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Cloning and in vitro evolution of a beta-galactosidase from a psychrophilic bacterium

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// Abstract
Lactose is a disaccharide found in most dairy products. Parallel to this a large percentage of the global population suffers from hypolactasia and is intolerant to the substance. The dairy industry is constantly developing new products that are free from lactose, to satisfy these individuals. Even though producers have come a long way in developing techniques that are efficient and cheap there is still improvements to be made. β-D-galactosidase have been proven to effectively remove lactase from dairy products, still further enhancement is presumed possible.

In this study the aim was to further explore and develop the properties of known β-D-galactosidase enzyme found in *Arthrobacter psychrolactophilus*. With the use of polymerase chain reaction and transformation techniques, the β-D-galactosidase coding *bglA* gene from *Arthrobacter psychrolactophilus* B7 was transformed into *Escherichia coli* BL 21 competent cells. Furthermore over expression of the rBglAp and spectrophotometric analysis was conducted to detect any successful transformations.

The transformation showed no indication of catalysis of lactose into its monomeric forms. Further verification of the techniques would have increased the success rate of the experiment. Detection methods like subcloning of the plasmid vector and selective media should be performed if the experiment is repeated.

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Abbreviations

AA	Amino acid
bp	Base pairs
CLS	Congenital lactase deficiency
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
gDNA	Genomic DNA
GMM	Genetically modified microorganisms
IPTG	Isopropyl β-D-thiogalactopyranoside
K_{cat}	Product formation rate
K_M	Substrate enzyme interaction
LAF	Laminar flow cabinet
LB	Luria-Bertani broth
LCT	Lactase gene
NCBI	National Center for Biotechnology Information
ONPG	Orto-Nitrophenyl-β-D-galactopyranoside
PCR	Polymerase chain reaction
rcf	Relative centrifugal force
TB	Terrific Broth
TBE	Tris base, boric acid and EDTA buffer
TSB	Trypticase soy broth

1 Background

1.1 Purpose of the bachelor thesis

With present techniques like PCR and transformation there is potential to improve the properties of genes and the enzymes that they express. The aim of the current study is to transform and further explore the enzymatic properties of the β-D-galactosidase enzyme expressed by *Arthrobacter psychrolactophilus* B7. To the writer's knowledge no previous articles on the improvement of the β-D-galactosidase from this strain has been attempted.

1.2 Lactose and intolerance

Lactose is a disaccharide sugar found in most mammal´s milk. The molecule consists of glucose and galactose molecules bound together by a β-1→4 glycosidic linkage (figure 1).

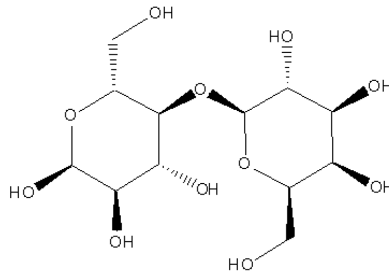

Figure 1. Molecule structure of lactose.

In the world approximately half of the population suffers from lactose intolerance also known as adult-type hypolactasia. There are some variations in the percentage of lactose intolerance, depending on population. The variation is a result of gene regulation. Intolerance is caused by an enzyme deficiency of lactase also known as β-D-galactosidase (E.C. 3.2.1.23) which catalyses lactose into its two sugar monomers. Like other digestive enzymes it is produced in the epithelium of the small intestine. When humans suffer from hypolactasia the consumed lactose is fermented by bacteria instead of being catalysed by the human β-D-galactosidase, which leads to symptoms like bloating, diarrhea and abdominal pain (Lember, 2012). Normally infants do not suffer from this type of deficiency and produce lactase enzyme, since without the enzyme infants would not be able to digest the lactose in the mother milk causing mal-nutrition. In some cases they can suffer from congenital lactase deficiency (CLS) and are then inhibited to produce lac-

tase due to a total lack of the lactase gene (*LCT*). During maturation the *LCT* gene is down regulated in most humans leading to hypolactasia (Järvelä, 2005).

1.3 Present techniques

There are other dairy products than milk that can be consumed by humans with hypolactasia, which include products like fermented dairies, aged cheese and butter. Because of the large population suffering from hypolactasia, dairy producers have developed ways to target consumers with hypolactasic condition and now have a range of lactose free products. The problem that has arisen with these products is the complexity of removing large quantities of lactose efficiently, resulting in high retail prices. Producers have tried to develop methods that are easy to perform commercially and remove lactose efficiently. Valio was one of the first companies to produce lactase hydrolysed products with their HYLA® (Hydrolysed Lactose) enzyme.

