0273-2289/00/84–86/0319/$12.25

Enhanced Alkaline Protease Production in Addition to α-Amylase via Constructing a *Bacillus subtilis* Strain

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Abstract

Bacillus subtilis Bios11 strain was previously isolated and identified. This strain naturally produces a high level of α-amylase. The multicopy (pS1) plasmid that carries the complete alkaline protease *aprA* gene was introduced to this host strain by transformation. The newly constructed strain was found to express the *aprA* gene and produces a high level of alkaline protease. The level of α-amylase production was not affected compared with the parent strain. The pS1 plasmid in the new host was proved to be segregationally and structurally stable, and the multicopy *aprA* gene was expressed at the stationary phase. This expression did not affect growth rate and sporulation frequency. Moreover, the level of α-amylase was maintained. Both alkaline protease and α-amylase enzymes were purified using a single-step affinity chromatography column. The use of the newly constructed strain would be valuable to the enzyme industry and would promote recycling of some food-processing wastes.

Index Entries: α-Amylase; *aprA* gene; *B. subtilis*; alkaline protease.

Introduction

Bacillus subtilis has great potential as an extremely useful system for gene cloning and expression of homologous and heterologous genes. Several aspects of *B. subtilis* are of great interest for potential industrial applications: *B. subtilis* cells have no known pathogenic interaction with humans or animals, secrete homologous and heterologous proteins, have the ability to grow on either simple or complex media and at relatively high temperature, and have historical interest for the production of many industrial and food products *(1)*.

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The instability of *B. subtilis* cultures containing recombinant plasmids is considered to be a major problem in industrial applications *(2–5)*. Recently, we constructed several *B. subtilis* strains by introducing the multicopy plasmid (pS1) that carries the complete alkaline protease *aprA* gene *(2)* into various *B. subtilis* hosts for two purposes: first, to reduce or overcome the plasmid instability problem and, second, to enhance the alkaline protease production in some *B. subtilis* hosts that normally produce no or a low level of this enzyme but secrete a high level of other industrially important enzymes such as α-amylases and lipases. *B. subtilis* BioS11 host strain, which naturally produces a high level of α-amylase, has been previously isolated and identified *(6)*. The multicopy (pS1) plasmid was introduced to this host strain by transformation. Our results indicated that the newly constructed strain expressed the *aprA* gene and produced an enhanced level of alkaline protease. Furthermore, the pS1 plasmid was found to be segregationally and structurally stable for a cultivation period up to 7 d in the absence of any selection pressure. Moreover, the expression of the *aprA* gene did not affect growth rate and sporulation frequency of the host, and the level of α-amylase was maintained.

Materials and Methods

Bacterial Strains and Plasmids

B. subtilis BioS11 host strain has been isolated and identified previously *(6)*. This strain naturally overproduces α-amylase enzyme and was used as a recipient strain for the multicopy pS1 plasmid. The pS1 plasmid (6.7 kbp) is a pUB110 derivative plasmid that carries the complete alkaline protease *aprA* gene and a kanamycin resistance marker *(2)*.

Media

Bacterial strains were activated and grown on peptone yeast extract (PY) medium *(7)*. PY medium consists of 10 g of Bacto peptone, 5 g of yeast extract, and 5 g/L of NaCl. PA medium, PY supplemented with 1.5% agar agar, was used in the plasmid stability experiments. Sporulation medium (modified Schaeffer's medium, 2XSG *[8]*) was used to determine the sporulation frequency of the bacterial strains. This medium was also used to monitor the production level of alkaline protease and α-amylase. 1XSG skim milk kanamycin medium was exactly like 2XSG except that the amounts of peptone and beef extract were reduced to half and skim milk was added to 1%, agar to 1.5%, and kanamycin to 5 μg/mL of medium.

Bacterial Transformation

The pS1 plasmid was prepared, purified, and introduced to *B. subtilis* BioS11 competent cells through a bacterial transformation procedure similar to that described previously *(9)*. Transformants, kanamycin-resistant colonies, were screened using PA kanamycin plates. Clones were tested to

have the correct plasmid by using a rapid miniscreen DNA method *(9)*. Colonies were checked for carrying complete *aprA* gene by growing them on 1XSG skim milk kanamycin medium.

