A newly isolated strain, 38C-2-1, produced alkaline and thermostolerant α-amylases and was identified as *Bacillus halodurans*. The enzymes were purified to homogeneity and named α-amylase I and II. These showed molecular masses of 105 and 75 kDa respectively and showed maximal activities at 50–60°C and pH 10–11, and 42 and 38% relative activities at 30°C. These results indicate that the enzymes are thermostolerant. The enzyme activity was not inhibited by a surfactant or a bleaching reagent used in detergents. A gene encoding α-amylase I was cloned and named *amyI*. Production of AmyI with a signal peptide repressed the growth of an *Escherichia coli* transformant. When enzyme production was induced by the addition of isopropyl β-D(-)-thiogalactopyranoside in the late exponential growth phase, the highest enzyme yield was observed. It was 45-fold that of the parent strain 38C-2-1.

**Key words:** alkaline amylase; *Bacillus halodurans*; thermostolerance

Various stresses, for example, temperature, pH, sodium concentration, and so on, are factors that greatly influence the growth of microorganisms, and tolerance of these factors is very important for their survival in their environments. Extremophiles produce enzymes showing activities in the extreme environments where they live, and they survive in those environments. Although the enzymes show high stability and interesting properties, their application in industrial fields is limited by their specific properties and the cost of maintaining extreme conditions. Recently, microorganisms, which show tolerance of a wide range of environmental factors, have been noticed as producers of enzymes showing activities in a wide range of reaction conditions with a view to application. For example, Vogel et al. cloned a gene encoding pH- and salt-tolerant cellulase from a metagenomic library, and halotolerant *Bacillus subtilis* strain EP-133 is reported to produce two halotolerant extracellular proteases and one halotolerant intracellular protease.

Thermostolerant microorganisms are also important for their applications in industrial fields because they can be grown in a moderate temperature range (30–50°C), and cooling and heating costs for cultivation are lower than for extremophiles and psychrophiles. In addition, thermostolerant enzymes, which are produced by thermostolerant microorganisms and are expected to show high activities in a wide temperature range related to their growth temperature, are very useful in the application of enzymes in industrial fields. In particular, a thermostolerant character is attractive in the use of enzymes as additives in detergents, because washing is performed using water that is hot or cold. Since protease came into use as an additive in detergents, various enzymes, such as lipases and cellulases, have been added to detergents, and the use of enzymes in detergents has increased from year to year. Amylolytic...
enzymes are also thought to make good additives, for example, to the detergents used in dish-washing machines. Enzymes used as additives to detergents must show high activity under alkaline conditions, because washing is performed on the alkaline side in many cases. In addition, the enzymes must also be resistant to surfactants and bleaching reagents. In the previous studies, it was reported that some Bacillus strains produced alkaline, thermostable, and unique α-amylases.5–9 For example, α-amylase from Bacillus strain KSM-K38 showed high resistance to chelating reagents,7 and Bacillus halodurans strain LBK 34 produced five alkaline, thermostable α-amylases.9 The effects of bleaching reagents on α-amylase activity, however, are unknown. Hence we tried to isolate a bacterium producing thermotolerant α-amylase activity at both 30 °C and 50 °C under an alkaline condition, and examined the effects of surfactants and bleaching reagents commonly used in detergents.

In this paper, we report the isolation of a new alkaline, thermotolerant α-amylase-producing bacterium, and purification and characterization of two α-amylases, α-amylase I and II. α-Amylase I showed resistance to a surfactant and a bleaching reagent commonly used as additives to detergents. A gene encoding α-amylase I was cloned and expressed in E. coli.

Materials and Methods

Strains and culture conditions. Strain 38C-2-1 was isolated from a soil sample in Bangkok, Thailand. The basal medium used in the isolation and cultivation of strain 38C-2-1 was composed of two separately prepared solutions. Solution A contained 5 g of soluble starch (Nacalai Tesque, Kyoto, Japan), 0.3 g of yeast extract S (Nippon Seiyaku, Tokyo), 1 g of K$_2$HPO$_4$, 2 g NaCl, 4 g Na$_2$CO$_3$, and deionized water in 860 ml total volume, with the final pH of 10. Solution B contained 1 g of NH$_4$NO$_3$, 0.2 g MgSO$_4$.7H$_2$O, and 140 ml deionized water. Autoclaved solutions A and B were mixed. Strain 38C-2-1 was cultivated at 30 °C and 50 °C with shaking at 105 rpm, and amylolytic activities in the culture supernatants were measured.

