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Efficient *Escherichia coli* expression systems for the production of recombinant β-mannanases and other bacterial extracellular enzymes

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Two *Escherichia coli* expression systems based on T7 RNA polymerase promoter (pET system) and tac promoter (pFLAG system) have been used for the production and secretion of recombinant β-mannanases from Bacillus sp. Both *Escherichia coli* OmpA signal peptide and native Bacillus signal peptide could be used efficiently for the secretion of recombinant enzymes into periplasmic space and culture media. The genes could be induced for overexpression with 0.1–1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when the OD_{600} of the culture broth reached 0.6–1.5. The recombinant enzymes could be harvested from whole cell lysate, perimplasmic extract or culture broth after induction for 4–20 hours. Since the enzyme is C-terminally tagged with hexahistidine, the recombinant enzymes could be conveniently purified to apparent homogeneity by one-step immobilized-metal affinity chromatography (IMAC) using Ni-NTA resins. The characteristics of purified recombinant β-mannanases from *B. licheniformis* and *B. subtilis*, which share 78% amino acid identity, are slightly different. These systems should be applicable for the production of various recombinant bacterial extracellular enzymes.

Key words: *Escherichia coli*, expression, bacteria, extracellular, enzyme, recombinant, mannanase, OmpA, tac promoter, T7 promoter

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Escherichia coli Expression Systems

Escherichia coli is the most commonly used bug for the production of numerous proteins for a wide variety of purposes. Thousands of genes encoding heterologous proteins from eubacteria, archaea and many eukaryotic proteins that do not need posttranslational modification have been successfully expressed in *Escherichia coli* using different expression vectors. Two of the most commonly used *Escherichia coli* overexpression systems are based on the induction from T7 promoter and *tac* promoter (**Fig. 1**).

An expression based on the promoter of bacteriophage T7 gene 1 RNA polymerase, which is an extremely processive DNA-dependent RNA polymerase with only 20-nucleotide long promoter requirement, was first developed in 1986 by W. F. Studier and B.A. Moffatt,[1] and further developed to become widely known as the pET System, owned by Novagen Company.[2,3] In pET vectors, target genes are cloned under the control of strong bacteriophage T7 transcription and translation signals. The gene expression is induced by providing a source of T7 RNA polymerase in the host cell such as *Escherichia coli* BL21 (DE3) that has been genetically engineered to incorporate the gene for T7 RNA polymerase, the lac promoter and the lac operator in it's genome. Therefore, gene expression can be induced by lactose or isopropyl beta-D-thiogalactoside (IPTG) via activation of the transcription of the T7 RNA polymerase gene. Novagen's pET System has continuously expanded and now includes over 42 pET vector types and 15 different host strains to suit different purposes of gene expression.

P*tac* promoter is a strong promoter, which is a hybrid of the *Escherichia coli* trp and lac UV5 promoters, a mutated version

