Songklanakarin J. Sci. Technol.
40 (4), 776-783, Jul.–Aug. 2018

http://www.sjst.psu.ac.th

Original Article

Bioconversion of cassava starch to bio-ethanol in a single step by co-cultures of *Amylomyces rouxii* and *Saccharomyces cerevisiase*

Pa-nga Yeunyaw[1] and Thalisa Yuwa-amornpitak[2*]

[1] *Graduate School, Mahasarakham University, Kantharawichai, Maha Sarakham, 44150 Thailand*

[2] *Department of Biotechnology, Faculty of Technology, Mahasarakham University, Kantharawichai, Maha Sarakham, 44150 Thailand*

Received: 1 December 2015; Revised: 23 September 2016; Accepted: 17 April 2017

Abstract

Single step ethanol production by co-cultures of amylolytic fungus and *S. cerevisiase* TISTR 5088 was the main purpose of this study. The most effective fungus that could convert starch at high concentrations and gain high reducing sugar content was selected. A morphological study and internal transcribed spacer sequence analysis identified *Amylomyces rouxii* YTH3 which was submitted to GenBank (accession number KM215272). The fungus rapidly hydrolyzed highly viscous starch at concentrations of 20% and 25% to fermenting sugar at 115.94±0.99 g/l and 159.72±4.08 g/l respectively. In a co-culture system, it was found that starch concentrations and yeast inoculum times influenced ethanol production. Whenever a higher starch concentration was used, the inoculum time should be extended. However, 73.68±1.81 g/l of ethanol from 20% cassava starch medium (theoretical yield 87.46%) was produced when the optimum time of adding the yeast was at 24 h.

Keywords: bioethanol, amylolytic fungi, cassava starch, co-cultures, ITS sequence

1. Introduction

Cassava is a root crop with a high yield. Thailand produces around 32.35 million tons per year (Office of Agricultural Economics, 2015). It is a staple food in many developing African countries. In Thailand the crop is grown for the export market. During the last decade Thailand was the largest exporter of dried cassava in the world (Newby, 2016). The cassava root contains more starch by dry weight than any other food crop. The starch is easy to extract by water, using a simple technique (Ayoola *et al*., 2013). The applications of cassava starch are many and varied for the food industry and non-food products. Cassava starch is used as a raw material to produce glucose. The carbohydrates in starchy plants, such as cassava, are not directly consumed by most yeasts and bacteria. They must first be depolymerized to fermentable sugars by acid (Fontana *et al*., 2008) or enzymes (Christiana & Eric, 2010; Ueda *et al*., 1981). The simple sugars (glucose and maltose) are very important C-sources for microbial conversion to various products such as ethanol, lactic acid, amino acid, and astaxanthin. Hydroxymethylfurfural (HMF) and furfural are generated as by-products during starch hydrolysis by acid treatment. It was found that 0.2% of 5-HMF and furfural strongly inhibited microbial growth and oxidative metabolism when added to the culture media (Sanchez & Bautista, 1988). A furfural concentration of 1.5 g/l influenced respiration and growth of *Pichia stipitis* and reduced the ethanol yield and productivity by 90.4% and 85.1%, respectively (Agbogbo & Wenger, 2007). Furthermore, the catabolic reduction charge and the anabolic reduction charge decreased in the presence of furan aldehyde HMF and furfural, respectively (Ask *et al*., 2013).

Although enzymes are convenient to use and starch is easily hydrolyzed by amylases and glucoamylase, at the industrial level the starch hydrolysis process using these enzymes operates at high temperatures and requires a long cooling time before adding the yeast inoculum (Ramachan-

*Corresponding author
Email address: ythalisa@yahoo.com

dran *et al*., 2013). A single step using a fungus for starch hydrolysis and co-culture with yeast saves time and energy consumption.

Tan-koji or loog-pang (a dried fungal starter) and some soils are major sources of starch amylolytic fungi and various fungal strains of *Rhizopus, Aspergillus, Penicillium,* and *Amylomyces* can be isolated. However, the most efficient strain for starch hydrolysis and high liberation of reducing sugars needs to be investigated. Due to maximum enzyme yield and high glucose formation, *Aspergillus fumigatus* KIBGE-IB33 was selected and converted cassava starch to ethanol in a double fermentation process (Pervez *et al*., 2014). Until recent years, the combination technique of the combined hydrolysis and fermentation step was widely used in the ethanol fermentation process and called simultaneous saccharification and co-fermentation (SSCF) (Ado *et al*., 2009; Itelima *et al*., 2013; Suriyachai *et al*., 2013; Swain *et al*., 2013). A study by Paschos *et al*. (2015) of the high solid content of wheat straw (26% dry matter content) produced 58 g/l of ethanol in simultaneous saccharification and fermentation by co-cultures of *Fusarium oxysporum* (solid culture) and *Saccharomyces cerevisiae*. SSCF was a better technique to convert the complex forms of carbohydrates to fermenting sugars such as glucose, and xylose to ethanol. Besides the selection of strains of hydrolysis molds, potential feedstock and the appropriate process must be developed for economic investment.

In this study, an amylolytic fungus was selected that rapidly hydrolyzed starch solutions and produced the highest level of reducing sugars. Cassava starch at both lowand high concentrations was gelatinized and used as the substrate. The time to add the yeast inoculum was studied in a co-culture system. The fermentation process starting by amylolytic fungus inoculation to convert the starch to reducing sugars and after that the yeast inoculum was added at appropriate times.

