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Glycosylation of small molecules by recombinant sucrose phosphorylase

MASTER'S THESIS

to achieve the university degree of

Diplom-Ingenieur

Master's degree programme: Biotechnology

submitted to

Graz University of Technology

Supervisor

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Graz, October 2016

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ACKNOWLEDGEMENTS

First of all, I want to thank Univ.-Prof. Dipl.-Ing. Dr. techn. Bernd Nidetzky for the opportunity to work at the Institute of Biotechnology and Biochemical Engineering at Graz University of Technology within a cooperation project together with the “Austrian Centre of Industrial Biotechnology” (“acib”).

Especially, I would also thank my both supervisors, Ass.-Prof.in Dipl.-Ing.in Dr.in Regina Kratzer and Dr. Rama Gudiminchi for the patience and guidance over the whole time working for the master thesis.

In deep respect, I wanted to thank my wife, Dipl.-Ing.in Verena Ruß and my parents, Stefan and Gabriele Plaschg for their patience and financial support, because otherwise I weren´t able to study.

Last but not least, I also want to thank all my friends, who accompanied my lifeline over the whole student life.

For my wonderful wife, Verena

Contents

Glycosylation of small molecules by recombinant sucrose phosphorylase

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Abstract

The application of AA-2G as fine chemical for cosmetic and pharmaceutical purpose challenges the search for new synthesis routes by enzymes for large scale production. Therefore, we investigated the optimization and scale-up of recombinant *BIS*Pase production in *E. coli* BL21 (DE3). The first step was plasmid selection at deep-well plate scale, followed by shake flask cultivation. Finally, the enzyme was produced in a stirred glass bioreactor with a working volume of 1.5 L. Samples were taken at certain time points to determine AA-2G formation, enzyme expression with SDS-PAGE and enzymatic activity, as well as volumetric yield. After upscaling to bioreactor level, optimization approaches were applied to restrain plasmid loss and increase the production of AA-2G.

1. Introduction

Sucrose phosphorylases are classified as members of glycosyl transferase family GH13.[1] These enzymes catalyze the reversible conversion of sucrose and phosphate into glucose-1-phosphate and D-fructose.[2] Sucrose phosphorylases are known from the microbial sources *Leuconostoc mesenteroides*, *Bifidobacterium adolescentis* and *Bifidobacterium longum, Pelomonas saccharophila, Pseudobutyrivibrio ruminis, Shewanella putrefaciens* and *Streptococcus mutans*.[3,4–6] Protein structures from the homodimeric *Bifidobacterium adolescentis* sucrose phosphorylase have been determined by X-ray crystallography.[7,8]

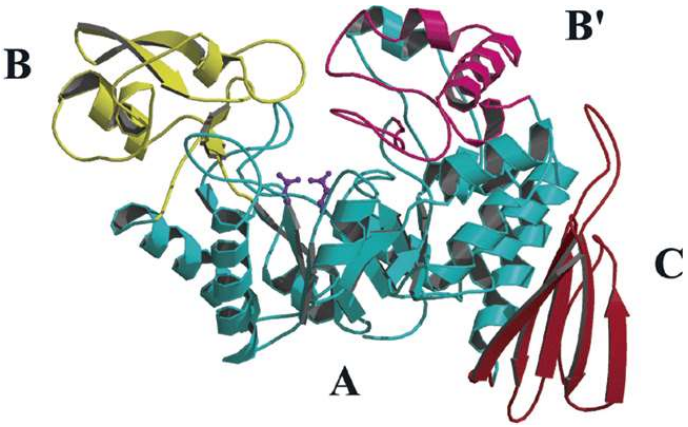

Figure 1. Richardson representation of the monomeric subunit of homodimeric *Ba*SPase. Domain A is indicated in blue, domain B in yellow, domain B' in magenta and domain C in red. The catalytic active site residues are shown in purple (taken from Sprogøe et al. 2004).[7]

The crystal structure of sucrose phosphorylase from *Bifidobacterium adolescentis* (*Ba*SPase) suggests four domains. Domain A comprises a $(\beta/\alpha)_8$-barrel, which is characteristic for family GH 13. The residues of the catalytic active-site (Asp^{192} and Glu^{232}) are located at the tips of β-sheets 4 and 5 in this barrel. Domain B contains two antiparallel β-sheets and two short α-helices. In contrast, domain B' is formed by a long

and a short α-helix. Additionally, the C-terminal domain (domain C) forms a single five-stranded antiparallel β-sheet.[7] Recombinant sucrose phosphorylase from *Bifidobacterium longum* has been shown to use a broad range of monomeric sugars as acceptor molecules.[5] The enzyme also catalyzes the conversion of sucrose and L-ascorbic acid (L-AA) into ascorbic acid 2-glucoside (AA-2G) and D-fructose, however at a very low rate. The product was detected by HPLC and LC-MS.[6,9] L-AA is a water soluble essential nutrient well known as vitamin C (VC).[10] In addition, VC is widely used in food, cosmetic and pharmaceutical industry.[11] However, L-AA is sensitive towards oxidation, especially at higher temperatures (Figure 2).[6] The degradation of L-AA leads to reduced biological activity.[10]

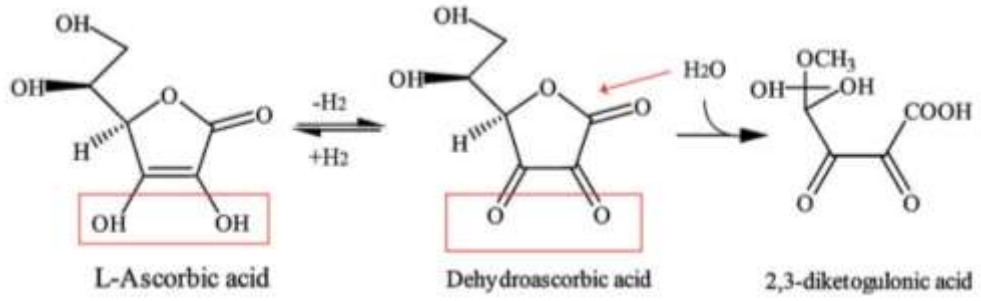

Figure 2. Degradation process of soluble L-ascorbic acid in H_2O. A derivatized molecule could block the oxidation of L-ascorbic acid to 2,3-diketogluconic acid (taken from Han et al. 2012).[10]

The degradation of L-AA hampers potential applications in food and other industries. Therefore, it is necessary to produce derivatives to increase the stability of ascorbic acid. Examples for derivatives are ascorbic acid sulfate, ascorbyl palmitate or ascorbyl glucosides.[12–14] However, most of these derivatives are not satisfactorily characterized with regard to anti-oxidation effects and L-ascorbic acid release *in vivo.* AA-2G was investigated in more detail. Its structural integrity enhances antibody production and collagen synthesis. AA-2G as L-AA derivative has hence potential in maintaining skin

elasticity and repairing skin damages.[15] The production of AA-2G in economically competitive amounts is of great interest and enzymatic transglycosylation reactions are promising synthesis routes.[6,14,16] Alternative biosynthetic routes include cyclodextrin glucanotransferase (CGTase) and glucansucrase. Gudiminchi et al. (2016) increased the yield of AA-2G by favoring the disproportionation route with the use of cyclodextrin glucanotransferase (CGTase). Figure 3 depicts the possible routes towards AA-2G by CGTase.[16] Kim et al. (2010) performed the glucosylation of L-AA to AA-2G with novel glucansucrase from *Leuconostoc lactis.* The enzyme was cloned into *E. coli* BL21 (DE3). The strain harboring the expression plasmid was cultivated and protein expression was induced with IPTG. The purified enzyme was used for the glucosylation of L-AA to AA-2G.[17]

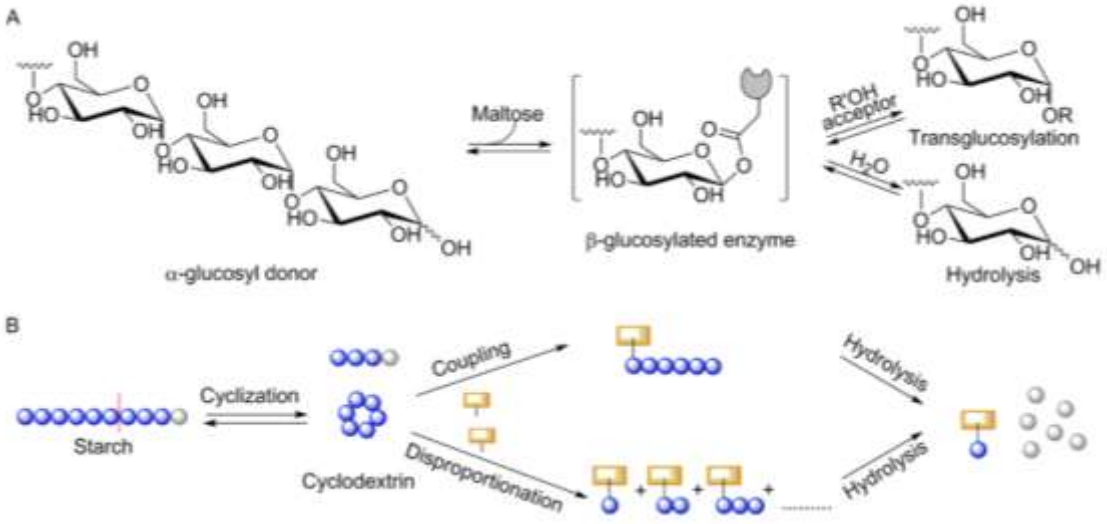

Figure 3. Catalytic routes towards AA-2G by using cyclodextrin glucanotransferase (CGTase). Panel A, reaction by a double displacement mechanism. The α-glucosyl donor (starch) is hydrolyzed resulting in a β-glucosylated enzyme intermediate. The glucosylated enzyme transfers the glucosyl moiety to the acceptor L-AA, leading to AA-2G. Panel B, starch (blue circles) gets cyclized to cyclodextrin (Figure 2B). Then the α-glucosylated donor molecule attaches by a coupling or disproportionation reaction to the acceptor (yellow frame). This finally leads to hydrolyzed AA-2G molecules at the end of the pathway by using cyclodextrin glucotransferase (taken from Gudiminchi et al. 2014).[16]

In this study we investigated the production of recombinant sucrose phosphorylase for the conversion of sucrose and L-AA to AA-2G and D-fructose. Main aim of this study was the scale-up and process intensification of sucrose phosphorylase expression. We used the vectors pCHEM21 and pQE_ara, expression plasmids that were developed at the Austrian Centre of Industrial Biotechnology (acib GmbH), and compared them to the commercially available pQE30 vector. Additionally, we used a whole cell catalyst for the production of AA-2G in small scale. We started with comparison of enzyme expression in deep-well plate cultivations. Cultivation was then scaled up to shake

flasks in order to verify expression levels of recombinant sucrose phosphorylase. We further used bioreactors for large scale cultivation and optimized process parameters thereof.

2. Materials and Methods

2.1 Chemicals and strains

Supplemented lysogeny broth (LB+) was used as growth medium for shake flask and bioreactor cultivation. Besides the standard LB components (5 g/L NaCl (Carl Roth), 10 g/L yeast extract (Carl Roth), 5 g/L peptone (Carl Roth)) 0.25 g/L NH_4Cl (Merck KgaA), 3 g/L K2HPO4 (Carl Roth), 6g/L KH2PO4 (Carl Roth) and 1 mL/L trace elements were added. The trace elements contained 4g/L $FeSO_4$*7 H_2O (Merck KgaA), 1g/L $MnSO_4$*H_2O (Merck KgaA), 0.73g/L $CoCl_2$*6 H_2O (Merck KgaA), 0.1g/L H_3BO_3 (Sigma Aldrich), 0.095g/L $CuSO_4$ (Merck KgaA) and 0.2g/L $ZnSO_4$*7 H_2O (Merck KgaA). Additionally, 100 µL/L PPG (Sigma Aldrich) was added as antifoam agent and 115 mg/L ampicillin (Carl Roth) as selection marker. For the deep well-plate cultivation LB was used without any trace elements.

E. coli BL 21 (DE3) was used as strain with recombinant sucrose phosphorylase from *Bifidobacterium longum*, cloned into pCHEM21 as expression system, a vector system that was developed at the Austrian Centre of Industrial Biotechnology (acib GmbH) within an EU project called CHEM21 as pQE_ara, an arabinose induced system, which was developed at acib GmbH, too. Additionally, pQE30 (QIAGEN AG) was used for deep-well plate experiments, too.

The inducer IPTG and L-arabinose were from Carbosynth and Tokyo Chemical Industry Co, Ltd., respectively. For the production of AA-2G, the substrates L-AA and D-

sucrose (1 M and 1.5 M) were obtained from Tokyo Chemical Industry, Ltd. The AA-2G standard with a purity of 99.8% was obtained from Tokyo Chemical Industry, Ltd., too.

For protein analysis with SDS-PAGE, 10 mM HEPES (Carl Roth)/Bug Buster (Novagen) (10% v/v) solution, DTT as reducing agent, a loading dye from Invitrogen and 10000x diluted benzonase (Sigma Aldrich, B2371) was used. For the insoluble protein after cell lysis, 6M urea (Carl Roth) was applied.

For the enzymatic ass *Bl*SPase activity, lysis buffer for cell lysis containing 100 mM citrate buffer, 50 mM NaCl, 1 mM EDTA and 0.5 mM DTT was prepared. Afterwards phosphoglucomutase (Sigma Aldrich, P3397) and glucose 6-phosphate dehydrogenase (Sigma Aldrich, G8404) was used for the NADH dependent assay. For the diluted 10x phosphoglucomutase, pH 6.0, a 3.2 M $(NH_4)_2SO_4$, containing 0.01% (w/v) EDTA disodium salt dihydrate (Carl Roth) was prepared. As well as for phosphoglucomutase, a solution of 3.2 M $(NH_4)_2SO_4$, pH 7.5, containing 50 mM Tris (Carl Roth) and 1 mM magnesium chloride (Carl Roth) was prepared for glucose-6-phosphate dehydrogenase. The crude enzyme extract was diluted afterwards in certain concentrations in a buffer solution, comprising 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$, 10 mM EDTA and 10 µM Glc1,6-bisP (Sigma Aldrich). 1.2 M D-sucrose (Tokyo Chemical Industries, Ltd.) in buffer solution was used as substrate solution for the assay and NAD^+ (20 mg/mL, Carl Roth) was prepared.

For protein determination via Bradford assay, a Roti-Quant (Carl Roth) solution 5x diluted was used.

2.2 Deep-well plate cultivation

The *E. coli* BL21 (DE3) cells were cultivated under conditions of sucrose phosphorylase expression in deep-well plates (VWR). Inducer concentration (0.1, 0.55, 1 mM IPTG; 0.0015, 0.015, 0.15 mM L-arabinose) and D-sucrose concentration (0, 0.5, 1.0 mM) were varied as shown in table 1. Three identical plates were prepared for induction at 25°C, 30°C and 37°C.

Table 1: Basic grid of design of experiments to compare expression conditions in deep-well plate cultivation. The abbreviations Suc and Ara stand for D-sucrose and L-arabinose. Therefore, the letters and numbers are characterizing the exact position in the deep well-plate, starting with B2 and ending with G10.

		2	3	4	5	6	7	8	9	10
pQE30	**B**	0 mM Suc 0.1 mM IPTG	500 mM Suc 0.1 mM IPTG	1000 mM Suc 0.1 mM IPTG	0 mM Suc 0.55 mM IPTG	500 mM Suc 0.55 mM IPTG	1000 mM Suc 0.55 mM IPTG	0 mM Suc 1 mM IPTG	500 mM Suc 1 mM IPTG	1000 mM Suc 1 mM IPTG
	C	0 mM Suc 0.1 mM IPTG	500 mM Suc 0.1 mM IPTG	1000 mM Suc 0.1 mM IPTG	0 mM Suc 0.55 mM IPTG	500 mM Suc 0.55 mM IPTG	1000 mM Suc 0.55 mM IPTG	0 mM Suc 1 mM IPTG	500 mM Suc 1 mM IPTG	1000 mM Suc 1 mM IPTG
pCHEM21	**D**	0 mM Suc 0.1 mM IPTG	500 mM Suc 0.1 mM IPTG	1000 mM Suc 0.1 mM IPTG	0 mM Suc 0.55 mM IPTG	500 mM Suc 0.55 mM IPTG	1000 mM Suc 0.55 mM IPTG	0 mM Suc 1 mM IPTG	500 mM Suc 1 mM IPTG	1000 mM Suc 1 mM IPTG
	E	0 mM Suc 0.1 mM IPTG	500 mM Suc 0.1 mM IPTG	1000 mM Suc 0.1 mM IPTG	0 mM Suc 0.55 mM IPTG	500 mM Suc 0.55 mM IPTG	1000 mM Suc 0.55 mM IPTG	0 mM Suc 1 mM IPTG	500 mM Suc 1 mM IPTG	1000 mM Suc 1 mM IPTG
pQEara	**F**	0 mM Suc 0.0015% Ara	500 mM Suc 0.0015% Ara	1000 mM Suc 0.0015% Ara	0 mM Suc 0.015% Ara	500 mM Suc 0.015% Ara	1000 mM Suc 0.015% Ara	0 mM Suc 0.15% Ara	500 mM Suc 0.15% Ara	1000 mM Suc 0.15% Ara
	G	0 mM Suc 0.0015% Ara	500 mM Suc 0.0015% Ara	1000 mM Suc 0.0015% Ara	0 mM Suc 0.015% Ara	500 mM Suc 0.015% Ara	1000 mM Suc 0.015% Ara	0 mM Suc 0.15% Ara	500 mM Suc 0.15% Ara	1000 mM Suc 0.15% Ara

500 µL of standard lysogeny broth (LB) medium supplemented with 100 mg/L ampicillin was used as cultivation medium. The medium was inoculated with *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase and incubated at 37°C and 120 rpm (Infors HT Multitron standard) until an OD_{600} ~ 0.8-1.0 was reached (typically approx. after 1.5 h cultivation time). Upon induction, the three different plates were incubated at 25°C, 31°C or 37°C. After 15 h, OD_{600} was determined and the cells were centrifuged for 20 min, 4000 rpm and 4°C (Eppendorf 5810R). The supernatant was discarded and the cell pellet re-suspended in 500 µL of the substrate solution containing 1200 mM L-AA, 800 mM D-sucrose (pH 5.2). After 24 h of incubation in the dark at 50°C and 120 rpm (Infors HT Multitron standard) cells were separated by centrifugation. The supernatants were diluted 100-fold in micro titer plates for HPLC analysis (Greiner bio-one). The analysis of AA-2G was performed on a Shimadzu HPLC system (Sampler SIL-20A, Oven CTO-20A, Solvent Delivery Unit LC-20A, System Controller CBM-20A, Degasser DGU-20A, UV-VIS detector SPD-M20A, RID detector RID-10A). Analysis was performed using a Cation H Micro-Guard pre-column followed by an Aminex HPX-87H column and H_2SO_4 (20 mM; stock solution from Carl Roth) was used as eluent at a flow of 0.4 mL/min. The total run time per sample was 23 min at 25°C temperature. Retention time of AA-2G was 12.5 min under these conditions. HPLC analysis was performed using authentic standards of AA-2G. The concentration of AA-2G in mmol per gram cell dry weight was calculated afterwards and plotted onto a graph over time.

2.3 Shake flask cultivation

1 L and 300 mL baffled Erlenmeyer shake flasks were filled with 200 and 50 mL of medium (main culture and pre-culture). The medium was a LB medium supplemented with 5.5 g/L glucose and 115 mg/L ampicillin. 50 mL pre-cultures of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase were incubated at 30°C and 120 rpm overnight (Sartorius

Certomat BS 1). The main cultures were inoculated to an OD_{600} of 0.01 and likewise incubated at 37°C and 120 rpm. At OD_{600} ~ 0.8-1.0, enzyme expression was induced with 1 mM IPTG and cultures were incubated at 25°C and 120 rpm. At the induction point, and 2 h, 4 h, 6 h, 18 h, 20 h, 25 h post induction, 1 mL samples were taken for protein expression analysis. In parallel, samples from a non-induced main culture were taken. Samples were centrifuged for 10 min at 4°C to collect the biomass as compacted pellet (Eppendorf 5427R). The wet biomass weight was determined and cells were lysed with 10 mM HEPES/BugBuster (Carl Roth, Novagen) solution prior to protein expression analysis. 25 h post induction, the remaining biomass was harvested from the flasks. The wet biomass was weighed and suspended in cell re-suspension buffer (100 mM citrate buffer, pH 5.2, containing 50 mM NaCl, 1 mM EDTA and 0.5 mM DTT) prior to mechanical cell lysis using a French press.