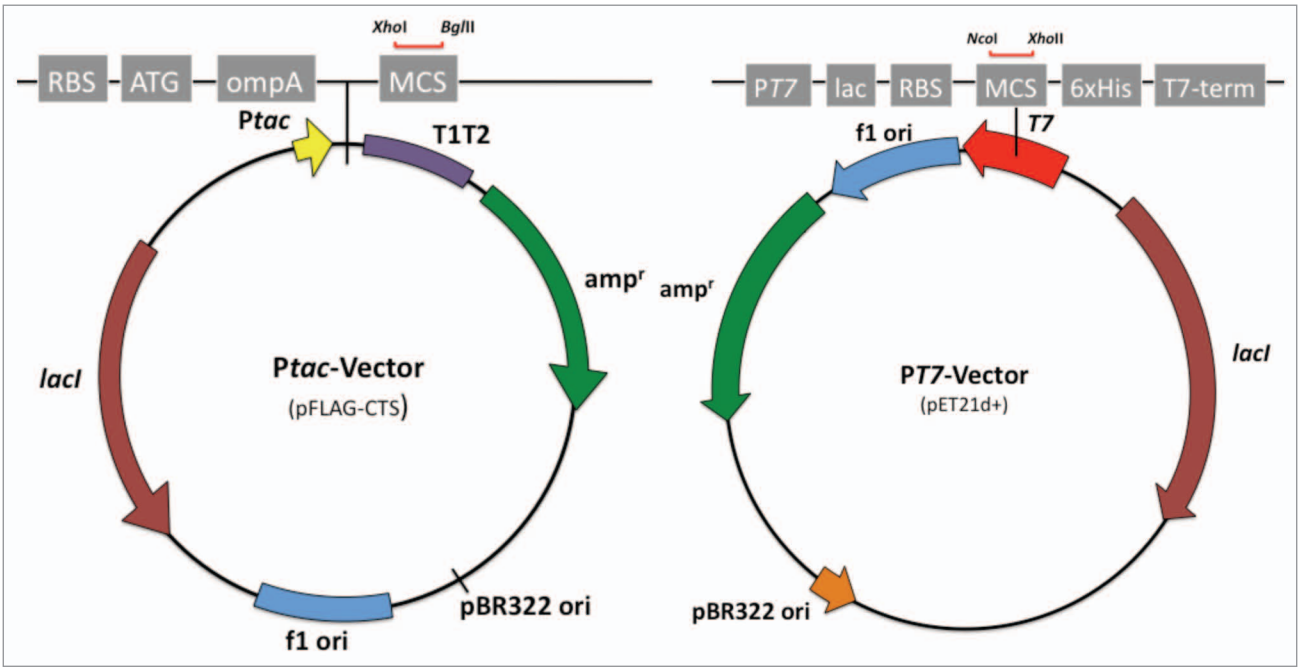

Figure 1. Map of the two expression vectors. The structure of plasmids for expression and secretion of recombinant bacterial extracellular enzymes in this study are illustrated. Left part shows the map of P*tac*-based vector. The vector carries genes for ampicillin resistance (amp^r) for selection and maintainance of the plasmid and the lac repressor (lacI), which is over produced from lacI^p promoter and represses tac prometer. T1T2 is the terminator sequence. Presence of the ompA signal sequence in front of the multiple cloning site (MCS) allows periplasmic secretion of the recombinant enzyme. In this study, *B. subtilis* β-mannanase gene fused with C-terminal 6x His followed by a stop codon was cloned into *Xho* I and *Bgl* II restriction sites of pFLAG-CTS (Sigma). Right part illustrates the T7 promoter (P*T7*)-based expression system or pET system. The pET-21d(+) vectors was used in our studies. The cloning/expression region of the coding strand is transcribed by T7 RNA polymerase, provided by *Escherichia coli* BL21 host. The vector carries genes for ampicillin resistance (amp^r) for selection and maintenance of the plasmid and the lac repressor (lacI), which represses lac promoters on the plasmid and *Escherichia coli* genome. *B. licheniformis* β-mannanase gene was cloned into *Nco* I and *Xho* I restriction sites, in-frame with 6x His tag on the vector.

of the lac promoter whose basal activity is dramatically less sensitive to intracellular levels of cyclic AMP.[4] Protein expression can be induced by lactose or IPTG as a de-repressor of the tac promoter in any established *Escherichia coli* expression host. Whereas pET system is designed for highly selective and tight regulation of gene expression, P*tac* system is useful for the controlled expression of foreign genes at high levels in *Escherichia coli*. The higher efficiency of tac promoter with respect to either one of the parental promoters might be because in contrast to the trp and the lac UV5 promoters, the tac promoter has not only a consensus -35 sequence but also a consensus Pribnow box sequence.[4]

Previous work in our laboratory has demonstrated that both T7 and tac promoter-based expression systems could be used for production and secretion of different extracellular hydrolytic enzymes.[5] These include enzyme alpha amylase,[5] chitinase,[6,7] and chitosanase (manuscript in preparation) from Bacillus sp. However, since the P*tac* system is more flexible than pET system, i.e., a wide variety of *Escherichia coli* hosts (e.g., DH5α, Top10, DH10B, TG1, K12) and various concentration of IPTG ranging from 0.0–1.0 mM can be used for heterologous gene expression; therefore, recent reports on expression and characterization of recombinant *B. licheniformis* chitinase (ChiA)[6,7] and β-mannanase (ManB)[8] from our laboratory were done using this system.

