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Isolation and Characterization of Cold-adapted Strains Producing β-Galactosidase

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β-Galactosidase is extensively employed in the manufacture of dairy products, including lactose-reduced milk. Here, we have isolated two gram-negative and rod-shaped cold-adapted bacteria, BS 1 and HS 39. These strains were able to break down lactose at low temperatures. Although two isolates were found to grow well at 10°C, the BS 1 strain was unable to grow at 37°C. Another strain, HS-39, evidenced retarded growth at 37°C. The biochemical characteristics and the results of 16S rDNA sequencing identified the BS 1 isolate as *Rahnella aquatilis*, and showed that the HS 39 strain belonged to genus *Buttiauxella*. Whereas the *R. aquatilis* BS 1 strain generated maximal quantities of β-galactosidase when incubated for 60 h at 10°C, *Buttiauxella* sp. HS-39 generated β-galactosidase earlier, and at slightly lower levels, than *R. aquatilis* BS 1. The optimum temperature for β-galactosidase was 30°C for *R. aquatilis* BS-1, and was 45°C for *Buttiauxella* sp. HS-39, thereby indicating that *R. aquatilis* BS-1 was able to generate a cold-adaptive enzyme. These two cold-adapted strains, and most notably the β-galactosidase from each isolate, might prove useful in some biotechnological applications.

Keywords: cold-adapted, β-galactosidase, *Rahnella aquatilis*, *Buttiauxella* sp.

Some microorganisms are capable of growth in unusual environmental conditions, including the high temperatures of volcanic hot springs, the low temperatures of polar regions, high pressures in deep seas, very high salt concentrations, or very high and low pH values (Fujiwara, 2002). These microorganisms can be divided into three groups: psychrophiles, mesophiles, and thermophiles, depending on their optimal growth temperatures. With the exception of mesophiles, which grow at mild temperature ranges, from 20 to 45°C, the other two types of microorganisms, thermophiles and psychrophiles, are classified as extremophiles (Cavicchioli and Thomas, 2000).

Thermophiles can proliferate at temperatures of above 45°C, and generate valuable biotechnological resources, including enzyme Taq DNA polymerase for use in the polymerase chain reaction (Saiki *et al.*, 1988), or thermostable α-amylase (Laderman *et al.*, 1993) and cellulase (Bronnenmeier *et al.*, 1995), which are employed in certain industrial processes, owing to their unusual properties. The geothermal and deep-sea thermal environments capable of supporting the growth of thermophiles are somewhat limited; however, more than 80% of the biosphere is characterized by cold habitats, such as in the Arctic, the Antarctic, high-alpine soils, cold deserts, permafrost soils, abysses, and deep sea waters (Cavicchioli and Thomas, 2000). Recently, many microorganisms adapted to cold conditions have been isolated (Margesin *et al.*, 2003; Groudieva *et al.*, 2004). These microorganisms are either psychrotolerant (psychrotrophic) or psychrophilic. Psychrophilic organisms have an optimal growth temperature at approximately 15°C or lower, a maximal growth temperature at approximately 20°C, and a minimal growth temperature at 0°C or below. However, psychrotolerant organisms thrive at temperatures close to the freezing point of water, but evidence the fastest growth rates at temperatures above 20°C (Morita, 1975; Cavicchioli *et al.*, 2002).

Psychrophiles and psychrotolerant strains most likely generate cold-adapted enzymes, which evidence low thermal stability, and also display highly specific activity and catalytic efficiency at low temperatures, for optimal adaptation to cold habitats (Feller *et al.*, 1997). Cold-adapted microorganisms have recently become the focus of a great deal of attention as sources

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of enzymes with potential for novel biocatalysts with the above-mentioned properties. Recently, a host of cold-active enzymes have been detected in cold-adapted strains (Feller *et al.*, 1997; Marshall, 1997; Gerday *et al.*, 2000; Groudieva *et al.*, 2004). Cold-active enzymes may have important biotechnological applications in the food industry, as well as in biomass conversion, bioremediation, and molecular biology protocols, inasmuch as running processes at low temperatures reduces the risk of contamination by mesophiles, and also saves energy (Gerday *et al.*, 2000). In addition, they can be inactivated readily by mild heat treatment.

