

Utilization of Stalks Waste of Sorghum to Produce Bioethanol by Using *Saccharomyces cerevisiae* and *S. cerevisiae*-*Pichia stipitis* Consortium

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ABSTRACT

Sweet Sorghum (*Sorghum bicolor* (L.) Moench) stalks as lignocellulosic agricultural biomass residues are one of the agricultural wastes that do not have economic value and are abundant enough in Indonesia. Compared to sugar cane, total sugar in the sorghum stalk had almost same juice yield. On the basis of the sufficient total sugar content in the sorghum crop residues, sorghum stalk is one of the promising potential sources for bioethanol production. This study was conducted to determine the optimum substrate concentration and microorganism capability to fermentation sorghum stalks using Separated Hydrolysis and Fermentation (SHF) method. The pretreatment conducted in this study was carried out using physical and chemical methods. The sorghum stalks were treated with chopping, drying, grinding and separate with the concentration of 25 grams (5%) and 50 grams (10%), then 0.25% H₂SO₄ was added and heated at the temperature of 121°C for 10 minutes. The enzymatic hydrolysis method using *T.viride* and *A.niger* was performed. After hydrolysis, 20% *S.cerevisiae* CC 3012 or 20% consortium *S.cerevisiae* CC 3012-*P.stipitis* were added for the fermentation process. The data obtained in this research pertained to the value of lignin, cellulose, hemicellulose, reducing sugar, C, N, P, and bioethanol. The results of this study indicated the highest ethanol yield is produced by 50 g substrate as the optimum substrate using the consortium of *S. cerevisiae* CC 3012-*P. stipitis* for 24 hours of fermentation.

Keywords: bioethanol, consortium, hydrolysis, lignocellulosic, sorghum

INTRODUCTION

Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a type of cereal plant that has a great potential to be developed in Indonesia because it has an extensive planting area. In general, the sorghum stem waste is not utilized, whereas it has a high sugar content that can be utilized as raw material to produce bioethanol (Pabendon et al., 2012). The liquid in the stem of sorghum contains approximately 70% water, the sucrose content ranged from 12.30–17.40%, the reducing sugar between 0.50–1.00% and the rest are organic and inorganic compounds (Shiringani and Friedt, 2009). The bioethanol production technology that

employed to date still uses a lot of sugar cane or grains and flour-based as the main substrate, which consequently affects the food supply and causes higher food prices (Cifuentes et al., 2014). However, due to its negative impact on food prices and food security, the interest in bioethanol production is currently shifting to lignocellulosic biomass (Zabed et al., 2016). The sorghum stem is suitable for bioenergy-producing plants because it is not competitive with food crops (Almodares and Hadi, 2009; McIntosh and Vancov, 2010). The use of bioethanol as a fuel can also reduce the CO₂ emissions of and the emissions of such contaminants as SO_x and NO_x. This is because bioethanol has the characteristics that

can improve the fuel quality and performance of the vehicle (Farrel et al., 2006). Bioethanol is an option because it can be continuously produced either by fermentation or by chemical synthesis.

The characteristics of bioethanol produced are the most important factors for choosing raw materials, where its must be able to produce a large amount of bioethanol, so that the production process must be more effective. Naturally, the three main components of the lignocellulose are not stand alone, but are incorporated in the matrix. The crystalline cellulose and the hemicellulose structure complement each other with lignin that is covalently bound to hemicellulose (Rastogi and Shrivastava, 2017). As a result of this structure, cellulose is isolated by hemicellulose and lignin (Sommerville et al., 2004), so the bond is difficult to break. Therefore, it is necessary to conduct pretreatment and hydrolysis process before the fermentation takes place.

The use of *S. cerevisiae* in the ethanol production has been widely developed in several countries. This is because *S. cerevisiae* can produce large amounts of ethanol and has a high tolerance to alcohol, but it is unable to ferment pentose sugars such as xylose (Wahlbom and Hahn-Hägerdal, 2002) to become one of the constraints of its utilization. *S. cerevisiae* is unable to ferment pentose sugars especially xylose because it lacks the functional lines of xylose assimilation encoded by XYL1 and XYL2 genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) (Ha et al., 2010). Some yeasts are known to ferment xylose, one of them is *P.stipitis* (Bayrakci and Kocar, 2014) which have XYL1 and XYL2 genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) (Ha et al., 2010), so *P.stipitis* was used in this study to form a consortium with *S.cerevisiae* and is expected to produce better ethanol. The objectives of this study were to determine the most optimum amount of sorghum stalks substrate and analyzing single microorganisms (*S. cerevisiae* CC 3012) compared to consortiums (*S. cerevisiae* CC 3012-*P. Stipitis*) in producing bioethanol.

