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β-GALACTOSIDASE ACTIVITY OF *MEIOTHERMUS RUBER* CELLS

JÓZEF SYNOWIECKI[1,3], IZABELA SINKIEWICZ[2], AGATA ZAKRZEWSKA[1], KATARZYNA GRUBIAK[1] and OLGA PIETROW[1]

[1]Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdańsk University of Technology, ul. Gabriela Narutowicza 11/12, 80-233 Gdańsk, Poland

[2]Department of Engineering and Catering Technology, Warsaw University of Life Sciences, ul. Nowoursynowska 159c, 02-776 Warsaw, Poland

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ABSTRACT

Freeze-dried cells of *Meiothermus ruber* catalyses cleavage of o-nitrophenyl-β-D-galactopiranoside (oNPβ-gal) and conversion of lactose into glucose and galactose. The permeabilization with 2% toluene, 20% ethanol and 20% acetone increased enzymatic activity from 74.87 U/g of lyophilized cells up to 129.44, 114.38 and 90.19 U/g, respectively. Ethanol was an effective permeabilizing agent and its efficiency was dependent on the concentration, the incubation time and incubation temperature. The K_m values for the untreated and permeabilized cells were 2.94 mM and 2.26 mM but V_{max} values were 122 μmol/min and 193 μmol/min, respectively. The optimum pH for the β-galactosidase activity in the untreated and permeabilized cells were 6.5 and optimum of temperatures 65C. The stability of enzymatic activity in *M. ruber* cells incubated for 1 h at pH 6.5 was almost unchanged at temperatures below 65C.

PRACTICAL APPLICATIONS

The current study shows that freeze-dried permeabilized cells of *Meiothermus ruber* can be used instead of isolated β-galactosidase. It leads to elimination of expensive purification procedures and assures longer half-life time of enzyme activity. Application of such biocatalyst for lactose hydrolysis in milk products for human consumption is limited until designation of the Generally Recognized As Safe status of *Meiothermus ruber*. It can be employed, however, for other purposes, e.g., for production of alkyl glycosides, a group of non-ionic surfactants with a variety of applications in detergents, cleaning agents, personal care products and pharmaceuticals.

INTRODUCTION

Thermostable β-galactosidases or β-glucosidases are useful for hydrolysis of lactose (Lac) or for oligosaccharide synthesis (Moracci *et al*. 2001; Splechtna *et al*. 2002; Synowiecki and Maciuńska 2002; Ladero *et al*. 2003; Kim *et al*. 2006; Synowiecki 2008). Compared with β-galactosidases from mesophilic sources the application of their thermostable counterparts causes: enhanced reaction velocity, reduced risk of microbial contamination, increased substrate solubility and reduced product inhibition (Petzelbauer *et al*. 1998). Furthermore, reduced activity of the thermostable enzymes at low temperature makes it possible to control the reaction by cooling.

During the last decades, there has been considerable interest in the direct use of intracellular enzymes of intact microbial cells (Groboillot *et al*. 1994; Ramakrishna and Prakasham 1999). Their application eliminates expensive procedures of enzyme isolation and purification and cause longer half-life of enzyme activity, by reason of protective effect of the cell interior (Duetz *et al*. 2001). However, for high enzymatic activity of whole cells, the efficient methods for reducing the barrier-like action of the cell envelope should be used (Felix 1982; Ni and Chen 2004). Cells ought to be permeabilized in such a way that substrates and products molecules can freely enter and leave the cells without significant enzyme leakage. Although biotransformations based on whole cells are common, their use for enzymatic processing of Lac received not very much attention (Siso *et al*. 1992; Siso and Doval 1994; Carvalho Lins and Rocha Leao 2002). Recently, the wet cells of *Kluyveromyces lactis* and recombinant cells of *Escherichia coli* with plasmid-encoded expression β-glycosidase

from *Pyrococcus woesei* were used for this purpose (Kamrat and Nidetzky 2007; Lee *et al.* 2004).

