UNIVERSITI PUTRA MALAYSIA

EFFECTS OF XYLANO-PECTINOLYTIC ENZYMES BY Bacillus subtilis ADI1 ON BIOBLEACHING OF KENAF (Hibiscus cannabinus L.) PULP

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FBSB 2016 32

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By

MUHAMMAD HARIADI NAWAWI

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

June 2016

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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June 2016

Chairman : Wan Zuhainis Saad, PhD
Faculty : Biotechnology and Biomolecular Sciences

Kenaf (*Hibiscus cannabinus*) is an annual non-wood plant which has shown great potential as an alternative source of papermaking fibre. In the pulp and paper industry, due to growing public concern about the environment, technological changes in the bleaching processes have been rising. The use of chemical additives during bleaching process to improve the brightness of paper causes the effluent to contain some chlorinated organic compounds that have shown mutagenic activity. The use of combined xylanase and pectinase in pulp bleaching may assist in enhancing the brightness of paper and could reduce harmful pectins produced in the effluent and thus rendering a more eco-friendly papermaking process as well as improvements in the paper quality. The aim of this research was to study the production of xylano-pectinolytic enzymes from a bacterial isolate *Bacillus subtilis* ADI1 and to examine the effectiveness of using xylano-pectinolytic enzymes on kenaf pulp in biobleaching. The characteristics of enzymes being sought in papermaking are higher production, higher optimal pH and temperature, good stabilities under these conditions and finally a low associated cellulase production. *Bacillus subtilis* ADI1, as the potentially high extracellular xylanase and pectinase producer, was isolated from composting and rotting area around UPM Serdang, Malaysia (Biorefinery Complex, UPM, Selangor). The effects of pH, temperature, inoculum concentration, agitation speed and concentration of rice bran on xylanase and pectinase production were studied. Optimum pH and temperature for xylano-pectinolytic enzymes production by *Bacillus subtilis* ADI1 was at pH 8 and 50°C. Highest xylanase and pectinase production was from 3% (v/v) inoculum concentration and agitation speed of 200 rpm. Maximum xylanase and pectinase production was obtained from 3% (w/v) of rice bran while higher and lower concentration of rice bran resulted in poor enzyme production. *Bacillus subtilis* ADI1 is an alkalophilic thermotolerant bacteria which under optimal cultural conditions, can produce 5.25 times more xylanase and 1.6 times more pectinase than prior to optimisation study (non-optimized condition). Meanwhile, the xylano-pectinolytic enzymes stabilities were studied at optimal pH and temperature for enzyme activities. Xylano-pectinolytic enzymes of *Bacillus subtilis* ADI1 resulted in the optimum conditions of pH 8 and temperature 50°C. The enzymes showed considerable thermostability (stable up to 2 to 3 hours at 40°C) and pH stability (stable up to 3 hours at pH 8). Thus, xylano-pectinolytic enzymes of *Bacillus subtilis* ADI1 are suitable for application in biobleaching of pulp. Optimal enzymatic processes were studied, the xylanase-pectinase doses required in biobleaching pretreatment were 15 and 7.5 U/g of oven dried pulp, and the effective retention time needed for pretreatment was 180 min. The optimised enzymatic processes significantly improved the pulp properties and showed that the ISO brightness increased from 71.14% (untreated) to 84.18% (treated) and reduced the chlorine consumption by 15.5% reduction. In conclusion, xylano-pectinolytic enzymes from *Bacillus subtilis* ADI1 showed interesting biotechnological characteristics that are suitable for application in pulp and paper industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Sarjana Sains

KESAN ENZIM XYLANO-PEKTINOLITIK OLEH *Bacillus subtilis* ADI1 PADA BIOLUNTUR PULPA KENAF (*Hibiscus cannabinus* L.)

