TECHNISCHE UNIVERSITÄT MÜNCHEN

Freising – Weihenstephan

Lehrstuhl für Technologie der Brauerei II

Defining and Optimization of Satho Production Technology

Lumprai Srithamma

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LIST OF ABBREVIATIONS

ml	milliliter
g	gram
mg	milligram
µg	microgram
µm	micrometer
mm	millimeter
cm	centimeter
N	normality
M	molarity
mM	millimolar
°C	degree Celsius
%	percent
%w/w	percent weight by weight
%w/v	percent weight by volume
%v/v	percent volume by volume
HPLC	High Performance Liquid Chromatography
TCA cycle	Tricarboxylic acid cycle
rpm	round per minute
bp	base pair
DNA	Deoxyribonucleic acid
rDNA	ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
PCR	Polymerase Chain Reaction
rt PCR	real time Polymerase Chain Reaction
EDTA	Ethylenediaminetetra acetic acid
$MgCl_2$	Magnesium chloride
EtBr	Ethidium bromide
NaCl	Sodiumchloride
Conc.	concentration
CFU	colony forming unit
Alc.	alcohol
∅	diameter
TSS	total soluble solid
TA	total acidity
ATP	Adenosinetriphosphate
dNTP	deoxynucleotidetriphosphate

I. RATIONALE AND PROBLEM

Satho, the Thai traditional non-distilled alcoholic beverage, generally it was called Thai rice wine, has been made for centuries by the local farmers of the Isaan (Northeastern) region of Thailand. It was produced from starchy glutinous or sticky steamed rice by fermentation process.According to the regulation, it's contained not more than 15 % v/v of ethanol which characterised by cloudy or clear, sweet, sour and alcoholic with distinctive aroma. Fermentation was promoted either by indigenous start culture known as loogpang, which consisted mainly of mould and yeast or pure culture. (Thai Community Product Standard 3/2003). Starch and other components in rice were depolymerised yielding the sugars, amino acids and fatty acids which would be further converted to alcohol and other by products. Some water was added during process at the properly ratio with create favour condition for yeast fermentation. Satho has been produced for long time about 800 years by traditional process and it was widely used for celebrations, festivals and ceremonies through country.

Figure 1. Some samples of satho products

Since the Thai government stop monopolized the production of fermented alcoholic beverage in 2001, there were around 5,000 small satho producers situated mostly in northeast and north part which produced more than 14,000,000 litres per year using traditional and simplicity of the process as illustrated in Figure 2 (Excise Department, Thailand).The increased exposure and availability has resulted in satho's popularity under the Thai government's One Tambon One Product program, OTOP (the government sponsored economic development program). The fermented home and cottage industries chose to produce tax-paid satho as their OTOP product under the names such as "Siam Satho", "Ruan Rak", "Glu Pli" and others as represented in Figure 1.However, nowsaday, only around 20 producers are still in this business (Excise Department, Thailand) cause of lack in scientific knowledge concern resulting in difference quality and often has a short shelf life. As satho production consists of biological process using complex microbial in loogpang accompany with the new advances in biotechnology was not being applied affect from the strong regulation by the government in the past, consequently, limited knowledge of key microorganism, their interaction and fermentation process resulted of low quality products. Therefore, to be substantial, scientific knowledge and quality control techniques must be applied as well, indigenous mixed start culture has to be studied whereas traditional fermentation process has to be defined and optimized. In addition, alternative satho

fermentation using rice malt to avoid limitation of mould used would be studied and characterised.

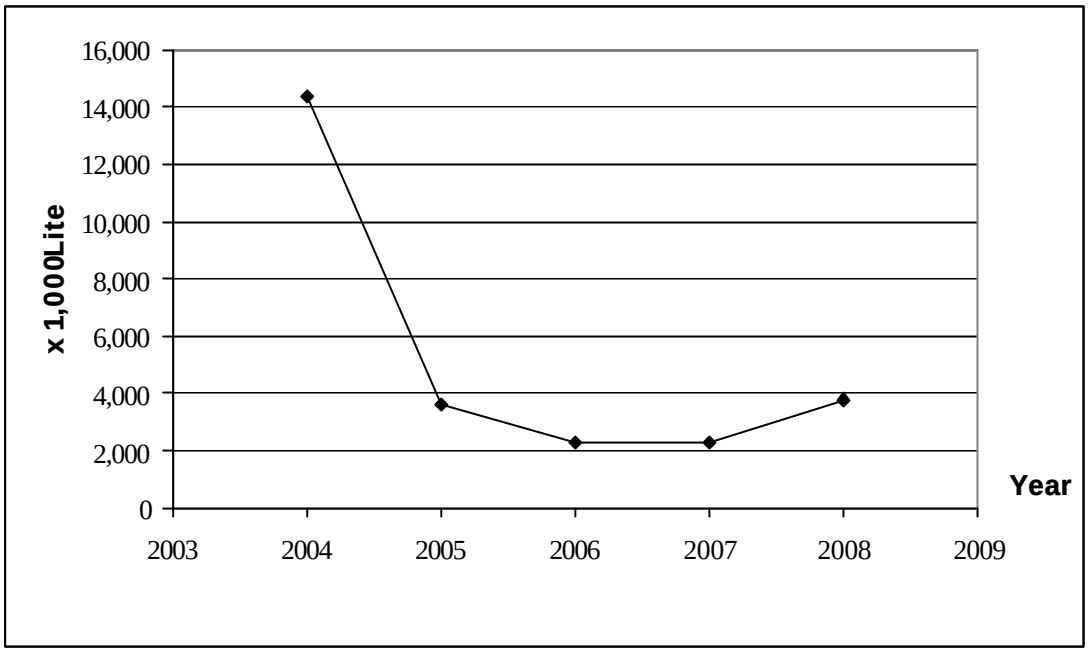


Figure 2. Quantity of satho production during 2004-2008

II.OBJECTIVES

The objective in this research was not an ecological study looking for the occurrence and distribution of all microorganisms present in loogpang, but it was aimed at maximizing productivity and finding highly productive strains. Therefore, only a limited selection of yeast and mould isolates that showed significant abilities for *satho* production were studied. Finished product obtained from various forms of start culture and substrates used for fermentations were characterised. These results might be specified in overview quality of this product in order to increase and stabilised quality. Therefore the objectives of this study were;

1. To isolated, identified, and characterised of high proficiency yeast and mould in selected loogpang samples prior the condition of satho fermentation has been optimised.
2. To characterised of satho produced by alternative fermentation using various kinds of start culture and substrates.

III. INTRODUCTION

3.1 Rice

Rice, *Oryza sativa L.*,a short living plant related to grass which a member of the family Poaceae, the second most widely cultivated cereal in the world, after wheat was produced and consumed around 90% of the world's rice in Asia which almost 60% in China and India. In 1999, total milled rice 550 MMT was produced from leading rice producers, China, India, Indonesia, Bangladesh, Vietnam, Thailand, Burma, Japan, Philippines, Brazil and United States. In the world market of rice,

Thailand is one of the fifth rice exporters.Rice and world rice exporters were presented in Figure 3.

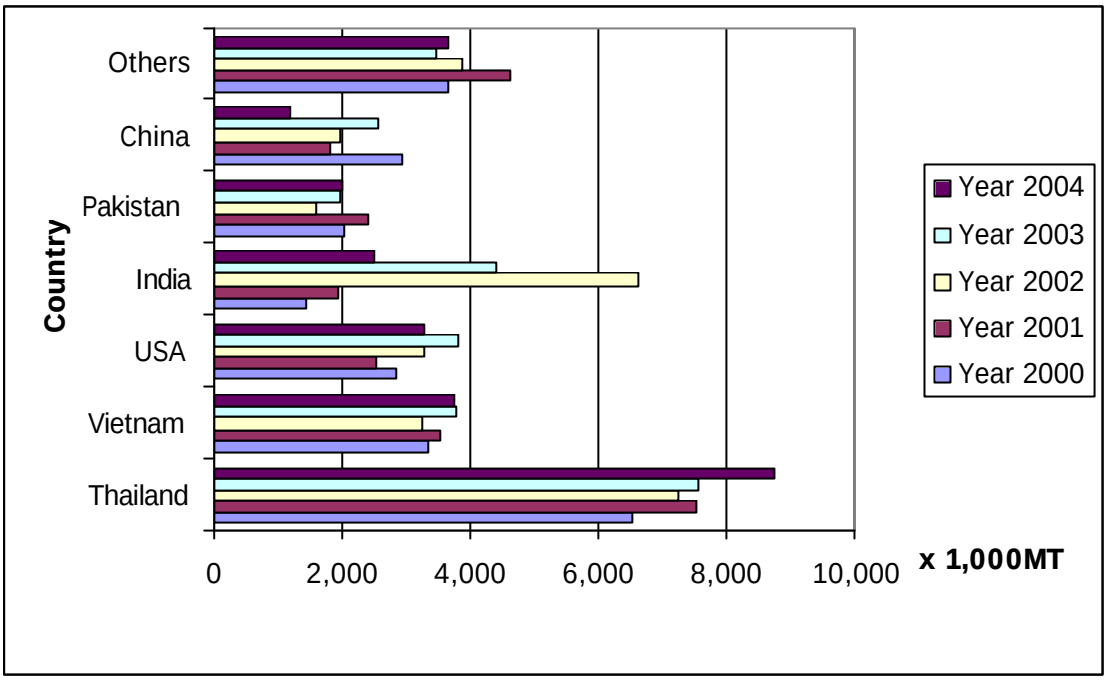


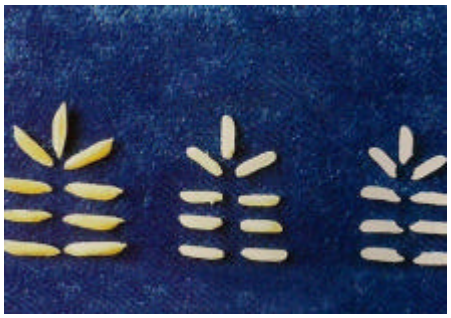
Figure 3. World rice exporters (x 1, 000 MT). **Source:** USDA

Moreover, rice was widely used in the world for the production of alcoholic beverageas sake in Japan, shaoshin-chi in China, satho in Thailand and other miscellaneous beverages in Eastern Asia. Rice was also used as adjunct in the production of other alcoholic beverages such as beer which were widely used in many countries except in Germany, where the German Reinheitgebot or Purity Law is effect for beer brewing for domestic consumption.

Figure 4. Rice field in Thailand

There are three main varieties of rice in the world, Indica (long grain), Japonica (round grain) and Javanica (medium grain). Indica varieties were predominating in tropical area in Indochina, Thailand, India, Pakistan, Brazil and Southern USA. Whereas Japonica rice was cultivated in northern China, Japan,

Korea, Taiwan and California. Javanica was grown only in Indonesia. Some famous varieties of rice were represented in Figure 5.



Surin 1 (Indica variety)

Thai Jasmine rice (Indica variety)

Japanese rice (Japonica variety)

Black rice (Indica variety)

Brown rice (Indica variety)

Figure 5. The famous rice varieties

Rice is harvested from field in form of paddy rice which one kernel contains of husk which is the outermost layer consists mostly of silica and cellulose. The next is bran layers, a thin bran film, consists mainly of fibre, protein, vitamin B and fat, the most nutritious part of rice kernel. The innermost part is rice kernel, 2 types of starch, amylose and amylopectin is presented. At the base of each grain is embryo which will grow to actual new plant. Overview of rice kernel was presented in Figure 6.

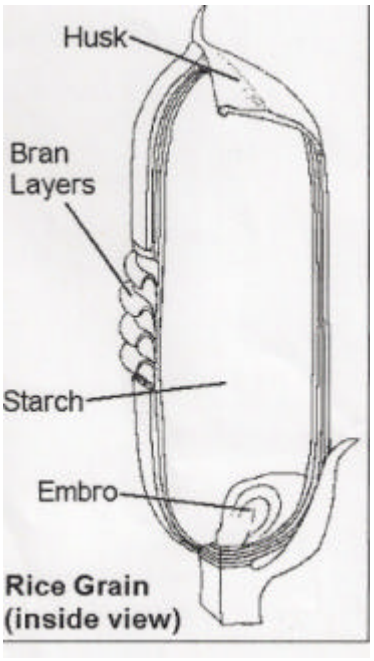


Figure 6. Inside view of rice grain. **Source:** www.therice.org

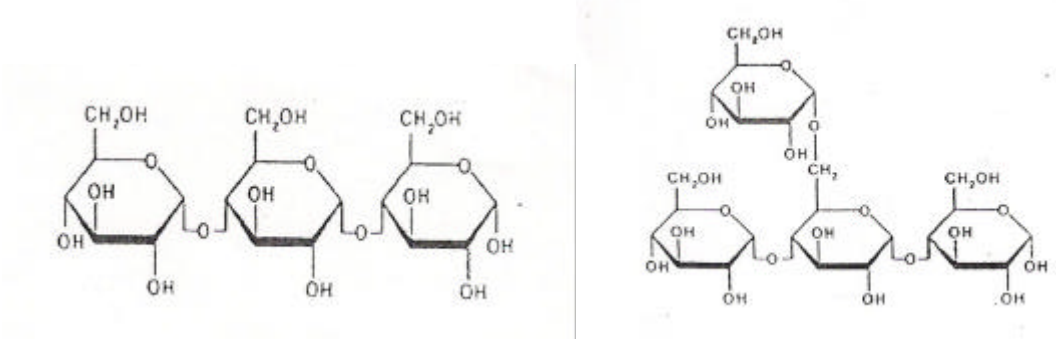
Rice composition

When concern about rice nutrition, it's rich in complex carbohydrates (starch and fibre), the main source energy, low in fat, contains some protein and plenty of B vitamins, thiamine and niacin, as summarised in Table 1. Rice contains low sodium content but contains useful of potassium and rice is gluten free. The main components of rice were carbohydrate, protein, fat and other inorganic substances.Starch in form of amylose and amylopectin structure as represented in Figure 7, was the largest constituent of rice and account for almost all of its carbohydrates. Amylose and amylopectin are built of glucose residues with different structures and consequently differ in the physichochemical change during steaming and saccharification by certain enzymes.

Table1. Rice Nutrition Facts

Nutrition facts serving:100g	White rice	Jasmine	Brown	Glutinous
Calories (Kcal)	361	355	362	355
Moisture, water (g)	10.2	11.9	11.2	11.7
Total fat (g)	0.8	0.7	2.4	0.6
Dietary fibre (g)	0.6	0.8	2.8	0
Calcium (mg)	8	5	12	7
Phosphorus (mg)	87	65	255	63
Potassium (mg)	111	113	326	0
Sodium (mg)	31	34	12	0
Vitamin B1 (mg)	0.07	0.12	0.26	0.08
Vitamin B2 (mg)	0.02	0.02	0.04	0.03
Niacin (g)	1.8	1.5	5.5	1.8
Protein (g)	6	6.1	7.4	6.3
Carbohydrate(g)	82.0	81.1	77.7	81.0

Source: Thai Food Composition Table (1999), Institute of Nutrition Research, Mahidol University, Thailand



Amylose Amylopectin

Figure 7. Structure of amylose and amylopectin of rice

Amylose consists of 200-400 α-glucose units linked in an unbranched helical chain by oxygen bridges at the 1,4-positions whereas amylopectin consists predominantly of a glucose units linked at their 1,4-positions by oxygen bridges. However, there is also 1, 6-linnked spaced 15 to 30 glucose units apart and so the amylopectin molecules look like brache tree and they may contain up to 6000 glucosr residues. Other minor constituents, such as sucrose, fructose and glucose were found

in trace amount (Momose, 1979).In saccharification; the amylases produced mainly by mould in start culture decomposed starch to form sugars, and then turned to alcohol by yeast.

Protein content of rice was affected by cultural conditions as well as rice variety, normally varies from 6-8% in brown rice. Milling reduced the protein content gradually; rice milled to 75% of the milling ratio usually contained 5-6% protein. In saccharification of satho, as well as mash fermentation of sake, acid protease produced by mould decomposed proteins to form peptides, which were further depolymised into amino acids by acid decarboxypeptidase. Some amino acids provided as growth supplement for yeast whereas some were assimilated by yeast and give satho or sake its full and heavy taste. On the other hand, an excess of these components was often negative quality of product with a rough taste, deepens the color, and accelerated deterioration of its quality.

Focus on lipid or fat content, brown rice contained about 2% crude fat (extractable with ethyl ether) and about 0.4% of fat-by-hydrolysis, composed of phospholipids, proteolipids, and other lipids. Because of crude fat existed mainly in the germ and the aleurone layer, milling reduced it greatly to below 0.1%. However, crude fat composed mainly of fatty acids and their glycerides; of which two unsaturated fatty acids; linoleic, oleic and one saturated fatty acid; palmitic acid were the main components. During the steaming process of rice, some glycerides decomposed to liberated fatty acids and free fatty acids. Consequently, steamed rice contained about half the amount

of crude fat before steaming (Ishikawa and Yoshizawa,1974).Concentration of fatty acids in medium was largly affected the formation of aroma ester; isoamyl acetate during fermentation by yeast (Yoshizawa,1976).Therefore, milling and steaming procedures of rice made a big contribution to the quality of sake, as well as satho.

Inorganic substances in brown rice consisted mainly of phosphorus, potassium or magnesium in the germ and aleurone layer which could be easily removed by milling.These substances played important role to promote the growth of mould, yeast and accelerated fermentation; therefore excess amount often negatively affecte the quality of product.Potassium was eluted from grains when rice was steeped in water,its content of the steamed rice was affected by the conditions of washing and steeping.During storage of milled rice, some inorganic substances; potassium moved from the surface of the kernel to the inner part. Consequently, white rice stored for a long time contained more potassium than rice milled before storage (Kubo, 1960; Yoshizawa *et, al.,* 1973a). Potassium is required in particular for the metabolism of carborhydrate and supports all enzymes reaction which proceeds with ATP. Whilst sodium activates enzymes; plays a considerable role in the transport of substances through the cell membrane, calcium slows down degeneration of yeast and supports break formation, if there is an efficiency of calcium, magnesium or manganese can be take its place.

Whereas the aroma of grapes was largely contributes the flavor of wine, rice has neither a characteristic flavor nor a profound effect on the flavor of satho, which was affected, instead, by the complicated procedure of production. Therefore, rice properties such as ease of milling, steaming and high yield of sugars when its starch was saccharified have been considered important in estimating its quality. As well as sake, rice which contained large amount of proteins, lipids and minerals, was considered undesirable for satho making. Many studies have been done to determine

the qualities of rice suitable for sake brewing. The several properties have been selected as being the most important in estimating the quality of white rice as an ingredient of sake brewing. These were; the weight of 1,000 grains, moisture content, protein and potassium, water absorbability in the steeping of rice in water, and the amount of sugars and amino acid formed in the saccharification of steamed rice by specific enzyme preparation (Yoshizawa *et al.*, 1973b, Hanamoto *et al.*, 1978, Iemura and Fujita, 1982a).

Generally, for sake and satho making, milled white rice was prepared by washing and steeping usually took 3-20 hours in water then the excess water was drained prior to be steamed for around 30-60 minutes. During washing and steeping, the grains absorb water to about 25-30% of their original weights which promoted penetration of heat into the grains easily when they were steamed. The velocity of water absorption differed with rice varieties and milling ratios (Akai, 1963; Saijo *et al.*, 1968). In washing and steeping step, sugars and some minerals; potassium ions, were eluted from the grains (Noshiro and Aoki, 1957, Kanoh, 1962, Yoshizawa and Ishikawa, 1974a), whereas calcium and iron were absorbed into the grains (Takase and Murakami, 1965).During steaming, starch in rice grains changed to the α-form, and protein was denatured, becoming resistant to enzyme action. To obtain completely steamed rice, the grains were usually steamed for 30-60 minutes. However, to removed fatty acids effectively from the grains; it took more than 45 minutes (Yoshizawa *et al*., 1979b).

For making satho, in contrast with sake, there were limit researches study about the required quality of rice. However, Juliano (1998) recommended that preferred properties of rice for rice wine making were low in amylose, protein and fat content. Indica glutinous rice, contained low amylose;0-2%, was mostly directed consumed and more used as an ingredient in sweet dishes, snack brewing industry and different rice products than japonica glutinous due to its tenderness. Studied in physicochemical of milled and cooked milled rice properties of Thai glutinous rice varieties, RD4, RD6 and Niaw San Pathong by Kongseree (1979) indicated that RD6 was accepted for rice wine making. Studying on effect of 3 rice varieties; RD6, RD10 and Niaw San Pathong on satho quality concluded that highest score (3.71 ± 0.81 from 5) of sensory test and highest alcohol produced (15.00%v/v) was obtained from RD6 variety after 9 days of fermentation.This report was confirmed by Wongpiyachon (1995), RD6, among 4 rice varieties; Khao Dawk Mali 105, TCC7, TCC12 and RD21, gave the highest in ethanol content after 21 days fermentation and yield the maximum volume which about 1.40 l/kg milled rice. Some of industrial rice varieties; RD6, Niaw San Pathong and Khao Dawk Mali 105 were illlustrated in Figure 8.

Niaw San Pathong

KDML105

Figure 8. Some of industrial rice varieties in Thailand

3.2 Start culture

Start cultures used for traditional rice fermentation or related products in Asia were known as Chinese yeast cake or *chu* in China, *murcha* or *marchaa* in Nepal and India, *loogpang* in Thailand, *ragi* in Indonesia, *bubod* in Phillipines (Hesseltine *et al.*, 1988b) and *nurok* or *nuruk* in Korea (Park *et al.*,1977).It was prepared by various traditional method using mixed culture from microorganism present in the natural environment, equipment, substrate through the repeated used of the inocula originating from a previous fermentation (Hesseltine 1965,1983., Verachtert and Dawoud,1990) or the previously prepared start mother culture. The association of yeast and lactic acid bacteria was known from a wide variety of traditional food and beverage fermentation (Soni *et al.*, 1985., Adegoke and Babalola, 1988., Sakai and Caldo, 1985).Haard *et al.*, (1999) reported the microorganism found in these start culture as summarised in Table 2 which consisted mainly of yeast, mucorales fungi and lactic acid bacteria belonging to be *Pediococcus* and *Streptococcus* as dominant (Hesseltine and Ray, 1988).

Table 2. Start culture for traditional rice fermentation in Asia

Country	Name	Ingredients	Shape	Microorganism
China	Chu	Wheat, barley, millet, rice (whole grain, grits or flour)	Granular or cake	*Rhizopus, Amylomyces*
Korea	Nuruk	Wheat, rice, barley (whole grains or flour)	Large cake	*Aspergillus, Rhizopus,* yeast
	Meju	Soybean (whole seed)	Large cake	*Aspergillus, Bacillus*
Japan	Koji	Wheat, rice (whole grains, grits or flour)	granular	*Aspergillus*
Indonesia	Ragi	Rice (flour)	Small cake	*Amylomyces, Endomycopsis*
Malaysia	Ragi	Rice (flour)	Small cake	*Amylomyces, Endomycopsis*
Phillippines	Bubod	Rice,glutinous rice (flour)	Small cake	*Mucor,Rhizopus, Saccharomyces*
India	Marchaa	Rice	Flat cake	*Hansenula anomala, Mucor fragilis, Rhizopus arrhizus*
Thailand	Loogpang	Rice bran	Powder/ Flat cake	*Amylomyces, Aspergillus*

Source; Haard *et al*., (1999)

The most prevalent mould species found were *Mucor* as high as $2x10^6$ ml, *Rhizopus* $2.5x10^6$ ml *and Amylomyces* at the level of $5x10^4$ ml from 56, 53 and 70 strains respectively obtained from traditional start culture in 7 regions (India, Indonesia, Thailand, Taiwan, China, Philippines and Nepal).These isolates were *Mucor circinelloides, Mucor indicus, Amylomyces rouxii* (formerly, *Chlarmydomucor oryzea*), *Rhizopus microsporus and Rhizopus oryzea* (Hesseltine and Ray 1988a).The mould flora in amylolytic oriental fermentation starters reported by Hesseltine (1988b) was represented in Table 3.

Table 3. Species of moulds in amylolytic oriental fermentation starters

Starter	Country	Species Found
Chinese yeast	Indochina	*A.rouxii*
	Indochina	*M.cambodja, M.rouxii*
	Formosa	Like *R.oligosporus*
	China	*R.chinensis,R.tritici*
	Singapore	*M.rouxii*
	Cambodia	*M.cambodja,M.rouxii*
	?*	*R.delemar*
	Formosa	*M.rouxii, M.javanicus, R.formosensis,R.oryzae, R.oligosporus var.glaber,R.chinensis var.rugosporus*
	Vietnam	*R.tonkinensis*
Yeast of Sikkim	India	*M.rouxii,M.praini*
Bakhar	India	*M.racemosus*
Chin-Yao	China	*R.hangchow,R.liquefaciens*
		R.formosaensis var.chlamydosporus
	China	*R.chungkuoensis*
		R.pseudochinensis
		R.candidus,R.salebrosus
		R.albus,R.humilis
	China	*R.chiuniang,R.shanghaiensis*
		R.niveus
Peka	Formosa	*R. Peka I, R.Peka II*
	Formosa	*R.salebrosus var.instriatus*
Chin-chu	Taiwan	*R.tienchiuliensis*
Ragi	Indonesia	*R.oryzae, Chlamydomucor oryzae*
	Indonesia	*M.javanicus*
	Indonesia	*M.dubius*
	Indonesia	*A.rouxii*
	Malaysia and Java	*C.javanicus*
	Indonesia	*R.chlamydosporus, C.oryzae*
	Indonesia	*M.rouxii, C.oryzae, A.rouxii*
	Indonesia	*M.rouxii,R.oryzae,C.oryzae*
	Malaysia and Java	*C.javanicus*
	Java	*R.javanicus R.semarangensis,*
	Java	*R.achlamydosporus, Chlarmydomucor*

* Country and origin unknown

Source: adapted from Hesseltine (1988b.)

All strains of *Rhizopus* sporulated on synthetic malt agar medium (SMA) whereas some strains sporulated heavily that the colonies appear gray on calcium carbonate plates. Table 4 represented the growth, acid production and starch hydrolysis of all strains of *Rhizopus* isolated from traditional start culture.

