

SCREENING, IDENTIFICATION AND CHARACTERIZATION OF *ARTHROBACTER* SPECIES BACTERIUM PRODUCING EXTRACELLULAR β -GALACTOSIDASE

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Introduction

β -Galactosidase (β -D-galactosidegalactohydrolase, β -D-galactohydrolase, EC 3.2.1.23) commonly also known as lactase is the enzyme that typically catalyzes hydrolysis of β -1,4-D-galactosidic linkages in β -D-galactosides, including disaccharide lactose, with glucose and galactose as end reaction products. Yet, this enzyme is able to catalyze oligomerization of galactosides, i.e. to provide synthesis of oligosaccharides, in particular galactooligosaccharides via the galactosyl transfer reaction.

β -Galactosidase is traditionally used in dairy industry for removal of lactose from milk products designed for lactose-intolerant people. It is also applied for enzymatic conversion of milk/cheese whey (a by-product of milk processing), which is formed in increasing amounts, has a high polluting load, and therefore represents a tremendous environmental problem. At present only nearly 50% of total accumulated world whey is turned into several value-added products – glucose/galactose syrups and galactooligosaccharides distinguished by proven prebiotic properties and potential application in the manufacturing of food and feed ingredients, pharmaceuticals, and other biologically active compounds [1–7].

Synthesis of β -galactosidase is a widely distributed property of plants, animals and microorganisms of different taxonomic groups. Among them only several microbial cultures show biotechnological significance as enzyme producers. The most extensively used β -galactosidases of commercial importance originated mainly from the yeasts (*Kluyveromyces* spp.), fungi (*Aspergillus* spp., *Penicillium* spp.) and to a lesser degree from bacteria (*Bacillus* spp.) [8]. As a rule, β -galactosidases produced by fungi are extracellular proteins, in contrast to yeast and bacterial enzymes having intracellular localization. In addition, the former is the most catalytically active at high acidity of reaction medium (pH 2.5–5.4) while the latter preferably function in neutral medium or near (pH 6.0–8.0). Depending on the nature of lactose-containing substances, pH values range from ~ 3.5–5.6 for acid whey to 6.8–7.0 for whole milk.

Before studies carried out by Loveland team there were no reports on β -galactosidases of *Arthrobacter* genus bacteria not utilizing lactose as a carbon source [9]. So far β -galactosidase activity was detected in some psychrotolerant and psychrophilic representatives of above-mentioned bacterial group, including different strains of *A. psychrolactophilus* [10–12] and *Arthrobacter* spp. [9; 13–20]. A weak production of intracellular β -galactosidase with very low specific activity is their main drawback.

Recently we found the collection strain *Arthrobacter* sp. BIM B-2242 which actively grew on agar media with lactose as a sole source of carbon and energy. In the present study we have characterized the ability of the strain to produce the β -galactosidase in liquid medium with lactose.

Methods

Strain *Arthrobacter* sp. BIM B-2242 was isolated from rice field soil (Krasnodar region, Russia) and maintained in the Belarussian Collection of Non-pathogenic Microorganisms, Institute of Microbiology, Belarus National Academy of Sciences (BCM).

Bacterial culture was maintained on slants of agar containing: peptone, 10 g·L⁻¹; yeast extract, 5 g·L⁻¹; NaCl, 5 g·L⁻¹; glucose, 5 g·L⁻¹, and agar, 15 g·L⁻¹. The initial pH was adjusted to 7.2–7.4

with 1 M NaOH and the medium was sterilized at 121°C for 20 min. The culture was grown on agar slants at 27–29°C for 72 h.

For screening of β -galactosidase-producing activity in the studied bacterium, the agar medium containing: peptone, 10 g·L⁻¹; yeast extract, 5 g·L⁻¹; NaCl, 5 g·L⁻¹; lactose, 10 g·L⁻¹, agar, 15 g·L⁻¹, and 40 mg·L⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) as indicator of β -galactosidase was used. Plates with bacterial culture spread onto surface of agar medium were incubated at 27–29°C for 72 h. Colonies of lactose-utilizing and β -galactosidase-producing bacterium turned blue due to enzymatic cleavage of colorless X-Gal into galactose and 5-bromo-4-chloro-3-hydroxyindole, which is spontaneously dimerized and oxidized into intensely blue product, 5,5'-dibromo-4,4'-dichloro-indigo.

