African Journal of Biotechnology Vol. 9(42), pp. 7122-7127, 18 October, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.933
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

Ragi tapai and *Saccharomyces cerevisiae* as potential coculture in viscous fermentation medium for ethanol production

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Accepted 30 August, 2010

A comparison study on the ethanol production from 20% (w/v) of unhydrolyzed raw cassava starch using *Saccharomyces cerevisiae* and *Candida tropicalis* was performed and compared with the commercialized ragi tapai. The findings showed that *S. cerevisiae*, *C. tropicalis* and ragi tapai produced 23, 20 mg/l and 26 g/l of ethanol in 72 h, respectively. Subsequent coculturing of the two best performing strains namely ragi tapai and *S. cerevisiae* were performed to improve ethanol production and to reduce the accumulation of inhibitory concentration of reducing sugar with 10% (w/v) unhydrolyzed raw cassava starch. The coculture of ragi tapai with *S. cerevisiae* using the unhydrolyzed raw starch in a single step-fermentation produced an ethanol concentration of 35 g/l when the starch was inoculated with ragi tapai and cocultured with *S. cerevisiae.* The yield was 46% higher than the one inoculated with ragi tapai only (24 g/l). The glucose concentration was maintained at a low concentration in the coculture medium as compared to the medium with pure ragi tapai. The findings suggested that coculture of ragi tapai with *S. cerevisiae* is capable of enhancing the ethanol production and prevention of the inhibitory effect of reducing sugars on amylolytic activity.

Key words: Cassava starch, ethanol, *Candida tropicalis*, ragi tapai, *Saccharomyces cerevisiae*, single-step bioconversion.

INTRODUCTION

Ethanol is known as a much cleaner motor fuel than petrol or gasoline. It is also used as an octane enhancer that gives low emissions, thereby reducing the green house effect. It is therefore able to provide solutions for a variety of complex problems related to energy and environment. Ethanol not only would mitigate air pollution but also could decrease imported oil and refined gasoline, thus creating energy security and varied energy portfolio (Lin and Tanaka, 2006). Furthermore, ethanol industry would be beneficial in the agriculture sector besides being advantageous for social benefits (Demirbas, 2006).

Most of the ethanol produced from sugar cane comes from Brazil or from corn produced in the United State of America. In Asia, cassava (*Manihot esculenta*) or tapioca, a perennial woody shrub has attracted more interest in this industry. Dai et al. (2005) reported that cassava fuel ethanol has higher energy efficiency than those of gasoline, diesel fuel and corn fuel ethanol but less than that of biodiesel. Using cassava starch as substrate in ethanol production will reduce the cost of ethanol production since cassava plants are abundant, cheap and can easily be planted in tropical countries such as Malaysia, Indonesia, etc. Thus, this study on cassava plants for biofuel production enhancement is so relevant that it can replace other raw materials such as palm oil in biofuel production and it will also bring about a significant economical impact on the producing nations.

For ethanol production, direct fermentation of starch using amylolytic yeasts offers an alternative to the conventional multistage use of commercial amylase enzymes

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for liquefaction and saccharification followed by fermentation with yeast (Abuzied and Reddy, 1986; Verma et al., 2000; Knox et al., 2004). However, the amylolytic yeasts capable of efficiently hydrolysis starch are very few (Knox et al., 2004). Ragi tapai or ragi tape' serves as an alternative for this setback. Ragi tapai is a dry-starter culture prepared from a mixture of rice flour, spices and water or sugar cane juice/extract (Merican and Quee-Lan, 2004). It is usually used to ferment cassava and glutinous rice into 'tapai or tape', a popular Malaysian delicacy, normally consumed as dessert. In the study of Hesseltine et al. (1988), out of the 41 starter samples from seven Asian countries, at least one yeast and one *Mucoraceous* mold (*Mucor, Rhizopus, or Amylomyces*) were present with one or two types of cocci bacteria in every sample of the dry starter. Several researchers (Merican and Quee-Lan, 2004; Hesseltine et al., 1988) presented cell count of ragi from different origins and showed that the fungal count is between 8×10^7 and 3×10^8 cell/g, yeast count is between 3×10^6 and 3×10^7 cell/g while bacterial count is less than 10^5 cell/g. Although wide range of organisms has been found in ragi, only a few genera were found present in the tapai or tape. This indicated that most of the other organisms are contaminants. Merican and Quee-Lan (2004) also listed the yeasts and *mucorale* that were present in tapai from the ragi list.

