ABSTRACT

Commercial β-galactosidases exhibit undesirable kinetic properties regarding substrate affinity ($K_M$) for lactose) and product inhibition ($K_I$ for galactose). An in silico screening of gene sequences was done and identified a putative β-galactosidase (BgaPw) from the psychrophilic bacterium Paenibacillus wynnii. The cultivation of the wild-type P. wynnii strain resulted in very low β-galactosidase activities of a maximum of 150 nkat_NPGal/L medium. The recombinant production of BgaPw in Escherichia coli BL21(DE3) increased the yield about 9,000-fold. Here, a volumetric activity of 1350.18 ± 11.82 µkat_NPGal/Lculture was achieved in a bioreactor cultivation. The partly purified BgaPw showed a pH optimum at 7.0, a temperature maximum at 40°C and an excellent stability at 8°C with a half-life of 77 d. Kinetic studies with BgaPw were done in milk or in milk-imitating synthetic buffer, so-called Novo buffer, respectively. Remarkably, the $K_M$ value of BgaPw with lactose was as low as 0.63 ± 0.045 mM in milk. It was found that the resulting products of lactose hydrolysis, namely, galactose and glucose, did not inhibit the β-galactosidase activity of BgaPw but instead showed a striking activating effect in both cases (up to 144%). In a comparison study in milk, lactose was completely hydrolyzed by BgaPw in 72 h at 8°C, whereas 2 other known β-galactosidases were less powerful and converted only about 90% of lactose in the same time. Finally, the formation of galactooligosaccharides (GOS) was demonstrated with the new BgaPw, starting with pharmaco-lactose (400 g/L). A GOS production of about 144 g/L was achieved after 24 h (36.0% yield).

Key words: β-galactosidase, Paenibacillus, lactose hydrolysis, galactooligosaccharides

INTRODUCTION

β-Galactosidases (E.C 3.2.1.23) are abundant glycoside hydrolase enzymes which catalyze the hydrolysis of a glycosidic bond between a terminal nonreducing β-D-galactoside unit and an aglycon moiety (Vera et al., 2020; Niu et al., 2017). β-Galactosidases are widely used in the dairy industry to catalyze 2 types of reaction: (I) the hydrolysis of lactose to D-glucose and D-galactose to produce lactose-depleted products, and (II) the transgalactosylation for the synthesis of galactooligosaccharides (GOS) or lactulose (Ambrogi et al., 2021; Yan et al., 2021). The GOS are prebiotic components which can be applied as food ingredients to stimulate the activity of beneficial microorganisms in the colon (Vera et al., 2016; Vera et al., 2020). The ratio between lactose hydrolysis and GOS synthesis depends strongly on the lactose concentration, the reaction temperature and the intrinsic enzyme properties (de Andrade et al., 2020).

β-Galactosidases can be derived from microbial sources, such as bacteria, fungi and yeasts (Husain 2010; Zhou et al., 2021). Fungal β-galactosidases commonly work in an acidic pH range from 2.5 – 5 and are, therefore, most effective for the hydrolysis of lactose in acidic products such as acid whey (Husain 2010). Since β-galactosidases from bacteria generally have a high enzyme activity, good stability and high production yield, they have been most widely used for lactose hydrolysis. β-Galactosidases from yeasts are active at a rather neutral pH between 6.0 and 7.0 and have been used to produce lactose-free milk due to their high hydrolytic activity. Up to now, β-galactosidases used in the food industry are obtained mostly from filamentous fungi, such as Aspergillus niger and oryzae, the yeast Kluyveromyces lactis, and the bacteria Bacillus circulans and Bifidobacterium bifidum (Godoy et al., 2016; Maksimainen et al., 2012; Rico-Díaz et al., 2017).

However, the identification of novel β-galactosidases with suitable characteristics for use in the dairy industry is still of great importance. Notably, the reaction product galactose is an inhibitor of most microbial β-galactosidases, competing with lactose.
LX) was done in milk. (enommetage β-galactosidase M1 and Opti-Lactase comparison study with 2 other known β-galactosidases β-galactosidase kinetic was investigated in detail and a the lactose, glucose and galactose concentrations on the lactose hydrolysis and GOS formation. The influence of file, temperature optimum and kinetic performance in major industrial relevant parameters, such as pH pro-

β-galactosidase was characterized by determining its E. coli

prominantly in . After partial purification, the new in the wild-type bacterium itself and then recombi-

native β-galactosidase formation was investigated for industrial applications at low temperatures. First, the native β-galactosidase formation was investigated in the wild-type bacterium itself and then recombi-

nantly in E. coli. After partial purification, the new β-galactosidase was characterized by determining its major industrial relevant parameters, such as pH profile, temperature optimum and kinetic performance in lactose hydrolysis and GOS formation. The influence of the lactose, glucose and galactose concentrations on the β-galactosidase kinetic was investigated in detail and a comparison study with 2 other known β-galactosidases (enommetage β-galactosidase M1 and Opti-Lactase LX) was done in milk.

MATERIALS AND METHODS

Chemicals, enzymes and kits

All chemicals were of analytical grade and purchased from Sigma Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany), Gerbu Biotechnik GmbH (Heidelberg, Germany) or Appli chem (Darmstadt, Germany). Hexokinase/Glucose-6-phosphate dehydrogenase was purchased from Megazyme International Ireland (Wicklow, Ireland). The commercial reference Opti-Lactase LX was obtained from Opti-ferm GmbH (Oy-Mittelberg, Germany) containing the β-galactosidase from Kluyveromyces lactis. The metagenome β-galactosidase M1 was produced in E. coli and prepared as described in the literature (Er-

ich et al., 2015). The enzymes required for molecular biological work were purchased from NEB (Frankfurt, Germany). Agarose was obtained from SERVA Electro-phoresis GmbH (Heidelberg, Germany). The UHT milk containing 1.5% fat (fettarme, haltbare Weidemilch 1.5%, Schwarzwaldmilch GmbH, Freiburg, Germany) was obtained from a local supermarket.