Enzymatic hydrolysis with soluble enzymes has been proven to be one of the most effective methods for removal of lactose in dairy products. In this process lactase enzymes are simply added to the batch of milk, either before or after heat treatment. Two other methods that are commercially used are membrane and chromatographic techniques. Chromatographic techniques use molecular interaction to separate lactose from milk products. Through adhesive resin in the colon lactose is separated from the milk. Different types of resins can be used like ion exchange, hydrophobic interaction and affinity chromatography. Separation with chromatography is highly specific and the industry uses combinations of the resin to obtain optimal effect. Membrane techniques use β-D-galactosidase enzymes to hydrolyse lactose in the same way as soluble enzymes, the enzyme is bound to a membrane and milk is passed over the membrane surface to separate lactose from products. The techniques suffer from some drawbacks, only approximately 80% of the lactose is removed and high microbial growth due to high temperatures can contaminate the product. Apart from the two other problems, glucose and galactose formation can sweeten the product. Furthermore their reducing ability increases the risk of reactions with amino acids (AA) creating Maillard reaction miscolouring the milk brown. The risk is even greater if the lactase is added before heat treatment (Harju *et al.*, 2012).

1.4 Enzymes and organisms

Taking techniques like these into consideration producers still have chosen to use hydrolysing enzymes to separate the two monosaccharides. Furthermore the enzymes are extracted from various microorganisms. Some organisms produce the enzyme naturally and are referred to as native producers and others are transformed into producing β-D-galactosidase enzymes, so called genetically modified microorganisms (GMM). One commercially used microorganism for β-D-galactosidase production is the yeast *Kluyveromyces lactis* (commercially avalible from chr. Hansen as HA-lactase). Moreover mould like *Aspergillus*, bacteria like *Echerichia coli* among many other organisms carry the genes for β-D-galactosidase or can be transformed into expressing the enzyme.

Most of the microorganisms that are used for production of β-D-galactosidase are mesophilic and are cultivated in temperatures that range in between 35-45 ^{o}C. Due to the conditions in which these microbes grow, their β-D-galactosidase enzymes have a catalytic optimum in that range as well. For the industry a catalytic optimum at 35-45 ^{o}C will have a negative effect on the production of lactose free dairy products. One of the effects is an increased microbial growth that can contaminate the product. To avoid probability of microbial growth the process temperatures can be lowered, on the other hand the catalytic efficiency of the enzymes are decreased resulting in a longer process time. To compensate for increased process time higher volumes of catalyst are added, increasing the retail prices of lactose free products (Panesar *et al.*, 2010).

Both scientists and the industrial sector are searching for new, better enzymes for commercial use. Enzymes like the ones synthesised by *Kluyveromyces*, *Pseudoalteromonas*, *Flavobacterium*, *Arthrobacter* among many others species. These cold active β-D-galactosidases have the properties desired by the dairy industry. Properties like optimal activity in neutral pH, as well as high product formation rate (K_{cat}) and substrate enzyme interaction (K_M) at <10 ^{o}C. Equally important is the enzymes ability to bind specifically to lactose and that interaction with glucose, galactose, Na^+ or Ca^{2+} does not constrain the enzymatic activity. Out of these microorganisms the *Arthrobacter psychrolactuphilus* is one of the strains that meet these criteria (Wierzbicka-Woś *et al.*, 2011). All these factors will eventually indirectly or directly affect the Michaelis-Menten kenetic model that will determine the effectiveness of the enzyme.

1.5 *Arthrobacter psychrolactophilus*

Several reports on the Gram-negative *Arthrobacter psychrolactuphilus* strains have been published, showing that their β-D-galactosidase effectively hydrolyse lactose during milk processing conditions. Depending on the strain of bacteria, several types of β-D-galactosidase enzymes have been

identified. Genes like the *bglA* (Nakagawa *et al.*, 2003), *isozyme 13*, *15* (Gutshall *et al.*, 1995), *galA* and *galB* (Ke Xu, 2011) are some of them, out of these the *bglA* gene is considered favourable. In contrast to the other genes the *bglA* gene in the *Arthrobacter psychlactophilus* is long with 3084 bp. When translated the *bglA* gene generates a β-D-galactosidase protein consisting of 1028 amino acid (AA) (Nakagawa *et al.*, 2007).

