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Original Article

Immobilization of mutated xylanase from *Neocallimastix patriciarum* in *E. coli* and application for kraft pulp biobleaching

Imobilização de xilanase mutada de *Neocallimastix patriciarum* em *E. coli* e aplicação para polpa kraft

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Abstract

As an important enzyme, xylanase is widely used in the food, pulp, and textile industry. Different applications of xylanase warrant specific conditions including temperature and pH. This study aimed to carry out sodium alginate beads as carrier to immobilize previous reported mutated xylanase from *Neocallimastix patriciarum* which expressed in *E. coli,* the activity of immobilization of mutated xylanase was elevated about 4% at pH 6 and 13% at 62 °C. Moreover, the immobilized mutated xylanase retained a greater proportion of its activity than the wide type in thermostability. These properties suggested that the immobilization of mutated xylanase has potential to apply in biobleaching industry.

Keywords: xylanase, immobilization, thermostable, alkali tolerant, sodium alginate, biobleaching.

Resumo

Como importante enzima, a xilanase é amplamente utilizada na indústria alimentícia, de celulose e têxtil. Diferentes aplicações de xilanase garantem condições específicas, incluindo temperatura e pH. Este estudo teve como objetivo realizar grânulos de alginato de sódio como carreador para imobilizar xilanase mutada relatada anteriormente de *Neocallimastix patriciarum* que expressa em *E. coli*, a atividade de imobilização da xilanase mutada foi elevada em cerca de 4% em pH 6 e 13% a 62 °C. Além disso, a xilanase mutada imobilizada reteve uma proporção maior de sua atividade do que o tipo amplo em termoestabilidade. Essas propriedades sugerem que a imobilização da xilanase mutada tem potencial para aplicação na indústria de biobranqueamento.

Palavras-chave: xilanase, imobilização, termoestável, tolerante a álcalis, alginato de sódio, biobranqueamento.

1. Introduction

Xylan, a component of hemicelluloses which are the second most abundant plant fraction available in nature, accounts for as much as 30% of the dry weight of some plant tissues (Ebringerová and Heinze, 2000). Biodegradation of xylan requires the action of several enzymes, among which xylanases (1,4-β-D xylan xylanohydrolas, EC 3.2.1.8) play a key role (Biely et al., 1997). More studies indicate microbial xylanases can be widely applied in the biobleaching industry, food and beverage industries and animal feeding industry (Atalah et al., 2019; Campioni et al., 2019).

Due to the important industrial application, many xylanase genes have been identified in bacteria and fungi (Chadha et al., 2019). Xylanases are grouped into glycosidase families based on the primary structure of the catalytic domains and are normally reported in glycosidase families 10 and 11 (Jeffries, 1996; Collins et al., 2005).

Until now, xylanase still has some obstacles in the application of pulp bioleaching industries, which makes it unable to be widely used in this field. Normally, free xylanase is often hampered by its reusability and limited by high production costs, low enzyme activity and high consumption of xylanase. But immobilized enzymes were allowed the enzyme to be reused in multiple cycles to lower the production costs and overcome such technical drawbacks (Pal and Khanum, 2011; Zhao et al., 2019).

To use xylanase in immobilization had been studied previously, such as metallic hollow spheres and magnetic graphene oxide (Steudler et al., 2020; Paz-Cedeno et al., 2021). However, when compared with these supports, sodium alginate, due to its low cost, ease of use, and non-toxicity is the one of best candidate to use as carrier (Taqieddin and Amiji, 2004; Catana et al., 2005; Bhushan et al., 2008; Jampala et al., 2017). A method of

