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Effects of Environmental Conditions on Expression of *Bacillus subtilis* α-Amylase in Recombinant *Escherichia coli*

SHIN PYONG K.[1], SEUNG H. NAM[2] AND JIN HO SEO*[2]

[1]*Korea Institute of Science and Technology, Seoul 130-650, Korea*
[2]*Department of Food Science and Technology, Research Center for New Biomaterials in Agriculture, Seoul National University, Suwon 441-744, Korea*

The expression of *Bacillus subtilis* α-amylase from the *phoA-amyE* fusion gene in recombinant *E. coli* was investigated under various environmental conditions. The overexpression of cloned α-amylase caused retardations in cell growth and synthesis of alkaline phosphatase (AP) from the chromosomal *phoA* gene. The change of culture temperature from 37°C to 30°C increased the specific activities of both α-amylase and β-lactamase by six and two times, respectively, whereas the AP activity remained unchanged. The experiments with chlorampenicol (a translation inhibitor) suggested the enhancement of α-amylase activity at 30°C, and this was partly due to the stability of α-amylase itself. The further decrease of the temperature to 25°C slowed down both the cell growth and cloned-gene expression rate. The α-amylase activity showed a maximum at pH of 7.4 while alkaline phosphatase was most effectively produced at pH of 8.3.

In order to develop viable processes for producing industrial enzymes by recombinant DNA technology, a systematic strategy has to be employed in an attempt to improve productivity. The selection of a proper cloning system is a key element for the high productivity of a recombinant DNA system. In the design of expression vectors, care has to be taken for maintaining the stability of the product protein against proteases. Among the key factors determining the overall productivity, the product stability is very important especially when the product is heterologous to the host cell. It has been reported that many foreign proteins in recombinant microorganisms are often very unstable and degraded very rapidly (11, 14, 16). The secretion of a product protein out of the cytoplasmic space of the cell can be one of the potential solutions associated with the instabilty of the product protein since it can prevent or minimize the degradation of the product protein by cellular proteases. Furthermore, periplasmic proteins are normally only 4% of total cell proteins (19); therefore, transport of a product protein to the periplasmic space may facilitate the purification of the product from other cellular proteins. Secretion of a heterologous product can generally be achieved at a molecular level by combining the structural gene of the product protein with the signal sequence of a native secreted protein (3, 7, 8).

After a cloning system is selected, the next step should be to find an optimal operating condition for the chosen system. Although protein secretion enhances product stability against cellular proteases, it is known that some proteases are also present in the periplasmic space (10) whose activities depend upon the culture temperature. Furthermore, the rate of mRNA degradation in *E. coli* decreases with the culture temperature (13). Culture pH is known to affect the secretion and consequently the product stability of a product protein (5).

This work was undertaken to investigate the expression and secretion of *Bacillus subtilis* α-amylase from the *phoA-amyE* fusion gene cloned on a multicopy plasmid in *E. coli* under different environmental conditions. The structural gene of *Bacillus subtilis* α-amylase (*amyE*) was fused with the promoter and the signal sequence region of the *E. coli* phoA gene. This fusion allowed the expression and secretion of α-amylase under the control of an inorganic phosphate in the medium (25). Specific emphasis was placed on the kinetics of α-amylase expression and the effects of its expression on the cell growth and product formation under different culture temperatures and pH.

*Corresponding author
Key words: α-Amylase, recombinant *E. coli*, fermentation

MATERIALS AND METHODS

Bacterial Strain and Plasmid

Escherichia coli K-12 strain BW3414, Δ(*argF-lacal) 205(U169) pho-499*, was used as the host (27). The α-amylase structural gene (*amyE*) of *B. subtilis* was inserted into a plasmid behind a gene fragment containing the promoter and signal sequence region of the *E. coli phoA* gene (pAMYA) (25). As a result, the expression and secretion of α-amylase were regulated by inorganic phosphate concentration in the medium. The plasmid pAMYA also contained an ampicillin resistance marker as illustrated in Fig. 1. The pAMYA plasmid was constructed and kindly donated by Dr.Yoda of the Department of Agricultural Chemistry at the University of Tokyo, Japan. Two other strains (BW13704 and BW13706) used in this study have been described elsewhere (24).

