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Full Length Research Paper

Isolation and characterization of a psychrophilic bacterium producing cold active lactose hydrolyzing enzyme from soil of Mt. Himalaya in Nepal

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Psychrophilic bacteria, which grew on lactose as a carbon source, were isolated from soil of Island Peak area at Mt. Himalaya in Nepal, and identified. 148 bacterial isolates were isolated via X-gal hydrolysis on plate. Among these, three isolates, referred to as KNOUC 401, 402 and 403, displayed good cold-adapted growth and activity for hydrolysis of *o*-nitrophenyl- -D-galactopyranoside (ONPG) and lactose in cell free extracts. Strain KNOUC403 showing negative activity in -hemolysis was selected and identified. The strain was gram-positive bacilli, non-motile, catalase-positive and aerobic. Optimum growth was done at 20°C and pH 6.5~6.8. Anteiso-$C_{15:0}$ (68.14%) was predominant in the fatty acid composition of cell. The physiological and biochemical properties, cellular fatty acid composition and sequence of 16S rDNA, suggest that strain KNOUC403 indicate a taxonomic affiliation to *Arthrobacter psychrolactophilus*. The optimal conditions for ONPG hydrolysis of cell free extracts from the strain were 15°C and pH 6.0, and the enzyme was stable at 4°C for 42 h. Zymogram assay of cell free extract from the strain in nondenaturing polyacrylamide gel showed a distinct band of X-gal hydrolysis.

Key words: Psychrophilic bacteria, *Arthrobacter psychrolactophilus,* cold-active lactose hydrolyzing enzyme, Mt. Himalaya.

INTRODUCTION

Recently a wide diversity of cold adapted microorganisms have been found in various cold environments (Groudieva et al., 2004), and their enzymes attracted interests because of their biotechnological potential offering economical and ecological advantages such as energy saving, function in cold environment, minimizing undesirable chemical reactions that occur at high temperature, and inactivating easily by heating when required (Russell, 1988; Margesin and Schinner, 1999; Margesin et al., 2002; Cavicchioli et al., 2002). Owing to the fact that cold completely penetrates microorganisms, all components including enzymes of psychrophiles living in cold environment must be suitably adapted. Therefore psychrophiles can produce cold adapted enzymes which are highly flexible in structure enabling increased complementarity between the active site and substrate at a low energy cost resulting in high catalytic activity at low temperature and labile at elevated temperature (Cavicchioli et al., 2002). A variety of useful cold active enzymes have been found in psychrophiles (Feller et al., 1997; Marshall, 1997; Gerday et al., 2000; Groudieva et al., 2004).

Cold active enzymes are attractive in food industry, e.g. processing of fruit juices and milk, as there is an increasing industrial trend to treat foodstuffs under low temperature conditions in order to avoid changes in taste and nutritional value, and to save energy (Margesin and Schinner, 1994; Russel, 1998). Cold active enzyme hydrolyzing lactose to glucose and galactose at refrigerating temperature is an important food-industrial enzyme, because it can be used to produce lactose hydrolyzed milk for the lactose intolerant people, and can also be used to convert lactose in whey, a by-product of

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cheese industry, to more readily fermentable glucose and galactose. Until now, some psychrophilic bacteria producing -galactosidase have been reported, but none of them have been hitherto used practically in the food industry, meaning that more study is required to find better microbial source. The aim of this study was to isolate a psychrophilic bacterium, from Mt. Himalaya of Nepal, producing the cold-active lactose hydrolyzing enzyme practically useful in food industry.

MATERIALS AND METHODS

Isolation and cultivation condition of microorganisms

A soil sample was collected from each of the 39 regions of Island Peak area (27°55'N, 86°56'E) of 4,500 to 6,000 m height in Mt. Himalaya of Nepal by using sterilized awls, sterilized spoons and sterilized Falcon tubes. To cultivate the psychrophilic bacteria, 5 g of soil samples were added to 45 ml of Brain heart infusion (BHI; Difco Laboratories, Detroit, Mich) broth containing 1% (w/v) lactose, and incubated at 4°C aerobically by shaking (200 rpm) for 30 days. 1 ml of cells grown in this enrichment were spread onto BHI agar containing 1% (w/v) lactose and 0.01% (w/v) 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside (X-gal; Duchefa Biochemei, Holland). After incubation at 4°C for 15 days, blue colonies were selected, then cultivated in brain heart infusion broth or agar for identification and determining properties of lactose hydrolyzing enzyme.

