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Laboratory standardization of procedure for the preparation of Zou - a fermented beverage made from rice

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Abstract

Zou or Yu is a distilled product of rice beer prepared using *Albizzia* bark. Albizzia bark was found to provide an ecological niche to a mold and a few yeast strains. The mold strain was found to be responsible for the alcoholic fermentation of rice. The strain was identified as *Mucor indicus* Lendner. For the fermentation process a starter culture called hamei (khai) was prepared by inoculating aqueous mixture of gamma sterilized rice and bark powders with *M. indicus* culture. Hamei was used as inoculum for the fermentation of rice slurry. The fermentation was carried out for 5 days at ambient temperature (26 ± 2°C). After completion of the fermentation, the beer was distilled at 78°C. The ethanol yield was quantified by gas chromatographic and alcohol dehydrogenase assay methods.

Key words: *Albizzia,* hamei, *Mucor indicus,* Rice, fermentation, Zou.

Introduction

Albizzia spp. (*A. myriophylla* and *A. kalkora*) plants are widely distributed in north-eastern region of India, especially in the state of Manipur. *Albizzia* belongs to the Leguminosae family. It is a tree or climber with rough dark bark. *A. myriophylla* has also been reported from the southern provinces of Vietnam where it is locally named as 'Cay Song Ran'[1]. In the state of Manipur (India) bark powder from *A. myriophylla* and *A. kalkora* is used to make rice cake, locally called as 'hamei' or khai. This rice cake is used to ferment cooked rice to make the beverage. The rice beer is locally named as Zou or Yu. Starch is the major constituent of rice and makes up 90% of the rice on a dry-weight basis[2]. Nutritive value of fermented foods and drinks using rice products has been reviewed[3, 4,5,6,7,8,9,10,11,12,13,14,15]. Fermented rice food products were classified into three categories [11]. These include solid, paste, and liquid. The solid-state fermented products include starter types such as *pekka*, *anka* (China), *ragi* (Indonesia), *Koji* (Japan), predigested "yellow rice" (Ecuador) [15], and bread like foods [10]. Paste products include *miso* (Japan), and *chiang* (China). The liquid products are *shao-hsing* wine (China), *sake* (Japan), and rice vinegar [5,11,16]. Present study deals with the laboratory standardization of the traditional alcoholic fermentation of rice using *Albizzia* bark.

Materials and Methods

Preparation of bark powder: Fresh bark was procured from the *Albizzia* plants and sun dried. Before crushing it was kept in an oven at 100°C for 2 h to remove the moisture. Dried bark was crushed to powder using a mechanical grinder. The powder was stored at ambient temperature for the experimental purpose.

Isolation and identification of microorganisms from Albizzia bark powder: Albizzia bark powder was suspended in sterile saline (0.85%) and serially diluted using the same, plated on yeast extract-peptone-dextrose-agar (YPDA) plate (pH 5.7) and incubated at 26 ± 2°C for 48 h. YPDA consists of yeast-extract (1%), peptone (2%), dextrose (2%), and agar (2%). Well-isolated, putative yeast and mold colonies were selected, re-streaked on YPDA plate, and single isolated colonies were preserved for identification and fermentation experiments. For estimating the total microbial load present in the bark powder it was suspended in saline, diluted, plated on Luria-Bertani (LB) agar (pH 7) plates, and incubated at 26 ± 2°C for 48 h. At the end of incubation total microbial load was determined taking dilution factor in consideration.

Preparation of hamei: Hamei, also called khai, was prepared using bark and rice powders. Powders were gamma sterilized using cobalt-60 gamma source in a package irradiator (AECL, Canada) (dose-rate 32 Gy/min). The radiation dose delivered for complete sterilization was 25 kGy. Isolated mold and yeast strains were grown separately in YPD broth at 26 ± 2°C on a rotary shaker (150 rpm). 1ml of the overnight grown culture was centrifuged at 10,000 g for 5 min, washed with sterile distilled water, and suspended in the same. For making a cake, 12.5 g of rice powder, 1 to 5% (125 mg to 625 mg) of bark powder, 1 ml of washed microbial culture, and 11.5 ml of sterile distilled water were mixed together and incubated in a sterile petri-plate at 26 ± 2°C for 5 days. The fermented cake (hamei) was used as a source of inoculum for alcoholic fermentation of rice.

