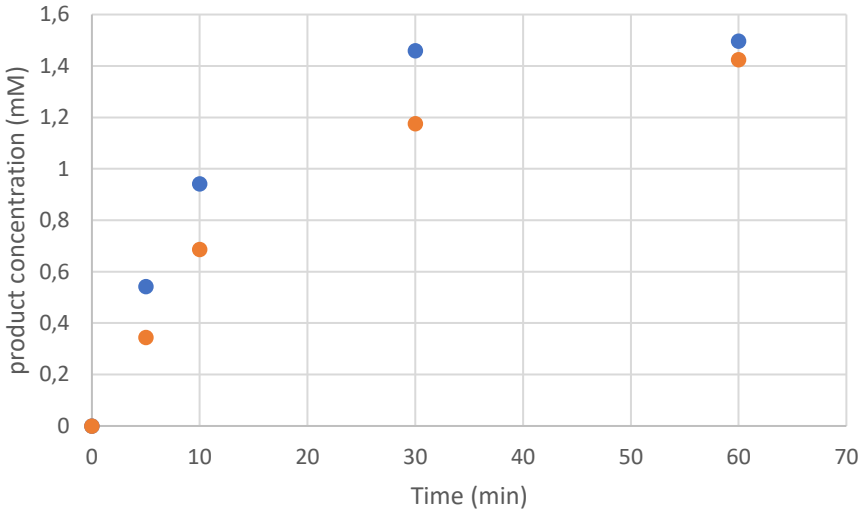


Figure 28. Time course conversion of CDP-Glc to CDP-Man using G206A (blue) and D204E (orange). The substrate concentration was 4 mM, and the enzyme concentration was 5.0 mg/mL.

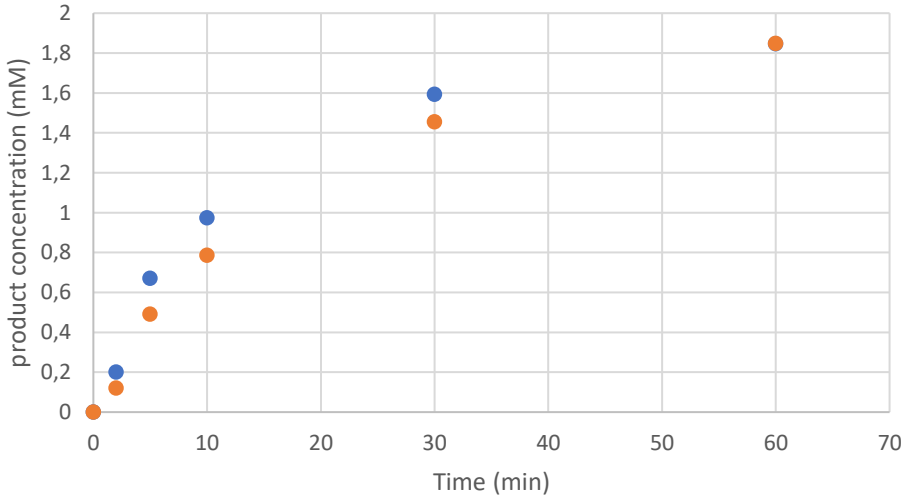


Figure 29. Time course conversion of CDP-Lyx to CDP-xyl using G206A (blue) and D204E (orange). The substrate concentration was 4 mM, and the enzyme concentration was 0.2 mg/mL.

