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INVESTIGATION OF CULTURE CONDITIONS FOR RECOMBINANT XYLANASE A PRODUCTION AND ITS ENZYMATIC HYDROLYSIS OF AGRICULTURAL WASTES

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SUMMARY

Xylanase A of *Aspergillus niger* DSM 1957 was successfully expressed in strain *Pichia pastoris* GS115/pXlnA in YP medium induced by methanol. Molecular weight of the recombinant xylanase A was 35 kDa, that was consistent with the theoretical calculation and the enzyme activity in the culture was 7310 U/mL. Maximal xylanase activity (11180 U/mL) was gained after culturing the recombinant yeast for 120 hours in the present of 1% methanol. Among of seven media (BMMY, MMY, MM, YPM, YPTM, YPTCM, and YP) utilized for the yeast culture, the highest activity of the produced recombinant xylanase A (21620 U/mL) was reached in BMMY medium, while the lowest activity (1410 U/mL) was found in YPTCM medium. At the appropriate conditions, the recombinant xylanase A activity was 2.96 folds higher than that expressed in normal conditions. The conditions for recombinant xylanase A enzymatic hydrolysis of several agricultural wastes were also investigated. The results showed that in appropriate conditions (40ºC, 24 hours, substrate concentration of 40 mg), the highest amount of reducing sugars produced from cob, rice bran and soybean meal substrates were 0.617 ± 0.002 µmol/mL, 0.663 ± 0.002 µmol/mL, and 0.814 ± 0.003 µmol/mL, respectively. Overall, with these distinctive properties, the recombinant xylanase A may initially become a potential candidate for various industrial applications.

Keywords: Activity, agricultural wastes, *Pichia pastoris* GS115/pXlnA, optimal, xylanase A

INTRODUCTION

Xylanases hydrolyze 1,4-β-D-xylosidic bonds in xylan structure of lignocelluloses biomass into xylose - a major carbon source for microbial cell metabolism. Xylanases are biosynthesized from vary microorganisms such as fungi, bacteria, yeasts, and seaweeds… (Polizeli *et al.*, 2005). While, xylanases produced by bacteria and actinomycetes (*Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp.) are active over a wide pH range of 5-9, with the optimum temperature range of 35ºC - 60ºC (Motta *et al.*, 2013). Xylanase from *Bacillus* spp. exhibits high activity at the alkaline pH and high temperature (Mandal, 2015). Fungi (*Aspergillus* sp.,

Fusarium sp., *Penicillium* sp.) are important producers of xylanase due to capable of secreting high quantity of xylanase into medium. Xylanase could be produced industrially by solid state fermentation (SSF) or submerged fermentation (SmF). Enzyme yield was much higher in SSF than in SmF (Zhao et al. 2019). However, SSF is currently only used at small extent for the production of enzymes and secondary metabolites due to several problems of technique as well as procedure. The discovery and development of new efficient xylanase have great significance for industries.

Recently, many studies over the world were conducted to clone and express xylanase from *A. niger* in *P. pastoris*. In 2006, Ping et al. successfully cloned a gene *xynB* encoding an acidophilic endo-β-1,4-xylanase from *A. niger* CGMCC1067, then successfully expressed in *P. pastoris* under control of the GAP promoter. The full gene of 745 bp includes an intron of 67 pb and encodes a protein containing 188 amino acids. However, pure recombinant xylanases appeared three bands at approximately 21, 30 and 35 kDa. The activity of recombinant enzyme reached 62 IU/mL, was higher than 50 times activity of nature enzyme from *A. niger*. Fang *et al.,* (2014) expressed xylanase GH11 from *A. niger* IA-001-xynB in *P. pastoris* using pPICZαA vector. The activity of the enzyme reached 1280 U/mL that was 19.39-fold compared to the wild xylanse. In a 10-liter bioreactor, recombinant xylanase activity reached 10035 U/mL after 114 hours.

