PART II - ISOLATION, SELECTION AND IDENTIFICATION OF MOLDS AND YEASTS FROM RAGI

1. Materials and methods

Microorganisms have been involved in the prepration of food for man from time inmemorial, such as in the fermentation of foods. Alcohol beverage production was invented in Egypt some 5000 to 6000 years B.C. (Huuber 1926, cited from Rose 1977), but it was only in 1680 that Leeuwenhoek observed microorganisms in fermenting beer, and it was two hundreds years later, in 1880, that Hansen began to use pure culture yeast in beer fermentation. He experienced that the presence of microorganisms different from the principal yeast which he called wild yeast in fermenting beer caused problems. Hansen then developed the yeast taxonomy, and thereafter Cuilermond, Kluyver, Stelling-Dekker, Lodder and Kreger-van Rij added valuable information on the properties of yeasts.

Schwann in 1837 called the yeast in the fermenting beer as "Zuckerpilze" or sugar fungus from which Saccharomyces originated and further introduced by Meyen in 1838.

The first scientist interested in the saccharifying mold found in the Chinese yeast was Calmette (1893) and named the mold Amylomyces rouxii. Afterwards, in 1894 Eikman, Vorderman, Went, Geerligs and others isolated and studied the saccharifying molds from ragi, and named them Chalamydomucor, Mucor, and Rhizopus.

A. *Isolation and selection of amylolytic molds*

To isolate molds that can attack starch, a selective medium containing only starch as a carbon source may be used. The molds having amylase enzymes will grow on it and degradethe starch into products such as dextrin, sugar, organic acids, alcohols, water, etc. Amylolytic molds producing glucose as the major product are desirable here besides their high liquifying capacities.

Materials and methods

The first step was to select good ragi by a fermentation test on glutinous rice substrate. Fifty-two commercial ragi samples, collected from different places in Java, Madura, Bali, Sumatera and Sulawesi, were used to inoculate glutinous rice substrate (50 g glutinous rice and 75 ml water in plastic

bag, autoclaved) at the concentration of 1% ragi powder and incubated at room temperature (24-28°C) for 3 days. The tape product was tasted and observed for the liquifaction ability. The ragi samples which were able to produce soft texture, high amount of liquid and desirable quality of sweetness in the fermentation product were selected for the study.

The selected ragis were then plated in starch agar (soluble starch 1.0%, aspargine 0.05%, $(NH_4)_2SO_4$ 0.04%, yeast extract 0.04%, K_2HPO_4 0.03%, $MgSO_4$ 0.01%, $FeSO_4$ and $MnSO_4$ trace/100ml, and agar 1.2-1.5%) at dilution of 10^{-4} to 10^{-6}. All mold colonies that grew very well on this medium were isolated. From these isolates giant colonies were made by inoculating on Starch Agar plates of 15 ml medium per petri-dish of 8.5 cm diameter at distant spots. Incubation was done at ambient temperature for one day. The developed colonies were flooded with Iodine 1% solution (I_2:KI=1:2). Clearing zones surrounding the colonies were measured and the clearance intensity estimated visually. The molds producing wide and intense clearing zones were selected and kept on PDA slants.

The final step was test of the fermentation activity in glutinous rice substrate. For this purpose pure mold culture ragi were made of the selected molds as follows: 10 g heat sterilized rice-flour (18 hours at 105°CO, 10 ml sterile water and one slant of one week old mold culture was mixed in a petri-dish and incubated at room temperature for 3 days, dried in oven at 42-43°C for 2 days and finely ground in mortar, aseptically. This ragi powder at 0.1% concentration was used to inoculate into glutinous rice substrate and incubated at room temperature for 3 days. The ragi molds producing the very sweet and juicy tape were selected.

The selected molds were then subjected to the amylase- activity test. The saccharifying and liquifying amylase activities were measured as follows. One loop of one week old mold culture was inoculated into 50 ml of starch broth medium (soluble starch 0.5%, polypeptone 0.25%, malt extract 0.15%, and pH of 5.5) in 200 ml volume erlenmeyer flask. Then incubated at 30°C on a rotary shaker at the rate of 75 rpm. At interval of two days the enzymatic activities of the broth culture were estimated by the procedure described below.

Assay of liquifying amylase activity (modified blue value method): are-action mixture, which was composed of 0.5ml of 0.2 M acetate buffer having a pH of 6.0 containing 10 mM calcium acetate and 1 ml of 0.1% starch solution, was incubated at 40°C for 10 minutes. Afterwards, 0.5 ml of suitably diluted enzyme preparation was added to the mixture, and the reaction was allowed to proceed for 30 minutes. The reaction was stopped by the addition of 0.5 ml of 0.5 N HCl. Then, 1 ml of 0.001 N I_2/KI solution was added to the mixture. The volume was made up to 10 ml with distilled water, and the resulting blue color was measured by Shimazu Doubble Beam Spectrophotometer UV-200S at the wave-length 700 nm. One unit of liquifying amylase activity was defined as the activity which caused 10% decreased in optical density per minute under the above conditions.

Assay of saccharifying amylase activity: a reaction mixture, which contained 3 ml of 2% starch solution and 2 ml of 0.2 M acetate buffer with pH 6.0, was incubated at 40°C for 10 minutes. One milliliter of the suitably diluted enzyme preparation was added to the mixture, and the reaction was allowed to proceed for 10 minutes. Two milliliters of Fehling B solution was added to stop the reaction, then the content of reducing sugar was estimated according to the Fehling method. One unit of saccharifying amylase activity was defined as the activity which produced 1 mg of glucose for 1 minute under the above conditions.

Fehling method: 2ml of Fehling A solution was added to the reaction mixture containing 2 ml of Fehling B solution, then the mixture was heated in boiling water for 15 minutes. The mixture was stood until the temperature came down to below 25°C. After 2 ml of 30% KI solution and 2 ml of 25% sulfuric acid were added to the mixture, the mixture was titrated with N/20 sodium thiosulfate under the administration of 1-2 drops of 1% starch solution for indicator. The endpoint was the disappearance of the blue color.

The cell growth of mold and pH changes in the starch broth culture for amylase activity tests were also recorded. The pH values were measured by Horiba pH meter/M7. The cell growth was estimated by centrifuging the mycelial cells at 3000 rpm for 30 minutes, washing the cell mass with sterile water, drying it in the oven at 60°C for 24 hours and finally weighing it.

The saccharifying and liquifying amylase activities of the molds at different pH levels were obtained in the same way mentioned above, except that of the substrate, which was adjusted to different pH values: a portion of soluble starch solution (0.1%) was dissolved in Mallvine buffer solutions ranging from pH 3.0 - 8.0.

B. Identification of the selected molds

The molds were grown on synthetic Mucor agar medium (dextrose 4 g, KH_2PO_4 0.05g, $MgSO_4$ 0.025 g, vitamin B_1-HCl 0.05 mg, agar 1.5%, distilled water 100 ml) and incubated at 26°C for one week. Microscopic examinations were made on the colonies.

C. Isolation and selection of alcohol fermentative yeasts

Certain ragi contain yeasts capable to ferment sugars to alcohol. From such ragi it may be expected to get good alcohol producing yeasts. However, good alcohol producing yeast not necessarily would produce good aroma or ester compounds. For brem wine fermentation yeast with both high alcohol and good ester producing capacity is desired.

Material and methods

Fifty-two ragi samples collected were subjected to the fermentation test: the tube containing 8 ml of YM broth-3% glucose and an inverted Durham tube each were inoculated with 0.1 g of ragi, and incubated at room temperature (24-28°C) for 1-2 days. The gas production after 1 day of incubation indicated high fermentation activity of the ragi. From these fermenting broth culture, the dominating yeasts were isolated by the plating method on YM agar and incubated at room temperature for 2 days. Several of the developed colonies were picked up at random for further analysis.

The fermentation activity of yeast in YM broth containing 20% glucose and 20% maltose was conducted as follows: into 8 ml of YM broth containing each of the sugars one loop of 2-3 days old yeast culture was inoculated and incubated at room temperature for 2 days. The gas formation was observed. The yeast producing high amount of gas in both sugars were selected, and were then subjected to the alcohol formation capacity test and ester formation test.

The alcohol formation test was carried out in YM broth containing 10 or 20% glucose and maltose in which the yeast was inoculated, and incubated at room temperature for 3 days. The alcohol % produced in the broth was determined by the distillation method. A 100 ml broth was distilled and the distillate was filled up to 100 ml volume. Then, an alcohol-meter was used to measure the alcohol %. A standard curve was made from known concentrations of alcohol and alcohol reading values at a definit temperature. The actural alcohol % values could be derived from the equation from the standard curve : $y=0.80x - 0.41$, where x is the alcohol % reading.

Lodder method for ester formation test was followed. YM agar containing 1% glucose was inoculated with one loop of yeast culture and plated in a petri-dish, and incubated at 28°C for 2 days. The lid of the petri-dish was slightly opened and smelled. A score of 1 to 10 was given.

Then, the selected yeasts were tested for alcohol production in black glutinous rice substrate. Glutinous rice and water (1:1.5) was autoclaved and inoculated with AM26 mold-ragi at 0.1% concentration, incubated at room temperature for 6 days and the syrup squeezed out. To a 100 ml of diluted syrup (syrup: water=1:1) of 17% Brix in an erlenmeyer flask was inoculated with 3 loops of each of the selected yeats, incubated at room temperature for one week, analysed for alcohol % (v/v) and the aroma scored. The yeasts capable of producing high concentration of alcohol and good aroma in the black rice were selected as a productive yeast.

D. The identification of the selected yeasts

For the identification of yeasts, the studies on the morphological and physiological characteristics of the yeasts were conducted according to the Lodder manual (1970) entitled "The yeast, a taxonomic study".

The morphological characteristics include the shape and size of the cell, the shape, the size and the number of spores formed by the cell, the ability of the yeast to form pseudo- or true-mycelium, the shape and size of the mycelium, and the pellicle formation in broth medium. The physiological properties indicate the ability of the yeast to assimilate certain carbon compounds, to ferment certain sugars, to utilize certain nitrogen sources, to require certain vitamins and to grow at elevated temperature and

in high osmotic pressure. The ability of the yeast to assimilate carbon compounds is very useful for taxonomic studies, especially if fermentation is weak or absent.

Materials and methods

The shape and size of the yeast cell was observed by growing the yeast in 5 ml of yeast extract malt extract broth medium (yeast extract 0.3%, peptone 0.5%, glucose 1%, malt extract 0.3%, and the pH was adjusted to 5.5-5.7) at 27°C for 2-3 days. A drop of the culture was mounted on a slide and a cover glass was put on it, and to avoid movements of the suspension by currents, the cover glass was sealed by the nail-polish. Observation was made under the microscope at 400x magnification.

The YM broth yeast culture above was also observed for pellicle formation. After incubation period of 2 days a pellicle or film layer development near the surface would be formed by a strong aerobic yeast.

The test on the ability of the yeast to grow at high temperatures was made by growing the yeast on YM agar slant and incubating it at 37°C for 1-2 days. Good growth could be observed when the temperature is still within tolerance.

The tolerance of the yeast to high osmotic pressure was tested by inoculating 1 loop of the yeast culture into 5 ml YM broth containing 50% and 60% glucose (YM base broth consisted of yeast extract 0.3%, malt extract 0.3%, peptone 0.5%; 50% glucose YM broth was prepared of 20 ml YM base broth and 20 g of glucose, and 60% glucose YM broth was made of 20 ml YM base broth and 30 g of glucose), pH 5.5 incubated at 27°C for 7-10 days. Yeast that can withstand high osmotic pressure would grow during this incubation period.

Filamentation could be stimulated by anaerobic condition of growth of the yeast. The ability of yeast to produce hyphae was observed by the slide culture technique as follows: in a petri-dish, a slide and cover glass were placed on top of a U-shape glass rod and 5 ml water was added at the bottom of the dish. Then the set was sterilized in autoclave at 15 psi for 15 minutes and cooled. Aseptically, using a sterile forceps to hold the slide, the slide was immersed in a melting Potato Dextrose Agar medium

(potato extract 0.4%, glucose 2.0 %, and agar 1.5%, pH 4.5-5.0), for a few seconds and cooled, with a result of the formation of a thin layer of PDA medium on the slide. The yeast to be tested was inoculated by streaking a straight line on the agar layer, then half of the streak was covered by the sterile cover glass to create an anaerobic condition for the yeast growth. Incubation was done at 27°C for 2-3 weeks. The edge or the margin of the colonies was observed under the microscope, magnified 400x, to examine any development of mycelium. Photographs were taken.

Spore formation in yeast may be enhanced by growing the yeast in a medium with limited nutrients. Such media includes Fowell Agar (Na-acetate trihydrate 0.3-0.5% and agar 1.5%, pH 6.5-7.0) and Gorodkova Agar modified (peptone 1%, glucose 0.1%, NaCl 0.5% and agar 2.0%). The yeast was grown on these media, and spore formation was observed after 2-3 weeks of incubation at 27°C. The wet mount of the yeast culture was examined microscopically.

For testing of sugar-fermentation, carbon assimilation and utilization, vitamin requirements and nitrogen assimilation, special preparation of yeast culture suspension is required. The most important step is to avoid any contamination of sugars from previous medium in which the yeast has been cultured. It was necessary to wash out the yeast cells so that any possible sugar carried with the cells may be eliminated. The procedure was as follows: the yeast was precultured in Wasserman tube containing 5 ml of YM broth at 27°C for 2 days, then it was centrifuged at 3000 rpm for 20 minutes. The cells sedimented at the bottom were washed twice with 5ml 0.85% NaCl solution using a Thermonics automatic thermomixer model 7M-104, and centrifuged. The cells were then resuspended in 5 ml 0.85% NaCl solution and the cell suspension was used to inoculate the fermentation test media. But, for testing the C and N utilization and the vitamin requirement, one drop of this suspension was suspended in 5 ml of 0.85% NaCl solution; and one drop of this diluted suspension was used for inoculating to the media. The fermentation media was prepared as follows: yeast extract 0.45%, peptone 0.75% and the tested sugar 3% dissolved in distilled water, was dispensed 5 ml into test tubes containing iverted Durham tubes. The sterilization of these sugar-containing media was at 100°C for 20 minutes. The C-compounds used were: glucose, galactose, -methyl D-glucoside, sucrose, maltose, cellobiose, trehalose, lactose, melebiose, raffinose, melezitose,

inulin and soluble starch. The preparation of C-assimilation media: Difco yeast N base 0.67% and tested C-compound 0.5% dissolved in destilled water, was dispensed 5 ml into test tube. The C-compounds were: glucose, galactose, α-methyl-D-glucoside, sucrose, maltose, cellobiose, trehalose, lactose, melebiose, raffinose, melezitose, inulin, soluble starch, L-sorbose, salicin, arbutin (hydroquinone β-glucoside), D-xylose, D-arabinose, L-arabinose, D-ribose, L-rhamnose, ethanl, glycerol, erythritol (erythrite), dulcitol (galactitol), D-mannitol, D-sorbitol, inositol, DL-lactic acid, succinic acid and citric acid. Preparation of N-assimilation media: Difco yeast C base 0.585% and NH_4SO_4 0.15% for NH_4-assimilation test, and yeast C base 0.58% and KNO_3 0.78% for nitrate assimilation test, whereas yeast C base 0.585% only was for N-requirement test. Five ml media was dispensed into each test tube.

Fermentation of the carbon compounds by the yeast was indicated by the formation of gas after 1-3 days of incubation at 27°C. And the growth of yeast in the assimilation media was shown by the turbidity of the culture broth compared with the control blank.

Results and discussion

A. Isolation and selection of amylolytic molds

Of the 52 ragi tested, only 13 were able to make a satisfactory fermentation product with very sweet taste and very soft texture (Table 6), 7 ragi produced adequate sweetness and softness, and 7 ragi could not make tape at all. Of the 14 very good ragi, 6 ragi were made by large factories and 8 were home made. Whereas of the 7 poor ragi, 2 were made in factories and 5 were made in the homes.

The 20 ragi were plated for isolation purpose along with 4 lower quality ragi with the hope to get good amylolytic molds. From the 24 ragi, only 88 mold isolates were able to grow well on starch agar medium. But, their degradation of starch to sugars, as shown by the clearance of the blue color of the medium upon flooding by iodine-solution, was limited to only 18 molds (Table 7).

Table 6. Ragi samples, their origin and fermentation product characteristics regarding sweetness and softness.

