# การหมักไบโอเอทานอลจากกากตะกอนเยื่อกระดาษโดยเชื้อจุลินทรีย์เดี่ยวและเชื้อจุลินทรีย์ผสม

# Comparative Bioethanol Fermentation from Paper Sludge by Monoculture and Co-culture

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**บทคัดย่อ**

วัตถุประสงค์ของการศึกษานี้เพื่อเปรียบเทียบการผลิตเอทานอลจากกากตะกอนเยื่อกระดาษโดยการทำให้เป็นน้ำตาลแยกจากการหมัก (SHF) และการทำให้เป็นน้ำตาลพร้อมการหมัก (SSF) โดยเชื้อเดี่ยว และเชื้อผสมของ *Saccharomyces cerevisiae* KM 5221 และ *Kluyveromyces marxinus* 5057 กากตะกอนเยื่อกระดาษถูกบดละเอียดโดยเครื่องบดหยาบอัลตร้าเซนตริฟิกัลป์มิลล์ และดิซค์มิลล์ ร่วมกับการปรับสภาพที่ 121 องศาเซลเซียสความดัน 15 ปอนด์ต่อตารางนิ้ว 30 นาทีในกรดซัลฟูริก 0.1 โมลาร์ ไฮโดรไลเสทมีน้ำตาลรีดิวซ์และกลูโคส 10.08 และ 5.41 กรัมต่อลิตร โดยการย่อยด้วยเซลลูเลส 30,000 หน่วยต่อกรัมคิดเป็น 66.67 เปอร์เซ็นต์ การหมักแบบ SHF โดย *K. marxinus 5057* และ *S. cerevisiae* KM 5221 ผลิตเอทานอล 14.52 และ 13.42 กรัมต่อลิตรตามลำดับ การหมักแบบ SSF โดย *K. marxinus 5057* และ *S. cerevisiae* KM 5221 ผลิตเอทานอล 7.94 และ 7.34 กรัมต่อลิตรตามลำดับ การหมักแบบ SHF โดย *S. cerevisiae* KM 5221 และ *K. marxinus 5057* ในอัตราส่วน $3\times10^{2}$: $4\times10^{4}$ CFUต่อมิลลิลิตร ผลิตเอทานอล (16.06 กรัมต่อลิตร) สูงกว่า *S. cerevisiae* KM 5221 $4\times10^{3}$ และ *K. marxinus 5057* $4\times10^{3}$ CFUต่อมิลลิลิตร และโดยเชื้อเดี่ยว การหมักแบบ SSF ของเชื้อผสม ในอัตราส่วน $3\times10^{2}$: $4\times10^{4}$ CFUต่อมิลลิลิตรผลิตเอทานอลต่ำกว่าการหมักแบบ SHF การศึกษานี้แสดงว่าเชื้อยีสต์ผสมผลิตเอทานอลสูงกว่าเชื้อยีสต์เดี่ยว

**คำสำคัญ:** การตะกอนเยื่อกระดาษ, เอทานอล, การหมัก, ยีสต์, การทำให้เป็นน้ำตาล

**Abstract**

The objectives of this study were to produce ethanol from pretreated paper sludge by separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) and to compare ethanol production by monoculture of *Saccharomyces cerevisiae* KM 5221 and *Kluyveromyces marxinus* 5057 and a co-culture of *S. cerevisiae* KM 5221 and *K. marxinus 5057*. Recycle paper sludge (RPS) was dried and milled into

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small pieces by blender, ultracentrifugal mill and disc mill and pretreated at 121 °C under vapor pressure of 15 lb/inch$^2$ for 30 min with 0.1 M sulfuric acid. In hydrolysate of pretreated RPS, the reducing sugar and glucose concentration were 10.08 g/L and 5.41 g/L and enzymatic digestibility of 66.67% cellulase loading 30,000 CMC Unit/g. In SHF, the ethanol production by *K. marxinus 5057* and *S. cerevisiae* KM 5221 and were 14.52 g/L and 13.42 g/L, respectively, while in SSF, the ethanol concentrations were 7.94 g/L and 7.34 g/L, respectively. In SHF, the inoculums ration of $3\times10^2$ CFU/mL : $4\times10^4$ CFU/mL of *S. cerevisiae* KM 5221 and *K. marxinus 5057*, respectively, produced ethanol concentration (16.06 g/L) higher than that of the inoculums ration *S. cerevisiae* KM 5221 of $4\times10^3$ CFU/mL and *K. marxinus 5057* of $4\times10^3$ CFU/mL and monoculture while in SSF, the ethanol concentration of co-culture ratio $3\times10^2$ CFU/mL : $4\times10^4$ CFU/mL of *S. cerevisiae* KM 5221 and *K. marxinus 5057*, respectively, was lower than that of in SHF. The results indicated that each strain of yeast was able to produce ethanol from RPS and the co-culture produce greater amounts of ethanol and higher yields than that fermented by monoculture.

