Utilization of yeast as peel-biocatalyst for mango wine production
5.1 INTRODUCTION

Wine-making is a very oldestendeavour because of the natural character of the product. Traditional wine fermentation technology uses freely suspended yeast cells in batch bioreactors using a variety of yeast strains to make different wines. Crushing of the grapes is all that is necessary to initiate fermentation; yeasts originating from the surface of the berries immediately proliferate, increasing from several thousand to several million cells/mL of juice as they ferment. The transformation of juice results in the production of wine. The sugars and other nutrients present in the liquid phase must diffuse into the yeast cell to be metabolized by different enzymes into ethanol and other flavour compounds which must diffuse out of the yeast cell into the liquid phase. The functional integrity of the cell membrane plays a key role in determining the viability characteristics of the yeast cells and their metabolic activity.

This biological reaction (a reaction due to the involvement of yeast) can take place in two different ways:

- A system where the microorganisms are suspended in the medium (homogeneous) for example, the fermentations in wine-making or in brewing.
- A system where the microorganisms are not free in the medium but where they are attached to a support, giving a two-phase system. For example, bio-films.
- In the controlled use of microorganisms in an industrial application, like alcoholic fermentation, traditionally free cells of microorganisms were used, which existed prior to knowledge of the biological nature of the reaction.

Over the last two decades or so, a new development in fermentation technology, using immobilized yeast cells for the continuous production of wine has gradually emerged worldwide. Earlier research has established the superior characteristics and advantages of using immobilized yeast cells to continuously produce ethanol in fixed bed and fluidized bioreactor systems. The large number of reports on yeast cell immobilization highlights the importance and interest in this new technology as we enter the twenty-first century. Continuous wine fermentation using immobilized cells
will perform better than the existing mainstream fermentation technology by reducing the time to produce a finished beer, reducing inventory, reducing floor space, and reducing product variation.

5.1.1 Cell immobilization — what and why?: Whole cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity" (Karel et al., 1985), and is a process that often mimics what occurs naturally when cells grow on surfaces or within natural structures. Immobilization systems are applied to enzymes, cellular organelles, microbial, animal and plant cells.

In general Immobilized cell technology offers several important advantages comparatively to the traditional fermentation using free cells such as higher cell densities per unit bioreactor volume that result in very high fermentation rates, the reuse of the same biocatalysts for prolonged periods, the development of continuous process that may be operated beyond the nominal washing-out flow rate, and smaller bioreactor volumes that may decrease capital costs (Pilkington et al., 1998). Especially, in the case of alcoholic beverages production these advantages include:

- Prolonged activity and stability of the biocatalyst due to the protective effect of the immobilization support against physicochemical effects.
- Higher cell densities in the bioreactor leading to higher productivity, shorter fermentation times and elimination of non-productive cell growth phases.
- Increased tolerance to high substrate concentration and reduced end-product inhibition.
- Feasibility of low-temperature fermentation leading to improved product quality.
- Easier product recovery with no need for separation and filtration steps, thus reducing cost for equipment and energy demands.
- Reduction of microbial contamination risk due to high cell densities and fermentation activity.
• Ability to use smaller bioreactors with simplified process designs and therefore lower capital costs.

5.1.2 Effects of cell immobilization: Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization, although it is difficult to predict the type and magnitude of such changes. Comparative studies on immobilized and free cells showed effects on activation of yeast energetic metabolism, increase in storage polysaccharides, altered growth rates, increased substrate uptake and product yield, lower yield of fermentation by-products, higher ploidy and RNA content, lower intracellular pH values, increased tolerance against toxic and inhibitory compounds and increased invertase activity. Among these effects the most prominent are those on growth and physiology, metabolic activity, stress tolerance, and flavour formation (Kourkoutas et al., 2004).

5.1.3 Methods of cell immobilization: Numerous biotechnological processes are advantaged by cell immobilization and therefore several such techniques and support materials have been proposed, which can be divided into four major categories (Kourkoutas et al., 2004).

