Low Temperature Enzymatic Hydrolysis (LTEH) and Fermentation for Bioethanol Generation from Suweg (Amorphophallus campanulatus B) Starch

by Hargono Hargono

Low Temperature Enzymatic Hydrolysis (LTEH) and Fermentation for Bioethanol Generation from Suweg (*Amorphophallus campanulatus B*) Starch

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Abstract.The ethanol generation through low temperature enzymatic hydrolysis (LTEH) of Suweg (*Amorphophallus campanulatus B)* starch followed by anaerobic fermentation using *Saccharomyces cerevisiae*has been studied. Granular starch hydrolyzing enzyme (GSHE) as Stargen ^{TM} 002 was used in the hydrolysis to degrade starch into reducing sugar at 30°C and pH 4. The concentration of Suweg starch was 200 g/L, concentration of enzyme were 1; 1.5 and 2% (w/w), respectively. The fermentation was carried out at pH 4.5 and 30 °C for 72 h employing yeast concentration of $1gL^{-1}$. The ideal state of the procedure was fermentation utilizing concentration of Suweg starch 200 g L^{-1}, concentration enzyme 1.5% (w/w), pH 4.5 and 30°C, for 60 h, which came about reducing sugar grouping of 31.32 g/L and further used for ethanol generation. It was discovered that most extreme ethanol fixation and profitability 13.12 g L^{-1} and 0.3727 $gL^{-1}h^{-1}$, separately. Although the result shows that Suweg starch is a potential raw material for ethanol generation, further investigations are required in both generation sustainability and techno-economical considerations.

1. Introduction

Being a perfect, safe, earth neighborly and sustainable power source asset, bioethanol is a potential option in contrast to the consistently exhausting petroleum derivatives [1]. Bioethanol can be delivered from different kinds of farming items, for example, glucose, sucrose, lignocellosic, starch and alga biomass through hydrolysis and aging procedure. Because of its high ethanol profitability, high ethanol resilience and capacity to process wide scope of sugars, Saccharomyces cerevisiae has been turned out to be the most well-known yeast relegated in the bioethanol generation [2]. Unfortunately, generation of bioethanol utilizing yeast regularly experiences a few difficulties in yeast maturation, which restrain ethanol generation, for example, high temperature, high ethanol fixation and the capacity to age pentose sugars [3].

Starch is one of the vital wellsprings of carbon and vitality in world, and in this manner displays enormous market request and applications in industry. It very well may be utilized to create numerous profitable sustenance items in the nourishment handling industry, for example, maltose, glucose, fructose, glucose-fructose syrups, natural acids, amino acids, and so on [4]. While most industrial starches are sourced from corn, cassava, wheat and potato, some other underutilized botanical sources remain hidden. Suweg (*Amorphophallus campanulatus B*) is a starchy root crop that is grown almost entirely in the low land tropics, including Indonesia, Malaysia and Thailand. This tuber is a decent

wellspring of vitality, sugar, starch, proteins and additionally minerals normal healthful profile contains starch (11% - 18%) [5].

Hydrolysis of starch frees a specific measure of reducing sugars, which is a important substrate in generating bioethanol [6]. The convention method of converting starch into glucose required two-advance hydrolisis step: liquefaction process and saccharification process. In liquefaction, the gelatinization process is advanced with using high temperature (90-100°C) and in abundant of water, changing over semi-crystalline starch molecule end up indistinct [7-8]. This process need high energy, so that increase the generation cost of starch-based ethanol, the energy used from those two stage process is arround 30%-40% of the aggregate energy needed in ethanol generation of starch [9]. Robertson *et al.* (2005) assessed that the decrease in energy utilization reached 10%-20% in ethanol generation [10]. Several authors had investigated the enzymatic hydrolysis of various tuber starches. The examinations consolidated the hydrolysis profile of nearby and delicately warm treated custard and sweet starches at sub gelatinization temperature [7], coordinate difference in cassava and sweet potato roots slurry into glucose and fructose syrups [8] also the generation of glucose and fructose syrup from different tuber starches [9]. However, as far as literature survey being carried out no study has been dealt to the effect of enzyme concentration at low temperature for Suweg starch on ethanol generation.

