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Fermentative Behavior of Saccharomyces Strains During Guava (*Psidium Guajava* L) Must Fermentation and Optimization of Guava Wine Production

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Abstract

Two different strains of *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 were evaluated in the production of guava fruit wine. Guava must concentrations were adjusted to 22°Brix with sucrose solution, and batch fermentations were performed. For optimization of guava wine fermentation various parameters, such as the osmotolerance, alcohol tolerance, inoculum size, Initial pH of the medium, amount of SO_2, amount of diammonium phosphate and Incubation temperature were studied for both the strains. For guava wine production *Saccharomyces cerevisiae* NCIM 3095 gave much better results as compare to *Saccharomyces cerevisiae* NCIM 3287.

Keywords: Guava must; *Saccharomyces cerevisiae* NCIM 3095 & 3287; Brix; Ethanol

Introduction

Guava *(Psidium guajava L.)* is one of the most important commercial fruit crops in India consumed locally. It is a good source of ascorbic acid, pectin, sugars and certain minerals. Its skin and flesh colours vary from variety to variety depending on the amount and type of pigments. Tropical Fruit juices have become important in recent years due to the overall increase in natural fruit juice consumptions as an alternative to the traditional caffeine-containing beverages such as coffee, tea or carbonated soft drink [1,2]. Guava, with its widely appreciated flavor and aroma, is able to compete in the market, either as guava juice or as mixtures with other juices or guava wine. However, raw guava juice is turbid, gray in color, very viscous and tends to settle during storage, and therefore, it must be clarified prior to commercialization [3]. The extraction of fruit juice with help of the pectinase enzyme and optimization is studied for apple [4], banana [5], guava [6] and pineapple fruits [7] by this it is easy to extract the maximum juice and then directly going for storage or wine production.

Grapes and apples have been widely applied to ferment beverages [8] the use of other fruits, such as orange [9] cacao [10], mango [11], gabiroba [12], cajá [13], kiwi [14],and in the production of wine has been recently demonstrated. Generally, fruits contain quantities of sugar that can be used by yeast during the fermentation process. In addition to the inherent characteristics of fruit (pH values, sugar contents and nitrogen contents), other factors must be taken into account during fruit wine production. The initial sugar concentrations, fermentation temperatures, SO_2 concentrations and specific yeast strains are key factors in determining successful fermentative processes of fruit wine [15,16].

Since the beginning of the 1980s, the use of *Saccharomyces cerevisiae* yeast starters has been extensively applied in the industrial and homemade beverage production processes. Currently, most of the wine production processes rely on *S. cerevisiae* strains that allow rapid and reliable fermentations, reduce the risk of sluggish or stuck fermentations and prevent microbial contaminations [17]. Yeast starter cultures that are specifically selected for the winemaking process on the basis of scientifically verified characteristics typically complement and optimise the raw material quality and individual characteristics of the wine, creating a more desirable product [18]. Generally, wines produced with selected yeasts have a higher quality than wines produced by spontaneous fermentation [19].

In modern winemaking, specific yeast strains have been preferentially used to guarantee the desired quality of the product. Yeasts are the prominent organisms involved in wine production and determine several characteristics of the wine, including the flavour, by a range of mechanisms and activities (Fleet, 2003).

Guava (*Psidium guajava* L.) wine is the product of anaerobic fermentation by yeast in which the sugars are converted into alcohol & carbon dioxide. Ethanol production from guava pulp is reported [20] still there is no study found in literature for guava must fermentation for guava fruit wine production. Therefore, the aim of the present study was to optimization of the fermentation parameter for guava wine production and comparison of the two strains for guava fruit wine production.

Materials and Methods

Raw material

Guava fruits were purchased from local market. The fruits were washed with fresh water. Guava was crushed completely to make guava pulp and guava pulp was treated with pectinase enzyme to extract the juice.

Chemicals

Citric acid, tartaric acid, Diammonium phosphate, Sucrose, Dextrose, Potassium metabisulfite, Sulfuric acid, Peptone, yeast extract were procured from Merck India Ltd Mumbai.

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Culture

Saccharomyces cerevisiae NCIM 3095 and NCIM 3287 were obtained from Institute of Microbial Technology, Chandigarh. The inoculum was prepared by inoculating loopful culture into 250 ml conical flask containing medium broth (100 ml) under sterile conditions. The flasks were kept on shaker (180 rpm) at room temperature for 48 hours. Then culture was isolated from the media by centrifugation at 4000 rpm for 20 min. After that the culture was washed with saline and mixed with 100 ml sterile saline solution. This solution was used as the standard stock inoculum for experimental work. Optical density was measured at 660 nm and the number of cells was counted with help of haemocytometer.

Medium

Culture medium recommended by IMTECH for *Saccharomyces cerevisiae* was as follows; Yeast extract 3 g/L, peptone 20 g/L and Dextrose 20 g/L. The pH of media was adjusted to 6.5 prior to sterilization. Sterilization was done by autoclaving at 121°C and 15 psi pressure. For the production yeast biomass, the flasks containing media were inoculated with a loop of yeast from the slants and kept on orbital shaker at 180 rpm at 25°C temperature.

Hand refractometer

Hand refractometer of range 0 to 32 was used to determine the °Brix of the juice and wine.

Apparatus for anaerobic fermentation

For anaerobic fermentation long neck conical flasks were used. The conical flask was closed using a rubber cork pierced with a glass tube. End of the glass tube was fitted with rubber tubing, the other end of which was immersed in water facilitating unidirectional flow of CO_2 and thus maintaining the perfect anaerobic condition.