Rice fermentation: Different rice varieties including, the local varieties (KD 2-6-3, RCM-9) from Manipur and polished market varieties (Kolam) were used in different sets of experiments. Rice was removed from husk, cleaned by air blow, and cooked in 5 vol of water in a pressure cooker. Cooked rice (50 g) was cooled, and mixed with single cake (hamei) i.e. fermented mother culture and incubated for 5 days at ambient temperature (26 ± 2°C). After incubation, fermented rice was centrifuged at 6000 g for 20 min twice to remove the rice debris.

Analysis of the fermented rice beer: The supernatant obtained after centrifuging the fermented rice was double distilled at 78°C. 60 ml of the distillate was obtained from one set of fermentation batch. Each fermentation batch consisted of 50 g of raw rice, cooked in 250 ml of water, cooled and mixed with a single cake, prepared from 12.5 g of rice powder and 2% of bark powder, unless specifically mentioned. Distilled product was analysed using GCMS, and quantified by ADH assay method.

Gas chromatography and Mass spectroscopy analysis of distillate: Headspace volatiles of the distillates[17] were subjected to GC/MS analysis using a Shimadzu QP 5050A gas chromatography / mass spectrometry instrument. The instrument was equipped with a GC-17A gas chromatograph and provided with DB-1 (Dimethyl polysiloxane, J&W Scientific) capillary column (length 30 m, internal diameter 0.25 mm, and film 0.25 mm). The operating conditions were: column temperature programmed from 60^0C to 200^0C at the rate of 4^0C/min, held at initial temperature and at 200^0C for 5 min and further to 280^0C at the rate of 10^0C/min held at final temperature for 20 min. Injector and interface temperatures were maintained at 210^0C and 230^0C, respectively. Helium was used as carrier gas. Ionisation voltage was 70 eV. Electron multiplier voltage was 1 kV. Compounds of interest were identified by comparing their mass fragmentation pattern with that of standard spectra available in the spectral library (Flavour and Fragrance and Wiley/ NIST libraries) of the instrument as well as by comparing the retention time of standards injected separately under the same conditions.

Quantification of ethanol in distilled product: The distillate, containing mainly ethanol was analysed by GCMS analysis. The actual ethanol content was quantified by ADH (alcohol dehydrogenase) assay[18]. For ADH assay the distillate was diluted to 1000 times (10 ml to 10 ml) using sterile distilled water and from this diluted solution 10 ml was analysed in 1ml reaction mixture. Thus, effective dilution in final reaction mixture was $1:10^5$.

Formula for NADH+H$^+$ estimation: According to Beer Lambert's law, NADH+H$^+$ conc. (mM) = Absorbance/Molar extinction coefficient x Path length (cm) = A/6.22 x 1= A/6.22. NADH+H$^+$ formation was estimated by measuring the absorbance at 340 nm. Alcohol conc. is equimolar to NADH+H$^+$ conc.

$$RCH2OH + NAD^+ \xrightarrow{ADH} RCHO + NADH + H^+$$

Ethanol tolerance of M. indicus: The mold was grown overnight in 10 ml of YPD (pH 5.7) broth at ambient temperature in a rotary shaker (150 rpm). The culture was centrifuged at 10,000 g for 5 min and the pellet was washed with equal vol of 0.85% saline. The pellet was suspended in the same solution. The cell suspension was mixed with different concentrations of ethanol (Hayman, UK) varying from 5 to 50%, and incubated for 2 h or more. The treated cell-suspension was serially diluted using saline, plated on LB agar plate, and incubated at 26 ± 2^OC for 48 h before the viable colony forming unit number was counted.

Temperature tolerance of M. indicus : The organism from actively growing culture plate was streaked on YPDA plate in different replicates. The plates were incubated at various temperatures ranging from 4^oC to 60^oC for 48 h. At the end of incubation period, growth was checked.

pH tolerance of M. indicus : The organism from actively growing culture plate was streaked on YPDA plates of different pH ranging from pH 5 to 10. The plates were incubated at 26 ± 2^oC for 48 h. At the end of incubation period, growth was checked. For pH less than 5, assay was performed in YPD broth, because agar was not able to solidify perfectly at pH 4 and below.

