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Constitutive Overexpression of the Endoxylanase Gene in *Bacillus subtilis*

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Abstract A strong constitutive P_{JH} promoter from *Bacillus* was applied to overexpress the endoxylanase gene in *B. subtilis*. The expression plasmid, pJHKJ4, was designed to contain the P_{JH} promoter and endoxylanase promoter (P_B), and introduced into *B. subtilis* DB104. Through batch fermentation of the transformant cell on a maltose medium, endoxylanase was produced in a growth-associated manner as the predominant protein. The total activity reached about 600 unit/ml at the end of the cultivation, which corresponded to 698 mg endoxylanase protein/l with a specific activity of 860 unit/mg protein. It was also found that the segregational plasmid instability was less than 30% and most of the endoxylanase activity was detected in the culture medium. This result suggests that the secretory production of endoxylanase can be significantly enhanced with the use of the P_{JH} promoter and high-cell density culture techniques, quantitatively as well as qualitatively.

Key words: Endoxylanase, constitutive expression, P_{JH} promoter, *Bacillus subtilis*, plasmid stability

Xylan is a major component of the cell walls of monocots and hardwoods, representing up to 35% of the dry weight of these plants. The hydrolysis of xylan is of considerable interest for various biotechnological applications (biobleaching, food, animal feed, etc) [1, 14]. Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains. Because of these side chains, the complete hydrolysis of xylan requires the action of several xylanolytic enzymes such as endoxylanases, β-D-xylosidases, α-glucuronidase, α-arabinofuranosidase, and esterase [1, 13]. Among these enzymes, endo-β-1,4-xylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37) are known to be the two main enzymes involved in the degradation of the xylan backbone. Recently, the endoxylanase gene from *Bacillus* sp. was cloned and expressed in *E. coli* and *B. subtilis* [4, 5]. Even though the strong P_{JH} promoter was used in the expression of the endoxylanase and β-glucosidase in the *Bacillus* system [4, 9], the expression levels were somewhat low and there was no quantitative assessment of the segregational plasmid instability. In this study, the P_{JH} promoter-based expression system is examined on a fermentor scale, and the feasibility for the commercial production of endoxylanase in *B. subtilis* is considered.

B. subtilis DB104 (*hisH nprR2 nprE18 aprE*) [6] was used as the host cell. The endoxylanase expression plasmid, pJHKJ4, was constructed as previously described [4]. The open reading frame (ORF) of the endoxylanase gene (639 bp) in pJHKJ4 was transcribed using two promoters; its own promoter (P_B) [8] and the strong *Bacillus* promoter (P_{JH}) located in the upstream region from P_B. The transformation of *B. subtilis* DB104 with the pJHKJ4 plasmid was carried out according to the method of Contente and Dubnau [3]. A Luria-Bertani (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar medium containing kanamycin (25 μg/ml) was used for the selection of the transformants.

The *B. subtilis* DB104 cell harboring the pJHKJ4 plasmid was precultured at 37°C in 10 ml of LB+kanamycin medium to an optical density of 1.0 at 660 nm (OD_{660}). The second preculture was conducted in a 500 ml baffled-flask containing 50 ml of LB+kanamycin medium, followed by inoculation into a fermentor (KFC, Korea). The fermentation medium was MLB medium (2% maltose, 1% tryptone, 1% yeast extract, 0.5% NaCl). The culture pH was controlled at 7.0 with 50% NH_4OH and 3 N HCl. The dissolved oxygen level was maintained at above 30% air saturation by automatically adjusting the agitation speed within a range of 300 to 800 rpm.