Ragi code	Brand name and place of origin/sampling	Characteristics of fermentation product
A	"NKL" Solo, Denpasar-Bali	sweet, quite soft
B	"Roda Mas R"Solo, Denpasar-Bali	sweet sour, soft
C	"Dewa", Sampang-Madura no	not sweet, quite soft
D	"NKL" Solo, Sampang-Madura	sweet, very soft
E	"Manalagi", Sampang-Madura	not good tape
F	Bondowoso, Surabaya	not good tape
G	"NKL" Solo, Malang	very sweet, very soft
H	"Roda" Solo, Malang	very sweet, soft
I	"Burung gelatik", Malang	sweet, quite soft
J	"66", Madiun	very sweet, very soft
K	"Roda Mas R" Solo, Semarang	not sweet, soft
L	"Leo" Solo, Semarang	sweet, soft
M	"NKL" Solo, Magelang	sweet, soft
N	Palembang-Sumatera	not sweet, quite soft
O	Sopeng-Sulawesi	not sweet, not soft
P	Sukabumi, Bogor	sweet sour, soft
Q	"Matahari/cakra", Bandung	sweet sour, soft
R	Bengkulu-Sumatera	sweet, soft
S	"Roda Mas R" Solo, Solo	sour-sweet, quite soft
T	"Roda Mas ABC" Solo, Solo	sweet, soft
U	Cugenang/Cianjur	very sweet, very soft
V	"Pohong Padi" Solo, Solo	very sweet, very soft
W	Lasem	very sweet, very soft
X	"Jempol", Solo	very sweet, soft
Y	"Roda Mas R" Solo, Solo	sweet, alcoholic, soft
Z	Cianjur	sweet sour, very soft
AA	Cibadak/Cipanas (singkong)	quite sweet, quite soft
AB	Cibadak/Cipanas (Ketan)	quite sweet, not soft
AC	Kuningan	sweet, not soft
AD	Cisaat/Cicurug	rather sweet, quite soft
AE	Gg Cincau/Bogor	not sweet, sour, quite soft
AF	Depok/Cicurug	not sweet, not soft

AG	Awirarangan/Cianjur	sweet, quite soft
AH	Cipanas/Cirebon	slimy, spoiled
AI	Cimangkit/Ciapus	rather sweet, not soft
AJ	Cugenang/Cianjur	slimy, spoiled
AK	Lembursitu/Sukabumi (singkong)	not sweet, hard
AL	Lembursitu/Sukabumi (ketan)	not sweet, hard
AM	Cibadak/Cipanas	very sweet, very soft
AN	Kebonjati/Sukabumi	sweet, very soft
AO	Jl. Labuan/Sukabumi	sweet, soft
AP	Jatinegara (bikangambon)	not sweet, hard
AQ	Caringin/Ciawi	slimy, spoiled
AR	Banjarrejo/Malang	not sweet, sour, quite soft
AT	"Gedang" Solo	not sw-et, hard
AU	Krasak-ageng/Pekalongan	very sweet, very soft
AV	Kebon-pedes/Sukabumi	very sweet, very soft
AW	Cugenang/Cianjur	quite sweet, not soft
AX	Pemalang/Pekalongan	very sweet, very soft
AY	Cicurug	sweet, soft
CB	Rawa-belut/Cipanas	very sweet, very soft
AS	Suger/Malang	hard, sour

Table 7. Mold isolates from selected ragi and their clearance intensities upon starch-iodine test

Mold code	Clearance intensity	Mold code	Clearance intensity	Mold code	Clearance Intensity
D1	-	Y4	++	AU5	+
D5	-	Y5	-	AU7	+
H1	-	W1	-	AU11	-
H2	-	W2	++++	AU12	-
G1	-	W3	-	AU13	-
G2	-	Z1	+++	AU19	-
J1	-	Z2	-	AV1	++
J2	-	AA1	-	AV2	+++
T2	-	AA3	-	AV3	+
T4	+	AB2	-	AV4	-
T5	-	AB6	++	AV5	++
U1	+	AC1	++	AV6	-
U3	++	AC2	-	AV11	-
U4	-	AC3	++	AX1	-
U5	-	AD1	++	AX2	++++
V1	-	AD4	-	AX3	+
V3	+	AE1	-	AX4	+
V4	-	AE2	-	AX6	-
V5	-	AF1	-	AX7	++
V6	-	AG1	-	AX8	+++
S2	++	AM1	+	AX9	+
S4	+++	AM2	+	AX11	++
S5	++	AM3	+	AX21	-
S6	++	AM26	+++	AX22	+
X1	-	AN1	+	CB1	-
X2	-	AN2	+	CB3	+++
X3	-	AU1	+	CB4	++
Y1	-	AU2	-	CB5	-
Y2	-	AU3	+++	CB6	++
Y3	-	AU4	+	CB9	+++

Table 8. Selected molds and their tape production

Mold code	Tape product characteristics
U3	no good tape
Z1	sweet, very soft
Y4	no good tape
W2	very sweet, pleasant aroma, very soft
S2	no good tape
S4	sweet firm tape
S5	sweet with some sourness,soft
S6	no good tape
AB6	sweet aromatic, very soft
AM26	very sweet, very soft, aromatic
AU3	very sweet with some sourness, very soft, alcoholic
AV2	very sweet, very soft, mild aroma
AX2	sweet, soft
AX8	sweet, soft
CB3	very sweet, aromatic, very soft, slightly sour
CB4	very sweet slightly sour, aromatic, soft
CB6	very sweet, soft
CB9	very sweet, very soft

From further test as seen in Table 8, only 4 molds out of the 18 selected molds were able to make excellent tape, very sweet and very soft product. Those molds were W2, AV2, AU3 and CB3.

Several molds having quite good clearance in starch agar-iodine test, such as U3, Y4, S2 and S6, could not make a good tape. Perhaps, this mold produces only gluco-amylase without any liquifying amylase.

The amylase activity tests showed that the AU3 and CB3 molds had the highest activities. The pattern of saccharifying amylase activity of the 4 molds were quite different, whereas that of liquifying amylase activity of the molds except AU3 were approximately similar (Figure 27).

During growth in starch broth, the pH of broth dropped from 5.5 to a minimum of 3.4 to 3.5 after 6 to 10 days incubation and the cell mass increased rapidly after 4 to 6 days incubation (Figure 28) and AV2 being the highest in mycelial mass.

As seen in Figure 27, the pH drop is accompanied by the decrease in the liquifying amylase activity in all the broth cultures. But, the saccharifying amylase is quite stable; it starts to decrease in activity after the pH reaches 3.4.

The effect of pH on the amylase activities are shown in Figure 29. At pH 4.5 the activity of both saccharifying and liquifying amylase is best for AV2 and AU3. But, for W2 the optimum pH for the amylase activities is 5.0. Therefore, to get good efficiency of the amylases, the pH of the substrate during fermentation need be maintained between 4.5-5.0.

B. Identification of the selected molds

The molds did not sporulate on conventional media. On synthetic Mucoragar the colony reaches a height of up to 1 cm. The sporangiophore commences from both vegetative- and aerial-mycelia and it rarely branches. The shape of the sporangium is globular to oval. The size of the sporagium is 100-230 microns in diameter. The sporangium is colorless at early stage, but later it turns to brown. No sporangios-

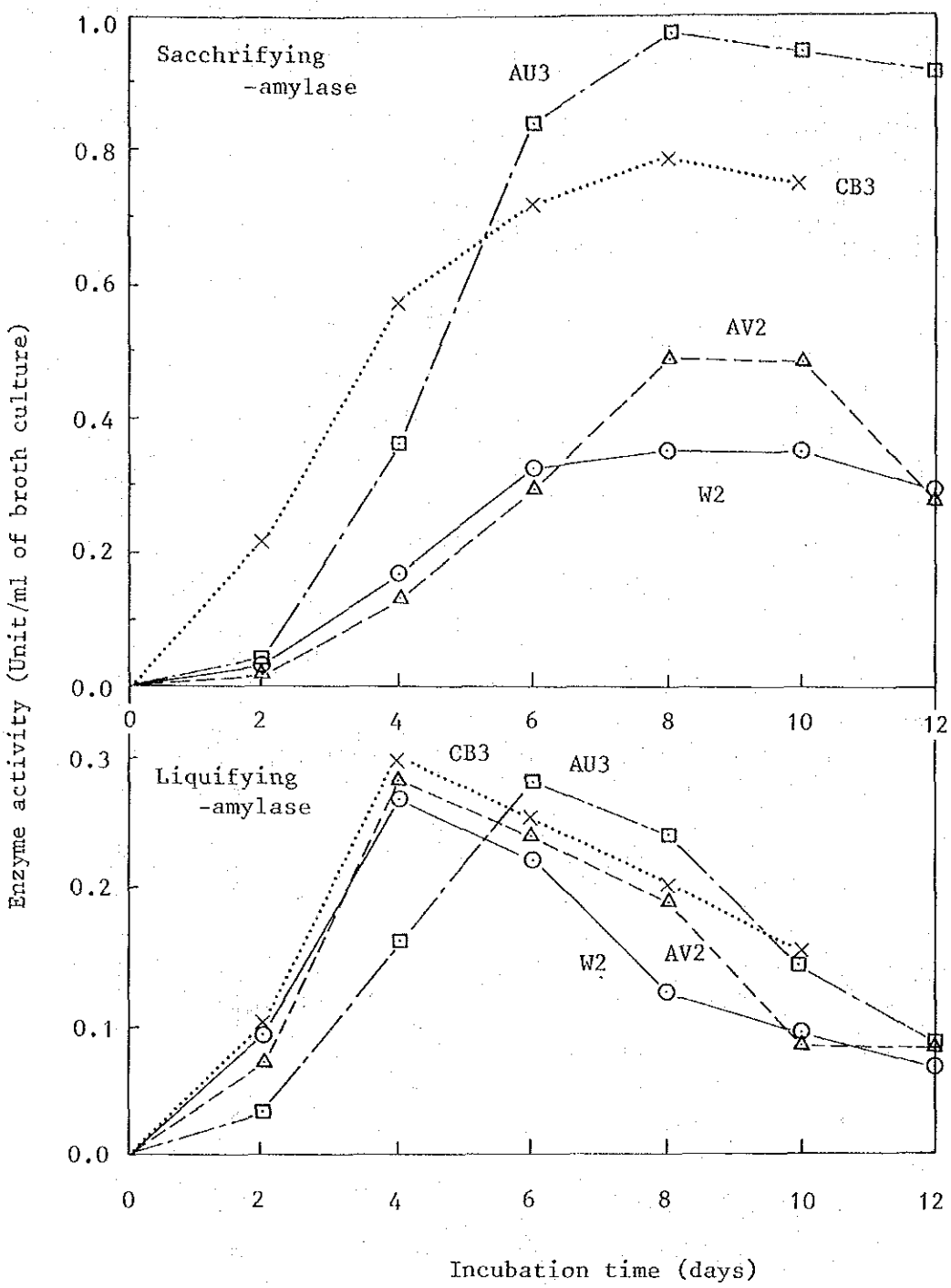


Figure 27. The amylase activities of W2, AV2, AU3 and CB3 molds during incubation in Starch broth.

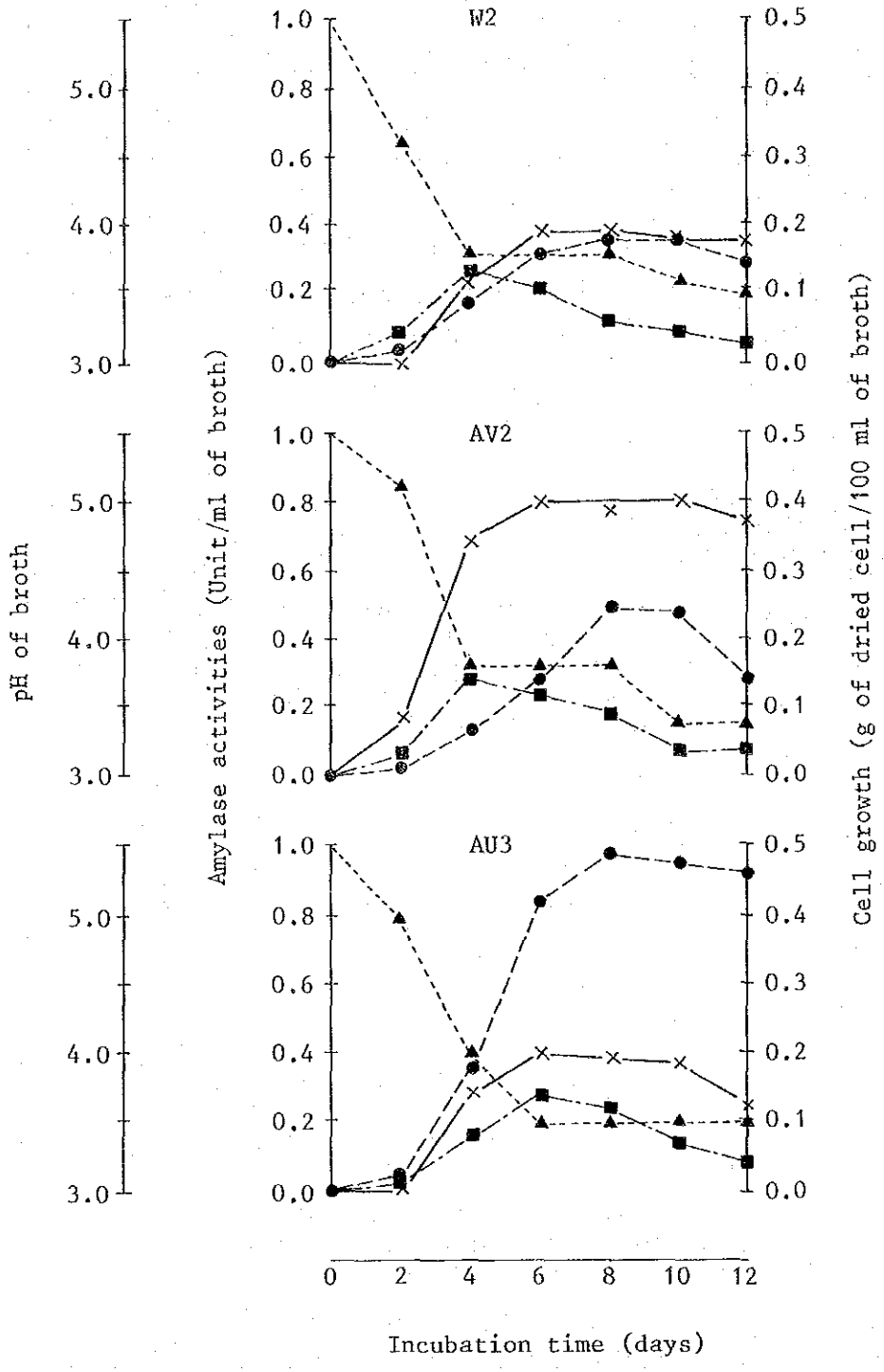


Figure 28. Changes in activities of saccharifying-amylase (●—●), liquifying-amylase (■—■) and pH (▲—▲) during cell-growth (×—×) of W2, AV2 and AU3 molds in Starch broth medium at 28°C.

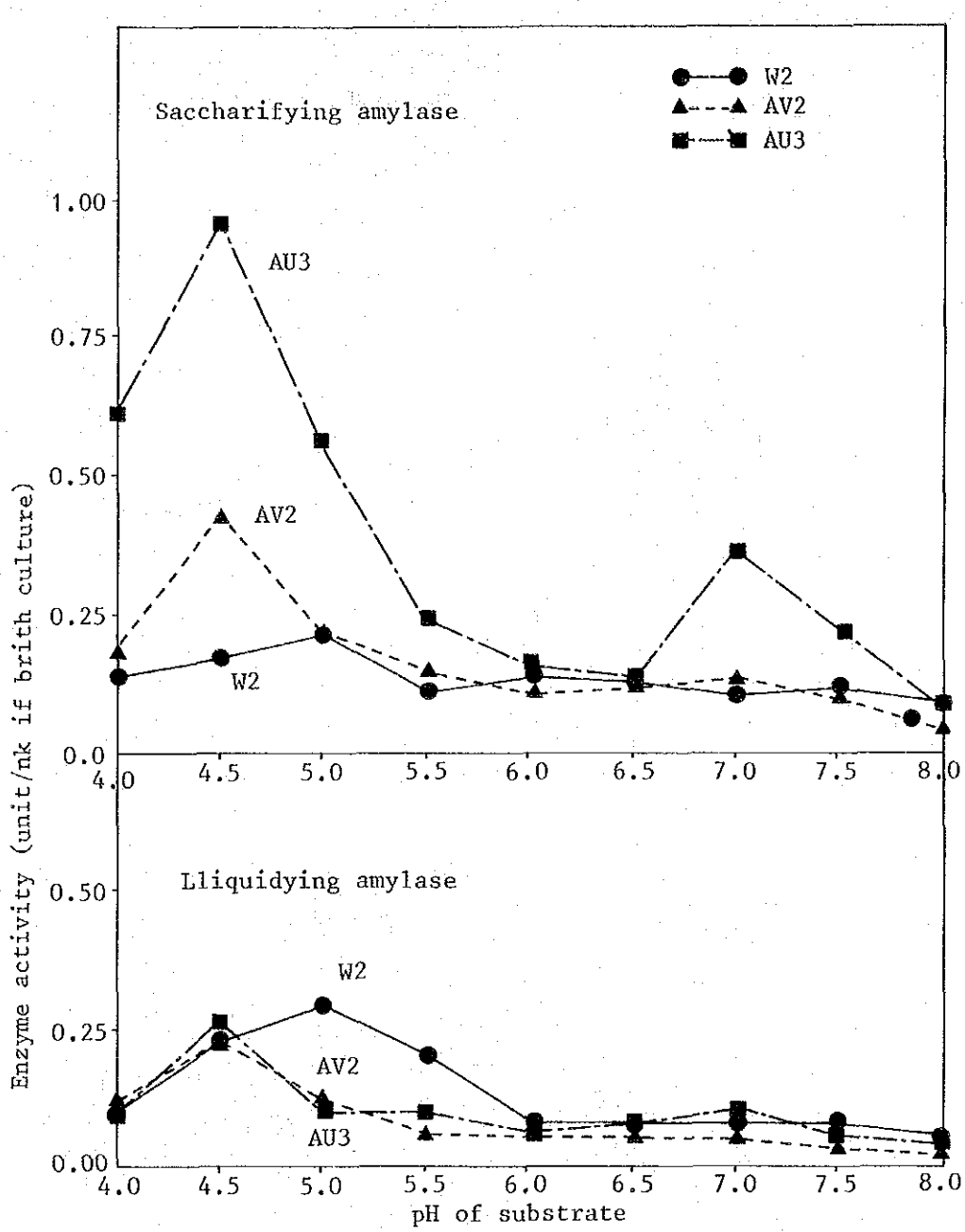


Figure 29. The saccharifying and liquifying amylase activities of W2, AV2 and AU3 molds at different pH values of Starch broth medium.