2.3.1 Determination of cell growth and specific growth rate μ

We specified cell growth with UV/VIS spectrophotometry at 600 nm (OD_{600}). Samples were taken at inoculation and after 1 h, 2 h, 3 h (induction point), 5 h, 7 h, 9 h, 21 h, 23 h and 28 h of cultivation time. Samples were diluted with 0.9 % NaCl (w/v). We derived the specific growth rate from the integrated form of autocatalytic growth $\frac{dx}{dt} = \mu x$, which is $x_{t_1} = x_{t_o} e^{\mu(t_1 - t_o)}$. x stands for the cell concentration in g/L. μ is known as specific growth rate (1/h). x_{t_1} and x_{t_o} are values for the cell concentration in g/L at the time points t_o and t_1. t_1 and t_0 are commonly defined as time points at the end and beginning of the determination or measurement.[18] Here, we used OD_{600} values instead of cell dry weights leading to $OD_{600t_1} = OD_{600\ t_0} e^{\mu(t_1 - t_0)}$.

2.3.2 Analysis of protein expression with SDS-PAGE

Biomass pellets obtained from shake flask cultivation were re-suspended in 10 mM HEPES/Bug Buster solution (Carl Roth/Novagen). The solution with benzonase (Sigma Aldrich, B2371) was prepared as described previously in the chemicals and strains section.

After incubation for 20 minutes, 900 rpm and room temperature (Eppendorf Thermo-mixer comfort), the cell debris were centrifuged for 15 min at 4°C and 13000 rpm (Eppendorf centrifuge 5427R). The supernatant was transferred into fresh 1.5 mL Eppendorf tubes. The remaining pellet/insoluble protein was treated with 6 M urea. Directly after resuspension of the remaining pellet, the mixture was centrifuged for 5 min at 4°C and 13000 rpm. The supernatant of the insoluble protein fraction was transferred again in a fresh tube. Soluble protein concentrations were determined by using a DeNovix DS-11 UV/VIS spectrophotometer at 180/160 nm ratio. Due to non-reliable measurements of unfolded proteins, the same concentration was assumed corresponding to soluble protein. Therefore, 20 µg protein in 10 µL of volume was loaded onto SDS gel. The volume of sample and ddH_2O was variated, due to different protein concentrations of the samples after cell lysis. Additionally, 1 µL DTT (Carl Roth) and 2.5 µL of NuPAGE loading dye were added. Gels were run in 1x NuPAGE MOPS SDS running buffer in the vertical Invitrogen NuPAGE Novex system at 220 V for 55 min. After staining with Coomassie Blue (Carl Roth) the 55 kDa *Bl*SPase was visible.

2.3.3 Determination of volumetric and specific activity

Biomass was re-suspended in 100 mM citrate buffer with 50 mM NaCl, 1 mM EDTA and 0.5 mM DTT (pH 5.2) in a ratio of 1 g wet biomass per 5 mL citrate buffer. Cells were mechanically disrupted by three passages through a pre-cooled French press (American Instrument Company) operated at an applied pressure of 1000 psi. Then,

the non-soluble cell debris was centrifuged for 30 min at 4°C and 4000 rpm (Eppendorf 5810R). The cell-free extract was aliquoted in 1 mL samples to Eppendorf tubes (1.5 mL) and stored at -20°C for use. Cell-free extracts containing *Bl*SPase were diluted appropriately for the D-sucrose based multi-enzyme assay.

2.3.4 Photometric *Bl*SPase activity assay

A cascade reaction consisting of *Bl*SPase, phosphoglucomutase and glucose 6-phosphate dehydrogenase (Glc 6-DH) provided the basis for the assay. In detail, 1.1 M D-sucrose in 50 mM potassium phosphate buffer (pH 7.0) was converted by recombinant *Bl*SPase to glucose 1-phosphate and D-fructose. Glucose 1-phosphate was further converted to glucose 6-phosphate by phosphoglucomutase. Finally, glucose 6-phosphate was oxidized to gluconolactone 6-phosphate by NAD^+-dependent glucose 6-phosphate dehydrogenase (Glc 6-DH). NADH formation was monitored for 15 min at 340 nm on a Beckman DU-800 spectrophotometer. phosphoglucomutase (Sigma Aldrich, P3397) was 10-fold diluted into 3.2 M ammoniumsulfate solution, containing 0.01% EDTA disodium salt dihydrate at pH 6. Glucose 6-phosphate dehydrogenase (Sigma Aldrich, G8404) was diluted 10-fold in 3.2 M $(NH_4)_2SO_4$ solution, containing 50 mM Tris and 1 mM magnesium chloride at pH 7.5. In general, the same assay was used corresponding to Goedl et al (2007).[4]

2.3.5 Determination of protein concentration

The protein concentration was determined spectrophotometrically at 595 nm by the standard method according to Bradford.[19] Roti-Quant (Carl Roth) was diluted 1:5 with ddH_2O according to the supplier. The sample was diluted to approximately 0.1 to 1.0 mg/mL protein. 20 µL of the sample was added to 1000 µL of the Roti-Quant solution, vortexed and incubated for 10 min at room temperature. Citrate buffer was used as

blank sample. A calibration curve prepared with 0.1 to 1.0 mg/mL bovine serum albumin (BSA) was used to calculate the exact protein concentration. The volumetric activity ($\frac{U}{mL}$) divided by the protein concentration ($\frac{mg}{mL}$) results in the specific activity ($\frac{U}{mg}$).

2.4 Bioreactor cultivation

Four flasks with 50 mL LB+ with 115 mg/L ampicillin were used for inoculation with *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase of the pre-culture. They were cultivated overnight at 30°C and used for inoculating the bioreactor at and OD_{600} of 0.5 (start OD_{600}). A "Lab Infors® Labfors 3" cascade 3 L bioreactor with a working volume of 1.5 L was used for all bioreactor cultivation studies. 1 mM IPTG was used for induction.

2.4.1 Determination of cell growth and specific growth rate µ

Samples were taken at 0 h, 3 h, 6 h, 9 h and 15 h of cultivation time. Cell growth and specific growth rate was determined as described in 2.3.1. In addition, the cell dry weight from 5 mL cultivation broth was determined after 1.25 h (induction point), 6 h and 15 h cultivation time. Cells were pelleted by centrifugation for 30 min at 4°C and 4000 rpm (Eppendorf 5804R); the supernatant was discarded. Pellets were washed in 5 mL of 0.9 % NaCl (w/v) and centrifuged. Washed cells were re-suspended in 1 mL ddH_2O and transferred into a pre-weighed GC vial prior to drying at 105°C overnight. Cell dry weights were calculated to g/L.

2.4.2 Production of L-ascorbic acid-α-D-glucoside (AA-2G) by whole cells

Synthesis of AA-2G from 800 mM D-sucrose and 1200 mM L-ascorbic was performed in a volume of 500 µL in 1.5 mL Eppendorf tubes. Therefore, samples were taken from the bioreactor cultivation broth at the induction point (1.25 h cultivation time), and 2 h, 4 h, 6 h and 10 h post induction. The fresh cells were centrifuged for 10 min. at 13400 rpm and 4°C to obtain a compact pellet. Cell pellets were either stored at -20°C or

directly used. Cells were re-suspended in 500 µL ddH_2O and thereof 100 µL cell suspension was transferred into a fresh Eppendorf tube for the conversion. 400 µL substrate solution of 1000 mM D-sucrose and 1500 mM L-ascorbic acid (pH 5.2) was added to the 100 µL cell suspension. Reaction mixtures were incubated for 24 h at 50°C and 400 rpm (Eppendorf Thermomixer comfort). After centrifugation of cells for 10 min at 4°C and 14000 rpm, Eppendorf 5418) supernatants were transferred into fresh Eppendorf tubes. Samples were diluted up to 100-fold and analyzed by HPLC as described under 2.2.

Catalyst productivity in mmol AA-2G per g_{CDW} (cell dry weight) was calculated: the concentration of AA-2G in mM was obtained by using the formula

$$AA-2G\ [mM]\ = \frac{(AA-2G\ peak\ area)\times 100}{7{,}000{,}000}.$$

Afterwards we correlated the mM AA-2G with the cell dry weight in g/L to calculate the yield $Y_{px,,}$ which explains the yield of mmol AA-2G per gram cell dry weight.

The obtained cell dry weight is divided by five, because only 100 µL of the gained cell suspension of 500 µL was added to the total whole reaction mixture.

Afterwards the following formula was used for calculation of Y_{px} to gain mmol AA-2G/g_{CDW}:

$$Y_{PX} = \frac{mM\ AA-2G}{\frac{g_{CDW}}{L}}$$

2.4.3 Analysis of protein expression with SDS-PAGE

The procedure was performed as described under 2.3.2. Samples taken at the induction point (1.25 h cultivation time), 3 h, 6 h, 9 h and 15 h post induction were analyzed.

2.4.4 Determination of volumetric and specific activity

Samples were taken after 6 h (7.25 h cultivation time) and 15 h post-induction. The determination of the protein concentration was performed as described in 2.3.5. For the calculation of the volumetric yield, the volume of the 5 mL citrate buffer in the sample of harvested cells was considered:

$$1\ g\ biomass\ \times 5\ mL\ citrate\ buffer = 5\ mL\ of\ citrate\ buffer$$

$$\frac{U}{mL}\ (determined\ volumetric\ activity) \times mL\ of\ citrate\ buffer = Total\ U$$

$$\frac{Total\ U\ in\ sample\ \times 1000\ mL\ fermentation\ broth}{sample\ volume\ from\ bioreactor\ in\ mL} = \frac{U}{L}\ fermentation\ broth$$

2.5 Optimization of bioreactor cultivation

2.5.1 Further ampicillin addition at the induction point

The same methods were used as for standard batch bioreactor cultivation. Samples were taken for cell growth and cell dry weight determination at inoculation (only OD_{600}), after 0.5 h, 1.5 h (both only OD_{600}), 1.75 h (induction point), 3.75 h, 5.75 h, 7.75 h, 9.75 h, 21.75 h and 26.75 h cultivation time. The samples for all additional experiments were taken at induction point, after 2 h, 4 h, 6 h, 8 h, 20 h and 25 h post-induction. For the determination of volumetric and specific activity an aliquot of harvested cells after 25 h post-induction was taken. Ampicillin (115 mg/L) was added at inoculation and induction time.

2.5.2 Use of carbenicillin instead of amplicillin

Monitoring of cell growth, protein expression analysis, production of AA-2G by whole-cell catalysis and determination of volumetric and specific activity was done. Samples

for cell growth and cell dry weight determination were taken at inoculation, after 1 h, 1.25 h (induction point), 4.25 h, 7.25 h, 10.25 h and 16.25 h cultivation time. For whole-cell conversions, protein expression analysis by SDS-PAGE and volumetric activity, specific activity and volumetric yield determination samples were taken at similar time points.

2.5.3 Addition of 250 mM D-sucrose to the fermentation broth

Monitoring of cell growth, protein expression analysis, production of AA-2G by whole-cell catalysis and determination of volumetric and specific activity was done. Samples for cell growth determination (OD_{600} and cell dry weight) were taken at inoculation, after 1 h (both only OD_{600}), 1.5 h (induction point), 4.5 h, 7.5 h, 10.5 h and 16.5 h, 26.5 h and 31.5 h cultivation time. Samples of time points after the induction were also used for production of AA-2G by whole-cell catalysis and analysis of protein expression by SDS-PAGE. For the determination of the volumetric and specific activity and volumetric yield samples after 6 h, 15 h and 30 h post-induction were analyzed.

2.5.4 Induction of protein expression at higher biomass concentration with further ampicillin addition at the induction point

Monitoring of cell growth, protein expression analysis, production of AA-2G by whole-cell catalysis and determination of volumetric and specific activity was done. Samples for cell growth determination (OD_{600} and cell dry weight) were taken at inoculation, after 1 h, 2 h (all three only OD_{600}), 3 h (induction point), 6 h, 9 h and 12 h and 18 h cultivation time. The sample at the induction point and post-induction samples were used for production of AA-2G by whole-cell catalysis, analysis of protein expression by SDS-PAGE and for determination of volumetric and specific activity as well as volumetric yield.

3. Results and discussion

3.1 Deep-well plate cultivation

We compared the heterologous expression of *Bl*SPase from three different vectors transformed into *E. coli* BL21 (DE3). The vectors pCHEM21 and pQE_ara, expression systems that were developed at the Austrian Centre of Industrial Biotechnology (acib GmbH) were compared to commercially available pQE30 from QIAGEN. For the comparison, an optimization of induction conditions with regard to inducer concentration and induction temperature was performed. Additionally, a possibly positive effect of the native substrate sucrose on the functional expression (soluble versus insoluble) of *Bl*SPase was tested. Vector maps of the plasmids are shown in Figure 3. All three vectors encode the gene for β-lactamase. Expression of the β-lactamase enables bacteria to easily cleave the lactam ring in the molecule.[20] pQE_ara and pQE30 have an additional chloramphenicol resistance marker gene. The encoded chloramphenicol acetyl transferase transfers an acetyl moiety from the co-factor acetyl-S-CoA to the chloramphenicol molecule, which leads to 3-acetoxychloramphenicol. The derivate loses its inhibition function.[21] pQE30 (*t5* promoter) and pCHEM21 (*tac1* promoter) carry both the classical lac operator and are hence inducible by IPTG. The *lac* promoter system is tightly regulated and under control of the LacI repressor protein.[22,23] Due to similarity with the *lac* promoter, IPTG as inducer is able to block the LacI repressor protein, which cannot bind on the promoter region to block the RNA polymerase for transcription of recombinant *Bl*SPase. Instead of *lac* promoter systems, *tac* promoter systems as *tac1* in pCHEM21 are much more efficient, due to the strong -35 box from the tryptophan promoter in the expression system.[22]

The pQE_ara system is induced by addition of arabinose. The regulator protein in the arabinose operon, AraC, blocks the transcription of a certain protein (in our case *Bl*SPase). AraC binds to the *araI* and *araO* site, the DNA forms a loop which prevents RNA polymerase from binding to the promoter. When arabinose is present, then AraC cannot bind in the region and the expression of *Bl*SPase starts.[24–27]

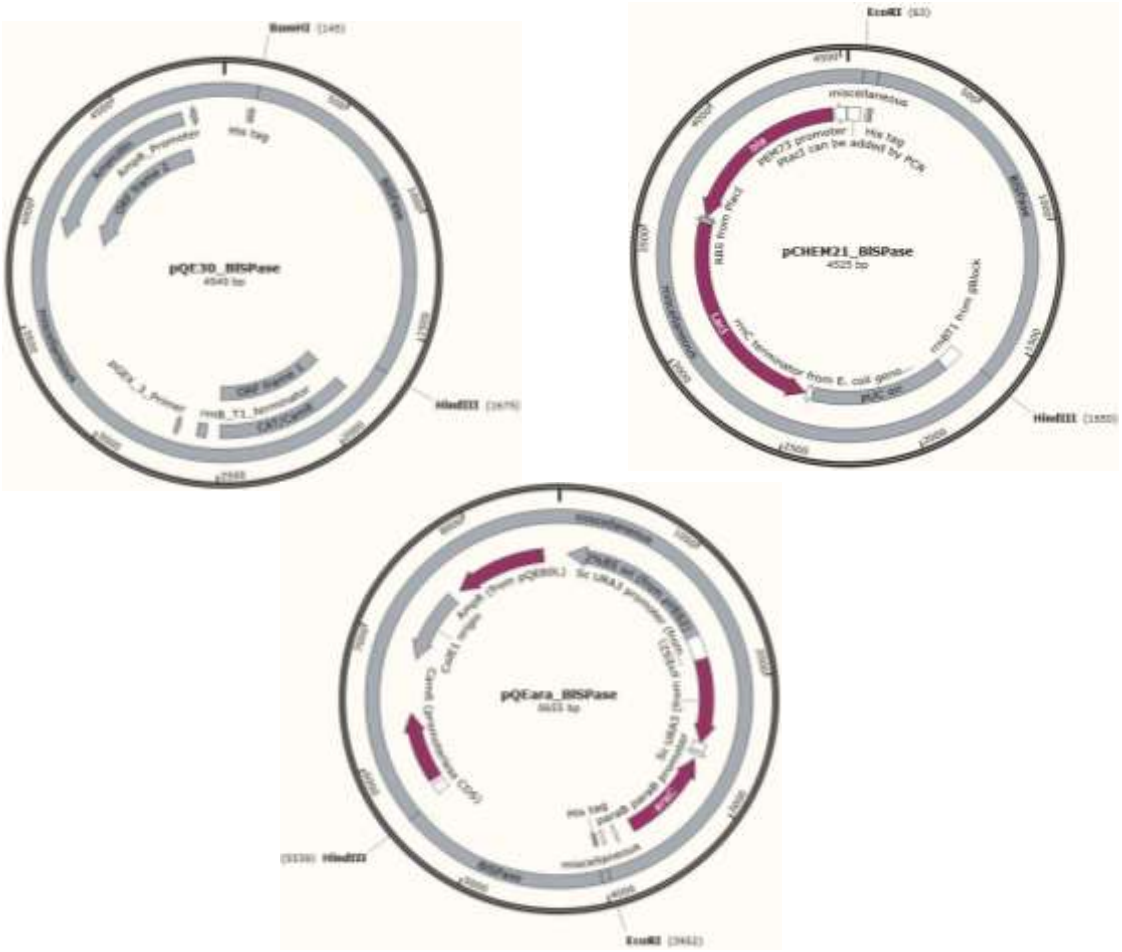

Figure 4. Comparison of the three vector systems used to express *Bl*SPase in deep-well plate cultivation.

The functional expression of the enzyme was verified by conversions of 800 mM D-sucrose and 1200 mM L-AA to AA-2G using cells obtained from deep-well plate culti-

vations as catalysts. The amount of AA-2G obtained after 24 h of conversion are summarized in Figure 5; tables with all detailed numbers are provided in the "Supplementary Information" part.

3.1.1 Effect of the plasmid

Panels 1, 4, 7 in Figure 5 show AA-2G formation (AA-2G in mmol per g_{cdw}) for cells harboring pQE30, panels 2, 5, 8 show results for pCHEM21 and panels 3, 6, 9 results for pQE_ara. pCHEM21 is the most promising vector system. We were able to produce more than double the amount of AA-2G with the whole cells carrying pCHEM21 compared to pQE30 and pQE_ara vector systems at 25°C as induction temperature (panels 1, 2, 3). Especially in combination with 500 mM D-sucrose as additive in the cultivation medium, the amount of AA-2G synthesized by whole cells harboring pCHEM increased up to 6-fold in contrast to cells with pQE30 or pQE_ara. Whole cells carrying pQE30 led to nearly 60 mmol of AA-2G per g_{cdw} (gram cell dry weight) of catalyst under most of the conditions. Cells that carry pCHEM21 produced over 300 mmol AA-2G per g_{cdw} under induction conditions of 1 mM IPTG and 500 mM D-sucrose as additive. Highest conversion of 100 mmol AA-2G per g_{cdw} was obtained with the arabinose inducible system pQE_ara at 0.15 % of arabinose, 500 mM D-sucrose and 25°C induction temperature.

