Isolation and Identification of a Strain Producing Cold-Adapted β-Galactosidase, and Purification and Characterisation of the Enzyme

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Abstract

Liu W.Y., Shi Y.W., Wang X.Q., Lou K. (2008): **Isolation and identification of a strain producing cold-adapted β-galactosidase, and purification and characterisation of the enzyme**. Czech J. Food Sci., **26**: 284–290.

Enzymes with high specific activities at low temperatures have potential uses in the food industry. Cold-adapted microorganisms are potentially useful sources of cold-active enzyme. To find cold-adapted β-galactosidase, we isolated several cold-adapted microorganisms from glacier zone soil. One cold-adapted β-galactosidase producing strain was obtained. The biochemical characteristics and the results of 16S rDNA sequencing identified the strain as *Rahnella aquatilis*. The enzyme was purified by column chromatography after which a single protein band migrating near 60 kDa was observed by means of SDS-PAGE. The β-galactosidase was optimally active at 35°C and at pH 6.5 when assayed with *o*-nitrophenyl-β-D-galactopyrano-side as substrate. The enzyme activity was sensitive to temperatures above 40°C and was undetectable at 45°C. Metal ions $Mn^{2+}$and K^{+} activated the enzyme while Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+} inhibited the activity. The enzyme was also assayed for lactose hydrolysis. When milk is treated with the enzyme at 30°C for 2 h, the degree of lactose hydrolysis can reach 80%. It has, thus, potential applications in the food industry.

Keywords: psychrotrophic microorganism; cold-adapted β-galactosidase; lactose hydrolysis

Lactose is abundant in milk, but it cannot be directly taken up by humans. However, cold-adapted β-galactosidase that hydrolyses lactose to glucose and galactose is an important food-industrial enzyme, because it can be used to remove lactose from milk at a low temperature so that it can be consumed by people who are lactose intolerant, and also to convert lactose in whey, a by-product of the cheese industry, from a pollutant to more readily fermentable glucose and galactose.

Cold-adapted microorganisms are potentially useful sources of cold-adapted enzymes as they are capable of growing in extreme low-temperature environments, such as polar regions, deep seas, jokul groups, and glaciers. Studies on the isolation of cold-adapted β-galactosidase producing

Supported by the Xinjiang Academy of Agricultural Sciences, Youth Fund No. 2005Q02.

strains have been performed from 1993 to now (Loveland *et al*. 1993,1994; Trimbur *et al*. 1994; Hoyoux *et al*. 2001; Fernandes *et al*. 2002; Coker *et al*. 2003, 2006; Nakagawa et al. 2003, 2006a, b, 2007; Turkiewica *et al*. 2003; Hubert *et al*. 2005; Park *et al*. 2006)..

In this study, we report on *Rahnella aquatilis* strain14-1, obtained from glacier zone soil in Xin-Jiang province of China. We have examined the growth and physiology of the strain. The strain can grow at 0°C but it does not form isolated colonies at 37°C. The ability to grow near 0°C is a characteristic of both psychrotrophic and psychrophilic microorganisms. Psychrotolerants grow optimally at temperatures around 20°C to 25°C, and may have upper limits as high as 40°C. In contrast, true psychrophiles are specifically adapted to the low-temperature growth and have optimal growth temperature below 15°C and the upper limits below 20°C (Morita 1975). The optimum growth temperature for our isolate was 20°C, so it belongs to the psychrotolerants. Therefore, we purified the enzyme from the intracellular fraction of the strain and studied the characteristics of irs cold-adapted β-galactosidase.

MATERIAL AND METHODS

Screening and isolation conditions. Soil samples were collected from glacier zone soil in XinJiang province of China. Approximately 1 g of the sample was added to 100 ml of 0.85% saline solution, diluted to 10^{-5}, and spread on tryptic soy agar (TSA) plates with 0.5% lactose as the sole carbon source and containing 0.1% of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, TaKaRa, Japan). The incubation was carried out at 15°C. After approximately 2 to 3 days, the colonies which appeared blue were collected and streaked onto medium. Once the isolates were purified, the strain producing enzyme of high activity was obtained by fermentation in a shaking flask.