This article presents the study concerning β-galactosidase activity of the freeze-dried cells of aerobic, gram-negative, thermophilic bacteria, which belong to *Meiothermus* genus (Nobre *et al.* 1996). This dark red pigmented and no endospore-forming microorganism isolated from hot springs grows well at 55C on a media containing peptone and yeast extract (Teneiro *et al.* 1995). To the best of the present knowledge, this is the first report of the possible use of lyophilized cells of *Meiothermus ruber* as a biocatalyst with β-galactosidase activity. The application of whole *M. ruber* cells as biocatalyst for Lac hydrolysis in milk products provided for human consumption is limited, because the Generally Recognized As Safe (GRAS) status of this bacterium is still not designated. However, it can be employed for other purposes, e.g., for production of alkyl glycosides a group of non-ionic surfactants with a variety of applications in detergents, cleaning agents, personal care products and pharmaceuticals (Das-Bradoo *et al.* 2004).

MATERIALS AND METHODS

Microorganism, Cultivation and Cells Permeabilization

Meiothermus ruber (DSM 1279) was cultivated under aerobic conditions on liquid media (pH 8.0) containing 0.5% of peptone bio-Trypcase (bio Merieux, Craponne, France), 0.1% of yeast extract (Difco Laboratories, Lawrence, KS) and 0.1% of starch. The growth media were sterilized for 30 min at 121C. The inoculum was prepared by incubation (48 h, 55C) of lyophilized *M. ruber* cells with 5 mL of the growth medium. The cultures were then incubated at 55C in Erlenmeyer flasks with a working volume of 1 L with the agitation rate of 160 rpm. After 24 h of growth without additional aeration, the cells were harvested by centrifugation at 9,000× g for 15 min and washed with deionized water.

In order to prepare permeabilized cells of *M. ruber* the harvested cells were re-suspended in 200 mL of ethanol solution (10–40%, v/v), stirred (160 rpm) for 10 min, 30 min or 60 min at 20, 30, 40 and 50C, centrifuged at 9,000× g for 15 min, washed twice with deionized water and freeze-dried. Controls included cells re-suspended in water. Cells permeabilized at standard toluene concentration of 2% were used as reference (Somkuti *et al.* 1996).

The leakage of β-galactosidase and other proteins from permeabilized cells was determined by extraction (30 min, 60C) of the cells with phosphate citrate buffer (pH 6.5). Proteins concentration in the extract was assayed according to Bradford (1976) using bovine serum albumin as a standard.

Enzyme Assay

The activity of β-galactosidase was determined according procedure of Craven *et al.* (1965), using 5 mM solution of *o*-nitrophenyl-β-D-galactopyranoside (*o*NPβ-gal) in 0.1 M phosphate citrate buffer (pH 6.5 adjusted at 65C). The assays were initiated by addition of the desired quantity of lyophilized cells, suspended in 0.2 mL of 0.1 M phosphate citrate buffer to 2.8 mL of substrate pre-incubated for 2 min at 65C. The reaction at 65C was terminated after 5 min by 1 mL of 1 M Na_2CO_3 solution and the cells were removed by centrifugation at 9,000× g for 5 min. A blank containing buffer instead of the cell suspension was used to correct the thermal hydrolysis of *o*NPβ-gal. The absorbance of *o*-nitrophenol released during reaction was measured at 420 nm and was converted to its concentration using a molar absorption coefficient of $4.5 \times 10^3 \times M^{-1}$. One unit of β-galactosidase activity (U) is defined as the amount of the cells required to release 1 μmol of *o*-nitrophenol per minute under the indicated assay conditions. The relative enzyme activity (%) was defined as the percentage of the maximal value of activity.

Catalytic Properties and Thermostability of the Lyophilized Cells

The pH-dependence on β-galactosidase activity in the intact cells of *M. ruber* was determined at 65C using 5 mM *o*NPβ-gal solution in 0.1 M phosphate citrate buffers within the pH range of 5.0–8.0. The pH of the buffers was adjusted at 65C. The temperature-dependence on the cells activity was assayed within the range of 55–80C. To examine the stability of β-galactosidase in the cells against thermal denaturation, the cells suspended in 0.1 M phosphate citrate buffer (pH 6.5) were incubated at various temperatures (ranging from 45 to 80C) for 60 min. After incubation the samples were cooled in ice and 0.2 mL of the suspension (containing 1.5 mg of the cells) were used for determination of remaining activity at 65C and pH 6.5. To evaluate the pH stability, the pH of 0.1 M phosphate citrate buffers used for incubation of the cells (55C, 1 h) was varied between 4.5 and 8.0. The effect of substrate concentration on the reaction rate was determined using 1–20 mM *o*NPβ-gal solutions in 0.1 M phosphate citrate buffer (pH 6.5). K_m and V_{max} were calculated from the Lineweaver–Burk plot, where (V) and (S) are the rate of the reaction and substrate concentration, respectively.