2. Materials and Methods

2.1 Starch

Cassava starch powder was purchased from the market (Red Cat Brand, Kriangkrai LTD., Thailand). The moisture content of the cassava powder was 10.07%±0.03. The starch was hydrolyzed with acid. The reducing sugar gained was 82.35% (the data not showed).

2.2 Microorganisms

Seventeen fungal strains isolated from loog-pang and various sources were selected and used in this study: 11 strains of *Aspergillus* sp. (*Aspergillus* sp. MSUBT1, *Aspergillus* sp. MSUBT4, *Aspergillus* sp. MSUBT5, *Aspergillus* sp. MSUBT6, *Aspergillus* sp. MSUBT7, *Aspergillus* sp. MSUBT8, *Aspergillus* sp. MSUBT9, *Aspergillus* sp. MSUBT10, *Aspergillus* sp. MSUBT11, *Aspergillus* sp. MSUBT12, and *Aspergillus* sp. MSUBT13), 2 of *Aspergillus niger* (*A. niger* TISTR 3063, and *A. niger* TISTR 3254), 3 of *Rhizopus* sp. (*Rhizopus* sp. MSUBT2, *Rhizopus* sp. MSUBT3, *Rhizopus* sp. MSUBT14), and 1 of *Amylomyces* sp. YTH3. Sub-cultures were individually cultivated on potato dextrose agar (PDA) plates and incubated at 30 °C for 4-5 days. All of them were kept on PDA slants and stored at 4 ^{o}C, except for *Amylomyces* sp.YTH3 which was cultivated and kept on yeast extract-malt extract (YM) agar. Yeast *Saccharomyces cerevisiae* TISTR 5088, *A. niger* TISTR 3063, and *A. niger* TISTR 3254 were purchased from Thailand Institute of Scientific and Technological Research, Thailand. Yeast was grown on a YM agar slant and kept as stock in the refrigerator. Yeast and fungi were transferred to fresh medium every 2 weeks.

2.3 Inoculum preparation

2.3.1 Mycelium inoculum

Five discs of each fungal strain grown on PDA plates for 4-5 days were transferred into 10 ml of a sterile medium containing (w/v) 0.2% peptone, 0.2% yeast extract, 0.1% $MgSO_4.7H_2O$, 0.2% $(NH_4)_2HPO_4$,and 1% glucose. The culture broth was incubated at 30 °C in a rotary shaker at 200 rpm for 2 days and then used as the inoculum. However, the *Amylomyces* strain was prepared as a rice koji starter (10 g cooked sticky rice, supplemented with 0.1% $MgSO_4.7H_2O$, 0.2% $(NH_4)_2HPO_4$, and 10 ml distilled water). One disc of the fungal strain grown on a YM agar plate was placed at the top of sticky rice and then incubated at 30 °C for 2 days before use as koji inoculum.

2.3.2 Yeast inoculum

A single colony of yeast growing on YM agar medium (24 h) was transferred to 10 ml of YM broth and incubated on a rotary shaker at 30 ^{o}C, 150 rpm for 24 h. It was added to 90 ml of YM broth and incubated at the same condition for 24 h before use as the starter (10% v/v).

2.4 Hydrolysis at low and high cassava starch medium

Cassava starch medium at a low concentration of 6% (w/v) and higher concentrations of 10, 15, 20, and 25% containing 0.2% peptone, 0.2% yeast extract, 0.1% $MgSO_4.7H_2O$, and 0.2% $(NH_4)_2HPO_4$ were adjusted to pH 5 before gelatinization and sterilization. The inoculum of individual fungal strains as mycelium in both the broth and koji were prepared as above, inoculated into the cassava starch medium, and then incubated at a rotation speed of 200 rpm for 96 h at 30 °C. Reducing sugar residue was analyzed (Miller, 1959). The experiments were performed in triplicate.

2.5 Time scale of yeast inoculation in co-culture fermentation

Cassava starch media at various high concentrations were prepared as detailed above. Co-culture fermentation was started when koji starter of *A. rouxii* YTH3 was added to the cassava starch medium. The yeast inoculum was then added at various time intervals of 0 h, 12 h, 24 h, and 36 h. All flasks were incubated at 30 °C at a rotation speed of 150 rpm. The fermentation broth was collected and centrifuged. Ethanol concentration and reducing sugars were determined from the supernatant. All experiments were conducted in triplicate.

2.6 Co-culture study in fermenter

The addition of yeast at 12 h and 24 h was performed in a 2-liter fermenter (BIOSTAT®B B. Braun, Germany). The fermentation process was started by adding 10% (w/v) koji starter of *A. rouxii* YTH3 in the fermenter that contained 1 liter of 20% sterilized cassava medium. Air was supplied for the mycelium growth at 0.05 vvm. The temperature was maintained at 30 °C and the agitation speed was 200 rpm. After 12 h or 24 h, yeast seedling was inoculated and the air supply was stopped. The fermentation temperature and agitation were continuously controlled. Ethanol concentration and reducing sugars were determined (Miller, 1959) from the supernatant at various times. The experiments were conducted in duplicate. Ethanol concentrations were analyzed by gas chromatography (Shimadzu model GC-14A) using a PEG-25M column at 200 °C and a flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of 1.75 kg/cm^2 with an injection volume of 1 µl. Isopropanal 2% (v/v) was used as the internal standard.

