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Production of Bioethanol from Seed Media of Kelengkeng Fruits (*Dimocarpus longan*) using Acid Hydrolysis Method and Its Purification using Distillation Method

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Abstract

This research was conducted to determine the level of ethanol production from longan seed media waste with parameters of seed media weight and acid concentration. Bioethanol production is carried out by means of fermentation in media that has been added with inoculum *Saccharomyces cerevisiae*, yielding bioethanol that was then purified through simple methods. Quantitative analysis of ethanol content using a spectrophotometer with potassium dichromate reagent and all analyzes were repeated three times. The results of the study showed that the best variations in the concentration of hydrolyzed acid and seed weight of the media were found at a concentration of 4% (ethanol yield: 2.942%) and media seed weight of 5 grams (ethanol yield: 1.838%), respectively. The concentration of ethanol in the produced distillate is still relatively low, ranging from 1.75% to 2.89% (v/v), and its purity decreases as the total fermentation volume increases.

Keywords: Dimocarpus longan, bioethanol, acid hydrolysis, saccharomyces cerevisae, destilation purification

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Abstrak (Indonesian)

Penelitian ini dilakukan untuk menentukan tingkat produksi etanol dari limbah media biji longan dengan parameter berat media biji dan konsentrasi asam. Produksi bioetanol dilakukan dengan cara fermentasi dalam media yang telah ditambahkan inokulum *Saccharomyces cerevisiae* dengan hasil bahwa bioetanol tersebut dimurnikan menggunakan metode distilasi sederhana Analisis kuantitatif kandungan etanol menggunakan spektrofotometer dengan reagen asam dikromat kalium dan seluruh analisis dilakukan pengulangan sebanyak 3 kali. Hasil penelitian menunjukkan bahwa variasi terbaik dalam konsentrasi asam terhidrolisis dan berat biji media ditemukan pada konsentrasi 4% (hasil etanol: 2,942%) dan berat biji media 5 gram (hasil etanol: 1,838%), masing-masing. Konsentrasi etanol dalam distilat yang dihasilkan masih tergolong rendah, yaitu berkisar antara 1,75% hingga 2,89% (v/v), dan kemurniannya menurun seiring meningkatnya volume total fermentasi.

Kata Kunci: Dimocarpus longan, bioethanol, hidrolisis asam, Saccharomyces cerevisae, pemurnian destilasi

INTRODUCTION

The global recovery following the pandemic, combined with ongoing geopolitical tensions, has accelerated the depletion of non-renewable energy resources, putting long-term energy security at risk [1,2]. Indonesia faces a significant challenge: its fossil fuel reserves are projected to be exhausted within the next 12 years, highlighting the urgent need for the development of renewable energy alternatives [3]. Biomass derived from agricultural organic waste offers a promising solution, as these byproducts can be converted into renewable fuels through fermentation processes [4]. Bioethanol, a key product of fermentation, has emerged as a highly viable

alternative fuel, typically produced from carbohydrate-rich feedstocks using *Saccharomyces cerevisiae* [5-7]. Various fruit seeds, including those from avocado, durian, jackfruit, and dates, have been successfully utilized for bioethanol production, demonstrating the potential of this category of substrates. The purity of bioethanol must meet specific standards based on its intended application, and achieving high purity requires effective purification methods, such as distillation [8-12].

Kelengkeng, or longan (*Dimocarpus longan*), is a tropical fruit extensively cultivated in Southeast Asia, particularly in Indonesia, which produced 46,096 tonnes in 2022 [13]. This volume generates considerable seed waste that is presently discarded, resulting in resource depletion and environmental stress [14]. Longan seeds comprise 44.9–49.5% (w/w) starch, in addition to proteins, fats, and minerals, signifying a substantial carbohydrate content amenable to conversion into fermentable sugars [15–20]. Consequently, longan seeds constitute a promising but underexploited feedstock for bioethanol production.

Previous works on the valorisation of longan waste said only low-quality fruit pulp and whole fruit waste were used with hydrothermal pretreatment followed by enzymatic hydrolysis [19-22]. Nguyen et al. [23] proved the feasibility of bioethanol production from longan fruit waste of low quality with ethanol levels from 14.32 to 16.74 g/L. However, no studies have explored longan seed as the only substrate for bioethanol production, and no studies have explored acid hydrolysis as the primary method of saccharification of this material. This gap is relevant as longan seeds are structurally different from the fruit pulp; they are mostly starchy and not cellulosic, and the presence of tannins and other phenolic compounds may require specific hydrolysis techniques [22]. Furthermore, the dilute acid hydrolysis has been successfully applied to other starchy seeds, such as avocado, which can produce up to 6.46% v/v ethanol [10]. However, the simplicity and cost-effectiveness of this method have not been evaluated for longan seeds.

