Advances in Biological Sciences Research, volume 15
Proceedings of the 10th International Seminar and 12th Congress of Indonesian Society for Microbiology (ISISM 2019)

Modification and Optimization of Low-cost Medium for Recombinant Alkalothermophilic Xylanase Production from *Pichia pastoris* KM71

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ABSTRACT
Xylanase is an enzyme that can degrade xylan into xylooligosaccharides by cleaving to 1, 4-β-D-xylosidic bonds and has high potential in industrial applications. In the previous study, recombinant *Pichia pastoris* via pPICZ-alpha vector has been constructed, and the yeast produced xylanase originally came from *Bacillus halodurans* CM1. Recombinant xylanase production from *Pichia pastoris* using standard medium has been conducted. However, if a larger scale has to be done using this standard medium, it will not be feasible, due to the high cost of the standard medium. Therefore, in this study, the composition of standard media was substituted with a low-cost substrate. Modification of production medium was conducted by replacing pure glycerol with technical glycerol as carbon source, while peptone and yeast extract as organic nitrogen source were substituted with soybean hydrolysate (18% (w/v) total N content) and rice bran (14.63% (w/v) total N content), respectively, and ammonium sulphate as an additional source of inorganic nitrogen. Use of technical glycerol 1% (v/v) and a mixture of 15 g/100 mL soybean hydrolysate, rice bran hydrolysate 30g /100 mL, and 2.5% (w/v) ammonium sulphate were found to be the most suitable medium that gave high volumetric activity (1383.9 U/mL), specific activity (861.7 U/mg), protein concentration (1.606 mg/mL), and dry cell weight (43.3g/L).

Keywords: *Xylanase, Pichia pastoris, Recombinant.*

1. INTRODUCTION

Xylanase is the most important enzyme in the hydrolysis process of polysaccharide xylan [1], which is one of the main components of hemicelluloses and is known as the second most abundant renewable resource after cellulose [2]. Because of its remarkable ability to hydrolyze xylan and its extensive use, the industrial demand for xylanase is increasing [3]. Therefore, industrial-scale of xylanase production is required to meet the demands of the industrial market.

Center for Bioindustry Technology, Agency for The Assessment and Application of Technology (BPPT), has succeeded in isolating *Bacillus halodurans* CM1 strain and producing alkalothermophilic xylanase from it [4, 5]. They also succeeded in the cloning of alkalothermophilic endoxylanase gene and carrying out the production of the recombinant xylanase using *Pichia pastoris* KM71 yeast as an expression cell. However, the composition of the media used in this experiment is costly [6].

The cost of cultivation media in the production of recombinant proteins from *P. pastoris* is the main factor that needs to be considered and optimized for larger-scale production [7]. Hence, it is interesting to formulate a suitable culture medium using low-cost raw materials. Our previous study has reported the development of an inoculum to increase enzyme production in *P. pastoris* KM71 using basal salts medium (BSM) medium as a culture medium (unpublished data). The results showed

that recombinant enzymes produced in flask shaken at 30°C and pH 5.0 showed relatively low activities (19 ± 0.039 U/mL) (unpublished data). In other reports, the medium modification showed an increase of enzyme activity from 118 to 204 U/mL at the flask scale [8]. The growth and recombinant protein production level in recombinant *P. pastoris* must depend on various conditions and composition of the culture medium [9].

At an industrial scale, the cost of enzyme production depends on the cost of growth and production medium used in the process [9]. Previous studies have reported about the fermentation using raw glycerol as substitution for pure glycerol that can be metabolized by yeast strains [10]. To minimize the medium costs, the crude glycerol has been used as a carbon source in *P. pastoris* culture medium to increase the cell concentration in the growth phase [11, 12]. Furthermore, soybean meal and rice bran have been used as organic nitrogen sources because they provide rich nutrients sources but inexpensive costs [12, 13].