Medium and Cultivation

A MOPS(3-[N-Morpholino]propanesulfonic acid) minimal medium (18) containing 2 g/l of glucose was employed for this study. Cells were grown at a given temperature and pH under aerobic conditions in a fermentor (New Brunswick Scientific, Model F-1000). The initial concentration of inorgnic phosphate in the growth medium was 0.1 mM K_2HPO_4 for derepression of the *phoA* gene. To minimize the effects of plasmid instability, ampicillin (Sigma) was added to all cultures at 100 mg/l.

Analysis

Cell growth was monitored by measuring optical density at 600 nm with a spectrophotometer (Milton Roy, Model 601). Glucose concentration was measured with a glucose analyzer (YSI Inc. Model 27). Assay methods for inorganic phosphate concentration in the medium and alkaline phosphatase (AP) activity were the same as described previously (24).

The activity of α-amylase was determined by the method developed by Fuwa (9) and modified by Yamaguchi (28). Two milliliters of 0.5% soluble starch (Fisher) in 40 mM phosphate buffer (pH 6.0) was preincubated for 10 minutes in a water bath at a temperature of 40℃, and 1 m*l* of an appropriately diluted enzyme solution was added to the starch solution. After incubation for 4 minutes at 40℃, 0.1 m*l* aliquot of the reaction mixture was added to 2.5 m*l* of the iodine reagent (100 times dilution of 0.5% I_2-5% KI solution). The optical density at 700 nm was measured after the mixture stood at room temperature for 10 minutes. One unit of activity was defined as the amount of enzyme which hydrolyzes 0.1 mg of soluble starch per minute at 40℃.

β-lactamase activity was measured by the method of Sawai (22). The enzyme in 2.5 m*l* of 0.1 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) was preincubated at 30℃ for 5 minutes in an assay tube to which 0.5 m*l* of substrate solution (100 μmol of penicillin-G [Sigma] in the same buffer) was added. After incubation at 30℃ for an appropriate period, the enzyme reaction was stopped by adding 5 ml of the iodine reagent (20 times dilution of 0.16 M I_2-1.2 M KI solution) with rapid mixing. After the mixture stood at room temperature for 10 minutes, the absorbance at 490 nm was measured. One unit of enzyme activity was defined as that quantity which hydrolyzes 1 μmol of substrate per minute under the conditions used. The specific activity of each enzyme was defined as the total enzyme activity divided by the optical density of the culture.

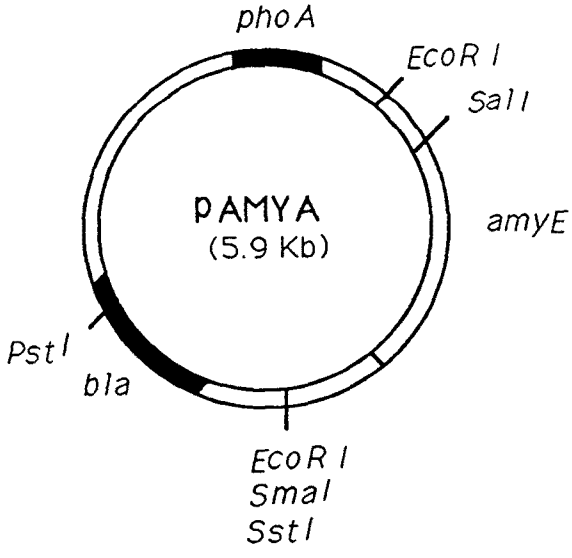

Fig. 1. Genetic map of plasmid pAMYA.

RESULTS

Influence of the Expression of *B. subtilis* α-Amylase on the Cell Growth and the Gene Expression

The fusion of the structural gene of the *Bacillus subtilis* α-amylase with the promoter and signal sequence region of the *E. coli phoA* gene permitted α-amylase expression and secretion into the periplasmic space under the control of inorganic phosphate concentration in the medium. About 95% of total α-amylase activity was found in the periplasmic space of the cells, indicating successful secretion into the periplasmic space of the *E. coli* cell (25). The expression pattern of the *phoA-amyE* fusion gene on the multicopy plasmid was compared with that of the *phoA* gene coding for alkaline phosphatase (AP) in the chromosome. The expression and secretion of both AP and α-amylase was directed by the same promoter and signal sequence of the *E. coli phoA* gene,

