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High level extracellular production of a truncated alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5 in *Escherichia coli* by the optimization of induction condition and fed-batch fermentation

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Abstract To improve the extracellular production of alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5 in *Escherichia coli*, two truncated recombinant mannanases (32a-ManAR2 and 22b-ManAR2) were obtained. Compared with the full-length mannanases (32a-ManAR1 and 22b-ManAR1), the truncated mannanases not only showed higher secretion rate, but also exhibited higher thermostability and alkalistability. The K_m value (11 mg/mL) of 32a-ManAR2 was higher than that (1.46 mg/mL) of 32a-ManAR1. The specific activity of 22b-ManAR2 was 2.7 times higher than that of 22b-ManAR1. 22b-ManAR2 showed the highest k_{cat}/K_m value of 602.7 ml/mg s. The parameters of induction for recombinant mannanase production of *E. coli* BL21 (pET32a-*manAR2*) and *E. coli* BL21 (pET22b-*manAR2*) were subsequently optimized. The yield of soluble mannanase was found to be enhanced with lower induction temperature (25 °C), lower IPTG concentration (0.01–0.05 mM), and Triton X-100 supplement (0.1 %) in a shake flask. Moreover, a one-time feeding strategy and Triton X-100 supplement were applied in production of 22b-ManAR2 in a 10 L fermentor. The productivity of the total soluble mannanase reached 9284.64 U/mL with the extracellular rate of 74 % at 46 h of fermentation, which was the highest productive level of alkaline β-mannanase in recombinant *E. coli* to date.

Keywords Alkaline β-mannanase · Truncation · *Escherichia coli* · Triton X-100 · One-time feeding

Introduction

Endo-β-1,4-mannanase (EC 3.2.1.78) is a critical kind of hemicellulolytic enzyme, since it catalyzes the random hydrolysis of β-1,4-mannosidic linkages in the backbone of β-mannan, glucomannan, and galactomannan [20]. Alkaline β-mannanase, which could keep high catalytic activity under the alkaline conditions, has a wide application prospect in paper-making, detergent, and the textile industry [4]. Alkaline β-mannanase was identified for the first time from an alkaliphilic *Bacillus* sp. AM001 [1]. Subsequently, several alkaline β-mannanases from the alkaliphilic microorganisms *Bacillus* sp. strains, including JAMB602, JAMB750, I633 and N16-5, *Bacillus circulans* strains CGMCC1554 and CGMCC1416, *Bacillus agaradhaerens* and *Bacillus nealsonii* PN-11, have been obtained and characterized [2, 4, 10, 12, 14, 19–21]. Of all the characterized alkaline β-mannanases, the one from alkaliphilic *Bacillus* sp. N16-5 showed the optimum catalytic condition at 70 °C and pH 9.5 [14]. Its high stability at alkaline condition (pH 8.5–10) allows it to have more potential for application in extreme alkaline industrial processes. However, the yield of alkaline β-mannanase by alkaliphilic *Bacillus* sp. N16-5 has been at a low level [13]. Some eukaryotic expression systems such as *Pichia pastoris* and *Kluyveromyces cicerisporus* have been used

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to improve the productivity of the *Bacillus* sp. N16-5 alkaline β-mannanase [16, 23]. Nevertheless, problems at the industrial scale such as low productivity, high production costs and complex fermentation bioprocess still prevented the industrial production of the *Bacillus* sp. N16-5 alkaline β-mannanase. Therefore, prokaryotic expression of alkaline β-mannanase by an *Escherichia coli* expression system may provide more competitive choices.

Escherichia coli is the most widely used prokaryotic host because it has been best characterized in terms of molecular genetics, physiology and expression system [5]. The productivity and secretion capability of heterologous proteins by a recombinant *E. coli* depend upon many factors, including the genetic properties of host strain, expression vector, signal peptide, process control strategies (medium composition, secretion mode, and feeding strategy), and the nature of the protein [9, 17, 18]. Most notably, the low productivity of foreign proteins in recombinant *E. coli* mainly resulted from the lack of suitable secretive and feeding strategies. In recent reports, several attempts on gene truncation at the C terminus of the glycoside hydrolase gene to improve the extracellular yield of the recombinant protein have been proven to be valid [12, 16]. Meanwhile, various feeding strategies have been developed for the production of foreign protein by recombinant *E. coli* [8, 24].

In this work, four recombinant *E. coli* BL 21 (DE3) expression systems (pET32a-*manAR1*, pET32a-*manAR2*, pET22b-*manAR1*, and pET22b-*manAR2*) were constructed by different expression plasmids pET32a (+) and pET22b (+) using full-length and truncated gene of the alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5, respectively. The truncated recombinant mannanases 32a-ManAR2 and 22b-ManAR2 showed the same pH (pH 9.5) and temperature (70 °C) optima as its full-length protein (32a-ManAR1 and 22b-ManAR1), but exhibited higher pH stability, thermal stability and extracellular activity. Thus, this study has focused on the high-level expression of the alkaline β-mannanase in *E. coli* BL21 (pET32a-*manAR2*) and *E. coli* BL21 (pET22b-*manAR2*) by the optimization of induction condition in shake flask. Furthermore, the total production of 22b-ManAR2 in a 10 L fermentor can reach 9284.64 U/mL with the secretion rate of 74 % by one-time feeding and Triton X-100 supplement. The engineered strain *E. coli* BL21 (pET22b-*manAR2*) has exhibited enormous potentiality for industrial production of alkaline β-mannanase.

Materials and methods

Construction of recombinant strains

The mannanase genes *manAR1* and *manAR2* were separately amplified by PCR from pMANA [14]. Both of them were being cut off the signal peptide encoding gene, and *manAR2* was a truncated gene without the encoding gene of carbohydrate binding domain (CBD). The plasmids pET32a (+) and pET22b (+) were used to construct the recombinant expression plasmids pET32a-*manAR1*, pET32a-*manAR2,* pET22b-*manAR1*, and pET22b-*manAR2*. For the PCR primers and the PCR amplification conditions (Supplementary Table S1), see the supplementary materials available online. *E. coli* DH5α and *E. coli* BL21 (DE3) were used for gene cloning and protein expression, respectively. The recombinant *E. coli* BL21 harboring pET32a-*manAR1*, pET32a-*manAR2,* pET22b-*manAR1*, and pET22b-*manAR2* was ultimately obtained by the chemical transform method [22].

