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A novel cold-active β-D-galactosidase with transglycosylation activity from the Antarctic *Arthrobacter* sp. 32cB – Gene cloning, purification and characterization

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ABSTRACT

A gene encoding a novel β-D-galactosidase from the psychrotolerant Antarctic bacterium *Arthrobacter* sp. 32cB was isolated, cloned and expressed in *Escherichia coli*. The active form of recombinant β-D-galactosidase consists of two subunits with a combined molecular weight of approximately 257 kDa. The enzyme's maximum activity towards *o*-nitrophenyl-β-D-galactopyranoside was determined as occurring at 28 °C and pH 8.0. However, it exhibited 42% of maximum activity at 10 °C and was capable of hydrolyzing both lactose and *o*-nitrophenyl-β-D-galactopyranoside at that temperature, with K_m values of 1.52 and 16.56 mM, and k_{cat} values 30.55 and 31.84 s^{-1}, respectively. Two units of the enzyme hydrolyzed 90% of the lactose in 1 mL of milk at 10 °C in 24 h. The transglycosylation activity of *Arthrobacter* sp. 32cB β-D-galactosidase was also examined. It synthesized galactooligosaccharides in a temperature range from 10 to 30 °C. Moreover, it catalyzed the synthesis of heterooligosaccharides such as lactulose, galactosyl-xylose and galactosyl-arabinose, alkyl glycosides, and glycosylated salicin from lactose and the appropriate acceptor at 30 °C. The properties of *Arthrobacter* sp. 32cB β-D-galactosidase make it a candidate for use in the industrial removal of lactose from milk and a promising tool for the glycosylation of various acceptors, especially those which are thermosensitive.

1. Introduction

Glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. These enzymes are currently classified into 133 families (www.cazy.org/Glycoside-Hydrolases.html). β-D-Galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23) catalyze the hydrolysis of terminal non-reducing β-galactose residues in β-D-galactosides and they are categorized within the glycoside hydrolase (GH) families 1, 2, 35 and 42. β-D-Galactosidases are widely distributed in nature and can be isolated from various sources, such as plants, animal organs, archaea, bacteria, yeasts, and fungi. Most of them are produced by microorganisms, including mesophiles, thermophiles, hyperthermophiles and psychrophiles.

The cold-active β-D-galactosidases found in cold-adapted microorganisms offer potential for the development of new industrial applications and are therefore the subject of intensive study. Some microorganisms harbor more than one gene encoding enzyme with β-D-galactosidase activity. A psychrotolerant *Arthrobacter* sp. B7 produces three β-D-galactosidase isozymes belonging to the GH families 2, 35 and 42 [1–4]. *Arthrobacter* sp. ON14 possess two genes encoding β-D-galactosidases belonging to GH2 and GH42 [5]. Two different cold-active β-D-galactosidases, members of GH35 and GH42, have been isolated from a lactic acid bacterium, *Carnobacterium piscicola* BA [6,7]. β-D-Galactosidases belonging to the GH family 42 have been obtained from psychrotolerant bacteria of the genus *Planococcus* and *Arthrobacter* [8–10]. Cold-active β-D-galactosidases classified into the GH2 family have been produced by *Arthrobacter* sp., *Pseudoalteromonas* sp., *Paracoccus* sp., *Flavobacterium* sp. and *Alkalilactibacillus ikkense* [11–21]. Moreover, cold-adapted β-D-galactosidase not assigned to any of the known GH families was obtained from *Halomonas* sp. S62 [22]. It has been shown that cold-active β-D-galactosidases belonging to the GH family 2 have the greatest potential for use in industry, since they demonstrate the highest activity towards natural substrate lactose.

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The major industrial application of β-D-galactosidase is in the production of low-lactose milk for people with lactose malabsorption. Lactose malabsorption and symptoms of milk product intolerance are the most common alimentary tract disorders. However, dairy produce is the primary source of calcium for many individuals, and a low intake of those products is thus associated with a higher risk of fractures or even of osteoporosis in the presence of other risk factors for this disease [23]. New technologies to produce lactose-reduced or lactose-free dairy products have therefore been developed, including lactose hydrolysis or separation [24]. The process of enzymatic lactose hydrolysis in milk is simple and requires no special equipment in dairy plants. Generally, it is conducted under refrigerated conditions for approximately 24 h using soluble β-D-galactosidase isolated from mesophilic yeast *Kluyveromyces lactis*. The main disadvantage of this enzyme is its low activity under process conditions, since it is optimally active at approximately 50 °C and exhibits no more than 10% of its maximum activity at temperatures below 10 °C (Ha-Lactase product information, Chr. Hansen A/S, Denmark). As a result, new β-D-galactosidases displaying high activity at low temperatures are still being sought.

Another important application of β-D-galactosidase is the synthesis of galactooligosaccharides (GOS) from lactose. Many different di-, tri-, tetra- and pentasaccharides have been identified as products of the enzymatic transgalactosylation of lactose catalyzed by β-D-galactosidase [25]. Galactooligosaccharides are non-digestible oligosaccharides (NDOs); they are not digested by the enzymes of the small intestine, but are fermented by bacteria in the large intestine. GOS are applied as functional food ingredients which provide the host with many health benefits, such as low cariogenicity, low caloric content, insulin independent metabolism, and stimulation of the growth and metabolism of beneficial colonic microorganisms of the genus *Bifidobacterium* and *Lactobacillus*; in other words, prebiotic properties. These microorganisms have been linked to increased resistance to infection and diarrhoeal disease, and stimulation of immune system activity, as well as protection against cancer. Bifidobacteria are also known to excrete a range of water soluble vitamins [26,27]. In addition to their prebiotic properties, the ability of GOS to inhibit the adherence of enteropathogenic *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to tissue culture cells has also been described [28,29]. The production of galactooligosaccharides from lactose by means of microbial β-D-galactosidases has been conducted with many enzymes originated from mesophiles, thermophiles and hyperthermophiles, but studies on GOS synthesis catalyzed by β-D-galactosidases from psychrophiles and psychrotolerant microorganisms are scant [30]. The formation of GOS during lactose hydrolysis has only been noted for cold-active enzymes from *A. psychrolactophilus* F2 [31], *Arthrobacter* sp. C2-2 [14], and *A. ikkense* [21]. Yet the low temperature of GOS synthesis offers some advantages, such as the elimination of nonenzymatic browning products formed at high temperatures, for instance, as well as its energy saving potential.

β-D-Galactosidases have been also used to produce heterooligosaccharides (HOS) by way of the transfer of galactosyl moiety to sugars other than lactose or its hydrolysis products, glucose and galactose. The best-known heterooligosaccharide is lactulose (4-*O*-β-D-galactopyranosyl-β-D-fructofuranose). It exhibits prebiotic properties and, like GOS, it is applied as a functional food ingredient. Lactulose is also used in the treatment of constipation and hepatic encephalopathy, and is applied in the diagnosis of colonic disorders by means of the hydrogen breath test. Currently lactulose is industrially produced by means of alkaline isomerization of lactose. However, this process leads to the formation of a certain amount of by-products as a result of sugar degradation under harsh conditions, namely a pH 10.5–11.5 and temperatures of between 70 and 100 °C. The enzymatic synthesis of lactulose offers much milder conditions and can be conducted in crude lactose materials such as whey or whey permeate [32]. Heterologous galactosyl transfer catalyzed by β-D-galactosidase yields a virtually unlimited diversity of oligosaccharides [33–39]. However, none of cold-active β-D-galactosidases have yet been used for this purpose.

The biosynthesis of alkyl glycosides belonging to the group of nonionic surfactants which can be used in the manufacture of detergents, cosmetics, and pharmaceuticals has also been conducted using microbial β-D-galactosidases [40,41]. Moreover, β-D-galactosidases have been shown to be capable of catalyzing the transglycosylation of various chemicals such as antibiotics [42], ergot alkaloids [43] and flavonol glycoside myricitrin [44].

This study was conducted with a view to obtaining a cold-active β-D-galactosidase exhibiting not only high hydrolytic activity towards lactose, but also the capability of transferring glycosyl. To this end, a psychrotolerant *Arthrobacter* sp. 32cB was isolated from a sample of Antarctic soil and a gene encoding a β-D-galactosidase of the GH family 2 was cloned, expressed in *E. coli*, purified and characterized. The recombinant enzyme showed a high efficiency in the hydrolysis of lactose in milk under refrigerated conditions. It was also able to produce GOS at relatively low temperatures. Moreover, it tolerated a wide range of acceptors and catalyzed the transglycosylation of saccharides, alcohols and complex chemicals such as salicin.

2. Materials and methods

2.1. Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Biosynth AG (Switzerland). *o*-Nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucouronide, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-L-arabinopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, L-arabinose, D-xylose, lactulose, dithiothreitol (DTT), ethylenediaminetetraacetic acid sodium salt (EDTA), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and tris(2-carboxyethyl)phosphine (TCEP) were supplied by Sigma (USA). Salicin was obtained from AppliChem GmbH (Germany). Peptone K, yeast extract and bacteriological agar were purchased from BTL (Poland). All other chemicals were supplied by POCH (Poland).

