BIOETHANOL PRODUCTION FROM RESIDUAL STARCH OF SAGO (Metroxylon sagu Rottb.) HAMPAS

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FBSB 2015 26

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2015

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

BIOETHANOL PRODUCTION FROM RESIDUAL STARCH OF SAGO (*Metroxylon sagu* Rottb.) HAMPAS

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Chair: Professor Suraini Abd Aziz, PhD

Faculty: Biotechnology and Biomolecular Sciences

Bioethanol has received renewed attention recently due to the uncertainties of oil price, elevated greenhouse gas emission, and the need for increased energy security and diversity. In the state of Sarawak, Malaysia, the waste from sago starch extraction process known as sago hampas is a starchy waste material which is commonly discharged directly into the river. It was revealed about 50 – 60% of residual starch is still trapped within sago hampas during starch production process from sago pith. It was estimated that one ton of sago hampas is formed from every ton of sago starch (dry weight basis) produced. Hence this study is carried out targeting the residual starch of sago hampas as a source for glucose production which can be used as the carbon source for the production of environmental friendly bioethanol.

Initially, attempts to extract the starch were made through various approaches such as steeping, retrogradation, steaming and boiling. Boiling was preferable due to the condition of the starch which is ready to be used for saccharification process and less process time was needed for glucose production. The study was then focused to maximize the recovery of glucose (80 g/L) from residual starch in sago hampas through enzymatic hydrolysis process utilizing the commercial enzyme, Dextrozyme. The load of sago hampas at 7% (w/v) was seen to be suitable for the hydrolysis process. However lower glucose concentration (27.79 g/L) as well as less hydrolysis yield (35.73%) was obtained. The amount of substrate loading during the enzymatic hydrolysis seems to be the main obstacle to obtain high glucose yield. Thus, an alternative method (cycles I, II and III) which involved reusing the hydrolysate for subsequent enzymatic hydrolysis cycles was introduced. Greater improvement of glucose concentration (138.45 g/L) and higher conversion yield (52.72%) was achieved after completing cycle III of the hydrolysis process.

The capability of CBY to metabolize glucose from SHH for generating high concentration of ethanol was observed to be suitable at 9 h of pre-germination time. Results showed that 40.30 g/L of ethanol was produced, whereas 0.48 g/g of

ethanol yield was generated and 93.29% of fermentation efficiency was achieved at an initial glucose concentration of 84.57 g/L after 16 h of fermentation. In the study on effects of various initial glucose concentrations, highest ethanol fermentability with respect to ethanol yield (0.50 g/g) and fermentation efficiency (98.00%) were obtained at 100 g/L glucose. By-products such as acetic acid, lactic acid and glycerol were also produced. Higher concentration of glycerol (9.98 g/L) was observed when initial glucose concentration was increased up to 250 g/L.

Yeast-based bioethanol production always considers the supplementation of nitrogen to enhance the fermentation reaction and yeast extract was commonly used. Thus in order to increase the capacity of SHH as an alternative substrate for bioethanol production, utilizing other type of nitrogen source such as urea and ammonium sulfate was also carried out in this study. Urea and ammonium sulfate were found to be feasible in using CBY for bioethanol production. The bioethanol yield was found to be 0.44 g/g and 0.42 g/g when urea and ammonium sulfate was applied as the nitrogen source with fermentation efficiency at 86.27% and 82.35%, respectively. Supplementation of selected metal ionic compounds such as calcium, magnesium and zinc for CBY in SHH was found to be unnecessary as non-significant profile of ethanol production and glucose consumption was observed. The recycle strategy for conducting enzymatic hydrolysis process shows advantage for generating high glucose concentration due to its ability to accommodate up to 21% (w/v) of substrate load. The glucose in hydrolysate was metabolized efficiently by CBY during ethanol fermentation, thus exhibits the capability of sago hampas to be an alternative cheap substrate for the renewable bioethanol production.

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

PENGHASILAN BIOETANOL DARIPADA KANJI HAMPAS SAGU (*Metroxylon sagu* Rottb.)

