Carina Frank, BSc BA

Expression optimization and characterization of α1,2-fucosyltransferases for the production of human milk oligosaccharides with focus on *Helicobacter* enzymes

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Univ.-Prof. Dipl.-Ing. Dr.techn Bernd Nidetzky

Institute of Biotechnology and Biochemical Engineering

Dipl.-Ing. Dr.techn. Barbara Petschacher

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Abstract

Fucosyltransferases are essential enzymes for the fucosyl transfer from GDP-L-fucose to acceptor glycans in synthesis of human milk oligosaccharides (HMOs). These oligosaccharides are present at high concentrations in human milk and confer beneficial health effects to the neonate. The most abundant HMOs are fucosylated. Optimized fucosylation is therefore also of industrial interest aiming at supplementation of baby formula with HMOs. Since fucosylated HMOs show limited availability by chemical production routes, biotechnological production based on bacterial fucosyltransferases creates new opportunities. The enzymatic synthesis of two of the most abundant HMOs, 2´-fucosyllactose and lacto-N-fucopenatose I, involves α1,2-fucosyltransferases (α1,2-FucT). However, some general shortcomings of bacterial α1,2-FucTs as low soluble expression yields and low activities need to be overcome. To enhance expression yields in this study the fusion partner NusA was N-terminally linked with the target proteins to mediate their solubility. The α1,2-fucosyltransferases from *Helicobacter pylori*, *Helicobacter mustelae* and *Bacteroides fragilis* were expressed with this fusion strategy which allowed futher purification and characterization. Specific activities on lactose and lacto-*N*-tetraose (LNT) were compared, temperature and storage stabilities were tested. Additionally, the fusion tag was removed via TEV cleavage, to reveal insight into the influence of the fusion partner. Inhibition by sugar nucleodtides was preliminarily investigated. 2'-fucosyllactose was produced with *Hp*FT with maximum conversion yields of 77% starting from 10 mM lactose. Characterization of donor hydrolysis rates upon increase of acceptor substrate showed, that with lactose concentrations higher than 50 mM more than 95% conversion can be achieved.

Keywords: Fucosyltransferases; Fucosyllactose; Human milk oligosaccharides; Fusion protein; TEV cleavage

Kurzfassung

*α*1,2-Fucosyltransferasen (*α*1,2-FucT) sind verantwortlich für den Transfer des Fucosylrests von GDP-fucose zum Akzeptormolekül und bilden dabei fucosylierte humane Milch-Oligosaccharide. Diese humanen Milch-Oligosaccharide sind in hoher Konzentration in der Muttermilch zu finden und besitzen positive, gesundheitsfördernde Effekte für Neugeborene. Fucosylierte Oligosaccharide zeigen die häufigste Vorkommnis in der Muttermilch und sind daher von großem biologischem Interesse. *α*1,2-FucTs katalysieren die Synthese von 2´-Fucosyllactose und lacto-*N*-fucopentaose I, welche nur limitierend zur Verfügung stehen. Daher ist die bakterielle Synthese fucosylierter HMOs von großer biotechnologischer Bedeutung. Die Expression dieser FucT-Gene in *E. coli* geht allerdings mit Schwierigkeiten, wie geringe Löslichkeit und niedrige Aktivitätsraten, einher. In dieser Arbeit werden verschiedene Strategien präsentiert, um diese Probleme zu lösen. Um die Löslichkeit zu erhöhen wurde der Fusionspartner NusA an die zu kodierende Sequenz der FucTs gehängt. Verschiedene Fucosyltransferasen von *Helicobacter pylori*, *Helicobacter mustelae* und *Bacteroides fragilis* wurden mit dieser Strategie exprimiert. Um den Einfluss des Nus-tags besser interpretieren zu können, wurde dieser mithilfe einer TEV Protease entfernt. Des Weiteren wurden die Aktivitäten mit Laktose und LNT als Substrate und auch die Umsetzungen miteinander verglichen. Mit 10 mM Laktose als Startkonzentration wurde mit *Hp*FT eine maximale Umsetzung von 77 % in 2´-Fucosyllaktose erreicht. Charakterisierungsexperimente bezüglich der Donorhydrolyse zeigten, dass mit Laktosekonzentrationen höher als 50 mM eine Umsetzung von mehr als 95 % erreicht werden konnte.

Schlagwörter: Fucosyltransferasen; Fucosyllaktose; Humane Milch-Oligosaccharide; Fusionsprotein; TEV Schnitt

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List of Abbreviation

2´-FL	2´-fucosyllactose
ATP	Adenosine 5'-triphosphate
BSA	bovine serum albumin
CIP	Alkaline Phosphatase, Calf Intestinal
DTT	1,4-dithiothreitol
FucT	Fucosyltransferase
GDP	5`-guanosine-diphosphate
GDP-fucose	Guanosin 5'-diphospho-β-L-fucose
GTP	Guanosine 5`-triphosphate
HMO	human milk oligosaccharide
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LNFP I	lacto-*N*-fucopenatose I
LNT	Lacto-*N*-tetraose
NAD	β-Nicotinamide adenine dinucleotide sodium salt
NADH	β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate
PEP	Phosphoenolpyruvate
PK/LDH	Pyruvate kinase/Lactate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBAB	Tetra-n-butylammonium bromit
TEV	tobacco etch virus
TLC	Thin layer chromatography

1 Introduction

Human milk is considered as the gold standard for infants to grow and develop. With 5-15 g/L human milk oligosaccharides (HMOs) are the third most abundant solids in breast milk after lactose and lipids. HMOs form complex sugars, which are strongly correlated to the health benefits of breastfeeding in infants and show a prebiotic effect, which is called the “bifidus factor” (Bode, 2012). They also have a potential to protect against diseases like diarrhoea, cholera or respiratory-tract infections (Fields and Demerath, 2013; Morrow et al., 2005) based on anti-adhesive properties. Moreover, HMOs act as immunemodulators and antimicrobials. Around 200 different HMOs have been identified so far. In general, HMO structures are based on five monosaccharides as elementary units, D-glucose (Glc), D-galactose (Gla), D-acetylglucosamine (GlcNAc), L-fucose (L-Fuc) and N-acetyl-neuraminic acid (Neu5Ac). All HMOs contain lactose, which forms the reducing end and can be elongated with the disaccharide units lacto-N-biose (type I) or lactosamine (type II) through *β*1,3 or *β*1,6 glycosidic linkages (Bode, 2012; Petschacher and Nidetzky, 2016). Lactose or the elongated oligosaccharide chain can be either fucosylated or sialylated. Fucosylated HMOs are a major component of human milk. The two major fucosylated HMOs are 2´-fucosyllactose and lacto-*N*-fucopentaose I (LNFP I), both of which contain an *α*1,2-linkage (Petschacher and Nidetzky, 2016).

Oligosaccharide concentrations in milk of most farm animals are up to 1000-fold lower than in human milk, especially fucosylated oligosaccharides are present at very low amounts only (Oliveira et al., 2015). Accordingly, extraction from animal milk is not a profitable option for the large-scale production of HMOs. Chemical synthesis would be an alternative route but due to the extensive need of protecting group chemistry to achieve the demanded regioselectivity, chemical synthesis is a very challenging process (Petschacher and Nidetzky, 2016; Kretzschmar and Stahl, 1998). Biotechnological synthesis routes, in particular fermentative production with engineered whole cell biocatalysts are currently the only option that can satisfy the requirements for an economically viable production of 2´-fucosyllactose (Chin et al., 2016; Chin et al., 2017; Bych et al., 2019).

The biocatalytic synthesis of fucosylated glycoconjugates can be performed either by fucosidases, engineered fucosidases called fucosynthases or fucosyltransferases (FucTs). Fucosidases and fucosynthases show higher activity rates and good expression. However, due to their high hydrolase activity towards both the donor substrate and the product, fucosidase based syntheses struggle to achieve high conversion yields (Petschacher and Nidetzky, 2016). Moreover, typical donor substrates are expensive, unstable or even toxic. Thus, highly regioselective FucTs with high regioselectivity and high conversion yields due to reduced hydrolysis side activity are commonly applied for HMO synthesis. They catalyse the fucosyl transfer from donor substrate guanosine-diphosphate L-fucose (GDP-L-fucose) to acceptor molecules to form fucosyloligosaccharides with α-glycosidic linkages (Lee et al., 2015; Albermann et al., 2001). Depending on the site of fucose addition, fucosyltransferases are categorized into α1,2, α1,3/4 and α1,6 fucoslytransferases (Ma et al., 2006). α1,2-fucosyltransferases are used to synthesize 2´-fucosyllactose and LNFP and are classified into glycosyltransferase family 11.

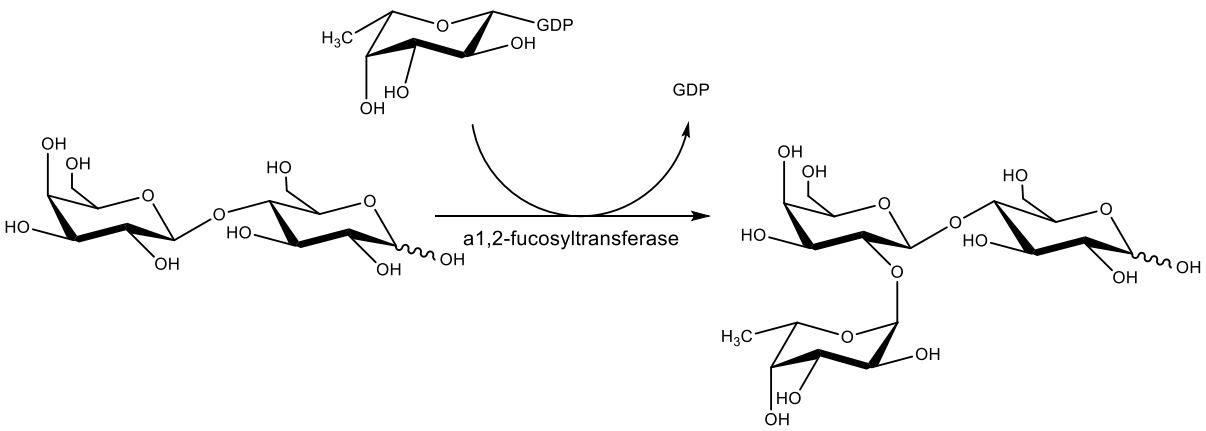

Figure 1: Fucosylation reaction catalysed by α1,2-fucosyltransferase. The enzyme catalyzes the transfer of the fucosyl group from the donor substrate GDP-fucose to the acceptor substrate lactose via an α1,2-linkage, forming the trisaccharide 2´-fucosyllactose.

Mammalian FucTs are membrane bound proteins which are difficult to express solubly. Therefore, the use of bacterial fucosyltransferases creates new opportunities. Expression levels of bacterial FucTs were found to be higher and are sufficient for whole cell production of 2´-fucosyllactose to titers of approximately 20 g/L (Baumgärtner et al., 2013). However, only few prokaryotic fucosyltransferases were so far expressed soluble in high enough levels to allow purification in sufficient amounts for detailed enzymatic characterization

(Petschacher and Nidetzky, 2016), among them are *Hp*FT from *H. pylori* (Stein et al., 2008; Lee et al., 2015), WbgL from *E. coli* O126 (Engels and Elling, 2014), *Te*FT from *Thermosynechococcus elongatus* (Zhao et al., 2016), *Ce*FT from *Caenorhabditis elegans* (Zheng et al., 2002), WcfB from *Bacteroides fragilis* (Chin et al., 2017), *Se*FT from *Salmonella enterica* (Gierlinger, 2017) or *Cl*FT from *Campylobacter lari* (this thesis). In addition to rather low solubility, α1,2-fucosyltransferases have a generally low activity in the mU/mg range which makes high expression levels even more important for efficient catalysis. Low solublity is caused by aggregation and accumulation of the recombinant protein in inclusion bodies (Lee et al., 2015). In order to prevent or decrease protein aggregation during protein production in *E. coli* fusion partner proteins can be used. These fusion partners are very stable protein molecules, solubly expressed and are linked with the target proteins to mediate their solubility. The fusion partner can also contain a protease recognition sequence between the tag and the recombinant protein which allows the removal of the tag. There are different solubility-enhancing proteins described, like the maltose-binding protein (MBP) or the N utilization substance A (NusA). MBP is a 42 kDa protein whereas the NusA-tag is a 55 kDa affinity tag (Young et al., 2012). According to Nallamsetty and Waugh (2006) both fusion partner show comparable solubility-enhancing properties.

In this study, the general applicability of the NusA-tag to enhance soluble expression or α1,2-FucTs was tested. Therefore, the genetic sequence coding for the tag was attached as a fusion partner to several fucosyltransferase coding sequences. The *E. coli* protein NusA is described to improve the soluble production of fusion proteins but also to increase the expression level (Costa et al., 2014; Gierlinger, 2017).

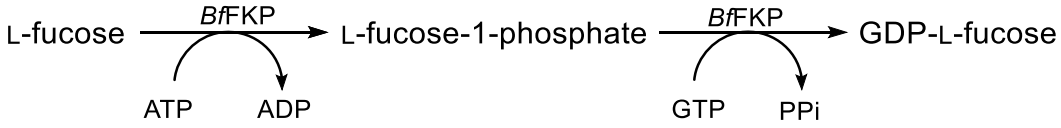

Figure 2: Synthesis of GDP-fucose via the salvage pathway. Conversion of L-fucose to GDP-L-fucose is catalysed by the enzyme *Bf*FKP from *Bacteroides fragilis* 9343.

Gierlinger (2017) already tested the soluble expression of four α1,2FucTs by adding the NusA-tag, which clearly showed a beneficial effect. This study presents a detailed characterization of the so far in literature not in detail described α1,2-fucosyltransferase from *H. mustelae*

(Huang et al., 2017; Nozomu Kamada and Satoshi Koizumi, 2004), which could be expressed in soluble form by addition of a N-terminal NusA-tag. *Hm*FT was further tested to be functionable in the enzymatic synthesis of fucosylated HMOs. WcfB from *Bacteroides fragilis* was also tagged with the fusion partner NusA, however, it was not sufficient to enhance the expression of the enzyme.

*Hm*FT was compared to *Hp*FT regarding activity, stability and in enzymatic 2'-fucosyllactose synthesis. Moreover, a comprehensive donor substrate hydrolysis study was conducted with *Hp*FT to reveal additional insight into the influence of the acceptor substrate concentration on the transfer/hydrolysis ratio. Additionally, the fusion partner NusA was removed from *Hp*FT via a TEV protease in order to clarify the influence of the NusA fusion tag on the specific activity of the fucosyltransferase.

A second part of this thesis dealt with the production of GDP-L-fucose, as the high price of commercially available GDP-fucose hampers detailed enzymatic characterization of FucTs. As stated in Figure 1 for synthesizing fucosylated HMOs the donor substrate GDP-fucose is required, which is naturally available in prokaryotes and eukaryotes. In general GDP-fucose can be produced via the *de novo* or the salvage pathway (Andrianopoulos et al., 1998; Becker and Lowe, 2003). Beginning with GDP-D-mannose the *de novo* pathway is a three step reaction which contains a GDP-D-mannose 4,6-dehydratase and a GDP-keto-6-deoxymannose 3,5-epimerase/4-reductase (Becker and Lowe, 2003).

In the salvage pathway the heterologously enzyme FKP (L-fucokinase/L-fucose-1-phosphate guanylyltransferase) from *Bacteroides fragilis* 9343 is used for the conversion of L-fucose to GDP-fucose via the intermediate L-fucose-1-phosphate. Hereby, the C-terminal part converts L-fucose to L-fucose-1-phosphate and the N-terminal domain is responsible for the second step where the intermediate reacts to GDP-fucose (Coyne et al., 2005; Jung et al., 2019). The enzymatic production of the donor substrate GDP-fucose by the bifunctinal L-fucokinase/L-fucose-1-phosphate guanylyltransferase (FKP) via the salvage pathway is also described in this work with focus on chromatographic purification.

2 Material and methods

2.1 General material

Plasmids were purified with Promega WizardPlus SV Miniprep DNA kit (Fitchburg, WI, USA). PCR purification and agarose gel extraction was carried out with EXTRACTME DNA GEL-OUT KIT from BLIRT S. A. (Gdansk, Poland). Primers were ordered at Integrated DNA Technologies (Coralville, IA, USA). The standard 1 kb DNA ladder, restriction enzymes and alkaline phosphatase (Calf intestinal) were obtained from New England Biolabs GmbH. 2'-fucosyllactose (purity: > 95 %, HPLC) was ordered at Isosep (Tullinge, Sweden), GDP-L-fucose (purity: > 90 %, HPLC) was ordered at Carbosynth (Berkshire, UK). The PageRuler^{TM} Prestained Protein Ladder and the PageRuler^{TM} Unstained Protein Ladder for SDS-Page were ordered at Thermo Fisher Scientific. All other chemicals were purchased from Carl Roth (Karlsruhe, Germany) and Sigma Aldrich (St. Louis, MO, USA) in analytical grade.

2.2 Genes, plasmids and strains

Table 1 gives an overview of the used genes, plasmid and strains.

Table 1: List of used genes, plasmids and strains in this work.

Genes	Description
*Hp*FT	α1,2-fucosyltransferase from *H. pylori*, Uniprot accession number J0C6Y0 with mutations W42G, G43S and P124S and codon exchange of the Pro4-sequence (CCC to CCA)
WbgL	α1,2-fucosyltransferase from *E. coli*, Uniprot accession number E2DNL9
*Cl*FT	α1,2-fucosyltransferase from *C. lari*; Uniprot accession number A0A0A8HWP7
*Hm*FT	α1,2-fucosyltransferase from *H. mustelae*; Uniprot accession number D3UIY5
WcfB	α1,2-fucosyltransferase from *B. fragilis*; Uniprot accession number Q9XDK2

TEV*Te*FT	*α*1,2-fucosyltransferase from *T. elongates*, basing on Uniprot accession number Q8DGK1, with R5H and E289G containing a TEV cleavage site
TEV*Hp*FT	*α*1,2-fucosyltransferase from *H. pylori*, Uniprot accession number J0C6Y0 with mutations W42G, G43S and P124S and codon exchange of the Pro4-sequence (CCC to CCA) containing a TEV cleavage site
*Bf*FKP	L-fucokinase/L-fucose-1-phosphate guanylyltransferase from *B. fragilis* 9343; Uniprot accession number Q58T34.
Plasmids	
pMCSG7	Bacterial expression vector with T7 promoter, adds N-terminal His-tag and TEV protease site, ampicillin resistance
pET22b(+)	Bacterial expression vector with T7 promoter, adds C-terminal His-tag, ampicillin resistance
pC21e1	Bacterial expression vector with tac promoter, ampicillin resistance
Strains	
BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] Δ hsdS* *λ DE3 = λ sBamHio Δ EcoRI-B int::(lacI::PlacUV5::T7 gene1)* *i21 Δnin5*
JM109	*end*A1*, rec*A1*, gyr*A96 *thi, hsd*R17 (r_k^- ,m_k^+), *rel*A1, *sup*E44, *Δ(lac-pro*AB), [F' *tra*D36*, pro*AB, *laqI*^{q}ZΔM15]
TOP10F'	F- *mcrA,* Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15 Δ *lac*X74, *rec*A1, *ara*D139, Δ(*araleu*)7697, *gal*U, *gal*K, *rps*L (StrR), *end*A1, *nup*G

2.3 Cloning and Modification

To clone the coding sequence for *Hm*FT and WcfB from the pET22b(+) vector into the pC21e1 vector with N-termnal NusA-tag and Strep-tag for further affinity purification the coding sequences were amplified using the primers *Hm*FT_Fwd and *Hm*FT_Rev for *Hm*FT and WcfB_Fwd and WcfB_Rev for WcfB. pC21e1 was amplified with the primers pC21e1_Fwd and pC21e1_Rev. Reaction mixtures for amplification by Q5 DNA polymerase contained 10 µM of each primer, 10 mM dNTPs and 1 ng/µL of the template DNA. The PCR mixtures were subjected to an initial denaturation at 98°C for 30 seconds, which was followed by 30 cycles of amplification (denaturation at 98°C for 30 seconds, annealing at 58°C for 30 seconds, and

elongation at 72°C for 4 minutes), and then final elongation at 72°C for 5 minutes. The resulting PCR products were separated using agarose gel and purified for cloning. The purified DNA was ligated into the pC21e1 vector using NEBuilder HiFi DNA Assembly Cloning Kit with a vector:insert ratio of 1:2. After ligation the recombinant plasmids pC21e1_*Hm*FT and pC21e1_WcfB were obtained and transformed into *E. coli* Top10F'. The insert was verified by DNA-sequencing. For expression experiments pC21e1_*Hm*FT and pC21e1_WcfB were transformed into *E. coli* JM109. In order to store host cells carrying the plasmid for long term, glycerol stocks were prepared and stored at -70°C.

Table 2: List of used primers in this work.