Table 4. *Rhizopus* strains from traditional start culture

Start culture	Source	Growth at 37 °C	Clearing of Calcium carbonate	Starch hydrolysis	Identification
murcha	India	+	-	+	*R.microsporus*
look-pang	Thailand	+	-	+	*R.microsporus*
ragi	Indonesia	+	-	+	*R.oryzae*
ragi	Indonesia	+	+	+	*R.oryzae*
Chinese yeast	Taiwan	+	+	+	*R.oryzae*
Kaoliang starter	Taiwan	+	+	+	*R.oryzae*
Kaoliang starter	Taiwan	+	No growth	+	*R.oryzae*
Kaoliang starter	Taiwan	+	-	+	*R.oryzae*
ragi	Indonesia	+	+	+	*R.oryzae*
ragi	Indonesia	+	-	+	*R.oryzae*
R.tonkiensis	China	+	+	+	*R.sp*
R.tonkiensis	China	+	-	+	*R.sp*
Chinese yeast	Taiwan	+	+	+	*R.sp*
Chinese yeast	Taiwan	+	+	+	*R.sp*
Chinese yeast	Taiwan	+	+	+	*R.sp*
Chinese yeast	Taiwan	+	-	+	*R.oryzae*
Chinese yeast	Taiwan	+	-	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.oryzae*
bubod	Philippines	+	+	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.oryzae*
bubod	Philippines	+	+	+	*R.sp*
bubod	Philippines	+	No growth	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.sp*
bubod	Philippines	+	-	+	*R.microsporus*
bubod	Philippines	+	-	+	*R.microsporus*
bubod	Philippines	+	-	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.oryzae*
bubod	Philippines	+	-	+	*R.oryzae*
R.stolonifera,ragi, F.Wolf	Indonesia	+	No growth	+	*R.sp*
R.chinensis, Chinese yeast	Taiwan	+	-	+	*R.microsporus*

Focus on yeast, *Saccharomycopsis fibuligera* (formerly *Endomycopsis fibuligera*), *Saccharomyces malanga, Saccharomyces cerevisiae* and *Pichia anomala* werereported to found by Hesseltine and Kurtzman (1990). Saccharifying yeast species belongs to saccharomycopsis predominate was suggested that they were involved in the amylolytic fermentation (Tamang and Sarkar, 1996). It was generally assumed that two types of yeast were involved in jaanr, the sweet-sour alcoholic drink from rice in Himalayan region of India; amylolytic yeasts, mostly *Saccharomycopsis*

degraded starch to glucose, while alcohol producing yeasts, *C.glabrata, P.anomala* and *S.bayanus* which lack in amylolytic activity grew rapidly on the resultant glucose to produce ethanol (Tamang and Sarkar, 1996; Thapa, 2002) On the other hand, *S.capsularis, S.fibuligera* and *P.burtonii* were enable to hydrolyzed starch but unable to produce alcohol. None of yeast strains presented in marchaa were found to capable of both amylolytic activity and alcohol producing (Tsuyoshi *et al.,* 2005).Table 5.represented the microflora of the yeast isolates in marcha samples from different sources, along with their activity profiles for amylolysis and ethanol production.

Table.5. Selection of yeast strains isolated from marchaa of Sikkim, India

Place of collection	Strain	*Group	Amylolytic activity	Ethanol production
Gangtok,market-1	*C.glabrata*	II	-	++
	P.anomala	III	-	+
	S.capsularis	IV	++	-
Gangtok,market-2	*S.capsularis*	IV	++	-
	S.bayanus	I	-	+++
	S.fibuligera	IV	++	-
Gangtok,market-3	*C.glabrata*	II	-	++
	P.anomala	III	-	+
	S.capsularis	IV	++	-
Sang village	*S.bayanus*	I	-	+++
	S.fibuligera	IV	++	-
Aho village	*C.glabrata*	II	-	++
	P.anomala	III	-	+
	S.capsularis	IV	++	-
Jhosing village	*C.glabrata*	II	-	++
	P.burtonii	IV	++	-
	P.anomala	III	-	+

Source: Tsuyoshi *et al*., (2005)

* based on physiological features using API test
Code in table: +++, strong; ++, good; +, moderate

Investigation of microorganism presented in loogpang samples, start culture for making satho in Thailand were summarized that *S.fibuligera*, starch degrading yeast which revealed the strong amylolytic activity was a common species found (Limtong *et al.,* 2002). Whereas *Saccharomyces spp.* has been found only in some samples (Chatisantien, 1977, Chaowsungkete, 1978 and Thanh *et al.*,1999). Among yeast isolates presented in these loogpang samples only some species play important roles in product formation (Lotong, 1994; Haard *et al*., 1999). Though there were some reports concerning with microorganism in loogpang and their possible role or involved in product formation but only few isolates of them were collected, isolated and maintained properly. Most samples of loogpang comprised mainly of *S.fibuligera*, this might be due to low quality of these samples or long term storage in undesirable conditions (Limtong *et al.,* 2002). Though most of *S.fibuligera* isolates could produce only low concentration of ethanol they might contribute to a certain extent of ethanol production or might have another role in contribution of desirable flavor of the products. This was agree with the previous studied by Rojas *et al.*,(2001) which

concluded that non-saccharomyces yeast might contribute to flavor or aroma formation in the alcoholic beverage.

Loogpang, a Thai term for dry form of "fermentation starter" for produce of traditional fermented products from starchy raw materials; *kao-mag* (alcoholic sweetened rice) and *lao* (rice wine) (Lotong, 1992). I t was a dry, round-to-flattened, creamy white to dusty white, solid ball-like starter which various in diameter and weight, ranging from 2-5cm in diameter and from 5-15 gram in weight as depicting in Figure 9 is known commonly as "Chinese yeast cake" to the Western people (Limtong *et al.*, 2002).

Figure 9. Collected loogpang samples from northeastern part of Tha iland

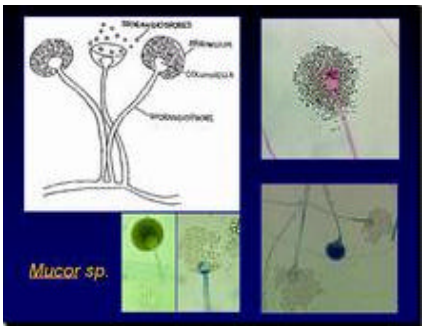
Generally, as summarized in Table 6, which modified by Lotong (1994) it was prepared from rice flour with various kind of spices; garlic, ginger, pepper, galangal, red dry chilli etc., and water was added to created the mixture prior to be kneaded into flat cake of varying size and shapes before the previous mother culture was sprinkling then placed individually on a platform suspended below the ceiling. These were left to ferment for 1-3 days prior to be sun-dried for 2-3 days and store in a dry place for more than a year (Thapa, 2002). Rice flour was used for fungal mycelium holding and carbon source whilst water was added to create the favor condition for growth of microorganism. The undesired microorganism was inhibited by the addition of various kinds of spices (unpublished data). One the other hand, some spices promoted the growth of desired microorganism by providing carbon and nitrogen source or vitamins (Thanonkeo, 2005).Wanapu (2002) reported that satho produced using loogpang with and without any spices added was not different in overall quality.

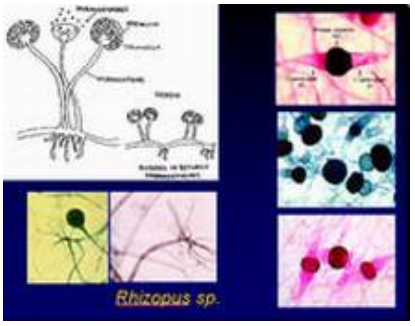
Table 6. Some of loogpang preparation fomula (modified by Lotong, 1994)

No.	Components	Quantity
1	Rice flour (g)	400
2	Garlic (g)	6
3	Ginger (g)	6
4	Galanga (g)	6
5	Liqourice(g)	6
6	Pepper (g)	6
7	Long peper (g)	2
8	Water	*

* Water was added just for ball or cake making, not measured and old seeding was added at 5 g per 1000g rice flour

Normally, it was apparently mixed cultures of moulds, yeast and bacteria with certain localities native herbs were added (Batra and Millner, 1974; Saono, 1982; Lotong, 1998 and Thanh *et al*., 1999). When focus on presented of mould in loogpang, the main 6 genus; *Rhizopus, Chlarmydomucor*, *Mucor*, *Aspergillus, Penicillium* and *Monilia* were found in collected loogpang and satho samples during 1959-1977 was reported by Chaowsungkete (1978). Puangveerakul (2000) recommened that high efficiency of mould for satho production were presented in 3 genus; *Aspergillus sp*., *Mucor sp*. and *Rhizopus sp*. Whereas previous studied by Sintara (2001) showed that mould presented in loogpang samples was found at the level of 10^4-10^5 cells/g which *A.rouxii* and *Rhizopus spp.* were always found in all samples of loogpang produced in Thailand, (Lotong, 1994 and Sintara, 2001). As well, studied by Karuwanna(2002) reported that acid producing and high activity amylase enzyme producing mould presented in loogpang samples were belonging to the class zygomecetes; *Rhizopus, Amylomyces* and *Mucor*. However, these genus performed incompleted saccharifying activity whilst *Aspergillus sp* which was belonging to the class deuteromycetes could complete saccharification. The recently report by Rattanapreedakul (2006) confirmed the previous studied which 3 isolates of mould; *A.rouxii*, *A.oryzea* and *R.oryzea* from 57 obtained isolates presented strong amylolytic activity in the range of 0.69-8.97 unit/ml as amylase activity.





Rhizopus sp. *Amylomyces sp.* *Mucor sp.*

Figure10. Major mould types in loogpang samples

Concerning of yeast consisted in loogpang samples; the main 2 groups; starch degrading or filamentous type (*Saccharomycopsis sp.)* and non-starch degrading yeast or Saccharomyces type (family *Saccharomycetaceae* and *Cryptococcaceae*) presented in the collected loogpang and satho samples during 1959-1977 was reported by Chaowsungkete (1978). Previous studying by Sintara (2001) and Rittipaeng (2004) suggested that yeast was frequently found at the level of 10^5-10^6 CFU/g which *S.fibuligera* and *P.anomala* were predominants. However, Mahintarathep (1991) indicated that the main yeast isolates found were *S.cerevisiae* and *S.fibuligera* which *S.cerevisiae* was more often found. Studying by Rittipaeng (2004a and 2004b), indicated that *C.pellicolosa, C. guilliermondi, C.tropicalis* and *P.anomala* were found in loogpang samples produced in northern and northeastern part of Thailand.While studying by Rattanapreedakul (2006) showed that 2 out 30 isolates of yeast revealed high in alcohol fermenting ability were identified as *S.cerevisiae* and *C.pelliculosa*. Limtong *et al.*, (2002) and Sujaya *et al.*, (2004) conclude that *S.fibuligera*, not only performed amylolytic and ethanol producing activity but also posses protease activity whilst lack of lipolytic and killer activities (Dedsungranond, 2003).

Not only mould and yeast presented in loogpang but lactic acid bacteria was also reported to found in the range of 10^4-10^7 CFU/g mostly was *Pediococcus pentasaceus* (Rittipaeng, 2004b). However, *Lactobacillus para paracasei, L.brevis, L.lactis lactis, P.acidilactic* were the dominants in loogpang produced in the north and northeastern part of Thailand (Rittipaeng, 2004b). The advantages of this presentation created the favor condition and inhibit growth of some contaminants by lowering the pH which beneficial for yeast fermentation.However, some of undesired microorganism; *Acetobacter sp., Gluconobacter sp., Lactobacillus sp., Bacillus sp., Fusarium sp.,* and *Pichia sp.* was usually presented in loogpang (Danvirutai, 2005).

Cause of it was produced under non-sterilised condition at home-scale with different distinguish formula for each producer, consequently, it was diversify and difficult to acquire due to its preparation process was known only a certain household with limited knowledge of some key microorganism, microbial community with their combined physiology, interactions and enzymatic activities were responsible for biochemical and nutritional changes that occur in the substrates of fermented food and beverages (Steinkraus,1982; Hesseltine and Wang,1967; Wood and Hodge,1985; Wood,1981). Figure 11 represented the alcohol producing ability of some loogpang samples collected from various sources of producers.

Figure11. Ability to produce alcohol by different source of loogpang samples

However, some research has attempted to investigate, most of the literature only referred to the present of microorganism and classified or identification following by biochemical changes with no indication of interaction. Only a few researches have studied systematically of these microbial interactions of the fermented rice beverage in within or between; yeast- yeast, yeast-bacteria, yeast-fungi in defined media (Gobbetti *et al.*,1994a, Kennes *et al.*, 1991b) but none of them have been studied in detail.

Different methods have been developed to identify yeast species by molecular biology technique using the information contained in the specific regions as depicting in Figure 12.Yeast isolates were identified base on the determination and comparison of the nucleotide sequences in specific regions which corresponding to the domains D1 and D2 located at the 5' end of the gene 26S (Kurtzman and Robnett, 1998) and the gene 18S made the technique very useful to assign an unknown yeast to a specific species when the percentage of homology of its sequences is over or similar to 99% (Kurztman and Robnett, 1998).

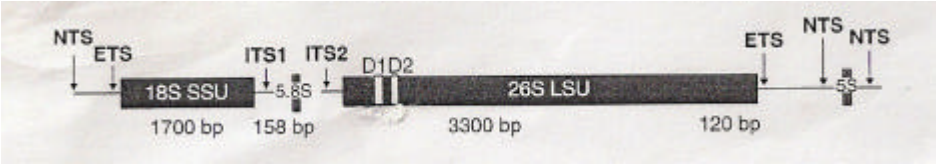


Figure 12. Structure of nuclear ribosomal DNA

Moreover, modern technologies of automatic sequencing technique together with the development of the DNA PCR, which enables direct sequencing of the

interested regions made this technique relatively quick to use, as outlined in Figure 13. The domain under consideration was amplified by PCR starting off with total DNA. Then PCR products were purified to eliminate the primers and the excess of deoxynucleotides. The four fluorescent dyes were used to identify each of the bases (A, G, C and T) in the automatic sequence systems. The DNA fragments marked in terms of their size and were simultaneously excited by a laser, producing an emission that was different for each of the dyes. The signals generated were later transformed by software into peaks of color, each of which corresponds to a nucleotide.

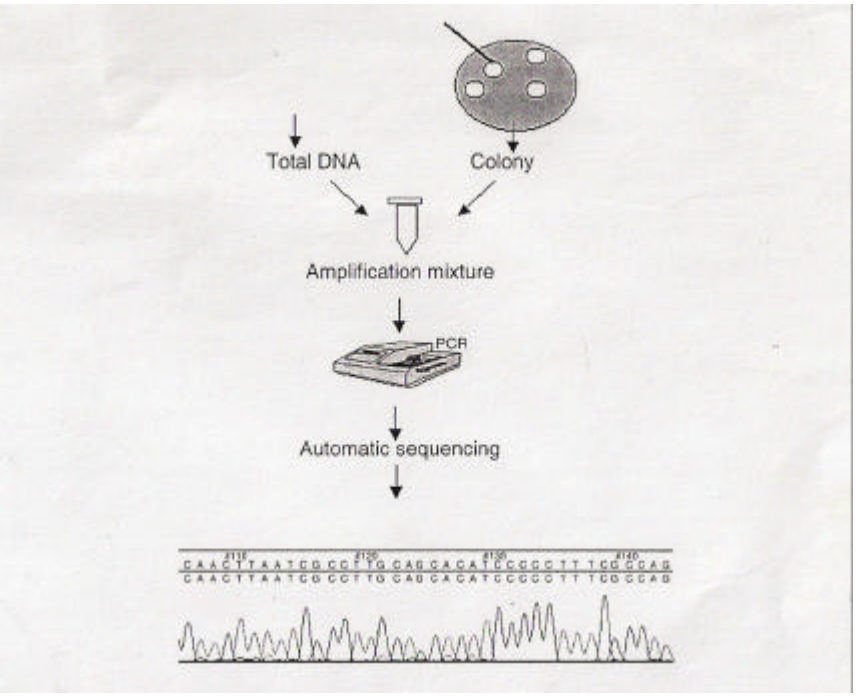


Figure13. Identification of yeast species based on PCR amplification and subsequent sequencing of ribosomal regions

3.3 Microbiology of Satho

As well as loogpang, various kinds of indigenous starter samples were isolated and identified, then the dominant mold, yeast, and bacterial species in commercial starter was establishing to attempts for reproduced a typical rice fermentation, lao-chao. The resulst of one experiment was illustrated in Figure 7. Preliminary fermentation studies were conducted using rice and commercial bubod and ragi starter. At the end of 24 hours at 30 °C glutinous rice was well digested and liquefied and good lao-chao was made. On the other experiment, both Chinese yeast and ragi were tested with glutinous and long-grain rice. Both types of inoculum on glutinous rice produced liquefaction and had a pleasant, sweet-sour aroma. Both types of starter gave the same fermentation product regardless of the type of rice used (Hesseltine, 1998).

Table 7. Combination of yeast, bacteria and fungi to make Lao-Chao

Name	Source	Liquefaction	Sporulation	Like LaoChao	Odor/Taste
S. fibuligera	Bubod	+	-	-	yeasty
S.fibuligera	Ragi	-	+	-	yeasty
S.fibuligera	Ragi	-	+	-	yeasty
R.chinensis	Chinese yeast	-	+	-	foul
R.oryzae	Ragi	-	+	-	none
A.rouxii	Bubod	+	-	-	Pleasant (sweet tasting, not sour)
A.rouxii	Ragi	-	-	-	Like grain
M.circinelloides	Bubod	-	+	-	Like grain
M.indicus	Bubod	-	+	-	Like grain
S.faecalis	Ragi				
P.pentasaceus	Ragi				

Source: Hesseltine (1999)

As satho fermentation was responsible by traditional mixed culture which mould, yeast and some bacteria were play important role (Tamang and Sarkar, 1996). In saccharification step, starch was depolymerised by mould amylase by generate mycelium over and penetrate into rice grains in wide-mouth tank with ¼ steamed rice was coarsely pack to create aerobic condition for mould activity with carried out in the first 4 days of fermentation yielding glucose, dextrin and other low molecular weight sugars,enabling to assimilate to alcohol and carbondioxide.Some amylolytic yeast; *Saccharomycopsis sp.* (Tamang and Sarkar,1996; Thapa, 2002) mostly *S.fibuligera* was common yeast found in loogpang samples in the range of 10^5-10^6 CFU/g(Sintara, 2001 and Jithpranee, 2004b) involved in starch degrading whilst alcohol producing yeast; *Saccharomyces spp*. has been presented only in some samples (Chatisantien,1997, Chaowsungkete,1978 and Thanh *et al.*, 1999) and grown rapidly on the resultant glucose to produce ethanol.When water was then added on day 4 of saccharification, mould which was strictly aerobic microorganism was stop growing whilst alcohol fermentation by alcohol producing yeast was carried out as represented in Figure 14. Though *Saccharomyces spp*. has been reported to be effective on alcoholic producing ability (Rattanapreedakul, 2006), at the beginning of fermentation, *S.fibuligera* was perform before disappear later cause of low alcohol tolerance (Danvirutai, 2005).However, as showed in Figure 15, fermentation was further carried out by alcohol producing yeast mostly; *S.cerevisiae* until completely finish.

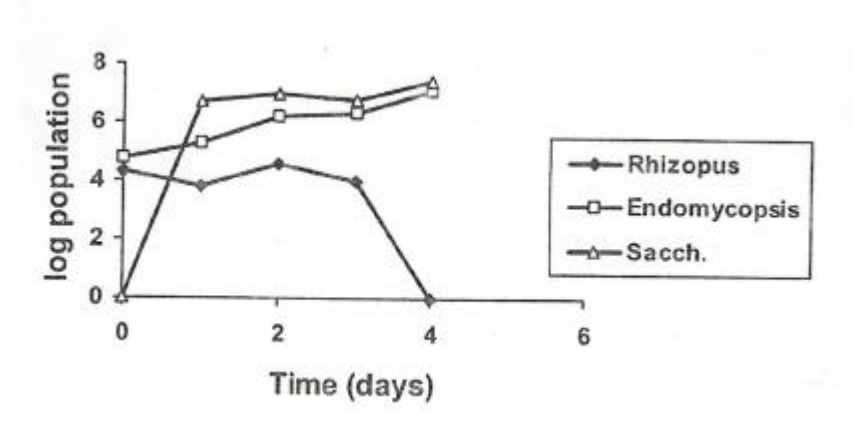
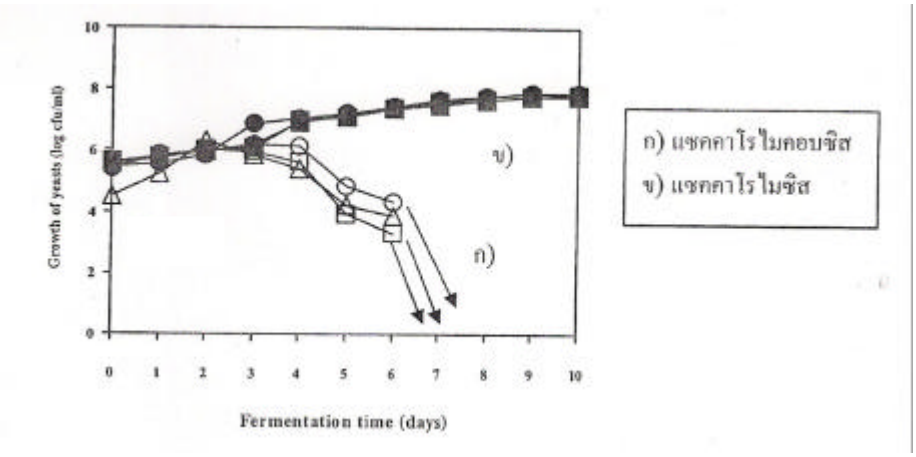


Figure 14. Growth of microorganism during satho fermentation



?) *Saccharomycopsis sp.* ? **)** *Saccharomyces sp***.**

Figure 15. Growth of *S.fibuligera* and *S.cerevisiae* from 3 sources during fermentation

3.4 Production process of satho

Conventional of satho fermentation using loogpang is simultaneously consisting primarily of saccharification and alcoholic steps which responsible by enzymes produced from mould and yeast, overview stage as illustrated in Figure 16.

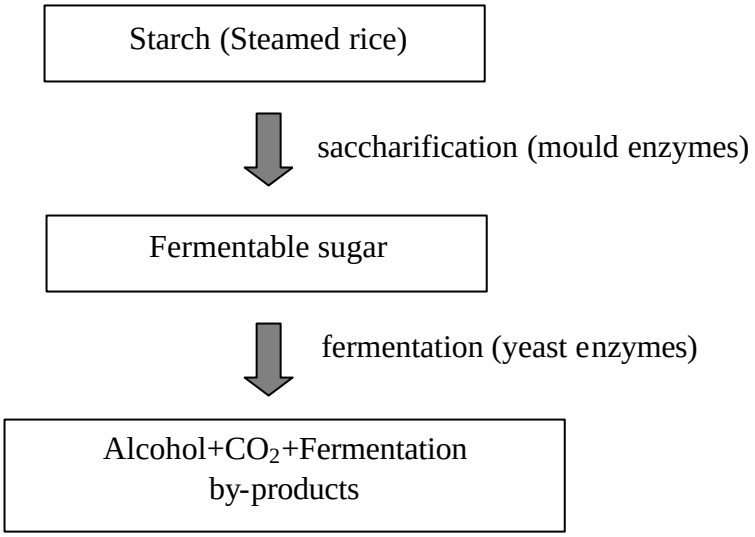


Figure 16. Main steps of satho fermentation

3.4.1 Saccharification

Saccharification or aerobic fermentation is the first step of satho production which promoted by amylolytic enzymes produced from mould and some yeast varieties in loogpang (Limtong *et al.*, 2002). As represented in Figure 17, starch content in rice has been liquefied firstly by α- and β-amylase to dextrin prior to be degraded by glucoamylase resulting in glucose as final product. Alpha-amylase (EC 3.2.1.1; α (1, 4)-glucan gluconohydrolase) from mould *A.niger, A.oryzea, R.oryzea* and starch degrading yesat; *S.fibuligera* was endoenzyme, random hydrolyze α- 1, 4 linkage in amylose and amylopectin constituents of starch structure to dextrins and oligosaccharides. The main source is obtained from *A.niger* and *A.oryzae* which frequentlt grown on semi-solid medium at temperature close to 30 °C and yielding, suitable enzyme levels in 24-48 hours (Beckhorn, 1967). As well, β-amylase (EC 3.2.1.2; α (1, 4)-glucan maltrohydrolase) was exoenzyme which hydrolyze α-1, 4 linkage for each two glucose units from non-reducing end. Therefore maltose was the product from hydrolysis. However, this enzyme could not degrade α- 1, 6 linkages in amylopectin. While exoenzyme; glucoamylase (EC 3.2.1.3; α (1, 4)-glucan glucohydrolase) was responsible for degradation of α-1, 4 and α-1, 6 linkages in limit dextrin to glucose as final product. This enzyme was mainly produced by *A.niger, A.oryzea, A.awamori*, and *R.niveus* (Arima,1964).In this step, not only glucose and other fermentable sugar but also lactic acid and other organic acids.Gluconic and citric were main acid produced by *A .niger* group, many species of *Penicillium, Gliocadium* and mycelial yeast, *S.fibuligera* (Ward, 1967). These acids would created optimum pH for yeast activity in the next step and protect growth of some contaminants (Karuwanna, 2002). Furthermore, lipase and protease activity produced from mould; *A.saitoi, A.niger, A.oryzea, Trametes sanguineara* and *Mucor pusillus* also occurred in this step (Keavy *et al.*, 1972). However, *Rhizopus spp.* was known as acid protease producing fungi (Wang and Hesseltine, 1970) whilst *A.oryzea* could produce all acids, neutral and alkaline types of protease. *Rhizopus arrhizus var.delema, Rhizopus sp.* and *Penicillium roqueforti* produced lipase whereas protease was also produced from *A. melleus, A.saitoi* and *Rhizopus sp.* These enzymes activities resulting from certain mould species in this step which provided a favour condition for yeast in the next step; alcoholic fermentation.

