การหมักเอทานอลจากไฮโดรไลเซทของแป้งมันสำปะหลังโดยยีสต์ทนร้อน *Pichia kudriavzevii* โดยการเพาะเลี้ยงแบบแบตช์และเฟดแบตช์

Ethanol Fermentation from Cassava Starch Hydrolysate by Thermotolerant Yeast *Pichia kudriavzevii* using Batch and Fed-batch Cultures

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บทคัดย่อ

การหมักเอทานอลโดยยีสต์ทนร้อน *Pichia kudriavzeii* PBB511-1 จากแป้งมันสำปะหลังที่ถูกไฮโดรไลต์ด้วยรา *Talaromyces flavus* 21-3 ที่ปริมาณแป้งมันสำปะหลัง 30 เปอร์เซ็นต์โดยน้ำหนัก ปรับพีเอชเริ่มต้นเท่ากับ 7.5 บ่มแบบเขย่าที่ความเร็ว 130 รอบต่อนาที ที่อุณหภูมิ 30-31 องศาเซลเซียส เป็นเวลา 7 วัน รา *T. flavus* 21-3 ไฮโดรไลต์แป้งมันสำปะหลังได้น้ำตาลรีดิวซ์เข้มข้น 60-80 กรัมต่อลิตร เมื่อหมักเอทานอลโดยยีสต์ *P. kudriavzeii* PBB511-1 ในถังหมักขนาด 7 ลิตร ที่อุณหภูมิ 45 องศาเซลเซียส พบว่าในการหมักแบบแบตช์ สภาวะที่หมักเอทานอลได้ดีที่สุดคือสภาวะที่อัตราเร็วการกวนเท่ากับ 400 รอบต่อนาที ที่ปราศจากการให้อากาศ ให้เอทานอลเข้มข้น 45.44 กรัมต่อลิตร อัตราเร็วในการผลิตเท่ากับ 0.87 กรัมต่อลิตรต่อชั่วโมง และให้ค่าผลผลิตทางทฤษฎีเท่ากับ 49.39 เปอร์เซ็นต์ ส่วนการหมักแบบเฟด-แบตช์ ให้เอทานอลน้อยกว่าการหมักแบบแบตช์ โดยพบว่าการเติมอาหารใหม่ลงไปในถังหมัก 1 ครั้ง ให้ผลการหมักเอทานอลดีกว่าการเติมอาหารใหม่ 2 และ 3 ครั้ง โดยให้เอทานอลเท่ากับ 24.85 กรัมต่อลิตร อัตราเร็วในการผลิตเท่ากับ 0.69 กรัมต่อลิตรต่อชั่วโมง และให้ค่าผลผลิตทางทฤษฎีเท่ากับ 27.01 เปอร์เซ็นต์ ดังนั้นรา *T. flavus* 21-3 และยีสต์ *P. kudriavzevii* PBB511-1 น่าจะมีอิทธิพลต่อการผลิตเอทานอลเชื้อเพลิงมากขึ้นในอนาคต

คำสำคัญ: การหมักเอทานอล, อุณหภูมิสูง, แป้งมันสำปะหลัง, *Pichia kudriavzevii*, *Talaromyces flavus*

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ABSTRACT

Ethanol fermentation of *Pichia kudriavzeii* PBB511-1 was completed after the saccharification period through the cassava starch hydrolysate by *Talaromyces flavus* 21-3 which produced 60-80 gL^{-1} of reducing sugar with the following conditions: 30% (w/v) cassava starch, initial pH at 7.5, temperature at 30-31°C and a shaking speed of 130 rpm for 7 days. The fermentation process began by adding yeast inoculum, then allowed to ferment in a 7-L jar fermenter at 45°C in cassava starch hydrolysate media. The batch fermentation that showed the best condition was an agitation speed of 400 rpm without aeration. The ethanol concentration reached 45.44 gL^{-1}, a productivity of 0.87 $gL^{-1}h^{-1}$ and a yield of 49.39% of theoretical yield. A fed-batch fermentation was also performed, but it was not achieved as batch fermentation. The results showed supplementation for a 1 time of substrate was better than supplemented for 2 and 3 times. It produced 24.85 gL^{-1} of ethanol, a productivity of 0.69 $gL^{-1}h^{-1}$ and a yield of 27.01% of theoretical yield. Therefore, *T. flavus* 21-3 and *P. kudriavzevii* PBB511-1 could become more influential on ethanol fuel production.