Growth kinetics of M. indicus in the hamei (cake) and fermentation broth: Hamei was incubated at ambient temperature for 5 days. On each day 1 g of cake was cut using a sterile scissor, and suspended in 5 vol of sterile water. 1 ml of aliquot from the suspension was serially diluted using sterile saline (0.85%) and plated on YPDA plates. The plates were incubated at 26 ± 2^oC for 48 h. The no. of viable colony forming unit was counted at the end of incubation and CFU/g was calculated taking dilution factor in consideration. Similarly, mold counting was also performed from fermentation broth on different days of incubation by withdrawing 1 ml of sample, serially diluting with saline (0.85%) and plating on YPDA plates.

Results and Discussion

Microbiological study of bark powder: Total microbial load present in the bark powder was found to be around $9x10^3$ cfu/g. Altogether five putative yeast or mold like strains were isolated from the bark powder of both *Albizzia* spp when plated on YPDA plates. Out of these, three were confirmed to be yeast, one mold, and one bacterial strain. These isolates were got identified at the Institute of Microbial Technology (IMTECH), Chandigarh (India) and deposited there. Identification no. and name of the organisms are mentioned in Table 1.

Alcoholic fermentative ability of the yeast and mold isolates: Fig. 1 shows the process flow chart for the laboratory production of zou. All the isolates were checked for their rice fermentation abilities by gas chromatographic analysis of the fermented product. Interestingly, only the mold isolate was able to produce ethanol amongst all the five isolates screened (Fig. 2A and 2B). The peak observed by GCMS analysis of distillate obtained through rice fermentation using mold (*M. indicus*) inoculated hamei, was found to match with the ethanol peak available in the GCMS library (Fig. 2A). Fermentation using yeast isolates did not yield any resembling peak in GCMS analysis (Fig. 2B). The results indicate that the mold stain probably possesses both amylolytic and saccharolytic activities and that is why it was able to degrade the starch present in rice to glucose, and ferment glucose to alcohol. Yeasts are known to have fermentative ability but lack in amylolytic activity and hence, are unable to carry out the alcoholic fermentation of rice alone.

Study of factors affecting ethanol yield: The experiments were conducted to know whether ethanol yield gets affected by changing the mode of inoculum in the fermentation broth. The inocula used were: (1) Mold inoculated cake (hamei), or (2) Overnight YPD broth grown mold, and bark powder directly mixed in the fermentation broth. Amongst these hamei was found to give better product compared to the other one (Fig.3). Bark powder was found to be helpful during preparation of starter culture i.e. hamei, because it provided the mild acidic environment optimal for the growth of inoculated *M. indicus*. Concentration of bark powder in the cake was optimized to get the maximum ethanol yield. Cake made by using 1 to 3% of bark powder was able to give the best yield of ethanol (Fig.4). Rice: water ratio at the time of cooking that has provided the best yield of ethanol was found to be 1:5 (data not shown). Different rice varieties including market polished rice were used in making cake as well as in fermentation broth, and the ethanol yield was estimated at the end of the fermentation. Local rice varieties were found to be slightly better source of ethanol production compared to the market polished rice (Fig.5). For alcohol estimation cake was harvested on different days of incubation. It was observed that even one-day-old cake was good enough as inoculum (data not shown). This may be due to fast growing nature of *M. indicus*.

Growth kinetics of M. indicus: The growth profile of *M. indicus* in the cake and the fermentation broth was also studied. Count of *M. indicus* in the cake was found to be $3x10^6$ cfu/g at the end of 24 h of incubation, which increased to $5x10^6$ cfu/g after 96 h of incubation. Similarly, in the fermentation broth initial (24 h) count was $2.3x10^6$ cfu/ml, which increased to $5x10^6$ cfu/ml at 96 h of incubation. The result indicated that around two to three fold increase in the number of colony forming unit was observed during fermentation either in starter culture or in the fermentation broth.

