Journal of Biotechnology 210 (2015) 27–37

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Novel high-performance metagenome β-galactosidases for lactose hydrolysis in the dairy industry

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ARTICLE INFO

Article history:
Received 15 May 2014
Received in revised form 5 May 2015
Accepted 23 June 2015
Available online 26 June 2015

Keywords:
β-Galactosidase
Metagenome
Lactose hydrolysis
Lactose-free milk

ABSTRACT

The industrially utilised β-galactosidases from *Kluyveromyces* spp. and *Aspergillus* spp. feature undesirable kinetic properties in praxis, such as an unsatisfactory lactose affinity (K_M) and product inhibition (K_I) by galactose. In this study, a metagenome library of about 1.3 million clones was investigated with a three-step activity-based screening strategy in order to find new β-galactosidases with more favourable kinetic properties. Six novel metagenome β-galactosidases (M1–M6) were found with an improved lactose hydrolysis performance in original milk when directly compared to the commercial β-galactosidase from *Kluyveromyces lactis* (GODO-YNL2). The best metagenome candidate, called "M1", was recombinantly produced in *Escherichia coli* BL21(DE3) in a bioreactor (volume 35 L), resulting in a total β-galactosidase M1 activity of about 1100 $\mu kat_{oNPGal,37\,^\circ C}\ L^{-1}$. Since milk is a sensitive and complex medium, it has to be processed at 5–10 °C in the dairy industry. Therefore, the β-galactosidase M1 was tested at 8 °C in milk and possessed a good stability ($t_{1/2}$ = 21.8 d), a desirably low apparent $K_{M,lactose,8\,^\circ C}$ value of 3.8 ± 0.7 mM and a high apparent $K_{I,galactose,8\,^\circ C}$ value of 196.6 ± 55.5 mM. A lactose hydrolysis process (milk, 40 $nkat_{lactose}\ mL_{milk,8\,^\circ C}^{-1}$) was conducted at a scale of 0.5 L to compare the performance of M1 with the commercial β-galactosidase from *K. lactis* (GODO-YNL2). Lactose was completely (>99.99%) hydrolysed by M1 and to 99.6% (w/v) by *K. lactis* β-galactosidase after 25 h process time. Thus, M1 was able to achieve the limit of <100 mg lactose per litre milk, which is recommended for dairy products labelled as "lactose-free".

1. Introduction

β-Galactosidases (E.C. 3.2.1.23) catalyse the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. One of the main applications is lactose hydrolysis in milk and milk products, where the β-galactosidase cleaves lactose into D-glucose and D-galactose (Pereira-Rodríguez et al., 2012). The enzymatic hydrolysis of lactose is an important biotechnological process in the food industry. The technological and sensorial characteristics of food can be improved by hydrolysing lactose. The hydrolysis of lactose in ice cream, for instance, improves the creaminess significantly. Moreover, the monosaccharides formed by lactose hydrolysis increase the sweetness of the products (Mlichová and Rosenberg, 2006). The enzyme is used in the dairy industry to produce lactose-free milk products, which is a suitable approach to overcome the lactose intolerance problem affecting humans worldwide (Harju et al., 2012). The authorities in Scandinavian countries set the limit of residual lactose content for products labelled as "lactose-free" to below 10 mg lactose per 100 g of product (<0.01% (w/w)). The GDCh (Gesellschaft Deutscher Chemiker e.V.) in Germany also recommends similar criteria for this type of lactose-free declaration (GDCh, 2005). According to these criteria, the lactose content of products with this label must be lower than 100 $mg_{lactose}\ kg^{-1}$ or L^{-1} of the dairy product (<0.01%). Thus, β-galactosidases should be at hand which possess industrially suitable properties at reasonable costs.

A considerable number of studies have been published over the past few decades investigating β-galactosidases from different sources for lactose hydrolysis (Panesar et al., 2006). Generally, the β-galactosidases utilised in the dairy industry were obtained from

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http://dx.doi.org/10.1016/j.jbiotec.2015.06.411

Kluyveromyces spp. or *Aspergillus* spp. and applied for industrial applications mainly as soluble, or rarely as immobilised, enzyme preparations (Husain, 2010; Novalin et al., 2005; Oliveira et al., 2011).

The kinetic properties of β-galactosidases utilised in the food industry still do not fit the processing conditions required optimally (Ansari and Satar, 2012; Harju et al., 2012). They are all, for example, significantly inhibited by the hydrolysis-generated product D-galactose (K_I value is too low), whereas the substrate affinity to lactose is not beneficial (K_M value is not low enough). Therefore, it is of great economic interest to discover β-galactosidases with improved processing characteristics for their application in milk systems.

Only 0.1–1% of the bacterial species in nature are calculated to be cultivable using conventional methods (Handelsman, 2005). Thus, an auspicious source for finding novel β-galactosidases with industrially desirable properties is the metagenome. Metagenomics has been in the spotlight since the 1990s (Handelsman et al., 1998). Briefly, only the DNA is isolated from environmental samples (e.g. soil) instead of living microorganisms. It is cut into shape and cloned into well-known vector/host systems (e.g. *Escherichia coli*) allowing the expression of enzymes from the non-cultivable organisms. This so-called metagenomic approach has proven to be an efficient tool for the discovery of novel biocatalysts (Gans et al., 2005; Lorenz and Eck, 2004). Metagenomic libraries have been screened for a wide range of enzymes, such as lipases, proteases, dehydrogenases, nitrile hydratases and oxidoreductases (Nacke et al., 2011; Steele et al., 2008). A novel metagenome-derived β-galactosidase was found by Wang et al. (2010), who screened a metagenomic library and found a new β-galactosidase which they characterised and tested for lactose hydrolysis in milk at temperatures between 4 and 25 °C over a time period of only 1 h. Thus, no evidence could be presented that this metagenome enzyme was really able to achieve a lactose hydrolysis down to 100 $mg_{lactose}$ L^{-1} milk in a reasonable time.

In literature, the best lactose conversion yields by using free or immobilized enzymes were in the range of 70–99% (Cieśliński et al., 2005; Horner et al., 2011; Nakagawa et al., 2007; Pan et al., 2010). However, the lactose content in milk of less than 100 $mg_{lactose}$ L^{-1} could not be achieved with these hydrolysis yields, since milk contains about 45–50 $g_{lactose}$ L^{-1} and a 99% hydrolysis yield would result in 450–495 $mg_{lactose}$ L^{-1}.

In our study, a metagenomic library from German soil samples was screened for β-galactosidase activity. Novel β-galactosidases were found using a three-step activity-based screening strategy and were expressed in *E. coli*. The best metagenome β-galactosidases were tested for lactose hydrolysis in milk at 8 °C and compared to a commercial reference β-galactosidase from *K. lactis* called GODO-YNL2 (Shusei Co, Ltd., Tokyo, Japan), since this enzyme is often utilised in the dairy industry today. The most promising metagenome β-galactosidase was then characterized concerning process-relevant and kinetic parameters. The enzyme activities and the kinetic parameters were determined with the natural substrate lactose at 8 °C, because it is known that the synthetic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) leads to dissimilar enzyme activities and kinetic parameters (Hildebrandt et al., 2009).

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical reagent grade and purchased from Merck (Darmstadt, Germany), Sigma–Aldrich (Steinheim, Germany), Gerbu (Heidelberg Wieblingen, Germany) or Carl-Roth (Karlsruhe, Germany).

Water was purified by reverse osmosis and passed through a Millipore Mili-Q unit. The commercial reference GODO-YNL2 (Shusei Co, Ltd., Tokyo, Japan) containing the β-galactosidase from *Kluyveromyces lactis* was used for comparison studies.

T4 DNA-ligase and hexokinase/glucose-6-phosphate-dehydrogenase were purchased from Roche Diagnostics (Mannheim, Germany). All restriction enzymes were acquired from New England Biolabs (Frankfurt, Germany). HotStar HiFidelity Polymerase was purchased from Qiagen (Hilden, Germany).

The chromatography resin BioFox 40 IDA_{low} and the Bioline HR glass column (300 × 10 mm) were kind gifts from KNAUER Wissenschaftliche Geräte GmbH (Berlin, Germany).

