Kor. J. Microbiol. Biotechnol.
Vol. 34, No. 4, 318–322 (2006)

Production of Intracellular Invertase from Alkalophilic and Thermophilic *Bacillus* sp. TA-11 in the Recombinant *E. coli*

Yi Sung-Hun[1], Dae-Hyung Lee, Jae-Duck No, Jae-Won Lee, Dae-Hyoung Lee, and Jong-Soo Lee*
Department of Life Science and Genetic Engineering, Paichai University, Daejeon 302-735 Korea
[1]Department of Food Science and Agricultural Chemistry, Macdonald College of McGill University, 21, 111 Lakeshore, Saint-Anne-De-Bellevue, Quebec, Canada H9X 3V9

The intracellular invertase gene of alkalophilic and thermophilic *Bacillus* sp. TA-11 which was isolated from compost was cloned and expressed in *E. coli* HB101 using pUC19 as a vector. The invertase of the recombinant *E. coli* (pYC17) was maximally produced when it was incubated at 37°C for 9 h in a SY medium containing 0.25% sucrose, 0.5% yeast extract, 0.1% each of K_2HPO_4 and KH_2PO_4, with an initial pH of 8.0. The final enzyme activity under the above condition was 47.7 U per ml of cell-free extract.

Key words: Intracellular invertase, alkalophilic and thermophilic *Bacillus* sp. TA-11

Invertase (*β*-fructofuranosidase : *β*-D-fructofuranoside fructohydrolase : EC 3.2.1.26) hydrolyzes sucrose to glucose and fructose. It also catalyzes transfructosylation reaction with sucrose as substrate, resulting in the formation of various isomers of ketose type trisaccharides (fructosyl sucrose) [1-4]. It is widely used in food and medical industries for the production of fructose syrup and fructooligosaccharides which function as a low-calorie sweetener, a bifidus growth factor and an anticarious substance [4,5].

The studies of the invertase have been performed extensively in various microorganisms [6-11] and plants [12-18] and its molecular mass were also various such as 58 kDa from *Zymomonas mobilis* [19], 135 kDa and 270 kDa from *Saccharomyces* sp. [1,3], 340 kDa from *Aspergillus niger* [9], 48 kDa from *Streptococcus mutans* [20], 52 kDa from *Arthrobacter* sp. K-1 [8]. However, there is little information concerning either molecular biological and biochemical studies for the mass production of intracellular invertase in the recombinant through the molecular cloning of the invertase gene. Only single gene encoding both the intracellular and extracellular invertase from yeast [21], invertase gene of *Streptococcus salvivarius* [10] and sucrase gene from *Bacillus subtilis* [7] have been cloned and sequenced. Three structural genes in *Bacillus subtilis* are known to be induced by sucrose: *sacA*, *sacB*, and *sacP* which code for sucrase and levansucrase, and a membrane component of the PEP-dependent phosphotransferase system of the sucrose transport, respectively. Regulatory loci of *sacS* and *sacT* control the expression of *sacA* and *sacP* [7].

In previous paper, a potently invertase-producing alkalophilic and thermotolerant *Bacillus* sp. TA-11 was isolated from compost and its optimal condition for invertase production were also investigated [4]. In this study, cloning of the intracellular invertase gene from alkalophilic and thermophilic *Bacillus* sp. TA-11 and expression in *E. coli* HB101 were investigated for the mass production of alkalophilic and thermotolerant invertase. Moreover, the production condition of intracellular invertase from the recombinant *E. coli* was also optimized.

*Corresponding author
Tel/Fax: 82-42-520-5388
E-mail: biotech8@pcu.ac.kr

Materials and Methods

Bacterial Strains, Plasmids and Cultivation

Alkalophilic and thermophilic *Bacillus* sp. TA-11 as described in a previous paper [4] was used as a donor strain of the invertase gene. *E. coli* HB101 was used as a host for cloning. pGEMEX-1 and pUC19 (Promega, U.S.A.) were used as vectors for cloning and subcloning, respectively, of the invertase gene. SY medium (1.0% sucrose, 0.6% yeast extracts, 0.1% each of K_2HPO_4 and KH_2PO_4, pH 9.5) was

used for study of invertase production and its molecular cloning. Transformamants was grown in LB medium containing ampicillin (100 μg/ml) at 37°C.

