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

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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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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)