Lactose is abundant in mammalian milk. It is comprised of galactose and glucose connected by β-1,4 glycosidic bonds, and this disaccharide is known to be hydrolyzed into monomeric sugar by β-galactosidase in bacteria. β-Galactosidase has attracted attention from researchers and dairy product manufacturers due primarily to its ability to remove lactose from milk, so that it can be consumed by people afflicted with lactose intolerance. It can also remove the lactose from whey, which is generated in the cheese industry, and is associated with environmental problems (Triveni, 1975; Loveland *et al.*, 1994). β-Galactosidase also participates in the synthesis of oligosaccharides which modify intestinal microflora (Yanahira *et al.*, 1998), and the removal of plant saccharides from fruit beverages. The best-studied and useful β-galactosidase thus far is the *Escherichia coli* enzyme, which is coded for by the *lacZ* gene. However, a host of other important genes coding for cold active β-galactosidase have been detected in *Arthrobacter* sp. (Gutshall *et al.*, 1995), *Carnobacteium* sp. (Coombs and Brenchley, 1999), *Planococcus* sp. (Sheridan and Brenchley, 2000), and even in yeasts (Nakagawa *et al.*, 2006).

In this study, we attempted to isolate psychrophilic or psychrotrophic microorganisms which were able to hydrolyze lactose at low temperatures, and to monitor growth behavior at a variety of temperatures, and to characterize the production and properties of β-galactosidase.

Materials and Methods

Screening and growth properties

Samples were collected near cattle sheds in the Chungchung and Kyungsang regions of Korea, between December 2004 and February 2005, and were maintained at 4°C in order to increase the probability of finding psychrophilic or psychrotrophic microorganisms. Approximately 1 g of each of the samples was added to 9 ml of 0.85% saline solution, diluted to 10^{-4}, and spread on LBL agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% lactose, 1.5% Bacto agar; pH 7.2) plates, which were then incubated at 10°C until the isolates formed colonies. The colonies were streaked at least three times to ensure purity. The screening of microorganisms generating cold-adaptive β-galactosidase was conducted on LBL agar plates containing 32 ug/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Duchefa Biochemie, Holland). Growth at different temperatures was studied in L broth containing 2% lactose as an additional carbon source, via the inoculation of cells from a turbid seed culture.

Morphological and biochemical characterization

The morphology of the isolated bacterial strains was assessed under a phase contrast microscope (Nikon, Japan) with cells grown in the exponential and stationary phases. The strains were characterized with regard to their biochemical properties, using the BioMérieux Vitet gram-negative identification card (GNI+) in accordance with the manufacturer's instructions. The results were read and interpreted using the GNI+ software, version R09.01 (bioMérieux, USA), after 8 h of incubation.

16s rDNA gene amplification and sequencing

Genomic DNA was acquired from the isolates via the bead beating method. 1 ml of harvested cells were suspended in 0.3 ml of lysis buffer (500 mM Tris-HCl, 10% sodium dodecyl sulfate, 100 mM NaCl, pH 8.0), and vortexed vigorously for 3 min with 0.5 g of 0.1 nm Zirconia glass beads (Biospec, USA). The resulting lysates were extracted with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol). The DNA was precipitated with 2 volumes of ethanol and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8).

The 16S rDNA gene was amplified from the chromosomal DNA of the isolated strains as a template via the polymerase chain reaction (PCR). PCR was conducted in a 50 ul reaction mixture containing 50 ng of template DNA, 2 pmol of each primer, 2.5 mM each dNTP, 1.25 U of Takara Ex Taq DNA polymerase (Takara Biomedicals, Japan) using a 2720 Thermal cycler (Applied Biosystems, USA) with the following program; 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min and 20 sec. The primers used were as follows: forward, 27F (*E. coli*; 5'-AG AGTTTGATCMTGGCTCAG-3') and reverse, 1492R (*E. coli*; 5'-TACGGYTACCTTGTTACGACTT-3'). The products were purified using a cleaning kit (Bioneer, Korea), and sequenced at SolGent Co. Ltd. (Korea) with the appropriate primers.