This study is the first investigation for bioethanol production from longan seeds by acid hydrolysis to fill these gaps. Key innovations include a new feedstock (longan seeds), a cost-effective saccharification method distinct from previous hydrothermal-enzymatic methods, and the systematic optimisation of acid concentration and media weight. The objectives were to produce bioethanol under optimised conditions, purify it by distillation, and provide a basic methodology for the sustainable valorisation of waste and the promotion of renewable energy in Indonesia.

MATERIALS AND METHODS

Materials

The raw materials used in this study were longan seeds, yeast strain SH5209 *Saccharomyces cerevisiae*, yeast extract, peptone, dextrose, bacto agar, Ammonium Sulfate $(NH_4)SO_4$ food grade, Merck potassium dihydrogen phosphate (KH_2PO_4), Hydrochloric Acid (HCl), Potassium Dihydrogen Phosphate (KH_2PO_4), Magnesium Sulphate ($MgSO_4.7H_2O$), Acetate Buffer pH 4 (Technical), Potassium Dichromate ($K_2Cr_2O_7$), Sulfuric Acid (H_2SO_4), Standard Absolute Ethanol and distilled water which all the chemical reagent from Merck. Instruments used in study were spectrophotometer Visible (Thermo Fisher), Autoclave (Al-American), Incubator (Memmert), Stirring hotplate (CIMAREC), Grinder MG-3000D (Orion).

Preparation of longan seeds as a fermentation medium

Crush 1 kg of longan seeds and dry them in the sun until a constant weight is obtained and then grind them. Media parameters in the study used *D. longan* seed weight and acid concentration, each parameter had 5 different variations, namely 1 gram to 5 g longan seeds and 1% to 5% HCl concentration. The media was autoclaved for 30 minutes at 121˚C and after autoclaving, the pH of the media was adjusted to 4-6 using 1 N NaOH as the fermentation formula.

Fermentation media and bioethanol fermentation [23]

Fermentation media was made with the composition of yeast extract 2 gram, $(NH_4)_2SO_4$ 2 gram, $MgSO_4.7H_2O$ 2 gram, KH_2PO_4 4 gram put into 250 mL Erlenmeyer and dissolved with 100 mL distilled water. Then sterilized using an autoclave at temperature 121℃ for 15 minutes. Then the fermentation process uses yeast *Saccharomyces cerevisiae*. Bioethanol fermentation process resulted from hydrolysis was carried out with the composition of the addition of longan seeds after the hydrolysis process as much 10 mL, 10 mL of inoculum medium, and 100 mL of fermentation medium then put into Erlenmeyer and incubated using a 150 rpm shaker at 30˚C. Fermentation was carried out for 4 days, with samples taken every 24 hours by taking 2 mL of sterile solution to determine the ethanol content using a spectrophotometer.

Percentage bioethanol content [23]

The results of the fermentation are then filtered using filter paper Whatman 40. The filtering results were then taken as much as 1 mL, added with 12 mL of $K_2Cr_2O_7$ reagent, 15 mL of distilled water, and

buffer acetate pH 4 as much as 5 mL and stirred until homogeneous. Mixing results then taken 20 mL and put into a 50 mL measuring flask, heated using a water bath for 20 minutes (T = 62.5 ºC). After finishing the solution allowed cooling, then diluted with distilled water up to the mark. The diluted solution is then measured using a spectrophotometer UV-Vis with λ_{max} = 599 nm. Previously, a calibration curve was made for measuring the ethanol content by measuring the standard ethanol concentration at concentrations of 0%, 2%, 4%, 6%, 8%, and 10% (% v/v).

Separation of ethanol from the fermentation medium

Separation of ethanol using a distillation apparatus. The fermented product is put into a 500 mL distillation flask, and the distillation is carried out at 80˚C. Distillate fraction containing ethanol then it will be put into the refrigerator. Ethanol will be tested qualitatively by adding acid dichromate reagent and quantitatively tested by acid dichromate method to determine the purity of the fermented ethanol.