Key words: ethanol fermentation, high-temperature, cassava starch, *Pichia kudriavzevii*, *Talaromyces flavus*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is not mainly food for Thai people, but it is considered as one of the most famous industrial crops in Thailand. The roots of cassava are utilized by making dry chips, pellets and starch which were applied in the food, beverages, paper, glue and ethanol industries. The most of plantation are belonging to the Northeastern region, then followed by Central (including Eastern) and Northern region of the country, respectively. In 2020, the total area of cassava plantation in Thailand was 9,439,009 rai and calculated with the total production are approximate 29 million tons per year (Office of agriculture economic, 2021). Cassava can grow on sandy or loamy soil which had low fertilizer. Moreover, it can be tolerant for drought climate. The price of this plant is quite low (the cassava chip was 260 USD per ton in May, 2021) (Thai tapioca development institute, 2021), so the farmers who do the farming are suffered and worse living. Therefore, cassava is one suitable source to feed stock for ethanol production. It is not only help farmer to get better lives but also can prevent the crisis of fossil fuel energy.

In Thailand, the temperature of ethanol fermentation can be very high as a result of high temperatures during daytime. In order to save yeast in the ethanol fermentation, a cooling system must be applied to cool down the fermentation temperature. In addition, using high efficiency thermotolerant yeast in the ethanol fermentation can reduce the cost of the cooling system and can minimize the risk of contamination with other microorganisms in the fermentation (Limtong *et al*., 2007; Yuangsaard *et al*., 2013; Kaewkrajay *et al*., 2014; Pongcharoen *et al.*, 2021).

Nowadays, it was reported that *Pichia kudriavzevii* or better known in the anamorphic stage, as *Issatchenkia orientalis*, can be isolated naturally. The morphologically, biochemically and physiologically characteristics of this yeast species was described by Kurtzman *et al.* (2011). After 3 days at 25°C, the cells are ovoid to elongate (1.3-6×3.3-14 µm) and occur singly or in pairs. Growth is butyrous and light-cream colored. Glucose is fermented. Glucose, ethanol, glycerol, DL-lactate, succinate, citrate, D-glucosamine, N-acetyl-D-glucosamine are assimilated. Growth at 37 and 40°C is present. Therefore, this yeast species is capable to be used in a high temperature fermentation. In this year, Pongcharoen *et al.* (2021) reported that the yeast *P. kudriavzevii* strains NUPHS34 and NUPHS33 produced the highest ethanol concentration at 40 and 45°C. They produced 69.79±1.54 gL^{-1} and 61.51±1.01 gL^{-1} of ethanol, respectively. Kaewkrajay *et al.* (2014) proposed *Pichia kudriavzevii* which was isolated from

soil sample in Chonburi province, Thailand. This yeast produced the highest ethanol yield after 36 h with fermentation at 45°C. It produced 37 gL^{-1} ethanol, with a productivity of 1.03 $gL^{-1}h^{-1}$ and a yield of 40% of the theoretical yield by shaking flask cultivation. Moreover, Dhaliwal *et al.* (2011) successfully isolated *P. kudriavzevii* from sugarcane juice and reported the success of producing ethanol at 40°C, it was found that ethanol concentration reached 71.9 gL^{-1}, a productivity of 4.0 $gL^{-1}h^{-1}$. In addition, by leaving alkali-treated cotton stalks and ozone-treated cotton stalks to ferment with *P. kudriavzevii* HOP-1, the results showed that ethanol concentrations were 19.82 gL^{-1} and 10.96 gL^{-1}, respectively (Kaur *et al*., 2012). Under the fermentation of cassava starch hydrolysate in a 2.5-L jar fermenter at 40°C with *P. kudriavzevii* DMKU 3-ET15 isolated from traditional fermented foods, ethanol concentration reached 7.86% (w/v) after 24 h of the fermentation, productivity of 3.28 $gL^{-1}h^{-1}$ and yield of 85.4% of theoretical yield (Yuangsaard *et al*., 2013).

This research aims to study ethanol production at high temperature by *P. kudriavzevii* PBB511-1, which was proposed to be the efficacy to produce ethanol at 45°C by shaking flask cultivation. The optimization at the up-scale was continuously studied in the bioreactor and the cassava starches were digested with *T. flavus* 21-3, which was previously reported to efficiency produce amylases. In doing so, the separate hydrolysis and fermentation (SHF) was studied. These data will provide a base line information for the simultaneous saccharification and fermentation (SSF) strategy. Co-culture of both strains can be support the concept of reducing cost for fuel ethanol production.

