β-Galactosidase-Producing Thermophilic Bacterium, *Thermus thermophilus* KNOUC114: Identification of the Bacterium, Gene and Properties of β-Galactosidase

Jong Kun Ahn (Corresponding author)
Department of Agricultural Sciences
Korea National Open University, Seoul 110-791, Republic Korea
Tel: 82-2-3668-4630 E-mail: ajk@knou.ac.kr

Eun Sook Nam
Department of Chemical and Biomolecular Engineering
Sogang University, Seoul 121-742, Republic of Korea
Tel: 82-2-3274-4822 E-mail: microbe-nam@hanmail.net

Hyun Bong Choi
Department of Agricultural Sciences
Korea National Open University, Seoul 110-791, Republic Korea
Tel: 82-2-3668-4630 E-mail: chb3102@naver.com

Jeong Hyun Lim
Department of Agricultural Sciences
Korea National Open University, Seoul 110-791, Republic Korea
Tel: 82-2-3668-4630 E-mail: junghyuni@yahoo.co.kr

Hyun Jin Park
Department of Agricultural Sciences
Korea National Open University, Seoul 110-791, Republic Korea
Tel: 82-2-3668-4630 E-mail: wasrenai@knou.ac.kr

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Abstract

A β-galactosidase-producing bacterium, strain KNOUC114 isolated from a hot spring was identified, and its gene of β-galactosidase and properties of the enzyme were studied. The strain KNOUC114 showed typical properties of genus *Thermus* with phenotypic characteristics of rod-shape (0.2x3.5μm), Gram negative, non-motile, endospore not observed, forming yellow-pigmented colonies, growing aerobically and optimally at 68-70℃. The strain could grow at the temperature above 80℃, which is a typical characteristic of *Thermus thermophilus*. The main cellular fatty acids of KNOUC114 were isobranched-$C_{17:00}$ and $C_{15:00}$ fatty acids that are the predomonant acyl chains of the strains of genus *Thermus*. In pylogenetic analysis based on 16S rDNA sequence, the strain KNOUC114 was finally identified as *Thermus thermophilus* species, and named as *Thermus thermophilus* KNOUC114. The β-galactosidase gene of KNOUC114 (*KNOUC114β-gal*) was cloned and expressed in *Eschericia coli*. *KNOUC114β-gal* was composed of 1938bp encoding 645 a.a with deduced MW. of

72,784 dalton. The purified recombinant β-galactosidase of KNOUC114 (KNOUC114β-gal) reacted optimally at pH 5.7 and 85℃, possessed good activity at the pH of raw milk and the temperature of HTST for raw milk, and was stable at the temperature of HTST and at the pH of raw milk, meaning that KNOUC114β-gal is suitable to be used for hydrolyzation of lactose in raw milk during HTST pasteurization of raw milk.

Keywords: β-galactosidase-producing bacterium, *Thermus thermophilus*, Gene of β-galactosidase

1. Introduction

Thermophilic bacteria can be used in industrial and biotechnological process owing to the production of thermozymes which are active and stable at high temperature. Since the discovery of *Thermus aquaticus* in 1969 (Brock and Freeze 1969), thermophiles prosper at 60-80℃ and hyperthermophiles optimally growing at temperature higher than 80℃ were found in geothermal areas(Degryse et al. 1978) and deep sea hydrothermal vents (Stetter 1982). *Thermus* (Otsu et al. 1998), and *Geobacillus* (Soliman 2008) were widely investigated for the thermostable and thermophilic enzymes useful in industry, and archaea such as *Pyrococcus* (Wanarska et al. 2005) and *Sulfolobus* (Pisani et al. 1990) were also studied. Thermozymes are industrially favorable due to long half life because of the high stability of its tertiary structure, high reaction rate, high solubility of substrate and hygienic safety(Vielle et al. 1996: Li et al. 2005), and thermozymes expressed in *Esherichia coli* can be purified easily by heat precipitation of host proteins (Vian et al. 1998). Thermophilic and thermostable β-galactosidase (EC. 3.2.1.23) is the one applicable in food industry. β-Galactosidase catalyzes the hydrolysis of β-D-1,4-galactopyranosides including lactose in milk. The enzyme functioning well at pasteurization temperature of raw milk has greater possibility to ensure the hygienically outstanding quality of lactose hydrolyzed milk useful for lactose intolerant persons prevalent in the world, especially in East Asia and Africa. An extremely thermostable β-galactosidase produced by a hyperthermophilic archaea of *Pyrococcus woesei* active up to 110℃ and optimally at 93℃ was reported (Dabrowski et al. 1998). Enzymes from hyperthermophiles have a strong advantage of excellent thermostability, however the utility of hyperthermophilic enzymes can be limited in industrial application due to their poor activity at temperature compatible with the stability of substrates, end products and industrial process. Therefore β-galactosidase highly resistant to heat but displaying lower optimum temperature is more useful for hydrolysis of lactose in milk than hyperthermophilic β-galactosidase. Some thermophilic β-galactosidases have been isolated from thermophilic bacteria of *Saccharopolyspora rectivirgula* (Nakao et al. 1994), *Thermoanaerobater ethanolicus*(Foknia and Velikodvorskaya (1997), *Thermus* sp.(Koyama et al. 1990; Ohtsu et al. 1998; Kang et al. 2005), *Thermotoga maritima*(Kim et al. 2004), *Alicyclobacillus acidocaldarius* (Gul-Guven et al. 2007) and *Geobacillus stearothermophilus* (Soliman 2008), however they have not been used in industry yet, and all of commercially available β-galactosidases are optimally active at temperature lower than pasteurization temperature. More studies are required to find β-galactosidases of different properties fit for diverse milk products such as raw milk, sweet whey, acid whey and butter milk.

