J. Microbiol. Biotechnol. (2003), **13**(6), 892–896

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

High-Level Expression and Secretion of *Bacillus pumilus* Lipase B26 in *Bacillus subtilis* Chungkookjang

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Received: May 19, 2003
Accepted: August 24, 2003

Abstract High-level expression of the lipase B26 gene from *Bacillus pumilus* was achieved using *Bacillus subtilis* Chungkookjang isolated from the Korean traditional fermented bean paste, Chungkookjang. For the secretory production of recombinant lipase B26 in a *Bacillus* host system, pLipB26 was constructed by ligating the lipase B26 gene into the recently designed *Escherichia coli-Bacillus* shuttle vector, pLipSM, and that was then transformed into *B. subtilis* Chungkookjang. Among the various vector, medium, and host combinations, *B. subtilis* Chungkookjang harboring the pLipB26 exhibited the highest lipase activity in PY medium, and *B. subtilis* Chungkookjang secreted two times more enzymes than *B. subtilis* DB104 under the same condition. When *B. subtilis* Chungkookjang harboring the pLipB26 was cultured in a 5-l jar-fermentor containing 2 l of a PY medium, the maximum lipase activity (140 U/ml) and production yield (0.68 g/l) were obtained during the late exponential phase from a cell-free culture broth. Although *B. subtilis* Chungkookjang also secreted extracellular proteases at the late exponential phase, these results suggested the potential of *B. subtilis* Chungkookjang as a host for the secretory production of foreign proteins.

Key words: Secretion, high-level expression, lipase B26, shuttle vector pLipSM, *Bacillus subtilis* Chungkookjang

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of the ester bonds of long-chain acylglycerol into glycerol and fatty acids at the oil-water interface. As such, lipases have many potential commercial applications and have already been applied as catalysts in various industrial biotechnologies, such as food additives, medicines, clinical reagents, detergents, and cosmetics. The applicability of lipases is due to the following reasons. First, lipases have a variety of properties in regards to fatty acid specificity, stereospecificity, and positional selectivity of triglycerides. Second, lipases can catalyze numerous reactions, for example, the hydrolysis and synthesis of ester bonds and ester group exchanges. Third, lipases are widely distributed in plants, animals, and microorganisms, including fungi and bacteria. In particular, microbial extracellular lipases are extremely diverse in their enzymatic properties and substrate specificity [2, 5– 10, 12, 26, 27].

Several lipases from *Bacillus* have already been applied biotechnologically due to their versatility [11, 16, 17, 24]. In particular, lipase B26 from *Bacillus pumilus* is an interesting enzyme that can hydrolyze long-chain triglycerides. Kim *et al.* [13] reported that lipase B26 exhibits interfacial activation and Ca^{2+}-independent thermostability and activity. Ca^{2+}-independency of this lipase B26 offers many advantages, such as full hydrolytic activity even in the presence of Ca^{2+}-chelating component in laundry detergents.

Despite many previous attempts to overexpress lipases in *E. coli*, most of the expressed enzymes have been accumulated as inclusion bodies inside the cells. Therefore, the secretory production of lipases in *Bacillus* is currently considered as the best method. Furthermore, one of the great advantages of using *B. subtilis* for cloning and as an expression host instead of *E. coli* is its ability to secrete proteins directly into the culture medium and accumulate

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them to a high level in a relatively pure state. Previously, Kim *et al.* [13] reported that the lipase B26 gene expressed as inclusion bodies in *E. coli* was successfully expressed using a *Bacillus* expression system, although with a relatively low productivity of approximately 8 U/ml.

Accordingly, since the overexpression of lipase B26 is necessary for industrial application, the current study attempted to produce lipase B26 by optimizing the expression vector system and culture conditions. In addition, for application in the food industry, the high-level expression of lipase B26 was particularly attempted in *Bacillus subtilis* Chungkookjang isolated from the Korean traditional bean paste, Chungkookjang [1], which was employed as a secretory production host. This is the first comprehensive study to evaluate the production of lipase B26 by utilizing a food-grade microorganism and various operating variables.

