Karina Maria Gierlinger, BSc

Expression optimisation and characterisation of α1,2-fucosyltransferases

Master's Thesis

Graz University of Technology

Institute of Biotechnology and Biochemical Engineering
Head: Univ.-Prof. Dipl.-Ing. Dr.techn. Bernd Nidetzky

Supervisor: Univ.-Prof. Dipl.-Ing. Dr.techn. Bernd Nidetzky
Dipl.-Ing. Dr.techn. Barbara Petschacher

Graz, October 2017

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Abstract

Bacterial α1,2-fucosyltransferases find application in biotechnological production of fucosylated human milk oligosaccharides. Human milk oligosaccharides show beneficial effects on the microbial flora of the infant's gut. 2'-fucosyllactose and lacto-N-fucopenatose I are the most abundant oligosaccharides in breast milk and main targets for biotechnological synthesis. A main drawback of bacterial α1,2-fucosyltransferases is the generally low solubility in the cytoplasm of *Escherichia coli*, the mainly used expression host. In this paper, a strategy to increase the solubility of the enzymes by adding the fusion tag NusA is presented. By application of the NusA fusion strategy and variation of other expression parameters, the share of α1,2-fucosyltransferases was increased up to 20% of whole cell protein in the host *E. coli* JM109. While several α1,2-fucosyltransferases were characterised one by one so far, this study presents the first direct comparison of bacterial fucosyltransferases from an identical expression and analysis setup. α1,2-fucosyltransferases from *E. coli*, *Helicobacter pylori* and *Thermosynechococcus elongatus* and the so far uncharacterised α1,2-fucosyltransferases from *Salmonella enterica* were compared with focus on activity on the substrates lactose and LNT as well as pH and temperature optima. For the *H. pylori* fucosyltransferase, kinetic parameters and hydrolysis rates were determined.

Keywords: Fucosyltransferases; Human milk oligosaccharides; Expression optimisation; Fusion protein

Kurzfassung

Bakterielle α1,2-Fucosyltransferasen finden Anwendung in der biotechnologischen Produktion von fucosylierten humanen Milcholigosacchariden. Milcholigosaccharide zeigen einen positiven Effekt auf die mikrobielle Darmflora von Kleinkindern. Zu den häufigsten humanen Milcholigosaccharide gehören 2'-Fucosyllactose und Lacto-N-Fucopenatose I und daher sind diese für die biotechnologische Herstellung interessant. α1,2-Fucosyltransferasen weisen jedoch nur eine geringe Löslichkeit im Zytoplasma von *E. coli*, dem häufigsten Expressionswirten, auf. In dieser Arbeit wird eine Strategie präsentiert, um durch das Anhängen des Fusionstags NusA die Löslichkeit zu erhöhen. Mit dieser Strategie und dem Variieren weiterer Parameter wurde der Anteil der α1,2-Fucosyltransferasen am Gesamtprotein im Wirt *E. coli* JM109 auf bis zu 20% erhöht. Obwohl bisher verschiedene α1,2-Fucosyltransferasen charakterisiert wurden, ist ein direkter Vergleich der Enzyme durch die verschieden gewählten Bedingungen und Proteinkonstrukte schwierig. Um diese Daten besser interpretieren zu können, wurden die Gensequenzen der bereits charakterisierten Enzyme Wbgl, HpFt und TeFt und der unbeschriebenen SeFt in dasselbe Vektorkonstrukt mit einem NusA-Tag kloniert, exprimiert und bezüglich ihrer Aktivität mit den Substraten Laktose und LNT verglichen. Weites wurde die kinetischen Parameter für StrepNusHpFt bestimmt.

Schlagwörter: Fucosyltransferase; Humane Milcholigosaccharide; Fusionsprotein; Expressionsverbesserung

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List of Abbreviations

2′-FL 2′-fucosyllactose

CFE cell free extract

CIP calf intestine phosphatase

CPEC circular polymerase extension cloning

DMSO dimethylsulfoxide

DTT 1,4-dithiothreitol

GDP-fucose guanosine 5′-diphospho-β-L-fucose

GST glutathione S-transferase

HMO human milk oligosaccharide

HPAEC-PAD high-performance anion exchange-pulsed amperometric detection

IPTG isopropyl b-D-thiogalactopyranoside

LB Luria-Bertani

LDH lactate dehydrogenase

LNFP I lacto-*N*-fucopenatose I

LNT lacto-*N*-tetraose

MBP maltose binding protein

PEP phosphoenolpyruvate

PK/LDH pyruvate kinase/lactate dehydrogenase

PK pyruvate kinase

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBAB tetra-n-butylammonium bromide

1 Introduction

Breast feeding offers important health benefits for infants (Bode, 2012). A major contribution to the health-beneficial effects is given by human milk oligosaccharides (HMOs), which function as prebiotics in the human colon (Yu et al., 2013). While some microorganisms, like *Bifidobacterium longum*, which are related to positive health effects on human's gut, can metabolize HMOs and use them as sole carbon source, unfavourable bacteria are not able to utilize HMOs and their growth is inhibited (Sela and Mills, 2011). HMOs also prevent attachment of pathogens to the infant's gut as they resemble glycoconjugates on the epithelian cell surfaces. HMOs decoy viruses, bacteria or protozoa to bind to the soluble HMO instead of the epithelian glycan oligosaccharides and thus support washing out the pathogens of the gut (Newburg et al., 2004). Moreover, there is evidence for antimicrobial and immune response modulating properties of HMOs (Bode, 2012; Gonia et al., 2015; Kuntz et al., 2008).

So far, around 200 HMOs are identified. The core structure consists of lactose elongated either with *N*-acetyllactosamine or lacto-*N*-biose in a β1,3- or β1,6- linkage. The glycan chain can be fucosylated or sialylated, while two of the three most abundant HMOs are fucosylated. These are 2'-fucosyllactose and lacto-*N*-fucopenatose I (LNFP I), occurring in a concentration of around 1.5 g/L in human breast milk (Petschacher and Nidetzky, 2016; Bode, 2012; Thurl et al., 2010). The fucosyl group is connected in an α1,2-linkage in both oligosaccharides. The production of HMOs is challenging. Oligosaccharide concentrations in milk of most farm animals including bovine milk are up to 1000-fold lower than in human milk with especially low content in fucosylated

variants (Oliveira et al., 2015). Chemical synthesis needs extensive use of protecting groups to enable regio-selective glycosidic linkages (Kretzschmar and Stahl, 1998). A biotechnological approach involving site-selective enzymes presents a sustainable and less expensive alternative (Gavrilescu and Chisti, 2005). For the production of fucosylated HMOs in an enzymatical process, either fucosidases or fucosyltransferases are used. Fucosidases have next to the transfucosylation activity a high hydrolysis activity of both the donor substrate and the product.

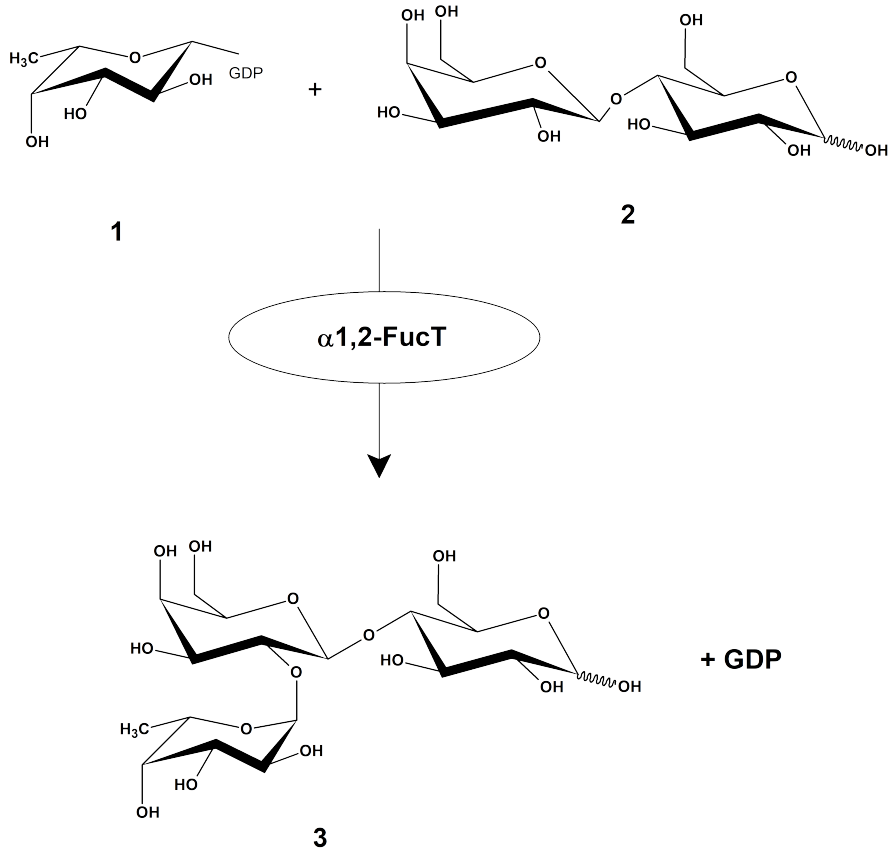

Figure 1.1: Reaction scheme of the transfer of the fucosyl group from the donor substrate GDP-L-fucose (1) to the acceptor substrate lactose (2) resulting in the production of 2'-fucosyllactose (3) catalysed by an α1,2-fucosyltransferase.

Only around 30-40% conversion yield could be reached with wildtype enzymes, while single mutations can increase the yield up to around 70% (Ze-

uner et al., 2014). An alternative to fucosidases are fucosynthases, where the nucleophilic residue is replaced with a non-nucleophilic residue and lowers therefore the hydrolysis of the product (Cobucci-Ponzano et al., 2009). Nevertheless, a main disadvantage of these enzymes is the use of expensive and sometimes toxic or unstable substrates, therefore fucosyltransferases are a preferable alternative for HMO production. Fucosyltransferases catalyse the transfer of the L-fucosyl group from the donor substrate GDP-β-L-fucose to an acceptor substrate. Depending on the formation of the linkage, it can be distinguished between α1,2-fucosyltransferases, α1,3 -fucosyltransferases and α1,4-fucosyltransferases. α1,2-fucosyltransferases can therefore be used for the production of 2'-fucosyllactose and LNFP. These enzymes are most abundant in glycosyltransferase family 11 (Petschacher and Nidetzky, 2016, Ma et al., 2006). So far, only few α1,2-fucosyltransferases have been cultivated and expressed in *E. coli*. Some examples for characterised α1,2-fucosyltransferases are Wbgl from *E. coli* O126 (Engels and Elling, 2014), which is described to have an activity of 250 mU/mg using lactose as acceptor substrate, FutC from *H. pylori*, which is also active with lactose at a specific activity of 80 mU/mg (Stein et al., 2008, Baumgartner et al., 2013), Te2Ft from *T. elongatus* (Zhao et al., 2016), which has a high specific activity towards lacto-*N*-tetraose (LNT) or WbiQ from *E. coli* O127 (Pettit et al., 2010). Though α1,2-fucosyltransferases were recently reviewed (Petschacher and Nidetzky, 2016), no comparative study of these enzymes produced by one single expression system and evaluated under identical reaction conditions was done so far.

Despite some advances, α1,2-fucosyltransferases still have the main drawbacks of a generally low activity and low solubility. A cause for the low solubility of the enzymes is aggregation of the protein due to improper folding leading to the formation of inclusion bodies (Lee et al., 2015). An abundant strategy to increase the expression levels of proteins is the attachment of a well expressed fusion protein. These large protein partners, like a maltose binding protein (MBP)- or a glutathione S-transferase (GST)-tag seem to have a beneficial effect on the solubility of the protein (Albermann et al., 2001, Engels and Elling, 2014, Zhang et al., 2010). A novel construct in combination with fucosyltransferases is the

attachment of a NusA-tag. NusA is a 55 kDa protein derived from *E. coli* and considered to not only increase the level of solubility but also to have a high expression level (Davis et al., 1999). It also seems to stabilise the fused protein better than a compared GST-tagged construct (De Marco et al., 2004).

Besides the usage of fusion proteins, varying expression parameters can have a high impact on the solubility of an expressed enzyme (Rosano and Ceccarelli, 2014). Considering the cultivation conditions, the choice of the media has a main influence on the growth of the cells. While LB-media is the most common for growth of *E. coli*, it does not lead to high cell densities because of only scant amounts of carbohydrates and cations (Sezonov et al., 2007). Alternatives are richer broths with an increased supplementation like TB-media or an autoinduction media (Studier, 2005). Another point to consider in order to reach high expression levels is the selection marker. Ampicillin is degraded very fast in cell cultures (Korpimäki et al., 2003). Therefore, in case of reduced selection pressure, non-plasmid carrying cells are not affected by a metabolic load due to heterologous protein production and outgrow plasmid carrying cells. A second addition of ampicillin at the time point of induction can increase selection pressure and lead to a higher enzyme yield. Furthermore, a slower rate of protein production can also improve proper folding of the protein. Slower protein production is achieved by lowering the expression temperature, which decreases the formation of aggregation (Vera et al., 2007, Schein and Noteborn, 1988). Another reason for insufficient protein solubility can be misfolding due to inappropriate redox conditions in the *E. coli* cytoplasm. The *E. coli* strain Origami B carries mutations in *trxB* and *gor*, and therefore might favour disulphide bond formation and correct folding of the protein (Derman et al., 1993).

The main focus of this study was the improvement of the expression and solubility of α1,2-fucosyltransferases, which was achieved by adding a fusion protein and expression parameter optimization. A further target was to compare α1,2-fucosyltransferases expressed from the same vector construct and under equivalent environmental parameters and therefore be able to investigate their use in the production of human milk oligosaccharides.

2 Material and methods

2.1 General methods and materials

For plasmid purification, the Thermo Scientific GeneJet Plasmid Miniprep kit (Waltham, MA, USA) and the Promega WizardPlus SV Miniprep DNA (Fitchburg, WI, USA) preparation systems were used. PCR purification and gel purification were performed by Thermo Scientific GeneJet PCR purification and gel extraction kit or the Promega Wizard SV gel and PCR clean-up system. Primers were ordered at Integrated DNA Technologies (Coralville, IA, USA). The pET41b(+)_Wbgl, pET41b(+)_SeFt, pET43.1b(+)_Wbgl and pET43.1b(+)_SeFt templates were ordered at GeneScipt (Piscataway, NJ, USA). pMCSG7_TeFt and pMCSG7_HpFt were kindly provided by Galab Laboratories GmbH (Hamburg, Germany). PCR steps were performed by a high-fidelity polymerase by Thermo Fisher and New England Biolabs GmbH, respectively. For restriction digestion, Thermo Fisher Restriction Enzymes were used. Further used enzymes were Thermo Fisher FastAP Thermosensitive Alkaline Phosphatase and Thermo Fisher T4 DNA ligase. The materials for cultivation and activity measurement were provided by Carl Roth (Karlsruhe, Germany) and Sigma Aldrich (St. Louis, MO, USA). 2'-fucosyllactose and GDP-L-fucose were ordered at Isosep (Tullinge, Sweden).

2.2 Genes, plasmids and strains

In table 2.1, an overview of used genes, plasmids and strains is shown.

Table 2.1: List of used genes, plasmids and strains in this work.

Genes	Description
HpFt	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)
SeFt	α1,2-fucosyltransferase from *S. enterica*, Uniprot accession number: Q5UHB1
TeFt	α1,2-fucosyltransferase from *T. elongatus*, basing on Uniprot accession number Q8DGK1, with R5H and E289G
Wbgl	α1,2-fucosyltransferase from *E. coli*, Uniprot accession number E2DNL9
Plasmids	
pMCSG7	Bacterial expression vector with T7 promoter, adds N-terminal His-tag and TEV protease site, ampicillin resistance
pET41b(+)	Bacterial expression vector with T7 promoter, adds N-terminal GST-tag followed by a His-tag and a second C-terminal His-tag, enterokinase site, kanamycin resistance
pET43.1b(+)	Bacterial expression vector with T7 promoter, adds N-terminal NusA-Tag, followed by a His-tag and a second C-terminal His-tag, enterokinase site, 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* Δ*nin5*
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]

2.3 pET43.1b(+)-constructs

The used primers can be seen in table 2.2. To reclone the coding sequences for TeFt and HpFt from the pMCSG7 vector into the pET43.1b(+) vector, in which a N-terminal NusA-tag is provided, the coding sequences were amplified using the primers TeFuctfw and TeFuctrv for TeFt and nHpinNusfw and HpFuctrev for HpFt, respectively.

The PCR was started at an annealing temperature of 62 °C for 12 cycles with a decrease of 0.5 °C per cycle, and 58 °C afterwards for 25 cycles. Further, DMSO at 5% (v/v) was added. dNTPs were usually used in a concentration of 200 mM, the primers at a concentration of 0.5 µM and the template at a concentration around 0.5 ng/µL. After the amplification of the inserts, a CPEC PCR was performed by combining pET43.1b(+) as backbone and the coding sequences for TeFt and HpFt, respectively. The CPEC basing on Quan and Tian, 2011 was performed with a modified touchdown PCR, including a decrease in annealing temperature of one degree from 70 °C for ten cycles. Afterwards, ten cycles were performed at 65 °C constantly. The PCR assay was performed by a Phusion polymerase and 5 % DMSO was used. After the PCR, the template plasmid was digested with *Dpn*I for 1 h at 37 °C.