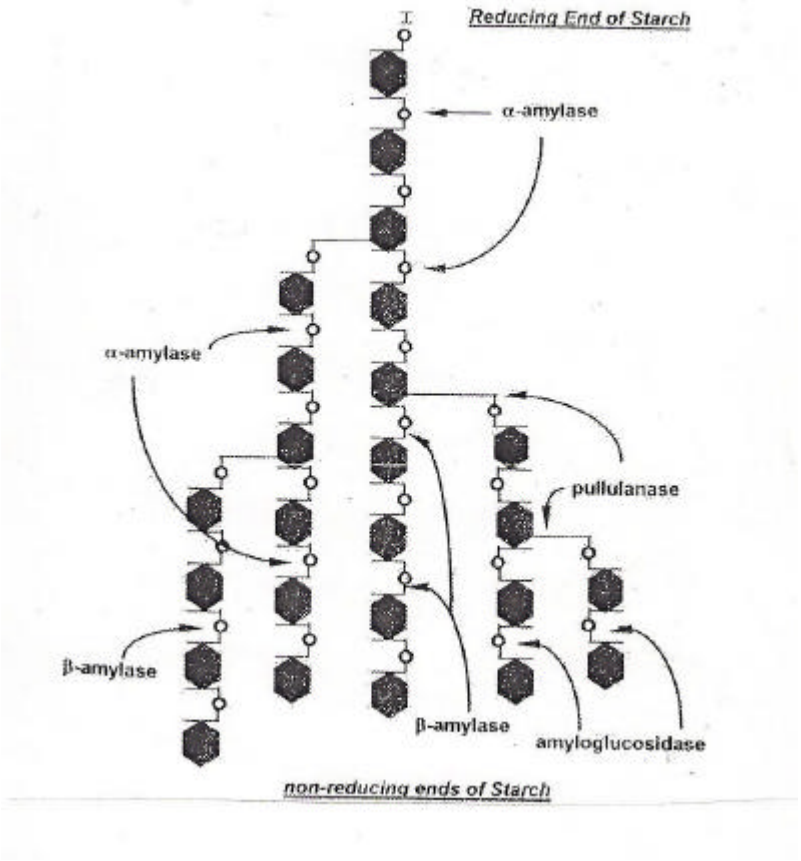


Figure17. Action pattern of starch-degrading enzymes.

Mould amylases are relatively stable and continue to act in alcoholic step throughout fermentation. In addition, protein in the rice grains is hydrolyzed by protease and carboxypeptidase to peotides and amino acids, some of which are assimilated successively by yeast. Eighteen different amino acids in sake have been identified; these compounds might interact with various other substances to give smoothness and balance to the taste of product. In brewing barley malt has normally been used in quantities as α- and β- amylase to ensure the hydrolysis of starch. In spirit production, bran cultures of *A.awamori* and submerged culture of *A.niger* have been used as source of saccharifying enzymes on sorghum (Reed, 1966).The other important of fungal enzymes are transglycosylation, β- glucanase, lactase and invertase. Transglycosylation is transferring enzyme which transfer of glycosyl group from a donor molecule such as a disaccharide to water acting as acceptor molecule. At high concentration of sugars enzymes normally acting as hydrolases may act as transglycosylases and produce oligosaccharides such as iso- maltose and iso-maltotriose. This enzyme activity is undesirable and so as to minimize in glucose production from starch which preparation from *Aspergillus* and *Rhizopus* species (Kooi, Harjes and Gilkison, 1962; Hurst and Turner, 1962). Fungal β- glucanase is the enzyme which improve the filtration characteristic of beer (Enkelund, 1972) with the hydrolysis of the β- glucas linked by β- 1, 4 and β- 1, 3 glycosidic links which not susceptible to hydrolysis by amylase and amyloglucisidae. Fungal lactase produce from *A.niger* and *A.oryzae* hydrolysed the β- 1, 4 gkycosidic link of the disaccharide lactose yielding glucose and galactose giving a sweeter and more soluble product (Pomeranz, Robinson and Shellenberger, 1963). Invertase may be obtained from

mould; *A.niger, A.oryzae* and other fungi with hydrolyse α-glucoside and β-glucoside of sucrose to glucose and fructose which is sweeter and more soluble than sucrose. These systematic names and Enzyme Commission number of all mention carbohydrases are concluded in Table 8.

Table.8 Systematic nomenclature and enzyme Commission numbers for industrial carbohydrases

Enzyme	Enzyme Commission number	Systematic nomenclature
α-Amylase	EC.3.2.1.1	α-1,4- Glucan 4-glucanohydrolas
β-Amylase	EC.3.2.1.2	α- Glucan maltohydrolase
Amyloglucosidase	EC.3.2.1.3	α-Glucan glucohydrolase
Invertase (β-fructofuranosidase)	EC.3.2.1.26	β-D-Fructopuranoside fructohydrolase
Lactase (β- galactosidase)	EC.3.2.1.23	β-D-Galactoside galactohydrolase
β- Glucanase	EC.3.2.1.6	β-1,3(4) Glucan glucanohydrolase
Transglycosylases	EC.2.4.1 enzymes, hexosyltransferases - but hydrolytic carbohydrases may show this activity.	

However, starch saccharification using mould isolates or mixed- culture in form of loogpang, sometime off flavour like mouldy flavour was presence or even some species of mould could produce alflatoxin (Sripathomsawat, 1981 and Wanapu, 2004). To overcome this problem, amylase enzymes from another source such as rice malt by mashing process as well as in beer brewing might be applied.

3.4.2 Alcoholic fermentation

When some water was added after saccharification, which created anaerobic condition for yeast fermentation which *Saccharomyces sp*. and *Saccharomycopsis sp.* played an important role to convert fermentable sugar to ethanol, carbondioxide and various kinds of fermentation by products such as succinic acid, malic acid and flavour compounds. At the beginning of this step *S. fibuligera* was predominant for a short time before responsible by *S.cerevisiae* until finish fermentation. Fermentation profile of satho and ethanol fermentation by yeast through glycolysis pathway was represented in Figure 18 and 19 respectively.

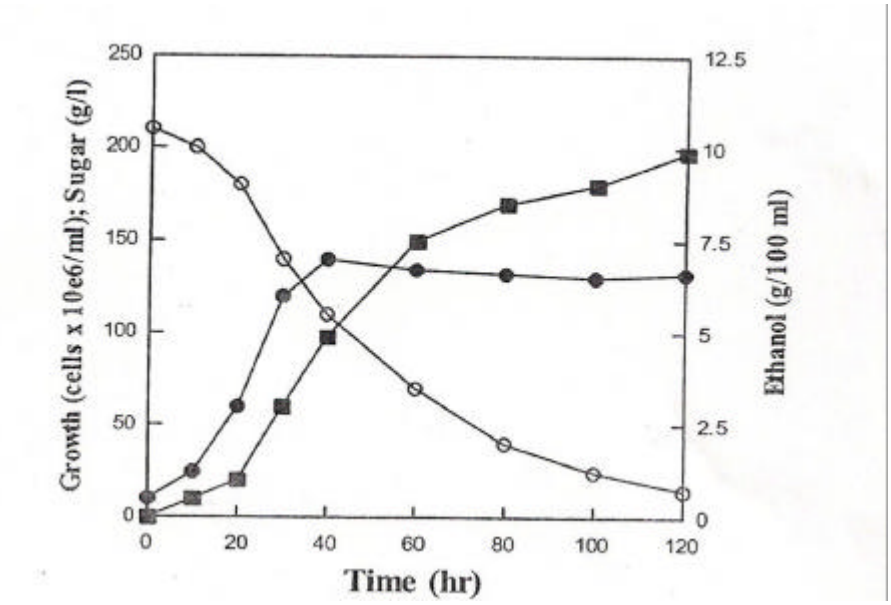


Figure 18. Ethanol production by yeast in satho fermentation

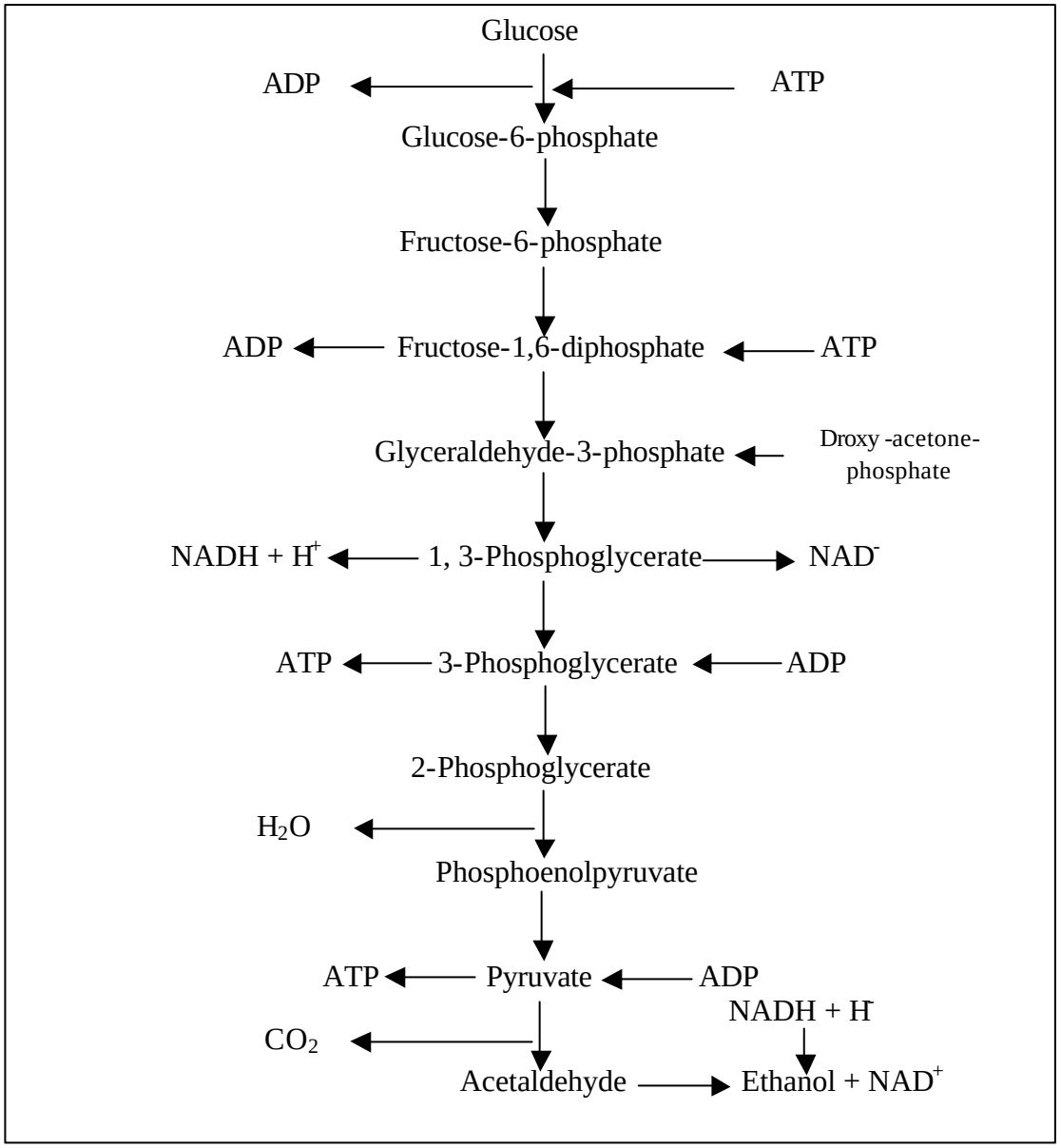


Figure 19. Ethanol fermentation by yeast through glycolysis pathway.

Furthermore, some of peptides which obtained from acid protease produced by mould in saccharification step and further decomposed to amino acids by acid decarboxypeptidase were assimilated by yeast and gave its full and heavy taste. For example; leucine, valine and isoluecine were assimilated by yeast to form higher alcohol; isoamyl alcohol, isobutanol, and 2-methyl butanol, respectively. In addition, isoamylalcohol was further reacted with acetyl CoA to form isoamylacetate, one of the main flavour components contributing to the aroma of sake (Karuwanna, 2000). On the other hand, excess of amino acids often gave the product a rough taste, deepens color, and accelerated deterioration of its quality. Figure 20 show a general overview of the correlation between yeast metabolism and the production of some important flavour components.

Figure 20. Basic fermentative metabolism and production of flavour-active metabolites by *S.cerevisiae.*

Carbonyl compounds; of more than 200 carbonyl compounds that have been detected in beer, acetaldehyde and the vicinal diketone diacetyl were the most important ones.Acetaldehyde, with its typical apple and grassy flavour, a by-product of glycolysis was an important metabolic intermediate (Boulton and Quain, 2001, Fliktweert *et al*., 1996, Roy and Dawes, 1987, Zubay,1993) resulted from pyruvate decarboxylase activity.It was mostly formed during the active growth phase of yeast and believed that the majority of acetyl CoA formation under fermentative conditions occurs via acetaldehyde, which can be converted into ethanol by alcohol dehydrogenase, but also to acetate by aldehyde dehydrogenase. Acetate can then be converted into acetyl CoA by acetate thiokinase.Diacetyl (2, 3- butanedione); was generated as represented in Figure 21, which an overflow product of valine biosynthesis from pyruvate with intermediate,α-acetolactate formed by α-acetolactate synthase Ilv2p. When metabolic flux through the subsequent part of the pathway was slow, α-acetolactate concentration increase and leak out of yeast cell, where it was

chemically converted into diacetyl (Debourg, 2000, Goossens *et al*., 1987) .It has a low flavour threshold with a butter-like aroma which highly undesired flavour in beer, however, could be part of the positive bouquet of some wine (Boulton and Quain, 2001, Hammond, 1993, Meilgaard, 1975a).

Figure 21. Diacetyl production, adapted from Hammond (1995)

Diacetyl is the most important immature beer aroma, above the threshold value it gives beer unclean, sweetish to revolting taste, and responsible for the aroma butter in high concentration.The breakdown of these vicinal diketones occurs parallel to other maturation reactions during beer conditioning process and it therefore nowsaday regarded as the essential criterion for the state of matruration of beer (Kunze, 2004). The diacetyl and pentanedione formdione by yeast cells which is favoured by several factors: temperature (increase greatly with increasing temperature), yeast concentration in maturing step, etc.

Diacetyl → Acetoin → Butanedione

Butanedione has a very high t hreshold and the amount formed is not perceived in beer.Acetaldehyde occurs as a normal intermediate product in alcoholic fermentation which responsible for the ‘ green’ young beer flavour ans also referred to as clear or musty aroma. The formation of acetaldehyde is increased by rapid, high fermentation temperature and infected wort in case of beer.

Fusel oils or higher alcohols; the secondary yeast metabolites produced in the highest amount with relatively high threshold value (10-600 mg/l). It was impart alcohol-like, bitter, chemical or flowery flavours which contribute to the overall aroma of the fermented beverages, mainly because of the synergistic matrix effect.The formation of higher alcohol was closely link to both amino acid catabolism

and anabolism.In catabolic pathway, amino acids were taken up from medium and formed α-keto after transamination and decarboxylation which could be further reduced to generate fusel oils.This was unique for amino acid catabolism through TCA cycle of *S.cerevisiae* which amino acid could only serve as a nitrogen source, while in other eukaryotes they function both as nitrogen and mostly carbon source (Dickinson,2003, Ehrlich,1907, Hammond,1993). Furthermore,the other following factors would create formation of higher alcohols;high fermentation temperature (Pinal *et al.*, 1997), presented of unsaturated fatty acids and sterols in medium (Taylor *et al.*, 1979) and high concentration of fermentable sugars as found in high gravity brewing (Engan,1972b, Szlavko, 1974, Youngish and Stewart,1999). In some cases, the availability or cellular synthesis rate of each amino acid affected the synthesis of the corresponding fusel alcohols (Hernandez-Orte *et al.*, 2002, Hirata *et al.*, 1992).However, the advantage of optimum concentration of higher alcohols was not only synergistic matrix effect but it was also one of the two substrates for volatile ester formation. For sake, the higher alcohols contains fairly large amount of n-propanol, i-butanol and i-amyl alcohol derived from glucose and partly from amino acids and sake yeast depending on amount and kinds of nitrogen source in mash, fermentation temperature and yeast strains used. Almost of higher alcohol is formed during the primary fermentation and cannot be removed, therefore must be adjusted by control the fermentation condition with increasing the yeast pitching rate, fermentation and pitching at low temperature, avoid movement of young beer by stirring or pumping which increase oxygen concentration and sufficient amino acids content in wort. Concentration of higher alcohols above 100 mg/l damages the flavour and acceptability of beer. The content in pale beer is 60-90 mg/l (Kunze, 2004).

Esters; the most important group of flavour compounds was recognised as one of the main components responsible for aroma and smell of fruits and flowers (Aharoni *et al*., 2000, Dudarev *et al.*, 1998, Harada *et al*., 1985, Homatidou, *et al.*, 1992, Wyllie and Leach,1990, Yahyaouii *et al*., 2002).Volatile esters were trace compounds but extremely important for the flavour profile in fermented beverages which responsible for the desirable fruity, candy and perfume-like aromas of the fermented beverages(Dufour *et al.*, 2002, Meilgaard,1975a).The most important aroma-active esters could be divided into 2 groups (Verstreppen, Derdelinckx and delvaux,2003) ;acetate esters and ethyl esters or medium chain fatty acids esters(MCFA esters). The most important flavour-active acetate esters in fermented beverages were ethyl acetate (solvent-like aroma), isoamyl acetate (fruity, banana aroma) and phenyl ethyl acetate (flowery, rose, honey). While the latter group had a characteristic sour apple flavour, these were included ethyl caproate (ethyl hexanoate; C_6 fatty acid), ethyl caprylate (ethyl octanoate; C_8 fatty acid) and ethyl caprate (ethyl decanoate; C_{10} fatty acid). The threshold values of important active ester aroma were represented in Table 9.

Table 9. Average concentrations and threshold values of some important aroma-active esters in larger beer

Component	Conc.range (mg/l)	Average conc. (mg/l)	Threshold value (mg/l)	Flavour description
Ethyl acetate	8-32	18.4	21-30	Fruity, solvent-like
Isoamyl acetate	0.3-3.8	1.72	0.6-1.2	Banana, pear
Ethyl caproate	0.05-0.3	0.14	0.17-0.21	Apple, aniseed
Ethyl caprylate	0.04-0.53	0.17	0.3-0.9	Apple
Phenyl ethyl aetate	0.10-0.73	0.54	3.8	Rose,honey, sweet

Generally, the most refined sake contains higher concentration of esters; ethyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate, isoamyl acetate,low concentration of nitrogenous compounds, organic acids and sugars, all of which contribute to provide the special flavour and taste (Sujaya *et al.*, 2004). Whereas beer contains about 60 different esters, however, only about 6 are of much important for the flavour properties; ethyl acetate, isoamyl acetate, isobutyl acetate, β-phenylacetate, ethyl caproate and ethyl caprylate. Ester formation is closely related to the oxygen supply to the yeast which affects on fat synthesis as well as respiration and fermentation. Ester formation is, therefore, a very complicated process which is not easy to influence. However, in overview, ester production is increasing by increase above 13% wort concentration, increase aeration, high fermentation temperature and increase movement during fermentation and maturation.Basically, two factors are important for the rate of ester formation; the concentration of the two substrates, acyl CoA and fusel alcohol, and the total activity of the enzymes involved in the formation and break down of the respective ester (Verstrepen *et al.*, 2003) as depicting in Figure 22.Some of the most important aroma active esters in beer are illustrated in Figure 23.

Figure 22. Biochemical background of yeast ester production

Figure 23. The most important aroma active esters in beer

Sulphur-containing compounds and organic acids; A number of sulphur-containing compounds and organic acids contributed both directly and indirectly to the flavour profile of fermented beverages.Hydrogen sulphide (H_2S) and sulphur dioxide (SO_2) were the important of sulphur compounds originated from metabolism of sulphur-containing amino acids of yeast (Boulton and Quain, 2001, Dufour, 1991 and Vermenulen and Collin, 2002).In addition,, dimethyl sulphide (DMS) might be arise from the spontaneous thermal decomposition of S-methyl methionine during malting and mashing, in case of beer, and the reduction of dimethyl sulphoxide (DMSO) in the fermenting yeast cells,DMS possesses an unpleasant 'cooked vegetable' flavour which could contribute to the fullness, but it was totally undesirable in high concentrations (Annes,1981, Annes and Bamforth,1982, Meilgaard, 1975a).The group of organic acids which involved in flavour profile of fermented beverages could be divided into two categories: medium chain fatty acids (MCFAs;C_6-C_{12}), which were toxic for yeast cells and other organic acids which shorter or longer carbon skeletons, this group comprised of the organic acids derived from pyruvate or TCA cycle intermediates as well as long-chain fatty acids present in yeast lipids (Boulton and Quain, 2001, Hammond,1993).The latter, included malate, citrate,2-oxoglutarate, succinate and acetate, which all contribute to product sourness and were partially responsible for the pH drop during fermentation (Coote and Kirsop, 1974, Klopper *et al.*, 1986). Some acids were precursors for the important flavour compounds; α- acetolactate as precursor of diacetyl and MCFAs, which were co-substrates in the formation of some ethyl esters. The formation of organic acids originating from carbohydrate metabolism depended mainly on rapid and vigorous fermentation whilst free MCFAs occurred mainly when cells shift from aerobic to anaerobic conditions.This was in agreement with the observation that the MCFAs found in MCFA esters originated mostly from long-chain fatty acid anabolism (Taylor and Krisop,1977). Table 10, represented the evaluation of the aroma compounds in beer.

Table 10. Evaluation of the aroma compounds in beer (according to Miedaner)

Aroma compounds	Conc.range (mg/l)	Flavour threshold(mg/l)	Aroma impression
Higher alcohols;			
2-methyl propanol	5-20	10-(200)	alcohol
2-methyl butanol	10-20	10-(65)	alcohol,solvent
3-methyl butanol	35-70	30-(70)	alcohol,banana
2-phenylethanol	10-20	28-(125)	roses
	30-50		(in wheat beer)
Esters:			
ethyl acetate	5-30	25-30	fruity, boiled sweet
isobutyl acetate	0.1	0.4-(1.6)	fruity, banana
isoamyl acetate	0.5-2.5	1-1.6	fruity, banana
ethyl butyrate	0.3	0.4	papaya, apple
ethyl hexanoate	0.1-0.3	0.12-0.23	apple, fruity
ethyl dodecanoate	0.02	3.5	soapy, estery
ethyl lactate	0.1-0.5	250	fruity, strawberry
	0.4-0.8		(boil.acidification)
Organic acids:			
butyric acid	0.2-0.6	1.2-2.2	cheesy, rancid
isovaleric acid	0.5-1.2	1.5-1.6	cheesy, old hops
octanoic acid	3-10	10-13	ioly
decanoic acid	0.8	10	rancid
dodecanoic acid	0.1-0.5	6	soapy
Vicinal diketones:			
diacetyl	0.1	0.1-0.15	sweet, unpleasant
acetoin	3	8-20	fruity
Sulpur compounds:			
dimethyl sulphide	0.03-0.12	0.10-0.12	vegetable, musty

In addition, Glycerol and fatty acids presented in this step which resulted from steaming process of rice and lipase activity during saccharification by mould were largely affected on the formation of aroma esters; isoamyl acetate by yeast during fermentation (Yoshizawa, 1976).Some of unsaturated fatty acids, linoleic or oleic incorporated into yeast cells and located mainly at the cell membrane (Ishikawa and Yoshizawa, 1979) suppressed isoamyl acetate formation by alcohol-acetyltransferase in yeast during fermentation and in ageing step (Thurston, Quain and Tubb 1982) of sake processing. These data was illustrated in Table 11 (Ishikawa and Yoshizawa, 1979, 1984; Yoshioka and Hashimoto, 1983).As alcoholic fermentation was anaerobic; yeast usually preferred incorporated fatty acids into the cells instead of synthesizing them. Consequently, the composition of fatty acids in the yeast cells and the ratio of the amount of saturated fatty acids to that of unsaturated ones reflect the composition.

Table 11. Ester formation by sake yeast in the fatty-acids-added medium [a,b]

Added fatty acid (0.5mM)	Ethanol (%)	Isoamyl alcohol (ppm)	Ethyl acetate (ppm)	Isoamyl acetate (ppm)
Blank	4.0	141	9.8	1.7
Stearic acid	4.5	145	11.6	2.1
Oleic acid	3.9	125	6.4	0.8

[a]Sake yeast Kyokai 7 was inoculated (inoculum size, 10^6 cells/ml) in the me dium containing glucose, hydrolyzes casein amino acids, and albumin as an emulsifier , incubated at 25° C, 3 days
[b] Data from Yoshizawa (1976)

Therefore, rice preparation (milling and steaming), fermentation temperature, yeast strain, pH, initial sugar content and aeration (Rankine and Bridson, 1971) were critical factors which play important role of contribution to the quality of product. This was confirmed that the fermentation was the heart of the production process (Boulton *et al*., 1996, Kruger, 1998a, Kruger, 1998b, Lambrechts and Pretorius, 2000, Meilgaard, 1975a, Meilgaard, 1975b, Meilgaard, 1991).

3.4.3 Downsteam processing

These steps were including all activities after fermentation which consists primarily of separation, filtration, maturation, pasteurisation, blending and bottling.Fermented mash was separated, conventional by squeezing in cheese cloth, the turbid satho was then clarified and lees were separated to avoid deterioration from yeast cells autolysis resulting in solid contains mainly of starch, protein and yeast cells. However filter press following by membrane filtration would be applied for fine satho in mass scale production. For satho maturation; neither aging condition nor aging time was studied and reported. For sake, it took 3-8 months aging at temperature 13-15 °C and 0-5 °C for common sake and fine ones, respectively.In order to kill some bacterial contaminants to ensure product quality, pasteurisation was carried out at 60-65°C for a short time prior to be bottled. Fine satho from each batch was blended to provide uniform quality prior to be pasteurised and bottled.

Traditional satho production by small producers was carried out as following; milled rice was washed before has been soaked overnight, excess water was drained. Soaked rice was cooked in rice steam cooker, then cooked rice was removed the slime by washing with tap water. Moist cooked rice was mixed with ground lookpang from the old seeding, left it ferment at the room temperature for around 3-5 days before water was then added for alcoholic fermentation which take for 7-21 days depending alcohol required. After finished alcoholic fermentation, solid and fermented broth has been separated by squeezing with cheese cloth. Normally, it was consumed directly after solid separation as fresh beverage. Overall of satho production was presented in Figure 24.

