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RESEARCH NOTE

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Heterologous Expression of α-Amylase Gene of *Bifidobacterium adolescentis* Int57 in *Bacillus polyfermenticus* SCD

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Abstract *Bacillus polyfermenticus* SCD was transformed by the recombinant shuttle vector for *Bacillus* and *Escherichia coli* containing 3 antibiotic resistant genes and an α-amylase gene from *Bifidobacterium adolescentis* Int57. The α-amylase gene fused to a secretion sequences was expressed under the control of the promoter of amylase gene from *B. subtilis* var. *natto*. The recombinant plasmid was maintained stably in the transformants producing the α-amylase. The enzyme was secreted to outside of the cell and showed the similar enzyme activity as that of *Bacillus subtilis* BD170 under the same conditions of pH and growth temperature. Because of the relatively easy transformation and the secretion of the enzyme, the transformants of *B. polyfermenticus* SCD may give a new strategy in the production of foreign genes.

Keywords: *Bacillus polyfermenticus* SCD, *Bifidobacterium*, α-amylase gene, heterologous expression

Introduction

Bacillus polyfermenticus, commonly called as 'Bispan' strain, produces a bacteriocin characterized as polyfermenticin which inactivates several strains such as *Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Bacillus pumilis, Bacillus subtilis*, and *Listeria monocytogenes* (1). Several studies suggested that the *B. polyfermenticus* SCD is significantly similar to *B. subtilis* strains in terms of morphological and biochemical properties (2). Although considerable informations have been accumulated concerning the microecology, taxonomy, and microphysiology of *B. subtilis*, relatively little research has been reported on the genetic analysis of *B. polyfermenticus* SCD. However, the *B. polyfermenticus* SCD is still distinct from *B. subtilis* strains. The former is capable of metabolizing lactose and produces a larger amount of acetic acid and lactic acid from glucose and lactose, respectively, than the latter does (3). *B. polyfermenticus* SCD has been also used as a probiotic bacteria, because the bacteria not only competes and suppresses unhealthy fermentation in human intestine, but also produces a number of beneficial health effects of their own (2, 4-9). The genus *Bacillus* includes a variety of industrially important species, which are commonly used as hosts in the bioindustries (10, 11). In this point of view, it may be attractive to express a foreign gene in *B. polyfermenticus* SCD as host for the production of available enzymes, which is not produced in the cells. Especially for the applications in bioindustries, the genetic transformation of the bacteria may be important. However, as far as we know, the transformation of *B. polyfermenticus* SCD has not been reported. The genetic transformation is important to develop and production of new substances in *B. polyfermenticus* SCD as host in bioindustries, because the bacteria produces some beneficial compounds and is not toxic to human.

In this study, we describe the transformation of *B. polyfermenticus* SCD by using the α-amylase gene cloned newly in our previous work from *Bifidobacterium adolescentis* Int57 (12). It was observed that the amylase gene in *B. polyfermenticus* SCD was good expressed. The expression of amylase can give benefits for the application commercially in digestion of starch, because the production of amylase in *B. polyfermenticus* SCD may not arise the conflict in toxic compounds against human health.

Materials and Methods

Bacterial strains, plasmids, and media *B. polyfermenticus* SCD and *B. subtilis* BD170 was used as host bacteria for the expression of α-amylase gene from *B. adolescentis* Int57. *Escherichia coli* DH5α was used for recombinant DNA manipulation. The bacteria strains were routinely grown 37°C in LB medium [1%(w/v) tryptone, 0.5%(w/v) yeast extract, 1%(w/v) NaCl], and transformants were selected and grown in the same medium supplemented with 50 μg/mL of kanamycin. For solid medium, 1.5%(w/v) agar was added. The *B. subtilis/E. coli* shuttle vector pRB373 (13) and the plasmid p8A1 (14) were used for the construction of the recombinant plasmid containing α-amylase gene. The competent cells for the transformation were prepared from *B. polyfermenticus* SCD and *B. subtilis* BD170 cultured in the medium SPMM-I [0.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, 1.4% K_2HPO_4, 0.1% sodium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, 0.2% casamino acid] and SPMM-II, supplemented with 0.5 mM $CaCl_2$

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and 1 mM $MgCl_2$ to SPMM-I.