2.2. Isolation of metagenomic DNA

Genomic DNA from different sampling sites near Zwingenberg (Germany) was prepared, as described in Gabor et al. (2012).

2.3. Metagenome library construction

High-copy plasmid-based metagenome libraries were constructed from these habitat samples. Therefore, DNA fragments of 2–10 kb were obtained by partially digesting metagenomic DNA with *Alu*I. After sizing by agarose gel electrophoresis and electroelution, fragments were ligated into a *Sma*I-digested dephosphorylated pBCS-PvegII vector. This vector (Supplemental S1) is a derivative of the commonly used pBC16 plasmid (Bernhard et al., 1978). Modifications of this vector include the replacement of the tetracycline by a chloramphenicol resistance marker, the addition of a pUC ori and the introduction of the strong constitutive vegII promoter of *Bacillus subtilis* up-stream of the *Nde*I cloning site to allow the initiation of transcription in both *B. subtilis* and *E. coli*. Ligation mixtures were used to transform *E. coli* DH5α (Fisher Scientific) by electroporation. Altogether, 5340 Mb DNA was isolated from several soil samples. The average insert size was 4 kb, which results in a total theoretical number of about 1335,000 clones for the screening.

2.4. Screening for β-galactosidase activity

A three-step activity-based screening strategy was applied to the metagenome clones (Fig. 1).

2.4.1. First screening step using X-Gal agar plates

As described in patent EP2530148 (A1) (Niehaus and Eck, 2012), the metagenome clones were cultivated on LB agar plates containing 12.5 μg mL^{-1} chloramphenicol and 80 μg mL^{-1} 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) for the first screening step. Positive clones with presumed β-galactosidase activity showed a blue colour on the agar plates, which resulted from the hydrolysis of X-Gal.

2.4.2. Second screening step with lactose as a substrate

In a second screening step, the positive clones from the first screening step were selected, cultivated and disrupted, as described in patent EP2530148 (A1) (Niehaus and Eck, 2012). The β-galactosidase activity was measured with lactose as a substrate at 8 °C. Therefore, a lactose solution (160 μL; 0.2 M) in potassium phosphate buffer (0.1 M, pH 6.75) was combined with 40 μL cell-free extract. The reaction was stopped by transferring 95 μL reaction mixture to 190 μL perchloric acid (1 M) after 1 h. The sample was centrifuged (740 × *g*) at 4 °C for 10 min after neutralisation with KOH (2 M). The D-glucose released was determined with the hexokinase/glucose-6-phosphate-dehydrogenase assay (HK assay)

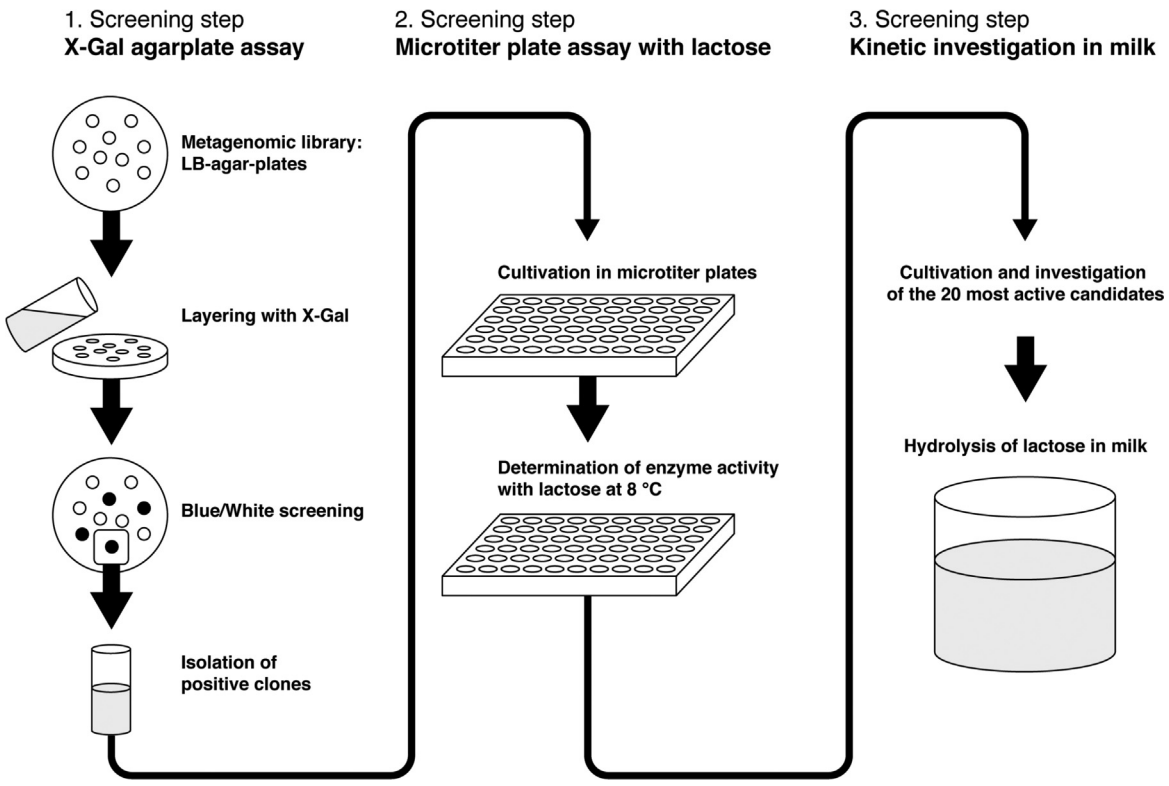

Fig. 1. Scheme of the three-step screening strategy for novel metagenome β-galactosidases.

by photometric measurement (340 nm) in microtiter plates, following the manufacturer's protocol for the D-glucose/D-fructose test kit (R-Biopharm AG, Darmstadt, Germany; product code 10 139 106 035).

2.4.3. Third screening step: Enzymatic lactose hydrolysis in milk

The lactose hydrolysis in milk ("Schwarzwälder H-Weidemilch, 3.8%" obtained from Schwarzwaldmilch GmbH, Germany) was performed with an operating volume of 30 mL at 8 °C in 100 mL flasks. Equal amounts of β-galactosidase activity (0.83 $nkat_{lactose,8\,°C}$ mL^{-1}) from the twenty most active metagenome enzymes from the second screening step and GODO-YNL2 (reference) were added to milk which contained 46 ± 0.5 g L^{-1} lactose and 0.1% (w/v) sodium azide (for the prevention of microbial growth). Samples (0.5 mL) were taken every 24 h. The enzymatic reaction in the samples was stopped with 1 mL of perchloric acid (1 M). After centrifugation of the samples at 7000 × *g* and 4 °C for 10 min (Eppendorf centrifuge 5417R), the samples were neutralised with potassium hydroxide (2 M) and cooled on ice for 10 min. Finally, the samples were filtered through a 0.45 μm PTFE filter and analysed by HPLC (high performance liquid chromatography; see Section 2.12).

2.5. Analysis of β-galactosidase genes

The genes of the six best metagenomes of β-galactosidase (M1 to M6) were sequenced. Database homology research was performed using the "Basic Local Alignment Search Tool" (BLAST, http://blast.ncbi.nlm.nih.gov) for analysis. Furthermore, the pairwise alignment of the metagenome was performed by using the ClustalW2 programme (http://www.genome.jp/tools/clustalw/). The nucleotide sequences of M1 to M6 are available in the GenBank database (M1: KM651981, M2: KM651982, M3: KM651983, M4: KM651984, M5: KJ792469; M6: KJ792470). In addition, the sequences for M1–M4 (M1: 3J_33, M2: 3J_37, M3: 3J_11, M4: 186B1/330C6) have already been published in patent EP2530148 (A1) (Niehaus and Eck, 2012).

2.6. Subcloning of the best metagenome β-galactosidase M1

In order to obtain a higher amount of enzyme, the best metagenome β-galactosidase M1 was subcloned into the expression vector pET20b. The construction of the expression vector was performed using *E. coli* XL1-blue and standard molecular biology techniques (Sambrook and Russell, 2001). The M1 β-galactosidase gene was amplified by polymerase chain reaction (PCR) using the template pBCS-3J-33(M1) with the primers M1_for_*Nde*I (5′-agagtctgcatatgcgacaaaagcttgttta-3′) and M1_rev_*Xho*I (5′-actaatctcgagaatggtgcgaaacgtaaag-3′). The PCR product was digested with *Nde*I and *Xho*I and ligated to a *Nde*I/*Xho*I-treated pET20b vector with a six-histidine tag using T4-Ligase to generate pET20b-M1.

