Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 5, 369-373 (1988)

Cloning and Expression of Thermostable Alpha-amylase Gene in *Escherichia coli* from *Bacillus licheniformis ATCC 27811*

Kim, I.C.[1], S.Y. Jang[1], J.H. Cha[1], Y.H. Ko[1], K.H. Park[1*] and H.M. Rho[2]

[1]*Department of Food Science and Technology, Seoul National University Suwon, 440-744, Korea*
[2]*Department of Zoology, Seoul National University, Seoul, 151-742, Korea*

Bacillus licheniformis ATCC 27811이 생산하는 내열성 α-amylase 유전자의 Cloning 및 발현

김인철[1]·장소영[1]·차재호[1]·고영환[1]·박관화[1*]·노현모[2]

[1]서울대학교 식품공학과 [2]동물학과

The gene for thermostable alpha-amylase from the thermostable bacterium *Bacillus licheniformis* has been cloned and expressed in *Escherichia coli.* The Alpha-amylase producing ***E. coli* cells contained a 7.4 kb chimeric plasmid (pTA 322) which was composed of the vector pBR 322 and a 3.1 kb EcoRI fragment of *B. licheniformis* DNA. The alpha-amylase from cloned fragement was shown to be indistinguishable from that of *B. licheniformis* in the optimum temperature of 90°C, heat stability and the pH stability. The foreign gene was expressed efficiently in *E. coli* and stably maintained.**

Alpha-amylase hydrolyzes the 1,4-alpha-glycosidic linkage in starch and it found in animals, plants and microorganisms. Alpha-amylase producers of commercial importance include *Bacillus subtilis*(1), *Bacillus licheniformis*(2), *Bacillus acidocaldarius*(3), and *Bacillus stearothermophilus*(4). It has been known that alpha-amylases secreted from *B. acidocaldarius, B. stearothermophilus,* and *B. coagulans,* were thermostable. The alpha-amylase from *B. licheniformas* has an optimum temperature of 90°C and can be used at the temperatures up to 110°C. In starch industry, the development of thermostable bacterial amylases which could operate at temperatures above 95°C enables the starch liquefaction process continuous.

The molecular cloning of thermostable alpha-amylase has been studied by many workers(5, 6). Cornelis *et al.*(5) cloned the alpha-amylase gene from *B. coagulans* into *E. coli* and the alpha-amylase was found to retain the thermostability. Palva (6) cloned *B. amyloliquefaciens* alpha-amylase into *B. subtilis* and the activity increased 2500 fold the activity secreted by *B. amyloliquefaciens.* Recently, the genes coding for the alpha amylase of *B. licheniformis*(7) were cloned into *B. subtilis* and the enzyme from *B. subtilis* was also heat stable. However, the productivity and the industrial application of the alpha-amylase of the transformant has not been studied intensively.

In this study, the gene of alpha-amylase from *B. licheniformis* ATCC 27811 was cloned and expressed in *E. coli.* The properties of alpha-amylase produced by the transformant were also characterized with regard to the possibility of an industrial application.

Materials and Methods

Microorganisms

B. licheniformis ATCC 27811 was obtained from the American Type Culture Collection (ATCC) and used as donor strain of thermostable alpha-amylase gene. *E. coli* HB 101 was used as host strain. SH broth(2) containing 4% soluble

Key words: Alpha-amylase, ther.. ostable enzyme, cloning of alpha-amylase.
*Corresponding author

starch, 0.35% $(NH_4)_2HPO_4$, 0.6% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% sodium citrate and 0.008% $CaCl_2 \cdot 2H_2O$ was used to produce alpha-amylase. The transformants were screened on solid LB medium containing 1.5% agar, 1% soluble starch and 50ug/ml of ampicillin.

Preparation of plasmid DNA and chromosomal DNA

Plasmid DNA was prepared by the rapid alkaline extraction method(8) and chromosomal DNA was isolated by the spool method(9).

DNA cloning

B. licheniformis DNA and the vector pBR 322 were cleaved with EcoRI. The mixture of the digested DNA of *B. licheniformis* and pBR 322 were annealed in buffer containing 20mM of Tris-HCl (pH 7.6), 10 mM of $MgCl_2$, 10mM of dithiothreitol, 0.6mM of ATP and ligated by adding 0.1 units of T_4-DNA ligase at 12.5 °C for 24 hours.