Oleh

DAYANG SALWANI BINTI AWANG ADENI

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Pengerusi: Professor Suraini Abd Aziz, PhD

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Bioetanol adalah bahan api yang mendapat perhatian sejak kebelakangan ini akibat daripada ketidakstabilan harga minyak, berleluasanya pembebasan gas daripada rumah hijau serta keperluan tenaga yang semakin bertambah. Di Sarawak, Malaysia, hampas sagu adalah biomas yang masih mengandungi kanji, yang kebiasaannya dibuang ke dalam sungai berdekatan hasil daripada aktiviti pemprosesan kanji sagu. Komponen utama hampas sagu ialah sisa kanji iaitu 50% – 60% daripada jumlah berat keringnya. Kajian sebelum ini mendapati bagi setiap tan penghasilan kanji sagu, sejumlah satu tan hampas sagu dibuang ke dalam sungai yang mengakibatkan pencemaran alam sekitar. Kajian ini dijalankan dengan tujuan menggunakan sisa kanji daripada hampas sagu sebagai sumber untuk menghasilkan bioetanol bersifat mesra alam. Peringkat awal kajian adalah mengekstrak kanji menggunakan beberapa kaedah iaitu rendaman, pengewapan, retrogradasi dan pendidihan. Kaedah pendidihan dipilih kerana kanji sagu berada dalam fasa gelatin pada suhu tersebut dan bersedia untuk proses sakarifikasi, disamping menjimatkan masa untuk menghasilkan glukosa. Fokus utama kajian ini adalah penghasilan glukosa yang berkepekatan 80 g/L menggunakan sisa kanji yang telah diekstrak, melalui kaedah hidrolisis menggunakan enzim komersial iaitu Dextrozyme. Didapati hampas sagu yang berkepekatan 7% (w/v) sesuai digunakan semasa proses hidrolisis berenzim tetapi kepekatan glukosa yang diperolehi adalah rendah (27.79 g/L), diikuti juga dengan hasil penukaran hidrolisis yang rendah iaitu 35.73%. Tindakbalas hidrolisis berenzim ke atas larutan hampas sagu didapati tidak efektif apabila jumlah substrat melebihi 7% (w/v) kerana masaalah penyerapan air yang tinggi oleh lignoselulosa sagu yang mengakibatkan aliran pemindahan haba dan jisim terjejas. Oleh itu kaedah kitar semula hidrolisat daripada hidrolisis pertama untuk kegunaan hidrolisis berikutnya diperkenalkan untuk tujuan menambah kepekatan glukosa. Didapati kepekatan glukosa dalam hidrolisat meningkat kepada 138.45 g/L, menunjukkan sebanyak 52.72% hasil penukaran selepas kitaran ke III.

CBY yang melalui fasa tumbesaran dalam media inokulum selama 9 jam didapati sesuai untuk penghasilan bioetanol. Hasil fermentasi menggunakan glukosa yang

kepekatan asal 84.57 g/L menunjukkan 40.30 g/L bioetanol dihasilkan dengan nisbah hasil penukaran substrat kepada produk ialah 0.48 g/g manakala nilai kecekapan fermentasi adalah 93.29%. Kajian ke atas kesan kepekatan asal glukosa berbeza-beza dalam hidrolisat dijalankan untuk melihat toleransi yis bagi tujuan meningkatkan penghasilan bioetanol. Didapati hidrolisat dengan kepekatan glukosa sebanyak 100 g/L menunjukkan hasil bioetanol yang memuaskan di mana kecekapan fermentasi mencapai 98.00% manakala nisbah hasil bioetanol terhadap glukosa yang digunakan adalah 0.50 g/g. Produk sampingan seperti asid asetik, asid laktik dan gliserol juga turut dihasilkan bersama bioetanol. Kepekatan gliserol didapati meningkat apabila kepekatan asal glukosa ditingkatkan ke 250 g/L.