Figure 30. The mold growth at early stage CB3.

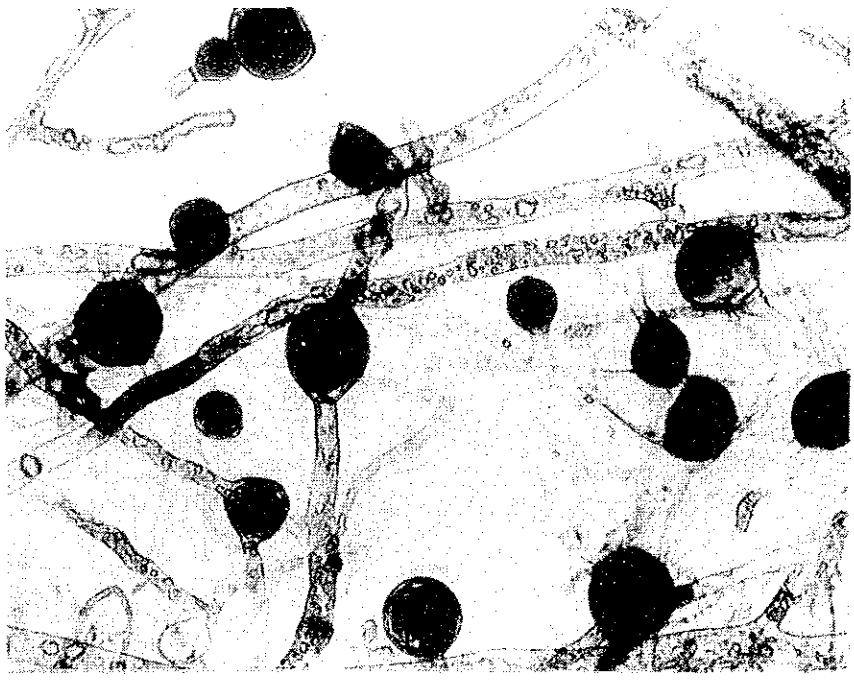


Figure 31. The chlamidospore of CB3 mold.

Table 9. Fermentation test of ragi samples, in 3% glucose YM broth

Ragi	Fermentation test	Ragi	Fermentation test
A	–	AA	–
B	++	AB	+
C	+	AC	–
D	–	AD	–
E	++	AE	++
F	+	AF	+++
G	–	AG	+
H	++	AH	–
I	+	AI	+
J	+	AJ	–
K	+++	AK	–
L	+	AL	++
M	–	AM	–
N	++	AN	++
O	++	AO	+
P	–	AP	+
Q	+	AQ	++
R	+	AR	–
S	+++	AS	++
T	+++	AT	+
U	+++	AU	++
V	+	AV	–
W	+	AW	–
X	+	AX	+
Y	+++	AY	+
Z	–	RM	+++

Table 10. Fermentation test in 20% glucose and 20% maltose YM broth

Ragi	Glucose	Ragi	Glucose	Maltose	Ragi	Glucose	Maltose
B1	+	T8	++		U11	++	-
B2	++	S21	++	++	U13	+	-
B3	+	S22	++	+	Y1	+++	+++
B4	++	S23	++	++	Y2	+++	+++
C1	-	S24	+	+	Y3	+++	++
E1	++	S25	++	++	Y4	+++	+++
E2	++	S26	+++	+++	Y5	+++	+++
E3	++	S27	+++	+++	Y6	++	+++
F1	-	H6	-	-	Y7	+++	++
G1	-	H7	-	-	Y8	+++	++
H1	+	K6	++	++	Y9	+++	++
H2	+	K7	++	+	Y10	++	+++
H3	+	K8	++	+	AF1	-	-
I2	++	K9	++	+	AF2	-	-
K1	+++	N5	++	-	AF3	-	-
K2	+++	N6	++	-	AF4	-	-
K3	+++	N7	++	-	AF5	-	-
L1	-	N8	++	+	AF6	-	-
N1	++	N9	++	+	AF7	+	-
N2	+	N10	++	+	AF8	+	-
N3	++	U1	-	-	AF9	+	-
N4	+	U2	-	-	AE1	++	-
O1	-	U3	-	-	AE2	++	++
O2	-	U4	+	-	AE3	++	++
O3	-	U5	+	-	AE4	++	++
Q1	+	U6	+	-	AE5	-	-
R1	-	U7	-	-	AE6	-	-
S5	++	U8	-	-	RM1	+++	+++
S6	++	U9	+	-			
T7	++	U10	+	-			

Table 11. The alcohol % produced in 10 and 20% sugar, and ester-score by yeasts

Yeast	% alc.*	ester score	Yeast	% alcohol in**		aroma score in	
				glucose	maltose	glucose	maltose
B2	8.5	6	Y1	11.8	10.0	5	5
B4	9.0	6	Y2	12.2	10.6	5	5
E1	9.0	4	Y3	11.4	10.1	5	5
E2	10.0	6	Y4	13.2	7.8	5	7
E3	8.3	6	Y5	13.4	10.6	8	5
H1	10.0	8	Y6	12.3	9.3	5	5
H2	7.6	2	Y7	12.2	10.6	5	5
H3	8.5	6	Y8	12.0	9.0	5	5
I2	10.0	4	Y9	14.0	10.2	5	5
K1	9.5	8	Y10	12.1	8.0	7	6
K2	9.0	8	K6	13.3	13.0	6	6
K3	10.2	10	K8	12.8	8.5	6	6
N1	9.0	4	K9	12.5	10.2	6	7
N2	6.8	2	N8	3.8	0.0	8	5
N3	9.5	8	N9	5.1	0.2	8	5
N4	4.8	2	N10	7.3	ND	7	6
Q1	5.6	4	AE2	12.2	7.0	5	5
S5	8.0	2	AE3	12.8	1.0	5	7
S6	8.0	8	AE4	12.5	7.3	6	6
T7	8.0	8	DA3	11.8	0.4	6	6
T8	8.0	6	RM1	+++	+++	8	6
			S26	14.0	10.6	6	6
			S27	13.6	13.0	6	6

* from 10% glucose-YM broth

** from 20% sugar-YM broth

Table 12. Alcohol production and aroma score by the yeast in black glutinous rice syrup of 17% Brix.

Yeast	Alcohol %	Aroma score
N3	11.4	8 (aromatic)
I2	11.0	6 (alcoholic)
S26	11.4	4 (plain)
S27	11.0	4 (plain)
Y5	11.8	8 (alcoholic and aromatic)
K3	11.0	8 (*pleasant, nice aroma*)
RM1	11.7	9 (very odoriferous)

pore is produced. An apophisis is found at the lower part of the sporangium with the shape of a wedge, a scallop or a club. Rhizoids and stolons are scarcely found. The chlamydospores are produced abundantly in both vegetative- and aerial-mycelia, which occur singly or in chains. The shape of the chlamydospore is globular, oval and cylindrical with the size of (15-145) x (15-105) micron. The chlamydospore has a thick wall, 2-8 microns in thickness.

Since these molds produce many chlamydospores in vegetative- and aerial-mycelia, an apophisis is found at the lower part of the sporangium, and their habitat is ragi, so these molds are identified as: Amylomyces rouxii. Calmette emend, Ellis, Rhodes et Hesseltine (Figure 30, 31).

c. Isolation and selection of alcohol yeasts from ragi

The results of the fermentative test of 52 ragi samples are presented in Table 9. From this test, it showed that only 7 ragi had very strong fermentative activities, whereas 16 ragi were not fermentative at all, which means that no alcohol yeast was present in such ragi.

From the 20 fermentative ragi 88 yeasts were isolated, and only 15 yeasts were strongly fermentative (Table 10). Nine-teen were non-fermentative.

Alcohol production in 10-20% glucose or maltose by the yeasts and their aroma score are shown in Table 11. Since maltose is present in some quantity in saccharified rice, the maltose fermentation is of importance. K6 yeast was good in alcohol formation from maltose, but unfortunately, in glucose broth it did not produce a pleasant aroma, and so it was not selected. RM1, on the other hand, was selected since it was dominant in a good brem wine fermentation.

Table 12 shows the production of alcohol and aroma in black glutinous rice syrup of 17%. From this test, 4 yeasts were selected due to their good alcohol and aroma production. Those were K3, N3, Y5 and RM1. Three of them, K3, Y5 and RM1, originated from "B" ragi, and N3 was isolated from a ragi made in Palembang (Sumatera).

D. Identification of the selected yeasts

The cell shape and cell size of the yeasts K3, N3, Y5 and RM1 are shown in Figure 32. All of these yeasts were able to produce ascospores, 2-4 spores per cell. They also formed pseudomycelium on PDA (Figure 34) (Figure 33). No pellicle or film layer was formed by these yeasts.

The results of the test for physiological characteristics are given in Table 13. By consulting the Lodder's key for yeast identification (1970), these yeasts all were identified as Saccharomyces cerevisiae.

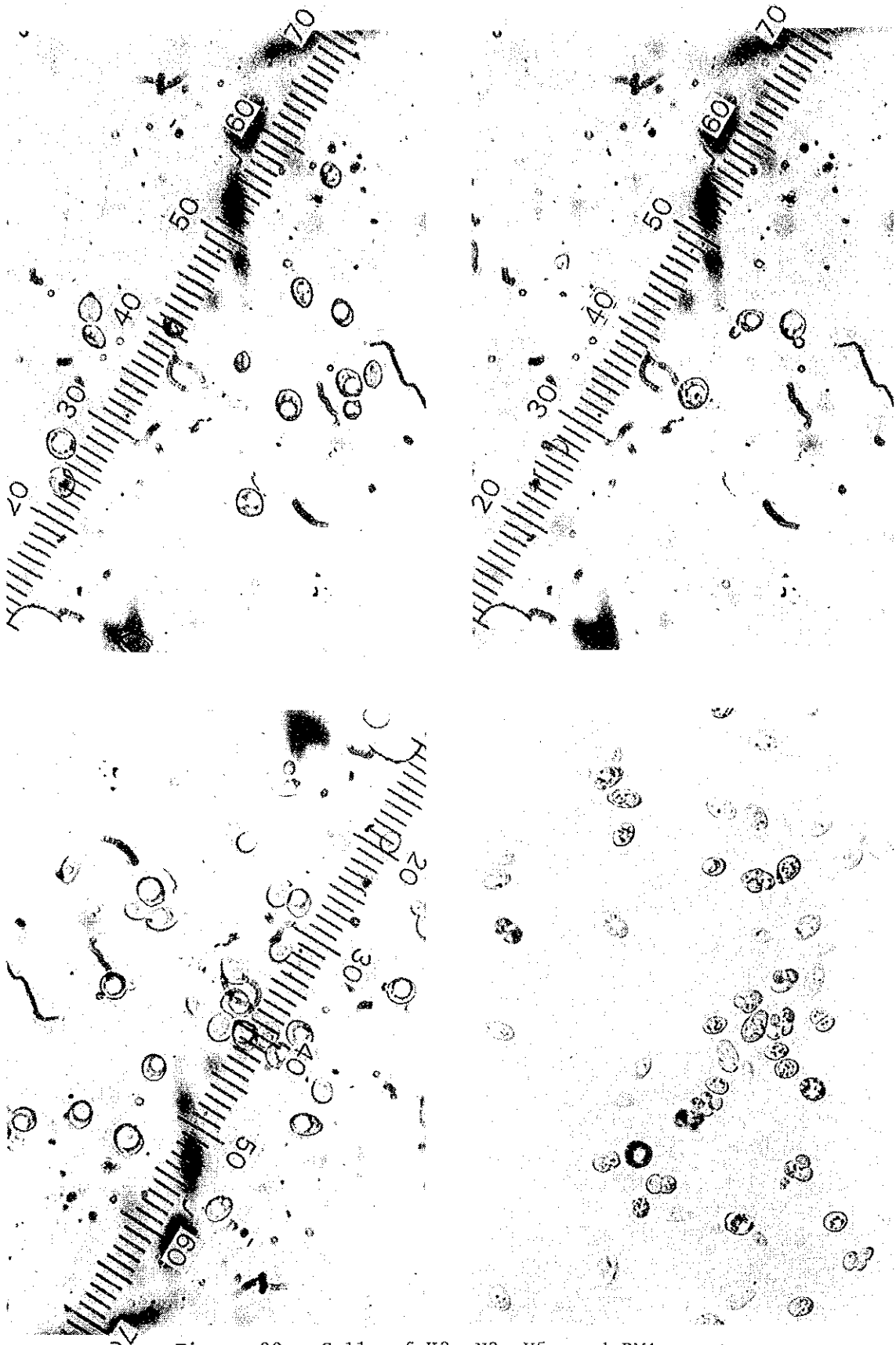


Figure 32. Cells of K3, N3, Y5, and RM1 yeast in 2 days old YM broth (400x: 1 scale - 1.5 micron)

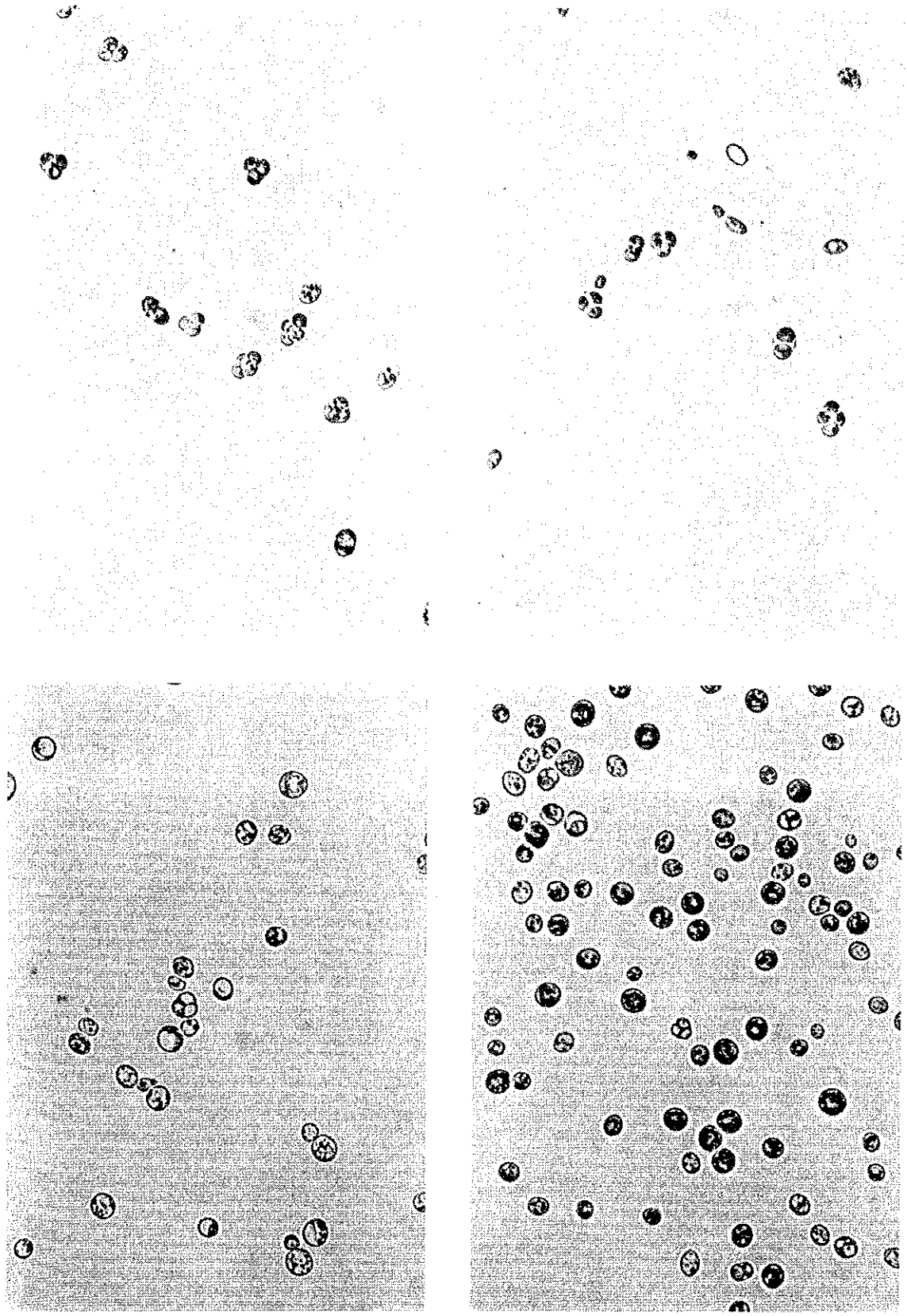


Figure 33. Spore formation in K3, N3, Y5 and RM1 yeast cells.

