Plant Pathol. J. 24(2) : 191-201 (2008)

The Plant Pathology Journal

Differential Proteomic Analysis of Secreted Proteins from Cutinase-producing *Bacillus* sp. SB-007

Yeon-Hee Ban[1,†], Miri Jeon[2,†], Jihee Yoon[3], Jae-Min Park[1], Hyun-Ju Um[1], Dae-Soon Kim[1], Seung-Ki Jung[4], Keun-Young Kim[4], Jeewon Lee[5], Jiho Min[3]* and Yang-Hoon Kim[1]*

[1]School of Life Science, Chungbuk National University, Cheongju 361-763, Korea
[2]College of Agriculture, Life & Environmental Sciences, Chungbuk National University, Cheongju 361-763, Korea
[3]Graduate School of Semiconductor and Chemical Engineering, Chonbuk National University, Jeonju 561-156, Korea
[4]Bioresource Inc., Suncheon 540-843, Korea
[5]Department of Chemical and Biological Engineering, Korea University, Sungbuk-Gu, Seoul 136-701, Korea

(Received on January 15, 2008; Accepted on March 1, 2008)

***Bacillus* sp. SB-007 was isolated from pea leaves harvested from the southwestern parts of South Korea through screening on a minimal medium containing 0.2% purified cutin for its ability to induce the cutinase production. However, no cutinase was produced when it was grown in a minimal medium containing 0.2% glucose. A proteomic approach was applied to separate and characterize these differentially secreted proteins. The expression level of 83 extracellular proteins of the cutinase-producing *Bacillus* sp. strain SB-007 incubated in a cutinase-induced medium increased significantly as compared with that cultured in a non cutinase-induced medium containing glucose. The extracellular proteome of *Bacillus* sp. SB-007 includes proteins from different functional classes, such as enzymes for the degradation of various macromolecules, proteins involved in energy metabolism, sporulation, transport/binding proteins and lipoproteins, stress inducible proteins, several cellular molecule biosynthetic pathways and catabolism, and some proteins with an as yet unknown function. In addition, the two protein spots showed little similarities with the known lipolytic enzymes in the database. These secreted proteome analysis results are expected to be useful in improving the *Bacillus* strains for the production of industrial cutinases.**

Keywords **:** *Bacillus* sp, cutinase activity, secreted proteome profiling, two-dimensional gel electrophoresis

Cutinases are serine hydrolases that degrade cutin, a cuticular polymer of higher plants that is composed of hydroxy and epoxy fatty acids usually with *n*-C16 and *n*-C18. Unlike lipases and esterases, the hydrolytic activity of cutinases does not depend on interfacial activation. In addition, cutinases have an easier access to the substrate and exert hydrolytic activity (Kim et al., 2003; Martinez et al., 1992). The roles of fugal and bacterial cutinases are to depolymerize this polymer by hydrolyzing the esters bonds and undergo enzymatic degradation as one of the first steps in the infection process (Cristina et al., 1998; Wilhelm et al., 1993). The irruption of higher plants by phytopathogenic fungi is based on the secretion of extracellular cutinases. Therefore, cutinase inhibitors may prevent the fungal penetration of plants and prevent infection (Soliday and Kolattukudy, 1983). Because cutinases appear to play an important role in the virulence of fungi and bacteria, several studies have examined its physiological and biochemical properties (Lin and Kolattukudy, 1980). Certain micro-organisms can survive using cutin as their sole carbon source by producing extracellular cutinolytic enzymes. Microbial cutinases have been isolated and characterized with respect to their biochemical, structural, and functional properties (Egmond and de Vlieg, 2000; Kim, 2003; Poulsen et al., 2006). These originate from different sources, including fungi (e.g. *Fusarium solani* f. s. *pisi*, *Magnaportha grisea*, and *Colletotrichum gloeosporioides*) and bacteria (e.g. a phyllosphere-inhabiting fluorescent *Pseudomonas putida* and *P. mendocina*, cohabiting with a nitrogen-fixing bacteria, and *Corynebacterium* sp.) (Chen et al., 2006; Cristina et al., 1999; Kolattukudy, 1984; Petersen et al., 1997; Um et al., 2007).

In recent years, the esterification and transesterification activities of cutinases have been studied extensively and might be applied advantageously to chemical synthesis (Cristina et al., 1999; Gerard et al., 1993). Cutinases have also been used as lipolytic enzymes in laundry or dish-washing detergents to efficiently remove immobilized fats and oils (Flipsen et al., 1998). Further potential uses include applications in the dairy industry to hydrolyze milk fat, and

*Corresponding authors.
YH Kim. Phone) +82-43-261-3575, FAX) +82-43-264-9600
E-mail) kyh@chungbuk.ac.kr
J Min. Phone) +82-63-270-2436, FAX) +82-63-270-2306
E-mail) jihomin@chonbuk.ac.kr
†These authors contributed equally to this work.

various uses in the oleochemical industry (Cristina et al., 1999). On the other hand, methods for preparing cutinolytic enzymes have been developed in order to increase the pharmacological effect of agricultural chemicals (Poulose and Kolattukudy, 1998). Cutinases have also been shown to degrade environmental toxic pollutants without the toxic cellular effects observed with a yeast esterase (Kim et al., 2002, 2005). In addition, the cutinases from *Fusarium moniliforme* and the yeast *Cryptococcus* sp. have been used to degrade biodegradable plastics (i.e. polycaprolactone (PCL), polylactic acid (PLA), and polybutylene succinate (PBS)) in an attempt to overcome the disposal problems associated with synthetic plastic waste (Murphy et al., 1998; Shimao, 2001). Due to these properties, cutinases have significant applied potential in environmental friendly biotechnology industries. Therefore, it is important to isolate and study novel cutinolytic microorganisms and cutinases. In this study, a cutinase-producing *Bacillus* sp. SB-007 was isolated from pea leaves in the mid-west area of South Korea.

Several *Bacillus* strains are known to secrete a number of proteins into the extracellular medium. This ability has been exploited in the fermentation industry for a long time, particularly for the production of industrial enzymes (Voigt et al., 2006). Previous studies showed that the highest level of protein secretion in *B. subtilis* was observed when the cells were grown in a complex medium (Antelmann et al., 2001). Moreover, cells grown in a minimal medium secrete considerable lower amounts of protein (Hirose et al., 2000), and most extracellular proteins have been identified after entry into the stationary growth phase. Recently, extended proteomics (along with the ever-increasing amount of protein sequence data and enhanced MS technology) has proven to be a powerful tool for identifying proteins in complex mixture. Furthermore, it is appropriate to study the changes in proteins expression in an organism under a variety of environmental conditions (Chu et al., 2000; Yoshida et al., 2001). Although there have been some reports on a comprehensive view of the physiological state and responses of the metabolism of *Bacillus* strains in various culture conditions using proteomics tools (Coppee et al., 2001; Haike et al., 2004; Vilain and Brozel, 2006), a proteomic approach for building a quantitative dynamic analysis and the metabolic synthetic pathway is still at the early stages of developments.

The aim of this study was to obtain a more comprehensive understanding of the expression patterns of the proteins secreted from *Bacillus* sp. SB-007. Significant differences in the proteins secreted between the cutinase-induced culture and non cutinase-induced culture were examined in detail using quantitative and comparative proteome analysis. The cutin-induced proteins were identified, and their functional characteristics were also explained by peptide mass fingerprinting (PMF) using automated matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry and the ExPASy database.

