Songklanakarin J. Sci. Technol.
33 (2), 163-170, Mar. - Apr. 2011

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Selection of Thai starter components for ethanol production utilizing malted rice from waste paddy

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Received 24 November 2010; Accepted 30 April 2011

Abstract

The use of mixed herbs in Thai rice wine starter (*Loog-pang*) were investigated in order to directly maintain the efficiency of the microbial community (*Saccharomycopsis fibuligera*, *Amylomyces* sp., *Gluconobacter* sp. and *Pediocccus pentosaceus*). The optimum formula was galanga, garlic, long pepper, licorice, and black pepper at the ratio of 0.5:8:1:4:1, respectively. Previously, waste paddy has been used directly as a renewable resource for fuel ethanol production using solid state fermentation (SSF) with *Loog-pang*. In this study, hydrolyzed malted rice starch was used as the sole nutrient source in submerged fermentation (SmF) to enhance the process yield. The maximum ethanol productivity (4.08 g/kg waste paddy h^{-1}) and the highest ethanol concentration (149±7.0 g/kg waste paddy) were obtained after 48 hrs of incubation. The results indicated that starch saccharification provided a higher ethanol yield (48.38 g/100g sugar consumed) than SSF. In addition, the efficiency of ethanol fermentation was 67% which is similar to that of the malted rice made from normal paddy (68%). This result suggests that waste paddy could be used as an alternative raw material for ethanol production.

Keywords: *Loog-pang*, waste paddy, saccharification, ethanol productivity, submerged fermentation

1. Introduction

Cereal grains are an important source for fuel ethanol production (Wang *et al.*, 2007). Waste paddy rice of germinated seeds is under water during the harvest in flooded areas. The rice seed are lying on the ground and the sprouts are under water. This gives the strong smell (Stafford *et al.*, 2005), and the waste paddy rice can not be used for human consumption or for beverage production. Waste paddy rice is usually used as feed for ducks and geese. Asia has the world's largest harvested rice area with over 90% of global production (Najafi *et al.*, 2009). The Food and Agricultural Organization (FAO) reported as approximately 5% of global rice production is produced in Thailand. Recently, the Ministry of Agriculture and Cooperatives in Thailand reported that the paddy fields have been severely affected by flooding. Thus, it resulted in a drastic yield reduction during the harvest in 2008 with >10% (4.15 M Tg) of rice yield. This amount could efficiently produce 178 M gallon of bioethanol.

More efficient and inexpensive technology should be developed for ethanol production from starch crops for farm and industrial use. Therefore, the experiment was designed to determine the value of waste paddy rice for ethanol production using the indigenous fermented starter (*Loog-pang*).

"*Loog-pang*" is a fermentation starter from Thailand, which is a mixed culture of mold, yeast and bacteria for traditional alcoholic products from raw rice. *Loog-pang* is made from rice flour mixed with a variety of herbs and spices,

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including a powdered starter from a previous batch (Lotong 1998). Herbs and spices serve as an antibacterial medium in alcohol fermentation preventing microbial spoilage or fermentation failure (Dung 2006). This study selected natural herbs to test their efficiency. Nowadays, the process of *Loog-pang* preparation is limited to only some families because the recipes are kept secret and inherited from one generation to another.

In this study, isolation and identification of the microbial community in *Loog-pang* AU2, which could be maintained efficiently using herbal mix, were carried out. The aim of the research was to investigate the influence of herbs found in *Loog-pang* on ethanol production from waste paddy rice. The feasibility of enhancing the ethanol productivity and ethanol yield were performed in submerge fermentation by improving starch hydrolysis by controlling the hydrolytic enzymes in the germinated seeds.

2. Materials and Methods

2.1 Waste paddy and normal paddy

Waste paddy rice of germinated Thai rice seeds (*Oryza sativa* L.) of the RD29 (non-waxy) variety was collected from flooded areas in Phitsanulok Province, Thailand (October 2008). The abnormal seeds were removed, while the germinated seeds were washed with water and sun-dried prior to storage at 4°C. The germinated seeds were composed of 14.5% amylose, 18.7% reducing sugar, 5.8% protein and 12-13% moisture.