The culture broths withdrawn during the batch fermentation were appropriately diluted and then spread on an LB plate. Next, 200 colonies on the LB plate were transferred to the selective medium (LB+kanamycin plate) by toothpicking. The ratio of the number of colonies on the selective plate to the number of colonies transferred was defined as the

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plasmid stability (%). The residual concentration of maltose was measured using the dinitrosalicylic acid method [11]. The endoxylanase activity was determined by measuring the amount of reducing sugar liberated from the oat spelts xylan [11]. The intracellular activity of endoxylanase was determined with cell lysates obtained by lysozyme treatment [12]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute at 60°C and pH 6.6. The protein concentration was determined by the Bradford method [2] using bovine serum albumin as the standard. The culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel (12%, w/v, gel) electrophoresis (SDS-PAGE) as described by Laemmli [10]. The electrophoresed gel was scanned by an Image Analyzer (Image Master VDS, Pharmacia Biotech., U.S.A.). For zymogram analysis of endoxylanase, an acrylamide gel containing 0.5% (w/v) oat spelts xylan was used. After electrophoresis, the gel was washed in Triton X-100 (2.5%, v/v) for 1 h at room temperature to remove any SDS and to restore the enzyme activity, and then incubated in 0.1 M phosphate buffer (pH 6.6) for 4 h at room temperature. The endoxylanase band of the gel was detected by staining with 0.1% (w/v) Congo red for 15 min. The purified endoxylanase was obtained by $(NH_4)_2SO_4$ precipitation, ultrafiltration, and CM52 cation-exchange chromatography [4].

When examined in the flask cultures as preliminary studies, glucose and maltose as the carbon source and yeast extract as the nitrogen source were found to be the most effective for cell growth and endoxylanase expression (data not shown). When the concentration of glucose was increased from 0.5% to 5%, the highest activity of extracellular endoxylanase (166 unit/ml) was observed with 2% glucose. In the case of maltose, the endoxylanase

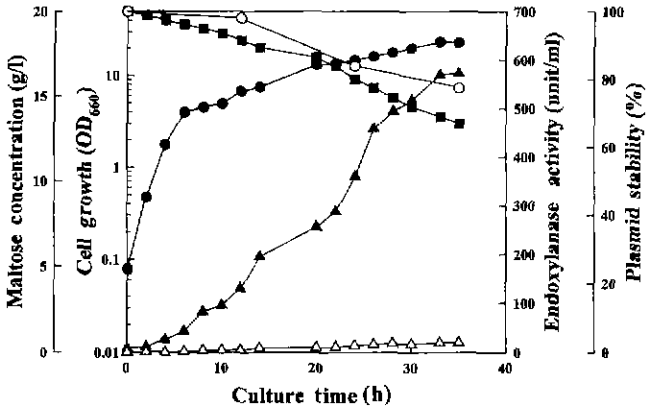

Fig. 1. Time profiles of cell growth, plasmid stability, maltose consumption, and endoxylanase expression by batch fermentation of *B. subtilis* DB104 harboring pJHKJ4 on MLB medium.
Symbols: (●), cell growth; (○), plasmid stability; (■), residual maltose concentration, (▲), extracellular endoxylanase activity; (△) intracellular endoxylanase activity

expression remained stable at 180 unit/ml, regardless of the concentration of maltose. It was also found that a higher concentration of the yeast extract increased the cell growth and endoxylanase expression. However, the highest endoxylanase activity per unit cell mass (29.8 unit/ml, OD_{660}) was obtained with 1% yeast extract. Consequently, the batch fermentation medium in the fermentor was designed to contain 2% maltose and 1% yeast extract (MLB medium).

