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Effect of Furfural, Hydroxymethylfurfural and Acetic Acid on Indigeneous Microbial Isolate for Bioethanol Production

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Abstract: Yeast isolate of Bekonang has been being applied for many years in ethanol home industry of Bekonang, Central Java, Indonesia. Its tolerance against furfural, Hydroxymethylfurfural (HMF) and acetic acid as the most by product components in acid hydrolysate was investigated. Isolate of Bekonang was cultivated on 50 g glucose L^{-1} at 30°C for 48 h under aerobic condition. Each flask was added with different concentrations of furfural (0.25, 0.5, 1, 1.5 g L^{-1}), HMF (0.5, 1, 2, 3 g L^{-1}) and acetic acid (0.75, 1.5, 3, 4.5, 6 g L^{-1}). During fermentation, glucose consumption, ethanol yield and productivity were examined and compared with a reference. The results show that was completely consumed in the media with addition of furfural and HMF up to 0.5 g L^{-1} as well as in the presence of acetic acid up to 6 g L^{-1}. Addition of 0.5 g L^{-1} of furfural and HMF only decreased both ethanol yield and productivity for <10%. However in the presence of furfural and HMF at 1 g L^{-1}, the ethanol yields were decreased by 26 and 18.45%, respectively and ethanol productivities were decreased by 73 and 71%, respectively. Additions of acetic acid up to 1.5 g L^{-1} result in increasing both ethanol yield and productivity up to 6.96 and 6.89%, respectively. However, higher concentrations of acetic acid up to 1.5 g L^{-1} caused decreased ethanol yield and productivity. Addition of acetic acid in all tested concentrations results in acceptable range of ethanol yield and productivity compared with the reference. These findings show that furfural and HMF were inhibitors to the Isolate of Bekonang, while acetic acid can behave as a friend or foe depending on its concentration.

Key words: Acetic acid, effect, furfural, hydroxymethylfurfural, isolate of bekonang, productivity, ethanol yield

INTRODUCTION

With the inevitable depletion of oil source coupled with environmental problems, bioethanol has been gaining interest as an alternative fuel. Recently, lignocellulosic biomass is considered as an attractive feedstock since it is the most abundant reproducible on the earth. Its low cost allows a significant reduction of production cost and result in increasing interest of lignocellulose conversion. The first process to produce ethanol from lignocellulosic biomass is hydrolysis which breaks the cellulose and hemicellulose polymers into fermentable sugars. Nowadays, at least two hydrolysis methods that are still of interest for producing ethanol from lignocellulose i.e., dilute acid and enzymatic hydrolysis. Dilute acid hydrolysis is more commonly applied due to its economically feasible prior to enzymatic hydrolysis. However, one of the major drawbacks of dilute acid hydrolysis is formation of by products that have potentiality to inhibit fermenting microorganism and may severely decrease the ethanol production rate from the hydrolysate (Taherzadeh *et al*., 1997). There are four major groups of by product compounds produced during hydrolysis including sugar degradation products (furfural and HMF), lignin degradation products (phenolic compounds) compounds derived from lignocelluloses structure (acetic acid) and heavy metal ions (iron, chromium and nickel) (Parajo *et al*., 1996).

After hydrolysis, the next process is fermentation in which fermentable sugars produced in hydrolysis process are further converted into ethanol using microorganism. By far, among the ethanol-producing yeasts, the industrial working horse *Saccharomyces cerevisiae* is the most well known and most widely used yeast in industry and research for ethanol fermentation. This yeast can gow both on simple hexose sugars such as glucose and on the disaccharide sucrose. *S. cerevisiae* is also generally recognized to be safe as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread. In addition,

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S. cerevisiae has high tolerance against ethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass. In Indonesia, for many years, several small ethanol home industries such as in Bekonang have been exploiting *S. cerevisiae* for producing ethanol from molasses. According to Andriani (1993), yeast isolated from home industry in Bekonang (further called Isolate of Bekonang) had glucose and ethanol tolerance as high as 60 and 7.5%, respectively.

In a previous research, we found Isolate of Bekonang performed the best with respect in ethanol production from lignocellulosic hydroysates among the other studied microorganisms. Based on the before mentioned results, it is of interest to further study this microorganism to gain information whether the Isolate of Bekonang is a potential microorganism with desirable properties for this purpose. Therefore, this research aimed to investigate the effect of some well-known inhibitors present in dilute-acid hydrolysates such as furfural, HMF and acetic acid.

