BIOCONVERSION OF CASSAVA ROOTS TO HIGH PROTEIN PRODUCT FOR ANIMAL FEED

Miss Podjana Chumkhunthod

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2000

ISBN

การเปลี่ยนมันสำปะหลังให้เป็นผลิตภัณฑ์ที่มีโปรตีนสูงเพื่อใช้เป็นอาหารสัตว์

นางสาว พจนา ชุ่มขุนทด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2543

ISBN

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อาจารย์ที่ปรึกษา: ศ. ดร. นันทกร บุญเกิด, 56 หน้า ISBN

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปลี่ยนมันสำปะหลังให้เป็นผลิตภัณฑ์ที่มีมูลค่าเพิ่มสูงขึ้น โดยการเพิ่มปริมาณโปรตีนเพื่อที่จะใช้เป็นอาหารสัตว์ ด้วยวิธีการใช้เชื้อจุลินทรีย์ในกลุ่มของเชื้อราที่มีความสามารถในการผลิตเอนไซม์อะมิเลส การดำเนินงานได้กระทำโดยการแยกเชื้อราและยีสต์จากกากมันสำปะหลัง ข้าวหมาก และลูกแป้งจากหลายแหล่ง เชื้อที่แยกได้นำมาทดสอบประสิทธิภาพการย่อยแป้งโดยวิธีตรวจสอบด้วยไอโอดีนในอาหารที่มีแป้งเป็นส่วนประกอบพบว่า เชื้อราประเภทสร้างสายใยที่แยกได้ให้ชื่อว่า SUT1 ซึ่งอยู่ ในจีนัส *Chlamydomucor* สามารถย่อยมันสำปะหลังดิบได้ดี ค่ากิจกรรมของอะมิเลสพบว่าสูงกว่าเชื้อกลุ่มอื่นที่นำมาทดสอบ กล่าวคือมีค่า α และ β อะมิเลส 2.32 หน่วยและ 0.69 หน่วย ตามลำดับ จึงเลือกจุลินทรีย์ดังกล่าวในการศึกษาครั้งนี้ ผลการทดลองหมักมันสำปะหลังที่ผ่านและไม่ผ่านการนึ่งด้วยไอน้ำ ภายใต้สภาวะอุณหภูมิห้องโดยไม่ปรับค่าความเป็นกรดด่างของวัตถุดิบ ซึ่งโดยปกติวัสดุหมักมีค่า pH ในช่วง pH 5-7 พบว่าเชื้อรา SUT1 สามารถย่อยมันสำปะหลังที่ผ่านการนึ่งได้ดีกว่ามันสำปะหลังที่ไม่ผ่านการนึ่ง เมื่อวัดปริมาณน้ำตาลรีดิวส์ได้ค่าสูงสุดที่ 680.07 มิลลิกรัมต่อกรัม หลังการหมักเป็นเวลา 5 วัน เมื่อใช้มันสำปะหลังสดนึ่งเป็นซับสเตรด และได้นำเชื้อดังกล่าวทำการผลิตเป็นหัวเชื้อในรูปลูกแป้งของเชื้อผสมระหว่างเชื้อรา SUT1 กับเชื้อยีสต์ *Candida utilis* พบว่าสามารถลดจุลินทรีย์ในกลุ่มของแบคทีเรียที่ปนเปื้อนได้ 5 log และพบว่าสามารถเพิ่มปริมาณโปรตีนให้สูงขึ้นถึง 18.3% เมื่อมีการปรับปริมาณยูเรียที่ใช้ให้เหมาะสมในการเป็นแหล่งไนโตรเจนที่ 1% เพื่อลดต้นทุนการผลิตจึงได้พัฒนาการหมักแบบ Non- aseptic solid state fermentation ในถังหมักขนาด 540 ลิตรต่อไป จากการศึกษาขั้นต้นพบว่าได้โปรตีนที่ปริมาณ 15.3% และมีอะมิโนไนโตรเจน 11% ซึ่งมีปริมาณสูงเพียงพอที่สามารถนำไปใช้เป็นอาหารสัตว์ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2543

ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
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PODJANA CHUMKHUNTHOD : BIOCONVERSION OF CASSAVA ROOTS TO HIGH PROTEIN PRODUCT FOR ANIMAL FEED.
THESIS ADVISOR: PROF. NANTAKORN BOONKERD, Ph.D. 56 PP. ISBN

This study was aimed at producing protein-enriched animal feed from cassava roots by the conversion of cassava using amylase-producing fungi. Mold and yeast which produce amylase were isolated from cassava wastes, khao-mak and various mold-brans (look-pang). It was found that the filamentous fungi strain no. SUT1 which most likely belongs to the genus *Chlamydomucor* was proved to be the best amylase producing strain. This fungi exhibited high α- and β-amylase activities at 2.32 and 0.69 units, respectively. Pretreatment of cassava was done by steaming and non-steaming. The cassava fermentation was conducted in solid state using urea as the nitrogen source. Under room temperature and uncontrolled pH, which stands commonly at between pH 5-7, steamed cassava was saccharified better than non-steamed cassava. Reducing sugars were obtained at 680.07 mg/g from steamed raw cassava after 5 days of fermentation when using inoculum in the form of look-pang. Then dry inoculum of mixed culture between *Chlamydomucor* SUT1 and *Candida utilis* was developed, it was found that the bacterial contamination was reduced in 5 log. The protein content from this fermentation condition which was amended with 1.0% urea was reached maximum at 18.3%. To reduce the production cost, non aseptic solid state fermentation in size of 540-L was recommended. After preliminary test, protein content could be obtained at 15.3% with composed of 11% amino nitrogen that high enough to use for animal feed in further.

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ACKNOWLEDGEMENT

The Authors wishes to thank all who gave assistance in this work.

Special appreciation is due to: Professor Dr. Nantakorn Boonkerd and Assistant Professor Dr. Sureelak Rodtong for their advice and his critical review of manuscript. Assistant Professor Dr. Neung Teaumroong for continuous encouragement, helpful discussion and reading manuscript. Miss Apinya Rattanajit for her technical assistance. All staffs in Biotechnology Laboratory for helpful suggestion and friendship. Suranaree University of Technology for financial support. My family for good looking after and taking a good care for my life.

Podjana Chumkhunthod

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LIST OF ABBREVIATIONS

amino-N	amino nitrogen
ca.	calibration
cm	centimeter
conc.	concentration
°C	degree Celsius
D.W.	distilled water
DW	dry weight
et al.	et alia (and others)
e. g.	for example
etc.	et cetera, and others
Fig.	Figure
g	gram
hr	hour
min	minute
mg	milligram
ml	milliliter
mM	millimolar
N	Normal
%	percent
pp.	page
L	liter
rpm	round per minute
SSF	Solid state fermentation
SCP	Single cell protein

CHAPTER I

INTRODUCTION

As widely recognized that Thailand is one of the biggest cassava producers for the world. Cassava production has been mainly produced in the North Eastern region of country. The processing capacity is more than 20 million tons each year. The majority of total products have been exported as animal feed to European countries. Besides exportation, it has still remained in high amounts for utilizing in the country. For domestic uses, cassava is mainly served as raw materials for flour production. In general processing, large amount of its waste are always produced because starch is generally used in various industries. It is also the major feedstock in fermentation industries such as sweetener production e.g. glucose and High Fructose Syrup (HFS), dextrin and maltose (Balagopalan, 1988). To utilize surplus cassava and its waste, cassava can be converted into high valued products by saccharification into glucose by amylolytic enzymes alpha-amylase, glucoamylase and pullulanase from microorganisms. Many of these starch-degrading enzymes are usually non-toxic and safe to handle for animal feed.

1.1 Main utilization of cassava and its waste

1.1.1 Food: Generally fresh cassava root is consumed after boiling or roasting as vegetable. In some West African countries, cassava is boiled and pounded with boiled plantain, to form an elastic dough called foo-foo, which is consumed with vegetable and meat soups (Brauman et al., 1996). Peeled cassava roots are also often sliced, dried and ground into flour. Kokonte in Ghana is also prepared in the same method except that the slices, dried roots are not ground immediately after drying. Mostly, cassava is eaten in the form of a roasted granular product, prepared from peeled , grated and fermented cassava roots, known as gari in West Africa. In south America and Latin America, a product called farinha de mandioca is very popular (Adam, 1993). It is similar to gari, except that considerably less fermentation is allowed to take place in its preparation. Chickwangue is another African product, prepared by soaking cassava in water from 2-7 days until it was soften, after which the root is peeled and mashed.

The fibers are removed, and the resulting paste of firm and elastic consistency is wrapped in palm and banana leaves (Asiedu, 1989). In Philippines cassava is made into landing or cassava rice. Popular indigenous fermented foods made from cassava are summarized in Table1.

Table1. Indigenous fermented foods from cassava

Product	Geography	Substrate	Microorganism(s)	Nature of product	Product Use
Banku	Ghana	Maize, cassava	Lactic acid bacteria, yeast	Dough	Staple
Chichwangue	Congo	Cassava roots	Bacteria	Paste	Staple
Gari	West Africa	Cassava roots	*Corynebacterium manihot*, *Geotrichum candidum*	Wet paste	Eaten fresh as staple with stews,vegetables
Lafun	West Africa, Nigeria	Cassava root	Bacteria	Paste	Staple food
Tape	Indonesia	Cassava or rice	*Sacchromyces cerevisiae*, *Hansenula anomala*, *Rhizopus oryzae*, *Chlamydomucor oryzae*, *Mucor* sp., *Endomycopsis fibuliger* (*Saccharomycopsis* sp.)	Soft solid	Eaten fresh as staple

Complied from Beuchat (1991), Hesseltine (1992) and Steinkraus (1996)

1.1.2 Feeds: Cassava is processed in the form of pellets and chips which are a source of energy in animal feeds. For the production of cassava chips in tropical, the fresh roots are washed, peeled and cut into slices 3-6 cm long. Then the slices are dried on large concrete surfaces in the open air. Pellets are made from chips; dried chips are ground and hardened into cylindrical pellets about 2 cm long and up to 1 cm in diameter. The most prominent exporting countries of cassava chips and pellets are Thailand, Malaysia and Indonesia, exporting mostly to European Community and the United States of America.

1.1.3 Industrial products: Besides cassava flour production, cassava root is also an important raw material for several non-food industries such as monosodium glutamate (MSG) which is an important flavoring agent in Asian cooking. The low amylose, high amylopectin content of cassava starch give it necessary viscosity property for high quality adhesives and for use in the paper and textile industries. Various industries use it as a binding agent because it is an

inexpensive source of starch. Cassava starch is also used for the production of dextrin, which are utilized in glues production. Another industrial product made from cassava is fuel alcohol (ethanol) that has been produced in Brazil.

1.2 Composition and nutritional value of cassava

The chemical composition of cassava roots varies with maturity, variety, cultural practices, storage environment and region. Cassava roots contain 30-40 percent dry matter, of which starch and sugar are the predominant components.

The cassava root has an average composition of 60-65% moisture, 30-35% carbohydrate, 0.2-0.6% other extractives, but extremely low in protein (1-2% crude protein). However, the roots are rich in calcium and vitamin C. The roots contain significant amounts of vitamin C about 35 mg per 100 g fresh weight (Cock, 1982; Balagopalan, 1988 and Asiedu, 1989). Although cassava and its waste have low nutrients but high carbohydrate content. It can be converted by microbial enzymes to be carbon source for biomass protein production. If protein content could be increased by microbial biomass, it might be served as animal feed, consequently the capital cost of animal feed could be reduced. The main amino acids present in cassava protein are arginine, histidine, isoleucine, leucine and lysine. However, sulphur amino-acids are deficient. In addition to these constituents, cassava roots contain a concentration of prussic acid in the range of 10-490 mg/kg root (Asiedu, 1989).

Cassava starch composes of two types of polymers of alpha-D-glucose so called amylose and amylopectin with linear and branched structures, respectively (Menezes, 1978). The enzymatic processes for converting starchy raw materials into glucose are liquefaction with $\propto$, β-amylase and saccharification using glucoamylase. The α-and β-amylase randomly hydrolyze $\propto$-(1, 4) linkage to liquefy starch and mainly produce maltose, but they do not hydrolyze $\propto$-(1, 6) linkages therefore starch is not completely broken down. To complete starch hydrolysis; exoamylase, amyloglucosidase or glucoamylase should be used to attack $\propto$-(1, 4) linkages from the nonreducing end releasing D-glucose molecules (Fig. 1). The hydrolysis also proceed on $\propto$-(1, 6) bond, but is much more slowly than $\propto$-(1, 4) linkages (Suckling, 1990). Amyloglucosidase acts synergistically in raw starch hydrolysis. The synergistic actions retarded by a decrease in the molecular weight of the substrate. Therefore, screening of microorganisms

producing high α-and β-amylase were investigated to enhance bioconversion of cassava for enriched feed production. These amylases derived from either bacteria or molds. For example, *Aspergillus awamori*, *Endomycopsis* sp., *Saccharomyces cerevisiae* var. *diastaticus*, *Bacillus megaterium*, *B. stearothermophilus* and *B. subtilis* etc. (Takahashi et al., 1978; Charoensiri et al., 1990 and Fujio and Elegado, 1993 and Zeikus, 1991).

Sreeramamurthy (1945) reported that digestibility of uncooked cassava starch is 48.3%, while cooked starch has a digestibility of 72.9% and it was digested to a greater extent by enzyme. Therefore, cassava can be converted to higher value animal feed not only by enrichment it with biomass protein but also increasing in digestibility.

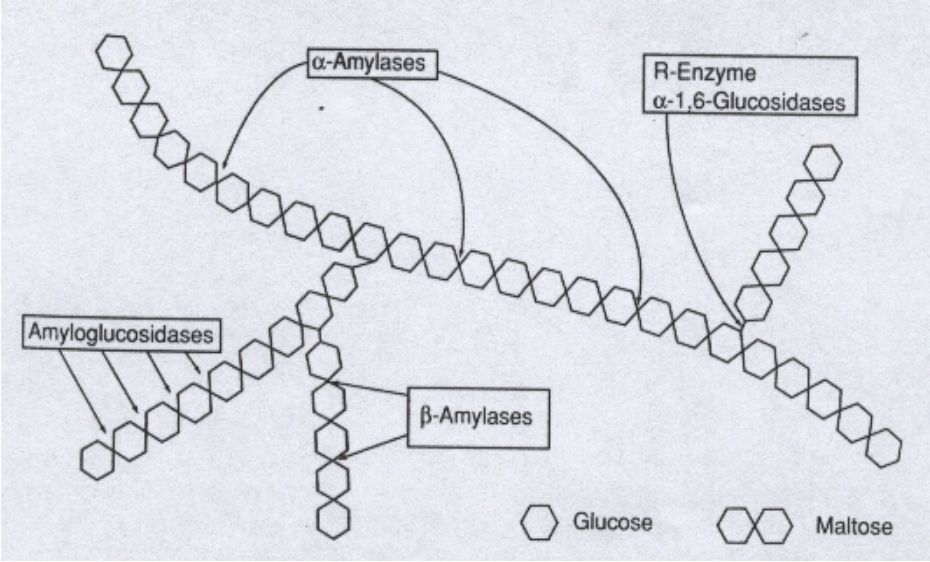


Fig. 1. Site of action of different amylase enzymes on starch (From: Helmut, 1998)

1.3 Toxicological status

Cassava contains two major cyanogenic glycoside: linamarin and lotaustralin. Both glycosides are hydrolyzed to produce hydrocyanic or prussic acid (HCN), a poison, when they come in contact with the enzyme linamarase, which is released when the cells of cassava roots are ruptures. The degradation of linamarin and the subsequent production of cyanide were depicted in Fig. 2.

