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Enhanced Production of Maltotetraose-producing Amylase by Recombinant *Bacillus subtilis* LKS88 in Fed-batch Cultivation

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Recombinant *Bacillus subtilis* LKS88[pASA240] containing the amylase gene from *Streptomyces albus* KSM-35 was exploited in fed-batch cultivation for mass production of maltotetraose-producing amylase. The effects of dissolved oxygen, additional organic nutrients (peptone and yeast extract) and mixed carbon sources (glucose plus soluble starch) on amylase production were examined in fed-batch operations in an effort to determine the optimum conditions for a maximum amylase productivity. Under the optimum conditions, maximum amylase activity was about 4.2 times higher than that obtained in batch cultivations, indicating that mass production of maltotetraose-producing amylase could be accomplished in fed-batch cultivation of the recombinant *B. subtilis* strain.

Maltotetraose consisting of four glucose molecules with α-1,4 glucosidic linkage is one of malto-oligosaccharides generated from partial hydrolysis of starch by amylases (30). Since maltotetraose has a number of physicochemical advantages over sugar (20, 28, 30), the demand of maltotetraose in the food industry is growing very rapidly. However, a major drawback for industrial applications is its high price due to the high cost of enzymes used.

Bacillus species may be useful microorganisms for the production of recombinant proteins at a low cost (4), since *Bacillus* species can secrete proteins in large amounts into the extracellular medium (3, 4, 6, 7, 13, 15-18, 21-26, 29). Secreted proteins are generally much easier and less costly to purify than intracellular proteins; and moreover, the secreted proteins exist as a soluble and biologically active form instead of an intracellular insoluble form. In addition, many *Bacillus* species are nonpathogenic to humans or animals because of having no pyrogenic lipopolysaccharides in contrast to *Escherichia coli*, and thus large scale cultivation processes for *Bacillus* species are well developed.

Fed-batch cultivation is an effective method to enhance product yields and productivity compared with batch and continuous cultures. The production phase in fed-batch cultivation can be elongated by controlling environmental parameters such as nutrient concentrations. Thus, fed-batch cultivation techniques have been exploited for *B. subtilis* to produce a desired product at high volumetric productivity. In fed-batch cultivation of recombinant *B. subtilis*, a nutrient feeding strategy needs to be determined to repress spore formation and production of metabolic inhibitors and to enhance expression and secretion of a desired product. Feeding strategies depend on medium pH or dissolved oxygen (DO). A prepared medium is fed to stabilize an abrupt pH or DO shift based on appropriate feeding modes such as intermittent feeding, gradient feeding, exponential feeding and continuous feeding.

In this work, the expression pattern of the amylase gene in recombinant *B. subtilis* LKS88 deficient in intrinsic amylase production capability and secreting 95% of the expressed recombinant protein to the medium (9) was investigated in fed-batch cultivation. Maltotetraose-producing amylase, originally from *S. albus* KSM-35, can hydrolyze starch mainly to maltotetraose (55%) and is stable at pH 6.0~8.0 and enzymatically active up to 60°C (1). Specifically, the effects of DO, nitrogen sources and carbon sources on recombinant amylase production were characterized in fed-batch fermentations in order to enhance both cell mass and amylase production.

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MATERIALS AND METHODS

Bacterial Strain and Plasmid

The recombinant *B. subtilis* LKS88 containing the plasmid pASA240, a derivative of the plasmid pUB110, was used. The plasmid pASA240 contains the *amyR2*

promoter and signal sequence ahead of the structural gene of amylase from *S. albus* KSM-35 (8). Kanamycin (15 μg/ml) was used for selection.