2.2. Isolation and characterization of bacterial strain exhibiting β-D-galactosidase activity

A 5 g of Antarctic soil collected in the neighbourhood of the Henryk Arctowski Polish Antarctic Station on King George Island, in the Southern Shetlands (62°10′ S, 58°28′ W) was dissolved in 45 mL of water containing 0.5 g of marine salt. After decantation 100 μL of the supernatant was spread out on LAS agar plates (0.5% peptone K, 0.25% yeast extract, 1% marine salt, 1.5% bacteriological agar) supplemented with X-gal (40 μg mL^{-1}) and IPTG (24 μg mL^{-1}). The plates were incubated at 20 °C for 72–120 h. After incubation the colonies producing β-D-galactosidase turned blue.

Growth properties of the analyzed strain were determined in an LBS medium (0.5% peptone K, 0.25% yeast extract, 1% marine salt). The proteolytic, lipolytic and amylolytic activities were examined

at 25 °C on LAS agar plates containing skimmed milk, tributyrin and starch, respectively.

The β-D-galactosidase activity towards lactose was determined by HPLC. The bacterial strain was grown in an LBS medium supplemented with 1 mM IPTG at 25 °C for 48 h with agitation (200 rpm). The cells were then harvested by centrifugation (10,000 × *g*, 20 min, 4 °C) and the cell pellet was washed with a 20 mM potassium phosphate buffer with a pH of 7.0. Cell lysis was achieved by grinding with aluminium oxide (Sigma, USA). After grinding, a 20 mM potassium phosphate buffer with a pH of 7.0 was added and the sample was centrifuged (10,000 × *g*, 30 min, 4 °C) to remove cell debris and aluminium oxide. The supernatant thus obtained was mixed with milk (UHT, 2% fat) in a ratio of 2 to 8 and incubated at 10 °C for 24 h. 20% H_2SO_4 was then added at a ratio of 17 μL to 1 mL and the sample was centrifuged (10,000 × *g*, 30 min, 4 °C) to remove denatured proteins. The quantities of lactose, D-glucose and D-galactose were determined using an Aminex HPX-87H column (Bio-Rad, USA), 5 mM H_2SO_4 as a mobile phase and the Agilent 1200 Series chromatograph with Refractive Index Detector.

2.3. Identification of the 32cB strain

Genomic DNA from strain 32cB was used as a template to amplify the 16S rDNA gene with the primers fD2 5′ CCGAATTCGTCGACAACACGGCTACCTTGTTACGACTT 3′ and rP1 5′ CCCGGGATCCAAGCTTAGAGTTTGATCCTGGCTCAG 3′ [45]. A PCR reaction was performed in a mixture containing 0.2 μM of each primer, 0.2 μg of genomic DNA, 200 μM of each dNTP, and 1 U of DNA polymerase *Hypernova* (DNA-Gdańsk II, Poland) in 1× PCR buffer (10 mM Tris–HCl pH 8.8, 50 mM KCl, 3 mM $MgCl_2$, 0.15% Triton X-100). The reaction mixture was incubated for 3 min at 94 °C, followed by 30 cycles at 94 °C for 1 min, 72 °C for 2.5 min, and a final incubation of 5 min at 72 °C, using a thermal cycler TGradient (Biometra, Germany). The PCR product was purified from an agarose gel band using a DNA Gel-Out kit (A&A Biotechnology, Poland), cloned into a pJET1.2/blunt vector (Fermentas, Lithuania) and sequenced (Genomed, Poland).

2.4. Isolation and sequencing of the β-D-galactosidase gene from Arthrobacter sp. 32cB

The gene encoding the β-D-galactosidase from *Arthrobacter* sp. 32cB was isolated using the PCR technique. In order to obtain a partial sequence of the β-D-galactosidase gene from *Arthrobacter* sp. 32cB, sequences encoding β-D-galactosidases of *Arthrobacter chlorophenolicus* A6 [GenBank: CP001341], *Arthrobacter psychrolactophilus* F2 [GenBank: AB243756], *Arthrobacter* sp. FB24 [GenBank: CP000454], *Arthrobacter* sp. 20B [GenBank: FJ217701], *Arthrobacter* sp. SB [GenBank: AY327444], *Arthrobacter* sp. C2-2 [GenBank: AJ457162] and *Arthrobacter* sp. ON14 [GenBank: HM178943] obtained from the GenBank database, were aligned using the ClustalX program, version 1.8. On the basis of the alignment, degenerated primers ArthrBgF 5′ GTGGTGGCTSCCBGGSATCTTCCG 3′ and RBgalIn 5′ TCRTTGCCSAGSGACCACAT 3′ were designed and synthesized. The PCR reaction was performed in a mixture containing 0.2 μM of each primer, 0.2 μg of *Arthrobacter* sp. 32cB genomic DNA, 200 μM of each dNTP, and 5 U of DNA polymerase *Taq* (EURx, Poland) in 1× PCR buffer (50 mM Tris–HCl pH 9.0, 50 mM NaCl, 1.5 mM $MgCl_2$). The reaction mixture was incubated for 3 min at 95 °C, followed by 30 cycles at 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, and a final incubation of 5 min at 72 °C. The PCR product thus obtained was then purified, cloned into the pJET1.2/blunt vector (Fermentas, Lithuania) and sequenced. The GenomeWalker™ Universal Kit (Clontech Laboratories, USA) was then used to obtain the 5′ and 3′ ends of *Arthrobacter* sp. 32cB β-D-galactosidase gene. The *Arthrobacter* sp. 32cB genomic DNA was first digested with the *Eco*RV or *Eco*47III restriction endonuclease and ligated to the GenomeWalker Adaptor. Two PCR amplifications were then performed. The primary reaction was carried out using adaptor-ligated genomic DNA fragments as a template and two primers; the outer adaptor primer (AP1) provided in the kit and 32cBgN1 primer 5′ GAGGATGCCTTCGCCTGTGATGATGTGGTCGTAGC 3′ or 32cBgC1 primer 5′ CCAGTGGGAGGATGCCCTGGTAGACCGCAT 3′, designed on the basis of the partial sequence of *Arthrobacter* sp. 32cB β-D-galactosidase gene previously obtained. The reaction mixture also contained 200 μM dNTPs and 1 U of DNA polymerase Marathon (A&A Biotechnology, Poland) in 1× Marathon buffer. DNA amplification was performed using the following parameters: (94 °C – 0.5 min, 72 °C – 3 min) 7 cycles, (94 °C – 0.5 min, 67 °C – 3 min) 32 cycles and 67 °C for 7 min after the final cycle. The primary PCR mixture was then used as a template for a secondary PCR with the nested adaptor primer (AP2) provided in the kit and 32cBgN2 primer 5′ CCGGCGTGGACGAAGACGTCGTCAATGCCG 3′ or 32cBgC2 primer 5′ GCATGCGCCGCACGGTTGAGCGCGACAAGA 3′. The nested PCR mixture also contained 200 μM dNTPs and 1 U of DNA polymerase Marathon in 1× Marathon buffer. DNA amplification was performed using the following parameters: (94 °C – 0.5 min, 72 °C – 3 min) 5 cycles, (94 °C – 0.5 min, 67 °C – 3 min) 20 cycles and 67 °C for 7 min after the final cycle. In the third step the nested PCR products were purified from an agarose gel bands, cloned into the pZErO-2 vector (Invitrogen, USA) and sequenced. Following this, three fragments of *Arthrobacter* sp. 32cB genomic DNA sequences were alignment and the full sequence of the β-D-galactosidase gene was obtained.

2.5. Construction of the E. coli expression system for the production of Arthrobacter sp. 32cB β-D-galactosidase

On the basis of the known sequence of the β-D-galactosidase gene from *Arthrobacter* sp. 32cB (GenBank accession no. KJ439699), the specific primers for PCR amplification were designed and synthesized. The gene was amplified using forward primer F232cBNco 5′ TCTACC**ATG**GCTGTCGAAACACCGTCCGCGCTGGCGGAT 3′, and reverse primer R32cBHind 5′ TGACAAGCT**TCA**GCTGCGCACCTTCAGGGTCAGTATGAAG 3′ (containing *Nco*I and *Hin*dIII recognition sites, underlined). The start and stop codons are given in bold. The PCR reaction mixture contained 0.2 μM of each primer, 0.2 μg of *Arthrobacter* sp. 32cB genomic DNA, 200 μM of each dNTP, and 5 U of DNA polymerase *Taq* (EURx, Poland) in 1× PCR buffer (50 mM Tris–HCl pH 9.0, 50 mM NaCl, 1.5 mM $MgCl_2$). The reaction mixture was incubated for 3 min at 95 °C, followed by 5 cycles at 95 °C for 1 min, 65 °C for 1 min, 72 °C for 3 min and 20 cycles at 94 °C for 30 s, 72 °C for 4 min, and a final incubation of 5 min at 72 °C using a thermal cycler TGradient (Biometra, Germany). The PCR product was then purified and digested with *Eco*RI endonuclease (Fermentas, Lithuania). The restriction fragments were separated by electrophoresis, purified from an agarose gel bands using a DNA Gel-Out kit (A&A Biotechnology, Poland), digested with *Nco*I or *Hin*dIII endonucleases (Fermentas, Lithuania) and cloned into a pBAD/Myc-His A vector (Invitrogen, USA) digested with *Nco*I and *Hin*dIII restriction enzymes. The resulting recombinant plasmid pBAD-Bgal 32cB containing the *Arthrobacter* sp. 32cB β-D-galactosidase gene under the control of the P_{BAD} promoter was used to transform chemically competent *E. coli* TOP10 cells (Invitrogen, USA). The clones were selected on Luria-Bertani agar plates supplemented with ampicillin (100 μg mL^{-1}), X-Gal (40 μg mL^{-1}) and L-arabinose (200 μg mL^{-1}). The plates were incubated at 37 °C for 12 h and then transferred to 22 °C. After 10–12 h of incubation at 22 °C the recombinant colonies producing β-D-galactosidase turned blue. Plasmid DNA from positive transformants was isolated

using the Plasmid Miniprep DNA Purification Kit (EURx, Poland) and sequenced (Genomed, Poland).