2.7. Production of M1 in a stirred tank reactor

E. coli BL21(DE3) was transformed with pET20b-M1 prior to the recombinant expression. The cultivation was realized using 2YT medium (16 g peptone, 5 g NaCl, and 10 g yeast extract per L) with D-glucose (2% w/v) and ampicillin (100 μg mL^{-1}) in a techfors bioreactor (Infors AG, Bottmingen, Switzerland; operating volume 35 L) at 500 rpm, air gassing *vvm* = 2 (pO_2 > 30%) and pH 7.0, controlled with 12.5% (v/v) NH_4OH and 0.66 M H_3PO_4. The cells were cultivated at 37 °C up to an OD_{600} of 10. Then, the culture broth was cooled to 30 °C and isopropyl-β-D-1-thiogalactopyranoside (IPTG; 0.4 mM) was added to induce the expression of the recombinant protein. Cells were harvested by centrifugation (8000 × *g*, 4 °C, 10 min) after 15.5 h. These cells were used for cell-disruption and purification, as described below.

During cultivation, the bio dry mass was determined gravimetrically. Therefore, samples were centrifuged (10,000 × *g*, 4 °C,

5 min), washed twice with saline and dried at 40 °C and 10 mbar in a RVC 2-33 IR vacuum centrifuge from Christ (Osterode, Germany). The residual D-glucose concentration of the medium was determined with the hexokinase/glucose-6-phosphate-dehydrogenase assay (see Section 2.4.2). The enzyme activity was determined with *o*NPGal as a substrate (see Section 2.9.1).

The expression and purification (see Section 2.8) of M1 was analysed by SDS-PAGE using 8% and 12% polyacrylamide gels, according to the method of Laemmli (1970). The molecular weight marker Protein Ladder (10–250 kDa, New England Biolabs Inc., Ipswich, MA, USA) was used as a reference. Gels were stained with Coomassie Brilliant Blue. The protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as the standard (quadruplicate measurement).

2.8. Purification of the metagenome β-galactosidase M1

After cell-disruption, the β-galactosidase M1 was purified by fractionated ammonium sulphate precipitation. Additionally, M1 was further purified using a Ni^{2+}-affinity chromatography step for the determination of the kinetic constants and the influence of D-glucose and D-galactose on the enzyme activity.

2.8.1. Cell-disruption

A cell suspension (30% w/v) was made in potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM). This suspension was conducted through a bead mill (Dyno®-mill, containing 0.1–0.2 mm glass beads). Afterwards, the suspension was centrifuged (13,000 × *g*, 4 °C, 20 min). The cell-free extract (supernatant) obtained was used for purification of the β-galactosidase M1.

2.8.2. Ammonium sulphate precipitation

In the first purification step, ammonium sulphate was added to the cell-free extract by stirring at 4 °C to obtain a salt saturation of 30% (w/v). After centrifugation (13,000 × *g*, 4 °C, 20 min), the pellet was discarded and more ammonium sulphate was added to the supernatant to obtain a final salt saturation of 45% (w/v). After further centrifugation (13,000 × *g*, 4 °C, 20 min), the supernatant was discarded and the pellet was dissolved in potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM) and dialysed (dialysis tubing cellulose membrane, Sigma Aldrich, Steinheim, Germany) against this buffer at 8 °C for 24 h. This enzyme preparation was used for lactose hydrolysis in milk at 8 °C and for the biochemical characterisation of the M1 (optimal pH, optimal temperature).

2.8.3. Ni^{2+}-affinity chromatography

The ammonium sulphate pellet precipitated was dissolved in potassium phosphate buffer (100 mM, pH 6.75) with NaCl (150 mM) and dialysed against this buffer, as described above. The dialysed solution was used for further purification using an Äkta FPLC system (Äkta FPLC, GE Healthcare, Germany). The flow rate was 1 mL min^{-1}. Eluted protein was detected at 280 nm.

A volume of 20 mL was injected via a Superloop™ (GE Healthcare, Germany) onto a Ni^{2+}-charged BioFox 40 IDA_{low} (KNAUER, Berlin) filled column (Bioline HR glass column (300 × 10 mm), KNAUER, Berlin; 1 column volume (CV) = 4.7 mL) for each chromatography run ($n = 4$). The chromatography resin had been equilibrated previously with buffer A (potassium phosphate buffer (100 mM, pH 6.75) with NaCl (150 mM)). Unbound protein was washed out with buffer A over eight CV. Bound protein was eluted by a linear gradient from 0 to 50% with buffer B (buffer A plus imidazole (500 mM)) over three CV and was held for a further two CV. Finally, the column was cleaned by 100% buffer B over two CV. Fractions (1 mL) were collected during the chromatography process and tested for β-galactosidase activity (*o*NPGal-assay; see Section 2.9.1). Active fractions were pooled and desalted to potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM) using PD-10 columns (GE Healthcare, Germany). This enzyme preparation was used for the determination of the kinetic constants and the influence of D-glucose and D-galactose on the enzyme activity.

2.9. Assays for β-galactosidase activity determination

The β-galactosidase activity was determined by different methods, depending on the needs of the particular experiment. One katal was defined as the amount of enzyme that catalyses the release of 1 mol *o*-nitrophenol from *o*NPGal (*o*-nitrophenyl-β-D-galactopyranoside) per s or the release of 1 mol D-glucose from lactose per s. All assays were performed at least in triplicate.

2.9.1. β-Galactosidase activity using oNPGal as a substrate

During the bioreactor cultivation, the β-galactosidase activity of the cell-free extract was measured at 405 nm with the substrate *o*NPGal at 37 °C. The reaction was performed in the kinetic mode of a spectral photometer containing a temperature-controlled cuvette (Ultrospec 3000, Amersham Bioscience, Freiburg, Germany). An amount of 0.6 mL *o*NPGal solution (50 mM) in potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM) was mixed with 0.5 mL of the same buffer and preincubated at 37 °C for 5 min. The reaction was started with 0.1 mL enzyme solution.

The *o*NPGal assay was modified and performed on a smaller scale prior to the kinetic measurements. A volume of 100 μL *o*NPGal solution (50 mM) in potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM) was mixed with 80 μL of the same buffer and preincubated at 37 °C for 5 min. The reaction was started with 20 μL enzyme solution and incubated at 37 °C for 120 s. The reaction was terminated by adding 200 μL precooled Na_2CO_3-solution (1 M). After centrifugation (13,000 × *g*, 4 °C, 5 min), the absorption was measured at 405 nm.

The effect of D-glucose and D-galactose on the enzyme activity of purified M1 and GODO-YNL2 was determined with a modified *o*NPGal assay. Synthetic milk ultra filtrate (SMUF), according to Pessela et al. (2003), was used to simulate the salt composition of the milk. A volume of 100 μL *o*NPGal solution (50 mM) in SMUF was mixed with 80 μL SMUF containing 25–500 mM of D-glucose, D-galactose or a mixture of both sugars (equimolar: e.g. MIX-50 containing 50 mM D-glucose and 50 mM D-galactose) and preincubated at 37 °C for 5 min. The assay was performed as described above.

2.9.2. β-Galactosidase activity using lactose as a substrate

The lactose assays were performed at different temperatures, depending on the particular experiment. A lactose stock solution (400 mM in $H_2O_{bidest.}$) was used. This solution (600 μL) was mixed with 500 μL potassium phosphate buffer (100 mM, pH 6.75; standard buffer) and $MgCl_2$ (5 mM), and preincubated at the respective temperature for 5 min. The reaction was started with 100 μL enzyme solution. After 90 s, a 100 μL sample of the reaction mixture was taken and transferred into 150 μL of perchloric acid (1 M) in order to stop the enzymatic reaction. After centrifugation (13,000 × *g*, 4 °C, 10 min), 160 μL of the supernatant was added to 55 μL potassium hydroxide (2 M). After a second centrifugation step (13,000 × *g*, 4 °C, 10 min), the D-glucose of the clear supernatant released was quantified enzymatically (HK assay, see Section 2.4.2). This enzyme assay was used for the determination of the kinetic parameters. It is the recommended official method of the "Lebensmittel- und Futtergesetzbuch" (LFGB) in Germany

2.10. Investigations of pH, temperature properties and kinetic constants

The pH and temperature profiles, temperature stabilities and kinetic constants of the β-galactosidases were investigated with lactose as a substrate.