This project presents two objectives. The first objective is to compare ethanol production from unhydrolyzed raw cassava starch by two different types of yeasts and commercialized ragi tapai. The other objective is to investigate the hypothesis that coculturing of the dry starter ragi tapai with *Saccharomyces cerevisiae* might have improved ethanol production and amylolytic activity. The single step bioconversion from unhydrolyzed cassava starch into ethanol will not only reduce the cost of enzymes that is normally used in liquefaction and saccharification steps but it will also reduce the substrate inhibition, especially on yeast cells.

MATERIALS AND METHODS

Yeast strains and culture conditions

The yeast strains used in this experiment were *S. cerevisiae* (industrial yeast) and *Candida tropicalis* from American Type Culture Collection (ATCC 20026). The cocultures used were commercialized ragi tapai obtained from local store and industrial yeast, *S. cerevisiae*. The culture medium contained 0.1% (w/v) peptone and distilled water with no other nutrient added. The medium was autoclaved at 121°C for 15 min. The dry starter and yeasts were then placed in the medium and incubated at 37°C at 200 rpm for 25 - 30 min before inoculation.

Batch fermentation

Fermentation with yeast strains

Sixteen of 100 ml shake flasks containing 30 ml of 20% (w/v) cassava flour in distilled water (containing 1 mM CaCl) for each yeast strain were prepared. The flour was added to preheated water (60°C) and stirred for 5 min. After that, the temperature was increased to 80°C for 1 h and then reduced to 30°C. The yeast was inoculated into the flasks containing flour and the flasks were placed in incubated shaker at 50 rpm and 30°C.

Fermentation with cocultures

Ten percent of cassava flour was mixed in preheated water (60°C) in a sterile jacketed fermentor for 5 min. The temperature was raised to 70°C and mixed for 1 h. The temperature was then maintained at 30°C to be inoculated with: i) ragi tapai, ii) cocultured ragi tapai and *S. cerevisiae* at zero hour and, iii) ragi tapai followed by *S. cerevisiae* after 1 h. Each (ragi tapai and *S. cerevisiaei*) contains 5% (w/w) of inoculation. The agitation was maintained at 50 rpm. The fermentation was conducted in 2 L bioreactor with 10% (w/v) of flour mixture due to some limitations of the bioreactor system used.

Analytical method

Samples were collected at every 2 h interval for the first 12 h which consist of 6 data and another 10 data at every 6 h for the next 60 h. A total of 16 data were collected for the entire run of 72 h. The samples were centrifuged at 5000 rpm for 30 min and the supernatant was analyzed using SUPELCOGEL C-610H column on an high performance liquid chromatography (HPLC) (WATERS) equipped with a refractive index detector to determine the concentration of ethanol, glucose and oligosaccharides. The column was eluted at 30°C with 0.1% H_3PO_4 at 0.5 ml min^{-1}.

RESULTS AND DISCUSSION

Fermentation using yeast strains

From ethanol concentration profile shown in Figure 1, the maximum ethanol produced by ragi tapai from 20% (w/v) of unhydrolyzed raw cassava starch was 26 g/l while production of ethanol from *S. cerevisiae* was 23 mg/l and *C. tropicalis* was about 20 mg/l. Ragi tapai had the highest ethanol produced due to the mixed culture present in the ragi, whereas *C. tropicalis* which has been reported to have amylolytic activity (Azoulay et al., 1980) produced ethanol from starch at a very slow rate (Knox et al., 2004; Jamai et al., 2006). Despite the fact that *S. cerevisiae* is non-amylolytic yeast and was reported to be unable to hydrolyze starch (Abuzied and Reddy, 1986; Jamai et al., 2006), 23 mg/l ethanol was detected in the fermented product from direct fermentation of raw unhydrolyzed starch by *S. cerevisiae* in this work. This shows that *S. cerevisiae* has the ability of producing ethanol but at very low rate.