Batch fermentation of the bacterium was carried out in 250 ml Erlenmeyer flasks, each containing 50 ml of the medium, on rotary shaker (180 rpm) at 27–29°C for 24–72 h. The liquid medium used for β -galactosidase production included: peptone, 10 g·L⁻¹; yeast extract, 5 g·L⁻¹; MgSO₄·7H₂O, 1 g·L⁻¹; K₂HPO₄ 3 g·L⁻¹ and lactose, 15 g·L⁻¹ as a carbon source. The initial pH was adjusted to 6.7–6.9 with 1 M NaOH and the medium was autoclaved at 121°C for 20 min.

Water suspension of bacterial cells (2 vol. %) with optical density (OD₅₄₀) 0.2±0.01 grown on peptone yeast agar at 27–29°C for 72 h was used as inoculum.

Periodically, under sterile conditions, samples were withdrawn and analyzed for growth, β -galactosidase activity and protein content.

Growth was monitored by measuring the optical density of a sample at 540 nm and the reading was converted into dry cell weight using a standard curve. Biomass was expressed in mg of dry cell weight per 1 ml of cultural liquid (mg·ml⁻¹). When growth was terminated, cells from 50 ml cultural liquid were separated by centrifugation at 8 000 g for 20 min at 4 °C, washed three times with distilled water, and used to obtain cell-free extracts.

Cell-free extracts of harvested bacterial cells were prepared by sonification twice for 1.5 min in ice bath using UDM-10 (Techpan, Poland) in 0.2 M sodium-phosphate buffer, pH 7.0. The residual insoluble cell debris were discarded after centrifugation at 8 000 g for 20 min at room temperature and sterilized through 0.2 μ m filter. Cell-free supernatants of cultural medium and cell extracts were used as sources of extra- and intracellular β -galactosidase, respectively.

β -Galactosidase activity was assayed using o-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate according to [21]. One unit of enzyme activity was defined as 1 μ mol of o-nitrophenol released from the substrate in 1 min at pH 7.0 and 40°C. The release of o-nitrophenol was measured spectrophotometrically at 420 nm using Ultrospec 2100 pro (GE Healthcare, USA). Activity of extracellular enzyme was expressed in U·ml⁻¹ cultural medium, U·mg⁻¹ biomass (biomass productivity) or U·mg⁻¹ protein (specific activity). The enzyme activity was measured in triplicate, with experimental errors below 5 %.

Amplification and sequencing of 16S rRNA genes from *Arthrobacter* sp. BIM B-2242 were performed by methods described earlier [22]. Reference sequences of *Arthrobacter* spp. type strains were obtained from GenBank database. Phylogenetic analysis was conducted in MEGA4 [23]. Reference sequences of *Arthrobacter* spp. type strains were obtained from GenBank database. Multiple alignments were performed by using CLUSTALW. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The evolutionary distances were computed using the Maximum Composite Likelihood method and presented in the units of the number of base substitutions per site. A phylogenetic tree was constructed by using the neighbor-joining method. A neighbor-joining bootstrap method was used to obtain confidence levels for the neighbor-joining analysis, with a bootstrap dataset of 1000.

The determined nucleotide sequence for 16S rRNA gene of *Arthrobacter* sp. BIM B-2242 reported in this work was deposited under name *Arthrobacter sulfonivorans* BIM B-2242 in the GenBank database under accession number KF537271.1.

In experiments, cell-free supernatant of bacterial cultural liquid partly purified by ultrafiltration with an Ultrafree-MC filter unit (nominal molecular weight limit, 100kDa; Millipore, Bedford, Mass.) was used as a crude β -galactosidase preparation.