Monitoring Bacterial Growth and Enzymatic Activity of Alkaline Protease and α-Amylase

Bacterial growth was monitored by measuring the absorbance of the *B. subtilis* BioS11 and *B. subtilis* BioS11 (pS1) cultures at 420 nm, which highly correlated with viable cell count. The activity of the extracellular alkaline protease and α-amylase was monitored throughout the growth of the bacterial strains. Cells were activated by growing them overnight on PA plates at 37°C. Fresh colonies were then transferred to 50 mL of 2XSG medium and allowed to grow at 37°C with shaking to the indicated time, at which 1.5 mL of the growing culture was centrifuged in a microcentrifuge at 3500*g* for 2 min. The supernatants were used to determine the activity of both alkaline protease and α-amylase.

Alkaline Protease Activity

The activity of the alkaline protease was determined according to the method of Sutar et al. *(10)*. One unit of enzyme activity was equal to the amount of enzyme that liberated 1 μmol of tyrosine from 10 mg of casein per 30 min at 37°C.

α-Amylase Activity

α-amylase activity was determined according to the method of Manning and Campbell *(11)*. One unit of α-amylase was equal to the amount of enzyme that hydrolyzed 5 mg of soluble starch per 30 min at 30°C.

Plasmid Stability

Plasmid stability was determined as described previously *(2)*. Cultures were renewed every 12 h using fresh PY medium. At the indicated time, a portion of the culture was plated on PA plates, and the resulting colonies were screened for their resistance to kanamycin (segregational stability) by growing them on PA km plates. Kanamycin-resistant colonies were also tested for the presence of complete *aprA* gene by transferring them onto 1XSG skim milk kanamycin plates (structural stability).

Sporulation Frequency

Sporulation frequency was determined as described previously *(12)*.

Purification of Alkaline Protease and α-Amylase

Alkaline protease and α-amylase enzymes, produced by the newly constructed strain, were purified using $(NH_4)_2SO_4$ selective precipitation (65% saturation) followed by a single-step affinity chromatography column *(13)*. Protein concentration was determined as described previously *(14)*.

