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Research article

Effect of culture condition on high-level expression of recombinant β-mannanase from *Bacillus circulans* NT 6.7 in *Escherichia coli* and application in mannan hydrolysis

Kwankanit Intaratrakul[a,b], Sunee Nitisinprasert[a,b], Suttipun Keawsompong[a,b,*]

[a] *Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.*

[b] *Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand (CASAF, NRU-KU, Thailand).*

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Abstract

Expression of β-mannanase from *Bacillus circulans* NT 6.7 in *Escherichia coli* BL21(DE3) was increased by optimizing the culture condition. β-mannanase activity of 131 U/mL was obtained using modified M9NG as the cultivation medium. Optimum induction temperature at 37°C gave highest mannanase activity of 731 U/mL. Mannanase activity of 1,309 U/mL was obtained using 10 mM lactose induction. Expression of β-mannanase under this optimum condition increased 18.4-fold, while production cost decreased 5-fold compared with the conventional condition using LB medium and IPTG induction at 18°C. β-mannanase activity increased to 2,114 U/mL under fermentation in a 5-L fermenter with pH controlled at 7. This recombinant enzyme hydrolyzed copra meal and LBG into manno-oligosaccharides as mainly mannotriose and mannotetraose.

Introduction

β-mannanase is an endohydrolase that catalyzes random hydrolysis of β-1,4-mannosidic linkages of mannan-based polysaccharides to yield short and long chain manno-oligosaccharides (Puls, 1997). This enzyme is used in several industrial applications including biobleaching of pulp and paper, reducing the viscosity of coffee extract during instant coffee processing, improvement of animal feed, and pretreatment of lignocellulosic materials for bioethanol production. Moreover, β-mannanase has gained interest in the production of manno-oligosaccharides (MOS) prebiotics from mannan-based substrates that can be used in food and feed applications (Moreira and Filho, 2008; Chauhan et al., 2012; Yamabhai et al., 2014).

The β-mannanase-producing strain *B. circulans* NT 6.7 was isolated from soil collected from coconut factories in Thailand. Its β-mannanase can efficiently hydrolyze copra meal, a mannan-rich by-product from coconut milk and coconut oil processing, with high abundance of galactomannan giving different types of manno-oligosaccharides. These were shown to promote the growth of probiotic bacteria while inhibiting pathogenic bacteria (Phothichitto et al., 2006; Pangsri et al., 2015). Moreover, the β-mannanase gene from *Bacillus circulans* NT 6.7 was cloned and successfully expressed in *E. coli* BL21(DE3). This recombinant enzyme showed high specificity for galactomannan (Piwpankaew et al., 2014), indicating that the recombinant enzyme had potential for MOS production from copra meal.

* Corresponding author
E-mail address: fagisuk@ku.ac.th (S. Keawsompong)

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The enzyme expression in *E. coli* is affected by several factors. Medium cost can substantially affect overall process economics. The composition of the culture medium can significantly affect product concentration, yield, volumetric productivity, the ease and cost of downstream product separation (Kennedy and Krouse, 1999). Induction temperature is also important and low temperatures ranging between 18 to 30°C have been reported to increase protein solubility (Song et al., 2008; Voulgaridou et al., 2013; Fathi-Roudsari et al., 2018). Conversely, induction at 37 to 42°C led to high protein expression (Yan et al., 2004; Collins et al., 2013; Ashayeri-Panah et al., 2017). IPTG is commonly used as an inducer for pET-based expression systems; however, it is inapplicable for the large-scale production because of economic reasons. Therefore, here, lactose was used as the inducer for β-mannanase expression in *E. coli* and effects of culture condition including culture medium and induction temperature were described.

Several reports outline MOS production through the enzymatic hydrolysis of mannanases from mannan-rich substrates. Prebiotic properties were mostly reported as MOS obtained from yeast cell walls (*Saccharomyces cerevisiae*). In hens, numbers of *Bifidobacterium* spp. and *Lactobacillus* spp. increased after treatment by a diet supplemented with MOS (Fernandez et al., 2002) and populations of *Clostridium perfringens* and *E. coli* decreased in MOS treatment groups (Kim et al., 2011). Moreover, specific immunity in cows was enhanced by MOS supplementation (Franklin et al., 2005).