Chemicals

All chemicals used were of analytical grade. Glucose, dinitrosalicylic acid, the electrophoresis reagents, acetonitril, cesium chloride, ethidium bromide, dithiothreitol and RNase were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Sucrose, ampicillin, calcium chloride, and agarose were procured from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Ammonium persulfate was purchased from Bio-Rad Lab. (California, U.S.A.) and triphenyl tetrazolium chloride (TTC) from Acros Organics (New Jersey, U.S.A.). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG) and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim (Mannheim, Germany), and the T4 DNA ligase and all restriction enzymes were sourced from Promega (Madison, U.S.A.). Bacto-tryptone, Bacto-peptone, beef extract, yeast extract, trypticase and Bacto-agar were purchased from Difco Lab (Detroit, Michigan, U.S.A.).

Assay of Invertase Activity

The invertase activity was assayed by measuring the amount of reducing sugar released from sucrose as the substrate [4]. The assay mixture contained 0.1 M sucrose, 0.1 M phosphate buffer (pH 6.5), and 0.2 ml of an enzyme solution in a total volume of 1 ml. After incubation for 2 h at 37°C, the reducing sugar formed in the reaction mixture was measured through a dinitrosalicylic acid method [22]. One unit of activity was defined as the amount of enzyme required to produce reducing sugar equivalent to 1 μmol of glucose per min.

Cloning of Invertase Gene from *Bacillus* sp. TA-11

Bacillus sp. TA-11 was grown to log phase in SY broth at 50°C while shaking. The chromosomal DNA of *Bacillus* sp. TA-11 was isolated using the methods of Rodriguez [23] with a slight modification and purified by treatments of RNase, isopropanol, or phenol/chloroform. Chromosomal DNA of *Bacillus* sp. TA-11 was partially digested with EcoR I, BamH I and Hind III, successively, and ligated with pGEMEX-1 digested with same restriction enzyme and further treated with calf intestinal alkaline phosphatase (CIP). Ligation of the chromosomal DNA (0.6 μg) was carried out by adding T4 DNA ligase in a ligation buffer with a final volume of 10 μl for 14 h at 14°C.

Competent cells of *E. coli* HB101 for transformation were routinely prepared using the method of Rodriguez [23]. The ligated DNA sample was added to 100 μl of competent cells and mildly mixed. After standing on ice for 60 min, the cell suspension was heated at 42°C for 90 sec and then chilled briefly on ice. The mixture was diluted into an appropriate volume of LB broth and incubated at 37°C for 60 min without shaking. The cells were plated on selective plates containing ampicillin and triphenyl tetrazolium chloride (TTC) [23] and incubated at 37°C for 24 h. The red colonies were selected from the agar plate as invertase positive clones. The inserted DNA of the positive clone was digested with EcoR I and inserted into pUC 19 which was digested with EcoR I and transformed into *E. coli* HB101. A clone showing a red color on the LB/TTC/ampicillin plate was selected.

Results and Discussion

Cloning of Invertase Gene from Alkalophilic and Thermophilic *Bacillus* sp. TA-11

A gene library of *Bacillus* sp. TA-11 genomic DNA was constructed in *E. coli* HB 101 and a positive clone, showing a red color on the LB medium supplemented with TTC and strong invertase activity was selected. The inserted DNA (pYC17) of the positive clone was subcloned into a plasmid vector, pUC19, which was digested with EcoR I. Following this, the recombinant DNA was transformed into *E. coli* HB101. A clone which showed a red color on the LB medium containing TTC and ampicillin as well as a high level of invertase activity(25.5 U/ml-cell free extract) was selected and designated as the recombinant *E. coli* (pYC17). Meanwhile, extracellular invertase activity of the recombinant *E. coli* (pYC17) was not determined.