Media and feeding solutions

Luria–Bertani (LB) medium containing 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl, was used for seed cultivation. For the shake flask cultivation, terrific broth (TB) medium consisting of yeast extract (24 g/L), tryptone (12 g/L), glycerol (5 g/L), KH_2PO_4 (2.31 g/L), and K_2HPO_4 (12.54 g/L) was used. The medium for recombinant mannanase production in a 10 L fermentor was a modified semisynthetic medium [24], which contained (g/L): tryptone 30.0, yeast extract 20.0, NaCl 5.0, Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.5, NH_4Cl 0.5, $(NH_4)_3C_6H_5O_7$ 1.08, K_2HPO_4 14.6, $NaH_2PO_4{\cdot}2H_2O$ 3.6, $MgSO_4{\cdot}7H_2O$ 2.0, glycerol 10.0, and trace metal solution 1.0 ml/L, pH 7.0. The trace mental solution consisted of 10 g/L of $FeSO_4{\cdot}7H_2O$, 2.25 g/L of $ZnSO_4{\cdot}7H_2O$, 1.0 g/L of $CuSO_4{\cdot}5H_2O$, 0.38 g/L of $MnSO_4{\cdot}H_2O$, 0.23 g/L of $Na_2B_4O_7{\cdot}10H_2O$ and 3.1 g/L of $CaSO_4{\cdot}2H_2O$. All the media used for the recombinant strain cultivation were supplemented with 100 μg/mL of ampicillin. The feeding solutions for the 10 L fermentor cultivation contained (g/L): glycerol 500, $MgSO_4{\cdot}7H_2O$ 3.4, yeast extract 50, tryptone 50.

Cultivation conditions for shake flask

For seed cultivation, 40 μL of glycerol stock (kept in −80 °C) was inoculated into the 20 mL LB medium supplemented with 100 μg/mL of ampicillin in a 100 mL shake flask. The seed culture was cultivated at 37 °C and 220 rpm in a rotary shaker for 10 h. One milliliter of seed culture was then added into a 20 mL TB medium in a 100 mL shake flask at 25 °C and shaken at 200 rpm. After 4 h of cultivation, IPTG was added to induce target protein expression for another 24 h. Samples were collected and analyzed for OD_{600nm} and enzyme activities at certain time intervals.

Cultivation conditions for a 10 L fermentor

The seed culture was prepared by inoculating 100 μL of frozen glycerol stock strain into 100 mL LB medium (100 μg/mL ampicillin) in a 250 mL shake flask. The cultivation was performed at 37 °C with 200 rpm shaking for 8–10 h. A 10 % (v/v) seed culture was then inoculated into 3 L of semisynthetic medium (100 μg/mL ampicillin) for fed-batch cultivation in a 10 L fermentor (BIOSTAT Bplus, Germany). It was cultured at 37 °C until glycerol exhausted detecting by a sudden increase in both DO and pH. Then a constant speed feeding at 0.6 g/L h of glycerol or one-time feeding by 16 g/L of glycerol was performed. Meanwhile, the IPTG concentration of 0.05 mM was added into the medium and temperature was decreased to 25 °C. Finally, stop feeding and 0.1 % of Triton X-100 was added into the medium for the release of recombinant mannanase at 36 h of fermentation. During the whole process, the pH was kept at 7.0 by automatic addition of ammonia solution (25 %, v/v). To control the DO level at 20–30 % of air saturation, the agitation speed was varied from 200 to 800 rpm. The air flow rate was kept at 1.8 L/min. Antifoam was added manually when necessary.

Purification and SDS-PAGE analysis of the recombinant mannanases

The recombinant mannanases were harvested by cell disruption or direct centrifugation, and then loaded onto a Ni^{2+}-NTA agarose (Novagen, Madison, WI) column for purification [22]. Bound protein was eluted with 200 μL elution buffer (300 mM sodium chloride, 50 mM sodium phosphate, 150 mM imidazole, pH 7.5). The protein concentration was analyzed by the method of Bradford [3]. SDS-PAGE was performed on a 5 % stacking and a 12 % separating gel, and protein bands were visualized by staining with Coomassie Brilliant Blue G250 (Sigma).

Enzyme assay

The method for determining the activity of the recombinant mannanases follows the method described previously [14] with some modification. The reaction mixture consisting of 450 μL of 0.5 % locust bean gum solution in glycine-NaOH buffer (20 mM, pH 9.5) and 50 μL of appropriately diluted enzyme extract were incubated at 70 °C for 10 min. The reaction was terminated by adding 500 μL of 3, 5-dinitrosalicylic acid and placing the mixture in a boiling-water bath for 10 min. The control contained the same reagents, but the reaction was terminated before adding enzyme. After cooling, the absorbance of the reaction mixtures was measured against the control at 540 nm in a spectrophotometer. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of the reducing sugar (mannose) per minute.

Characterization of the recombinant mannanases

The optimum temperature and pH of the purified recombinant mannanases were determined by assaying the mannanase activity at different temperatures (40–80 °C) and various pH values (pH 3.0–11.0). The buffers used here were as follows (at a concentration of 20 mM): acetic acid sodium buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 9.5–11.0). Thermal stability was determined by incubating the recombinant mannanase in 20 mM glycine–NaOH buffer (pH 9.5) at various temperatures (40–80 °C) for 3 h. Similarly, to assess its pH stability, the recombinant mannanase was incubated in buffers at various pH values (pH 3.0–11.0) at 70 °C for 3 h. Then the residual enzyme activity was shown in order to analyze the thermal or pH stability. The kinetic parameters (K_m, V_{max} and k_{cat}) were estimated from a Lineweaver–Burk double reciprocal plot of mannanase activity at various concentrations (0.1–1 %, w/v) of locust bean gum under optimum reaction conditions for 10 min.

