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INHIBITION OF THE GROWTH OF TOLERANT YEAST *Saccharomyces cerevisiae* STRAIN I136 BY A MIXTURE OF SYNTHETIC INHIBITORS

Penghambatan Pertumbuhan Ragi Toleran Saccharomyces cerevisiae *Strain I136 Menggunakan Campuran Inhibitor Sintetis*

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ABSTRACT

Biomass from lignocellulosic wastes is a potential source for bio-based products. However, one of the constraints in utilization of biomass hydrolysate is the presence of inhibitors. Therefore, the use of inhibitor-tolerant microorganisms in the fermentation is required. The study aimed to investigate the effect of a mixture of inhibitors on the growth of *Saccharomyces cerevisiae* strain I136 grown in medium containing synthetic inhibitors (acetic acid, formic acid, furfural, 5-hydroxymethyl furfural/5-HMF, and levulinic acid) in four different concentrations with a mixture of carbon sources, glucose (50 $g.l^{-1}$) and xylose (50 $g.l^{-1}$) at 30ºC. The parameters related to growth and fermentation products were observed. Results showed that the strain was able to grow in media containing natural inhibitors (BSL medium) with μmax of 0.020/h. Higher level of synthetic inhibitors prolonged the lag phase, decreased the cell biomass and ethanol production, and specific growth rate. The strain could detoxify furfural and 5-HMF and produced the highest ethanol (Y(p/s) of 0.32 $g.g^{-1}$) when grown in BSL. Glucose was utilized as its level decreased in a result of increase in cell biomass, in contrast to xylose which was not consumed. The highest cell biomass was produced in YNB with Y (x/s) value of 0.25 $g.g^{-1}$. The strain produced acetic acid as a dominant side product and could convert furfural into a less toxic compound, hydroxyl furfural. This robust tolerant strain provides basic information on resistance mechanism and would be usefull for bio-based cell factory using lignocellulosic materials.

[Keywords: inhibitors, growth profile, S*accharomyces cerevisiae*, yeast]

ABSTRAK

Biomassa dari limbah lignoselulosa berpotensi sebagai sumber produk biologi. Namun, salah satu kendala pemanfaatan hasil hidrolisis biomassa adalah adanya senyawa inhibitor sehingga penggunaan mikroorganisme tahan inhibitor sangat diperlukan dalam proses fermentasi. Penelitian ini bertujuan untuk mengetahui pengaruh campuran inhibitor terhadap Saccharomycess cerevisiae *strain I136 yang ditumbuhkan dalam medium yang mengandung campuran inhibitor sintetis (asam asetat, asam format, furfural, 5-hydroxymethylfurfural/5-HMF, dan asam levulinat) dalam empat konsentrasi yang berbeda dengan sumber karbon glukosa (50 $g.l^{-1}$) dan xilosa (50 $g.l^{-1}$) pada suhu 30ºC. Pengamatan dilakukan terhadap parameter yang terkait dengan pertumbuhan strain ini dan produk hasil fermentasi. Hasil penelitian menunjukkan bahwa strain I136 tahan terhadap media BSL dengan nilai μmax 0,020/h. Peningkatan konsentrasi inhibitor dalam medium memperpanjang fase lag serta menurunkan produksi biomassa sel, produksi etanol, dan laju pertumbuhan spesifik. Strain ini mampu mendetoksifikasi senyawa furfural dan 5-HMF dan menghasilkan etanol tertinggi dengan nilai (Y(p/s) 0,32 $g.g^{-1}$ ketika ditumbuhkan dalam media BSL. Glukosa dapat digunakan yang ditandai dengan menurunnya konsentrasi glukosa dan meningkatnya konsentrasi sel biomassa. Sebaliknya, xilosa tidak digunakan dan konsentrasinya tetap sekitar 50 $g.l^{-1}$. Produksi biomassa sel tertinggi dicapai ketika strain ini ditumbuhkan dalam media YNB dengan nilai Y(x/s) 0,25 $g.g^{-1}$. Strain ini menghasilkan asam asetat sebagai produk samping yang dominan dan dapat mengubah furfural menjadi senyawa yang kurang toksik, yaitu hidroksi furfural. Hasil penelitian ini memberikan informasi awal tentang mekanisme toleransi dan berguna sebagai pabrik sel untuk produk biologi dengan menggunakan materi dari bahan berlignoselulosa.*

[Kata kunci: inhibitor, profil pertumbuhan, ragi, S*accharomyces cerevisiae*]

INTRODUCTION

Concerns on sustainability and environment are the most important reasons for driving force research on bio-based chemicals and bioenergy. In particular, lignocellulosic waste is considered as the most potential raw material for sustainable industry due to its abundantly available and low cost to meet economic value of bio-based products (Riyanti 2009, Sinumvayo et al. 2015). The annual global production of lignocellulose was approximately 50 billion tones (Claasen et al. 1999), indicating a sufficient amount of materials to develop environmentally friendly and sustainable biofuel production (Naik et al. 2010; Sims et al. 2010). Biofuel by-products from lignocellulosic biomass could be utilized further for soil amendmend to substitute chemical fertilizer (Singla and Inubushi 2014).