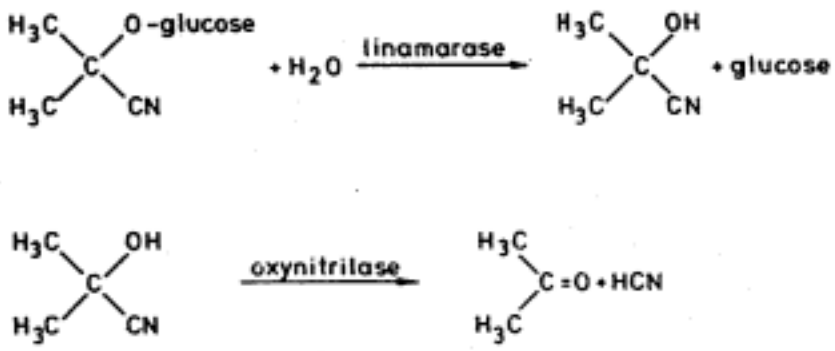


Fig. 2. The degradation of linamarin (From: Asiedu,1989)

On digestion, cyanogenic glucosides present in the cassava root are broken down and cyanide can be released. Therefore, these have to be detoxified before consumption. This can be achieved in a variety ways but it is often done by washing the cassava in clean water or by fermenting it. The prussic acid in particular is lethal if the concentration more than about 0.1g of the food eaten by an individuals at any one time. In general, three standard methods of detoxification are employed: (a) microbial detoxification through fermentation; (b) decomposition of the glycosides by heating them; and (c) rupture of the roots to allow intimate interaction between linamarase and the glycoside, then expressing or volatilizing the resultant products of hydrolysis (Ruffle, 1998 and Kanjanape, 1995).

1.4 Cassava as a sole carbon source

Cassava composes of starch (polysaccharide) which is the major source of carbohydrates. From saccharification process, endoamylase randomly hydrolyzes ∝-(1, 4) linkage to produce maltose. However, these enzymes cannot hydrolyze ∝-(1, 6) linkages so starch is not completely broken down. When it is combined with exoamylase that removes glucose one unit at a time from the non-reducing at ∝-(1, 4), ∝-(1, 6) as ∝-(1, 3) position, thus glucose could be produced. (Galliard, 1987). These enzymes have been found in various fungi and yeasts.

1.4.1 Essential amylolytic strains

The essential amylase producers range from *Aspergillus oryzae* used in Japanese sake manufacture, to molds of the genera *Mucor* and *Rhizopus*, to *Amylomyces rouxii* and others that play an important roles in the fermentation.

Pichyangkura and Kulprecha (1977) examined 52 samples of mold bran (look-pang) from various parts of Thailand. The look-pang was used as a starter in tape like products and for production of alcoholic rice wines. All starters contained filamentous fungus *Amylomyces*. Samples from the north contained also *Amylomyces* and *Aspergillus* while from central part contained five genera: *Amylomyces*, *Rhizopus*, *Aspergillus*, *Mucor* and *Absidia*. For the sample from the South contained *Amylomyces* and *Rhizopus*. The present study was undertaken to determine if the species of fungi found in the various look-pang from the orient were alike or whether different species occurred between such diverse areas as Thailand and Indonesia.

Interest in traditional food among local food scientists has resulted in the improvement of many local traditional foods popular only in the home town country. One such food is "khao-mak" a sweet fermented food made from glutinous rice and usually consumed as a dessert or a snack. Many efforts have been concentrated on the selection of the desired microorganisms, the preparation of pure culture inoculum, scale up of inoculum by the entrepreneur, and adaptation of microbiological principle and modern technology in the processing steps incorporating hygiene and sanitation and modern packaging for longer shelf life and attractive presentation.

In the acceptance of mold-fermented foods, cassava roots could be processed in the same manner of khao-mak that is well known as the fermented rice product of Thailand. It is also known as lao-chao by the chinese. Glutinous rice is first steamed and cooked, then mixed with a small amount of commercial starter known as look-pang. Filamentous fungi may also play a role in the khao-mak preparation. According to Hesseltin (1992) and Chatisatienr (1978), members of the mucoraceous fungi, including *Rhizopus oryzae*, *R. chinensis* and *Chlamydomucor oryzae* can be consistently isolated from loa-chao. *Endomycopsis*, one of the few yeasts capable of utilizing starch, is also integral part of necessary microflora. The starters are generally complex mixtures of essential and nonessential microorganisms depending on the conditions of fermentation. The basic starchy substrates can be converted either to alcoholic beverages or to sweet/sour alcoholic pastes.

1.4.2 Fermented cassava as a model for saccharification

Fermented cassava has been reported as tape ketella in Indonesia. The product is white appearance, soft texture and pleasant, sweet alcoholic aroma and flavor. In the traditional process, fermentation is initiated by the addition of powdered ragi made from rice flour containing the desired fungi. In practical sense, yeasts and molds naturally present in the environment and on equipment used to manufacture tape serve as inocula for preparing ragi. For the preparation, cassava is steamed or cooked until soft, spread in thin layers in bamboo trays, inoculated with powdered ragi, covered with a banana or other suitable leaf and allowed to fermented for 1 to 2 days before consumption. Several researchers have studied the microorganisms essential for tape fermentation. The presence of amylolytic molds and alcohol-producing yeast appears to be necessary for preparing good tape. Cronk et al. (1977) investigated the basic biochemical changes that occur during a typical tape ketan fermentation under pure culture condition. *Amylomyces rouxii* (formerly *Chlamydomucor oryzae*) in combination with eight strains of yeast that had been isolated from ragi-tape, with particular emphasis on *Endomycopsis burtoni*, were evaluated. It was demonstrated that the use of pure culture starters for tape fermentation on industrial scale by using a mixture of *Chlamydomucor oryzae* and *E. fibuliger* originally isolated from Indonesian ragi had good fermentation characteristics (Shaver and Batajoo, 1991 and Hesseltine, 1992).

1.5 Dry inoculum "Look pang" as starter culture

Microbial starter cultures are actually conducted for fermentation process. In the west and wherever culture collections exist, it is sometimes possible to obtain pure cultures for specific fermentations. However, there is usually some expense involved, and in villages and outlying areas the expertise and equipment for handing pure cultures are generally not available.

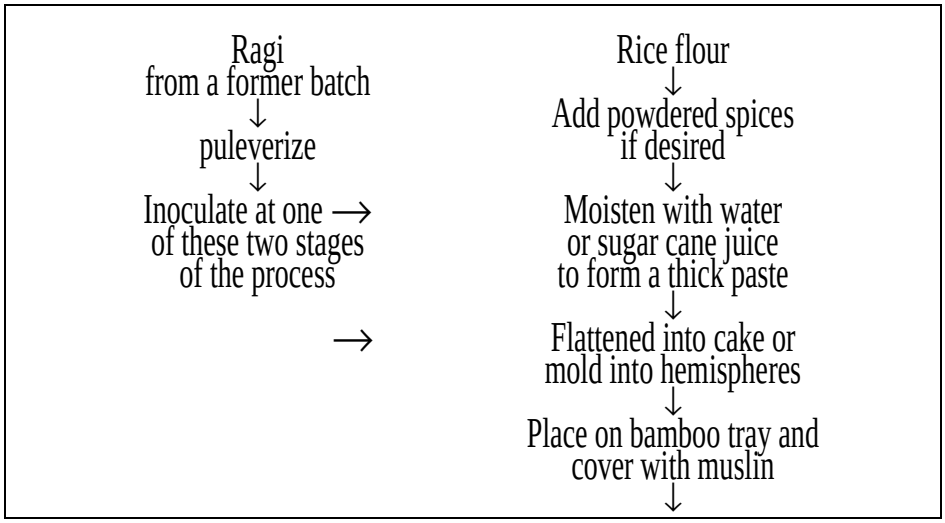
Name in the literature include Indonesian ragi-tape'; ragi *peuyeum* (for cassava); ragi *beras*; Malaysian ragi *tapai*; Malaysian *jui-paing*; Thai *look-pang* (grown on bran); Philippines *bubod levadura*: Chinese "ch'u", *levure chinoise*, *javanische* ragi, *Chinesische hefe*; Indian *bakhar*, *murcha*, *ranu*, or *u-y-iat*. The term used in India for the millet *Eleusine coracana* is also "ragi"; the two should not be confused. The name following ragi indicates use of the starter; thus, ragi-tempe is a Tempe inoculum, ragi-tape is a tape inoculant, and ragi-samsu is an

inoculum for Malaysian samsu-rice wine (Hesseltin, 1992; Penny, 1988; Quee-Lan and Zahara, 1989; Shaver and batajoo, 1991).

Except for Thai look-pang, in which the organisms are grown on bran, the predominant form of the ragi-type starters is small (3-6 cm), round, flattened cakes of rice flour on which the desired microorganisms have grown. The cakes are air- or sun-dried. Dehydration sometimes occurs simultaneously with growth of the organisms. Sometimes the rice cakes have a hole at the center so that they can be hung on a wire and displays in the marketplace.

Look pang-type inocula are available in the markets of most Asian countries. They are generally produced by household or village manufactures using closely guarded recipes. The size and shape of the rice cake, as well as the particular spices added, are the manufacturer's trademarks.

Procedures of the preparation of Indonesian ragi and Malaysian ragi tapai is given in Fig. 3. Rice flour may be mixed with dry powdered ginger, pepper, chili, garlic, cane sugar, etc. The exact additives vary along with the manufacturer. The mixture is moistened with water or alternatively sugar cane juice in northern Malaysia, either before or after moistening, or in some cases both. The mixture is inoculated with dry powdered ragi from previous batches. The cakes, which may be 3 cm in diameter and 0.5 to 1-cm thick when flattened, are placed on a bamboo tray and incubated for several days at ambient temperature followed by dehydration to preserve the cakes until needed. The air- or sun- dried ragi cakes preserve the essential microorganisms for several months at room temperature in the tropics. (Saono et al., 1974)



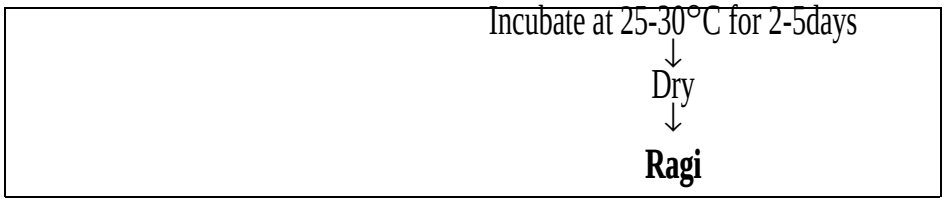


Fig. 3. Flow diagram of Ragi preparation (From Steinkraus, 1996)

In the Philippines, bubod (bubud) levadura is made by several processes. It is made either from glutinous rice, mixtures of glutinous and ordinary rice, or from rice flour. The rice is washed thoroughly, soaked overnight, drained, and ground in a stone mill. The mash is mixed with pureed ginger root and wild rood plant and molded into balled or flattened pieced of various lengths and thickness. In the Bontoc process, no ginger or unweed root is added, and this process, no inoculum is added. In the other processes, the bubod cakes are inoculated with previously prepared powdered bubod. Incubation at room temperature continues for at least 3 days during which time the desired microorganisms grow and the cakes become dehydrated. In the Ifugao process, the rice grains are roasted before soaking. The spices are added to some ragis contribute other microorganisms or may inhibit development of undesirable microorganisms (Steinkraus, 1996 and Wolf, 1997).

1.6 Protein enriched feed production

Utilization of cassava peels as substrate was performed for crude protein formation. Effects of pH, incubation temp. moisture levels and inorganic supplementation of mash on crude protein formation were studied. They found that protein production was highest in mash supplemented with urea, Temp. of 30°C, pH of 5.5 and moisture content of 130% were found as the optimum conditions (Tai and Mbongo, 1994) .

Single cell protein (SCP) production and organic substance reduction in the effluent are the aims of most bioconversion processes. *Candida* species as *C. utilis*, *C. arborea* and *C. tropicalis* are most successful for cell mass production (Balagopalan et al., 1988; De Mot, 1990; Boze, Moulin and Galzy, 1991). For microbial biomass protein production, Symba process have been proposed. This process has been used at industrial scale. Combination of amylolytic strain and *Candida utilis* are used in mixed culture condition. The latter hydrolysis starch to glucose and *C. utilis* grows on this substrates. Biomass protein could be obtained. Although many reports demonstrated that biomass production by using solid state fermentation (Daubresse et al., 1987), the protein yield was still not high enough, thus rendering modification conditions of fermentation may also be conducted to increase product yield.

In recent years, there has been increasing interest in the use of solid state fermentation processes as alternatives to submerged fermentation such as batch, continuous and fed-batch

fermentation etc. because it has lower energy requirements, produce less waste water and partly because of environmental concerns regarding the disposal of solid wastes (Lonsane and Ramash, 1990). Thus, solid state fermentation is considered to be conducted in this study for bioconversion of cassava roots for biomass protein production.

Solid state fermentation (SFF) has been widely used in the production of traditional oriental foods and alcoholic beverage: khao-mak in Thai, tempeh in Indonesia, soy sauce and sake in Japan etc. Hence, SSF has played an important role in the traditional food-making processes of oriental countries. The advantages of SSF as compared with submerged fermentation that make it interested to use in this study are; 1) SSF is relatively inadequate to bacterial contamination since bacterial growth is restricted by low water activity, serious contamination on a solid medium rarely occurs, 2) Treatment of the fermented residue is very simple since the moisture content of the fermented residue is very low, it can be dried and used as animal feed and 3) lower waste treatment and energy cost (Sato and Sudo, 1999; Hosobuchi and Yoshikawa, 1999).

