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Bioethanol Production from Various Agricultural Waste Substrate using *Saccharomyces cerevisiae*

Amellia Septhiani Faustine, Rustini, Akmal Djamaan*
Faculty of Pharmacy, Universitas Andalas, Padang 25163, West Sumatra, Indonesia

Abstract
Background*:In recent years, oil production has continued to decline due to the limited availability of raw materials. Raw material reserves for petroleum production continue to decrease. Meanwhile the need for oil continues to increase. The existing fuel oil also creates environmental problems due to overuse. One of them is the effect of greenhouse gases which can blanket the earth so that the air is trapped in the atmosphere and causes the earth to get warmer. If this condition is allowed to continue, it will result in global climate change. If the climate changes, more natural disasters will arise. This will obviously cause serious problems in the future. Therefore, alternative solutions are being sought to make petroleum from other renewable sources. One of the renewable fuels as a substitute for petroleum is bioethanol. Bioethanol can be produced by fermentation using microorganisms, one of which is Saccharomyces cerevisiae. This yeast can be fermented with several substrates from agricultural waste so that it is environmentally friendly. Bioethanol can be used as a substitute for petroleum which is about 5-10% more efficient, so bioethanol is an important thing for further development. The development of bioethanol production as a renewable alternative energy must be supported by research on the sources of raw materials that can be converted into bioethanol. This review compares the journals of bioethanol production from various agricultural waste substrates using Saccharomyces cerevisiae strains ATCC 36858 and TISTR 5596.*
Materials and methods*: In preparing this review article, the methods used were sourced from literature studies from international journals in the last 10 years (2010-2020). In addition, in the preparation of this review article, data search was also carried out using online media with keywords, namely ethanol production using Saccharomyces cerevisiae.*
Results*: All strains of Saccharomyces cerevisiae produced significantly different concentrations of ethanol. ATCC 36858 produces higher ethanol than TISTR 5596. Agricultural waste that can be used for bioethanol fermentation using Saccharomyces cerevisiae strains ATCC 36858 and TISTR 5596 are carob extracts (with differences in fermentation times of 36 hours, 48 hours and 12 hours), tea, date palm extract, sugarcane leaves, cassava starch pulp, cassava pulp lignocellulosic fiber, Thai mission grass, straw, and rambutan with the amount of ethanol production respectively 40.1 g/L, 44.51 g/L, 24.51 g/L, 1.75 g/L, 16 g/L, 4.71 g/L, 9.9 g/L, 11.9 g/L, 16 g/L, 9.021 g/L, and 10.8 g/L.*
Conclusion*: From this article, the best substrate for ethanol production is carob extract with the highest ethanol yield of 44.51 g / L fermentation time of 48 hours.*
Keywords*: bioethanol, fermentation, Saccharomyces cerevisiae.*

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I. Introduction

Petroleum and other fuels such as natural gas and coal come from the decomposition of the remains of living things. The process of its formation takes a very long time so that it includes natural resources that cannot be renewed. The current supply of petroleum continues to decrease from time to time. The limited source of raw materials for oil production is the main cause. Meanwhile, the need for petroleum continues to increase in line with the increase in the world's population. The use of fuel oil has resulted in an environmental crisis. The combustion of fuel produces gases such as carbon dioxide (CO_2), carbon monoxide (CO), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), and methane (CH_4) which cause an increase in the concentration of greenhouse gases in the atmosphere resulting in a warming phenomenonon global warming. Global warming will also result in world climate change.

Demand for fuel oil, which is inversely proportional to the scarcity of existing raw material sources and awareness of global climate change, encourages the development of alternative energy sources to replace petroleum. One of them is bioethanol. Ethanol is the alcohol most commonly used in everyday life. Ethanol which is produced through a fermentation process using these microorganisms is called bioethanol. Bioethanol is an alternative fuel that can reduce carbon dioxide emissions by up to 18%. There is no difference between

ordinary ethanol and bioethanol. It's just that what distinguishes it is the raw material for manufacture and the manufacturing process.