Elgharbi *et al.* (2015) successfully cloned and expressed *XAn11* encoding β-1,4-endoxylanase from *A. niger* US368 in *P. pastoris* using pGAPZαB vector. The maximum activity of the recombinant enzyme in medium was 41 U/mL, about three times more than the activity of wild-type strain. The pure enzyme (24 kDa) had specific activity of 910 U/mg. Although the optimal condition for enzyme activity was at pH 4 and 50°C, the enzyme could be stable over a wide pH range and resistant to some detergents and organic solvents. In 2020, Long et al. cloned the xylanase GH10 gene from *A. niger* BE-2 (XynC) and expressed it in *P. pastoris* GS115. The recombinant enzyme exhibited activity of 1650 U/mL medium, and was active in a wide range of pH from 4.5 to 7.0 (optimal at pH 5.0) and at 55°C (Long *et al.,* 2020). Another study also succeeded in expression of *xylB* gene encoding xylanase family GH11 from *A. niger* BCC14405 in *P. pastoris* KM7. Recombinant XylB showed a high specific activity of 3852 U/mg and worked optimally at 45°C, pH 6.0 (Aiewviriyasakul *et al.,* 2021).

Several studies on recombinant xylanases have been published such as xylanase A (Do *et al.,* 2013), xylanase G2 from recombinant strain *P. pastoris* GS115 (Nguyen *et al.,* 2009), xylanase G from recombinant *A. niger* (Do *et al.,* 2021). With the aim to have new xylanase with high activity, stability and versatile application, the recombinant strain *P. pastoris* GS115/pPXlnA was generated using a fragment of a gene encoding xylanase A from *A. niger* DSM1957. In this study, the appropriate conditions and culture medium were investigated to improve the biosynthesis of recombinant xylanase A and evaluate the effectiveness of recombinant enzyme on the hydrolysis of agricultural wastes.

MATERIALS AND METHODS

Strains and research materials

The recombinant *P. pastoris* GS115/pXlnA strain was used for production of xylanase A from *A. niger* DSM1957. The strain was created by cloning a gene of 984 bp (code EU848304 in genebank) from *A. niger* DSM1957, encoding xylanase 327 amino acids into pPICZαA at *Eco*RI, *Xba*I then transforming the obtained plasmid into *P. pastoris* GS115 strain.

Reagents

Birchwood xylan was purchased from Biochemika, 3,5,5-dinitrosalisilic acid (DNS), SDS were supplied from Sigma-Aldrich (St. Luis, USA). Peptone, yeast extract, D-xylose from Sigma (Japan). Acrylamide from Bio Basic Canada Inc (Canada).

Media

Media used for xylanase expression in the *P. pastoris* GS115/pXlnA: YP medium (1% yeast extract, 2% peptone supplemented with 0.5% methanol); BMMY medium (1.34% YNB, 4.10^{-5} biotin, 0.1 M phosphate buffer, pH 6.0, 0.5% methanol); MMY medium (YP; 1.34% YNB, 4.10^{-5} biotin, 0.5% methanol); MM medium (1.34% YNB, 4.10^{-5} biotin, 0.5% methanol); YPM medium (YP, 0.5% methanol); YPTM medium (YPM; 0.01% triton-X 100); YPTCM medium (YPTM, 1% casamino acid)

Activation of the yeast strain

P. pastoris GS115/pXlnA was reactivated on YPD agar at 28°C for 3-5 days, then grown in liquid YPD at 28°C, 200 rpm for 16-18 hours.

Expression of recombinant XlnA in *P. pastoris*

Colonies of *P. pastoris* GS115/pPXlnA were initially cultured in 5 mL of YP medium supplemented with 0.5% glycerol, under the constant agitation at 200 rpm at 30°C for about 16 h until OD_{600} reached between 2 and 6. Then the cultures were centrifugated to harvest the cells and the cells were resuspended in 50 mL of YP medium supplemented with 0.5% (v/v) methanol to OD_{600} =1. Every 24 h, 1 mL of sample was collected and 0.5% methanol was added once to maintain induction. The expression solution was centrifugated at 8000 rpm and 4°C for 5 min to remove the cells, then precipitated 10 times with methanol for SDS-PAGE analysis on 12.5% polyacrylamide gel.