Results and discussion

Expression of the recombinant mannanases

The alkaline β-mannanase from alkaliphilic *Bacillus* sp.N16-5 consists of 493 amino acids. And there is a 32-amino acid signal peptide at its N terminus. Domain analysis of its mature peptide showed that it was composed of a cellulase catalytic domain belonging to GH5 (residues 45–288), two segments of linker sequences (residues 33–44 and residues 289–351), and a carbohydrate binding domain (CBD) at 352–493 residues (Fig. 1a). Several previous reports have demonstrated that the truncated enzyme cutting off the terminal non-catalytic domain could usually cause higher expression and higher secretion than the full-length protein [11, 16]. According to the three-dimensional structure of this alkaline β-mannanase generated via Swiss-model (http://swissmodel.expasy.org/), a linker sequence (residues 289–351) located between the catalytic domain and the CBD. To keep the integrity of protein folding, the linker sequence was retained in the truncated enzyme. Therefore, the full-length recombinant mannanase (ManAR1) and the truncated recombinant mannanase (ManAR2) were separately expressed in *E. coli* BL21 (DE3) in this study (Fig. 1a). Because two expression plasmids (pET32a and pET22b) were used, four recombinant mannanases have been expressed in *E. coli* BL21 (DE3) named 32a-ManAR1, 32a-ManAR2, 22b-ManAR1 and

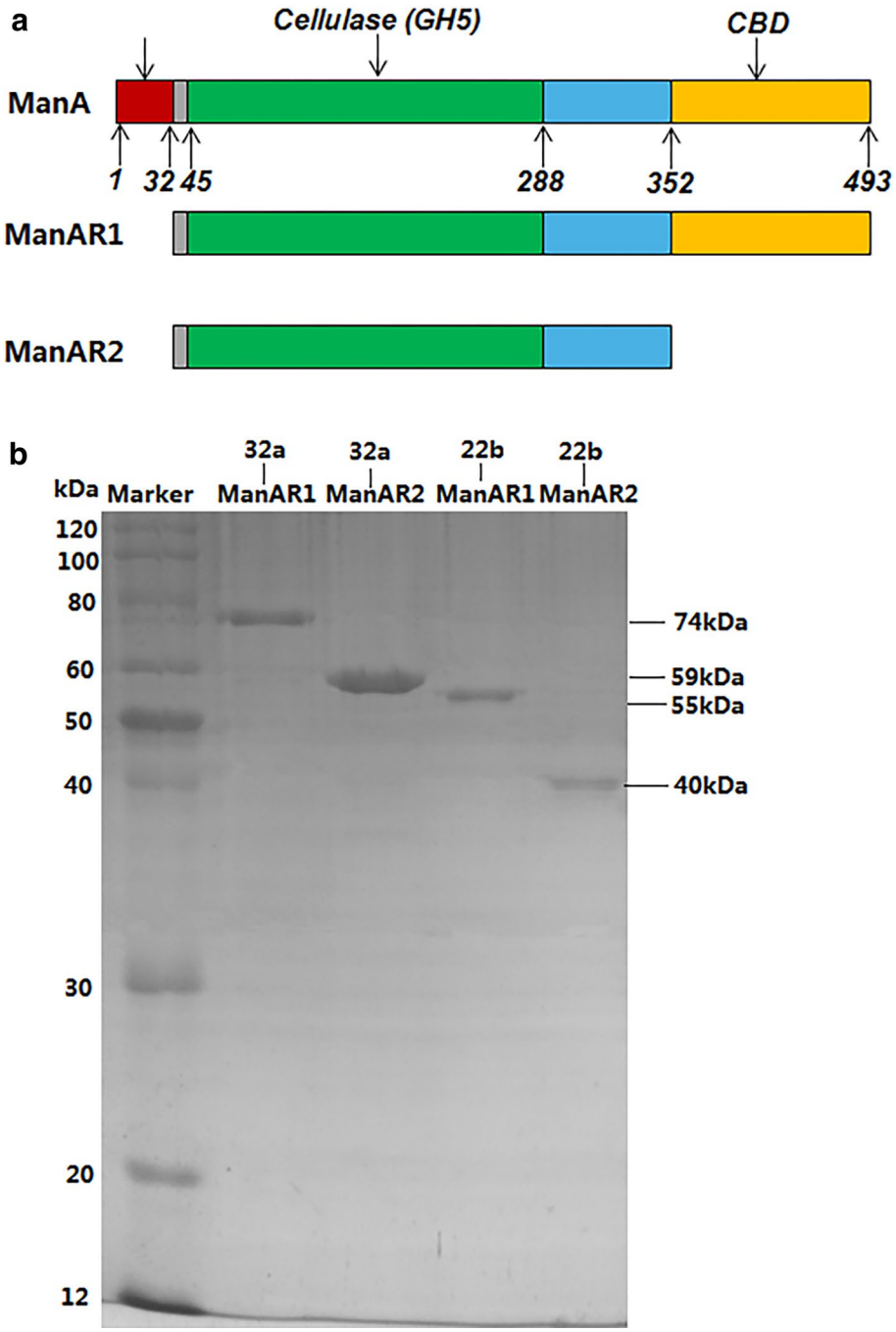

Fig. 1 Schematic and SDS-PAGE analysis of ManAR1 and ManAR2. **a** Schematic representation of ManA, ManAR1 and ManAR2 constructions. All of them shared the same core catalytic domain. Amino acid residues of each domain were labeled. **b** SDS-PAGE analysis of the purified recombinant mannanases 32a-ManAR1, 32a-ManAR2, 22b-ManAR1, and 22b-ManAR2

22b-ManAR2, respectively (Fig. 1b). Moreover, the purified recombinant mannanases showed different molecular weights. The molecular weights of the truncated mannanases 32a-ManAR2 and 22b-ManAR2 were 15 kDa smaller than those of their full-length mannanases 32a-ManAR1 and 22b-ManAR1, respectively. The recombinant protein 32a-ManAR1 and 32a-ManAR2 contained an N-terminal extension of 36 additional residues (trixB protein with a molecular weight of about 19 kDa) due to the inherent sequences of pET32a (Fig. 1b). All of the recombinant mannanases have been produced from the corresponding engineering strain in shake flask fermentation. Each of the recombinant strain could secrete recombinant mannanase into the medium at different degrees (Table 1). However, 22b-ManAR1 and 22b-ManAR2 showed higher ratio of extracellular activity than 32a-ManAR1 and 32a-ManAR2,because of the guidance by a signal peptide in the plasmid pET22b (Table 1). Further, as a truncated