Identification of the Products of Lac Conversion

The products of Lac conversion catalyzed by freeze dried cells of *M. ruber* (60C, pH 6.5) were separated by high-

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performance liquid chromatography (HPLC) using Rezex RPM Monosaccharide Pb^{+} column (Phenomenex, Torrance, CA) and refractive index detector (La Chrom L-7490, Merck, Darmstadt, Germany). The samples purified by the cells separation, thermal precipitation (110C) of the residual proteins, adsorption on charcoal, centrifugation (8,000× g) for 15 min and filtration on a 0.2-μm Puradisc™ filter (Whatman Inc., Florham Park, NJ) were passed through a column using water as the mobile phase at a flow rate of 0.4 mL/min. The column temperature was 65C. Lac, glucose (Glc) and galactose (Gal) were used as standards at concentrations of 10 mg/mL.

Cell Immobilization

The permeabilized cells (75 mg) were suspended in 20 mL 0.15 M NaCl solution, mixed with 40 mL of 4.5% sodium alginate solution and slowly injected at room temperature into 0.2 M $CaCl_2$. The cells entrapped in calcium alginate granules (0.5–0.7 mm i.d.) were washed with distilled water on filter paper (Whatman) and used for determination of β-galactosidase activity at conditions given earlier.

Preparation of Crude Enzyme

The frozen wet cells (10 g) of *M. ruber* were disrupted for 15 min in a refrigerated mortar with 20 g of Alumina A-5 (Sigma, St. Louis, MO). During extraction 70 mL of 0.01 M phosphate buffer (pH 6.2) containing 1 μmol/mL of dithiotreitol was gradually added. The resulting suspension was centrifuged at 9,000× g for 15 min and the supernatant was concentrated by ultrafiltration on a Centriplus Centrifugal Filter Device with Ultracel-YM membrane 30 kDa cut-off (Millipore, Billerica, MA).

RESULTS AND DISCUSSION

The HPLC identification of the products obtained after lactose conversion catalyzed by freeze-dried (lyophilized) cells of *Meiothermus ruber* shows generation of Glc and Gal (Fig. 1). Thus, the whole cells of this bacterium give attention because of their possible utilization in the processing of lactose-containing fluids. The cells of thermophiles offer an interesting alternative to that belonging to mesophiles because enhanced reaction temperature leads to better diffusion of substrate through cell envelope as well as to improvement of lactose solubility and higher resistance of reaction media against microbial contamination. However, to avoid changes in milk taste and aroma caused by Maillard reaction products, the moderate thermophiles (e.g., *M. ruber*) producing β-galactosidase with optimal activity at temperatures below 80C should be used.

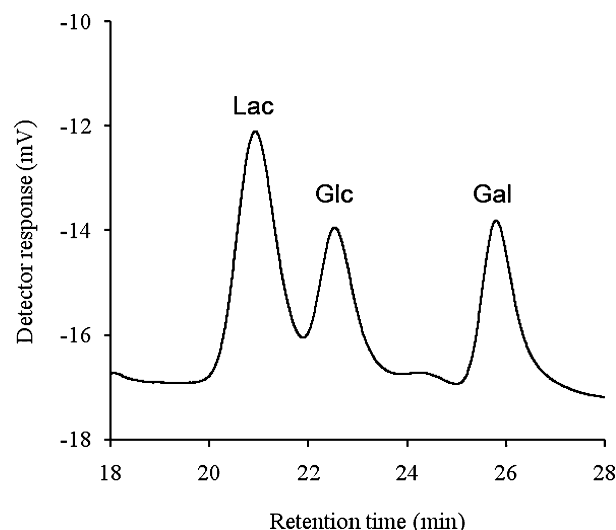

FIG. 1. CHROMATOGRAM OF THE PRODUCTS (GLUCOSE [Glc], GALACTOSE [Gal]) OBTAINED AFTER 15 MIN CONVERSION OF 30 mM LACTOSE (Lac) IN PHOSPHATE CITRATE BUFFER (pH 6.5) AT 60C
The products were determined using Rezex RPM Pb^{+} column and refractive index detector (La Chrom L-7490, Merck).

