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Wine Production by Guava Piece Immobilized Yeast from Indian Cultivar Grapes and its Volatile Composition

[1]L.V.A. Reddy, [2]Y.H.K. Reddy and [1]O.V.S. Reddy
[1]Department of Biochemistry, Sri Venkateswara University, Tiruapti India 517 502
[2]Department of Microbiology, Osmania University, Hyderabad India 500 007

Abstract: Suitability of guava pieces for the preparation of immobilized yeast biocatalyst for wine production was investigated by using *Saccharomyces cerevisiae* in repeated batch fermentation. The fermentation rate and other parameters were compared with free yeast cells at different temperatures. The volatile compounds, methanol, ethyl acetate, 1-propanol, isobutanol and amyl alcohols which, formed during fermentation were analyzed with the help of GC-FID. The concentrations of ethyl acetate and methanol were not more than 100 mg L^{-1} in all cases, indicating an improvement in the product. There are no changes in cell metabolism of immobilized yeast. Preliminary sensory tests established the fruity aroma, fine taste and the overall improved quality of the produced wines.

Key words: Wine production, immobilization, repeated batch fermentation, guava pieces

INTRODUCTION

Production of wine is a very old finding because of the natural character of the product. It defined as an alcoholic beverage obtained from grape juice using yeast as a fermenting organism (Kunkee and Goswell, 1977). In the mid of 19th century application of immobilized enzymes were developed and the applications of this technique was introduced in the field of analysis and industrial production of biomolecules (Kennedy *et al*., 1976). In wine making procedure also brought many changes from its traditional methods with the progress of biotechnology (Jones *et al*., 1981). In recent years, cell immobilization techniques have become increasingly important and are being successfully applied in industrial process such as the production of alcohols (ethanol, butanol and isopropanol), organic acids (including malic, citric, lactic and gluconic acids), enzymes (cellulase, amylase, lipase and others) and biotransformation of steroids for hormone production, waste water treatment and food applications, beer and wine production (Mensour *et al*., 1997; Pilkington *et al*., 1998; Kourkoutas *et al*., 2001).

Production of wine using immobilized yeast in repeated batch fermentation and high cell density reactors have several advantages over the traditional fermentation by free cells, such as increase in productivity and reduction in cost of product. The host for yeast immobilization for wine making is always biologically inert (Silton and Gaddy, 1980). Many investigators proposed many supports for immobilization of yeast cells like alginate and other inorganic materials. In alcohol fermentations there is a wide variety of supports are used by many investigators (Mensour *et al*., 1997; Pilkinton *et al*., 1998; Divies *et al*., 1994). But in wine fermentation the immobilization in low cost is the big problem because the alginate and organic materials are proved inappropriate for wine production and they are not in use. Bardi and Koutinas (1994) have been reported the delignified cellulosic material for wine fermentation.

In this situation the fruits are the alternative for supports in wine fermentations. Previously few investigators were done good piece of work in this field they used apple pieces, grape skins and quince and pear pieces as solid supports wine production in repeated batch and continuous processes (Pilkington *et al*., 1998; Bardi and Koutinas, 1994; Mallouchos *et al*., 2002; Kourkoutas *et al*., 2003; Mallios *et al*., 2004). In the present study the suitability of guava pieces as support for wine fermentation by *Saccharomyces cerevisiae* was investigated. The biocatalyst prepared by using guava fruit was used in batch and manually packed bed reactor using a glass cylinder.

MATERIALS AND METHODS

Yeast strain: *Saccharomyces cerevisiae* CFTRI (101) culture was procured from Central Food Technology Research Institute, Mysore (CFTRI, India). The culture was maintained on MPYD agar (Maltose 0.3%, Peptone

Corresponding Author: Dr. O.V.S. Reddy, Associate Professor, Department of Biochemistry, Sri Venkateswara University, Tiruapti India 517 502

0.5%, Yeast extract 0.3% and Dextrose 1% agar 2%) and stored at -20°C. Ripe grapes were obtained at local market and used to prepare must. Good quality of guava fruits were brought from local market and used as immobilizing material.

Inoculum preparation: The inoculum was prepared by inoculating the slant culture into 10 mL test tube incubated overnight then 3% inoculum transferred to 100 mL of the sterile MPYD liquid medium taken in 250 mL flask and grown it on a rotary shaker (100 rev m^{-1}) for 48 h. The culture was harvested by centrifugation at 2000 x g for 5 min, washed twice with phosphate buffer saline (PBS; 200 mM phosphate containing 600 mM KCl, pH 6.0).

Preparation of grape must: Grapes (Banglore blue *cvl*) were procured from local market Tirupati (India). The grapes are washed with 20 ppm chlorine solution and then crushed with dejuicer. The final concentration of sugar was adjusted to 20% (w/v). The must was used without any nutrient addition or adjustments and sterilized at 121°C for 15 min before use.