(Milled sticky rice)

(Steam cooking)

⇓

(*Loogpang*)

(Steamed rice)

⇓

(Hand mixing)

⇓

(Transfer into fermentation jar)

Figure 24. Overall of traditional satho production process

(Fermentation)

(Siphon)

⇓

(Pasteurization)

⇓

(Finished product)

Figure 24. Overall of traditional satho production process (continue).

Sake, as well as *satho,* a traditional alcoholic beverage in Japan produced using the certain isolates of mould and sake yeast by fed-batch fermentation. Sugar released from steamed rice and koji were fermented gradually until the alcohol content reached nearly 20 % v/v from about 40% sugars needed (Yoshizawa and Ishikawa, 2004). To reach around 20% v/v alcohol, Koji , which was comparable to malt used in beer brewing, was a culture of mould, *Aspergillus oryzea* grown on and within steamed rice grains, which accumulated various kinds of more than 50 enzymes involved in sake brewing was applied (Yoshizawa and Ishikawa, 2004) . The most important were α-amylase, glucoamylase and acid protease. These enzymes were relatively stable and function throughout fermentation.Saccharification and alcoholic fermentation were occurring simultaneously. Alcoholic fermentation was responsible by inoculated pure yeast culture. Seed mould, seed mash, steamed rice,

koji and water were prepared and divided successively into 3 batches over 4 days. The fermentation took 15-25 days after the addition of the final batch. Lactic acid 0.5% solution was added at the first addition to avoid bacterial contamination and to promote the growth of the inoculated yeast. After alcoholic fermentation, solid was withdrawn and fermented broth was stabilised for one week at 4 °C before lee was separated and aged. Matured sake would be smooth taste and a golden pale yellow colour was occur before blend, alcohol was adjusted (14-16%), treated with activated charcoal (if necessary) followed by filtration, pasteurisation and bottling (Yoshizawa and Ishikawa, 2004)

As a common characteristic of fermented drinks such as beer, wine and derivatives consisted of a complexity and diversity of flavour compounds which many more aromas have not yet been discovered or described (Ebeler, 2001, Lambrect and Pretorius, 2000, Meilgaard, 1975a, Meilgaard, 1975b, Meilgaard, 1991, Pisarnitskii, 2001). The reason for this complexity lied in the production process of fermented drinks; multiple ingredients were used in a complex process that induced a multitude of chemicals and biochemicals conversions (Kruger, 1998a, 1998b). Moreover, the production of fermented beverages was a long process which took several weeks or months until it was consumed leading to the chemical components, including flavour substances, were slowly broken down, converted or formed.This study, therefore mainly focus on the flavour of fresh products obtained from various conditions of start culture and substrates used which would be basic overview of the quality satho production process.

IV. MATERIALS AND METHODS

4.1. Equipments and other materials

- Steam rice cooker
- Autoclave
- Hot air oven
- Desssicator
- KRUESS HRN-16 Hand refractometer No.256
- Thoma cell count chamber, dept 0.100 mm, area 0.0025 mm^2
- Microscope,standard 25, Zeiss,Jena
- 12 mm diameter Cork borrer
- Density Meter DMA 35N, Anton Paar GmbH
- PH meter WTW pH 525
- Hot plate with stirrer
- MEBAK-miller; Universal-Buehler (for coarse milling with 1.00 mm size)
- MEBAK-miller, Buehler-Miag (for fine milling with 0.2 mm size)
- BENDER and HOBEIM mashing apparatus
- Tecator ß- Glucan 5700 Analyzer, Tecator, Sweden
- SCABA 5610 Automatic Beer Analyzer
- Hellige Neo-Komparator for colour evaluation
- Kjeldahl digestion and distillation apparatus
- Pilot brewery
- HPLC
- Minishaker for 1.5 and 2.0 ml safe lock tubes, IKA MS-1
- Spectophotometer, 3100 pro, Ultraspec, Cambridge, UK
- Insta Gene Matrix, Bio-Rad, Hercules, CA, USA
- Thermocycler PTC-100, MJ research, Biozym, Oldendorf

- Horizontal Electrophoresis-Einheit HE33, Hoefer
- UV-Transilluminator Reprostar, CAMAG, Berlin
- Membrane filter Minisart 0.45µm, Sartorius, Goettingen
- Laboratory balance
- Laboratory glassware

4.2. Microorganism

- Reference strains of yeast; Y-SUT and mould M-SUT was kindly supported from the School of Biotechnology, Suranaree University of Technology, Nakhon ratchasima, Thailand

4.3 Culture media

- Dichloran Rose Bengal Chloramphenicol (DRBC) medium
- Yeast extract-malt extract agar (YM agar)
- Basal medium for lipolytic and proteolytic activity test (Lodder and Kreger van Rij, 1952)
- Starch agar medium
- Calcium carbonate medium

4.4. Chemicals

- Lugol's iodine solution
- Standard 0.01 N Sodiumhydroxide
- Standard chemicals for HPLC
- Chemicals for protein and soluble nitrogen determination by Kjeldahl method
- 20% Lactic acid solution
- Primer, Operon Biotechnologies, Cologne
- Ultrapure dNTP set, Amersham Biosciences, Freiburg
- Taq-Polymerase: Hot star Taq, Qiagen, Hilden
- PCR-buffer (10x), $MgCl_2$, Qiagen, Hilden
- Agarose Seakem LE 50001, FMC Bioproducts, Rockland, USA
- TAE-buffer; 0.04 M Tris-acetate, 1mM EDTA, 0.5 µg EtBr/ml,pH 8.0
- 0.05% Bromphenolblue, 250 mM EDTA, 55% Saccharose
- 100 bp-Leiter, Amersham Biosciences, Freiburg

4.5. Milled rice samples

Thai glutinous milled rice, RD6 variety from Pathum Thani Rice Research Centre; Pathum Thani, Thailand, was used as substrate for conventional fermentation in form of steamed rice and saccharified rice liquid for semi-conventional fermentation.

4.6. Rice malt samples

Thai rice malt, 6 varieties, namely SUT-1(San Pathong), SUT-2 (Pathum 60), SUT-3 (Glutinous black rice), SUT- 4 (Jasmine rice), SUT-5 (RD 6) and SUT-6 (normal black rice) were prepared as represented in Figure 25 by the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (SUT), Nakhonratchasima, 30000, Thailand prior to be shipped to laboratory of Lehrstuhl fuer Technologie der Brauerei II in Technische Universitaet Muenchen-Weihenstephan, Germany.

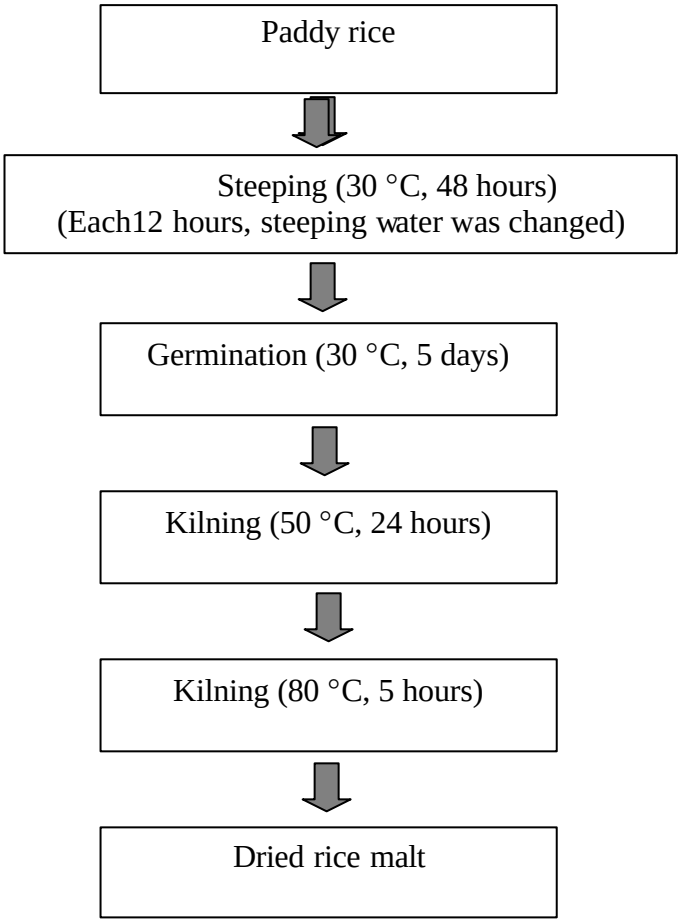


Figure 25. Preparation of rice malt samples

The quality of rice malt samples were analysed as diagram in Figure 26, in terms of extract content, protein, soluble N and Hartong 45 according to standard method for malt analysis, colour was determined using Hellige Neo-Komparator for color evaluation, whlist β-glucan was analysed using Tecator ß-Glucan 5700 Analyzer.The good quality of rice malt was considered to select base on crucial parameters; extract content and soluble nitrogen.Rice wort was prepared at the pilot brewery of the Lehrstuhl fuer Technologie der Brauerei II, Technische Universitaet Muenchen-Weihenstepahn (TUM -Weihenstephan),Germany, using modified standard mashing program as illustrated in Figure 27, in oder to increase extract and soluble N content.

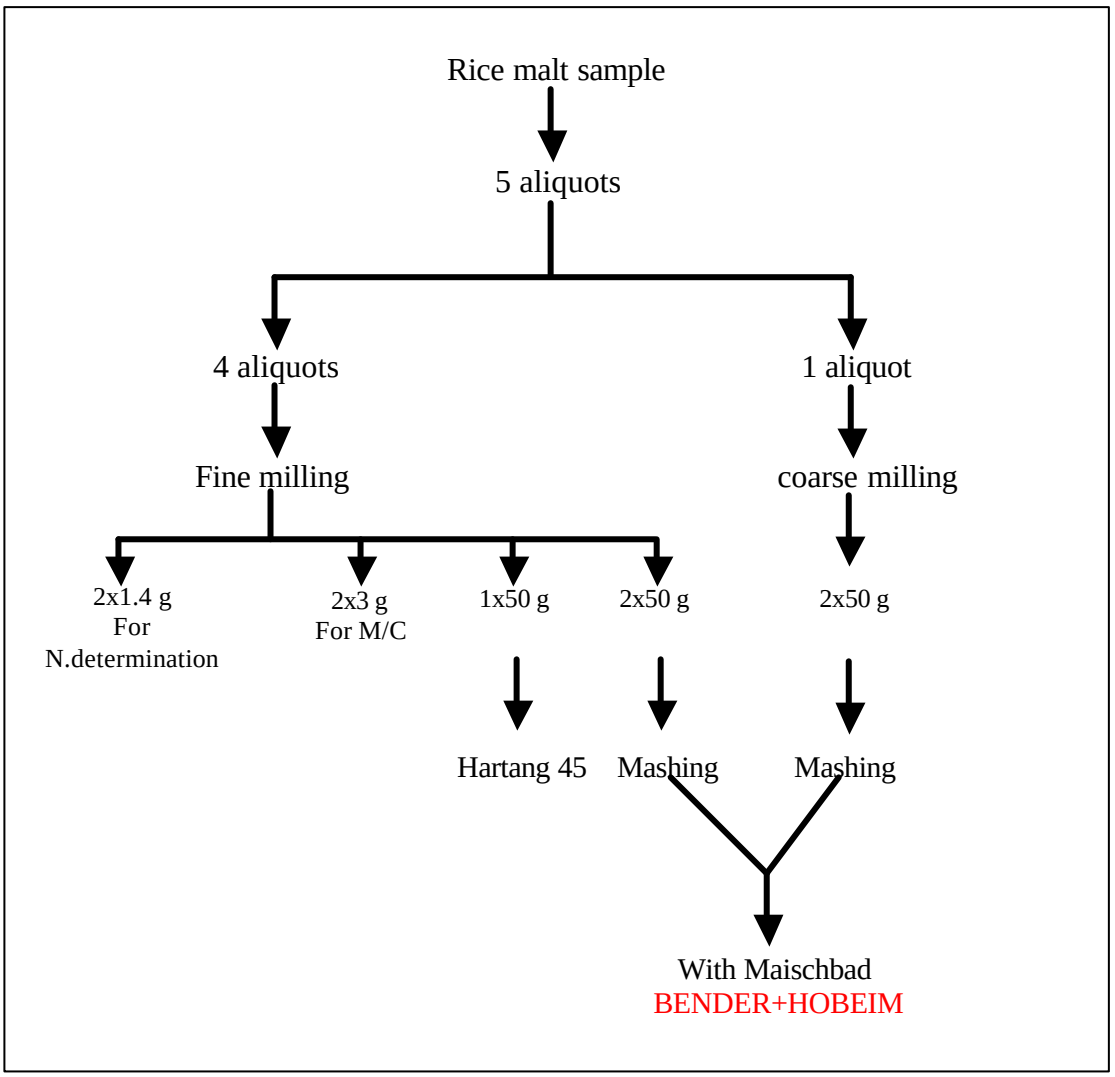


Figure 26. Diagram of rice malt analysis

Five kilograms of rice malt was milled using MEBAK Universal-Buehler (for coarse milling with 1.00 mm size) and MEBAK-miller, Buehler-Miag (for fine milling with 0.2 mm size) prior to be mashed with 17.5 litre of 45 °C water, temperature profile was 50 °C, 55 °C and 60 °C with increasing rate 1 °C/min, holding time for each temperature was 20 minutes before raise to 70 °C, stand for 60 minutes as illustrated in Figure 27, then mashing out before filtered by folded filter paper with 320 mm diameter, Whatman Schleicher & Schuell 2555 ½ (Whatman GmbH, Dassel, Germany) and rice wort was boiled. Fresh rice wort was then formulated with barley wort prior to be used as substrate for alternative fermentation.

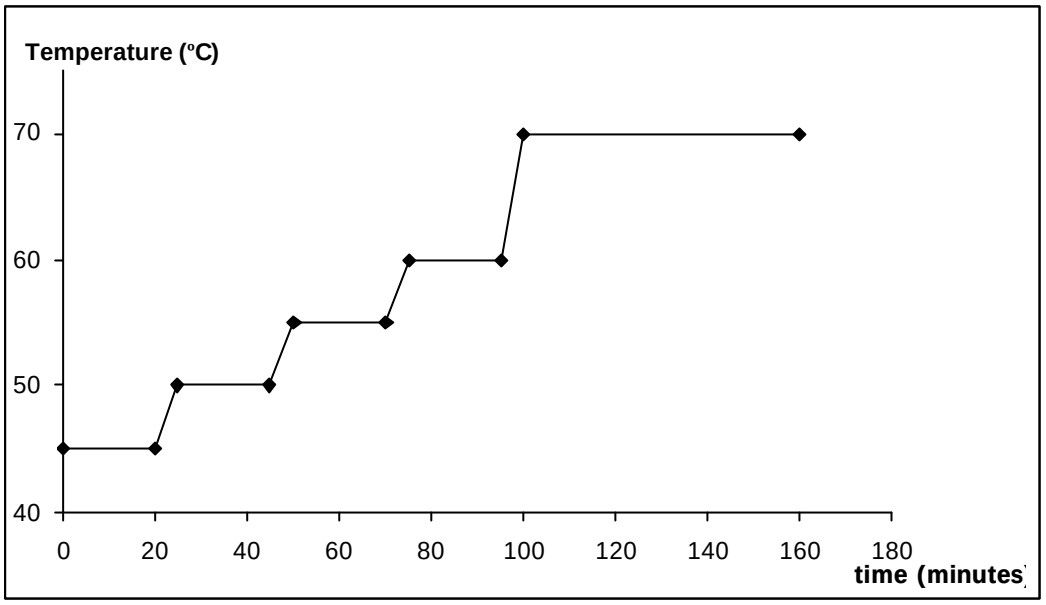


Figure 27. Modified mashing profile for rice malt

4.7. Collection and screening of loogpang samples

Eleven samples of lookpang were collected during 05.02.2004 –10.02.2004 from 9 provinces where were the main satho producing areas as illustrated in Figure 28. Ten samples from northeast; code LPNA-1, LPNA-2 and LPNA-3 were collected from Nakhonratchasima, 1 each sample code LPMA-1, LPBU-1, LPSA-1, LPYA-1, LPSR-1, LPSU-1and LPLO-1 from Mahasarakham, Buriram, Sakhonnakorn, Yasothorn, Srisaket, Surin and Loie respectively. Another one, CH-1 was collected from Chaingmai, the northern part of Thailand.They were collected from markets and direct from small producers in the villages as finished loogpang in dried form, round-to-flattened, creamy white to dusty white colour, and solid ball which range in 2.76 – 5.07 cm diameter which average 3.97cm. Each sample was packed in polyethylene bag and kept in 4-6 ° C refrigerators during studying.

Figure 28. Collecting areas of loogpang samples

All samples were screened based on ability to produce good quality satho in conventional process. Finished product from each treatment was observed by focus group in overall quality; taste, flavour, aroma and colour using 5 point scale Hedonic Test. The desired quality of satho products were selected for further analysed in alcohol content and fermentation by- products; acetaldehyed, ethylacetate, n-propanol, i-butanol, i-amylacetate, amylalcohol, diacetyl and 2, 3 pentandione. Microorganism consisted in the sample of loogpang which yielding the desired quality of satho was enumerated, isolated and functionality test. The high proficiency

isolates of yeast were identified using real time PCR (Brandl, 2006), Kurtzman and Robnet (1998). As well, the high saccharifying activity of mould isolates were identified by sequencing of the ITS1-5.8S-ITS2 rDNA (White *et al.,* 1990, Fernandez-Esparinar *et al.,* 2000, 2006), whereas the other isolates were identified to the genus level by morphological characteristic using agar block slide culture technique.

4.7.1 Conventional fermentation

Satho was prepared in the traditional way; milled rice was soak in water (rice: water ratio 1:2) overnight at room temperature, excess water was drained off through a stainless steel screen prior to be steamed in rice cooker for 15-20 minutes. Then steamed rice was wash with tap water, moisture content was determined and adjust to 73% (Shrestha, Nand and Rati, 2002) before 125 g of steamed rice was introduced to 1 litre Erlenmeyer flask for sterilise at 121 °C for 15 min. Aseptically well mixed with 0.5 g of ground inoculum before incubated at 30°C for 4 days, soft and juicy rice was present, solid content was withdrawn by squeezing with cheese cloth. Total soluble solid (TSS) of the slightly turbid liquid was measured before adjusted to 22° Brix with sterilised water or sugar. The pH value of the solution was determined and adjusted to 3.5-3.6 with 20% lactic acid solution.Whilst total acidity (TA) of the solution was determined as lactic acid (%w/v) according to Thyagaraja *et al*., (1992). Alcoholic fermentation was carried out at 30 °C until completely stop fermentation which would take around 10-14 days. Lee was separated; fresh satho has been stabilized in cold storage at 4°C for 1 week before filtered, pasteurized and bottling. Overall quality of finished satho has been evaluated by trained focus group prior to be analyzed in alcohol content (%v/v) and the profile of fermentation by- products; acetaldehyde, ethylacetate,n-propanol,i-butanol,i-amylaceatate,amylalcohol,diacetyl and 2,3-pentanedione.

However, as it was still not clear of the desired amount of chemical compositions of satho product, therefore, the product reference was screened from 5 producers in the market by sensory evaluation of the focus group.

4.7.2 Enumeration and isolation

Yeast and mould in superior selected loogpang samples were enumerated and isolated using serial dilution standard plate count method (FDA-BAM, 2001). Pure isolate of yeast and mould were further tested in their functionalities according to Dung *et al.,* (2005).

Pure isolates of mould and yeast were grown on YM agar at 30 °C for 5 and 2 days respectively before maintain at 4 °C for studying. Moreover, pure isolate of mould, M-SUT and yeast, Y-SUT were used as reference for satho fermentation. A suspension of cells or spores was made by adding 5 ml of sterilised 0.85% NaCl solution onto each slant. Desired amount of spore or cell was prepared under microscope using Thoma cell counting chamber.

4.7.3. Screening and identification of mould isolates

Mould isolate was screened using following criteria; hydrolysis of starch or starch degradation, starch saccharification, lipolytic activity, proteolytic activity and acid production test.

4.7.3.1. Hydrolysis of starch or starch degradation

A piece of growing mould pure culture was cut by 12 mm diameter cork borer before transferred onto the centre of a plate of rice-starch agar medium, incubated at 30°C for 48-72 hours. The degree of enzymatic activity was based on rate of the appearance of lytic zone and size of clear zone after flooding agar surface with

Lugol's iodine solution for 1 minute, diameter of clear zone and colony were measured.

4.7.3.2. Starch saccharification

Spore suspension of mould isolate was inoculated to sterilised glutinous rice at 10^6 spores/ g steamed rice before incubated at 30 °C for 4 days. Sample of liquid was taken daily, pH, total acidity (TA) and total soluble solid (TSS) were investigated.

4.7.3.3. Lipolytic activity

Lipolytic activity of mould isolates was tested on the same basal medium but supplemented with 0.5% calcium carbonate and 50 ml of soybean oil and incubated at 30° C for 4 days. Lipolytic activity presented as the appearance of a zone containing greenish blue granules around after flooding agar surface with saturated copper sulphate solution (saturated.CuSO4).

4.7.3.4. Proteolytic activity

Proteolytic activity was tested in proteolytic activity test medium which observed daily for a period of up to 6 days at 30°C of incubation as the presence of a clear zone around the colony on an opaque white background.

4.7.3.5. Acid production test

Acid production test of mould isolates were examined using calcium carbonate medium. A piece of growing mould was cut by cork borrer and then inoculated onto the centre of medium prior to be incubated at 30°C for 4 days. If acid was produced, clearing occur around and beneath the mould colonies.

Mould isolate which resulted highest amount of total soluble solid from starch saccharification was further tested in saccharification capacity using the following condition;

- Spore inoculums; 10^4, 10^5, 10^6 spores/g steamed rice
- Incubation time; 2, 3, 4 day
- Incubation temperature; 30, 40 °C

Previous studying on effect of incubation temperature on starch saccharification by Dung *et al.*,(2005) indicated that saccharification was not occur at 20°C, therefore 30 and 40°C would be used in this studying.

Each treatment was done in duplicates whilst total soluble solid, pH and total acidity were determined at the end of saccharification. The selected high performance isolates were further identified in molecular level using ITS1-5.8S –ITS2 rDNA sequencing according to White *et al.,* (1990) and Fernandez- Esparina *et al.,* (2000, 2006) . Whereas the other isolates were identified to the genus level by morphological characteristic using agar block slide culture technique.

4.7.4. Screening and identification of yeast isolates

Yeast isolates were screened base on ability to produce alcohol and tolerance of alcohol present in liquid medium.

4.7.4.1. Ability to produce alcohol

Yeast isolates were tested in ability to produce alcohol in batch culture using fermentation medium and saccharified rice liquid as substrate. The preparation of saccharified rice liquid was modified from Dung *et al.,* (2005) as following;

Fifty gram of milled glutinous rice was soaked in 60 ml of distilled water in a 250 ml Erlenmeyer flask for 4 hours. After soaking, the mixture was steamed in an autoclave for 1 hour at 100 °C. The steamed rice was cooled to 35-40 °C then inoculated with pure culture of reference isolate, M-SUT which has been grown for 5 days at 30°C on a slant of YMA and suspended in a sterilised physiological salt solution. Inoculation was carried out at 10^5 CFU/ g steamed rice and missed well

before incubated at 30°C for 4 days, then separated and squeezing to obtain a slightly turbid liquid. This liquid was adjusted in total soluble solid to 22 % w/w with sterilised distilled water, divided in 70 ml in 100 ml Erlenmeyer flask aliquot prior to be steamed at 115°C for 10 minutes. Yeast inoculums of each isolate which previously cultured in YM broth medium, shaking 170 rpm at 30 °C for 18 hours (Limthong *et al.*, 2002) was inoculated at 10^6 cells/ml rice liquid before incubate at 30°C for 5 days. Alcohol content, pH and total acidity of fermented broth were measured at the end of fermentation.

4.7.4.2. Tolerance of yeast to alcohol present in liquid medium

Pure ethanol was added to 50 ml sterilised saccharified rice liquid at the levels of 0%, 5%, 10%, 15% and 20% w/v, yeast suspension at10^4 cells/ml by microscopic count was inoculated. Number of viable yeast cells at start and after 3 days of fermentation at 30 °C was measured by plate counting on YM agar.

All isolates of yeast were identified using specific real time PCR assay for *S.cerevisiae* using primers; Sc-f (**5'**CAAACGGTGAGAGATTTCTGTGC-**3'**),Sc-r (**5'**GATAAAATTGTTTGTGTTTGTTACCTCTG-**3'**) and the 200 nM of the fluorescent probe Scer (**5'**-FAM-ACACTGTGGAATTTTCATATCTTTGCAACTT-BHQ1-**3'**) on a iCycler IQ (Biorad) (Brandl, 2006) whereas non *Saccharomyces* was identified by sequencing of the D1/D2 domains of the 26S rRNA gene using NL-1 (50-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (50- GGTCCGTGTTTCAAGACGG) as primers (Kurtzman and Robnet, 1998). A phylogenetic tree was reconstructed by neighbor-joining algorithm.

4.8 Satho fermentation

Feasibility study of high quality satho production was carried out using various kinds of start culture and substrates. Chemical compositions; alcohol content and some fermentation by-products; carbonyl compounds (acetadehyde and vicinal diketones), ester compounds (ethyl acetate and isoamyl acetate) and higher alcohols(n-propanol, i-butanol and amylalcohol) which affected on taste and flavour were used for consideration were monitored. There were two treatments carried out;

4.8.1 Satho fermentation using steamed rice

Three forms of start cultures were applied for fermentation; conventional using the selected loogpang, pure culture of mould and yeast isolate, semi- conventional using pure isolate of mould accompanied by the selected loogpang. Steamed rice was prepared as mention above. The condition for saccharification and alcoholic fermentation was used as previously optimized.

4.8.2. Satho fermentation using rice wort

Alternative fermentation of satho using indigenous start culture and pure isolate of the selected yeast in formulated rice wort was investigated. Among rice malt varieties, black normal rice (SUT-6) was selected for mashing due to high content in extract and soluble nitrogen content. Standard mashing program was modified for rice malt to increase extract and soluble nitrogen content. Five kilograms of rice malt was ground prior to be mashed with 17.5 litre of 45 °C water, temperature profile was 50 °C, 55 °C and 60 °C with increasing rate 1 °C/min, hold at each temperature for 20 minutes before raise to 70 °C, hold for 60 minutes, then mashing out before lautering.