Physiological features of Albizzia bark: Bark powder was found to be acidic in nature and i.e. why probably mold and yeast have selected *Albizzia* as their natural habitat (Fig.6). The pH range of bark powder was found to vary from 4.8 to 5.1 (Fig. 6). Even increasing the bark powder concentration from 1 to 10% did not make significant change in the pH. The result indicates that the compound(s) responsible for acidic nature of bark are weakly acidic in nature. Fig. 7 shows the comparative photograph of the cake prepared from sterilized and non-sterilized bark powders. The figure clearly indicates the contamination occurred in the non-sterilized one. The radiation sterilization treatment removed the microorganism/s that would have interfered with the alcoholic fermentation of rice.

Morphological and physiological features of M. indicus: Fig.8 shows the colony morphology of the *Mucor indicus* isolate. The colony was colorless and displayed hairy appearance typical of the mold. The diameter of the overnight grown colonies was found to vary from 1.4 to 2.5 cm, which increased significantly on further incubation. The optimum growth temperature of the mold was found between 26 and 35^{O}C. It was not able to grow at temperature of 10^{O}C and below. At 15^{O}C its growth was found to be poor. At 40^{O}C and above the organism was not able to grow and lost its viability. *M. indicus* was found to grow well between the pH ranges of 5 to 9. At pH 10, the growth was found to be slow. At pH 4 and 3 some growth was observed after 48 h and 72 h of incubation, respectively. Even at pH 2, its viability was not lost, however, it was not able to grow at this pH.

Ethanol tolerance of M. indicus: Since mold was found to produce high yield of ethanol, its tolerance limit to ethanol was determined. A concentration dependent ethanol tolerance was observed when *M. indicus* was treated with different concentrations of ethanol and incubated for 24 h before plating was carried out. It was found to be completely tolerant to 5% ethanol (Fig. 9A). At 10 to 15%, a concentration dependent decrease in cell viability was observed, however 20% concn. was found to be lethal and not a single viable colony was observed (Fig. 9A). The effect of duration of ethanol treatment on cell viability was also studied. Even at 20% concn. of ethanol, some viability was observed when treatment duration was reduced (Fig. 9B). At 25% concn. a few viable colonies were found when they were treated for 2 h only. Increasing the treatment period for 6 h, resulted in complete loss of the cell viability. Thus, alcoholic fermentation using *Albizzia* bark to produce rice beverage was standardized as per the flow chart (Fig. 1). Mold (*M. indicus*) was found to be the key factor for the alcoholic fermentation. Radiation sterilization of bark and rice powders was introduced in the process to get rid of unwanted microorganism(s). However, cooking of rice would take care of the contamination in rice. Acidic nature of the bark powder seems to be responsible for its natural selection as habitat by mold as well as for the optimal growth of *M. indicus* in the starter culture. The characterization of the acidic factor in the bark is of further interest.

