
UNIVERSITI PUTRA MALAYSIA

# EVALUATION OF XYLANO-PECTINOLYTIC ENZYMES AND OIL PALM EMPTY FRUIT BUNCH PULP BIOBLEACHING BY LOCAL ISOLATE OF *Bacillus* spp.

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**By**

**MUHAMMAD HARIADI NAWAWI**

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**EVALUATION OF XYLANO-PECTINOLYTIC ENZYMES AND OIL PALM EMPTY FRUIT BUNCH PULP BIOBLEACHING BY LOCAL ISOLATE OF *Bacillus* spp.**

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**March 2021**

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The growing public concern about the environment in the pulp and paper industry has resulted in an increase demand of technological changes in the bleaching processes. The use of combined xylanase and pectinase in pulp bleaching could assist in enhancing the brightness of paper and reduce harmful chemical and 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 and characterisation of xylano-pectinolytic enzymes from a bacterial isolate *Bacillus amyloliquefaciens* ADI2 and to examine the effectiveness of using xylano-pectinolytic enzymes on OPEFB pulp in biobleaching. *Bacillus amyloliquefaciens* ADI2, as the potentially high extracellular xylanase and pectinase producer, which was isolated out of 86 isolates, from composting and rotting areas around Selayang in Selangor, Malaysia. RSM analysis demonstrated the optimum pH and temperature for xylano-pectinolytic enzymes production by *Bacillus amyloliquefaciens* ADI2 was at pH 8.38 and 28°C. Optimum inoculum size was at 4% (v/v) and agitation speed of 94 rpm with banana peel as the best substrate. *Bacillus amyloliquefaciens* ADI2 is an alkalophilic thermotolerant bacterium which under optimal cultural conditions, can produce 1.34 times more xylanase and 5.96 times more pectinase than prior to the optimisation study (OFAT). Xylano-pectinolytic enzymes were successfully purified with the recovery of 33.45% and 47.86% for xylanase and pectinase, respectively, weighing around 11 kDa (xylanase) and 31 kDa (pectinase). Xylanase and pectinase were also regarded to be active in broad ranges of pH particularly in alkaline conditions (pH 7.5-10) and being thermally active in the near thermophilic state (40-50°C), enzyme production can be further increased by the addition of $Ca^{2+}$. Xylanase activity was enhanced by EDTA, glycine, and T-80, while no stimulation of activity was observed for pectinase. These enzymes also exhibited high binding affinities ($K_m$) towards xylan beechwood (1.48 mg/mL - xylanase) and polygalacturonic acid (0.96 mg/mL - pectinase). Optimal enzymatic processes for pretreatment of biobleaching were studied, in which the xylanase-pectinase doses required were 15 and 19.2 U/g of oven dried pulp, and the effective retention time needed for pretreatment was 180 min. The recommended pH for pretreatment and temperature

were pH 8.5 and 40°C, respectively. In the $D_1EpD_2$ process, the optimised enzymatic processes significantly improved the pulp properties and showed that the kappa number decreased from 122.8 (control) to 89.5 (treated) compared to 100.8 (untreated). Furthermore, the treated handsheet showed 11.25% enhancement in brightness with 28% compared to the untreated handsheet with 24.85%, which subsequently resulted in an 11.25% reduction of chlorine or chemical consumption. On the other hand, treated OPEFB pulp improved the quality of paper, where there were gains of 30% in tensile strength, 19.4% in bursting index, 20.9% in tearing index contrasted to conventional bleaching. In conclusion, xylano-pectinolytic enzymes from *Bacillus amyloliquefaciens* ADI2 showed interesting biotechnological characteristics that are suitable for application in the pulp and paper industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**PENILAIAN ENZIM XYLANO-PEKTINOLYTIK DAN BIOLUNTUR PULPA TANDAN BUAH KOSONG KELAPA SAWIT OLEH ISOLAT TEMPATAN *Bacillus* spp.**