Table 1 Expression of the recombinant mannanases

	32a-ManAR1	32a-ManAR2	22b-ManAR1	22b-ManAR2
Activity of extracellular mannanase (U/mL)	21.86 ± 1.32	43.29 ± 1.51	41.28 ± 1.21	43.12 ± 1.43
Activity of intracellular mannanase (U/mL)	113.62 ± 3.11	131.91 ± 3.21	44.47 ± 1.45	28.98 ± 1.38

The fermentation was performed in shake flasks under the original induction condition. LB medium was used for the cultivation of the recombinant strains. Data are presented as mean ± SD ($n = 3$)

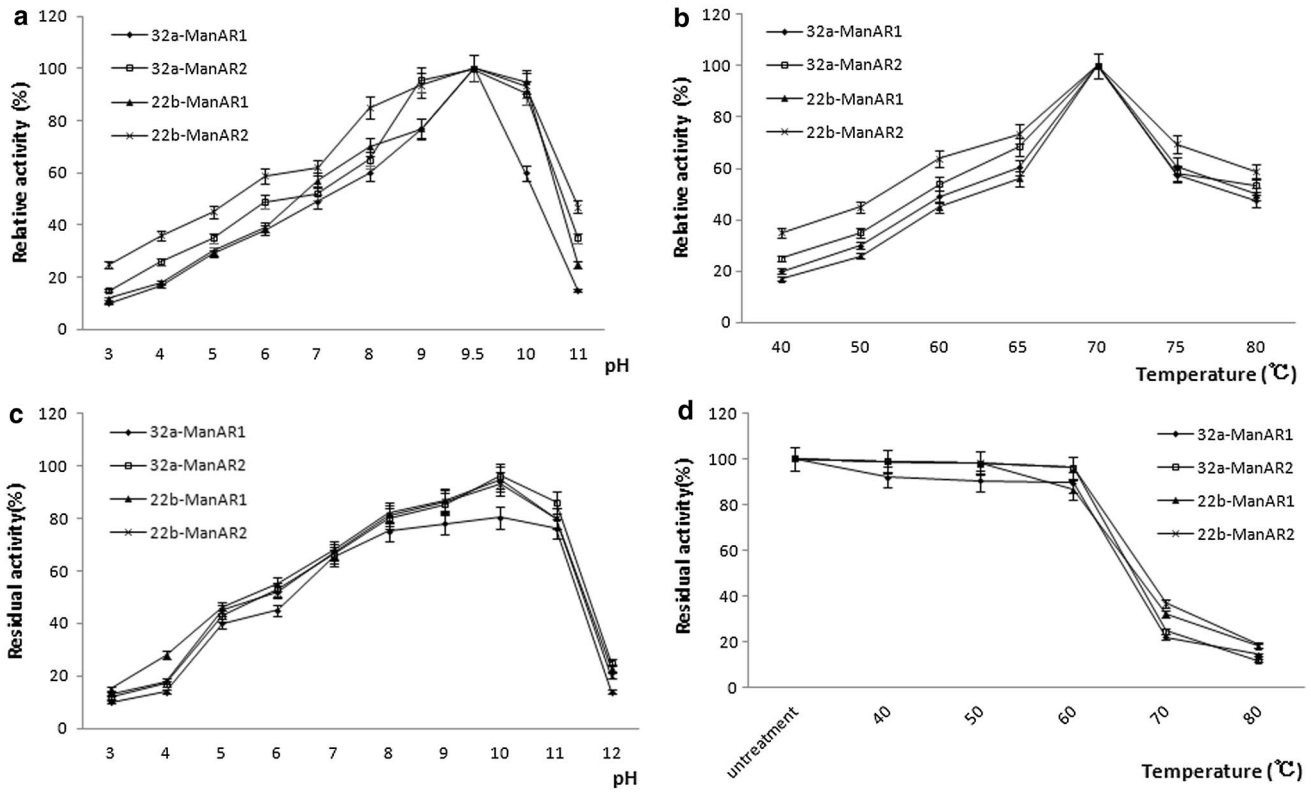

Fig. 2 Enzymatic activity and stability of the recombinant mannanases at various pH values and temperatures. **a** the optimum pH of the recombinant mannanases; **b** the optimum temperature of the recombinant mannanases; **c** the pH stability of the recombinant mannanases at 70 °C and various pH values for 3 h; **d** the temperature stability of the recombinant mannanases at pH 9.5 and various temperatures for 3 h. Data are presented as mean ± SD ($n = 3$)

mannanase, 22b-ManAR2 showed 59.8 % extracellular activity that is 1.2 times higher than that of 22b-ManAR1 (Table 1). In addition, 32a-ManAR1 and 32a-ManAR2 showed visibly higher total mannanase activity than 22b-ManAR1 and 22b-ManAR2, though the majority of mannanase activities were intracellular (Table 1). Similarly, as a truncated mannanase, 32a-ManAR2 showed the highest total mannanase activity of 175.2 U/mL, which is 1.3 times higher than that of 32a-ManAR1 (Table 1). Consequently, as truncated mannanase, both 32a-ManAR2 and 22b-ManAR2 showed higher level of total mannanase activity and extracellular activity than each corresponding full-length mannanases 32a-ManAR1 and 22b-ManAR1, respectively. These results indicate that the truncated mannanases 32a-ManAR2 and 22b-ManAR2 with improved extracellular activity are more suitable for industrial production [6, 15].