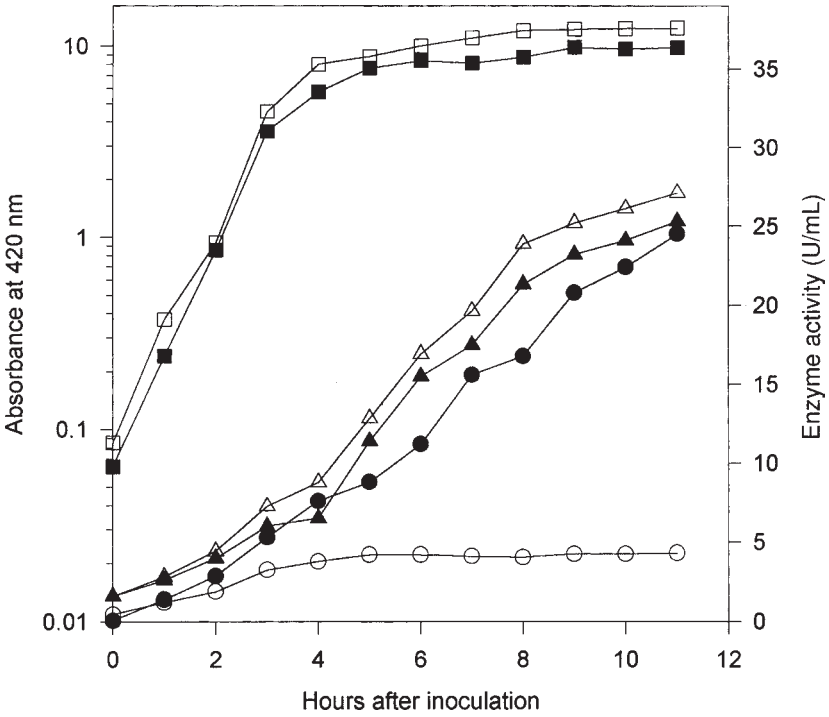

Fig. 1. Monitoring alkaline protease and α-amylase enzymes produced by *B. subtilis* BioS11 and *B. subtilis* BioS11 (pS1) cells. Cells were cultivated on 2XSG medium and allowed to grow at 37°C to the indicated times. □, △, and ○: absorbance at 420 nm, α-amylase activity, and alkaline protease activity for *B. subtilis* BioS11 strain; ■, ▲, and ●: the same parameters for *B. subtilis* BioS11 (pS1).

Results and Discussion

Monitoring Enzymatic Activity of Alkaline Protease and α-Amylase

The enzymatic levels of the extracellular alkaline protease and α-amylase of both *B. subtilis* BioS11 and *B. subtilis* BioS11 (pS1) cells, when grown on 2XSG medium, were monitored as described in Materials and Methods. The 2XSG medium was used to give a short exponential phase and an extended stationary phase. Expression of the cloned *aprA* gene in the newly constructed *B. subtilis* BioS11 (pS1) cells started late at approximately T_2, i.e., 2 h after the end of the exponential phase. The level of alkaline protease activity was more than fivefold higher than that of the parent strain after about 10 h of growth at 37°C on 2XSG medium (Fig. 1). This level reached about 20-fold after 24 h of growth. Data obtained are quite similar to those obtained when the expression of the cloned *aprA* gene was monitored on the growth of *B. subtilis* DB100 (pS1) cells on 2XSG medium *(2)*. On the other hand, the enzymatic levels of α-amylase produced by the newly constructed and the parent strains were almost the same. The level of α-amylase secreted by the parent strain looks higher than that of the

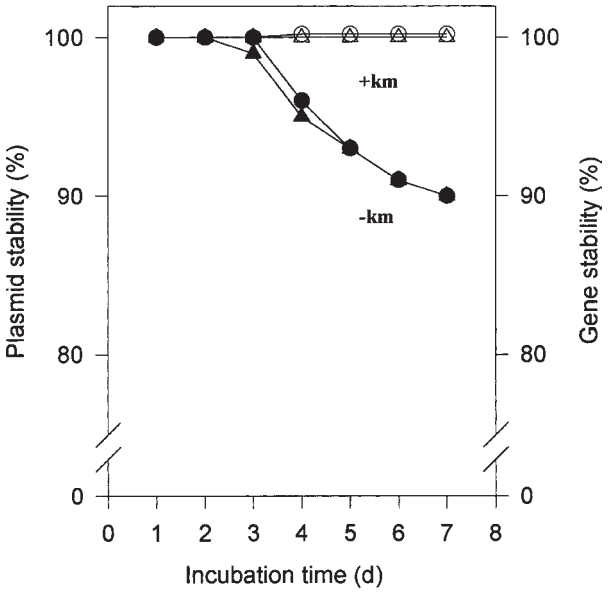

Fig. 2. Stability of the pS1 plasmid harbored by *B. subtilis* BioS11 (pS1) cells. ○ and △, plasmid and gene stability, respectively, when cells were grown on PY kanamycin medium; ● and ▲, plasmid and gene stability, respectively, when cells were grown on PY alone (without stress).

newly constructed strain since the growth of the parent strain, in this experiment, was not much greater than that of the constructed strain (Fig. 1). The level of α-amylase activity, as units/log colony-forming units, for both strains was quite similar. However, the full expression of the multicopy *aprA* gene did not affect the level of α-amylase, probably because the cloned *aprA* gene is a homologous gene.

