Efficient *E. coli* expression systems for the production of recombinant \( \beta \)-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 \( \beta \)-mannanases from Bacillus sp. Both *E. 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-\( \beta \)-D-\( \lambda \)-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 \( \beta \)-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.

**E. 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 *E. coli* using different expression vectors. Two of the most commonly used *E. 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 *E. coli* trp and lac UV5 promoters, a mutated version 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.
that it is worthwhile to try the condition when induction with IPTG is prolonged until the cell density reaches as high as OD600 = 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 E. 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 E. 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.

### 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 OD600 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 as a de-repressor of the tac promoter in any established E. 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 E. 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.

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. These include enzyme alpha amylase, chitinase, 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 E. 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) and β-mannanase (ManB) from our laboratory were done using this system.

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') for selection and maintenance of the plasmid and the lac repressor (lacI), which is over produced from lacI promoter and represses lac promoter. 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 (PT7)-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 E. coli BL21 host. The vector carries genes for ampicillin resistance (amp') for selection and maintenance of the plasmid and the lac repressor (lacI), which represses lac promoters on the plasmid and E. 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.
Bacillus β-mannanase

β-mannanase 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. In this addendum to the previously published work on expression and characterization of B. licheniformis β-mannanase, 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 GGT 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 NcoI and XhoI 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, E. coli BL21 (DE3) was transformed with the recombinant plasmid, designated pETManBub. Gene expression was done as previously described. 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. Zymogram analysis showing active 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. 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.
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 \textit{B. licheniformis} and \textit{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 \textit{B. subtilis} seemed to need longer incubation time to produce M2 and mannose. In addition, less M4 were generated when β-Mannanase from \textit{B. subtilis} was used (Fig. 3). All of the methods for biochemical characterization has previously been described. 

**Conclusion**

In this addendum to our recently published paper about the production and characterization of recombinant \textit{B. licheniformis} β-mannanase, we provide additional information on the properties of related recombinant enzymes from \textit{B. subtilis}. Both enzymes were produced using two equally efficient \textit{E. coli} expression systems, namely pET and pTac-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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**References**

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. Std: a standard mixture of M1–M6; G1: galactose blank; 2 min, 5 min, 10 min, 15 min, 30 min, 60 min, 12 h are the reaction products after incubation at 2, 5, 10, 15, 30, 60 minutes and 12 hours 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

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