RESULTS AND DISCUSSION

Bioethanol product from fermentation on variations B. longan seeds weight and acid hydrolysis concentration

The ethanol content produced by the yeast *Saccharomyces cerevisiae* is produced through the conversion of reduced sugars which are hydrolyzed by hydrochloric acid (HCl). Longan seeds (*Dimorcapus longan*) contain 5.08 grams of glucose, 4.85 gram of fructose and 0.18 gram of sucrose in every 100 gram of longan seeds [24]. The sugar content is sufficient for the growth of fermenter microorganisms that require at least a 5-carbon sugar source, including xylose, arabinose, lactose, galactose [25-28]. glucose and fructose are the most efficient carbon sources to use [29]. In addition, Li *et al*. [19] reported that the content of longan seeds contains the minerals Calcium (Ca = 0.13%), Magnesium (Mg = 0.16%), Sodium (Na = 54 mg/Kg), Potassium (K = 0 .41%), and Phosphorus (P = 0.16%) which are the main components required by the growth of fermenter microorganisms [30]. The primary polysaccharide in longan seeds is starch (44.9–49.5% w/w) [18], and HCl hydrolysis targets the glycosidic bonds in starch to release fermentable sugars.

Fermentation was carried out to convert the hydrolysate substrate from longan seeds into ethanol using the yeast *Saccharomyces cerevisiae*. The initial pH of the medium was adjusted to 5.0 ± 0.2, and the fermentation was conducted at a constant temperature of 30°C, which falls within the optimal range for S. *cerevisiae* (30–37 °C) [31]. Samples were taken aseptically every 24 h (daily interval) for four consecutive days. Each collected sample was immediately stored in a refrigerator at 4 °C to halt any further metabolic activity, and all samples were analyzed simultaneously at the end of the fermentation period using a UV-Vis spectrophotometer. The ethanol content was determined spectrophotometrically using the acid dichromate method. In this assay, ethanol reduces Cr(VI) (dichromate ion, orange-red) to Cr(III), causing a distinct color change from orange-red to green [23]. Reducing sugar concentrations were not directly measured in this study, but the ethanol yields imply that starch hydrolysis successfully provided fermentable sugars.

Analysis of the ethanol content was carried out using variations in the weight of longan seeds hydrolyzed with hydrochloric acid (HCl) and variations in several concentrations. The purpose of this hydrolysis is to hydrolyze the polysaccharides, especially starch, in longan seeds into simple sugars so that the yeast *Saccharomyces cerevisiae* gets nutrition from these sugars and converts these sugars into ethanol [32]. These simple sugars are usually in the form of sucrose, fructose, galactose and glucose [33,34]. Hydrochloric acid (HCl) also functions to hydrolyze the glycosidic bonds of starch, and may act on any residual cellulose, hemicellulose, and lignin so that they become sugar compounds that can be used for the conversion of bioethanol in *Saccharomyces cerevisiae* [35]. Data analysis of ethanol content in longan seeds with variations in weight and HCl concentration can be seen in **Figure 1** and **Figure 2**.

The ethanol content in the fermented *D. longan* seeds (**Figure 1**) shows the variation which is the largest percentage is weight of *D. longan* seeds 5 gram in second day with an ethanol percentage of 1.838% while the smallest percentage is found in the weight of 1 gram on the fourth day with an ethanol percentage of 0.292%. The data also shows a relationship between fermentation and media weight of *D. longan* seeds, that the more longan seeds used for fermentation, the greater the ethanol content produced. This is in accordance with research reported by Mardawati *et al* [36] regarding the effect of bioethanol substrate concentration which showed that the increasing substrate of the fermentation media the higher the ethanol content formed due to the increasing sugar nutrient content which was successfully hydrolyzed for the growth of the yeast *Saccharomyces cerevisiae*. Xin *et al* [37] revealed that the growth of the yeast *Saccharomyces cerevisiae* requires reduced sugar, this was concluded from the fact that the longer the incubation time, the less reduced the sugar component in the fermentation medium.

