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BIOHYDROGEN PRODUCTION FROM SAGO HAMPAS BY Clostridium butyricum A1

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By

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Faculty : Biotechnology and Biomolecular Sciences

The hydrogen has been applied in fuel cell vehicle and expected to shift toward the technologies that produce no net greenhouse gas effects. Biohydrogen production from biomass is now attracting many researchers in developing a renewable, clean and environmental friendly biofuel. The availability of abundant biomass from various sources could possibly be an advantage for the production of biohydrogen as a competitive energy carrier in the future. There are vast choices of possible types of biomass that can be subjected as the carbon source for the production of biohydrogen including starch based and lignocellulosic biomass. Sago industry is one of the possible source of biomass since the industry is producing large quantities of starch and lignocellulosic biomass. Statistically, a single sago starch processing mill has produced 7 ton/day of sago *hampas*. Thus, this study aimed to produce biohydrogen from sago biomass by locally isolated biohydrogen producer and to optimize the production of biohydrogen using statistical approach.

The locally isolated biohydrogen producer *Clostridium butyricum* A1 was successfully isolated from landfill soil. This strain produced a biohydrogen yield of 1.90 mol H_2/mol glucose with productivity of 170 mL/L/h using pure glucose as substrate. The highest cumulative biohydrogen collected after 24 h of fermentation time was 2468 mL/L-medium. Biohydrogen fermentation using sago *hampas* hydrolysate generate higher biohydrogen yield (2.65 mol H_2/mol glucose) compared to sago pith residue (SPR) hydrolysate at 2.23 mol H_2/mol glucose. A higher biohydrogen productivity of 1757 mL/L/h was obtained when using sago *hampas* hydrolysate much higher when compared to pure glucose at 170 mL/L/h. In this study, the new isolate *C. butyricum* A1 together with the use of sago biomass as the substrate is a promising technology for future biohydrogen production. Optimization of biohydrogen production from sago *hampas* hydrolysate by *C. butyricum* A1 was conducted using four variables including temperature, sugar concentration, initial pH and inoculum size. This study has applied central composite design (CCD) and artificial neural network (ANN) as the optimization

step. As a result, three out of four variables have given significant effects on the production of biohydrogen from sago *hampas* hydrolysate; which are temperature, sugar concentration and pH. Using ANN, pH was found to be the most significant variable with the relative importance of 73.6%. The optimum conditions given by ANN with respect to optimized biohydrogen yield of 2.92 mol of H_2/mol of glucose are 39°C, pH 8, initial glucose concentration at 13 g/L and 13% (v/v) inoculum size. As conclusions, biohydrogen production from sago *hampas* by *C. butyricum* A1 has successfully conducted and optimized.

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

PENGHASILAN BIOHIDROGEN DARIPADA HAMPAS SAGU OLEH *Clostridium butyricum* A1

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Hidrogen telah diaplikasikan dalam kenderaan sel fuel dan dijangka akan menggerakkan teknologi yang tidak menghasilkan kesan gas rumah hijau. Penghasilan biohidrogen daripada biomas telah menarik minat ramai penyelidik dalam melestarikan penghasilan bahan api biologi yang boleh diperbaharui, bersih dan mesra alam. Ketersediaan biomas yang banyak daripada pelbagai sumber menjadi kelebihan pada penghasilan biohidrogen sebagai pembawa tenaga yang lebih berdaya saing pada masa hadapan. Pelbagai jenis biomas boleh dijadikan sumber karbon untuk penghasilan biohidrogen, termasuklah biomas berasaskan kanji dan lignoselulosa. Industri pemprosesan sagu adalah salah satu sumber biomas berpotensi kerana industri ini menghasilkan biomas berasaskan kanji dan lignoselulosa. Secara statistiknya, satu kilang pemprosesan sagu kanji telah menghasilkan sisa empulur sagu sebanyak 7 tan/hari. Tujuan kajian ini adalah untuk menghasilkan biohidrogen daripada biomas sagu oleh mikroganisma penghasil biohidrogen pencilan tempatan dan meningkatkan penghasilan biohidrogen menggunakan pendekatan statistik.