MATERIALS AND METHODS

Bacillus subtilis Strain and Plasmids

pMB26 harboring the lipase B26 gene from *B. pumilus* was donated by Kim [13]. *Bacillus subtilis* DB104, *Bacillus subtilis* 168 [23], and *Bacillus subtilis* Chungkookjang [1] were used as hosts, and the *E. coli-Bacillus* shuttle vector, pLipSM, which was constructed by the BioLeaders Corp., was used for the high-level secretory overexpression of lipase B26. The *E. coli-Bacillus* shuttle vector, pLipSM, contained a *Hpa*II promoter, *rrn*B transcription terminator, and multi-cloning site downstream from the Lip signal (Fig. 1).

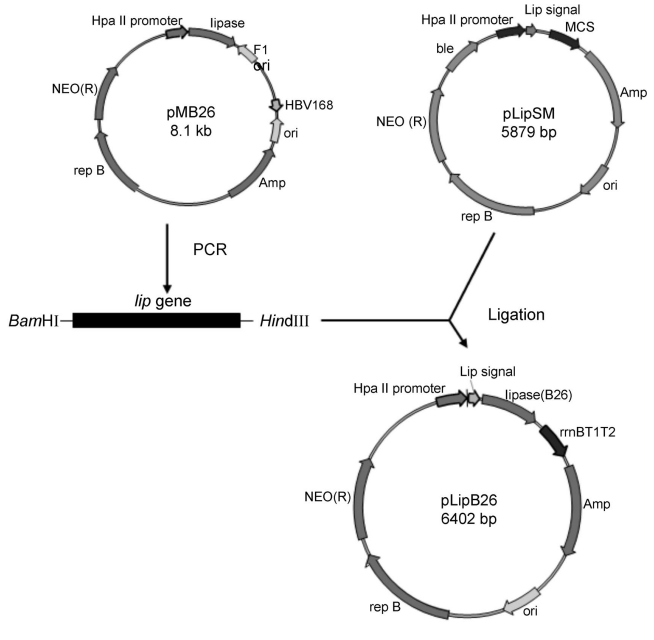

Fig. 1. Construction of recombinant plasmid pLipB26 for overproduction of lipase B26. See Materials and Methods for details.

Construction of Recombinant Plasmid, pLipB26

After preparation of the plasmid DNA, pMB26, a 543 bp mature *lip* coding region without a 102 bp signal sequence was amplified by a PCR using specially designed oligonucleotides, including *Bam*HI and *Hin*dIII restriction sites upstream and downstream, respectively. The sequence of each primer was as follows: forward primer, 5'-gcgggatccgctgagcataat-ccggttg-3' and reverse primer, 5'-tccaagcttattaattcgtattctg-tcctcc-3'. The amplified product was ligated at the same restriction enzyme sites (*Bam*HI and *Hin*dIII) of the shuttle vector, pLipSM, and transformed into *E. coli* JM83 [28], after which the resulting plasmid was isolated from the transformed *E. coli* host.

Transformation of pLipB26 into *Bacillus* Strains

Electrocompetent cells of *Bacillus* were prepared by the method described by Matsuno *et al.* [22], and the pLipB26 was transformed into the *Bacillus* strains by electroporation. Next, the electroporated cell suspension was incubated at 37°C for 3 h, and it was then spread on an LB-TBN (tributyrin) agar plate supplemented with 10 μg/ml of kanamycin and incubated overnight at 37°C [14]. The colonies surrounded by a clear zone were selected and, for confirmation of the lipase B26 gene sequence, the plasmids harboring the lipase B26 gene were isolated from the transformants by the alkaline lysis method and sequenced [25].

Media

For the overproduction of lipase B26 in *Bacillus*, a PY medium was used having the following composition: 0.2% (w/v) K_2HPO_4, 0.2% (w/v) KH_2PO_4, 4% (w/v) polypeptone, 0.5% (w/v) beef extract, 0.5% (w/v) yeast extract, 2% (w/v) glucose, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.0001% (w/v) $ZnSO_4 \cdot 7H_2O$, 0.0001% (w/v) $MnSO_4 \cdot 7H_2O$, and 0.001% (w/v) $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 7.2 with KOH. An LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] agar plate containing 1% emulsified tributyrin (Sigma Chemical Co., U.S.A.) was used to visually confirm any lipolytic activity [14, 15].

