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Bioethanol production from *Yarrowia lipolytica* Po1g biomass Yeshitila A. Tsigie a, Chung-Hsan Wu a, Lien Huong Huynh

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Cantho University, 3-2 Street, Cantho City, Viet Nam c Department of Chemical Engineering, Widya Mendala Catholic University, Surabaya, Indonesia article info Article history: Available online 29 November 2012 Keywords: Acid hydrolysis Bioethanol Glucose Subcritical water *Yarrowia lipolytica* Po1g abstract Bioethanol production from the yeast *Yarrowia lipolytica* biomass was studied. The effects of temperature (90–150 °C) and H₂SO₄ concentration (2–15% w/w) on the saccharification of biomass at a hydrolysis time of 1 h were investigated. A maximum glucose concentration of 35.89 g/L can be produced from defatted biomass using 6% H₂SO₄ at 120 °C. Subcritical water (SCW) pretreatment has negligible effect on maximizing glucose yield. Only 14.53 g/L glucose can be produced using 6% H₂SO₄ at 120 °C if un-defatted biomass was used. The highest ethanol concentration achieved was 13.39 g/L with a corresponding ethanol yield of 0.084 g/g dry biomass (0.38 g ethanol/g glucose). Ó 2012 Elsevier Ltd. All rights reserved. 1. Introduction Increasing concern for rapid population growth, industrializa- tion,

**21the security of oil supply and the negative impact of fossil fuels on
 environment have put pressure on society to find**

renew- able fuel alternatives. Even though different renewable energy alternatives have been suggested, the most commonly practiced and environmentally friendly renewable fuel today is ethanol. Earlier researches were basically focused on ethanol production from sugar or grain.

27Conventionally, bioethanol is produced from the fermentation of starchy

27materials or sucrose-containing feed- stocks such as corn and sugar cane.

**8The yields of bioethanol from corn and sugar are high and the techniques
 are mature, however, this technology increases the risk of causing global
 food shortage**

(Chen et al., 2010). Consequently,

14research efforts have become more focused on low-cost

lignocellulosic feedstock

14originating mainly from agricultural and forest residues along with herba-
ceous materials and municipal wastes.

46Lignocellulosics are the most abundant biomass available on earth,
comprising mainly of cellulose and hemicellulose.

Ferment- able sugars such as glucose and xylose can be released from ligno- cellulosics (Lebeau et al., 1997). Lignocellulosic biomass conversion to bioethanol has long been pursued for its potential to provide an alternative renewable energy source that can substitute fossil fuels. It was suggested that

12future large-scale use of ethanol will most certainly have to be based on
production from lignocellulosic materials. Lignocellulosic substances such as
agricultural

wastes ↑

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091 are attractive feedstocks for bioethanol production since they

17are cost effective, renewable and abundant

(Sarkar et al., 2012). Wastes from vegetables were also used as feedstocks for a di- luted acid hydrolysis process using sulfuric acid as a catalytic. Sug- ars in fresh and processed vegetable wastes (residues from tomato, red pepper, beans, artichoke, lentils and chickpeas) are widely available and easily obtainable and they can be considered as po- tential feedstocks for bioethanol production (del Campo et al., 2006). According to a review, the utilization of algal biomass

17for bioethanol production is undoubtedly a sustainable and eco- friendly
approach for renewable biofuel production suggesting that more

attention is needed for the efficient use of these easily culti- vable microorganisms to generate bioethanol (John et al., 2011). One of the major challenges in bioethanol production is to optimize the integration of process engineering, fermentation technology, enzyme engineering and metabolic engineering (Hahn- Hägerdal et al., 2006).

20The pretreatment step has been recognized as a technological bottleneck
for the cost-effective development of bioprocesses from lignocellulosic
materials

(Conde-Mejia et al., 2012). It has been reported that the extraction and saccharification of easily fermentable carbohydrates from dif- ferent sources such as weed, grasses, saw dust, municipal solid waste, waste paper or pulp and paper mill wastes is the necessary step in the production of bioethanol. Cell disruption is often re- quired to recover the intracellular products of microalgae, such as oil and starch for biodiesel and ethanol production, respectively, as well as added-value compounds (Brennan and Owende, 2010).

3Proper pretreatment can increase concentrations of ferment- able sugars
after enzymatic saccharification, thereby improving the efficiency of the
whole process.