Enzymatic properties of the recombinant mannanases

The four recombinant mannanases were purified to electrophoretic homogenous states for identification of their enzymatic properties, respectively. All the recombinant mannanases showed the same pH (pH 9.5) and temperature (70 °C) optima (Fig. 2a, b), which coincided with the native alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5 [15]. All of them showed high alkaline stability at pH 8–11 for 3 h and high thermostability at 60 °C

Table 2 Kinetic parameters of the recombinant mannanases

	Specific activity (U/mg)	V_{max} (μmol/min mg)	K_m (mg/mL)	k_{cat}/K_m (mL/mg s)
32a-ManAR1	745.46 ± 1.59	909.09 ± 0.98	1.46 ± 0.02	125.94 ± 1.34
32a-ManAR2	971.12 ± 1.19	3333.33 ± 1.29	11 ± 0.06	22.04 ± 0.75
22b-ManAR1	1195.93 ± 1.9	2000 ± 1.06	13.8 ± 0.12	17.88 ± 0.25
22b-ManAR2	3225.32 ± 2.9	5000 ± 2.08	4 ± 0.09	602.70 ± 1.98

Locust bean gum (Sigma) was used as the substrate. Data are presented as mean ± SD ($n = 3$)

for 3 h, respectively (Fig. 2c, d). Moreover, compared with 32a-ManAR1 and 22b-ManAR1, the truncated mannanases 32a-ManAR2 and 22b-ManAR2 exhibited higher relative activity at each test pH and temperature (Fig. 2a, b). Similarly, the pH stability of 32a-ManAR2 showed 1.07–1.2 times higher residual activity at pH 8–11 than that of 32a-ManAR1 (Fig. 2c). 22b-ManAR2 showed the same pH stability with 22b-ManAR1 (Fig. 2c). When the recombinant mannanases were incubated at 60 °C for 3 h, the residual activities of 32a-ManAR2 and 22b-ManAR2 showed 7.3–10.4 % higher than that of 32a-ManAR1 and 22b-ManAR1, respectively. Thus, the deletion of CBD at C terminal of this alkaline β-mannanase could increase its pH stability and thermal stability. These results showed that the CBD of this alkaline mannanase not only affects its substrate recognition and binding, but also affects the enzymatic properties. Pan et al. also found that the truncated mannanase showed higher stability than its full-length enzyme [16]. All of the recombinant mannanases showed high specific activity against locust bean gum (Table 2). Due to deletion of CBD, the K_m value (11 mg/mL) of 32a-ManAR2 was higher than that (1.46 mg/mL) of 32a-ManAR1. However, the specific activity (971.12 U/mg) of 32a-ManAR2 was higher than that (745.46 U/mg) of 32a-ManAR1 (Table 1). Likewise, the specific activity of 22b-ManAR2 was 2.7 times higher than that of 22b-ManAR1. From these results, the specific activities of the four recombinant mannanases showed negative correlation with their molecular weights. Lower molecular weights of mannanase may cause higher specific activities. More interestingly, the K_m value (4 mg/mL) of 22b-ManAR2 was lower than that (13.8 mg/mL) of 22b-ManAR1. It indicates that the CBD is not the only deciding factor for the substrate affinity of the alkaline mannanase. The k_{cat}/K_m value of 22b-ManAR2 was up to 602.7 ml/mg s, which indicates the highest catalytic efficiency. The kinetic parameters of the same enzyme expressed, respectively, from pET32a (+) and pET22b (+) were different, due to the difference of the disulfide bond formation controlled by the N-terminal trixB protein. According to the data in Table 2, the N-terminal trixB protein from pET32a (+) could decrease the V_{max} of the recombinant mannanases. In conclusion, the truncated mannanases 22b-ManAR2 and 32a-ManAR2 possessing better enzymatic properties showed more appropriate for application in industry [6, 15].

Effects of induction temperature and shaking speed on the production of soluble mannanase in shake flasks

The recombinant *E. coli* BL21 (pET32a-*manAR2*) and *E. coli* BL21 (pET22b-*manAR2*) were selected for further testing to determine the optimum production conditions of the truncated mannanases (32a-ManAR2 and 22b-ManAR2) in a shake flask. In general, the growth of recombinant *E. coli* cells at low temperatures increases the solubility of the recombinant proteins by preventing the formation of insoluble inclusion bodies (IBs) [7]. Thus, to optimize the induction temperature for the production of recombinant mannanases, the recombinant *E. coli* cells expressing 32a-ManAR2 and 22b-ManAR2 were induced at 20 °C, 25 °C, 30 °C, and 37 °C in TB medium, respectively. As shown in the results (Fig. 3a, b), high induction temperature had a negative effect on the production of soluble mannanases, and the optimum induction temperature was 25 °C for both the two recombinant *E. coli* cells. At an induction temperature of 25 °C, the total soluble mannanase activities of 32a-ManAR2 and 22b-ManAR2 were 4208.11 U/mL and 3201.86 U/mL, respectively. The extracellular mannanase activity of 22b-ManAR2 taken up to 51.44 % of the total soluble mannanase activity. In addition, the impacts of shaking speed on cell growth and soluble mannanase production were investigated at an induction temperature of 25 °C. When the shaking speed was increasing from 160 to 200 rpm, the soluble mannanase activities of both the recombinant mannanases (32a-ManAR2 and 22b-ManAR2) were increasing accordingly (Fig. 3c, d). However, when the shaking speed was higher than 200 rpm, the soluble mannanase activities of both 32a-ManAR2 and 22b-ManAR2 began to decline, which may be due to the fact that more IBs were formed at high shaking speed.