Investigation of culture conditions for high expression of recombinant XlnA

Sample collection time

To determine time point for harvesting the highest XlnA at the culture, the recombinant *P. pastoris* strain was cultivated in YPM medium, pH 7.0, shaken at 200 rpm at 28°C, sampled and induced by 0.5% (v/v) methanol every 24 hours. Cell density and enzyme activity were determined at different time points.

Inducer concentration

The recombinant strain was cultivated for expression of XlnA in liquid YP medium containing methanol at concentrations of 0.5; 1; 1.5; 2% at 28°C, in 120 hours, pH 7.0 shaken at 200 rpm under the optimized conditions of temperature. Then the cultures were collected to determine cell density and activity of xylanase.

Expression media

To select the appropriate medium for xylanase A expression, the recombinant *P. pastoris* strain was cultured in 7 different media (BMMY, MMY, MM, YPM, YPTM, YPTCM and YP). The cultures were collected to determine cell density and recombinant xylanase A activity.

Xylanase quantification

The xylanase activity was quantified by spectrophotometric determination according to Miller (Miller 1959). A mixture of 100 µL of the crude xylanase was incubated with 400 µL of 0.5% (w/v) birchwood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55ºC for 5 min. The reaction was terminated by adding 1.25 mL of DNS reagent. The mixture was then measured at the absorbance of 540 nm. D-xylose was used as standard. Under the standard assay conditions, one unit of xylanase activity was the amount of enzyme that catalyzed the hydrolysis reaction of the substrate xylan to a reducing saccharides equivalent to 1 ηmol of xylose per minute under a certain condition.

Evaluation of the hydrolysis capacity of recombinant xylanase

Evaluation of xylanase enzyme's ability to hydrolyze substrates was conducted as describing by Li et al. (Li et al. 2018): The reaction of enzyme solution (50 U) with substrates as agricultural by-products (including 40 mg of cob substrates, rice bran, soybean meal-dry powder). The agricultural straws were merged into 2 mL of 100 mM sodium phosphate buffer, pH 6.5 was occurred when being mixed well and incubated at 40°C in 24 hours. 500 µl of

this fluid was transferred into a new Eppendorf with 1250 mL of DNS and boiled for 5 minutes. For control sample, rXlnA enzyme was replacing by buffer supplementation. The amount of reducing sugars released was detected by reaction with DNS and that was measured by spectrophotometry at 540 nm.

Determination of protein content and SDS-PAGE electrophoresis

Polyacrylamide gel was used for protein electrophoresis at a concentration of 12.5% according to Laemmli (Laemmli 1970). Protein amount was determined by Bradford method (Bradford 1976).

Data analysis/Statistical analysis method

The data were statistically analyzed by MS Excel 2016. Values were expressed as ± SD (mean value, SD is standard deviation).

RESULTS AND DISCUSSION

Expression of recombinant xylanase of the strain *Pichia pastoris*

P. pastoris GS115/pXlnA were cultured in YPM medium at 28°C and 200 rpm and induced with 0.5% methanol every 24 hours. The culture supernatant was collected periodically to detect XlnA activity.

Figure 1. Analysis of xylanase activity in culture from *P. pastoris* GS115/pXlnA strain by agar diffusion test. (Cont): 100 µL pre-induced sample; 1-2: 100 µL induced samples (diluted xylanase solution at 1, 20 times, respectively).

Enzyme diffusion on agar plate was conducted to check the enzyme activity in the culture. The results showed that culture supernatant of the strain exhibited activity to hydrolyze xylan substrate to create bright zone around the wells containing culture supernatant at different dilution serials 1, 20 times (Figure 1). The xylanase activity in the culture was 7310 U/mL and the specific activity was 21200 U/mg protein after induction of 120 hours.

