Co-expression of Xylanase and Mannanase in Pichia pastoris and Analysis of Enzyme Production Postprint

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Abstract

Xylanase and mannanase are two important hemicellulases and also represent significant feed enzyme preparations. In this study, a co-expression recombinant plasmid pPICZ A/DSB-ManA for xylanase DSB and mannanase ManA was constructed using the method of generating multiple copies via in vitro tandem expression cassettes in the Pichia pastoris expression system. This recombinant plasmid was electroporated into the host strain Pichia pastoris X33 to obtain the recombinant strain X33/DSB-ManA that co-expresses both enzymes, achieving their co-secretory expression. Following induced expression, the enzyme activities of xylanase and mannanase reached 273.6 U/mL and 256.8 U/mL, respectively, corresponding to 30.4% and 73.4% of those from the individually expressing recombinant strains X33/DSB and X33/ManA. Enzymatic property analysis demonstrated that the optimal reaction temperature for both DSB and ManA was 75°C, with good thermal stability maintained within the 45°C-75°C range, retaining over 60% of maximum enzyme activity. The optimal pH for DSB was 6.5, while that for ManA was 6.0. Under conditions of pH 3.0 and 40°C, ManA retained over 80% of maximum enzyme activity after 1 h of treatment, whereas DSB retained over 50% after 1 h. Both DSB and ManA exhibited good tolerance to various metal ions and chemical reagents (at a concentration of 1 mM), maintaining over 60% enzyme activity. This study successfully accomplished the co-expression of different enzymes using a single strain, providing a theoretical foundation for the production and application research of complex enzyme feed additives.

Full Text

Co-expression of Xylanase and Mannanase in *Pichia pastoris* and Enzymatic Analyses

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Abstract

Xylanase and mannanase are two important hemicellulases and feed enzyme preparations. In this study, we constructed a recombinant plasmid pPICZ A/DSB-ManA containing both the xylanase DSB gene and mannanase ManA gene using the in vitro tandem expression cassette method in the *Pichia pastoris* expression system. The recombinant plasmid was then electroporated into host strain *Pichia pastoris* X33 to obtain the co-expression recombinant strain X33/DSB-ManA, which successfully achieved co-secretory expression of both enzymes. After induction, the enzyme activities of xylanase and mannanase in the supernatant reached 273.6 U/mL and 256.8 U/mL, respectively, corresponding to 30.4% and 73.4% of the activities observed in the single-expression recombinant strains X33/DSB and X33/ManA.

Enzymatic characterization revealed that both DSB and ManA exhibited an optimal reaction temperature of 75°C and maintained over 60% of their maximum activity across the temperature range of 45°C to 75°C. The optimal pH values were 6.5 for DSB and 6.0 for ManA. Under conditions of pH 3.0 and 40°C, ManA retained more than 80% of its maximum activity after 1 hour of treatment, while DSB maintained over 50% activity under the same conditions. Both enzymes demonstrated good tolerance to various metal ions and chemical reagents at a concentration of 1 mM, preserving more than 60% of their enzymatic activity. This study successfully accomplished the co-expression of different enzymes in a single strain, providing a theoretical foundation for the production and application research of compound enzyme feed additives.

Keywords: Xylanase; Mannanase; *Pichia pastoris*; Co-expression; Enzymatic characteristics

Introduction

Xylanase and mannanase are two important hemicellulases. Xylanase (endo-1,4- -xylanase, EC 3.2.1.8) degrades xylan into xylobiose, xylooligosaccharides, and small amounts of xylose [1], playing a key role in xylan degradation. In the CAZy classification system, xylanases belong to families 5, 7, 8, 10, 11, and 43,

with families 10 and 11 being the most predominant. These enzymes catalyze hydrolysis through a retaining mechanism that preserves the original configuration of the anomeric carbon, with two glutamic acid residues participating in the complete catalytic mechanism [2]. -mannanase (-1,4-mannanase, EC 3.2.1.78) is the key enzyme for degrading mannan and belongs to the endo-hydrolases that can degrade manno-oligosaccharides and manno-polysaccharides containing -1,4-mannosidic bonds [3]. -mannanases primarily belong to glycoside hydrolase families 5 and 26 [4]. Although -mannanases from different families exhibit differences in their catalytic domain sequences, they all adopt a TIM barrel structure and follow a retaining catalytic mechanism.

