

Liquor Quality of Rice Wine as Affected by Yeast Strains Isolated from Coconut and Palmyrah Sap

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ABSTRACT. The suitability of pure cultures of four high yielding isolates of palm wine *Saccharomyces*, (C1, C3 and C8 from coconut sap and P1 from palmyrah sap) for rice fermentation based on their capacity to produce compounds responsible for aroma and flavor was studied. The maximum ethanol content produced by fermenting rice under ambient temperature ($28\pm 2^{\circ}\text{C}$) ranged from 11.1 to 15.2%. The volatile compounds in the headspace of four rice wines showed that ethanol, ethyl acetate, acetaldehyde, isoamyl alcohol and two other unknown compounds were the most prominent. Ratio between ethyl acetate to isoamyl alcohol could be considered as a valuable criterion for selecting a yeast isolate. The lowest ratio was evident in rice wine produced using the isolate C3. In addition, rice wine produced using the isolate C3 had the highest content of acetaldehyde, which could impart the fruity aroma. Furthermore, slower rate of ethanol production by the isolate C3 in comparison to the other three isolates is advantageous as rice fermentation is a batch process, which is carried out slowly over a period.

INTRODUCTION

Sake or rice wine is a traditional alcoholic beverage in Japan, becoming popular in the world. The characteristic features of sake brewing are the use of koji, a culture of *Aspergillus oryzae* on steamed rice grains that saccharifies the rice starch, and fermentable yeast, mainly *Saccharomyces cerevisiae* that converts the fermentable sugars into alcohol and carbon dioxide in which both proceed simultaneously (Kodama *et al.*, 2002).

Yeast forms an array of chemicals, like higher alcohols, esters, aldehydes and acids in addition to ethanol, all that attribute sake its body, nuances of fragrance, flavor and aroma. These chemical compounds are present in varying quantities, depending on the yeast strain, temperature at which fermentation takes place and nitrogen compounds and lipids in the raw materials (Akiyama *et al.*, 1978). *Gingo* sake contains more pleasant aroma compounds than ordinary sake, and requires the use of highly polished rice and fermentation at a low temperature for a long term (Ichikawa, 2002). The choice of yeast for rice fermentation should therefore directly match with the temperature of fermentation. In Sri Lanka, palm wine yeasts can be successfully used at room temperature in rice fermentation yielding 10.5 to 13.5% ethanol (Wellala *et al.*, 2004). Their suitability in industrial palm wine production also has been well studied, where the flavor volatiles are of major concern

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(Uzochukwu *et al.*, 1994; Uzochukwu *et al.*, 1999; Samarajeewa *et al.*, 1981). Integrated contribution of many esters and higher alcohols, produced by palm wine yeasts irrespective of raw materials, is associated with characteristic palm wine aroma (Uzochukwu *et al.*, 1994), which is prominent in rice wine brewed by them. Higher alcohols such as amyl and isoamyl alcohol and esters such as ethyl caproate, isoamyl acetate and ethyl butyrate that attribute to favorable aroma, which are found to be common constituents of fruit juices (Flath and Forry, 1970; Macial *et al.*, 1986), are also products of palm wine yeasts.

Use of pure cultures of selected strains of palm wine *Saccharomyces*, which possess favorable biochemical potential with delicate aroma and flavor properties, in fermentation of rice under tropical ambient temperature would be beneficial in industrial application. This study was undertaken to screen one such strain out of previously isolated four high alcohol yielding palm wine *Saccharomyces* (Wellala *et al.*, 2004) based on their capacity to produce compounds responsible for aroma and flavor.

MATERIALS AND METHODS

Preidentification of yeasts

Three yeast isolates from coconut and one isolate from palmyrah sap (Wellala *et al.*, 2004) were further subjected to taxonomic preidentification by using lysine as a sole source of nitrogen (Martinez *et al.*, 2004).