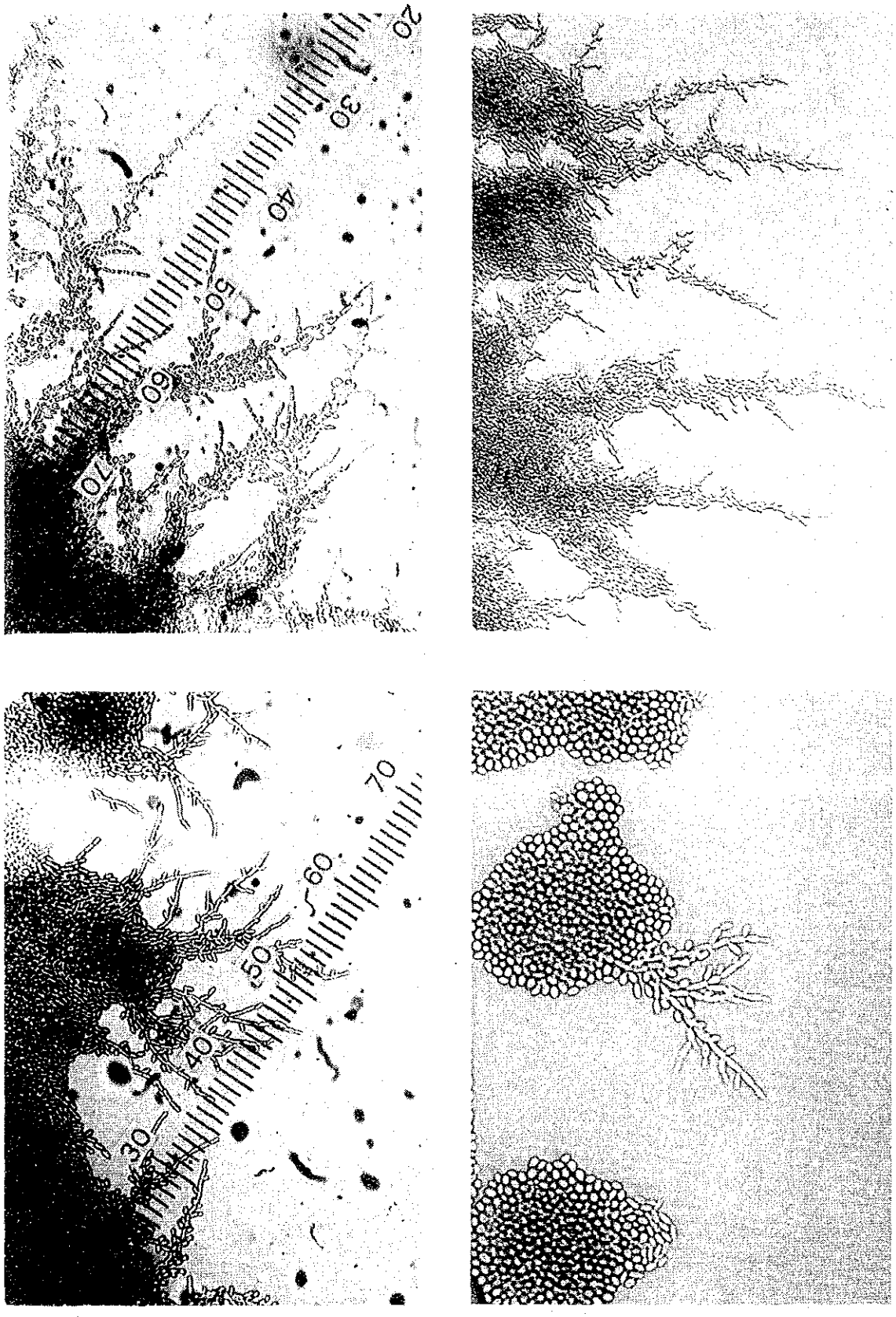


Figure 34. Pseudomycelium formation by K3, N3, Y5 and RM1 yeasts on PDA.

Morphological characteristics of the selected fermentative yeasts

Yeast	Cell shape	Cell size (micron)	Spore shape	Spore number/cell	Pseudomycelium form'on
K3	spherical to oval	(2.3-4.5)x(3.8-5.3)	spherical	2 - 4	+
N3	oval to ellipsoidal	(2.3-4.5)x(3.0-5.3)	spherical	2 - 4	+
Y5	oval to ellipsoidal	(2.3-4.5)x(3.8-5.3)	spherical	2 - 4	+
RM1	spherical to oval	(2.3-4.5)x(3.0-5.0)	spherical	2 - 4	+

Physiological characteristics of the selected yeasts

Fermentation of:	gal	α-meth D-gluc	sucr	malt	cell	treh	lact	meleb	raff	melez	inul	sol starch
Yeast K3	+	-	+	+	-	+s	-	-	+1/3	-	-	-
N3	+	-	+	+	-	-	-	-	+1/3	-	-	-
Y5	+	-	+	+	-	-	-	-	+1/3	-	-	-
RM1	+	+s	+	+	-	-	-	-	+1/3	-	-	-

Utilization of:	gal	α-meth D-gluc	sucr	malt	cell	treh	lact	meleb	raff	melez	inul	sol starch
Yeast K3	+	+s	+	+	-	+	-	-	+	+	+	-
N3	+	-	+	+	-	+s	-	-	+	+	+	-
Y5	+	-	+	+	-	+	-	-	+	+	+s	-
RM1	+	+s	+	+	-	+	-	-	+	+	+s	-

Table 13. Physiological characteristics of the selected fermentative yeasts (continued)

Utilization of:	L-sor	D-xyl	D-ara	L-ara	D-ri	L-rha	Eth'ol	Glyo'ol	Eryt'ol	Ino'ol	Dul'ol	Man'ol	Sor'ol
Yeast K3	–	–	–	–	–	–	+	+	–	–	–	–	–
N3	–	–	–	–	–	–	+	+	–	–	–	–	–
Y5	–	–	–	–	–	–	+	+	–	–	–	–	–
RM1	–	–	–	–	–	–	+	+	–	–	–	–	–

Utilization of:	Sal	Arb	Lact acid	Suc acid	Citr acid	Nitrate	Ammo-nium	Growth in medium: N2-free	Growth in medium: Vitamin-free	glucose 50%	glucose 60%	Temperature 37°C
Yeast K3	–	–	+	–	–	–	+	–	+s	+s	–	+
N3	–	–	+	–	–	–	+	–	+s	+s	–	+
Y5	–	–	+	–	–	–	+	–	+s	+s	–	+
RM1	–	–	+	–	–	–	+	–	+s	+s	–	+

	Splitting of Arbutin	Glyloheximide resistance (1ppm)	Pellicie formation
Yeast K3	–	–	–
N3	–	–	–
Y5	–	–	–
RM1	–	–	–

PART III - EFFECT OF RAGI SPICES ON RAGI MYCOFLORA

Some spices such as garlic and cinnamon have a harmful effect on microorganisms. (Frazier and Westhoff 1978). Garlic inhibited Rhizopus cryzae. And, ginger inhibited the amylolytic molds Mucor and Clamydomucor but not the alcohol yeast Saccharomyces cerevisiae (Soedarsono 1970). Therefore perhaps that ginger was used in bubod for basi preparation, since in this basi preparation no amylolytic mold is needed and the only microorganism necessary is the S. cerevisiae yeast. (Sanches 1981)

In the survey of ragi making (Part II), it was seen that red chillies concentration may have an effect on the fermentativity of ragi.

Materials and Methods

Ragi was prepared of 40 g of rice flour and a single ragi spice of 5 g red-chillies (12.5%), 7.5 g garlic (18.75%), or 15 g laos root (37.5%). Rice flour in petri dish was oven-heated at 120°C for 1 hour. The red-chillies, garlic and laos were washed by detergent, soaked in 95% alcohol for 15 minutes and rinsed throughly by sterile water (3x) and drained. Then it is ground in motor aseptically, mixed with 35 ml sterile coconut juice of 2.9% Brix and inoculated by 3 main groups of ragi microorganisms separatedly: (1) Y5, Saccharomyces cerevisiae, isolated from "B" ragi from Solo; (2) H13, Endomycopsis fibuligera, isolated from "C" ragi, Solo; (3) W2, Amylomyces sp., isolated from ragi from Lasem. Incubated at 28-30°C for 48 hours and afterwards dried at 42-44°C for 48 hours, and stored at ambient temperature. Analysis of the microbial retention and contamination was done after 0 and 10 weeks of storage by Plating method on Starch Agar (SA), Yeast extract Malt extract Agar (YMA) with pH 5.5, and Glucose Yeast extract Peptone Agar (GYPA) containing 1% $CaCO_3$ and having pH of 6.5.

Results and Discussion

The results are given in Table 14. During incubation period, red-chillies (at 6% concentration in the dough) stimulated the growth of all ragi mycoflora tested, Saccharomyces cerevisiae, Endomycopsis fibulgera and Amylomyces sp., but also yeast contaminants perhaps coming from the red-chillies inner-parts. However, garlic (9% in the dough) and laos (16.5% in the dough) proved to inhibit these ragi mycoflora. Further, during storage of dried ragi, both

garlic and laos decreased the ragi mycoflora after 10 weeks. Also garlic seemed to support the contamination and growth of lactic-acid bacteria in ragi, which may not be desirable if in high quantity. Refering to Soedarsono's (1972) investigation, garlic was inhibiting Rhizopus oryzae, Aspergillus niger and Bacillus subtilis. Therefore garlic may be of use to avoid growth of the unwanted A. niger and B. subtilis in ragi, but it would be harmfull to the desired ragi mycoflora also.

Table 14. The numbers of ragi mycoflora Y5, H13 and W2, and contaminants in ragi containing red chillies, garlic, and laos, compared to blank ragi, after 0 and 10 weeks of storage.

Ragi mycoflora	Blank		Red Chillies		Garlic		Laos	
	0 wk	10 wk	0 wk	10 wk	0 wk	10 wk	0 wk	10 wk
Y5	$8x10^5$	$3x10^6$	$5x10^6$	$1x10^6$	10^4	$1x10^2$	$4x10^5$	$2x10^2$
H13	$9x10^6$	$2x10^8$	$4x10^7$	$1x10^7$	10^4	10^2	10^3	$1x10^1$
W2	$1x10^4$	$2x10^4$	$4x10^4$	$7x10^4$	$6x10^4$	$1x10^2$	$3x10^3$	10^2
Bacterial-contaminants								
Y5	10^4	10^2	10^3	10^2	10^1	L 10^4	10^4	10^3
H13	10^4	10^2	10^4	10^2	10^1	L 10^4	10^3	10^3
W2	10^4	10^4	10^4	(Y 10^6)	10^1	L 10^4	10^4	10^3

L: Lactic-acid bacteria

Y: non-filamentous yeast able to utilize starch

In the blank ragi, where no spices was added, higher number of ragi mycoflora was retained during storage. This means that making ragi would be better in the absence of spices, if conducted under aseptic conditions and provided cultures as a microbial source is used. In ragi preparation, the presence of spices such as cinnamon, pepper, adas, chillies, laos and garlic seemed to serve mainly as a source of some microorganisms and as a selective stimulator and inhibitor for certain microorganisms.

PART IV - EFFECTS OF STORAGE TEMPERATURES ON THE VIABILITY AND ACTIVITY OF MOLD AND YEAST IN RAGI

It is known that the quality of ragi decreased during storage. Old ragi can not produce good tape and the tape produced from such ragi would taste sour and smell sour. The shelf-life of ragi is about 2-3 months at room temperature. Certain microorganisms can not survive in ragi balls, others may have survived, causing the imbalance of fermentation by such ragi.

From an experiment (Saono and Tresnalarawaty 1982) on yeasts isolated from ragi showed that the amylolytic filamentous yeast Candida intermedia was resistant and increased in numbers during storage at ambient temperature (28-30°C) whereas the fermentative non-filamentous Saccharomyces cerevisiae decreased drastically.

The study whether lower temperatures could improve the shelf-life of ragi microorganisms was of importance here.

Materials and Method

Strains W2, AV2 and AU3 of the amylolytic mold A. rouxii were isolated from home-made ragi of Lasem, Sukabumi, and Pekalongan, respectively. Fermentative yeast Saccharomyces cerevisiae strain K3 was isolated from factory-made "B" ragi from Solo.

Preparation of single-culture mold ragi: 10 g of rice flour in petri dish was heated in oven at 105°C for 18 hours, then a one-week old mold culture on Potato Dextrose Agar slant was scraped off by a small spatula and 12 ml sterile water was poured into the already cooled rice flour, and mixed throughly by using a spoon into a thick dough. This was carried out in aseptic condition. Afterwards the ragi mixture was incubated at 28-30°C for 3 days, and dried at 42-45°C for 2 days. For each strain of mold 4 petri dishes of ragi were made.

Preparation of mixed-cultures of mold and yeast ragi: it was the same as for single-culture mold ragi, except that here a one-week old yeast culture on Yeast extract Malt extract Agar slant was added besides the mold culture. The yeast culture was scraped off by loop into the sterile water prior to the scraping of the mold culture.

The method of storage of ragi: The dried ragi of 4 petri dishes were gound together in mortar aseptically and mixed homogenously, thereafter it was devided into 3 petri dishes for storage at 3 different temperature conditions: (1) freezer temperature of -14 to -11°C, (2) refrigerator temperature of 5 to 10°C, and (3) room temperature of 25 to 30°C. The petri dish containing the ragi was wrapped in clean paper and put in plastic bag during storage period of 8.5 months.

The viability determination of microorganisms in ragi was done by Plating-method on PDA and YMA media after quantitative dilution procedure of the ragi sample, incubated at 25-28°C for 1 day for mold count and 2 days for yeast colonies count.

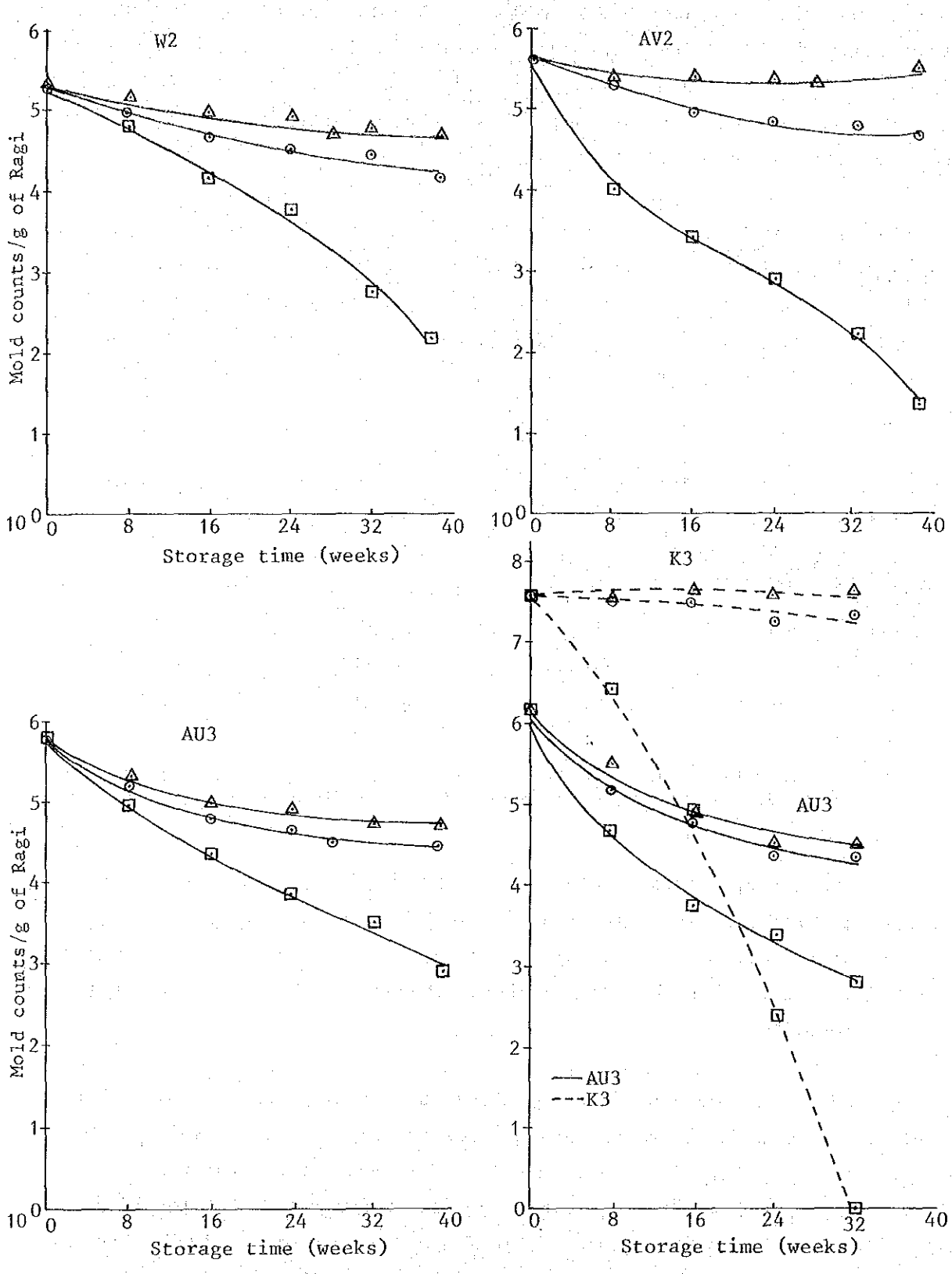
The aw-measurement of ragi was done by Durotherm Aw Wertmesser, Lufft (West Germany).