Determination of ethanol

Ethanol in a dilute sample can be separated from other wine components by Gas Chromatography. To improve quantification, 2-propanol (used as internal standard) solution was used to quantitatively dilute the sample. The peak area ratio for the two chromatographic peaks is compared with the area ratio obtained from injection of standard ethanol-internal standard mixture [21].

Result and Discussion

Osmotolerance studies

10 flasks were taken containing about 100ml of growth medium.

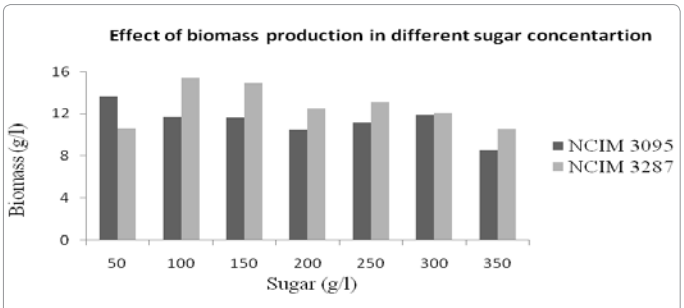

Figure1: In osmotolerance study experiment effect of sugar concentrations on biomass production of Saccharomyces cerevisiae NCIM 3095 and NCIM 3287 in osmotolerance tolerance studies.

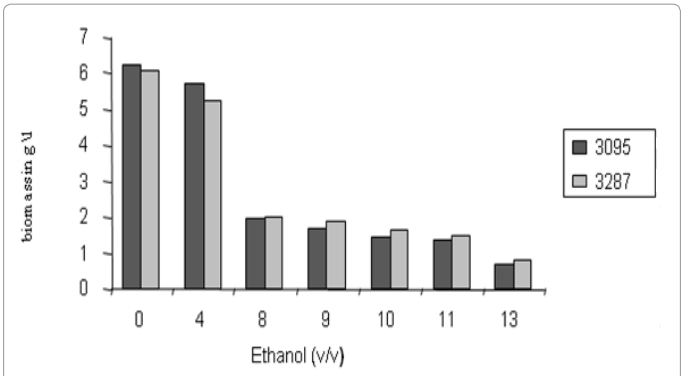

Figure 2: In Alcohol tolerance study experiment effect of ethanol concentrations on biomass production of *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 in alcohol tolerance studies.

All the flasks were inoculated with a loop of *saccharomyces cerevisiae* NCIM 3095 strain and shaken on a shaker at 180 rpm. Absorbance of all the flasks were regularly noted at 660 nm. After the lapse of lag phase, i.e. 6 hrs, each flask was added with different concentration of sucrose. A control contained 0% of sucrose. In other flasks, sucrose was added in the range 12% to 28% (w/v), the addition of sucrose was varied in the increment of 2%. Similar experiments were carried out for the other yeast strain, *saccharomyces cerevisiae* NCIM 3287. The variations in absorbance for all the flasks were continuously monitored. Figure 1 showing the osmotolerance study for the strain NCIM 3095.Higher the initial sugar concentration is not good for yeast because of higher osmotolerance.

As shown in Figure 1 osmotolerance study for NCIM 3287, both the strains showed sugar tolerance level in the range of 20 to 22% (w/v). Higher sugar concentrations inhibited the growth of yeasts, which may be accounted for due to effects of high osmotic pressure on yeast cells. Even at higher sugar concentration, growth to a limited extent was observed for both the strains. So on the basis of osmotolerance studies 22°Brix is decided for guava must concentration for fermentation.

Alcohol tolerance studies

Typical growth curves for the yeast strains, *S. cerevisiae* NCIM 3095 and S. cerevisiae NCIM 3287, showed that the lag phase consisted of about 5 Hrs. 10 flasks were taken containing about 100 ml of growth medium. All the flasks were inoculated with a loop of *S. cerevisiae* NCIM 3095 strain and shaken on a shaker at180 rpm. Absorbance of all the flasks were regularly noted at 660 nm. After the lapse of lag phase, i.e. 6 hrs, each flask was added with different concentration of alcohol. A control contained 0% of alcohol. In other flasks, ethanol was added in the range 0% to 13% (v/v), the addition of ethanol was varied in the increment of 2%. The total volume after addition of alcohol was 100 ml for all of the flasks. Similar experiments were carried out for the other yeast strain, *S. cerevisiae* NCIM 3287. The variation in absorbance for all the flasks were continuously monitored. After 48 hours the total biomass (dry cell wt) productions in different alcohol concentration calculated.

Figure 2 shows the production of biomass (g/l) for *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 subjected to different concentration of ethanol ranging from 0 to 13% (v/v). The ethanol was added after the lapse of lag phase, i.e., 6 hrs. It can be seen from the Figure 1 that biomass production is higher in the alcohol concentration from 0 to 4% (v/v) it mean both the strains are growing very well in the media, alcohol up to 4 % not have much effect on yeast growth. When initial alcohol concentration is increased up to 8 % (v/v) than

reduction in biomass production around 200%, for both the strain but still growth is there Both the strain showing good alcohol tolerance up to 6 %. Figure 2 shows that for higher alcohol concentration strain NCIM 3287 showing better tolerance than NCIM 3095.When concentration of alcohol was increased to 6% and beyond, yeast growth was drastically inhibited. But growth, though inhibited, was observed for alcohol concentrations up to the levels as high as 18%. The alcohol inhibition is a classical example for product inhibition during fermentation. Ethanol at higher concentration has a denaturing affect on proteins, and it affects the enzyme activity, for example on glycolytic enzymes, membrane transportation systems etc.

