UNIVERSITI PUTRA MALAYSIA

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Thesis Submitted in Fulfilment of the Requirement for the
Degree of Master of Science in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia

June 2001

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

PRODUCTION OF ETHANOL BY GENETICALLY MODIFIED *SACCHAROMYCES CEREVISIAE* USING SAGO STARCH AS SUBSTRATE

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Faculty: **Food Science and Biotechnology**

The performance of direct fermentation of sago starch to ethanol by three strains of genetically modified *Saccharomyces cerevisiae*, YKU 107 (expressing α-amylase), YKU 131 (expressing glucoamylase) and YKU 132 (expressing α-amylase and glucoamylase), was investigated. In fermentation using 2 L fermenter, we found that strains YKU 107, YKU 131 and YKU 132 was capable to hydrolyse 83.45%, 67.45% and 71.9% of sago starch, respectively. However, only strain YKU 131 has the ability to produce significant amount of ethanol (2.16 gL^{-1}) from sago starch. The superiority of strain YKU 131 as compared to strain YKU 107 and YKU 132 was found to be correlated with its ability to secrete glucoamylase. *S. cerevisiae* YKU 131 was selected for further studies. To investigate the performance of *S. cerevisiae* YKU 131 in ethanol production using various types of starch, initial starch concentration was fixed at 20 gL^{-1} Among different types of starch investigated, corn starch was the most susceptible to the glucoamylase, followed by sago, cassava, potato and rice starch. The maximum ethanol concentration was accumulated after 60 hours of fermentation, with the highest in corn starch (2.76 gL^{-1}), followed by the sago, cassava, potato and rice starch, which was 2.39, 2.03, 1.62 and 1.11 gL^{-1}, respectively. Sago starch was selected as carbon source for further studies. From Response Surface Methodology, there was significant interaction between yeast extract and sago starch; peptone and sago starch; as well as yeast extract and peptone. The optimum values for the tested variables for the maximum yield of ethanol were pH = 5.06; yeast extract = 11.86 gL^{-1}; peptone = 23.98 gL^{-1}; and sago starch = 37.19 gL^{-1}. The maximum predicted yield of ethanol was 0.224 g/g, which was in close agreement with the real experiment. The optimum C/N ratio for the recombinant yeast was 7.93, at which 4.70 and 10.13 gL^{-1} of ethanol was produced from 20 and 40 gL^{-1} of initial starch concentration, respectively. At 40 gL^{-1} of initial starch concentration, the theoretical yield of ethanol from utilised sago starch was 72.59%, which is considered compatible. However, only 62.30% of sago starch was utilised. The optimum batch fermentation strategy for maximum ethanol production was aeration (0.1 vvm) at the first 24 hours, with the switching of temperature from 30 to 34°C at the aeration shutoff point. The ethanol concentration produced from 40 gL^{-1} of initial starch concentration was 13.35 gL^{-1}, with approximately 67.98% of sago starch utilisation. The theoretical yield of ethanol on the basis of hydrolysed starch was 87.5%. In the fed-batch fermentation, the sago starch concentration was maintained at 20 - 40 gL^{-1} by intermittent addition of starch. The ethanol concentration after 180 hours was 28.15 gL^{-1}.

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

PENGHASILAN ETANOL OLEH *SACCHAROMYCES CEREVISIAE* YANG TERUBAHSUAI SECARA GENETIK MENGGUNAKAN KANJI SAGU SEBAGAI SUBSTRAT

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Pengerusi: **Dr. Suraini Abd. Aziz**