Primer	Sequence
pC21e1_Fwd	TAA AAG CTT AGG CAT CAA ATA AAA CGA AAG GC
pC21e1_Rev	GAG CTC CGG ACT CTT GTC GTC G
*Hm*FT_Fwd	CAA GAG TCC GGA GCT CGA TTT TAA GAT TGT GCA AGT G
*Hm*FT_Rev	GAT GCC TAA GCT TTT AAG ATT TTG TCT CAA ATT GGG
WcfB_Fwd	CAA GAG TCC GGA GCT CTT ATA TGT AAT TTT ACG TGG
WcfB_Rev	GAT GCC TAA GCT TTT ACA TAT TCT TCT TTC TTT TCC

For the removal of the NusA-tag, *Hp*FT was cloned into the vector pC21e1, which contains a TEV cleavage site. Therefore, pC21e1_*Hp*FT and the vector containing the TEV cleavage site (pC21e1_TEV*Te*FT) were digested with *Sac*I and *Bam*HI. The digested *Hp*FT and the vector backbone were purified from agarose gel and ligated using T4 DNA Ligase. After ligation the desired recombinant construct was verified by DNA sequencing. Afterwards the heterologous host *E. coli* JM109 was transformed with the plasmid pC21e1_TEV*Hp*FT for overexpression of the α1,2-fucosyltransferase. Cells were grown and purified as stated below in sections 2.4 and 2.5.

2.4 Cultivation

Transformants of *E. coli* JM109 pC21e1_*Hm*FT, *E. coli* JM109 pC21e1_WcfB, *E. coli* JM109 pC21e1_*Hp*FT, *E. coli* JM109 pC21e1_TEV*Te*FT, *E. coli* JM109 pC21e1_TEV*Hp*FT, *E. coli* JM109 pC21e1_*Cl*FT and *E. coli* BL21 (DE3) pET16b_*Bf*FKP were grown in 500 mL Erlenmeyer flasks containing 50 mL LB medium with 100 µg/mL ampicillin and incubated overnight at 37 °C with agitation (100 rpm). For the overproduction cells were grown in 1000 mL Erlenmeyer flasks with 250 mL LB medium at 37 °C and 100 rpm to an OD_{600} of 0.6-0.8. After induction with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), expression was proceeded for 18-20 h at 25 °C. TEV*Hp*FT was expressed at 18 °C. Subsequently, cells were harvested by centrifugation (5,000 rpm, 20 min, 4°C) and the pellets were resuspended in washing buffer [100 mM Tris, 150 mM NaCl, 1 mM EDTA (pH 8)] and stored at -20 °C until further usage.

*Bf*FKP was cultivated and expressed as stated above. In contrast to the fucosyltransferases, *Bf*FKP was resuspended in binding buffer containing 20 mM $NaPO_4$, 500 mM NaCl and 20 mM Imidazole (pH 7.4).

2.5 Enzyme purification and SDS-PAGE

2.5.1 Sonication

The thawed suspension was disrupted by sonication (2 sec. pulse, 4 sec. pause, 60 % amplitude for 6 minutes) and then cell lysate was obtained by centrifugation at 15,000 rpm for 60 min at 4 °C. The clear lysate (soluble fraction) was used for further purification steps.

2.5.2 Glass beads

Cells were disrupted using glass beads (0.5 mm dia, Cat. No. 11079105, Biospect Products, Bartlesville, OK 74005, USA). The cell suspension and the glass beads were mixed in a 2:1 ratio (250 mL). The whole solution was then disrupted by alternating between 1 min vortexing and 1 min resting on ice. This was repeated 5 times. The disrupted cells were then centrifuged for 10 min at fullspeed and 4°C. The supernatant was used for further applications like SDS-PAGE.

Strep Tag:

The protein purification was performed by the ion affinity chromatography on an ÄktaPrime plus system (GE Healthcare, Little Chalfont, UK) equipped with a 5 mL Gravity flow Strep-Tactin Sepharose column from IBA (Göttingen, Germany). The enzyme was bound at a constant flow rate of 2.0 mL/min using the washing buffer. The protein was eluted by a concentration of 2.5 mM desthiobiotin in the elution buffer [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA]. The desired fractions were pooled and concentrated by using VivaSpin® tubes with a 30 kDa cut-off membrane (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Afterwards the solvent was exchanged to a Tris-HCl (pH 7.0) buffer. The purified protein solution was aliquoted, shock-frozen in liquid nitrogen and stored at -20 °C and -70 °C.

His Tag:

For His-tag affinity purification of *Bf*FKP two 5 mL His-Trap colums from GE Healthcare were used on an ÄktaPrime plus system (GE Healthcare). The protein was bound at a flow rate of 0.5 mL/min. After removing the unbound proteins, *Bf*FKP was eluted using a 100 mL gradient from 0 to 150 mM imidazole at a flow rate of 2 mL/min. To remove the imidazole and to concentrate the protein, ultrafiltration with a 30 kDa VivaSpin® tube (Sartorius) was applied and the buffer was exchanged to 50 mM Tris-HCl (pH 7.6). For better stabilization through storage 10 % glycerol and 0.02 % Tween 80 were added. The protein was then aliquoted, shock-frozen in liquid nitrogen and stored at -20 °C.

The protein concentration was determined with two different methods. For an overall quantification of proteins a spectrophotometer (DeNocvix Wilmington, USA) was used and the concentration was measured at 260 nm and 280 nm. For the Bradford (Bradford, 1976) Roti-quant (Roth, Karlsruhe, Germany) was used as protein dye. Therefore 490 µL were mixed with 10 µL of sample. The absorbance was measured at 595 nm and the protein concentration was determined through a BSA standard calibration curve.

Protein expression level was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with precast Thermo Fisher NuPAGE Bolt 10 % Bis-Tris Plus Gels

and Coomassie Blue staining. The molecular mass was determined by using a prestained or unstained Thermo Fisher PageRuler standard as protein ladder.

2.6 GDP-L-fucose synthesis

The nucleotide sugar GDP-fucose was synthesized via the salvage pathway. In this system, the bifunctional enzyme *Bf*FKP from *Bacterioides fragilis* L-fucokinase/L-fucose-1-phosphate guanylyltransferase (FKP) catalyses the formation of GDP-fucose from fucose, adenosine 5'-triphosphate (ATP) and guanidine 5'-triphosphate (GTP). Therefore, 10 mM L-fucose, 15 mM GTP, 10 mM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$ in 50 mM Tris-HCl (pH 7.6) were mixed together to a total volume of 18 mL. The solution was adjusted to pH 7.6 and by adding 56 U iPPase and 1 mg/mL *Bf*FKP the reaction was started and incubated at 37 °C for 21 hours under slightly stirring.

After the synthesis of the nucleotide sugar downstreaming was processed in 4 main steps (Fürpaß, 2018; Schmölzer et al., 2017):

1. Nucleotide hydrolysis with phosphatase (CIP-digest)
   The pH was adjusted to 7.0 and then 10 U/mL alkaline phosphatase were added and the mixture was incubated for 16 hours at 30 °C under slightly stirring.
2. Ion exchange chromatography (IEC)
   Residual GDP and GMP were removed by anion exchange. The binding buffer (A) contained 20 mM NaOAc (pH 4.3) and the elution buffer (B) contained 1 M NaOAc (pH 4.3). The IEC columns (2 x 1 mL) were packed with Toyopearl Super Q-650M (Tosoh Bioscience). GDP-fucose was eluted with a flow rate of 0.7 mL/min and the wavelength was set to 254 nm. All relevant fractions were pooled and evaporated to a volume of about 10 mL. The GDP-fucose containing solution was stored on ice at 4 °C overnight.

3. Size Exclusion Chromatography (SEC)
   To separate sugar and salt a size exclusion was performed. As mobile phase dH_2O was used. The flow rate was set to 2 mL/min. The elution of salt-free GDP-fucose was

monitored at 254 nm. Relevant fractions were pooled and the volume was reduced via the rotary evaporator.

4. Lyophilisation

   The GDP-fucose containing solution was freeze-dried with liquid nitrogen and vacuum was applied. Hereby the residual water was sublimated and the pure GDP-fucose was gained as white powder.

2.7 Activity Measurement

2.7.1 Activity of α-1,2-fucosyltransferases

To maintain the assay condition constant, all activity measurements were performed at 37 °C and monitored at 340 nm in a microtitre plate reader using the pyruvate kinase/lactate dehydrogenase (PK/LDH) system based on Gosselin et al. (1993). The reaction mechanism contains three important steps. The first step includes the catalytic conversion of the donor substrate GDP-fucose into 2´-fucosyllactose and GDP with the presence of the acceptor substrate lactose. The released GDP then reacts with PEP to pyruvate and GTP by a pyruvate kinase. The last step contains the conversion of pyruvate into lactate by lactate dehydrogenase using NADH as reducing cofactor.

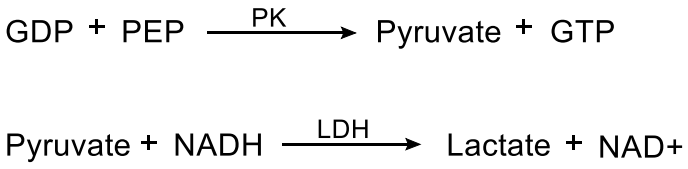

Figure 3: Reaction mechanism of the coupled PK/LDH assay. The release of GDP was coupled to NADH oxidation via pyruvate kinase (PK) and lactate dehydrogenase (LDH) and measured photometrically at 340 nm.

The release of GDP is equivalent to the oxidation of NADH and is therefore detectable on a BMG Labtech Fluostar Omega Platereader. The reaction was started by adding 0.15 mg/mL enzyme. The absorbance at 340 nm was then monitored for a period up to 30 minutes.

The reaction mixture contained 1 mM phosphoenolpyruvate (PEP), 0.66 nicotinamide adenine dinucleotide (NADH), 5 mM $MgCl_2$, 20 mM KCl, 10 mM lactose or 1 mM LNT, 1 mM GDP-

fucose, 5 U pyruvate kinase/lactatdehydrogenase in 100 mM Tris-HCl (pH 7.0) in a total volume of 150 µL. Pyruvate kinase and lactate dehydrogenase were not limiting. Prior adding the fucosyltransferase, the reaction was kept at 37 °C for 15 minutes to let free GDP react.

2.7.2 Activity of *Bf*FKP

To determine the activity of the enzyme the GDP-fucose synthesis was monitored over 60 minutes by HPLC. The assay was performed in a 500 µL reaction mixture containing 5 mM L-fucose, 10 mM GTP, 5 mM ATP, 5 mM $MgCl_2$, 3 U iPPase and 0.11 mg/ml *Bf*FKP in 50 mM Tris-HCl (pH 7.6) and mixed with 350 rpm at 37 °C. Samples were taken after 0, 5, 10, 15, 30, 45 and 60 minutes. 40 µL of the sample were stopped with 40 µL ice-cold ACN (1:1 pre-mixed with H_2O) and measured on HPLC.

2.8 Determination of temperature stability

The assay was performed as described above in section 2.7.1. 0.15 mg/mL enzyme were incubated with 25 mM $MgCl_2$, 100 mM KCl, 50 mM lactose in 100 mM Tris-HCl (pH 7.0) in a range from 25 °C to 37 °C for 60 minutes. Samples of the enzyme containing solution were taken after 0 min, 15 min, 30 min, 45 min and 60 min incubation, mixed with 1 mM GDP-fucose, 0.66 mM NADH, 1 mM PEP and 5 U PK/LDH, which was incubated separately at 37 °C for 15 minutes and measured photometrically at 37 °C in order to monitor the enzyme stability.

2.9 Determinaton of storage stability

The assay was performed as described in section 2.7.1. Enzymes were stored at -20 °C in a concentration of 2.2 mg/mL and the storage stability of purified enzymes was measured photometrically at 37 °C after different time points (directly after purification and after 3, 8, 14 and 16 weeks).

2.10 Removal of the affinity tag

To determine the influence of the NusA-tag on the specific activity of the *α*1,2-fucosyltransferases, the tag was cleaved off by using a TEV protease from Sigma Aldrich (St. Louis, MO, USA), encoded by the tobacco etch virus. Therefore, *Hp*FT was cloned into pC21e1, which contains a TEV cleavage site. After successful cloning, the TEV cleavage site is located between the NusA-tag and the coding sequence for *Hp*FT.

The optimal time required for cleavage was examined by comparing the cleavage product after different incubation times via SDS-PAGE. For all further TEV cleavages several parameters were kept constant; the concentration of the purified protein (TEV*Hp*FT) (7.2 mg/mL), a protein:TEV ratio of 1:100, the addition of 1 mM DTT and 0.5 mM EDTA, the cleavage temperature (4 °C) and the incubation time (14 h). After 14 h incubation the cleavage was checked on a SDS-PAGE and the specific activity was measured as described in section 2.7.1.

2.11 Methods for enzyme inactivation

Analytics were developed regarding the stopping methods of enzymatic reactions. Different inactivation methods including dramatic pH change, heating the entire sample solution and adding methanol or acetonitrile were tested to get the best option for measuring the formed product 2´-fucoslylactose. Acidification is a method were the pH was shifted using 2 M HCl. Afterwards 2 M NaOH was added to neutralize the sample solution. For heat inactivation the samples were rapidly boiled with pre-heated Tris-HCl (pH 7.0) buffer in a ratio 1:1 in a Thermomixer at 95 °C for 5 minutes. Methanol was also used to inactivate the enzyme activity. Therefore, methanol and the sample were mixed in a ratio 1:1 at 4 °C for 30 minutes. Another method was using ice-cold 50 % acetonitrile, where the sample:acetonitrile ratio was 1:4. After denaturation the residual activity was measured photometrically as described in section 2.7.1.

2.12 Conversion assays and HPLC analysis

2.12.1 Conversion assay for 2´-FL production

For the conversion experiment of lactose to 2'-fucosyllactose, the assay consisted of 1-5 mM GDP-fucose, 10 mM lactose, 10 mM $MgCl_2$ and 1U CIP in 100mM Tris-HCl (pH 7.0) and was incubated at 30 °C. 0.15-0.5 mg/mL enzyme were added to start the reaction. The reactions were stopped by heat inactivation at 95 °C for 5 minutes at several time points and the samples were centrifuged for 30 min at 16,100 g afterwards.

2.12.2 Thin layer chromatography (TLC)

For thin layer chromatography, samples were taken at defined points in time. 2 µL of the end products and standards were spotted directly onto a Silica gel 60 GF_{254} TLC plate (Merck, Darmstadt, Germany). The plate was developed twice to the top using 1-butanol/ethanol/water in a ratio of 2:1:1 (v/v/v) as mobile phase. After development the sugar components were vizualized by staining with a thymol-sulphuric acid reagent (0.5 w/v thymol in 95 v/v ethanol and 5 v/v concentrated sulphuric acid). For detection, the rf-values were compared to commercially available standards of the expected products.

2.12.3 HPLC

The quantification of lactose and 2'-fucosyllactose was performed on a Merck-Hitachi LaChrome HPLC System (Darmstadt, Germany) equipped with a Merck-Hitachi LaChrome L-7250 autosampler, a Merck L-7490 RI detector and a Biorad Aminex *HP*X87-H column (Biorad, Richmond, CA, USA). The mobile phase was 5 mM sulphuric acid and was eluted at a flow rate of 0.3 mL/min and 65 °C.

The consumption of GDP-fucose was monitored using a Shimadzu HPLC with a Kinetex C18 column (phenomenex, Torrence, CA, USA) in a reversed phase chromatography (Kyoto, Japan). The mobile phase consisted of TBAB with 6.5 % acetonitrile and was eluted at a flow rate of 2 mL/min. Analytes were detected at 262 nm.

2.12.4 Influence of the lactose concentration

Various lactose concentrations (1, 5, 10, 20, 50 and 100 mM of lactose) were added to the conversion assay mixture, to test the donor hydrolysis during 2'-fucosyllactose production by StrepNus*Hp*FT. The reaction mixture was the same as described in section 2.12.1. 0.15 mg/mL *Hp*FT was added. The mixture was incubated at 30 °C for 4 hours. Samples were taken at the beginning and after 4 hours and stopped by heat inactivation.

2.12.5 Inhibition

Due to the fact, that GDP-fucose hydrolyses and therefore can contain GDP, GMP or Guanosine, the eventual inhibition effect was studied. This was done by conversion experiments containing reagents as described in section 2.12.1. 0.15 mg/mL *Hp*FT was added. The only differences were, that additionally 3 mM GMP, GDP or Guanosine were added respectively and no CIP was used in the reaction mixture. Samples were taken at time point 0 and after 2 hours. The enzyme assay was then stopped by heat inactivation. The results were examined via TLC.

Furthermore, the inhibition effects of GTP, GMP, Guanosine and ATP were also determined photometrically. Thereby the photometer assay was examined as described in section 2.7.1. Additionally, 3 mM GTP, GMP, Guanosine, ATP or 10 mM GTP, ATP were added respectively and the oxidation of NADH was then detected.

3 Results and discussion

The access to α1,2-fucosylated human milk oligosaccharides on a large scale is hampered by a lack of α1,2-fucosyltransferases that show high activity and can be obtained in large amounts (Zhao et al., 2016). Due to hampered availability of enzymes and their dependence on expensive donor substrates α1,2-fucosyltransferases are also not well characterized. This study examined expression optimization by addition of an N-terminal fusion tag (NusA) as general methodology for soluble α1,2-fucosyltransferases expression, which allows purification and characterization of novel α1,2-fucosyltransferases. Expression of *Hm*FT, *Cl*FT and WcfB was tested. While WcfB could not be expressed and *Cl*FT showed low activity with LNT as acceptor and no activity with lactose, *Hm*FT was identified as a useful biocatalyst for 2'-FL production. *Hm*FT was compared with the already characterized α1,2-fucosyltransferases *Hp*FT regarding activity, stability and application in conversion experiments. As *Hp*FT was confirmed as most active biocatalsyst for 2'-FL production, *Hp*FT was further characterized concerning donor hydrolysis. Inhibition by nucleotides was preliminarily studied. Part of the thesis involved also GDP-L-fucose production and in detail investigation of efficiencies in GDP-L-fucose purification steps.

3.1 Optimization of expression

Since the expression of N-terminal His-tagged WcfB and *Hm*FT in *E. coli* BL21 (DE3) from pET22b(+) yielded very low protein levels (Figure 4, A), the FucT coding sequences were cloned into pC21e1 with a sequence coding for a StrepII-tag for further purification directly followed by the NusA-tag N-terminally added to the FucTs for solubility enhancement (Figure 4, B). The new construct was then expressed in *E. coli* JM109. The expression was tested in standard shake flask cultivations. Glass beads were used to lyse the cells and the expression of the fucosyltransferases was checked by SDS-PAGE. For calculating the intensity of the bands ImageJ was used.

The expression of the NusA-tagged fucosyltransferases showed considerably better results and the yield of StrepNus*Hm*FT amounted to 23 % of total soluble protein. Expression of *Cl*FT

worked also sufficiently with the NusA-tag. The beneficial effect of the NusA-tag was already shown by Gierlinger (2017), where four different fucosyltransferases showed higher expression yields when tagged with the fusion partner NusA. With results from this study fusion with the Nus-Tag could be confirmed as widely applicable strategy for soluble expression of α1,2-fucosyltransferases. However, for StrepNusWcfB there was no beneficial effect of the NusA-tag observed. No expression was detectable in the soluble protein fraction.

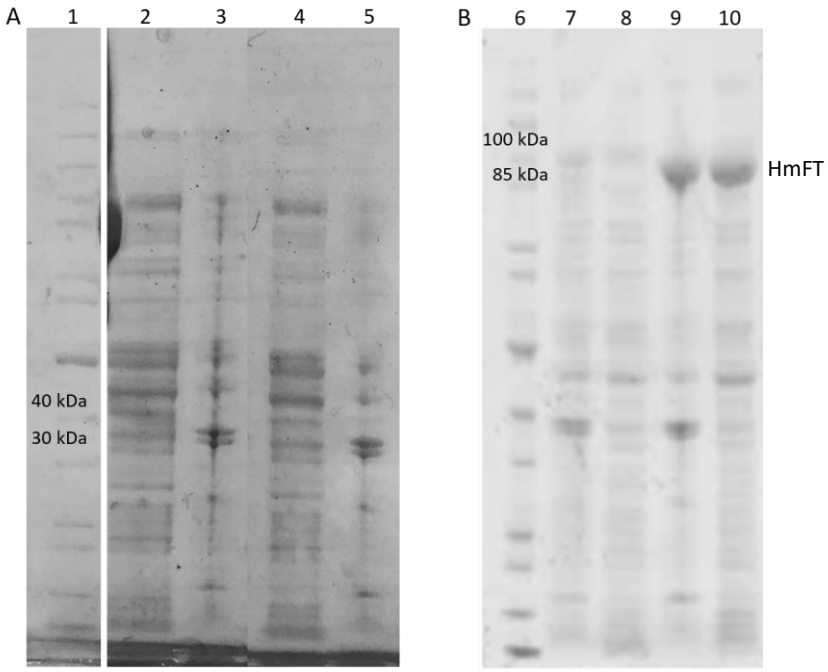

Figure 4: (A) Expression of pET22b(+)_WcfB (33.85 kDa) and pET22b(+)_*Hm*FT (33.41 kDa) without NusA-tag. 1: unstained protein ladder, 2 + 3: *Hm*FT soluble and pellet fraction. 4 + 5: WcfB soluble and pellet fraction. **(B)** Expression of StrepNus*Hm*FT (94.0 kDa) and StrepNusWcfB (94.1 kDa). 6: unstained protein ladder, 7 + 8: WcfB pellet and soluble fraction, 9 + 10: *Hm*FT pellet and soluble fraction.

