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2nd Generation Ethanol by Zygomycetes Fungi at Elevated Temperature

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Abstract

Seven zygomycetes tempe isolates were screened for ethanol production at 40°C, 45°C, 50°C. All the isolates were able to produce ethanol at 40°C with the yields of 0.39-0.47 g/g on glucose and three isolates were able to produce ethanol at 45°C with the yields of 0.22-0.26 g/g on glucose. No isolate could grow and produce ethanol at 50°C. One isolate was selected and used for ethanol production from untreated and fungal-pretreated oil palm empty fruit bunch (OPEFB), untreated and fungal-pretreated oat straw as well as Avicel by non-isothermal simultaneous saccharification and fermentation (NSSF). This NSSF included hydrolysis with cellulase and β-glucosidase for 24 h at 45°C, followed by simultaneous saccharification and fermentation using the selected isolate at 40°C for 96 h. The NSSF of untreated and fungal-pretreated OPEFB, untreated and fungal-pretreated oat straw, and Avicel resulted in 14.10%, 14.25%, 27.28%, 49.88%, and 63.77% ethanol compared to the theoretical yield.

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Keywords: Ethanol; Oil Palm Empty Fruit Bunch; Oat Straw; Tempe; Zygomycetes

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1. Introduction

Ethanol is now the dominant biofuel in the global market. Currently, it is produced from sugars and starch-based materials, while lignocelluloses are the forthcoming raw materials for the second generation of bioethanol production in the future [1, 2]. Oil palm empty fruit bunch (OPEFB) and oat straw are lignocellulosic waste materials that are available in large amount, and thus very potential to be used as feedstock for bioethanol production. Indonesia is one of the largest oil palm producers in the world, produced 101.7 million metric tons of oil palm fruit in 2011 and accumulated about 23.4 million tons of OPEFB [3]. Oat is mainly found in Europe and North America. Its annual global production reached 24.2 million tons with oat straw estimated to be available in 11 million tons per year [4].

For efficient conversion of lignocelluloses to ethanol, it is essential to perform a pretreatment process in order to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials [5]. Biological pretreatment using white-rot fungi have been intensively investigated for several purposes due to its mild environmental condition and low energy requirement [6]. White-rot fungi produce ligninolytic enzymes, which could degrade lignin efficiently [7].

In searching for ethanol-producing microorganisms, zygomycetes attracts interest to be a good alternative ethanol producer. Zygomycetes are primarily saprophytic, capable of growing in limited conditions and capable of assimilating different kind of sugars. In Indonesia, some species belonging to zygomycetes have been used in the production of tempe (fermented soybean) for centuries. Tempe has been widely consumed by Indonesian people and for the species used in tempe production could thus be considered as GRAS (generally recognized as safe) microorganisms [8]. Our previous studies showed that zygomycetes have several advantages over *Saccharomyces cerevisiae* such as (a) capable of utilizing xylose, (b) capable of producing ethanol at 37 ^{o}C with comparable yield and productivity to *S. cerevisiae* at 30 ^{o}C, (c) its biomass contains chitosan, which can be extracted for useful applications [9, 10, 11].

In this work, several zygomycetes tempe isolates were investigated to evaluate their performance in growing at elevated temperatures. Fermentation at elevated temperature has potential to reduce the amount of cellulase required for enzymatic hydrolysis in simultaneous saccharification and fermentation (SSF), accelerate fermentation rate, and reduce the risk of contamination. The fungal isolates were grown and screened under anaerobic condition at 40°C, 45°C, and 50°C on glucose medium. One isolate with the best performance was used for ethanol production from OPEFB and oat straw using non-isothermal simultaneous saccharification and fermentation.

2. Materials and Methods

2.1. Fungal isolates

Seven different zygomycetes isolated from tempe inoculums in Yogyakarta, Indonesia (R8, R10, R17, R21, R23, A1, 41) were used in this work [10]. The fungi were maintained on potato glucose agar (PGA) medium containing peptone 10 g/L, agar 20 g/L, D-glucose 40 g/L. Cultures were grown aerobically at 30^{o}C for 5 days and stored at 4^{o}C. Spore suspension was prepared by addition of 3.5 mL sterile distilled water into slant and shaking it vigorously.