Fermentable sugars from lignocelluloses can be exposed through several pre-treatment technologies including sulphuric acid treatment, alkaline treatment, steam explosion and enzymatic degradation (Hu and Ragauskas 2012; Saini et al. 2015; Wi et al. 2015). However, simple sugars produced by pretreatment of lignocelluloses generate a variety of inhibitory compounds by further degradation of simple sugars (Zha et al. 2014), and it is recognized as the greatest constraint in lignocelluloses industrialization. Research on various areas for utilizing sugars from lignocellulosic materials for valuable products, such as isolation of new microbes, gene studies, genetic manipulation and kinetic evaluation have long been conducted (Jeon et al. 2009; Riyanti and Rogers 2009a, 2009b; Riyanti 2011).

The growth of most robust microbe for fermentation, *Saccharomyces cerevisiae,* was inhibited by compounds resulted from lignocelullosic hydrolisate such as aldehydes, ketones, phenols and organic acids (Palmqvist and Hahn-Hagerdal 2000; Li et al. 2015). These inhibitory aldehyde compounds have been given more attention during the last two decades due to their toxicity to the fermentative microbe's cells. The aldehyde compounds such as 5-hydroxymethyl furfural (5-HMF), furfural, methyl glyoxal, vanillin and glycol aldehyde are the main inhibitors present in the hot-compressed water-treated lignocelluloses (Yu et al. 2007; Jayakodi et al. 2011; Caspeta et al. 2015), while furfural is the most toxic compound in the lignocellulosic hydrolysate (Heer and Sauer 2008).

S. cerevisiae is the most robust microorganism for ethanol cell factory compared to other microbes due to its morphological advantages (Barnet 2003). However, the natural yeast is sensitive to inhibitors resulted from lignocellulosic hydrolysate materials. Aims to sought inhibitor-tolerant yeast have been reported by adaptation to toxic compound and genetic engineering. Few publications reported that tolerant yeast produced low ethanol in the presence of low concentrations of furfural and HMF (Taherzadeh et al. 2000; Liu et al. 2004). A recombinant inhibitor-tolerant *S. cerevisiae* strain $D5A^{+}$ could grow in a medium consisting of 60% (v/v) non-detoxified hydrolysate from triticale straw, supplemented with 20 $g.l^{-1}$ xylose as a carbon source, in semi-aerobic batch cultures. In the same medium, this strain exhibited a slightly lower maximum specific growth rate (μmax = 0.12 ± 0.01 h−1) compared to TMB3400, with no ethanol produced by the latter strain (Smith et al. 2014; Ohgren et al. 2006).

The objective of this study was to investigate the effect of a mixture of synthetic inhibitors on the growth of *S. cerevisiae* I136. The result would be usefull for further survival mechanism study of microbes toward inhibitors.

MATERIALS AND METHODS

Yeast Strain and Cell Culture Preparation

Saccharomyces cerevisiae strain I136 was maintained in Yeast Potato Dextrose (YPD) medium containing 10 g yeast extract, 20 g bacto peptone, 20 g glucose and 15 g agar per liter (Ausubel et al. (eds) 2003). The strain was obtained from and deposited at the Faculty of Chemichal Engineering, Kobe University, Kobe, Japan.

Seed Inoculum

The single colony of strain I136 from YPD agar plate was cultured in 150 ml erlenmeyer flask containing 12.5 ml YNB broth medium and incubated overnight at 30°C, 150 rpm for seed culture. This pure I136 culture was then used for further assay.

Chemicals, Medium Preparation and Fermentation Conditions

Furfural and 5-HMF were purchased from Wako (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. The purity of both furaldehydes was 95%. Furfuraldehyde was immediately prepared by dissolving in dimethyl sulfoxide (DMSO) as 2M stock solutions after the bottle was opened to prevent oxidation.

The batch cultivations were carried out in 250 ml conical flasks equipped with rubber stoppers for aerobic cultivation. Fermentation media with four degrees of complex inhibitor strength, namely medium I, II, III and IV, and control medium using natural inhibitors (steamed high pressure treated-bagase/Bagase Sugar Lysate, BSL), and control medium without inhibitors (Yeast Nitrogen Base, YNB) were used in this experiment. Medium IV contained 6.7 YBS without amino acid, 50 g glucose, 50 g xylose, 40 mg adenine sulfate salt, and a mixture of inhibitors: 60 mM acetic acid, 30 mM formic acid, 60 mM furfural, 10 mM 5-HMF, and 5 mM leuvinic acid in 1 L. YNB medium consisted of 50 g glucose, 50 g xylose, 6.7 g YNB without amino acid and 40 mg adenine salt. The experiment was carried out three times as replication. The inhibitor concentrations used in this experiment were shown in Table 1.