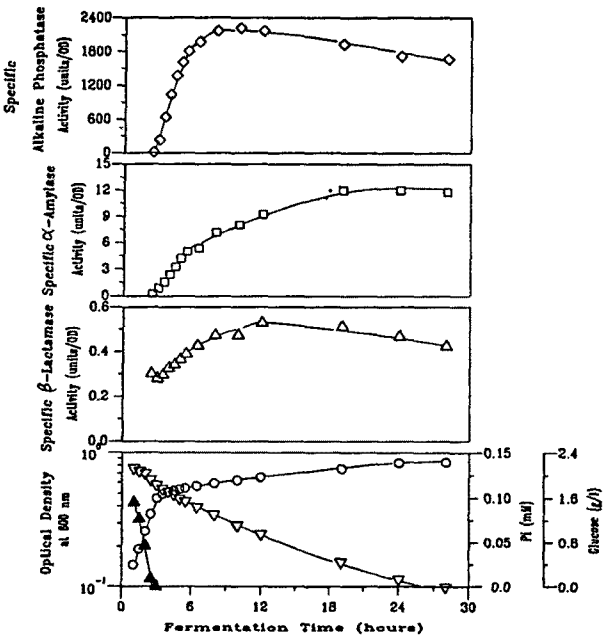

Fig. 2. Time trajectories of optical density (○), glucose concentration (▲), inorganic phosphate concentration (▽), specific alkaline phosphtase activity (◇), specific α-amylase activity (□), and specific β-lactamase activity (△).

E. coli strain BW3414/pAMYA was grown in a MOPS medium with 0.1 mM initial inorganic phosphate at 37℃ and pH 7.4. The specific enzyme activity was defined as a total activity divided by the culture OD.

but the *phoA* gene was located at different cloning sites.

The dependence of inorganic phosphate (Pi), glucose, optical density (OD), specific activity of AP, α-amylase, and β-lactamase on the fermentation time at an initial phosphate concentration of 0.1 mM at 37℃ is displayed in Fig. 2. After inorganic phosphate in the medium was exhausted in 3 hours, the expression of both AP and α-amylase was initiated with a slow increase in cell mass. The same type of dependency of cell growth and enzyme expression on inorganic phosphate concentration was also observed in other *phoA*-directed expression systems (24). The growth rate of this α-amylase expression system after phosphate starvation, however, was far lower than that of the recombinant strain (BW13704) which produced β-galactosidase from the *phoA-lacZ* fusion gene on the multicopy plasmid (plasmid pDK110). The specific growth rates of the α-amylase producing (BW3414/pAMYA) and β-galactosidase producing strains (BW13704) after phosphate depletion were estimated as 0.075 and 0.28 (hr^{-1}), respectively. Even though the copy number of the plasmid pAMYA was approximately 10 times less than that of pDK110 (23), the expression and secretion of α-amylase showed deleterious effects on the cell growth compared to the expression of β-galactosidase, which is a cytoplasmic enzyme. In addition, inhibition to cell growth was found to be more severe in the shake flask culture (data not shown). The BW3414/pAMYA strain stopped growing in one or two hours after phosphate depletion. Although the reasons are not clear, it seems that the overexpression of the secreted protein α-amylase may greatly disturb the secretion of other membrane proteins which are necessary for cell divisions, causing the cessation of cell growth.

The expression modes of alkaline phosphatase (AP) and α-amylase under derepressed conditions were investigated in more detail in order to explore growth inhibition phenomena. The expression of both enzymes was controlled by the same promoter; however, their expression patterns were quite different from each other. As soon as inorganic phosphate concentrations in the medium fell to zero, both enzymes were expressed at the maximum rate for 3.5 hours. After this period, the AP expression rate decreased gradually and the maximum specific AP activity was obtained in 4.5 hours. On the other hand, the α-amylase expression continued for 12.5 hours with a rather reduced rate as compared to the initial rate. A possible reason for this difference might have been the competition for secretion sites between the two enzymes. Both enzymes were expressed in the cytoplasmic space and then secreted through the cytoplasmic membrane. As the number of the secretion sites at the cytoplasmic membrane is constant (12), the overexpression of α-amylase seems to interfere with the secretion of other secreted proteins, including AP. The effect of the competition for the secretion sites on the enzyme activity was also observed for β-lactamase, another secreted protein. The specific activity profile of β-lactamase was similar in shape to that of AP, as if β-lactamase were also under the control of inorganic phosphate concentration in the medium. However, when the total β-lactamase activity in the culture was plotted against fermentation time, it increased at a constant rate for an initial period of six hours regardless of inorganic phosphate concentrations (Fig. 2). This was consistent with the fact that β-lactamase is expressed constitutively. The specific activity of β-lactamase reached a maximum value at around 12 hours of fermentation. The expression rate for β-lactamase changed almost at the same time when the expression rate of both enzymes (AP and α-amylase) changed, suggesting that the competition for the secretion sites between the secreted proteins became severe after 6.5 hours of fermentation. Saturation of the secretion pathway by the overexpression of a secreted protein has also been reported by many other research groups

