XYLANASE PRODUCTION BY Penicillium oxalicum T3.3 USING RICE STRAW AS SUBSTRATE FOR BIOBLEACHING OF BAMBOO PULPS

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By

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Chairman : Associate Professor Umi Kalsom bt. Md Shah, PhD
Faculty : Biotechnology and Biomolecular Sciences

Recently, xylanase has raised high attention in pulp and paper industry due to their bleach-boosting properties, which assist bleaching process. Xylanase can improve the performance of pulp. It is a hydrolytic enzyme that can degrade the linear polysaccharide beta-1,4-xylan into xylose. Thus, the aims of this research are to select potential xylanase-producing fungi with enhance xylanase production and to evaluate the performance of the xylanase in assisting the biobleaching process of bamboo pulps. Four fungi species namely *Penicillium oxalicum* T3.3, *Aspergillus niger* ATCC 6275, *Colletotrichum gliosporoides* and *Pycnoporus sanguineus* were screened for xylanase production based on the observation of clear zone formation on Malt Extract Agar (MEA) containing xylan. *P. oxalicum* T3.3 and *A. niger* ATCC 6275 showed larger clear zone formation on the agar plate. These fungi were grown in shake flask by submerged fermentation containing rice straw as substrate. *P. oxalicum* T3.3 showed highest xylanase activity (65.89 U/mL) with lowest carboxymethyl cellulase (CMCase) (1.88 U/mL) and filter paperase (FPase) activity (0.16 U/mL) after 4 days fermentation at 30°C. The cultural conditions for xylanase production by *P. oxalicum* T3.3 was investigated under various concentration of rice straw, initial pH, temperature, agitation speed, nitrogen sources and additional of surfactants. The best cultural conditions was determined by *one-factor-at-a-time* method. By optimizing rice straw concentration at 1% (w/v), initial pH at 6, temperature 35°C, agitation speed at 150 rpm, using yeast extract as the nitrogen source, and addition of Tween 80 as surfactant, the highest xylanase production of 79.12 U/mL was obtained. That is 20% improvement compared to that before optimization. The xylanase was found more stable at pH range of 4.0 to 7.0 and temperature from 45°C to 55°C. The enzyme retained 72% and 51% of its activity after incubation for 2 h and 4 h, respectively at 50°C and pH 6. At -80°C, xylanase was still above its half life for 2 weeks. Biobleaching of bamboo pulps with xylanase was most effective at an enzyme dose of 5 U/g oven dried pulp at pH 7 and incubated in water bath at 50°C. Biobleaching process increased paper brightness by

7.51 points at optimum conditions. Results of chemical composition of biobleached bamboo pulps revealed that lignin content reduced by 25% with only slightly reduction of holocellulose and α-cellulose content by 0.5% and 12% compared to chemically bleached bamboo pulp. Thus, cellulase-poor xylanase produced from *P. oxalicum* T3.3 grown on rice straw has high potential for biobleaching application in pulp and paper industry in terms of technical and biological performance and economical aspects.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN XYLANASE OLEH *Penicillium oxalicum* T3.3 MENGGUNAKAN JERAMI PADI SEBAGAI SUBSTRAT UNTUK AGEN BIOPELUNTURAN PULPA BULUH

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Baru-baru ini, xilanase menarik perhatian industri pulpa dan kertas disebabkan oleh ciri-ciri meransang peluntur, yang mana boleh membantu proses meluntur. Xilanase boleh meningkatkan prestasi pulpa. Ianya adalah enzim hidrolitik yang mana boleh menguraikan polisakarida linear beta-1,4-xylan kepada xylosa. Oleh itu, tujuan kajian ini adalah untuk memilih kulat berpotensi sebagai penghasil xilanase, menentukan keadaan kultur terbaik yang meningkatkan penghasilan xilanase dan untuk menilai prestasi xilanase dalam membantu biopelunturan pulpa buluh. Empat spesis kulat iaitu *Penicillium oxalicum* T3.3, *Aspergillus niger* ATCC 6275, *Colletotrichum gliosporoides* dan *Pycnoporus sanguineus* telah disaring untuk penghasilan xilanase berdasarkan pemerhatian pembentukan zon jelas di atas Agar Ekstrak Malt (MEA) yang mengandungi xylan. *P. oxalicum* T3.3 and *A. niger* ATCC 6275 menunjukkan pembentukan zon jelas lebih besar di atas agar. Kulat ini dihidupkan dalam kultur cecair mengandungi jerami padi sebagai substrat. *P. oxalicum* T3.3 menunjukkan aktiviti xilanase paling tinggi (65.89 U/mL) dengan paling rendah carboxymethyl selulase with (CMCase) (1.88 U/mL) dan filter paperase (FPase) activity (0.16 U/mL) selepas 4 hari fermentasi pada 30°C. Keadaan kultur penghasilan xilanase oleh *P. oxalicum* T3.3 dikaji di bawah pelbagai kepekatan jerami padi, pH awal, suhu, kelajuan goncangan, sumber nitrogen dan penambahan surfaktan. Penentuan keadaan kultur dilakukan dengan kaedah satu faktor pada satu masa. Dengan mengoptimumkan kepekatan substrat pada 1% dan pH awal pada 6, suhu 35°C , kelajuan gonjangan pada 150 rpm, ekstrak yis sebagai sumber nitrogen, dan penambahan Tween 80 sebagai surfaktan, xilanase yang tinggi dihasilkan sebanyak 79.12 U/mL. Oleh itu, 20% penambahan berbanding sebelum pengoptimuman. Xilanase dicirikan dan didapati lebih stabil pada julat pH 4.0 hingga 7.0 dan suhu dari 45°C hingga 55°C. Enzim boleh mengekalkan 72% dan 51% aktivitinya selepas 2 dan 4 jam inkubasi pada 50°C dan pH 6. Pada suhu -80°C, xilanase masih di atas separuh hayat untuk 2 minggu. Biopelunturan pulpa buluh dengan xylanase adalah paling berkesan pada dos enzim 5 U/g ketuhar kering pulpa, pada pH 7 and inkubasi dalam air pada 50°C.