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Keupayaan penghasilan etanol daripada kanji sagu dengan menggunakan tiga strain *Saccharomyces cerevisiae* yang diubahsuai secara genetik, iaitu YKU 107 (menghasilkan α-amilase), YKU 131 (menghasilkan glukoamilase), dan YKU 132 (menghasilkan α-amilase dan glukoamilase) telah dilakukan. Dalam eksperimen yang menggunakan 2 L fementor, didapati *Saccharomyces cerevisiae* YKU 107, YKU 131 dan YKU 132 beruapaya menghidrolisiskan 83.45%, 67.2% dan 71.9% kanji sagu, masing-masing. Walau bagaimanapun, hanya strain YKU 131 berupaya menghasilkan jumlah etanol yang signifikan (2.16 gL^{-1}) daripada kanji sagu. Kelebihan YKU 131 berbanding dengan YKU 107 and YKU 132, adalah berkaitrapat dengan keupayaannya menghasilkan glukoamilase. *S. cerevisiae* YKU 131 telah dipilih untuk kajian yang selanjutnya. Dalam kajian penghasilan etanol daripada pelbagai jenis kanji, kepekatan kanji ditetapkan pada 20 gL^{-1}. Di antara pelbagai kanji tersebut, kepekatan ethanol terkumpul yang maksima selepas 60 jam ialah daripada jagung (2.76 gL^{-1}), diikuti dengan kanji sagu, ubi kayu, ubi kentang dan beras, dengan kepekatan etanol 2.39, 2.03, 1.62 dan 1.11 gL^{-1} masing-masing. Kanji sagu dipilih sebagai sumber karbon untuk kajian selanjutnya. Dengan menggunakan Kaedah Respons Permukaan, terdapat interaksi yang signifikan di antara ekstrak yis dengan kanji sagu; pepton dengan kanji sagu; dan ekstrak yis dengan pepton. Nilai optima untuk pembolehubah yang telah dikaji bagi menghasilkan perolehan etanol yang maksima ialah pH = 5.06; ekstrak yis = 11.86 gL^{-1}; pepton 23.98 gL^{-1} dan kanji sagu = 37.19 gL^{-1}. Perolehan etanol maksima yang diramalkan ialah 0.224 g/g, di mana ia menghampiri dengan perolehan sebenar dari eksperimen. Nisbah C/N optima untuk yis terubahsuai strain YKU 131 ialah 7.93, di mana 4.70 dan 10.13 gL^{-1} etanol telah dihasilkan daripada 20 dan 40 gL^{-1} kepekatan awal kanji sagu masing-masing. Pada 40 gL^{-1} kepekatan awal kanji sagu, perolehan teori etanol dari kanji sagu terhidrolisis ialah 72.59%, ini dianggap memadai. Namun demikian, hanya 62.30% daripada jumlah kanji sagu dapat dihidrolisiskan. Strategi fermentasi sesekelompok yang optima bagi penghasilan etanol maksima ialah pengudaraan pada 24 jam yang pertama, diikuti dengan perubahan suhu dari 30 ke 34°C pada takat penghentian pengudaraan (0.1 vvm). Etanol yang terhasil daripada 40 gL^{-1} kepekatan awal kanji sagu ialah 13.35 gL^{-1}, dengan kira-kira 67.98% daripada jumlah kanji sagu yang telah digunakan. Dalam fermentasi suapan-sesekelompok, kepekatan kanji sagu dikekalkan pada 20 – 40 gL^{-1} dengan penambahan kanji secara berselang. Kepekatan etanol yang terhasil selepas 180 jam ialah 28.15 gL^{-1}.

ACKNOWLEGDEMENTS

I am truly indebted to my supervisor, Dr Suraini Abd Aziz for accepting me, without any biotechnology background, as her post-graduate student Without her giving me the opportunity, my studies in fermentation technology might not be possible I also would like to express my appreciation, from the bottom of my heart, to my co-supervisors, Assoc Prof Dr Arbakariya Ariff, Dr Hirzun Mohd Yusof and Prof Dr Mohamed Ismail Abdul Karim, for their invaluable guidance, never ending patient, kind assistance and time Their many useful suggestions and comments have been a great help

My gratitude also goes to Mr Rosli for supporting my laboratory work Special appreciation is also expressed to Kak Mai, and all my friends, for their help, support and encouragement

Last but not least, I would like to take this opportunity to thank my family for their endless love, care and supports