2.2. Substrate for non-isothermal simultaneous saccharification and fermentation (NSSF)

The substrates for NSSF were (a) untreated OPEFB, (b) pretreated OPEFB, (c) untreated oat straw, and (d) pretreated oat straw. Pure cellulose Avicel was also used as reference. The OPEFB was obtained from an oil palm mill in Sumatra, Indonesia, while the oat straw was purchased from local pet shop in Borås, Sweden. The pretreatment applied on OPEFB and oat straw was fungal pretreatment using *Pleurotus floridanus LIPIMC* for 28 days according to Isroi *et al.* [12]. The untreated OPEFB and the untreated oat straw were milled and sieved through 40 mesh screens, and kept in an airtight container at room temperature before use while the pretreated materials were kept in freezer and freeze-dried before being milled. Table 1 presents the cellulose content of the lignocellulosic materials, which were determined according to NREL methods [13]. The cellulose content in Avicel was 99.7% according to the supplier.

Table 1. Composition of OPEFB and Oat Straw Before and After Fungal Pretreatment

Material	% Components		
	Lignin	Hemicellulose	Cellulose
Untreated OPEFB	34.32	23.94	39.13
Untreated oat straw	16.30	24.33	46.30
Pretreated OPEFB	35.16	20.83	34.17
Pretreated oat straw	17.60	16.1	40.3

2.3. Anaerobic fermentation for the selection of fungal isolates

The isolates were cultivated under anaerobic condition in 150 mL medium in 300 mL E-flask equipped with loop trap at 40, 45, and 50°C for 60 h. The medium consisted of (in g/L): Glucose 50, Yeast Extract 5, KH_2PO_4 5, $MgSO_4.7H_2O$ 0.75, $(NH_4)_2SO_4$ 7.5, and $CaCl_2.2H_2O$ 3.5. One mL of spore suspension containing $5x10^5$ spores was added into each flask as inoculum. Pure nitrogen gas was sparged into the medium in the beginning of the cultivation and during the sampling. The metabolites profile was examined using HPLC.

2.4. Non-isothermal simultaneous saccharification and fermentation (NSSF)

For the purpose of NSSF, a solution of 3% (w/v) solid substrates in 67.5 mL of 50 mM citrate buffer at pH 4.8 was autoclaved in E-flasks capped with aluminium foil. Cellulase of 15 FPU/g (celluclast 1.5 L, Novozyme, Denmark) and β-glucosidase of 30 IU/g (Novozyme 188) were added and incubated for 24 h at 45°C. The mixture was then supplemented with 7.5 mL medium containing (g/L): yeast extract, 5; $(NH_4)_2SO_4$, 7.5; KH_2PO_4, 3.5; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 1. The medium was inoculated with 1.0 mL of spore suspension (2.6×10^6 spores), cultivated at 40°C for 4 days. During this second step, the flasks were equipped with anaerobic loop-trap and sterile plastic tube with sterile 0.2 μm filter and clamp for taking samples. Nitrogen gas filtered with sterile 0.22 μm filter was sparged into the flasks in order to provide anaerobic condition. The metabolites's profiles were traced using HPLC.

2.5. Analysis

The liquid samples were analyzed by HPLC (Waters, Milford, USA) equipped with UV (Waters 2487) and RI detector (Waters 2414). Glucose, glycerol, ethanol, acetic acid, and lactic acid were analyzed on an Aminex HPX-87H column (Bio-Rad, USA) at 60°C with 5 mM sulfuric acid as eluent. The biomass of the fungi was measured at the end of the experiment, where it was filtered with filter paper, washed with distilled water, and dried for 24 h at 105 ± 3°C.

3. Results and Discussion

3.1. Screening of Zygomycetes for Ethanol Production at 40°C and 45°C.

Seven zygomycetes isolates were investigated on their ability to produce ethanol at 40°C, 45°C, and 50°C and the results are presented in Table 2. High glucose consumption in the range of 75.48-92.27% was observed for all fungal isolates at 40°C. The glucose consumption decreased to 4.66-25.59% in the fermentation at 45°C. Further increasing temperature of fermentation to 50°C resulted in no glucose consumption by the fungi. Ethanol was produced by all fungal isolates in fermentation at 40°C with ethanol yields in the range of 0.39-0.47 g/g. It was only isolates "R8", "A1", and "41" that were able to produce ethanol at 45°C with ethanol yields decreased to the range of 0.22-0.26 g/g. Since glucose was not consumed by the isolates at 50°C, no ethanol was either produced. Glycerol