3.1.2 Effect of sucrose

Cells from cultivation conditions 2, 5, 7 were cultivated without D-sucrose, cells from conditions 3, 6, 8 with 500 mM D-sucrose and cells from conditions 4, 7, 10 with 1000 mM D-sucrose. No product was detected with cells from cultivations with 1000 mM D-sucrose. Highest product concentrations were obtained with plasmids pCHEM21 and pQE_ara with 500 mM D-sucrose in the cultivation broth. *BIS*Pase expression from

pQE30 was hardly affected by addition of 500 mM D-sucrose. The question remains why D-sucrose had such a significant effect on enzyme production, since literature suggests that *E. coli* BL21 is not able to utilize D-sucrose as a substrate and has no permease to transport the D-sucrose in the cell. Bruschi et al. (2014) reported the use of D-sucrose as carbon source to produce a short peptide surfactant called DAMP4 in *E. coli*. They expressed a second plasmid with the *csc*AKB operon to facilitate D-sucrose metabolism.[28] The thereby engineered *E. coli* W has the genetic setup to transport sucrose into the cells and convert it to fructose-6-phosphate that can be isomerized to glucose-6-phosphate. *csc*B is coding for the sucrose permease in the cell wall, while *csc*A codes for an invertase, *csc*K for a D-fructokinase. The gained fructose-6-phosphate can be isomerized by phosphoglucomutase to glucose-6-phosphate, which is able to enter the glycolysis road to produce energy like ATP.[29]

Still, D-sucrose could have a stabilizing effect on the enzyme over the long cultivation time of approximately 15 h (overnight culture) in the deep-well plate. D-sucrose cannot be actively taken up by the cells, however, the long cultivation times results in old cells with more permeable cell walls.[30] This permeability may facilitate D-sucrose to enter the cells and stabilize the protein at the end of the cultivation.[30–32] This might be especially important when the specific growth rate (µ) is decreasing to zero but the cells are still expressing protein.[9,30,33] Important in this context is the tremendous decrease of cell growth upon the addition of 500 mM or 1000 mM D-sucrose. The effect is especially relevant with cells containing the pCHEM21 vector system. The decrease is between 2 and 5-fold (OD_{600} from 10) compared to cell growth without D-sucrose (OD_{600} from 2 to 5). Two research groups investigated the effect of sucrose on µ. Cheng et al. (2011) varied the sucrose concentration from 0 to 500 mM to study osmotic stress on the cAMP level in the cell. Additionally, they investigated cell growth of *E. coli* wildtype and

crp and *crp/lacI* mutants in the presence of D-sucrose. *crp* codes for the cAMP reactive protein, which is involved in the regulation of the lac operon. The µ of the wild-type decreased approximately 2-fold when 500 mM D-sucrose was in the growth medium. The engineered strains were able to grow as fast as the cells without sucrose.[34] Record et al. (1998) also examined the effect of osmotic stress on the growth of *E. coli* cells. The obtained results support our assumption that, especially in undefined LB medium, there is a dramatic decrease of cell growth dependent on the D-sucrose concentration.[35]

3.1.3 Effect of induction temperature

Induction temperature turned out as further important factor determining *BIS*Pase expression. Panels 1, 2, 3 show AA-2G expression at 25°C, panels 4, 5 ,6 at 31°C, and panels 7, 8, 9 at 37°C. Highest AA-2G concentration was obtained in panel 2, condition 9. There, cells carrying the pCHEM21 vector were grown in the presence of 500 mM D-sucrose, induced with 1 mM IPTG at an induction temperature of 25°C. It is generally known that a lower temperature promotes the expression of functional protein by minimizing inclusion body formation (e.g. Hannig et al., 1998).[36] Our results follow this general rule, 25°C as induction temperature led to more active cells and highest AA-2G formation compared to 31°C and 37°c as induction temperature. Only the arabinose induced system pQE_ara shows better conversion results, when 37°C as induction temperature was applied. In combination with 500 mM D-sucrose as additive in the growth medium, 0.15 % of arabinose as inductor 230 mM of AA-2G per g_{cdw} was obtained at 37°C. We decided to use cells carrying the pCHEM21 vector in further shake flask cultivations. Best AA-2G concentrations were obtained with this strain construct when 1 mM IPTG as inducer and an induction temperature of 25°C was used.

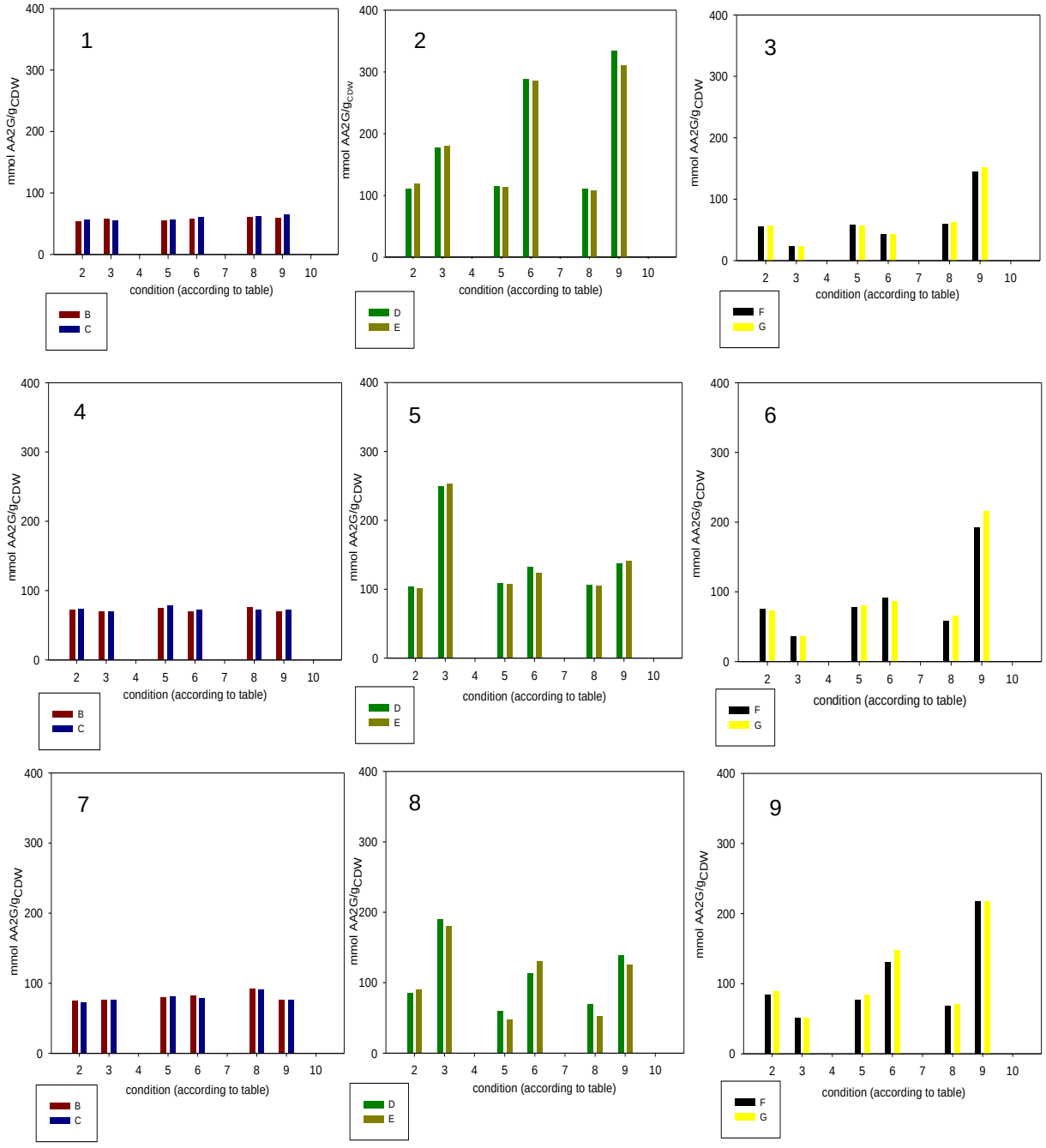

Figure 5. Screening for *BIS*Pase expression conditions. Shown is AA-2G formation per g cell dry weight (mmol AA-2G per g_{cdw}) obtained from whole-cell conversions of 800 mM D-sucrose and 1200 mM AA-2G after 24 h. Experiments were done in duplicate. The x-axis shows the condition numbered in the deep-well plate (Table 1). Panels 1, 4, 7 relate to vector pQE30, panels 2, 5, 8 to pCHEM21, and panels 3, 6, 9 to

pQE_ara. Panels 1, 2, 3 show results from 25°C induction temperature, panels 4, 5 ,6 from 31°C and panels 7, 8, 9 from 37°C.

3.2 Shake flask cultivation

Shake flask cultivation was used as a further step towards cultivation in the bioreactor. The effect of 1 mM IPTG on the cell growth, protein expression and specific cell activity on cells harboring pCHEM21 was studied. As described in the "Materials and Methods" part, we used a glucose-supplemented LB-medium (5 g/L glucose) in the pre- and main cultures to gain more biomass. In Figure 6 cell growth with and without induction by 1 mM IPTG is shown. In both cases an OD_{600} of ~ 12 was attained after 28 h. There is a clear difference in cell growth between induced and non-induced cells. The main difference was in the growth rate in the exponential phase (μ_{max}). We obtained a μ_{max} of 0.47/h, calculated from 5 h to 9 h of cultivation time for the non-induced cells and a μ_{max} of 0.13/h, calculated from 5 h to 21 h of cultivation time for induced cells. Different growth temperatures are one reason for differing μ_{max} values.[30,36] Cells were cultivated at 25°C after induction and at 37°C without induction. A second reason is the induction itself, as protein expression is highly energy consuming, which reduces the cell growth rate. The higher the protein expression, the lower is μ.[37]

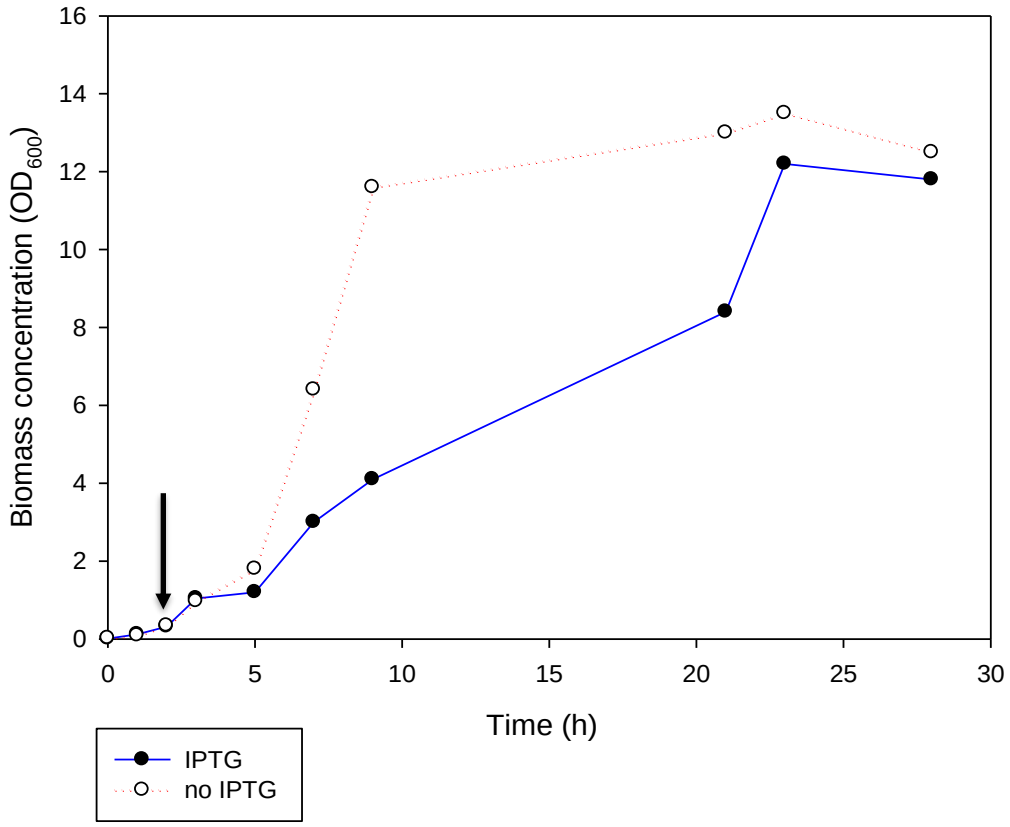

Figure 6. Cell growth of induced and non-induced *E. coli* BL 21 (DE3) harboring pCHEM21 in shake flask cultivation. The blue line with black dots indicates growth of induced cells; the dotted red line with white dots the non-induced cells. The black arrow shows the induction point of induced cells.

Additionally, we determined the protein expression with SDS-PAGE and the specific activity (U/mg) after mechanical cell lysis. Figure 7 shows the expression of recombinant *BIS*Pase in the soluble and insoluble fraction. At the beginning of induction (0-4 h), the enzyme is mainly found in the soluble fraction. After 6 h of induction, the expressed enzyme was distributed into half soluble and half insoluble fraction (inclusion bodies). Solubility might be increased by the co-expression of chaperons like DnaK or GrpE in *E. coli*.[38] Instead of co-expressing chaperones, certain chemicals for refolding to yield more soluble protein are reported.[39] One example might be the effect of D-

sucrose on functional protein expression seen in the present study in deep-well plate cultivations.

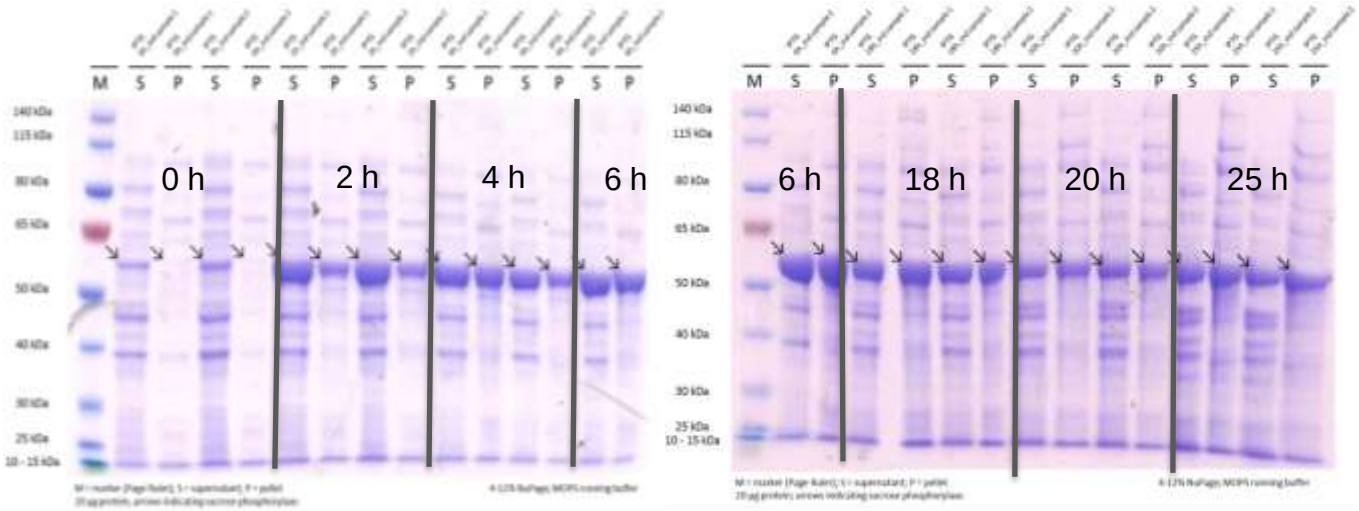

Figure 7. SDS-PAGE of *BI*SPase expression in shaken flask cultivation of *E. coli* BL21(DE3) harboring pCHEM21. The protein expression was traced over the induction time. Samples were taken at the induction point (0 h) and after 2 h, 4 h, 6 h, 18 h, 20 h and 25 h post induction. The abbreviations M, S and P are coding for marker, soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *BI*SPase at 55 kDa. All samples were determined in duplicates.

For a comprehensive comparison we also analyzed *BI*SPase expression of the non-induced cells over the cultivation time. The *tac1* promoter in the pCHEM21 is tightly regulated. However, in Figure 8 a slight *BI*SPase overexpression band is seen. In the beginning mainly soluble recombinant sucrose phosphorylase is expressed, but also traces of insoluble protein are increasing over cultivation time. The amount of recombinant *BI*SPase is about 10 % of the *E. coli* BL21 (DE3) soluble protein (non-induced cells) as estimated from the gel picture in Figure 8.

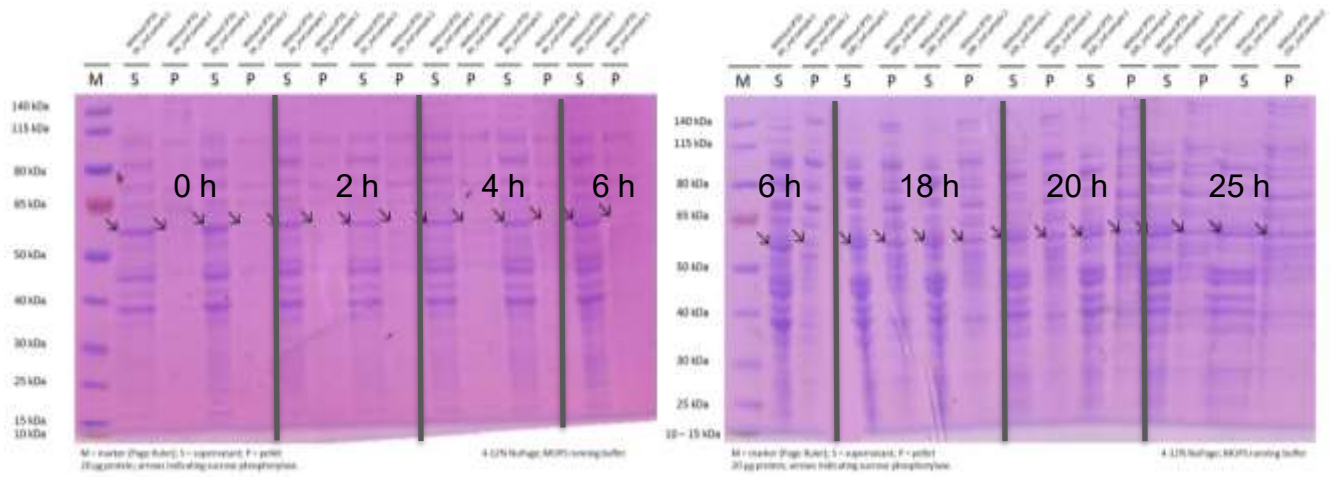

Figure 8. Leaky expression of *BIS*Pase from *E. coli* BL21 (DE3) harboring pCHEM21. Samples were taken at the induction point (0 h) and after 2 h, 4 h, 6 h, 18 h, 20 h and 25 h post induction. Those cells were not induced, but the same samples were taken compared to Figure 7. The abbreviations M, S and P are coding for marker, soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *BIS*Pase at 55kDa. All samples were determined in duplicates.

Induced cells yielded *BIS*Pase activity of 19 U/mg. In addition, we determined a *BIS*Pase activity of 1.2 U/mg for non-induced cells. The approximately 20-fold increase in protein activity upon induction is also seen in *BIS*Pase band on SDS-PAGE (Figures 7 and 8).

3.3 Upscaling to bioreactor cultivation

Bioreactor cultivation was optimized to scale-up and intensify biomass production focusing on a high volumetric yield in U/L. Therefore, several cultivation conditions were tested. The “standard batch cultivation” used 1 mM IPTG as inductor, 115 mg/L ampicillin, 25°C as induction temperature and no addition of D-sucrose. In addition, the effect of 250 mM D-sucrose in the cultivation medium, a later induction point and further antibiotic addition were tested. Cultivations were monitored by cell growth determination (cell dry weight and OD_{600}). Furthermore, protein expression, enzymatic activity of lysed cells (photometric) and whole-cell activities (conversion of 800 mM D-sucrose and 1200 mM L-AA to AA-2G) were analyzed.

3.3.1 Cell growth and specific growth rate μ_{max}

We determined cell growth and calculated μ_{max}. An end-OD_{600} of 32.5 was reached after 18 h of batch bioreactor cultivation, which equals a cell dry weight of approximately 8 g/L. μ_{max} of 0.28/h was calculated as overall specific cell growth rate between 4.25 h and 10.25 h cultivation time.

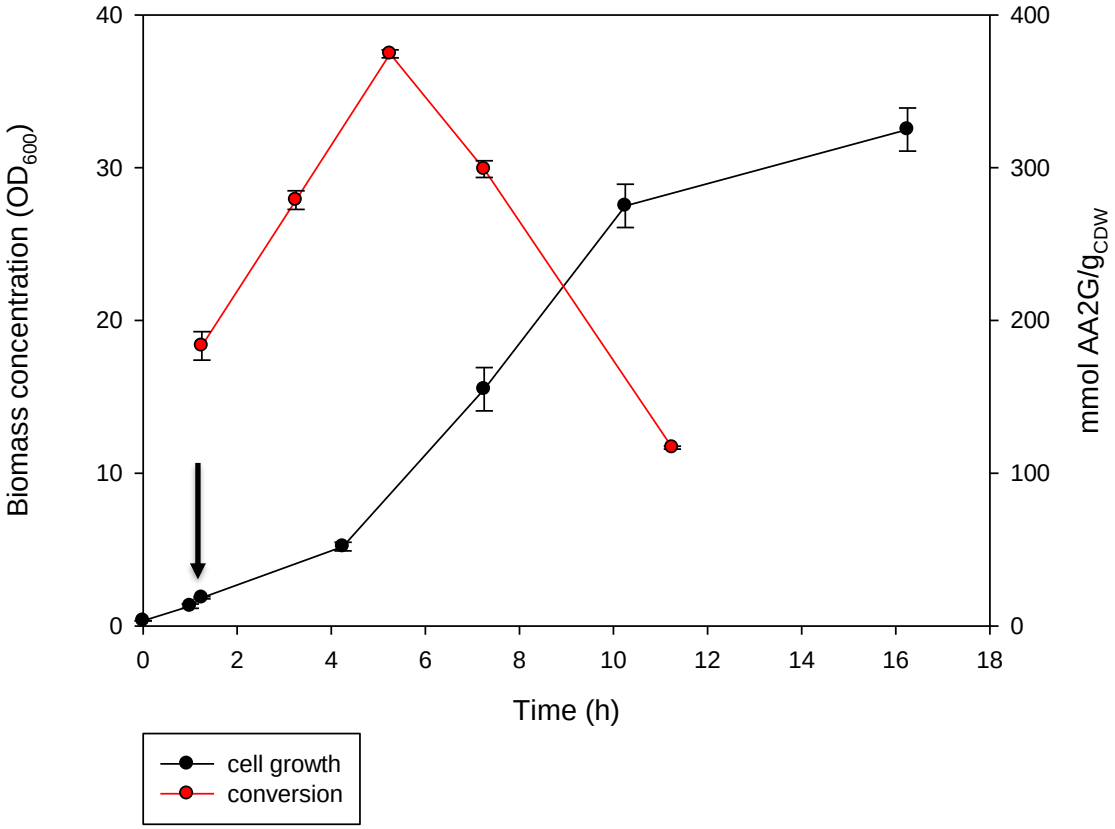

Figure 9. Biomass concentration (OD_{600}) and whole-cell catalyzed formation of mmol AA-2G per g_{cdw}. Standard conditions: At an OD_{600} of 1.8 (1.25 h, an arrow is indicating the induction point) the cells were induced with 1 mM IPTG. Black dots and black line show OD_{600}. For whole cell conversions samples were taken out of the bio-reactor and used to convert 800 mM D-sucrose and 1200 mM L-AA to AA-2G; red dots and red line show mmol AA-2G per g_{cdw} formation by the whole cells.