The enzymatic activity of the intact cells depends on diffusion rate of substrate and product through cell envelope. It can be increased by freeze-drying, which leads to pore formation in the cytoplasmic membrane (Jirkú 2004). Enhancement of membrane permeability caused by freeze-drying is generally a consequence of lypolysis, lipids oxidation and changes in water activity during lyophilization (Castro *et al.* 1996; Castro *et al.* 1997).

The present study showed that the cells permeabilized only by freeze-drying had β-galactosidase activity (measured at 60C towards *o*NPβ-gal) of 74.87 ± 5.96 U/g. Further increase of activity can be achieved by preliminary degradation of the cytoplasmic membrane with organic solvents (Shutte and Kula 1990). Treatment *M. ruber* cells with toluene leads to 1.73-fold increased activity of lyophilized biomass. Similar value (1.56) had this factor calculated using data reported by Canovas *et al.* (2005) for gram-negative mesophile *Escherichia coli* permeabilized with 2% of toluene. Similarity of these results suggests that increased termostability of the cell membranes of *M. ruber* did not significantly influence permeabilization efficiency. Toluene is the agent most frequently used (Felix 1982). However, their application in the food processing is restricted and in further study it was replaced by ethanol, allowed as a food additive. The permeabilization of *M. ruber* in relation to β-galactosidase activity showed that treatment (35C, 30 min) of the cells with 20% ethanol was only 11.6 ± 1.1% less effective as the standard permeabilization procedure (35C, 30 min) with toluene.

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TABLE 1. EFFECT OF PERMEABILIZING AGENTS ON THE β-GALACTOSIDASE ACTIVITY IN LYOPHILIZED CELLS OF *M. RUBER*

		β-galactosidase activity, U/g of cells:		
Permeabilizing agent	Concentration (%)	Before permeabilization (a)	After permeabilization (b)	b/a
Toluene	2	74.87 ± 1.04	129.44 ± 5.10	1.73
Ethanol	10	78.03 ± 2.46	86.13 ± 2.39	1.10
Ethanol	20	78.03 ± 2.46	114.38 ± 4.70	1.46
Ethanol	30	78.03 ± 2.46	105.30 ± 3.92	1.35
Ethanol	40	78.03 ± 2.46	94.23 ± 4.72	1.20
Acetone	20	70.22 ± 0.69	90.19 ± 2.72	1.28

The results are mean values of three replicates ± standard deviation.

TABLE 2. EFFECT OF PERMEABILIZATION TEMPERATURE AND CONTACT TIME WITH 20% ETHANOL ON β-GALACTOSIDASE ACTIVITY (U/g) OF LYOPHILIZED *M. RUBER* CELLS

	Permeabilization temperature (C)			
Exposure (min)	20	30	40	50
10	100.59 ± 1.26	109.21 ± 1.23	110.12 ± 1.05	91.01 ± 2.01
30	104.28 ± 0.73	113.25 ± 0.68	113.05 ± 1.56	94.35 ± 0.99
60	112.58 ± 1.15	118.23 ± 2.35	118.09 ± 0.75	101.86 ± 0.58
90	114.22 ± 1.93	118.15 ± 1.43	–	–

The results are mean values of three replicates ± standard deviation.

Similarity of permeabilization efficacy of *Streptococcus thermophilus* with toluene and ethanol was also reported by Somkuti *et al*. (1996).

The level of β-galactosidase activity of permeabilized cells increased with alcohol concentration and the maximum was reached with 20% ethanol. Permeabilization with 30% or higher ethanol solutions resulted in a decrease in enzyme activity (Table 1). The time of permeabilization also influenced β-galactosidase activity and the highest values were achieved after 30–60 min of treatment (Table 2). Contact time longer than 30 min only little influenced on enzyme activity.

Permeabilization with ethanol slightly influenced on release of β-galactosidase and other proteins during extraction of *M. ruber* cells with buffer used for determination of enzyme activity (Table 3). The results of this study showed that obtained extract contained only 4.28–5.55% of proteins and had 2.59–3.32% of β-galactosidase activity exhibited by crude enzyme prepared by disruption of the cells, according to Materials and Methods section. Despite the fact that *M. ruber* is red pigmented the extract of the cells obtained at conditions of enzymatic reaction remains colorless.

