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Synthesis of benzyl β-D-galactopyranoside by transgalactosylation using a β-galactosidase produced by the over expression of the *Kluyveromyces lactis LAC4* gene in *Arxula adeninivorans*

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ABSTRACT

The *LAC4* gene of *Kluyveromyces lactis* encoding for β-galactosidase was overexpressed in the yeast *Arxula adeninivorans* to produce the enzyme, which can be used for the synthesis of β-D-galactosides. These compounds play a major role as precursors for the synthesis of glycolipids and glycoproteins in medicine or for the production of tensides.

The Xplor®2 transformation/expression platform was used because it enabled stable integration of the gene in the *Arxula* genome and the production of high levels of the enzyme. The recombinant β-galactosidase, fused with C-terminal His-tag region (Lac4-6hp), was purified by precipitation with ammonium sulphate and FPLC using hydroxylapatite. The enzyme exhibited optimal activity at 37 to 40 °C, pH 6.5 in 50 mM sodium acetate buffer. Activity was measured by the formation of *p*-nitrophenol at 405 nm from the hydrolyzed chromogenic substrate, *p*-nitrophenyl-β-D-gal. Biochemical characterization included the calculation of K_M and apparent k_{cat} values of the enzyme. The formation of benzyl β-D-gal by 0.1 U enzyme from *A. adeninivorans* with transgalactosylation was six times higher than that for the prokaryotic enzyme from *E. coli*. Moreover, the partially purified enzyme was used for the selective hydrolysis of allyl β-D-gal in a mixture of allyl β- and allyl α-D-gal, with 4 g l^{-1} being hydrolysed within one day by 1 U ml^{-1}. Thus, the recombinant β-galactosidase produced in *A. adeninivorans* is of potential interest for the enzymatic synthesis of benzyl β-D-gal and other galactosides as well as the selective hydrolysis of anomeric mixtures and could be used to replace difficult chemical procedures.

1. Introduction

Beta-galactosidases (EC 3.2.1.23) are enzymes that are widespread in bacteria, yeast, filamentous fungi, plants and animals [1]. They are used for the catalysis of hydrolytic and transglycosylation reactions. The hydrolytic reaction is used industrially for the hydrolysis of lactose in milk and dairy products to make them suitable for lactose intolerant people [2] and the transglycosylation reaction is used for the synthesis of galactosylated products like di- and oligosaccharides [3,4] and alkyl β-D-galactosides [5,6]. In this reaction, a glycosyl donor, such as lactose in case of β-galactosidase, is hydrolyzed concomitant with the formation of an intermediate of the enzyme and a glycosyl-group, which is then transferred to another sugar or an aglycon such as different alcohols. The reaction allows the production of elongated products [7], which are important precursors for the synthesis of glycolipids and proteins required in medicine [8] or for the production of detergents such as octyl β-D-gal [9]. Enzymatic synthesis is preferable to chemical synthesis, because it is stereo- and regioselective. It also does not require blocking of OH-groups, activation of the glycosidic OH-group and the separation of the anomeric mixture. Additionally, the biological process is less difficult than the chemical process.

Depending on the application, different properties of the enzymes are required. For example, the hydrolysis of lactose from milk is best done using cold-stable enzymes such as those from *Arthrobacter* sp. 32c, *Arthrobacter psychrolactophilus* or *Pseudoalteromonas haloplanktis*, which catalyze the reaction at low temperatures [10–12]. In contrast, biocatalysts with resistance to organic solvents are ideally used for the linkage of a non-sugar alcohol with galactose. Beta-galactosidases from *Aspergillus oryzae*,

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Kluyveromyces lactis and *Sulfolobus acidocaldarius* expressed in *Escherichia coli* and other organisms have been used for this reaction [3,5,6]. Currently, *K. lactis* β-galactosidase is a commercial product, which is used in the food industry (Maxilact, DSM Food Specialities, Delft, The Netherlands).

In the present work, the *LAC4*-gene from *K. lactis* was expressed in the yeast *A. adeninivorans* (syn. *Blastobotrys adeninivorans*) using the Xplor®2 transformation/expression platform, which allows the introduction of a single DNA cassette either as a Yeast rDNA Integrative Expression Cassette (YRC) or a Yeast Integrative Expression Cassette (YIC) into the genome. This platform has already been successfully applied to the construction of transgenic *A. adeninivorans* strains that secrete high levels of recombinant *Klebsiella*-derived phytase, recombinant human interferon α and recombinant tannase [13–15]. This work has produced a yeast strain that produces β-galactosidase, which was then used for the synthesis of benzyl β-D-gal, an important precursor for glycolipids and proteins [8]. Currently, this compound is difficult to procure, so its synthesis at high levels is of industrial interest.

2. Materials and methods

2.1. Strains and culture conditions

E. coli XL1 blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F'*proAB lacI*^{q} *ZΔM*15 Tn10 (Tet^r)]) obtained from Invitrogen (USA), served as the host strain for bacterial transformation and plasmid isolation. The strain was grown on LB medium (Sigma, USA) supplemented with 75 mg l^{-1} ampicillin (Applichem, Germany) or 75 mg l^{-1} kanamycin (Roth, Germany) for selection.

In this study, the auxotrophic mutants *A. adeninivorans* G1212 (*aleu2 ALEU2::atrp1* – [16]), MS1006 (*aleu2 ALEU2::atrp1 ALEU2::aade2* – this work) and the wild-type *A. adeninivorans* strain LS3 were used. LS3 was originally isolated from wood hydrolysates in Siberia and deposited as *A. adeninivorans* SBUG 724 into the strain collection of the Department of Biology of the University of Greifswald [17]. All strains were grown at 30 °C under non-selective conditions in a complex medium (YEPD) or under selective conditions in yeast minimal medium supplemented with 20 g l^{-1} glucose or lactose as carbon source and 43 mM $NH_4H_2PO_4$ (YMM-glucose-$NH_4H_2PO_4$, YMM-lactose-$NH_4H_2PO_4$) or 43 mM $NaNO_3$ (YMM-glucose-$NaNO_3$, YMM-lactose-$NaNO_3$) as nitrogen sources [18,19].

Agar plates were prepared by adding 16 g l^{-1} agar to the liquid medium.

Cultures of *A. adeninivorans* expressing *LAC4-6H* (see below) and the control strain, G1212/YRC102, were grown in glucose and lactose media and sampled at regular intervals. The samples were used to determine biomass by centrifuging 2 ml yeast culture, washing the pellet with 1 ml water and then drying by lyophilization. The dried cells were weighed and the biomass (dcw) expressed as g l^{-1} of growth medium. The samples were also used to assay intracellular β-galactosidase activity (U l^{-1} culture) using *p*NP-β-D-gal as substrate, and to calculate the β-galactosidase (Lac4-6hp) output Y (P/X) (U g^{-1} dcw).