Materials and Methods

Isolation of cutinase-producing microorganism and culture conditions. The cutinase-producing *Bacillus* sp. SB-007 used in this study was isolated from the leaves of peas (*Pisum sativum* L) and identified to the species level using API 50 CH (BioMerieux, Marcy l'Etoile, France) and the Vitek BACIL card with Bergey's *Manual of Systematic Bacteriology* (Staley et al., 1989; Williams et al., 1989). This organism was maintained on NBY (0.2% yeast extract and 0.8% nutrient broth) agar plates and stored at 4°C, while the stock was maintained in 10% glycerol at −80°C. A single colony of each isolate grown on NBY agar plates was inoculated into 5 ml of a NBY broth and cultured at 30°C. After 24 h, 1.5 ml of the culture broth was inoculated into three different flasks containing 100 ml of mineral defined (MD) medium (K_2HPO_4 0.5 g/l, Ferric ammonium citrate 0.5 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 5.5 mg/l, $FeCl_2 \cdot 6H_2O$ 13.5 mg/l, $MnCl_2 \cdot 4H_2O$ 1 mg/l, $ZnSO_4 \cdot 7H_2O$ 3.6 mg/l, $CuCl_2 \cdot 2H_2O$ 0.43 mg/l, $CoCl_2 \cdot 6H_2O$ 0.60 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 0.60 mg/l, Citric acid 4 g/l, L-glutamic acid 2 g/l), MDG (MD + glucose 20 g/l) and MDC (MD + cutin 20 g/l) medium, respectively, and grown for 12 days with constant shaking at 500 rpm. The cutin from the apple cultivar, Golden Delicious, was prepared using the same procedure described by Um et al. (2007). The cells were harvested every 24 h by centrifugation at 18,700×g and 4°C for 20 min. The extracellular fractions were used to estimate the cutinolytic activity.

Cutinolytic enzyme assays and cutin degradation test. The cutinase activity was determined spectrophotometrically using *p*-nitrophenyl butyrate (PNB, Sigma, USA) and *p*-nitrophenyl palmitate (PNP, Sigma, USA) as substrates (Kim et al., 2003). The hydrolysis reactions were performed on a 96-well microplate at 30°C for 4 min. Each well contained 200 µl of an enzyme/substrate solution (solution A), consisting of 106.7 µl of a phosphate buffer (0.1 M, pH 8.0), 13.3 µl Triton X-100 (4 g/L), 13.3 µl of a cell-free culture broth, and 66.7 µl of the substrate (PNB or PNP) solution. The PNB or PNP solutions were prepared with variable concentrations ranging from 0 µg/l to 1000 µg/l. The initial velocity (i.e. the initial maximum rate of change in absorbance (ΔOD_{405nm} per second)) was measured using a Bio-Rad 96 well microplate reader (Bio-Rad, USA). Enzyme-free solution A was used as a blank. Since the

eight wells in each of the 12 columns of the 96-well microplate contained an equal amount of enzyme and substrate, the eight initial velocities measured for each column were used to calculate the average initial velocity for a specific reaction condition, which resulted in a standard deviation <5%.

The degradation of cutin into water-soluble products was examined by measuring the concentration of water-soluble total organic carbon (TOC) in a cell-free culture broth at 30°C using a TOC analyzer (Rose Mount DC-18, Germany).

Preparation of the extracellular protein fraction. Immediately after harvesting the bacterial cells at 12 days, the culture supernatants (added protease inhibitor cocktail tablets) obtained from Boehringer Mannheim GmbH (Germany) were centrifuged at 7,000 rpm for 20 min at 4°C. The resulting supernatant was filtered through a 0.2 μm membrane. The proteins were precipitated with PlusOne 2-D Clean-up kit (Amersham Biosciences, USA). The aggregated proteins were resolved using rehydration solutions (8 M urea, 0.5%(v/v) Triton X-100, 1.5% dithiothreitol (DTT), 0.5%(v/v) IPG buffer pH 3-10, 0.005% orange G dye). The protein concentration was determined using a Bio-Rad (USA) protein assay kit with bovine serum albumin as the standard.

Two-dimensional gel electrophoresis and image analysis. The first dimension of 2-D electrophoresis was carried out on a Pharmacia Biotech IPGphor Electrophoresis System at 20°C. A linear pH4-7-immobilized pH-gradient (IPG) gel strip (18 cm, Amersham Pharmacia Biotech, Sweden) was rehydrated overnight by placing the strip gel-side-down in the sample-containing the rehydration solution in an IPGphor strip holder (Amersham Pharmacia Biotech, Sweden) and covering it with a DryStrip Cover Fluid (Amersham, Sweden Pharmacia Biotech, Sweden). The sample was loaded on the silver-stained gels at an extracellular protein concentration of 45 μg. The first dimension was run at 20°C under the following conditions: A five-phase program was used for the analytical and preparative gels. The first phase was set at 500 V for 2 h, the second phase was set at 1,000 V for 30 min. and the third and fourth phases were set at 2,000 V and 4,000 V for 30 min, respectively. The last phase was set to 8,000 V to 70,000 Vhs. After electrophoresis, the strips were stored at −80°C or used directly for the second dimensional electrophoresis. Subsequently, the IPG gel strip was equilibrated for 15 min in an equilibration solution (1.5 M Tris-HCl, pH 8.8 50 mM, Urea 6 M, Glycerol 30%(v/v), SDS 2%, Bromophenol blue trace) containing 1% DTT and 2.5% Iodoacetamide. The second dimensional separations were carried out by running 12.5% SDS-PAGE gels (PROTEAN II xi cell system, Bio-rad) in a 4°C cold chamber. The IPG strips were embedded on top of the gels with 1% agarose. Electrophoresis was carried out at 30 mA/gel for 12 h until the bromophenol blue indicator reached the bottom of the gel. The gels were fixed and silver stained using a slight modification of the method reported elsewhere (Thierry, 1999). The molecular mass of the proteins were assigned by the molecular weight markers (Seeblue plus2, Invitrogen). The stained gels were scanned using EPSON EXPRESSION1680 Pro (EPSON, Korea) and analyzed using Progenesis PG200 v2006 from Nonlinear Dynamics. The spots were quantified after normalization, and the spot volume (pixel intensity X area) is expressed as the percentage of the total volume of the spots on the gel. In this study, four to eight independent protein preparations were made, and at least four gels for each preparation were run, silver stained and analyzed.

MALDI-TOF mass spectrometric analysis and protein identification. MALDI-TOF mass spectrometry analysis was performed using the previous protocol (Majoul et al., 2003). Enzymatic digestions were carried out overnight at 37°C in stationary incubator using 10-15 μg/ml of sequencing-grade modified trypsin (Promega, USA) in 25 mM ammonium bicarbonate (pH 8.0). The in-gel-digested peptide fragments were extracted from the gel pieces using a solution prepared by adding 5% v/v trifluoroacetic acid (TFA) to 50% v/v acetonitrile followed by vortexing for 1 h. After repeating three times, the solute materials including the peptide fragments were dried by vacuum centrifugation. A Ziptip column (Millipore, USA) containing C18 resin fixed at the end of the tip was used to eliminate the impurities in the samples. The peptide solution was prepared with an equal volume of a saturated α-cyano-4-hydroxy-cinnamic acid solution in 50% ACN/0.1% TFA on the sample plate of the MALDI-TOF mass spectrometer. Protein analyses were carried out using a MALDITOF mass spectrometry system (Voyager DE-STR, PE Biosystem, USA). The spectra were calibrated using matrix and tryptic autodigestion ion peaks as the internal standards. The peptide mass fingerprints were analyzed using the MS-Fit (http://www.prospector.ucsf.edu/). The identification of a protein with the respective theoretical parameters (pI, molecular mass) was accepted if the peptide mass matched with a mass tolerance of 10 ppm. The accessibility of such data has been revolutionized through the use of internet protocols such as SWISS-2D-PAGE (http://www.expasy.org/) and NCBI (hsttp://www.ncbi.nlm.nih.gov/).