Bioethanol is an ideal substitute for fuel for several reasons. First, bioethanol has a higher octane number, which is 108.6 compared to gasoline. The high octane number will prevent explosion during combustion.Then the efficiency of gasoline is increased by 10% by blending gasoline-ethanol in a ratio of 60:40. Second, the result of a cleaner combustion because it contains oxygen so that the resulting CO gas emissionsare low and can reduce air pollution levels. That way, the environment will be clean and humans can breathe clean air and avoid diseases that may be caused by burning fuel. Third, the result of the combustion of bioethanol is slightly cooler than other fuels so that it can extend the life of the vehicle engine. Fourth, it has high efficiency which can increase energy. Fifth, bioethanol fuel can expand the market for farmer products, especially the sugar sector. If bioethanol is produced on a large scale by industry, it will require raw materials such as sugar cane, cassava, corn and others. This benefits farmers because they can easily sell their crops to the industry so that the harvested raw materials will not be wasted and the price will not drop in the market. Meanwhile, for the industry it self will certainly be able to create jobs and absorb a lot of labor.

An alternative source of raw material that can be used for ethanol production is agricultural waste. This is due to its abundance, low prices and does not cause environmental problems. Agricultural waste and organic waste can be converted into ethanol because of their high chemical content as an alternative source of raw materials for bioethanol production. Ethanol production from agricultural waste has several advantages. First, reduce waste disposal. Waste can be reprocessed as a medium or substrate for the growth of microorganisms to produce ethanol so that it is more environmentally friendly. Second, it saves ethanol production costs. The relatively high raw material prices cause the high ethanol production costs. Therefore, an alternative raw material for ethanol production is sought so that it can reduce ethanol production costs. This alternative can be found in agricultural waste which is very easy to obtain in Indonesia.

The raw materials for bioethanol production are classified into three groups, namely sugar, starch and cellulose. Sources of sugar derived from cane sugar, beet sugar, molasses, and fruits can be directly converted into ethanol. Sources of starchy materials such as corn, cassava, potatoes and plant roots must first be hydrolyzed into sugar. The source of cellulose comes from wood, agricultural waste, pulp and paper mill wastewhich all have to be converted into sugar with the help of mineral acids[1]. The process of bioethanol production consists of three major stages, namely the conversion of polysaccharides into simple sugars, fermentation, and distillation (ethanol refining process).

In recent years, ethanol production from agricultural waste has attracted a lot of attention due to its simple, readily available and environmentally friendly processes. *Saccharomyces cerevisiae* is the most commonly used bioethanol producing microorganism. Therefore, this article aims to compare which substrates better at producing high concentrations of ethanol.

II. Materials and Methods

In preparing this review article, the method used was based on literature studies from official books and international journals in the last 10 years (2010-2020). In addition, in the preparation of this review article, data search was also carried out using online media with keywords, namely ethanol production using *Saccharomyces cerevisiae*.

III. Results

Potential microorganisms such as mold / yeast can be used in the production of ethanol, which the process is environmentally friendly and has economic value. A wide variety of substrates and treatment conditions are used in the ethanol production process.

Table 1. **Ethanol production with *S.cerevisiae* from the substrate and treatment conditions**

Strain *S.cerevisiae*	Substrate	Pretreatment	Enzymatic Hydrolysis	Long Fermentation	Ethanol Produced (g/L)	References
ATCC 36858	Carob extract	Immobilization		36 hours	40.10	[2]
ATCC 36858	Carob extract	-	Processed immediately	48 hours	44.51	[3]
ATCC 36858	Carob extract	-	Raw unprocessed enzymes	12 hours	24.51	[4]
ATCC 36858	Tea	H_2SO_4	Cellulose	48 hours	1.75	[5]
ATCC 36858	Date palm extract	-	Processed immediately	72 hours	16	[6]

TISTR 5596	Sugarcane leaves	H_2SO_4, $Ca(OH)_2$	Cellulose	72 hours	4.71	[7]
TISTR 5596	Cassava starch pulp	-	Amylase and glucoamylase	48 hours	9.9	[8]
TISTR 5596	Cassava pulp lignocellulosic fibers	H_2SO_4, $Ca(OH)_2$	Cellulose	48 hours	11.9	[8]
TISTR 5596	Thai mission grass	NaOH	Cellulose, hemicellulose	24 hours	16	[9]
TISTR 5596	Straw	H_2SO	Cellulose	25 hours	9.021	[10]
TISTR 5596	Rambutan	-	Raw unprocessed enzymes	14 days	10.8	[11]

IV. Discussions

Ethanol has been produced from various substrates derived from agricultural waste (table 1). One example is carob extract (a type of legume)[2,3,4], tea[5], date palm extract[6], sugarcane leaves[7], cassava starch[8], Thai mission grass[9], straw[10], and rambutan juice[11]. Ethanol production is carried out by fermentation using microorganisms. In this article, we use *Saccharomyces cerevisiae* yeast with two types of strains, namely ATCC 36858 and TISTR 5596. The *Saccharomyces cerevisiae* strain was obtained from the American Type Culture Collection (ATCC) and the Thailand Institute of Scientific and Technological Research (TISTR).