The effect of pH on β -galactosidase activity was determined in 0.04 M universal buffer (phosphoro-acetic-boric) in pH range 2.0–10.0 at 40°C, with ONPG as the substrate. Thermal optimum of enzyme activity was determined in 0.2 M sodium-phosphate buffer (pH 7.0) as the most preferable for *A. sulfonivorans* β -galactosidase assay (data not presented) at temperatures ranging from 20°C to 70°C.

The thermostability of β -galactosidase was tested after incubating enzyme during 60 min in 0.04 M universal buffer (phosphoro-acetic-boric) at temperatures 20°C, 30°C, 40°C, 50°C in pH range 5.0–8.0 followed measuring of residual activity under standard assay conditions (0.2 M sodium-phosphate buffer, pH 7.0; 40°C).

Protein concentration was determined according to Bradford [24] using BSA as standard.

Separation of extracellular bacterial proteins was carried out by electrophoresis under non-denaturing conditions (without sodium dodecyl sulfate) according to Laemmli [25]. For this, samples of cell-free supernatants were placed on 12% polyacrylamide gelplate in duplicate. After electrophoresis using 0.025 M Tris-glycine (pH 8.5) as an electrode buffer for 1.5–2 h at 20 mA current per plate, the gels were washed with distilled water and cut into two identical parts. The first of them was stained according to Fairbanks [26] with Coomassie Brilliant Blue R-250 for visualization of all extracellular proteins produced by the bacterium. The second part of the gel was incubated at 37°C for 2–3 h in the 0.2 M sodium-phosphate buffer (pH 7.0) containing X-Gal for *in situ* detection of proteins with β -galactosidase activity. Enzyme proteins were identified in gel as blue bands of 5,5'-dibromo-4,4'-dichloro-indigo formed in reaction of enzymatic hydrolysis of colorless X-Gal.

Experiments and assays were carried out in triplicates. Results were analyzed by Microsoft Excel 2010 and presented as average values, with standard deviation below 0.01.

Results and discussion

Characterization and identification of the strain Arthrobacter sp. BIM B-2242. Despite the opinion that bacteria of *Arthrobacter* genus are not utilizing lactose as a sole source of carbon nutrition [9] we have sorted out the representative of this taxonomic group of prokaryotes – *Arthrobacter* sp. BIM B-2242 which actively grew on agar media with this disaccharide.

Strain *Arthrobacter* sp. BIM B-2242 is a Gram-positive, non-motile bacterium, with rod-coccus growth cycle. It is an obligate aerobe, mesophile, hemoorganotroph with respiratory type of metabolism. It does not form endospores and displays optimum conditions for growth at temperature 27–29°C and pH 7.0. The strain is positive in catalase, protease, amylase, urease, and lipase activities. It assimilates lactose, D-glucose, D-galactose, rhamnose, saccharose, arabinose, sorbitol, pectin, starch, organic acids as carbon and energy sources. It utilizes the following nitrogen sources: yeast extract, peptone, bactopecton, tripton, gelatin, casein, sodium caseinate, urea, amino acids, ammonium salts of inorganic acids and nitrates. The strain liquefies gelatin and does not peptonize milk.

To identify species of β -galactosidase-producing bacterium *Arthrobacter* sp. BIM B-2242, about 1.5 kb fragment of 16S rRNA gene was amplified and sequenced. The deciphered nucleotide sequence shared 99.4–99.5% identity with 16S rRNA gene of *Arthrobacter sulfonivorans* DSM 14002T. The sequence similarities between the studied strain and type strains of the other closest species *Arthrobacter defluvii*, *A. equi*, *A. oryzae*, *A. oxydans*, *A. pascens*, *A. phenanthrenivorans*, *A. polychromogenes*, *A. ramosus* were 97.9–98.3%.

In the neighbor-joining phylogenetic tree (figure 1) the *Arthrobacter* sp. BIM B-2242 strain formed robust cluster with the type strain of *A. sulfonivorans*. Similar topologies were obtained by the maximum parsimony and minimum evolution methods. The results of phylogenetic analysis indicated that the studied strain BIM B-2242 of β -galactosidase-producing bacterium belongs to *A. sulfonivorans* species.

