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Synthesis and purification of two nucleotide sugars: UDP-β-L-rhamnose and GDP-β-L-fucose

MASTER'S THESIS

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Abstract

Nucleotide sugars are monosaccharides attached to a nitrogenous base and to mono-, di- or tri phosphates. Nucleotide sugars present an activated form of the monosaccharide and can act as glycosyl donors for glycosylation reactions. The most numerous in nature of these activated sugars are nucleoside diphosphate sugars (NDP-sugars). In this thesis enzymatic synthesis of two nucleoside diphosphate sugars, GDP-β-L-fucose and UDP-β-L-rhamnose, was studied.

GDP-β-L-fucose acts as a sugar donor for the fucosylation of glycan structures and free oligosaccharides, such as human milk oligosaccharides, by fucosyltransferases. The synthesis of GDP-β-L-fucose can be carried out, *inter alia*, via the salvage pathway. In this case, L-fucose is metabolized to L-fucose-1-phosphate and then processed further to GDP-β-L-fucose. In the bacterium *Bacteroides fragilis* 9343, an enzyme was discovered which accomplishes both conversion steps (L-fucokinase/L-fucose-1-phosphate guanylyltransferase, *Bf*FKP). This thesis describes the enzymatic production and purification of GDP-β-L-fucose with *Bf*FKP. Enzymatic conversion of L-fucose to GDP-β-L-fucose was optimized with regard to substrate and enzyme concentrations and manganese addition. Optimized conversion reached 91 % conversion yield. An optimized purification procedure including a digestion with a phosphatase, an anion exchange chromatography step, a size exclusion step and freeze drying is described and yielded 28.3 mg GDP-β-L-fucose (in dried form). The identity of GDP-β-L-fucose was confirmed using a reference standard (analyzed by HPLC with a UV detector) and by ^{1}H NMR.

UDP-β-L-rhamnose acts as a sugar donor for rhamnosylations, e.g. of flavonoids. The sugar is obtained by conversion of UDP-α-D-glucose to UDP-β-L-rhamnose by the enzyme *At*RHM2 from *Arabidopsis thaliana*, which is a UDP-rhamnose synthase. The focus of this side project was the expression of the enzyme and first conversion trials.
Different expression constructs for *AtR*HM2 were cloned with restriction site cloning or via CPEC method. The cloning of two constructs with C-terminal His tag was successful, as was the expression in *E.coli BL21* (DE3) but the purification failed (done via immobilized metal affinity chromatography). As a consequence the conversion reaction was done with cell free extract. As UDP-β-L-rhamnose was not available as standard reference no clear statement could be done by analysis with CE (UV detector) whether UDP-β-L-rhamnose was synthesized.

Kurzfassung

Nukleotidzucker sind Monosaccharide, die an eine stickstoffhaltige Base und an Mono-, Di- oder Triphosphate gebunden sind. Nukleotidzucker präsentieren eine aktivierte Form des Monosaccharids und können als Glycosyldonoren für Glycosylierungsreaktionen dienen. Die am häufigsten vorkommenden aktivierten Zucker sind Nucleosiddiphosphat-Zucker (NDP-Zucker). In dieser Arbeit wurde die enzymatische Synthese von zwei Nucleosiddiphosphat-Zuckern, GDP-β-L-Fucose und UDP-β-L-Rhamnose, untersucht.

GDP-β-L-Fucose fungiert als Zuckerdonor für die Fucosylierung von Glykanstrukturen und freien Oligosacchariden, wie zum Beispiel menschliche Milcholigosaccharide, wobei Fucosyltransferasen als Katalysator dienen. Die Synthese von GDP-β-L-Fucose kann unter anderem über den Salvage Pathway erfolgen. In diesem Fall wird L-Fucose zu L-Fucose-1-Phosphat metabolisiert und dann weiter zu GDP-β-L-Fucose umgewandelt. In dem Bakterium *Bacteroides fragilis* 9343 wurde ein Enzym entdeckt, das beide Umwandlungsschritte (L-fucokinase/L-fucose-1-phosphate guanylyltransferase, *Bf*FKP). bewerkstelligt. Diese Arbeit beschreibt die enzymatische Produktion und Reinigung von GDP-β-L-Fucose mittels *Bf*FKP. Die enzymatische Umwandlung von L-fucose zu GDP-β-L-Fucose wurde in Bezug auf Substrat- und Enzymkonzentrationen und Manganzugabe optimiert. Die optimierte Umsetzung ergab 91% Ausbeute. Das Reinigungsverfahren wurde optimiert und die folgenden Schritte wurden ausgeführt: Nukleotid-Verdau mit einer Phosphatase, Anionenaustauschchromatographie, Größenausschlusschromatographie und Gefriertrocknen. So wurden 28,3 mg GDP-β-L-fucose in Pulverform gewonnen. Die Identität von GDP-β-L-fucose wurde unter Verwendung eines Referenzstandards (HPLC Analyse mit einem UV-Detektor) und durch ^{1}H NMR bestätigt.

UDP-β-L-rhamnose agiert als Zuckerdonor für Rhamnosylierungen, z.B. von Flavonoiden. Das Enzym *At*RHM2 aus *Arabidopsis thaliana* (UDP-Rhamnosesynthase) katalyisert die Umwandlung von UDP-α-D-glucose zu UDP-β-L-rhamnose. Im Fokus dieses Nebenprojekts standen die Expression dieses Enzyms und erste Umsetzungsversuche. Die verschiedenen Expressionskonstrukte von *At*RHM2 wurden mittels Restriktionsenzymen oder mit Hilfe der CPEC Methode kloniert. Die Klonierung von zwei Konstrukten mit C-terminalem His-Tag war erfolgreich, ebenso wie die Expression in *E. coli* BL21 (DE3), aber die Reinigung mittels immobilisierter Metallaffinitätschromatographie schlug fehl. Als Konsequenz wurde die Umwandlungsreaktion mit zellfreiem Extrakt durchgeführt. Da UDP-β-L-Rhamnose als Standardreferenz nicht verfügbar war, konnte durch die Analyse mit der Kappilarelektrophorese (mit UV-Detektor) keine Aussage getroffen werden, ob UDP-β-L-rhamnose synthetisiert wurde.

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Part I

Synthesis and purification

of GDP-β-L-fucose

1 Introduction

Nucleotide sugars are monosaccharides which are linked to a nitrogenous base and to one to three phosphate groups. In this activated form nucleotide sugars act as glycosyl donors for glycosyl transferases. The numerous majority of nucleotide sugars are nucleoside diphosphate sugars (NDP-sugars). This part of the thesis deals with the nucleotide sugar GDP-β-L-fucose.

GDP-β-L-fucose acts as a donor for fucosylation and is therefore a substrate for fucosyltransferases (Niittymäki et al., 2004). These fucosyltransferases need nucleotide-activated sugar molecules to transfer the sugar moiety to the acceptor (Albermann et al., 2000), which can be a glycan structure of proteins or a free oligosaccharide

The general known pathway for the production of GDP-β-L-fucose in bacteria is a three step reaction with a GDP-mannose 4,6-dehydratase (Gmd, EC 4.2.1.47) and a fucose synthase with a combined epimerase and NADPH-dependent reductase activity (Fcl, EC 1.1.1.271) starting from GDP-β-D-mannose (Andrianopoulos, Wang, & Reeves, 1998). This pathway is also known in humans with Gmd and a similar fucose synthase (FX protein) (Tonetti et al., 1996). In general this *de novo* pathway is available in eukaryotic and prokaryotic cells.

In the salvage pathway, the L-fucose is absorbed exogenously and processed further (Becker & Lowe, 2003). First via an intermediate by means of a fucokinase (EC 2.7.1.52) and ATP (Park, Pastuszak, Drake, & Elbein, 1998) and then to GDP-β-L-fucose with a fucose-1-phosphate guanylyltransferase (EC 2.7.7.30) and GTP (Pastuszak et al., 1998). For a long time it was assumed that this pathway would only be present in eukaryotes. But in 2005 Coyne, Reinap, Lee and Comstock detected a protein in *Bacteroides fragilis* 9343 which had the ability to convert exogenous L-fucose to GDP-β-L-fucose.

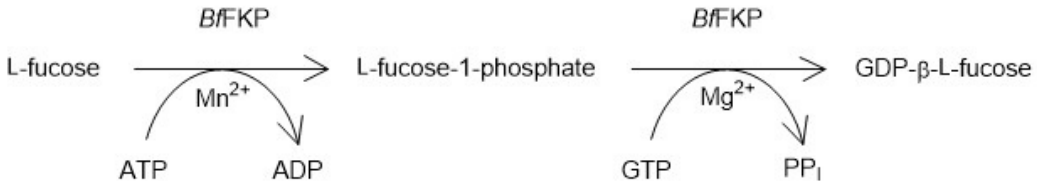

Figure 1.1: Illustration of the salvage pathway for the conversion from L-fucose to GDP-β-L-fucose by the enzyme *Bf*FKP found in *Bacteroides fragilis* 9343. Figure adapted from (Coyne et al., 2005).

The C-terminal part is responsible for the first step where the L-fucose reacts to the intermediate L-fucose-1-phosphate. The N-terminal domain converts the intermediate to GDP-β-L-fucose (Coyne et al., 2005).

This enzyme which takes over the two reaction steps was named Fkp (L-fucokinase/L-fucose-1-phosphate guanylyltransferase, UniProtKB - Q58T34).

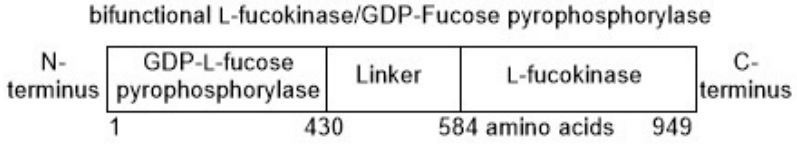

Figure 1.2: Domains of *Bf*FKP. The N-terminal fucose-1-phosphate guanylyltransferase (1 - 430 aa) is connected via a linker (153 aa) with the C-terminal L-fucokinase (584 - 949 aa). Figure adapted from Liu et al. (2011).

A sequence alignment with mammalian proteins (Niittymäki et al., 2004) gave the information, that the N-terminal part is 20% identical with the human GDP-β-L-fucose pyrophosphorylase and that the C-terminal domain is very similar to the mammalian fucokinase (Liu et al., 2011). The function of the linker between both domains with 153 amino acids is currently unknown.

Cheng et al. (2014) examined the *Bf*FKP enzyme concerning the structure. They assumed that the linker contributes to the stability of the enzyme. When carrying out gel filtration and ultracentrifugation experiments they found out that *Bf*FKP is a trimeric molecule in solutions. An electron cryomicroscopy of the enzyme showed two parallel objects. So they concluded based of their obtained results “that the structure of the higher oligomer of FKP may be constructed by two trimers of FKP” (Cheng et al., 2014).

Some kinetic data of the enzyme *Bf*FKP are published by Tsai et al. (2013) and Wang et al. (2009) which are stated in Table 1.1.

Table 1.1: Published kinetic data for the steps which *Bf*FKP is catalyzing. Data from Tsai et al. (2013) and Wang et al. (2009).

	Tsai et al. (2013)	Wang et al. (2009)	Tsai et al. (2013)	Wang et al. (2009)
Substrate	K_m [mM]		k_{cat} [s^{-1}] / [min^{-1}]	k_{cat} [s^{-1}] / [min^{-1}]
L-fucose	0.05	0.045	7.8 / 469	6.1 / 365
ATP	3.3	1.080	12 / 722	7.5 / 448
L-fucose-1-phosphate	0.04	0.030	0.9 / 54	14.6 / 878
GTP	1.08	0.012	1.6 / 96	13.3 / 800

The K_m values for L-fucose and L-fucose-1-phosphate are very similar in both publications. They also agree that ATP has the higher K_m value compared to GTP. But the values and the ratio between the K_m of ATP and GTP are not in agreement.

Tsai et al. (2013) are stating that both K_m values are over 1 and in a ratio of 3:1 (ATP over GTP). Wang et al. (2009), however, published lower values and a ratio of 90:1 (ATP over GTP). The next difference between both papers is the rate of conversion of the two steps. Wang et al. (2009) determined that the second step from the intermediate to the nucleotide sugar is the faster one (about twice the rate). Whereas Tsai et al. (2013) examined that the

first step from L-fucose to L-fucose-1-phosphate is the faster one (approximately 7 to 8 times faster).

In both papers roughly the same working conditions and assays were used. Both steps were determined separately. They measured the fucokinase activity with a fluorometric assay which is based on the production of ADP using the pyruvate kinase/lactate dehydrogenase coupled enzymatic assay for NADH consumption. So the principle is the same but they used other concentrations of the compounds and other temperatures. The second step was followed by the production of pyrophosphate using the same EnzCheck Pyrophosphate Assay Kit from Invitrogen. Again some differences concerning concentration and temperature were used (Tsai et al., 2013 and Wang et al., 2009).

Also the specific activity of *Bf*FKP was determined and published by Tsai and Wang: (I) 16.18 U/mg for the first step and 78.69 U/mg for the second step (Tsai et al., 2013) and (II) 4.5 U/mg for the whole conversion reaction (Wang et al., 2009).

Concerning the downstream processing there are different processes for the purification of GDP-β-L-fucose stated in literature.

Table 1.2: Summary of downstream processing methods of GDP-β-L-fucose. Different publisher, the synthesis methods, the method of purification and the gained yield of the nucleotide sugar are stated.

Publisher	Synthesis	Purification	Yield [mg]	Yield [%]
Gokhale, Hindsgaul, & Palcic, 1990	Chemical	Ion exchange, gel filtration, lyophilization	155	27
Stiller & Thiem, 1992	Salvage pathway	Ion exchange, gel filtration, lyophilization	3	22
Albermann, Distler, & Piepersberg, 2000	*De novo* pathway	Prep. HPLC, gel filtration, membrane anion exchange chromatography, gel filtration, lyophilization	78	75*
Hoh, 2004	*De novo* pathway	Ultrafiltration, anion exchange chromatography, nanofiltration, lyophilization	76	63
Zhao, Guan, Cai, & Wang, 2010	Salvage pathway	Alkaline phosphatase, gel filtration, lyophilization	150 - 250	26 - 43*
Engels & Elling, 2014	Salvage pathway	Alkaline phosphatase, gel filtration	25	43

* Values calculated from the protocol and mg product given by the authors

As one can assume from Table 1.2, the processes used for purification are all more or less similar, except for Hoh et al, who used a combination of ultra and nanofiltration and chromatography.

The aim of this part of the master thesis was to produce the nucleotide sugar GDP-β-L-fucose in high purity for further use as donor substrate for fucosyltransferases. Therefore the

enzyme *Bf*FKP should be expressed and purified. With the pure enzyme the synthesis of GDP-β-L-fucose had to be carried out and optimized. After a successful scale up of the enzymatic conversion the purification process needed to be established. In the end the nucleotide sugar should be present in high purity in powder form. This established downstream process should also be described in a purification method protocol to be available for future purifications by coworkers.

2 Materials and Methods

Following materials and methods were used during the master thesis, when not described otherwise.

Materials and devices which were used for the general lab work are listed in Table 2.1.

Table 2.1: Materials and devices which were used for the general lab work.

Materials	Company	Comments
Reaction tubes: 1.5 mL, 2 mL	Eppendorf AG, Hamburg, Germany	
Reaction tubes: 15 mL, 50 mL	Sarstedt AG & Co, Nümbrecht, Germany	
Syringe: 5 mL, 10 mL	B.Braun Melsungen AG, Melsungen, Germany	
Devices	**Company**	**Comments**
Bioair Auro 2000 Lamina Flow	EuroClone S.p.A., Milan, Italy	Sterile workbench
DeNovix DS-11	DeNovix Inc., Wilmington, DE, USA	Spectrophotometer
Eppendorf Centrifuge	Eppendorf AG, Hamburg, Germany	Centrifuge for 1.5 mL and 2 mL reaction tubes
Eppendorf Centrifuge 5415 R	Eppendorf AG, Hamburg, Germany	Centrifuge for 15 mL and 50 mL reaction tubes
Eppendorf Thermomixer comfort	Eppendorf AG, Hamburg, Germany	Heater and shaker for 1.5 mL and 2 mL reaction tubes
Finnpipette 0.5 - 10 µL	Thermo Fisher Scientific, Waltham, MA, USA	Adjustable volume pipette
Heidolph Relax Vortex	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany	Vortexer
MR Hei-Standard	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany	Magnetic stirrer
peqpette 2 - 20 µL, 10 - 100 µL, 20 - 200 µL, 100 - 1000 µL, 1 - 5 mL	Peqlab Biotechnologie GmbH, Erlangen, Germany	Adjustable volume pipettes
Varioklav Dampfsterilisator	HP Medizintechnik GmbH, Oberschleißheim, Germany	Sterilizer

2.1 Cultivation and expression

Chemicals, materials and devices used for cultivation and expression are stated in Table 2.2. The vector pET16b-*Bf*FKP was provided by the Comstock Group from Boston, more details on the construct can be found in: Human symbionts use a host-like pathway for surface fucosylation; Coyne et al. (2005).

Table 2.2: Chemicals, materials and devices used for expression of *Bf*FKP from pET16b in *E. coli* BL21 (DE3).

Chemicals	Company	Comments
Agar Agar	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Ammonium chloride (NH_4Cl)	Merck KGaA, Darmstadt, Germany	
Ampicillin sodium salt	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at 4°C
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck KGaA, Darmstadt, Germany	
Phosphoric acid (H_3PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Polypropylenglycol (PPG)	Sigma Aldrich, St. Louis, MO, USA	
Potassium hydroxide (KOH)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Trace elements	-	Composition from the institute
Tryptone (Pepton aus Casein)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Yeast extract	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
α-D-glucose monohydrate	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Materials	**Company**	**Comments**
Centrifuge beaker: 500 mL	-	
Measuring cylinder	VITLAB GmbH, Grossostheim, Germany	
Petri dish, 92x16 mm	Sarstedt AG & Co, Nümbrecht, Germany	
Shake flasks: 250 mL, 1 L	SCHOTT Austria GmbH, Vienna, Austria	
Sterile filter, CA 0.20	Sarstedt AG & Co, Nümbrecht, Germany	
Devices	**Company**	**Comments**
Biochrom WPA CO8000	Biochrom Ltd., Cambridge, UK	Cell Density Meter

Infors HT RC-406	Infors AG, Bottmingen Switzerland	Shaker 30°C room
Pilot shaker	Adolf Kühner AG, Basel, Switzerland	Shaker 37°C room
Sorvall Evolution RC Centrifuge with SLC 3000 rotor	Thermo Fisher Scientific, Waltham, MA, USA	Centrifuge for 500 mL beaker

2.1.1 Cultivation and expression in shake flasks

E. coli BL21 (DE3) carrying pET16b-*Bf*FKP was streaked onto a LB-Amp agar plate (recipe as stated below plus 15 g/L agar) and was incubated overnight at 37°C.
LB medium was prepared according to the following recipe: 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. The components were mixed together, filled up to the appropriate amount with deionized water and the pH was adjusted to 7. The medium was filled up in shake flasks of size 250 mL and 1 L. The flasks were autoclaved at 121°C for 20 minutes.

The ONC was prepared in a shake flask with LB-medium and ampicillin and was inoculated with one grown colony of the desired strain. Incubation took place overnight at 37°C and 100 rpm. The optical density was measured at a wavelength of 600 nm (OD_{600}) and was added to the main culture to reach a final concentration of OD 0.05. The growth of the cells at 37°C and 100 rpm was monitored until a value of 0.6 to 0.8 optical density was reached. After a cool down of the broth to about 4°C the expression was induced via an inducer. In case of the T7 system the reagent IPTG (Isopropyl β-D-1-thiogalactopyranoside, 0.2 mM) was used. The time of expression lasted about 15 hours or more. The cells were harvested by centrifugation for about 30 minutes at 4000 rpm (Thermo Sorvall Evolution RC Centrifuge with SLC 3000 rotor for volumes up to 300 mL). The supernatant was decanted and the pellets were resuspended in buffer (buffer for His-tag purification, see Table 2.7) and merged. The suspension was aliquoted in desired amounts, centrifuged again and after removing of the supernatant the weight of the pellets was annotated. The pellets were stored at -20°C.

2.1.2 Cultivation and expression in a 2 L fermenter

The general mechanism of cultivation and expression was the same as in the shake flasks. One big difference was the composition of the media, for the ONCs it is stated in Table 2.3. The three media were mixed and autoclaved separately. Additionally a measuring cylinder, deionized water, PPG, 2 M KOH, 1 M H_3PO_4 and pipette tips were autoclaved.

Table 2.3: Composition of components for 2 ONCs (300 mL each) for expression of pET16b-*Bf*FKP in *E. coli* BL21 (DE3). The cultivation device was a 2 L fermenter.

Media	Components	Final concentration	Amount for 600 mL	Volume [mL]
A	Glucose [1]	5.5 g/L	3.3 g	120
B	Tryptone [1]	10 g/L	6 g	300
	Yeast extract [1]	5 g/L	3 g	
	NaCl [1]	5 g/L	3 g	
	NH_4Cl [1]	1 g/L	0.6 g	
	$MgSO_4.7H_2O$ [1]	0.25 g/L	0.15 g	
	Trace elements [1]	1 mL/L	0.6 mL	
C	K_2HPO_4 [1]	3 g/L	1.8 g	180
	KH_2PO_4 [1]	6 g/L	3.6 g	

[1] Chemicals are stated in Table 2.2. The components were dissolved in deionized water.

The media were combined under sterile conditions in shake flasks so that there were two ONCs with 300 mL each. One grown single colony was used for inoculation of the two overnight cultures (streaked out onto a LB-Amp plate the day before). Ampicillin was added to a final concentration of 100 µg/mL and incubation took place at 30°C and 100 rpm overnight.

Preparation of the media for the main culture can be seen in Table 2.4. The media were mixed and autoclaved separately. The 2 L tank was assembled and media B was filled in for autoclaving. The pH electrode was calibrated before autoclaving.

Table 2.4: Composition of components for the main culture for expression of pET16b-*Bf*FKP in E coli BL21 (DE3). The cultivation device was a 2 L fermenter.

Media	Components	Final concentration	Amount for 2 L	Volume [mL]
A	Glucose [1]	22 g/L	44 g	300
B	Tryptone [1]	10 g/L	20 g	1000
	Yeast extract [1]	5 g/L	10 g	
	NaCl [1]	5 g/L	10 g	
	NH_4Cl [1]	1 g/L	2 g	
	$MgSO_4.7H_2O$ [1]	0.25 g/L	0.5 g	
	Trace elements [1]	1 mL/L	2 mL	
C	K_2HPO_4 [1]	3 g/L	6 g	300
	KH_2PO_4 [1]	6 g/L	12 g	

[1] Chemicals are stated in Table 2.2.

After autoclaving the fermenter tank was installed and connected to the control unit. The oxygen electrode was polarized overnight at 10°C nitrogen flushing. After bringing the system at 37°C the tank was flooded with oxygen to accomplish the calibration, preliminaries were continued.

The cell density of the ONCs was measured and the needed amount for the final concentration of OD_{600} of 0.1 was calculated.

Media A and C, ampicillin, PPG and deionized water (the volume of the later added ONC was left blank) were added in a sterile way with an inoculation flask. The pH control was started and set to a value of 7. The stirrer and the oxygen supply were geared to each other.
As the oxygen signal and the pH were stable the ONC was added with the inoculation flask. Immediately before the addition a sample was withdrawn and was used as blank and for dilution. Right after the inoculation another sample was withdrawn and set as time point zero. Every 30 minutes a sample was taken and the cell density was determined. At an OD_{600} of 1.8 - 2 the broth was cooled down to induction temperature (25°C). The expression was induced with 0.2 mM IPTG. The induction took place overnight for at least 15 hours.
For the cell harvest the same procedure as with the shake flasks described under section 2.1.1 was used.

2.2 Cell lysis

Two different mechanical methods were used for cell lysis. For breaking up the cells with the French Press (French Pressure Cell Press, American Chemical Society, Washington D.C., USA) a pellet was thawed on ice and mixed one on one (or in a ratio of 1:2) with binding buffer for His-tag purification. The pellet was re-suspended completely and the cells were passed through the press at about 1500 psi (~10.5 MPa). This procedure was repeated at least three times with cooling steps in between. The suspension was centrifuged in 1.5 mL reaction tubes to pellet cell debris (13200 g, 40 minutes, 4°C). The supernatant, namely cell free extract (CFE), was pooled and used for further experiments. To examine the fraction of insoluble protein one pellet was further analyzed. The removed supernatant was replaced by 6 M Urea (re-suspension of pellet fraction).
The second method was the sonication (Sonic Dismembrator, Ultrasonic processor; Fisher Scientific (Austria) GmbH, Vienna, Austria). The pellet was thawed and re-suspended in maximal 30 mL binding buffer for His-tag purification. The cells were transferred in a small glass or plastic beaker and installed at the sonication station. It was important to cool the cells during the sonication, as heat was released during the process. The conditions for lyses were 2 seconds pulse and 4 seconds pause at amplitude of 60% with the big tip (10 mm diameter). The time for pulse was set to 3 to 6 minutes, depending on the volume. The suspension was divided onto 1.5 mL reaction tubes as it was the same procedure from here on as already described above with the French Press.

2.3 Evaluation of protein content

For determination of crude lysate and purified protein in a solution the method of Bradford was used (Bradford, 1976). Therefore a standard calibration curve with BSA (Albumin Fraktion V, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and the consequent regression equation were needed. The following concentrations of standards (BSA diluted with ultrapure

water) were prepared in duplicates: 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL 0.5 mg/mL, 0.6 mg/mL and 0.8 mg/mL. For the reaction 20 µL of each standard was mixed with 1 mL of the reaction solution (Roti-Quant, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in a semi-micro cuvette (Sarstedt AG & Co, Nümbrecht, Germany) and incubated for about 10 minutes at room temperature. The absorbance at a wavelength of 595 nm was measured at the Spectrophotometer DU 800 (Beckman Coulter, Brea, CA, USA).
The workflow for measuring the protein content was the same as with the standards described above. 20 µL of the protein containing solution (most of the time diluted, as the range of absorption of the spectrophotometer was between 0.1 and 0.8) was mixed with 1 mL of the reaction solution, incubated for 10 minutes at room temperature and was measured on the spectrophotometer (595 nm).

2.4 SDS-PAGE analysis of *Bf*FKP

To separate proteins according to their molecular mass a SDS-PAGE analysis was performed. Sample preparation for NuPAGE® Bis-Tris Mini Gels (Thermo Fischer Scientific, Waltham, MA, USA) is stated in Table 2.5.

Table 2.5: Sample preparation for SDS-PAGE analysis. Components were mixed and incubated at 70°C for 10 minutes. The gel was loaded with 10 µL of the reaction solution.

Components	Volume [µL]
Sample	10 (diluted to protein concentration of 1 mg/mL)
NuPAGE® LDS sample buffer, 4-fold [1]	10
50 mM Dithiothreitol (DTT) [2]	4
ultrapure water	16
Total	40

[1] Sample buffer from Thermo Fischer Scientific, Waltham, MA, USA; [2] DTT: 1,4-Dithiothreitol from Carl Roth GmbH + Co. KG, Karlsruhe, Germany

The cell free extract and the pellet fraction (protein content measured as described in section 2.3), were diluted to a concentration of 1 mg/mL. With this initial concentration, the bands could be seen with sufficient intensity on the gel. A prestained and an unstained PageRuler™ protein ladder were used as standards (Thermo Fischer Scientific, Waltham, MA, USA) and were loaded with 5 µL on the gel. The gel electrophoresis was run at 150 V for about an hour. The run was finished as the colored frontline reached the bottom of the gel. The gel was stained with a staining solution (IET Gel Staining Solution; Bio-Rad Laboratories, Inc.; Hercules, CA; USA) for about 30 minutes. The destaining was mostly done overnight or as long as the color needed to be washed out. Therefore a few changes of destaining solution were necessary.

2.5 Purification of the enzyme

The purification of *Bf*FKP was done via the N-terminal His10-tag (plasmid map can be seen in the appendix). The method of the purification was an immobilized metal ion affinity chromatography (IMAC) performed at the ÄKTAprime plus (liquid chromatography system from GE Healthcare, Chicago, IL, USA). All chemicals, materials and devices used for the purification are stated in Table 2.6.

Table 2.6: Chemicals, materials and devices for the purification of *Bf*FKP via the N-terminal His10-tag.

Chemicals	Company	Comments
Disodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Ethanol, 96%	Lactan Ges.m.b.H & CO.KG, Graz, Austria	Flammable
Ethylendiamintetraacetat (EDTA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Imidazole	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Corrosive
Nickel(II)sulphate ($NiSO_4$)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Cancerogen! Stored at 4°C
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Materials	**Company**	**Comments**
Cellulose acetate filter, pore size of 0.45 µm	Sartorius Stedim Biotech, Göttingen, Germany	
HiTrap HP column, 5 mL	GE Healthcare, Chicago, IL, USA	
Vivaspin Turbo 15, 10 kDa cut of, 15 mL	Sartorius, Göttingen, Germany	
Devices	**Company**	**Comments**
ÄKTAprime plus	GE Healthcare, Chicago, IL, USA	

The 5 mL HisTrap HP column was stripped and reloaded with a special order of deionized water, binding buffer and nickel(II)sulphate (0.1 M) like stated in the instruction of GE healthcare (used buffers are stated in Table 2.7).

The sample was loaded on the column and the His10-tagged protein built a complex with Ni^{2+}. All other proteins which were also expressed in *E. coli* were washed out.

Table 2.7: Buffer preparation for His-tag purification.
The buffers were prepared as recommended in the instruction of GE healthcare. All buffers were filtered and degassed.

	Binding buffer pH 7.4	Elution buffer pH 7.4	Stripping buffer pH 7.4
Components	Concentration [mM]		
Sodium phosphate [1,2]	20	20	20
NaCl [2]	500	500	500
Imidazole [2]	20	500	-
EDTA [2]	-	-	50

[1] The pH of sodium phosphate was adjusted by mixing the acidic (Na_2HPO_4) and the basic (NaH_2PO_4) components together. [2] Chemicals are stated in Table 2.6.

The elution was done with a gradient of elution buffer (Table 2.7) from 0% to 50% in 20 mL at a flow rate of 1 mL/min. The fractions where the signal of the eluted protein was depicted were pooled (signal detection via UV-Vis detector at 280 nm). To get rid of the high amount of imidazole a buffer exchange was done via ultracentrifugation (3 washing steps with storage buffer in a Vivaspin Turbo 15 ultrafiltration unit). The storage buffer was TRIS/HCl, 50 mM, pH 7.6. The protein was aliquoted in 100 µL and stored at -20°C. The result was analyzed by SDS-PAGE and the protein content was determined.

2.6 Synthesis of GDP-β-L-fucose

The reaction scheme of the conversion from L-fucose to GDP-β-L-fucose is shown in section 1. The reaction mixture was adapted from Wang et al. (2009) and Engels & Elling (2014). All used chemicals for the synthesis are represented in Table 2.8.

Table 2.8: Chemicals and devices used for the synthesis of GDP-β-L-fucose.

Chemicals	Company	Comments
Acetonitrile (ACN)	Sigma Aldrich, St. Louis, MO, USA	
ATP (Adenosine 5'-triphosphate di-sodiumsalt)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
GTP (Guanosine 5'-triphosphate di-sodiumsalt)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Hydrochloride (HCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
L-fucose	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Manganese chloride dihydrate ($MnCl_2.2H_2O$)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
PPase	from Institute	Inorganic pyrophosphatase
TRIS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Devices	**Company**	**Comments**
Agilent 1200 series	Agilent Technologies, Inc.; Santa Clara, CA, USA	HPLC with UV detector
Shimadzu 20A series	Shimadzu Handelsgesellschaft m.b.H., Korneuburg, Austria	HPLC with UV detector

Preliminary studies were done by another student from the institute who started to work with the enzyme *Bf*FKP (Details in: Production and purification of GDP-fucose by using the bifunctional enzyme Fkp, November and December 2015, Gerald Rinner).

The first setup for the conversion reaction of this thesis is stated in Table 2.9. All components except the enzyme and the PPase were mixed together and the solution was adjusted to pH 7.6. The enzyme and the PPase were added afterwards to start the conversion reaction which lasted for 24 hours. The thermo mixer was set to 37°C and 350 rpm. Different time points were chosen for sampling. Therefore 40 µL of the sample was stopped with 40 µL ice cold ACN (mixed with ultrapure water in a ratio of 1:1) and measured on the HPLC. The whole reaction was stopped by heat.

Table 2.9: Composition of the batch for synthesis of GDP-β-L-fucose.
For the preparation of the stocks the chemicals were dissolved in TRIS/HCl, 50 mM, pH 7.6. The conversion reaction took place at 37°C and 350 rpm over 24 hours with sampling at different time points in between.

Components	Stock	Final concentration	Volume [µL]
L-fucose [1]	100 mM	5 mM	25.0
GTP [1]	100 mM	10 mM	50.0
ATP [1]	100 mM	5 mM	25.0
$MgCl_2$ [1]	100 mM	5 mM	25.0
PPase [1]	6 U/µL	3 U	0.5
*Bf*FKP [1]	0.6 mg/mL	0.12 mg/mL [2]	100.0
TRIS/HCl, pH 7.6 [1]	50 mM	-	274.5
Total	-	-	500.0

[1] Chemicals are stated in Table 2.8. [2] corresponds to 30 mU/mL

The GTP and ATP stocks were additionally measured via the spectrophotometer and the HPLC to determine the concentration more precise. The used wavelengths therefore were 256 nm for GTP and 259 nm for ATP at the spectrophotometer and 262 nm at the HPLC.

To further optimize and proof this combination of chemicals and their concentration different conversion settings were chosen. The different variations were:

- Change of reaction temperature to 30°C
- Varying of enzyme concentration: 0.12 mg/mL, 0.22 mg/mL and 0.45 mg/mL
- Addition of $MnCl_2$
- Variation of L-fucose concentration: equimolar or in excess
- Alteration of ATP concentration: equimolar or in excess
- Alteration of GTP: equimolar or in excess
- Change of the molarity of the buffer: 50 mM or 200 mM
- Use of L-fucose, ATP and GTP at equimolar conditions
- Usage of enzyme in crude extract and crude lysate

The optimal reaction mixture was used for up scaling experiments. The volume was increased whilst the molar concentration stayed the same. The raise was done over 1 mL and 2 mL (performed in a 2 mL reaction tube on the thermo mixer) to 10 mL and 15 mL (in 15 mL reaction tubes either on a shake wheel or on the bigger shaker). 15 mL and 20 mL were also tried in a small beaker with a stirrer bar on a magnetic stirrer.

The molar concentration was increased 5 mM to 10 mM of L-fucose as shown in Table 2.10.

Table 2.10: Composition of the optimized and upscaled batch for synthesis of GDP-β-L-fucose.
For the preparation of the stocks the chemicals were dissolved in TRIS/HCl, 50 mM, pH 7.6. The conversion reaction took place at 37°C and at low activity of magnetic stirrer bar over 24 hours with sampling at different time points in between.

Components	Stock	Final concentration	Volume [mL]
L-fucose [1]	100 mM	10 mM	2.0
GTP [1]	100 mM	15 mM	3.0
ATP [1]	100 mM	10 mM	2.0
$MgCl_2$ [1]	100 mM	10 mM	2.0
$MnCl_2$ [1]	100 mM	10 mM	2.0
PPase [1]	2.8 U/µL	56 U	0.02
*Bf*FKP	-	0.22 mg/mL [2]	-
TRIS/HCl, pH 7.6 [1]	50 mM	-	up to 20
Total	-	-	20

[1] Chemicals are stated in Table 2.8. [2] corresponds to 55 mU/mL

2.7 Measure of activity of *Bf*FKP

To determine the activity of the enzyme a reaction mixture similar to that for the synthesis was used. The difference to the standard reaction mixture (see Table 2.9) was the concentration of the enzyme, which was set to 0.11 mg/mL to gain a long linear increase of GDP-β-L-fucose concentration. The time points for sampling were: 0, 5, 10, 15, 30, 45 and 60 minutes. 40 µL of the sample was stopped with 40 µL ice cold ACN (premixed with ultrapure water in a ratio of 1:1) and measured on the HPLC. The determined concentration of GDP-β-L-fucose was plotted against time and out of the slope of regression line the enzymatic activity was calculated.