As an effort to find a suitable β-galactosidase fit for operation at pasteurization temperature in dairy industry, we isolated a thermophilic bacterium, strain KNOUC114 growing well at 70℃ and showing excellent β-galactosidase activity (Nam et al. 2004). In this study the strain was identified, and gene of β-galactosidase and properties of the enzyme were investigated. And the results are being reported.

2. Materials and Methods

2.1 Cultivation of bacteria

Strain KNOUC114 isolated from a hot spring in the area of Golden springs in New Zealand (Nam et al. 2004) was cultivated in the liquid medium of ATCC1598 (Alfredsson et al. 1985) at 70℃ aerobically by shaking at 200rpm, or at the solid medium of ATCC1598(Alfredsson et al. 1985) for identification or for preparation of chromosomal DNA. *Escherichia coli* JM109 and *Escherichia coli* JM109 (DE3) were cultured in Luria-Bertani (LB) medium at 37℃ aerobically by shaking at 200rpm for cloning and expression of gene respectively.

2.2 Identification of strain KNOUC114

2.2.1 Morphological and physiological properties

The morphology of strain KNOUC114 was observed under scanning electron microscope (SEM). Physiological properties presented in Table 1 were examined following the methods of Santos et al. (1989) and Manaia and da Costa (1991).

2.2.2 Composition of cellular fatty acids

Wet cell mass of KNOUC114 was harvested on solid medium, and cellular fatty acid methyl esters were obtained by saponification, methylation and extraction by the methods presented by Kuykendall et al. (1988). The fatty acids were analyzed by model 6890 GC system (Hewlett Packard, USA), and identified by the standard MIS Library generation Software (Microbial. ID INC. USA).

2.2.3 16S rDNA sequence and phylogenetic analysis

Chromosomal DNA from KNOUC114 was isolated, PCR amplification of 16S rDNA using chromosomal DNA as template was performed, and 16S rDNA sequencing was carried out by the method described by Rainey et al. (1996). Universal primers of fD1 (5'agagtttgatcctggctcag3') and rD1 (5'acggctaccttgttaccactt3') were used to amplify the 16S rDNA in PCR. The sequence of 16S rDNA was aligned against the previously determined sequences of genus *Thermus* available from the Ribosomal Data of GenBank. The evolutionary tree for the dataset was constructed using the neighbor-joining method (Saitou and Nei 1987).

2.3 Cloning, sequencing β-galactosidase gene

β-Galactosidase gene of strain KNOUC114 (*KNOUC114β-gal*) was amplified by PCR using chromosomal DNA of strain KNOUC114 as template and redundancy primers. Redundant primers were prepared on the basis of β-galactosidase gene structure of *Thermus* sp. A4 (Ohtsu et al. 1998) and *Thermus* sp. T2 (Vian et al. 1998). The nucleotide sequence of forward primer was 5'atgYtSggcgtttgYtaYtacc3', and the sequence of reverse primer was 5'tcatgYctcctcccaSacg3'. The PCR product, purified by agarose electrophoresis and Geneclean Kit method (Bio101, USA), was ligated into pGEM-T Easy plasmid (Promega, USA) and transformed into *Escherichia coli* JM109. The transformed *E. coli* was cultured and screened on LB plate containing ampicilline at the concentration of 100μg/ml. The DNA sequence was determined using Big Dye Automatic sequencer AB1377 band PE9600 Thermocycler (Perkin Elmer, USA). Amino acid sequence analysis based on DNA sequence, and homology search were performed using the World Wide Web server from BLAST search of NCBI.

2.4 Recombinant expression of β-galactosidase gene and purification of the enzyme

Vector for expression, constructed by inserting the cloned gene into pET-5b (Promega, USA) regulated by *Lac*UV5 promoter, was transformed to *E. coli* JM109 (DE3), and cultured on LB solid medium containing ampicillin (100μg/ml) at 37℃. After heating colonies grown on the LB solid medium at 70℃ for 3 hr., Z-buffer (Ausubel et al. 1998) containing 1.4mg of X-gal/ml was poured over those colonies, and the colonies were incubated for 3hr. more at 70℃. The plasmid in the dead blue colony was transformed into *Escherichia coli* JM109 (DE3) and used to confirm the expression of β-galactosidase gene by SDS-polyacrylamide gel electrophoresis (PAGE) and X-gal hydrolysis in zymogram assay. *E. coli* JM109 (DE3) expressing *KNOUCβ-gal* was cultivated in LB broth containing ampicillin (100μg/ml) at 37℃ aerobically by shaking on 200rpm for 18 hr. Cells were harvested by centrifugation at 8000g for 10min. at 4℃, washed 2 times with Na-phosphate buffer (0.02M, pH 7.0), suspended in the same buffer, and sonicated. Cell debris was eliminated by centrifugation at 12000g for 20min at 4℃. The cell free extracts was heated in a water bath of 70℃ for 40min to denature host proteins, and the denatured proteins were removed by centrifugation at 14000g for 20 min. at 4℃. The clarified supernatant was eluted through Superdex 200pg column (GE Healthcare, Sweden) for gel filtration by Na-phosphate buffer (20mM, pH 7.0, NaCl 0.15M). The fractions having activity of ONPG hydrolysis were pooled and loaded onto Resource Q column (GE Healthcare, Sweden) for ion exchange chromatography, and eluted with a linear gradient of 0-0.2M NaCl in Tris-HCl buffer (20mM, pH 8.2). The active pool from Resource Q column was added to HiTrap Phenyl HP column (GE Healthcare, Sweden) for hydrophobic interaction chromatography, and eluted using a linear gredient of 0.25-0 M ammonium sulfate in Na-phosphate buffer (50mM, pH 7.0). The active fractions from HiTrap Phenyl HP column were dialyzed to Na-Phosphate buffer (50mM, pH 6.8), and used for further study.

