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Full Length Research paper

The making of pomegranate wine using yeast immobilized on sodium alginate

Sevda S. B.* and Rodrigues L.

Department of Food Engineering and Fermentation Technology, University Institute of Chemical Technology, Matunga, Mumbai -400019, India.

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The wine yeast *Saccharomyces cerevisiae* NCIM 3095 was immobilized in sodium alginate beads as a biocatalyst in/for pomegranate wine making. The immobilized biocatalyst was suitable for pomegranate must fermentation at ambient temperatures. In order to optimize immobilization conditions, a study was conducted using various concentrations of alginate, cell loading and bead diameter. The optimized parameters were alginate concentration 3% (w/v), initial cell loading 8 g/100 ml and cell bead diameter of 3 mm. In comparison to free cells, the rate of fermentation by immobilized cell proved to be greater, showing suitability for fruit wine production.

Key words: Pomegranate wine, immobilization, *Saccharomyces cerevisiae* NCIM 3095, sodium alginate.

INTRODUCTION

India is the second largest producing country of pomegranates after Iran. Pomegranates (*Punica granatum* L.) are rich in polyphenols, specifically ellagic acid and punicalagins, both of which can act as potent antioxidants. Ellagic acid is found in the red arils (seeds) of the pomegranate, as well as in other red-coloured berries. It is the punicalagins, however, that have come to the forefront of research. Punicalagins are found only in the outer skin of the pomegranate, and are estimated to have twice the antioxidant capability of red wine and green tea. Pomegranate juice is mainly used as a health drink. However, most phytochemicals can be found in the rind of the fruit (Bakoyianis et al., 1992; Adsule et al., 1992). Pomegranate juice containing good amount of sugar hence the pomegranate wine containing good amount of alcohol as well as good color.

Pomegranate wine is the product of anaerobic fermentation by yeast in which the sugars are converted into alcohol and carbon dioxide (Adsule et al., 1995). Fruit juice is full of sugar, which could cause weight gain. Consumption of wine, to get the benefits better, should be limited to a glass or two a day, as yeast converts some of the sugar during fermentation into alcohol. But the net loss of carbons going from sugar to alcohol is small (as carbon dioxide). These carbons get burned in body or get converted into fat. 100 mL of wine contains around 70 kcal, whereas 100 mL of pressed pomegranate juice will contain around 60 kcal. The soluble polyphenolic content of pomegranate juice (0.2 to 1.0%) includes anthocyanins, catechins, tannins, and gallic and ellagic acids (Aviram et al., 2000). Kulkarnai and others reported that the antibacterial action of pomegranate juice varied with variety and depended on the contents of phenolic compounds, pigments, and citric acid.

The use of immobilized cells in the making of fruit wine is a rapidly expanding research area with potentially greater advantages as compared to free cell systems (Kourkoutas et al., 2005). An upsurge of interest in cell immobilization for alcoholic beverages and potable alcohol production has been taking place recently. This is mainly due to the numerous advantages that cell immobilization offers including enhanced fermentation productivity, feasibility of continuous processing, cell

*Corresponding author. E-mail: sevdasuraj@gmail.com. Tel: 91 22 3361111/2222, +91 9990576049. Fax: 91 22 33611020.

and downstream processing (Stewart et al., 1986). Cell immobilization may also protect cells against shear force. Industrial use of immobilized cells is still limited; however, further application will depend on the development of immobilIzation procedures that can be readily scaled-up (Amutha et al., 2001). Cell immobilization in alcoholic fermentation is an attractive and rapidly expanding research area because of its technical and economical advantages compared to the free cell system (Kocher et al., 2006).

The reason for using immobilized cells in fruit wine making is to increase fermentation productivity (Riboulet et al., 1986; Kourkoutas et al., 2004; Fumi et al., 1988). Commercial preparations of selected yeasts immobilized on alginates (Colagrande et al., 1994; Iconomou et al. 1996**)** or confined by microfiltration membranes (Bardi et al., 1996) are available for sparkling wine production for secondary fermentation in the bottle according to the traditional Champagne method. The use of immobilized cells in food fermentation processes has been extensively studied due to the well-known technical and economic advantages of immobilized cells compared to free cell systems (Kourkoutas et al., 2001). To satisfy the demand for clean technologies and consumer acceptance, various food grade as natural materials like cellulose (Ezquerro et al., 2005; Fumi et al., 1987) gluten (Silva et al., 2002), and fruit pieces (Silva et al., 2003; Lemonnier et al., 1989) have been proposed as yeast immobilization supports for use in wine making and brewing processes.