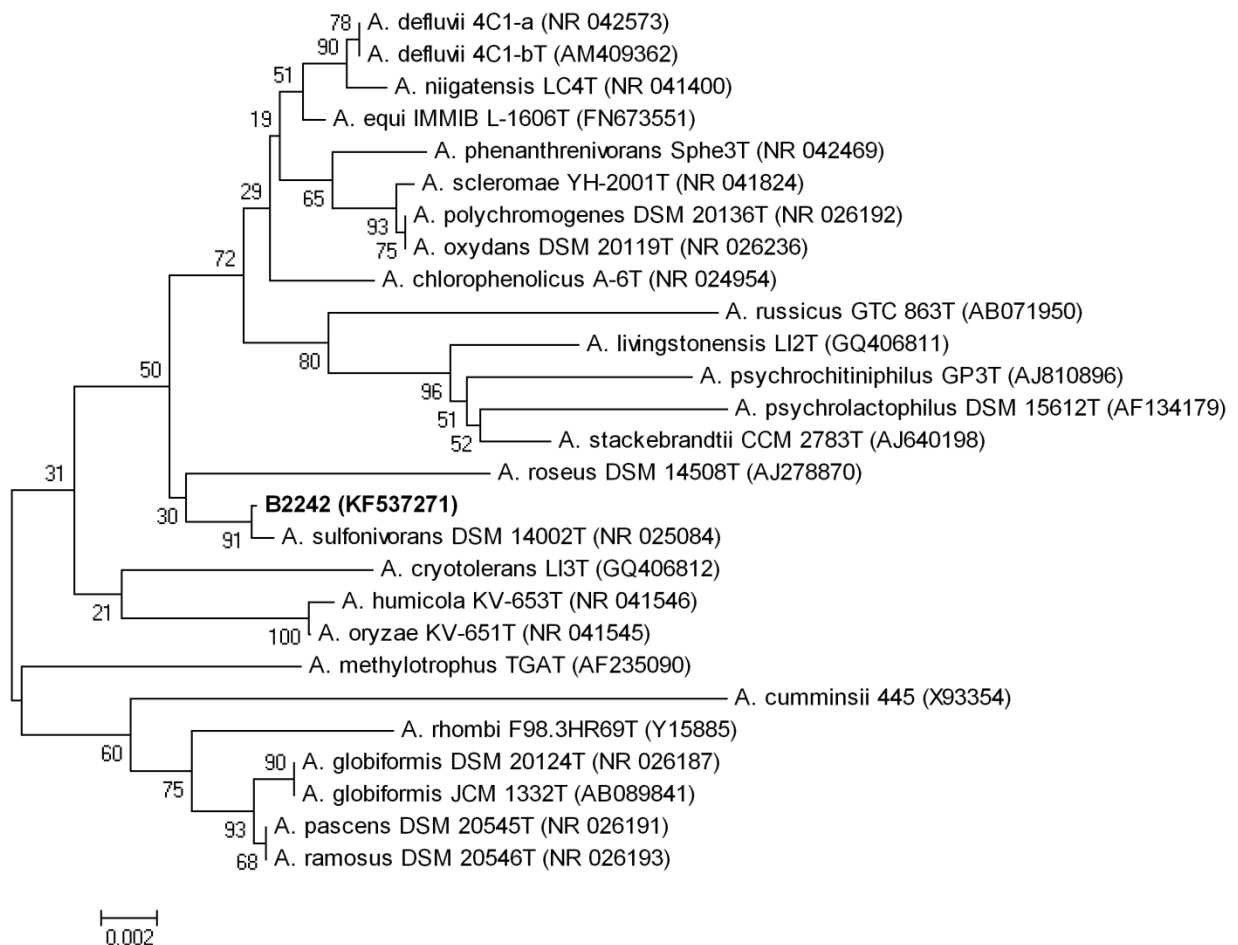


Figure 1 – Neighbor-joining phylogenetic tree, showing the relatedness between the strain B2242 and the type strains of the genus *Arthrobacter*. The GenBank accession numbers are given in parenthesis (www.ncbi.nlm.nih.gov). There were 1064 positions in comparison dataset. Bootstrap values were calculated based on 1000 resamplings. Scale bar, 0.002 substitutions per nucleotide position.