Optimization of percent inoculum

Six different concentrations of inoculum, 2%, 4%, 8%, 12%, 16% and 20% (v/v) of the standard stock inoculum was added in the juice and anaerobic fermentation was carried out. TSS was checked daily as °Brix. Before inoculation each flask containing the juice was added with additives like Diammonium phosphate (DAP), Sucrose, Potassium metabisulfite (KMS). DAP was added to pasteurized juice as a nitrogen and phosphorous source. The juice was adjusted to Brix of 22° using sucrose. pH level was adjusted to pH 3.5 by adding citric acid. For SO_2, KMS equivalent to 100 ppm of SO_2 was added. 173.4 ppm of KMS is equivalent to 100 ppm of SO_2. The flasks were shaken intermittently to evolve dissolved CO_2 thus facilitating the fermentation. After fermentation alcohol analysis was done for each of the flask. Figure 3(a) depicts the effect of different levels of standard stock inoculum of *Saccharomyces cerevisiae* NCIM 3287 on reduction of TSS. This shows the variation in TSS % during fermentation period and it also shows

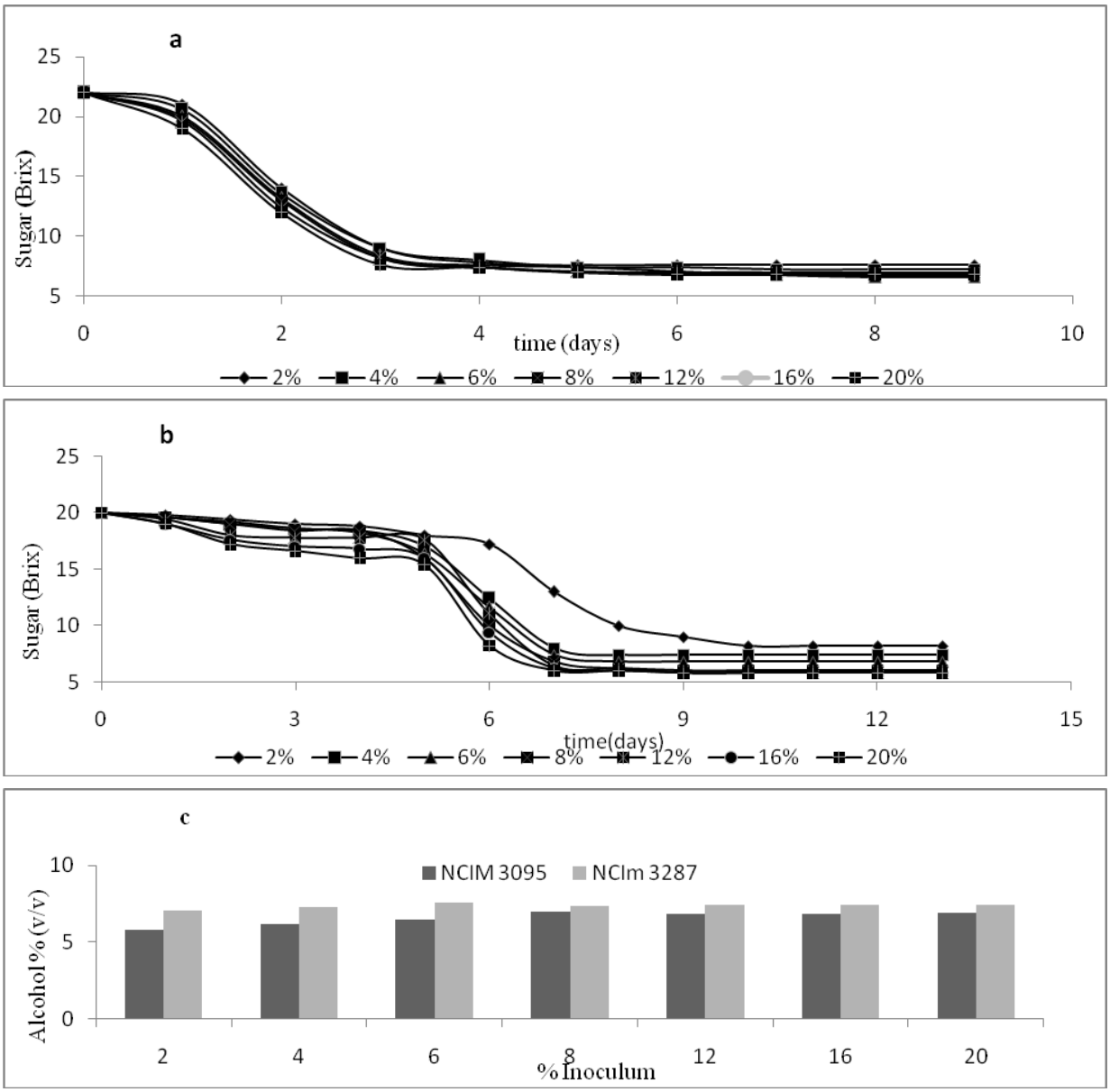

Figure 3: Decrease in sugar level (TSS) with fermentation time for guava wine production at different inoculum percentage for fermentation with (a) *Saccharomyces cerevisiae* NCIM 3095 and (b) *Saccharomyces cerevisiae* NCIM 3287. Comparison of alcohol productions by varying inoculum percentage of (c) Saccharomyces cerevisiae NCIM 3095 and NCIM 3287in guava wine production.