2.7 Genotypic identification

Chromosomal DNA was extracted from the fungal mycelium and cultured on YM medium for 2 days. The internal transcribed spacer (ITS) DNA between 16S rRNA and 23S rRNA was amplified by polymerase chain reaction (PCR) techniques using the primers ITS1 (5´-TCCGTAGG-TGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGA-TATGC-3´). The PCR product of the ITS region was directly sequenced using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and a model v3.1 automatic sequencer (Applied Biosystem, USA). The nucleotide sequences of the ITS region for closely related strains were retrieved from the GenBank database and aligned using ClustalW2 (Larkin *et al*., 2007). The phylogenetic trees were constructed with MEGA V6.0 using the neighbor-joining method. The confidence values of the tree branches were determined by bootstrap analyses for 1,000 replicates (Tamura *et al*., 2013).

2.8 Statistical analysis

The data were analyzed by ANOVA test using SPSS version 17. The difference values were indicated by a probability of $P<0.05$.

3. Results and Discussion

3.1 Fungal hydrolysis at low starch content

After testing on starch agar medium (results not shown), all fungi were tested by submerged culture in 6% cassava starch medium and the results are shown in Figure 1. The cassava starch was converted to simple sugars during fungi growth and the remaining reducing sugars increased over time, with the highest values recorded at 96 h. Only two strains of *A. niger* TISTR 3254, and *Amylomyces* sp. YTH3 showed the highest sugar liberation after 24 h which then decreased. The reducing sugar residue was the highest at day 1 at 70.86±0.23 g/l and 38.26±0.03 g/l from *Amylomyces* sp. YTH3 and *A. niger* TISTR 3254, respectively. The difference results from *Aspergillus* sp. MSUBT1 showed an increase of sugar liberation with time. Reducing sugar at day 2 to day 4 was higher than day 1 and the highest was 29.14±5.49 g/l at day 3. In addition, the starch solution was clear. The three fungal strains of *A. niger* TISTR 3254, *Amylomyces* sp. YTH3 and *Aspergillus* sp. MSU BT1 were selected and examined at higher concentrations of cassava starch solution.

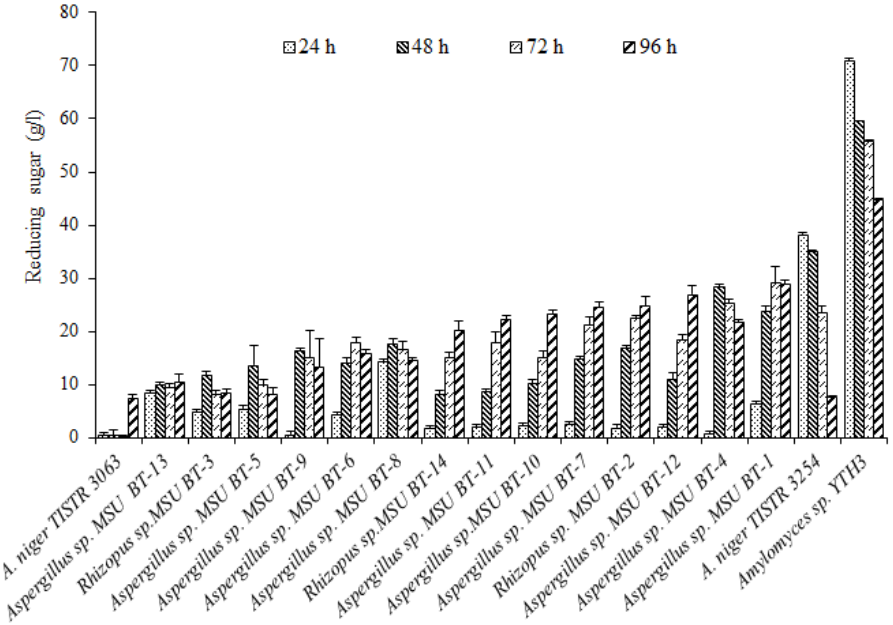

Figure 1. Sugar residues remaining at various times were derived from 6% hydrolyzed cassava starch medium by 17 fungal strains.

3.2 Fungal hydrolysis at high starch concentration

Cassava starch at concentrations between 10% and 25% were used to select the most efficient fungus that could hydrolyze starch to produce the highest quantity of reducing sugar remaining in the broth. The results are shown in Figure 2. Starch concentrations at 10% and 15% were hydrolyzed perfectly (no remaining solidified starch) by the three fungal strains (Figures 2A and 2B). Only *Amylomyces* sp. YTH3 was able to liquefy and saccharify 20% and 25% cassava starch (Figure 2C). Since *A. niger* TISTR 3254 and *Aspergillus* sp. MSU BT1 were unable to hydrolyze starch at the higher concentrations of 20% and 25%, there was no reducing sugar liberation signal from both fungi (Figure 2C). Sugar remaining by *Amylomyces* was higher than either of the *Aspergillus* strains. High levels of remaining sugars (>100 g/l) were obtained from the 10% and 25% starch concentrations at 24 h by *Amylomyces*. This finding indicated that *Amylomyces* sp. YTH3 was the most efficient for starch hydrolysis.