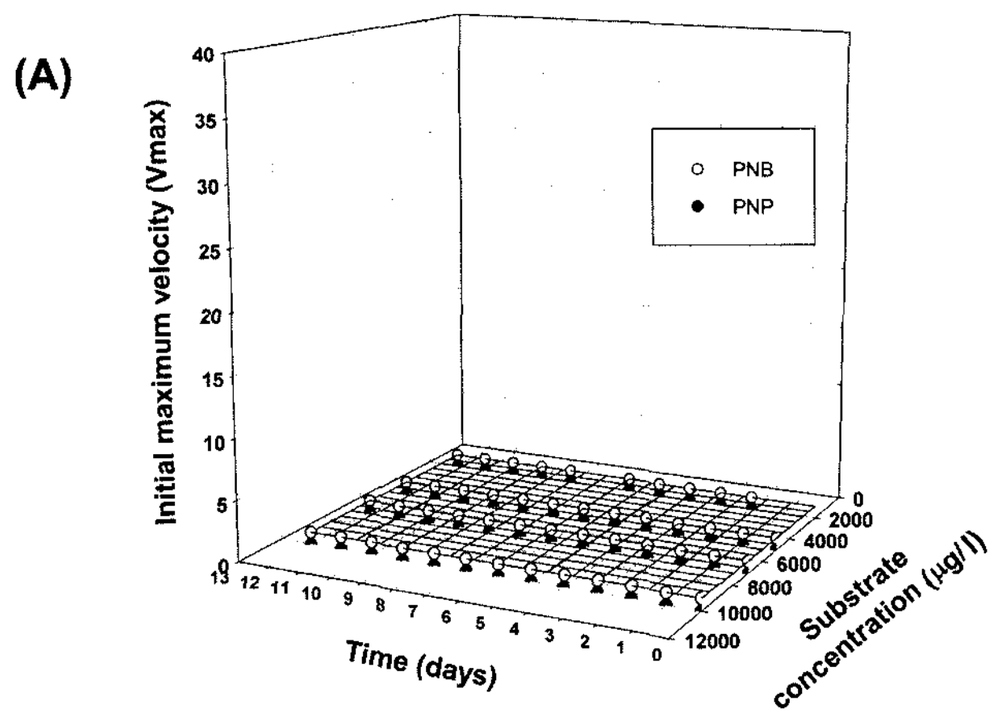
Results and Discussion

Identification of the selected bacterial isolate. SB-007,

which showed promising cutinase activity, was selected for identification. The taxonomic schemes and criteria to determine the isolate followed the Bacteriology Committee of the American Phytopathogenical Society criteria (Schaad, 1988). The isolate was identified as a *Bacillus* species strain based on the biochemical, morphological, and cultural characteristics (data not shown). The isolate was also confirmed by 16S rRNA sequences analysis using the NCBI BLAST searching tool. The sequence showed more than 99% homology with the reference sequence (data not shown).

Cutinase activity and cutin degradation by *Bacillus* sp. SB-007. *Bacillus* sp. SB-007 showed the best growth on the MDC medium (MD medium+cutin) but relatively poor growth on the MD medium alone (Fig. 1). As shown in Fig. 1, the growth rate of *Bacillus* sp. in the cutinase-induced culture was increased significantly after 2 days. However, in the MDG medium (MD medium+glucose), the growth of *Bacillus* sp. SB-007 was only half that of that observed in the cutinase-induced cultivation. Previous studies showed that substrate-specific hydrolysis and the high accessibility to PNB are intrinsic properties of cutinase only, which have never been observed in hydrolysis by lipases and esterases (Kim et al., 2003). These distinct properties of cutinase might have applications in the rapid and easy isolation of natural cutinases from different microbial origins. The time-course hydrolytic activities of the cell-free supernatant from the *Bacillus* sp. SB-007 culture broth on the two *p*-nitrophenyl esters, PNB and PNP, were estimated over a range of substrate concentrations (Fig. 2). The hydrolytic activities in this study were defined as the initial maximum rate of hydrolysis at each substrate concentration, as previously described (Kim et al., 2003).

During the growth of *Bacillus* sp. SB-007 in the MDC medium, the hydrolytic enzyme activity on PNB increased rapidly after 3 days and was maintained at a high level until the end of cultivation. The initial maximum rate of PNB hydrolysis (V_{max}) in the MDC medium was up to 15 times higher than that in the MD medium (Fig. 2A and 2C), whereas the hydrolytic activity (V_{max}) on PNP was quite



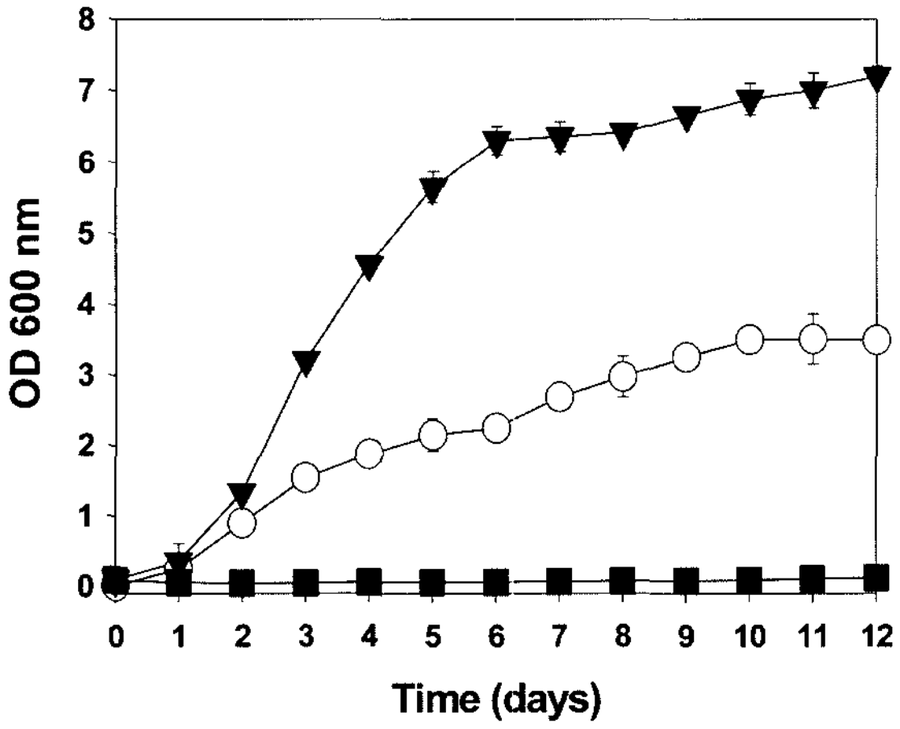


Fig. 1. Growth measured by culture turbidity (OD_{600}) in MD (■), MDG (MD+glucose 20 g/l) (○), and MDC (MD + cutin 20 g/l) (▼) medium.