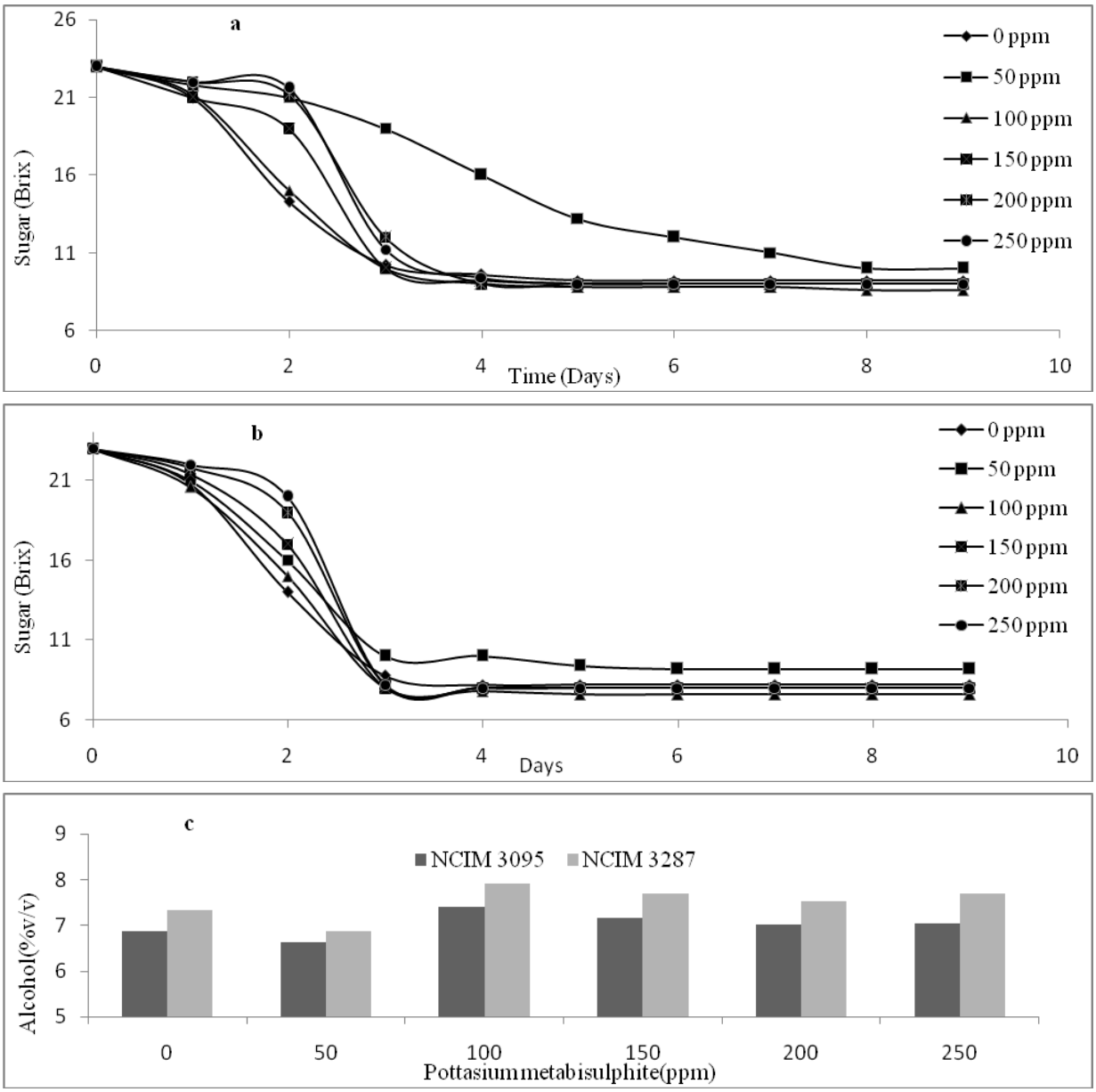

Figure 4: Decrease in sugar level (TSS) with fermentation time for guava wine production at different concentration of SO_2 for fermentation with (a)*Saccharomyces cerevisiae* NCIM 3095 and (b) *Saccharomyces cerevisiae* NCIM 3287.Comparison of alcohol production with varying ppm of potassium metabisulphate for(c) *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 for guava wine.

how the different inoculum sizes affect on the rate of fermentation. As it can be seen from the figure, higher the inoculum size higher will be the initial fermentation rate. At 2% inoculation it was slowest and decrease in sugar level is from 22% to 14.0 after initial two days of fermentation. At 20% inoculum it was fastest indicating drastic decrease in sugar concentration from 22% to 12% after initial two days.

Experiments with higher inoculum size rapidly reached the completion of fermentation and at the later stage of fermentation the decrease in TSS were slower and it was more or less equal in all the cases. Figure 3(b) depicts the effect of different levels of standard stock inoculum of *Saccharomyces cerevisiae* NCIM 3095 on reduction of TSS. Alcohol production increased with increasing in inoculum size up to 8% (v/v). Higher levels of inoculum gave almost same amount of alcohol content, such as 8% inoculation gave 7.05% of alcohol content, while 20% inoculum concentration gave 7.04% alcohol. From this it can be concluded that as the concentration of yeast inoculum is increased, yeast converted more sugars to alcohol, while at higher concentration yeast was not able to utilize more sugar for conversion. The alcohol yield was increased with increase in inoculum concentration up to 6 % for NCIM 3287 and up o 8 % for NCIM 3095. From these results it can be concluded that the optimum level of inoculation for *Saccharomyces cerevisiae* NCIM 3287 is 6 % and 8 % for NCIM 3095 for guava wine production. Figure 3(c) shows comparison of alcohol production from two different strains of yeasts at different levels of inoculum concentration. The graph shows that the maximum alcohol 7.56 (% v/v) produced by NCIM 3287 compare to 7.05 (%v/v) by NCIM 3095.