Conversion of glucose as well Y.A. Tsigie et al. / Bioresource Technology 145 (2013) 210–216 211

49 **as xylose to ethanol needs some new fermentation technologies, to make the whole process cost effective**

(Sarkar et al., 2012). Different methods of biomass pretreatment that have been studied include steam explosion of wheat straw (Bellido et al., 2011), acid hydrolysis of wood biomass and ionic liquids-based chemical hydrolysis of Chlorella biomass (Zhou et al., 2011). A previous study showed that subcritical water assisted pretreatment can increase the amount of extractable lipids from yeast cells by two fold (Tsigie et al., 2012a). For algal cell wall disruption, both physical and chemical methods have been tested. Chemical lysis is a method where chemical agents (acid or alkaline) must be added in order to hydrolyze biomass into its constituent molecules (Lee et al., 2010). After the disruption, carbohydrates are released from the intracellular medium requiring further processing to obtain monosaccharides (Brennan and Owende, 2010). Currently, biomass to ethanol conversion technology relies mainly on chemical and enzymatic treatments. Chemical hydrolysis

6 **of biomass with dilute sulfuric acid has long been recognized as a critical step for removing the hemicellulosic fraction from ligno-cellulosic substrate to economize the biological conversion of cellulosic biomass to ethanol. However, the**

pentose sugar-rich acid hydrolysate also contains toxic byproducts such as furfural, hydroxy methyl furfural (HMF) and phenolics, which significantly affect yeast cell metabolism during fermentation

3 (Chandel et al., 2007; Palmqvist and Hahn-Hägerdal,

2000). Although various detoxification

23 **methods have been investigated for the removal of fermentation inhibitory compounds**

3 (Chandel et al., 2007; Palmqvist and Hahn-Hägerdal,

2000), overliming, activated charcoal adsorption and base neutralization methods are widely used (Gupta et al., 2009; Miyafuji et al., 2003; Tsigie et al., 2011). The ascomycetous *Yarrowia lipolytica* is

9 **one of the non-conventional yeasts used as a model for the study of protein secretion, dimorphism, hydrophobic substrate degradation, biolipid production and other related fields**

(Fickers et al., 2011). Previous studies about lipid and biodiesel production from this yeast biomass also showed its potential applications in biofuel research (Tsigie et al., 2012a,b, 2011). In this study, *Y. lipolytica* Po1g biomass was used

25 **as a feedstock for glucose and bioethanol production. In acid hydrolysis and**

subcritical water pretreatment, the effects of temperature, acid concentration and biomass loading on the amount of glucose were investigated and the optimal conditions were determined. 2. Methods 2.1. Materials *Y. lipolytica* Po1g was obtained from Yeastern Biotech Company (Taipei, Taiwan). Ethanol Red *Saccharomyces cerevisiae* was purchased from Fermentis (Marcq en Baroeul Cedex, France). Ethanol (95%) and methanol (HPLC grade, 99.9% purity) were supplied by Echo, Taiwan. Acetonitrile (99.9% purity, HPLC grade) and furfural (99% purity) were obtained from Sigma-Aldrich (St. Louis, USA) while 5-hydroxymethylfurfural (5-HMF, 98% purity) was purchased from Alfa Aesar Company (Lancaster, UK). Agar pure powder, anhydrous ACS reagent grade, D(+)-glucose, D(+)-xylose (99% purity), L(+)-arabinose (99% purity), potassium hydroxide pellets, sodium hydroxide pellets, dinitrosalicylic acid (99% purity) and potassium sodium tartrate (99% purity) were obtained from Acros Organics (NJ, USA). Glacial acetic acid (99.7% purity) was bought from Mallinckrodt (Austin, USA) and sulfuric acid (98% purity) was obtained from Scharlau (Barcelona, Spain). Peptone and yeast extract as nitrogen sources for microbial growth were supplied by Bacto (Mt Pritchard, Australia). Different types of filter papers were purchased from Advantec (Dublin, USA). 2.2. *Y. lipolytica* Po1g biomass production *Y. lipolytica* Po1g cells were placed on a sterilized agar plate containing a medium composed of

39 **10 g/L yeast extract, 20 g/L pep- tone, 20 g/L glucose, and 20 g/L agar.** Then **the**

plate was incubated at 26 °C over night and stored in a refrigerator at 4 °C until use. A 24 h small scale culture was prepared by inoculating a single colony of *Y. lipolytica* Po1g into a