Bacterial strains and genomic DNA

Escherichia coli XL1 Blue (Merck KGaA, Darmstadt, Germany) and E. coli BL21(DE3) (Novagen, Madison, USA) were utilized for the host cloning and T7 expres-

sion work, respectively. The strain and the genomic DNA of Paenibacillus wynnii DSM18334 were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

Cultivation of Paenibacillus wynnii

P. wynnii DSM1834 was cultivated in medium 1, 2, 3 and 4, respectively (Supplements, Table S1). To induce native β-galactosidase expression, 20 g/L of lactose were supplied to each media. A volume of 20 mL overnight culture was used to inoculate 400 mL of the respective medium in non-baffled shake flasks. Cells were grown at 22°C for 8 h and cultivation was monitored by analyzing the optical density (OD$_{600nm}$), pH and β-galactosidase activity (see below). The bioreactor cultivation was done in a Minifors fermenter (Infors HT, Einsbach, Germany) with an operating volume of 2 L. Before inoculating the bioreactor, 2 precultures (5 and 200 mL) were done at 22°C. The following parameters were chosen for the cultivation: 200 – 300 rpm, air gassing $v_{vm}$ = 0.5, and pH 7. The pH was adjusted using 2 $M$ NaOH and 2 $M$ H$_3$PO$_4$. Samples were taken during the cultivation to analyze the optical density (OD$_{600nm}$) and β-galactosidase activity (see below).

Construction of the expression vector

The construction of the expression vector for the β-galactosidase from P. wynnii (WP_036650547.1; S1) was based on the genomic DNA sequence of the β-galactosidase from P. wynnii DSM18334 (European Molecular Biology Laboratory: EMBL coding: KGE19535.1) available at the EMBL, European Bioinformatics Institute. One nucleotide was exchanged for cloning, but this did not result in an amino acid exchange. The cloning of the bgapw gene in the NdeI/ XhoI site of pET20b was obtained by a homologous recombination strategy according to Jacobus and Gross (2015). Therefore, the vector and the gene were amplified separately by PCR. The PCR products were purified by gel purification. A volume of 100 ng vector
and 50 ng gene DNA were premixed in a volume of 10 µL of H2O for the homologous recombination. The transformation of this mixture in E. coli XL1 Blue cells was carried out by heat shock. The construct pET20b_bgapw resulted with a C-terminal His6-tag in frame due to the cloning strategy chosen. The construct was transformed in E. coli BL21(DE3) cells for the expression of the P. wynnii β-galactosidase (BgaPw).

The gene sequence of bgapw and the primers for the amplification can be found in the Supplemental Material (S2 and Table S2).

Production of the recombinant BgaPw in E. coli

E. coli BL21(DE3) pET20b_bgapw was cultivated in 2YT medium (tryptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L) containing glucose (20 g/L) supplemented with ampicillin (100 µg/mL) in a Biostat E Fermenter (B Braun AG, Melsung, Switzerland) with an operating volume of 3.5 L. Three precultures (5, 30, 300 mL) were done at 37°C before inoculating the bioreactor. The following parameters were chosen for the cultivation: 300 rpm, air gassing vvm = 2 and pH 7. The pH was adjusted using 2 M NaOH and 2 M H3PO4. Samples were taken during the cultivation to analyze the optical density (OD600nm), the bio dry mass and the glucose concentration. The cells were cultivated at 37°C up to an OD600nm of 5. The temperature was then decreased to 30°C to minimize the formation of inclusion bodies, and recombinant protein expression was induced by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After further cultivation for 9 h at 30°C, the cells were harvested using a continuous working centrifuge (CEPA Rapid centrifuge Type GLE, flow rate 350 mL/min, 36,000 x g, 4°C). The harvested cells were washed twice with saline, centrifuged (6,000 x g, 4°C, 10 min) and stored at −20°C.

Additionally, samples (10 mL) were taken at various time points during the cultivation to determine the β-galactosidase activity (see below) after cell disruption. The samples were centrifuged (8,000 x g, 10 min, 4°C) and the cell pellets were resuspended in saline. After centrifugation (see above), the cell pellets were resuspended in potassium phosphate buffer (100 mM, pH 6.75) with MgCl2 (5 mM). The cell disruption was realized by sonification (UP200S ultrasonic processor, Dr. Hielcher, Berlin, Germany; cycle 0.5, amplitude 95%; 10 cycles containing 1 min disruption, 1 min break) on ice. The cell debris was separated by centrifugation (8,000 x g, 20 min, 4°C) to obtain the cell-free extract.

Purification of BgaPw

The recombinant BgaPw with a hexa histidine-tag was purified by nickel affinity chromatography using an ÄKTA FPLC system (GE Healthcare, München, Germany). Accordingly, a 25% (wt/vol) cell suspension in binding buffer (BB), consisting of phosphate buffer (100 mM, pH 6.75) with MgCl2 (5 mM) and NaCl (150 mM), was prepared for cell disruption (see above). After centrifugation (see above), the supernatant was filtered through a 0.45 µm membrane filter.