**Keywords:** paper sludge, ethanol, fermentation, yeast, saccharification

## Introduction

The major cost in the fermentative production of fuel ethanol is that of the substrate.[1] To reduce production cost, cheap raw materials, such as industrial wastes, should be used.[2] Bioethanol is being considered as a high potential liquid fuel due to the limited amount of natural resources.[3] Especially bioethanol produced from non-food lignocellulosic waste products could be an environmentally-friendly alternative.[4]

In Thailand, approximately 70 ton per day of RPS is produced. RPS is the solid waste stream of the papermaking industry containing the short cellulose fibers, which leave the process. The waste stream usually is deposed off, which has a significant cost-increasing factor on the paper production. Another option for utilizing the organic content of this waste stream is heat and electricity generation by direct combustion. However, the high water and inorganic matter content, which can be as high as 30 wt% dry matter, would result in substantial energy loss.[5] Producing a value added product, such as fuel ethanol from the cellulose existing in the RPS, could provide an economically more attractive option. Due to the high and rather accessible cellulose content (50–60%) of RPS, it could be a potential feedstock for fuel ethanol production.[5]

In general, cellulosic materials are resistant to bioconversion and require a pretreatment to increase their digestibility and make cellulose more accessible to the cellulolytic enzymes. The main components of lignocellulose are cellulose, hemicelluloses and lignin. The combination of lignocelluloses and lignin resulted in complex structure around the cellulose must be removed or modified before hydrolysis of cellulose.[6]

Pretreatment processes include physical, chemical, biological and combination of these methods. Dry-defibrated mill[7] and dilute acid hydrolysis has been successfully developed for pretreatment of cellulosic. Typical sulfuric acid

concentration for hemicellulose hydrolysis are in the range 0.5-1.5% and temperatures above 121-160°C. Hemicellulose is a branched polymer, which cellulose and hemicelluloses to monomeric sugars for ethanol fermentation, the low cost of materials together with an efficient conversion of an sugars to ethanol could potentially decrease the production cost of ethanol produced from materials.[8] Co-fermentation of hexoses and pentoses to ethanol is a prime research target. This process can also be carried out by SHF and SSF. Therefore, RPS as substrates can be served as an affordable and renewable low cost raw material for ethanol production. The objectives of this study were to develop the dilute-acid pretreatment process for enzymatic hydrolysis of RPS to fermentable sugars and to assess the ethanol yield from RPS by mono and co-fermentation in SHF and SSF process.

## Methodology

RPS from the factory was washed by tap water and dried at 90°C in hot-air oven for 2 days. Generally, objective of the biomass pretreatment is to make the cellulose more accessible to enzyme. In this study, the dry RPS was milled into small pieces by a blender, an ultracentrifugal mill and a disc mill. The milling pretreatments either with blender rotating at 1,000 rpm or ultra-centrifugal mill rotating at 6,000 rpm or Disc mill rotating at 12,000 rpm were investigated for enable production of the fermentable sugars from that, enzymatic hydrolysis. The milled RPS was pretreated with 0.1 M, 1 M and 2 M sulfuric acid at 121°C under pressure of 15 lb/inch$^2$ for 30 min. The pH of pretreated RPS suspension were adjusted to 5.5 for fermentation process. After pretreatment, the accessibility of the cellulose to cellulase enzyme is increased.[9] The consists of pentoses such as xylose and arabinose and it also contains hexoses such as glucose, mannose and galactose. In the conversion of reducing sugars, glucose conversions and ethanol yields were evaluated after 48 h of fermentation of the RPS pretreated with disc mill by monoculture, and co-culture in different ratios of *S. cerevisiae* KM 5221 and *K. marxinus* 5057. The co-culture (CFU/mL) ratio of 8 x $10^2$ : 4 x $10^4$, 4×$10^3$ : 4×$10^3$, (3×$10^2$ : 4×$10^4$ and 0 : 8x$10^6$ were cultivated at 30°C pH 5.5 in fermentation process by SHF and SSF.