2.2. Transformation procedures, recovery of stable *A. adeninivorans* strains, and isolation of nucleic acids

E. coli and *A. adeninivorans* cells were transformed according to Böer et al. [14]. Stable yeast transformants were obtained after a sequence of passages on selective and non-selective media [20].

Isolation of plasmid and chromosomal DNA and DNA restriction were carried out as previously described [21].

2.3. Construction of a double auxotrophic *A. adeninivorans* mutant

The parasexual processes of the imperfect phase of *A. adeninivorans* were used to produce the double auxotrophic *atrp1 aade2* mutant. First, the auxotrophic mutants, *A. adeninivorans* G1216 (*aleu2 aade2::ALEU2* – [22]) and G1212 (*aleu2 atrp1::ALEU2* – [16]), were fused by the spheroplast technique [23]. The resulting heterozygous diploid *A. adeninivorans* fp1006 (*aleu2 aleu2 AADE2 aade2::ALEU2 ATRP1 atrp1::ALEU2*) was then induced with benomyl to undergo mitotic segregation as described by Samsonova et al. [23]. Since the two auxotrophic markers are located on different chromosomes (*ATRP1* on chromosome 2, *AADE2* on chromosome 1) they segregate independently of each other. The double mutant that was selected (*aleu2 atrp1::ALEU2 aade2::ALEU2*) and designated *A. adeninivorans* MS1006, was used as the host for the introduction of LAC4-6H expression modules (see next section).

2.4. Construction of *LAC4* expression plasmids

The *LAC4*-gene fused with the His-tag encoding sequence at the 3′-end (*LAC4-6H*) was expressed in *A. adeninivorans* G1212 and MS1006. The open reading frame (ORF) was amplified by PCR from chromosomal DNA of *K. lactis* JA6 [24] using primers that incorporated flanking *Eco*RI and *Not*I cleavage sites (primer 1,5′- C**GAATTC**ATGTCTTGCCTTATTCCTGA -3′, nucleotide positions 1-20, *Eco*RI restriction site in bold type; and primer 2,5′- ATATTA-**GCGGCCGC**CTATTAGTGGTGGTGATGATGGTGTTCAAAAGCGAGAT-CAAAC -3′, nucleotide positions 3057-3078, *Not*I restriction sites in bold type). The amplified *Eco*RI-*Not*I *LAC4-6H* gene fragment, which corresponds to the complete ORF with a 3′-fused sequence encoding hexahistidin-tag, was inserted into the plasmid pBS-TEF1-PHO5-SS (flanked by *Spe*I-*Sac*II restriction sites) between the *A. adeninivorans* derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator [13].

Construction of plasmids with one LAC4-6H expression module required the insertion of *TEF1* promoter-*LAC4-6H* gene-*PHO5* terminator flanked by *Spe*I-*Sac*II restriction sites into the plasmid Xplor2.2 or Xplor2.4 to generate Xplor2.2-TEF1-LAC4-6H-PHO5 and Xplor2.4-TEF1-LAC4-6H-PHO5. The 25S ribosomal DNA (rDNA) target sequences are interrupted by the selection marker module (*ALEU2* promotor-*ATRP1m* or *AADE2*) and *Eco*47III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion [22].

Construction of plasmids bearing two β-galactosidase expression modules, required *Mlu*I restriction sites to be added: one behind the *Sac*II restriction side and the other in front of the *Spe*I restriction side in the *TEF1* promoter-*LAC4-6H* gene-*PHO5* terminator fragment by PCR (primer 3, 5′- TATAC-TAGTTGACTACGCGTCTCGACTTCAATCTATAATCAGTC -3′, primer 4, 5′- TATACTAGTTAACTCCGGACTCGACTTCAATCTATAATCAGTC -3′, primer 5, 5′- TATACCGCGGCGGCCCCAGCTTGCATGCCTGCAGA-3′, primer 6, 5′- GGATCCGCGGCCGAACGCGTAGCTTGCATGCCTGCA-GATTTTAATC -3′). The resulting fragments were inserted into Xplor2.2 to get Xplor2.2-*Spe*I-TEF1-LAC4-6H-PHO5-*Mlu*I-*Sac*II and Xplor2.2-*Spe*I-*Mlu*I-TEF1-LAC4-6H-PHO5-*Sac*II. Expression module was cut out from Xplor2.2-*Spe*I-*Mlu*I-TEF1-LAC4-6H-PHO5-*Sac*II by *Mlu*I/*Sac*II and inserted into the vector Xplor2.2-*Spe*I-TEF1-LAC4-6H-PHO5-*Mlu*I-*Sac*II.

2.5. Assay for determination of β-galactosidase activity

The assay for the determination of the β-galactosidase activity was performed in 50 mM sodium acetate buffer pH 6.5 containing the substrate *para*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-D-gal), 1 mg ml^{-1}. The reaction was started by the addition of the enzyme solution. After 10 min incubation at 37 °C with shaking,

the reaction was stopped with 200 g l^{-1} K_2CO_3 (half of the reaction volume). It was centrifuged for 10 min at 16,000 × g and the absorption of the supernatant was measured at 405 nm.

The concentration of the hydrolyzed *p*NP-β-D-gal was determined from a *p*NP/K_2CO_3 calibration curve.

One Unit (1 U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol *p*NP by the hydrolysis of *p*NP-β-D-gal per min at 37 °C, pH 6.5.

2.6. Determination of molecular mass and K_M of β-galactosidase

The determination of the β-galactosidase molecular mass was done by gel filtration using Superdex™ 200 (Amersham Biosciences, UK). The flow rate was 1 ml min^{-1} and fractions of about 1 ml were collected for 152 min (buffer: 50 mM sodium phosphate pH 6.5 + 0.15 M NaCl). A calibration curve was constructed using bovine serum albumin, ovalbumin, catalase and alcohol dehydrogenase as standards.

The K_M value for the substrate *p*NP-β-D-gal was determined using the Tate and Reynolds method [25]. The solution was buffered at pH 6.5 with 50 mM sodium acetate buffer, containing 10 mM α-cyclodextrin. The reaction was followed for two min and the increase in absorbance from the complex formation was recorded. From these data, enzyme kinetics were calculated, and Michaelis–Menten and Hanes plots were drawn. Determinations were done in duplicate. The extinction coefficient of *p*NP in the buffer was determined via Beer–Lambert equation ($A = \varepsilon \cdot c \cdot d$, where A = absorbance, b = path length (cm) and c = concentration (M)).