A 5 mL sample was injected into the Ni2+ affinity column (His60 Superflow Resin TAKARA, 1 column volume (CV) = 10 mL) at a flow rate of 1 mL/min. The column was previously equilibrated with 2 CV BB. Unbound protein was eluted for 2 CV using 90% (vol/vol) BB and 10% (vol/vol) elution buffer (EB; BB + 250 mM imidazole). Bound protein was eluted by a linear gradient (4 CV) to 100% EB. The flow rate for the elution was 2 mL/min and eluted protein was detected by an UV detector at λ = 280 nm and fractionated in 2.5 mL fractions. The fractions in which an UV signal was detected were pooled. The fractions were desalted to potassium phosphate buffer (100 mM, pH 6.75) with MgCl2 (5 mM) using PD-10 columns (GE Healthcare, München, Germany). Afterward, the fractions were analyzed for β-galactosidase activity (see below). Active fractions were pooled and stored at 4 – 8°C until further use.

Assays for the β-galactosidase activity determination

The β-galactosidase activity was determined using either the synthetic substrate o-nitrophenyl-β-D-galactopyranoside (oNPGal) or the natural substrate of the enzyme lactose. One katal was defined as the amount of enzyme that catalyzes the release of 1 mol o-nitrophenol from oNPGal per second or the release of 1 mol D-glucose from lactose per second. All assays were performed at least in triplicate.

β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (oNPGal) as a substrate

The enzyme activity of β-galactosidase was determined in 1.2 mL scale using oNPGal as substrate at 37°C. A final concentration of 25 mM oNPGal dissolved in potassium phosphate buffer (100 mM, pH 6.75) with MgCl2 (5 mM) was used for the assays. The substrate and enzyme solution were preheated separately. The reaction was performed in a temperature-controlled cuvette using a spectrophotometer by adding enzyme solution to oNPGal solution. The increase of absor-
bance at 405 nm as result of o-nitrophenol release was measured for 2 min. The activity was calculated from the slope of the straight line. The amount of the o-nitrophenol released was determined using a calibration curve (range: 0.0167 – 1.67 mM o-nitrophenol).

The oNPGal assay was also used in a smaller scale with modifications. Therefore, 100 µL oNPGal solution (50 mM) in potassium phosphate buffer (100 mM, pH 6.75) with MgCl₂ (5 mM) was mixed with 80 µL of the same buffer and preincubated for 5 min at 37°C. The reaction was started by the addition of 20 µL enzyme solution which was separately preincubated. After 2 min incubation at 37°C and 900 rpm the reaction was stopped by the addition of 200 µL Na₂CO₃ (1 M). After centrifugation (13,000 x g, 5 min, 4°C), the supernatant (240 µL) was transferred to a 96-well microtiter plate and absorbance was measured at 405 nm. The amount of the o-nitrophenol released was determined using a calibration curve (range: 0.0063 – 2.5 mM o-nitrophenol).

β-galactosidase activity using lactose as a substrate. The lactose assay was used for the determination of the pH, temperature maximum and stability, and the investigation of the kinetic parameters. Depending on the study, the assay was performed at different temperatures and in different buffers (see experiment). The implementation of the standard assay is described in the following.

A lactose solution (400 mM in potassium phosphate buffer [(100 mM, pH 6.75) with MgCl₂ (5 mM)] and enzyme solution was preincubated separately at the corresponding temperature in a thermal shaker. The enzymatic reaction was started by adding 100 µL of the β-galactosidase sample to 100 µL lactose solution with buffer. The reaction was carried out for a defined period at the corresponding temperature. The reaction was stopped by adding 300 µL HClO₄ (1 M) to the enzyme-substrate-mix. The mix was kept on ice for 5 min before it was centrifuged at 13,000 x g and 4°C for 5 min. An amount of 160 µL of the supernatant was transferred to 75 µL KOH (2 M) to neutralize the solution. The neutralization step was checked using pH paper and the pH was adjusted if necessary. The solution was kept on ice for another 5 min before it was centrifuged again (13,000 x g, 4°C, 5 min). The glucose concentration of the supernatant was determined enzymatically, as described below.

### Determination of glucose

The D-glucose released was determined enzymatically with the hexokinase/glucose-6-phosphate dehydrogenase assay (HK assay) by photometric measurement (340 nm) in microtiter plates. For the determination, 235 µL TRA buffer (triethanolamine hydrochloride (181.1 mM), NADP⁺ (1.65 mM), ATP (3.66 mM) and MgSO₄ x 7 H₂O (3.65 mM)) and 15 µL sample were added to a microplate. Mixing was then performed in the microplate reader and absorbance A1 was read after 3 min. Subsequently, 5 µL hexokinase/glucose-6-phosphate dehydrogenase (Megazyme) was added and mixed again. The A2 was measured after 25 min. The glucose concentration was determined using a calibration curve (range: 0.05 – 2 g/L glucose monohydrate)

### Investigations of pH, temperature and stability

The investigation of the pH-optimum, temperature maximum and temperature stability was implemented using the lactose standard assay (see above). For the determination of the pH-optimum the β-galactosidase activity was determined in a pH range of 5 – 9 at 37°C. Citrate buffer (pH 5 – 6), potassium phosphate buffer (pH 6 – 8) and Tris-HCl buffer (pH 8 – 9) with a concentration of 100 mM were used.