2.4 pC21e1-constructs

To put the genes under the control of a tac-promoter, the CDSs, including the sequence for the NusA-tag, were cloned into the pC21e1 vector. This was achieved by amplifying the CDSs using NusEcoRIfw as a forward primer and HindWbglrv for NusWbgl, HindSeFtrv for NusSeFt and TeHindIIIrv for NusTeFt. The inserts were amplified at an annealing temperature of 62 °C and cut with *Eco*RI and *Hind*III.

For addition of an N-terminal Strep-tag, the primers StrepNusfw and StrepNusrv were ordered. As program, a ramp from 72 – 58 °C decreasing by 2 °C

Table 2.2: List of primers and nucleotide sequence. Restriction sites are written in bold.

Primer	Sequence
TeFuctfw	CGC GGG TTC TGG TAC GAT TGA TGA CGA CGA CAA GAG TCC G**GA GCT C**AT CAT TGT GCA TCT CTG TGG
TeFuctrv	TTT TAT CAG CCT AGG AAC GCC CAA CTT AAT TAA CAT TAG TGG TGG TGG TGG TGG TG**C TCG AG**C ACA ATC CAT CCA GGA CAA TAC
nHpinNusfw	GAC GAC AAG AGT CCG **GAG CTC** GCT TTT AAA GTG GTG CAA ATT TGC GGG
HpFuctrev	TTT TAT CAG CCT AGG AAC GCC CAA CTT AAT TAA CAT TAG TGG TGG TGG TGG TGG TG**C TCG AG**A GCG TTA TAC TTT TGG GAT TTC A
NusEcoRIfw	GAT GAT **GAA TTC** ATT AAA GAG GAG AAA TTA ACT ATG AAC AAA GAA ATT TTG GCT GTA GTT GAA GCC G
HindWbglrv	GTT GTT **AAG CTT** TTA ACA CGA GCT ATG TTT ATC CAC G
HindSeFtrv	GTT GTT **AAG CTT** TTA TTT TTT AAT TCT TAT CCA ACT TTC TG
TeHindIIIrv	GTT GTT **AAG CTT** TTA CAC AAT CCA TCC AGG ACA ATA CAG GTC TC
StrepNusfw	GTT AAT TAA GCC TTT CTC GAA CTG CGG GTG GCT CCA GCT AGC CAT AGT TAA TTT CTC CTC TTT AAT **GAA TTC** AAA TTG TTA TCC GC
StrepNusrv	ATG GCT AGC TGG AGC CAC CCG CAG TTC GAG AAA GGC TTA ATT AAC AAC AAA GAA ATT TTG GCT GTA GTT GAA GCC G
HpCpecrv	CTG AGC CTT TCG TTT TAT TTG ATG CCT **AAG CTT** TTA AGC GTT ATA CTT TTG GGA TTT CAC CTC AAA ATG G

for every other second for ten cycles was performed, followed by 58 °C for ten cycles and 62 °C for ten cycles. For this reaction, the Q5 polymerase was used with 20% Q5 enhancer. The template plasmid was digested with *Dpn*I for 1 h at 37 °C after the PCR.

As HpFt carries an internal *Hind*III restriction site in its nucleotide sequence, an approach using restriction enzymes could not be used. Therefore, a CPEC assay with primers nHpinNusfw and HpCpecrv used. The setup of the PCR was the same as for the pET43.1b(+)-strategy. As template for the backbone for the CPEC, pC21e1_StrepNusTeFt construct was used.

2.5 Transformation and storage

The plasmids were transformed in *E. coli* Top 10F' after cloning by electrophoresis. After reisolation, generated constructs were checked in size on an agarose gel after colony PCR and restriction digestion and were sequenced. For expression experiments, pET41b(+)- and pET43.1b(+)-plasmids were transformed in *E. coli* BL21 (DE3) Gold and pC21e1-constructs in *E. coli* JM109. For long term storage, glycerol stocks of the plasmid carrying host cells were prepared and stored at −70 °C.

2.6 Cultivation and expression

Shake flask cultures were done with LB-media using 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per litre media. TB media consisted of 12 g of tryptone, 24 g of yeast extract and 5 g of glycerol in 900 mL dH_2O, with the addition of 100 mL 89 mM phosphate buffer after autoclaving. Autoinduction media was prepared according to Studier, 2005. To prevent contamination, ampicillin was added in a concentration of 100 mg/L. 50 mL shake flask cultures were used as preculture and grown overnight at 37 °C at 100 rpm. To the main

culture in 1 L shake flasks with 250 mL medium, the preculture was added to an OD_{600} of 0.01. The main culture was incubated at 37 °C and 100 rpm until an OD_{600} of 0.6 - 0.8. The main culture was cooled for 20 minutes and afterwards induced with 0.1 mM IPTG. The cultivation was prolonged overnight at 25 °C. The cells were harvested for 25 minutes at 4,400 g. The cell pellet was resuspended in 10 ml 50 mM TRIS/HCl per gram cell wet weight. Cell disruption was performed using sonication. The cell debris and supernatant were separated by centrifugation at 16,100 g. The pellet fraction was dissolved in 6 M urea. The pellet fraction and the cell free extract were stored at −20 °C.

2.7 Enzyme purification and SDS-PAGE

The purification of the fucosyltransferases was performed by affinity chromatography. Strep-tagged proteins were purified by an IBA Gravity flow Strep-Tactin Sepharose column (Göttingen, Germany). The cell free extracts were diluted with a washing buffer, consisting of 100 mM TRIS/HCl, 150 mM NaCl and 1 mM EDTA at pH 8.0, in a ratio of 1:2. The proteins were eluted with 2.5 mM desthiobiotin and stored at −20 °C. The protein concentration was determined with the Bradford method (Bradford, 1976). Roth Roti-quant (Karlsruhe, Germany) was used as a protein dye. 1 mL was mixed with 20 µL of sample. The absorbance of the samples was measured at 595 nm and the protein concentration calculated basing on a BSA standard calibration curve. SDS-PAGE analysis of protein expression was done with precast Thermo Fisher NuPAGE Bolt 10% Bis-Tris Plus Gels and Coomassie Blue staining. For determination of the molecular mass, Thermo Fisher PageRuler prestained or unstained served as protein ladder. Quantitative analyses of the SDS-PAGE were performed using ImageJ 1.50i provided by the National Institutes of Health, USA.

2.8 Thin layer chromatography

For TLC, into a 10 µL assay of 1 mM GDP-fucose and 1 mM acceptor substrate in 50 mM TRIS/HCl pH 7.6, 1 µL of enzyme solution was added. After incubation for several hours at 30 or 37 °C, 2 µL were spotted on a silica matrix at different timepoints. As eluent, 1-butanol, ethanol and water in a ratio of 2:1:1 were used. For detection of the oligosaccharides, a thymol-sulphuric acid reagent (0.5 g thymol in 95 ml ethanol and 5 ml concentrated sulphuric acid) was sprayed on the silica surface and heated. For detection, the r_f-values were compared to commercially available standards of the expected products.

2.9 Photometric assay

A coupled LDH/PK assay was used for activity measurement of the enzymes based on Gosselin et al., 1994. Initial rate data was determined by measuring the decrease in NADH at 340 nm on a BMG Labtech Fluostar Omega Platereader with a temperature controlled cell holder. The reaction was started using an appropriate enzyme concentration for a range between 0.001-0.1 ΔA/min. The observed initial rate data was constant for $\geq$ 1 min and blanks and controls without acceptor substrates recorded.

The standard assay contained 1 mM GDP-fucose, 10 mM lactose or 1 mM of LNT as acceptor, 0.66 mM NADH, 5 mM $MgCl_2$, 1 mM PEP, 20 mM KCl, 5 U PK and 5 U of LDH in 100 mM TRIS/HCl (pH 7.0) in a total volume of 150 µL and was performed at 37 °C. For StrepNusTeFt and StrepNusSeFt, 20 mM $MgCl_2$ were used. For StrepNusWbgl, 2 mM $MnCl_2$ was used instead of $MgCl_2$ and 1 mM DTT was added. The assay was performed at pH 7.6 and 30 °C according to the characteristics determined by Engels and Elling, 2014. The enzymes were used in a range between 0.1 - 1 mU. An enzyme activity of 1 U is defined as the decrease of 1 µmol NADH per minute. Lactate dehydrogenase and pyruvate

kinase were not limiting. Before the addition of the fucosyltransferase, the reaction was kept a few minutes at reaction temperature to let free GDP react.

2.10 pH and temperature optima determination

For the determination of the optimal pH conditions of StrepNusSeFt, the photometric standard assay in citrate buffer, MES, TRIS/HCl and HEPES overlapping at a range of pH 5 - 8 was used. The temperature optimum was measured using the standard photometric assay at temperatures between 30 - 45 °C.

2.11 Conversion assays, HPLC and HPAEC-PAD analysis

2.11.1 Conversion assays

For the conversion experiment of lactose to 2'-FL, the assay consisted of 2 mM GDP-fucose, 10 mM lactose, 5 mM $MgCl_2$ and 1 U CIP in 100 mM TRIS/HCl (pH 7.0) at 37 °C. For StrepNusWbgl, $MnCl_2$ was used instead of $MgCl_2$ and 1 mM DTT was added. The reaction for StrepNusWbgl was performed at 30 °C and at pH 7.6. Between 0.1 - 1 mU enzyme was added to start the reaction. The reactions were stopped by heating for 5 minutes at 95 °C at several time points and the samples were centrifuged for 10 min at 16,100 g afterwards.

For the conversion of LNT to LNFP I, the assay consisted of 1 mM GDP-fucose, 1 mM LNT and 1 U CIP in 50 mM TRIS/HCl, pH 7.0.

2.11.2 HPLC

The detection 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 HPX87-H (Biorad, Richmond, CA, USA). The samples were eluted with 5 mM sulphuric acid using a 60 minutes method at a flow rate of 0.3 mL/min and an oven temperature of 65 °C.

For analysis of GDP-fucose, a Kinetex C18 column (Phenomenex, Torrence, CA, USA) in a reversed phase chromatography was used on Shimadzu HPLC (Kyoto, Japan). The absorbance of the analytes was detected at 262 nm. As eluent, TBAB with 6.5% acetonitrile was used at flow rate of 2 mL/min. The method lasted for 3 minutes. The oven temperature was set at 40 °C.

2.11.3 HPAEC-PAD

For the detection of LNFP I, a high-performance anion exchange-pulsed amperometric detection (HPAEC-PAD) analysis was performed using a Dionex (Dionex Corporation, Sunnyvale, CA) and a CarboPac PA10 column. The eluent was a mixture of 25% NaOH, 71% of deionized water and 4% of NaAc and ran for 40 minutes.

To determine the concentration of the produced oligosaccharides, a standard calibration curve was set up by using commercially available HMOs (Isosep, Tullinge, Sweden). Control assays were performed without the acceptor substrate.

2.12 Apparent kinetic data analysis of StrepNusHpFt

Apparent kinetic parameters for conversion of lactose (K_m for lactose, v_{max}) to 2'-fucosyllactose (2'-FL) with StrepNusHpFT were calculated from the initial rate measurements with a photometric assay. The lactose concentration was varied between 1 - 100 mM lactose at a steady GDP-fucose concentration of 1 mM. The kinetic constants were calculated using Sigma Plot 10 (SPSS, Erkrath, Germany) by non-linear regression.

3 Results and discussion

Microbial α1,2-fucosyltransferases in general lead to low levels of soluble protein when standard expression in *E. coli* is used. This study examines expression optimization by addition of a fusion protein and variation of expression conditions.

3.1 Expression optimisation of α1,2-fucosyltransferases with N-terminal fusion protein

As solubility of fucosyl transferases upon fusion of a GST-tag (Wbgl and SeFt) or a 6xHis-tag (HpFt and TeFt) was low when expressed in *E. coli* JM109 (figure 3.1), a novel construct with fusion of an N-terminal NusA-tag to the α1,2-fucosyltransferases was tested. For purification, a StrepII-tag was added in front of the NusA-tag. The construct was expressed from a pC21e1 FTO 4.0-vector under control of a tac-promoter. Expression was tested in shake flask cultivations containing 250 mL LB-media. After induction at OD_{600} 0.6-0.8 with 0.1 mM IPTG and 16 hours of expression, the cells were lysed by ultrasonication and fucosyltransferase expression was checked by SDS-PAGE in pellet and soluble fraction. The intensity of the bands was calculated using ImageJ. Cytosolic levels of four α1,2-fucosyltransferases from *E. coli* (Wbgl), *H. pylori* (HpFt), *T. elongatus* (TeFt) and *S. enterica* (SeFt) when expressed with GST- or His-tag in comparison to expression with NusA tag are shown in 3.1.

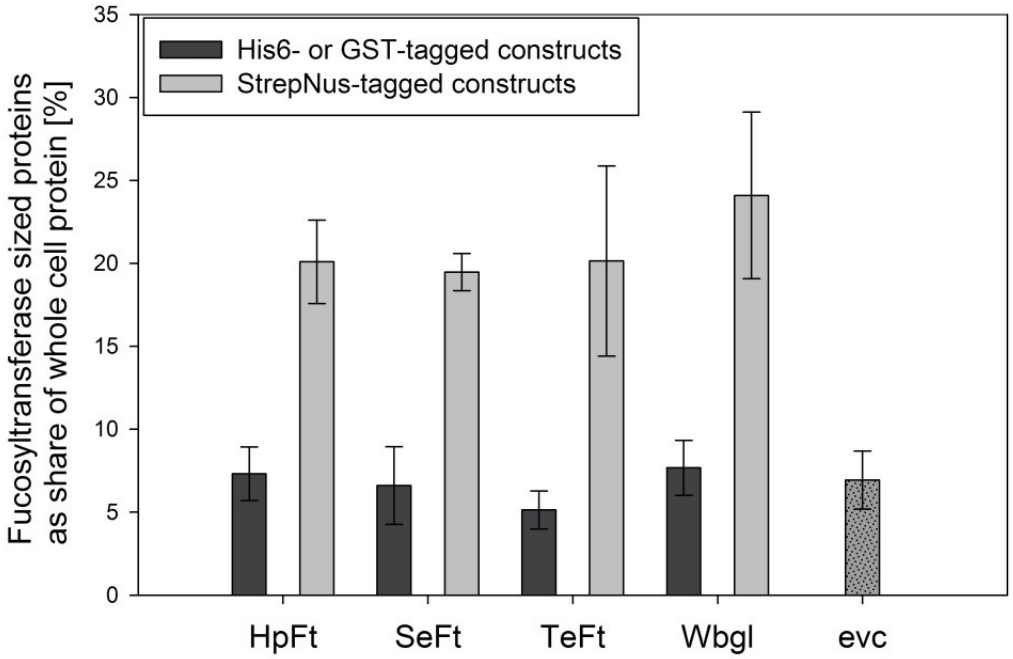

Figure 3.1: Estimated enzyme yield of 2'-fucosyltransferases expressed with different fusion tags. The cultivation experiment was performed in 250 mL shake flasks. HpFt and TeFt were expressed with a His6-tag in *E. coli* BL21(DE3) Gold or with a Strep- and NusA-tag in *E. coli* JM109. SeFt and Wbgl were expressed in *E. coli* BL21(DE3) Gold with a GST-tag and in *E. coli* JM109 with a Strep- and NusA-tag. evc: empty vector control, using a pC21e1 vector expressed in *E. coli* JM109, which shows the background proteins of *E. coli* at the expected sizes of the α1,2-fucosyltransferase. The expression of all constructs was performed in triplets beside GSTWbgl and GSTSeFt, which were performed in double measurements.

The beneficial effect of the NusA-tag can be clearly seen for all comprised fucosyltransferases. While expression of GST- or His6-tagged enzymes is insignificant compared to the empty vector control, the NusA-expressed protein is clearly better soluble. Expression of NusA-tagged α1,2-fucosyltransferases reached around 20% of the soluble protein fraction.

With a whole cell protein yield of around 200 mg/L shake flask culture, the

estimated enzyme yield of the α1,2-fucosyltransferases is around 40 mg/L. This shows a drastic increase in case of the α1,2-fucosyltransferase of *H. pylori*, where in a comparable result in literature, 1 mg/L could be reached with a His6-tagged enzyme (Stein et al., 2008). A similar protein yield (31 mg/L) could be reached by Engels and Elling, 2014 with a His6Prop-tagged enzyme.

3.2 Expression optimisation of α1,2-fucosyltransferases by variation of expression parameters

Variations in expression parameters were investigated to further optimise the expression level. A main factor for improved and efficient expression is the expression time, so fucosyltransferase expression levels after three different expression periods were compared. The benchmark enzyme StrepNusHpFt in *E. coli* JM109 was used for the test. The experiment was performed in LB-medium using 50 mL shake flasks. The cultures were incubated for 4, 16 and 24 hours after induction. Expression levels were analysed by SDS-PAGE and the yield of fucosyltransferase per liter of culture estimated from intensity of the bands and total protein concentrations. Result can be seen in table 3.1.