As a solution for the conventional method, the low temperature enzymatic hydrolysis (LTEH) is being contemplated. In fact, the investigation of this process is not recent [12-14] but only a few researchers have intensively researched this area [8,15]. Granular Starch Hydrolyzing Enzyme (GSHE) can specifically process crude starch granule at low temperature. As an outcome, GSHE essentially decrease the energy required for hydrolysis step [11]. However, there are still difficulties to be handled in order to streng then the competitiveness of this LTEH. First, lower temperature utilized generally create contamination. Second, the starch granules remain solid and after that, the degradation reaction occur in liquid-system, where the related mass exchange is an outstanding restriction [11]. As an outcome, larger amount of enzymes and longer occasions are required in order to finish transformation process to glucose [16]. According to Zhang *et al.* who researched the simultaneous reaction of saccharification and fermentation (SSF) of liquefied sweet potato obtained 112 gL^{-1} of ethanol in 24 h [17]. Bialas *et al.* reported the highest ethanol concentration of about 100gL^{-1} after 60 h during SSF of granular corn starch [18].

The target of this investigation were to observe the impact of concentration of enzyme on the reducing sugar using GSHE at low temperature and to observe the impact of reducing sugar concentration (product of hydrolysis) on ethanol generation and productivity during separated hydrolysis and fermentation (SHF).

2. Materials and Method

2.1. Raw materials, chemicals, enzyme and microorganism
Suweg (*Amorphophallus campanulatus* B)

A 10 month old Suweg tuber was acquired from Wonogiri region in Central Java- Indonesia.
Starch extraction

The procedure for extraction of starch from Suweg tuberused in this study was the same as that previously used by Hargono [19].
Chemicals

Potassium sodium tartrate tetrahydrate and 3,5-Dinitrosalicylic acid (by Merck), NaOH (98%, Merck), Na_2SO_3 (98.5%, Merck), H_2SO_4 (98.5%, Merck), sodium acetat buffer (Merck), glucose (99.5%, Merck) and ethanol (99.5%, Merck), $(NH4)_2HPO_4$, $MgSO_4.7H_2O$ and yeast extract were purchased from Sigma-Aldrich Indonesia.

Enzyme

GSHE as Stargen ™ 002 was acquired from Genencor International (USA) [20]. These enzyme comprise *Aspergillus kawachii* α-amylase revealed in *T. reesei* and glucoamylase from *T. reesei*. The specific gravity of from 1.13-1.16 g m/L and ideal pH went from 4.0-4.5. The suggested temperature 20-40°C and the minimum activity is 570 GAU g^{-1}. One glucoamylase unit (GAU) is the number of enzyme that discharge 1 g of reducing sugar measured as glucose every hour from dissolvable starch substrate under the states of the examine.

Microorganism

Dry pastry yeast S. *cereviceae* (pastry's yeast, Mauri Pan) was obtained from nearby pastry shop in Kabita, Semarang, Indonesia and kept in cooler till utilize. S. *cereviceae* was scattered in clean water at room temperature at a concentration of 10 g/L (g dry pastry yeast/litre of DI water) and 10 mL of this was utilized inoculum without no further cultivation and added to 90 mL of maturation medium to get 10% (v/v) portion. Prior to vaccination, the cup and medium were sanitized by operating autoclave at 121°C, 0.5 h. The temperature and agitation speed were kept up steady.

2.2. Enzymatic hydrolysis experiment

Enzymatic hydrolysis experiments were done aseptically in 250 mL Erlenmeyer flasks installed vertically onto a rotary shaker. Suweg starch of concentration 200 g/L was used in this research. The pH of the starch suspension was change in accordance with 4 (in 50 mM sodium acetate buffer). Before including enzyme, the mixtures were preheated for 1 h at 62 °C in a responding water bath as suggested by GSHE maker [20]. After that, the enzyme at 1, 1.5 and 2% (w/w) were added carefully into the right flasks. The mixtures were mixed and hatched at 80±1°C in thermostatic water bath heater with non stop stirring of 100 rpm for 15 min. The slurry obtained from each flask was cooled until 30±1°C (room temperature) and incubation was proceeded for 18 h. Samples were periodically 3 hours withdrawn from the flask and genereously subjected reducing sugar determination [21]. Samples were centrifuged (100 Hz,4°C and 10 min) to obtain filtrate, then it can be analyzed by using Spectrophotometer.