(1, 4, 12, 29).

Effect of Temperature

To study the effects of culture temperature on the expression of *Bacillus subtilis* α-amylase in *E. coli*, we compared the expression patterns of three enzymes (alkaline phosphatase, α-amylase, and β-lactamase) at three different temperatures: 25, 30, and 37℃. As summerized in Table 1, specific growth rates were also determined from the exponential phase of fermentations at each of these temperatures. As expected, specific growth rates were increased with the increase of temperatures that ranged from 25℃ to 37℃.

Cells grown at 30℃ exhibited a similar qualitative dependence of inorganic phosphate (Pi), glucose, OD, AP, α-amylase, and β-lactamase on fermentation time at an initial phosphate concentration of 0.1 mM to the dependence at 37℃ (Fig. 3). The reduction of the growth temperature from 37℃ to 30℃, however, resulted in a six-fold increase in the specific activity of α-amylase, a two-fold jump in the specific activity of β-lactamase, but only a slight increase in the AP specific activity (Table 1). The expression rate of β-lactamase at 30℃ was similar to that at 37℃, whereas the rate was kept constant for 18 hours which was far longer than the period at 37℃. In the case of α-amylase, the expression rate at 30℃ was approximately five times higher than that at 37℃ (data not shown). The increase in both α-amylase and β-lactamase expression levels by lowering culture temperatures from 37 to 30℃ can be attributed to many factors such as plasmid copy number increase (gene dosage effect), stability during the post-transcriptional processes (this term was used to include mRNA,translation intermediates, and precursor molecules of a secreted protein which contain a signal peptide along with the main polypeptide), and product protein stability. Among these, Oka *et al.* (20) have studied the expression of human epidermal growth factor (hEGF) in *E. coli* using a *phoA*-directed expression system. They found that product protein stability contributed to the increase in the product protein at lower temperatures. Chesshyre and Hipkiss (6) also showed that the lesser accumulation of interferon (IFN) α-2 in recombinant *E. coli* at 37℃ than at 25℃ was due to the high degradation rate of IFN -2 at 37℃. Koizumi and his co-workers (15) identified the gene dosage effect as the most significant factor for the enhancement of penicillinase productivity in recombinant *Bacillus stearothermophilus* with a decrease in cultivation temperatures.

Among the possibilities described above, the product protein stability was tested by using chloramphenicol

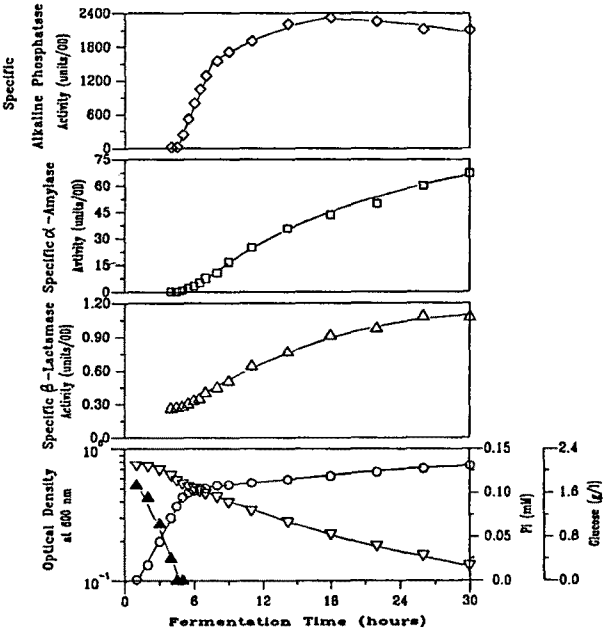

Fig. 3. Profiles of optical density (○), glucose concentration (▲), inorganic phosphate concentration (▽), specific alkaline phosphatase activity (◇), specific α-amylase activity (□), and specific β-lactamase activity (△) vs. cultivation time.