Synthesis and localization of β -galactosidase. A dark blue color of colony appearing during *A. sulfonivorans* BIM B-2242 growth on lactose-containing agar medium with X-Gal indicates probable β -galactosidase activity of the strain.

In fact, β -galactosidase activity was found in the extract of bacterial cells grown in medium with lactose for 72 h and in cell-free cultural liquid (table 1).

Table 1 – Cell growth and synthesis of intra- and extracellular β -galactosidase by *A. sulfonivorans* BIM B-2242 in the medium with lactose as a sole carbon source

Cell dry weight, mg·ml ⁻¹	Cell-free cultural liquid					Cell extract			
	Protein concentration, mg·ml ⁻¹	β -galactosidase activity				Total protein, mg	β -galactosidase activity		
		U·ml ⁻¹	Total, U	Biomass productivity, U·mg biomass ⁻¹	Specific activity, U·mg protein ⁻¹		Total, U	Biomass productivity, U·mg biomass ⁻¹	Specific activity, U·mg protein ⁻¹
7.8±0.39	0.15±0.0075	15.20±0.76	760.0±38.0	1.95±0.975	101.3±5.065	6.3±0.315	0.96±0.048	0.12±0.006	0.15±0.0075

Furthermore, it was revealed that the percentage of total intracellular β -galactosidase activity (0.96 U in biomass recovered from 50 ml of cultural liquid) to extracellular one (760.0 U in 50 ml cell-free cultural liquid) was 0.12% only. Specific activity of extracellular β -galactosidase quantified as 101.3 U·mg⁻¹ was 675.3 times higher than that of intracellular enzyme (0.15 U·mg⁻¹).

To determine whether *A. sulfonivorans* BIM B-2242 β -galactosidase has extracellular localization or is secreted into the medium during cells autolysis, a time course experiment were carried out to monitor enzyme activity in cell free cultural liquid and in cell extracts (figure 2).

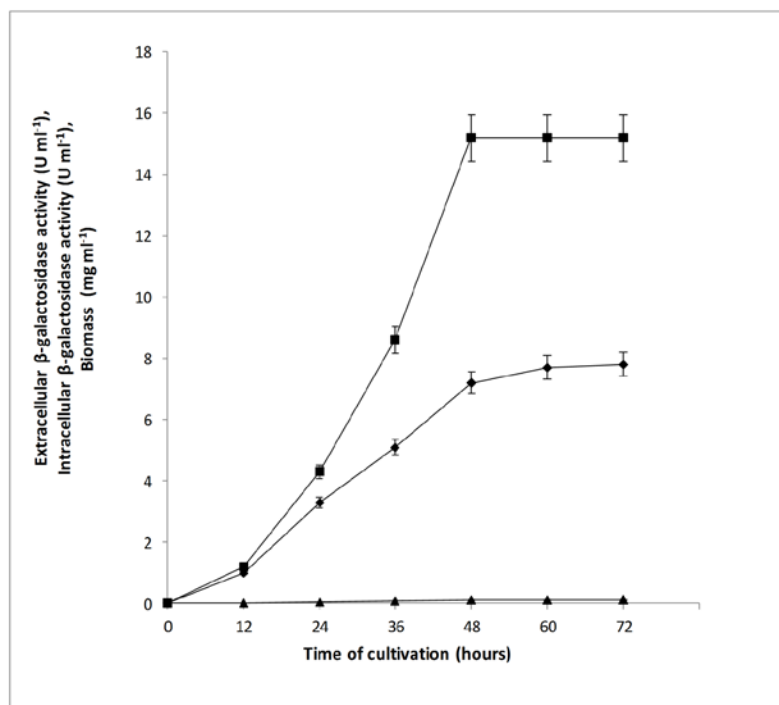


Figure 2 – *A. sulfonivorans* BIM B-2242 biomass growth (◆) and extra- (■) and intracellular (▲) β -galactosidase synthesis in medium with lactose (1.5%)