The β-galactosidase concentration was determined using a Coomassie stained SDS-PA gel for the calculation of k_{cat} [26].

2.7. Protein analysis

SDS-PAGE and Western analyses were performed as described by Kunze et al. [26]. Western blots were treated with an anti-His-tag specific primary antibody produced in rabbits (Antibodies online GmbH, Germany) and a goat anti-rabbit IgG alkaline phosphatase conjugate (BIO-RAD, USA), and subsequently stained by incubation with NBT/BCIP substrate (Roche Diagnostics, Switzerland).

The dye-binding method of Bradford [27] was used for protein quantification (BIO-RAD, USA), with bovine serum albumin used as the standard.

2.8. TLC for the analysis of lactose, glucose and galactose

The analysis of lactose, glucose and galactose in the supernatant of different yeast strains was done by thin-layer chromatography (Polygram SIL G/UV_{254} activated by 0.1 M boric acid and then heated to 130 °C for 30 min) using acetic acid and n-butanol, 1:1, as the solvent and 1% 4-methoxybenzaldehyd, 4% sulphuric acid, 95% ethanol for development.

2.9. Enzymatic synthesis of benzyl β-D-gal by transgalactosylation

The enzymatic synthesis of benzyl β-D-gal by transgalactosylation was done in a total volume of 500 μl. Lactose was dissolved in the purified enzyme preparation (0.1 U) and water. The reaction was started by the addition of benzyl alcohol in a ratio of water: benzyl alcohol 9:1 (v/v). It was incubated at 35 °C with shaking for one day. Then the sample was centrifuged for 10 min, 16,000 × g and enzyme activity was stopped by heating the supernatant for 5 min at 95 °C. Other solvents (acetone, acetonitrile, tert. butanol or dioxane) if present, were evaporated until only the benzyl alcohol was left. Methanol was then added to give a total volume of 500 μl. The sample was centrifuged again and the supernatant was analyzed by HPLC with a UV-detector (column: Kromasil 100 C18, MZ Analysentechnik, Mainz, Germany, 150 × 3.0 mm, S: 5 μm, mobile phase: acetonitrile and water with 11.53 g l^{-1} phosphoric acid, gradient: 0–5 min, 5% acetonitrile, 5–33 min 5–95% acetonitrile; 33–38 min 95% acetonitrile; 38–48 min 5% acetonitrile, flow: 0.4 ml min^{-1}, temperature: 24 °C). Ethyl benzoate in methanol was used as an internal standard for quantification.

2.10. Selective hydrolysis of allyl α-D-gal and allyl β-D-gal

4 g l^{-1} of allyl α-D-gal and allyl β-D-gal was incubated with 1 U ml^{-1} enzyme in a total volume of 625 μl at 37 °C with shaking for 24 h. A sample was removed at various times and the reaction stopped by heating for 5 min at 95 °C. It was mixed with an internal standard (glycerin, 50 mg ml^{-1}) and analyzed with an HPLC fitted with a refractive index detector (column: YMC-Pack Polyamine II, YMC, 250 × 4.6 mm, S: 5 μm, 12 nm, mobile phase: acetonitrile and water, 85:15, flow 1 ml min^{-1}, temperature: 24 °C).

3. Results

3.1. Generation of a β-galactosidase producing yeast strain

The Xplor®2 transformation/expression platform was used for the generation of a β-galactosidase producing yeast strain because it allows the construction of resistance marker-free transformants [14]. The system is based on a bacterial vector backbone, with yeast selection markers and expression modules inserted between two 25S rDNA segments. After the construction of the plasmids in *E. coli*, all bacterial sequences are removed by *Asc*I and *Sbf*I restriction. The choice of the restriction enzyme determined whether the rDNA fragment flanks the expression cassette (*Asc*I) or not (*Sbf*I), so that the β-galactosidase gene can be integrated in the yeast genome as a Yeast rDNA Integrative Cassette (YRC) or a Yeast Integrative Cassette (YIC).

The first step in producing a β-galactosidase producing yeast strain was to ligate one or two of the amplified *LAC4-6H* genes with ATRP1m and AADE2 selection marker modules (Fig. 1). Linearized DNA containing one or two LAC4-6H expression modules and the ATRP1m selection marker module without rDNA sequences (YIC102-LAC4-6H, YIC102-2LAC4-6H) or with rDNA sequences (YRC102-LAC4-6H, YRC102-2LAC4-6H) were integrated in the genome of the *atrp1* auxotrophic mutant strain *A. adeninivorans* G1212. In addition, the double mutant strain *A. adeninivorans* MS1006 [*atrp1, aade2*] was co-transformed with YRC102-2LAC4-6H and YRC104-2LAC4-6H or YIC102-2LAC4-6H and YIC104-2LAC4-6H. Thus, recombinant yeast strains containing one, two or four copies of the LAC4-6H expression module were generated.

All cassettes were successfully transformed in both yeast strains with around 96 transformants per cassette. After stabilization of the transgenic yeast strains by passaging (see Materials and methods), no loss of YRCs or YICs was observed after cultivation on non-selective medium for a period of 30 days.

3.2. Expression of LAC4-6H gene in A. adeninivorans

Transgenic yeast strains carrying one LAC4-6H expression module were grown in YMM-glucose-$NaNO_3$ at 30 °C for 48 h. Since LAC4-6H does not contain an obvious secretion sequence, the recombinant protein should be localized intracellularly. Whole-cell extracts were analyzed for the presence of recombinant Lac4-6hp activity in the initial screen by testing the supernatant of whole cells, disrupted cells and the supernatant of the disrupted cells for β-galactosidase activity. Most activity was detected in the supernatant of the disrupted cells, suggesting the enzyme is located intracellularly and is soluble. However, one third of the activity was also detected in the disrupted cells (activity of the intracellular soluble fraction excluded).