Regarding the maximum temperature, the β-galactosidase activity was determined in a temperature range between 5 and 50°C with potassium phosphate buffer (100 mM, pH 7). In addition, the enzyme solutions were incubated at 2 different temperatures (8 and 30°C) for 246 h (11 d) to determine the temperature stability. The data were fitted to first-order plots and analyzed, with the first-order reaction constants (k) determined by linear regression of ln residual activity versus the incubation time (Supplements Figure S4). The activity half-life (t₁/₂) was calculated using the following equation: 

\[ t_{1/2} = \ln 2 / k \]

### Determination of the influence of Novo buffer

Synthetic Novo buffer (trisodium citrate 2.7 mM, citric acid 7.91 mM, K₂HPO₄ 2.99 mM, KH₂PO₄ 10.84 mM, KOH 19.43 mM, MgCl₂ 4.08 mM, CaCl₂ 5.1 mM, Na₂CO₃ 3.33 mM) was designed after Pes-sela et al. (2003) to imitate the composition of milk. The investigation of the influence of Novo buffer on the β-galactosidase activity was carried out at 37°C and pH 7 with lactose as a substrate. The substrate solution lactose was dissolved in Novo buffer, as described above. The final lactose concentration was 200 mM.

### Determination of kinetic parameters

The Michaelis Menten constant Kₘ of the β-galactosidase BgaPw with lactose as the substrate was determined at 8°C. The lactose concentration varied from 0.76 to 97.5 mM. The respective volume of Novo buffer (see above) was used since the natural
lactose concentration of milk (about 140 mM) had to be reduced. The kinetic parameter \( K_M \) was analyzed using the Enzyme Kinetic Models in SigmaPlot 12.5 (Systat Software, Inc., San Jose, USA).

**Influence of galactose and glucose on the β-galactosidase activity**

The influence of galactose on the hydrolysis activity was determined with BgaPw, the commercial β-galactosidase Opti-Lactase (Optiferm GmbH, Oy-Mittelberg, Germany) and the metagenome β-galactosidase M1 at 8°C in Novo buffer (see above). The β-galactosidase activity was determined with a lactose concentration of 125 or 50 mM with the addition of galactose (125 or 200 mM). The β-galactosidase activity was determined as described above.

The effect of glucose and galactose on the enzyme activity of BgaPw was ascertained with a modified oNPGal assay. A volume of 100 µL oNPGal solution (50 mM) in Novo buffer was mixed with 80 µL Novo buffer containing 25 – 500 mM of glucose, galactose or a mixture of both sugars (equimolar: e.g., MIX-50) containing 50 mM glucose and 50 mM galactose and preincubated at 37°C for 5 min. The assay was performed as described above.

**Hydrolysis of lactose in milk**

The hydrolysis of lactose in milk was carried out in a scale of 31.5 mL. A volumetric enzyme activity of β-galactosidase BgaPw, Opti-Lactase and M1 of 2.7 nkat\(_{\text{lactose, 40°C}}\)/mL\(_{\text{milk}}\) was used. The hydrolysis of lactose took place under constant mixing with the aid of a magnetic stirrer over a period of 149 h in the cold room (\( T = 5 – 8^\circ\text{C} \)). As the analysis period was 6.5 d, 0.1% (wt/vol) sodium azide was added to the milk to prevent the growth of other microorganisms. During the turnover period, 200 µL samples were taken at regular intervals and stopped with perchloric acid and neutralized with potassium hydroxide, as described above. The samples were analyzed by high-performance liquid chromatography (HPLC) (lactose content). The HPLC was done on an Agilent Series 1100 HPLC system (Agilent Technology, Santa Barbara, USA) coupled with a low-temperature evaporative light scattering detector (ELSD Sedex 85LT, Sedere, Alfort ville Cedex, France) at 50°C and 3 bar. A Shodex HILICpak VG-50 4E column (4.6 × 250 mm, 5 µm, Shodex, Showa Denko K.K., Japan) was used to determine the concentration of lactose. The column was eluted with 75% (vol/vol) acetonitrile, 15% (vol/vol) methanol and 10% (vol/vol) double-distilled water. The program sequence of the HPLC is attached in the supplementary information. The flow rate was set to 0.75 mL/min, the injection volume was 2.5 µL and the total run time was 32 min.

**Calculation of lactose conversion and GOS yield**

The lactose conversion describes how much lactose was processed by the β-galactosidase during the enzymatic conversion of lactose in buffer. In this case, the HPLC was done with the same HPLC system and the same ELSD detector. Lactose, glucose and galactose were separated by using 2 consecutive carbohydrate Ca\(^{2+}\)-HPLC columns (250 × 8 mm; CS, Langerwehe, Germany). The columns were tempered to 85°C using the Croco-Cil HPLC column oven (SCO Seitz Chromatographie Produkte GmbH, Weiterstadt, Germany). The system was operated with a pressure of 80 bar. An automated sampler applied a 10 µL sample to the columns. Elution was carried out with a constant flow rate of 0.5 mL/min of H\(_2\)O\(_{\text{dd}}\) for 35 min. Fructose was used as an internal standard. The limit of detection of lactose, glucose and galactose is 0.13 g/L each.

The lactose conversion and GOS yield was calculated by the following equation:

\[
X = \left( \frac{c_{\text{lac,t}} - c_{\text{lac,0}}}{c_{\text{lac,0}}} \right) \times 100\% ,
\]

with:

- \( X \) = lactose conversion [%]
- \( c_{\text{lac,t}} = \) lactose concentration at the beginning [g/L]
The GOS yield describes the GOS concentration as a percentage of the initial lactose concentration.

\[ Y = \frac{c_{\text{lac},0} - (c_{\text{lac},t} + c_{\text{gal},t})}{c_{\text{lac},0}} \times 100\% \]

With:
- \( Y \) = GOS yield [%]
- \( c_{\text{lac},0} \) = lactose concentration at the beginning [g/L]
- \( c_{\text{lac},t} \) = lactose concentration at time t [g/L]
- \( c_{\text{gal},t} \) = Sugar concentration at time t [g/L]

**RESULTS**

The metagenome \( \beta \)-galactosidase M1 showed promising kinetic properties for lactose hydrolysis in milk (Erich et al., 2015). Thus, a BLAST search with the M1 sequence was done and a promising sequence, annotated as \( \beta \)-galactosidase (see Supplemental S1), from the bacterium *Paenibacillus wynnii* (DSM18334) was chosen that possessed a sequence identity to M1 of about 77%. Further investigations of this gene product should prove whether the corresponding protein is truly a \( \beta \)-galactosidase (BgaPw) and which biochemical properties the new \( \beta \)-galactosidase has.