Penghasilan bioethanol berasaskan yis sebagai sumber nitrogen bagi meningkatkan tindakbalas fermentasi selalunya menggunakan ekstrak yis. Bagi meningkatkan kapasiti hidrolisat hampas sagu sebagai alternatif substrat, sumber nitrogen lain seperti urea dan ammonium sulfat telah dikaji. Hasil bioetanol didapati 0.44 g/g dan 0.42 g/g , manakala kecekapan proses fermentasi adalah 86.27% dan 82.35%, apabila urea dan ammonium sulfat digunakan. Sebaliknya, supplementasi bahan logam berion terpilih iaitu kalsium, magnesium dan zink bersama SHH didapati tidak perlu kerana tiada perubahan signifikan pada profil bioetanol yang dihasilkan oleh CBY. Strategi mengitar semula hidrolisat untuk digunakan dalam proses hidrolisis berikutnya ternyata boleh menghasilkan glukosa berkepekatan tinggi kerana 21% (w/v) jumlah substrat digunakan. Glukosa yang terhasil bersama-sama hidrolisat digunakan oleh CBY secara efisien ketika fermentasi bioetanol, menunjukkan hampas sagu boleh dijadikan sumber alternatif untuk penghasilan bioetanol yang diperbaharui.

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim. In the name of Allah Taala, the most gracious, most merciful. Shalawat and rememberance for the Holy Prophet, Muhammad S.A.W.

Alhamdulillah, praise to Allah for this golden opportunity, studying PhD under the supervision of Professor Dr Suraini Abd Aziz, the most outstanding supervisor I ever met, who constantly guiding, encouraging, advising and caring. Special thanks also to the panel of supervisory committee, Professor Dr Kopli Bujang and Professor Dr Mohd Ali Hassan for their responsibilities as well as time and energy spent in improving this report.

Special thanks to Ms Rubena, Mr Ugam, Assoc. Prof. Dr Cirilo, Mr Leo, Mr Rajuna, Mr Amin, Mr Ben, Mdm Ting Woei, Prof Fasihuddin for lending their help in a way to make this study more meaningful and successful. Not to forget to all EB group members in UPM–lecturers and students, especially to Mdm Siren and Dr Ezyana, many thanks for the advice, your concern as well as knowledge sharing.

Last but not least, utmost thanks to my beloved husband, Burhanudin Abu Bakar, as well as my mum, my mum in law, all family members and definitely to Kak Na, Kak Wa and Haseef, for their steadfast support, understanding and sacrifices in making this journey to reach its destination.

Wassalam and Thank You.

I certify that a Thesis Examination Committee has met on 27 January 2015 viva voce to conduct the final examination of Dayang Salwani binti Awang Adeni on her thesis entitled "Bioethanol Production from Residual Starch of Sago (*Metroxylon sagu* Rottb.) Hampas" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
AMG	Amyloglucosidase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Bioethanol	Biological Ethanol
BOD	Biological Oxygen Demand
$CaCl_2.2H_2O$	Calcium chloride dehydrate
CBY	Commercial Bakers' Yeast
CFU	Colony Forming Units
CG	Commercial glucose
COD	Chemical Oxygen Demand
CRAUN	Crop Agricultural Research Centre
LCDA	Land Custody of Development Authority
DNS	Dinitrosalicyclic Acid
DOE	Department of Environmental
E_f	Fermentation efficiency
H_2SO_4	Sulphuric acid
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
NaOH	Sodium hydroxide
NADH	Nicotinamide Adenine Dinucleotide

NDF	Neutral Detergent Fiber
OD	Optical Density
PBS	Phosphate buffered saline solution
Q_p	Product volumetric productivity
rpm	revolution per minute
SEM	Scanning Electron Microscope
SHH	Sago Hampas Hydrolysate
TMTC	Too many to count
TFTC	Too few to count
PDA	Potato Dextrose Agar
$Y_{p/s}$	Product yield based on substrate
YPG	Yeast Peptone Glucose Agar
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

