

Isolation and Characterization of Cold-adapted bacteria Producing Lactose hydrolyzing enzyme isolated from soils of Nome area in Alaska

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The cold-adapted bacteria producing lactose hydrolyzing enzyme were isolated from the soils of Nome area in Alaska. The strain KNOUC601 was rod, stained negatively in Gram stain. It grew optimally at 30°C and pH 8.0-8.5, and utilized glucose, maltose, sucrose, mannose, galactose, lactose as carbon source. This strain was aerobic asporogenic bacilli, immobile, catalase positive and oxidase negative. Based on physiological and biochemical properties, the strain could be presumably identified as *Rahnella* sp., finally as *Rahnella aquatilis* by phylogenetic analysis based on 16S rDNA sequence, and the strain was named as *Rahnella aquatilis* KNOUC601. The optimal conditions for ONPG hydrolysis of cell free extract from the strain were 15°C and pH 7.8. It retained activity higher than 90% of maximum activity at refrigerate temperature. Zymogram assay of cell free extract from the strain in nondenaturing polyacrylamide gel showed a distinct band of X-gal hydrolysis. The strain *Rahnella aquatilis* KNOUC601 has high possibility to produce lactase available for practical use at refrigerate temperature.

Keywords: Alaska, cold-adapted bacteria, lactose hydrolyzing enzyme, *Rahnella aquatilis*

INTRODUCTION

The Earth is a cold planet. About 85% of the biosphere is permanently exposed to temperatures below 5°C. Cold habitats span from the Arctic to the Antarctic, from high-mountains to the deep ocean. The major fraction of this low-temperature environment is represented by the deep sea, followed by permafrost (24% of land surface) and other cold environments such as cold soils (especially subsoils), deserts, lakes, and caves (Bakermans, 2007). Cold environments are colonized by a wide diversity of microorganisms, including bacteria, archaea, yeasts, filamentous fungi and algae. To thrive successfully in low-temperature environments, these microorganisms have evolved a complex range of structural and functional adaptations. Adaptations include the production of cold-active enzymes with high catalytic efficiency at low temperatures, the incorporation of unsaturated fatty acids

in cell membranes to maintain membrane fluidity, and the production of cold-shock protein synthesis at low temperatures (Gerday and Glansdorff, 2007; Margesin et al., 2008). A variety of psychrotrophic bacteria, belonging to different genera, produce extracellular enzymes such as protease, lipase, amylase, phosphates and β -galactosidase. Cold tolerant microorganisms and their enzymes have wide range of applications in biotechnology, dairy and food industry, biotransformation, environmental bioremediation, molecular biology, diagnostics, biosensors and bioreporters (Ramana et al., 2000; Fujiwara, 2002; Bertus, 2003).

Among the above enzymes, cold-active β -galactosidase (EC 3.2.1.23), which hydrolyzes lactose to glucose and galactose, is one of the important food-industrial enzymes. It can be used to degrade lactose for several purposes, e.g., (i) removal of lactose from refrigerated milk for people who are lactose intolerant, and (ii) conversion of the lactose to glucose and galactose, which are more fermentable sugars than lactose, in whey. Some cold-active β -galactosidases have

been reported (Hoyoux et al., 2001; Fernandes et al., 2002; Coker et al., 2003; Karasova-Lipovova et al., 2003; Nakagawa et al., 2003; Turkiewicz et al., 2003; Cieslinski et al., 2005; Park et al., 2006; Liu et al., 2008). However there is no cold-active β -galactosidase practically used yet.

This study was performed to isolate a microorganism that is able to produce lactose hydrolyzing enzyme active at refrigerate temperature and available for practical use.

MATERIALS AND METHODS

Screening and Cultivation Condition Soil samples were collected from 15 regions of Nome area in Alaska. To cultivate the psychrophilic bacteria, soil samples were added to the Brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich) containing 1% (w/v) lactose, and incubated at 4°C aerobically by shaking (200rpm) for 30 days. 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- β -D-galactopyranoside (X-gal; Duchefa Biochemie, Holland). After incubation at 4°C for 15 days, blue colonies were selected, and then cultivated in Brain heart infusion broth and agar for identification and determining properties of lactose hydrolyzing enzyme.