Preparation of koji

Rice (Bg 358) was washed with water for thirty seconds followed by further washing in running water for thirty more seconds, soaking in water for seven minutes, draining of excess water from rice for forty five minutes, steaming for one hour and cooling down to 30-35⁰C. One gram of tane-koji (Sooriyamoorthy *et al.*, 2004) was sprinkled on one kg of rice (Bg 358) and incubated at 35⁰C for two days. During incubation, the heap of rice grains was mixed after 24 h. On the second day of incubation, grains looked-like white frosting with uniform growth of the fungal mycelia were packaged in airtight plastic boxes and stored at -18⁰C.

Preparation of starter cultures

Culture medium (100 ml) for each yeast strain was prepared in 250 ml Erlenmeyer flasks, containing; glucose (BDH Laboratories) 5 g, Bactopectone (Difco) 1 g and Yeast extract (Difco) 1 g in 0.1M KH₂PO₄ in which pH was adjusted to 4.5 using 0.1M HCl (Wellala *et al.*, 2004). After sterilizing the medium at 121⁰C for 15 minutes and cooling down to about 80⁰C, potassium metabisulphite (KMS) was added to obtain a final concentration of 100 ppm. A loopfull of cells of yeast strains from the PDA slants maintained at 6⁰C was inoculated at room temperature (28±2⁰C) on an orbital shaker (SO1, Bibby Sterlin, UK) at 100 rpm to obtain about 10⁷ cells/ml. Cell counts were taken by using a haemocytometer (Superior, West Germany).

Rice fermentation

Four palm wine yeast isolates, three (C1, C3 and C8) from coconut sap and one (P1) from palmyrah sap, selected based on the amount and rate of ethanol production during rice fermentation (Wellala *et al.*, 2004) were further assessed for their ability to produce aroma and flavor compounds in a completely randomized design with triplicate. As described by Wellala *et al.* (2004), rice fermentation was carried out using 70% polished, steamed rice (Bg 358), koji and spring water obtained from the Kandy District and ingredients were added in five successive batches every other day in proportions given in Table 1. They were kept at room temperature under semi-aerobic conditions during successive additions of ingredients and under semi-anaerobic conditions thereafter, until a constant weight was reached. At the end, unfermented solids were pressed out by squeezing the fermented liquid through cheesecloth, and the resulted filtrate was further filtered through Whatman No 1 filter papers using a Buckner funnel. The clear filtrate was bottled, pasteurized at 65°C for 20 minutes and stored in a refrigerator until further use.

Determination of aroma compounds using headspace gas chromatography

Headspace analysis was carried out in triplicate. Glass vials with self-sealing septa containing 5 ml rice wine were allowed to stand at 50°C for 30 minutes in a water bath. Headspace gas (3 ml) was withdrawn using a gas tight syringe, injected into the gas chromatograph (Shimadzu model GC-14B), and the volatile constituents were separated on a custom 2 m x 5 mm x 2.6 mm glass column (Carbopack BAW) and N₂ gas at a flow rate of 40 ml/min. The detector used was a flame ionization detector. Oven temperature was maintained at 105°C. Both injector and detector temperatures were 150°C.

Table 1. Successive addition of ingredients for sake type rice fermentation.

Ingredient	Starter (1 st day)	3 rd Day	5 th Day	7 th Day	9 th Day (final addition)	Total
Total rice (g)	7.00	14.0	26.50	44.5	8.0	100.0
Koji rice (g)	2.25	4.0	6.25	7.5	-	20.0
Residual rice (g)	4.75	10.0	20.25	37.0	8.0	80.0
Spring water (ml)	7.75	12.5	31.75	67.5	8.0	127.5
Yeast starter (ml)	5.00	-	-	-	-	5.0

Determination of liquor quality

The ethanol content of fermented liquid was determined in triplicate according to the standard AOAC (1995) method, using isopropyl alcohol as an internal standard. The residual sugar was estimated in triplicate by the anthrone test (Khoshkhoo *et al.*, 1994). Total soluble solids (TSS) and pH were measured in triplicate using a Refractometer (Atago N-1E, Japan) and pH meter (IM-40S TOA Electronics, Japan), respectively.

