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Synthesis of 2'-Fucosyllactose in one pot coupled with an efficient cofactor regeneration system

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Abstract:

Human Milk Oligosaccharides (HMOs) are highly abundant in human milk and are correlated to health benefits for infants. 50 to 80% of these oligosaccharides in human milk are fucosylated. The HMO 2'-Fucosyllactose (2'FL) is one of the sugars with the highest concentration in human milk, but could not be supplemented to formulae for bottle-fed children so far due to a shortage in economic production methods. Biotechnological approaches for 2'FL synthesis *in vitro* exist but their applicability is limited by some bottlenecks. The cheap supply of GDP-L-fucose (GDP-Fuc) - required as donor substrate for the fucosylation by α1,2-fucosyltransferases – is one barrier for a powerful *in vitro* production system. To overcome this limitation a one-pot reaction system was developed by coupling a fucosyltransferase enzyme from *Helicobacter pylori* (*Hp*Ft) and a bifunctional L-fucokinase/GDP-Fuc pyrophosphorylase from *Bacteroides fragilis* (*Bf*FKP). The integration of an efficient regeneration system based on two polyphosphate kinases (*Sm*PPK and *Aj*PPK) ensures the supply of the cofactors ATP and GTP required for the GDP-Fuc synthesis by *Bf*FKP. The cofactors are formed out of their corresponding NMPs and are regenerated during the reaction (GTP ~14 times, ATP ~28 times) from the cheap cosubstrate polyphosphate. This one-pot reaction system enables to synthesize about 13.3 mM of 2'FL (6.5g/L) within 24 hours.

Chapter 1: Manuscript

1. Introduction:

Human Milk Oligosaccharides (HMOs):

Human Milk Oligosaccharides (HMOs) are unconjugated glycans highly abundant in human milk. So far more than one hundred HMOs have been identified, but not every woman is able to synthesise the same HMOs. Also the amount and the composition of HMOs vary during the time of lactation.[1,2,3] 10-15 g of HMOs are present in one litre of human milk, which is about 100 - 1000 fold higher than the concentration in bovine milk. More and more studies postulate the health benefits of this complex sugars for infants, many of them are unique to humans.[4,5]

HMOs are composed of five monosaccharide building blocks: D- glucose (Glc), D-galactose (Gal), D-acetylglucosamine (GlcNAc), L-fucose (L-Fuc) and the sialic acid derivative N-acetyL-neuraminic acid (Neu5Ac). In HMOs lactose builds the reducing end. The HMOs can be divided into a type 1 or a type 2 chain, which depends on the elongation with Galß1-3GlcNAc (type 1 chain) or Galß1-4GlcNAc (type 2 chain). Further the HMO backbone or elongated chain can be fucosylated or sialylated. [4,6] The carbohydrate 2'-Fucosyllactose (2'FL) is a major component of HMOs. An enzyme called fucosyltransferase 2 (FUT2) can catalyse the addition of L-Fuc in an α1-2 linkage on HMOs. The concentration of 2'FL with about 1.84g/L is one of the highest regarding to the HMOs found in human milk.[3,6] But about 30 % of the Caucasian women do not express actively the FUT2 enzyme. They are called Nonsecretors and their milk lacks α1-2 fucosylated HMOs like 2'FL.[5,7–10]

HMOs of the human milk are the first prebiotics taken up by babies in their diet. Most HMOs enter the gastrointestinal tract in high concentrations and provide as substrates a growth advantage for some bifidobacterial species like *Bifidobacterium longum subsp. infantis*. The presence of this bacteria appears to have a beneficial effect to the infant. Other bacteria like *Clostridium*, *Enterococcus, Streptococcus* and *Escherichia coli* are not able to metabolise milk oligosaccharides that efficiently and grow slower or not all.[11–13]

Antiadhesive effects were also associated with HMOs. Pathogens often use glycan structure to attach to the epithelial cell surface. HMOs act as soluble decoy receptors prohibiting the

binding to glycans on the host cell, therefore reduce the risk of infections caused by viral or bacterial attachment. Bacterial diarrhea is one of the most common cause of infant mortality. Mother milk containing high levels of 2'FL lowers the number of diarrhea infections in infants caused by *Campylobacter jejuni*, which is the most common cause for diarrhea.[14]

Beside these effects it is supposed that HMOs act as antimicrobial preventing bacterial proliferation and therefore can be considered as natural template for the development of antibiotics in future.[5]

Production of fucosylated HMOs:

Chemical synthesis of HMOs is associated with extensive use of protection groups and multistep reactions.[10] Therefore a biotechnological route using fucosyltransferase catalysed reactions is a valuable alternative to produce fucosylated HMOs. The fucosyltransferases belong to the class of "Leloir" glycosyltransferases as they use GDP-L-fucose (GDP-Fuc) for the transfer of the fucosyl residue onto the acceptor. They are also known for high selectivity and low hydrolysis activity on the donor substrate and fucoside products synthesized. One disadvantage is the very expensive donor substrate GDP-Fuc especially for *in vitro* reactions. To overcome this problem multi enzyme cascade reactions, which synthesize GDP-Fuc *in situ*, starting with L-Fuc have been developed (reviewed from Petschacher et al 2016).[6] In these one pot (OP) reactions mostly the bifunctional L-fucokinase/GDP-Fuc pyrophosphorylase of *Bacteroides fragilis* (*Bf*FKP) is involved in the supply of GDP-Fuc, but for this reaction the expensive cofactors ATP and GTP in stochiometric amounts are necessary. *In vivo* synthesis of fucosylated HMOs presents a possibility to overcome this problem. [15,16] The economic feasibility of *in vitro* systems for enzymatic fucosylations is enhanced by involving a recycling system for ATP and GTP.

Polyphosphate kinases (PPK):

A regeneration system based on PPKs and Polyphosphate (PolyP) is interesting due to the availability and the low price of PolyP in comparison to the commonly used phosphoenolpyruvate (PEP), which is the most expensive phosphate donor.[17,18] The phosphate donor inorganic polyphosphate (PolyP) is a linear polymer of ten to hundred phosphate (P_i) residues linked by high energy phosphoanhydride bonds and has been found in all living organisms. [19]

PPKs can be divided into two families PPK1 and PPK2. The enzymes belonging to PPK1 family favour the catalysis of the PolyP synthesis using nucleoside triphosphates (NTP) as substrate. The PPK2 enzymes preferentially catalyse the phosphorylation of nucleoside monophosphate (NMP) or nucleoside diphosphate (NDP) and PolyP degradation. Further the PPK2 family can be divided into three subfamilies (class I, II and II) based on their preference using mono and/or dinucleotides as phosphate acceptor substrates. Class I and II catalyse the PolyP dependent phosphorylation of NMP and NDP, respectively. Class III is catalysing phosphorylation of NMP and NDP as well. [20–22]

Focus of the thesis:

The focus of this work is the synthesis of the α1-2 fucosylated HMO 2'FL coupled with the integration of an efficient and cheap regeneration system to reduce production costs. To achieve this aim, an enzyme cascade was established to synthesise 2'FL by a one-pot reaction (OP reaction). The expensive donor substrate GDP-Fuc for the fucosyltransferase is supplied within the one pot by the addition of the bifunctional *Bf*FKP enzyme from *Bacteroides fragilis*. For the conversion of the initial substrate L-fucose to GDP-Fuc the cofactors GTP and ATP are needed, which were generated from their corresponding NMP or NDP by the PPKs from *Sinorhizobium meliloti* (*Sm*PPK) and *Acinetobacter johnsonii* (*Aj*PPK) in the beginning of the reaction. During the reaction, the utilized cofactors are regenerated by the PPKs. The inorganic pyrophosphatase (iPPase) from *Escherichia coli* hydrolyses the generated by-product pyrophosphate to accelerate the GDP-Fuc production. The fucosyltransferase from *Helicobacter pylori* (*Hp*Ft) utilizes the GDP-Fuc for the fucosylation of lactose.

2. Materials:

PolyP was obtained from Sigma-Aldrich (Vienna, Austria) and Merck (Darmstadt, Germany) and the nucleotides were from Carbosynth (Berkshire, UK). The sugars 2'-Fucosyllactose and GDP-fucose were purchased from Isosep (Tullinge, Sweden), L-fucose from Roth (Karlsruhe, Germany). The His-Trap FF columns were from GE Healthcare (Vienna, Austria), the StrepTrap HP columns and the chemicals for the Strep-tag affinity chromatography were from IBA (Göttingen, Germany). The Vivaspin concentrator tubes (10 kDa cut-off) were from Sartorius (Göttingen, Germany). SDS-PAGE (NuPAGE Bis- Tris Precast Gels) and the protein ladder (PageRuler unstained) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The protein dye Roti-quant was from Roth. A Kinetex C18 column from Phenomenex (Aschaffenburg, Germany) and CarboPac PA10 column from Thermo Fisher Scientific were purchased. All other chemicals and reagents used in this master thesis were obtained from Roth, Merck or Sigma-Aldrich.

3. Methods:

3.1 Protein Expression:

Polyphosphate kinases (PPK):

The cultivation and expression of *Sm*PPK and *Aj*PPK was done in shake flask cultures with LB media containing 10 g/L tryptone, 5g/L NaCl and 5 g/L yeast extract. Precultures of the expression host were cultivated in 70 mL of LB selection media (100 µg/mL kanamycin) in shake flasks overnight at 37°C at 100rpm. The main culture of 300 mL (LB selection media) was induced with the preculture to an OD_{600} of 0.05 and incubated to a maximum OD_{600} of 0.8 at 37°C and 100 rpm. After cooling down for 20 minutes, expression was induced by the addition of IPTG to 0.5 mM at 18°C overnight. Cells were harvested by centrifugation for 30 minutes at 5000rpm. The pellet was dissolved in 50 mM Tris-HCl buffer (pH 7.6) containing 500 mM of NaCl and 20 mM of imidazole. The cell solution was frozen at -20°C or directly sonicated for enzyme purification.

***Bf*FKP:**

The cultivation and expression of *Bf*FKP was also done in shake flasks and LB media, but with some differences. In contrast to the PPKs, the volume of the pre- and main culture was 50 mL and 250 mL respectively. The selection media contains 100 µg/mL ampicillin and the expression was induced by a final concentration of 0.2 mM of IPTG. The protein expression was performed at 25°C overnight.

***Hp*Ft:**

The same procedure as for *Bf*FKP was applied for the expression and cultivation of *Hp*Ft. The differences were the change of the IPTG concentration to 0.01 mM by inducing the main culture and that the pellet was dissolved in a buffer containing 100 mM of Tris, 150 mM of NaCl and 1 mM of EDTA at pH 8.0.

3.2 Enzyme Purification:

The cell solution was thawed and sonicated or sonicated directly after harvesting the cells. After disrupting the cells and removing cell debris, the clear lysate was used for purification.

His Tag:

For His- tag affinity purification of *Sm*PPK, *Aj*PPK and *Bf*FKP two 5 mL His-Trap columns were used on an ÄktaPrime plus system (GE Healthcare) cooled at 4°C.

The enzyme was bound at a constant flow rate of 0.5 mL min^{-1} of a 50 mM Tris-HCl buffer (pH 7.6) containing 500 mM of NaCl and 20 mM of imidazole. After removing the unbound proteins, the bound protein was eluted using a constant 100 mL long gradient from 20 to 250 mM imidazole at a flow rate of 2 mL min^{-1}. After the first purification a step gradient elution was established. A step at 50 mM of imidazole to elute the weak unspecific bound proteins and then a step at 250 mM of imidazole to elute the enzyme was applied. To remove the imidazole and to concentrate the enzyme, ultrafiltration with a Vivaspin concentrator tube was applied until the imidazole concentration was under 1 mM of imidazole. Aliquots were stored at 4°C or -20°C respectively.

Strep Tag:

For Strep- tag affinity purification of iPPase and *Hp*Ft two 5 mL StrepTrap HP columns were connected in series on an ÄktaPrime plus system cooled at 4°C. The enzyme was bound at a

constant flow rate of 1.5 mL min^{-1} using a Buffer (pH 8.0) containing 100 mM of Tris, 150 mM of NaCl and 1 mM of EDTA. The protein was eluted by a step gradient of 2.5 mM of desthiobiotin at a flow rate at 3 mL min^{-1}. To concentrate the enzyme, ultrafiltration with a Vivaspin concentrator tube was applied. Aliquots were stored at 4°C or -20°C respectively.

The protein concentration was determined using the Bradford method [23]. As protein dye Roti-quant was used. 1 mL of the of the dye was mixed with 20 µl of sample. The absorbance was measured at 595 nm and the protein concentration was determined through a BSA standard calibration curve.

The purity of the proteins was analysed by SDS-PAGE. For the determination of the molecular mass a protein ladder (PageRuler unstained) was applied and the gel was stained with Coomassie Blue.

3.3 Activity assays:

Analysis of nucleotides and GDP-fucose:

To observe the nucleotides and GDP-Fuc during the activity assays and the OP reactions, samples were withdrawn and stopped by addition of ACN to 40 vol % and analysed on a HPLC (Shimadzu, Kyoto, Japan; Agilent, Santa Clara, United States) using a Phenomenex Kinetex C18 column. A flow rate of 2 mL/min was applied with a mobile phase of 6.5 % acetonitrile and 93.5 % of a TBAB buffer (40 mM TBAB, 20 mM KH_2PO_4, pH 5.9). The oven temperature was set to 40°C and the absorbance at 262 nm was monitored with a multiple wavelength detector.

Analysis of the sugars:

For the detection of the sugars, samples were stopped by heating for 5 min at 90 °C or by mixing 1:1 with 50 mM of NaOH. The sugars were analysed on a high-performance anion exchange-pulsed amperometric detection (HPAEC-PAD) system (Dionex Corporation, Sunnyvale, USA) using a CarboPac PA10 column. The eluent contains a mixture of 50 mM of NaOH and 25 mM of NaOAc. The sugar concentration in the sample was determined using a calibration curve with commercial available sugars 2'FL and L-Fuc.

Activity of *Bf*FKP:

The synthesis of GDP-Fuc was monitored over 1 hour by HPLC to determine the volumetric activity. The assay was performed in a 250 µl reaction mixture containing 5 mM of L-fucose, 10 mM of ATP, 5 mM of GTP, 5 mM of $MgCl_2$, 1.4 U of iPPase and 0.2 mg/mL *Bf*FKP in 50 mM Tris/HCl (50 mM, pH 7.6) mixed with 350 rpm at 37°C. To get the same conditions as in the OP reaction the $MgCl_2$ concentration was adjusted to 10 mM and 50 mM of PolyP was added.