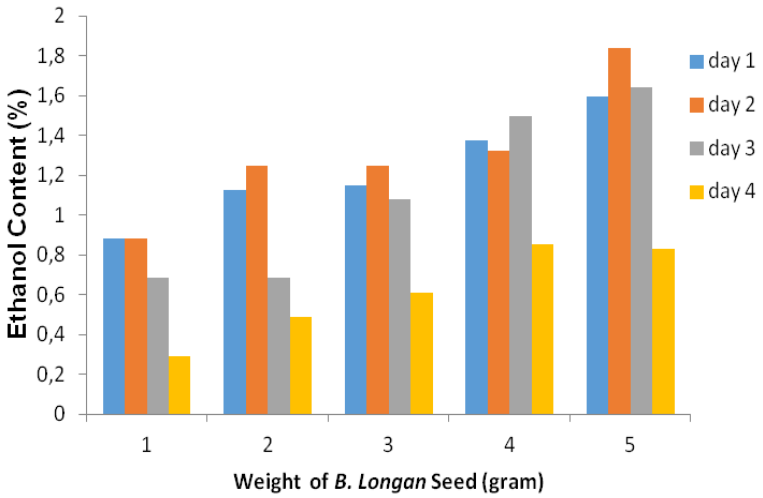

Figure 1. Graph of ethanol content of weight variation for 4 days

Ethanol production is also affected by the concentration of the hydrolyzing component with strong acid. Hydrochloric acid is the most often used for sugar hydrolysis because of the efficiency and ease of obtaining this component [38,39]. Hydrolysis of starch is carried out by strong acids by breaking the glycosidic bonds, so that these polysaccharides become simpler sugars. Analysis data from the influence of strong acid hydrolyzers with ethanol production from the yeast fermentation of *Saccharomyces cerevisiae* can be seen in **Figure 2**.

The ethanol content shown in **Figure 2** indicates that the highest fermentation yield from longan seeds was observed on the first day using a 4% HCl concentration (v/v), reaching 2.942%. In contrast, the lowest yield occurred on the fourth day with a concentration of 5% HCl (v/v) resulting in an ethanol content of 0.906%. Ethanol production at 1% and 2% HCl concentrations decreased on the third day. For HCl concentrations of 3% and 5%, a steady decline was observed starting on the second day. Interestingly, a distinct fluctuation pattern was observed at a 4% HCl concentration; the ethanol content initially decreased on the second day but experienced a brief (transient) increase on the third day before eventually declining again on the fourth day. This overall decline in ethanol production, particularly at higher acid concentrations (4% and 5%), is attributed to the demand for reducing sugars required for the growth of *Saccharomyces cerevisiae*, which may prompt the yeast to utilize ethanol or convert it into other metabolites [40]. Additionally, the accumulation of carbon dioxide (CO_2) as a byproduct of fermentation can potentially inhibit yeast growth, which also contributes to this decline [41]. The decrease in ethanol yield at the highest acidity level (5% HCl) results in the formation of lignocellulose degradation products, such as furfural and 5-hydroxymethylfurfural (HMF), which are typically formed during intensive acid pretreatment processes. The study documented that, as in similar biomass conversion studies, this furan derivative acts as a potent inhibitor that can disrupt yeast glycolysis, damage mitochondrial membranes, and trigger the accumulation of reactive oxygen species (ROS), thereby significantly reducing the fermentation efficiency of *Saccharomyces cerevisiae*. Unusual results observed at a 4% HCl concentration on day 3 indicate an attempt to metabolize or evade this structural inhibitor, before nutrient depletion or toxicity effects eventually became dominant again.

Monitoring bioethanol production from longan seeds using *Saccharomyces cerevisiae* was conducted daily through the fourth day. The highest production was observed in the 4% acid concentration treatment on the first day. This was based on the amount of ethanol produced compared to ethanol production at other concentrations. The addition of 4% HCl efficiently breaks the glycosidic bonds in complex sugars; however, at a 5% concentration, the sugar content begins to break down into carbon, causing a decrease in reducing sugars. Excessive acid hydrolysis degrades sugars into byproducts specifically furfural and inhibits the fermentation process and yeast growth. This aligns with research in the field of bioethanol from the report by Ahmad *et al*. [42], which states that the higher the concentration of hydrolyzing acid used, the more sugar is obtained during the hydrolysis process. A decrease in sugar production may occur after the optimal phase due to the depletion of sugar, which serves as a nutrient for the yeast. An increase in reducing sugar levels resulting from acid hydrolysis will affect the growth of *Saccharomyces cerevisiae*

yeast; in this case, the yeast will produce the enzyme zymase, which converts glucose or other simple sugars into ethanol [20]. The mechanism scheme of sugar transport and metabolism in *S. cerevisiae* [43] can be seen in **Figure 3**.