However, for application in the food industry, further research is needed to obtain cells immobilized on a support that is, more hygienic for food, cheap, abundant in nature, suitable for low temperature fermentation, and will lead to an improvement of the aroma and taste of the final product. Many researchers have proposed various supports for cell immobilization in the wine-making process (Kosseva et al., 1998; Chen et al., 2010).

Thus, there has been growing interest in the direct use of immobilized cells as a means of catalysis. Thus, immobilized cells were used for production of useful compounds by various types of bioreactions, such as transformation, synthesis or degradation.

Still there is no study found in literature by using immobilized cell for pomegranate wine fermentation. Therefore, the aim of the present study was to investigate the suitability of immobilized cells entrapped in alginate beds for alcoholic fermentation and pomegranate wine making, as well as the influence of alginate concentration, initial cell loading and cell bed diameter for pomegranate wine production.

MATERIALS AND METHODS

Chemicals

The Na-alginate was purchased from Sdifine chemicals, Mumbai Calcium chloride was bought from HiMedia. Sucrose (Extra pure grade) was purchased from Merck Ltd.

Yeast and fermentation substrate

Saccharomyces cerevisiae wine yeast (NCIM 3095) was used in all experiments. Yeast cultures stored in slants were reactivated in yeast extract, peptone and dextrose medium (YEPD) for 48 h at 25°C.

Fermentations were carried out on pomegranate must. Before the start of fermentation, the pomegranate must was added to 0.15% potassium metabisulphate, 50 ppm diammonium phosphate and sugar (to make 22° Brix). The optimization of all these above-listed parameter for pomegranate wine production is done for free cell experiments (Sevda et al., 2001). All experiments were performed in 250-ml shake flasks in duplicates.

Preparation of Na-alginate solution

Different concentrations of Na-alginate solution were prepared by dissolving Na-alginate in hot water. The solution was cooled to the room temperature. The appropriate amount of the cell suspension is mixed with Na-alginate solution. Mixing is done with cyclone mixer for 5 ml or less solution. In case of higher suspension volume, n orbital shaker was used.

Cell immobilization

Immobilized beads were prepared by drop-wise extruding cell-alginate suspension in 0.1 M $CaCl_2$ solution with help of needle and syringe. To form even size and perfectly spherical beads, drop wise extrusion was done from approximately 28 cm height. Needles with different size (#18, #22, #24) were used to vary diameter of the beads.

Analytical method

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 (Zoecklein et al., 1995).

RESULTS AND DISCUSSION

Effect of alginate concentration

To study the effect of alginate concentration on alcohol production, alginate solutions of 1 to 5% were prepared. To each 40 ml alginate solution with different alginate concentration, 10 ml of cell suspension containing 9 g/L of yeast cells was added. The anaerobic fermentation was carried with 8 g of immobilized beads and 100 ml of pomegranate juice for the production of alcohol. The reduction in TSS during pomegranate wine fermentation by yeast cells immobilized on alginate beads with varying concentration of sodium alginate is shown in Figure 1A. In 3% alginate concentration, there was maximum