MATERIALS AND METHODS

Raw Material Preparation

Raw materials preparation was done by separating the sorghum stalk from the roots. Once

separated, the stalk of sorghum was washed and stem liquid squeezed, then stem liquid was stored at 4°C. The remaining pulp of sorghum stalks that had been squeezed, was cut into small pieces with a size of ± 5 mm. The samples were then dried at 60°C in an oven for 48 hours, then crushed. The crushed biomass was mixed with the sorghum stem liquid, and then the cellulose, hemicellulose, and lignin contents were measured.

T. viride and *A. niger* Preparation

Potato Dextrose Agar (PDA) in the amount of 3.9 g was poured to 100 mL aquadest, and dissolved by hot plate heating. The fluid in the amount of 7 mL was put into the reaction tube, and then sterilized using an autoclave at 121°C and 1 atm pressure. The microorganisms were taken from the preparation in one needle of inoculation, implanted into PDA media and incubated in the incubator. Furthermore, *T.viride* and *A.niger* inoculants were taken from PDA and implanted into Potato Dextrose Broth (PDB) medium. The pH of the media was adjusted using 0.2 M acetate buffer, to suit the living conditions of the microorganisms at pH 5. The proliferation of *T.viride* and *A.niger* in PDB media was performed by shaking at 180 rpm at room temperature. After 3 days, *T.viride* and *A.niger* were ready for use in the hydrolysis process.

Fermentation was performed using *S. cerevisiae* CC 3012 and consortium of *S. cerevisiae* CC 3012 and *P. stipitis* with a ratio of 2:1. Prior to using *S. cerevisiae* CC 3012 and *P. Stipitis*, they were inoculated into PDB media, then shaken for 1 day at 180 rpm; its Optical Density (OD) was subsequently measured until it reached $OD_{600} = 1 = 0.33 \times 10^8$ CFU (Myers et al., 2013).

Acid Pretreatment

Acid concentration, temperature and treatment time are important factors affecting the efficiency of acid pretreatment (Choudhary, 2013). The pretreatment was carried out by physical and chemical methods. The sorghum stalks were powdered, and then 2% H₂SO₄ was added. Each reactor was added in the amount of 5 ml H₂SO₄/gram substrate (Mood et al., 2010) and heated at 121°C for 10 minutes (Choudary et al., 2013). After pretreatment, the aquadest was added until the volume became 500 mL.

Separated Hydrolysis Fermentation (SHF) Process

Hydrolysis was carried out for three days and followed by fermentation process for three days. The utilized reactor had a capacity of one liter with a working volume of 500 mL. Hydrolysis was carried out by utilizing *T. viride* and *A. niger* mixed with a ratio 2:1. The microorganisms used were three days old. The inoculant was added to the sample with a concentration of 2.5% per 500 mL of medium (w/v). The fermentation used *S.cerevisiae* CC 3012 and another variable consortium of *S.cerevisiae* CC 3012 and *P.stipitis* with ratio 2:1, and pH maintained at 5, as can be seen in Table 1. During the process, pH, temperature, glucose, and microorganisms were measured every 24 hours. The other variables were the number of substrate, 25 and 50 grams. The control reactor was prepared with the addition of *T.viridae* and *A.niger*, but without the addition of fermentor microorganisms.

Parameters Analysis

Cellulose, hemicellulose, and lignin were analyzed using the Datta Method and reducing sugar was measured with Nelson Samogyi Method. The bioethanol concentration was measured at 24, 48 and 72 hours after fermentor microorganisms added by means of the Gas Chromatography Method. The measurements of Carbon, Nitrogen, and Phosphorus (C, N, and P) also need to be done to determine the characteristics of the substrate and nutrient adequacy for the growth of microorganisms.