MATERIALS AND METHODS

Microorganism: The microorganism was isolated from ethanol home industry in Bekonang, Surakarta, Central Java by using plating method. The yeast isolate was maintained on agar plates made of yeast extract 4.5 g L^{-1}, bacterial peptone 7.5 g L^{-1}, bacterial agar 20 g L^{-1} and D-glucose 20 g L^{-1} as additional carbon source.

Inoculum preparation: One loopfull of culture was first gown in 50 mL of cotton-plugged conical flasks on a shaker of 25 rpm at 30°C. The gowth medium was a defined medium with liquid volume of 10 mL. The medium contained per liter of medium solution: D-glucose 50 g, $(NH_4)_2$ SO_4 7.5 g, KH_2PO_4 3.5 g, $MgSO_4.7H_2O$ 0.75 g, $CaCl_2.2H_2O$ 1 g and Yeast extract 5 g. After incubation for 24 h, first inoculum was further inoculated in 300 mL of cotton-plugged conical flasks on a shaker of 25 rpm at 30°C. The liquid volume was 100 mL. The growth medium was a defined above. After incubation for 24 h, 110 mL of inoculum was obtained.

Cultivation conditions: Aerobic batch cultivations were carried out in 300 mL cotton-plug erlenmeyer flasks placed in a shaker bath at 25 rpm and temperature was set at 30°C for 48 h. The total liquid volume was 100 mL containing 50 g glucose L^{-1}, 1 mL inoculum, 7.5 g $(NH_4)_2$ SO_4 L^{-1}, 3.5 g KH_2PO_4 L^{-1}, 0.75 g $MgSO_4.7H_2O$ L^{-1}, 1 g $CaCl_2.2H_2O$ L^{-1}, 5 g yeast extract L^{-1} and different concentrations of inhibitor including furfural (0.25, 0.5, 1, 1.5 g L^{-1}), HMF (0.5, 1, 2, 3 g L^{-1}) and acetic acid (0.75; 1.5; 3; 4.5; 6 g L^{-1}). Samples were withdrawn at certain times.

Analytical methods: Glucose was analyzed by high pressure liquid Chromatogaphy (Beckman) on an Aminex HPX-87H coloumn with effluent flow rate of 0.5 mL min^{-1}. Concentration of glucose was determined from a refractive index detector. Ethanol was determined using Gas Chromatogaphy (Porapak P, Shimadzu 8A). Prior to glucose and ethanol analysis, samples were centrifugated at 6000 rpm for 15 min and were further kept at -20°C.

RESULTS AND DISCUSSION

Effect of furfural on ethanol yield and productivity: The effects of furfural on glucose consumption and ethanol production are shown in Table 1 and Fig. 1. In the absence of furfural as well as in the presence of furfural up to 0.5 g L^{-1}, glucose was completely consumed. However, while 1 and 1.5 g L^{-1} of furfural added to the medium, only 34.94 and 1.93% of glucose was assimilated, respectively. This results show that addition of furfural >0.5 g L^{-1} significantly decrease glucose consumption. In term of ethanol yield and productivity, additions of furfural up to 0.5 g L^{-1} decreased ethanol yield and productivity from 0.44-0.41 and from 0.48-0.44, respectively (Table 1). Those results show that addition of furfural up to 0.5 g L^{-1} only decreased 7.5% both ethanol yield and productivity compared to the

Table 1: The effect of furfural in batch cultivation of Isolate of Bekonang in synthetic media with 50 g L^{-1} glucose for 48 h

Added furfural (g L^{-1})	Glucose consumption (%)	Ethanol production (g L^{-1})	Ethanol productivity (g L^{-1} h)	Ethanol yield (g g^{-1})
Reference[a)]	100.00	22.90	0.48	0.44
0.25	100.00	21.28	0.44	0.42
0.50	100.00	21.19	0.44	0.41
1.00	34.94	6.14	0.13	0.33
1.50	1.93	0.53	0.01	0.28