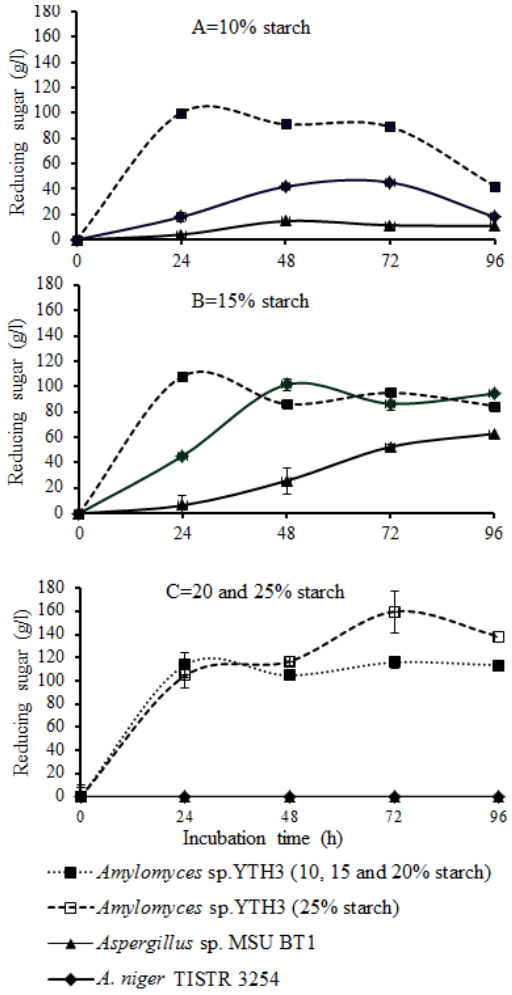

Figure 2. Levels of remaining reducing sugars were derived from *Amylomyces* sp. YTH3, *A. niger* TISTR 3254, and *Aspergillus* sp. MSU BT1 in 100 ml of starch medium at various concentrations. A=10%, B=15%, C=20% and 25% starch content.

3.3 Effects on ethanol production from timing of yeast inoculum

Cassava starch at 15%, 20%, and 25% concentrations were used for ethanol production by co-culture fermentation. Initially, 10% (w/v) koji of *Amylomyces* sp. YTH3 was used to inoculate and hydrolyze the cooked starch. To convert sugars into ethanol, 10% (v/v) yeast starter was added at 0 h, 12 h, and 24 h into 15% starch medium and at 12 h, 24 h, and 36 h into 20% and 25% starch mediums for solidification. Since the 20% and 25% cooked starch mediums were very viscous and solidified when they cooled down, more time was required to hydrolyze the solidified starch by the amylolytic fungi. Therefore at 20% and 25% starch medium, the yeast inoculum was added after 12 h when the fungus was added. The times of adding the yeast starter affected the amount of sugar residues and ethanol production. The sugar residues and ethanol production profiles from the various starch concentrations are shown in Figure 3. At the lowest starch content of 15%, the highest level of sugar residues 108.24±3.35 g/l was achieved by adding yeast inoculum at 24 h (Figure 3A). However, the sugar residues from 15% and 20% starch mediums were lower than those derived from 25% starch medium at the same inoculation time. The sugar residues derived from 20% starch medium were 82.47±8.67, 117.53±10.04, and 128.66±13.39 g/l at the yeast inoculation times of 12 h, 24 h, and 36 h, respectively (Figure 3B). Sugars li-berated from the 25% starch concentration were 93.73±2.05, 130.81±7.99, and 140.58±9.42 g/l at the yeast inoculation times of 12 h, 24 h, and 36 h, respectively (Figure 3C).

Ethanol production from 15% starch medium increased and was the highest at the yeast inoculation time of 12 h (Figure 3A). The ethanol production profile from adding the yeast at 12 h reached the highest level of 49.79±4.24 g/l within 48 h of the fermentation process. The ethanol production from adding the yeast at 24 h was slightly higher than at 12 h; however, the ethanol profile took more time (88 h) to reach the maximum value of 51.02±0.13 g/l, while the lowest profile of ethanol production was observed from adding the yeast at 0 h. Similar results were also found at higher starch concentrations of 20% and 25%. The ethanol production profile decreased as the yeast inoculation time increased. The starch concentration of 25% was more difficult to hydrolyze than the 20% starch concentration. The highest ethanol yield of 57.45±4.04 g/l was achieved from a concentration of 20% starch at a yeast inoculation time at 12 h. The lowest ethanol concentration of 46.96±2.31 g/l was obtained from adding the yeast at 36 h. Consequently, the levels of ethanol production from the 25% starch medium were 53.90±9.88, 51.97±0.54, and 46.36±0.92 g/l for yeast inoculation times at 12 h, 24 h, and 36 h, respectively. The optimal time to add the yeast to convert starch into sugar was investigated. It was found to depend on the starch concentration and sugar consumed by both the yeast and the fungus. Even though the starch content at 25% was viscous and rigid, *Amylomyces* sp. YTH3 hydrolyzed it efficiently. However, ethanol concentrations from the 25% starch were lower than the 20% concentration of starch (Figures 3B and 3C) because of the remaining solidified starch in the system.