Escherichia coli XL1-Blue was used in molecular biological experiments and in the production of α-amylase by a cloned gene. E. coli was cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml) at 37 °C with shaking. E. coli strains carrying α-amylase genes were cultivated at 30 °C with shaking in the gene expression experiments.

Morphological and phenotypic characterization. Morphological, physiological, and biochemical characters, such as Gram stain, flagella type, spore formation, catalase activity, oxidase activity, and the oxidation-fermentation test, were determined by classical methods.11) The 16S rRNA sequence was determined according to a previous paper.12) The DDBJ/EMBL/GenBank accession number for the determined sequence of a partial 16S rRNA gene from strain 38C-2-1 is AB274919.

Enzyme assay. Amylolytic activity was routinely assayed at 50 °C in a 0.5 ml reaction mixture consisting of 0.4 ml of 1.0% (w/v) soluble starch in 100 mm glycine–NaOH buffer (pH 10) and 0.1 ml of suitably diluted enzyme. The reducing sugar produced was measured by the Somogyi–Nelson method. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar as glucose per min under the assay conditions. Protein concentrations were measured by the method of Lowry.

Purification of enzymes. All purification steps were performed at 4 °C. The culture was harvested at the early stationary growth phase and centrifuged at 11,000 × g for 10 min to obtain the supernatant as an α-amylase source (fraction 1, 13.8-liter). Activated charcoal (138 g) was added to fraction 1, and the mixture was stirred for 30 min on ice. The mixture was filtered, and the filtrate was collected (fraction 2, 13.8-liter). Fraction 2 was divided into six portions, and each aliquot was loaded onto a DEAE-cellulose column (1.6 × 12.5 cm) previously equilibrated with 20 mm Tris–HCl buffer (pH 8.0) (buffer A). After the column was washed with buffer A, containing 0.15 M NaCl, enzymes were eluted with a linear gradient of 0.15–0.7 M NaCl in buffer A and active fractions were pooled (fraction 3, 900 ml). Fraction 3 was dialyzed against buffer A. The dialyzed sample was loaded onto a DEAE-Toyopearl 650S column (1.5 × 15 cm) equilibrated with buffer A. After the column was washed with buffer A, containing 0.15 M NaCl, one peak with amylolytic activity was eluted with a linear gradient of 0.15–0.7 M NaCl in buffer A, and the active fractions were collected (fraction 4, 60 ml). After dialysis against buffer A, fraction 4 was brought to 2.0 M NaCl and loaded onto a Phenyl-Toyopearl 650M column (1.6 × 11 cm) equilibrated with buffer A containing 2.0 M NaCl. Proteins were eluted with a linear gradient of 2.0–0 M NaCl in buffer A, and the active fractions were pooled (fraction 5, 60 ml). Polyacrylamide gel electrophoresis (PAGE) was performed to verify the purity of the enzyme. Proteins were stained with Coomassie Brilliant Blue R-250, and amylolytic activity in the gel was detected by activity staining, which was performed as follows: After electrophoresis, the gel was incubated in 100 mm glycine–NaOH buffer (pH 10) containing 1% soluble starch on ice for 1 h. The enzyme reaction was performed at 60 °C for 15 min. The gel was washed with deionized water three times, and soaked in Lugol’s iodine solution. Preparative native-PAGE was performed using a Nihon Eido NA-1800 disc preparative electrophoresis apparatus (Nihon Eido, Tokyo) according to the manufacturer’s instructions, because fraction 5 contained two proteins showing amylolytic activity. The
eluted fractions (0.5 ml/tube) were pooled, and the purity of the active fractions (fraction 6) was verified again by analytical native-PAGE.