MATERIALS AND METHODS

1. Preparation of medium

The cassava starches were analyzed as the moisture by following the Thai Industrial Standards TISI no. 52 number 6.4 method (Charoenrat, 2002). The percentage of starch was analyzed by the using color method (Saelee, 1993). After that the media was prepared by adding 30% of cassava starch with initial pH at 7.5 and then sterile by autoclaving at 121°C for 15 min. The sterile media were used for cultivation thermotolerant fungi *T. flavus* 21-3 to allow digestion of cassava starch by incubation with shaking speed of 130 rpm at 30-31°C for 7 days. The fungal culture was filtrated through the filter paper (Whatman no. 4) and immediately kept at -20°C. The aliquot was analyzed reducing sugar concentration by the Nelson-Somogyi method (Nelson, 1944)

2. Ethanol fermentation in a fermenter

The starter medium contained 20 gL^{-1} reducing sugar, 0.5 gL^{-1} $(NH_4)_2SO_4$, having an initial pH of 4.5 and autoclaved at 121°C for 15 min. A loopful of *P. kudriavzeii* PBB511-1 cells was added into 100 ml inoculum media in 250 ml Erlenmeyer flask and shaking speed of 150 rpm at room temperature for 24 h.

The inoculum was transfer at the rate of 5% to the cassava starch hydrolysate which contained 180 gL^{-1} reducing sugar, 0.5 gL^{-1} $(NH_4)_2SO_4$, 0.5 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4$. $7H_2O$ and 1 gL^{-1} yeast extract, having an initial pH of 4.5. Batch and fed-batch fermentations were cultivated at 45°C in a 7-L jar fermenter with a 4-L working volume. The sample was taken every 4-12 h until 72 h to analyze growth, ethanol concentration and sugar consumption.

The conditions of batch fermentation studied are 1) an agitation speed of 300, 400 and 500 rpm without the aeration, 2) an agitation speed of 300, 400 or 500 rpm with the aeration rate of 0.2 vvm throughout the fermentation.

The fed-batch fermentations were performed following Sringiew method (Sringiew, 2005). The substrate was added into the 3 types of fed-batch fermentation by observed sugar concentration. Firstly, the substrate was supplemented for 1 time by adding 50% of total reducing sugar with an initial volume of 3-L. Second, the substrate was supplemented for 2 times by adding 33% of total reducing sugar with an initial volume of 2.5-L. Final substrate was supplemented for 3 times by adding 25% of total reducing sugar with an initial volume of 2.5-L.

3. Analytical methods

The cell growth was determined by measuring the optical density at the wavelength

of 660 nm with the spectrophotometer (Shimadzu; UV-pharmaspec 1700) after washing with 0.1 M HCl at least 3 times and was centrifuged at 3500 rpm for 5 min. The cell sediment was then resuspended with 0.1 M EDTA pH 7.

The ethanol concentration was analyzed by gas chromatography (Varian star 3600 GC apparatus) equipped with a flame ionization detector (FID) and the capillary column (0.32 mm ID, 60 m) of DB-wax coated at 70°C. The injector and detector maintained a temperature of 200°C. Nitrogen gas was used for carrier samples with a flow rate at 4 ml min^{-1}. The 4% of n-propanol was used as an internal control (Komagata and Ohmomo, 1984). The ethanol concentration was calculated from the ratio of ethanol and propanol area and was compared with the standard. The ethanol productivity and the fermentation yield have been calculated according to Morimura *et al.* (1997).

The sugar concentration was determined by measuring in accordance with the Nelson -Somogyi method (Nelson, 1944). The high sugar concentration was diluted with distilled water until the optical density was valued at the wavelength of 520 nm in the range of 0.1-1.0. The sugar concentration of the samples was compared with the glucose standard.

RESULTS AND DISCUSSION

1. Cassava starch hydrolysate

Subjected to hydrolysate of cassava starch with *T. flavus* 21-3, concentrated at 30% (w/v), initial pH at 7.5, temperature at 30-31^{o}C and a shaking speed of 130 rpm for 7 days, it was found that *T. flavus* 21-3 had digested cassava to sugar around 60-80 gL^{-1}, which was quite much but it was still not enough to be used as source of ethanol fermentation. According to *P. kudriavzevii* PBB511-1, suitable condition for ethanol fermentation is the medium containing 180 gL^{-1} reducing sugar, 0.5 gL^{-1} $(NH_4)_2SO_4$, 0.5 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4$. $7H_2O$, 1.0 gL^{-1} yeast extract and pH at 4.5 (Kaewkrajay *et al.*, 2014). Therefore, the glucose was added into the main medium until the initial sugar concentration reached 180 gL^{-1}.