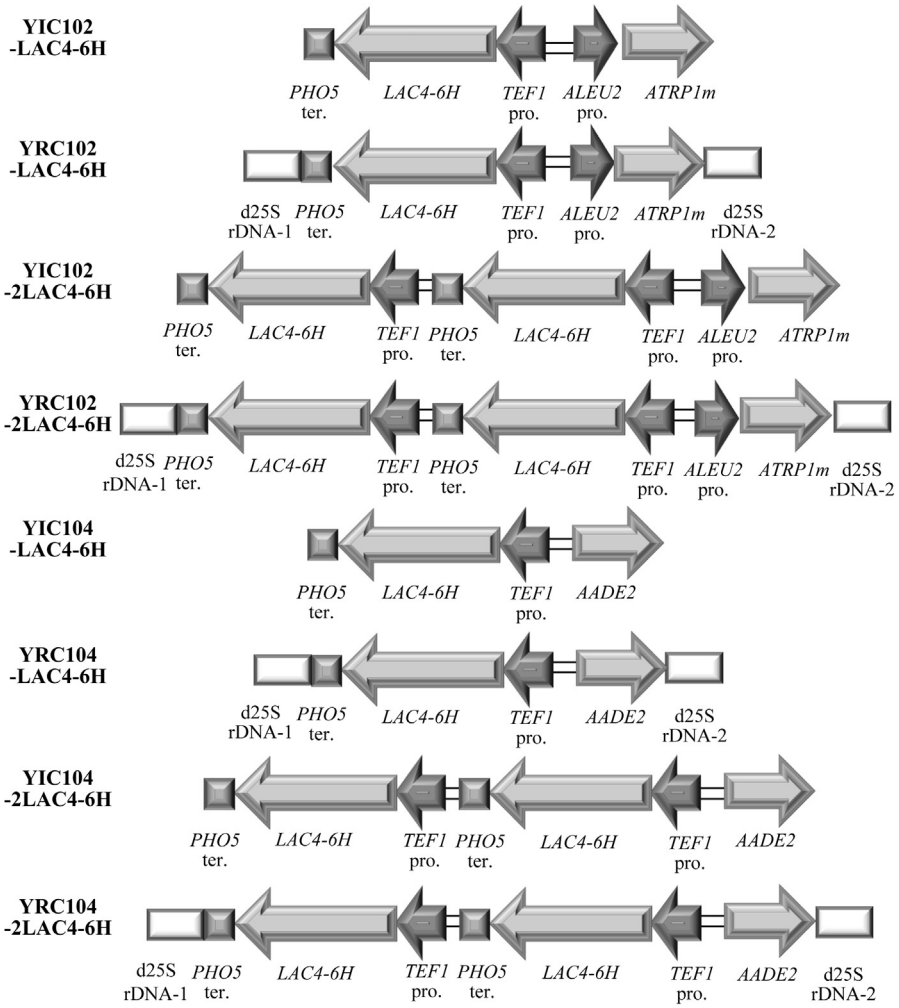

Fig. 1. Physical maps of the YRCs and YICs used for transformation of *A. adeninivorans* G1212 and MS1006. Cassettes contain the selection marker *ATRP1m* fused to the *ALEU2* promoter (YIC102-LAC4-6H, YRC102-LAC4-6H, YIC102-2LAC4-6H, YRC102-2LAC4-6H) or the selection marker *AADE2* (YIC104-LAC4-6H, YRC104-LAC4-6H, YIC104-2LAC4-6H, YRC104-2LAC4-6H) together with one or two copies of the expression module *TEF1* promoter – *LAC4-6H* gene – *PHO5* terminator. In addition YRCs (*Asc*I fragments) are flanked by 25S rDNA sequences for targeting, whereas YICs (*Sbf*I fragments) contain only the selection marker and expression modules.

Highest activities of 0.2 to 0.3 U per mg protein were achieved by transforming YRC102-2LAC4-6H and YRC104-2LAC4-6H and YRC102-LAC4-6H and YRC104-LAC4-6H in *A. adeninivorans* MS1006. The activity of the transformants increased with the number of expression modules (data not shown).

One transgenic *A. adeninivorans* strain with high recombinant Lac4-6hp activity was analyzed in a time-course experiment. The strain was cultured in different media and temperatures for 96 h, i.e. under conditions that allow high-level expression from the *TEF1* promoter and where cultures enter the stationary phase after approx. 24 h (Fig. 2). Cell extracts were then analyzed for the presence of Lac4-6hp.

In all culture conditions used, maximal dry weight was achieved after 24 h and remained constant until the end of the experiment. In contrast to negative control strains (*A. adeninivorans* G1212/YRC102 without LAC4-6H expression modules), recombinant enzyme activity was detected in all transformants with LAC4-6H expression modules. Culture conditions however influenced enzyme production with no enzyme activity detectable in YMM-glucose-$NH_4H_2PO_4$ (Fig. 2 a), while activities of up to 110 U l^{-1} (7 U g^{-1} dcw) were detected in YMM-glucose-$NaNO_3$ grown cells (Fig. 2 b). However, transformants grown on a rich medium (YEPD) produced 90 U l^{-1} (4 U g^{-1} dcw) after 48 h with no increase on further incubation (Fig. 2 c). Increasing the cultivating temperature from 30 to 37 °C (Fig. 2 d) and the addition of more or less glucose (Fig. 2 e, f) did not increase the yield.

3.3. Growth of the β-galactosidase producing yeast strain on lactose

A. adeninivorans cannot grow on media which contains lactose as the sole carbon source. It was expected that transformants would however grow on lactose because β-galactosidase will hydrolyse lactose to glucose and galactose.

Growth of the yeast strain MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H was observed in liquid culture with lactose or glucose whereas the control strain G1212/YRC102 only grew on glucose. The optical density at 600 nm increases for both strains in glucose containing YMM-glucose-$NaNO_3$ 50-fold and is stable for several days. In contrast, growth is not detectable in the control strain on lactose media at seven days while the MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H population doubled reaching its maximum at three days (Fig. 3 a).

The supernatant from the lactose medium was analyzed for lactose, glucose and galactose by thin-layer chromatography (Fig. 3 b). Lactose was present in the supernatant of the control strain indicating it was not metabolized, whereas for MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H strain, lactose concentration decreased to zero

