Preliminary study of bioethanol production by *Saccharomyces cerevisiae* BTCC12 utilizing hydrolysis products of *Dioscorea hispida* tubers

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**Abstract.** *Dioscorea hispida* is an underutilised plant despite its high carbohydrate content. This study used *D. hispida* starch as the raw material for bioethanol production by employing Separated Hydrolysis Fermentation (SHF) technique. Initially, the starch was used as the sole carbon source for *Aspergillus awamori* KT-11 to produce amylases. The optimum enzyme activity (7.4 U/ml) was observed at 96 h fermentation. The enzymes were further used to hydrolyse *D. hispida* starch. The optimum condition for the hydrolysis was achieved at 6 h when using a mixture of starch and enzyme with a ratio of 1:6 (w/v). Thin-layer chromatography results showed that the main hydrolysis product was glucose. The hydrolysates were then used by *Saccharomyces cerevisiae* BTCC12 as the medium for bioethanol fermentation. HPLC analysis showed that the optimum ethanol concentration was 0.37 g/L after 6 h fermentation while the reducing sugar concentration was only 2.9% of the initial. The results provide a solid basis for further studies to optimise bioethanol fermentation utilising *D. hispida* as the substrate.

1. **Introduction**

The growing of the world population and the industrial expansion have caused a continuous increase in global energy consumption. The search for alternative energy is needed because fossil fuel reserves continue to decline. One of the potential renewable energies is bioethanol, and its use may reduce the burden to the environment. Many studies have utilized biomass as the media for bioethanol fermentation [1][2][3].

The first-generation bioethanol production from biomass has caused competition between food and energy usage. At present, most bioethanol production in the United States, European Union and China uses corn, while countries in the tropical region such as India, Brazil and Colombia use sugar cane [1]. The second-generation bioethanol production utilizes lignocellulosic materials, which can also be hydrolysed to simple sugars. The hydrolysis yield may reach 70%, and the hydrolysates can be used as substrates for bioethanol fermentation [2][3]. Currently, the production of bioethanol from microalgae biomass provides a better option and may increase energy security and preserve ecological balance [4].

Bioethanol fermentation is carried out by initially hydrolyzing the biomass, either using acids or enzymes [5], to produce simple sugars. Enzymatic hydrolysis is far more efficient than acid hydrolysis.

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and reduces pollution due to minimum waste and simple downstream processing. The sugars, mostly glucose, are then converted to ethanol through a fermentation process. One of the microorganisms that is often used in the fermentation is \textit{Saccharomyces cerevisiae} [6].

Finding a suitable biomass source may tackle the problem of using staple food as media for bioethanol fermentation. It could be derived from agricultural waste or plants that are rich in cellulose or starch. \textit{Dioscorea hispida} is an underutilised carbohydrate-rich plant. It has a high starch content, reaching 70\% of the total dry weight. The plant can be easily cultivated in various types of soil and is reported not to have natural pests [7]. Because of its high level of starch, \textit{D. hispida} is potential as the carbon sources for bioethanol fermentation.

Therefore, this study aims to utilize \textit{D. hispida} tubers as raw material for bioethanol fermentation. The tubers were enzymatically hydrolyzed using amylases produced by \textit{Aspergillus awamori} KT-11. The strain produces complex amylases, which directly hydrolyzes raw starch without undergoing a gelatinization process [8]. The hydrolysis products were then used as a medium for bioethanol fermentation by \textit{S. cerevisiae} BTCC12. The results may provide preliminary information for bioethanol production by utilizing \textit{D. hispida} tubers.

2. Methods

2.1. Sample and microorganisms
\textit{D. hispida} tubers were collected from Montasik area in Aceh Besar district. Freshly peeled tubers were sliced and soaked in water containing 2\% sodium chloride for 24 h. The soaking solution was regularly changed. The slices were later washed several times with distilled water, dried at 70 °C for 24 h and sieved with 100 mesh sieve. Microorganism used in this study were \textit{A. awamori} KT-11 and \textit{S. cerevisiae} BTCC 12, which were culture collections of the Research Centre for Biotechnology, Indonesia Institute of Sciences.