Di bawah keadaan yang optimum, proses biopelunturan meningkatkan kecerahan kertas sebanyak 7.51 mata. Keputusan komposisi kimia pulpa buluh biopelunturan menunjukkan kandungan lignin berkurang sebanyak 25% dengan hanya sedikit pengurangan kandungan holoselulosa dan alpha-selulosa sebanyak 0.5% dan 12% berbanding pulpa pelunturan. Oleh itu xilanase rendah selulase dihasilkan oleh *P. oxalicum* T3.3 ditumbuhkankan atas jerami padi mempunyai potensi besar dalam aplikasi biopelunturan dalam perusahaan pulpa dan kertas dari segi teknikal dan prestasi biologikal serta aspek ekonomi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CAZY	Carbohydrate-Active Enzyme
CE	Carbohydrate Esterase
CMC	Carboxymethycellulose
CMCase	Carboxymethycellulase
CTMP	Chemi-Thermo-Mechanical Pulping
DNS	Dinitrosalicylic Acid
E.C.	European Commission
FOA	Food and Agriculture Organization
FPase	Filter Paperase
GH	Glycoside Hydrolysis
ISO	International Organization for Standardization
LSD	Least Significant difference
MADA	Muda Agriculture Development Authority
MARDI	Malaysian Agricultural Research and Development Institute
MEA	Malt extract Agar
NAG	N-acetylglucosamine
ORF	Open Reading Frame
PDA	Potato Dextrose Agar
PGW	Pressure Groundwood Pulping
pI	Isoelectric Point
RSM	Response Surface Methodology
SAS	Statistical Analysis Software
SD	Standard Deviation
TAPPI	Technical Association of the Pulp and Paper Industry
TIM-barrel	Triosephosphate Isomerase-barrel
TMP	Thermo-mechanical Pulping
UPM	Universiti Putra Malaysia
USDA	United States Department of Agriculture

LIST OF SYMBOLS

%	Percentage
:	Ratio
<	Less than
>	More than
°C	Degree celcius
µg/mL	Microgram/mililiter
µL	Microliter
$C_6H_5O_7Na_3.2H_2O$	Sodium citrate
cm	Centimeter
CO_2	Carbon dioxide
$CoCl_2$	Cobalt chloride
$FeSO_4.7H_20$	Ferrous sulphate heptahydrate
g	Gram
g	Gravity
h	Hour
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
IU/g	Unit/gram
IU/mL	Unit/mililiter
kDa	Kilodalton
L	Liter
M	Molar
Mg/mL	Miligram/mililiter
mL	Mililiter
mM	Milimolar
mM	Milimolar
$MnSO_4.H_2O$	Zinc sulphate heptahydrate
N	Normality
$Na_2HPO_4.7H_2O$	Dibasic sodium phosphate
NaCl	Sodium chloride

$NaClO_2$	Sodium chlorite
NaOH	Sodium hydroxide
Nm	Nanometer
Rpm	Revolution per minute
U/o.d.	Unit/oven dry
v/v	Volume/volume
w/v	Weight/volume
β	Beta