After 4 h of lag phase, the induced cells were in the exponential growth phase for 6 h, (time points 4 to 10 h). They reached the stationary phase at time point 10 h. At time point 16 h glucose was consumed, checked with glucose test stripes and verified by a tremendous decreasing stirrer speed in the cascade. Therefore, the cells were harvested. Decreasing exponential growth due to the induction is very common. For example, Larentis et al. (2014) expressed a leptospiral protein called LigB, causing a

zoonosis called leptospirosis, in *E. coli* BL21 (DE3) and observed different growth behavior compared to non-induced cells at the same temperature. While the non-induced cells grew exponentially, the induced cells decreased in growth.[40] We saw the same differences in the growth behavior between non-induced and induced cells in shake flask cultivations (Figure 6).

Additionally, to cell growth measurement by adsorption at 600 nm, we determined the gram cell dry weight to 8 g_{cdw}/L at the end of the cultivation, corresponding to 0.24 g_{cdw} L^{-1} OD_{600}^{-1}.

3.3.2 Formation of AA-2G by whole-cell catalysis

The production of AA-2G by whole cells expressing *BIS*Pase is a promising and cheap synthesis route. Cell samples taken during the bioreactor cultivation were used to convert 800 mM D-sucrose and 1200 mM L-AA to AA-2G (Figure 9). Highest AA-2G concentrations (in mM AA-2G/g_{cdw}) were obtained at 3.75 h post-induction (5 h cultivation time). Afterwards a dramatic drop of AA-2G formation was observed. The rapid increase of catalytic activity until 3.75 h post-induction followed by a rapid decrease is reflected in the SDS-PAGE shown in Figure 10. The main reason for decreasing cell activity is most probably plasmid loss over time in the bioreactor. Ampicillin is hydrolyzed leading to plasmid loss and hence lower protein expression.[41] Several strategies are known to circumvent plasmid loss e.g. the use of more stable β-lactam antibiotics, such as carbenicillin instead of ampicillin, or the use of other types of antibiotics like kanamycin.

Another reason might be increasing mass transfer limitation over the cultivation time. The mass transfer limitation is increasing over biomass concentration. This means, that the limitation is higher, when the cell concentration is higher, which leads to a

lower amount of AA-2G in mmol/g_{cdw}. For example, Doran (2013) explains that, the mass transfer coefficient k_L multiplied with the surface area a and known as $k_L a$ and used to calculate the mass transportation rate N_A is highly influenced by high viscosity and in our case due to 1.2 M D-sucrose the pH adjustment with 10 M NaOH the effect of mass transfer limitation is reinforced. The reason is the thicker film at the thicker solid/liquid fluid film, compared to liquids with less viscosity.[42]

However, low cell wall permeability is a major drawback in whole-cell catalysis, leading to activity loss up to 98 %. Kratzer et al. (2015) reported in their review about cell wall dependent mass transfer limitations encountered in NAD(P)H-dependent bioreductions.[43] Several approaches are reported to circumvent mass transfer limitations by the cell wall. One example is cell wall permeabilization by the antibiotic nonapeptide polymyxin B.[44] Until now, we did not investigate cell wall permeabilization for AA-2G synthesis by the use of additives or, more simply, by cell lyophilization.

3.3.3 Analysis of *BIS*Pase expression in *E. coli* BL21 (DE3)

The protein expression was analyzed by SDS-PAGE. The highest protein expression was obtained after 6 h of induction (7.25 h cultivation time) and correlates with the results obtained from conversion studies. Afterwards the expression is decreasing over the whole cultivation time, most probably due to plasmid loss. At the end of cultivation, when the glucose was consumed, the protein expression nearly reached the same level as at the induction point. Lower protein expression at the end of the cultivation correlates with lower cell activities and hence lower AA-2G formation (lower mmol AA-2G per g_{cdw}). *BIS*Pase expression prior to induction indicates a leaky promoter.

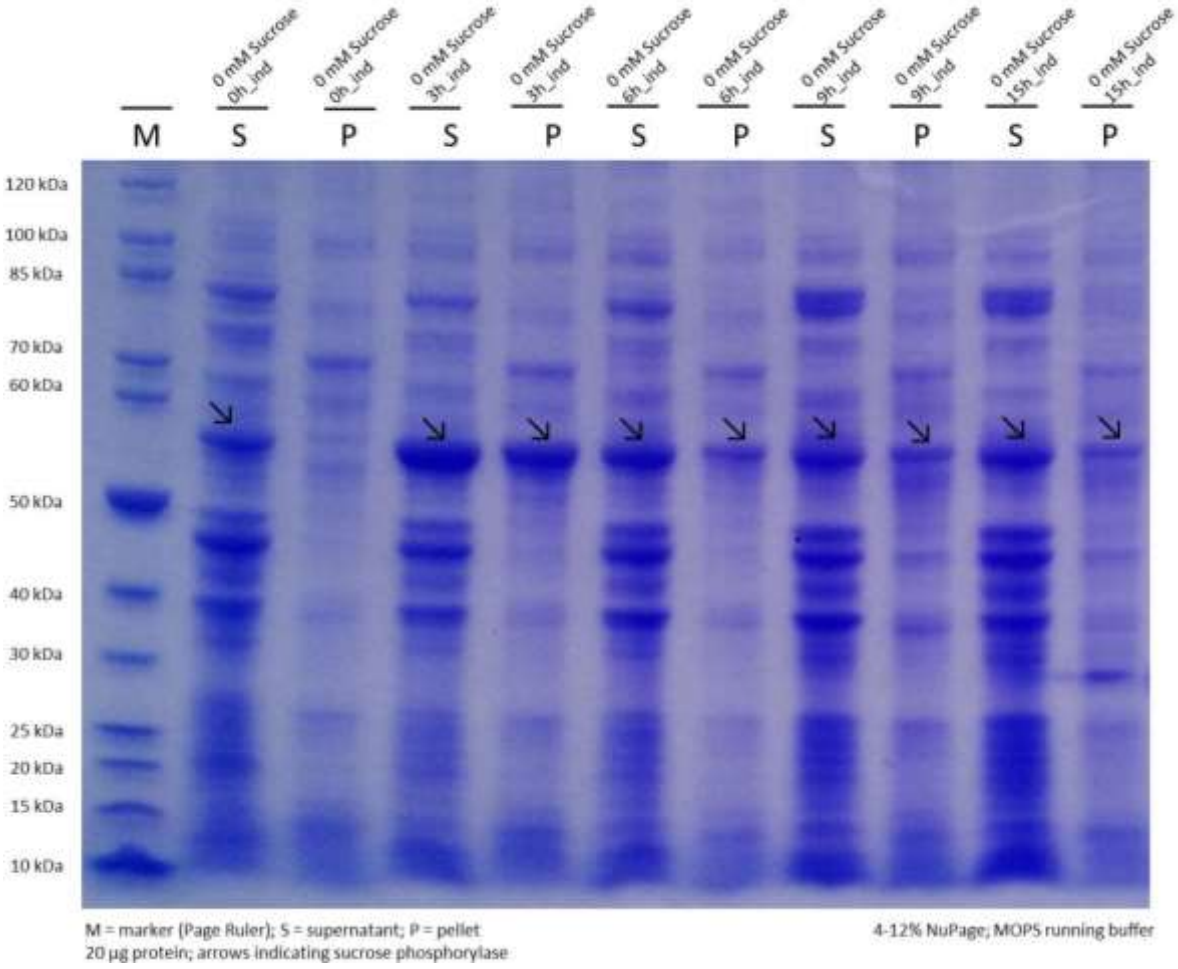

Figure 10. SDS gel of *BI*SPase expression in bioreactor cultivation. The protein expression was traced over the induction time. Samples were taken at the induction point (0 h; 1.25h cultivation time) and at 3 h, 6 h, 9 h, 15 h post-induction. The abbreviations M, S and P are coding for marker, soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *BI*SPase at 55 kDa. 20 µg protein was used per SDS slot.

The fraction of the insoluble protein decreased over the cultivation time (Figure 10). At 3 h post-induction (4.25 h cultivation time) the ratio is nearly equal. Afterwards and until cell harvest at 15 h post-induction the insoluble-protein fraction decreased. This was not seen in the shake flask cultivation. There the ratio of soluble to insoluble protein was constant over the cultivation time. Implementation of pH control in the bioreactor

cultivation is one of the main differences to the uncontrolled shaken flask cultivation. Acetate formation and transportation out of the cell by diffusion causes a pH drop in the medium that significantly influences protein expression.[45]

To stabilize and/or optimize the protein expression several approaches are known. One is the integration of the target gene into the genome of *E. coli* BL21 (DE3). A novel, highly effective multigene editing technique to integrate target genes is CRISPR/Cas9. Jiang et al. (2015) published a multigene editing approach in *E. coli*. They worked with plasmids called pTARGET and integrated several genes into the genome of *E. coli*. The use of subgenomic RNA (sgRNA) as vector system enables target gene integration into the CRISPR box of the genome of *E. coli*.[46] However, plasmid construction is not an easy task, which makes genomic integration labor-intensive.

3.3.4 Enzymatic activity of *Bl*SPase in the cell-free extract

After cell harvest and mechanical cell lysis with French press, the enzymatic activity was determined with a sucrose based NADH-dependent assay. Samples at 6 h post-induction (7.25 h cultivation time) and 15 h post-induction (end of bioreactor cultivation) were analyzed to follow activity loss in the cell free protein extract over cultivation time. We determined a volumetric activity of 1200 $U/mL_{cell\text{-}free\ extract}$ at 6 h post-induction and 615 $U/mL_{cell\text{-}free\ extract}$ at 15 h post-induction. After determination of protein concentration, a specific activity of 80 U/mg at 6 h post-induction and 61.5 U/mg at 15 h post induction was calculated. In addition, a volumetric yield of 112,100 U/L at 6 h post-induction and 122,350 U/L at 15 h post-induction was determined. The highest specific activity was determined at 6 h post-induction. Also protein expression (SDS-PAGE) and the level of AA-2G formation was the highest at this time point. After 6 h of induction expression and cell activity decreased (Figures 9 and 10). There is a clear dis-

crepancy between the whole-cell activities at 6 h and 15 h post induction and the corresponding specific activities. One explanation might be an increase in the mass transfer limitation of the cells over time. Mass transfer limitations, due to high biomass that is increasing fluid viscosity[42], does not hamper the formation of glucose 1-phosphate and D-fructose in the cell free extract, compared to whole cell catalysis. Another reason for the slow decrease of specific activity compared to whole-cell catalysis could be the different reactions that were catalyzed by the whole cells and by the cell-free extract. The specific activity of *Bl*SPase in the cell-free extract relates to attaching phosphate on the 1-OH group of D-glucose. We have no information about the formation of AA-2G in the cell-free extract compared to whole cells. Kwon et al. (2007) produced AA-2G by transglucosylation with a *Bl*SPase expressed in *E. coli* BL21 (DE3)[6,9]. In these publications no numbers for the velocity of the reaction are mentioned.[6] But Goedl et al. (2007) determined the specific activity for recombinant sucrose phosphorylase from *Leuconostoc mesenteroides* (*Lm*SPase) in dependence of induction temperature. For 25°C induction temperature, they reported 50 U/mg, for 30°C 20 U/mg and for 37°C 15 U/mg of *Lm*SPase in the crude cell extract of *E. coli* DH10B.[4] In comparison the expression of *Bl*SPase in *E. coli* BL21 (DE3), as reported here, is competitive.

3.4 Optimization of bioreactor cultivation

Plasmid loss, due to antibiotics degradation over cultivation time is one of the main reasons for cell-activity loss besides protein degradation.[20,47,48] Therefore, formation of inclusion bodies turned out as a further factor decreasing functional *Bl*SPase production. Several strategies were tested to restrain plasmid loss and increase cell activity. Based on conditions in the "standard batch cultivation", we used further ampicillin addition at the induction point, replacement of ampicillin by the more stable carbenicillin, addition of 250 mM D-sucrose and induction at a higher biomass concentration to optimize and stabilize protein expression of *Bl*SPase over cultivation time.

3.4.1 Additional ampicillin at the induction point

Ampicillin was used as antibiotic to prevent plasmid loss over cultivation time. We used the same conditions as in the standard batch cultivation but added a second dose of ampicillin at the induction point.

3.4.1.1 Cell growth and specific growth rate μ_{max}

Cell growth and biomass formation was monitored by OD_{600} measurements over cultivation time (Figure 11). Based on the growth curve we calculated a μ_{max} of 0.13/h for the maximum growth between the time points 1.75 h and 21.75 h. After cultivation when the glucose was depleted, we harvested 6.7 g_{cdw}/L, which equals 0.21 g_{cdw} L^{-1} OD_{600}^{-1}.

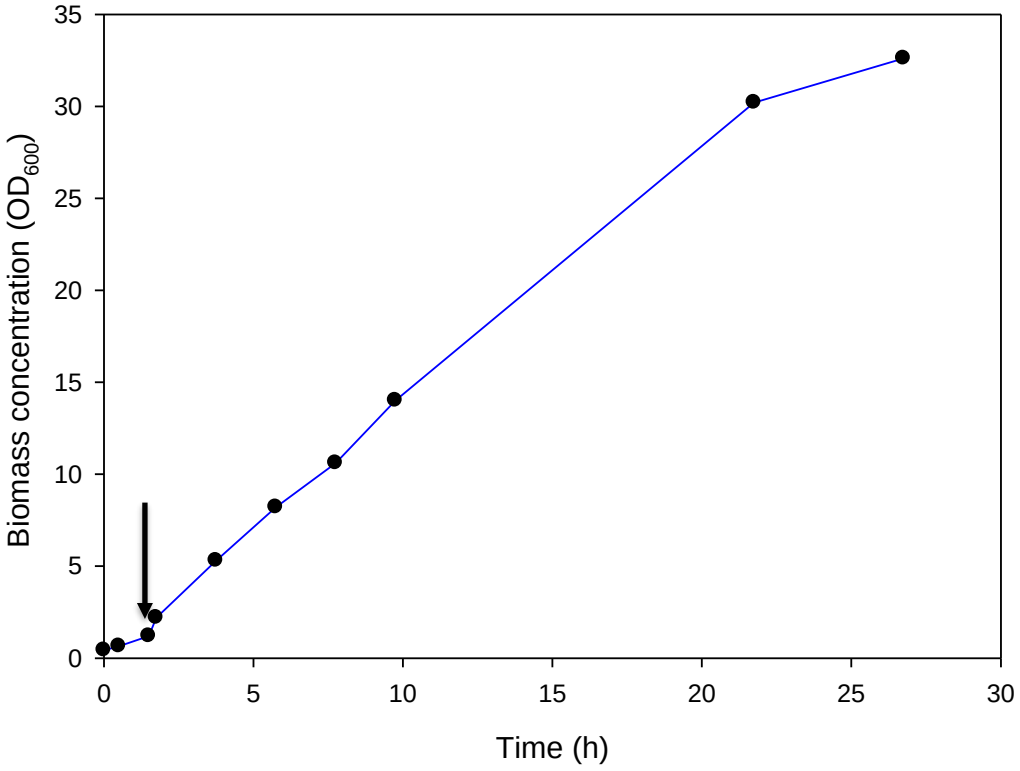

Figure 11. Growth curve of *E. coli* BL21 (DE3) expressing *BIS*Pase. Conditions: At an OD_{600} of approximately 2 (1.75 h, an arrow is indicating the induction point) the cells were induced with 1 mM IPTG; at the induction point further ampicillin was added. Black dots and the blue line show the increasing biomass concentration.

Compared to Figures 6 and 9 a significant lag phase is missing, but the specific growth rate μ is half of the standard batch cultivation. The higher antibiotic concentration leads to higher plasmids copy numbers, however, this correlation is not so trivial. Cunningham et al. (2009) reported that the huge load of β-lactamase expression triggers metabolic burden. The resources used for expression of antibiotics resistance gene hampers plasmid replication and expression of the protein of interest.[49]

3.4.1.2 Production of AA-2G by whole-cell catalysis

In order to exclude an effect of cell concentration we tried to dilute the cells to an OD_{600} of 2 (induction OD_{600}) after sampling. No validate trend can be observed, due to high

scattering values. Therefore, no statement can be made about the production of AA-2G. The raw data in double determination can be observed in the supplementary information part.

3.4.1.3 Expression of *BIS*Pase in *E. coli* BL21 (DE3)

The expected, stable expression of recombinant *BIS*Pase over cultivation time was not observed (Figure 12).

Protein expression was increasing up to 8 h post-induction and decreased afterwards. Additionally, the insoluble protein fraction (marked as pellet P in Figure 12) was much higher in relation to the standard batch cultivation (Figure 10).

Due to excessive destaining in the third gel showing samples from the end of the bioreactor cultivation (20 and 25 h post-induction), the expression is not displayed uniformly. But still it seems that enzyme expression is decreasing at the end of bioreactor cultivation. Furthermore, the ratio of insoluble to soluble protein is nearly constant.

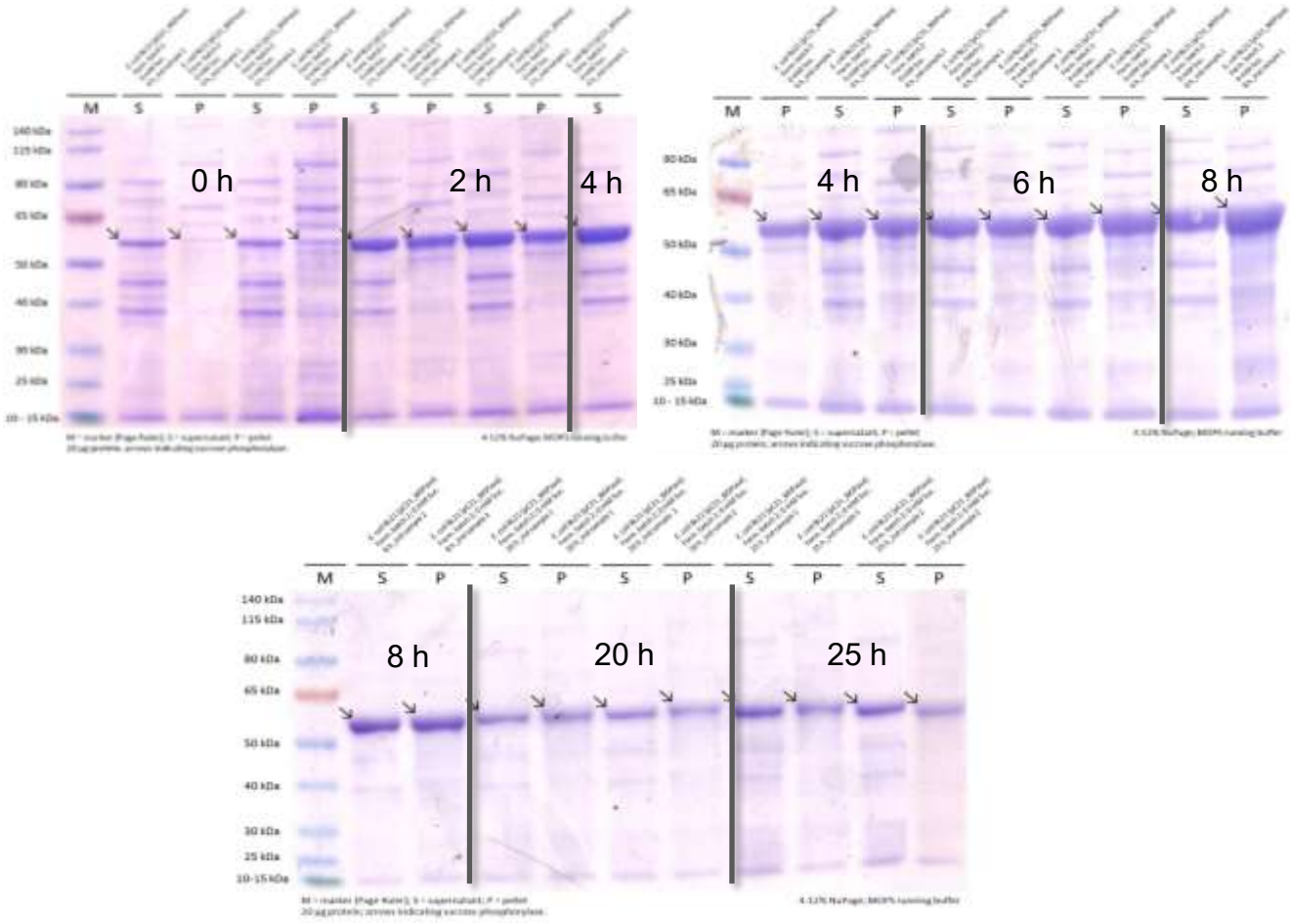

Figure 12. SDS gel of *BI*SPase expression in bioreactor cultivation with further ampicillin addition at the induction point. M, S and P are standing for the molecular marker, soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *BI*SPase at 55kDa. 20 µg protein was used per SDS slot.