Anti-nutritional factors are prevalent in grain feedstuffs such as corn, soybean meal, and barley, as well as their by-products, with xylan and mannan being two important examples. Supplementing feed with xylanase and mannanase can disrupt plant cell walls, reduce anti-nutritional effects, and improve digestibility and nutrient absorption. Multiple studies have demonstrated that compound enzyme preparations are more effective than single enzyme supplements in feed [5]. Currently, most feed compound enzymes are produced through physical mixing, which requires the separate production of multiple individual enzymes and involves complex manufacturing processes. Using genetic engineering methods to co-express multiple enzymes in the same host strain enables single-strain fermentation to produce multiple enzymes, thereby simplifying the fermentation process. Huang et al. [6] co-secreted phytase and mannanase in *Pichia pastoris*, obtaining a recombinant yeast strain that could simultaneously produce both enzymes with good stability, showing no significant difference in enzyme activities after ten generations. Wu et al. [7] constructed a *Pichia pastoris* recombinant strain co-expressing phytase and endoglucanase, which simultaneously secreted both enzymes with activities reaching 39.4% and 56.2% of the single-expression strains, respectively, yet still achieving high levels of 13,726.7 U/mL and 364.5 U/mL.

The *Pichia pastoris* expression system has developed into an ideal protein expression platform and is widely used for heterologous protein production [8-9]. This study utilized this expression system to achieve co-secretory expression of two hemicellulases (xylanase and mannanase) and analyzed their enzymatic properties, providing a theoretical basis for the production and application of compound enzyme feed additives.

Materials and Methods

1.1 Strains and Plasmids

The plasmids pAO815hr/DSB (containing the xylanase DSB gene) and pAO815hr/ManA (containing the mannanase ManA gene) were constructed and preserved in our laboratory. The expression vector pPICZ A, *Pichia pastoris* X33, and *Escherichia coli* Top10 were also maintained in our laboratory.

1.2 Reagents

Restriction endonucleases, Prime STAR Max DNA polymerase, T4 DNA ligase, and DNA markers were purchased from Takara Bio (Dalian, China). Plasmid mini-prep kits, DNA gel extraction kits, and PCR cleanup kits were obtained from AxyPrep (Shanghai, China). Substrates including oat xylan and locust bean gum were purchased from Sigma-Aldrich. Peptone and yeast extract were from Oxoid. Yeast nitrogen base without amino acids (YNB), sorbitol, and agar were from Biosharp. Zeocin was from Invitrogen. All other reagents were of analytical grade from domestic or international sources.

1.3 Construction of Co-expression Recombinant Plasmid

Using plasmids pAO815hr/DSB and pAO815hr/ManA as templates, the DSB and ManA genes were amplified by PCR with primers P1/P2 and P3/P4, respectively (Table 1). The PCR products were digested with EcoRI/NotI and KpnI/XbaI, respectively, and inserted into pPICZ A to obtain the recombinant plasmids pPICZ A/DSB and pPICZ A/ManA. Based on these two recombinant plasmids, the co-expression plasmid was constructed using the in vitro tandem expression cassette method for multimerization. Specifically, the pPICZ A/ManA plasmid was double-digested with the compatible enzymes BglII and BamHI to obtain the ManA expression cassette containing the AOX1 promoter, terminator, and -signal peptide sequence for secreted expression. Simultaneously, the recombinant plasmid pPICZ A/DSB was single-digested with BamHI. The ManA expression cassette was then ligated with the linearized pPICZ A/DSB vector to obtain the DSB and ManA co-expression recombinant plasmid pPICZ A/DSB-ManA. The construction scheme is illustrated in Figure 1 [Figure 1: see original paper]. The resulting plasmid was verified by double digestion and sequenced by Wuhan Tsingke Biotechnology Co., Ltd.