**\( \beta \)-galactosidase production in the wild-type *P. wynnii***

Some \( \beta \)-galactosidases, which are commercially available as technical enzyme preparations, are produced by their native hosts as claimed by the companies. Consequently, at the beginning, the native production of the new \( \beta \)-galactosidase was investigated in the wild-type strain *P. wynnii* DSM 18334 using lactose as the carbon and energy source in the medium. No relevant information is available in the literature regarding how to grow *P. wynnii* in suspension culture, either in shaking flasks or in a bioreactor. Thus, different media and cultivation conditions were tested empirically (Figure 1). Preliminary experiments demonstrated that the best growth of the Antarctic bacterium *P. wynnii* took place at 22°C (data not shown). Spore formation started after ~24 h of the cultivations (microscopic images are shown in supplemental, Figure S1) in the shaking flask experiments shown in Figure 1A, and the cultivations were stopped after 66 h. However, only cells grown in medium 3 showed reasonable growth up to an OD of 1.0 (Figure 1A). By contrast, *P. wynnii* cells cultivated in media 1, 2 and 4 reached an OD of only between 0.3 and 0.45. Here, the pH dropped down to <5.8 during the cultivations. Medium 3 contained bis-tris salt as a buffer system and the pH was kept constant around 6.6. Medium 1 contained no nitrogen source, because Rodriguez-Diaz et al. (2005) reported that *P. wynnii* fixes nitrogen, medium 2 contained some yeast extract (1 g/L). The intracellular \( \beta \)-galactosidase activities of the biomasses from the shaking flask cultivations were determined between 25 and 150 nkat\(_{\text{NPGal}}\)/L\(_{\text{medium}}\).

Finally, *P. wynnii* was also cultivated in a bioreactor with a working volume of 2 L using medium 4 for 240 h (Figure 1B). Probably bis-tris could be also used as a nitrogen source from *P. wynnii* as well but, for a bioreactor cultivation bis-tris was decided to be too exotic and medium 4 with NH4Cl as nitrogen source was chosen. However, the log phase of the cultivation was inexplicably very long (9 d) and, at the end, a disappointingly low \( \beta \)-galactosidase activity of only 5.5 nkat\(_{\text{NPGal}}\)/L\(_{\text{medium}}\) was determined after 216 h. Hence, a change to the recombinant BgaPw production in *E. coli* was decided since the upscaling of the native BgaPw production in a bioreactor was clearly unsuccessful.

**Recombinant production and purification of BgaPw***

The gene *bgapw* was cloned into the expression vector pET20b for the recombinant production of the \( \beta \)-galactosidase in *E. coli* BL21 (DE3). The recombinant *E. coli* BL21(DE3) pET20b-BgaPwHis\(_6\) strain was cultivated in a bioreactor with a working volume of 3.5 L using 2YT\(_{\text{Amp}}\) medium with 2% (wt/vol) glucose as a carbon source (Figure 2). The expression of *bgapw* was induced by the addition of IPTG (0.5 mM) at an optical density (OD\(_{600}\)) of 5. A maximal OD\(_{600}\) of around 25 could be reached after 12 h of cultivation. This corresponded to a bio dry mass of 11.8 g/L. The maximal growth rate \( \mu_{\text{max}} \) was 0.22 h\(^{-1}\) and the biomass yield coefficient \( Y_{X/S} \) was 0.29. The maximum \( \beta \)-galactosidase activity of 1,350 ± 12 µkat\(_{\text{NPGal}}\)/L\(_{\text{culture}}\) was obtained after 8 h of cultivation. This was about 9,000-fold more than with the native *P. wynnii* strain (max. 150 nkat/L). The cells finally harvested comprised an intracellular \( \beta \)-galactosidase activity of 1,184 ± 43 µkat\(_{\text{NPGal}}\)/L\(_{\text{culture}}\). The recombinantly produced BgaPw possessed a C-terminal His\(_6\) tag. Thus, the cell-free crude extract was purified by Ni\(^{2+}\) affinity chromatography (Supplements Figure S2). The specific \( \beta \)-galactosidase activity of the combined active elution fractions increased by a factor of 2.4, from 20.33 to 45.61 nkat\(_{\text{NPGal}}\)/g\(_{\text{protein}}\), and resulted in a total activity of 1.27 nkat\(_{\text{NPGal}}\)/L\(_{\text{culture}}\) corresponding to a purification yield of 86.1%. A strong protein band was recognized at 120 kDa on the SDS PAGE, which corresponded to the theoretical molecular mass of the \( \beta \)-galactosidase of 119.3 kDa (Supplements, Figure S3).
Determination of relevant parameters for a potential industrial application

Considering a subsequent application of the enzyme in the food industry, the relevant parameters, such as the pH, temperature profile and temperature stability, were investigated.