The fermentation activity of ragi: 50 g black glutinous rice and 75 g destilled water in heat resistant polyethylene bag was autoclaved at 15 p.s.i. for 15 minutes, cooled, and inoculated by 0.125 g ragi, then incubated at 25-28°C for 6 days. The soft fermented rice was squeezed, and the liquor produced was measured volumetrically, and further analyzed for reducing-sugar concentration (Lane-Eynon method), pH (Hitachi Horiba M7II-pH meter), Titratable Acidity, Alcohol content (distillation method), and the waste or residue was weighed and its moisture content determined by drying at 105°C.

The amylase-activity test of mold in ragi: a 300 mg of ragi was inoculated into 50 ml of starch broth medium (soluble starch 0.5%, polypeptone 0.25%, malt extract 0.15%, yeast extract 0.15%, and pH adjusted to 5.5) in a 200 ml volume erlenmeyer flask. Then incubated at 30°C in a rotary shaker at the rate of 75 rpm. At interval of 2 days the enzymatic activities of the culture were estimated by the procedure described in Part II).

Results and discussion

The viabilities of the molds in the rice flour medium or ragi during storage at different temperatures are shown in Figure 35. The molds @w, AV2, and AU3 had the same mode of viability during 9 months in a rice medium as single culture. At room temperature all molds were greatly decreased in numbers, from 1.3×10^5,



The viabilities of molds (W2, AV2, AU3) in ragi and combined mold (AU3) and yeast (K3) in ragi during storage at different temperatures of Room ( ), Refrigerator ( ) and Freezer ( ).

$4.0x10^5$, and $1.5x10^5$ to $1.4x10^2$, $2.0x10^1$, and $5.0x10^2$ for W2, AV2 and AU3 respectively; whereas at refrigerator temperature all molds were most viable with viable counts of 4.6×10^4, $3.1x10^5$, and $4.8x10^4$ after 39 weeks. And, the freezer temperature was less suitable than the refrigerator temperature, with remaining mold counts of $1.2x10^4$, $5.6x10^4$, and $2.5x10^4$.

Figure 35 shows the viability of mold AU3 and yeast K3 when present as the mixed culture in the ragi. Here, the number of mold decreased more rapidly than in single culture, after 32 weeks. Interestingly, the yeast K3 in the ragi kept at room temperature decreased very rapidly and after 32 weeks K3 had died off. But, at refrigerator and freezer temperatures K3 remained in fairly viable state.

Table 15. The viability of mold and yeast in mixed and single cultured

	Initially	Room	Refrigerator	Freezer
		(after 32 weeks storage)		
Mixed				
AU3	$2.1x10^6$	$6.0x10^2$	$3.1x10^4$	$1.9x10^4$
K3	$4.1x10^7$	$0.0x10^0$	$5.5x10^7$	$2.4x10^7$
Single				
AU3	$1.5x10^5$	$3.0x10^3$	$6.0x10^4$	$4.8x10^4$

The aw-values of the ragi before and after the drying process are presented in Table 16. After drying, upon standing in the oven for 6 hours the ragi absorbed moisture from the suroundings during cooling time so that the a_w-value increased due to the high humidity in Bogor (85-95%). The a_w of ragi increased from 0.81 to 0.85-0.87.

After 32 weeks of storage the aw-values of ragi differed due to storage conditions. The ragi kept in freezer had the lowest available-water values, whereas the ragi stored in incubator at room temperature had the highest values. The observation of the humidity of the storage places indicated that the RH of room was ranging 75-95%, in refrigerator 55-60%, and in freezer was 100%. The available moisture may have affected the viability of the microorganisms, especially when combined with temperature. At high temperature and high available-moisture condition (the room), the viability of microorganisms was

lowest. Perhaps due to the high rate of metabolism of the microorganisms. However, the refrigerator-stored ragi was a little higher in viability even though it had a rather high available moisture content, compared to the freezer-stored ragi.

Table 16. The a_w-values of ragi before drying, after drying and cooling, and after 32 weeks of storage at different temperature

Before drying	0.92-0.95
After drying at 42-45°C for 2 days in oven	0.81-0.83
After cooling for 6 hours	0.84-0.86

The a_w-values of ragi after 32 weeks storage at different temperature conditions

	After 32 weeks storage at temperatures of:		
Mold/yeast	Room	Refrigerator	Freezer
W2	0.89	0.87	0.83
AV2	0.89	0.86	0.83
AU3	0.88	0.85	0.84
AU3 + K3	0.91	0.85	0.84
Range	0.88-0.91	0.85-0.87	0.83-0.84

These Amylomyces molds had a much shorter shelf-life of only 3-5 weeks when kept on potato dextrose agar slants. It seemed that there are protective agents present in rice, which made the molds to survive longer than in the synthetic medium.

According to Ponte and Teen (1978), the low viability of yeast cells in the dry state during storage remained a problem, and several chemical protective agents have been suggested.

The results of the fermentation activities of the stored ragi are given in Table 17 and Figure 36. All mold ragi when stored at a lower temperature (freezer and refrigerator) for 8 months produced larger amounts of reducing sugar than the ones stored at higher temperature. This means that the low

emperature had preserved the saccharification activity of the mold. It is shown that the number of molds in the ragi did not correlate with the amount of reducing sugar produced. Freezer stored ragi having lower mold count produced higher quantity of reducing sugar than the refrigerator stored ragi.

Further, the mold count also did not correlate with the quantity of liquor produced. Room temperature stored ragi having very low viable count of mold (AU3, W2) showed very high liquifaction activity and lower residual waste.

The residual waste may be an indication of the activity of the mold's enzyme that attack the rice substrate. The more active the enzyme the less residue is remained. The quantity of residue was reversely parallel with the yield of liquor produced. The more liquor the mold produced the less residual waste remained.

For AV2 the freezer storage was the best. At this temperature its production of reducing sugar and volume of liquor was highest and residue was lowest.

Whereas for W2, the lower the temperature the higher the production of reducing sugar, the lower the liquor volume and the higher the higher the residual waste.

But for AU3, the refrigerator temperature was the best, since its liquor yield was largest and leaving lowest residue.

The Brix values indicate the soluble solids concentration in the liquid, and, most of the soluble solids in the syrup is sugar. A sugar analysis of syrup liquor by the high performance liquid chromatography Hitachi model 365 (Saono 1981), showed that the sugar in the syrup obtained from the mold fermentation of black and white glutinous rice, bitter and sweet cassava was principally glucose, very low amounts of maltose, dextrin and arabinose (Figure 37).

The pH values of the liquor (Table 18) indicated that the K3 yeast reduced the pH of the liquor, but, the titratable acidity was not significantly affected. The brem produced by AU3-K3 was quite higher in pH and much lower in titratable acidity than the brem produced by the commercial ragi, the "B" ragi. The taste of the brem derived from this commercial ragi was destinctively sour. As already known from previous chapter (Table 5), this ragi contained considerable numbers of lactics. So, lactic acid fermentation may have taken place in this brem.

The results of the amylase-activity tests are shown in Figure 38. It shows that the room temperature stored ragi of W2 mold had the highest activities in both saccharifying and liquifying amylase. Therefore, among the three levels of storage temperature, the room temperature ragi was expected to produce the highest amount of reducing sugar and the largest volume of liquor. However, referring to the histogram of the reducing sugar produced by W2 ragi in Figure 36, the total reducting sugar produced in the fermented mass was unpredictably lowest with the room stored ragi. This controversial result was perhaps due to the difference in nature of the media used for the tests. For the amylase activity test the starch broth was used, whereas for the fermentation test the black glutinous rice. There may be another possibility, that the sugar produced by this ragi had been further degraded into other fermentation products due to the over-activity of the enzymes.

And, it may be concluded that the high liquifying-amylase activity of mold in stored ragi correspond with its capacity to produce the largest volume of liquor, lowest residual waste and highest moisture obtained from the rice grain (Table 17).

Table 27. The amount of sugar and liquor produced and residue remained of 6 days fermentation product of black glutinous rice* by 8.5 months old ragi stored at different temperatures.

Ragi condition	Mold-count/ g ragi	Reduging sugar/125g sustrate (g)	Liquor yield (ml)	Residue dry weight (g)	g Moisture** derived from mold attack on rice grain
W2:		%			
Room	1.4x10	22.80 (18.24)	82.25	11.41.	25.11
Refr.	4.6x10	24.53 (19.62)	79.65	12.30	21.98
Freez.	1.5x10	26.03 (20.82)	77.10	12.64	18.34
AV2:					
Room	2.0x10	20.20 (16.16)	68.20	15.39	19.99
Refr.	4.1x10	22.55 (18.04)	76.30	13.19	19.49
Freez.	5.6x10	27.63 (22.10)	77.06	13.09	20.02
AU3:					
Room	5.0x10	21.93 (17.54)	79.95	11.95	20.14
Refr.	4.8x10	23.18 (18.54)	80.50	11.33	20.33
Freez.	2.5x10	25.15 (20.12)	75.75	13.50	18.61
Control+	0	0.21 (0.17)	0.0	43.30	0.00

* 50 g black glutinous rice + 75 g water, autoclaved.

** Total liquid - liquid in control (calculated approximate value)

\+ Substrate* not inoculated by ragi (as control), autoclaved:

weight before autoclaving 125.0 g

weight after autoclaving 123.8 g

% moisture of control 65.03%

weight of moisture in control 80.51 g

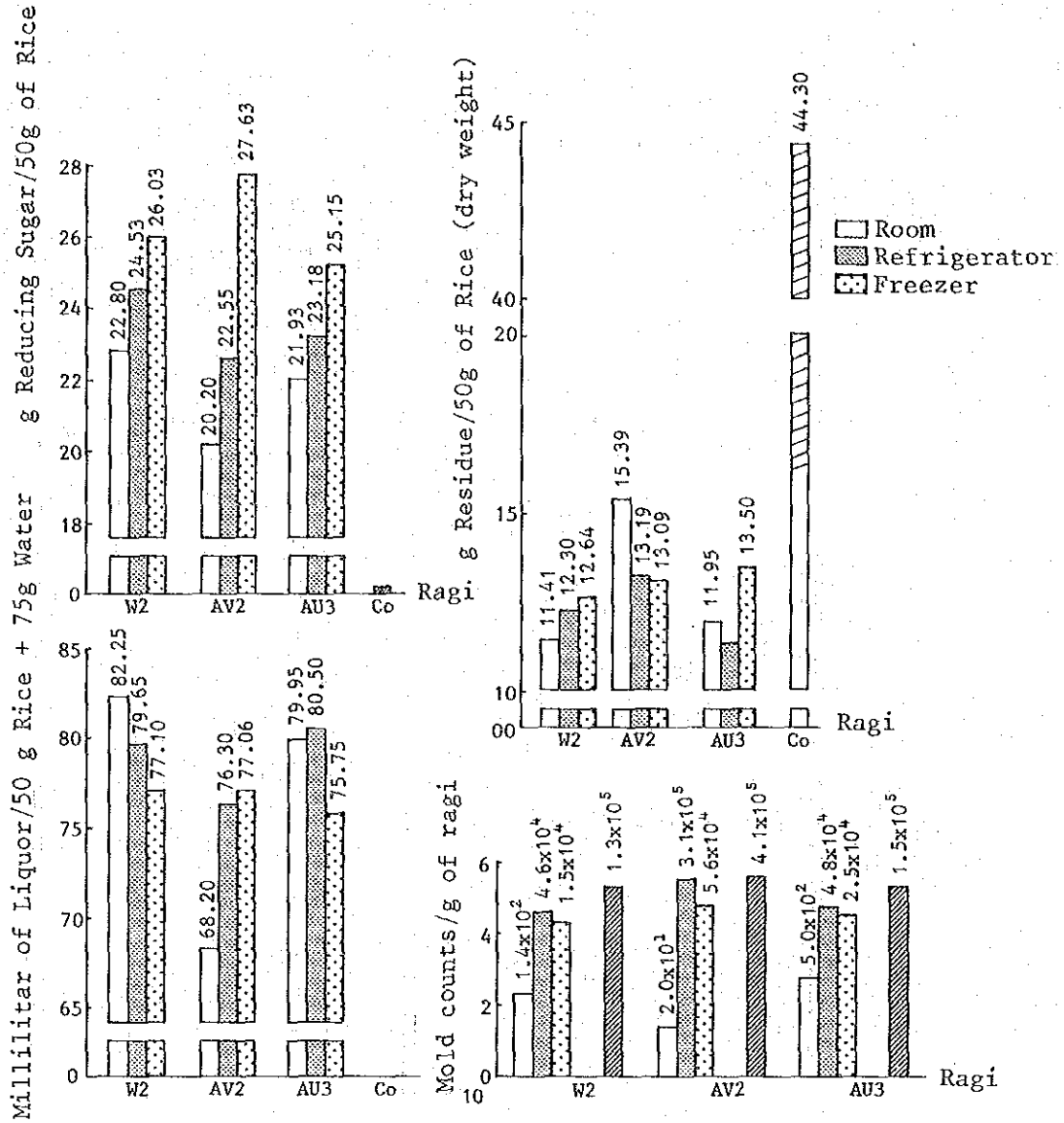


Figure 36. The total reducing-sugar and volume of liquor produced from black glutinous rice substrate, and residue remained unattacked by 8.5 months old ragi (W2, AV2, AU3) stored at Room ( ), Refrigerator ( ) and Freezer ( ) temperatures as compared to controle of no ragi ( ) , and the mold count of the ragis as compared to initial count ( ).