Supplementation of natural nitrogenous material like chicken dung, pine apple peel, groundnut, etc. for enhancing the fermentation of cassava has been attempted (Balagopalan, 1988). It was observed that direct fermentation of cassava with *Aspergillus*, *Neurospora*, and *Rhizopus* could increase the protein content (Khor et al., 1977). Among the nitrogenous supplements tested, pineapple peel at 25% elevated the protein to 4 to 5% while mixture of 12.5% pineapple peel and 12.5% chicken dung elevated the protein to 7%. Soybean and groundnut were found to be better additives to facilitate protein enrichment.

Yuthavong and Gibbons (1994) reported that using urea as nitrogen source, maximum growth of *C. eichhorniae* in solid state process was observed. From their study, corncobs were mixed in fermentation for ventilation and after one week of incubation, 12-19% of protein yield were obtained.

Reade and Gregory (1975) found that with urea as the nitrogen source, no pH control was necessary in simple, non aseptic and low cost process for the conversion of cassava by using *Aspergillus fumigatus* in submerged fermentation.

Aspergillus sp. N-2 was selected as the best producer and its extracellular glucoamylase production was investigated. Conditions for the production were optimized for both liquid and

solid cultures, but solid state was proved to be more efficient than liquid culture (Tani, Vitchuporn and Jaroon, 1986).

Protein enrichment of cassava by *A. oryzae* in solid state fermentation has been studied. (Zvauya and Muzondo, 1993) During fermentation there was an initial increase in pH to 5. after 10 hr followed by a gradual decrease to 3.1. Amylolytic enzyme were active in the stationary phase reaching peak after 50 hr of fermentation, while yeasts and lactic acid bacteria increased during the early stages of fermentation and then leveled off. After 50 hr of fermentation protein content had increased from <2%-19%, while starch content had decreased from 80 to 4g/100g substrate.

Solid state fermentation for protein enrichment of cassava were tested on pilot units in Burundi (Central Africa) (Daubresse, Ntbashrwa, Gheysen and Meyer, 1986), provides enriched cassava contained 10.7% of dry matter protein versus 1% before fermentation. Cassava chips, proceed into granules of 2-4 mm diameter, are moistened (40% water content) and steamed. After cooling to 40°C, cassava is mixed with nutritive solution containing the inoculum (*Rhizopus oryzae*) and providing the follow per 100 g dry matter: 3.4 g urea, 1.5 g KH_2PO_4, 0.8g $MgSO_4.7H_2O$, and 22.7g citric acid. For fermentation, cassava with ca., 60% moisture content, was spread in a thin layer (2-3 cm thick) on perforated trays and slid into an aerated humidified enclosure. The incubation lasts 65 hr. The production of protein enriched cassava is 3.26 Kg dry matter/m^2 tray.

Solid state fermentation of cassava using *Rhizopus* spp. has been reported. Glucoamylase production was higher on raw cassava than on cooked cassava. After 48 hr of fermentation, the protein content was increased from 1.75% to 11.3% (Soccol, 1994).

Cephalosporium eichhorniae 152, soil filamentous fungus was isolated and used as the protein source for animal feed production. This thermophilic, obligately acidophilic filamentous grew well at 45°C and pH 3.8 and could use cassava as substrate for biomass production (Charoensiri et al., 1990).

Effects of initial moisture content (400, 450, 550, and 600 g/kg), fermentation temp. (30, 35, 40, and 45°C), and inoculum concentration (2×10^6, $2\times10^7$and 2×10^8spores/g) on protein enrichment of cassava meal were studied using 3 *Aspergillus* spp. (*A. niger*, *A. oryzae* var. oryzae CBS102.07, and *A. hennbergii* CBS118.35) (Zvauya and Muzondo, 1994). Optimum conditions

for protein enrichment was found at 550g/kg initial moisture, 40°C and inoculum conc. of 2×10^7 spores/g substrate.

1.7 Yeasts as a source of proteins

This process was aimed at conversion of starch into yeast cell mass by growing *C. utilis* in conjunction with an amylase-producing fungal species. The enzyme activity of the latter converted the starch into lower saccharides, predominantly glucose, which the fast–growing *C. utilis* then was able to use for biosynthesis of cell substance. The low–molecular–weight sugars (glucose, maltose, etc.) were assimilated by *C. utilis* as soon as they are formed (Litchfield, 1991).

Among microorganisms considered as possible food sources, yeast has attracted perhaps the greatest interest. It represents, in fact, the only sort of microbial protein that has in the past been used as a food to an extent worth mentioning. As a feed, yeast (*Candida*, *Sacchromyces*) has a well established place for many decades (Kockva-kratochivilova, 1990; Berry, 1989 and DeMot, 1990). The technology for yeast production is well known, as is yeast composition and nutrition value. So yeast may be one of the few types of microbial cell substances that could in the near future be used as food to an extent large enough to be consequence in the world food situation.

This investigation was aimed at elucidation the extensive screening of potential amylolytic microorganism strains and developed high efficient dry inoculum preparation method of mixed cultures of combined between amylolytic strain and *Candida* species. This inoculum was used as starter cultures in food fermentation based upon starchy substrates such as rice and cassava roots. Solid state fermentation was also performed in saccharification process to enhance biomass protein yield in protein-enriched feed production.

1.8 Objectives

1). To screen for the potential amylolytic strains of microorganisms to be used for conversion of cassava roots to high protein product for animal feed.

2). To obtain optimal conditions for biomass protein production using solid state fermentation.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potato dextrose agar (PDA) was purchased from Difco (Detroit, MI, USA). Others medium were prepared according to a previously published procedure.

2.1.1 Iodine solution

Dissolved 0.66 g iodine in distilled water 165 ml

2.1.2 DNS reagent

Dissolved 10 g DNS and 19 g NaOH in 1,416 ml of distilled water with warming and vigorously stirring. Dissolved 306 g sodium potassium tartate in 500 ml D.W. that contained 7.6 g phenol and 8.3 g sodium metabisulfite. The two solution were mixed and kept in brown bottle.

2.1.3 Reagents for the Kjeldahl method

Concentrated sulphuric acid

Catalyst ($CuSO_4$: K_2PO_4) in ratio 1:10

32% Sodium hydroxide solution

4% Boric acid

0.1 N Hydrochloric acid

2.1.4 Reagent formaldehyde nitrogen

Saturated potassium oxalate solution

Phenolphthalein

35% Formaldehyde solution; pH 9

0.1 N Sodium hydroxide solution

2.1.5 Reagent Ammoniacal nitrogen

Magnesium oxide

0.1 N Sodium hydroxide

4% Boric acid

Mixed indicator was prepared by dissolved 0.1 g bromocresol green and 0.2 g of methyl red with 100 ml of ethyl alcohol.

2.2 Culture medium

2.2.1 Cassava starch agar

Cassava starch	20	g
Yeast extract	3	g
$(NH_4)_2SO_4$	5	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1	g

Final pH 6 at room temperature

The composition were suspended in 1 liter distilled or deionized water and heated to boil until dissolve completely. The medium was dispensed into tubes or flasks as desired then sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

2.2.2 Raw cassava medium

Dried cassava	10	g
Yeast extract	3	g
$(NH_4)_2SO_4$	5	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1	g

Final pH 5 at room temperature

The composition were suspended in 1 liter distilled or deionized water and heated to boil until dissolve completely. The medium was dispensed into tubes or flasks as desired then sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

2.2.3 Cassava broth for amylase activity

Dried cassava	80	g
$(NH_4)_2SO_4$	2	g
$MgSO_4.7H_2O$	0.25	g
KH_2PO_4	0.5	g
$CaCO_3$	2.5	g

All component accept dried cassava were suspended in 1 liter distilled or deionized water and heated to boil until dissolve completely. The medium was dispensed into tubes or flasks as desired then sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

2.3 Isolation and collection of microorganisms

Microbial strains were isolated from cassava solid waste that obtained from local factories, khao-mak and mold bran (look-pang). The starch utilizing microorganism strains were isolated from the samples using a single colony isolation technique on 2% starch agar medium. For mold and yeast isolation, enrichment culture was carried out at 30°C in the synthetic medium containing raw cassava as the sole of carbon source. One gram of sample was added into the medium and incubated at 30°C with shaking for 72 hrs, an aliquot of the culture was streaked on to a nutrient agar plate containing 2% cassava starch. Colonies grew on starch agar medium were picked. Thirty seven strains were isolated from the sample. Cultures were maintained on 1% starch agar slants for further screening.

2.4 Screening and selecting of starch utilizing strains

For the primary screening, the isolated microorganisms were cultured for amylase production by patching the cultures to raw starch agar plate surface and allowed them to grow for 3 days at 30°C. Amylolytic zones corresponding to enzymatic degradation of starch were detected by staining the plates with iodine solution. Colonies exhibiting large clear zone were selected and cultured again on starch agar plate.

Enzymatic hydrolysis of cassava starch were tested in liquid medium. The selected strains were grown in 50 ml of starch broth containing dried cassava 8% by shaking at room temperature, 200 rpm. Samples were collected after 36 hr. After centrifugation, the supernatant was recovered as a source of secreted enzymes and frozen at -20°C until assayed. Amylase activities were determined by 3,5 dinitrosalicyclic acid micro method (DNS). One unit of enzyme activity was defined as the amount that liberated 1 μM of glucose per 30 min per ml of enzyme sample at 30°C. The selected stains were used for biomass protein production. The selected stains were cultured on starch agar slant for 72 hr then 1 ml of cell or spore suspension was inoculated into plate containing 50 g of autoclaved glutinous rice. Samples were collected after incubation everyday until 4 days. The extracellular amylolytic activity that produced during fermentation of glutinous rice was determined by measuring the amount of reducing sugars producing from substrate using DNS micro method. Physical property and aroma changes were recorded. The best strains were identified from its morphological and physiological properties.

The color and the appearances of colonies on plates were also observed. Identification of these mold and yeast strains were performed based on recommendation of Alexopoulos, Mims and Blackwell, 1996; and Kurtzman and Fell, 1998 respectively.

2.5 Dry inoculum preparation (Look-Pang)

The selected strain was grown in 2% cassava starch slants at 30°C for 4 days. Before inoculation, 5 ml of distilled water was added then cell suspension was adjusted to approximately 10^8 cells/ml). To prepare the dry inoculum, rice was cleaned and then the moisture in the rice was allowed to equilibrate by tempering for several hours before further processing. The water was changed every hour to retard contamination of lactic acid bacteria. The grain was ground with a blender for 5-10 min. at high speed. The suspension was filtered through cheesecloth and starch was obtained after water removal. Several types of spices; licorice, cinnamon, rose scarlet, rhizome, garlic, ginger, long pepper and pepper were used at 0.1 percent of rice flour. Stiff dough was made from rice flours, spices, culture suspension and water. The dough was stored under room temperature for 2-3 hr. After fermentation, the dough was formed into small loaves in diameters of approximately 1-1.5 cm, incubated at room temperature for 24 hr before air-dried (Fig. 4). Inoculum balls were stored in paper bags and kept in refrigerator at 8°C before used as the inoculum for different trials. Shelflife of inoculum was always determined monthly by enumeration of viable culture.

2.6 Efficiency tests for cassava hydrolysation and biomass protein production (lab scale)

The solid state fermentation was performed at room temperature in 250-ml beaker. Cassava fraction between 1-2 cm was added after treatment by steaming for 15 min. The inoculum powder at 0.4% was inoculated and mixed with substrates then incubated for a period of time. The fermentation product was determined for reducing sugars by DNS Micro method to indicate amylase activity. For protein content, micro Kjeldahl method was carried out.

To obtain the efficient fermentation before scale up in large scale using 540-L fermentor, the optimal conditions of fermentation were accomplished. Varying parameters such as type of substrate, urea concentration and inoculum sizes to find their effects on pH, moisture, reducing sugars, crude protein content or even amino nitrogen were tested in lab scale.

Fig. 4. Flow diagram of development of dry inoculum preparation (look-pang) of mixed culture of *Chlamydomucor* SUT1 and *Candida utilis*

2.7 Biomass protein production (Process scale up)

After fermentation in lab scale, the large scale fermentation was conducted in solid state fermentor 540-L (60×150 cm, 60 cm in depth) as shown in Fig.5. Gelatinization was carried out by steaming for 15 min with 50 Kg of processed raw cassava. The fraction of cassava between 3-4 cm was investigated. After gelatinization, the cassava was left to cool then the nitrogen source solution of 1% urea and powder of dry inoculum were mixed by hand. Heavy inoculum of mixed culture dry inoculum between *Chlamydomucor* SUT1 and *C. utilis* was added at 4%. The cassava was spread out in uniform 10-12 cm thick layer on the tray. Then the fermentation was undertaken in non-sterile conditions (pasteurization), non pH and temperature control for a period of time. Ventilation for this fermentation system could be provided by opening the lid (natural convection). This was also sufficient to permit removal of excess heat that generated from culture's metabolism in the fermentor. Changes in protein, pH, moisture, reducing sugar and amino acid were measured everyday during the fermentation. The fermented cassava was put out to dry on a tray in the sun before reduced into flour for the purpose of animal feed production.

(a) **(b)**

Fig. 5. Photos of 540-L fermentor that made from stainless steel (3 wheels drive), comportable for movement, there are tube and its connection line for steaming a: Front view, b: Inside Fermentor have sieve to contain substrate during sterile and fermentation.

2.8 Enzymatic assay

Measurement of ∝-amylase activity was performed in test tubes by adding 0.5 ml enzyme solution (diluted if necessary) to a 0.5 ml substrate solution containing 0.0067 M NaCl and 1% soluble starch in 100 ml of 0.02 M phosphate buffer (pH 6.9). After 10 mins incubation at 30°C, the reaction was terminated by adding 0.5 ml 3, 5-dinitrosalicylic acid solution and quickly placing the reaction tubes into a boiling water bath for 5 min. The reaction tubes were then cooled in running tap water and diluted with 4 ml double-distilled water. The extent of product formation by enzyme reaction was monitored at 540 nm absorbance (A_{540}). Glucose was used as a standard. One unit of ∝-amylase activity was defined as the amount of enzyme required to liberate one μM of glucose/min. or in terms of milligrams of maltose ($C_{12}H_{22}O_{11}.H_2O$) liberated in 1 minutes at 30°C by 1 ml. of the enzyme solution (Bernfeld, 1951), even though, in the case of amylase action, the actual reaction products are dextrin rather than maltose. Specific activity was expressed as amylase activity per milligrams of protein. Protein is determined according to Lowry et al., 1951. For β-amylase 0.016 M acetate buffer, pH 4.8 were investigated. Glucoamylase activity, it was assayed in the same manner as α-amylase except that the reaction was carried out in 0.05 M sodium acetate buffer, pH 4.5. One glucoamylase unit was defined as the amount of enzyme that releases 1 μM glucose/min. (Tan, Febguson and Cariton, 1984)

2.9 Analytical methods

2.9.1 Moisture

The dry matter and the moisture content were determined by drying at 105°C in the oven. (AOAC, 1990).