2. Batch fermentation

Batch fermentation based on yeast *P. kudriavzevii* PBB511-1 under a temperature control of 45°C found that;

Batch fermentation I: In the first 12 h of fermentation, there was a maintained airless condition and agitated continuously at a speed of 300 rpm, the yeast growth was low, but the growth increased gradually till 24 h. The growth increased dramatically from 48 to 72 h, which presented an optical density of 5.6-6.7, by measuring at a wavelength of 660 nm. In terms of reducing sugar, the amount of sugar gradually decreased in the first 24 h and significantly dropped during 24 to 32 h from 141 gL^{-1} to 81 gL^{-1}. The amount of sugar remained in the fermentation container at 54 gL^{-1}. In terms of ethanol base, the ethanol produced was 1.6 gL^{-1} at 12 h and sharply increased to 12.36 gL^{-1} at 24 h till 72 h. A concentration of ethanol gradually increased to the highest rate at 25.28 gL^{-1}, a production rate of 0.35 $gL^{-1}h^{-1}$, and a fermentation yield of 27.48% of theoretical yield. (Table 1, Figure 1A)

Batch fermentation II: Maintaining an agitation speed of 400 rpm and no air supplied, yeast grew very slow during the first 8 h but after 12 h, growth continuously increased. However, the growth decreased as it reached 72 h and optical density was 3.64. In terms of reducing sugar consumption, it was found that the amount of reducing sugar decreased slightly during the first 12 h but after this period the amount of reducing sugar dramatically dropped. At 72 h, the amount of reducing sugar remaining in the fermentation was 65 gL^{-1}. Subjected to this fermentation, it was found that yeast started to produce ethanol when reaching 8 h which produced 1.43 gL^{-1} of ethanol and dramatically increased to 29.55 gL^{-1} as it reached 34 h. After this the ethanol concentration still increased and reached the maximum at 45.44 gL^{-1} as it reached 52 h. The fermentation provided a production rate at 0.87 $gL^{-1}h^{-1}$, a yield of 49.39% of theoretical yield (Table 1, Figure 1B).

Batch fermentation III: At a maintained agitation speed of 500 rpm with no air supplied, it revealed that the overall grow rate of yeast was very slow. The grow rate of the yeast started to climb up after 12 h of fermentation and continuously increased till 72 h. It was found that at 56 h the optical density measured at the wavelength of 660 nm reached the maximum rate of 2.83. The low cell biomass was seen at high agitation rate. In 2019, Santharam *et al*. (2019) reported that the yeast biomass loss when using more agitation rates than 150 rpm. They suspected that the low heat evolution rate and thereby less cumulative heat at high agitation speed may be attributed to biomass loss caused by shear stress. Shear stress had negative effects at high agitation rate. In part, the reducing sugar consumption did not provide a good performance, and there was a reducing sugar of 99 gL^{-1} remaining in the fermenting tank as measured at the end of the fermentation. The highest ethanol concentration was 30.52 gL^{-1} at 48 h, production rate at 0.64 $gL^{-1}h^{-1}$ and yield at 33.17% of the theoretical yield. As the fermentation continued, the ethanol concentration decreased and dropped to 25.95 gL^{-1} at 72 h of fermentation (Table 1, Figure 1C).

Subjected to an agitation speed of 400 rpm without air supply, the *P. kudriavzevii* PBB511-1 could produce the highest ethanol. Regarding the test results, the agitation speed at 400 rpm was selected for the further study with an air supply case.

Batch fermentation IV: By maintaining an agitation speed at 400 rpm with an air supply at 0.2 vvm, it was found that within the period of the first 12 h of fermentation, yeast could grow very slow. However, its growth slightly increased in the period between 24-32 h of fermentation and increased sharply as the fermentation reach 36 h. Moreover between 0-32 h of fermentation, it was found that concentration of reducing sugar decreased gradually and significantly dropped after 36 h of fermentation. The reduction of reducing sugar was related to the growth of yeast. At the end of the 72 h of fermentation, the reducing sugar in the fermentation tank remained at 84 gL^{-1}. During fermentation, it started to produce ethanol at 2.31 gL^{-1} at 8 h of fermentation and ethanol gradually increased thereafter. Till the 36 h of fermentation, production rate of ethanol was the highest at 30.58 gL^{-1}, ethanol production rate at 0.85 $gL^{-1}h^{-1}$, and yield at 33.24% of theoretical yield. However, it was shown that by allowing the fermentation to continue till the 72 h, ethanol concentration was 24.23 gL^{-1}. Considering the growth of yeast between 36-72 h of fermentation, yeast growth was very low and turbidity level of yeast cells reduced after 72 h of fermentation (Table 1, Figure 1D)