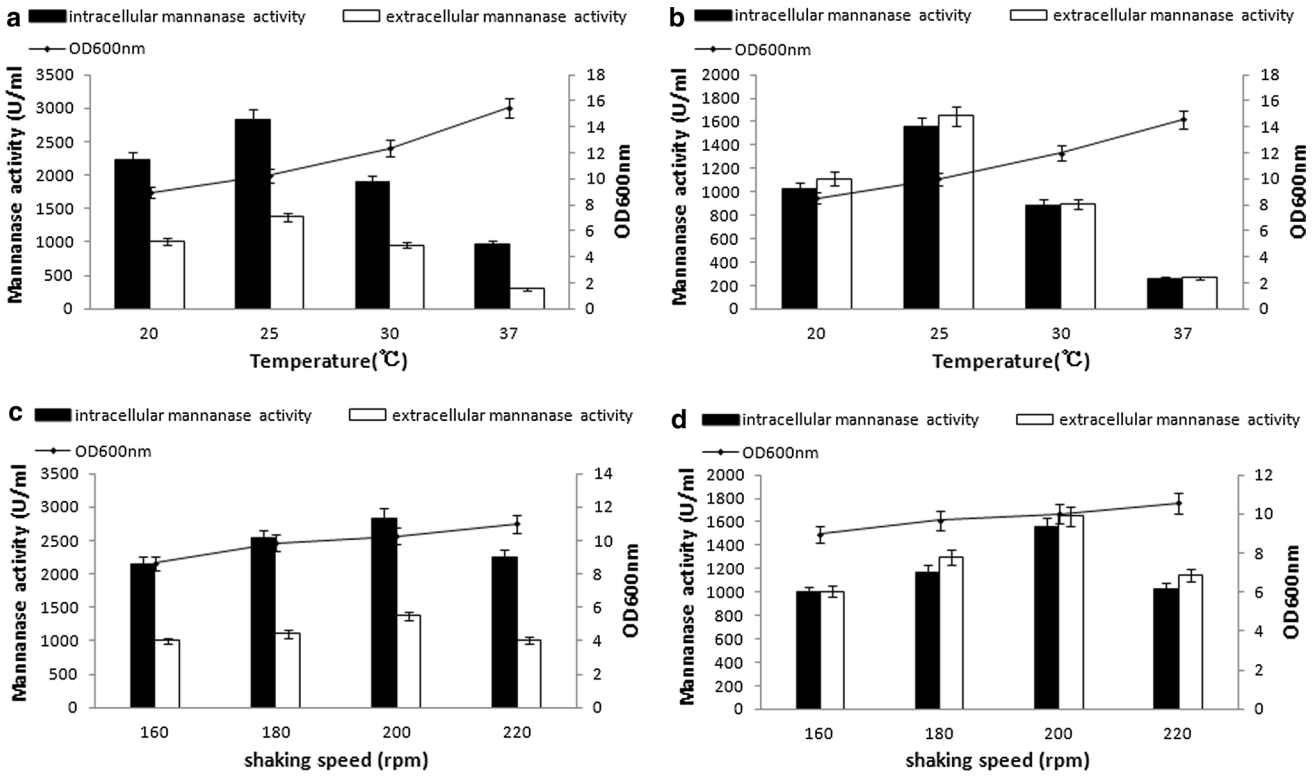

Fig. 3 Comparison of cell growth and mannanase activity at different induction temperature and shaking speed. **a** The impacts of induction temperature on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET32a-*manAR2*) in shake flasks; **b** the impacts of induction temperature on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET22b-*manAR2*) in shake flasks; **c** the impacts of different shaking speed on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET32a-*manAR2*) in shake flasks; **d** the impacts of different shaking speed on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET22b-*manAR2*) in shake flasks. Data are presented as mean ± SD ($n = 3$)

Effect of IPTG concentrations on the production of soluble mannanase in shake flasks

Because both of pET32a and pET22b were T7 promoter-based expression plasmids, different concentrations of IPTG were used to induce the expression of recombinant mannanases through *E. coli* BL21 (pET32a-*manAR2*) and *E. coli* BL21 (pET22b-*manAR2*), respectively. For the recombinant *E. coli* BL21 (pET32a-*manAR2*), both the OD_{600nm} and soluble mannanase activity were declined after the concentrations of IPTG were increased to higher than 0.01 mM (Fig. 4a). Meanwhile, for the recombinant *E. coli* BL21 (pET22b-*manAR2*), although the cell growth (OD_{600nm}) was declining along with the increasing concentrations of IPTG, the soluble mannanase activity of 22b-ManAR2 was increasing along with the rising concentrations of IPTG at a certain range (0–0.05 mM) (Fig. 4b). At the IPTG concentration of 0.05 mM, there was a balance between the cell growth and soluble mannanase production, and the highest soluble mannanase activity (4702.07 IU/mL) was achieved. Further, the extracellular rate (extracellular activity/total soluble activity) of 22b-ManAR2 was up to 58.2 %.

Effect of osmolytes on the production of soluble mannanase in shake flasks

Previous studies reported that the addition of osmolytes in the cell can significantly increase the permeability of cell membrane [7, 8]. Because of that the extracellular rate of the recombinant enzyme can be increased and at the same time can lead to an increase in the total yield of the recombinant enzyme. In this study, the effects of osmolytes (proline, glycine, betaine, Tween-80, Tween-20 and Triton X-100) on the production of soluble mannanase were investigated. As shown in Fig. 5a and b, the addition of glycine, Tween-20, or Triton X-100 in the medium led to higher ratio of extracellular mannanase activity (32a-ManAR2 and 22b-ManAR2) than that of control. The addition of Tween-80 only increased the extracellular activity of 32a-ManAR2, and the addition of betaine only caused increasing extracellular activity of 22b-ManAR2.

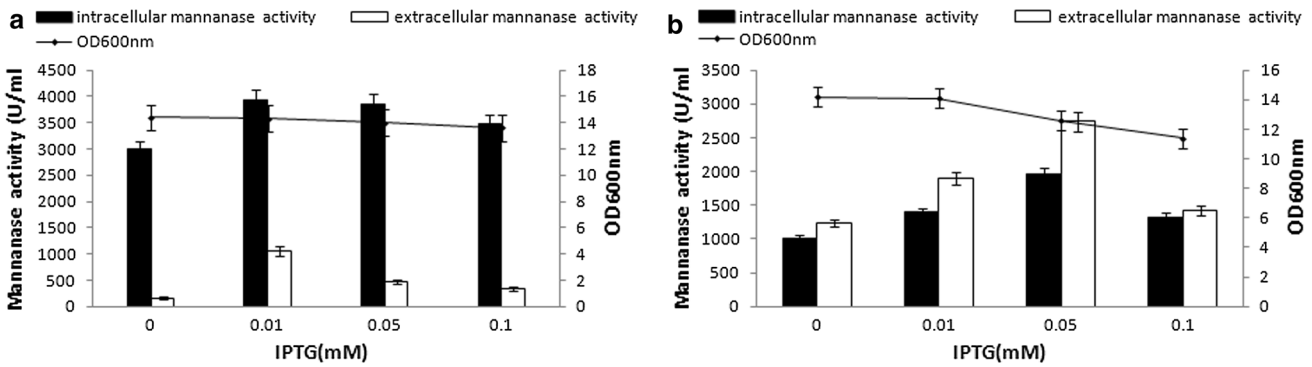

Fig. 4 Comparison of cell growth and mannanase activity at different IPTG concentrations. **a** The impacts of IPTG concentrations on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET32a-*manAR2*) in shake flasks; **b** the impacts of IPTG concentrations on cell growth and soluble mannanase production of the recombinant *E. coli* BL21 (pET22b-*manAR2*) in shake flasks. Data are presented as mean ± SD ($n = 3$)

Moreover, for both of 32a-ManAR2 and 22b-ManAR2, the addition of Triton X-100 led to the highest extracellular mannanase activity. Therefore, Triton X-100 was chosen for further studies to improve the extracellular rate of recombinant mannanase.

