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Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems

Montarop Yamabhai *, Suphap Emrat, Sirima Sukasem, Puntarika Pesatcha, Nanthnit Jaruseranee, Bancha Buranabanyat

School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand

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Abstract

Bacillus spp. are Gram-positive bacteria that secrete a large number of extracellular proteins of industrial relevance. In this report, three *Bacillus* extracellular hydrolytic enzymes, i.e., alpha-amylase, mannanase and chitinase, were cloned and over-expressed in Gram-negative *Escherichia coli*. We found that both the native signal peptides and that of *E. coli* outer membrane protein, OmpA, could be used to direct the secretion of the recombinant enzymes. The expressed enzymes were observed as clearing zones on agar plates or in zymograms. Determination of enzyme activities in different cell compartments suggested that the ability of the enzymes to be secreted out into the culture medium depends on the time of induction, the type of the signal peptides and the molecular mass of the enzymes. After overnight induction, most of the enzyme activities (85–96%) could be harvested from the culture supernatant. Our results suggest that various signal peptides of *Bacillus* spp. can be recognized by the *E. coli* secretion machinery. It seems possible that other enzymes with similar signal peptide could be secreted equally well in *E. coli* expression systems. Thus, our finding should be able to apply for cloning and extracellular production of other *Bacillus* hydrolytic enzymes as well as other proteins.

Keywords: Secretion; Enzymes; *Bacillus* sp.; Expression; *Escherichia coli*

1. Introduction

Protein secretion in bacteria is one of the important subjects in biological researches in the past decade. The secretion system of the Gram-negative bacterium *Escherichia coli* has been extensively studied and much is known about the molecular mechanism of protein secretion (for review see Baneyx, 1999; Choi and Lee, 2004; Mergulhao et al., 2005). On the contrary, the understanding of protein secretion in Gram-positive bacteria such as *Bacillus* species have only recently been emerging and are still less characterized (Schallmey et al., 2004; Westers et al., 2004). Even though the structure of the cell membrane and cell wall of *E. coli* and *Bacillus* spp. are significantly different, several lines of evidence have suggested that the molecular mechanisms for protein export of both types of bacteria are related and share a number of similar features (Meens et al., 1993; Simonen and Palva, 1993). For example, both of their exported proteins are synthesized by membrane bound-ribosomes, use a proton motive force to transport the protein, and the structure of the signal peptides of both *Bacillus* and *E. coli* are quite similar (Simonen and Palva, 1993).

Enzymes or biocatalysts are the key components of industrial biotechnology. Their abilities to convert the substrates to desired products at mild conditions and in greatly specific manners are essential for sustainable development of various industries including food, textile and pharmaceuticals (Alcalde et al., 2006). Both *E. coli* and *Bacillus* species are attractive systems for the production of enzymes and other secretory proteins in industry (Alcalde et al., 2006; Olempska-Beer et al., 2006; Schallmey et al., 2004). Secretion of recombinant proteins to the culture medium or periplasm has several advantages over intracellular production such as facilitated downstream processing, higher product stability and solubility, increased biological activity, and correct folding and processing. *E. coli* expression systems have long been used for the production of recombinant proteins either intracellulary or extracellulary (Baneyx, 1999;

* Corresponding author. Tel.: +66 44 224152 4; fax: +66 44 224150.
E-mail address: montarop@sut.ac.th (M. Yamabhai).

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Mergulhao et al., 2005; Pines and Inouye, 1999; Sorensen and Mortensen, 2005). Overexpression of recombinant enzymes in *E. coli* allows the engineering of the enzymes for the study of structure-function relationships by site-directed mutagenesis as well as improvement of the enzymes properties using directed evolution technology (Alcalde et al., 2006; Arnold and Moore, 1997; Kaur and Sharma, 2006). The main limitation of using *E. coli* for secretion of recombinant proteins is that not every protein can be secreted efficiently (Mergulhao et al., 2005). Moreover, *E. coli* expression systems are not common for large-scale production of products that are used in food industry because of its potential pathogenicity, only *E. coli* K-12 has been approved for GRAS status by USA-FDA (Olempska-Beer et al., 2006). *Bacillus* species are important sources and have long been used for the production of secretory enzymes for industrial purposes (Schallmey et al., 2004). Since many *Bacillus* species are non-pathogenic and have high secretion capacity, they are another attractive system for secretion of recombinant enzymes to be used in food industries (Olempska-Beer et al., 2006). The drawback of using *Bacillus* systems for secretion of recombinant proteins is that they also secrete a large number of hydrolytic enzymes including proteases into the medium, which will complicate downstream processing and effect product stability (Simonen and Palva, 1993). In addition there is still limited knowledge of their molecular genetics and the mechanism of protein secretion, when compared to *E. coli*. Thus, in many cases, it will be advantageous to overexpress *Bacillus* hydrolytic enzymes in *E. coli* expression systems.

