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SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF SAGO HAMPAS INTO BIOBUTANOL BY Clostridium acetobutylicum ATCC 824

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By

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Chairman : Mohamad Faizal Ibrahim, PhD
Faculty : Biotechnology and Biomolecular Sciences

The increasing prices of the petrol have driven the researchers towards the utilisation of various renewable resources for biofuel production. Renewable resources such as sago hampas composed of 86.3% potential sugars from starch and lignocellulosic materials with only 3.3% of lignin. High carbohydrate contents, low percentage of lignin content and no pretreatment process is required, make the sago hampas as a promising feedstock for biofuel production including biobutanol. Biobutanol can be produced through acetone-butanol-ethanol (ABE) fermentation by Clostridium species. Conventional separate hydrolysis and fermentation (SHF) provides desired amount of sugars but requires multiple processing steps and long processing duration. Therefore, simultaneous saccharification and fermentation (SSF) approach was carried out in biobutanol production. SSF process combines saccharification and fermentation in a single vessel, thus reduces steps, costs and time in biobutanol production. Improvement in SSF process was done due to 'solid effect' when high substrate concentration was used with the aim of giving better biobutanol productions.

This research highlights on potential to convert our country's underutilised sago hampas into sustainable biobutanol. The optimisation of the saccharification to produce high fermentable sugars yield that affects SSF of biobutanol production was conducted. Enzymatic saccharification of sago hampas was performed by three different approaches, which were the saccharification of sago hampas using 71.4 $U/g_{substrate}$ of Dextrozyme glucoamylase, 20 $FPU/g_{substrate}$ of Acremonium cellulase and mixture of both. Results showed that, mixture of Dextrozyme glucoamylase and Acremonium cellulase gave the highest reducing sugars concentration with 67.0 g/L. Saccharification of sago hampas was conducted at the conditions needed for acetone-butanol-ethanol (ABE) fermentation (37°C, 150 rpm, anaerobic condition) produced up to 63.2 g/L of reducing sugars. The normal SSF process by *Clostridium acetobutylicum* ATCC

824 produced 6.75 g/L of ABE with biobutanol concentration of 3.81 g/L and yield of 0.11 g/g_{sugar}. Then, sequential saccharification and simultaneous fermentation (SSSF) was conducted to reduce the solid load in SSF. However, the biobutanol concentration and productivity produced were low about 0.83 g/L and 0.00 g/L.h. In order to improve the biobutanol concentration and productivity, delayed simultaneous saccharification and fermentation (DSSF) was introduced. DSSF has better efficiency since the enzymes and microbe were operated at their optimal conditions. This fermentation generated a biobutanol concentration of 4.62 g/L and 0.5-fold higher biobutanol productivity than normal SSF. In this study, it suggested that the DSSF has the potential to be implemented for the production of biobutanol from sago hampas.

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

SAKARIFIKASI DAN FERMENTASI SERENTAK HAMPAS SAGU KEPADA BIOBUTANOL OLEH *Clostridium acetobutylicum* ATCC 824

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Harga pasaran petrol semasa yang kian meningkat telah mendorong penyelidik ke arah penggunaan pelbagai sumber yang boleh diperbaharui untuk pengeluaran bahan api biologi. Sumber yang boleh diperbaharui seperti hampas sagu mengandungi 86.3% gula potensi daripada kanji dan bahan lignoselulosa dengan lignin hanya 3.3%. Kandungan karbohidrat yang tinggi, peratusan kandungan lignin yang rendah dan tiada proses prarawatan yang diperlukan, membuatkan hampas sagu mempunyai potensi sebagai stok suapan untuk pengeluaran bahan api biologi termasuk biobutanol. Biobutanol boleh dihasilkan melalui fermentasi aseton-butanol-etanol (ABE) oleh spesies Clostridium. Proses konvensional hidrolisis dan fermentasi terasing (HFT) boleh memberikan jumlah gula yang diingini, tetapi mempunyai beberapa langkah pemprosesan dan mengambil jangka masa yang lama untuk diproses. Oleh itu, pendekatan sakarifikasi dan fermentasi serentak (SFS) telah dijalankan dalam pengeluaran biobutanol. Proses SFS menggabungkan sakarifikasi dan fermentasi didalam satu bekas, justeru itu ianya mengurangkan langkah, kos dan masa dalam pengeluaran biobutanol. Penambahbaikan dalam proses SFS dilakukan oleh sebab 'kesan pepejal' apabila substrat berkepekatan tinggi digunakan untuk tujuan pengeluaran biobutanol yang lebih baik.