2.3. Fermentation experiment

The chosen concentration of substrate, enzyme and also the best pretreatment were settled so as to assess maturation conditions in fermentation process. Fermentation process take places in a 1.2 L reactor, equipped with temperature and pH control. The volume of the system was approximately 1L. The pH of the medium was set at 4.5 during the cultivation by careful addition of 3 M NaOH. The fermentation medium was added by many nutrients, such as $(NH4)_2HPO_4$ 0.5 g L^{-1}, $MgSO_4.7\ H_2O$ 0.025 gL^{-1} and yeast extract 1.0 gL^{-1} for 15 h in an incubator-shaker at 37°C and 80 rpm. Next, the dry yeast was added (5 g L^{-1}), the experiment run during 72 h. Samples were collected after 12, 24, 36, 48, 60 and 72 h, and analyzed for ethanol using gas chromatography and reducing sugar by Spectrophotometer. Productivity of ethanol (Q_{eth}) for the first hour of fermentation can be determined equation (1):

$$Q_{eth}(t) = \frac{C_{eth,t} - C_{eth=0}}{t} \tag{1}$$

where Q_{eth} is the ethanol productivity (g/$L^{-1}h^{-1}$), $C_{eth,t}$ is the ethanol concentration (g L^{-1}) at the time t and $C_{eth,t=0}$ is the mass concentration of ethanol before adding yeast to the medium while t (h) is the time of fermentation.

The yield parameter of conversion reducing sugar to ethanol (Yp/s) was calculated by the equation (2) [22].

$$P = -Y_{P/S}S + \left(P_0 + Y_{P/S}S_0\right) \tag{2}$$

where P is the concentration of product (g/L), S is the concentration of substrat at the time t (g /L), P_0 is initial concentration of product (g L^{-1}) and S_0 is initial concentration of substrate (g /L).
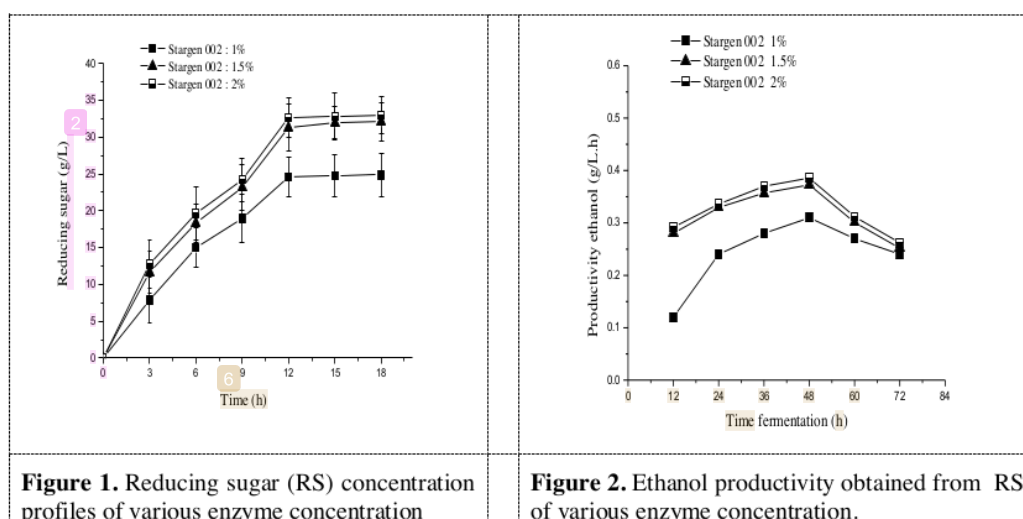
2.4 Analytical methods

