β-Galactosidase Gene of *Thermus thermophilus* KNOUC112 Isolated from Hot Springs of a Volcanic Area in New Zealand: Identification of the Bacteria, Cloning and Expression of the Gene in *Escherichia coli* *

E. S. Nam, J. W. Choi, J. H. Lim, S. K. Hwang, H. J. Jung, S. K. Kang[1], K. K. Cho[2], Y. J. Choi[1] and J. K. Ahn*
Department of Agricultural Science. Korea National Open University, Seoul 110-791, Korea

ABSTRACT : To isolate the β-galactosidase producing thermophilic bacteria, samples of mud and water were collected from hot springs of avolcanic area near Golden Springs in New Zealand. Among eleven isolated strains, the strain of KNOUC112 produced the highest amounts of β-galactosidase at 40 h incubation time (0.013 unit). This strain was aerobic, asporogenic bacilli, immobile, gram negative, catalase positive, oxidase positive, and pigment producing. Optimum growth was at 70-72°C, pH 7.0-7.2, and it could grow in the presence of 3% NaCl. The main fatty acids of cell components were iso-15:0 (30.26%), and iso-17:0 (31.31%). Based on morphological and biochemical properties and fatty acid composition, the strain could be identified as genus *Thermus*, and finally as *Thermus thermophilus* by phylogenetic analysis based on 16S rRNA sequence. So the strain is designated as *Thermus thermophilus* KNOUC112. A gene from *Thermus thermophilus* KNOUC112 encoding β-galactosidase was amplified by PCR using redundancy primers prepared based on the structure of β-galactosidase gene of *Thermus sp.* A4 and *Thermus sp.* strain T2, cloned and expressed in *E. coli* JM109 DE3. The gene of *Thermus thermophilus* KNOUC112 β-galactosidase(KNOUC112β-gal) consisted of a 1,938 bp open reading frame, encoding a protein of 73 kDa that was composed of 645 amino acids. KNOUC112β-gal was expressed as dimer and trimer in *E. coli* JM109 (DE3) *via* pET-5b. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 11 : 1591-1598)***

Key Words : Hot Springs, β-galactosidase, *Thermus thermophilus*, Cloning and Expression

INTRODUCTION

Many thermophiles, whose optima temperature for growth 45-80°C, are present in hot springs as well as other thermal environments (Campbell and Pace, 1968). These organisms offer some major advantages for industrial and biotechnological process. Many of which reproduce rapidly and efficiently at high temperatures, their enzymes are capable of catalyzing biochemical reactions at high temperatures and are generally more stable than enzyme from mesophlies, thus self-life of the enzyme can be longer. The enzymes stable at high temperature generally represent high stability not only to heat, but also to other protein denaturants such as detergent and organic solvents. Because of those properties mentioned above, thermostable enzymes are utilized extensively in industry (Thomas and William, 1986)

β-Galactosidase (EC 3.2.1.23) catalyzes not only hydrolysis of β-D-galactopyranosides, such as lactose, but also a trans-galactosylation reaction that produces galacto-oligosaccharides (Onishi and Tanaka, 1995). The enzyme is useful in dairy industry for prevention of lactose crystallization in frozen and condensed milk products, for decreasing water pollution caused by whey from cheese production, and for remedy of lactose intolerance (Kern and Struthers Jr., 1966). Galacto-oligosaccharides, enzymatic trans-galactosylation reaction products from lactose, have become of interest for human health. Galacto-oligosaccharides are recognized as a growth-promoting factor for intestinal bifido bacteria (Ohtsuka et al., 1989), which are helpful for maintenance of human health (Hughes and Hooner, 1991). Thermostable β-galactosidase can be a good tool for the efficient use of lactose and for the difficulties caused by lactose.

Many β-galactosidases from microbial sources have been reported (Dumortier et al., 1994; Brady et al., 1995; Choi et al., 1995; Berger et al., 1997), but studies on the thermostable β-galactosidase is not much. From the late of 1990', gene of highly thermostable β-galactosidase began to be reported, and Ohtsu et al. (1998) and Vian et al. (1998) purified and characterized a thermostable β-galactosidase from *Thermus sp.* A4 and *Thermus sp.* strain T2 respectively. Still more research is required to find better thermostable β-galactosidase.

Accordingly, in this study a β-galactosidase producing thermophilic bacteria from hot spring was isolated and identified. And the β-galactosidase gene of the bacteria was

* This Study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (1998-2002).
** Corresponding Author: J. K. Ahn. Tel: +82-2-3668-4630, Fax: +82-2-3668-4187, E-mail: ajk@knou.ac.kr
[1] School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea.
[2] Department of Animal Resource Technology, Jinju National University, Jinju 660-758, Korea.
Received March 17, 2004; Accepted July 20, 2004

cloned and expressed in *E. coli*.