**Determination of molecular masses.** The molecular masses of the subunits of the enzymes were determined by SDS–PAGE. An LMW peptide calibration kit for SDS–PAGE (Amersham Biosciences, Piscataway, NJ) was used for size markers. The native molecular masses of the purified α-amylases were determined by the method of Hedrick and Smith by native-PAGE. A substrate and amylose were from Wako Pure Chemical and pullan were purchased from Nacalai Tesque; dextran purity of the active fractions (fraction 6) was verified by SDS–PAGE. An LMW peptide calibration kit for SDS–PAGE was also used for size markers. A native molecular mass of 600 kDa was used for size markers.

**Substrate specificity.** Amylopectin, glycogen, and pullulan were purchased from Nacalai Tesque; dextran and amylose were from Wako Pure Chemical and Sigma-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively. A substrate and amylose were from Wako Pure Chemical and Sigmal-Aldrich (St. Louis, MD) respectively.

**Preparation of total DNA, polymerase chain reaction (PCR), and sequencing.** Total DNA from *B. halodurans* 38C-2-1 was prepared by the method of Strauss and used in the construction of gene libraries and PCR as a template. To amplify a partial α-amylase I gene, primers 1f (5′-TYTTGCGCCARTG) and 1r (5′-CCCC-TYTTYTGCCARTG) were designed on the basis of the conserved amino acid sequences of several α-amylases (accession nos. AY528737, X55452, and AB026834). The conserved amino acid sequences, FWVGE and HWQKMG, corresponded to amino acid sequences of residues 366 to 371 and residues 499 to 504 respectively of Amy34 from *B. halodurans* LBK 34.9) PCR was performed with Taq DNA polymerase (New England Biolabs, Beverly, MA) at 95°C (1 min), 45°C (2 min), and 72°C (1 min) for 35 cycles. The amplified fragment was purified with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany) and inserted into pGEM-T easy vector (Promega, Madison, WI). The resulting recombinant plasmid was introduced into E. coli XL-1 Blue by the method of Hanahan, and the plasmid was recovered from transformant cells with a FlexiPrep kit (Amersham Biosciences), and sequenced using a Thermo sequencing labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Bioscience). The reaction mixture was analyzed with a Shimadzu DSQ-2000. DNA sequencing (Shimadzu, Kyoto, Japan).

**Expression of the α-amylase gene.** Two primers, Sf (5′-GCCCTAAAAATGGGACAAAGG) and ΔSf (5′-GAG-GAAATATGTACTCAAGGTGGCGACGCAGCA), were designed to amplify α-amylase genes with or without a region encoding a signal peptide on the basis of the determined nucleotide sequence (Fig. 1, panel B). Primer Cr (5′-ATCGAAAACCATTTATGCTC) was used as a reverse primer. The α-amylase gene with or without the signal peptide–corresponding DNA region was amplified by PCR (95°C for 1 min, 42°C for 2 min, and 72°C for 4 min for...
35 cycles) with Taq DNA polymerase and recovered from gel slices with a QIAEX II gel extraction kit after 1% agarose gel electrophoresis. The recovered DNA fragments were ligated into pGEM-T easy vector, and the resulting recombinant plasmids were introduced into E. coli XL1-Blue. Transformants were isolated on LB plates without isopropyl $\beta$-D-thiogalactopyranoside (IPTG). Plasmids pSIG5 and pΔSIG17, which carried the amyI gene with and without the signal peptide-corresponding DNA region respectively, were obtained and sequenced to ensure that the cloned genes possessed the original amyI sequence. Transformants carrying pSIG5 and pΔSIG17 were cultivated in 50 ml of LB medium, and $\alpha$-amylase production was induced by the addition of IPTG at a final concentration of 1 mm in the early or late exponential growth phase. Transformant cells were harvested in the stationary phase by centrifugation, and then suspended in buffer A at a ratio of 10 ml to 1 g (wet weight) of cells. The suspended cells were disrupted with a Kubota 201 M ultrasonic oscillator (Kubota, Tokyo) at 180 W for 5 min. The supernatants were recovered as cell extracts by centrifugation, and amyloytic activity was assayed.