![Diagram of yeast immobilization methods](image)

**Fig. 5.1.** Basic methods of yeast immobilization: (1) attachment to a surface, (2) entrapment within a porous matrix, (3) self-aggregation, and (4) containment behind a barrier.
5.1.3.1 **Immobilization on solid carrier surfaces:** Attachment or adsorption on solid carriers by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier. Such carriers are DEAE-cellulose, wood, sawdust, delignified sawdust, inorganic materials (e.g. mineral kissiris, γ-alumina, polygorskite, montmorillonite, hydromica, porous porcelain, porous glass), etc. Solid materials like glass or cellulose can also be treated with suitable chemicals to enhance their adsorption ability.

5.1.3.2 **Entrapment within a porous matrix:** Entrapment in a porous matrix in which cells either penetrate or grow until their mobility is obstructed, or the porous material is formed *in situ* into the cell culture. An example is the entrapment into polysaccharide gels such as alginates, κ-carrageenan, agar, chitosan, polygalacturonic acid, gelatin and collagen.

5.1.3.3 **Cell flocculation (Aggregation):** Self-aggregation of cells by natural flocculation to form a larger unit, or the property of cells in suspensions to adhere in clumps and sediment rapidly, or by cross-linking agents (artificially induced).

5.1.3.4 **Mechanical containment behind a barrier:** Cell containment behind barriers, either by use of microporous membranes, or by entrapment of cells in microcapsules, or by cell immobilization on the interaction surface of two immiscible liquids. Natural materials like delignified cellulosics (sawdust), gluten pellets, pieces of fruit, brewer’s spent grains, whole grains, grape skins, etc., have been used as cell immobilization supports in food fermentation processes with great success.

Fig. 5.2 Cell immobilization by entrapment  
Fig. 5.3. Cell flocculation
Fig 5.4 Cell immobilization by mechanical containment

However, an immobilized cell system should have the following properties for large scale industrial application:

✓ The carrier material must be nontoxic, readily available and affordable,
✓ The system should be efficient, easy to operate and give good yields,
✓ The carrier material should allow for high cell loading and physical strength,
✓ The cells should have a prolonged viability in the support.

5.2 REVIEW OF LITERATURE

In the 1960s, work on immobilized enzymes was developed and numerous industrial applications were proposed and used in the fields of analysis (enzyme electrodes) and the industrial production of biomolecules (Chihata 1978). The major advantage motivating this research was the reduced manufacturing cost of the product. Enzymatic preparations can be used in the continuous processes, and it is possible to increase reactor productivity with the use of concentrated enzymes. The prior purification of enzymes is a costly process.

Divies (1975, 1977) showed the possibility of immobilizing microorganisms in an included form while maintaining their viability over long periods in order to carry out multienzyme transformations with a food technology application. Since then, research in the field of immobilized cells has been intense, (Webb et al 1986) although industrial transfer of this technology has remained minimal.

Wine-making involves two principal operations: first, preparation of the grape must to tailor its composition and to maintain the qualities of the grape at harvest; and,
second, conducting microbial fermentation through rational exploitation of the biochemical activities of yeasts and or lactic acid bacteria.