Morphological and biochemical characterisation. Microscopic identification was done by performing Gram staining with cells grown in the exponential phase. The biochemical properties of the strain were determined using the Biomerieux Vitek 32 gram-negative identification card (GNI+, BioMerieux, France). The assays were done twice, and if the results were marginal, they were repeated for the third time.

16S rDNA gene amplification and sequencing. Genomic DNA was acquired from the strain using the bacteria genome extraction kit (50 preps, HuaShun, Shanghai, China). The 16S rDNA gene was amplified from chromosomal DNA by polymerase chain reaction (PCR). PCR was conducted in a 25 µl reaction mixture containing 0.5 µl of template DNA, 0.5 µl of each primer, 11 µl sterilised water, 1.25 U of TaKaRa Ex Taq DNA polymerase, 0.4mM each dNTP and 4mM Mg^{2+}, using the following program: 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and the extension at 72°C for 2 min and 30 seconds. The primers used were as follows: 27F (5-AGAGTTT ATCCTGGTCAG-3') and reverse, 1541 (5'-AAGGAGGTGATCCAGCC-3'). The PCR products were sequenced at Sangon (Shanghai, China) with the appropriate primers.

Phylogenetic analysis of 16S rDNA. The 16S rDNA gene sequence found was compared with those from the national center for biotechnology information (NCBI) database (http:www.ncbi.nlm/nih.gov) and aligned using the Clustax W program (Thompson *et al*. 1997). The phylogenetic tree for the data set was created via the neighbour-joining method using the Mega 3 program (Kumar *et al*. 2004). The stability of relationships was evaluated via bootstrap analyses of the neighbour-joining data (Saitou & Nei 1987).

Enzyme purification. Stain 14-1 was grown in 30 ml tryptic soy broth (TSB) at 20°C for 10 hours. This culture was inoculated into 2000 ml of TSY medium (15 g of tryptone, 5 g of soya peptone, 3 g of yeast extract, 5 g of NaCl, and 5 g of K_2HPO_4 per liter) containing 2.5 g lactose per 100 ml. The cultures were incubated for 48 h, and the cells were harvested by centrifugation at 10 000 g at 4°C for 10 min and were then resuspended in 200 ml distilled water. The cells were disrupted with a sonifer, and the debris was removed by centrifugation at 14 000 g and 4°C for 20 minutes.

Ammonium sulfate was gradually added to the crude extract to 20% saturation, and the mixture was incubated for 4 h and centrifuged at 14 000 g and 4°C for 20 min. The pellet was discarded, and additional ammonium sulfate was added to 60% saturation. After the static incubation overnight, the solution was centrifuged again, and the supernatant was discarded. The pellet was resuspended in 50 ml of 0.02M Tris-HCl buffer (pH 6.5). The sample was applied to a a Sephadex G-25 (Sigma, USA) column previously equilibrated with 0.02M Tris-HCl buffer (pH 6.5). The enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min.

The active fraction was applied to a DEAE-cellulose anion exchanger column (Sigma, USA) and eluted using a stepwise increase of NaCl concentration from 1% to 5% at a flow rate of 0.3 ml/min. After DEAE-cellulose anion exchange column chromatography, the fraction possessing β-galactosidase activity was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue.

Enzyme assays. β-galactosidase activity was determined by measuring the hydrolysis of 0.25% *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 0.1M sodium phosphate buffer, pH 6.5. The reaction mixture was incubated at 30°C for 10 min, and the enzyme reaction was terminated with 0.8 ml 0.5% Na_2CO_3. The absorbence of the mixture was measured at 420 nm. One unit (U) of activity was defined as the amount of enzyme that produced 1 μmol of *o*-nitrophenol (ONP) per minute. On the other hand, hydrolysis of lactose was measured by detection of the produced glucose. To 1 ml milk containing 5% lactose was added 0.5 ml of the purified β-galactosidase, and the solution was incubated at 30°C for 4 hours. We detected the produced glucose every 0.5 hours. Protein was determined by the Bradford method using bovine serum albumin (BSA) as the standard (Bradford 1976).