2.5 Assay of enzyme activity and properties

Activity of β-galactosidase was assayed by measuring hydrolysis of o-nitrophenyl-β-D- galactopyranoside (ONPG) (Miller 1972) in Na-phosphate buffer (0.05M, pH 6.8) at 75℃, unless described otherwise. Enzyme activity was tested in triplicate. One unit of enzyme activity was defined as the activity hydrolyzing 1μmole of ONPG per min. under the presented conditions. Effect of pH and temperature on β-galactosidase activity and thermostability were investigated. Effect of pH on enzyme activity was evaluated at the pH ranging from pH 4.3 to 7.6. Na-citrate buffer (0.05M) was used for pH 4.3 to 6.3, and Na-phosphate buffer (0.05M) was used for pH 6.0 to 7.6. To examine the effect of temperature on enzyme activity, activities were tested in Na-phosphate buffer (0.05M, pH 6.8) at the temperatures from 63℃ to 95℃. Thermostability of enzyme was determined by

measuring the residual activity during heating the enzyme (15μg/ml) in Na-phosphate buffer (0.05M, pH 6.8) for 120 min. at the temperatures from 75℃ and 95℃. Effect of pH on thermostability of β-galactosidase was determined at pH from 4.7 to 7.7 by measuring the residual activity after heating the enzyme (15μg/ml) at 85℃ or 90℃ for 30 min. in the buffer of each pH. Na-citrate buffer (0.05M) was used for pH 4.7 to 6.3, and Na-phosphate buffer (0.05M) was used for pH 6.5 to 7.7.

2.6 Protein and zymogram assay

Protein concentration was decided by Bio-Rad DC protein assay (Bio-Rad, USA) with bovine serum albumin as standard. Purity of purified enzyme was confirmed by SDS-PAGE of 7.5% acrylamide and Experion automated electrophoresis system of Experion pro260 analysis kit(Bio-Rad, USA). For zymogram assay, native-PAGE gel of 7.5% acrylamide was soaked in Z-buffer (Ausbel et al. 1998) containing X-gal (1.4mg/ml) at 70℃ for 15min. SDS-PAGE and native-PAGE were performed by the method of Laemmli (1970).

3. Results and Discussion

3.1 Identification of bacterium

Morphological and physiological properties of KNOUC114 are presented in Table 1. The strain KNOUC114 was rod-shaped (0.2×2-3.5μm), Gram negative, non-motile, endospore not observed, formed yellow-pigmented colonies, grew aerobically and optimally at 68-70℃. All those above properties of KNOUC114 are fit to the ones of genus *Thermus* (da Costa et al. 2001). Almost all species of *Thermus* formed yellow-pigmented colonies, and several carotenoids of thermozeaxanthins and thermobiszeaxanthins were identified in the yellow pigments of *Thermus* strain HB-27 (Yokoyama et al. 1995, 1996). The majority of *Thermus* strains have a maximum growth temperature slightly below 80℃ (Mania and da Costa 1991), however Degryse et al. (1978) suggested that strains of *Thermus thermophilus* could be distinguished from all other species of genus *Thermus* by their ability to grow at above 80℃, and *Thermus thermophilus* HB8 was capable of growth at above 80℃ (Manania et al. 1994). Strain KNOUC114 could grow at 85℃. Generally most of the strains of *Thermus* isolated in marine hot spring grew in the medium containing 3-4% NaCl, whereas the strains isolated from inland hydrothermal areas did not grow at the NaCl concentration above 1% (Manaia and da Costa 1991 ; Manaia et al. 1994). However *Thermus thermophilus* HB8 from inland hot spring in Japan was reported to grow in the medium of 3% NaCl (Manania et al. 1994), and strain KNOUC114 isolated from inland hot spring in New Zealand could also grow at salinity of 3%. Utilization of carbohydrates by strains of *Thermus* is diverse, however most strains were not able to assimilate pentose with the exception of some strains of *Thermus thermophilus* (Williams and da Costa 1992). As in Table 1 strain KNOUC KNOUC114 utilized pentoses of arabinose and xylose. Those above morphological and physiological characteristics are suggesting that the strain KNOUC114 would be presumably identified as *Thermus thermophilus*.