Phylogenetic analysis of the isolated strains

The 16S rDNA sequences obtained in this study were

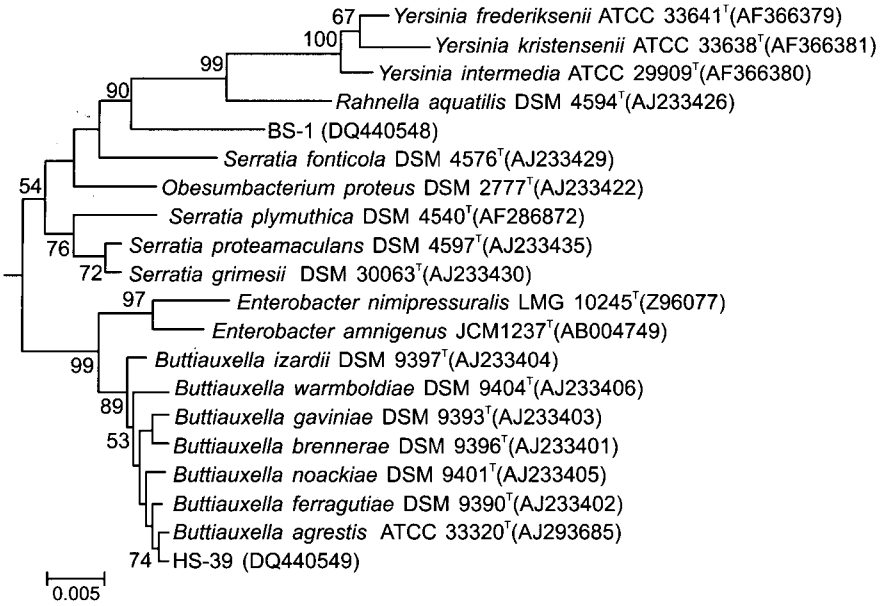

Fig. 1. Phylogenetic tree of the 16S rDNA sequences of BS-1, HS-39, and other taxa. The tree was rooted via a neighbor-joining method, and numbers within the dendrogram indicate the occurrence (%) of the branching order in 1000 bootstrapped trees (only values in excess of 50 are shown). *Bacillus subtilis* was utilized as an outgroup (not shown), and the GenBank accession number is shown in parentheses.

assembled with SeqMan software (DNAStar, USA), and utilized for phylogenetic analysis. The 16S rDNA gene sequences of the related taxa were acquired from the GenBank database at the National Center for Biotechnology Information (USA). Multiple alignments were conducted using the ClustalX program (Thompson *et al.*, 1997), and gaps were edited using the BioEdit program (Hall, 1999). The evolutionary distances were calculated with the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree for the data set was created via the Neighbor-joining method (Saitou and Nei, 1987) using the Mega 3 program (Kumar *et al.*, 2004). The stability of relationships was evaluated via bootstrap analyses of the neighbor-joining data, on the basis of 1,000 replications (Felsenstein, 1985).

β-Galactosidase assays

β-Galactosidase production was evaluated via measurements of the rate of hydrolysis of 2-nitrophenyl-β-D-galactopyranoside (ONPG; Duchefa Biochemie, Holland) in Z buffer (60 mM $NaHPO_4{\cdot}7H_2O$, 40 mM $Na_2HPO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 50 mM β-mercaptoethanol; pH 7.0) for 10 min at 15°C, using the crude cell lysates from cells which were time-course incubated at 10, 20, and 30°C. The optimal enzyme activity temperatures were determined via 10 min of incubation of the cell lysates in 1 ml of Z buffer with 0.2 ml of 10 mM ONPG at temperatures ranging from 0°C to 60°C. The enzyme reactions were halted via the addition of 0.5 ml 1 M Na_2CO_3. Hydrolysis of the 2-nitrophenyl group was detected at

Table 1. Biochemical properties of the isolated bacteria

Principal component	BS-1	HS-39
2,2,4'-Trichloro-2'-Hydroxydiphenylether	–	–
Glucose	+	+
Peptone, Tryptophan	+	+
Acetamide	–	–
Esculin	+	+
Indoxyl-β-D-Glucoside	+	+
Urea	–	–
Citrate	–	–
Malonate	–	+
Tryptophan	–	–
Polymyxin B	–	–
Lactose	+	+
Maltose	–	+
Mannitol	+	+
Xylose	–	+
Raffinose	+	–
Sorbitol	–	–
Sucrose	+	–
Inositol	–	–
Adonitol	–	+
ρ-coumaric	–	–
Sodium Thiosulfate	–	–
O-Nitrophenyl-β-D-Galactopyranoside	+	+
Rhamanose	–	+
L-Arabinose	+	+
Glucose	+	+
Arginine	–	+
Lysine Decarboxylase	–	–
Ornithine	–	–
Oxidase	–	–

+, positive reaction; –, negative reactionreaction

420 nm, and a unit of activity was defined as described by Miller (1972).