Oleh

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Dalam industri pulpa dan kertas, kebimbangan orang ramai terhadap alam sekitar menjadikan perubahan teknologi dalam proses pelunturan semakin meningkat. Penggunaan gabungan xylanase dan pektinase dalam pelunturan pulpa dapat membantu meningkatkan kecerahan kertas dan mengurangkan pektin berbahaya yang dihasilkan dari dalam efluen maka terhasillah proses pembuatan kertas yang lebih mesra alam serta berlaku penambahbaikan dalam kualiti kertas. Tujuan penyelidikan ini adalah untuk mengkaji pengeluaran dan pencirian enzim xylano-pektinolytik dari isolat bakteria *Bacillus amyloliquefaciens* ADI2 dan untuk mengkaji keberkesanan penggunaan enzim xylano-pektinolytik terhadap pulpa OPEFB dalam bio-pelunturan. *Bacillus amyloliquefaciens* ADI2, sebagai pengeluar xylanase dan pektinase ekstraselular yang berpotensi tinggi dimana telah diasingkan daripada 86 isolat, ia didapati dari kawasan pengkomposan di sekitar Selayang yang terletak di Selangor, Malaysia. Analisis RSM menunjukkan pH dan suhu optimum bagi pengeluaran enzim xylano-pektinolitik oleh *Bacillus amyloliquefaciens* ADI2 adalah pada pH 8.38 dan 28°C. Kepekatan inokulum yang optimum adalah 4% (v/v), dengan kelajuan agitasi 94 rpm dan menggunakan kulit pisang sebagai substrat adalah kondisi yang terbaik. *Bacillus amyloliquefaciens* ADI2 merupakan bakteria thermotoleran alkalofilik dimana apabila berada di bawah keadaan yang optimum, ia boleh menghasilkan 1.34 kali lebih banyak xylanase dan 5.96 kali lebih pektinase daripada sebelum pengoptimuman kajian (OFAT). Enzim xylano-pektinolytik berjaya disucikan dengan pemulihan 33.45% dan 47.86% untuk xylanase dan pectinase, dengan berat sekitar 11 kDa (xylanase) dan 31 kDa (pektinase). Xylanase dan pektinase juga dianggap aktif dalam julat luas pH terutamanya dalam keadaan alkali (pH 7.5-10) dan juga aktif dalam keadaan yang hampir dengan suhu thermofilik (40-50°C), pengeluaran enzim boleh terus ditingkatkan dengan penambahan $Ca^{2+}$. Xylanase dipertingkatkan oleh EDTA, glisin dan T-80 manakala tiada rangsangan aktiviti berdasarkan pemerhatian bagi pektinase. Enzim-enzim ini juga mempamerkan pertalian mengikat ($K_m$) yang tinggi ke atas xylan kayu beech (1.48 mg/mL - xylanase) dan asid polygalakturonik (0.96 mg/ml - pektinase). Bagi proses optimum enzimatik untuk pra-rawatan bioluntur yang dikaji, dos xylanase-pektinase yang diperlukan adalah 15 dan

19.2 U/g pulpa kering oven, dan masa pengekalan yang berkesan untuk pra-rawatan adalah 180 min. Kadar pH yang disarankan untuk pra-rawatan ialah 8.5 dan suhu 40°C. Dalam proses $D_1EpD_2$, proses enzim yang dioptimumkan meningkatkan sifat pulpa dan menunjukkan bahawa bilangan kappa menurun dari 122.8 (kawalan) ke 89.5 (dirawat) berbanding dengan 100.8 (tidak dirawat). Di samping itu, pulpa yang dirawat menunjukkan peningkatan sebanyak 11.25% kecerahan dengan 28% berbanding 24.85% pada pulpa yang tidak dirawat. Secara tidak langsung ia telah menunjukkan pengurangan penggunaan klorin sebanyak 11.25%. Pada masa sama, pulpa OPEFB yang dirawat menunjukkan kualiti kertas yang meningkat, di mana terdapat peningkatan sebanyak 30% dalam kekuatan tegangan, 19.4% dalam indek pecah, 20.9% dalam indek pemedih berbanding dengan pelunturan secara konvensional. Kesimpulannya, enzim xylano-pektinolitik dari *Bacillus amyloliquefaciens* ADI2 menunjukkan ciri-ciri bioteknologi menarik yang sesuai untuk diaplikasikan dalam industri pulpa dan kertas.