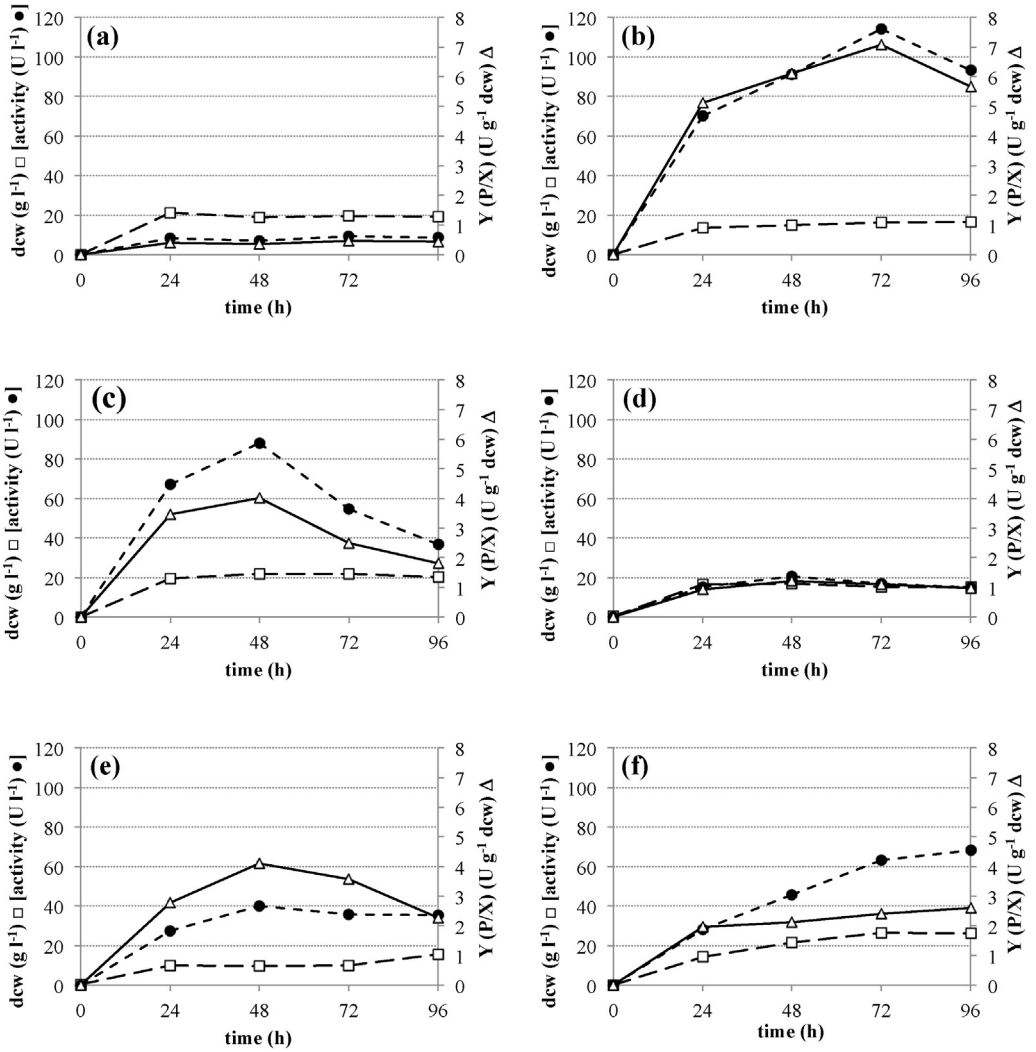

Fig. 2. Time-courses of expression of *LAC4-6H* by transgenic strains of *A. adeninivorans* grown in various media. Transformant MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H was cultured in shake-flasks for 96 h in (a) YMM-glucose-$NH_4H_2PO_4$ at 30 °C, (b) YMM-glucose-$NaNO_3$ at 30 °C, (c) YEPD at 30 °C, (d) YMM-glucose-$NaNO_3$ at 37 °C, (e) YMM-glucose (10 g l^{-1})-$NaNO_3$ at 30 °C, (f) YMM-glucose (50 g l^{-1})-$NaNO_3$ at 30 °C.

after three days. Glucose and galactose were not detectable in the supernatant indicating that they had been metabolized by the cells.

Analysis of the *Arxula* genome (manuscript in preparation), revealed that a gene for lactose permease is not present, suggesting that there is no mechanism available for the transport of lactose into the cell as there is in *K. lactis*. In this species, lactose is transported into the cell and cleaved intracellularly by Lac4p to glucose and galactose. However, the existence of another lactose transporter cannot be excluded [28].

3.4. Determination of the optimum temperature and pH

The optimum temperature and pH of the β-galactosidase activity was investigated using cells grown for 24 h on YMM-glucose-$NaNO_3$.

The soluble intracellular fraction was tested with the following buffers: 50 mM sodium acetate (pH 3.5 to 7), 50 mM sodium citrate (pH 3.5 to 6.5), 100 mM sodium phosphate (pH 6 to 8), 100 mM TRIS-HCl (pH 7 to 9) to determine the optimal pH. Highest activity was detected at pH 6 to 7 in sodium acetate. The optimum temperature at pH 6.5 was 40 °C, with 80% of activity present at 35 °C and 42 °C.

Table 2
Kinetic constants of β-galactosidase Lac4-6hp synthesized *in A. adeninivorans* MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H for *p*NP-β-D-gal.

K_M in mM	7.42
k_{cat} in s^{-1}	137.47
k_{cat}/K_M in mM^{-1} s^{-1}	18.53

3.5. Purification of β-galactosidase

The purification of the β-galactosidase was necessary to avoid cross-reactions, which would disrupt the transgalactosylation of

Table 1
Summary of β-galactosidase purification.

Step	Protein in mg	A_{total} in U	Yield in %	A_{sp} in U ml^{-1} of protein	Fold purified
Crude extract	133	12.8	100	0.096	1
Precipitation	94.3	9.3	72.7	0.099	1
Sephadex G25	54.1	8.3	64.8	0.153	1.6
Hydroxylapatite	14	7.2	56.3	0.514	5.4