In this report, three extracellular hydrolytic enzymes from *Bacillus* species, namely alpha-amylase, chitinase and mannanase, have been cloned and expressed in *E. coli.* The activities of the enzymes in different compartments of the cell were analyzed. We found that both the native and *E. coli* signal peptides could be used to direct the secretion of the recombinant enzymes into the periplasmic space and into the culture medium. These results suggest that various signal peptides of *Bacillus* can be recognized by the *E. coli* secretion machinery. We speculate that other enzymes with similar signal sequences could be secreted equally well in *E. coli*. Thus, our finding should be able to apply for the cloning and expression of other *Bacillus* hydrolytic enzymes in *E. coli* expression system. Moreover, these systems may be useful for the production of other recombinant proteins as well.

2. Materials and methods

2.1. Molecular cloning of recombinant enzymes

Bacillus licheniformis 13 (Veith et al., 2004) and *Bacillus licheniformis* 8785 were obtained from DSM German Culture Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Bacillus subtilis* strain 168 (Kunst et al., 1997) was obtained from American Type Culture Collections. The bacteria were grown and kept in M1 medium. Genes of the enzymes used in this study were cloned by a PCR-based method. The primers were designed from previously published data according to the nucleotide sequence of the amylase, chitinase and mannanase genes (NCBI accession numbers AAU22245, AAU21943, Z99107, respectively), compatible with the cloning sites of the pET21d(+) (Novagen) or pFLAG-CTS expression vectors (Sigma). Thirty cycles of PCR were performed using *Pfu* DNA polymerase (Promega).

The templates for the construction of the recombinant enzymes used in this study were obtained from genomic DNA of *B. licheniformis* DSM 8785 (for recombinant amylase); *B. licheniformis* DMS 13 (for recombinant chitinase) and *B. subtilis* 168 (for recombinant mannanase). The amplified PCR products were ligated into pET21d(+) or pFLAG-CTS expression vectors as appropriate. For protein expression, all the constructs in pET vectors were transformed into *E. coli* strain BL21 (DE3) (Novagen), whereas the constructs in pFLAG vectors were expressed in *E. coli* strain Top10 (Invitrogen).

2.2. Assay of enzyme activities on agar plates

The activity of the recombinant enzymes was assayed on agar plates containing 100 μg/ml ampicillin, 0.1–1.0 mM IPTG and appropriate substrates. Freshly transformed cells were spotted onto the plates and incubated at 37 °C overnight. *E. coli* colonies expressing recombinant amylase were spotted onto minimal agar plates containing 1% soluble starch. Hydrolytic clear zones were observed after the plates were stained with iodine. *E. coli* cells expressing recombinant chitinase were spotted onto LB agar containing 0.2% colloidal chitin from crab shells (Sigma, product number C9752). Hydrolytic clearing zones were observed by staining the plates with 2% Congo red. *E. coli* colonies expressing recombinant mannanase were spotted onto LB agar plates containing 0.5% locus bean gum. Hydrolytic clear zones were observed after the plates were stained with iodine solution or 2% Congo red. Every plate was also spotted with *E. coli* cells harboring the empty vector as negative control. The clear zone of native enzymes from *B. licheniformis* and *Bacillus subtilis* were observed by growing the bacteria on M1 + 2% potato extract plate containing appropriate substrates overnight.

2.3. Zymograms

The activities of various enzymes were assayed in polyacrylamide gels containing the appropriate substrates. Enzyme preparations were run on an SDS-PAGE gel containing 0.01% glycol chitin, 1% soluble starch or 0.25% locus bean gum for the analysis of chitinase, amylase or mannanase, respectively. For the analysis of chitinase, the protein samples were mixed in the loading buffer (Laemmli buffer) without reducing agent and briefly heated at 100 °C in a heat block (Eppendorf) for 3 min before being separated using a 12% polyacrylamide gel containing 0.01% glycol chitin. After electrophoresis, the gel was rinsed with de-ionized water briefly, then soaked in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 at 37 °C for 2 h under shaking condition. Then the gel was rinsed with de-mineralized water, and stained with 0.01% Calcofluor White M2R in 0.5 M Tris–HCl (pH 8.9). Protein bands exhibiting chitinase activity were visualized under a UV transil-