The water content in Suweg starch be measured with AOAC 991.43 method [23]. The starch content be measured by Ewer's polarimetriv technique using hydrochloric acid [24], while the reducing sugar value was estimated utilizing dinitrosalicylic-acid technique [21]. Reagent containing fluid arrangement of 1% 3,5-dinitrosalicylic-acid, 0.05% natrium sulfit, 20% natrium kalium tartrate and 1% NaOH solution was included in proportion 3:1 to the samples in glass tubes, wiggled in incubated in a boiled water bath for 8-min. After that, samples were being soaked in an icy water bath for about 5 min, preceding to measure absorbance at 540-nm by utilizing a UV-visible spectrophotometer (UV160A, SHIMADZU, Kyoto, Japan). Glucose (0-5 g/L) was utilized as standard references; so that, reducing sugar concentrations was reported as g/L. A standard curve was made through estimating the known glucose arrangements absorbance at 570-nm, while ethanol generation by fermentation was estimated by gas chromatography (Shimadzu, Kyoto, GC 2010) , out fitted with a flame ionization detector (FID) and N_2 was utilized bearer gas. Both the injector and identifier were kept up at 200 and 220°C, and the segment temperature was acclimated 170°C, utilizing 1 %, 3 %, 5 %, and 7 % (v/v) ethanol standard.

3. Results and Discussion

The Suweg starchcontains moisture 10.38%, starch 78.24% and non starch materials (11.38%) by weight. The starch is important component which will be degraded to reducing sugar by enzymatic hydrolysis. Later, the reducing sugar is used as a raw material for bioethanol generation through fermentation.

3.1 Effect of enzyme concentration on reducing sugar

The effect of concentration Stargen^{TM} 002 (1, 1.5 and 2%) on reducing sugar at concentration of Suweg starch 200 gL^{-1}, pH 4 and 30°C are given in Figure 1. The time of hydrolysis under studies range from 0 to 18 hours, while the true to archive time a maximum reducing sugar was 12 h and further. The concentration reducing sugar maximum obtained were 24.62, 31.32 and 32.68 g/L, respectively. Then after 12 hours, the hydrolysis of Suweg was constant rate at 15 to 18 h.

Figure 1. Reducing sugar (RS) concentration profiles of various enzyme concentration	**Figure 2.** Ethanol productivity obtained from RS of various enzyme concentration.

Sawai *et al*. reported the hydrolisis of sweet potato starch using ß-amylase concentration. The effect of ß-amylase concentration on reaction rate constant (k) increased with concentration up to 5.56 units mL^{-1} and then became almost constant [25]. Earlier, Hargono *et al*. reported that the reducing sugars acquired from harsh cassava and Gadung starches with concentration of enzyme 1.5%, concentration of starch 200 g/L at pH 4, 30 °C were 41.7 g/L and 9.08 g/L, respectively [19]. Shanavas *et al*. reported the reducing sugar made from hydrolysis of cassava starch using Stargen ^{TM} 002 at pH 4.5 and 30°C.The highest reducing sugar concentration was 98.3 gL^{-1} when 100 mg enzyme was used to hydrolyze 10% (w/v) starch [26].
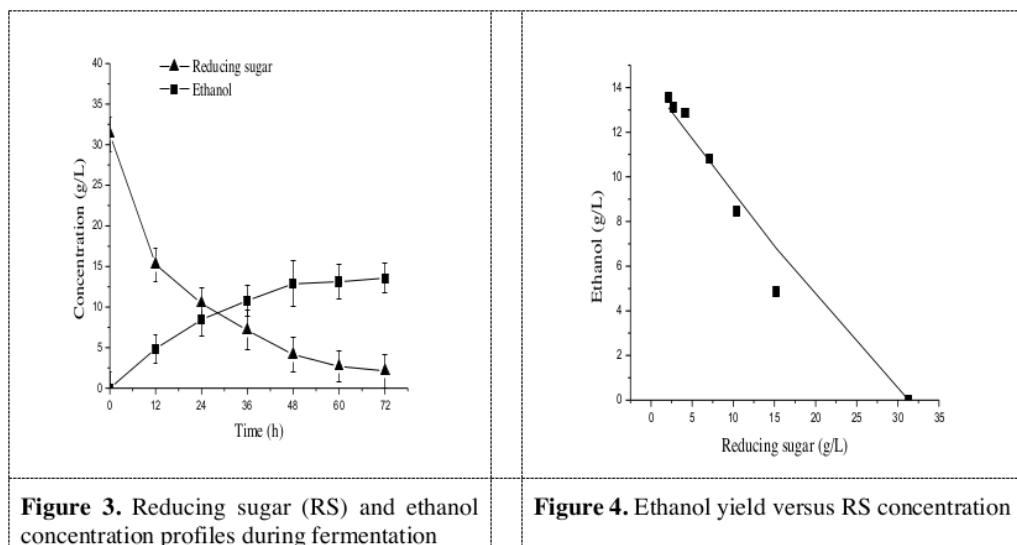
The velocity of the enzymatic reaction is directly straightforwardly corresponding to the concentration of the enzyme. However, this condition only applies to certain enzyme concentrations and after that the enzyme does not affect the velocity reaction [27]. This happens because the velocity of the enzymatic reaction is affected by the active enzyme concentration, which is an enzyme that binds to a cofactor. As only the soluble starch will form bindings with enzyme, the amount of enzyme used for hydrolysis is basically limited. The hydrolysis was conducted at low temperature suggesting that the solubility of Suweg starch (as the member of tuber crops) was definitely low. Further increase of enzyme concentration may trigger competition between enzyme-enzyme to reach the starch molecules.