Table 1 The maximum ethanol concentration, productivity and fermentation yield of yeast Pichia kudriavzevii PBB511-1 using batch and fed-batch cultures

Conditions	[EtOH]max (gL^{-1})	Time (h)	Productivity ($gL^{-1}h^{-1}$)	Fermentation yield (% of theoretical yield)
Batch fermentation				
Batch I	25.28	72	0.35	27.48
Batch II	45.44	52	0.87	49.39
Batch III	30.52	48	0.64	33.17
Batch IV	30.58	36	0.85	33.24
Fed-batch fermentation				
Fed-batch I	24.85	36	0.69	27.01
Fed-batch II	24.08	28	0.86	26.17
Fed-batch III	21.60	24	0.90	23.48

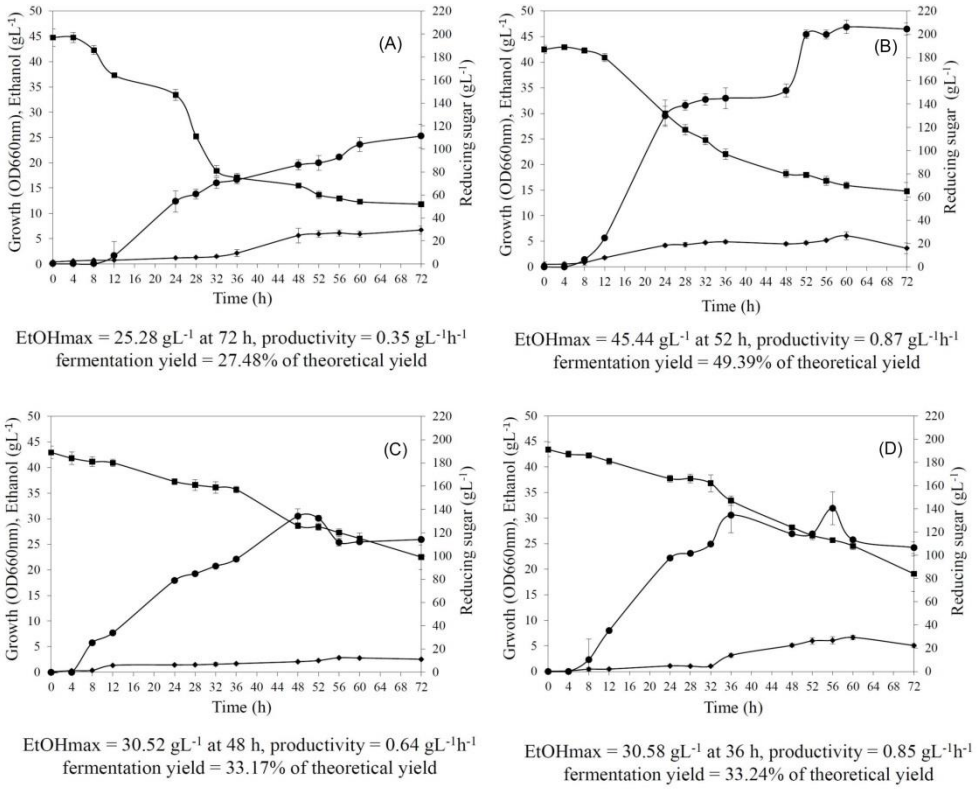

Figure 1 Growth (♦) Ethanol concentration (●) and reducing sugar concentration (■) by batch fermentation at 45ºC of *P. kudriavzevii* PBB511-1 (A) agitation speed of 300 rpm without aeration; (B) agitation speed of 400 rpm without aeration; (C) agitation speed of 500 rpm without aeration; (D) agitation speed of 400 rpm with aeration 0.2 vvm.