2.2. Production of amylases from \textit{A. awamori} KT-11
One colony of \textit{A. awamori} KT-11 from a solid PDA medium was cultured at 30 °C and 150 rpm in a liquid medium made from 4\% \textit{D. hispida} flour, 0.24\% urea, 0.036\% MgSO$_4$.7H$_2$O, 0.08\% Triple Super Phosphate (TSP) pH 5. The fermentation broth was sampled at 72, 96, and 120 h. Crude amylases were extracted by centrifugation at 12000×g for 10 min at 4°C. The supernatant containing the enzymes was assayed by the Nelson-Somogyi method [9] at 500 nm using a standard curve of glucose (300 – 1000 µg/mL). All analysis was done in triplicate. One enzyme unit (U) is defined as the amount of enzyme required to release 1 µg/mL reducing sugar per minute under the assay conditions. The supernatant showing the highest amylases activity was used to hydrolyze \textit{D. hispida} starch.

2.3. Hydrolysis of \textit{D. hispida} starch by amylases from \textit{A. awamori} KT-11
The starch and the supernatant containing the enzyme were mixed with 1:6 dan 1:9 ratio (w/v) and the pH adjusted to 5.0. The mixture was incubated in a shaker water bath at 60°C, 150 rpm for 3, 6, and 9 h. The mixture was then centrifuged 10000×g for 10 min at 4°C. The supernatant was assayed by DNS method [10] at 540 nm using a standard curve of glucose (0.1 – 0.4 mg/mL). All analysis was done in triplicate. One enzyme unit (U) is defined as the amount of enzyme required to release 1 µg/mL reducing sugar per minute under the assay conditions. The supernatant showing the highest amylases activity was used to hydrolyze \textit{D. hispida} starch.

2.4. Bioethanol fermentation
Bioethanol was produced by \textit{S. cerevisiae} BTCC 12 in a medium containing 17.5 mL of 2\% Yeast Nitrogen Base (YNB), 5 mL of the hydrolysis product, and 2.5 mL \textit{S. cerevisiae} BTCC 12 (OD = 1). The pH medium was set to 4.8 by citrate buffer. Fermentation was conducted at 30°C and 150 rpm. Samplings were performed at 3, 6, 9, 24 and 48 h. Ethanol and reducing sugar concentrations were determined using High-Performance Liquid Chromatography (Shimadzu, LC-20AD pump, RI RID-
10A detector, Aminex HPX-87H column). Beforehand, the fermentation broth was centrifuged at 10000×g for 10 min. The supernatant was run at a flow rate of 0.6 mL/min using 5 mM H$_2$SO$_4$ as the mobile phase [11].

3. Results and discussion

3.1. Production of amylases from *A. awamori* KT-11

This study used amylase produced by *A. awamori* KT-11 to hydrolyze *D. hispida* starch, which was used as a medium for bioethanol fermentation by *S. cerevisiae*. Fermentation was carried out for 120 h, with sampling carried out every 24 h after the second day. The highest amylase activity (7.4 U/mL) was observed at 96 h of fermentation. Enzyme activity at 72 and 120 h was 4.8 and 5.4 U/mL, respectively (Figure 1). *A. awamori* KT-11 was also reported to be able to produce amylase in cassava media. The highest amylase activity was achieved faster at 72 h. The amylase activity (69.3 U/mL) almost 10-fold higher compared to that of this study [8]. These data indicate that *A. awamori* KT-11 was likely to produce amylase with better activity in complex carbohydrates than in dissolved starch as used in this study. Amylase produced by *A. awamori* KT-11 at 96 hours was later used to hydrolyze *D. hispida* starch.

![Figure 1. Amylase activity produced by *A. awamori* KT-11 on a medium containing 4% *D. hispida* starch. Fermentation was conducted at pH 5, 30°C and 150 rpm](image)

3.2. Hydrolysis *D. hispida* flour by amylases from *A. awamori* KT-11

*D. hispida* starch was then hydrolyzed to provide a medium for bioethanol fermentation. The starch and supernatant containing crude enzymes were mixed with a ratio of 1: 6 and 1: 9 (w/v). Hydrolysis was carried out for 3, 6, and 9 h. The highest reducing sugar concentration (0.43 mg/mL) peaked at 6 h of hydrolysis on the substrate with a ratio of 1: 6. The reducing sugar concentrations in the 1:9 medium were lower than those in the 1:6 medium. However, the reducing sugar levels in both media were practically the same at 9 h (Figure 2). The highest hydrolysis yield in this study was 43% at 6 h and in the 1:6 medium. The yield was lower than the hydrolysis using α-amylase/glucoamylase from *Aspergillus niger* to *Solanum lycocarpum* starch, which produces 92% yield [1]. The yield was also lower than the hydrolysis results using complex amylases from *A. awamori* to Babassu flour, with a yield of 87% [12].
3.3. Sugars profile after hydrolysis

The type of sugars produced by hydrolysis of both 1:6 and 1:9 media was qualitatively evaluated by TLC. Figure 3 shows that hydrolysis converted starch to mostly glucose, indicated by the thick spots with $R_f$ values parallel to the glucose standard. The hydrolysis of the 1:6 medium seems to produce more glucose than the 1:9 medium. Apart from glucose, all conditions also produce maltose. Glucose was also present at 0-hour condition, which may have originated from the supernatant from the previous experimental stage.