Production Condition of the Invertase in the Recombinant *E. coli*

Table 1 show the effect of the concentration of sucrose on the invertase production in the recombinant *E. coli* (pYC17). The invertase activity increased as the level of sucrose increasing to 0.25%, and then decreased as the sucrose level increased further in concentration. However,

Table 1. Effects of sucrose on invertase production in the recombinant *E. coli* (pYC17).

Sucrose (%)	Cell growth (A_{660})	Invertase activity (U/ml-cell free extract)
0.1	1.0	16.9
0.25	1.2	27.8
0.5	1.0	26.9
1.0	1.1	25.0
1.5	1.1	21.3
3.0	1.2	19.4

*Cultivation was carried out as described in Bacterial strains, plasmids and cultivation of Material and Method at 37°C for 12 h.

Table 2. Effects of yeast extract on invertase production in the recombinant *E. coli* (pYC17).

Yeast extract (%)	Cell growth (A_{660})	Invertase activity (U/ml-cell free extract)
0.1	1.0	9.5
0.3	1.2	13.9
0.5	1.8	27.8
0.7	1.4	25.0
1.0	1.4	23.0

*Cultivation was carried out as described in Bacterial strains, plasmids and cultivation of Material and Method at 37°C for 12 h.

the cell growth did not change as the sucrose concentration increased : therefore, the optimum concentration of sucrose for enzyme production was 0.25% (7.3 mM). This is a lower concentration than that of the parent strain (1.0%) [4].

To investigate the effect of the yeast extract concentration on invertase production, yeast extract was added to the SY medium at various concentrations and the invertase activity of the cell-free extract were determined. As shown in Table 2, the invertase was maximally produced at 0.5% of yeast extract, similar to the parent strain (0.6%) [4].

The effect of the initial pH of the medium on the production of invertase was examined in various pH levels of SY medium (Fig. 1). The optimum initial pH of the medium for the production of invertase was pH 8.0 (27.8 U/ml) and significant levels of enzyme (24.5 U/ml) was also produced at pH 9.0. These results are similar with those of the parent strain (*Bacillus* sp. TA-11) [4].

The optimal culture temperature for enzyme production was 37°C. Over this temperature, enzyme production and cell growth were inhibited markedly (Fig. 2). This temperature was lower than that of parent strain, alkalophilic and thermophilic *Bacillus* sp. TA-11 (50°C) [4].

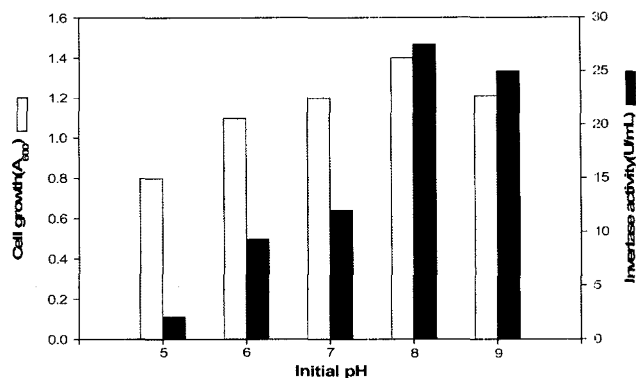

Fig. 1. Effects of initial pH of medium on invertase production from the recombinant *E. coli* (pYC17). *Cultivation was carried out as described in Material and Method at 37°C for 12 h.

