MICROBIOLOGY INDONESIA
ISSN 1978-3477

Volume 2, Number 3, December 2008
p 101-106

REVIEW

Recent Developments in the Bioconversion of Lignocelluloses into Ethanol

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Ethanol has been commercially produced using sugars derived from sugarcane and corn. Recently, research has been focused on the development of thermotolerant and ethanol-tolerant yeast or bacteria that are able to produce ethanol efficiently, as well as the development of lignocellulosic materials as the carbon sources of fermentation. Utilization of lignocellulosic materials as fermentation substrate is promising since they are available in large amounts, renewable and relatively cheap. A lignocellulose biomass is a complex mixture of carbohydrate polymers. In order to develop an efficient process, there have been many attempts to obtain more efficient ways in the conversion of lignocelluloses to ethanol, including pretreatment, enzymatic hydrolysis of lignocelluloses and direct co-culture fermentation. This paper describes the production process of ethanol from starch-containing material, recent developments on the enzymatic bioconversion of lignocelluloses into sugars and their subsequent fermentation into ethanol and the possible recombination of microbes for the direct conversion of lignocelluloses into ethanol.

Key words: lignocelluloses, ethanol, bioconversion

Energy consumption is increasing in the world. However, the amount of fossil oil is decreasing due to the fact that it is unrenewable. Alternative renewable energy, which is more environmentally friendly, is a top priority for many researchers and industries. Such new energy sources are expected to reduce the use of fossil oil or to replace it in the future and to minimize the green house effect that had caused global warming.

Currently, Indonesia consumes approximately 215 million litres of fossil oil per day, while the domestic production is only 178 million litres per day and the remainder has to be imported (Dartanto 2005). In contrast, Indonesian oil reserves will only be available until 2030 (Shintawaty 2006). Facing these facts, the Indonesian Ministry of Research, Science and Technology has emphasized the need for research of renewable energy sources and the Indonesian government also supports the development and commercialization of renewable bioenergy. One of the potential alternative bioenergy sources is bioethanol. This energy sources is very promising, since the mix of ethanol and gasoline (gasohol) was proven to reduce the emission of the harmful gas and substances. The cost of production of bioethanol is also the same as, or tends to become lower than, the cost production of fossil oil (Shintawaty 2006).

State of the Art of Bioethanol Production

Ethanol is by far the cleanest market-feasible renewable fuel. Ethanol can be produced chemically from petroleum or biologically by microbial conversion (bioconversion) of sugars through fermentation. In 2003, about 95% of the ethanol in the world was produced by the fermentation method and 5% by the chemical synthesis method. The fermentation method basically uses the following three steps: the formation of a solution of fermentable sugars, the fermentation of these sugars into ethanol and the separation and purification of the ethanol, usually by distillation (Fig 1).

In general, sugar, starch and cellulose are potential ethanol feedstocks (Fig 1). The technology of ethanol production using sugar and starch is well established and currently being commercialized, e.g. ethanol production from sugarcane in Brazil, ethanol production from maize grain in the USA, or ethanol production from cassava and molasses in Indonesia. However, sugar and starchy feedstocks are in the human food chain, thus they are relatively expensive.

The use of cellulosic or lignocellulosic materials is more promising, since they are renewable, available in abundant amounts and inexpensive. However, there is still lack of low-cost technology for the commercial production of bioethanol from lignocellulose. The compactness and complexity of lignocelluloses makes them much more difficult than starch to be enzymatically degraded into fermentable sugars. Starch is a plant storage compound consisting of glucose linked via β-1,4 and α-1,6 glycosidic linkages (amylose and amylopectin), whereas lignocellulose is a plant structural compound mainly consisting of cellulose, which is exclusively glucose linked via -1,4 glycosidic bonds. Because of the â-1,4 linkages, cellulose is very crystalline, rigid and compact, making it very resistant to biological attack (Gray *et al.* 2006). Hence, the cost of producing ethanol from biomass is higher than that from starch.

This review describes recent developments in the bioconversion process from lignocelluloses to bioethanol and the related problems.