Oleh

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Kenaf (*Hibiscus cannabinus*) merupakan tumbuhan bukan kayu tahunan yang telah menunjukkan potensi yang besar sebagai sumber alternatif gentian pembuatan kertas. Dalam industri pulpa dan kertas, timbul kebimbangan di kalangan orang ramai mengenai alam sekitar yang kini membawa kepada perubahan dan perkembangan teknologi dalam proses pelunturan. Penggunaan bahan kimia semasa proses pelunturan untuk meningkatkan kecerahan kertas menyebabkan efluen mengandungi beberapa sebatian organik berklorin yang telah menunjukkan aktiviti mutagen. Penggunaan kombinasi xylanase dan pektinase dalam pelunturan pulpa boleh membantu dalam meningkatkan kecerahan kertas dan boleh mengurangkan pektin-pektin berbahaya yang terhasil di dalam pengeluaran efluen dan dengan itu menjadikan proses pembuatan kertas menjadi lebih mesra alam dan meningkatkan kualiti pengeluaran kertas. Tujuan penyelidikan ini adalah untuk mengkaji pengeluaran enzim xylano-pektinolitik daripada isolat bakteria am *Bacillus subtilis* ADI1 dan untuk memeriksa keberkesanan bioluntur menggunakan enzim xylano-pektinolitik pada pulpa kenaf, kerana kenaf (*hibiscus cannabinus*) memainkan peranan utama dalam industri "hijau", dengan seratnya menghasilkan pulpa yang sangat baik membuatkan kilang kenaf menjadi terkemuka bagi industri kertas. Sifat-sifat enzim yang dicari dalam pembuatan kertas adalah: pengeluaran enzim yang lebih tinggi, pH dan suhu optima yang lebih tinggi, kestabilitian enzim yang baik di bawah syarat-syarat ini dan akhirnya pengeluaran selulase yang rendah. Oleh itu, enzim dari bakteria yang akan disasarkan dalam kajian ini. *Bacillus subtilis* ADI1 telah diasingkan sebagai xylanase luar sel yang berpotensi tinggi dan pengeluar pektinase yang dikumpul dari kawasan kompos dan reput di sekitar UPM Serdang, Malaysia (Kompleks kilang biopenapisan, UPM, Selangor), jelas mempunyai daya katabolit terhadap xylan dan pektin alam semula jadi. Kesan pH, suhu, kepekatan inokulum, kelajuan goncangan dan kepekatan dedak beras untuk xylanase dan pektinase untuk pengeluaran optimum telah dikaji. pH optimum untuk pengeluaran enzim xylano-pektinolitik oleh *Bacillus subtilis* ADI1 adalah pada pH 8 dan mencapai suhu optimum pada 50°C. Pengeluaran tertinggi xylanase dan pektinase berlaku pada kepekatan inokulum 3% (v/v) dan pengeluaran enzim tertinggi berlaku pada goncangan 200 rpm.

Perbezaan kepekatan dedak beras menunjukkan pengeluaran maksimum xylanase dan pektinase adalah pada 3% (w/v) manakala kepekatan yang lebih tinggi dan lebih rendah menyebabkan pengeluaran enzim berkurangan. *Bacillus subtilis* ADI1 adalah bakteria tahan haba alkalofilik yang di bawah keadaan optimum seperti yang dinyatakan sebelum ini, boleh menghasilkan 5.25 kali lebih xylanase dan 1.6 kali lebih pektinase daripada basal medium (bukan kondisi optimal). Sementara itu, kestabilan enzim xylano-pektinolitik turut dikaji dan merangkumi kesan suhu, pH dan juga kestabilan pada keadaan optimum. Enzim xylano-pektinolitik milik *Bacillus subtilis* ADI1 menghasilkan suhu yang tinggi dan nilai pH yang bersamaan dengan keadaan optimum untuk pH (pH 8) dan suhu (50°C). Enzim menunjukkan kestabilanthermo yang baik (stabil selama 2 hingga 3 jam pada suhu 40°C) dan kestabilan pH (selama 3 jam). Oleh itu, ini menjadikan enzim xylano-pektinolitik *Bacillus subtilis* ADI1 adalah sesuai untuk digunakan di dalam proses bio-luntur pulpa.. Proses enzim yang optimum telah dikaji menyebabkan dos xylanase-pektinase yang diperlukan dalam rawatan awal bioluntur adalah 15 dan 7.5 U/g pulpa kering ketuhar, dan masa tahanan yang berkesan diperlukan untuk rawatan awal adalah 180 min. Proses-proses enzim dioptimum bagi memberikan peningkatan yang ketara dalam sifat-sifat pulpa dan menunjukkan peningkatan kecerahan ISO dari 71.14% (tidak dirawat) kepada 84.18% (telah dirawat) dan menyebabkan kurang penggunaan klorin berkurangan sebanyak 15.5%. Oleh itu, *Bacillus subtilis* ADI1 dan enzim xylano-pektinolytik yang telah mempunyai sifat-sifat bioteknologi yang dikehendaki kelihatan sesuai untuk kegunaan industri pulpa dan kertas.