2.6. Production and purification of the recombinant Arthrobacter sp. 32cB β-D-galactosidase

Expression of the *Arthrobacter* sp. 32cB β-D-galactosidase gene was performed in the *E. coli* LMG194 cells carrying the pBAD-Bgal 32cB plasmid.

The cells were grown for 18 h at 37 °C in LB medium (1% peptone K, 0.5% yeast extract, 1% NaCl) containing 100 μg mL^{-1} ampicillin. Then 20 mL of culture was added to 1 L of LB medium containing 100 μg mL^{-1} ampicillin and cultivated at 30 °C to OD_{600} of 0.5. 20% L-arabinose solution was then added to the final concentration of 0.02% and cultivation was continued for 15 h to OD_{600} of 3.8 ± 0.2. The culture was then centrifuged (6000 × *g*, 15 min, 4 °C), the cell pellet was resuspended in 50 mL of buffer A (20 mM potassium phosphate buffer pH 6.0 containing 50 mM KCl), and the cells were disrupted by sonication.

After centrifugation (12,000 × *g*, 35 min, 4 °C), the supernatant was applied onto a Fractogel EMD DEAE weak anion exchanger purchased from Merck (Germany) (60 mL column) equilibrated with four volumes of buffer A. The column was washed with three volumes of buffer A and the recombinant β-D-galactosidase was eluted with a linear gradient of potassium chloride (50–1050 mM) in the same buffer (four volumes of the column). Fractions containing β-D-galactosidase were pooled, dialyzed against buffer A and loaded onto a Resource Q strong anion exchanger purchased from GE Healthcare Life Sciences (Sweden). The 6 mL column had previously been equilibrated with four volumes of buffer A and the elution was performed with a linear gradient of potassium chloride (50–850 mM) in the same buffer (four volumes of the column). Fractions containing *Arthrobacter* sp. 32cB β-D-galactosidase were pooled, dialyzed against 20 mM potassium phosphate buffer pH 7.5 containing 150 mM KCl and loaded onto a Superdex™ 200 10/300 GL column (GE Healthcare Life Sciences, Sweden). The elution was performed using the same buffer. The purified protein was dialyzed against 40 mM potassium phosphate buffer pH 7.5 and concentrated using Amicon Ultra-15 30K Centrifugal Filter Device (Merck Millipore Ltd., Ireland). Glycerol was then added to the final concentration of 50% and the enzyme was stored at −20 °C until used. The enzyme can be stored without loss of activity for at least two years.

The concentration of purified protein was determined by the Bradford method [46] using bovine serum albumin (BSA) as a standard.

The molecular mass of the native *Arthrobacter* sp. 32cB β-D-galactosidase was estimated by gel filtration using a Superdex™ 200 10/300 GL column (GE Healthcare Life Science, Sweden), and bovine thyroglobulin (669 kDa), apoferritin from horse spleen (443 kDa), β-amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase from bovine erythrocytes (29 kDa) purchased from Sigma (USA) as standards.

2.7. Characterization of the Arthrobacter sp. 32cB β-D-galactosidase hydrolytic activity

The activity of purified *Arthrobacter* sp. 32cB β-D-galactosidase was determined using ONPG as a substrate. One unit of β-D-galactosidase activity was defined as being the quantity of enzyme releasing of 1 μmol *o*-nitrophenol per min at 28 °C and pH 8.0.

To determine the effect of temperature on the β-D-galactosidase's activity, the enzyme (0.8 U mL^{-1}) was incubated in 20 mM potassium phosphate buffer pH 8.0 containing 1 mg mL^{-1} ONPG at 5–55 °C for 10 min. The hydrolysis was halted by heating of the reaction mixtures at 95 °C for 5 min and the absorbance was measured at 405 nm.

For the thermal stability studies, the enzyme was incubated at various temperatures for different periods of time and the residual β-D-galactosidase activity was then measured at 28 °C and pH 8.0.

For pH-activity studies, hydrolysis of ONPG (1 mg mL^{-1}) was performed in 20 mM citrate-sodium phosphate buffer pH 4.0–6.5, 20 mM potassium phosphate buffer pH 6.0–8.0 and 20 mM glycine–sodium hydroxide buffer pH 7.8–10.0 at 28 °C for 5 min. The hydrolysis was halted by the addition of 1.5 M Na_2CO_3 to the final concentration of 450 mM and the absorbance was measured at 405 nm.

The effects of various metal ions and EDTA on recombinant enzyme activity were measured by assaying the enzyme in 20 mM HEPES pH 8.0 containing 5 mM $CaCl_2{\cdot}2H_2O$, $CoCl_2{\cdot}6H_2O$, $MgCl_2{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, $NiCl_2{\cdot}6H_2O$ or EDTA at 28 °C for 5 min. The hydrolysis of ONPG was halted by heating of the reaction mixtures at 95 °C for 5 min and the absorbance was measured at 405 nm.

The effects of selected reagents on the β-D-galactosidase activity were measured by assaying the enzyme in 20 mM potassium phosphate buffer pH 8.0 containing 10 mM cysteine, DTT, glutathione or TCEP at 28 °C for 5 min. The hydrolysis of ONPG was halted by the addition of 1.5 M Na_2CO_3 to the final concentration of 450 mM and the absorbance was measured at 405 nm.

Substrate specificity was estimated using *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucouronide, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-L-arabinopyranoside, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-α-D-glucopyranoside. Activity determination was carried out under standard conditions in 20 mM potassium phosphate buffer pH 8.0 at 28 °C. Reactions stopped after 5 min by the addition of 1.5 M Na_2CO_3 to the final concentration of 450 mM.

To determine the kinetic parameters, the hydrolysis of ONPG and lactose was performed in 20 mM potassium phosphate buffer pH 8.0 containing 1–5 mM ONPG and 1–20 mM lactose at 10, 20 and 30 °C. To estimate the amount of glucose released during lactose hydrolysis the Glucose oxidase/peroxidase reagent (Sigma, USA) was used. The K_m and V_{max} values were obtained using the Lineweaver–Burk equation.

The effects of D-glucose and D-galactose on the β-D-galactosidase activity were measured by assaying the enzyme in 20 mM potassium phosphate buffer pH 8.0 containing 5 mM ONPG and 5–150 mM D-glucose or 5–50 mM D-galactose at 20 °C for 5 min. The hydrolysis of ONPG was halted by the addition of 1.5 M Na_2CO_3 to the final concentration of 450 mM and the absorbance was measured at 405 nm. The K_i values were determined at 20 °C using 20 and 50 mM concentrations of the inhibitor D-galactose with lactose and ONPG as substrates.

Analysis of the hydrolysis of lactose in milk by the *Arthrobacter* sp. 32cB β-D-galactosidase was performed using 1 or 2 U of the enzyme per 1 mL of milk (UHT, 2% fat). The reaction mixtures were incubated at 10 °C for 24 h. After each 2-h period for 12 h and after 24 h, 1 mL samples were collected and the hydrolysis was halted by the addition of 20% H_2SO_4. Lactose, D-glucose and D-galactose concentrations were determined by HPLC.

2.8. Characterization of the Arthrobacter sp. 32cB β-D-galactosidase transglycosylation activity

GOS synthesis was performed using 1 or 2 U of the *Arthrobacter* sp. 32cB β-D-galactosidase per 1 mL of 20 mM potassium phosphate buffer pH 8.0 containing 29–584 mM lactose. The reaction

mixtures were incubated at 10, 20 and 30 °C for 24 h. After each 2-h period for 10 h and after 24 h, 0.1 mL samples were collected and the enzyme was inactivated by incubation at 95 °C for 5 min. Afterwards samples were diluted and products were separated by thin layer chromatography. Aliquots of 1 μL were applied on TLC plates (Merck, Germany) and separated using 1-butanol:ethanol:2-propanol:water (2:3:3:2) as a mobile phase. Detection was achieved by spraying with cerium-molybdate stain (sodium molybdate 3.74 g, cerium sulfate 1.21 g, sulfuric acid 6.3 mL, distilled water 83.5 mL) and heating at 100 °C.