Plasmid Stability

Several reports have described segregational and structural instabilities of plasmids in *B. subtilis (1–5,15,16)*. The stability of the pS1 plasmid in the newly constructed strain was determined as mentioned in Materials and Methods. Since expression and stability of the cloned *aprA* gene are very related and the expression of the gene was late during stationary phase, a plasmid stability experiment was conducted with cells grown at stationary phase in which *aprA* gene is fully expressed. Figure 2 illustrates the stability of the pS1 plasmid hosted by *B. subtilis* BioS11 (pS1) cells when grown for different times on PY medium with and without stress (5 μg of kanamycin/mL of medium). The plasmid was almost 100% stable when selection was applied. However, when cells were grown on PY medium without kanamycin, plasmid stability reached 90% after 7 d when cultures were renewed every 12 h (Fig. 2). The reason for this could have been that the pS1 plasmid was maintained stably whereas the *aprA* gene itself was deleted or rearranged. This was not the case, however, since all kanamycin-

Table 1
Sporulation Frequency of *B. subtilis* Strains Used[a]

Strain	Number of spores/mL
B. subtilis BioS11	5.73×10^8
B. subtilis BioS11 (pUB110)	5.71×10^8
B. subtilis BioS11 (pS1)	5.76×10^8

[a]Sporulation frequency was determined as described in the text. The pS1 is a pUB110 derivative plasmid that carries the complete *aprA* gene as well as a kanamycin resistance marker *(2)*.

resistant colonies also showed a high level of protease activity on 1XSG skim milk kanamycin plates. Thus, the recombinant pS1 plasmid is both segregationally and structurally stable (Fig. 2).

We reported previously the stability of this plasmid when it was harbored by the *B. subtilis* DB100 host strain. Plasmid stability reached only 66% after 4 d when cells were grown at 37°C on PY medium without kanamycin *(2)*. It is obviously clear that the plasmid-host interaction plays an important role in plasmid stability and that the newly constructed *B. subtilis* BioS11 (pS1) has proved to be a good host to harbor the pS1 plasmid. Consequently, enhanced proteolytic activity in addition to α-amylase activity will always be maintained.

Effect of High Copy Numbers of aprA *Gene on Growth Rate and Sporulation Frequency*

There are several reports concerning the inhibition of sporulation and/or metabolic rate of growth by the expression of high copy numbers of certain *B. subtilis* genes *(9)*. Growth rates of *B. subtilis* BioS11, *B. subtilis* BioS11 (pUB110), and *B. subtilis* BioS11 (pS1) strains were almost identical, indicating that the high copy number of the *aprA* gene did not affect growth rates. Moreover, sporulation frequency (number of spores/mL) of these clones was similar (Table 1). These results are in agreement with previous reports *(2,9,12)*.

Purification of Alkaline Protease and α-Amylase

Alkaline protease and α-amylase were purified using $(NH_4)_2SO_4$ selective precipitation (65% saturation) followed by a single-step affinity chromatography (activated CH-Sepharose 4B) column. This column uses the dye 4-(4-aminophenylazo) phenylarsenic acid, which can be coupled to an activated Sepharose 4B matrix at one site and to the subtilisin (alkaline protease) on the other site *(13)*. A specific interaction of the alkaline protease with the dye leads to a reversible inactivation of the enzyme.

The data in Table 2 illustrate that both alkaline protease and α-amylase were selectively purified using $(NH_4)_2SO_4$ to 43.6- and 41.2-fold, respectively. The two enzymes were purified further using the single-step affinity chromatography column. α-Amylase was eluted first since it did

Table 2
Purification Table of Alkaline Protease
and α-Amylase Enzymes Produced by Newly Constructed *B. subtilis* BioS11 (pS1) Strain

Purification step	Total protein (mg/mL)	Alkaline protease			α-amylase		
		U/mL[a]	U/ (mL·mg protein)[b]	Fold purification[c]	U/mL[d]	U/ (mL·mg protein)[b]	Fold purification[c]
Cell-free supernatant	8.0	12.0	1.5	1.0	19.4	2.43	1.0
$(NH_4)_2SO_4$ precipitation and dialysis	5.2	340.0	65.38	43.6	520.0	100.0	41.2
Affinity chromatography	1.2	180.0	150.0	100.0	—	—	—
	2.2	—	—	—	406.0	184.5	76.0

[a]One unit of enzyme activity is equal to the amount of enzyme that liberates 1 μmol of tyrosine from 10 mg of casein per 30 min at 37°C.
[b]Specific activity.
[c]Fold purification was derived by dividing specific activity at any step by that of the initial step.
[d]One unit of enzyme activity is equal to the amount of enzyme that hydrolyzes 5 mg of soluble starch per 30 min at 30°C.