luminator. To prepare the zymograms of amylase, the enzyme preparations were mixed in the loading buffer (Laemmli buffer) without reducing agent and briefly heated at 100 °C in a heat block (Eppendorf) for 3 min before being separated using 10% polyacrylamide gels containing 0.12% soluble starch. After electrophoresis, the gel was rinsed with de-ionized water briefly, then soaked in 2.5% Triton X-100 for 1 h at 4 °C, then the gel was incubated in sodium phosphate buffer pH 6.5 at 45 °C for 3 h. The gel was then rinsed with de-mineralized water, and stained with iodine solution. Protein bands exhibiting amylase were observed as clear bands on the black background. For the analysis of mannanase, a zymogram of mannanase activities was generated by in-gel activity assay using 0.25(locust bean gum as substrate copolymerized with polyacrylamide. The enzyme samples were mixed with the loading buffer (Laemmli buffer) in the absence of reducing agent, and then applied on a 10% (w/v) polyacrylamide gel. After electrophoresis, the gel was rinsed with de-ionized water briefly, then soaked in 2.5% Triton X-100 for 1 h at 4 °C, then the gel was incubated in 100 mM sodium phosphate buffer (pH 7.0) for 1 h at 50 °C. The gel was then stained with 0.1% Congo red solution with gentle shaking for 1–2 h prior to destained with 1 M NaCl for 2–3 h and thereafter was placed in 5% acetic acid. Mannanase activity was detected as clear zones against red (after staining with Congo red) or blue background (after soaking in 5% acetic acid).

2.4. Expression and preparation of recombinant enzymes from various compartments

Freshly transformed *E. coli* harboring appropriate recombinant constructs were inoculated into 5 ml of LB broth containing 100 μg/ml of ampicillin at 37 °C for 16 h. After that, 1 ml of overnight culture was inoculated into 200 ml of LB broth containing 100 μg/ml ampicillin and grown at 37 °C until the optical density (O.D.) at 600 nm reached 0.5. Then, IPTG was added into the culture broth to a final concentration of 1 mM. The culture was then incubated at 26 °C (room temperature) for 4 h, or overnight (20 h). Fifty milliliters of the culture was then collected and chilled in an ice box for 5 min and centrifuged at 8000 rpm for 10 min at 4 °C to collected cells and supernatants. To extract the periplasmic content, the cells were resuspended in 2.5 ml of cold (4 °C) spheroplast buffer [100 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 8000 × *g* at 4 °C for 10 min and resuspended in 1.0 ml of ice-cold sterile water supplemented with 1 mM $MgCl_2$ and incubated on ice for 5 min with frequent shaking. The supernatant of nearly 1.0 ml was then collected by centrifugation at 8000 × *g* at 4 °C for 15 min as the periplasmic fraction. To extract the cell lysate, the precipitated cells from the previous step were washed once with lysis buffer (50 mM Tris–HCl + 0.5 mM EDTA), resuspended in 1 ml of lysis buffer and sonicated (Ultrasonic Processor; 60 amplitude, pulser 6 s, for 2 min) on ice. The cell debris was then spun down at 10,000 × *g* and the supernatant of nearly 1.0 ml was collected as the cell lysate.

2.5. Determination of enzymatic activities

2.5.1. Alpha-amylase assay

The assay was performed by adding 50 μl of enzyme sample into 500 μl of 1% (w/v) soluble starch (Sigma, product number S9765) in 50 mM potassium phosphate buffer (pH 7.0) and then incubated at 50 °C for 10 min. The enzyme hydrolysis reaction was stopped by adding 200 μl of 3,5-dinitrosalicylic acid (DNS) reagent. The amount of reducing sugar was determined by reading the optical density at 540 nm with a spectrophotometer (Nelson, 1944; Smogyi, 1952). One unit of enzyme is defined as the amount of enzyme which liberates 1 μmol reducing sugar (using D-Glucose as a standard) per minute under the experimental conditions.

2.5.2. Chitinase assay

The standard chitinase activity assay was performed according to a previously published protocol (Frandberg and Schnurer, 1994). Ten microliters of protein sample was added into 100 μl of 0.18 mM *p*-NP-$(GlcNAc)_2$ (Sigma, product number N6133) in 50 mM sodium phosphate buffer, pH 6.0 and incubated at 37 °C in a Thermomixer (Eppendorf) for 1 h. The reaction was terminated by adding 10 μl of 1 N NaOH, and the amount of *p*-nitrophenol released from *p*-NP-$(GlcNAc)_2$ was measured by recording an absorbance at 405 nm. One unit of enzyme is defined as the amount of enzyme which liberates 1 μmol *p*-nitrophenol per minute at the experimental conditions.

2.5.3. Mannanase assay

Mannanase activity was assayed using the DNS method. The substrate, 0.5% locus bean gum (Sigma, product number G0753), was dissolved in 50 mM sodium phosphate buffer, pH 6.0 by homogenizing at 80 °C, heating to the boiling point, cooled and stored overnight with continuous stirring, after which insolubles were removed by centrifugation. The enzyme solution (0.1 ml) was then incubated with 0.9 ml of the substrate solution at 50 °C for 5 min. The reducing sugar liberated in the enzyme reaction was assayed by adding 3 ml of DNS-reagent (Nelson, 1944; Smogyi, 1952), boiling for 5 min, cooling and measuring the absorbance at 540 nm. One unit of enzyme is defined as the amount of enzyme which liberates 1 μmol reducing sugar (using D-Mannose as a standard) per minute under the experimental conditions.