Lactulose, galactosyl-xylose and galactosyl-arabinose were synthesized using 1 or 2 U of the enzyme per 1 mL of 20 mM potassium phosphate buffer pH 8.0 containing 29–438 mM lactose and the same concentrations of D-fructose, D-xylose and L-arabinose, respectively. The reaction mixtures were incubated at 30 °C for 24 h with intensive shaking. Transgalactosylation reactions were terminated by thermal denaturation of the β-D-galactosidase. Samples were then diluted, applied on TLC plates modified by spraying with 30 mM boric acid and two times resolved in a system acetonitrile:water (7:2). Detection was achieved using cerium-molybdate stain.

The synthesis of alkyl glycosides was performed using 1 or 2 U of purified β-D-galactosidase per 1 mL of 20 mM potassium phosphate buffer pH 8.0 containing 29–438 mM lactose and the same concentrations of 2-propanol, 1-butanol and 1-hexanol, respectively. The reaction mixtures were incubated at 30 °C for 24 h with intensive shaking. Transgalactosylation reactions were terminated by thermal denaturation of the enzyme. Samples were diluted, applied on TLC plates and resolved in a system A 1-butanol:acetone:water (4:1:1). Plates were then dried and resolved in a system B 2-propanol:ethyl acetate:water (2:2:1). Detection was achieved using cerium-molybdate stain.

Glycosylated cyclohexanol and salicin were synthesized under standard conditions. Products were separated using butanol:ethanol:water (5:3:2) as a mobile phase and detected using cerium-molybdate stain.

All the measurements were taken and the experiments carried out in triplicate.

2.9. Nucleotide sequences accession numbers

The 16S rDNA and β-D-galactosidase gene sequences reported in this article have been deposited in the GenBank database and assigned Accession Nos. KJ439698 and KJ439699, respectively.

3. Results

3.1. Isolation, characterization and identification of strain 32cB

Two bacterial strains exhibiting β-D-galactosidase activity on agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were isolated from Antarctic soil sample, and named 20 and 32cB. The activity of the enzyme in the cell extracts was confirmed using milk as a substrate. After 24 h of incubation at 10 °C, the yields of lactose hydrolysis were 1 and 25% for isolate 20 and 32cB, respectively. Thus the 32cB strain was selected for further studies. It also exhibited amylase activity, but lipase/esterase and protease activities were absent. The cells were Gram-positive and displayed a rod-coccus life cycle. The agar colonies were circular, smooth and creamy in colour. The optimum growth of strain 32cB was observed at 24–26 °C and no growth occurred at 37 °C. Hence, isolate 32cB can be categorized as a psychrotolerant microorganism.

The genus of strain 32cB was assessed on the basis of the 16S ribosomal DNA gene sequence. Alignment of the 16S rDNA gene of isolate 32cB (GenBank, accession no. KJ439698) with the sequences available in the GenBank database demonstrated that the strain 32cB should be classified as an *Arthrobacter* sp., and that its closest relative is *Arthrobacter oxydans* DSM 20119 (99.9% identity, 100% query coverage).

Table 1
Comparison of the amino acid sequence of *Arthrobacter* sp. 32cB β-D-galactosidase with amino acid sequences of GH2 β-D-galactosidases from bacteria of the genus *Arthrobacter* and *Escherichia coli*.

Organism	GenBank accession no.	Identity (%)	Similarity (%)
Arthrobacter phenanthrenivorans Sphe3	ADX74853	82.4	93.0
Arthrobacter chlorophenolicus A6	ACL41674	77.1	89.6
Arthrobacter sp. FB24	ABK05292	70.7	84.8
Arthrobacter sp. C2-2	CAD29775	46.1	69.6
Arthrobacter sp. B7	AAA69907	46.0	69.6
Arthrobacter sp. ON14	ADJ18283	46.2	69.1
Arthrobacter psychrolactophilus F2	BAF33372	45.2	68.9
Arthrobacter sp. 20B	ACI41243	44.3	67.3
Arthrobacter sp. SB	AAQ19029	43.8	67.4
Escherichia coli	ABN72582	36.0	60.4

A homology search was performed using version 3 of the FASTA program at the EBI.

3.2. Cloning, expression and purification of Arthrobacter sp. 32cB β-D-galactosidase

The degenerated primers ArthrBgF and RBgalIn were designed on the basis of the sequences encoding β-D-galactosidases belonging to the glycoside hydrolase family 2 from *A. chlorophenolicus* A6, *A. psychrolactophilus* F2, *Arthrobacter* sp. FB24, *Arthrobacter* sp. 20B, *Arthrobacter* sp. SB, *Arthrobacter* sp. C2-2, and *Arthrobacter* sp. ON14. The 699 bp DNA fragment obtained by conducting a PCR using those primers was a putative internal fragment of *Arthrobacter* sp. 32cB β-D-galactosidase gene. The unknown parts of the β-D-galactosidase gene adjacent to the known sequence were found using the GenomeWalker™ Universal Kit (Clontech Laboratories, USA). Analysis of the sequences obtained revealed an open reading frame consisting of 3033 bp, which encoded a 1010 amino acid protein with a calculated molecular mass of 109,648.4 Da and a theoretical p*I* of 4.92 (ProtParam; ExPASy Proteomics Server). A homology search, performed using version 3 of the FASTA program at the European Bioinformatics Institute, displayed an 82.4% amino acid identity and a 93.0% similarity with β-galactosidase/β-glucuronidase from *Arthrobacter phenanthrenivorans* Sphe3. The *Arthrobacter* sp. 32cB β-D-galactosidase also showed a high homology with β-D-galactosidases from *A. chlorophenolicus* A6 and *Arthrobacter* sp. FB24 (Table 1). A conserved domain search found three classical domains in the *Arthrobacter* sp. 32cB β-D-galactosidase. The first is a GH family 2 sugar binding domain (Glyco_hydro_2_N, residues 54–224). The second is a GH family 2 TIM barrel domain (Glyco_hydro_2_C, residues 312–609). The third is a β-galactosidase small chain (Bgal_small_N, residues 730–1006) [47]. The multiple sequence alignment of *Arthrobacter* sp. 32cB β-D-galactosidase (32cB-B-gal) with its counterparts from psychrotolerant *Arthrobacter* sp. C2-2 (C221-B-gal) and mesophilic *E. coli* (EC-B-gal) revealed some conserved regions and two essential catalytic residues, namely E441 and E517, corresponding to the E442 and E461 of *Arthrobacter* sp. C2-2 β-D-galactosidase and the E521 and E537 of *E. coli* β-D-galactosidase (Fig. 1) [48].

In order to produce and investigate the biochemical properties of *Arthrobacter* sp. 32cB β-D-galactosidase, a recombinant pBAD-Bgal 32cB plasmid was constructed and used for the expression of the β-D-galactosidase gene in the *E. coli* LMG194 strain, which

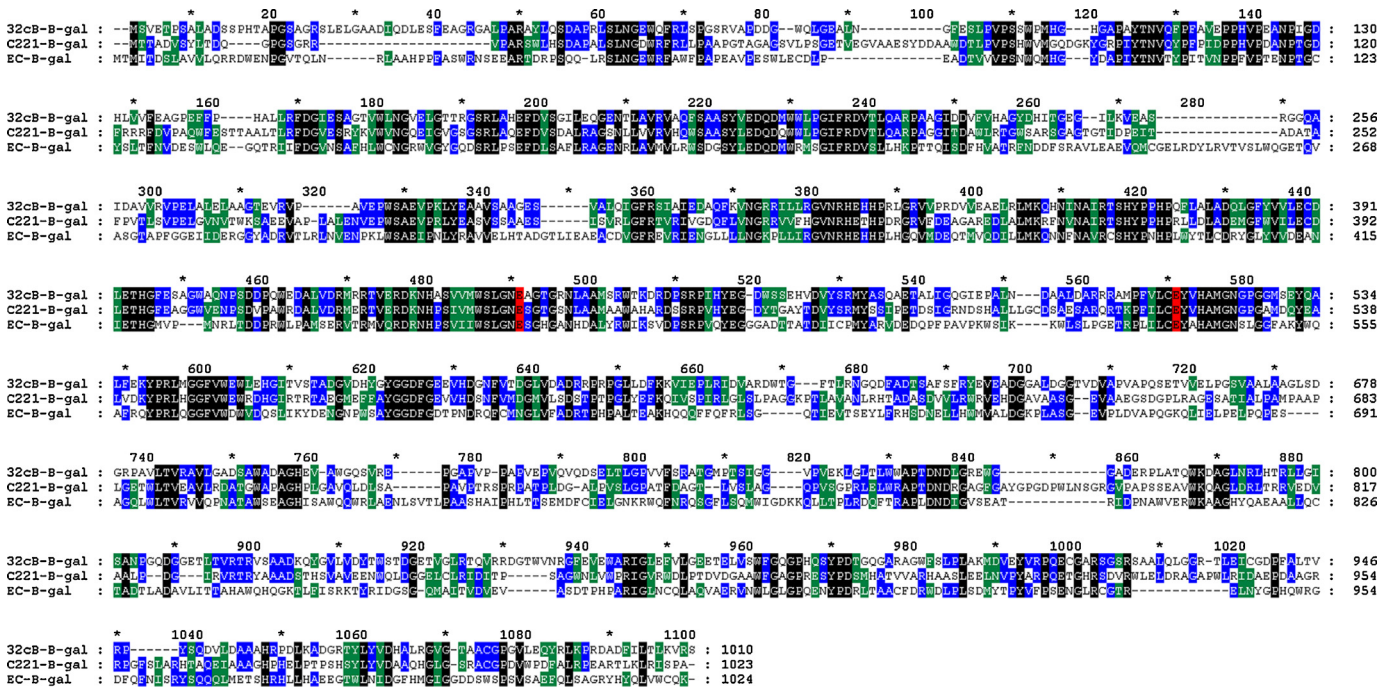

Fig. 1. Multiple sequence alignment of *Arthrobacter* sp. 32cB β-D-galactosidase with β-D-galactosidases from *Arthrobacter* sp. C2-2 and *E. coli*. 32cB-B-gal–β-D-galactosidase from *Arthrobacter* sp. 32cB, C221-B-gal–β-D-galactosidase from *Arthrobacter* sp. C2-2, EC-B-gal–β-D-galactosidase from *E. coli*. Three levels of conserved residues are indicated by black (100%), blue (80%) and green (60%) backgrounds. Catalytic residues are shaded red. The alignment was performed using Clustalx 2.0.11 program. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