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| | |
|---|---|
| °C | Degree celcius |
| AOX | Adsorbable organic halides |
| *B. amyloliquefaciens* | *Bacillus amyloliquefaciens* |
| *B. subtilis* | *Bacillus subtilis* |
| CMC | Carboxymethylcellulose |
| DP | Degree of polymerisation |
| ECF | Elemental chlorine-free |
| FRIM | Forest Research Institute Malaysia |
| *g* | Gravitational force |
| h | Hour |
| ha | Hectare |
| L | Litre |
| MARDI | Malaysian Agriculture Research and Development Institute |
| min | Minutes |
| mL | Millilitre |
| NA | Nutrient agar |
| OA | Orthogonal array |
| 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

Xylanases and pectinases have a broad range of different applications in biotechnology such as digestion of animal feed, food processing, brewing technology, as well as the pulp and paper industries (Bajpai, 2018; Romanowska et al., 2006; Silverside et al., 2006; Selinheimo et al., 2006; Reid and Ricard, 2000; Wong et al., 1996). It is crucial to study their functional aspects in specific activities on the effects of pH and temperature and their stabilities, which provide a great deal of choice in their potential usage, as the pulp and paper industry demands that the enzymes used be stable in the industrial process spectrum, with a wide pH and temperature range (Bhardwaj et al., 2019; Arora et al., 2009; Iyer and Ananthanarayan, 2008). Other than that, the main bottleneck of inclusive use of enzymes in industry is attributable to their high cost of enzymes production. To obtain economic feasibility, production of enzymes and microbial growth is essential to be optimised. There are a variety of optimal parameters need to be studied in enzymes biosynthesis such as media compositions, environmental conditions, and variations of the producing strain.

In pulp and paper industry, oil palm empty fruit bunch (OPEFB; or EFB) is another example of non-wood fibre resource that can be a good solution to replace the main fibre resources for pulp and paper making, mainly in Malaysia. Apart from edible oil, oil palm is the most significant product produced from Malaysia and the country acts as a leading exporter and producer to many other countries. In 2012, Malaysia itself produced relatively 83 million tonnes of lignocellulosic biomass from the oil palm industry including trunks, fronds, empty fruit bunches, palm shells, and palm oil mill effluent, as reported by Rizal et al. (2018) and is expected to increase the annual production of more than 100 million tonnes by 2020 to meet the robust demand from worldwide for palm oil. Oil palm empty fruit bunches is good lignocellulosic biomass and has the potential to yield up to 73% fibres compared to other oil palm biomass (Wirjosentono et al., 2004). It has also become an accessible resource for natural fibre substitution. OPEFB fibre offers various benefits in addition to the capability to be eco-friendly and its renewable nature, in which it proposes numerous advantages in terms of its low cost, low density, low specific gravity, recyclability, biodegradability, high specific strength, low energy consumption during processing and better thermal and insulating properties (Faizi et al., 2016; Rozman et al., 2004). Since OPEFB has numerous benefits to offer and is deemed to be one of the most abundant biomass materials along with the alpha-cellulose content of 60.6% (Wanrosli et al., 2004), the efforts to convert this material into value-added products have gained significant interests (Rohaizu & Wanrosli, 2015), especially in the papermaking industry (Mahanim et al., 2017; Daud & Law, 2011).