Fig. 2. Initial maximum rate of PNB and PNP hydrolysis in the extracellular culture broth of *Bacillus* sp. SB-007 that was cultivated in MD (A), MDG (B) and MDC (C) medium.

low within the same range of substrate concentrations, even in the MDC medium (Fig. 2C). This shows the same substrate-specificity upon the hydrolysis of PNB and PNP as that observed in previous studies on fungal and bacterial cutinases (Kim et al., 2003). Through the PNB/PNP hydrolysis assay, *Bacillus* sp. SB-007 was found to produce a highly inducible cutinase when grown in a MDC medium containing 1% purified cutin from the apple cultivar, Golden Delicious, but did not produce any cutinase when grown in the MDG media only. This concurs with Kolattukudy (1998) in that the presence of glucose in the medium significantly repressed the cutinase activity. Therefore, the results shown in Fig. 2 strongly suggest that *Bacillus* sp. SB-007 hydrolyzed cutin effectively using the induced cutinase in an extracellular culture broth. The water-soluble total organic carbon (TOC) level in the MDC medium was more than 20 times higher than in the YM medium (Fig. 3). This suggests that cutin is significantly degraded and is converted to water soluble products by cutinase. The TOC concentration was the same in both the cell-free MD and MDC fresh medium for 12 days (Fig. 3), which suggests that the increase in the TOC level was due to the cutinase produced by *Bacillus* sp. SB-007.

Secreted proteome profiling of *Bacillus* sp. SB-007. Two-DE was used to examine the characteristics of the overall secreted protein expression resulting from the two different growth media (MDG vs. MDC). Prior to 2-DE analysis, *Bacillus* sp. SB-007 was cultivated in a 100 ml flask using cutin and glucose as the carbon source (Fig. 1). The culture supernatants were taken at 12 days after harvesting the bacterial cells. 2-DE was repeated four to eight times for each protein sample. For comparative image analysis, an average gel image was prepared from three gels showing a high degree of reproducibility using Progenesis PG200 v2006 (Nonlinear Dynamics). Through comparative analysis of the 2-DE results from the cutinase-induced zmedium and non cutinase-induced medium containing glucose, the change in the expression levels of the secreted protein (as measured by the protein spot intensity on the 2-DE gels) was monitored and compared for the cutin-induced proteins (Fig. 4). Based on image analysis, a total of 282 and 208 protein spots were counted on an average gel image from the MDG and MDC medium, respectively. Comparative gel analysis showed that 83 spots of the large extracellular proteins in the cutinase-induced culture showed higher intensity than that from the non cutinase-induced culture. In particular, 27 protein spots from the 83 spots were only

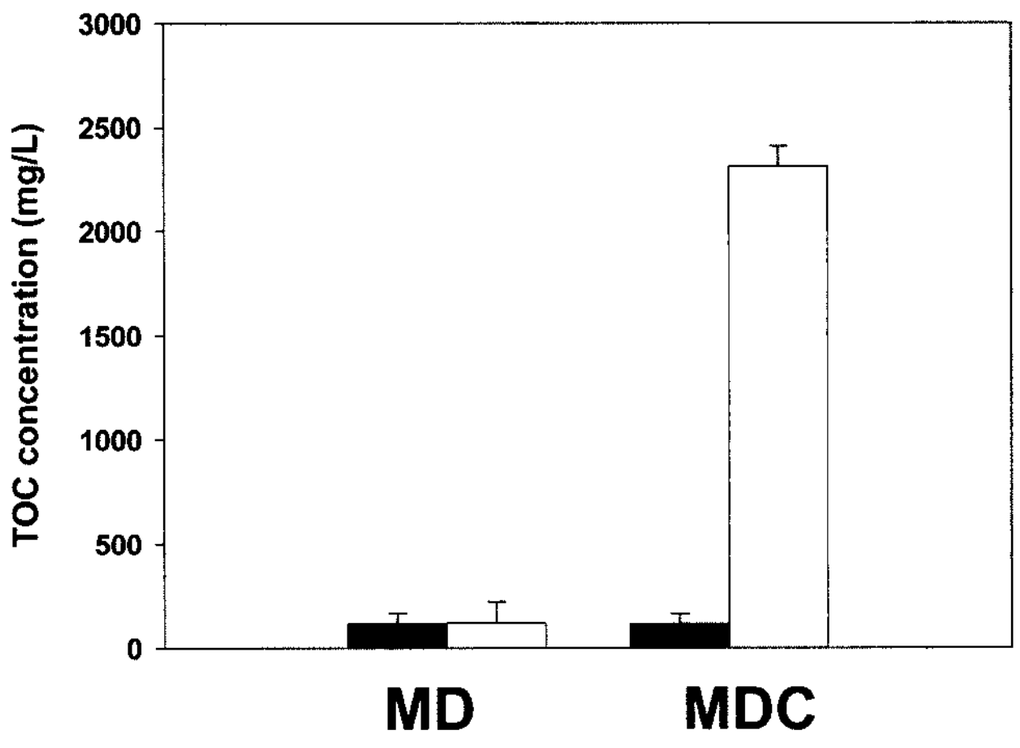


Fig. 3. Total organic carbon (TOC) concentrations measured in the culture broth of *Bacillus* sp. SB-007 when grown in DM and DMC medium. The black and white columns represent the TOC concentrations in the cell-free fresh medium and after 12-days, respectively.

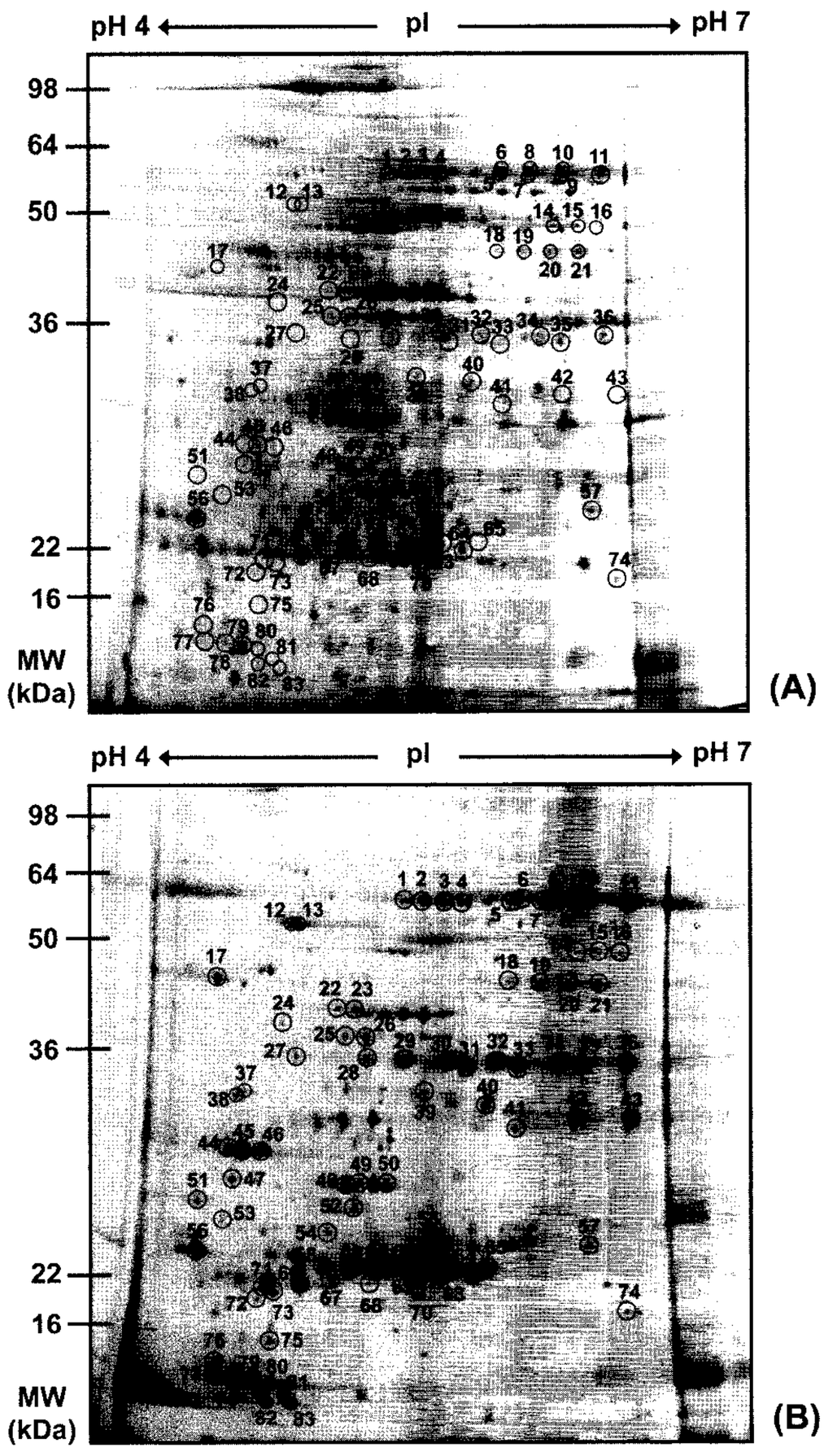


Fig. 4. Silver-stained 2-D SDS PAGE gels showing the 83 protein spots listed in Table 1. (A) non cutinase-induced medium containing glucose; (B) cutinase-induced medium.