Induction Condition

In general, it is recommended that the induction of gene expression should be done at log-phase growth or when the optical density (OD) at 600 nm of the cell culture reaches 0.5–0.6. Unexpectedly, in many cases, we found that for both β-mannanases and chitinases from Bacillus sp., the highest yields could be achieved if the inductions were done when the OD_{600} reached approximately 1.5. This might be because the enzymes are not toxic to the cell, thus higher cell numbers for enzyme expression could be obtained. Therefore, we suggested that it is worthwhile to try the condition when induction with IPTG is prolonged until the cell density reaches as high as OD_{600} = 1.0–1.5, and vary the induction time before harvesting the enzyme. The optimal duration for induction varies from 3 to 20 h, depending on the induction temperature, concentration of IPTG, location of the enzyme in different compartment (cytosol, periplasm or culture broth), and most importantly, the nature of each enzyme.

The strains of *Escherichia coli* that were employed for overexpression of genes encoding recombinant enzymes from pET and P*tac*-based vectors were BL21 (DE3) and Top10, respectively. BL21 (DE3), a B strain *Escherichia coli* harboring a

bacteriophage lambda lysogen with bacteriophage 21 immunity (hence the name BL21) is much healthier than the prevalent K-12 strains routinely used in molecular biology. Therefore, it can grow more robustly and potentially improved levels of protein expression.[1] In addition, this strain is deficient in both the lon protease and the membrane bound protease OmpT, which can improve yields of the recombinant enzymes. DE3 indicates the cryptic λDE3 lysogen harboring a single copy of T7 gene 1 (T7 RNA polymerase) under the placUV5 promoter, allowing tight regulation of gene expression with IPTG. This feature is essential for the induction of pT7-based vector.

The genotype of Top10 strain *Escherichia coli* (Invitrogen) is highly similar to DH10B strain.[9] It has been reported that DH10B has a 13.5-fold higher mutation rate than the wild-type *Escherichia coli* K-12 strain MG1655.[9] Both DH510B and Top10 *Escherichia coli* cells have been used extensively for high efficiency cloning, plasmid propagation and maintenance of large plasmids. The protocol from Invitrogen (One Shot® Top10, version M, 6 April 2004) indicated that Top10 *Escherichia coli* doesn't require IPTG to induce expression from lac promoter. This could be the reason why we could observe high levels of gene expression after induction with only 0.1 mM IPTG using P*tac*-based vector. When compared with DH5α, we found that Top10 *Escherichia coli* is more suitable for recombinant enzymes production.

Secretion of Recombinant Enzymes

The attractive aspect of the expression systems reported in our studies is the ability to harvest the enzyme from culture supernatant, periplasmic extract or whole cell lysate. Previous report in our laboratories have demonstrated that both *Escherichia coli* ompA signal peptide and native signal peptides of Bacillus extracellular hydrolytic enzymes could be used efficiently to direct the secretion of the recombinant enzymes into the periplasmic space and eventually leaked out into the culture medium.[5] The amino acid sequence of the signal peptides of *B. subtilis* β-mannanase is 26-amino acids long (MFK KHT TSL LII FLL ASA VLA KPI EA), whereas that of *Escherichia coli* OmpA consists of 21 amino acids (MKK TAI AIA VAL AGF ATV AQA). Both 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,[10] which is commonly used for the secretion of recombinant enzymes into the periplasmic space. However, the mechanism for secretion of proteins into the extracellular space and hence in the culture medium is still unclear.[10] 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. In fact we have found that Bacillus enzymes that are not extracellular, i.e., β-galactosidase and glutaminase couldn't secrete efficiently using OmpA signal peptides (manuscripts in preparation). This could be because it has been shown that not only the signal peptide is required for the secretion of the protein, but also the sequence of the mature protein especially at the N-terminus is also important for secretion efficiency.[11,12] Therefore, we emphasize that the expression system in this study is only appropriate for bacterial extracellular enzymes.