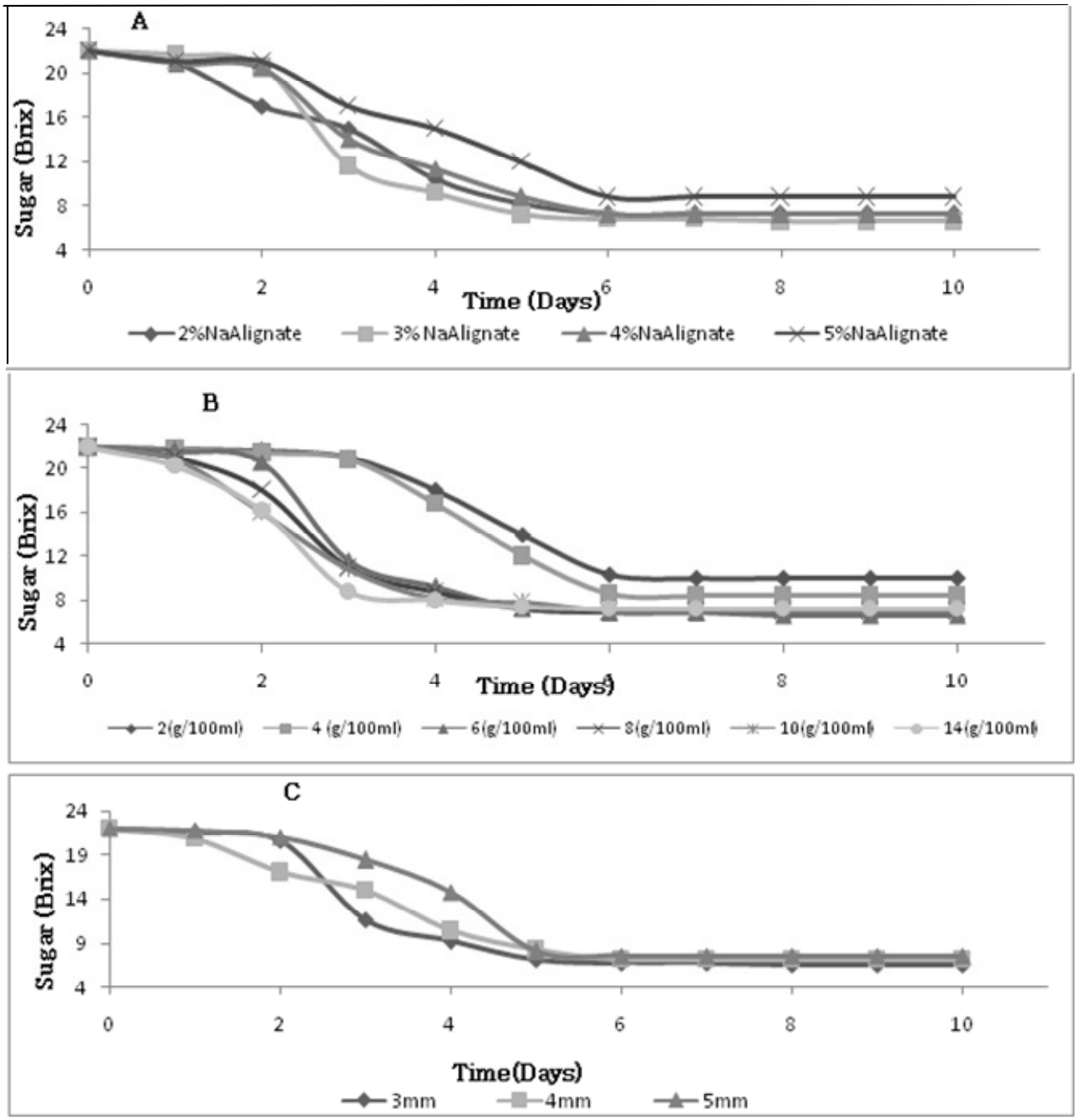

Figure 1. Study on reduction of TSS (degree brix) during pomegranate wine fermentation by immobilized yeast cell NCIM 3095 with alginate beads with (A) Effect of alginate concentration. (B) Effect of initial cell loading (C) Effect of bead diameter of alginate beads and (n = 2, all experiments done in duplicate and average value is used).

reduction in TSS during fermentation. Figure 1B depict the effect of alginate concentration on production of alcohol by immobilized yeast cells *S. cerevisiae* NCIM 3095. As alginate concentration was varied from 1 to 5%, it caused significant effect on alcohol production: alginate 2 and 3% shows maximum alcohol production 7.8 %v/v. Lower alginate concentration have problem of cell leakage and the beads with higher alginate concentrations had good mechanical properties as compared to the low concentrations. Mechanical properties like hardness, mechanical strength etc are essential to operate them in a reactor. This would be expected since there should be a defined pore structure that is dependent on the type of gel material and on the gel concentration. Since the gel forms a quasi-solid structure, it is expected to hinder transport of the solute