3.4.1.4 Enzymatic activity of *BI*SPase in the cell-free extract

At the end of the cultivation (26.75 h) cells were mechanically disrupted using a French press. The activity of *BI*SPase was determined spectrophotometrically in the cell-free extract by a coupled enzyme assay. The protein concentration was determined by the method of Bradford. A volumetric activity of 280 U/mL and a protein concentration of 8.4 mg/mL were obtained. The specific activity was calculated to 33 U/mg. The specific activity is 46 % lower as compared to the standard batch cultivation. Hence, further addition of ampicillin at the induction point does not improve the production of *BI*SPase

activity. Therefore, we determined 46,374 U/L as volumetric yield after 26.75 h of cultivation time.

3.4.2 Replacement of ampicillin by carbenicillin

Carbenicillin has the same antibiotic effect but a 3-fold higher stability as ampicillin.[47] It is widely used to circumvent plasmid loss and accompanied decreased protein expression. Therefore, we tried to restrain plasmid loss by using carbenicillin instead of ampicillin.

3.4.2.1 Cell growth and specific growth rate μ_{max}

Cell growth was determined spectrophotometrically at 600 nm (OD_{600}). A specific growth rate μ_{max} of 0.17 h^{-1} was determined between 10.25 h and 16.25 h cultivation time. In addition, the cell dry weight was determined at the end of the bioreactor cultivation. We reached 12 g/L, which equals 0.20 $g_{cdw}\ L^{-1}\ OD_{600}^{-1}$.

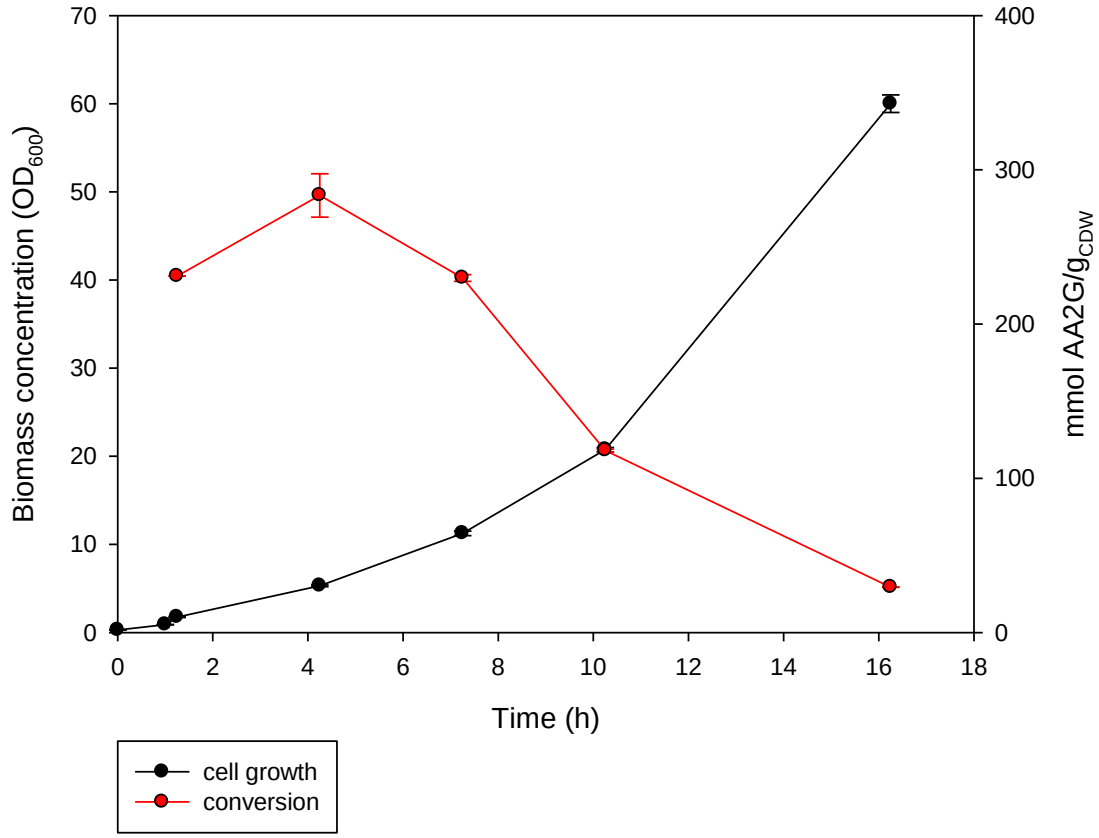

Figure 13. Cell growth of *E. coli* BL21 (DE3) expressing recombinant *BIS*Pase and whole-cell formation of AA-2G. The cells were induced with 1 mM IPTG after 1.25 h of cultivation time indicated by an arrow. Samples for conversion were taken at the induction point, 3 h, 6 h, 9 h and 15 h post-induction. The cell growth is indicated in the black line and the samples for conversion are indicated in red.

In comparison to all other bioreactor cultivations the biomass increased dramatically over cultivation time up to an OD_{600} of 60, which could be either an indicator for failed induction or microbial contamination. In correspondence, a dramatic drop of AA-2G conversion was observed.

3.4.2.2 Production of AA-2G by whole-cell catalysis

We obtained the highest concentration of 283 mM AA-2G per g_{cdw} 3 h post-induction. 15 h post-induction an AA-2G concentration of 28 mM AA-2G g_{cdw} was obtained, which indicates a dramatic cell-activity loss.

3.4.2.3 Expression analysis of recombinant *BIS*Pase in *E. coli* BL21 (DE3)

The SDS-PAGE shows a deviating protein-band pattern compared to previous bioreactor cultivations and shaken flask cultivations (Figure 14). This finding suggests a microbial contamination during cultivation. The size of the most dominant protein band is at 55 kDa and therefore the *BIS*Pase. However, the *BIS*Pase expression was much weaker compared to all previous cultivations. In addition, the β-lactamase with a molecular weight of approximately 40 kDa is also strongly expressed. Two main reasons could be responsible for the contamination: non-sterile working during bioreactor inoculation and non-sterile sampling. The medium itself seemed sterile as no microbial growth was observed prior to inoculation. Furthermore, an infection during sampling seems unlikely as the protein-band pattern deviated from previous cultivations already in the first sample. Therefore, it seems that the contamination occurred at inoculation of the medium.

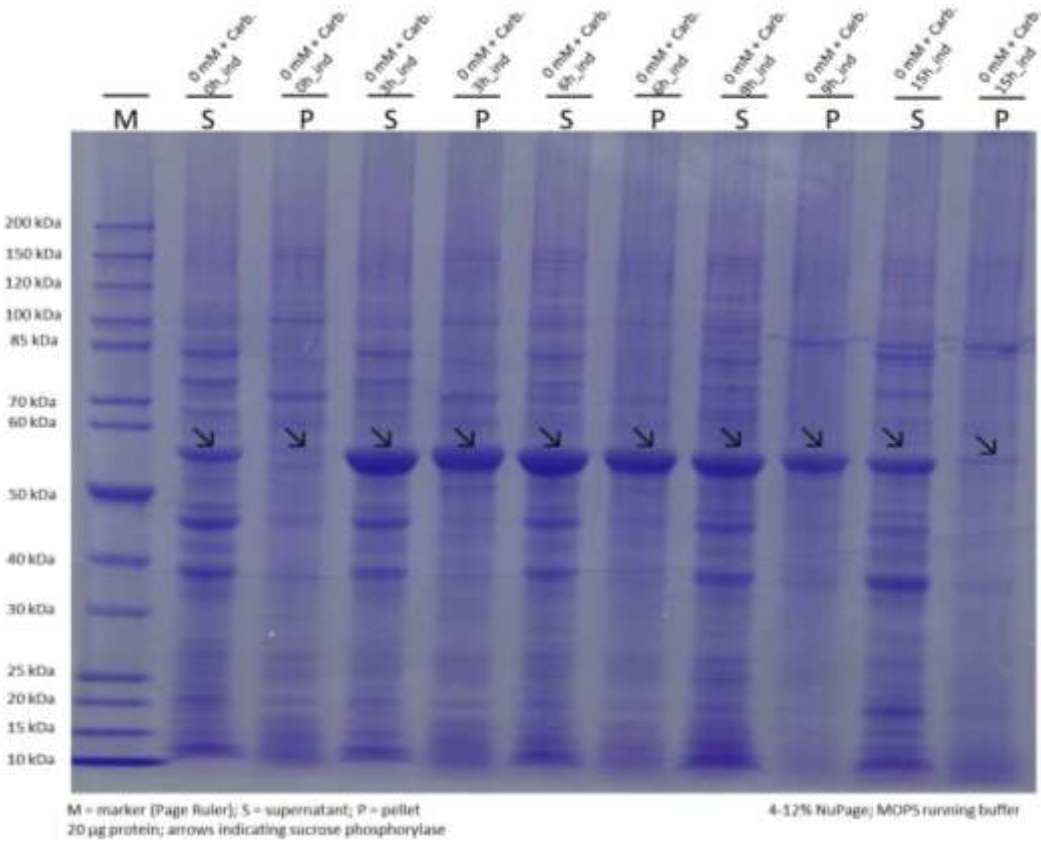

Figure 14. SDS gel of *Bl*SPase expression in bioreactor cultivation with carbenicillin instead of ampicillin. The abbreviations M, S and P are coding for marker (PageRuler/Prestained Protein Ladder from Thermo Scientific), soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *Bl*SPase at 55 kDa. 20 µg protein was used per SDS slot.

3.4.2.4 Specific activity of *Bl*SPase in the cell-free extract

Enzyme activity in the cell-free extract was assayed spectrophotometrically by a coupled activity assay. We analyzed the cells of the samples taken at 6 and 15 h post-induction. At 6 h post-induction we obtained 26 U/mL volumetric activity and 18 U/mg specific activity. After 15 h post induction we obtained 31 U/mL as volumetric activity and 12 U/mg as specific activity. Due to obvious microbial contamination the volumetric activity after 6 h is approximately 1,712 U/L and after 15 h post-induction 14,348 U/L fermentation broth.

3.4.4 Addition of 250 mM D-sucrose to the cultivation medium

Promising productivities of AA-2G formation obtained with whole cells from deep-well plate cultivation prompted addition of D-sucrose to the bioreactor medium. Cells grown and induced in the presence of 500 mM D-sucrose showed in combination with the pCHEM21 vector system the best productivity results in terms of mmol AA2G per g_{cdw}. in deep-well cultivation (Table 1, Figure 5).

3.4.4.1 Cell growth and specific growth rate μ_{max}

The cell growth curve is depicted in Figure 15. We obtained ~0.08 h^{-1} for μ_{max} as parameter for specific cell growth. After 16.25 h of cultivation cells were harvested at a biomass concentration of 5.0 g_{cdw}/L. A ratio of 0.20 g_{cdw} L^{-1} OD_{600}^{-1} was determined. In general, the growth phase was extended due to addition of D-sucrose. Growth was most probably impaired by osmotic stress.[35]

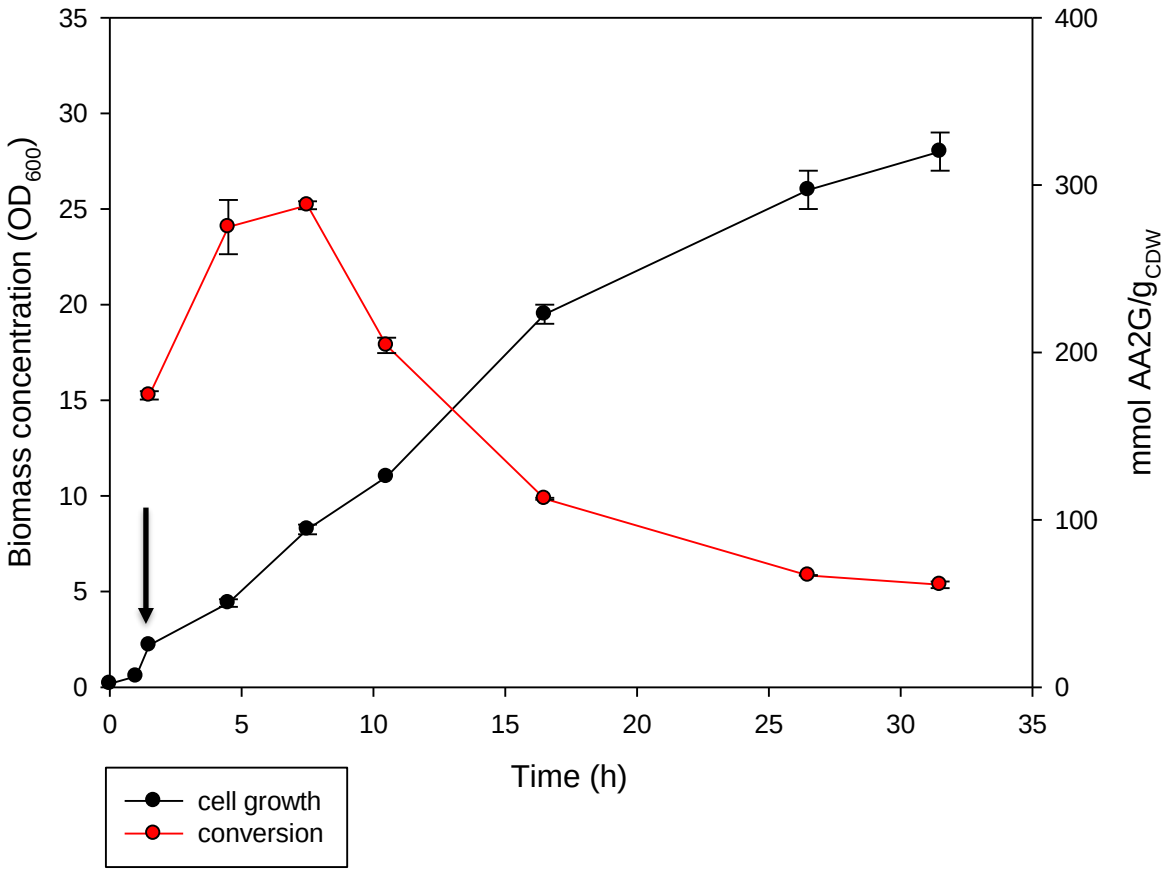

Figure 15. Biomass concentration (OD_{600}) and whole-cell catalyzed formation of mM AA-2G per g_{cdw}. Conditions: At an OD_{600} of 1.8 (1.5 h, an arrow is indicating the induction point) the cells were induced with 1 mM IPTG. Additionally, 250 mM of D-sucrose was added to the medium. Black dots and black line show OD_{600}. For whole cell conversions samples were taken out of the bioreactor and used to convert 800 mM D-sucrose and 1200 mM L-AA to AA-2G; red dots and red line show AA-2G formation in mmol/g_{cdw}.

3.4.4.2 Production of AA-2G by whole-cell catalysis

At 6 h post-induction the cells were able to produce 300 mmol AA-2G per g_{cdw}. After 3 h post-induction the specific cell productivity decreased gradually. Cells were harvested after 31.5 h of cultivation (30 h post-induction). Therefore, the production rate of AA-2G is 6-fold less at the end of the cell cultivation. This indicates a strong decrease

in protein expression in correlation to cell growth. From 9 h post-induction the amount of AA-2G produced by samples was dramatically decreased to 50 mmol AA-2G per g_{cdw}. Compared to the standard batch cultivation the addition of 250 mM D-sucrose showed no prevention of plasmid loss and accompanied stabilization of protein expression.

3.4.4.3 Expression analysis of recombinant *BIS*Pase in *E. coli* BL21 (DE3)

The SDS-PAGE showed an increase in soluble *BIS*Pase expression until 9 h post-induction (10.5 h cultivation time). Afterwards *BIS*Pase expression decreased dramatically. In addition, the amount of insoluble protein is also increasing up to 9 h post-induction. Afterward 9 h post-induction the amount of soluble protein is decreased on account of the insoluble protein. Hence, ratios of soluble compared to insoluble *BIS*Pase bands suggest formation of inclusion bodies. Furthermore, thinner *BIS*Pase bands indicate plasmid loss over time, especially after 15 h post-induction.

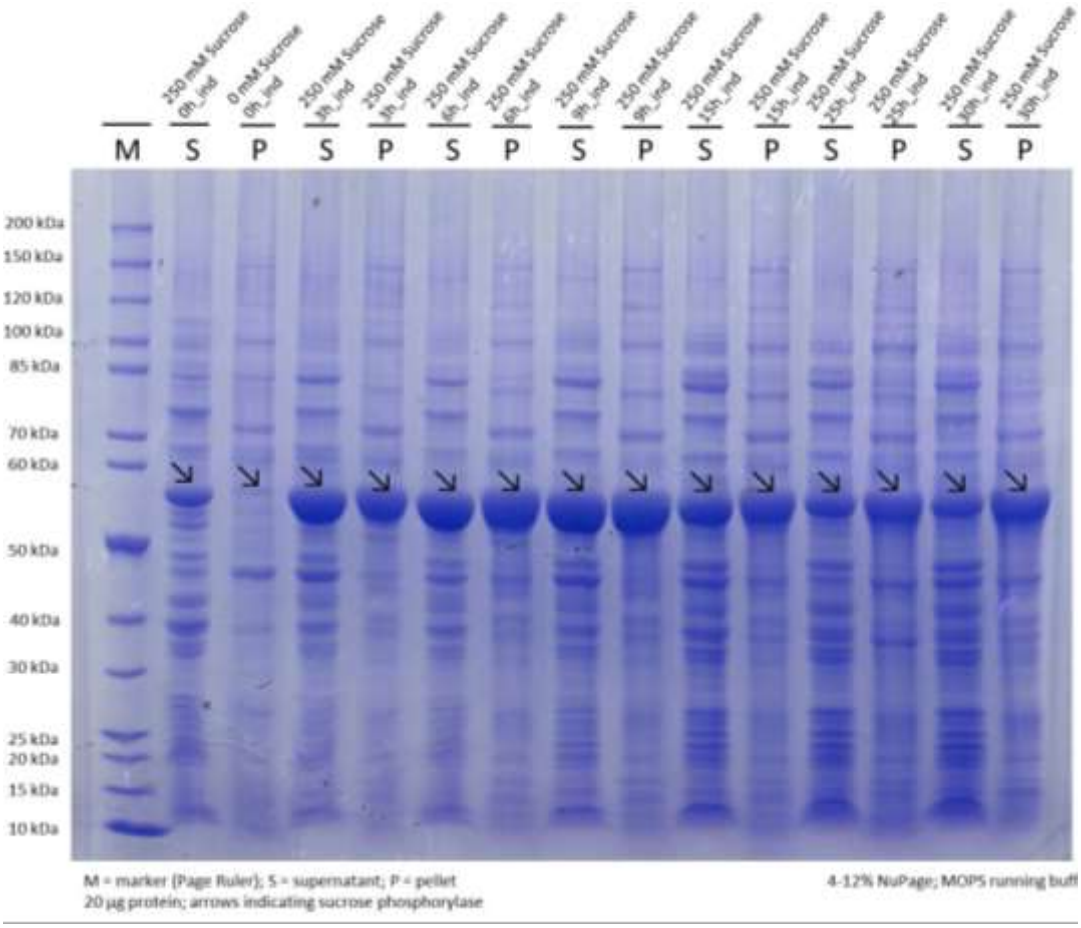

Figure 16. SDS gel of *Bl*SPase expression in bioreactor cultivation with addition of 250 mM D-sucrose. The abbreviations M, S and P are coding for marker (PageRuler/Prestained Protein Ladder from Thermo Scientific), soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *Bl*SPase at 55 kDa. 20 µg protein was used per SDS slot.