5.2.1 Application in fermentation: Many papers deal with the use of immobilized cells of yeasts (usually *S. cerevisiae*) to achieve the alcoholic fermentation of musts (red or white). The main purpose is always to ensure better control of this important step in wine-making: low-temperature fermentations, improvement of organoleptic characteristics, increase of reaction rates, good achievement of sugar consumption, etc. Gorff (1988) patented a process using yeast cells immobilized on derivatized cellulose and later Divies *et al.*, (1990) patented a process to entrap the yeast cells in calcium alginate beads. The same year Sarishvili *et al.*, (1990) described a “technology for manufacture of dry red wines with immobilized yeast” and the cells were immobilized on beech, oak or polyethylene and the authors observed that the quality of wines was improved. Malik *et al.*, (1991) then tried ten different strains immobilized in alginate and noticed a reduction in their acidification potential compared with that of unbound cells. However for industrial wine production, the selection of a suitable support for cell adsorption is important because a number of factors are known to influence the cell support interactions such as nature of support and microbial cell, environmental conditions (Margaritis and Merchant, 1984) and hence further research was concentrated to obtain cells immobilized on a support that is more hygienic for food, cheap, abundant in nature, suitable for low temperature fermentation, and which would lead to an improvement of the aroma and taste to the final product. To satisfy these prerequisites, various natural supports have been proposed for ambient and low-temperature wine making. But most of the studies on this subject are due to the Dept. of the Chemical University of Patras (Greece). Bakoyianis *et al.*, (1992) had reported the use of a psychrophilic and alcohol-resistant yeast strain immobilized on *kissiris* in a continuous process for making wine at low temperature. Later Argiriou *et al.*, (1996) showed that this yeast strain was more efficient if some preservation treatments at 0°C were made. Bardi and Koutinas (1994) described experiments where different supports were tested as well as different conditions of fermentation: immobilization of cells on delignified cellulose and use of them in 55 repeated batch cultures at low (10°C) or
room (30°C) temperature: the main result was that the fermentation rates are increased (threefold) compared with those for free cells. Also the stability of the biocatalyst was proven. Bakoyianis et al., (1998) using cells of *S. cerevisiae* immobilized on different supports (alumina, kissiris and alginate) compared the volatile by-products obtained at different temperatures in a continuous process. It was observed that the levels of 1-propanol, isobutyl alcohol and amyl alcohols were less than those synthesized by free cells for all supports and temperatures studied. Further many supports have been used such as gluten pellets (Bardi et al., 1996), dried figs (Bekatorou et al., 2002), grape skins (Mallouchos et al., 2002), fruit pieces like apples, pears, raisin berries (Kourkoutas et al., 2001; Mallios et al., 2004; Tsakiris et al., 2004b), brewer’s spent grains (Athanasis et al., 2007), corn starch gels (Kandylis et al., 2008), potato pieces (Kandylis and Koutinas, 2008), cork pieces (Tsakiris et al., 2010), watermelon rind pieces and sugarcane pieces (Reddy et al., 2008; 2010b) etc. Furthermore, cell immobilization was applied for the production of wide variety of fermented beverage products such as batch wine-making of white wines (Yajima and Yokotsuka, 2001) and red wines (Tsakiris et al., 2004a), for continuous wine fermentation (Kourkoutas et al., 2002), secondary wine fermentations like malolactic fermentation (Kosseva et al., 1998), Cider (Cabranes et al., 1998), Immobilization of deacidifying yeast *Schizosaccharomyces pombe* was also used for malic acid elimination from musts and wines (Ciani, 1995), mead, an alcoholic beverage made from fermented honey (Quereshi and Tamhane, 1986), brewing of beer (Nedovic et al., 2005), fermented cheese (Kourkoutas et al., 2006), fermented probiotic milk (Kourkoutas et al., 2005), distillates production (Loukatos et al., 2003). In addition to the above products, immobilization technology have been applied to fruit wine-making like fermentation of fresh sugar cane juice (Fukushima and Hatakeyama, 1983), fermentation of ripe Cavendish banana fruit pulp (Del Rosario and Pamantong, 1985), production of alcoholic beverages from different fruit juices like peach, plum and cherry (Quereshi, and Tamhane, 1985), Persimmon and Kiwifruit wine (Ohta et al., 1989), Umeshu (a liqueur made from Japanese apricot fruit) (Takatsuji et al. 1992), watermelon wine (Nakada, 1990). Eventhough several number of natural immobilization supports were tried for
wine-making or for other fermented beverages, their usage was limited to their abundance and cost effectiveness.