Table 3.1: Protein content and fucosyltransferase yield after different expression durations.

expression duration [h]	total protein content in CFE [mg/L]	estimated fucosyltransferase yield [mg/L]
4	46	3
16	178	40
24	216	37

An expression period longer than 16 hours had no beneficial effects on the soluble fucosyltransferase level. On the other hand, a too short expression

time results in an insufficient overall protein yield. Therefore, an overnight expression for 16 hours is optimal and was used as standard expression time for further cultivations. To further optimise the expression level, influences of temperature, expression host strain and media were investigated for the expression of StrepNusHpFt. Expression in *E. coli* JM109 was compared at 15 °C and 25 °C, in LB media, TB media and autoinduction media (all at 25 °C) and to expression in *E. coli* OrigamiB at 25 °C in LB-medium. Additionally, the influence of a second addition of ampicillin (100 mg/L) was tested. The results can be seen in figure 3.2.

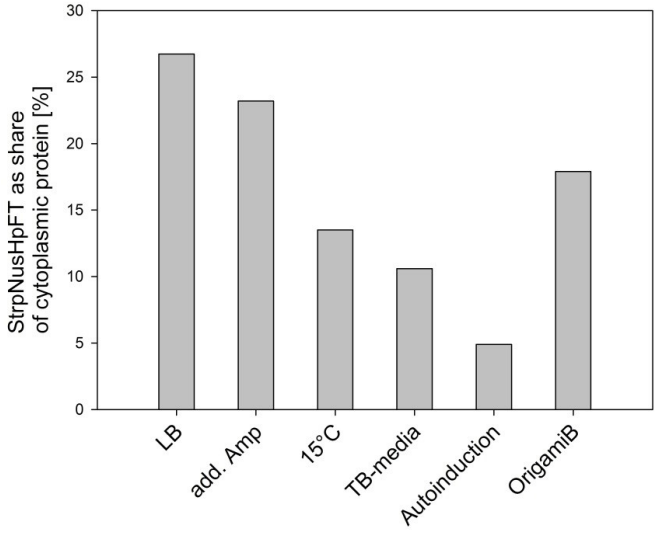

Figure 3.2: Estimated enzyme yield of StrepNusHpFt with varied expression parameters. 15 °C: expression at 15 °C; OrigamiB: Expression with *E. coli* OrigamiB as host strain; addAmp: second addition of ampicillin at time point of induction; TB-media: expression in TB-media; Autoinduction: expression in autoinduction media; LB: expression in LB-media at 25 °C in *E. coli* JM109 as host strain

Growing *E. coli* JM109 in LB-media and induction with 0.1 mM IPTG at 25 °C showed the best result. With these parameters, NusA-tagged fucosyltransferase expression accounted for around 30% of the total soluble protein fraction. The addition of ampicillin for a second time at the time point of induction gave

nearly the same result. As the higher ampicillin concentration keeps the selection pressure on the bacteria cells high and therefore supports growth of cells containing the resistance marker-carrying plasmid, this method was chosen as standard method for all further cultivations. Expression in Origami B strain had no positive influence on the solubility of the protein. The fucosyltransferases contain around eight cysteines. It seems that the improper folding of speculative sulphur bonds due to the reducing conditions of the cytoplasm are not the main reason for the reduced solubility. The expression at 15 °C did not increase the expression level of soluble fucosyltransferase. Due to the low induction temperature, the cell growth was also reduced. This reduction led unexpectedly to a decrease in the overall yield of the enzyme from 30 to 18% of total soluble protein. Cultivation in rich media also had no positive effect on protein expression. The TB- and autoinduction media indeed led to a very high OD_{600}, but at the same time expression levels of the fucosyltransferase were reduced to 30-50% compared to the LB experiment.

In summary, while other variations of expression parameters did not show a further increase in expression compared to standard expression set-up, the addition of NusA as highly soluble fusion partner increased expression of the fucosyltransferase drastically.

3.3 Preliminary activity check in the CFE

Due to the unknown influence of the NusA-tag on the active site of fucosyltransferases and the unknown characteristics of SeFt, preliminary activity of the enzymes with two substrates, lactose and LNT, was examined in cell free extracts. Reaction mixtures were analysed via TLC using a mixture of 1-butanol, ethanol and water as an eluent. Activities of the StrepNus-tagged enzymes with lactose and LNT as acceptor substrates are shown qualitatively in table 3.2. As *E. coli* JM109 was chosen as host which is deficient in β-galactosidase activity, no hydrolysis of the substrate lactose was expected in the cell free extract.

Table 3.2: Preliminary activity tests of fucosyltransferases in CFE, products detected by TLC

Enzyme	Lactose	LNT
StrepNusHpFt	++	++
StrepNusSeFt	-	+
StrepNusTeFt	-	+
StrepNusWbgl	+	+

The experiment showed that all enzymes were active. All tested enzymes were active with LNT as substrate, while with lactose only StrepNusHpFt and StrepNusWbgl showed the production of 2'-fucosyllactose. This confirms literature data, where the same acceptor specificities are found for lactose and LNT (Engels and Elling, 2014; Zhao et al., 2016; Stein et al., 2008; Albermann et al., 2001). The reaction of StrepNusHpFt appeared to be more active on TLC than the other enzymes. 2'-fucosyllactose production by StrepNusHpFt and StrepNusWbgl in the CFE was also verified on HPLC (results not shown).

3.4 Enzyme purification

The enzymes were purified using affinity chromatography. For GST- and His6-tagged proteins, purification was not pursued, as the expressed protein yields in the crude extract were too low. For the Strep-NusA-tagged constructs, concentrations of purified protein per liter cultivation could be reached as seen in table 3.3.

The gained protein yield is lower than expected. Given the fact, that the protein yield of the whole cell lied between 100 - 200 mg/L, the theoretical yield of the purified enzyme would lie around 20 - 40 mg/L, as seen in chapter 3.1. This difference might be caused by an unsuccessful binding of the StrepII-tag to the StrepTactin sepharose. As visible in figure 3.3, a large part of the enzyme can be found in the flow through. Optimising it by adding another purification step

Table 3.3: Enzyme yield of several α1,2-fucosyltransferases in a pC21e1-vector with StrepII-tag and NusA-tag.

Enzyme	Size [kDa]	Enzyme yield [mg/L]
StrepNusWbgl	97.5	7
StrepNusTeFt	95.9	9
StrepNusSeFt	100.1	7
StrepNusHpFt	95.2	11

or a second purification of the flow through, the protein yield could probably be further improved.

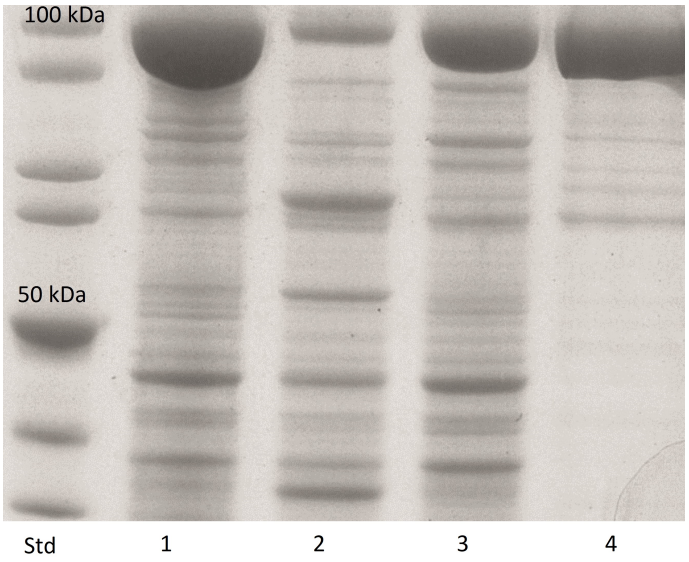

Figure 3.3: SDS-PAGE of StrepNusSeFt. Std: Thermo Fisher PageRuler Unstained Protein ladder; 1: pellet fraction; 2: soluble fraction, diluted 1:10; 3: unbound protein after purification; 4: purified enzyme

Compared to other expression strategies of α1,2-fucosyltransferases, the yield of StrepNusHpFt showed a promising result. Using the NusA-tag leads to a 10-fold increase in protein yield when being compared to a His6-tagged α1,2-FucT of *H.*

pylori in literature, where around 1 mg/L could be reached (Stein et al., 2008). In comparison to a His6Prop-tagged Wbgl (protein yield 31 mg/L, Engels and Elling, 2014) and a MBP-tagged TeFt (protein yield 16 mg/L, Zhao et al., 2016), the gained yields of the expressed and purified StrepNus-constructs were lower. An improved protein purification might lead to an increase in protein yield as the compared number from literature was within the theoretical share of the α1,2-fucosyltransferases to whole cell protein.

3.5 Activity measurement and conversion

The activity of purified fucosyltransferases was measured photometrically by using a coupled lactate dehydrogenase/pyruvate kinase assay, where the decrease of NADH was detected at 340 nm. The assay set-up was identical for all enzymes, but adjusted for the characteristics of each enzyme in temperature, pH and metal ion dependency. Measured initial rate data are shown in table 3.4 in comparison to published data from characterisations of single fucosyltransferases.

Concerning the substrate specificity, the results confirm literature data with HpFt being active on LNT and lactose (Albermann et al., 2001; Stein et al., 2008; Tsai et al., 2013), while TeFt was already described to be highly active with LNT but not with lactose (Zhao et al., 2016). WbgL showed an almost 30-fold higher specific activity for LNT compared to lactose although due to cost reasons, only 1 mM of LNT but 10 mM of lactose were used as substrate. Specific activity of Wbgl with LNT has not been reported before. Compared to published data, for TeFt with LNT and Wbgl with lactose the here measured activities of StrepNus-tagged enzymes are clearly lower. This is very drastic in case of Wbgl, where only 2.5% of the published activity could be found. A reason for that might be the size and influence of the fusion-tag. As the NusA-fusion partner is very large in molecular mass, it could have influences on the active site of the proteins and therefore causes the reduced activity. Efforts to cleave the Nus-tag off after purification at an enterokinase cleavage

Table 3.4: Published and measured activities of α1,2-fucosyltransferases.

Enzyme	Substrate	Published spec. activity [mU/mg]	Measured spec. activity [mU/mg]
HpFt	LNT (1 mM)	55 (2 mM lactose) [1]	380
	Lactose (10 mM)	30 - 80 (2 - 3 mM lactose) [2]	260
SeFt	LNT (1 mM)	-	55
TeFt	LNT (1 mM)	720 (10 mM LNT) [3]	90
Wbgl	Lactose (10 mM)	250 (5 mM lactose) [4]	6
	LNT (1 mM)	-	160

[1] Stein et al., 2008; Tsai et al., 2013
[2] Stein et al., 2008; Albermann et al., 2001
[3] Zhao et al., 2016
[4] Engels and Elling, 2014

site were not successful. Also, the exchange of the enterokinase with a TEV cleavage site did not show any better accessibility. The reason might be steric hindrances due to the two large compounds of the protein construct with both the fusion tag and the α1,2-fucosyltransferase itself. For StrepNusHpFt, the detected activity with 10 mM lactose was around 4-fold higher compared to the so far published activities, with 1 mM LNT around 7-fold. This effect is even more outstanding considering the differences in molar masses when comparing the specific activities, as StrepNusHpFt is around thrice the size of an only His6-tagged HpFt. This increase in activity could be either caused again by the unknown influence of the fusion tag on the protein structure, but also in the different measurement methods. While Albermann et al., 2001 measured the synthesised product, the LDH/PK assay detects via coupled NADH formation the released GDP, which also can originate from donor hydrolysis. While donor hydrolysis activity measured with the photometric assay without acceptor substrate ranged from around 5-10% of the NADH formation rate detected with an acceptor substrate present for StrepNusWbgl and StrepNusTeFt, it goes up

to around 20% for StrepNusSeFt. Using StrepNusHpFt, even up to around 60% of the donor substrate is cleaved. Further analysis of donor hydrolysis for HpFt is described below.

Table 3.5: Measured hydrolysis rate in mU/mg of purified α1,2-fucosyltransferases using the standard photometric assay.

	StrepNusWbgl	StrepNusTeFt	StrepNusSeFt	StrepNusHpFt
Hydrolysis rate [mU/mg]	0.33	4	14	76
Share on specific acitiy [%]	6	4	21	29

With LNT as acceptor substrate, the first time characterised StrepNusSeFt showed an activity of 55 mU/mg. The product formation was also verified by HPLC for 2'-fucosyllactose and HPAEC-PAD analysis for LNT. GDP-decrease was monitored on HPLC as well.

Conclusively, in comparison of the four here tested Nus-tagged fucosyltransferase constructs, StrepNusHpFt showed the highest activity for both tested substrates.

StrepNusHpFt was further characterised by following the time course of conversion reactions in 1 mL assays by product detection on HPLC (figure 3.4). 5 mM lactose and 1.4 mM GDP-fucose were used as starting conditions in a standard assay and incubated at 37 °C for 24 hours, with samples taken at several time points. After 2 hours, the maximum product concentration was reached, resulting in 0.2 mM of 2'-fucosyllactose. This leads to a 15% conversion yield (2'-fucosyllactose concentration after 24 hours compared to initial GDP-fucose concentration). Nevertheless, all GDP-fucose seemed to be consumed after 5 hours. When comparing the results to another experiment with 1 mM initial acceptor concentration, an even lower conversion yield was achieved. Only 0.07 mM 2'-fucosyllactose could be produced, resulting in only 9% conversion yield. Again, all GDP-fucose was consumed. The difference is particularly

obvious when comparing the synthesis rates of 2'-fucosyllactose of the first two hours of the assays. While at the assay containing 5 mM lactose 0.2 mM 2'-fucosyllactose have been produced, only 0.032 mM 2'-fucosyllactose could been found at the reaction assay with 1 mM lactose.
A further aspect appearing is a decrease in lactose visible throughout nearly all conversion experiments. This might be due to some rest activity of a β-galactosidase, which could not be separated completely after purification. Further investigations have to be done concerning this issue, but as the reaction stops after a decrease of around 3 mM at all experiments, it is supposed to not have an influence on the conclusion of the work (conversion experiments shown in chapter 6). To exclude that the hydrolysis of GDP-fucose is not a natural decoy, the decrease of donor substrate without enzyme was measured. The rate lies around 0.2 µmol/min and is therefore far below the GDP-fucose decrease measured in previous experiments.
Conclusively, the experiments indicated on the one hand a presumptive hydrolysis of the donor substrate and on the other hand the influence of the lactose concentration on the conversion yield of the product and led therefore to re-evalutation experiments of the K_m-value.

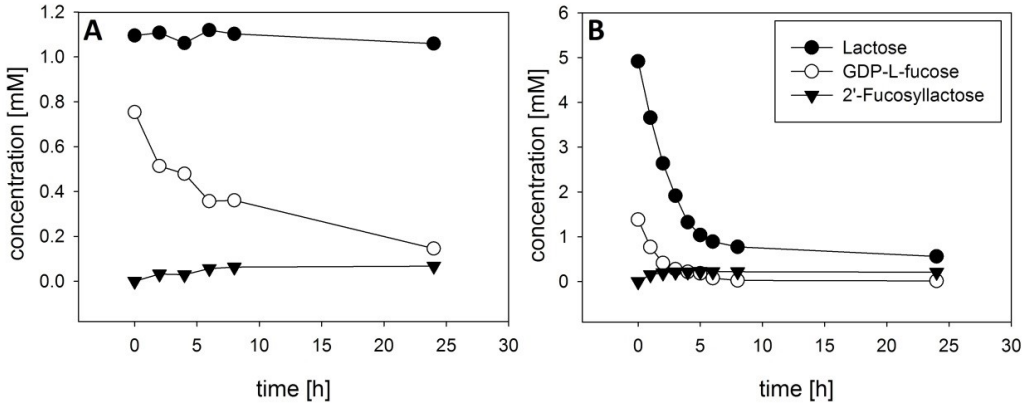

Figure 3.4: Conversion experiments for the production of 2-fucosyllactose using StrepNusHpFt. The assays were performed with 1 mM (left) and 5 mM (right) initial lactose concentration, leading to 9% and 15% conversion yield, respectively, with respect to the initial GDP-fucose concentration.

3.6 Determination of apparent K_m-values for StrepNusHpFt

Albermann et al., 2001 measured a K_m of 168 µm for lactose. Considering the increase in the synthesis rate with higher lactose concentration, the K_m was assumed to be higher for StrepNusHpFt. The apparent K_m-value was determined for StrepNusHpFt varying the lactose concentration between 1 - 100 mM with results shown in table 3.6.

The K_m-value is higher as the previously published for untagged HpFt (Albermann et al., 2001). The discrepancy between the former published K_m and the now measured could again be caused by the unknown influence of the fusion tag. The catalytic efficiency k_{cat}/ K_m for lactose of 1.2 min^{-1} mM^{-1} is generally speaking low, but higher as comparable values for α1,2-fucosyltransferases like Wbgl with 0.76 min^{-1} mM^{-1} (Engels and Elling, 2014) or WbsJ with 0.006

Table 3.6: Apparent kinetic data of StrepNusHpFt for the acceptor substrate lactose measured using the standard photometric assay with fixed GDP-fucose concentrations (1 mM) and varying lactose concentration (between 1 - 100 mM).

K_m [mM]	k_{cat} [min^{-1}]	k_{cat} / K_m [min^{-1} mM^{-1}]
48	63	1.3

min^{-1} mM^{-1} (Li et al., 2008). The higher K_m-value would be another explanation for the higher measured activity, compared to Albermann et al., 2001. Measuring around 1 mM lactose concentration lead to an specific activity of around 80 mU/mg, similar to Albermann's assay. Due to the differences in the molar masses, an increase in specific activity of StrepNusHpFt compared to a His6-tagged HpFt is still significantly visible.