Statistical Analysis

ANOVA (CRD) and LSD mean separation procedure were performed to estimate the variability, and for mean separation, respectively. The SAS computer package was used for data analysis and the p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Inability to use lysine as a sole nitrogen source further confirmed that all isolates obtained from coconut and palmyrah sap were *Saccharomyces cerevisiae*. The rate of ethanol produced during fermentation by each yeast isolate after the final addition of ingredients was calculated by using the equation reported by Tabera *et al.* (1985) based on drop of weight of the culture medium (Fig. 1). As shown in Figure 1, C3 of the third morphological group (Wellala *et al.*, 2004) showed the lowest rate of ethanol production throughout the fermentation period while C8, C1 and P1 of group 1 showed higher rates. These results further confirmed the relationship between the morphological characteristics and the rate of ethanol production of two morphologically different yeast groups described previously, where the first and third morphological groups having round and ellipsoidal shaped single cells exhibited comparatively fast and slow rates of ethanol production, respectively (Wellala *et al.*, 2004).

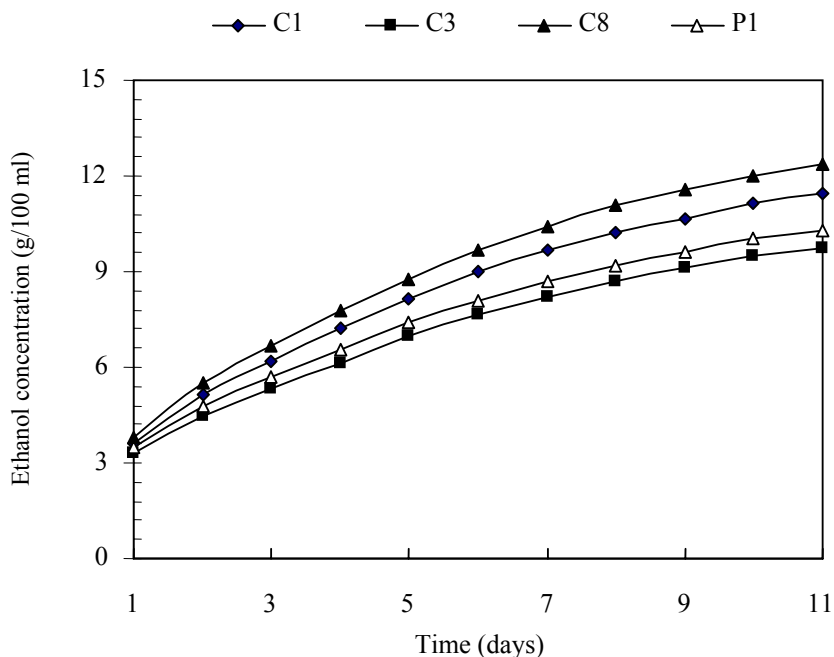


Fig. 1. Rate of ethanol production during rice fermentation as affected by yeasts isolated from coconut (C1, C3 and C8) and palmyrah (P1) sap.

The rice wine was clear and light yellow in color. Its chemical properties are presented in Table 2. The average ethanol content of rice wine produced by the four yeast isolates ranged from 11.1 to 15.2%, which was in agreement with results reported previously (Wellala *et al.*, 2004). Among the four yeast isolates, the isolate C3 produced rice wine with significantly higher levels of reducing sugar followed by C8, P1 and C1. This high level of sugars in rice wine is a beneficial property in sake brewing, as it contributes to the characteristic taste of sake (Yoshizama and Kishi, 1994). However, there was no clear relationship between the ethanol and the total sugar contents of the four rice wines produced by the four yeast isolates (Table 2). The pH of the rice wines varied from 4.19 to 4.50 resembling that of Japanese sake.

Table 2. Physico-chemical properties of rice wine produced by yeast isolates at the end of batch fermentation at 28±2°C.

Isolate	Ethanol (% v/v)	TSS (°Brix)	Total sugar (%)	pH
C1	11.1±0.5 ^b	6.1±0.1 ^a	3.8±0.1 ^b	4.41±0.01 ^b
C3	14.6±0.3 ^a	6.3±0.1 ^a	4.3±0.2 ^a	4.25±0.02 ^c
C8	15.2±0.4 ^a	6.3±0.2 ^a	4.0±0.1 ^b	4.50±0.01 ^a
P1	14.2±0.4 ^a	6.1±0.1 ^a	3.9±0.1 ^b	4.19±0.02 ^d

Note: Each value represents mean±SD of triplicate. Means in each column followed by the same letter are not significantly different ($p < 0.05$).