The glucose concentration profile presented in Figure 1 shows that both yeast strains and ragi tapai are capable of hydrolyzing raw cassava starch into glucose but at different rates. In 72 h of fermentation, the glucose concentration in medium inoculated with ragi tapai only was still high which was at 66 g/l available for further

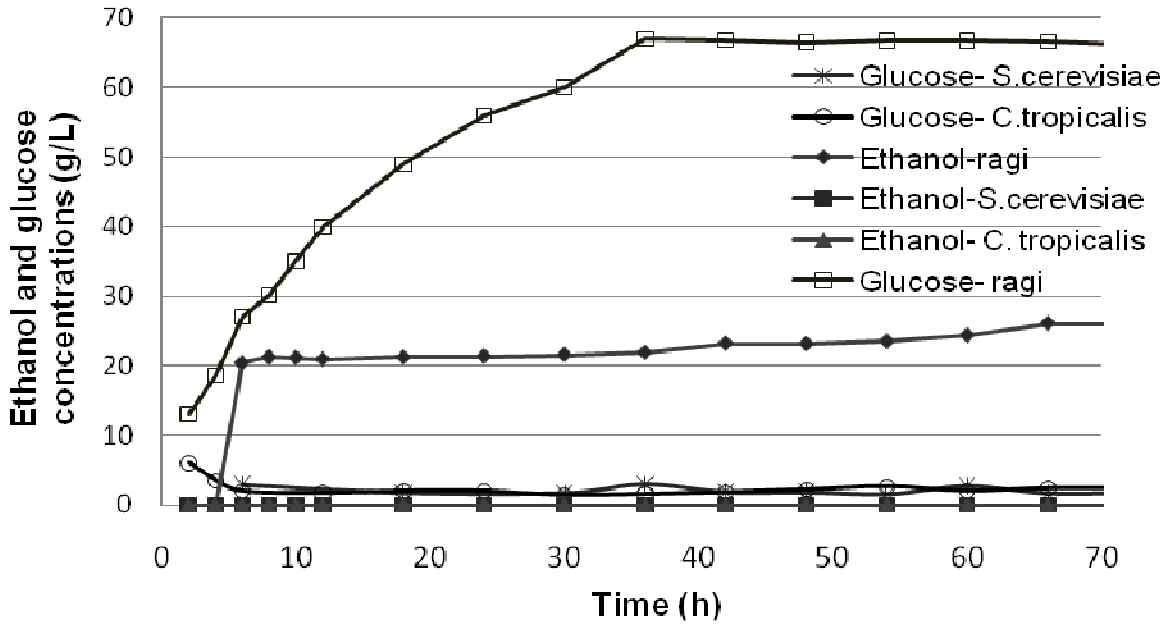

Figure 1. The ethanol and hydrolyzed glucose concentration produced by yeast strains and ragi tapai.

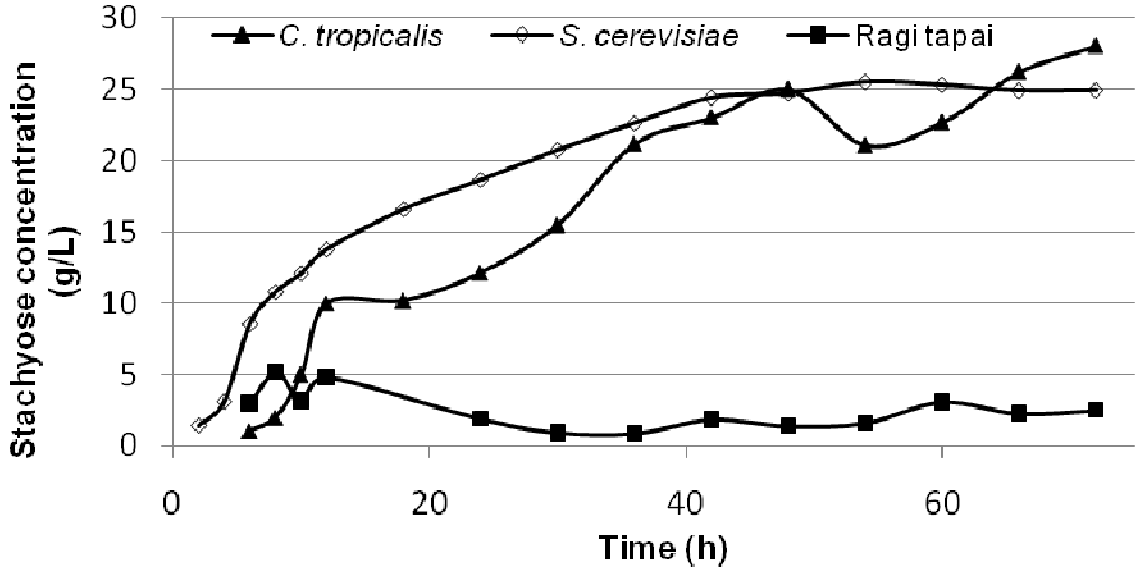

Figure 2. Stachyose concentration produced by yeast strains and ragi tapai.

conversion to ethanol. However, both *S. cerevisiae* and *C. tropicalis* were maintained at low glucose concentration which is below 7 g/l of glucose. The low rates clearly indicated that both strains have low hydrolyzing capability as compared to ragi tapai.