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covalent immobilization of xylanase was attached to the surface of alginate beads through (Li et al., 2007, Ortega et al., 2009, Zhang et al., 2010). The glutaraldehyde is one of the most frequently used linker for enzyme immobilization, a chemical covalent bond is formed between the functional groups on the enzyme protein molecule and the reactive group on alginate beads, forming an immobilized product with a strong binding force to ensure the stability of the enzyme during the catalytic reaction (Zhao et al., 2019, Alnoch et al., 2020). In present study, we have used sodium alginate beads as carrier to immobilize previous reported mutated xylanase (XynM) which expressed in *E. coli*, whose characteristics have been analyzed. In previous report, XynM was studied and shown a relatively high specific activity 10,128 µmol glucose·min^{-1}(mg protein)$^{-1}$at pH 6.0. It was more thermostable at 60°C and alkaline tolerant at pH 10.0 than the wild-type xylanase (Chen et al., 2001). Based on these characteristics, we have designed an immobilization method to use sodium alginate beads as carrier to immobilize the XynM.

XynM is a potential candidate according to the previous data shown a better activity in alkaline pH. In order to further improve the performance of XynM and reduce the amount of enzyme, the immobilization study could be introduced. In paper industry, chlorine is often to use to treat the pulp for bleaching. The main role of chlorine in bleaching is to convert the residual lignin in the pulp into water or alkali-soluble products (Balakshin et al., 2001). During the kraft process, part of the xylan was transferred to the fiber surface. After the pulping process, a large amount of xylan is present in the fiber. To this end, xylanase can be used to treat these xylan, biobleaching is eco-friendly method (Sridevi et al., 2016; Zhang et al., 2019), xylanase pretreat kraft pulp can help minimize the usage of chlorine in the process of delignification, reduce environmental damage and improve bleaching performance. However, the bottle neck for biobleaching is that xylanase should be stable, active at extreme condition and low cost of enzymes. (Kolar et al., 1983; Shoham et al., 1992; Nagarathnamma & Bajpai, 1999; Bajpai, 2004; Gordon and Rosenblatt, 2005). In present study we have used sodium alginate to immobilize XynM, it might potentially reduce the cost of usage of enzyme.

2. Materials and Methods

2.1. Chemicals

Beechwood xylan, 3, 5-dinitrosalicylic acid (DNS), and D-xylose were purchased from Yuanye Ltd. (Shanghai, China). All other chemicals and solvents used in this work were of analytical grade and obtained from Aladdin Ltd (Shanghai, China).

2.2. Xylanase cloning, expression and purification

The template for the construction of the XynM used in this study was synthesized according to previous reported sequence (Chen et al., 2001). The fragment of XynM of *Neocallimastix patriciaru*gene was amplified using primers F (5'–AAAAACATATGCAGAGCTTTTGTAG–3') and R (5'–TTTTTCTCGAGTTAATCGCCAATGT–3'). PCR was performed in a 0.5 mL Eppendorf tube. The 100 µL reaction mixture was incubated for 5 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final incubation for 10 min at 72 °C. The restriction enzyme sites are NdeI and XhoI. The amplified PCR products were ligated into pET-28a (+) expression vector as appropriate. All the constructs in pET vectors were transformed into *E. coli* BL21 (DE3).

A single and fresh bacterial colony harboring the plasmid was used to inoculate 10 mL of LB medium containing 50 µg/mL of kanamycin. The culture was then incubated at 37 °C overnight. 300 mL of LB media was inoculated with 3 mL of overnight culture and grown at 37 °C until the OD_{600} reached 0.5, the cells were induced by adding 1 M stock solution of isopropyl-β-D-thiogalactopyranoside (Final concentration: 0.1 mM) and further incubated at 16 °C about 14 h. Following induction, the cultures were centrifuged (6000 × g; 10 min; 4 °C). Prior to protein purification, protein stock solution contained phosphate buffer, pH 8 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) from the cell lysate was obtained by sonication of cells followed by centrifugation at 13,000 × g for 10 min at 4 °C.