3.2 Productivity of ethanol during fermentation at various of enzyme concentrations

The results of productivity of Suweg starch fermentation as calculating equation (1) are exhibited in Figure 2. The volumetric productivity of ethanol (Q_{eth}) was evaluated during 72 h of fermentation of concentration of starch 200 g L^{-1} at concentration of enzyme 1, 1.5 and 2%. The highest of Q_{eth} for Suweg starch during 48 h of fermentation were obtained 0.310, 0.373 and 0.386 g/L.h.

3.3 Reducing sugar concentration and ethanol formation by SHF

Based on concentration of enzyme (1.5%) as well as the best pretreatment, the reducing sugar concentration and ethanol formation by SHF using *S. Cerevisiae* during 72 h are shown in Figure 3.

Figure 3. Reducing sugar (RS) and ethanol concentration profiles during fermentation

Figure 4. Ethanol yield versus RS concentration

The initial reducing sugar concentration used for fermentation was (31.32 gL^{-1}). In the beginning of fermentation (up to 12 h) it decreased sharply and followed by a constant decrease as the fermentation reached 48 h (4.14gL^{-1}). This situation changed to gradual reduction of reducing sugar concentrationuntil at the end fermentation. A maximum ethanol concentration (13.12 gL^{-1}) was obtained at 60 h fermentation. At 60 h the growth of *S. Cerevisiae* may has stopped and the stationary phase started when almost all reducing sugar was used up and ethanol generation became slower. The profile of reducing sugar and ethanol was similar to Nadir *et al*.who studied the fermentation of cassava using *S.cerevisiae* [28].

The results of yield parameter of conversion reducing sugar to ethanol (Yp/s) calculated by equation (2) are shown in Figure 4. As shown in Figure 4, the value of Yp/s is 0.481 g g^{-1}. Bialas *et al*.observed that theYp/s for simultaneous saccharification and fermentation of corn starch to ethanol was 0.4759 g g^{-1} [29]. Their result was in good agreement with the results obtained by Kroumov *et al*. [30] and Davis [31], who studied the SSF process of alcohol generation from gelatinized wheat flour using enzyme amyloglucosidase and the yeast *Saccharomyces cerevisiae*.

4. Conclusion

The enzyme concentration and the time required for maximum reducing sugar formation are part of crucial issues in ethanol generation. In this work, 12 h and 1.5% were found to be the time and enzyme concentration for Suweg starch hydrolysis to achieve maximum reducing sugar concentration. Fermentation of this reducing sugarusing 1 g L^{-}1. *Saccharomyces cerevisiae* achieved highest ethanol productivity of 0.373 g L^{-1}h^{-1} at 48 h. The maximum ethanol concentration (13.2 g/L) was achieved after fermentation last for 60 h and the value of yield of reducing sugar to ethanol was 0.481 g/g.

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