Morphological and Biochemical Characterization of Microorganism Isolates showing cold adaptive growth and X-gal hydrolysis were investigated for Gram staining, morphological, biochemical, and physiological properties. Cells were grown on BHI agar to determine growth conditions of various temperatures (5-50°C) and in BHI broth to determine pH (4.0-8.0) condition for growth. pH of media was adjusted with HCl or NaOH. Acid production from carbohydrate, and utilization of sole carbon sources were determined using API20E and API 50CH test strips (Bio-Merieux Inc., France). For the test of hemolysis, the colonies were streaked on sheep blood agar (KOMED. Co. Ltd). After incubation at 15°C for 3 days, β -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.

16S rDNA Sequence Determination and Phylogenetic Analysis Isolation of genomic DNA, PCR amplification of the 16S rDNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. (1996). Universal primers of fD1 (5'-gagttgacccgctcag-3') and rD1 (5'-agaaaggaggtgacccagcc-3') were used for PCR. Sequence reaction products were purified by ethanol precipitation and electrophoresis, 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 *Rahnella* available from the Ribosomal Database Project (Maidak et al., 1996). The phylogenetic tree for the dataset was inferred using the neighbor-joining method (Saitou and Nei, 1987). The PHYLIP package (Felsenstein, 1993) was used for constructing the tree.

Assay of Lactose Hydrolyzing Enzyme Activity microorganisms were harvested by centrifugation at 8,000 X g for 10 min 4°C, suspended in sodium phosphate buffer (0.01M, pH 6.8), washed 2 times by the same buffer, suspended in the same buffer, and sonicated at 4°C. Cell debris was eliminated by centrifugation at 12,000g at 4°C for 20 min. The cell free extracts were used for the assay of lactose hydrolyzing enzyme activity. Lactose hydrolyzing enzyme activity was determined by measuring the rate of hydrolysis of o-nitrophenyl- β -D-galactopyranoside (ONPG) or lactose as substrate. Hydrolysis activity for ONPG was determined by the method of Miller (1972) except that the reaction was performed at 4°C for 2 hrs. The hydrolysis of lactose was assayed by measuring the release of glucose from lactose. The reaction mixture consisted of 1.6ml skim milk (lactose conc., 4.7%) and 0.4ml 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 505nm. One unit of enzyme activity for hydrolysis of lactose is the activity of hydrolyzing 1 μ mol of lactose per day by cell free extracts from 1ml of culture that was concentrated to 8 of A₆₀₀.

Influence 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 50°C) in sodium phosphate buffer (0.01M, pH 6.8). Effect of pH on enzyme activity was evaluated at pH ranging from 4.3 to 9.7 at 4°C. Na-acetate buffer (0.01M) was used for the pH from 4.3 to 6.04, Na-phosphates buffer (0.01M) was used for the pH from 6.04 to 7.91, and Tris-HCl was used for pH 7.91 to 9.71. Stability of enzyme was investigated by residual activity during incubation of enzyme in sodium phosphate buffer (0.01M, pH 6.8) for 10 days at 4°C and 37°C.

Zymogram Analysis of Lactose Hydrolyzing Enzyme Activity Zymogram assay for the intracellular fractions from selected strain was performed. After native-PAGE of intracellular fraction on 10% (w/v) polyacrylamide gel (Laemmli, 1970) the gel was incubated in the solution of 0.25mM X-gal dissolved in Z-buffer (Trimbur et al., 1994) at 4°C for 2 hours. Hydrolysis of X-Gal was confirmed by blue band within the polyacrylamide gel.