40 **medium containing yeast extract (10 g/L), peptone (20 g/L) and glucose (20 g/L).** After that, **a**

large scale culture was prepared by inoculating a 24 h culture seed into an Erlenmeyer flask containing 250 mL YPD medium made of

43 **yeast extract (10 g/L), peptone (20 g/L) and glucose (20 g/L)** with an inoculum **to** YPD medium ratio of

1–10 (v/v). The culturing was carried out at 26 °C in an orbital shaker incubator. Cell mass was harvested by centrifugation. The harvested cells were washed with deionized (DI) water and dried in a freeze dryer (Free Zone 2.5 Benchtop model 7670520, Labconco, USA). All media and flasks were autoclaved at 121 °C for 40 min before use. 2.3. Lipid extraction and cell wall composition determination

33 **The dried biomass was ground into powder by using** an agate mortar **and**

pestle. For lipid extraction, the biomass powder was put in a cellulosic thimble

7 **and placed in a Soxhlet extractor. Extraction was carried out** for 10 h **using**

350 mL methanol and hexane (1:1 v/v). The residue after extraction was dried in an oven at 50 °C. Cellulose and hemicellulose contents of the dried biomass were determined according to the

38 **method of the National Renew- able Energy Laboratory (Sluiter et al.,**

2008). 2.4. Subcritical water (SCW) pretreatment of biomass Freeze-dried defatted biomass sample (1.0 g) was put in a sub- critical reactor and 10 mL DI water was added. Nitrogen was intro- duced into the reactor until pressure in the reactor reached 20 bar. The mixture in the reactor was then heated to a pre-determined temperature (100, 120, 150 or 180 °C). After 1 h, pressure in the reactor was reduced to ambient pressure and the

16 **reactor was then cooled down to room temperature.** Solid residue in **the** mixture **was** collected **and**

freeze-dried for 2 days and stored at 4 °C for fur- ther study. 2.5. Acid hydrolysis of dry biomass The dried biomass was hydrolyzed with different biomass to sulfuric acid ratios (1:8, 1:10, 1:12 and 1:15 w/w) at different tem- peratures (90, 100, 121, 135 and 150 °C) and different acid concen- trations (2, 4, 6, 10 and 15 wt.%). The reaction time was set at 1 h. 2.6. Neutralization of hydrolysate The biomass acid hydrolysate was constantly stirred at room temperature while KOH (1 M) was slowly added until pH of the solution was 6.0. Then the solution

16 **was centrifuged to remove** pre- cipitate. **The supernatant**

was then

4 **filtered through a 0.22 μ m PVDF syringe filter**

(Tokyo, Japan) and stored at 4 °C. 2.7. Ethanol production Ethanol Red[®] *S. cerevisiae* cells were maintained on a sterilized YPD agar plate

26 containing yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) and agar (20 g/L). The plate was

then

32 incubated in an orbital shaker incubator at 37 °C for 24 h

and then stored at 212 Y.A. Tsigie et al. / Bioresource Technology 145 (2013) 210–216 4 °C. A single colony of *S. cerevisiae* was incubated in a YPD medium at 37 °C for 1 h and then transferred (inoculum to medium ratio = 1:10, v/v) to a flask containing a medium composed of the hydrolysate (with total glucose concentration = 35.89

15 g/L), peptone (20 g/L) and yeast extract (10 g/L). Then, it was incubated in

an orbital shake incubator at 37 °C. During fermentation, sample was taken for analysis of glucose and ethanol concentration at 0, 3, 6, 9, 12, 24 and 48 h. 2.8. Sugar analysis Concentrations of D-glucose, D-xylose and L-arabinose in the hydrolysate were determined by a HPLC equipped with a PU-1580 pump (Jasco, Japan), a DG-4400 degasser (Phenomenex, USA), an Alltech 2000 ELSD detector (Alltech, USA) and a Zorbax NH2

30 column (5 μm, 250 × 4.6 mm, Agilent Technologies, USA). The

16 sample was diluted appropriately with DI-water, filtered through a

40.22 μm PVDF syringe filter and then injected into the HPLC.