3.7 Donor hydrolysis by StrepNusHpFt

To test the rate of donor hydrolysis of StrepNusHpFt and exclude low conversion rates due to enzyme degradation, a reaction setup aiming at full conversion of GDP-fucose was chosen. In an assay consisting of 10 mM lactose and 2.8 mM GDP-fucose with 2 mU StrepNusHpFt, the conversion took 6 hours at 37 °C. As a decrease in the conversion rate could be seen after 2 hours, 2 mU fresh enzyme was added. After 4 hours, the entity of GDP-fucose was consumed. At that time point, 0.88 mM 2'-fucosyllactose was produced. A conversion of 34% of GDP-fucose to 2'-fucosyllactose was reached. Literature reported yields for comparable assays of 44% with Wbgl (Engels and Elling, 2014) or 65% using HpFt (Albermann et al., 2001).

A factor which should also not be underestimated and seems to be a reason for the low conversion is the hydrolysis rate, as already mentioned in chapter 3.5. Especially at low acceptor concentrations, the hydrolysis of GDP-fucose has a high impact. While the ratio between synthesis of the product and hydrolysis of

the donor substrate lies at 0.125 at a lactose concentration of 1 mM, it grows up to 0.35 at 10 mM lactose, measured on HPLC, as visible from the data in table 6.14 and 6.16.

To test, if donor hydrolysis during 2'-fucosyllactose production by StrepNusH-pFt could be overcome using a high lactose concentration, the conversion yield towards 2'-fucosyllactose was determined using various concentrations of lactose (figure 3.5).

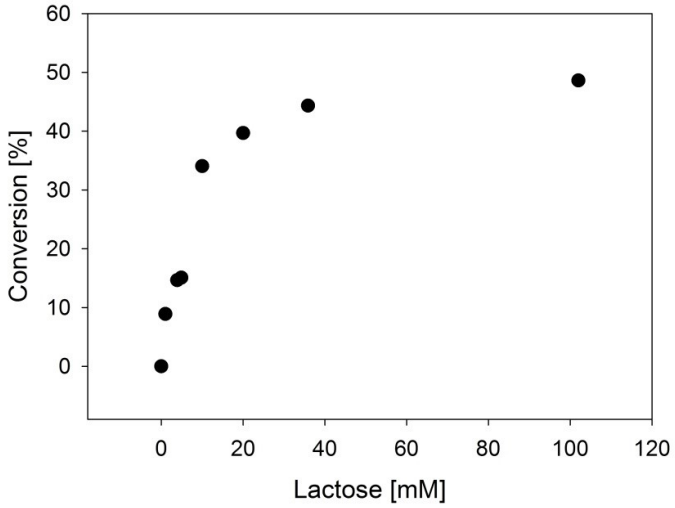

Figure 3.5: Conversion rates calculated of produced 2'-fucosyllactose to donor substrate GDP-fucose, with increasing lactose concentrations.

As seen in figure 3.5, even at high acceptor concentration, the hydrolysis of the donor substrate GDP-fucose could not be overcome completely, which leads to the assumption that the enzyme always performs to some degree a certain ratio of "error hydrolysis" instead of the fucosyl transfer, independent on the offered substrate level. At least 50% of the donor substrate was hydrolysed. This effect was also described for other glycosyl transferases (Sugiarto et al., 2012; Zhang et al., 2010). For α1,2-fucosyltransferases, Stein et al., 2008 reported that the hydrolysis activity of an α1,2-FucT of *H. pylori* vanished at the addition of oligosaccharides. A reason this could not be observed in our experiments might

be, that lactose is a weak acceptor substrate compared to other oligosaccharides. Again, the influence of the fusion-tag must be considered. To overcome error hydrolysis, addition of ATP (Zheng et al., 2008), periodic addition of the donor (Zhang et al., 2010) or using a structure-based site directed mutagenesis approach would be considerable (Sugiarto et al., 2012).

Albeit the high K_m and the hydrolysis rate, StrepNusHpFt, due to the high specific activity, is the best choice for 2'-fucosyllactose production in the comparison of the protein constructs of the four α1,2-fucosyltransferases.

3.8 Biochemical characterisation of StrepNusSeFt

Biochemical characteristics for an α1,2-fucosyltransferase from *S. enterica* have not been reported before. pH- and temperature optima were determined and are shown in comparison to published data for α1,2-fucosyltransferases HpFt, TeFt and Wbgl in table 3.7.

Table 3.7: pH- and temperature optima of α1,2-fucosyltransferases.

Enzyme	pH	temperature	Reference
HpFt	5	50 °C	Stein et al., 2008 Tsai et al., 2013 Albermann et al., 2001
TeFt	6.5	40 °C	Zhao et al., 2016
Wbgl	7.2 - 7.6	27 °C	Engels and Elling, 2014
SeFt	7	40 °C	This study

StrepNusSeFt has optima at very mild reaction conditions. With a temperature optimum of 40 °C and pH 7.0 in TRIS/HCl buffer the use of the enzyme could be considered in one-pot conversion systems coupled to other enzymes or

whole cell systems. The activity declines to 25% after 6 months of storage at −20 °C. Despite the moderate activity, StrepNusSeFt is a potential candidate for production of LNFP I.

4 Conclusion and outlook

This study revealed an effective strategy for increasing soluble expression of α1,2-fucosyltransferases. A clear increase in solubility of a set of four transferases was visible by using NusA-tagged protein constructs instead of un- or GST-tagged. Furthermore, expression conditions were optimised to increase the enzyme yield, which was best in LB-media at 25 °C. With the here presented expression strategy, expression levels of StrepNus-tagged constructs of Wbgl, SeFt, TeFt and HpFt up to 20% of total soluble cell protein could be achieved which are considered not to be the bottle-neck in a biotechnological production of fucosylated human milk oligosaccharides. The activity of these four α1,2-fucosyltransferases was tested with different assays using lactose and LNT as substrate and for the first time directly compared to each other and to literature data. StrepNusWbgl and StrepNusTeFt reached with 6 and 90 mU reached only between 5 and 10% of the in literature published activity, where other fusion tagging strategies were used. StrepNusHpFt reached a specific activity of 260 mU/mg for lactose and 380 mU/mg. This is the highest so far published activity for StrepNusHpFt for both substrates. Further experiments indicated that only around 50% of GDP-fucose can be converted to 2'-fucosyllactose using StrepNusHpFt as biocatalyst. This is due to hydrolysis of the donor substrate GDP-fucose, which can be decreased but not totally overcome by conversions at high lactose concentration. To be able to work under optimised conditions for each enzyme, temperature and pH-optima were determined for StrepNusSeFt which are located at 40 °C and pH 7.

The NusA-tag has clearly positive effect on the solubility of the enzymes. Concerning influence on the transferase activity and donor hydrolysis, its effect

has to be further investigated. StrepNusHpFt was shown to have an acceptable activity for the production of 2'-fucosyllactose. Nevertheless, compared to other glycosyltransferases, the activity is still low and an improvement is desirable. An effective way therefore would be the determination of the protein structure followed by site directed mutagenesis. Beside sequence optimisation, the screening for novel α1,2-fucosyltransferases could be considered. A main drawback in screening is that no high-throughput assay is available so far. To increase the product yield, a whole cell strategy or a change to a eukaryotic expression system might be investigated.

5 Appendix

Nucleotide sequence of StrepNusWbgl:

ATGGCTAGCTGGAGCCACCCGCAGTTCGAGAAAGGCTTAATTAACAACAAAGAAATTTTG
GCTGTAGTTGAAGCCGTATCCAATGAAAAGGCGCTACCTCGCGAGAAGATTTTCGAAGCA
TTGGAAAGCGCGCTGGCGACAGCAACAAAGAAAAAATATGAACAAGAGATCGACGTCCGC
GTACAGATCGATCGCAAAAGCGGTGATTTTGACACTTTCCGTCGCTGGTTAGTTGTTGAT
GAAGTCACCCAGCCGACCAAGGAAATCACCCTTGAAGCCGCACGTTATGAAGATGAAAGC
CTGAACCTGGGCGATTACGTTGAAGATCAGATTGAGTCTGTTACCTTTGACCGTATCACT
ACCCAGACGGCAAAACAGGTTATCGTGCAGAAAGTGCGTGAAGCCGAACGTGCGATGGTG
GTTGATCAGTTCCGTGAACACGAAGGTGAAATCATCACCGGCGTGGTGAAAAAAGTAAAC
CGCGACAACATCTCTCTGGATCTGGGCAACAACGCTGAAGCCGTGATCCTGCGCGAAGAT
ATGCTGCCGCGTGAAAACTTCCGCCCTGGCGACCGCGTTCGTGGCGTGCTCTATTCCGTT
CGCCCGGAAGCGCGTGGCGCGCAACTGTTCGTCACTCGTTCCAAGCCGGAAATGCTGATC
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GCTCGCGATCCGGGTTCTCGTGCGAAAATCGCGGTGAAAACCAACGATAAACGTATCGAT
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GGTGGCGAGCGTATCGATATCGTCCTGTGGGATGATAACCCGGCGCAGTTCGTGATTAAC
GCAATGGCACCGGCAGACGTTGCTTCTATCGTGGTGGATGAAGATAAACACACCATGGAC
ATCGCCGTTGAAGCCGGTAATCTGGCGCAGGCGATTGGCCGTAACGGTCAGAACGTGCGT
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CAACTTTTTCAGTTCTCATTTGGGTATGCGCTTTCCAAAATTAATGGGACACCATTATAT
TTTGATATAAGTCATTATGCTGAAAATGATGATCATGGTGGTTACAGGCTAAACAATCTA
CAAATTCCAGAGGAATATTTACAGTATTACACACCAAAAATTAATAATATTTATAAATTT
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Nucleotide sequence of StrepNusHpFt:

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5 Appendix

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Nucleotide sequence of StrepNusTeFt:

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Nucleotide sequence of StrepNusSeFt:

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Amino acid sequence of StrepNusWbgl:

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Amino acid sequence of StrepNusHpFt:

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Amino acid sequence of StrepNusTeFt:

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Amino acid sequence of StrepNusSeFt:

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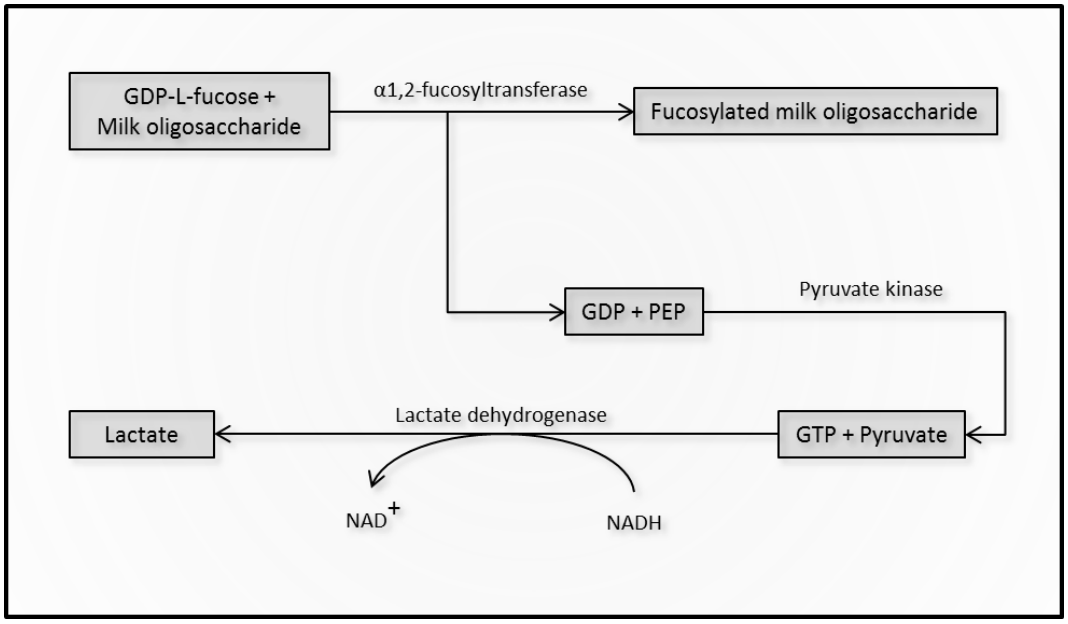

Figure 5.1: Reaction scheme of PK/LDH assay

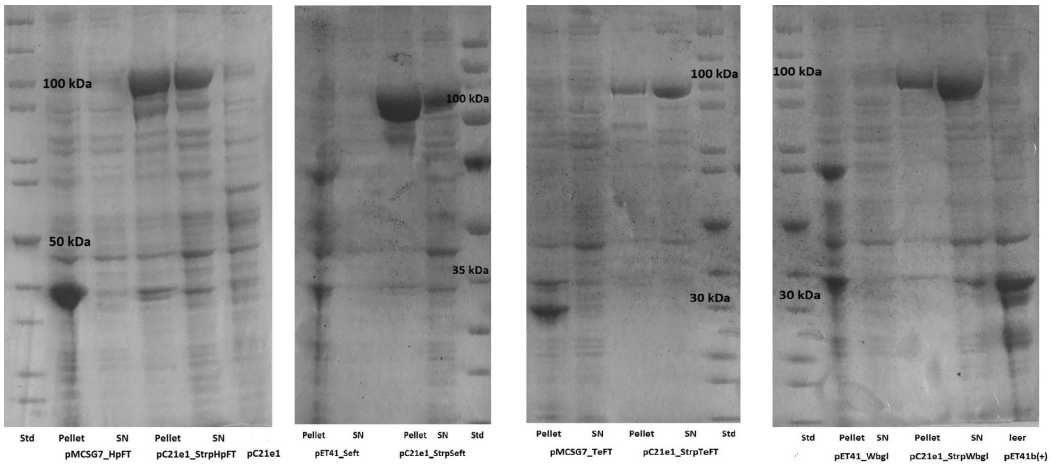

Figure 5.2: SDS-PAGE for comparison of expression level and solubility of fucosyltransferases with varying N-terminal fusion tags, expressed in 50 mL LB-shake flask culture. Std: Standard, Pellet: insoluble fraction, SN: cell free extract. pMCSG7_FT: His6-tagged fucosyltransferase, pET41_FT: GST-tagged fucosyltransferase, pC21e1_FT: Strep- and NusA-tagged fucosyltransferase.

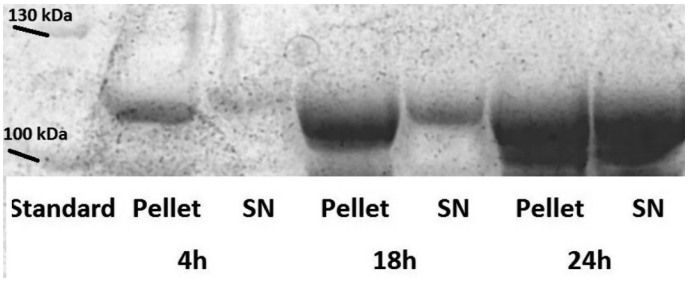

Figure 5.3: SDS-PAGE of different expression durations of StrepNusHpFt. Expression was performed at 25 °C in 50 mL LB-shake flask cultures and expressed for 4, 18 and 24 hours. Pellet: insoluble fraction, SN: cell free extract.

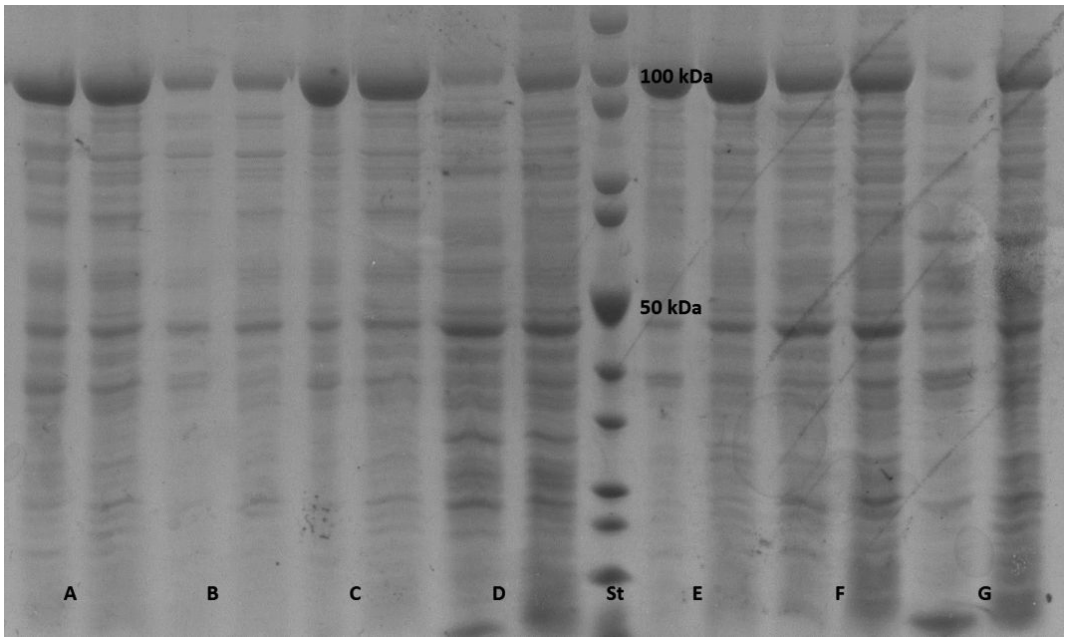

Figure 5.4: SDS-PAGE of different expression parameters of StrepNusHpFt. The expression took place in 50 mL shake flask cultures. A: expression in LB-media at 25 °C in *E. coli* JM109, B: expression at 15 °C, C: second addition of antibiotics at time point of induction, D: expression in autoinduction media, E: expression strain *E. coli* Origami B, F: expression in TB-media. The first lane of each assay shows the insoluble fraction, the second the cell free extract.