The bioethanol production process consists of several stages, namely pretreatment, saccharification, fermentation, distillation and purification[4]. The pretreatment stage consists of hydrolysis which converts carbohydrates into reduced sugars[12]. Hydrolysis is a type of decomposition reaction of a substance using water to break the bonds of the substance. Fermentation is at the heart of ethanol production. *Saccharomyces cerevisiae* is the yeast that is most widely used in ethanol production because it has several advantages, namely being able to tolerate a wide range of pH with acid optimum so that it is less susceptible to infection, tolerance of ethanol is better than other microorganisms, fast fermentation rate, insensitivity to temperature and substrate concentration[13, 14].

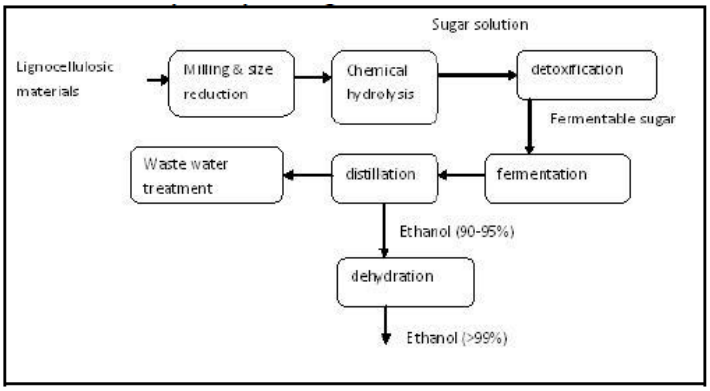

Figure. 1 Bioethanol production process from lignocellulosic materials[15].

In general, the biethanol production process from lignocellulose consists of several steps as shown in Figure 1. The hydrolysis process in an acidic atmosphere will break the polysaccharide bonds[15]. The advantage of acid hydrolysis is that it reduces contamination, the hydrolysis reaction is much faster because the sugar from hydrolysis does not inhibit the hydrolysis process itself so that the resulting ethanol is higher[15]. In addition, this method does not require a pretreatment process and can reduce production costs and speed up the bioethanol production process.

Bioethanol production consists of two generations, namely the first generation and the second generation. The first generation is bioethanol which consists of sugar (sugar cane, molasses) or starch-patian (cassava, corn)[17]. While the second generation is bioethanol which is produced from lignocellulosic biomass[16,17]. Sources of lignocellulosic biomass include agricultural waste (corn cobs, straw), plantation waste (fruit peels), and organic waste[17]. Due to the abundance of lignocellulosic biomass sources and a lot of waste, it becomes an alternative solution for producing bioethanol on a large scale. However, there are challenges in the production of this second generation of bioethanol, namely enzyme hydrolysis (the presence of lignin content causes a decrease in the hydrolysis rate)[17]. This condition can be overcome by a pretreatment process. To increase the ability of enzyme hydrolysis, before the fermentation, delignification or removal of lignin is carried out.

Enzyme hydrolysis is a critical step in determining the successfull conversion of biomass containing lignocellulose to bioethanol[18, 19].

Several studies on ethanol production from carob extract using the same strain (ATCC 36858) resulted different final amounts of ethanol. This can be affected by different fermentation conditions such as pH, temperature, inoculum size, agitation rate, nitrogen source and pretreatment. The length of fermentation also affects the amount of ethanol produced. In this research, carob extract with a fermentation time of 48 hours showed higher results compared to fermentation which was only carried out for 12 hours or 36 hours[3]. Fermentation with controlled pH resulted in a slightly higher ethanol productivity than without pH control[3]. In general, the maximum ethanol yield and growth rateare in pH- controlled fermentation. Therefore, based on the results of ethanol fermentation with carob extract of *Saccharomyces cerevisiae*, the optimal pH was in the range 5.0 - 5.5[3]. The PH was controlled by automatic pH addition in a bioreactor with NaOH 4 N[2]. Ethanol production is completely inhibited when the pH is below 4.0 or below the optimal value due to the production of organic acids which results in lower ethanol production rates in uncontrolled pH fermentation[3]. This shows that pH has a significant effect on ethanol production.