2.8 Analysis on HPLC

The quantitative and qualitative analysis of the nucleotide sugar GDP-β-L-fucose was done by HPLC. A kinetex reversed phase C-18 column was used with TBAB buffer as mobile phase (40 mM TBAB, 20 mM sodium phosphate, pH 5.9). The components were separated at a flow of 2 mL/min with 6.5% ACN and detected with a UV detector at a wavelength of 262 nm. The method lasted for 10-12 minutes and the oven temperature was set to 40°C.

Chemicals, materials and devices used for HPLC analysis are written down in Table 2.11.

Table 2.11: Chemicals, materials and devices used for analysis of GDP-β-L-fucose.

Chemicals	Company	Comments
Acetonitrile (ACN)	Sigma Aldrich, St. Louis, MO, USA	Irritant, flammable
ADP (Adenosine 5′-diphosphate)	Sigma Aldrich, St. Louis, MO, USA	Stored at -20°C
AMP (Adenosine 5′-monophosphate)	Sigma Aldrich, St. Louis, MO, USA	Stored at -20°C
ATP (Adenosine 5'-triphosphate di-sodiumsalt)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
Dipotassium hydrogen phosphate (K_2HPO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
GDP (Guanosine 5'-diphosphate)	Sigma Aldrich, St. Louis, MO, USA	Stored at -20°C
GDP-β-L-fucose	IsoSep, Tullinge, Sweden	Stored at -20°C
GMP (Guanosine 5'-monophosphate)	Sigma Aldrich, St. Louis, MO, USA	Stored at -20°C
GTP (Guanosine 5'-triphosphate di-sodiumsalt)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Tetra-n-butylammonium bromide (TBAB)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Harmful
Materials	**Company**	**Comments**
Crimp caps, 11 mm	Markus Bruckner Analyse Technik, Linz, Austria	Lid for HPLC vials
Crimp Neck Vial, 1.5 mL, clear glass	Markus Bruckner Analyse Technik, Linz, Austria	Vials for HPLC
Kinetex C-18 column	Phenomenex Ltd. Deutschland, Aschaffenburg, Germany	Reversed phase column
Micro inserts, 0.1 mL, clear glass, 31x6 mm	Markus Bruckner Analyse Technik, Linz, Austria	Inserts for micro volume for HPLC vials
Devices	**Company**	**Comments**
Agilent 1200 series	Agilent Technologies, Santa Clara, CA, USA	HPLC with UV detector
Shimadzu 20 series	Shimadzu Handelsgesellschaft m.b.H., Korneuburg, Austria	HPLC with UV detector

For the qualitative and quantitative analysis on the HPLC some standards were needed. GMP, GDP-β-L-fucose, GDP and GTP were mixed together in one sample for qualification; each compound had a final concentration of 1 mM. AMP, ADP and ATP were mixed together in one sample for qualification; each compound had a final concentration of 1 mM. The GDP-β-L-fucose standard was needed for quantification. The following concentrations were prepared and a standard calibration curve was created: 0.5 mM, 1 mM, 2 mM and 4 mM.

2.9 Purification of GDP-β-L-fucose

All chemicals, materials and devices used for the purification of GDP-β-L-fucose are stated in Table 2.12.

Table 2.12: Chemicals, materials and devices used for the purification of GDP-β-L-fucose.

Chemicals	Company	Comments
Acetic acid, 99-100%,	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Flammable, corrosive
Acetonitrile (ACN)	Sigma Aldrich, St. Louis, MO, USA	Irritant, flammable
Alkaline phosphatase, calf intestinal (CIP)	New England Biolabs, Ipswich, MA, USA	Stored at -20°C
Ethanol, 96%	Lactan Ges.m.b.H & CO.KG, Graz, Austria	
Liquid nitrogen	AIR LIQUIDE AUSTRIA GmbH, Schwechat, Austria	
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	In form of platelets
Toyopearl Super Q-650M	Tosoh Bioscience, Griesheim, Germany	
Materials	**Company**	**Comments**
AEC column	-	Stationary phase: Toyopearl Super Q-650M
Cellulose acetate filter, pore size of 0.45 µm	Sartorius Stedim Biotech, Göttingen, Germany	
Loop, 5 mL	-	
Needle, long, 100 Sterican, 0,80 x 120mm, 21G x 4 ¾”	B.Braun Melsungen AG, Melsungen, Germany	
Needle, short, 100 Sterican, 0,90 x 40mm, 20G x 1 ½”	B.Braun Melsungen AG, Melsungen, Germany	
Round-bottom flask	Schott Austria GmbH, Vienna, Austria	
SEC column	GE Healthcare (Little Chalfont, UK)	Stationary phase: Sephadex G-10
Syringe: á 2 mL, á 10 mL	B.Braun Melsungen AG, Melsungen, Germany	
Vivaspin® Turbo 15, 15 mL, cut-off: 10 kDa	Sartorius AG, Göttingen, Germany	Rinse twice with deionized water prior the first use, store on buffer
Devices	**Company**	**Comments**
Agilent 1200 series	Agilent Technologies, Inc.; Santa Clara, CA, USA	HPLC with UV detector
ÄKTA FPLC	GE Healthcare (Little Chalfont, UK)	Software: Unicorn, by Amersham Biosciences,
ÄKTAprime plus (FPLC)	GE Healthcare (Little Chalfont, UK)	Software: PrimeView

Lyophilizer	B. Braun Biotech International GmbH, Melsungen, Germany	
Metrohm 691	Metrohm, Herisau, Switzerland	pH meter
Motor unit (CVC 2000)	vacuubrand GmbH + Co KG, Wertheim, Germany	Power supply for rotary evaporator
Rotary evaporator (Laborota 4000)	Heidolph Instruments, Schwabach, Germany;	
Shimadzu 20A series	Shimadzu Handelsgesellschaft m.b.H., Korneuburg, Austria	HPLC with UV detector
Vacuum pump, MZ2CNT	vacuubrand GmbH + Co KG, Wertheim, Germany	

The downstream processing of the synthesized nucleotide sugar was done in 4 main steps:

(I) CIP digest – digestion of nucleotide tri~, di~ and monophosphates

The solution was adjusted to pH 7 and the alkaline phosphatase (2 – 10 U/mL) was added. The minimal incubation time was 8 hours and the maximal time was 16 hours overnight at 30°C.

(II) Anion exchange (AEC) – separation of GDP-β-L-fucose from other phosphate groups

Two different columns with different binding capacities were used. The small 1 mL column bound about 30 mg of phosphate (all phosphate residues are binding, capacity determined by researchers from the institute) and the 20 mL column had a binding capacity of about 1 g. The mobile phases were a binding buffer (20 mM NaOAc, pH 4.3) and an elution buffer (1 M NaOAc, pH 4.3).

Packing of AEC column: The AEC columns were packed with Toyopearl Super Q-650M from Tosoh Bioscience.

Run: Conditions: flow rate: 1 mL/min, wavelength: 254 nm; Three different ways of the elution process to gain the GDP-β-L-fucose were tried: (I) a gradient was set from 0% to 70% buffer B in 40 mL, (II) the concentration of buffer B was set to 15% to elute the monophosphates and then the concentration was set to 50% to elute the GDP-β-L-fucose or (III) a gradient was set from 0% to 40% buffer B in 40 mL and at the raising of a peak the concentration was set on hold and the gradient was continued at the end of the peak. The different fractions of the occurring peaks were analyzed on the HPLC to determine the GDP-β-L-fucose containing fractions. These fractions were pooled into a round bottom flask and held on ice until the volume reduction at the rotary evaporator started. The volume was reduced to about 2 - 8 mL.

(III) Size exclusion (SEC) – separation of nucleotide sugar and salt

Conditions: mobile phase: deionized water, flow rate: 1.5 mL/min, wavelength: 254 nm; About 25 mg of nucleotide sugar can bind to the resin of the column (determined by researchers from the institute). The separation of sugar and salt was according to their size. The fractions with the nucleotide sugar were pooled and were held on ice. The volume of the collected fractions was reduced to about 10 mL via the rotary evaporator.

(IV) Lyophilization – freeze drying of the nucleotide sugar

The remaining 10 mL of GDP-β-L-fucose containing solution was shock frozen with liquid nitrogen. The round bottom flask with the thin frozen layer of GDP-β-L-fucose and water was stuck on the lyophilizer and the vacuum was applied. The process was finished as the resulting powder in the flask was on room temperature, this usually lasted overnight.

These steps of purification were mainly adapted from Lemmerer, Schmölzer, Gutmann, & Nidetzky (2016) and G. Zhao et al. (2010).

2.10 ^{1}H NMR

The gained GDP-β-L-fucose was analyzed by ^{1}H NMR. Therefore the nucleotide sugar was dissolved in D_2O. The spectrum was recorded on a Varian (Agilent) INOVA 500-MHz spectrometer (Agilent Technologies, Santa Clara, United States) using VNMRJ 2.2D software. ^{1}H NMR spectrum was measured at 499.98 MHz on a 5 mm indirect detection PFG-probe. The water signal was PFG-probe pre-saturated by a shaped pulse, using standard pre-saturation sequence with relaxation delay 2 s; 90° proton pulse; acquisition time 2.049 s; spectral width 8 kHz; number of points 32 k, temperature 30°C. The process of analysis was done with the help of Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Hansjörg Weber at the Institute of Organic Chemistry.

3 Results and Discussion

3.1 Cultivation and expression

Cultivation and expression of pET16b_*Bf*FKP in *E. coli* BL21 (DE3) was done in shake flasks (in volumes between 1.5 L and 3 L). It was done in LB medium with ampicillin and the cells were induced with IPTG (0.2 mM) at 25°C overnight.

To determine the protein content the following standard curve was created (Figure 3.1).

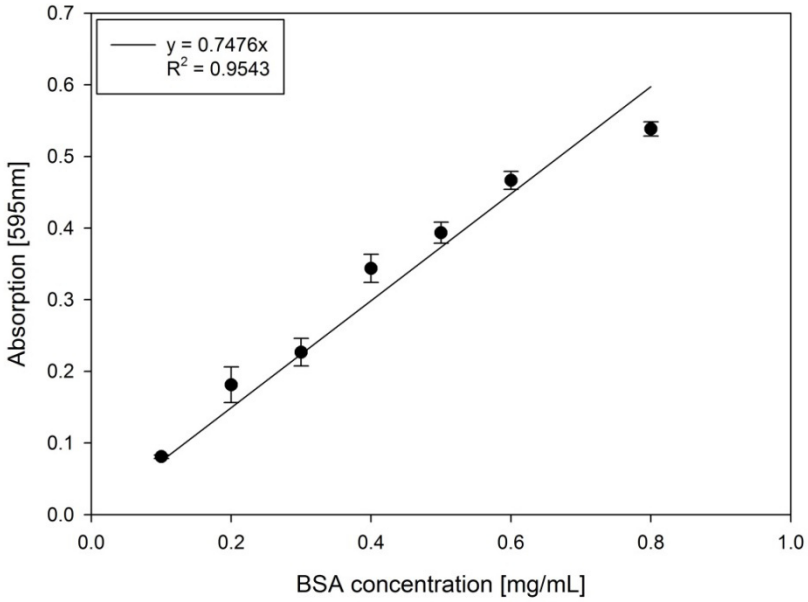

Figure 3.1: Standard curve with BSA.
This curve was used for the calculation of the protein content with the method of Bradford as described in section 2.3. Values were measured in quadruplets. Linear equation: y=0.7476x, determination coefficient: R^2=0.9543

With this data the total protein content and the concentration of the purified protein were calculated. The protein content was needed for SDS-PAGE analysis as the best result was reached by loading samples with an initial concentration of 1 mg/mL. In Figure 3.2 one can see the CFE and the pellet in different concentrations. The SDS-PAGE analysis confirmed a good over expression of *Bf*FKP with a size of 108 kDa.

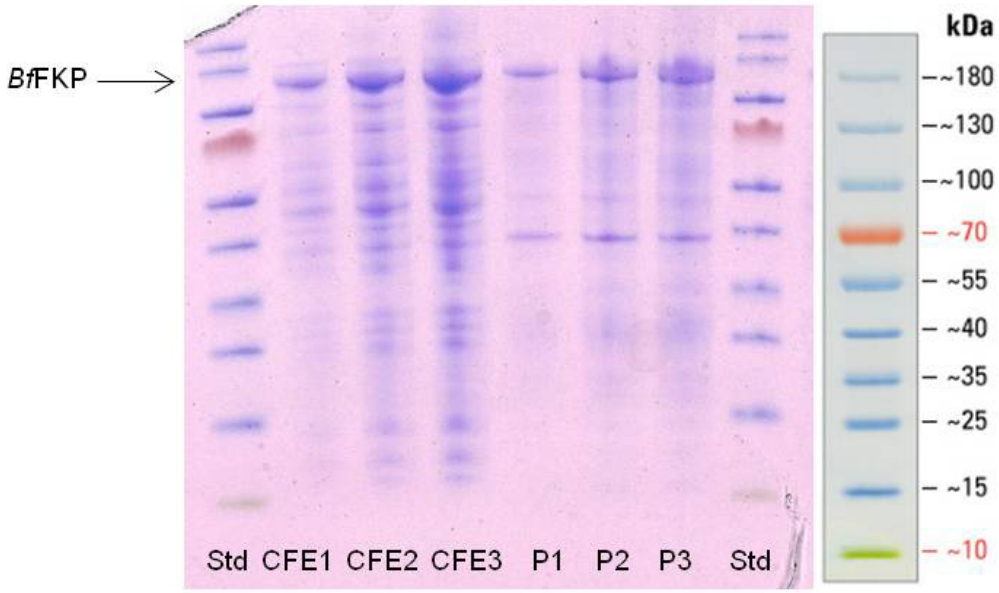

Figure 3.2: SDS-PAGE analysis of the CFE and the pellet fractions of *Bf*FKP expressed in *E. coli* BL21 (DE3).
Std: Prestained PageRuler™ protein ladder, 5 µL; CFE1: cell free extract with a concentration of about 0.02 mg/mL, CFE2: cell free extract with a concentration of about 0.04 mg/mL, CFE3: concentration of cell free extract with a concentration of about 0.1 mg/mL, P1 to P3: pellet fractions with different concentrations (adapted to the concentrations of CFE1 to CFE3).

The concentration of the band at 108 kDa was measured with ImageJ 1.50i (computer software, National Institutes of Health, Bethesda, MD, USA) and could be stated as about 15% of the total protein content.

In the following Table 3.1 all done cultivations are summarized regarding the overall protein yield, the produced biomass, the procedure of cell disruption and the cultivation device.

Table 3.1: Summary of all done cultivations of pET16b-*Bf*FKP in *E. coli* BL21 (DE3).
Overall yield of total protein, the total produced biomass (wet weight), the method of cell lysis and the cultivation device is stated.

	Protein [mg] / culture [L]	Biomass [g] / broth [L]	Cell lyses	Device
1	148.0	5.0	French Press	Shake flask
2	122.0	4.8	Sonication	Shake flask
3	368.2	4.8	Sonication	Shake flask
4	88.8	4.3	Sonication	Shake flask
5	570.0	29.2	French Press	Fermenter
6	204.5	5.9	French Press	Shake flask
7	86.7	3.6	Sonication	Shake flask
8	266.6	3.6	Sonication	Shake flask
9	555.3	5.0	Sonication	Shake flask

The cell lysis with the French Press showed a good result, as the most of the enzyme was in the cell free extract as seen in Figure 3.2. The enzyme which was left behind could either be insoluble enzyme, enzyme which was attached to the cell wall and was therefore also in the pellet fraction or come from still not lysed cells.

As already mentioned sonication was also used as method for cell lysis. The efficiency was not compared within one experiment, but it seemed that is had the same or sometimes the better efficiency. One mentionable advantage of the sonication is that the handling is easy with small volumes of about 10 mL (which is more difficult with the French Press). And it is also a time saving method.

The cultivation and expression in the 2 L fermenter was not successful as the afterwards performed synthesis experiments showed no activity of *Bf*FKP - no GDP-β-L-fucose was produced. The SDS-PAGE analysis showed a band at the right size of 108 kDa but it looked like that there was more expressed enzyme in the pellet fraction as usual. This would suggest a higher fraction of insoluble enzyme. One reason could be that there occurred a problem with folding. As in the paper of Cheng et al. (2014) stated (and already summarized in the introduction) *Bf*FKP is suggested to be a trimeric molecule and that two trimeric molecules are together a unity. So it could be, that for example the growth was too fast for the correct folding and led to inactivation (Burgess, 2009).
The conversion with the enzyme out of the 2 L fermenter was done two times to make sure that there was really no activity. Because the cultivation and expression was successful enough in the shake flasks, there were no further examinations concerning the problem with the cultivation in the fermenter conducted.

3.2 Purification of enzyme

For purification of *Bf*FKP via the N-terminal His10-tag the CFE was loaded on the HisTrap column. A chromatogram of the purification can be seen in Figure 3.3. The blue curve represents the absorption at a wavelength of 280 nm and the green curve shows the concentration of the elution buffer. The pink straight line depicts the start of injection, where the sample was loaded onto the column. Peak 1 is the flow through, where all components which were not binding to the column gave a signal. The peaks marked with 2 are air bubbles or signal disturbances. The peak of interest is number 3. This is the signal of the enzyme eluted at a concentration of about 150 mM imidazole.

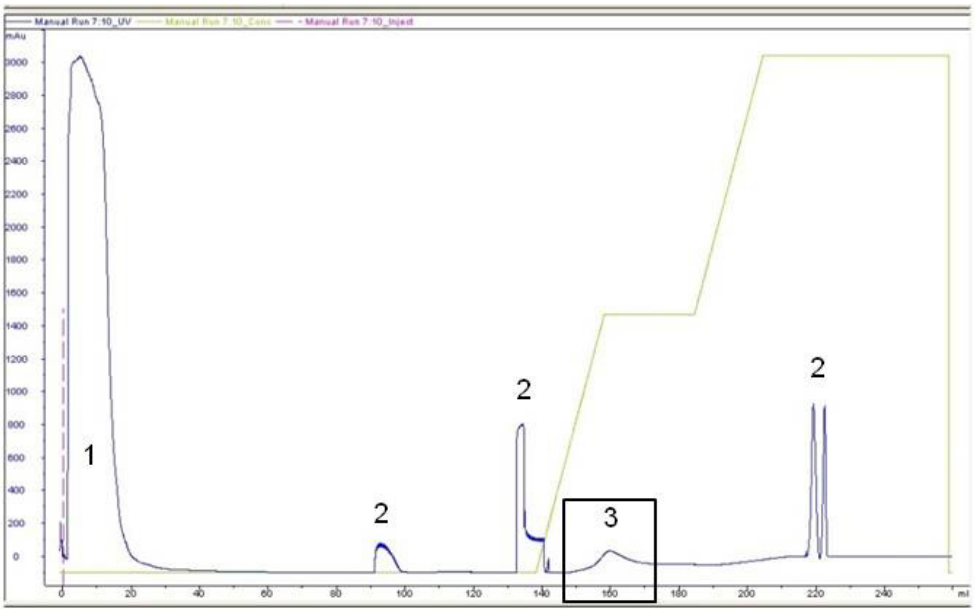

Figure 3.3: Chromatogram of time course of purification of *Bf*FKP via the His10-tag with IMAC.
The blue curve represents the absorption at 280 nm and the green curve shows the concentration of the elution buffer. The pink straight line depicts the start of injection, where the sample was loaded onto the column. Peak 1: flow through, peak 2: air bubbles or signal disturbances, peak 3 in the black box: signal of the enzyme eluted at an imidazole concentration of about 150 mM

The elution buffer was exchanged to TRIS/HCl, 50 mM, pH 7.6 and the resulting enzyme solution (about 2 - 3 mL) was portioned in 100 µL aliquots and stored at -20°C. The protein content was determined via Bradford.

In this purification example the whole protein content of the cultivation was 192.6 mg and the amount of the purified enzyme was 19.5 mg (shown in Figure 3.4). This is an actual yield of 10.1% (Table 3.2) in comparison to the theoretical estimated 15% with the computer software. This would imply a loss of enzyme of about 32.5%. This loss can be explained by the impreciseness of the densitometric protein determination, for example. Or some of the enzyme could also get lost during purification.

The reached isolated yield of 10% is near the average of all own performed cultivations. Where the lowest yield reached was 0.2% and the highest was 19.1%, as stated in Table 3.2.

Table 3.2: Summary of all purifications of *Bf*FKP.
The protein concentration in the present volume is stated, as well as the purified protein yield regarding to the volume of the fermentation broth and the overall isolated yield.

	Volume [mL]	Protein concentration [mg/mL]	Purified protein [mg] / broth [L]	Yield [%]
1	3	6.5	0.5	10.1
2	3	0.7	4.5	0.2
3	3	1.0	3.0	2.3
4	3	1.3	2.3	1.7
5	1	5.0	0.2	4.7
6	5	1.2	4.2	19.1
7	5	1.4	3.6	9.2

SDS-PAGE analysis was used to determine the purity of the enzyme containing solution. As seen in Figure 3.4 it seemed to be a very pure fraction of *Bf*FKP. Also the flow through was checked if there was any unbound His-tagged enzyme in it.

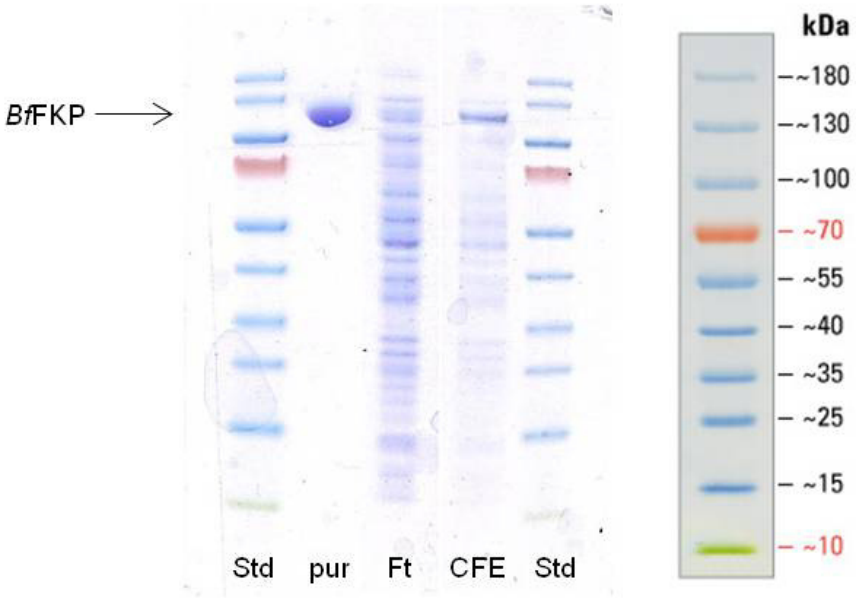

Figure 3.4: SDS-PAGE analysis of the purified enzyme (~108 kDa), the flow through and the CFE of *Bf*FKP expressed in *E. coli* BL21 (DE3). Std: Prestained PageRuler^{TM} protein ladder, pur: purified enzyme, Ft: flow through, CFE: cell free extract

3.3 Synthesis of GDP-β-L-fucose

In this section the results of (relevant) optimizations of the synthesis of GDP-β-L-fucose is presented. The starting point, which is the standard conversion before optimization, is stated in detail in Table 2.9, with an initial concentration of 5 mM L-fucose, 5 mM ATP, 10 mM GTP and 10 mM magnesium. All optimizations were based on this synthesis reaction, and only the compound changed is given in the following figures.

As shown in Figure 3.5 the reaction temperature of 37°C was more effective than 30°C. This result was considered in the next reactions (1.6-fold increase), which were done at 37°C.

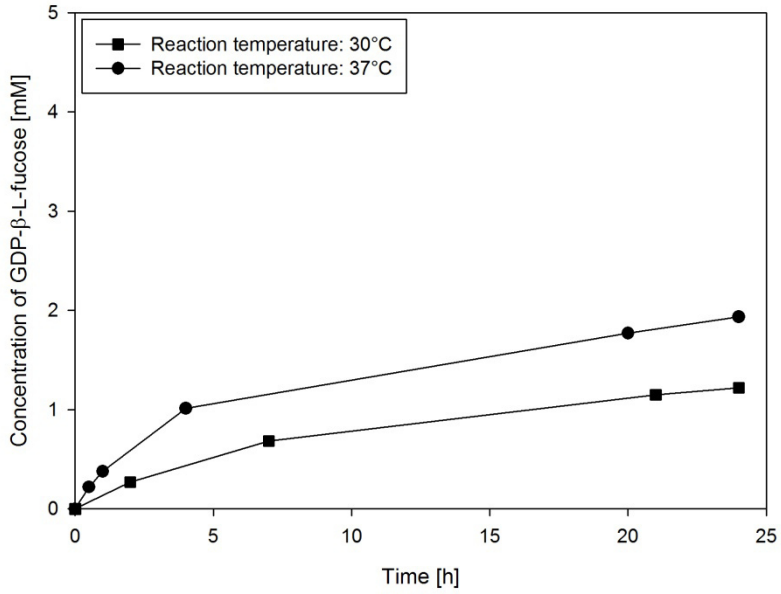

Figure 3.5: Synthesis of GDP-β-L-fucose with different temperatures. The reaction temperature of 37°C is more effective. Yield at 30°C reaction temperature: 1.2 mM; Yield at 37°C reaction temperature: 1.9 mM

As shown in Figure 3.6 an increase of the enzyme concentration from 0.12 mg/mL to 0.45 mg/mL yielded in a 1.7-fold higher product amount. But the increase of the enzyme concentration by more than 3 times was in no relation to the gained product yield, therefore the concentration of 0.12 mg/mL was maintained.

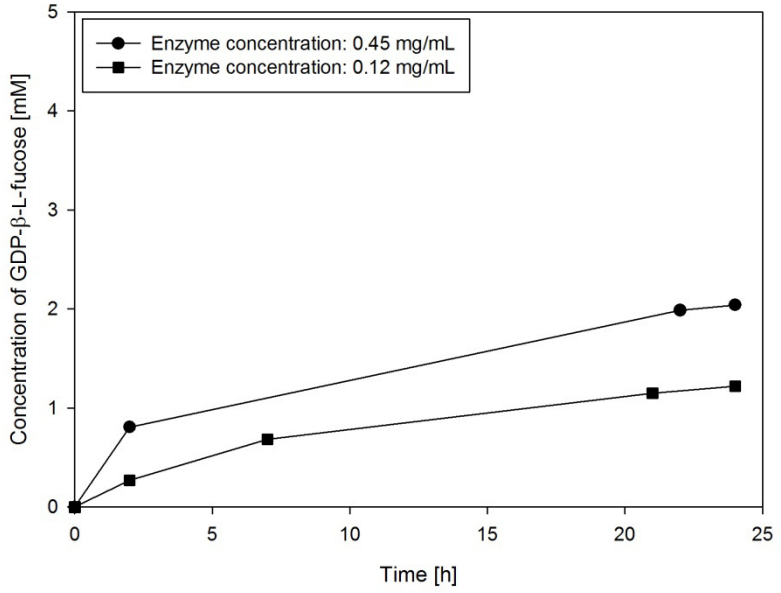

Figure 3.6: Synthesis of GDP-β-L-fucose with enzyme concentrations of 0.12 mg/mL (GDP-β-L-fucose yield: 1.1 mM) and 0.45 mg/mL (GDP-β-L-fucose yield: 1.9 mM).

As the addition of 10 mM manganese resulted in exactly the doubled product amount (1.2 mM in comparison to 2.4 mM), the addition of manganese was used in all next batches (Figure 3.7).

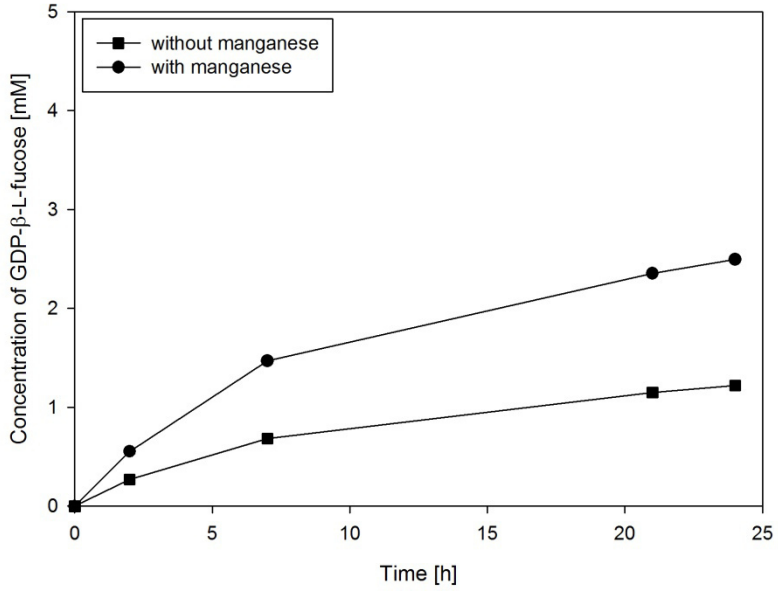

Figure 3.7: Synthesis of GDP-β-L-fucose with and without additional manganese as second divalent ion (1.2 mM GDP-β-L-fucose in comparison to 2.4 mM).

Another synthesis reaction with increased enzyme concentrations was done (Figure 3.8). And this time the doubled enzyme concentration gave about 1.7-fold increased product yield. Therefore the concentration of 0.24 mg/mL was used for all next batches.

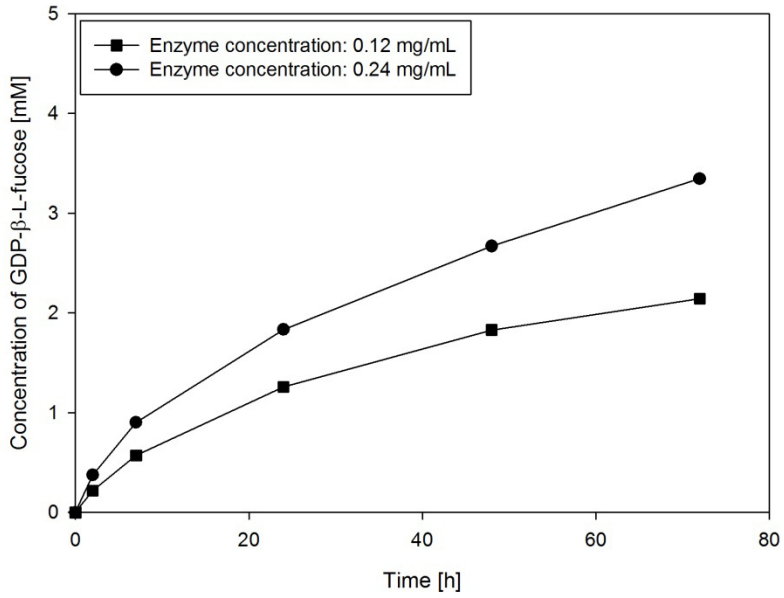

Figure 3.8: Synthesis of GDP-β-L-fucose with different enzyme concentrations. Yield with 0.12 mg/mL enzyme concentration: 2.0 mM GDP-β-L-fucose; yield with 0.24 mg/mL enzyme concentration: 3.2 mM GDP-β-L-fucose

The increased concentration of ATP from 5 mM to 10 mM speeded up the reaction, but ended in the same amount of GDP-β-L-fucose, as illustrated in Figure 3.9. The limiting substrate in both cases was fucose (5 mM).

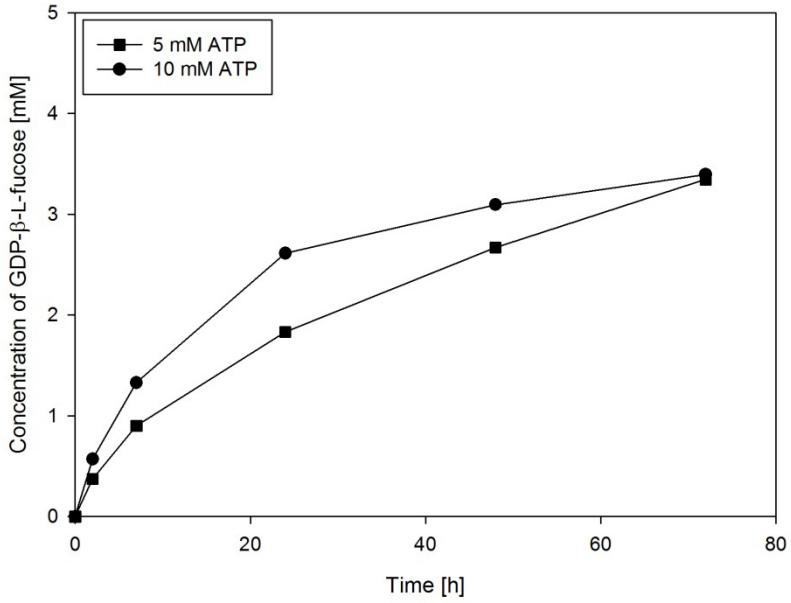

Figure 3.9: Synthesis of GDP-β-L-fucose with different concentrations of ATP but the same yield of GDP-β-L-fucose after 72 h.

Based on the published K_m values (stated in the introduction) the higher concentration of ATP (10 mM) is three to ten times higher as the K_m value. This would mean that it was closer to a saturated range than 5 mM and that would further explain the higher initial reaction velocity. As ATP as compound is rather expensive and time was not an important parameter (at least not at these short time periods) the lower concentration of ATP was used for the further optimization reactions.
The expanded reaction time to 72 hours was due to the fact, that the enzyme was already stored for longer time at -20°C and thereby showed a decreased specific activity.

As both reactions at 5 mM and 10 mM L-fucose (with 5 mM ATP and 10 mM GTP) gave almost identical results (Figure 3.10), it was confirmed that no excess of fucose was needed. So for further batches the L-fucose concentration of 5 mM was maintained.

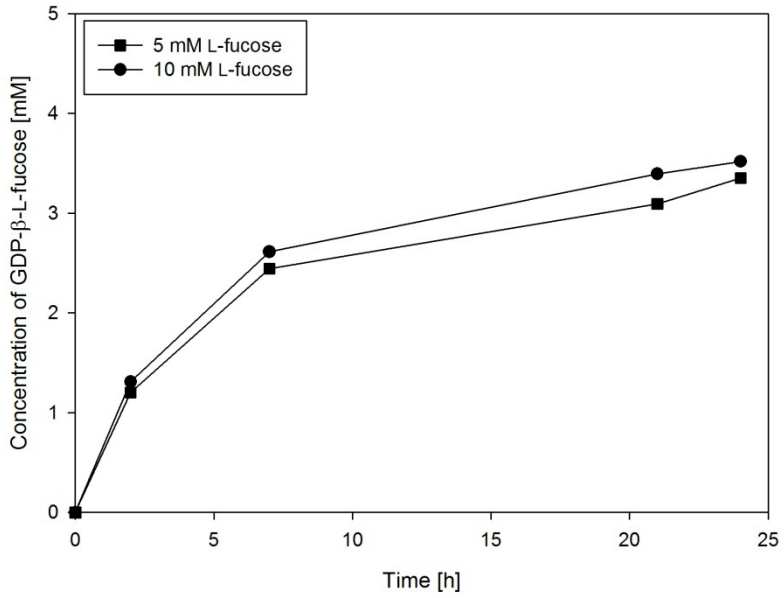

Figure 3.10: Synthesis of GDP-β-L-fucose with different concentrations of L-fucose. 5 mM L-fucose or 10 mM L-fucose with 5 mM ATP and 10 mM GTP

Because of the very different values published for the K_m values of ATP (1.080 mM and 3.330 mM) and the need for higher titers for product isolation an increase of the starting concentrations was tested (10 mM L-fucose, 10 mM ATP, 15 mM GTP, 10 mM $MgCl_2$ and 10 mM $MnCl_2$). The result can be seen in Figure 3.11. Against the expectations that for saturating conditions an ATP concentration of 10 mM would be needed, the batch with the 5 mM starting concentration of ATP (5 mM L-fucose, 10 mM GTP) had the same conversion within the first 2 hours as the batch starting with 10mM ATP (10 mM L-fucose, 15 mM GTP). This would mean that no higher concentrations of substrate and cofactors would be needed to reach saturating concentrations but rather that higher concentrations could already be inhibiting again. However, the experiment should be remeasured with more datapoints at the beginning of the conversion. In any case, for high product titers higher substrate amounts are necessary, which could alternatively be added stepwise.