Seed culture of 1.2 ml was used to inoculate the 12 ml fermentation medium to give 10% concentration. Fermentation was conducted in a shaker incubator at 150 rpm, 30^{0}C for 48 hours. A sample of 500 µl was taken every 3 hours during fermentation. The samples were then centrifuged 5000 g at 4^{0}C for 5 min, and then 450 µl supernatant were transferred into a High-Performance Liquid Chromatography (HPLC) vial for further analysis.

Table 1. Composition of inhibitors in four different concentrations.

Inhibitors	Inhibitor concentration (mM)					
	BSL	YNB	Medium I	Medium II	Medium III	Medium IV
Acetic acid	24	0	24	30	36	60
Formic acid	10	0	12	15	18	30
Furfural	5.7	0	24	30	36	60
5- HMF	0.5	0	4	5	6	10
Levulinic acid	3.1	0	2	2.5	3	5

Cell Staining and Examination

Cells were stained using propidium iodine (PI) (Annexin V-FITC apoptosis detection kit, AbcamR). Cells from 100 µl broth culture were sedimented using centrifugation at 5000 rpm for 2 min. After cells wash with 100 µl PBS, the 100 µl binding solution and 1 µl Anexin VFITC were added, resuspended and incubated for 15 min at 30^0C, 150 rpm in DeepWell Maximizer, Taitec (Bio Shaker M-BR022UP) shaker incubator. At a final step, PI (1 µl) was added and incubated for 5 min at 150 rpm, 30^oC using DeepWell Maximizer, Taitec (Bio Shaker M-BR022UP) shaker incubator. The cells were then examined under microscope (Biozero BZ-8000) with Nikon Plant APO 40 x 0.95 lenz.

Fermentation Product Analysis

Cell biomass was monitored using UV mini-1240, UV-VIS Spectrophotometer, Shimadzu with OD_{600} measurement. The standard curve for OD_{600} reading and dried cells was determined earlier for conversion of OD_{600} reading during fermentation. Sugar consumption (glucose and xylose) and fermentation products such as ethanol, acetic acid and lactic acid were assayed using HPLC Shimadzu machine LC2010, equipped with ISEP ICE-COREGEL 87H3 column (Biorad, Hercules, USA) at 80°C. Elution of analytes was done at a flow rate of 0.4 ml.min^{-1} using H_2SO_4 as the mobile phase. Sample injection was conducted at 20 µl per sample according to the manual for ISEP ICE-COREGEL 87H3 column (Biorad, Hercules, USA).

Inhibitor concentrations were analyzed using a gas chromatograph-mass spectrometer (GC-MS) (QP2010 Ultra, Shimadzu) with a DB-FFAP column (60 m × 0.25 mm i.d., 0.50-µm film thickness) Agilent Technologies, CA, USA. The column temperature was maintained at 80°C. Peak areas were normalized to the internal standard and used for representing the abundance of furans in the samples.

Data Analysis

Data analysis was performed using exel program (@ Microsoft 2017). Error bars were added for the standard deviation as follow:

$$\mathbf{s} = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where x represents each value in the population, x is the means of the sample, Σ is the sum (total), and n-1 is the number of values in the sample minus 1.

The biomass was sampled each hour for 48 hour. One ml of culture was dried in a vaccume dryer for 2 x 24 hour and then weighed using a fine balance to make calibration curve for biomass vs OD_{600}. Biomass produced was then calculated based on the biomass calibration curve.

The growth kinetic parameters of strain I136 were estimated using the following formula. Specific growth rate (µmax) = 1/x (dx/dt); x = cell mass concentration (g.l^{-1}), t = time. Growth yield coefficient = Yx/s = dx/ds; x = cell mass concentration (g.l^{-1}), s = substrate (g.l^{-1}). Product yield coefficient Yp/s = dp/ds; p = product (ethanol) (g.l^{-1}), s = substrate (g.l^{-1}).

RESULTS AND DISCUSSION

Growth Profiles of Strain I366

The growth of strain I136 in media containing four different concentrations of synthetic inhibitors (acetic acid, formic acid, furfural, 5-HMF and leuvinic acid) showed that strain I136 grew well in natural medium from liquefied lignocellulosic material, BSL (Figure 1 F) containing natural inhibitors (Table 1). This means that the strain is tolerant to natural inhihitors. The rate of glucose consumption varied depending on the inhibitor concentrations in the medium. In medium without inhibitors (YNB), the strain used glucose to start the fermentation and continued to use it upto 12 hrs after incubation (Figure 1 A).