3. Results

3.1. Cloning and expression of Bacillus hydrolytic enzymes in E. coli

PCR cloning was used to generate the recombinant enzymes containing either their native signal peptides or signal peptide from *E. coli* outer membrane protein, OmpA. Genes encoding precursor enzymes containing native signal peptides were cloned into the pET21d(+) expression vector under the control of T7 promoter; whereas genes for mature enzymes fused with the *E. coli* OmpA signal sequence were cloned into the pFLAG-CTS expression vector under the control of *tac* promoter. All of

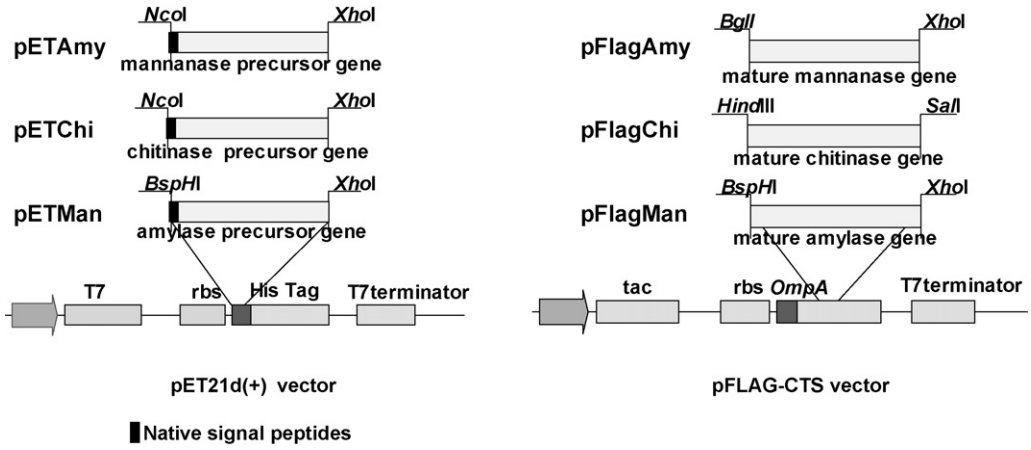

Fig. 1. Map of constructs used in this study. The different genes of precursors of *Bacillus* amylase, chitinase and mannanase containing their native signal peptides were cloned into pET21d(+), whereas genes of the mature enzymes fused to *E. coli* OmpA signal peptides were cloned into pFLAG-CTS vector. The genes in pET2d(+) were under the control of T7 promoter, whereas those of pFLAG-CTS were under the control of *tac* promoter.

the recombinant enzymes in either pET21d(+) or pFLAG-CTS vector could be induced for overexpression by isopropyl-beta-D-thiogalactopyranoside (IPTG) in *E. coli* BL21 (DE3) or Top10, respectively. The maps and names of all of the constructs used in this study are illustrated in Fig. 1. The signal peptides of the three *Bacillus* hydrolytic enzymes share 7–23% similarity. They are 26–32 amino acids long, containing basic N-termini, followed by stretches of hydrophobic residues, and polar C-regions. The OmpA signal peptide of *E. coli* is 21 amino acid long and has similar feature as those of the *Bacillus* hydrolases as shown in Fig. 2.

3.2. Secretion of the recombinant enzymes

Secretion of the recombinant enzymes in *E. coli* was observed on agar plate and by in-gel activity staining (zymogram). We found that all of the enzymes containing either their native signal peptides or the OmpA signal could be highly expressed and efficiently secreted out of the cells as shown as hydrolytic clear zones on agar plates containing appropriate substrates (Fig. 3). These results suggested that both of the *Bacillus* native signal peptides and *E. coli* signal peptide were able to direct the secretion of the enzymes.

Based on zymogram analysis, the enzymes were found in the cell lysate (cytoplasm + periplasm) as well as in the culture media (Fig. 4). This result is in contrast to the secretion of native enzymes from *Bacillus* spp., where the enzymes were only found in the culture supernatant (Fig. 4, lanes 5 and 6). We speculate that high-level expression of recombinant enzymes might have saturated the secretion machinery of *E. coli*, resulting in accumulations of the enzymes in the cytoplasm.

3.3. Activities of the recombinant enzymes in various compartments

In order to determine the ability of the cells to secrete different recombinant enzymes, the activities of the enzymes in various compartments were quantified at different periods after induction. The enzymatic activities in cell lysate, periplasm and culture media were measured at 4 and 20 h after induction with IPTG. The total and relative activities of recombinant enzymes containing either the native signal peptide or the *E. coli* OmpA signal peptide in different compartments at various times after induction are reported in Table 1. Routinely, 1.3 U/ml of amylase, 0.004 U/ml of chitinase and 9 U/ml of mannanase were obtained after an overnight cultivation in shaken flasks under the induction condition described in Section 2. When the relative enzyme activities and their distribution over different compartment were analyzed, it appeared that the results were varied depending on the type of the construct and the time after induction. At 4 h after induction, differences in the secretion efficiencies of amylase and chitinase could be observed. We found