This study is aimed at determining the suitability of a medium containing crude glycerol, soybean meal, rice bran, and ammonium sulphate for substituting a medium with high purity commercial glycerol, peptone, and yeast extract, respectively, for production of recombinant xylanase by *Pichia pastoris*.

2. MATERIAL AND METHODS

2.1 Microbial strain

The genetically engineered *Pichia pastoris* KM71 clone 3E which is transformed by the pPICZαA plasmid containing the *Bacillus halodurans* CM1 alkalothermophilic xylanase gene under methanol inducible *AOX1* promoter was provided by Center for Bioindustrial Technology, Agency for The Assessment and Application of Technology, LAPTIAB-BPPT, Pusiptek-Serpong, Tangerang Selatan [6]. The recombinant yeast was maintained on YPD (2% (w/v) peptone, 1% (w/v) yeast extract, 10% (w/v) glucose and zeocin) agar. The expression of the xylanase as a secretory product was controlled under the methanol inducible *AOX1* promoter and repressed by glycerol.

2.2 Preparation of standard medium

The standard medium for starter was BMGY (2% (w/v) peptone, 1% (w/v) yeast extract, and 1% (v/v) glycerol). This mixture was sterilized by autoclaving at 121ºC for 15 minutes, then cooled down and yeast nitrogen base (YNB), and 100 mM potassium phosphate buffer pH 6 were added. For xylanase production BMMY medium (2% (v/v) peptone and 1% (v/v) yeast extract) was used, where the same procedure as BMGY was conducted. Induction to express the xylanase gene was done by replacing glycerol with 2% (v/v) methanol. This methanol concentration was used for induction in all experiments.

2.3 Preparation of crude glycerol

The crude glycerol (75% (v/v) was sterilized first by autoclaving at 121ºC for 15 minutes. Then the crude glycerol was added to the medium accordingly to give the desired final glycerol concentration (v/v) ignoring the impurities. The crude glycerol was adjusted to pH 7.0 prior to addition to the culture medium.

2.4 Preparation of soybean meal and rice bran hydrolysates

Soybean meal and rice bran were obtained from Sleman regency, Yogyakarta, Indonesia, and separately treated in 1 N sulfuric acid (H_2SO_4) at 33% (w/v). Each mixture was autoclaved at 121°C for 40 min, cooled down. The distilled water added to the required volume as well as 10 N NaOH to set the pH to 7.0. The suspension was then filtered and the filtrate stored at 4°C until use. The total nitrogen content was analyzed by the Kjeldahl method.

2.5 Medium optimization

The effect of nutrient composition, in terms of the concentration of crude glycerol, soybean meal, rice bran hydrolysates, and ammonium sulfate, on the growth and production of recombinant xylanase by *P.pastoris* was investigated in that order as follows.

For investigation of the best concentration of crude glycerol for the initial growth of *P. Pastoris* KM71 clone 3E, the concentration was varied in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, and 1% glycerol (v/v)) by substitution of the pure glycerol with the crude glycerol at a final glycerol variations concentrations of 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5% (v/v). *P. pastoris* was cultured on each of these media (50 mL) in a 250 mL baffled flask at 30°C with 250 rpm agitation for 21-24 h until the optical density values (OD_{600}) reached 11-14 OD/mL, and then of the yeast dry cell weight (DCW) was measured.

For xylanase production by this recombinant *P. pastoris*, the yeast was first grown in the optimum crude glycerol concentration in BMGY medium (BMcGY), harvested by centrifugation (6000 rpm, 10 min), washed in sterilized water and then resuspended and transferred into 100 mL BMMY medium (as per BMGY) for induction of the *AOX-1* promoter. The cell suspension was then grown in 250-mL baffled flasks at 30°C with shaking at 250 rpm for 96 h. For each selected methanol concentration, the same amount (final concentration) of fresh methanol was added to the culture each day (0, 24, 48 and 72 h). At 12, 24 h and then every 24 h thereafter a 1 mL aliquot of the cell suspension was removed to

Table 1. Effect of crude glycerol concentration as a substitute for pure glycerol in BMGY media.