Activity of *Aj*PPK and *Sm*PPK:

The phosphorylation of GMP or GDP was monitored over 1 hour by HPLC for the determination of the volumetric activity. Because of the preference of the PPK to phosphorylate adenosine nucleotides, the phosphorylation of the guanosine nucleotides is determining for the volumetric activity. The assay was performed in a 250 µl reaction mixture containing 5 mM of GMP and AMP or GDP and ADP, 10 mM $MgCl_2$, 50 mM of PolyP and 0.005 mg/mL *Sm*PPK or 0.1mg/mL *Aj*PPK in 50 mM Tris/HCl (50 mM, pH 7.6), mixed with 350 rpm at 37°C.

Activity of *Hp*Ft:

The synthesis of 2'FL was monitored over 1 hour by HPAEC-PAD to determine the volumetric activity. The assay was performed in a 300 µl reaction mixture containing 1 mM of GDP-Fuc, 5 mM of lactose, 5 mM of $MgCl_2$ and 0.2 mg/mL *Hp*Ft in Tris/HCl (50 mM, pH 7.6), mixed with 350 rpm at 37°C.

3.4 Other experimental methods:

Determination of pH value:

To optimise the pH value, OP reactions of total 500 µl containing L-Fuc (10 mM), ADP and GDP (5 mM), $MgCl_2$ (10 mM), PolyP (50 mM) and lactose (10 mM) were shaken at 350 rpm and 37°C. A 50 mM Tris/HCl buffer was used for the reactions at pH 7.6 and 7.0. To obtain pH 6 and 5.5 in the OP reaction a 50 mM MES buffer was used. The reaction was catalysed by 0.05 mg/mL *Sm*PKK, 0.3 mg/mL *Bf*FKP, 0.02 mg/mL *Hp*Ft and 0.5 U iPPase. The samples were stopped by heating for 10 min at 90°C and were centrifuged to separate denatured enzymes. The supernatant was analysed by HPLC and HPAEC-PAD.

PEP/PK vs. PolyP/*Sm*PPK:

The PK/LDH enzyme mixture from rabbit muscle (Sigma-Aldrich) was used for the regeneration of the NTPs in the PEP/PK system instead of *Sm*PPK. OP reactions of 350 µl were examined in a 50 mM Tris/HCl buffer (pH 7.6) at 350 rpm and 37°C. The solution contains 10 mM of L-Fuc, 2 mM of ADP and GDP, 10 mM of $MgCl_2$, 20 mM of PolyP/PEP, 10 mM of lactose. The reaction was catalysed by 0.05 mg/mL *Sm*PPK, 0.3 mg/mL *Bf*FKP, 0.4 mg/mL *Hp*Ft and 0.5 U iPPase. The PEP/PK system contains 2U PK/LDH instead of *Sm*PPK. The samples were stopped by heating 10 min at 90°C and were centrifuged to get rid of the enzymes. The supernatant was analysed by HPLC and HPAEC-PAD.

Influence of the initial cofactor concentration (A):

Various initial cofactor concentrations (2, 4 and 8 mM of NDP) were added to the OP reaction, to observe their impact. To an OP reaction of 500 µl containing 10 mM of L-Fuc, 10 mM of $MgCl_2$, 10 mM of lactose, 50 mM of PolyP and either 2,4 or 8 mM of ADP and GDP in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 40 mU of *Bf*FKP, 10 mU of *Hp*Ft and 0.5 U iPPase were added. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by heating the samples for 10 min at 90°C and then centrifuged to denature the enzymes. The supernatant was analysed by HPLC and HPAEC-PAD.

Influence of the initial cofactor concentration (B):

Various initial cofactor concentrations (1, 2 and 4 mM of NMP) were added to the OP reaction, to observe the impact on the system, which comprises also *Aj*PPK. To an OP reaction of 500 µl containing 10 mM of L-Fuc, 10 mM of $MgCl_2$, 10 mM of lactose, 50 mM of PolyP and either 1, 2 or 4 mM of AMP and GMP in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 80 mU of *Aj*PPK, 40 mU of *Bf*FKP, 10 mU of *Hp*Ft and 0.5 U iPPase were added. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by mixing 1:1 with 50 mM of NaOH.

Influence of the lactose concentration:

Various lactose concentrations (10, 20, 30, 40 and 50 mM of lactose) were added to the OP reaction, to observe the impact on the system. To a OP reaction of 500 µl containing 10 mM of L-Fuc, 10 mM of $MgCl_2$, 50 mM of PolyP, 1 mM of AMP and GMP and either 10, 20, 30, 40 and 50 mM of lactose in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 120 mU of

*Aj*PPK, 80 mU of *Bf*FKP, 40 mU of *Hp*Ft and 0.5 U iPPase were added. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by mixing 1:1 with 50 mM of NaOH.

Optimisation of the enzyme amount:

The *Bf*FKP and the *Hp*Ft amount was increased to exhaust the regeneration system and to increase the productivity. To an OP reaction of 500 µl containing 10 mM of L-Fuc, 10 mM of $MgCl_2$, 50 mM of PolyP, 1 mM of AMP and GMP and either 10 mM of lactose in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 120 mU of *Aj*PPK, 40 or 80 mU of *Bf*FKP, 10 or 40 mU of *Hp*Ft and 0.5 U iPPase were added. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by mixing 1:1 with 50 mM of NaOH.

Optimisation of the cofactor concentration:

The initial cofactor concentrations were varied to find best composition for an efficient and productive OP reaction. To an OP reaction of 500 µl containing 10 mM of L-Fuc, 10 mM of $MgCl_2$, 50 mM of PolyP, 30 mM of lactose in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 12 mU of *Aj*PPK, 80 mU of *Bf*FKP, 30 mU of *Hp*Ft and 0.5 U iPPase were added. for reaction (A) 1 mM of AMP and GMP, for reaction (B) 0.5 mM of AMP and GMP, for reaction (C) 0.5 mM AMP and 1 mM of GMP and for reaction (D) 0.25 mM of AMP and 1 mM of GMP was supplied as initial cofactor concentration. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by mixing 1:1 with 50 mM of NaOH.

OP synthesis of 2'FL:

The best reaction conditions and parameters like enzyme amount, initial cofactor and sugar concentration which were determined in previous experiments, were combined to enhance the 2'FL productivity and the regeneration efficiency in the OP system. To an OP reaction of 500 µl containing 20 mM of L-Fuc, 10 mM of $MgCl_2$, 0.5 mM of AMP, 1 mM of GMP, 50 mM of PolyP, 50 mM of lactose in a 50 mM Tris/HCl buffer (pH 7.6) 120 mU of *Sm*PPK, 12 mU of *Aj*PPK, 120 mU of *Bf*FKP, 80 mU of *Hp*Ft and 0.5 U iPPase were added. The mixture was incubated at 37°C and shook with 350 rpm. The samples were stopped by mixing 1:1 with 50 mM of NaOH.

4. Results:

4.1 OP reaction with one polyphosphate kinase:

The aim of this study was to establish a functional OP reaction system for 2'FL production, which comprises the generation of GDP-Fuc, the synthesis of 2'FL and the regeneration of the required cofactors. Fig. 1 shows the scheme of the OP reaction, which starts with L-fucose, ADP, GDP and lactose as substrates and ends with 2'FL. The *Sm*PPK enzyme belongs to class I of the PPK2 family and can catalyse the phosphorylation of NDP to NTP.[24] The focus in this first part of this thesis was to investigate and determine the optimal reaction conditions like pH, cofactor concentration and to understand the characteristics and interactions between the reactions in the designed OP reaction system.

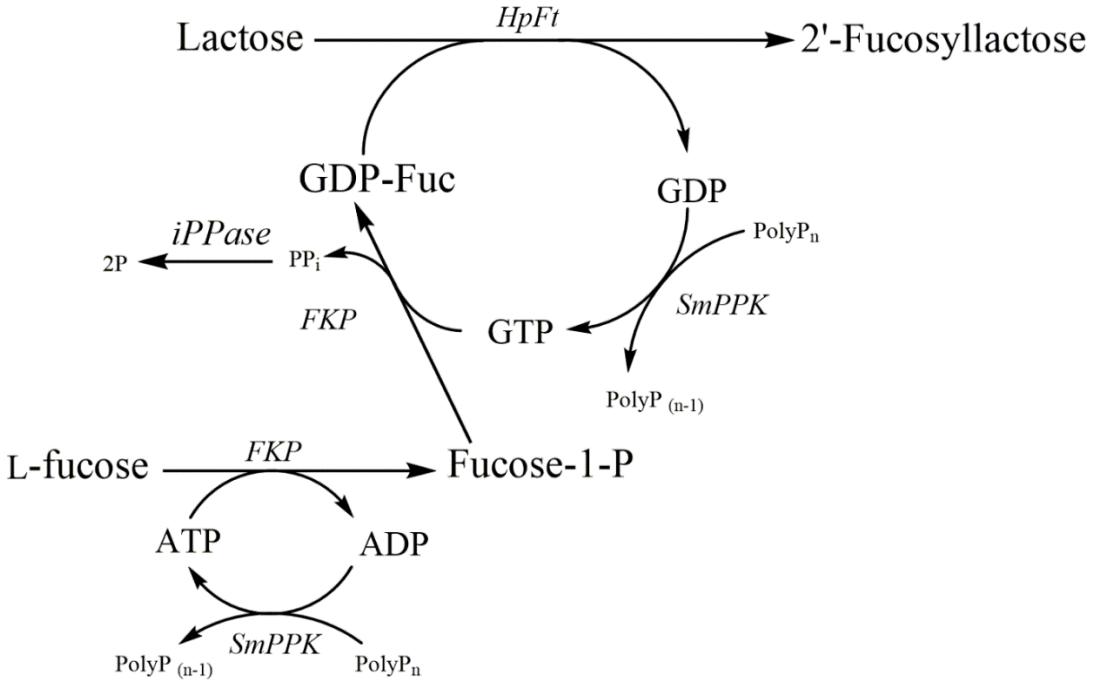

Fig. 1: Schematic figure of the one-pot reaction including the regeneration system. L-fucose is converted via the reaction intermediate Fucose-1-phosphate to GDP-fucose by bifunctional L-fucokinase/GDP-Fuc pyrophosphorylase from *Bacteroides fragilis (Bf*FKP). The inorganic pyrophosphatase (iPPase) from *E.coli* catalyses the hydrolysis of the by-product PPi. The fucosyltransferase from *Helicobacter pylori (Hp*Ft*)* catalyses the fucosylation of lactose, GDP-Fucose acts as donor. The polyphosphate kinase from *Sinorhizobium melilot* (*Sm*PPK) as regeneration system catalyses the phosphorylation of the NDPs.

4.1.1 pH optimum:

To select the best condition for the OP reaction pH values between 5.5 and 7.6 were chosen to determine the effect on the synthesis of 2'FL and GDP-Fuc. The published pH optimum of *Hp*Ft is at pH 5 and the pH optimum of *Bf*FKP at 7.6.[25,26] The *Sm*PPK enzyme was added in an excessive amount to overcome limitation of NTPs in one of the reactions. 6 hours after the reaction start 2'FL can be detected in the samples at pH 7.6, 7 and 6. GDP-Fuc was synthesised in all samples from the beginning (B) and was available for the fucosylation of lactose by *Hp*Ft. In our OP reaction system, the GDP-Fuc synthesis works best in a pH range of 7-7.6 (Fig. 2 B), but also the reactions with a lower pH show a permanent production of GDP-Fuc over a reaction period of 24 hours.

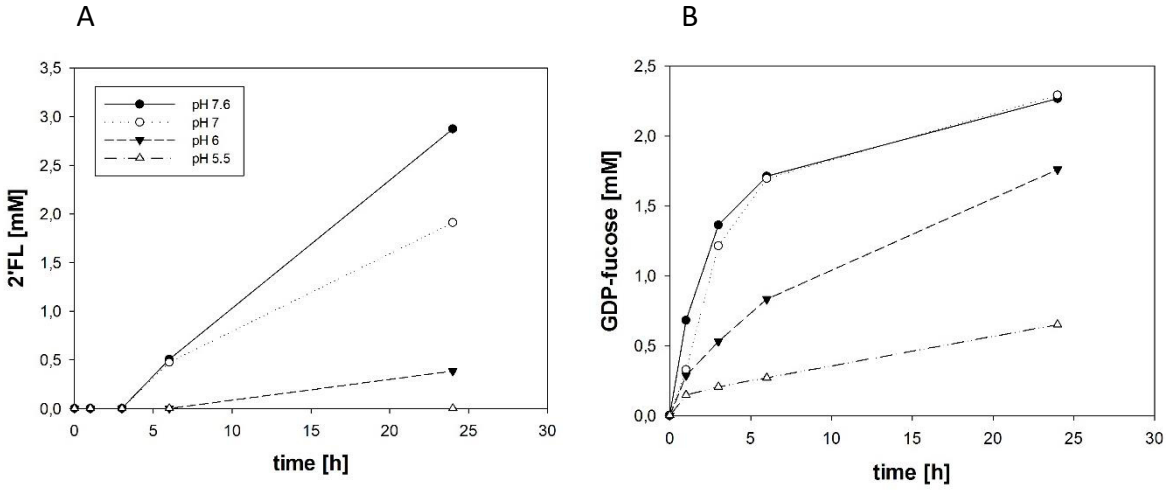

Fig. 2: Effect of the pH value on the synthesis of 2'FL (A) and GDP-fucose (B) in a typical OP reaction over a period of 24 hours. The assays were performed in 50 mM buffer at respective pH (Tris/HCl pH 7.6 and 7, MES pH 6 and 5.5). 0.05 mg/mL *Sm*PKK, 0.3 mg/mL *Bf*FKP, 0.02 mg/mL *Hp*Ft and 0.5 U iPPase were incubated at 37°C with L-fuc (10 mM), ADP and GDP (5 mM), $MgCl_2$ (10 mM), PolyP (50 mM) and lactose (10 mM) at 350 rpm. The higher the pH value the higher is the productivity of the reaction intermediate (GDP-Fuc) and of the product (2'FL).

More interesting is the fact, that 2'FL was not synthesised at a pH at 5.5, which is near the optimum described in literature, although the reaction intermediate GDP-Fuc was available in the OP reaction. The higher the pH value, the higher is the productivity of 2'FL and GDP-Fuc in the OP reaction system. The most productive conditions for this OP reaction system is clearly at a pH of 7.6. The sample at a pH of 7 shows 67% of the productivity of the sample at pH 7.6 over a period of 24 hours. Due to this results a pH value of 7.6 was determined as the most promising condition for this OP reaction system.