Results and Discussion

Isolation of psychrotrophic strains and phylogenetic analysis

Due to the paucity of reports regarding useful cold-adapted enzymes and the industrial application of the psychrophilic and psychrotrophic strains, we attempted to isolate cold-adapted strains, and to detect the production of cold-adapted β-galactosidase from the isolates. Approximately 48 bacterial isolates were screened via X-gal hydrolysis on plates at 10°C during the winter of the year 2004. Among these, two isolates, referred to as BS 1 and HS 39, displayed cold-adapted growth and high intracellular β-galactosidase activity in the crude extracts. We selected these two strains for further analysis. Microscopic examination showed that BS 1 and HS 39 were rod-shaped during the exponential phase, and gram-stained preparations of these strains were gram-negative (data not shown).

Due to our interest in two isolated microorganisms, we have attempted to determine their phylogenetic relationships. We amplified nearly the complete sequence (1,465 nucleotides) of the 16S rDNA gene via PCR, determined its sequence, and then examined the relevant phylogenetic relationships via the Neighbor-joining method. The isolated BS 1 was clustered to a type strain, *Rahnella aquatilis,* and type strains in *Yersinia* spp. According to the phylogenetic tree, BS 1 was more closely related to *R. aquatilis* than were *Yersinia* spp. (Fig. 1). In addition, the 16S rDNA sequences from BS 1 evidenced similarities of 99.7% and 98.2 % to those of *Rahnella* genosp. 3 and the *R. aquatilis* type strain from the Genbank database, respectively. HS 39 was clustered with many *Buttiauxella* spp. type strains (Fig. 1). Comparison of the 16S rDNA sequence with all bacterial sequences available in the GenBank database using the Blast program 2.2.14 revealed high degrees of similarity (between 99.1 and 99.7%) to the different species of the *Buttiauxella* genus.

Biochemical and growth characterization

The results of biochemical characterization, obtained using a GNI+ kit, are shown in Table 1. The BS 1 and HS 39 isolates were similar in that they evi-