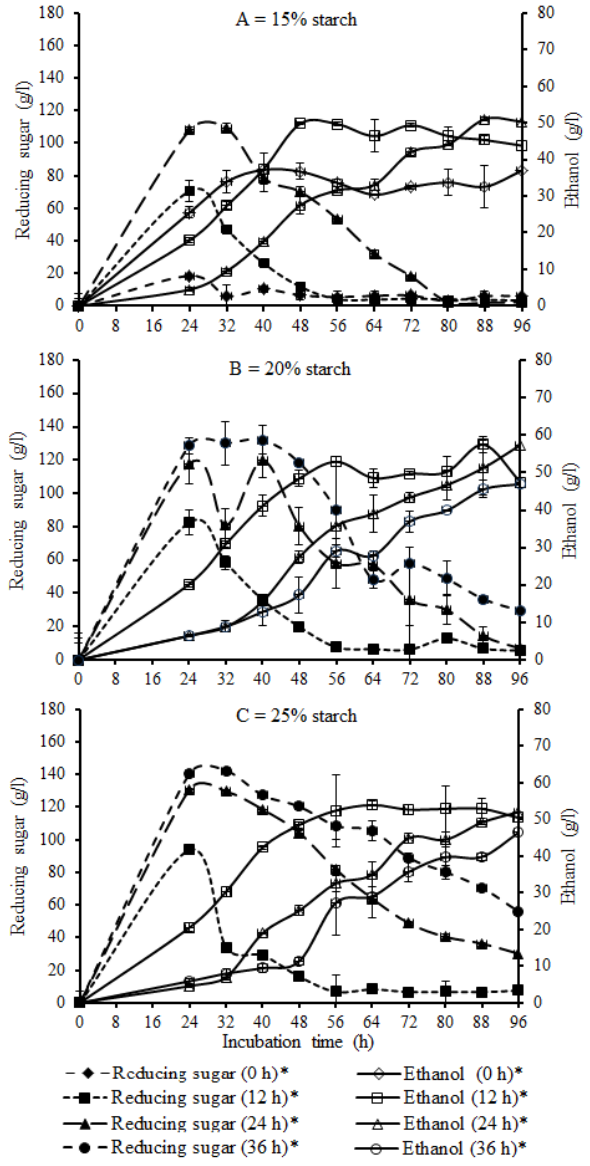

Figure 3. Effects on ethanol production from timing of adding yeast from various concentrations of cassava starch by coculturing *Amylomyces* sp.YTH3 and *S. cerevisiae* TISTR 5088 in a shake flask at 30 °C and 150 rpm. The yeast inoculation times are shown in the parenthesis as (h*). Starch content: A=15%, B=20%, and C=25%.

3.4 Ethanol production by co-cultures in a fermenter

The results implied that a starch concentration of 20% was the optimal concentration for ethanol production. However, the most suitable time to add the yeast in the co-culture process should be examined further in a bioreactor. The results are shown in Figure 4. The active fermentation process was started with *Amylomyces* sp. YTH3 that hydrolyzed cassava starch into fermenting sugar. It was found that the remaining sugars at 24 h were at the highest levels of 92.47±1.60 and 113.52±22.77 g/l when the yeast was added at 12 h and 24 h respectively. The remaining sugar in the fermentation broth when the yeast was added at 24 h was higher than at 12 h. At the fermentation time of 80 h, the levels of residual sugar from both inoculation times were low at the same value and nearly zero. Ethanol was produced immediately after adding the yeast inoculum. Ethanol concentration gradually increased and become constant at an average of 59.66±3.25 g/l at 88 h when yeast was added at 12 h. In addition, between the fermentation time of 12 h and 64 h, the ethanol content derived from adding the yeast at 12 h was higher than at 24 h and after that it seemed to be constant and slightly low at the end of the fermentation process. When yeast was added at 24 h, the ethanol concentration gradually increased continuously and reached 73.68±1.81 g/l at the fermentation time of 96 h. This result indicated that the time of adding the yeast has an effect on the remaining sugar and ethanol production. However, it was clear that ethanol yields were related to the timing of adding the yeast (Table 1), especially when the fermentation time was longer than 40 h. In addition it was also found that the highest ethanol yields from adding the yeast at 12 h and 24 h were 0.381 g ethanol/g sugar and 0.453 g ethanol/g sugar at the fermentation time of 88 h and 96 h, respectively. The percent theoretical yields of adding the yeast at 12 h and 24 h were 70.82% and 87.46%, respectively. The results implied that adding the yeast at 24 h was the optimal time for the co-culture process between *Amylomyces* sp. YTH3 and S. *cerevisiae* TISTR 5088.

Ethanol production from co-culturing of ragi tapai (a mixture starter of mold and yeast) and *S. cerevisiae* was studied by Azmi *et al*., (2010). They also found that the time of adding the yeast affected ethanol production. Ethanol production from co-culturing between ragi tapai and *S. cerevisiae*, ragi tapai (after 1 h adding ragi tapai) from 10% gelatinized cassava starch (70^{o}C for 1 h) was 35 g/L at 72 h, and higher than zero time adding yeast with ragi tapai (27 g/l at 72 h). Based on a study by Ogbonna *et al*. (2010), they found that the highest ethanol content (58 g/l) was gained from 10% starch using co-cultures of *Aspergillus awamori* (10 g koji prepared from 7.5 g rice grain with 2.5 g rice bran) and *S. cerevisiae* (0.3 g).