Production of Lipase B26 in *Bacillus* Strains

The recombinant *Bacillus* strains harboring pLipB26 were cultivated in the PY medium supplemented with 10 μg/ml of kanamycin at 30°C with shaking at 180 rpm for 24 h. For mass production of the enzyme, the transformants were cultured in a 5-l jar-fermentor containing 2 l of the PY medium under the following operating conditions: initial pH 7.2, temperature 30°C, and agitation speed 600 rpm. To inhibit the protease activity secreted into the culture medium during the cultivation of the recombinant *Bacillus* strains, the following protease inhibitors were added to the culture medium: 1 μM Pepstatin (Boehringer Mannheim GmbH, Germany), 0.1–1 mM

phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., U.S.A.), 5–100 μM tosyl phenylalanyl chloromethyl ketone (TPCK) (Boehringer Mannheim GmbH, Germany), and 1 and 5 mM ethylenediamine-tetraacetic acid (EDTA) (Sigma Chemical Co., U.S.A.). Five-ml samples were withdrawn from the culture broth at regular intervals, and cell-free culture broth was used to assay the lipolytic and proteolytic activities.

Lipase Activity Assay

The activity of the lipase B26 produced was measured by the pH titration method and spectrophotometric method. In the case of the pH titration method, the lipase activity was assayed by titrating the free fatty acids released by the hydrolysis of olive oil (Junsei Chemical Co., Japan) using the pH stat method [20]. After an appropriate amount of the enzyme solution was added to 20 ml of the substrate emulsion, the decrease of the pH due to the release of fatty acids was determined by titrating with 10 mM NaOH to a titration end point of pH 8.5 using a pH titrator (718 Stat Titriono, Metrohm, Switzerland) for 4 min at 35°C. One unit of lipase activity was defined as the amount of enzyme liberating 1 μmole of fatty acid per min.

For the spectrophotometric detection of lipase activity, *p*-nitrophenyl caprate (Sigma Chemical Co., U.S.A.) was used as the substrate. The enzyme activity was then assayed by spectrophotometrically detecting the product, *p*-nitrophenol, at 405 nm. One unit of activity with *p*-nitrophenyl caprate as a substrate was defined as 1 μmole of *p*-nitrophenol released per min [4].

Assay of Protease Activity

The proteolytic activity of the cell-free culture broth was measured by the enhanced method according to the manufacturer's instructions using a Universal Protease Substrate (Roche, Germany) and by determining the absorbance of resorufin-labeled peptides at 574 nm. One unit of enzyme was defined as the amount of enzyme to increase absorbance at 574 nm by 0.01 per min.

RESULTS AND DISCUSSION

Construction and Transformation of pLipB26

For the effective production of lipase B26, the lipase B26 gene was amplified by a PCR using the plasmid pBM26 (donated by H. K. Kim from KRIBB) as the template. The 543 bp amplified DNA fragment was then cloned into the *Bam*HI-*Hin*dIII site of pLipSM, constructed by the BioLeaders Corp., for the secretory production of the recombinant proteins in *E. coli* and *Bacillus* (Fig. 1). The insertion of the lipase B26 gene into pLipSM was confirmed by agarose gel electrophoresis, and the resulting plasmid was named pLipB26. The recombinant plasmid pLipB26 harboring the lipase B26 gene was transformed into the *Bacillus* host strains, *B. subtilis* 168^T, *B. subtilis* DB104, and *B. subtilis* Chungkookjang by the electroporation method. After transforming pLipB26 into the hosts, the transformants were spread onto LB agar plates containing tributyrin, and several transformants forming a clear zone around the colony were selected for the secretory production of lipase B26 (Fig. 2).