Temperature of the ELSD detector and nitrogen flow rate were 80 °C and 2 mL/min, respectively. The column temperature was 25 °C. A mixture of acetonitrile and water

42 (80:20, v/v) was employed as the mobile phase with a flow rate of 1 mL/min. The injection volume was

25 μL.

35 The concentrations of D-glucose, D-xylose and L-arabinose were calculated by

using calibration curves obtained from standard solutions of these compounds.

33 The amount of total reducing sugars in the hydrolysates was measured by the Miller method

(Miller, 1959)

38 based on a colorimetric reaction between sugars and dinitrosalicylic acid.

The samples were monitored at 540 nm using a spectrophotometer (V-550, Jasco, Japan).

22 The concentration of total sugars was calculated by using calibration curve obtained from

standard D-glucose solution. 2.9. Inhibitor analysis Concentrations of 5-hydroxymethylfurfural (5-HMF) and furfural in the hydrolysate were determined by a HPLC equipped with a PU-2089 pump (Jasco, Japan) combined with a degasser, an UV-2077 detector (Jasco, Japan) and a Luna

30C-18 column (5 μ m, 250 \times 4.6 mm, Phenomenex, USA). The

sample was diluted appropriately with DI-water, filtered through a

40.22 μ m PVDF syringe filter and then injected into the HPLC

32under a column temperature of 25 $^{\circ}$ C. Acetonitrile: water:

acetic acid (11:88:1,

24v/v) with a flow rate of 0.8 mL/min was the mobile phase. The injection volume was 25 μ L. The absorption wavelength was 276 nm.

The concentrations of 5-HMF and furfural were calculated by using calibration curves obtained from standard solutions of these compounds. 2.10. Ethanol analysis The concentration of ethanol was determined by a GC 14B (Shimadzu, Japan) with a FID detector and a Stabilwax-DA column (fused silica, polar phase; crossbonded carbowax polyethylene glycol, Restec, USA). The

34sample was filtered through a 0.22 μ m PVDF syringe filter and

then injected into the GC. The column temperature was 40 $^{\circ}$ C while

47the injector and detector temperatures were set at 200 $^{\circ}$ C and 250 $^{\circ}$ C, respectively. The

injection volume was 1 μ L.

22The concentration of ethanol was determined by using a calibration curve obtained from

standard ethanol solutions.

353. Results and discussion 3.1. Composition of

defatted *Y. lipolytica* P01g biomass Lignocellulosic composition of biomasses differs depending on the type and origin of the biomass. Plant cellulose and hemicellulose contents range from 25% to 58% and 10% to 35%, respectively (Mohamed et al., 2010; Schädel et al., 2010). In this study, cellulose and hemicellulose contents in the dried, defatted yeast biomass was found to be 43.09 and 2.81 wt.%, respectively. It has also been reported that carbohydrate composition of microalgae varies from strain to strain and a significant amount of carbohydrates was found to be glucose suggesting

45the species with a high potential for use as a fermentation feedstock for bioethanol production.

Green algae species have 20–40 wt.% cellulose and 20–50 wt.% hemicellulose suggesting that different species have different lignocellulose compositions (Balat, 2011). In this study, the high cellulose content of *Y. lipolytica* biomass relative to its lignocellulose content suggests the existence of high concentration of glucose relative to pentose sugars upon hydrolysis. 3.2. Effect of hydrolysis temperature An increase in temperature in hydrolysis results in higher reaction rate, hence shorter time to reach high glucose concentration. But if the temperature is too high, sugar will be degraded into compounds such as 5-HMF and furfural that are toxic for *S. cerevisiae* in fermentation. Fig. 1 shows the effect of temperature on the hydrolysis of biomass. It can be seen that

5glucose is the most abundant sugar in the hydrolysate. Both xylose

and arabinose concentrations are very low (0.90 g/L and 1.43 g/L, respectively). Hence total sugar concentration is almost equivalent to glucose concentration in the hydrolysate. Hydrolysis at higher temperature (120 °C) yielded higher glucose concentration (35.89 g/L) than those obtained at lower temperatures (1.37 g/L and 10.89 g/L for 90 °C and 100 °C, respectively). Too high temperature (135 °C and 150 °C) not only resulted in reduced glucose concentrations (27.58 g/L and 10.98 g/L, respectively) but also increased the inhibitor concentrations. 5-HMF and furfural are the decomposition products of hexoses and pentoses, respectively. 5-HMF and furfural concentrations are very low at temperature up to 100 °C. At 120 °C, HMF and furfural concentrations are