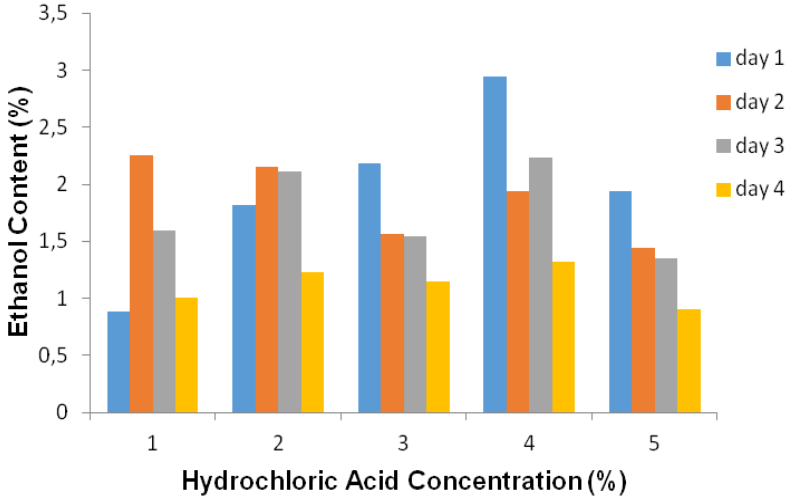

Figure 3. Graph of ethanol content of variations in acid concentration for 4 days.

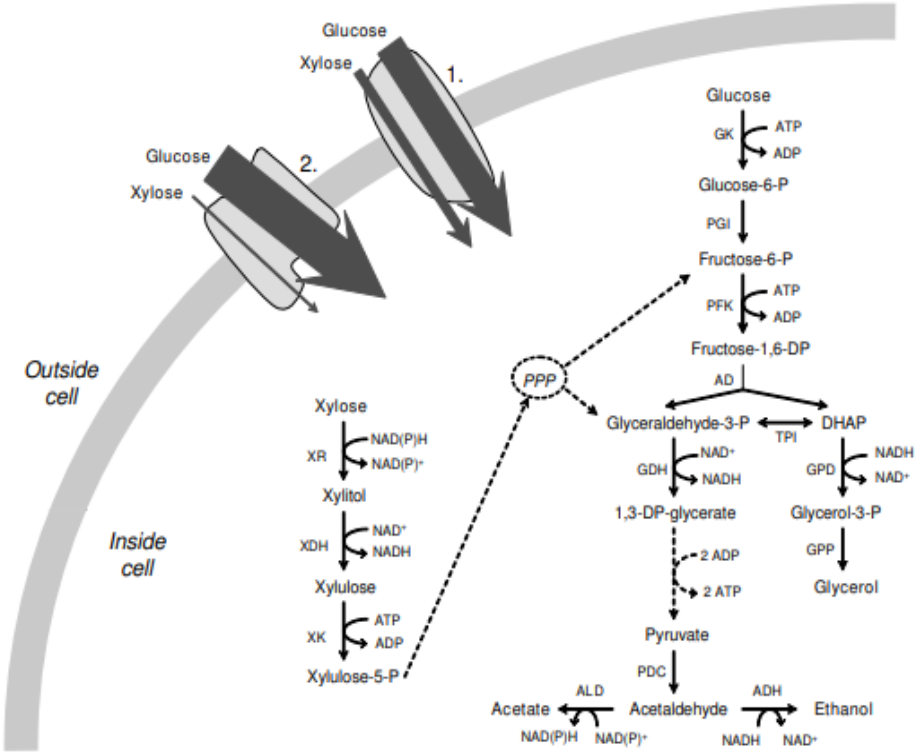

Figure 2. Simplified scheme of sugar transport and metabolism in *S. cerevisiae*. 1. Low and medium affinity hexose carriers. 2. High affinity hexose carriers. (Abbreviations: PPP, pentose phosphate pathway; XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulokinase; GK, glucokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; AD, aldolase; TPI, triose phosphate isomerase; GDH, glyceraldehyde- 3-P dehydrogenase; GPD, glycerol-3-P dehydrogenase; GPP, glycerol-3-phosphatase; PDC, pyruvate decarboxylase; ALD, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase) [43]

Table 1. Ethanol content in fermented longan seeds through the fermentation method by the yeast *Saccharomyces cerevisiae*