1.6 Techniques

There are many techniques for analysing, characterising and further developing the properties of enzymes such as the β-D-galactosidase. With the use of standardised methods like cell lysis and polymerase chain reaction (PCR) genes can be amplified from genomic DNA of a bacterium. A crucial step when using PCR techniques is the design of primers. Both the melting temperature (T_M) and GC content (GC%) is of importance. GC% values should range between 40-60% and an optimal T_M value should be in the range of 52-58 ^{o}C. In addition to these aspects the use of flanking ends on the primers increase the affinity, when the gene is inserted through ligation into a plasmid vector (Abd-Elsalam, 2003).

To transfer the amplified gene from one organism to another a plasmid vector can be used. Vectors usually are designed to have an antibiotic resistance coding region; which increases selectivity when incubating the transformed organism. Through transformation the plasmid vectors genetic material is transferred from one organism to another. The *Escherichia coli* BL 21 competent cells are specially designed to accept vectors and their genetic material. When a competent cell has accepted a vector containing the genetic material it can be over expressed into proteins. To ensure a high yield of protein the transformed bacteria is incubated in enrichment media, when overexpressing enzymes separate media with certain ingredients are preferably used to avoid inhibition of the translation. Isopropyl β-D-thiogalactopyranoside (IPTG) is used to trigger over expression in the recombinant bacteria, resulting in high amounts of intercellular proteins (Bruno Miroux, 1996). The intercellular enzymes are extracted and can be analysed with various techniques. Spectrophotometry is on suitable method, using orto-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate the catalytic activity of the enzyme can be measured. A method like this generates a Michaelis Menten curve which is used for calculating K_{cat} and K_m of an enzyme (Nakagawa *et al.*, 2007).

In order to improving K_{cat} and K_m of an enzyme error prone PCR with a low or no proofreading polymerase can be used. By altering the nucleotide sequence of the gene new or enhanced properties can be obtained (Whittall & Sutton, 2009).

2 Materials and methods

In the flow chart (figure 2) a simplified overview of the methods that were used is displayed.

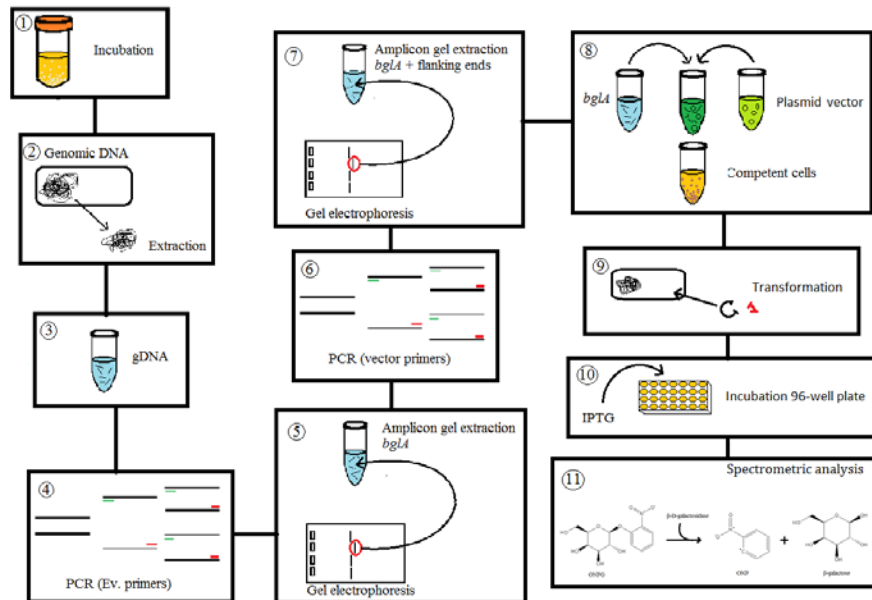

Figure 2. Systematic flow chart describing how extraction, amplification and transformation was performed when genetically modifying *E. coli* BL 21.

2.1 Incubation

Arthrobacter psycholactophilus strain B7 (DSMZ DSM No. 15612) was incubated in trypticase soy broth (TSB), (Maine Manufacturing) at 28 °C at a neutral pH of 7.0-7.2. The cultures were incubated for 48h in a shake incubator before visible growth was observed.