As illustrated in Fig. 2, the optimum temperature for β-galactosidase activity exhibited by lyophilized cells and cells freeze-dried after permeabilization with ethanol was about 75C. Figure 3 shows the retention of β-galactosidase activity in *M. ruber* cells incubated for 1 h at various temperatures. The enzymatic activity was very stable in phosphate citrate buffer (pH 6.5) at temperatures below 65C. The increase in

TABLE 3. EFFECT OF ETHANOL CONCENTRATION ON THE LIBERATION OF PROTEINS AND β-GALACTOSIDASE FROM PERMEABILIZED CELLS OF *M. RUBER*

Ethanol concentration (%)	Extracted proteins (mg/g dry basis of the cells)*	β-galactosidase activity in the extract (U/g dry basis of the cells)
0	10.42 ± 0.31	1.94 ± 0.21
10	11.98 ± 0.19	1.98 ± 0.12
20	12.59 ± 0.82	2.43 ± 0.33
40	13.50 ± 0.47	2.49 ± 0.11

* Cells permeabilized after lyophilization were extracted (30 min, 60C) with 0.1 M phosphate-citrate buffer (pH 6.5).
Results are mean values of three replicates ± standard deviation.

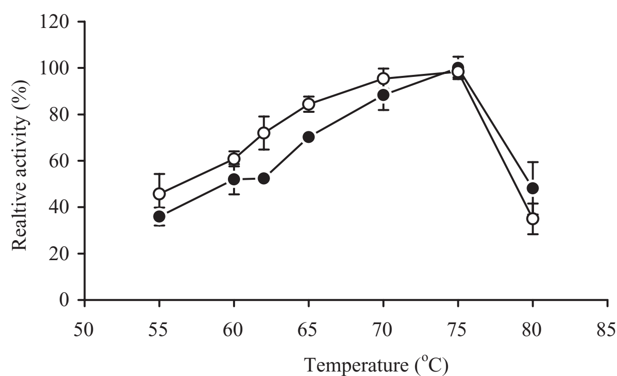

FIG. 2. EFFECT OF TEMPERATURE AT pH 6.5 ON ACTIVITY OF β-GALACTOSIDASE IN UNTREATED (●) AND PERMEABILIZED CELLS OF *MEIOTHERMUS RUBER* (○) ON *O*-NITROPHENYL-β-D-GALACTOPYRANOSIDE Untreated and permeabilized cells were freeze-dried before determination of enzymatic activity. The results are mean values of three determinations.

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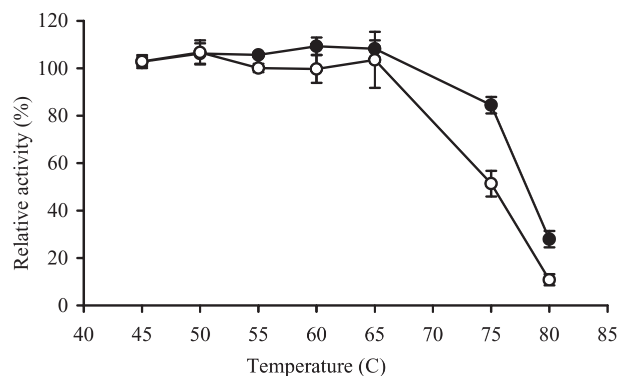

FIG. 3. STABILITY OF β-GALACTOSIDASE ACTIVITY IN UNTREATED (●) AND PERMEABILIZED (○) CELLS INCUBATED FOR 1 h UNDER DIFFERENT TEMPERATURES IN 0.1 M PHOSPHATE CITRATE BUFFER (pH 6.5)
The results are mean values of three replicates.