The ligated DNA was transformed into *E. coli* HB101 and the transformants were selected on LB plates containing ampicillin (50 μg/ml). The ampicillin-resistant colonies were toothpicked onto a solid LB plate containing 1% soluble starch. A 5 ml overlay of plating medium containing 3 mg of D-cycloserine was added to the plates. After overnight incubation at 37 °C, alpha-amylase producing colonies were detected by addition of a solution of 2.5% Lugol's iodine. Positive colonies were surrounded by a clear zone on the agar medium.

Enzyme assay

Alpha-amylase activity was estimated by the modified Fuwa method(10). The substrate solution containing 0.5 ml of 1% soluble starch and 0.25 ml of 0.05M Tris-HCl buffer (pH 7.5) was prewarmed at 50 °C for 5 min and 0.25 ml of the enzyme solution was added and incubated at 50 °C for 30 min. The reaction was terminated by adding 3 ml of DNS solution(11). After boiling the reaction mixture for 5 min, absorbance at 550 nm was measured. One unit of enzyme was defined as the amount of the enzyme causing an increase of 1.0 absorbance/30 min under the described conditions.

Production of alpha-amylase

B. licheniformis ATCC 27811 was precultured in LB broth for 25 hours at 50 °C and 4 ml of the preculture solution was transferred to 1 L-Erlenmeyer flask containing 200 ml of SH medium and cultivated at 50 °C for 7 days. The cells were harvested by centrifugation at 10000 × g and ammonium sulfate was added to the supernatant up to 80% saturation. The precipitate was suspended in 12.5 ml of 50 mM of Tris-HCl (pH 7.5) and dialyzed against the same buffer.

Transformant cells containing the alpha-amylase gene of *B. licheniformis* were cultured in 200 ml of LB medium at 37 °C for 24 hours and harvested by centrifugation at 10000 × g. The cells were suspended in 12.5 ml of 50 mM Tris-HCl (pH 7.5) and sonicated. The cell debris was discarded by centrifugation at 20000 × g and the supernatant was used as crude enzyme.

Liquefaction of starch

2.5 ml of alpha-amylase solution was added to the starch slurry of 30% (pH 6.8) containing 50 mM calcium ion. The reaction mixture was heated up to 110 °C in steam jacket and held for 5 min, and then cooled to 95 °C and hold for 1 hour. Dextrose equivalents (DE) of the resulting hydrolysate were measured by cryoscope (Fiske, U.S.A)

Results and Discussion

Cloning the gene for alpha-amylase

The chromosomal DNA from *B. licheniformis* and plasmid pBR 322 were digested with EcoRI,

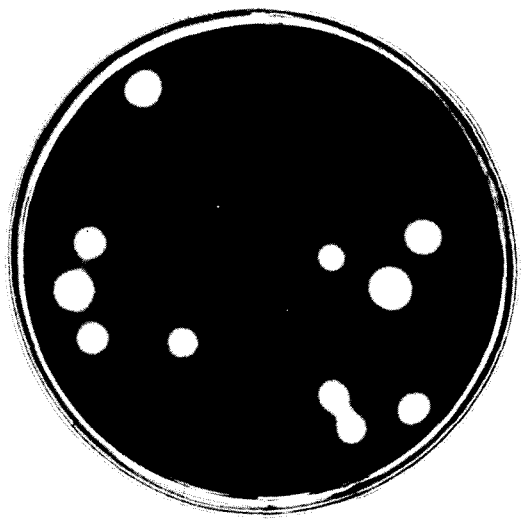

Fig. 1. Screening of *E. coli* HB101 harbouring recombinant plasmid pTA 322.