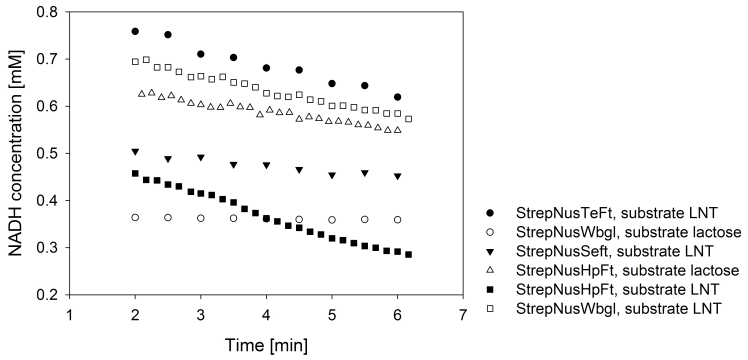

Figure 5.5: PK/LDH-assay for comparison of the activities of fucosyltransferases. 10 mM lactose or 1 mM LNT were used as acceptor substrates. Filled circle: activity of StrepNusTeFt using LNT as a substrate; open circle: activity of StrepNusWbgl using lactose as substrate; filled triangle: activity of StrepNusSeFt using LNT as substrate; open triangle: activity of StrepNusHpFt with lactose as substrate; filled square: activity of StrepNusHpFt with LNT as substrate; open square: activity with StrepNusWbgl using LNT as substrate.

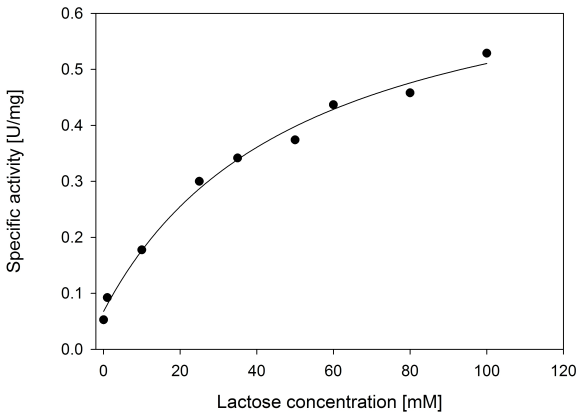

Figure 5.6: Apparent K_m-value determination of StrepNusHpFt with GDP-fucose as fixed substrate (1 mM) and varying lactose concentration (between 1 - 100 mM). Enzyme activity was determined using the standard PK/LDH-assay at pH 7.0 and 37 °C.

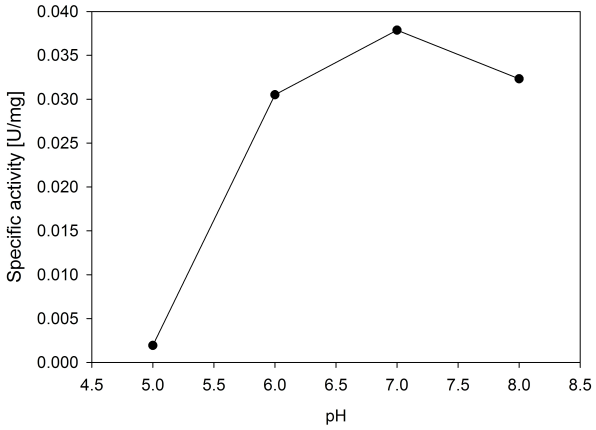

Figure 5.7: Measured pH-optimum of StrepNusSeFt using PK/LDH-assay at 37 °C. pH 5-6: Citrate, pH 6-7: MES, pH 7: TRIS/HCl, pH 7-8: HEPES

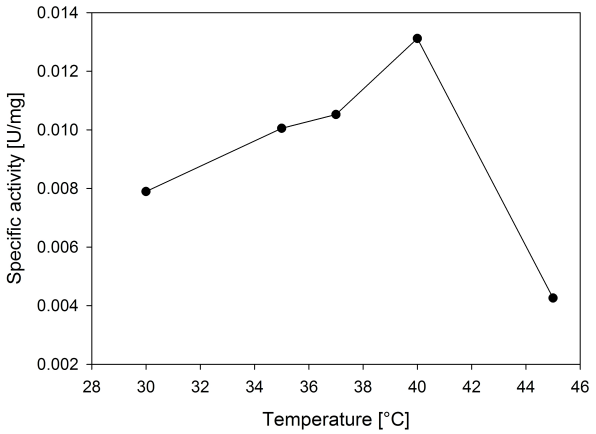

Figure 5.8: Measured temperature optimum of StrepNusSeFt using PK/LDH-assay at pH 7.0. The decline in activity is explained due to the instability of the enzyme over a six month storage at −20 °C.

Table 5.1: List of used devices.

Devices	
Applied Biosystems 2720 Thermal Cycler	Thermal Cycler
BioRad Modell 200/ Gel Doc 2000 system	Agarose gel system
Thermo Sorvall Evolution RC Centrifuge with SLC 3000 rotor	Centrifuge
Fisher Scientific Sonic Dismembrator	Sonifier
Thermo Fisher XCell Sure Lock Mini Cell	Electrophoresis system
BioRad MicroPulser	Electroporator
Eppendorf 5415R	Centrifuge
BMG Labtech Fluostar Omega	Platereader
DeNovix DS-11+	Spectrometer
Shimadzu 20A series	HPLC
Merck L-7000 series	HPLC
Dionex 50 series	HPAEC-PAD

6 Supplementary data

Table 6.1: Calculation of the intensity of the protein bands of His6HpFt with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	3591.77	65684.00	5.47	7.32	1.61
2	37363.864				
3	24728.37				
1 (FT)	9905.77	139794.17	7.09		
2	65067.156				
3	64821.245				
1 (FT)	16616.376	176795.83	9.40		
2	87317.248				
3	72862.203				

Table 6.2: Calculation of the intensity of the protein bands of StrepNusHpFt with ImageJ. The first peak is the fucosyltransferase, the other peaks the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	22178.24	108513.46	20.44	20.09	2.52
2	6846.00				
3	79489.22				
1 (FT)	51891.92	307930.16	16.85		
2	5743.23				
3	250295.00				
1 (FT)	28273.32	122979.16	22.99		
2	6678.21				
3	55657.14				
4	32370.50				

Table 6.3: Calculation of the intensity of the protein bands of His6TeFt with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	16307.48	433587.51	3.76	5.14	1.15
2	327895.87				
3	89384.16				
1 (FT)	14184.96	278640.58	5.09		
2	164624.09				
3	99831.53				
1 (FT)	17466.68	265685.12	6.57		
2	161697.10				
3	86521.34				

Table 6.4: Calculation of the intensity of the protein bands of StrepNusTeFt with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	25702.61	188740.73	13.62	20.15	5.73
2	28641.62				
3	134396.50				
1 (FT)	26360.25	136866.06	19.26		
2	1744.21				
3	108761.61				
1 (FT)	78227.15	283828.44	27.56		
2	17144.24				
3	188457.05				

Table 6.5: Calculation of the intensity of the protein bands of GSTSeFt with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	6194.21	145161.70	4.27	6.60	2.34
2	128403.78				
3	10563.71				
1 (FT)	19947.57	223075.87	8.94		
2	66314.85				
3	136813.45				

Table 6.6: Calculation of the intensity of the protein bands of StrepNusSeFt with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	43684.14	214326.60	20.38	19.48	1.11
2	136023.72				
3	34618.74				
1 (FT)	44533.02	248672.13	17.91		
2	1564.82				
3	202574.28				
1 (FT)	49804.19	247309.86	20.14		
2	7104.34				
3	190401.34				

Table 6.7: Calculation of the intensity of the protein bands of GSTWbgl with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	11630.38	193345.21	6.02	7.68	1.66
2	155130.76				
3	26584.07				
1 (FT)	22781.88	244013.29	9.34		
2	68036.01				
3	153195.40				

Table 6.8: Calculation of the intensity of the protein bands of StrepNusWbgl with ImageJ. The first peak is the fucosyltransferase, the second and third peak the area before and after the fucosyltransferase.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1 (FT)	64776.37	219115.61	29.56	24.10	5.02
2	33388.53				
3	120950.71				
1 (FT)	27955.73	110534.44	25.29		
2	314.75				
3	82263.96				
1 (FT)	50953.84	292035.99	17.45		
2	20507.79				
3	220574.35				

Table 6.9: Calculation of the intensity of the protein bands of empty vector control with ImageJ.

Peak	Peak Area	Whole protein peak area	share of fucosyl-transferase	Mean value	Standard dev.
1	6630.76	76304.71	8.69	6.94	1.75
2	5744.52				
3	63929.43				
1	6750.67	130114.26	5.19		
2	6789.14				
3	116574.46				

Table 6.10: Expression of the fucosyltransferase StrepNusHpFt and the theoretical share of it to the whole cell proteins, calculated from the bands of a SDS-PAGE using ImageJ.

	shake flask volume [mL]	CWW [g]	protein in CFE [mg/mL]	CFE volume [mL]
15 °C	50	0.38	0.71	10
Origami B	50	0.4	1.25	10
add. Amp.	50	0.45	1.51	10
TB-media	50	1	1.66	10
Autoinduction	50	1	1.99	10
LB-media	50	0.5	1.4	10
empty vector control	250	1.5	2	10

	protein [mg]	protein / L shaking flask [mg]	band intensity	estimated amount of protein/ L shake flask culture [mg]
15 °C	7.1	142	17.8	25
OrigamiB	12.5	250	22.2	56
add. Amp	15.1	302	27.5	83
TB-media	16.6	332	14.9	49
Autoinduction	19.9	398	9.2	37
LB-media	14	280	31.04	87
empty vector control	20	80	4.3	3

Table 6.11: Absorbance values measured photometrically in continuous manner at 340 nm for the detection of the apparent K_m-value of StrepNusHpFt with steadious GDP-fucose concentration and variation in lactose concentration. The title of the columns contains the used lactose concentrations in mM.

Time [min]	10	10	25	35	35	50	60	60	80	80	100	100
0	1.0037	0.9802	1.0821	1.0877	1.0643	1.0673	1.1291	1.104	1.1363	1.0809	1.1241	1.1007
0.25	1.0114	0.9698	1.0757	1.0967	1.0619	1.0636	1.1168	1.0927	1.1387	1.0763	1.1214	1.0899
0.5	0.9998	0.9639	1.0703	1.0848	1.056	1.0528	1.1132	1.0903	1.1264	1.0814	1.1423	1.0909
0.75	1.0016	0.954	1.0665	1.084	1.0601	1.0527	1.1113	1.0804	1.1379	1.0668	1.1135	1.0854
1	0.9951	0.9517	1.0661	1.0786	1.0583	1.0461	1.0933	1.0808	1.1234	1.0626	1.1099	1.086
1.25	0.9808	0.9518	1.0628	1.0765	1.0538	1.0373	1.0898	1.0695	1.116	1.0471	1.0939	1.0736
1.5	0.9739	0.9485	1.0535	1.0701	1.0431	1.0339	1.083	1.0603	1.1053	1.0382	1.0856	1.0609
1.75	0.9705	0.9333	1.0427	1.0613	1.0332	1.0309	1.0735	1.0552	1.0848	1.0369	1.0739	1.0513
2	0.9751	0.9258	1.0366	1.0991	1.0233	1.0195	1.068	1.0453	1.0939	1.0246	1.0668	1.0447
2.25	0.9742	0.9246	1.0289	1.0661	1.0179	1.012	1.0521	1.0364	1.0742	1.0173	1.0609	1.0341
2.5	0.9674	0.9252	1.0201	1.0454	1.0039	1.0008	1.0543	1.0281	1.0704	1.0085	1.0554	1.0284
2.75	0.9623	0.9212	1.0134	1.0402	0.9933	1.0007	1.0396	1.0204	1.0524	0.9996	1.0303	1.0279
3	0.9628	0.9155	1.0057	1.0301	0.9859	0.9939	1.0349	1.0104	1.0454	0.99	1.0264	1.0072
3.25	0.9596	0.9142	0.9912	1.0208	0.9769	0.9789	1.0283	1.0005	1.0313	0.9818	1.012	0.9968
3.5	0.9621	0.9115	0.9869	1.0081	0.9697	0.9675	1.0218	0.9906	1.0212	0.9746	0.9988	0.9857
3.75	0.9555	0.9089	0.9749	1.0034	0.9557	0.9579	1.0149	0.9788	1.0109	0.9605	0.9894	0.9831
4	0.9542	0.9009	0.9623	0.9899	0.9437	0.9509	1.0377	0.9728	1.0028	0.9518	0.9794	0.971
4.25	0.9486	0.8952	0.9553	0.972	0.9297	0.942	0.9955	0.9603	0.983	0.9411	0.9637	0.9606
4.5	0.9406	0.8907	0.9344	0.9587	0.9187	0.9273	0.9784	0.943	0.9784	0.9271	0.9564	0.942
4.75	0.9408	0.8871	0.935	0.9517	0.9148	0.9171	0.9649	0.9378	0.961	0.919	0.9437	0.9315
5	0.936	0.8838	0.9221	0.9434	0.9018	0.9062	0.9614	0.9246	0.9588	0.9073	0.9344	0.9162
5.25	0.9332	0.883	0.9196	0.9283	0.8935	0.8908	0.957	0.9108	0.9432	0.896	0.9229	0.9065
5.5	0.9307	0.8792	0.9142	0.9218	0.8835	0.8824	0.9464	0.9025	0.9412	0.8863	0.9197	0.8924
5.75	0.9207	0.8798	0.9114	0.9104	0.8796	0.8713	0.9296	0.884	0.9166	0.8716	0.9007	0.8813
6	0.9133	0.8736	0.8978	0.9001	0.872	0.8581	0.926	0.8718	0.9203	0.8594	0.8912	0.8677
6.25	0.9089	0.8718	0.8954	0.8873	0.8817	0.8413	0.9132	0.8611	0.9044	0.8471	0.8738	0.8564

6.5	0.9058	0.8625	0.8908	0.8769	0.8794	0.834	0.9101	0.8446	0.9088	0.8338	0.8632	0.8496
6.75	0.9037	0.8602	0.8849	0.8731	0.8643	0.8168	0.923	0.8268	0.8936	0.8154	0.8533	0.8332
7	0.8978	0.8541	0.8733	0.863	0.843	0.8011	0.9165	0.8126	0.8749	0.7987	0.8365	0.8239
7.25	0.8911	0.8512	0.867	0.8558	0.8291	0.796	0.89	0.7966	0.8502	0.7797	0.8229	0.8113
7.5	0.8882	0.8472	0.8572	0.8457	0.8156	0.7735	0.8667	0.7736	0.8373	0.76	0.813	0.7961
7.75	0.8817	0.8421	0.8572	0.84	0.8086	0.7685	0.8635	0.7596	0.8482	0.7477	0.7968	0.7816
8	0.8797	0.8356	0.8464	0.8377	0.7995	0.7595	0.8639	0.7428	0.8362	0.7267	0.7834	0.761
8.25	0.8727	0.8317	0.8344	0.8222	0.7939	0.7487	0.8594	0.7317	0.8082	0.722	0.7659	0.7366
8.5	0.8668	0.8254	0.8308	0.8121	0.7857	0.7369	0.8171	0.7188	0.7917	0.7188	0.7487	0.7172
8.75	0.8646	0.8174	0.8236	0.802	0.7749	0.7272	0.7938	0.7033	0.7809	0.7059	0.7255	0.6919
9	0.8605	0.8131	0.8246	0.8028	0.7675	0.7252	0.7745	0.6905	0.7628	0.6747	0.7052	0.6804
9.25	0.8552	0.808	0.8152	0.7862	0.7601	0.7165	0.7625	0.6802	0.7506	0.6618	0.6834	0.6703
9.5	0.8456	0.804	0.799	0.7773	0.7465	0.7094	0.75	0.6703	0.7408	0.6524	0.6658	0.6414
9.75	0.8444	0.8029	0.7932	0.7697	0.7413	0.7	0.7441	0.6626	0.7206	0.6439	0.6518	0.6308
10	0.836	0.7965	0.7831	0.7645	0.7306	0.6909	0.7466	0.649	0.6955	0.6382	0.633	0.6157
10.25	0.8353	0.7873	0.77	0.7501	0.7233	0.6875	0.724	0.6389	0.6717	0.6248	0.6214	0.5992
10.5	0.8248	0.778	0.7608	0.7428	0.7142	0.6764	0.6908	0.6309	0.6643	0.6228	0.6084	0.5778
10.75	0.8195	0.779	0.7556	0.7363	0.7089	0.6701	0.675	0.6217	0.6642	0.6117	0.5885	0.5672
11	0.8143	0.7713	0.7447	0.7219	0.6956	0.6599	0.6593	0.6066	0.6458	0.6016	0.5566	0.5439
11.25	0.811	0.7652	0.7316	0.7144	0.6857	0.6549	0.6493	0.5971	0.6288	0.5907	0.5457	0.5321
11.5	0.8028	0.761	0.7228	0.7052	0.6824	0.6472	0.6366	0.5853	0.6138	0.5819	0.5288	0.5151
11.75	0.7959	0.7546	0.7139	0.6999	0.6728	0.6336	0.6218	0.5789	0.5911	0.571	0.5183	0.5091
12	0.793	0.7463	0.7009	0.687	0.6655	0.6158	0.608	0.5652	0.5779	0.5619	0.5107	0.4884
12.25	0.7917	0.7465	0.6966	0.6761	0.6568	0.6115	0.599	0.5552	0.5688	0.5532	0.5062	0.4775
12.5	0.7787	0.7438	0.6888	0.6645	0.6453	0.6026	0.5871	0.5444	0.5419	0.5459	0.4772	0.4634
12.75	0.7803	0.7448	0.6775	0.6538	0.6374	0.5907	0.5787	0.5352	0.5314	0.5396	0.4625	0.4483
13	0.774	0.7399	0.6707	0.6455	0.6279	0.5818	0.5689	0.5201	0.5162	0.5207	0.4393	0.4333
13.25	0.7662	0.7279	0.6575	0.6326	0.6172	0.5688	0.556	0.5105	0.4967	0.5049	0.4221	0.4164
13.5	0.7607	0.72	0.6503	0.63	0.6117	0.5594	0.5457	0.5038	0.489	0.4994	0.4081	0.406
13.75	0.7584	0.7123	0.6417	0.6167	0.5988	0.5508	0.5401	0.4911	0.4779	0.4845	0.3939	0.3933
14	0.7528	0.706	0.628	0.6123	0.5876	0.5401	0.5347	0.4765	0.463	0.4689	0.3795	0.3792

14.25	0.7463	0.7032	0.6277	0.6015	0.5791	0.5335	0.5227	0.4707	0.4581	0.4608	0.3675	0.3629
14.5	0.7366	0.6987	0.6112	0.587	0.5656	0.5167	0.5077	0.4524	0.4446	0.4453	0.353	0.3492
14.75	0.7301	0.7006	0.6015	0.5779	0.5603	0.51	0.5015	0.4413	0.4298	0.4421	0.3388	0.3367
15	0.7315	0.6899	0.5942	0.5716	0.5509	0.4963	0.4929	0.4312	0.418	0.4225	0.3246	0.3246
15.25	0.7292	0.6881	0.5832	0.5573	0.5389	0.487	0.4794	0.42	0.4112	0.4146	0.3096	0.308

Table 6.12: Equation for fitting of the Michaelis-Menten-curve.