[a)]no inhibitor compound

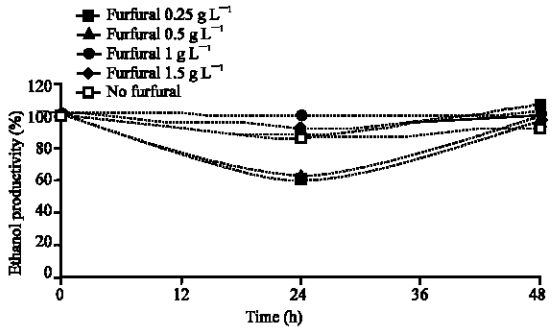

Fig. 1: Reslative ethanol productivity in batch cultivation of Isolate of Bekonang in synthetic media containing 50 g L^{-1} glucose as the carbon and energy sources at different furfural concentrations

reference. However, additions of furfural at concentration >0.5 g L^{-1} significantly decreased both ethanol productivity and ethanol yield for 73 and 26%, respectively. It means that Isolate of Bekonang can tolerate furfural in fermentation medium up to 0.5 g L^{-1}. According to Nigam (2001), furfural concentration of 1.5 g L^{-1} could decrease ethanol yield and ethanol productivity of *Pichia stipitis* up to 90.4 and 85.1%, respectively.

Meanwhile, at the same concentration, it decreased ethanol yield and ethanol productivity of Isolate of Bekonang only for 37.57 and 97.7%.

This shows that Isolate of Bekonang has comparable tolerance with another yeast against furfural. Furfural has been found to decrease the specific gowth rate, the volumetric (Azhar *et al*., 1981), the cell-mass yield on ATP and specific ethanol productivities (Palmqvist *et al*., 1999).

According to Banerjee (1981) furfural directly inhibited ADH that might have contributed to acetaldehyde excretion. The accumulation of intracellular acetaldehyde has been suggested responsible to the lag phase in growth (Palmqvist *et al*., 1999). It is due to the synthesis of new enzymes or co-enzymes for furfural reduction.

Effect of HMF on ethanol yield and productivity: The effect of HMF on glucose consumption and ethanol production are shown in Table 2 and Fig. 2. In the absence of any inhibitor as well as in the presence of HMF up to 0.5 g L^{-1}, glucose was completely consumed. However, addition of HMF at higher concentration results in declining glucose consumption. Increasing of HMF concentration also result in declining of ethanol yield and productivity. Similar with furfural, addition of 0.5 g HMF L^{-1} decreased both ethanol yield and ethanol productivity for <10%.

Meanwhile, the presence of 1 g HMF L^{-1} in the medium could decline up to 71.42% of ethanol productivity and 18.45% of ethanol yield. This results show that HMF has negative effect or inhibit both on ethanol yield and productivity. This is in agreement with another study reported by Alves *et al.* (1998) in which cell growth and fermentation of *Saccharomyces cereviseae* was inhibited by 1 g HMF L^{-1} (Alves *et al*., 1998). *Saccharomyces cerevisiae* was reported had ability to convert furfural and HMF during fermentation. Furfural is converted into furfuryl alcohol or furoic acid by action of the enzyme alcohol dehydrogenase and by the enzyme aldehyde dehydrogenase, respectively (Taherzadeh *et al*., 2000a). Meanwhile, HMF is converted by *S. cereviceae* into HMFAL (5-hydroxymethyl-furfural-alcohol) (Taherzadeh *et al*., 2000b). The conversion rate of HMF is lower than furfural. This might be due to lower membrane permeability of HMF (Larsson *et al*., 1998).

Table 2: The effect of HMF in batch cultivation of Isolate of Bekonang in synthetic media with 50 g L^{-1} glucose for 48 h

Added HMF (g L^{-1})	Glucose consumption (%)	Ethanol production (g L^{-1})	Ethanol productivity (g L^{-1} h)	Ethanol yield (g g^{-1})
Reference[a)]	100.00	22.90	0.48	0.44
0.5	100.00	21.03	0.44	0.41
1.0	34.05	6.55	0.14	0.36
2.0	25.12	1.72	0.04	0.11
3.0	100.00	1.67	0.03	0.03

[a)]no inhibitor compound

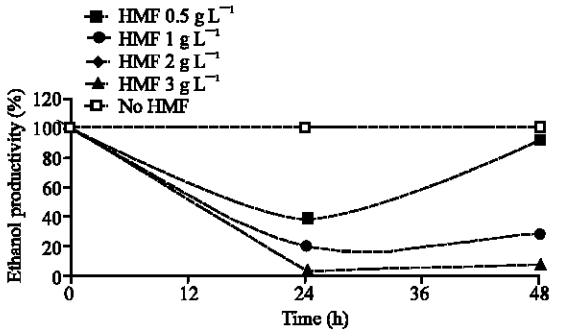

Fig. 2: Relative ethasnol productivity in batch cultivation of Isolate of Bekonang in synthetic media containing 50 g L^{-1} glucose as the carbon and energy sources at different HMF concentrations

Effect of acetic acid on ethanol yield and productivity: The effect of acetic acid added to the medium are shown in Table 3 and Fig. 3. As shoen in Table 3, both in the absence and in the presence of acetic acid up to 6 g L^{-1} glucose were completely consumed. This shows that acetic acid added to the fermentation medium up to 6 g L^{-1} was not enough to decrease glucose consumption.