Bioethanol is refers to the ethanol that produced using a renewable biomass (Savaliya et al., 2013). The use of bioethanol as a biofuel is both renewable and environmentally friendly. In prediction, bioethanol production will reach 100 billion litres in 2015. The demand for those amounts is met when using first generation bioethanol crops such as sugar cane and corn (Puri et al., 2013). The research into new carbon sources such as industrial by-products and agricultural residues for use in bioethanol production is needed in order to sustain for the successful development of renewable biofuels (Lima-Costa et al., 2012). However more effective and economical process has to be explored to enhance the competitive capacity of agricultural waste in the production of fuel ethanol against traditional grain-based fuel ethanol (Shen et al., 2012). High ethanol yields in a short fermentation time are an economically relevant factor in industrial ethanol production, however this is dependent on the yeast strain, type of process (batch or fed-batch), cell density, temperature and sugar concentration and enrichment of the medium with the proper nutrients, along with other factors that influence the microbial activity (Laluce et al., 2009).

The waste to wealth concept research was practiced actively over last few years, which widely focusing on bioethanol production from lignocellulosic biomass. In recent, the hydrolysis of lignocellulosic biomass is aiming on obtaining high sugar concentrations in the hydrolysate instead of high conversion yields (Puri et al., 2013). Thus, high solid loading was required during hydrolysis stage in order to generate high concentrations of sugars. Higher sugars concentration will concomitantly increase ethanol concentration theoretically when considering fermentation by yeast. Larsson and Zacchi (1996) claimed that the ethanol with concentration of 40 g/L or above probably able to minimize the continuous distillation cost of ethanol, means able to cut cost downstream processing. Thus at least 80 g/L of initial sugar solution is required for fermentation, which is equivalent to 20% of initial lignocellulosic total solid content (Larsen et al., 2008). With respect to this issue, Yang et al. (2011) proposed that working at high-solid concentrations is an important parameter for enzymatic hydrolysis as it will influences the energy balance and economic viability of bioethanol production. However Zheng et al. (2009) claimed that enzymatic hydrolysis always hindered by low reaction rate, high cost of enzyme as well as low concentration of product. Thus, Chandra et al. (2011) proposed that operating enzymatic hydrolysis using high insoluble solid consistency might help to overcome the problem, however many aspects have to be considered as high insoluble solid will increased viscosity, poor heat transfer caused by rheological properties of dense fibrous suspension will minimize conversion and shear problem will lowering the enzymes activity.

Sago hampas is a starchy waste material created from sago starch processing industry which abundantly found in the state of Sarawak, Malaysia. The sago

starch processing industry is well established, as the sago flour was fast becoming one of the important agricultural export commodities (Karim et al., 2008). It was approximately 51,000 tons of starch exported mainly to Peninsular Malaysia, Japan, Singapore and other countries (Department of Agricultural Sarawak, 2011). However, sago starch extraction process that commonly involves debarking, rasping, sieving, settling, washing and drying are still insufficient thus some residual starches remain embedded in the sago hampas (Karim et al., 2008). The waste from sago starch industries are one type of lignocellulosic waste material available in large quantities but of no commercial value (Akmar and Kennedy, 2001).

It was revealed by the manufacturers that approximately one ton of sago hampas is formed from every ton of sago starch produced (dry weight basis) (Lai et al., 2013). According to Manan et al. (2003), approximately 60 thousand tons (dry weight) of sago hampas is available annually from eight sago mills in Mukah, Sarawak. The residual starch is found to be 40-50% yielding which equivalent to the amount of 24-30 thousand tons of dry starch, hence, equal to half of the total starch produced per year in Sarawak.