Crude glyserol concentration (v/v)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Dry cell weight (g/L)
0%	217.727 (± 11.404)	0.837	260.127	14.600
0.5%	1526.102 (± 94.084)	1.290	1183.025	20.400
1%	4137.611 (± 39.915)	1.633	2533.748	37.800
1.5%	4027.538 (± 11.974)	1.594	2526.687	28.200
2%	3350.167 (± 91.803)	1.506	2224.546	27.600
2.5%	3437.661 (± 87.812)	1.550	2217.846	42.200

Table 2. Effect of soybean meal hydrolysate as a substitute for peptone in BMGY and BMMY media.

Soybean meal hydrolysate (g/100mL)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Dry cell weight (g/L)
0	155.449(± 1.595)	0.773	201.098	31.8
10	2272.160 (± 62.699)	1.358	1673.167	32.4
15	3002.577 (± 21.945)	1.537	1953.531	40.6
23.2	2758.181 (± 27.431)	1.530	1802.733	33.2
30	2610.768 (± 5.486)	1.442	1810.519	38.2
35	2645.682 (± 32.917)	1.496	1768.504	40.1
40	2560.337 (± 10.972)	1.461	1752.455	36.9

ascertain the extracellular xylanase activity. After 72 h, the fermentation was stopped, and the culture was sampled and determined for yeast DCW, protein concentration, enzyme activity, and specific activity.

The substitution effect of peptone with soybean meal hydrolysate (at 0, 10, 15, 23, 30, 35 and 40 (w/v)) on *P. pastoris* growth in the BMcGY medium with optimal glycerol concentration, and on the growth of cells and xylanase production level in BMMY medium was then evaluated as mentioned above. As organic nitrogen source, the replacement of both peptone and yeast extract in the BMGY and BMMY media with a combination of soybean meal and rice bran hydrolysates were then evaluated, but using the optimal soybean hydrolysate level and varying the rice bran hydrolysate at 0, 10, 15, 20, 25, 30, 35, and 40 (w/v) in place of the yeast extract. Finally, the optimal modified BMcGY and BMMY media were supplemented with ammonium sulphate at 0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) as an inorganic nitrogen source for the recombinant *P. pastoris* growth and xylanase production.

2.6 Enzyme assay

The evaluation of the extracellular xylanase activity in the culture supernatant assay was performed by monitoring the hydrolysis of xylan as the total reducing sugar, evaluated using the dinitrosalicylic acid (DNS) method [14]. Each reaction contained 450 µL of 2% (w/v) oat spelt xylan in 0.05M Tris-HCl buffer (pH 9.0) and 50µL of the diluted test enzyme solution. The reaction was incubated at 60°C for 5 min and then 750 µL of DNS reagent was added, incubated in a boiling water bath for 5 min, and then cooled. Thereafter, the absorbance was measured at 540 nm to estimate the quantity of reducing sugars produced in the assay. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose (as reducing sugar) per minute under the above assay conditions [15]. Xylose (0-2 mg/mL) was used as calibration curve.

2.7 Measurement of protein concentration

The protein concentration was determined by the Bradford method (1976) [16]. A total of 20 µL of the crude enzyme was reacted with 1 mL of a solution of

Bradford reagent. The mixture was homogenized with vortex and incubated at room temperature for 15 minutes and measuring the absorbance at 595 nm. Bovine serum albumin (0-1 mg/mL) was used as a standard.

2.8 Determination of yeast cell concentration

The yeast DCW was determined by centrifugation (6000 rpm for 5 min) of 5 mL of the culture broth, washing the cells with distilled water, and then drying at 105°C for 24 h. DCW was then calculated from the obtained mass (g/L). Moreover, the concentration of the cell yeast was estimated in terms of the optical density (OD) of the suspension at 600 nm (OD_{600}).