10.24 g/L and 0.17 g/L,

respectively, but increased to 1.12

29g/L and 0.43 g/L, respectively at 135 °C. However,

at 150 °C, 5-HMF and furfural concentrations

29decreased to 0.75 g/L and 0.35 g/L, respectively while the

concentrations of other unknown products increased as observed from HPLC results (data not shown). Based on total sugar concentration and inhibitor concentration, 120 °C was chosen as the optimum hydrolysis temperature. It was reported that at fixed acid concentration and reaction time, glucose concentration decreased when temperature is higher than the optimum temperature due to the degradation of glucose (Palmarola-Adrados et al., 2005). Similar results were obtained when *G. amansii* (a macroalgae) was used in the sulfuric acid catalyzed hydrolysis (Jeong et al., 2012). 3.3. Effect of sulfuric acid concentration

31It was observed that increasing acid concentration resulted in higher glucose

concentration but degradation also occurred when acid concentration was too high. Fig. 2 shows the effect of acid concentration on the hydrolysis of defatted biomass.

34A maximum glucose concentration of 35.89 g/L was obtained

when 6% sulfuric acid was used. At acid concentration higher than 6%, not only lower glucose concentration was obtained but an increment in inhibitor concentration was observed. 5-HMF was not detected when using 2% sulfuric acid. At 6%, the concentration of 5-HMF

36and furfural were 0.24 g/L and 0.19 g/L,

respectively. When acid concentration was raised to 10%, the concentration of HMF

36and furfural became 0.30 g/L and 0.24 g/L,

respectively. Y.A. Tsigie

28et al. / *Bioresource Technology* 145 (2013) 210–216 213 Fig. 1. Effect of

temperature on glucose, 5-HMF and furfural concentrations. Reaction conditions: 6% sulfuric acid, 1 h, biomass to acid solution ratio 1:10 (w/w). Defatted biomass without SCW pretreatment was used. Fig. 2. Effect of sulfuric acid concentration on glucose, 5-HMF and furfural concentrations. Reaction conditions: 120 °C, 1 h, biomass to acid solution ratio 1:10 (w/w). Defatted biomass without SCW pretreatment was used. A previous study reported that in the hydrolysis of sugarcane bagasse, increasing HCl concentration from 1% to 2.5% increased

41the total sugar concentration in the hydrolysate from 9.89 to

21.38 g/L (Tsigie et al., 2011). However, when acid concentration was raised to 3%, total sugar concentration obtained decreased to 17.71 g/L. This was caused by the decomposition of hexose to 5-HMF

and pentose to furfural. The highest 5-HMF and furfural concentrations were obtained (0.83 and 0.15 g/L, respectively) when 3% HCl was used. In the hydrolysis of *G. amansii* (a macroalgae) biomass, increasing sulfuric acid concentration from 3% to 4% resulted in sharp increases of glucose decomposition products such as

185-HMF, formic acid and levulinic acid by 23.6%, 64.7% and 51.2%,

respectively while

18glucose yield decreased by 12.5%

(Jeong et al., 2011). 3.4. Effect of biomass loading A change of biomass to acid solution ratio from 1:8 to 1:12 did not result in significant differences in the amount of glucose produced. However, at a ratio of 1:15, glucose concentration decreased significantly as shown in Fig. 3. Similar results were reported when

Glucose (g/L)	40	HMF (g/L)	0.39	35	30	Glucose (g/L)	25	0.26	20	15	0.13	10	
HMF (g/L)	5	0	0.00	1:8	1:10	1:12	1:15	Biomass to acid solution ratio (w/w) Fig. 3. Effect of biomass to acid solution ratio on glucose, HMF and Furfural concentrations. Reaction conditions: 6% sulfuric acid, 120 °C, 1 h. Defatted biomass without SCW pretreatment was used. 214 Y.A. Tsigie					