Morphological, biochemical and physiological properties

Isolates showing good growth and good X-gal hydrolysis on BHI agar, and high activity for hydrolysis of ONPG and lactose were investigated for Gram staining, morphological, biochemical, and physiological properties. Cell was grown in BHI broth to determine growth conditions of various temperatures (5 to 40°C) and pH (4.0 to 8.0). pH of media was adjusted with HCl or NaOH. Acid production from carbohydrate and utilization of sole carbon sources were determined using API 20E and API 50CH test strips (Bio-Merieux Inc., France). For the test of hemolysis, a colony was streaked on sheep blood agar (KOMED. Co. Ltd). After incubation at 15°C for 3 days, -hemolytic activity was detected by lysis and complete digestion of red blood cell contents surrounding colony. All biochemical and physiological tests were done at 15°C for 3 days

Composition of cellular fatty acid

The cell biomass for cellular fatty acid composition analysis was collected from BHI agar plates after incubation at 15°C for 3 days. Cells were harvested, and the cellular fatty acids were saponified, methylated and extracted, following the instructions in the manual for Sherlock Microbial Identification System (MIDI, USA). The fatty acids were analyzed by gas chromatography (Hewlett Packard 6890, USA) and identified using the microbial identification software package (Sasser, 1990).

16S rDNA sequence determination and phylogenetic analysis

Isolation of genomic DNA, PCR amplification of the 16S rDNA gene and sequencing of the purified PCR product were carried out as described by Rainey et al. (1996). Universal primers of fD1 (5'-gagtttgatcctggctcag-3') and rD1 (5'-agaaaggaggtgatccagcc-3') were used for PCR. PCR product was purified by ethanol precipitation, and sequenced with a model 377 Genetic Analyzer (Perkin-Elmer Co.). The 16S rDNA sequence obtained in this study was aligned against the previously determined sequences of the genus of *Arthrobacter* available from the Ribosomal Database Project (Maidak et al., 1996). The phylogenetic tree for the data set was inferred using the neighbor-joining method (Saitou and Nei, 1987). The PHYLIP package (Felsentein, 1993) was used for constructing the tree.

Assay of lactose hydrolyzing enzyme activity

Microorganism was cultivated at 4°C aerobically by shaking (200 rpm) for 7 days to the late of log phase, and harvested by centrifugation at 8,000 X g for 10 min at 4°C, suspended in sodium phosphate buffer (0.01 M, pH 6.8), washed 2 times by the same buffer, suspended in the same buffer, and sonificated at 4°C. Cell debris was eliminated by centrifugation at 12,000 g and 4°C for 20 min. The cell free extracts were used for assay of lactose hydrolyzing enzyme. Lactose hydrolyzing enzyme activity was determined by measuring the rate of hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (ONPG) or lactose as substrate. The procedure of Miller (1972) for hydrolysis of ONPG was used. An aliquot of cell free extracts (0.5 ml) was added to 2.5 ml of ONPG (0.04 M) dissolved in sodium phosphate buffer (0.01 M, pH 6.8) and incubated at 4°C for 2 h. The reaction was stopped by addition of 3 ml of 0.5 M Na_2CO_3 dissolved in H_2O and the absorbance at 420 nm was measured.

One unit of enzyme activity for hydrolysis of ONPG was defined as the activity hydrolyzing 1 mol of ONPG per min by cell free extract from 1 ml of culture that was concentrated to 8 of A_{600}. The hydrolysis of lactose was assayed by measuring the release of glucose from lactose. The reaction mixture consisted of 1.6 ml of skim milk (lactose conc., 4.7%) and 0.4 ml of cell free extracts. The mixture was incubated at 4°C for 5 days followed by heating in boiling water for 2 min. After centrifugation, glucose concentration in the supernatant was determined by the colorimetric method with a commercial Glucose C-II test kit (Wako Chemical Co., Japan) at 505 nm. One unit of enzyme activity for hydrolysis of lactose is the activity of hydrolyzing 1 mol of lactose per day by cell free extracts from 1 ml of culture that was concentrated to 8 of A_{600}. Zymogram assay for the cell free extracts of a selected strain was performed. After native-PAGE of cell free extracts on 10% (w/v) polyacrylamide gel (Laemmli, 1970), the gel was stained with 0.25 mM X-gal (Trimbur et al., 1994) at 4°C for 2 h. Hydrolysis of X-Gal was confirmed by blue band within the polyacrylamide gel.