MATERIALS AND METHODS

Isolation of β-galactosidase producing thermophilic bacteria

β-Galactosidase producing thermophilic microorganisms were isolated from mud and water collected near by Golden Springs in New Zealand, as described below. To cultivate the thermophilic microorganisms, 1 g of mud sample or 1 ml of water sample from hot spring was added to the medium of ATCC 1598 (Alfredsson et al., 1985) and incubated at 70°C aerobically by shaking (200 rpm) for 48 h. One liter of this medium was composed of Bacto-tryptone, 2.5 g; yeast extract, 2.5 g; nitrilotriacetic acid, 100.0 mg; $CaSO_4 \cdot 2H_2O$, 40 mg; $MgCl_2 \cdot 6H_2O$, 200 mg; 0.01 M Fe-citrate, 0.5 ml; trace element soln, 0.5 ml; phosphate buffer (pH 7.2). The trace element solution had the following composition (/L): nitrilotriacetic acid, 12.8 g; $FeCl_2 \cdot 4H_2O$, 10.0 g; $MnCl_2 \cdot 4H_2O$, 0.5 g; $COCl_2 \cdot 6H_2O$, 0.3 g; $CuCl_2 \cdot 5H_2O$, 50 mg; $Na_2MoO_4 \cdot 2H_2O$, 50 mg; H_3BO_3, 20 mg; $NiCl_2 \cdot 6H_2O$, 20 mg; pH adjusted to 7.2.

To screen β-galactosidase producing bacteria, the cultures were streaked onto ATCC 1598 solid medium containing 1% (v/w) lactose and 0.02% (v/w) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase (X-gal). After incubation at 70°C for 48 h, blue colonies were selected, then cultivated in ATCC 1598 liquid medium for identification and testing β-galactosidase activity.

Assay of β-galactosidase activity

Microorganisms were harvested by centrifugation at 8,000×g for 10 min at 4°C, suspended in sodium phosphate buffer (10 mM, pH 6.8), washed 2 times by the same buffer and sonificated 5 times of 30 sec (100 Hz). Cell debris was eliminated by centrifugation (12,000×g) for 20 min at 4°C.

The cell free extract was used for the assay of β-galactosidase activity using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate (Craven et al., 1965). β-Galactosidase activity was determined by release of *o*-nitrophenol from 0.04 M ONPG dissolved in sodium phosphate buffer (100 mM, pH 6.8). An aliquot of cell free extract (0.5 ml) was added to 2.5 ml of ONPG solution and incubated at 70°C for 10 min. The reaction was stopped by addition of 3 ml of Na_2CO_3 (0.5 M), and the absorbance at 420 nm was measured. One unit of enzyme activity is defined as the activity hydrolyzing 1 μmol of ONPG per min by cell free extract from 1 ml of culture.

Identification of β-galactosidase-producing bacteria

Morphology and physiological characteristics : The bacterial strain showing the highest β-galactosidase activity was selected, then tested for Gram staining, morphological, biochemical, and physiological properties. The morphology of the isolated bacteria was observed under scanning electron microscope (SEM). Catalase reaction, oxidase reaction, NaCl tolerance, casein hydrolysis, starch hydrolysis, gelatin liquefaction, H_2S production, citrate utilization, urease production, ONPG hydrolysis, indole production, nitrate reduction, and acid production from carbohydrates were tested. Most tests were examined as applied by Santos et al. (1989) and Manaia and da Costa (1991). The pigment producing property was determined by carotenoide production (Brock and Brock, 1967). All biochemical and physiological tests were done at 70°C for 48 h.

Composition of cellular fatty acid : Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation and extraction as described by Kuykendall et al. (1988). The fatty acid methly esters were separated by using a model 5890 GC system (Hewlett Packard) equipped with a flame ionization detector fitted with a 5% phenyl methyl silicone capillary column (0.2 mm ×25 m). The carrier gas was H_2 of high-purity, the column head pressure was 60 kPa, the septum purge was 5 ml/min, the column split ratio was 55:1 and the injection port temperature was 300°C. The temperature program was run from 170 to 270°C at the rate of 5°C/min. The numerical analysis of fatty acid methyl esters and fatty acid profiles were performed by using the standard MIS Library Generation Software (Microbial ID Inc.).

16S rRNA sequence determination and phylogenic analysis : Isolation of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. (1996). Universal primers of fD1 (5'agagtttgatcctggctcag3') and rD1 (5'acggctaccttgttaccactt3') were used to amplify the gene of 16S rRNA. Sequence reaction products were purified by ethanol precipitation and electrophoresis with a model 377 Genetic Analyzer (Perkin-Elmer, USA.). The 16S rRNA sequences obtained in this study were aligned against the previously determined sequences of the genus of *Meiothermus*/*Thermus* sequences available from the Ribosomal Database Project (Maidak et al., 1996). The evolutionary 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.

Isolation, cloning and sequence determination of β-galactosidase gene

β-galactosidase gene was amplified by Touch Down PCR in gene cycler (Bio-Rad, Japan). PCR was performed using redundancy primers and chromosomal DNA of the

Table 1. Origin of strains isolated near by Golden springs in New Zealand

Area	Strain No.	Temperature (°C)	pH	β-galactosidase production at 40 h incubation time (unit)
Waikite	KNOUC106	75-80	10	NA
Waiotapu	KNOUC107	65-90	10	NA
	KNOUC108	75	6.0-7.0	NA
	KNOUC109	75-80	4.0-5.0	NA
	KNOUC110	75-80	5.0	0.008
Orakeikorako	KNOUC111	75-80	7.0	0.012
	KNOUC112	60-75	4.0	0.013
	KNOUC113	50-70	5.0	0.003
	KNOUC114	60-70	5.0	0.012
Mud pool	KNOUC126	70-80	3.5	NA
	KNOUC127	75	3.5	NA