Barley wort with and without hop was kindly supported by Weihenstephan-State Brewery, TUM-Freising, Weihenstephan, Germany. Various formulas of wort were prepared as represented in Table 12 prior to be divided in 70 ml in 100 ml Erlenmeyer flask alquot and steamed at 115°C for 10 min, cooled down and each sample of loogpang was ground and inoculated at the level of 0.4% w/v. On the other

hand, the selected yeast isolates at the level of 10^6 cells/ml was applied as pure culture. Fermentation was carried out at 30°c for 4 days. Fermented broth obtained from each treatment was analysed.Rice wort and rice-wort mixed with and without hop was prepared as illustrated in Table 12.

Tabe 12. Rice-wort mixed substrates preparation

Formulas	Composition (%)		Properties	
	Rice wort	Barley wort	Extract (%)	pH
S1	100	0	8.5	5.82
S2	80	20	9.0	5.62
S3	60	40	9.5	5.41
S4	50	50	10.0	5.28
S5	0	100	12.0	4.84

Remark;

- Formulas S* as well as in table 3 but mixed with barley wort without hop
- Total volume for each formula was 70 ml in 100 ml Erlenmeyer flask
- All treatments were done in triplicate

4.9 Quality evaluation

Finished product of satho obtained from each treatment has been characterised based on chemical compositions in terms of alcohol content and fermentation by-products; acetaldehyde, ethylacetate, n-propanol, i- butanol, i - amylacetate, amylalcohol, diacetyl and 2, 3-pentandione.

Sensory of the finished satho product obtained from each treatment was evaluated by trained focus group using a five-point Hedonic scale based on physical appearance, mouthfeel, aroma, taste, sweetness, sourness, alcoholic flavour and overall quality which modified from Shrestha, Nand and Rati (2002) using the following scale;

1. No flavour, no taste → poor
2. Less flavour, less taste → average
3. Alcoholic flavour, bitter taste → good
4. Good flavour, sweet, less sour, pleasant taste, less bitter → very good
5. Good flavour, less sweet, less sour, pleasant taste, less bitter → excellent

4.10 Analysis

4.10.1 Isolation and Identification of yeast and mould

Yeast and mould in the selected indigenous start culture was enumerated and isolated using serial dilution standard plate count method using DRBC medium(FDA-BAM, 2001). High proficiency of yeast isolates were identified by specific real time PCR assay for *S.cerevisiae* using 400 nM of the Primer Sc-f **(5´**CAAACGGTGAGAGATTTCTGTGC-**3´),** Sc-r **(5´**GATAAAATTGTTTGTGTTTGTTACCTCTG-**3´)** and 200 nM of the fluorescent probe Scer **(5´**-FAM-ACACTGTGGAATTTTCATATCTTTGCAACTT-BHQ1-**3´**) were added to the 1x Qantitect probe Master Mix (Qiagen) which performed on a iCycler IQ (Biorad) using the following conditon; 40 PCR cycles with annealing at 60 °C for 1

minute, denaturation at 95 °C for 1 second and preliminary denaturation at 95 °C for 10 minutes (Brandl, 2006).The amplification of the D1/D2 domain of the 26S rRNA gene was applied for non-*Saccharomyces* yeast performed for 36 PCR cycles with annealing at 52 °C, extension at 72 °C for 2 minutes and denaturation at 94 °C for 1 minute using the primers NL-1 (50-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (50-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnet, 1998). A phylogenetic tree was reconstructed by neighbor-joining algorithm.As well, the high performance of mould isolates were identified by sequencing of ITS1-5.8S-ITS2 rDNA according to White *et al.,* (1990) and Fernandez-Esparina *et al.,* (2000, 2006). However, the other mould isolates presented in the selected loogpang samples were identified to the genus level by morphological characteristics and microscopy using agar block slide culture.

4.10.2 Chemicals analysis; Moisture content was determined according to ASBC (1992), total acidity (TA) was estimated titrimetrically according to Thyagaraja *et al.,* (1992), total soluble solid (TSS) was measured using KRUESS HRN-16 Hand refractometer No.256 and pH were determined by pH meter WTW pH 525.Whereas alcohol and extract contents were analysed using SCABA 5610 Automatic Beer Analyser, as well, ß- glucan in rice wort was analysed using Tecator ß- Glucan 5700 Analyser. Fermentation by-products; acetaldehyde, ethylacetate, n-propanol, i butanol, i - amylacetate and amylalcohol were analysed according to MEBAK III 1.1.1 whilst diacetyl and 2, 3-pentandione were detected according to MEBAK III 1.2.1.

All data was further analysed using SPSS 9.05 for windows program with one way ANOVA. The mean difference was significant at the 0.05 level with 95% confidence interval.

V. RESULTS

5.1. Screening of loogpang samples

5.1.1. Conventional fermentation; Satho produced from conventioanl fermentation using 3 sources of samples; LPMA-1, LPBU-1, and LPNA-2 gave pleasant in overall quality at 4.0, 4.5 and 5.0 respectively, which code LPNA-2 has highest between those of them. While the others range mostly at 3.0 and presented off-flavour. The chemical properties of satho samples produced from these three loogpang samples were analysed and compared with the reference product, data was represented in Table 13.

Table 13. Overall quality of satho produced by conventional fermentation

Treatment no.	Source of loogpang	Overall appearance	Overall taste/flavour
1.	LPNA-1	3.0	3.0
2.	LPMA-1	4.0	4.0
3.	LPBU-1	4.5	4.5
4.	LPSA-1	2.0	3.0
5.	LPNA-2	5.0	5.0
6.	LPNA-3	3.0	3.0
7.	LPYA-1	3.0	3.0
8.	LPSR-1	3.0	3.0
9.	LPCH-1	3.0	1.0
10.	LPSU-1	3.0	2.0
11.	LPLO-1	3.0	3.0

When compared with the reference sample, they were same in alcohol content. Three categories of fermentation by-products; carbonyl compounds (acetaldehyde, diacetyl and 2, 3-pentanedione), higher alcohols (n-propanol, i-butanol and amylalcohol) and volatile esters (ethyl acetate and i-amyl acetate) which affected taste and flavour of the product were compared. As depicting in Table 14 and Figure 29, among the all three product samples, they contained not different for all test parameters.Whereas, significantly different was found between product sample and reference in concentration of carbonyl compounds, esters and total acidity. The high quantity of carbonyl compound and low amount of esters content were presented in product samples.

Table 14. Comparison of chemicals content between satho obtained from conventional fermentation and reference product sample

Test parameters	Source of Loogpang			Reference satho product
	LPMA-1	LPBU-1	LPNA-2	
Sensory test (overall score)	4.0	4.5	5.0	4.5
Alcohol (%v/v)	10.94	11.00	10.56	9.89
pH	3.34	3.46	3.42	4.76
Total acidity (%w/v)	1.27	1.03	1.28	0.70
Acetaldehyde (mg/l)	34.37	39.09	46.59	19.50
Diacetyl, total (mg/l)	0.15	0.43	0.11	0.15
2,3- Pentanedione, total (mg/l)	0.02	0.03	0.01	0.01
Ethylacetate (mg/l)	7.86	6.82	7.01	51.5
i- Amylacetate (mg/l)	< 0.1	< 0.1	< 0.1	0.3
n- Propanol (mg/l)	41.59	41.18	41.29	34.7
i- Butanol (mg/l)	70.02	76.36	89.30	86.6
Amylalcohol (mg/l)	104.57	87.09	123.39	105.5

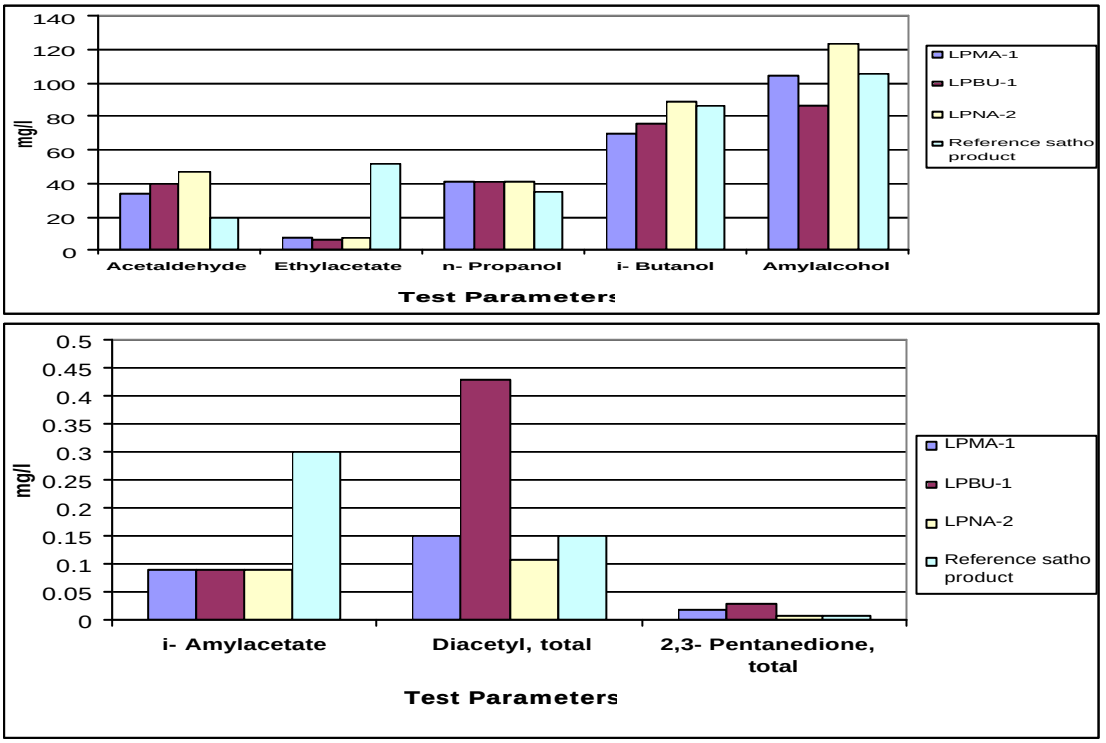


Figure 29.Comparison of fermentation by-products of satho obtained from conventional fermentation and reference product sample

5.1.2. Enumeration and Isolation

Number of yeast presented in all 3 samples fall in the range of $4.64x10^5$-$1.28x10^7$ CFU/g which 16 isolates were obtained whereas 10 isolates of mould was achieved in the range of $1.17x10^5$ - $5.09x10^6$ CFU/g. The results of enumeration and isolation of yeast and mould in the 3 selected of loogpang samples was represented in Table 15.

Table15. Enumeration and isolation of yeast and mould

Sample code	Yeast		Mould	
	No. of yeast (CFU/g)	No. of isolate (isolates)	Mould count (CFU/g)	No. of isolate (isolates)
LPMA-1	$4.64x10^5$	5	$5.09x10^6$	4
LPBU-1	$5.20x10^5$	5	$1.17x10^5$	4
LPNA-2	$1.28x10^7$	6	$5.50x10^5$	2

5.2. Screening test for mould isolates

As represented in Table 17, the average of mould count in 10 isolates obtained were 1.92 x 10^6CFU /g which only some isolates of each loogpang sample were presented as high amount number; MMA-1/61, MBU-1/53 and MNA-2/40 originated from loogpang LPMA-1, LPBU-1 and LPNA-2, respectively. In order to achieve the high proficiency isolates, therefore, 10 isolates of mould were studied in their functionality; ability to produce acids, starch hydrolysis, saccharification, proteolytic and lipolytic activity.

Table16. Enumeration and isolation of mould

Source of Loogpang	Mould count (CFU/g)	No. of isolate (isolates)	Isolate code	Mould count (CFU/g)
LPMA-1	5.09 x 10^6	4	MMA-1/60	5.00 x 10^3
			MMA-1/61	5.00 x 10^6
			MMA-1/66	8.50 x 10^4
			MMA-1/67	3.0 x 10^3
LPBU-1	1.17 x 10^5	4	MBU-1/53	1.00 x 10^5
			MBU-1/54	5.00 x 10^3
			MBU-1/55	5.00 x 10^3
			MBU-1/56	6.70 x 10^3
LPNA-2	5.50 x 10^5	2	MNA-2/39	5.00 x 10^4
			MNA-2/40	5.00 x 10^5

5.2.1. Functionality of mould isolates

Acid production, starch hydrolysis, saccharification proteolytic and lipolytic activity of mould isolates were tested. All isolates of mould originated from LPMA-1 and LPNA-2 together with one isolate from LPBU-1; MBU-1/54 were acid producing isolates. Focus on hydrolysis activity,all isolates presented high activity whereas starch saccharification was not presented for isolate MBU-1/53 and MBU-1/55 which originated from start culture, LPBU-1 even high hydrolysis on starch medium occurred. This indicated that starch degradation in starch agar medium was correlated with a-amylase whereas the efficiency of saccharification was correlated closely with the glucoamylase enzyme (Underkofler, 1976). Therefore successful in starch hydrolysis was not a guarantee for good saccharification (Araujo *et al.*, 2004). All isolates presented proteolytic activity on test medium whilst all 3 loogpang samples consisted both negative and positive of lipolytic activity. For saccharification, all isolates were starting to release liquid after 2 days of incubation. At the end of saccharification, highest total soluble solid was found in isolate code MNA-2/39 obtained from starter code LPNA-2 on the level of 39.0% w/w. This data was summarised in Table 17.

Table17. Summary of functionality of mould isolates

Isolate code	**Acid production**	**Proteolytic activity**	**Lipolytic activity**	****Starch hydrolysis**	**Starch saccharification (°Brix)**
MMA-1/60	+	+	+	1.12	36.0
MMA-1/61	+	+	-	1.00	28.2
MMA-1/66	+	+	-	1.00	21.0
MMA-1/67	+	+	+	1.00	37.5
MBU-1/53	-	+	+	1.08	*
MBU-1/54	+	+	+	1.09	28.5
MBU-1/55	-	-	-	1.09	*
MBU-1-56	-	+	+	1.06	38.4
MNA-2/39	+	+	+	1.10	39.0
MNA-2/40	+	+	-	1.08	30.0
M-SUT	**+**	+	+	1.00	38.4

*saccharification not occurred

**** expressed as ∅ of clear zone/∅ of colony

In term of comparison, saccharification using mixed culture in form of loogpang and pure isolate originated from the same source, total soluble solid achieved from those mixed culture were higher than pure isolate for all treatments;

data was summarised in Table 18 and Figure 30. This indicated that not only one isolate but also possible starch degrading yeast was responsible for degradation or synergistic activity among mould-mould or yeast-mould occurred which has to be further in detail investigated. When focus on pH and total acidity, using mixed-culture as loogpang were lower pH and higher total acidity than those pure isolates in all treatments were occur. This show that the purity of culture, culture consists of acidifying bacteria which always found in starchy start culture(Hesseltine,1988), the pH usually becomes considerably lower than 3.5. This result was agreed with previous study by Dung, *et al.*, (2005). Whereas pH of saccharified liquid produced from pure culture originated from same sources were high. The presence of these bacteria might due to the conditions of producing the starter were favour or they might develop later during saccharification when glucose was present. However, Ellis *et al*., (1976) reported that some of fungi regularly found in amylolytic starters can also produce lactic acid from sugar.

Table 18. Comparison of starch saccharification between loogpang and pure isolates

Start culture code	TSS (°Brix)	pH	Total acidity (%w/v)	Juice volume (ml/100g rice)
***LPMA-1**	**43.80**	**3.26**	**1.56**	**69.96**
MMA-1/60	36.0	4.45	0.60	65.00
MMA-1/61	28.2	3.59	1.19	52.73
MMA-1/66	21.0	3.58	1.00	69.10
MMA-1/67	37.50	3.32	1.46	56.36
***LPBU-1**	**39.90**	**3.82**	**0.96**	**69.96**
MBU-1/54	28.2	3.53	1.25	47.29
MBU-1/56	38.4	4.43	0.56	42.73
***LPNA-2**	**41.10**	**3.44**	**1.70**	**71.68**
MNA-2/39	39.0	4.06	0.81	67.73
MNA-2/40	30.0	3.63	1.02	45.78
M-SUT	38.4	4.36	0.63	67.73

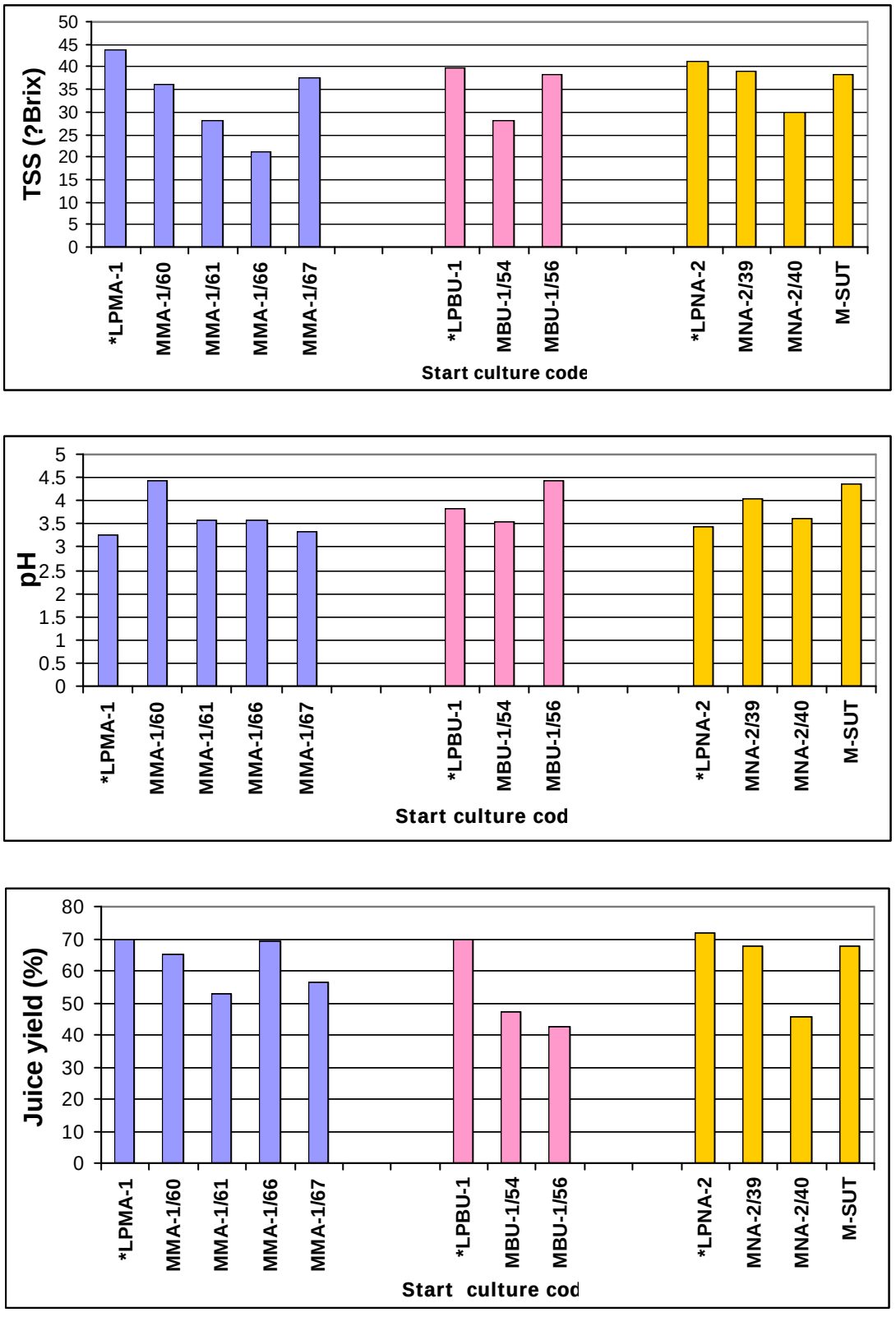


Figure 30. Starch saccharification using loogpang and pure isolates

Table 19. Functional comparison of high proficiency mould isolates

Isolate code	Proteolytic activity	Starch hydrolysis	Saccharification (°Brix)	Lipolytic activity	Acid production
MNA-2/39	+	1.10	39.00	+	+
MBU-1/56	+	1.12	38.4	+	-
MMA-1/60	+	1.12	36.00	+	+
MMA-1/67	+	1.00	37.50	+	+
M-SUT	**+**	1.00	38.4	+	+

5.2.1.4. Saccharification capacity of the selected mould isolates

Four isolates of mould; MNA-2/39, MBU-1/56, MMA-1/60, MMA-1/67, were selected to study of incubation temperature on saccharification property. Previous studied by Dung *et al*., (2005) indicated that no liquid was form for all treatments at 20 °C due to the poor growth of mould. While liquid was produced and released at 30 and 40°C.Therefore, incubation temperature at 30 and 40 °C were used for this studying with inoculating level at10^5 spores/g for 4 days.

All isolates were completely saccharified at 30 °C resulting in high total soluble solid which presented higher than 30% w/w, pH was higher than 3.00 for all treatments which fall in the range between 4.06-4.45 except for isolate code MA-1/67 was 3.32. Juice yield was range between 42.73-67.73%. At 40°C of saccharification; total soluble solid was not differently significant compared with 30 °C, in contrast, pH of most isolate was lower than at 30°C However, significantly difference was found in juice yield between 30 and 40°C, all isolates showed low juice yield volume at 40°C. Appearance of cooked rice after incubated at 40°C for 4 days was found in slurry form; this was too difficult to separated or filtering. Therefore, incubation temperature at 30 °C was used for studying the effect of inoculums size on saccharifying property of selected 4 isolates of mould.

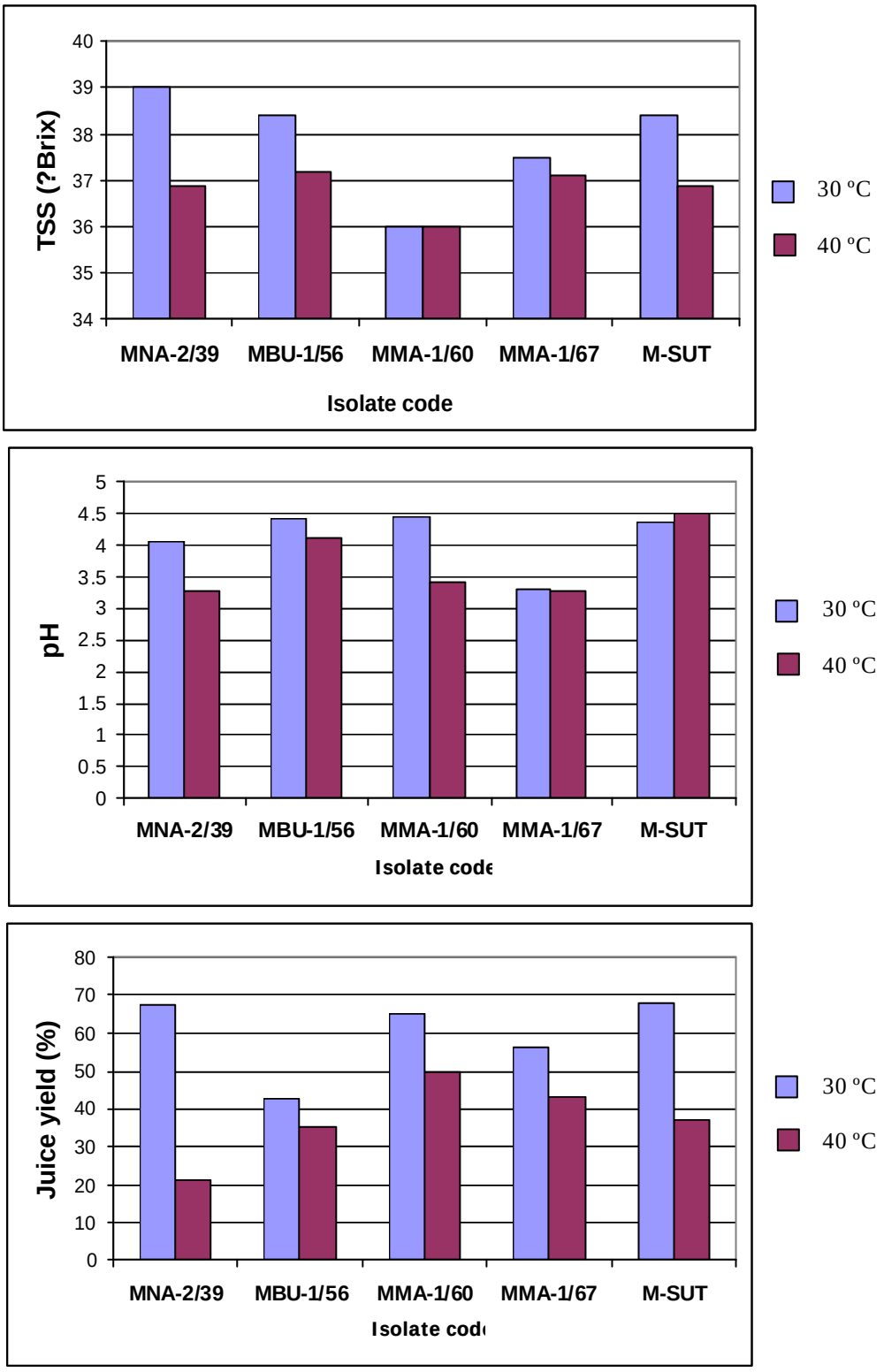


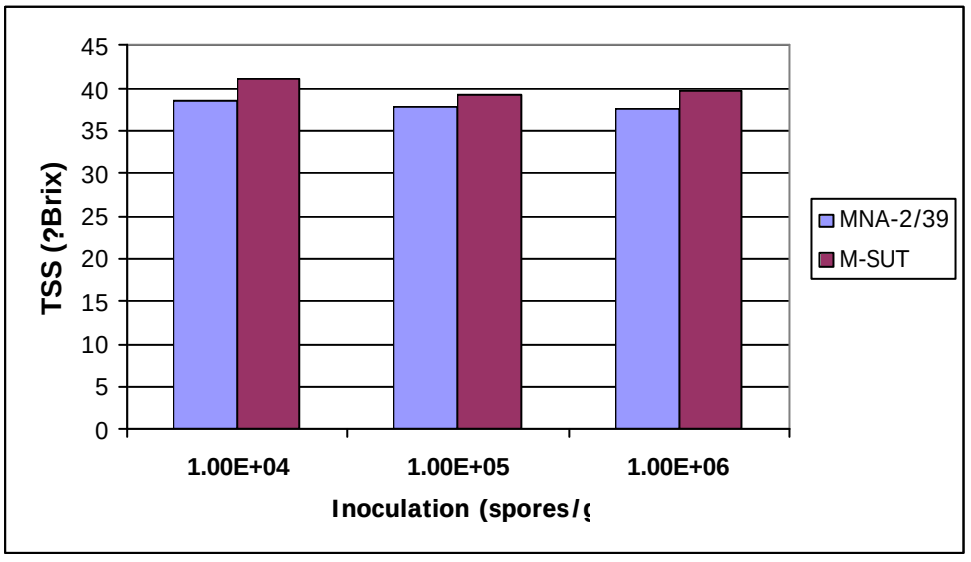
Figure 31. Effect of temperature on saccharification profile

Isolate code NA-2/39 which presented highest saccharifying property was selected for studying effect of inoculation level on saccharification profile. The result

showed that on day 2 of incubation with inoculation level of 10^4 and 10^5 spores/g steamed rice, saccharification was not presented, only soft rice with white mycelium occurred.Whereas, inoculums levels at 10^6 spores/g steamed rice, sacchrification started and liquid was present. All inoculation levels were continuing saccharified before finished on day 4. At the end of incubation, all treatments were not differently significance in total soluble solid whereas total acid was produced more at high inoculums level at 10^6 spores/g steamed rice than at low inoculation level.The result was represented inTable 20 and Figure 32.