and lactic acid were the most important by-products produced by all the fungal isolates. The yields of glycerol from fermentation at 40°C and 45°C were in the range of 0.055-0.086 g/g and 0.043-0.069 g/g, respectively. The corresponding yields of lactic acid were 0.013-0.080 g/g and 0.058-0.082 g/g. The biomass was measured at the end of fermentation and the yields were 0.039-0.053 g/g and 0.007-0.015 g/g at 40°C and 45°C, respectively.

It is shown that the higher temperature, the lower glucose consumed by the fungal isolates. Accordingly, the production of ethanol and biomass decreased. It was reported that high temperature could cause heat stress for microorganisms that further influence the metabolism and the nutrient uptake [14]. For the fermentation of OPEFB and oat straw using NSSF, isolate "41" was selected due to its good performance in fermentation at 40°C and 45°C compared to the other isolates.

3.2. Non-Isothermal Simultaneous Saccharification and Fermentation

NSSF was carried out by 24 h-enzymatic hydrolysis at 45°C followed by 96 h simultaneous saccharification and fermentation (SSF) at 40°C. The most important results are summarized in Table 3. The enzymatic hydrolysis of the untreated OPEFB and oat straw resulted in 2.72% and 6.56% digestibility. The fungal pretreatment resulted in improved digestibility to 3.07% for OPEFB and 27.70% for oat straw. The digestibility of pretreated oat straw even exceeded the digestibility of Avicel as the reference. Fungal pretreatment had a significant effect on oat straw rather than OPEFB. OPEFB had higher lignin and hemicelluloses content compared to oat straw (Table 1). Both lignin and hemicelluloses act as physical barriers that restrict access of cellulose to degrade cellulose. In addition, OPEFB has crystallinity index of 0.81 [15], whereas the crystallinity index of oat straw is 0.575 [16]. This further stresses the fact that OPEFB had lower digestibility compared to that of oat straw.

Table 2. Glucose Consumption and the Yield of Ethanol, Glycerol, Lactic Acid, and Biomass by the Fungal Isolates in Anaerobic Fermentation at 40°C and 45°C for 60h

Isolates Code	Temp. (*°C*)	Glucose Consumption (*%*)	Ethanol Yield (*g/g glucose consumed*)	Glycerol Yield (*g/g glucose consumed*)	Lactic Acid Yield (*g/g glucose consumed*)	Biomass Yield (*g/g glucose consumed*)
R8	40	75.48	0.42 ± 4.04	0.082 ± 10.24	0.080 ± 15.74	0.041 ± 1.26
	45	7.55	0.22 ± 6.95	0.044 ± 7.39	0.058 ± 14.45	0.007 ± 0.64
R10	40	90.11	0.47 ± 3.76	0.086 ± 1.24	0.079 ± 34.35	0.039 ± 2.18
	45	17.32	0	0	0	0.010 ± 4.93
R17	40	91.84	0.42 ± 3.56	0.060 ± 18.24	0.067 ± 30.11	0.046 ± 6.47
	45	11.43	0	0	0	0.007 ± 4.22
R21	40	79.77	0.39 ± 3.71	0.085 ± 6.97	0.019 ± 6.78	0.044 ± 2.96
	45	19.96	0	0	0	0.010 ± 2.8
R23	40	80.06	0.41 ± 1.60	0.070 ± 13.53	0.013 ± 7.80	0.053 ± 5.38
	45	4.66	0	0	0	0.012 ± 3.83
A1	40	87.00	0.45 ± 1.24	0.055 ± 1.86	0.075 ± 25.79	0.044 ± 2.68
	45	5.38	0.24 ± 32.84	0.069 ± 22.22	0.082 ± 22.20	0.013 ± 0.89
41	40	92.27	0.41 ± 3.01	0.067 ± 23.26	0.076 ± 10.02	0.043 ± 4.12
	45	25.59	0.26 ± 9.16	0.043 ± 7.29	0.079 ± 13.13	0.015 ± 3.96