secreted into the cutinase-induced medium, as shown in Fig. 4B. These differences in the total amount of proteins secreted per cell show that some proteins specifically related to the production of cutinases were probably induced by cutin and were repressed in the glucose containing minimal medium. The variation in the level of protein synthesis was estimated comparatively by defining the spot intensity of the proteins visualized at the MDG medium as unity (Table 1). Different intensity ratios >1 indicate the cutin-responsive induction of protein expression. The proteins were identified by MALDI-TOF analysis for each protein spot and the subsequent PMF using the *Bacillus* ExPASy database, as listed in Table 1. Of these 83 proteins, 62 were known or their functions were predicted by a database search. These include 4 enzymes related to the energy metabolism, 6 sporulation-specific proteins, 9 transport/binding proteins or lipoproteins, 2 proteins related to protein translations, 8 enzymes related to amino acid biosynthesis pathways, 3 proteins related to membrane bioevergetics, mobility and chemotaxis, 6 enzymes associated with competence for nucleotide biosynthesis and transports, 6 stress-induced proteins, and 18 cellular functional proteins. The functions of the remaining 21 proteins are unknown.

As shown in Table 1 and Fig. 4, the expression level of the 6 sporulation-related proteins (spots 7, 18, 65, 74, 81, 50) was significantly up-regulated in the cutinase-induced culture. At least 4% of the *B. subtilis* genome is dedicated to the process of sporulation, germination and outgrowth (Sinchaikul et al., 2002). In *B. subtilis*, expression of the genes for extracellular enzymes, such as a-amylase, several proteases, and hydrolytic enzymes, is linked temporally to the initiation of sporulation (Honjo et al., 1990; Masayama et al., 2007). The sporulation and expression of these degradative-enzyme genes are regulated under catabolite repression (Sinchaikul et al., 2002). On the other hand, the expression level of spore coat protein A (CotA), which is involved in the brown pigmentation during sporogenesis, was more than 4 times higher in the cutinase-induced culture compared with the results from the culture grown in the non cutinase-induced culture. This means that in this study, the brown pigmentation of cell-free culture broth caused technical problems in removing the color during the purification step and unexpected difficulties during isoelectric focusing of the 2-DE experiments. Most bacterial lipolytic enzymes, such as lipase and esterase, are extracellular enzymes that must be translocated through the membranes to reach their final destination. Therefore, they have an N-terminal signal sequence that mediates their secretion. *B. subtilis* also secretes two lipases, LipA and LipB, into the culture medium (Dartois et al., 1992; Eggert et al., 2000). These enzymes have been characterized in detail through biochemical, genetic, and structural studies (Dartois et al., 1992; Eggert et al., 2000, 2001). Although extracellular proteins were already identified in the phosphate-, glucose-, and amino acids-starved *B.* subtillis cells and a detailed analysis of the secretome of *B. subtillis* upon heat, ethanol, and oxidative stress are currently underway (Antelmann et al., 2003, 2004; Buttner et al., 2001; Voigt et al., 2006), the cutinase from *B. strains* have never been reported. For this reason, identification of the cutinase secreted from *Bacillus* sp. SB-007 is severely limited using 2-DE and PMF due to the insufficient construction of the proteome databases for protein identification. Interestingly, through secreted proteome analysis of *Bacillus* sp. SB-007, it was observed that two lipolytic enzyme-like protein spots (spots 12 and 13), which were identified as para-nitrobenzyl esterase (PnbA) from the *B. subtilis* ExPASy database, were only expressed in the cutinase-induced culture, as shown in Fig. 4 and Table 1. Detailed studies of these lipolytic enzymes (spots 12 and 13) from *Bacillus* sp. SB-007 for clear identification using N-terminal amino acid sequencing are currently underway.

Previous studies reported that some amino acids, arginine, glutamic acid, histidine, leucine, isoleucine, methionine, threonine, tryptophan and valine, are required for the production of lipolytic enzymes during the growth of *Streptococcus faecalis* (Chander and Ranganathan, 1975). The expression of eight metabolic enzymes (spots 1, 6, 22, 26, 28, 33, 48, 56) out of the 83 proteins was strongly induced in the cutinase-induced culture. The level of dihydroxyacid dehydratase (IlvD) and ketol-acid reductoisomerase (IlvC) synthesis involved in the biosynthesis of valine and isoleucine were 9 and 41 times higher in the cutinase-induced culture than in the non cutinase induced culture. It was assumed that a significant amount of the key metabolic precursor related to valine and isoleucine biosynthesis might accumulate in the cutinase-induced culture, which would direct the overall metabolic flow toward the valine and isoleucine biosynthetic route. Furthermore, the other key enzymes for the metabolism of L-methionine, i.e. S-adenosylmethionine synthetase (MetK), Methylthioribose-1-phosphate isomerase (MtnA), and 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase (MtnD), were oversynthesized in the cutinase-induced culture growth. There were also several transporting/binding proteins and lipoproteins (spots 14-15, 25, 30, 39, 44, 47, 59) present in the cutinase-induced extracellular medium. These proteins were present at high levels in the extracellular proteome of the stationery growing *Bacillus* sp. SB-007 in MDC medium. The twin-arginine translocation pathway (Tat), ABC transporters and several transport pathways are secretion pathways for special purposes, and have been found to transport only a few proteins in *B. subtilis* (Jongbloed et al.,

Table 1. The 83 proteins of *Bacillus* sp. SB-007 showing significant differences in extracellular protein level between the cutin-induced culture and glucose-containing culture.