2.9.2 Crude protein

Total nitrogen was determined by the Kjeldahl method. The proteins were expressed in terms of total real nitrogeneous matter (TRNM) and obtained by multiplying by 6.25 by following AOAC, 1990.

2.9.3 pH

The pH was measured in a mixture obtained by homogenization of 1 g sample diluted in 5 ml distilled water using a Denver Instrument pH meter, model AP5.

2.9.4 Reducing sugars

DNS colorimetric determination of reducing sugars (James, 1995). Alkaline 3,5-dinitrosalicylic acid (DNS) forms a red-brown reduction product, 3-amino-5-nitrosalicylic acid, when heated in the presence of a reducing sugars. The intensity of the color developed at 540 nm was used to determine the available carbohydrate content of the food following hydrolysis of carbohydrate to reducing sugars.

The samples (1 ml) were added into 3.0 ml of the reagent then mixed well. The mixtures were heated at 100°C for 10 min. After rapid cooling to room temperature, the absorbance were determined at 540 nm. Reducing sugars content were calculated along with standard curve of glucose.

2.9.5 Determination of amino nitrogen

Amino acid nitrogen was determined as the difference value between formaldehyde nitrogen with ammoniacal nitrogen in gram of sample by method of TISO, 1983.

2.9.5.1 Formaldehyde nitrogen

Although proteins are too weak to be titrated directly with alkali but if formaldehyde is added, it reacts with the $-NH_2$ groups to form the methylene-amino($-N-CH_2$) group, and the carboxyl group is available for titration. The procedures were performed as followed;

Weigh 0.5 g of sample into breaker 50 ml containing 10 ml of D.W. Neutralized the solution to a faint color with 0.1 N Sodium hydroxide solution from a burette. Add 10 ml of formaldehyde solution then continued the titration until pH of solution was adjusted to pH 9. Record the volume of alkali solution that used for pH adjustment from pH 7- pH9.

Calculation

$$\text{Formaldehyde nitrogen (mg/g)} = \frac{14\times(\text{ml of NaOH})\times(\text{N of NaOH})\times 20}{\text{Weight of sample}}$$

2.9.5.2 Ammoniacal nitrogen

Weigh 0.5-1 g of sample into distillation tube with containing 40 ml D.W. Then 1.5 g of magnesium oxide was added before place the tube into distillation unit. Place the conical flask containing 25 ml of boric acid (containing mixed indicator) under the outlet tube of condensor. Titrate with 0.1 N of hydrochloric acid to a purplish end-point

Calculation

$$\text{Ammonaical nitrogen (mg/g)} = \frac{5.6\times(\text{ml of NaOH})\times(\text{N of NaOH})}{\text{Weight of sample}}$$

CHAPTER III

RESULTS AND DISCUSSION

3.1 Screening of amylolytic microbial strains

Out of the 122 microbial strains isolated from various mold brans and solid waste samples from factories in the local area, 37 culture strains of molds, yeasts and bacteria were able to grow on cassava starch agar medium and gave clear zone of starch hydrolysis when iodine test was performed (Table 2). Because the isolated strains could produce extracellular amylase enzyme for starch breakdown. Efficiency of culture in starch hydrolysation was compared by using size diameter of clear zone of each strains. From the results, 6 isolated strains provided larger clear zone diameter which markedly different than others were mold SUT1, mold SS, yeast 3A, yeast G, yeast 69 and yeast 4A. It was noticed that mold showed higher ability in starch utilization than yeast and bacteria as previous study (Chatisatenr, 1978). Typical mold bran was a appropriate source of amylase producing strains because it contained many different culture in both mold and yeast that significantly grew and produced amylase. That was commonly found in khao-mak manufacture. Beside that some high efficiency strains were also found from cassava solid waste and spoiled raw cassava. It was surprised that only yeast strains were found in khao-mak. This could be explained that after early stage in starch hydrolysis of fermentation. All mold was quickly autolysis themselves because it could not stand in high concentration of glucose and low pH (Boze, Moulin and Galzy, 1991). So only yeast which could fermented glucose and tolerance to that surrounding condition could be survived. From this part, it could be concluded that mold SUT1 is the best strain that gave highest clear zone at 36 mm.. Even those 6 strains demonstrated highly efficient in starch utilization but for food and feed production, morphology characteristics of culture must also be considered and realized because it was important and could directly effected to the texture and sensory of product. So colony characteristics has been reported in Table 3.

Table 2. Clear zone diameter on starch agar in preliminary screening test for cassava starch hydrolysation ability of microorganisms.

Original source	Isolate no.	Clear zone (mm.)	Culture type
Cassava solid waste	2	2	Mold
	4	10	Mold
	5	10	Mold
	6	9	Bacteria
	7	1	Mold
	8	1	Bacteria
	9	2.5	Bacteria
	11	2.0	Mold
	13	0.8	Bacteria
	14	2	Bacteria
	19	7	Bacteria
	20	6	Bacteria
	23	1.1	Mold
	24	1.4	Yeast
	25	1.2	Bacteria
	29	1.0	Bacteria
	60	3.0	Yeast
	64	1.0	Yeast
	68	0.6	Bacteria
	69	19.4	Yeast
	72	2.3	Bacteria
	73	2.5	Mold
	79	0.6	Bacteria
	85	11	Bacteria
	94	11	Bacteria
	105	1.2	Bacteria
Sake cake	AA	8.1	Yeast
Loa mold bran	BB	4.6	Yeast
	CC	2.8	Yeast
Khao mak	1A	3.0	Yeast
	1B	3.0	Yeast
	2A	5.0	Yeast
Typical mold bran	3A	21.0	Yeast
	4A	19.0	Yeast
	G	20.0	Yeast
	SUT1	36.0	Mold
Spoiled raw cassava	SS	32.9	Mold

Table 3. Colony characteristics of isolated strains

Isolate no.	Spores	Mycelium	Colony color	Surface colonies plate culture																								
				Form					Elevation					Surface					Margin					Optical character			
				Punctiform	Circular	Irrgular	Filamentous	Rhizoid	Effuse	Flat	Raised	Convex	Pulvinate	Smooth	Rough	Concentrically	Contoured	Radiately ridged	Entire	Undulate	Lobate	Erose	Filamentous	Opaque	Translucent	Dull	Glistening
2	-	-	White	/				/																	/	
4	/ Green	/																								
5	/ Black	/	Yellow																							
6			White		/					/				/					/							/	
7		/	Orange					/																		
8			Green		/									/												/
9			Yellow		/									/												/
11	/ Black	/	black																							
13			Orange				/		/					/									/		/		
14			White		/								/			/			/							/	
19			Cream	/						/																/	
20			White																							/	
23	/ Black	/	Black																							
24			White		/																					/	
25			Orange			/			/					/					/							/	
29			White	/					/					/						/						/	
60			White	/							/				/								/	/		/	
64			Cream	/					/					/						/							/
68			Yellow	/					/					/					/								/
69			White		/								/				/						/	/			
72			Yellow	/						/				/						/							/
73			Orange																								

Table 3. (continued)

Isolate no.	Spores	Mycelium	Colony color	Surface colonies plate culture																							
				Form					Elevation					Surface					Margin					Optical character			
				Punctiform	Circular	Irrgular	Filamentous	Rhizoid	Effuse	Flat	Raised	Convex	Pulvinate	Smooth	Rough	Concentrically	Contoured	Radiately ridged	Entire	Undulate	Lobate	Erose	Filamentous	Opaque	Translucent	Dull	Glistening
79			Yellow	/					/					/					/								/
85			Yellow			/			/							/						/				/	
94			Cream			/			/						/							/				/	
105			Yellow	/								/		/						/							/
AA			Yellow			/				/				/					/								/
BB			White	/						/								/	/							/	
CC			White	/					/					/					/							/	
1A			White	/					/					/					/								/
1B			White	/					/					/					/								/
2A			White	/						/				/				/									/
3A			White				/						/			/							/			/	
4A			Cream	/					/					/						/							/
G			White		/										/			/					/	/			
SUT1		/																									
SS		/ White																									

Table 4. Confirming culture type by gram staining technique

Isolate no.	Morphology	Cell size(mm)	Gram staining	Culture type
6	Rod	0.3×0.5	G+	Bacteria
8	Cocci	0.1-0.3	G-	Bacteria
9	Rod	0.3×0.5-0.7	G-	Bacteria
13	Rod	0.3×0.5	G-	Bacteria
14	Rod	0.3×0.5	G-	Bacteria
19	Rod	0.3×0.5	G+	Bacteria
20	Rod	0.1-0.3×0.4-0.5	G-	Bacteria
24	Globose	0.7-1.0		Yeast
25	Cocci	0.1-0.3	G-	Bacteria
29	Rod	0.5×1	G+	Bacteria
60	Cylindroidal	1-3×6-7		Yeast
64	Globose	2-7		Yeast
68	Cocci	0.1-0.3	G-	Bacteria
69	Globose	3-7		Yeast
72	Cocci	0.5-1	G-	Bacteria
79	Cocci	0.1-0.3	G-	Bacteria
85	Rod	0.1-0.3×0.7-1	G variable	Bacteria
94	Rod	0.1-0.3×0.5-1	G variable	Bacteria
105	Rod	0.1-0.3×0.5-1	G-	Bacteria
AA	Globose	2		Yeast
BB	Globose	2-5		Yeast
CC	Globose and ellipsoidal	5-7		Yeast
1A	Globose and ellipsoidal	2-3		Yeast
1B	Ellipsoidal	4-5		Yeast
2A	Ellipsoidal	2-3		Yeast
3A	Globose and ellipsoidal	5-7		Yeast
4A	Ellipsoidal	2-3		Yeast
G	Ellipsoidal	3-5		Yeast

Note. Starch hydrolysis bacteria 14 isolates :

Coccibacilli, G-	5	isolates
Rod, Gram positive	3	isolates
Rod, Gram negative	6	isolates

From their morphological characteristics, it was reported that several genus of mold and yeast had been found. The yeasts mostly seemed to be in group of *Saccharomyces* and *Candida*. Molds consisted of *Aspergillus*, *Rhizopus* and also *Chlamydomucor* that produced spore color in black, green and no color respectively (Alexopoulos, Mims and Blackwell, 1996). To distinguish between yeast and bacteria, the results was confirmed by the results from gram staining as shown in Table 4 that among the bacterial strains, they were gram-negative, coccobacilli 5 isolates; gram positive, rod-shaped 3 isolates and gram negative, rod-shaped 6 isolates. Some of isolated bacterial strains which frequently found in cassava solid waste were *Bacillus.* That because the

genus *Bacillus* were also common , as they are widely spread in nature and have relatively simple nutrition requirements. This also caused from cassava solid waste contained high water that suitable for growth of the bacteria, particularly *Lactobacillus* species and to a lesser degree of *Streptococcus* spp. (Beuchat, 1991). In addition some of them seemed to be in group of *Acetobacter* that was gram negative and commonly found in food fermentation (Holt et al., 1994).

All of the selected strains were tested for their capability of starch hydrolysis by preliminary fermentation test with glutinous rice from the initial hypothesis that cassava starch composed of 16-18% amylose that not much different from starch of rice. If microorganism could produce amylase, they could hydrolyze glutinous rice. Preliminary fermentation studies were conducted using glutinous and pure culture of the selected strains. At the end of 24 hr at room temperature glutinous rice was well digested and liquefied and good quality of khao-mak was obtained. Softening, starchiness and odor of product were recorded everyday. Reducing sugars of product was also detected.

Results in Table 5. showed that sweet sour alcoholic rice pastes could be obtained from many microbial strains. For examples: pure culture of mold 7, bacteria 13, yeast 64, yeast 69, mold 4 and mold 5. Although these strains could liquefy starch and provided product that similar to khao-mak but some of them gave only small amounts of reducing sugars when compared with typical mold brans and visible growth of some cultures had been observed. Due to these factors could affect to the result in next step of biomass protein production. So only mold SUT1 was selected. It proved to be the best amylolytic strain that gave the highest reducing sugars at 560 mg/g when compared with typical mold brans which provided reducing sugars at only 425 mg/g. Moreover, it gave higher efficiency than the other reference strains such as *Saccharomycopsis fiburigera*, *Endomycopsis fiburigera* and *Candida krusei* etc. *S. fiburigera* and *E. fiburigera* were known as amylolytic strains that simply found in bubod from Philippines (Steinkraus, 1996 and Wolf, 1997) and look-pang, respectively. For *C. krusei*, it was the strain that commonly found in food fermentation which could utilized starch as substrate (Beuchat, 1991). Based on these results, the strain of mold SUT1 most likely be a strain containing very high performance in starch hydrolysis which almost 56% of conversion efficiency could be performed.

Table 5. Preliminary test of pure culture in glutinous rice fermentation (khao-mak making)

Culture strain	Time (days)	Appearance of fermented sticky rice				
		Visible growth of culture	Softening	Starchiness	Odor	Reducing sugar(mg/g)
Control (no culture)	1			Dry	Spoiled +	
	2		++	Wet +	Spoiled ++	
	3		+++	Wet ++	Spoiled +++	18
Typical mold bran	1		+	Dry	Fermented ++	
	2		++	Wet +++	Fermented +++	
	3		++++	Wet ++++	Fermented ++++	425
Mold no. 2	1		-	-	-	
	2	/	-	-	-	
	3	/	-	-	-	-
Mold no. 4	1		+	-	-	
	2		++	-	-	
	3		++	Wet +++	Fermented rice ++	110
Mold no. 5	1		+	-	-	
	2		+	-	-	
	3	/	+	-	Alcohol ++	52
Bacteria no. 6	1		+++	Wet +++		
	2		+++	Wet +++		
	3		+++	Wet +++		182
Mold no. 7	1		++	Wet +		
	2		++++	Wet ++++	Fermented ++	
	3		++++	Wet ++++	Fermented ++	99
Bacteria no. 8	1		+	Wet +		
	2		++	Wet ++		
	3		+++	Wet +++	Spoiled +	63

Table 5. (continued)

Culture strain	Time (days)	Appearance of fermented sticky rice				
		Visible growth of culture	Softening	Starchiness	Odor	Reducing sugar(mg/g)
Bacteria no. 9	1		+	Wet +		
	2		+++	Wet +++		
	3		+++	Wet +++		118
Mold no. 11	1		++	Dry	Fermented rice ++	
	2	/	+++	Wet +++	Fermented rice +++	
	3	/	+++	Wet ++++	Fermented rice, ++++, Acid +	89
Bacteria no. 13	1		+	Dry	Fermented ++	
	2		++	Wet +++	Fermented +++	
	3		++++	Wet ++++	Fermented ++++	49
Bacteria no. 14	1		-	-	-	
	2	/	-	-	-	
	3	/	-	-	-	86
Bacteria no. 19	1		+	-	-	
	2		++	-	-	
	3		++	Wet +++	Fermented rice ++	18
Bacteria no. 20	1		+	-	-	
	2		+	-	-	
	3	/	+	-	Alcohol ++	71
Mold no. 23	1		+++	Wet +++		
	2		+++	Wet +++		
	3		+++	Wet +++		73
Yeast no. 24	1		++	Wet +		
	2		++++	Wet ++++	Fermented ++	
	3		++++	Wet ++++	Fermented ++	ND.