Optimization of SO_2

Five different levels of SO_2 concentration were studied namely, 0, 50, 100, 150, 200, 250 ppm and 300 ppm of SO_2. SO_2 was added in the form of Potassium metabisulfite (KMS). 100 ppm of potassium metabisulfite is equivalent to 173.4 ppm of KMS. Figure 4(a) reduction in sugar content with variation in time at different added SO_2 levels for *Saccharomyces cerevisiae* NCIM 3095. SO_2 added in the form of KMS acted as an antimicrobial agent. A control experiment with 0 ppm of SO_2 is also shown in the figure. As SO_2 level was increased, it can be seen from the graph that the curve for 50 ppm is coinciding with the control (0 ppm) indicating that 50 ppm SO_2 was less significant as an antimicrobial agent.

Higher fermentation rates were observed at lower doses of SO_2 and vice versa. With increase in SO_2 levels growth of the yeast was inhibited and hence slower rates of fermentation were observed at higher levels of SO_2. 100 ppm of KMS is giving higher reduction in TSS for a constant rate of fermentation. Figure 4(b) show reduction in sugar content with variation in time at different added SO_2 levels for *Saccharomyces cerevisiae* NCIM 3287. As the SO_2 level increase initial fermentation rate is decreases. 100 ppm of KMS level showing the maximum reduction in TSS for guava wine production by NCIM 3287. At higher amount of KMS also affects the growth of yeast itself. Figure 4 (c) showing the comparative ethanol production by both the strain different level of KMS. *Saccharomyces cerevisiae* NCIM 3287 showing the maximum alcohol production up to 7.91 %(v/v) at 100

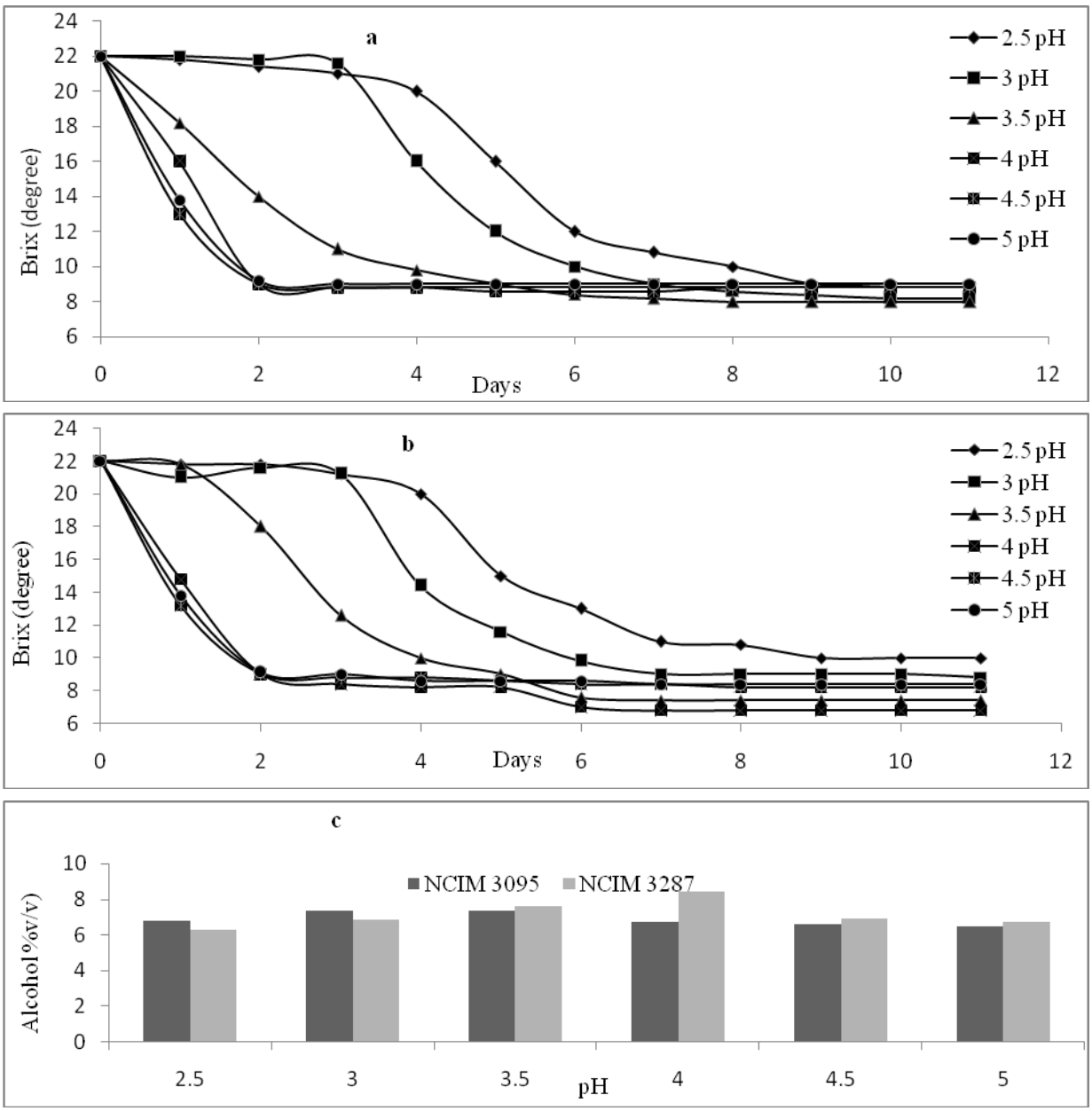

Figure 5: Decrease in sugar level (TSS) with fermentation time for guava wine production at different pH levels of fermentation for (a) *Saccharomyces cerevisiae* NCIM 3095 and (b) *Saccharomyces cerevisiae* NCIM 3287. Comparison of alcohol production with varying pH for(c) *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 for guava wine production.