Bakteria pencilan tempatan penghasil biohidrogen, *Clostridium butyricum* A1 telah berjaya dipencilkan daripada tanah tapak pelupusan. Bakteria ini telah berjaya menghasilkan biohidrogen sebanyak 1.90 mol H_2/mol glukosa dan 170 mL/L/j produktiviti dengan menggunakan glukosa tulen sebagai substrat. Biohidrogen kumulatif tertinggi yang telah dicatatkan selepas 24 j fermentasi adalah 2468 mL/L-media. Fermentasi biohidrogen menggunakan hidrolisat hampas sagu telah menghasilkan biohidrogen yang lebih tinggi (2.65 mol H_2/mol glukosa) berbanding hidrolisat sisa empulur sagu (SES) (2.23 mol H_2/mol glukosa). Kadar penghasilan biohidrogen yang tinggi juga telah dicatatkan dengan menggunakan hidrolisat hampas sagu iaitu 1757 mL/L/j berbanding dengan menggunakan glukosa tulen dengan produktiviti 170 mL/L/j. Dalam kajian ini, *C. butyricum* A1 bersama dengan penggunaan biomas sagu sebagai substrat menjanjikan satu teknologi yang berpotensi untuk penghasilan biohidrogen di masa hadapan. Pengoptimuman penghasilan biohidrogen daripada hidrolisat hampas sagu oleh *C.*

butyricum A1 telah dijalankan dengan berasaskan 4 pembolehubah, termasuklah suhu, kepekatan gula, pH awal dan saiz inokula. Kajian ini telah menggunakan *central composite design* (CCD) dan *artificial neural network* (ANN) di dalam proses pengoptimuman. Daripada hasil kajian, tiga dari empat pembolehubah telah memberikan kesan yang signifikan pada penghasilan biohidrogen daripada hidrolisat hampas sagu; iaitu suhu, kepekatan gula dan pH. Berdasarkan analisis menggunakan ANN, pH merupakan pembolehubah yang memberikan kesan yang paling ketara dengan kepentingan relatif sebanyak 73.6%. Keadaan yang optimum telah diberikan oleh ANN untuk penghasilan 2.92 mol H_2/mol glukosa ialah 39°C, pH 8, 13 g/L kepekatan glukosa dan saiz inokula 13% (v/v). Kesimpulannya, penghasilan biohidrogen daripada sisa empulur sagu oleh *C. butyricum* A1 telah berjaya dijalankan dan dioptimumkan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment 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

A.	*Aspergillus*
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
BPNN	Back Propagation Neural Network
Btu	British Thermal Unit
C.	*Clostridium*
CCD	Central Composite Design
CCS	CO_2 Capture and Storage
CDW	Cell Dry Weight
CH_4	Methane
CO	Carbon Monoxide
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
DAS	Department of Agricultural Sarawak
DNA	Deoxyribonucleic Acid
DNS	3,5−Dinitrosalicylic Acid
dNTPs	Deoxyribonucleotide triphosphates
E.	*Enterobacter*
E.	*Escherichia*
EC	Enzyme Commission
EIA	Environmental Impact Assessment
GC	Gas Chromatography
g	G force
h	Hour
H_2	Hydrogen
H_2S	Hydrogen Sulphite

HCl	Hydrochloric Acid
HHV	Higher Heating Value
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
j	*Jam*
$MgCl_2$	Magnesium Chloride
min	Minute
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NDF	Neutral Detergent Fiber
OPDC	Oil Palm Decantor Cake
OPEFB	Oil Palm Empty Fruit Bunches
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
POME	Palm Oil Mill Effluent
PSA	Pressure Swing Adsorption
R^2	Correlation coefficient
RCM	Reinforced Clostridia Medium
rDNA	Ribosomal DNA
rpm	Rotation Per Minute
RSM	Response Surface Methodology
RSS	Residual Sum of Square
SAS	Sarawak Agriculture Statistic
sp.	Species
SPR	Sago Pith Residue
SRM	Steam Reforming of Methane
TKN	Total Kjedahl Nitrogen
UPM	Universiti Putra Malaysia
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solid