By optimizing induction conditions, one can manipulate the localization of the enzyme. For example, overnight induction leads to more accumulation of the enzyme in culture supernatant, whereas harvesting an enzyme from periplasmic space and cytosol is most appropriate after 3–4 h of induction with IPTG. Under the strong induction with IPTG, a significant amount of enzyme was located in the cytosol, possibly due to the saturation of the *Escherichia coli* expression system.[13] Therefore, the enzyme could be harvested from whole cell lysate, which includes enzyme from both cytosolic and periplasmic fractions. Harvesting enzyme from each compartment has different advantages. Whole cell lysate contains the highest concentration of the enzyme, whereas crude enzyme from periplasmic extract usually has highest specific activity because much less total proteins exist when compared to whole cell lysate, facilitating purification step. In addition, enzyme transported into the periplasmic space is usually correctly folded and fully active. More importantly, the extracellular location of the enzyme is of interest for large-scale cultivations as it can facilitate downstream processing because cell disruption is not necessary.

Bacillus β-mannanase

β-mannanse is the common name for mannan endo-1,4-β-mannosidase or 1,4-β-D-mannan mannanohydrolase (E.C. 3.2.1.78). This enzyme catalyzes the random hydrolysis of β-1,4-mannosidic linkages in the main chain of β-mannans. It is valuable in various biotechnological applications, especially those related to renewable resource utilization.[14] In this addendum to the previously published work on expression and characterization of *B. licheniformis* β-mannanase,[8] we reported the biochemical characteristics of *B. subtilis* β-mannanase that was over-produced using pET-based expression system. The Gene of the *B. subtilis* β-mannanase were cloned by a PCR-based method, using primers; B.subManfw: 5'-CTG TGC CCA TGG GGT TTA AGA AAC ATA CGA TCT CTT TGC TC-3' and B.subManrv: 5'-CTG TGC TCG AGC TCA ACG ATT GGC GTT AAA GAA TCA CC-3'. These primers were designed from the published genomic database of *B. subtilis* str. 168 for the gene *ydhT,* encoding hypothetical protein BSU05880 (NCBI accession number NP_388469). The gene was cloned into the *Nco*I and *Xho*I restriction sites of the vector pET-21d (+) (Novagen) such that the recombinant enzyme was fused with a hexahistidine tag on the vector at its C-terminus. To express the enzyme, *Escherichia coli* BL21 (DE3) was transformed with the recombinant plasmid, designated pETManBsub. Gene expression was done as previously described.[8] Recombinant enzymes from culture broth, periplasmic extract and cytosol (as shown on an SDS-PAGE on **Fig. 2**, right part of the original paper for this addendum) were collected according to the previously published protocol.[5] Zymogram analysis showing active