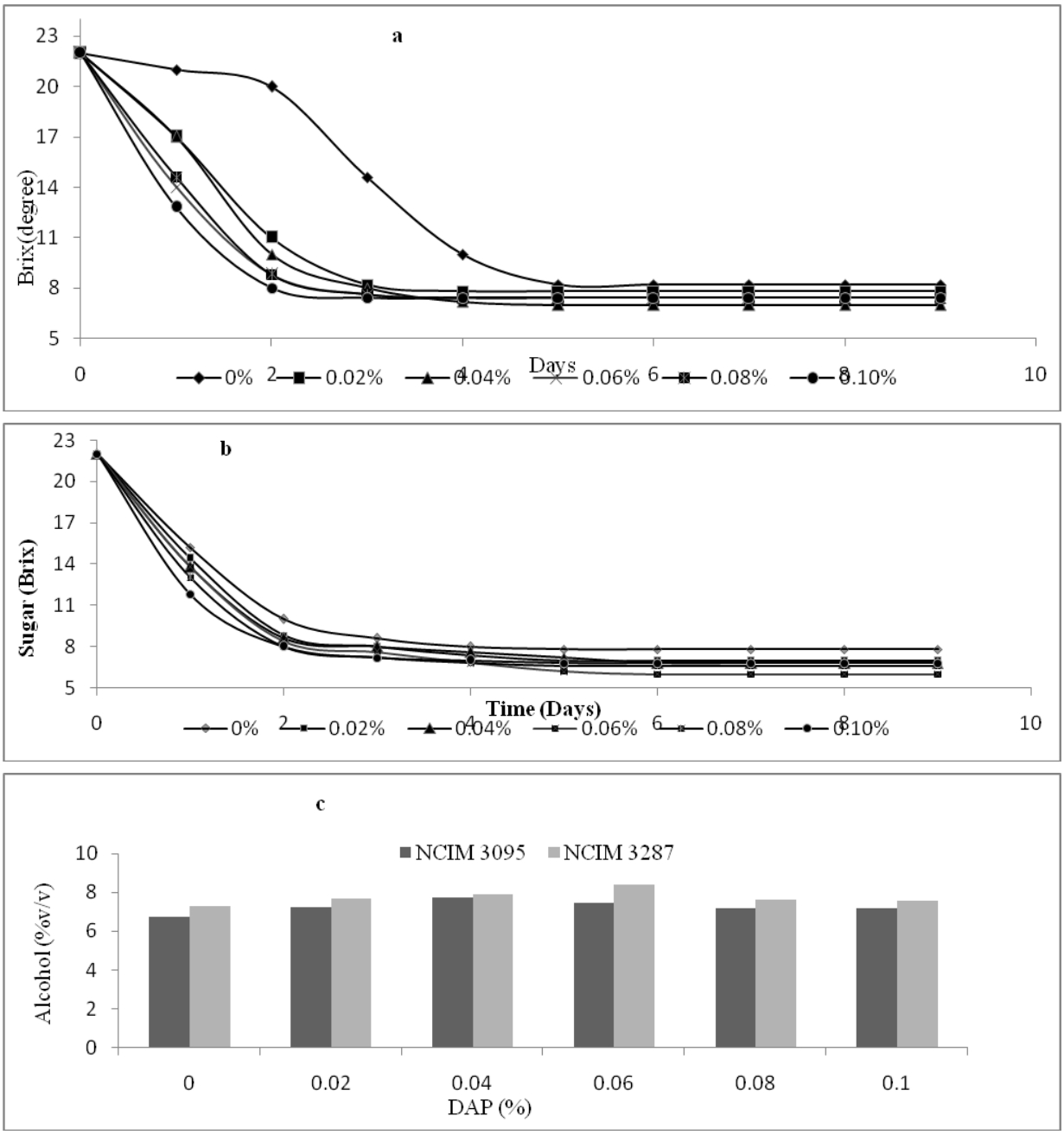

Figure 6: Decrease in sugar level (TSS) with fermentation time for guava wine production at different concentration of DAP for fermentation with (a) *Saccharomyces cerevisiae* NCIM 3095 and (b) *Saccharomyces cerevisiae* NCIM 3287.Comparison of alcohol production with varying DAP(%) for (c) *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 for guava wine production.

ppm of potassium metabisulfite. *Saccharomyces cerevisiae* NCIM 3095 produce s maximum 7.40 % (v/v) alcohol at 100 ppm of potassium metabisulfite.