To verify the close relation of KNOUC114 with genus *Thermus* and species *Thermus thermophilus*, composition of cellular fatty acids were analyzed. As in Table 2, the main fatty acids of KNOUC114 were isobranched-$C_{17:00}$ and $C_{15:00}$ fatty acids reaching about 59.32% of total fatty acid, and anteiso-branched $C_{15:00}$ and $C_{17:00}$ fatty acids were found to be present in considerably high content of 20.42%. Nobre et al. (1996a) reported that iso and anteiso branched-$C_{15:00}$ and $C_{17:00}$ fatty acids are the predominant acyl chains of the strains of genus *Thermus*. And the major fatty acids of *Thermus thermophilus* HB8 (Chung et al. 2000) were isobranched-$C_{15:00}$ and $C_{17:00}$ fatty acids, same with the fatty acid composition of strain KNOUC114, implying that strain KNOUC114 is chemotaxanomically close to *Thermus thermophilus*.

To search the phylogenetic position, 16S rDNA sequence was determined and it was found to be composed of 1379bp as in Figure 1. The structure of 16S rDNA was compared with sequences of the strains of genus *Thermus* in Ribosomal Database of NCBI, and the phylogenetic tree was constructed using the neighbor-joining method (Saito and Nei 1987) as in Figure 2. The phylogenetic tree is showing that the 16S rDNA of KNOUC114 is same with that of *Thermus thermophilus* AT-62 (GenBank Accession No. L09660) and has the similarity of 99.9% with that of *Thermus thermophilus* HB8 (GenBank Accession No. AB603517). The strain KNOUC114 was different in phenotypic characteristics on utilizing lactose, galactose, mellibiose, and fructose with *Thermus thermophilus* AT-62 (Manaia et al. 1994), and distinct from *Thermus thermophilus* HB8 (Manania and da Costa 1991; Manania et al. 1994) in nitrate reduction and utilization of arabinose, galactose, lactose, raffinose, glycerol, malate and arginine. Therefore strain KNOUC114 is a strain independant from *Thermus thermophilus* AT-62 and *Thermus thermophilus* HB8, though its 16S rDNA structure is same or highly similar with those of *Thermus thermophilus* AT-62 and *Thermus thermophilus* HB8.

Accordingly, on the basis of morphological, physiological and 16S rDNA structure, strain KNOUC114 was identified as *Thermus thermophilus* and named as *Thermus thermophilus* KNOUC114.

3.2 Gene of β-galactosidase

The β-galactosidase gene of KNOUC114 (*KNOUC114β-gal*; GenBank Accession No. DQ887182) was composed of 1938bp encoding 645 a.a, with deduced MW. of 72,784 dalton. The β-galactosidase of KNOUC114 (KNOUC114β-gal) conserves two glutamic acid residues at 141st and 312nd amino acid in its polypeptide that were known to participate in the catalytic reaction of the β-galactosidase from *Bacillus stearothermophilus* (Henrissat et al. 1995), suggesting that KNOUC114β-gal can be classified to the family 42 of glycosyl hydrolases (Henrissat and Bairoch 1993; Ohtsu et al. 1998; Vian et al. 1998). Amino acid sequence of KNOUC114β-gal showed 99% and 77% identity with the β-galactosidases of *Thermus* sp. A4 (GenBank Accession No. D85027; Ohtsu et al. 1998) and *Thermus* sp. T2 (Genbank accession No. Z93773; Vian et al. 1998) respectively whose genes were refered for preparation of the redundant primers used to amplify *KNOUC114β-gal* by PCR. KNOUC114β-gal has homology of 93% and 78% in amino acid sequence with those of *Thermus* sp. IB-21 (Genbank Accession No. AY130259; Kang et al. 2005) and *Thermus brockianus* strain IT1360(GenBank accession No. AF135398) respectively. All the β-galactosidase genes of *Thermus* strains presented above are composed of 1938bp encoding 645 a.a. However *Thermus oshimai* produced a β-galactosidase composed of 632 amino acids (GenBank Accession No. GQ404016). The β-galactosidases of *Meiothermus silvanus* DSM9946(Genbank accession No. CP002042) and *Meiothermus ruber* DSM1279(GenBank accession No. CP001743), that form a sister line of descent with the species of genus *Thermus* in pylogenetic analysis (Nobre et al. 1996b) with which strain KNOUC114 shares about 86% similarity in 16S rDNA sequence, have identities of 64% and 66% in amino acid sequence respectively with KNOUC114β-gal, and they are composed of 645 a.a and 646 a.a respectively. The β-galactosidase of *Escherichia coli*, a mesophilic bacteria, produced a β-galactosidase of 1,023 amino acids (GenBank Accession No. V00296) that is larger than KNOUC114β-gal. *Pyrococcus woesei*, a hyperthermophilic archaea, produced a β-galactosidase of 510 amino acids (GenBank Accession No. AF043283) that is smaller than KNOUC114β-gal. However a hyperthermophilic bacteria, *Thermotoga maritime* produced a β-galactosidase of large molecule composed of 1100 amino acids (Kim et al. 2004).

3.3 Expression of gene and purification of recombinant β-galactosidase

KNOUC114β-gal ligated in pET-5b was expressed intracellularly in *Eschericia coli* JM109 (DE3). In SDS-PAGE, cell free extracts of *Eschericia coli* JM109 (DE3) harboring *KNOUC114β-gal* in pET-5b showed a new band of about 73kDa corresponding to the deduced Mw. of KNOUC114β-gal (Figure 3a). And in zysmogram assay, it was found that *KNOUC114β-gal* was expressed as one active form(Figure 3b). *Escherichia coli*(Wickson and Huber 1970) and *Kluyveromyces fragilis*(Kulikova et al. 1972) produced their β-galactosidase as a form of tetramer, and *Streptococcus lactis* 7962 produced two active forms of monomer and dimer(Mcfeters et al. 1969).