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Ethanol Bioconversion in Indonesia and Asia: Challenges and Opportunities

Indonesia has a wide range of agricultural land and forestry that supplies agricultural and forestry residue as an abundant source of inexpensive raw materials for the production of biofuels and high-value bioproducts. Indonesia also has a wide range of biodiversity, which is expected to be rich in microorganisms living in extreme environments such as hot springs, volcanoes and geothermal sites. Those environments are potential habitats for extremophilic lignocellulose-utilizing microorganisms whose thermostable enzymes are useful for ethanol bioconversion.

The total potential bioethanol production from lignocellulosic crop waste was predicted to be about 16 times higher than the current world level of ethanol production. Having a large population with rice as the staple crop, Asia has the biggest potential for bioethanol production. Asia could produce up to 291 Gl bioethanol per year. Rice, wheat straw and corn stover are the most favorable bioethanol feedstocks in Asia (Seungdo and Bruce 2004). Likewise, in Indonesia rice straw and corn stover will become a potential feedstock for lignocellulose ethanol production since the staple food of the population is rice and maize.

Despite the above mentioned facts, Indonesia is behind some other developing countries like Thailand and Brazil in bioethanol production. Brazil at present is the number one producer of bioethanol in the world. In 2005, Brazil produced 14.7 billion liters of bioethanol from 5.5 million hectares of sugarcane and in 2015 this production level is predicted to double (Dorfler 2008). Thailand's production of ethanol in 2006 was 1 million kl (Yoosin and Sorapipatana 2007).

In 2005 Indonesia has produced more than 133 kl of bioethanol from molasses by major companies like: PT. Indo Acidatama Chemical, PT. Bukitmanikam Subur Persada, PT. Molindo Raya Industrial and PT. Rhodia Manyar (Table 1). Center for Starch Technology of the Agency for Application and Assessment of Technology has developed a pilot scale for ethanol production from cassava, using enzymatic hydrolysis of cassava starch using á-amylase and glucoamylase followed by the fermentation of the produced glucose, with the production capacity of 8 Kl per day (Panaka and Yudiarto 2007). The production cost of this bioethanol is relatively low compared with the same amount of fossil oil without subsidy. This pilot plant also can be operated by using molases as raw material.

Table 1 Ethanol production in Indonesia (2005)

Company	Production		
	Location	Capacity (kL/year)	Feedstocks
PT. Aneka Kimia Nusantara	Mojokerto	5 000	Molasses
PT. Basis Indah	Sulawesi	1 600	Molasses
PT. Bukitmanikan Subur Persada	Lampung	51 282	Molasses
PT. Indo Acidatama Chemical	Surakarta	42 000	Molasses
PT. Madu Baru	Yogyakarta	6 720	Molasses
PT. MolindoRaya Industrial	Malang	10 000	Molasses
PT. Perkebunan Nusabtara IX	Bondowoso	6 000	Molasses
PT. Rhodia Manyar	Gresik	11 000	Molasses
B2TP-BPPT	Lampung	~ 30	Cassava

Source: Panaka and Yudiarto (2007).

Techno-Economic Barrier for Production of Bioethanol from Lignocellulosic Materials

The production of ethanol from lignocellulose usually involves the following general steps: (i) pretreatment of the raw material into a hydrolysable substrate; (ii) the enzymatic hydrolysis reaction that converts the lignocellulosic materials into fermentable sugars; (iii) conversion of the fermentable sugars into ethanol using yeast fermentation; separation and concentration of ethanol product from the by-products and wastes (Fig 1).

Lignocellulose consists of cellulose (insoluble fibers of â-1,4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans and glucans) and lignin (a complex polyphenolic structure) (Saha 2003). This cellulose and hemicellulose in lignocellulose are contained in bundle-like structures, with lignin acting like a glue to bond the bundles together. Xylan, a main component of hemicellulose, is a complex polysaccharide consisting of a backbone of