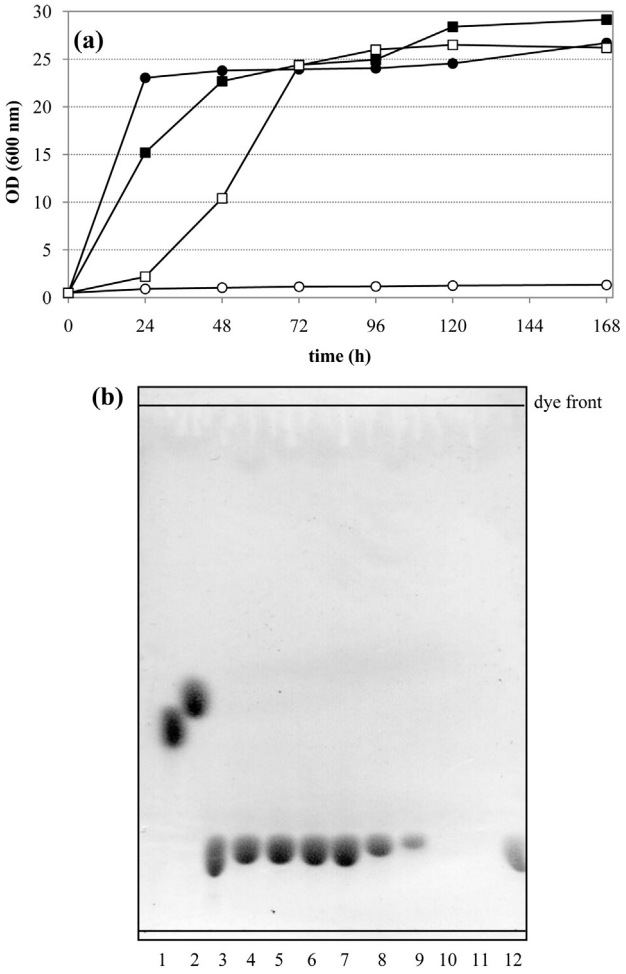

Fig. 3. Growth of *LAC4-6H* expressing *A. adeninivorans* and control strain G1212/YRC102 on glucose and lactose containing media. (a): Transformant MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H (■, □) and control strain (●, ○) were cultured in shake flasks for 168 h in YMM-glucose-$NaNO_3$ (■, ●) and YMM-lactose-$NaNO_3$ (□, ○). At the specified times, the optical density at 600 nm was determined. (b): (1) reference galactose, (2) reference glucose, (3) reference lactose, (4–6) control strain cultured in shake flasks for 24, 48 and 72 h in YMM-lactose-$NaNO_3$, (7–9) MS1006/YRC102-LAC4-6H - YRC104-LAC4-6H cultured in shake flasks for 24, 48 and 72 h in YMM-lactose-$NaNO_3$, (10) YMM-lactose-$NaNO_3$.

lactose with benzyl alcohol. A conventional purification method was used because the purification by Ni-NTA (Novagen, UK) via His-tag was not successful, probably due to steric hindrance.

Cells were harvested by centrifugation (5 min, 3000 × g) and suspended in 1 ml 50 mM sodium acetate buffer, pH 6.5, per g wet weight. Cells were disrupted by grinding under liquid nitrogen and the cell debris was centrifuged (20 min, 6000 × g) to remove all insoluble cellular components. Host proteins were removed from the crude extract by fractionated precipitation using ammonium sulphate from 0 to 50% at 0 °C. The salt was added over 10–15 min and it was incubated for 2 h, before the precipitated proteins were separated (30 min, 3500 × g). Seventy percentage of ammonium sulphate was added to the supernatant to precipitate the β-galactosidase. The centrifuged protein pellet, which contained most of the β-galactosidase activity, was solubilized in 1/25 the volume of the crude extract. Further purification with Sephadex-G25 (Pharmacia, Sweden) was used to eliminate most of the salts and small proteins. The pooled fractions with β-galactosidase activity were loaded onto a hydroxylapatite column (CHT5-I, Bio-Rad, USA) with potassium phosphate running buffer, pH 6.5. The enzyme was eluted by increasing the buffer concentration (5–500 mM) and 1 ml

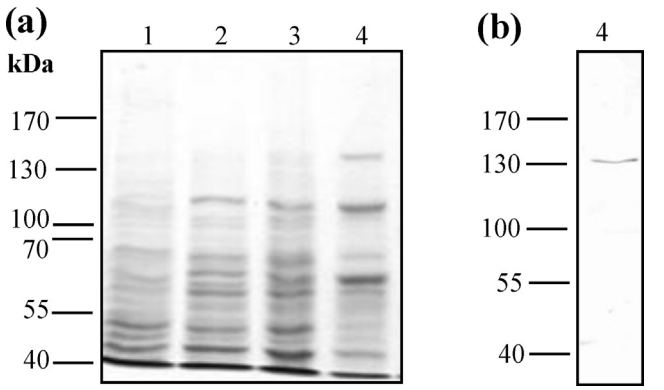

Fig. 4. Purification of Lac4-6 hp with ammonium sulphate precipitation, Sephadex G-25 and hydroxylapatite. (a) The samples containing 20 μg of protein were fractionated by electrophoresis on 8% SDS-PA gel. The different steps of the purification are shown: (1) crude extract, (2) precipitation, (3) Sephadex-G25 and (4) hydroxylapatite. (b) Western Blot of the purified enzyme preparation (4) of the SDS-PA gel shown in Fig. 4 a.

fractions were collected over 73 min (flow of 1 ml min^{-1}). The yield of the purification process was 56.3% of the crude extract protein content (Table 1).

Although ammonium sulphate precipitation concentrated the enzyme, the increase in the specific activity was only 3%, which was caused by loss of the enzyme by as much as 27.3%. However, desalting the enzyme preparation with Sephadex G25 increased the activity from 0.099 U mg^{-1} to 0.153 U mg^{-1} protein. The most efficient purification step was hydroxylapatite, which eliminated many proteins and increased the specific activity to 0.514 U mg^{-1} protein (5.4 fold) and resulting in a band of approximately 130 kDa in an SDS-PA gel (Fig. 4). The identity of this band was confirmed with a Western blot with rabbit anti poly-histidine antibody. The *LAC4*-gene product has a calculated molecular mass of 118 kDa, adding to the evidence that the band is the product of the over-expressed gene.

3.6. Effects of cations

Phosphate and acetate with a monovalent cation are frequently used to buffer enzyme reactions, e.g., sodium acetate, sodium phosphate and potassium phosphate. Here sodium acetate gave the highest enzyme activity. The reaction of the enzyme with *p*NP-β-D-gal in the presence of 1.5 mM Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Mo^{2+}, Zn^{2+} was performed to determine the influence of these ions on β-galactosidase activity. Ca^{2+} and Cu^{2+} decreased the activity whereas Co^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} increased the activity by 130 to 200%. Mo^{2+}, Fe^{3+} and Ni^{2+} had no influence.

3.7. Parameters of the purified β-galactosidase

The approximate molecular mass of the native β-galactosidase was obtained by gel filtration on a Superdex™ 200 column. Beta-galactosidase activity was confined to a peak with a M_r of 160–450 kDa (maximum activity at 300 kDa). These results suggest that the main active form of the enzyme is dimeric.

The kinetic constants of the purified protein were determined photometrically, as described in Materials and methods using *p*NP-β-D-gal as substrate. The apparent k_{cat} and k_{cat}/K_M values were estimated from a SDS-PA gel of the protein to determine the concentration of the enzyme. These constants are summarized in Table 2.

Cross reactivity with other substrates such as *p*NP-α-D-gal, *p*NP-β-D-glc and *p*NP-α-D-man were eliminated by purification (data not shown).