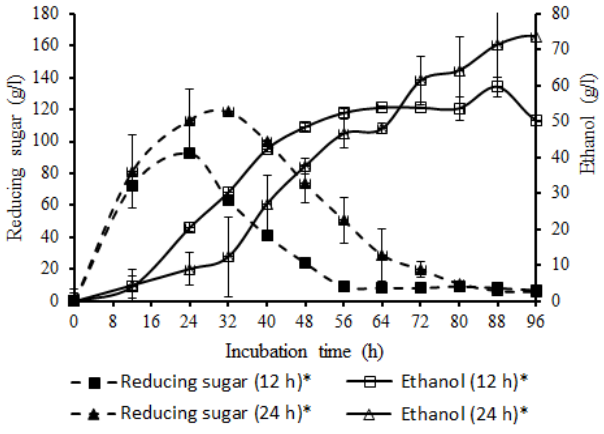

Figure 4. Effects on ethanol production from timing of adding yeast in a batch fermentation of 1 liter from 20% cassava starch medium by co-cultures of *Amylomyces* sp. YTH3 and *S. cerevisiae* TISTR 5088. After 12 h or 24 h of koji starter inoculation, yeast starter cultures were inoculated.

Table 1. Ethanol yield at various times of batch fermentation process at different times of adding yeast to 20% cassava starch medium by co-cultures between *Amylomyces* sp. YTH3 and *Saccharomyces cerevisiae* 5088 were compared.

Fermentation time, h	Ethanol yield, g/g sugar*	
	Time of adding yeast, 12 h	Time of adding yeast, 24 h
24	0.284±0.0354[a]	0.196±0.061[b]
32	0.299±0.0095[ab]	0.293±0.085[ab]
40	0.342±0.0015[a]	0.422±0.0018[b]
48	0.344±0.0069[a]	0.417±0.0057[b]
56	0.336±0.0058[a]	0.412±0.038[b]
64	0.346±0.0048[a]	0.371±0.0018[b]
72	0.344±0.001[a]	0.417±0.025[b]
80	0.345±0.0019[a]	0.421±0.0013[b]
88	0.381±0.0139[a]	0.446±0.0065[b]
96	0.319±0.0132[a]	0.463±0.0042[b]

* Ethanol yields represent average and standard error (n=2).

Table 2 shows the comparative ethanol production from various C-sources by the SSCF process. Fungus played an importance role for the conversion of carbohydrate complexes both in starch and lignocellulose to fermenting sugar. Both solid state and submerged cultures were studied. The greater ethanol yield and lower cost of the process by *in-situ* enzyme production from living fungal cells were beneficial for industrial use. Paschos *et al*. (2015) found that a 19% higher ethanol production was achieved from a solid culture of *F. oxysporum* than a submerged culture. However, the fungal strain and the process were the main parameters that affected the ethanol yield.

4. Morphology and Phylogenetic Tree

The morphology between *Amylomyces* sp. YTH3 and *Rhizopus* sp. is clearly different. Based on the taxonomic study by Ellis *et al*. (1976), the mycelium of the former is coenocytic. Chlamydospores are present and abundant inside the mycelium with a lack of rhizoids and poor sporangia. The mycelium on an agar plate is white when young and turns grayish to brown when it ages. *Rhizopus* sp. produces black spores and also lacks rhizoids and stolons (Ellis, 1985). *Amylomyces* is a monotypic genus, with only one species of *A. rouxii* (Ellis *et al*., 1976). Therefore, *Amylomyces* sp. YTH3 should be re-named as *A. rouxii* YTH3. Many previous studies reported that the DNA sequence of *A. rouxii* is similar to *R. oryzae*. It is possible that *A. rouxii* is a mutation product of *A. oryzae* (Hesseltine, 1983). The phylogenetic tree of the ITS sequences among *A. rouxii* YTH3 and the sequences retrieved from the GenBank are shown in Figure 5. The *Penicillium coraligerun* sequence was used as an outgroup. *A. rouxii* YTH3 nucleotide sequence is most similar (above 99%) to the genera *Rhizopus*, *Mucor*, and *Amylomyces*. However, the name of *Mucor rouxii* was changed to *Amylomyces rouxii* (*Mucor amylomyces rouxii* in trinomial) in 1882. Nowadays, the old name of *Mucor rouxii* is still used (William & Akiko, 2015).

The rRNA-ITS sequence of *A. rouxii* was closely related to *Rhizopus* sp. It is possible that *A. rouxii* was a mutant of *R. oryzae* with greatest similarity at 95% (Abe *et al*., 2006). *A. rouxii* YTH3 was placed in clade A (Figure 5), including *Mucor*, *Amylomyces*, and *Rhizopus*. Abe *et al*. (2006) reported that *A. rouxii* clustered with *R. oryzae*. They recommended that *R. oryzae* and *A. rouxii* should be reclassified. They also developed a new method to screen the types of *R. oryzae* by organic acid production. They found that *A. rouxii* was placed in the same clade with *R. oryzae* as a lactic acid producer in the phylogenetic tree. The nucleotide sequence of *A. rouxii* YTH3 has been submitted in GenBank under accession number KM215272.

Table 2. Studies of ethanol production in simultaneous saccharification and co-fermentation (SSCF) by fungal strains and *Saccharomyces cerevisiae* from various starch sources and lignocellulosic materials

Fungal strain/ type of fermentation	Time, day	Temperature, °C	Ethanol, g/l	Ethanol yield, $Y_{P/S}$	Raw Material (concentration), %	Reference
Scheffersomyces stipitis BCC15191 (submerged culture)	3	33.1	28.6	0.28	Rice straw 10% (w/v)	Suriyachai *et al.* (2013)
Trichoderma sp. (solid state culture)	3	30	43	0.17	Sweet potato 25% (w/v)	Swain *et al*. (2013)
Aspergillus niger GS4 (submerged culture)	4	35	36	0.45	Cassava starch 8% (w/v)	Ado *et al.* (2009)
Fusarium oxysporum F3 (solid state culture)	5	30	58	0.22	Wheat straw 26% (w/v)	Paschos *et al.* (2015)
Aspergillus niger (submerged culture)	7	30	79	0.87	Corn cobs 9% (w/v)	Itelima *et al.* (2013)
Amylomyces rouxii YTH3 (submerged culture)	4	30	74	0.45	Cassava starch 20% (w/v)	This study