In addition to the production of glucose during fermentation, stachyose (a tetrasaccharide containing glucose, fructose and two galactose units) (Vaclavik and Christian, 2008) is also produced during the hydrolysis of starch. Other oligosaccharides such as maltoheptaose, maltohexaose, maltopentaose (highest sugar monomer), maltotetraose and isomaltotriose (lowest sugar monomer) may be produced too.

Figure 2 shows the stachyose concentration profile. It was consistently produced at high rate during both fermentations by *C. tropicalis* and *S. cerevisiae* but lowest for ragi tapai which was maintained at below 5 g/l throughout the fermentation. The stachyose concentration produced by *C. tropicalis* was at about 28 g/l in 72 h of fermentation. As for *S. cerevisiae*, the maximum

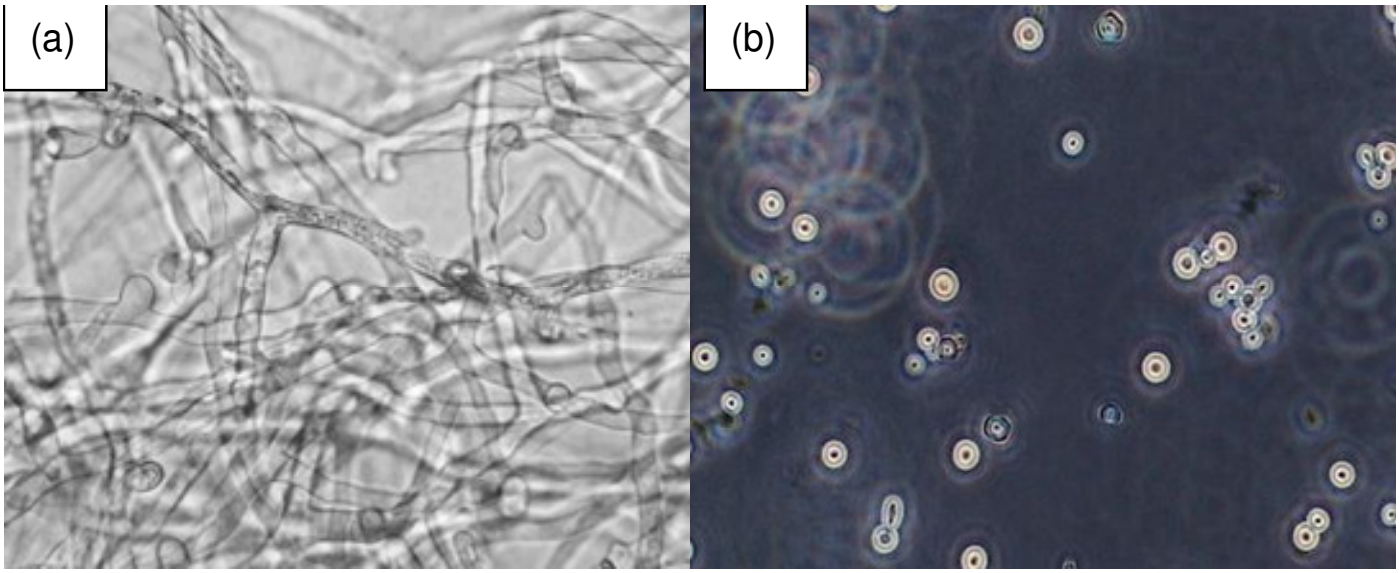

Figure 3. (a): Image of mold captured from 72 h of fermentation medium with ragi tapai only. (b): Image of yeast at 72 h of fermentation from 1 h coculture medium with no presence of mold.

stachyose production was 25 g/l towards the end of fermentation. On average, the stachyose concentrations from both fermentation involving *S. cerevisiae* and *C. tropicalis* were higher if compared to ethanol and glucose concentrations presented in Figure 1. This indicates that both yeasts inefficiently hydrolyzed the raw starch to glucose, while ragi tapai almost completely hydrolyzed starch into glucose which subsequently produed higher ethanol.