Equation	$f = y_0 + \frac{a * x}{b + x}$
y_0	0.0569
a	0.6681
b	47.6909
R^2	0.9907

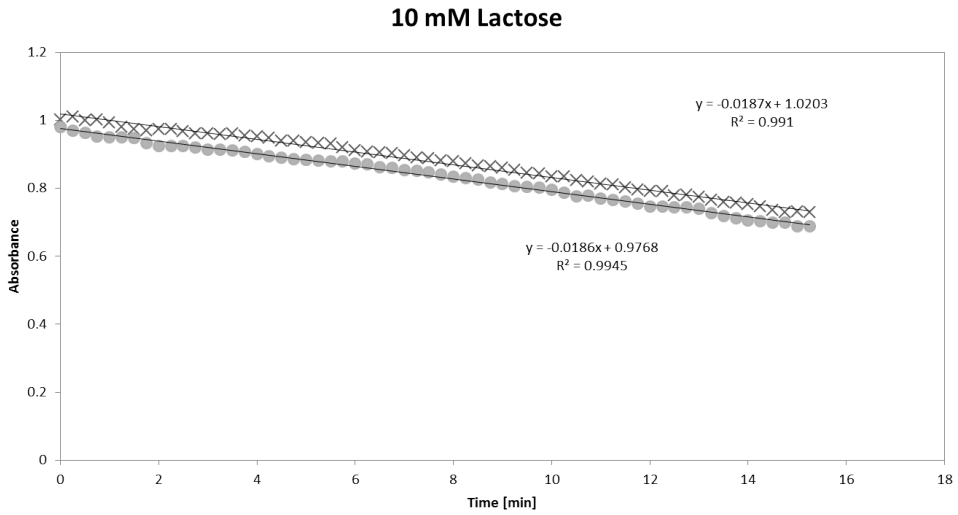

Figure 6.1: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 10 mM lactose concentration.

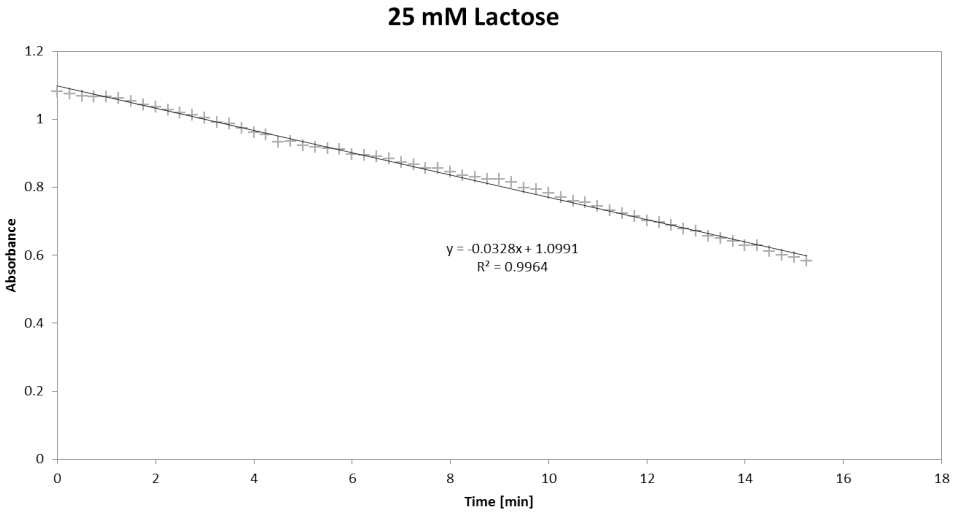

Figure 6.2: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 25 mM lactose concentration.

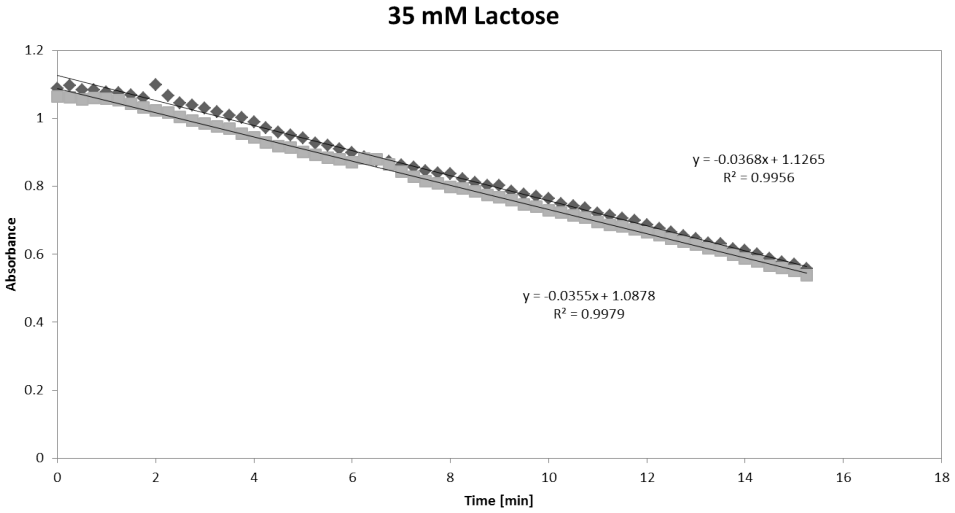

Figure 6.3: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 35 mM lactose concentration.

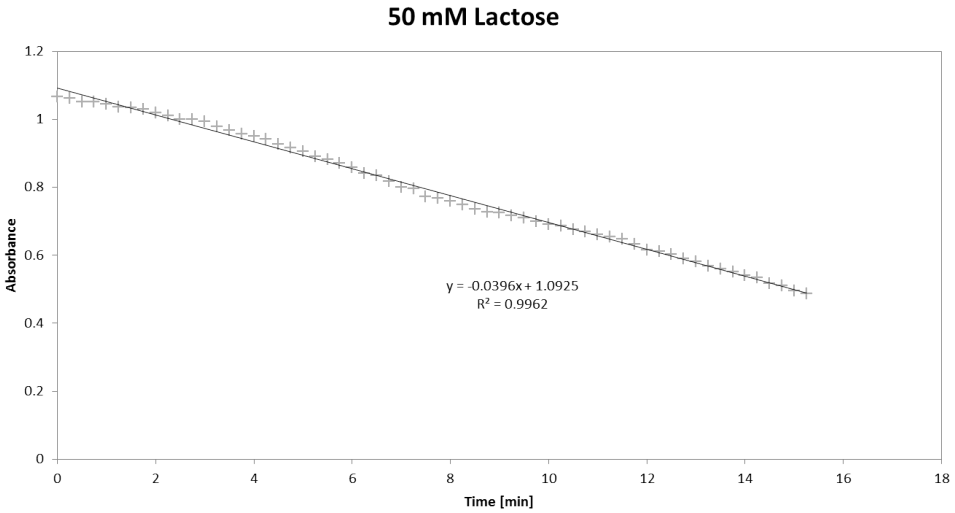

Figure 6.4: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 50 mM lactose concentration.

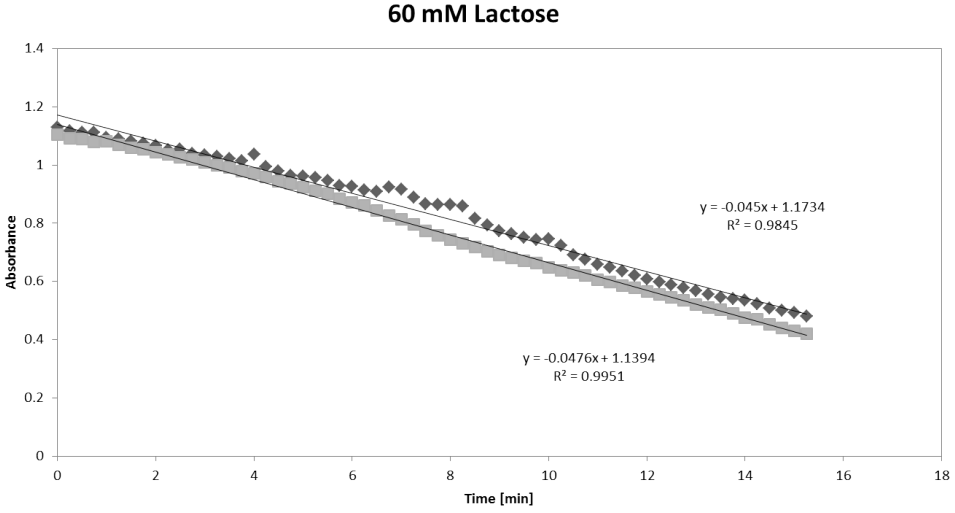

Figure 6.5: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 60 mM lactose concentration.

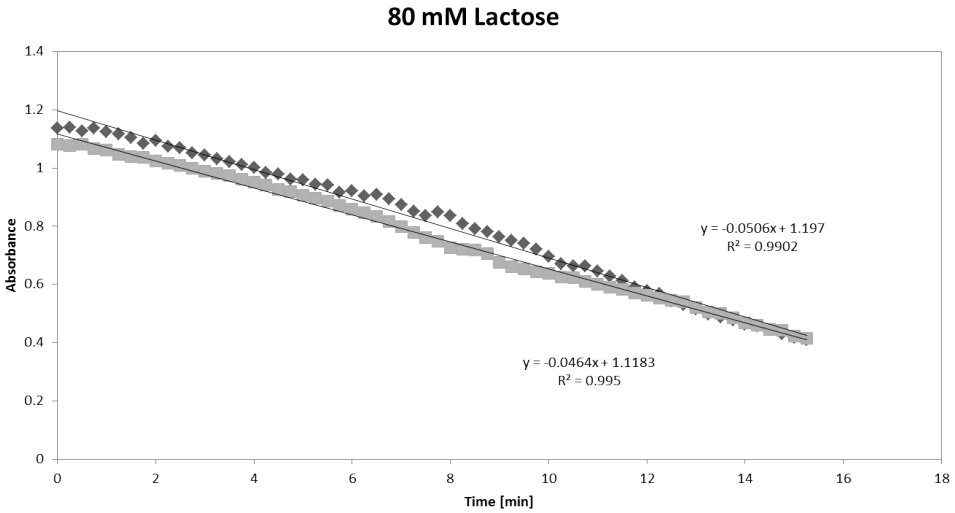

Figure 6.6: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 80 mM lactose concentration.

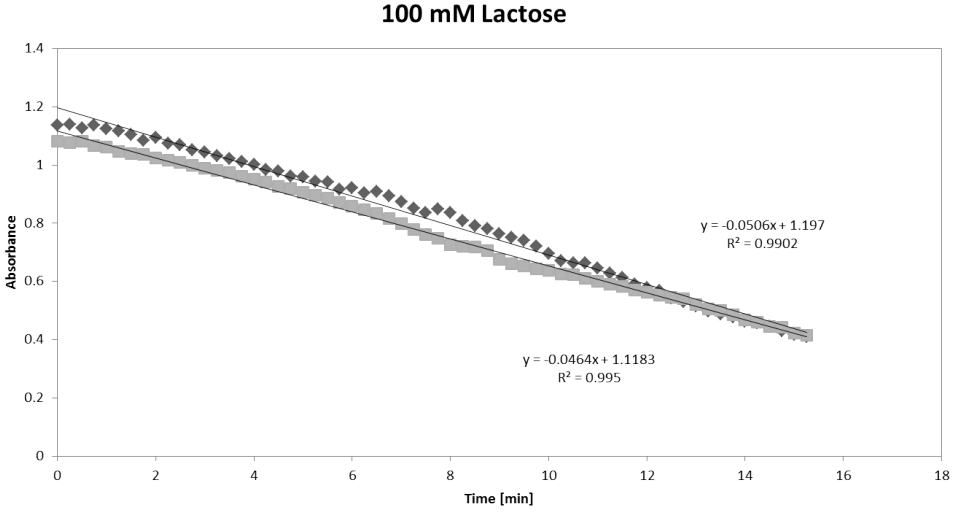

Figure 6.7: Absorbance over time for the photometric detection of the specific activity of StrepNusHpFt for the calculation of the apparent K_m-value with at 100 mM lactose concentration.

Table 6.13: Conversion of lactose over reaction with different lactose starting concentration measured on HPLC using Aminex H87-column.

Name	Peak area at reaction start	Peak area at reaction end	Name	Conc. at reaction start [mM]	Conc. at reaction end [mM]
1	136432	96006	1	1.701	1.060
4	243790	161992	4	3.915	2.564
5	306084	70190	5	4.916	0.564
10	1706020	1425590.4	10	13.699	10.603
20	2505852	1533073.5	20	20.121	14.383
36	4468800	2898004.2	36	35.883	28.690
102	12713308	8173523.4	102	102.085	91.320

Table 6.14: Conversion of GDP-fucose over reaction with different lactose starting concentration measured on HPLC using Aminex H87-column.

Name	Peak area at reaction start	Peak area at reaction end	Name	Conc. at reaction start [mM]	Conc. at reaction end [mM]
1	125218	24156	1	0.75	0.15
4	508040	0	4	3.06	0.00
5	203764	2010	5	1.38	0.01
10	429484	13330	10	2.59	0.08
20	431706	0	20	2.60	0.00
36	457460	0	36	2.75	0.00
102	441864	0	102	2.66	0.00

Table 6.15: Conversion of 2′-fucosyllactose over reaction with different lactose starting concentration measured on HPLC using Aminex H87-column. The 5 mM experiment was a performed on another day than the other values with a lower GDP-fucose starting concentration as could be seen in 6.14. As the conversion yield fits into the data, the 5 mM data set was included.

Name	Peak area at reaction start	Peak area at reaction end	Name	Conc. at reaction start [mM]	Conc. at reaction end [mM]
1	0	12140	1	0.00	0.07
4	0	171166	4	0.00	0.68
5	0	51812	5	0.00	0.21
10	0	196546	10	0.00	0.88
20	0	220266.9	20	0.00	1.04
36	0	228402.3	36	0.00	1.13
102	0	231535.5	102	0.00	1.30

Table 6.16: Slope of standard calibration curve of 1 mM of substance used for calculation of the above concentrations.