4.1.2 PEP/PK vs. PolyP/*Sm*PPK:

To estimate roughly the ability and capability of the PPK regeneration system, it was compared with the PEP/PK system. The regeneration system based on phosphoenolpyruvate (PEP) as phosphate donor can be employed to phosphorylate NDP to NTP. Pyruvate Kinase (PK) catalyses the transfer of the phosphoryl group to the NDP in this common regeneration system.[17,18] But PEP is compared to PolyP a very expensive phosphate donor.

To compare the PPK with the PEP system, two OP reactions as similar as possible either with PEP or PolyP as polyphosphate donor were performed at the same conditions. The enzymes were added in a surplus to overcome limitation effects. In the first 6 hours both samples behave nearly the same related to the 2'FL synthesis, but after 24 hours the system based on PPK and PolyP increases the difference in the 2'FL concentration (Fig. 3).

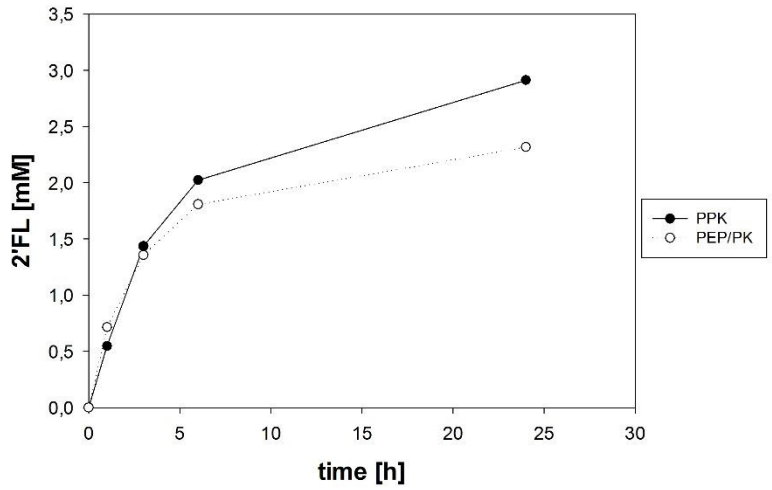

Fig. 3: Synthesis of 2'FL over time in a OP reaction using PPK system vs. PEP/PK. Reaction conditions for the OP reactions: 10 mM L-fuc, 2 mM ADP and GDP, 10 mM $MgCl_2$, 20 mM PolyP/PEP, 10 mM lactose at 37°C, pH 7.6 and 350 rpm. The assay was catalysed by 0.05 mg/mL *Sm*PPK, 0.3 mg/mL *Bf*FKP, 0.4 mg/mL *Hp*Ft and 0.5 U iPPase. The PEP/PK system contains 2U PK/LDH instead of *Sm*PPK.

In the first 6 hours of reaction the amount of ATP and GTP was moderately higher in the reaction with PEP due to a higher reaction equilibrium on the NTP side, but had no positive influence on the synthesis of 2'FL. After 24 hours the amount of GTP and ATP decreases in both assays, but more significantly in the reaction with the PEP/PK system (Fig. S 6). The PPK system shows a higher stability under these certain conditions. It was observed that the enzymatic activity of PK is decreased to 50 % over 24 hours of incubation at 37°C.[27] Beside

the less expensive phosphate donor the PolyP/*Sm*PPK system shows a higher 2'FL productivity.

4.1.3 Influence of the initial cofactor concentration (A):

A decreased enzymatic activity caused from instability under storing conditions was observed for several of the applied enzymes. The enzyme amount supplied to the OP reaction was calculated by the protein concentration in the previous OP reactions. To overcome instability issues, the addition of the enzymes was now calculated accurately by determination of the volumetric activity of each enzyme before starting the OP reaction. The result was a higher reproducibility and more efficient OP reaction system.

The influence of the initial cofactor concentration was investigated by supplying various initial cofactor concentrations to the OP reaction (Fig. 4). In the first 6 hours of reaction the productivity in all samples was nearly the same, then the 2'FL concentration drifts in the samples. The results after 24 hours indicate, that a higher initial cofactor concentration lead to a higher productivity. The reason is, that due to the higher initial cofactor concentration a higher GTP and ATP concentration is steadily available in the OP reaction. The outcome is a higher concentration of GDP-Fuc (Fig. S 7), which leads to a higher productivity. Fig. 4 shows that the productivity in the sample with 8 mM of GDP and ADP was 38% higher than in the sample with 2 mM of ADP and GDP. This demonstrate obviously, the influence of a fourfold higher cofactor concentration (8 mM vs. 2 mM). The results of the reaction with 4 mM of the cofactors confirms the hypothesis, that a higher cofactor concentration leads to a higher productivity.

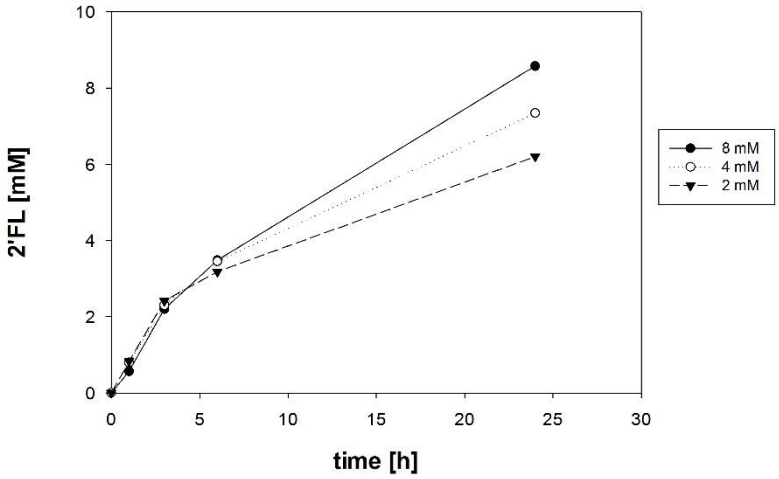

Fig. 4: Influence of various initial cofactor concentrations on the 2'FL synthesis. The initial cofactor concentration was either 2, 4 or 8 mM GDP and ADP. 120 mU *Sm*PPK, 40 mU *Bf*FKP, 10 mU *Hp*Ft and 0.5 U iPPase were incubated in a 50 mM Tris/HCl buffer (pH 7.6) with 10 mM L-fuc, 10 mM $MgCl_2$, 10 mM lactose, 50 mM PolyP and various cofactor concentrations at 37°C and 350 rpm.

Besides the productivity, the regeneration efficiency is also an important criterion to determine the most promising condition. Regarding the regeneration times of the cofactors, the 2 mM NDP sample was the most efficient. In this sample NTPs can be regenerated at least 3 times comparing with 1.8 times (4 mM NDP) and 1 time (8 mM NDP) of the other samples.

To get a valuable OP reaction it is necessary to find a reaction condition, which enhances productivity and regeneration efficiency as well.

4.2 OP reaction with two polyphosphate kinases:

In order to establish a more efficient regeneration system starting from the nucleoside monophosphates the *Aj*PPK enzyme was also integrated in the OP reaction. The *Aj*PPK enzyme is a class II PPK, which catalyses the PolyP depended phosphorylation of nucleoside monophosphates to their corresponding diphosphates.[28] The enzyme enables to start with GMP and AMP as initial cofactors, which are about tenfold cheaper than GDP and ADP. Moreover, NMP generated as by-product during the reaction can be regenerated (Fig. S 8). The focus was to find the best reaction conditions to combine a high 2'FL productivity and an efficient regeneration system in one reaction system.

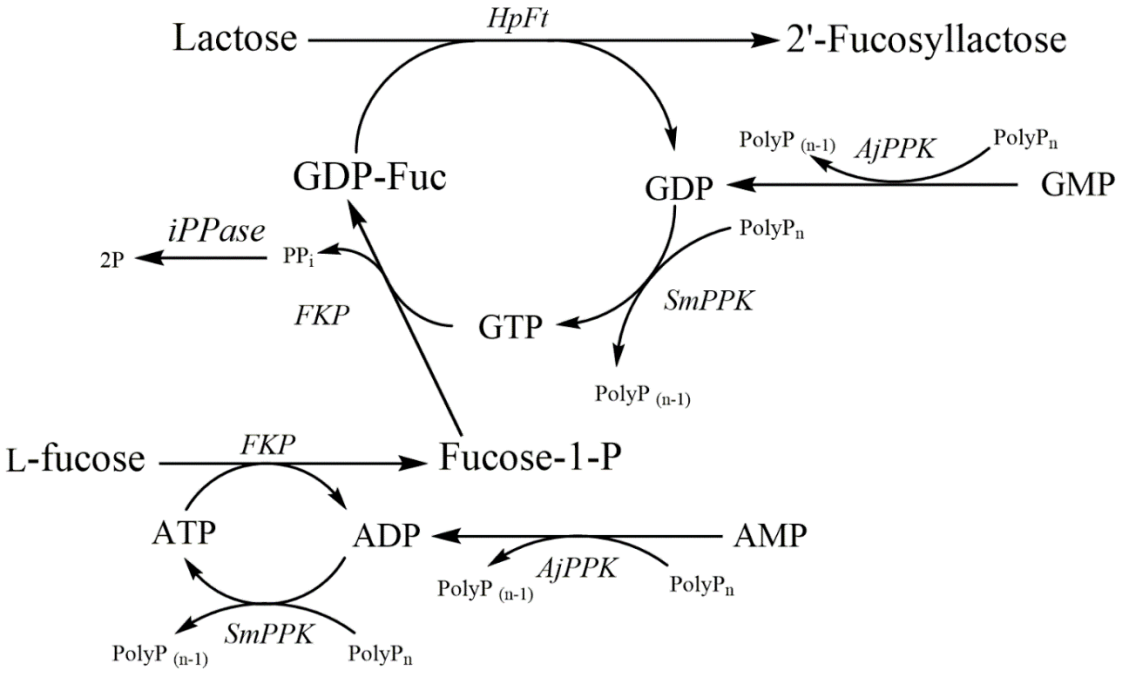

Fig. 5: Schematic figure of the one-pot reaction including the *Aj*PPK enzyme. In comparison to Fig. 1 the regeneration system is enlarged by a second PPK from *Acinetobacter johnsonii* (*Aj*PPK), which enables to start the reaction with NMPs instead of NDPs.

4.2.1 Influence of the initial cofactor concentration (B):

Various initial cofactor concentrations were supplied to the OP reaction, to determine the influence of the cofactors on the regeneration system enlarged by *Aj*PPK.

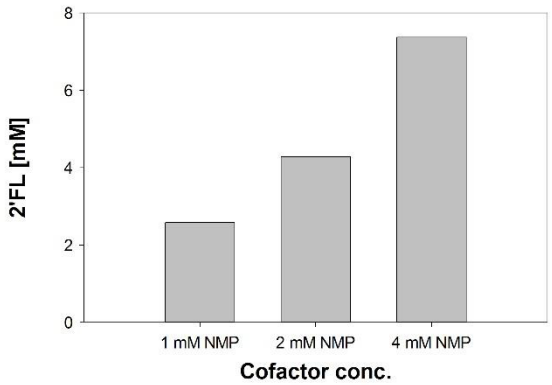

Fig. 6: Influence of the cofactor concentration on the OP reaction with *Aj*PPK and *Sm*PPK. 120 mU *Sm*PPK, 80 mU *Aj*PPK, 40 mU *Bf*FKP, 10 mU *Hp*Ft and 0.5 U iPPase were incubated for 24 hours with 10 mM L-fuc, 10 mM $MgCl_2$, 10 mM lactose, 50 mM PolyP and either 2,4 or 8 mM of ADP and GDP in a 50 mM Tris/HCl buffer at pH 7.6, 37°C and 350 rpm.

To compare the regeneration efficiency in the different assays the value "nPO_4" was introduced, which describes the number of phosphate groups transferred. A value of 1 nPO_4

means that 1 mM of NMP was converted to 1 mM of NDP. The standard value "regeneration cycles" describes only the regeneration from NDP to NTP. The value nPO_4 includes also the conversion from NMP to NDP.

Table 1: Productivity and regeneration efficiency in a OP reaction with *Aj*PPK and *Sm*PPK. Various initial cofactor concentrations were applied in the assays (1, 2 and 4 mM of NMP). The table shows the results of Fig. 6 and contrasts the productivity to the regeneration efficiency after 24 h.

	2'FL [mM]	**nPO_4**
1 mM NMP	2.6	7.1
2 mM NMP	4.3	6.3
4 mM NMP	7.4	5.7

It was possible to catalyse the phosphorylation of AMP and GMP to their corresponding nucleoside triphosphate in one pot by coupling the enzymes *Aj*PPK and *Sm*PPK. Further on the reaction intermediate GDP-Fuc and the product 2'FL were synthesised. The productivity was increasing with the cofactor concentration (7.4 mM vs. 2.6 mM of 2'FL). The results show the same influence on the OP reaction system as with the *Sm*PPK enzyme alone (Fig. 4). Regarding the regeneration system, the OP reaction with the lowest initial cofactor concentration was the most efficient one. To compare the regeneration efficiency in the different assays the value "nPO_4" was introduced, which describes the number of phosphate groups transferred. A value of 1 nPO_4 means that 1 mM of NMP was converted to 1 mM of NDP. The standard value "regeneration cycles" describes only the regeneration from NDP to NTP. The value nPO_4 includes also the conversion from NMP to NDP (Table 1). By looking on the regeneration cycles GTP was regenerated at least 2.6 times in the reaction with 1 mM NMP compared to 1.5 times in the reaction starting with 4 mM NMP. The cofactors in the sample with an initial cofactor concentration of 2 mM were regenerated about 2 times.
This result showed, that the regeneration system itself is more efficient using low levels of cofactors, but the productivity is less good.

4.2.2 Optimisation of the enzyme amount:

Regarding to the results showed in Fig. 6 the regeneration system itself look promising, because no major inhibition or instability factors were observed within 24 hours of reaction. To enhance the productivity, the amount of *Bf*FKP and *Hp*Ft in the reaction was increased. The amount of *Bf*FKP was doubled, the amount of *Hp*Ft was quadrupled.