Table 1. Hydrolysis of ONPG and lactose of isolated strains from Nome area in Alaska

No.	Strain No.	Hydrolysis of	
		ONPG ¹⁾	Lactose ²⁾
1	KNOUC601	2.668	8.39
2	KNOUC602	0.255	N.D
3	KNOUC603	0.242	N.D
4	KNOUC604	0.417	N.D
5	KNOUC605	0.475	N.D
6	KNOUC606	2.686	N.D
7	KNOUC607	2.753	N.D
8	KNOUC608	2.674	N.D
9	KNOUC609	2.669	N.D
10	KNOUC610	2.665	N.D
11	KNOUC611	2.669	N.D
12	KNOUC612	0.199	N.D
13	KNOUC613	0.201	N.D
14	KNOUC614	2.683	N.D
15	KNOUC615	2.655	N.D
16	KNOUC616	2.679	N.D
17	KNOUC617	2.675	N.D
18	KNOUC618	2.693	N.D
19	KNOUC619	2.679	6.16
20	KNOUC620	2.706	4.22
21	KNOUC621	2.695	N.D

N.D: Not detection

¹⁾ One unit of enzyme activity is defined as the activity hydrolyzing 1 μ mol of ONPG per min by cell free extract from 1ml of culture whose A₆₀₀ is 8.²⁾ One unit of enzyme activity is the one hydrolyzing 1 μ mol of lactose per day at 4°C by cell free extracts from 1ml of culture whose A₆₀₀ is 8.

RESULTS

Lactose hydrolyzing enzyme producing bacterial strains were isolated from 15 samples of soils collected from Nome area in Alaska. 21 strains were screened via X-gal hydrolysis on plate at 4°C for 15days. Among those, KNOUC601, KNOUC619 and KNOUC620 showed good growth at low temperature, 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 (Table 1). The strains of KNOUC601, KNOUC619 and KNOUC620 were characterized for their physiological and biochemical properties to get information on the suitability for using in food industry.

As in Table 2, KNOUC601, KNOUC619 and KNOUC620 were presumably identified as *Rahnella* sp, *Vibrio* sp. and *Yersinia* sp. respectively based on the properties of Gram staining, shape, spore formation,

motility, catalase, V-P test, nitrate reduction and oxidase (Holt et al., 1994). And all of them were negative in hemolysis, and the Strain KNOUC601 showing the highest activity of lactose hydrolysis was selected for further study. The sequence of 16S rDNA of strain KNOUC601 determined as 1,498bp 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 KNOUC601 with the sequences in Ribosomal Database Project (Maidak et al., 1996) and at NCBI, demonstrated that strain KNOUC601 should be classified as *Rahnella* sp., and its closest relative was *Rahnella aquatilis* (98-100%). Therefore, strain KNOUC601 was identified as *Rahnella aquatilis*, and the strain was named as *Rahnella aquatilis* KNOUC601.

Cell free extracts of strain KNOUC601 was tested for the properties of lactose

Table 2. Physiological and biochemical properties of the strain KNOUC 601, KNOUC619 and KNOUC620

Characteristics	KNOUC601	KNOUC619	KNOUC620
Gram staining	-	-	-
Shape	Rod	Rod	Rod
Motility	-	+	-
Spore formation	-	-	-
Optimum temp for growth	30°C	30°C	30°C
Optimum pH for growth	8.0-8.5	8.0	8.0
Growth at 4°C	+	+	+
Catalase	+	+	+
Oxidase	-	+	-
ONPG hydrolysis	+	+	+
PNPG hydrolysis	+	+	+
Nitrate reduction	+	-	-
Gas production	-	-	-
H ₂ S formation	-	-	-
Indol production	-	-	-
Citrate utilization	-	-	-
Urease	-	-	-
Hemolysis	-	-	-
Esculin hydrolysis	+	-	+
V-P	+	-	-
Growth on MacConkey agar	+	-	-
Utilization of			
D-glucose	+	+	+
D-mannitol	+	+	+
D-maltose	+	+	-
D-rhamnose	+	+	-
D-sucrose	+	-	+
D-mannose	+	-	+
D-galactose	+	-	+
D-lactose	+	+	+
D-raffinose	+	-	+
D-sorbitol	+	+	-
N-acetyl-glucosamine	+	+	-
Inositol	-	+	-
Gluconate	+	-	-
Xylitol	-	+	-
Phenylacetate	-	-	+
Saccharose	+	+	-
Genus	<i>Rahnella</i> sp.	<i>Vibrio</i> sp.	<i>Yersinia</i> sp.