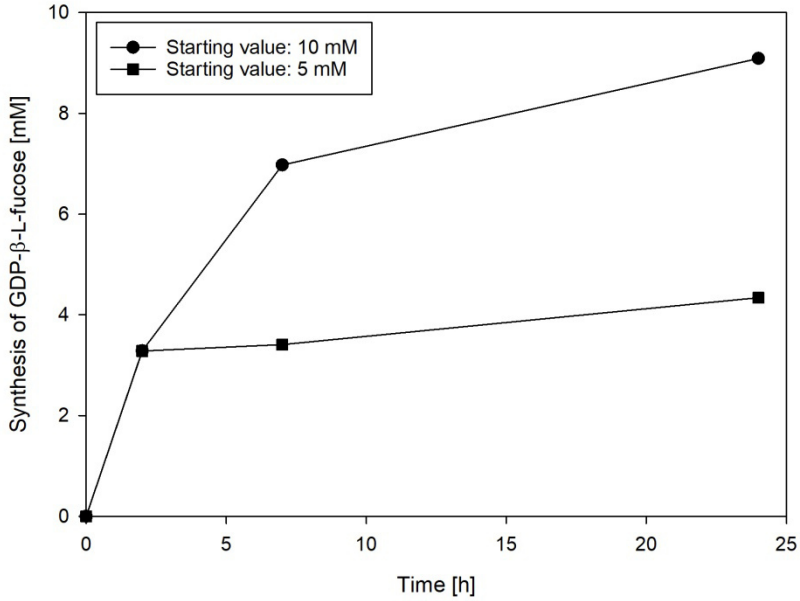

Figure 3.11: Synthesis of GDP-β-L-fucose with different starting values. First batch (sqares): 5 mM L-fucose, 5 mM ATP, 10 mM GTP; second batch (circles): 10 mM L-fucose, 10 mM ATP, 15 mM GTP

As GTP was the most expensive reagent in the reaction mixture, it was important to test again if the excess of GTP was really necessary. In Figure 3.12 it is shown, that the excess of GTP results in nearly 100% conversion of L-fucose to GDP-β-L-fucose in comparison to about 50% with equimolar conditions of L-fucose, ATP and GTP.

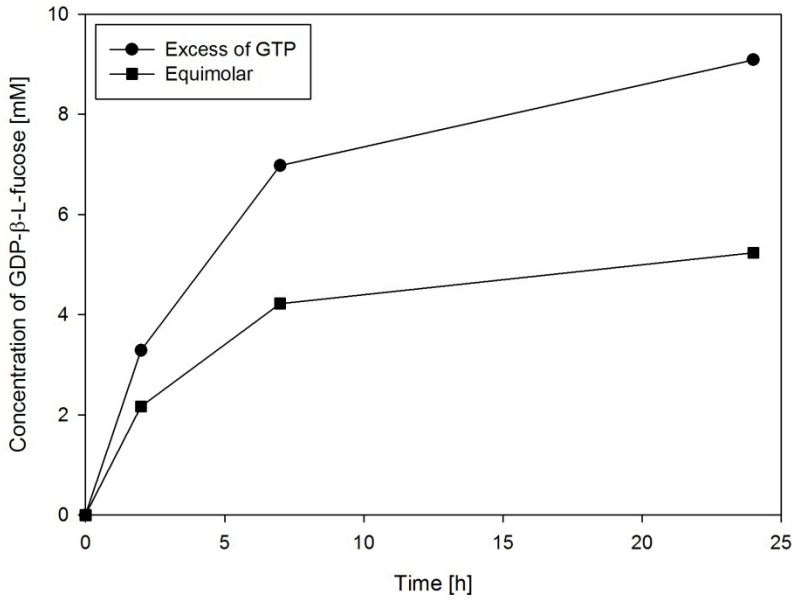

Figure 3.12: Synthesis of GDP-β-L-fucose with different concentrations of GTP. Equimolar conditions: 10 mM L-fucose, 10 mM ATP, 10 mM GTP: resulted in 5.1 mM GDP-β-L-fucose; excess of GTP: 10 mM L-fucose, 10 mM ATP, 15 mM GTP, resulted in 9.2 mM GDP-β-L-fucose

At the upscale of the concentration (from 5 mM L-fucose, 5 mM ATP and 10 mM GTP to 10 mM L-fucose, 10 mM ATP and 15 mM GTP) GTP was not doubled. In Figure 3.12 it was

shown that with the 1.5-fold concentration a yield of 91% GDP-β-L-fucose was reached (10 mM would be 100%).

At the volumetric scale up from 500 µL over steps of 1 mL, 2 mL, 5 mL, 10 mL up to 20 mL the question of the best reaction vessel arose. The reaction mixture tended to flocculate a little bit and got milky. The best reaction vessel tested was a small beaker with a lid and with a small magnetic stirrer which was moving very slowly. With this practical option the best conversion and the least milky solution were gained. Another method was a 15 mL (or 50 mL) reaction tube spinning slowly in a wheel. But for the further bigger batches the small beaker was more feasible. As shown in Figure 3.13 there was just a little difference in the yielded nucleotide sugar with the different vessels.

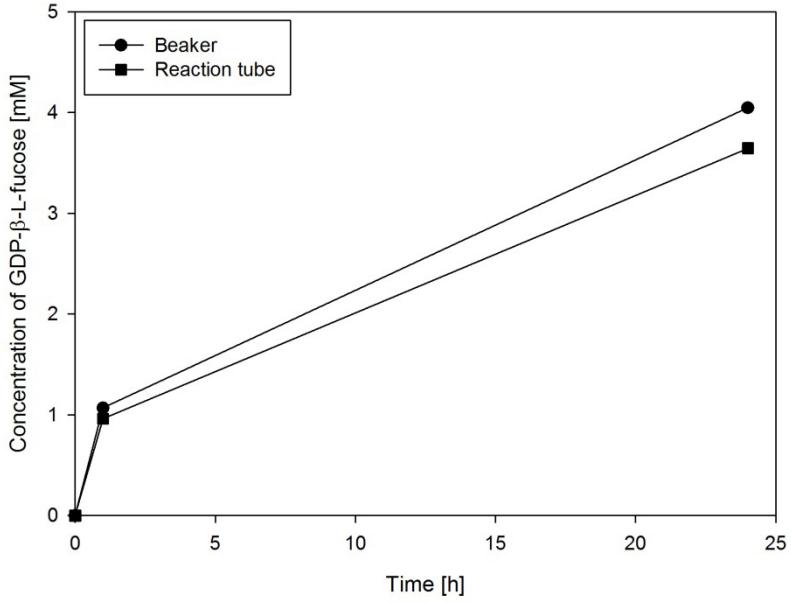

Figure 3.13: Synthesis of GDP-β-L-fucose at a volumetric scale up (20 mL) with different reaction vessels.

Moreover synthesis with CFE instead of the purified protein was tried. This was not successful. ATP and GTP were consumed, but no GDP-β-L-fucose was produced, or present anymore at the end of the reaction. So this time and material saving method was not further pursued.

In Figure 3.14 two conversion experiments with different starting concentrations are shown over 24 hours of reaction time. The consumption of ATP und GTP and the synthesis of GDP-β-L-fucose are given. In Figure 3.14 A the starting concentrations were 5 mM L-fucose, 5 mM ATP and 10 mM GTP. In Figure 3.14 B one can see the reaction course with higher starting concentrations: 10 mM L-fucose, 10 mM ATP and 15 mM GTP, which are the optimized conditions.

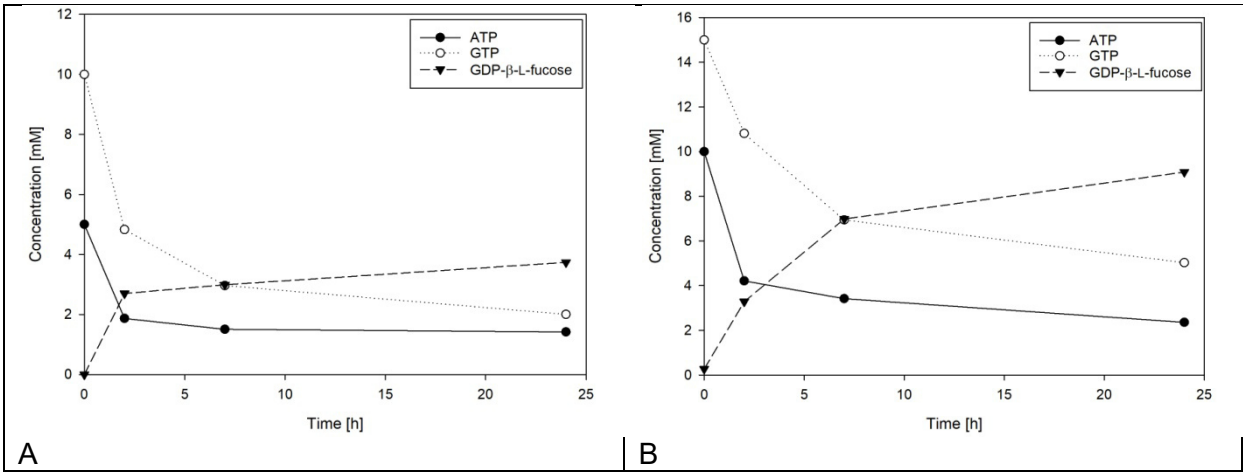

Figure 3.14: Chromatograms of the consumption of the co-substrates ATP and GTP and the synthesis of GDP-β-L-fucose over 24 hours.
A: Starting concentration: 5mM L-fucose, 5 mM ATP and 10 mM GTP. B: Starting concentration: 10 mM L-fucose, 10 mM ATP and 15 mM GTP.

With starting concentrations of 5 mM fucose, 5 mM ATP and 10 mM GTP conversion reached 74.6 % after 24 hours based on the starting fucose concentration.
With 10 mM fucose, 10 mM ATP and 15 mM GTP 90.9 % conversion was reached after 24 hours based on the starting fucose concentration.

In the introduction the different K_m values found in literature for reaction of *Bf*FKP with ATP and GTP were quoted. For ATP values of 3.3 mM (Tsai et al., 2013) and 1.08 mM (Wang et al., 2009) were published. For GTP values of 1.08 mM (Tsai et al., 2013) and 0.012 mM (Wang et al., 2009) were determined.
In the experiment starting with 5 mM ATP and 10 mM GTP after 2 hours of reaction about 1.9 mM ATP and 4.8 mM GTP are left. These values are coming close to the K_m values stated in literature, so there were no saturating conditions anymore, which could explain decreased reaction rates. However, as ATP conversion slows down severely in both reaction setups after two hours, in one case the remaining ATP concentration being below 2 mM in the other case 4 mM, the reason for enzyme inactivation might rather be denaturation of the enzyme or inhibition by ADP.

In the case of GTP the saturated conditions are reached for both possible K_m values given in literature with both starting concentrations (10 mM and 15 mM). The clear decrease in reaction rate for reaction with GTP after two hours can result from an even higher K_m value then given in literature or from inhibition for example by GDP-β-L-fucose. A limitation in L-fucose-1-phosphate due to a decrease in fucokinase activity will also lead to a decreased activity in the GDP-β-L-fucose forming step. K_m values for L-fucose-1-phosphate given in literature are 30 to 40 µM measured at room temperature.

In both experiments more GTP was consumed than GDP-β-L-fucose was produced, especially at the experiment starting with 5 mM ATP and 10 mM GTP. On the one hand GTP is hydrolyzed to GDP rather easily. Moreover, it was shown before that the enzyme does not necessarily have to take the phosphate group from the ATP for the first step, but could also take GTP as a co-substrate.

Beside the synthesis of GDP-β-L-fucose also the rate of the reaction steps was of interest. In the introduction varying results for the identification of the rate limiting step was stated. Tsai et al. (2013) published that the fucokinase step had a higher k_{cat}, where ATP was needed. The conversion from L-fucose to L-fucose-1-phosphate was stated to be about 7 to 8 times faster than the second step. In contrast, Wang et al. (2009) showed that the conversion from L-fucose-1-phosphate to GDP-β-L-fucose (GTP consumption) had a two times higher k_{cat} as the first step. Data from the experiment shown in Figure 3.14 B suggest that conversion of fucose with ATP is faster than conversion of fucose-1-phosphate with GTP even at concentrations (10 mM ATP, 15 mM GTP) probably not as saturating for conversion with ATP as for conversion with GTP (literature gives K_m values of 3.3 mM for ATP for the fucosekinase activity and 1.1 mM for GTP for guanylyltransferase activity, K_m values for L-fucose-1-phosphate are uniformly stated to be < 50 µM). The intermediate L-fucose-1-phosphate probably accumulates in the first two hours of the reaction and is than further converted to GDP-β-L-fucose. In the first two hours a GDP-β-L-fucose building rate of 0.029 mM/min was detected, while ATP was used with a rate of 0.049 mM/min. At an already very low fucosekinase activity of 0.005 mM between 2 and 7 hours the GDP-β-L-fucose rate decreased to 0.016 mM/min.

3.4 Measurement of activity of *Bf*FKP

The first measurement of the activity of *Bf*FKP is illustrated in Figure 3.15. The reaction mixture was a composition of compounds as stated in Table 2.9. The enzyme concentration of 0.11 mg/mL was chosen to gain a long enough linear range of product increase for rate evaluation

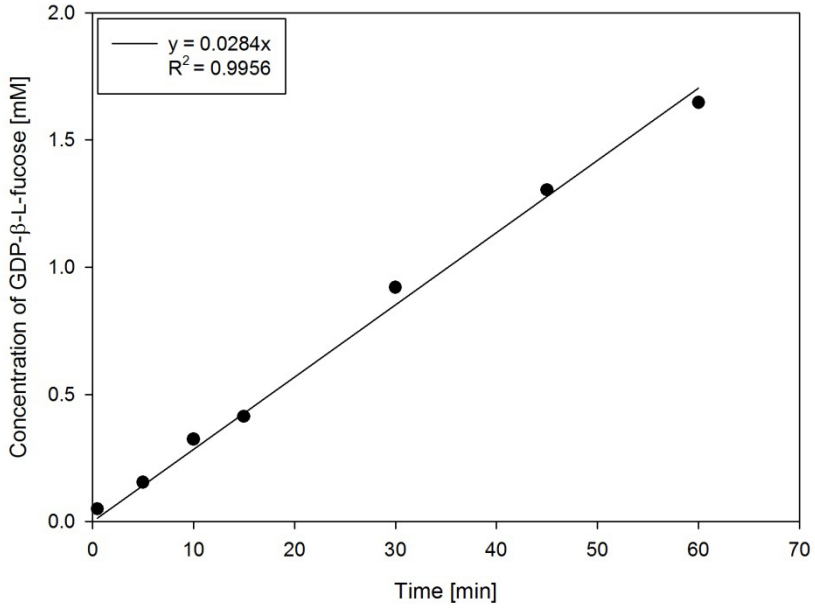

Figure 3.15: Activity measurement of *Bf*FKP.
Enzyme concentration: 0.11 mg/mL, despite of that standard conditions (5 mM L-fucose, 5 mM ATP, 10 mM GTP, 10 mM $MgCl_2$ and 10 mM $MnCl_2$); analyzed on HPLC; time points of reaction: 0.30 min, 5 min, 10 min, 15 min, 30 min and 60 min;

Analysis of the product increase was done via HPLC as described under section 2.8. The activity measurement gave the following regression equation for calculation: $y = 0.0284x$ The activity of the enzyme was 0.258 U/mg or 0.0284 U/mL. In comparison to the stated specific activity of 4.5 U/mg (Wang et al., 2009), the reached activity is about 17 times lower. The construct of Wang was also in a pET16 vector and was also provided from the Comstock group from Boston (USA) (but the conditions were not exactly the same, as the substrate concentrations were equimolar (5 mM of L-fucose, ATP and GTP)).

The stability during storage at -20°C was verified by two further activity measurements. After 6 days the activity showed no decrease as the specific activity stayed exactly the same. Another 6 days later the enzyme lost about 4% of its initial activity, which is negligible.

Gerald Rinner, a colleague from the institute who was also working with *Bf*FKP had stability problems during storage at -20°C over longer time. He determined that the addition of 10% glycerol and 0.02% Tween® 80 was stabilizing the enzyme. If this enzyme is to be stored for a longer time, the addition of these chemicals can be considered.

3.5 Analysis on HPLC

The method for analysis on the HPLC is stated above in section 2.8. The following diagrams show the start and end point of synthesis.

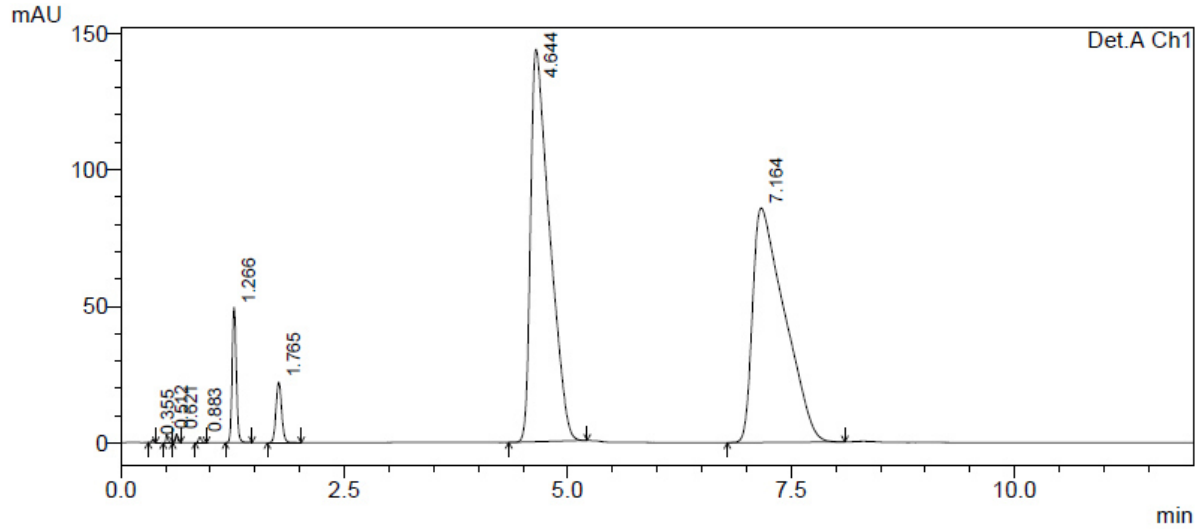

Figure 3.16: Chromatogram of HPLC analysis with a Kinetex C-18 column (reversed phase) on the Shimadzu system.
Starting point of conversion from L-fucose (10 mM) to GDP-β-L-fucose. Peak assignment: 0.355 min: guanosin, 0.512 min: GMP, 0.621 min: AMP, 0.883 min: GDP-β-L-fucose, 1.266: GDP, 1.765 min: ADP, 4.644 min: GTP (15 mM), 7.164 min: ATP (10 mM). Conditions: mobile phase = 93.5% TBAB buffer and 6.5% ACN, 2 mL/min, 10 minutes, 40°C

Compounds eluted with the following order: GMP, AMP, GDP, ADP, GTP and ATP. The desired product GDP-β-L-fucose eluted between AMP and GDP after about 0.8 to 0.9 minutes (Figure 3.17).

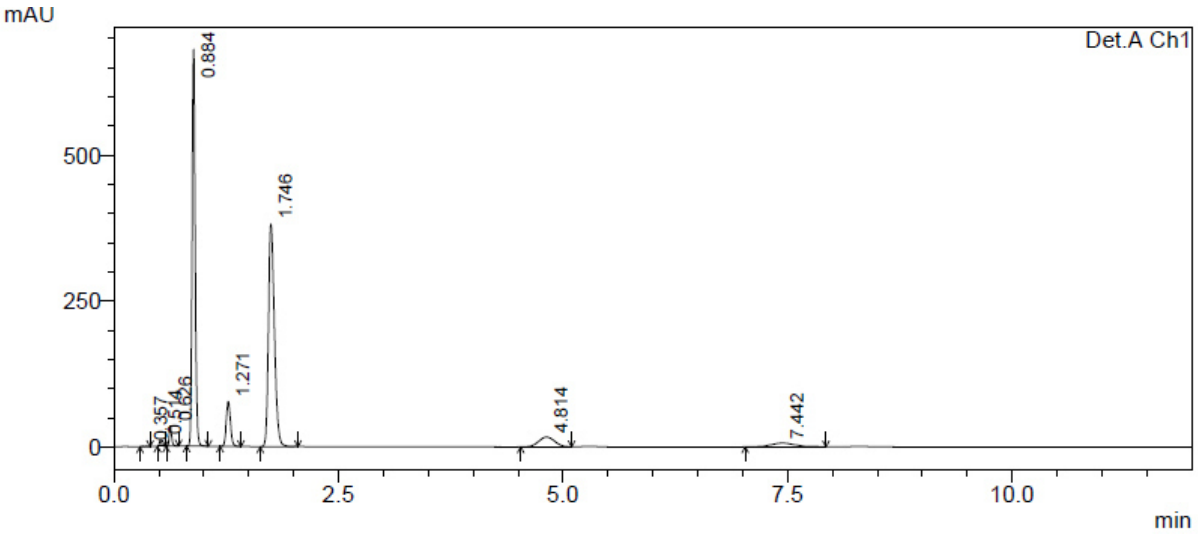

Figure 3.17: Chromatogram of HPLC analysis with a Kinetex C-18 column (reversed phase) on the Shimadzu system.
Chromatogram after 24 hours of conversion from L-fucose to GDP-β-L-fucose. Peak assignment: 0.357 min: guanosin, 0.514 min: GMP, 0.626 min: AMP, 0.884 min: GDP-β-L-fucose, 1.271: GDP, 1.746 min: ADP, 4.814 min: GTP, 7.442 min: ATP. Conditions: mobile phase = 93.5% TBAB buffer and 6.5% ACN, 2 mL/min, 10 minutes, 40°C

When comparing Figure 3.16 and Figure 3.17 one can see that the peaks of ATP and GTP were getting less and a peak between AMP and GDP arose. In comparison to the standard from Isosep this could be identified as GDP-β-L-fucose. Another identification was done by ^{1}H NMR analysis, which is stated below in a separate section. Also the consumption of ATP and GTP was an evidence for the synthesis of the nucleotide sugar.

3.6 Purification of GDP-β-L-fucose

As already stated the downstream processing of GDP-β-L-fucose consists of 4 essential steps, which are (I) CIP digest, (II) anion exchange, (III) size exclusion and (IV) lyophilization.

(I) The optimization of CIP digest to get rid of the nucleotide tri~, di~ and monophosphates is described below with different examples.

(II) The chromatogram of AEC is illustrated in Figure 3.18. In this case a step gradient was used to elute the wanted and the not wanted components. The first step was 15% buffer B to elute AMP and GMP (peak 2). The second step at 50% buffer B was to elute the wanted nucleotide sugar (peak 3). In the end the concentration of buffer B was set to 100% to elute possible impurities or compounds which could still stuck on the column. The loading and elution steps were done more than once.
The best way to conduct the AEC process is stated below with different examples.

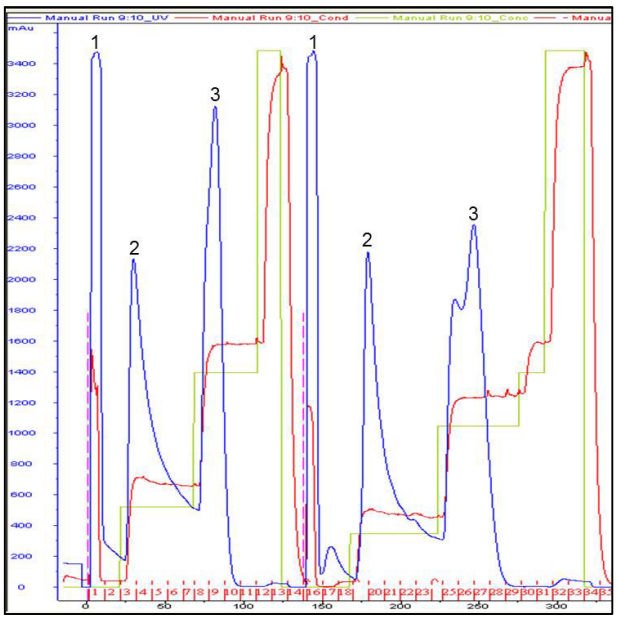

Figure 3.18: Chromatogram of anion exchange (AEC) with two 1 mL columns.
Blue curve: absorption at 254 nm [mAU], green curve: concentration of buffer B [%], red curve: conductivity [mS/cm], pink line: time point of injection; Peak 1: flow through containing adenosine and guanosine, Peak 2: AMP and GMP, Peak 3: GDP-β-L-fucose; x-axis: mL, y-axis: mAU, mobile phase: buffer A: loading buffer and buffer B: elution buffer, stationary phase: Toyopearl Super Q-650M

The GDP-β-L-fucose containing fractions from two runs were pooled and the volume was reduced via the rotary evaporator.

(III) The third step, the size exclusion, is pictured in Figure 3.19. This step was used to separate the nucleotide sugar from the salt concerning size. The GDP-β-L-fucose eluted first (blue line in Figure 3.19) and the salt eluted afterwards or overlapping (brown curve in

Figure 3.19). The smaller the injected volume, the sharper was the peak in blue and there was less overlapping of sugar and salt. Therefore the loaded volume was between 1 mL and 2 mL. No different experiments were made, since the size exclusion worked well from the beginning.

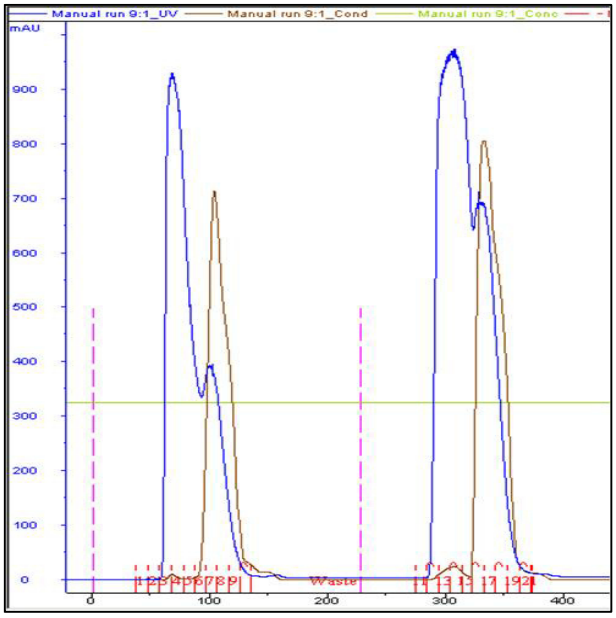

Figure 3.19: Chromatogram of size exclusion (SEC).
Blue curve: absorption at 254 nm [mAU], this shows the nucleotide sugar; brown curve: conductivity [mS/cm], this illustrates the salt; pink line: time point of injection; x-axis: mL, y-axis: mAU; mobile phase: deionized water; stationary phase: Sephadex G-10

All GDP-β-L-fucose containing fractions were pooled and the volume was reduced to about 10 mL via the rotary evaporator.

(IV) In Figure 3.20 the process of lyophilization is shown. The remaining 10 mL of solution in the round bottom flask were shock frozen with liquid nitrogen and stuck on the lyophilizer. The vacuum was applied and after one night some powder was left in the round bottom flask.

Figure 3.20: Process of lyophilization.
Left figure: Start of lyophilization; there was a thin layer of ice inside the round bottom flask and a vacuum was applied. Right figure: dried product in powder form; if the round bottom flask was at room temperature, the freeze drying process was finished.

The final product was analyzed by HPLC, see therefore Figure 3.21. One can see that there is no absolute purity, as there is guanosin, GMP and GDP in the solution (the powder was diluted in TRIS/HCl buffer).

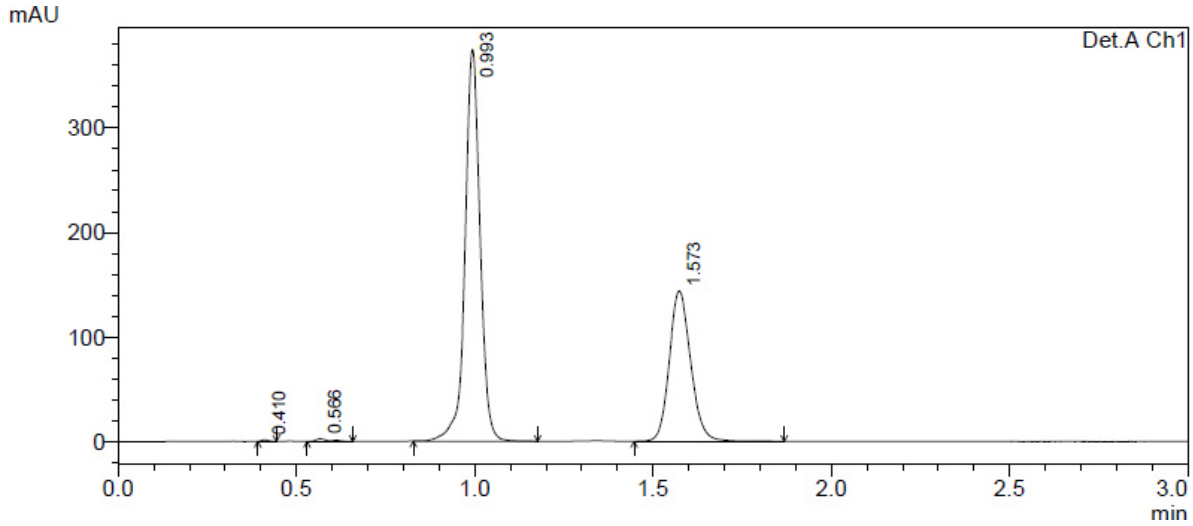

Figure 3.21: Chromatogram of HPLC analysis with a Kinetex C-18 column (reversed phase) on the Shimadzu system.
Chromatogram after the purification process of GDP-β-L-fucose. Peak assignment: 0.410 min: guanosin, 0.566 min: GMP, 0.993 min: GDP-β-L-fucose, 1.573: GDP. Conditions: mobile phase = 93.5% TBAB buffer and 6.5% ACN, 2 mL/min, 10 minutes, 40°C

In the following tables the most relevant and best tracked purifications are stated, explained and discussed.

The yields of GDP-β-L-fucose shown in Table 3.3 are stated in milligram and percent over the whole purification process. Also the loss of each evaluated step is declared. The different steps were distributed over several days.

Table 3.3: Purification of GDP-β-L-fucose.
Listed values gained during the steps of synthesis and purification. Synthesis: value at the end of the reaction, prior to CIP: after separation of *Bf*FKP and storage until CIP digest (no values available), CIP: value after the digest with the alkaline phosphatase, AEC: value after the anion exchange, SEC: value after the size exclusion, prior to lyophilization: after volume reduction for freeze-drying process (no values available), lyophilization: total weight of the gained product, yield: amount of nucleotide sugar in the gained powder

	10 mL batch, max. yield: 31.67 mg			
	Yield [mg]	Yield [%]	Loss per step [%]	Comments
Synthesis	31.26	100.00	0.00	
Prior to CIP	-			
CIP	21.88	69.99	29.94	elongated digest
AEC	6.55	20.95	49.04	1x1 mL column
SEC	3.96	12.66	8.29	60% Nt-sugar
Prior to lyophilization	-			
Lyophilization	7.40	23.67	-	no powder, gelatinous, 50% Nt-sugar
Total *	3.70	11.83	-	

* This value represents the actual proportion of gained GDP-β-L-fucose

The high loss of nucleotide sugar during the CIP digest can be explained by the elongated time of digestion. The CIP is only working properly if the pH and the temperature are right. Otherwise the alkaline phosphatase is getting unspecific and can harm the GDP-β-L-fucose instead of reducing the nucleotide tri~, di~ and monophosphates. In this case the amount of the alkaline phosphatase was 2 U/mL. As not all nucleotide tri~ and diphosphates were digested overnight the incubation time was elongated. This resulted in the digest of the nucleotide sugar instead of the nucleotide tri~ and diphosphates.

The anion exchange was in all purifications the step with the most loss. But the smaller the amount of nucleotide sugar the more loss was expected.

All relevant fractions of SEC were pooled and the volume was reduced to about 3 mL. These were transferred in a reaction tube and were freezed at -70°C overnight.

On the next day the tube was put into the lyophilizer. The end product of 7.40 mg of a gelatinous like substance was about half GDP-β-L-fucose and half GDP. This suggested that a hydrolysis reaction took place during SEC or during the storage at 4°C until volume reduction. Also the slow freezing method overnight at -70°C favored the hydrolysis process.

The distribution of the purification steps over several days resulted in a high loss of nucleotide sugar. As a consequence the processes of AEC, SEC and the start of lyophilization were done on one day (Table 3.4).

Table 3.4: Purification of GDP-β-L-fucose.
Listed values gained during the steps of purification. Different batches were pooled to gain an appropriate amount of nucleotide sugar to purify. Synthesis: value at the end of the reaction (no values available as different batches were pooled), prior to CIP: starting point of CIP digest, CIP: value after the digest with the alkaline phosphatase, AEC: value after the anion exchange, SEC: value after the size exclusion, prior to lyophilization: after volume reduction for freeze-drying process (no values available), lyophilization: total weight of the gained product in powder form, yield: amount of nucleotide sugar in the gained powder

	23 mL pooled batches			
	Yield [mg]	Yield [%]	Loss per step [%]	Comments
Synthesis	-			
Prior to CIP	84.47	100.00	0.00	
CIP	81.19	96.12	3.88	
AEC	55.86	66.13	29.99	2x1 mL column
SEC	51.47	60.94	7.86	
Prior to lyophilization	-			
Lyophilization	45.00	53.28	7.66	about 48% Nt-sugar
Total *	21.60	25.57	-	

* This value represents the actual proportion of gained GDP-β-L-fucose

In the purification which is stated in Table 3.4 4 U/mL of the alkaline phosphatase (CIP) was added. It did not harm the nucleotide sugar because there were no problematic conditions. But the concentration of the alkaline phosphatase was too low to degrade all nucleotide diphosphates in time.

Since it was not possible to separate all nucleotide diphosphates from the nucleotide sugar with anion exchange, there was a larger proportion of impurities from the outset. In more detail there were 24% ADP in the solution from the beginning and also 7% of GDP. The part of ADP stayed the same and the percentage of GDP increased: (I) after SEC: 10% GDP, (II) after the final volume reduction for lyophilization: 14% and (III) after lyophilization: 24% GDP. The remaining 4% of impurities were GMP, AMP, GTP and ATP.

As can be seen in the experiments on pH optimum (Table 3.7), GDP-β-L-fucose was less stable at acidic pH. The pH at AEC and at SEC was relatively low. The storage in deionized water at 4°C after SEC was therefore not optimal and could evoke the hydrolysis process.