The chromatograms representing the volatile compounds of the headspace of four rice wines are shown in Figure 2. The identities of the peaks as numbered in chromatograms and proportions of compounds revealed by peak areas are listed in Table 3. Ethanol, ethyl acetate, acetaldehyde, isoamyl alcohol and two other unknown compounds were the most prominent volatile compounds found in the headspace of rice wines. However, peak areas representing their relative composition in the headspace can be slightly affected by changes of warming temperature and warming time of the vials (Akiyama *et al.*, 1978). Ethanol is the main and final product of fermentation and isoamyl and isobutyl alcohols are some intermediate products of amino acid synthesis (Miyake, 1993). According to them, most of the increased enzyme activities and ester production are carried out during the stationary phase of the growth of yeasts as a consequence of an increase in activities of both alcohol acetyltransferase and the synthesis-related activity of esterases. After that, the production of esters decreases as the hydrolysis related activity of esterases strongly increases (Miyake *et al.*, 1993). Ethyl acetate is one of the main volatile ester compounds in sake and in other alcoholic beverages. It can contribute positively to wine aroma at low concentrations, but is considered undesirable at higher concentrations because of low thresholds of 12.5 mg/l (Bartowsky *et al.*, 2003).

Table 3. Volatile aroma compounds of rice wine determined using headspace gas chromatography.

Peak No.	Compound	Peak area (%)			
		C1	C3	C8	P1
1	Acetaldehyde	0.310±0.070	0.540±0.050	0.440±0.140	0.480±0.080
2	Methanol	0.025±0.001	0.024±0.001	0.023±0.005	0.025±0.001
3	Ethanol	90.41±0.270	92.98±0.320	90.69±0.860	91.00±0.870
4	Ethyl acetate	2.300±0.290	1.090±0.130	2.670±0.850	3.460±0.290
5	Unknown 1	0.300±0.010	0.180±0.010	0.220±0.050	0.230±0.020
6	Unknown 2	3.630±0.280	2.720±0.160	3.250±0.380	2.190±0.140
7	Isoamyl alcohol	2.890±0.170	2.280±0.200	2.510±0.310	2.430±0.020
	Others (traces)	0.135±0.003 (18 ^a)	0.186±0.005 (26 ^a)	0.197±0.001 (20 ^a)	0.185±0.004 (15 ^b)
	Ethyl acetate/Iso-amyl alcohol	0.830±0.110	0.480±0.089	1.080±0.430	1.140±0.110

Note: Each value represents mean±SD of triplicate. ^a - No of peaks (average).

Ethyl acetate in combination with some other higher alcohols and esters such as propanol, isobutanol, octanol and heptyl acetate, produced by palm wine yeast, especially *Saccharomyces* is responsible for characteristic palm wine aroma (Uzochukwu *et al.*, 1994), which is not acceptable in rice wine. However, ethyl acetate in combination with relatively higher amounts of isoamyl alcohol and isobutyl acetate does not impart any unpleasant aroma (Akiyama *et al.*, 1978). The use of rice with a higher polishing ratio causes an increase in isoamyl alcohol content in the sake mashes, and this is presumed to be due to increase in the uptake of amino acids, which are used as precursors of higher alcohols by the yeast cells (Furukawa *et al.*, 2003). Teramoto *et al.* (1993) also have shown that, rice wine produced with powder obtained from sprouting rice as the saccharifying agent gave lighter and pleasant aroma due to the presence of relatively lower levels of ethyl acetate compared to isoamyl alcohol. Moreover, rice wine produced with barley malt as the saccharifying agent resulted in rice wine containing large amounts of ethyl acetate compared to isoamyl alcohol with heavy, complicated and unpleasant aroma (Teramoto *et al.*, 1993). These findings suggest that the ratio between ethyl acetate to isoamyl alcohol could be considered a valuable criterion in selecting a yeast isolate among the four used in this study for producing rice wine with acceptable aroma and flavor.