Expression of StrepNus*Hp*FT and StrepNusTEV*Hp*FT in *E. coli* JM109 was compared at 18 °C and 25 °C (Figure 5), while the other cultivation parameters were kept unchanged. Regarding the intensity of the bands on the SDS-PAGE, expressing StrepNus*Hp*FT at 25 °C (slot 6 + 7) showed the best results. The expression of soluble StrepNus*Hp*FT at 25 °C was twice as high than at 18 °C. However, expressing StrepNusTEV*Hp*FT at 25 °C (slot 8 + 9) showed an expression yield of only 7 % of total protein, leading to the assumption that the sequence between Nus-tag and protein is crucial for a high expression yield. Here, by decreasing the

expression temperature for StrepNusTEV*Hp*FT to 18 °C (slot 4 + 5) an increase in the overall yield of the enzyme to 18 % was obtained.

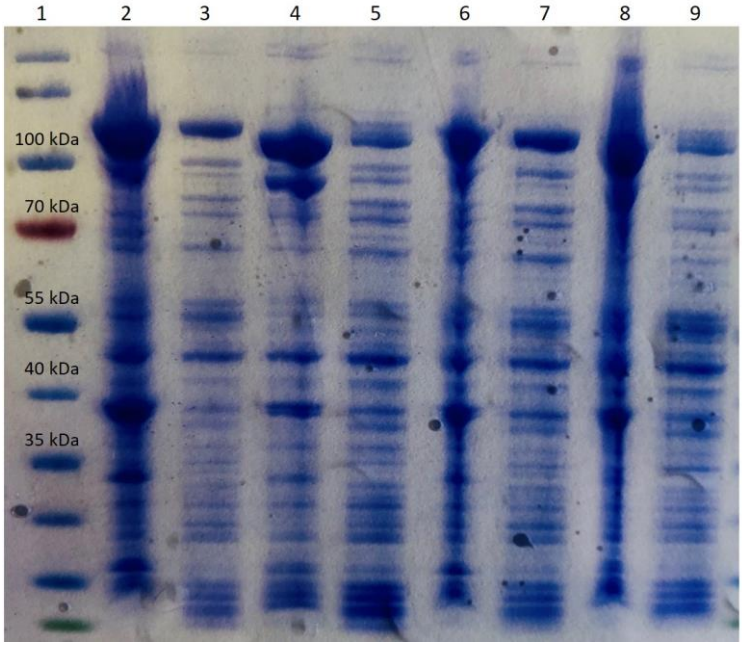

Figure 5: SDS-PAGE of different expression temperatures for StrepNus*Hp*FT and StrepNusTEV*Hp*FT. 1: pre-stained protein ladder, 2 + 3: *Hp*FT expressed at 18 °C (pellet and soluble fraction), 4 + 5: TEV*Hp*FT expressed at 18 °C (pellet and soluble fraction), 6 + 7: *Hp*FT expressed at 25 °C (pellet and soluble fraction), 8 + 9: TEV*Hp*FT expressed at 25 °C (pellet and soluble fraction).

Summarizing, the addition of the fusion partner NusA for improving the solubility increased the expression of StrepNus*Hm*FT significantly. However, StrepNusWcfB was not expressible in the soluble fraction. Additionally, for StrepNusTEV*Hp*FT the yield of soluble enzymes was increased by lowering the expression temperature from 25 °C to 18 °C.

3.2 Enzyme purification

3.2.1 α1,2-fucosyltransferases

The overexpressed α1,2-fucosyltransferases were purified to approximate homogeneity by Strep-tag affinity chromatography, as confirmed by SDS-PAGE. The obtained yield of the α1,2-fucosyltransferases per litre cultivation is shown in Table 3.

3.2.2 *Bf*FKP

The His-tagged *Bf*FKP enzyme was purified by His-tag affinity chromatography by using a stepwise purification. Therefore, the imidazole concentration was increased to 150 mM to elute the purified *Bf*FKP enzyme. The yield is shown in Table 3.

Table 3: Enzyme yields after 16 hours of expression and purification by affinity chromatography.

Enzyme	Size [kDa]	Enzyme yield [mg/L]
StrepNus*Hp*FT	95.2	5.9*
StrepNusTEV*Hp*FT	91.8	13.2
StrepNus*Hm*FT	94.0	16.0
StrepNusWbgL	95.8	1.7
StrepNus*Cl*FT	94.5	2.3
StrepNusWcfB	94.1	not expressible
*Bf*FKP	108.0	48.5

*Gierlinger (2017): 11 mg/L

The enzyme yield of StrepNus*Hp*FT is around 5.9 mg/L, which shows an increase when compared to literature results, where Stein et al. (2008) reached a yield of about 1 mg/L with a His6-tagged enzyme. However, (Gierlinger, 2017) achieved an expression yield of StrepNus*Hp*FT of about 11 mg/mL. The construct with the TEV cleavage site between the fusion partner NusA and the α1,2-fucosylltransferase (StrepNusTEV*Hp*FT) reached a 2.2 fold higher expression yield of 13.2 mg/L. According to Figure 5 the expression of StrepNusTEV*Hp*FT was half as good as StrepNus*Hp*FT, however, the overall enzyme yield after purification showed compareable results in best cases. For further purifications therefore, the flow through should be analysed via SDS-PAGE to check if protein is lost during affinity purification. Expression yields for StrepNusWbgL and StrepNus*Cl*FT were comparatively low. Therefore, these two fucosyltransferases were only used for checking the activity but not for further experiments.

The amount of purified *Bf*FKP was 48.5 mg/L which is about 2.5 times higher than comparable results, where 19.5 mg/L could be reached with the same expression procedure (Fürpaß, 2018).

3.3 GDP-L-fucose synthesis

The purification of enzymatically synthesized GDP-L-fucose was done by CIP digest, anion exchange (IEC), size exclusion (SEC) and lyophilisation. The standard reaction mixture is stated in section 2.6.

CIP digest

In order to remove the nucleotide tri, di and monophosphates calf intestinal alkaline phosphatase, calf intestinal was added to the solution. Results were analysed via HPLC. No nucleotide tri, di or monophosphates were left in the solution.

IEC

In Figure 6 the chromatogram of the anion exchange purification step of the GDP-fucose preparation is shown. A step gradient to 50 % of B was used to elute the GDP-fucose then the concentration of B was set to 100 % to elute possible impurities. Loading and elution was performed several times.

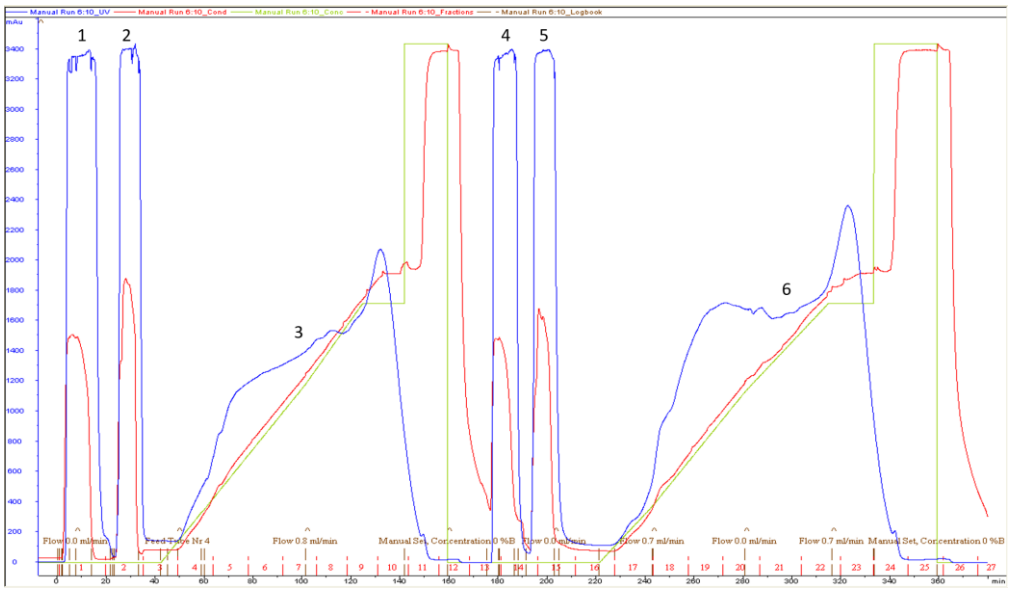

Figure 6: Chromatogram of anion exchange with 2x1 mL columns. Red curve: conductivity [mS/cm], blue curve: absorption at 254 nm [mAU], green curve: concentration of B [%], pink line: time point of injection. Peaks 1, 2, 4 and 5: flow through containing adenosine and guanosine, Peaks 3 and 6: GDP-fucose containing solution. x-axis: min, y-axis: mAU; mobile phase: buffer A (loading buffer) and buffer B (elution buffer), stationary phase: Toyopearl Super Q-650M.

The fractions were analysed via HPLC using a Kinetex C18 column. GDP-fucose containing fractions (peaks 3 and 6) were pooled and the volume was reduced on the rotary evaporator and stored at 4 °C overnight.

SEC

In Figure 7 the third step of the downstream process is illustrated. Size exclusion chromatogrtaphy was used to desalt the nucleotide sugar. The injection was performed 5 times and 2 mL were loaded onto the column. First, the GDP-fucose eluted (blue curve), followed by the salt (brown curve). The size exclusion worked well, which can be seen in the chromatogram below. Fractions were analysed via HPLC using a Kinetex C18 column. GDP-fucose containing fractions were pooled and the volume was reduced on the rotary evaporator.

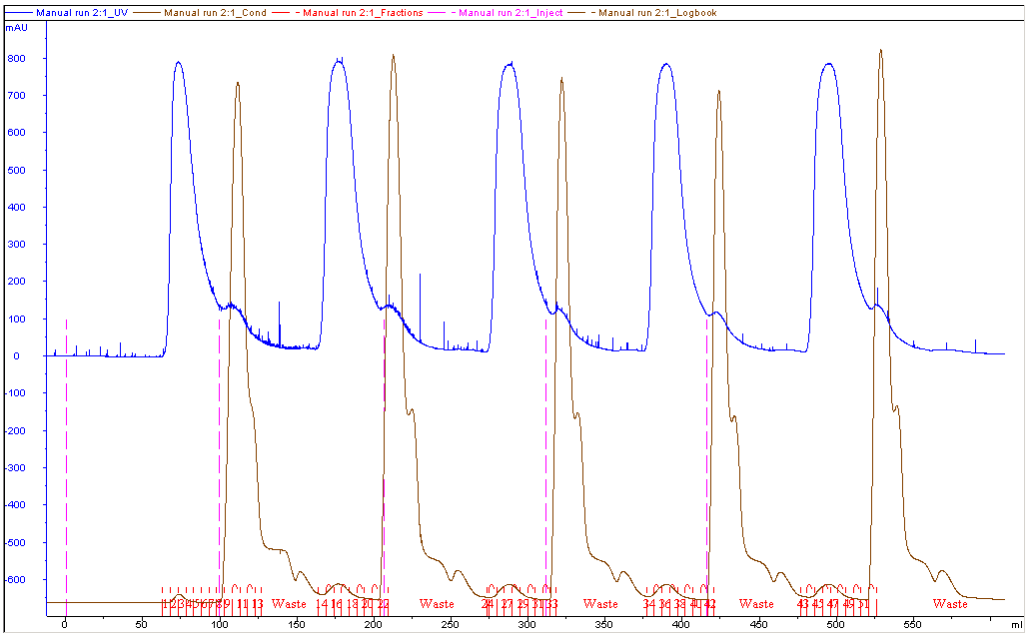

Figure 7: Chromatogram of size exclusion. Pink line: time point of injection, blue curve: absorption at 254 nm [mAU], brown curve: conductivity [mS/cm], which indicates the elution of the salt. X-axis: mL, y-axis: mAU; mobile phase dH_2O, stationary phase: Sequadex G-10.

Lyophilisation

The last crucial step was the lyophilisation. The concentrated GDP-fucose containing solution was shock frozen with liquid nitrogen and by applying vacuum over night the residual liquid was removed from the final product resulting in a white powder.

The yields of GDP-fucose at the different purification steps were measured via HPLC and can be seen in the tables below.

Table 4: Purification of GDP-fucose. Listed values measured via HPLC during synthesis and purification steps. Synthesis: at the end of the reaction, prior CIP: value prior CIP digest, CIP: after CIP digest with alkaline phosphatase, IEC: after the anion exchange step, SEC: after size exclusion, lyophilisation: total weight of gained product, yield: amount of nucleotide sugar in the gained powder. Purity: 99.7 % (detected via HPLC).

	yield [mg]	yield [%]	loss [%]
Synthesis	97.82	100.00	0.00
prior CIP	89.90	91.90	8.10
CIP	85.54	87.45	4.45
IEC	58.69	60.00	27.45
SEC	48.52	49.60	10.40
Lyophilization	28.80	*29.44	20.16
Yield	28.68	29.32	0.12

* high loss due to handling issues

Table 5: Purification of GDP-fucose. Listed values measured via HPLC during synthesis and purification steps. Synthesis: at the end of the reaction, prior CIP: value prior CIP digest, CIP: after CIP digest with alkaline phosphatase, IEC: after the anion exchange step, SEC: after size exclusion, lyophilisation: total weight of gained product, yield: amount of nucleotide sugar in the gained powder. Purity: 71 %. (detected via HPLC).

	yield [mg]	yield [%]	loss [%]
Synthesis	150.66	100.00	0.00
prior CIP	128.32	85.17	14.83
CIP	119.32	79.19	5.97
IEC	48.30	32.06	47.14
SEC	32.69	21.69	10.36
Lyophilization	14.00	9.29	12.40
Yield	9.94	6.60	2.69

The highest losses for both batches were obtained at the anion exchange step. Relevant fractions after IEC were collected and the volume was reduced. The GDP-fucose containing solution was then put on ice and stored at 4 °C overnight. SEC was performed on the following

day. No losses were registered during the storage overnight, which leads to the assumption that GDP-fucose is stable in 0.5 M NaOAc (pH 4.3).

Relatively high amounts of GDP-fucose were lost at the lyophilisation step. This was due to some handling issues with the lyophilisator. Besides these two main losses it can be seen that all other downstream process steps are not problematic and are comparable between the two different batches. Consequently, the use of a simple drying without lyophilisation could be an attractive alternative. However, compared to the published yield of Engels and Elling (2014) of 43 % the gained yield especially for the second batch was very low, where a yield of only 6.6 % was reached.

To sum up, the anion exchange step is very critical and high losses were reported. The usage of more suitable columns or optimizations regarding the pH of the mobile phase for the IEC could reduce such high losses and further on would lead to yield improvements. Differences in purity of the two purification batches can be explained by the duration of lyophilisation, which was longer for the second batch. It might be that GDP-fucose, when on room temperature after drying undergoes degradation.

Due to the very time-consuming production of GDP-fucose it has to be considered generally if the own synthesis is worthwhile. However, acquiring the substrate by purchase is associated with relatively high costs. A calculation of own costs including working hours should be done to evaluate the benefit from in-house production of GDP-L-fucose.

3.4 Activity measurement

3.4.1 α1,2-fucosyltransferases

To determine the activity of the purified α1,2-fucosyltransferases, a photometric assay with coupled enzyme reactions was used, where finally the decrease of NADH was detected at 340 nm. For all enzymes the same assay set-up was used. Initial rate measurements were used to calculate the specific activity, which is given in Table 6.

Table 6: Measured and published activity of the given fucosyltransferases. Measured specific activities were gained using Nus-tagged FucTs.

Enzyme	Substrate	Published spec. activity [mU/mg]	Measured spec. activity [mU/mg]
*Hp*FT	1 mM LNT	55 (2 mM lactose) [1]	1350
	10 mM Lactose	30-80* (2-3 mM lactose) [2]	380
*Hm*FT	1 mM LNT	-**	880
	10 mM Lactose	-**	200
WbgL	1 mM LNT	-	52
	10 mM Lactose	200 [3]	n.d.
*Cl*FT	1 mM LNT	-	29
	10 mM Lactose	-	n.d.

[1] Stein et al., 2008; Tsai et al., 2013

[2] Stein et al., 2008; Albermann et al., 2001

[3] Engels and Elling, 2014

*activity was detected by measuring the incorporation of radioactive label from GDP-L-fucose into reaction product with His-tagged enzyme, hence does not include donor hydrolysis

***Hm*FT described in patent US20040219553A1 -> no activity given

As stated above *Hp*FT is active on LNT (Type I antigen structure) and lactose, which was also shown in previous studies (Albermann et al., 2001; Stein et al., 2008). The highest activities were obtained by using LNT as acceptor substrate. For *Hp*FT the activity with LNT was 3.6 fold higher than with lactose. For *Hm*FT a 4.4-fold higher activity was reached with LNT. WbgL and *Cl*FT were only active when using LNT. No activity was detectable with lactose. However, Engels and Elling (2014) obtained a specific activity of 200 mU/mg with 10 mM lactose and WbgL.

Especially activities with the Nus-tagged *Hp*FT are higher than the published activities. It can therefore be assumed that the fusion partner NusA might have some influences on the specific activities of the α1,2-fucosyltransferases. For HpFT higher activity compared to literature reported values was detected while for WbgL, an enzyme that is already used for industrial 2'-FL production, the Nus-tagged form showed no activity on lactose anymore.

To clarify the influence of the tag on HpFT the NusA-tag was removed by TEV protease of the fusion product and the specific activity was measured afterwards, which is described in section 2.7.1.

3.4.2 Activity of *Bf*FKP

The activity of *Bf*FKP was measured as stated in section 2.7.2. 0.11 mg/mL enzyme was added to the reaction mixture.

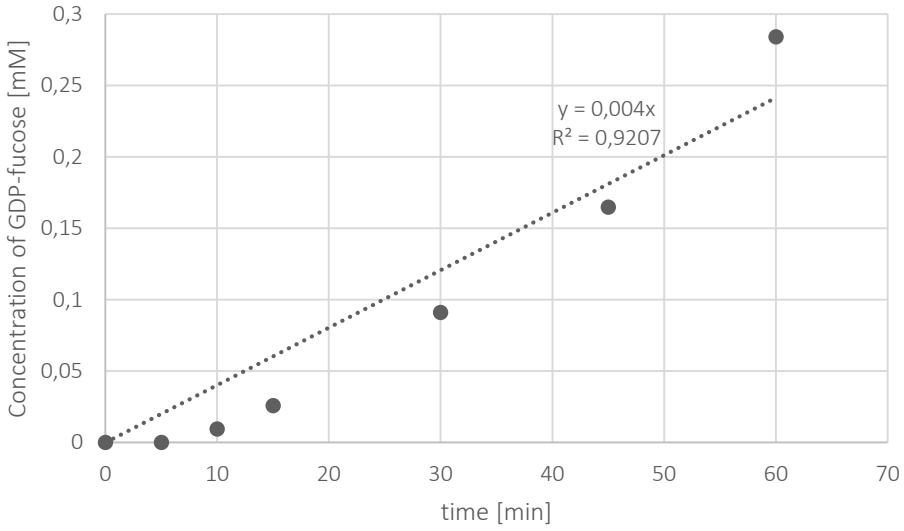

Figure 8: Activity measurement of *Bf*FKP. 5 mM L-fucose, 10 mM GTP, 5 mM ATP, 5 mM $MgCl_2$, 3 U iPPase and 0.11 mg/ml *Bf*FKP in 50 mM Tris-HCl (pH 7.6), mixed with 350 rpm at 37 °C. Samples were taken at 0, 5, 10, 15, 30, 45 and 60 minutes and analysed on HPLC.

The synthesis of GDP-fucose was measured via HPLC and is stated in Figure 8. Interestingly, there was no GDP-fucose formation within the first 5 minutes, only after 10 minutes product synthesis started and the enzyme seems to gain an increasing activity over time. Ignoring this increase of activity over time, a linear regression equation (y = 0.004x) would give an activity of the enzyme of 42.7 mU/mg. However, Wang et al. (2009) reached an activity of 4.5 U/mg. As the final product is built in two steps, an increasing accumulation of Fucose-1-phosphate might lead to an increased rate of the second step towards GDP-fucose. Incubating the enzyme before addition of GTP with ATP and fucose could be a solution to overcome this problem when measuring a total specific activity and is therefore demanded. However, for GDP-fucose production in an overnight reaction immediate optimal reaction rates are of no

importance and all substrates can be added from the beginning. For upscaled GDP-fucose syntheses more enzyme was used to get higher product concentrations.

3.5 Temperature stability

In order to test the optimal temperature for further conversion experiments enzyme stability at varying temperatures were tested. Therefore, the enzymes were incubated with 25 mM $MgCl_2$, 100 mM KCl, 50 mM lactose in 100 mM Tris-HCl (pH 7.0) at different temperatures (25, 30, 37 °C) and mixed at 300 rpm. The reaction mixture is stated in section 2.8. As conversion experiments were performed over 60 minutes and longer, the incubation time for stability tests was also 60 minutes. After incubation the specific activities of *Hp*FT and *Hm*FT were determined photometrically in standard activity assay setup (37°C), which is shown in Figure 9 and Figure 10.