Within this purification process volume reduction took place more often after SEC. As soon as there were about 50 mL of GDP-β-L-fucose containing solution the volume was reduced and then stored on ice until the next fractions were ready for volume reduction (the same round bottom was used again). Therefore, the sugar was increasingly exposed to 40°C. This may have strengthened the hydrolysis. The two facts of low pH and high temperature could be the explanation for the large hydrolysis effect (see pH experiments in Table 3.7).

To freeze the nucleotide sugar as quickly as possible (and stop hydrolysis), it was treated with liquid nitrogen and then stuck immediately on the lyophilizer.

The problems with the CIP digest were overcome with the purification illustrated in Table 3.5.

Table 3.5: Purification of GDP-β-L-fucose.
Listed values gained during the steps of synthesis and purification. Synthesis: value at the end of the reaction, prior to CIP: after separation of *Bf*FKP and storage until CIP digest, CIP: value after the digest with the alkaline phosphatase, AEC: value after the anion exchange, SEC: value after the size exclusion, prior to lyophilization: after volume reduction for freeze-drying process, lyophilization: total weight of the gained product, yield: amount of nucleotide sugar in the gained powder

	20 mL batch, max. yield: 63.33 mg			
	Yield [mg]	Yield [%]	Loss per step [%]	Comments
Synthesis	48.41	100.00	0.00	
Prior to CIP	45.59	94.17	5.83	
CIP	45.54	94.07	0.10	
AEC	20.87	43.11	50.96	1x1 mL column
SEC	8.36	17.27	25.84	lost fractions
Prior to lyophilization	8.33	17.21	0.06	
Lyophilization	5.00	10.33	6.88	about 90% Nt-sugar
Total *	4.50	9.30	-	

* This value represents the actual proportion of gained GDP-β-L-fucose

The addition of the alkaline phosphatase was adapted to the data from Lemmerer et al. (2016) and due to personal experience from the previous experiments. Therefore 10 U/mL of alkaline phosphatase were added and incubated overnight at 30°C (pH 7, for about 14 hours). No nucleotide tri~ and diphosphates were left in the solution.
Some percent of nucleotide monophosphates, which could be separated via anion exchange, were present. So there was no problem with impurities and also hydrolysis effects were low (about 10%).
A problem during SEC occurred where about a half of the gained GDP-β-L-fucose was lost, which explains the high loss in Table 3.5. The rest of the purification went according to plan, there were no delays and the steps went as fast as possible.

The purification stated in Table 3.6 started with a very good conversion rate of 98.5%.

Table 3.6: Purification of GDP-β-L-fucose.
Listed values gained during the steps of synthesis and purification. Synthesis: value at the end of the reaction, prior to CIP: after separation of *Bf*FKP and storage until CIP digest, CIP: value after the digest with the alkaline phosphatase, AEC: value after the anion exchange, SEC: value after the size exclusion, prior to lyophilization: after volume reduction for freeze-drying process, lyophilization: total weight of the gained product, yield: amount of nucleotide sugar in the gained powder

	20 mL batch, max. yield: 126.66 mg			
	Yield [mg]	Yield [%]	Loss per step [%]	Comments
Synthesis	124.73	100.00	0.00	
Prior to CIP	124.32	99.67	0.33	
CIP	115.76	92.81	6.86	
AEC	67.39	54.03	38.78	one big column
SEC	39.28	31.49	22.54	
Prior to lyophilization	34.77	27.87	3.62	
Lyophilization	33.30	26.70	1.17	85% Nt-sugar
Total *	28.31	22.69	-	

* This value represents the actual proportion of gained GDP-β-L-fucose

The CIP digest was proofed to be optimized. As the starting concentrations were scaled up in a ratio of 1.67 also the concentration of CIP was adjusted. Therefore 16.7 U/mL of alkaline phosphatase were added. This resulted in the digest of all nucleotide tri~ and diphosphates and most of the nucleotide monophosphates.
For easier handling a bigger column for AEC was used with a binding capacity of 1 g of phosphate groups. Afterwards it seemed not to be better as with the small columns, as the loss of product was greater. It had been shown that it was better to use the complete binding capacity of the columns to achieve a better product yield.
A problem with the big column and only one elution process was that there was a limitation concerning SEC. A maximal amount of 25 mg of the nucleotide sugar can be loaded on the column at each run. At the time point of loading no actual measured and calculated values were available and the column was loaded for about 5 times (to avoid an overload). This loading and elution processes took its time and the nucleotide sugar was a longer time in the surrounding of low pH and high salt concentrations. The hydrolysis process started and after SEC there were already 10% of GDP in the sample. This fact explains the greater loss during SEC compared to the previous runs.
After lyophilization 15% of GDP were present.

Beside the well documented purifications there were also other tries. The first purification for example resulted in 4.5 mg GDP-β-L-fucose in the form of white powder. At the preparing of a stock with this powder it was already observed that the calculated concentration did not correspond to the actual concentration of the stock.
Besides the other impurities, there was also the ability of the presence of moisture-donating L-fucose. It is reported, that L-fucose increases the hydration and the elasticity of human skin (Isnard, Fodil-Bourahla, Robert, & Robert, 2004). L-fucose is therefore used in various cosmetics such as, for example, skin creams. The German company Jennewein Biotechnology GmbH specializes in this, among others.
This ability had been noticeable during the experiments since the stock solutions always had a lower concentration than assumed. L-fucose would possibly absorb the moisture from the air and thereby the stock is more diluted.

Another purification yielded in 26.08 mg powder with hardly any GDP in it and with just little other impurities. But as a stock was made, the hydrolysis process started and nearly all GDP-β-L-fucose was degraded. In this case the powder was dissolved in ultrapure water and the pH was rather low. It had been shown that it was better to dissolve the nucleotide sugar in buffer in order to adjust the pH, which prevents the hydrolysis.

To evaluate the best pH in combination with temperature the nucleotide sugar was dissolved in buffer with different pH values and stored at different temperatures (see Table 3.7).

Between the pH values of 4 and 8 the nucleotide sugar is quite stable as there was a maximal loss of about 3%. One problem was the storage of GDP-β-L-fucose at 4°C in ultrapure water where the pH was about 3.

This fact also explained the hydrolysis during purification. The low pH of the binding and elution buffer during AEC in combination with high salt concentrations was harmful for the nucleotide sugar. The GDP-β-L-fucose was eluted at about 300 mM sodium acetate and at the volume reduction step this salt concentration was raised to about 3 M.

Table 3.7: Stability of GDP-β-L-fucose at different pH values.
GDP-β-L-fucose was dissolved in buffer with different pH values and stored at different temperatures, to determine the best storage conditions. Dissolving the nucleotide sugar in ultrapure water seemed to be the worst idea. The other pH values and temperatures are quite the same, but the least loss gave the sample with a pH of 7.

	4°C			-20°C			-70°C		
	Day 0	Day 1	Day 17	Day 0	Day 1	Day 17	Day 0	Day 1	Day 17
	Stability of GDP-β-L-fucose [%]								
ultrapure water (~ pH 3)	100	94.30	88.92	100	98.32	93.93	100	98.54	98.26
pH 4	100	99.09	98.90	100	98.48	98.35	100	98.21	98.09
pH 5	100	98.76	98.56	100	98.17	98.14	100	98.17	98.01
pH 6	100	98.03	97.72	100	99.18	98.99	100	99.17	99.06
pH 7	100	99.83	98.90	100	99.92	99.89	100	99.74	99.71
pH 8	100	98.47	97.91	100	99.43	99.06	100	99.33	99.10

[1] The pH of the solution was about 3.

Nunez, O'Connor, Rosevear, & Barker (1981) found out, that not only the pH but also higher temperatures can harm the GDP-β-L-fucose. At the combination of pH 3 and 37°C the nucleotide sugar showed a half life of about 7 hours. Therefore it is also important to keep the time of volume reduction as short as possible, as this takes place at 40°C.

3.7 ^{1}H NMR

The produced GDP-β-L-fucose was analyzed additionally with ^{1}H NMR. Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Hansjörg Weber made the measurements and with the additional data from G. Zhao et al. (2010) the sample could be identified as GDP-β-L-fucose (spectrum shown in Figure 3.22).

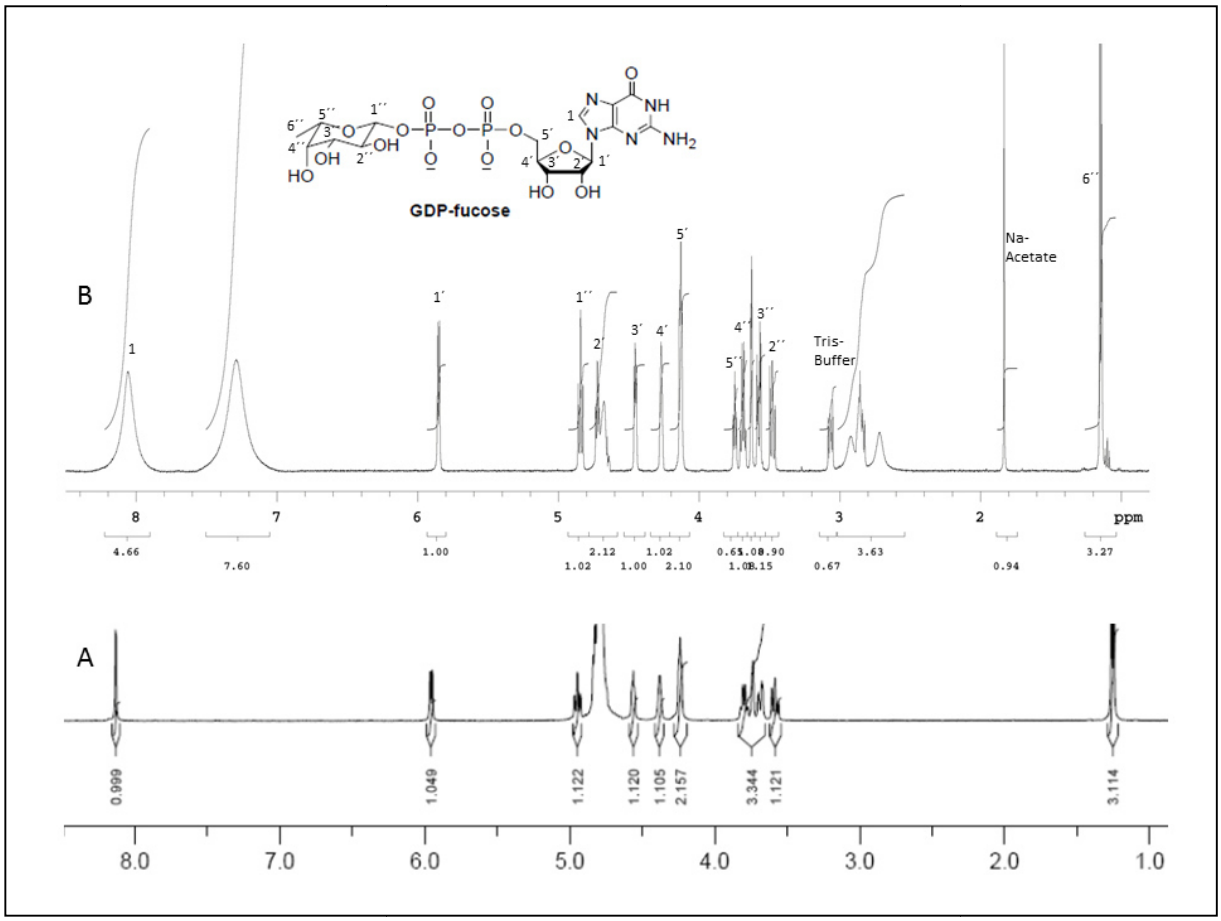

Figure 3.22: Comparison of ^{1}H NMR spectrum of GDP-β-L-fucose. A: spectrum of GDP-β-L-fucose published by Zhao et al., (2010) B: spectrum of GDP-β-L-fucose synthesized in this thesis. The peaks in B are assigned with numbers corresponding to the numbers in the structure.

1´´ can be identified as fucose (triplet) and 6´´ is the methyl group. The peak at 8 ppm is the nitrogenous base guanine. The more broaden peak, compared to the reference spectrum can be explained by the (maybe paramagnetic) coordination of some cations to the phosphate group. Another reason for the broaden peak at 7 and 8 ppm is the presence of sodium acetate and TRIS buffer in the solution and therefore there are interchangeable protons (Figure 3.22_B). All other peaks are also comparable with the reference spectrum (Figure 3.22_A).

4 Conclusion and Outlook

The cultivation and expression of *Bf*FKP from a pET16 vector in *E. coli* BL21 (DE3) was unproblematic. The purification of the enzyme yielded a maximum of about 19.5 mg purified protein from one purification and a maximum of 4.5 mg purified protein per 1 L of cultivation broth.

The synthesis of GDP-β-L-fucose was optimized by doing the conversion at 37° C with equimolar amounts of ATP and 1.5 fold surplus of GTP compared to fucose and under addition of 10 mM magnesium and manganese. Both in the small scale and in the larger

scale (20 mL) high conversion numbers were gained. The conversion yield reached in maximum 91% in 24 hours starting from 10 mM fucose, 10 mM ATP and 15 mM GTP.

Separation of peaks for HPLC analysis could be optimized by using a reversed phase column and a low concentration of ACN (analysis conditions: mobile phase: 93.5 % TBAB buffer and 6.5 % ACN, 2 mL flow per minute, analysis time: 10 minutes, oven temperature: 40°C).

The purification of the nucleotide sugar was rather a challenge. After optimization the described purification procedure is now feasible to be done in one day, but one must be familiar with the protocol, which is stated in Part II of this master thesis. It is also suggested to do the purification together with a second person. The following optimized yields were reached: (I) CIP digest optimized: loss of 4%, (II) major loss during AEC: 30%, (III) unproblematic SEC: loss of 5% and (IV) loss during lyophilization: 1%. Summarizing one can say that it is possible to have a total loss of 40% and therefore could have 60% product recovery.
Within this thesis this yield was not reached within the same purification process, but it was shown, that it is theoretically possible. The maximal overall yield reached was 28.31 mg dried GDP-β-L-fucose. Published yields gained via the salvage pathway and similar purification methods are in the range of 3 to 250 mg with a maximum of 43% purification yields (Stiller & Thiem, 1992; Zhao et al., 2010; Engels & Elling, 2014). Best results after synthesis via the *de novo* pathway were 78 mg GDP-β-L-fucose at 75% yield after purification (Albermann et al., 2000).
The most problematical issue is the stability of the nucleotide sugar. Stability can be compromised by pH, salt concentration and temperature. The time span where the GDP-β-L-fucose is exposed to the non-optimal conditions is very important and should be kept as short as possible.
The high salt concentration, which is at about 3 M after AEC and volume reduction, is harmful for the nucleotide sugar, therefore it is important to separate the salt from the sugar as fast as possible. Low pH of the aqueous solution might support the hydrolysis to GDP and L-fucose. To prevent the nucleotide sugar from hydrolysis it is necessary to start the process of lyophilization as soon as possible after SEC and volume reduction.

The goal of the purification would be to reach the theoretically possible best yields of each step in one process. Also further optimization would be pleasant to reach more than 60% isolated yield. In order to improve this, one could more optimize the pH of the mobile phases, or obtain more suitable columns for AEC and SEC in terms of volume and binding capacity.

5 Appendix

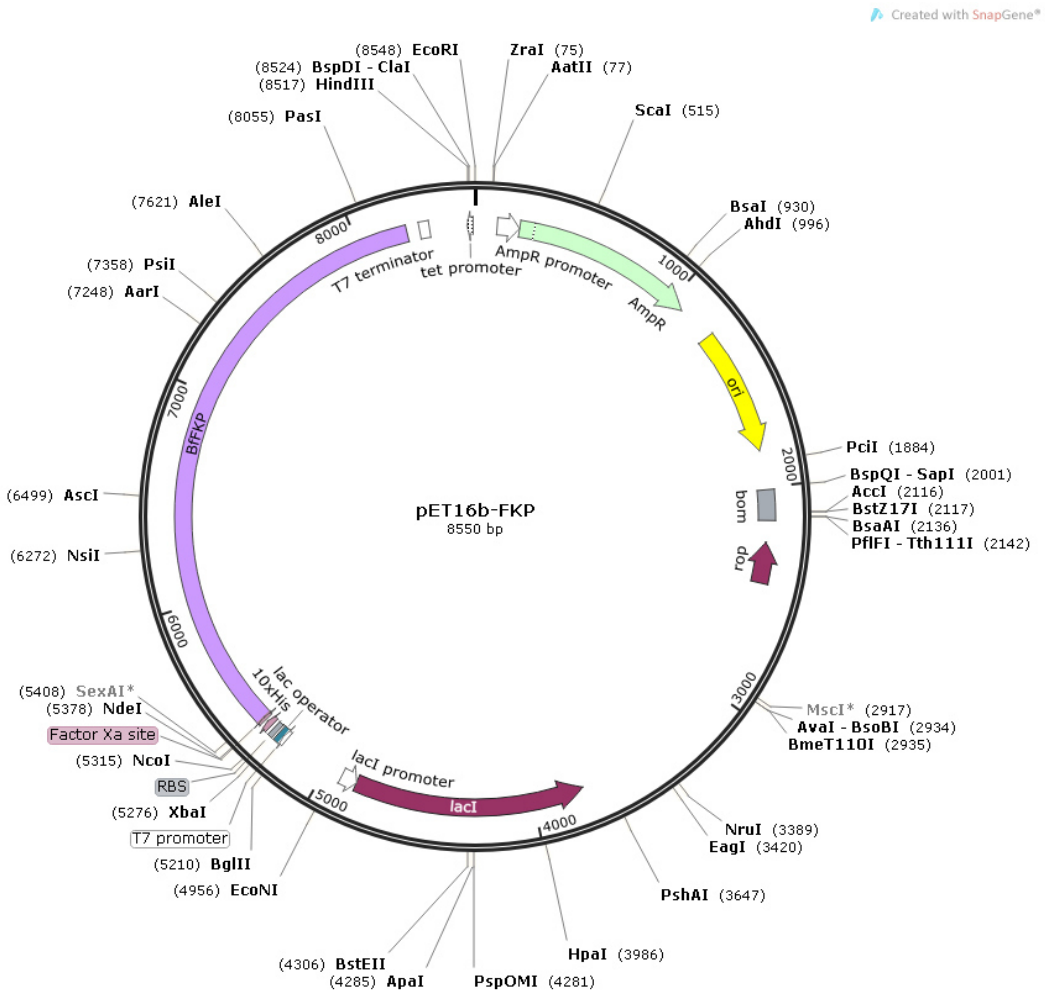

Figure 5.1: Plasmid map of *Bf*FKP in a pET16 vector

Sequence of *Bf*FKP, According to UniProtKB-Q58T34:

catatgcagaaactgctgagcctgccgagcaacctggtgcagagctttcatgaactggaacgcgtgaaccgcaccgattg
gttttgcaccagcgatccggtgggcaaaaaactgggcagcggcggcggcaccagctggctgctggaagaatgctataacg
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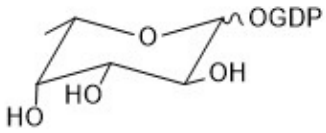

GDP-β-L-fucose
$C_{16}H_{23}O_{15}P_2$
517.29 g/mol

Figure 5.2: Structure of GDP-β-L-fucose

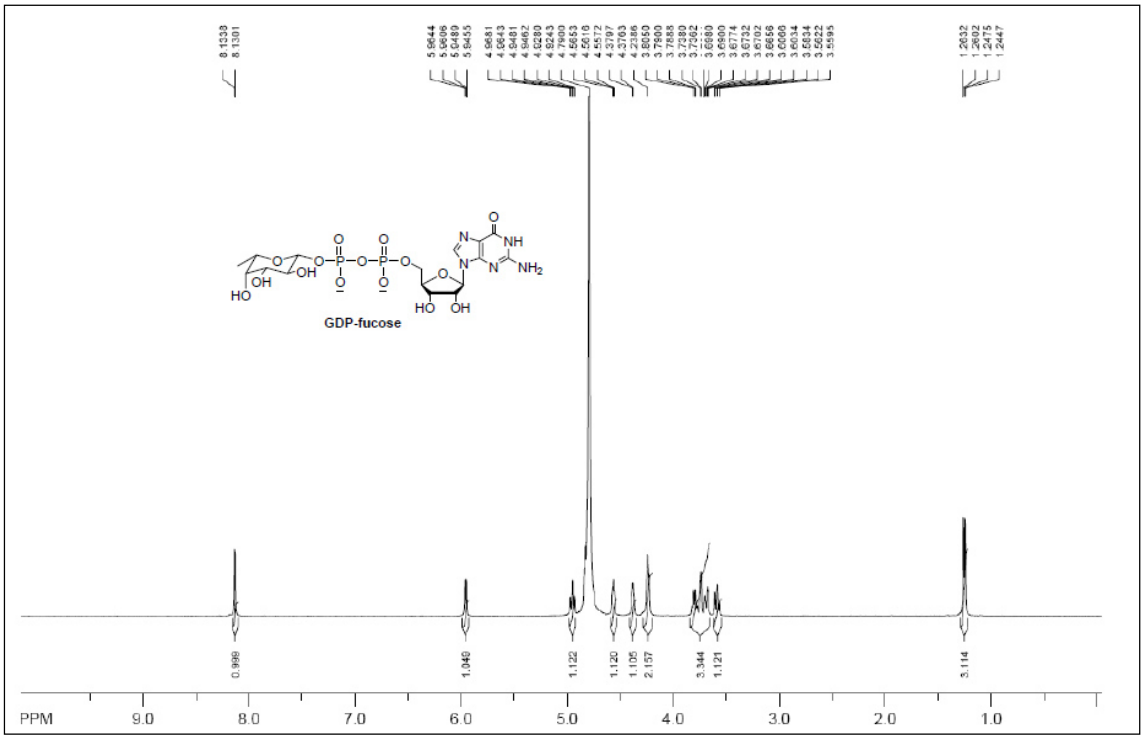

Figure 5.3: ^{1}H NMR spectrum stated in the supplementary data of G. Zhao et al., 2010

Part II

SOP for synthesis and purification

of GDP-β-L-fucose

6 Synthesis of GDP-β-L-fucose (1 - 2 days)

The enzyme *Bf*FKP is catalyzing the reaction from L-fucose to GDP-β-L-fucose via the intermediate L-fucose-1-phosphate (see Figure 6.1).

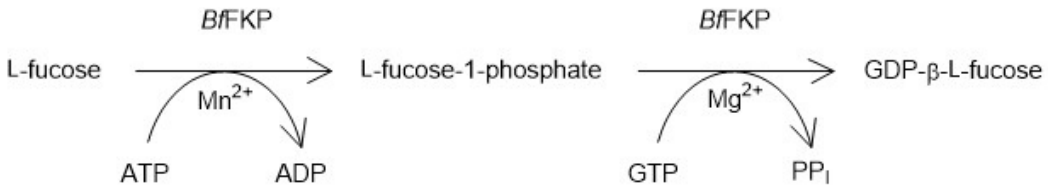

Figure 6.1: Reaction equation catalyzed by the bifunctional enzyme FKP.

All chemicals, materials and devices used for the synthesis of GDP-β-L-fucose are stated in Table 6.1.

Table 6.1: Chemicals, materials and devices for the synthesis of GDP-β-L-fucose.

Chemicals	Materials	Devices
Acetonitrile (ACN)	Small beaker with a lid	Spectrophotometer
ATP (Adenosine-5'-triphosphate di-sodiumsalt)	Reaction tubes: 1.5 mL	Magnetic stirrer
GTP (Guanosine-5'-triphosphate di-sodiumsalt)	Reaction tubes: 15 mL	HPLC with UV-detector
Hydrochloride (HCl)	---	---
L-fucose	---	---
Magnesium chloride ($MgCl_2$)	---	---
Manganese chloride ($MnCl_2$)	---	---
Inorganic phosphatase (PPase)	---	---
TRIS	---	---

Solutions:

- TRIS/HCl buffer, 50 mM, pH 7.6, 0.5 L
  - Molar mass of TRIS: 121.14 g/mol
    - Weigh 3.03 g TRIS in a beaker and dissolve it in 0.5 L deionized water. Adjust the pH with HCl to 7.6.
    - Filtrate with a cellulose acetate filter with a pore size of 0.45 µm
- Preparation of the stocks for reaction mixture (needed volumes can be seen in Table 6.2):
  - L-fucose:
    - Molar mass: 164.16 g/mol
    - To prepare a 100 mM stock weigh in 0.0328 g and dilute it in 2 mL ultrapure water
  - GTP:
    - Molar mass: 621.18 g/mol
    - To prepare a 100 mM stock weigh in 0.1801 g and dilute it in 2.9 mL ultrapure water

- GTP and ATP stocks need to be controlled via spectrophotometer and HPLC. Therefore dilute the stocks 1:50 and 1:100 (about 10 µL are needed) and measure it (duplicates) at a UV/Vis spectrophotometer. Start from a 1:10 dilution (about 60 µL). For GTP use a wavelength of 256 nm and for ATP use 259 nm. With the help of the Lambert-Beer law and the known extinction coefficient of GTP and ATP in acidic surrounding the concentration of the stocks can be calculated. $E = \varepsilon \cdot c \cdot d$ where E is the extinction, ε is the extinction coefficient (ε_{GTP}: 12.4 L/mmol/cm, ε_{ATP}: 14.7 L/mmol/cm), c is the concentration one wants to know and d is the layer thickness which the device takes automatically into account in the measured microvolume of 2 µL. Additionally the stocks are also measured on the HPLC (method described below). Therefore take 50 µL of a 1:10 dilution centrifuge it for about 5 minutes, transfer 40 µL into a HPLC tube and inject 1 µL. So one gets the result how much nucleotide tri~, di~ and monophosphates are in the stock. Take this into account and adapt it to the reaction mixture for synthesis.

- ATP:
  - Molar mass: 551.10 g/mol
  - To prepare a 100 mM stock weigh in 0.1102 g and dissolve it in 2 mL ultrapure water
- $MgCl_2.6H_2O$
  - Molar mass of 203.30 g/mol
  - To prepare a 100 mM stock weigh in 0.0407 g and dissolve it in 2 mL ultrapure water
- $MnCl_2.2H_2O$
  - Molar mass of 161.88 g/mol
  - To prepare a 100 mM stock weigh in 0.0324 g and dissolve it in 2 mL ultrapure water

The reaction mixture for the synthesis of GDP-β-L-fucose is given in Table 6.2.

Table 6.2: Reaction mixture for the synthesis of GDP-β-L-fucose. The amount of enzyme must be calculated, based on the calculated yield of the purification.

Components	Stock	Final concentration	Volume [mL]
L-fucose [1]	100 mM	10 mM	1.8
GTP [1]	100 mM	15 mM	2.7
ATP [1]	100 mM	10 mM	1.8
$MgCl_2$ [1]	100 mM	10 mM	1.8
$MnCl_2$ [1]	100 mM	10 mM	1.8
PPase [1]	2.8 U/µL	50.4 U	0.018
*Bf*FKP	-	0.22 mg/mL [2]	-
TRIS/HCl, pH 7.6 [1]	50 mM	-	up to 18
Total	-	-	18

[1] Chemicals are stated in Table 6.1. [2] Or use about 1 U to 1.5 U.

Procedure for the synthesis of the nucleotide sugar:

Day 1:

1. Mix all components (except the enzyme and the PPase) in a small beaker with a lid, add a stirrer bar and bring it to room temperature. Adjust the pH to 7.6 either with HCl (10%) to lower the pH or with NaOH (5 M) to increase the pH. After reaching the pH add the PPase.
2. Prepare a 1.5 mL reaction tube with 25 µL ice cold 50% ACN (diluted with ultrapure water).
3. Now the reaction can be started by addition of the enzyme. Just right after mixing take a sample of 25 µL and mix it with the prepared ACN, so that the reaction is stopped. Vortex and store the sample at -20°C.
4. Heat up the reaction solution to 37°C and let it react for the next 21 hours under slight stirring (with magnetic stirrer).
5. After the reaction time of 21 hours, take a sample for HPLC analysis. The reaction solution can get milky and can flocculate therefore centrifuge the solution in a reaction tube about 10 minutes and then transfer into an ultrafiltration device to get rid of the enzyme. Be aware that the flow through is used for further work up, as the nucleotide sugar is in there.
6. The maximal yield could be 10 mM GDP-β-L-fucose at 100% conversion in 18 mL, which corresponds to 114 mg (sodium salt).
7. Continue with the next section: Purification of GDP-β-L-fucose
   NOTE: Hold the GDP-β-L-fucose containing solution on ice all the time after the reaction has been stopped!
8. Here is a time point for a break. The GDP-β-L-fucose containing solution can be stored at -20°C.

7 Purification of GDP-β-L-fucose (2 – 3 days)

This section describes the purification of GDP-β-L-fucose via ÄKTAprime plus and ÄKTA FPLC (adapt the handling if using another liquid chromatography system). After the synthesis of the nucleotide sugar the other components in the reaction mixture have to be removed.

The procedure for purification is stated below. The different steps are:

- CIP digest – about 14 hours over night
- AEC, rotary evaporator, SEC – about 12 hours
  - To master this step in the best possible way, it makes sense to work together, since the sections are complex and overlap in time.
- Lyophilisation: overnight

All chemicals, materials and devices used for the purification of GDP-β-L-fucose are stated in Table 7.1.

Table 7.1: Chemicals, materials and devices used for the purification of GDP-β-L-fucose.

Chemicals	Materials	Devices
Acetic acid, 99-100%,	AEC column (1 mL)	Liquid chromatography system
Acetonitrile (ACN)	Loop, 5 mL	Lyophilizer
ADP (Adenosine 5′-diphosphate)	Needle, long, 0.80 x 120mm	pH Meter
Alkaline phosphatase, calf intestinal (CIP)	Needle, short, 0.90 x 40mm	Rotary evaporator + motor unit
AMP (Adenosine 5′-monophosphate)	Reaction tube: 15 mL	HPLC with UV detector
ATP (Adenosine-5'-triphosphate di-sodiumsalt)	Round-bottom flask	Vacuum pump
Ethanol, 96%	SEC column	---
GDP (Guanosine-5'-diphosphate)	Syringe: á 2 mL, á 10 mL	---
GDP-β-L-fucose	Toyopearl Super Q-650M	---
GMP (Guanosine-5'-monophosphate)	Reaction tubes: 1.5 mL	---
GTP (Guanosine-5'-triphosphate di-sodiumsalt)	Cellulose acetate filter, pore size of 0.45 µm	---
Liquid nitrogen	Ultrafiltration device, 15 mL, cut-off: 10 kDa	---
Sodium hydroxide (NaOH)	---	---
Tetra-n-butylammonium bromide (TBAB)	---	---

Solutions:

- deionized water: filtrate with a cellulose acetate filter with a pore size of 0.45 µm, 1 L and 2 L are needed
- Binding buffer: 20 mM sodium acetate (NaOAc), pH 4.3
  - Dilute the 1 M elution buffer (see below) before pH adjustment to 20 mM and adjust the pH to 4.3 with NaOH
- Elution buffer: 1 M NaOAc, pH 4.3
  - Molar mass of acetic acid: 60.05 g/mol, density: 1.05 g/cm^3,
  - The following formula can be used to take the density into account: $c = \frac{\rho}{M}$ where c is the concentration of the solution, ρ is the density and M is the molar mass
  - Dissolve 57.14 mL acetic acid in 942.86 mL deionized water and adjust the pH to 4.3 with NaOH (about 30 platelets are needed)
  - Filtrate with a cellulose acetate filter with a pore size of 0.45 µm
- Ethanol, 20%
  - Dilute the ethanol (96%) with deionized water
  - Filtrate with a cellulose acetate filter with a pore size of 0.45 µm

- TBAB buffer, 1 L (40 mM TBAB, 20 mM potassium phosphate, pH 5.9)
  - Molar mass of TBAB: 322.4 g/mol, dissolve 12.90 g in 0.8 L deionized water,
  - Molar mass of K_2HPO_4: 174.18 g/mol, molar mass of KH_2PO_4: 136.09 g/mol, prepare stock solutions with deionized water of 100 mM each and mix the basic (K_2H) and acidic (KH_2) compounds together (roughly in a ratio of 1 to 11) to gain a pH of 5.9
  - Add 0.2 L of the potassium phosphate solution to the TBAB solution
  - Filtrate with a cellulose acetate filter with a pore size of 0.45 µm
  - Prepare 2 L of TBAB buffer.
- Liquid nitrogen
  - Handle with care! Always use protective goggles!

Preliminaries:

- Packing of column for AEC:
  - Pack two AEC columns with Toyopearl Super Q-650M from Tosoh Bioscience. Therefore take the 1 mL columns and open them on one end. Rinse the columns with deionized water and clean them from the resin.
  - Pipette the new resin (which needs to be shaken well before pipetting) into the entire columns. Take a 2 mL syringe and attach it with an adapter to the closed end. Slightly pull the syringe to remove a little bit of water and to make the resin more compact. Fill the space then again with resin and put the lids back on the columns and close them. Make sure that there are no air bubbles.
  - The columns are now ready to use. The side where the column was open is the end. State the flow direction directly on the column with an arrow.
  - One AEC column has a binding capacity of about 30 mg of phosphate (all phosphate due are binding). As all phosphate groups are binding the initial concentration of ATP and GTP counts as calculation basis.

Table 7.2: Calculation of phosphate groups in the reaction mixture

Compound	Concentration [mM]	Volume [mL]	Molar mass [g/mol]	Phosphate groups [mg]
ATP	10	18	551.14	99.21
GTP	15	18	520.15	140.44
Total	-	-	-	239.65

As a consequence of this result: two AEC columns must be loaded four times to exploit the binding capacity and to utilize the whole GDP-β-L-fucose containing solution.

- Preparation of a standard calibration curve for HPLC (for quantification and qualification) measured with the method described below:
  - GMP (363.22 g/mol), GDP-β-L-fucose (633.31 g/mol), GDP (443.20 g/mol), GTP (621.18 g/mol) each 1 mM end concentration in one sample (for qualification), prepare about 60 µL for multiple measurements
  - AMP (347.22 g/mol), ADP (427.20 g/mol), ATP (551.10 g/mol) each 1 mM end concentration in one sample (for qualification), prepare about 60 µL
  - GDP-β-L-fucose: 1 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM and 12 mM, prepare about 50 µL of each concentration; measure in duplicates, evaluate the results with spreadsheet software and create a standard calibration curve for quantification
  - The standards for qualification should be measured every time samples are measured. The order of the elution of the compounds will never change (Adenosine, Guanosine, AMP, GMP, GDP-β-L-fucose, GDP, ATP and GTP) but the retention times could vary a little bit depending on the buffer and the age of the column for example.

HPLC method:

- Stationary phase: C 18 kinetex column – reversed phase
- Mobile phase: 93.5% TBAB buffer (described above) and 6.5% acetonitrile
- Wavelength: 262 nm, flow rate: 2 mL/min, time: 10 to 12 minutes (adapt after measurement of standards), injection volume: 1 µL (if not otherwise stated), oven temperature: 40°C
- HPLC vial with insert: 40 µL minimal volume; for sampling: take 50 µL, centrifuge it for 5 minutes and then transfer 40 µL to the HPLC vials to prevent solids from reaching the column

Procedure for the purification of GDP-β-L-fucose:

Day 1 - evening:

1. Take the GDP-β-L-fucose containing solution and adjust the pH to 7 with 0.1 M HCl, wear gloves and protective goggles whenever adjusting the pH. Take a sample (25 µL sample diluted with 25 µL 50% ACN, centrifuge and transfer 40 µL into the HPLC vial) of the GDP-β-L-fucose containing solution and measure it on the HPLC. This is the starting point of the digest with the alkaline phosphatase (Calf Intestinal, CIP).
2. Add 10 U/mL of the alkaline phosphatase and let the solution incubate at 30°C overnight under slightly stirring.

   NOTE: If one is adjusting the concentration of the substrates to another scale also the addition of the amount of alkaline phosphatase must be adjusted!