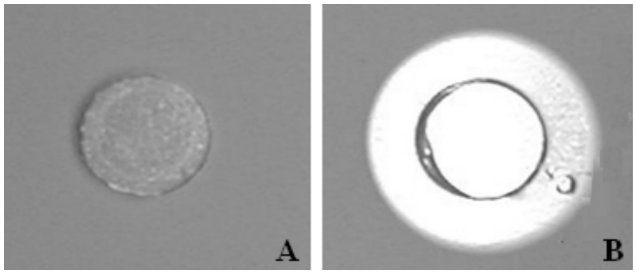

Fig. 2. Lipase activity on Luria-Bertani (LB) agar plate containing tributyrin of (A) *B. subtilis* Chungkookjang used as control and (B) *B. subtilis* Chungkookjang harboring pLipB26.

Expression of Lipase B26

To test which hosts and plasmids were suitable for the overproduction of lipase B26, various combinations of plasmids and host cells were tested. The plasmids pMB26 and pLipB26 were introduced into the *Bacillus* strains DB104 and Chungkookjang, and the transformants were cultured to a stationary phase in 1 l Erlenmeyer flasks containing 250 ml of an LB or PY medium at 30°C and 180 rpm. As shown in Fig. 3, the PY medium was better than the LB medium for cell growth and the secretary production of lipase in *Bacillus* and, when the same pLipB26 vector and PY medium were used, *B. subtilis* Chungkookjang produced two times more enzyme than *B. subtilis* DB104. It was also found that *B. subtilis* Chungkookjang harboring pLipB26 exhibited higher lipase activity than *B. subtilis* Chungkookjang harboring pMB26.

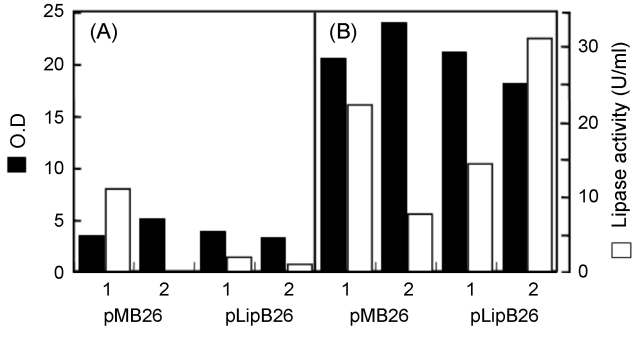

Fig. 3. Comparison of cell growth and lipase activity.
The recombinant hosts (1) *B. subtilis* DB104 and (2) *B. subtilis* Chungkookjang harboring pMB26 or pLipB26 were cultured in 1-l Erlenmeyer flasks containing 200 ml of (A) LB or (B) PY media.

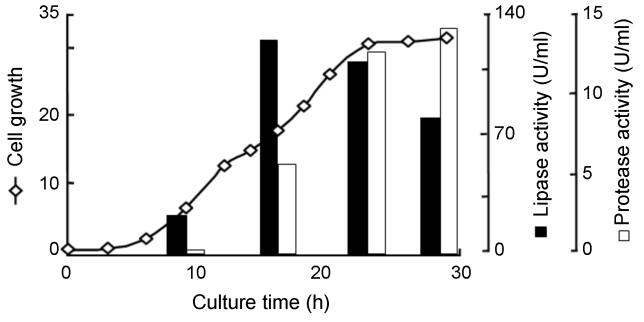

Fig. 4. Production of lipase B26 in *B. subtilis* Chungkookjang harboring the pLipB26.
The cells were cultivated in Erlenmeyer flask containing 200 ml of the PY medium at 30°C and 180 rpm.

Among the various vector, media, and host combinations, the cell growth reached about 25 OD at 600 nm and the lipase activity was about 30 U/ml, when *B. subtilis* Chungkookjang harboring pLipB26 was cultivated in the PY medium. Therefore, the combination of *B. subtilis* Chungkookjang, pLipB26, and the PY medium was identified as the best for the production of lipase B26; however, degradation of lipase B26 was also observed. As shown in Fig. 4, the lipase activity reached a maximum value (about 135 U/ml) after 16 h of cultivation, then gradually decreased with time. Conversely, protease activity was initially detected after 10 h and then rapidly increased with cell growth. It was also found that the protein band at 21 kDa on SDS-PAGE gradually disappeared over time due to the proteases secreted from the host, *B. subtilis* Chungkookjang (data not shown). In a previous study, it was suggested that the loss of lipase activity over time was due to proteolytic degradation of lipase [18, 19, 21]. Therefore, it was not surprising that the extracellular lipase was subjected to proteolytic attack.