Yeast cell immobilization: The method used for preparation of guava pieces biocatalyst was previously described by Kourkoutas *et al.* (2001). Two hundred gram of guava fruits are made into 2-3 cm pieces, sterilized at 121°C for 15 min. These pieces were taken into a wide mouth 500 mL conical flask then 200 mL of culture medium contain yeast cells O.D of 2 at 590 nm. pH was adjusted to 5 and leave for 8-12 h for fermentation. The fermented broth was decanted to remove the un immobilized yeast cells. The biocatalyst prepared by this method was used for the repetitive batch fermentation and also for continuous fermentation. In batch fermentation the biocatalyst was washed twice with 200 mL of grape must after every fermentation.

For the determination of yeast cell number 10 g of wet guava pieces were taken from the fermentation conical flask during fermentation. The pieces are homogenized with mechanical mortar and pestle 90 mL of ¼ strength Ringer solution (De Pina and Hogy, 1999). The cells were counted after appropriate dilution by using a Neubauer improved heamocytometre. 3×10^6 yeast cells gm^{-1} guava fruit pieces were attached.

Scanning electron microscopy: For the microscopic studies samples were transferred to vials and fixed with glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed 25 aqueous osmium tetraoxide in the same buffer for 2 h. After the post fixation the samples were dehydrated in series of graded alcohol and dried to critical point drying with Electron Microscopy Science CPD unit, then the dried samples were mounted over the stubs with double sided conductivity tape. Finally, apply a thin layer of platinum over the sample using an automated sputter coater (JEOL JFC-1600) for about 90 sec, then scanned the samples under scanning electron microscope (model: JEOL-JSM 5600) at various magnifications (De Pina and Hogg, 1999; Kourkoutas *et al*., 2003).

Fermentation: Repeated batch fermentations were carried out by 200 g of biocatalyst prepared as above were used to ferment 400 mL of grape must. The biocatalyst was kept submerged in case of attach fermentation by means of plastic netting. The fermentation was conducted at various temperatures like 15, 20, 25, 30 and 35°C. The end point of fermentation s are detected with measuring of residual sugars less than 2%.

Methods of analysis: The reducing sugar content was estimated by Shaffer and Somogyi method (Shaffer and Somogyi (1995). Ethanol and other metabolites (methanol and total esters) were determined with the help of Gas Chromatography (Antony, 1984).

Gas chromatographic method: The fermented samples were centrifuged at 5000 X g for 10 min. The supernatant was used for ethanol analysis. An agilent systems Model 6890 plus instrument was used and conditions were as follows: Carbopack-B 80/120 mesh glass column (6 ft /2 m; 2 mm ID;1/4 mm), nitrogen used as a carrier gas with a flow of 20 mL min^{-1}) and the eluted compounds were detected by Flame Ionization Detection (FID), for this the fuel gas was hydrogen with a flow rate of 40 mL min^{-1} and the oxidant was air, with a flow rate of 400 mL min^{-1}. In this n-propanol was used as internal standard (Antony, 1984).

RESULTS AND DISCUSSION

Repeated batch fermentation: The prepared biocatalyst was used in about 15 repeated batch fermentations. Every time the biocatalyst was washed and used. The yeast strain used had the advantage of alcohol tolerance. The fermentation results are summarized in (Table 1). All the fermentations were carried out using guava supported biocatalyst with same initial concentration of sugar (20% w/v). The obtained wine contained alcohol at concentrations similar to dry and table wines (9.5-12% v/v). The fermentations carried out continuously for 100 days without any significant loss of the biocatalic activity. At 10°C the fermentations were completed in 4 days while at 30°C it takes only 36 h. Both wine and

Table 1: Fermentation parameters obtained in repeated batch fermentations at different temperatures

Temp (°C)	Repeated batches	Initial Sugar (%)	Fermentation time (h)	Ethanol concentration % (v/v)	Ethanol concentration (g L^{-1})	Ethanol productivity (g L^{-1} h)	R. sugars (g L^{-1})	Free cell concentration (g L^{-1})	Total acidity (g L^{-1})	Volatile acidity (g L^{-1})
30 (C)	3	21.0	72	12.0	95.0	1.32	tr	5.3	4.1	0.06
30	1	20.2	72	11.0	87.0	1.21	tr	7.2	5.4	0.08
30	2	20.0	35	12.0	95.0	2.71	0.5	6.4	5.9	0.06
30	3	20.1	24	12.0	95.0	3.96	o.6	5.5	6.0	0.10
25 (C)	3	20.0	100	10.0	79.0	0.79	0.4	5.0	3.8	0.09
25	4	20.2	70	10.0	79.0	2.63	0.5	6.2	5.2	0.11
25	5	20.5	55	10.5	83.0	3.32	1.0	5.0	5.6	0.12
25	6	20.0	48	10.2	81.0	2.31	0.2	5.5	4.9	0.15
20 (C)	3	20.0	120	9.5	75.0	0.63	1.2	4.3	4.1	0.15
20	7	20.3	83	10.5	83.0	1.51	0.4	7.0	4.3	0.19
20	8	20.1	65	10.0	79.0	1.58	0.3	6.0	5.5	0.15
20	9	20.6	42	10.4	82.5	1.96	0.8	7.5	4.7	0.16
15 (C)	3	20.2	180	9.3	77.4	0.52	1.5	4.2	3.2	0.20
15	10	20.4	120	11.2	88.5	1.77	tr	4.5	4.0	0.20
15	11	20.2	90	10.9	86.2	1.57	tr	5.0	5.0	0.18
15	12	20.0	96	10.0	85.0	1.89	0.4	4.2	4.2	0.15