* NA: Negligible activity.

isolated bacteria. Redundancy primers were prepared on the basis of gene structures of β-galactosidase from *Thermus sp.* A4 (Ohtsu et al., 1998) and *Thermus* T2 (Vian et al., 1998). The nucleotide sequence of forward primer was 5'atgYtSggcgtttgYtaYtacc3', and that of reverse primer was 5'tcatgYctcctcccaSacg3'. DNA fragment produed in PCR was isolated by agarose electrophoresis and Geneclean Kit method (Bio101, USA), ligated into pGEM-T Easy (Promega, USA) and cloned in *Escherichia coli* JM109. Luria-Bertani (LB) medium was used for culturing *E. coli* JM109. Ampicilline was added at the concentration of 100 μg/ml to screen *E. coli* JM109 transformed by pGEM-T Easy harboring the DNA fragment. Sequence of the DNA fragment was determined using Big Dye Automatic sequencer ABI377 (Perkin Elmer, USA) and PE9600 Thermocycler (Perkin Elmer, USA). The DNA and amino acids sequence analysis was performed by the DNASIS software system. The homology search was done using the World Wide Web server from BLAST search maintained at National Center for Biotechnology Information (NCBI).

Expression of β-galactosidase gene

Expression plasmid was constructed by inserting the isolated gene into pET-5b (Promega, USA) regulated by *Lac*UV5 promoter. *E. coli* JM109 (DE3) was transformed with the pET-5b harboring the gene and cultured on LB solid medium containing ampicillin (100 μg/ml) at 37°C. After heating colonies formed on the LB solid medium at 70°C for 3 h, Z-buffer (Ausubel et al., 1998) containing 1.4 mg of X-gal/ml was poured over those colonies and those colonies were incubated 3 h at 70°C. Blue colony was picked and used to confirm the expression of β-galactosidase gene. The confirmation of expression was performed by cell free extracts ONPG hydrolysis at 70°C and by X-gal hydrolysis of native PAGE gel soaked and incubated at 70°C in Z-buffer of X-gal (1.4 mg/ml).

Electrophoresis

Observation of DNA and protein was performed by agarose electrophoresis and polyacrylamide gel electrophoresis (PAGE) respectively. Agarose electrophoresis was done on 1% agarose gel. SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) on 7.5% polyacrylamide slab gel, and native polyacrylamide gel electrophoresis was done on the same way with that of SDS polyacrylamide gel electrophoresis expect without addition of SDS and without denaturation of sample before electrophoresis.

RESULTS AND DISCUSSION

Isolation of β-galactosidase-producing thermophilic bacteria

β-galactosidase-producing bacterial strains were isolated from 11 samples of mud and water collected from 11 hot springs of 4 regions near by Golden springs in New Zealand (Table 1). The temperatures of those hot springs were 50 to 90°C, where thermophilic organisms may highly predominate. The range of sample pH was from 3.5 to 10. From each of 11 samples, a colony of blue color showing hydrolysis of X-gal on ATCC 1598 solid medium was isolated. Those 11 strains were tested for β-galactosidase activity at 40 h incubation time using ONPG as substrate. Among 11 strain, the strain of KNOUC112 (0.013 unit) isolated from a hot spring of Orakeikorako was selected due to its highest β-galactosidase activity.

Identification of β-galactosidase-producing thermophilic bacteria

Morphological and biochemical characteristics : The morphological and biochemical characteristics of the strain KNOUC112 were summarized in Table 2. The strain KNOUC 112 was found to be a Gram-negative, non-motile, non-spore forming bacteria, and it formed yellow-pigmented colonies. As shown in Figure 1, it was rod-shaped (0.2×2.0~3.5 μm). The optimum growth temperature was about 70~72°C and growth did not occur below 45°C

Table 2. Morphological and physiological characteristics of strain KNOUC112

Characteristics	Reaction	Characteristics	Reaction
Gram reaction	-	Utilization of	
Cell shape	Rod	Glucose	+
Motility	-	Mannitol	+
Size (μm)	0.2-0.4	Inositol	+
Spore formation	-	Sorbitol	+
Color of colony	Yellow	Sucrose	+
Optimum temperatures	68-70	Mellibiose	+
Optimum pH	7.0-7.2	Amygdalin	+
Growth at/in		Arabinose	+
80°C	+	Galactose	+
85°C	+	Saccharose	+
1% NaCl	+	Trehalose	+
3% NaCl	+	Lactose	+
5% NaCl	-	Fructose	+
Catalase	+	Xylose	-
Oxidase	+	Mannose	+
Nitrate reduction	+	Ducitol	+
Voges-Proskauer reaction	+	Adonitol	+
Indol production	-	Raffinose	+
Hydrogen sulfide formation	+	Glycerol	+
Citrate utilization	-	Erythrol	+
Hydrolysis of		Malate	+
Starch	+	Citrate	-
Casein	+	Propionate	+
ONPG	+	Pyruvate	+
Urease	+	Formate	+
Gelatin liquefaction	+	Glutamate	+
Oxidation-Fermentation	Oxidation	Arginine	+
Growth on nutrient plate	-	Lysine	+
		Ornitine	+
		Tryptophane	+