The pH profile was investigated in the range of pH 5 to 9. The maximum activity was detected at pH 7 (Figure 3A). While the relative activity in the alkaline range at pH 9 was about 40%, no activity could be detected in the acidic range at pH 5. However, the influence of the buffer salt was recognized. At pH 6, a relative activity of 22% was measured in phosphate buffer, while only 2% was determined with citrate buffer. Thus, the β-galactosidase of *P. wynnii* (BgaPw) will be suitable for lactose conversion in milk or sweet whey at around pH 6.7, as it exhibited high activity in the range of pH 6.5 (67%) to 7 (100%).

The temperature profile and the temperature stability were determined in phosphate buffer (100 mM, pH 7.0). The maximal activity was determined at 40°C (Figure 3B). While a high activity was still measurable at 45°C, no activity could be determined at 50°C. A relative β-galactosidase activity of about 25% was measurable at 5°C, which is advantageous for the application of lactose conversion in milk because it is often carried out at about 8 – 10°C in industrial processes. The temperature stability was measured at 8 and 30°C. The latter temperature is 10°C under the estimated maximum activity and that is the preferred temperature for the application of many enzymes as it combines the enzyme activity and stability properties at their best. The BgaPw showed an outstanding stability at 8 and 30°C with half-lives of 77 and 18 d, respectively.

Kinetics and the influence of galactose and glucose on BgaPw activity

The apparent Michaelis-Menten constant $K_M$ was investigated in milk which was diluted using the so-called “Novo buffer” (Pessela et al., 2003). The Novo buffer was designed to reproduce the ionic composition of milk. However, the Novo buffer does not account for the reproduction of the milk proteins although proteins would have an influence on the activity results (Greenberg and Mahoney, 1984). Thus, Novo buffer is a compromise focusing on the ionic strength of milk. The activity of the BgaPw (at 37°C, pH 7) measured in Novo buffer was 57% in comparison to the phosphate
buffer normally used. The dilution with Novo buffer was necessary to obtain lower lactose concentrations than milk originally contains (about 140 mM), which was required for the kinetic experiments determining the $K_M$. The resulting $K_M$ value for lactose in milk diluted with Novo buffer was determined to be as low as 0.63 ± 0.045 mM (Figure 4) and no substrate inhibition was observed.

Next, kinetic studies were undertaken to investigate a possible product inhibition by galactose or glucose. The influence of galactose on the BgaPw activity was done at 2 different lactose concentrations (125 and 50 mM) and 2 different galactose concentrations (125 and 200 mM). Two other β-galactosidases known from literature, so-called M1 from metagenome (Erich et al., 2015) and Opti-Lactase LX from *Kluyveromyces lactis* (Optiferm GmbH, Oy-Mittelberg, Germany), were used for comparison. The experiments were done under the same conditions in Novo buffer at 8°C and pH 6.7.

Table 1 shows the relative enzyme activities of the 3 β-galactosidases. The BgaPw was the only one which was activated by galactose in all 3 approaches (from 108 to 115%). The metagenomic M1 was second best, and was slightly inhibited by galactose at equimolar concentrations to lactose of 125 mM (96.7%) but also activated in the 2 other cases (101 and 105%). By contrast, Opti-Lactase LX from *K. lactis* was clearly inhibited by galactose in all 3 approaches.

In addition, the influence of both products, galactose and glucose, on the β-galactosidase activity of BgaPw was investigated in more detail (Figure 5). Surprisingly, glucose achieved an even higher activation, up to about 144%, on BgaPw than galactose. Thus, both products showed an activating effect on BgaPw.

**Lactose hydrolysis in milk**

Finally, a comparative lactose hydrolysis study with the 3 β-galactosidases mentioned previously was done in milk on a 30 mL scale at 8°C (Figure 6). An equal amount of β-galactosidase activity was used (2.7 nkat$_{\text{Lactose, sC/mL}}$). Complete hydrolysis of lactose was determined with BgaPw after 72 h. By comparison, M1 and Opti-Lactase performed worse, reaching only about 90% lactose hydrolysis in the same time. Thus, BgaPw seems to be an attractive alternative for industrial use.

**GOS Formation**

The feature of transgalactosylation activity using BgaPw was studied with pharma-lactose (400 g/L, 1.17 M) in potassium phosphate (100 mM, pH 6.75), MgCl$_2$ (5 mM) at 40°C for 24 h. The time course of the lactose, glucose and galactose concentrations analyzed is shown in Figure 7. The lactose concentration decreased constantly over the reaction time to a final concentration of 131 g/L (383 mM), thus, 65.5% lactose was converted. The glucose and galactose concentration increased differently over the reaction time, whereby more free glucose than galactose was released (89.5 g$_{\text{glucose/L}}$, 497 mM; 39.0 g$_{\text{galactose/L}}$, 216 mM). Hence, a theoretical formation of GOS of about 144 g/L was achieved (36.0% yield).
A new β-galactosidase (BgaPw) from a psychrophilic bacterium was produced recombinantly in *E. coli* and partially characterized with a focus on the lactose conversion in milk. The BgaPw showed good activity, a high substrate affinity to lactose and a superior stability in milk at a low temperature and neutral pH. The activation of BgaPw by galactose and glucose was exceptional (Fischer and Lutz-Wahl 2020).

Enzymes derived from psychrophilic microorganisms are often efficient catalysts at low temperatures and possess a great potential for biotechnological applications. Compared with mesophilic and thermophilic homologs, psychrophilic (cold-adapted) enzymes are expected to have a greater structural flexibility to increase the catalytic rate constant and a higher degree of conformational complementarity with substrates to allow a decrease in activation energy at low temperatures (Arcus and Mulholland 2020). Several cold-active β-galactosidases have been tested for their performance of lactose hydrolysis in milk at low temperatures (2 – 10°C) and resulted in a wide variety of hydrolysis yields between 5 and 100% (Mangiagalli and Lotti 2021).