RESULTS

Isolation and identification of cold-adapted β-galactosidase producing strain

Isolate 14-1 was selected for further study because the cells grew at low temperatures and hydrolysed the chromogen X-gal which was the indicator of β-galactosidase activity. Microscopic examination showed that strain 14-1 was rod-shaped during the exponential phase, and gram-stained preparation of the strain was gram-negative. It grew at temperatures from 0°C to 30°C but did not form colonies on TSA medium at 35°C. The results of the biochemical characterisation, obtained using a GNI+ card, are shown in Table 1. Our results were closely cosistent with the previously published biochemical profiles of *R. aquatilis* (Holt *et al*. 1994).

To examine the phylogenetic position of our isolate, we amplified nearly the complete sequence (1451 nucleotides) of the 16S rDNA gene via PCR, determined its sequence, and then examined the relevant phylogenetic relationships via the neighbour-joining method. The isolated bacteria was clustered to a type strain, *Rahnella aquatilis* (Figure 1). In addition, the 16S rDNA sequence from strain 14-1 evidenced similarities of 99% to that

Table 1. Biochemical properties of the isolated bacteria

Principal component	Strain 14-1	Principal component	Strain 14-1
Glucose	+	Sorbitol	–
Peptone, tryptophan	+	Sucrose	+
Acetamide	–	Inositol	–
Esculin	+	Adonitol	–
Indoxyl-β-D-glucoside	+	*p*-Coumaric	–
Urea	–	Sodium thiosulfate	–
Citrate	+	*o*-Nitrophenyl-β-D-galactopyranoside	+
Malonate	–	Rhamnose	+
Polymyxin B	–	L-Arabinose	+
Lactose	+	Glucose	+
Maltose	+	Arginine	–
Mannitol	+	Lysine decarboxylase	–
Xylose	+	Ornithine	–
Raffinose	+	Oxidase	–

+ positive reaction; –,negative reaction

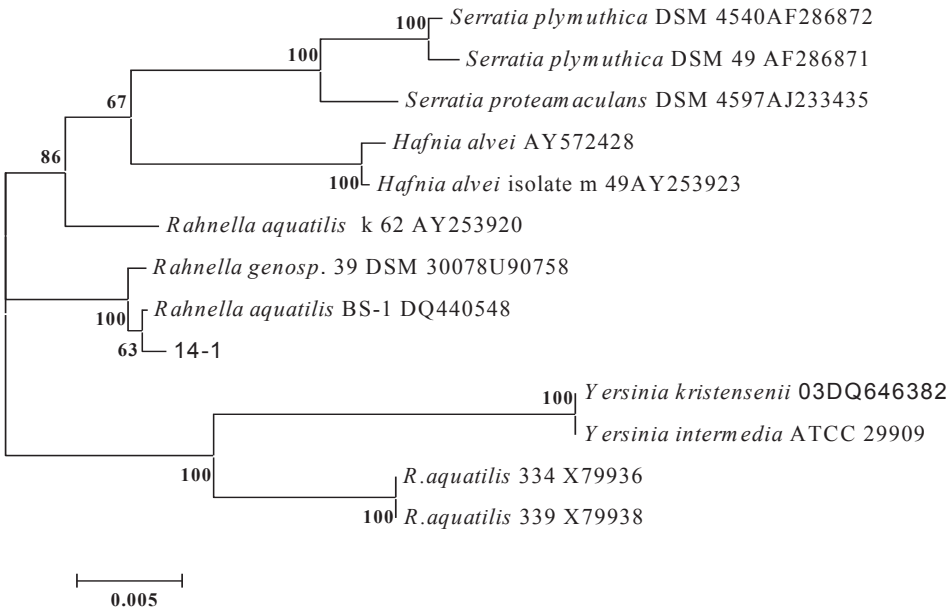
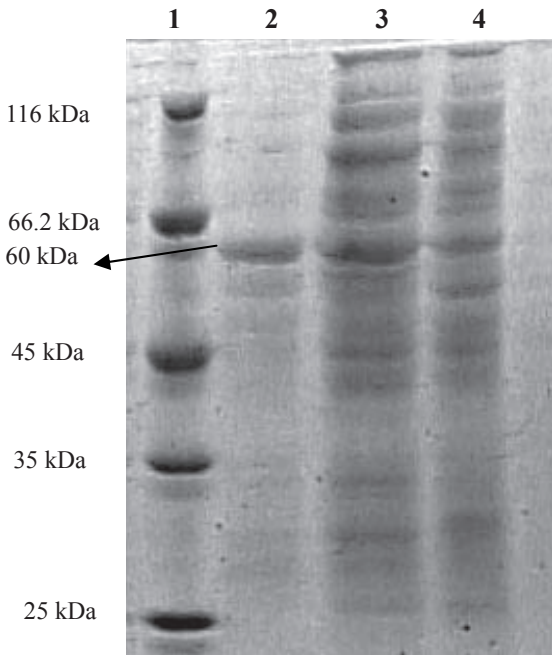