Preliminaries for Day 2 done on Day 1:

1. Turn on the ÄKTAprime plus system (cooled system) and conduct a system wash with filtered deionized water.
2. Then install the AEC columns in the system by considering the flow direction (flow rate 0.2 mL/min). Also install a 5 mL loop.
3. The columns and the loop should first be washed with 2 to 3 CVs filtered deionized water and then with 2 CVs elution buffer to be sure that there are no impurities on the columns (flow rate: 1 mL/min – pressure: 0.5 MPa, which is the maximal pressure limit for the columns). Also check if the pressure value is in range and if the columns are dense.

   NOTE: The easiest way to wash the loop is to fill a 10 mL syringe with the respective solution and load it with the content. 2 to 3 loop volumes are recommended.
4. Then wash the columns and the loop with 2 to 3 CVs binding buffer and conduct a system wash with deionized water and wash the loop and the column with 2 to 3 volumes deionized water (the ÄKTA system should not be on buffer overnight). Turn off the system and the computer and leave the columns and the loop installed.
5. Turn on the ÄKTA FPLC system and let it start up. Also turn on the computer and open the program “Unicorn” (details for the program see above). Conduct a system wash with filtered deionized water. Water will be the only mobile phase for the separation of the nucleotide sugar and the salt. Install the SEC column and a 2 mL loop at the ÄKTA FPLC. The SEC column should be washed with about 5 column volumes filtered deionized water (~300 mL). Let it run overnight with 0.4 mL/min. Set the wavelength to 254 nm. This can be done directly at the ÄKTA FPLC on the right side at the UV detector. To confirm it follow the instructions under “For base line”.

   NOTE: Take 2 L of filtrated deionized water to make sure, that it is enough of the mobile phase for the whole purification process.

Day 2:

1. Take a sample in the morning and measure it on the HPLC to have the endpoint of the digest (25 µL sample stopped with 25 µL acetonitrile, centrifuge and transfer 40 µL into a HPLC vial).
2. There are no nucleotide tri~, di~ and (a very low amount of) monophosphates present in the solution. Centrifuge the solution (~10 minutes) as it gets milky and flocculate and remove the enzyme through ultracentrifugation with a centrifugal concentrator column (Vivaspin, 15 mL). Centrifuge for 10 minutes and then fill in the rest of the solution and centrifuge another 10 minutes. The sugar is collected in the lower part of the column.

   NOTE: Not all monophosphates can be degraded into phosphate and the appropriate nucleoside. At some time point the concentration of phosphate is too high and inhibits the activity of the alkaline phosphatase totally.

3. Fill up the volume to 20 mL with TRIS/HCl buffer and store the GDP-β-L-fucose containing solution at 4°C.
4. In the meantime turn on the ÄKTAprime plus system and conduct a system wash with having tube A in binding buffer and tube B in elution buffer. Fill the device with about 40 reaction tubes á 15 mL.
5. Start a manual run by setting the flow rate with buffer A to 1 mL/min and the pressure to 0.5 MPa. Set the wavelength of the system to 254 nm (on the right side at the UV detector) and do an “Autozero”. Fill the loop with binding buffer and by getting constant signals for the UV absorption the system is ready to use.
6. Check if the valve position is at the load position and load the loop with a syringe a little bit more than 5 mL of the GDP-β-L-fucose containing solution (also take a sample for HPLC analysis: 25 µL sample + 25 µL ultrapure water, centrifuge and transfer 40 µL to a HPLC vial). Try to avoid air bubbles in the syringe and therefore in the loading loop (a little bit of the solution should remain in the syringe to avoid problems with air reaching the column and the system). Change the valve position to “Inject”. The content of the loop will now be loaded on the column. Set the “Fraction Size” to 10 mL as the UV signal starts to increase so that the flow through can be collected. After about 7 mL (= 7 minutes) set the valve to “Load” again (as the whole fill of the loop is on the column now). The flow through contains all fractions which cannot bind to the column (guanosine and adenosine, seen as a big peak). Pool the fractions and take a sample to analyze it on the HPLC to check if there is any unbound GDP-β-L-fucose in it (40 µL undiluted sample without centrifugation). If this is not the case it can be thrown away. If there is any GDP-β-L-fucose in it store it on ice for another loading and elution cycle.
7. As the UV signal is stable again (after 3 to 5 column volumes, during the washing the flow can be set to 1.5 mL/min) the elution process can be started. Therefore set a gradient from 0% to 30% buffer B (elution buffer) in 40 mL and feed tube. The monophosphates AMP and GMP which are often remaining in the solution are eluted first. So the first peak should contain mostly these two compounds. Take a sample of each fraction for analysis on the HPLC to determine the content (40 µL without dilution without centrifugation; inject 2 µL at the HPLC).
8. Feed tube as the UV signal is increasing for the second peak and press the button “hold” (the concentration of buffer B is not increasing anymore to avoid unnecessary higher salt concentration). This occurring peak is now the GDP-β-L-fucose. Take a sample of the first fraction for HPLC analysis (40 µL without dilution without centrifugation; inject 2 µL at the HPLC).
9. Pool all relevant fractions from the peak in an appropriate round-bottom flask, take a sample for HPLC analysis (40 µL without centrifugation) and store it on ice.
   NOTE: The flask should only be filled to its half volume.

10. Continue (button “hold/cont.”) and let the gradient finish. As the UV signal is stable again (during the washing the flow can be set to 1.5 mL/min) set the concentration of B to 100% for 2 to 3 column volumes, then decrease the concentration of B to 0% again and wash with 2 to 3 CVs.
11. Start the next cycle of loading and elution (until no GDP-β-L-fucose containing solution is left, about 4 times).

    NOTE: The eluted fractions containing GDP-β-L-fucose of each cycle should be held on ice and should be further processed as fast as possible, as the high salt concentration after AEC could be harmful for the GDP-β-L-fucose.
12. Let the rotary evaporator cool for at least 10 minutes with water. The temperature of the water bath should be set to 40°C. As the round-bottom flask hangs on the rotary evaporator the speed of rotation can be set to 200 and the pressure can be applied (valve for air supply must be closed). The starting point can be around 120 mbar and the endpoint could be around 30 mbar. Go down in steps of 10 mbar (leave it about 20 to 30 seconds at each step) and take care at the range between 75 and 55 mbar, to avoid boiling delay (steps of 5 mbar recommended). The fluid should be reduced to approximately 2 mL. Transfer the solution into a 2 mL reaction tube and store it on ice.

    NOTE: Each volume reduced sample should be hold on ice and also should be further processed as fast as possible as the high salt concentration could harm the GDP-β-L-fucose.
13. The ÄKTA FPLC system with the already installed and washed SEC column (inclusive 2 mL loop) should be set to a wavelength of 254 nm (check if this is the case). Confirm the wavelength by “Autozero” again. The signal should be stable and then the loading of the column can start. Fill the devices for the tubes with ice and with about 20 tubes á 15 mL.
14. Set the flow rate to 2 mL/min and check if the valve position is at “Load”. Then load maximal 2 mL of GDP-β-L-fucose containing solution (for easier handling put a short needle on the 2 mL syringe to get the solution from the 2 mL reaction tube into the syringe). The maximal amount of GDP-β-L-fucose in this volume should not exceed 25 mg (can be calculated via analysis of HPLC results) as this is the loading maximum of the column.

    NOTE: There is a relatively high salt concentration in the solution. Therefore it could be that the salt is precipitating. If this is the case just dilute the solution with some deionized water until the salt is dissolved again.
15. As the volume of the column is about 60 mL and the flow rate is 2 mL/min the signal should increase after about 30 minutes. Set the fraction size to 10 mL as the UV signal increases. The chromatogram shows the absorption signal and the low conductivity for the nucleotide sugar and then high conductivity of the salt. Therefore one can distinguish

between sugar and salt. As the conductivity for the salt is increasing switch to a next tube (“Feed Tube”). From the first peak on collect all fractions but pool only the fractions with nearly no salt in it (should be cooled every time!). There is the possibility to pool and reload the fractions where sugar and salt are overlapping to minimize the loss of GDP-β-L-fucose. At the time point where the first UV signal is starting to decrease one can set the valve to “Load” position and load the next 2 mL. Repeat the procedure until there are no samples left.

NOTE: Always keep the samples with GDP-β-L-fucose in it on ice!

16. Weigh an empty 500 mL round-bottom flask on the micro scale and state the weight directly on the flask. Also mark the volume for 10 mL (as done with the smaller round-bottom flask). Pool all relevant fractions (take a sample: 40 µL without dilution) and start to minimize the volume on the rotary evaporator. In the end a volume of about 10 mL should be left in the flask. Take a sample for HPLC analysis (20 µL sample + 20 µL ultrapure water without centrifugation).
17. Prepare the lyophilizer for his tasks.

    NOTE: Instructions are needed to operate the system.
18. The remaining solution of volume reduction is now ready to be shock frozen with liquid nitrogen. Take a sample for HPLC analysis (20 µL sample + 20 µL ultrapure water without centrifugation). Then put the round bottom flask back on the rotary evaporator. Remove the water bath (carefully) and put a polystyrene box with liquid nitrogen at the place. Let the round bottom flask rotate slowly and immerse it into the liquid nitrogen until the whole solution is frozen.

    NOTE: Wear protective goggles and be very careful when handling liquid nitrogen.
19. The flask with the thin frozen layer of GDP-β-L-fucose and water should now be stuck on the lyophilizer and the vacuum can be applied. Let it last overnight.

    NOTE: Make sure that the vacuum is really applied and that the round-bottom flask is fixed.

<u>Day 3:</u>

1. In the round bottom flask should now be some white powder. Normally the drying process should be finished overnight. One can check the temperature of the flask. If it is cold, give the drying process some more time. If it is at room temperature the process is finished. Weigh the flask with the powder inside and determine the resulting amount of powder.
2. Remove the powder from the flask with a spatula into a weighing boat and then aliquot the powder in 1.5 mL reaction tubes with about 5 mg each and store it at -80°C.
3. The round-bottom flask can then be rinsed with 500 µL TRIS/HCl buffer so that the remaining powder is not wasted. Transfer it into a 1.5 mL reaction tube and take a

sample for analysis on the HPLC (20 µL sample + 20 µL ultrapure water without centrifugation). Store it also at -80°C.

4. Evaluate all analyzed samples and calculate the yield of GDP-β-L-fucose of each step. The expected isolated yield is about 57 mg.

8 Troubleshooting

Table 8.1: Problems and their solutions or explanation.

Problem	What to do
The CIP digest does not work properly.	Control the pH – is it at 7? If not, adjust.
	If there are still monophosphates in it, stop the digest – they can be separated via AEC. At some point, the CIP is inhibited by the amount of phosphate.
The GDP-β-L-fucose containing solution is milky after the CIP digest.	The nucleosides adenosine and guanosine are slightly soluble and cause the milky solution.
GDP-β-L-fucose is in the flow through.	No problem. Store it on ice and load the samples again in the next run.
The peaks at the AEC are not separated.	Prolong the gradient for example for 10 minutes.
There is some GDP-β-L-fucose in the first peak with AMP and GMP.	Discard the fractions. The purity of the product is more important than the yield.
Double peaks are occurring where the fractions contain the same compound.	This doesn´t matter. The double peaks are occurring because of the adapter between the columns.

9 Appendix

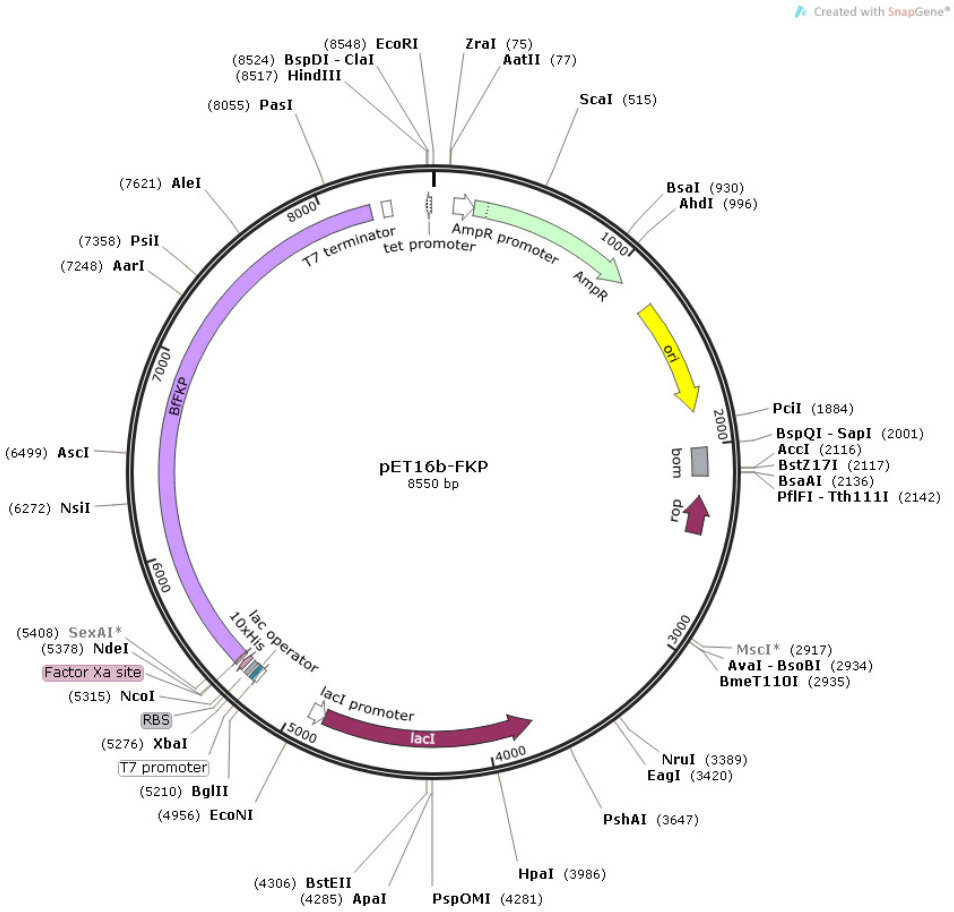

Figure 9.1: Plasmid map of pET16b_*Bf*FKP

Part III

Synthesis of

UDP-β-L-rhamnose

10 Introduction

Nucleotide sugars are monosaccharides which are linked to a nitrogenous base and to one to three phosphate groups. The numerous majority of nucleotide sugars are nucleoside diphosphate sugars (NDP-sugars) (Bar-Peled and Neill, 2011). This part of the thesis deals with the nucleotide sugar UDP-β-L-rhamnose.

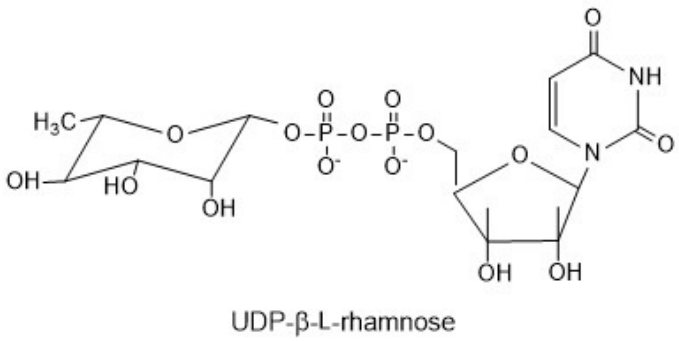

Figure 10.1: Structure of UDP-β-L-rhamnose.

UDP-β-L-rhamnose is used in plants and bacteria for the synthesis of cell wall polysaccharides (Ridley et al., 2001; De Bruyn et al., 2015). In vascular plants these polysaccharides are also very important for the development and growth (Ridley et al., 2001), whereas in bacteria they are necessary for survival and the interaction between bacteria (Dong et al., 2003). This nucleotide sugar is also a donor for the synthesis of flavonoid O-rhamnosides, which are precursor for alternative antibiotics for viruses, bacteria and fungi (De Bruyn et al., 2015) and have also effects to help fight against cancer (Kim et al., 2012).

The biosynthetic pathway to synthesize the NDP-rhamnose is different in bacteria and plants. In bacteria the rhamnose is linked with thymidine instead of uracile in plants. The production of dTDP-β-L-rhamnose is done by three enzymes (RmlB, RmlC and RmlD) and three conversion steps starting from dTDP-α-D-glucose (Dong et al., 2003). In plants these three steps are done by (I) UDP-glucose 4,6-dehydrogenase (comparable to RmlB), (II) UCP-4-keot-6-deoxy-D-glucose 3,5 epimerase (comparable to RmlC) and (III) UDP-4-keto-L-rhamose 4-keto reductase (comparable to RmlD) (Kamsteeg, Brederode, & Nigtevecht, 1978).

In Figure 10.2 the synthesis of UDP-β-L-rhamnose in plants is shown.

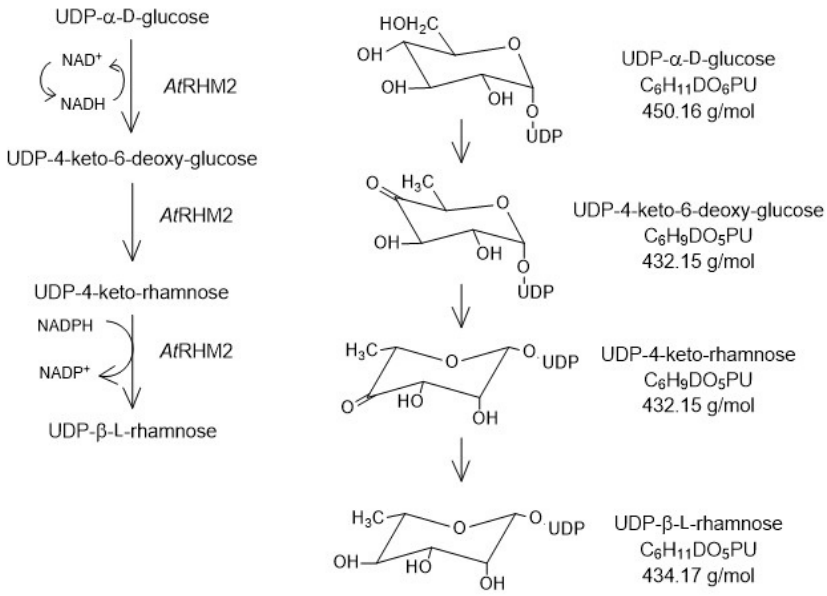

Figure 10.2: Conversion of UDP-α-D-glucose to UDP-β-L-rhamnose in plants showing the structure of each compound.

In the plant *Arabidopsis thaliana* only one enzyme is taking over the role to convert UDP-α-D-glucose to UDP-β-L-rhamnose (Oka, Nemoto, & Jigami, 2007) , as shown in Figure 10.3.

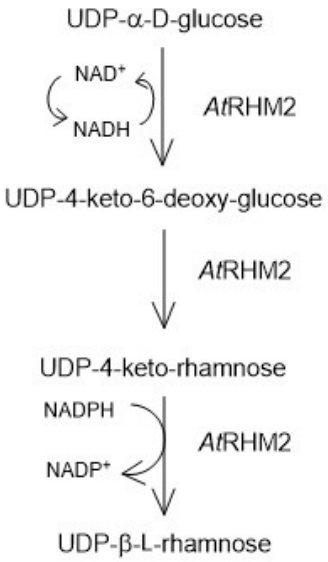

Figure 10.3: Biosynthetic pathway for the synthesis of UDP-β-L-rhamnose. Adapted from Oka et al. (2007).

The enzyme *At*RHM2 (UDP-rhamnose synthase 2) can be subdivided into two parts: the N-terminal part and the C-terminal part, this is illustrated in Figure 10.4.

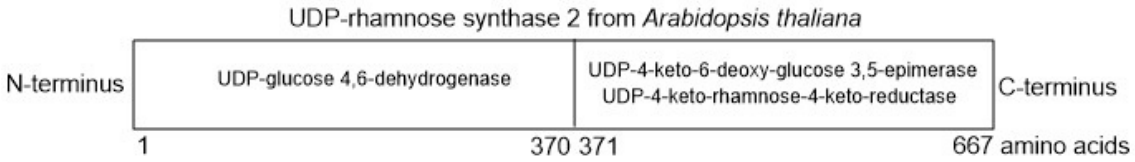

Figure 10.4: Partitioning of *At*RHM2. The N-terminal domain takes over the part of UDP-glucose 4,6-dehydrogenase and the C-terminal domain takes over the parts of the UDP-4-keto-6-deoxy-glucose 3,5-epimerase and UDP-4-keto-rhamnose-4-keto-reductase.

The N-terminal part from amino acid 1 to 370 takes over the first reaction from UDP-α-D-glucose to UDP-4-keto-6-deoxy-glucose. The C-terminal domain from amino acid 371 to 667 is taking the next two steps to produce UDP-β-L-rhamnose via the intermediate UDP-4-keto-rhamnose.

As can be seen in Figure 10.3 there is a binding of the co-factors (NAD(P)(H)). Oka et al. (2007) suggested that these bindings are important for the activity and for the stability of *At*RHM2. They compared the sequences of the two domains of the protein and ascertained that both parts have a Rossmann fold for binding NAD(P)(H) (Rossmann & Argos, 1978) and two sets of conserved YXXXK motifs (which are catalytic sites in short chain dehydrogenase/reductase family proteins, Duax, Ghosh, & Pletnev, 2000).

The aim of this part of the master thesis was the synthesis of UDP-β-L-rhamnose. Cloning experiments should be done to find a good construct for overexpression and purification of *At*RHM2. Cultivation should take place in *E. coli* BL21 (DE3). The synthesized nucleotide sugar was planned to be analyzed by capillary electrophoresis.

11 Materials and Methods

Following materials and methods were used during the master thesis, when not described otherwise.

Materials and devices which were used for the general lab work are listed in Table 11.1.

Table 11.1: Materials and devices which were used for the general lab work.

Materials	Company	Comments
Reaction tubes: 1.5 mL, 2 mL	Eppendorf AG, Hamburg, Germany	
Reaction tubes: 15 mL, 50 mL	Sarstedt AG & Co, Nümbrecht, Germany	
Syringe: 5 mL, 10 mL	B.Braun Melsungen AG, Melsungen, Germany	
Devices	**Company**	**Comments**
Bioair Auro 2000 Lamina Flow	EuroClone S.p.A., Milan, Italy	Sterile workbench
DeNovix DS-11	DeNovix Inc., Wilmington, DE, USA	Spectrophotometer
Eppendorf Centrifuge	Eppendorf AG, Hamburg, Germany	Centrifuge for 1.5 mL and 2 mL reaction tubes
Eppendorf Centrifuge 5415 R	Eppendorf AG, Hamburg, Germany	Centrifuge for 15 mL and 50 mL reaction tubes
Eppendorf Thermomixer comfort	Eppendorf AG, Hamburg, Germany	Heater and shaker for 1.5 mL and 2 mL reaction tubes
Finnpipette 0.5 - 10 µL	Thermo Fisher Scientific, Waltham, MA, USA	Adjustable volume pipette
Heidolph Relax Vortex	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany	Vortexer
Metrohm 691	Metrohm Inula GmbH, Vienna, Austria	pH meter
MR Hei-Standard	Heidolph Instruments GmbH & Co.KG, Schwabach, Germany	Magnetic stirrer
peqpette 2 - 20 µL, 10 - 100 µL, 20 - 200 µL, 100 - 1000 µL, 1 - 5 mL	Peqlab Biotechnologie GmbH, Erlangen, Germany	Adjustable volume pipettes
Varioklav Dampfsterilisator	HP Medizintechnik GmbH, Oberschleißheim, Germany	Sterilizer

11.1 Cloning

The original construct of pET43.1b(+)-*At*RHM2 had a His6-tag between the N-terminal Nus-tag and the sequence of the enzyme. This purification tag was hardly accessible and therefore the construct was modified to gain an accessible tag for the purification of the enzyme.

Enzymes, chemicals, materials and devices used for cloning are given in Table 11.2.

Table 11.2: Enzymes, chemicals, materials and devices which were used for cloning.

Enzymes	Company	Comments
*Dpn*I	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
FastAP	Thermo Fisher Scientific, Waltham, MA, USA	Thermosensitive Alkaline Phosphatase
FastDigest *Nde*I	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
FastDigest *Xho*I	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
*Nde*I	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Phusion High-Fidelity Polymerase	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Q5® High-Fidelity DNA Polymerase	New England Biolabs, Ipswich, MA, USA	Stored at -20°C
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
*Xho*I	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Chemicals	**Company**	**Comments**
Acetic acid, 99-100%,	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Flammable, corrosive
Agarose	Peqlab Biotechnologie GmbH, Erlangen, Germany	
Buffer O, 10x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
dNTP´s mix, 10 mM	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Ethylendiamintetraacetat (EDTA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
FastDigest Green buffer, 10x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
GeneRuler™ DNA ladder mix	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
HD Green Plus DNA Stain	INTAS Science Imaging Instruments GmbH, Göttingen, Germany	Stored at -20°C
Loading dye, 6x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Phusion HF buffer, 5x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Q5® High GC enhancer, 5x	New England Biolabs, Ipswich, MA, USA	Stored at -20°C
Q5® Reaction Buffer, 5x	New England Biolabs, Ipswich, MA, USA	Stored at -20°C

T4 DNA Ligase Buffer, 10x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
Tango Buffer, 10x	Thermo Fisher Scientific, Waltham, MA, USA	Stored at -20°C
TRIS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Materials	Company	Comments
Electroporation cuvettes	Cell Projects Ltd, Kent, United Kingdom	
GeneJET Gel Extraction Kit	Thermo Fisher Scientific, Waltham, MA, USA	
GeneJET PCR Purification Kit	Thermo Fisher Scientific, Waltham, MA, USA	
MF-Millipore™ DNA Filter Paper	Merck KGaA, Darmstadt, Germany	
Wizard® Plus SV Minipreps DNA Purification System	Promega, Fitchburg, WI, USA	
Wizard® SV Gel and PCR Clean-Up System	Promega, Fitchburg, WI, USA	
Devices	Company	Comments
Electrophoresis power supply, model 200	Biorad, Hercules, CA, USA	
Electrophoresis chamber	Biorad, Hercules, CA, USA	
Gel Doc 2000	Biorad, Hercules, CA, USA	with the attendant analysis software Quantity 1-D
MicroPulser™ Electroporator	Biorad, Hercules, CA, USA	
Microwave	-	
MyCycler	Biorad, Hercules, CA, USA	Thermal cycler
Thermal Cycler 2720	Applied Biosystems, Waltham, MA, USA	

The original construct of pET43.1b(+)-*At*RHM2 was modified via classical cloning methods and the CPEC method. Overall the cloning of 4 different constructs was attempted.
The classical method included the following steps: (I) plasmid isolation of the vector and the insert to be modified, (II) PCR with the insert as template – for introduction of modifications, (III) plasmid isolation after PCR, (IV) restriction with the respective restriction enzymes, (V) dephosphorylation of the vector, (VI) preparative agarose gel plus isolation of DNA out of the gel, (VII) ligation of vector and insert and afterwards desalting of ligation, (VIII) transformation in electro competent cells, for example *E. coli* TOP10 F', (IX) identification of positive clones via restriction and agarose control gel, (X) sequencing of putative positive clones and (XI) preparation of glycerol stocks for storage at -80°C (if the right construct was gained).

Ad (II): The modification of the insert was mostly done via PCR. The basic components were: buffer, dNTP´s, one forward primer, one reverse primer, template DNA, DNA polymerase

and ultrapure water. The standard conditions for the run in a thermal cycler are given below (Table 11.3).

Table 11.3: Standard protocol for the conditions of a PCR run in a thermal cycler.

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	5-10 seconds	25-35
Annealing	45-72	10-30 seconds	
Extension	72	15-30 seconds per kbp	
Final extension	72	5-10 minutes	1
Hold	4-10	∞	

Ad (IV and VI): The successful restriction was controlled via an agarose gel. This 1% agarose gel was prepared according to the following recipe: 0.6 g agarose, 60 mL 1-fold TAE buffer and 3 µL dye. A *BioRad Model 200 electrophoresis power supply* was used and for imaging the gel the *Gel Doc 2000* with the attendant analysis software *Quantity 1-D* was needed.

Ad (IV and IX): Vector and insert were digested with the same restriction enzymes.

Ad (VIII): A standard batch of ligation of vector and insert is illustrated in Table 11.4.

Table 11.4: Standard batch for the ligation of vector and insert.
The components are mixed and incubated at 4°C or 16°C overnight.

Components	Volume [µL]
Vector	x (100 ng)
Insert	y (3-fold excess on molar level)
Ligase buffer, 10-fold	2
Ligase	1 (1 U)
Ultrapure water	up to total
Total	20

11.1.1 Cloning of Strategy 1

The cloning experiments of pET43.1b(+)-*At*RHM2 started with the removal of the N-terminal Nus-tag and the removal of a C-terminal stop codon for the addition of a C-terminal His6-tag (see the original and the modified construct in the appendix). Therefore the classical cloning steps as listed in section 11.1 were used if not otherwise stated.
The following primers were used for the modification of *AtR*HM2: (I) forward primer, NdeIAtRHM2fw, 5´-3´: GTTGTTCATATGGATGATACTACGTATAAGCCAAAG and (II) reverse primer, XhoIAtRHM2rv, 5´-3´: GTTGTTCTCGAGGGTTCTCTTGTTTGGTTCAAAG. Primer design was done on the homepage of IDT (Integrated DNA Technologies, Leuven,

Belgium) where the primers were also purchased. The sequence map was opened with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA).

The composition for a successful PCR reaction for the modified insert (*At*RHM2 without the sequences of the N-terminal Nus-tag and the C-terminal stop codon (TAA) for expression of a C-terminal His6-tag) is stated below in Table 11.5.

Table 11.5: Composition for a successful PCR for the modification of the insert for strategy S1 (*At*RHM2 without the N-terminal Nus-tag and with a removed C-terminal stop codon for expression of a C-terminal His6-tag).

Components	Concentration	Final concentration	Volume [µL]
Phusion High-Fidelity Polymerase [1]	2 U/µL	0.02 U/µL	0.5
Phusion HF buffer [1]	5-fold	1-fold	10.0
Forward primer (NdeIAtRHM2fw) [2]	10 µM	0.5 µM	2.5
Reverse primer (XhoIAtRHM2rv) [2]	10 µM	0.5 µM	2.5
Template (pET43.1b(+)-*At*RHM2_original)	8.7 ng/µL	0.17 ng/µL	1.0
dNTP´s [1]	10 mM	0.2 mM	1.0
Ultrapure water	-	-	32.5
Total	-	-	50.0

[1] Chemicals are stated in Table 11.2. [2] Sequence of the primers are stated in section 11.1.1.

The corresponding PCR program with the sample composition of Table 11.5 is given in the next table (Table 11.6).

Table 11.6: PCR program with the sample composition of Table 11.5 for the modification of the insert for strategy S1 (*At*RHM2 without the N-terminal Nus-tag and with a removed C-terminal stop codon for expression of a C-terminal His6-tag).

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	30
Annealing	65	30 seconds	
Extension	72	30 seconds	
Final extension	72	7 minutes	1
Hold	4	∞	

The vector pET43.1b(+) and the PCR product (insert) were digested under standard conditions and ligation was done with the restriction enzymes *Nde*I and *Xho*I. Therefore the following composition of compounds was chosen as summarized in Table 11.7.

Table 11.7: Composition for ligation of pET43.1b(+) and *At*RHM2_S1 both digested with *Nde*I and *Xho*I. The components were mixed and incubated at 16°C for about 17 hours.

Components	Volume [µL]
pET43.1b(+)	10 (30 ng)
*At*RHM2_S1	3 (3-fold excess on molar level)
T4 DNA Ligase Buffer, 10-fold	3
T4 DNA Ligase	1 (1 U)
Ultrapure water	13
Total	30

Transformation into electro competent *E. coli* TOP10 F´ cells took place and the resulting putative positive clones were controlled via restriction with *Nde*I and *Xho*I. To check successful cloning, plasmid DNA was isolated from a single colony and was sent to Microsynth for sequencing (see section 11.2 Sequencing).
This construct was named pET43.1b(+)-*At*RHM2_S1.
A glycerol stock was made and the construct was also re-transformed into *E. coli* BL21 (DE3) for cultivation.

11.1.2 Cloning of Strategy 2

The second strategy was to change the vector backbone. The classical cloning steps as listed in section 11.1 were used if not otherwise stated. The DNA sequence of the enzyme with the Nus-tag should be cloned into the pET-STRP3 vector (see appendix for plasmid map), to purify the enzyme via a Strep-tag. The pET-STRP3 backbone was gained from pET-STRP3_OsCGT by restriction with *Nde*I and *Xho*I. The *At*RHM2 coding insert was obtained via restriction of the original construct pET43.1b(+)-*At*RHM2 with *Nde*I and *Xho*I. Classical ligation was done with the following different conditions listed in Table 11.8.

Table 11.8: Different conditions for ligation of pET-STRP3 with *At*RHM2_original.
The conditions were differing in temperature, incubation time and the excess of insert on molar level. The rest of the ligation mixture stayed the same as stated in Table 11.4.

Conditions	Temperature [°C]	Time [h]	Vector [ng]	Excess of insert on molar level
1	16	15	104	3-fold
2	22	0.5	98	3-fold
3	22	0.5	98	4-fold
4	22	1	111	3-fold
5	4	8	100	3-fold
6	4	15	100	3-fold

As this classical method was not successful the CPEC (Circular Polymerase Extension Cloning; Quan & Tian, 2009) method or a variation thereof was done.

To add an overlap to the insert which is complementary to the vector the following primers were used: (I): forward primer, 5´-3´: ACCCGCAGTTCGAGAAAGGCTTAA TTAACCATATGAACAAAGAAATTTTGGCTGTAGTTGAAG and (II): reverse primer, 5´-3´:

AGTGGTGGTGGTGGTGGTGCTCGAGTTAGGTTCTCTTGTTTGGTTCAAAGACG. Primer design was done on the homepage of IDT (Integrated DNA Technologies, Leuven, Belgium) where the primers were also purchased. The sequence map was opened with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA).

Table 11.9: Composition for a PCR for the modification of the insert for strategy S2 (*At*RHM2_original with a large overlap to the pET-STRP3 vector).

Components	Concentration	Final concentration	Volume [µL]
Phusion High-Fidelity Polymerase [1]	2 U/µL	0.02 U/µL	0.5
Phusion HF buffer [1]	5-fold	1-fold	10.0
Forward primer (NdeIAtRHM2fw) [2]	10 µM	0.5 µM	2.5
Reverse primer (XhoIAtRHM2rv) [2]	10 µM	0.5 µM	2.5
Template (pET43.1b(+)-*At*RHM2_original)	7.3 ng/µL	0.2 ng/µL	1.0
dNTP´s [1]	10 mM	0.2 mM	1.0
Ultrapure water	-	-	32.5
Total	-	-	50.0

[1] Chemicals are stated in Table 11.2. [2] Sequence of the primers is stated in section 11.1.2.

The corresponding PCR program with the sample composition of Table 11.9 is described in the next table (Table 11.10).

Table 11.10: PCR program with the sample composition of Table 11.9 for the modification of the insert for strategy S2 (*At*RHM2_original with a large overlap to the pET-STRP3 vector).

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	30
Annealing	72	120 seconds	
Extension	72	30 seconds	
Final extension	72	7 minutes	1
Hold	4	∞	

The pET-STRP3 vector was linearized by digestion with *Nde*I and *Xho*I. The mixture of components for CPEC is shown in Table 11.11.

Table 11.11: Composition for CPEC of linearized pET-STRP3 vector and *At*RHM2 (+ Nus-tag) with an overlap to the vector.

Components	Concentration	Volume [µL]
Phusion High-Fidelity Polymerase [1]	2 U/µL	0.5
Phusion HF buffer [1]	5-fold	10.0
Vector (pET-STRP3)	22.7 ng/µL	4.0 (90.8 ng)
Insert (*At*RHM2 with an overlap to the vector on both sides)	63.0 ng/ µL	11.2 (8-fold excess on molar level
dNTP´s [1]	10 mM	1.0
DMSO [1]	-	2.0 (4%)
Ultrapure water	-	24.3
Total	-	50.0

[1] Enzymes and chemicals are stated in Table 11.2.