1.4 Construction of Recombinant Strains Co-expressing Xylanase and Mannanase and Their Induced Expression

The recombinant plasmid pPICZ A/DSB-ManA was concentrated ten-fold using a vacuum centrifugal concentrator (to approximately 50 g) and electroporated into *Pichia pastoris* X33 competent cells. Transformants were screened on YPDS plates containing Zeocin. Positive transformants were identified by colony PCR using primers P1/P2 and P3/P4. For enzyme production, positive transformants were inoculated into 25 mL BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 1% glycerol, 4×10 % biotin, 0.1 mol/L potassium phosphate buffer pH 6.0) and cultured at 30°C with shaking at 250 rpm for 24 h. Cells were harvested by centrifugation and resuspended in 25 mL BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 1% methanol, 4×10 % biotin, 0.1 mol/L potassium phosphate buffer pH 6.0) to an initial OD of 1.0. Induction was performed at 30°C with shaking at 250 rpm for 144 h, with 1% methanol added every 24 h and 0.5 mL samples collected at each time point. The collected samples were centrifuged, and the supernatants were used for enzyme

activity assays. Transformants with high enzyme activities were selected as the co-expression recombinant strain X33/DSB-ManA. Additionally, recombinant plasmids pPICZ A/DSB and pPICZ A/ManA were separately transformed into X33 to obtain the single-expression control strains X33/DSB and X33/ManA. All experiments were performed in triplicate.

Xylanase activity was determined by the DNS method [10] using xylan as substrate. One unit of xylanase activity was defined as the amount of enzyme required to release 1 mol of xylose per minute from 0.5% xylan substrate under optimal temperature and pH conditions. Mannanase activity was also measured by the DNS method [11] using locust bean gum as substrate. One unit of mannanase activity was defined as the amount of enzyme required to release 1 mol of mannose per minute from 0.5% mannan substrate under optimal conditions.

1.5 SDS-PAGE Analysis of Enzymes from Co-expression Recombinant Strain

After induction of the co-expression recombinant strain GS115/DSB-ManA, the fermentation supernatant was analyzed by SDS-PAGE according to the protocol described in *Molecular Cloning: A Laboratory Manual* (2nd edition).

1.6 Enzymatic Characterization

1.6.1 Determination of Optimal Temperature The activities of xylanase (at pH 6.5) and mannanase (at pH 6.0) from the co-expression recombinant strain were measured at temperatures of 60°C, 65°C, 70°C, 75°C, 80°C, and 85°C. The highest enzyme activity was defined as 100%, and the relative activities at different temperatures were calculated. All experiments were performed in triplicate.

1.6.2 Thermal Stability Assay Appropriately diluted enzyme solutions were incubated at temperatures ranging from 45°C to 85°C (at 5°C intervals) for 30 min, then rapidly cooled on ice. The residual activities of xylanase and mannanase were measured, with the activities of untreated enzyme solutions defined as 100%. The relative residual activities after treatment at different temperatures were calculated. All experiments were performed in triplicate.

1.6.3 Determination of Optimal pH A 50 mmol/L sodium phosphate-citrate buffer system was prepared across pH 4.5–7.5. Substrate solutions of 0.5% xylan and 0.5% locust bean gum were prepared using the respective buffers. Enzyme solutions were diluted with the corresponding pH buffers, and activities were measured at 75°C. The highest enzyme activity was defined as 100%, and relative activities at different pH values were calculated. All experiments were performed in triplicate.

1.6.4 pH Stability Assay Two pH conditions were selected to approximate animal gastric fluid (pH 3.0) and intestinal environment (pH 6.0). Enzyme solutions diluted with the respective pH buffers were incubated at 40°C for 3 h, with 0.5 mL samples taken every 30 min to measure residual xylanase and mannanase activities. The activities of untreated enzyme solutions were defined as 100%, and the relative residual activities over time were calculated. All experiments were performed in triplicate.

1.6.5 Effects of Metal Ions and Chemical Reagents on Enzyme Stability Metal ion and chemical reagent solutions were added to 0.5% xylan and 0.5% locust bean gum substrates to achieve final concentrations of 0.5 mM and 1 mM. Enzyme activities were measured using these treated substrates, with the activities of untreated controls defined as 100%. The relative activities under different treatments were calculated. All experiments were performed in triplicate.