Table 3. The Yield and Maximum Concentration of Ethanol and By-Products in Non-isothermal Simultaneous Saccharification and Fermentation (NSSF) of Untreated and Fungal Pretreated Lignocellulosic Materials and Avicel with Isolate "41"

Material	Digestibility (%)[a]	Max Produced Glucose (g/L)	Max ethanol concentration (g/L)	Max ethanol yield (%)[b]	Max glycerol yield (mg/g)[c]	Max Acetic Acid yield (mg/g)[c]	Max Lactic Acid yield (mg/g)[c]
Avicel	24.95 ± 3.50	8.27 ± 1.16	10.77 ± 0.95	63.77 ± 5.65	78.72	17.15	43.46
Untreated OPEFB	2.72 ± 0.87	0.91 ± 0.29	2.40 ± 0.21	14.10 ± 1.25	33.01	23.94	0
Pretreated OPEFB	3.07 ± 0.08	1.02 ± 0.03	2.42± 0.44	14.25 ± 2.58	35.37	0	0
Untreated oat straw	6.56 ± 1.40	2.19 ± 0.47	4.64 ± 0.13	27.28 ± 0.77	49.68	48.29	0
Pretreated oat straw	27.70 ± 0.11	9.23 ± 0.04	8.48 ± 0.28	49.88 ± 1.63	121.55	0	0

[a] Digestibility = [max produced glucose (g/L)]/[1.111 x dry weight of biomass (g/L) x *F*] x 100, where *F* = cellulose fraction in biomass

[b] Max ethanol yield = [max produced ethanol (g/L)]/[0.51 x 1.111 x dry weight of biomass (g/L) x *F*] x 100, where *F* = cellulose fraction in biomass

[c] Max yield = [max produced by-product (g/L)]/[1.111 x dry weight of biomass (g/L) x *F*] x 1000, where *F* = cellulose fraction in biomass

In the SSF period, glucose concentration (with the exception of untreated and pretreated OPEFB) was still high in the first 6 h of SSF. At 12 h, the glucose was kept zero (data not shown). The fastest rate of ethanol production during SSF by isolate "41" was obtained within 12 h. With the exception of Avicel, the experiments of SSF lasted for 4 days, but ethanol concentration was almost already constant within the last 2 days (data not shown). It can be concluded that the isolate "41" needs only 2 days to reach the maximum ethanol concentration from OPEFB and oat straw. Meanwhile, ethanol production from Avicel was still slowly increasing after 96 h of SSF. This means that glucose was still being produced and directly consumed by the fungus to produce ethanol. The highest ethanol concentration was obtained from Avicel, which was 10.77 g/L (Table 3). The corresponding yield was 63.77% from the theoretical value. This yield is in the order with the previous reports in SSF of Avicel using *S. cerevisiae* and *Rhizopus oryzae* [17]. Compared to *S. cerevisiae*, the selected fungus has an advantage as it could grow up to 45°C as it was shown in Section 3.1. Furthermore, significant improvement in ethanol yield was obtained from the oat straw by fungal pretreatment. However, this was not the case for OPEFB where the ethanol yields were similar for untreated and pretreated OPEFB. The ethanol yields from OPEFB were lower than that from oat straw. This result is reasonable since oat straw had a higher digestibility than OPEFB (Table 3). Therefore, any improvement in the pretreatment process will enhance the yield of ethanol from OPEFB as well as oat straw. Glycerol was the main by-product of SSF by the fungus. The highest glycerol yield of 121.55 mg/g was obtained from pretreated oat straw. The other by-products detected were acetic acid and lactic acid. The yields of acetic acid were in the range of 17.15-48.29 mg/g. Lactic acid was only detected on fermentation of Avicel with the yield of 43.46 mg/g.

4. Conclusion

Seven zygomycetes tempe isolates were investigated to produce ethanol on glucose medium at 40°C, 45°C, and 50°C. All fungal isolates were capable in producing ethanol at 40°C, while three isolates could grow and produce ethanol at 45°C. None of the isolates could grow at 50°C. One fungal isolate, which showed good ethanol production at 40°C and 45°C was selected and further used as the fermenting microorganism of pretreated OPEFB and oat straw. The selected isolate showed good ethanol yield on the fermentation of oat straw. The low ethanol yield from OPEFB was due to the low digestibility of OPEFB after fungal pretreatment.

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