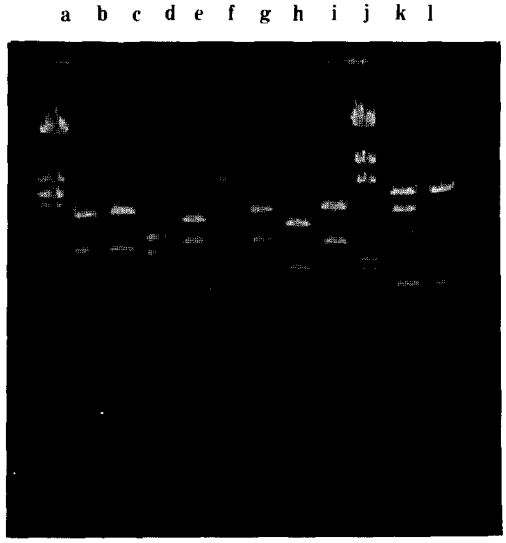

Fig. 2. Restriction patterns of the recombinant plasmid pTA 322.

a : λDNA + EcoRI, b : pTA322 + HindIII + EcoRI
c : pTA322 + HindIII, d : pTA322 + PstI
e : pTA322 + BamHI + EcoRI, f : pTA322 + BamHI
g : pTA322 + EcoRI, h : pTA322 + SalI + EcoRI
i : pTA322 + SalI, j : λDNA + HindIII
k : pTA322 + EcoRI + ClaI, l : pTA322 + ClaI

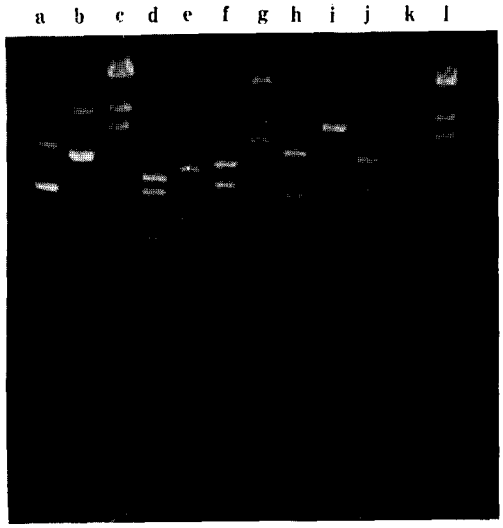

Fig. 3. Restriction patterns of the recombinant plasmid pTA 322.

a : intact pBR322 b : intact pTA322
c : λDMA + HindIII, d : pTA322 + PstI + BamHI
e : pTA322 + PstI + EcoRI,
f : pTA322 + EcoRI + BamHI
g : λDNA + EcoRI, h : pTA322 + PstI
i : pTA322 + BamHI, j : pTA322 + EcoRI
k : pBR322 + EcoRI, l : λDNA + HindIII

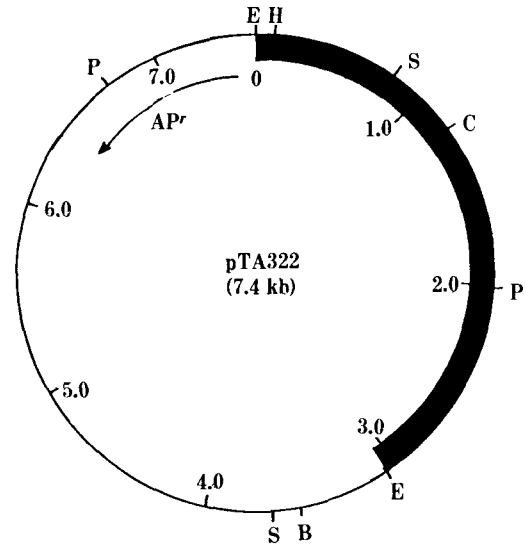

Fig. 4. Restriction map of plasmid pTA322.
Thick line indicates the insert containing α-amylase gene. Restriction sites, E: EcoRI, P: PstI, H: HindIII, C: Cla I, S: SalI, B: BamHI

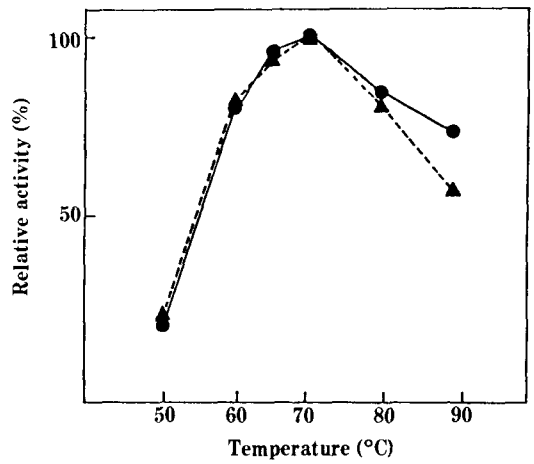

Fig. 5. Temperature-activity profile of α-amylase from *B. licheniformis* ATCC 27811 (▲—▲) and *E. coli* HB101 containing pTA322 (●—●).