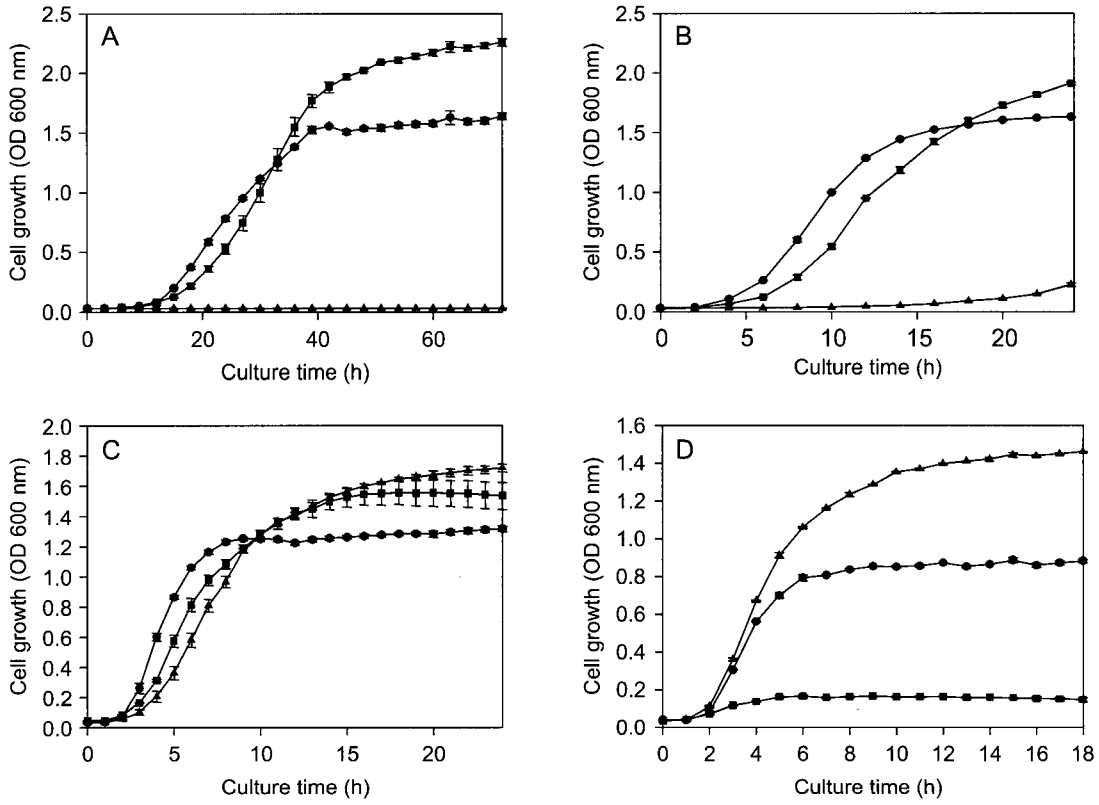

Fig. 2. Growth of psychrotolerant isolates, BS-1 and HS-39.
The cells were grown in L broth supplemented with 2% lactose at (A) 10°C, (B) 20°C, (C) 30°C, and (D) 37°C by shaking at 150 rpm. The closed square (■-■), closed circle (●-●), and open triangle (▲-▲) represent BS-1, HS-39, and the control strains *E. coli* DH5α transformed with pBluescript SK II', respectively. Standard deviations from the mean of three independent assays are indicated by error bars.

denced an ability to catabolize and generate acid with lactose, glucose and L-arabinose. Although we noted some differences in the utilization of several carbon sources, our results were closely consistent with previously published biochemical profiles of *R. aquatilis* (Holt *et al.*, 1994). In accordance with the results regarding the similarity of the 16S rDNA sequences as well as the relevant microbiological properties, we classified BS 1 as *R. aquatilis*. In the case of the HS 39 strain, the GNI+ software interpreted it as *Leclercia adecarboxylata* according to its biochemical properties, although there was a discrepancy in terms of the 16S rDNA similarity results. As the presence of arginine dihydrolase and an inability to produce acid from raffinose and sucrose are the key features that distinguish the genus *Buttiauxella* from *Leclercia* (Muller *et al.*, 1996), we concluded that BS1 belongs to *Buttiauxella* sp. rather than to *Leclercia* sp. Nonetheless, the exact identification of HS 39 will require more data in the future.

We then determined the optimal growth temperature for our two isolates, using culture medium L broth containing 2% lactose, which was determined to be the optimal growth medium. Although *Escherichia coli*, a mesophilic control strain, was unable to grow at 10°C, our *R. aquatilis* BS 1 and *Buttiauxella* sp. HS 39 isolates thrived at this temperature (Fig. 2). Whereas *E. coli* could only begin to grow after 20 h of incubation at 20°C, *R. aquatilis* BS 1 and *Buttiauxella* sp. HS 39 strains had reached a nearly stationary phase at this time. Interestingly, the growth of *R. aquatilis* BS 1 halted when incubated at 37°C, and the HS 39 strain could grow to half of the optical density of mesophilic strains. These results indicate that our *R. aquatilis* BS 1 and *Buttiauxella* sp. HS 39 isolates were cold-adapted psychrotrophic strains. In addition, *R. aquatilis* BS 1 was found to be more sensitive to higher temperatures than was HS 39.

Time course production of β-galactosidase by temperature

The production of β-galactosidase was checked in the two isolated strains at a variety of incubation temperatures (10, 20, and 30°C) (Fig. 3). Mesophilic *E. coli* evidenced only a little enzyme activity when incubated for 12 h at 30°C. By way of contrast, our two isolates, *R. aquatilis* BS 1 and *Buttiauxella* sp. HS 39, evidenced much higher enzyme production under the same conditions than did *E. coli*. Although the production of β-galactosidase from *R. aquatilis* BS 1 was highest when it was incubated for 60 h at 10°C, the *Buttiauxella* HS-39 strain evidenced the highest levels of β-galactosidase production when incubated either for 12 h at 20°C or for 48 h at 10°C. As the isolates evidenced favorable growth (Fig. 1) and high