The recombinant KNOUC114β-gal produced in *Eschericia coli* JM109(DE3) was purified by heat precipitation of host protein, gel filtration, ion exchange chromatography and hydrophobic interaction chromatography. After the final step of hydrophobic interaction chromatography by HiTrap Phenyl HP column, the enzyme could be purified to 87.3 fold, and 20.9% of total activity in cell free extracts was recovered (data not shown). In Experion automated electrophoresis the purity of purified enzyme and Mw. of its subunit were confirmed to be 97.9%, and 70.43 kDa respectively as presented in Figure 4(b).

3.4 Characteristics of recombinant β-galactosidase

Effects of pH and temperature on the activity of KNOUC114β-gal are presented in Figure 5. The enzyme showed good activity at pH 5.0 to 6.5. and highest activity at pH 5.7. At the pH of raw milk, it retained about 50% of optimum activity. At 85℃ the enzyme showed maximum activity. However, Arrhenius plot, as in Figure 5(c), is showing that the logarithmic value of enzyme activity increased linearly from 63℃ only to 80℃ meaning that there was denaturation on the enzyme at 85℃. Arrhenius activation energy of KNOUC114β-gal calculated by linear regression analysis from 63 to 80℃ was 40.8kJ/mol that is similar with those of thermophilic enzymes optimally active at temperature higher than 85℃ (Dion et al. 1999; Kengen et al. 1993). Around the temperatures of LTLT and HTST, KNOUC114β-gal displayed fairly good activity. β-Galactosidase of *Thermus* sp. A4 was optimally active at pH 6.5 and its activity increased as the temperature was raised to 90℃ (Ohtsu et al. 1998), that are much different with those of KNOUC114β-gal though their amino acid sequences are 99% homologous each other. Optimum pH and temperature for β-glacosidases of *Thermus* sp. T2 (Vian et al. 1998) and *Thermus* sp. IB-21 (Kang et al. 2005) were pH 5.0 and 90℃, and pH 5.0-6.0 and 90℃ respectively that are different a little with those of KNOUC114β-gal. Thermostability of KNOUC114β-gal was investigated at the temperature from 75 to 95℃ in Na-phosphate buffer (0.05M, pH 6.8) as in Figure 6(a). KNOUC114β-gal was

stable at 75℃. At 80℃ the enzyme retained its full activity in 30min. and 90% of initial activity in 2hr. At 90℃ the half life of KNOUC114β-gal was 2 hr. KNOUC114β-gal was more stable than the β-galactosidase of *Thermus* sp. T2 that lost 50% of activity in 1 hr. at 70℃ (Vian et al.,1998). The half life of β-galactosidase produced by *Thermus* sp. A4 was 1 hr. at 90℃ (Ohtus et al. 1998). Effect of pH on the thermostability of KNOUC114β-gal was examined by heating at 85℃ or 90℃ for 30min. in buffers of pH 4.7 to 7.7, and the results are presented in Figure 6(b). KNOUC114β-gal showed good stability at neutral pH of 6.3 to 7.0, and at the pH lower than 6.3 and higher than 7.0 the stability decreased.

4. Conclusion

Based on the morphological and physiological properties, cellular fatty acid composition and phylogenetic analysis, the β-galactosidase producing strain KNOUC114 isolated from a hot spring in the area of Golden springs in New Zealand was identified as a strain of *Thermus thermophilus*. By the results on the stability and activity profile of KNOUC114β-gal at different pH and temperatures, this enzyme is suitable for hydrolysis of lactose in raw milk and mildly acidic whey during pasteurization.

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Table 1. Phenotypic characteristics of strain KNOUC114

Characteristics	**KNOUC114**	**Characteristics**	**KNOUC114**
Cell shape	Rod	Mannitol	+
Cell size	0.2×2-3.5μm	Inositol	+
Motility	-	Sorbitol	+
Spore	-	Rhamnose	+
Gram reaction	-	Sucrose	+
Color of colony	Yellow	Mellibiose	+
Optimum temperatures	68~70℃	Amygdalin	+
Optimum pH	7.0-7.2	Arabinose	+
Growth at/in		Galactose	+
80℃	+	Saccharose	-
85℃	+	Trehalose	-
1% NaCl	+	Lactose	+
3% NaCl	+	Fructose	-
5% NaCl	-	Xylose	+
Catalase	+	Mannose	+
Oxidase	+	Ducitol	+
Nitrate reduction	+	Adonitol	+
Voges-Proskauer reaction	+	Raffinose	+
Indol prodcution	-	Glycerol	+
Hydogen sulfide formation	+	Erythrol	+
Citrate utilization	-	Malate	+
Hydrolysis of		Citrate	-
Starch	+	Propionate	+
Casein	+	Pyruvate	+
Urease	+	Formate	+
Gelatin liquefaction	+	Glutamate	+
Hydrolysis of ONPG	+	Arginine	+
Oxidation-Fermentation	Oxidation	Lysine	+
Growth on nutrient plate	-	Ornithin	+
Utilization of Glucose	+	Tryptophane	+