is *lac*Z deficient. The recombinant enzyme was purified by means of ion exchange chromatography, using weak and strong anion exchangers, and by gel filtration. After the purification, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue, a protein band was observed migrating near 116 kDa (Fig. 2, lane 4). This concurred with the molecular mass of *Arthrobacter* sp. 32cB β-D-galactosidase deduced from the nucleotide sequence of the gene (109.6 kDa). The overexpression system and purification method applied were quite efficient, yielding 18 mg (Table 2) of *Arthrobacter* sp. 32cB β-D-galactosidase from 1 L of *E. coli* culture. The molecular mass of the native enzyme, as estimated by gel filtration, was 256.6 kDa. It has thus been assumed that the *Arthrobacter* sp. 32cB β-D-galactosidase is probably a dimeric protein.

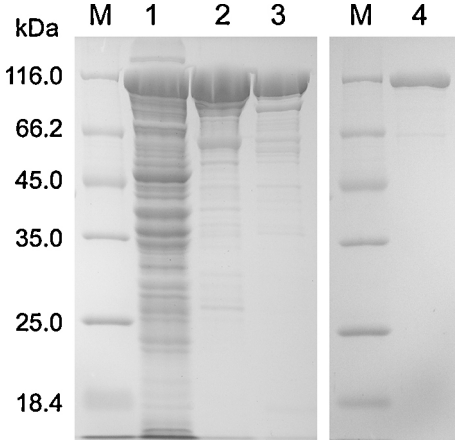

Fig. 2. SDS-PAGE analysis of the fractions obtained by expression and purification of *Arthrobacter* sp. 32cB β-D-galactosidase. Lane M – Unstained Protein Molecular Weight Marker (Fermentas): 116, 66.2, 45, 35, 25, 18.4 and 14.4 kDa, lane 1 – cell extract of *E. coli* LMG194 + pBAD-Bgal 32cB after β-D-galactosidase gene expression, lane 2 – purified β-D-galactosidase after ion exchange chromatography on Fractogel EMD DEAE column, lane 3 – purified β-D-galactosidase after ion exchange chromatography on Resource Q column, and lane 4 – purified β-D-galactosidase after gel filtration.

3.3. Properties of *Arthrobacter* sp. 32cB β-D-galactosidase

The investigation into the effect of temperature on *Arthrobacter* sp. 32cB β-D-galactosidase showed that the highest hydrolytic activity during 10 min of incubation with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate occurred at 28 °C. The enzyme exhibited over 95% of maximum activity in a temperature range of 26–31 °C, and maintained 31–42% of maximum activity at 5–10 °C (Fig. 3A).

Table 2
Summary of the purification of recombinant *Arthrobacter* sp. 32cB β-D-galactosidase obtained from 1 L of *E. coli* LMG194 culture.

Purification step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Cell extract	510.0	7490.0	14.7	1.0	100
Fractogel EMD DEAE	63.0	6800.0	107.9	7.3	12.4
Resource Q	35.0	5600.0	160.0	10.9	6.9
Gel filtration	18.0	3840.0	213.3	14.5	3.5

The enzyme activity was measured with ONPG as a substrate.

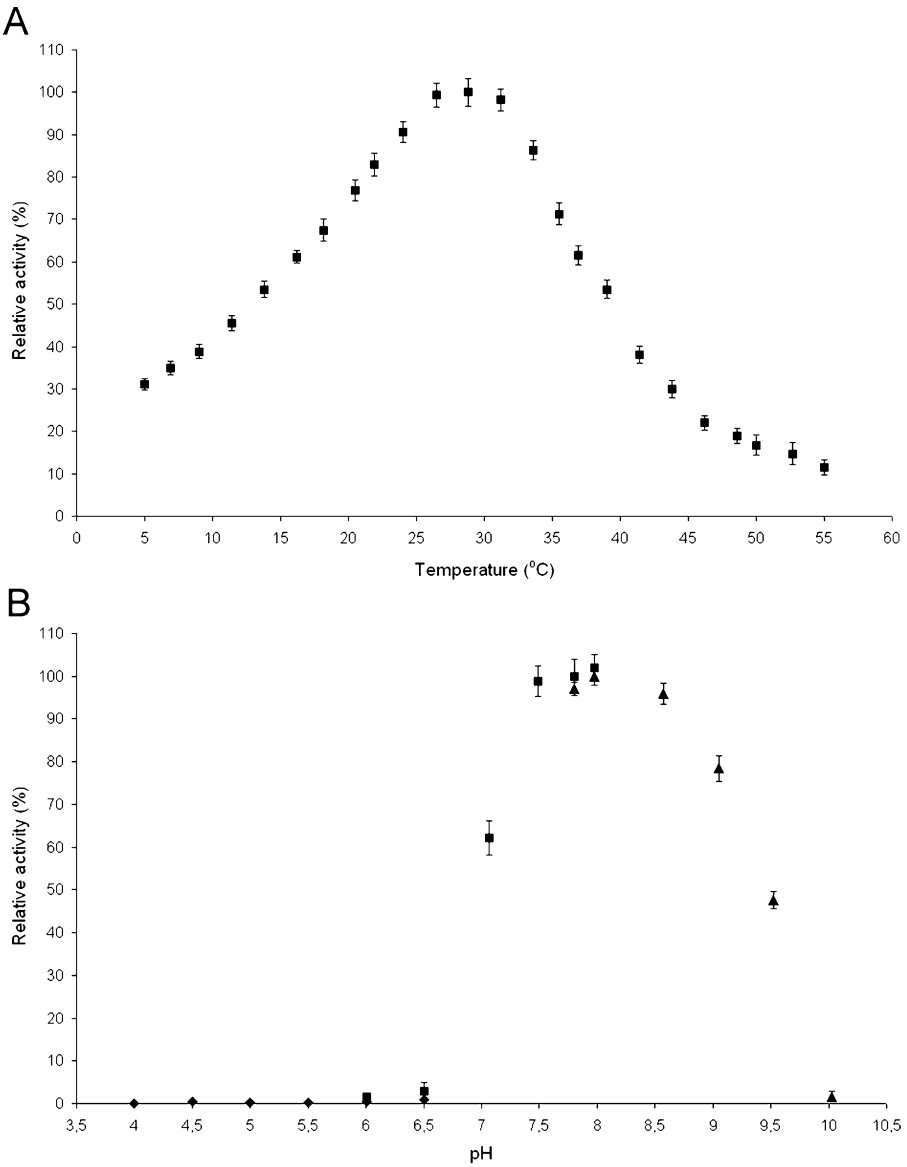

Fig. 3. Effects of temperature (A) and pH (B) on activity of recombinant *Arthrobacter* sp. 32cB β-D-galactosidase. Panel A: reaction mixtures containing 0.8 U mL^{-1} 32cB-B-gal and 1 mg mL^{-1} ONPG in 20 mM potassium phosphate buffer pH 8.0 were incubated at temperatures from 5 to 55 °C for 10 min. Panel B: reaction mixtures containing 0.8 U mL^{-1} 32cB-B-gal and 1 mg mL^{-1} ONPG in 20 mM citrate-sodium phosphate buffer pH 4.0–6.5 (♦), 20 mM potassium phosphate buffer pH 6.0–8.0 (■) and 20 mM glycine–sodium hydroxide buffer pH 7.8–10.0 (▲) were incubated at 28 °C for 5 min.

The *Arthrobacter* sp. 32cB β-D-galactosidase exhibited high stability at a temperature of 30 °C and a pH of 8.0, given that approximately 80% of its initial activity was retained after 8 h of incubation (Fig. 4A). At 42 °C, over 50% of initial activity was lost in an incubation period of 5 min and the enzyme was inactivated by 5 min at 44 °C (Fig. 4B).

In order to determine the optimum pH for recombinant β-D-galactosidase activity, the hydrolysis of ONPG was performed at various pH values and at 28 °C. The enzyme exhibited maximum activity at a pH of 8.0 and maintained over 95% of maximum activity in a pH range of 7.5–8.5 (Fig. 3B).