2.10.1. Optimal pH, optimal temperature and temperature stability

The β-galactosidase activity was measured in a pH range between 5 and 9 and in a temperature range between 8 and 60 °C. All buffers for the pH profile had a concentration of 100 mM. The following buffers were used: citrate buffer (pH 5–6), potassium phosphate buffer (pH 6–8) and Tris–HCl buffer (pH 8–9). The temperature profile was determined with potassium phosphate buffer (100 mM, pH 6.75) containing $MgCl_2$ (5 mM). The assay temperature ranged from 8 to 60 °C. In addition, the enzyme solutions were incubated at two different temperatures (8 °C and optimal temperature) for 48 h to determine the temperature stability. The residual enzyme activities of the samples were measured at 37 °C. The data were fitted to first-order plots and analysed, with the first-order rate constants (k) determined by linear regression of ln residual activity versus the incubation time. The activity half-life ($t_{1/2}$) was calculated using the following equation: $t_{1/2} = \ln 2/k$.

2.10.2. Determination of kinetic parameters

The Michaelis–Menten kinetic parameters (K_M and K_I) of the metagenome β-galactosidases M1 (purified) and GODO-YNL2 were determined at 8 °C. The kinetic parameters were calculated by non-linear regression fitting with the Enzyme Kinetics Module of SigmaPlot 12.5 (Systat Software, Inc,. San Jose, CA).

For determining the apparent K_M value in buffer, the lactose concentrations in the reaction mixture ranged from 5 to 200 mM. For determining the apparent K_I value, the galactose concentrations ranged from 100 to 400 mM. Each galactose concentration was tested at five different lactose concentrations (5–200 mM).

The apparent kinetic parameters K_M and K_I were also investigated in diluted milk. A dilution was needed to obtain various appropriate lactose concentrations in the range of 5–62.5 mM in the final reaction mixture. The milk was diluted with H_2O_{bidest}. The D-galactose concentration for the determination of the K_I value ranged between 100 and 400 mM. For this, D-galactose was added to the milk. All kinetic experiments were carried out with β-galactosidase activity ranging between 10 and 20 $nkat_{lactose,8\,°C}$ $mL_{total\ sample}^{-1}$.

2.11. Comparison of the lactose hydrolysis in milk of M1 and GODO-YNL2 on a larger scale

Large-scale lactose hydrolyses were performed in a table bioreactor (Infors AG, Bottmingen, Switzerland) using the metagenome β-galactosidase M1 and GODO-YNL2. The bioreactor was coupled to a cooling circulator (Julabo F12, JULABO Labortechnik GmbH, Seelbach, Germany) and contained an operating volume of 500 mL. The lactose hydrolysis in milk was investigated at 8 °C over 24 h with an enzyme activity equal to 40 $nkat_{lactose,8\,°C}$ mL^{-1}. After 24 h, the temperature was raised to 40 °C for one hour to increase the activity of the β-galactosidases. When this temperature step was included, a residue lactose concentration below 0.1 g L^{-1} was achieved. Samples (0.5 mL) were taken after 0, 1, 4, 10, 24, 24.5 and 25 h and prepared as described above (see Section 2.4.3).

2.12. Quantification of lactose via HPLC

HPLC analysis was carried out on a Spectra System (Thermo Fisher Scientific, Dreieich, Germany) coupled with an evaporative light scattering detector (ELSD, SEDEX 75 SEDERE, France). Three REZEX Ca^{2+} columns (300 × 7.8 mm, 5 μm; Phenomenex Aschaffenburg, Germany) were used in sequence for the resolution of the sugars. Water was used as an isocratic mobile phase. The flow rate was set to 0.5 mL min^{-1}, the injection volume was 20 μL and the total runtime was 55 min.

The HPLC method of Erich et al. (2012) was used for the quantification of lactose at contents below 100 mg L^{-1}. The limit of detection for lactose with this method (UV detection) was estimated at 2 mg L^{-1} and the limit of quantification was 6 mg L^{-1}.

3. Results

3.1. Screening of the metagenomic library for β-galactosidase activity

The screening started with more than one million metagenome clones (theoretical estimation), which were cultivated on LB agar plates containing X-Gal. A blue colour around a bacterial colony indicated potential β-galactosidase activity. This amounted to 354 single colonies. After the second screening step, using lactose as a substrate, the amount of β-galactosidase activity-positive clones was reduced to 97. The third screening step was intended to investigate the performance of the novel metagenome β-galactosidases for their lactose hydrolysis in milk at 8 °C. As described above, equal amounts of β-galactosidase activity (0.83 $nkat_{lactose,8\,°C}$ mL^{-1}) from the twenty most active metagenome enzymes from the second screening step and GODO-YNL2 (reference) were added to milk (30 mL). Six metagenome candidates (M1–M6) showed a better lactose hydrolysis performance in milk than the β-galactosidase reference GODO-YNL2. They achieved lower levels of remaining lactose after a conversion time of 250 h (Fig. 2). The other fourteen candidates (M7–M20; not illustrated in Fig. 2) demonstrated a poorer performance than GODO-YNL2. The host strain *E. coli* DH5α of the metagenome enzymes was cultivated under the same conditions and no background β-galactosidase activity was detected with lactose as a substrate at 8 °C.

3.2. Analysis of the β-galactosidase genes of the best candidates

A BLASTx search in the databases of NCBI (http://blast.ncbi.nlm.nih.gov) exposed that M1–M4 are members of the family 2 glycosyl hydrolases and M5 and M6 are members of the family 1 glycosyl hydrolases. The most similarity to M1 is shown by the β-galactosidase sequence from *Paenibacillus wynnii* (strain JDR-2) with an identity of 77% (M1). M2 and M3 show the highest identity of 97% to the next neighbours, which are the β-galactosidase from *Paenibacillus* sp. FSL H7-0357 (M2) and from *Paenibacillus* sp. FSL R5-192 (M3). The next neighbour to M4 is the β-galactosidase from *Paenibacillus vortex* (74% identity) and to M5, the β-glucosidase from *Paenibacillus stellifer* (73% identity). The sequence of M6 shows an identity of 63% to the next neighbour, *Acaryochloris* sp. CCMEE5410 β-galactosidase. The pairwise alignment of the amino acid sequences of M1–M4 depicts identities between 42.9% (M2/M3) and 66.3% (M1/M4), whereas M5 and M6 depict an identity of 44.3%. However, the novel M1–M6 sequences show quite low identity to other metagenome β-galactosidases, such as ZD410 (highest identity of 17.2% with M6; Wang et al., 2010) and Gal308 (highest identity of 18.6% with M2; Zhang et al., 2013), respectively.