Ragi tapai and *S. cerevisiae* cocultured fermentation

Ragi tapai was chosen based on its ability to produce higher glucose and ethanol yields from starch directly as presented in the previous section. From microscopic observation, even though ragi tapai contains a mixture of microorganisms, only the presence of yeast and mold were found in the medium at the end of the fermentation. Interesting to note is the fact that when co-cultured with S. *cerevisiae*, the dominant microorganism was yeasts and no mold was observed as illustrated in Figure 3a and b. This phenomenon may be due to insufficient oxygen in the fermentation broth and an increase in other micro-organisms such as *S. cerevisiae* during the cocultured fermentation which utilized most of the oxygen for the process. The reduction in the oxygen concentration creates the anaerobic condition in the fermentation medium. Due to the absence of oxygen, the *Mucoraceous* mold could not produce sporangia (Hesseltine and Featherston, 1985) and failed to survive in the medium.

From the ethanol concentration profile shown in Figure 4, it is shown that the maximum ethanol concentration from ragi tapai inoculation only produced 24 g/l ethanol. When simultaneously cocultured at time zero, the ethanol production increased to 32 g/l at 55 h which gave rise to 33% higher yield compared to monoculture with 27 g/l at 72 h. The ethanol loss might be due to ethanol been converted into other byproducts. Among all three batches, the highest ethanol concentration obtained was from the sequential co-culture of ragi tapai and *S. cerevisiae*. This combination produced maximum ethanol of 35 g/l. This was 46% higher than the yield obtained with monoculture of ragi tapai at 24 g/l. The coculture after 1 h allows the mixed culture to hydrolyze the raw starch into glucose and made them available for *S. cerevisiae* to subsequently ferment them into ethanol. While in the simultaneous coculture, though *S. cerevisiae* is capable of hydrolyzing starch into stachyose, it is not efficient for the strain to convert stachyose into glucose. As a result, ethanol is not produced in desirable amount. Thus, further study on the glucose production at optimum coculture time is beneficial in maximizing ethanol production.

The most prominent type of reducing sugar observed from the HPLC analysis in this study was glucose. The highest glucose concentration obtained was 31 g/l, which occurred as early as 30 h after inoculation with ragi tapai as illustrated in Figure 4. The amount of ethanol produced at that time was 21 g/l ethanol. The glucose concentration was about half of the amount obtained from the previous findings when 20% (w/v) of tapioca flour was fermented in shake flask. In both simultaneous coculture and sequential coculture, the glucose concentration obtained was less than 6 g/l and it was maintained at that level for the entire fermentation process. This shows that *S. cerevisiae* utilizes glucose immediately to produce ethanol before it accumulate and correspondingly inhibit the fermentation process by osmotic pressure on the

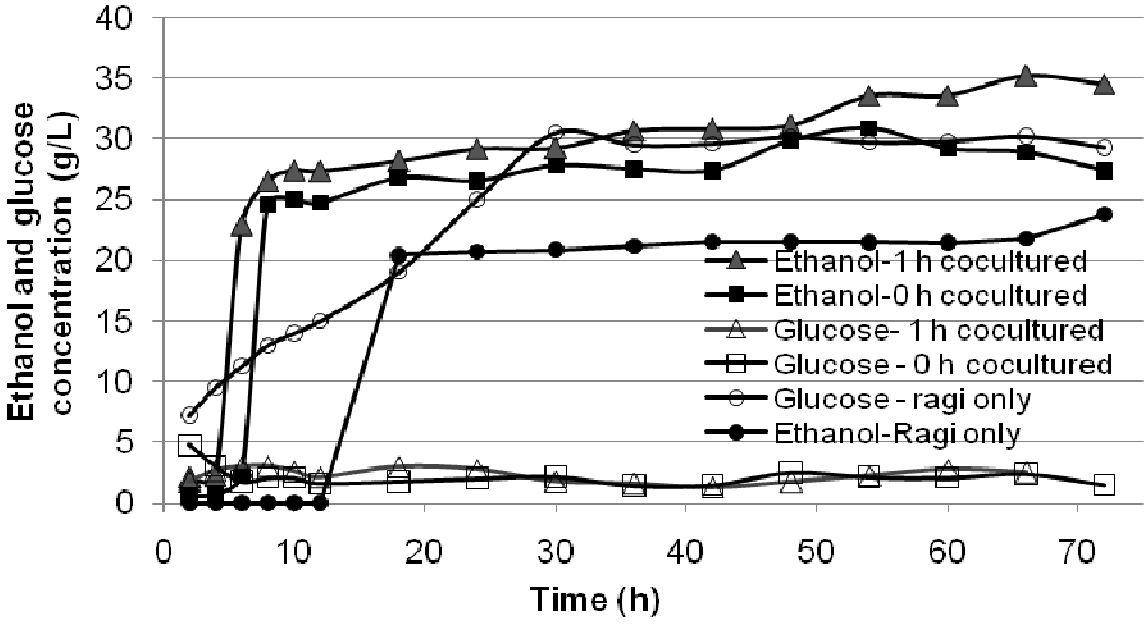

Figure 4. The ethanol and glucose concentration produced in bioreactor.