+, positive reaction; -, negative reaction

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGAAAGTAGCTTGCTACTTTGCCGCGAGCGGC
 GGACGGGTGAGTAATGTCTGGGAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACCTC
 GAAAGAGCAAAGTGGGGGATCTTCGGACCTCAGCCATCGGATGTGCCAGATGGGATTAGCTAGTAGGTGAGGTAATG
 GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAAGCCACACTGGAAGTGAGACACGGTCCAGACTCCTAC
 GGGAGGCAGCAGTGGGGAATTTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGG
 GTTGTAAAGCACTTTCAGCGAGGAGGAAGGCATCATACTTAATACGTGTGGTGATTGACGTTACTCGCAGAAGAAGCACCG
 GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAG
 GCGGTTTGTAAGTCAGATGTGAAATCCCCGCGCTTAACGTGGGAAGTGCATTTGAACTGGCAAGCTAGAGTCTTGTA
 GGGGGGTAGAATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
 CAAAGACTGACGCTCAGGTGCGAAAAGCGTGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAACGATGTC
 GACTTGAGGTTGTGCCCTTGAGGCGTGGCTTCCGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGAAG
 GTTAAACTCAAATGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTT
 ACCTACTCTTGACATCCACGGAATTCGCCAGAGATGGCTTAGTGCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGT
 CGTCAGCTCGTGTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCCTTTGTTGCCAGCAGTAATGGTG
 GGAACCTCAAAGGAGACTGCCGGTGATAACCGGAGGAAGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGG
 GCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGT
 CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATAGCTTC
 CCGGGCCTTGATACACCCGCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCG
 CTTACCACCTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCTGC

Figure 1: 16S rDNA sequence of KNOUC601 (1,498bp)

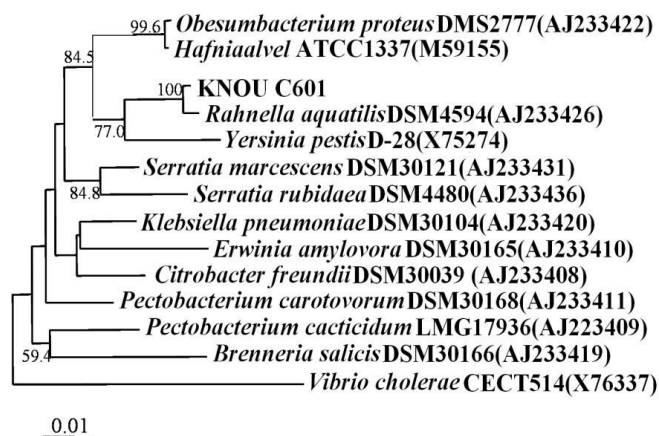


Figure 2: Phylogenetic tree based on 16S rDNA sequences showing the positions of strain KNOUC601, the representative of some other related taxa. Bootstrap values (1000replications) are shown as percentage at each node only if they are 50% or greater. Scale bar represents 0.01 substitution per nucleotide position.

hydrolyzing enzyme at various pHs and temperatures to confirm the possibility of the isolate for a source of cold active lactose hydrolyzing enzyme. The enzyme was active in a broad range of temperatures (5 to 60°C), showing maximum activity at 15°C as in Figure 3. The enzyme exhibited ca. 90.1% of maximal activity at 4°C, and less than 20.9% of maximal activity at 50°C. The effect of pH on enzyme activity is shown in Figure 4. The enzyme was active at broad range of pH (between 4.3-9.7), and the maximum activity was observed at pH 7.8. It retained more than 70% of maximum activity between pH 6.5 and pH 9.0. To determine the stability of enzyme at 4°C and 37°C, the cell free extracts in Na-phosphate buffer (0.01M, pH6.8) was incubated at those temperatures for 10 days and residual activity were tested during incubation. There was no change in activity for hydrolysis of ONPG for 10 days at 4°C, and at 37 °C it was stable only for a day (Figure 5). It lost 30% activity in

10 days at 37°C.

To confirm the existence of lactose hydrolyzing enzyme in crude cell free extracts of strain KNOUC601, zymogram assay for X-gal hydrolysis was performed. There was a distinct band of X-gal hydrolysis (Figure 6).