ACKNOWLEDGEMENT

The author wishes to express his deepest appreciation to all the people who have inspired, advised and assisted him during this study. Special recognition and sincere appreciation are extended to his supervisors; Dr. Wan Zuhainis Saad, Assoc. Prof Dr. Rosfarizan Mohamad, and Professor Dr. Paridah Md. Tahir, for their guidance, encouragement and constructive criticism throughout the study.

He would like to express his utmost thanks to the staff of the Mycology Laboratory in the Faculty of Biotechnology and Biomolecular Sciences; Laboratory in Pulp and Paper Technology in Faculty of Forestry and Institute of Tropical Forest and Forest Product (INTROP) 3; Microscopy Units, Institute of Biosciences, Universiti Putra Malaysia; Laboratory of Wood Chemistry Division of Forest Research Institute of Malaysia (FRIM), for their assistance in facilitating him and giving him full cooperation such as using laboratory facilities and equipment during his study.

He would also like to take this opportunity to thank his valuable coursemates and friends for their help, encouragement and comment. Finally and most importantly, he would like to thank his family members for their encouragement and continuous full support throughout the course of his study. All his achievements would not be possible without them.

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENT	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF ABBREVIATIONS	xvii

CHAPTER

1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	3
	2.1 Kenaf	4
	2.1.1 History	4
	2.1.2 Botanical Aspect on Kenaf	4
	2.1.3 Physical Properties of Kenaf	4
	2.1.4 Chemical Properties of Kenaf	5
	2.1.4.1 Cellulose and its Composition in Kenaf	5
	2.1.4.2 Xylan and its Composition in Kenaf	6
	2.1.4.3 Pectin and its Composition in Kenaf	7
	2.1.4.4 Lignin and its Composition in Kenaf	7
	2.1.4.5 Extractives	8
	2.2 Pulp And Paper Industry	8
	2.2.1 Pulp and Paper Industry in Malaysia	9
	2.3 Non-Wood As Raw Material For Pulp Paper	10
	2.3.1 Fibre Morphology Non-Wood Plants in Papermaking	11
	2.3.2 Kenaf in Papermaking	11
	2.4 Biotechnology In Pulp & Paper Industry	12
	2.4.1 Biobleaching in Pulp and Paper Industry	12
	2.4.1.1 Xylanase & Its Performance in Bleaching	15
	2.4.1.2 Pectinase & Its Performance in Bleaching	16
	2.5 Enzymes Synthesis	18
	2.5.1 Mode of Production for Enzymes Production	18
	2.5.2 Rice Bran as Substrate for Enzymes Production	19

3 ISOLATION, SCREENING AND IDENTIFICATION OF XYLANO-PECTINOLYTIC BACTERIA FROM COMPOST 20
3.1 Introduction 20

3.2 Materials And Methods 20
3.2.1 Isolation of Bacteria from Compost Area 21
3.2.2 Primary Screening of Xylano-Pectinolytic Enzymes Producing Bacteria 21
3.2.3 Secondary Screening of Xylano-Pectinolytic Enzymes Producing Bacteria 21
3.2.4 Analytical Methods 22
3.2.4.1 Assays for Xylanase, Pectinase and Cellulase Enzymes 22
3.2.4.2 Determination of Soluble Protein 22
3.2.4.3 Determination of Reducing Sugar Content 22
3.2.5 Statistical Analysis 23
3.2.6 Microorganisms and Maintenance 23
3.2.7 Identification of Bacteria 23
3.2.7.1 Molecular Identification using 16S rDNA Sequencing 23

3.3 Results And Discussion 23
3.3.1 Isolation of Bacteria from Compost 23
3.3.2 Screening of Xylano-Pectinolytic Enzymes Producing Bacteria 25
3.3.3 Identification of Bacterial Culture using 16S rRNA Gene Sequence Analysis 30