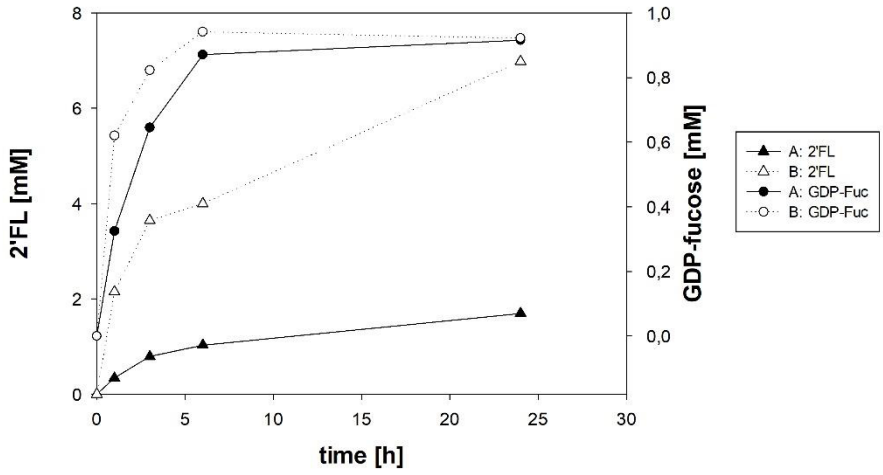

Fig. 7: Synthesis of 2'FL and GDP-fucose in a OP reaction over 24 hours with various enzyme amounts. Black symbols display the OP reaction with 10 mU *Hp*Ft and 40 mU *Bf*FKP (A). White symbols display the OP reaction with 40 mU *Hp*Ft and 80 mU *Bf*FKP (B). The dots display the concentration of the reaction intermediate GDP-Fuc. The triangles show the concentration of the product 2'FL. Reaction conditions: 10 mM L-fuc, 10 mM $MgCl_2$, 50 mM PolyP, 1 mM AMP and GMP,10 mM lactose incubated with 120 mU *Sm*PPK, 120 mU *Aj*PPK, 0.5 U iPPase and 40 or 80 mU of *Bf*FKP, 10 or 40 mU of *Hp*Ft at 37°C and 350 rpm in a 50 mM Tris/HCl buffer (pH 7.6).

The OP reaction (B) with the fourfold *Hp*Ft amount synthesised about 3.5-fold more 2'FL than the reaction (A) with the fourfold lower enzyme amount. In contrast, despite doubling the amount of *Bf*FKP the course of GDP-Fuc formation was in both reactions nearly the same, except a slightly faster synthesis in the first three hours in reaction B (Fig. 7). But 6 hours after starting the reaction, the GDP-concentration in both reactions was almost the same and stayed at a high level (more than 90 % of initial GMP is converted to GDP-Fuc).

The increase of the *Bf*FKP enzyme amount provides directly a high level of GDP-Fuc, which is then steadily present in the OP reaction. This indicates, that the regeneration system, composed of *Aj*PPK and *Sm*PPK, generates rapidly GTP and ATP and is not a limiting factor in the enzyme cascade for the supply of the donor substrate. It depends only on the total activity of *Bf*FKP added to the OP reaction. The number of nPO_4 was also increased from 6 to 16 (A vs. B), which also points out the good functionality of the regeneration system. In terms of regeneration efficiency, GTP and ATP was regenerated in reaction B at least 7 times, compared to 1.7 times in reaction A.

The 3.5-fold enhanced productivity is almost directly proportional to the 4-fold increase of the supplied *Hp*Ft amount, indicating that the activity of *Hp*Ft is the bottleneck in the OP reaction. The high level of GDP-Fuc and the flat slope of the 2'FL curve in both reactions

after 6 hours, compared to the slope at the beginning, are indicating a decrease of the *Hp*Ft activity during the reaction.

To sum up, the higher enzyme concentration increased significantly the productivity. Because of the higher productivity the regeneration efficiency was also improved. Another point to consider is the loss of the *Hp*Ft activity during the OP reaction.

4.2.3 Influence of the lactose concentration:

The K_m-value of the *Hp*Ft enzyme for the acceptor substrate lactose was measured by Albermann et al. (2001) and determined with 168 µM.[29] Karina Gierlinger determined in her master thesis (2017) an apparent K_m-value for the Nus-tagged *Hp*FT with a photometric assay, which detects transfer and hydrolysis in one. An apparent K_m-value of 54 mM for lactose was determined. HPLC analysis of GDP-fucose and HPAE-PAD analysis of 2'-FL in conversion experiments with increasing lactose concentrations revealed, that the donor substrate GDP-Fuc was hydrolysed in substantial amounts by the *Hp*Ft enzyme. Increasing lactose concentrations could supress donor hydrolysis partly and increase 2'FL yields drastically but even at high lactose concentrations conversion of more than 50% GPD-fucose to 2'FL lactose was not possible. Stein et al. also reported an enzymatic hydrolysis of the donor substrate GDP-Fuc, which was inhibited by the addition of an acceptor substrate[25,29]. To determine the influence of the lactose concentration on the OP reaction with the Nus-tagged *Hp*Ft enzyme construct, various lactose concentrations were applied (10 – 50 mM).

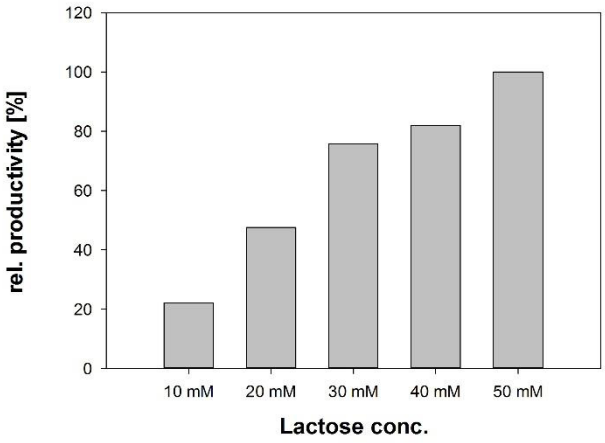

Fig. 8: Influence of the lactose concentration on the productivity. OP reactions from 10 mM of lactose to 50 mM of lactose were observed after 24 hours. Reaction conditions: 10 mM L-fuc, 10 mM $MgCl_2$, 50 mM PolyP, 1 mM of AMP and GMP,) 120 mU *Sm*PPK, 120 mU *Aj*PPK, 80 mU *Bf*FKP, 40 mU *Hp*Ft, 0.5 U iPPase in 50 mM Tris/HCl buffer at pH 7.6, 37°C and 350 rpm.

The productivity was enormously increased by offering a higher lactose concentration. 10 mM of lactose had only about 20 % of the productivity than the reaction with 50 mM of lactose (Fig. 8). The results let assume that the high hydrolysis rate of GDP-Fuc also has a huge impact on the productivity of the OP reaction.

4.2.4 Optimisation of the cofactor concentration:

The results of Fig. 6 and Fig. 7 demonstrate, that the productivity can be enhanced by adjusting the enzyme amount and the initial lactose concentration. Furthermore, it shows the high potential of the regeneration system composed of *Aj*PPK and *Sm*PPK. To exhaust the efficiency of the regeneration system lower initial cofactor concentrations were supplied to the OP reaction. Because of the more than tenfold lower K_m-values of the PPK enzymes for AMP and ADP compared to their corresponding guanosine nucleotides at almost similar conditions, it was tried to supply a lower initial concentration of AMP (Fig. 9). [24,30]

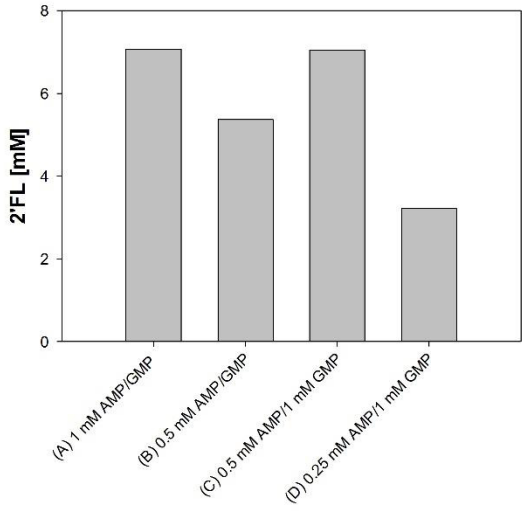

Fig. 9: Optimisation of the initial cofactor concentration on the synthesis of 2'FL over 24 hours. OP reactions with different initial cofactor concentrations were monitored over a period of 24 hours. The figure shows the result after 24 hours. (A) 1mM AMP and GMP; (B) 0.5 mM AMP and GMP; (C) 0.5 mM AMP and 1mM GMP; (D) 0.25 mM AMP and 1 mM GMP. 10 mM L-fuc, 10 mM $MgCl_2$, 50 mM PolyP, 30 mM lactose were incubated with 120 mU *Sm*PPK, 12 mU *Aj*PPK, 80 mU *Bf*FKP, 30 mU *Hp*Ft and 0.5 U iPPase in a 50 mM Tris/HCl buffer at pH 7.6, 37°C and 350 rpm.

The most productive reactions were (A) and (C), which synthesised about 7 mM of 2'FL within 24 hours (Fig. 9). In contrast to that, reaction (B) reached within 24 hours only 75%,

reaction (D) only 45% of the 2'FL concentration in reaction (A) and (C). The best conditions regarding the regeneration system were observed in sample (B) and (C), which achieved a nPO_4 value of 23. Reaction (D) obtained an acceptable nPO_4 value with 18, but the productivity of 2'FL was not satisfying compared to (A) and (C).

In conclusion, the NMP mix in reaction (C) was the most efficient and productive OP reaction system. GTP was regenerated 7 times, ATP 14 times at least. The decrease of the initial AMP concentration to 0.5 mM had no negative influence on the productivity. On the contrary the regeneration system is more efficient, since the nPO_4 value was increased by 45% compared to reaction (A), which was the standard condition so far. These results prove that the cofactor composition comprise high productivity with an efficient regeneration system.

4.2.5 OP synthesis of 2'FL:

The best reaction conditions determined in previous OP reactions were combined to maximise the parameters productivity and regeneration efficiency. The course of 2'FL production shows a strong increase in the first hours of the reaction that cannot be maintained over the reaction time. At the end 24 hours after reaction start an average concentration of 13.3 mM of 2'FL was synthesised in the OP reaction, which was examined in triplicates (Fig. 10). A concentration of about 6.5 g/L of 2'FL and a space time yield of 0.27 g L^{-1} h^{-1} was reached under these optimised conditions. Related to the regeneration efficiency a nPO_4 value of about 42 was calculated. The cofactor GTP was regenerated 14 times, ATP at least 28 times.

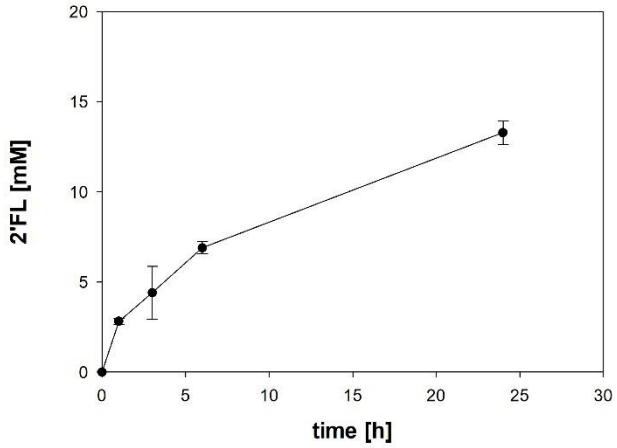

Fig. 10: Enzymatic OP production of 2'FL including a GDP-fucose regeneration system. The OP reaction was examined in triplicates, the arithmetic mean and the standard deviation is displayed in the figure. Reaction conditions: 20 mM L-fuc, 10 mM $MgCl_2$, 0.5 mM AMP, 1 mM GMP, 50 mM PolyP, 50 mM lactose incubated with 120 mU *Sm*PPK, 12 mU *Aj*PPK, 120 mU *Bf*FKP, 80 mU *Hp*Ft and 0.5 U iPPase in a 50 mM Tris/HCl buffer at pH 7.6, 37°C and 350 rpm.

The course of the GDP-Fuc concentration which is the *Bf*FKP product as well as the donor for the fucosyltransferase reaction is increasing during the whole reaction (Fig. 11). At the end of the OP reaction the highest GDP-Fuc concentration (0.88mM) during the OP reaction was observed. The high level at the end and the flatter slope of the 2'FL concentration shows that the *Hp*Ft activity is limiting and decreasing over time.

The graph of the ATP concentration displays that the concentration can be maintained over the whole reaction. More than 60% of the initial AMP was converted to ATP shortly after starting the reaction and this level was continuously present in the OP reaction. This high level at beginning compared to the GTP concentration demonstrates the preference of the PPKs for the adenosine nucleotides clearly. The GTP concentration reached the highest level with about 0.3 mM after 3 hours and decreases to about 0.05 mM after 24 hours. The reason for that is not a low activity of the PPKs but the high concentration of GDP-Fuc, which is not utilized anymore in that amount as at the beginning of the reaction.

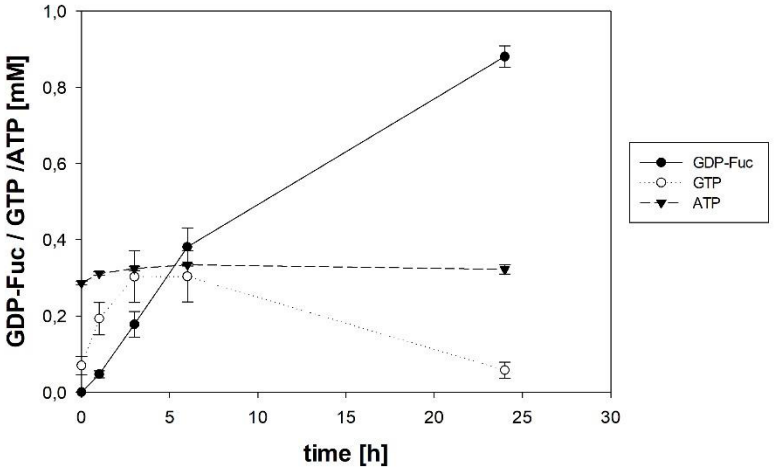

Fig. 11: The concentration of the reaction intermediate GDP-Fuc and the cofactors during the OP reaction with the highest productivity and regeneration efficiency. The OP reaction was examined in triplicates, the arithmetic mean and the standard deviation is displayed in the figure. Reaction conditions: 20 mM L-fuc, 10 mM $MgCl_2$, 0.5 mM AMP, 1 mM GMP, 50 mM PolyP, 50 mM lactose incubated with 120 mU *Sm*PPK, 12 mU *Aj*PPK, 120 mU *Bf*FKP, 80 mU *Hp*Ft and 0.5 U iPPase in a 50 mM Tris/HCl buffer at pH 7.6, 37°C and 350 rpm.