Table 20. Effect of inoculums size on saccharification profiles

Isolate code.	Inoculum level (spores/g)	Day 2			Day 3			Day 4		
		TSS (°Brix)	TA (%w/v)	pH	TSS (° Brix)	TA (%w/v)	pH	TSS (° Brix)	TA (%w/v)	pH
MNA-2 /39	10^4	-	-	-	40.50	0.65	3.75	38.70	0.72	3.69
	10^5	-	-	-	36.90	0.64	3.83	37.8	0.63	3.62
	10^6	34.50	0.97	3.67	36.00	1.04	3.72	37.50	1.11	3.73
M-SUT	10^4	-	-	-	40.80	0.54	4.04	41.10	0.79	4.15
	10^5	-	-	-	38.10	0.50	4.15	39.30	0.77	4.26
	10^6	31.80	0.64	4.04	37.35	0.82	3.95	39.75	0.87	3.96



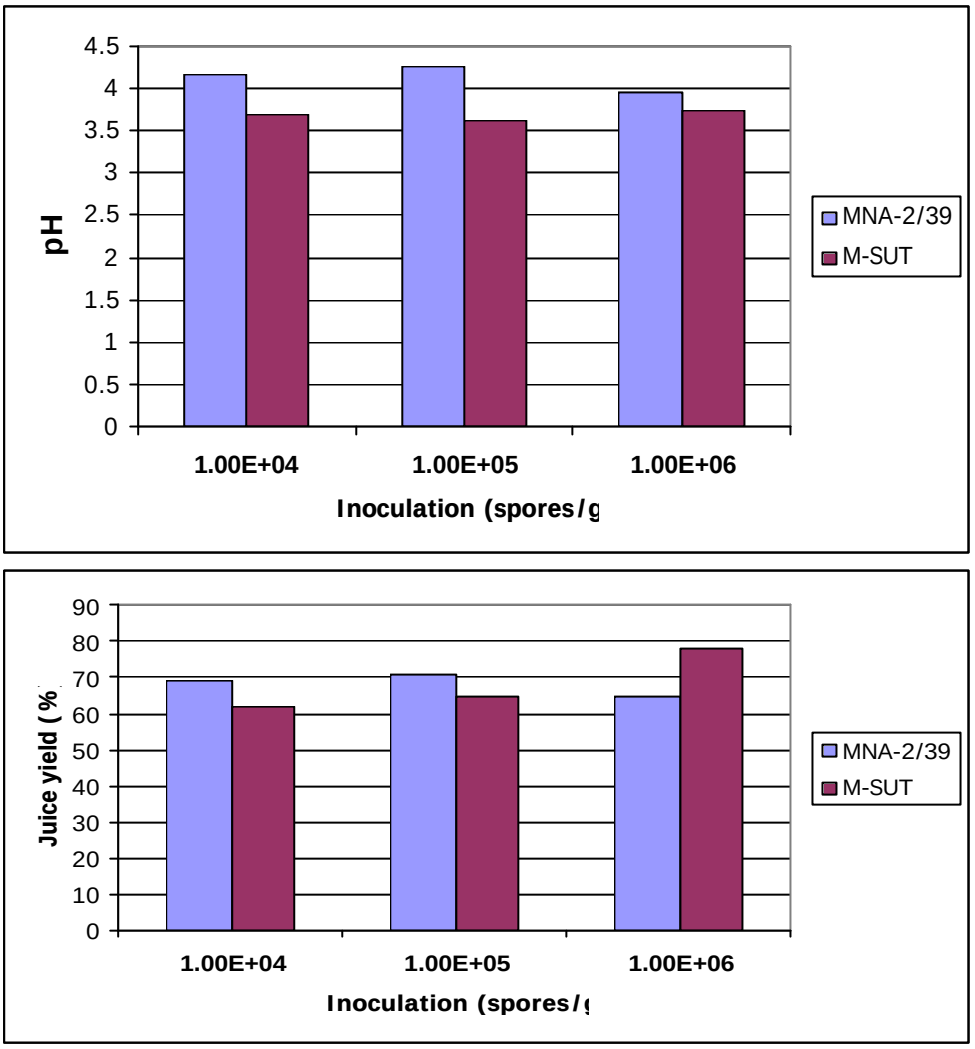


Figure 32. Effect of inoculums size on starch saccharification

5.2.2. Identification of mould isolates

There were 5 types of mould found in 10 isolates originated from the 3 selected loogpang samples; *R.oryzea, Fusarium sp., Mucor sp., Penicillium sp.,* and *Aspergillus sp.* which *R.oryzea* presented in all samples even at low level but it was the main isolate responsible for starch hydrolysis and saccharification.However, isolate MBU-1/56 which negatively in acid producing, but it was also identified belonging to *R.oryzea*, this might be different in sub-species level. The other mould types were found in some samples with one isolate of *Penicillium sp* presented in loogpang sample code LPBU-1. Summary of mould identification was depicting in Table 21.

Table 21. Summary of mould isolates found in the selected loogpang samples.

No.	Isolate code	Frequency found (CFU/g)	Saccharifiction ability (TSS, °Brix)	Identification	Identification method
1.	**MNA-2/39**	$5.00X10^4$	39.0	*R.oryzea*	ITS1-5.8S-ITS2-SEQ
2.	MNA-2/40	$5.00X10^5$	30.0	*Fusarium sp.*	morphological
3.	MBU-1/53	$1.00x10^5$	*	*Mucor sp.*	morphological
4.	MBU-1/54	$5.00x10^3$	28.5	*Penicillium sp.*	morphological
5.	MBU-1/55	$5.00x10^3$	*	*Aspergillus sp.*	morphological
6.	**MBU-1/56**	$6.70x10^3$	38.4	*R.oryzea*	ITS1-5.8S-ITS2-SEQ
7.	**MMA-1/60**	$5.00x10^3$	36.0	*R.oryzea*	ITS1-5.8S-ITS2-SEQ
8.	MMA-1/61	$5.00x10^6$	28.2	*Aspergillus sp.*	morphological
9.	MMA-1/66	$8.50x10^4$	21.0	*Mucor sp.*	morphological
10.	**MMA-1/67**	$3.00x10^3$	37.5	*R.oryzea*	ITS1-5.8S-ITS2-SEQ
11.	M-SUT	-	38.4	*R.oryzea*	ITS1-5.8S-ITS2-SEQ

5.3. Screening test for yeast isolates

Three samples of loogpang; LPMA-1, LPBU-1 and LPNA-2 were selected for further studied cause of their ability to produced good quality satho. Among 3 samples, total yeast cells was found in the range of $4.64 \times 10^5 - 1.28 \times 10^7$ CFU/g with total 16 isolates were obtained from LPMA-1, LPBU-1 and LPNA-2 at amount of 5, 5 and 6 isolates respectively. This data was illustrated in Table 22.

Table 22. Enumeration and isolation of yeast

Source of Loogpang	Total yeast cell number (CFU/g)	No. of isolates found (isolates)	Isolate code	Frequency of cell number found (CFU/g)
LPMA-1	4.64 x 10^5	5	YMA-1-/32	1.31 x 10^5
			YMA-1/33	9.00 x 10^4
			YMA-1/35	4.50 x 10^4
			YMA-1/36	9.30 x 10^4
			YMA-1/38	1.05 x10^5
LPBU-1	5.20 x 10^5	5	YBU-1/26	1.30 x 10^5
			YBU-1-/27	1.48 x 10^5
			YBU-1/28	1.60 x10^3
			YBU-1/29	2.40 x 10^5
			YBU-1/30	1.24x10^4
LPNA-2	1.28 x 10^7	6	YNA-2/5	1.28 x 10^7
			YNA-2/6	1.00 x 10^3
			YNA-2/7	1.00 x 10^3
			YNA-2/8	1.20x10^4
			YNA-2/9	1.20 x10^3
			YNA-2/10	1.00x10^4

5.3.1. Identification and ability to produce alcohol

As represented in Table 23 and Figure 33, all the selected loogpang samples consisted mainly of *S.fibuligera* and *S.cerevisiae* which *S.fibuligera* was predominant. Moreover, *S.malanga* and *I.oreintalis* were presented in LPBU-1 and LPNA-2 respectively.This results agree with previous studied by Chatisantien (1977), Chaowsungkete (1978). Dijen, (1972), Saono, (1982), Lotong, (1998), Thanh *et al.*, (1999) and Limtong *et al.*, (2002). Studying on alcohol producing, revealed that *S.fibuligera* produced relatively low alcohol with average1.89%v/v whilst *S.malanga* and *I.orientalis* produced 2.85 and 4.18%v/v respectively. For these studied of loogpang samples, therefore, indicated that *S.cerevisiae* in loogpang was the main isolate responsible for alcohol producing in satho which average of 7.59%v/v.

Table 23. Summary of yeast identification and abilty to produce alcohol

Source	Starter code	Alcohol produced (%v/v)	Identification	Identification method
LPMA-1	LPMA-1	8.59	-	
	YMA-1/32	2.34	*S.fibuligera*	D1/D2 26S-SEQ
	YMA-1/33	2.34	*S.fibuligera*	
	YMA-1/35	2.44	*S.fibuligera*	
	YMA-1/36	2.36	*S.fibuligera*	
	YMA-1/38	7.46	*S.cerevisiae*	D1/D2 26S-SEQ real time PCR
LPBU-1	LPBU-1	8.92	-	
	YBU-1/26	1.51	*S.fibuligera*	D1/D2 26S-SEQ
	YBU-1/27	2.66	*S.fibuligera*	
	YBU-1/28	1.46	*S.fibuligera*	
	YBU-1/29	7.55	*S.cerevisiae*	D1/D2 26S-SEQ real time PCR
	YBU-1/30	2.85	*S.malanga*	D1/D2 26S-SEQ
LPNA-2	LPNA-2	8.75	-	
	YNA-2/5	1.84	*S.fibuligera*	D1/D2 26S-SEQ
	YNA-2/6	7.78	*S.cerevisiae*	D1/D2 26S-SEQ real time PCR
	YNA-2/7	1.93	*S.fibuligera*	D1/D2 26S-SEQ
	YNA-2/8	4.18	*I.orientalis*	
	YNA-2/9	7.63	*S.cerevisiae*	D1/D2 26S-SEQ real time PCR
	YNA-2/10	1.83	*S.fibuligera*	D1/D2 26S-SEQ

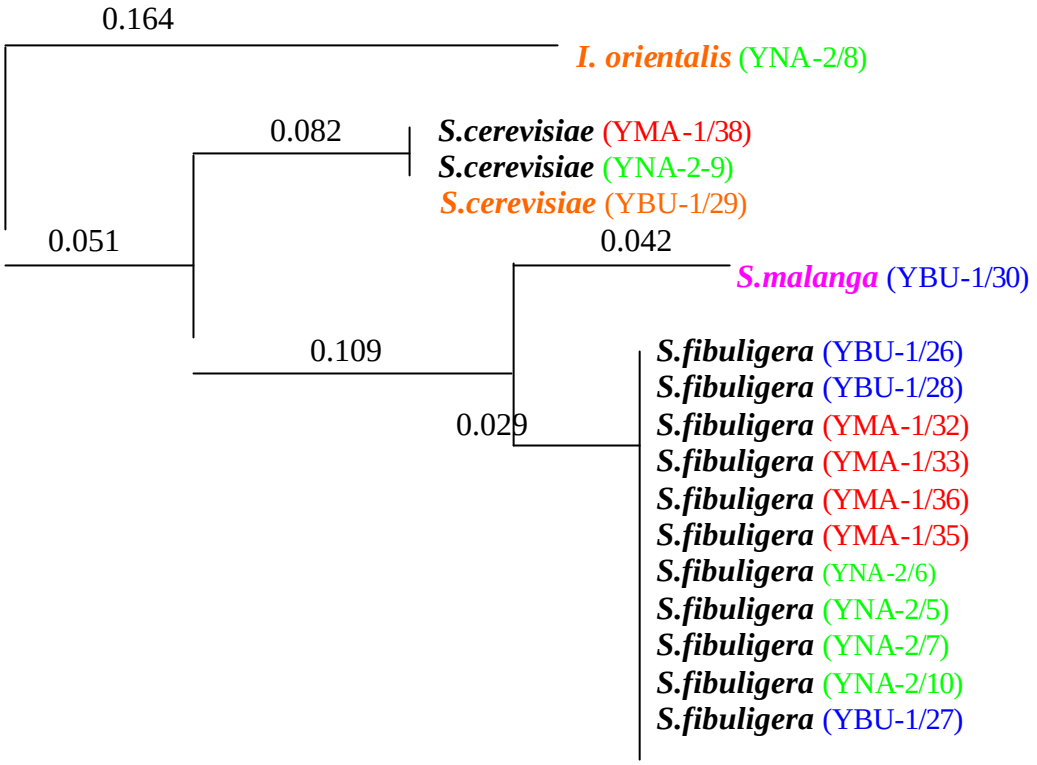


Figure 33. Phylogenetic tree of yeast isolate found in loogpang samples

5.3.2 Tolerance of yeast to alcohol present in liquid medium

Isolate YBU-1/26 as *S.fibuligera*, YNA-2/6 as *S.cerevisiae* and WHS no.68 were selected for examined of alcohol tolerance presented in medium in form of single isolate and co-culture between *S.fibuligera* and *S.cerevisiae* for investigate the interaction on alcohol tolerance.The data in Table 24 showed that high tolerance of alcohol presented in medium was maximum at 15 %v/v found in *S.cerevisiae* from LPNA-2 and reference isolate from SUT whilst *S.fibuligera* alone and mixed with *S.cerevisiae* was disappeared at this concentration. This property related to the characteristic of satho fermentation, in conventional using mixed culture in form of loogpang, *S.fibuligera* presented at the beginning of alcoholic fermentation, later decreased and substituted by *S.cerevisiae* as previously recommended by Danvirutai (2005).

Table 24. Alcohol tolerance of selected yeast isolates.

Isolate no.	Cell number(CFU/ml)				
	Alc. 0%w/v	**Alc. 5%w/v**	**Alc. 10%w/v**	**Alc. 15%w/v**	**Alc. 20%w/v**
S.fibuligera	$4.27x10^5$	$1.93x10^5$	$9.70x10^2$	0	0
S.cerevisiae	$4.00x10^6$	$4.53x10^6$	$6.43x10^5$	$2.35\ x10^2$	0
S.fibuligera + *S.cerevisiae*	$8.73x10^6$	$1.54x10^5$	$6.57x10^3$	0	0
WHS.no.68	$8.50x10^5$	$1.13x10^5$	$1.05x10^2$	0	0
Y-SUT *(S.cerevisiae)*	$1.38x10^6$	$2.78x10^6$	$2.63x10^5$	$2.95x10^2$	0

Remark; cell number at day 0 = $1.06x10^4$ cells/ml

5.4 Satho fermentation using steamed rice

5.4.1 Pure isolate of mould *R.oryzea* and yeast *S.cerevisiae* were used for pure culture fermentation whereas the high efficiency selected of loogpang samples; LPMA-1, LPBU-1 and LPBU-1 were used for conventional fermentation. For semi-conventional fermentation; pure isolate of *R.oryzea* was applied for saccharification and the selected 3 sources of loogpang samples were inoculated for alcoholic fermentation. The condition for saccharification by mould was inoculation level at 10^6 spores/g steamed rice, incubated for 4 days at 30° C. On the other hand, inoculum of *S.cerevisiae* at the level of 10^6cells/ml was inoculated for alcoholic fermentation which carried out at 30° C for one week. The results was concluded in Table 25, 26,27 and Figure 33,34 and 35 for pure culture fermentation, conventional and semi-conventional, respectively.

Table 25. Summary of chemical contents of satho produced by pure culture

Test Parameters	*S.cerevisiae* (Y – SUT)	WHS no. 68	*S.cerevisiae* (YNA-2/6)
Alcohol content (%v/v)	7.55	7.02	8.60
pH	3.60	3.82	3.79
*Acetaldehyde (mg/l)	18.50	48.70	48.40
Diacetyl, total (mg/l)	0.66	0.60	0.19
2, 3 – Pentandione, total (mg/l)	0.19	0.06	0.05
*Ethylacetate (mg/l)	11.30	5.40	4.90
*i – Amylacetate (mg/l)	1.20	0.90	0.70
*n – Propanol (mg/l)	27.30	27.10	31.90
*i – Butanol (mg/l)	70.60	161.50	56.30
*Amylalcohol (mg/l)	102.90	141.60	102.20

- Base on 10% v/v alcohol content

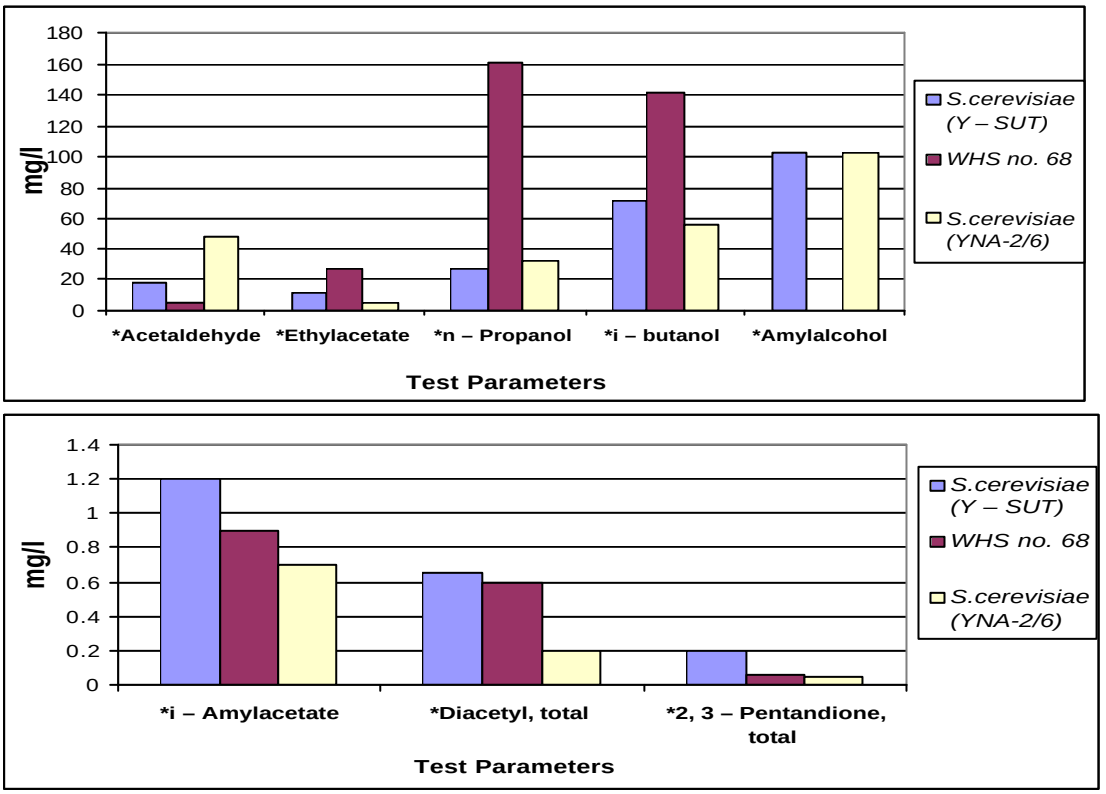


Figure 34. Fermentation by-products of satho produced by pure isolates

Table 26. Summary of chemical contents of satho produced by conventional

Test parameters	Source of Loogpang		
	LPMA-1	**LPBU-1**	**LPNA-2**
Sensory test (overall score)	4.0	4.5	5.0
Alcohol (%v/v)	10.94	11.00	10.56
pH	3.34	3.46	3.42
Total acidity (%w/v)	1.27	1.03	1.28
Acetaldehyde (mg/l)	34.37	39.09	46.59
Diacetyl, total (mg/l)	0.15	0.43	0.11
2,3- Pentanedione, total (mg/l)	0.02	0.03	0.01
Ethylacetate (mg/l)	7.86	6.82	7.01
i- Amylacetate (mg/l)	< 0.1	< 0.1	< 0.1
n- Propanol (mg/l)	41.59	41.18	41.29
i- Butanol (mg/l)	70.02	76.36	89.30
Amylalcohol (mg/l)	104.57	87.09	123.39

Table 27. Summary of chemical contents of satho produced by semi-coventional fermentation

Test Parameters	Source of loogpang		
	LPMA-1	**LPBU-1**	**LPNA-2**
Alcohol content (%v/v)	8.59	8.92	8.75
pH	3.44	3.43	3.46
Acetaldehyde(mg/l)	15.3	15.9	13.7
Diacetyl, total(mg/l)	0.21	0.18	0.19
2, 3 – Pentandione, total (mg/l)	0.05	0.03	0.06
Ethylacetate (mg/l)	37.3	38.7	36.9
i – Amylacetate(mg/l)	3.3	3.5	3.3
n – Propanol(mg/l)	44.7	47.6	45.0
i – Butanol(mg/l)	169.5	167.6	154.5
Amylalcohol(mg/l)	155.4	141.8	136.6

All treatments were quite the same in alcohol content whereas WHS.no 68 produced higher amount of not carbonyl compounds than *S.cerevisiae* (Y-SUT) but also alcohol. Alcohol production by various forms of start culture in batch fermentation using steamed rice substrate was concluded in Table 27.

Table 28. Amount of alcohol produced by various forms of start culture

No.	Type of starter used		Alcohol produced (%v/v)	pH	Type of fermentation
	Saccharification	Alcoholic fermentation			
1	LPMA-1	LPMA-1	10.94	3.34	conventional
2	LPMA-1	YMA-1/38	8.14	3.40	semi-conventional
3	MMA-1/67	LPMA-1	8.59	3.44	semi-conventional
4	MMA-1/67	YMA-1/38	7.55	3.60	pure culture
5	LPBU-1	LPBU-1	11.00	3.46	conventional
6	LPBU-1	YBU-1-29	7.96	3.47	semi-conventional
7	LPBU-1/56	LPBU-1	8.92	3.43	semi-conventional
8	MBU-1/56	YBU-1/29	7.46	3.64	pure culture
9	LPNA-2	LPNA-2	10.56	3.42	conventional
10	LPNA-2	YNA-2/6	8.10	3.43	semi-conventional
11	MNA-2/39	LPNA-2	8.75	3.46	semi-conventional
12	MNA-2/39	YNA-2/6	7.78	3.68	pure culture

For semi-conventional fermentation of satho using the selected high performance of 3 loogpang samples; LPMA-1, LPBU-1 and LPNA-2 in saccharification step following by applied of pure isolate of S.cerevisiae as inoculum for alcoholic fermentation. Fermented broth was then analysed, data was shown in Table 28 and Figure 35. All samples achieved from each treatment contained the same amount of chemical properties with average content were 15.21, 39.83 and 302.73 mg/l, respectively.