However, few studies exist regarding production and determination of prebiotic properties of MOS prepared from plants; particularly hydrolyzed by a recombinant β-mannanase. Therefore, here, copra meal and locust bean gum (LBG) hydrolysis by a recombinant β-mannanase produced from *E. coli* were performed with the aim to understand hydrolysis patterns and further use these hydrolysis products as prebiotics.

Materials and Methods

Bacterial strains and cultivations

E. coli/Man6.7, a recombinant *E. coli* BL21(DE3) harboring plasmid pET-21d/*man6.7*, constructed as described by Piwpankaew et al. (2014) was used in this study. Cells were grown in 100 mL of Luria-Bertani (LB) medium with 100 µg/mL of ampicillin. Cultures were incubated in an orbital shaker at 37°C, 200 rpm until the OD_{600nm} ~ 1.0. IPTG was added to a final concentration of 1 mM and the cultures were then incubated at 18°C.

Effect of culture medium

Cultivation media used were LB (0.5% yeast extract, 1% tryptone and 1% NaCl), M9NG (25 mM NH_4Cl, 25 mM KH_2PO_4, 50 mM Na_2HPO_4, 2 mM $MgSO_4$, 1% N-Z-amine type A, 0.5% glycerol, 0.5% NaCl, 0.05% glucose), and trace mix (0.004 mM $CaCl_2$, 0.0004 mM $CuCl_2$, $NiCl_2$, Na_2MoO_4 and H_3BO_3, 0.002 mM $ZnSO_4$, $MnCl_2$, 0.01 mM $FeCl_3$) (Sadaf et al., 2007), Modified M9NG (25 mM NH_4Cl, 25 mM KH_2PO_4, 50 mM Na_2HPO_4, 2 mM $MgSO_4$, 1% peptone, 0.5% glycerol, 0.5% NaCl, 0.05% glucose), trace mix (0.004 mM $CaCl_2$, 0.0004 mM $CuCl_2$, $NiCl_2$, Na_2MoO_4 and H_3BO_3, 0.002 mM $ZnSO_4$, $MnCl_2$, 0.01 mM $FeCl_3$), and a high biomass medium (HBM) (15 g/L glucose, 6.6 g/L $(NH_4)_2HPO_4$, 20.1 g/L KH_2PO_4, 1.7 g/L $MgSO_4.7H_2O$) (Nikerel et al., 2006). All media were supplemented with filter sterilized ampicillin at a final concentration of 100 µg/mL. Overnight cultures of recombinant *E. coli*/Man6.7 were inoculated into 100 mL of each medium at a final concentration at 1% (v/v). Cultures were incubated at 37°C and 200 rpm until the OD_{600nm} reached 1.0. Then, protein expression was induced by adding IPTG to a final concentration of 1 mM. After induction, cultivations were carried out at 18°C with 200 rpm. Culture samples were taken at 3 h intervals to determine cell growth by total plate count technique. After centrifuging at 8,000 rpm for 10 min to remove bacterial cells, the supernatant was collected to measure mannanase activity under standard conditions by the dinitrosalicylic acid (DNS) method.

Effect of induction temperature

The effects of induction temperature at 18°C, 37°C and 42°C on β-mannanase expression were determined. The inoculum was transferred into flasks containing 100 mL of modified M9NG medium and grown until OD_{600nm} ~ 1.0. Then, IPTG at final concentration of 1 mM was added and cultures were incubated at 18°C, 37°C and 42°C. Culture samples were collected to determine cell growth and measure mannanase activity as described above.

Effect of lactose induction

Recombinant *E. coli*/Man6.7 was cultivated in modified M9NG medium until OD_{600nm} ~ 1.0. Then, cultures were induced with 1 mM IPTG and various concentrations of lactose (1 mM, 5 mM, 10 mM, 15 mM and 20 mM) to determine the effect of inducer on β-mannanase expression. The cultures were subsequently incubated at 37°C and 200 rpm along the induction period. Culture samples were collected to determine cell growth and measure mannanase activity as described above.