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B.lichen13Amy          -MKQQKR--LYARLLPLLFALIFLLPHSAAAA      29
B.lichen8785Amy        -MKQQKR--LYARLLTLLFALIFLLPHSAAAA      29
B.subMan               --M-FKKH-TISLLIIFLLASAVLA-KPIEA-      26
Rec.B.subMan           --MGFKKH-TISLLIIFLLASAVLA-KPIEA-      27
B.lichen13/8785Chi     MLINK-SKKFFVFSFIFVMMLSLSFVNGEVAKA-    32
E.coliOmpA             ----MKKT--AIAIAVALAGF-A-TVAQA---      21
                             .*      : :    : .    :
Consensus                  +  ++      ψψψψψψψψψψψψ  ρρ ρ
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Fig. 2. Sequence alignment of signal peptide. The amino acid sequences of the signal peptides of the three *Bacillus* hydrolytic enzymes in this study are 26–32 amino acids long. The signal peptide of *E. coli* OmpA is 21 amino acid long. Recombinant signal peptide of *B. subtilis* mannanase contains an additional G residue after the start codon due to the engineering into the expression vector. All of the signal peptides contain basic N-termini (+), hydrophobic regions (ψ) and polar C-regions (ρ). The alignment was performed using CLUSTAL W (1.83) multiple sequence alignment program.

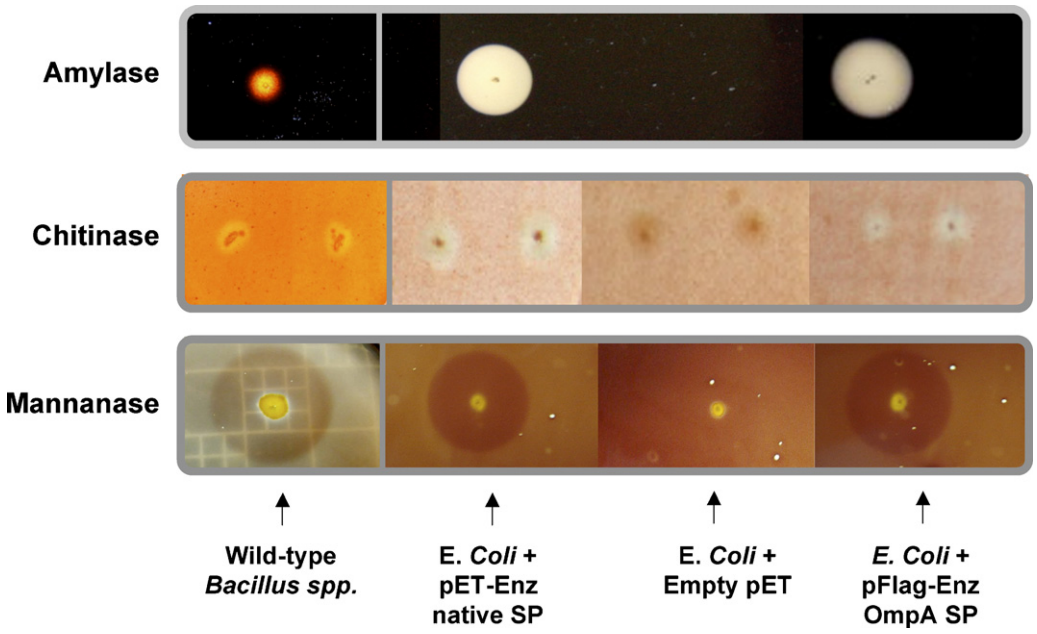

Fig. 3. Enzyme activity assay on agar plates. *Top panel illustrates the assay for amylase activities.* From left to right, hydrolytic zone of wild-type *B. licheniformis* DSM 8785 on M1 agar containing 1% soluble starch; clearing zone of *E. coli* expressing recombinant amylase from *B. licheniformis* containing the native signal peptide (pETAmy), empty pET21d(+); and *E. coli* expressing recombinant amylase from *B. licheniformis* fused to the OmpA signal peptide (pFlagAmy) on minimal plate containting 1% soluble starch. All the plates were stained with iodine solution. *Middle panel illustrates the assay for chitinase activities.* From left to right; hydrolytic zone of wild-type *B. licheniformis* DSM 13 on M1 agar plate containing 2% colloidal chitin; clearing zone of *E. coli* expressing recombinant chitinase from *B. licheniformis* the containing native signal peptide (pETChi); empty pET21d(+); and *E. coli* expressing recombinant chitinase from *B. licheniformis* fused to the OmpA signal peptide (pFlagChi) on LB agar plate containing 0.2% colloidal chitin. Plates were stained with 2% Congo red. *Bottom panel illustrates the assay for mannanase activities.* From left to right; hydrolytic zone of wild-type *B. subtilis strain 168* on M1 agar plate containing 1% locus bean gum; clearing zone of *E. coli* expressing recombinant mannanase from *B. subtilis* containing its native signal peptide (pETMan); empty pET21d(+); and *E. coli* expressing recombinant mannanase from *B. subtilis* fused to the OmpA signal peptide (pFlagMan) on LB agar plate containing 0.5% locus bean gum. The M1 plate was stained with iodine solution whereas LB plates were stained with 2% Congo red.