C = Control with free yeast cells

ethanol productivities were some fold higher than in traditional fermentations and were significantly affected by temperature ($p>0.05$), at low temperatures (15°C), an improvement of fermentation time and productivity was observed. This may probably due to adaptation of the immobilized cells (Table 1).

Wine and ethanol productivity was slightly reduced after first three-repeated batch fermentations. This may be due to difficulty in nutrient transfer, since a decrease in the guava biocatalyst was observed and, therefore, yeast cells were not uniformly spread throughout the whole mass of must. Therefore, the first and second batches were carried out with 300 and 250 mL, respectively and the subsequent batches with 200 mL. This decrease was probably due to the utilization of the guava sugar by the yeast cells. The guava pieces volume remained stable after seventh or eighth batch. There is no decrease in volume until the end of fermentations, which was mainly due to the residual cellulosic content of guava, an unfermentable constituent (Fig. 1). Although there was significant loss of guava volume and the possible transfer of guava constituents to the wines, pretreatment was not required. Because, most guava constituents, glucose, fructose, organic acids, phenolic compounds and others which are generally present in red wine. This may also contribute to the flavour and distinctive aroma of the product and therefore it might be useful. The viability of yeast cells was high in immobilized cells. Since the cell number in the immobilized cells increased as a result of yeast cells are detached from support and grew in the medium solution, which initially no yeast cells were present in the media (Jouenne *et al*., 1992). The appearance of yeast cells in the medium occurs after 12 h of fermentation. Free cell biomass concentrations for the duration of the experiment at all temperatures ranged

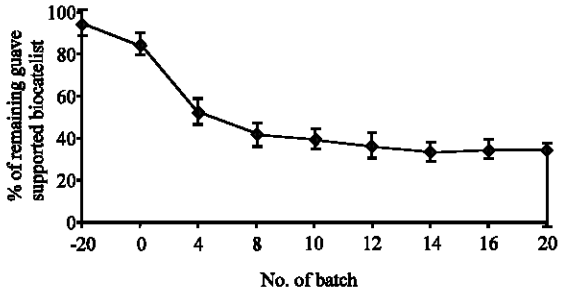

Fig. 1: Reduction in guava volume as function of batch number

from 0.3 to 12.8 g L^{-1}. But the cell mass in the immobilized pieces are maintained constantly. It may be due to the new cells were absorbed by the support (Kourkoutas *et al*., 2003). Both the total and the volatile acidities of produced wine was similar to table and dry wines.

Electron microscopic examination of the yeast immobilized on guava pieces showed the proliferation of the yeast cells within the biocatalyst tissue structure (Fig. 2a and b). Immobilization of the yeast on the support may have occurred as a result of hydrogen bonding, entrapment of the cells in guava pieces and also the van der Waals forces. Similar results have been observed in apple pieces yeast biocatalyst (Kourkoutas *et al*., 2001).

Variation in fermentation performance: The rate of fermentation was increased in low temperature (15°C) and this in turn is decreased the time that required for fermentation. The sugars were completely converted to ethanol (2 g L^{-1}). Biocatalyst was produced higher amount of ethanol and glycerol than free cells. Glycerol may be high in immobilized cell medium due to the

Fig. 2a: Scanning electron microscopic image of inside the guava piece

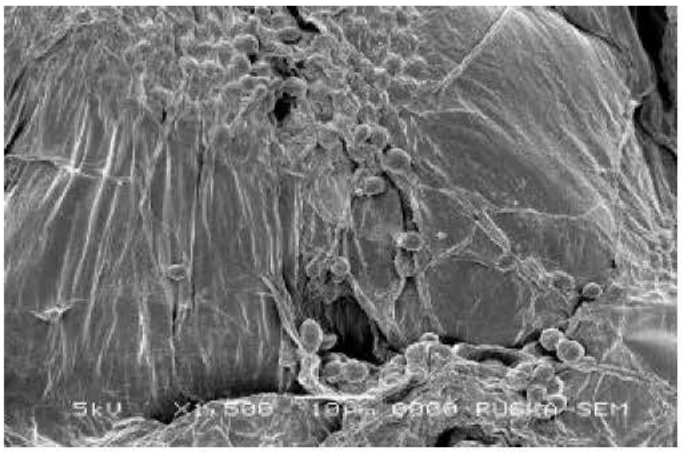

Fig. 2b: Scanning electron microscopic image of guava piece surface

immobilized cells subjected some stress in addition to this the biocatalyst gave low percent of sugars to medium. This may also contribute for formation of high amount of ethanol by biocatalyst.