Purification of His-tagged fusion XynM was carried out using Ni-NTA column (Beyotime, China) according to manufacturer's instructions. Briefly, the column was first washed with 10 ml of distilled water and then equilibrated with 10 mL of lysis buffer. The cell supernatant was loaded on a Ni-NTA column. Then, the column was washed with 30 ml of wash buffer contained phosphate buffer, pH 8 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). After that, the recombinant XynM was eluted using elution buffer contained phosphate buffer, pH 8 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole). Finally, the purity of the XynM was analyzed by SDS-PAGE stained with Coomassie Brilliant Blue. Protein concentration was determined by Bradford assay using BSA as a standard (Cheng et al., 2016).

To minimize imidazole and buffer effected downstream experiment, elution fractions were pooled together and concentrated to 100µL by Millipore Ultra Centrifugal Filter (10kDa cut-off) after confirmation by SDS-PAGE.

2.3. SDS-PAGE

SDS-PAGE was performed using a 10% (w/v) polyacrylamide gel. Enzyme samples from each step was mixed with loading buffer and boiled at 100 °C for 40 s prior to electrophoresis. After the separation of the enzyme samples. The gel that contained the samples and molecular markers was stained in Coomassie Brilliant Blue R-250 and de-stained with water.

2.4. Sodium alginate beads preparation

The sodium alginate beads were produced by dropping a 2% (w/v) sodium alginate solution through a syringe into 0.02M $CaCl_2$ solution as previously reported (Pal and Khanum, 2011). The ratio between the alginate and the $CaCl_2$ solution was 1:2 (v/v). The beads were subsequently stored at 4 °C overnight to allow them to harden.

The alginate beads were further activated by dipping them into a 2% w/v glutaraldehyde solution. The activation

process was carried out at room temperature under orbital stirring for 4 h using a ratio of 1:10 beads: glutaraldehyde solution (Pal and Khanum, 2011). After activation, the beads were thoroughly washed to remove any unbound glutaraldehyde, the beads were ready to use.

2.5. Xylanase immobilization

The filtrated culture was divided into 100 aliquot partsto calculate the amount of immobilized XynM for further use. The aliquots parts mixed with the activated beads at a ratio of 1:1 (v/w). The enzyme and bead mixture were then mixed at 200 rpm for 2 h using an orbital shaker. The free XynM in the solution has been analyzed in order to calculate the yield of immobilized XynM. After activation, the beads were thoroughly washed by distilled water.

2.6. Xylanase assay and protein determination

XynM activity was measured by the method of Khanna and Gauri (Khanna and Gauri, 1993). The solution of beech wood xylan (1%) and the enzyme preparation was carried out at 50 °C for 10 min. The standard reaction mixture contained 100 µL dissolved xylan substrates with 50 mM sodium acetate buffer (pH 5), 100µL enzymes/beads and 800 µL buffer (50 mM sodium acetate). The reaction was stopped by adding 1000 µL of 3,5-dinitrosalicylic acid (DNS) reagent and boiled for 30 min. The reaction mixture was measured at 540 nm.

One unit of xylanase is defined as the amount of enzyme required to hydrolyze xylan to form 1 µmol of reducing sugar as xylose equivalent/min under the assay conditions. All the experiments were done in triplicate and the results are expressed as mean ± SD.

2.7. Effects of pH and temperature on the activity and stability of free and immobilized enzymes

The activity and stability of immobilized XynM were assayed at different temperatures and pH values by the enzyme assay, as described above. The effect of temperature on the xylanase activity and stability was evaluated at temperature range of 30-80 °C (pH 5). The effect of pH on the activity and stability of free and immobilized XynM were investigated at different pH: sodium acetate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), and Tris–HCl buffer (pH 8.0–9.0) at 50°C.

2.8. Effect of xylanase on waste paper pulp

The paper pulp was prepared according to previous report (Subramaniyan and Prema, 2002). Immobilized xylanase-treated pulp was prepared by taking 5% (W/V) of pulp and treated with an enzyme dose of 20-100 U mL^{-1} per gram of pulp for a time period of 1 h at 50 °C with gentle shaking. The treated pulp was filtered, thoroughly washed and dried at 50 °C for 3 h. A control was added without enzyme treatment. Kappa number was used to evaluate the efficiency of treatment process. The brightness of the pulp was calculated by the method of Jordan and Popson (1994). The reducing sugar in supernatant was measured by the DNS method and chromophores and hydrophobic compounds were measured at 237 and 465 nm (Manimaran and Vatsala, 2007), expected releasing from lignin and hemicelluloses and improving the brightness.