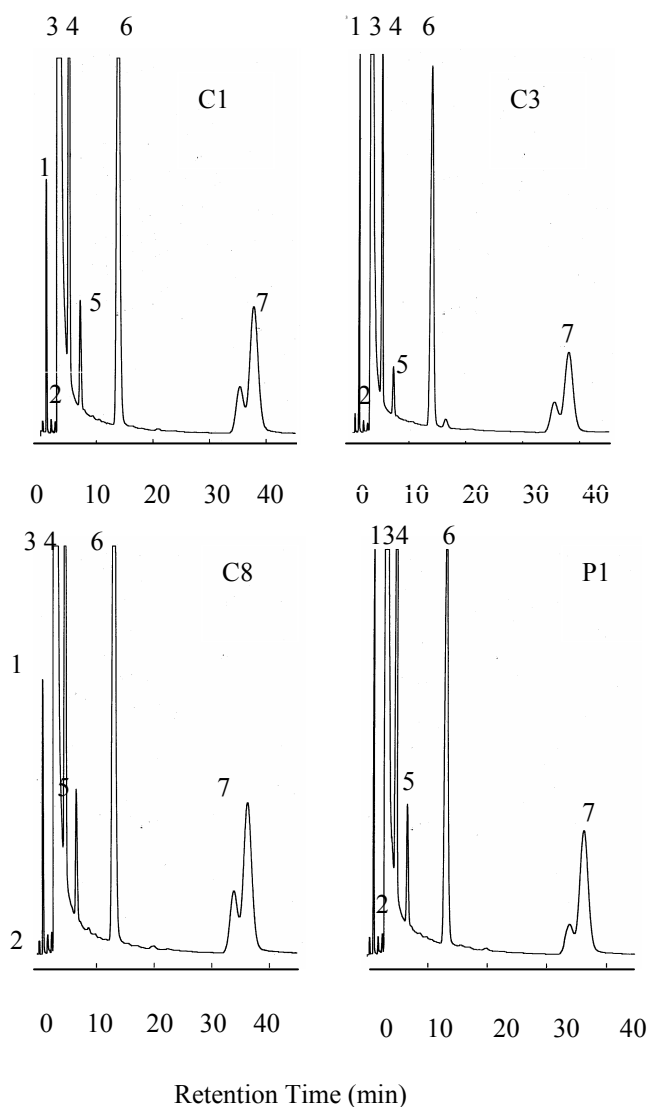


Fig. 2. Headspace GC chromatographs of rice wine prepared using yeast strains isolated from coconut (C1, C3 and C8) and palmyrah (P1) sap.

The lowest ratio between ethyl acetate to isoamyl alcohol was evident in rice wine produced using the isolate C3. Therefore, the isolate C3 can be considered better than the other three isolates in producing rice wine of good liquor quality. Moreover, pure acetaldehyde possesses a pungent irritating odor, but at dilute concentrations, it gives a pleasant fruity aroma. It is an intermediate product of alcohol fermentation and one of the

sugar metabolites widely used in artificial fruit flavors such as apple, apricot, banana and peach (Miyake and Shibamoto, 1993). As rice wine produced using the isolate C3 had the highest content of acetaldehyde (Table 3), suitability of C3 in obtaining rice wine of good liquor quality was further revealed. Furthermore, slower rate of ethanol production by the isolate C3 in comparison to the other three isolates (Fig. 1) is another advantage as rice fermentation is a batch process where fermentation is carried out slowly on a time span.

CONCLUSIONS

The ethanol content of rice wine produced using the four yeast strains isolated from coconut and palmyrah sap ranged from 11.1 to 15.2%. Ethanol, ethyl acetate, acetaldehyde, isoamyl alcohol and two other unknown compounds were the most prominent headspace volatiles of rice wine. The C3 isolate was the best, based on liquor quality and slow rate of ethanol production, among the four yeast isolates for rice fermentation.