GDP-fucose	166094
Lactose	124537
2′-Fucosyllactose	248164

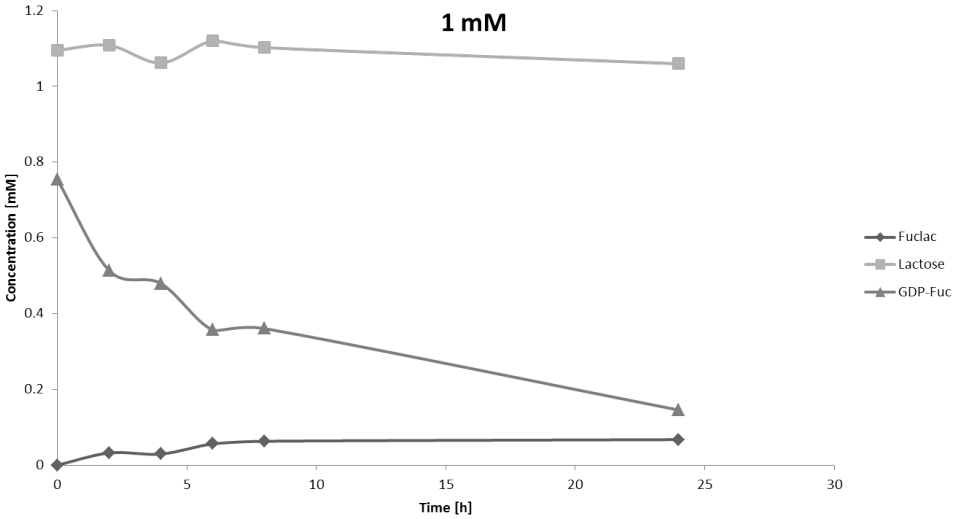

Figure 6.8: Conversion of substrates and products over time with 1 mM lactose at reaction start.

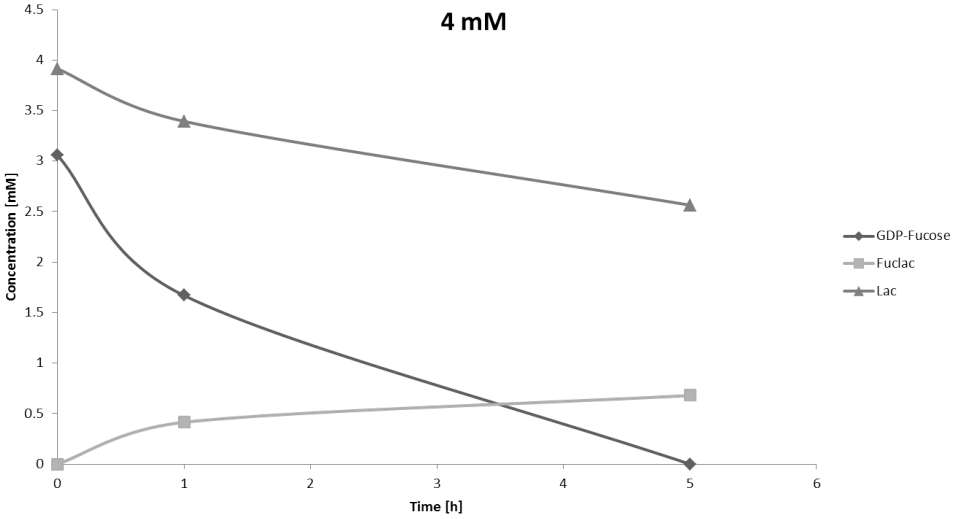

Figure 6.9: Conversion of substrates and products over time with 4 mM lactose at reaction start.

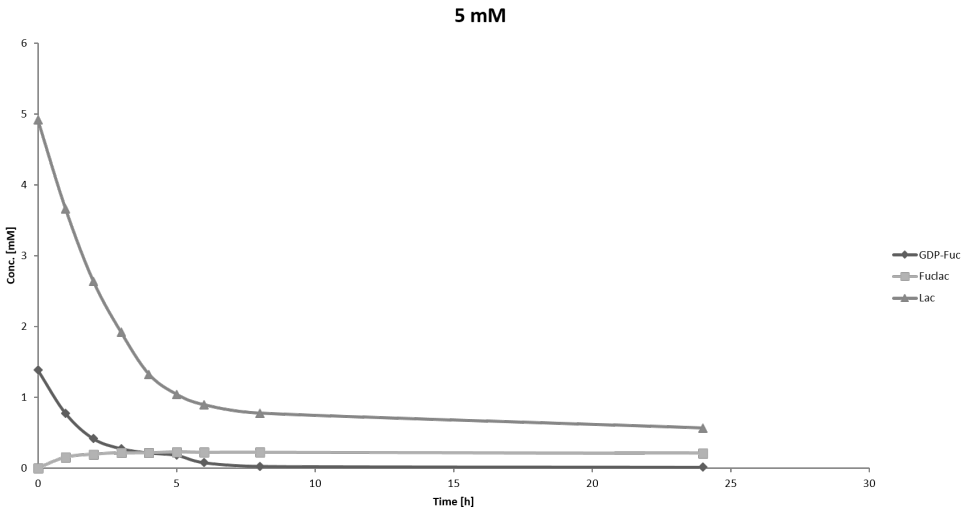

Figure 6.10: Conversion of substrates and products over time with 5 mM lactose at reaction start.

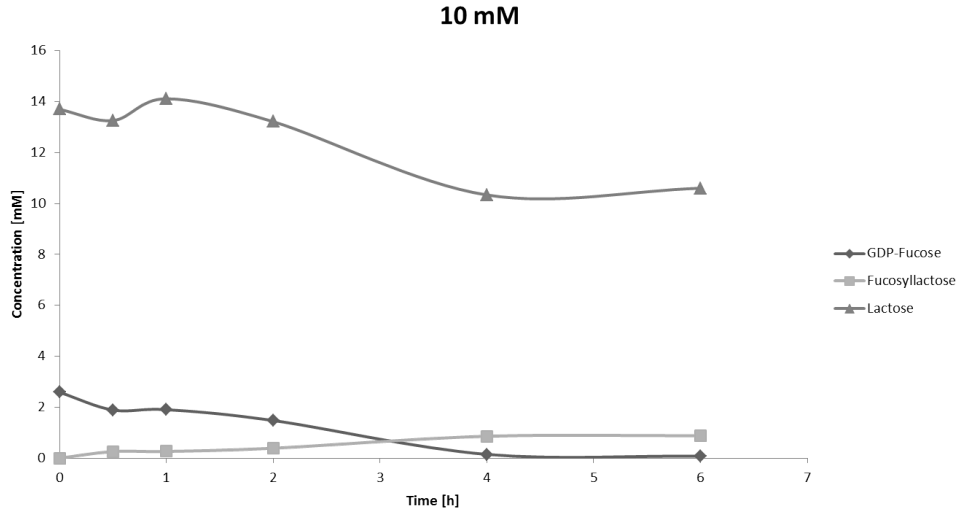

Figure 6.11: Conversion of substrates and products over time with 10 mM lactose at reaction start.

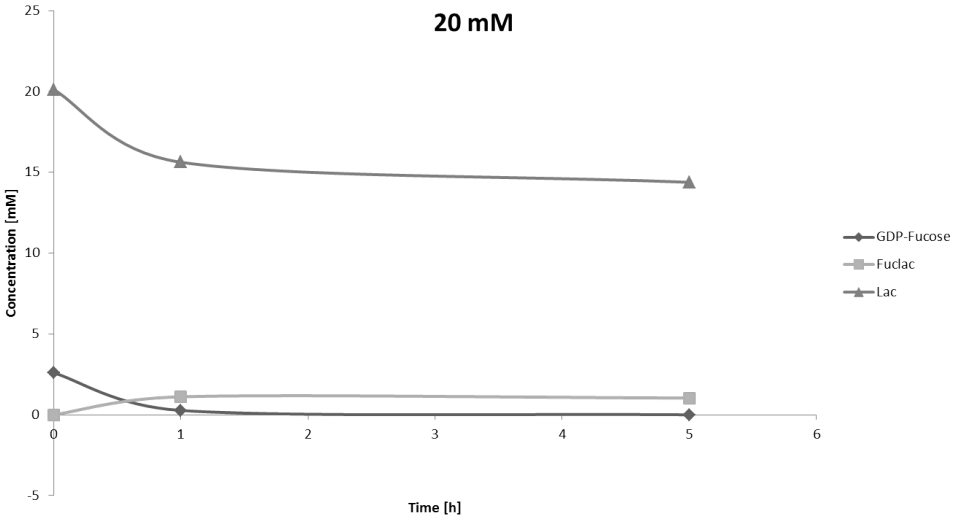

Figure 6.12: Conversion of substrates and products over time with 20 mM lactose at reaction start.

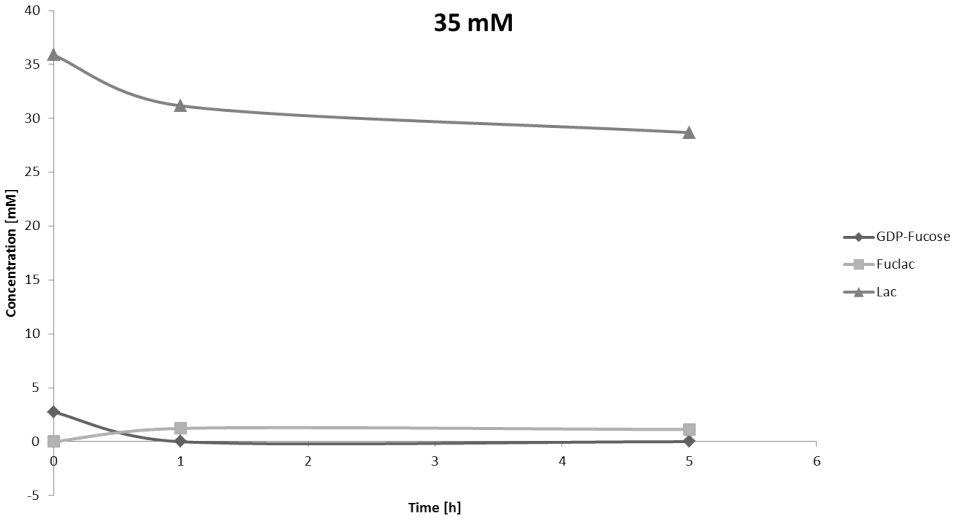

Figure 6.13: Conversion of substrates and products over time with 35 mM lactose at reaction start.

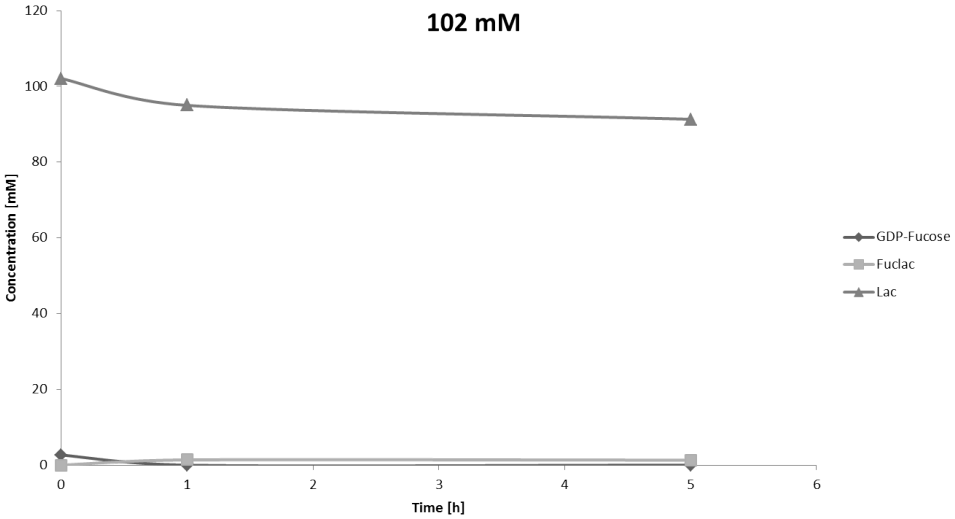

Figure 6.14: Conversion of substrates and products over time with 100 mM lactose at reaction start.

Table 6.18: Further Experiment for the determination of the temperature optimum of Strep-NusSeFt using a photometric assay.

Time [min]	37 °C	40 °C	45 °C
2.12	1.022	0.961	0.956
2.20	1.021	0.96	0.955
2.28	1.02	0.959	0.953
2.37	1.018	0.96	0.952
2.45	1.017	0.96	0.954
2.53	1.017	0.957	0.953
2.62	1.016	0.957	0.952
2.70	1.014	0.956	0.951
2.78	1.014	0.955	0.95
2.87	1.013	0.956	0.951
2.95	1.012	0.955	0.95
3.03	1.011	0.954	0.95
3.12	1.01	0.953	0.951

3.20	1.009	0.953	0.95
3.28	1.009	0.951	0.951
3.37	1.006	0.951	0.952
3.45	1.005	0.95	0.95
3.53	1.004	0.95	0.949
3.62	1.002	0.949	0.949
3.70	1.003	0.949	0.948
3.78	1.002	0.948	0.949
3.87	1.001	0.948	0.95
3.95	1	0.947	0.948
4.03	0.999	0.947	0.948
4.12	0.998	0.947	0.949
4.20	0.997	0.946	0.948
4.28	0.996	0.946	0.948
4.37	0.996	0.946	0.949
4.45	0.996	0.945	0.948
4.53	0.996	0.945	0.948
4.62	0.994	0.945	0.948
4.70	0.994	0.945	0.948
4.78	0.994	0.944	0.949
4.87	0.992	0.944	0.948
4.95	0.992	0.944	0.948
5.03	0.992	0.945	0.948

Table 6.17: Determination of the temperature optimum of StrepNusSeFt using a photometric assay.

Time [min]	35 °C	37 °C	40 °C	45 °C
2.00	1.7563	1.1523	1.1319	0.9613
2.17	1.7573	1.1516	1.1304	0.9621
2.33	1.7562	1.1521	1.1308	0.96
2.50	1.7545	1.1511	1.1299	0.9612
2.67	1.7549	1.1506	1.1296	0.9577
2.83	1.7542	1.1482	1.12803	0.9584
3.00	1.7556	1.1473	1.1307	0.9565
3.17	1.7545	1.1476	1.1264	0.9567
3.33	1.7543	1.1479	1.1276	0.9554
3.50	1.7505	1.1459	1.1233	0.9582
3.67	1.7484	1.1468	1.1231	0.9542
3.83	1.7469	1.1447	1.1223	0.9567
4.00	1.7478	1.1447	1.1252	0.9546
4.17	1.7511	1.1458	1.1224	0.9546
4.33	1.7484	1.1446	1.1213	0.9513
4.50	1.7476	1.1443	1.1243	0.9534
4.67	1.7445	1.1456	1.1239	0.9523
4.83	1.7439	1.1446	1.1241	0.9506
5.00	1.7428	1.1453	1.1196	0.9496
5.17	1.7427	1.1433	1.1197	0.9504
5.33	1.7435	1.1421	1.1201	0.9506
5.50	1.7423	1.1419	1.1164	0.9511
5.67	1.7431	1.1404	1.1174	0.9499

Table 6.19: Further experiments for the determination of the temperature optimum of Strep-NusSeFt using a photometric assay.

Time [min]	30 °C	30 °C	55 °C
2.00	1.0305	1.1194	1.219
2.25	1.029	1.1167	1.2203
2.50	1.0267	1.12	1.2215
2.75	1.0359	1.1099	1.2216
3.00	1.0307	1.1154	1.2197
3.25	1.0306	1.1111	1.2117
3.50	1.0275	1.1056	1.2142
3.75	1.0219	1.1136	1.2064
4.00	1.0265	1.1176	1.2057
4.25	1.0208	1.1127	1.2027
4.50	1.0229	1.1136	1.2014
4.75	1.0219	1.1093	1.1962
5.00	1.0119	1.1044	1.1964
5.25	1.0214	1.1022	1.1989
5.50	1.0119	1.1026	1.1909
5.75	1.0084	1.1037	1.1913

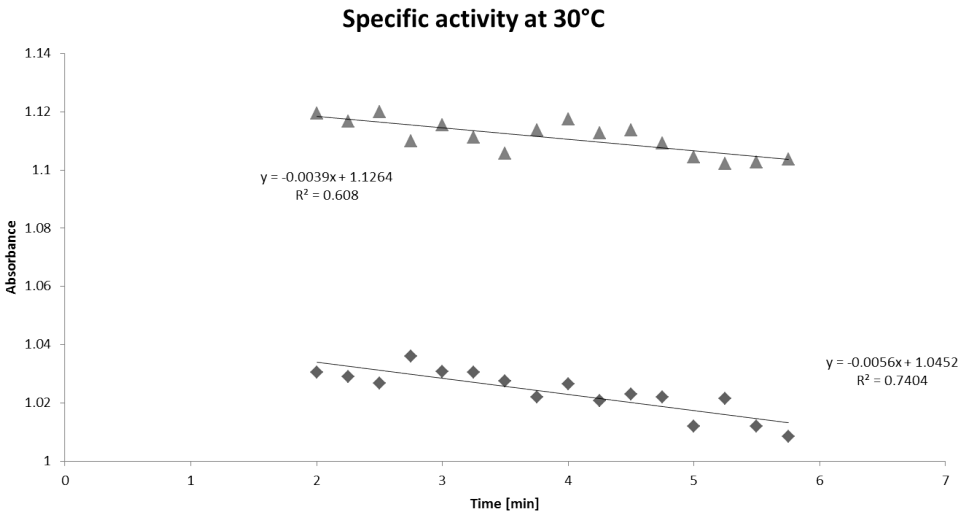

Figure 6.15: Specific activity of StrepNusSeFt at 30 °C measured photometrically.