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μ_{max}	Maximum specific growth rate of yeast
$Y_{x/s}$	Yield of cell on the basis of hydrolysed starch
$Y_{p/s}$	Yield of ethanol on the basis of hydrolysed starch
dS/dt_{max}	Maximum starch hydrolysis rate during fermentation
G_{max}	Maximum glucose concentration during fermentation
P_{max}	Maximum ethanol concentration during fermentation
Y^1	Yield of ethanol on the basis of utilised starch concentration
Y^2	Yield of ethanol on the basis of initial starch concentration
t_m	Fermentation time, the time needed to reach the maximum ethanol concentration
X_m	Maximum cell concentration
X_{sp}	Cell concentration at the aeration shutoff time
S_{sp}	Starch concentration at the aeration shutoff time
P_m	Maximum ethanol concentration during fermentation
P_m/IS	Yield of ethanol on the basis of initial starch concentration
P_m/US	Yield of ethanol on the basis of utilised starch concentration
ρ_m	Maximum ethanol productivity

CHAPTER 1

INTRODUCTION

Ethanol is monohydric primary alcohol. It is the most commonly utilised solvent next to water in chemical industry. The main applications of ethanol are (1) as a potable alcoholic beverage, (2) as an intermediate chemical for cosmetics, pharmaceuticals etc., and (3) as a feedstock for the production of other chemicals, such as PVC, chlorinated solvent, acetic acid etc. In future, ethanol has a great potential to substitute the petroleum as a more environmental friendly fuel. Ethanol could be produced either synthetically by direct hydration of ethylene which is a product of natural gas, or by fermentation process which involved the conversion of fermentable sugar into ethanol by microorganism such as *Zymomonas mobilis* and *Saccharomyces cerevisiae*. Except for human consumption, synthetic ethanol is widely used due to its lower production costs. However, the synthetic ethanol is relied on non-renewable resources. Therefore, if ethanol is to be widely used as a chemical feedstock and as fuel in future, the production of ethanol via fermentation process using renewable biomass is essential.

The use of ethanol based fuel is not a new concept. In fact, during the Second World War alcohol has been extensively used as petroleum fuel substitute especially in Europe. However, the use of alcohol as fuel was overlooked due to large supply and relatively low cost of petroleum after the war. During 1970s, the fuel crisis had stimulated new research into applications and bioconversion techniques as well as re-assessment of the environmental advantages of using alcohol fuels. Brazil is one of the pioneer countries in large-scale use of alcohol fuels because of the abundant sugarcane in the country, as raw material readily for ethanol fermentation. In 1980, 10% of all new cars produced in Brazil were required by law to be powered by straight ethanol. Today, Brazil is the world's leader in the use of ethanol as an automobile fuel. More than 11 billion litres of ethanol for fuel are produced each year. About 15% of the vehicles with spark ignition engines run on neat ethanol, and the rest use a blend of 20% ethanol in gasoline. Since 1979, Americans have driven over 3 trillion kilometres on ethanol-blended fuels, which is actively encouraged by governments and some environmentalists in US. In Canada, ethanol-blended gasoline is now available at over 700 gas bars from Quebec to the Pacific, including the Yukon Territory (Ethanol Information Centre, 2000). These governments have offered tax incentives to encourage the development of fermentation ethanol (or bio-ethanol) industry for a number of reasons, including reduced dependence on imported foreign fossil-fuels; increased market opportunity for agricultural crops; reduced the emission of environmental hazard pollutants, e.g. carbon monoxide, carbon dioxide, ground level ozone-causing gaseous etc (Ethanol Information Centre, 2000).

The renewable resources that can be used in ethanol fermentation are (1) sugar bearing materials, such as sugarcane, molasses etc., (2) starchy materials, such as cassava, corn, potatoes, sago etc., and (3) cellulose mainly from wood. Traditionally, ethanol fermentation is relied on sugar-rich substrate, mainly sugarcane, because of their carbohydrate is in fermentable sugar form. However, sugarcane is expensive and not continuously available because it is a seasonal crop that required high-quality agricultural land to grow. Thus, the production of ethanol from starch crops and cellulose has great economic advantages due to their abundance and lower costs. In comparison to cellulose, starchy materials were proven currently more feasible as substrates for ethanol production.