**Results**

**Isolation and identification of strain 38C-2-1**

Strain 38C-2-1 was isolated as a microorganism grown in a medium containing soluble starch as the sole carbon and energy source at 30°C and 50°C. It produced a halo around a colony on the plate containing soluble starch by the addition of Lugol’s iodine solution. In addition, the cultural supernatant of strain 38C-2-1 showed 42% relative activity at 30°C as compared with that at 50°C. It was a Gram-positive, spore-forming rod of $0 \div 7 \div 0 \div 9 \div 3 \div 0 \div 2$ mm, motile with peritrichous flagella, oxidase-positive but catalase-negative, and it utilized D-glucose, D-fructose, D-galactose, D-mannose, sucrose, and lactose. These properties correspond to those of *Bacillus halodurans*.

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(accession nos. AB031212 and AJ302709). Strain 38C-2-1 was identified as *B. halodurans* on the basis of these results.

**Purification of α-amylases and their molecular properties**

Table 1 shows a summary of typical enzyme purification. The preparation obtained by Phenyl-Toyopearl column chromatography contained two proteins with amylolytic activity (Fig. 2, panels A and B). Preparative electrophoresis was performed to separate the two α-amylases from each other. The final preparation showed a single protein band on an analytical native polyacrylamide gel, and the enzymes were named α-amylase I and II (Fig. 2, panel C). The specific activities of purified α-amylase I and II were 130 and 18 units/mg respectively. The apparent molecular masses of α-amylase I and II were estimated to be 110 and 87 kDa respectively. The subunit sizes of α-amylase I and II were estimated to be 100 and 75 kDa respectively. These results indicate that the enzymes are monomers with molecular masses of 100 and 75 kDa.

**General properties of the purified enzymes**

Panel A in Fig. 3 shows the effects of temperature on α-amylase I and II activities. α-Amylase I showed maximum activity at 50–60°C, and the maximum activity of α-amylase II was observed at 50°C. When the enzyme activities were assayed at 30°C, α-amylase I and II showed 42% and 38% relative activities of those at 50°C. In addition, α-amylase I showed 36% relative activity even at 20°C. These results indicate that α-amylase I and II are thermotolerant enzymes. The maximum activities of α-amylase I and II were observed at pH 10–11 and pH 10 respectively (Fig. 3, panel B). α-Amylase I retained more than 95% of its activity after heating at 50°C for 30 min; α-amylase II, on the contrary, lost 58% of its activity (Fig. 3, panel C). α-Amylase I was stable in a range of pH 6 to 11, and retained more than 80% of its activity (panel D). The pH stability of α-amylase II was not examined, because this enzyme showed lower thermostability than α-amylase I and was disadvantageous for application in industrial fields. The amino terminal amino acid sequence of α-amylase I was SQGGEQQSFSXDH. The sequence of α-amylase II was not determined because the amino terminus was blocked.

**Effects of inhibitors, denaturing agents, metal ions, surfactant, and bleaching reagent on α-amylase I activity**

As shown in Table 2, α-amylase I was completely inhibited by EDTA. Among the tested metal ions, Hg²⁺, Fe²⁺, Ni²⁺, Zn²⁺, and Pb²⁺ inhibited α-amylase I activity. Sulfhydryl reagents such as PCMB and monoiodoacetic acid showed no significant inhibition.
Amylase I was active in the presence of 0.58 mM NOBS and 1.3 mM sodium percarbonate (200 ppm for each chemical, Table 2). These are commercially used as a surfactant and a bleaching reagent in detergents. The enzyme also retained 83% of its activity after treatment with 0.58 mM NOBS and 1.3 mM sodium percarbonate at 40°C for 30 min (Table 2).

Substrate specificity of α-amylase I
Among the tested substrates, α-amylase I showed the highest activity toward amylopectin (121% relative to that for soluble starch), and significant activity toward glycogen (104%) and amylose (30%). No hydrolytic activity toward pullulan or dextran was observed. These results indicate that α-amylase I hydrolyzes not the 1,6-glycosidic linkage, but rather the 1,4-glycosidic linkage of polysaccharides.