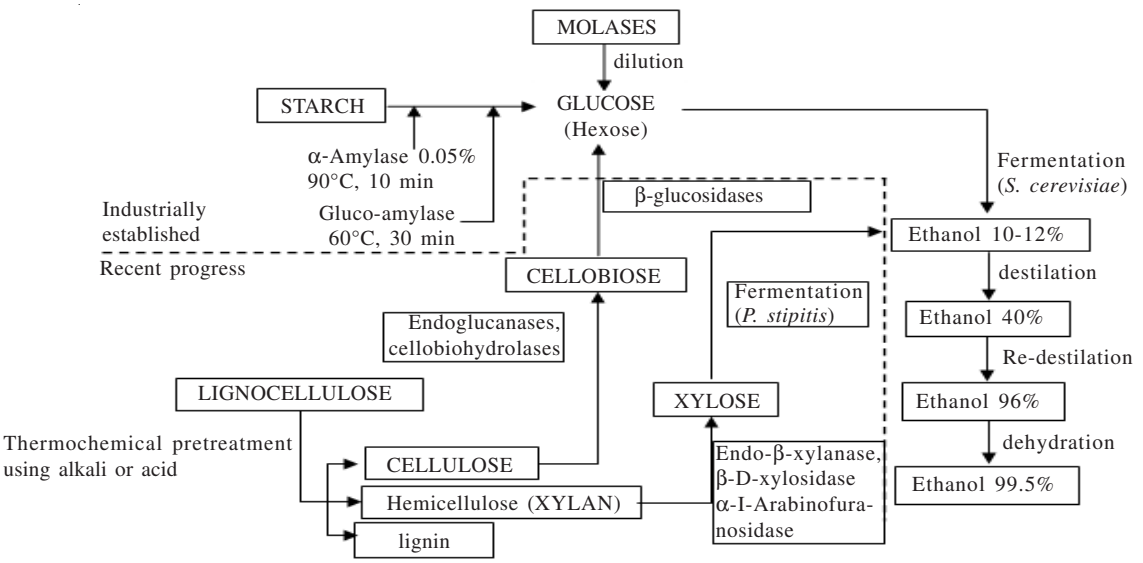

Fig 1 State of the art of bioethanol production.

â-1,4-linked xylopyranoside, which is partially substituted with acetyl, glucuronosyl and arabinosyl side chains. Lignocellulose materials differ in their proportions of cellulose, hemicellulose and lignin. The polysaccharide content in various biomass feedstocks is as showed in Table 2. Typical biomass contains 30-50% cellulose (glucan), up to 25% hemicellulose (xylan) and 10-25% lignin. Based on this very compact and rigid structure, lignocellulose is more difficult to disrupt than starch; in other words it needs a more complicated effort in pretreatment, especially if an environmentally friendly process, such as enzymatic hydrolysis, is required to achieve a reasonable rate and extent of hydrolysis. The objectives of pretreatment are to reduce crystallinity and to increase the available surface by maximum destruction of fiber structure and interaction between the cellulose molecules.

Pretreatments of lignocellulosic materials have been the major techno-economic barrier for bioethanol production from lignocellulose biomass. For the last two decades, hemicellulose content of lignocellulosic materials in the ethanol bioconversion have not been paid enough attention. In the 1980s the focus was on the cellulosic materials only. At that time, Iotech Canada used high pressure and temperature steam (steam explosion) to increase the digestibility of straw or hardwoods. Another available pretreatment is alkali dilution treatment (the Beckmann process). The material is treated with 1% NaOH at 45°C for 3 h. This treatment is very effective for straw or bagasse. In some cases concentrated alkali treatment is more suitable. Straw is treated with 20% NaOH and the residue is then neutralized (Wilson and Pigden 1964). This treatment resulted in more digestible cellulosic biomass for ruminant feed.

Considering the relatively high content of hemicelluloses in addition to cellulose, for the efficient utilization of biomass, the conversion of xylan as well as cellulose is required. A study clearly showed the necessity of utilizing the pentose fraction for ethanol production to obtain satisfactory process economy (Galbe *et al.* 2007; Sassner *et al.* 2008). The challenge is that these polysaccharides exhibit differential reactivity to thermal, chemical and biological processing. Hence, several thermochemical pretreatment methods have been developed to improve the digestibility of this polysaccharides (Kurakake *et al.* 2001; Kim and Holtzapple 2005; Kim and Lee 2005).

Chemical pretreatment combined with high temperature treatment can vary from very acidic to alkaline, thereby giving different effects upon the major constituents in the biomass. For instance, the acidic pretreatments will hydrolyze the hemicellulose fraction by leaving the cellulose and lignin intact, but more enzymatically digestible in the residual solids (Lloyd and Wyman 2005). The most common approaches utilize concentrated H_2SO_4, though other strong acids have also been tried. This pretreatment offers potential for large-scale processing. However the problem using this concentrated acid is the high amount of lime needed for neutralization. This would be come an environmental problem and lead to additional costs for treatment. In the acidic method, the pentose sugar will be dissolved already and without good controlling these dissolved sugars will become unwanted products (furfurals in the liquid phase) that cannot be converted into ethanol and also have deleterious effects on fermenting microorganisms.