Media and Cultivation Methods

Ten ml LB medium at pH 7.0 was precultured at 37°C and used for inoculation. As described in the previous paper (8), the production medium composition was as follows (per liter): glucose 10 g; peptone (Difco) 7 g; yeast extract (Difco) 5 g; Na-citrate 3 g; glutamic acid 0.5 g; K_2HPO_4 12 g; $(NH_4)_2SO_4$ 1.2 g; $MgSO_4 \cdot 7H_2O$ 1 g; NH_4Cl 0.2 g; $CaCl_2$ 40 mg; $FeSO_4 \cdot 7H_2O$ 40 mg; $MnSO_4 \cdot H_2O$ 7 mg; $CoCl_2 \cdot 6H_2O$ 4 mg; $MoNaO_4 \cdot 2H_2O$ 2 mg; $ZnSO_4 \cdot 7H_2O$ 2 mg; $AlCl_3 \cdot 6H_2O$ 1 mg; $CuCl_2 \cdot H_2O$ 1 mg; H_3BO_3 0.5 mg.

Fed-batch cultivation with an initial volume of 0.8 liter was carried out in a 2.5-liter jar bioreactor (KF-5L, Korea Fermentation Co.) at 37°C by inoculating 10 ml of the preculture. The medium pH was maintained at 7.0 with 5% ammonia and 2 N HCl. In the presence of glucose, the DO level is gradually lowered as cell mass increases, so the desired DO level was achieved by regulating agitation speed and air supply rate. When glucose was depleted in the medium, the DO level increases very sharply and fed-batch cultivation was initiated by feeding the prepared medium solutions to the bioreactor by a peristaltic pump and carried out with the DO-stat strategy in an intermediate feeding mode. Glucose solutions were fed to maintain 10 g/l glucose concentration in the medium. After the glucose solution was added in the medium, the DO level returned rapidly to the set level.

Cell and Carbon Concentrations

The optical density of the cultivation medium was measured with a spectrophotometer at 600 nm (UV-2201, Shimadzu, Japan) and converted to cell concentration with a calibration curve based on dry cell weight (DCW). One unit of optical density corresponds to 0.31 g DCW/l.

Glucose concentrations in the culture were determined by a glucose analyzer (1500G, YSI, U.S.A.) and the concentrations of maltose and reducing sugars were determined by the modified 3,5-dinitrosalicylic acid (DNS) method (2).

Amylase Activity

After centrifugation at 7,000×g for 5 min, 0.1 ml of cell-free supernatant was mixed with 0.9 ml of 1% (w/v) soluble starch as a substrate, dissolved in 50 mM phosphate buffer at pH 7.0 and incubated at 50°C for 10 min. Five (5) ml of modified DNS solution was added to terminate the enzyme reaction. After boiling for 5 min, the sample was cooled in ice water and its absorbance was measured using a spectrophotometer at 570 nm. The absorbance was converted into maltose concentration based on the absorbance-concentration correlations. One unit of amylase was defined as the amount of amylase required to generate 1 μmol of maltose per min at 50°C.

Plasmid Stability

Diluted cell suspension was spread on LB agar plates (yeast extract 5 g/l, tryptone 10 g/l, NaCl 10 g/l, agar 15 g/l). After the plates were incubated at 37°C for 10 h, the colonies were transferred to LBSK agar plates (yeast extract 5 g/l, tryptone 10 g/l, NaCl 10 g/l, soluble starch 10 g/l, agar 15 g/l, kanamycin 15 μg/ml). After 8 h of incubation at 37°C, 5 ml of iodine solution of 52 g of KI and 2.03 g of I_2 dissolved in one liter distilled water was spread on the LBSK plate to calculate the total colonies and those showing transparent halos.

Catabolic Products

Acetic acid concentration was measured by HPLC (Knauer, Germany) using a RI detector with a fermentation monitoring column (model 125-0115, Bio-Rad, U.S.A.). The column temperature was 65°C and the mobile phase (5 mM H_2SO_4) was flowed at 0.8 ml/min. Twenty (20) μl of sample was injected and the peak heights were converted to concentration data using peak height-concentration correlations.