Production of β-mannanase in 5-L fermenter

Fermentation of recombinant *E. coli*/Man6.7 was performed in a 5-L fermenter using 3-L of modified M9NG. The pH was automatically controlled at 7 by adding 5 M NaOH and 5 M HCl. Cultivations were performed at 37°C at 300 rpm and 2 vvm. Then, 10 mM lactose was added when cell OD_{600nm} ~ 1.0. Culture samples were collected to determine cell growth and measure mannanase activity as described above.

Mannan hydrolysis and product analysis

Mannan hydrolysis was studied at 50°C using LBG and defatted copra meal as a substrate. The hydrolysis reaction of 1 mL contained

of 5 mg of the respective substrate incubated and 5 U of recombinant β-mannanase in 50 mM potassium phosphate buffer, pH 6.0. Hydrolysis products were determined by thin-layer chromatography (TLC) performed on silica gel 60 glass plates (Merck, Germany). A solution of 1-butanol:acetic acid:water at a ratio 2:1:1 (v:v:v) was used as a mobile solvent. The spots were visualized by spraying onto silica plates with 10% sulfuric acid in ethanol and further heating at 110°C for 15 min (Pongsapipatana et al., 2016). Mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6) (Megazyme, Ireland) were used as standards.

Enzyme assay

The assay to determine mannanase activity was composed of 100 μL of 1% LBG in 50 mM potassium phosphate buffer, pH 6.0 and 100 μL of the appropriately diluted enzyme. Reaction mixtures were incubated at 50°C for 60 min. The amount of sugar released in the reaction was determined by the DNS method using D-mannose as the standard (Miller, 1959). One unit of β-mannanase activity is defined as the amount of enzyme that liberates 1 μmol of mannose equivalents per minute under assay conditions.

Results and Discussion

Effect of culture medium

Culture medium is an important factor regarding the expression of a heterologous protein and can significantly affect cell growth, product concentration, yield and production cost (Kennedy and Krouse, 1999). Luria Broth (LB) is a general culture medium for protein expression in *E. coli* systems; however, high expression levels were also obtained from other complex and chemically defined media (Studier, 2005; Sadaf et al., 2007). Complex media consisting of a protein hydrolysate such as peptone, tryptone and N-Z-amine type A have been widely used for recombinant protein expression in *E. coli*. Successful use of M9NG, a culture medium consisting of N-Z-amine type A, for recombinant protein expression in *E. coli* expression systems has been reported in several publications (Sadaf et al., 2007; Ikram et al., 2009; Naz et al., 2010). This compound acts as a nitrogen source in the cultivation medium. However, N-Z-amine is expensive which adversely affects the cost of enzyme production. In this study, a modified M9NG culture medium was developed whereby the N-Z-amine type A was substituted by a peptone for cultivation of *E .coli*/Man6.7.

Recombinant *E. coli*/Man6.7 was grown in different culture media including LB, M9NG, modified M9NG and HBM to examine the optimum medium for recombinant β-mannanase expression. β-mannanase activity and cell growth are shown in Table 1. High mannanase activity was obtained from a complex medium including M9NG, modified M9NG and LB as sufficient nutrients in these culture media enabled high growth rate. The number of ribosomes per cell of *E. coli* in the rich medium increased with increasing growth rate. Peptide elongation rate also increased and resulted in high protein synthesis (Farewell and Neidhardt, 1998). HBM medium showed the lowest specific growth rate (data not shown). The high glucose content in this medium might cause catabolic repression of β-mannanase expression and result in low mannanase activity. At the end of cultivation, the number of cells in M9NG and modified M9NG were less than in other culture media. This indicated that β-mannanase in cells was released into culture medium after cell death and lysis, thereby increasing extracellular mannanase. Here, an optimum culture medium for recombinant *E. coli*/Man6.7 was determined as modified M9NG with maximum mannanase activity of 131 U/mL, approximately 2-fold higher than cultivation in LB. Another implication of these results is halving production cost by substituting the N-Z-amine type A with peptone.