Figure 1. Phylogenetic tree of the 16S rDNA sequences of 14-1 and other taxa

of *Rahnella aquatilis* BS1 type strain from GenBank database (Figure 2). In accordance to its biochemical properties and phylogenetic analysis, we concluded that the strain 14-1 belongs to *Rahnella aquatilis*.



Lanes: 1 – molecular marker, 2 – pooled fractions after DEAE-cellulose chromatography, 3 – pooled fractions after sephadex G-25 chromatography, 4 – crude enzyme

Figure 2. SDS-PAGE of strain 14-1 β-galactosidase containing samples at different stages of purification

Characterisation of β-galactosidase

Because we thought that the enzyme from strain 14-1 may be a novel type of cold-adapted β-galactosidase, we attempted to purify the β-galactosidase from strain 14-1. The purification procedure is summarised in Table 2. After the DEAE-cellulose anion exchange column chromatography, the fraction containing β-galactosidase gave a single band sodium in10% SDS-PAGE, which corresponded to a molecular mass of ca. 60 kDa (Figure 2).

The optimum temperature for ONPG hydrolysis by the *Rahnella aquatilis* 14-1-β-galactosidase is 35°C. The enzyme from the strain 14-1 displays approximately 40% of maximum activity at 15°C (Figure 3). It is stable for 6 h at temperatures as low as 30°C and undergoes rapid denaturation above 40°C (Figure 5). The carbohydrase is completely inactivated within 1.5 h at 45°C. The optimum pH range of the enzyme activity is 6.5 to 7.0 (Figure 4). The activity of the strain 14-1 β-galactosidase is slightly enhanced by Mn^{2+} (only 19.8% rise in activity), some metal ions (5mM), such as Cu^{2+}, Zn^{2+}, Al^{3+}, and Fe^{3+} reduce it significantly (to 5.65%–28.02% of the initial activity) (Table 4), while K^{+}, Li^{+}, Mg^{2+}, Na^{+}, and Ca^{2+} have no remarkable impact on the enzyme activity (Table 3).

Lactose hydrolysis in milk by the enzyme

The lactose hydrolysis activity of the purified enzyme in milk was examined, 0.5 ml of enzyme could hydrolyse about 80% of lactose in 1 ml of

Table 2. Purification of strain 14-1 β-galactosidase

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	2456	32	76.75	1	100
$(NH_4)_2SO_4$ (60%)	2217.2	18.8	117.9	1.53	90
Sephadex G-25	1869.6	13.96	133.4	1.73	76
DEAE-cellulose	617.76	1.92	321.7	4.19	25