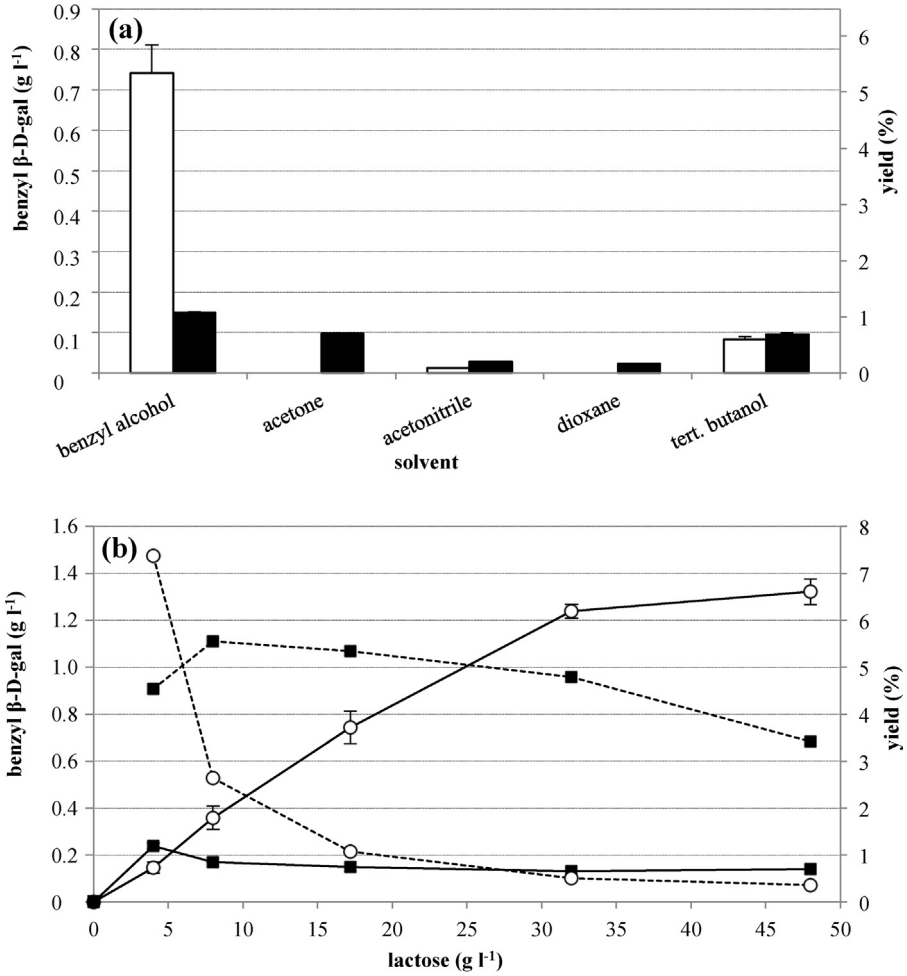

Fig. 5. Optimization of the enzymatic synthesis of benzyl β-D-gal by partially purified β-galactosidase from *A. adeninivorans*. (a) Enzymatic synthesis of benzyl β-D-gal by 0.1 U β-galactosidase from *A. adeninivorans* (white) and *E. coli* (black) at 35 °C in different reaction systems (10% water, 58% solvent, 32% benzyl alcohol) were tested. (b) Enzymatic synthesis of benzyl β-D-gal by 0.1 U β-galactosidase from *A. adeninivorans* (white) and *E. coli* (black) at 35 °C in benzyl alcohol: water, 9:1 with different lactose concentrations were tested. Solid lines show the synthesized benzyl β-D-gal concentration, dashed lines the yield of the product related to the used lactose concentration.

3.8. Enzymatic synthesis of benzyl β-D-gal by transgalactosylation

Different parameters such as reaction system and substrate concentration were optimized for the synthesis of benzyl β-D-gal by transgalactosylation from lactose by the *Arxula* β-galactosidase. Commercially available β-galactosidase lacZp from *E. coli* was used as a comparison.

Different reaction systems were investigated to obtain optimal conditions for the synthesis of benzyl β-D-gal. First the acceptor alcohol, benzyl alcohol, itself was used and second, solvents such as acetone, acetonitrile, 1,4-dioxan and *tert.* butanol, which increase the solubility of lactose were introduced (10% water, 58% solvent + 32% benzyl alcohol) (Fig. 5.a). The highest concentration of benzyl β-D-gal produced by both enzymes was however found with benzyl alcohol only. 0.75 g l^{-1} benzyl β-D-gal was synthesized with 0.1 U yeast β-galactosidase while 0.1 U *E. coli* β-galactosidase yielded 0.15 g l^{-1} in 24 h. The partial replacement of benzyl alcohol by different solvents to improve the solubility of lactose reduced the benzyl β-D-gal concentration drastically or completely for the *A. adeninivorans* β-galactosidase and a smaller decrease in the product yield was seen with the *E. coli* enzyme.

Different concentrations of lactose were tested to determine the effect on yield (Fig. 5b). The concentration of benzyl β-D-gal could be increased from 0.74 to 1.3 g l^{-1} by increasing lactose concentration to 40 g l^{-1} however the maximum yield of 5.5% was achieved at 8 g l^{-1}.

In contrast raising lactose concentration did not increase the product concentration for the *E. coli* enzyme.

3.9. Selective hydrolysis of the anomeric mixture allyl α-D-gal and allyl β-D-gal

The enzyme also selectively hydrolyses allyl β-D-gal in the anomeric mixture of allyl α- and β-D-gal. A mixture of the two anomers at a concentration of 4 g l^{-1} was incubated with 1 U ml^{-1} purified β-galactosidase (Fig. 6). The allyl β-D-gal anomer was selectively hydrolysed by the purified enzyme preparation with no cross reactivity to the α-anomer. In 60 min 30% of the β-anomer was hydrolysed by the enzyme. After 24 h most of the allyl β-D-gal was hydrolysed, whereas allyl α-D-gal remained at its initial concentration.

4. Discussion

In this communication, the over-expression of the *K. lactis* his-tagged β-galactosidase gene *LAC4* in the yeast *A. adeninivorans*, and its potential for the enzymatic synthesis of benzyl β-D-gal is described.

The Xplor®2 transformation/expression platform [14] enables the integration of the gene as yeast ribosomal DNA (rDNA)

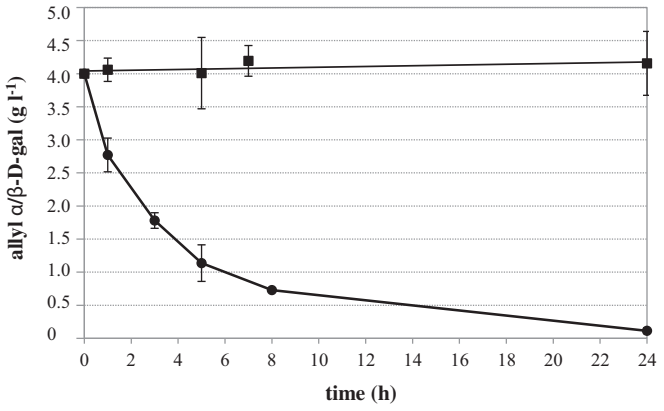

Fig. 6. Selective hydrolysis of allyl α-D-gal and allyl β-D-gal mixture by 1 U ml^{-1} β-galactosidase from *A. adeninivorans*. The reaction was done in 50 mM sodium acetate buffer, pH 6.5, containing allyl α-D-gal (■) and β-D-gal (●), 4 g l^{-1}. A sample was taken at various times and the reaction stopped by heating. It was analyzed by HPLC as described in Materials and methods.