Volatile by-products: Formation of high alcohols is decreased with the temperature decrease. The propanol and isobutanol were significantly decreased with temperature decrease (Table 2). The product formed with low concentrations of higher alcohols is good in quality (Kourkoutas *et al*., 2001). The guava fruit biocatalyst results are comparable and confirmed the results of previous reports such as immobilization on apple, quince fruit and delignified cellulose materials.

Ethyl acetate was analyzed due to its effect on the organoleptic characteristics of the wine. Concentrations up to 88 mg L^{-1} were detected but in most cases it was low (Table 2). Ethyl acetate concentration was increased with temperature decrease. There is no vinegar odour in final product with high concentrations of ethyl acetate. It contributes to increase the wine flavour and taste.

The acetaldehyde concentration in wines usually ranges from 13 to 40 mg L^{-1} but reaches 75 mg L^{-1} (Kourkoutas *et al*., 2003). In present studies it ranges from 12 to 50 mg L^{-1} (Table 2). Methanol, which derived from methylated pectic substances (pectines) by the action of pectic esterases, was from 40 to 90 mg L^{-1}, which is lower than the usual range in wines (100-200 mg L^{-1}). This was high in first 3 to 4 repeated batch fermentations because of pectines from guava pieces. The low concentrations of methanol is considered advantageous, as this compound is undesirable.

Table 2: Effect of temperature on the production of volatile (mg L^{-1}) in the fermentation of grape must using immobilized yeast in guava pieces biocatalyst#

Temp(°C)	Batch	Acetaldehyde	Ethyl acetate	1-propanol	Isobutanol	Amyl alcohols	Methanol
30	1	18	40	16	65	220	90
30	2	25	52	13	63	221	53
30	3	50	55	19	58	210	65
25	4	12	53	17	55	195	47
25	5	20	60	14	60	180	51
25	6	22	58	20	49	190	54
20	7	27	48	22	45	173	52
20	8	19	65	18	42	150	46
20	9	24	62	23	37	155	41
15	10	28	70	25	50	166	60
15	11	30	77	30	48	140	62
15	12	25	88	28	53	120	70
30 (C)	3	15	23	28	50	200	74
25 (C)	3	23	31	23	42	167	61
20 (C)	3	20	45	25	35	130	55
15 (C)	3	32	40	15	38	103	48

= Values presented in the table are mean of five experiments. C = Control with free yeast cells

Table 3: Sensory evaluation results of mango wine obtained by the free and guava immobilized yeast cells

Test	Wine from free cells	Immobilized on guava pieces
Aroma	7.2±0.84	7.8±0.58
Taste	5.8±1.04	7.1±0.63

Continuous fermentation: Continuous fermentation was carried out to investigate the operational stability and suitability of the immobilized yeast strain 101 on guava pieces in this present work. The biocatalyst, which was optimized and adapted to low temperatures (15°C) used for the continuous fermentation. The yeast was bound on both surface and with in the pieces. Continuous fermentation was carried out for 40 days. The system was operated at both 15 and 20°C. The guava-supported biocatalyst was stable in both mechanically and physically during fermentation.

Sensory evaluation: After the adjustments made to the basic wine, sensory tests were conducted. Ten well-experienced panelists from previous wine experiments. Tests were conducted in morning times only. The panelists were instructed to record each judgement by ticking 0-10 grades (0 not acceptable and 10 excellent). The results were compared with local commercial wine and free cell wine 9 control). The preliminary taste investigation characterized the new wine as a novel, special type of wine, with a pleasant, soft aroma and fruity taste (Table 3).

CONCLUSIONS

With the results obtained in the present study it can concluded that the mixed effects of temperature and immobilization produces wines with better ratio of esters to alcohols which finally gives more fruity character to the produced wines. Guava immobilized yeast biocatalyst contributes as a good and effective support for alcoholic fermentation both at low and room temperatures. They are very cheap, readily available of food grade and there is no need to special pretreatment before their use. The immobilization on guava pieces not negatively affected the fermentation. The immobilized biocatalyst shows better stability and increases the fermentation rate, particularly at low temperatures, which makes its use possible in industries.

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