Table 5. (continued)

Culture strain	Time (days)	Appearance of fermented sticky rice				
		Visible growth of culture	Softening	Starchiness	Odor	Reducing sugar(mg/g)
Bacteria no. 25	1					
	2		+			
	3		++			-
Bacteria no. 29	1		+	Wet +		
	2		++	Wet ++		
	3		+++	Wet +++	Spoiled +	70
Yeast no. 60	1		+	Wet +		
	2		+++	Wet +++		
	3		+++	Wet +++		100
Yeast no. 64	1		++	Dry	Fermented rice ++	
	2	/	+++	Wet +++	Fermented rice +++	
	3	/	+++	Wet ++++	+Acid ++++,+	124
Bacteria no. 68	1		+	Dry	Spoiled +	
	2		++	Wet ++	Spoiled +	
	3		+++	Wet +++	Spoiled ++	107
Yeast no. 69	1		+++	Wet +++	Fermented rice ++	
	2		+++	Wet +++	+Alcohol +++	
	3		++++	Wet ++++	Fermented ++++	158
Yeast no. 72	1	/	+	Dry		
	2		++	Wet +		
	3		+++	Wet ++	Fermented +	196
Mold no. 73	1		+	Wet +	Fermented +	
	2		+++	Wet +++	Fermented +	
	3		+++	Wet +++	Fermented +	124
Bacteria no. 79	1	/				
	2		+	Wet +		
	3		+++	Wet +++		105

Table 5. (continued)

Culture strain	Time (days)	Appearance of fermented sticky rice				
		Visible growth of culture	Softening	Starchiness	Odor	Reducing sugar(mg/g)
Yeast no. 85	1		+++	Wet +		
	2		++++	Wet ++		
	3		++++	Wet +++		141
Bacteria no. 105	1		+++	Wet +++		
	2		+++	Wet +++		
	3		+++	Wet +++		139
Yeast 3A	1	/white			Spoiled +	
	2		+	Wet +	Spoiled +	
	3		+	Wet +	Alcohol ++	-
Yeast G	1					
	2		+	Wet ++	Fermented ++	
	3		+	Wet ++	Alcohol ++	ND.
Mold Sut1	1					
	2		++	Wet ++	Fermented rice ++	
	3		++++	Wet ++++	++++	560
Mold SS	1					
	2	/	-	Dry		
	3	/	-	Dry		325
Mold 4 (as *Rhizopus*)	1	/ Gray spores and mycelium	+	Wet +	Fermented +	
	2	/ full plate	+	Wet +	Fermented rice ++	
	3	/ full plate	+	Wet +	Fermented rice ++	325
Mold 5 (as *Aspergllus*)	1	/	+++	Wet +++	Fernented ++	
	2	/	+++	Wet +++	Fernented ++	
	3	/ full plate	+++	Wet +++	Fernented ++	375

Table 5. (continued)

Culture strain	Time (days)	Appearance of fermented sticky rice				
		Visible growth of culture	Softening	Starchiness	Odor	Reducing sugar(mg/g)
Saccharomycopsis fiburigera	1		+	Wet ++		
	2		++	Wet +++	Fermented +++	
	3		+++	Wet +++	Alcohol ++	85
Endomycopsis fiburigera	1			Wet ++	Fermented +	
	2		++	Wet +++	Fermented +++	
	3		+++	Wet +++	Alcohol ++	70
Aspergillus sp. TISTR 3063	1	/ Orange				
	2	Orange and yellow	+			
	3	Orange and yellow	++			136
Candida famata TISTR 5098	1	Cream				
	2	Orange and cream	+		Fermented +	
	3	Orange and cream	+			170
C. uitlis TISTR 5001	1	Cream				
	2	Orange				
	3	Orange				170
C. tropicalis TISTR 5087	1	Cream and orange	+			
	2		++	Wet +	Fermented rice	
	3		++	Wet +		17
C. krusei TISTR 5099	1		+			
	2		++	Wet +++	Fermented rice +++	
	3		+++	Wet +++	Fermented rice +++	118

Furthermore, the amounts of α-, β-amylase and glucoamylase amounts were also investigated and the results were summarized in Table 6.

Table 6. Production of μ-amylase and b-amylase activity by the selected strains cultured in 8% cassava broth

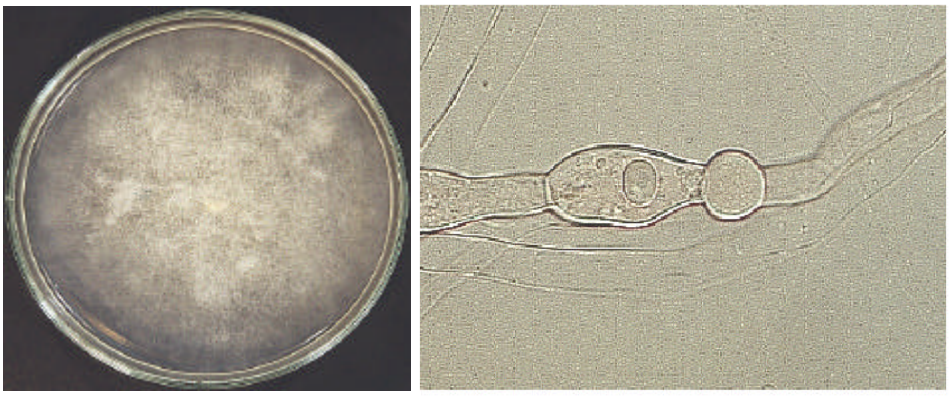
Culture Strain	Total Protein (mg)	Alpha amylase		Beta amylase		Glucoamylase	
		Total act. (Units)	Specific act. (Units/mg)	Total act. (Units)	Specific act. (Units/mg)	Total act. (Units)	Specific act. (Units/mg)
Typical mold bran	0.0875	0.76	10.21	ND	ND	1.51	18.49
Yeast G	0.0350	0.61	27.47	0.48	21.09	1.14	40.13
Yeast 69	0.0390	0.32	16.76	0.07	7.35	ND	ND
Yeast 4A	0.1125	0.03	0.55	ND	ND	0.25	2.45
Yeast 3A	0.0275	0.44	29.64	ND	ND	ND	ND
Mold SUT1	0.0800	2.32	31.08	0.69	6.95	ND	ND
Rhizopus spp.	0.0450	0.55	19.07	ND	ND	0.25	5.26
Penicillium sp.	0.0575	1.39	28.53	ND	ND	0.88	18.8
E. fiburigera	0.0500	0.97	25.09	0.69	16.32	2.15	47.35
S. fiburigera	0.0240	1.70	87.08	ND	ND	2.52	117.84

U = 1 unit of α, β amylase defined as 1μmol of maltose that liberated at 30°C pH 6.9 and 4.8 respectively,
U = 1 unit of glucoamylase defined as 1μmol of glucose that liberated at 37°C pH 4.5
ND = not detected

Mold SUT1 performed the highest α-and β-amylase activities when compared with other filamentous fungal strains included investigated reference strains that had been reported that was important in look-pang. Maximum activity was detected at 2.32 and 0.69 units respectively but glucoamylase production was not detected which was markedly different from others. In contrast with amylase specific activity was in the medium range. If we focused on the specific acitivity, it was found that specific activity produced by α-amylase produced by mold SUT1 was lower than 2 times that produced by *Saccharomycopsis fiburigera.* This was similar to results of specific activity of β-amylase obtained from yeast G that was higher than mold SUT1. It showed that amount of amylase which was produced by yeasts was less than mold but its efficiency per unit of enzyme was greater than mold. However, for saccharification the total activities were the most important parameters and needed to obtained the highest reducing sugars yield for supplying as energy for *C. utilis* in biomass production stage. *S. fiburigera* were also described in their amylase activity because it could produced α-amylase and glucoamylase but it was not selected to use because 1) In biomass production if glucose concentration was excess the yeast would switched mode of metabolism from respiration to ethanol fermentation that resulting

decreased in cell growth that affected straight to biomass yield (Berry, 1989) and 2) *C. utilis* also had ability in utilization of disaccharide such a maltose (Kockova-kratochvilova, 1990). Thus only high α-, β-amylase production were necessary for the stage of starch hydrolysation. So mold SUT1 should be selected to use in further experiment.

When the filamentous fungal strain was observed by microscopy (Fig. 6) It was found that mold SUT 1 is in-group of non-septate mycelium fungal, spores are scattered on mycelium. Strain of this mold varies from ones without sporangia and posse only chlamydospores to one that produce considerable numbers of abortive-type sporangia that identified as *Chlamydomucor*. This genus had also been reported that found and plays important role in khao-mak manufacture. (Chatisatiern, 1978; Pichyangkura and Kulprecha, 1977).



(a) (b)

Fig. 6 Morphology of *Chlamydomucor* SUT1 (a) *Chlamydomucor* SUT1 on PDA after 2 days incubation, (b) Spores and mycelium of *Chlamydomucor* SUT1 under microscope (40X)

3.2 Effect of temperature on cell growth

The effect of temperature on amylase production (Table 7) was determined by varying the incubation temperature from 30°C, 35°C and 40°C when growing the selected culture strains on 2% cassava starch agar. After iodine test, it was found that most of strains performed the largest clear zone at 30°C. Some of them could produce the highest amylase activity at 35° C. For *Chlamydomucor* SUT1, the decrease in amylase production measured was significantly

effected by temperature. Decrease in growth and enzyme production was observed at 40°C with 57.74%.

Table 7. Effect of temperature on amylase activity on cassava starch agar plate.

Culture	Clear zone (cm.) from edge of colony		
	30 °C	35 °C	40 °C
Yeast G	0.745	0.431	0.428
Yeast No.69	0.521	0.538	0.584
Yeast 3A	0.631	0.656	0.528
Mold SS	1.74	0.26	0.16
Chlamydomucor SUT1	3.20	3.50	1.50
Endomycopsis fiburgera	0.603	0.534	0.412
Saccharomycopsis fiburigera	0.287	0.575	0.325

It was known that this culture strain was not active in high temperature and optimal temperature of maximum amylase production was performed at 35°C. However, temperature at 30°C was still optimized for this mold because only 8.57% of efficiency was reduced. The growth and enzyme production pattern of *Chlamydomucor* SUT1 was similar to both reference strain of *E. fiburigera* and *S. fiburigera* that the ability in amylase excretion was lower when temperature increased to 40°C. The results were differed from yeast 69 that amylase production remained fairly constant even in such evaluated temperature.

3.3 Effect of substrate and its pretreatment for conversion of cassava roots into glucose (saccharification)

Initial experiments were conducted to assess the usefulness of raw and dried cassava as substrate. During growth of the fungus, the cassava polysaccharides were extensively degraded with a concomitant increase in the total reducing sugars (Tan, Ferguson and Carlton,1984). The effect of raw and cooked starch on saccharification was determined by measurement of reducing sugars production obtained after 4 days of cassava fermentation (Fig. 7). Steaming of raw materials were found very effective in utilizing by microorganisms. The reducing sugars trended to be increased in steamed substrate. In contrast with non-steamed substrate that the reducing sugars production was almost could not be observed. Since steaming could reduced the contamination thus initial bacteria was also reduced in high amount by steaming for 15 min. This condition promoted the growth of inoculated pure culture of *Chlamydomucor* SUT1 because

competition state from bacteria was decreased at the early stage. Another advantage was that the molecular size of starch was decreased that facilitate amylase activity.

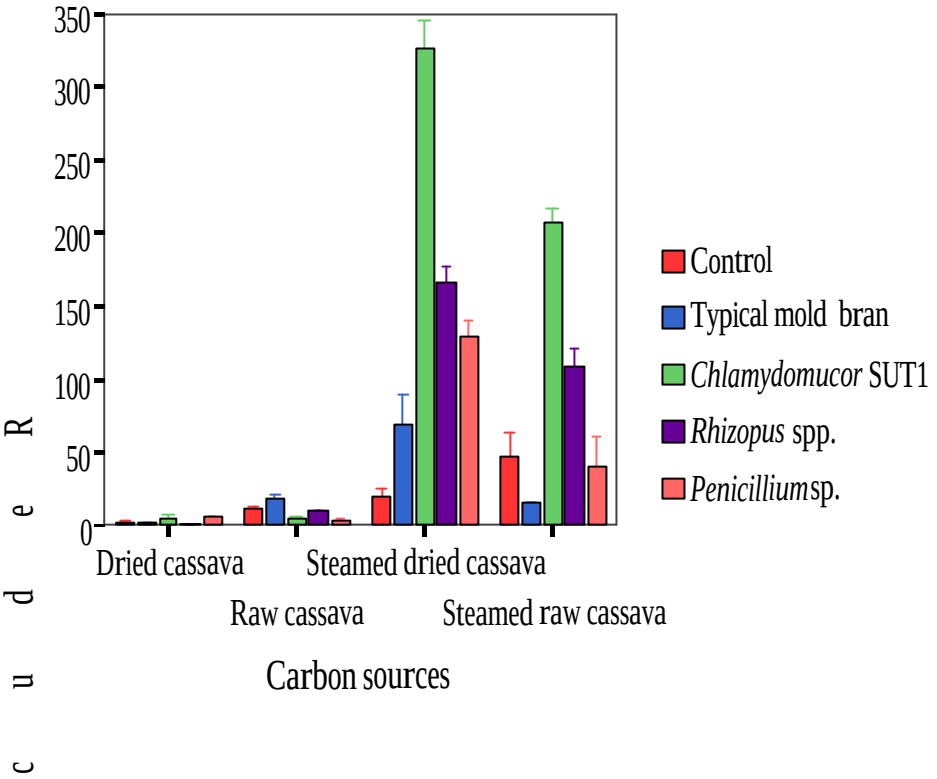


Fig. 7. Effects of various carbon sources and its pretreatment on saccharification.