Effect of induction temperature

The effect of induction temperature at 18°C, 37°C and 42°C was determined. Mannanase activity and cell growth after induction at different temperatures are presented in Fig. 1. The most suitable temperature for induction was 37°C producing highest mannanase activity at 731 U/mL, possibly due to the T7-based expression system being suppressed at low temperature (Shin et al., 1997; Collins et al., 2013). Farewell and Neidhardt (1998) reported that peptide elongation increased in response to increasing temperature. The number of functional ribosomes and peptide elongation rate at 15°C were lower than at 37°C. Cells contained a pool of nontranslating ribosomes, contributing to the idea of a defect in protein synthesis initiation at low temperature. Our results concurred with a previous report that productivity of silk-elastin-like protein increased when induction temperature increased to 37–42°C. Conversely, highest biomass production was observed at a lower temperature (25°C) (Collins et al., 2013). Yan et al. (2004) reported that the expression of target recombinant proteins induced at 37°C was noticeably higher than those at 28°C. Our results also indicated cell death after 3 h of induction at 37°C and mannanase activity in the culture supernatant then increased rapidly. Other secretory proteins have also been reported to cause cell death (Kurokawa et al., 2001; Fu et al., 2005). A hyper-expression of a secretory exoglucanase in *E. coli* caused cell death within 60–100 min (Fu et al., 2005). Moreover, Low et al. (2012) reported that extracellular secretion of *E. coli* caused cell lysis due to overproduction of recombinant protein. Therefore, β-mannanase accumulated in cells was released into the culture medium resulting in an increase in extracellular mannanase.

Table 1 β-mannanase activity and cell growth in different culture media after induction for 18 h. Cultivations were performed at 37°C and switched to 18°C after induction by 1 mM IPTG.

Culture medium	Activity (U/mL)	Cell growth (logCFU/mL)
LB	71	7.2
M9NG	116	5.0
Modified M9NG	131	4.9
HBM	59	6.9

Effect of lactose induction

The β-mannanase gene from *B. circulans* NT 6.7 was expressed in *E. coli* BL21(DE) under control of the T7 promoter and the lac operator of plasmid pET21d. IPTG is most commonly used as an inducer. However, it is expensive and not suitable for large-scale production. Also, IPTG must be removed from the induced products using complicated methods because of its toxicity. Lactose has also been reported to induce this promoter (Yildirim and Mackey, 2003). Low cost and non-toxicity make lactose a practical potential source for recombinant protein expression. Thus, experiments were performed to determine the viability of substituting IPTG by lactose as an inducer for recombinant β-mannanase expression.

Lactose has previously been used as an inducer for gene expression in *E. coli*-pET expression system by several authors (Gombert and Kilikian, 1998; Kotik et al., 2004; Yan et al. 2004; Sadaf et al., 2007; Ikram et al., 2009; Naz et al., 2010; Cheng et al., 2011). Here, cultivations were performed using 100 mL of modified M9NG medium and cells were induced by 1 mM IPTG or different concentrations of lactose. At the same concentration of 1 mM, lactose showed better induction than IPTG. These results corresponded to Yan et al. (2004) who found that lactose was able to induce the expression of rHpaA, rUreB, rLTB and rLTKA63 with higher efficiency than IPTG.

Moreover, expression of recombinant β-mannanase increased as a function of lactose concentration. Highest mannanase activity obtained from induction with 10 mM lactose was 1,309 U/mL and not significantly different from induction with 15 and 20 mM lactose (Fig. 2). Thus, 10 mM lactose was sufficient for induction of β-mannanase expression. Comparison of β-mannanase activity obtained from this optimum and conventional condition (cultivation in LB medium and induction by 1 mM IPTG at 18°C) showed an increase of 18.4-fold while production cost decreased 5-fold.

Production of β-mannanase in 5-L fermenter

Previously, we found that higher β-mannanase activity was obtained using pH-controlled cultivation at pH 7. Optimum aeration and agitation were 2 vvm and 300 rpm, respectively (data not shown). These results emphasized that environmental factors such as pH, oxygenation and agitation rates are also important for protein expression in the *E. coli* expression system. Fermentation was performed in a 5-L bioreactor containing modified M9NG medium with cultivation under this optimum condition. After induction with 10 mM lactose and incubation at 37°C, highest mannanase activity was 2,114 U/mL, 30-folds higher than obtained by cultivation in a shake flask under conventional conditions (Fig. 3).