Normal paddy rice of the RD29 variety contains 29.6% amylose and 62% amylopectin, 7.33% protein and husk of 27.6 g/100g paddy seeds (Palawisut *et al.*, 2008). These seeds were germinated for three days after soaking with water and maintained at 30°C under aerobic conditions (Saman *et al.*, 2008). Then, the germinated seeds were dried in an oven at 50°C for 24 hrs and stored at 4°C. The germinated seeds were composed of 17.3% amylose, 20% reducing sugar, 6.5% protein, and 10-12% moisture.

2.2 Thai traditional starter (*Loog-pang*)

Loog-pang AU2 from Authaithanee Province in Thailand (August 2008) was used throughout the experiments. The preliminary results showed that *Loog-pang* AU2 exhibited the highest ethanol productivity in rice wine making. *Loog-pang* AU2 must be kept dry at an ambient temperature and used within three months.

2.3 Isolation and identification of microorganisms in *Loog-pang*

The *Loog-pang* was aseptically kept in plastic bags, freeze-dried and stored at -40°C before being tested by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The strain of fungi was identified based on morphological characteristics (Samson *et al.*, 2004), while the bacteria and yeast were identified by amplifying partial sequences of ribosome encoding genes. Direct sequencing of 16S rDNA was use for testing the bacteria (Kawasaki *et al.*, 1993) and the sequences of the D1/D2 domain of 26S rDNA were used for the yeasts (Kurtzman and Robnett, 1998b; Saitou and Nei, 1987). These were compared using the BLASTn homology search.

2.4 Screening of herb parameters using Plackett-Burman design

A Plackett-Burman design for eight experiments, which includes the effects of five numerical parameters (galanga, garlic, long pepper, licorice, and black pepper) at two levels (-1 and +1) (Table 1 and 2) (Plackett and Burman, 1944), was used for screening. Each ratio of herbs (per 100g of *Loog-pang* dry wt.) was mixed with a powdered starter of old *Loog-pang* (15.5g dry wt.), rice flour (72-77 g), and water (40-50 ml). The inoculated dough with a final moisture content of 45% (w/w) was incubated at 30°C for 48 hrs. The experiments were performed in triplicated experiment. The fermented dough was dried in an oven at 45°C for 48 hrs, to reach a moisture content of 20% (w/w) (Lotong 1998). The starter dough was tested for ethanol production in waste paddy rice using SSF, and each treatment was performed duplicated. Samples were withdrawn every two hours.

2.5 Ethanol production in solid state fermentation (SSF)

Ethanol production from waste paddy rice was prepared in the traditional Southeast Asian style. Waste paddy rice from germinated seed was dehusked using a paddy dehusker (Gear type, India). Then, 400 g of unpolished rice was soaked in 500 ml water for an hour, steamed for 1 h and cooling down. The cooked rice was rinsed with 10% (w/v) $Ca(OH)_2$ and then mixed with 10 g of powdered starter (*Loog-pang*). For saccharification, the mixture was transferred to a wide-mounted bottle, closed with a cap and incubated at 30°C. This process allows saccharification of rice to be occurred. The sugar concentration of the fermented rice syrups was reduced to 200-220 g/l by adding 0.25 L water gently and ethanol fermentation was carried out under static incubation at 30°C for one week.

2.6 Optimization of the mashing process

Waste paddy rice from germinated seed was ground using a hammer mill (Type SM-1, Germany) and sieved through a 300 μm pore size. The saccharification process was performed at various concentrations of ground malted rice (50, 75, 100, and 125 g/l dry wt.). The ground malted rice was weighed at 10 g, 15 g, 20 g and 25 g (dry wt.), 200 ml of 0.3 M $CaCl_2.2H_2O$ was added and the pH was adjusted to 6.0 with 1 M of lactic acid. The mash was cooked at 80°C for 2 hrs in a stirred fermentor (150 rpm).

Table 1. The variety of herbs and spices at two levels was used in the Plackett-Burman design for *Loog-pang* preparation (g/100g of *Loog-pang* dry wt.).