5. Discussion:

HMOs in human milk have beneficial effects for infants. The HMOs in the gastrointestinal tract act as selective growth factor for specific bacteria like *Bifidobacteria* and *Lactobacilli*, which compete for nutrient supply with potential pathogens.[31] *Bifidobacteria* together with other infant associated bacteria create an environment favourable for commensals by producing short-chain fatty acids and other metabolites so called post-biotics, which have inhibitory effects on potentially harmful bacteria.[32] 2'FL is one of the most abundant HMOs in human milk and promotes the growth of *Bifidobacteria* selectively.[33] In addition it shows anti-adhesive effect against *Campylobacter jejuni*. This organism is the most common cause of bacterial diarrhea.[14] These biological activities and the fact that formula for bottle fed babies could so far not be supplemented with 2'FL because HMOs are not available in the needed amounts shows that an efficient production system for 2'FL is required.

The here described production of 2'FL from fucose by cascading action of *Bf*FKP and *Hp*FT in one pot includes enzymatic recycling of GDP. The combination of a polyphosphate based recycling system using two different PPKs to enable a start from nucleoside monophosphates is new in the field of enzymatic HMO production.

A detailed evaluation of reaction parameters was done to optimize 2'FL yields. A pH of 5.0 was reported in literature to be the optimum for the *Hp*FT activity, but was not observed to be the optimum pH for 2'FL production in our one-pot system (Fig. 2).[25] The pH optimum of GDP-fucose production was determined to be 7.6, which fits together with the published pH optimum for *Bf*FKP.[26] It was determined that a pH of 7.6 is the best reaction condition for our OP reaction regarding the GDP-Fuc supply for the fucosyltransferase reaction and the 2'FL productivity. The reason for the different pH optimum could be that a more acidic pH decreases the GDP-Fuc synthesis, which influences the *Hp*FT activity negatively by a lower concentration of the donor substrate, decreased *Hp*FT stability or increased GDP-fucose hydrolysis at lower pH.

A high initial lactose concentration increased enormously the 2'FL productivity (Fig. 8). The result of this assay indicates, that addition of the acceptor substrate inhibits the hydrolysis of the donor substrate GDP- Fuc. Stein et al. (2008) also observed enzymatic hydrolysis of GDP-Fuc, which was shifted by the presence of a sugar donor.

The tenfold lower K_m value of the PPK enzymes for AMP and ADP compared to their corresponding guanosine nucleotides, enabled reduction of the AMP concentration to half the concentration of GMP (from 1.0 mM to 0.5 mM) in the OP reaction without decreasing the 2'FL productivity (Fig. 9).[24,30] Thereby the GDP-Fuc synthesis was also hardly impeded by the lower AMP concentration. The lower initial cofactor concentration enhances the efficiency of the regeneration system and thereby can reduce total costs.

A low initial cofactor concentration and a high lactose concentration combined high productivity with an efficient regeneration system (Fig. 8, Fig. 9).

In summary it was possible to develop a cost-efficient high potential regeneration system based on two PPKs. In our system two PPKs are used not only for ATP recycling but for GTP recycling as well. In the best case the cofactor GTP was regenerated at least 14 times, ATP at least 28 times. The calculation of this values is based on the synthesis of 2'FL and does not include any form of cofactor hydrolysis, which was monitored in the OP reactions (Fig. S 8). The data of the best OP reaction shows, that the regeneration system is fully functional after 24 hours and would be probably capable of performing more recycling events than could be shown in this experiment. In the first 3 hours of reaction the GDP-Fuc concentration is low and the 2'FL productivity high, at the end the of the reaction vice versa (Fig. 10, Fig. 11). The rising GDP-Fuc concentration and the decreasing 2'FL productivity over the whole OP reaction indicates a high stability of the regeneration system and a declining *Hp*FT activity during the OP reaction. In our setup the GDP-Fuc supply based on the recycling system appeared not to be a bottleneck, the low specific activity and the instability of the fucosyltransferase during the reaction were limiting factors in the OP reaction system.

A comparable sugar nucleotide regeneration system was published by Tsai et al. (2013). They synthesised Allyl-Globo H by the enzymes *Bf*FKP and *Hp*FT in a OP reaction and used pyruvate kinase for the regeneration of ATP and GTP. A turnover number of 20 for GTP was observed.[34] The combination of *Aj*PPK and *Sm*PPK was also examined by Mordhorst et al. (2017), which developed a supply cascade for ATP starting from adenosine and AMP. AMP was converted to ATP by the action of the two PPKs, ATP and adenosine form AMP and ADP. adenosine and AMP could be supplied in a 10.000:1 ratio for full conversion of adenosine. The supply cascade was used for the production of S-adenosylmethionine (SAM). The number of recycling events for ATP calculated by dividing the amount of SAM formed by the AMP amount supplied was more than 1500. The system was also applied in a whole

regeneration cycle for SAM in a methylation reaction. Here the regeneration system was active for 11 ± 3 cycles.[35] In another example in the field of regeneration systems based on PPKs, ATP was regenerated about 40 times from AMP for the synthesis of acetyl-CoA. A membrane associated PPK from *M.xanthus* for AMP phosphorylation and a PPK from *E.coli* for ADP phosphorylation were coupled for the regeneration.[30] Meng et al. (2016) used a PPK from *Rhodobacter sphaeroides* for the regeneration of ATP from ADP to produce 5-aminolevulinic acid by cell free multi-enzyme catalysis. The ATP was regenerated at least 5.4 times until the reaction end. [36]

In the best reaction about 13.3 mM or 6.5 g/L of 2'FL was synthesized within 24 hours in one pot without any purification steps of reaction intermediates or donors. Compared to so far reported literature values for *in vitro* synthesis the concentration of 2'FL was enhanced by a factor of 3.[37] Chin et al. (2015) obtained 6.4g/L of 2'FL with an *in vivo* fed batch fermentation over 80 hours.[16] By a whole cell production system Baumgärtner et. al. (2013) achieved a final 2'FL concentration of about 20g/L, half of the product was accumulated intracellularly, in a whole cell fermentation after 35.5 hours.

6. Conclusion:

In this study we illustrated the possibility to produce fucosylated products *in vitro* without the need to start from the expensive donor substrate GDP-fucose. For the first time a PPK driven recycling system based on cheap polyphosphate was used for ATP and GTP recycling at the same time. The NTP regeneration system was coupled to the action of *Bf*FKP for GDP-fucose formation and *Hp*FT for fucose transfer to lactose in one pot. The integration of a second PPK (*Aj*PPK), which can convert NMP to NDP led to valuable improvements for the OP reaction system. The combination of *Aj*PPK and *Sm*PPK enables the formation of the cofactors ATP and GTP from AMP and GMP, which are required for the GDP-Fuc synthesis, in an OP reaction. Looking at the economical side, NMPs are cheaper than their corresponding NDPs and NTPs. A big advantage by using a second PPK is the reutilization of NMPs, which are generated as by-product during the OP reaction. For adding a second PPK the reaction concept must not be changed by adding another substrate or cofactor. Both enzymes use PolyP as phosphate donor and can perform at similar salt concentrations, pH values and temperatures and have the same substrate preference (adenosine nucleotides > guanosine nucleotides).[24,30] A final 2'FL titer of 6.5 g/L and a space time yield of 0.27 g/L/h are the highest so far reported values for *in vitro* 2' FL production. In a whole cell system a three and two times higher titer and space time yield could be achieved, but the product isolation from *in vivo* reactions is usually more challenging than from enzymatic reactions.

This study shows that *in situ* formation and regeneration of ATP and GTP by coupling two PPKs is a highly efficient and cost-efficient way to supply cofactors for HMO fucosylation. To increase the 2'FL productivity for *in vitro* OP reactions systems further investigations on the stability of *Hp*Ft should be carried out or a better suited fucosyltransferase used.

Chapter 2: Supporting Information for the Manuscript

Purification of *Sm*PPK:

The His-tagged *Sm*PPK enzyme construct has a size of 34.8 kDa and was eluted in all collected fractions (A-D). A step gradient of 50 mM imidazole was applied to remove the weak unspecific bound proteins. Then a second step with 250 mM of imidazole was used to elute the desirable protein. Fig. S 1 (A) shows, that the purity of the enzyme is increasing with the fraction number. Especially fraction A shows a high amount of unspecific bound proteins, which were eluted first. The volumetric activity of the fractions B, C and D was determined and in consequence fraction C was further used for the OP reactions.

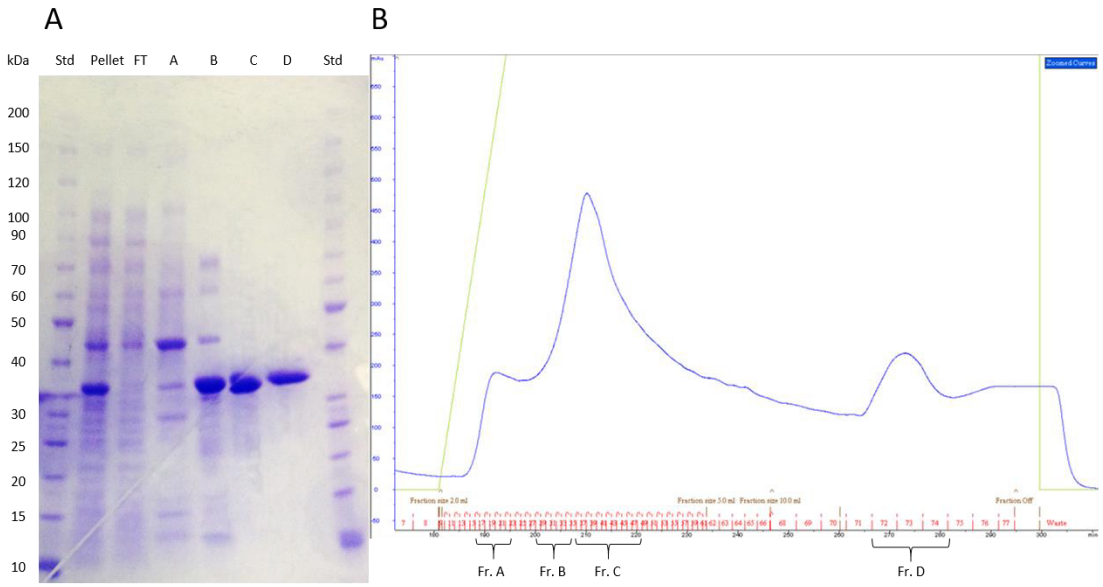

Fig. S 1: Purification of *Sm*PPK. SDS-PAGE (A) and UV signal (B, blue) for the purification of His-tagged *Sm*PPK. The eluted samples were collected and separated in Fraction A, B, C and D. The fractions were concentrated and analysed with the Flow Through (FT) and Pellet fractions by SDS- Page. The *Sm*PPK enzyme with a size of 34.8 kDa was eluted in all fractions (A-D), but the purity was increasing in the later eluting fractions.

Purification of *Aj*PPK:

The His-tagged *Aj*PPK enzyme construct has a size of 55.8 kDa and was eluted without any visible impurities in fraction A (Fig. S 2). A gradient from 20 mM to 250 mM of imidazole was applied to elute the enzyme.

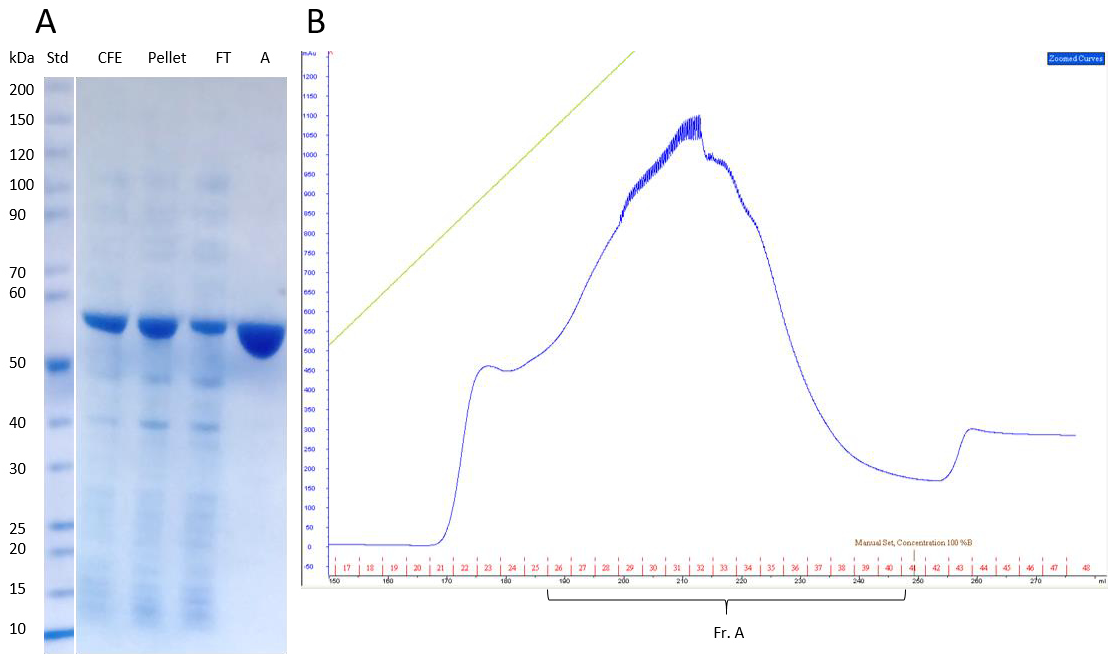

Fig. S 2: Purification of *Aj*PPK. SDS-PAGE (A) and UV signal (B, blue) for the purification of His-tagged *Aj*PPK. The eluting samples were collected and pooled (fraction A). Fraction A was concentrated and analysed with the Flow Through (FT) and Pellet fraction by SDS-PAGE. The *Aj*PPK enzyme with a size of 55.8 kDa is eluted with a high purity in fraction A.

Purification of *Bf*FKP:

The His-tagged *Bf*FKP enzyme construct has a size of 108 kDa and was eluted in a step purification. The first purification step with an imidazole concentration of 50mM was applied to remove all unspecific bound proteins. For the second purification step the imidazole concentration was increased to 250 mM to elute a pure *Bf*FKP enzyme (Fig. S 3).