Sorotan penting dalam kajian ini ialah potensi menukarkan biojisim sagu negara kita yang kurang digunakan untuk ditukarkan kepada biobutanol yang lestari. Pengoptimuman sakarifikasi untuk menghasilkan hasil gula fermentasi yang tinggi boleh memberi kesan terhadap SFS untuk menghasilkan biobutanol telah dijalankan. Sakarifikasi enzimatik hampas sagu telah dilaksanakan melalui tiga pendekatan berbeza iaitu, sakarifikasi hampas sagu menggunakan 71.4 $U/g_{substrat}$ Dextrozyme glucoamilase, 20 $FPU/g_{substrat}$ Acremonium selulase dan campuran kedua-duanya sekali. Keputusan menunjukkan bahawa, campuran Dextrozyme glucoamilase dan Acremonium selulase memberikan kepekatan gula tertinggi iaitu sebanyak 67.0 g/L. Sakarifikasi hampas sagu dijalankan pada

keadaan yang diperlukan untuk fermentasi aseton-butanol-etanol (ABE) (37°C, 150 rpm, keadaan anerobik) menghasilkan gula penurun sehingga 63.2 g/L. Proses SFS normal oleh *Clostridium acetobutylicum* ATCC 824 menghasilkan 6.75 g/L ABE dengan kepekatan biobutanol 3.81 g/L dan hasil 0.11 g/g_{gula}. Selepas itu, sakarifikasi berturutan dan fermentasi serentak (SBFS) dilaksanakan untuk mengurangkan beban pepejal di dalam SFS. Walaubagaimanapun, kepekatan dan daya pengeluaran biobutanol yang terhasil adalah rendah dengan masing-masing sebanyak 0.83 g/L dan 0.00 g/L.h. Untuk penambahbaikan kepekatan dan daya pengeluaran biobutanol, sakarifikasi dan fermentasi serentak tertunda (SFST) telah diperkenalkan. SFST mempunyai kecekapan yang lebih baik oleh sebab enzim dan mikrob dikendalikan pada keadaan optimal mereka. Fermentasi ini menjana 4.62 g/L kepekatan biobutanol dan memberikan 1.5 kali ganda lebih tinggi daya pengeluaran daripada SFS normal. Dalam kajian ini, ia mencadangkan bahawa SFST mempunyai potensi untuk dilaksanakan sebagai pengeluar biobutanol daripada hampas sagu.

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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

ADF	Acid detergent fibre
ADL	Acid detergent lignin
ABE	Acetone-butanol-ethanol
C.	*Clostridium*
CDW	Cell dry weight
DSSF	Delayed simultaneous saccharification and fermentation
DNS	3,5-Dinitrosalicyclic acid
EC	Enzyme commission
GC	Gas chromatography
HPLC	High performance liquid chromatography
NDF	Neutral detergent fibre
OPDC	Oil Palm Decanter Cake
RCM	Reinforced Clostridial Medium
rpm	Rotation per minute
$	US Dollar
SAS	Statistical analysis system
sp.	Species
SPR	Sago pith residue
SSF	Simultaneous saccharification and fermentation
SSSF	Sequential saccharification and simultaneous fermentation
UPM	Universiti Putra Malaysia
UV-Vis	Ultraviolet-visible