It was found that activity of the enzyme detected in cultural liquid at early log-phase of bacterial growth, rose quickly achieving 28% ($4.3 \text{ U} \cdot \text{ml}^{-1}$) of its peak value by 24 h of cultivation and reached maximum level ($15.2 \text{ U} \cdot \text{ml}^{-1}$) in period of 36–48 h. It should be noted that by that time *A. sulfonivorans* BIM B-2242 growth was not terminated and there were no indications of biomass lysis. In addition, intracellular level of β -galactosidase activity fluctuated insignificantly (0.05 – $0.12 \text{ U} \cdot \text{ml}^{-1}$) during the whole fermentation period.

Gel-electrophoresis of native extracellular proteins produced by bacterium in medium with lactose (1.5%) was performed in 12% PAGE. According to the data obtained (figure 3) two active molecular forms of β -galactosidase were observed on zymograms upon 24 h of fermentation and their ratio changed by 48 h. These enzyme proteins corresponded to dominant bands in PAGE pattern of total native extracellular proteins (figure 3).

In accordance with the obtained results we stated that *A. sulfonivorans* BIM B-2242 synthesizes a truly secretory β -galactosidase, in contrast with other prokaryotes producing intracellular enzymes. Moreover, the studied strain forms enzyme protein with the highest specific activity ever described. For comparison, this characteristic of crude and purified enzyme proteins produced by different strains of *Arthrobacter* genus varies in the range 0.05 – $3.6 \text{ U} \cdot \text{mg}^{-1}$ and 0.5 – $121.7 \text{ U} \cdot \text{mg}^{-1}$, respectively [12, 13, 19]. The exception is *Bacillus* sp. MTCC 3088 generating high level of extracellular β -galactosidase, up to $20 \text{ U} \cdot \text{ml}^{-1}$ by 12th day of cultivation [27]. Though the authors refer *Bacillus* sp. MTCC 3088 β -galactosidase to extracellular enzyme, it should be recognized that secretory form of enzyme protein emerged after long cultivation period correlating with the completion of growth, destruction of cells and, consequently, the release of the enzyme accumulated in the cells. The specific activity of *Bacillus* sp. MTCC 3088 β -galactosidase is also essentially lower than *A. sulfonivorans* BIM B-2242 enzyme specific activity and it does not exceed $1.23 \text{ U} \cdot \text{mg}^{-1}$ protein in cultural liquid or $44.4 \text{ U} \cdot \text{mg}^{-1}$ protein in purified form [28].

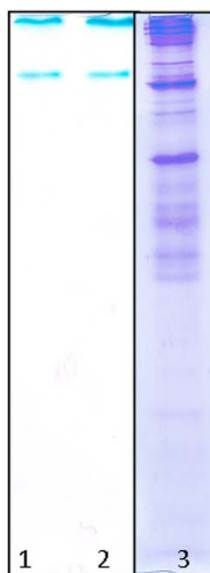


Figure 3 – Zymograms (1, 2) and electrophoregram (3) of native proteins, extracellularly produced by *A. sulfonivorans* BIM B-2242 by 24 (1) and 48 h (2, 3) in medium with lactose (1.5%)

Characterization of biochemical properties of Arthrobacter sp. BIMB-2242 β -galactosidase. A crude β -galactosidase of *A. sulfonivorans* BIM B-2242 has optima at 42.5°C and pH 7.0 (figure 4). At optimal temperature catalytic activity of the enzyme drops two-fold as compared to maximum at pH 6.0 and 8.0 and it is not detected at pH 5.0 and 9.0. These results are in agreement with data on properties of some β -galactosidases derived from *Arthrobacter* species [11; 15; 29].