and above 85°C. The range of pH for growth was from 5.0 to 8.5, and it reproduced optimally at pH 7.0~7.2. The strain KNOUC112 was catalase-positive and showed growth in medium containing 1-3% NaCl. It could utilize rhamnose, sucrose, mellibiose, amygdalin, galactose, saccharose, trehalose, lactose, fructose, mannose, ducitol, adonitol, raffinose, glycerol, erythrol, malate, propionate, pyruvate, formate, glutamate, arginine, lysine, and ornitine, but not xylose. Alfredsson et al. (1985) reported that most thermophilic bacteria utilized many kinds of monosaccahide, pyruvate and glutamate, and hydrolyzed casein. All strains of genus *Thermus* formed yellow-pigmented colonies, were Gram-negative rods and not motile, did not from spores, and their optimum growth temperatures were about 70°C (Brock and Brock, 1967). *T. thermophilus* could be distinguished from all other species of the genus *Thermus* by their ability to grow at temperatures above 80°C (Degryse et al., 1978) and in medium containing 2-3% NaCl (Manaia and da Costa, 1991). The strain KNOUC112 showed same properties with those of genus *Thermus* reported by Brock(1984) for morphology and optimum

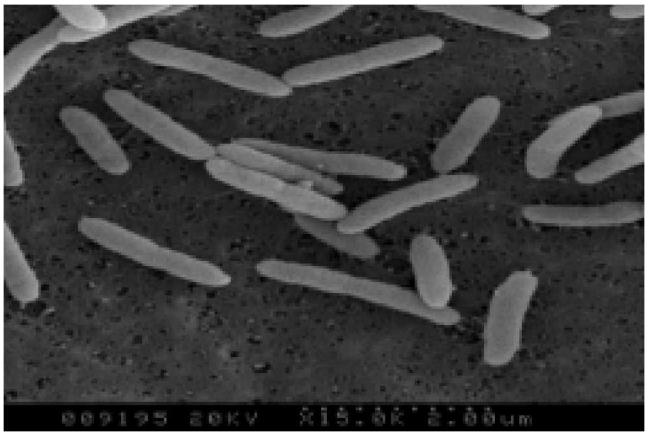

Figure 1. Scanning electron micrograph of the isolated strain KNOUC112 (X 20,000).

Table 3. Composition of cellular fatty acids of the strain KNOUC112

Fatty acid	Contents (%)
13:0 iso	0.66
14:0 iso	1.33
14:0	0.75
15:0 iso	30.26
15:0 anteiso	13.42
16:0 iso	7.71
16:0	5.06
17:0 iso	31.31
17:0 anteiso	8.65
18:0 iso	0.84
Total	100

growth temperature, and it coincides with *Thermus thermophilus* reported by Mania and da Costa (1991) in growth above 80°C and in the medium containing NaCl of 3%. The carbon-source utilization of KNOUC112 also closely resembles those of other *Thermus* strains (Degryse et al., 1978) with the only difference of utilizing malate as a single carbon source by this isolate.

Composition of cellular fatty acid : To ascertain the relatedness of strain KNOUC112 to *Thermus* and *Thermus thermophilus*, composition of cellular fatty acids was analyzed as in Table 3, the cellular fatty acids of strian KNOUC112 were composed of iso-$C_{13:0}$ (0.66%), iso-$C_{14:0}$ (1.33%), $C_{14:0}$ (0.75%), iso-$C_{15:0}$ (30.26%), antiso-$C_{15:0}$ (13.42%), iso-$C_{16:0}$ (7.71%), $C_{16:0}$ (5.06%), iso-$C_{17:0}$ (31.31%), anteiso-$C_{17:0}$ (8.05%) and iso-$C_{18:0}$ (0.84%). The main fatty acids of KNOUC112 were iso-$C_{17:0}$ and iso-$C_{15:00}$ reaching about 61.57% of total fatty acids. Nobre et al. (1996) reported that *Thermus* strains had high levels of iso-$C_{15:0}$ and iso-$C_{17:0}$, and low level of iso-$C_{16:0}$. The major fatty acids of *T. thermophius* HB8 (Chung et al., 2000) were iso-$C_{15:0}$ (32.4%) and iso-$C_{17:0}$ (41.1%). The strain KNOUC112 showes similar fatty acids composition with the results above reported by Nobre et al. (1996) and Chung et al. (2000), meaning that KNOUC112 is

Table 4. Levels of 16S rRNA similarity for strain KNOUC 112, the type strains of *Thermus* species and representatives of some related taxa