28et al. / Bioresource Technology 145 (2013) 210–216 Fig. 4. Effect of SCW pretreatment temperature on

glucose, 5-HMF and furfural concentrations. Reaction conditions: biomass to acid solution ratio 1:10, 120 °C, 6% sulfuric acid, 1 h. Defatted biomass was used. microalgal biomass was hydrolyzed through acid pretreatment (Harun and Danquah, 2011). From Figs 1–3, it can be observed that the effect of temperature on glucose concentration as well as concentration of inhibitors is more pronounced than the effect of acid concentration. The results are similar to those reported in the hydrolysis of potato skin using phosphoric acid as the catalyst in which it was found that degradation of sugars was mainly affected by temperature (Lenihan et al., 2010). Under the optimum conditions (biomass to acid solution ratio 1:10, hydrolysis temperature 120 °C, sulfuric acid concentration 6%), the hydrolysate obtained

5in this study contained 35.89 g/L glucose, 0.24 g/L

HMF and 0.17 g/L furfural. 3.5. Effect of SCW pretreatment Fig. 4 shows the effect of temperature during SCW pretreatment on acid-catalyzed hydrolysis of defatted biomass. Glucose concentration increased with temperature from 100 to 120 °C. However, further increase in temperature resulted in decreasing glucose concentration. It was found that SCW pretreatment did give higher glucose concentration while inhibitor concentration was much higher than that without SCW pretreatment. It was reported that glucose recovery of enzymatic hydrolysis was enhanced if oil palm fronds were SCW pretreated (Goh et al., 2010). But according to the results of this study, SCW pretreatment was unsuccessful for biomass of *Y. lipolytica*. SCW pretreatment is Table 1 Effect of lipid extraction on the concentrations of glucose, HMF and furfural.

Reactions

48were carried out at 120 °C, 1 h and a solid to liquid ratio of 1: 10 (g/mL)

on biomass without SCW pretreatment. Acid concentration Concentration (g/L) (% w/w) Glucose HMF Furfural 2 7.51 ± 0.82a 14.86 ± 0.25b 4 13.84 ± 1.78 16.92 ± 1.20 6 14.53 ± 2.17 35.89 ± 1.90 10 14.41 ± 2.07 28.27 ± 1.67 0.013 ± 0.006 ND 0.093 ± 0.015 0.04 ± 0.00 0.160 ± 0.000 0.24 ± 0.01 0.213 ± 0.012 0.30 ± 0.03 0.167 ± 0.012 0.19 ± 0.00 0.097 ± 0.006 0.09 ± 0.01 0.123 ± 0.005 0.17 ± 0.00 0.177 ± 0.021 0.24 ± 0.01 ND = not detected. a Non-defatted biomass. b Defatted biomass. considered to be a hydrolysis without using acid as catalyst. Further acid hydrolysis of the pretreated biomass reduced glucose concentration and raised inhibitors concentration. The cell wall of yeast may not be as rigid as that of lignocellulosic biomass of plant origin, which contains lignin. Thus yeast cell wall does not require subcritical water pretreatment. If yeast cell is pretreated, it results in too high degree of hydrolysis and significant sugar degradation. 3.6. Effect of lipid extraction *Y. lipolytica* is an oleaginous microorganism which can accumulate large amount of lipid in its biomass. The effect of biomass lipid removal on the amount of glucose and inhibitors concentrations in the hydrolysate was investigated and the results are shown in Table 1. It can be seen that glucose concentration is much lower in the hydrolysate of non-defatted biomass than in that of defatted biomass. The biggest difference in glucose concentration is 21.36 g/L which occurred at an acid concentration of 6%. Apparently the presence of lipid in biomass had negative effect on hydrolysis. Harun et al. (2010) used the biomass of microalgae *Chlorococum* sp. as substrate for bioethanol production through fermentation by 40 35 30 Glucose Concentration (g/L) Ethanol 25 20 15 10 5 0 0 12 24 36 48

23 **Time (h) Fig. 5. Time courses of glucose and ethanol concentration in fermentation.**

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1 **et al. / Bioresource Technology 145 (2013) 210–216 215 Table 2 Sugar and**

ethanol concentrations using various biomass and with different pretreatment or hydrolysis methods. Microorganism Pretreatment conditions Maximum glucose Maximum ethanol Ethanol yield Reference conc. (g/L) conc. (g/L) C. saccharoperbutylacetonicum Chlamydomonas reinhardtii Chlorella biomass Chlorococcum humicola Yarrowia lipolytica Po1g Pretreatment with H₂SO₄ or NaOH, 90 °C, 30 min 0.2% enzyme, 55 °C, 30 min 2% HCl, 2% MgCl₂, 180 °C, 10 min 1% H₂SO₄, 140 °C, 25 min 6% H₂SO₄, 120 °C, 60 min 8.92 1.24 5.46 11.73 46.17 22.60 – 7.2 35.89 13.39 0.14 g/g total sugar 0.235

25 **g/g microalgae 0.47 g/g glucose 0.52 g/g microalgae 0.38 g/g**

glucose Ellis

31 **et al. (2012) Choi et al. (2010) Zhou et al. (2011) Harun and Danquah (2011)**

This study Saccharomyces bayanus.