3.3. Production of the metagenome β-galactosidase M1

Since M1 showed the most favourable properties in the comparative milk hydrolysis, only this candidate was investigated further. To characterise and confirm the suitability of M1 for lactose hydrolysis in milk, a larger amount of the enzyme was produced using

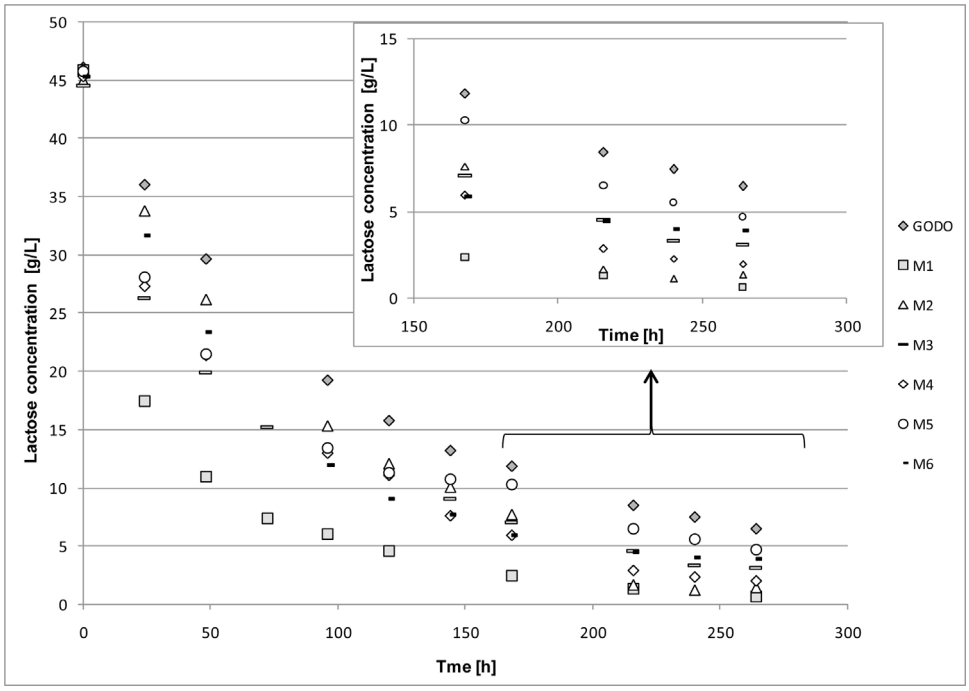

Fig. 2. Hydrolysis of lactose in milk (30 mL scale) with six metagenome β-galactosidases (M1–M6) and the commercial enzyme preparation GODO-YNL2 at 8 °C.

the vector/host system pET20b-M1/*E. coli* BL21(DE3). The cultivation was carried out in 2YT medium with 2% (w/v) glucose in a bioreactor with 35 L working volume. The cultivation is shown in Fig. 3. The recombinant *E. coli* pET20b-M1 was initially grown at 37 °C for 2.5 h. Induction was initiated with IPTG at an OD_{600} of 10 and the temperature was lowered to 30 °C in order to avoid inclusion body formation. The highest β-galactosidase activities were measured in the stationary phase (range: $963.0 \pm 1.9 - 1{,}089 \pm 3$ $\mu kat_{oNPGal,37\,^{\circ}C}\ L_{culture}{}^{-1}$). The cells were harvested after a cultivation time of 15.5 h.

The expression of M1 was investigated by gel electrophoresis experiments (SDS-PAGE). The PAGE gel with samples of the cell-free extracts of *E. coli* BL21(DE3) wild-type and *E. coli* BL21(DE3) pET20b-M1 at various cultivation times is shown in Fig. 4. The over-expressed M1 appeared as a clearly visible protein band with an apparent mass of about 120 kDa (see Fig. 4). This result is in agreement with the in silico mass predicted of 119.77 kDa (including His_6-tag). No protein band of that molecular mass was seen on the SDS-PAGE performed with the protein pellet after cell disruption (data not shown). Thus, no formation of inclusion bodies by the β-galactosidase M1 was observed.

3.4. Purification of M1 and comparison with GODO-YNL2

3.4.1. Partial purification by ammonium sulphate precipitation

An enzyme purification using column chromatography is not common for applications in the food industry due to the high costs of this process step (Stressler et al., 2015). Therefore, the

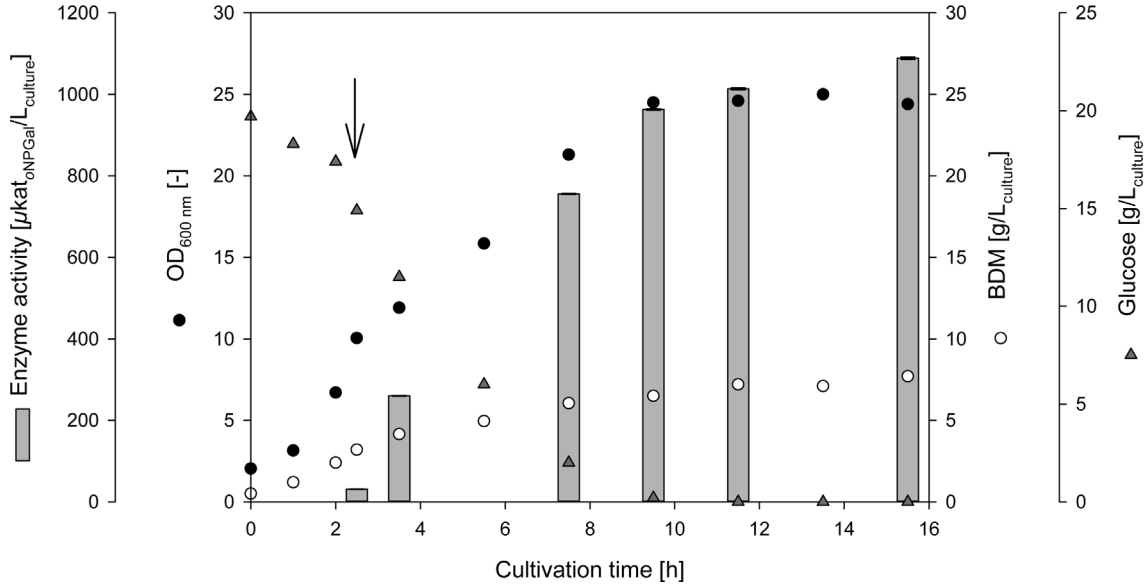

Fig. 3. Cultivation of *E. coli* BL21(DE3) pET20b-M1 for metagenome-β-galactosidase M1 production. Bioreactor at 35 L scale, arrow indicates induction with IPTG and temperature shift; enzymatic activity was measured with *o*NPGal at 37 °C, BDM = Bio Dry Mass.

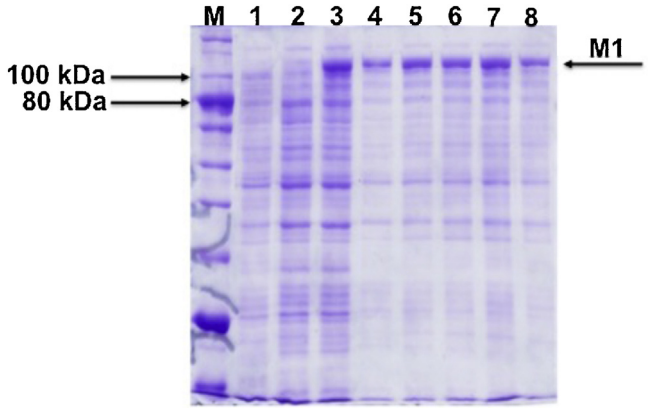

Fig. 4. SDS-PAGE analysis of the expression of the metagenome-β-galactosidase M1 in *E. coli* BL21(DE3). 12% gel; M: Protein Ladder 10–250 kDa; 1: *E. coli* BL21(DE3) wild-type (reference); 2: *E. coli* BL21(DE3) pET20b-M1 before induction; 3–8: *E. coli* BL21(DE3) pET20b-M1 1, 5, 7, 9, 11 and 13 h after induction; Coomassie stained.

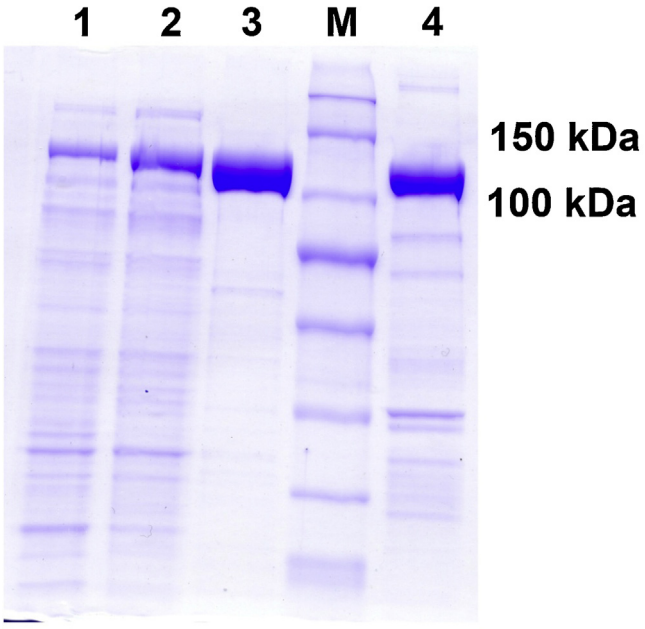

Fig. 5. SDS-PAGE analysis of the purification of M1 and comparison to GODO-YNL2. 8% gel; M: Protein Ladder 10–250 kDa; 1: M1 after cell disruption (cell-free extract); 2: M1 after ammonium sulphate precipitation; 3: M1 after purification with Ni^{2+}-affinity chromatography; 4: GODO-YNL2 (commercial enzyme preparation); Coomassie stained.