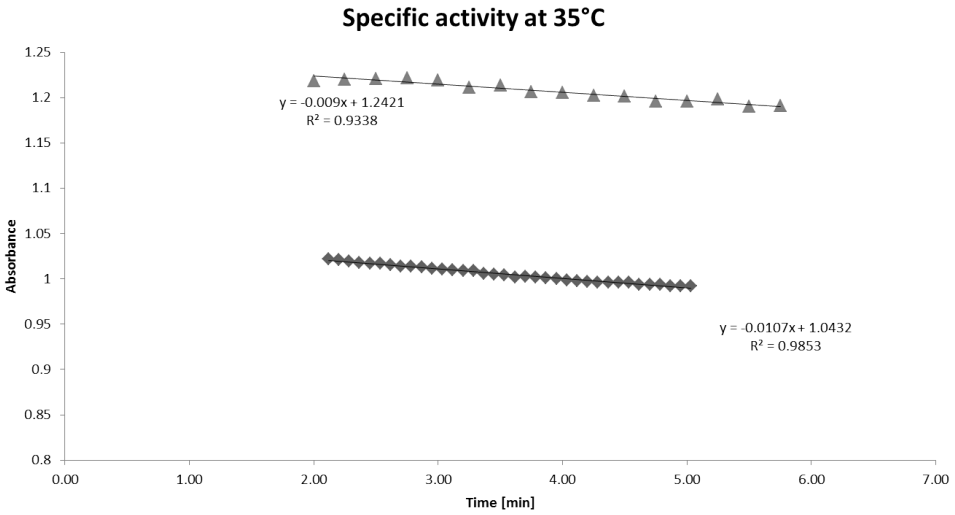

Figure 6.16: Specific activity of StrepNusSeFt at 35 °C measured photometrically.

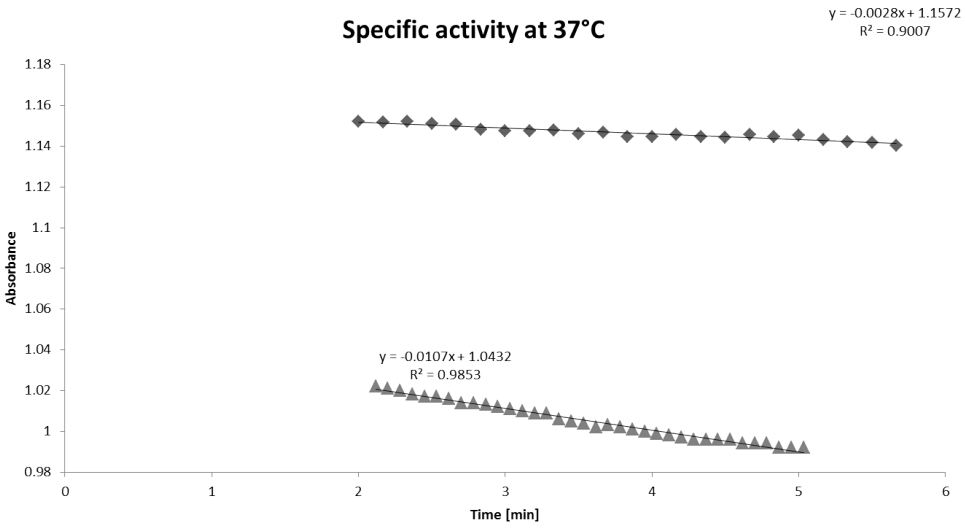

Figure 6.17: Specific activity of StrepNusSeFt at 35 °C measured photometrically.

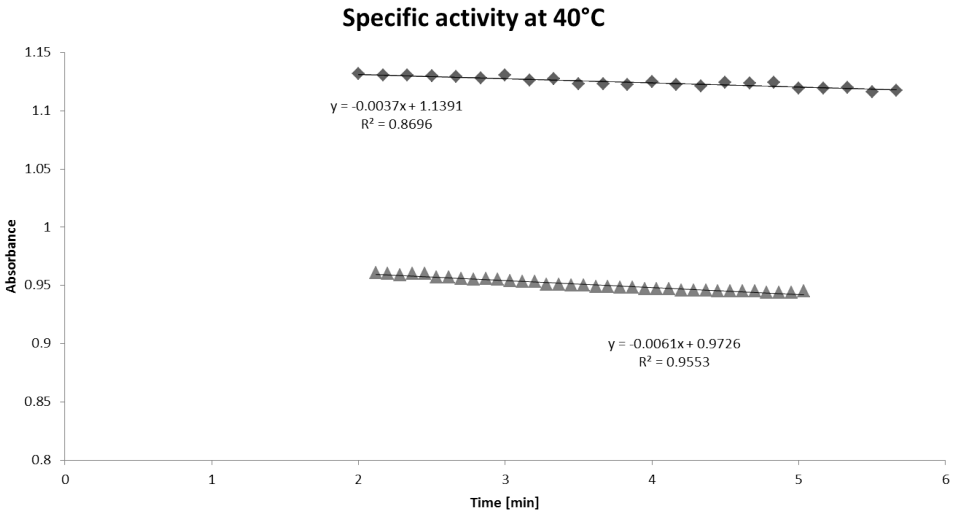

Figure 6.18: Specific activity of StrepNusSeFt at 35 °C measured photometrically.

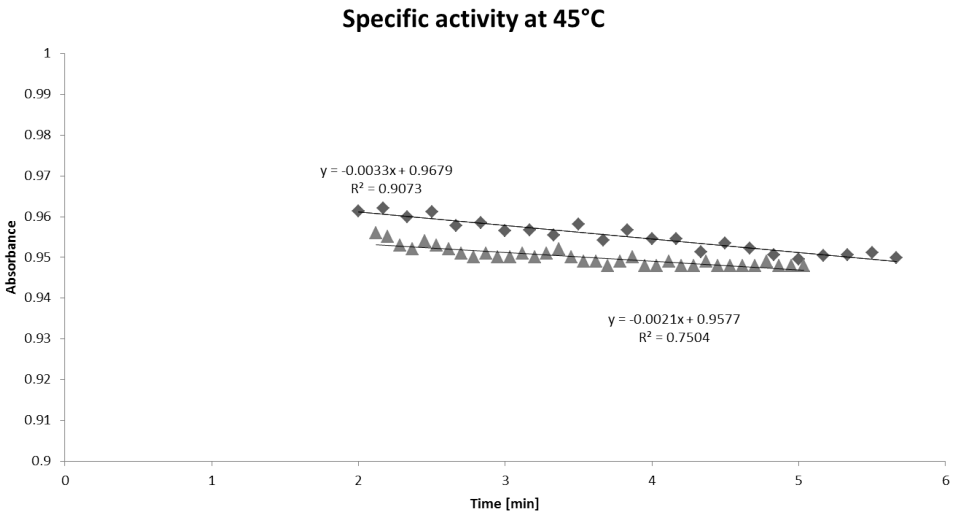

Figure 6.19: Specific activity of StrepNusSeFt at 45 °C measured photometrically.

Table 6.20: Determination of the pH optimum of StrepNusSeFt using a photometric assay.

Time [min]	Citrate pH 6	MES pH 6	HEPES pH 7	MES pH 7	TRIS/HCl pH 7	HEPES pH 8
0.00	1.4631	0.8471	0.7231	0.6341	0.9850	0.5442
0.18	1.4660	0.8360	0.7185	0.6349	0.9827	0.5419
0.37	1.4763	0.8375	0.7183	0.6276	0.9675	0.5361
0.55	1.4478	0.8244	0.7081	0.6178	0.9491	0.5290
0.73	1.4611	0.8276	0.7011	0.6158	0.9414	0.5275
0.92	1.4519	0.8161	0.6997	0.6110	0.9250	0.5206
1.10	1.4432	0.8076	0.6870	0.6037	0.9178	0.5190
1.28	1.4205	0.7957	0.6851	0.6035	0.9070	0.5156
1.47	1.4155	0.7873	0.6792	0.5940	0.8983	0.5093
1.65	1.4112	0.7766	0.6706	0.5917	0.8903	0.5043
1.83	1.4138	0.7682	0.6702	0.5878	0.8813	0.5019
2.02	1.3931	0.7506	0.6570	0.5816	0.8746	0.4976
2.20	1.3934	0.7467	0.6507	0.5734	0.8597	0.4958
2.38	1.3907	0.7391	0.6542	0.5721	0.8560	0.4924

2.57	1.3759	0.7322	0.6442	0.5656	0.8482	0.4841
2.75	1.3621	0.7235	0.6398	0.5616	0.8436	0.4833
2.93	1.3955	0.7205	0.6346	0.5600	0.8329	0.4786
3.12	1.3751	0.7147	0.6341	0.5579	0.8279	0.4768
3.30	1.3472	0.7074	0.6261	0.5502	0.8186	0.4773
3.48	1.3526	0.7083	0.6221	0.5481	0.8200	0.4705
3.67	1.3414	0.7007	0.6179	0.5423	0.8055	0.4628
3.85	1.3385	0.6946	0.6107	0.5399	0.8100	0.4634
4.03	1.3289	0.6890	0.6084	0.5369	0.7902	0.4614
4.22	1.3235	0.6841	0.6029	0.5341	0.7793	0.4555
4.40	1.3020	0.6804	0.5992	0.5272	0.7782	0.4562
4.58	1.3080	0.6763	0.5965	0.5301	0.7794	0.4550
4.77	1.3041	0.6707	0.5932	0.5242	0.7710	0.4507
4.95	1.2961	0.6632	0.5872	0.5220	0.7569	0.4444
5.13	1.3213	0.6618	0.5849	0.5163	0.7530	0.4474
5.32	1.3088	0.6557	0.5816	0.5119	0.7433	0.4448
5.50	1.2629	0.6486	0.5711	0.5074	0.7409	0.4377
5.68	1.2593	0.6457	0.5711	0.5069	0.7451	0.4409
5.87	1.2502	0.6352	0.5677	0.4986	0.7306	0.4367
6.05	1.2430	0.6374	0.5691	0.5022	0.7246	0.4365
6.23	1.2379	0.6341	0.5611	0.5004	0.7157	0.4354
6.42	1.2383	0.6308	0.5575	0.4952	0.7150	0.4340
6.60	1.2296	0.6228	0.5525	0.4959	0.7042	0.4296
6.78	1.2145	0.6217	0.5513	0.4883	0.7026	0.4291
6.97	1.2179	0.6177	0.5512	0.4866	0.6957	0.4264
7.15	1.2109	0.6141	0.5506	0.4860	0.6914	0.4236

Table 6.21: Further experiments for the determination of the pH optimum of StrepNusSeFt using a photometric assay.

Time [min]	Citrate pH 5	MES pH 6
0.00	1.1801	0.8082
0.25	1.1790	0.8027
0.50	1.1762	0.7829
0.75	1.1794	0.7726
1.00	1.1799	0.7550
1.25	1.1791	0.7499
1.50	1.1768	0.7294
1.75	1.1749	0.7195
2.00	1.1714	0.7103
2.25	1.1738	0.6958
2.50	1.1710	0.6905
2.75	1.1730	0.6804
3.00	1.1696	0.6781
3.25	1.1709	0.6622
3.50	1.1682	0.6455
3.75	1.1686	0.6375
4.00	1.1706	0.6252
4.25	1.1696	0.6173
4.50	1.1670	0.6159
4.75	1.1647	0.6014
5.00	1.1677	0.5900
5.25	1.1671	0.5836

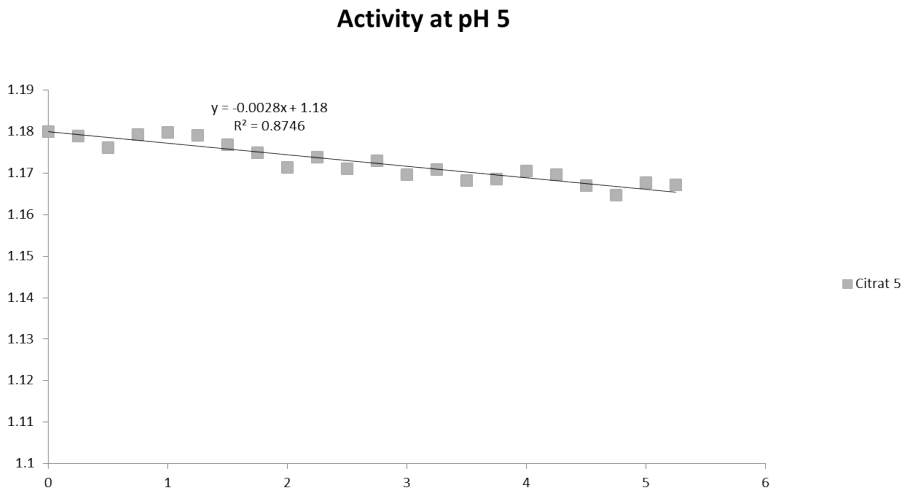

Figure 6.20: Specific activity of StrepNusSeFt at pH 5 measured photometrically.

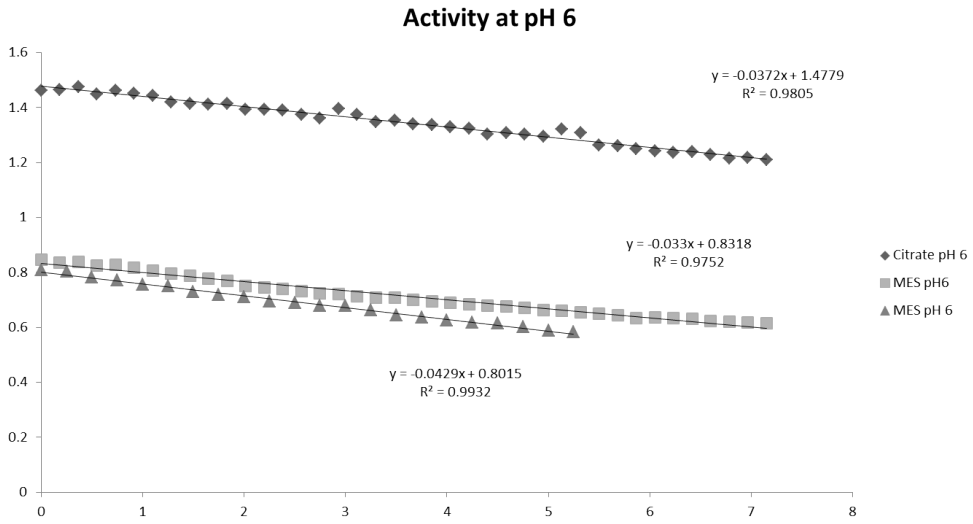

Figure 6.21: Specific activity of StrepNusSeFt at pH 6 measured photometrically.

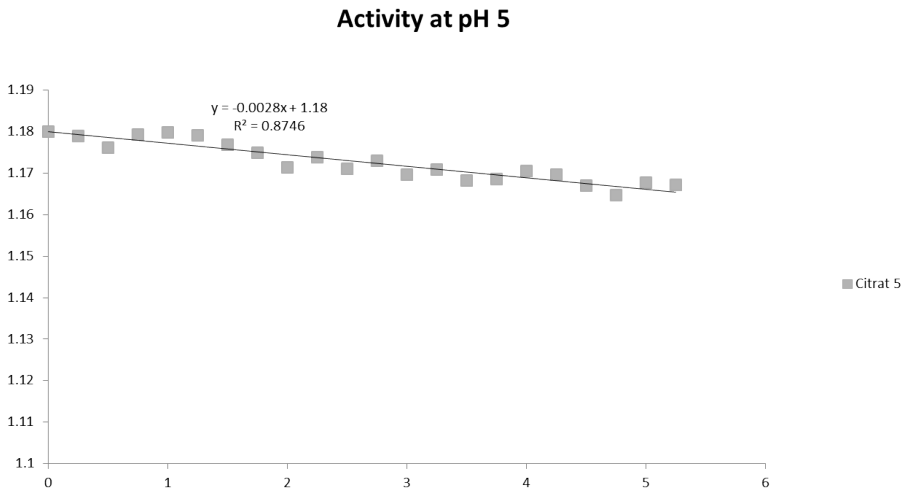

Figure 6.22: Specific activity of StrepNusSeFt at pH 7 measured photometrically.

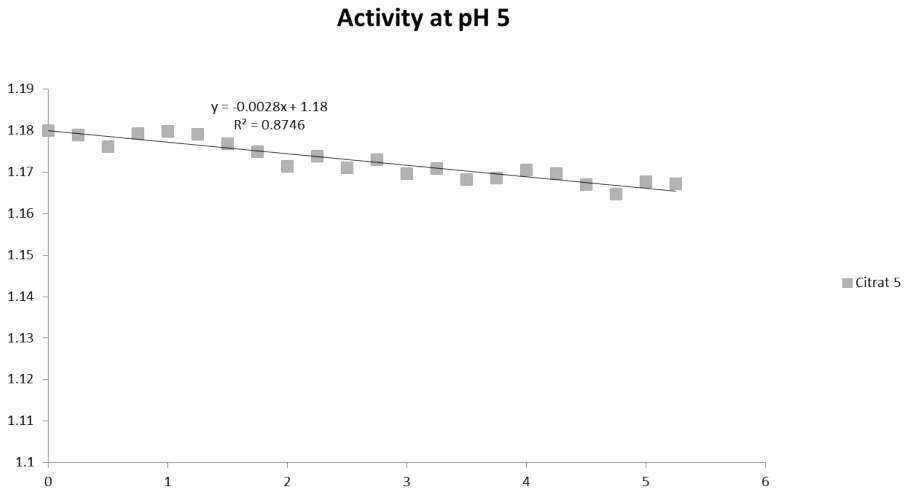

Figure 6.23: Specific activity of StrepNusSeFt at pH 8 measured photometrically.

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