3.4.4.4 Specific activity of *Bl*SPase in the cell-free extract

Enzyme activity in the cell-free extract was assayed spectrophotometrically by a coupled activity assay. After 6 h post-induction (7.5 h cultivation time) we determined a volumetric activity of 343 U/mL and a protein concentration of 12.25 mg/mL. The corresponding specific activity was calculated to 28 U/mg in the cell-free extract. Additionally, we determined a volumetric yield of approximately 40,000 U/L cultivation broth. At 15 h post-induction a volumetric activity of 140 U/mL and a protein concentration of 7.8

mg/mL was determined. The corresponding specific activity was 18 U/mg. A volumetric yield of 14,000 U/L was reached. After 30 h post induction (cell harvest) we determined a volumetric activity of approximately 57 U/mL and a protein concentration of 9.3 mg/mL. Therefore, the specific activity was 6.1 U/mg. We yielded 7,700 U/L cultivation broth after cell harvest. The decrease of volumetric yield from 15 to 30 h post-induction suggests protein degradation after freezing the centrifuged cells after cell harvest at -20°C and thawing for homogenization with french press for activity measurements of the cell free crude extract. Generally, the volumetric yield should increase, due to increasing biomass concentration over cultivation time.

3.4.5 Shift of induction to a higher biomass concentration with additional ampicillin

We used an OD_{600} of 12 to induce the cells to overcome the decrease of functional protein expression observed within a few hours after induction.

3.4.5.1 Cell growth and specific growth rate μ_{max}

We determined a cell dry weight of 7 g_{cdw}/L at the end of the cultivation. The ratio of cell dry weight to OD_{600} was 0.3 $g_{cdw}\ L^{-1}\ OD_{600}^{-1}$. A specific growth μ_{max} of 1.1 h^{-1} before induction was measured. After induction (3 h cultivation time) the cell growth did not increase as compared to early induction (Figure 17). Therefore, after induction the cells expressed protein on account of cell growth.

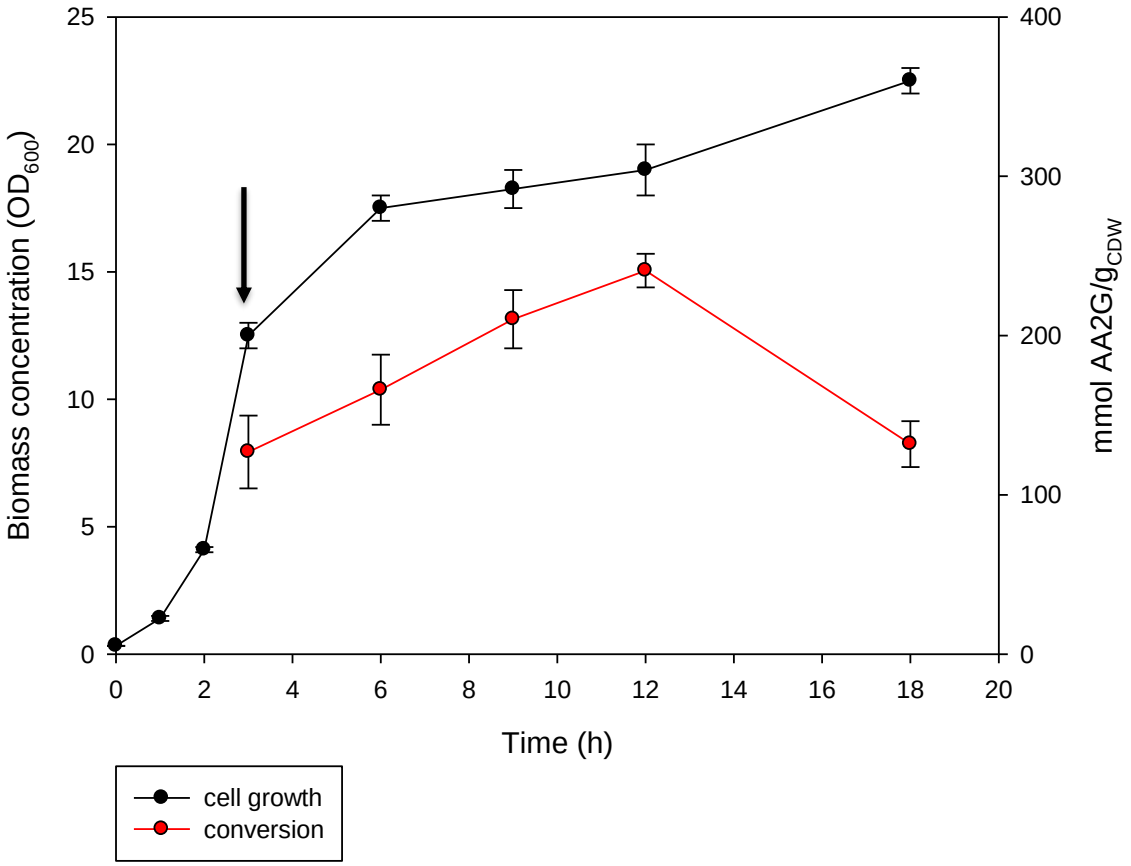

Figure 17. Cell growth of *E. coli* BL21 (DE3) *BIS*Pase and whole-cell production of AA-2G. Biomass concentration (OD_{600}) and whole-cell catalyzed formation of mmol AA-2G per g_{cdw} is shown. Conditions: At an OD_{600} of 12.5 (3 h, an arrow is indicating the induction point) the cells were induced with 1 mM IPTG. Additionally, 115 mg/L ampicillin was added to the medium. Black dots and black line show OD_{600}. For whole cell conversions samples were taken out of the bioreactor and used to convert 800 mM D-sucrose and 1200 mM L-AA to AA-2G; red dots and red line show AA-2G formation in mmol/g_{cdw}.

The biomass concentration at the end of the cultivation was lower compared to the standard batch bioreactor cultivation. The low biomass yield is the main drawback of this strategy, but could be circumvented by the application of a fed-batch strategy. A

fed-batch cultivation would combine the positive effects of high biomass formation and late protein expression.

3.4.5.2 Production of AA-2G by whole-cell catalysis

The production of AA-2G (in mmol AA-2G per g_{cdw}) did not reach the same level as compared to the standard batch fermentation. However, it was possible to prevent fast plasmid loss shortly after the induction. The amount of AA-2G is increasing over the whole induction time, except for the last sample at 15 h post-induction. The drop in AA-2G formation was much slower, instead of 4-fold decrease seen in the standard batch cultivation, a 2-fold decrease was observed.

Mass transfer limitation, due to high biomass and high viscosity of the substrate solution, could have a tremendous influence in AA-2G formation, too.[42]

These results suggest a fed-batch strategy to combine the benefits of higher biomass concentration and late induction point.

3.4.5.3 Expression analysis of recombinant *BIS*Pase in *E. coli* BL21 (DE3)

Almost all protein was expressed in soluble form, as displayed in Figure 17. The ratio between soluble and insoluble protein was much better compared to all other cultivations and confirms the chosen induction strategy. The trend seen in whole-cell activities is also seen in the *BIS*Pase expression band on SDS-PAGE. After the chosen induction point of OD_{600} 12.5 the *BIS*Pase expression was weaker compared to other bioreactor cultivation approaches but steadily increasing. Therefore, plasmid loss was restrained by shifting the induction to higher biomass and the accompanied ampicillin addition. The low biomass concentration could be overcome by a fed-batch approach.

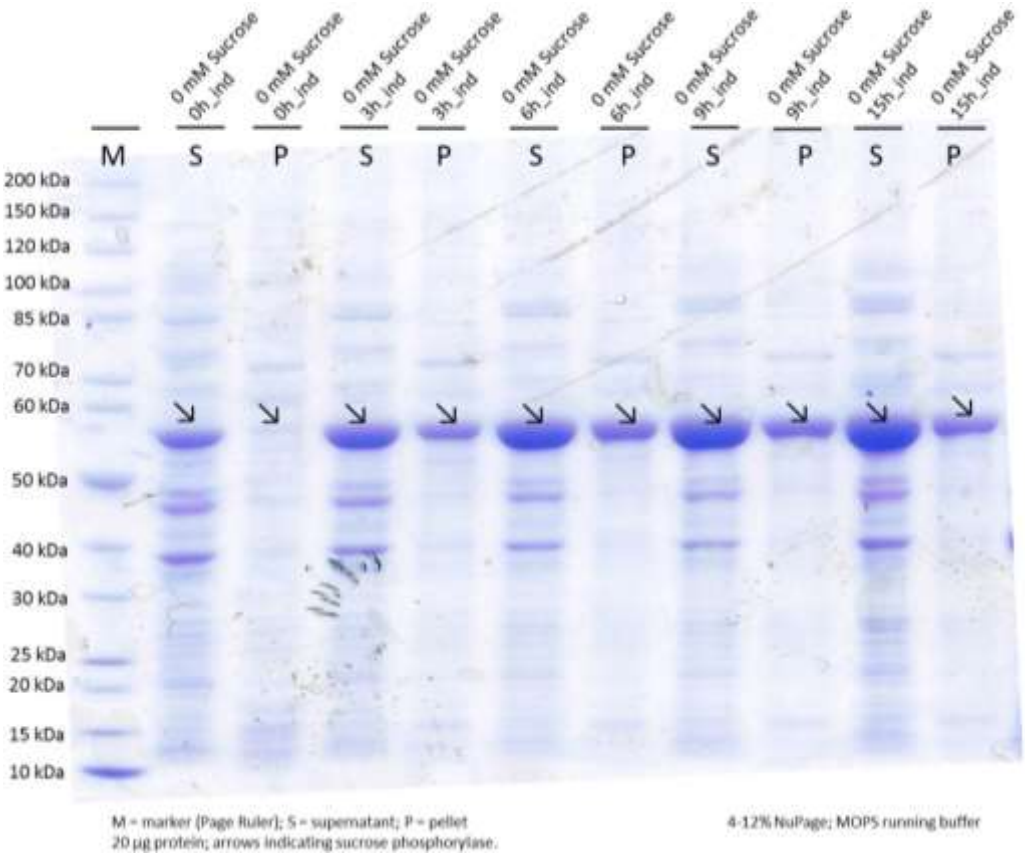

Figure 18. SDS gel of *Bl*SPase expression in bioreactor cultivation with induction at high OD_{600}. The abbreviations M, S and P are coding for marker (PageRuler/Prestained Protein Ladder from Thermo Scientific), soluble protein and pellet (insoluble protein). The arrows are indicating recombinant *Bl*SPase at 55 kDa. 20 µg protein was used per SDS slot.

3.4.5.4 Specific activity of *Bl*SPase in the cell-free extract

We determined volumetric activity, specific activity and volumetric yield at 3 h, 6 h, 9 h and 15 h post-induction. After 3 h post-induction we measured 620 U/mL as volumetric activity and a protein concentration of 8.9 mg/mL. We calculated a specific activity of 70 U/mg and a volumetric yield of 54,150 U/L a. After 6 h post-induction 900 U/mL for the volumetric activity and 15 mg/mL as protein concentration was determined. Therefore, we obtained 60 U/mg as specific activity and 55,360 U/L as volumetric yield. After 9 h post-induction we got 700 U/mL as volumetric activity and 11 mg/mL as protein

concentration. We calculated 87 U/mg as specific activity and 71,040 U/L as volumetric yield. After cell harvest (15 h post-induction) the obtained volumetric activity was 890 U/mL. Additionally, we determined 8.5 mg/mL as protein concentration in the cell-free extract. By calculation, we obtained 104 U/mg as specific activity and 124,180 U/L as volumetric yield.

Late induction, at an OD_{600} of 12.5, led to increased specific activity over the whole cultivation time in the cell free extract, due to less plasmid loss and low amounts of insoluble protein. We could yield nearly the same volumetric activity compared to standard batch cultivation, due to less biomass over cultivation time.

5. Conclusion and Outlook

In this research study, we were able to optimize and scale-up the production of recombinant *BIS*Pase in *E. coli* BL21 (DE3). The first step was plasmid selection at the deep-well plate scale. Three plasmids, namely, pQE30, a vector system from QIAGEN, pCHEM21 and pQE_ara (L-arabinose induced), both developed by the Austrian Centre of Industrial Biotechnology (acib GmbH), were compared in correspondence to the application of different D-sucrose concentrations in the cultivation medium. Therefore, pCHEM21 was used as suitable vector system for all further experiments, due to promising results.

After shake flask cultivation to detect the promoter leakiness of pCHEM21, a standard bioreactor cultivation with a working volume of 1.5 L was applied for the scale up of enzyme expression. Therefore, obvious limitations in enzyme activity, protein expression and conversion by whole cell catalysis, due to plasmid loss and inclusion body

formation needed to be circumvented either by ampicillin boosting at induction or replacing of carbenicillin. Also the addition of 250 mM D-sucrose in the fermentation and shift of induction to higher biomass concentration was applied.

A promising strategy to restrain plasmid loss and increase the production of AA-2G at later sample time points of bioreactor cultivation was the late induction of protein expression. The protein expression was lower at the beginning, compared to standard batch cultivation, but increasing over time instead of decreasing, except at cultivation end, probably due to mass transfer limitation, resulting from higher biomass concentration in combination with high viscosity of the substrate. Therefore, fed-batch fermentation to combine the effect of higher biomass and higher specific activity should be considered to optimize the production of recombinant *Bl*SPase. This approach could be one adjustable screw for the production of AA-2G by whole cell catalysis, too.

Another opportunity, especially to increase AA-2G formation by whole cell catalysis is the controlled permeabilization of the cell wall, as already mentioned beside decreasing fluid viscosity. Decreasing fluid viscosity can be reached for example by application of lower substrate concentrations, especially of D-sucrose. One strategy for long term industrial production of whole cell catalyzed AA-2G could be continuous processing with lower substrate concentrations for conversion.

Table 2. Key numbers of all bioreactor cultivations. The following table shows all key numbers of all bioreactor cultivation approaches, including all cultivation conditions, the induction OD_{600}, the biomass concentration (OD_{600}) at cultivation end, the highest formation of AA-2G after certain conversion hours (24 h or 4 h) and highest specific activity. Additionally, the volumetric yields after 6 h and at cultivation end are displayed.

Cultivation conditions	Induction OD_{600}	End OD_{600}	Highest mmol AA-2G/g_{cdw}	Highest Specific activity (U/mg)	Volumetric yield after 6 h (U/L)	Volumetric yield at cultivation end (U/L)
0 mM Sucrose	1.8	32.5	375 (24 h)	80	112,100	122,350
0 mM Sucrose + Amp	2.2	32.6	----------	33	----------	46,374
0 mM Sucrose + Carb	1.8	60	292 (4 h)	18	1,712	14,348
250 mM Sucrose	2.2	28	296 (4 h)	18	40,000	7,700
0 mM Sucrose (induction shift + Amp)	12.5	22	230 (4 h)	104	71,040	124,180

As in table 2 displayed, the best volumetric yield was obtained with an induction shift to an OD_{600} of 12.5 and addition of ampicillin (115 mg/L) at induction point. Therefore, optimization by the use of fed-batch fermentation is an interesting approach to overcome plasmid loss and inclusion bodies formation.

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Supplementary information

A Deep-well plate cultivation

Table A.1. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pQE30_*Bl*SPase (induction temperature 25°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position B (AA-2G area)	Position C (AA-2G area)	mmol AA-2G/g_{cdw} Position B	mmol AA-2G/g_{cdw} Position C
2	104797184	103004813	53	57
3	105665848	107203397	57	55
4	0	0	0	0
5	109354397	109801036	55	56
6	106802042	109124536	58	61
7	0	0	0	0
8	112019180	107937103	61	62
9	110526851	112476492	59	65
10	0	0	0	0

Table A.2. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase (induction temperature 25°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position D (AA-2G area)	Position E (AA-2G area)	mmol AA-2G/g_{cdw} Position D	mmol AA-2G/g_{cdw} Position E
2	155133500	156462967	110	119
3	136721664	139727010	177	180
4	0	0	0	0
5	159872388	155641563	114	113
6	99134501	98472047	288	286
7	0	0	0	0
8	154424496	155746966	111	108
9	93007920	87394377	334	310
10	0	0	0	0

Table A.3. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pQE_ara_*Bl*SPase (induction temperature 25°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position F (AA-2G area)	Position G (AA-2G area)	mmol AA-2G/g_{cdw} Position F	mmol AA-2G/g_{cdw} Position G
2	105710237	108642367	56	57
3	65517621	67939171	24	24
4	0	0	0	0
5	124277456	124812548	58	57
6	102507684	106610808	42	43
7	0	0	0	0
8	148178547	155416542	59	63
9	116340969	125604212	145	152
10	0	0	0	0

Table A.4. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pQE30_*Bl*SPase (induction temperature 31°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position B (AA-2G area)	Position C (AA-2G area)	mmol AA-2G/g_{cdw} Position B	mmol AA-2G/g_{cdw} Position C
2	125948131	124488458	73	74
3	110449709	108770558	69	70
4	0	0	0	0
5	121246894	126828915	75	78
6	113679224	107726272	70	73
7	0	0	0	0
8	123935361	124189984	76	72
9	112052211	107862559	69	72
10	0	0	0	0

Table A.5. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase (induction temperature 31°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position D (AA-2G area)	Position E (AA-2G area)	mmol AA-2G/g_{cdw} Position D	mmol AA-2G/g_{cdw} Position E
2	144805302	146278778	103	101
3	117436877	119953395	249	253
4	0	0	0	0
5	146130921	147878655	108	108
6	107683349	105687275	132	123
7	0	0	0	0
8	141946958	142275839	106	105
9	109953587	105870713	137	142
10	0	0	0	0

Table A.6. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pQE_ara_*Bl*SPase (induction temperature 31°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position F (AA-2G area)	Position G (AA-2G area)	mmol AA-2G/g_{cdw} Position F	mmol AA-2G/g_{cdw} Position G
2	128010641	124514152	183	178
3	81568734	85497165	117	122
4	0	0	0	0
5	141961262	140833650	203	201
6	112259698	115392746	160	165
7	0	0	0	0
8	145010394	149397788	207	213
9	135389968	136678665	193	195
10	0	0	0	0

Table A.7. AA-2G formation in mmol per g_{cdw} with *E. coli* BL21 (DE3)_pQE30_*Bl*SPase (induction temperature 37°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position B (AA-2G area)	Position C (AA-2G area)	mmol AA-2G/g_{cdw} Position B	mmol AA-2G/g_{cdw} Position C
2	133891728	134335166	191	192
3	126249744	125557385	180	179
4	0	0	0	0
5	135412981	138306322	193	198
6	131172820	126724064	187	181
7	0	0	0	0
8	139497110	140945540	199	201
9	131407854	131811096	188	188
10	0	0	0	0

Table A.8. AA-2G formation in mmol per g_{cdw} at 37°C with *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase (induction temperature 37°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position D (AA-2G area)	Position E (AA-2G area)	mmol AA-2G/g_{cdw} Position D	mmol AA-2G/g_{cdw} Position E
2	146390121	149313391	209	213
3	132644538	131124400	189	187
4	0	0	0	0
5	93985721	74898386	134	107
6	127802464	126126265	183	180
7	0	0	0	0
8	107720493	77934915	154	111
9	127226014	126178674	182	180
10	0	0	0	0

Table A.9. AA-2G formation in mmol per g_{cdw} at 37°C with *E. coli* BL21 (DE3)_pQE_ara_*Bl*SPase (induction temperature 37°C)

Position in deep well plate (AA-2G area)			mmol AA-2G/g_{cdw}	
Number (deep-well plate)	Position F (AA-2G area)	Position G (AA-2G area)	mmol AA-2G/g_{cdw} Position F	mmol AA-2G/g_{cdw} Position G
2	145388455	146843127	208	210
3	98706888	102557490	141	147
4	0	0	0	0
5	150624999	153697341	215	220
6	140183308	148115429	200	212
7	0	0	0	0
8	161496685	170316351	231	243
9	151790274	151672716	217	217
10	0	0	0	0

B Shake flask cultivation

Table B.1. Values for cell growth of induced and non-induced *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (induced)	OD_{600} (non-induced)
0	0.01	0.02
1	0.12	0.09
2	0.32	0.34
3	1.04	0.97
5	1.2	1.8
7	3	6.4
9	4.1	11.6
21	8.4	13
23	12.2	13.5
28	11.8	12.5

C Upscaling to bioreactor cultivation

Table C.1. OD_{600} and cell dry weight values for cell growth of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (1)	OD_{600} (2)	Cell dry weight (g/L) (1)	Cell dry weight (g/L) (2)	g L^{-1} OD_{600}^{-1} (1)	g L^{-1} OD_{600}^{-1} (2)
0	0.35	0.34				
1	1.4	1.2				
1.25	1.8	1.9	1.48	1.54	0.80	0.80
4.25	5	5.4				
7.25	14.5	16.5	4.64	4.1	0.30	0.26
10.25	28.5	26.5				
16.25	31.5	33.5	8.12	7.94	0.25	0.24

Table C.2. AA-2G formation in mmol/g_{cdw} of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Sample time point (h of cultivation)	AA2G Peak	mM AA2G	mmol AA2G/g_{cdw}	mean mmol AA2G/g_{cdw}
1.25 (1)	998978	14	174	183
1.25 (2)	1106180	16	193	
3.25 (1)	4276189	61	273	279
3.25 (2)	4467502	64	285	
5.25 (1)	7759858	111	372	375
5.25 (2)	7867176	112	377	
7.25 (1)	8825338	126	305	299
7.25 (2)	8506943	122	294	
11.25 (1)	8554590	122	118	117
11.25 (2)	8416345	120	116	