that the *E. coli* OmpA signal peptides seemed to be better in directing the secretion of the recombinant enzymes to the culture media when compared to the native signal peptides from *Bacillus* spp. (68.2/90.4% of total activity found in the medium for amylase, and 81.3/84.3% for chitinase). However, there is no significant difference in the secretion of recombinant mannanase when either the native (94.4% of total activity found in the medium) or the OmpA signal peptides (93.9%) were used. After overnight induction, no significant difference was evident for the different types of signal peptide with regard to the secretion

Table 1
Enzymes in various compartments

Enzyme/type of signal peptide (SP) (vector)	Induction time	Enzyme activity (%)*		
		Cytoplasm	Periplasm	Culture broth
Amylase/native SP (pETAmy)	4 h	17.9 [4.59]	13.9 [3.58]	68.2 [17.50]
	Overnight	3.6 [2.64]	3.8 [2.76]	92.6 [67.50]
Amylase/OmpA SP (pFlagAmy)	4 h	3.3 [1.48]	6.3 [2.81]	90.4 [40.50]
	Overnight	2.9 [1.90]	3.3 [2.15]	93.8 [60.50]
Chitinase/native SP (pETChi)	4 h	14.6 [0.018]	4.1 [0.005]	81.3 [0.100]
	Overnight	8.1 [0.019]	6.8 [0.016]	85.1 [0.200]
Chitinase/OmpA SP (pFlagChi)	4 h	8.4 [0.015]	7.3 [0.013]	84.3 [0.150]
	Overnight	7.0 [0.016]	6.1 [0.014]	86.9 [0.200]
Mannanase/native SP (pETMan)	4 h	2.3 [8.44]	3.3 [12.22]	94.4 [347.0]
	Overnight	1.7 [8.27]	2.4 [11.43]	95.9 [461.0]
Mannanase/OmpA SP (pFlagMan)	4 h	2.6 [8.78]	3.5 [11.60]	93.9 [311.5]
	Overnight	2.1 [9.65]	2.5 [11.83]	95.4 [446.0]

Routinely, 1.3 U/ml of amylase, 0.004 U/ml of chitinase and 9 U/ml of mannanase were obtained after an overnight cultivation in shaken flask under the induction condition described in Section 2.

* Unit of enzyme from 50 ml of culture medium.