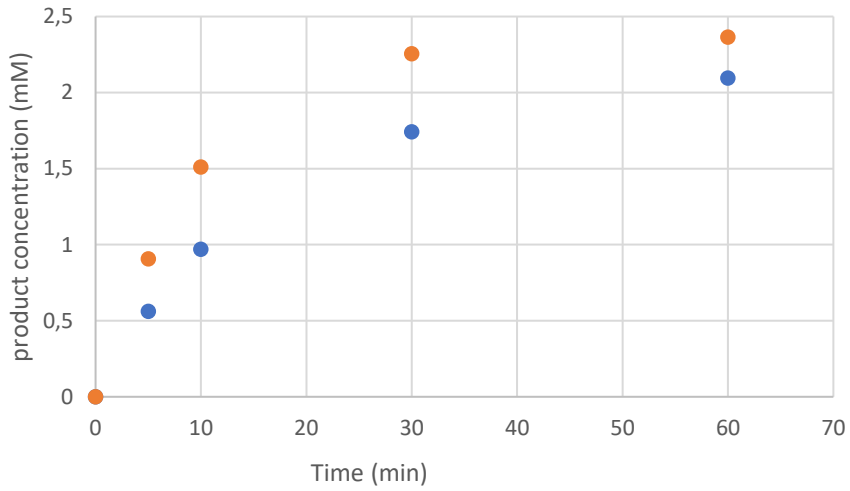


Figure 30. Time course conversion of CDP-Man to CDP-Glc using G206A (orange) and D204E (blue). The substrate concentration was 4 mM and the enzyme concentration was 5.0 mg/mL.

When data was collected, specific activity was calculated from Figures 27-30. All calculated specific activities are showed in Table 19.

Table 19. The specific activities of wild-type *Ta*CPa2E and mutants

Enzyme	Substrate	Specific activity U/mg
D204E	CDP-Man	0.02
D204E	CDP-Lyx	0.41
D204E	CDP-Glc	0.014
G206A	CDP-Man	0.02
G206A	CDP-Glc	0.018
G206A	CDP-Lyx	0.52
wild-type *Ta*CPa2E	CDP-Lyx	0.27
wild-type *Ta*CPa2E	CDP-Man	0.02

When specific activity of wild-type *Ta*CPa2E for CDP-Man was compared to the activity for CDP-Lyx, the reaction rate was increased 14x with an equilibrium: 64 % CDP-Xyl (*gluco-*

conf.) : 36 % CDP-Lyx (*manno*-conf.). This confirmed the proposed hypothesis that C6 removal would lead to enhanced reaction rate.

Mutants activity was confirmed using CDP-Glc. G206A showed 2x times higher specific activity for CDP-Glc then D204E. Both mutants showed same activity for CDP-Man in comparison to the wild-type.

Mutants activity for CDP-Lyx was higher compared to wild-type. D204E showed almost 1.5x, and G206A 2x higher specific activity for CDP-Lyx when compared to the wild-type. Further research is needed to explain this, but the potential reason for this is better adaptability of the CDP-Lyx to the active site of the mutants. Also, it is interesting to compare the activity of the mutants against CDP-Lyx and CDP-Man. G206A showed 27.5x increase in reaction rate when CDP-Lyx was used instead of CDP-Man. D204E showed 20.63x increase in reaction rate when CDP-Lyx was used instead of CDP-Man. This corresponds with previously confirmed hypothesis that C6 removal would lead to enhanced reaction rate in wild-type *Ta*CPa2E.

Reactions with 1.0 mg/mL wild-type *Ta*CPa2E and 3 mM CDP-4-F-4-deoxy-D-Glc showed no results (Figure 31 A). However, when 5.0 mg/mL mutants were used with same CDP-4-F-4-deoxy-D-Glc concentration, low activity was found after 60 min (Figure 31 A and 31 B). When G206A was used, conversion to the fluorinated *manno*-config. sugar was 3.77 % and when D206E was used, conversion was 5.63 %. The activity has been shown to exist but is low, probably because of low purity. Further purification of obtained CDP-4-F-4-deoxy-D-Glc and activity testing with pure substrate is needed.