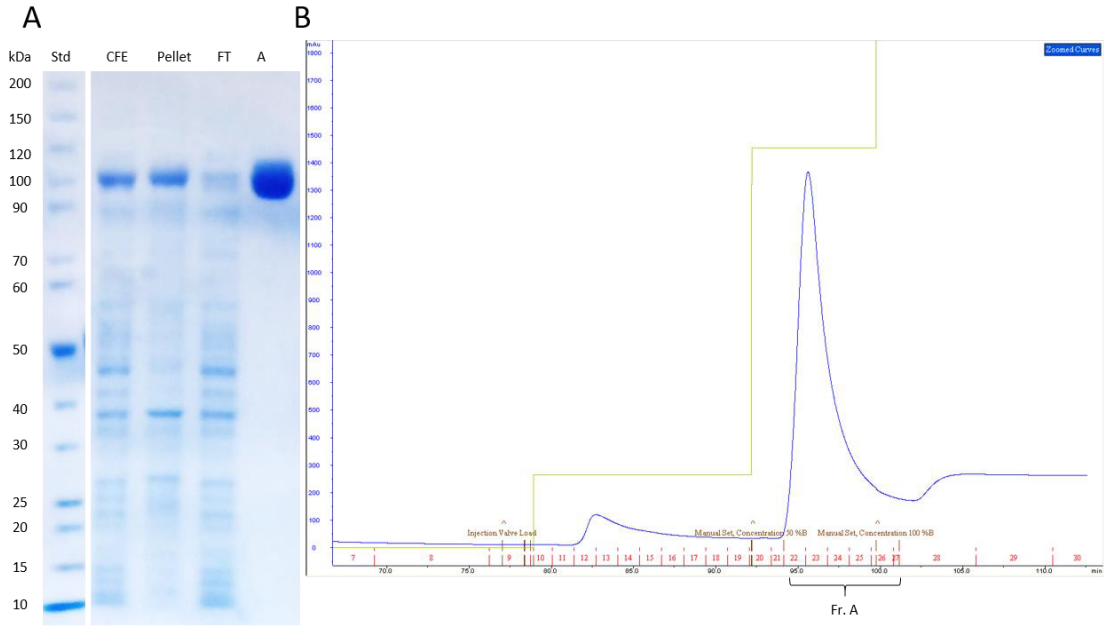

Fig. S 3: Purification of *Bf*FKP. SDS-PAGE (A) and UV signal (B, blue) for the purification of His-tagged *Bf*FKP. The eluting samples were collected and pooled (fraction A). Fraction A was concentrated and analysed with the Flow Through (FT) and Pellet fraction by SDS-PAGE. The *Bf*FKP enzyme with a size of 108 kDa is eluted in fraction A.

Purification of *Hp*Ft:

The StrepNus*Hp*Ft construct has a size of 95.2 kDa and was eluted in one single peak. Beside the strong band presenting the *Hp*Ft enzyme, a second band below is visible on the SDS-PAGE (Fig. S 4 A), which is also present in the CFE. This impurity was present in every purification and could be a truncated *Hp*Ft enzyme, that was not translated to the end. About 16 mg of purified *Hp*Ft can be isolated out of 1 L cell culture.

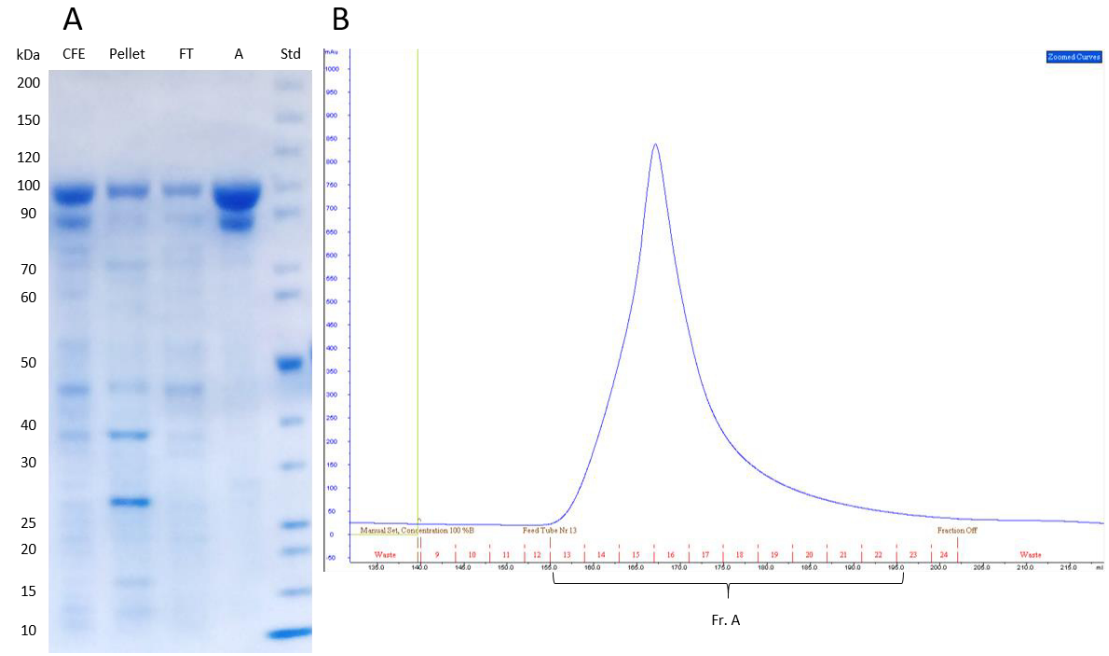

Fig. S 4: Purification of *Hp*Ft. SDS-PAGE (A) and UV signal (B, blue) for the purification of Strep-tagged *Hp*Ft. The eluting samples were collected and pooled (fraction A). Fraction A was concentrated and analysed with the Flow Through (FT) and Pellet fraction by SDS-PAGE. The *Bf*FKP enzyme with a size of 95.2 kDa is eluted in fraction A.

Stabilisation of *Bf*FKP:

The purified *Bf*FKP enzyme was not stable under common storage conditions at 4°C and -20°C without additives. After a few days at -20°C the enzyme formed gel like structures, which obviously originates from denatured enzyme and show decreased specific activity (Fig. S 5). It is known that freezing of enzymes can induce various stresses, which result in irreversible protein aggregation and loss of activity.[38] The enzyme might denature during the adsorption of proteins onto the ice surface. It was demonstrated, that freezing of proteins goes in hand with a loosening of the native fold, which is largely reversible by thawing but in some cases the initial catalytic activity cannot be reached again.[39,40]

The *Bf*FKP enzyme was stabilised by the addition of the nonionic surfactant Tween 80 and Glycerol. The formulation contains in total a concentration of 10% Glycerol and 0.02% Tween

80. Glycerol is a common cryoprotectant, which helps to prevent the formation of ice crystals.[41,42] Tween 80 has a protective effect on a protein solution and enhances significantly the recovering activity after the freeze-thawing cycle. It is supposed, that Tween molecules can cover the ice crystal surface and thereby reduce the ice-protein interaction.[43]

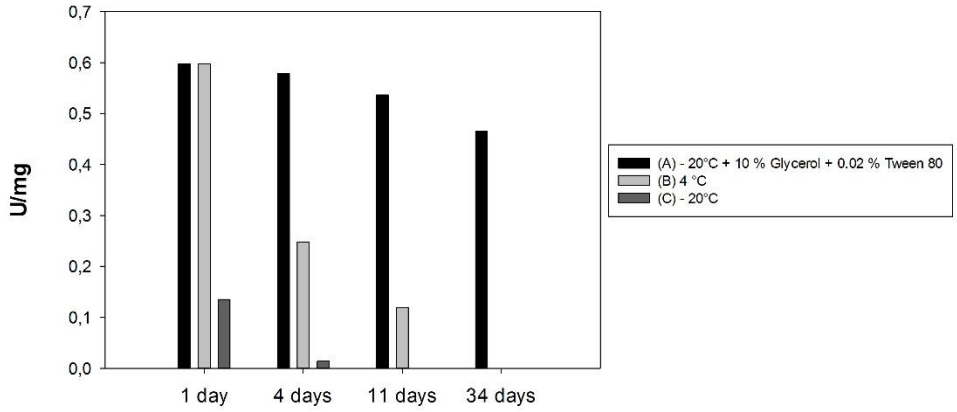

Fig. S 5: Storage stability of the purified *Bf*FKP under various conditions. Three storing methods were studied: (A) -20°C, 10 % Glycerol and 0.02 % Tween 80; (B) 4°C; (C) -20°C. Reaction conditions: 5 mM L-fuc, 5 mM $MgCl_2$, 10 mM ATP, 5 mM GTP, 10 mM lactose incubated with 0.5 U iPPase and 0.2 mg/mL *Bf*FKP at pH 7.6, 37°C and 350 rpm in a 50 mM Tris/HCl buffer. The formulation with 10% Glycerol and 0.02 % Tween 80 stabilizes the enzyme significantly (A). Without these additives, the enzyme activity decreases rapidly stored at 4°C (B) and -20°C (C).

One day after freezing the *Bf*FKP enzyme the recovered specific activity in formulation (A) was nearly 4.5-fold higher than in the pure enzyme fraction (C) without additives. Four days later the recovered specific activity of sample (A) was 41-fold higher than in sample (C). This result shows the stabilising effect of the additives. Over a period of more than four weeks the specific activity is decreased by about 28% in the fraction stored with 10% of glycerol and 0.02% of Tween 80. The enzyme stored without additives showed no activity after 4 weeks of storage. Compared to the other storing conditions, the formulation with glycerol and tween 80 is preferred for long time storage (Fig. S 5). The protective effect can be certainly improved by further investigation, which study the ideal concentration of the additives and the cooling rate.

PEP/PK vs. PolyP/*Sm*PPK:

The cofactors (GTP/ATP) in the PEP/PK reaction decreased significantly between 6 -24h (Fig. S 6). The GDP-Fuc concentration was relative stable, but declines slightly. In contrast to that, the concentrations of GTP and ATP of the PolyP/*Sm*PPK reaction were lower at the

beginning, but stayed after 24 hours at a much higher level than in the PEP/PK system. These facts showed, that the PolyP/*Sm*PPK regeneration system is more stable than the PEP/PK system under these specific reaction conditions and enzyme mixture present in the OP reaction. It was observed that the enzymatic activity of PK is decreased to 50 % over 24 hours of incubation at 37°C.[27]

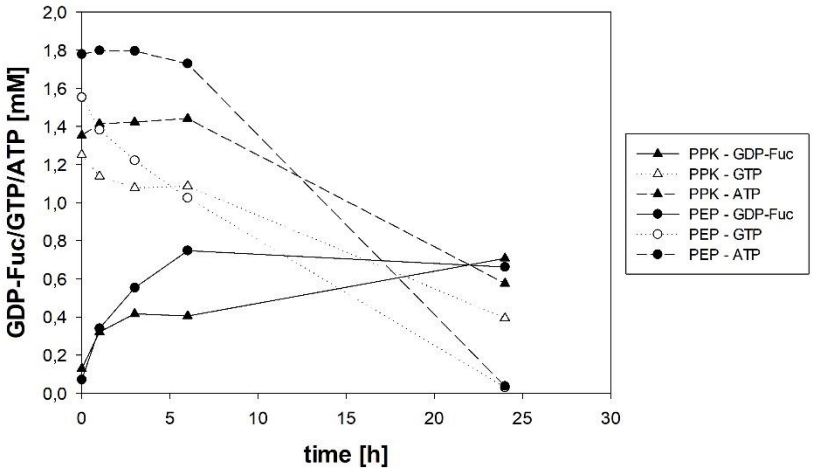

Fig. S 6: GDP-Fuc /GTP/ ATP concentrations in a OP reaction with either PEP/PK or PolyP/*Sm*PPK regeneration system over a period of 24 hours. The triangles display the PPK system, the dots the PEP system. Reaction conditions for the OP reactions: 10 mM L-fuc, 2 mM ADP and GDP, 10 mM $MgCl_2$, 20 mM PolyP or PEP, 10 mM lactose at 37°C, pH 7.6 and 350 rpm. The assay was catalysed by 0.05 mg/mL *Sm*PPK, 0.3 mg/mL *Bf*FKP, 0.4 mg/mL *Hp*Ft and 0.5 U iPPase. The PEP/PK system contains 2U PK/LDH instead of *Sm*PPK. The samples were measured at the same time points as the 2'Fl concentration (Fig. 3).

Influence of the initial cofactor concentration:

The reason for the elevated GDP-Fuc concentration in the samples with a higher initial cofactor concentration is that a higher concentration of GTP and ATP is steadily present in the sample. Fig. S 7 illustrates that GDP-Fuc concentration is proportional higher by increasing the initial cofactor concentration

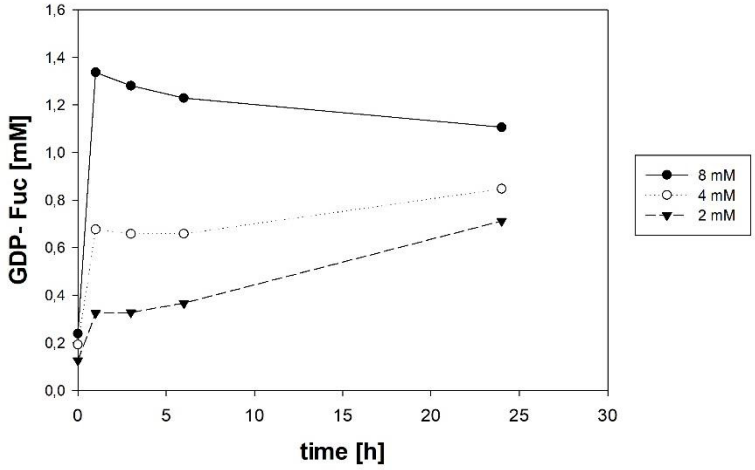

Fig. S 7: GDP-Fuc concentration in OP reactions with a different initial cofactor concentration Either 2 mM, 4 mM or 8 mM of ADP and GDP were supplied in the beginning of the reaction. 120 mU *Sm*PPK, 40 mU *Bf*FKP, 10 mU *Hp*Ft and 0.5 U iPPase were incubated in a 50 mM Tris/HCl buffer (pH 7.6) with 10 mM L-fuc, 10 mM $MgCl_2$, 10 mM lactose, 50 mM PolyP and various cofactor concentrations at 37°C and 350 rpm. The GDP-Fuc concentration was determined at the same time points as the 2'Fl concentration (Fig. 4).

The relative amount of AMP and GMP is increasing during the reaction in all three samples independently from the initial cofactor concentration. Especially the relative GMP amount raised during the OP reaction from about 4 % to 12 %, which cannot be used for the regeneration anymore (Fig. S 8). The course of the AMP displays the same characteristic in all reactions by increasing slightly from about 4% to 7.5%. This result shows that during the reaction an amount of nucleotides, which should not be underestimated, cannot be utilised by the regeneration system anymore.