integrative expression cassettes (YRCs) and yeast integrative expression cassettes (YICs) with a selection marker and one, two or four expression modules in the genome of auxotrophic *A. adeninivorans* strains. A yeast strain was created that produces around 100 U l^{-1} cell culture (7 U g^{-1} dcw) in shake flasks. In comparison, González Siso [29] produced 500 to 700 U ml^{-1} batch culture of *K. lactis* by fermentation on milk whey. They defined 1 U as hydrolysis of 1 μg *ortho*-nitrophenyl β-D-gal. If this value is converted to the unit definition used here, it is around 2000 U l^{-1} cell culture. However, in general, there is a 10 to 100-fold increase in the production of an overexpressed gene on transfer from shake flask to bioreactor and it is expected that the yield of β-galactosidase would increase similarly. Moreover, the *LAC4*-gene can be cloned two more times in the Xplor 2.2 and 2.4 vectors so that vector can contain four expression modules resulting in the gene integrating up to eight times in the genome of *A. adeninivorans* MS1006, which would lead to a higher expression level [14]. The substitution of the promoter with a partially deleted promoter could also increase the β-galactosidase-productivity of the yeast strain [16]. Because of *Arxula*'s capacity to use a broad range of substrates, this strain should offer an inexpensive method to produce the enzyme.

Enzyme localized in the intracellular soluble fraction (70%) was used for the determination of optimal pH and temperature. Maximal hydrolysis of *p*NP β-D-gal was found in sodium acetate buffer, pH 6.5 which is similar to the *K. lactis* β-galactosidase, which shows highest activity at around pH 7 [30,31]. The *E. coli* enzyme has the same pH optimum, however an increase or decrease in pH of about 0.5, halves the activity [32]. This is due to local changes of the charged amino acids within the protein at pH 6.5 [33]. In contrast, the enzyme from *A. adeninivorans* shows almost the same activity from pH 6.5 to 7.5, which means that there could be some structural differences in the protein, perhaps caused by the C-terminal His-tag.

K. lactis enzyme has an optimum temperature of around 35 °C, which is contrast to the enzyme produced in *Arxula* and in *E. coli* where the optimal temperature is around 37 to 40 °C [32].

The concentration of Lac4-6hp precipitated with ammonium sulphate, desalted with Sephadex G25 and hydroxyl apatite increased 5.4 fold to 0.514 U mg^{-1} protein.

Becerra et al. [34] purified the β-galactosidase of *K. lactis* by gelfiltration, ion exchange chromatography and ultrafiltration and achieved an 11-fold concentration, whereas Dickson et al. [30] used centrifugation and precipitation steps with DEAE Sephadex and hydroxylapatite, (as used here) and got an 80-fold concentration to 139,000 U mg^{-1} protein. Although these purifications were much more effective than the method used here, there were more purification steps, resulting in higher activity losses. Our enzyme was however sufficiently pure to permit its use for enzymatic synthesis.

The enzyme, identified by Western blot with anti-poly Histidine-antibody, had a molecular mass of around 130 kDa which was more than the expected 118 kDa calculated from the amino acid sequence. These results however agree with those of Becerra et al. [34] and Dickson et al. [30] who identified major bands around 135 and 124 kDa in SDS-PA gel and concluded that the enzyme exists as dimer from determination of the native molecular mass [27,32]. They and others also found that tetramers of the enzyme are possible [30,34–36].

The activity of the *K. lactis* β-galactosidase is stimulated by divalent cations as Mg^{2+} and Mn^{2+}. In contrast Ca^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} inhibit the hydrolysis of *p*NP β-D-gal. There was no influence on activity by Co^{2+} and Fe^{2+} [30,37]. *A. adeninivorans* β-galactosidase responded similarly with the exceptions that Ni^{2+} does not influence activity, while Co^{2+} and Zn^{2+} double the rate of the hydrolytic reaction.

The Michaelis–Menten constant of the *A. adeninivorans* enzyme is around 7.42 mM for the hydrolysis of the substrate *p*NP-β-D-gal. A K_M-value for the enzyme from *K. lactis* was not found, however others have determined the K_M-value for *o*NP β-D-gal, which is 1.5 mM lower than that of the recombinant biocatalyst [30,31] indicating that the *K. lactis* β-galactosidase has a higher affinity for *o*NP β-D-gal, than the enzyme from *A. adeninivorans*. The apparent k_{cat} and k_{cat}/K_M of the recombinant enzyme are 137.47 s^{-1} and 18.53 mM^{-1} s^{-1}.

The β-galactosidase produced by *A. adeninivorans* and the commercially available enzyme from *E. coli*, were tested for the synthesis of benzyl β-D-gal via transgalactosylation with lactose as substrate. The reaction was carried out in benzyl alcohol as solvent with 10% water. After 24 h at 35 °C, the level of benzyl β-D-gal synthesized by the yeast enzyme was around five times higher than it was with the *E. coli* enzyme. This is possibly due to the reaction conditions, because some enzymes need much more water to remain active [38,39].

The specificity of the enzyme was high without cross reactivity to glucose derivatives or α-galactosides.

The replacement of some of the benzyl alcohol with acetone, acetonitrile, dioxan or *tert.* butanol was not successful, although there was a positive effect using *tert.* butanol and acetone if the reaction was done in the absence of water [5]. This effect is, however, not due the solvent but to reducing the water content in the reaction so that the secondary hydrolysis of the benzyl β-D-gal is reduced.

In addition, an increased concentration of lactose doubled the concentration of benzyl β-D-gal produced, although this did not occur with the *E. coli* β-galactosidase. Stevenson et al. [5] demonstrated that the reaction rate of the benzyl β-D-gal synthesis rises if the concentration of lactose increases from 0.5 to 1.5 M with a doubling of the yield after 15 min incubation with *K. lactis* β-galactosidase.

5. Conclusion

The production of the recombinant β-galactosidase was achieved in *A. adeninivorans*. The enzyme can be used effectively for the enzymatic synthesis of benzyl β-D-gal by transgalactosylation of lactose. Optimal production occurs at relatively low enzyme concentrations at 35 °C in benzyl alcohol and water, 9:1, with up to 40 g l^{-1} lactose and results in 1.3 g l^{-1} benzyl β-D-gal. Our results also show that β-galactosidase can be used for the selective hydrolysis of anomeric mixtures such as allyl β-D-gal and allyl α-D-gal.

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