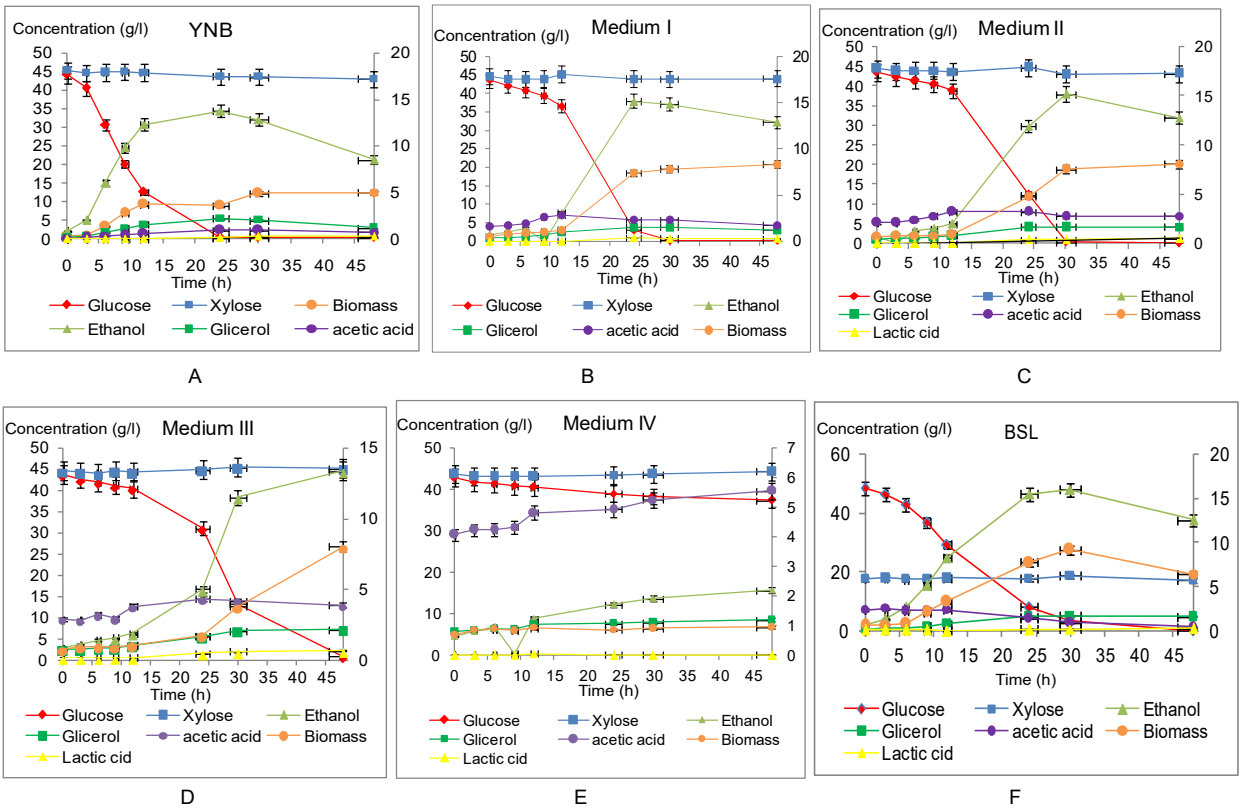

Fig. 1. Growth profiles of *Saccharomyces cerevisiae* strain I136 in different inhibitor-containing media. A = medium without inhibitor (YNB), B = medium I, C = medium II, D = medium III, E = medium IV, F = BSL.

Figure 1 (A-E) shows that higher concentrations of synthetic inhibitors prolonged the lag phase during fermentation process. In medium I, with the least inhibitor concentration, the strain started to utilize glucose rapidly after the lag phase at 12 hrs, and used up all the glucose at about 24 hrs. Similarly, in medium II the lag phase was upto 12 hrs, and then the strain started to uptake the glucose for cell growth and used up at about 30 hrs. In medium III (Figure 1 D), the strain used glucose slowly after the lag phase at 12 hrs, and uptaken all the glucose in the medium at 48 hrs after fermentation. In medium IV (Figure 1 E), containing the highest inhibitor concentration, the strain did not grow properly as the cell biomass did not increase during fermentation process, and the sugar carbon was not utilized. Interestingly in medium containing natural inhibitor (BSL) (Figure 1 F), the strain uptook glucose directly when the fermentation process started, but the strain experienced slugish lag phase compared to that grown in medium without inhibitors (YNB) (Figure 1A). Lag-phase is the period before the growth takes place, which is mainly influenced by the initial media composition. A longer lag-phase indicates the presence of compounds that inhibit the growth (Liu 2004; Zha et al. 2014). During this phase, the strain may adapt to the medium either by degrading or converting its compounds. Glucose of about 50 g/l^{-1} in the BSL medium was taken up after 35 hrs. Ethanol was produced as a result of glucose utilization and the growth increased exponentially after 12 hrs of fermentation. In medium IV, the strain was not able to utilize both sugars, glucose and xylose. and growth was stopped as the cells deceased. Dong et al. 2016 reported that yeast cells perform a programmed cell death to response environmental stress such as acetic acid treatment. Observation under a microscope showed that the death cells would be red as a result of Pi absorbtion, while the healthy cells remained colorless (Figure 2). PI is a fluorescent intercellating agent which cannot cross the membrane of live cells, therefore it is commonly used for differentiating between necrotic and healthy cells (Lecoeur 2002)

Herr and Sauer (2008) reported that inhibitors affected cell growth resulting in longer lag phase. Furan furfural inhibits at least three enzymes in the central carbon metabolism (Modig et al. 2002). According to Heer and Sauer (2008), furfural concentrations in different lignocellulosic raw materials and hydrolysis processes ranged from 5.5 to 30 mM, leading to the inhibition of

yeast growth. Lag phases of various yeast strains due to the presence of inhibitors ranged from 7 to 90 hrs. Strain resistance was based on its capacity to remain viable in a toxic environment during prolonged furfural induced lag phase. In this experiment, the media contained higher concentrations of furfural, i.e. 24, 30, 36 and 60 mM in medium I, II, III and IV, respectively. The long lag phase suggested that the cells adapted to the inhibitors for growth and survival. Moreover, the strain was not able to utilize xylose as a carbon source as its concentration remained at 50 $g.l^{-1}$ at the end of cultivation period.