Effect of temperature and pH on lactose hydrolyzing enzyme

The effect of temperature on the activity of cell free extracts for ONPG hydrolysis was analyzed by measuring the enzyme activity at various temperatures (5 to 40°C) in sodium phosphate buffer (0.01 M, pH 6.8) . Effect of pH on enzyme activity was evaluated at pH ranging from 4.3 to 7.8 at 4°C was used for the pH from 4.3 to 6.0, and Na-phosphates buffer (0.01 M) was used for the pH from 6.0 to 7.8. Stability of enzyme was investigated by residual activity during incubation of cell free extracts in sodium phosphate buffer (0.01 M, pH 6.8) for 42 h at 4 and 37°C.

RESULTS

Isolation of strain producing cold active lactose hydrolyzing enzyme

Psychrophilic bacteria, which grow and hydrolyze lactose

Table 1. Physiological and biochemical properties of isolated strains.

Characteristics \ Strains	KNOUC401	KNOUC402	KNOUC403
Gram staining	-	+	+
Shape	Rod	Rod	Rod
Motility	-	+	-
Spore formation	-	+	-
Optimum temp for growth	20°C	20°C	20°C
Optimum pH for growth	6.8	6.5-6.8	6.5-6.8
Growth at 4°C	+	+	+
Growth at 37°C	+	+	-
Catalase	+	+	+
Oxidase	+	+	-
ONPG hydrolysis	+	+	+
PNPG hydrolysis	+	+	+
Nitrate reduction	-	-	-
Gas production	+	-	-
H_2S formation	-	-	-
Citrate utilization	+	-	-
Growth on MacConkey agar	+	-	-
Utilization of			
D-glucose	+	+	+
D-mannitol	-	-	-
D-maltose	+	+	-
D-rhamnose	+	+	-
D-mannose	-	-	-
D-galactose	-	-	-
D-lactose	+	+	+
D-fructose	-	-	-
D-adonitol	-	+	-
D-raffinose	+	+	-
D-sorbitol	+	+	-
Sucrose	+	-	-
Xylitol	+	+	-
Ducitol	+	+	+
Gelatin	-	-	-
Sorbose	-	-	+
Glycerol	+	+	-
Erythrol	-	-	-
Genus	*Aeromonas*	*Bacillus*	*Arthrobacter*

+, Positive reaction; -, Negative reaction.

at low temperature, were isolated from the soil of Island Peak area at Mt. Himalaya in Nepal. 148 bacterial isolates were isolated via X-gal hydrolysis after soil samples were fortified in BHI broth, spread and incubated on BHI agar at 4°C. Among these, three isolates, referred to as KNOUC 401, KNOUC402 and KNOUC403, showed good growth at low temperature (4°C), formed distinct blue colony on BHI plate containing X-Gal, and their cell free extracts showed practically useful activity for hydrolysis of ONPG and lactose.

Identification of strain KNOUC403

The strains of KNOUC401, KNOUC402 and KNOUC403 were characterized for their physiological and biochemical properties to get information on their suitability for use in the food industry. KNOUC401, KNOUC402 and KNOUC403 were presumably identified as *Aeromonas*, *Bacillus* and *Athrobacter* respectively by phenotypic features as in Table 1. Isolate KNOUC 403 was negative for β-hemolysis on sheep blood agar, but

Table 2. Properties of preliminarily selected strains.