Results

2.1 Construction of Co-expression Recombinant Plasmid

Using plasmids pAO815hr/DSB and pAO815hr/ManA as templates, the DSB gene (~600 bp) and ManA gene (~800 bp) were amplified by PCR with primers P1/P2 and P3/P4, respectively (Fig. 2a [Figure 2: see original paper]). The PCR products were double-digested and ligated into pPICZ A to obtain the recombinant plasmids pPICZ A/DSB and pPICZ A/ManA. Restriction analysis of both recombinant plasmids showed bands of the expected sizes (Fig. 2b). The co-expression recombinant plasmid pPICZ A/DSB-ManA was then constructed using the compatible enzymes BglII and BamHI via the in vitro tandem expression cassette method. Double digestion verification of pPICZ A/DSB-ManA yielded two bands of correct sizes: the DSB-ManA tandem expression cassette (~4.8 kb) and the vector backbone (~1.9 kb), confirming successful construction (Fig. 2c). All recombinant plasmids were sequenced by Wuhan Tsingke Biotechnology Co., Ltd., and the sequences were verified as correct.

2.2 Construction of Recombinant Strains and Analysis of Enzyme Production

The recombinant plasmid pPICZ A/DSB-ManA was transformed into *Pichia pastoris* X33, and the co-expression recombinant strain X33/DSB-ManA with the highest enzyme activities was selected. The control strains X33/DSB and X33/ManA were obtained by separately transforming pPICZ A/DSB and pPICZ A/ManA into X33. Following shake-flask fermentation for 144 h, the average xylanase activity of eight X33/DSB transformants was 900 U/mL (Fig. 3 Figure 3: see original paper), while the average mannanase activity of eight X33/ManA transformants was 350 U/mL (Fig. 3(b)). In contrast, the X33/DSB-ManA strain produced maximum xylanase and mannanase

activities of 273.6 U/mL and 256.8 U/mL, respectively (Fig. 4(b) [Figure 4: see original paper]), representing 30.4% and 73.4% of the activities from the single-expression strains. Furthermore, the growth curve of X33/DSB-ManA was consistent with that of the host strain X33 (Fig. 4(a) [Figure 4: see original paper]), indicating that co-expression of DSB and ManA had no significant effect on host cell growth. These results demonstrate that DSB and ManA were successfully co-secreted in the same host strain, and although enzyme activities were reduced compared to single-expression strains, both enzymes still exhibited relatively high activity.

SDS-PAGE analysis of the X33/DSB-ManA fermentation supernatant revealed two distinct bands of approximately 23 kDa and 30 kDa (Fig. 5 [Figure 5: see original paper]), consistent with the predicted molecular weights of DSB and ManA, further confirming that the GS115/DSB-ManA strain could simultaneously secrete both hemicellulases.

2.3 Enzymatic Properties of the Co-expressed Enzymes

2.3.1 Optimal Temperature and Thermal Stability The optimal temperature for both xylanase and mannanase from X33/DSB-ManA was 75°C, classifying them as thermophilic enzymes (Fig. 6(a) [Figure 6: see original paper]). Both enzymes maintained over 60% relative activity across the temperature range of 60°C to 85°C. To assess thermal stability, the fermentation supernatant was treated at various temperatures for 30 min. The results showed that ManA retained over 90% relative activity after treatment at 45°C to 75°C, while DSB maintained over 60% relative activity under the same conditions (Fig. 6(b) [Figure 6: see original paper]). Feed processing involves high-temperature treatments such as pelleting, extrusion, and drying (above 70°C), which typically affect enzyme activity to varying degrees. The excellent thermal stability of DSB and ManA represents a significant advantage for feed enzyme production and demonstrates promising application potential.

2.3.2 Optimal pH and pH Stability The optimal pH for DSB and ManA was 6.5 and 6.0, respectively (Fig. 7(a) [Figure 7: see original paper]). DSB maintained over 60% relative activity at pH 4.5–7.5, while ManA retained over 50% relative activity across the same pH range. To evaluate pH stability under conditions approximating the animal gastrointestinal tract, the fermentation supernatant was treated at pH 3.0 (gastric) and pH 6.0 (intestinal) at 40°C for 3 h. The results showed that both DSB and ManA maintained stable activity without significant decline at pH 6.0. At pH 3.0, ManA retained over 80% activity after 1 h and 50% activity after 2.5 h, while DSB activity decreased more rapidly with prolonged treatment but still maintained over 50% activity after 1 h (Fig. 7(b) [Figure 7: see original paper]). These results indicate that both enzymes can maintain relatively high activity under acidic conditions, which is beneficial for resisting the acidic environment of the animal gastrointestinal tract when used as feed additives.