*Paenibacillus wynnii* sp. nov. is a newly discovered species which was isolated from Antarctic soil in 2005 and was able to grow at 4°C (Rodríguez-Díaz et al., 2005). The optimal cultivation parameter of this *Paenibacillus* species is at temperatures around 20°C and a neutral pH.
The study of $K_{M,\text{lactose}}$ value for BgaPw was done in both milk and Novo buffer. The latter has a comparable salt concentration to milk and was used to adjust the lactose concentration desired by diluting the milk. The BgaPw showed a 6-fold lower $K_{M}$ value in milk than the metagenome β-galactosidase M1 (Erich et al., 2015). Other β-galactosidases belonging to the genus Paenibacillus were described in the work of Benešová et al. (2009) and Liu et al. (2017) (see Table 2). The β-galactosidase from P. thiaminolyticus, which had a sequence identity of 33% with the sequence of BgaPw, showed a very low affinity for the substrate lactose ($K_{M} = 206 \pm 5 \text{ mM}$). However, the determination was performed at pH 6 in buffer and at 37°C (Benešová et al., 2010). A higher $K_{M}$ value of 43.27 ± 1.84 mM was also determined in another study with the β-galactosidase PbGal2A from P. barengoltzii, which has a sequence identity of 36% to BgaPw. In this case, the determination of the kinetic data also took place under optimal conditions for PbGal2A (45°C, pH 7.5, phosphate buffer) (Liu et al., 2017). Another β-galactosidase GalM found in an aquatic habitat metagenome (Thakur et al., 2022) and the β-galactosidase from Lactococcus lactis (Vincent et al., 2013) demonstrated comparable $K_{M}$ values in buffer systems below 1 mM. However, the conditions, for example, pH, temperature and buffer salts, under which the $K_{M}$ determinations were carried out differed from each other.

The aspect of product inhibition plays a decisive role when it comes to the industrial application of an enzyme. Here, a slightly increase in β-galactosidase activity was observed with BgaPw in the presence of galactose up to 200 mM, while the relative activity remained constant with M1 and decreased with the β-galactosidase from K. lactis (Opti-Lactase LX). A comparable study was performed with the β-galactosidase GalM, although here, the synthetic substrate oNPGal was used instead of lactose and the studies were done under defined buffer conditions. No inhibition was detected here either in the presence of glucose or galactose (up to 200 mM). In the study from Kim et al. (2004) the influence of different lactose and galactose concentrations on the β-galactosidase activity of the recombinant β-galactosidase from K. lactis was also investigated but under different conditions (pH 8.0 and 37°C). Galactose showed an inhibitory effect at low concentrations, whereas at higher concentrations of galactose (50 and 100 mM) and lactose (100 and 200 mM) no inhibition was seen. The effect of glucose was also investigated in the study from Kim et al. (2004) using the synthetic substrate oNPGal. Here, a slight activation was observed at low oNPGal concentrations (5 mM) and glucose concentrations of up to 50 mM, but this did not improve when the glucose concentration...
### Table 2: Comparative analysis of β-galactosidases reported previously