![Figure 2](image1.png)

**Figure 2.** Reducing sugar concentration after hydrolysis of *D. hispida* starch using amylases from *A. awamori* KT-11 at starch: enzyme ratio of 1:6 dan 1:9 (w/v) at pH 5.0. Hydrolysis was conducted at 60°C and 150 rpm.

![Figure 3](image2.png)

**Figure 3.** Qualitative sugar identification after hydrolysis 3, 6, 9 and 24 h using Thin Layer Chromatography Silica Gel GF60 (70 - 230 mesh). The elution buffer was a mixture of n-butanol: acetic acid: distilled water in a 2:1:1 ratio. The spots were developed by spraying Diammonium Phosphate (DAP) and heating at 120°C for 10 min.

This results showed that amylases from *A. awamori* KT-11 could hydrolyse raw starch without undergoing the gelatinization process. This sort of amylase reduces energy requirements for gelatinization, liquefaction and saccharification processes, thereby reducing production costs [13].
About 10% of amylases can degrade raw starch. They are known to have a starch-binding domain (SBD) that is responsible for binding and degrade raw starch that general amylases do not have [14].

3.4. Bioethanol fermentation

The results of starch hydrolysis for 6 h on the substrate with a ratio of 1:6 and 1:8 were then used as a medium for bioethanol fermentation, using the Separated Hydrolysis and Fermentation (SHF) technique. This technique hydrolyzes starch separately and uses hydrolysis products as the substrate for fermentation [15]. Fermentation was carried out using *S. cerevisiae* BTCC 12 by facultative anaerobic fermentation at 30°C. The concentrations of the glucose consumed and the ethanol produced was determined by HPLC.

Figure 4 shows that during fermentation, *S. cerevisiae* BTCC 12 consumed glucose quickly and converted it to ethanol. On the 1:6 substrate, the initial glucose concentration was 2.9 mg/mL, and about half of it remained after 6 h of fermentation. At the same time, the highest ethanol concentration was produced (0.37 mg/mL). After the glucose depleted, ethanol concentration also decreased (Figure 4A). A different trend is seen in the fermentation using the 1:9 substrate. Glucose was not immediately consumed by *S. cerevisiae* BTCC 12. The concentration after 6 h was still about 90% of the initial. Ethanol concentration reached a peak after 6 h, although the value was only about 17% of that produced in the 1:6 medium (Figure 4B).

![Figure 4](image-url)

**Figure 4.** Concentrations of glucose and ethanol during separated hydrolysis fermentation by *S. cerevisiae* BTCC 12 on media resulting from the hydrolysis of *D. hispida* starch (A) 1:6 ratio (B) 1:9 ratio.

The ethanol concentration and the yield produced in this study were far lower than those produced by fermentation using *S. cerevisiae* using reducing sugars resulting from the hydrolysis of amylases produced by Aspergillus sp MZA, with the ethanol concentration and yield were 65.7 g/L and 91%, respectively [13]. Nevertheless, this study shows that the SHF method could convert reducing sugars resulting from the hydrolysis of *D. hispida* starch for the production of bioethanol.

4. Conclusion

The *D. hispida* starch was optimally converted by amylases from *A. awamori* KT-11 into glucose at 6 h incubation, with reducing sugar level of 0.94 mg/mL. Separation using TLC shows that the reducing sugars were predominantly glucose. The hydrolysis product was able to provide an adequate carbon source for bioethanol fermentation using *S. cerevisiae* BTCC12. The glucose consumption and bioethanol formation were inversely correlated, where the highest bioethanol concentration was produced when the glucose concentration in the media depleted. Amylases produced by *A. awamori* KT-11 could hydrolyse raw starch without the need to undergo a gelatinization process, which reduces
the cost in its application. The results suggest that optimization of ethanol fermentation needs further investigations. Also, efforts to increase the yield need to be done to scale-up the production.

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