To test reusability, the immobilized enzyme was washed by distilled water, and then added to a fresh reaction solution to start a new enzymatic reaction cycle. Experiments were repeated for eight cycles. The highest activity measured under the corresponding temperature or pH ranges was designated as 100%, and the activities at all the remaining temperatures and pHs were values proportional to that highest activity.

3. Results

3.1. Purification

SDS-PAGE analysis showed a high degree of purity following purification by Ni-NTA affinity chromatography. The line 1 has indicated the XynM has been well expressed; the flow through fraction (Line 2) has shown that most of XynM was binded with Ni-NTA resin; during the washing step there was no XynM has been lost (Line 3). The purified XynM are well purified and present in lanes 4-6 of the gel (Figure 1).

3.2. Optimum pH and temperature

The yield of immobilized XynM is 58% according to the calculation of the uncoupling XynM which present in the supernatant. In order to know the characteristic of immobilized XynM, we have tested the free/immobilized XynM in different pH (3~9) and temperature (30 °C~80 °C). Our results suggested that the optimum pH of immobilized XynM has 4% higher activity than free XynM (Figure 2). In alkaline pH range the immobilized XynM behaved better, it suggested that the immobilization formation enhance the tolerance in alkaline condition. This characteristic also

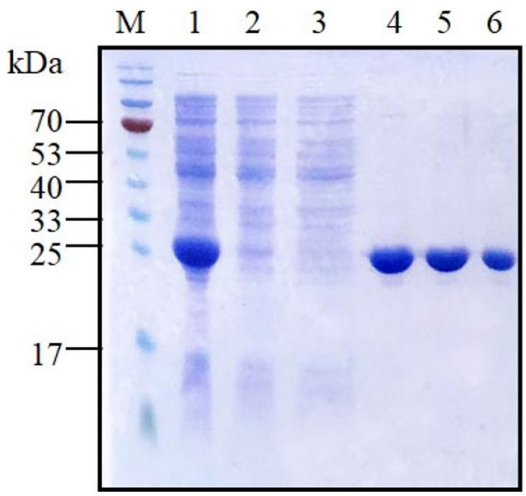

Figure 1. SDS-PAGE analysis of affinity purification steps of the His-tagged recombinant XynM from *Neocallimastix patriciarum* from *E. coli* cell lysate (Lane 1), flowthrough (Lane 2), wash (Lane 3) and elutions 1–3 (Lane 4-6).