ligated and transformed into *E. coli* HB 101. About 5000 ampicillin resistant colonies were selected for harbouring a recombinant DNA. The transformants were grown on LB media. After cell lysis, alpha-amylase positive colonies were selected by iodine staining (Fig. 1). Plasmid DNA was isolated from the alpha-amylase positive colony and re-transformed into *E. coli* HB 101. It was confirmed that the chimeric plasmid contained the gene coding

alpha-amylase (Fig. 1).

Characterization of the cloned alpha-amylase gene

The recombinant plasmide (pTA 322) was isolated from the alpha-amylase producing transformant, and digested with EcoRI. The 3.1 kb insert was confirmed by agarose gel electrophoresis (Fig. 2, lane g). For mapping the insert, the chimeric plasmid pTA 322 was digested with Hind III, Bam HI, Pst I, Sal I and Cla I, and the fragments were electrophoresed on a agarose gel. The insert has a single restriction endonuclease site for Hind III, Sal I, Cla I and Pst I (Fig. 2 and 3), but the site for Bam HI was not found. The restriction endonuclease map of pTA 322 is indicated in Fig. 4.

Properties of alpha-amylase produced by *E. coli* HB 101/pTA 322

The temperature dependence of alpha-amylase produced by *E. coli* HB 101 harbouring pTA 322 was compared with that of the donor strain, *B. licheniformis* ATCC 27811. Both enzymes exhibited similar temperature profile and the temperature optimum of 70 °C (Fig. 5). In the presence of Ca^{++} ion, the temperature optimum of both alpha-amylases was shifted to 90 °C (Fig. 6). The pH stability has been also examined (Fig. 7). In the pH stability, no significant differences between both enzymes were observed. The above results suggested that the alpha-amylases produced by the transformant and donor strain were very similar in their enzymatic properties.

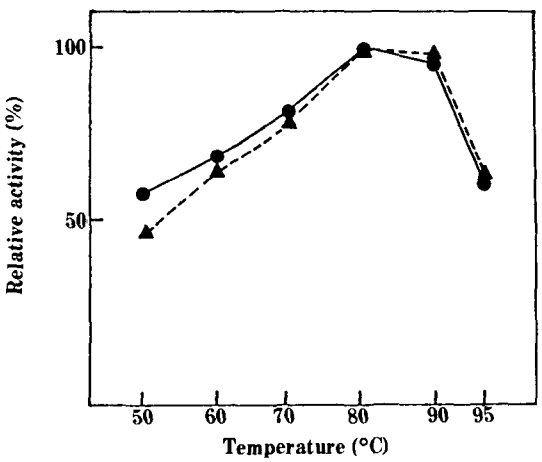

Fig. 6. Temperature-activity profile of α-amylase from *B. licheniformis* ATCC 27811 (▲—▲) and *E. coli* HB101 containing pTA322 (●—●) in the presence of 25 mM calcium ion.

Starch liquefaction and productivity

To ensure the thrmostability and liquefaction ability of the enzyme the starch slurry of 30% was hydrolyzed by alpha-amylase of the transformant and dextrose equivalents of the starch were compared with those by the commercial enzyme, Termamyl® at a same level of enzyme concentration. The final DEs of hydrolyzed starch by alpha-amylase from *E. coli*/pTA 322 and Termamyl® were 25.5 and 23.4, respectively.

The alpha-amylase activities produced by the transformant and the donor strain, *B. licheniformis* ATCC 27811 were shown in Table 1. *E. coli* HB 101 did not produce the amylolytic activity. However, an approx. 100-fold increase of the alpha-amylase activity was achieved in *E. coli* HB 101/pTA 322.