Construction of recombinant plasmid for an amylase gene expression The shuttle vector pRB373, which contains 2 replication origins, antibiotic resistant genes (ampicillin, bleomycin, and neomycin), T_1 and T_0 transcriptional terminators for foreign gene expression, and multiple cloning sites (MCS) (15), was used to construct vector for amylase gene expression in *B. polyfermenticus* SCD and *B. subtilis* BD170. The plasmid p8A1 was digested with *Bam*HI and *Xba*I. The 500 bp fragment harboring promoter and secret signal sequence was inserted into the same enzyme restriction sites of pRB373. The constructed plasmid pKY1 was further digested by *Kpn*I and *Sac*I to insert the amylase gene of *B. adolescentis* Int57. The recombinant plasmid pKYamy1 containing the amylase gene was fragmented by the digestion with *Sma*I and *Eco*RV, and then self-ligated to remove the other restriction enzyme sites. The resulting plasmid was designated as pKYamy2. The construction and restriction map of the recombinant plasmids above were listed in Fig. 1.

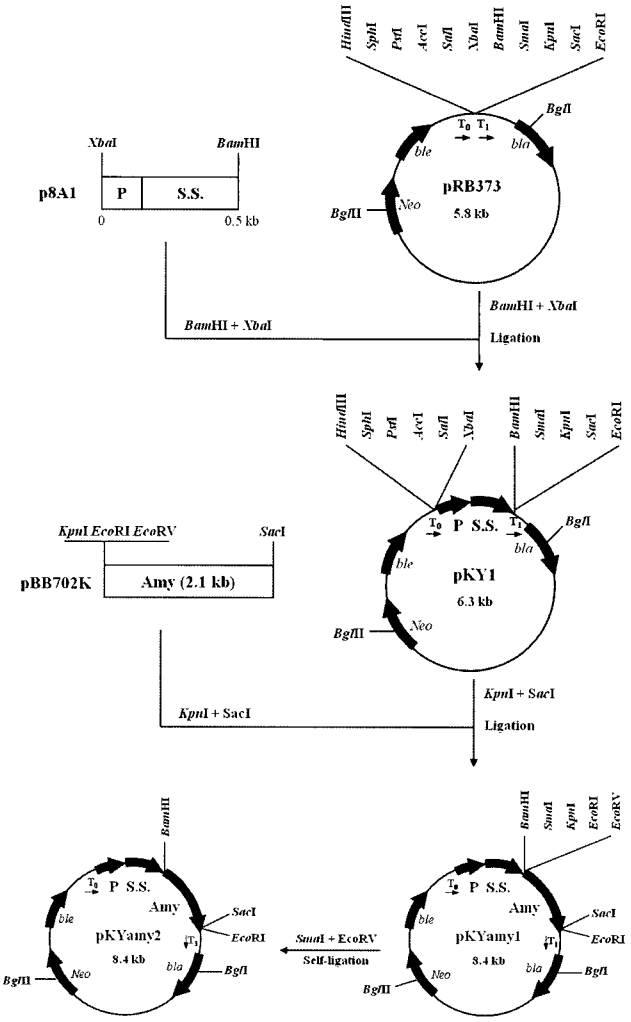

Fig. 1. Construction of the vector pKYamy2. The P and S.S. indicate promoter and signal sequence of α-amylase from *B. subtilis* var. *natto*, respectively. Amy, α-amylase gene of *B. adolescentics* Int57; T_0 and T_1, transcriptional terminators; bla, ampicillin resistant gene; ble, bleomycin resistant gene; neo, neomycin resistant gene.

Determination of minimal inhibitory concentration (MIC) To determine the MIC of antibiotic against *B. polyfermenticus* SCD, 1×10^5 cells were spread on LB plates containing serial concentrations of kanamycin (0.4, 0.8, 1.6, 3.2, 6.4, 15.5, 25, and 50 μg/mL). After incubation at 37°C overnight, the MIC was determined through the formation of colonies.

Transformation of *B. polyfermenticus* SCD Transformation was done with a modification of the method introduced by Sadaie and Kada (16). *B. polyfermenticus* SCD was inoculated in 5 mL of 0.5 × LB broth and precultured at 37°C for 24 hr. Eighteen mL of SPMM-I broth were inoculated with 2 mL of the precultured cells, and cultured at 37°C for 150 min with vigorous shaking until it entered early stationary phase in the growth curve. The culture was added to 20 mL of prewarmed SPMM-II broth and incubated at 37°C for 90 min at 180 rpm. One mL of cell suspension was mixed with plasmid DNA and the mixture was incubated at 37°C for 40 min at 180 rpm. Then, 2 mL of LB broth was added and incubated with the same speed at 37°C for 1 hr. Transformed cells were centrifuged at 3,000×g for 10 min. The pellet was resuspended with 100 μL of sterilized water and spread on LB agar plates containing 50 μg/mL of kanamycin and 1% (w/v) soluble starch. The amylase-producing colonies were detected by the addition of iodine solution [5%(w/v) I_2, 5%(w/v) KI].