2.2 Genomic DNA extraction

2.2.1 Cell lysis

In this step a laminar flow cabinet (LAF) was used to avoid contamination. With the use of a chargeswitch genomic DNA mini bacteria kit (Invitrogen Ref. CS11301) genomic DNA from the bacteria cells could be extracted. First 0.5 ml incubated cultures were centrifuged at a relative centrifugal force of 14 000× *g* for 10 minutes, the supernatant was then removed from the tube. A volume of 5 µl of lysozyme and 100 µl of resuspension buffer were added to the tube containing the pellet; carefully the tube was vortexed to evenly distribute the cells. The cells were incubated for 10 minutes at 37 °C. After incubation 500 µl of lysis buffer was mixed with 10 µl of protein-

ase K and the tubes were inverted a few times. Finally the sample was incubated for 90 minutes at 55 ^{o}C.

2.2.2 DNA purification

A magnetic bead kit from Invitrogen (ChargeSwitch® gDNA mini bacteria kit No. CS11301) was used in the extraction of the DNA, 40 µl of magnetic bead suspension was added to each Eppendorf tube and carefully mixed by pipetting up and down, after the sample had been mixed, 300 µl of binding buffer was added to the sample. The magnetic beads were collected after settling for 1 minute in a magnetic rack and the supernatant was then removed with a pipette. In addition 1 ml of wash buffer was added to the sample and the solution was gently pipetted up and down 3 times. Once more the tube was placed in the magnetic rack to absorb the beads carrying the genomic DNA and the supernatant was carefully removed. This step was repeated twice. After adding 200 µl of elution buffer the sample was incubated for 5 minutes at room temperature. In the final step the beads were collected and once more the supernatant containing genomic DNA was separated, the separated supernatant was diluted twenty times and then stored in a fresh Eppendorf tube at 4 ^{o}C.

2.2.3 Storage and concentration

In addition the absorbance was measured on a spectrophotometer (Pharmacia biotech Ultrospec 4000 UV/Visible Spectrophotometer) to determine yield and purity of the genomic DNA sample before proceeding with PCR. A volume of 1.98 ml of 0.5×tris HCl buffer mixed with 20 µl of genomic DNA sample was measured at 230 (X), 260(Y), 280(Z) and 320(A) nm. Next the absorbance values were inserted into the formula (X-A)/(Y-A) = Purity. For the yield the formula (X-A)×20×50 = Yield, to calculate weight the formula yield×0.1 = µg was used. Samples were stored in 4 ^{o}C for daily use and -20 ^{o}C for later analysis.

2.3 PCR and Gel electrophoresis

2.3.1 Primer construction

To quantify the *bglA* gene an AccuPOL DNA Polymerase kit (VWR) was used. Two primer pairs were created for the *bglA* gene, primer pair (A) and primer pair (B). Both pairs were designed using National Center for Biotechnology Information (NCBI) primer designing tool (Owczarzy *et al.*, 2004). All the primers had to be shortened in the 3´ end to match the desired properties. For reverse primer B the first three nucleotides were removed as well, to ensure acceptable T_M and GC% values. The exact sequence of the *bglA* gene that was used hade the accession number AB243756. Nucleotide sequences, T_M and GC% are shown in table 1. Primers A were designed without flanking ends, primers B were designed with flanking meaning that an extra sequence is deliberately added to the primer. These ends increase specificity when transforming the *bglA* gene into the competent cells. The

T_M (X) value for the forward primer and the reverse primer was inserted to the formula $((X_1+X_2)/2)-Y$ for calculation for the optimal annealing temperature during PCR. X_1 = forward primer, X_2 = reverse primer, Y = 5°C.

Table 1. Sequence, length and properties for primers used for amplification

Primers	Sequence 5´→3´	Length	T_M	GC%
A Forward	TGGGGGCGTCTGCAAAGCATG	21	66.15	61.90
A Reverse	CAGTGCGGAGAAGCGCAGTACCAG	24	67.67	62.50
B Forward	CATCATCACCACATCATGGGGGCGTCTG	28	71.76	60.00
B Reverse	GTGGCGGCCGCTCTATTACAGTGCGGAGAA	30	73.04	60.00

2.3.2 PCR

The PCR reactions were carried out on a MJ research PTC-200 Peltier thermal cycler. The extracted genomic DNA was mixed with 5 µl of 10× PCR buffer, 4 µl of 10 mM deoxynucleotide triphosphates (dNTP) mix, 1 µl of 50 mM $MgCl_2$, 10 µl of 10 µM forward primer and 10 µl of 10 µM reverse primer, 3 µl of 39 µg/ml genomic DNA, polymerase 2.5 U/µl and then deionized water was added to make up a total volume of 50 µl of PCR mix, the components were added in that specific order inside a LAF cabinet. For the PCR with primer pair B 10 µl of DNA was used, the amount was unknown.