Unlike the effects of furfural and HMF, increasing of acetic acid concentration up to certain level cause an increase of both ethanol yield and productivity. In the presence of acetic acid at 0.75 and 1.5 g L^{-1} cause an increase of 2.5 and 6.9%, respectively for both ethanol productivity and yield compared to the reference. However, additions of acetic acid into fermentation medium on concentration >1.5 g L^{-1} cause the reduction of ethanol yield and productivity. As shown in Table 3, addition of acetic acid up to 6 g L^{-1} still resulted in acceptable range for both ethanol yield and productivity. It shows that Isolate of Bekonang can

Table 3: The effect of acetic acid in batch cultivation of Isolate of Bekonang in synthetic media with 50 g L^{-1} glucose for 48 h

Added acetic acid (g L^{-1})	Glucose consumption (%)	Ethanol production (g L^{-1})	Ethanol productivity (g L^{-1} h)	Ethanol yield (g g^{-1})
Reference[a)]	100	22.90	0.48	0.44
0.75	100	23.47	0.49	0.46
1.5	100	24.48	0.51	0.48
3.0	100	22.87	0.48	0.44
4.5	100	20.98	0.44	0.41
6.0	100	22.08	0.46	0.43

[a)]No inhibitor compound

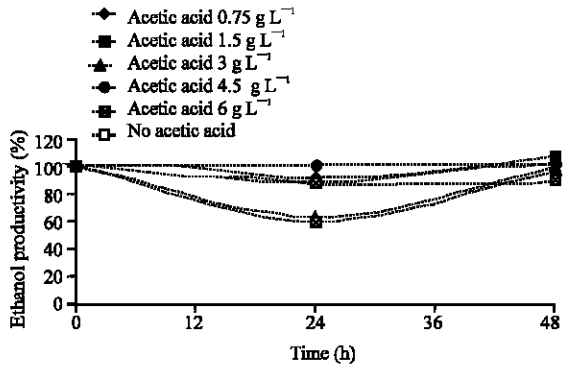

Fig. 3: Relative ethanol productivity in batch cultivation of Isolate of Bekonang in synthetic media containing 50 g L^{-1} glucose as the carbon and energy sources at different acetic acid concentrations

tolerate the presence of acetic acid up to 6 g L^{-1} in the fermentation medium at pH of 4 up to 5. The results show that acetic acid has both positive and negative effect depending on its concentration. It can be explained by the mechanisms of acetic acid inhibition. According to the uncoupling mechanism, inflow of acetic acid resulted in the drop of intracellular pH and it is neutralized by the action of the plasma membrane ATPase which pumps protons out of the cell (Verduyn *et al*., 1992).

In this study, additional ATP must be generated by increasing ethanol production under anaerobic condition at the expense of biomass formation (Viegas and Sa-Correia, 1991).

Therefore, the presence of acetic acid up to certain level resulted in elevating ethanol production. At high acid concentrations, the proton pumping capacity of the cell is exhausted resulting in depletion of the ATP content, dissipation of the proton motive force and acidification of the cytoplasm (Imai and Ohono, 1995). According to reduction of ethanol yield and productivity, Isolate of Bekonang can tolerate furfural, HMF and acetic acid up to 0.5, 0.5 and 6 g L^{-1}, respectively. This shows that Isolate of Bekonang tolerated higher concentration of acetic acid than other studied inhibitors.

CONCLUSION

The result of current research shows that furfural and HMF were inhibitor compounds on cultivation of Isolate Bekonang. Meanwhile, acetic acid can behave as a friend or foe depending on its concentration. Isolate of Bekonang is relatively tolerant against most of the hydrolysis by products present in dilute acid hydrolyzates including furfural, HMF and acetic acid by having comparable tolerance with other ethanol producer microorganisms i.e., *Pichia stipitis*. In this study, the effect of each by product was investigated separately, therefore further study needed to investigate the potential synergic of all the by product compounds as it is found in the lignocellulosic hydrolyzates.

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