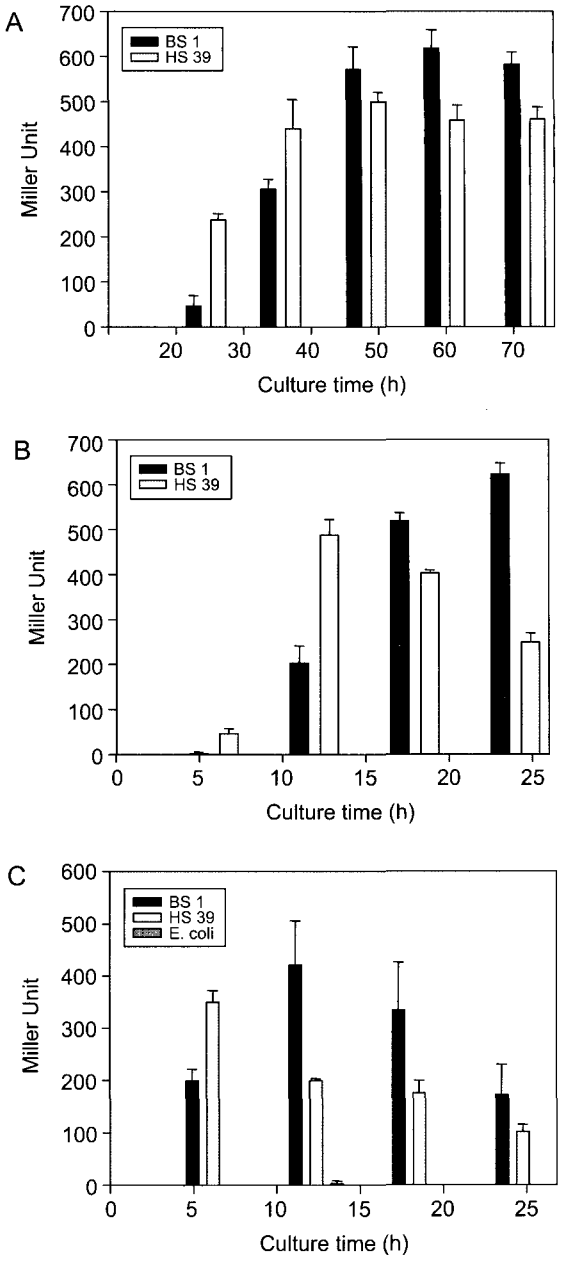

Fig. 3. β-Galactosidase production in psychrotolerant isolates on the basis of growth temperature. The strains were grown in L broth supplemented with 2% lactose at 10°C (A), 20°C (B) and 30°C (C), and the enzyme activities were determined for 10 min at 15°C. Standard deviations from the mean of three independent assays are indicated by error bars.

enzyme production (Fig. 3) at low temperatures, these two isolates may prove useful in whey treatment in natural environments.

The optimal temperature for enzyme activity

The activity levels of β-galactosidase generated by cell extracts grown at 30°C were determined, in order to ascertain the optimal enzyme temperature. Whereas

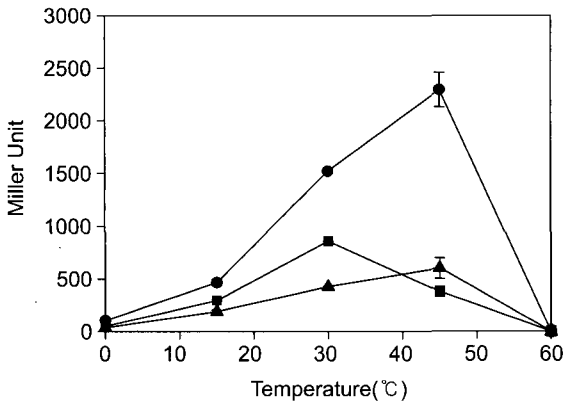

Fig. 4. The activities of β-galactosidase from psychrotolerant isolates. The strains were cultured to log phase in L broth supplemented with 2% lactose at 30°C and the enzyme activities were determined for 10 min at each temperature in the horizontal axis, except for 0°C, at which activity was determined for 15 min. The symbols for the strains are the same as shown in Fig. 2. Standard deviations from the mean of three independent assays are indicated by error bars.

R. aquatilis BS 1 evidenced the highest activity levels at 30°C. The *Buttiauxella* HS 39 strain evidenced maximal activity at 45°C, similar to that of *E. coli* (Fig. 4). Taken together, *R. aquatilis* BS 1 evidenced adaptive growth at lower temperatures than did HS 39 (Fig. 2), and produced β-galactosidase that was more cold-adaptive than HS 39. Ohgiya *et al.* (1999) have classified β-galactosidase into three groups, in accordance with their thermolability and catalytic properties. The β-Galactosidase generated by *R. aquatilis* BS 1 can be assigned to group 2, members of which evidence higher activity at low temperatures and a greater degree of heat sensitivity than has been observed with equivalent mesophilic enzymes. The enzyme generated by *Buttiauxella* sp. HS 39 can be assigned to group 3, members of which evidence higher activity at low temperatures, but thermostability similar to those of mesophilic enzymes. The β-galactosidase generated by the two cold-adapted strains can be employed not only in bioremediation for areas contaminated with whey, which is a waste by-product of cheese-making, but also in the manufacture of low-lactose milk.

Acknowledgments

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