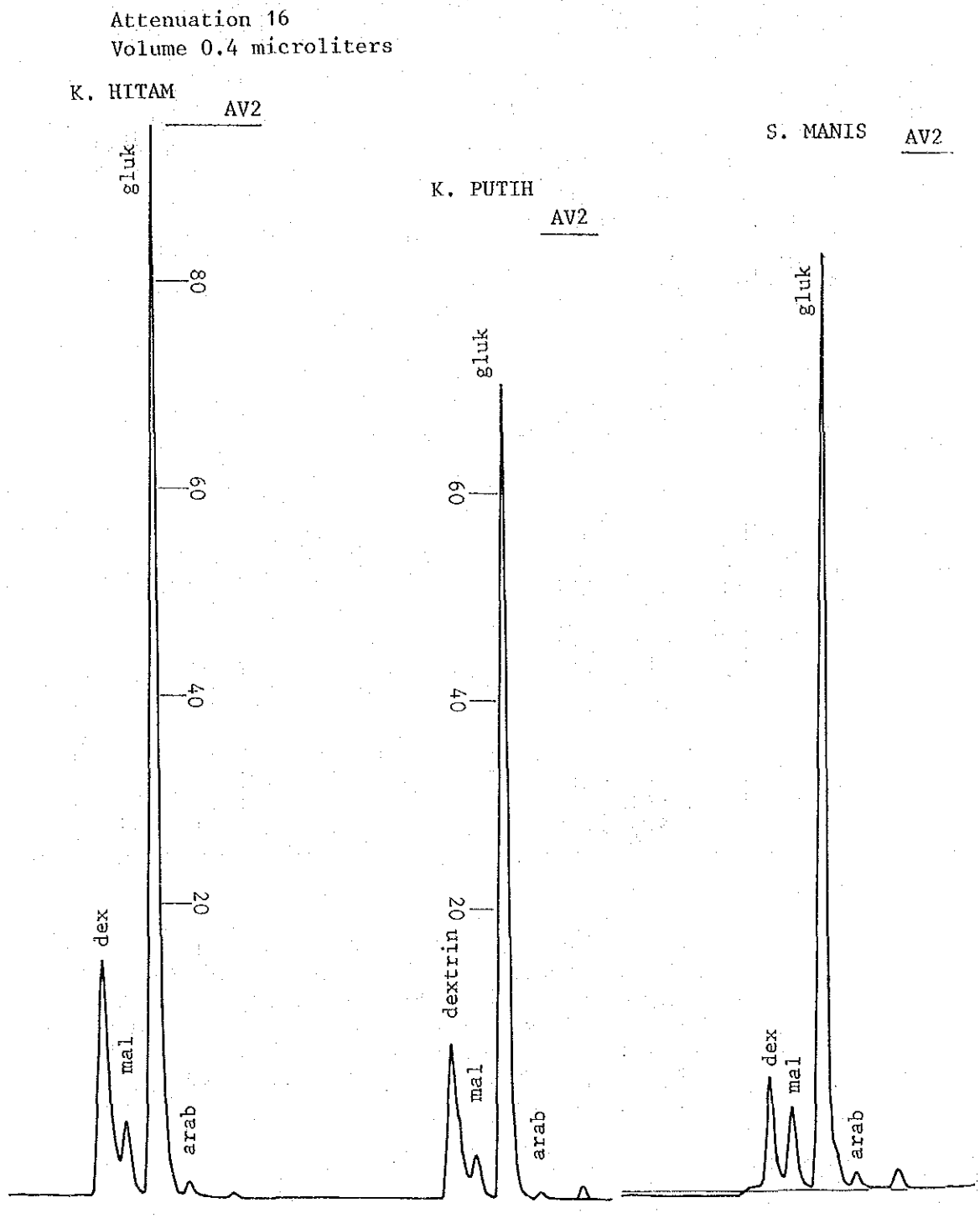


Figure 37. The HPLC-chromatogram of the analysis of sugars in syrup liquor produced by AV2 ragi on black glutinous rice (k. hitam), white glutinous rice (k. putih) and cassava (s. manis) substrates.

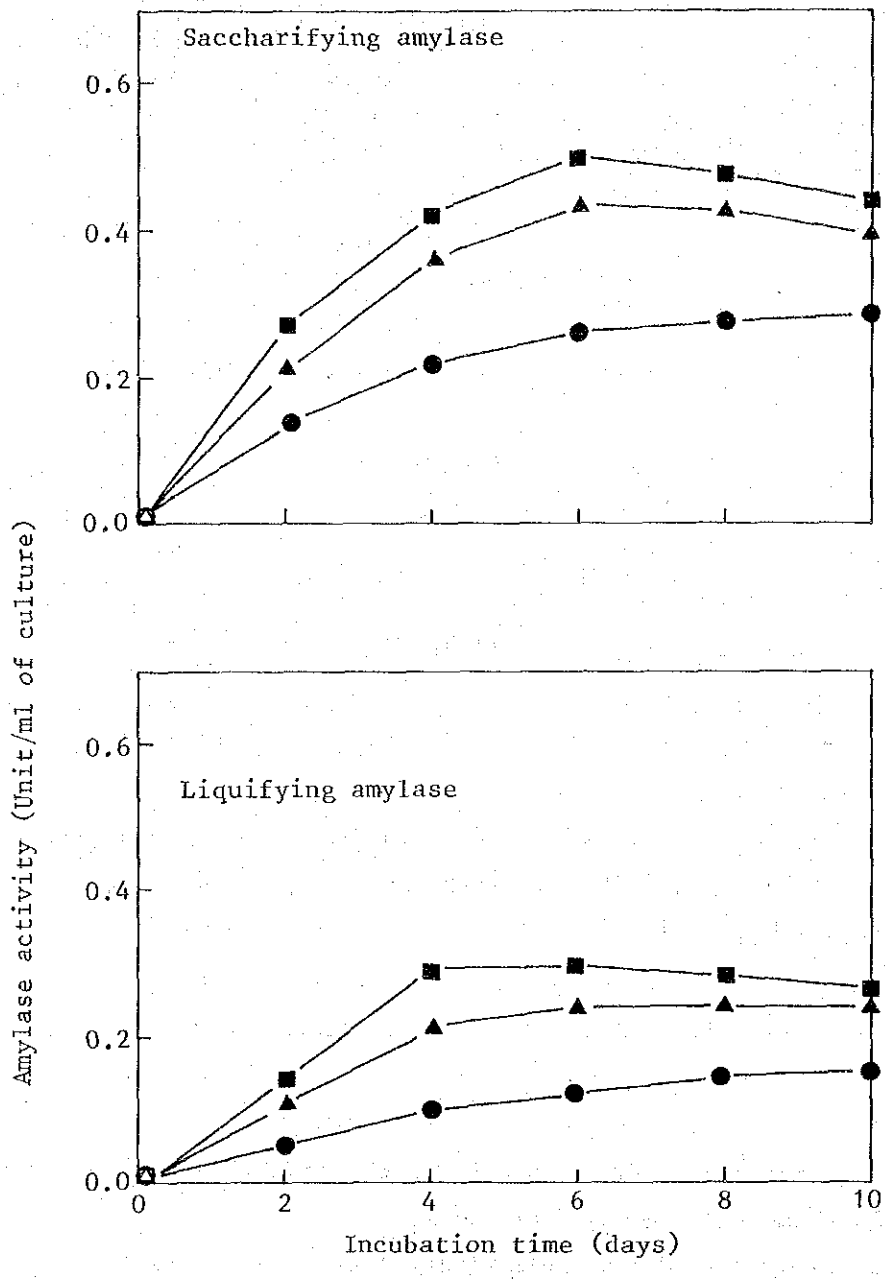


Figure 38. The amylase activities of 8.5 months old ragi stored at Room ( ), Refrigerator ( ), and Freezer ( ) temperatures, in Starch broth medium at 2, 4, 6, 8, and 6, 8, and 10 days incubation periods.

Table 18. The yield of liquor of 5 days fermentation of black glutinous rice* by 8 months old ragi stored at different temperatures, and the properties of the liquor.

Ragi condition	ml liquor yield/125 g	Reducing sugar(%)	Brix (%)	pH	ml 0.01N NaOH/ /10ml liquor (T.A.)	Taste
W2:						
Room	72.8	22.02	26.8	5.15	8.58	sweet
Refr.	70.5	27.24	29.0	5.50	9.59	sweet
Freez.	71.5	28.69	29.2	5.50	7.67	sweet
AV2:						
Room	63.5	14.73	28.2	4.15	10.10	sour, sweet
Refr.	68.5	24.29	29.4	5.30	7.57	v. sweet
Freez.	71.5	26.67	29.8	5.32	6.36	v. sweet
AU3:						
Room	68.5	24.06	28.3	5.31	8.08	sweet
Refr.	71.5	23.40	28.0	5.00	10.18	sweet
Freez.	69.0	21.95	28.0	5.18	11.31	sweet
AU3+K3:						
Room	69.5	21.49	28.4	4.89	10.30	sweet
Refr.	70.5	8.78	17.8	4.85	8.08	v.alcoholic
Freez.	73.0	10.81	18.2	4.85	8.58	alcoholic
Roda Mas R**	72.5	8.78	18.0	3.94	15.65	sour alcoholic

* 50 g black glutinous rice + 75g water, autoclaved. (125 g substrate of cooked rice)

** The commercial ragi used as control.

v = very

PART V - THE EFFECT OF TEMPERATURE AND MICROORGANISMS ON THE BREM WINE FERMENTATION

Temperature influences the rate of metabolism of the microorganism, and hence temperature may affect the activity of the microorganism in the fermentation. The temperature during the preparation of brem wine using traditional method is not controlled, which ranges from 25 to 32°C.

The microorganisms responsible for the development of the aroma components in most wines are the yeasts. But from the results in Part IV, the saccharified rice by mold alone showed specific aroma which may contribute to the aroma of the brem wine.

Materials and methods

A. The effect of temperature on brem wine fermentation

The effect of temperature on brem fermentation: 100 g of black sticky rice + 150 g of water in a container was autoclaved at 15 p.s.i. for 15 minutes, cooled, and inoculated with 250 mg ragi containing mold R. formosaensis, AV2 and CB3 separatedly. Incubation was conducted at both temperatures of 25°C and 30°C for each mold fermentaion. The incubation period was 6 days. The liquid syrup produced was squeezed out and weight. Further, 75 ml of syrup was inoculated by 1 ml yeast suspension of S. cerevisiae Steinberg at a density of 8.6×10^9 cell/ml and 1 ml RM yeast at 1.5×10^{10} cells/ml, seperatedly. This is a two-steps process. The alcohol yeast is added after the saccharification process is completed, incubated also at 2 levels of temperatures, 25 and 30°C for 7 days and the cells was let to settle down for another 7 days.

Determination of reducing sugar % was done by the Somogy method and the Brix % values was obtained by the Atago hand-refractometer.

Formol-nitrogen analysis: in erlenmeyer flask 10 ml sample was diluted with 90 ml H_2O, added with 1 ml phenophthalein indicator, and titrated by 0.1N NaOH until pink color re-developed. Then, after the addition of 5 ml of formaline where-upon the pink color disappeared, then it was further

titrated by 0.1N NaOH until it turned pink (a ml). At the same time blank was run (b ml). Calculation: amino type nitrogen = (a-b) x 1.4 mg/10 ml sample, and therefore % Formol N = (a-b) x 0.014g/100 ml.

Titratable acidity (ml of 0.1N NaOH/10 ml sample) was determined as follows: in an erlenmeyer flask 10 ml sample was diluted with 90 ml distilled water, 1 ml phenolphthalein was added and titrated with 0.1N NaOH until the pink color re-developed. A blank of 100 ml distilled water was run at the same time. The pH values were measured by Horiba pH-meter M7.

B. The contribution of the mold and yeast to the aroma

The fermentation was carried out as described in Part A of this chapter, and the aroma components analyzed by the gas chromatography described below.

The aroma components was determined by standard extraction method: 30 ml of sample was extracted by ether-pentane (2:1), where it was concentrated on Widmer columm at 33°C and so gas chromatography sample was obtained. Gas chromatography operating conditions of ethyl ester: instrument Hitachi G.C. -663, column PEG-20 M (10%) 2 mm diameter x 4 m Glass column, temperature of columm 80-180°C, Injection 230°C F/D 250°C and sample volume 1 microliter. And, Gas chromatography operating conditions of alcohol: instrument Hitachi G.C. -663, column PEG 1500 (10%) 3mm diameter x 2 ml glass column, temperature of column 90°C, Injection 180°C F/D 180°C and sample volume 1 microliter.

Results and discussion

A. Effect of temperature on brem fermentation

The results in Table 19 shows that at lower temperature of 25°C the yield of liquor was better for ragi molds CB3 and AV2, however, the R. Formosaensis at 30°C gave a higher yield. Therefore, R. formosaensis could be recommended for the production of brem wine in the tropics.

The reducing sugar concentration in the saccharified liquor produced by R. formosaensis was much higher than those by CB3 and AV2 molds. But, the ethanol analysis indicated that quite high amount of ethanol had been formed by those ragi molds. CB3 and AV2 molds seemed to have the alcohol fermenting ability. Cronk (1975) observed that Clamydomucor oryzae produced a maximum of 5.6% of ethanol at 4 days fermentation of tape taken.

As expected, the yeast fermentation increased the ethanol content, decreased the sugar content, decreased the pH and increased the ml total acidity, but the formol nitrogen content was decreased in R. formosaensis liquor and increased in ragi molds liquors.

Higher fermentation temperature of 30°C, compared to lower of 25°C, had the tendency to result in higher formol nitrogen, higher ethanol, and higher remaining sugar concentrations in the brems.

B. Contribution of myoflora to the aroma components

The results in Table 20 shows that the mold R. formosaensis alone could produce considerable amount of ethyl acetate, and the yeast activities then decreased this component.

Iso-butyl alcohol, pellargonic acid, caprillic acid and an unknown compound having a retention time close to ethyl laurate were highly produced by the ragi molds CB3 and AV2, and in lesser quanity by the R. formosaensis. The yeast fermentation had a tendency to increase these compounds to several folds.

It can be concluded that both the molds and the yeasts contributed to the aroma components formation in brem wine.

Table 19. Characteristics and yield syrup and brem wine produced from different ragi molds and yeats, compared to the commercial ragi, at 25 and 30°C.

Ragi mold-yeast	Temp. (°C)	g Yield*	Ethanol (%)	Reducing sugar %	% Brix	TA ml	pH	Formol N %
R. for**	25	154.84	1.14	27.97	30.4	3.96	5.04	0.0105
	30	159.21		27.74	30.4	3.96	5.23	0.0161
CB3	25	147.05	5.78	16.00	22.3	5.66	4.80	0.0035
	30	145.10		17.97	20.1	6.22	4.80	0.0077
AV2	25	150.52	5.52	18.20	23.8	5.61	4.90	0.0084
	30	144.02		18.13	21.0	6.77	4.80	0.0084
R.for-St***	25		8.62	5.32	14.5	7.62	4.45	0.0063
	30		9.62	7.93	15.8	7.12	4.55	0.0084
R.for-RM1	25		7.87	8.80	16.4	7.32	4.49	0.0070
	30		7.96	16.36	19.0	7.32	4.50	0.0126
CB3-St	25		6.44	8.76	16.8	7.07	4.60	0.0105
	30		9.17	7.84	16.1	6.47	4.78	0.0147
CB3-RM1	25		7.53	4.42	13.8	6.97	4.72	0.0161
	30		8.99	9.68	17.2	6.92	4.68	0.0196
AV2-St	25		10.62	7.76	16.2	6.97	4.80	0.0119
	30		8.56	10.19	18.0	6.92	4.78	0.0112
AV2-RM1	25		6.33	7.84	16.0	6.07	4.68	0.0084
	30		7.02	10.39	18.1	7.22	4.62	0.0119
Ragi B	25	110.04	6.98	13.16	15.4	10.43	4.23	0.0140
	30	125.04	5.81	21.44	19.2	12.34	4.23	0.0126

* R. for: Rhizopus formosaensis

** St: Saccharomyces cerevisiae

*** per 100 g rice and 150 g water of substrate

Table 20. The concentration of aroma components (ppm) in black rice by mold saccharified liquor with and without yeast fermentation.

Aroma components	R. foromosaensis			CB3			AV2		
	-	St	RM1	-	St	RM1	-	St	RM1
Unknown	-	0.85	-	1.81	-	0.52	0.91	-	-
Unknown	1.58	-	-	-	-	-	5.22	-	-
Acetaldehyde	11.49	93.22	19.11	2.31	23.03	27.14	52.13	45.47	16.62
Acetone	10.71	28.54	16.76	+	11.28	20.97	0.95	36.54	14.21
Ethyl-acetate	65.28	16.37	11.34	4.29	7.52	7.57	15.43	11.54	6.92
iso-Butyl alcohol	16.76	694	828	125.37	304	120.86	271	353	63.95
Unknown	340	190	150	64.46	87	157.51	96.31	201	62.48
iso-Amyl alcohol	19.42	200	140	157.87	145	269	11.33	457	124.69
Ethyl-caproate	+	+	1.63	+	+	+	+	+	+
Unknown	36.15	69.70	44.07	88.65	36.23	110.58	54.71	96.45	81.27
Unknown	0.78	3.98	0.42	3.54	1.36	3.97	2.15	2.05	-
iso-Butyl-caproate	4.47	17.86	92.81	22.01	4.38	9.57	2.06	2.81	3.87
n-Caprilic acid	387.48	1112.56	1145.56	1318.24	295.70	513.83	421.51	732.62	680
Unknown	10.45	8.07	-	-	-	-	22.54	30.28	73.26
Pellargonic acid	232.76	340.69	307.06	441.07	167.82	320.83	405.31	682.39	650
Unknown	-	-	-	2.49	-	2.29	-	-	-
Ethyl-caprate	1.30	8.18	90.23	9.55	-	+	1.65	2.99	2.83
Unknown	+	1.34	-	0.34	2.38	+	+	-	+
Unknown	28.56	0.07	27.59	26.47	-	+	22.11	35.28	67.93
Unknown	+	35.70	44.85	+	-	5.34	37.44	46.36	123.17
Unknown	+	-	-	+	-	+	+	-	+
Unknown	34.47	102.66	76.04	120.08	28.96	88.40	60.12	88.52	260.62
Ethyl-laurate	0.67	3.50	6.74	0.33	+	0.82	11.97	18.93	+
Unknown	87.66	37.38	+	461.06	53.82	520.47	229.65	409.58	401
Unknown	1.62	373.82	+	1.64	13.53	49.66	34.99	0.49	118.39

PART VI - IMPROVED METHOD IN THE PREPARATION OF BREM RAGI

Brem wine fermentation is principally carried out by certain ragi, that is the "B" ragi. This brand of ragi is the best here fore brem making, since it contains the alcohol-aroma microorganisms which are not available in most other commercial ragi. However, from time to time this ragi would make poor product of brem wine due to its variations in microbial population.