3. RESULT AND DISCUSSION

In this study, an attempt to reduce the cost of cultivation media was evaluated to obtain efficient

Table 3. Effect of rice bran hydrolysate as substituted for yeast extract in BMGY and BMMY media

Rice bran hydrolysate (g/100mL)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Dry cell weight (g/L)
0	17.908 (± 0.798)	0.418	42.841	12.9
10	133.638 (± 10.902)	0.508	263.067	23.2
15	136.608 (± 19.319)	0.514	265.774	27.7
20	242.331 (± 17.639)	0.652	371.673	30.2
24,9	283.313 (± 12.600)	0.723	391.858	31.5
30	700.858 (± 12.600)	1.060	661.187	38.3
35	614.736 (± 8.400)	0.961	639.683	32.6
40	343.005 (± 2.100)	0.794	431.996	36.9

xylanase production by *Pichia pastoris*. The main effect of the low-cost media (carbon and nitrogen sources) on the growth and enzyme production was observed [17].

3.1 Cultivation of Pichia pastoris KM71 in BMGY medium utilizing crude glycerol to replace commercially pure glycerol

Glycerol is commonly used as the main carbon source that provides energy to increase the production of *Pichia pastoris*' metabolite [18]. By utilizing crude glycerol in growth and production media, the highest level of enzyme activity was obtained at 1% (v/v) glycerol concentration, and 37.8% dry cell weight (DCW), with a specific activity of 2533.748 U/mg (Table 1). The concentrations below and above 1% show that xylanase production was not optimal, as previously reported that the crude glycerol concentration above or below 1% (v/v) resulted in lower yeast yields [12]. This happened because glycerol is needed as a carbon source in a certain amount to optimally stimulate cell growth and xylanase production. Based on Invitrogen (2010) [19], the optimal concentration of glycerol is needed at a concentration of 1%. When more than 1%, it will make the cells, unable to capture carbon as a source of nutrition, so it is no longer able to secrete xylanase optimally. Thus, *P. pastoris* was initially inoculated in BMGY medium with 1% (v/v) crude glycerol replacing commercial glycerol (hereafter referred to as BMcGY). Also, the economic saving would still be obtained since, as compared to the pure glycerol (US$155.92 per kg), the crude glycerol was cheaper (US$2 per kg). In many industrial fermentations, crude glycerol has been used as the carbon source [20]. For biotechnological applications, it may make it economically feasible as a cheaper carbon source.

3.2 Growth of recombinant Pichia pastoris **KM71** *and production of xylanase in BMGY and BMMY medium utilizing organic nitrogen sources*

Nitrogen sources play an important role in microorganism growth and metabolite production [18]. Based on the Kjeldahl method, the total nitrogen content of soybean meal and rice bran hydrolysates was 0.84% and 0.65% (w/v), respectively; whereas that of peptone and yeast extracts were 18% and 14.63% (w/v), respectively.

3.2.1 *Soybean meal hydrolysate replacement for peptone.*

Soybean meal is considered to be one of the most nutritious plants because of its protein content [21]. However, macromolecular proteins need to be further degraded into small molecular peptides and even become amino acids before they are absorbed and utilized by microbes. Therefore, the hydrolysis process is needed to reach amino acids and absorbable peptides [22]. Utilizing the soybean hydrolysate as a substitute for peptone in BMcGY and BMMY medium was able to support the growth of P. pastoris [13], with an increasing yeast yield being obtained with an increasing soybean meal hydrolysate concentration up to a maximum yeast yield and xylanase production at 15 g/100 mL soybean hydrolysate (Table 2). This is comparable with enzyme activity, protein content, specific activity, and dry cell weight (DCW) obtained with the peptone containing BMcGY medium. Therefore, the medium both BMcGY and BMMY were modified to contain 15 g/100 mL soybean meal hydrolysate in place of peptone (hereafter, we designated as BMcGYS and BMMYS, respectively).