E. coli strain BW3414/pAMYA grown in a MOPS medium with 0.1 mM initial inorganic phosphate at 30℃ and pH 7.4.

Table 1.Effects of culture temperature on the specific growth rates and the maximum specific activities of alkaline phosphatase, α-amylase and β-lactamase.

Temperature (℃)	Specific growth rate[a]	Specific activity (Units/OD)		
	(hr^{-1})	alkaline phosphatase	α-amylase[b]	β-lactamase
25	0.15	1110	58.8(35.0[c])	0.80
30	0.39	2318	67.1(25.5[c])	1.08
37	0.63	2211	11.1	0.53

[a] Measured at the exponential phase

[b] Since specific activity increased continuously during fermentation at both 25 and 30℃, the specific activity of the last sample of each culture was considered as a maximum.

[c] Values represent the fermentation time in hours from Pi depletion to the time when the sample was taken.

which can block the translation processes in the cell and hence stop *de novo* protein synthesis. Cells at the same culture stage were treated with the drug and cultivated at two different temperatures. First, cells were grown in a high phosphate (2.0 mM) medium for 24 hours in a shake flask and harvested by centrifugation. The cell pellet was resuspended in the same medium without phosphate. The resuspended cells were cultivated at 30℃ for six hours and then split into two flasks. After the addition of chloramphenicol (10 μg/ml) to each flask, the cultures continued for 10 hours at 30℃ and 37℃. During the cultivation, samples were taken to determine the activities of the two enzymes.

Fig. 4 illustrates the results of the chloramphenicol addition experiment. Since the addition of chloramphenicol may change the size and shape of the cell, we compared total enzyme activities for both enzymes expressed at 30℃ and 37℃ along with those for a control culture at 30℃ without chloramphenicol. At 30℃, α-amylase activity remained almost constant for 10 hours after the drug was added, while the enzyme activity at 37℃ decreased at appreciable rates. This suggests that α-amylase is unstable at 37℃. The β-lactamase activity remained constant for 10 hours at 30 and 37℃, indicating that this enzyme is rather stable at both temperatures. Even though α-amylase is unstable at 37℃, this is still not enough to explain the six-fold increase in α-amylase activity at 30℃ compared to that at 37℃. This observation is very interesting since α-amylase is generally known as a thermostable enzyme.

For α-amylase, the possibility of the variation of either the stabilty during the post-transcriptional processes or the plasmid copy number with temperature changes, and the subsequent effect on the enzyme expression rate, remains to be elucidated. We have previously observed that when an *E. coli* native enzyme β-galactosidase was expressed from a strain containing the *phoA-lacZ* fusion gene on the multicopy plasmid (BW13704), the expression rate at 30℃ was almost the same as that at 37℃ (23), suggesting that the effects of the plasmid copy number change with temperature variation on the total