CHAPTER 1

INTRODUCTION

Recent increase in demand of energy and depletion of fossil fuel has diverted the attention of researchers towards the utilisation of various renewable resources for the production of biofuel worldwide (Schmidt & Weuster-Botz, 2012). Biobutanol is considered as a better liquid biofuel than bioethanol with promising potential to replace petrol without modification on current distribution and engine system. It has high hydrophobicity property which makes it safer and suitable to be applied directly or blending with petrol for fuel. (Ndaba et al., 2015). Besides, biobutanol is less corrosive and non-hygroscopic, which make it easier to preserve as it has high tolerance to water contamination in petrol blend (Al-Shorgani et al., 2011). Biobutanol has high energy content and high boiling point which prolong the burning process in motor engine. However, the production of biobutanol has several limitations including high cost of fermentation substrate, small amount of biobutanol obtained and its toxicity property that inhibit cell growth and fermentation (Ezeji et al., 2007a; Lee et al., 2008).

Malaysia is a notable tropical country with various plantation industries including oil palm, rubber, paddy, cocoa and sago. The agricultural sector in Malaysia has become major contributors to national growth, income and export earnings, generates about 12% of gross national income (GNI) (Istikoma et al., 2015). Sarawak is the biggest sago planting areas in Malaysia and is presently the biggest exporter of sago in the world. Besides, the sago starch ranks as the fourth highest export value of agricultural products in Malaysia from 2003 until 2012 (Statistics of Sago, 2012). Sarawak exports sago products to Japan, Thailand, Singapore and even to Peninsular Malaysia itself. In current practice, most of the sago wastes (180 tonnes/year) produced in sago mill are usually dumped in open land or directly disposed to a nearby river, which contributes to a serious environmental problem due to its slow degradability and high starch content (Abd-Aziz, 2002; Apun et al., 2000). These abundant wastes have high potential to be served as substrate for fermentation including biobutanol production, yet it is underutilised till now (Awg-Adeni et al., 2010).

Solid waste from sago (sago hampas) contains starch and lignocellulosic material with high total carbohydrate composition and low percentage of lignin (Jenol et al., 2014). Thus, no pretreatment is required prior to saccharification process, and this is useful to reduce the operational cost as compared to other lignocellulosic biomass. Sago pith residue (SPR) attained after hydrolysis of sago hampas by amylase consist of (on a dry weight basis); cellulose (37%), hemicellulose (20%) and lignin (6%) (Linggang et al., 2012). This lignocellulosic material can be further hydrolysed into a mixture of sugar monomers using cellulase. Previous studies that have been conducted using sago hampas only focused on the utilisation of strachy component (sago hampas) or lignocellulosic component (SPR) as biobutanol fermentation substrate (Linggang et al., 2013; Liew et al., 2006; Madihah et al., 2001). Recently, several studies were

conducted on starch-based material such as corn (Pfromm et al., 2010), cassava (Thang et al., 2010), konjac waste (Shao & Chen, 2015) and lignocellulosic-based material such as king grass (Gallego et al., 2015), oil palm empty fruit bunch (Ibrahim et al., 2015), corn stover (Wang & Chen, 2011), oil palm decanter cake (Abdul Razak et al., 2013), switch grass (Qureshi et al., 2010b), wheat straw (Qureshi et al., 2008b) and many other lignocellulosic substrates for biobutanol production.

Biobutanol production can be produced by two different approaches which are petrochemical and biological route. Petrochemical route is based on propylene oxo synthesis, reppe synthesis and crotonaldehyde hydrogenaration from propylene, carbon monoxide and hydrogen to yield 1-butanol (García et al., 2011; Green, 2011). Presently, this route is used in the industry due to the cheaper process compared to biological route. However, it is not practical in the future as scientific developments in synthetic biotechnology and microbiology has a sudden improvement in optimising the process technology of the biological route, making it economically equivalent to petrochemical processes. The biological route is performed via acetone-butanol-ethanol (ABE) fermentation by *Clostridium* species in the present of carbohydrate monomers (Ezeji et al., 2007b). The ABE fermentation was discovered since 1861 and keep on improving to challenge the butanol production through petrochemical route. The improved ABE process generates high n-butanol purity with 45% higher carbon footprint that is compared to petrol-based butanol (Speight, 2015). The theoretical biobutanol yield calculated based on the stoichiometry of ABE fermentation is about 0.6 g/g.