2.3.3 Stability in the Presence of Metal Ions and Chemical Reagents
The effects of various metal ions and chemical reagents on enzyme activities are summarized in Table 2 and Table 3 . For mannanase ManA, Cu^{2} , Fe^{3} , Mn^{2} , Co^{2} , Pb^{2} , EDTA, and -mercaptoethanol exhibited varying degrees of inhibition, with Cu^{2} , Fe^{3} , and Co^{2} showing significant inhibition only at higher concentrations. Mg^{2} , Li , Ni^{2} , and SDS had minimal effects on ManA activity, while Ca^{2} significantly activated the enzyme. For xylanase DSB, Cu^{2} , Mn^{2} , Mg^{2} , Pb^{2} , SDS, and EDTA showed inhibitory effects, whereas Fe^{3} , Co^{2} , Li , Ni^{2} , Ca^{2} , and -mercaptoethanol had minimal impact, and Co^{2} slightly promoted DSB activity at low concentrations. During feed processing, raw materials contain various metal elements, and different types and ratios of trace metal ions are added to meet animal growth requirements, which can affect enzyme activity and stability. The results in Table 2 and Table 3 demonstrate that DSB and ManA possess good tolerance to most metal ions and chemical reagents, which is advantageous for maximizing their catalytic activity as feed additives.

Discussion

Xylanase and mannanase are important feed enzyme preparations. Supplementing feed with compound enzyme preparations containing both enzymes can promote feed nutrient utilization, improve animal growth performance, and enhance animal health [12-17]. However, most compound enzymes are currently produced through physical mixing, which requires the separate production of multiple individual enzymes and involves complex manufacturing processes. In this study, we selected *Pichia pastoris* X33 as the host strain and used genetic engineering technology to construct the co-expression recombinant plasmid pPICZ A/DSB-ManA. The recombinant plasmid was then transformed into *Pichia pastoris* X33 to obtain the co-expression strain X33/DSB-ManA. Shake-flask fermentation results showed that after 144 h of induction, xylanase and mannanase activities reached 273.6 U/mL and 256.8 U/mL, respectively, demonstrating successful co-expression of both enzymes at relatively high activity levels. For industrial compound enzyme production, this approach simplifies the fermentation process and reduces production costs.

We compared the enzyme activities of the co-expression strain X33/DSB-ManA with those of the single-expression strains X33/DSB and X33/ManA. After 144 h of shake-flask fermentation, X33/DSB exhibited xylanase activity of 900 U/mL, while X33/ManA showed mannanase activity of 350 U/mL. In contrast, X33/DSB-ManA produced xylanase and mannanase activities of 273.6 U/mL and 256.8 U/mL, respectively, corresponding to 30.4% and 73.4% of the single-expression levels. These results indicate that co-expression of DSB and ManA in the same host led to reduced maximum enzyme activities compared to single-expression strains, suggesting that the host' s maximum expression capacity may have an upper limit [7,18]. Notably, the decrease in DSB activity (~70%) was more pronounced than that in ManA activity (~30%), indicating that co-

expression had a greater impact on DSB activity. This may be attributed to selective high expression of one gene when both genes use the same promoter [19], though the specific mechanisms require further investigation.

Both co-expressed enzymes in this study were thermophilic, with optimal reaction temperatures of 75°C. After treatment at 75°C for 30 min, both enzymes retained over 60% relative activity, demonstrating excellent thermal stability. This characteristic is crucial for addressing enzyme inactivation during high-temperature pelleting in industrial enzyme preparation and meets the requirements of industrial production. Additionally, both enzymes exhibited good acid resistance. At pH 3.0 and 40°C, ManA retained over 80% activity after 1 h, while DSB maintained over 50% activity. This indicates that both enzymes can resist the acidic environment of the animal gastrointestinal tract when administered as feed additives, suggesting promising applications in the feed industry.

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Note: Figure translations are in progress. See original paper for figures.