Cloning of the gene encoding α-amylase I
A partial α-amylase I gene was amplified by PCR and used as a probe (probe A) to clone a gene encoding α-amylase I (Fig. 1, panel A). In the first colony hybridization, a 1.3-kb EcoRI fragment was cloned, and other fragments encoding α-amylase I were cloned by subsequent colony hybridizations. As a result, a 3,419-bp nucleotide sequence was determined from the

<table>
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<th>Compound</th>
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<td>83</td>
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<td>Sodium percarbonate + NOBS</td>
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</tr>
</tbody>
</table>

*Sodium percarbonate + NOBS was assayed after treatment with 1 mM of reagents or metal ions at 40°C for 30 min. Sodium percarbonate and NOBS were used at final concentrations of 1.3 and 0.58 mM (200 ppm for each chemical) respectively.

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cloned fragments, and a 2,874-bp open reading frame (ORF) encoding 958 amino acid residues was found in it. The molecular mass of the deduced amino acid sequence was 107 kDa, which was similar to the molecular mass, 110 kDa, of α-amylase I purified from strain 38C-2-1. In addition, the amino terminal amino acid sequence determined from the purified enzyme was found in the deduced amino acid sequence of the ORF (Fig. 1, panel B). Furthermore, homologues of the ORF did not exist on the genome of strain 38C-2-1, because only a single positive band appeared in each Southern blot analysis preceding colony hybridization (data not shown). On the basis of these results, it was concluded that the ORF encoded α-amylase I from *B. halodurans* 38C-2-1, and it was named *amyI*. The deduced signal peptide sequence consisted of 35 amino acid residues, and a predicted initiation codon, ATG, was located 4-bp downstream of a potential ribosome-binding site (Fig. 1, panel B). The nucleotide sequence of *amyI* showed 99.8 and 98.1% identities to those of the alkaline and maltohexaoase-forming α-amylases from *B. halodurans* C-125 (accession no. AP001518) and LBK 34,9) and the deduced amino acid sequence of *amyI* also showed 99.8 and 98.1% identities to those of the α-amylases from *B. halodurans* C-125 (protein ID, BAB04132.1) and LBK 34,9) and 79% identity to G6-amylase from *Bacillus* sp. H-167 (accession no. X55452).

Expression of amyI

Table 3 shows enzyme productivity in the *E. coli* recombinant strains and the wild strain, *B. halodurans* 38C-2-1, and their growth. When AmyI without a signal peptide region was produced in an *E. coli* transformant that carried pSIG17, growth and enzyme productivity under inducible conditions were similar to that of a non-inducible transformant in spite of the induction time. These results indicate that enzyme production is kept at a constant level and independent of growth. The growth of a transformant carrying plasmid pSIG5 was inhibited by the addition of IPTG, as compared with the growth of non-inducible cells.

AmyI with a signal peptide was found as an intracellular enzyme and was not secreted in any growth phase. The *amyI* gene was expressed at a low level in the absence of IPTG (1.4 (U/ml)/OD₆₆₀). Depending on growth, enzyme yield and productivity increased in transformants carrying plasmid pSIG5, and when enzyme production was induced in the late exponential growth phase, the highest enzyme yield and productivity (10 U/ml and 3.3 (U/ml)/OD₆₆₀) showed increases of 45- and 28-fold, as compared with those of the wild strain, 38C-2-1 (0.22 U/ml and 0.12 (U/ml)/OD₆₆₀ respectively).

**Discussion**

In this study, we isolated *B. halodurans* 38C-2-1, which produced alkaline and thermotolerant α-amylase I and II with molecular masses of 105 and 75 kDa. It has been reported that *Bacillus* strains produce α-amylases with different molecular masses in culture. For example, five α-amylases, AmyI to V, with different molecular masses were found in the culture of alkaliphilic *Bacillus* strain GM8901, and AmyII to V were produced by proteolytic degradation of AmyI with the largest molecular mass.22) When the N-terminal amino acid sequence of α-amylase II was analyzed, no peak showing the existence of an α-amino residue appeared, although the N-terminal amino acid sequence of α-amylase I, with a larger molecular mass than α-amylase II, was determined. These results suggest that α-amylase I and II are fundamentally different in biosynthesis and secretion in the parent strain, 38C-2-1.