Mango (Mangifera indica L.) is one of the most important tropical fruits, accounting for 54.2% of the total mangoes produced worldwide and is considered as "the king of fruits". It is highly perishable seasonal fruit and hence one of the methods for processing and preserving mango is to ferment the juice, which has high carbohydrate content (20-24 °Bx) into wines. The edible pulp makes up 33-85% of the fresh fruit. It is processed into various products like slices, nectar, jams, freeze dried powder, pickles etc., however, production of wine from mango is one of the alternative ways to exploit and convert surplus production into a value-added product (Reddy and Reddy, 2005; Kumar et al., 2009).

During processing of mango, peel is a major byproduct up to 7-24%, and according to Larrauri, et al., (1996) by-products of industrial mango processing may account 35-60% of the total fruit weight and represent a serious disposal problem. The utilization of the mango kernels as a source of fat, natural antioxidants, starch, flour and feed has been extensively investigated. The use of mango peels for the production of biogas and dietary fiber has been described; however, studies on peels are scarce. Animal feeding is the usual application of these wastes, although they can also be used to obtain more valuable products like good quality pectins (Pedroza-Islas et al., 1994).

Mango peel is rich in fiber, phytochemicals such as carotenoids, antioxidant polyphenols, vitamins, anthocyanins, dietary fiber, sugars, trace minerals and volatile compounds etc., and provides a pleasant characteristic aroma similar to that of its fruit (Ajila et al., 2007a, Ajila et al., 2007b). Larrauri et al., (1997) had shown the antioxidant activity of mangoes peel fibers. Ajila and Rao (2008) had reported the protective action of mango peel extract against hydrogen peroxide induced oxidative damage in rat erythrocytes. An investigation by Parmar and Kar (2009) has demonstrated the pharmacological importance of mango peel extracts with respect to its possible regulation of tissue lipid peroxidation (LPO), thyroid dysfunctions, lipid and glucose metabolism. Kim et al., (2010) had evaluated the ability of mango peel extract
to function as an antioxidant and anticarcinogenic agent. Hence, it is safe and inexpensive, comprising an interesting new support for cell immobilization for wine fermentation. The production of wine or any other beverages using cells entrapped in mango peel has not been attempted, and it is a very attractive proposition, due to the full compatibility of this support and advantages to select immobilization material which contains potentially bioactive compounds. Therefore, the aim of the present study was to investigate the suitability of immobilized cells entrapped in mango peel for mango wine fermentation and wine making at various temperatures, as well as the influence of the immobilized biocatalyst on the volatile composition of the produced wines.

5.3 MATERIALS AND METHODS

5.3.1 Yeast strain and inoculum preparation: The wine yeast, *Saccharomyces cerevisiae* (S.C), a generous gift from Prof. Roberto Ambrosoli, University of Turin, Italy, was used in the experiments. The culture was maintained on MPYD agar slants as discussed in section 2.3.3.

5.3.2 Preparation of mango juice: Ripe mango (*Mangifera indica* L.) fruits of *Rumani* cultivar were procured from the local fruit market in Tirupati, Andhra Pradesh, South India and were processed and homogenized according to (Reddy and Reddy, 2005) as shown in section 2.3.1 and 2.3.2.

5.3.3 Yeast cell immobilization: Mango peel from *Banginapalli* cultivar was obtained by peeling off the fruits manually and the ideal ones were selected, cut into small pieces (3×5 cm, 200g) and sterilized by autoclaving at 121°C for 15 min. These pieces were taken into a 1000 mL glass cylinder and fermented with 400 mL of culture medium containing yeast cells with optical density (O.D.) of 1 at 590 nm and then allowed to ferment for 8-12 h. The fermented broth was decanted to remove the unimmobilized yeast cells. The biocatalyst prepared by this method was used for the repetitive batch fermentation and the biocatalyst was washed twice with 200 mL mango juice after each batch of fermentation.
5.3.4 Repeated batch Fermentation: Repeated batch fermentations were carried out by 100 g of mango peel biocatalyst for 1000 mL of mango juice into a glass cylinder for fermentation. The fermentation was carried out separately at various temperatures (15, 20, 25 and 30°C) and no stirring was performed during any stage of the fermentation. The end point of the fermentation was detected by measuring of residual sugars of less than 2 g/L. The fermented liquid was decanted and the support was washed twice with 200 mL of the medium that was used for wine production. The volume of the biocatalyst in the bioreactor and volatiles were determined in all fermentations performed and the effect of temperature was monitored during the repeated fermentations.