DISCUSSION

In this study we report on *Rahnella aquatilis* KNOUC601 producing cold adapted lactose hydrolyzing enzyme, isolated from soil of Nome area in Alaska. We examined the physiological and biochemical properties. Strain KNOUC601 could grow at 4°C and did not form colony at 40°C. Morita (1975) defined that psychrotolerants grew optimally at temperatures around 20 to 25°C and might have upper limit as high as 40°C, however psychrophiles were specifically adapted to low-temperature growth and

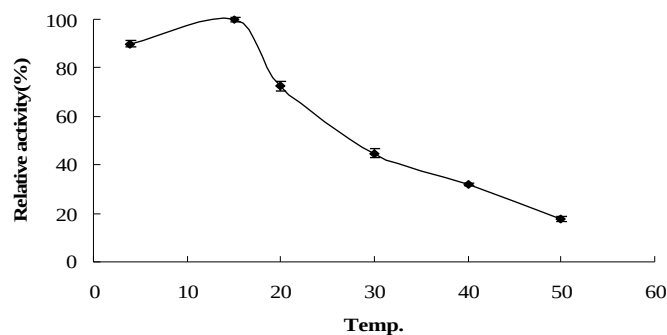


Figure 3. Effect of temperature on the activity of lactose hydrolyzing enzyme in cell free extracts of strain KNOUC601
 * Effect of temperature on enzyme activity was analyzed in Na-phosphate buffer (0.01M, pH 6.8). Values are means of triplicates $\pm$ SD.
 * Values are means of triplicates $\pm$ S.D.

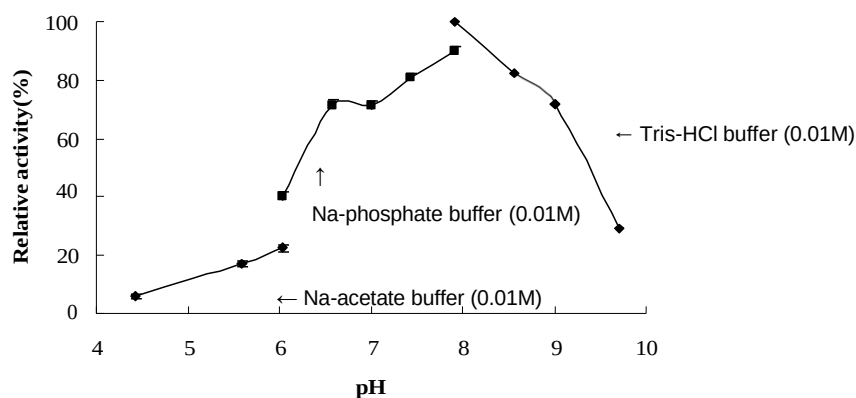


Figure 4. Effects of pH on the activity of lactose hydrolyzing enzyme in cell free extracts of strain KNOUC601
 * Effect of pH on enzyme activity was evaluated at 4°C in Na-acetate buffer (0.01M, pH4.3-6.04), Na-phosphate buffer (0.01M, pH6.04-7.91) and Tris-HCl buffer (0.01M, pH7.91-9.71). Values are means of triplicates $\pm$ SD.

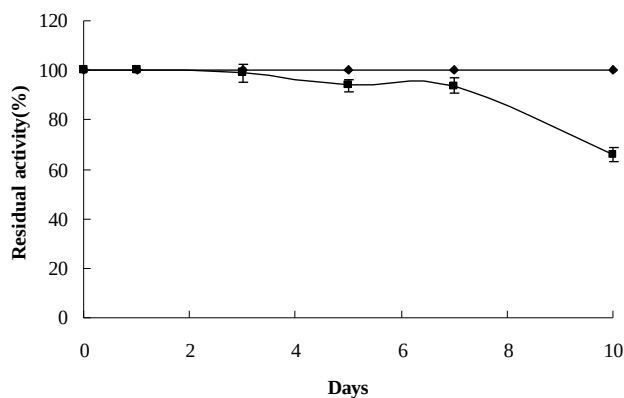


Figure 5. Stability of lactose hydrolyzing enzyme in cell free extracts of *Rahnella aquatilis* KNOUC601 at 4°C (♦) and 37°C (■) in Na-phosphate buffer (0.01M, pH6.8)
 * Cell free extracts of KNOUC601 was incubated in Na-phosphate buffer (0.01M, pH 6.8)
 * Values are means of triplicates $\pm$ S.D.