Hashim *et al.* characterized α-amylase from *B. halodurans* LBK 34 and reported that the enzyme was alkaline and thermostable, but the enzyme was produced by *E. coli* carrying a cloned α-amylase gene and fused with an extrapeptide containing a hexa-histidine tag.9)
We characterized two α-amylases from the parent strain and found that the B. halodurans enzymes are originally alkaline and thermostolerant. In addition, α-amylase I showed enzyme activity in the presence of 0.58 mM NOBS and 1.3 mM sodium percarbonate (200 ppm for each chemical), which are commercially used in detergents as a surfactant and a bleaching reagent. α-Amylase I also showed resistance to treatment with 0.58 mM NOBS and 1.3 mM sodium percarbonate (Table 3). Although α-amylase I from strain 38C-2-1 shows properties similar to those of alkaliphilic and thermostable α-amylases previously reported, the effects of these chemicals on their enzyme activity and resistance are not known.5-9,22 These properties of α-amylase I, therefore, show that the enzyme is suitable for use as a detergent additive.

α-Amylase I activity was completely lost after treatment with 1 mM EDTA (Table 2). It has been reported that α-amylases, including alkaline α-amylases, need Ca$^{2+}$ for maintenance of their higher-order structure and enzyme activity.6,22 When α-amylase I was incubated with 1 mM Ca$^{2+}$, enzyme activity increased, although other metal ions, except for Mg$^{2+}$, inhibited α-amylase I activity or showed no effects on it. These results suggest that Ca$^{2+}$ plays important roles in α-amylase I. Sulfhydryl reagents such as PCMB and monooiodoacetic acid showed no significant inhibition as reported alkaline α-amylases,5,6 indicating that sulfhydryl groups are not important in the catalysis of α-amylase I.

We cloned amyI encoding α-amylase I in four DNA fragments, and determined the complete nucleotide sequence of amyI. The deduced amino acid sequence of amyI showed 98.1% identity to that of an alkaline-active maltohexaose-forming α-amylase from strain LBK 34,9 and 18 nonidentical amino acid residues were found between those sequences. Thirteen amino acid residues in them were crowded into a sequence of 41 amino acid residues at positions of Ser$^\text{170}$, Arg$^\text{170}$, Thr$^\text{582}$, Gln$^\text{625}$, and Glu$^\text{831}$. The replacement of the thirteen amino acid residues in this region might be related to differences in resistance to proteolytic enzymes, because the isoform pattern of strain 38C-2-1 enzymes (Fig. 2, panel B) was different from that of strain LBK 34 enzymes, which showed five clear zones with amyloytic activity.9 α-Amylase I from strain 38C-2-1 might be more resistant to proteolytic enzymes than the LBK 34 enzymes.

When we started to clone amyI, we tried to clone the amyI gene from a gene library consisting of fragments of more than 5-kb DNA produced by partial digestion with Sau3AI. Although some positive clones were obtained by colony hybridization using probe A, all the clones lacked a 5′-terminal region containing a sequence encoding a signal peptide region and the putative ribosome-binding site AGGA (data not shown). This finding suggests that α-amylase I with the signal peptide is toxic to E. coli cells and greatly affects their growth. Considering these results, we cloned the four different fragments separately to determine the nucleotide sequence of amyI. Our assumption was supported by the observation that the growth of the transformants was repressed by induction of production of AmyI with the signal peptide (Table 3). Production of AmyI without the signal peptide was low in spite of the conditions of induction, although higher productivity was expected than that of AmyI with the signal peptide because of the decrease in toxic effects due to the signal peptide (Table 3). AmyI without the signal peptide might be unstable in E. coli cells, and might be degraded by proteolytic enzymes, or might exist as an inclusion body in the cells.

When enzyme production was induced in the late exponential growth phase, the productivity of AmyI with the signal peptide increased 28-fold as compared with that of B. halodurans 38C-2-1 in the late exponential phase. Other overexpression systems, e.g., pET overexpression systems using the T7 promoter, might be available for improvement of enzyme productivity. Further efforts are needed to increase enzyme productivity.

Acknowledgment

This work was supported by the Japan Society for the Promotion of Science’s (JSPS) Core University Program.

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