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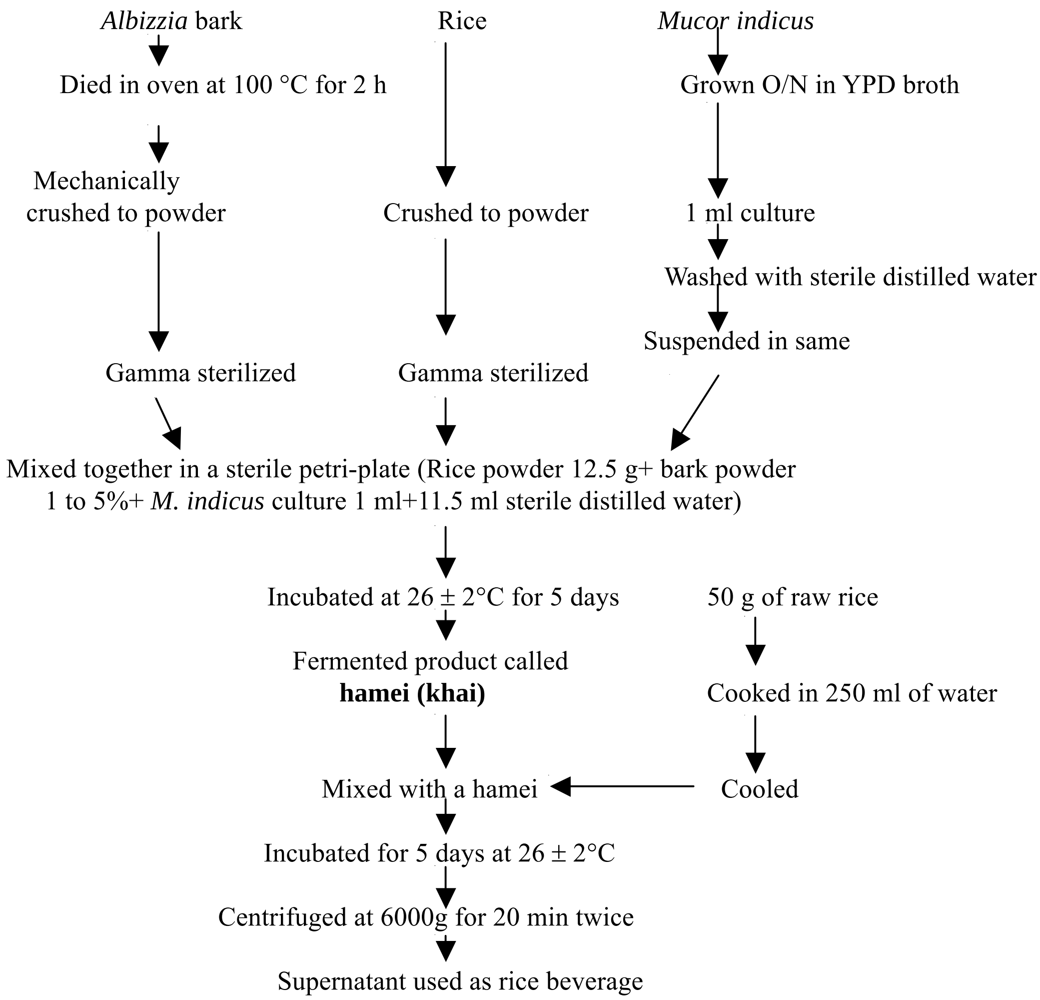

Figure 1. Process flow chart for the laboratory production of zou.

Table 1. Microbial isolates from *Albizzia* bark powder.

Isolate	Source	Name	ID NO.	General features
Mold	*A. kalkora*	*Mucor indicus Lendner*	MTCC 4349	Colorless, Fast growing, Sporulating
Yeast	*A. myriophylla*	*Rhodotorula mucilaginosa*	MTCC 4449	Pink colored, Medium growing
Yeast	*A. kalkora*	*Rhodotorula mucilaginosa*	MTCC 4450	Pink colored, Medium growing
Yeast	Cake	*Candida parapsilosis*	MTCC 4448	White colored
Bacteria	*A. kalkora*	*Streptococcus* sp.	Not provided	Colorless, slow growing