However, reducing sugars in non-steamed materials was very low. This was also suggested that *Chlamydomucor* SUT1 could not hydrolyze starch in form of non-cooked substrate. The maximum reducing sugars were produced at 327.44 mg/g DW after 4 days of incubation in steamed dried cassava by pure culture of *Chlamydomucor* SUT1. For steamed raw cassava reducing sugars were increased much slower and reached only 207.3 mg/g DW at 4th day of cultivation. It produced reducing sugars much higher than *Penicillium* sp. and *Rhizopus* spp. The reasons to explain this result were these references which obtained from look-pang for fermented rice production might not have ability in cassava starch hydrolysis so small amounts of reducing sugars was provided or *Chlamydomucor* SUT1 produced amylase and hydrolyzed cassava starch in higher rate than those reference strains. However, *Chlamydomucor* SUT1 was

the best culture in cassava fermentation. From this finding it could be concluded that steamed cassava were suitable substrate in solid state fermentation of cassava than non-steamed materials.

Reducing sugars obtained from steamed dried cassava was higher than in steamed raw cassava in all culture strains (included references strains). In contrast with control system that reducing sugars was detected at lower level. Even it was not highly significant. However, It could explain that in non sterilized condition, some contaminants could easily be performed during incubation and steamed raw cassava was more preferably for microbes than those steamed dried cassava which higher numbers of contaminated microorganisms had already been presented.

It was also shown in Fig.7 that the reducing sugars production from look-pang of *Chlamydomucor* SUT1 at maximum level was higher than the typical mold bran in both types of steamed substrate. This could be described that typical mold from local area which less quality control for maintaining the uniformity and quality of look-pang. Moreover, this was depend on the shelf life of look-pang before distribution. So it clearly indicated that look-pang from the selected strain gave more efficient than typical mold bran that made from several mixed culture types.

3.4 Dry inoculum preparation

From dry inoculum preparation, it was revealed that after dry inoculum of mixed culture of *Chlamydomucor* SUT.1 and *C. utilis* was developed. Culture number of *Chlamydomucor* SUT1 could be detected in high numbers at 10^6 CFU/g and cell numbers of *C. utilis* were obtained at 9.45×10^7 CFU/g in the inoculum. Very low level of bacterial contaminant was detected. This might be due to only single starch utilizing of mold SUT1 grew in the carrier (look-pang), some reducing sugars were accumulated and able to promote growth of other microbes. In contrast, when mixed cultures of *Chlamydomucor* SUT1 and *C. utilis* were developed, after the mold hydrolyzed starch, the yeast could use remaining glucose as carbon source for biomass production that promoted the lack of nutrient condition for other microbial contaminants. This was not only useful for overcoming the problem of contamination but *C. utilis* also beneficially provided some alcohol that enhanced flavor as well as protein yield of product in the same time. Another reason that could explain was the yeast *C. utilis* could

reproduce themselves rapidly than mold which performed high capacity in competition with bacteria resulted in 5.97 log CFU/g reduction of bacteria. From the experiment, look-pang of

single culture of *Chlamydomucor* SUT1 had to be developed first. Then followed with mixing of *C. utilis* culture to obtain dry inoculum of mixed culture as shown in Fig. 8.

(a)

(b)

Fig. 8. Photos of dry inoculum (a) Single culture inoculum of *Chlamydomucor* SUT1 (b) mixed culture dry inoculum of *Chlamydomucor* SUT1 and *C. utilis*

From the appearance of dry inoculum, some different visible characteristics could be observed. The dry inoculum developed from single culture of *Chlamydomucor* SUT1 had less porous than using mixed culture because after pure culture of *C. utilis* was added in to the dough, yeast reproduced themselves rapidly and produced more CO_2. However, both of these inocula showed high ability in saccharification.

3.5 Saccharification efficiency of *Chlamydomucor* SUT1- bran

For cassava fermentation with two types of substrate; steamed raw and steamed dried cassava, It was clearly shown that highest efficiency was obtained in steamed raw cassava (Fig. 9). Reducing sugars were assumed as oligosaccharides and simple sugars such as glucose and maltose (Fennema, 1985 and Gerhartz, 1990) and they were reached maximum at 680.07 mg/g DW after 5 days of fermentation. While using steamed dried cassava as carbon source, maximum-reducing sugars produced at 380 mg/g DW at 3 days after inoculation. Look-pang produced from single culture of *Chlamydomucor* SUT1 gave higher efficiency than using pure culture. It was possible that the microorganisms in look-pang that have solid rice flour as carrier could be protected themselves from environmental changed at initial stage. Another reason, the culture in dry inoculum stay in starving-like condition, when enriched substrate were reached, they should utilize substrate in high rate for growth and reproduction.

If emphasize on type of substrate for look-pang SUT1, steamed raw cassava was better than steamed dried cassava due to raw cassava that prepared from whole cassava root by chopping and steamed supposed to have lower impurities material than steamed dried cassava. It could also possible that steaming condition unable to get rid of all bacterial contaminant from initial so after fermentation the contaminants could reproduce themselves as fast as inoculated culture. Therefore, reducing sugars were lower in steamed dried cassava. Product from steamed raw cassava was appeared in white, soft texture, sweet and alcohol aroma as the previously reported (Beuchat, 1991 and Chatisatienr, 1978).

In addition, when control which no culture treatment was investigated, low level of reducing sugars were detected. It was meant that the solid state fermentation using steamed substrate in non sterilized condition was not disturbed by bacterial contamination which was normally found as a main problem in solid state fermentation.

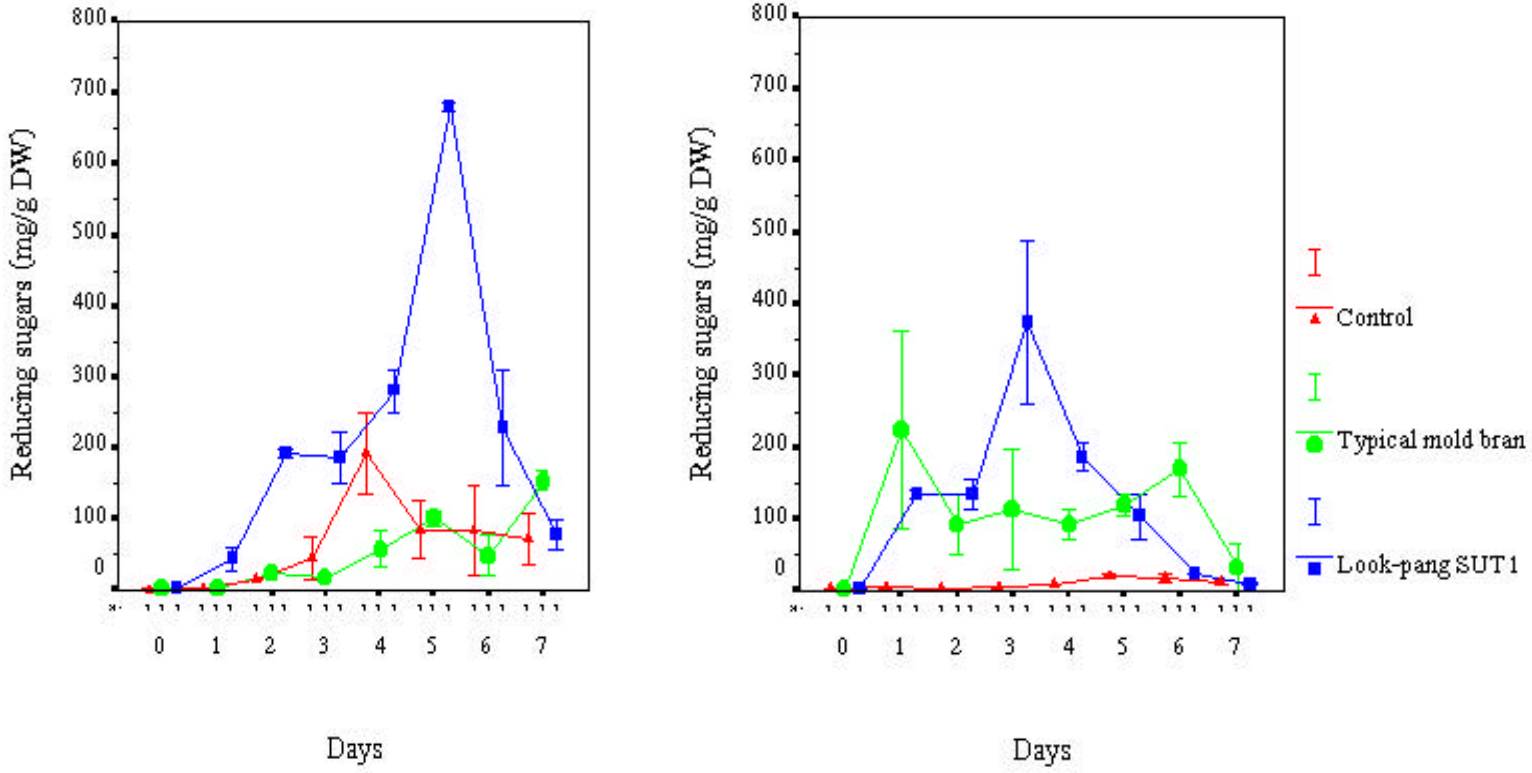

Reducing sugars (mg/g DW)
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7
Days
Reducing sugars (mg/g DW)
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7
Days
Control
Typical mold bran
Look-pang SUT1

Fig. 9. Efficiency of dry inoculum *Chlamydomucor* SUT1 on saccharification of steamed raw and steamed dried cassava in 250-ml beakers with 50 g substrate.

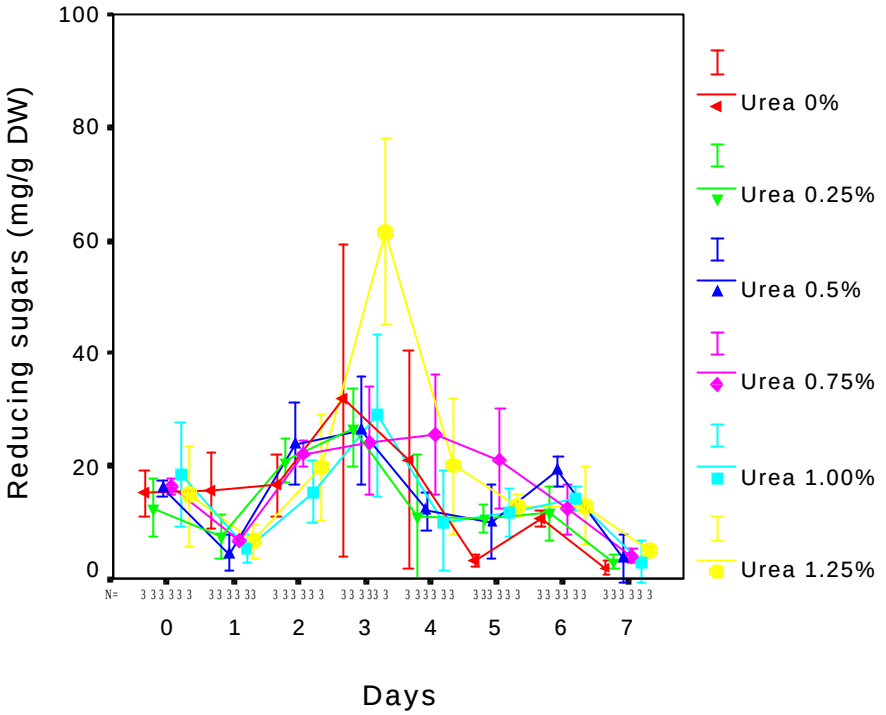
3.6 Effects of nitrogen source concentration on biomass production

The time courses of reducing sugars, crude protein, pH and moisture content when using 50g steamed raw cassava as substrate, 0.4% inoculum sized and various urea concentrations: 0%, 0.25%, 0.5%, 0.75%, 1.00% and 1.25% were illustrated in Fig. 10 (a, b, c, d). The incubation periods was prolonged for 1 week. The result showed that the reducing sugars were decreased in the 1st day and then dramatically increased and reached maximum level at the 3rd day of incubation for all urea concentration. This could be explained that at the 1st day of incubation, the culture could used initial reducing sugars which were obtained from steaming for growth. But after *Chlamydomucor* SUT grew up, it could produced α and β-amylase to utilize cassava by themselves that promoted the reducing sugars content in this stage of saccharification. This was very useful for *C. utilis* that would assimilate glucose and maltose as sole of carbon source for growth and reproduced themselves. During the growth of *C. utilis*, they consumed reducing sugars very fast. So reducing sugars were rapidly decreased at the end of fermentation (Fig. 10a). While crude protein yield was slowly increased and reached maximum at the end of fermentation period (Fig. 10b). It was suggested that the starch-degrading enzymes were very well synthesized in a short time at saccharification stage resulted in promotion of the cell growth of *C. utilis* due to assimilation of glucose. Crude protein production was increased when urea concentrations were applied up to 1.00% and 1.25%, at which the maximum crude protein content was reached, and remained constant at a concentration of 1.25%. For pH of substrate, it showed that pH profile was dramatically dropped in the first 2-3 days and became constant at the 3rd day by standing between pH 5.5-6 after the urea was added at 0.75% (Fig. 10c). It was clearly shown that when using urea as nitrogen source, pH control was not necessary as reported in previous work (Reade and Gregory, 1975). While moisture content did not differ at all in every urea concentrations but it trended to be decreased at the last day because the effect of evaporation time (Fig. 10d). When various urea concentration were used, it was found that the concentrations of 1% and 1.25% were the most effective (Fig.11) in providing nitrogen source for maximizing the amounts of amylase production that directly related to the second process of biomass production after first stage of saccharification.