The following cycles were used for primer pair A. *Initiation*: one cycle at 95 °C for 2 min, *Denaturation:* 30 cycles at 95 °C for 45 sec, *Annealing*: 62 °C for 30 sec, *Elongation*: 72 °C for 7 min. *Final Elongation* was set for 72 °C in 10 min and a *Final hold* at 4 °C.

The following cycles were used for primer pair B. *Initiation*: one cycle at 95 °C for 2 min, *Denaturation*: 30 cycles at 95 °C for 45 sec, *Annealing*: 68.4 °C for 30 sec, *Elongation*: 72 °C for 7 min. *Final Elongation* was set for 72 °C in 10 min and a *Final hold* at 4 °C.

After amplifying the gene, mutation of the sequence was conducted using a mutagenesis kit (Aglient technologies genemorph II EZClone domaine mutagenesis kit No. 200552). For this step a PCR tube was filled with 5 µl of 10× mutazyme reaction buffer and 2 µl of 10 mM dNTP mix. In addition to mutazyme and dNTP 1.35 µl of 10 µM reverse primer B was added and 1.37 µl of 10 µM forward primer B. To calculate the right volume of primer for the mix the following formula was used. µg/umol was converted into ug/L then 125ng/(µg/L) = uL of the primer needed. Moreover 20 µl of DNA sample (amount unknown) and 1 µl of 2.5 U/µl mutazyme II DNA polymerase were transferred to the PCR mix. Last deionized water was added to make up a total volume of 50 µl of PCR mix. The same temperatures, time and number of cycles as for primer pair B was used.

2.3.3 Gel electrophoreses

The PCR product was analysed by agarose gel electrophoresis, a VWR 300V power source and a VWR box (Ref. 700-0034). The gel contained 30 µl of 0.5x tris base, boric acid and EDTA mix buffer (TBE), 270 mg (0.9%) of agarose and 5 µl of 10 ug/ml ethidium bromide. 2 µl of bromophenol blue was added to the samples and the voltage set to 90 volt for 90 minutes, the gel was evaluated under a UV light (UVP high performance ultraviolet transilluminator Model: TFM-20).

Visible bands were cut out from the gel and moved to an Eppendorf tube under sterile conditions. With a Purelink™ quick gel extraction & PCR purification combo kit (Invitrogen No. K220001) the amplification product was extracted. First the gel piece containing the desired gene was weighed in an Eppendorf tube, the weight was converted to µl and then multiplied by 3. The amount of L3 buffer that was added to the gel sample equals this number. Next the gel piece was incubated for 10 minutes at 55 ^{o}C. The dissolved gel piece was transferred into a separation column together and was then centrifuged at 12200× *g* for 1 minute. The supernatant was discarded and 500 µl of wash buffer was added, then the sample was centrifuged twice, first at 12,200× *g* for 1 minute and then at 15000× *g* for 3 minutes. The supernatant was discarded and 50 µl of elution buffer was added, a final centrifugation of the sample separated the DNA from the mobile face. The extracted DNA was stored in 4 ^{o}C. The sample was then used during the second PCR with primer pair B.

Extraction of the amplified gene from PCR with primer pair B was done with the Purelink™ quick gel extraction & PCR purification combo kit (Invitrogen No. K220001).

All samples were stored in 4 ^{o}C except for the genomic DNA which was stored in -20 ^{o}C.

2.4 Transformation

When transforming the extracted gene into competent cells from the strain *Escherichia coli* BL 21. A Expresso® T7 cloning kit N-His (Lucigen No. 49001-1) was used. Initially the competent cells and vector were thawed on ice. The vector was pulse centrifuged briefly. 2 µl of vector and 1 µl of DNA were mixed, the mix was transferred into a new tube and the sample was incubated on ice for 30 minutes. After incubation the cells were heat shocked in a water bath set at 42 ^{o}C for 45 seconds then placed on ice again for 2 minutes. 960 µl of recovery medium were added to the Eppendorf tubes. The tubes were placed on a shake incubator set at 250 rpm for one hour. After incubation 100 µl of the sample was spread on Luria-Bertani Broth (LB) plates containing 50 µg/ml kanamycin sulphate (kana). Each plate was incubated for 24 hours at 37 ^{o}C.