Table 2 Cellulose, hemicellulose, and lignin content in various sources of biomass

Feedstock	Composition (%)		
	Cellulose	Hemicellulose	Lignin
Corn stover	36.4	22.6	16.6
Wheat straw	38.2	24.7	23.4
Rice straw	34.2	24.5	23.4
Switchgrass	31.0	24.4	17.6
Poplar	49.9	20.4	18.2

Source: Wiseloge *et al.* (1996).

The alkaline treatments tend to have a relatively better effect on the lignin component and leave both important hemicelluloses and cellulose intact (Kim and Holtzapple 2005; Kim and Lee 2005). The alkaline pretreatment has been used for many years as a means of improving the texture of cellulose textiles and to improve the nutritive value of forage and forest residues for feeding ruminants. The treatment of cellulose-containing residues with low concentrations of alkali makes them considerably more susceptible to enzymatic and microbiological conversion and is very important for the alcoholic fermentation of these materials. The alkaline methods may result in high concentrations of acetate in the hydrolysate. The recent focus is on the development of low-cost reactors and processes such that pretreatment becomes a relatively small portion of the total ethanol production costs.

Developed countries like the USA through its Department of Energy is focusing on supporting lignocelluloses conversion research and development. This helps to make cellulosic ethanol cost competitive compared with petroleum by 2012 by supporting acid or enzymatic hydrolysis of lignocellulose to sugars with subsequent fermentation to ethanol (US DOE 2006).

Enzymes for Lignocelluloses Saccharification: Their Production and Protein Engineering

Saccharification is a process by which the pretreated lignocellulosic material is converted to soluble hexose and pentose sugars, which are further used for yeast fermentation. Enzymatic hydrolysis is a better option than chemical degradation, since enzymes cannot break the sugar unit. After comparing different lignocellulosic materials as potential substrate for ethanol production, a research group showed the necessity of utilizing the pentose fraction for ethanol production to obtain an economically efficient process (Galbe *et al.* 2007). Therefore, cellulase and hemicellulase, which break the polysaccharides of the lignocellulosic materials, are very important subjects for future study.

There are three types of enzymes related to cellulose degradation: (i) endoglucanases (EC 3.2.1.4), which cleave internal β-1,4-glucosidic bonds; (ii) exoglucanases (EC 3.2.1.91), which act on the reducing and non-reducing ends of cellulose chains to produce short-chain cello-oligosaccharides; (iii) β-glucosidases (EC 3.2.1.21), which

hydrolyze soluble cello-oligosaccharides (e.g. cellobiose) to fermentable glucose.

In hemicelluloses degradation more enzymes are needed, including enzymes that break down both â-1,4-xylan (xylanases, EC 3.2.1.8 and â-xylosidases, EC 3.2.1.37) and various side chains (α-l-arabinofuranosidases, EC 3.2.1.55; α-glucuronidases, EC 3.2.1.139; acetylxylanesterases, EC 3.1.1.72; ferulic acid esterases, EC 3.1.1.73 and α-galactosidases, EC 3.2.1.22). The cellulases and hemicellulases are structurally related to glycosylhydrolase families.

Before the advancement of protein engineering using recombinant DNA technology, the costs of both pretreatment and saccharification are functions of scale. For systems in which mechanical or chemical pretreatment is followed by fermentation, large-volume operations would be attractive in industrialized countries as a means of reducing unit costs. However, many enzymes for saccharification were found and engineered to achieve the desired properties and at present this has already decreased the cost of saccharification. Recently, many new cellulases and hemicellulases from both bacterial and fungal sources have been isolated (Sunna and Bergquist 2003; Huang *et al.* 2005). Moreover, significant progress has been made in the cost reduction of cellulases production, particularly for the extracellular cellulases. For example, cellulases commercialized by Genencor and Novozymes has reported to 30-fold cost reduction, that means the enzyme costs drop from USD 5.00 to below USD 0.20 per gallon of ethanol produced (Gray *et al.* 2006). This cost reduction was achieved by a combination of enzyme engineering and fermentation process development.