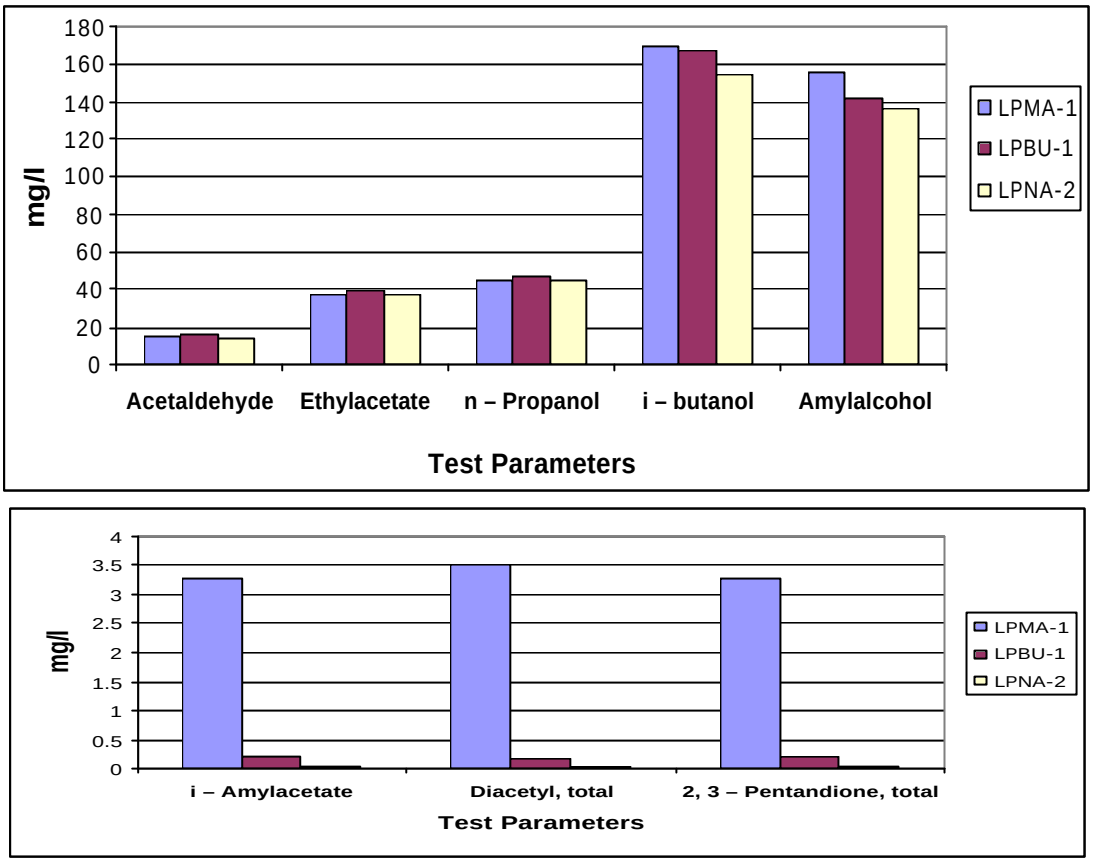


Figure 35. Fermentation by-products of satho produced by semi-coventional fermentation

As represented in Table 29 and Figure 37, highest alcohol content was found in conventional fermentation. Whereas fusel alcohols produced at average at 354.3 mg/l for both samples produced from conventional and semi-conventional fermentation. Lowest content of fusel alcohols was found in satho produce using pure culture. This results show that, under the same fermentation condition, pure isolate of *S.cerevisiae* alone was not the main isolate responsible for fusel alcohol formation. On the other hand, the other isolates of mould and yeast in loogpang might be involved; this was confirmed in conventional and semi-conventional fermentation which loogpang was applied.The largely different was the amount of ester compounds produced from semi-conventioanl which presented more higher than in conventional fermentation. This might be not only the role of the other microorganism include lactic acid bacteria which reported to found in loogpang by some researcher (Hesseltine and Ray, 1988) but also the result of microbial interaction.

Table 29. Summary of chemical composition of satho produced by various forms of start culture

Test Parameters	Type of fermentation		
	Conventional	Semi-conventional	Pure culture
Alcohol(%v/v)	10.83	8.41	7.60
Acetaldehyde(mg/l)	40.0	15.0	24.3
Diacetyl, total(mg/l)	0.23	0.19	0.67
2, 3 – Pentandione, total (mg/l)	0.02	0.03	0.21
Ethylacetate (mg/l)	7.2	37.6	26.8
i -Amylacetate(mg/l)	< 0.1	3.3	0.8
n – Propanol(mg/l)	41.4	45.8	25.6
i – Butanol(mg/l)	78.6	163.9	65.4
Amylalcohol(mg/l)	105.0	144.6	96.5

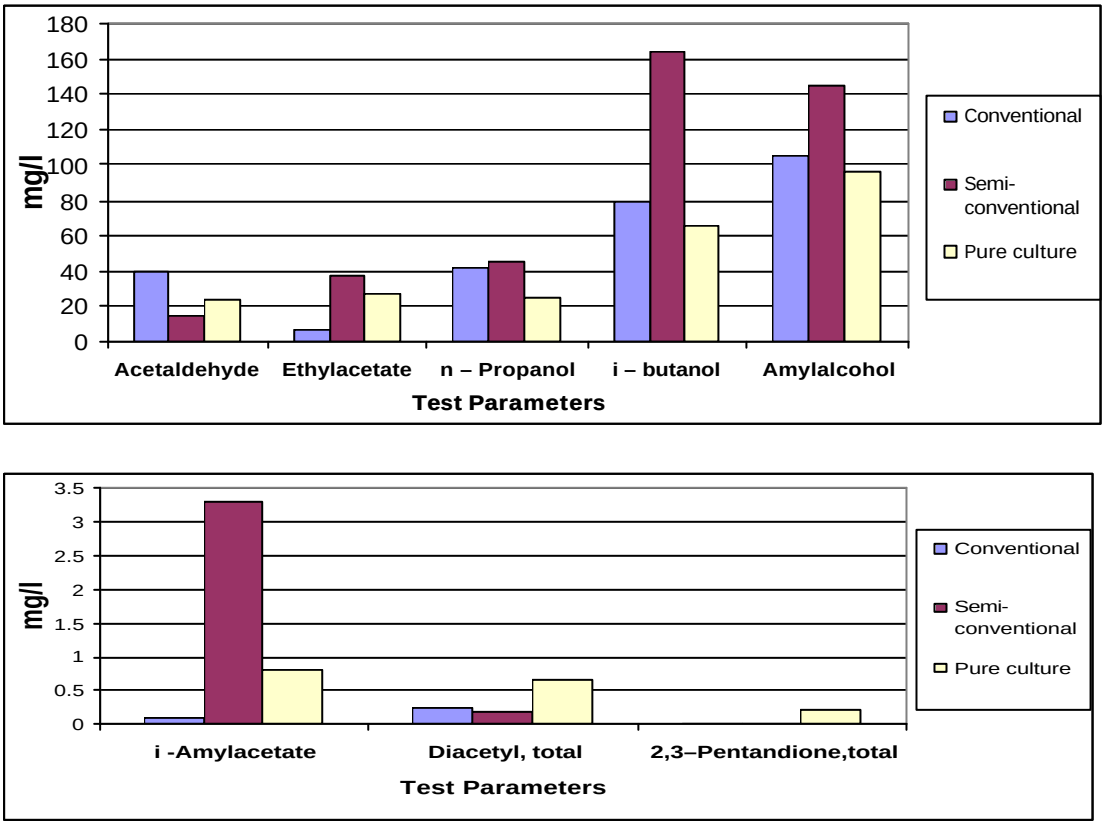
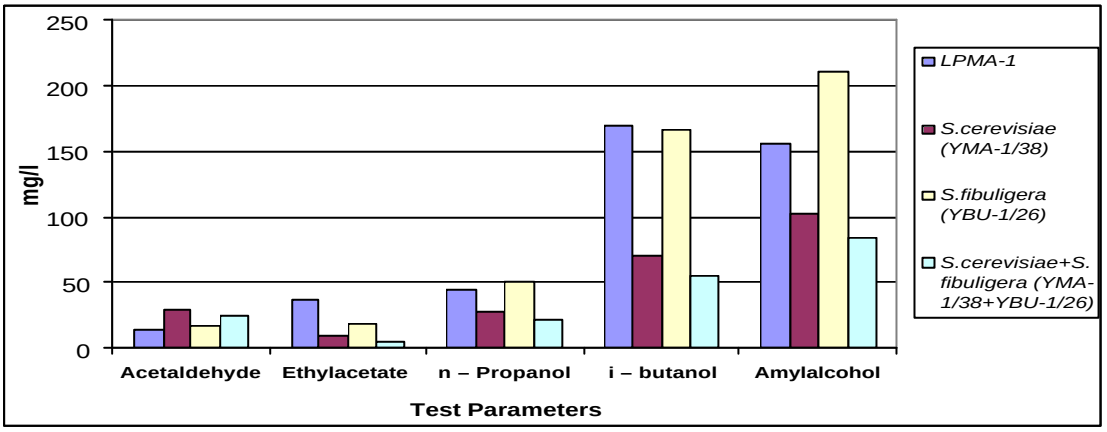


Figure 36. Fermentation by-products of satho produced by various forms of start-culture

As represented in Table 30 and Figure 36, alcoholic fermentation using pure isolate of *S.fibuligera* alone was not accepted in overview cause of low alcohol produced. Moreover, this isolate produced higher amount of ester and fusel oils than the other two treatments by pure isolate used whilst they decreased when mixed-culture of *S.cerevisiae* and *S.fibuligera* was applied. However, the selected loogpang, LPMA-1 produced highest amount of alcohol and ester compounds when compared with using in form of pure culture.

Table 30. Comparison of chemical profiles of satho produced between semi-conventional and pure isolates

Test Parameters	LPMA-1	Pure isolate of yeast		
		S.cerevisiae **(YMA-1/38)**	***S.fibuligera*** **(YBU-1/26)**	***S.cerevisiae*** + ***S.fibuligera***
Alcohol content (%v/v)	**8.59**	7.46	1.51	7.78
pH	**3.44**	3.64	3.91	3.68
Acetaldehyde (mg/l)	**15.3**	29.62	17.2	24.8
Diacetyl, tota l(mg/l)	**0.21**	0.54	3.51	0.80
2, 3 – Pentandione, total (mg/l)	**0.05**	0.15	0.07	0.30
Ethylacetate (mg/l)	**37.3**	10.1	18.5	5.4
i – Amylacetate(mg/l)	**3.3**	0.8	1.9	0.5
n – Propanol(mg/l)	**44.7**	27.6	51.7	22.0
i – Butanol(mg/l)	**169.5**	70.9	166.2	54.8
Amylalcohol(mg/l)	**155.4**	102.8	210.6	83.8



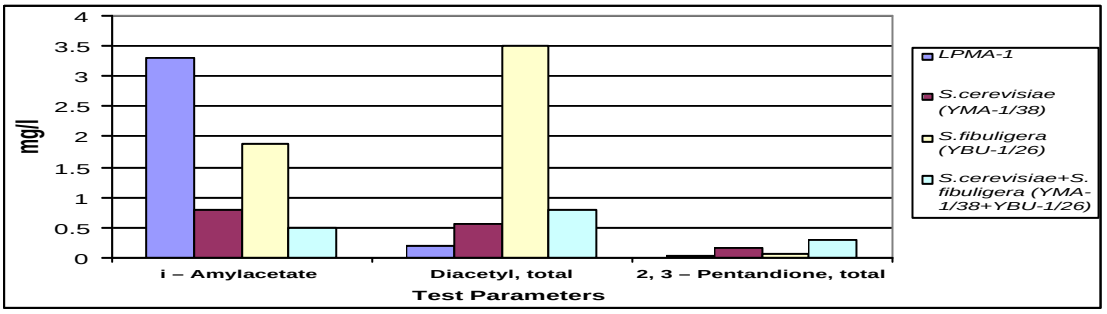


Figure 37. Comparison of fermentation by-products of satho produced between semi-conventional and pure isolates

In overview based on the basic of chemicals content as represented in Table 31,Satho produced from semi-conventional using pure isolate of mould; MNA-2/39 which identified as *R.oryzea* for saccharification and loogpang LPNA-2 for alcoholic fermentation contained lowest and highest amount of carbonyl compound and ester compounds respectively compared with conventional and pure culture fermentation. Whereas highest amount of carbonyl compound presented in satho produced in conventional. Focus on ester compounds; smallest amount was produced in conventional eventhough the same loogpang was used fro semi-conventional fermentation. For pure culture fermentation, ester compounds produced from pure isolate of *S.cerevisiae* and *S.fibuligera* alone was higher than produced from mixed of both.

Table 31. Conclusion of chemical profiles of satho produuced by various kinds of fermentation.

Test Parameters	**Type of fermentation**			
	Conventional	Semi-conventional	Pure culture fermentation	
			S.cerevisiae	*S.cerevisiae* + *S.fibuligera*
Alcohol (%v/v)	10.83	8.75	7.46	7.78
pH	3.41	3.44	3.64	3.68
Acetaldehyde(mg/l)	40.02	14.97	29.62	24.80
Diacetyl, total(mg/l)	0.23	0.19	0.54	0.80
2, 3 – Pentandione, total (mg/l)	0.02	0.05	0.15	0.30
Ethylacetate (mg/l)	7.23	37.63	10.10	5.40
i – Amylacetate(mg/l)	<0.1	3.3	0.8	0.5
n – Propanol(mg/l)	41.35	45.77	27.6	22.0
i – Butanol(mg/l)	78.56	163.87	70.9	54.8
Amylalcohol(mg/l)	105.02	144.6	107.8	83.8

5.5. Satho fermentation using rice wort

Rice wort and rice wort-base substrates were formulated and fermented using loogpang and pure isolate of yeast in the optimum condition which predetermined.

5.5.1. Quality of rice malt samples

Properties of 6 rice malts varieties were presented in Table 32

Table 32. Quality of 6 rice malts varieties.

Tested Parameters	Rice varieties					
	***SUT-1**	***SUT-2**	***SUT-3**	****SUT-4**	***SUT-5**	****SUT-6**
Water content (%)	7.4	6.8	7.3	7.0	6.9	7.4
Extract, fine (%)	62	63.3	61.9	66.8	62.8	58.4
Extract, coars (%)	60.1	61.5	60.8	64.8	61.1	55.9
Extract difference	1.9	1.8	1.1	2.0	1.7	2.5
Wort colour (EBC)	2.6	2.4	11.5	2.6	2.5	11.5
Boiled wort colour (EBC)	3.7	3.6	12.5	3.6	3.6	14.5
Protein (%)	6.3	7.0	7.7	6.4	5.9	10
Soluble N content (gN/100g malt)	0.351	0.387	0.414	0.35	0.346	**0.436**
VZ 45	10-15	10-15	30-35	10-15	10-15	30-35
Filtration time (min)	>60	>60	>60	>60	29	19
Hartong 45 (%)	39.6	31.2	-	31.1	43.3	33
Hartong 65 (%)	83.4	90.1	89.8	96.1	87.6	93.8
Turbidity (EBC)	8.86	4.7	8.79	6.5	4.01	2.7
pH	5.85	5.88	5.78	5.74	5.91	5.81
ß-glucan (mg/100gmalt)	0	0	0	0	0	0

- *were glutinous rice
- ** were normal rice,
- SUT-3 and SUT-6 were glutinous and normal black rice;

SUT- 6 was selected for further studying of mashing cause of highest soluble N content. In addition, it was attractive brown-red colour with good smell. By the way, due to low in extract yield, standard congress mashing has be modified. Modified mashing procedure was adjusted in rice malt: water ratio to be 3.5:1.0 (standard mashing is 5:1) and temperature profile was adjusted as in 4.6, quality of modified rice malt was presented in Table 35.

Table 33. Quality of the selected rice wort; SUT-6

Parameters	Content
Extract content, fine (%)	60.00
Extract content, coarse (%)	59.90
Colour (EBC)	11.5
Protein (%)	10.50
Soluble N (gN/100g malt dry basis)	0.668
Hartong 45 (%)	39.7
ß – Glucan (mg/100g malt dry basis)	<15

Rice wort prepared from rice malt SUT-6 was used as substrate for alternative satho fermentation in various formulas as following;

Table 34. Rice wort mixed substrate formulation.

Items	Formulas				
	S1	S2	S3	S4	S5
Rice wort (%)	100	80	60	50	0
Barley wort (%)	0	20	40	50	100
Total soluble solid, TSS (°Brix)	8.5	9.0	9.5	10.0	12.0
pH	5.82	5.62	5.41	5.28	4.84
Adjusted TSS (°Brix)	22.00	22.00	22.00	22.00	22.00

5.5.2. Fermentation using rice wort mixed substrates

Fermentation was carried out using the selected high performance loogpang samples; LPMA-1, LPBU-1 and LPNA-2 with rice wort mixed substrates, data was illustrated in Table 35.

Table 35. Ability of loogpang to produce alcohol in rice wort mixed substrates

No.	Source of start culture	100 % Rice wort		80% RW with hop		80% RW without hop	
		Alc. (%v/v)	pH	Alc. (%v/v)	pH	Alc. (%v/v)	pH
1	**LPMA-1**	**7.28**	**3.89**	**7.52**	**3.95**	**7.28**	**3.81**
2	**LPBU-1**	**7.48**	**3.63**	**7.68**	**3.91**	**7.19**	**3.41**
3	**LPNA-2**	**7.07**	**3.80**	**7.40**	**3.92**	**7.34**	**3.63**

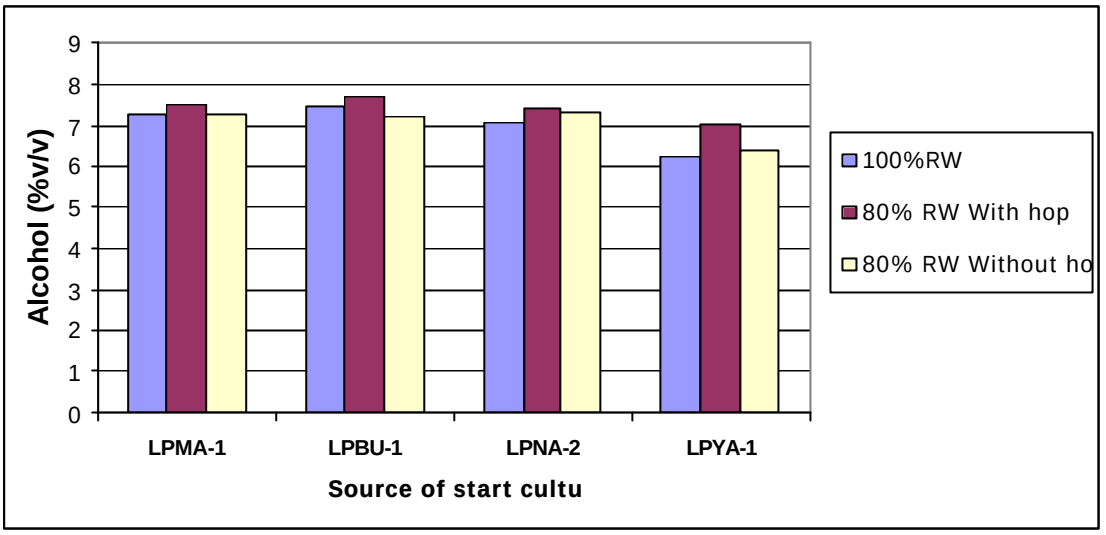


Figure 38. Ability to produce alcohol of the selected loogpang in rice wort

The results showed that hop presented in the fermented medium did not have any affect on ability to produce alcohol by all source of loogpang.

Superior pure isolates of yeast originated from LPBU-1, code; YBU-1/29 was selected for studying ability to produce alcohol using various formulas of substrate.

Table 36. Ability to produce alcohol of pure isolate in rice wort substrate

No.	Isolate no.	Substrate formula	Fermented broth analysis		pH
			Alcohol (%v/v)	Ferment ability (%)	
1.	***S.cerevisiae* (YBU-1/29)**	**S1**	3.47	62.2	4.48
2.		**S2**	4.94	52.8	4.22
3.		***S2**	5.55	58.8	4.10
4.	***S.cerevisiae* (Y-SUT)**	**S1**	6.51	66.3	4.08
5.		**S2**	6.06	60.7	4.13
6.		***S2**	3.56	37.6	4.54
7.	**WHS no.68**	**S1**	1.11	15.5	4.80
8.		**S2**	5.71	66.3	3.93
9.		***S2**	2.60	27.7	4.35

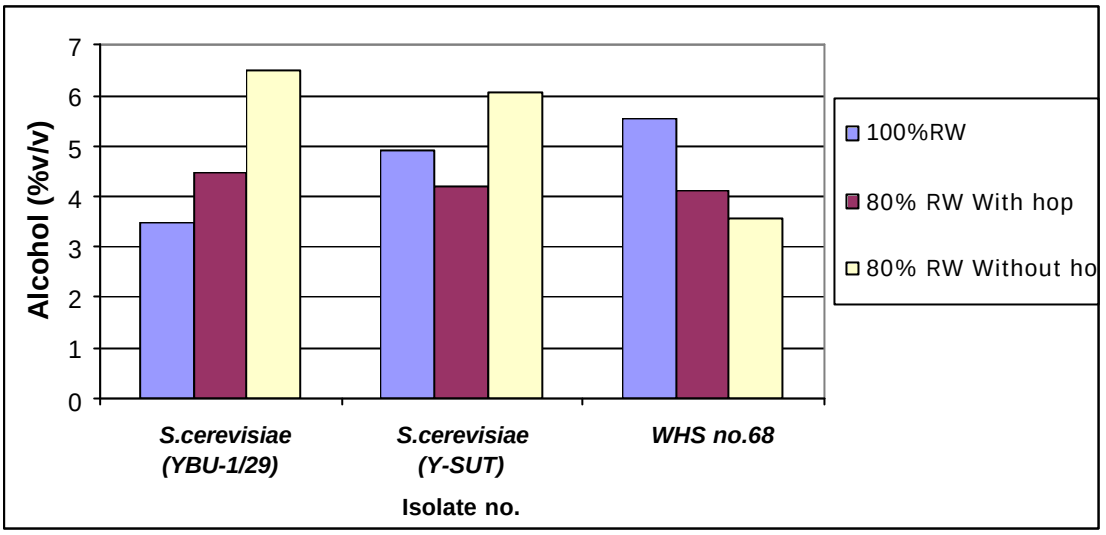


Figure 39. Ability to produce alcohol of pure isolate in rice wort substrate

Table 37. Ability of the selected loogpang to produce alcohol in various kinds of substrates

No.	Source of start culture	Type of substrate	Fermented broth analysis		
			Alcohol (%v/v)	Fermentability (%)	pH
1	LPMA-1	SRL	8.59	85.2	3.44
		*RWM	7.28	79.2	3.81
		100%RW	7.28	72.6	3.89
2	LPBU-1	SRL	8.92	86.9	3.43
		*RWM	7.19	75.7	3.41
		100%RW	7.48	75.1	3.63
3	LPNA-2	SRL	8.75	85.9	3.46
		*RWM	7.34	78.5	3.63
		100%RW	7.07	70.4	3.80
4	LPYA-1	SRL	8.54	83.5	3.43
		*RWM	6.39	69.5	3.30
		100%RW	6.22	69.6	3.31

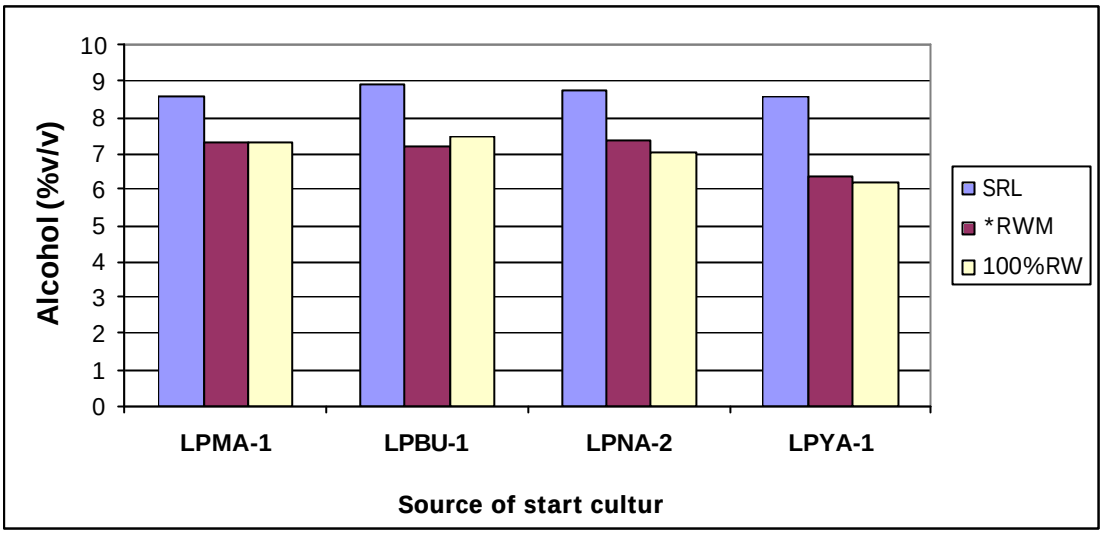
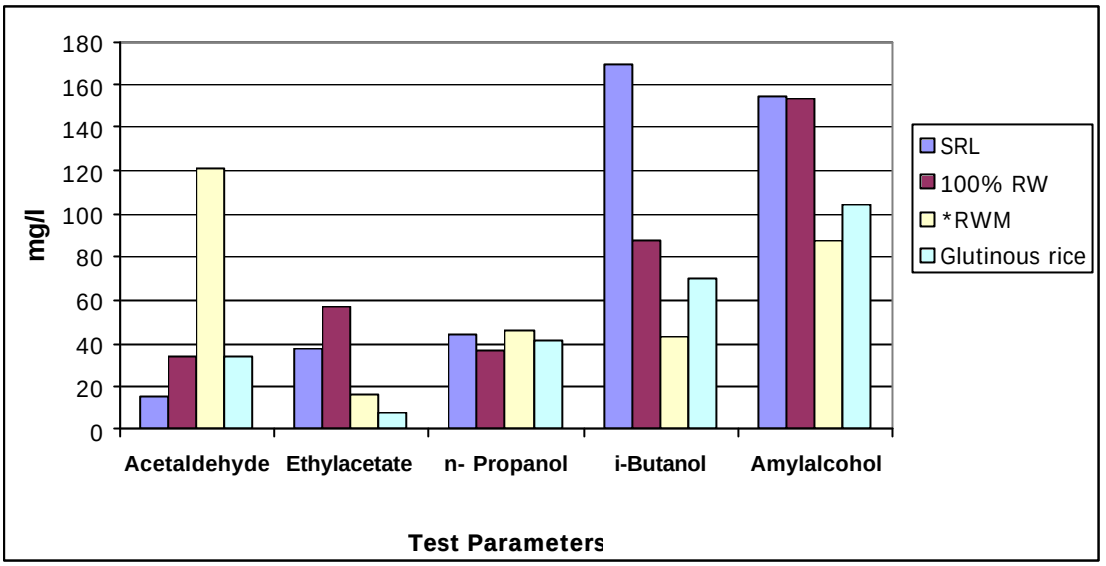


Figure 40. Ability of the selected loogpang to produce alcohol in various kinds of substrates

Among these 4 types of substrates used; saccharified rice liquid, 100% rice wort , rice wort mixed with and without hop and steamed glutinous rice, high ester content was found in 100% rice wort for all 3 loogpang samples which fall in the range of 54.6-61.9 mg/l whilst fusel alcohol presented highest amount in satho produce using saccharified rice substrate.