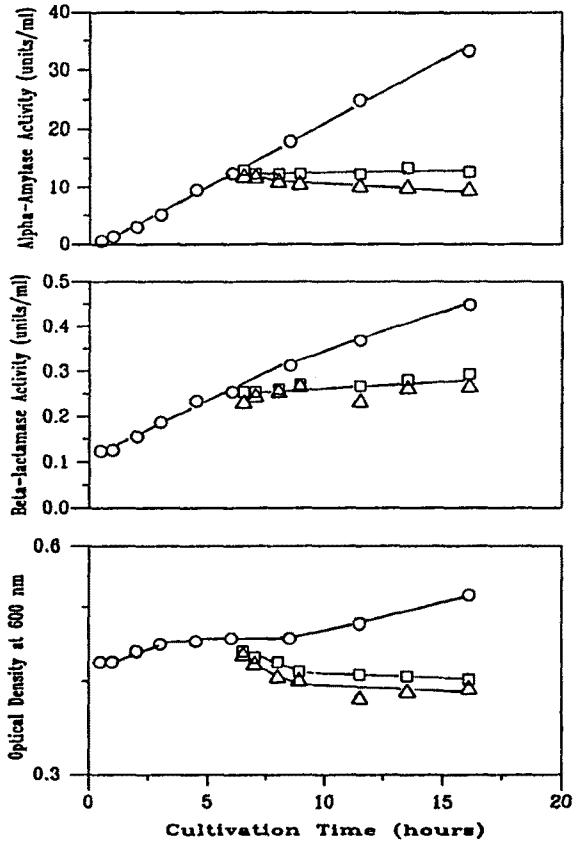

Fig. 4. Changes of optical density, α-amylase acitvity, and β-lactamase activity vs. cultivation time.
The shake flask culture of the *E. coli* BW3414/pAMYA strain was carried out at two temperatures (30 and 37℃) after chloramphenicol (10 μg/ml) addition. Symbols are the values at 37℃ (△) and at 30℃ (□). (○) represents the data obtained for the control experiment.

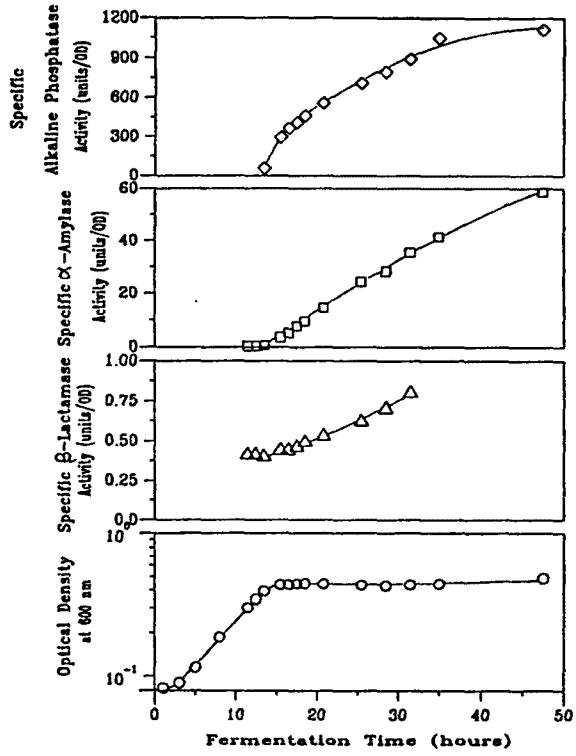

Fig. 5. Plot of optical density (○), specific alkaline phosphatase activity (◇), specific α-amylase (□) and specific β-lactamase activity (△) vs. cultivation time.
E. coli strain BW3414/pAMYA was grown in a MOPS medium with 0.1 mM initial inorganic phosphate at 25℃ and pH 7.4.

enzyme expression rate may not be significant, if any. The comparison of the intensity of the bands for plasmid DNA on a gel also led to a similar conclusion. The plasmid DNA was isolated from cells grown at 30℃ and 37℃ by a miniscreen method (21), and then gel electrophoresis (agarose, 0.7%) was performed on the plasmid DNA. Bands representing plasmid DNA from both temperatures did not show any significant difference in intensity (data not shown), which was consistent with our previous assumption of no change of plasmid content with temperature change. As a result, we concluded that the increase in the stability of the enzyme itself at the final place partly contributes to the enhancement of α-amylase expression at 30℃ compared to that at 37℃, and the change of plasmid copy number with temperature changes had no significant effect on the enhancement.

When the culture temperature was further lowered to 25℃, cells showed a rather different behavior of growth and gene expression from those at 30℃ or 37℃. During the derepression of both AP and α-amylase, cells did not grow at all for at least 30 hours (Fig. 5). Although specific AP activity increased continuously with a decreasing rate for 35 hours after gene expression was initiated, its final expression level was far lower than that at either 30 or 37℃ (Table 1). The α-amylase specific activity increased at a constant rate for 30 hours and reached the level that was obtained in 21 hours of derepression at 30℃. The reduction of culture temperature to 25℃ caused severe inhibition of both cell growth and α-amylase formation rate.

Effect of pH

Several studies have shown that the culture pH affects the expression of secreted proteins in *E. coli*. Chang and her co-workers (5) who studied the pH effects on the production of human growth hormone (hGH) in *E. coli*, revealed that pH 7.0 is optimal for expression of secreted hGH, and they also showed that both acidic and alkaline media have negative effects on foreign protein expression. Contrary to this, Atlan *et al.* (2) showed that *E. coli* lky mutants produce extracellular AP most effectively at pH 8.3 with the least release of the cytoplasmic enzyme β-galactosidase in an LB medium. Later, they also reported that an *E. coli* AP constitutive mutant carrying a plasmid encoded *phoA* gene excreted AP into the growth medium in a pH-dependent fashion and that pH 8.4 was the optimum for excretion of AP (16). Marten and Seo (17) found that the expression and secretion of cloned invertase in *S. cerevisiae* also depends upon culture pH. All of these results suggest that the pH effects on the expression and secretion of secreted proteins may depend upon the characteristics of both host cells and target proteins.