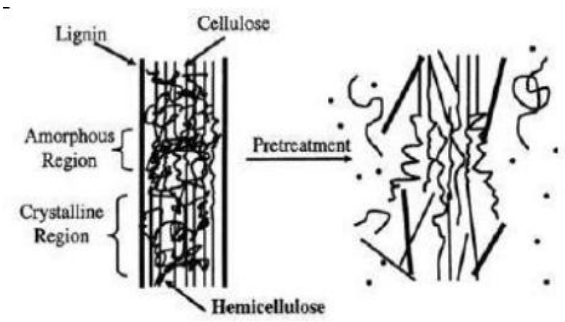

Figure2. Schemes of lignocellulosic biomass pretreatment[15].

The pretreatment or delignification process is an important step in the production of bioethanol from lignocellulosic biomass. The purpose of pretreatment is to open the lignin structure which is very tight so that it can be passed by water and the cellulose enzymes are easier to break down polysaccharide polymers into sugar monomers[15]. If pretreatment is not carried out, lignocellulose is difficult to hydrolyze because the structure of the lignin itself is very strong in protecting the cellulose in it. The presence of lignin and hemicellulose makes cellulose difficult to hydrolyze[15]. Therefore, pretreatment is very important to do to open the structure of lignin and hemicellulose (as in figure 2).

The ethanol fermentation of the carob extract with various inoculums (1%, 3%, 5%) was used to determine the effect of inoculum size on ethanol fermentation[3]. The ethanol concentration increases with the length of fermentation. The increase in inoculum size results in an increase in biomass concentration, maximum consumption rate and maximum growth rate[3]. These results indicate that the size of the inoculum has a significant effect on ethanol production. The optimal inoculum size for maximum ethanol production is 3% which is proven to be acceptable in the ethanol fermentation process[3]. Temperature also affects the ethanol produced. The research shows the result that higher ethanol was obtained at higher temperatures[6].

Fermented Carob extract without the addition of nutrients (nitrogen source, nutrients) was at the lowest value[3]. The addition of an alternative nitrogen source as a substitute for the yeast extract to the fermentation medium resulted in an acceptable amount of ethanol[3]. Therefore it is necessary to add nutrients to the carob extract. Agitation rate is an important parameter for nutrient transfer in fermentation, especially when using immobilized cells. The optimal agitation rate in fermentation is 150 rpm because the beads do not break at this speed and the amount of ethanol produced is slightly higher[1]. Ethanol fermentation was optimized at initial sugar concentration, pH, and agitation rate using Response Surface Methodology (RSM)[3]. The ranges used for the initial sugar concentration are 4-10 Bx, for pH 5.0-6.0, and for agitation 100-200 rpm[3].

The ethanol production from the carob extract of *Saccharomyces cerevisiae* by immobilization showed quite good results[2]. Immobilization (impaired physical mobility) is a state of limited motion so that the cells are trapped in the matrix. Ca-alginate or Na-alginate is used as an immobilizing agent[20]. The results showed that the Ca-alginate concentration and the number of immobilized cells had a significant effect on the final ethanol yield[2]. Higher Ca-alginate concentrations result in lower ethanol[2]. Higher concentrations of Ca-alginate can delay the consumption of glucose, fructose, and sucrose because the surface of Ca-alginate crystals contains denser aggregations, thereby reducing surface area and making it difficult for glucose to penetrate into cell pores[2]. The optimal concentration of Ca-alginate is 2%[2]. The ethanol fermentation value for immobilized cells is higher than free cells because cells that cannot move are more durable than free cells[2].

Yeast has various cell shapes, such as round, oval, cylindrical, triangular, pseudomycelium and so on. Saccharomyces cerevisiae yeast has a single cell form with a length of 1-5 μm to 20-50 μm, and a width of 1-10 μm[21] (Figure 3). Cell age and environmental conditions affect the size and shape of yeast cells[21]. Different cell wall characteristics of microorganisms will affect the effectiveness of their mobilization[21]. A good quality of immobilization can be provided by a support material depending on which support material is more suitable for the immobilized cell, such as the suitability of the number of hydrophilic groups between the support material and the yeast cell[21].