The survey on the methods of ragi preparation and the brem wine production, indicates that there is a need to develop a new improved method of ragi preparation, so that a more consistant and better quality of brem wine can be obtained.

Earlier experiments proved that it seemed possible and even desirable to abstain from using spices in ragi making as to eliminate undesirable microorganisms present in the spices. Furthermore, to incorporate selected productive strains of mold and yeast in ragi may improve brem wine fermentation process.

Materials and method

Ragi making by improved method: Firstly, the seed for ragi was made by heating of 20 g rice flour in a petri-dish in oven at 105°C for 18 hours, and after cooling, it was inoculated with one slant of one week mold culture on potato dextrose agar scraped by spatula in 9 ml of sterile distilled water, and 3 loupe of 3 day old yeast culture on yeast extract malt extract agar suspended into another 9 ml of sterile water containing no or 0.2 g saccharose. Then, they were mixed thoroughly with a spoon and incubated at 30°C for R. formosaensis and 25-28°C for the ragi molds AU3 and CB3, for a period of 2-3 days: with 1 day in closed condition and 2 days in slightly open condition. The mold covered rice dough was dried in drying oven at 42-44°C for 2 days. This process was done aseptically. This seed ragi was used to inoculate a larger batch of 500 g of rice flour at the rate of 5 g of seed ragi and 450 g of water with 5 grams of cane sugar that has been cooked. These ingredients were mixed and spread on an aluminium foil into thin layer of 0.5-1.0 cm thick, covered with an aluminium foil and kept at room temperature for 3 days with 1 day covered and 2 days slightly open. Prior to drying, the molded dough was cut into squares of 3 cm x 3 cm to facilitate the drying process.

The mold and yeast counts were done by plating method on yeast extract malt extract agar medium (this medium was better for counting ragi molds than potato dextrose agar by giving higher counts and restricted growth of colonies and not too fast spreading of mycelial growth) after quantitative dilution of the finely ground ragi, aseptically. Incubation was done at 27°C for 1 day for mold and 2 days for yeast.

Brem making from improved ragi: 100 g of black glutinous rice and 150 g of water in a container was autoclaved at 15 p.s.i. for 15 minutes, cooled, and inoculated by 250 mg of ragi containing mold and yeast. Incubated at 30°C (for ragi containing R. formosaensis and "B" ragi) and 25-27°C (for ragi containing AU3 and CB3 molds) for 7 days. Then the saccharified rice was pressed to squeez out the liquor. The liquor was transfered into a screw-capped jar for further alcoholic fermentation at 25°C for 14 days. The brem wine was aged for 2 months at refrigerator temperature (5-8°C). Analyses were done for ethanol % (Gas chromatography after extraction method, as in the analysis of aroma components in Part V - B), reducing sugar % (Somogy method), formol nitrogen, ml of total acidity, and pH as in Part V - A.

Results and discussion

Ragi by the improved method

During the preparation of ragi, it was observed that R. formosaensis and S. cerevisiae strains grew well at 30°C and the aeration during incubation needed to be controlled by closing the petri-dish for 1 day and then opening it slightly for 2 days. However, the ragi molds AU3 and CB3 needed a lower temperature of 25-27°C for good growth. It was also noticed that the addition of sugar was harmful to the CB3-RM1 ragi; it produced a watery ragi after 3 days of incubation period. But, the presence of sugar did not cause any harm to the ragi containing R. formosaensis and the wine yeasts.

Level of heat treatment givento the rice flour had an effect on the sporulation of R. formosaensis. Flour that had been heated at 105°C for 6 hours did not cause the growth mold to sporulate, while the heat treatment at 120°C for 4 hours which caused browning of the flour resulted in sporulation of this mold after 3 days of incubation at 30°C. And plating of both flour on glucose yeast extract malt extract agar showed no bacterial growth nor noticeable odor. Therefore, for ragi making the extend of heating at 105°C for 6 hours would be

adequate.

The microbial counts of ragi are presented in Table 21. It is shown here that the number of fermentative yeasts were quite in improved ragi that in the commercial ragi, but the mold counts were lower. However, in Part IV it was proved that even at a low counts (10^2) W2 mold had a higher saccharifying and liquifying activities than at higher counts (10^4). Because, in a suitable medium or substrate a Mucoraceour mold can spreads rapidly.

In the commercial ragi, a great number of filamentous yeast were found besides lactic-acid bacteria. The presence of these microorganisms may be undesirable since they both could lower the pH of the brem wine too much.

The numbers of fermentative ragi yeasts K3 and RM1 in the improved ragi were much higher than the wine yeasts. It seemed that the development in rice flour of ragi yeasts was better than the wine yeasts.

Table 21. Microbial counts of improved ragi and the B ragi, per gram of ragi.

Ragi	Mold	Yeast		Lactic acid bacteria
		true	filamentous	
R.for-S.sake	$2x10^4$	$6.4x10^5$	0	0
R.for-S.steinberg	$2x10^4$	$1.1x10^5$	0	0
R.for-S.taiken	$3x10^3$	$2.1x10^7$	0	0
CB3-RM1	$2x10^4$	$1.9x10^7$	0	0
AU3-K3	$8x10^4$	$1.7x10^8$	0	0
B ragi	$5x10^5$	$1.0x10^5$	$2.2x10^8$	$4x10^4$

Table 22 shows that the brem produced by ragi CB3-RM1 and AU3-K3 had better alcohol content than the brem produced by ragi R. formosaensis - S.cerevisiae steinberg, sake or taiken, and also "B" ragi. The degree of alcohol production may be due to the combined action of the yeast and the mold.

The % Brix values were quite high; this % Brix indicates all the soluble solids present in the brem, including the undermented sugars.

Table 22. Characteristics of the brem wine made from improved ragi, compared to the B ragi, bubod and sake.

Ragi	Ethanol %	Reducing sugar %	% Brix	Formol N %	TA ml	pH
R.fos-S.sake	7.91	9.85	17.7	0.0154	6.82	4.81
R.for-S.steinb	7.28	4.92	14.0	0.0154	7.87	4.81
R.fos-S.taiken	9.82	10.44	19.0	0.0211	5.82	5.05
CB3-RM1	10.83	7.43	15.8	0.0112	6.82	4.84
AU3-K3	10.68	5.17	14.2	0.0105	6.22	4.97
B ragi	8.70	8.02	17.5	0.0070	10.03	4.19
Philipine bubod*	12.5- 19.1	0 - 5.44	8-9	0.0045- 0.0257	6.55- 22.49	3.3- 4.9
Japanese sake**	15.0	4.20		0.0288	1.52	

* Tanimura et al. 1968, Del Rosario 1980

** Hayashida et al. 1968

The acidity of brem could be seen from the ml NaOH titration value and pH value. The ml T.A. of brem from improved ragis were much lower and the pH quite higher than the brem produced by "B" ragi. Perhaps this higher acidity by "B" ragi was due to the lactic-acid bacteria and fillamentous yeasts present in this ragi as shown earlier in Table 5.

The formol nitrogen in brem produced by the improved ragis were quite higher than in brem by "B" ragi, and quite lower than in Japanese sake. Formol nitrogen indicates the amino type nitrogen or amino acids present in the brem. The amino acid are formed during fermentation from the protein in the rice substrate by the attack of protease from mycoflora, and, amino acids on the other hand are used up by the yeast for its growth and in the reactions to form higher-alcohols and esters.

The results of the aroma components analysis given in Table 23 shows that the total analysable aroma components was highest in CB3-RM1 brem (1952.32 ppm) and lowest in "B" ragi (1089.75 ppm). Ethyl acetate was quite high in "B" ragi and CB3-RM1 brems, iso-butyl alcohol was distinctively high in CB3-RM1 and Rfor-sake brems, and an unknown compound*, iso-amyl alcohol and ethyl caproate were very high in CB3-RM1 and AU3-K3 brems. Propyl alcohol was not detected in the brems, whereas methyl alcohol was present only in Rfor-steinberg and Rfor-sake only.

Cronk et al (1979) studied the higher alcohols produced by ragi micro-organisms in tape ketan fermentation; the largest amount of fusel oil produced were iso-amyl alcohol and iso-butanol, but no n-propanol was detected. Amylomyces rouxii produced 275 ppm, A. rouxii Endomycopsis fibulgera 558 ppm, A. rouxii- Candida sp. 618 ppm, and A. rouxii-Hansenula sp. 248 ppm of ethyl acetate after 8 days.

The famous Batavia arak, a distilled rice wine from fermentation by ragi, was highly demanded in Europe (Raffles 1830; Prinsen Geerligs 1905). This arak contained high amount of volatile acids and esters, aldehyde, furfural, and higher-alcohols as analysed by Prinsen Geerligs (Table 23).

* that has a retention time very close to iso-amyl alcohol

And, De Kruyff (1909) suggested the yeasts responsible for the good aroma of Batavia arak to be the now called Saccharomyces pombe and Willia indica, whereas in the lower quality Cheribon arak were S. cerevisiae and Hansenula anomala.

Table 23. The composition of Batavia and Cheribon arak (in ppm).

	Batavia	Cheribon
free acids	1500	1290
Esters	2464	1936
aldehyde	594	ND
furfural	50	100
higher-alcohols	250	300
alcohol, % volume	60.7%	59.1%
free acids of formic	90	20
acetic	1250	610
butyric	110	20
capric	70	50
ethyl ester of formic	50	20
acetic	1480	670
butyric	50	20
capric	90	60

Brem, similar to Cronk's tape ketan, has a much higher concentration of higher alcohoos than sake, this may distinguish the brem wine from sake. Brem is made of completely different substrate and fermented by different microorganisms, also the method of preparation differed from that of sake. The higher-alcohols or fusel-oil in brem wine may have been derived from the proteins in the unpolished rice, through the action of the proteases of the microorganisms. Yoshizawa (1980) described the mechanism of the formation of higher-alcohols from glucose, amino acids and acetic acids.

Rice for sake preparation is polished 25-30%, but for the highly refined sake it is polished up to 50% (Kodama 1970). This polishing is to remove a great part of the protein, since high concentrations of protein in the substrate cause off-flavor development in the sake (Kodama and Yoshizawa 1977). Higher-alcohols in sake are considered unpleasant. Also in grape wines higher-

alcohols give an unpleasant flavor, but they are usually present at such low concentration so that they are not necessarily unfavorable, and may even contribute to the quality of the wine (Kunkee and Amerine 1970).

The principal higher-alcohols in sake are n-propanol 120 ppm, isobutanol 64 ppm, iso-amyl alcohol and active amyl alcohol 170 ppm and phetanol 75 ppm. The esters in sake are ethyl acetate 20-120 ppm, iso-butyl acetate 0.2-1.5 ppm, ethyl butyrate 0.5-5.0 ppm, iso-amyl acetate 2-10 ppm, ethyl caproate 2-10 ppm, ethyl caprilate 5-10 ppm, ethyl caprate 10 ppm, ethyl pelargonate 3-5 ppm, ethyl laurate 2-11 ppm, ethyl lactate 2-5 ppm and phenethyl acetate 5-8 ppm (Kodama and Yoshizawa 1977). According to these two authors, the formation of aroma components depends on the concentration of amino acids in the fermentation mash, temperature of fermentation and the yeast strain. And, that aeration may induce di-acetyl and higher-alcohols formation (Yoshizawa 1980).

The brem wine produced from CD3-RM1 ragi had a nice grape juice aroma and made it desirable. However, the brem wine from AU3-K3 was less acceptable due to its strong aroma. And, the flavor of the brem wine made from R. formosaensis and the wine yeasts S. cerevisiae steinberg, taiken and sake were all prefered than the commercial ragi's.

"B" ragi wine had the highest ethyl acetate content, which might be attributed to the H. anomala present in that ragi. Cronk et al's (1979) experiment showed that Hansenula sp. produced very high concentration of ethyl acetate, 354-369 ppm, in tape ketan fermentation. According to Kunkee and Amerine (1970), Saccharomyces sp., Torulaspora sp. and Torulopsis sp. generally produced ethyl acetate at low rate, whereas Kloeckera apiculate, S. ludwigii, and especially Pichia sp. and Hansenula sp. produced ethyl acetate at high rate. However, too high ethyl acetate content in wine, over 225 ppm, was considered to give an aroma of spoiled fruit (Kramer and Twigg 1966).

Table 23. The concentration of aroma components (ppm) in brem-wine produced by improved ragi, compared to Roda Mas R ragi.

Aroma components	Roda Mas R	Pfor-stein	Pfor-Sake	Rfor-taiken	AU3-K3	CB3-RM1
Acetaldehyde	18.33	17.98	27.71	46.13	35.35	86.83
Acetone	6.45	16.84	30.21	34.82	19.37	54.44
Ethyl-acetate	92.58	15.54	20.70	21.89	19.29	60.83
Methyl-alcohol	-	21.76	36.83	-	-	+
Propyl-alcohol	-	-	-	-	-	-
iso-Butyl alcohol	397.58	272.95	650.38	299.01	344.41	737.61
Unknown	166.58	184.91	206.84	187.75	252.54	301.30
n-Butyl alcohol	-	-	-	-	-	-
iso-amyl alcohol	157.97	151.91	150.33	171.16	430.82	234.69
Unknown	0.70	1.33	1.36	0.94	1.06	0.91
Ethyl-caproate	114.60	241.14	178.68	229.76	429.88	317.69
Unknown	2.37	2.51	1.17	4.19	1.15	0.21
iso-Butyl caproate	4.77	4.57	0.09	0.07	-	+
Unknown	1.37	1.79	1.10	0.11	+	1.82
Unknown	0.33	0.28	-	-	-	-
n-Caprilic acid	60.59	126.75	38.89	68.12	18.65	35.51
Unknown	-	1.07	0.14	-	-	-
Pelargonic acid	0.40	0.63	4.62	4.97	+	-
Unknown	+	9.36	-	8.65	-	-
Unknown	0.10	6.68	11.82	-	1.47	4.02
Unknown	2.46	0.45	-	-	-	-
Unknown	2.35	-	-	-	0.58	0.41
Ethyl-caprate	0.30	0.90	0.58	0.90	0.05	+
Unknown	1.73	0.29	+	0.21	-	-
Unknown	2.35	0.68	0.33	-	0.44	3.45
Unknown	0.09	0.87	0.49	2.28	3.98	3.13
Unknown	5.34	5.14	1.67	0.96	1.77	5.04
Unknown	4.97	3/46	7.27	0.50	1.20	-
Ethyl-phenyl acetate	13.67	19.40	24.22	10.56	60.75	18.90
Ethyl-laurate	0.83	0.28	3.06	-	2.24	0.91
Unknown	30.88	57.38	77.57	101.55	126.04	84.61
Unknown	0.06	-	-	-	-	-
Total	1089.75	1166.85	1476.06	1194.31	1751.04	1952.32

SUMMARY

Ragi is principally used as a starter for the fermentation of tape, brem wine, brem dry cake, badek and arak in Indonesia. Ragi originated from China (Vorderman 1893) was brought into Indonesia by Chinese settlers arriving in the country since the 9th century. However, studies on ragi were started in 1894, Went and Geerligs 1894, Went and Geerligs 1895) due to their interest in the famous Batavia arak which was made by ragi fermentation.

Ragi contains several important microorganisms. Amylomyces, Mucor, and Rhizopus molds are necessary in the saccharification and liquifaction of starch. Saccharomyces cerevisiae and Hansenula anomala yeasts are responsible for the alcohol and good aroma production. The filamentous yeasts. Saccharomycopsis and Candida, may affect the saccharification, odor and sourness of the product. The lactics produce acidity in the product.

There are variations in the quality of ragi from batch to batch, even by the same maker, since the microbial population coming from the ingredients are uncontrollable. This causes the inconsistency in the quality of the fermentation product by such ragi.

The improvement of ragi especially for brem wine fermentation is specially considered here. Because, brem wine is used on many occasions: as a welcome drink for state guests by the president of the Indonesian government, a welcome drink for tourists in Bali and in offerings to the Hindu Gods. Further, it may in the future become an important export commodity like Batavia arak in the 19th century.

The quality of brem wine is inconsistent, and the analyses of brem wine available on the market shows that the alcohol content ranges from 2.8 to 10.0%, acidity from 0.067 to 0.218% as acetic acid, reducing sugar from 17.3 to 26.3% and the residual extract from 24.67 to 39.42%.

The shelf-life of ragi at room temperature is around 2-3 months, after which period no good fermentation product could be expected. Perhaps, non-viability of important microorganism or imbalance of the microbial population occurs in old ragi.