19 **A maximum ethanol concentration of 3.83 g/L was obtained from 10 g/L of lipid-extracted microalgae debris. The lipid-extracted microalgae**

gave 60% higher ethanol concentration than the dried and intact microalgae. The high glucose concentration from lipid free biomass compared to biomass with lipid might be because the acid catalysis facilitated lipid removal prior to biomass hydrolysis for sugar production. 3.7. Fermentation and ethanol production Every organism must find in its environment all nutrients required for energy generation and cellular synthesis. Factors

13 **such as carbon source, nitrogen source, C/N molar ratio and culture conditions (temperature and pH) have significant influences on cell growth, glucose consumption and ethanol accumulation of**

ethanol fermentation microorganism (Tsigie et al., 2011). For *S. cerevisiae*, the most important and common carbon source is glucose. If the *S. cerevisiae* used in fermentation is not genetically modified, it can use glucose, but not xylose and arabinose, as the carbon source and generate ethanol under anaerobic condition. In this study, Ethanol Red *S. cerevisiae* was employed in the fermentation process using *Y. lipolytica* biomass hydrolysate as the medium. Fig. 5 shows the time courses of glucose and ethanol concentration during fermentation. After 12 h most glucose was consumed while ethanol concentration reached 12.53 g/L. After 48 h, 98.05% of glucose was consumed and final ethanol concentration was 13.39 g/L, corresponding to a yield of 0.38

37 **g ethanol/g glucose. The saccharification yield was 0.224 g glucose /g dry biomass and the ethanol yield was 0.084 g ethanol/g**

dry weight. A maximum conversion of 0.115 g glucose/g corn fiber was reported when corn fiber was subjected to alkali pretreatment (Shrestha et al., 2010). Mathew et al. (2011) obtained a maximum glucose yield of 0.441 g/g biomass when oilseed rape straw was subjected to alkali pretreatment. This study gave a maximum glucose

5 **yield of 0.224 g/g dry biomass and productivity of 0.37 g glucose /h/**

L. 3.8. Comparison with other studies In this work, the glucose containing hydrolysate from *Y. lipolytica* biomass was used as the medium in the fermentation by *S. cerevisiae* with the final goal of producing ethanol. Table 2 shows comparison of results of this study with those reported in literature. The highest ethanol concentration (22.60 g/L) was obtained when *Chlorella* biomass was hydrolyzed (2% HCl and 2.5% MgCl₂) to give a glucose concentration of 46.17 g/L in the hydrolysate. On the other hand, acid or base

pretreatment of *Clostridium saccha- roperbutylaceticum* biomass resulted in 8.92 g/L glucose and led to 1.24 g/L maximum ethanol production.

41 In this study, the a glucose concentration of 35.89 g/L

was obtained when defatted biomass was hydrolyzed with 6% sulfuric acid at 120 °C for 1 h. After 48 h fermentation, the ethanol concentration achieved was 13.39 g/L. 4. Conclusion Under optimum conditions (120 °C, biomass to acid solution ratio of 1:10, 6 wt.% sulfuric acid), the hydrolysate contained 35.89 g/L glucose. Temperature was the most important parameter that affected hydrolysis. Glucose was the predominant sugar in the hydrolysate indicating its availability for microbial fermentation. During fermentation, glucose concentration

15 decreased to 0.7 g/L and ethanol concentration

reached 13.39 g/L after 48 h and

44 98.05% of glucose was consumed; corresponding to a yield of 0.38 g ethanol/g glucose.