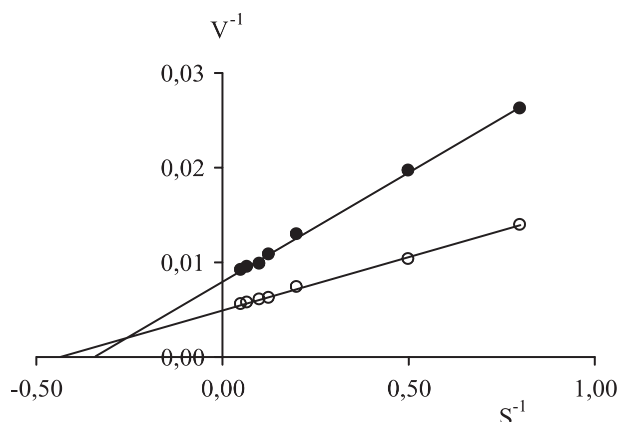

FIG. 5. LINEWEAVER–BURK PLOT FOR *O*-NITROPHENYL-β-D-GALACTOPYRANOSIDE HYDROLYSIS CATALYZED BY UNTREATED (●) AND PERMEABILIZED (○) CELLS OF *MEIOTHERMUS RUBER*

residual activity after 1 h incubation of the cells within the range of 45–70C could be a consequence of the conformational change of the protein. This fact was also indicated in case of thermostable β-galactosidase isolated from *Sulfolobus solfataricus* (Pisani *et al.* 1990). On the other hand, almost complete inactivation of *M. ruber* cells was observed after preincubation at 80C.

The highest activity of permeabilized and untreated cells was achieved at pH 6.5 and both preparations indicated about 60% of maximal activity between pH 5.2–7.5 (Fig. 4). This makes the application of intact *M. ruber* cells to lactose hydrolysis in whey possible. Such biocatalyst is more stable in comparison with the free enzyme and can be reused as well as immobilized to a solid support, e.g., by entrapment in calcium alginate gel. To determine the effect of pH on the stability of β-galactosidase in permeabilized *M. ruber*, the cells were incubated for 1 h at 55C and at different pH

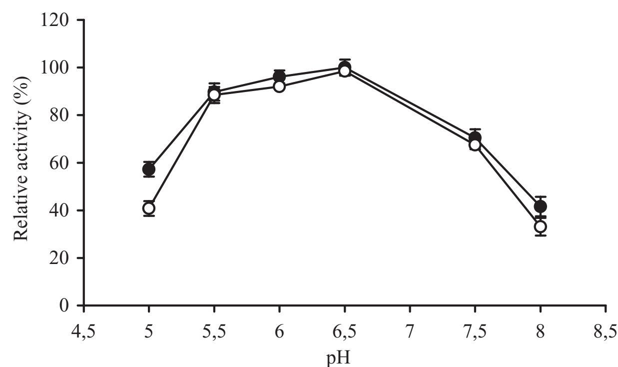

FIG. 4. EFFECT OF pH ADJUSTED BY 0.1 M PHOSPHATE CITRATE BUFFER ON ACTIVITY OF β-GALACTOSIDASE IN UNTREATED (●) AND PERMEABILIZED CELLS (○) OF *MEIOTHERMUS RUBER*
The reaction temperature was 65C. Results are mean values of three replicates.

values adjusted by phosphate citrate buffers. The highest remaining activity had the cells incubated at neutral pH and relatively low decrease of activity (not exceeding 15%) was observed at pH values ranged between 5.5 and 7.5 (data not shown).

Comparative studies were performed to determine the K_m and V_{max} values for β-galactosidase both in permeabilized or untreated cells of *M. ruber*. The effect of *o*NPβ-gal concentration on hydrolysis rate was examined at pH 6.5 and 60C. This study showed the increase of V_{max} value for permeabilized cells (from 122 μmol/min for untreated cells up to 193 μmol/min after their permeabilization) whereas permeabilization diminished the K_m value (from 2.94 mM to 2.26 mM) (Fig. 5). The data presented earlier confirmed that permeabilization decreased the diffusional restrictions.

Freeze-dried, permeabilized cells, entrapped in calcium alginate beads, were used for repeated batch conversion of *o*NPβ-gal. The results of this study showed that β-galactosidase activity of the cells decreased continuously, and after seven separate reactions the residual activity was 49.5% of the initial value.

CONCLUSIONS

The presented results indicate that the biocatalyst with β-galactosidase activity obtained by permeabilization of *Meiothermus ruber* cells seems to be useful for industrial lactose conversion. According to our knowledge, no previous research on β-galactosidase activity of permeabilized cells of *M. ruber* has been reported. The operational stability of enzyme activity and the low cost make whey processing possible at a temperature that minimizes undesired microbial contamination of the reaction mixture and leads to increased reaction rate.

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