Table 2. Cellular fatty acid composition of strain KNOUC114

Fatty acid	Contents (%)
13:0 iso	0.53
14:0 iso	1.12
14:0	0.61
15:0 iso	26.98
15:0 anteiso	11.75
16:0 iso	8.55
16:0	6.74
17:0 iso	32.34
17:0 anteiso	8.67
18:0 iso	1.28
18:1 w9c	0.41
18:1	0.61
19:0 iso	0.41
Total	100

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   1  GGTGAACGCT GGCGGCGTGC CTAAGACATG CAAGTCGTGC GGGCCGCGGG GTTTTACTCC GTGGTCAGCG GCGGACGGGT GAGTAACGCG TGGGTGACCT
 101  ACCCGGAAGA GGGGGACAAC CCGGGGAAAC TCGGGCTAAT CCCCCATGTG GACCCGCCCC TTGGGGTGTG CCAAAGGGCT TTGCCCGCTT CCGGATGGGC
 201  CCGCGTCCCA TCAGCTAGTT GGTGGGGTAA TGGCCCACCA AGGCGACGAC GGGTAGCCGG TCTGAGAGGA TGGCCGGCCA CAGGGGCACT GAGACACGGG
 301  CCCCACTCCT ACGGGAGGCA GCAGTTAGGA ATCTTCCGCA ATGGGCGCAA GCCTGACGGA GCGACGCCGC TTGGAGGAAG AAGCCCTTCG GGGTGTAAAC
 401  TCCTGAACCC GGGACGAAAC CCCCGAGGAG GGGACTGACG GTACCGGGGT AATAGCGCCG GCCAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGCGC
 501  GAGCGTTACC CGGATTCACT GGGCGTAAAG GGCGTGTAGC TCAGGCTAGA CGGTGGGAGA GGGTGGTGGA ATTCCCGGAG TAGCGGTGAA ATGCGCAGAT
 601  ACCGGGAGGA ACGCCGATGG CGAAGGCAGC CACCTGGTCC ACCCGTGACG CTGAGGCGCG AAAGCGTGGG GAGCAAACCG GATTAGATAC CCGGGTAGTC
 701  CACGCCCTAA ACGATGCGCG CTAGGTCTCT GGGTCTCCTG GGGGCCGAAG CTAACGCGTT AAGCGCGCCG CCTGGGGAGT ACGGCCGCAA GGCTGAAACT
 801  CAAAGGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGAAGAACCT TACCAGGCCT TGACATGCTA GGGAACCCGG
 901  GTGAAAGCCT GGGGTGCCCC GCGAGGGGAG CCCTAGCACA GGTGCTGCAG GCCGTCGTCA GCTCGTGCCG TGAGGTGTTG GGTTAAGTCC CGCAACGAGC
1001  GCAACCCCCG CCGTTAGTTG CCAGCGGTTC GGCCGGGCAC TCTAACGGGA CTGCCCGCGA AAGCGGGAGG AAGGAGGGGA CGACGTCTGG TCAGCATGGC
1101  CCTTACGGCC TGGGCGACAC ACGTGCTACA ATGCCCACTA CAAAGCGATG CCACCCGGCA ACGGGGAGCT AATCGCAAAA AGGTGGGCCC AGTTCGGATT
1201  GGGGTCTGCA ACCCGACCCC ATGAAGCCGG AATCGCTAGT AATCGCGGAT CAGCCATGCC GCGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC
1301  ACGCCATGGG AGCGGGCTCT ACCCGAAGTC GCCGGGAGCC TACGGGCAGG CGCCGAGGGT AGGGCCCGTG ACTGGGGCG
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Figure 1. 16S rDNA sequence of strain KNOUC114

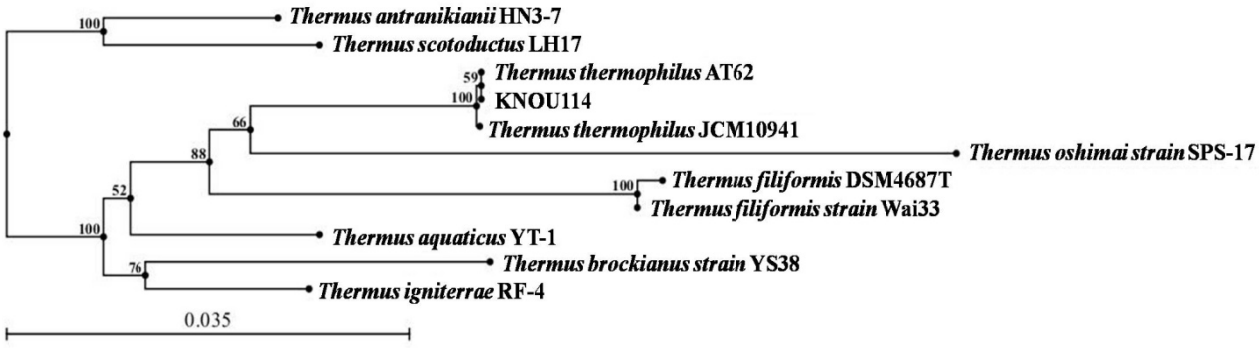

Figure 2. Phylogenetic tree based on 16S rDNA sequences showing the position of strain KNOUC114 among the strains of selected type strains of *Thermus* species. Scale bar represent 0.035 substitution per nucleotide position