Factors influencing bioethanol production include temperature, sugar concentration, pH, fermentation time, agitation rate and inoculum size (Zabed et al. 2014). Ethanol production increased rapidly when the strain started to utilize the glucose. The maximum ethanol yield (Y (p/s)) was acheived when the strain was grown in BSL medium (0.32 $g.g^{-1}$) (Table 2). In medium without inhibitor, the maximum ethanol production of 11 $g.l^{-1}$ was reached at 12 hrs after fermentation. In medium I, ethanol increased slightly to 12 $g.l^{-1}$ at 24 hrs, and in medium II and III the maximum ethanol was produced at 30 hrs after fermentation. In medium IV, ethanol was not produced because the inhibitors totally inhibited the cell growth.

Growth Kinetic Parameters on Batch Fermentation

The YNB medium provided the best growth for the strain with the highest maximum specific growth rate (µmax) of 0.08/h compared to that in other media containing inhibitors. Biomass coefficient determines the efficiency of conversion of substrate to biomass which was reflected in the Y(x/s) values (Table 2). The highest biomass production was achieved when the strain was grown in YNB (without inhibitors) with Y (x/s) value of 0.25 g g^{-1} and then the biomass production decreased as a result of increasing inhibitor concentration in medium I, II, III, and IV (Table 2). This result was supported by the highest specific growth rate in medium without inhibitors (YNB) compared to that in medium containing inhibitors. Harsh conditions during biomass pretreatment processes cause sugars and lignin in biomass hydrolysates to degrade, forming products that posses inhibitory effects towards fermenting hosts, resulting in reduced growth and productivity (Palmqvist and Hahn-Hägerdal 2000; Panagiotopoulos et al. 2011; Zha et al. 2011).

Product formed in batch fermentation can be expressed in Y(p/s) (Table 2). The highest product formation was achieved in BSL medium, indicating that the strain was tolerant to natural inhibitors in the medium. This medium is a liquid biomass resulted from hydrolysis using steam explotion method which contained complex natural inhibitors. Increasing concentrations of synthetic inhibitors (from medium I to medium IV) decreased the biomass yield Y(x/s). The highest biomass yield was achieved when the strain was grown in medium without inhibitor (YNB) as shown in Table 2. Increasing inhibitor concentrations in the medium also decreased the fermentation product (Y(p/s)). Palmqvist and Hahn-Hägerdal (2000) reported that inhibitors in the lignocellulosic hydrolysate limited efficient utilization of hydrolysates for ethanol production. The highest ethanol yield was obtained when the strain was grown in BSL medium containing natural inhibitors with the Y(p/s) value of 0.32 $g.g^{-1}$, suggesting that ethanol production was not inhibited by the presence of natural inhibitors. The growth of most ethanol producing microbes is inhibited by inhibitors present in the lignocellulosic hydrolysate (Palmquist and Hahn–Hagerdal 2000), however the studied strain was not affected by natural

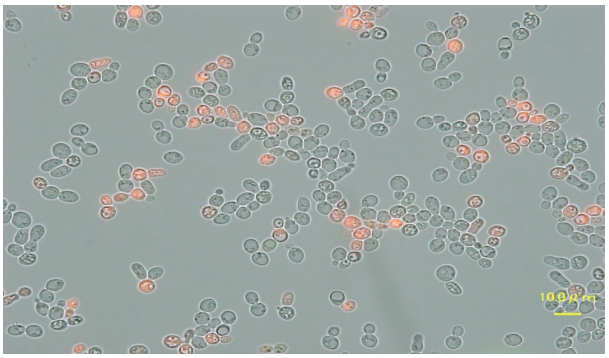

Fig. 2. *Saccharomyces cerevisiae* strain I136 examination under fluorescent microscope after staining using propidium iodine (PI); the cells previously were grown in medium containing synthetic inhibitors; transparent cells = healty cells, red cells = death cells.

Table 2. Growth kinetic parameters of *Saccharomyces cerevisiae* strain I136 in different media containing synthetic inhibitors.

Medium	Y(x/s)	Y(p/s)	µmax (/h)
	($g.g^{-1}$)		
BSL	0.20	0.32	0.020
YNB	0.25	0.28	0.080
I	0.17	0.30	0.062
II	0.16	0.30	0.058
III	0.16	0.26	0.052
IV	0.09	0.22	0.008

Y(x/s) = cell biomass yield based on substrate utilization, Y(p/s) = ethanol yield based on substrate utilization, S = substrate (glucose), µmax = specific growth rate.

inhibitors in the BSL medium.