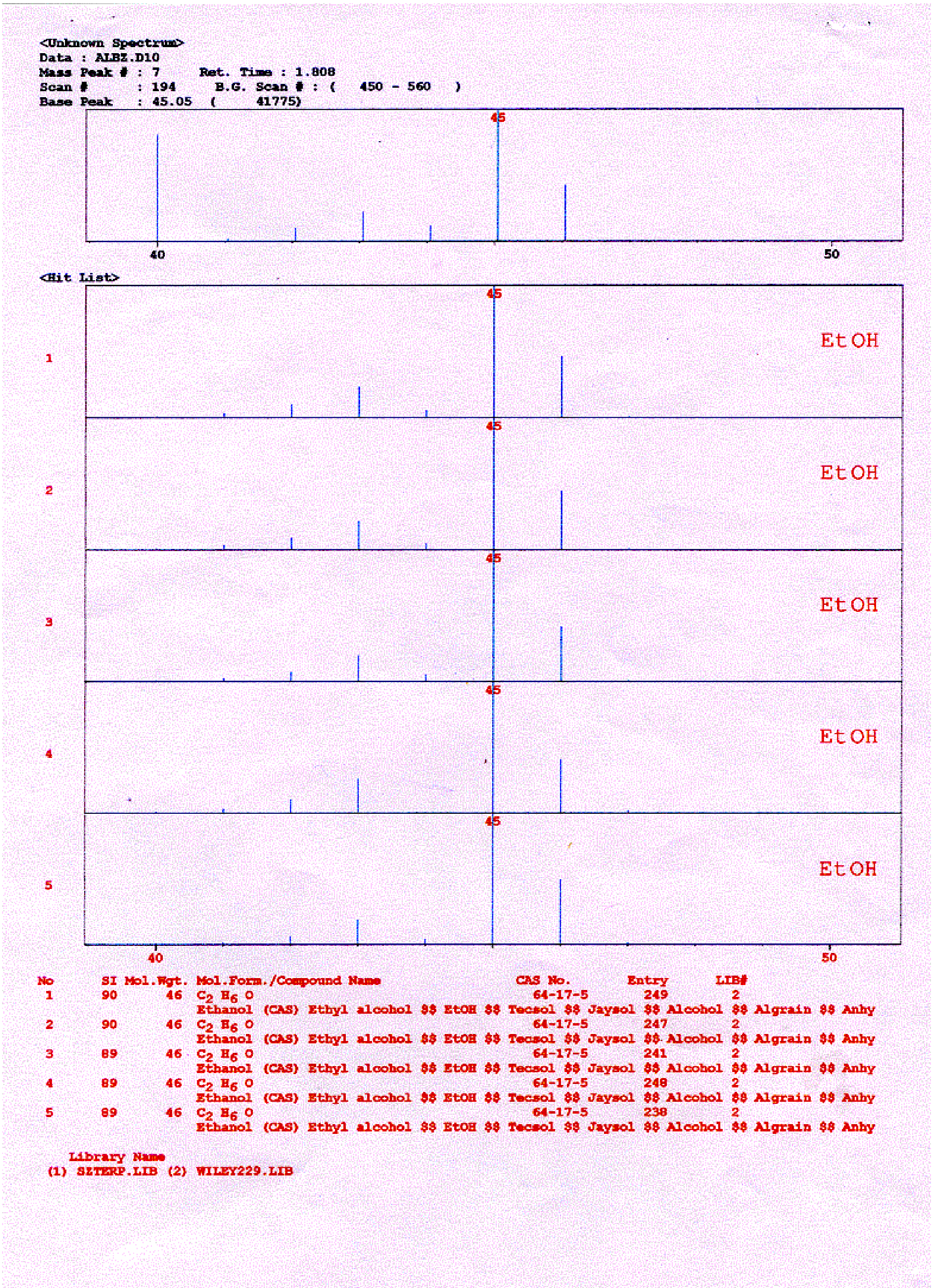

Figure 2a. GCMS profile of the distillate obtained using *Mucor indicus*.