Parameters	Common name	Scientific name	level	
			-1	1
1	Galanga (wet wt.)	*Alpinia galangal* Swartz	0.4	0.8
2	Garlic (wet wt.)	*Allium sativum*	3.0	6.0
3	Long pepper (dry wt.)	*Piper retrofractum* Vahl	0.75	1.5
4	Licorice (dry wt.)	*Glycyrrhizs glabra* Linn	1.5	3.0
5	Black pepper (dry wt.)	*Piper nigrum* Linn	0.75	1.5

Table 2. Plackett-Burman design for eight experiments with observed results the ethanol production (g/kg waste paddy dry wt.) at 90 hrs of incubation in SSF.

Experiment	Galanga	Garlic	Long pepper	Licorice	Black pepper	Ethanol Production
1	-1	-1	-1	1	-1	41
2	-1	1	-1	-1	1	53
3	-1	-1	-1	-1	-1	99
4	1	-1	1	-1	1	97
5	1	1	1	-1	1	29
6	-1	1	-1	1	-1	128
7	1	1	1	1	-1	110
8	1	-1	1	1	1	101

The mashing process was conducted at different temperatures (50°C, 60°C, 70°C, 80°C, 90°C and 100°C) for 2 hrs in a stirred fermentor at 150 rpm. The mixture contained 20 g (dry wt.) of ground malted rice and 200 ml of 0.3 M $CaCl_2.2H_2O$. The samples were centrifuged at 8,000 rpm, 4°C for 10 min (Sorvall RC5, USA) and the supernatant was analyzed for reducing sugar using the DNS method (Miller, 1959).

2.7 Acid pretreatment

After mashing under proper conditions, the acid hydrolysis of the remaining flour in the slurry was optimized by adjusting the pH (1.0 and 2.0) with 1.0 N HCl and by incubating at different temperatures (80°C, 90°C and 100°C) for 2 hrs in a stirred fermentor (150 rpm). The mixture was neutralized to pH 6.0 using 1.0 N NaOH and then centrifuged at 8,000 rpm, at 4°C for 10 min. The supernatant was analyzed for reducing sugar and sterilized at 121°C for 10 min before being used as a culture broth.

2.8 Ethanol production in submerged fermentation (SmF)

Three liters of the hydrolysate of the malted rice of waste paddy (reducing sugar concentration of 20% w/v) was transferred to a 5 L fermentor (B-Braun, Germany). The inoculum solution was prepared from 10 g powdered starter (*Loog-pang* AU2) in 150 ml of the hydrolysate and stirred at 150 rpm at 30°C for 30 min. The hydrolysate was adjusted to pH 6.0 with 1 M of lactic acid. The inoculum solution was added and the solution was incubated at 30°C with agitation (150 rpm) for 20 hrs, followed by 40 hrs of a static incubation. Samples were withdrawn every two hours.

2.9 Analytical techniques

The cultured supernatant was analyzed for reduced sugar using DNS method (Miller, 1959). The starch content was determined colorimetrically at 620 nm in an iodine solution (Pintado *et al.*, 1999). The protein content was determined by the Kjeldahl method (AOAC, 2006). Ethanol analysis was determined using gas chromatography (Varian CX 3800, USA) with a capillary column**.** The percentage of saccharification of starch in the substrate was calculated (Jeya *et al.*, 2009). The efficiency of the fermentation was calculated by dividing the sugar consumed during fermentation by the initial sugars and multiplying the results by 100 (Mohanty *et al.*, 2009).