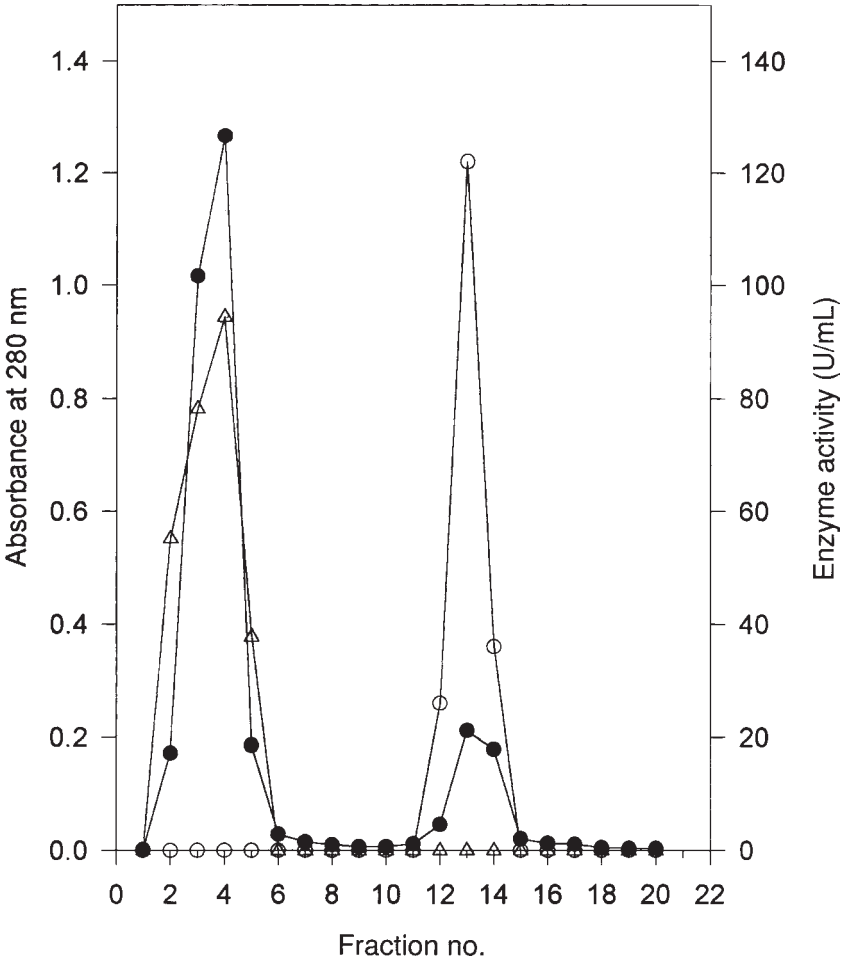

Fig. 3. Fractionation pattern of α-amylase and alkaline protease enzymes on using an activated CH-Sepharose 4B (affinity chromatography) column. ●, △, and ○: absorbance at 280 nm (protein content), α-amylase activity (U/mL), and alkaline protease activity (U/mL), respectively.

not bind to the column (Fig. 3). Fractions 1–6 were eluted with 20 m*M* acetate buffer, pH 5.9, containing 5 m*M* $CaCl_2$ and harbored all the α-amylase activity. Alkaline protease was then eluted with 10 m*M* Tris-HCl, pH 8.9, containing 5 m*M* $CaCl_2$ and 0.1 *M* NaCl starting with fraction 10. The protein peaks (absorbance at 280 nm) overlapped with the activity peaks of both α-amylase and alkaline protease. Moreover, the alkaline protease activity peak was very narrow, which was expected since the single-step column was proven to be a very specific column for alkaline protease. The enzyme was purified almost 100-fold (Table 2). Our data indicate that the use of the newly constructed strain would be valuable to the enzyme industry and would promote recycling of some food-processing wastes.

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