The modified medium containing soybean meal hydrolysate (BMcGYS) used for P. pastoris starter cultivation, and BMMYS medium for xylanase production, after induction with methanol, could support xylanase production, with a maximum level (3002.577 U/mL or 1953.531 U/mg) (Tabel 2). The standard medium gave 5823.801 U/mL or 3324.42 U/mg xylanase activity. Although the activity was less than that of standard, however, they are comparable in the same digit. Therefore, soybean meal hydrolysate has the potential to replace peptone.

Table 4. Effect of ammonium sulphate concentration in BMGY and BMMY media.

Ammonium sulphate concentration (v/v)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Dry cell weight (g/L)
0.0%	289.846 (± 20.159)	0.498	582.021	17.1
0.1%	293.410 (± 15.119)	0.522	562.088	21.8
0.5%	281.531 (± 11.760)	0.514	547.726	22.5
1.0%	301.725 (± 10.080)	0.639	472.184	24.8
1.5%	565.438 (± 18.479)	1.033	547.375	35.9
2.0%	808.957 (± 18.479)	1.526	530.116	38.1
2.5%	1383.898 (± 8.400)	1.606	861.705	43.3
3.0%	939.625 (± 5.040)	1.573	597.346	43.7

3.2.2 Rice bran hydrolysate to replace yeast extract.

The rice bran hydrolyzate used as a substitution for yeast extract in BMcGY and BMMY, and we named the medium as BMcGYSR and BMMYSR. A report described that these mediums were able to support the growth of P. pastoris [13]. In our study, the optimal concentration of rice bran hydrolysate concentration that gave maximal xylanase activity (700.858 U/mL) was at 30 g/100 mL (Table 3).

Although the xylanase activity using this medium was less than that of using the standard medium, but still the possibility of financial savings was obtained. Thus, the hydrolysate mixture of soybean meal and rice bran can be used as a low-cost nitrogen source for enzyme production.

3.3 Growth of Pichia pastoris KM71 *and xylanase production in BMGY and BMMY medium using inorganic nitrogen sources*

Supplementing ammonium sulphate in the BMcGYSR and BMMYSR medium, as an inorganic nitrogen source was able to increase the enzyme activity, protein content, dry cell weight (DCW), and specific activity. The optimal ammonium sulphate concentration that gave maximal xylanase activity was 2.5% (w/v) (Table 4). The BMcGYSR and BMMYSR supplemented by ammonium sulphate were named as BMcGYSRA and BMMYSRA, respectively.

The modified BMcGYSRA and BMMYSRA media could increase yeast growth, xylanase activity, and protein content compared to media without ammonium sulfate. A study reported that ammonium salt is a good source of nitrogen for yeast growth, hence fermentation media is often equipped with inexpensive inorganic nitrogen, such as ammonium sulfate. However, to increase yeast growth, it is not enough if only ammonium salt is used, because cells need growth factors such as amino acids, vitamins, and other nutrients that have specific roles in catalytic or structural reactions [23].

4. CONCLUSION

A low-cost medium for recombinant xylanase production from recombinant *Pichia pastoris* KM71 was proposed. The 1% (v/v) crude glycerol, 15 g/100 mL (w/v) soybean meal, and 30 g/100 mL (w/v) rice bran hydrolysates were investigated to replace the 1% (v/v) pure glycerol, 2% (w/v) peptone, and 1% (w/v) yeast extract in standard BMGY and BMMY media, respectively. Supplementation of 2.5% (w/v) ammonium sulphate in this low medium increased xylanase activity. The media will provide a feasible economic advantage of recombinant enzymes by *Pichia pastoris* for the industrial-scale production, however, still, the further experiment related to the optimization of fermentor physical condition for production must also be conducted.

ACKNOWLEDGMENTS

This work was funded by the INSINAS Research Incentives Program of Indonesia Ministry of Research, Technology, and Higher Education in the year 2019-2020 granted to IH. The authors thank Lina Mulyawati and Haniyya for their technical assistance.

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