3. Results and Discussion

3.1 Isolation and identification of microorganisms in *Loog-pang*

The community of microorganisms in the *Look-pang* AU2 was identified and is listed in Table 3. Some of the

Table 3. Identification of microorganisms in *Loog-pang* AU2 compared to the species diversity of traditional Asian fermentation starters to produce rice wine.

local name, Country	Microorganisms	References
Loog-pang, Thailand	**Yeast;** *Saccharomycopsis fibuligera* **Mould;** *Amylomyces* sp., *Mucor* sp., *Rhizopus* sp. **Bacteria;** *Gluconobacter* sp., *Pediococcus pentosaceus*	This work
Loog-pang, Thailand	**Yeast;** *Saccharomycopsis fibuligera, Saccharomyces cerevisiae*, *Pichia anomala*, *P. burtonii*, *P. fabianii*, *P. Mexicana*, *P. heimii, Issatchenkia orientalis, Candida rhagii, C. glabrata*, *Torulaspora globosa, Rhodotorula philyla*, *Trichosporon asahii, T. delbrueckii* **Mould;** *Amylomyces* sp., *Actinomucor* sp., *Aspergillus niger, Aspergillus* sp., *Mucor* sp., *Monascus* sp., *Penicillium* sp., *Rhizopus* sp.,	Limtong *et al.* (2002;2005)
Banh men, SR Vietnam	**Yeast;** *Saccharomycopsis fibuligera, Saccharomyces cerevisiae, Issatchenkia* sp., *Pichia anomala, P. ranongensis, Candida tropicalis, Clavispora lusitaniae* **Mould;** *Amylomyces* sp., *Absidia corymbifera, Rhizopus oryzae, R. microsporus,* **Bacteria;** *Acetobacter orientalis, A. pasteurianus, Bacillus subtilis, B. circulans, B. amyloliquefaciens, B. Sporothermodurans, Lactobacillus plantarum, L. brevis, Pediococcus pentosaceus, Weissella confusa, W. Paramesenteroides,*	Thanh *et al.* (2008)
Ragi tapé, Indonesia	**Yeast;** *Candida tropicalis, Saccharomycopsis fibuligera* **Mould;** *Amylomyces rouxii, Rhizopus oryzae*, *Mucor indicus*	Abe *et al.* (2004)
Sake Koji, Japan	**Yeast;** *Saccharomyces cerevisiae* **Mould**; *Aspergillus oryzae, Rhizopus* sp., *Mucor* sp., *Amylomyces* sp.	Kotaka *et al.* (2008)

organisms found in this study are similar to those previously reported (Limtong *et al.*, 2002; Abe *et al.*, 2004; Limtong *et al.*, 2005; Thanh *et al.*, 2008; Kotaka *et al.*, 2008). *S. fibuligera* was found to be the principle yeast species and is the main amylase producer in the *Loog-pang*. This result was similar to those found in a traditional Vietnamese starter (*Banh men*). Other amylase producers in the *Loog-pang* were *Rhizopus* sp. and *Amylomyces* sp. However, Mucoraceae, amylolytic microorganisms involved in traditional alcohol fermentations, were predominant in *Look-pang*, *Ragi tapé*, and *Sake Koji*, but not in *Banh men*. The sample had a microbial community dominated by molds and yeasts at cell densities of 3.8×10^6 and 5.1×10^6 cfu/g sample, respectively.

In addition, the species of acetic acid producing bacteria was *Gluconobacter* sp., which can grow in an oxygenated environment. Although not verified empirically, the role of lactic acid bacteria (*Pediococcus pentosaceus*) in traditional alcohol fermentation starter is often thought to be an acidification that favors the growth of yeasts and filamentous fungi while suppressing the growth of food spoiling bacteria (Gandjar 1999).

3.2 Screening of herbs parameters using the Plackett-Burman design

The screening of herbs in the *Loog-pang* was carried out based on reviews of existing literature (Lotong 1998; Dung *et al.*, 2006) and the experiences of traditional rice wine makers in Thailand. After 48 hrs of incubation, the fermented dough was evaluated for ethanol fermentation. Ethanol production at 90 hrs of incubation in SSF showed the largest variability of production rate in the experiments, which were used to calculate the statistic analysis of the effects (Figure 1a). The Pareto chart revealed that ca. 80% of the cumulative effect in critical parameters was due to two parameters: licorice and black pepper (Figure 1b). The other parameters, galanga, garlic, and long pepper were found to have a less critical effect.