No.	Gene name	Protein name	pI	MW (kDa)	Accession Number[a]	% Sequence coverage[b]	Protein level ratio[c]
Energy metabolism							
21	*melA*	Alpha-galactosidase	5.9	49264	O34645	36	+54.3
31	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase 1	5.0	35676	P09124	32	+4.8
46	*pta*	Phosphate acetyltransferase	4.7	34636	P39646	25	+36.5
80	*ptsH*	Phosphocarrier protein HPr	4.7	9119	O69250	39	+++[d]
Sporulation							
7	*cotA*	Spore coat protein A	6.0	58759	P07788	33	+4.1
18	*spsK*	Spore coat polysaccharide biosynthesis protein	5.9	48650	P39631	25	+20.9
50	*tasa*	Tasa protein	5.3	28800	Q5ND72	37	+++[d]
65	*spo0A*	Stage 0 sporulation protein A	6.0	29540	P06534	14	+++[d]
74	*cotO*	Spore coat protein O	6.4	25445	O31622	32	+6.0
81	*gerPA*	Probable spore germination protein	4.7	7541	O06721	34	+++[d]
Transport/binding proteins and lipoproteins							
14	*rbsA*	Ribose import ATP-binding protein	6.2	54532	P36947	30	+1.4
15	*rbsA*	Ribose import ATP-binding protein	6.2	54532	P36947	30	+2.5
16	*rbsA*	Ribose import ATP-binding protein	6.2	54532	P36947	31	+10.1
25	*ycdH*	YcdH	5.0	38400	O34966	25	+3.3
30	*yfiY*	YfiY protein	5.6	36700	O31567	17	+48.5
39	*mntA*	Manganese-binding lipoprotein (Precursor)	5.2	34300	O34385	34	+2.6
44	*pstS*	Phosphate-binding protein (Precursor)	4.6	32600	P46338	36	+36.7
47	*yhcJ*	Uncharacterized lipoprotein (Precursor)	4.7	29400	P54594	34	+27.4
59	*artP*	Arginine-binding extracellular protein (Precursor)	5.1	28162	P54535	15	+2.5
Protein translations							
35	*rpsB*	30S ribosomal protein S2	6.3	27817	P21464	34	+26.3
76	*rplL*	50S ribosomal protein L7/L12	4.4	12611	P02394	24	+++[d]
Amino acid biosynthesis, degradiaon, and transport							
1	*ilvD*	Dihydroxyacid dehydratase	5.4	59549	P51785	32	+8.7
6	*hutU*	Urocanate hydratase	5.7	60601	P25503	30	+6.3
22	*metK*	S-adenosylmethionine synthetase	5.0	43882	P54419	21	+++[d]
26	*mtnA*	Methylthioribose-1-phosphate isomerase	5.0	38703	O31662	26	+5.0
28	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	5.2	37917	P23715	31	+++[d]
33	*ilvC*	Ketol-acid reductoisomerase	5.4	37302	P37253	25	+40.6
48	*speE*	Spermidine synthase	5.1	31184	P70998	37	+3.8
56	*mtnD*	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	4.4	20680	O31669	29	+5.8
Membrane bioenergetics, mobility, and chemotaxis							
2	*tlpC*	Methyl-accepting chemotaxis protein	5.1	61785	P39209	32	+15.1
5	*ymdA*	UPF0144 protein	5.5	58920	O31774	34	+2.5
45	*flgG*	Flagellar basal-body rod protein	4.6	28000	P23446	24	+35.8
Nucleotide biosynthesis and transport							
3	*abfA*	Alpha-N-arabinofuranosidase 1	5.5	57061	P94531	23	+4.3
4	*abfA*	Alpha-N-arabinofuranosidase 1	5.5	57061	P94531	33	+7.2
10	*hsdBM*	Modification methylase BsuBI	6.0	57198	P33563	31	+10.9
11	*hsdBM*	Modification methylase BsuBI	6.0	57198	P33563	19	+14.1
49	*bsn*	Extracellular ribonuclease Precursor	5.1	30500	O32150	23	+8.4
53	*adk*	Adenylate kinase	4.5	23972	P16304	30	+++[d]

Table 1. Continued

No.	Gene name	Protein name	pI	MW (kDa)	Accession Number[a]	% Sequence coverage[b]	Protein level ratio[c]
Metabolism of ribose							
37	*rbsK*	Ribokinase	4.6	30986	P36945	25	+++[d]
Metabolism of lipid and fatty acid							
38	*fabD*	Malonyl CoA-acyl carrier protein transacylase	4.5	33880	P71019	28	+++[d]
Stress-induced proteins							
27	*yhdN*	General stress protein 69	4.8	37156	P80874	24	+++[d]
55	*sigB*	RNA polymerase sigma-B factor	5.4	29995	P06574	34	+4.8
64	*ycdF*	Glucose 1-dehydrogenase 2	5.7	27625	P80869	16	+10.2
72	*dps*	General stress protein 20U	4.4	16451	P80879	37	+3.6
75	*yfkM*	General stress protein 18	4.7	18720	P80876	36	+++[d]
82	*cspB*	Cold shock-like protein	4.6	7200	Q45097	22	+6.7
Metabolism of phosphate							
24	*phy*	3-phytase (Precursor)	4.7	42000	P42094	22	+++[d]
Pharge-related function							
66	*xkdM*	Phage-like element PBSX protein	4.7	16400	P54332	25	+4.6
Other functions							
12	*pnbA*	para-nitrobenzyl esterase	5.0	53987	P37967	14	+++[d]
13	*pnbA*	para-nitrobenzyl esterase	5.0	53987	P37967	11	+++[d]
19	*albA*	Antilisterial bacteriocin subtilosin biosynthesis protein	6.1	51546	P71011	35	+41.8
20	*cypC*	Cytochrome P450 152A1	6.5	48110	O31440	22	+14.4
32	*ccpA*	Catabolite control protein A	5.1	36784	P25144	24	+117.3
36	*ppnK2*	Probable inorganic polyphosphate/ATP-NAD kinase 2	6.5	30102	O34934	24	+47.4
40	*folD*	Bifunctional protein	5.6	30534	P54382	27	+28.8
41	*fmt*	Methionyl-tRNA formyltransferase	5.9	34823	Q9K9Y6	31	+++[d]
43	*folE*	GTP cyclohydrolase I	6.3	21074	P19465	16	+++[d]
58	*azoR1*	FMN-dependent NADH-azoreductase 1	5.0	22830	O35022	23	+2.9
62	*ndk*	Nucleoside diphosphate kinase	5.6	16816	P31103	36	+2.7
67	*pyrR*	Bifunctional protein	5.0	20119	P39765	25	+++[d]
77	*trxA*	Thioredoxin	4.3	11254	P14949	23	+41.4
83	*comZ*	ComG operon repressor	4.7	7214	O32437	26	+++[d]
Unknown							
8	*ykqC*	UPF0036 protein	5.9	61517	Q45493	31	+10.4
9	*ytdP*	YtdP	6.4	58994	O30503	25	+1.6
17	*ypiA*	TPR repeat-containing protein	4.3	48321	P54389	39	+++[d]
23	*ydaJ*	YdaJ protein	5.0	41300	O31486	26	+6.6
29	*yxjG*	Uncharacterized protein	5.3	37861	P42318	28	+16.9
34	*yfhM*	YfhM protein	6.1	32606	O31581	38	+55.9
42	*ykpB*	YkpB protein	6.1	33419	O31717	37	+++[d]
51	*ygaI*	Hypothetical 29.7 kd protein	4.3	29557	P71088	36	+++[d]
52	*yvbY*	YvbY protein	5.2	26128	O32259	15	+8.8
54	*ywfL*	Uncharacterized protein	5.0	29335	P39648	28	+5.0
57	*yuaE*	YuaE protein	6.2	18967	O32078	37	+13.7
60	*yjbC*	YjbC protein	5.1	22956	O31601	26	+8.9
61	*ypjP*	Uncharacterized protein	5.4	23400	P54172	22	+18.0
63	*yjcG*	UPF0477 protein	5.6	19518	O31629	28	+8.4
68	*ytxH*	Uncharacterized protein	5.1	16543	P40780	14	+++[d]
69	*ysnB*	Putative metallophosphoesterase	5.3	19011	P94559	31	+++[d]

Table 1. Continued

No.	Gene name	Protein name	pI	MW (kDa)	Accession Number[a]	% Sequence coverage[b]	Protein level ratio[c]
70	*ysnB*	Putative metallophosphoesterase	5.3	19011	P94559	29	+++[d]
71	*ykuQ*	YkuQ protein	4.7	24838	O34981	31	+++[d]
73	*ykuQ*	YkuQ protein	4.7	24838	O34981	21	+++[d]
78	*ynfE*	Uncharacterized protein	4.3	10244	Q45069	25	+104.9
79	*yugE*	Uncharacterized protein	4.4	10065	O05234	32	+7.0

[a]Acession code refers to the SWISS-2DPAGE database
[b]Mass tolerance in protein identification through PMF experiments was 10 ppm
[c]Protein expression level in cutin-induced medium compared with glucose-containing medium. Expression level in MDG was set at 1.
[d]These proteins were only detected in cuntin-induced culture.