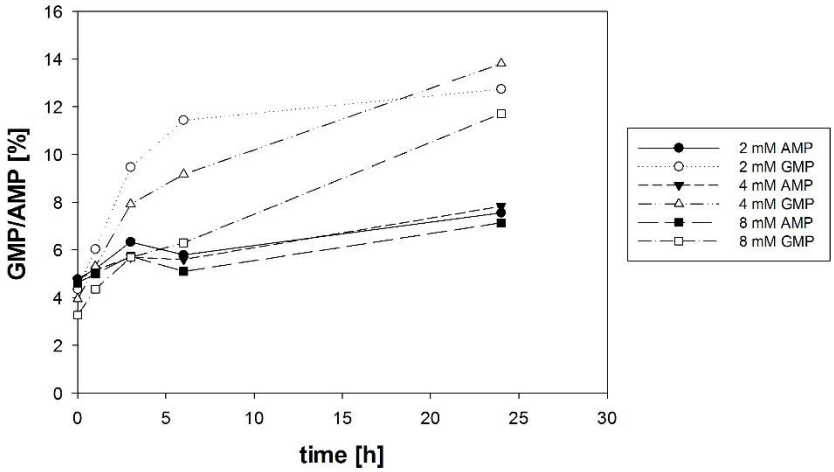

Fig. S 8: Relative amount of AMP/GMP during the reaction. In all three OP reactions (2 mM, 4 mM and 8 mM) the nucleoside monophosphate amount was monitored. 120 mU *Sm*PPK, 40 mU *Bf*FKP, 10 mU *Hp*Ft and 0.5 U iPPase were incubated in a 50 mM Tris/HCl buffer (pH 7.6) with 10 mM L-fuc, 10 mM $MgCl_2$, 10 mM lactose, 50 mM PolyP and various cofactor concentrations at 37°C and 350 rpm.

Chapter 3: Additional Experiments to characterize the OP reaction

Influence of the PolyP:

The impact of two variants of PolyP was examined in a standard conversion reaction starting with ADP and GDP, which were phosphorylated by *Sm*PPK to their corresponding nucleoside triphosphate. The figure Fig.S2 1 shows the relative amount of ATP and GTP after 24 hours of reaction. In the reaction with the PolyP (A) from Sigma Aldrich 52% of the initial ADP and 51% of the initial GDP was phosphorylated to ATP respectively GTP. In contrast to that, it was possible to increase the relative amount of phosphorylated ATP and GTP to 78% and 76% in the same reaction with the other PolyP (B) from Merck.

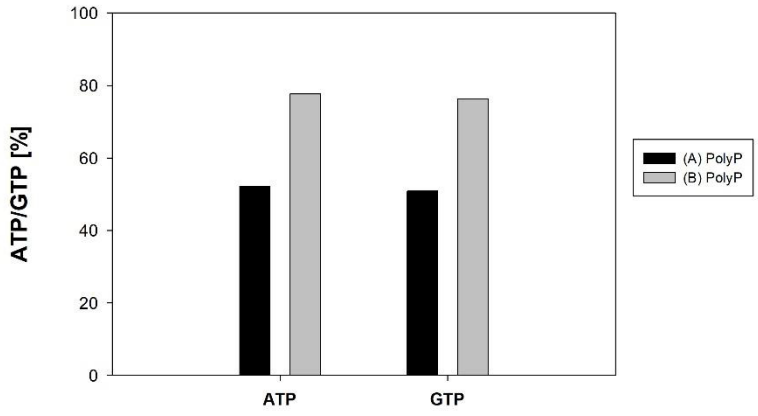

Fig.S2 1: Influence of the PolyP. The figure shows the relative amount of ATP/GTP in after 24 hours of reaction. Two different PolyP were investigated (A) from Sigma, (B) from Merck. The reaction contains 5 mM GDP and ADP, 10 mM $MgCl_2$, 50 mM PolyP, 0.05mg/mL *Sm*PPK and was performed in 50 mM Tris-HCl at pH 7.6 and 37°C.

It was reported that higher PolyP chain lengths increase the phosphorylation activity of the PPKs. A preferred PolyP chain length of 25 to 50 for the class III PPK2 enzyme from *M.ruber* was examined.[22]. Therefore, the chain length of the (A) and (B) PolyP was observed via phosphor NMR. It showed, that the amount of internal phosphate residues in (B) was about 5 times higher than in (A). The manufactures of (B) specify a PolyP chain length of about 25 for their product. In conclusion to the phosphor NMR, PolyP (A) have a chain length of about 5.

From this result, together with the investigations of Motomura et al. (2014) it can be concluded that the *Sm*PPK as a class I PPK2 enzyme also prefer higher PolyP chain lengths. [22] Another aspect to consider is the effect of the PolyP concentration on the activity and reaction equilibrium. To observe this issue, a standard phosphorylation reaction catalysed by *Sm*PPK was monitored with three different PolyP concentrations. The relative amount of the nucleoside triphosphates at 1, 2 and 24 hours after reaction start is displayed on Fig.S2 2. It shows that a low PolyP concentration (25mM) increases the initial activity, but the level cannot be hold over 24 hours. The highest PolyP concentration somehow inhibits the phosphorylation. The sample with a PolyP concentration of 50 mM shows the most promising performance regarding maintenance of a high NTP level and initial activity. This result confirms that a higher concentration of PolyP inhibits the reaction rate.[36] Possible reason is the key role of the cofactor Mg^{2+} for the phosphorylation activity. A high concentration of PolyP might chelate with Mg^{2+} to a higher extent and thereby reduce the *Sm*PPK activity.[36,44]

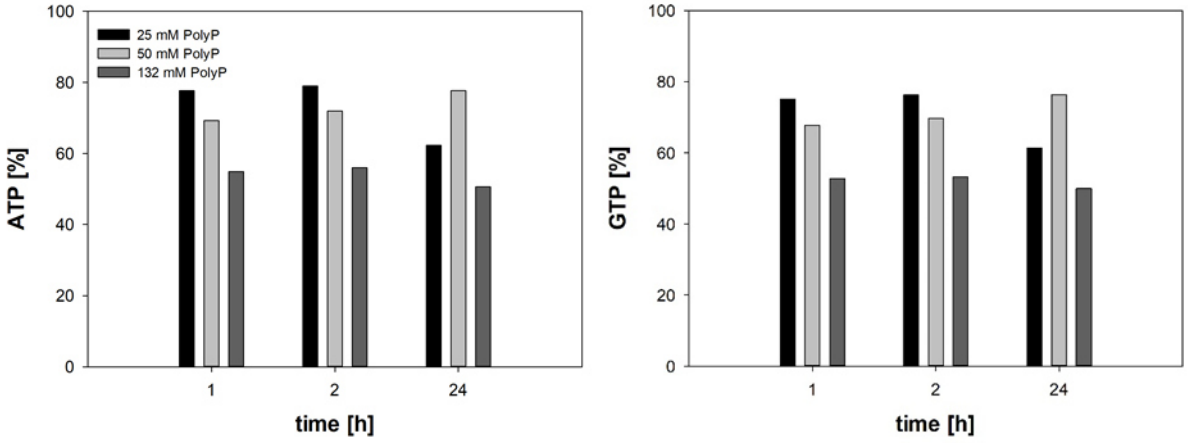

Fig.S2 2: Effect of the PolyP concentration on the activity and reaction equilibrium. The reaction system contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM GDP and ADP, 0.05 mg/mL *Sm*PPK at a pH of 7.6 and 37 °C. The relative amount of ATP and GTP was determined after 1,2 and 24 hours.

Degradation of sugars:

It was observed, that sugar degradation occurred in the OP after 48 hours of reaction. Especially lactose and L-fucose were affected, 2'FL appeared to be quite stable. The concentration of this substrate sugars decreased significantly between 24 and 48 hours after the reaction start, but the 2'FL concentration did not increase. It is also possible, that the

degradation of sugars occurs during the whole reaction, but can only be detected at a later reaction phase, when the reaction rate of the enzymes is slower and does not cover the degradation anymore. As it is displayed in Table S2 1 somehow the sugars disappear for unexplained reasons. An additional peak for glucose or galactose, which should be the case if lactose is degraded, was also not detected in the chromatogram.

Table S2 1: Typical sugar degradation in a standard OP reaction. The reaction contains 10 mM L-fucose, 10 mM $MgCl_2$, 2 mM ADP and GDP, 10 mM lactose, 40 mM PolyP, 0.5 U iPPase, 0.05 mg/mL *Sm*PPK, 0.3 mg/mL *Bf*FKP, 0.4 mg/mL *Hp*Ft in a 50 mM Tris-HCl buffer (pH 7.6) at 37°C and 350 rpm. In the first 24 hours the synthesis of 2'FL fits at least roughly to the decrease of the substrates. After 24 hours the concentration of all sugars decreases without any hint.

time [h]	L-fucose [mM]	Lactose [mM]	2'FL [mM]
0	10.54	11.53	0.00
1	9.01	10.80	0.94
3	7.00	8.85	2.11
6	5.45	7.74	2.97
24	2.87	5.30	4.33
48	1.54	1.66	3.95

To find out which enzyme or which interaction led to this degradation, each reaction step of the OP reaction was performed itself.

First it was tested out if there are sugar degradation or precipitation effects originating from reaction conditions or substrate composition, because the reaction contains PolyP and $MgCl_2$, which could chelate at a too high PolyP concentrations. [44,45]

Table S2 2: One-pot reaction without enzymes. Reaction (A) is without $MgCl_2$, reaction (B) is a standard OP reaction composition: 10 mM L-fucose, 10 mM $MgCl_2$, 2 mM ADP and GDP, 10 mM lactose, and 20 mM PolyP in 50 mM Tris-HCl buffer at pH 7.6, 37°C and 350 rpm. Triplicates of each assay were performed. *This assay was sterile filtered before the reaction start and was measured only after 48 hours to exclude a contamination.

time [h]	**A**			**B**		
	L-fucose [mM]	Lactose [mM]	2'FL [mM]	L-fucose [mM]	Lactose [mM]	2'FL [mM]
0	10.53	8.66	0.38	10.00	8.06	0.37
24	10.36	8.23	0.37	10.20	7.90	0.37
48	10.66	7.96	0.38	11.10	8.34	0.41
48*				10.42	7.60	0.35

Table S2 2 shows, that the sugar concentrations in the samples varying during a reaction period of 48 hours, but obviously, no sugar degradation occurred. The slight differences in the sugar concentration are originated by the measurement system. Before starting the

reaction one sample was withdrawn from the master mix for reaction (B) and was sterile filtered to guarantee, that the reaction is not contaminated by other bacteria. Based on these results, precipitation of the sugars generated by sample composition or decrease of sugars caused by bacteria containment can be excluded.

The regeneration system with the *Sm*PPK enzyme was also investigated itself. The Table S2 3 shows the sugar concentration during the phosphorylation reaction, which supplies cofactors required for the GDP-Fucose synthesis. The sugars in the sample are not involved in the phosphorylation of the NDPs, so the concentration should not vary unless the *Sm*PPK enzyme is responsible for the sugar degradation.
In general, the sugar concentration switches up and down in both reactions, but a remarkable decrease of sugars as in Table S2 1 cannot not be confirmed.

Table S2 3: Regeneration system with *Sm*PPK. Reaction (A): standard composition of the regeneration system, Blank: same sample composition without *Sm*PPK. Duplicates of each sample were performed. The calculated mean is displayed in the table. The assays contain 10 mM L-fucose, 10 mM $MgCl_2$, 2 mM ADP and GDP, 5 mM lactose and 20 mM PolyP in 50 mM Tris-HCl buffer and was executed at pH 7.6, 37°C and 350 rpm with 0.05 mg/mL *Sm*PPK.

time [h]	A		Blank	
	L-fucose [mM]	Lactose [mM]	L-fucose [mM]	Lactose [mM]
0	9.10	3.24	10.49	3.72
1	9.47	3.14	10.78	3.90
24	10.06	3.22	9.29	3.52
48	10.33	3.03	8.91	3.36

Possible sugar degradation effects originating from the *Bf*FKP enzyme, which is also involved in the GDP-Fucose synthesis was also investigated. Further an assay with the *Bf*FKP enzyme in combination with the PPK regeneration system, that represent the first step in the OP reaction, was examined. The sugar L-fucose acts as a substrate in this synthesis catalysed by *Bf*FKP. Lactose is not directly involved and was only added to observe a possible sugar decrease during this reaction.
As it can be seen in Table S2 4, the L-fucose concentration is decreasing during the reaction, because it is part of the GDP-fucose synthesis. By considering the initial concentration of the cofactors (2 mM in both samples) it is not possible to convert the consumed 4 mM of L-fucose completely to GDP-fucose. This difference in the mass balance was observed in the period between 24 and 48 hours after reaction start. Within the first 24 hours it fits together

tolerably. Lactose was also decreased in the period between 24 and 48 hours in both reactions, but not in that extent as seen in Table S2 1.

Compared to the previous assays (Table S2 2; Table S2 3) a clear degradation of sugars, especially of L-fucose, can be observed. But nevertheless, this negative mass balance regarding the sugars cannot be explained, because potential and unknown by-products were not detected after 48 hours of reaction.

Table S2 4: First step of the one-pot reaction – GDP-Fuc synthesis. Reaction (A): *Bf*FKP with ATP and GTP as cofactor; reaction (B): *Bf*FKP with *Sm*PPK, PolyP, ADP and GDP as initial cofactor. Duplicates of each sample were performed. The calculated mean is displayed in the table. Both assays contain 10 mM L-fucose, 10 mM $MgCl_2$, 2 mM ATP and GTP in (A) or 2 mM of ADP and GDP in (B), 4 mM lactose, 0.3 mg/mL *Bf*FKP and 0.5 U iPPase. (B) additionally comprise 20 mM of PolyP and 0.05 mg/mL *Sm*PPK. Both assays were executed in 50 mM Tris-HCl at pH 7.6, 37°C and 350 rpm.

time [h]	A		B	
	L-fucose [mM]	Lactose [mM]	L-fucose [mM]	Lactose [mM]
0	10.54	4.30	10.48	4.21
1	9.89	4.11	10.00	4.09
24	9.16	4.15	8.36	4.13
48	5.99	3.19	6.08	3.40

The focus was only on the enzymes, which are involved in the fucosyltransferase reaction, the second step in the OP reaction. Reaction (B) was executed to observe the whole second step in the OP reaction. The fucosylation of lactose generates GDP as by-product, which can be regenerated to GTP by *Sm*PPK. GTP is used as a cofactor for the synthesis of GDP-fucose. **Table** S2 5 displays the sugar concentration over the reaction. Due to a calculation error by providing the lactose, 1.5-fold of the expected lactose concentration was added in the samples at the reaction start. Consequently, the standard curve for the determination of the lactose concentration was inaccurate for that lactose concentration in the samples, which leads to an imprecisely measurement. That also explains the high amount of lactose in the sample after 48 hours. Apart from this sample the other results fit together in a manner that a sugar degradation originating from these two enzymes in this constellation can be excluded also by considering the measurement inaccuracy. If degradation effects like shown in Table S2 1 would occur the lactose concentration after 48 hours should be considerably lower.