However, growing public concerns about the environment are giving rise to technological changes in the bleaching processes. The effluent from the conventional bleaching process contains some chlorinated organic compounds that show mutagenic

activity (Kumar, 2020; Bajpai, 2012a; Bajpai et al., 2006; Eriksson and Kirk, 1985). During conventional pulp bleaching, chlorinated phenolic compounds are produced. These compounds are highly resistant to biodegradation, thus contributing to environmental pollution.

With increasing environmental awareness, ligninase and hemicellulase (xylanases) had 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 was also present in the pulp fibres, which required pectinase to degrade pectin (Satapathy et al., 2020; Yang et al., 2008). Pectinase is effective in biobleaching of mixed hardwood and bamboo kraft pulps due to its ability to depolymerise polymers of galacturonic acid. This lowered the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Garg et al., 2016; Dhiman et al., 2009; Ahlawat et al., 2007; Viikari et al., 2001; Reid and Ricard, 2000). Nowadays, xylanase is commonly used as a physical barrier to the access of bleaching chemicals since it can remove the lignin-carbohydrate complex (Tao et al., 2019; Kapoor et al., 2008; Beg et al., 2000b; Viikari, 1994). In addition, pectinase is known to depolymerise 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 combined use of xylanase and pectinase from microbes in pulp bleaching may be able to reduce harmful chemical produced in the effluent, thus rendering the papermaking process more eco-friendly and improving the quality of paper production.

Due to high-cost production and the absent of a robust enzyme, there is a greater need to search for alternative enzymes which are cheaper and efficient in papermaking process. Isolating a local bacteria that capable of producing xylano-pectinolytic enzyme with high yield production and having characteristic properties to survive in the harsh industrial level processing, such as active and stable in a broad range of temperature and pH, plus, offering a better performance and eco-friendly process compared to conventional method, could make a significant development in pulp and paper industry. This finding will aid and help the pulp and paper industry in Malaysia that are mainly using OPEFB pulp in paper production to grow rapidly and compete with the global market.

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

The aim of this study was focused on the identification of xylano-pectinolytic enzymes producing bacteria with optimum enzymes production and characterisation, and to evaluate the effectiveness of xylano-pectinolytic enzymes as the oil palm empty fruit bunch (OPEFB) pulp biobleaching agent.

Therefore, the specific objectives of this study are as follows:

a) to isolate, screen, and identify xylanase and pectinase enzymes-producing bacteria from garden soil, decayed plant material, plant composting material, vegetable waste and fruit waste,
b) to determine the optimum conditions for enhanced production of xylano-pectinolytic enzymes by the isolated bacteria using OFAT and RSM techniques,
c) to purify and characterise xylano-pectinolytic enzymes from isolated bacteria, and
d) to investigate the effect of xylano-pectinolytic enzymes in pretreatment on the physical properties of the bleached OPEFB pulp.

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Muhammad Hariadi bin Mohd Nawawi was born in Taiping, Perak on May 21st, 1990. He attended his primary education in Sekolah Kebangsaan Seksyen 20, Shah Alam (1997-2002) and secondary education in Sekolah Menengah Kebangsaan Seksyen 4 Kota Damansara, Petaling Jaya, Selangor (2003-2007). He enrolled his foundation into a 2-years matriculation programme at Perak Matriculation College (KMPk) (2008-2010). Immediately following foundation from KMPk, he further his study in Bachelor of Science (Honours) Microbiology in Universiti Putra Malaysia for 3 years and graduated in 2013. After that, he decided to pursue his Master degree in Applied Microbiology in Universiti Putra Malaysia, from 2013-2016. In 2016, he registered as a Ph.D candidate and continued his Doctoral degree in Enzyme Biotechnology at the same university.

## LIST OF PUBLICATIONS

**Journal** (Submitted/Published)**:**

Nawawi, M. H., Mohamad, R., Tahir, P. M., & Saad, W. Z. (2017). Extracellular Xylano-pectinolytic Enzymes by *Bacillus subtilis* ADI1 from EFB's Compost. *International Scholarly Research Notices*. https://doi.org/10.1155/2017/7831954.

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