For examination of the metal ion requirements, the purified 32cB-B-gal was assayed in the presence of 5 mM Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Ni^{2+} ions. The enzyme was strongly activated by Mg^{2+} and strongly inhibited by Co^{2+}, Ni^{2+}, and Mn^{2+} ions. It was also inhibited by a chelator of divalent cations ethylenediaminetetraacetic acid sodium salt (EDTA). It is important to note is that the activity of the *Arthrobacter* sp. 32cB β-D-galactosidase was only partially inhibited by Ca^{2+} ions. Moreover, the enzyme was completely inactivated by the strong reducing agent tris(2-carboxyethyl)phosphine (TCEP) (Table 3).

The substrate specificity studies demonstrated that *Arthrobacter* sp. 32cB β-D-galactosidase hydrolyzed only two chromogenic substrates out of the ten tested. The enzyme exhibited a high activity

Table 3
Effects of metal ions and selected reagents on *Arthrobacter* sp. 32cB β-D-galactosidase activity.

Metal ion/EDTA (5 mM)	Relative activity (%)	Reducing agent (10 mM)	Relative activity (%)
None	100	None	100
Mg^{2+}	162 ± 6.3	DTT	117 ± 5.1
Ca^{2+}	77 ± 2.5	Cysteine	65 ± 2.7
Co^{2+}	36 ± 2.1	Glutathione	30 ± 2.2
Ni^{2+}	22 ± 1.8	TCEP	0
Mn^{2+}	17 ± 1.4		
EDTA	37 ± 2.0		

The enzyme activity was measured with ONPG as a substrate.

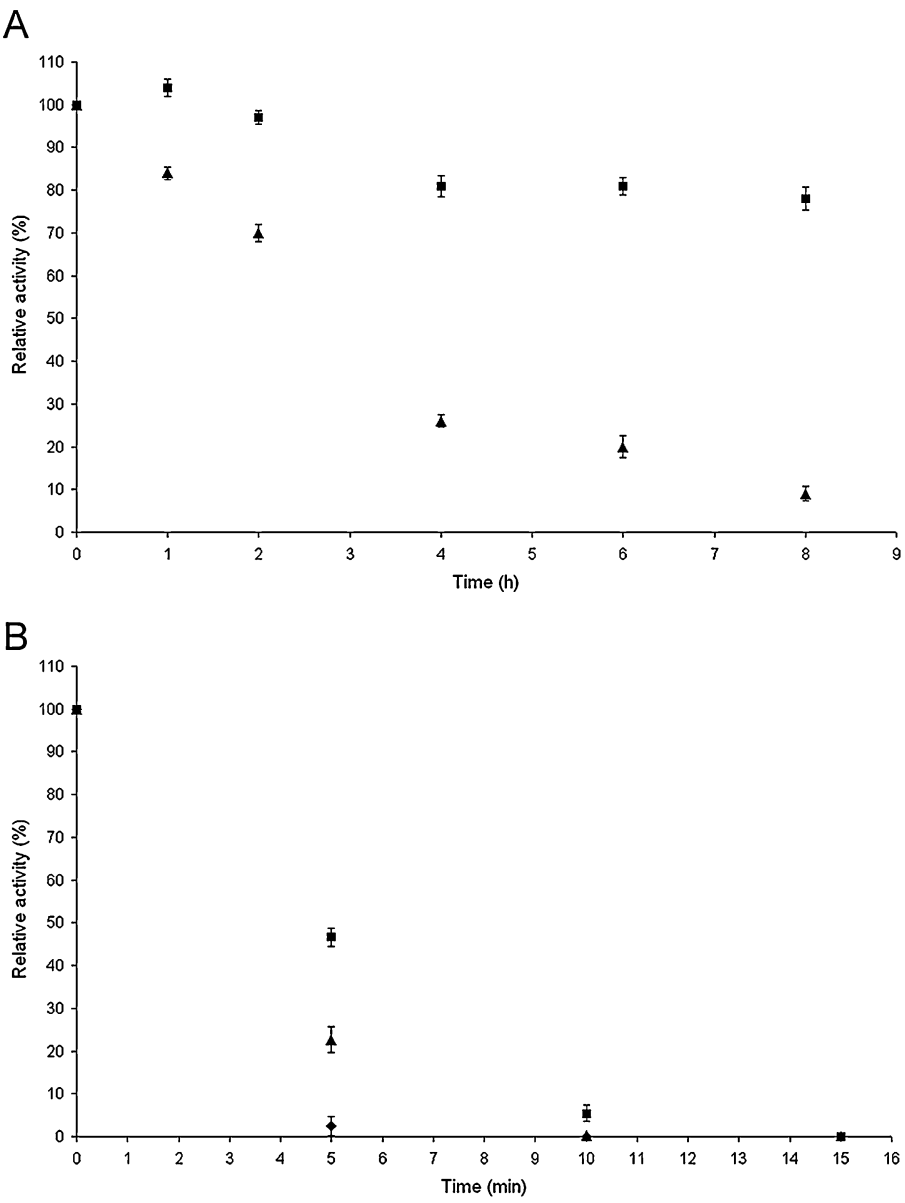

Fig. 4. Effect of temperature on stability of recombinant *Arthrobacter* sp. 32cB β-D-galactosidase. Panel A: the enzyme was incubated at 30 (■) or 35 °C (▲) for 1–8 h and then assayed at 28 °C. Panel B: the enzyme was incubated at 42 (■), 43 (▲) or 44 °C (♦) for 5–15 min and then assayed at 28 °C.

towards *p*-nitrophenyl-β-D-galactopyranoside (PNPG) and a very weak activity towards *p*-nitrophenyl-β-D-fucopyranoside (about 4% of the PNPG activity). It showed less than 1% of the PNPG activity with *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucouronide, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-L-arabinopyranoside, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-α-D-glucopyranoside.

The kinetic parameters of *Arthrobacter* sp. 32cB β-D-galactosidase for ONPG and lactose as substrates were determined at 10, 20 and 30 °C. The enzyme exhibited an one order of magnitude higher catalytic efficiency (k_{cat}/K_m) towards synthetic substrate (Table 4).

The *Arthrobacter* sp. 32cB β-D-galactosidase activity towards ONPG (5 mM) was not inhibited by D-glucose in concentrations up to 150 mM, but it was inhibited by D-galactose. The activity was reduced twofold in the presence of 50 mM monosaccharide. The K_i values for D-galactose determined at 20 °C using ONPG and lactose as substrates were 24.12 and 14.80 mM, respectively. D-Galactose was a competitive inhibitor given that V_{max} values for ONPG and lactose were unchanged, while K_m values increased from 1.58 to 5.00 mM for ONPG and from 14.67 to 32.86 mM for lactose.

Experiments on the hydrolysis of lactose in milk by *Arthrobacter* sp. 32cB β-D-galactosidase revealed that 70 or 90% of lactose was digested by 1 or 2 U of the enzyme per 1 mL of milk over 24 h at a temperature of 10 °C, respectively.

The transglycosylation activity of *Arthrobacter* sp. 32cB β-D-galactosidase was tested using lactose as a substrate. GOS synthesis was conducted at 10, 20 and 30 °C in reaction mixtures containing up to 584 mM of lactose. The efficient synthesis of tri- and tetrasaccharides was achieved in 292–584 mM of lactose solutions containing 2 U mL^{-1} β-D-galactosidase after an incubation period of 24 h at 10 and 20 °C or of 6 h at 30 °C. Moreover, in the reaction mixtures incubated at 20 and 30 °C, some quantities of pentasaccharides were also detected (Fig. 5).

Arthrobacter sp. 32cB β-D-galactosidase was used to catalyze the synthesis of heterooligosaccharides from lactose and different monosaccharides, namely, D-fructose, D-xylose and L-arabinose.

Table 4
Kinetic parameters of *Arthrobacter* sp. 32cB β-D-galactosidase.