The enzyme solution (filter – sterilized Acellulase enzyme (Genecor) 30,000 CMC unit/g solubilized in citrate – phosphate buffer pH 5.0) was used for hydrolysis of pretreated RPS. The pretreated sample was supplemented with additional nutrients to give a base medium composition of $MgSO_4$•7HgO,1 g/L; $(NH_4)_2SO_4$,2 g/L; $KH_2PO_4$, 0.5 g/L; yeast extract, 1 g/L. The enzymatic hydrolysis was run at the same time as fermentations in 100 mL erlenmeyer flasks containing 50 mL of the mixture. In monoculture fermentation, both microorganisms *S. cerevisiae* KM 5221 and *K. marxinus* 5057 and co-culture (CFU/mL) ratios of 8 x $10^2$ : 4 x $10^4$, 4×$10^3$ : 4×$10^3$ , 3×$10^2$ : 4×$10^4$ and 0 : 8x$10^6$ were used. The culture was incubated in shaking incubator with 50 rpm for at 30°C for each culture in SHF and SSF methods and sampled the medium every 4 h. The glucose content was measured using glucose oxidase/peroxidase assay. The amount of reducing sugar was determined using the dinitrosalicylic acid (DNS) method.[10] Ethanol concentration was measured by gas-chromatography (GC-17A, Shimadzu) using a chromosorb 101 column and a flame ionization detector.[11]

**Statistical analysis**

Data were analyzed using one-way analysis of variance (ANOVA) using the SPSS 16 program, coupled with Duncan multiple range test. The data were expressed as means ± standard deviation of the mean. Differences were considered significant at $P < 0.05$ level.

## Results and Discussion

The productions of reducing sugars and glucose from un-milled RPS pretreated with 0.1 M, 1 M and 2 M sulfuric acid at 121°C under pressure of 15 lb/inch$^2$ for 30 min are shown in Table 1. The highest yield of reducing sugar was 1.38 g/L obtained from 2 M sulfuric acid with disc mill. So, the dilute-acid pretreatment with disc mill is more efficient for enzymatic hydrolysis as compared to steam. Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment showed high reaction rates and significantly promoted cellulose hydrolysis.[12] Typical sulfuric acid concentrations for hemicelluloses hydrolysis were in the range 0.5-1.5% and temperatures above 121-160°C.

The dried RPS of substrate composed of 60.09% cellulose, 11.18% hemicelluloses, 9.74% lignin. Generally, Biomass cannot be saccharified by enzymes without a pretreatment because the lignin in plant cell walls forms a barrier against enzyme attack.[13] The pretreatment will reduce the lignin content and the crystallization of cellulose and increase the surface area for enzymatic hydrolysis. The results show that pretreatment by different concentration of sulfuric acid without enzyme did not provided significantly different .reducing sugars from milled RPS by blender, ultra-centrifugal mill and disc mill. After enzyme hydrolysis, .reducing sugars and glucose from milled RPS by disc mill provided significantly higher ($P$<0.05) than that from blender and ultra-centrifugal mill. Reducing sugars and glucose from RPS pretreated with disc mill provided maximum yields of 9.13 g/L and 5.42 g/L,

**Table 1** Yields of reducing sugar and glucose from pretreated RPS by dilute $H_2SO_4$ and enzymatic hydrolysis

| Pretreatment with $H_2SO_4$ (M) | Reducing sugar (g/L)** | | | Glucose (g/L)** | | |
|---|---|---|---|---|---|---|
| | Blender | Ultra-centrifugal Mill | Disc Mill | Blender | Ultra-centrifugal Mill | Disc Mill |
| 0.1* | 0.77±0.04$^{aA}$ | 1.07±0.12$^{aB}$ | 1.31±0.14$^{aC}$ | - | - | - |
| 1.0 * | 0.80±0.02$^{aA}$ | 1.27±0.09$^{aB}$ | 1.35±0.12$^{aB}$ | - | - | - |
| 2.0* | 0.81±0.01$^{aA}$ | 1.29±0.16$^{aB}$ | 1.38±0.07$^{aB}$ | - | - | - |
| 0.1 | 5.76±0.07$^{bA}$ | 6.04±0.14$^{bB}$ | 9.13±0.12$^{bC}$ | 3.21±0.16$^{D}$ | 3.66±0.11$^{E}$ | 5.42±0.07$^{F}$ |