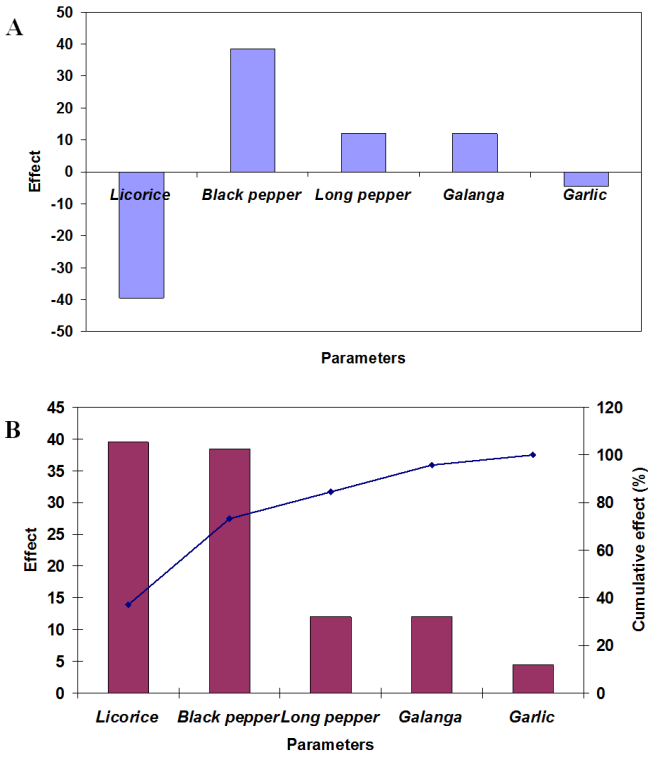

Figure 1. (A) Effect profile of the eight experiments from table 1 and 2; (B) Pareto chart of initial screening on five herbs.

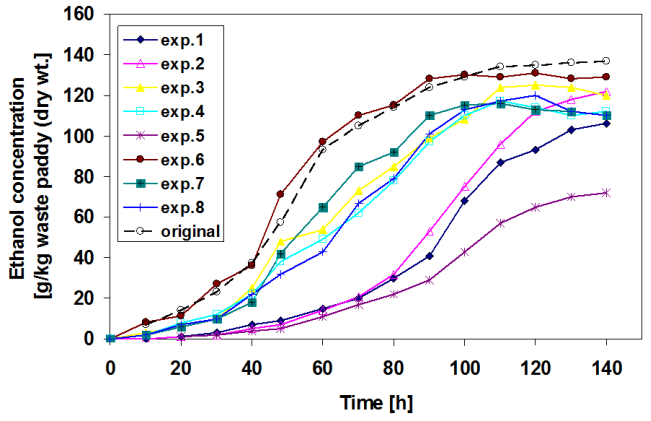

Figure 2. Ethanol production of malted rice from waste paddy in SSF of the eight experiments of Plackett-Burman design from table 1 and 2 in comparison with the old *Loog-pang* AU2 (old LP.).

Thus, the *Loog-pang* from experiment no. 6 (Table 2 and Figure 2) showed ethanol production equal to the original *Loog-pang* AU2. Figure 2 shows that the maximum amount of ethanol produced in the SSF was obtained from ca. 128 g/kg mated rice from waste paddy after 90 hrs of incubation and the highest amount of reducing sugar reached ca. 300 g/kg malted rice from waste paddy after 48 hrs of incubation. Otherwise, the percentage of saccharification and the efficiency of ethanol fermentation in SSF were 42.8% and 57.7%, respectively.

3.3 Optimization of rice starch saccharification

Figure 3 shows starch saccharification. The slurry contained the highest level of reducing sugars under mashing conditions at 80°C, pH 6.0 for 2 hrs in a 100g/l substrate concentration. The maximum reducing sugar concentration (347.7±7.0 g/kg malted rice from waste paddy) was 47.8± 1.5 % saccharification. This process resulted in a higher sugar contents in the slurry than in liquid SSF and less incubation time.