Consequently, several kinds of protease inhibitors were added to the culture broth to prevent the proteolytic degradation of lipase B26 over time (Table 1). The protease inhibitors PMSF, Pepstatin, EDTA, and TPCK were added to the culture broth after 0 and 9 h of cultivation, and the lipase and protease activities were measured after 24 h of cultivation. No increase in the lipase activity was observed (Table 1). When the aspartic protease inhibitor Pepstatin or serine protease inhibitor PMSF was added to the culture medium, the cell growth was unaffected, yet lipase activity was inhibited without any effect on protease activity. Meanwhile, when the metalloprotease inhibitor EDTA or chymotrypsin-like serine protease inhibitor TPCK was added to the culture medium, both lipase and protease activities decreased, while cell growth was almost completely inhibited with the addition of 5 mM EDTA or 0.1 mM TPCK. Accordingly, it was concluded that the best way to produce lipase B26 in *B. subtilis* Chungkookjang was to harvest after 16 h of cultivation without using any protease inhibitors.

Table 1. Effect of protease inhibitors on lipolytic and proteolytic activities.

Protease inhibitor	Relative lipolytic activity (%)	Relative proteolytic activity (%)
None	100	100
Pepstatin 1 μM	88.6	118
EDTA 1 mM	49.6	18.6
EDTA 1 mM[a)]	72.9	37.2
EDTA 5 mM	–[b)]	–[b)]
EDTA 5 mM[a)]	58.5	11.6
PMSF 1 mM	94.6	153
PMSF 1 mM[a)]	20.3	116
TPCK 0.1 mM	–[b)]	–[b)]
TPCK 0.1 mM[a)]	43.7	8.1

Lipolytic and proteolytic activities were measured after cultivation of the cells in the presence of the protease inhibitors Pepstatin, PMSF (phenylmethylsulfonyl fluoride), EDTA (ethylenediamine-tetraacetic acid) and TPCK (tosyl phenylalanyl chloromethyl ketone).
[a)]The inhibitors were added after 9 h cultivation of cells.
[b)]No growth.

Mass Production and Purification of Lipase B26

For the mass production of lipase B26, the recombinant *Bacillus* hosts harboring pLipB26 were cultivated in a 5-l jar-fermentor containing 2 l of PY medium supplemented with kanamycin to a final concentration of 10 μg/ml, and the results are shown in Table 2. As expected, the newly isolated *B. subtilis* Chungkookjang was the best strain for the secretory production of lipase B26, and after 16 h of cultivation, lipase activity reached its highest level at about 140 U/ml and production yield was about 0.8 g/l. When comparing with the results previously reported by Kim *et al.* [13], the lipase activity was increased about 17-fold from 8 to 140 U/ml, while the yield (0.68 g/l) was about 13 times higher than that (about 0.06 g/l) obtained by Kim *et al.* These results demonstrated that isolated *B. subtilis* Chungkookjang had a strong ability to secrete proteins and could be used as a suitable host system for secretory production of recombinant proteins inspite of the presence of proteolytic activity.

Table 2. Production of lipase B26 in jar-fermentor.

Bacillus subtilis strains	Cell growth (Abs. at 600 nm)	Lipolytic activity (U/ml)	Proteolytic activity (U/ml)
168^T	17	38	0.9
DB104	15	22	0.4
Chungkookjang	20	140	3.3

The recombinant *Bacillus* strains harboring pLipB26 were cultivated in 5-l jar-fermentors containing 2 l of a PY medium. The culture condition and activity assay of lipase and protease is described in Materials and Methods.

Acknowledgment

This work was supported by a Research Grant from Kookmin University.

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