β-galactosidase M1 was partially purified only for the later experiments concerning food process-relevant characteristics, such as optimal pH and temperature, and temperature stability. This partially purified enzyme preparation was consistently used for the lactose hydrolysis in milk on a larger scale. The volumetric activity of M1 determined was 4700 ± 100 $nkat_{lactose,30\,°C}$ mL^{-1} (705 ± 14 $nkat_{lactose,8\,°C}$ mL^{-1}) after a fractionated ammonium sulphate precipitation of the cell-free extract. The total protein content was 42 ± 1.7 mg mL^{-1}. By comparison, the activity of the commercial β-galactosidase GODO-YNL2 was $65{,}600 \pm 980$ $nkat_{lactose,30\,°C}$ mL^{-1} ($11{,}808 \pm 12$ $nkat_{lactose,8\,°C}$ mL^{-1}) and its protein content was 48 ± 1.7 mg mL^{-1}. Thus, GODO-YNL2 is a highly concentrated β-galactosidase preparation (see Fig. 5). It is about 14-fold higher in volumetric activity than the β-galactosidase M1 preparation produced after salt precipitation.

Due to the fact that the host strain used, *E. coli* BL21(DE3), is not β-galactosidase negative, its background activity was determined.

Table 1
Optimal pH, optimal temperature and temperature stability (half-life $t_{1/2}$) of the novel metagenome β-galactosidase M1 and the commercial β-galactosidase preparation GODO-YNL2. (Determination in buffer with the substrate lactose, experiments were done in triplicate, standard deviation of the enzyme activities determined <5%; see Supplemental S2–S4).

		Temperature properties		
			Half-life $t_{1/2}$ [d]	
	Optimal pH [–]	Optimal temp. [°C]	At optimal temp.	At 8 °C
GODO-YNL2	6.5	40	5.0	30.3
M1[a]	7	37	3.0	21.8

[a] Partially purified (ammonium sulphate precipitation).

Therefore, *E. coli* BL21(DE3) wild type was cultivated and processed as described for the M1 production. No background β-galactosidase activity was measured with lactose as a substrate at 8 °C. A negligible background β-galactosidase activity of 100 ± 9 $nkat_{lactose}$ mL^{-1} was detectable at 30 °C (2% of the M1 activity at this temperature).

3.4.2. Further purification of M1 by Ni^{2+}-affinity chromatography

In contrast to an application in the food industry, a protein should be purified before the functional properties (Camper and Viola, 2009), such as enzyme kinetics (Stressler et al., 2014), can be investigated. Here, M1 was further purified by Ni^{2+}-affinity chromatography, as seen in Fig. 5. This figure shows that the purified enzyme preparation of M1 had a higher purity than industrially applied GODO-YNL2. The specific enzyme activity of M1 after purification increased about sixfold up to 99.12 $nkat_{lactose,8\,°C}$ $mg_{protein}^{-1}$. However, the specific activity of GODO-YNL2 (246 $nkat_{lactose,8\,°C}$ $mg_{protein}^{-1}$) was still about 2.5 times higher than the specific activity of the purified metagenome β-galactosidase M1.

3.5. Determination of process-relevant parameters of M1 and GODO-YNL2

The pH and temperature profiles of enzymes determine their application area in the food industry. Thus, these properties were ascertained for the metagenome β-galactosidase M1 and were compared with the commercial enzyme preparation GODO-YNL2. All data were determined with lactose as the substrate. The results are shown in Table 1 and Supplemental S2–S4. Both enzymes tested possessed an optimal pH between 6.5 and 7.0, which enables them for an application in milk or sweet whey (both: pH 6.7). Regarding the optimal temperature, M1 was most active at 37 °C. GODO-YNL2 showed a slightly higher optimal temperature (40 °C). Since industrial lactose hydrolysis in milk is carried out at low temperatures (below 10 °C) and the β-galactosidases must be thermally inactivated afterwards at pasteurisation temperatures (above 65 °C), the optimal temperature of M1 was appropriate for food applications.

The stability of the *β*-galactosidases was measured at 8 °C and at their particular optimal temperature (see Table 1 and Supplemental S4), respectively. M1 showed a sufficient temperature stability ($t_{1/2}$ 21.8 days at 8 °C and $t_{1/2}$ 3 days at 37 °C). However, the commercial enzyme GODO-YNL2 was more stable ($t_{1/2}$ 30.3 days at 8 °C and $t_{1/2}$ 5 days at 40 °C). The residual activities of M1 and GODO-YNL2 were also examined at 8 °C after 48 h. Here, M1 and GODO-YNL2 showed a residual enzyme activity of 85% and 90%, respectively.

In summary, all results suggest that both enzyme preparations are suitable for lactose hydrolysis in milk.

3.6. Lactose hydrolysis in milk on a larger scale

Both β-galactosidase preparations M1 and GODO-YNL2 were compared under industrial-like conditions in a scale of 0.5 L milk

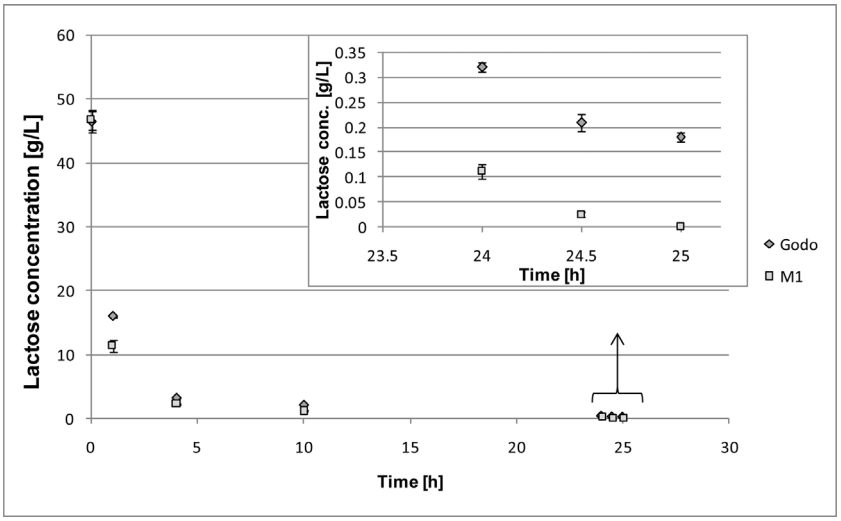

Fig. 6. Hydrolysis of lactose in milk (500 mL scale) with the metagenome β-galactosidase M1 and the commercial β-galactosidase GODO-YNL2 at 8 °C for 25 h.

concerning their ability to hydrolyse lactose (see Fig. 6). M1 was used as a partially purified enzyme preparation due to the higher resemblance to commercial preparations and, therefore, increased relevance for industrial applicability. An equal amount of β-galactosidase activity was used (40 $nkat_{lactose,8\,°C}$ mL_{milk}^{-1}). The initial lactose concentration of the milk was 46 ± 0.5 g L^{-1}. The β-galactosidase M1 exhibited a better performance in lactose hydrolysis from the beginning (see Fig. 6). After 60 min, 75% of the milk lactose had already been hydrolysed with M1 compared to 67% with GODO-YNL2. However, the total conversion of lactose after 25 h was most important (see small illustration in Fig. 6). In comparison to M1 leaving lactose amounts well below 50 mg L^{-1}, GODO-YNL2 still left about 180 mg lactose L^{-1}. Thus, the recommended level for "lactose-free" labelling (below 100 mg L^{-1}) was reached with the novel β-galactosidase M1 (below the limit of quantification, see Erich et al., 2012). A higher enzyme amount than 40 $nkat_{lactose,8\,°C}$ mL_{milk}^{-1} would be needed to reach this desired result with the commercial reference GODO-YNL2.