Strains	% similarity in												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1 Strain KNOUC 112													
2 *Thermus thermophilus* ATCC27634 (HB8) T	99.9												
3 *Thermus antranikianii* DSM12462T	95.1	95.1											
4 *Thermus aquaticus* ATCC25104T	96.4	96.4	96.5										
5 *Thermus brockianus* NCIMB12676T	95.0	95.0	95.1	95.6									
6 *Thermus filiformis* ATCC43280T	94.4	94.4	94.6	93.6	94.6								
7 *Thermus igniterrae* DSM12459T	95.0	95.0	95.1	95.5	97.1	97.3							
8 *Thermus oshimai* NCIMB13400T	93.4	93.4	93.6	92.3	92.5	92.2	91.8						
9 *Thermus scotoductus* DSM8553T	94.7	94.7	94.8	97.8	96.1	95.9	94.0	96.2					
10 *Meiothermus ruber* DSM1279T	87.2	87.2	87.6	87.4	87.2	87.0	86.8	87.7	85.2				
11 *Meiothermus silvanus* DSM9946T	86.1	86.1	86.3	86.7	86.7	87.3	85.9	86.8	86.0	85.9			
12 *Meiothermus chliarophilus* DSM9957T	86.7	86.7	87.1	86.5	86.3	86.2	86.2	86.7	85.6	86.3	90.7		
13 *Deinococcus radiodurans* DSM20539T	80.4	80.4	80.8	80.6	80.6	81.3	81.3	80.7	80.8	80.5	81.3	82.3	

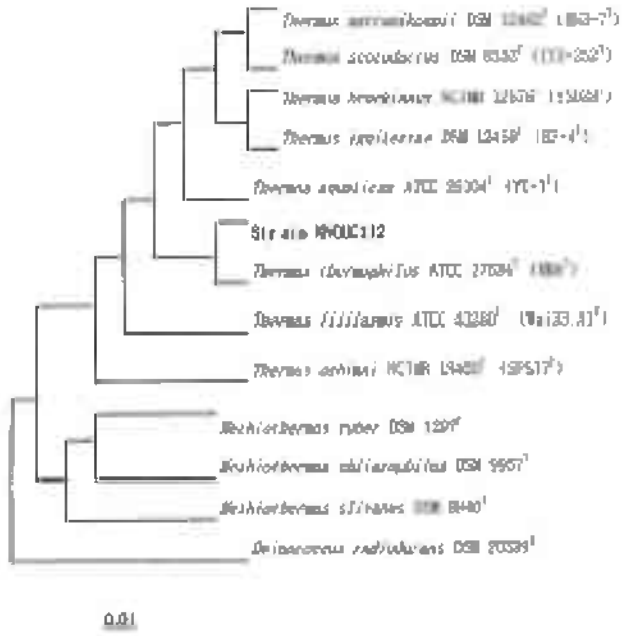

Figure 2. Phylogenetic tree based on 16S rRNA sequences showing the positions of strain KNOUC112, the type strains of *Thermus* species and the representatives of some other related taxa. Scale bar represents 0.01 substitution per nucleotide position.

chemotaxonomically closed to *T. thermophilus*.

16S rRNA Sequence determination and phylogenetic analyses : The phylogenetic position of the strain KNOUC112 was determined by 16S rRNA sequence. 16S rRNA sequence of KNOUC 112 was compared with sequences in Ribosomal Database Project (Maidak et al., 1996) and NCBI database. The 16S rRNA of strain KNOUC112 was determined as 1,441 bp. The strain KNOUC112 was demonstrated by value for DNA-DNA reassociation with the type strains of the genus *Thermus* and representatives of some related texa (Table 4). The phylogenetic tree constructed using the neighbor-joining method (Felsenstein, 1993) is shown in Figure 2. The scale bar represents 0.01 substitutions per nucleotide position. It showed a higher degree of DNA-DNA homology with *Thermus thermophilus* ATCC 27634 (99.9%), the 16S rRNA gene sequence of which was described previously by Williams et al. (1995).

Therefore, on the basis of its morphological, biochemical and physiological characteristics, strain KNOUC 112 was identified as *Thermus thermophilus*, and the strain is named as *T. thermophilus* KNOUC112.

β-galactosidase gene

DNA fragment of about 2 kb was produced by PCR. The fragment was a β-galactosidase gene consisting of a 1,938 bp open reading frame, encoding a protein of 645 amino acids. The deduced molecular weight of the protein is 72,896 dalton. The nucleotide sequence of the DNA fragment named as KNOUC112β-gal and it's deduced amino acid sequence are shown in Figure 3. KNOUC112β-gal has high homology of 99% and 83% with those of *Thermus sp.* A4 (Gene bank accession No. g3157465, Ohtsu et al., 1998) and *Thermus sp.* strain T2 (Gene bank accession No. g2765752, Vian et al., 1998) respectively whose genes were referred for the preparation of redundancy primers used to amplify the β-galactosidase gene of KNOUC112 in PCR. KNOUC112β-gal also has high identity of 90% and 74% with those of *Thermus sp.*, *Thermus sp.* IB-21 (Gene bank accession No. g25989599) and *Thermus brockianus* (Gene bank accession No. g4928634) respectively. All *Thermus spp.* above have β-galactosidase of 645 a.a, but the β-galactosidases of *Pyrococcus woesei* (Dabrowski et al., 2000), *Bacillus stearothermophilus* (Gene bank accession No. g80200), *Thermotoga maritime* (Moore et al., 1994) and *E. coli* (Jacobson, 1994) are composed of 510, 672, 1,037, 1,023 a.a respectively that are much different with those of *Thermus thermophilus* KNOUC112 and other *Thermus spp.*