Substrate	Temperature (°C)	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
ONPG	10	1.52 ± 0.12	16.74 ± 1.05	30.55 ± 2.12	20.16
	20	1.58 ± 0.31	31.20 ± 1.91	56.95 ± 2.41	35.98
	30	1.51 ± 0.15	50.38 ± 2.70	91.95 ± 2.70	60.74
Lactose	10	16.56 ± 0.55	17.44 ± 1.20	31.84 ± 2.04	1.92
	20	14.67 ± 0.88	31.58 ± 1.55	57.64 ± 2.58	3.93
	30	16.18 ± 1.05	42.04 ± 2.10	76.74 ± 2.72	4.74

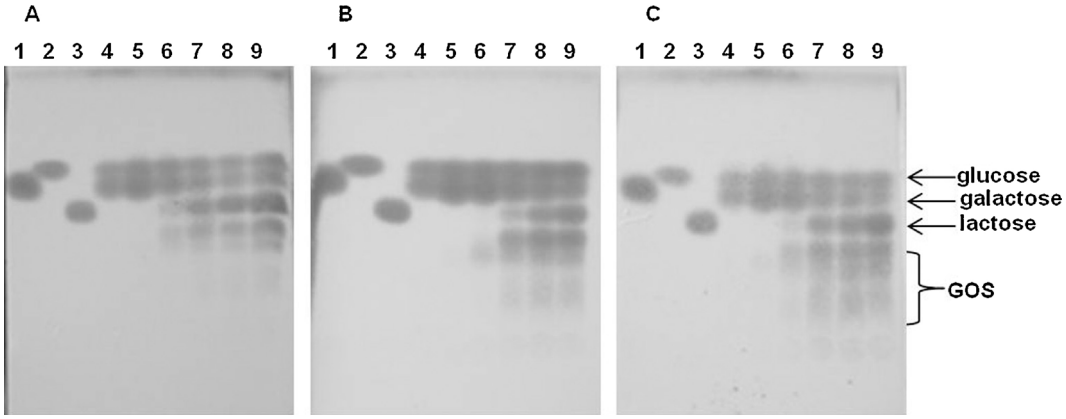

Fig. 5. TLC of products of GOS synthesis catalyzed by *Arthrobacter* sp. 32cB β-D-galactosidase. Reaction mixtures containing lactose as a substrate and 2 U mL^{-1} *Arthrobacter* sp. 32cB β-D-galactosidase were incubated at 10 and 20 °C for 24 h (A and B) or 30 °C for 6 h (C). Lane 1 – D-galactose, lane 2 – D-glucose, lane 3 – lactose, lane 4 – reaction mixture containing 29 mM lactose, lane 5 – reaction mixture containing 58 mM lactose, lane 6 – reaction mixture containing 146 mM lactose, lane 7 – reaction mixture containing 292 mM lactose, lane 8 – reaction mixture containing 438 mM lactose, and lane 9 – reaction mixture containing 584 mM lactose.

The highest yield of lactulose (galactosyl-fructose) was achieved after 8 h incubation at 30 °C (Fig. 6). The similar results were obtained during synthesis of galactosyl-arabinose and galactosyl-xylose.

In addition, it was found that the Antarctic *Arthrobacter* sp. 32 cB β-D-galactosidase catalyzes the synthesis of alkyl glycosides from lactose and alcohols such as 2-propanol, 1-butanol, 1-hexanol and cyclohexanol. The enzyme showed the highest transferase activity towards 1-butanol (Fig. 7A). Yields of other derivatives were lower, owing to steric hindrance by bulky groups in 2-propanol (Fig. 7B) and the relatively low solubility in water of 1-hexanol (Fig. 7C). Cyclohexanol was found to be an inhibitor for the enzyme, as evidenced by the low quantities of lactose hydrolysis products at higher concentrations of this alcohol in the reaction mixtures. As a result, the transglycosylation product was barely detectable (Fig. 7D).

To investigate the transglycosylation activity of *Arthrobacter* sp. 32cB β-D-galactosidase further, a salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside) was used as an acceptor of galactosyl moiety. The enzyme was active towards salicin as a substrate and large quantities of the product were obtained after 8 h of transglycosylation at 30 °C in the reaction mixtures containing 146–292 mM concentrations of lactose and salicin (Fig. 8).

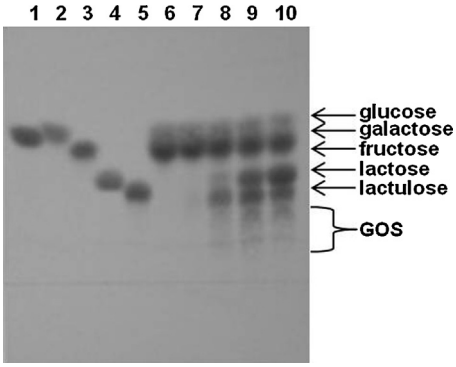

Fig. 6. TLC of products of lactulose synthesis catalyzed by *Arthrobacter* sp. 32cB β-D-galactosidase. Reaction mixtures containing lactose and D-fructose as substrates, and 2 U mL^{-1} *Arthrobacter* sp. 32cB β-D-galactosidase were incubated at 30 °C for 8 h. Lane 1 – D-galactose, lane 2 – D-glucose, lane 3 – D-fructose, lane 4 – lactose, lane 5 – lactulose, lane 6 – reaction mixture containing 29 mM of each substrate, lane 7 – reaction mixture containing 58 mM of each substrate, lane 8 – reaction mixture containing 146 mM of each substrate, lane 9 – reaction mixture containing 292 mM of each substrate, and lane 10 – reaction mixture containing 438 mM of each substrate.

4. Discussion

The article has described the cloning, purification and characterization of β-D-galactosidase belonging to the GH family 2 from the psychrotolerant Antarctic bacterium *Arthrobacter* sp. 32cB. This enzyme exists in the solution as a dimer, similarly to the β-D-galactosidase from *Paracoccus* sp. 32d, although most of the cold-active GH2 β-D-galactosidases characterized to date are tetramers (Table 5). The main industrial β-D-galactosidase from *K. lactis* is active in both dimeric and tetrameric form; however, the dominant active form of the enzyme is dimeric [49].

The *Arthrobacter* sp. 32cB β-D-galactosidase is optimally active at relatively low temperatures ranging from 26 to 31 °C, with maximum activity at 28 °C, and it exhibits over 40% of maximum activity at 10 °C. Moreover, it can be easily inactivated by incubation at moderate temperature, namely 5 min at 44 °C. Almost all the cold-active β-D-galactosidases of the GH2 family are optimally active at temperatures above 10 °C and only one enzyme, which was isolated from psychrophilic bacterium *A. psychrolactophilus* F2, exhibits maximum activity at 10 °C (Table 5). On the other hand, the *Arthrobacter* sp. 32cB β-D-galactosidase is stable at 30 °C and below for at least 8 h and it can be purified, transported and stored

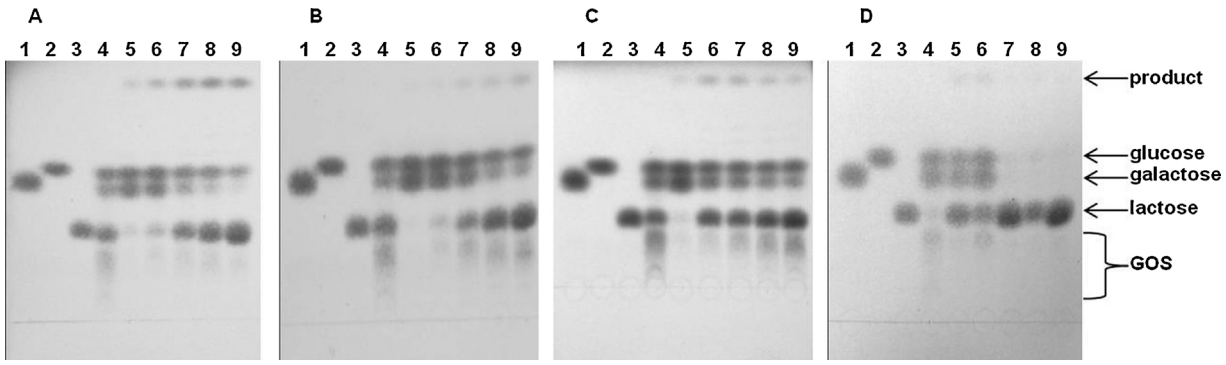

Fig. 7. TLC of products of alkyl glycosides synthesis catalyzed by *Arthrobacter* sp. 32cB β-D-galactosidase. Reaction mixtures containing lactose and 1-butanol (A), 2-propanol (B), 1-hexanol (C) or cyclohexanol (D) as substrates, and 2 U mL^{-1} *Arthrobacter* sp. 32cB β-D-galactosidase were incubated at 30 °C for 8 h. Lane 1 – D-galactose, lane 2 – D-glucose, lane 3 – lactose, lane 4 – GOS, lane 5 – reaction mixture containing 29 mM of each substrate, lane 6 – reaction mixture containing 58 mM of each substrate, lane 7 – reaction mixture containing 146 mM of each substrate, lane 8 – reaction mixture containing 292 mM of each substrate, and lane 9 – reaction mixture containing 438 mM of each substrate.

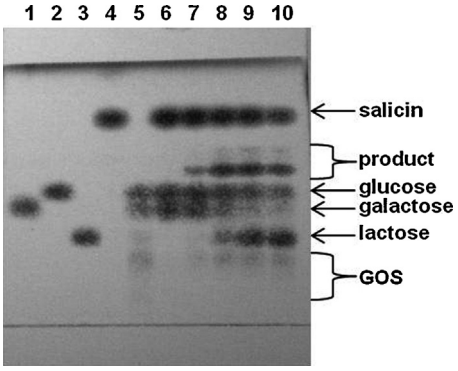

Fig. 8. TLC of products of salicin glycosylation catalyzed by *Arthrobacter* sp. 32cB β-D-galactosidase. Reaction mixtures containing lactose and salicin as substrates and 2 U mL^{-1} *Arthrobacter* sp. 32cB β-D-galactosidase were incubated at 30 °C for 8 h. Lane 1 – D-galactose, lane 2 – D-glucose, lane 3 – lactose, lane 4 – salicin, lane 5 – GOS, lane 6 – reaction mixture containing 29 mM of each substrate, lane 7 – reaction mixture containing 58 mM of each substrate, lane 8 – reaction mixture containing 146 mM of each substrate, lane 9 – reaction mixture containing 292 mM of each substrate, and lane 10 – reaction mixture containing 438 mM of each substrate.

for a short time at room temperature without significant loss of activity, which is important for industrial enzymes. In contrast, the *Arthrobacter* sp. SB β-D-galactosidase irreversibly dissociates into inactive monomers at 25 °C [15].