Amylase activity assay Amylase activity was measured by the method of Miller (17) with some modifications. An aliquot of 1 mL was taken from a bacteria culture and centrifuged. Twenty μL of the supernatant was directly transferred to the enzyme reaction mixture to test the enzyme activity. The pellet was washed twice with distilled water, reuspended with 50 mM acetate buffer (pH 5.5), and sonicated on ice. After centrifugation at 12,000× g for 20 min, the supernatants were used as intracellular enzyme solution. Each solution was used for determination of amylase activity. The reaction mixture containing 250 μL of 1%(w/v) soluble starch in 50 mM acetate buffer (pH 5.5) and 230 μL of the same buffer was prewarmed at 50°C for 5 min. The enzyme solution (20 μL) was added to the prewarmed solution and the mixture was incubated for 30 min. The reaction was stopped by adding 1.5 mL of dinitrosalicylic acid (DNS) solution. After boiling for 5 min in a water bath, the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme librating reducing sugar equivalent to 1 μmol of maltose from starch per min at the same conditions.

Preparation of plasmid and growth curve The plasmid was isolated from *Bacillus* and *E. coli* by using the modified method of Birnboim and Doly (18) and was carried out on 0.8% agarose gel. The growth of the cell was measured photometrically at 600 nm and proposed in Fig. 3.

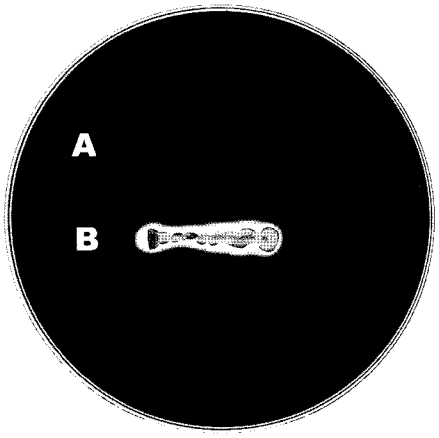

Fig. 2. Clear zone by the amylase expressed in *B. polyfermenticus* SCD after the staining with I_2-KI solution. A, *B. polyfermenticus* SCD (pKY1) [control]; B, *B. polyfermenticus* SCD (pKYamy2).

Results and Discussion

Minimal inhibitory concentration (MIC) The MIC (19) was determined on LB agar plates containing kanamycin. The activity of kanamycin to the *B. polyfermenticus* SCD was measured in terms of its ability to inhibit microbial growth. *B. polyfermenticus* SCD was resistant to a low level of kanamycin (3.25 μg/mL), but susceptible to a high level (>6.25 μg/mL) (data not shown). Therefore, we confirmed that *B. polyfermenticus* SCD had a high susceptibility against the high level of kanamycin.

Transformation of *B. polyfermenticus* SCD *B. polyfermenticus* SCD was transformed by the pKYamy2 plasmid and selected on LB agar medium supplemented with 50 μg/mL of kanamycin and 1%(w/v) soluble starch. The transformants producing the amylase could be recognized by forming clear zone after the addition of the iodine solution (Fig. 2).

Amylase assay expressed in *B. polyfermenticus* SCD The *B. polyfermenticus* SCD transformed with pKYamy2 was cultured at 37°C for 24 hr long in 200 mL LB-broth medium under vigorous shaking. An aliquot of 2 mL was taken at 2 hr intervals, and 1 mL was used for the photometrical measure at 600 nm for the cell growth analysis. After the centrifugation of the rest another aliquot of 1 mL, the amylase activity was observed both in culture supernatant and in disrupted pellet as described. The enzyme activity after the culture for 24 hr was estimated about 0.21 U/mL in intracellular and 1.74 U/mL in extracellular above (Fig. 3A). The activity in the supernatant was higher than that of the pellet. This result indicated that the enzyme was secreted to outside of the cell membrane. The secretion of the enzyme may be due to the secretion signal sequence fused to the amylase gene. Little secretion was observed in the amylase gene without the control of the 500 bp fragment. As shown in Fig. 3A, the bacteria still produced and secreted the enzyme in the culture of 24 hr long. The production of the enzyme suggested in