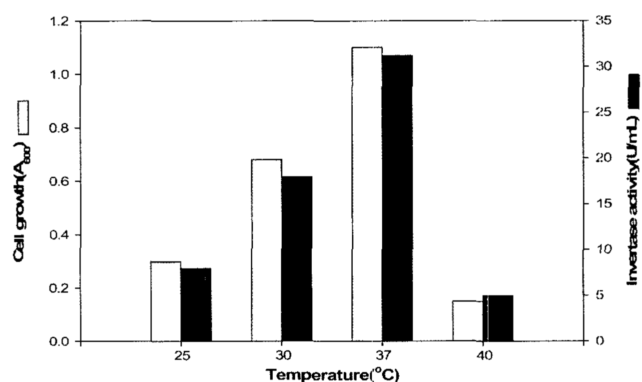

Fig. 2. Effects of cultural temperature on invertase production from the recombinant *E. coli* (pYC17). *Cultivation was carried out as described in Material and Method at 37°C for 12 h.

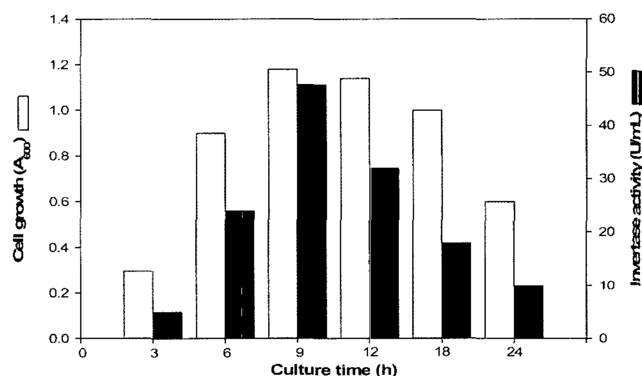

Fig. 3. Time course for production of invertase from the recombinant *E. coli* (pYC17)

The time course of the enzyme production was determined under the optimal culture conditions for the invertase production. As shown in Fig. 3, the maximal levels of cell growth and enzyme production (47.7 U/ml) were observed after cultivation for 9 h, whereas the

maximal enzyme production in the parent strain was reached after cultivation for 48 h [4]. Less culture time for invertase production compared to the parent strain is very important in terms of invertase productivity. Hence, the recombinant *E. coli* (pYC17) can be more useful for the mass production of invertase and useful as well in the food industry compared to the parent strain.

재조합 대장균에서 호알칼리성, 고온성 *Bacillus* sp. TA-11의 세포내 Invertase의 생산

이성훈[1] · 이대형 · 노재덕 · 이대형 · 이종수
배재대학교 생명유전공학과, [1]맥길대학교 식품 · 농화학과

알칼리와 고온에 대한 내성을 가진 invertase를 대량생산하기 위하여 호알칼리성이며 고온성 세균인 *Bacillus* sp. TA-11의 세포내 invertase 유전자를 pUC 19 벡터를 이용하여 *E. coli* HB101에 클로닝 시키고 발현시켜 invertase를 강력하게 생산하는 재조합 *E. coli* (pYC17)를 얻었다. 재조합 *E. coli*(pYC17)를 이용한 invertase 생산 최적조건을 검토한 결과 *E. coli*(pYC17)를 0.25% sucrose, 0.5% yeast extract, 0.1% K_2HPO_4와 0.1% KH_2PO_4을 함유한 SY 배지(초기 pH 9.0)에 접종하여 37°C에서 9시간 배양하였을 때 친주보다도 많은 47.7 U/ml-cell free extract의 invertase가 생산되었다.