Table 38. Fermentation by-products in various kinds of substrate using LPMA-1

Test Parameters	**Type of substrates**			
	SRL	**100% RW**	***RWM**	**Glutinous rice**
Acetaldehyde (mg/l)	15.3	34.5	121.8	34.37
Diacetyl(mg/l)	0.21	0.32	0.29	0.15
2,3- Pentanedione, total,(mg/l)	0.05	0.11	0.11	0.02
Ethylacetate(mg/l)	37.3	57.1	16.5	7.86
i-A mylacetate(mg/l)	3.3	4.8	1.1	<0.1
n- Propanol(mg/l)	44.7	36.3	46.3	41.59
i-Butanol(mg/l)	169.5	87.8	43.1	70.02
Amylalcohol(mg/l)	155.4	153.6	87.0	104.57



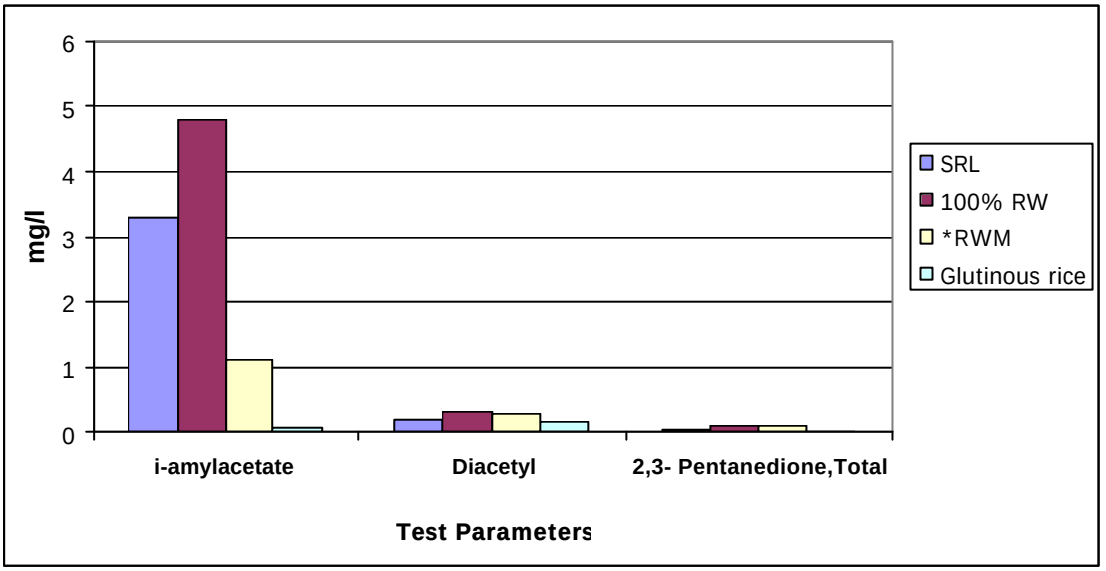


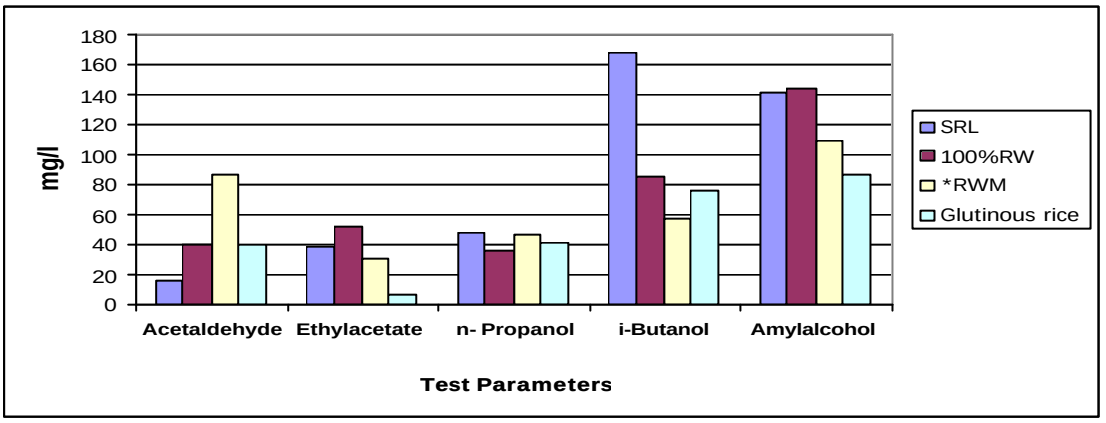
Figure 41. Fermentation by-products in various kinds of substrate using LPMA-1

Remark;

SRL = Saccharified rice liquid
*RWM = Rice wort mixed, S2 formula
100%RW = 100% rice wort, TSS was adjust to be 22 (°Brix)

Table 39. Fermentation by-products in various kinds of substrate using LPBU-1.

Test Parameters	Type of substrates			
	SRL	**100%RW**	***RWM**	**Glutinous rice**
Acetaldehyde (mg/l)	15.9	40.0	86.7	39.09
Diacetyl (mg/l)	0.18	0.31	0.42	0.43
2,3- Pentanedione,total(mg/l)	0.03	0.07	0.08	0.08
Ethylacetate (mg/l)	38.7	52.1	30.5	6.82
i-Amylacetate (mg /l)	3.5	4.8	2.1	<0.1
n- Propanol (mg/l)	47.6	36.8	46.6	41.18
i-Butanol (mg/l)	167.6	86.1	58.1	76.36
Amylalcohol (mg/l)	141.8	144.9	109.1	87.09



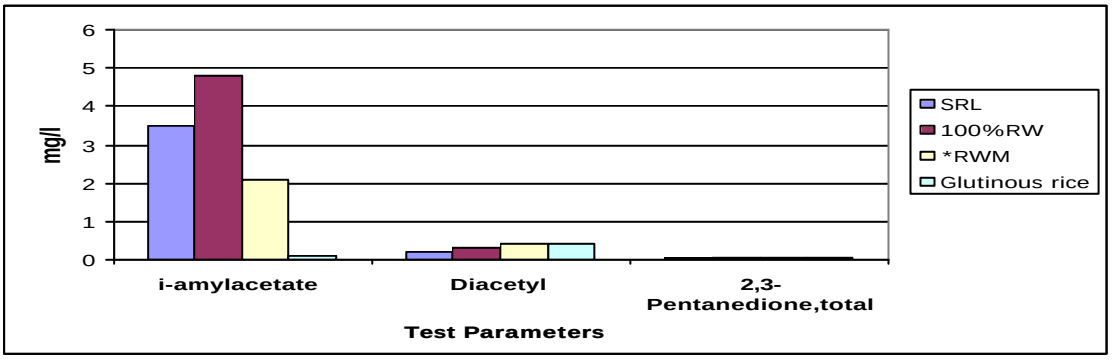
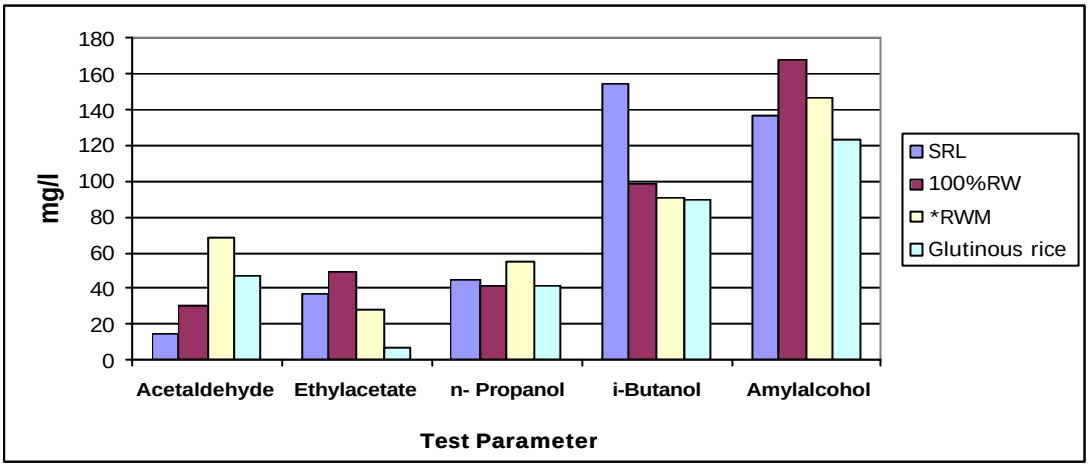


Figure 42. Fermentation by-products in various kinds of substrate using LPBU-1

Table 40. Fermentation by-products in various kinds of substrate using LPNA-2

Test Parameters	Type of substrates			
	SRL	**100%RW**	***RWM**	**Glutinous rice**
Acetaldehyde (mg/l)	13.7	30.3	68.9	46.59
Diacetyl(mg/l)	0.19	0.31	0.41	0.11
2,3Pentanedione,total(mg/l)	0.03	0.08	0.11	0.01
Ethylacetate(mg/l)	36.9	49.2	27.8	7.01
i-Amylacetate(mg /l)	3.3	5.4	2.2	<0.1
n- Propanol(mg/l)	45.0	41.4	55.7	41.29
i-Butanol(mg/l)	154.5	98.3	90.5	89.30
Amylalcohol(mg/l)	136.6	168.0	146.2	123.39



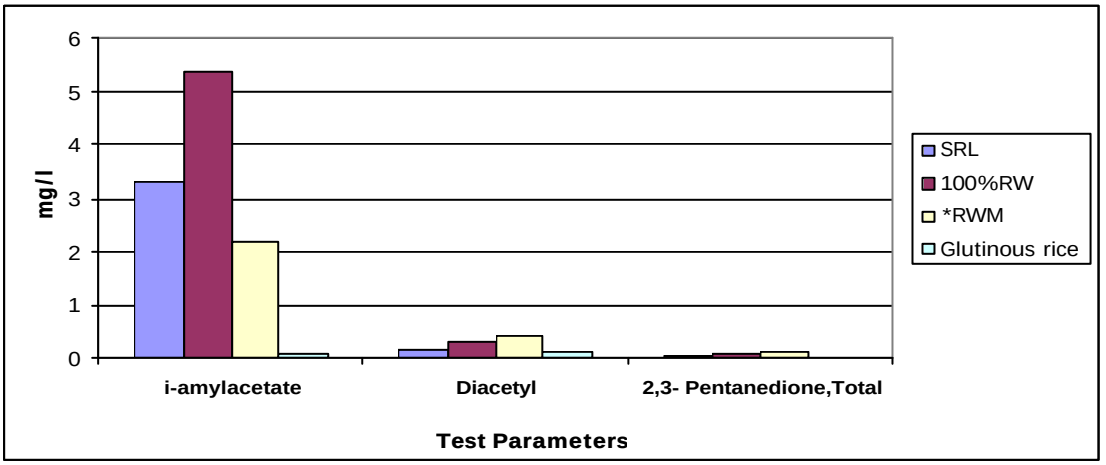


Figure 43. Fermentation by-products in various kinds of substrate using LPNA-2.

Table 41. Conclusion of chemicals composition of satho produced by various kinds of fermentation

Test Parameters	Types of fermentation				Ref.product
	conventional	Semi - conventional	Pure culture	Rice wort	
Alcohol content (%v/v)	10.83	8.75	7.46	7.01	9.89
pH	3.41	3.44	3.64	3.65	4.76
Acetaldehyde (mg/l)	40.02	14.97	29.62	34.5	19.5
Diacetyl,total (mg/l)	0.23	0.19	0.54	0.32	0.15
2, 3 – Pentandione,total (mg/l)	0.02	0.05	0.15	0.11	0.01
Ethylacetate (mg/l)	7.23	37.63	10.10	57.1	51.5
i – Amylacetate (mg/l)	<0.1	3.3	0.8	4.8	0.3
n – Propanol (mg/l)	41.35	45.77	27.6	36.3	34.7
i – Butanol (mg/l)	78.56	163.87	70.9	87.8	86.6
Amylalcohol (mg/l)	105.02	144.6	107.8	153.6	105.5

VI. DISCUSSION

6.1 Screening of loogpang samples; Three out 11 of samples were selected based on overall quality of satho produced by focus group discussion using 5 point scale of Hedonic Test. The three selected satho products produced using these loogpang samples were analyzed in chemical composition compared with the selected reference famous product from market. The comparison showed that significantly different was found between product samples and reference in the concentration of carbonyl compounds, esters and total acidity as depicting in Table 14 and Figure 28.This might be the resulting from immature as well as source of form of start culture used in product samples. Whereas, for reference, it would be matured and pure culture of yeast and mould were applied in mass production. This was confirmed that complex community of microbial in loogpang and maturation step play an important role and affected either positively or negatively on the product quality. However, for application these loogpang samples for mass production scale, care must be done on the way of their preparation and ingredients, completed periodic examination of microorganism presented, genetic fingerprint and standard fermentation pattern trials.

6.2 Enumeration, Isolation and Identification; each of the selected loogpang sample consisted of the diversity of yeast and mould population which confirmed the previous studied by the several reports. Focus on their functionalities of satho fermentation, mould; *R.oryzea* and yeast, *S.cerevisiae* were the main isolates responsible for saccharification and alcoholic fermentation respectively. This finding was agree with the former studied by Rattanapreedakul (2006) whilst in contrast with Limtong *et al.*, (2002) recommended that instead of *S.cerevisiae* but *P.anomala* and *I.orientalis* were high alcohol producing yeast found in loogpang.This indicated the diversity of loogpang produced by various producers using various kinds of ingredients which directly affected on microbial community and finally on product quality. For isolation of yeast and mould in loogpang samples, in order to achieved as many isolates as possible, other media such as dichloran 18% glycerol agar (DG18; Hocking,1981), malt yeast 50% glucose (MY50 G; Pitt and Hocking,1985), or malt yeast 5% sodiumchloride 12% glucose (MY 5-12; Pitt and Hocking,1985) should be applied for xerophilic fungi or halophiles. Focus on mould isolate found; 2 isolates of *Aspergillus sp* presented in 2 samples (LPMA-1 and LPBU-1), these isolates must to be further identified, which selective medium for *A. flavus* and *A. parasiticus*, the aflatoxin producers using Aspergillus flavus and parasiticus agar (AFPA; Pitt *et al*., 1983) might be basically used. Production of high performance loogpang, periodic examination of microorganism and the way of preparation to prevent growth of undesired or toxin producing microbial together with scientific knowledge must to be applied. Whereas lactic acid bacteria which might be involved must be monitored and their interaction among them during production process. Moreover, some of lactic acid bacteria which reported to found in these start cultures (Hesseltine and Ray, 1988) should be investigated even the exactly role was unknown. Whereas the other isolates of mould presented in the selected loogpang samples should be identified in molecular level which could be defined the exactly function on the production process.

6.3 Satho fermentation; for batch fermentation using steamed rice as substrate, semi- conventional fermentation using pure isolate of mould;*R.oryzea* for saccharification following by the selected loogpang samples,LPMA-1,LPBU-1 or LPNA-2 for alcoholic fermentation might give full taste of flavour compared with conventional and pure isolate based on chemical profiles. This results indicated that not only pure isolate of *S.cerevisiae* or *S.fibuligera* but also the role of other microbial such as lactic acid bacteria or the resulting from microbial interaction; yeast-yeast, mould-mould, yeast-mould etc., Whereas, fermentation using 100 % rice wort substrate together with the high quality selected loogpang samples performed highest amount of ester compounds compared with the other 3 types of substrates; saccharified rice liquid (semi-conventional fermentation), rice wort mixed with 20% barley wort and steamed glutinous rice in conventional fermentation. This might be the resulting from suitable combination of rice wort as well as mixed culture of microbial consisted in the selected loogpang. This research attempted to produce high quality of satho product by defining and optimized the production process in overview using scientific knowledge on interdisciplinary of biotechnology focus on main chemical compositions which affected on product quality. However, in order to achieved high product quality, the principal ingredients; rice and the selected loogpang which were the crucial factors must be further intensively investigated in detail on their diversities. The suitable rice variety for satho making in form of steamed rice and rice wort for alternative fermentation has to be studied in term of physicochemical change and the way of effective preparation as well as for making

sake in Japan. Whereas the high performance of loogpang samples must be investigated in the way of preparation which affected on product quality, all presented microorganism, yeast, mould and might be bacteria has to be periodic identified and monitored on their interaction. However, the dynamic of microorganism involved in all steps of production process, as well as, biochemical profiles must be monitored for control the course of the fermentation process which leads to the production of end product with the desired properties. Moreover, based on chemical compositions in overview, as satho produced from alternative fermentation using rice wort contained of high volatile esters, this might be the good signal for intensively studied in order to replace sacccharification step by mould. This would be possible when all important factors; rice, microorganism and fermentation condition were optimized. Malting, mashing and fermentation technology accompanied with quality control system has to be applied. Focus on finished product quality; maturation which consisted of the complex reaction of formation and break-down of some fermentation by-products must be studied as well, chemical profiles of all detail attributes must be defined by sensory evaluation using Quantitative Descriptive Analysis (QDA).

VII. CONCLUSION

The fermented beverage product from rice using indigenous start culture, satho, is the result of complex interaction of microorganism, especially yeast and mould. From 3 out 11 of loogpang samples which gave the accepted product quality in overall consisted of 10 and 16 isolates of mould and yeast respectively. Among mould isolates, high efficiency of hydrolysis and starch saccharification was identified belonging to *R.oryzea* whilst yeast; *S.fibuligera* and *S.cerevisiae* were presented for all samples which *S.fibuligera* predominated.Whereas, *S.cerevisiae* which found 25% of all isolates obtained was the main isolate responsible for alcoholic fermentation in satho fermentation. High performance of loogpang for satho making was considered to present of mould, *R.oryzea* and yeast, *S.cerevisiae*, therefore. The production process of high quality satho has been optimized using various kinds of start culture and substrates. Fermentation using pure culture of mould *R.oryzea* and yeast, *S.cerevisiae* were carried out using 10^6 spores/g steamed rice, for 4 days at 30 °C and for 2 weeks of alcoholic fermentation resulting in 37.8 ° Brix of total soluble solid and 8.60 %v/v of alcohol. Chemical composition; alcohol, acetaldehyde, diacetyl, 2,3-pentanedione, ethyl acetate, i-amyl acetate,n- propanol, i-butanol and amylalcohol which mainly affected of taste and flavour were considered of satho produced. For batch culture; 3 types of fermentation; conventional, semi-conventional and pure culture using steamed rice as substrate were carried out.The results showed that satho produced by semi-conventional fermentation using pure isolate of *R.oryzea* which previously isolated for saccharification following by high quality of loogpang; LPMA-1, LPBU-1 or LPNA-2 for alcoholic fermentation contained higher amount of ester compounds, as well, fusel alcohols than the others. Whereas, satho produced under unlimited substrate could increased not only alcohol but also ester compounds formation.To avoid saccharification by mould; rice wort prepared from suitable rice malt variety accompanied with appropriate mashing program might be applied as alternative substrates for produce satho. Among various studied substrates; satho produced from 100% rice wort using all high performance loogpang samples gave highest content of ester compounds which fall in the range of 54.6-62.9 mg/l whilst highest amount of fusel alcohols presented in satho produced from saccharified rice liquid substrate. Therefore, in the basic overview of chemical

composition, high quality of satho could be produced either by semi-conventional using steamed rice or high quality rice wort using the selected high quality of loogpang. Howevern for overall taste and aroma, the matrix effect model of this product has to be intensively defined.

VIII. SUMMARY

High quality of satho production has been studied using various kinds of start cultures and substrates. High performance of mould and yeast isolates were achieved from the 3 selected out of 11 loogpang samples, collected from the main satho producing areas, based on their saccharifying and alcohol producing ability respectively.Identification method using real time PCR S.c.assay and D1/D2 26s rDNA sequencing was carried out for yeast whilst for mould, ITS1-5.8s-ITS2 rDNA was sequenced.There were 5 types of mould; *R.oryzea, Mucor sp, Aspergillus sp, Penicillium sp* and *Fusarium sp*. which *R.oryzea* presented and responsible for hydrolysis of starch and saccharification of all the selected high proficieny loogpang samples.Whereas yeast,were belonging to *S.fibuligera,S.cerevisiae,S.malanga* and *I.oreintalis* which *S.fibuligera* was predominants found in all samples.However, *R.oryzea* and *S.cerevisiae* were the main isolates responsible for the principal of satho production step; saccharification and alcoholic fermentation respectively.Saccharification using 10^6 spores/g steamed rice at 30 ° C for 4 days resuled in 37.8 °Brix of total soluble solid.Satho produced from various kinds of start culture and substrates were characterrised in their chemicals composition; alcohol content and the important 3 groups of fermentation by-products; carbonyl compounds(acetaldehyde,diacetyl and 2,3-pentandione),esters (ethylacetate and isoamyl acetate) and higher alcohols (n-propanol, i-butanol and amylalcohol) which mainly affected either directly or indirectly of taste, aroma and flavour of product.Satho produced in batch culture with steamed rice using pure isolate of *R.oryzae* for saccharification and the selected high quality loogpang; LPMA-1, LPBU-1 or LPNA-2 for alcoholic fermentation contained average 40.9mg/l of ester compounds, which higher than satho obtained from conventional fermentation (7.2 mg/l) and fermentation using pure culture (27.6 mg/l). Fed-batch fermentation using pure culture increased not only amount of ester compounds from 10.9 mg/l to 35.9 mg/l but also fusel alcohols which largely increased 44.78%. For alternative fermentation, which saccharification by mould was replaced by mashing of rice malt, using rice wort fermented with the mixed culture in the selected loogpang, LPMA-1, LPBU-1 or LPNA-2 showed closely of chemical profiles with the selected referenced product which contained highest amount of ester compounds ,among the studied substrates, presented in the range of 54.6-61.9 mg/l.This would be the feasibility to study for alternative processing for satho with could be overcome or diminished the disadvantage of mould saccharification,substituted by appropriate high quality rice wort fermented with mixed culture in high quality loogpang.

IX. ZUSAMMENFASSUNG

Das chemische Profil verschiedener Satho Produkte, die mit unterschiedlichen Substraten und Starterkulturen hergestellt wurden, wurde analysiert. Aus 11 loogpang Proben 9 verschiedener Quellen wurden 3 Proben bezueglich ihrer resultierenden Produkqualitaet ausgewaehlt und daraus 10 Schimmelpilz und 16 Hefestaemme isoliert. Die Verzuckerung und Alcoholbildungseigenschaften wurde zur Auswahl von hoch effektiven Schimmelpilz und Hefeisolaten herangezogen.Die Hefeisolate wurden ueber 16S-rDNA –Sequenzierung and real time PCR als S.fibuligera, *S.cerevisiae*, S.malaga and Isaatchenkia orientalis identifizert, wobei S.fibuligera die dominierende Art war. *S.cerevisiae* produzierte die hoechsten Alkoholkonzentrationen. Die Schimmelpilzisolate mit den hoechsten Verzucherungseigenschaften wurden ueber ITS1-5.8-ITS2 rDNA Sequenzierung als *Rhizopus oryzea* identifizert. Die uebrigen Schimmelpilzisolate wurden morphologisch als *Aspergillus sp. Mucor sp, Fusarium sp, and Peniciium sp.* Identifizert. Satho Produkte, die durch Starterkulturen-Fermentation, semikonventioneller. Fermentation und konventioneller Fermentation u aus den Substraten gedaemfter Reis und Reiswuezer hergestellt wurden, wurden bezueglich der chemischen Parameter Alkoholgehalt und drei Gruppen von Gaerungsnebenprodukten analysiert. Hierbei wurden Carbonylkomponenten (Acetaldehyde und vicinale Diketones), Ester(Ethyl acetate und Isoamylacetate) und hoehere Alkohole analysiert (iso-Butanol and Isoamylalkohol). Mit dem gedampften Reis als Sustrate,zeigte Satho aus der semikonventionellen Fermentation die hoechsten Esterkonzenr aenliche chemische Profile und wtrationen mit 40.96 mg/l (Starterkultur-Fermentation 7.23 mg/l konventionelle Fermentation 10.90 mg/l). Die Alternative Satho Fermentationen mit Rieswuerze als Substrat unf ausgewaehlten loogpang Proben zeigten zum Referenzprodukt sehr ahnliche chemische Profile und wies zudem die hoechste Esterkonzentration mit 61.9 mg/l auf.

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IX. APPENDIX

Medium; Composition per litre of each medium was prepared as following;

1. Basal medium (Lodder and Kreger Van Rij, 1952)

$(NH_4)SO_4$	5 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Yeast extracts	5 g
Washed agar	25 g

For yeast: 1% of tapioca starch or glutinious rice flour was added
For mould; 1% of soluble starch was added

2. Lipolytic activity;
Lipolytic activity was tested on the same basal medium but supplemented with 0.5% $CaCO_3$ and 50 ml of soybean oil

3. Proteolytic activity medium

Glucose	20 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Skim milk	10 g

4. Yeast extract-malt extract agar

Agar	20 g
Malt extracts	3 g
Yeast extracts	3 g
Peptone	5 g
Glucose	10 g

5. Yeast extract-malt extract broth

Malt extracts	3 g
Yeast extracts	3 g
Peptone	5 g
Glucose	10 g

6. Fermentation medium

Glucose	180 g
Peptone	5 g
Malt extracts	3 g
Yeast extracts	3 g

7. Plate count agar

Tryptone	5 g
Yeast extracts	2.5 g
Glucose	1 g
Agar	15 g

8. Yeast-malt extract starch agar (YMS agar)

Agar	20 g
Malt extracts	3 g
Yeast extracts	3 g
Peptone	5 g
Soluble starch	10 g

9. Dichloran Rose Bengal Chloramphenicol medium (DRBC)

Mycological peptone	5 g
Glucose	10 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Dichloran (2-6-dichloro-4-nitro-aniline)	0.002 g
Rose Bengal	0.025 g
Chloramphenicol	0.1 g
Agar	15 g

Preparation; suspened the ingredients except chloramphenicol in the water and bring to the boil to dissolve completely. Add 10 ml of 1% ethanolic solution of chloramphenicol, mix and sterilize by autoclaving at 121°C for 15 minutes. Cool to below 50 °C and dispense 15 ml amounts into steriled Petri dishes. Use immediately or store at 2-6 °C in the dark until required.

Chemicals:

1. Lactphenol-cotton blue solution

Lactic acid	20 ml
Phenol crystal	20 g
Glycerol	40 ml
Methylene blue	0.05 g
Distilled water	20 ml

2. Lugol's iodine solution

Iodine (I_2)	6.70 g
Potassium iodide (KI)	6.70 g
Distilled water	1000 ml

3. Bufferred phosphate diluent (BPD)

Stock solution:

Dissolved 34 g KH_2PO_4 in 500 ml distilled water, pH was adjusted to 7.2 (with about 175 ml 1 N NaOH) diluted to 1 litre.

Diluent:

Diluted 1.25 ml of stock solution to 1 litre with distilled water