RESULTS AND DISCUSSION

Determination of Dissolved Oxygen Level for DO-stat

Two different levels of dissolved oxygen (DO) (30 and 50% of air saturation) were examined in fed-batch cultivations using the basal medium as described in Materials and Methods and a 500 g/l glucose solution as a feeding medium. When a glucose is depleted in the medium, the DO level increases very sharply concurring with an increase in medium pH, suggesting that DO or pH can be exploited as a control indicator for determining the glucose feeding time.

As shown in Fig. 1, cell growth continued during glucose feeding with an increase in amylase activity. After 40 h, the maximum dry cell weight (DCW) and amylase activity in 30% DO were 43.4 g/l and 136.7 U/ml, respectively. The concentration of acetic acid, a product of glucose catabolism and also an inhibitor of metabolic activities, reached 10.9 g/l.

The higher DO level, 50%, has the same feeding strategy as the above cultivation. The maximum cell concentration of 51.7 g/l and amylase activity of 92.5 U/ml were obtained, which are 19% higher and 32% lower than those of the 30% DO cultivation. In addition, acetic acid concentration reached 15.9 g/l, which is 46% higher compared with the 30% DO cultivation. This observation could be explained in that sufficient oxygen supply and successive glucose feeding in the medium turned the cellular activities toward the direction of cell growth instead of amylase production, resulting in higher acetic acid concentrations. High acetic acid concentrations would be detrimental to metabolic activities of

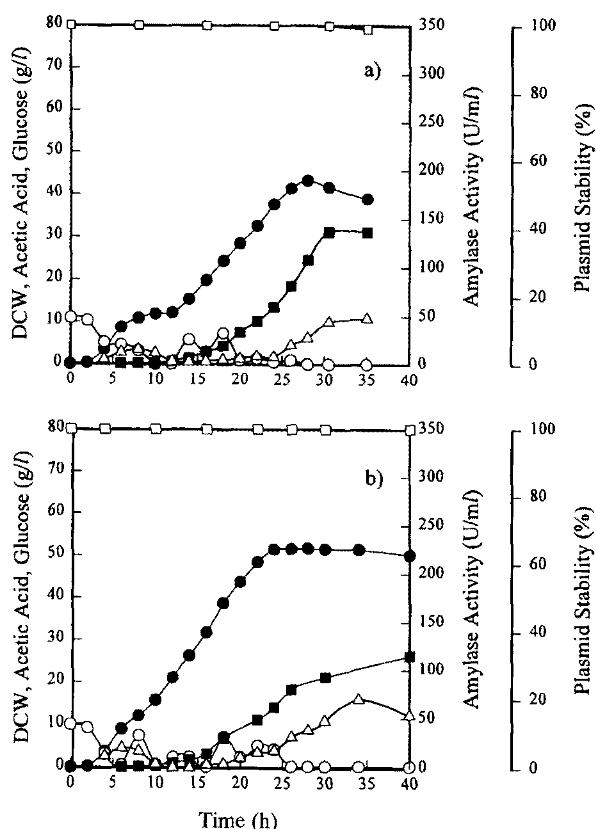

Fig. 1. Profiles of cell growth, maltotetraose-producing amylase production, plasmid stability, glucose, and acetic acid in fed-batch cultivations of recombinant *B. subtilis* LKS88 grown at 37°C and pH 7.0. The DO levels in the culture medium were kept at (a) 30% and (b) 50%.
□, plasmid stability; ■, amylase activity; △, acetic acid; ○, glucose; ●, dry cell weight.

amylase-producing *B. subtilis* LKS88. Mas *et al.* (11) had reported that 7 g/l of acetic acid is enough to inhibit the cell growth of *B. polymyxa*. These results indicate that amylase production by *B. subtilis* LKS88[pASA240] is reciprocal to cell growth and that a low DO level (30%) is more beneficial to amylase production than a high DO level (50%). A DO level was maintained at 30% in the following fed-batch cultivations.