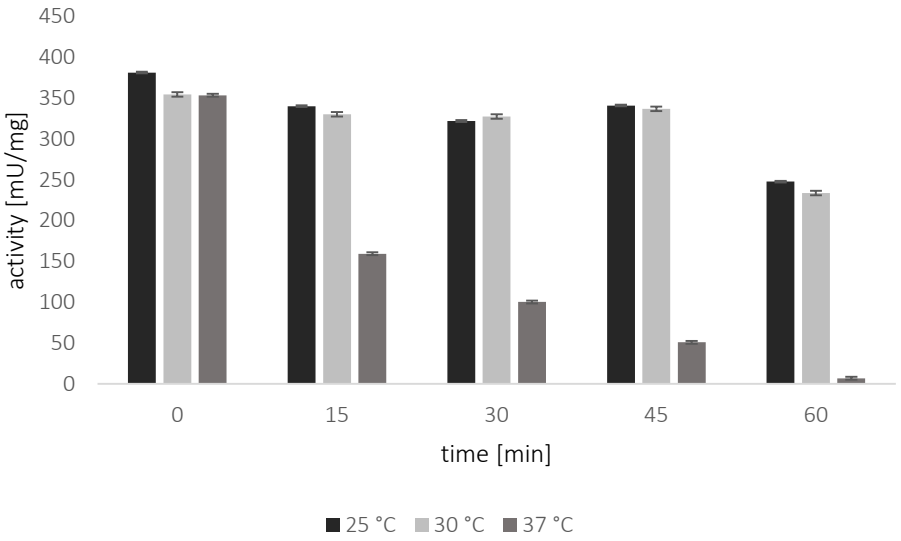

Figure 9: Effect of different temperatures on *Hp*FT activity. Measured photometrically after incubation for 60 minutes at indicated temperatures with 1 mM PEP, 0.66 mM NADH, 5 mM $MgCl_2$, 20 mM KCl, 5 U PK/LDH, 10 mM lactose, 1 mM GDP-fucose in 100 mM Tris-HCl (pH 7.0).

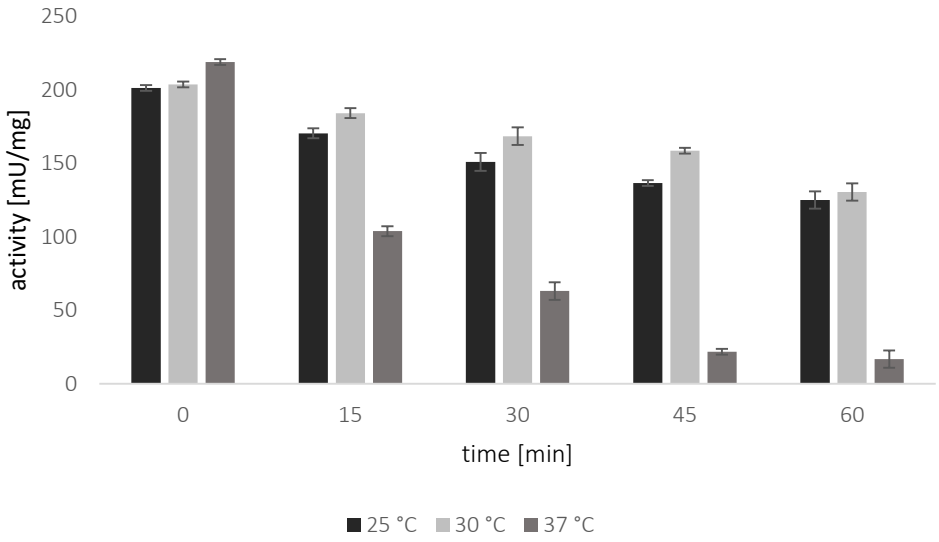

Figure 10: Effect of different temperatures on *Hm*FT activity. Measured photometrically after incubation for 60 minutes at indicated temperatures with 1 mM PEP, 0.66 mM NADH, 5 mM $MgCl_2$, 20 mM KCl, 5 U PK/LDH, 10 mM lactose, 1 mM GDP-fucose in 100 mM Tris-HCl (pH 7.0).

Both, *Hp*FT and *Hm*FT showed a dramatic activity drop of about 50 % already after 15 minutes when incubated at 37 °C. Only little differences were obtained when incubated at 25 °C and 30 °C. After 60 minutes for both enzymes the specific activity at 25 °C and 30 °C was around 63 % of the starting activity, whereas at 37 °C the remaining activity was almost 0 and enzyme precipitation was already observed. This high temperature will influence the photometer assay only to a minor extent, as only the first period (usually the first 2-3 minutes, maximum up to 5 minutes) is used for determining initial rates in activity assays of the FucTs.

Albermann et al. (2001) conducted reactions at 37 °C, this is why the temperature stability in this study was also tested at 37 °C but due to the given findings, further conversion experiments with *Hp*FT and *Hm*FT were performed at 30 °C in order to avoid enzyme denaturation and to achieve high conversion yields. The temperature for photometrical measurements was kept at 37°C to allow comparison with literature data.

3.6 Storage stability

To test the activity of α1,2-fucosyltransferase from *H. pylori* and *H. mustelae* over an extended storage time, the enzymes were stored at -70 °C without any stabilization substances in

enzyme concentrations of 2.2 mg/mL. The activity was repeatedly measured over 16 weeks and the results are stated in Figure 11.

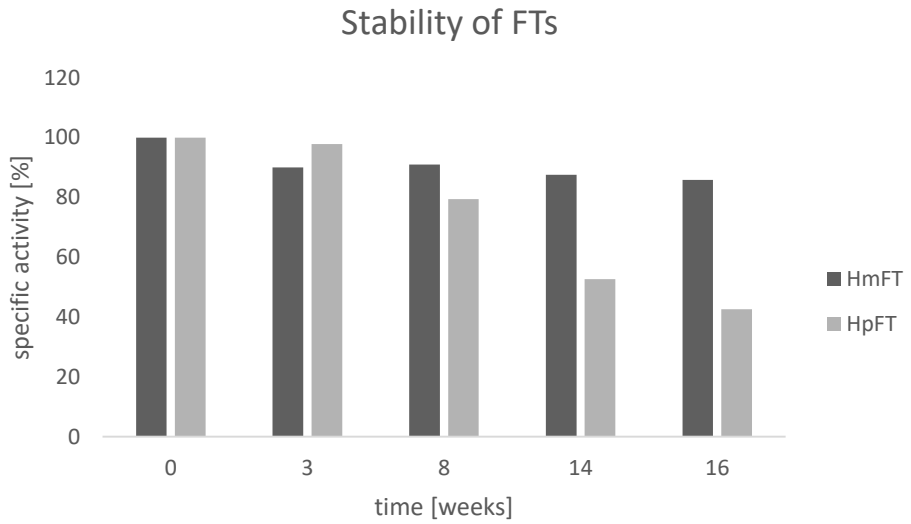

Figure 11: Specific activity [percentage] of *Hp*FT and *Hm*FT over time. The activity was measured photometrically at 340 nm at 37 °C. The reaction mixture contained 1 mM PEP, 0.66 NADH, 5 mM $MgCl_2$, 20 mM KCl, 10 mM lactose, 1 mM GDP-fucose, 5 U PK/LDH in 100 mM Tris-HCl (pH 7.0). 100 % *Hp*FT correspond to 380 mU/mg. 100 % *Hm*FT correspond to 201 mU/mg.

Whereas the specific activity of *Hm*FT remains nearly the same over time (only 14 % loss in 4 months), *Hp*FT showed activity losses after 16 weeks of storage at -70°C. Starting with a specific activity of 380 mU/mg only 43 % were remaining after 16 weeks. However, the activity of *Hp*FT after 16 weeks was nearly the same as the activity of *Hm*FT.

Conclusively, *Hm*FT showed a better storage stability in comparison to *Hp*FT. However, since the starting activity of *Hp*FT is almost twice as high in total remaining specific activity *Hm*FT is still outperformed by *Hp*FT for the first 2.5 months of storage. Therefore, freshly prepared *Hp*FT would be the preferred enzyme if looking for high specific activity, whereas for long-term storage *Hm*FT seems more favourable. Since the activity of *Hm*FT remains stable over time, the usage for experiments seems to be preferable, as specific activity measurements do not need to be calculated previous to each experiment. Stability in conversion experiments would need further investigation to gain a clear insight if *Hm*FT is in general more stable than *Hp*FT.

3.7 Removal of affinity tag

As stated in section 3.4.1, specific activities of Nus-tagged constructs were higher compared to published data where no NusA-tag was used. In order to check the influence of the NusA-tag on the specific activity, the fusion tag was removed by TEV-induced digestion. To determine the required incubation time for an efficient cleavage, different durations were compared. Furthermore, different amounts of added TEV protease were compared, to get the best conditions for cleaving off the NusA tag. One reaction mixture was carried out with 10 µL, a second one with 15 µL of TEV protease (commercial variant from Sigma-Aldrich). The cleavage efficiency after 14, 24 and 48 hours was examined via SDS-PAGE, which can be seen in Figure 12.

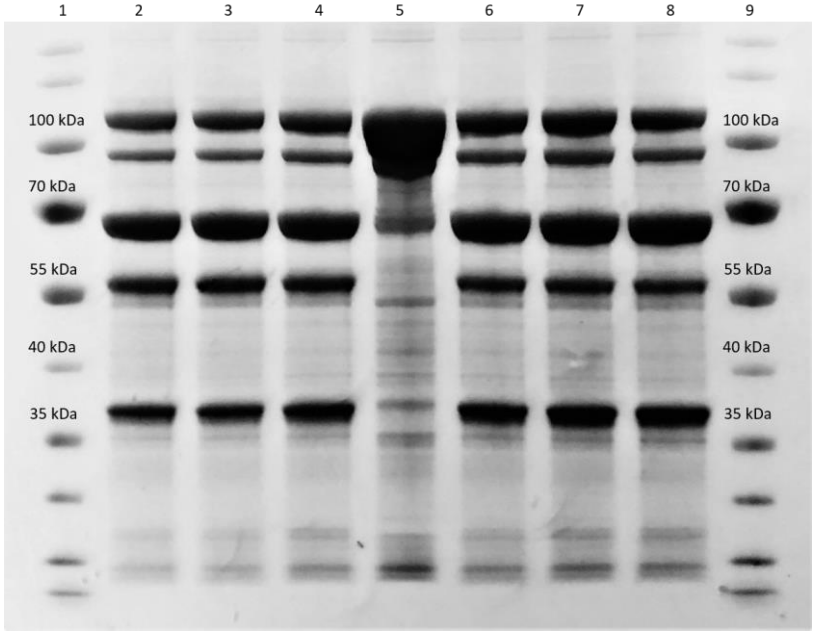

Figure 12: SDS-PAGE of TEV cleavage of recombinant TEV*Hp*FT with a 52 kDa TEV protease. Lane 1+9: pre-stained protein ladder, 2-4: cleavage with 15 µL TEV protease (14, 24, 48 h incubation at 4°C), 5: purified TEV*Hp*FT, 6-8: cleavage with 10 µL TEV protease (14, 24, 48 h incubation at 4°C). ~100 kDa: StrepNusTEV*Hp*FT, ~60 kDa: NusA-tag, ~55 kDa:TEV protease, ~35 kDa: TEVHpFT without NusA-tag.

As seen in the upper figure, double bands appear around 100 kDa. This is true for all purified TEV*Hp*FT and *Hp*FT constructs and can eventually be explained by frameshifts due to codon exchange. In Figure 12 these double bands belong to the uncleaved TEV*Hp*FT. Bands around 60 kDa indicate the cleaved Nus-tag. The TEV protease is visible at around 55 kDa and the lowest bands belong to the cleaved *Hp*FT without the Nus-tag.

According to the ImageJ results for band intensity determination there were no clear differences detectable regarding incubation time and the amount of added TEV protease. With all different cleavage conditions around 70 % of the added TEV*Hp*FT were cleaved. However, further cleavages were incubated for 14 hours and 10 µL of TEV protease were added to the reaction mixture. Based on Figure 12 it can be seen that for all combinations the cleavage was not performed completely. There was still uncleaved StrepNusTEV*Hp*FT in the reaction mixture, even after 48 hours (lines 4 and 8).

Another in-house produced TEV protease preparation (35 kDa) was alternatively used to execute the cleavage, which is shown in Figure 13. With this preparation the tag could almost completely cleaved off, about 9 % of the TEV*Hp*FT remained uncut. In order to calculate the activity of *Hp*FT without the NusA tag, the cleaved *Hp*FT from Figure 13 was added to the reaction assay directly from the cleavage reaction mixture and activity was measured photometrically. After the TEV cleavage the activity was lower, but still 76 % of the activity of the uncut construct were observed.

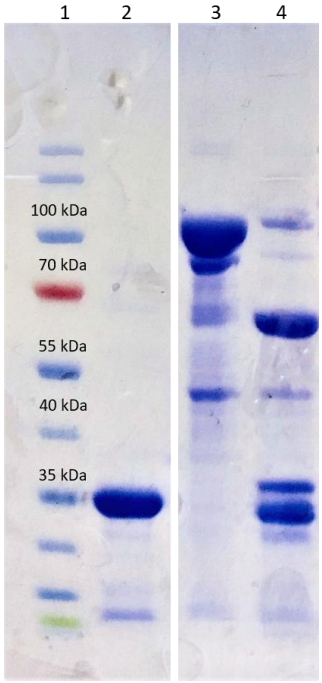

Figure 13: SDS-PAGE of TEV cleavage of recombinant TEV*Hp*FT with a 35 kDa TEV protease. Lane 1: pre-stained protein ladder, 2: TEV protease, 3: purified TEV*Hp*FT, 4: cleavage with 10 µL TEV protease. ~100 kDa: StrepNusTEV*Hp*FT, ~60 kDa: NusA-tag, ~35 kDa:TEV protease, ~35 kDa: TEVHpFT without NusA-tag.

Table 7: Specific activities measured photometrically with 1 mM GDP-fucose and 10 mM lactose. Activities of StrpNus*Hp*FT and StrpNusTEV*Hp*FT were measured after purification. The specific activity of *Hp*FT in the cleavage solution was measured after TEV cleavage.

	Activity [mU/mg]
StrpNus*Hp*FT	380.0
StrpNusTEV*Hp*FT	382.4
*Hp*FT in cleavage solution	292.9
*Hp*FT (referred to cleaved *Hp*FT)	1,360.0

Since the cleavage was never performed completely it is difficult to say, if the remaining specific activity refers to the uncut construct or to the cleaved *Hp*FT without NusA-tag. Therefore, intensities were used to calculate the activity of the cleaved *Hp*FT. Referred to the intensity of the uncleaved TEV*Hp*FT (9 % of total protein) and the cleaved *Hp*FT (19 %) the activity of HpFT without the Nus-tag was calculated to be 1.36 U/mg. This signifies an increase of about 256 % when compared to the activity of TEV*Hp*FT. This would implicate a negative effect of the fusion partner NusA but also an unexpectidly high activity for untagged HpFT, which needs further confirmation. The overall activity of 292 mU/mg in the solution with cleaved *Hp*FT was still clearly higher than published activities (Stein et al., 2008, Albermann et al., 2001).

Further optimization of cleavage and separation of *Hp*FT from the cleavage solution could be envisaged. Purifying *Hp*FT after cleavage via a His and a Strep column to get the FucT in a pure form would be an option.

3.8 Enzyme inactivation

Different methods for denaturation and inactivation of the α1,2-fucosyltransferases were investigated which comprised boiling, addition of MeOH or ACN, and acidification. For all four methods the inactivation was totally accomplished resulting in no residual activity of the enzyme, which was confirmed photometrically. Since it was important to obtain almost undiluted samples with high product concentrations for further HPLC analyses, heat inactivation was further on used to stop the enzymatic assay. By boiling the samples the lowest dilution rate (1:2) was possible. Accordingly, for all conversion assays boiling was used

as enzyme inactivation method. Using MeOH or ACN resulted in too high dilutions, whereby samples were unquantifiable as peaks were not integrable anymore.

3.9 Conversion experiments for 2'-FL production

Performance of StrepNus*Hp*FT and StrepNus*Hm*FT was also characterized in conversion experiments, where the production of 2´-fucosyllactose was measured. Therefore, 1–1.3 mM GDP-fucose and 10 mM lactose were used to produce 2´-fucosyllactose over time at 30 °C. Samples were taken at several time points and product formation was detected on HPLC. The first conversion assays were performed over 60 minutes and 0.5 mg/mL enzyme without adding CIP.

For *Hp*FT (Figure 14) the GDP-fucose (1.3 mM) was consumed completely after 30 minutes, whereas at the conversion with *Hm*FT (Figure 15) the whole GDP-fucose was already consumed after 10-15 minutes. These data also fit to the maximum product formation, which is achieved after similar conversion durations. For *Hp*FT this leads to a formation of 2´-fucosyllactose of 1 mM and for *Hm*FT of 0.93 mM, resulting in a 77 % and 70 % conversion yield. Gierlinger (2017) reached a maximum conversion yield of 15 % with *Hp*FT after 2 hours with similar reaction conditions. Conclusively, within this study the conversion yield of *Hp*FT was increased fivefold.

Donor hydrolysis is an important factor, which has to be considered. In Figure 14 and Figure 15 it can be seen that the GDP release gives higher GDP concentrations than the 2´-fucosyllactose formation. The difference between these two concentrations can be described by donor hydrolysis, where GDP is released but the fucosyl-transfer is not performed. Donor hydrolysis partly is caused by chemical decay of GDP-L-fucose but to a higher extent occurs as a side activity of the furcosyltransferases in parallel to the transfer reaction. At the given assay conditions *Hp*FT showed a hydrolysis rate of about 22.5 %, which means that from the available donor substrate 22.5 % are lost by hydrolysis while the other 77.5% were used for GDP transfer. This hydrolysis effect was also described in literature, where Gierlinger (2017) reported a photometrically measured hydrolysis rate of 29 % for *Hp*FT. *Hm*FT showed a hydrolysis rate of about 23.6 %.

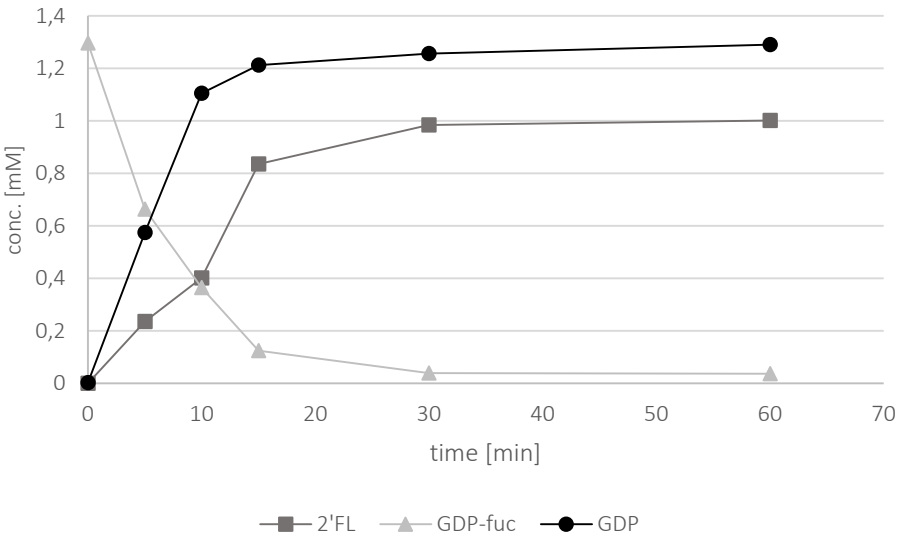

Figure 14: Conversion experiment for the production of 2'-fucosyllactose using StrpNus*Hp*FT. The assay was performed with 1.3 mM GDP-fuc, 10 mM lactose, 10 mM $MgCl_2$, 0.5 mg/mL *Hp*FT in 100 mM Tris-HCl (pH 7.0). Duration: 1 hour at 30 °C. 77 % conversion yield.

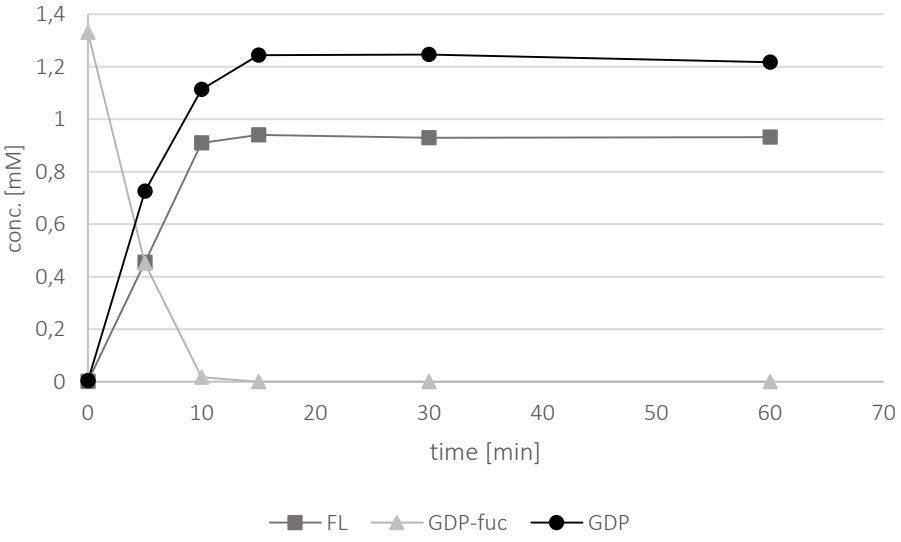

Figure 15: Conversion experiment for the production of 2'-fucosyllactose using StrpNus*Hm*FT. The assay was performed with 1.3 mM GDP-fuc, 10 mM lactose, 10 mM $MgCl_2$, 0.5 mg/mL *Hm*FT in 100 mM Tris-HCl (pH 7.0). Duration: 1 hour at 30 °C. 70 % conversion yield.