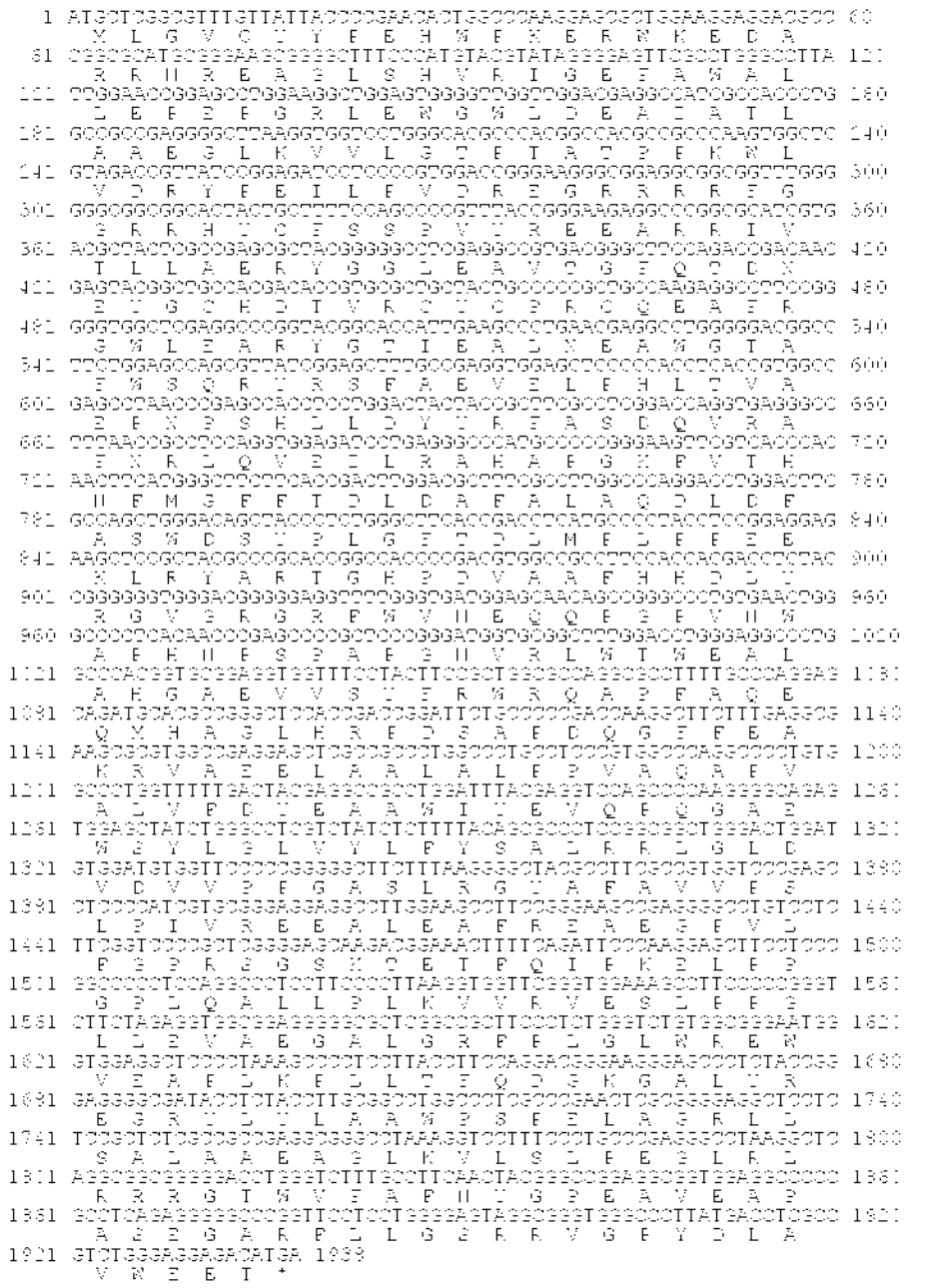

Figure 3. DNA sequence of *Thermus thermophilus* KNOUC112 β-galactosidase gene (KNOUC 112β-gal) and deduced amino acid sequence.

Expression of β-galactosidase gene

Colony of *E. coli* JM109 (DE3) transformed by pET-5b carrying the KNOUC112β-gal hydrolyzed X-gal in solid LB medium at 70°C, and it's β-galactosidase activity of hydrolyzing ONPG was detected in the cell free extract of *E. coli* JM109(DE3) showing that the β-galactosidase was produced intracellularily. In the gel of SDS-PAGE, there was a new protein of about 73 kDa (Figure 4-C line) fitting with the deduced molecular weight by the nucleotide sequence of KNOUC112β-gal. In native PAGE, the cell free extract of *E. coli* having KNOUC112β-gal showed no protein of 73 kDa (Figure 5-C line), but of about 140 kDa (dimmer) and about 210 kDa (trimer). The dimmer and trimer showed activity of X-gal hydrolysis (Figure 5-D line), and the activity of dimer was much stronger than that of trimer. It means that KNOUC112β-gal was expressed as active form of dimer and trimer in *E. coli* JM109 DE3, and the dimer may be the main product of KNOUC112β-gal. *E. coli* β-galactosidase (Wickson and Huber, 1970) and *Kluyveromyces fragilis* β-galactosidase (Kulikova et al., 1972) had activity when they were tetramer. Monomer and dimer of *Streptococcus lactis* 7962 β-galactosidase were active (Mcfeters et al., 1969).