Figure 1 shows the result of the batch culture of *B. subtilis* harboring the pJHKJ4 plasmid. The endoxylanase expression continued to increase during the slow-growing period, beginning after 6 h, and reached a maximum level of 570 unit/ml at the end of the cultivation. The intracellular endoxylanase activity observed was less than 30 unit/ml throughout the entire culture period, indicating that the majority (>95%) of endoxylanase was efficiently secreted into the culture medium. This higher expression level of endoxylanase than the flask culture seems to be due to the high cell concentration (23.5 OD_{660}) caused by sufficient and well-controlled oxygen supply in the fermentor culture. Considering the specific activity of 860 unit/mg-protein [4], the total amount of expressed endoxylanase was approximately 700 mg-endoxylanase protein/l. The pJHKJ4 plasmid was not extractable from the kanamycin-sensitive cells. Therefore, the plasmid was segregationally lost during the growth of the recombinant cells, and the fraction of plasmid-free cells was maintained at less than 30% even at the end of the cultivation. The very poor consumption of maltose over the batch fermentation suggests two possibilities: an inherent deficiency in the assimilation of maltose (or carbon) by the *Bacillus* host cell or the presence of growth-limiting factor(s) in the medium. The observation that neither glucose instead of maltose nor supplementation with excess amounts of amino acids and vitamins improved the carbon utilization and cell growth (data not shown), suggests an existence of a metabolic defect(s) in carbon (maltose and glucose) utilization of the *B. subtilis* DB104 host cell.

For SDS-PAGE and zymogram analyses, six microliters of the culture supernatants at indicated times were directly loaded on the gel. As shown in Fig. 2A, the endoxylanase band of 20.4 kDa was the major protein among the extracellular proteins and the band intensity increased with the culture time, as well as the endoxylanase activity (Fig. 1). Since the activity was only detected in the protein band of 20.4 kDa (Fig. 2B), the protein bands with higher molecular weights, such as 25.5, 42.2, and 61.7 kDa, are uncharacterized proteins produced by *B. subtilis*. When the gel was scanned, the endoxylanase band corresponded to 65% of the total extracellular protein. Based on a total extracellular protein concentration of 1.0 g/l, the concentration of the secreted endoxylanase protein was calculated to be 650 mg/l, which correlated well with the protein concentration quantified by the specific activity of endoxylanase. This expression level was superior to that of staphylokinase

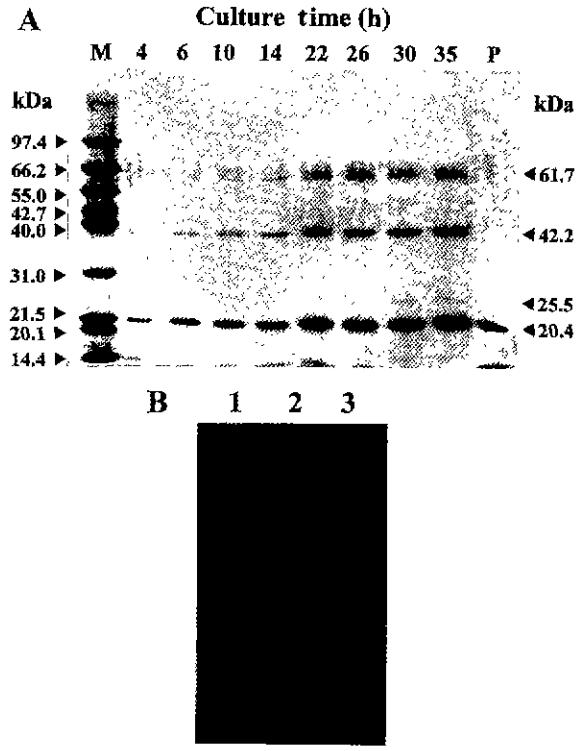

Fig. 2. SDS-PAGE (A) and zymogram (B) analyses of extracellular endoxylanase. Six microliters of the culture supernatant from each culture times were directly loaded on a 12% gel.
In (A), lane M, protein molecular weight markers, lane P, purified endoxylanase. In (B); lane 1, supernatant at 26 h, lane 2, supernatant at 35 h; lane 3, purified endoxylanase.

directed by the strong constitutive promoter, P43 [15], and cyclodextrin glucanotransferase directed by the α-amylase promoter, *amy*R2 [7].

Consequently, the present work demonstrates that the P_{HI} promoter can be applied as an efficient expression system for the overproduction of endoxylanase as well as other cloned-gene products in *B. subtilis*. Optimization of the host cells and fed-batch culture will be focused on in the future work.

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