Table S2 5: Second step of the OP reaction. Reaction (A): *Hp*Ft with GDP-fucose; Reaction (B): *Hp*Ft with GDP-fucose, *Sm*PPK and PolyP. Both assays contain 10 mM lactose, 10 mM $MgCl_2$, 5 mM GDP-fucose, 0.4 mg/mL *Hp*Ft. (B) additionally comprise 20 mM of PolyP and 0.5 mg/mL *Sm*PPK. Both assays were executed in 50 mM Tris-HCl at pH 7.6, 37°C and 350 rpm.

Time [h]	A		B	
	Lactose [mM]	2'FL [mM]	Lactose [mM]	2'FL [mM]
0	16.70	0.00	16.12	0.00
1	15.82	1.74	15.03	1.62
24	14.95	2.53	14.53	2.89
48	18.65	3.26	13.97	2.87

To sum up, the samples with the *Bf*FKP enzymes showed a clear indication of a sugar degradation in the period between 24 and 48 hours after reaction start (Table S2 4). But in these samples only the concentration of L-fucose was decreasing in that amount of the typical sugar degradation shown in Table S2 1, whereas the lactose concentration just declines slightly. One possible reason might be some impurities in the isolated enzymes, which cause the sugar degradation and were not detected on the SDS-gel (Fig. S 4). But the *Hp*Ft enzyme shows no sugar degradation effects in the assays (Table S2 5).

If sugars are degraded, the resulting by-products should be detected, which was not the case. The only explanation are precipitation effects not visible in the solution, which only occur if all substrates, cofactors and enzymes of the OP reaction are in one sample and may interact in an unknown way, that leads to sugar degradation.

Modification of *Hp*Ft:

The amino acid substitution in *Hp*FT was carried out for a more efficient expression. The gene contains a 14 C tract, which led to translational frame shift during the expression. This frame shift causes the co-expression of an inactive truncated form.[46] To prevent this inactive truncated form the elimination of three consecutive CCC codons was carried out.[47] The plasmid pC21e1 FTO 4.0 with the StrepNus*Hp*Ft construct was transformed in *E.coli* TOP 10F' by Karina Gierlinger in her master thesis (2017). This plasmid was isolated and amino acid substitutions were introduced by QuikChange Site- Directed Mutagenesis.[48] The PCR was performed with the primers 5'-CACCCTACCACCACCACCCGAAAATGGAAATAATAAAAAAAAAG-AGG – 3' and 3'-CATTTTCGGGTGGTGGTGGTAGGGTGAAGGTTTGCTTGATTAAAG-5' (the exchanged bases are underlined), a Q5 Polymerase and 5 % DMSO. The PCR product was transformed in electrocompetent *E.coli* TOP10F' and the construct was verified by DNA

sequencing. For the enzyme expression the verified construct was transformed in *E.coli* JM109 cells.

OP reaction with addition of *Hp*Ft:

The aim of this experiment was to maximise the productivity and regeneration efficiency by providing a fresh *Hp*Ft enzyme, which activity and stability appeared to be a limiting factor. Regarding to the results shown in Fig. 10 the enzyme *Hp*Ft was constantly fed to the OP reaction. The 2'FL productivity was high in the first 3 hours of the reaction (Fig.S2 1). After 3 hours the productivity declines strongly or rather hardly any 2'FL was synthesised by considering the standard deviation. Around 14 mM of 2'FL was synthesised in the OP reaction, which is no improvement in comparison to Fig. 10.

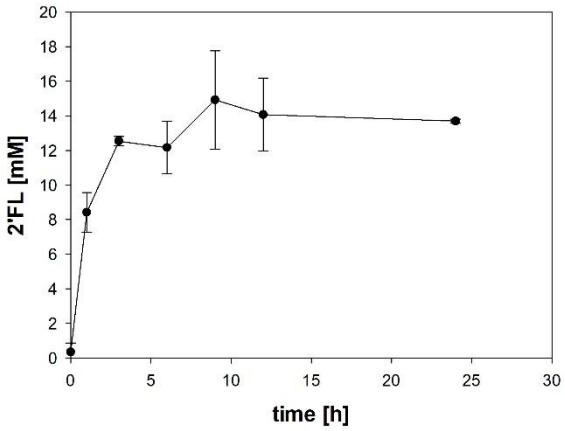

Fig.S2 3: 2'FL synthesis over 24 hours in a fed batch like OP reaction. At reaction start the OP reaction contains 80 mM L-Fuc, 15 mM $MgCl_2$, 0.8 mM AMP, 1.6 mM GMP, 80 mM PolyP, 100 mM lactose in a 50 mM Tris/HCl buffer (pH 7.6). 120 mU *Sm*PPK, 30 mU *Aj*PPK, 120 mU *Bf*FKP, 80 mU *Hp*Ft and 0.5 U iPPase were added to start the reaction. The mixture was incubated at 37°C and 350 rpm. After 1, 3, 6, 9, 12 and 24 hours samples were withdrawn and analysed. The reaction was fed with 30 mU of fresh *Hp*Ft enzyme 3, 6, 9 and 12 hours after the reaction start. After 12 hours the OP reaction contains 50 mM L-Fuc, 10 mM $MgCl_2$, 0.5 mM AMP, 1 mM GMP, 50 mM PolyP and 62 mM lactose. The OP reaction was examined in duplicates and the arithmetic mean and standard deviation were displayed in the figure.

The GDP-Fuc and cofactor concentration was also monitored and is displayed in Fig.S2 4. The GDP-Fuc concentration is increasing in the first 6 hours of the reaction. The low GDP-Fuc concentration at the first timepoints can be de explained by the high productivty of 2'FL in this period. After 9 hours the graph begins to decline and after 24 hours GDP-Fuc was not detected anymore. The ATP concentration is relatively stable but also started to decrease

after 9 hours. The GTP concentration is high in the first period, then started to decline with the increase of the GDP-Fuc and then stays at a constant level till the end.

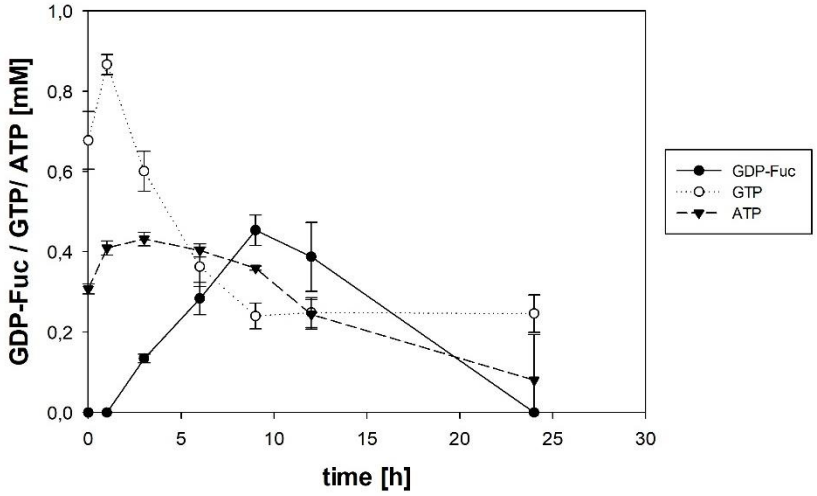

Fig.S2 4: GDP-Fuc and cofactor concentration over 24 hours in a fed batch like OP reaction. The withdrawn samples were analysed at the same timepoints as in Fig.S2 3 by HPLC to observe the concentrations of GDP-Fuc, GTP and ATP. At reaction start the OP reaction contains 80 mM L-Fuc, 15 mM $MgCl_2$, 0.8 mM AMP, 1.6 mM GMP, 80 mM PolyP, 100 mM lactose in a 50 mM Tris/HCl buffer (pH 7.6). 120 mU *Sm*PPK, 30 mU *Aj*PPK, 120 mU *Bf*FKP, 80 mU *Hp*Ft and 0.5 U iPPase were added to start the reaction. The mixture was incubated at 37°C and 350 rpm. After 1, 3, 6, 9, 12 and 24 hours samples were withdrawn and analysed. The reaction was fed with 30 mU of fresh *Hp*Ft enzyme 3, 6, 9 and 12 hours after the reaction start. After 12 hours the OP reaction contains 50 mM L-Fuc, 10 mM $MgCl_2$, 0.5 mM AMP, 1 mM GMP, 50 mM PolyP and 62 mM lactose. The OP reaction was examined in duplicates and the arithmetic mean and standard deviation were displayed in the figure

The courses of the GDP-Fuc, GTP and ATP concentration are going up and down during the OP reaction (Fig.S2 4). One reason is the lowering of the concentrations up to 62% of the initial concentration by feeding the reaction with the fresh *Hp*Ft enzyme. Also, all enzymes involved in the GDP-Fuc supply and regeneration of cofactors were diluted massively during the reaction. The lower concentration in combination with the dilution of the enzymes are an explanation for the drop down of the concentrations. At the end GTP and ATP are available in the OP reaction and should be utilized by *Bf*FKP for the GDP-Fuc synthesis. A reason for the drop down of the GDP-Fuc could be the constant addition of fresh *Hp*Ft enzyme, but Fig.S2 3 shows no significant increase of the 2'FL in that period. The high protein concentration after 12 hours, caused by the addition of *Hp*Ft, might influence the productivity negatively. The *Hp*Ft concentration was increased from 3.7 mg/mL to 8.5 mg/mL. Together with the other enzymes and the PolyP a very viscous solution was the result. To sum up, regarding to the standard deviation the measurement did not supply valid

or trustful data. At the best, the trend of the graph can be evaluated and it shows that no significant improvement of the 2’FL titer compared to Fig. 10 can be observed.

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Appendix:

Genes:

The *Bf*FKP enzyme from *Bacteroides fragilis* 9434 (Uniprot: Q58T34) was cloned in pET16.b vector and then transferred in *E.coli* BL21 strain.

The *Hp*Ft enzyme in a pMCSG7 vector (Uniprot: J0C6Y0 with W42G, G43S and P124S) was kindly provided by Galab Laboratories GmbH (Hamburg, Germany). The cloning work was done by Karina Gierlinger as part of her Master thesis (2017).

The PPK2 enzymes were kindly received in pET- 28a vectors with an N-terminal His-tag from Prof. Jennifer Andexer (Institute of Pharmaceutical Sciences, University of Freiburg, Germany). The cloning work was done by Sandra Kulmer as part of her Master thesis.[45]

***Aj*PPK**

ATGGACACCGAAACCATTGCAAGCGCAGTTCTGAATGAAGAACAGCTGAGCCTGGATCTGATTGAAGCACAGTATGCACT
GATGAATACCCGTGATCAGAGCAATGCAAAAAGCCTGGTTATTCTGGTTAGCGGTATTGAACTGGCAGGTAAAGGTGAAG
CAGTTAAACAGCTGCGTGAATGGGTTGATCCGCGTTTTCTGTATGTTAAAGCAGATCCGCCTCACCTGTTTAATCTGAAACA
GCCGTTTTGGCAGCCGTATACCCGTTTTGTTCCTGCCGAAGGTCAGATTATGGTTTGGTTTGGTAATTGGTATGGTGATCTG
CTGGCAACCGCAATGCATGCAAGCAAACCGCTGGATGATACCCTGTTTGATGAATATGTTAGCAACATGCGTGCCTTTGAA
CAGGATCTGAAAAATAACAATGTGGATGTGCTGAAAGTGTGGTTTGATCTGAGCTGGAAAAGCCTGCAGAAACGTCTGGA
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TGCAGATGCAATGCGTTGTGATGATCGTAAAGTTGTGATTGCGTTTGAAGGTATGGATGCAGCCGGTAAAGGCGGTGCAA
TTAAACGTATTGTTAAAAAACTGGATCCGCGTGAGTATGAGATTCATACCATTGCAGCACCGGAAAAATATGAACTGCGTC
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GGTTGAACGTGTTGAAGGTTTTGCAACCGAAGTTGAATGGCAGCGTGCCTATGCAGAAATTAATCGCTTTGAAAAAAATCT
GAGCAGCAGCCAGACCGTGCTGATCAAATTTTGGCTGGCAATTGATAAAGATGAACAGGCAGCACGTTTTAAAGCACGTG
AAAGCACACCGCATAAACGCTTTAAAATCACCGAAGAGGATTGGCGCAATCGTGATAAATGGGATGATTATCTGAAAGCC
GCAGCAGATATGTTTGCACATACCGATACCAGCTATGCACCGTGGTATATTATCAGCACCAATGATAAACAGCAAGCCCGT
ATTGAAGTTCTGCGTGCAATCCTGAAACAACTGAAAGCGGATCGTGATACCGATTAA

***Sm*PPK:**

ATGGCCCTGGATGAAGCACCGGCAGAAGCACGTCCGGGTAGCCGTGCAGTTGAACTGGAAATTGATGGTCGTAGCCGCA
TTTTTGATATTGATGATCCTGATCTGCCGAAATGGATTGATGAAGAGGCATTTCGTAGTGATGATTATCCGTATAAAAAAA
AACTGGATCGCGAAGAGTATGAGGAAACCCTGACCAAACTGCAGATCGAACTGGTTAAAGTTCAGTTTTGGATGCAGGCA
ACCGGTAAACGTGTTATGGCAGTTTTTGAAGGTCGTGATGCAGCAGGTAAAGGTGGTGCAATTCATGCAACCACCGCAAA
TATGAATCCGCGTAGCGCACGTGTTGTTGCACTGACAAAACCGACCGAAACCGAACGTGGTCAGTGGTATTTTCAGCGTTA
TGTTGCAACCTTTCCGACCGCAGGCGAATTTGTTCTGTTTGATCGTAGCTGGTATAATCGTGCCGGTGTTGAACCGGTTATG
GGTTTTTGTACACCGGATCAGTATGAACAGTTTCTGAAAGAAGCACCGCGTTTTGAAGAAATGATTGCCAATGAAGGTATC
CACCTGTTCAAATTCTGGATTAACATTGGTCGTGAGATGCAGCTGAAACGTTTTCATGATCGTCGTCATGATCCTCTGAAAA
TTTGGAAACTGAGCCCGATGGATATTGCAGCACTGAGCAAATGGGATGATTATACCGGCAAACGTGATCGTATGCTGAAA
GAAACCCATACCGAACATGGTCCTTGGGCAGTTATTCGTGGTAATGATAAACGTCGCAGCCGTATTAATGTTATTCGTCAC
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