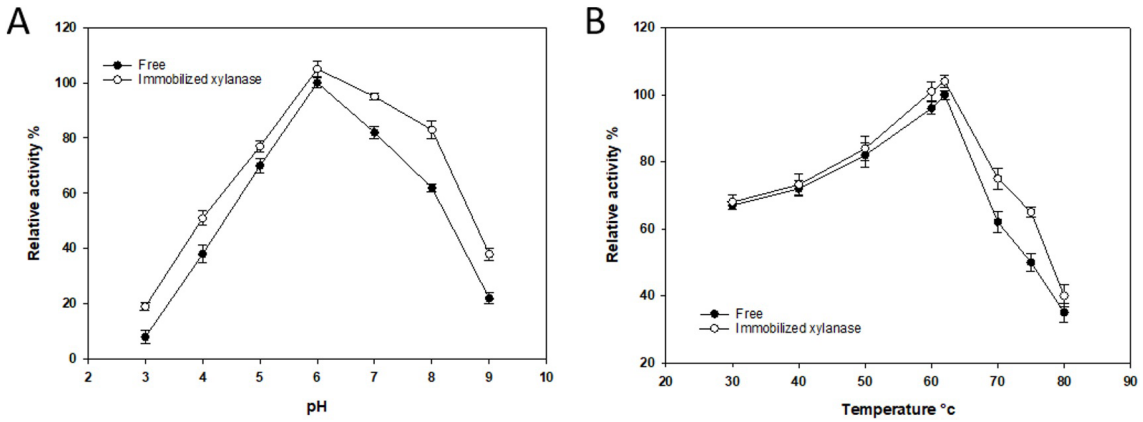

Figure 2. Influence of pH and temperature on the activity of the recombinant XynM from *Neocallimastix patriciarum*.

observed in acidic range, immobilized XynM has about 10% higher activity than free XynM.

In the meantime, the optimum temperature has been tested as well, the results indicated that the optimum temperature are the same at 62 °C, immobilized XynM shown 3.8% higher activity than free XynM (Figure 2). However, at 70 °C the immobilized XynM shown a better activity around 13% higher. immobilized XynM still shown about 10% higher activity to compare free XynM.

3.3. Thermostability

Optimum temperature study shown that immobilized XynM has better tolerance in higher temperature, it may have indicated that thermostabiltiy will also be improved. We have tested the challenged temperature range from 50°C-70°C, the result has been proved the immobilized XynM has at least 20 minutes longer thermostability at 70°C (Figure 3). From 50°C to 60°C the thermostablity of immobilized XynM has been improved, the related activity has been elevated 1-7% to compare with free XynM.

3.4. Application in pulp biobleaching and reusability

In bioleaching process, immobilized XynM treated pulp samples were studied, Kappa number and brightness were obtained. After immobilized XynM treatment, a decrease in kappa and a brightness increase were observed, suggested that lignin has been released. In addition, compared with the untreated example (Table 1), the Kappa number with various amount of immobilized XynM was reduced, and the brightness was improved, which proved that catalytic efficiency in the reaction. The increased chromophores (λ237) and hydrophobic compounds (λ465) were also observed, it suggested that immobilized XynM removed these compounds efficiently as well (Table 1). Meanwhile reducing sugar were increased has been observed, it confirmed the pulp fibers were broken down and sugar released when compare with untreated sample.

In order to know the reusability of immobilized XynM, the test has been performed as well. The results suggested that after 7 cycles immobilized the XynM still main about 55% activity (Figure 4).

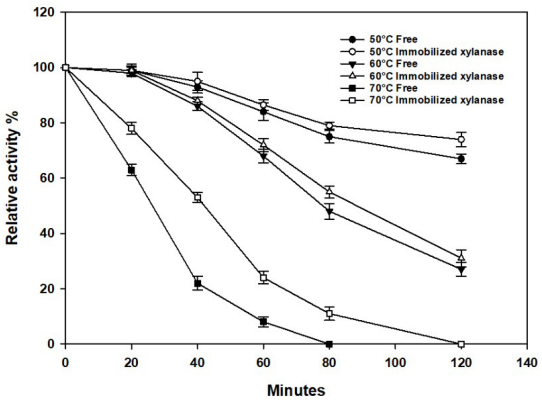

Figure 3. Relative activities of the recombinant XynM from *Neocallimastix patriciarum* from stability assay.

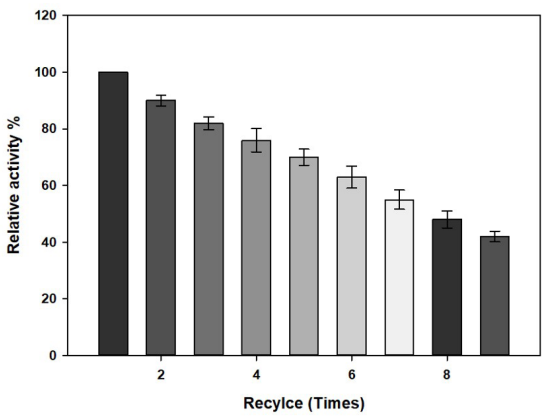

Figure 4. The reusability of the immobilized recombinant XynM from *Neocallimastix patriciarum*.