Fed-batch Cultivations with Additional Organic Nutrients

Yeast extract and peptone commonly used as medium components can provide many compounds necessary for cell growth and protein production. It is well known that organic nitrogen sources are more advantageous for cell growth and enzyme production than inorganic nitrogen sources (5, 10, 27). Thus, it is reasonable to expect that yeast extract and peptone are influential medium components on cell growth and enzyme production. Yoo *et al.* (31) and Park (19) showed that yeast extract could enhance enzyme production in *Bacillus* species. In addition, the lag phase of cell growth was much shorter in the presence of yeast extract than in the yeast extract-free medium (12, 19). For *E. coli*, the yields of biomass and recombinant enzyme were higher in the medium enriched with additional yeast extract (10, 14), but addition of excess yeast extract yielded detrimental effects on both cell growth and enzyme production (10). Yeast extract and peptone have also been found to affect metabolite production and uptake. Moon and Parulekar (12) observed that the maximum acetic acid concentration decreased with increasing yeast extract concentration. In another study, the influences of yeast extract and peptone on acetate utilization and enzyme stability were also examined (14). Yeast extract promoted acetate utilization, but did not prevent a decrease in enzyme activity. In a culture with peptone, enzyme activity was stabilized, but acetate was not utilized. With both components, all positive effects including acetate utilization and stable enzyme activity were observed.

In order to experimentally verify the hypothesis that yeast extract and peptone are able to improve maltotetraose-producing amylase production in recombinant *B. subtilis*, the effects of yeast extract and peptone on cell growth and amylase production were examined by supplementing yeast extract and peptone to the growth medium. Adequate ratio of yeast extract and peptone was determined to 5 : 7 from our previous result (8). After cultivation for 6 h or so, first 30 ml of concentrated yeast extract and peptone solution was fed and the same amounts of the other solutions were fed four times with 4-h intervals. Total amounts of the supplied yeast extract and peptone were in the ranges from 0 to 20 g and from 0 to 28 g, respectively, as shown in Table 1. Hereinafter, the term "Medium Quality (MQ)" refers to the amounts of the supplied yeast extract and peptone.

The experimental results were obtained as expected; maximum DCW and amylase activity increased with increasing amounts of yeast extract and peptone up to certain levels and the maximum DCW and amylase activity increased in proportion to the medium quality up to 10 g of yeast extract and 14 g of peptone (designated as MQ III) (Fig. 2). The maximum DCW and amylase activity of the cultivation supplied with 10 g of yeast extract and 14 g peptone were 53 and 21%, respectively, higher than those cultivated without supplemental yeast extract and peptone (designated as MQ I). Similar to the results of Li *et al.* (10), excess supply of yeast extract and peptone was detrimental to both cell growth and amylase production; the cultivation supplied with 20 g of yeast extract and 28 g of peptone (designated as MQ IV) showed about 5% and 19% lower maximum DCW and amylase activity, respectively, than those of MQ III. The specific

Table 1. Comparison of cell grwoth and amylase production of recombinant *B. subtilis* LKS88 grown in various Medium Quality.

Medium quality		Maximum dry cell weight (g DCW/l)	Yield coefficient (g dry cell/g glucose)	Maximum amylase activity (U/ml)	Specific growth rate[a] (h^{-1})
MQ I	YE[b] 0 g + Pep[c] 0 g	43.4	0.27	136.7	0.27
MQ II	YE 5 g + Pep 7 g	56.4	0.26	141.9	0.28
MQ III	YE 10 g + Pep 14 g	66.5	0.29	166.0	0.27
MQ IV	YE 20 g + Pep 18 g	63.0	0.30	134.1	0.28

[a]During exponential growth phase. [b]Yeast extract added to bioreactor. [c]Peptone added to bioreactor.