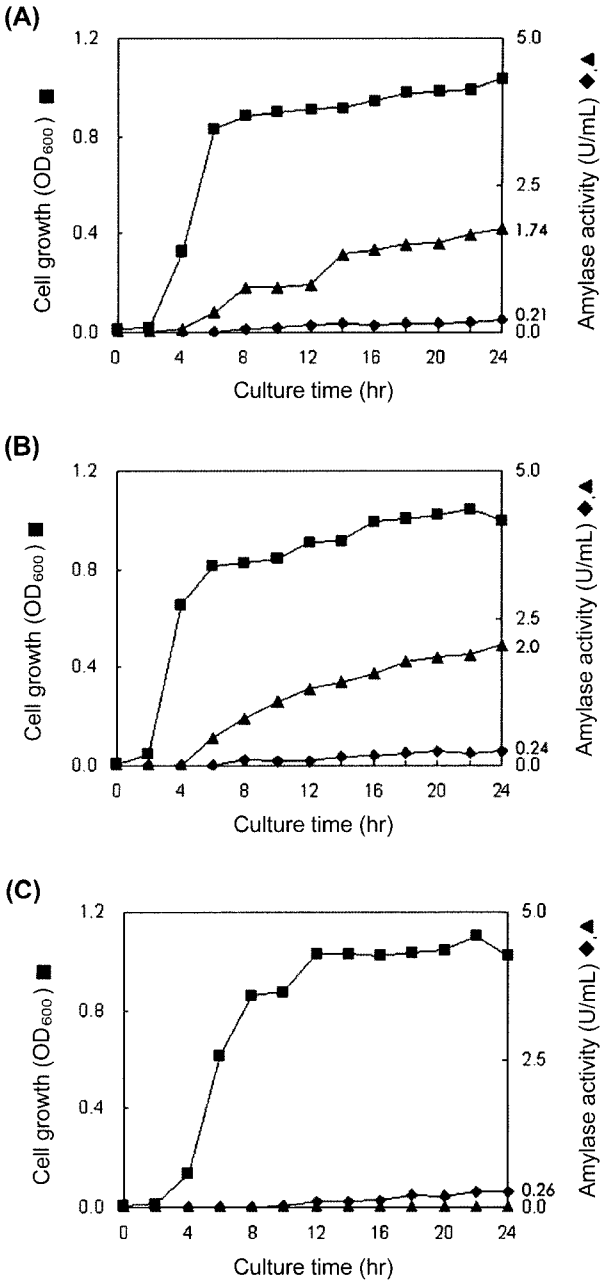

Fig. 3. Production of the α-amylase during the culture of the bacteria transformed with pKYamy2. (A) *B. polyfermenticus* SCD, (B) *B. subtilis* BD170, (C) *E. coli* DH5α. ■, cell growth measured in terms of absorbance at 600 nm; ▲, amylase activity in the culture medium; ◆, amylase activity in the sonicated cells.

another words that the transferred shuttle plasmid was stably maintained in *B. polyfermenticus* SCD, *B. subtilis* BD170, and *E. coli* were also transformed with the vector plasmid pKYamy2 for the controls of the gene expression and secretion. The culture and enzyme activity assays were performed by the same method in *B. polyfermenticus* SCD. The expression of the gene and secretion in *B. subtilis* BD170 were similar to those in *B. polyfermenticus* SCD (Fig. 3B). However, the gene was only expressed, but not secreted in *E. coli* (Fig. 3C). The signal sequence

is active in *B. subtilis* BD170, but not in *E. coli*. Regarding the results above, *B. subtilis* BD170 may be also used for the production of the amylase, but the bacteria is not generally accepted as probiotics. *B. polyfermenticus* SCD and *Bifidobacterium* is well known as probiotics and useful for the health care. The amylase gene can be used easily for the transformation and the production of probiotics, because the origin of the gene is *Bifidobacterium* isolated from human.

In this point of view, the transformation of *B. polyfermenticus* SCD may be attractive in the production of the enzyme for the industrial and commercial purposes, especially for the production and improvement of probiotics. In addition, the amylase can be used for the production of bioenergy, such as ethanol. For the purpose the transformants of *B. subtilis* BD170 may be carefully recommended.

Acknowledgments

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