5.3.5 Determination of immobilized cells: Determination of immobilized cells on wet mango peel pieces and free cell concentrations were carried out by the method of (Reddy et al., 2008) with sterilized Ringer’s solution. Immobilization on the mango peels were confirmed before and after repeated batches by scanning under scanning electron microscope.

5.3.6 Viability determination: This was done according to the methodology discussed in section 2.3.4.

5.3.7 Determination of sugars, glycerol and acidity: Sugar concentrations, total acidity and volatile acidity were estimated according to methodology described in section 2.3.5. Glycerol was enzymatically determined by glycerol kinase method (Wieland, 1988) on diluted samples employing commercial kit from Megazyme, Ireland.

5.3.8 Determination of volatiles by gas chromatography: This was done according to the methodology described in section 2.3.11.

5.3.9 Scanning electron microscopy (SEM): The immobilized biocatalyst was washed and fixed with glutaraldehyde in phosphate buffer at 4°C for 4 h and dehydrated by using series of graded alcohol and dried in at a critical point in a Hitachi HCP-2(Japan) with CO₂. It was then coated with thin layer of platinum using an automated sputter
coater (Polaton, UK) for about 90 s and the samples were then scanned under scanning electron microscope (Model: Hitachi S520, Tokyo, Japan) at various magnifications at Indian Institute of Chemical Technology (IICT), Hyderabad.

5.3.10 Sensory evaluation: This was performed according to the methodology described in section 2.3.12.

5.3.11 Statistical analysis: This was performed using SPSS 12.0, as described in section 2.3.13.

5.4 RESULTS AND DISCUSSION

5.4.1 Immobilization and fermentation: In the present study, mango peels from seven different cultivars viz., Banginapalli, Alphonso, Neelam, Raspuri, Rumani, Sindhura, Totapuri were studied as immobilization support for mango wine production, of which the wine produced from the peels of cultivar Banginapalli, Alphonso and Sindhura had better aroma and taste, however, further work was carried out only with Banginapalli peel because of its abundance from near-by mango processing industries in Chittoor district. Some researchers have employed pieces of fruits such as apple, dried raisin berries and dried figs as supports for cell immobilization because it would lead to a product with superior taste and aroma because of the transfer of some of their aroma constituents into the wine. However, Mallouchos et al., (2002) have used grape skins as an immobilization support in repeated batch fermentations. The use of aforementioned fruit pieces other than mango peels may impart a complex aroma and flavor to the wine but, the peels from Banginapalli mango cultivar had intensified the unique aroma of the mango fruit, colour, flavour and taste of the final wine.

The mango cultivar Rumani, a low-priced fruit is abundantly available locally during the season with a juice yield of 560±5.2 ml/kg, medium pectin content (8-10 % w/w), and with a total sugar content of 14.3 to 15.5 %, however, the total sugar content in all the trials was adjusted to ~20% with commercial glucose. Preparation of mango wine from other high-priced cultivars like Banginapalli and Alphonso was also tried (Reddy and Reddy, 2009; Kumar et al., 2009). In the present study, the cultivar Rumani
was selected to exploit the low-priced mango fruits to produce a good quality mango wine which would be profitable to the farming community.

For immobilization of yeast cells, the pieces of mango peel were mixed with a liquid culture of yeast biomass and allowed to ferment for 12 h. Around this time about $3.4 \times 10^6 \pm 1.0 \times 10^6$ yeast cells were attached per gram of mango peel pieces. The prepared biocatalyst was washed and used for 12 repeated batch fermentations of mango juice for mango wine-making at room and low temperatures. The stability and productivity in the repeated batch fermentations and the leached out free cell concentrations are shown in Table 5.1.