After mashing the malted rice, the remaining starch in the slurry was digested continuously under acidic conditions (pH 1.0) at 100°C for 2 hrs. The result showed that the total reducing sugar in the slurry was increased to 520.5± 13.0 g/kg mated rice from waste paddy (ca. 74% saccharification) (Table 4 and 5). The waste paddy rice consisted of 27.6% husk which meant the yield of reducing sugar from waste paddy was about 719 g/kg of unpolished rice. That was close to the yield of reducing sugar from aging paddy

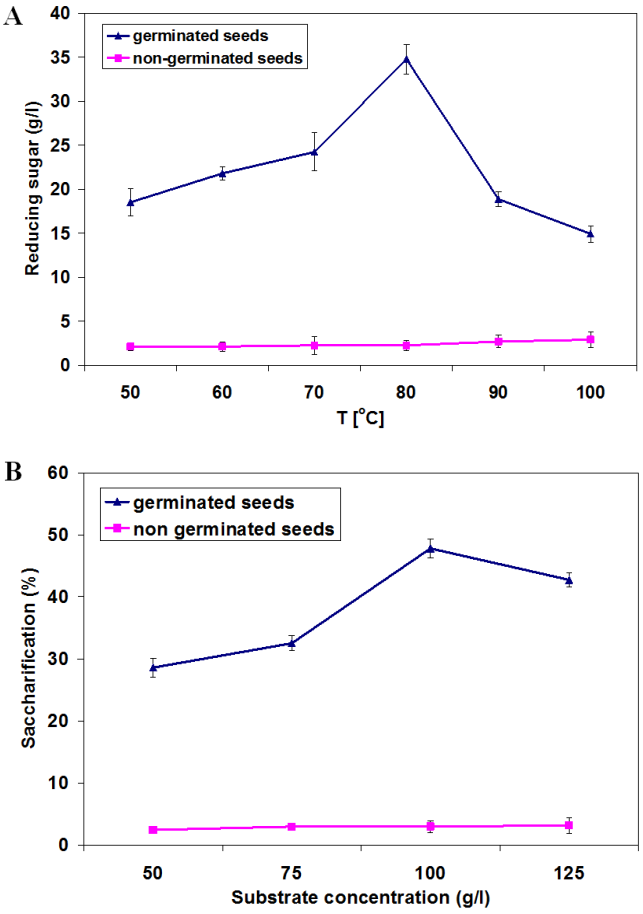

Figure 3. Optimal mashing of malted rice from waste paddy: (A) effect of temperature on reducing sugar production; (B) effect of substrate concentration on saccharification.

Table 4. Effect of acid concentrations of hydrolysis at 80°C on enhanced reducing sugar of geminated seeds from waste paddy and non-germinated seeds from normal paddy rice.

pH in solution	non-germinated seeds		germinated seeds	
	Reducing sugar (g/l)	Saccharification (%)	Reducing sugar (g/l)	Saccharification (%)
1.0	3.83±0.52	4.92±0.67	45.82 ±0.88	65.46±1.26
2.0	3.17±0.61	4.08±0.78	40.72 ±0.67	58.17±0.96
6.0	2.51± 0.63	3.23±0.81	35.27 ±1.03	50.39±1.47

Table 5. Effect of temperatures of acid hydrolysis at pH 1.0 on enhanced reducing sugar of geminated seeds from waste paddy rice.

T(°C)	Time of hydrolysis (1 hrs)		Time of hydrolysis (2 hrs)	
	Reducing sugar(g/l)	Saccharification(%)	Reducing sugar(g/l)	Saccharification(%)
80	39.74±0.62	56.77±0.89	45.82 ±0.88	65.46±1.26
90	41.18±1.11	52.95±1.57	48.15±1.18	68.79±1.69
100	44.61±1.22	63.73±1.74	52.05±1.30	74.36±1.86

which was 810.26±24 g/kg of unpolished rice after the saccharification process when adding commercial enzymes to hydrolyze for 32 hrs (Lu *et al.*, 2009).

3.4 Optimization of starter concentration

The appropriate dosage and maximum ethanol concentration are depicted in Figure 4. Ethanol production in SmF increased as the starter concentration increased. The surface plots of ethanol production indicate that the starter concentration should not exceed 3.33 g/l to produce the highest ethanol productivity of 4.54 g/kg malted rice from waste paddy h^{-1} in 24 hrs of incubation.