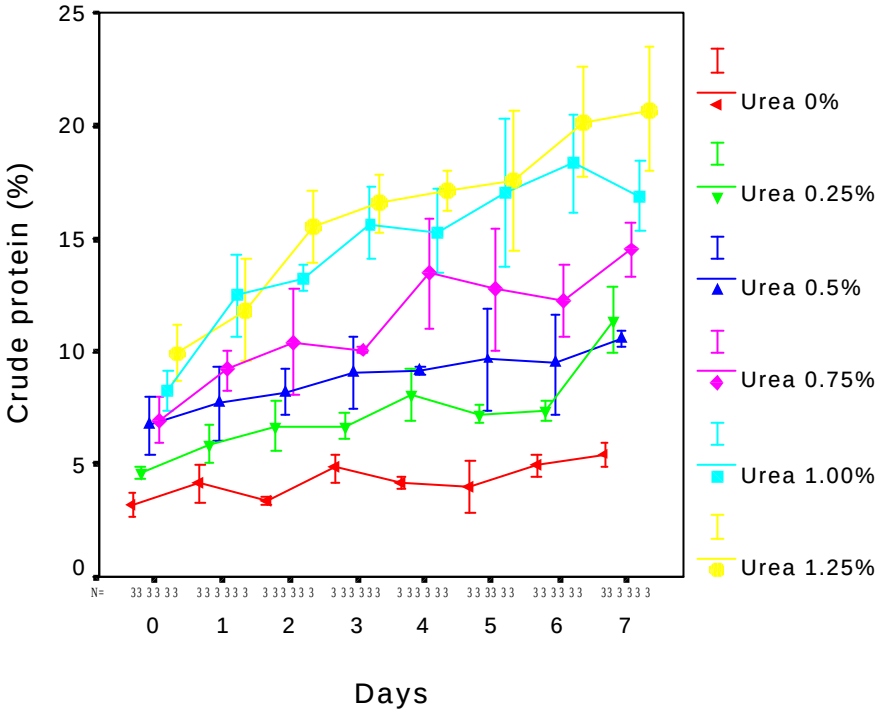
However, if comparing between the two levels of urea concentrations (1.00% and 1.25%) by emphasizing on crude protein yield. The highest yield was obtained at 10.06% when

amended with 1% urea (Fig.11). This was due to the microorganism could synthesized protein by using inorganic and organic nitrogen such as urea (Moo Young, 1986). To get a rid of the effects from excess urea in true protein yield in batch culture, determination of amino acid that responded to the real amounts of urea consumption for cell growth in biomass production, was also carried out. Amino-N was monitored for 7 days of fermentation by comparing between using 1.00% and 1.25% urea. The maximum amino-N was attained at 1% urea (Fig. 12). High significant differences of both levels of nitrogen were observed. It was confirmed that the solid state fermentation of cassava using 1% urea was the most effective for bioconversion of cassava to microbial protein production. Fig.12 was also shown that in non sterile condition, some contamination could be occurred during the fermentation and could be detected at the 3rd of incubation period. It demonstrated that at the 3rd day which maximum reducing sugars were produced, some contaminant could use reducing sugars in the substrate as carbon source and grew up rapidly that might competitive to *C. utilis*. Even though the amino-N content that could be assumed as microbial cells were detected in low level, but it trended to be increased quickly when the incubation time was prolonged. So to overcome and avoid contamination problem, the fermentation time should be considered and the quantity of inoculum must be increased in large scale which bacterial contamination was the major concern.

In addition, The results obtained from solid state fermentation especially reducing sugars varied quite widely. This variability could be depended on different factors such as heterogeneity of substrate. Gelatinization of raw cassava by steaming could not guarantee perfect uniformity in the cassava granules throughout the mass. The heating methods employed create temperature gradients and corresponding shifts in humidity levels which caused a lower degree of gelatinization in the center of the mass. The solid substrate itself was a main source of heterogeneousness in oxygenation by natural convection, humidity, temperature, nutritive salts and inoculum homogeneity (Daubresse, Ntibashirwa, Gheysen and Meyer, 1986). To achieve successful fermentations, it was suggested that the nitrogen and inoculum should be provided in sufficient quantity, it was important to ensure that all components were mixed well and good ventilation of oxygen should be obtained for whole fermentation.

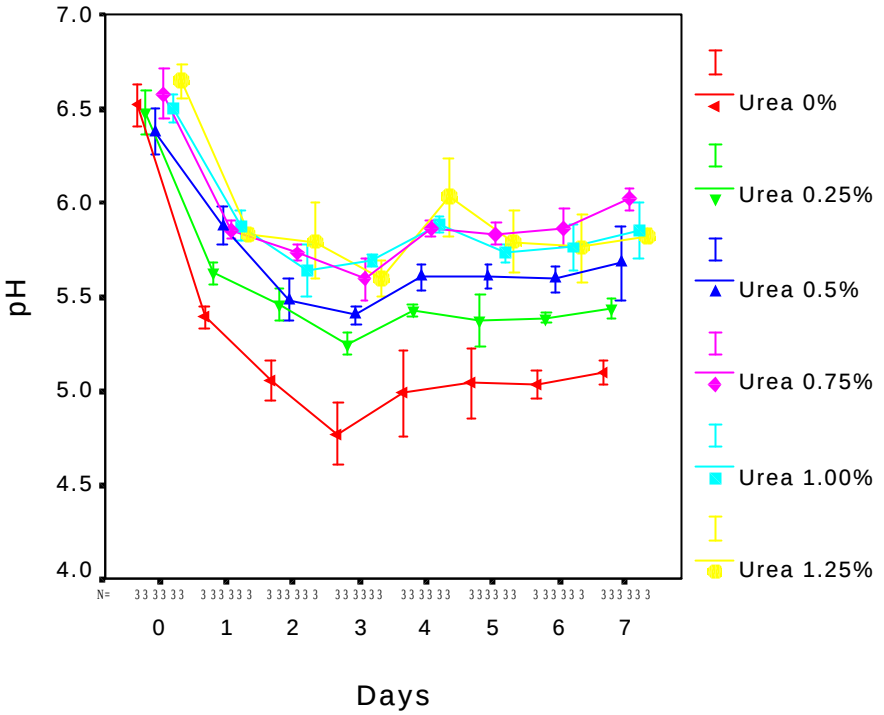


(a)

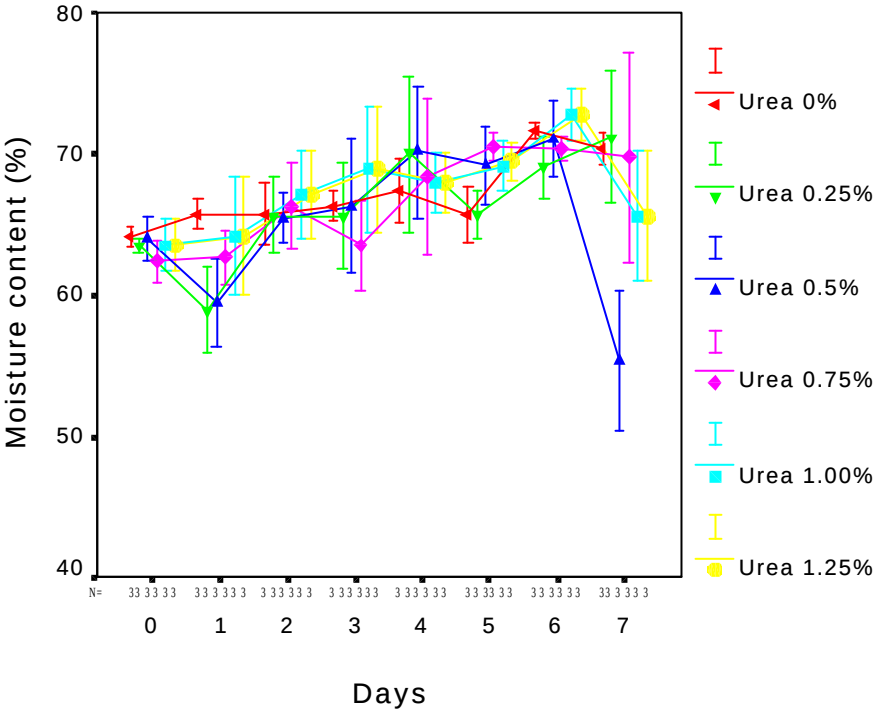


(b)

Fig. 10. Effect of various urea concentrations; 0%,0.25%, 0.5%, 0.75%, 1.00% and 1.25% on reducing sugars (a) and crude protein content (b) using 50g steamed raw cassava and 0.4% inoculum in 250-ml beakers.



(c)



(d)

Fig. 10. Effect of various urea concentrations; 0%,0.25%, 0.5%, 0.75%, 1.00% and 1.25% on pH (c) and moisture content (d) using 50g steamed raw cassava and 0.4% inoculum in 250 ml-beakers.

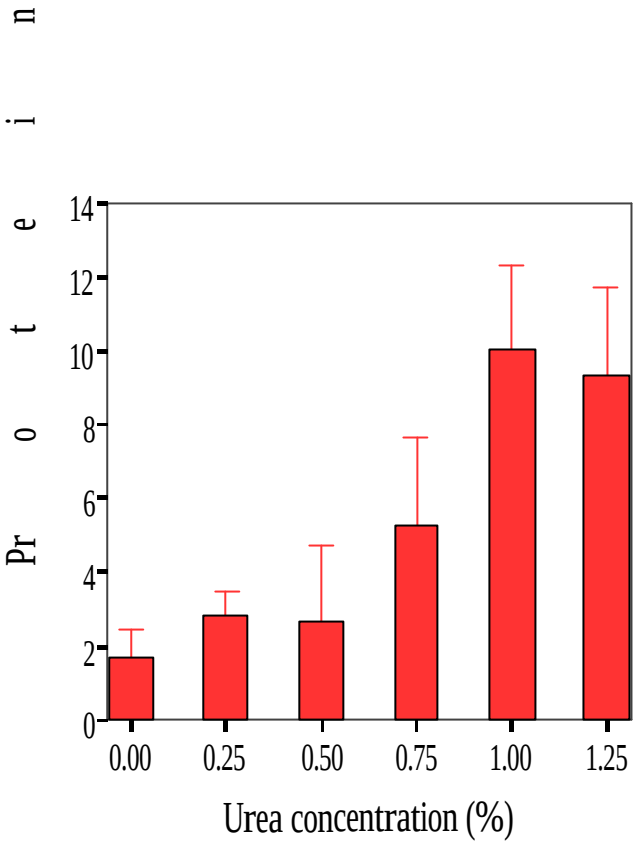


Fig. 11. Effects of various urea concentration on crude protein after 6 days of incubation.

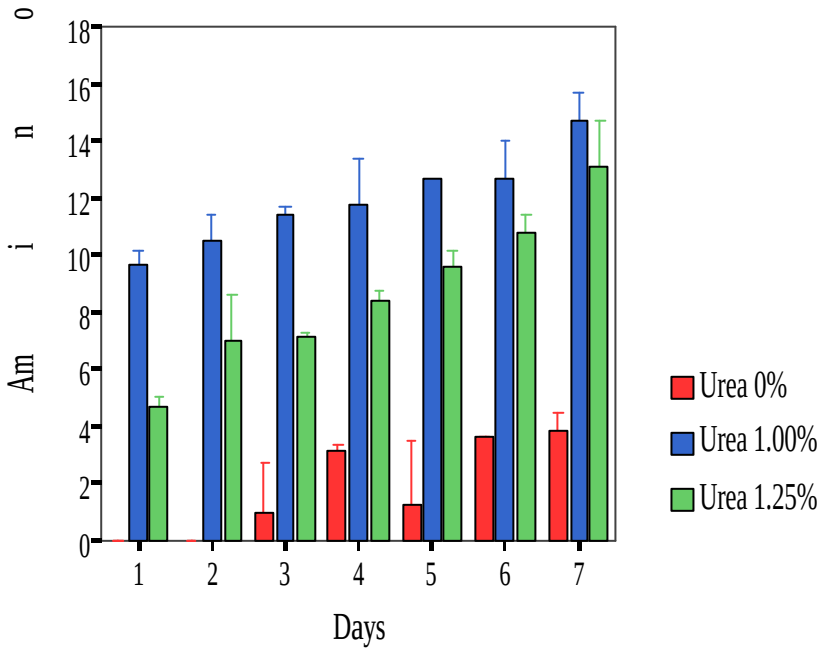


Fig. 12. Comparison of (a) crude protein and (b) amino-N obtained from using 1% and 1.25% of urea concentrations in 250-ml beakers containing 50 g steamed raw cassava at room temperature.

3.7 Protein production by solid state fermentation of *Chlamydomucor* SUT1 and *C. utilis*

Results obtained from optimization condition could be concluded that steamed raw cassava was the most suitable as carbon source and urea was considered to be the best nitrogen source at concentration of 1% by using dry inoculum of mixed cultures that necessary for further production.

Cassava fermentation was preliminary conducted in lab scale by using simple process, non-controlled pH and room temperature (30-35°C) incubation with initial moisture of 63%. The protein content of cassava, which generally is about 2 to 3% was raised to a maximum of 18.3% on basis of dry mass (Fig.13). This might be due to the losses of total solids and the synthesis of protein by the microorganisms (Cronk et al., 1977) after 6 days of cultivation. Almost all reducing sugars in the product was utilized by that time. Without pH control, pH level was slowly decreased from initial pH at 6.6 and maintained at pH 5.6 to 5.8 which was suitable for yeast growth. During fermentation the moisture was slowly increased, however, remained between 60% to 75% throughout experiment. During the culture was growing rapidly and crude protein content were reached maximum at 6^{th} day and gradually decreased at the end of fermentation. Reducing sugars were observed at very low level less than when single strain inoculum was performed. This was due to the results of mixed culture that *C. utilis* could use reducing sugars while starch was hydrolyzed. It was also possible that steaming could be only partially hydrolyzed starch resulted in low starting sugars that was detected at initial stage. During the fermentation period the cassava was soften and a sweet/sour alcoholic flavor was developed.

The results clearly suggested that mixing of dry inoculum powder before cultivation time could well promote more homogeneity of cultures which was directly effect on increasing crude protein content. The conversion of cassava starch to crude protein by mixed culture strains of *Chlamydomucor* SUT1 and *C. utilis* was comparable to that crude protein content level in similar published systems (Zvauya and Muzando, 1994; Reade and Gregory, 1975) and higher than that was reported by other authors who used raw cassava in solid state fermentation by culturing *Rhizopus* able to increase protein from 1.75-11.3% (Soccol, 1994). Another solid state fermentation that cassava was processed by moistening, steaming and mixing with nutrient solution containing urea, KH_2PO_4, $MgSO_4.7H_2O$ the protein could be increased to 10.7%

(Daubresse and Ntibashirwa, 1986). The method used in this research was more simply and less costly cost because no moisture adjustment, no pH control and cheap nitrogen source. Moreover, dry inoculum using in this study was proved to be the appropriate tool for bioconversion of cassava since only 0.4% of inoculum could be converted starch into almost 20% protein.