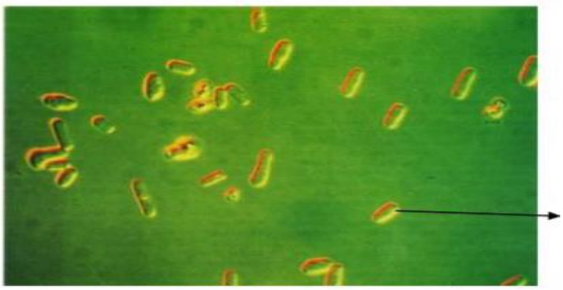

Figure 3. Cell form of *Saccharomyces cerevisiae* [21]

Tea processing waste (TPW) can be used as a renewable energy source for producing ethanol because it contains 13.60% cellulose, 32.16% hemicellulose, and 33.38% lignin[5]. TPW must be ground to increase hydrolysis efficiency. TPW is hydrolyzed with dilute acid then cooled to room temperature and filtered. Hydrolyzate is used for fermentation and stored at ± 4^{o}C[5]. The results of the degradation of cellulose, hemicellulose, and lignin formed at the pretreatment stage can inhibit the production of ethanol[5]. The inhibitors formed are hydroxymethylfurfural (HMF), acetic acid, formic acid and other phenolic compounds[5]. Inhibitors formed during hydrolysis can inhibit ethanol production such as inhibition of cell growth and sugar consumption during yeast cultivation[22]. There are several ways to reduce the inhibitor, such as: (a) repeated sequential fermentation so that the yeast can conform to the inhibiting chemical[22]; (b) over-liming can detoxify the inhibitor by storing it at a high pH[23]; (c) the addition of activated charcoal, due to its high adsorption capacity and also to shorten the fermentation time[24]; (d) anion exchanger[25]; (e) addition of reducing agents[26]; (f) evaporation[8]; (g) site detoxification by fermenting microbes[27]; (h) enzymatic treatment with peroxidase and laccase[28]; (i) membrane extraction[29]; and (j) solvent extraction[28].

About half of the world's lowquality dates production is not consumed[6]. Dates contain more than 75% reducing sugar (mostly glucose and fructose in about equal amount)[6]. These underutilized dates can be an opportunity to produce ethanol. Fermentation with *Saccharomyces cerevisiae* strain ATCC 36858 in date palm extract resulted in a high enough ethanol concentration. The results obtained show the number 16 g/L[6]. The date palm extract substrate was prepared with 1 L of deionized water to extract 400 g of dates, stirred in a water bath at 40^{o}C for 2 hours[6]. Then the extract is filtered twice to remove the fiber[6]. The substrate was sterilized by autoclaving at 121^{o}C for 15 minutes[6]. Deionized water is made by passing tap water, spring water, or distilled water through an electrically charged resin, where ion exchange occurs. The cations and anions in water are exchanged with H^+ and OH^- in the resin to produce H_2O.

Sugarcane leaves have been used as a substrate for ethanol production by the saccharification method and fermentation using *Saccharomyces cerevisiae* strain TISTR 55966. The dried sugarcane leaves are pretreated by suspending them in a dilute sulfuric acid solution or lime (calcium hydroxide)[7]. Optimization of pretreatment conditions by varying the original concentration of sulfate or lime. Then centrifuged to remove residual hydrolyzate or lime powder[7]. The optimum concentration of sulfuric acid was obtained at 1.5% while the optimum conditions for lime (calcium hydroxide) was 3%[7]. Before the fermentation process, sugarcane leaves that have been processed by pretreatment were autoclaved for 30 minutes at a temperature of 121^{o}C and scarified with a buffer solution (enzyme Accellerase 1000)[7]. Increasing the autoclave period can significantly reduce susceptibility to cellulose hydrolysis from sugarcane leaves by pretreatment using dilute sulfuric acid[7]. Whereas in the pretreatment using lime (calcium hydroxide) there was a decrease in the autoclave period resulted in a slight but significant increase in susceptibility to cellulose[7]. Thus, by comparing the 2 pretreatment conditions, it was found that sulfuric acid treatment resulted in greater susceptibility to cellulose hydrolysis in sugarcane leaves compared to pretreatment with lime (calcium hydroxia)[7]. Sugarcane leaves pretreated with dilute sulfuric acid gave higher ethanol yields than sugarcane leaves pretreated with peroxide base[7]. Sugarcane leaves pretreated dilute sulfuric acid (1.5% w/v) is recommended for hydrolysis with cellulose while suspended in pretreatment hydrolyzate for maximum ethanol yield[7].