When using *Hp*FT higher conversion yields were gained, therefore it was used for further conversion assays. The conversion with *Hm*FT showed an increase of GDP after 10 minutes,

although there was no remaining GDP-fucose in the reaction mixture. This is probably due to quantificational issues.

In order to analyse the performance of *Hp*FT in fucosyllactose formation at lower enzyme concentrations over an extended reaction time in a following assay less enzyme was added to the reaction mixture. Again 10 mM lactose but 1 mM GDP-fucose were used as starting conditions. 0.15 mg/mL *Hp*FT and 1U CIP were added and the reaction mixture was incubated at 30 °C for 6 hours (Figure 16).

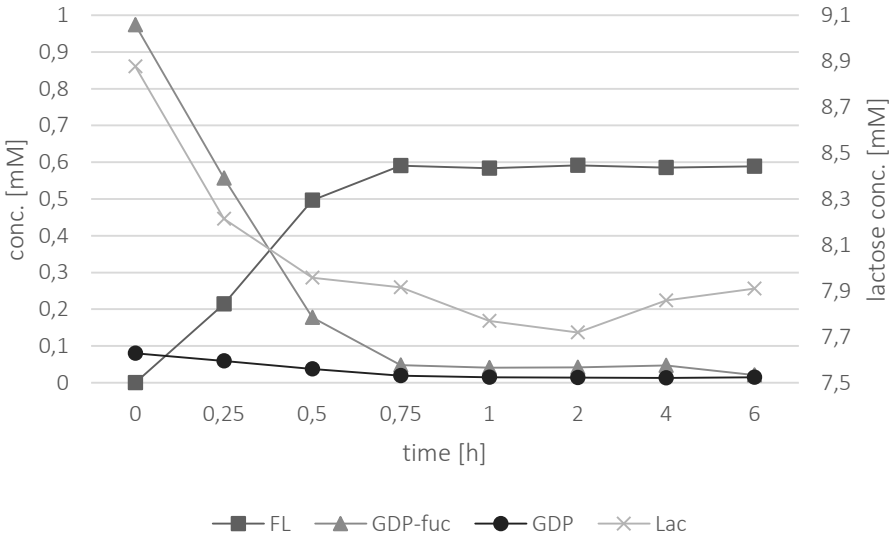

Figure 16: : Conversion experiment for the production of 2'-fucosyllactose using StrepNus*Hp*FT at 30 °C for 6 hours. The assay was performed with 1 mM GDP-fucose and 10 mM lactose, leading to 61 % conversion yield.

The lower amount of enzyme did not really effect the product formation. With 0.5 mg/mL *Hp*FT the maximum product concentration was reached after 30 minutes. By lowering the amount of *Hp*FT to 0.15 mg/mL the maximum product yield was reached after 45 minutes. This is also true when comparing the GDP-fucose data. 95 % of the GDP-fucose are consumed after 45 minutes. By calculating the activity from the first 30 minutes of the product formation curve results of about 110 mU/mg were achieved, which fits closely to the photometrical measured activity of *Hp*FT. After 6 hours 0.6 mM 2´-fucosyllactose were formed, which results in a 61 % conversion yield. This is lower when compared to the first conversion assay, where a yield of 77 % could be reached. The reason might be the prolonged duration of this experiment, which could go along with a chemical decay of GDP-L-fucose.

As CIP was added to the reaction, the GDP value should be zero over time, which was not true for the first 45 minutes. However, maximum accumulation concentrations were <0,1 mM which will not have a significant inhibitory effect. After 45 minutes GDP was degraded completely. Regarding the lactose quantification there are still some analytical problems which lead to scattering concentration results. As stated in Figure 16 the initial lactose concentration was 8.9 mM whereas 10 mM were added. A lactose decrease from 8.9 mM to 7.9 mM was analysed via HPLC, whereas only 0.6 mM product was formed. Accordingly, this decline was a little bit too high, which was referable to quantificational issues.

A further assay was performed with 0.15 mg/mL *Hp*FT, 10 mM lactose, 1 U CIP and 5 mM GDP-fucose. The reaction mixture was incubated for 6 hours at 30 °C. As shown in Figure 17 even after 6 hours no product formation was detectable. The GDP-fucose concentration remained relatively stable over time. Only a small decrease of about 12 % (0.6 mM) could be obtained after 6 hours of incubation. This finding could also be explained by chemical decay of GDP-L-fucose as already mentioned above.

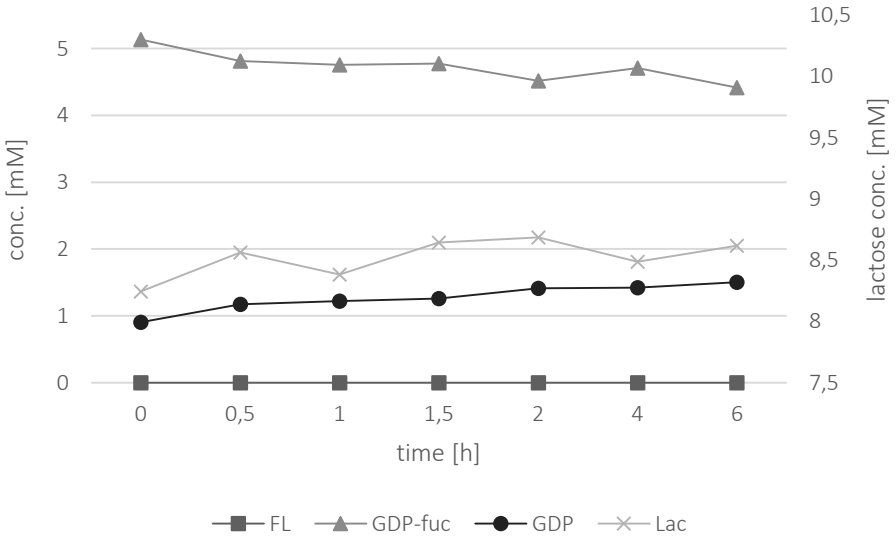

Figure 17: : Conversion experiment for the production of 2'-fucosyllactose using StrepNus*Hp*FT at 30 °C for 6 hours. The assay was performed with 5 mM GDP-fucose and 10 mM lactose, leading to no product formation.

Unexpectedly no 2´-fucosyllactose was formed within 6 hours of conversion. This observation can be explained by the lack of pH adjustment before adding the enzyme. The higher the GDP-fucose concentration the more the reaction mixture acidifies. In the assay with 5 mM GDP-

fucose the pH drops to 4.4, which is problematic for *Hp*FT. Stein et al. (2008) reported a pH optimum of about 5 with a sharp decline in activity between pH 4.0-5.0. The used CIP (NEB) has a pH optimum of around 8. Accordingly, the low pH in the reaction mixture results in precipitation and enzyme inactivation.

The results of the 5 mM conversion experiments clearly indicate that adjusting the pH is an essential and very important step when working with higher GDP-fucose concentrations.

3.10 Donor hydrolysis

Significant GDP-fucose hydrolysis activity of fucosyltransferases lower synthetic yields (Zhao et al., 2016; Gierlinger, 2017) but can be minimized by high acceptor substrate concentrations. Here, the rate of donor hydrolysis of *Hp*FT was determined at different lactose concentrations (1, 5, 10, 20 50 and 100 mM). In all set ups, a full conversion of GDP-fucose was achieved. After 4 hours the synthesis of 2´-FL was analysed on HPLC. (Figure 18).

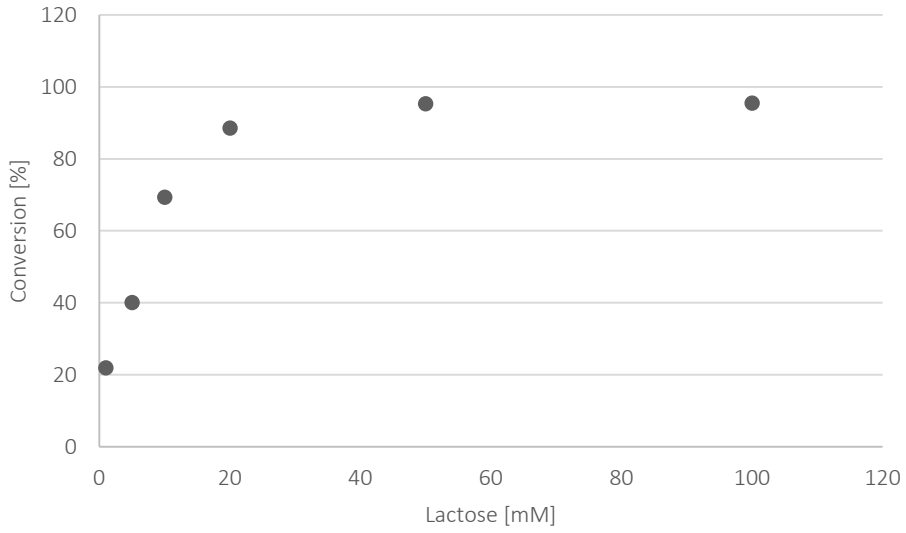

Figure 18: Influence of increasing lactose concentrations on the production of 2′-fucosyllactose. Reaction condition: 1 mM GDP-fucose, 1–100 mM lactose, 10 mM $MgCl_2$, 1 U CIP, 0.15 mg/mL *Hp*FT in 100 mM Tris-HCl (pH 7.0); 30 °C, 4 hours.

As seen in Figure 18 by increasing the lactose concentration to higher than 50 mM, the donor hydrolysis could be supressed almost completely and therefore the formation of 2´-fucosyllactose was increased more than 75 %, from 22% to over 95% conversion between 1 mM and 50 mM lactose starting concentration. With 100 mM lactose conversion rates were

not increased compared to 50 mM anymore. Gierlinger (2017) reported that at least 50 % of the donor substrate was hydrolysed when offering 100 mM lactose. In the current study the GDP-fucose hydrolysing activity was almost completely abolished by the addition of high concentrations of acceptor substrate, which was also reported by Stein et al. (2008). By offering 10 mM lactose the conversion yield in this series of experiments was around 70%, which is comparable to other conversion experiments using *Hp*FT.

Summarizing, the lactose concentrations higher than 50 mM could overcome the enzymatic hydrolysis of the donor substrate GDP-fucose almost entirely and led to over 95 % conversion of 1 mM GDP-fucose. There were no differences detectable between 50 mM and 100 mM lactose regarding the product formation. Accordingly, adding 50 mM acceptor substrate (lactose) would lead to significantly higher conversion yields in further conversion experiments.

3.11 Inhibition

In fucosyllactose synthesis reactions starting from GDP-L-fucose and leading to GDP as by-product inhibition of the fucosyltransferase by GDP or degradation products thereof is an important influence factor on finally reachable conversion yields. If the fucosyltransferase is to be combined with donor production from fucose by the action of *Bf*FKP, which depends on ATP and GTP, possible inhibition of the FucT by these compounds is also important to take into consideration in reaction design. To determine if the α1,2-fucosyltransferase *Hp*FT is inhibited by the guanosine nucleotides or guanosine, preliminary inhibition studies using GMP, GDP and guanosine were carried out. Inhibitory effects were examined via conversion experiments, where product formation after 4 hours was visualized by TLC (Figure 19). Additionally, inhibition effects of GTP, GMP, Guanosine and ATP were also measured photometrically to detect potential limitations of the *Hp*FT activity (Table 8).

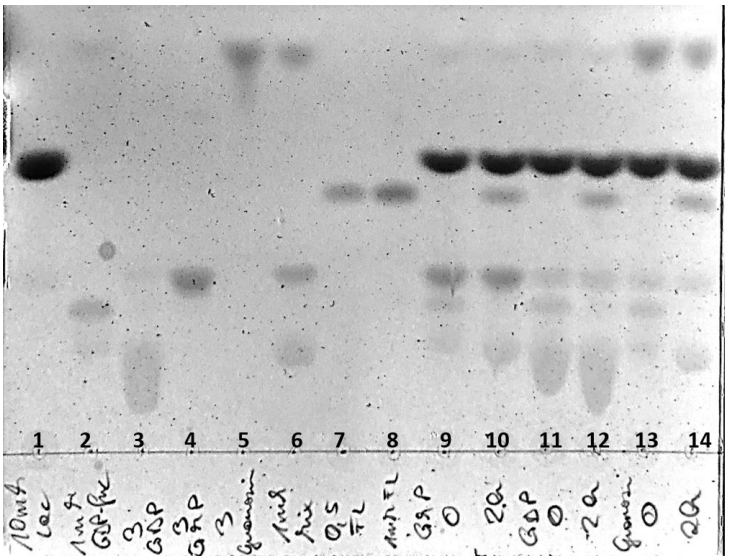

Figure 19: Thin liquid chromatography (TLC). Standard 1: 10 mM lactose, 2: 1 mM GDP-fucose, 3: 3 mM GDP, 4: 3 mM GMP, 5: 3 mM guanosine, 6: 1 mM GDP/GMP/guanosine mix, 7: 0.5 mM 2´-fucosyllactose, 8: 1 mM 2´-fucosyllactose. 9 + 10: reaction assay with 3 mM GMP at time point 0 and after 4 hours, 11 + 12: reaction assay with 3 mM GDP at time point 0 and after 4 hours, 13 + 14: reaction assay with 3 mM guanosine at time point 0 and after 4 hours.

The synthesis of 2´-fucosyllactose was detectable in all three conversion assays. With 3 mM GMP, GDP or guanosine product formation was detectable. According to the spotted 2´-fucosllactose standard on the TLC at least 0.5 mM product was formed with all three analogues. These results would indicate a possible inhibition effect. However, a yield of about 50 % was reached within this experiment. Ihara et al. (2006) reported that the mammalian α1,6-fucosyltransferase was competitively inhibited by GMP and Kötzler (2012) obtained data that showed a 10-fold higher affinity of GDP compared to that of GDP-fucose.
To sum up, hydrolysis products could affect the 2´-fucosyllactose formation. But with all three analogues product formation was possible.

Inhibition effects were also tested preliminarily by addition of nucleotides to the photometric assay. In Table 8 it can be seen that the activity of *Hp*FT is least affected by additionally 3 mM GMP, where 76.5 % residual activity was measured. By adding 3 mM guanosine to the photometer assay mixture the activity shows stronger inhibition and only 63.5 % of the original *Hp*FT activity are remaining. Nevertheless, on the TLC product formation with 3 mM guanosine was detectable. With 58.5 % and 53.4 % residual activity 3 mM GTP and 10 mM ATP show highest inhibiton of the activity of *Hp*FT. Interestingly by adding 10 mM GTP the activity

was increased by 14.2 %, which could not be further verified within the scope of this master thesis. Inhibition by GDP would have to be done in conversion experiments measured by HPLC.

Table 8: Inhibitory effects on the activity of *Hp*FT measured photometrically.

	mU/mg	%
*Hp*FT	274.19	100
*Hp*FT + 3 mM GTP	160.48	58.5
*Hp*FT + 3 mM GMP	209.88	76.5
*Hp*FT + 3 mM Guanosine	174.22	63.5
*Hp*FT + 10 mM ATP	146.30	53.4
*Hp*FT + 10 mM GTP	313.07	114.2

4 Summary

In this study an optimization of expressing soluble α1,2-fucosyltransferases was achieved, whereby detailed enzymatic characterization of FucTs was made possible. Expression of before not described *Hm*FT and *Cl*FT was achieved by adding the fusion partner NusA N-terminally to the transferase. Including results from this study, tagging with the NusA could be confirmed as successful strategy to assist soluble expression for in total six α1,2-fucosyltransferases by now. However, for StrepNusWcfb no expression could be achieved with this strategy.

Two Helicobacter α1,2-fucosyltransferases, *Hp*FT and *Hm*FT, were characterized in more detail. The expression temperature was optimized. Whereas expression results at 25 °C were doubled for *Hp*FT and *Hm*FT from about 10 % target enzyme in total protein of cell free extract to 20 %, for TEV*Hp*FT expression at 18 °C showed the best results with 18.3 % instead of 7.4 % at 25°C.

Furthermore, the specific activity of *Hp*FT and *Hm*FT was measured with lactose and LNT as acceptor substrate. StrepNus*Hp*FT reached 1,350 mU/mg when using LNT and 380 mU/mg with lactose. Compared to literature (30 – 80 mU/mg)(Stein et al., 2008; Albermann et al., 2001) this is the highest reported specific activity for *Hp*FT so far. When using *Hm*FT activities with LNT were around 880 mU/mg and with lactose around 200 mU/mg, which is lower than the activities measured with *Hp*FT but still higher compared to the other α1,2-fucosyltransferases. StrepNusWbgL and StrepNus*Cl*FT were only active on LNT but with 52 and 29 mU/mg they were not competitive and therefore not used for further experiments.

Since activities are clearly higher than published ones the influence of the fusion partner NusA was examined. By using the TEV protease the NusA-tag was cleaved off and the specific activity was then measured photometrically. A solution containing around 9% of total protein NusA-tagged *Hp*FT and 19% untagged *Hp*FT showed an activity of 293 mU/mg, which is lower than the fused construct (380 mU/mg) but still higher than published activities. When calculating the activity of the cleaved HpFT by using the band intensities determined via ImageJ, the specific activity of *Hp*FT without tag amounts to 1.36 U/mg. This signifies an increase of about 256 % and would implicate a negative effect of the fusion partner NusA on specific activity.

Storage stability experiments showed that *Hm*FT is more stable over time compared to *Hp*FT when stored at -80°C. Although *Hp*FT achieved higher activities, a constant activity decrease of around 50% over 16 weeks was registered. Further experiments were performed to determine the temperature stability of the two *α*1,2-fucosyltransferases. Results showed that a pre-incubation of the enzyme over 1 hour at 37 °C before application in the standard activity test reduces the activity to almost 0 % for both enzymes. *Hp*FT and *Hm*FT showed relatively constant specific activities when incubated at 30 °C. Due to this results long-term conversion assays should rather be performed at 30 °C than at 37 °C.

Conversion yields with *Hp*FT were increased from 15 % (Gierlinger, 2017) to 77 % (based on available donor concentration) starting from 10 mM lactose and 1 mM GDP-L-fucose. An almost 100 % conversion of GDP-L-fucose to 2´-fucosyllactose was reached with higher lactose concentrations (50 + 100 mM). Hence, hydrolysis of the donor substrate was supressed almost completely at increase acceptor concentrations.

Finally, inhibition effects of GMP, GDP and guanosine were tested. Results clearly indicate that product formation of about 50 % was observable within 4 hours with all three analogues on TLC. The activity of *Hp*FT was inhibited the most by adding 3 mM GTP and 10 mM ATP to the photometer assay. Here only 50 % residual activity was measured.

As the high price of commercially available GDP-L-fucose hampers detailed enzymatic characterization experiments, the donor substrate was synthesized. A purity of 99.6 % was reached, but substantial losses were obtained during downstreaming. Especially the anion exchange step is in great need of improvement. However, the overnight storage of the GDP-fucose containing solution after IEC resulted in no further losses. Therefore, the high salt concentration is not harmful for the nucleotide sugar.