The corresponding program in the thermal cycler can be seen below in Table 11.12.

Table 11.12: Program for the thermal cycler with the sample composition of Table 11.11.

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	3
Ligation	72-70 (each cycle decrease of 1°C)	4.5 minutes	
Final ligation	72	10 minutes	1
Hold	4	∞	

A batch similar to the first one (shown in Table 11.11) with about 120 ng vector, 2-fold excess of insert in a total volume of 20 µL was done. The program for the thermal cycler is stated in Table 11.13.

Table 11.13: Program for the thermal cycler with the sample composition of sample composition as stated above in the text.

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	25
Ramp annealing	70-55	0.1°C decrement per second	
Annealing	55	30 seconds	
Extension	72	1.5 minutes	
Final extension	72	5 minutes	1
Hold	4	∞	

As there were no positive results gained another High-Fidelity polymerase in combination with a High GC enhancer was used. This enhancer was recommended for amplicons with more than 65% GC content. The composition is illustrated in Table 11.14.

Table 11.14: Composition for CPEC of linearized pET-STRP3 vector and *At*RHM2 with an overlap to the vector.

Components	Concentration	Volume [µL]
Q5® High-Fidelity DNA Polymerase [1]	2 U/µL	0.5
Q5® Reaction Buffer [1]	5-fold	4.0
Q5® High GC enhancer [1]	5-fold	4.0
Vector (pET-STRP3)	20.93 ng/µL	5.0 (104.7 ng)
Insert (*At*RHM2 with an overlap to the vector on both sides)	241 ng/ µL	4.2 (3-fold excess on molar level
dNTP´s [1]	10 mM	1.0
Ultrapure water	-	1.3
Total	-	20.0

[1] Enzymes and chemicals are stated in Table 11.2.

The corresponding temperature profile for the thermal cycler can be seen in Table 11.15.

Table 11.15: Temperature profile for the thermal cycler with the sample composition as stated Table 11.14.

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	12
Annealing	71-60 (each cycle decrease of 1°C)	30 seconds	
Extension	72	3 minutes	
Final extension	72	2 minutes	1
Hold	4	∞	

11.1.3 Cloning of Strategy 3

The third cloning strategy was to clone the original *At*RHM2 into the pC21e1 FTO 4.0 vector with a Strep-tag. The vector had already a Nus-tag and thus only the sequence of the enzyme itself was cloned into the vector. Therefore the following primers were used: (I) forward primer, SacIAtRHM2fw, 5´-3´: GTTGTTGAGCTCATGGAT GATACTACGTATAAGCC and (II) reverse primer, HindIIIAtRHM2rv, 5´-3´: GTTGTTAAGCTTTTAGGTTCTCTTGTTTGGTTCAAAGACGTATTTG. Primer design was done on the homepage of IDT (Integrated DNA Technologies, Leuven, Belgium) where the primers were also purchased. The sequence map was opened with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA).

Table 11.16: Composition of PCR for the modification of the insert for strategy S3 (*At*RHM2_original in a pC21e1 FTO 4.0 vector with a Strep-tag).

Components	Concentration	Final concentration	Volume [µL]
Q5® High-Fidelity DNA Polymerase [1]	2 U/µL	0.04 U/µL	1
Q5® Reaction Buffer [1]	5-fold	1-fold	10
Q5® High GC enhancer [1]	5-fold	1-fold	10
Forward primer (SacIAtRHM2fw) [2]	10 µM	0.5 µM	2.5
Reverse primer (HindIIIAtRHM2rv) [2]	10 µM	0.5 µM	2.5
Template (pET43.1b(+)-*At*RHM2_original)	3.6 ng	0.07 ng	1
dNTP´s [1]	10 mM	0.2 mM each	1
Ultrapure water	-	-	22
Total	-	-	50

[1] Enzymes and chemicals are stated in Table 11.2. [2] Sequence of the primers in section 0.

The corresponding PCR program to the batch of Table 11.16 is shown in Table 11.17.

Table 11.17: PCR program with the sample composition of Table 11.16 for the modification of the insert for strategy S3 (*At*RHM2_original in a pC21e1 FTO 4.0 vector with a Strep-tag).

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	30
Annealing	62	30 seconds	
Extension	72	60 seconds	
Final extension	72	7 minutes	1
Hold	4	∞	

11.1.4 Cloning of Strategy 4

The fourth idea was to use the original construct but added an additional C-terminal His6-tag. Therefore the following primers were used: (I) NusNdeIfw, forward primer, AGATATACATATGAACAAAGAAATTTTGGC, designed for and (II) XhoIAtRHM2rv, reverse primer, GTTGTTCTCGAGGGTTCTCTTGTTTGGTTCAAAG, designed for strategy 1. The composition of reagents is stated in Table 11.18. Primer design was done on the homepage of IDT (Integrated DNA Technologies, Leuven, Belgium) where the primers were also purchased. The sequence map was opened with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA).

Table 11.18: Composition for a successful PCR for the modification of the insert for strategy S4 (*At*RHM2 without the N-terminal Nus-tag and with a removed C-terminal stop codon for expression of a C-terminal His6-tag).

Components	Concentration	Final concentration	Volume [µL]
Q5® High-Fidelity DNA Polymerase [1]	2 U/µL	0.02 U/µL	0.5
Q5® Reaction Buffer [1]	5-fold	1-fold	10.0
Forward primer (NusNdeIfw) [2]	10 µM	0.5 µM	2.5
Reverse primer (AtRHM2XhoIrv) [2]	10 µM	0.5 µM	2.5
Template (pET43.1b(+)-*At*RHM2_original)	4.7 ng	0.09 ng	1.0
dNTP´s [1]	10 mM	0.2 mM	1.0
ultrapure water	-	-	32.5
Total	-	-	50

[1] Chemicals are stated in Table 11.2. [2] Sequence of the primers in section 11.1.4.

The corresponding temperature profile for the thermal cycler is listed below in Table 11.19.

Table 11.19: PCR program with the sample composition of Table 11.18 for the modification of the insert for strategy S4 (*At*RHM2 without the N-terminal Nus-tag and with a removed C-terminal stop codon for expression of a C-terminal His6-tag).

Steps	Temperature [°C]	Time	Cycle(s)
Initial denaturation	98	30 seconds	1
Denaturation	98	10 seconds	30
Annealing	62	30 seconds	
	65	30 seconds	
Extension	72	100 seconds	
Final extension	72	5 minutes	1
Hold	4	∞	

The PCR product and the vector pET43.1b(+) were both digested with *Nde*I and *Xho*I. Ligation was done as stated in Table 11.20.

Table 11.20: Setting for ligation of pET43.1b(+) and *At*RHM2_S4.
The components were mixed and incubated at 22°C for 4 hours and then 3 hours at 4°C.

Components	Volume [µL]
pET43.1b(+)	8 (192 ng)
*At*RHM2_S4	18 (630 ng, 13-fold excess)
T4 DNA Ligase Buffer, 10-fold [1]	3
T4 DNA Ligase [1]	1 (1 U)
ultrapure water	0
Total	30

[1] Enzymes and chemicals are stated in Table 11.2.

Transformation into electro competent *E. coli* TOP10 F´ cells took place and the resulting putative positive clones were controlled via restriction with *Nde*I and *Xho*I. To check

successful cloning, plasmid DNA was isolated from a single colony and was sent to Microsynth for sequencing (see section 11.2 Sequencing).
This construct was named pET43.1b(+)-*At*RHM2_S4.
A glycerol stock was made and the construct was also re-transformed into *E. coli* BL21 (DE3) for cultivation.

11.2 Sequencing

For sequencing about 80 ng/µL plasmid DNA of the construct pET43.1b(+)-*At*RHM2_S1 (in *E. coli* TOP10 F´) were sent to Microsynth (Microsynth Austria GmbH, Vienna, Austria) with the following primers: (I) forward primer, pET-up (out of the standard primer list of Microsynth), 5´-3´: ATGCGTCCGGCGTAG and (II) reverse primer (order now primer, designed), 5´-3´: GCTAGTTATTGCTCAGCGG. Primer design was done on the homepage of IDT (Integrated DNA Technologies, Leuven, Belgium). The sequence map (see appendix) was opened and created with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA) and the evaluation was done with the free software “Serial Cloner” from Serial Basics.

For sequencing the construct pET43.1b(+)-*At*RHM2_S4 (in *E. coli* TOP10 F´) following primers were used: (I) forward primer, T7 (out of the standard primer list of Microsynth), 5´-3´: TAATACGACTCACTATAGG and (II) reverse primer, T7 term (out of the standard primer list of Microsynth), 5´-3´: TGCTAGTTATTGCTCAGCGG. The sequence map (see appendix) was opened and created with SnapGene® (computer program from GSL Biotech LLC, Chicago, IL, USA) and the evaluation was done with the free software “Serial Cloner” from Serial Basics.

The construct pET43.1b(+)-*At*RHM2_S4 in *E. coli* BL21 (DE3) was sequenced with 4 primers: (I) forward primer, T7 (out of the standard primer list of Microsynth), 5´-3´: TAATACGACTCACTATAGG, (II) forward primer, NusNcoIAtRHM2, ATAAACACACCATGGACATCGCC, (III) forward primer SacIAtRHM2fw, 5´-3´: GTTGTTGAGCTCATGGATGATACTACGTATAAGCC and (IV) reverse primer, T7term (out of the standard primer list of Microsynth), 5´-3´: TGCTAGTTATTGCTCAGCGG. The evaluation was done with the free software “Serial Cloner” from Serial Basics.

11.3 Cultivation and expression

*At*RHM2 was expressed in three different constructs. Chemicals, materials and devices used for cultivation and expression are stated in Table 11.21.

Table 11.21: Chemicals, materials and devices used for expression of *At*RHM2 from pET43.1b(+) in *E. coli* BL21 (DE3).

Chemicals	Company	Comments
Agar Agar	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Ampicillin sodium salt	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at 4°C
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Tryptone (Pepton aus Casein)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Yeast extract	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Materials	**Company**	**Comments**
Centrifuge beaker: 500 mL	-	
Petri dish, 92x16 mm	Sarstedt AG & Co, Nümbrecht, Germany	
Shake flasks: 250 mL, 1 L	Schott Austria GmbH, Vienna, Austria	
Sterile filter, CA 0.45	Sarstedt AG & Co, Nümbrecht, Germany	
Devices	**Company**	**Comments**
Biochrom WPA CO8000	Biochrom Ltd., Cambridge, UK	Cell Density Meter
Infors HT RC-406	Infors AG, Bottmingen Switzerland	Shaker 30°C room
Pilot shaker	Adolf Kühner AG, Basel, Switzerland	Shaker 37°C room
Sorvall Evolution RC Centrifuge with SLC 3000 rotor	Thermo Fisher Scientific, Waltham, MA, USA	Centrifuge for 500 mL beaker

11.3.1 Cultivation of *E. coli* BL21 (DE3) and expression of *At*RHM2 (original construct)

E. coli BL21 (DE3) carrying pET43.1b(+)-*At*RHM2 was streaked onto a LB-Amp agar plate (recipe as stated below plus 15 g/L agar) and was incubated overnight at 37°C.

LB medium was prepared according to the following recipe: 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl. The components were mixed together, filled up to the appropriate amount with deionized water and the pH was adjusted to 7. The medium was filled up in shake flasks of 50, 250 and 1000 mL. The flasks were autoclaved at 121°C for 20 minutes.

One grown single colony was used for inoculation of two overnight cultures. Ampicillin was added to a final concentration of 100 µg/mL. Incubation took place at 30°C and 100 rpm overnight. The main cultures were inoculated with the ONCs to an OD_{600} of 0.05 and ampicillin was added to a final concentration of 100 µg/mL. The main cultures were incubated at 37°C and 100 rpm until the cells were grown to an OD_{600} between 0.6 and 0.8. The broth was cooled down about 20 minutes at 4°C and induction with 0.2 mM IPTG followed. The cells were further incubated at 25°C and 100 rpm overnight for a minimum of 15 hours.

At the end of the induction phase the cells were harvested by centrifugation (4000 g, 20 minutes, 4°C). The supernatant was decanted and the pellets were resuspended in His-tag binding buffer (see Table 11.25) and merged. The suspension was aliquoted in desired amounts and centrifuged again. The pellets were stored at -20°C.

11.3.2 Cultivation of *E. coli* BL21 (DE3) and expression of *At*RHM2_S1

For the construct without the Nus-tag different induction conditions were tried. The general steps were the same as described under section 11.1. The cultivation took place in a small scale with 15 mL ONCs and 50 mL main cultures, with the conditions shown in Table 11.22. The cultivation was done twice.

Table 11.22: Expression of *At*RHM2_S1 form pET43.1b(+) cultivated in *E. coli* BL21 (DE3).
Experiments with variation of incubation temperature and IPTG concentration were done, each for 16 hours at 100 rpm.

Strain	Temperature [°C]	IPTG[1] [mM]
pET43.1b(+)-*At*RHM2_S1	37	0.2
	37	1.0
	25	0.2
	25	1.0
	15	0.2
	15	1.0

[1] Chemicals are stated in Table 11.21

11.3.3 Cultivation of *E. coli* BL21 (DE3) and expression of *At*RHM2_S4

The steps of cultivation were done as described with the original construct with 50 mL ONCs and 250 mL main cultures. The cells were induced with 0.2 mM IPTG and incubation took place at 25°C and 100 rpm overnight for about 15 hours.

11.4 Cell lysis

Two different mechanical methods were used for cell lysis. For breaking up the cells by sonication (Sonic Dismembrator, Ultrasonic processor; Fisher Scientific (Austria) GmbH, Vienna, Austria) the pellet was thawed on ice and re-suspended in maximal 30 mL binding buffer for His-tag purification. The pellet was re-suspended completely and the suspension was transferred in a small glass or plastic beaker and installed at the sonication station. It was important to cool the cells during the sonication, as heat was released during the process. The conditions for lyses were 2 seconds pulse and 4 seconds pause at amplitude of 60% with tip with 10 mm diameter. The time for pulse was set to 3 to 6 minutes, depending on the volume (the more volume, the more time). The suspension was centrifuged in 1.5 mL reaction tubes to pellet cell debris (13200 g, 40 minutes, 4°C). The supernatant, namely cell free extract (CFE), was pooled and used for further experiments. To examine the fraction of insoluble protein one pellet was further analyzed. The removed supernatant was replaced by 6 M Urea (re-suspension of pellet fraction).
The second method for cell lysis, especially for very small amounts, was the use of glass beads (0.5 mm glass beads, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) which was performed in 1.5 mL reaction tubes. The thawed pellets were re-suspended in buffer (MOPS buffer, pH 7.5, 250 mM), to gain an optical density of 100. 1 milligram of glass beads was added per milliliter. Over a time period of 20 minutes there was alternately one minute of mixing the solution on the vortexer and one minute of cooling the solution on ice. The solution was separated from the glass beads per pipetting and the rest of the procedure was the same as described above.

11.5 Protein content

For determination of crude lysate and purified protein in a solution the method of Bradford was used (Bradford, 1976). A standard calibration curve with BSA (Albumin Fraktion V, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and the consequent regression equation were needed. The following concentrations of standards (BSA diluted with ultrapure water) were prepared in duplicates: 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL 0.5 mg/mL, 0.6 mg/mL and 0.8 mg/mL. For the reaction 20 µL of each standard was mixed with 1 mL of the reaction solution (Roti-Quant, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in a semi-micro cuvette (Sarstedt AG & Co, Nümbrecht, Germany) and incubated for about 10 minutes at room temperature. The absorbance at a wavelength of 595 nm was measured at the Spectrophotometer DU 800 (Beckman Coulter, Brea, CA, USA).

The workflow for measuring the protein content was the same as with the standards described above. 20 µL of the protein containing solution (most of the time diluted, as the

range of absorption of the spectrophotometer was between 0.1 and 0.8) was mixed with 1 mL of the reaction solution, incubated for 10 minutes at room temperature and was measured on the spectrophotometer (595 nm).

11.6 SDS-PAGE analysis of *At*RHM2

To separate proteins according to their molecular mass a SDS-PAGE analysis was performed. Sample preparation for NuPAGE® Bis-Tris Mini Gels (Thermo Fischer Scientific, Waltham, MA USA) is stated in Table 11.23.

Table 11.23: Sample preparation for SDS-PAGE analysis.
The reaction solution was incubated at 70°C for 10 minutes and 10 µL were loaded on the gel.

Components	Volume [µL]
Sample	10 (diluted to protein concentration of 1 mg/mL)
NuPAGE® LDS sample buffer, 4-fold [1]	10
50 mM Dithiothreitol (DTT) [2]	4
ultrapure water	16
Total	40

[1] Sample buffer from Thermo Fischer Scientific, Waltham, MA, USA; [2] DTT (1,4-Dithiothreitol from Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

The cell free extract and the pellet fraction (protein content measured as described in section 11.5), were diluted to a concentration of 1 mg/mL. With this initial concentration, the bands had a sufficient intensity on the gel. A prestained and an unstained PageRuler^{TM} protein ladder were used as standards (Thermo Fischer Scientific, Waltham, MA, USA) and 5 µL were loaded on the gel. The gel electrophoresis was run at 150 V for about an hour. The gel was stained with a staining solution (IET Gel Staining Solution; Bio-Rad Laboratories, Inc.; Hercules, CA; USA) and destained with deionized water.

11.7 Stability of *At*RHM2

As the concentration of the MOPS buffer was rather high and the enzyme was exposed to this high concentration under a temperature of 30°C, a test for protein degradation was done. The degradation over time was monitored via SDS-PAGE analysis. The enzyme was dissolved in MOPS buffer, 250 mM, pH 7.5 and was incubated at 30°C over 3.5 hours. Sampling took place every 30 minutes and the samples were stored at -20°C and until they were loaded on the gel.

11.8 Purification of the enzyme

The purification of *At*RHM2_original, AtRHM2_S1 and AtRHM2_S4 was done via the His6-tag (plasmid maps for each construct can be seen in the appendix). The method of the

purification was an immobilized metal ion affinity chromatography (IMAC) performed at the ÄKTAprime plus (liquid chromatography system from GE Healthcare, Chicago, IL, USA).

Table 11.24: Chemicals, materials and devices for the purification of *At*RHM2_original, *At*RHM2_S1 and *At*RHM2_S4 via the His6-tag.

Chemicals	Company	Comments
Disodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Ethanol, 96%	Lactan Ges.m.b.H & CO.KG, Graz, Austria	Flammable
Ethylendiamintetraacetat (EDTA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Imidazole	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Corrosive
Nickel(II)sulphate ($NiSO_4$)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Cancerogen! Stored at 4°C
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Materials	**Company**	**Comments**
Cellulose acetate filter, pore size of 0.45 µm	Sartorius Stedim Biotech, Göttingen, Germany	
HiTrap HP column, 5 mL	GE Healthcare, Chicago, IL, USA	
Vivaspin Turbo 15, 10 kDa cut of, 15 mL	Sartorius, Göttingen, Germany	
Devices	**Company**	**Comments**
ÄKTAprime plus	GE Healthcare, Chicago, IL, USA	

The 5 mL HisTrap HP column was first stripped and then reloaded with a special order of deionized water, binding buffer and nickel(II)sulphate (0.1 M) like stated in the instruction of GE healthcare (used buffers are stated in Table 11.25).

The sample (on average about 20 mL CFE with a concentration of 8 to 10 mg/mL were used) was loaded on the column and the His6-tagged protein built a complex with Ni^{2+}. All other proteins which were also expressed in *E. coli* were washed out.

Table 11.25: Buffer preparation for His-tag purification.
The buffers were prepared as recommended in the instruction of GE healthcare. All buffers were filtered and degassed.

	Binding buffer pH 7.4	Elution buffer pH 7.4	Stripping buffer pH 7.4
Components	Concentration [mM]		
Sodium phosphate [1,2]	20	20	20
NaCl [2]	500	500	500
Imidazole [2]	20	500	-
EDTA [2]	-	-	50

[1] The pH of sodium phosphate was adjusted by mixing the acidic (Na_2HPO_4) and the basic (NaH_2PO_4) components together. [2] Chemicals stated in Table 11.24.

The elution was done with a gradient of elution buffer (Table 11.25) from 0% to 50% in 20 mL at a flow rate of 1 mL/min. The fractions where the signal of the eluted protein was depicted were pooled. To remove the high amount of imidazole a buffer exchange (washing 3 times with about 15 mL storage buffer) was done via ultracentrifugation (Vivaspin Turbo 15). The storage buffer was MOPS, 100 mM, pH 7.5. The protein was aliquoted in 100 µL and stored at -20°C. The result was analyzed by SDS-PAGE and the protein content was determined.

11.9 Synthesis of UDP-β-L-rhamnose

The reaction scheme of the conversion from UDP-α-D-glucose to UDP-β-L-rhamnose is shown in section 10. The reaction mixture was adapted from Oka et al. 2007. All used chemicals for the synthesis are represented in Table 11.26.

Table 11.26: Chemicals and devices used for the synthesis of UDP-β-L-rhamnose.

Chemicals	Company	Comments
Acetonitrile (ACN)	Sigma Aldrich, St. Louis, MO, USA	
Caffeine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
Hydrochloride (HCl)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
MOPS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
NAD	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at 4°C
NADPH	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
TRIS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
UDP-α-D-glucose	-	From Institute, stored at -20°C
Devices	**Company**	**Comments**
Hewlett Packard 3D CE Capillary Electrophoresis	HP Austria GmbH, Vienna, Austria	Capillary chromatography with UV/Vis diode array detector

The conversion time was calculated on the basis of the activity studies of Oka et al. (2007). with a theoretical activity of the enzyme (0.06 U/mg). An enzymatic activity of 0.06 U/mg was assumed and an enzyme concentration of 0.5 mg/mL. If 2 µL were used in a 10 µL batch, 0.5 mM UDP-α-D-glucose would be converted in 8.33 minutes. That is, if 3 mM UDP-α-D-glucose was used (as in the conversion reactions in this thesis), the reaction would take about 50 minutes.

In the following Table 11.27 the composition for the conversion reaction with the original construct of *At*RHM2 (CFE) is stated.

Table 11.27: Composition of the batch for synthesis of UDP-β-L-rhamnose with *At*RHM2_original in CFE.
For the preparation of the stocks the chemicals were dissolved in MOPS, 250 mM, pH 7.5. The conversion reaction took place at 30°C and 350 rpm over 96 hours with sampling at different time points in between. Time points for sampling were: 0, 10, 30, 60, 120 minutes and 24 hours. 5 µL were withdrawn and stopped with 50 µL ice cold ACN (premixed 1:1 with ultrapure water). After 96 hours the reaction was stopped by heat. A background control (with the CFE of an empty pET43.1b(+) vector) and a negative control (with buffer instead of enzyme) were done.

Components	Concentration [mM]	Final concentration [mM]	Volume [µL]
UDP-α-D-glucose [1]	30	3	5
NAD^+ [1]	60	6	5
NADPH [1]	60	6	5
MOPS buffer, pH 7.5	500	250	25
CFE with *At*RHM2_original	-	-	10 [2]
Total	-	-	50

[1] Chemicals are stated in Table 11.26. [2] 10 µL of enzyme corresponded to 0.75 OD_{600} units (no enzyme concentration available).

In two following batches the enzyme concentrations of 3.4 mg/mL and 1.6 mg/mL (3.75 OD_{600} units) were used. Additionally these batches had caffeine as an internal standard, see Table 11.28.

Table 11.28: Composition of the batch for synthesis of UDP-β-L-rhamnose.
For the preparation of the stocks the chemicals were dissolved in MOPS, 250 mM, pH 7.5. The conversion reaction took place at 30°C and 350 rpm over 120 minutes with sampling at different time points in between. Time points for sampling were: 0, 10, 30, 60 and 120 minutes. 5 µL were withdrawn and stopped with 50 µL ice cold ACN (premixed 1:1 with ultrapure water). After 120 minutes the reaction was stopped by heat. A background control (with the CFE of an empty pET43.1b(+) vector) and a negative control (with buffer instead of enzyme) were done.

Components	Concentration [mM]	Final concentration [mM]	Volume [µL]
UDP-α-D-glucose [1]	30	3	5
NAD^+ [1]	60	6	5
NADPH [1]	60	6	5
MOPS buffer, pH 7.5	500	250	25
CFE with *At*RHM2_original	-	-	9.5 [2]
Caffeine [1]	10	1	0.5
Total	-	-	50

[1] Chemicals are stated in Table 11.26. [2] In one batch 9.5 µL of enzyme corresponded to 3.4 mg/mL and in a second batch it corresponded to 1.6 mg/mL.

The conversion with *At*RHM2_S1 in the CFE was done with 10 OD_{600} units, the composition was the same as stated above in Table 11.28. Time points and implementation of sampling as stated above.

For the conversion with the construct of *At*RHM2_S4 in the CFE the concentration of the MOPS buffer was reduced to 100 mM und 20 µL enzyme were applied which corresponded to 3.8 mg/mL. Samples were withdrawn at 0, 5 and 24 hours (batch shown in Table 11.29).

Table 11.29: Composition of the batch for synthesis of UDP-β-L-rhamnose.
For the preparation of the stocks the chemicals were dissolved in MOPS, 100 mM, pH 7.5. The conversion reaction took place at 30°C and 350 rpm over 24 hours with sampling at different time points in between. Time points for sampling were: 0, 5, and 24 hours. 5 µL were withdrawn and stopped with 50 µL ice cold ACN (premixed 1:1 with ultrapure water). After 24 hours the reaction was stopped by heat. A background control (with pET43.1b(+) instead of enzyme) and a negative control (with buffer instead of enzyme) were done.

Components	Concentration [mM]	Final concentration [mM]	Volume [µL]
UDP-α-D-glucose [1]	30	3	5
NAD^+ [1]	60	6	5
NADPH [1]	60	6	5
MOPS buffer, pH 7.5	500	100	10
CFE with *At*RHM2_S4	-	-	20 [2]
Caffeine [1]	50	5	5
Total	-	-	50

[1] Chemicals are stated in Table 11.26. [2] 20 µL of CFE corresponded to 3.8 mg/mL.

11.10 Analysis with the capillary electrophoresis (CE)

The quantitative and qualitative analysis of the nucleotide sugar UDP-β-L-rhamnose and the components of the conversion reaction was done with a capillary electrophoresis with a UV-Vis diode array detector. A 56 cm x 50 µM fused silica extended light path capillary was used. The samples were measured at a wavelength of 262 nm for 10 to 12 minutes at 40°C. The mobile phase was a sodium tetra borate buffer, 20 mM, pH 9.3.

Table 11.30: Chemicals, materials and devices for the analysis of UDP-β-L-rhamnose and the components of the conversion reaction on the CE.

Chemicals	Company	Comments
Caffeine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	
NAD	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at 4°C
NADPH	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	Stored at -20°C
UDP-α-D-glucose	-	From Institute, stored at -20°C
Materials	**Company**	**Comments**
Crimp caps, 11 mm	Markus Bruckner Analyse Technik, Linz, Austria	Lid for CE vials
Crimp Neck Vial, 1.5 mL, clear glass	Markus Bruckner Analyse Technik, Linz, Austria	Vials for CE
Micro inserts, 0.1 mL, clear glass, 31x6 mm	Markus Bruckner Analyse Technik, Linz, Austria	Inserts for micro volume for CE vials
Devices	**Company**	**Comments**
Hewlett Packard 3D CE Capillary Electrophoresis	HP Austria GmbH, Vienna, Austria	Capillary chromatography with UV-Vis diode array detector

For the qualitative analysis on the CE different standards were needed. The standards were prepared according to their concentration in the reaction. Therefore NAD and NADPH were prepared as a 6 mM stock, UDP-α-D-glucose was diluted to a concentration of 3 mM and the caffeine standard had a concentration of about 5 mM. For UDP-β-L-rhamnose there were no small amounts commercially available, so there was no standard available for the analysis of the nucleotide sugar. All standards were dissolved in MOPS buffer, 250 mM, pH 7.5.

12 Results and Discussion

The aim of this smaller part of the thesis was to express *At*RHM2 with a tag for purification, purify the enzyme and do first conversion experiments. In the following chapters all results of the cloning experiments (strategy 1 to 4), the expression of the enzyme and the conversion reactions from UDP-α-D-glucose to UDP-β-L-rhamnose are given.

12.1 Cloning

The aim of the experiments done with pET43.1b(+)-*At*RHM2 was the modification of the construct to improve the accessibility of the purification tag. It was assumed, that the His6-tag between the sequences of the Nus-tag and the enzyme itself was hardly reachable (see plasmid maps in the appendix). Two of the four cloning strategies were successful, further information is stated in the next sections.

12.1.1 Cloning of strategy 1

The original construct of pET43.1b(+)-*At*RHM2 was isolated and used as template for modification via PCR (shown in Figure 12.1). The Nus-tag coding sequence and the C-terminal stop codon (TAA) were removed in order to add a C-terminal His-tag; the PCR product had a size of 2010 bp.

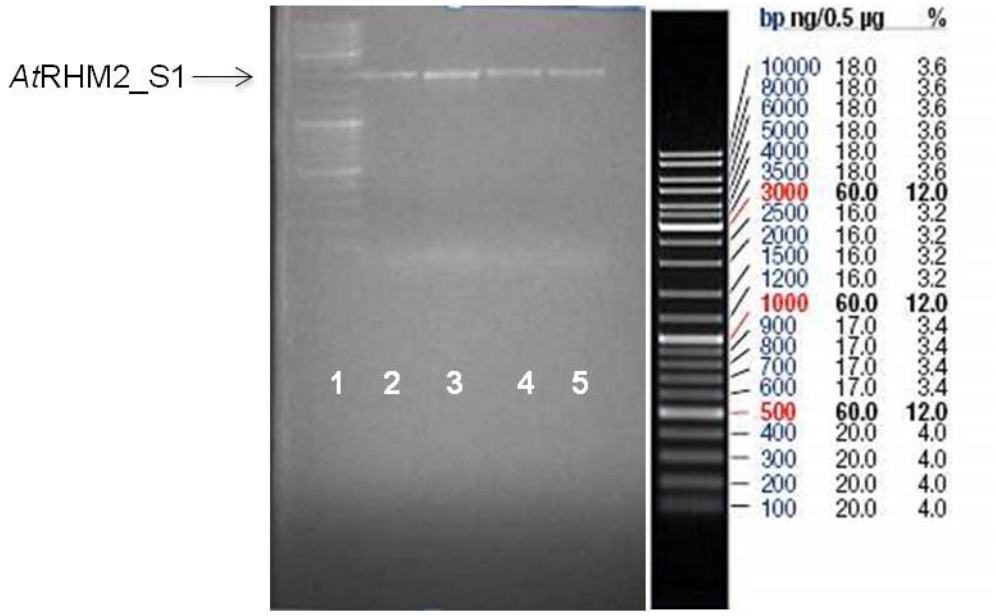

Figure 12.1: Control gel of PCR on a 1% agarose gel – Strategy 1.
Construct of *At*RHM2 without the N-terminal Nus-tag and without the C-terminal stop codon (TAA) with a size of 2010 bp. 1: GeneRuler™ DNA ladder mix, 6 µL; 2-5: PCR product, 2010 bp, conditions: 30 minutes, 80 V, 1x TAE-buffer

The original construct of pET43.1b(+)-*At*RHM2 and the PCR product were digested with the restriction enzymes *Nde*I and *Xho*I. The vector backbone and the PCR product were isolated, ligated and transformed into electro competent *E. coli* TOP10 F´ cells. The transformants were grown on LB-medium with ampicillin.

The successful ligation was verified by restriction of one isolated single colony with the restriction enzymes *Nde*I and *Xho*I and analyzed by 1% agarose gel electrophoresis, which can be seen in Figure 12.2.

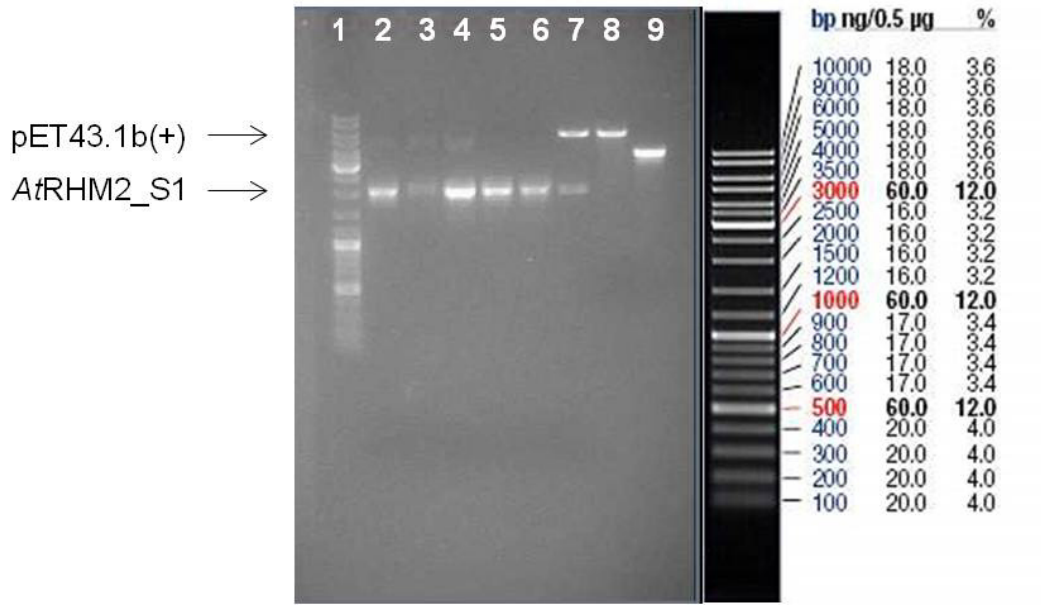

Figure 12.2: Control digestion with *Nde*I and *Xho*I of pET43.1b(+)-*At*RHM2_S1 transformants on a 1% agarose gel.
1: Standard, GeneRuler™ DNA ladder mix, 10 µL; 2-7: digested transformants, where lane 7 shows the right restriction pattern: pET43.1b(+) with a size of 5490 bp and modified *At*RHM2 with a size of 2003 bp; 8: linearized pET43.1b(+) vector backbone; 9: original *At*RHM2 with a size of 3656 bp; conditions: 30 minutes, 80 V, 1x TAE-buffer

Sequencing confirmed the wanted construct of pET43.1b(+)-*At*RHM2_S1. The construct was re-transformed into *E. coli* BL21 (DE3) for cultivation and expression. Glycerol stocks were made of both strains and stored at -80°C.

12.1.2 Cloning of strategy 2

Strategy 2 should have aimed in the change of vector backbone, from pET43.1b(+) to pET-STRP3. Cloning with the classical method came to no positive result. No positive clone was gained, as can be seen in Figure 12.3.