Area (M)	Strain No.	Hydrolysis of		Identification	Hemolysis
		ONPG (Unit[1)])	Lactose (Unit[2)])		
4700	KNOUC401	3.3394	11.54	*Aeromonas*	+
4950	KNOUC402	0.1467	1.04	*Bacillus*	+
5550	KNOUC403	0.23319	2.76	*Arthrobacter*	-

[1)] One unit of enzyme activity is defined as the activity hydrolyzing 1 mol of ONPG per min by cell free extract from 1 ml of culture whose A_{600} is 8.
[2)] One unit of enzyme activity is the one hydrolyzing 1 mol of lactose per day at 4°C by cell free extracts from 1 ml of culture whose A_{600} is 8.

Table 3. Composition of major cellular fatty acids of strain KNOUC403.

Fatty acid	Contents (%)
14:0 iso	5.73
15:0 iso	3.14
15:0 anteiso	68.14
16:0 iso	8.08
17:0 iso	4.21

KNOUC401 and 402 were positive (Table 2) . Therefore, we selected strain KNOUC403 for further study. For further identification of strain KNOUC403, the strain was tested for its cellular fatty acid composition and the sequence of 16S rDNA. The cellular fatty acids of KNOUC403 were composed of anteiso-$C_{15:0}$ (68.14%), iso-$C_{16:0}$ (8.08%), iso-$C_{14:0}$ (5.73%), anteiso-$C_{17:0}$ (4.21%), iso-$C_{15:0}$ (3.14%), with additional fatty acids comprising less than 1% (Table 3).

The sequence of 16S rDNA (GeneBank accession No. HQ610444) of strain KNOUC403 determined as 1,492 bp is shown in Figure 1, and the phylogenetic tree constructed by the neighbor- joining method (Felsenstein, 1993) is shown in Figure 2. Comparing 16S rDNA sequence of strain KNOUC403 with the sequences in Ribosomal Data base Project (Maidak et al, 1996) and NCBI, demonstrated that strain KNOUC401 should be classified as an *Arthrobacter* sp., and its closest relative was *Arthrobacter psychrolactophilus* (98 to 100%). Therefore, strain KNOUC403 was identified as *A. psychrolactophilus,* and the strain was named as *A. psychrolactophilus* KNOUC403.

Properties of lactose hydrolyzing enzyme

Cell free extracts of strain KNOUC403 was tested for the properties of lactose hydrolyzing enzyme related to pH and temperature to confirm the possibility as a source of cold active lactose hydrolyzing enzyme. The optimal temperature for hydrolysis of ONPG was 15°C (Figure 3a). The enzyme exhibited ca. 87% of maximal activity at 4°C, and the enzyme activity decreased to less than 80% of maximal activity with a further increase in temperature to 40°C. The optimal pH was pH 6.0, it exhibited activities higher than 80% of optimum activity at broad pH from 4.3 to 7.8 (Figure 3b), meaning that this strain produces cold active lactose hydrolyzing enzyme useful at wide range of pH. To determine the stability at 4 and 37°C, the cell free extracts in Na-phosphate buffer (0.01 M, pH 6.8) was incubated at those temperatures for 42 h and residual activity were tested during incubation. It was stable at 4°C but lost its all activity at 37°C in 42 h (Figure 4). To confirm the existence of lactose hydrolyzing enzyme in crude cell free extracts of strain KNOUC403, zymogram assay for X-gal hydrolysis was performed. There was a distinct band of X- gal hydrolysis (Figure 5). In native electrophoresis, the enzyme moved to the spot of around 130 kDa.

DISCUSSION

Cold-adapted lactose hydrolyzing enzymes of high activity at low temperature would be useful for hydrolysis of lactose in refrigerated milk; enabling it to be consumed by lactose-intolerant individuals, and for converting whey to a sweetener by hydrolyzing lactose to sweeter sugars of glucose and galactose. Psychrophilic bacteria living in cold environments, such as glacier silts, place of high altitude in Himalaya, Arctic soils and Antarctic environments, are potentially useful sources of cold-adapted enzymes. Among strains isolated from soil of perpetual snow area at Island peak region of Mt. Himalaya in Nepal, we selected the strain KNOUC403 showing the good growth and lactose hydrolyzing activity at 4°C, and no activity of β-hemolysis. The growth physiology of this strain, growing at 4~30°C with optimum growth temperature of 20~30°C and not growing over 37°C, is well fitted to define this strain as a psychrophile (Kocur et al., 1991) . The strain KNOUC403 has phenotypic characters of Gram positive, rod, nonsporing, nonmotile , and catalase negative, that are fitting to the genus *Arthrobacter* (Holt et al., 1994), as in Table 1. The

GATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCCCGCTTG
CGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTC
TGGGATAAGCCTTGGAAACGAGGTCTAATACTGGATATTGACTTTGCCTCGCATGG
GGTTTGGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTG
GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACG
GCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTT
GAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGT
TTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTAC
GGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATG
CGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGA
CGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAT
GCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAG
CTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAAC
GCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAATACCTGGAAACAGGTGCC
CCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTA
ATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGAGGACG
ACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGG
TACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTT
CGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA
TCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGT
CACGAAAGTTGGTAACACCCGAAGCCGGTGGCCTAACCCCCTTGTGGGGAGGGA
GCTGTCGAAGGTGGGACTGGCGATTGGGACTAAGTCGTAACAAGGTAGCCGTACC
GGAAGGTGC

Figure 1. 16S rDNA sequence of strain KNOUC403 (1,492 bp).

cellular fatty acid composition profile of KNOUC403 with anteiso-$C_{15:0}$ as a predominant fatty acid is typical for a member of genus *Arthrobacter* (Lee et al., 2003; Storms et al., 2003; Kotouckova et al., 2004; Margesin et al., 2004). Heyrman et al. (2005) reported that predominant fatty acid of the genus *Arthrobacter* was anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The predominant fatty acids of *A. psychrolactophilus* (sp. nov.) was also anteiso-$C_{15:0}$ that comprised 73% of total fatty acids (Loveland-Curtze et al., 1999). Therefore “Loveland-Curtze et al. (1999) it has been reported that several *Arthrobacter* strains produced cold-active -galactosidase (Coker et al., 2003; Nakagawa et al., 2003). *Arthrobacter* sp. 20B - galactosidase was optimally active at pH 6.0 to 8.0 and