According to some studies in the world, when expressing enzymes in different strains, the xylanase activities are different. Expression of xylanase from *A. usamii* in *E. coli* BL21 (DE3) with enzyme activity reached 49.6 U/mL (Zhou et al. 2008). Expression of xylanase G2 in *P. pastoris* GS115 reached 41 U/mL (Nguyen Thi Thu Thuy et al. 2009). In this research, we used recombinant strain *P. pastoris* GS115/pPXlnA for xylanase biosynthesis. Nowadays, besides *E. coli*, *P. pastoris* is one of the most preferred expression systems. *Pichia* expression system has several advantages over other expression systems such as inexpensive cost, rapid expression, growing easily to high cell density and effectively secretion of recombinant protein. This strain also provides potent promoters for inducible high-level protein production (Ahmad et al. 2014). The xylanase biosynthetic gene was obtained from strain *A. niger* DSM 1957 which is an enzyme-producing fungal strain with strong xylanase activity. After culturing, enzyme activity reached 7310 U/mL culture and 21200 U/mg protein. Compared to enzyme extracts from other recombinant xylanase-producing strains with the same expression system as *P. pastoris*, this is an considerably modest result (Do et al. 2013; Liu et al. 2006; Luo et al. 2010)

Investigation of conditions for enhanced expression of recombinant xylanase activity

Effect of harvesting time

To determine the suitable time for harvesting xylanase secreted in the medium, the recombinant strain *P. pastoris* GS115/pXlnA was shaken at 200 rpm in YPM medium, pH 7.0 at 28°C, and provided 0.5% (v/v) methanol every 24 hours for 144 hours.

Table 2. Recombinant xylanase activities in the culture that were harvested at different time points.

Harvesting time (h) after induction	**Xylanase activity (U/mL)** ($\bar{X}$±SD)	**Xylanase specific activity (U/mg protein)** ($\bar{X}$±SD)
24	2070 ± 26.6	7900 ± 12.4
48	2390 ± 2.3	12780 ± 30.2
72	6370 ± 4.6	14050 ± 12.3
96	7070 ± 15.0	18640 ± 14.0
120	**7290 ± 4.5**	**20190 ± 10.8**
144	6840 ± 13.6	15060 ± 28.1

From the data in Table 2, it is clear that the highest recombinant xylanase activity (7290 U/mL) was gained after culturing the cells for 120 h. Therefore, we chose to collect the enzyme in the culture supernatant at 120 h after the induction of 0.5% methanol for the following experiments. Results on SDS-PAGE electrophoresis showed that a sharp protein band located at ~35 kDa was observed in the cultures harvested after induction of 96, 120, 144 hours (lane 2, 4, 6, 7, 9, and 10). On the other hand, the control sample from the *P. pastoris* GS115 strain did not carry the xylanase gene (lane 1, 3, 5 and 8) did not have any band at this location (Figure 2).

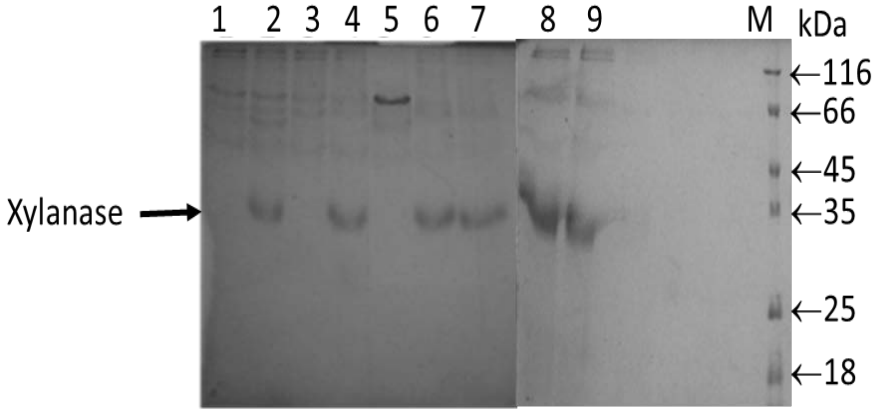

Figure 2. SDS-PAGE electrophoresis of proteins in the supernatant of cultures harvested every 24 hours. 1, 3, 5: Noninduced samples at 96h, 120h, and 144h, respectively; 2, 4, 6: Induced samples at 96h, 120h, and 144h, respectively; 7-9: Induced sample at 120 h; M: Marker (Fermentas).