Optimization of pH

Six different levels of pH were studied namely, pH 2.5,3.0, 3.5, 4.0, 4.5 and 5.0. pH was adjusted with citric acid. Figure 5(a) indicates the decrease in sugar level with time for wine fermentation at different initial pH using *Saccharomyces cerevisiae* NCIM 3095. It was observed that with increase in pH form 2.5 to 5.0 there was gradual increase in rate of fermentation. At pH 2.5, decrease in sugar level with time was slower than that of other higher pH. But at pH 4.0and pH 5.0, fermentation was almost similar. Higher rate of decrease in TSS at higher pH might be an indication that there could be activity of other microbes, as at higher pH, added SO_2 became less significant to prevent the growth of wild strains. For fruit wine production pH is also affects final texture & aroma of wine. At pH 3.5 showing the maximum conversion of sugar (TSS) to alcohol compare to others. Figure 5(b) indicates the decrease in sugar level with time for guava wine fermentation at different initial pH using *Saccharomyces cerevisiae* NCIM 3287. Here for lower pH initial fermentation rates are very slow and for higher pH like 4.5 and 5

initial fermentation rates are very higher after some time fermentation is almost stop in that. At pH 4 is given the maximum conversion of sugar level for NCIM 3287. Figure 5(c) shows the comparison of alcohol production by two different strains at different pH. For NCIM 3096 the maximum ethanol production is 7.32 % (v/v) at pH 3.5. For NCIM 3287 the maximum ethanol production is 8.43 % (v/v) at pH 4. So the strain NCIM 3287 gave better results than NCIM 3095.

Optimization of DAP (Diammonium phosphate)

Five different levels of SO_2 concentration were studied namely, 0, 0.02%, 0.04%, 0.06%, 0.08%, 0.10%(w/w) of DAP.

Figure 6 (a) shows the effect of addition of Diammonium phosphate (DAP) on reduction in sugar level with time using *Saccharomyces cerevisiae* NCIM 3095.DAP supplies nitrogen as well as phosphate to the yeast, which are necessary for the growth. The figure indicates that the fermentation was faster at DAP concentration of 0.04%. Any concentrations above or below this value showed decreased fermentation rates, implying that 0.04% DAP was optimum for growth whereas higher concentrations resulted in inhibition of growth and lower concentrations caused deficit of nitrogen and phosphate sources. Figure 6(b) shows the effect of addition of diammonium phosphate on reduction of sugar level with time using *saccharomyces cerevisiae* NCIM 3287. This shows the as the value of DAP is increases the rate

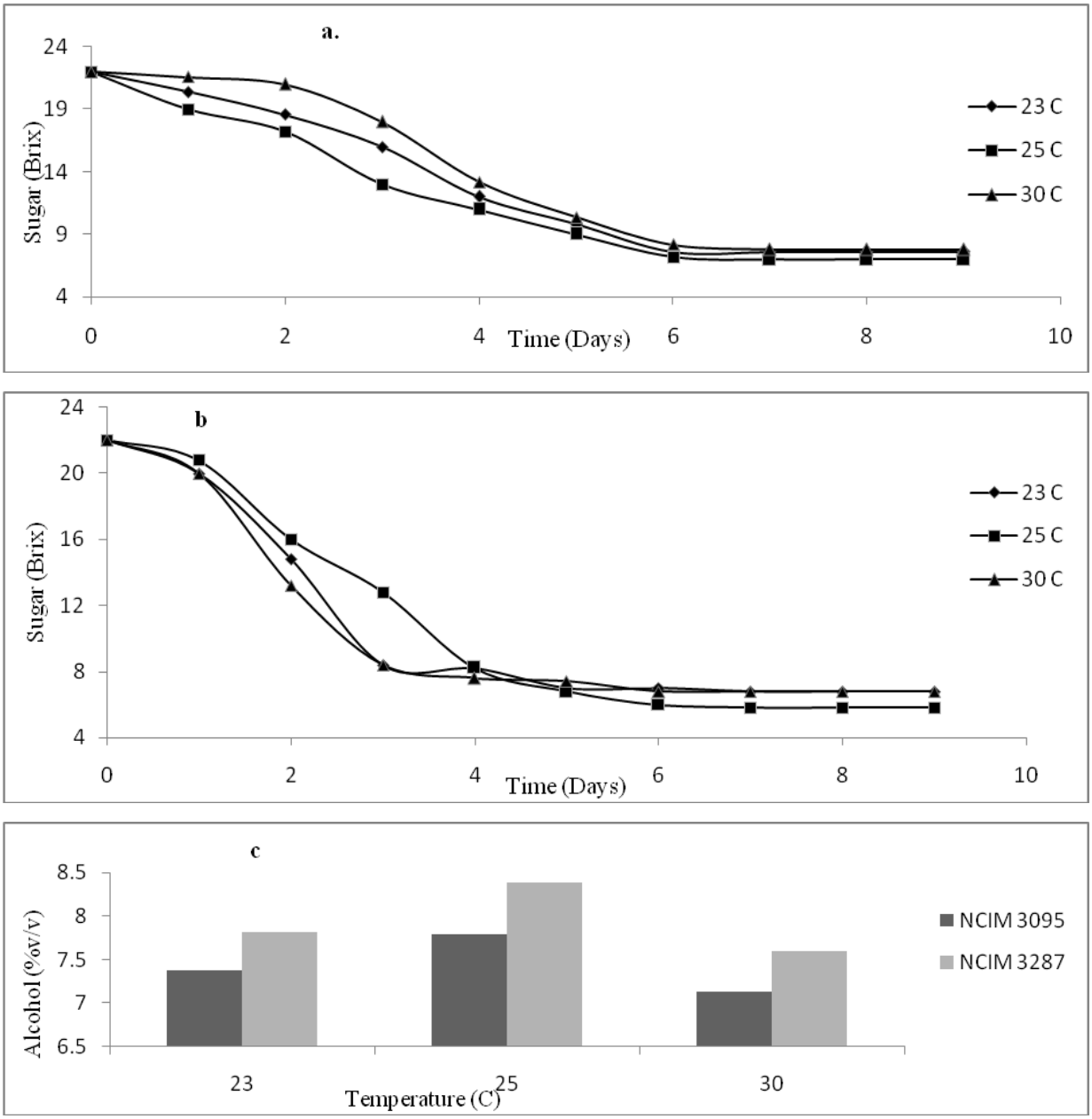

Figure 7: Decrease in sugar level (TSS) with fermentation time for guava wine production at different temperatures of fermentation of (a) *Saccharomyces cerevisiae* NCIM 3095 and (b) NCIM 3287.Comparison of alcohol production in guava wine production with varying temperature for(c) *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287.