96-well plates were used to further incubate the bacteria colonies; each well was filled with 300 µl of premade LB-kana-medium. One bacteria colony was inoculated to each well of the 96-well plate. Colonies were incubated for 24 hours at 30 °C on a shaker (250 rpm).

After 24 hours of incubation 30 µl of the medium was transferred to a new 96-well plate, each well containing 300 µl of Terrific Broth (TB) mixed with a concentration of 1 mM of IPTG. The plate was incubated at 30 °C for 18 hours with shaking (250 rpm). When the cultures had been incubated for 18 hours they were centrifuged at 1500× *g* for 10 minutes. Directly after centrifugation, the supernatant was removed from the wells. 100 µl of Bug-Buster ® HT protein extraction reagent was added to each well and the plate was incubated for 15 minutes on a shaker (250 rpm) in room temperature. The plate was centrifuged at 1600× *g* for 20 minutes at 4 °C, the pellet from each well was transferred to a third 96-well plate and analysed.

2.5 Spectrophotometric analysis

Each well on the 96-well plate was measured with a spectrophotometer (Devices model: Spectramax M2). The enzymatic activity of β-D-galactosidase was assayed using ONPG, at a concentration of 3 mg/ml. Before inserting the plate into the spectrophotometer 50 µl of substrate was applied to each well, directly after the substrate was applied 50 µl of the supernatant containing enzymes were added to each of the wells. Each well was measured with a 34 second interval at the optical density of 410 nm at 23.7 °C (Nakagawa *et al.*, 2007).

2.6 Verification with a commercial enzyme

15 µl of 3 mg/ml of commercially produced Lactrase GO (Oy Verman AB) was added to 1.5 ml of 3 mg/ml of ONPG mix for 60 minutes at 23.7 °C.

3 Results and discussion

In previous studies the catalysis of lactose into glucose and galactose with the use of cold active β-D-galactosidase, have been proven to function with good results. With the use of transformation, previous experiments have successfully introduced the *bglA* gene from *Arthrobacter psycholactophilus* into new expression systems. According to the results from these experiments the rBglAp enzyme catalysis ONPG with a K_M of 2.7 mM and K_{cat} of 12.7 s^{-1}. The temperature was 10 ^{o}C during the measurements (Nakagawa *et al.*, 2007). Improvement of the properties of the BglAp with mutagenesis has not been attempted in these analyses there for mutagenesis with error prone PCR were attempted to increase the K_M and K_{cat} for the BglAp.

3.1 Incubation

Initially the pre-ordered strain of *Arthrobacter psycholactophilus* B7 was incubated in order to create a sufficient amount of bacteria for the extraction of genomic DNA. According to previous research the bacteria had been incubated on peptone yeast agar containing peptone, glycerol, KCl and agar (Loveland-Curtze *et al.*, 1999). Other researchers that have incubated *Arthrobacter psycholactophilus* have been using TSB medium, which also was used in this experiment (Gutshall *et al.*, 1995; Nakagawa *et al.*, 2003, 2007). The strains B7, D2, D5, and D10 have also been incubated on 1% peptone, 0.2% lactose and 75% reconstituted seawater and been incubated at 5 ^{o}C (Loveland-Curtze *et al.*, 1999). Luria-Bertani medium (LSB) is another medium that have been used (Ke Xu, 2011; Wierzbicka-Woś *et al.*, 2011). What most media in these studies have in common is an oceanic salt content. Also when comparing previous studies the incubation temperature and time differed from the one used in this experiment, they used temperatures and time ranging between 5-10 ^{o}C for 8-10 days.

In this analysis a temperature of 28 ^{o}C was used according protocols supplied by the producer of the isolate German Collection of Microorganisms and Cell Cultures (DSZM) (Loveland-Curtze *et al.*, 1999). Incubation at 28 ^{o}C showed visible growth after 48 hours. Incubation for 72 hours resulted in an amount considered high enough to be extracted for further analysed. The time frame that was set for this analysis did not allowed for any testing of an optimal media, incubation time or temperature, to ensure selectivity of growth for the isolate. For that reason the risk of contamination of the incubated isolate cannot be ruled out. If further analysing *Arthrobacter psychrolactophilus* B7 some sort of detection technique should be used to ensure proliferation of the wright isolate. Method like API-tests or is an easy and cheap way to detect although less accurate. Other methods that have been used in previous studies of *Arthrobacter psychrolactophilus* are real time

PCR with fluorescent markers (Ke Xu, 2011) or a southern blot to identify certain DNA sequences (Gutshall *et al.*, 1995).