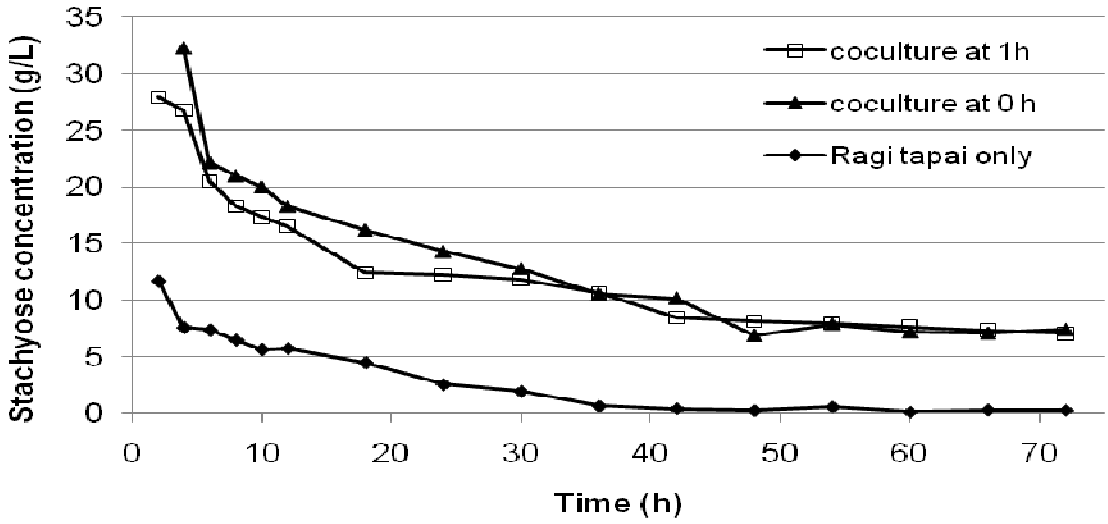

Figure 5. Stachyose concentration produced in bioreactor.

cells (Bai et al., 2008). As for the ethanol inhibition on cells, the amount of ethanol produced was only at 35 g/l which would not greatly affect the cell growth.

Besides glucose, oligosaccharides were also analyzed. Stachyose, the intermediate product, was consistently produced during all three fermentations as shown in Figure 5. In the fermentation process of ragi tapai, the stachyose concentration was the lowest but the concentration profile increased when ragi tapai was cocultured with *S. cerevisiae*. This might have been caused by the high cell numbers from extra 5% (w/w) *S. cerevisiae.* The yeast strain alone could have degraded starch into stachyose, subsequently to glucose and followed by the production of ethanol at slow rate as discussed in previous section. It might also be due to low glucose concentration of coculture medium which had initiated the amylolytic activity and thus resulted in more stachyose production.

Conclusion

The production of ethanol directly from the fermentation of cassava starch using ragi tapai was proven feasible.

The ethanol production can be enhanced by coculturing ragi tapai with *S. cerevisiae* which gave rise to an ethanol yield which is 46% higher than that without coculture. The glucose concentration was lower in the coculture and this had prevented the inhibitory effect of reducing sugars. Stachyose concentration profile shows that the process initiated the amylolytic activity to produce more ethanol while maintaining the sugar concentration at low level. Simultaneous single step bioconversion from unhydrolyzed cassava starch into ethanol will not only reduce the cost of enzymes that is used in liquefaction and saccharification steps but will also reduce the substrate inhibition, especially on yeast cells. Sugar which is released from fermentation of starch would be consumed immediately by yeast cells before it could accumulate. This will lead to a reduction of sugar concentration in the broth which subsequently reduces the inhibition of the fermentation process by osmotic pressure on the cells. Further studies, especially on the process optimization, are required to maximize the ethanol production from raw unhydrolyzed cassava starch using coculture ragi tapai and *S. cerevisiae.*

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