Table 1. Research Variable

Substrate (sorghum)	<i>S.cerevisiae</i> CC 3012	<i>S.cerevisiae</i> CC 3012 – <i>P.stipitis</i> consortium	Control
25 grams	R1	R2	R3
50 grams	R4	R5	R6

Table 2. Decreased of lignin, cellulose and hemicellulose

Lignocellulose Composition	Initial (%)	After Pretreatment (%)	After Hydrolysis and Fermentation (%)	Decreased (%)
Hemicellulose	57.99	50.33	48.51	16
Cellulose	17.41	8.20	7.80	55
Lignin	14.95	10.28	9.89	34

RESULTS AND DISCUSSIONS

Composition C, N, P of Sorghum Stalks

The characteristics of C, N, and P of sorghum stalks were analyzed to determine the availability of nutrient to be utilized by microorganisms. The result of the initial C/N ratio was 34.6, while the C/N ratio after the pretreatment process was 34.35. These results correspond to the ideal ratio of 20–30 (Lie et al., 2011). According to Lie et al. (2011), at the appropriate C/N ratio, the microbes get enough carbon for energy and nitrogen for protein synthesis. At high C/N ratio, the microbes will lack nitrogen as a nutrient source so that the decomposition process will run slowly. Conversely, if the ratio of C/N is low, inhibition by TAN and VFA occurs (Kayhanian, 1999), causing lower gas production. The measurement of C/N ratio before and after pretreatment shows a value of about 34, was suitable for the growth of microorganisms and their ability to decompose organic matter in the hydrolysis and fermentation process.

Composition of Hemicellulose, Cellulose and Lignin

The lignin, cellulose and hemicellulose content of sorghum stalks after pretreatment and fermentation can be seen in Table 2. The initial lignin content was 14.95%, it decreased to 10.28% after pretreatment and to 9.89% after fermentation. The cellulose content also decreased from 17.41% to 8.20% after pretreatment and became 7.80% after fermentation. The hemicellulose content decreased from 57.99% to 50.33% after pretreatment and remained 48.51% after hydrolysis and fermentation. The degradation of sorghum

stem occurred due to a combination of physical, acid and heating pretreatment and hydrolysis and fermentation enzymatically.

T. viride is excellent in producing cellulase enzymes and effective in complementing the degradation of lignocellulose composition (Neethu et al., 2012). *T. viride* has the ability to degrade lignin during the hydrolysis process. *A. niger* has the ability to produce cellulase enzymes to break down cellulose and hemicellulose into glucose (Daud et al., 2012). In addition, *A. niger* can also produce xylanase enzymes, which can degrade hemicellulose especially xylan, so that the bond between lignin and cellulose can be broken. The remaining lignin content is not degraded after the fermentation stage, still remaining as much as 9.89%, due to the presence of a bond between lignin and carbohydrates known as lignin-carbohydrate complexes (LCCs). Generally, lignin is never found in the simplest form among cell wall of polysaccharides, but always combines or binds with the polysaccharides (Daud et al., 2012). According to McIntosh and Vancov (2010) the hemicellulose, cellulose and lignin content were 27.00%, 32.40%, and 2.90% respectively, while in the research by Phuengjayaem and Teeradakorn (2011) the hemicellulose, cellulose and lignin content were 38.62%, 44.51%, and 6.18%. It indicates that the cellulose content was higher than hemicellulose, while the opposite was shown in this study. This is due to the constituent content of the sorghum rod depending on the type and place it grows. The amount of hemicellulose and cellulose content indicated that sorghum stalks have the potential to be used as a bioethanol feedstock.

The hydrolysis and fermentation process

Acid pretreatment caused hydrolysis of hemicellulose especially xylan contained in lignocellulose. The main product measured in this study was reducing sugar produced from the bioconversion of cellulose and hemicellulose. Hemicellulose can be degraded into xylose, mannose, acetic acid, galactose, glucose and others, while cellulose is converted into glucose. In this study, the reduction of sugar data, where lignocellulose contains reducing sugar, can reach 1%, while sucrose can reach up to 17%. The yield of reducing sugar formed during the hydrolysis and fermentation steps can be seen in Table 3. The combination of *T. viride* and *A. niger* used in the hydrolysis stage of this study strongly influences the formation of

reducing sugar. *T. viride* produces high cellulase enzyme and *A. niger* produced cellulase enzyme b-glucosidase; it improved the hydrolysis process efficiency (Idris et al., 2017) and converted cellulose into glucose (Sun and Cheng, 2002), so that the glucose concentration during hydrolysis continued to increase.

The reducing sugar concentration decreased with time due to the *S. cerevisiae* and *S. cerevisiae*-*P. stipitis* consortium utilized as a carbon source to produce bioethanol and other gases. *S. cerevisiae* fermented all of the hexose sugar of the sorghum stalks but were unable to use pentose sugar (Wahlbom and Hahn-Hägerdal, 2002). On the other hand, *P. stipitis* was able to degrade the xylose (pentose sugar) and all simple sugar compounds into ethanol (Van et al., 1988).