Since pretreatments, either acidic or alkaline, are usually performed at high temperature, the thermal stability as well as acidic or alkaline-stable xylanolytic and cellulolytic enzymes for subsequent hydrolysis are required. Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates, such as higher specific activity, decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance and increased flexibility with respect to process configurations. Those potencies lead to the reduction of the overall cost of the production process. The screening of thermostable cellulase in an extreme environment has been done by some researchers (Kashima *et al.* 2005; Zvereva *et al.* 2006). Several genetic engineering approaches have been performed to improve cellulase. For instance, a directed evolution to alter pH stability of *T. reesei* endoglucanase or DNA recombination with other cellulases to obtain more stable enzymes were conducted by Murashima *et al*. (2002) and Wang *et al*. (2005). Several group performed mutation using combination site-directed mutagenesis, error-prone PCR and DNA shuffling to generate variants of *T. reesei* cellobiohydrolases (Zhang *et al.* 2006). The variant enzyme genes were expressed in *Saccharomyces cerevisiae* and screened for improved thermal stability and thermal activity. One of the variant DNAs that gave the best thermal stability and thermal activity was integrated into the chromosome of *T. reesei* in place of the wild-type gene. As a result, this recombinant strain gave better performance compared to the parent *T. reesei* in the hydrolysis of pretreated cornstover.

An efficient hydrolysis of hemicelluloses requires a synergistic action of multiple enzymes. Further on, the development of commercially feasible hydrolysis of hemicellulases in lignocellulose is ready to be performed. For instance, cloning and overexpression of thermostable xylanolytic enzymes were reported (Damaso *et al*. 2003; Sunna and Bergquist 2003). The mixture of three thermostable cellulolytic enzymes (cellobiohydrolase, endoglucanase and â-glucosidase) and thermostable xylanolytic enzymes were stable at high temperature hydrolysis during an experiment on technical steam-pretreated-lignocellulosic materials of spruce and corn stover (Viikari 2007). In these studies, hemicellulases facilitated cellulose hydrolysis by exposing the cellulose fibers, thus making them more accessible.

Since the efficient way to obtain bioethanol is to utilize both-cellulose and hemicellulose, the development of non-acid pretreatments that do not dissolve the xylose sugar but leave lignin and cellulose intact is important. There is a development of non-acid pretreatment methods (Kurakake *et al.* 2001; Kim and Holtzapple 2005; Kim and Lee 2005), where the hemicellulose fraction remains intact, thus in this case hemicellulases are required. Current cellulases have weak hemicellulase activity and are not sufficient for complete conversion to monomer sugars, so hemicellulases that can work synergistically with this cellulase are required. There has been continued progress in understanding the structure/function of xylanases and hemicellulases (Fillingham *et al*. 1999; Ouyang *et al*. 2006). Many attempts to obtain endoxylanase in more efficient and cheaper procedures have been undertaken (Wu *et al.* 2006; Helianti *et al*. 2008). In the near future the research and development of low-cost, commercial hemicellulases that work synergistically with cellulases for bioethanol production is expected to be the prime task. The development of cheap and reliable enzymatic saccharification technologies using protein engineering-based research is necessary. This kind of research and development is now being undertaken by industrialized countries and the developing world should monitor progress and take advantage of the improvement made.

Metabolism Engineering for Ethanol Fermenting Microorganisms

Ethanol production from sugar derived from starch and sucrose usually utilize the yeast *S. cerevisiae* using a wild type strain that does not metabolize xylose. However, sugar derived from lignocellulose biomass is a mixture of hexoses (mainly glucose) and pentoses (mainly xylose), so that the wild-type strains of *S. cerevisiae* could not be applied in fermentation of both sugars. Researchers have conducted two approaches to increase fermentation yields of ethanol derived from lignocellulose biomass sugars (Gray *et al.* 2006). The first approach is improving the yeast or other natural fermentation microorganisms by inserting additional pentose metabolic pathways by genetic engineering. An example of this approach is xylose-metabolizing genes that have been introduced into wild-type ethanologens such as yeast and the bacterium *Zymomonas mobilis* (Ho *et al*. 1998; Jeffries and Jin 2004). Recombinant strains of *S. cerevisiae* with the

ability to co-ferment glucose and xylose have been constructed by inserting *Pichia stipitis* genes for an NADPH-dependent xylose reductase and a NAD+-dependent xylitol dehydrogenase and by enhancing the expression of the endogenous xylulokinase (Ho *et al*. 1998; Roca *et al*. 2003; Jeffries and Jin 2004). Although improved recombinant yeast strains have been developed to ferment biomass hydrolysates with this added xylose pathway (Jeffries and Jin 2004), the anaerobic cofermentation of glucose and xylose is still below commercial requirements.