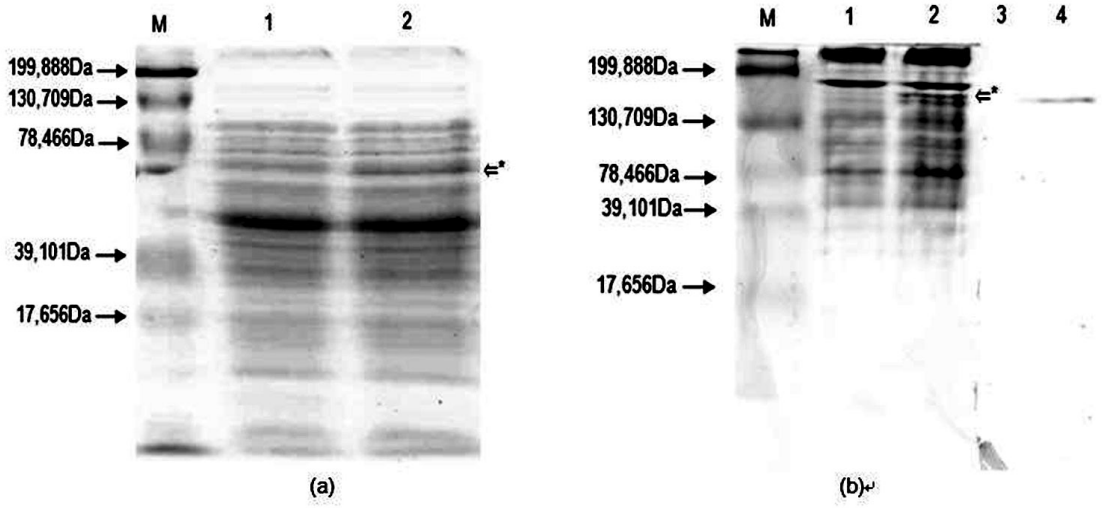

Figure 3. Expression of *KNOUC114β-gal* in *Escherichia coli* JM109(DE3)

(a)SDS polyacrylamide gel electrophoresis of 7.5% acrylamide : M-molecular weight marker, 1-Cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b, 2-Cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b harboring *KNOUC114β-gal*. (b)Native polyacrylamide gel electrophoresis of 7.5% acrylamide and zymogram assay : M-Molecular weight marker, 1-Cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b, 2-Cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b harboring *KNOUC114β-gal*., 3-Zymogram assay for hydrolysis of X-gal by cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b, 4-Zymogram assay for hydrolysis of X-gal by cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b harboring *KNOUC114β-gal.*

⇐*: KNOUC114β-gal

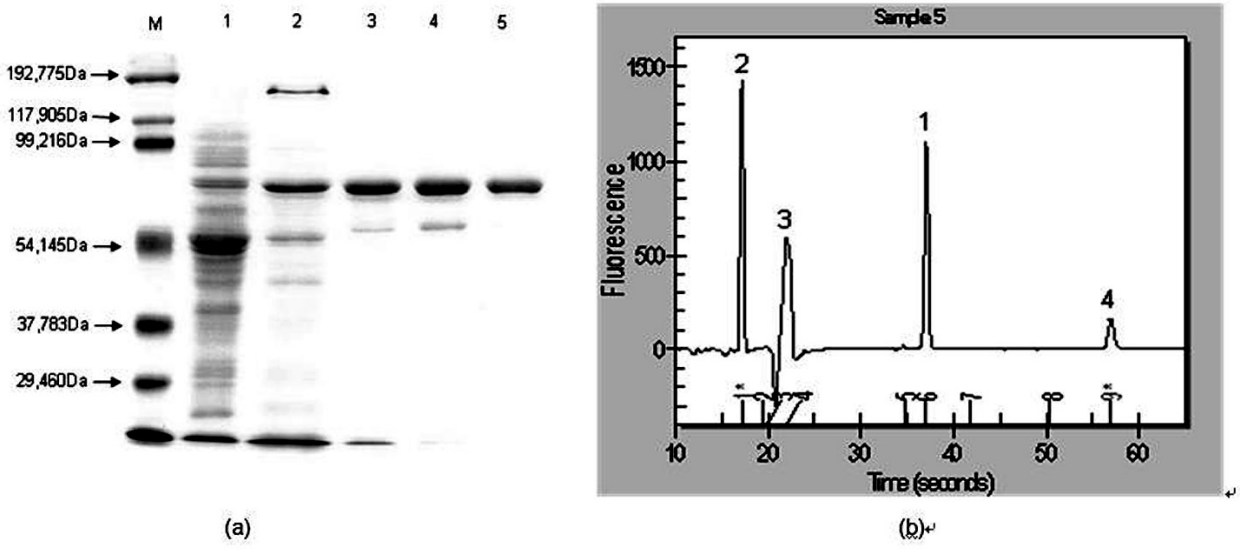

Figure 4. SDS polyacrylamide gel electrophoresis(a) and Experion electropherogram(b) of purified recombinant KNOUC 114β-galactosidase expressed in *Escherichia coli* JM109(DE3).

(a)M-Molecular weight marker, 1-Cell free extracts of *E. coli* JM109(DE3) transformed with pET-5b, 2-Soluble fraction of cell free extracts after heating at 70℃ for 40min., 3-After gel filtration on Superdex 200pg, 4-After ion exchange chromatography on Resource Q, 5-After hydrophobic interaction chromatography on HiTrap Phenyl HP. (b)1-Purified KNOUC114β-gal after hydrophobic interaction chromatography on HiTrap Phenyl HP, 2-Lower marker(1.2 kDa), 3-System marker, 4-Upper marker(260.0 kDa).