Table 5.1. Effects of different mango peels on quality of mango wine by immobilization

<table>
<thead>
<tr>
<th>Peel of mango cultivar</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Banginapalli</td>
<td>Wine with better aroma and taste was observed and the peel remained unchanged until the end of the fermentation</td>
</tr>
<tr>
<td>2. Alphonso</td>
<td>Wine with better aroma and taste was observed and the peel remained unchanged until the end of the fermentation</td>
</tr>
<tr>
<td>3. Neelam</td>
<td>A slight increase in total acidity was observed in first 5 batches and there was no significant difference in sensory attributes.</td>
</tr>
<tr>
<td>4. Raspuri</td>
<td>Mango peel was thin relatively and its shape was distorted after 3 repetitive batches</td>
</tr>
<tr>
<td>5. Rumani</td>
<td>Mango peel was thin relatively and its shape was distorted after 2 repetitive batches</td>
</tr>
<tr>
<td>6. Sindhura</td>
<td>Wine with better aroma and taste was observed and the peel remained unchanged until the end of the fermentation</td>
</tr>
<tr>
<td>7. Totapuri</td>
<td>The peel remained unchanged until the end of the fermentation but there was no significant difference in sensory attributes.</td>
</tr>
</tbody>
</table>
The morphology of the mango peel surface after the immobilization of yeast cells and their existence or attachment on the fibers of mango peel (biocatalyst) was proved by the electron micrographs (Figure 5.5 A and B). The predominance and proliferation of the yeast cells within the biocatalyst tissue structure could be viewed at higher magnification (Figure 5.5 C and D). Cells immobilized on mango peel were found to be suitable for mango wine-making at ambient temperatures and the biocatalyst appeared to have good operational stability (Figure 5.6).

Effective immobilization of yeast cells on mango peel biocatalyst was proved by the ability to perform successive repeated batch fermentations for ~5 months without any significant loss of the biocatalytic activity at different temperatures (15-30°C); although the support was washed after each batch to remove free cells, it showed yeast cells densely and homogenously adhered to the surface of the carrier support.

Adhesion of S. cerevisiae is essentially dependent upon electrostatic interactions between the support and the normally negatively charged cell surface and cell immobilization on peel pieces may take place either by natural entrapment into the porous pectin-cellulosic material of mango peel, or due to physical adsorption by electrostatic forces or covalent binding between the cell membrane and the carrier support (Tsakiris et al. 2004a).

5.4.2 Repeated batch fermentation: Repeated batch fermentations were conducted with entrapped and free cells separately at different temperatures (15, 20, 25 and 30°C). All the fermentations were carried out using mango peel supported biocatalyst with same initial concentration of sugar, ~20% (w/v) (Table 5.2). The residual sugar content was very low (ranging from none to 0.8 g/L), indicating that the biocatalyst was very active and suitable for alcoholic fermentation and the resultant mango wine contained alcohol concentrations similar to dry and table wines, 9.5-12% (v/v). It was found that the temperature mainly affected the fermentation rate. At 15°C the fermentation was completed in 72 h which is less time that is required for the natural fermentation of mango juice while at 30°C it took only 40 h. Both higher alcohols and ethanol productivities were higher in immobilized system than fermentation with free cell.
Electron micrographs showing the surface of mango peel immobilized with Yeast at various magnifications. (A) at $\times 500$, (B) at $\times 1000$, and (C) at $\times 2500$ and (D) After Fermentation.

Fig. 5.5. Scanning Electron Microscopy images of un-immobilized mango peel (control) and mango peel immobilized with yeast cells.
batches are significantly affected by temperature ($p < 0.05$). At low temperatures (15 and 20°C) an improvement of fermentation time and productivity were observed only after first two batches. This may be probably due to the adaptation of the immobilized yeast cells to the mango peel matrix.

Wine and ethanol productivity were slightly reduced after first three repeated batches. This may be due to difficulty in nutrient transfer, since there is a decrease in the mango peel biocatalyst and therefore, yeast cells were not uniformly spread throughout the mango peel. Therefore, the first and second batches were carried out with 400 ml and the subsequent batches with 300 ml. The volume of the mango peel pieces was weighed after every batch and a slight decrease in weight was observed up to 4 repeated batches.