Inhibitor Detoxification

Inhibitor detoxification positively correlated with fermentation period; the higher the concentration the longer the detoxification process (Figure 3A). Furfural concentration decreased according to the increasing concentrations of inhibitors in the medium and reached the zero level after 12 hrs, 24 hrs and 30 hrs in medium I, II, and III, respectively. In medium IV, the furfural decreased slowly and remained of about 25 mM at the end of fermentation process as the yeast cells stopped growing (μmax = 0.008/h). Our study result is in a good agreement with the previous findings, where several yeast strains could withstand in the presence of lignocellulosic inhibitor by detoxifying the toxic compound into a less toxic one. In this experiment, the toxic compound and the detoxified compound, furfuryl alcohol, were monitored (Figure 3A and 3B). Concentration of the less toxic compound resulting from furfural detoxification, furfuryl alcohol, increased in the fermentation process (Figure 3B). During the fermentation process, the aldehyde furfural and 5-HMF reduced to their corresponding less toxic alcohols (Palmqvist and Hahn-Hagerdal 2000; Jonsson et al. 2013).

Lignocellulosic hydrolysis lead to the dehydration of glucose and xylose to furfural and HMF, respectively, which are inhibitory compounds to yeast growth and alcohol fermentation. The most toxic compound in lignocellulosic hydrolysate is furfural (Jonsson et al. 2013; Field et al. 2015). This I136 strain could detoxify furfural into a less toxic coumpound, furfuryl alcohol (Figure 3), and the highest rate of detoxification was found in medium II (Table 3).

Detoxification rate was measured for two important inhibitors, furfural and 5-HMF (Table 3). The concentration of 5 HMF decreased during fermentation in medium I, II and III containing synthetic inhibitors. However, in medium IV its concentration remained stable and the cells were unable to grow properly due to inhibition (Figure IE). Leuvinic acid and formic acid

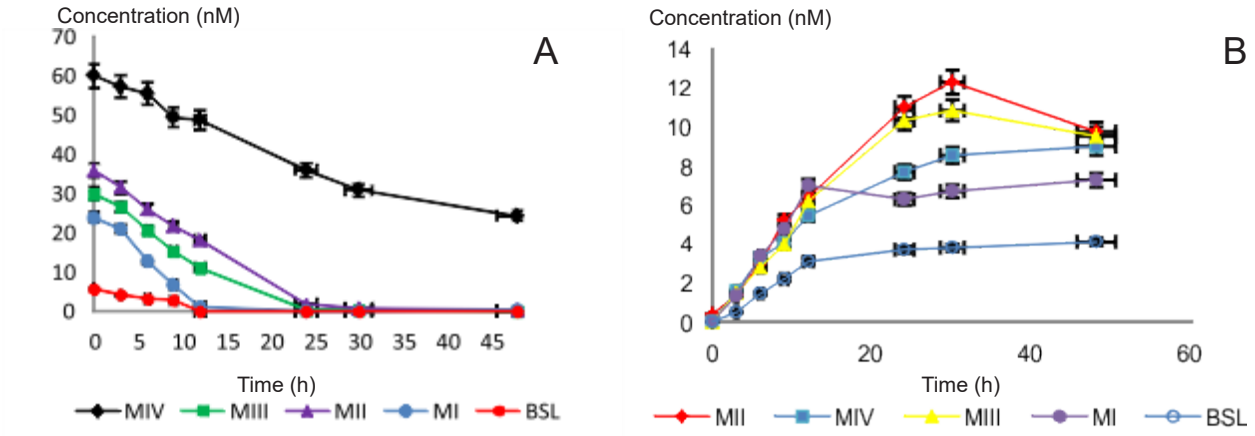

Fig. 3. Detoxification of furfural into furfuryl alcohol.

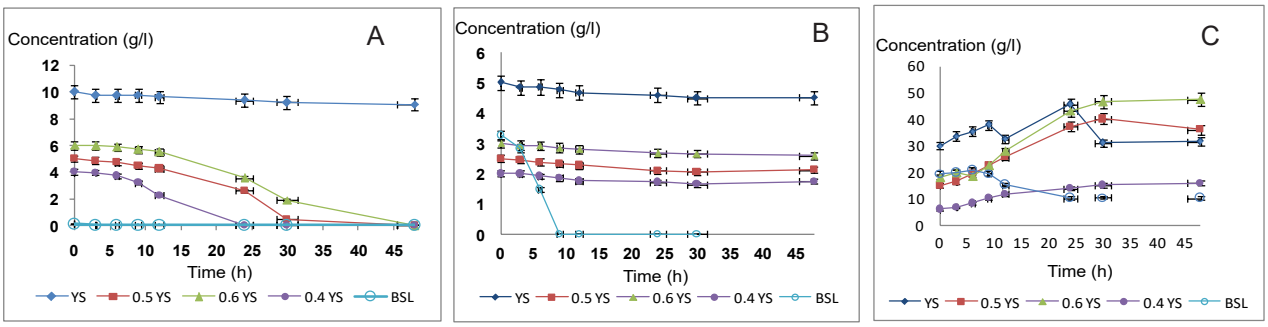

Fig. 4. The dynamic of inhibitors during fermentation process. A = 5-HMF, B = levulinic acid, C = formic acid.

concentration remained stable in the medium. It shows that *S. cerevisiae* enzymatically converts most of these toxic compounds into the less toxic ones. Multiple genes possibly involved in the conversion pathways coupled with nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Jayakodi et al. 2014).