Sample		Ethanol Content (%)			
Sample Variation	**Ammount/ Concentration**	**day 1**	**day 2**	**day 3**	**day 4**
Acid Concentration	1%	0.881	2.255	1.593	1.004
	2%	1.813	2.157	2.108	1.225
	3%	2.182	1.568	1.544	1.151
	4%	2.942	1.936	2.231	1.323
	5%	1.936	1.445	1.347	0.906
D. Longan Seed Media Weight	1 gram	0.881	0.881	0.685	0.292
	2 gram	1.126	1.249	0.685	0.488
	3 gram	1.151	1.249	1.077	0.611
	4 gram	1.372	1.323	1.494	0.856
	5 gram	1.593	1.838	1.642	0.832

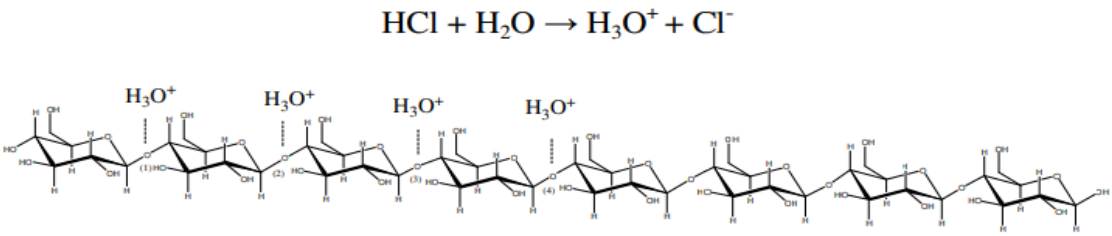

Figure 4. The hydronium ion attacks the cellulose molecule at positions 1, 2, 3 and 4

Research data on bioethanol production from longan seeds (**Table 1**) show that ethanol production with longan seeds is optimal at a powder weight of 5 grams and a hydrochloric acid (HCl) concentration of 4%. Fermentation is influenced by several factors such as incubation temperature, pH, oxygen, media components and substrate concentration [44]. Substrate concentration in seed media can be increased by acid hydrolysis in the media so that it becomes simple sugars [45]. Hydronium ions (H_3O^+) attack the glycosidic bonds in cellulose or complex carbohydrates with the mechanism shown in **Figure 4** [46]. The incubation temperature during fermentation affects the growth rate of *Saccharomyces cerevisiae* to convert sugar into bioethanol at a temperature of 30-37 ^oC [47,48]. pH determines the success of bioethanol production using yeast with the optimal pH for the growth of the yeast *Saccharomyces cerevisiae* in the pH range of 4-6 [49]. The reaction of sugar complex to alcohol and their metabolism from *Saccharomyces cerevisiae* can be seen in **Figure 5.**

The effect of decreasing the concentration of ethanol is also often associated with the length incubation of fermentation by *S. cerivisae* this can be attributed to several factors as stated point by Tse et al [7] which stated that the number of microorganisms caused by nutrients in the media has been greatly reduced and also caused by results that can inhibit the growth of microorganisms. Increasing and decreasing the number of microorganisms will increase the pH of the media. According to Permatasari *et al*. [50] stated that the most optimal fermentation time for the process of making bioethanol with the yeast *Saccharomyces cerevisiae* is 3 days. If fermentation is carried out for more than 3 days, the alcohol content may decrease. Its related to this research that the longer incubation time for fermentation by S. cerevisiae, the less ethanol content produced. Reducing the amount of alcohol caused by alcohol must be something else, for example esters. According to Hernandez *et al*. [51] the longer the fermentation time, the higher the microbial population, which eventually enters the death phase due to nutrient depletion and the accumulation of toxic ethanol.

Purification of ethanol fermented d. longan seeds from the yeast Saccharomyces cerevisiae

Purification of fermented ethanol is carried out by distillation method. The bioethanol fermentation was repeated by doubling the total volume of the resulting mixture (100 mL of fermented media mixture formula, 10 mL of inoculum medium, and 10 mL of longan seed

hydrolysis) to 2-3 times the initial volume. Fermentation was carried out based on the results of the optimal research that had been carried out, namely at a variation of 4% acid concentration and 5 gram of *D. longan* seed media with a fermentation time of 1 day. The results of ethanol purification data can be seen in **Table 2**.