REFERENCES

- Akiyama, H., Yoshizawa, K., Ouchi, K. (1978). Sake flavor and its improvements using metabolic mutants of yeast. pp. 229-248. *In*: Charalambous, G. (Ed). Analysis of Food and Beverages: Headspace Techniques, Academic Press, New York.
- AOAC (1995). Official Methods of Analysis, 16th edition. Vol. 2. Pp. 3. The Association of Official Analytical Chemist Inc., Arlington, USA.
- Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H. and Henschke, P.A. (2003). Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* 36: 307-314.
- Flath, R.A. and Forry, R.R. (1970). Volatile components of smooth Cayene pineapple. *J. Agric. Food Chem.* 18(2): 306-309.
- Furukawa, K., Yamada, T., Mizoguchi, H. and Hara, S. (2003). Increased alcohol acetyltransferase activity by inositol limitation in *Saccharomyces cerevisiae* in sake mash. *J. Biosci. and Bioengin.* 96(3): 382-396.
- Ichikawa, E. (2002). Isolation and Breeding of Brewing Yeast. Food Processing and Preservation Technology II, Biological Application Technology Department, Japan International Cooperation Agency.
- Khoshkhoo, N., Hedin, P.A. and McCarthy, J.C. (1994). Free sugars in root-knot nematode susceptible and resistant cotton plants roots and leaves. *J. Agric. Food Chem.* 42(2): 366-368.
- Kodama, S., Yamamoto, A., Matsunaga, A., Matsui, K., Nakagomi, K. and Hayakawa, K. (2002). Behaviour of D- and L- lactic acid during the brewing process of sake (Japanese rice wine). *J. Agric. Food Chem.* 50(4): 767-770.

- Macial, M.I., Hansen, T.J., Aldinger, S.B. and Labows, N. (1986). Flavor chemistry of cashew apple juice. *J. Agric. Food Chem.* 34(5): 927-934.
- Martinez, C., Gas, S., Lavin, A. and Ganga, M. (2004). Genomic characterization of *Saccharomyces cerevisiae* strains isolated from wine-producing areas in South America. *J. Appl. Microbiol.* 96: 1161-1164.
- Miyake, T. and Shibamoto, T. (1993). Qualitative analysis of acetaldehyde in foods and beverages. *J. Agric. Food Sci.* 41(9): 1968-1970.
- Samarajeewa, U., Adams, M.R. and Robinson, J.M. (1981). Major volatiles in Sri Lanka arrack, a palm wine distillate. *J. Food Technol.* 16: 437-444.
- Sooriamorthy, S.S., Silva, K.F.S.T., Gunawardhane, M. H. W. and Illeperuma, C. K. (2004). Isolation of indigenous *Aspergillus oryzae* for saccharification of rice wine. *Trop. Agric. Res.* 16: 121-127.
- Tabera, J., Iznola, M.A., Schnabel, I. and Garrido, J. (1985). Yeast strain screening method for a continuous process of alcoholic fermentation. *Biotechnol. Lett.* 7: 437-442.
- Teramoto, Y., Okamoto, K., Kayashima, S. and Ueda, S. (1993). Rice wine brewing with sprouting rice and barley malt. *J. Ferment. Bioeng.* 75(6): 460-462.
- Uzochukwu, S., Balogh, E., Tucknot, O.G., Lewis, M.J. and Ngoddy, P.O. (1999). Role of palm wine yeasts and bacteria in palm wine aroma. *J. Food Sci. Technol.* 36(4): 300-304.
- Uzochukwu, S.V.A. (1994). Volatile constituents of palm wine and palm sap. *J. Sci. Food Agric.* 64: 405-411.
- Wellala, C.K.D., Gunawardhane, M. H. W., Wijeratne, M. C. P. and Illeperuma, C. K. (2004). Fermentation of rice using yeast isolated from coconut and palmyrah sap. *Trop. Agric. Res.* 16: 114-120.
- Yoshizama, K. and Kishi, S. (1994). Rice in Brewing. pp. 619-645. *In*: Juliano, B.O. (Ed). *Rice Chemistry and Technology*, The American Association of Cereal Chemists, USA.