The yeast *Saccharomyces cerevisiae*, the main microorganism used for alcoholic fermentation, is widely used as a host cell for foreign gene products due to the abundance of information that available following by the early development of recombinant DNA techniques for *S. cerevisiae*. Besides, yeast is an eukaryotic cell that has an ability to produce mature foreign protein from plants or animals.

In this work, the recombinant *Saccharomyces cerevisiae* obtained from the host strain YKU 76 named YKU 107 (expressing α-amylase), YKU 131 (expressing glucoamylase) and YKU 132 (expressing α- and glucoamylase) were used for direct fermentation of sago starch to ethanol.

The objectives of the study are:

1) To investigate the performance of three recombinant *Saccharomyces cerevisiae* to produce ethanol using starches as substrate.

2) To determine the optimum media composition for direct conversion of starch into ethanol by the selected recombinant yeast.

3) To study the effect of C/N ratio and initial starch concentration on the performance of selected recombinant *S. cerevisiae* in direct ethanol fermentation.

4) To enhance to performance of selected recombinant by *S. cerevisiae* adjusting the aeration, agitation and the temperature during fermentation process.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of Ethanol

Ethanol is miscible with water in all proportions and is normally separated from water by distillation method. Ethanol forms a constant-boiling mixture, or azeotrope, with water that contains 95% ethanol and 5% water and boils at 78.15°C; since the boiling point of this binary azeotrope is below than that of pure ethanol (78.5°C), absolute ethanol cannot be obtained by simple distillation. However, if benzene is added to 95% ethanol, a ternary azeotrope of benzene, ethanol and water with boiling point of 64.9°C can be formed. Thus, the water in 95% ethanol could be removed by adding benzene and distilling off this azeotrope. Ethanol reacts with certain acid to form ester, e.g., with acetic acid it forms ethyl acetate. Ethanol can also be oxidise to form diethyl ether, or at higher temperature, ethylene (The Columbia Electronic Encyclopedia, 2000). Table 2.1 shows some properties of ethanol, compared to gasoline, diesel and fuel oil.

2.2 Industrial Importance of Ethanol

2.2.1 Ethanol Uses in Chemical Industry

Ethanol is a versatile chemical which technically can be used for a wide variety of applications both as an intermediate chemical and as a raw material for the production of other chemical products. As raw material, synthetic ethanol is normally used. Production of many small-volume chemical products normally involves production of ethylene first (from petroleum or natural gas), then its conversion into ethanol and finally production of the chemical product from ethanol by dehydration, dehydrogenation or oxidation. On the other hand, bio-ethanol is preferred over synthetic ethanol, for applications involving human consumption such as in pharmaceuticals, toiletries and cosmetics. As a result, ethanol from the fermentation process normally commands a premium price over synthetic ethanol. Figure 2.1 shows the simplified outline of ethanol-based chemical.

Table 2.1: Main Physical and Chemical Properties of Ethanol and Hydrocarbon Fuels

Property	Ethanol	Gasoline	Diesel	Fuel Oil
Formula	CH_3CH_2OH	C_4 to C_{12} Hydrocarbons	C_{14} to C_{19} Hydrocarbons	C_{20+} Hydrocarbons
Molecular Weight	46.1	100 – 105 avg.	240 avg.	NA
Composition (% weight)				
Carbon	52.2	85 – 88	85 – 88	85 – 87
Hydrocarbon	13.1	12 – 15	12 – 15	10 – 11
Oxygen	34.7	Neg.	Neg.	Neg.
Specific Gravity	0.79	0.72 – 0.78	0.83 – 0.88	0.88 – 0.98
Boiling temperature (°C)	78.5	27 – 225	240- 360	360 +
Calorific value (kcal/L)	5 048	7 700	8 738	8 795
Solubility in water (%)	Infinite	< 0.05	0	0

Note: NA: Not applicable
Neg: Negligable
Source: American Petroleum Institute (2000)