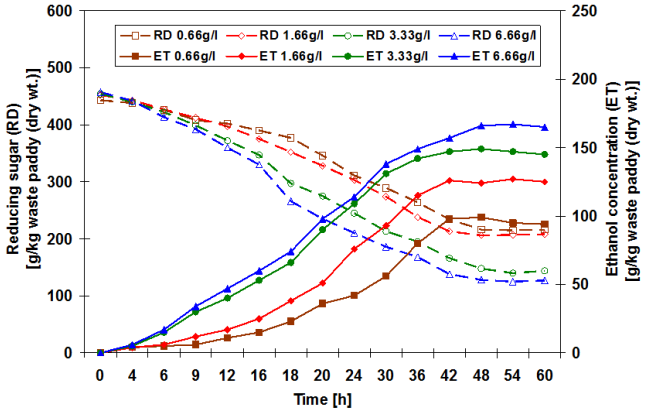

Figure 4. Effect of reducing sugar of hydrolysate of malted rice from waste paddy on ethanol production in submerged fermentation.

3.5 Ethanol production from waste paddy in SmF

The experiment was carried out in a fermentor with three liters of culture volume under optimal conditions. The ethanol formation profile for SmF, the final ethanol concentration, was 149±7.0 g/kg malted rice from waste paddy after 48 hrs of incubation. This was significantly higher than that obtained in SSF (130±7.8 g/kg malted rice from waste paddy) after 90 hrs of incubation (Figure 5). The higher of ethanol productivity and ethanol yield reached 4.08 g/kg waste paddy h^{-1} and 48.38 g/100g of sugar consumed, respectively. Therefore, the efficiency of ethanol fermentation from waste paddy (67%) was similar to that of regular paddy (68%). In this case, the growth of microorganisms in the *Loog-pang* might be limited by essential nutrients, or affected by inhibiting substances of the hydrolysate.

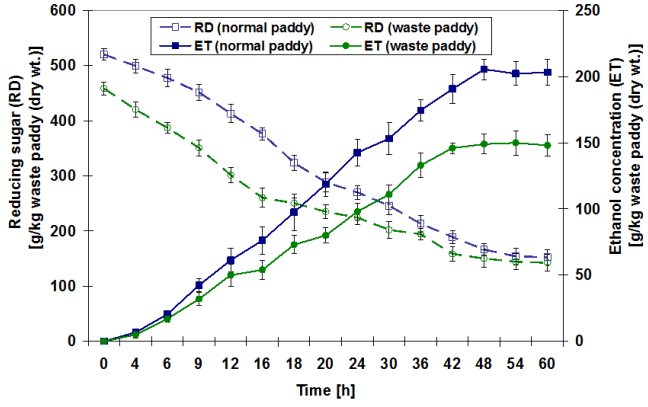

Figure 5. Production of ethanol from malted rice of waste paddy and normal paddy rice in submerged fermentation.

4. Conclusion

This study found that the secret ratio of herbal mix in

the starter components maintained a community of microorganisms in *Loog-pang*. The Plackett-Burman design can help to reduce the experimental runs and initial screenings of herbs such as: galanga, garlic, long pepper, licorice and black pepper. The results showed in this study that germinated seeds and malted rice of waste paddy are an economic alternative raw material for ethanol production by *Loog-pang*. For energy farm needs, conventional SSF can be used more efficiently in the direct fermentation of starch to ethanol avoiding the multi-step process and reducing energy consumption, but the period of incubation was twice as long as that of SmF. Moreover, starch saccharification of malted rice under optimal mashing and acidic pretreatment obtained higher sugar contents in the hydrolysate as the sole nutrient sources which obtained a higher ethanol production in SmF. Further studies in the malted rice hydrolysate could be performed to learn more about the essential nutrients for microorganisms to enhance growth and ethanol formation. Also, economic feasibility of ethanol production should be considered in future fermentation strategies.

Acknowledgments

This research was financially supported by the National Research Council of Thailand (NRCT) and Naresuan University, Thailand.

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