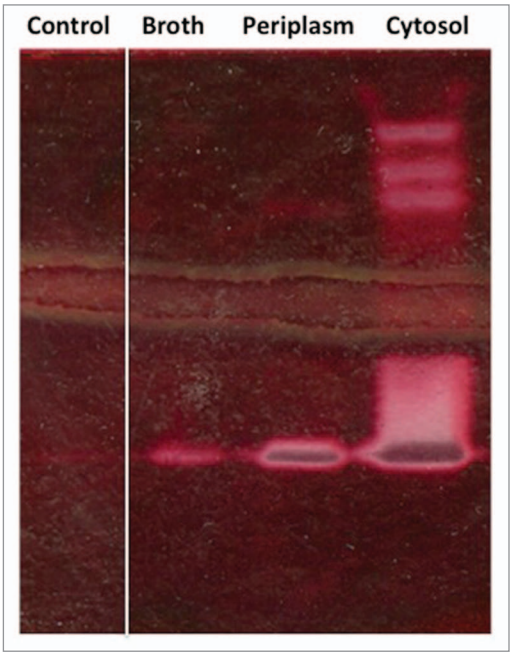

Figure 2. Zymogram analysis of the recombinant *B. subtilis* β-mannanase in three compartments. In gel activity staining was done as previously described.[8] Approximately 5–10 ng of total protein from various enzyme preparations were loaded onto each lane.

enzymes in all three compartments is shown in **Figure 2**. The present of active, hence correctly folded, enzymes in all compartments correspond to our previous reports on the measurement of enzyme activities in various compartments.[5,8] The recombinant enzyme could be purified to apparent homogeneity by one-step affinity chromatography using Ni-NTA agarose beads as demonstrated in **Figure 3** of the original paper for this addendum.[8]

Biochemical characterization of the *B. subtilis* β-mannanase revealed that the optimal temperature and pH of the enzyme for the 5-min assay was at 60°C and 6.0, respectively. The enzyme was stable up to 50°C after incubation for 30 min at pH 6.0 without substrate. The half-life time of activity, τ1/2 was 4 min at 60°C. The enzyme was stable within pH 2–10 after incubation for 30 min at 50°C, and only stable at pH 6.0 after incubation at 50°C for 24 h. These properties are slightly different from those of *B. licheniformis.* The optimal pH of *B. licheniformis* mannanase is wider (between pH 6.0–7.0) and more stable in alkaline condition; whereas *B. subtilis* β-mannanase was more stable at acidic condition (pH 2–10) in a 30-min assay. β-Mannanase from *B. licheniformis* is slightly more thermostable than those of *B. subtilis.* Comparison of the relative activity of β-mannanases from both strains of Bacillus is shown in **Table 1**. β-Mannanase from *B. subtilis* exhibited highest activity on pure 1,4-β-D-mannan followed by glucomannan prepared from konjac and the galactomannan locust bean gum (LBG); while *B. licheniformis* β-mannanase exhibited highest activity on konjac glucomannan followed by pure 1,4-β-D-mannan and galactomannan LBG. Both enzymes showed no activity against highly substituted galactomannan from guar gum and copra meal. However, we found that partial hydrolysis of copra meal after incubation occurred after incubation of this substrate with the enzyme for 2 to 3 days. These results indicated that β-mannanases from both *B. licheniformis* and *B. subtilis* preferred soluble and low-substituted mannan substrates. A slight difference in biochemical property and substrate specificity between the two enzymes is reflected in the results of product analysis by thin-layer chromatography as shown in **Figure 3**. Both enzymes generated various manno-oligosaccharide products (M2-M6) as well as mannose (M1) after incubation with LBG. However, the time course for the appearance of each product was slightly different. β-Mannanase from *B. subtilis* seemed to need longer incubation time to produce M2 and mannose. In addition, less M4 were generated when β-Mannanase from *B. subtilis* was used (**Fig. 3**). All of the methods for biochemical characterization has previously been described.[8]

Conclusion

In this addendum to our recently published paper about the production and characterization of recombinant *B. licheniformis* β-mannanase,[8] we provide additional information on the properties of related recombinant enzymes from *B. subtilis*. Both enzymes were produced using two equally efficient *Escherichia coli* expression systems, namely pET and P*tac*-based vectors. The fact that the two enzymes are highly similar (78 and 69% identity of amino acid and DNA sequence, respectively) and possess some unique favorable properties makes them ideal for improvement by directed evolution using DNA shuffling technique. Most importantly, we speculate that the two expression systems described in our studies will be applicable for expression of other bacterial extracellular enzymes as well.