Mannan hydrolysis

Analyses of hydrolysis products from defatted copra meal and LBG by TLC are shown in Fig. 4. Various types of manno-oligosaccharides were detected after hydrolysis for 1 h. The main products of copra meal hydrolysis were mannotriose and mannotetraose. Mannobiose and mannopentaose were also formed in small amounts, whereas mannose and mannohexaose were not detected. For LBG hydrolysis, the main products were mannotriose, mannotetraose and mannopentaose. Small amounts of mannohexaose, mannobiose and mannose were also formed. Our results contrasted with other bacterial mannanase experiments where the main hydrolysis product was mannobiose (Ghosh et al., 2015).

LBG is a pure galactomannan, whereas defatted copra meal contains galactomannan, mannan and cellulose (Balasubramaniam, 1976), and the ability of β-mannanase to hydrolyze the mannan backbone depends on the number and distribution of galactose substituents along the mannan backbone chain (Safitri et al., 2014). The ratio of mannose to galactose in copra meal and LBG is 14:1 and 4:1, respectively (Regalado et al. 2000; Moreira and Filho, 2008). Therefore, hydrolysis products obtained from copra meal and LBG were different.

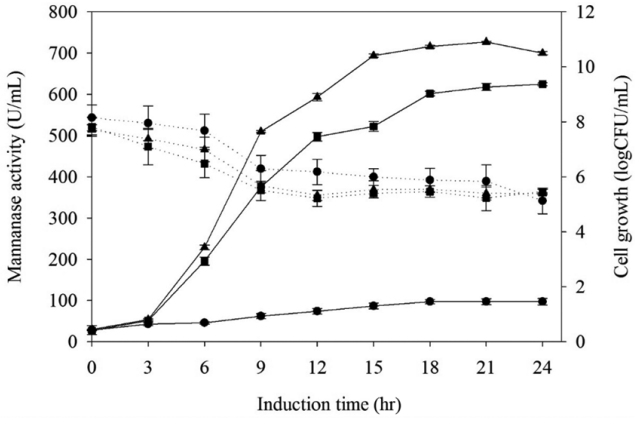

Fig. 1 Cell growth and mannanase activity after induction at different temperatures. Cultivations were performed in modified M9NG medium with induction by 1 mM IPTG. (┈) represents cell growth and (—) represents mannanase activity under 18°C (●), 37°C (▲) and 42°C (■).

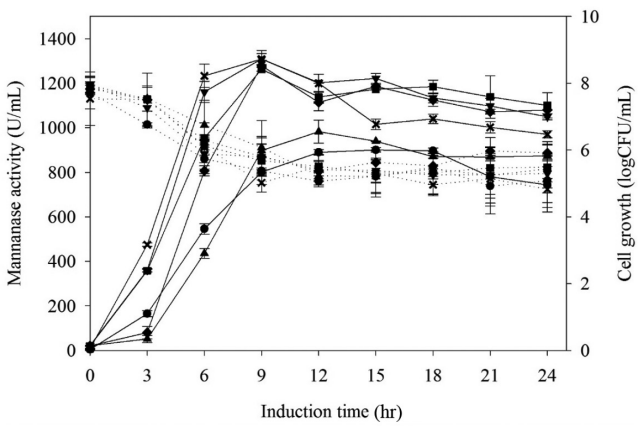

Fig. 2 Cell growth and mannanase activity after induction by 1 mM IPTG and different concentrations of lactose. Cultivations were performed in modified M9NG medium at 37°C. (┈) represents cell growth and (—) represents mannanase activity induced with 1 mM IPTG (●), 1 mM lactose (▲), 5 mM lactose (■), 10 mM lactose (▼), 15 mM lactose () and 20 mM lactose (×).