5 Appendix

Nucleotide sequence of StrepNus*Hp*FT (mutations are marked in bold)

ATGGCTAGCTGGAGCCACCCGCAGTTCGAGAAAGGCTTAATTAACAACAAAGAAATTTTGGCTGTAGTTGAAG
CCGTATCCAATGAAAAGGCGCTACCTCGCGAGAAGATTTTCGAAGCATTGGAAAGCGCGCTGGCGACAGCAA
CAAAGAAAAAATATGAACAAGAGATCGACGTCCGCGTACAGATCGATCGCAAAAGCGGTGATTTTGACACTTT
CCGTCGCTGGTTAGTTGTTGATGAAGTCACCCAGCCGACCAAGGAAATCACCCTTGAAGCCGCACGTTATGAA
GATGAAAGCCTGAACCTGGGCGATTACGTTGAAGATCAGATTGAGTCTGTTACCTTTGACCGTATCACTACCCA
GACGGCAAAACAGGTTATCGTGCAGAAAGTGCGTGAAGCCGAACGTGCGATGGTGGTTGATCAGTTCCGTGA
ACACGAAGGTGAAATCATCACCGGCGTGGTGAAAAAAGTAAACCGCGACAACATCTCTCTGGATCTGGGCAA
CAACGCTGAAGCCGTGATCCTGCGCGAAGATATGCTGCCGCGTGAAAACTTCCGCCCTGGCGACCGCGTTCGT
GGCGTGCTCTATTCCGTTCGCCCGGAAGCGCGTGGCGCGCAACTGTTCGTCACTCGTTCCAAGCCGGAAATGC
TGATCGAACTGTTCCGTATTGAAGTGCCAGAAATCGGCGAAGAAGTGATTGAAATTAAAGCAGCGGCTCGCG
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Nucleotide sequence of StrepNus*Hm*FT

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Nucleotide sequence of StrepNusWcfB

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Nucleotide sequence of StrepNusTEV*Hp*FT

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Nucleotide sequence of StrepNusWbgL

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Nucleotide sequence of StrepNus*Cl*FT

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Nucleotide sequence of *Bf*FKP

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Amino acid sequence of StrepNus*Hp*FT

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Amino acid sequence of StrepNus*Hm*FT

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Amino acid sequence of StrepNusWcfB

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Amino acid sequence of StrepNusWbgL

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Amino acid sequence of StrepNusTEV*Hp*FT

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Amino acid sequence of StrepNus*Cl*FT

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Amino acid sequence of *Bf*FKP

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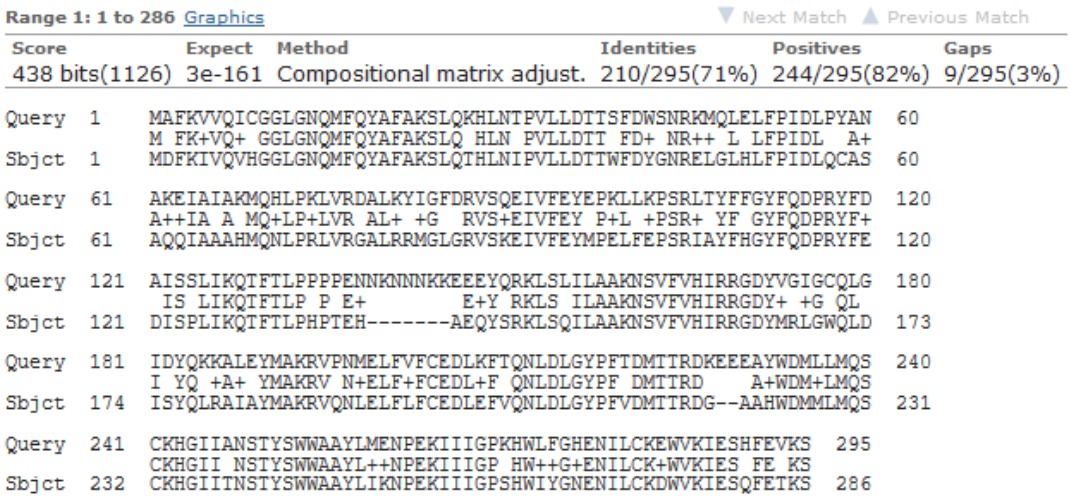
Sequence alignment of *Hp*FT and *Hm*FT



Range 1: 1 to 286 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
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            A++IA A MQ+LP+LVR AL+ +G  RVS+EIVFEY P+L +PSR+ YF GYFQDPRYF+
Sbjct  61   AQQIAAAHMQNLPRLVRGALRRMGLGRVSKEIVFEYMPELFEPSRIAYFHGYFQDPRYFE  120

Query  121  AISSLIKQTFTLPPPPENNKNNNKKEEEYQRKLSLILAAKNSVFVHIRRGDYVGIGCQLG  180
             IS LIKQTFTLP P E+         E+Y RKLS ILAAKNSVFVHIRRGDY+ +G QL
Sbjct  121  DISPLIKQTFTLPHPTEH--------AEQYSRKLSQILAAKNSVFVHIRRGDYMRLGWQLD  173

Query  181  IDYQKKALEYMAKRVPNMELFVFCEDLKFTQNLDLGYPFTDMTTRDKEEEAYWDMLLMQS  240
            I YQ +A+ YMAKRV N+ELF+FCEDL+F QNLDLGYPF DMTTRD    A+WDM+LMQS
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Query  241  CKHGIIANSTYSWWAAYLMENPEKIIIGPKHWLFGHENILCKEWVKIESHFEVKS  295
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Sbjct  232  CKHGIITNSTYSWWAAYLIKNPEKIIIGPSHWIYGNENILCKDWVKIESQFETKS  286
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Figure 20: Alignment of the coding sequences of *Hp*FT and *Hm*FT using the BLAST sequence alignment software. The protein sequences showed a homology of 71 %.

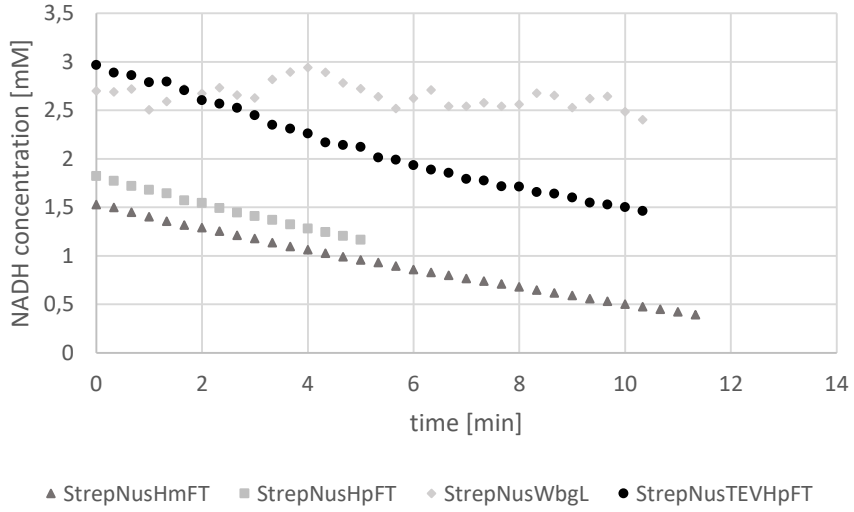

Figure 21: PK/LDH-assay to compare the specific activities of various *α*1,2-fucosyltransferases. 10 mM lactose was used as acceptor substrate. Triangle: activity of StrepNus*Hm*FT, square: activity of StrepNus*Hp*FT, diamond: activity of StrepNusWbgL, circle: activity of StrepNusTEV*Hp*FT.

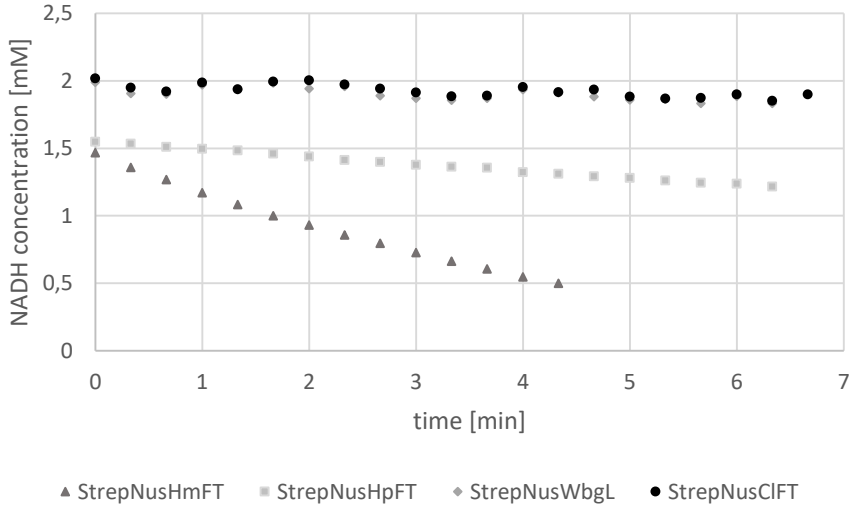

Figure 22: PK/LDH-assay to compare the specific activities of various *α*1,2-fucosyltransferases. 1 mM LNT was used as acceptor substrate. Triange: activity of StrepNus*Hm*FT, square: activity of StrepNus*Hp*FT, diamond: activity of StrepNusWbgL, circle: activity of StrepNus*Cl*FT.

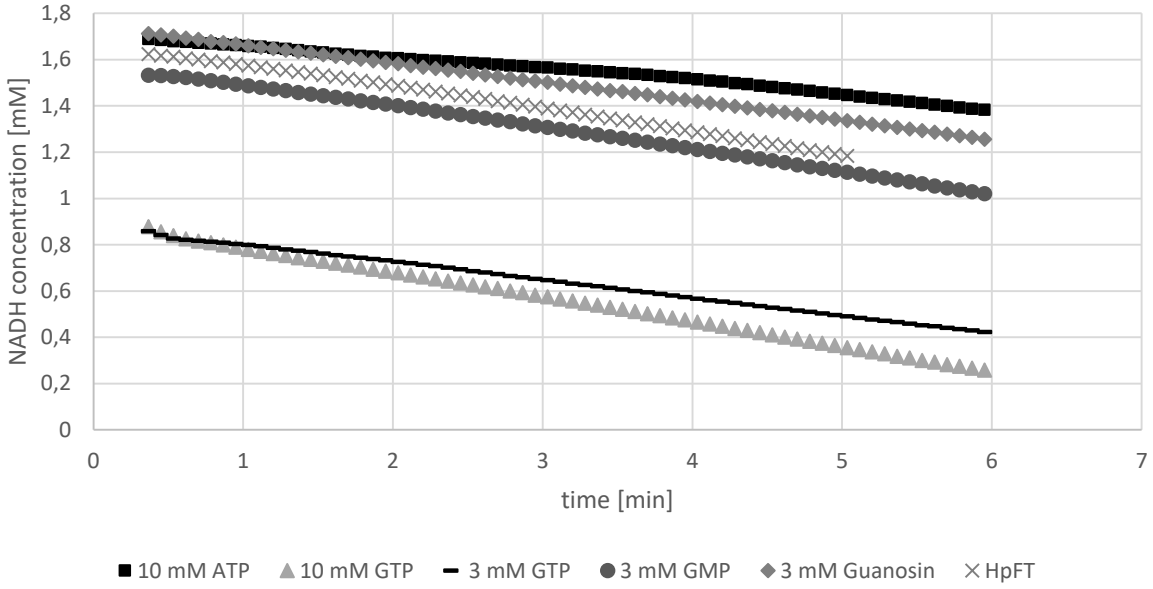

Figure 23: PK/LDH-assay to determine the inhibition effects of GTP, GMP, Guanosine and ATP on the activity of the *α*1,2-fucosyltransferase *Hp*FT.

Purification of *Hp*FT

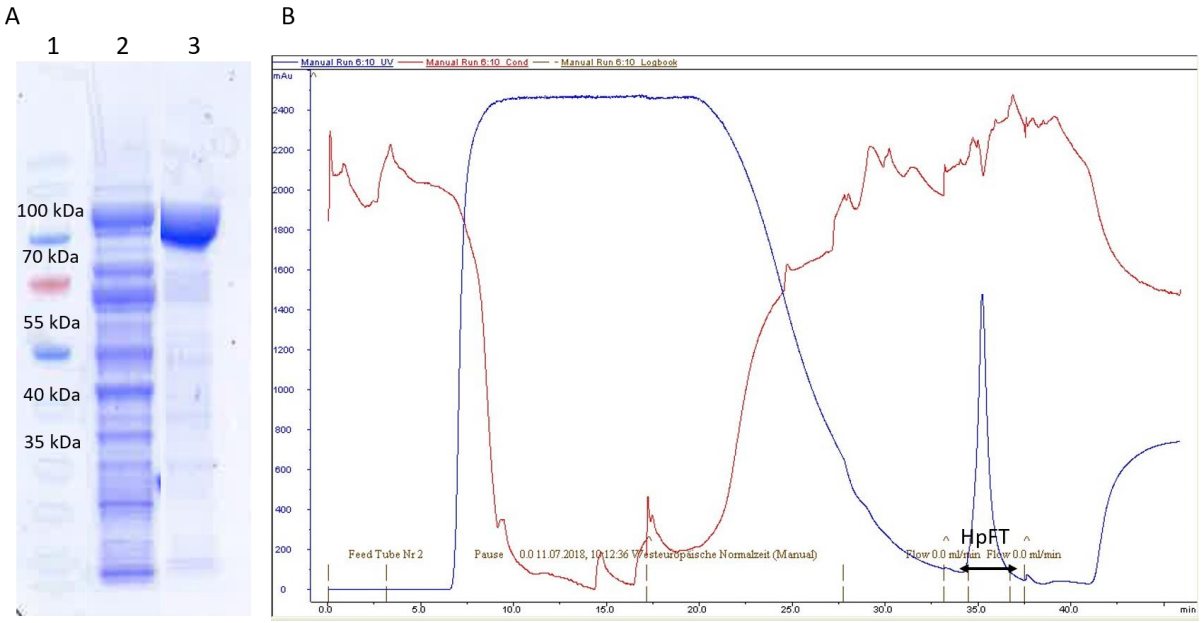

Figure 24: Purification of *Hp*FT. SDS-PAGE (A) and UV-signal (B, blue) and conductivity (red) for the purification of the Strep-tagged *Hp*FT. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: *Hp*FT soluble fraction, 3: *Hp*FT purified. The *Hp*FT has a size of 95.2 kDa.

Purification of *Hm*FT

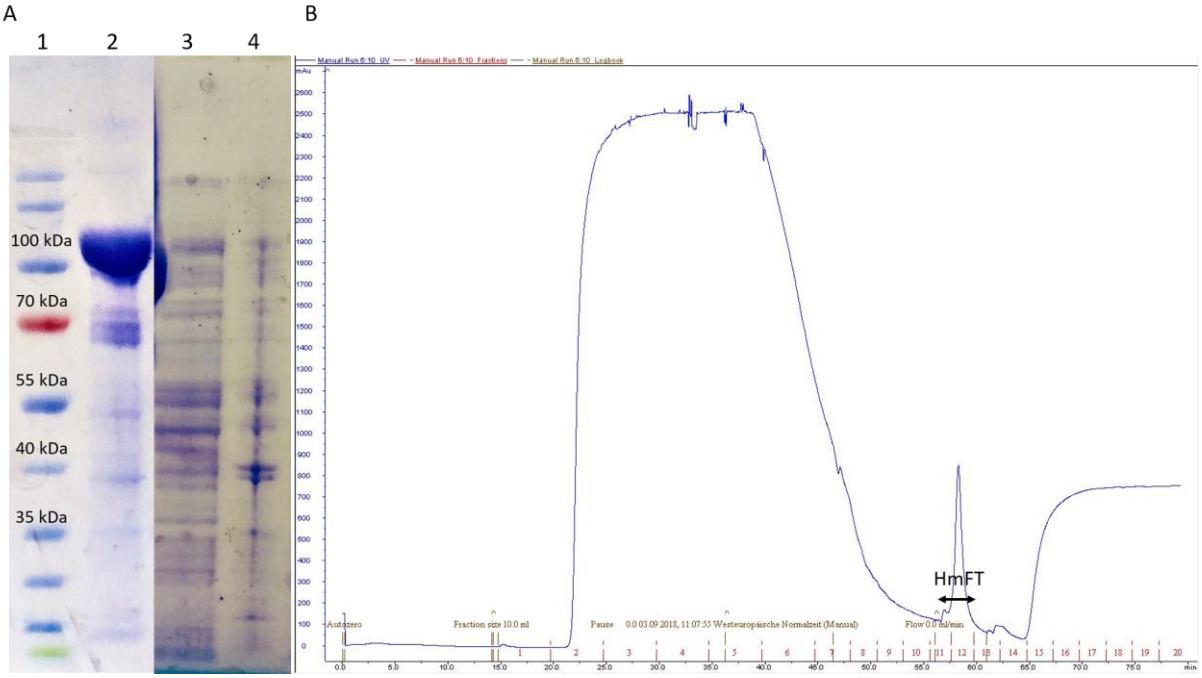

Figure 25: Purification of *Hm*FT. SDS-PAGE (A) and UV-signal (B, blue) for the purification of the Strep-tagged *Hm*FT. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: *Hm*FT purified, 3: *Hm*FT soluble fraction, 4: *Hm*FT pellet fraction. The *Hm*FT has a size of 94 kDa.

Purification of WbgL

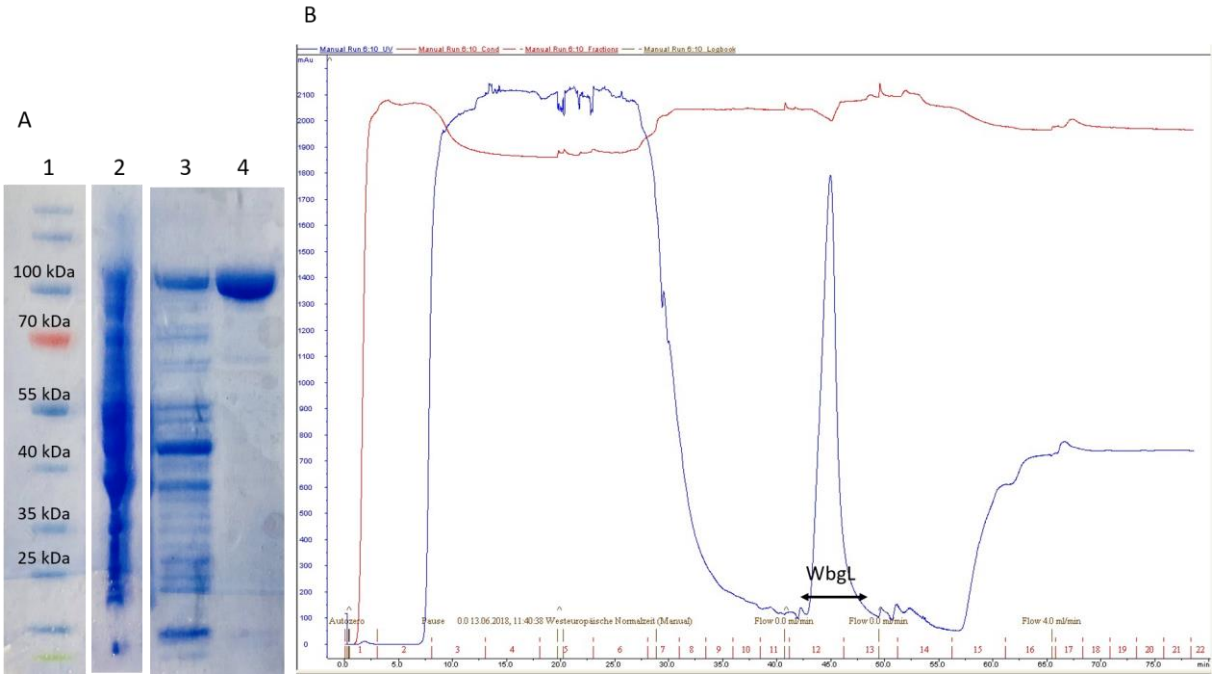

Figure 26: Purification of WbgL. SDS-PAGE (A) and UV-signal (B, blue) and conductivity (red) for the purification of the Strep-tagged WbgL. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: WbgL pellet fraction, 3: WbgL soluble fraction, 4: WbgL purified. The WbgL has a size of 95.8 kDa.

Purification of TEV*Hp*FT

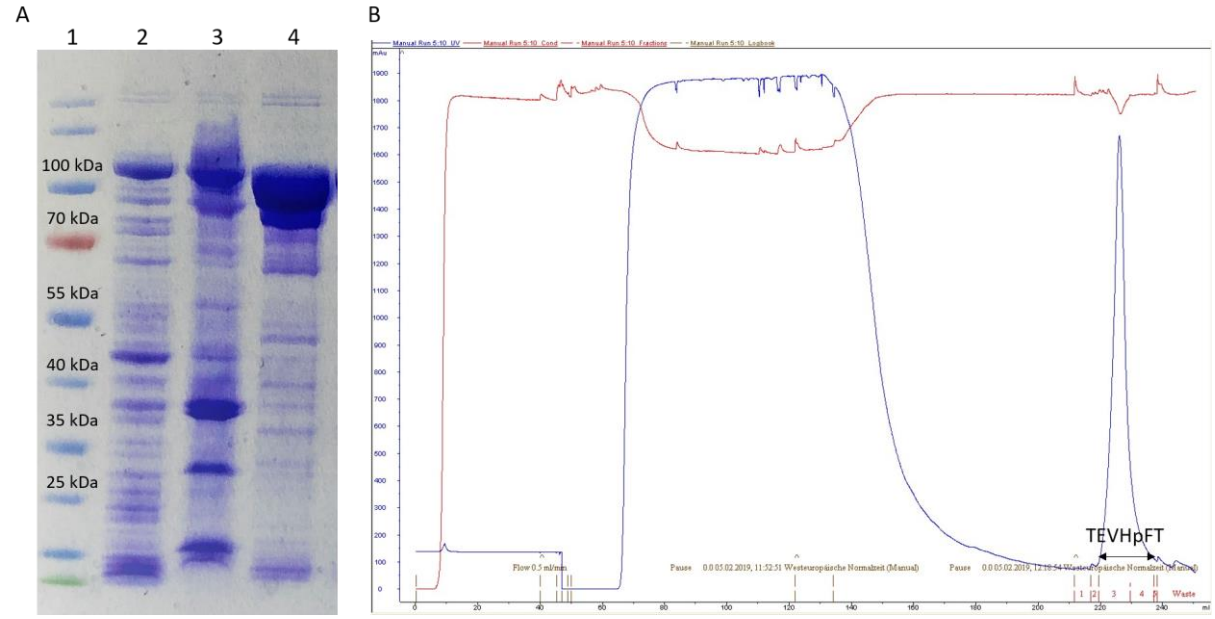

Figure 27: Purification of TEV*Hp*FT. SDS-PAGE (A) and UV-signal (B, blue) and conductivity (red) for the purification of the Strep-tagged TEV*Hp*FT. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: TEV*Hp*FT soluble fraction, 3: TEV*Hp*FT pellet fraction, 4: TEV*Hp*FT purified. The TEV*Hp*FT has a size of 91.8 kDa.