CHAPTER 1

INTRODUCTION

Nowadays the applications of xylanase have been diversified from textile to the pulp and paper industry (Dedhia et al., 2014). In the pulp and paper industry, large amounts of chlorine-based chemicals was used during bleaching process which are toxic, mutagenic and persistent. The chlorinated organic compound and absorble organic halides are produced when chlorine reacted with lignin, thus contributed as the major sources of environmental pollution (Yeasmin et al., 2011). Owing to this problem, public awareness has increased which forced the goverment to put stringent regulation concerning pulp and paper effluents (Dedhia et al., 2014). Hence, an environmental friendly technology is a need in the paper industry. In 1986, biobleaching which is basic idea of enzyme-aided bleaching was published. The usage of xylanase to treat pulp prior to bleach enhances the bleaching process by degrading the xylan present in the wood pulp and ease the release of lignin (Sharma et al., 2014). According to Boruah et al., (2015), pretreatment by using xylanase from *P.meleagrinum* can reduced lignin content, small amount of cellulose and increased the brightness of bamboo pulp. There are several conditions to ensure the successful of biobleaching, which is xylanase enzyme must be able to withstand high temperature and alkaline conditions. Besides, xylanase is preferably contain low or free-cellulase because high cellulase resulted in the lost of cellulose, degrade the quality of pulp and increase the cost of effluent treatment (Bhakyaraj, 2014).

Xylanase is an extracellular enzyme which is the main xylanolytic enzyme in degradation of β-1, 4 backbone of xylan in hemicellulose component of plant cell wall (Hatanaka, 2012). Degradation of xylan produced small fragments such as mono-, di- and trisaccharides of β-D-xylopyranosyl that are used by microorganism as main carbon source (Kanimozhi & Nagalakshmi, 2014). Different types of miroorganisms can produced xylanase enzyme such as bacteria, actinomyces and fungi except the mammals (Chandra et al., 2012). However, the microbial xylanase was preferred in xylanase production compared to other producers, because they meet industrial application (Goswami & Rawat, 2015). This is because, microbial sources are easy to handle for large production, has short generation time, are structurally stable, easy genetic manipulation and their availability (Sindhu et al., 2011). Due to high production level and extracellular secretion, filamentous fungi usually were used in industrial application (Selvarajan & Veena, 2017). There are many fungi producing xylanase such as *Aspergillus*, *Trichoderma*, and *Penicillium* (Burlacu et al., 2016). Thus, in this study *Penicillium oxalicum* T3.3, *Aspergillus niger* ATCC 6275, *Colletotrichum gliosporoides*, and *Pcynoporus sanguineus* were evaluated for xylanase production for application in biobleaching of bamboo pulp.

Due to inducible characteristic, xylanase requires inducer for maximum production of xylanase activity. In submerged fermentation, xylanase was induced by various sources of xylan (Pandey et al., 2014). Thus, when fungi are grown in medium containing xylan as carbon source, the production of xylanase also increased. The sources of xylan can be rice straw, beechwood xylan, and sugarcane bagasse. The level of xylanase production is different depend on the sources of xylanase (Motta et al., 2013). In Malaysia, 2.7 million tonnes of rice was produced and the ratio to rice straw is 1:1 (FOA 2015; MADA 2010). Besides the rice straw, Malaysia also produced a lot of oil palm trunk. The trunk's rich cellulose and hemicellulose content are promising feed stock for fermenting sugars (Ang et al., 2013). The rice straw were used as the source of the xylan in xylanase production in order to reduce the environment pollution and production cost.

From our finding, there are few research on the best cultural conditions for xylanase production by *Penicillium oxalicum* using submerged fermentation. Thus a study on the effect of cultural conditions such as concentration of rice straw, initial pH, temperature, agitation speed, nitrogen sources and additional of surfactants are important consideration to determine the best cultural condition for xylanase production. The most important aspects to reduce the production cost are optimization of media and process condition. The conventional methods of optimization included changing one parameter and fixing the others parameter at certain level (Dedhia et al., 2014). Optimization of *one-factor-at-a-time* techniques were used in submerged fermentation to produce high xylanase activity.

Xylanase is widespread in the market, but most widely available xylanases cannot withstand with the harsh conditions during industrial operation. In biobleaching technology, although xylanase from bacteria have higher values in this application compared to fungi, but they have low level enzyme activity. Thus, fungi are more preferable as producer when the crude enzyme were applied in application due to high level enzyme production (Selvarajan & Veena, 2017). Therefore, the aim of this research is to identify the potential of cellulase poor xylanase production by selected fungi using as rice straw, as an economical substrate to reduce the production cost and to evaluate the effectiveness of crude xylanase in assisting biobleaching process of pulp.

1.1 Objectives

The specific objectives of this study were as follows:

1. To select the potential fungal isolates from laboratory culture collection for xylanase production.
2. To optimize the cultural conditions for xylanase production by the selected fungus.
3. To evaluate the effectiveness of crude xylanase in assisting biobleaching process.

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