The utilization of starchy biomass for bioethanol production could prove to be simple and better alternative to solid waste management (Essaki et al., 2013). Unlike other agro-industrial wastes such as rice husk, wheat straw, cotton stalk and baggase which are primarily cellulosic, sago hampas is primarily starchy and therefore amenable to saccharification and subsequent fermentation to ethanol. The characterizations by Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) had confirmed that the sago hampas compound is a mixture of two components which were starch and lignocellulosic (Lai et al., 2013). On dry basis, sago hampas contains 58% starch, 23 % cellulose, 9.2% hemicellulose and 4% lignin (Linggang et al., 2012a). Currently, these residues which are mixed together with wastewater are either washed off into nearby streams or deposited in the factory's compound. These circumstances, in time, may potentially lead to serious environmental problems.

Several studies on the utilization of sago hampas have been attempted for the production of laccase (Kumaran et al., 1997; Rifat et al., 2003), reducing sugars and enzymes (Vickineswary and Shim, 1996; Apun et al., 2000; Shahrim et al., 2008; Kumoro et al., 2008) as well as carboxymethyl cellulose (Pushpamalar et al., 2006). However some studies on ethanol fermentation are using pure sago starch as raw material (Kim et al., 1992; Haska and Ohta, 1993; Pranamuda et al., 1995; Bandaru et al., 2006). In common practices, the sago starch has to be hydrolyzed into glucose by enzymes or acid before being used as substrate for ethanol fermentation. This study was carried out to initiate the utilization of sago hampas as raw material for ethanol fermentation. Since sago hampas is a starchy materials, thus first part of the study is require a reaction with water (hydrolysis) as well as amylolytic enzymes to break down the starch into glucose. Thus the hydrolysate that contains mixtures of sago hampas residue will acts as fermentation media to generate ethanol. The study mainly focused on how efficient would be the fermentation process by monitoring the ethanol profile with regards to their yield, volumetric productivity, and fermentation efficiency. The commercial baker's yeast was used in this study.

Ethanol industry commonly choose *Saccharomyces cerevisiae* to ferment glucose for generating ethanol, even after some genetic manipulations have been made (Bai et al., 2008). Research has revealed that many strains of *Saccharomyces cerevisiae*, instead of low cost, they can tolerate to significant amounts of ethanol (Tasic and Veljkovic, 2011), without any conditioning or genetic modification (Casey and Ingledew, 1986; Thomas and Ingledew, 1992). Even though wild type yeast is non-amylolytic strain, the availability of commercial enzymes will help hydrolyzate the starch initially.

The ethanol production by fermentation process is common, however the high production cost are still an obstacle to use ethanol as biofuel. Therefore, using economical carbon source for fermentation process is important when considering a commercial scale for bioethanol production (Tanaka et al., 1999; Tao et al., 2005). Besides, it is also important to improve the efficiency of the fermentation system to utilize the economical carbon source with a high ethanol production ability (Lin et al., 2012). On the other hand since the distillation cost per unit amount of ethanol produced is substantially higher at low ethanol concentration (Zacchi and Axelsson, 1989), several investigators have dealt with the idea of concentrating sugars solutions prior to fermentation (Oh et al., 2000; Iraj et al., 2002). Clearly it is necessary to improve the ethanol fermentation performance and to solve the problem between the concentration of ethanol produced and sugar added is an economically sustainable system is to be carried out.

The potential of producing bioethanol from sago hampas, as well as minimizing the river from pollution for environmental conservation awareness, provides alternate resolution for the factory to overcome problem on managing sago waste.
This study with it specific objective below provide some alternative solution to the above problem. The objectives of this study are:

1. To identify the effects of various methods for extracting residual starch in sago hampas.
2. To maximize the glucose concentration in the hydrolysate of sago hampas through enzymatic hydrolysis process using commercial enzyme preparation.
3. To determine the effects of selected parameters on bioethanol fermentation in sago hampas hydrolysate by commercial bakers' yeast.
4. To enhance the production of bioethanol from sago hampas using commercial bakers' yeast.

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LIST OF PUBLICATIONS

Awg-Adeni, D.S., Bujang, K.B., Hassan, M.A., and Abd-Aziz, S. (2013). Recovery of Glucose from Residual Starch of Sago Hampas for Bioethanol Production, BioMed Research International, Volume 2013, Article ID 935852, 8 pages.

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