3.4 Conclusions 31

4 OPTIMISATION OF CULTURAL CONDITIONS TO ENHANCE ENZYMES PRODUCTION AND PARTIAL CHARACTERISATION OF XYLANO-PECTINOLYTIC ENZYMES OF *BACILLUS SUBTILIS* ADI1 32
4.1 Introduction 32

4.2 Materials And Methods 33
4.2.1 The Selected Microorganism Used for Optimisation and Partial Characterisation Studies 33
4.2.2 Preparation of Culture Medium and Culture Cultivation for Optimisation and Partial Characterisation Studies 33
4.2.3 Production of Xylano-Pectinolytic Enzymes 33
4.2.4 Enzymes Assay for Xylanase, Pectinase and Cellulase Enzymes 34
4.2.5 Analytical Methods 34

4.2.6 Effect of Cultural Conditions on Xylanase and Pectinase Production by *Bacillus subtilis* ADI1 34
4.2.6.1 Effect of pH on Xylanase and Pectinase Production 34
4.2.6.2 Effect of Temperature on Xylanase and Pectinase Production 34
4.2.6.3 Effect of Inoculum Concentration on Xylanase and Pectinase Production 34
4.2.6.4 Effect of Agitation on Xylanase and Pectinase Production 35
4.2.6.5 Effect of Substrate Concentration on Xylanase and Pectinase Production 35
4.2.7 Partial Characterisation of Crude Xylano-Pectinolytic Enzymes 35
4.2.7.1 Effect of pH on Xylanase and Pectinase Activities and Stabilities 35
4.2.7.2 Effect of Temperature on Xylanase and Pectinase Activities and Stabilities 36
4.3 Results And Discussion 36
4.3.1 Effect of Cultural Conditions on Xylanase and Pectinase Production by *Bacillus subtilis* ADI1 36
4.3.1.1 Effect of pH on Xylanase and Pectinase Production 35
4.3.1.2 Effect of Temperature on Xylanase and Pectinase Production 38
4.3.1.3 Effect of Inoculum Concentration on Xylanase and Pectinase Production 39
4.3.1.4 Effect of Agitation on Xylanase and Pectinase Production 40
4.3.1.5 Effect of Substrate Concentrations on Xylanase and Pectinase Production 42
4.3.1.6 Comparison of Xylanase and Pectinase Production in the Optimised Cultural Conditions with Non-Optimised Conditions 43
4.3.2 Partial Characterisation of Crude Xylanases and Pectinases 44
4.3.2.1 Effect of Temperature on Activity and Stability of Crude Xylanase and Pectinase 44
4.3.2.2 Effect of pH on Activity and Stability of Crude Xylanase and Pectinase 47
4.4 Conclusion 50

5	**EFFECT OF XYLANO-PECTINOLYTIC ENZYMES BY *BACILLUS SUBTILIS* ADI1 IN BIOBLEACHING ON KENAF PULP**	51
	5.1 Introduction	51
	5.2 Material And Methods	52
	5.2.1 Pulp Sample	52
	5.2.2 Optimisation of Enzymatic Pretreatment Reaction Conditions	52
	5.2.3 Evaluation of the Pulp-Free Filtrate and the Pulp Sample	52
	5.2.4 Biobleaching and Chemical Bleaching of Kenaf Pulp	53
	5.2.5 Analytical Studies of Physical Properties of Kenaf Pulp	53
	5.2.6 Statistical Analysis	53
	5.3 Results And Discussion	54
	5.3.1 Optimisation of Enzymatic Pretreatment Processes	54
	5.3.2 Biobleaching using Xylano-Pectinolytic Enzymes on Kenaf Pulp	56
	5.4 Conclusion	57
6	**GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS FOR FUTURE STUDIES**	58
	6.1 General Discussion	58
	6.2 Conclusion	61
	6.3 Recommendations For Further Studies	62
REFERENCES		63
APPENDICES		86
BIODATA OF STUDENT		94
PUBLICATION		95

LIST OF TABLES

Table		Page
3.1	Morphological characteristics of isolates	24
3.2	Primary screening of selected isolates on agar plate with clear zone diameter	26
3.3	Growth profile and enzymes production by EFB-11 from initial screening	28
5.1	Effect of different enzyme doses on pulp biobleaching	54
5.2	Effect of different retention time on pulp biobleaching	55
5.3	Physical properties of enzymatic pretreated kenaf pulp	56