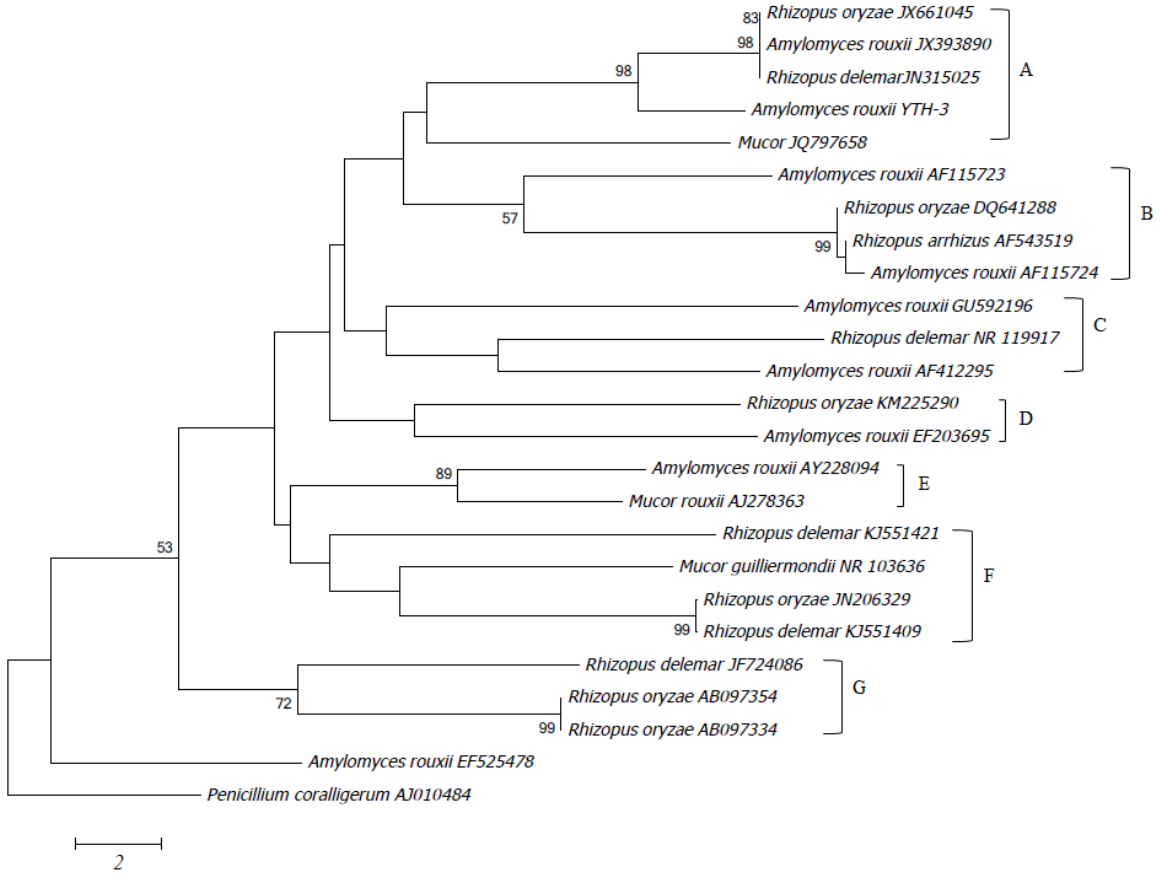

Figure 5. Neighbor-joining phylogenetic tree of ITS sequences of *Amylomyces rouxii* YTH3 and the most similar strains derived from GenBank data base were constructed with MEGA version 6. *Penicillium coralligerum* AJ010484 was used as outgroup. *Amylomyces rouxii* YTH3 was grouped in clade A.

5. Conclusions

Loog-pang is a popular source for screening starch amylolytic fungi. In this study, we successfully selected the highly efficient starch hydrolysis fungus *Amylomyces* sp. YTH3. It was identified as *Amylomyces rouxii* YTH3 by ITS sequence and submitted to GenBank under accession number KM215272. It is the most efficient fungus to digest and liquefy high starch content in a short time. The most suitable time to add the yeast was investigated for ethanol production in co-culture fermentation. It was found that the yeast inoculation time after amylolytic mold addition influenced ethanol production. This work showed the economic feasibility for the scale-up of ethanol production from the SSCF process by co-cultures of *A. rouxii* YTH3 and *S. cerevisiae* TISTR 5088 from cassava starch.

Acknowledgements

This study was supported by a grant from the Energy Conservation Promotion Fund, Project Research Grants Renewable Energy Plan 2554-2555 year, the Energy Policy and Planning Office, Ministry of Energy, Thailand. The authors are also grateful to the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand.

References

Abe, A., Oda, Y., Asano, K., & Sone, T. (2006). The molecular phylogeny of the genus *Rhizopus* based on rDNA sequences. *Bioscience Biotechnology and Biochemistry, 70*, 2387-2393.