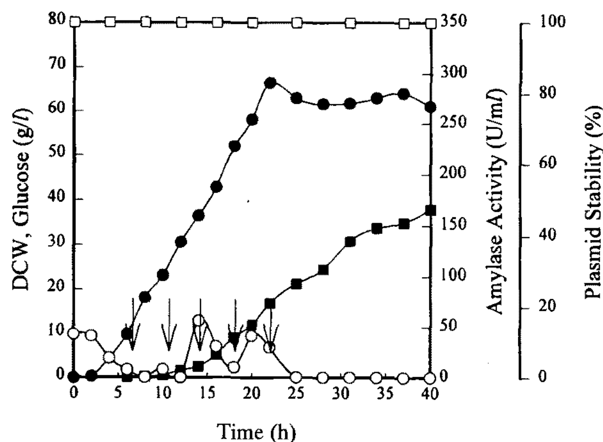

Fig. 2. Trajectories of cell growth, maltotetraose-producing amylase production, glucose, and plasmid stability in fed-batch cultivation in which the mixed solution of 10 g of yeast extract and 14 g of peptone was fed to the bioreactor five times with 4-h intervals. The cultivation condition was 37°C and pH 7.0. DO in the culture medium was kept at 30%. Arrows indicate the feeding time of yeast extract and peptone solution.

□, plasmid stability; ■, amylase activity; ○, glucose; ●, dry cell weight.

growth rate was independent of the amounts of the supplied yeast extract and peptone, which is somewhat expected since glucose used as a carbon source could be a factor in the growth rate.

Fed-batch Cultivations with Mixed Carbon Sources

In batch cultivation where soluble starch was substituted for glucose, the specific amylase activity increased about 1.4 times at the expense of specific growth rate. This indicates that glucose is a favorable carbon source for cell growth while soluble starch is a favorable carbon source for amylase production. Higher amylase production with soluble starch is probably due to the uptake and utilization of oligosaccharides generated from hydrolysis of starch by the secreted amylase. The mixture of glucose and starch was introduced in fed-batch modes of operation to induce a concurrent increase in cell growth by glucose and amylase production by soluble starch.

A partial substitution of soluble starch for glucose was carried out to increase amylase production, based on the previous results obtained in the batch cultivations. By increasing the percentage of soluble starch in the feeding solution, maximum DCW and amylase activity increased simultaneously (Table 2). The maximum DCW and amylase activity for 200 g/l of soluble starch were about 41% and 92%, respectively, higher than those of starch-free cultivation (Fig. 3). The specific growth rate decreased slightly as the soluble starch content increasd, since starch is a secondary carbon source. An increase in starch concentration to 200 g/l resulted in 19% enhancement in the product yield ($Y_{p/s}$) in comparison with the starch-free medium. The volumetric productivity also increased by approximately 46% compared with the starch-free medium.

Enhanced Production of Maltotetraose-producing Amylase in Fed-batch Cultivation

As described already, the best conditions for fed-batch

Table 2. The effects of soluble starch fraction in feeding solution as a substrate on cell growth and amylase production of recombinant *B. subtilis* LKS88.

Feeding solution	Maximum dry cell weight (g DCW/l)	Yield coefficient (g dry cell/g	Maximum amylase activity (U/ml)	Volumetric productivity (kU/l·h)	Specific growth rate[a] (h^{-1})
500 g/l glucose	43.4	0.27	136.7	4.48	0.27
400 g/l glucose + 100 g/l soluble starch	70.2	0.33	200.1	5.00	0.26
300 g/l glucose + 200 g/l soluble starch	77.7	0.30	262.4	6.56	0.24

[a]During exponential growth phase.