<table>
<thead>
<tr>
<th>Source</th>
<th>pH_{opt}</th>
<th>T_{max} [°C]</th>
<th>rel. EA at T_{max} [-10°C] [°C]</th>
<th>K_{MM, lactose} [mM]</th>
<th>Lactose hydrolysis in milk</th>
<th>GOS formation in milk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paenibacillus wynni</em></td>
<td>7.0</td>
<td>45</td>
<td>~25</td>
<td>0.34 ± 0.084 (buffer, 8°C)</td>
<td>&gt;99.7% (72 h, 8°C, 2.7 nkat/mL)</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td><em>Paenibacillus barengoltzii</em></td>
<td>7.5</td>
<td>45</td>
<td>ND</td>
<td>0.63 ± 0.045 (milk, 8°C)</td>
<td>Almost complete (2 h, 40°C, 83.3 nkat/mL)</td>
<td>+</td>
<td>(Liu et al., 2017)</td>
</tr>
<tr>
<td><em>Paenibacillus sp. 3179</em></td>
<td>8.0</td>
<td>50</td>
<td>~18</td>
<td>43.27 ± 1.87 (buffer, 45°C)</td>
<td>ND</td>
<td>+</td>
<td>(Thøgersen et al., 2020)</td>
</tr>
<tr>
<td><em>Paenibacillus thiaminolyticus</em></td>
<td>5.5</td>
<td>65</td>
<td>ND</td>
<td>206 (buffer, 37°C)</td>
<td>ND</td>
<td>+</td>
<td>(Benešová et al., 2010)</td>
</tr>
<tr>
<td>Metagenome M1</td>
<td>7.0</td>
<td>37</td>
<td>~37</td>
<td>14.3 ± 1.6 (buffer, 8°C)</td>
<td>98.4% (144 h, 8°C, 2.7 nkat/mL)</td>
<td>+</td>
<td>This study, (Erich et al., 2015)</td>
</tr>
<tr>
<td>Metagenome Gal_M</td>
<td>6 - 7</td>
<td>50</td>
<td>~20</td>
<td>0.7 (buffer)</td>
<td>Complete (4 h, 50°C, 33.3 nkat/mL)</td>
<td>—</td>
<td>(Thakur et al., 2022)</td>
</tr>
<tr>
<td>Marine Metagenome</td>
<td>7.0</td>
<td>50</td>
<td>~5</td>
<td>3.0 (buffer, 50°C)</td>
<td>80% (24 h, 10°C)</td>
<td>+</td>
<td>(Sun et al., 2022)</td>
</tr>
<tr>
<td><em>Arthrobacter</em></td>
<td>8.0</td>
<td>10</td>
<td>100</td>
<td>50 (buffer, 10°C)</td>
<td>ND</td>
<td>ND</td>
<td>(Nakagawa et al. 2006a)</td>
</tr>
<tr>
<td><em>psychrobactophilus F2</em></td>
<td>8.0</td>
<td>10</td>
<td>100</td>
<td>50 (buffer, 10°C)</td>
<td>ND</td>
<td>ND</td>
<td>(Nakagawa et al. 2006a)</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp. C2-2</td>
<td>8.0</td>
<td>30</td>
<td>ND</td>
<td>3.8 (buffer, 10°C)</td>
<td>86% (24 h, 10°C)</td>
<td>ND</td>
<td>(Yao et al., 2019)</td>
</tr>
<tr>
<td><em>Alteromonas</em> sp. ML117</td>
<td>8.0</td>
<td>40</td>
<td>ND</td>
<td>15 (buffer, 10°C)</td>
<td>ND</td>
<td>ND</td>
<td>(Li et al., 2020)</td>
</tr>
<tr>
<td><em>Planococcus</em> sp.</td>
<td>8.0</td>
<td>40</td>
<td>~10</td>
<td>15 (buffer, 10°C)</td>
<td>ND</td>
<td>ND</td>
<td>(Sheridan and Brenchley 2000)</td>
</tr>
<tr>
<td><em>Pseudoalteromonas</em></td>
<td>8.5</td>
<td>45</td>
<td>~8</td>
<td>2.5 (buffer, 25°C)</td>
<td>33% (5 h, 4°C, 23 nkat/mL)</td>
<td>ND</td>
<td>(Hoyoux et al., 2001)</td>
</tr>
<tr>
<td><em>Guehomyces pullulans</em></td>
<td>4.0</td>
<td>50</td>
<td>~25</td>
<td>50.5 (buffer, 10°C)</td>
<td>80% (96 h, 10°C, 166.67 nkat/mL)</td>
<td>ND</td>
<td>(Nakagawa et al. 2006b)</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> IL1403</td>
<td>6.0 - 7.5</td>
<td>15 - 55</td>
<td>~60 - 75</td>
<td>0.84 (buffer, 10°C)</td>
<td>98% (9 h, 4 and 10°C)</td>
<td>ND</td>
<td>(Vincent et al., 2013)</td>
</tr>
<tr>
<td><em>Klyuyveromyces lactis</em></td>
<td>6.5</td>
<td>40</td>
<td>~30</td>
<td>20.4 ± 1.9 (buffer, 8°C)</td>
<td>98.8% (149 h, 8°C, 2.7 nkat/mL)</td>
<td>+</td>
<td>This study (Erich et al., 2015)</td>
</tr>
</tbody>
</table>

1 detection limit of lactose is 0.13 g/L; 2 detection limit of lactose is not given; 3 data not shown; +: detected, -: not detected, n.d.: not determined.
further increased. Similar experiments were carried out in this study with BgaPw, but in Novo buffer at 8°C. Thereby, a slight increase in BgaPw activity (a factor of 1.18) was observed in the presence of galactose up to a concentration of 200 mM. An even higher activation was observed in the presence of glucose (a factor of 1.44). These results showed the promising potential of BgaPw to be used in lactose hydrolysis in milk at 8°C, which was done in comparison to M1 and the β-galactosidase from *K. lactis* (Opti-Lactase LX). As supposed, the BgaPw hydrolyzed >99.7% of the lactose in milk after 72 h, whereas the metagenome β-galactosidase M1 and the *K. lactis* β-galactosidase reached 89.7 and 90.2% of lactose hydrolysis, respectively. Furthermore, it would be interesting to investigate the GOS formation of the BgaPw at the end of the hydrolysis in the future.

The lactose was completely hydrolyzed after only 2 h in another case of lactose hydrolysis in diluted milk (about 32 g/L lactose) with the β-galactosidase from *P. barengoltzii*. But there, the hydrolysis was done very differently, with a more than 10-fold higher β-galactosidase activity in 1 mL scale at 40°C (Liu et al., 2017). Thus, the results are incomparable.

In another investigation, the β-galactosidase from *Lactococcus lactis* hydrolyzed 98% of lactose in milk over a 9 h period at 4 and 10°C (Vincent et al., 2013). However, a comparison is not possible due to missing information about the amount of enzyme used. The metagenome β-galactosidase Ga1M1, which also had a low $K_M$, was able to hydrolyze lactose to 95% after 24 h at 15°C (Thakur et al., 2022). Here, an activity of 33.3 nkat/mL was used, that is about 12-fold more than in this study with the BgaPw.

Lastly, the GOS formation was investigated quantitatively in the conversion of Pharma-lactose with BgaPw. A theoretical GOS yield of 36% was achieved. The β-galactosidase from *P. barengoltzii* also possessed transgalactosylation activity and achieved 47.9% GOS but, as mentioned above, under incomparable conditions (Liu et al., 2017). Other cold-active β-galactosidases also showed transgalactosylation activity but the GOS formation was only detected by thin-layer chromato- graphy (see Table 2).

In conclusion, the recombinant BgaPw was revealed to be a cold-active β-galactosidase with a sufficient activity of 25% at 5°C and a remarkable half-life of 77 d at 8°C. These evolutionary cold-adapted properties combined with a very low $K_M$, no product inhibition but activation, makes the BgaPw a strong competitor in the lactose hydrolysis of milk to all known β-galactosidases described in literature. In addition, the enzyme can also be used for GOS production.

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