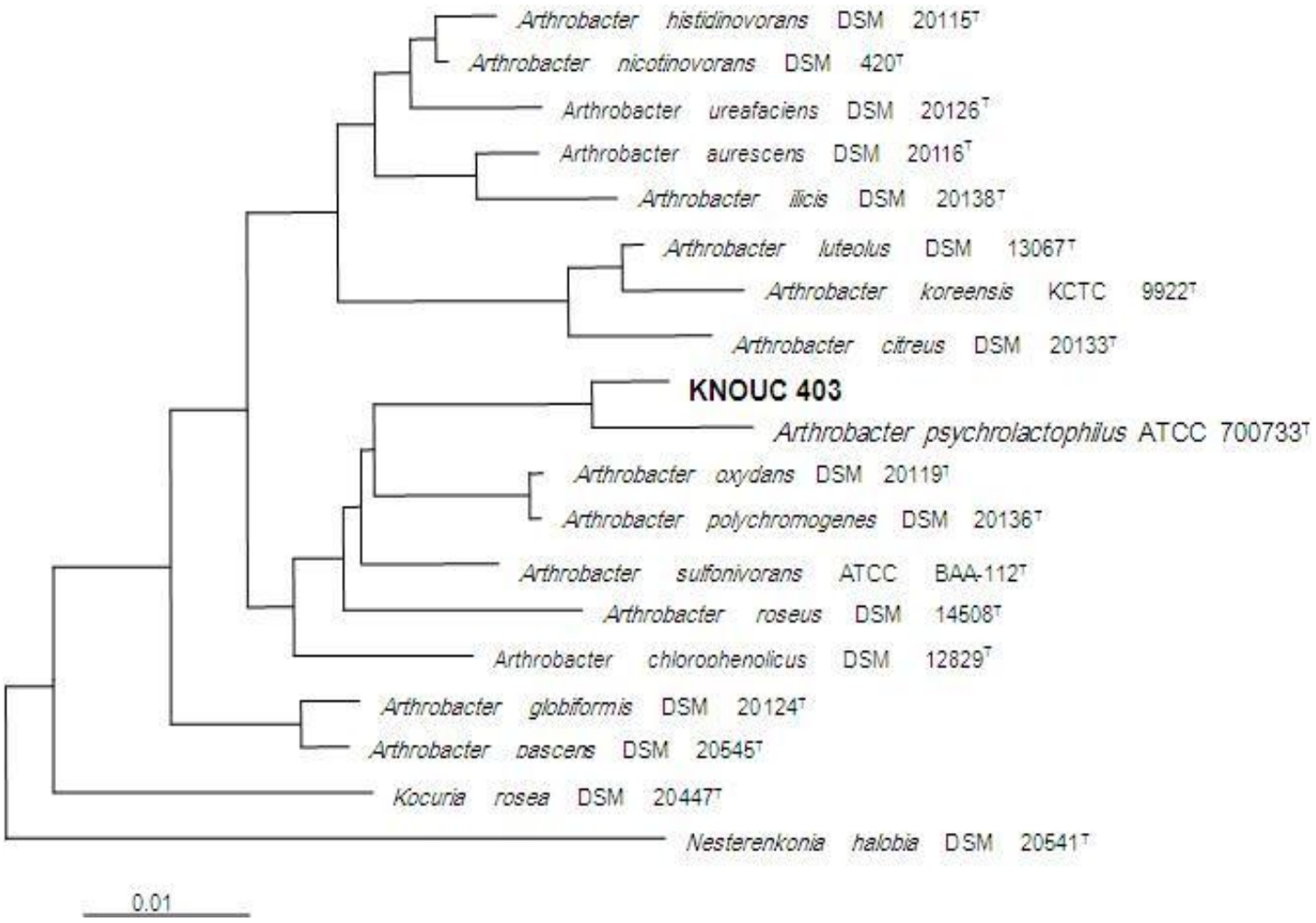
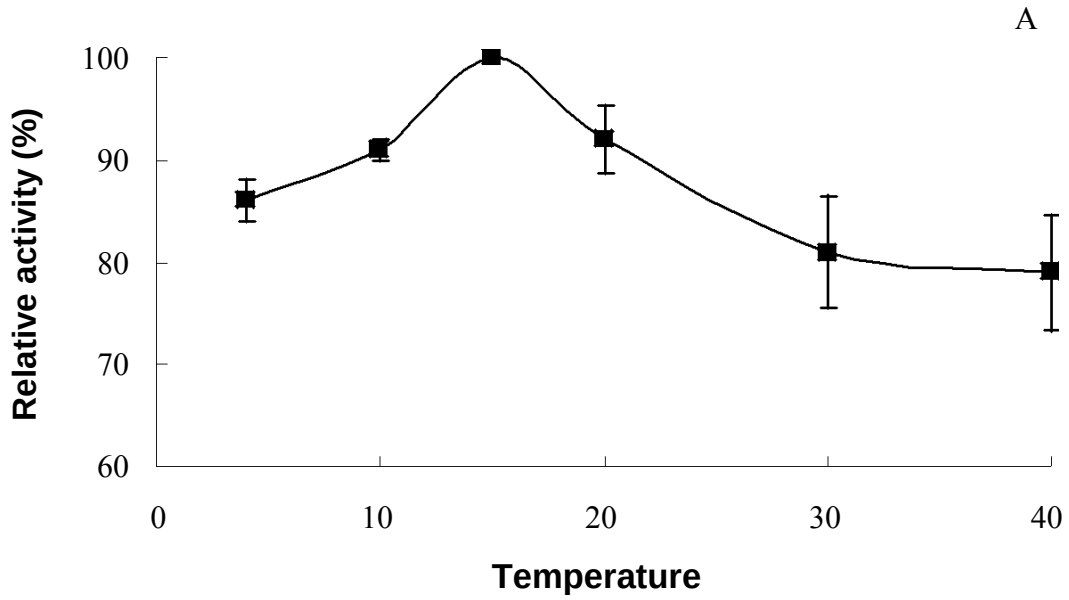


Figure 2. Phylogenetic tree based on 16S rDNA sequence showing the position of strain KNOUC403 as the type strain of *Arthrobacter* species and the representative of some other related taxa. Scale bar represents 0.01 substitutions per nucleotide.