The results showed that an agitation speed of 400 rpm without aeration, the yeast *P. kudriayzevii* PBB511-1 produced the highest ethanol concentration (45.44 gL^{-1} at 52 h). Kaewkrajay *et al*. (2014) reported that this strain could produce 42.4 gL^{-1} of ethanol after 48 h, at the rate of 0.88 $gL^{-1}h^{-1}$ and a yield of 46% of the theoretical yield when cultivated in fermenter with an agitation speed of 300 rpm and an aeration rate of 0.2 vvm throughout the fermentation. These results implied that both conditions were appropriately produced ethanol by this yeast strain. A recent study reported that the ethanol production from molasses by yeast *Saccharomyces cerevisiae* DMKU 3-S087 was carried out in a 5 L stirred tank fermenter. Batch fermentation was conducted without aeration, temperature and agitation were controlled at 40°C and 300 rpm, respectively. The highest ethanol concentration was 72.4 gL^{-1}, a productivity of 1.21 $gL^{-1}h^{-1}$ and a yield of 0.36 gg^{-1} at 60 h (Pattanakittivorakul *et al.*, 2019). Ethanol production from sugarcane bagasse hydrolysate in stirred-tank fermenter using *Scheffersomyces stipitis* NRRL Y-7124 and *Scheffersomyces shehatae* UFMG HM 52.2 was performed under controlled condition. Ethanol production from these two yeasts was favored by initial pH increase and agitation rate decrease. The maximum fermentation yield was attained at 100 rpm, initial pH 6.50 and under oxygen limited conditions (Dusśan *et al.*, 2016). In the current year, Krajang *et al.* (2021) studied the single-step ethanol production in 5 L fermenter using combination of raw cassava starch hydrolysis and fermentation. The fermentation was performed at 30 and 40°C without temperature control. Fermenter were agitated at the speed of 200 rpm for 72 h along with the fermentation. The final ethanol concentration at a temperature of 30°C was 70.92 gL^{-1} while maintaining a temperature of 40°C was 65.78 gL^{-1}.

It could be seen that temperature is one of the most important factors that affect ethanol production. Yuangsaard *et al.* (2013) conducted a study using *P. kudriavzevii* DMKU3-ET15 to ferment starch within a 2.5 L fermentation tank while maintaining a temperature of 40°C and an agitation speed at 300 rpm with air supply at 0.1 vvm. They found that ethanol produced was 7.39% (w/v) after 33 h, a productivity of 2.23 $gL^{-1}h^{-1}$ and a yield of 79.9% of the theoretical yield. Furthermore, *Kluyveromyces marxianus* DMKU 3-1042 was also used to ferment sugarcane juice under batch fermentation in a 5-L tank by maintaining temperature at 37°C and an agitation speed at 300 rpm with the air supply at 0.2 vvm. A production rate of ethanol at 6.43% (w/v), a productivity of 1.3 $gL^{-1}h^{-1}$ and a yield of 57.1% of theoretical yield were the results (Limtong *et al*., 2007).

3. Fed-batch fermentation

The fed-batch fermentations were done by using an agitation speed at 400 rpm without aeration. The starter and main media were prepared the same as the batch fermentation. The main media was supplied to fermentation tank 1, 2 and 3 times as 50, 33 and 25% of total reducing sugar, respectively.

Fed batch fermentation I: with 1 time feeding. It was found that within 12 h of fermentation, yeast growth was very slow but still higher that the growth of the batch fermentation. After 12 h, yeast gradually grew and became significantly greater when the fermentation reached 48 h. Within 12 h of fermentation, yeast was gradually accustomed to a new environment, yeast slowly consumed reducing sugar. Although the fed batch fermentation with 1 time feeding could reduce an initial condensation of reducing sugar to 50% (90 gL^{-1}), this condensation was still higher than that of the starter (20 gL^{-1}). After feeding at 12 h of fermentation, yeast consumed more reducing sugar and this consumption was very high as the fermentation reached 36 h. This was clearly linked to the growth of yeast at 48 h. At the end of fermentation at 72 h, the reducing sugar still remained at a high level at 100 gL^{-1}. In the case that a fermentation period was extended more than 72 h, the amount of remaining reducing sugar may reduce and growth of yeast increase; however, ethanol will not increase because the results showed that there was no sign of increase for ethanol after 36 h. Moreover, it appeared that yeast began to produce ethanol at 8 h rate at 10.38 gL^{-1} which producing ethanol at 11.71 gL^{-1}. At 24 h, ethanol increased significantly and reached the highest at 36 h that ethanol was produced at 24.85 gL^{-1}, production rate was 0.69 $gL^{-1}h^{-1}$ and yield was 27.01% of the theoretical yield (Table 1, Figure 2A).