The second approach is to improve ethanol yields by genetic engineering of microorganisms that already have the ability to ferment both hexoses and pentoses (Jeffries and Jin 2004). There are some examples of success in engineering of Gram-negative bacteria such as *Escherichia coli* and *Klebsiella oxytoca* that are naturally able to use a wide spectrum of sugars, including xylose, to produce ethanol (Dien *et al*. 2003). Although this approach has been experimentally successful, unfortunately rates and yields of ethanol on mixed sugars derived from lignocellulosic biomass have not been commercially feasible to date.

Compared to the purity of sugars derived from starch and sucrose, hydrolysates derived from lignocellulosic biomass contain fermentation inhibitors such as acetic acid, furfural, vanillin, etc. They must be removed when concentrations are too high or require the development of robust microbe strains that are resistant to the inhibitors. Some basic studies to make yeast more robust against these inhibitors have been conducted (Liu 2006; Endo *et al*. 2008). A study to identify the gene resistant to the vanillin, one of the most effective inhibitors in lignocellulose hydrolysates, allowed to make robust yeast (Endo *et al.* 2008).

In the future, breakthroughs in ethanol production from lignocellulosic biomass may come from the field of bioengineering. At present researchers are engineering microbes by incorporating genetic pathways not only from other microbes but also from plants and animals. Synthetic Genomics (Rockville, Maryland), founded by biotechnology pioneer Craig Venter, is attempting to produce a highly engineered "synthetic organism" that can perform multiple tasks well: efficiently break down cellulose like a bacterium, ferment sugar like a yeast and tolerate high levels of ethanol.

Beside the above mentioned research, there are also approaches to make the bioconversion of ethanol from lignocellulose in one integrative reactor. For example, after pretreatment of lignocellulose, this material is degraded through the co-fermentation of two yeast strains with the first strain already engineered to degrade cellulose and the second engineered to hydrolyze xylan and subsequently metabolise the xylose into ethanol (Fujita *et al*. 2004; Katahira *et al*. 2004). Since co-fermentation requires optimal conditions to adopt the speed of bioconversion and fermentation, a construction of recombinant mesophylic bacteria to produce thermophylic xylanase (Wu *et al*. 2006; Helianti *et al*. 2008) is a promising step to increase the efficiency of the bioconversion. Thus, the bioconversion of bioethanol could be done in one step (Koesnandar 2001). Although the research is still at the experimental level, this concept is interesting as it makes the bioconversion of lignocelluloses into ethanol more efficient and effective.

As a conclusion, Asian countries including Indonesia are the potential places to produce lignocellulosic ethanol. Many developments have been made in the past several years in all aspects of lignocellulose bioconversion into ethanol and to make lignocellulosic ethanol production more cost-competitive. To establish a commercially feasible process, a reduction in capital and operating costs of each of the unit operations is a must. During the last few years a large number of pretreatment methods have been developed, comprising methods working at low, medium and high pH. A pretreatment process that provides more digestible hemicellulose and cellulose but also offer further reduction of costs still needs to be explored. Enzyme costs have also been decreased by using recombinant DNA technology based on-protein engineering and other biochemical engineering process. However, further cost reductions are still expected and would come from tailored mixtures of enzymes with higher activities, better thermostability and improved acid or alkaline stability than the current commercial enzymes. Improvements have been made in yeasts and bacteria that are able to ferment multiple sugars into ethanol. However, efforts are still needed to produce more robust multiple sugar-fermenting microorganisms with higher productivity, so they can withstand the full-scale industrial process such as at high concentrations of inhibitors.

ACKNOWLEDGEMENT

We thank Agus Eko Tjahjono, Head of the Center for Starch Technology, Agency for Application and Assessment of Technology for his valuable discussion.

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