Table 2. Specific activities of alkaline phosphatase and α-amylase at different culture pH.[a]

pH	Alkaline phosphtase (Units/OD)	α-Amylase (Units/OD)
6.0	1136	38.4
7.4	1052	73.9
8.3	1864	68.4

[a]Culture conditions are described in the text.

To investigate the pH effects on the expression of α-amylase in *E. coli*, experiments were performed at three different pH values: 6.0, 7.4, and 8.3. To prevent pH fluctuation during cultivation, cells were initially grown overnight at a high phosphate (2.0 mM Pi) medium. Cells were harvested by centrifugation and resuspended in the phosphate-depleted medium adjusted to different pH. Since cells cannot grow well in the phosphate-depleted medium, we kept culture pH relatively constant by preventing the formation of organic acids during cell growth. These resuspended cells were grown at 30℃ for 23 hours, and then both AP and α-amylase activities were assayed. As summarized in Table 2, α-amylase was produced most effectively at pH 7.4, while alkaline phosphatase at pH 8.3. The results indicated the pH-dependent protein expression in *E. coli*. More research is in progress to explore the influence of culture pH on the expression and secretion of cloned proteins in *E. coli*.

DISCUSSION

We investigated the expression behavior of *Bacillus subtilis* α-amylase in *E. coli* under various culture conditions. The fusion of the α-amylase structural gene (*amyE*) with the *E. coli phoA* gene fragment which contains the promoter and the signal sequence allowed the expression and secretion of α-amylase under the control of an inorganic phosphate concentration in the medium. The overexpression of α-amylase after inorganic phosphate depletion caused severe growth inhibition. Furthermore, it resulted in a rather different activity profile for alkaline phosphatase, which has its biological activity only after secretion into the periplasmic space of the cell. The switching time of β-lactamase expression rates was almost identical to that of both AP and α-amylase, suggesting the saturation of the secretion pathway by the overexpression of α-amylase.

The reduction of culture temperature from 37℃ to 30℃ resulted in a six-fold increase in the specific activity of α-amylase and a two-fold enhancement of β-lactamase, while keeping the alkaline phosphatase specific

activity unchanged. The expression rates of α-amylase at the two temperatures were reduced to the same degree from their initial values by the competition for the secretion sites among the secreted proteins. This indicates that the competition for secretion sites was not the main reason for the decrease of α-amylase activity at 37℃ compared to that at 30℃. A set of experiments was carried out to estimate the effect of product degradation on such increments. The experiment using chloramphenicol (a translation inhibitor) showed that an increase in α-amylase stability at 30℃ partly contributed to the overexpression of α-amylase. The observation that an *E. coli* native enzyme β-galactosidase was expressed with identical rates at 30℃ and 37℃ from a strain containing the *phoA-lacZ* fusion gene on the multicopy plasmid (BW13704) excluded the possibility of gene dosage effects (the plasmid copy number changes with temperature) on the total gene expression rate. Other factors such as the stability of the product protein in post-transcriptional processes remain to be studied to characterize the enhancement of α-amylase at 30℃ compared to at 37℃. Interestingly, a shift of culture temperature to 25℃ slowed down the cell growth and cloned-gene expression rates.

The stability of β-lactamase at 30℃ and 37℃ was almost the same. Although the expression rate of β-lactamase at 30℃ was very similar to that at 37℃ in the recombinant *E. coli* strain BW3414/pAMYA, it remained unchanged for 18 hours at 30℃, which is far longer than the period at 37℃. This observation indicated that the competition for secretion sites between the secreted proteins was more severe at 37℃ than at 30℃, which resulted in a decrease of the β-lactamase level at 37℃.

Culture pH also showed a significant effect on the expression and secretion of both AP and α-amylase in *E. coli*. pH 7.4 was the best of three pH values (6.0, 7.4, and 8.3) for α-amylase production while AP was most effectively produced at pH 8.3.

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