Fed batch fermentation II: with 2 times feeding. It was found that yeast growth was less than that for the fed batch fermentation with 1 time feeding. Considering the consumption of reducing sugar during fermentation, reducing sugar decreased very quickly after the first feeding at 6 h of fermentation. The second feeding was made at 12 h and it was found that reducing sugar sharply dropped at 72 h of fermentation leaving the reducing sugar at 72 gL^{-1}. For ethanol production, it was started at 6 h of fermentation at the rate of 10.92 gL^{-1} and the production clearly increased after the second feeding at 24 h. The ethanol production climbed up to the highest point at 28 h with condensation at 24.08 gL^{-1} production rate of 0.86 $gL^{-1}h^{-1}$ and yield at 26.17% of the theoretical yield (Table 1, Figure 2B) Subjected to the results, it could be perceived that ethanol with the fed batch fermentation with 2 times feeding produced slightly lower than that with the fed batch fermentation with 1 time feeding, on that other hand the production rate of ethanol was higher than the fed batch fermentation with 1 time.

Fed batch fermentation III: with 3 times feeding which this test, yeast grew very fast within the 24 h of fermentation and thereafter. The increase of growth was slowed down at 48 h. Considering the amount of reducing sugar within the first 6 h of the fermentation, there was few reducing sugars used. The first feed was made at 6 h and resulted in a slight decrease of reducing sugar. The reducing sugar significantly lessened after the second feed at 12 h and the reduction rate was the highest when the third feed

was made at 24 h. At 72 h, it was found that the reducing sugar remaining was 82 gL^{-1}. For ethanol production, ethanol was first produced at 12 h at 11.86 gL^{-1}, and it increased very after the second feed at 24 h. Thereafter the amount of ethanol remained constant and reached the peak at 21.60 gL^{-1} at 24 h with production rate 0.90 $gL^{-1}h^{-1}$ and yield at 23.48% of theoretical yield (Table 1, Figure 2C). From the figure, it could be seen that ethanol production of this fed batch fermentation with 3 times feed was less than that for the 1 time and 2 times feed, but the production rate was greater than both fermentation periods.

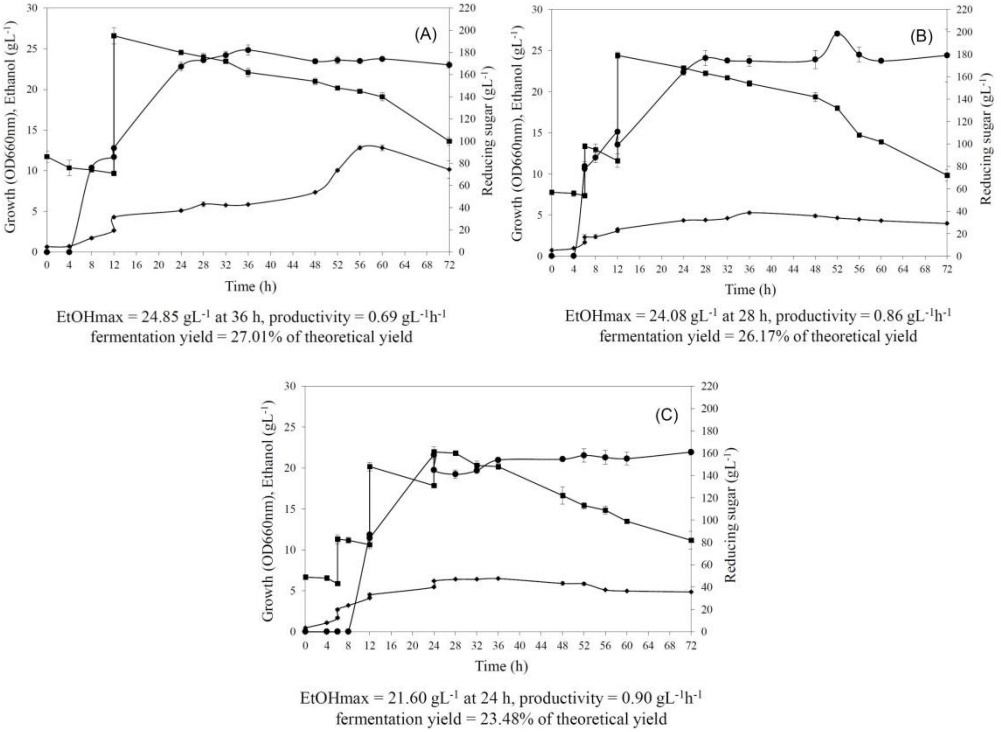

Figure 2 Growth (♦) Ethanol concentration (●) and reducing sugar concentration (■) by fed-batch fermentation at 45ºC of *P. kudriavzevii* PBB511-1 (A) one time feeding; (B) two times feeding; (C) three times feeding, by agitation speed of 400 rpm and without aeration through the fermentation.