REFERENCES

1. Abrams, B. B., R. Hckel, T. Mizunaga, and J. O. Lampen, 1978. Relationship of large and small invertase in *Saccharomyese*; Mutant selectively deficient in small invertase. *J. Bacteriol.* **135**: 809-817.
2. Arnold, W. N. 1972. Location of acid phosphatase and *β*-fructofuranosidase within yeast cell envelopes. *J. Bacteriol.* **112**: 1346-1352.
3. Babczinski, P. 1980. Partial purification, characterization and localization of the membrane-associated invertase of yeast. *Biochim. Biophys. Acta.* **614**: 121-133.
4. Choi, Y. J. and J. S. Lee. 1995. Production of *β*-fructofuransosidase from alkalophilic, thermophilic *Bacillus* sp. TA-11. *Korean J. Appl. Microbiol. Biotechnol.* **23**: 197-202.
5. Fitzgerald, R. J., D. M. Spinell, and T. H. Stoudt. 1968. Enzymatic removal of artificial plague. *Arch. Oral. Biol.* **13**: 125-128.
6. Dodyk, F. and A. Rothstein. 1964. Factors influencing the apperarance of invertase in *Saccharomyces cerevisiae. Arch. Biochem. Biophys.* **104**: 478-486.
7. Fouet, A., A. Klier, and G. Rapoport. 1986. Nucleotide sequence of the sucrose gene of *Bacillus subtilis. Gene.* **45**: 221-225.
8. Fujita, K., K. Hara, H. Hashimoto, and S. Kithata. 1990. Purification and some properties of *β*-furctofuranosidase I from *Arthrobacter* sp. K-1. *Agric. Biol. Chem.* **54**: 913-919.
9. Hirayama, M., N. Sumi, and H. Hidaka. 1989. Purification and properties of a fructooligosaccharides-producing fructofuranosidase from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* **53**: 667-673.
10. Houck, C. M., J. R. Pear, R. Elliott, and J. T. Perchorowicz. 1987. Isolation of DNA encoding sucrase genes from *Streptococcus salivarius* and partial characterization of the enzymes expressed in *Escherichia coli. J. Bacteriol.* **169**: 3679-3684.
11. Kidby, D. K. and R. Davies. 1970. Invertase and disulphide bridges in the yeast wall. *J. Gen. Microbiol.* **61**: 327-333.
12. Kim, B. M. 1980. Studies on invertase from Kroean ginseng, Panax ginseng C. A. Meyer. *Korean J. Food. Sci. Technol.* **12**: 1-5.
13. Krishnan, H. B., J. T. Blanchette, and T. W. Okita. 1985. Wheat invertase. *Plant Physiol.* **78**: 241-245.
14. Nakagawa, H., Y. Kawasaki, N. Ogura, and H. Takehata. 1971. Purification and some properties two types of *β*-fructofuranosidase from *tomato fruit*. *Agric. Biol. Chem.* **36**: 18-26.
15. Obenland, D., U. Simmen, T. Boller, and A. Wiemken. 1993. Purification and characterization of three soluble invertase from *barley leaves*. *Plant Physiol.* **101**: 1331-1339.
16. Porntaveewat, W., T. Takayanagi, and K. Yokotsuka. 1994. Purification and properties of invertase from Muscat Bailey A grapes. *J. Ferment. Bioeng.* **78**: 288-292.
17. Pressey, R. 1966. Separation and properties of potato invertase and invertase inhibitor. *Arch. Biochem. Biophys.* **113**: 667-674.
18. Unger, C., J. Hofsteenge, and A. Sturm. 1992. Purification and characterization of a soluble *β*-fructofuranosidase from *Daucus carota. Eur. J. Biochem.* **204**: 915-921.
19. Yanase, H., H. Fukushi, N. Ueda, Y. Maeda, A. Toyoda, and K. Toromura. 1991. Cloning, Sequencing, and characterization of the intracelluar invertase gene from *Zymomonas mobilis. Agric. Biol. Chem.* **55**: 1383-1390.
20. Tanzer, J. M., A. T. Brown, and M. F. Mclnerney. 1973. Identification, preliminary characterization, and evidence for reg-

ulation of invertase in *Streptococcus* mutants. *J. Bacteriol.* **116**: 192-202.

21. Ottolenghi, P. 1971. A comparison of five genetically distinct invertase from *Saccharomyces. Eur. J. Biochem.* **18**: 544-552.
22. Miller, G. L. 1959. Uses of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426-428.
23. Rodriguez, R. L. and R. C. Tait. 1983 Recombinant DNA techniques : Introduction. pp. 37-51. Addison-Wesley Pub. *co.* Massachusette.

(Received Aug. 18, 2006/Accepted Nov. 21, 2006)