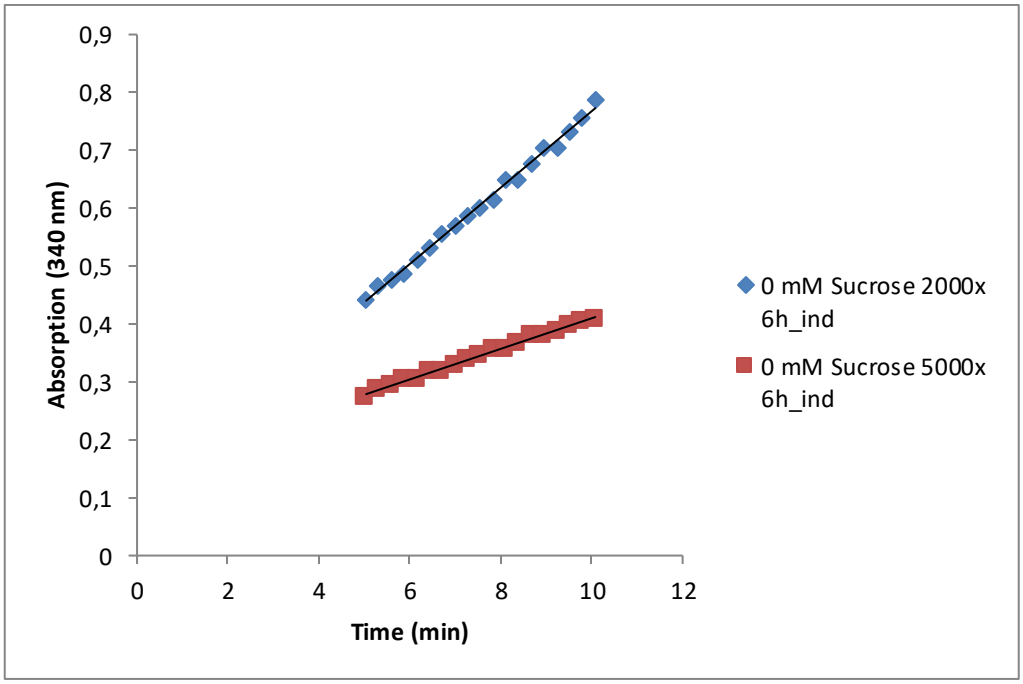

Figure C.1. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 6 h post induction

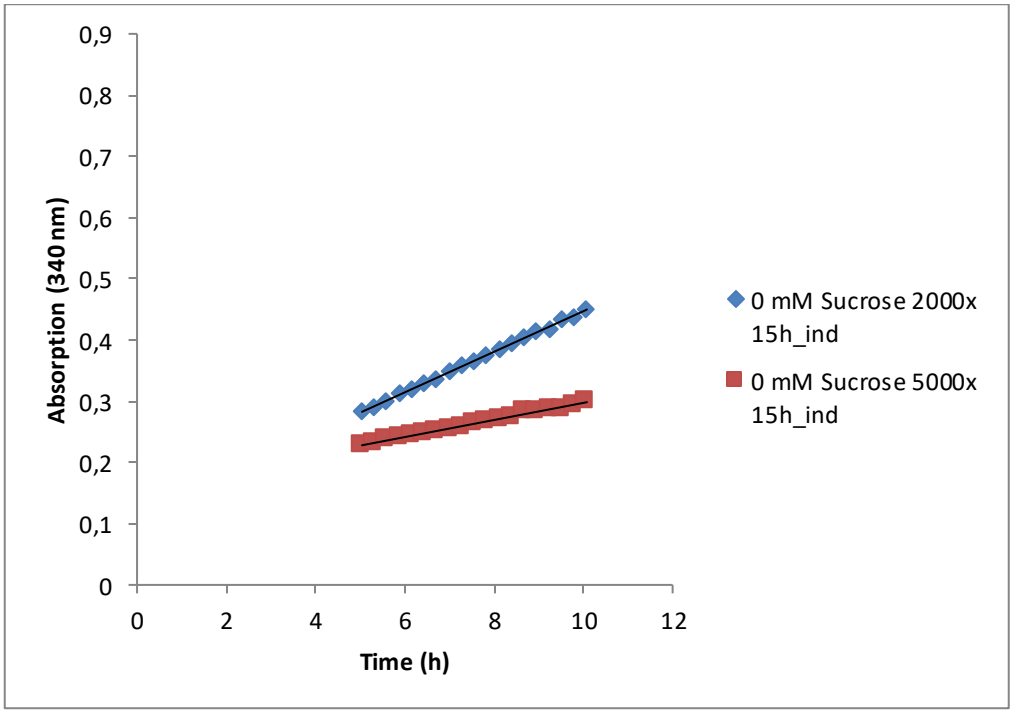

Figure C.2. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 15 h post induction

Table C.5. Raw data for calibration curve for protein concentration determination of recombinant *Bl*SPase with Bradford assay

BSA standard	Standard conc. (mg/mL)	Calculated concentration (mg/mL)	Difference (mg/mL)	Absorption (595 nm)
Standard 1	0.1			
Mean		0.0751		0,0782
replicate 1		0.0728	0,0272	0,0766
replicate 2		0.0773	0,0227	0,0797
Standard 2	0.2			
Mean		0.203		0,1656
replicate 1		0.2099	0,0099	0,1702
replicate 2		0.1962	0,0038	0,1609
Standard 3	0.3			
Mean		0.3181		0,2441
replicate 1		0.3245	0,0245	0,2485
replicate 2		0.3117	0,0117	0,2397
Standard 4	0.4			
Mean		0.3974		0,2983
replicate 1		0.3952	0,0048	0,2968
replicate 2		0.3996	0,0004	0,2998
Standard 5	0.5	.		
Mean		0.4917		0,3627
replicate 1		0.4917	0,0083	0,3627
replicate 2				
Standard 6	0.6			
Mean		0,616		0,4476
replicate 1		0,6269	0,0269	0,4551
replicate 2		0,605	0,005	0,4401
Standard 7	0.7			
Mean		0,7149		0,5152
replicate 1		0,7213	0,0213	0,5195
replicate 2		0,7085	0,0085	0,5108
Standard 8	0.8			
Mean		0,8099		0,5800
replicate 1		0,815	0,015	0,5835
replicate 2		0,8047	0,0047	0,5765
Standard 9	0.9			
Mean		0,9024		0,6432
replicate 1		0,9181	0,0181	0,6539
replicate 2		0,8867	0,0133	0,6325
Standard 10	1			
Mean		0,9716		0,6905
replicate 1		0,9716	0,0284	0,6905

replicate 2		0,9716	0,0284	0,6905

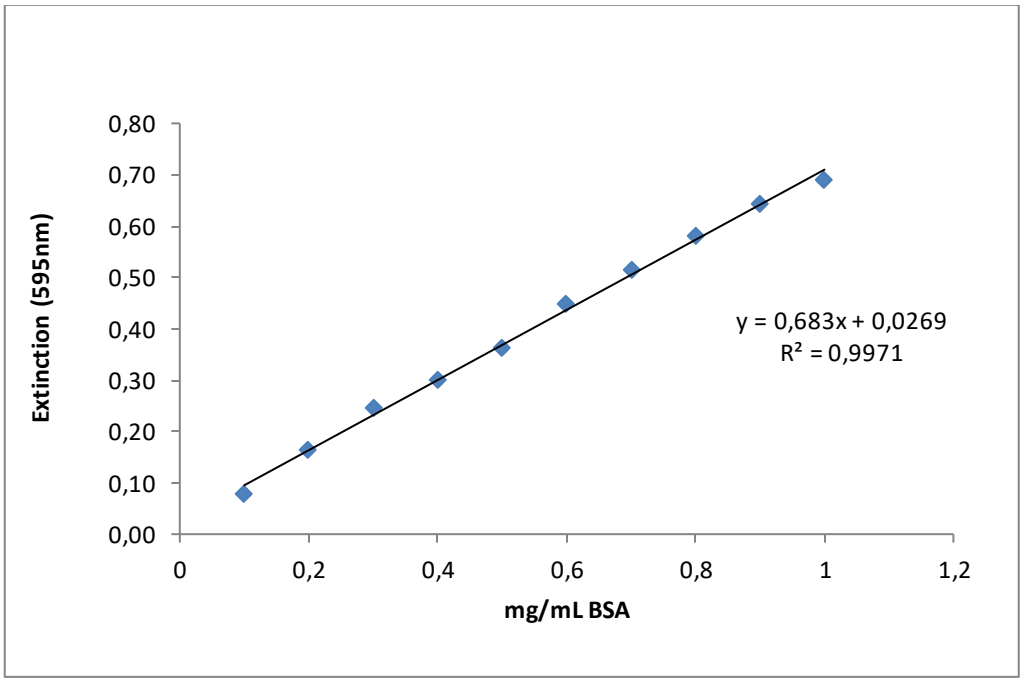

Figure C.3. Calibration curve with BSA standards from 0.1 – 1.0 mg/mL.

Table C.6. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 6h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0mM Sucrose 1:20 6h post induction	0.5913	0.5835	0.5874	0.683	0.027	0.82	16.41	15.02
0mM Sucrose 1:50 6h post induction	0.2283	0.198	0.2131	0.683	0.027	0.27	13.63	

Table C.7. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 15 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0mM Sucrose 1:20 15h post induction	0.4002	0.3449	0.37255	0.683	0.027	0.51	10.12	10.08
0mM Sucrose 1:50 15h post induction	0.1685	0.1597	0.1641	0.683	0.027	0.20	10.04	

D Optimization of bioreactor cultivation

D.1 Additional ampicillin at induction point

Table D.1.1. OD_{600} and cell dry weight values for cell growth of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (1)	OD_{600} (2)	Cell dry weight (g/L) (1)	Cell dry weight (g/L) (2)	g L^{-1} OD_{600}^{-1} (1)	g L^{-1} OD_{600}^{-1} (2)
0	0.43	0				
0.5	0.65	0.5				
1.5	1.2	1.5				
1.75	2.2	1.75	0.7	0.76	0.32	0.35
3.75	5.3	3.75	1.26	1.24	0.24	0.23
5.75	8.2	5.75	1.58	1.5	0.19	0.18
7.75	10.6	7.75	1.8	1.62	0.17	0.15
9.75	14	9.75	1.94		0.14	
21.75	30.2	21.75	5.36	4.84	0.18	0.16
26.75	32.6	26.75	6.76	6.7	0.21	0.21

Table D.1.2. AA-2G formation in mmol/g_{cdw} of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Sample time point (h of cultivation)	AA2G Peak	mM AA2G	mmol AA2G/g_{cdw}	mean mmol AA2G/g_{cdw}
1.75 (1)	2634839	38	419	377
1.75 (2)	2113317	30	336	
3.75 (1)	1541733	22	245	272
3.75 (2)	1877000	27	298	
5.75 (1)	1190067	17	189	189
5.75 (2)	944800	13	150	
21.75 (1)	3347890	48	532	519
21.75 (2)	3188452	46	507	
26.75 (1)	2924224	42	465	465
26.75 (2)	1249598	18	199	

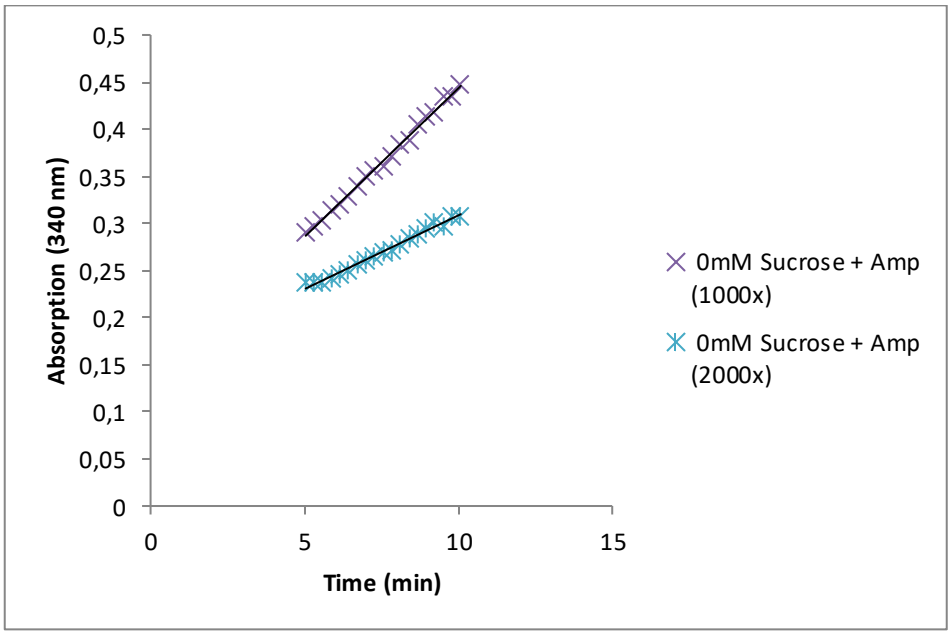

Figure D.1.1 Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 25 h post induction. Abbreviation Amp stands for ampicillin.

Table D.1.4. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 25 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0mM Suc. + Amp 1:20 25h post induction	0.3597	0.3874	0.3736	0.683	0.0269	0.52	10.40	8.43
0mM Suc. + Amp 1:50 25h post induction	0.1015	0.1118	0.1067	0.683	0.0269	0.13	6.46	

D.2 Replacement of ampicillin by carbenicillin

Table D.2.1. OD_{600} and cell dry weight values for cell growth of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (1)	OD_{600} (2)	Cell dry weight (g/L) (1)	Cell dry weight (g/L) (2)	g L^{-1} OD_{600}^{-1} (1)	g L^{-1} OD_{600}^{-1} (2)
0	0.3	0.28	0.3	0.34	0.17	0.19
1	0.88	0.91	1.04	0.88	0.20	0.17
1.25	1.8	1.7	1.6	1.6	0.14	0.14
4.25	5.2	5.4	3.48	3.58	0.17	0.17
7.25	11.5	11	11.96	12.22	0.20	0.20
10.25	21	20.5	0.3	0.34	0.17	0.19
16.25	59	61	1.04	0.88	0.20	0.17

Table D.2.2. AA-2G formation in mmol/g_{cdw} of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Sample time point (h of cultivation)	AA2G Peak	mM AA2G	mmol AA2G/g_{cdw}	mean mmol AA2G/g_{cdw}
1.25 (1)	1034942	15	231	231
1.25 (2)	1035675	15	231	
4.25 (1)	3619385	52	269	283
4.25 (2)	3997823	57	297	
7.25 (1)	5199097	74	232	230
7.25 (2)	5099038	73	228	
10.25 (1)	5880560	84	119	118
10.25 (2)	5781056	83	117	
16.25 (1)	4959787	71	29	29
16.25 (2)	4998625	71	30	

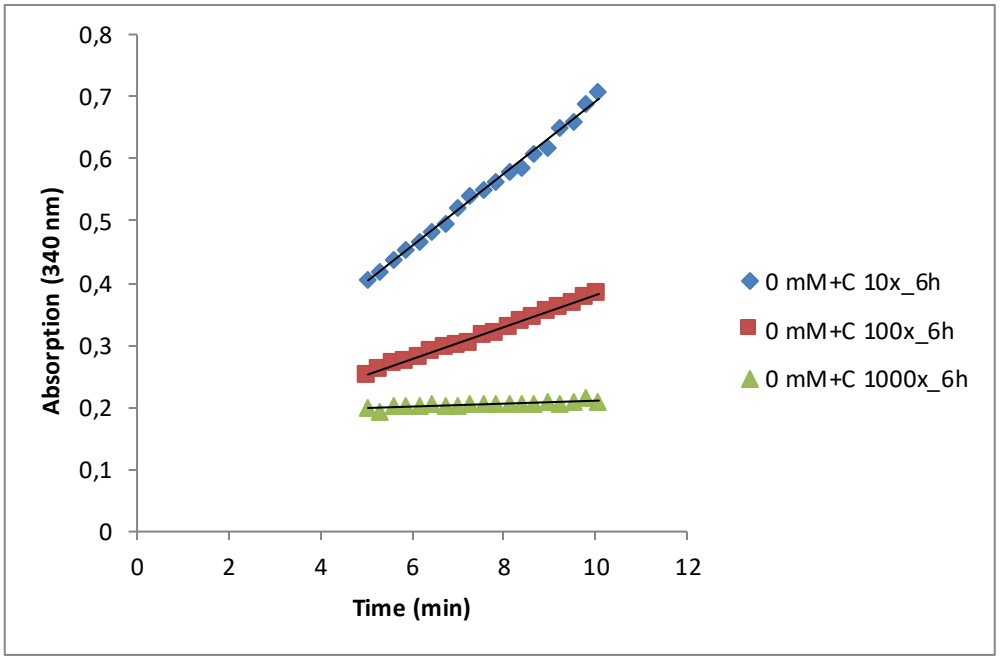

Figure D.2.1 Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 6 h post induction. Abbreviation C stands for carbenicillin

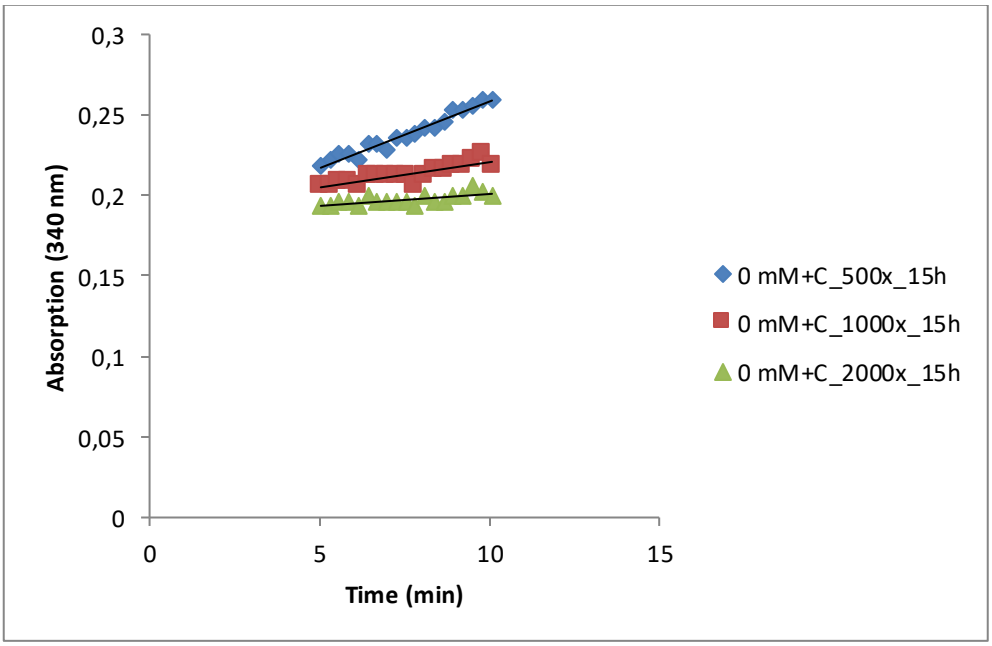

Figure D.2.2 Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 15 h post induction. Abbreviation C stands for carbenicillin

Table D.2.3. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 6 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0mM Suc. + Carb 1:20 6h post induction	0.1737	0.2734	0.2236	0.683	0.0269	0.29	2.88	2.25
0mM Suc. + Carb 1:50 6h post induction	0.0842	0.0804	0.0823	0.683	0.0269	0.08	1.62	

Table D.2.4. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 15 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0mM Suc. + Carb 1:20 15h post induction	0.1888	0.1656	0.1772	0.683	0.0269	0.22	4.40	3.13
0mM Suc. + Carb 1:50 15h post induction	0.0523	0.0525	0.0524	0.683	0.0269	0.04	1.87	

D.3 Addition of 250 mM D-sucrose

Table D.3.1. OD_{600} and cell dry weight values for cell growth of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (1)	OD_{600} (2)	Cell dry weight (g/L) (1)	Cell dry weight (g/L) (2)	g L^{-1} OD_{600}^{-1} (1)	g L^{-1} OD_{600}^{-1} (2)
0	0.18	0.18				
1	0.57	0.57				
1.5	2.2	2.2	0.48	0.44	0.22	0.20
4.5	4.2	4.6	1.02	1.06	0.23	0.24
7.5	8	8.5	1.42	1.5	0.17	0.18
10.5	11	11	1.96	2.42	0.18	0.22
16.5	19	20	3.9	3.9	0.20	0.20
26.5	27	25	4.96	5.1	0.19	0.20
31.5	27	29	5.04	5.04	0.18	0.18

Table D.3.2. AA-2G formation in mmol/g_{cdw} of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Sample time point (h of cultivation)	AA2G Peak	mM AA2G	mmol AA2G/g_{cdw}	mean mmol AA2G/g_{cdw}
1.5 (1)	1139264	16	177	174
1.5 (2)	1106864	16	172	
4.5 (1)	3767426	54	259	275
4.5 (2)	3635410	52	291	
7.5 (1)	5837357	83	286	288
7.5 (2)	5935045	85	290	
10.5 (1)	6400677	91	209	204
10.5 (2)	6120533	87	200	
16.5 (1)	6182041	88	113	113
16.5 (2)	6120533	87	112	
26.5 (1)	4693595	67	67	67
26.5 (2)	4714837	67	67	
31.5 (1)	4175760	60	59	61
31.5 (2)	4460090	64	63	

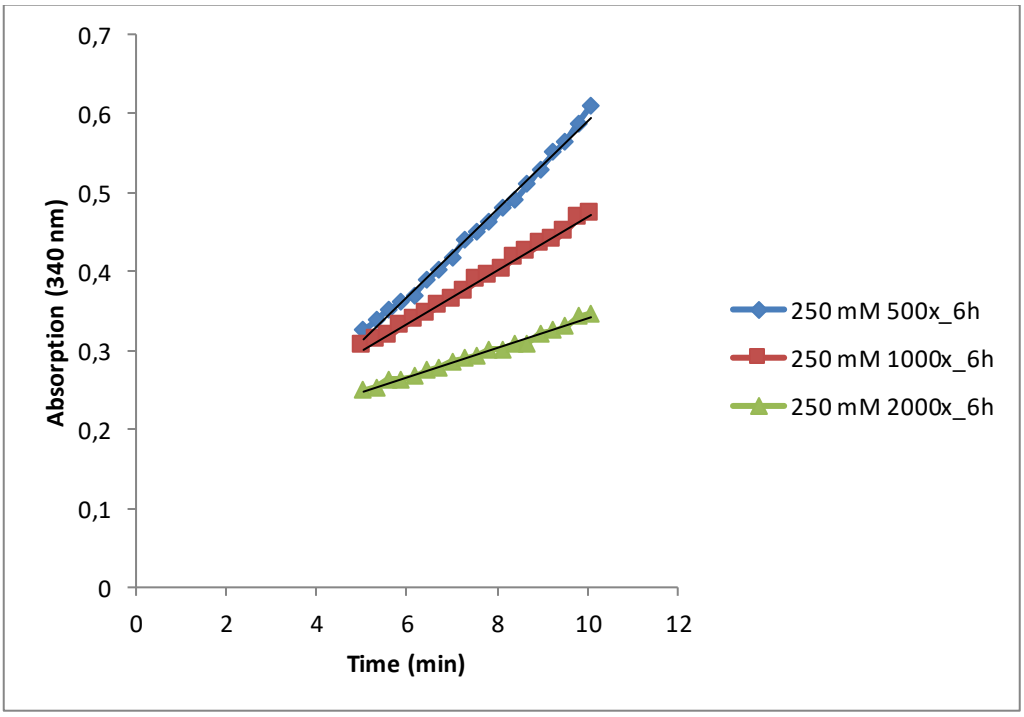

Figure D.3.1. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 6 h post induction.