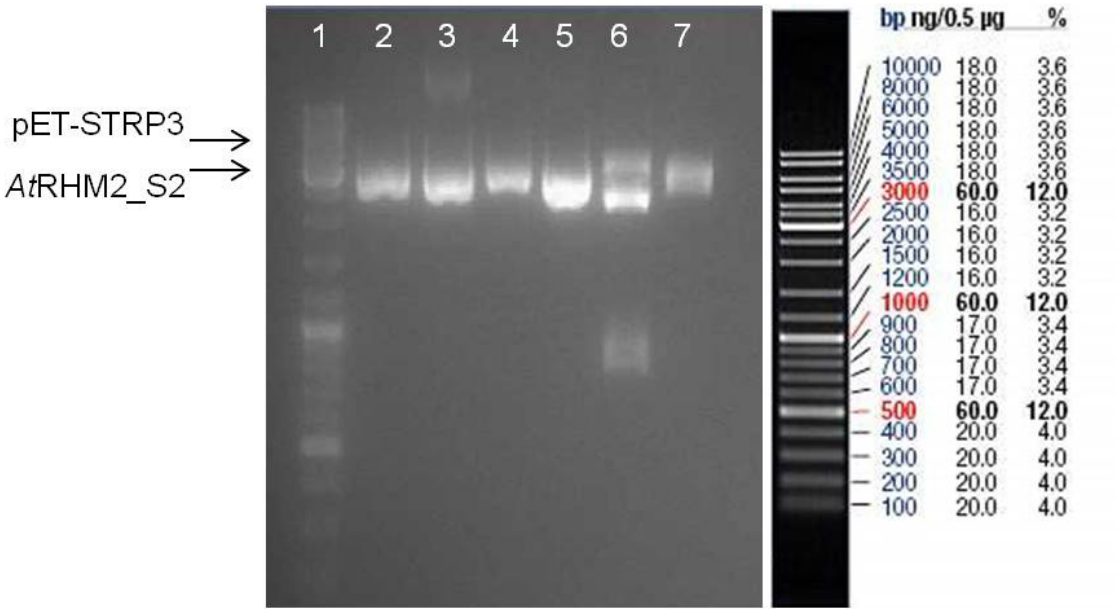

Figure 12.3: Control digestion with *Nde*I and *Xho*I of pET-STRP3-*At*RHM2_S2 transformants on a 1% agarose gel.
1: Standard, GeneRuler™ DNA ladder mix, 10 µL; 2-7: digested pET-STRP3_*At*RHM2_S2, where no lane showed the right restriction pattern with pET-STRP3 at a size of about 5278 bp and *At*RHM2 at a size of about 3656 bp; conditions: 30 minutes, 80 V, 1x TAE-buffer.

It was assumed, that the digestion with the restriction enzyme *Nde*I was problematic. At prolonged incubation time the restriction enzyme could get unspecific and could digest the end where it should only cut (hint is written in the instructions of Thermo Fisher Scientific). It was published that fragments digested with *Nde*I have a lower ligation efficiency and also need a lower ligation temperature (Chang, Ge, Lee, So, & Wang, 2005). Different ligation times and lower ligation temperatures were tried but no positive result was gained during the thesis.

The preparation for the CPEC method resulted in a PCR product (*At*RHM2 with a large overlap to the pET-STRP3 vector on both sides) of 3711 bp as seen in Figure 12.4.

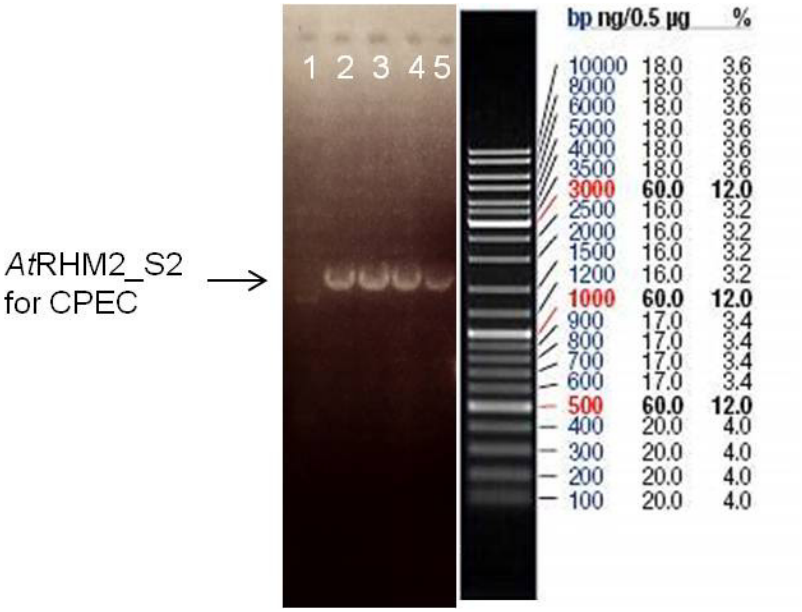

Figure 12.4: Control gel of PCR for CPEC on a 1% agarose gel – Strategy 2.
Construct of *At*RHM2 with a large overhang to the pET-STRP3 vector, 3711 bp 1: GeneRuler™ DNA ladder mix, 6 µL; 2-5: PCR product of modified *At*RHM2, conditions: 30 minutes, 80 V, 1x TAE-buffer.

The ligation of the linearized pET-STRP3 backbone (digested with *Nde*I and *Xho*I) and the PCR product failed. The assembling according to the CPEC method is similar to a PCR and more an extension rather a ligation. One control gel of control digestion after transformation in electro competent *E. coli* TOP10 F´ cells is illustrated in Figure 12.5.

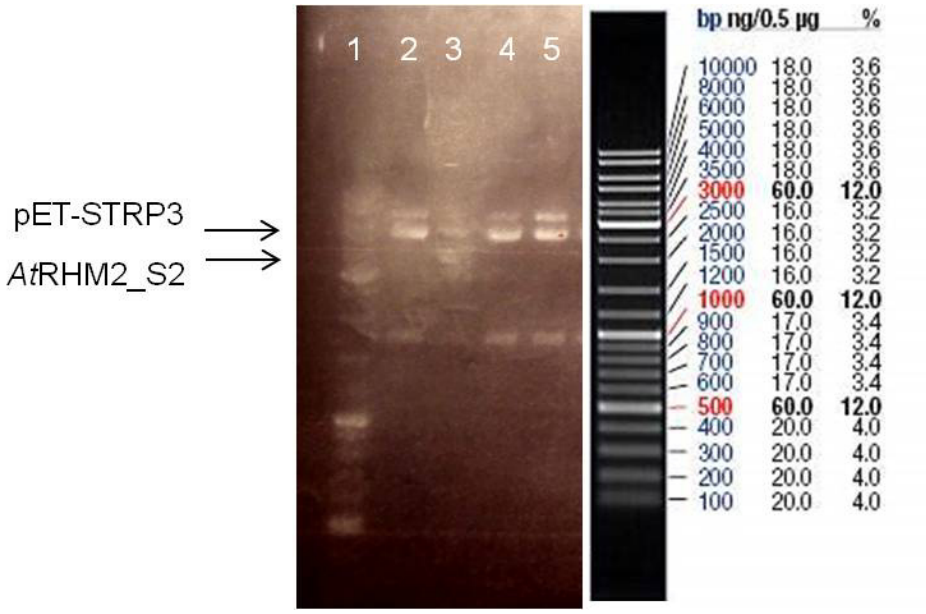

Figure 12.5: Control digestion with *Nde*I and *Xho*I of pET-STRP3-*At*RHM2_S2 transformants on a 1% agarose gel.
1: Standard, GeneRuler™ DNA ladder mix, 10 µL; 2-5: digested pET-STRP3-*At*RHM2_S2, where no lane showed the right restriction pattern with pET-STRP3 at a size of about 5278 bp and *At*RHM2 at a size of about 3656 bp; conditions: 30 minutes, 80 V, 1x TAE-buffer.

The wanted construct of strategy 2 was not gained.

Generally CPEC is a high efficient cloning method, with about 95-100% positive results, according to Quan & Tian, 2009, whether the circular vector is used or it is linearized. In this thesis a linearized vector was used. A CPEC with a circular vector should be done to test the chances of success.

12.1.3 Cloning of strategy 3

The idea of strategy 3 was to clone the enzyme into a pChem vector where a Nus-tag and a STRP-tag already existed. The amplified insert for this strategy can be seen in Figure 12.6.

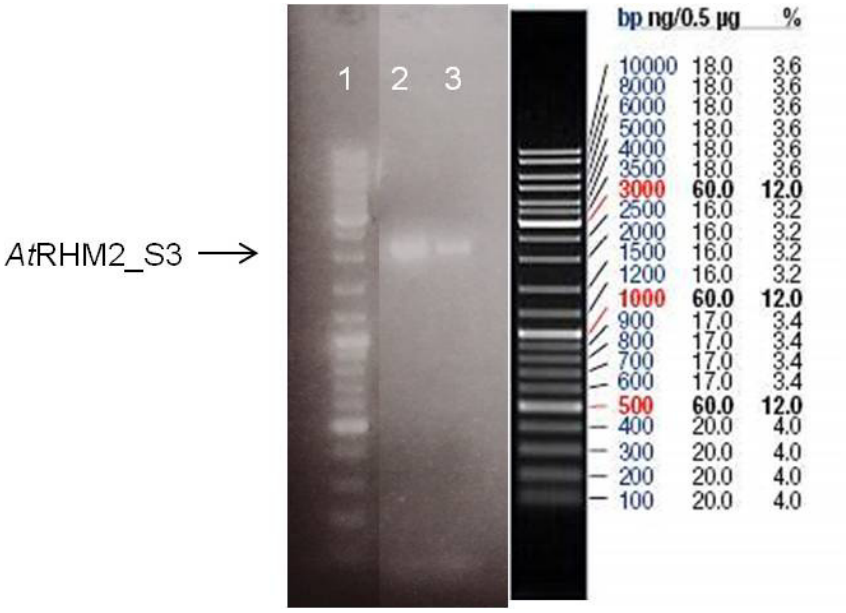

Figure 12.6: Control gel of PCR on a 1% agarose gel – Strategy 3.
Construct of *At*RHM2 with a size of 2016 bp. 1: GeneRuler™ DNA ladder mix, 6 µL; 2-3: PCR product of amplified *At*RHM2-S3, conditions: 30 minutes, 80 V, 1x TAE-buffer.

Simultaneously to the experiments for strategy 3 also the experiments for strategy 4 were done. As these experiments for strategy 4 leaded faster to success no more effort was done to get the construct 3 finished.

12.1.4 Cloning of strategy 4

For strategy 4 the original construct was extended with a C-terminal His6-tag for purification. The described composition of the PCR sample plus the corresponding PCR program as stated above in material and methods resulted in the following bands shown in Figure 12.7.

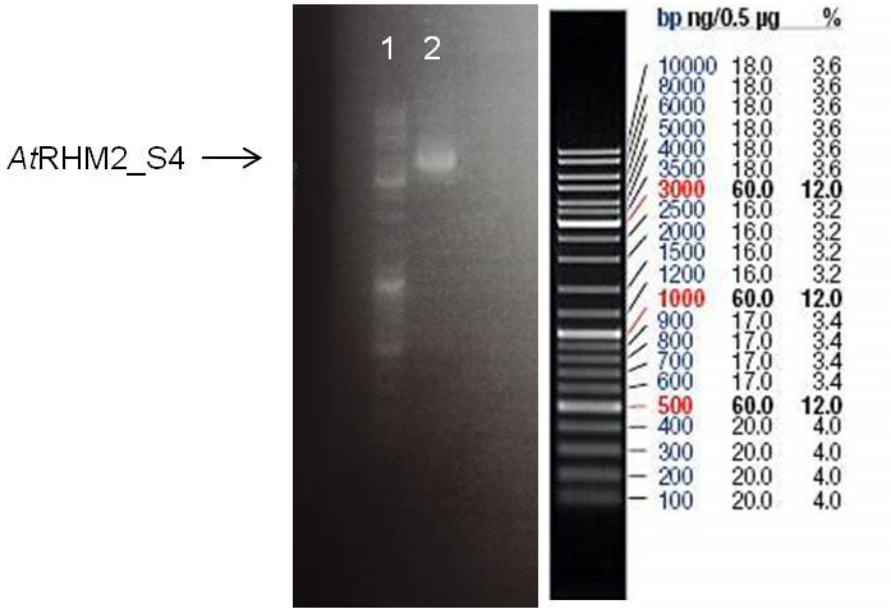

Figure 12.7: Control gel of PCR on a 1% agarose gel – Strategy 4.
Construct of *At*RHM2 with a removed C-terminal stop codon for expression of a C-terminal His6-tag. 1: GeneRuler™ DNA ladder mix, 6 µL; 2: PCR product of modified *At*RHM2_S4 with a size of 3667 bp, conditions: 30 minutes, 80 V, 1x TAE-buffer.

The original *At*RHM2 with a removed C-terminal stop codon to gain an additional C-terminal His6-tag had a size of 3667 bp.

The original construct of pET43.1b(+)-*At*RHM2 and the PCR product were digested with the restriction enzymes *Nde*I and *Xho*I. The vector backbone and the PCR product were isolated, ligated and transformed into electro competent *E. coli* TOP10 F´ cells. The transformants were grown on LB-medium with ampicillin.

The successful ligation was verified by restriction of one isolated single colony with the restriction enzymes *Nde*I and *Xho*I and analyzed by 1% agarose gel electrophoresis, which can be seen in Figure 12.8.

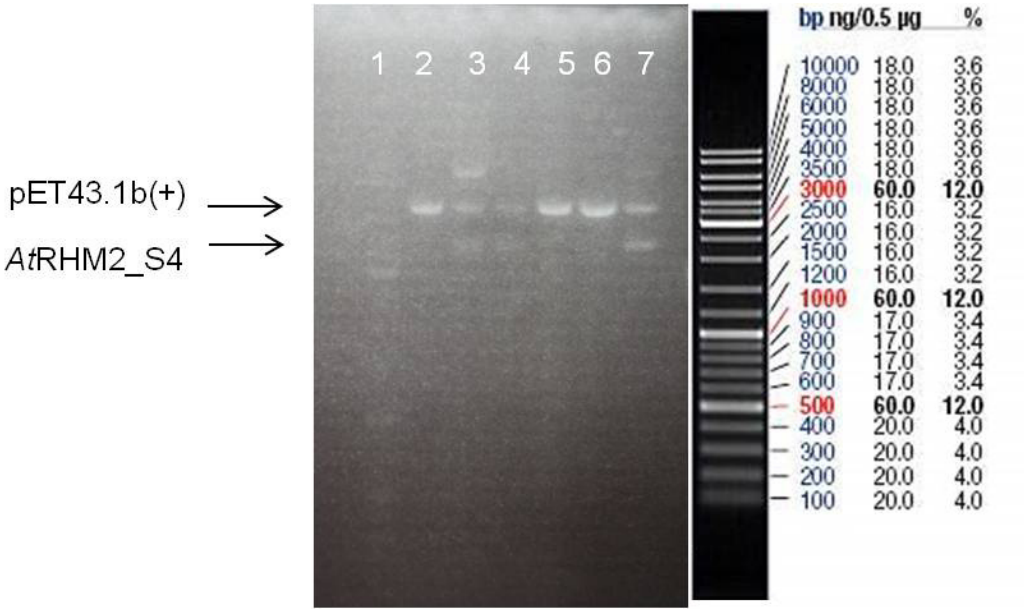

Figure 12.8: Control digestion with *Nde*I and *Xho*I of pET43.1b(+)-*At*RHM2_S1 transformants on a 1% agarose gel.
1: Standard, GeneRuler™ DNA ladder mix, 10 µL; 2-7: digested transformants, where lane 4 showed the right restriction pattern: pET43.1b(+) with a size 5490 bp and modified *At*RHM2 at a size of about 3653 bp; conditions: 30 minutes, 80 V, 1x TAE-buffer.

Sequencing confirmed the wanted construct pET43.1b(+)-*At*RHM2_S4 in *E. coli* TOP 10 F´. As seen below, the stop codon was removed for the expression of the C-terminal His6-tag.

```
Seq_1  445   ATACGTCTTTGAACCAAACAAGAGAACC---CTCGAGCACCACCACCACCACCACTAATG  389
             ||||||||||||||||||||||||||||   ||||||||||||||||||||||||||||
Seq_2  8581  ATACGTCTTTGAACCAAACAAGAGAACCTAACTCGAGCACCACCACCACCACCACTAATG  8640
```

The new construct was also re-transformed into *E. coli* BL21 (DE3) and the sequence was also verified by sequencing.

12.2 Cultivation and expression

Cultivation of *E. coli* BL21 (DE3) carrying pET43.1b(+)-*At*RHM2 was done in shake flasks and in reaction tubes. All cultivations of the different constructs were done in LB medium with ampicillin and the expression was induced with IPTG.

12.2.1 Expression of *At*RHM2 (original construct)

The expression of *At*RHM2_original took place in shake flasks. The volume of the first batch was 1.25 L and 0.25 L for a second one.

Table 12.1: Summary of the cultivations of *E. coli BL21* (DE3) carrying pET43.1b(+)-*At*RHM2_original and the reached biomass (wet weight) and total protein.

Number	Biomass [g]/ broth [L]	Pellet [g]	Total protein content [mg]	Cell lysis
1	4.3	2.5	49.2	Sonication
		0.5	35.9	Sonication
		2.4	166.3	Sonication
2	7.0	1.8	8.7	Glass beads

SDS-PAGE analysis was used to separate the proteins according to their molecular mass (see Figure 12.9).

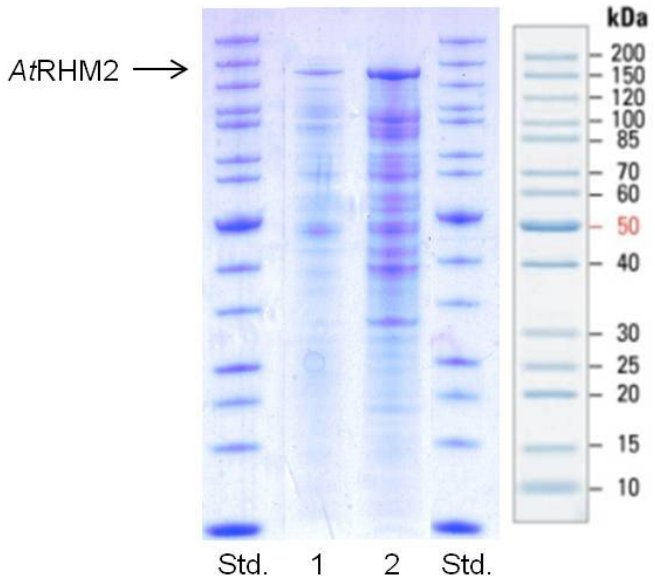

Figure 12.9: SDS-PAGE analysis of the expression of *At*RHM2_original (in the pET43.1b(+)-vector) in *E. coli* BL21 (DE3).
Std.: Unstained PageRuler^{TM} protein ladder, 5 µL; 1: Supernatant (CFE) of *At*RHM2_original in *E. coli* BL21 (DE3); 2: Pellet of *At*RHM2_original in *E. coli* BL21 (DE3) dissolved in 6M Urea

The overexpression of *At*RHM2_original can be seen clearly at a size of 135 kDa.

12.2.2 Expression of *At*RHM2_S1

The expression of *At*RHM2_S1 in *E. coli* BL21 (DE3) was done in shake flasks. In the following table (Table 12.2) the different conditions and the corresponding values for the biomass wet weight and the protein content is stated.

Table 12.2: Evaluation of biomass (wet weight) and protein content of the samples with the different expression conditions.

Conditions	Biomass [g]/ broth [L]	Total protein content [mg]
37°C, 1 mM IPTG	6.2	8.91
37°C, 0.2 mM IPTG	6.0	9.39
25°C, 1 mM IPTG	8.6	8.52
25°C, 0.2 mM IPTG	8.0	8.25
18°C, 1 mM IPTG	6.0	12.69
18°C, 0.2 mM IPTG	6.2	9.30

There was no readily identifiable overexpression of *At*RHM2_S1, as shown in Figure 12.10.

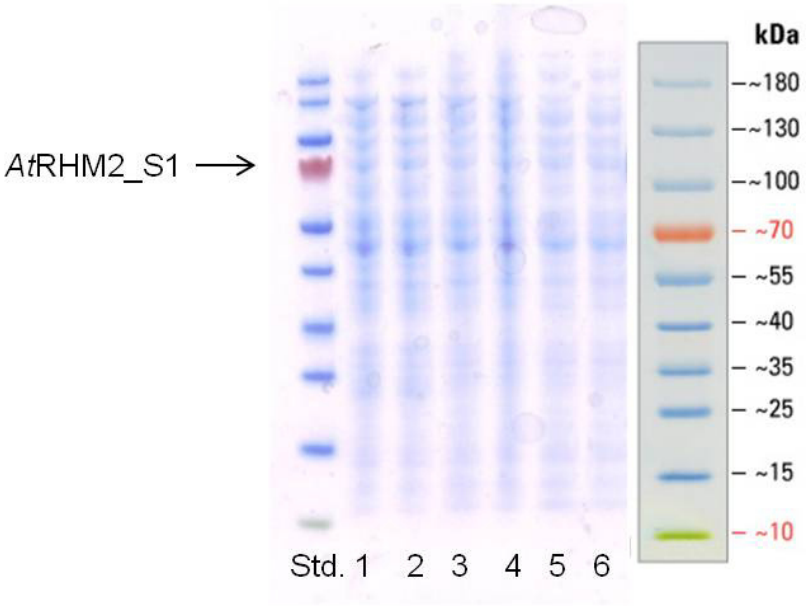

Figure 12.10: SDS-PAGE analysis of the expression of *At*RHM2_S1 (at 75 kDa) under different conditions.
Std.: Prestained PageRuler^{TM} protein ladder, 5 µL; 1: 37°C, 1 mM; 2: 37°C, 0.2 mM; 3: 25°C, 1 mM; 4: 25°C, 0.2 mM, 5: 18°C, 1 mM; 6: 18°C,0.2 mM

While no clear overexpression of AtRHM2 was detectable on the gel, induction conditions of 18°C and 0.2 mM IPTG were chosen for a larger cultivation batch to still try to purify the possibly expressed *At*RHM2_S1. Out of 1500 mL broth 5.79 g biomass was produced. 98 mg total protein was expressed. Sonication was done and the CFE was used for purification of *At*RHM2_S1 via the His6-tag.

12.2.3 Expression of *At*RHM2_S4

1500 mL broth was used for cultivation of *E. coli* BL 21 (DE3) with the strain pET43.1b(+)-*At*RHM2_S4. 8.18 g biomass was produced and 220 mg proteins were expressed. The cells were broken up via sonication and the CFE was used for the purification of *At*RHM2_S4 via the His6-tag.

12.3 Protein content

To determine the protein content the following standard curve was created (Figure 12.11).

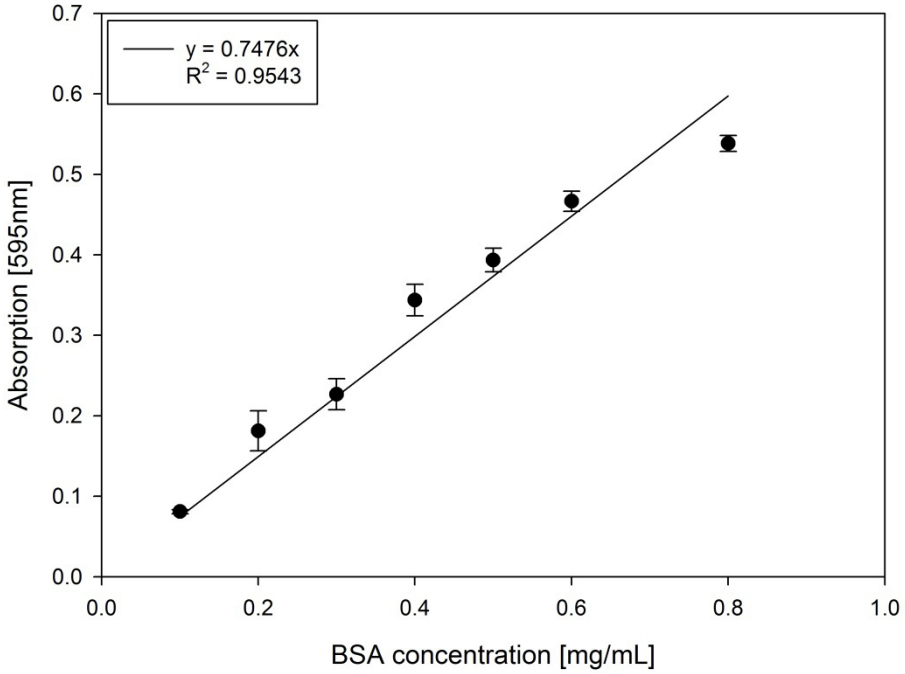

Figure 12.11: Standard curve with BSA.
This curve is used for the calculation of the protein content with the method of Bradford, as described in section 2.3. Values measured in quadruplets. Linear equation: y=0.7476x, determination coefficient: R^2=0.9543

With this data the total protein content and the purified protein content were calculated. The protein content was, among others, needed for SDS-PAGE analysis as the best result was reached by loading samples with an initial concentration of 1 mg/mL.

12.4 Stability of *At*RHM2

The enzyme is exposed to high salt concentrations and higher temperature at the conversion reaction. The enzyme is not degraded under these conditions, as seen in Figure 12.12. A possibly denaturation would not be seen on the gel.

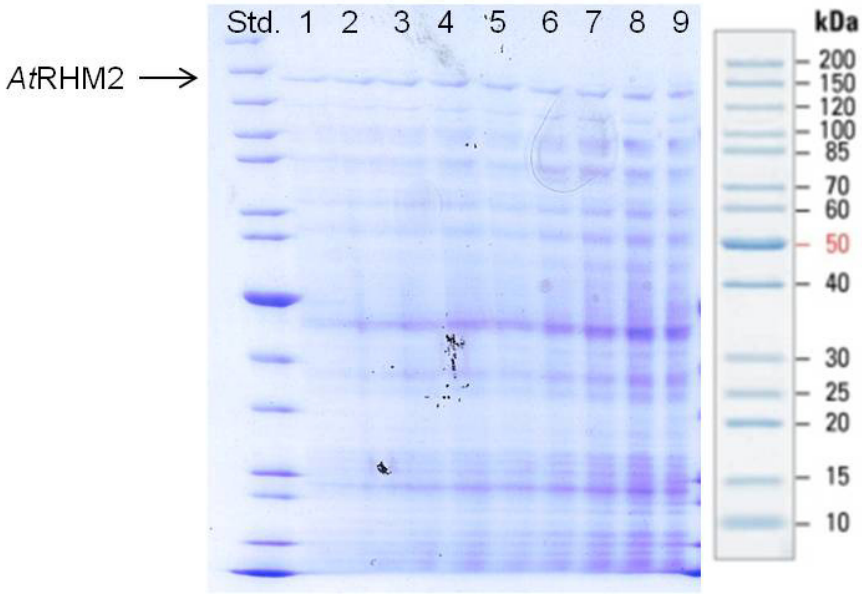

Figure 12.12: SDS-PAGE analysis of the stability of *At*RHM2.
Std.: Unstained PageRuler^{TM} protein ladder, 5 µL; Bands 1-9: *At*RHM2 at different time points in MOPS buffer, 250 mM, pH 7.5 and at 30°C; 1: starting point; 2: 10 minutes of incubation; 3: 30 minutes of incubation; 4: 60 minutes of incubation; 5: 90 minutes of incubation; 6: 120 minutes of incubation; 7: 150 minutes of incubation; 8: 180 minutes of incubation; 9: 210 minutes of incubation

12.5 Purification of enzyme

For purification of *At*RHM2_original via the internal His6-tag located between Nus-tag and AtRHM2 the CFE was loaded on the HisTrap column. The chromatogram of the purification can be seen in Figure 12.13. The blue curve represents the absorption at a wavelength of 280 nm and the green curve shows the concentration of the elution buffer. The pink straight line depicts the start of injection, where the sample was loaded onto the column. Peak 1 is the flow through, where all proteins which were not binding to the column gave a signal. The peak of interest is number 2. This is the signal of the enzyme eluted at a concentration of about 200 mM imidazole.

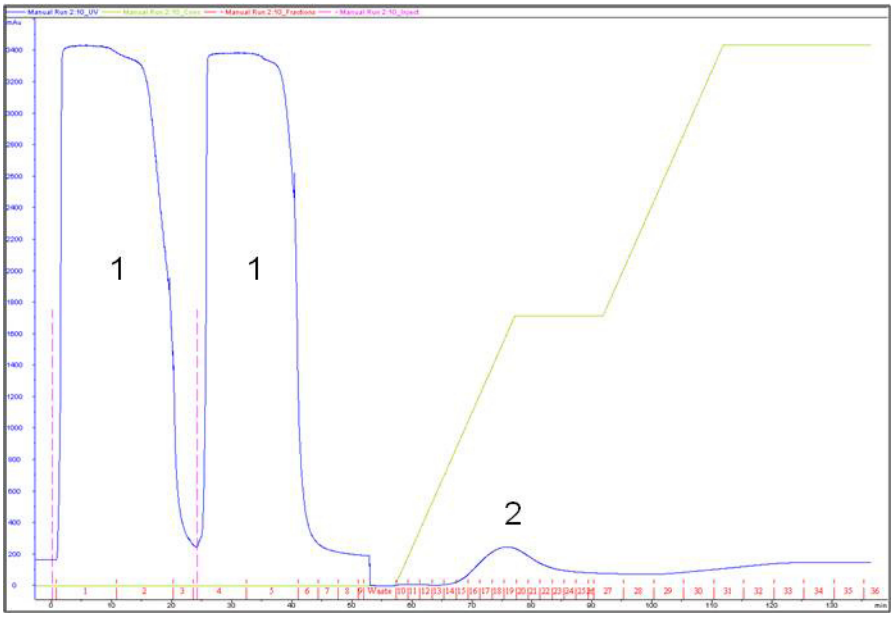

Figure 12.13: Chromatogram of the purification of *At*RHM2_original via the His6-tag with IMAC. The blue curve represents the absorption at 280 nm and the green curve shows the concentration of the elution buffer. The pink straight lines depict the start of injection, where the sample was loaded onto the column. Peak 1: flow through, peak 2: signal of the enzyme eluted at an imidazole concentration of about 200 mM.

Outgoing from 166 mg total protein content there was a yield of 15.31 mg purified protein. SDS-PAGE analysis showed the following pattern of the bands (Figure 12.14), which were not expected. The His6-tagged protein was as well in the purified fraction as in the flow through. Also other not His6-tagged proteins were in the purified fraction. In lane 1 at the height of 135 kDa should have been a clear single band. The broader band at the size of 60 to 70 kDa could be *At*RHM2 (75 kDa) without the Nus-tag (55 kDa).

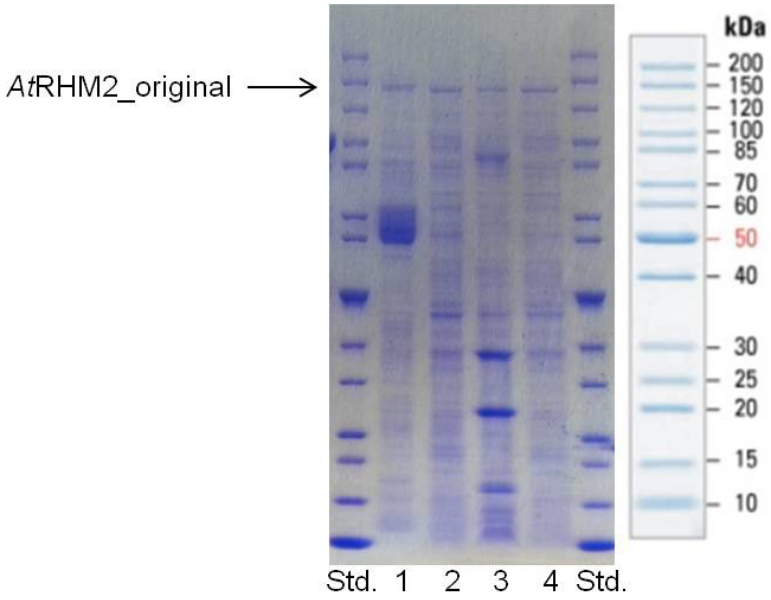

Figure 12.14: SDS-PAGE analysis of purified *At*RHM2_original in comparison to the CFE, the pellet and the flow through.
Std.: Unstained PageRuler^{TM} protein ladder, 5 µL; 1: purified protein, 2: CFE, 3: pellet fraction, 4: flow through

It seemed that there were binding problems of the His6-tag to the column resin. In the troubleshooting section of the HisTrap^{TM} HP manual of GE Health care (Instructions 71-5027-68 AK) there can be found some tips for further proceeding. The concentration of imidazole could be reduced in the binding buffer, for example, for the case that the concentration is too high for the binding of the His6-tag. Alternatively the concentration of NaCl could be increased to avoid nonspecific interactions. Another explanation could be the position of the His6-tag, as it was between the enzyme and the Nus-tag, and therefore hardly accessible.

The purification of *At*RHM2_S1 via the N-terminal His6-tag without the N-terminal Nus-tag in the front is illustrated in Figure 12.15.

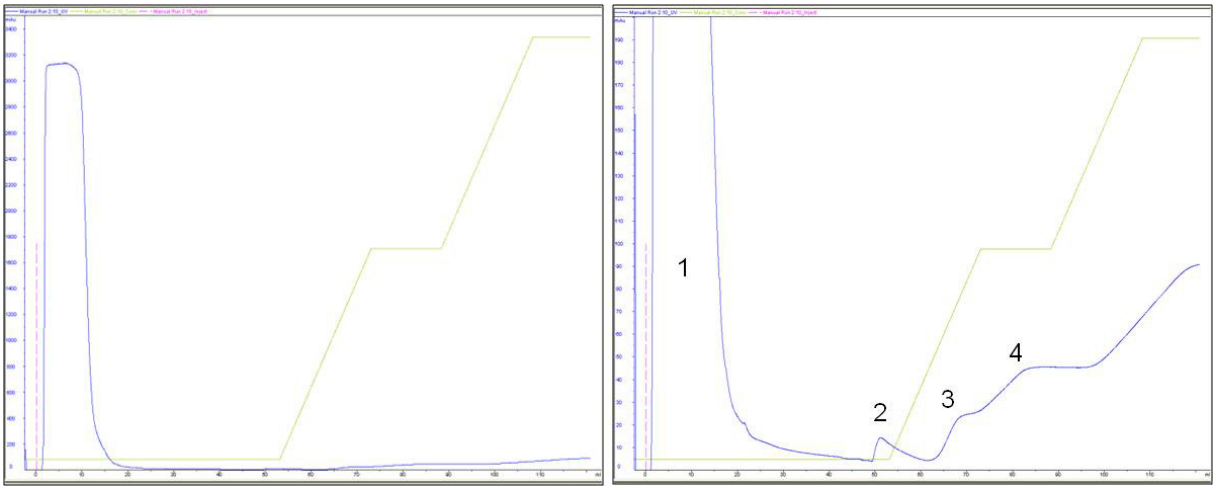

Figure 12.15: Chromatogram of the purification of *At*RHM2_S1 via the His6-tag with IMAC.
The blue curve represents the absorption at 280 nm and the green curve shows the concentration of the elution buffer. The pink straight lines depict the start of injection, where the sample was loaded onto the column. Left chromatogram: whole view; right chromatogram: zoom of elution peaks; peak 1: flow through, peak 2: protein concentration of 0.04 mg/mL, peak 3: protein concentration of 0.07 mg/mL, peak 4: protein concentration of 0.01 mg/mL

The overexpression of the enzyme without the Nus-tag was problematic and not very successful. Nevertheless a purification of the enzyme amount that was expressed was tried. As can be seen in the left chromatogram of Figure 12.15 the elution peak is not really noticeable in full view. Only at high zoom elution peaks can be seen (right chromatogram in the figure above). It was not clear which peak would be the enzyme itself, therefore all three peaks were collected and the protein content was measured. But the measurements were inexact because the values were out of the range of the spectrophotometer. The samples were not analyzed via SDS-PAGE and no further experiments were done.

At the purification of *At*RHM2_S4 the first gradient with elution buffer from 0 - 50% in 20 mL resulted in a distinct elution peak (shown in Figure 12.16, peak 2).