Purification of *Cl*FT

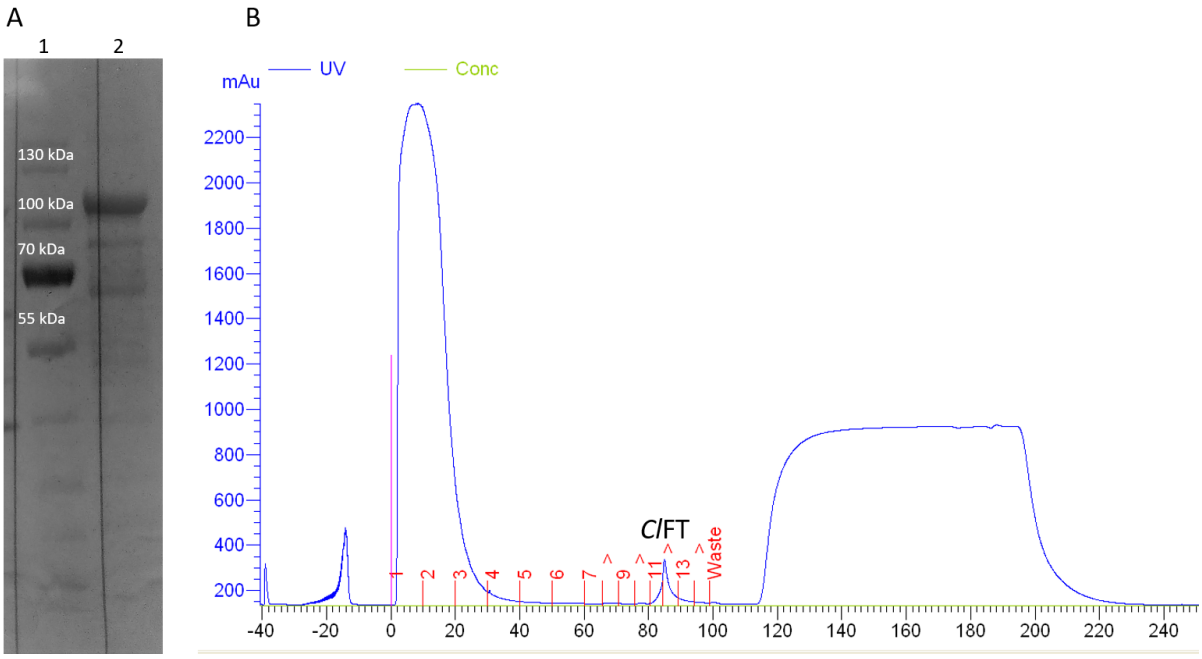

Figure 28: Purification of *Cl*FT. SDS-PAGE (A) and UV-signal (B) for the purification of the Strep-tagged *Cl*FT. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: *Cl*FT purified. The *Cl*FT has a size of 94.5 kDa.

Purification of *Bf*FKP

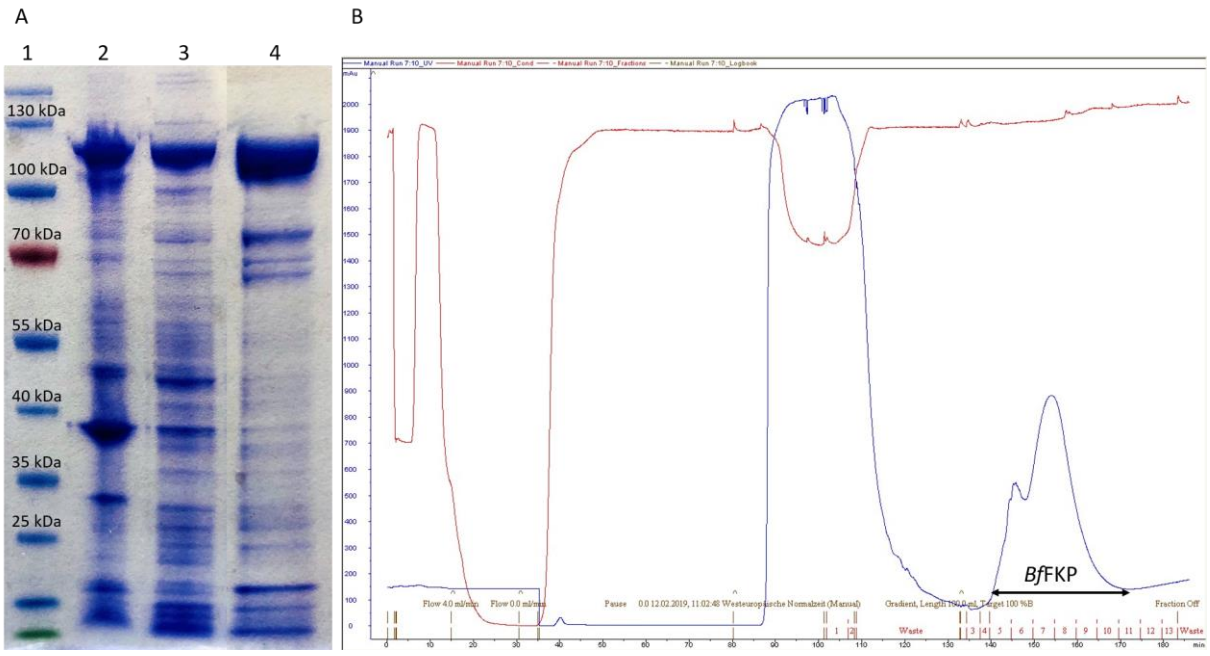

Figure 29: Purification of *Bf*FKP. SDS-PAGE (A) and UV-signal (B, blue) and conductivity (red) for the purification of the His-tagged *Bf*FKP. The eluted samples were collected, concentrated and analysed by SDS-PAGE. 1: prestained protein ladder, 2: *Bf*FKP pellet fraction, 3: *Bf*FKP soluble fraction, 4: *Bf*FKP purified. The *Bf*FKP has a size of 108 kDa.

IEC: GDP-fucose purification

At IEC 1 a stepwise gradient starting with 5 % B was executed. The concentration of B was then stepwise increased to at least 70 % B. Fractions 8 to 14 (peak 3) were collected for further purification steps. For the IEC 2 a gradient from 0 to 30 % B in 40 mL was chosen and fractions 27 to 32 were used for size exclusion. According to HPLC analysis fractions 33 to 37 contained solely GDP-fucose. For following GDP-fucose purifications these fractions should also be collected, which increases the total yield.

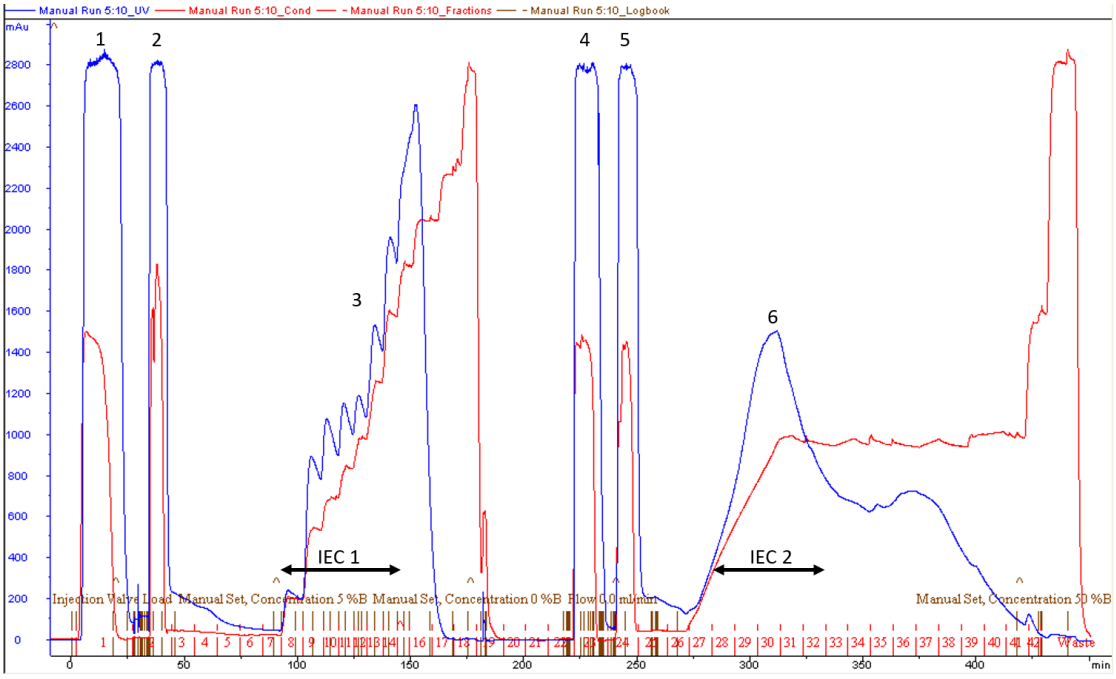

Figure 30: Chromatogram of anion exchange with 2x1 mL columns. Red curve: conductivity [mS/cm], blue curve: absorption at 254 nm [mAU], pink line: time point of injection. Peaks 1, 2, 4 and 5: flow through containing adenosine and guanosine, Peaks 3 and 6: GDP-fucose containing solution. x-axis: min, y-axis: mAU; mobile phase: buffer A (loading buffer) and buffer B (elution buffer), stationary phase: Toyopearl Super Q-650M.

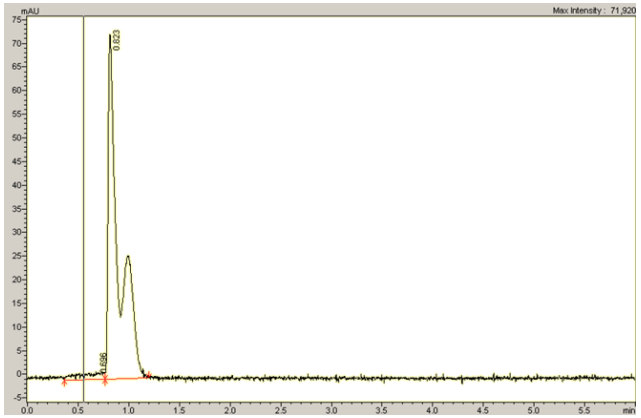

Figure 31: HPLC analysis of fraction 37 from the IEC monitored on Shimadzu using the conditions described in section 2.12.3. Double peaks were also obtained in standard solution.

SEC: GDP-fucose purification

For the SEC the injection was performed 6 times. Firstly, the GDP-fucose eluted (blue curve), followed by the salt (brown curve). The desalting of the nucleotide sugar worked well, no overlapping of the eluted GDP-fucose and the salt was obtained. GDP-fucose containing fractions were pooled and the volume was reduced on the rotary evaporator.

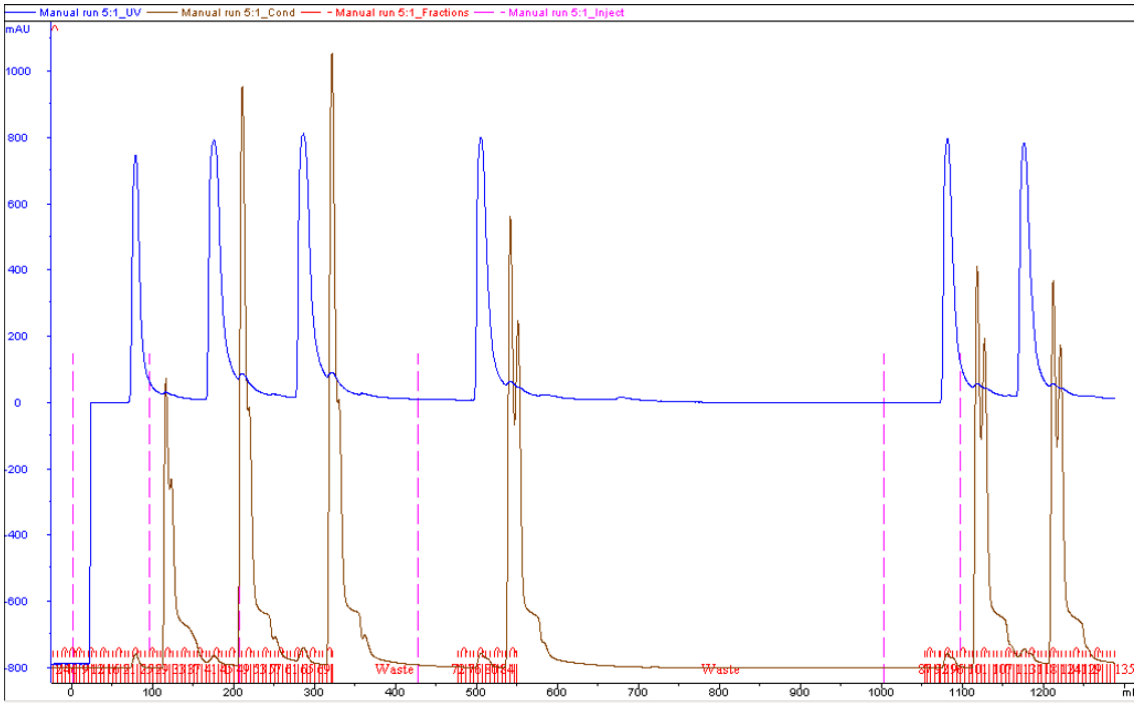

Figure 32: : Chromatogram of size exclusion. Pink line: time point of injection, blue curve: absorption at 254 nm [mAU], brown curve: conductivity [mS/cm], which indicates the elution of the salt. X-axis: mL, y-axis: mAU; mobile phase dH_2O, stationary phase: Sequadex G-10.

Conversion experiments for 2´-FL production at 37 °C

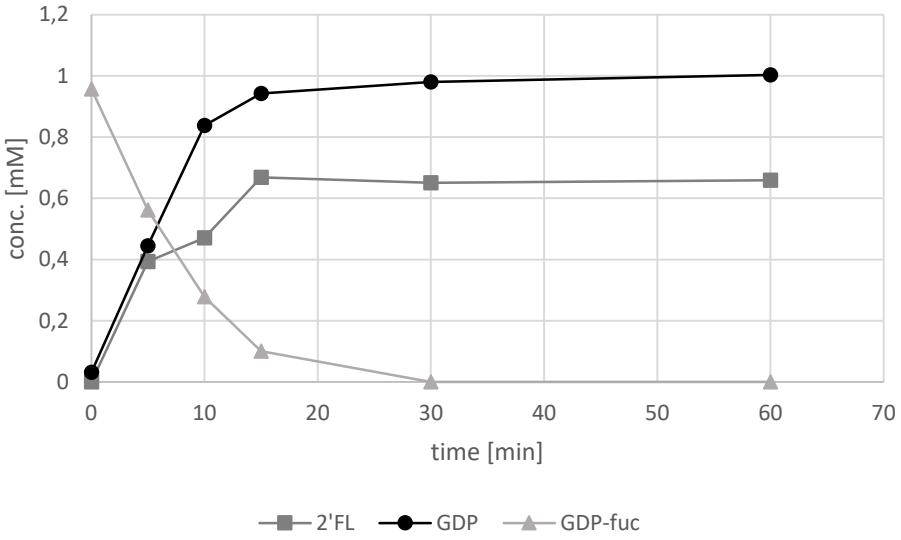

Figure 33: Conversion experiment for the production of 2'-fucosyllactose using StrpNus*Hp*FT. The assay was performed with 1 mM GDP-fuc, 10 mM lactose, 10 mM $MgCl_2$, 0.5 mg/mL *Hp*FT in 100 mM Tris-HCl (pH 7.0). Duration: 1 hour at 37 °C. 69 % conversion yield.

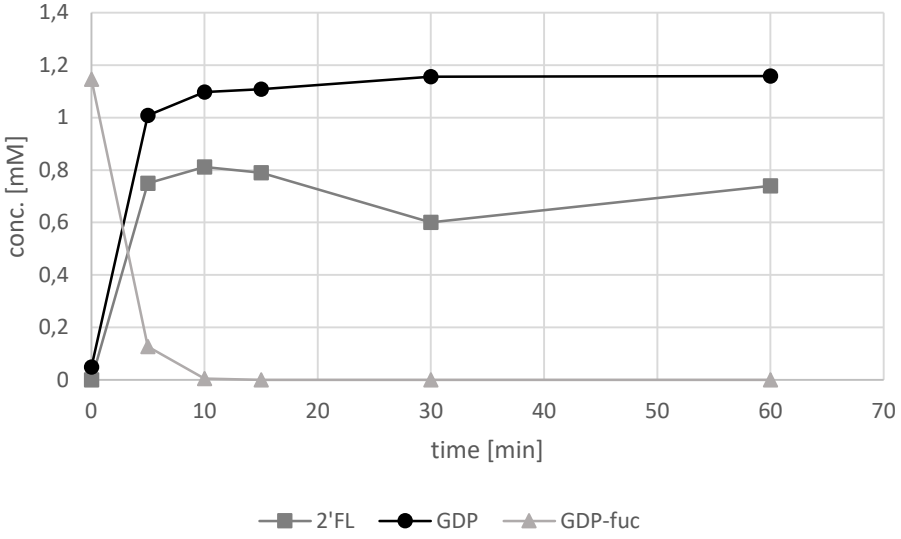

Figure 34: Conversion experiment for the production of 2'-fucosyllactose using StrpNus*Hm*FT. The assay was performed with 1 mM GDP-fuc, 10 mM lactose, 10 mM $MgCl_2$, 0.5 mg/mL *Hm*FT in 100 mM Tris-HCl (pH 7.0). Duration: 1 hour at 37 °C. 65 % conversion yield.

6 Supplementary data

Table 9: Determination of the intensity of the protein bands of StrepNusHpFT and StrepNusTEVHpFT with ImageJ to find the optimal expression temperature.

	peak area	[%]
*Hp*FT 18 °C	211858.876	100.0
	24363.7707	11.5
TEV*Hp*FT 18 °C	275569.119	100.0
	50429.1488	18.3
*Hp*FT 25 °C	247606.826	100.0
	50016.5789	20.2
TEV*Hp*FT 25 °C	194678.207	100.0
	14406.1873	7.4

Table 10: Determination of the intensity of the protein bands of the TEV cleavage with ImageJ. 15 µL TEV protease were used and different cleavage durations were tested.

	TEV 15µl 14h		TEV 15µl 24h		TEV 15µl 48h	
	peak area	[%]	peak area	[%]	peak area	[%]
sum	43274.725	100.0	50145.453	100.0	59116.273	100.0
uncut	6277.305	14.5	7517.134	15.0	8548.719	14.5
NusA	9911.426	22.9	10924.962	21.8	11226.205	19.0
TEV	8269.205	19.1	8874.79	17.7	9411.326	15.9
*Hp*FT	8100.205	18.7	8777.912	17.5	11064.447	18.7

Table 11: : Determination of the intensity of the protein bands of the TEV cleavage with ImageJ. 10 µL TEV protease were used and different cleavage durations were tested.

	TEV 10µl 14h		TEV 10µl 24h		TEV 10µl 48h	
	peak area	[%]	peak area	[%]	peak area	[%]
sum	74338.515	100.0	82477.021	100.0	76991.95	100.0
uncut	10060.184	13.5	11544.012	14.0	9174.355	11.9
NusA	13829.154	18.6	14241.033	17.3	14487.861	18.8
TEV	10517.912	14.1	10959.154	13.3	10578.619	13.7
*Hp*FT	13616.154	18.3	14458.154	17.5	13815.861	17.9

Table 12: Photometric assay for determination of specific activities of various α1,2-fucosyltransferases at 340 nm using 1 mM GDP-fucose and 10 mM lactose.