Biobutanol production through acetone-butanol-ethanol (ABE) fermentation by solventogenic *Clostridium* sp. frequently used monosaccharides including glucose, galactose and other sugars from biomass (Ezeji et al., 2007b). In the biobutanol production from biomass, the separate hydrolysis and fermentation (SHF) process is commonly used involving a separate process of (1) saccharification of biomass into sugar and (2) the ABE fermentation of the sugar produced into biobutanol. The simultaneous saccharification and fermentation (SSF) process that combined process (1) and (2) simultenously is currently taking interest for biobutanol production, as this process could reduce the number of processing steps, reduce the whole process duration and therefore improve process productivity while maintaining biobutanol production yield (Guan et al., 2016; Ibrahim et al., 2015; Sasaki et al., 2014; Qureshi et al., 2008a). Besides, the problem arises from the potential loss of sugar through multiple processing steps involve in SHF makes SSF to be more favourable. Moreover, the operations of SHF is more complex and have a longer cycle compared to SSF. Furthermore, SSF involves one-step fermentation method that uses less energy and materials to be performed (Zhu et al., 2012). Nevertheless, performing SHF ensures that both the enzyme and the microbe function under their optimal conditions, whereas performing SSF requires optimisation of the saccharification and fermentation condition.

The enzymatic saccharification of sago hampas for biobutanol production was performed at less than 10% of substrate concentration due to the inefficiency of mass and heat transfer (Awg-Adeni et al., 2013; Jenol et al., 2014; Linggang et al., 2013). Performing SSF in high solid loading can cause insufficient mixing, decrease in water availability and irreversible binding of adsorbed enzyme to the substrate. Therefore, high solid loading is a major mechanical obstacle in industrial application and little information can be obtained for both SHF and SSF. Thus, a comprehensive study and comparison of the SHF and SSF processes should be conducted, especially at the high substrate concentration which subsequently produce high sugars for the production of biobutanol. About 60 g/L of fermentable sugars is required to be converted into approximately 15 g/L solvent (Jesse et al., 2002), this can counter the challenges in producing high biobutanol yield with 25% conversion yield. In this study, biobutanol production from sago hampas at high substrate concentration through SSF and SHF were compared based on the action of multiple enzymes (glucoamylase and cellulase) to improve biobutanol yield. Recently, further development in SSF process using a new proposed process integration has been developed which have advantages over conventional SSF. The sequential saccharification and fermentation (SSSF) was introduced in order to reduce solid load in starchy lignocellulosic material by performing sequential saccharification. The SSSF undergo two-step process of enzymatic saccharification taking place in one vessel. The delayed simultaneous saccharification and fermentation (DSSF) involved operating both enzymes and microorganism at their optimal condition by the incorporate of the pre-saccharification process in DSSF (Liu et al., 2015). Thus, it will increase efficiency of saccharification and fermentation process and could reduce viscosity in high substrate concentration (Paulová et al., 2014).

However, very limited studies were reported on the saccharification of both starch and lignocellulosic biomass in SSF including in SSSF and DSSF for biobutanol production. Therefore, the feasibility of biobutanol production using sago hampas through these processes involving the combination of enzymatic saccharification by amylase and cellulase was depicted in this study. The potential of producing biobutanol from sago hampas, as well as maximising the underutilised sago hampas could provide alternative solution for the mill to overcome problem in managing sago waste.

Therefore, the objectives of this study were

1. To obtain high fermentable sugars concentration from sago hampas using Dextrozyme glucoamylase and Acremonium cellulase, and
2. To evaluate the suitable fermentation process to produce biobutanol from sago hampas.

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