Starch-producing root crops such as cassava can be fermented up to 30% to produce ethanol[30]. The production of ethanol from cassava starch (cassava pulp) has high potential due to the high residual content of starch and the small particle size of lignocellulosic fibers[8]. Cassava pulp contains high levels of starch and water

(75-80% w/w) which causes cassava slurry to rot quickly and cause environmental problems. To reduce environmental contamination problems, cassava pulp can be used as an alternative to ethanol production. In addition, lignocellulosic fibers obtained from the removal of starch from cassava pulp also have the potential to become a substrate for ethanol production[7]. Cassava has advantages in ethanol production,which are it can adapt well to various conditions of growth and rapid growth of cassava plants, making it easier to harvest each year[29]. The saccharification process was carried out on the substrate of cassava pulp starch and lignocellulose fibers of cassava pulp[8]. The hydrolyzate of starch from cassava slurry is clarified withalpha-amylase and glucoamylase, the glucose level obtained increases with increasing levels of glucoamylase which indicates a high degree of saccharification[8]. Hydrolyzedstarch was separated from lignocellulosic fibers by filtration and centrifugation, fermented into ethanol using *Saccharomyces cerevisiae* TISTR 5596 while lignocellulosic fibers were subsequently given pretreatment and then saccharified with cellulose to glucose[8]. The results showed that lignocellulosic fibers were most susceptible to cellulose[8].

Thailand mission grass has the potential to become lignocellulosic biomass in bioethanol production[9]. In the initial treatment stage, the grass is milled and given NaOH, the grass undergoes acid and enzymatic hydrolysis[9]. The glucose hydrolyzate from Thai mission grass was detoxified by evaporation to remove inhibiting compounds and degradation products[9]. The advantages of using evaporation detoxification are low cost, easy operation, and also that the glucose concentration in the hydrolyzate can be regulated[9]. Overliming at pH 10 produces the highest ethanol yield[9]. Among the various types of yeast, *Saccharomyces cerevisiae* TISTR 5596 with a yeast concentration of 10% v/v yields maximum ethanol yield at 16 g/L in 24 hours and is one of the fastest ethanol producing microorganisms compared to other *Saccharomyces cerevisiae* strains[9].

Saccharomyces cerevisiae TISTR 5596 can produce ethanol from hydrolysis of straw cellulose[10]. The results showed that the maximum amount of sugar from the hydrolysis reaction of synthetic cellulose in sulfuric acid was 3% v/v[10]. Then fermentation at 30^{o}C for 25 hours produces ethanol as much as 9.021 g/L[10]. To analyze the ethanol concentration, gas chromatography was used. The ethanol concentration is influenced by the glucose concentration, if the glucose produced is low, the ethanol concentration is also low[10].

Rambutan has a high sugar content (18-20%) so that it can be a good nutrient in yeast fermentation to produce ethanol[11]. Low quality rambutan fermented with *Saccharomyces cerevisiae* TISTR 5596 was able to produce 10.8 g/L of ethanol on the 8th day[11]. Fermentation was continued for 14 days and was able to maintain ethanol in the range of 10 g/L[11].

V. Conclusions

Saccharomyces cerevisiae strains ATCC 36858 produces higher ethanol than *Saccharomyces cerevisiae strains* TISTR 5596. Agricultural waste that can be used for bioethanol fermentation using *Saccharomyces cerevisiae* strains ATCC 36858 and TISTR 5596 are carob extract (with different fermentation times of 36 hours, 48 hours and 12 hours), tea, date palm extract, sugarcane leaves, cassava starch pulp, cassava pulp lignocellulosic fiber, Thai mission grass, straw, and rambutan with the amount of ethanol produced respectively 40.1 g/L, 44.51 g/L, 24.51 g/L, 1.75 g/L, 16 g/L, 4.71 g/L, 9.9 g/L, 11.9 g/L, 16 g/L, 9,021 g/L, and 10.8 g/L. The substrate that produced the highest ethanol was carob extract with 44.51 g/L fermentation time of 48 hours.

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