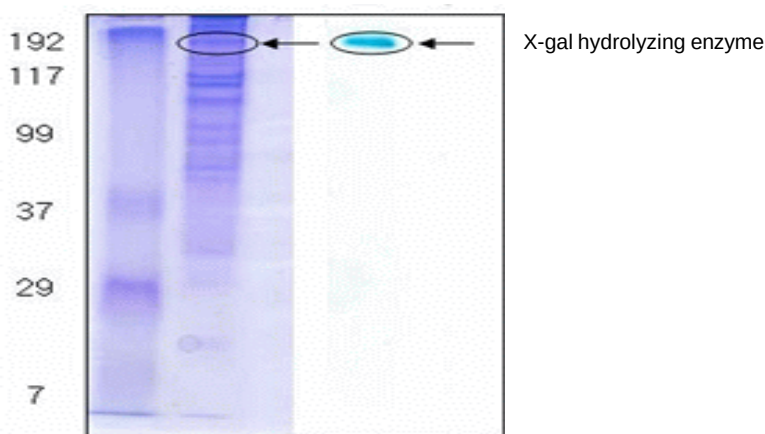


Figure 6. Native polyacrylamide gel electrophoresis of crude cellular extract from *Rahnella aquatilis* KNOUC601. The protein was analyzed on a 10% polyacrylamide gel. Ma: Kaleidoscope Polypeptide Standards Marker. 1: Gel after native polyacrylamide gel electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. 2: The gel was soaked in 0.1M X-gal solution (in Z-buffer) at 4°C for 2 hr

had optimal growth temperature below 15°C and upper limit below 20°C. The ability of strain KNOUC601 growing near 0°C is a characteristic of both psychrotolerants and psychrophiles. However the optimum growth temperature of this strain was 30°C, therefore it belongs to psychrotolerants. Physiological and biochemical properties of strain KNOUC601 was fit to be presumably identified as Genus *Rahnella*, and the phylogenetic analysis of 16S rDNA sequence from this isolate placed it in species of *Rahnella aquatilis*. The 16S rDNA sequence of strain KNOUC601 evidenced similarity of 100% and 99% to *Rahnella aquatilis* DSM4594 and *Rahnella aquatilis* BS1 type strain from GenBank database respectively.

The lactose hydrolyzing enzyme produced by *Rahnella aquatilis* KNOUC601 reacted optimally at 15°C that is much lower than the optimum temperature for growth (30°C) of this strain. Activity of this lactose hydrolyzing enzyme at 4°C was 90% of optimum temperature and it was stable for 10 days at 4°C meaning that this enzyme is useful at refrigerated temperature. β -Galactosidase of *Rahnella aquatilis* BS1 showed maximum activity at 10°C (Park et al., 2006). The optimum temperature of β -galactosidases from *Pseudomonas* sp. 22b (Turkiewicz et al., 2003) and *Arthrobacter psychrolactophilus* F2 (Nakagawa et al., 2006a, b) were reported to be 5°C and 25°C respectively. The lactose hydrolyzing enzyme of *Rahnella aquatilis* KNOUC601 showed highest activity at pH 7.9 that is similar with those of β -galactosidases produced by *Arthrobacter* sp. ON14 (Xu et al., 2011), and *Alkalilactibacillus ikkense* (Schmidt and Stouggard,

2010) whose optimum pH are 8.0 and 7.8 respectively. The lactose hydrolyzing enzyme of *Rahnella aquatilis* KNOUC601 had more than 80% activity of optimum pH at pH between 6.5 and 7.0, the pH of milk.

From these facts, we conclude that *Rahnella aquatilis* KNOUC601 is useful for production of cold active lactose hydrolyzing enzyme that can be applicable at refrigerated temperature in food industry including dairy industry.

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