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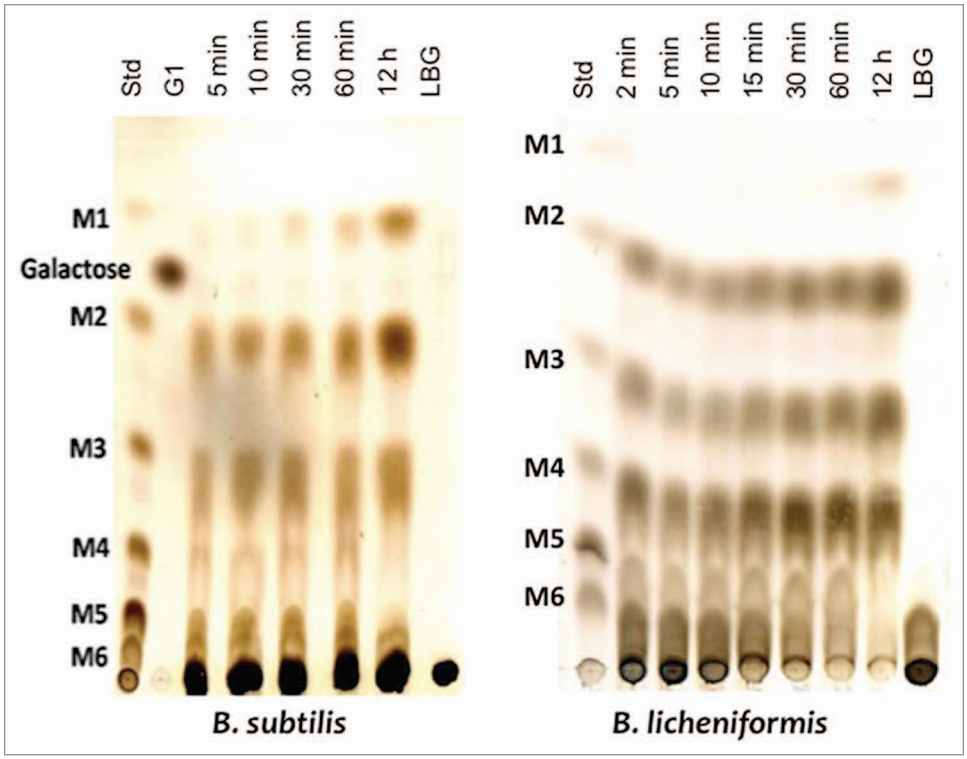

Figure 3. Thin layer chromatography analysis. Products from LBG hydrolysis using β-mannanases from (B) *subtilis* (left part) and *B. licheniformis* (right part) at various time points are illustrated. The hydrolysis reactions were done as previously described.[8] Std: a standard mixture of M1–M6; G_1: galactose blank; 2, 5, 10, 15, 30, 60 min, 12 h are the reaction products after incubation at 2, 5, 10, 15, 30, 60 min and 12 h at 50°C in 50 mM citrate buffer, pH 6.0, respectively; LBG: locust bean gum blank.

Table 1. Substrate specificities of β-mannanases from *B. subtilis* and *B. licheniformis*

Substrate	Activity (Unit/mg) Relative activity (%) R	
	B. subtilis	***B. licheniformis***
Glucomannan (Konjac)	962.1 ± 52.7 (128%)	2844.8 ± 44.7 (219%)
1,4-b-D-Mannan	2223.0 ± 9.0 (295%)	2159.1 ± 46.6 (166%)
Locust bean gum	635.0 ± 24.4 (100%)	1301.3 ± 37.2 (100%)
Guar gum	nd	nd
Copra meal	nd	nd

*The specific activity of mannan endo-1,4-β-mannosidase from *B. licheniformis* was determined under standard assay conditions using each substrate at a concentration of 5 g/l as previously described.[7] The relative activity with the standard substrate locust bean gum (high viscosity) was defined as 100%. nd, no apparent activity at standard assay condition was detected.

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