Discussion

The thermostable alpha-amylase gene from *B.*

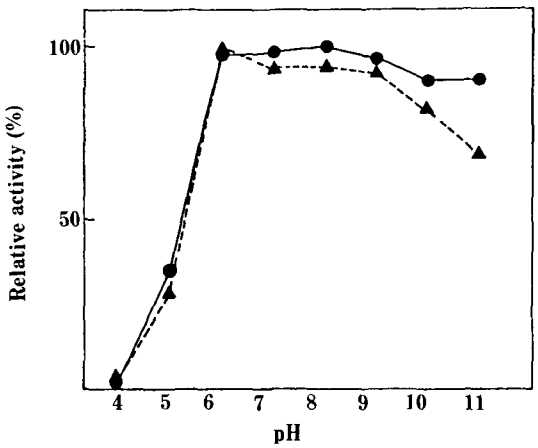

Fig. 7. Effect of pH on the stability of α-amylase from *B. licheniformis* ATCC 27811 (▲—▲) and *E. coli* HB101 containing pTA322 (●—●).

Table 1. Amounts of the α-amylase produced by *B. licheniformis* ATCC 27811 and *E. coli* HB101 containing pTA 322.

Strains	Enzyme activity (U/ml culture solution)
B. licheniformis ATCC 27811	1.20
E. coli HB101	0.0
E. coli HB101/pTA 322	119.50

licheniformis was transformed and expressed in *E. coli*. The transformed *E. coli* strain secreted alpha-amylase of the donor strain, *B. licheniformis* ATCC 27811. Since both enzymes were indistinguishable in temperature- and pH-dependence, reaction rate and thermostability.

Alpha-amylase produced by *E. coli* HB 101/pTA exhibited the temperature optimum of 90 °C in the presence of Ca^{++} ion. Yoon(12) has reported that the thermostability of alpha-amylase from *B. licheniformis* was increased in the presence of starch. Therefore we expect that the thermostability of alpha-amylase from the transformant can be increased in the presence of starch and used for brief periods at temperatures up to 110 °C. When dextrose equivalents of the starch liquefied by the transformant were compared with that by Termanyl^{R}, the enzyme of *E. coli*/pTA 322 was considered to be sufficient to liquefy starch almost completely at temperatures above 100 °C. Moreover, the culture time has been shortened to 5-6 days in the production of alpha-amylase in *E. coli*. In future the greater increase in the alpha-amylase production by *E. coli* can be obtained by the optimization of the fermentation conditions.

요 약

Bacillus licheniformis ATCC 27811의 염색체 DNA를 분리하며 제한효소인 Eco RI으로 부분절단하고, 동일 효소로 절단한 plasmid pBR 322에 ligation 시킨 뒤 *E. coli* HB 101에 형질전환시켜 alpha-amylase 형질을 보여주는 균주를 선별하였다.

선별된 형질전환체로부터 alpha-amylase를 분리하며, 원 균주인 *Bac. licheniformis*가 생산하는 alpha-amylase와 pH 및 온도특성을 비교하며 모균주와 같은 성질을 가졌음을 확인하였다. 재조합체 DNA로부터 얻은 Insert는 대략 3.1 kb 정도였고, Hind III, Cla I, Pst I, Sal I Site를 한개씩 가지고 있었다.

Acknowledgement

This research was supported by Sunkyong Agricultural Research Fund.

References

1. Yamane, K. and B. Maruo: *J. Bacteriol.*, **120,** 792 (1974).
2. Saito, N.: *Arch. Biochem. Biophys.*, **155,** 290 (1973).
3. Buonocore, V., Caporale, C., de Rosa M. and Gambacorta, A.: *J. Bacteriol.*, **128,** 515 (1976).
4. Pfueller, S.L. and W.H. Elliott: *J. Biol. Chem.*, **244,** 48 (1969).
5. Cornelis, P., C. Digneffe and K. Willemot: *Mol. Gen. Genet.*, **186,** 507 (1982).
6. Palva, I.: *Gene* **91,** 81 (1982).
7. Yuuki, T., Nomura T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N. and Udaka, S.: *J. Biochem.*, **98,** 1147 (1985).
8. Maniatis, T., Fritsch E.F. and Sambook, J.: *Molecular Clonings.* A Laboratory manuals, Cold Spring Harbor Laboratory (1982).
9. Dubnau, D. and Abelson, R.D.: *J. Mol. Biol.*, **56,** 209 (1971).
10. Fuwa, H.: *J. Biochem.*, **41,** 583 (1954).
11. Miller, G.L.: *Analytical Chem.*, **31,** 426 (1959).
12. Yoon, C.W.: *M.S. thesis,* Seoul National Univ. (1987).

(Received August 16, 1988)