Time [min]	StrepNus*Hm*FT	StrepNus*Hp*FT	StrepNusWbgL	StrepNusTEV*Hp*FT
0.000	1.5278	1.8206	2.6985	2.9653
0.167	1.4954	1.7992	2.7215	2.9312
0.333	1.4975	1.7699	2.6875	2.8862
0.500	1.4589	1.7369	2.7826	2.8978
0.667	1.4465	1.7175	2.7181	2.8603
0.833	1.422	1.6933	2.572	2.9065
1.000	1.4016	1.6799	2.5025	2.7887
1.167	1.3778	1.6477	2.5798	2.778
1.333	1.3547	1.6409	2.5912	2.7948
1.500	1.3494	1.592	2.6151	2.7688
1.667	1.3161	1.5697	2.7209	2.7057
1.833	1.296	1.5543	2.7836	2.6883
2.000	1.2882	1.5423	2.6737	2.6039
2.167	1.2641	1.5057	2.5987	2.5831
2.333	1.2517	1.4917	2.7332	2.5666
2.500	1.2313	1.4689	2.5753	2.5796
2.667	1.2103	1.4451	2.6564	2.5231
2.833	1.1989	1.4188	2.5357	2.4379
3.000	1.1776	1.407	2.627	2.4493
3.167	1.151	1.3814	2.7433	2.3931
3.333	1.1339	1.3696	2.8179	2.3491
3.500	1.1141	1.3373	2.8521	2.3842
3.667	1.0928	1.3226	2.8936	2.3108
3.833	1.087	1.2953	2.8387	2.2721
4.000	1.0628	1.2804	2.9387	2.259
4.167	1.0443	1.2537	2.8827	2.2224
4.333	1.0243	1.2424	2.8908	2.1679
4.500	1.0093	1.2153	2.7232	2.1291
4.667	0.99	1.2026	2.783	2.1397
4.833	0.9743	1.1788	2.8449	2.1417
5.000	0.9565	1.1631	2.7214	2.1208
5.167	0.944	1.1408	2.7831	2.1004
5.333	0.9285	1.1281	2.6386	2.0115
5.500	0.9094	1.1078	2.7042	1.9994
5.667	0.893	1.1028	2.5185	1.9877
5.833	0.8724	1.0879	2.4343	2.0074
6.000	0.8574	1.0791	2.6232	1.9321
6.167	0.8423	1.0624	2.6851	1.9589
6.333	0.8286	1.0505	2.7085	1.8867
6.500	0.8124	1.0381	2.5811	1.8571
6.667	0.7979	1.0318	2.5396	1.8544

6.833	0.7856	1.0216	2.5783	1.8449
7.000	0.7655	1.0139	2.5416	1.7921
7.167	0.7566	0.9984	2.5928	1.8147
7.333	0.7382	0.9892	2.5752	1.7749
7.500	0.7241	0.9806	2.5539	1.7604
7.667	0.7083	0.9729	2.5388	1.7149
7.833	0.6885	0.9571	2.6542	1.7039
8.000	0.6781	0.9573	2.5591	1.7102
8.167	0.6627	0.9439	2.7452	1.6806
8.333	0.646	0.9368	2.6753	1.6573
8.500	0.6307	0.9281	2.546	1.6619
8.667	0.6168	0.9208	2.6523	1.6384
8.833	0.6024	0.9063	2.5328	1.6306
9.000	0.5877	0.9013	2.5281	1.5979
9.167	0.5722	0.8886	2.7255	1.5695
9.333	0.5572	0.8812	2.6201	1.5451
9.500	0.545	0.8731	2.7514	1.5487
9.667	0.5287	0.8662	2.6412	1.526
9.833	0.5179	0.8601	2.7052	1.5263
10.000	0.5007	0.8482	2.4849	1.499
10.167	0.4876	0.8406	2.4973	1.4957
10.333	0.4746	0.833	2.4008	1.4625
10.500	0.4578	0.827	2.336	1.4517

Table 13: Photometric assay for determination of specific activities of various α1,2-fucosyltransferases at 340 nm using 1 mM GDP-fucose and 1 mM LNT.

Time [min]	StrepNus*Hp*FT	StrepNus*Hm*FT	StrepNusWbgL	StrepNus*Cl*FT
0.000	1.5493	1.4692	1.9894	2.0181
0.167	1.5308	1.4189	1.9257	1.9487
0.333	1.534	1.3593	1.9068	1.9489
0.500	1.5174	1.3135	1.9108	1.9438
0.667	1.51	1.2679	1.9011	1.9221
0.833	1.5087	1.2289	1.9834	1.9836
1.000	1.4962	1.1716	1.9714	1.9885
1.167	1.4993	1.1283	1.9286	1.9436
1.333	1.4849	1.0846	1.9357	1.9367
1.500	1.477	1.0502	1.9195	1.9471
1.667	1.4603	1.0016	1.9841	1.9948
1.833	1.4416	0.9645	1.9237	1.9433
2.000	1.4407	0.932	1.9429	2.0033
2.167	1.4246	0.8944	1.9107	1.925
2.333	1.4137	0.8589	1.959	1.973

2.500	1.4141	0.8281	1.9697	1.9854
2.667	1.3994	0.7958	1.8893	1.9416
2.833	1.3854	0.7642	1.9402	1.946
3.000	1.379	0.7289	1.8725	1.9129
3.167	1.3683	0.6998	1.9199	1.9401
3.333	1.3626	0.6638	1.8577	1.8847
3.500	1.3589	0.6342	1.9155	1.9504
3.667	1.3555	0.6059	1.871	1.8896
3.833	1.3471	0.5756	1.8576	1.8801
4.000	1.3236	0.5476	1.936	1.9554
4.167	1.3223	0.5225	1.8912	1.8941
4.333	1.3121	0.5002	1.911	1.9171
4.500	1.3051	0.4626	1.8628	1.8768
4.667	1.2922	0.4354	1.8829	1.9345
4.833	1.2834	0.4062	1.9005	1.9371
5.000	1.2808	0.3757	1.8596	1.8839
5.167	1.2719	0.3497	1.8835	1.9086
5.333	1.2628	0.3345	1.865	1.8693
5.500	1.2582	0.3263	1.8897	1.922
5.667	1.2447	0.3181	1.8338	1.8742
5.833	1.2383	0.312	1.8699	1.8997
6.000	1.2382	0.3125	1.885	1.8994
6.167	1.2214	0.3077	1.8279	1.8409
6.333	1.2171	0.3054	1.8334	1.852

Table 14: Synthesis of GDP-fucose over time measured on HPLC using Kinetex C18 for determination of *Bf*FKP activity. Samples were measured in duplicates.

Time [min]	Peak area	Conc. [mM]	Average conc. [mM]
0	0	0.000	0.000
	0	0.000	
5	0	0.000	0.000
	0	0.000	
10	5542	0.011	0.009
	3861	0.008	
15	15361	0.031	0.026
	10378	0.021	
30	49764	0.100	0.091
	41242	0.082	
45	86564	0.173	0.165
	78154	0.156	
60	142014	0.284	0.284

Table 15: Detection of 2´-fucosyllactose synthesis, GDP-fucose conversion and GDP release. 2´-fucosyllactose concentrations were measured on HPLC using Aminex H87-column. GDP-fucose and GDP concentrations were measured on HPLC using Kinetex C18. Conversion was performed with 1 mM GDP-fucose and 0.5 mg/mL StrepNus*Hp*FT.

	Peak area			Concentration [mM]		
Time [min]	2´-FL	GDP-fucose	GDP	2´-FL	GDP-fucose	GDP
0	0.00	1364768	118904	0.000	1.297	0.125
	0.00	1264608	111760	0.000	1.202	0.117
5	1685.54	698032	547792	0.263	0.663	0.575
	1341.03	699840	563352	0.209	0.665	0.592
10	2865.42	383896	1053056	0.447	0.365	1.106
	2279.74	267352	1115192	0.356	0.254	1.171
15	5302.48	130720	1246464	0.827	0.124	1.309
	5422.03	55256	1229408	0.846	0.053	1.291
30	6942.71	20784	1431864	1.083	0.020	1.503
	4576.58	41296	1319440	0.714	0.039	1.385
60	7194.54	33688	1466656	1.122	0.032	1.540
	5658.64	39128	1302456	0.882	0.037	1.368

Table 16: Slope of standard calibration curve for calculation of the above stated concentrations.

2´-fucosyllactose	6412.2
GDP-fucose	1052112
GDP	952356

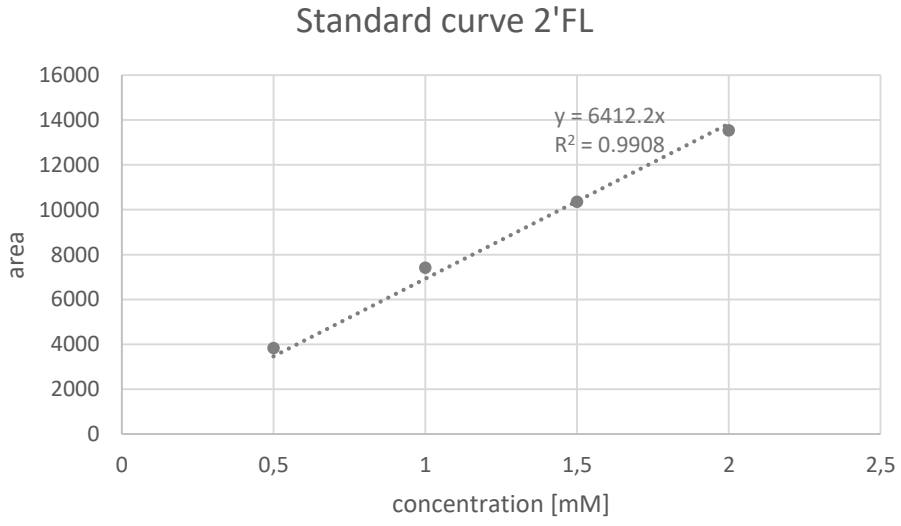

Figure 35: Standard curve of 2´-fucosyllactose measured on HPLC using Aminex H87-column.

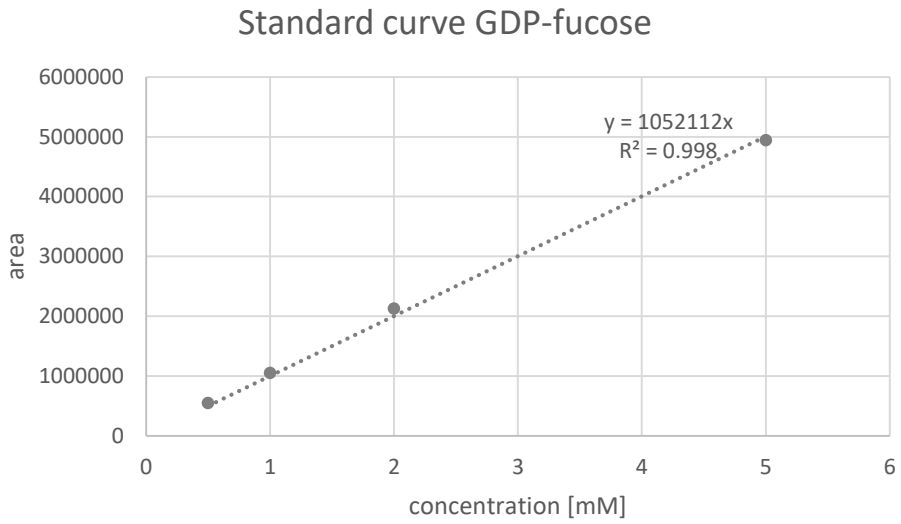

Figure 36: Standard curve of GDP-fucose measured on HPLC using Kinetex C18.

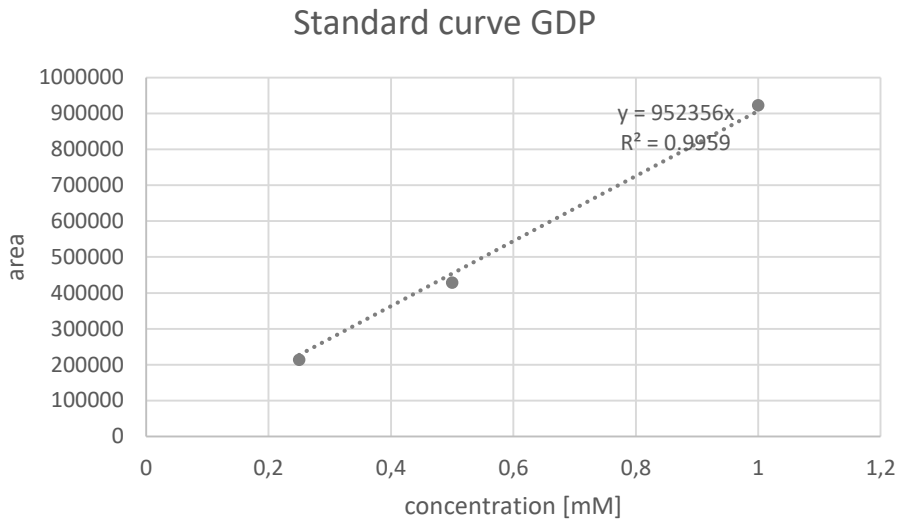

Figure 37: Standard curve of GDP measured on HPLC using Kinetex C18.

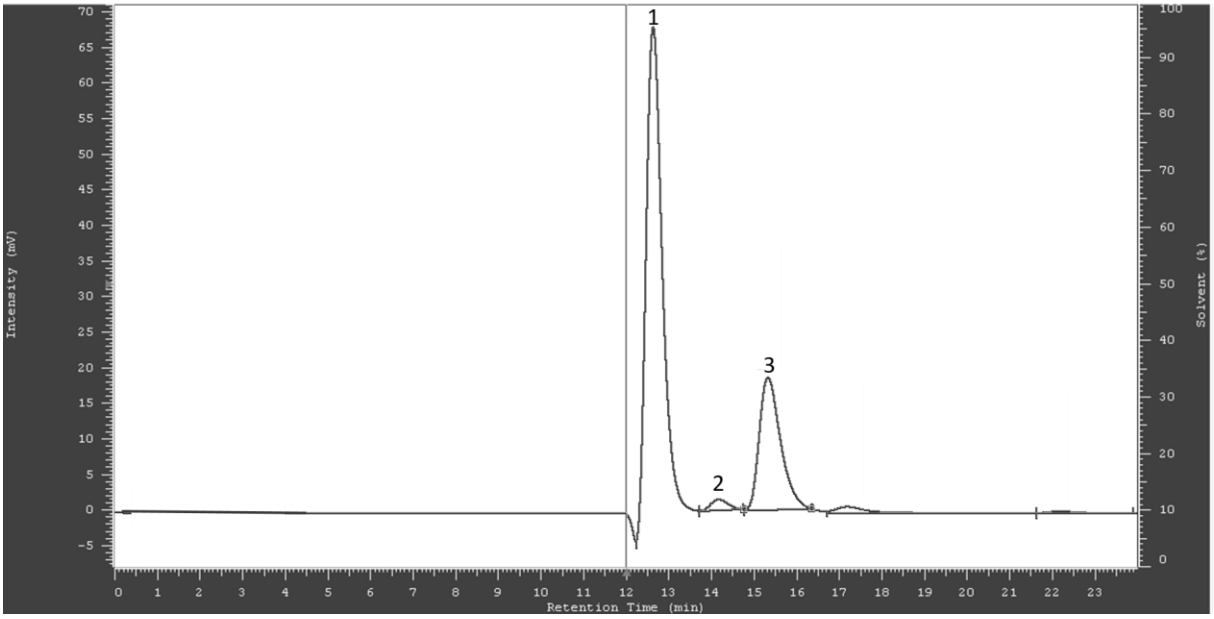

Figure 38: HPLC chromatogram of a conversion experiment with 1 mM GDP-fucose and 10 mM lactose starting concentration after 6 hours measured using Aminex H87-column. Peak 1: TRIS/HCl buffer pH 7.0, peak 2: 2´-fucosyllacotse, peak 3: lactose. x-axis: retention time [min], y-axis: intensity [mV].

Table 17: Detection of 2´-fucosyllactose synthesis, GDP-fucose conversion and GDP release. 2´-fucosyllactose concentrations were measured on HPLC using Aminex H87-column. GDP-fucose and GDP concentrations were measured on HPLC using Kinetex C18. Conversion was performed with 1 mM GDP-fucose and 0.5 mg/mL StrepNus*Hm*FT.

	Peak area			Concentration [mM]		
Time [min]	2´-FL	GDP-fucose	GDP	2´-FL	GDP-fucose	GDP
0	-	1205313.82	72953.85	-	1.205	0.073
	440.96	1457573.98	113593.85	0.152	1.458	0.114
5	1075.63	353456.911	704178.46	0.370	0.353	0.704
	959.63	549196.748	747353.85	0.330	0.549	0.747
10	2041.15	0	1100523.08	0.702	0.000	1.101
	2029.27	33450.4065	1127766.15	0.698	0.033	1.128
15	2215.97	0	1163784.62	0.762	0.000	1.164
	1988.62	0	1325520.00	0.684	0.000	1.326
30	2154.35	0	1124012.31	0.741	0.000	1.124
	2002.90	0	1369089.23	0.689	0.000	1.369
60	1910.92	0	1148110.77	0.657	0.000	1.148
	2259.77	0	1287440.00	0.777	0.000	1.287

Table 18: Slope of standard calibration curve for calculation of the above stated concentrations.

2´-fucosyllactose	2907.1
GDP-fucose	1000000
GDP	1000000

Table 19: Detection of 2´-fucosyllactose synthesis, GDP-fucose and latose conversion and GDP release. 2´-fucosyllactose and lactose concentrations were measured on HPLC using Aminex H87-column. GDP-fucose and GDP concentrations were measured on HPLC using Kinetex C18. Conversion was performed with 1 mM GDP-fucose and 0.15 mg/mL StrepNus*Hp*FT.

	Peak area				Concentration [mM]			
Time [h]	2´-FL	Lactose	GDP-fucose	GDP	2´-FL	Lactose	GDP-fucose	GDP
0	0	1401016	968749.56	252258.78	0.000	8.935	0.969	0.084
	0	1383206	979662.26	226810.26	0.000	8.821	0.980	0.076
0.25	32724	1254688	515357.76	174138.78	0.233	8.001	0.515	0.058
	27484	1321424	597835.62	180261.9	0.196	8.427	0.598	0.060
0.5	73242	1247436	131270.48	123264.06	0.522	7.955	0.131	0.041
	66270	1247968	224003.58	100627.86	0.472	7.959	0.224	0.034
0.75	83066	1239724	47606.92	50517.6	0.591	7.906	0.048	0.017
	82816	1242808	48488.74	62209.56	0.590	7.926	0.048	0.021
1	83468	1243094	42608.52	43641.18	0.594	7.928	0.043	0.015
	80564	1193182	39061.36	43899.72	0.574	7.609	0.039	0.015
2	80844	1214696	42825.78	44777.64	0.576	7.746	0.043	0.015
	85202	1205970	39791.24	39950.94	0.607	7.691	0.040	0.013
4	81594	1217598	47740.4	42997.62	0.581	7.765	0.048	0.014
	82876	1246762	46300.52	36097.02	0.590	7.951	0.046	0.012
6	83142	1246750	0	42672.12	0.592	7.951	0.000	0.014
	82386	1233730	41147.34	43637.46	0.587	7.868	0.041	0.015

Table 20: Detection of 2´-fucosyllactose synthesis, GDP-fucose and latose conversion and GDP release. 2´-fucosyllactose and lactose concentrations were measured on HPLC using Aminex H87-column. GDP-fucose and GDP concentrations were measured on HPLC using Kinetex C18. Conversion was performed with 5 mM GDP-fucose and 0.15 mg/mL StrepNus*Hp*FT.

	Peak area				Concentration [mM]			
Time [h]	2´-FL	Lactose	GDP-fucose	GDP	2´-FL	Lactose	GDP-fucose	GDP
0	0	1289426	5133640.8	2771461.4	0	8.223	5.134	0.924
	0	1296242	5138028.6	2654647.8	0	8.266	5.138	0.885
0.5	0	1353114	4706590	3655883.9	0	8.629	4.707	1.219
	0	1332686	4925809.6	3375202.5	0	8.499	4.926	1.125
1	0	1314284	4725662.02	3606218.2	0	8.382	4.726	1.202
	0	1314580	4791344.12	3730858.7	0	8.383	4.791	1.244
1.5	0	1354324	4784958.38	3802094.8	0	8.637	4.785	1.267
	0	1356266	4770460.18	3742338.6	0	8.649	4.770	1.247
2	0	1389858	4449547.28	4122915.1	0	8.863	4.450	1.374
	0	1333938	4588325.3	4351533.2	0	8.507	4.588	1.451
4	0	1330730	4721927.42	4195062.6	0	8.486	4.722	1.398
6	0	1376838	4411359.22	4572810.0	0	8.780	4.411	1.524
	0	1325884	4422734.84	4450074.2	0	8.456	4.423	1.483

Table 21: Slope of standard calibration curve for calculation of the above stated concentrations.

2´-fucosyllactose	140442
Lactose	156807
GDP-fucose	1000000
GDP	3000000

Table 22: Conversion of GDP-fucose with different lactose concentrations measured on HPLC using Aminex H87-column. Conversion was performed with 1 mM GDP-fucose and 0.15 mg/mL StrepNus*Hp*FT.

	Peak area			Concentration [mM]		
Lac. conc. [mM]	2´FL	lactose at reaction start	lactose at reaction end	2´-FL	lactose at reaction start	lactose at reaction end
1	32534.61	133420	89972	0.219	0.988	0.666
5	59499.63	689808	663952	0.400	5.110	4.918
10	103015.79	1328402	1265018	0.693	9.840	9.371
20	131606.00	2904808	2665438	0.885	21.517	19.744
50	141684.00	6906714	6890086	0.953	51.161	51.038
100	141884.00	12305950	11857064	0.954	91.155	87.830

Table 23: Slope of standard calibration curve for calculation of the above stated concentrations.

2´-fucosyllactose	148697
Lactose	135000

7 References

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