I. Survey of ragi and brem wine preparation

Ragi and brem wine are prepared by traditional methods. Ragi is made from rice flour and finely ground spices, such as garlic, laos rhizomes, red chillies, white pepper, cinnamon, black pepper and adas seeds molded into flat or round balls, inoculated by powdered ragi, incubated on rice straw layer and dried in the sun. Brem wine is made by ragi fermentation of steamed glutionous rice, the liquor derived is further fermented for alcohol development. Aging process is not always done.

The recipes for making ragi in the villages in West and Central Java are summarized below,

The percentage of ingredients in 24 ragi samples

Ingredients	No. of ragi	% (Compared to rice)
Rice Oryza sativa Linn	24	100.0
Garlic Allium sativum Linn	23	0.5 - 18.75
Laos tuber Alpinia galanga Sw	20	2.5 - 50.0
White pepper Piper nigrum Linn	20	0.05 - 6.25
Red Chillies Capsicum frustescens Linn	11	0.25 - 6.25
Cinnamon Cinnamomum burmani BL	5	0.05 - 3.5
Black pepper Piper retrofractum Vahl	3	0.3 - 2.5
Adas seed Foeniculum vulgare Mill	2	2.5 - 3.0
Sugar cane Saccharum Officinarum Linn	2	1.0 - 12.5
Lemon juice Citrus auranticum auranti-folia var fusca Linn	1	2.5
Coconut juice Cocos nucifera Linn	1	50.0

It is interesting to note that all brem wine makers use the ragi of certain brand made in Solo; this brand of ragi is considered to be the best for brem wine making. However, its inconsistency in the success of fermentation is known.

II. Isolation, selection and identification of amylolytic molds and fermentative yeasts from ragi.

Of the 52 ragi samples collected mainly in West and Central Java and some

in East Java, Madura, Bali, Sulawesi and Sumatera, only 13 samples were able to make very good fermentation product with very sweet taste and very soft texture. From these ragi samples, 90 molds were isolated and only 4 of these isolates gave excellent fermentation product with a high quality in both sweetness and softness: W2, AV2, AU3 and CB3. These molds were all identified as Amylomyces rouxii.

During the growth of the molds in starch broth medium, AU3 and CB3 showed higher saccharification and liquifaction activities. The pH of the broth dropped from 5.5 to 3.3-3.5 after 6-10 days from the begining of incuba tion, the cell mass increased rapidly after 4 days with AV2 being the highest in cell mass, and the optimum pH for amylase activities was between 4.5 to 5.0.

Of these 52 ragi, only 7 had very strong fermentative ability and 16 were isolated and only 13 of these isolates were strong fermentatives. Four strains having good aroma and alcohol productivity were slected: K3, N3, Y5 and RM1. These yeasts were all identified as Saccharomyces cerevisiae.

III. *Effect of spices on ragi microorganisms*

Red chillies at a concentration of 6.25% in ragi stimulated the growth of all ragi microorganisms tested: Saccharomyces cerevisiae Y5, Endomycopsis fibuligera H13 and Amylomyces rouxii W2. Garlic at 9.0% inhibited the yeasts S. cerevisiae and E. fibuligera but not the mold A. rouxii.

After 10 weeks storage at room temperature, the ragi containing red chillies retained high number of microorganisms, whereas the ones containing the garlic and laos decreased these microbial counts.

IV. Effect of storage temperature onthe viability and activity of mold and yeast in ragi

The viability of all the molds W2, AV2 and AU3 after 8.5 months storage room temperature decreased greatly, but they remained at the highest level in refrigerator storage. Among them, AU3 was the most resistant during

storage and AV2 the least resistant. With the presence of K3 yeast, lower surviving cells of AU3 were observed compared to those of AU3 kept without the presence of K3.

The saccharification activity of mold on glutinous rice substrate remained highest when stored at freezer temperatures (-14 to -11°C), however, their liquifying activities were not affected by the degree of temperature. The remaining residue or the substrate not attacked by the mold was in paralel with the mold's liquifying activity.

V. Improved method of ragi preparation

The ragi made by the improved method, using pure cultures of the selected molds and yeasts, CB3-RM1 and AU3-K3, had a much higher counts of fermentative yeast (10^7 and 10^8) but lower counts of mold (10^4 both) than the commercial ragi from Solo. This commercial ragi had a true-yeast counts of 10^5 and amylolytic mold counts of 10^5. No filamentous yeast and no lactic-acid bacteria were present in both of the new ragi, whereas in the commercial ragi these microorganisms were present in high numbers, 10^8 of filamentous yeast and 10^4 of lactics.

Brem wine made from CB3-RM1 and AU3-K3 ragi had a quite higher ethanol % (10.83 and 10.68%), lower reducing sugar % (7.43 and 5.17%), lower ml of titratable acidity (6.82 and 6.22 ml), higher pH values (4.84 and 4.97) and higher formol nitrogen % (0.-112 and 0.0105%) as compared to the commercial-ragi's wine. The wine made from the commercial ragi had an ethanol of 8.70%, reducing sugar of 8.02%, ml. T.A. of 10.03, pH of 4.19 and formol nitrogen of 0.0070%.

Brem wine produced from CB3-RM1 ragi had a grape aroma and more desirable than the commercial one. However, brem wine from AU3-K3 was not prefered due to its strong fusel-oil aroma.

CONCLUSION

The traditional method of ragi preparation could not control the micro-organisms coming from the ragi ingredients, hence, the quality of the fermentation product would vary greatly.

Storage of ragi at freezer temperatures and refrigerator temperatures for 8.5 months kept the yeast S. cerevisiae K3 and the mold A. rouxii AU3 highly viable and active in the saccharification of glutinous rice.

Improved method of ragi preparation for brem wine using pure cultures of productive mold and yeast isolated from ragi, A. rouxii CB3 and S. cerevisiae RM1, made it possible to eliminate the undesirable microflora from the ragi, and hence, produced a better quality of brem wine.

LITERATURE CITED

Akiyama, H. K. Yoshizawa and K. Ouchi. 1978. Sake flavor and its improvement using metabolic mutants of yeast. Pages 229-248 in G. Charalambous (ed.) Analysis of floods and beverages. Headspace techniques. Academic Press.

Asharie. 1896. Inlansche abortiva. Tijdschrift voor Inlandsche Geneeskunde: 31-32.

Beauchat, L.R. 1978. Traditional fermented food products. Pages 224-253 in L.R. Beauchat (ed). Food and beverage mycology. AVI Publishing Company

Boedijn, K.B. 1958. Notes onthe Mucorales of Indonesia. Sydowia, XII (1/6): 321-331.

Calmette, A. 1893. Contribution a l'etude des fermente de l'amidon La Levure chinoise. Centralblatt fur Bakteriologie, Band XIII: 273-275.

Chrzaszez, T. 1901. Die Chinesiche Hefe. Centralblatt fur Bakteriologie, Band VII: 326-327.

Cronk, T.C. 1975. Biochemical studies of an Indonesian fermented rice food - tape ketan. Ph. D. Thesis. Cornell University, Ithaca, New York. (Abstract)

Cronk, T.C., L.R. Mattick, K.H. Steinkraus and L.R. Hackler. 1979. Production of higher alcohols during Indonesian tape ketan fermentation. Applied and Environmental Microbiology, 37: 892-896.

Cronk, T.C., K.H. Steinkraus, L.R. Hackler, and L.R. Mattick. 1977. Indonesian tape ketan fermentation. Applied and Environmental Microbiology, 33: 1067-1073.

De Kruyff, E. 1909. De arak fabricatie te Batavia. Archief voor de Suikerindustrie in Nederlandsch Indie, 17e Jaargang: 151-160.

Del Rosario, R.R. 1980. Traditional Filipino fermented foods. Pages 71-78 in Food Industry Research and Development Institute. Proceedings Oriental fermented foods, Taipei, Taiwan, Republic of Chine.

Dhamcharee, B. 1981. Traditional fermented foods in Thailad. Pages 89-92 in S. Saono, F.G. Winarno, and D. Karyeli (ed.) Proceedings of a technical seminar of ASCA, Feb. 9-11, 1981, Medan, Indonesia.

Dwidjoseputro, D. 1970. Microbiological studies of Indonesian ragi. Ph. D. Thesis. Vanderbilt University, Nashville, Tennesee.

Dwidjoseputro, D. and F. T. Wolf. 1970. Microbiological studies of Indonesian fermented foodstuffs. Mycopathologia et Mycologia Applicate, 41: 211-222.

Eijkman, C. 1894. Over chineesche rijstgist en de arak-bereiding te Batavia, Geneeskundig Tijdschrift: 468-479.

Eijkman, C. 1894. Mikrobiologische uber die Arak-fabrikation in Batavia. Centralblatt fur Bakteriologie, Bank XIV: 97-103.

Ellis, J.J., L.J. Rhodes and C.W. Hesseltine. 1976. The genus Amylomyces. Mycologia, 68: 131-143.

Frazier, W.C. and D.C. Westhoff. 1978. Food microbiology AVI.

Hadisepoetro, E. S. S., N. Takada, and Y. Oshima. 1979. Microflora in ragi and usar. Journal of Fermentation and Technology, 57:251-259.

Heyne, K. 1950. De nuttige planten van Indonesia. Uitgev. W. van Hoeve.

Hayashida, M., R. Ueda and S. Teramoto. 1968. Studies on organic acids in sake brewing VII. J. Fermentation Technology 46(20: 77-91.

Hesseltine, C.W. 1965. A millenium of fungi, food, and fermentation. Mycologia, 57: 147-197.

Hesseltine, C.W. 1955. Genera of the Mucorales with note on their synonimy. Mycologia, 47: 344-363.

Kato, K., K. Kuswanto, I. Banno, and T. Harada. 1976. Identification of Endomycopsis fibulgera isolated from ragi in Indonesia and properties of its crystaline glucoamylase. Journal of Fermentation Technology, 54: 831-837.

Ko, S.D. 1965. Tinjauan terhadap fermented foods Indonesia. Pages 209-224 in R.M. Soemantri (ed.). Research di Indonesia 1945-1965, Volume II. Departemen Urusan Research Nasional Republik Indonesia.

Ko, S.D. 1972. Tape fermentation. Applied Microbiology, 23:976-978.

Ko, S.D. 1982. Indigenous fermented foods. Pages 15-38 in A.H. Rose (ed.). Economic Microbiology, Volume 7. Fermented foods. Academic Press, London.

Kodama, K. 1970. Sake yeast. Pages 225-282 in A.H. Rose and J.S. Harrison (ed.) The Yeast Volume 3. Yeast technology. Academic Press.

Kodama, K. and K. Yoshizawa. 1977. Sake. Pages 423-477 in A.H. Rose (ed.) Economic microbiology Volume 1. Alcoholic beverages. Academio Press.

Kozaki, M. 1976. Fermented foods and related microorganisms in South east Asia. Japanese Association of Mycotoxicology (2): 1-9.

Kramer, A. and B.A. Twigg. 1966. Fundamentals of quality control for the food industry. The AVI publishing Co.

Liu, I.S., T.C. Ni, and C.F. Huang. 1980. Studies on the modified method for the rice liquor fermentation. Pages 126-134 in Food Industry Research and Development Institute. Preceedings Oriental Fermented Foods, Taipei, Taiwan, Republic of China.

Lodder, J. (ed.). 1970. The yeasts, a taxonomic study. North Holland Publishing Company, Amsterdam.

Prinsen-Geerligs, H. C. 1905. De kwaliteit der Java-arak. Archief voor de Java-suikerindustire: 379-399.

Rachtamianto. 1980. Laporan survey brem Bali. Departemen Perdagangan dan Koperasi, Direktorat Standardisasi Normalisasi dan Pengendalian Mutu.

Raffles, Sir. T.S. 1817/1830. The History of Java. Volume I and II. John Murray, Albemarle-street.

Rose, A.H. 1977. History and scientific basis of alcoholic beverage production. Pages 1-43 in A.H. Rose (ed.). Economic microbiology, Vol. 1. Alcoholic Beverages. Academic Press.

Saito, K. 1904. Eine neue Art der Chinesichen Hefe. Centralblatt fur Bakteriologie, Band XIII: 153-161.

Sanchez. P. C. 1891. Studies on sugarcane wine (basi) in the Philipines. Ph. D. Thesis. Tokyo University of Agriculture, Tokyo.

Saono, J.K. D. and E. Tresnalarawaty. 1983. Ketahanan Candida intermedia dan Saccharomyces cerevisiae dalam ragi selama penyimpanan. Buletin Ilmu dan Teknologi Pangan (in press).

Saono, S., I. Gandjar, T. Basuki, and H. Karsono. 1974. Mycoflora of ragi and some other traditional fermented foods of Indonesia. Annales Bororrienses, V (4): 187-204.

Soedarmo, D. 1970. Training program on nutritional biochemistry as regards to taste and flavour and detection as well as identification of micro concentration of flavour components in foodstuffs. Technical Report I by T.N.O. Zeist, Utrecht, Nederland.

Soedarsono, J. 1972. *Some notes on "ragi tape" an inoculum for "tape"* fermentation. Ilmu Pertanian 1 (6): 235-241.

Soeratman, P.C. 1979. Cabinet fermentor dalam pembuatan brem. Departemen Perindustrian, Badan Penelitian dan Pengembangan Industri.

Steinkraus, K.H. 1981. Traditional food fermentation as industrial resourses. Pages 3-16 in S. Saono, F. G. Winarno, and D. Karyadi (ed.). Proceedings of a technical seminar of ASCA, Feb. 9-11, 1981. Medan, Indonesia.

Tabuh Rah. 1976. Keputusan seminar tafir agama Hindu ke III Tahun 1976. Diterbitkan seksi bimbigan masyarakat Hindu dan Budha, 1979.

Tanimura, W., P.C. Sanches and M. Kozaki, 1977. The fermented foods in the Philipines (Part I). Tapuy, rice wine. J. Agric. Sci. of Tokyo University of Agriculture 22 (1): 118-134.

Teysmann, J.E. 1875. Verslag eener botanische reis naar de Westkust van Borneo. Natuurkundig Tijdschrift voor Nederlandsch Indie, Deel, 35: 237-386.

Toyota, Y. and M. Kozaki. 1978. Microorganisms in ragi as starter in Indonesia: on lactic acid bacteria identification and characterization. Annual Meeting of the Agricultural Chemists Society of Japan. (Abstract).

Van Veen, A.G. 1972. Fermented rice foods. Pages 428-431 in D.F. Housten (ed.) Rice chemistry and technology. American Association of Cereal Chemists, Incorporation.

Vorderman, A.G. 1893. De lo-tsoe of rijstwijn. Tijdschrift voor Inlandsche Geneeskunde: 27-29.

Vorderman, A.G. 1893. Oorspronkelyke bijdragen: Analecta op bromatologisch gebied I. Geneeskundig Tijdschrift voor Nederlandsch Indie, Deel XXXIII: 23-397.

Wang, H.H. 1980. Environmental control in Chinese traditional food processing. Food Industries Monthly, 12 (12): 11-13.

Wang, H.H. 1980. A study of modernization of the brewing of kaoliang liquor. Pages 135-142 in Food Industry research and Development Institute. Proceedings Oriental fermented foods, Taipei, Taiwan, Republic of China.

Wehmer, C. 1900. Studien uber technische Pilze VIII. Der Javanische Ragi und seine Pilze. Centralblatt fur Bakteriologie, Band VI: 610-619.

Wehmer, C. 1901. Der Javanische Ragi und seine Pilze II. Central blatt fur Bakteriologie, Band VIII: 313-326.

Wehmer, C. 1904. Ueber Kugelhefe und Garung bei Mucor javanicus. Centralblatt fur Bakteriologie, Band XIII: 277-280.

Went, F.A. and H.C. Prinsen-Geerligs. 1894. Over suiker en alkoholvorming door organismen in verband met de verwerking der naproducten in de riest-suiker fabrieken. Archief voorde Javasuikerindustrie: 529-549.

Yoshizawa, K. 1980. Higher alcohols and esters in sake. J. of the Brewing Society of Japan 75(6): 451-457.

Zycha, H. and R. Siepmann. 1969. Mucorales, eine Beschreibung aller Gattungen und Arten dieser Pilzgruppe. Verlag von J. Cramer.

ACKNOWLEDGEMENT

My gratitude and sincere thanks are due to:

J.I.C.A., Ministry of Foreign Affairs, The Japanese Government, for awarding me the financial support during my study in Japan.

Dr. J. Sugi, President of SAEDA, for giving me the opportunity to study in Japan.

Bogor Agricultural University, Ministry of Education, Government of the Republic of Indonesia, for giving me the opportunity to complete my study.

Prof. M. Kozaki, The Tokyo University of Agriculture (TUA), for his kind guidance throughout this study.

Prof. A. Matsuyama, AP4 Project, J.I.C.A.-B.A.U., for his valuable advice and help.

Prof. K. Noshiro, TUA, for his kind advice.

Drs. A. Hosono, T. Baba, A. Tomomatsu, K. Koizumi and K.N. Chen, for their thoughtful help, assistance and cooperation.