![Fig. 5.6. Bioreactor with active fermentation using mango peel immobilized yeast biocatalyst.](image-url)
Table 5.2. Effect of the use of immobilized yeast on fermentation parameters at different temperatures

| Temp (°C) | Repeated batches | Initial | Fermentation | Residual | Sugar | Sugar | Ethanol | Ethanol | Ethanol | Ethanol | Free cell | Total | Volatile |
|-----------|------------------|---------|--------------|----------|-------|-------|---------|---------|---------|---------| concentration | acidity | acidity |
|           |                  | sugar (%)| time (h)     | sugar (g/L) | Conversion (%) | concentration % (v/v) | concentration (g/L) | productivity (g/L/h) | concentration (g/L) | (g/L) | (g/L) |
| 30(C)     | 3                | 20.8    | 74           | 0.9      | 95.7  | 11.0  | 88.0    | 1.19    | 6.2     | 2.1    | 0.15 |
| 30        | R1               | 20.4    | 61           | 0.2      | 99.0  | 11.8  | 94.4    | 1.55    | 3.1     | 2.9    | 0.20 |
| 30        | R2               | 20.2    | 43           | tr       | 99.5  | 11.9  | 96.0    | 2.23    | 4.1     | 2.4    | 0.14 |
| 30        | R3               | 20.6    | 28           | tr       | 99.5  | 11.5  | 92.0    | 3.29    | 4.4     | 3.1    | 0.12 |
| 25(C)     | 3                | 20.1    | 52           | 1.1      | 94.5  | 10.0  | 80.0    | 1.54    | 5.6     | 3.2    | 0.19 |
| 25        | R4               | 20.4    | 43           | 0.2      | 99.0  | 10.1  | 80.8    | 1.88    | 3.1     | 2.9    | 0.17 |
| 25        | R5               | 20.9    | 47           | 0.3      | 98.6  | 10.0  | 80.0    | 1.70    | 4.8     | 4.6    | 0.11 |
| 25        | R6               | 20.5    | 39           | tr       | 99.5  | 10.5  | 84.0    | 2.15    | 5.0     | 4.0    | 0.24 |
| 20(C)     | 3                | 20.6    | 71           | 1.4      | 93.2  | 9.42  | 75.3    | 1.10    | 5.5     | 3.5    | 0.26 |
| 20        | R7               | 20.1    | 52           | 0.8      | 96.0  | 10.7  | 85.6    | 1.65    | 3.0     | 2.1    | 0.14 |
| 20        | R8               | 20.7    | 50           | 0.4      | 98.1  | 10.1  | 80.8    | 1.62    | 4.3     | 3.6    | 0.16 |
| 20        | R9               | 21.1    | 35           | tr       | 99.5  | 10.0  | 80.0    | 2.29    | 4.7     | 4.5    | 0.20 |
| 15(C)     | 3                | 20.3    | 64           | 1.7      | 91.6  | 9.50  | 76.0    | 1.19    | 4.1     | 3.5    | 0.17 |
| 15        | R10              | 20.4    | 52           | 0.4      | 98.0  | 9.95  | 79.6    | 1.53    | 2.8     | 2.6    | 0.20 |
| 15        | R11              | 20.1    | 40           | 0.4      | 98.0  | 9.50  | 76.0    | 1.90    | 3.6     | 2.9    | 0.18 |
| 15        | R12              | 20.5    | 31           | tr       | 99.5  | 9.70  | 77.6    | 2.50    | 3.9     | 2.4    | 0.20 |

(C) = Control, fermentation batches with free yeast cells; (R) = Repeated batch fermentations with biocatalyst.