Effect of methanol concentration

We further investigated the effect of methanol concentration on recombinant xylanase A expression levels. The recombinant *P. pastoris* strain GS115/pXlnA was cultured in YPM medium, pH 7.0; 28°C for 120 hours, supplemented with methanol at concentrations of 0.5, 1, 1.5, and 2%. Expression cultures were collected at the 120th hour to determine xylanase activity.

The results (Table 3) showed that at the inducing concentration of 1% methanol, the xylanase enzyme activity in the culture was the highest (11180 U/mL, 22350 U/mg protein). The SDS-PAGE electrophoresis chromatogram showed that in all post-induction samples, a dark protein band of about 35 kDa appeared that was consistent with the theoretical calculation (Figure 3).

Table 3. Xylanase activity in the cultures at different methanol concentrations.

Inducer concentration	0.5% methanol	1.0 % methanol	1.5 % methanol	2.0 % methanol
Xylanase activity (U/mL)	7260 ± 4.4	**11180 ± 21.1**	8680 ± 0.1	8150 ± 5.9
Xylanase specific activity (U/mg protein)	17030 ± 10.2	**22350 ± 23.2**	17270 ± 0.21	20820 ± 32.3

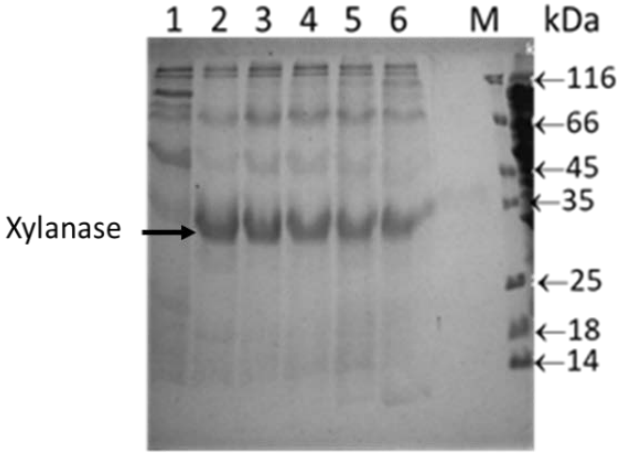

Figure 3. SDS-PAGE electrophoresis of proteins in the supernatant of cultures induced with different methanol concentrations. 1: Pre-induced sample; 2-4: 0.5%, 1%, 1.5% methanol respectively; 5-6: 2.0% methanol; M: Marker (Fermentas).

Effect of culture media

To select the appropriate medium for recombinant xylanase expression, the recombinant *P. pastoris* GS115/pXlnA strain was cultured in 7 different media (BMMY, MMY, MM, YPM, YPTM, YPTCM and YP).

The results (Table 4) showed that the BMMY medium gave the highest xylanase activity (25646 U/mL) and also cell mass (102.9 mg/mL). This medium was supplemented with an important vitamin as biotin aiming to raise the cell growth rate. This may be a reason for increasing recombinant xylanase production.

Table 4. Xylanase activity in different culture media containing 1% methanol for 120 h.

Medium	Cell mass (mg/mL)	Xylanase activity (U/mL)	Protein concentration (mg/mL)	Specific xylanase activity (U/mg)
BMMY	102.9 ± 1.98	21620 ± 12.1	0.843	25646 ± 0.14
YPM	48.7 ± 1.45	9600 ± 15.3	0.377	25464 ± 0.39
MMY	74.2 ± 1.22	14010 ± 9.6	0.625	22416 ± 0.14
MM	12.2 ± 0.32	1800 ± 2.9	0.104	17307.7 ± 1.15
YPCTM	15.6 ± 1.09	1410 ± 3.2	0.208	6778.8 ± 17.2
YPTM	9.5 ± 0.91	3210 ± 4.01	0.216	14861 ± 10.1
YP	47.62 ± 1.72	7290 ± 5.09	0.361	20194 ± 22.3

Evaluation of the hydrolysis capacity of recombinant xylanase A

After examining the conditions to improve the biosynthesis of recombinant xylanase from the strain *P. pastoris* GS115/pXlnA, the strain was cultivated in BMMY medium, induced by 1% methanol for 120 h. Fermentation solution was centrifuged at 10,000 rpm for 10 minutes, removed the precipitate, and collected the extracellular extract. This enzyme solution will be used to evaluate the ability to hydrolysis agricultural by-products. In order to determine the optimal incubating temperature for the recombinant xylanase enzymatic hydrolysis, the reactions were carried out at a temperature range of 30-45ºC. The reducing sugars released in samples were determined using method of Li *et al.* (2018)..