Bioethanol Yields of Sorghum Stalks Fermentation

The concentration of fermented bioethanol by using *S. cerevisiae* and *S. cerevisiae*-*P. stipitis* consortium can be seen in Figure 1. The results showed the highest average ethanol content formed at 24 hours, pH level 5 and temperatures around 32°C. Microorganisms did not only convert reducing sugars into bioethanol, but also glucose and fructose from the conversion of sucrose (Bizukoje and Ledakowicz, 2004). According to Wahlbom and Hahn-Hägerdal (2002), *S. cerevisiae* can produce ethanol optimally in the anaerobic facultative state at a temperature of 30–35°C and pH range of 4–5. The highest concentration of bioethanol, amounting to 3.61%, was produced by a consortium of *S. cerevisiae*-*P. stipitis* in the reactor with a substrate of 50 grams at 24 hours.

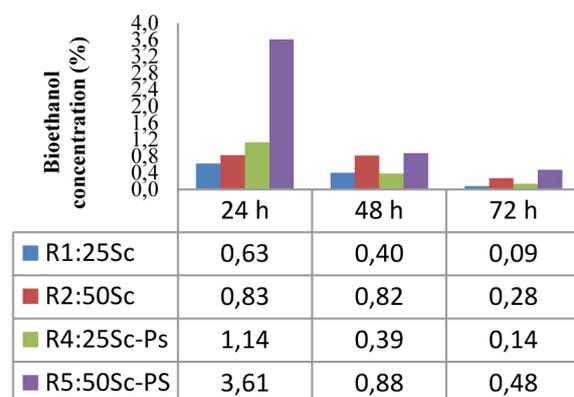


Figure 1. Bioethanol produced during fermentation

Fermentation by *S.cerevisiae* as a single microorganism, produced lower ethanol than a consortium of *S.cerevisiae*-*P.stipitis*. This is due to the ability of *S.cerevisiae* to ferment only the hexose sugar as well as the inability to ferment xylose and other pentose sugars commonly present in hemicellulose. On the other hand *P. stipitis* is able to ferment xylose and convert it to ethanol without producing xylitol (Nigam, 2002). *P. stipitis* complements the performance of *S.cerevisiae* so that the consortium can both produce better ethanol than using single microorganisms. This result is similar to that of Li et al. (2011) who state that the combined use of *S. cerevisiae* and *P.stipitis* is efficient in the production of bioethanol from lignocellulosic biomass.

The bioethanol content decreased in the 48th hour of fermentation in both research variables, because the nutrients begin to run out, so the microorganisms utilized the formed bioethanol and produced acid. This suggests a long relationship of fermentation and growth of yeast cells with the levels of bioethanol produced. The observation of microbial growth indicates that the exponential phase of *S.cerevisiae* and consortium occurs at 24 to 48 hours. On the other hand, at 48 to 72 hours, it begins to enter the stationary phase. In the stationary phase, where the nutrients are already depleted, the number of cells tends to stagnate to enter the phase of death. During the stationary phase, there will be the accumulation of toxin substances that can kill microorganisms (Griffin, 1996).

In the fermentation stage, furfural obtained from xylose degradation, carboxylic acid and acetic acid from hemicellulose decomposition as well as the phenol component of lignin degradation (Li et al., 2011), constitute microbial growth

inhibitors. The growth of microbial fermentation that did not increase significantly in the stationary stage indicates inhibited growth and is possible due to the presence of inhibitor compounds formed during the fermentation process.

Microbial Growth During Hydrolysis Process

According to Sun and Cheng (2002), the hemicellulose and cellulose polysaccharide are hydrolyzed by *T. viride* and *A.niger* as carbon sources to produce monomeric sugars such as glucose, xylose, arabinose, mannose, and galactose. The growth of *T. viride* and *A. niger* combination showed a significant increase at 48 hours, as can be seen in Figure 2. The growth of these microorganisms can be observed in all reactors. This is due to the substantial catabolism of the substrate used for growth, enzyme synthesis and the synthesis of other compounds (Ernes et al., 2014), along with the high sugar concentration produced at the hydrolysis stage. However, at 72 hours *T. viride* and *A. niger* begin to enter to the stationary period, because almost all the substrate hydrolyzed, so the process of substrate metabolism also decreased. This is inverse to the resulting in reducing sugar content, which during the exponential phase of sugar content continues to increase. Even until the 72nd hour, due to the process of cellulose metabolism into simple sugars by the enzyme cellulase of *T. viride* and *A. niger* activity.