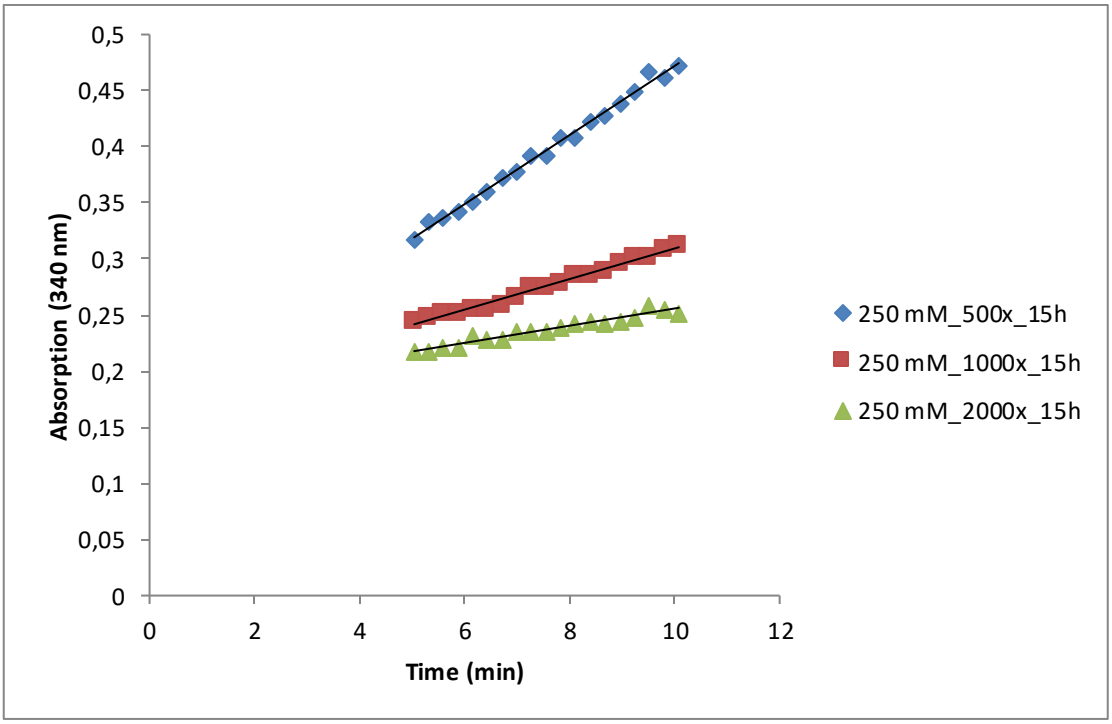

Figure D.3.2. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 15 h post induction.

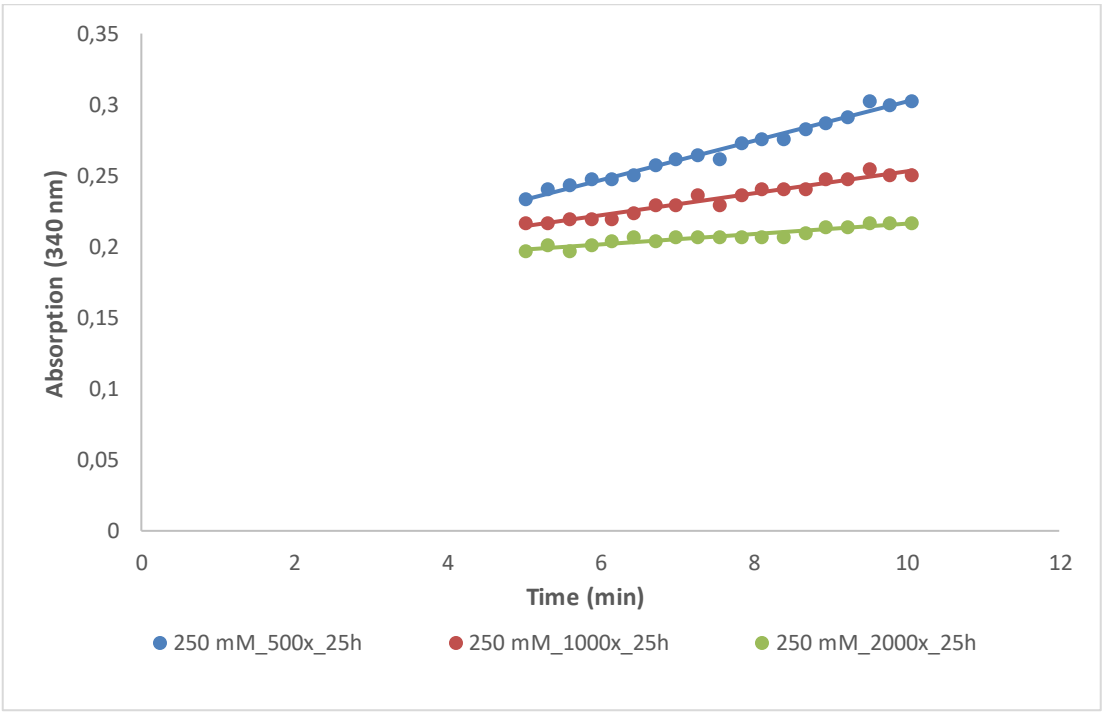

Figure D.3.3. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 25 h post induction.

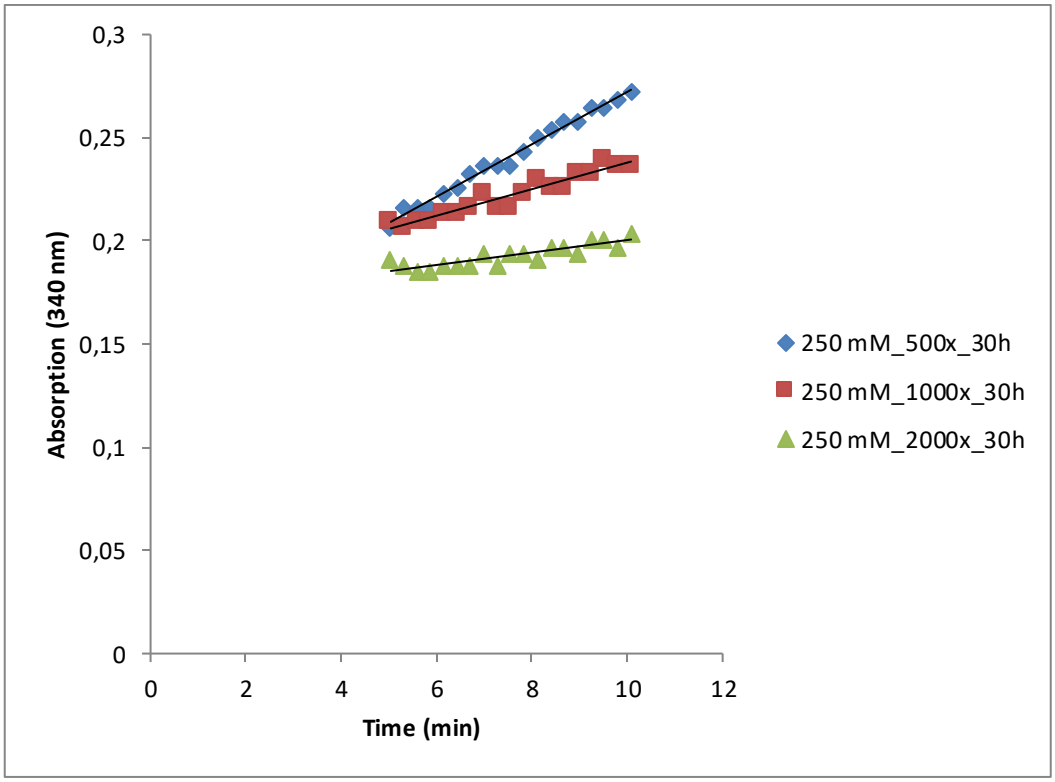

Figure D.3.4. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 30 h post induction.

Table D.3.3. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 6 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
250 mM Sucrose 1:20 6h post induction	0.47	0.4857	0.47785	0.683	0.0269	0.66	13.20	12.30
250 mM Sucrose 1:50 6h post induction	0.1797	0.1853	0.1825	0.683	0.0269	0.228	11.39	

Table D.3.4. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 15 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
250 mM Sucrose 1:20 15h post induction	0.3177	0.3364	0.32705	0.683	0.0269	0.44	8.79	7.65
250 mM Sucrose 1:50 15h post induction	0.1118	0.1201	0.11595	0.683	0.0269	0.13	6.52	

Table D.3.5. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 25 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
250 mM Sucrose 1:20 25h post induction	0.3518	0.3695	0.36065	0.683	0.0269	0.49	9.77	8.53
250 mM Sucrose 1:50 25h post induction	0.1091	0.1437	0.1264	0.683	0.0269	0.15	7.28	

Table D.3.6. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 30 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
250 mM Sucrose 1:20 30h post induction	0.346	0.3633	0.3547	0.683	0.0269	0.48	9.60	9.30
250 mM Sucrose 1:50 30h post induction	0.1387	0.161	0.1499	0.683	0.0269	0.18	9.00	

D.4 Shift of induction to a higher biomass concentration with additional ampicillin

Table D.4.1. OD_{600} and cell dry weight values for cell growth of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Time (h)	OD_{600} (1)	OD_{600} (2)	Cell dry weight (g/L) (1)	Cell dry weight (g/L) (2)	g L^{-1} OD_{600}^{-1} (1)	g L^{-1} OD_{600}^{-1} (2)
0	0.33	0.32	4.06	3.96	0.32	0.32
1	1.3	1.5	5.06	5.16	0.29	0.29
2	4	4.2	5.76	5.34	0.32	0.29
3	12	13	6.34	4.8	0.33	0.25
6	17	18	7.3	7.04	0.32	0.31
9	19	17.5	4.06	3.96	0.32	0.32
12	20	18	5.06	5.16	0.29	0.29
18	22	23	5.76	5.34	0.32	0.29

Table D.4.2. AA-2G formation in mmol/g_{cdw} of *E. coli* BL21 (DE3)_pCHEM21_*Bl*SPase

Sample time point (h of cultivation)	AA2G Peak	mM AA2G	mmol AA2G/g_{cdw}	mean mmol AA2G/g_{cdw}
3 (1)	5843388	83	104	127
3 (2)	8408271	120	150	
6 (1)	10301221	147	144	166
6 (2)	13450477	192	188	
9 (1)	14921226	213	192	210
9 (2)	17760416	254	229	
12 (1)	17953389	256	230	241
12 (2)	19600887	280	251	
18 (1)	14686769	210	146	132
18 (2)	11797044	169	118	

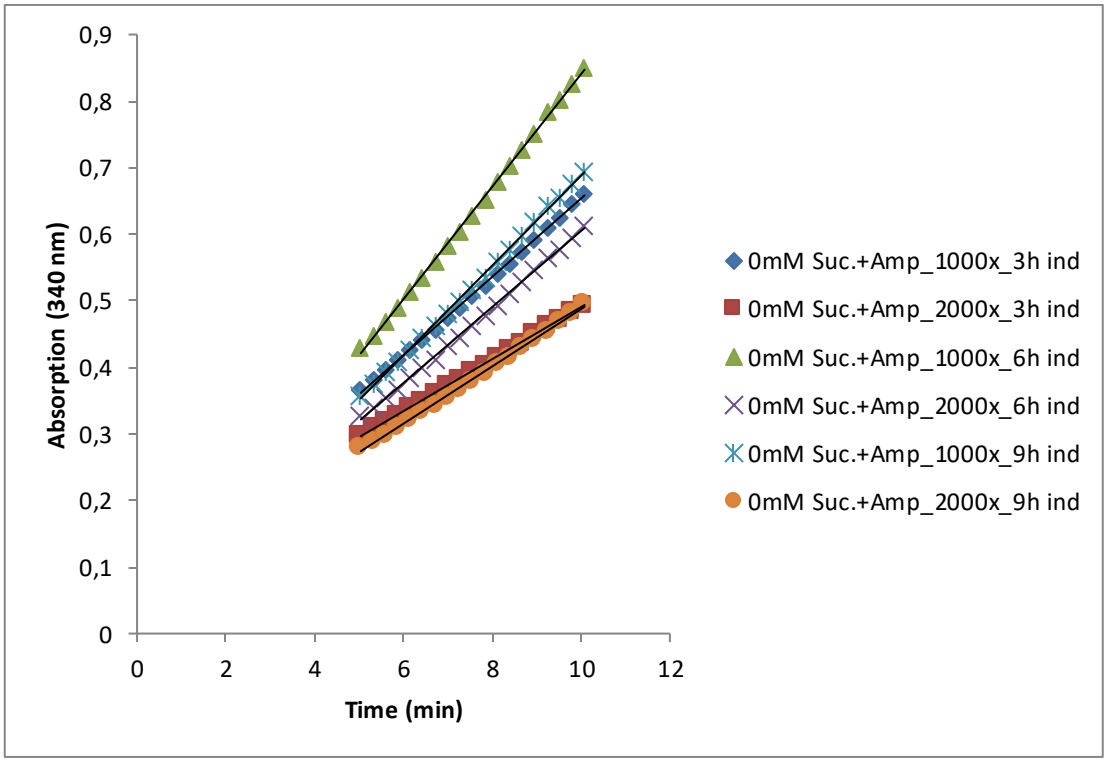

Figure D.4.1. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 3 h, 6 h and 9 h post induction. The abbrevations "Suc." and "Amp" stand for sucrose and ampicillin.

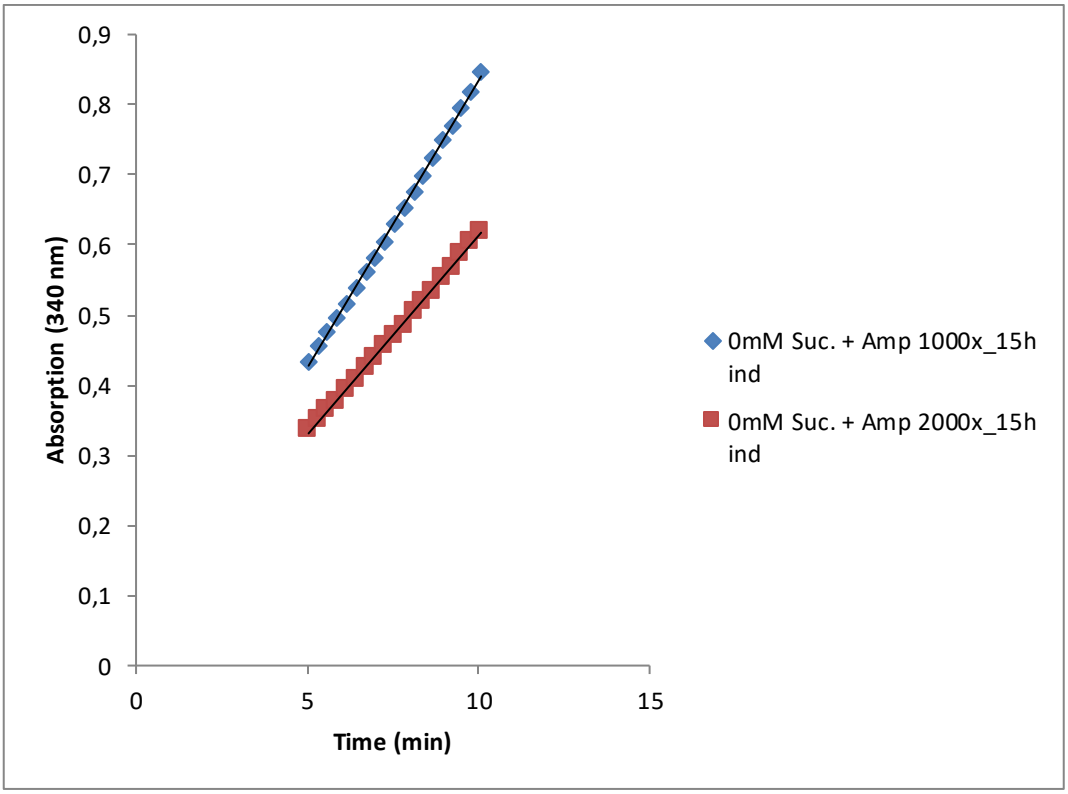

Figure D.4.2. Determination of NADH-dependent absorption over time for determination of volumetric activity of recombinant *Bl*SPase after 15 h post induction. The abbrevations "Suc." and "Amp" stand for sucrose and ampicillin.

Table D.4.3. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 3 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0 mM Sucrose 1:20 3h post induction	0.3617	0.3465	0.3541	0.683	0.0269	0.48	9.58	8.94
0 mM Sucrose 1:50 3h post induction	0.1361	0.1442	0.1402	0.683	0.0269	0.17	8.29	

Table D.4.4. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 6 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0 mM Sucrose 1:20 6h post induction	0.5627	0.5394	0.55105	0.683	0.0269	0.77	15.35	15.11
0 mM Sucrose 1:50 6h post induction	0.2121	0.2482	0.23015	0.683	0.0269	0.30	14.88	

Table D.4.5. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 9 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0 mM Sucrose 1:20 9h post induction	0.3562	0.4729	0.41455	0.683	0.0269	0.57	11.35	11.06
0 mM Sucrose 1:100 9h post induction	0.1009	0.0999	0.1004	0.683	0.0269	0.11	10.76	

Table D.4.6. Protein concentration determination of recombinant *Bl*SPase with Bradford assay after 15 h post induction

condition	Extinction (1)	Extinction (2)	mean extinction	slope	intercept	mg/mL protein	mg/mL protein (factor)	mean mg/mL protein
0 mM Sucrose 1:20 15h post induction	0.3403	0.3445	0.3424	0.683	0.0269	0.46	9.24	8.50
0 mM Sucrose 1:50 15h post induction	0.1351	0.131	0.1331	0.683	0.0269	0.16	7.77	