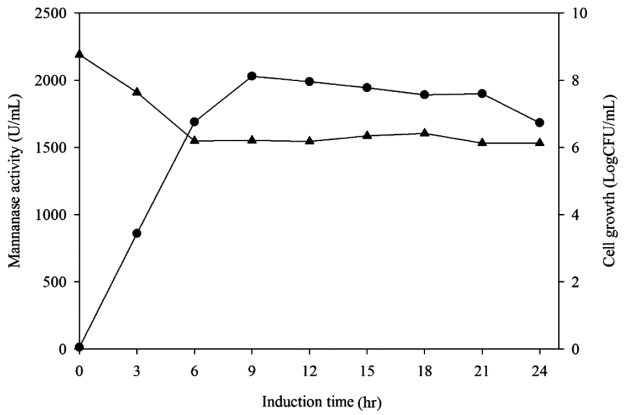

Fig. 3 β-mannanase production by *E. coli*/Man6.7 in a 5-L fermenter. Cultivation was performed with a working volume of 3 L of modified M9NG culture medium. Temperature and pH were controlled at 37°C and pH 7 during the fermentation period. (●) represents mannanase activity and (▲) represents cell growth.

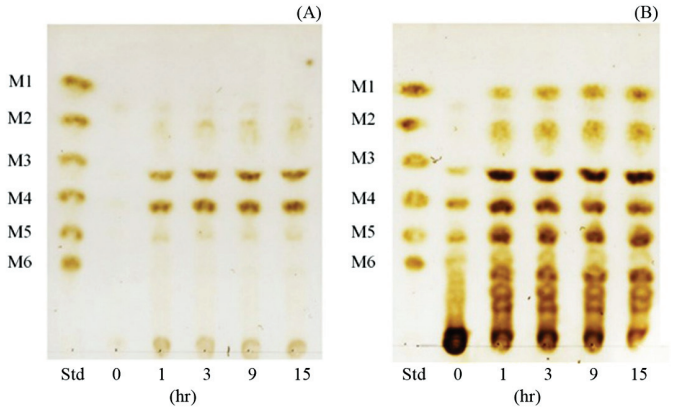

Fig. 4 TLC of (A) copra meal and (B) locust bean gum hydrolysis products formed by recombinant β-mannanase. The reaction consisted of 5 mg substrate incubated with 5 U of enzyme performed at 50°C, pH 6.

Our previous studies reported prebiotic properties of copra meal hydrolysate prepared by β-mannanase from *B. circulans* NT 6.7. Copra meal hydrolysate promoted growth of a probiotic strain *L. reuteri* KUB-AC5 (Pangsri et al., 2015; Rungruangsaphakun and Keawsompong, 2018) and led to an increase in the growth of chicken gut microbiota including the groups Lactobacillus, Enterobacter, and Enterococcus (Prayoonthien et al., 2018). Moreover, increasing numbers of *Lactobacillus* and *Bifidobacterium* were detected in *in vitro* fermentation of copra meal hydrolysate by human fecal microbiota (Prayoonthien, 2018).

Our results showed that hydrolysis products found in copra meal and LBG hydrolysis by recombinant β-mannanase were very similar to those obtained from *B. circulans* NT 6.7 β-mannanase as presented in Pangsri et al. (2015). Accordingly, a copra meal hydrolysate prepared by recombinant β-mannanase has potential for use as a prebiotic. Nevertheless, production of copra meal hydrolysate by this recombinant β-mannanase and its prebiotic properties has not yet been fully examined. Hydrolysis patterns obtained here will be used as preliminary data to optimize conditions of manno-oligosaccharide production for further determination of their prebiotic properties.

Here, increased recombinant β-mannanase expression in *E. coli*/Man6.7 was obtained by optimizing culture conditions of culture medium, induction temperature and inducer. Our results indicated optimum culture condition for β-mannanase expression using modified M9NG medium with induction by 10 mM lactose at 37°C. Under this optimum condition β-mannanase activity increased while production cost decreased compared with the conventional method. Activity also increased by fermentation in a 5-L fermenter, with pH controlled at 7 during cultivation. Moreover, this recombinant enzyme hydrolyzed galactomannan substrate into various types of manno-oligosaccharides with potential use as prebiotics.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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