Sringiew (2005) reported that by using yeast *K. marxianus* DMKU3-1042 to ferment 22% sugarcane juice and incubated at 37ºC, the results revealed that the fed batch fermentation with 2 times feed could produce more ethanol than that in the fed batch fermentation with 1 time and 3 times feed. Subjected to the results, ethanol was produced at 7.92% (v/v) and at 54 h of the fermentation. In addition, it was reported that fed batch fermentation SSF was done by using high dry matter corncob as the substrate. The fresh substrate was pretreated with diluted sulfuric acid-sodium hydroxide within a 6 L-reactor. The results showed that after test the final dry matter contest was 25% (w/v) which was set at the beginning at 19% and thereafter there were 3 additions made of 6% at 3, 6, and 12 portions during the first 24 h of the fermentation. After reaching 96 h of fermentation, it was found that every fed-batch produced ethanol more than 80 gL^{-1}, interval adding time at 4 h (label B) produced more ethanol than interval adding time 8 h (label A) and interval adding time 2 h (label C). Fermentation under label B, C and A categories produced ethanol at 84.7 gL^{-1}, 83.3 gL^{-1} and 80.2 gL^{-1} respectively (Zhang *et al*., 2010). With the fed-batch fermentation, sugar-cane blackstrap molasses was reported as being used to ferment for producing ethanol. The

fermentation was set in 14-L tank at temperature of 32 (±1) ℃ and initial volume of 3-L. During the fermentation, the sugarcane blackstrap molasses was added for 3 times at the 1, 2 and 3 h point which could be determined a time constant (K) as 0.08 or 1.6 h^{-1}. The results showed that the maximum ethanol yield was presented in the Test (n.) at 10, filling time at 3 h, time constant at 0 h^{-1}, which produced ethanol yield at 89.0% (Echegaray *et al.*, 2000). Sankh *et al*. (2013) reported that by using yeast oil, *P. kudriavzevii* MTCC 5493 extracted from rotten fruits, to produce biodiesel with the fed-batch fermentation in 26-L tank. The results showed that dry biomass yielded an oil yield of up to 33 gL^{-1} and 19% (w/w) respectively.

From the results shown above, it could be seen that the fed-batch fermentation with *P. kudriavzevii* PBB511-1 and 1 time cassava starch hydrolysate medium added produced ethanol at 24.85 gL^{-1} at 36 h which is higher than by adding for 2 and 3 times. Adding medium 2 times and 3 times produced ethanol at 24.08 gL^{-1} at 28 h and 21.6 gL^{-1} at 24 h, respectively. However, by adding the medium for 2 and 3 times, ethanol production rates were 0.86 and 0.9 $gL^{-1}h^{-1}$ which was slightly higher than that of 1 time medium added that has a production rate at 0.69 $gL^{-1}h^{-1}$. Therefore, the 1 time medium added for the fed-batch fermentation would be the appropriated for yeast strain PBB511-1.

CONCLUSIONS

Subjected to the *T. flavus* 21-3 strain, it found that fermentation of 30% cassava starch for 7 days, pH of 7.5, temperature at 30-31°C and a shaking speed of 130 rpm produced 60-80 gL^{-1} reducing sugar. Then, *P. kudriavzevii* PBB511-1 was performed for batch and fed-batch fermentation at 45°C. It was found that with agitation speed at 400 rpm with no air supply was the most appropriate for fermentation. The results showed that ethanol produced was 45.44 gL^{-1} after 52 h of fermentation, which could determine a productivity of 0.87 $gL^{-1}h^{-1}$ and a yield of 49.39% of theoretical yield. Subjected to the fed-batch fermentation of *P. kudriavzevii* PBB511-1, a result showed that a 1 time medium added could produce ethanol more than that of the 2 and 3 times medium added. With the 1 time medium added, ethanol was produced 24.85 gL^{-1} at 36 h which was determined a production rate of 0.69 $gL^{-1}h^{-1}$ and a yield of 27.01% of the theoretical yield.

Form the results above, *T. flavus* 21-3 and *P. kudriavzevii* PBB511-1 could become more influential on manufacturing ethanol fuel in Thailand, since the climate of Thailand is generally not suitable for yeast fermentation due to the high temperatures. Therefore, a cooling system and a germ contamination protection process is required to fulfill an ethanol fermentation under high temperatures. With these thermotolerant fungi and thermotolerant yeast, capable of high temperatures, it could be more advantageous for ethanol fuel manufacturing in Thailand.

SUGGESTIONS

The co-culture of these microorganisms should be studied in term of Simultaneous Saccharification and Fermentation (SSF). It might be improved and reduced cost of the ethanol fermentation process.

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