CONCLUSION

Saccharomyces cerevisiae I136 was capable of growing in medium containing synthetic inhibitors and produced ethanol. This strain was able to grow in natural medium from lignocellulosic hydrolysate, BSL, containing natural inhibitors and produced high ethanol. The inhibitors prolonged the lag phase of the strain and decreased the cell biomass and ethanol production. The strain stopped growing in medium containing high inhibitors (60 mM acetic acid, 30 mM formic acid, 60 mM furfural and 10 mM 5-HMF) and the cells died. Strain I136 was able to detoxify furfural into a less toxic compound, furfuryl alcohol.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J. A, Struhl, K., Wiley, C.J., Allison, R.D., Bittner, M. & Blackshaw, S. (2003) *Current Protocols in Molecular Biology*. Frederick M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A. Smith,K.S. (ed.) *Molecular Biology*. [Online] 1, John Wiley & Sons. Available from: doi:10.1002/mrd.1080010210.

Barnett, J.A. (2003) Beginnings of microbiology and biochemistry: The contribution of yeast research. *Microbiology*. [Online] 149 (3), 557–567. Available from: doi:10.1099/mic.0.26089-0.

Caspeta, L., Castillo, T. & Nielsen, J. (2015) Modifying yeast tolerance to inhibitory conditions of ethanol production processes. *Frontiers in Bioengineering and Biotechnology*. [Online] 3, 1–15. Available from: doi:10.3389/fbioe.2015.00184.

Claassen, P.A.M., van Lier, J.B., Lopez Contreras, A.M., van Niel, E.W.J., Sijtsma, L., Stams, A.J.M., de Vries, S.S. & Weusthuis, R.A. (1999) Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology*. [Online] 52 (6), 741–755. Available from: doi:10.1007/s002530051586.

Dong, Y., Hu, J., Fan, L. & Chen, Q. (2017) RNA-Seq-based transcriptomic and metabolomic analysis reveal stress responses and programmed cell death induced by acetic acid in Saccharomyces cerevisiae. *Scientific Reports*. [Online] 7, Nature Publishing Group. Available from: doi:10.1038/srep42659.

Field, S.J., Ryden, P., Wilson, D., James, S.A., Roberts, I.N., Richardson, D.J., Waldron, K.W. & Clarke, T.A. (2015) Identification of furfural resistant strains of Saccharomyces cerevisiae and Saccharomyces paradoxus from a collection of environmental and industrial isolates. *Biotechnology for Biofuels*. [Online] 8 (1), 33. Available from: doi:10.1186/s13068-015-0217-z.

Heer, D. & Sauer, U. (2008) Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain. *Microbial Biotechnology*. [Online] 1 (6), 497–506. Available from: doi:10.1111/j.1751-7915.2008.00050.x.

Hu, F. & Ragauskas (2012) Pretreatment and lignocellulosic chemistry. *BioEnergy Research*. [Online] 5 (4), 1043–1066. Available from: doi:10.1007/s12155.

Jayakody, L.N., Hayashi, N. & Kitagaki, H. (2013) Molecular mechanisms for detoxification of major aldehyde inhibitors for production of bioethanol by Saccharomyces cerevisiae from hot-compressed water-treated lignocellulose.In: Méndez-Vilas,A. (ed.) *Materials and processes for energy: communicating current research and technological developments*. Badajoz, Spain, Formatex Research Center, pp.302–311.

Jeon, Y.J., Fong, J.C.N., Riyanti, E.I., Neilan, B.A., Rogers, P.L. & Svenson, C.J. (2008) Heterologous expression of the alcohol dehydrogenase (adhI) gene from Geobacillus thermoglucosidasius strain M10EXG. *Journal of Biotechnology*. [Online] 135 (2), 127–133. Available from: doi:10.1016/j.jbiotec.2008.02.018.

Jönsson, L.J., Alriksson, B. & Nilvebrant, N.-O. (2013) Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*. [Online] 6 (16). Available from: doi:10.1186/1754-6834-6-16.

Lecoeur, H. (2002) Nuclear apoptosis detection by flow cytometry: Influence of endogenous endonucleases. *Experimental Cell Research*. [Online] 277 (1), 1–14. Available from: doi:10.1006/excr.2002.5537.