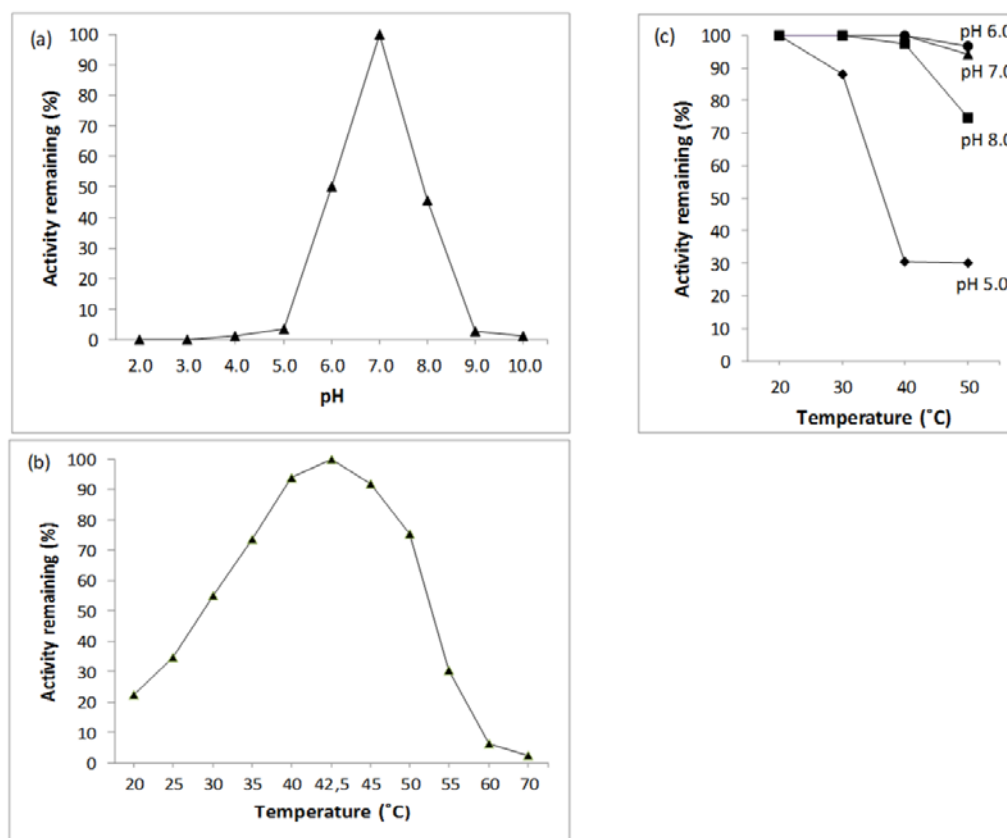


Figure 4 – Activity and stability of extracellular β -galactosidase *A. sulfonivorans* BIM B-2242. pH- (a) and thermostability (b) of β -galactosidase. (c) pH- and thermostability of β -galactosidase; symbols, pH: 5.0 (◆), 6.0 (●), 7.0 (▲), 8.0 (■)

At optimal pH value *A. sulfonivorans* BIM B-2242 β -galactosidase is stable after 60 min under 40°C, it loses 20% of activity at 50°C in 60 min, and it is totally inactivated at 60°C by 10 min and at 70°C – within a minute.

Enzyme keeps full activity for 15 min at 30°C and 40°C in pH range 6.0–8.0 and 6.0–7.0, respectively; at 50°C and pH 6.0–7.0 its activity is at least 94.2–96.8%. At pH 8.0 the enzyme retains 99.5% activity within 15 min at 40°C and up to 75% – at 50°C, while at pH 5.0 its residual activity at above-mentioned temperatures does not exceed 30%.

Conclusions

Summing up, analysis of experimental findings on properties of *A. sulfonivorans* BIM B-2242 β -galactosidase allows us to presume that this enzyme with unique for prokaryotes extracellular localization may find application in food industry for manufacturing lactose-free dairy products and in pharmacology as bioactive substance of medicines prescribed for patients suffering from lactase deficiency.

In our paper enzyme properties are the result of two isoenzymes action. Certainly, detailed studies are needed for origination of isoenzymes – genetic or posttranslational modification. Secretion mechanism and regulation of β -galactosidase synthesis in *A. sulfonivorans* BIM B-2242 will be studied in near future as well as characteristics of purified enzyme and multiple forms.

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