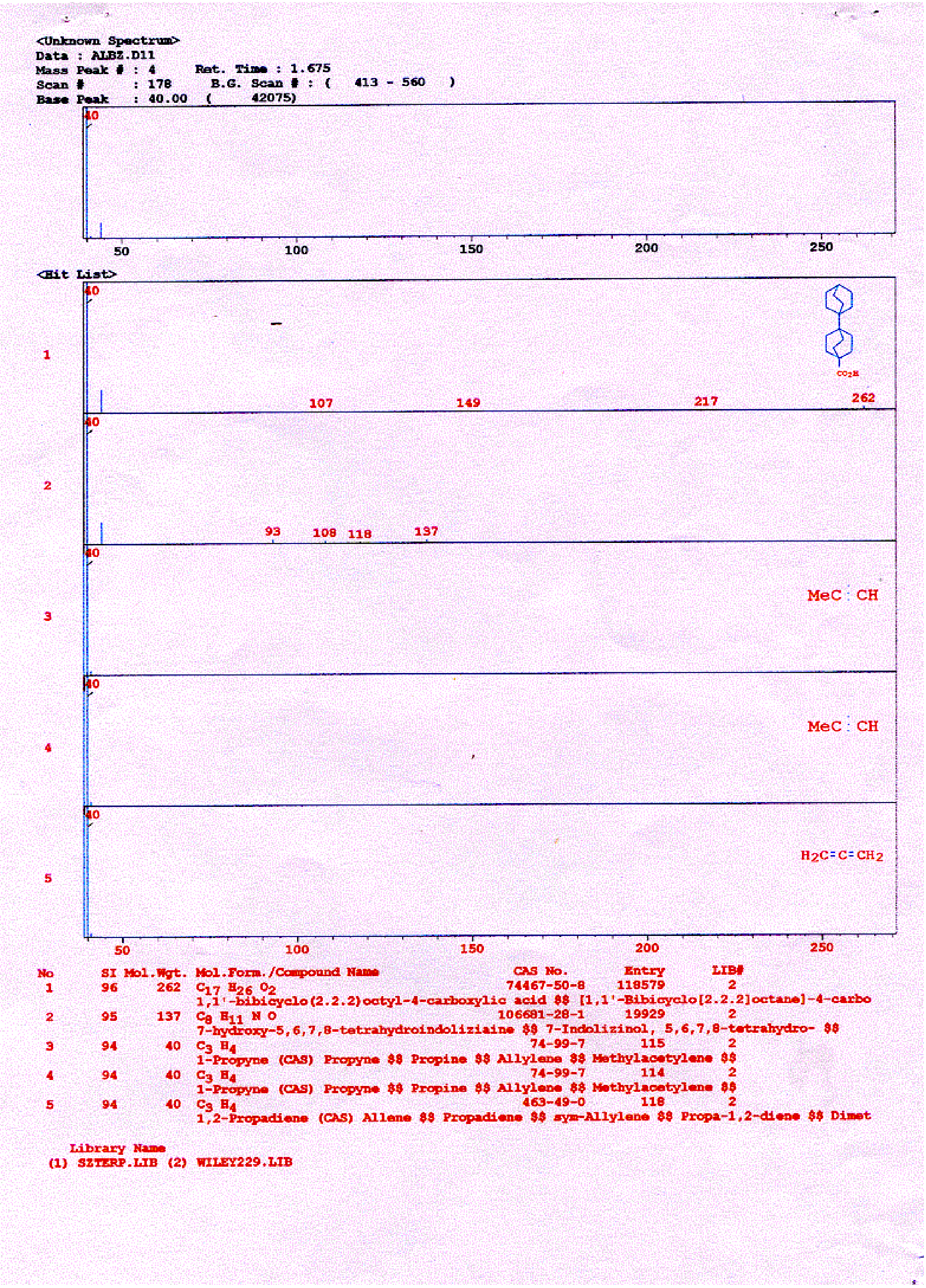

Figure 2b. GCMS profile of the distillate obtained using Yeast isolate.

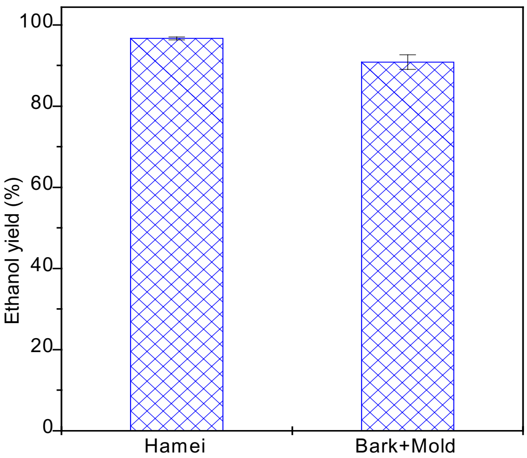

Figure 3. Effect of using hamei prepared with mold or barks powder and mold separately as inoculums in fermentation broth on ethanol yield.

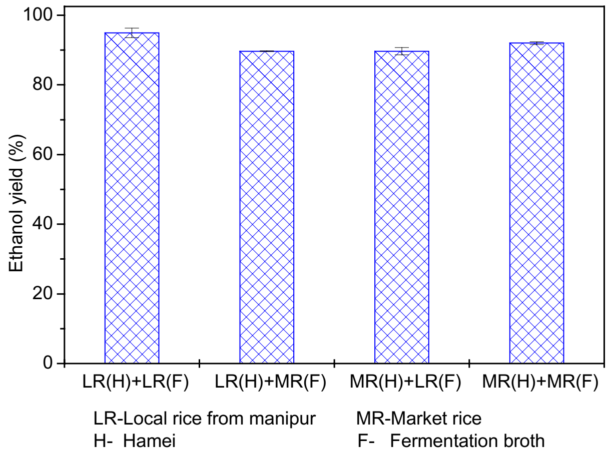

Figure 5. Graph showing effect of rice varieties on ethanol yield.