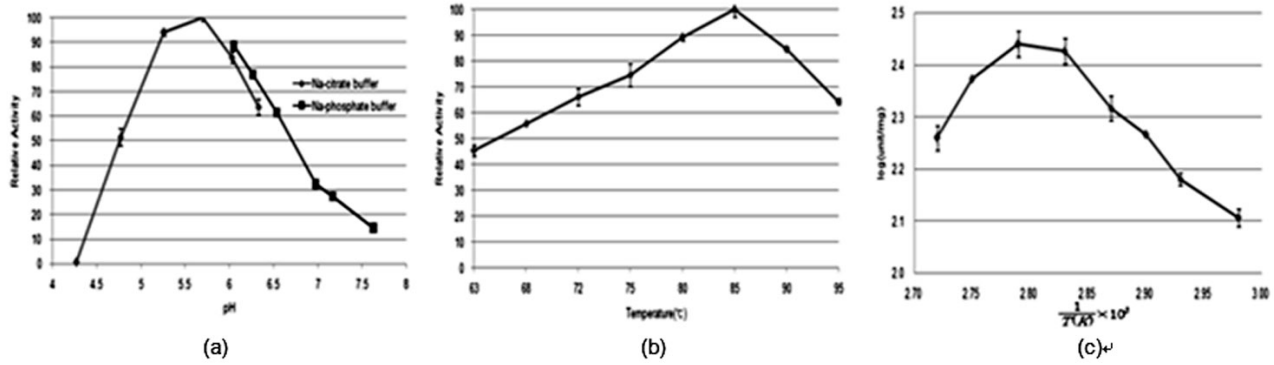

Figure 5. Effect of pH(a), temperature(b) and Arrhenius plot for the effect of temperature(c) on activity of recombinant KNOUC114 β-galactosidase expressed in *Escherichia coli* JM109(DE3)

* Values are means of triplicates ± S.D.

* Effect of pH on enzyme activity was evaluated at 75℃ in Na-citrate buffer(0.05M) of pH ranging from pH 4.3 to 6.3 and in Na-phosphate buffer(0.05M) ranging from pH 6.0 to 7.6.

* Effect of temperature on enzyme activity was tested in Na-phosphate buffer(0.05M, pH 6.8) at the temperatures from 63℃ to 95℃

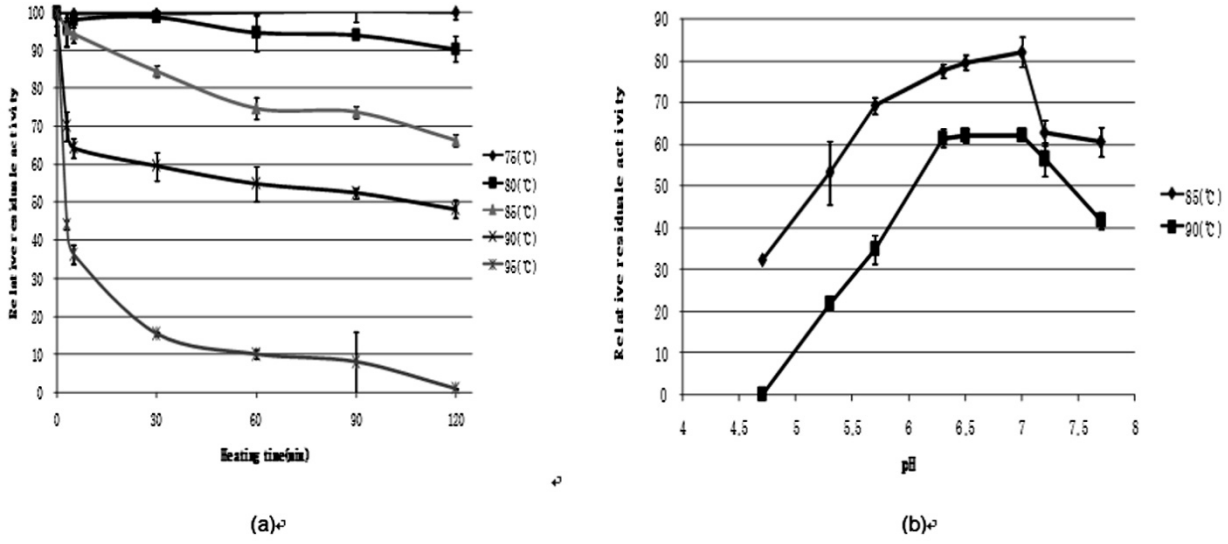

Figure 6. Thermostability(a) and effect of pH on thermostability(b) of recombinant KNOUC114 β-galactosidase expressed in *Escherichia coli* JM109(DE3)

* Values are means of triplicates ± S.D.

* Thermostability of enzyme was determined by the residual activity during heating the enzyme(15μg/ml) in Na-phosphate buffer(0.05M, pH 6.8) for 120 min. at the temperatures from 75℃ and 95℃

* Stability of β-galactosidase at pH from 4.7 to 7.7 was determined by measuring the residual activity after heating the enzyme(15μg/ml) at 85℃ and 90℃ for 30 min. in the buffer of each pH. Na-citrate buffer(0.05M) was usde for pH 4.7 to 6.3, and Na-phosphate buffer(0.05M) was used for pH 6.5 to 7.7.