CHAPTER 1

INTRODUCTION

Biohydrogen gas produced through fermentation process is an ideal alternative energy carrier that can be used as biofuel, due to it characteristics which are clean and sustainable bioenergy for the future. Biohydrogen is a simple gas with a molecular formula of H_2 and has high energy yield of 122 kJ/g, which is 2.75 times higher than fuels derived from petroleum (Claassen *et al*., 2010; Chong *et al*., 2009a; Pattra *et al.*, 2008). The combustion of hydrogen in engine as energy carrier is 50−70% more efficient than gasoline, hence the only byproduct generated is water (Pattra *et al*., 2008; Fields, 2003), thus subsequently reduce the release of carbon dioxide into the atmosphere.

At present, the industrial production of hydrogen is through several processes including steam reforming of methane, electrolytic (water electrolysis) and thermochemical reactions (Levin and Chahine, 2010; Steinfeld, 2005). The major problems among these available technologies are ineffective cost and high energy consumption. Therefore, this situation leads to edge novel hydrogen uncompetitive to replace our current reliance on fossil fuel. Hence, innovative and novel biohydrogen production via fermentation using cheap feedstock has now being attempted because, it needs less land for set up and not being affected by weather conditions (Ni *et al*., 2007).

In European countries, the “hyvolution” concept has been introduced in order to attain greener biohydrogen production. The concept is by exploiting bacteria that are capable in producing biohydrogen as byproduct during growth on biomass (Claassen *et al*., 2010). Besides, utilization of biomass as a fermentation feedstock can reduce the cost of raw material which contributed around 50–70% of total biohydrogen production cost. By this mean, it will also help biomass related industries in managing their biomass waste and to generate extra profit (Hassan *et al*., 2004).

Naturally, biomass present in various forms, including starch and lignocellulosic materials. The starch based biomass is composed of mainly starch, while the lignocellulosic biomass which is composed of 38-50% cellulose, 23-32% hemicellulose and 15-25% lignin (Perego and Bianchi, 2010). The polysaccharides of starch, cellulose and hemicellulose can be converted into simple sugars which are useful to be utilized as carbon sources for the fermentation process by microorganism. The bioconversion of biomass into simple sugars can be conducted through the hydrolysis process using specific enzyme. Utilization of carbon source recovered from the hydrolysis of biomass for biohydrogen production has been reported by several researchers including from palm oil mill effluent (Chong *et al*., 2009a), oil palm empty fruit bunch (Inayat *et al*., 2012), cassava wastewater (Sreethawong *et al*., 2010) and sweet potato starch residue (Yokoi *et al*., 2001) by exploiting various species of microorganisms.

Malaysia is blessed with favorable climate and abundant natural resources for commercial cultivation of crops such as sago palm. The Malaysian sago palm industry is one of the most important sago exporters in the world and exporting in the range of 55,000 to 65,000 tons/year of sago starch (Department of Statistic, Sarawak, 2012). The Department of Agriculture Sarawak (2010) has reported that the export value is increasing by 15–20% per year. In preliminary 2012, the export value of sago products was 48,314 tons compared to 2003 (40,780 tons) (Department of Statistic, Sarawak, 2012). Increment in production will significantly increase the number of waste generated from this industry, which may cause waste management problems and contribute to the environmental pollution. This industry has generated vast amount of wastes in the form of byproducts including sago bark, sago *hampas* and sago wastewater.