Data from the purification of ethanol shows that the fermented ethanol can be separated using the distillation method. A qualitative test to detect the correctness of purified ethanol was carried out by adding 12 mL of acid dichromate reagent. In the results of observations there was a color change in the solution from the initial color of dark red to dark green. Determination of ethanol content was carried out quantitatively and the results obtained were the purity of ethanol that was distilled with the larger the volume, the lower the purity. This is caused by the inclusion of other compounds which have lower boiling points than ethanol so that they are also mixed with the distilled ethanol. These data indicate a relatively low purity in ethanol purification considering that the purity of ethanol using the distillation method reaches 95-99.89% [52]. Ethanol purification in industry is more specific and more efficient on a large scale. Several methods are used to purify ethanol from fermentation products, including using pervaporation techniques with selective membrane modules [53,54], vacuum fermentation [55], gas striping [56], adsorption [57] and solvent extraction [58].

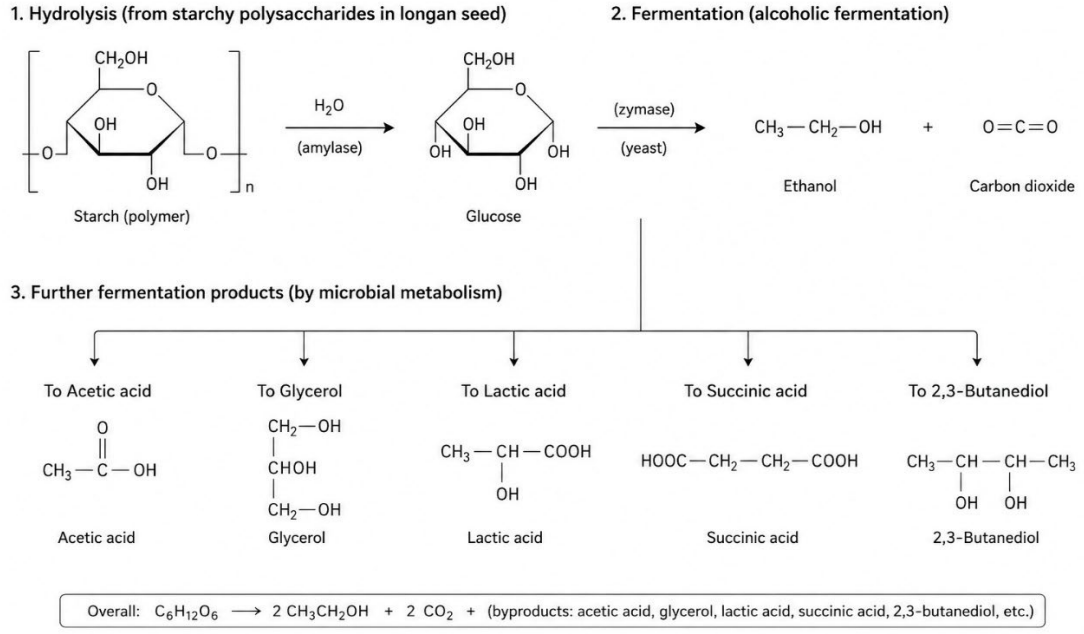

Figure 5. the reaction of sugar to alcohol

Table 2. Results of bioethanol purification by distillation method

Volume of Fermented sample (mL)	Fermentation Formula	Ethanol volume (mL)	Percentage of ethanol (%)	Percentage Purity of Ethanol (%)
120	100 mL MF 10 mL MI 10 mL MHBK	2,1	1,75	89,81
240	200 mL MF 20 mL MI 20 mL MHBK	6,7	2,79	87,99
360	300 mL MF 30 mL MI 30 mL MHBK	10,4	2,89	82,12

Note: MF = Fermentation Media; MI = inoculum medium; MHBK = *D. Longan* Seed Hydrolysis Media

CONCLUSION

Fermented bioethanol from *D. longan* seed media hydrolyzed with hydrochloric acid (HCl) was successfully carried out. Under the tested conditions, the highest ethanol concentrations were obtained at an HCl concentration of 4% (v/v) (ethanol concentration: 2.942% v/v) and a seed weight of 5 g (ethanol concentration: 1.838% v/v). However, these values represent individual optimum points from separate single factor experiments and should not be interpreted as a statistically confirmed optimal combination. Purification of bioethanol from fermented products was performed via simple distillation, yielding ethanol concentrations in the distillate ranging from 1.75% to 2.89% (v/v). The relatively low ethanol concentration in the distillate indicates that the purification process, while able to separate ethanol, was limited in its effectiveness. The purity tended to decrease as the total fermentation volume increased.

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