* without enzymatic hydrolysis

** Each value is expressed as mean ± SE (n = 3). Different superscript small letters (a, b, c…) within a column are significantly different ($P$ <0.05). Different superscript capital letters (A, B, C…) within a row are significantly different ($P$ <0.05).

respectively. So, this pretreatment method was optimal for the enzyme hydrolysis to fermentable sugars. The scanning electron microphotographs un-milled pretreated RPS was shown in Figure 1A. Figure 1B shows the scanning electron microphotographs of RPS pretreated by ultra-centrifugal milled. Figure1C shows the scanning electron microscropic image of structure RPS pretreated with disc mill. The microphotographs shows cellulose fiber mechanically damaged compared to those of intact RPS. The results show removed physical barriers of cellulose structure of RPS by disc mill pretreatment.

The average size of dry-defibrated disc mill pretreated RPS was 100-300 μm in length and 10 μm in thickness, ultra-centrifugal mill fiber was 700-950μm in length and 15-25 μm in thickness, while that of untreated fiber was 1-2 mm in length and 30 μm in thickness. The size of materials is 10-30 mm after chipping and 0.2-2 mm after milling or grinding.[14] Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity.[14] From the results, diluted acid with steam pretreatment is the most effective methods, characterized because of using low energy consumption. In waste materials; hemicellulose is most thermal-chemically sensitive. Hemicellulose solubilized into the water at temperature higher than 150°C. The various components, xylan can be extracted easily.[15] The results are shown in Table 2 and Figure 2.

Ethanol yields by SHF from RPS dry-defibrated pretreated with disc mill and dilute acid at 121°C for 30 min by monoculture and co-culture at 30°C pH 5.5 are shown in Figure 2. In Figure 3, production of ethanol from SSF of dry-defibrated RPS pretreated with disc mill and diluted acid fermented with monoculture of *S. cerevisiae* KM 5221 and *K. marxinus* 5057 were studied.

The ethanol concentration were 13.42 g/L and 14.52 g/L, respectively. Table 2 shows comparison ethanol yields from SHF and SSF process. The results showed that $C_E$ (Ethanol yield per unit biomass) of monoculture and co-culture by SHF and SSF (except co-culture by SSF) were not significantly different ($P$<0.05). The maximum ethanol yield or $P_{MAX}$ (16.06 g/L) of co-culture (*S. cerevisiae* KM 5221 $3\times10^2$ CFU/mL and *K. marxinus* $4\times10^4$ CFU/mL) by SHF showed was significantly highest ($P$<0.05) among the other fermentations. So, the optimum condition to produce the highest ethanol yield was obtained

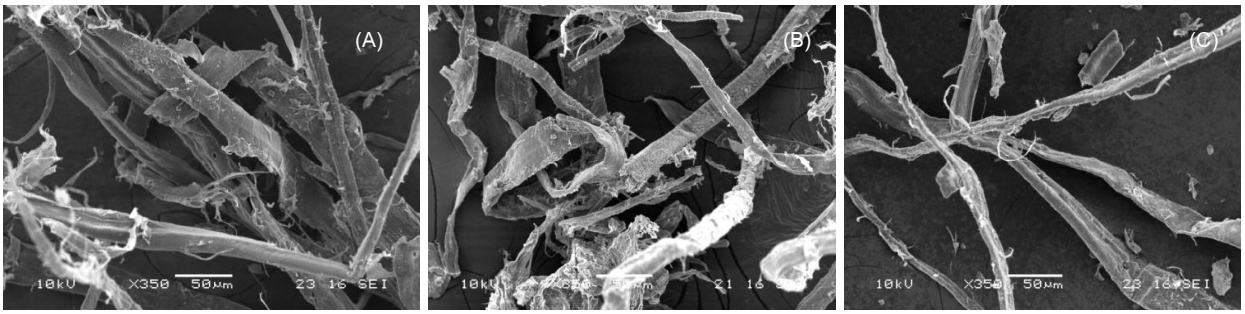


**Figure 1** Scanning electron microphotographs of RPS. The intact RPS (A) RPS pretreated with ultra-centrifugal mill (B) RPS pretreated with disc mill (C) were observed with a 350-fold magnification using a scanning electron microscope