Sago *hampas* contains (on a dry weight basis) 58% starch, 23% cellulose, 9.2% hemicellulose and 3.9% lignin (Awg-Adeni *et al*., 2012; Linggang *et al*., 2012; Ozawa *et al.,* 1998). The enzymatic conversion of starch present in sago *hampas* into glucose produces another biomass which is known as sago pith residue (SPR). Linggang *et al*. (2012) has reported that SPR composed of (on a dry weight basis) 37% cellulose, 20% hemicellulose and 6% lignin and cellulose and hemicellulose can be hydrolyzed into a mixture of fermentable sugars using enzyme called cellulase. These types of biomass have the potential to be used as a carbon source in the production of valuable products. Thus, a study on the utilization of sago *hampas* and SPR for biohydrogen production was conducted. A new local isolated strain *Clostridium butyricum* A1 was employed to convert fermentable sugars into biohydrogen to be compared with a biohydrogen producer, *Clostridium butyricum* EB6 isolated by Chong *et al.* (2009a).

A variety of biohydrogen-producing microorganisms has been documented by several studies. To date, *Clostridium* sp. is widely employed for biohydrogen production since this species is capable in converting hexose sugar to biohydrogen with a theoretical yield of 4 mol H_2/mol hexose. This is higher than the biohydrogen produced by *Enterobacter* sp. that has a yield of 1 mol H_2/mol hexose (Kotay and Das, 2008). However, several studies have been done for *Enterobacter* sp. since this species can achieve higher productivity than other reported species (Prasertsan *et al*., 2009; Kotay and Das, 2008; Chen *et al*., 2005). Jamil *et al.* (2009) has been reported on production of biohydrogen by *Rhodopseudomonas palustris* PBUM001 with biohydrogen yield 0.66 mL H_2/mL POME. *Clostridium* sp. has the capability to produce a biohydrogen yield in the range of 1.4–2.8 mol H_2/mol glucose (Levin and Chahine, 2010; Lin and Tanaka, 2006; Chen *et al*., 2005). The highest biohydrogen yield reported was 3.26 mol H_2/mol glucose by employing *C. butyricum* (Keskin and Hallenbeck, 2012). There are also several reports on the production of biohydrogen by *Thermotoga* sp. using biomass as substrate with a yield in the range of 1.1–2.0 mol H_2/mol glucose or other hexoses (Mars *et al*., 2010; Evvyernie *et al*., 2001).

However, biohydrogen production depends on various fermentation factors that correlate each other to improve biohydrogen yield. Due to the fact that, different substrate and Clostridia species employed in the fermentation produces different yield of biohydrogen, thus a tool that evaluate various fermentation factors at one time with statistical analysis are usually practiced. One of the most widely implemented tools is the response surface methodology (RSM) over the last two decades. RSM is an approach that implies the statistical technique based on the crucial technique of duplication and randomization, thus makes the optimization study easier (Baskar *et al*., 2008). Based on the review, RSM is an effective tool to manipulate the large number of variables, therefore many reports have been used RSM to improve fermentation condition (Balusu *et al*., 2005; Ezhumalai and Thangavelu, 2010). This tool has proven its ability to improve the biohydrogen yield using POME as the substrate, as conducted by Chong *et al*. (2009a; 2009b).

The artificial neural network (ANN) which is more advance and powerful statistical analysis tool is also able to optimize the fermentation parameters including for biohydrogen fermentation. One of the advantages of ANN is less time is required for development of extensive experimentation (Morteza *et al*., 2013). This situation is due to the fact that, it could be conducted with limited numbers of experiments to predict the degree of non-linearity. It is also capable of learning complex relationships without requiring the knowledge of the model structure (Mingzhi *et al*., 2009). According to Dutta *et al*. (2004), the ANN is a superior and more accurate tool in terms of modeling technique as compare to RSM because ANN depicts the nonlinearity of the model. Although, ANN has no ability to estimate the model equation similar to RSM, it still is able to estimate the response based on the trained data (Baş and Boyacı, 2007).

The study aims to produce the biohydrogen from sago biomass by *Clostridium butyricum* A1 with the specific objectives as follows:

1. To evaluate the suitability of sago *hampas* and sago pith residue for biohydrogen production by local isolates.

2. To compare biohydrogen production by *Clostridium butyricum* A1 and *Clostridium butyricum* EB6 using selected sago biomass.

3. To optimize biohydrogen production from selected sago biomass by selected *Clostridium* sp. using artificial neural network.

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