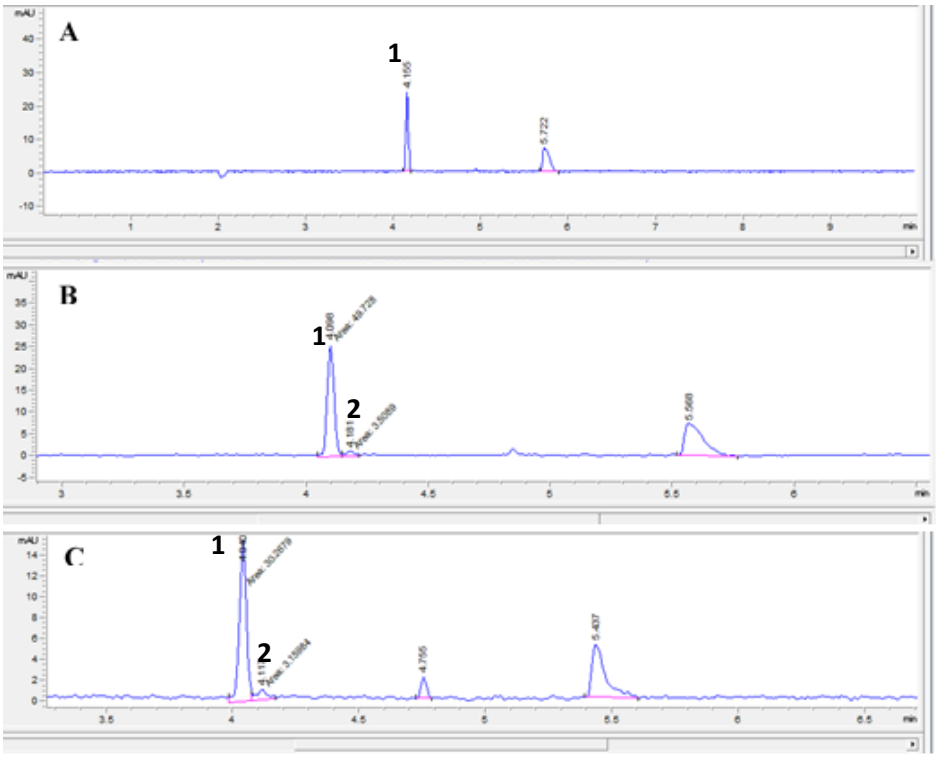


Figure 31. CE chromatograph for reaction mixture with *Ta*CPa2E and 3 mM CDP-4-F-4-deoxy-D-Glc. Number 1 indicates CDP-4-F-4-deoxy-D-Glc while number 2 indicates CDP-4-F-4-deoxy-D-Man; A) 1.0 mg/mL wild-type *Ta*CPa2E B) 5.0 mg/mL G206A. C) 5.0 mg/mL D204E.

5. CONCLUSIONS

The following conclusions were obtained from this research:

1. Mechanistic probes for investigating activity of CDP-Tyvelose 2-Epimerase-like enzyme from *Thermodesulfatator atlanticus* (*Ta*CPa2E) were synthesized. The synthesis was performed by employing two approaches - chemical and enzymatic. CDP-Lyxose, the mechanistic probe for characterization of C6 interaction with *Ta*CPa2E, was obtained by using chemical approach while CDP-4-Fluoro-4-deoxy-D-Glucose, the mechanistic probe for studying C4 interaction with the enzyme, was yielded by using enzymatic anomeric approach.
2. Efficient enzymatic synthesis of CDP-4-Fluoro-4-deoxy-D-Glucose was performed after two required enzymes - galactokinase from *Streptococcus pneumoniae* (GalKSpe4) and UDP-glucose pyrophosporylase from *Bifidobacterium longum* (UGPase), were produced in *E. coli* (DE3) LEMO21 and *E. coli* BL21 (DE3), respectively, and purified, and therefrom used in the manufacturing of the substrate (CDP-4-Fluoro-4-deoxy-D-Glucose).
3. Unfortunately, the synthesis of additional two interesting substrates for *Ta*CPa2E, CDP-Arabinose and CDP-Xylose, was not achieved when both approaches, chemical and enzymatic, were employed.
4. Besides wild-type *Ta*CPa2E, two active *Ta*CPa2E mutants - G206A and D204E were created and also used in characterization of C4/C6 interaction of corresponding substrates with the enzyme.
5. As probed with Capillary Electrophoresis, the highest specific activity towards CDP-Lyxose was determined for wild-type *Ta*CPa2E (0.27 U/ml) as well as for the two mutants - G206A (0.50 U/ml) and D204E (0.41 U/ml), while CDP-Mannose seems to be better accepted by the all three variants of the enzyme (0.02 U/ml for all three forms of the enzyme) than CDP-Glucose. Further research is needed to shed more light on CDP-Glucose - and CDP-4-Fluoro-4-deoxy-D-Glucose - enzyme interactions.
6. It might be concluded that *Ta*CPa2E has more affinity towards less bulky substrate - C5 CDP-activated substrate (CDP-Lyxose) than towards CDP-activated hexoses where among two C2 epimers CDP-Mannose was better accepted than CDP-Glucose.

6. LITERATURE

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STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

Magdaleen Matus

Name of student