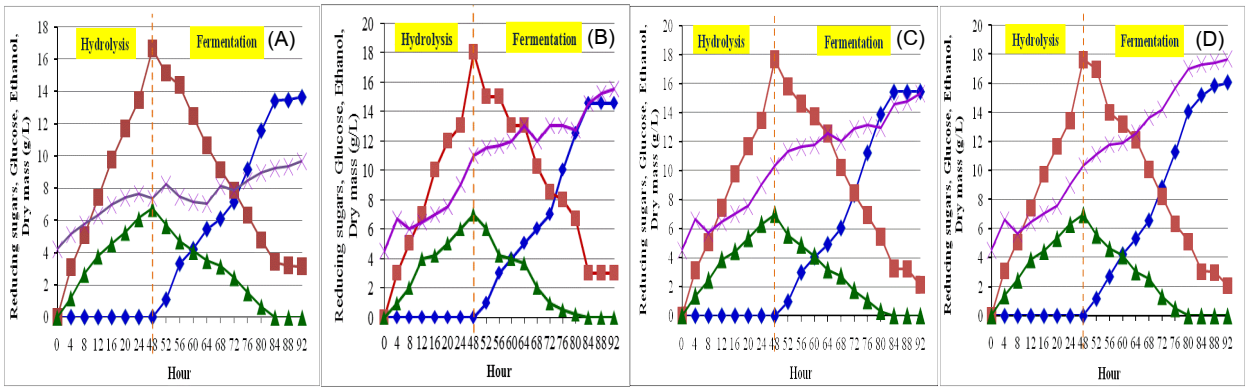


**Figure 2** Time courses of SHF of RPS by *S. cerevisiae* KM 5221 (A), *K. marxinus* 5057 (B), *S. cerevisiae* KM 5221 and *K. marxinus* 5057 $4\times10^{3}$ CFU/mL : $4\times10^{3}$ CFU/mL (C), *S. cerevisiae* KM 5221 and *K. marxinus* 5057 $3\times10^{2}$ CFU/mL : $4\times10^{4}$ CFU/mL (D). ■, ×, ♦ and ▲ stand for reducing sugars, biomass, ethanol and glucose, respectively

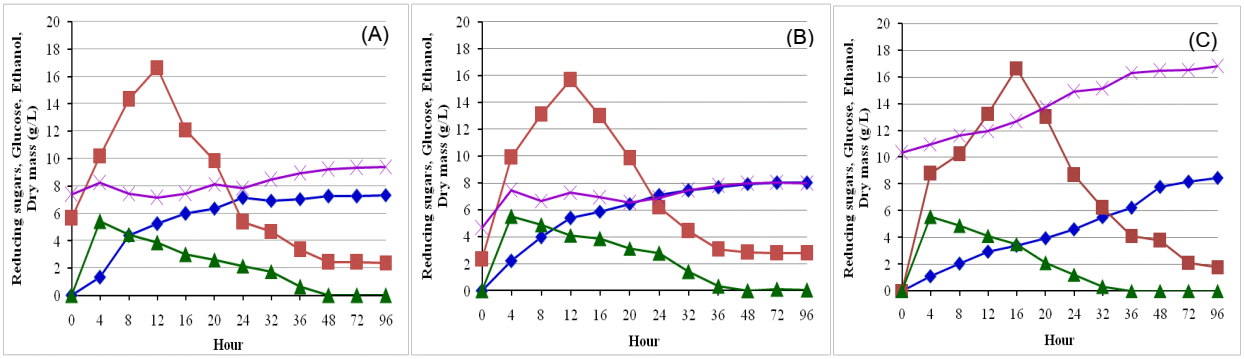


**Figure 3** Time courses of SSF of RPS by *S. cerevisiae* KM 5221 (A), *K. marxinus 5057* (B), *S. cerevisiae* KM 5221 and *K. marxinus* 5057 $3\times10^{2}$ CFU/mL : $4\times10^{4}$ CFU/mL (C). ■, ×, ♦ and ▲ stand for reducing sugars, biomass, ethanol and glucose, respectively

from SHF process by using the co-culture of *S. cerevisiae* KM 5221 and *K. marxinus* 5057 in the ratio of $3\times10^{2}$ : $4\times10^{4}$ CFU/mL, respectively.