of fermentation is increases in the starting 3 days, after at higher value fermentation is almost stops. At DAP 0.06 % is given the maximum conversion of sugar level for NCIM 3287. Figure 6(c) shows the comparison of alcohol produced at different concentration of DAP (%) for both the strains NCIM 3095 and NCIM 3287. For NCIM 3095 the maximum ethanol production is 7.74 % (v/v) at 0.04 % DAP. For NCIM 3287 the maximum ethanol production is 8.36 % (v/v) at 0.06% DAP.

Optimization of temperature

Three different levels of temperature were studied. Those are 23°C, 25°C and 30°C. The flasks were kept in temperature-controlled incubator and constantly monitored for sugar levels.

Data in graph in Figure 7(a) show the effect of changing temperature on fermentation rate and reduction in TSS and hence the fermentation rates for *Saccharomyces cerevisiae* NCIM 3095 in guava wine production. As the temperature increase initial fermentation rates are increased due to temperature that increased the enzyme activity of the metabolic pathway. At the same time higher temperatures have negative effect on stability of enzymes or any other biomolecules and decrease the enzyme activity. So after fourth days fermentation rates are lower at higher temperatures. So here at 25°C give the higher alcohol yield as well fermentation rate is also almost constant in all the way of process. For higher stability of fruit wines during aging is also lower temperature is preferred. Figure 7(b) showing the effect of temperature on fermentation rate and reduction in TSS in guava wine production by *Saccharomyces cerevisiae* NCIM 3287. This figure shows that at 25°C the maximum conversion of sugar comparative other temperature. Figure 7(c) shows the comparison of alcohol produced at different temperature for both the strains NCIM 3095 and NCIM 3287. For NCIM 3095 the maximum ethanol production is 7.784 % (v/v) at 25°C. For NCIM 3287 the maximum ethanol production is 8.396 % (v/v) at 25°C.

Phenolic compounds and fusel oils in guava fruit wines

Table 1 showing variation of wine intensity, hue, total anthocyanins and chemical ageing factor with different fermentation pH for *Saccharomyces cerevisiae* NCIM 3095 for guava wine production. Wine intensity is calculated by taking the absorbance at 420 and 520 nm and than sum of these, it determine the total color of wine.

pH	Wine Intensity	Wine hue	total antocyanins (mg\l)	Chemical age factor
2.5	0.83	4.57	86.600	4.723404
3	0.771	5.477	44.733	6.32
3.5	0.635	6.955	18.933	16.30769
4	0.819	7.7127	109.000	2.631579
4.5	0.728	8.838	14.200	21.27273
5	0.78	5.341	85.700	2.866667

Table 1: Variation of wine intensity, hue, total anthocyanins and chemical ageing factor with different fermentation pH for *Saccharomyces cerevisiae* NCIM 3095 for guava wine production.

pH	Wine Intensity	Wine hue	total antocyanins (mg\l)	Chemical age factor
2.5	0.764	7.907	15.167	14.5
3	0.815	3.88	105.900	4.263158
3.5	0.732	7.318	69.333	24.13793
4	1.104	7	4.400	45.33333
4.5	0.765	8	59.133	4.5625
5	0.87	5.084	139.100	2.835616

Table 2: Variation of wine intensity, hue, total anthocyanins and chemical ageing factor with different fermentation pH for *Saccharomyces cerevisiae* NCIM 3287 for guava wine production.

Wine hue is showing the stability of wine color. Table 2 shows the variation of wine intensity, hue, total anthocyanins and chemical age factor with different pH for NCIM 3287 for guava wine production. By these tables variation in chemical age factor is there at lower pH and higher pH.

Degree of polymerization of anthocyanins and tannins is used to determine the chemical age factor. Chemical age factor is degree to which polymeric pigment forms have replaced monomeric pigment forms. For NCIM 3095 gives maximum chemical age factor at pH 3.5 and 4.5, since the optimum pH is 3.5 for this strain so at this have good chemical age factor that is good for wine stability and flavour.

NCIM 3287 have maximum chemical age factor at pH 4 and also the optimum pH for guava wine production for this strain. Both tables show the variation in anthocyanins level for different pH. Qualitatively analysis for alcohols and fusel oils in guava wine shows the presence of acetaldehyde, methanol, ethyl acetate, ethanol, n-propanol, isobutyl alcohol, n-butanol and isoamyl alcohol.

Conclusion

The above study demonstrate that both the strain *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 are capable for guava wine production. After comparing the overall result *Saccharomyces cerevisiae* NCIM 3287 is giving better result comparing to NCIM 3095. Optimization fermentation parameter for *Saccharomyces cerevisiae* NCIM 3287 is fermentation temperature (25°C), pH (4), diammonium phosphate (0.06%) sulphur dioxide (100ppm) & 6 % inoculum level gave a better quality of guava wine.

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