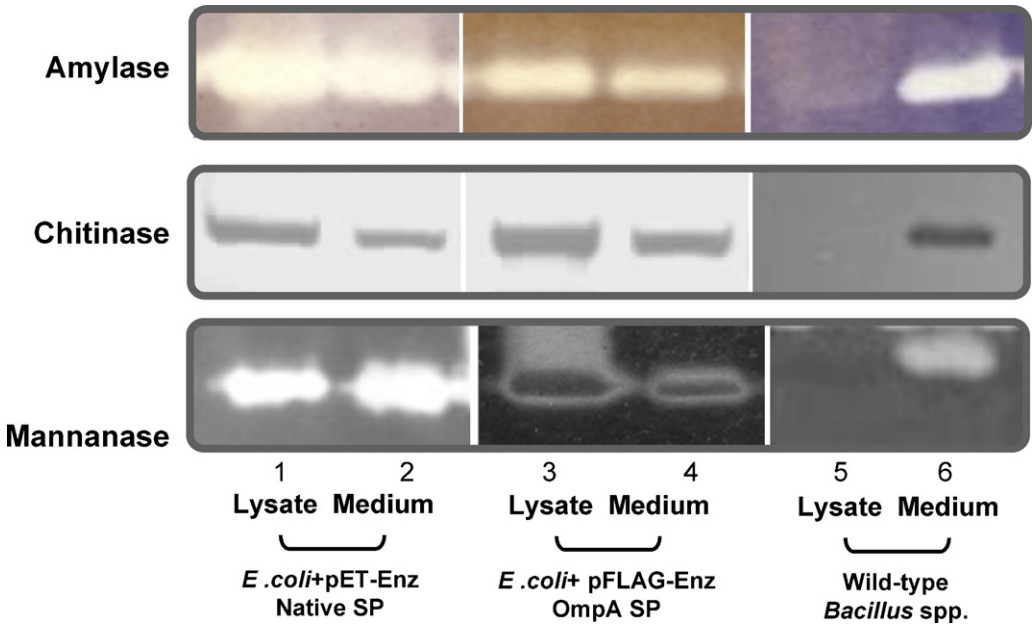

Fig. 4. Zymograms analysis *Top panel illustrates in-gel analysis of amylase activity*. Lanes 1 and 2 are hydrolytic bands of recombinant amylase from *B. licheniformis* containing its native signal peptide from cell lysate (cytoplasm + periplasm) or culture medium (4 h after induction), respectively; lanes 3 and 4 are hydrolytic bands of recombinant amylase from *B. licheniformis* containing the OmpA signal peptide from cell lysate or culture medium (4 h after induction), respectively; lanes 5 and 6 are hydrolytic bands of *B. licheniformis* wild-type amylase from cell lysate or culture medium (after cultivation in M1 broth for 72 h), respectively. *Middle panel illustrates in gel analysis of chitinase activity.* Lanes 1 and 2 are hydrolytic bands of recombinant chitinase from *B. licheniformis* containing its native signal peptide from cell lysate or culture medium (4 h after induction), respectively; lanes 3 and 4 are hydrolytic bands of recombinant chitinase from *B. licheniformis* fused with the OmpA signal peptide from cell lysate or culture medium (4 h after induction), respectively; lanes 5 and 6 are hydrolytic bands of wild-type *B. licheniformis* chitinase from cell lysate or culture medium (after cultivation in M1 broth for 72 h), respectively. *Bottom panel illustrates in gel analysis of mannanase activity.* Lanes 1 are 2 are hydrolytic bands of recombinant mannanase from *B. subtilis* containing its native signal peptide from cell lysate or culture medium (4 h after induction), respectively; lanes 3 and 4 are hydrolytic bands of recombinant mannanase from *B. subtilis* containing OmpA signal peptide from cell lysate or culture medium (4 h after induction), respectively; lanes 5 and 6 are hydrolytic bands of wild-type B. subtilis mannanase from cell lysate or culture the medium (after cultivation in M1 broth for 72 h), respectively. Please note that unequal amounts of proteins were loaded onto the gel to enhance visualization. The total protein concentrations from cell lysate were approximately 50-fold higher than those from culture medium.

efficiencies. The majority of the enzyme activities (85.1–86.9% of chitinase; 92.6–93.8% of amylase; and 95.4–95.9% of mannanase) could be found in the culture medium. The distributions of the enzymes in cytoplasm and periplasm were varied. After overnight induction, less than 10% of the enzyme activities were left in the cytoplasm. At 4 h after induction, only a small fraction (2.3–2.6%) of mannanase was left in the cytoplasm, whereas the recombinant amylase and chitinase were found to accumulate more in cytoplasm when the native signal peptide was used (17.9% of total activity for amylase and 14.6% for chitinase) than when OmpA was used (3.3% for amylase and 8.4% for chitinase) In periplasm, different amount of enzymes (2.4–2.5% of total activity of mannanase, 6.1–6.8% of chitinase and 3.3–3.8% of amylase) were found after overnight induction. However, there is no significant different in the ability of native or OmpA signal peptide on the secretion of the recombinant enzymes into the periplasmic space (3.8/3.3% of total acitivity for amylase, 6.8/6.1% for chitinase, 2.4/2.5% for mannanase). At 4 h after induction, less than 10% of the total acitivity of the recombinant enzymes were found in the periplasmic extract except for recombinant amylase that was fused to native signal peptide (13.9%).

We would like to note that the data above came from the analysis of recombinant amylases from *B. licheniformis* DSM 8785, recombinant chitinases from *B. licheniformis* DMS 13 and recombinant mannanases from *B. subtilis* 168. We found that there were no significant differences in the abilities of the *E. coli* to secrete recombinant enzymes from different strains of *Bacillus*, i.e., *B. subtilis*, or *B. licheniformis* (data not shown). Thus, only the data from these constructs were collected and reported for simplicity.

4. Discussion

Bacillus species are important sources and have long been used for the production of various industrial enzymes, mainly alpha-amylase and proteases, since these bacteria have a high secretion capacity (Schallmey et al., 2004), and many *Bacillus* species are non-pathogenic with GRAS (generally recognized as safe) status (Olempska-Beer et al., 2006). In this report, three *Bacillus* hydrolytic enzymes, i.e., alpha-amylase, mannanase and chitinase, containing their native or the *E. coli* OmpA signal peptides, have been cloned and expressed in Gram-negative *E. coli* to compare their secretion efficiencies. We found that active enzymes could be expressed highly and secreted out of the cells efficiently as shown by clearing zones on agar plates containing appropriate substrates or by in-gel activity staining. Determination of enzyme activities in different cell compartments revealed that after overnight induction (20 h), most of the enzyme activity (85–96%) could be harvested from the culture

medium. These hydrolases were produced and secreted equally well when their native signal peptides were replaced with those of the *E. coli* outer membrane protein, OmpA. Differences in secretion efficiencies of amylase and chitinase were observed at 4 h after induction. The *E. coli* OmpA signal peptide seemed to be more efficient in directing the secretion of the recombinant enzymes to the culture media (84–90%) when compared to the native signal peptides (68–81%). However, there is no significant difference in the secretion of recombinant mannanase when either native or OmpA signal peptides (94%) were used. The *E. coli* OmpA signal peptide was used for comparison in this study as there had been previous reports that the OmpA signal peptide could be used to direct the translocation of different enzymes across *B. subtilis* plasma membrane (Collier, 1994; Meens et al., 1993).