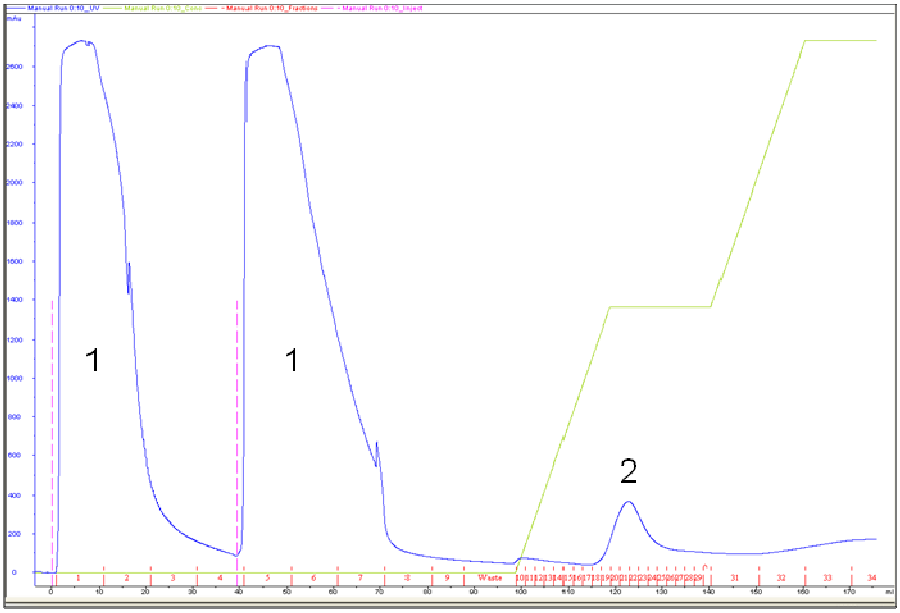

Figure 12.16: Chromatogram of the purification of *At*RHM2_S4 via the His6-tag with IMAC. The blue curve represents the absorption at 280 nm and the green curve shows the concentration of the elution buffer. The pink straight lines depict the start of injection, where the sample was loaded onto the column. Peak 1: flow through, peak 2: signal of the enzyme eluted at an imidazole concentration of about 250 mM.

The evaluation of the protein content gave the following results as stated in Table 12.3.

Table 12.3: Evaluation of protein content of cultivated *E. coli* BL21 (DE3) carrying pET43.1b(+)-*At*RHM2_S4.

Sample	Protein content [mg/mL]	Total volume [mL]	Total protein content [mg]
CFE	9.56	23	219.88
Flow through	4.29	10	42.92
Purified enzyme	5.47	1	5.47
Pellet	3.37	1.4	4.72

The evaluation gave 5.47 mg of purified enzyme. But at the SDS-PAGE analysis an already known pattern of the bands was represented (Figure 12.17).

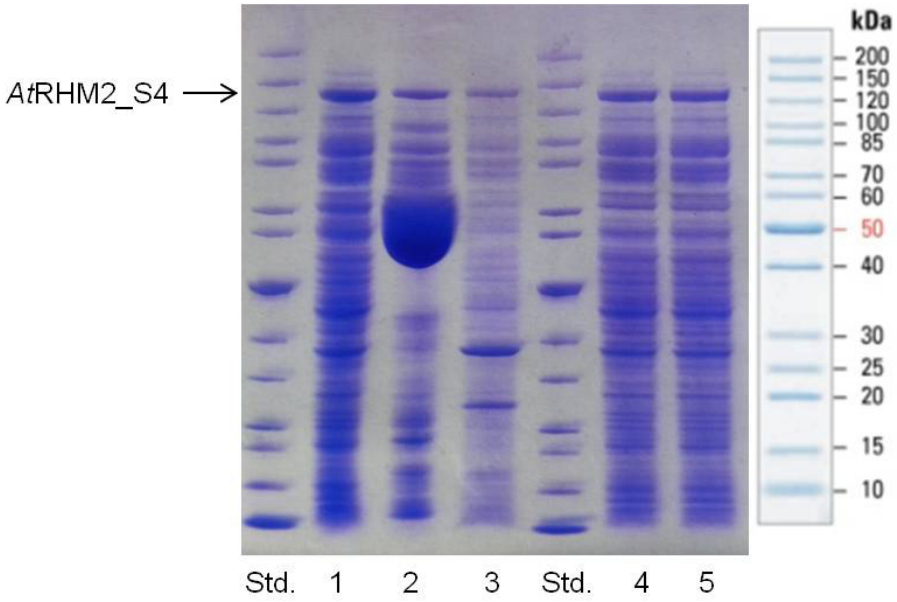

Figure 12.17: SDS-PAGE analysis of purified *At*RHM2_S4 in comparison to the CFE, the pellet and the flow through. Std.: Unstained PageRuler^{TM} protein ladder, 5 µL; 1: CFE; 2: purified protein, there should have been only one band at the size of 135 kDa; 3: pellet fraction; 4 & 5: flow through

The purification was again not successful. And it seemed that there was the same problem as with the purification of construct S1. Again there was some protein left in the flow through, a big band at a size of about 60 – 70 kDa and a lot of other proteins in the purified fraction. It was assumed, that not the His6-tag was the problem, because in the case of construct S4 there is additionally to the N-terminal His6-tag (which seemed to be hardly accessible) a C-terminal His6-tag.

To be sure, that the right construct was cultivated the clone was sequenced. And this gave the certainty that it was the wanted sequence. The problem must therefore found elsewhere. Some explanations were already written down at the discussion of purification of the original construct.

12.6 Synthesis of UDP-β-L-rhamnose

The conversion reactions to synthesize UDP-β-L-rhamnose out of UDP-α-D-glucose were all done at 30°C with the enzyme containing CFE (*At*RHM2 in pET43.1b(+) cultivated in *E. coli* BL21 (DE3)).

In the conversion shown in Table 11.27 it seemed that UDP-α-D-glucose was converted in the batch with the enzyme and also in the control reaction where an empty vector control was used. As reported by Oka et al. 2007 the UDP-β-L-rhamnose peak should be right before the UDP-α-D-glucose. Because of lack of the reference substance for UDP-β-L-rhamnose this could not be proofed in this thesis (but there were reference standards for all other compounds used in the batch).

In Table 12.4 the retention times and the possible association of the substances at the

beginning and at the end of the reaction are shown. At first glance, it seemed that UDP-β-L-rhamnose was produced. But there was a forward shift of retention time of about 0.15 minutes (seen at Caffeine (the first peak) and NAD^+ (the highest peak)). Therefore it is not really clear if the peak at 5.80 minutes is UDP-β-L-rhamnose or UDP-α-D-glucose.

Table 12.4: Relevant peaks at the start of conversion and at the end.

Time of conversion	10 minutes		120 minutes		
Peak	4	5	4	5	6
Retention time [min]	5.16	5.96	5.01	5.80	5.96
Peak area [mAU]	435.32	172.11	246.40	110.27	15.60
Substance	NAD^+	UDP-α-D-glucose	NAD^+	UDP-β-L-rhamnose *	UDP-α-D-glucose *

* Theoretical association of retention time and substances. No clear statement could be done because of lack of the reference substance UDP-β-L-rhamnose.

A similar situation was reported in the batch with an empty vector control: forward shift of retention time (about 0.19 minutes), peak at 5.80 minutes arose.

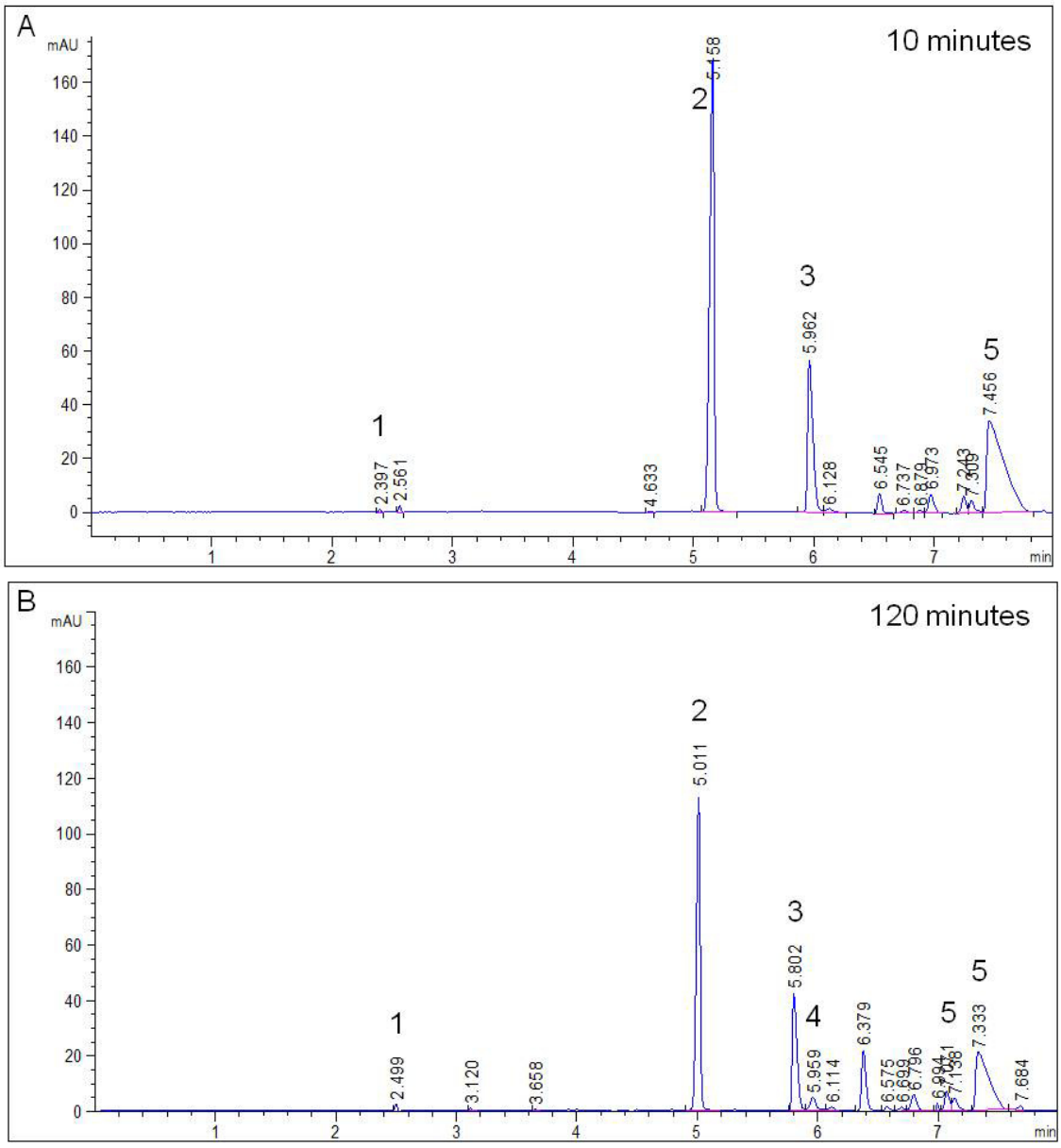

Figure 12.18: Chromatogram of analysis with the capillary electrophoresis of conversion reaction from UDP-α-D-glucose to UDP-β-L-rhamnose.
A: 10 minutes, B: 120 minutes; 1: Caffeine, 2: NAD, 3: UDP-α-D-glucose (*), 4: UDP-β-L-rhamnose (*), 5: NADP and NADPH; (*) Theoretical association of retention time and substances. No clear statement could be done because of lack of the reference substance UDP-β-L-rhamnose.

The result of the second batch which was described in Materials and Methods was similar than the result stated above. But a greater shift of about 0.52 minutes referred to the NAD^+ Peak occurred.

The third data analysis gave another picture. This time the shift in retention time was backwards for about 0.34 minutes referred to NAD^+.

The chromatogram of the fourth batch is depicted below in Figure 12.19. As one can see there is a variety of peaks. The easiest peak to identify is the sharp high NAD^+. The orientation outgoing on this peak is easier. But one cannot say if UDP-α-D-glucose is converted into UDP-β-L-rhamnose. The lack of reference substance, the shift of retention

time with each additional peak and the proximity of elution time of both sugars made the analysis very difficult.

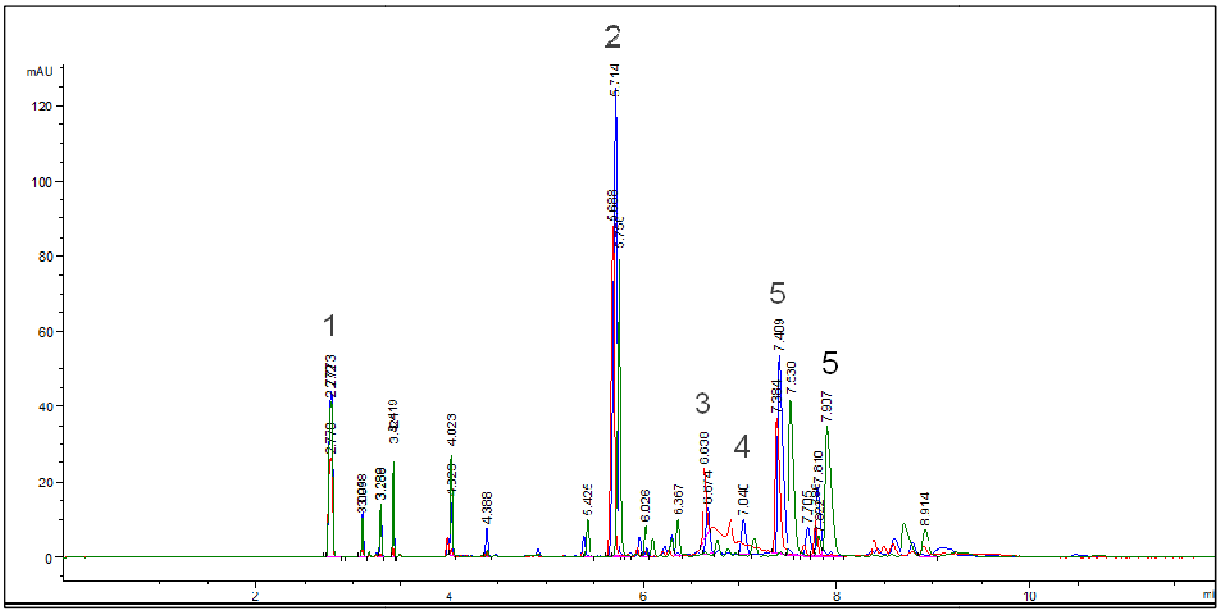

Figure 12.19: Chromatogram of analysis with the capillary electrophoresis. Overlay of chromatograms of sampling at 0 hours, 5 hours and 24 hours of conversion from UDP-α-D-glucose to UDP-β-L-rhamnose. Red line: 0 hours, blue line 5 hours, green line: 24 hours. 1: Caffeine, 2: NAD, 3: UDP-α-D-glucose, 4: NADH, 5: NADP and NADPH. Between the peaks 2 and 3 appeared new peaks, one of these could maybe be the wanted product UDP-β-L-rhamnose (but without a standard it is hard to define). Conditions: mobile phase: tetra borate 20 mM, pH 9.3; 40°C, 12 minutes

13 Conclusion and Outlook

The aim of this part of the thesis, the synthesis of UDP-β-L-rhamnose was not reached, however, the different steps undertaken are summarized below.

The expression of *At*RHM2_original with the Nus-tag was relatively uncomplicated. The purification whereas was not possible. SDS-PAGE analysis gave not the anticipated pattern. The desired protein was also present in the flow-through, and in addition other unexpected bands were seen in the purified fraction. There was an intense band at 60 to 70 kDa instead of 135 kDa. It was assumed, that the His6-tag was hardly accessible between the Nus-tag and the enzyme itself and therefore no purified protein was obtained.

The construct with the removed N-terminal Nus-tag and a C-terminal His tag gave no clearly detectable expression of *At*RHM2_S1. Thus the cloning strategy S1 where the N-terminal Nus-tag was removed was successful, but not suitable for expression.

Cloning strategy S2 (vector exchange to pET-STRP3) was not successful as the classical cloning method and the CPEC method did not result in the wanted construct. Strategy 3

(vector exchange to pChem vector) was not further pursued, because strategy 4 was promising, see below.
The construct of strategy 4 (Nus-*At*RHM2-His) was successfully cloned, the expression was satisfactory (since the Nus-tag was present) and the purification looked promising judged from the purification chromatogram. However, SDS-PAGE analysis revealed no purification of the full length protein but an enrichment of an unidentified protein of 60-70 kDa. Therefore, it was suggested that not only the location of the His6-tag could be the problem, but also the attachment of the tag to the resin of the column.
Therefore the composition of buffer and maybe pH should be adjusted for further purification attempts of Nus-*At*RHM2-His. There were already stated some suggestions for the changes of buffer, for example the change of concentration of imidazole and NaCl in binding and elution buffer.

The conversion reactions were carried out with the CFE of the cultivated original, S1 and S4 constructs in *E. coli* BL21 (DE3). The batch of the conversion was based on the experiments of Oka et al., 2007. It is to mention, that Oka et al. also used the CFE and not the purified protein. However, the conversion from UDP-α-D-glucose to UDP-β-L-rhamnose could not be demonstrated for sure.

Analysis was performed via capillary electrophoresis and the major drawback was the fact that no standard of UDP-β-L-rhamnose was commercially available, at least not in small quantities. Therefore the retention time of the wanted nucleotide sugar could only be guessed on the basis of the published data. Furthermore the background activities of other expressed proteins in the CFE were confusing, as the comparison of the chromatograms of the conversion reaction and the conversion control with an empty pET43.1b(+) vector did not make the result clearer.

To proof that there is a catalytic activity of *At*RHM2 the consumption rate of NADPH could be observed at a spectrophotometer at a wavelength of 340 nm. This experiment should be done with NAD and in the absence of NAD. Important would be that the blank also contains CFE but with an empty expressed pET43.1b(+) vector.

If the synthesis and purification of putative UDP-β-L-rhamnose would be feasible the nucleotide sugar could be analyzed by H^1 NMR (UDP-α-D-glucose and UDP-β-L-rhamnose are easily distinguished with this structure-elucidating method).

14 Appendix

Sequence of *At*RHM2:

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ATGGATGATACTACGTATAAGCCAAAGAACATTCTCATTACTGGAGCTGCTGGATTTATTGCTTCTCATGTTGCCAACAG
ATTAATCCGTAACTATCCTGATTACAAGATCGTTGTTCTTGACAAGCTGGATTACTGTTCAGATCTGAAGAATCTTGATC
CTTCTTTTTCTTCACCAAATTTCAAGTTTGTCAAAGGAGATATTGCGAGTGATGATCTCGTTAACTACCTTCTCATCACT
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CTAA
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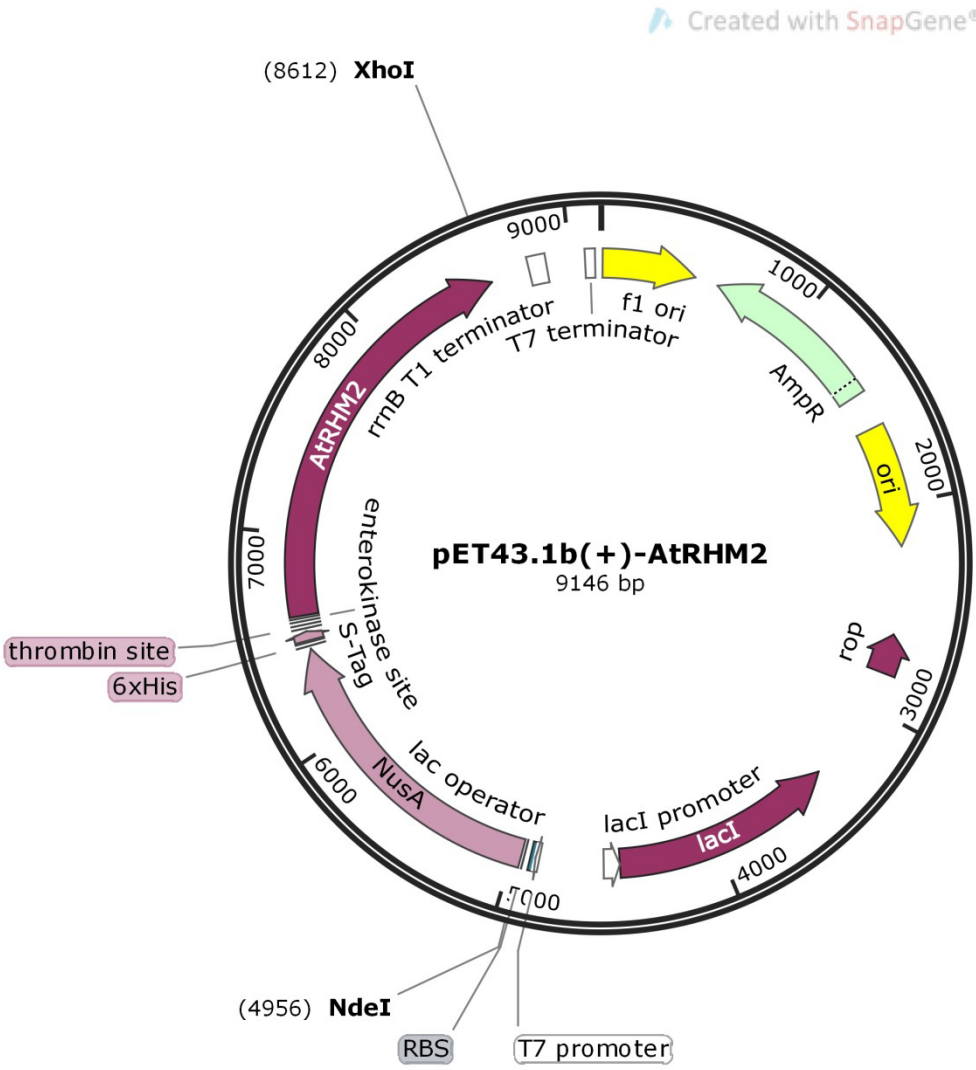

Figure 14.1: Plasmid map of the original construct of *At*RHM2.

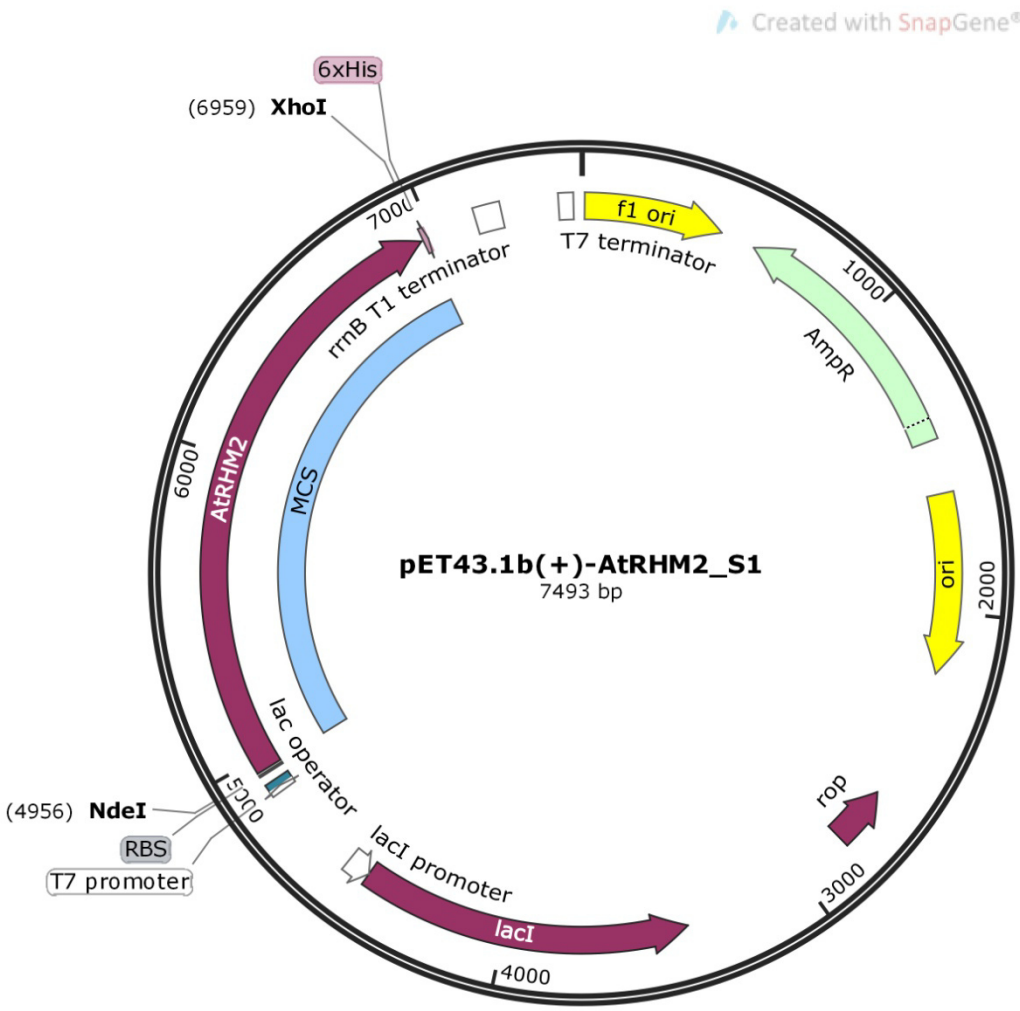

Figure 14.2: Plasmid map of strategy 1: *At*RHM2 without the N-terminal Nus-tag and with a C-terminal His6-tag.

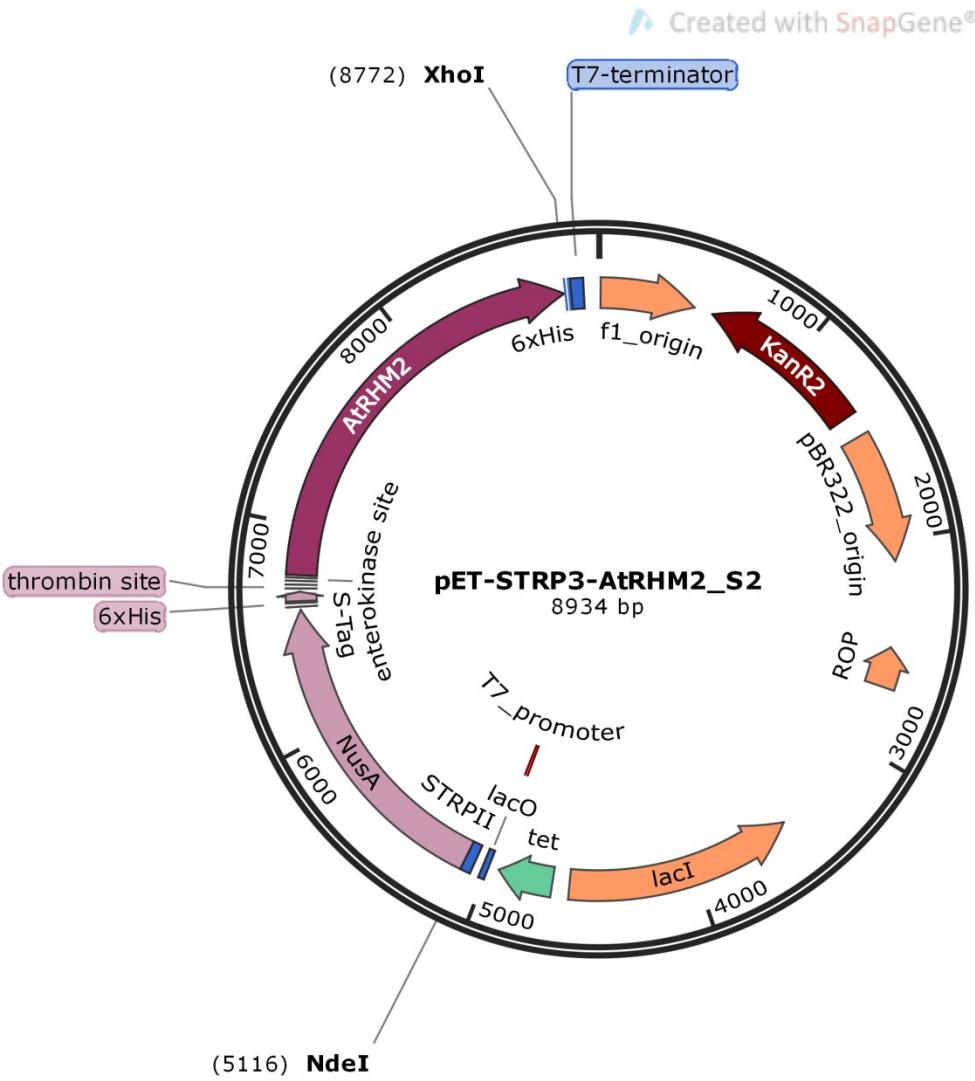

Figure 14.3: Plasmid map of strategy 2: *At*RHM2 with Nus-Tag in the pET-STRP3 vector.

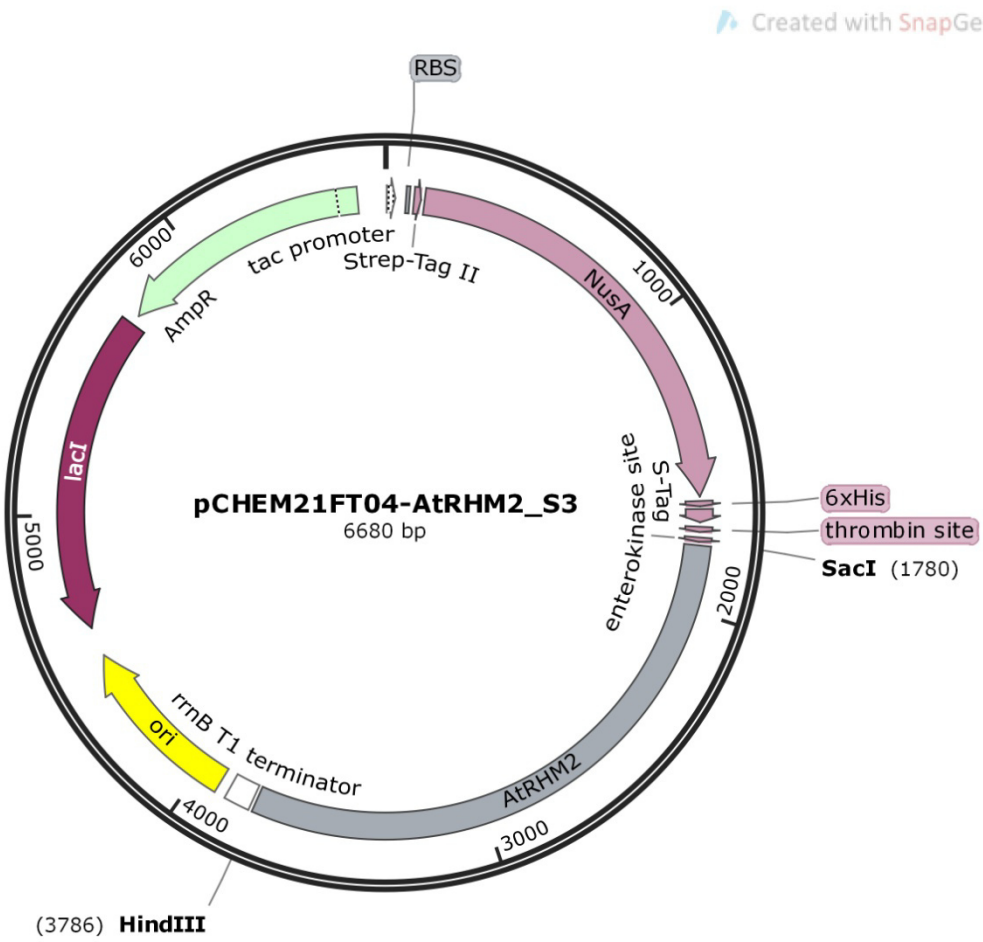

Figure 14.4: Plasmid map of strategy 3: *At*RHM2 in pCHEM FTO 4.0 vector with a N-terminal Strep-Tag and a N-terminal Nus-tag.

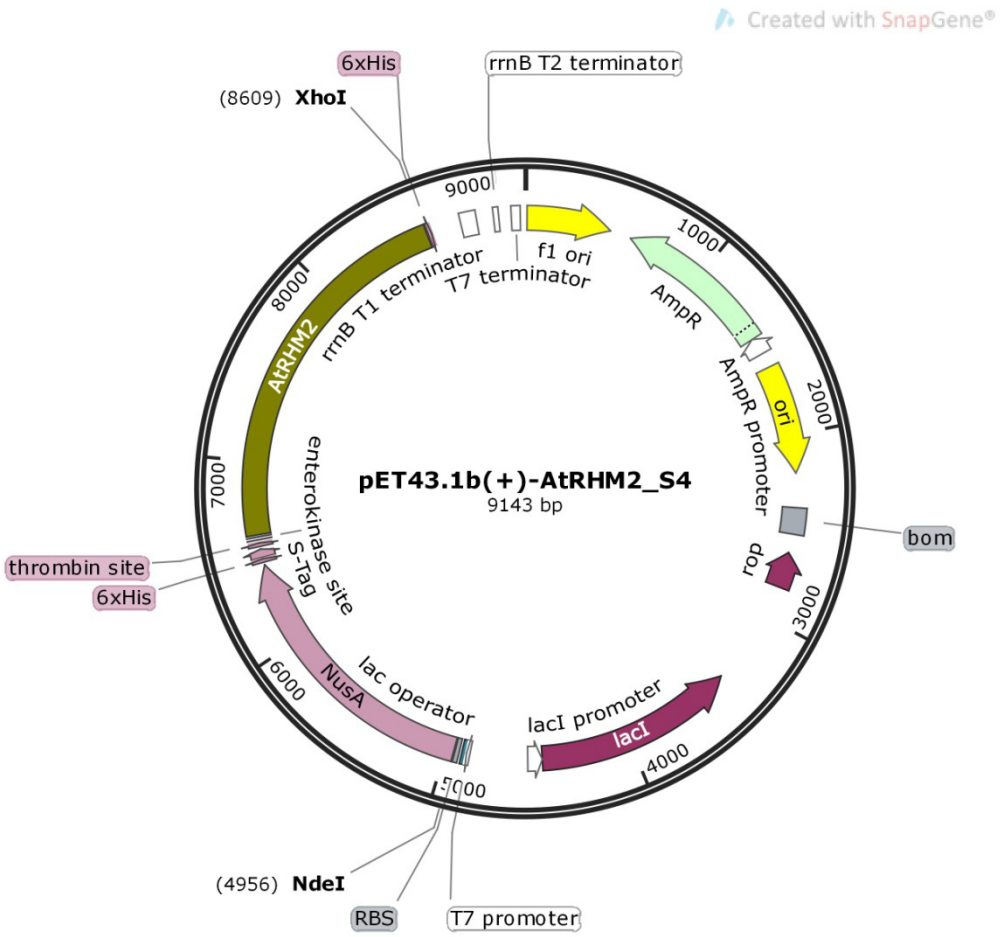

Figure 14.5: Plasmid map of strategy 4: *At*RHM2 with the N-terminal Nus-tag and a C-terminal His6-tag.

15 Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
AA	Amino acid
ACN	Acetonitrile
ADP	Adenosine 5'-diphosphate
AEC	Anion exchange
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
*Bf*FKP	bifunctional L-fucokinase/L-fucose-1-phosphate guanylyltransferase

CFE	Cell free extract
CIP	Calf intestinal alkaline phosphatase
CPEC	Circular polymerase extension cloning
CV	Column volume
dTDP	Deoxythymidine 5´-diphosphate
E. coli	*Escherichia coli*
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
HPLC	High performance liquid chromatography
IMAC	Immobilized metal ion affinity chromatography
Kbp	Kilo base pairs
K_m	Michaelis constant
LB medium	Lysogeny broth medium
LB-Amp	Lysogeny broth with ampicillin
NADH	Reduced nicotinamide adenine dinucleotide
NDP	Nucleotide diphosphate
Nt-sugar	Nucleotide sugar
OD_{600}	Optical density at 600 nm
ONC	Overnight culture
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion
TBAB	Tetra-n-butylammonium bromide

UDP Uridine 5´-diphosphate

16 References

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