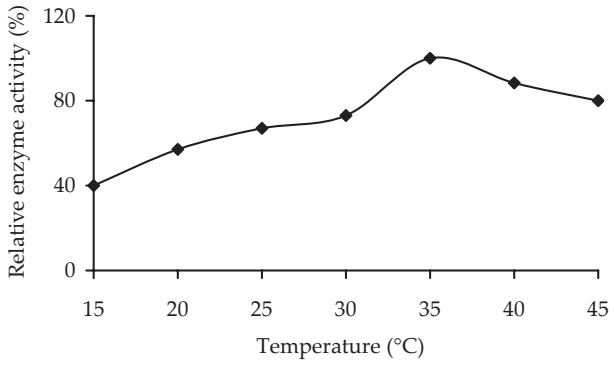


Figure 3. The influence of temperature on the activity of the enzyme

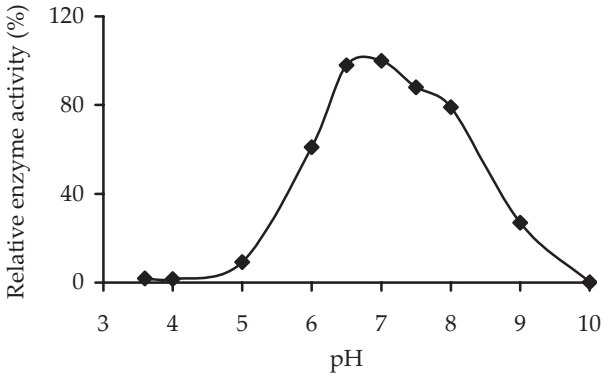


Figure 4. The influence of pH on the activity of the enzyme

milk within 2 h (Figure 6). Therefore, it seems that the β-galactosidase from *Rahnella aquatilis* 14-1 exhibits a good potential as an industrial cold-adapted enzyme.

Table 3. Effect of metal ions on strain 14-1 β-galactosidase activity

Metal ion	Enzyme activity (U/ml)	Relative enzyme activity (%)
None	12.74	100
Cu^{2+}	0.72	5.65
Mn^{2+}	15.26	119.8
Al^{3+}	1.41	11.07
K^{+}	12.84	100.8
Fe^{3+}	3.57	28.02
Li^{+}	12.69	99.6
Ca^{2+}	11.35	89.1
Zn^{2+}	0.923	7.24
Mg^{2+}	11.79	92.5
Na^{+}	11.60	91.05

DISCUSSION

Microorganisms that produce β-galactosidase have been extensively studied. The β-galactosidase gene from *E.coli* has been so well studied and exploited as a reporter gene, which is coded for by the l*acZ* gene. However, a number of other important genes coding for cold active β-galactosidase have been detected in *Arthrobacter* (Coker *et al*. 2003; Nakagawa *et al*. 2003), *Pseudoalteromons* (Hoyoux *et al*. 2001; Fernandes *et al*. 2002), *Rahnella aquatilis* (Park *et al*. 2006), and even in yeast (Nakagawa *et al*. 2006a, b). Cold-adapted β-galactosidase with high activity levels at low temperatures might prove to be useful for removing lactose from refrigerated milk enabling it to be consumed by lactose-intolerant individuals, and for converting lactose in whey into glucose and galactose.

In the searche for cold-adapted β-galactosidase, we isolated and classified a cold-adapted β-galactosidase producing strain 14-1. The phylogenetic analysis of the 16S rDNA sequence from this isolate placed it in the genus *Rahnella aquatilis*, which is consistent with its growth and morphological characteristics. We found no studies of β-galac-

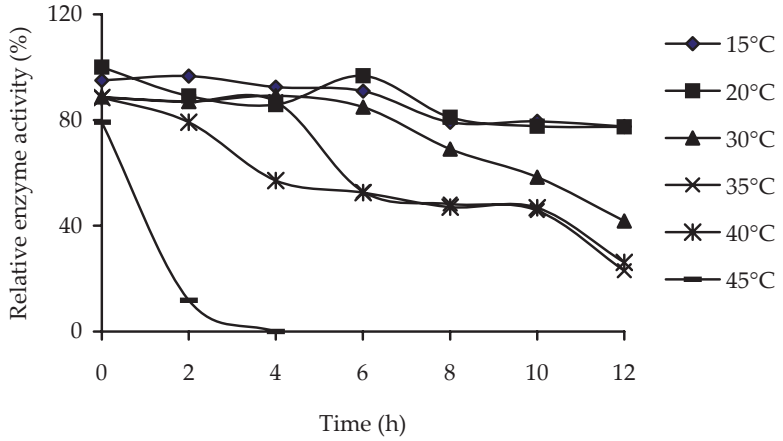


Figure 5. Temperature stability of the enzyme

tosidase from *Rahnella aquatilis* species except *Rahnella aquatilis* BS 1 (Park *et al*. 2006). The 16S rDNA sequence from strain 14-1 evidenced similarities of 99% to that of *Rahnella aquatilis* BS1 type strain from GenBank database. Further study of these and other strains will be required to help determine whether strain 14-1 is a new one.