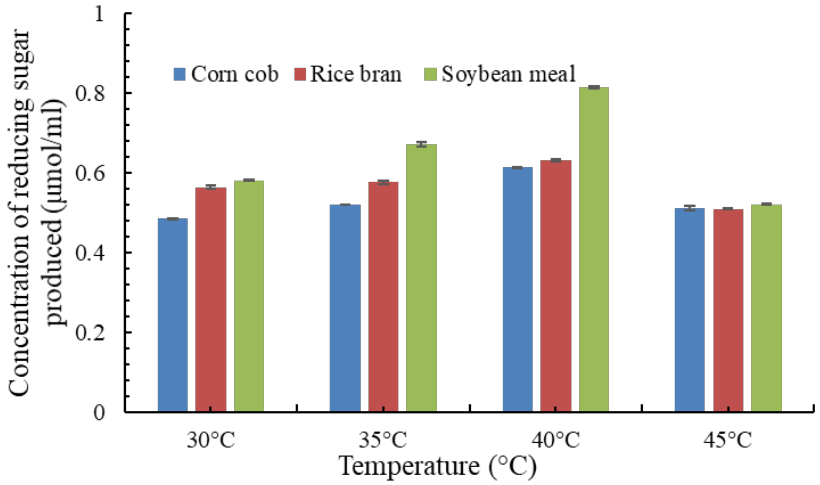

Figure 4. The effect of temperature on the hydrolysis of agricultural by-products by the recombinant xylanase after incubation time of 24 hours

The bar chart illustrates that the highest amount of reducing sugars was at a temperature of 40ºC, with values of 0.617±0.002 µmol/mL, 0.663±0.002 µmol/mL, and 0.814±0.003 µmol/mL respectively, corresponding with corn cob, rice bran, soybean meal (Figure 4). Therefore, an incubation temperature of 40ºC was chosen to conduct the experiment with the effect of incubation time on the ability to hydrolyze the substrates of xylanase.

According to several studies, Li et al. (Li et al. 2018) conducted a temperature effect study on recombinant xylanases from *Lentinula edodes* (rLeXyn11A) expressed in *Pichia pastoris* at a range of temperature as: 10, 20, 30, 40, 50, 60, 70, 80 and 90ºC. The research demonstrated that 40 ºC is optimal for recombinant xylanase (Li et al. 2018). In addition, Nguyen Thi Kim Thu (Nguyen, 2020) studied on recombinant xylanase from *A. niger* VTCC017/pANXlnG2 expressed in *P. pastoris* which hydrolyzed substrates of bagasse meal, wood pulp, and xylan. The experiment investigated on a range of temperature from 37 to 70ºC showed that 55ºC was the optimal temperature. In our experiment, the obtained temperature of 40°C is the most optimal. This may explain that the enzyme is biosynthesized in different strains, the hydrolysis of the substrates is different.

CONCLUSION

In this study xylanase A derived from *A. niger* DSM1957 was expressed successfully in *P. pastoris* GS115. The xylanase activity reached 7310 U/mL in YP medium supplemented with 0.5% methanol after induction of 120 hours. In 7 particular culture media, BMMY medium gave the highest ability to produce xylanase after the induction of 1% methanol, at 120 hours. The xylanase activity reached 21620 U/mL,

increased 2.96 fold compared to that of the original YPM medium. The xylanase exhibited significantly the hydrolysis of several agricultural wastes. Therefore, the reducing sugars content produced was 0.617 ± 0.002 μmol/mL, 0.663 ± 0.002 μmol/mL, and 0.814 ± 0.003 μmol/mL corresponding with the cob, rice bran and soybean meal substrates, respectively.

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