Li, H., Wu, M., Xu, L., Hou, J., Guo, T., Bao, X. & Shen, Y. (2015) Evaluation of industrial Saccharomyces cerevisiae strains as the chassis cell for second-generation bioethanol production. *Microbial Biotechnology*. [Online] 8 (2), 266–274. Available from: doi:10.1111/1751-7915.12245.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M.A., Kurtzman, C.P. & Gorsich, S.W. (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *Journal of Industrial Microbiology and Biotechnology*. [Online] 31 (8), 345–352. Available from: doi:10.1007/s10295-004-0148-3.

Modig, T., Liden, G. & Taherzadeh, M.J. (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal*. [Online] 363 (3), 769–776. Available from: doi:10.1042/bj3630769.

Naik, S.N., Goud, V. V., Rout, P.K. & Dalai, A.K. (2010) Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews*. [Online] 14 (2), 578–597. Available from: doi:10.1016/j.rser.2009.10.003.

Öhgren, K., Bengtsson, O., Gorwa-Grauslund, M.F., Galbe, M., Hahn-Hägerdal, B. & Zacchi, G. (2006) Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400. *Journal of Biotechnology*. [Online] 126 (4), 488–498. Available from: doi:10.1016/j.jbiotec.2006.05.001.

Palmqvist, E. & Hahn-Hägerdal, B. (2000) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of

inhibition. *Bioresource Technology*. [Online] 74 (1), 25–33. Available from: doi:10.1016/S0960-8524(99)00161-3.

Panagiotopoulos, I.A., Bakker, R.R., de Vrije, T. & Koukios, E.G. (2011) Effect of pretreatment severity on the conversion of barley straw to fermentable substrates and the release of inhibitory compounds. *Bioresource Technology*. [Online] 102 (24), Elsevier Ltd, 11204–11211. Available from: doi:10.1016/j.biortech.2011.09.090.

Riyanti, E.I. (2011) Beberapa gen pada bakteri yang bertanggung jawab terhadap produksi bioetanol. *Jurnal Penelitian dan Pengembangan Pertanian*. 30 (3), 41–47.

Riyanti, E.I. (2009) Biomassa sebagai bahan baku bioetanol. *Jurnal Penelitian dan Pengembangan Pertanian*. 28 (3), 101–110.

Riyanti, E.I. & Rogers, P.L. (2009a) Construction and expression of pet operon using shuttle vector for mesophilic and thermophilic bacteria. *Jurnal Agrobiogen*. 5 (1), 7–15.

Riyanti, E.I. & Rogers, P.L. (2009b) Kinetic evaluation of ethanol-tolerant thermophile Geobacillus thermoglucosidasius M10EXG for ethanol production. *Indonesian Journal of Agricultural Science*. 10 (1), 34–41.

Saini, J.K., Saini, R. & Tewari, L. (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech*. [Online] 5 (5), 337–353. Available from: doi:10.1007/s13205-014-0246-5.

Sims, R.E.H., Mabee, W., Saddler, J.N. & Taylor, M. (2010) An overview of second generation biofuel technologies. *Bioresource Technology*. [Online] 101 (6), Elsevier Ltd, 1570–1580. Available from: doi:10.1016/j.biortech.2009.11.046.

Singla, A. & Inubushi, K. (2014) Effect of biochar on CH4 and N2O emission from soils vegetated with paddy. *Paddy and Water Environment*. [Online] 12 (1), Springer Japan, 239–243. Available from: doi:10.1007/s10333-013-0357-3 [Accessed: 17 July 2017].

Sinumvayo, J.P., Ishimwe, N., Komera, I. & Niyomukiza, S. (2015) Ethanol biofuel production from lignocellulosic biomass by engineered saccharomyces cerevisiae. *Journal of Academia and Industrial Research*. 3 (10).

Smith, J., van Rensburg, E. & Görgens, J.F. (2014) Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant Saccharomyces cerevisiae harbouring xylose isomerase. *BMC biotechnology*. [Online] 14 (1), 41. Available from: doi:10.1186/1472-6750-14-41.

Taherzadeh, M.J., Gustafsson, L., Niklasson, C. & Lidén, G. (2000) Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology*. [Online] 53 (6), 701–708. Available from: doi:10.1007/s002530000328.

Yu, Y., Lou, X. & Wu, H. (2008) Some recent advances in hydrolysis of biomass in hot-compressed water and its comparisons with other hydrolysis methods. *Energy and Fuels*. [Online] 22 (1), 46–60. Available from: doi:10.1021/ef700292p.

Zabed, H., Faruq, G., Sahu, J.N., Azirun, M.S., Hashim, R. & Nasrulhaq Boyce, A. (2014) Bioethanol production from fermentable sugar juice. *The Scientific World Journal*. [Online] 2014. Available from: doi:10.1155/2014/957102.

Zha, Y., Westerhuis, J.A., Muilwijk, B., Overkamp, K.M., Nijmeijer, B.M., Coulier, L., Smilde, A.K. & Punt, P.J. (2014) Identifying inhibitory compounds in lignocellulosic biomass hydrolysates using an exometabolomics approach. *BMC Biotechnology*. [Online] 14 (22), BMC Biotechnology. Available from: doi:10.1186/1472-6750-14-22.