Because we thought that the enzyme from *Rahnella aquatilis* 14-1 may be a novel type of β-galactosidase, we attempted to purify this respective enzyme the purification of the enzyme was conveniently done by chromatography, using Sephadex G-25 and DEAE-cellulose columns, which is often used for the purification of β-galactosidase (Hoyoux *et al*. 2001; Fernandes *et al*. 2002; Turkiewicz *et al*. 2003; Akolkar *et al*. 2005). The enzyme was purified until a single band had been obtained in 10% SDS-PAGE, which corresponded to a molecular mass of cca 60 kDa. The comparison of this result obtained by SDS-PAGE with the molecular mass of another β-galactosidases, i.e. 116 kDa (Coker *et al*. 2003) of *Arthrobacter* β-galactosidase, 115 kDa of *Pseudoalteromonas* (Turkiewicz *et al*. 2003) 110 kDa and 130 kDa (Nakagawa *et al*. 2006a, b, 2007), suggests that the β-galactosidase from strain 14-1 is a novel enzyme.

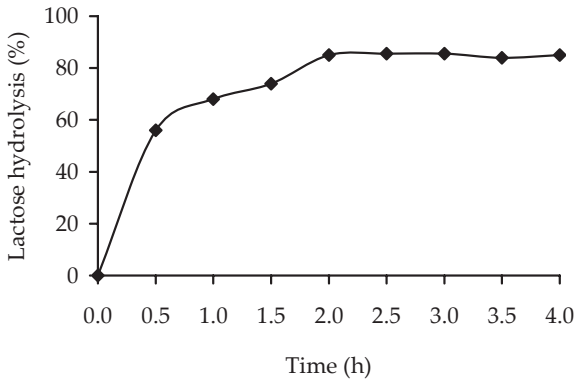


Figure 6. The influence of time on the percentage of lactose hydrolysis

The optimum temperature (Figure 3) of the enzyme activity of 35°C is 25°C below that of the known mesophilic β-galactosidase and 5°C below that of *Pseudoalteromonas* sp. 22b β-galactosidase (Turkiewicz *et al*. 2003), but 5°C above that of *Rahnella aquatilis* BS1 β-galactosidase (Park *et al*. 2006), and 25°C above that of *Arthrobacter psychrolactophilus* F2 β-galactosidase (Nakagawa *et al*. 2006a, b). Other cold-adapted β-galactosidases that have been reported exhibited optimum temperatures of around 30°C to 50°C. Thermostability studies of the activities of the isolates show that they are stable at low temperatures but labile when incubated above 45°C for 1.5 h (Figure 4). Judging from these findings, the β-galactosidase from *Rahnella aquatilis* 14-1 is a cold-adapted enzyme that is extremely heat-labile.

Rahnella aquatilis 14-1 β-galactosidase showed optimum activity at pH 6.5 to 7.0, which is different from those of *P. haloplanktis* β-galactosidase (8.5) (Hoyoux *et al*. 2001). *Pseudoalteromonas* sp. β-galactosidase (9.0) (Fernandes *et al*. 2002), and the β-galactosidase from *Arthrobacte*r (8.0) (Nakagawa *et al*. 2006a, b). Its pH stability range is relatively wide (6 to 9). The optimum pH value was 6.5 to 7.0, meeting the requirements for the milk treatment. The enzyme was able to hydrolyse about 80% of the lactose present in milk at 30°C within 2 hours. From these facts, we conclude that the β-galactosidase from strain 14-1 appears to be of advantage in the food industry.

Acknowledgement. We would like to thank Dr. Kai Lou and Prof. Pei-Hong Mao and the Xingjiang Extremity Environment Microorganism Laboratory.

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Received for publication February 22, 2008
Accepted after corrections April 17, 2008

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