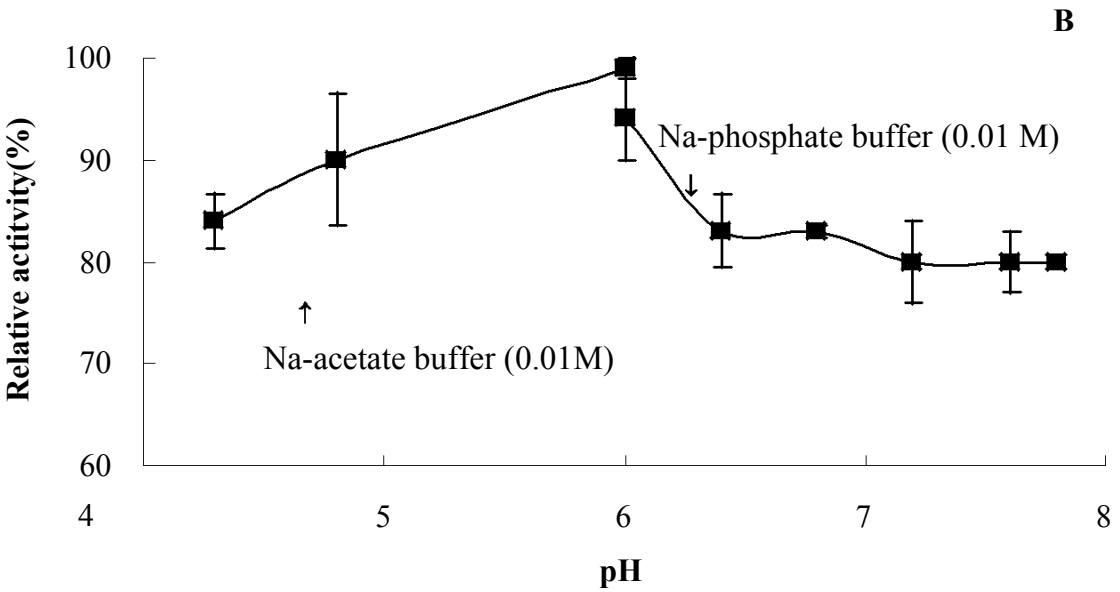


Figure 3. Effects of temperature (A) and pH (B) on the activity of lactose hydrolyzing enzyme in cell free extracts of *Arthrobacter psychrolactophilus* KNOUC403 for the hydrolysis of ONPG. *Effect of temperature on enzyme activity was analyzed in Na-phosphate buffer (0.01 M, pH 6.8) . *Values are means of triplicates ± S.D. *Effect of pH on enzyme activity was evaluated at 4°C in Na- acetate buffer (0.01 M, pH 4.3 to 6.3) and Na-phosphate buffer (0.01 M, pH 5.8 to 7.8), *values are means of triplicates ± S.D.

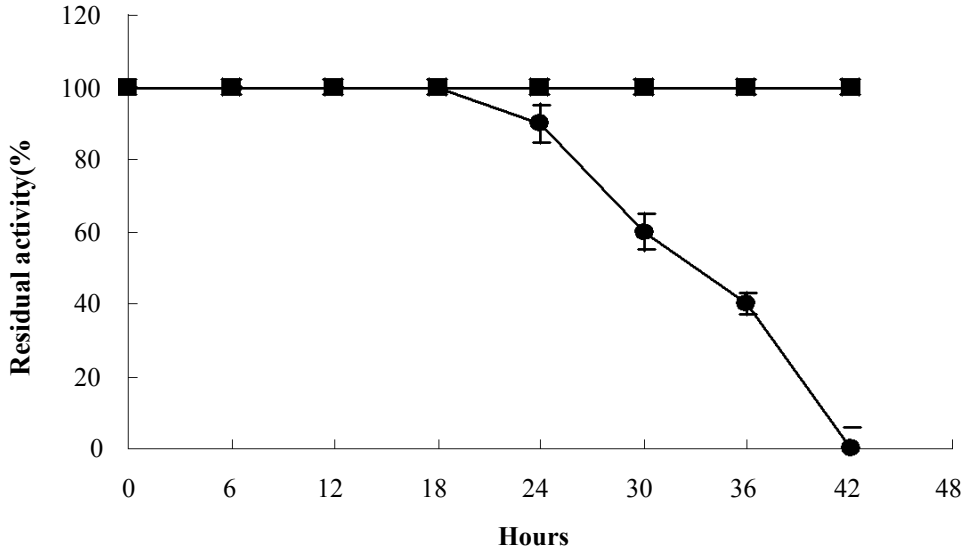


Figure 4. Stability of lactose hydrolyzing enzyme in cell free extracts of *Arthrobacter psychrolactophilus* KNOUC403 at 4°C ( ) and 37°C ( ) in Na-phosphate buffer (0.01 M, pH 6.8). *Values are means of triplicates ±S.D.