Ado, A. S., Olukotun, G. B., Ameh, J. B., & Yabaya, A. (2009). Bioconversion of cassava starch to ethanol in a simultaneous saccharification and fermentation process by co-cultures of *Aspergillus niger* and *Saccharomycescerevisiae*. *Science World Journal, 4*, 19-22.

Agbogbo, F. K., & Wenger, K. S. (2007). Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. *Journal of Industrial Microbiology and Biotechnology, 34*, 723-727.

Ask, M., Bettiga, M., Mapelli, V., & Olsson, L. (2013). The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*. *Biotechnology for Biofuels, 6*, 22-34.

Ayoola, A. A., Adeeyo, A. O., Efeovbokhan, C. V., & Olasimbo, D. A. (2013). Optimum hydrolysis conditions of cassava starch for glucose production. *International Journal of Advanced in IT and Engineering, 2*, 92-101.

Azmi, A. S., Ngohm, G. C., Mel, M., & Hasan, M. (2010). Ragi tapai and *Saccaromyces cerevisiae* as potential coculture in viscous fermentation medium for ethanol production. *African Journal of Biotechnology, 42*, 7122-7127.

Christiana, N. O., & Eric, C. O. (2010). Conversion of cassava flour to ethanol by sequential solid state and submerged cultures. *Process Biochemistry, 45*, 1196-1200.

Ellis, J. J., Rhodes, L. J., & Hesseltine, C. W. (1976). The genus *Amylomyces*. *Mycologia*, *68*, 131-143.

Ellis, J. J. (1985). Species and varieties in the *Rhizopus arrhizus – Rhizopus oryzae* group as indicated by their DNA complementarity. *Mycologia, 77*, 243-247.

Fontana, J. D., Mitchell, A. M., Molina, O. E., Gaitan, A., Bonfim, T. M. B., Adelmann, J., . . . Passos, M. (2008). Starch depolymerization with diluted phosphorric acid and application of the hydrolysate in astaxanthin fermentation. *Food Technology and Biotechnology, 46*, 305-310.

Hesseltine, C. W. (1983). Microbiology of oriental fermented foods. *Annual Review of Microbiology, 37*, 575-601.

Itelima, J., Ogbonna, A., Pandukur, S., Egbere, J., & Salami, A. (2013). Simultaneous saccarification and fermentation of corn cobs to bio-ethanol by co-culture of *Aspergillusniger* and *Saccharomyces cerevisiae*. *International Journal of Environmental Science and Development, 4*, 239-242.

Larkin, M. A.,Blackshields, G.,Brown, N. P.,Chenna, R., McGettigan, P. A., McWilliam, P. A., . . . Higgins, D. G. (2007). CLUSTAL W and Clustal X version 2.0. *Bioinformatics*, *23*, 2947-2948.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for derterminatoin of reducing sugar. *Analytical Chemistry, 31*, 426-428.

Newby, J. (2016). Cassava farms in Southeast Asia exposed to policy changes in global Carbohydrate market. Posted by Australian Center for International Agricultural Research (ACIAR). Retrieved from http://aciarblog.blogspot.com/2016/04/cassava-farmers-in- southeast-asia.html

Office of Agricultural Economics, Ministry of Agriculture and Cooperatives. (2015). Agricultural statistics. Retrieved from http://www.oae.go.th/production.html

Ogbonna, C. N., & Okolib, E. C. (2010). Conversion of cassava flour to fuel ethanol by sequential solid state and submerged cultures. *Process Biochemistry, 45*, 1196-1200.

Paschos, T., Xiros, C., & Christakopoulos, P. (2015). Simultaneous saccharification and fermentation by co-cultures of *Fusarium oxysporum* and *Saccharomyces cerevisiae* enhances ethanol production from liquefied wheat straw at high solid content. *Industrial Crops and Production, 76*, 793-802.

Pervez, S., Aman, A., lqbal, S., Naveed, N. S., & Qader, S. A. U. (2014). Saccharification and liquiefaction of cassava starch: an alternative source for the production of bioethanol using amylolytic enzymes by double fermentation process. *BMC Biotechnology, 14*, 49-58.

Ramachandran, V., Pujari, N., Matey, T., & Kulkarni, S. (2013). Enzymatic Hydrolysis for Glucose-A Review. *International Journal of Science, Engineering and Technology Research, 2*, 1947-1942.

Sanchez, B., & Bautista, J. (1988). Effect of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliemondii*. *Enzyme and Microbial Technology, 10*, 315-318.

Suriyachai, N., Weerasaia, K., Laosiripojana, N., & Champreda, V. (2013). Optimized simultaneous saccharification and co-fermentation of rice straw for ethanol production by *Saccaromyces cerevisiae* and *Scheffersomyce stipitis* co-culture using design of experiments. *Bioresource Technology, 142*, 171-178.

Swain, M. R., Mishra, J., & Thatoi, H. (2013). Bioethanol production from sweet potato (Ipomoea batatas L.) flour using co-culture of *Trichoderma* sp. and *Saccharomyces cerevisiae* in solid-state fermentation. *Brazilian Archives of Biology and Technology, 56*, 171-179.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.*Molecular Biology and Evolution, 30*, 2725-2729.

Ueda, S., Zenin, C. T., Monteiro, D. A., & Park, Y. K. (1981). Production of ethanol from cassava starch by a non-conventional fermentation method. *Biotechnology and Bioengineering, 23*, 291-200.

William, S., & Akiko, A. (2015). History of soybeans and soy foods in France (1665-2015). Retrieved from http:// www.soyinfocenter.com/pdf/180/Fran.pdf