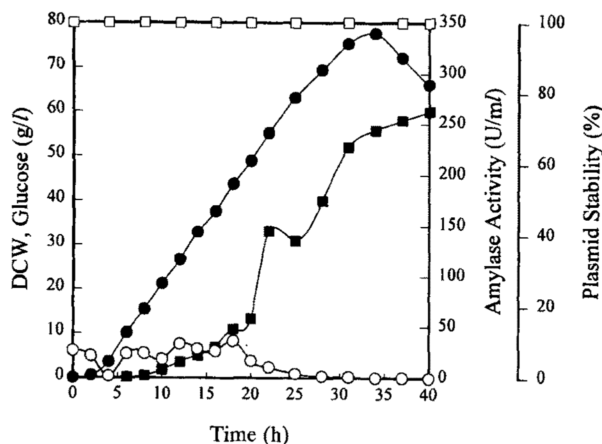

Fig. 3. Profiles of cell growth, maltotetraose-producing amylase production, glucose, and plasmid stability in fed-batch cultivation in which carbohydrate source fed to the bioreactor was the mixed solution of 300 g of glucose and 200 g of soluble starch dissolved in one liter distilled water. The cultivation condition was 37°C and pH 7.0. DO in the culture medium was kept at 30%.

□, plasmid stability; ■, amylase activity; ○, glucose; ●, dry cell weight.

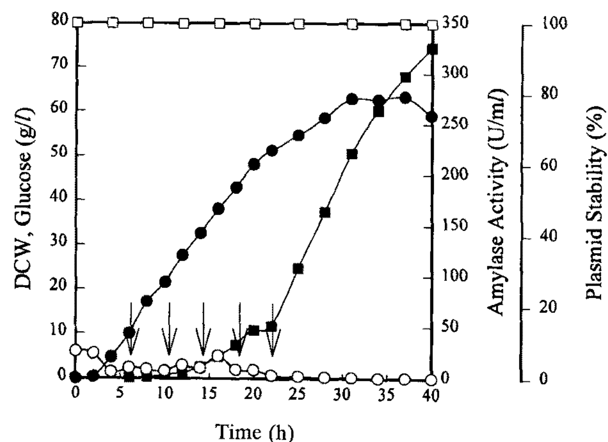

Fig. 4. Trajectories of cell growth, maltotetraose-producing amylase production, glucose, and plasmid stability in fed-batch cultivation in which carbohydrate source fed to the bioreactor was the mixed solution of 300 g of glucose and 200 g of soluble starch dissolved in one liter distilled water and the mixed solution of 10 g of yeast extract and 14 g of peptone was fed to the bioreactor five times with 4-h intervals. The cultivation condition was 37°C and pH 7.0. DO in the culture medium was kept at 30%. Arrows indicate the feeding time of yeast extract and peptone solution.

□, plasmid stability; ■, amylase activity; ○, glucose; ●, dry cell weight.

cultivations were determined to enhance recombinant amylase production in fed-batch cultivation; 30% DO, additional yeast extract (10 g) and peptone (14 g) and mixed carbon sources with glucose (300 g/l) and soluble starch (200 g/l). These conditions were employed synchronously in the fed-batch cultivation to magnify amylase production by inducing the synergestic effects. After 40 h of cultivation, the DCW and amylase activity reached 63.3 g/l and 326 U/ml, respectively (Fig. 4). The maximum amylase activity is about 24% higher than the highest value achieved in the preceding fed-batch cultivations and about 4.2 times higher than the highest activity (77.5 U/ml) of batch cultivations reported before (8). The volumetric productivity was 8.16 kU/l h, 24% higher than the highest value achieved in the preceding fed-batch cultivations. The specific growth rate was very close to those of the preceding fed-batch cultivations, as glucose was used as a primary carbon source in both cases.

In conclusion, the production of maltotetraose-producing amylase by recombinant *B. subtilis* could be significantly enhanced by using the fed-batch cultivation. In comparison with the highest amylase activity level in batch cultivations, about 4.2 times higher maximum amylase activity was achieved in fed-batch cultivation supplied with additional organic nutrients and mixed carbon sources. Our results show that mass production of maltotetraose-producing amylase at a low cost could be accomplished in fed-batch cultivation of *B. subtilis* LKS88[pASA240].

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