Like other cold-active β-D-galactosidases from GH family 2, the enzyme from *Arthrobacter* sp. 32cB shows high specificity towards β-D-galactopyranosides including lactose. It exhibits higher catalytic efficiency (k_{cat}/K_m) towards lactose than β-D-galactosidases from *A. psychrolactophilus* F2, *Arthrobacter* sp. SB and *Arthrobacter* sp. C2-2 (Table 5). The *Arthrobacter* sp. 32cB β-D-galactosidase is inhibited by D-galactose, which is the competitive inhibitor of many microbial β-D-galactosidases, but it is not inhibited by D-glucose. The similar behaviour was reported for the enzyme from *Kluyveromyces fragilis* (commercial Lactozym, Novo Nordisk, Denmark) [50,51]. D-Galactose was also a competitive inhibitor of cold-active β-D-galactosidase from *Arthrobacter* sp. SB [15]. In contrast, the enzyme from *Pseudoalteromonas* sp. 22b was inhibited by D-glucose, but not by D-galactose [52]. The *Paracoccus* sp. 32d β-D-galactosidase was inhibited by both monosaccharides [19], whereas neither D-glucose nor D-galactose had any effect on the *Arthrobacter* sp. C2-2 β-D-galactosidase activity [14]. Thus 32cB-B-gal has the potential for use in the removal of lactose from milk under refrigeration. Two units of this enzyme (9.4 μg) are capable of digesting 90% of the lactose in 1 mL of milk in 24 h at 10 °C. For comparison, 1 U of *A. psychrolactophilus* F2 β-D-galactosidase (30.0 μg) hydrolyzes about 80%, while 1 U of *Paracossus* sp. 32d β-D-galactosidase (24.4 μg) removes approximately 97% of lactose from 1 mL of milk under the same conditions [12,19]. The advantage of *Arthrobacter* sp. 32cB β-D-galactosidase is its high specific activity, which allows the use of small quantities of protein and thus reduces the cost of the process.

Table 5
Biochemical properties of cold-active GH2 β-D-galactosidases from various microorganisms.

Organism	Molecular mass (kDa)	Oligomeric state	T_{opt} (°C)	pH_{opt}	Thermal inactivation	Kinetic parameters in lactose hydrolysis			References
						K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	
Arthrobacter sp. 32cB	257 (109.6)[a]	2	28	8.0	5 min at 44 °C	16.56 (10 °C)	31.84	1.92	This study
Arthrobacter psychrolactophilus F2	548 (111.7)[a]	4	10	8.0	5 min at 50 °C	50 (10 °C)	18.0	0.36	[12]
Arthrobacter sp. ON14	NR (111.4)[a]	NR	15	8.0	20 min at 50 °C	NR	NR	NR	[5]
Arthrobacter sp. SB	463 (114.0)[a]	4	18	7.0	10 min at 37 °C	11.5 (20 °C)	5.2	0.53	[15]
Arthrobacter sp. 20B	460 (113.7)[a]	4	25	6.0–8.0	1 min at 60 °C	NR	NR	NR	[13]
Arthrobacter sp. B7	NR (111.0)[a]	NR	40	7.2	10 min at 50 °C	16 (30 °C)	NR	NR	[2]
Arthrobacter sp. C2-2	550 (110.8)[a]	4	40	7.5	10 min at 50 °C	344.2 (10 °C)	324	0.9	[14]
Alkalilactibacillus ikkense	NR (119.1)[a]	NR	20–30	8.0	5 min at 50 °C	NR	NR	NR	[21]
Paracoccus sp. 32d	161 (81.7)[a]	2	40	7.5	15 min at 50 °C	2.94 (10 °C)	43.23	15.06	[19]
Pseudoalteromonas sp. 22b	490 (117.1)[a]	4	40	6.0–8.0	2 min at 50 °C	3.3 (20 °C)	157	47.5	[17,18]
Flavobacterium sp. 4214	<66 (114.3)[a]	1	42	7.5	NR	NR	NR	NR	[20]
Pseudoalteromonas haloplanktis TAE79	>300 (118.1)[a]	4	45	8.5	NR	2.4 (25 °C)	33	13.7	[16]

NR, not reported.
[a] Molecular mass of the monomer, calculated from the amino acid sequence.

The article also presents a study carried out on the transglycosylation activity of *Arthrobacter* sp. 32cB β-D-galactosidase. The enzyme was capable of synthesizing galactooligosaccharides, namely tri-, tetra- and even pentasaccharides, at relatively low temperatures ranging from 10 to 30 °C. The formation of GOS during lactose hydrolysis has previously been noted for cold-active β-D-galactosidases from *A. psychrolactophilus* F2 [31], *Arthrobacter* sp. C2-2 [14] and *A. ikkense* [21]. In the case of recombinant *Arthrobacter* sp. C2-2 β-D-galactosidase, the formation of trisaccharides reached a plateau after approximately 10 h at 15 °C; however the concentration of tetrasaccharides was much lower than that of trisaccharides [14]. Similar results were obtained for recombinant *A. psychrolactophilus* F2 β-D-galactosidase. The product corresponding to trisaccharide was detected during the lactose hydrolysis in milk catalyzed by this enzyme, and its formation reached a plateau after around 10 h at 10 °C [31].

The *Arthrobacter* sp. 32cB β-D-galactosidase was also tested as a tool for the synthesis of alkyl glycosides. Like the β-D-galactosidase from the psychrotolerant *Pseudoalteromonas* sp. 22b [40], it was capable of catalyzing the formation of 2-propyl, 1-butyl, 1-hexyl and cyclohexyl galactopyranosides at 30 °C. The disadvantage of 32cB-B-gal is the relatively low yields of transglycosylation products. However, the authors suppose that the quantities of alkyl glycosides can be increased by optimization of the reaction conditions, for example by the addition of selected organic solvents, as in the case of *Pseudoalteromonas* sp. 22b β-D-galactosidase [40].

Moreover, it was found that, like some enzymes from mesophilic microorganisms, the recombinant *Arthrobacter* sp. 32cB β-D-galactosidase catalyzed the synthesis of heterooligosaccharides, namely lactulose, galactosyl-xylose or galactosyl-arabinose, and glycosylated salicin. β-D-Galactosidases from *Enetrobacter agglomerans* B1 and *E. cloacae* B5 catalyze glycosyl transfer from ONPG to various acceptors such as hexoses (glucose, galactose, mannose, fructose, sorbose), pentoses (arabinose, xylose), disaccharides (cellobiose, sucrose, trehalose), hexahydroxyalcohols (mannitol, sorbitol), cyclitol (inositol) and aromatic glycoside (salicin) [33,34]. The enzymatic synthesis, by *Aspergillus oryzae* β-D-galactosidase, of galactosyl-xylose from ONPG and xylose as substrates has been also reported [35]. Nevertheless, synthetic β-D-galactosidase substrate ONPG is useless as galactosyl donor in industrial processes. Hence, in our study the natural, plentiful and inexpensive β-D-galactosidase substrate lactose was used as the glycosyl donor in the synthesis of glycoconjugates. The production of lactulose from lactose and fructose has previously been investigated with several commercial enzymes such as *E. coli* β-D-galactosidase, *A. oryzae* β-D-galactosidase, *S. fragilis* β-D-galactosidase, and *K. lactis* β-D-galactosidase, with the enzyme from *K. lactis* exhibiting the highest lactulose productivity of the β-D-galactosidases tested [53].

5. Conclusions

The new β-D-galactosidase from psychrotolerant *Arthrobacter* sp. 32cB obtained and characterized in this study has interesting properties. It exhibits maximum activity towards the synthetic substrate ONPG at a temperature of 28 °C and a pH of 8.0, but retains over 40% of its maximum activity at 10 °C. It is highly active towards not only synthetic β-D-galactopyranosides, but also natural substrate lactose, and is only partially inhibited by calcium ions and D-galactose, one of the end products of lactose hydrolysis. These features make it an attractive proposition for the production of low-lactose dairy products under refrigerated conditions.

Furthermore, the enzyme shows transglycosylation activity at relatively low temperatures. It is capable of synthesizing galactooligosaccharides at temperatures ranging from 10 to 30 °C. At the latter temperature, it also catalyzes glycosyl transfer from lactose to various chemicals such as sugars (D-fructose, D-xylose and L-arabinose), alcohols (1-butanol, 1-hexanol, 2-propanol and cyclohexanol), and aromatic glycoside salicin. The *Arthrobacter* sp. 32cB β-D-galactosidase thus has the potential of being an interesting biocatalyst for the production of food additives, cosmetics, and pharmaceuticals, and can be particularly useful in the glycosylation of thermosensitive chemicals.

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