Different concentrations of Triton X-100 were added into the medium to improve the extracellular rate of recombinant mannanase. The results show that the maximum extracellular mannanase activity of 32a-ManAR2 and 22b-ManAR2 was both achieved at the concentration of 0.1 % (Fig. 5c, d). In addition, the effects of various Triton X-100 supplement times on mannanase production were further investigated. The optimum supplement time was found to be the initial point of induction, at which the extracellular mannanase rate reached 57.85–55.70 % for 32a-ManAR2 and 22b-ManAR2, respectively (Fig. 5e, f). At the optimum fermentation condition in shake flasks, the highest soluble mannanase activity of 32a-ManAR2 was 3646.21 U/mL, while the highest soluble mannanase activity of 22b-ManAR2 was up to 6823.38 U/mL. It is mainly because 22b-ManAR2 kept the rate of extracellular mannanase perpetually high, which improved the yields of total soluble mannanases (natively folded recombinant proteins) efficiently.

Improvement of mannanase production by fed-batch fermentation in a 10 L fermentor

Based on above tested fermentation parameters with some appropriate modifications, a scale-up fermentation of *E. coli* BL21 (pET22b-*manAR2*) for recombinant mannanase (22b-ManAR2) production was performed in a 10 L fermentor. The IPTG and Triton X-100 concentrations have been further optimized according to the biomass in a 1 L six-parallel fermentor (Supplementary Figure S1). The optimal IPTG concentration was 0.05 mM at OD_{600nm} = 15–25 of fermentation in the 1 L fermentor, and 0.1 % of Triton X-100 was enough to dissolve cells at a certain range of cell density (OD_{600nm} = 21–32). The data above could guide the scale-up fermentation in a 10 L fermentor. Moreover, unlike cultivation in shake flasks, the selection of nutrient feeding strategy was critical in fermentor cultivation because it affected the metabolic pathway fluxes, and consequently affected the cell density and the specific productivity of the recombinant proteins [9]. In this study, the effects of two glycerol feeding strategies on cell growth and recombinant mannanase production were investigated. First of all, when the initial glycerol was exhausted at 12 h of fermentation, a constant glycerol feeding (0.6 g/L h) started (Fig. 6a). During the fed-batch phase, both the growth of cells and the production of the recombinant mannanase were increasing slowly (Fig. 6a). The extracellular mannanase activity remained at a low level (Fig. 6a). Until the end of fed batch, at 36 h of fermentation, 0.1 % Triton X-100 was added into the medium, the total activity and extracellular activity of the recombinant mannanase were both increasing visibly along with the cells dissolving. At 48 h of fermentation, the total activity of recombinant mannanase was at 6909.44 U/mL. However, a one-time feeding by 16 g/L at 12 h of fermentation (the point of initial glycerol exhausted) caused continuously rapid growth of cells (Fig. 6b). Both the total mannanase activity and extracellular mannanase activity were increasing exponentially. In addition, when the fed glycerol was nearly exhausted at 36 h of fermentation, the addition of 0.1 % Triton X-100 caused a second promotion of the extracellular activity of the recombinant mannanase (Fig. 6b). At 46 h of fermentation, the highest total activity of recombinant mannanase was up to 9284.64 U/mL, and the extracellular mannanase activity was at 6892.42 U/mL, which consisted of 74 % of the total activity. To our best knowledge, both the total activity and extracellular activity of the recombinant