3.2 DNA extraction

Extraction of genomic DNA from the B7 strain with magnetic beads proved effective. After analysing the sample and to ensure a sufficient amount of genomic DNA, the calculated readings indicated a desired purity and yield which is shown in table 2. As seen in table 2 a yield of 39 µg/ml or 3900 ng was obtained. An amount ranging from 50-200 ng proved to be a sufficient amount of genomic DNA to further continue the experiment. Purity of the sample is also of importance, readings below 1.5 indicate high turbidity in the sample, organic compounds and chaotropic salts which can affect amplification. The obtained value of purity was 2.3 as displayed in table 2 (Hamid Kheyrodin, 2012).

Table 2. Purity, yield and amount of gDNA extracted from *Arthrobacter psychrolactophilus* B7

Genomic DNA extraction	
Purity	2.3
Yield (µg/ml)	39
Amount (ng)	3900

According to the data given by the spectrometric analysis the values were considered acceptable for further analysis.

3.3 PCR

The T_M values for the primers in this experiment hade values ranging just above 65 ^oC, values in that range increase the risk for secondary annealing and a probability of affecting the result of the amplification. Primer A and B as can be seen in table 1 have acceptable GC% values. These values should in further experiments be revised for better aneling (Abd-Elsalam, 2003). Although the primer design could have affected the result from the PCR, it should be noted that a lack of time and testing of the isolate could have led to amplification of the wrong gene from another strain of bacteria.

3.3.1 Primers A

After the amplification with primers A which did not have any vector flanking ends, several bands were observed and could be extracted from the gel. Knowing that the *bglA* gene is 3084 bp, an increased concentration of nucleotides was used to ensure a sufficient amount of building blocks to obtain a maximum yield of PCR product. As displayed in table 3 the purity measured from the extracted samples showed good yield and purity.

Table 3. DNA purity and yield for genomic DNA product after PCR

DNA amplicon		
Purity	2.1	2.3
Yield µg/ml	685	1080

3.3.2 Primers B

After amplifying the *bglA* gene with primer pair A, the gene template received from the PCR product was used in further amplifications. In figure 3 bands at approximately 3000 bp could be observed, the *bglA* gene is sequenced to 3084 bp and there for should give rise to a band matching a reference at that length. The ladder that was used for identifying the *bglA* gene in this experiment only had reference bands ranging up to 2000 bp, so an estimation of which band to extract hade to be made lowering the probability of extracting the sought gene. The primer pair B consisted of an 18 bp long sequence coding for vector flanking ends, which lower the affinity of the primer. Less than half of the primer was there for complimentary to the *bglA* gene at the first cycles of the PCR. Error prone PCR with the low proofreading polymerase failed, it was presumed that higher concentrations of primers were needed due to insufficient time. No further tests were conducted.

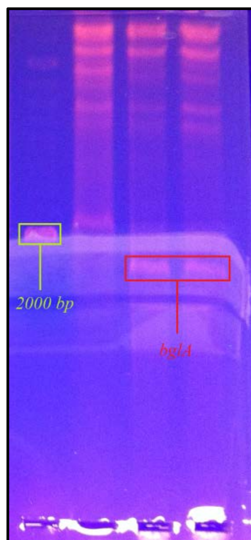

Figure 3. Photo of gel electrophoresis, showing bands at an estimated 3000 bp Region. Bands are market with a read box.

Visualizing the gel under strong UV light can mutate the nucleotide sequence if exposed for too long. For that reason the expression of the enzyme could have been affected.

3.4 Transformation

To verify the effectiveness of the rBglAp, *bglA* was introduced into an expression system. The *E. coli* BL21 is a proven organism for expressing transformed genes. Several colonies of transformed cells proliferated in the selective media and there for were presumed to have accepted the genetic material carried by the vector. The probability of an empty plasmid or the wrong insert should be eliminated by the reporter gene coding for kanamycin resistance as well as the vector flanking ends. Still there is a risk of an imperfect vector plasmids being accepted. Sequencing of the plasmid vector should have been used to verify a correct ligation of the sequences, ensuring a successful transformation of the *bglA* gene.

3.5 Spectrophotometric analysis

Interpretation of the results from the spectrophotometric readings did not produce the expected Michaelis Menten curve. Firstly an anticipated catalysis of ONPG into ONP and β-galactose could not be visually observed. A successful catalysis of ONPG into ONP will result in a yellow colour shift. To test the substrates function a commercially bought Lactrase GO enzyme tablet was used. A clear colour shift could be observed in the sample from translucent to yellow, verifying the substrate function. The expected mechanism is shown in figure 4.