3.7. Comparison of M1 and GODO-YNL2 concerning kinetic parameters and the influence of the lactose hydrolysis products on the enzyme activity

Although M1 and GODO-YNL2 showed nearly the same pH, temperature and stability characteristics, the performance of M1 during the lactose hydrolysis was better. A possible reason could have been the particular kinetic properties of the enzymes.

3.7.1. Kinetics

The apparent kinetic parameters K_M (affinity to lactose; see Supplemental S5 for Michaelis–Menten plots) and K_I (product inhibition by D-galactose) for both β-galactosidases were determined with lactose in buffer and in diluted milk at 8 °C. The diluted milk was chosen as an additional test matrix to simulate the behaviour of the enzymes when applied in the food industry. The results are presented in Table 2. Interestingly, the apparent kinetic parameters for M1 and GODO-YNL2 depended on the medium applied (diluted milk or buffer system) and displayed an opposing trend for each enzyme. The apparent K_M value decreased in the case of purified M1 from 14.3 ± 1.6 mM in buffered medium to 3.8 ± 0.7 mM in diluted milk. Contrarily, in the case of GODO-YNL2, an increase of the apparent K_M value was seen from 20.4 ± 1.9 mM in buffered medium to 30.9 ± 3.7 mM in diluted milk. Looking at the K_I values in buffer and diluted milk, M1 again performed more favourably than GODO-YNL2. In the case of GODO-YNL2, with an unfavourable low apparent K_I of 32.4 ± 3.9 mM in diluted milk, the product inhibition by D-galactose is already noticeable after about 25% lactose hydrolysis (35 mM D-galactose). Although the apparent K_I value for M1 decreased from 364.4 ± 49.6 mM in buffer to 196.6 ± 55.5 mM in diluted milk, the apparent K_I values for M1 were still higher than the theoretically possible D-galactose concentration in milk after total lactose hydrolysis (approx. 140 mM = 48 g L^{-1}). Thus, the product inhibition of M1 in milk restricts the lactose hydrolysis only marginally and only at a high lactose degree of hydrolysis towards the end of the process. However, it should be mentioned that these results explain the shift of the kinetic parameters in a milk-like environment. Further studies, using a more appropriate dilution medium than water (e.g. SMUF-buffer), should be carried out in the future.

Table 2
Apparent kinetic parameters K_M and K_I of the novel metagenome β-galactosidase (M1) and GODO-YNL2 (commercial β-galactosidase). (Experiments were carried out in triplicate, and data were calculated by non-linear regression fitting with the Enzyme Kinetics Module of SigmaPlot 12.5, Systat Software, Inc,. San Jose, CA).

		Buffer[a] [8 °C]	Diluted milk[b] [8 °C]
$K_{M,\ lactose\ [mM]}$	M1[c]	14.3 ± 1.6	3.8 ± 0.7
	GODO-YNL2	20.4 ± 1.9	30.9 ± 3.7
$K_{I,\ galactose\ [mM]}$	M1[c]	364.4 ± 49.6	196.6 ± 55.5
	GODO-YNL2	34.3 ± 2.9	32.4 ± 3.9

[a] Potassium phosphate buffer (100 mM, pH 6.75) with $MgCl_2$ (5 mM).
[b] H-Schwarzwaldmilch (3.8% fat) diluted with H_2O.
[c] Purified (ammonium sulphate precipitation and Ni^{2+}-affinity chromatography).

3.7.2. Investigation of product inhibition by D-glucose and D-galactose

In addition to D-galactose, the effect of released D-glucose on the enzyme activity of M1 and GODO-YNL2 during hydrolysis was also of interest. Due to the experimental setup (analysis), it was not possible to measure the influence of D-glucose with the natural substrate lactose. Therefore, *o*NPGal was used for the determination of β-galactosidase activity in the presence of D-glucose. In addition, the influence of D-galactose and the influence of an equimolar concentration of both D-glucose and D-galactose was tested. The influence of M1 and GODO-YNL2 on the enzyme activity is shown in Fig. 7. The enzyme activity of GODO-YNL2 decreased with increasing D-galactose concentration (150 mM: 81.1 ± 0.3% activity) as seen in Fig. 7A. This is in accordance with the results displayed

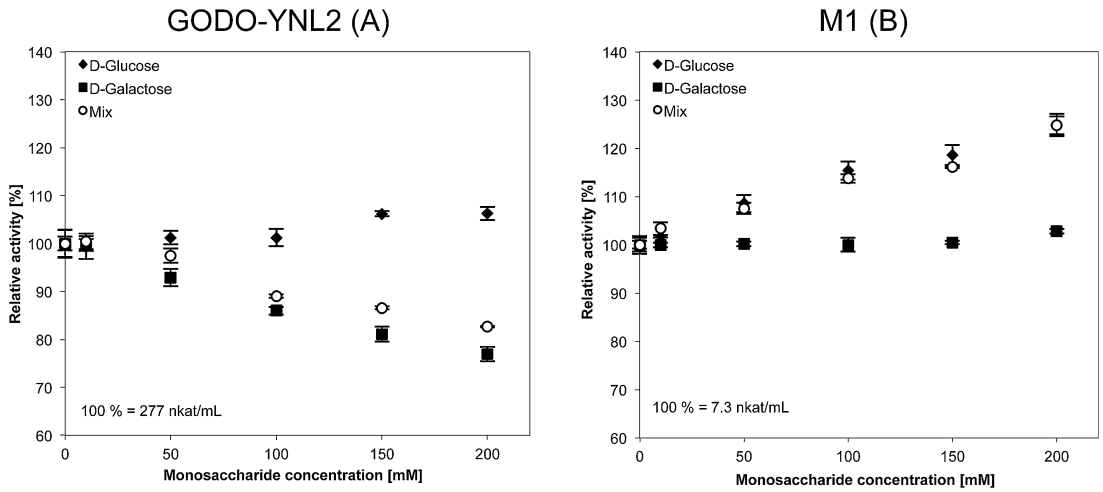

Fig. 7. Effect of added D-glucose, D-galactose or equimolar mixture of both sugars on the activity of GODO-YNL2 (A) and purified M1 (B). Enzymatic activity was measured with *o*NPGal at 37 °C; experiments were carried out in triplicate.

previously. By the addition of D-glucose, a minor activating effect of the enzyme activity was observed. However, this was only seen at high D-glucose concentrations. The equimolar mixture of both sugars showed that the activating effect of D-glucose resulted in a lesser reduction of the enzyme activity compared to the reduction resulting from the exclusive influence of D-galactose. However, as expected, the inhibiting effect of D-galactose was dominant. In the case of M1, the results were quite different. No inhibition was visible up to a concentration of 200 mM D-galactose. Furthermore, the presence of D-glucose showed a much stronger activating effect on the enzyme activity of M1 (at 150 mM D-glucose: 118.6 ± 2.1% activity) compared to GODO-YNL2. No further effect on the enzyme activity (at MIX-150: 116.1 ± 0.3% activity) was observed for the equimolar mixture of D-glucose and D-galactose, compared to the exclusive addition of D-glucose.

In conclusion, the kinetic data presented promoted the finding that M1 performed better in milk lactose hydrolysis than GODO-YNL2.

4. Discussion

4.1. Metagenome screening

The screening strategy is the most important factor when looking for novel enzymes with desirable properties. The general saying "you get what you screen for" (Schmidt-Dannert and Arnold, 1999) means, in the case of novel β-galactosidases for the food/dairy industry, that one should, firstly, utilise lactose as a screening substrate and, secondly, verify the novel enzymes under process conditions (medium, pH, temperature). The first screening step was carried out with an artificial substrate (X-Gal) for β-galactosidases, since the high number of metagenome clones (more than one million) needed to be managed. This principle was also successfully proven in other metagenome β-galactosidase screenings (Wang et al., 2010 Zhang et al., 2013). Nevertheless, it is likely that an unknown number of metagenome β-galactosidases could not be discovered because X-Gal did not fit into their active site, as the indoxyl residue is quite different in shape and functionality compared to natural hexose units. On the contrary, the fact that X-Gal cleaving metagenome enzymes do not all act on lactose was measured in our second screening step. In fact, the percentage of true β-galactosidases hydrolysing lactose was only 27.4% (97 from 354). The third screening step challenged the positive candidates under industrial process conditions (milk, 8 °C), and six candidates (about 6% of the true β-galactosidases) featured promising properties when compared to the commercial reference GODO-YNL2 (see Fig. 2). Thus, the three-step screening strategy proposed accomplished its objective.