Thermus thermophilus KNOUC112, in 40 h incubation

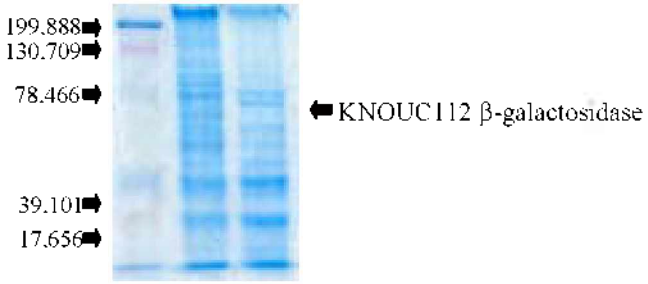

Figure 4. SDS-PAGE of proteins in *E. coli* JM109 (DE3) harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112β-gal) in pET-5b. A: Molecular weight marker, B: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b, C: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b harboring KNOUC112β-gal.

at 70°C, produced β-galactosidase of 0.013 unit of activity assayed at 70°C. And *E. coli* JM109 (DE3) having pET-5b harboring KNOUC112β-gal produced β-galactosidase of 4.0 unit in 20 h incubation at 37°C. Transferring KNOUC112β-gal from *Thermus thermophilus* KNOUC112 to *E. coli* JM109 DE3 *via* pET-5b increased β-galactosidase production about 250 times more.

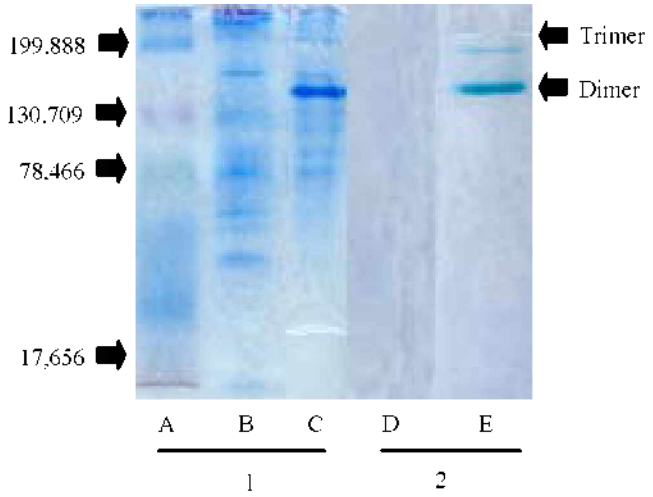

Figure 5. Native-PAGE of proteins in *E. coli* JM109 DE3) harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112β-gal). 1. Native-PAGE, A: Molecular weight marker, B: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b, C: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b harboring KNOUC112β-gal. 2. X-gal hydrolysis by Native-PAGE gel at 70°C, D: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b, E: Cell free extracts of *E. coli* JM109 (DE3) transformed with pET-5b harboring KNOUC112β-gal.

REFERENCES

Alfredsson, G. A., S. Baldursson and J. K. Krjstjansson. 1985. Nutritional diversity among *Thermus spp.* isolated from Icelandic hot spring. Syst. Appl. Microbiol. 6:308-311.

Asubel, F. M., R. Brent, R. E. Kingston, D. D. Moor, J. G. Seidman, J. A. Smith and K. Struhl. 1998. Current protocols in molecular biology. 13.6.5. Wiley.

Berger, J. L., B. H. Lee and C. Lacroix. 1997. Purification, properties and characterization of a high-molecular-mass β-galactosidase isoenzyme from *Thermus aquaticus* YT-1. Biotechnol. Appl. Biochem. 25:29-41.

Brady, D., R. Merchant, L. Michael and A. P. McHale. 1995. Isolation and partial characterization of β-galatoaisdase activity produced by a thermotolerant strain of *Kluyveromyces marxianus* during growth on lactose-containing media. Enz. Microbe. Technol. 17:696-699.

Brock, T. D. 1984. Genus *Thermus*. In: (Ed. N. R. Kriegand and J. G. Holt) Bergey's manual of systematic bacteriology, vol. 1. pp. 233-237. Williams & Wilkins. Baltimore.

Brock, T. D. and M. L. Brock. 1967. The measurement of chlorophyll, primary productivity, photophospholylation and macromolecules in benthicalgal mats. Limnology and Oceanography. 12:600-605.

Campbell, L. L. and E. Pace. 1968. Physiology of growth at high temperatures. J. Appl. Bacteriol. 31:24-35.

Choi, Y. J., H. Kim, B. H. Lee and J. S. Lee. 1995. Purification and characterization of β-galactosidase from alkalophilic and thermophilic *Bacillus sp.*TA-11. Biotechnol. Appl. Biochem. 22: 191-201.

Chung, A. P., F. A. Rainey, M. Valente, M. F. Nobre and M. S. da Costa. 2000. *Thermus sp.* Nov. and *Thermus antranikianii sp.* Nov., two new species from Iceland. Int. J. Syst. Evol. Microbiol. 50:209-217.

Craven, G. R., E. Steers and C. B. Enfinsen. 1965. Purification, composition and molecular weight of the β-galactosidase of *Escherichia coli* K-12. J. Bio. Chem. 240:2468-2477.

Dabrowski, S., G. Sobiewska, J. Maciunska, J. Synowiecki and J. Kur. 2000. Cloning, expression, and purification of the His6-tagged thermostable β-galactosidase from *Pyrococcus woesei* in *Escherichia coli* and some properties of the isolated enzyme. Protein Expression and Purification. 19:107-112.