2002; Tjalsma et al., 2004; van Dijl et al., 2001). Furthermore, the strong secretion of some proteins involved in the metabolism of nucleotide was observed (spots 27, 55, 64, 72, 75, 82). The induction of several proteins involved in the metabolism of nucleotides during grown in different media and different levels of nutrient starvation for *B. licheniformis* has been also described (Voigt et al., 2006).

It is possible that *Bacillus* sp. SB-007 expresses some of the exoenzyme genes and secretes the corresponding hydrolyting enzymes only when cultured on different carbon sources in the medium. It was observed that the synthesis of 3-phytase (Phy) and catabolite contoral protein A (CcpA) were strongly promoted in the MDC medium. Antelmann et al. (2003) reported the secretion of SacB and Phy, which were not observed in earlier experiments, when *B. subtilis* was grown in a medium containing maltodextrin as the carbon source. In addition, carbon catabolite repression is a global regulatory mechanism to coordinate the carbon metabolism in *Bacillus*. In the presence of the preferred sugar, glucose, the regulator, CcpA, represses the catabolic genes whose expression is essential for utilizing alternative carbon sources. Most of the genes encoding extracellular carbohydrate degrading enzymes are under the control of CcpA in *B. subtilis* (Martin-Verstraete et al., 1999; Voigt et al., 2006; Yoshida et al., 2001). Interestingly, a comparison with the secreted proteome analysis showed that the six stress inducible proteins (spots 27, 55, 64, 72, 75, 82) were significantly overexpressed in the cutinase-induced extracellular medium compared with the glucose-containing extracellular medium, as shown in Table 1 and Fig. 4. There are detailed reports on the temporal and spatial control of gene expression, intracellular communication, the effects of protein secretion stress, and the responses of nutrient starvation and/or changes on the composition of the extracellular proteome of *B. subtilis* (Antelmann et al., 2003). However, the mechanism for the secretion stress responses is still unclear (Voigt et al., 2007). Therefore, the (putative) chaperone-like and protease activities of the membrane-bound and extracellular forms of these proteins *in vitro* will need to be characterized in order to achieve a complete understanding of the function of the stress-related proteins of *Bacillus* sp. SB-007. Furthermore, it will be important to examine the role of the stress-related proteins in the biogenesis of the membrane and cell wall associated proteins of *Bacillus* sp. SB-007. This study also suggests that the synthesis of cytochrome P450 (cypC) was strongly activated in the cutinase-induced culture. It is well known that the cytochrome P450 families of *Bacillus* sp. can be used to oxidize a wide range of compounds for biomedical applications, including steroids and fatty acids, and xenobiotics, such as drugs and environmental chemicals (Sinchaikul et al., 2002).

In conclusion, this paper reports the isolation of cutinase-producing *Bacillus* sp. SB-007. The results demonstrate that the proteomic approach is a highly efficient method for examining the differential expression of the secreted proteins by *Bacillus* sp. SB-007 grown under cutinase-induced conditions. It is expected that more complete information on the physiological and functional characteristics of the overall secreted proteome from cutinase-producing *Bacillus* sp. SB-007 will be obtained through detailed analysis of the transcriptomic responses at the gene expression level. Further detailed studies of the biological function and mechanism of cutinase biosynthesis from *Bacillus* sp. SB-007 are currently underway. Moreover, these results will be important for the development of new agricultural and biomedical applications.

Acknowledgements

This study was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-331- D00103). This study was also supported by a research grant of the Chungbuk National University in 2007.

References

Antelmann, H., Darmon, E., Noone, D., Veening, J. W., Westers,

H., Bron, S., Kuipers, O. P., Devine, K. M., Hecker, M. and van Dijl, J. M. 2003. The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. *Mol. Microbiol.* 49:143-156.

Antelmann, H., Sapolsky, R., Miller, B., Ferrari, E., Chotani, G., Weyler, W., Gaertner, A. and Hecker, M. 2004. Quantitative proteome profiling during the fermentation process of pleiotropic *Bacillus subtilis* mutants. *Proteomics* 4:2408-2424.

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Diji, J. M. and Hecker, M. 2001. A proteomic view on genome-based signal peptide predictions. *Genome Res.* 11: 1484-1502.

Buttner, K., Bernhardt, J., Scharf, C., Schmid, R., Mader, U., Eymann, C., Antelmann, H., Volker, A., Volker, U. and Hecker, M. 2001. A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. *Electrophoresis* 22: 2908-2935.

Chander, H. and Ranganathan, G. 1975. Role of amino acids on the growth and lipase production of *Streptococcus faecalis*. *Experientia* 31:1263.

Chen, Z., Franco, C. F., Baptista, R. P., Cabral, J. M., Coelho, A. V., Rodrigues, C. J. Jr. and Melo, E. P. 2006. Purification and identification of cutinase from *Colletotrichum kahawae* and *Colletotrichum gloeosporioides*. *Appl. Microbiol. Biotechnol.* 73:1306-1313.

Chu, P. W., Yap, M. N., Wu, C. Y., Huang, C. M., Pan, F. M., Tseng, M. J. and Chen, S. T. 2000. A proteomic analysis of secreted proteins from xylan-induced *Bacillus* sp. strain K-1. *Electrophoresis* 21:1740-1745.

Coppee, J. Y., Auger, S., Turlin, E., Sekowska, A., Le Caer, J. P., Labas, V., Vagner, V., Danchin, A. and Martin-Verstraete, I. 2001. Sulfur-limitation-regulated proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study. *Microbiology* 147:1631-1640.

Cristina, M. L. C., Maria, R. A. B. and Joaquim, M. S. C. 1998. Cutinase structure, function and biocatalytic applications. *Electronic J. Biotechnol.* 1:160-173.

Cristina, M. L. C., Maria, R. A. B. and Joaquim, M. S. C. 1999. Cutinase: from molecular level to bioprocess development. *Biotechnol. Bioeng*. 66:17-34.

Dartois, V., Baulard, A., Schanck, K. and Colson, C. 1992. Cloning, nucleotide sequence and expression in Escherichia coli of a lipase gene from *Bacillus subtilis* 168. *Biochim. Biophys. Acta*. 1131:253-260.

Eggert, T., Pencreac'h, G., Douchet, I., Verger, R. and Jaeger, K. E. 2000. A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase. *Eur. J. Biochem.* 267:6459-6469.

Eggert, T., van Pouderoyen, G., Dijkstra, B. W. and Jaeger, K. E. 2001. Lipolytic enzymes LipA and LipB from *Bacillus subtilis* differ in regulation of gene expression, biochemical properties, and three-dimensional structure. *FEBS Lett*. 502:38-92.

Egmond, M. R. and de Vlieg, J. 2000. *Fusarium solani pisi* cutinase. *Biochimie* 82:1015-1021.

Flipsen, J. A. C., Appel, A. C. M., Van der Hijden, H. T. W. M. and Verrips, C.T. 1998. Mechanism of removal of immobilized triacylglycerol by lipolytic enzymes in a sequential laundry wash process. *Enzyme Micro. Technol.* 23:274-280.

Gerard, H. C., Fett, W. F., Osman, S. F. and Moreau, R. A. 1993. Evaluation of cutinase activity of various industrial lipases. *Biotechnol. Appl. Biochem.* 17:181-189.