LIST OF FIGURES

Figure		Page
2.1	a) Cell wall containing cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. b) Cross section of cell wall. c) Layers of the cell wall	14
3.1	Colony morphology of EFB-11	24
3.2	Gram's stain of EFB-11 under light microscope	25
3.3	Zone of clearance around the well containing bacterial suspension on xylan agar	26
3.4	Phylogenetic tree of *Bacillus subtilis* ADI1 with other *Bacillus* sp.	30
4.1	Pattern of enzyme activity in basal medium with different pH values (pH 5 to 11). Enzymes activity was monitored after 72 hours	37
4.2	Pattern of pH change in basal media with different initial pH values (pH 5 – 11) during growth of *Bacillus subtilis* ADI1 at 72 hours	38
4.3	Effect of temperature on xylanase and pectinase production by *Bacillus subtilis* ADI1 after 72 hours	39
4.4	Effect of inoculum concentration on growth and xylanase and pectinase production after 72 hours	40
4.5	Effect of agitation on xylanase and pectinase production by *Bacillus subtilis* ADI1 after 72 hours	41
4.6	Effect of different levels of rice bran (RB) on xylanase and pectinase production after 72 hours	42
4.7	Xylanase and pectinase production in control and optimized cultural conditions by *Bacillus subtilis* ADI1 after 72 hours	44
4.8	Effect of temperature on crude (●) xylanase and (■) pectinase activity. The reaction was carried out for 10 minutes at various temperatures (25-90°C) using 0.01 M NaOH glycine buffer (pH 8) and enzyme activity was expressed as a percentage of highest activity (40°C)	45
4.9	Effect of temperature on the stability of xylanase. Thermal stability was determined by preincubating the crude enzymes for 24 hours at (●) 40°C, (■) 55°C, (▲) 60°C, (×) 65°C and (+) 70°C.	46

The residual activities were monitored at 1-hour interval up to 5 hours under standard assay conditions

4.10 Effect of temperature on the stability of pectinase. Thermal stability was determined by preincubating the crude enzymes for 24 hours at (●) 40°C, (■) 55°C, (▲) 60°C, (×) 65°C and (+) 70°C. The residual activities were monitored at 1-hour interval up to 5 hours under standard assay conditions 47

4.11 Effect of pH on (●) xylanase and (■) pectinase activity. The enzyme was buffered with 0.01M citrate buffer (CP) (pH5-6.5), 0.01M phosphate buffer (P) (pH 6-8), 0.01M Tris-HCl buffer (TH) (pH 7.5-8.5) and 0.01M glycine-NaOH buffer (GN) (pH 9-10) and incubated for 10 minutes 48

4.12 Effect of pH on stability of xylanase. Stability studies were performed by incubating crude enzyme solution for 24 hours at pH (●) 6.5 (citrate phosphate buffer), (■) 7.0 (phosphate buffer), (▲) 8.0 (phosphate buffer), (×) 8.0 (tris-HCl) and (+) 9.0 (glycine-NaOH) 49

4.13 Effect of pH on stability of pectinase. Stability studies were performed by incubating crude enzyme solution for 24 hours at hours at pH (●) 6.5 (citrate phosphate buffer), (■) 7.0 (phosphate buffer), (▲) 8.0 (phosphate buffer), (×) 8.0 (tris-HCl) and (+) 9.0 (glycine-NaOH) 49

5.2 Handsheets produced after bleaching process. A) untreated, B) treated with xylano-pectinolytic enzymes 57

LIST OF ABBREVIATIONS

°C	Degree celcius
AOX	Adsorbable organic halides
B. subtilis	*Bacillus subtilis*
CMC	Carboxymethylcellulose
DP	Degree of polymerization
ECF	Elemental chlorine free
g	Gravitational force
h	Hour
ha	Hectare
MARDI	Malaysian Agriculture Research and Development Institute
min	Minutes
ml	Millilitre
NA	Nutrient agar
OD	Optical density
OPEFB	Oil palm empty fruit bunch
PGA	Polygalacturonic Acid
psi	pounds per square inch (pressure)
rpm	Rotation per minute
SmF	Submerged fermentation
sp.	Species
SSF	Solid-state fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
TCF	Total chlorine free
ton	Tonne
ton/ha	Tonne per hectare