The saccharification yield was 0.224 g glucose/g dry weight and the ethanol yield was 0.084 g ethanol/g dry weight. Acknowledgements This work was funded by National Science Council of Taiwan with Grant number NSC-98-2221-E-011-046-MY3. References Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. *Energy Conversion and Management* 52 (2), 858–875. Bellido, C., Bolado, S., Coca, M., Lucas, S., González-Benito, G., García-Cubero, M.T., 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. *Bioresource Technology* 102 (23), 10868–10874. Brennan, L., Owende, P., 2010. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* 14 (2), 557–577. Chandel, A.K., Kapoor, R.K., Singh, A., Kuhad, R.C., 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresource Technology* 98 (10), 1947–1950. Chen, W.-H., Tu, Y.-J., Sheen, H.-K., 2010. Impact of dilute acid pretreatment on the structure of bagasse for bioethanol production. *International Journal of Energy Research* 34 (3), 265–274. Choi, S.P., Nguyen, M.T., Sim, S.J., 2010. Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. *Bioresource Technology* 101 (14), 5330–5336. Conde-Mejía, C., Jiménez-Gutiérrez, A., El-Halwagi, M., 2012. A comparison of pretreatment methods for bioethanol production from lignocellulosic materials. *Process Safety and Environmental Protection* 90 (3), 189–202. del Campo, I., Alegría, I., Zazpe, M., Echeverría, M., Echeverría, I., 2006. Diluted acid hydrolysis pretreatment of agri-food wastes for bioethanol production. *Industrial Crops and Products* 24 (3), 214–221. Ellis, J.T., Hengge, N.N., Sims, R.C., Miller, C.D., 2012. Acetone, butanol, and ethanol production from wastewater algae. *Bioresource Technology* 111, 491–495. Fickers, P., Marty, A., Nicaud, J.M., 2011. The lipases from *Yarrowia lipolytica*: genetics, production, regulation, biochemical characterization and biotechnological applications. *Biotechnology Advances* 29 (6), 632–644. Goh, C.S., Lee, K.T., Bhatia, S., 2010. Hot compressed water pretreatment of oil palm fronds to enhance glucose recovery for production of second generation bio- ethanol. *Bioresource Technology* 101 (19), 7362–7367. Gupta, R., Sharma, K.K., Kuhad, R.C., 2009. Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulose ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. *Bioresource Technology* 100 (3), 1214–1220. 216 Y.A. Tsigie et al. / *Bioresource Technology* 145 (2013) 210–216 Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G., Zacchi, G., 2006. Bio-ethanol – the fuel of tomorrow from the residues of today. *Trends in Biotechnology* 24 (12), 549–556. Harun, R., Danquah, M.K., 2011. Influence of acid pre-treatment on microalgal biomass for bioethanol production. *Process Biochemistry* 46 (1), 304–309. Harun, R., Danquah, M.K., Forde, G.M., 2010. Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemical Technology and Biotechnology* 85 (2), 199–203. Jeong, T.S., Choi, C.H., Lee, J.Y., Oh, K.K., 2012. Behaviors of glucose decomposition during acid-catalyzed hydrothermal hydrolysis of pretreated *Gelidium amansii*. *Bioresource Technology* 116, 435–440. Jeong, T.S., Kim, Y.S., Oh, K.K., 2011. Two-stage acid saccharification of fractionated *Gelidium amansii* minimizing the sugar decomposition. *Bioresource Technology* 102 (22), 10529–10534. John, R.P., Anisha, G.S., Nampoothiri, K.M., Pandey, A., 2011. Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresource Technology* 102 (1), 186–193. Lebeau, T., Jouenne, T., Junter, G.A., 1997. Simultaneous fermentation of glucose and xylose by pure and mixed cultures of *Saccharomyces cerevisiae* and *Candida shehatae* immobilized in a two-chambered bioreactor. *Enzyme and Microbial Technology* 21 (4), 265–272. Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology* 101 (Suppl. 1), S75–S77. Lenihan, P., Orozco, A., O'Neill, E., Ahmad, M.N.M., Rooney, D.W., Walker, G.M., 2010. Dilute acid hydrolysis of lignocellulosic biomass. *Chemical Engineering Journal* 156 (2), 395–403. Mathew, A.K., Chaney, K., Crook, M., Humphries, A.C., 2011. Alkaline pre-treatment of oilseed rape straw for bioethanol production: evaluation of glucose yield and pre-treatment energy consumption. *Bioresource Technology* 102 (11), 6547–6553. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry* 31 (3),

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