Degryse, E., N. Glansdorff and A. Pierard. 1978. A comparative analysis of extreme thermophilic bacteria belonging to the genus *Thermus*. Arch. Microbiol. 117:189-196.

Dumortier, V. C. Brassart and S. Bouquelet. 1994. Purification and properties of a β-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. Biotechnol. Appl. Biochem. 19:341-354.

Felsenstein, J. 1993. PHYLIP: Phylogenetic Inference Package, version 3.5. Department of Genetics, University of Washington, Seattle, WA, USA.

Hughes, D. B. and D. G. Hooner. 1991. Bifidobacteria: their potential for use in American dairy products. Food Technol. 45: 64-83.

Jacobson, R. H., X-J. Zhang, R. F. DuBose and B. W. Matthews. 1994. Three dimensional structure of β-galactosidase from E. coli. Nature. 369:761-766.

Kern, F. Jr. and J. E. Struthers Jr. 1966. Intestinal lactose deficiency and lactose intolerance in adults. J. Am. Med. Assoc. 195:143-147.

Kulikova, A. K., A. S. Tikhomirova and R. V. Feniksova. 1972. Purification and properties of *Saccharomyces fragilis* β-

galactosidase. Biokhimiya. 37(2):405-419.

Kuykendall, L. D., M. A. Roy, J. J. O`Neil and T. E. Devine. 1988. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bacteriol. 38:358-361.

Kwak, H. S., J. B. Lee and J. Ahn. 2002. Treatment of microencapsulated β-galactosidase with zone: Effect on enzyme and Microoganism. Asian-Aust. J. Anim. Sci. 15:596-601.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Maidak, B. L., G. J. Olsen, N. Larson, R. Overbeck, M. J. MaCoughey and C. R. Woese. 1996. The Ribosomal Database Project (RDP). Nucleic acids Res. 24:82-85.

Manaia, C. M. and M. S. da Costa. 1991. Characterization of halotolerant *Thermus* isolate from shallow marine hot springs on S. Miguel, Azore. J. Gen. Microbial. 137:2643-2648.

Mcfeters, G. A., W. E. Sandine, R. R. Becker and P. R. Elliker. 1969. Some factors affecting association-dissiciation of β-galactosidase from *Streptococcus lactis* 7962. Canadian J. Microbio. 15:105-110.

Moore, J. B., P. Markiewicz and J. H. Miller. 1994. Identification and sequencing of the *Thermotoga maritima lacZ* gene, part of a divergently transcribed operon. Gene. 147:101-106.

Nobre, M. F., L. Carreto, R. Wait, S. Tenreiro, O. Fernandes, R. J. Sharp and M. S. da Costa. 1996. Fatty acid composition of the species of the genera *Thermus* and *Meiothermus*. Syst. Appl. Microbiol. 19: 303-311.

Ohtsu, N., H. Motoshima, K. Goto, F. Tsukasaki and H. Matsuzawa. 1998. Thermostable β-galactosidase from an extreme thermophilic, *Thermus sp.* A4: Enzyme purification and characterization, and gene cloning and sequencing. Biosci. Biotechnol. Biochem. 6298:1539-1545.

Ohtsuka, K., V. Benno, K. Endo, O. Ozawa, H. Veda, T. Uchida and T. Mitsuoka. 1989. Effects of 4-galactosyllactose intake on human fecal microflora. Bifidus. 2:143-149.

Onishi, N. and T. Tanaka. 1995. Purification and properties of a novle thermostable galacto-oligosaccharide-producing β-galactosidase from *Sterigmatomyces elviae* (BS8119). Appl. Environ. Microbiol. 61:4022-4025.

Rainey, F. A., N. Ward-Rainey, R. M. Kroppenstedt and E. Stackebrandt. 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. nov. Int. J. Syst. Bacteriol. 46:1088-1092.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstruction phylogenetic trees. Mol. Biol. Evol. 4:406-42

Santos, M. A., R. A. D. Williams and M. S. da Costa. 1989. Numerical taxonomy of *Thermus* isolated from hot springs in Portugal. Syst. Appl. Microbial. 12:310-315.

Thomas, K. N. and R. K. William. 1986. Industrial application of thermostable enzyme pp. 197-215. In: (Ed. T. D. Brock) Thermophiles, John Wiley & Sons. Inc. New York.

Vian, A., A. V. Carrascosa, J. L. Garcia and E. Corted. 1998. Structure of β-galactosidase gene from *Thermus sp.* strain T2: Expression in *Eschrichia coli* and purification in a single step of an active fusion protein. Appl. Environ, Microbiol. 64(6):2187-2191.

Wickson, V. M. and R. E. Huber. 1970. The non simultaneous dissociation and loss of activity of β-galactosidase in urea. Biochem. Biophys. Acta. 207:150-155.

Williams, R. A. D., K. E. Smith, S. G. Welch, J. Micallefand and R. J. Sharp. 1995. DNA relatedness of *Thermus* strains, description of *Thermus brokianus sp.* Nov., and proposal to reestablish *Thermus thermophilus* (Oshima and Imahori). Int. J. Syst. Bacteriol. 45:495-499.