CHAPTER 1

INTRODUCTION

The utilization of raw materials for pulp and paper production, such as non-wood plant fibers instead of wood fibers is rising in many developing and developed countries. From 1970 to the present time, the capacity of the non-wood plant fiber has increased two to three times compared to the capacity for producing wood pulp for papermaking (Dutt et al., 2009; Rowell et al., 1997). Due to the global demand for fibrous material, non-woods have become one of the important alternative sources for the 21st century, since there is a worldwide shortage of trees in many areas (Ashori and Bahreini, 2009).

Over the last decade, kenaf (*Hibiscus cannabinus L.)* a warm-season annual plant in the family of Malvaceae, which grows quickly, has attracted attention as a potential non-wood raw material for pulp and papermaking. In Malaysia's climate, kenaf managed to grow to height of 3.66-4.27 m for around five months, thus achieving yields of kenaf that vary from 2 ton/ha to 25 ton/ha (Mossello et al., 2010a; Paridah et al., 2009a; Fried 1999; Touzinsky, 1993) which is faster when compared to hardwood that requires several years to grow (Webber and Bledsoe, 2002; Kaldor et al., 1990). Kenaf can act as great alternative fibre crop in the pulp and paper industry since it can produce pulp and papers in comparable quality to those produced from wood (Ashori et al., 2006). Furthermore, kenaf is becoming a new important non-wood fibre crops for paper products because of the dwindling amount of virgin wood and forests cause these products to become more expensive (Monti and Alexopoulou, 2014).

In the pulp and paper industry, growing public concern about the environment is giving rise to technological changes in the bleaching processes. The effluent from the conventional bleaching process contains some chlorinated organic compounds that have shown mutagenic activity (Bajpai et al., 2006; Bajpai, 2005; Erikson and Kirk, 1985). During conventional pulp bleaching, chlorinated phenolic compounds are produced. These compounds are highly resistant to biodegradation, thus contributing to environmental pollution.

When Viikari et al. (1986) initially carried out the use of xylanases to depolymerize hemicelluloses from Kraft pulp prior to pulp bleaching, it showed a reduction of chemicals released. With increasing environmental awareness, ligninase and hemicellulase (xylanases) have been tested for biobleaching and resulted in low chlorine consumption as reported by Kaur et al. (2010). Instead of using xylanase to degrade xylan, pectin is also present in the pulp fibres which required pectinase to degrade pectin (Yang et al., 2008). The pectinases are effective in biobleaching of mixed hardwood and bamboo kraft pulps due to its ability to depolymerize polymers of galacturonic acid. This lowered the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Dhiman et al., 2009; Ahlawat et al., 2007; Viikari et al., 2001; Reid and Ricard, 2000). Nowadays, xylanases are commonly used as a physical barrier to the access of bleaching chemicals since it can remove lignin-carbohydrate complex (Kapoor et al., 2008; Beg et al., 2000a; Viikari et al., 1994). In addition, pectinase are known to depolymerize pectin (or polymers of galacturonic acid) that is present in xylan-pectin-lignin complexes on pulp fibres, subsequently reducing the requirement of peroxide bleaching and cationic demand of pectin solutions. Therefore, the use of xylanase and pectinase from microbes in combination in pulp bleaching may able to reduce harmful pectins produced in the effluent, thus rendering the papermaking process more eco-friendly and improving the quality of paper production.

No report has been published on the biobleaching of kenaf pulp by using concurrent xylanase and pectinase. To date, there is no report available regarding the simultaneous production of xylanase and pectinase in combination from the local Malaysian bacterial isolate for kenaf pulp biobleaching. One of the new and developing techniques is the replacement of chlorine-based bleaching stages with biobleaching using xylano-pectinolytic (ligninolytic) enzymes.

Therefore, the specific objectives of this study were:

a) to isolate, screen and identify xylanase and pectinase enzymes-producing bacteria from compost,
b) to determine the optimum cultural conditions for enhanced production of xylano-pectinolytic enzymes by the isolated bacteria, and
c) to investigate the effect of xylano-pectinolytic enzymes in pretreatment on the physical properties of the bleached kenaf pulp.

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