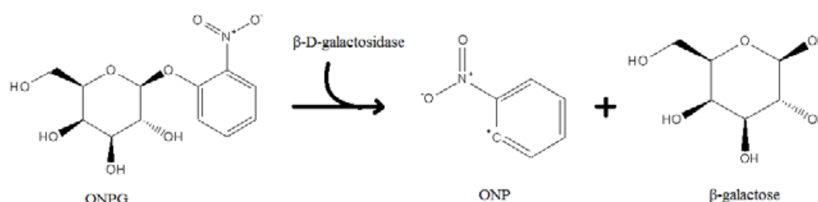

Figure 4. Mechanism for β–D-galactosidase catalysis of ONPG into ONP and β-galactose.

In figure 5 results from the spectrophotometric reading is plotted, each measurement has an interval of 36 seconds. Figure 5 does not show any catalysis of ONPG into ONP. If the substrate was to be catalysed a Michaelis Menten curve should have been plotted. One of the supposed reasons for the result could be the temperature setting of 24 °C due to faulty equipment. This is 14 °C outside the optimal catalytic temperature of the rBglAp (Nakagawa *et al.*, 2006).

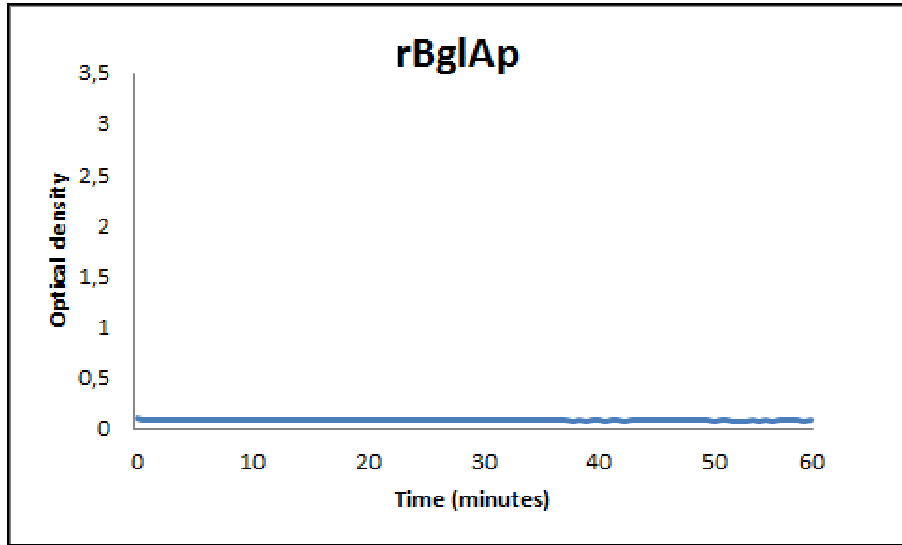

Figure 5. Optical density measurements for rBglAp catalysis of ONPG over time at the wave length 410 nm.

4 Conclusion

The industrial use of soluble enzymes has showed potential for improvement. With the use of techniques like mutagenesis, PCR, transformation and spectrophotometric analysis properties of β-D-galactosidas enzyme could be improved. The aim of the study was to transform the *bglA* gene from *Arthrobacter psycholactophilus* with a bacterial plasmid vector. Furthermore enhancement of lactose hydrolysing properties of the enzyme through error prone PCR were tested. With the use of vector flanking ends and reporter genes coding for kanamycin the risk of a failed transformation was reduced, still no hydrolysis of the rBglAp was measured. The assumed reasons for the result are time and insufficient verification techniques. In further studies methods should be complemented with other verifications techniques as well as selective media. Techniques like subcloning of the plasmid vector, southern blot of bacterial DNA and API-tests could also be used. This analysis has hopefully raised some pitfalls that might be encountered when further developing enzymes.

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6 Appendix

Table 4. Absorbance results for gDNA measurements at different wave length

gDNA absorbance				
Wave length	230 nm	260 nm	280 nm	320 nm
Absorbance	0.046	0.063	0.041	0.024

Table 5. Wave length and absorbance that was measured for gDNA amplicon

Amplicon absorbance				
Wave length	230	260	280	320
Sample 3.	0.103	0.216	0.105	0
Sample 6.	0.069	0.144	0.067	0.007