and, thus, reduce the diffusion coefficient. Na-alginate and $CaCl_2$ reacts and forms the Ca-alginate gels. This gel involves formation of a porous structure in the beads. Application of immobilized yeast in wine production decreased the fermentation time in comparison with the free cell. The sugar uptake rate of the immobilized yeast was always higher than that of the free yeast. The same phenomenon was also observed by different researchers when using immobilized yeast in alginate gel (D'Amore, 1989; Galazzo and Bailey, 1990), on pear pieces (Mallios, 2004). In addition, the biosynthesis of volatile acids of the immobilized yeast was lower than that of the free yeast. Low volatile acidity in young wine improved the flavour of the final product. According to Bardi et al. (1994) and Mallouchos et al. (2003), wine fermented by yeast immobilized on delignified cellulose or grape skin had a better flavour in comparison with the control sample using the free yeast. The increase in the Na-alginate concentration results in more dense structure. Thus higher alginate concentration decreases the pore size in the bead. It results in more retardation of substrate molecule that is, increase in internal mass transfer resistance and decrease in effective diffusivity of the substrate molecule and thus resulting in lower rates of fermentation.

Effect of cell loading

Cell loading was varied in the range of 2 g/100 ml to 20 g/mL with 3 mm bead diameter and 3% alginate concentration for pomegranate wine. The immobilized alginate beads were washed three times with 100 ml of saline to remove the cells, which can get leaked. Then 8 g of beads were mixed with 100 ml of pomegranate must, subjected to anaerobic fermentation and analyzed for alcohol content. Figure 1C represents effect of initial cell loading on reduction of TSS. Figure 2A indicates the effect of initial cell loading on alcohol production by immobilized system. As the cell loading was decreased from 18 g/100 ml to 6 g/ml, it had no effect on alcohol production whereas further reduction in cell loading from 6 to 2 g/ml decreased the alcohol production. The reason behind the low production rate can be attributed to lower cell density in the bead at low cell loadings (D'Amore, 1989). At optimum of 6 g/ml of cells, it shows maximum reduction in TSS with a constant rate of fermentation. The cells are present to such an extent that further increase in cell density will result in only marginal increase in productivity.

Effect of bead diameter

Beads with various sizes (2, 3 and 4 mm diameter) were prepared in 3% alginate solution. 10 ml of 9 g/l cell suspension was mixed in 40 ml of alginate solution. 8 g of these beads are added in 100 ml of fruit juice and subjected to anaerobic fermentation. After completion of fermentation, the samples were analyzed for alcohol content. Figure 2B represents the effect of cell bead diameter on alcohol production with immobilized cells. There was gradual decrease in alcohol production with increasing bead diameter. As the diameter of the bead is increased, the substrate molecule has to travel more to reach the center of the bead. At the same time, cells, present inside the matrix, react with the molecule and forms product. Due to product formation, outward flow of product towards outside bulk liquid stream starts. It additionally lowers the diffusion coefficient and internal mass transfer limitation occurs. It results in overall reduction in the production. Thus smaller beads (3 mm) have more surface area per unit volume and hence more productivity (Mallios, 2004). Figure 2C indicates the effect of cell bead diameter on alcohol production by immobilized cells. This shows that lower cell bed diameter results in higher alcohol production.

Conclusion

The above study demonstrates that sodium alginate can be used for the development of a cost effective wine-making process involving immobilization of yeast. They are a cheap, safe, and easily available raw material. Their use demands no pre-treatment and the immobilization technique is a natural process. The immobilized biocatalyst is operationally stable, which makes possible its use at commercial scale.

The immobilization of yeast cells in sodium alginate spherical beads was made by entrapment of cells in this. The biocatalyst was found effective for guava wine making, and the process was feasible using a modified bioreactor, designed to keep separately the grape skins from the immobilized biocatalyst. The good operational stability, proved by the performed repeated fermentation batches, will give the possibility to remove the biocatalyst after a longer time.

Various physical factors like alginate concentration, bead diameter and cell loading plays important role in the success of immobilized systems as all the factors has impact on the mass transfer rate of the substrate and product formed. The optimized parameters were alginate concentration 3 %w/v, initial cell loading 8 g/100 ml and cell bead diameter of 3 mm. Also, because of their shape and size, they could be applied in continuous processes and fluidized bed bioreactor systems.