Amino acid sequence analysis revealed that the signal peptides of the three *Bacillus* hydrolytic enzymes are 26–32 amino acids long, whereas that of *E. coli* OmpA consists of 21 amino acids. All of the signal peptides share similar features, which are a basic N-terminus, a central hydrophobic region and a polar C-terminal region. The sequences of the signal peptides suggest that the secretory pathway used for secretion of the enzymes is the type II, secB dependent pathway (Mergulhao et al., 2005). This pathway is commonly used for the secretion of recombinant enzymes into the periplasmic space. The mechanism for secretion of proteins into the extracellular space and hence in the culture medium is still unclear (Mergulhao et al., 2005). However, since more enzymes are found in the culture medium after overnight induction, we speculate that the enzymes may have leaked out of the periplasm after shaking for more than 20 h.

The molecular masses of the three hydrolytic enzymes used in this study are varied; amylase is approximately 59 kDa, chitinase is 65 kDa and mannanase is 41 kDa. It seems possible that the difference in the size of the enzymes could lead to the difference in their ability to secrete out into the culture media. Mannanase is the smallest enzyme and it was shown that it can be secreted the best (94% of the total activity found extracellularly at 4 h, and 95–96% after overnight induction), whereas 93–94% of total amylase activity, and 85–87% of total chitinase activity (the largest enzyme used in this study) were observed in the culture supernatant after overnight induction.

Not only is the signal peptide required for the secretion of the protein, the sequence of the mature protein especially at the N-terminus is also important for secretion efficiency (Brockmeier et al., 2006; Li et al., 1988). Since all of the hydrolytic enzymes from *Bacillus* in this study are extracellular enzymes, it is likely that the structure of the mature proteins is favorable for secretion. Taken together our results suggest that the differences in the secretion efficiency of the enzymes are depending on the time of induction, type of signal peptide and the molecular masses of the enzymes.

All of the recombinant enzymes used in this study were induced for overexpression with isopropyl-beta-D-thiogalactopyranoside (IPTG). This level of expression might not be optimal for efficient secretion of the enzymes, as it has been formerly reported that translational level is a critical factor for the secretion of heterologous proteins in *E. coli* (Simmons and Yansura, 1996). The overexpression of the recombinant enzymes might also explain why the enzymes were found in cytoplasm as well as in the culture media, which was in contrast to the secretion of native enzyme from *Bacillus* spp., where the enzymes were only found in the culture supernatant. The large amount of enzymes produced might have saturated the secretion machinery of *E. coli*. However, these effects were only significant at 4 h after induction, as after growing the cells overnight most of the enzymes could be found in the culture media. It is worthwhile to note that the promoters that were used to drive the expression of enzymes containing native or OmpA signal peptide were different. The T7 promoter was used to control the expression of enzymes containing native signal peptide, whereas the *tac* promoter was used to control the secretion of OmpA containing enzymes. Both of these promoters allowed the recombinant enzymes to be highly expressed when induced by IPTG as demonstrated in this study. However, we cannot rule out the possibility that the differences in the levels of expression of the recombinant enzymes might be the results of the differences in the promoters, which might lead to the differences in the secretion efficiency at 4 h after induction.

There have been previous reports on the secretion of native *Bacillus* hydrolytic enzymes in *E. coli*, i.e., subtilisin (Ikemura et al., 1987), alpha-amylase (Shahhoseini et al., 2003; Manonmani and Kunhi, 1999; Watanabe et al., 2006) and mannanase (Zhang et al., 2006). However, our results demonstrate the direct comparison of three recombinant *Bacillus* hydrolytic enzymes under similar experimental condition. Taken together we hypothesize that various signal peptides of *Bacillus* can be recognized by the *E. coli* secretion machinery, confirming the observation that the basic mechanism of protein translocation is highly conserved in Gram-positive and Gram-negative bacteria, despite the great difference in the structures of cell membrane and cell wall (Mergulhao et al., 2005; Simonen and Palva, 1993). Thus, from our data and the previous reports, it can be speculated that other enzymes with similar signal sequence can be secreted equally well using *E. coli* expression systems. Moreover, our results has suggested that both *E. coli* OmpA signal peptide and the three *Bacillus* signal peptides could equally efficiently direct the secretion of the recombinant proteins in the *E. coli* expression system. Since *E. coli* OmpA signal peptide has been shown to be able to direct the secretion of a wide variety of proteins (Mergulhao et al., 2005; Yamabhai and Kay, 1997), thus the three *Bacillus* signal peptide used in this study should be able to use for secretion of different recombinant proteins as well. Therefore, our finding should be able to apply for the cloning and extracellular production of not only other *Bacillus* enzymes but also for other proteins using *E. coli* expression system.

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