Microbial Growth During Fermentation Process

The results of microbial growth observation during the fermentation using *S. cerevisiae* and

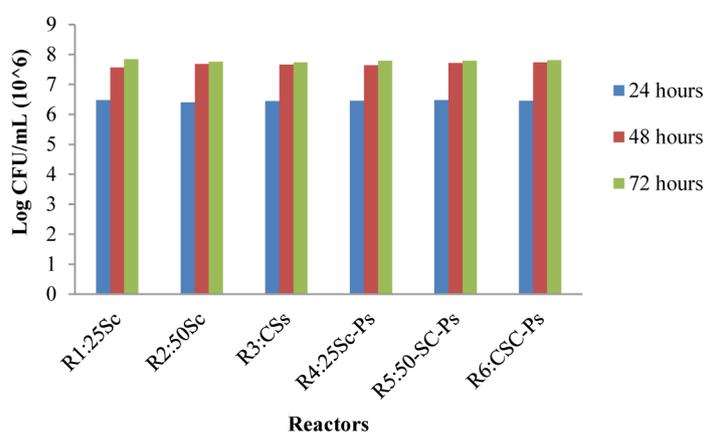


Figure 2. Microorganisms growth during Hydrolysis Process

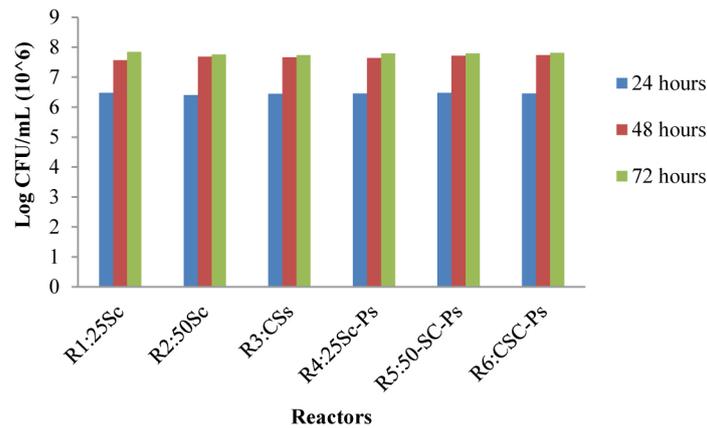


Figure 3. Microorganisms growth during Fermentation Process

consortium of *S.cerevisiae*-*P.stipitis* is shown in Figure 3. It significantly increased the cell growth at 48 fermentation hours. In this fermentation stage, *S.cerevisiae* produced ethanol from the hexose sugar contained in the substrate (Bayrakci and Kocar, 2014), while *P.stipitis* fermented hexoses and pentoses into ethanol (Ganguly et al., 2013). This rapid growth of microorganisms is in line with the increased bioethanol production, as can be seen in Figure 1. The growth of these microorganisms is also in accordance with the availability of nutrients as in Table 3. *S.cerevisiae* and *S.cerevisiae*-*P.stipitis* enter the stationary phase at 72 hours. In this phase, the reducing sugar content and bioethanol concentration decreased. The microorganisms metabolized bioethanol for growth, although the growth of microorganisms slowed down, but it still slightly increased until 72 hours, namely the stationary phase. The results showed that the most effective fermentation in the first 24 hours and the consortium of microorganisms produced the highest amount of bioethanol. This result is also supported by the growth of microorganisms, where the expansional growth occurs from the 24th hour to the 48th hour.

CONCLUSIONS

The results showed the highest bioethanol concentration was 3.61% in the reactor with 50-gram substrate and 24 hours fermentation time. The consortium of microorganisms (*S.cerevisiae*-*P.stipitis*) produced 4.37 times larger amount of bioethanol than single microorganisms (*S.cerevisiae*).

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Table 3. Reducing sugar yields

Substrate Quantity	Microorganism Type	Reactors	Hydrolysis			Fermentation		
			Reducing Sugar (mg/g)					
			24h	48h	72h	24h	48h	72h
25 grams	<i>S.cerevisiae</i>	R1	2.7	4.0	4.3	3.6	3.0	1.2
		R2	3.1	5.6	5.7	3.7	2.4	1.9
		R3(Control)	2.7	3.5	4.2	2.4	2.1	0.9
50 grams	<i>S.cerevisiae</i> + <i>P.stipitis</i>	R4	3.0	4.1	4.1	3.7	2.4	1.9
		R5	2.4	5.6	5.9	3.9	2.8	2.3
		R6(Control)	2.1	4.7	4.8	3.8	3.4	2.5

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