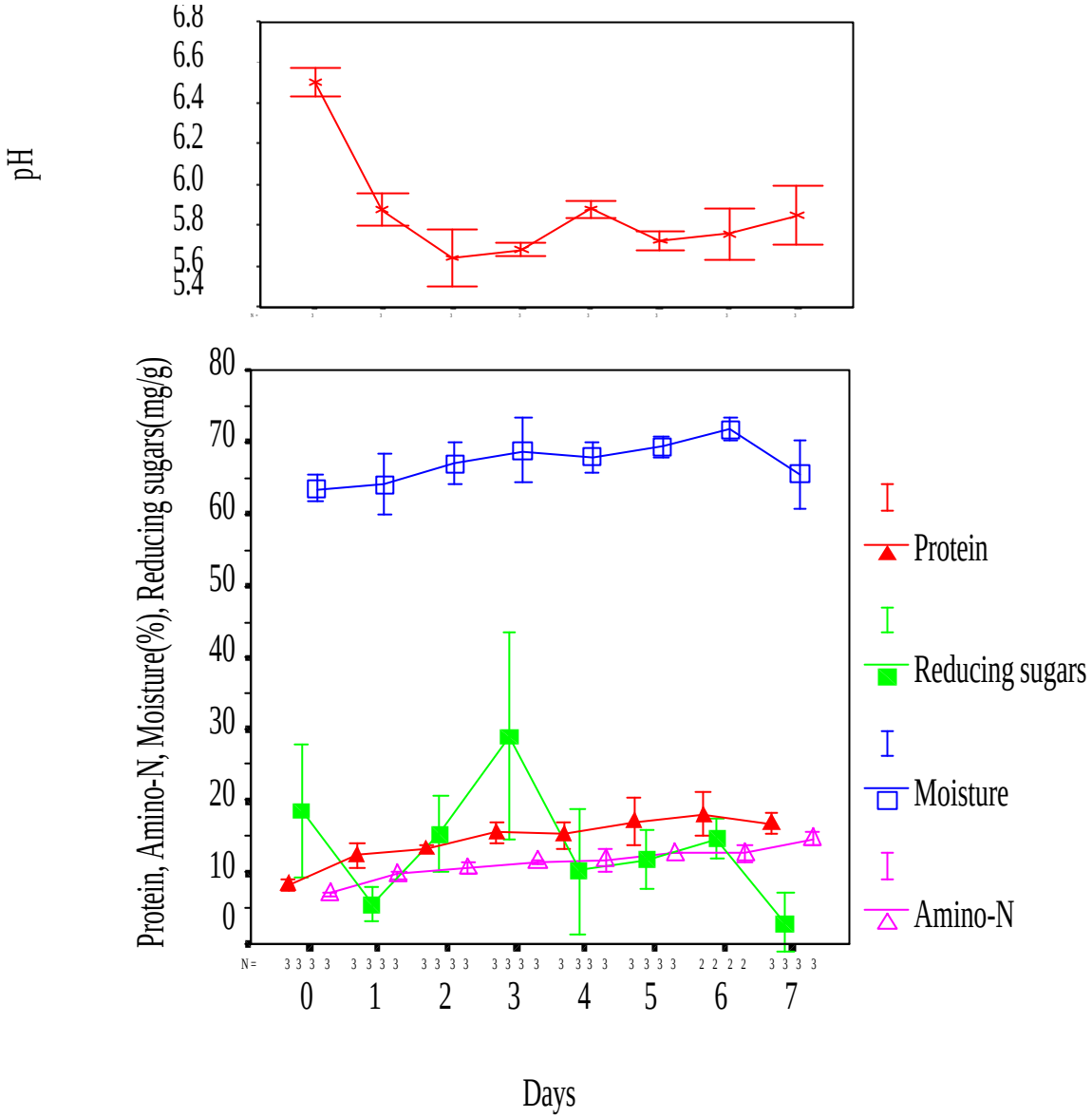


Fig. 13. Changes in reducing sugars, protein, amino nitrogen, pH and moisture content with time obtained from using 1% urea in breakers with 50 g steamed raw cassava by dry inoculum of *Chlamydomucor* SUT1 and *Candida utilis* at room temperature.

3.8 Process scale up

The biomass production was further evaluated in batch culture of 540-L fermentor. The 1% of urea was added at the initial of batch culture with 6 days of incubation period as presented in a typical time course in Fig. 14. The inoculum was increased from 0.4% to 4% to achieve the development of *Chlamydomucor* SUT1 and *C. utilis* by using heavy inoculum. Fermentation was performed without controlling pH. Although the raw cassava had not been sterilized, no development of contaminant microorganisms was observed from its appearance and total plate count.

Changes in reducing sugars, protein, pH and moisture were observed in the same pattern of the experiments obtained from lab scale. The bioconversion was proceeded and the highest reducing sugars were obtained at 32.37 mg/g in the 3rd day and they were completely utilized after 5 days of incubation time. This activity correlated with crude protein yield which was reached at the same time that reducing sugars were depleted. At the end of fermentation amino nitrogen and crude protein content were in the same level. It could be notified that the last day of fermentation, almost all organic and inorganic substances such as urea was converted into organic substances like amino nitrogen indicated that protein converted from cassava. It was interested to find that after using heavy inoculum technique coupled with 1% urea concentation which was optimized from lab scale, the maximum protein content was detected faster than expected at 15.3% on a dry basis which was composed of 11% amino acid in the 5th day. In general, the fermentation without pH control, the culture pH was usually rapidly fell below a level that the organism could tolerate. In contrast with this batch fermentation that pH was slightly dropped and remained at pH between 5.5-6.0 that was optimal condition for fungal growth. This was possibly due to the use of urea as nitrogen source resulted in maintaining pH level. These encourage results make it possible to envisage numerous applications of heavy dry inoculum of *Chlamydomucor* SUT1 and *C. utilis* as starter to reduce contamination and shorten time of traditional food fermentation in solid substrate processes. For moisture content, almost 70% moisture was in the product indicating liquefaction of starch hydrolysis efficiency. In parallel if consideration on total solid in product, only 30% solid was left in product that may limited product recovery. Therefore to overcome this problem, cassava fermentation should be

performed in large fermentor to obtain high amounts of product in commercial scale. The appearance of cassava during the process was shown in Fig. 15.

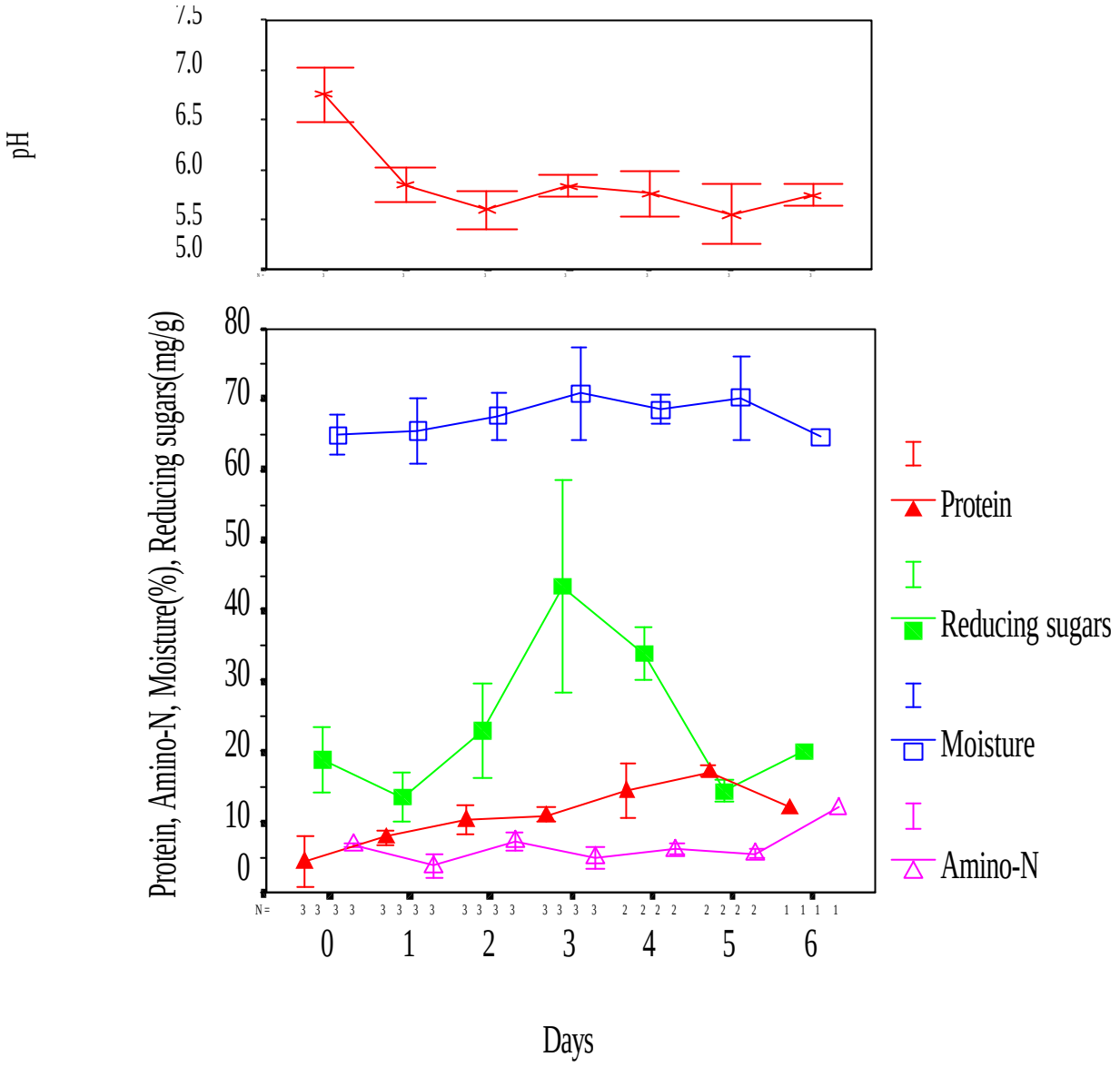
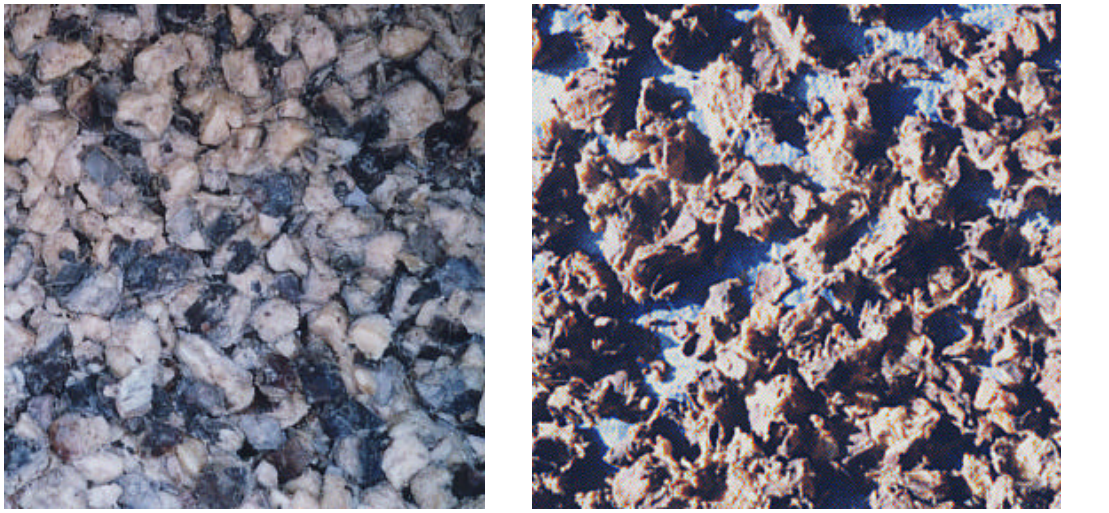


Fig. 14. Changes in reducing sugars, protein, amino nitrogen, pH and moisture content with time obtained from using urea concentration at 1% in 540-L fermentor with 50 Kg steamed raw cassava by dry inoculum of *Chlamydomucor* SUT1 and *Candida utilis* at room temperature.

(a) (b)



(c) (d)

Fig. 15. Photos show appearance changes of steamed raw cassava during fermentation in 540-L fermentor (a) the first day, (b) the second day, (c) the final day and (d) after sun drying.

CHAPTER IV

CONCLUSION

The present study demonstrated that the regular mold flora from look-pang in Thailand are *Chlamydomucor* SUT1, the same genus as found in fermented rice in other countries. It had high efficiency in glutinous rice as well as in cassava hydrolysation than others. Look pang from *Chlamydomucor* SUT1 was proved to be the best amylase producing inoculum that gave maximum reducing sugars yield at 680.07 mg/g DW in 5 days of incubation.

By comparing between different sources of substrate, steamed raw cassava was the most suitable substrate for solid state fermentation in biomass protein production. Dry inoculum preparation method was developed for cassava fermentation by using mixed culture of *Chlamydomucor* SUT1 and *Candida utilis*. The dry inoculum was not only promoted fast starch hydrolysis but also reduced the bacterial contamination. It was considered that this microorganism could utilized cassava as a sole carbon source and reducing sugars were produced in high amount which was useful for supplying energy for *C. utilis* in biomass protein production. Moreover, dry inoculum of mixed culture between *Chlamydomucor* SUT1 and *C. utilis* using in this study was proved to be the appropriate tool for bioconversion of cassava since only 0.4% of inoculum could be converted starch into 18.3% crude protein on a dry basis that high enough to use as enriched protein feed for animal such as cow and pig because the crude protein requirement of these animals are between 15-18% of dry matter. However, animal-feeding experiments are necessary before use.

The conversion of cassava starch to crude protein by mixed culture strains of *Chlamydomucor* SUT1 and *C. utilis* was comparable to that crude protein content level obtained from previous published systems (Zvauya and Muzando, 1994; Reade and Gregory, 1975) and higher than that was reported by other authors who used raw cassava in solid state fermentation by culturing *Rhizopus* able to increase protein from 1.75-11.3% (Soccol, 1994). Another solid state fermentation that cassava was processed by moistening, steaming and mixing with nutrient solution containing urea, KH_2PO_4, $MgSO_4.7H_2O$ the protein could be increased to 10.7%

(Daubresse and Ntibashirwa, 1986). The cassava fermentation technology which were developed under this project offered the advantages of being simple and reliable and requiring only a low capital investment. This means that application of the process can be envisaged at the scale of small units such as a stock farms and cooperatives. Preliminary tests were made in 540-L fermentor in which an average of 15.3% protein that composed of 11% amino-N was obtained. It was shown that this technology apparently presented rather simple and without operation control. In addition, using solid substrate fermentation give the inherent advantages, e.g. low energy requirements, less waste water and the growing need to dispose safety of solid waste materials, use of this technology is expected to increase in the future. Additional fermentation capacity and fermentation time would be considered to recover the problem of total solid lost that directly effect to product recovery and capital cost.

In the future research work, it is very interesting to develop dry inoculum of *Chlamydomucor* SUT1 with other microorganism culture. For example; *Saccharomysis fiburigera* to convert cassava into other value added product as ethanol for fuel mixture. This could not only increase consumption of cassava in our country but also decrease the price of fuel vehicles in the market. Moreover, the 540-L fermentor should be modified to achieve mixing efficiency of substrate, dry inoculum, nitrogen source and other compositions to promote fermentation efficiency.

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BIBLIOGRAPHY

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