4. Discussion

In previous study, the XynM has been characterized as more alkali tolerant XynM than wild-type xylanase, our characterization results are consistent with the previous study. With this purpose we have tried to express and use

Table 1. Comparison of kappa number and brightness of paper pulp.

Parameter	Kappa number	Brightness (ISO units)	Chromophoric compounds (λ 237)	Hydrophobic compounds (λ 465)	Reducing sugar (mg g^{-1})
Untreated	25.2	38.1	0.248	0.425	1.88
XynM (20 U g^{-1})	23.9	40.7	0.979	0.845	2.88
XynM (40 U g^{-1})	21.2	41.7	1.998	1.009	8.98
XynM (80 U g^{-1})	18.1	43.1	2.447	1.711	12.45
XynM (100 U g^{-1})	17.3	44.8	2.681	2.014	14.89

the purified XynM to immobilize in a cost-effective carrier. Sodium alginate could be an option which can be easily prepared. In the bleaching process that xylanase-aided bleaching of kraft pulps reduce the consumption of chlorine chemicals. We have designed an immobilization method which is to use sodium alginate beads. Sodium alginate beads is relatively big in size, eco-friendly, low cost, high loading capacity and easy prepared are widely used in immobilization study (Cheng et al., 2016).

From our study, we have characterized the immobilized XynM through various pH and temperature. The results indicated that immobilized XynM has 4% higher activity at pH 6 and the optimum pH has been increased about 3.8% when compare to free XynM. The immobilized XynM shown more alkali tolerant than free XynM. In the optimum temperature study, the immobilized XynM shown better activity at 62°C, even at 70 °C shown more stable than the free XynM. The stability test also confirmed the immobilized XynM is more stable. A similar study also observed that the immobilization of xylanase onto sodium alginate beads increases the activation energy from 28.50 J mol^{-1} K^{-1} to 39.38 J mol^{-1} K^{-1}, it suggested that xylanase immobilized onto sodium alginate beads could increase the enzyme stability and retains its activity (Jampala et al., 2017). The reason may explain as covalent bond formation between glutaraldehyde and XynM help strengthen the structure, can further help tolerate in extreme condition. According to our knowledge, the highest specific activities of the xylanases are around 15k~18k µmol glucose·min^{-1}· (mg protein)$^{-1}$. However, Xylanase from *Bispora sp.* MEY-1 with 18k µmol glucose·min^{-1}· (mg protein)$^{-1}$ specific activity shown no activity at pH 8 (Luo et al., 2009). In another study, xylanase from *Cellulomonas flavigen* only retained 5% activity which is around 200 µmol glucose·min^{-1}· (mg protein)$^{-1}$ at 65 °C (Kim et al., 2018). Our study shown a better activity and it might be also useful for the future engineering, according to the previous study to have a better performance at alkaline condition and industrial required temperature.

In order to prove our idea, we have used immobilized XynM to pretreat kraft pulp. In pulp pretreatment test, immobilized XynM increased reducing sugar released and brightness has been improved. The reusability results also further confirmed the stability. Above all, our study proved potential application in kraft pulp biobleaching industry.

5. Conclusion

In the present study, XynM from Neocallimastix patriciarum expressed in E. coli has been immobilized in sodium alginate beads. Immobilized XynM shown a higher activity at optimum pH and temperature. Immobilized XynM was applied in biobleaching pretreatment study was confirmed the possibility in kraft pulp pretreatment. To compare with similar study but in different enzymes such as cellulase and lacasse (Bankeeree et al., 2016; Li et al., 2019), our study offers a different evidence to prove the possibility of improving properties of xylanase through immobilization on sodium alginate beads and according the properties of improvement our application in pulp bleaching was well tested and offer a little insight of potential immobilized XynM application in paper industry.

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