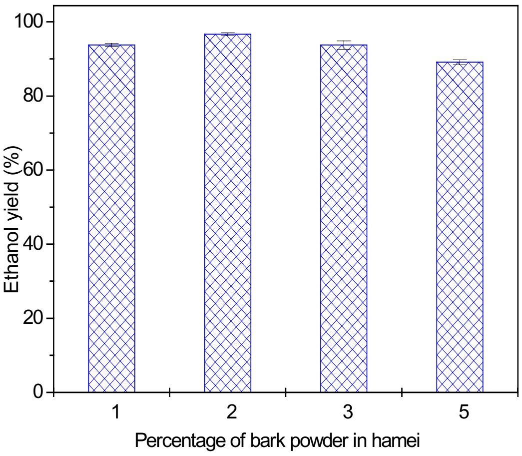

Figure 4. Effect of bark powder percentage in the cake on etha nol yield.

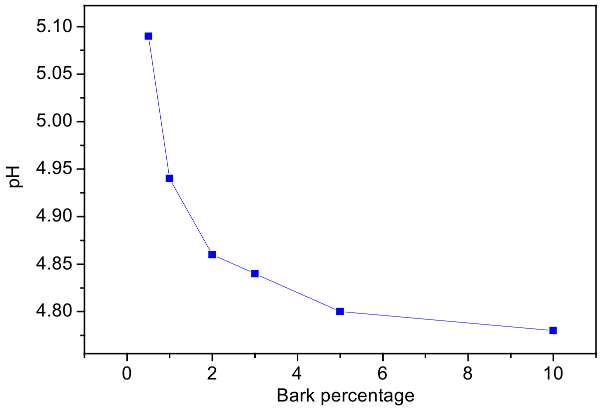

Figure 6. Graph showing acidic nature of *Albizzia* bark.

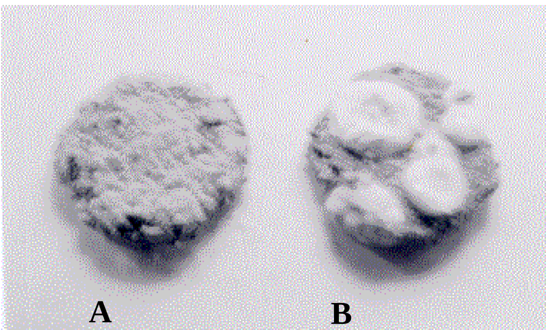

Figure 7. Showing comparative photograph of cake (hamei) prepared from (A) gamma sterilized rice and back powders,(B) non-sterilized rice and bark powders.

Figure 8. Colony morphology of *M. indicus*.

Fig. 9 (A)

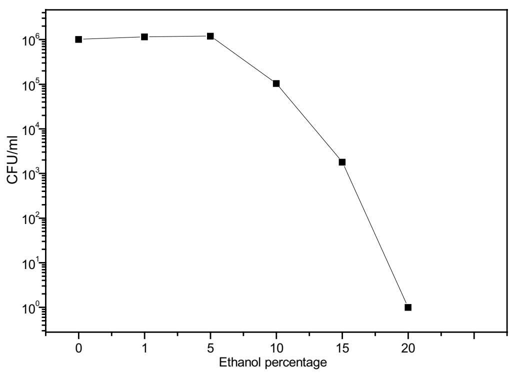

Figure 9A. Graph showing tolerance of *Mucor indicus* when cells were treated for 24 h with different concentrations of ethanol.

Fig. 9 (B)

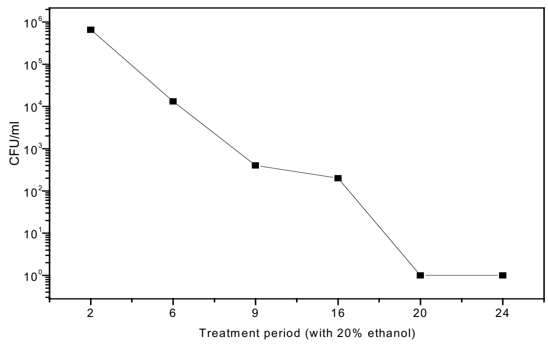

Figure 9B. Graph showing ethanol tolerance duration of *Mucor indicus* when cells were treated with 20% ethanol for different time period.