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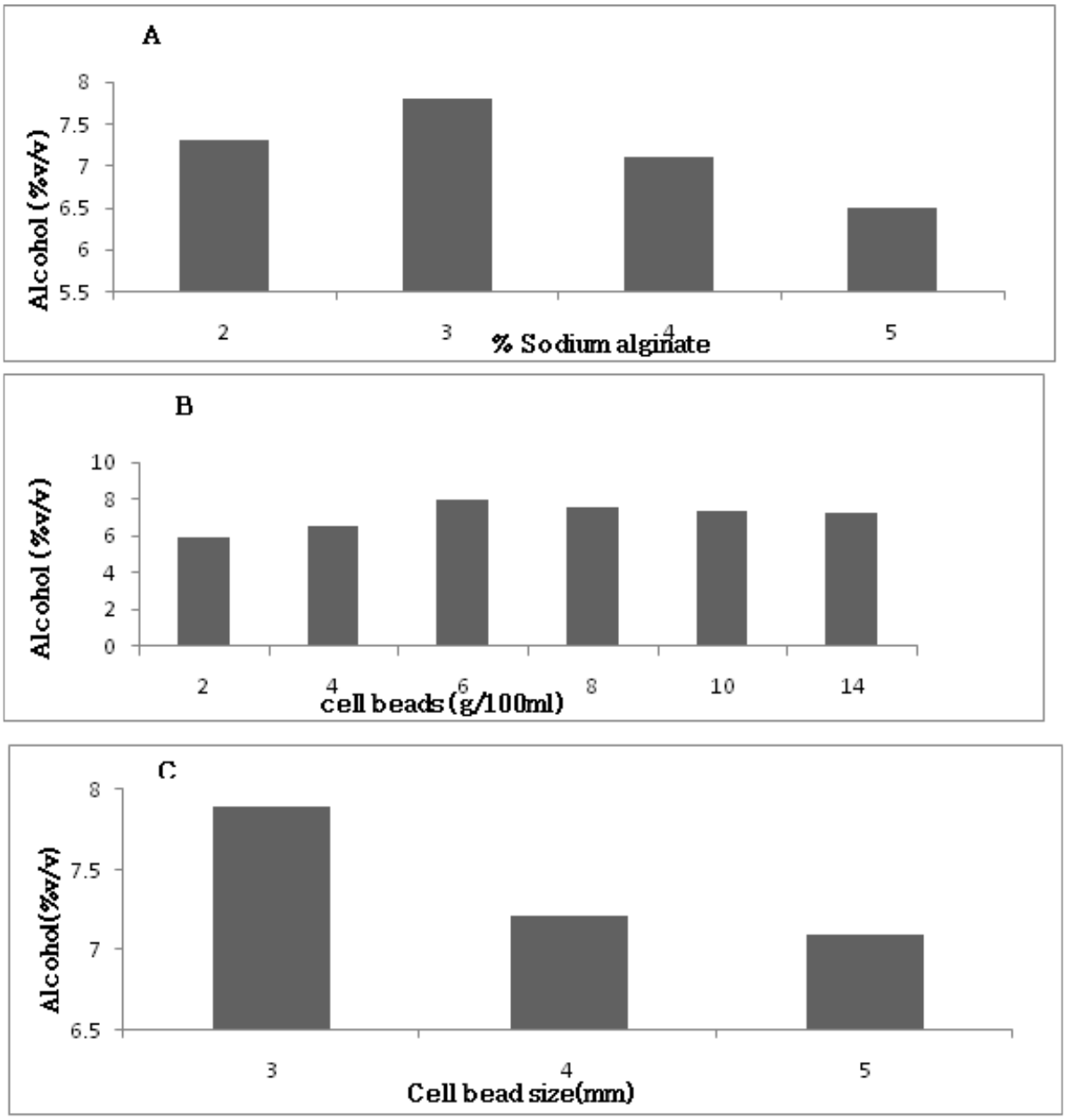

Figure 2. Immobilized yeast cells *Saccharomyces cerevisiae* NCIM 3095 with (A) Effect of alginate concentration (B) Effect of initial cell loading on production of alcohol and (C) Effect of bead diameter of alginate beads in guava wine production(n = 2, all experiments done in duplicate and average value is used).

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