Hirose, I., Sano, K., Shioda, I., Kumano, M., Nakamura, K. and Yamane, K. 2000. Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. *Electrophoresis* 146:65-75.

Honjo, M., Nakayama, A., Fukazawa, K., Kawamura, K., Ando, K., Hori, M. and Furutani, Y. 1990. A novel *Bacillus subtilis* gene involved in negative control of sporulation and degradative-enzyme production. *J. Bacteriol.* 172:1783-1790.

Jongbloed, J. D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., Pries, F., Quax, W. J., van Dijl, J. M., Braun, P. G. 2002. Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. *J. Biol. Chem.* 277:44068-44078.

Kim, D. H. 2003. Modulation of a fungal signaling by Hypovirus. *Plant Pathol. J.* 19:30-33.

Kim, Y. H., Ahn, J. Y., Moon, S. H. and Lee, J. 2005. Biodegradation and detoxification of organophosphate insecticide, malathion by *Fusarium oxysporum* f. sp. *pisi* cutinase. *Chemosphere* 60:1349-1355.

Kim, Y. H., Lee, J., Ahn, J. Y., Gu, M. B. and Moon, S. H. 2002. Enhanced degradation of an endocrine-disrupting chemical, butyl benzyl phthalate, by *Fusarium oxysporum* f. sp. *pisi* cutinase. *Appl. Environ. Microbiol.* 68:4684-4688.

Kim, Y. H., Lee, J. and Moon, S. H. 2003. Uniqueness of microbial cutinases in hydrolysis of p-nitrophenyl esters. *J. Microbiol. Biotechnol.* 13:57-63.

Kolattukudy, P. E. 1984. Cutinase from fungi and pollen. In: *Lipase,* ed. by B. Borgstrom and T. Brockman, pp. 471-504. Elsevier, Amsterdam.

Lin, T. S. and Kolattukudy, P. E. 1980. Structural studies on cutinase, a glycoprotein containing novel amino acids and glucuronic acid amide at the N terminus. *Eur. J. Biochem.* 106:341-351.

Majoul, T., Bancel, E., Triboi, E., Hamida, J. B. and Branlard, G. 2003. Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from total endosperm. *Proteomics* 3:175-183.

Martin-Verstraete, I., Deutscher, J., Galinier, A. 1999. Phosphorylation of HPr and Crh by HprK, early steps in the catabolite repression signalling pathway for the *Bacillus subtilis* levanase operon. *J. Bacteriol.* 181:2966-2969.

Martinez, C., De Geus, P., Lauwereys, M., Matthysens, G. and Cambillau, C. 1992. *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. *Nature* 356:615-618.

Masayama, A., Kuwana, R., Takamatsu, H., Hemmi, H., Yoshimura, T., Watabe, K. and Moriyama, R. 2007. A novel lipolytic enzyme, YcsK (LipC), located in the spore coat of *Bacillus subtilis*, is involved in spore germination. *J. Bacteriol.* 189:2369-2375.

Murphy, C. A., Cameron, J. A., Huang, S. J. and Vinopal, R. T.

1998. A second polycaprolactone depolymerase from *Fusarium*, a lipase distant from cutinase. *Appl. Microbiol. Biotechnol.* 50:692-696.

Petersen, M. T. N., Martel, P., Petersen, E. I., Drablos, F. and Pertersen, S. B. 1997. Surface and electrostatics of cutinases. *Methods in enzymology* Vol. 284, ed. by B. Rubin and E.A. Dennis, pp. 130-154. Academics, New York.

Poulose, A. J. and Kolattukudy, P. 1988. Enzymes as agricultural chemical adjuvants. European Patent 0,272,002 A1.

Poulsen, K. R., Sorensen, T. K., Duroux, L., Petersen, E. I., Petersen, S. B. and Wimmer, R. 2006. The interaction of *Fusarium solani pisi* cutinase with long chain spin label esters. *Biochemistry* 45:9163-9171.

Schaad, N. W. 1988. Laboratory Guide for Identification of Plant Pathogenic Bacteria 2nd Edition.

Shimao, M. 2001. Biodegradation of plastics. *Curr. Opin. Biotechnol.* 12:242-247.

Sinchaikul, S., Sookkheo, B., Topanuruk, S., Juan, H. F., Phutrakul, S. and Chen, S. T. 2002. Bioinformatics, functional genomics, and proteomics study of *Bacillus* sp. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 771:261-287.

Soliday, C. L. and Kolattukudy, P. E. 1983. Primary structure of the active site region of fungal cutinase, an enzyme involved in phytopathogenesis. *Biochem Biophys Res Commun.* 114:1017-1022.

Staley, J. T., Bryant, M. P., Pfennig, N. and Holt, J. G. 1989. Bergey's Manual of Systematic Bacteriology, Vol. 3, ed. by Williams and Wilkins, Baltimore.

Thierry, R. 1999. Silver staining of 2-D electrophoresis gels. *Methods Mol. Biol.* 112:297-311.

Tjalsma, H., Antelmann, H., Jongbloed, J. D., Braun, P. G., Darmon, E., Dorenbos, R., Dubois, J. Y., Westers, H., Zanen, G., Quax, W. J., Kuipers, O. P., Bron, S., Hecker, M., van Dijl, J. M. 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* 68:207-233.

Um, H. J., Seo, H. S., Min, J. H., Rhee, S. K., Cho, T. J., Kim, Y. H. and Lee, J. W. 2007. *Pseudozyma jejuensis* sp. nov., a novel cutinolytic ustilaginomycetous yeast species that is able to degrade plastic waste. *FEMS Yeast Res.* 7:1035-1045.

van Dijl, J. M., Bolhuis, A., Tjalsma, H., Jongbloed, J. D. H., de Jong, A. and Bron, S. 2001. Protein transport pathways in *Bacillus subtilis*: a genome-based road map. In: *Bacillus Subtilis and its Closest Relatvies: from Genes to Cells.*, ed. by A. L. Sonenshein, J. A. Hoch and R. Losick., pp. 337-355. American Society for Microbiology Press, Washington, DC, USA.

Vilain, S. and Brozel, V. S. 2006. Multivariate approach to comparing whole-cell proteomes of *Bacillus* cereus indicates a biofilm-specific proteome. *J. Proteome Res.* 5:1924-1930.

Voigt, B., Hoi le, T., Jurgen, B., Albrecht, D., Ehrenreich, A., Veith, B., Evers, S., Maurer, K. H., Hecker, M. and Schweder, T. 2007. The glucose and nitrogen starvation response of *Bacillus licheniformis. Proteomics* 7:413-423.

Voigt, B., Schweder, T., Sibbald, M. J., Albrecht, D., Ehrenreich, A., Bernhardt, J., Feesche, J., Maurer, K. H., Gottschalk, G., van Dijl, J. M. and Hecker, M. 2006. The extracellular proteome of *Bacillus licheniformis* grown in different media and under different nutrient starvation conditions. *Proteomics* 6:268-281.

Wilhelm, S. 1993. The role of cutinase in fungal pathogenicity. *Trends Microbiol.* 1:69-71.

Williams, S. T., Sharpe, M. E. and Holt, J. G. 1989. Bergey's Manual of Systematic Bacteriology, Vol. 4. ed. by Williams and Wilkins, Baltimore.

Yoshida, K., Kobayashi, K., Miwa, Y., Kang, C. M., Matsunaga, M., Yamaguchi, H., Tojo, S., Yamamoto, M., Nishi, R., Ogasawara, N., Nakayama, T. and Fujita, Y. 2001. Combined transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in *Bacillus subtilis*. *Nucleic Acids Res.* 29:683-692.