25°C (Bialkowska et al., 2009).

A -galactosidase of *Arthrobacter* sp. obtained from Antarctic dry valley soil was cold optimal at 18°C, showed 50% activity of optimum activity at 0°C, and was an unstable one, inactivated within 10 min at 37°C (Coker et al., 2003). *Arthrobacter* reported by Nakagawa et al. (2006) produced cold active -galactosidase with optimum temperature of 10°C and optimum pH of 8.0. However, two -galactosidases of *Pseudoalteromonas* sp., a psychrophile, were not cold active enzymes whose optimum temperature were 26°C (Fernandes et al., 2002) and 45°C (Hoyoux et al., 2001) showing that all

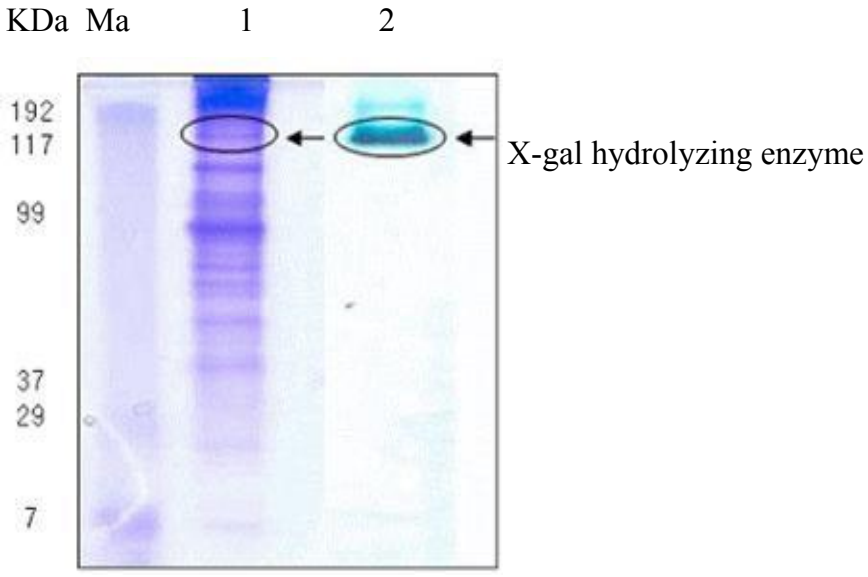


Figure 5. Native polyacylamide electrophoresis and zymogram of cell free extracts of strain *Arthrobacter psychrolactophilus* KNOUC 403. The protein was analyzed on a 10% polyacrylamide gel. Ma: Kaleidoscope polypeptide standards marker. 1: The gel was stained with Coomassie Brilliant Blue R-250. 2: The gel was soaked in 0.1 M X-gal solution (in Z-buffer) at 4°C for 2 h.

psychrophiles do not always produce cold active enzyme. The other -galactosidases of *Athrobacter* sp. B7 (Trimbur et al., 1994) and *Arthrobacter* sp. C2-2 (Karasova-Lipovova et al., 2003) were characterized also by high optimum temperature of 40°C. The lactose hydrolyzing enzyme in cell free extracts of strain KNOUC403 shows high activity at 4°C and in wide range of pH 4.3 to 7.8, is stable at 4°C, and has a moderate but valuable stability at 37°C, meaning that this strain has high possibility of producing cold active lactose hydrolyzing enzyme that is useful for hydrolysis of lactose in foods of wide range of pH at refrigerating temperature. Judging from these findings of physiological and enzymatic properties, *A. psychrolactophilus* KNOUC403 is a psychrophile of useful source, for a cold active lactose hydrolyzing enzyme that would have advantages for application in the food industry.

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