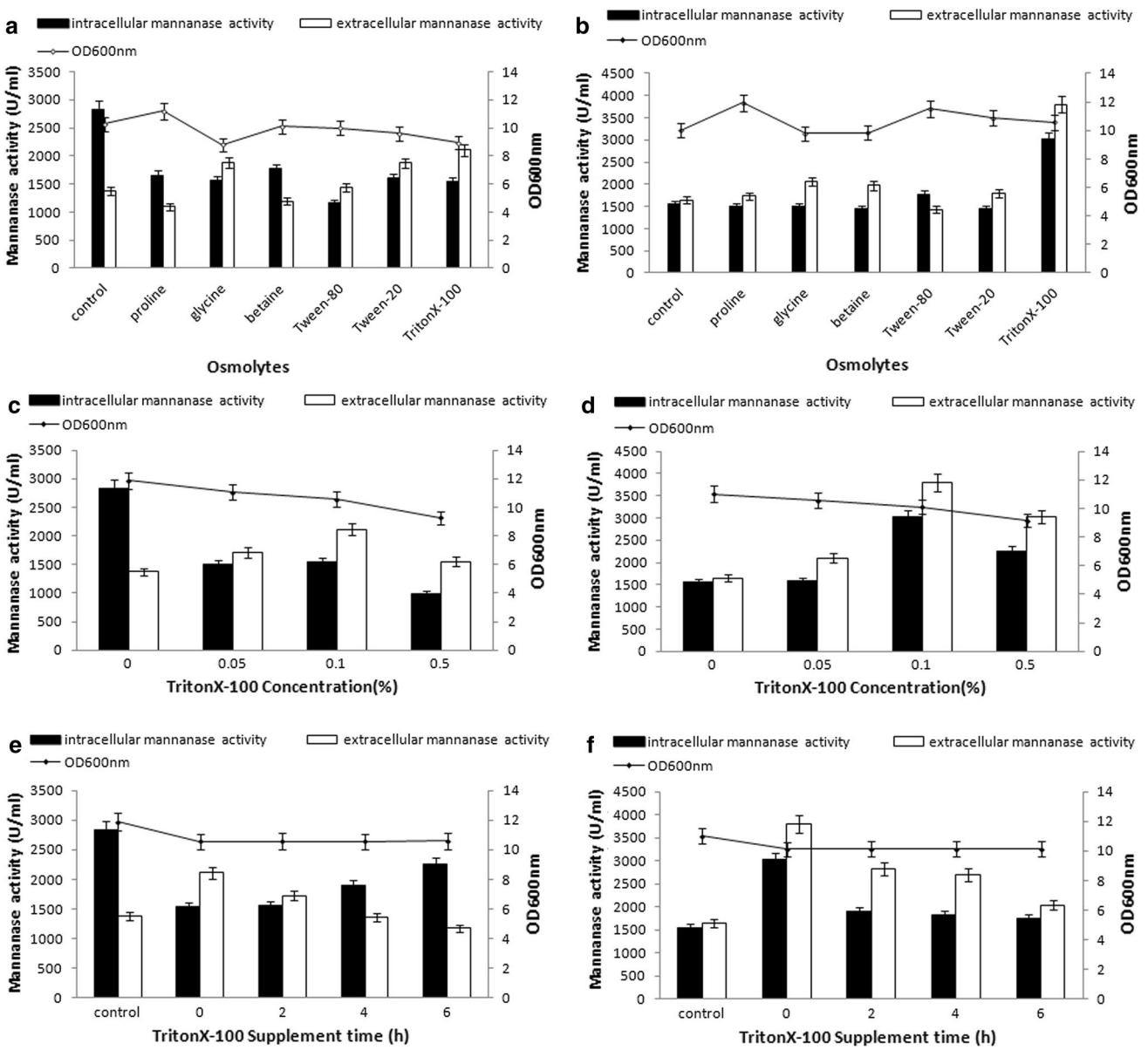

Fig. 5 Comparison of cell growth and soluble mannanase activity by addition of different osmolytes. **a** Different osmolytes (the concentration of each osmolyte was 0.1 %) for the yield of 32a-ManAR2; **b** different osmolytes (the concentration of each osmolyte was 0.1 %) for the yield of 22b-ManAR2; **c** the effects of different Triton X-100 concentrations on the yield of 32a-ManAR2; **d** the effects of different Triton X-100 concentrations on the yield of 22b-ManAR2; **e** the effects of different Triton X-100 supplement time on the yield of 32a-ManAR2; **f** the effects of different Triton X-100 supplement time on the yield of 22b-ManAR2. Data are presented as mean ± SD ($n = 3$)

mannanase obtained in this work were the highest production level in the recombinant *E. coli* expression system [6, 15]. Lin et al. [13] have achieved 310.1 U/mL of alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5 in the scale of a 5 L fermentor. Thereafter, a truncated alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5 has been expressed and secreted in *K cicerisporus* [16]. The maximum yield of recombinant mannanase reached 3795 U/mL in a 15 L fermentor [16]. Meanwhile, a process for efficient production of an alkaline β-mannanases from *Bacillus* sp. N16-5 was established by heterologous expression using *Pichia pastoris* [23]. The recombinant mannanase activity level reached 6336 U/mL within 84 h of fermentation in a 1 L fermentor [23]. Compared with the eukaryotic expressing system, the recombinant *E. coli* expression system showed higher expression level of the *Bacillus* sp. N16-5 alkaline β-mannanases (22b-ManAR2). In conclusion, the mannanase truncation and Triton X-100 supplement have

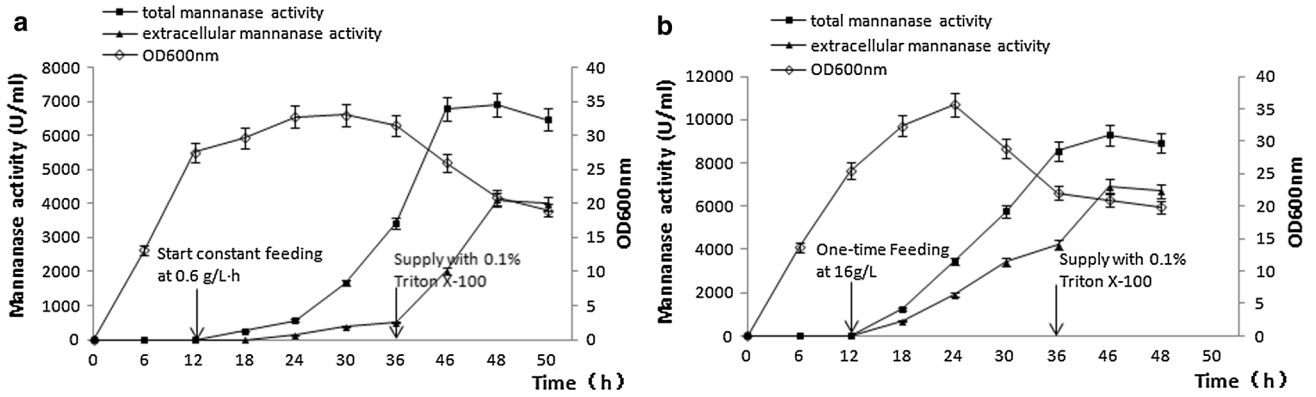

Fig. 6 Expression of 22b-ManAR22 in fed-batch cultivation in a 10 L fermentor. **a** Fed-batch cultivation using constant speed glycerol feeding at 0.6 g/L h; **b** fed-batch cultivation using one-time glycerol feeding at 16 g/L. Data are presented as mean ± SD ($n = 3$)

further enhanced the extracellular yield of the recombinant mannanase. Therefore, the recombinant *E. coli* BL21 (pET22b-*manAR2*) showed its great potential in industrial production of alkaline β-mannanase [6, 15].

Conclusion

In this study, the truncated recombinant mannanases (32a-ManAR2 and 22b-ManAR2) have achieved higher extracellular activity than that of their full-length enzymes by *E. coli* engineering strains. The higher alkaline stability at pH 8–11 and higher thermostability at 60 °C of the truncated mannanases render them more suitable for industrial application. The maximal soluble production and extracellular rate of 22b-ManAR2 in a 10 L fermentor with one-time feeding and 0.1 % Triton X-100 supplement were 9284.64 U/mL and 74 %, respectively. The convenience and efficiency of the fermentation process make the recombinant *E. coli* BL21 (pET22b-*manAR2*) a potential candidate for industrial production of alkaline β-mannanase.

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