Since screening step two was carried out at a pH of 6.75, similar to milk, the resulting metagenome enzyme M1 showed an optimal pH of 7 (see Table 1). The optimal neutral pH and the optimal temperature- of the β-galactosidase (37 °C) are quite common for mesophilic β-galactosidases reported in the literature (Ansari and Satar, 2012; Mahoney, 2002). The temperature stabilities of β-galactosidases are often investigated for only a few hours in the literature (Hoyoux et al., 2001; Hu et al., 2007; Wang et al., 2010). Thus, a comparison with literature data does not seem appropriate. Nevertheless, the M1 β-galactosidase was compared with GODO-YNL2 in our study at a process-relevant temperature of 8 °C over 48 h (Table 1). The extrapolated half-lives for both β-galactosidases (M1 21.8 days, GODO-YNL2 30.3 days) were more than sufficient for an industrial process, since the industrial lactose hydrolysis in milk operates no longer than 48 h.

4.2. Hydrolysis of lactose in milk

The milk lactose hydrolysis performance of M1 in milk was directly compared to GODO-YNL2 on a scale of 0.5 L. The hydrolysis was carried out at 8 °C for 24 h. Subsequently, the milk was heated for 1 h to 40 °C, similar to what would have been done in the dairy industry for pasteurisation. Under these conditions, the M1 β-galactosidase hydrolysed >99.9% of the lactose in milk after 25 h, whereas GODO-YNL2 (industrially utilised reference) reached 99.6% (w/v) after the same time. The metagenome β-galactosidase ZD410, which was investigated by Wang et al. (2010), could hydrolyse 4.2% of the lactose in milk at 4 °C after 60 min of incubation. Unfortunately, they did not investigate it for a longer period of time. In the study by Hoyoux et al. (2001), lactose hydrolysis was also performed for short time intervals. It was reported that a β-galactosidase from *Pseudoalteromonas haloplanktis* hydrolysed 33% of the lactose in milk at 4 °C after 50 min. Longer time intervals were investigated by Pan et al. (2010). In this study, the hydrolysis conversion of lactose in milk was 73% with a β-galactosidase from *Lactobacillus acidophilus* at 10 °C after 30 h. Thus, this hydrolysed milk sample still had a residual lactose content of 13.5 g L^{-1}. In other studies, cold-active β-galactosidases were also investigated for lactose hydrolysis in milk (Cieśliński et al., 2005; Nakagawa et al., 2007). With the recombinant cold-adapted β-galactosidase

from Antarctic bacterium *Pseudoalteromonas* sp. 22b, immobilised on chitosan beads, Cieśliński et al. (2005) achieved a lactose hydrolysis conversion of 90.5% at 15 °C after 28 h. The β-galactosidase activities of four commercial preparations were investigated by Horner et al. (2011) at 2 °C. Here, they obtained a maximum lactose hydrolysis conversion with GODO-YNL2 of 99.08% after 24 h.

4.3. Kinetic parameters and effects of D-glucose and D-galactose on the enzyme activity

Although the metagenome β-galactosidase M1 and GODO-YNL2 showed similar biochemical characteristics, the performance during the lactose hydrolysis in milk was better for M1. To find an explanation for this, the apparent kinetic parameters K_M and K_I of M1 and GODO-YNL2 were investigated in buffer and in milk diluted with water at 8 °C. Both M1 and GODO-YNL2 exhibited product inhibition by D-galactose in buffer and diluted milk. The apparent K_I of M1 obtained was higher than the maximum theoretical hydrolysis yield of D-galactose in milk (approx. 140 mM = 48 g L^{-1}). Wang et al. (2010) discovered the metagenome β-galactosidase ZD 410. However, the kinetics (K_M values) were only measured with various synthetic nitrophenyl-monosaccharides (chromogenic substrates) and, thus, were not comparable with our results using lactose. Zhang et al. (2013) reported a novel metagenome β-galactosidase Gal308 (optimal temperature with lactose 78 °C, pH 6.8), which possessed product inhibition by D-galactose (K_I = 238 mM) in buffer when *o*-nitrophenyl-β-D-galactopyranoside was used as the substrate. Zhang et al. (2013) also tested Gal308 for milk lactose hydrolysis at 65 °C over 60 min. At the end, a 93.6% hydrolysis rate was observed. A comparison with M1 is not possible from their data due to the different conditions and a shorter time-dependent course of observation. Comprehensive kinetic data from β-galactosidases of culturable strains, such as *K. lactis*, *Aspergillus niger* and others, were published by Mahoney (1997). The *K. lactis* β-galactosidase, which should be similar to our reference GODO-YNL2, was reported to possess a $K_{M,lactose}$ of 12–17 mM and a $K_{I,D\text{-}galactose}$ of 42 mM. This is in accordance with our data for GODO-YNL2 (see Table 2). In our work, the apparent K_M/K_I ratio for GODO-YNL2 is 0.6 (in buffer, 8 °C) and 1.0 (milk, 8 °C). It was calculated as Mahoney (1997) did for the evaluation of the hydrolysis potential for *K. lactis* (0.38 in buffer, 35 °C). Again, the data of Mahoney (1997) and ours in buffer correspond to each other, but the different temperatures should be kept in mind. The metagenome β-galactosidase M1 had favourably low apparent K_M/K_I ratios of 0.04 (in buffer, 8 °C) and 0.02 (milk, 8 °C). The rather large difference between M1 and GODO-YNL2, with M1 having apparent K_M/K_I ratios up to 50 times lower than GODO-YNL2, highlights the favourable properties of M1, especially for usage in milk. In summary, the apparent kinetic constants $K_{M,lactose}$ and $K_{I,D\text{-}galactose}$ of both β-galactosidases were highly dependent on the medium and, with the aspect of lactose hydrolysis in milk, they have to be determined in milk-like systems in order to allow a more realistic estimation for an industrial utilisation. No inhibitory effect was determined for M1 and GODO-YNL2 in the presence of D-glucose up to a concentration of 200 mM (Fig. 7; substrate: *o*NPGal). Even a slight activating effect was determined in the case of GODO-YNL2 and a more significant activating effect for M1. Therefore, the D-glucose released during the lactose hydrolysis should not negatively influence the performance of the enzymes. Thus, the D-glucose released during lactose hydrolysis will most probably not have a noticeable negative effect on M1 during lactose hydrolysis, especially in milk.

5. Conclusions

In conclusion, the theoretical total number of about 1.3 million metagenome clones were screened for novel β-galactosidases with potential applications in the dairy industry. The third screening step, since it allowed quantitative evaluation of the performance of the positive candidates in the target reaction environment, is especially highly recommended. The metagenome bore six novel β-galactosidases with previously unknown gene sequences which performed very promisingly in milk lactose hydrolysis when compared to an industrially utilised reference enzyme. The apparent kinetic constants of the most promising metagenome candidate M1 (K_M, K_I) were dependent on the environment and were of empiric character. M1 showed the most favourable kinetic properties in diluted milk. This novel metagenome β-galactosidase M1 was able to completely hydrolyse milk lactose in 25 h (>99.9% conversion).

Acknowledgments

The authors would like to thank Wolfgang Claaßen (University of Hohenheim, Germany) for his support during the cultivation and cell-disruption experiments. We also thank Karin Maierhofer (University of Hohenheim) for her highly appreciated support in the metagenome screening experiments. We express our thanks to KNAUER Wissenschaftliche Geräte GmbH (Berlin, Germany) for providing the chromatography resin and glass column. This research project was supported by the Federal Ministry for Economic Affairs and Energy (via AiF) and the FEI (Forschungskreis für Ernährungsindustrie e.V., Bonn): Project AiF 15,801N.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.06.411

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