SSF reduces equipment cost by performing the hydrolysis and fermentation in a single reactor and reducing the withstanding strong acid or other chemical using SSF technique provides the possibility to overcome the main disadvantage of enzymatic hydrolysis i.e. decreasing the enzyme loading and therefore the production cost. In spite of the economical advantage of SSF over separate hydrolysis and fermentation, the critical problem with SSF is different in temperature optimum of cellulose and fermentation microorganism.

**Table 2** Ethanol yields from SHF and SSF by monoculture of *S. cerevisiae* KM 5221 and *K. marxinus* and co-culture of *S. cerevisiae* KM 5221 and *K. marxinus* in different cell concentration. Monoculture consisted of $8\times10^6$ CFU/mL. Co-culture (A) consisted of *S. cerevisiae* KM 5221 $4\times10^3$ CFU/mL and *K. marxinus* $4\times10^3$ CFU/mL. Co-culture (B) consisted of *S. cerevisiae* KM 5221 $3\times10^2$ CFU/mL and *K. marxinus* $4\times10^4$ CFU/mL

| | Strain | $C_E$** | $P_{MAX}$ (g/L)** | Yp/s** |
|---|---|---|---|---|
| SHF | *S. cerevisiae* KM 5221 | 0.31±0.02[a] | 13.42±0.11[a] | 0.44±0.01[a] |
| | *K. marxinus* 5057 | 0.34±0.06[a] | 14.52±0.14[b] | 0.40±0.03[ab] |
| | Co-culture of *S. cerevisiae* KM 5221 and *K. marxinus* 5057 | | | |
| | (A) | 0.33±0.01[a] | 14.45±0.17[b] | 0.47±0.09[a] |
| | (B) | 0.34±0.071[a] | 16.06±0.12[c] | 0.49±0.07[a] |
| SSF | *S. cerevisiae* KM 5221 | 0.26±0.01[a] | 7.37±0.09[d] | 0.38±0.02[b] |
| | *K. marxinus* 5057 | 0.29±0.03[a] | 7.94±0.14[e] | 0.44±0.01[a] |
| | Co-culture of *S. cerevisiae* KM 5221 and *K. marxinus* 5057 | | | |
| | (B) | 0.19±0.02[b] | 8.45±0.12[f] | 0.49±0.06[a] |

$C_E$ Ethanol yield per unit biomass (g (g-biomass)$^{-1}$)

$P_{MAX}$ Maximum ethanol production (g/L)

Yp/s Production (ethanol) yield coefficient (g (g-total sugar)$^{-1}$)

** Each value is expressed as mean ± SE (n = 3). Different superscript small letters (a, b, c…) within a column are significantly different ($P$ <0.05).

The fermentation of SSF with by *S. cerevisiae* KM 5221 and *K. marxinus* 5057 showed maximum ethanol concentration of 7.37 g/L and 7.94 g/L after 48 h, respectively, while co-culture of *S. cerevisiae* KM 5221 $3\times10^2$ CFU/mL and *K. marxinus* 5057 $4\times10^4$ CFU/mL provided the maximum ethanol yield of 8.45 g/L (Table 2). Co-culture appreciably consumed (95.18 ± 0.68%) the sugar available in hydrolysate when compared with the monoculture.

Our studies indicated that the combined pretreatment of disc mill and diluted acid pretreatment at high temperature was recommended for RPS for enzymatic hydrolysis. The optimal condition to provide the maximum ethanol yield was attained by co-culture in SHF.

## Conclusions

The present results suggested that the complex pretreatment is sufficient for promoting the fermentation of RPS. A substantial increase in the bioconversion of cellulose to ethanol can be obtained by optimum pretreatment prior to enzymatic hydrolysis. The most efficient method of pretreatment was disc mill combined with diluted acid is confirmed to be effective for improving the enzymatic saccharification of RPS. The data suggested that co-culture enhanced the ethanol yield and productivity. Difference in temperature

optimum of cellulase and the fermenting microorganism in SSF process involved in less ethanol production compared to the optimum condition in SHF process.

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