Tr = Traces
This decrease was probably due to the utilization of the peel sugar by the yeast cells. The peel pieces volume remained stable after seventh or eighth batch and was not disrupted significantly and remained intact throughout the fermentation experiments, which was mainly due to the unfermentable residual ligno-cellulosic and pectin matrix of peel pieces (Figure 5.6). It was observed that the viability of yeast cells was high (>90%) in the biocatalyst at the end of the fermentation when compared to conventional fermentation. This may be due to tolerance of the immobilized yeast cells to various stresses like ethanol concentration and heat shock.

The yeast population increased during the repeated batch fermentations and enumeration of immobilized viable cells after immobilization resulted in a yeast cell population of 6.42 CFU/g of mango peel biocatalyst and the amount of cells retained on the biocatalyst was about threefold higher than the amount of free cells in the broth. As the cell number increased, the decrease of surface on immobilized material led to detachment of few yeast cells from the immobilizing support and subsequent growth in the medium solution, which initially was devoid of yeast cells. The appearance of the yeast cells was observed in the medium after 25 h of fermentation. The detached cell biomass concentrations ranged from 2.8 to 5.0 g/L for the entire duration of the experiment at different temperatures, this was in agreement with results obtained on wine produced from yeast cells entrapped in corn starch gel (Kandylis et al. 2008). But, the concentrations of the detached cells in to the mango wine in the present study were less when compared to wines produced from immobilized sugarcane pieces (Reddy et al. 2010b). However the cell mass in the immobilized pieces was maintained constantly (Figure 5.5). It may probably be due to the new cells which are also adsorbed to the support. A two fold increase in fermentation rate was observed with immobilized cells in low temperature (15°C) and this, in turn, shortened the fermentation time when compared to the free cells.

Total and volatile acidities were in the ranges of 2.1 to 4.6 and 0.11 to 0.26 (g/L) respectively which were within the normal limits of the dry wines (4-6 g/L). In the present study, fermentation temperature and immobilization support did not affect the volatile acidity and total acidity. Kourkoutas et al. (2001) reported that there was a little
increase of total acidity due to the transfer of apple acids to the wine prepared with yeast immobilized on apple pieces, but there was no increase in the total acidity throughout the study. However they observed that total acidity was lowered slightly as the temperature drops from 9 to 1°C. This reduction can be attributed to the increase of crystallization of tartrate salts with the decrease in temperature.

![Graph](image)

**Fig.5.7.** Reduction pattern of mango peel biocatalyst volume as function of batch number.

The chemical analyses of the mango wine showed that the produced wine was similar to dry table wines with respect to alcohol content and residual sugar content (Table 5.2) as it was generally known that dry wines contain residual sugars generally below 1.5 g/L consisting mostly of pentoses such as arabinose, rhamnose, and xylose (Soleas et al. 1997).

5.4.3 Volatile by-products: As mango peel pieces were proved to be a suitable support for mango wine-making, particularly easy to use for immobilization, the study of the aroma through determination of the most abundant volatile by-products in the wine was essential.
Table 5.3. Effect of the use of immobilized yeast on volatile compounds at different temperatures.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Batch</th>
<th>Acetaldehyde (mg/L)</th>
<th>Ethyl acetate (mg/L)</th>
<th>I-Propanol (mg/L)</th>
<th>Isobutanol (mg/L)</th>
<th>Amyl alcohols (mg/L)</th>
<th>Total higher alcohols†</th>
<th>Methanol (mg/L)</th>
<th>Total volatiles‡ (g/L)</th>
<th>Glycerol (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>R1-3</td>
<td>30.2±1.2a</td>
<td>27.7±2.4bc</td>
<td>12.9±0.8b</td>
<td>50.4±1.5c</td>
<td>262±3.8a</td>
<td>326±2.4</td>
<td>155±2.4c</td>
<td>384±4.9</td>
<td>8.9±1.2c</td>
</tr>
<tr>
<td>25</td>
<td>R4-6</td>
<td>31.2±0.8a</td>
<td>28.0±2.3abcd</td>
<td>16.3±1.7c</td>
<td>51.1±0.4d</td>
<td>234±3.6f</td>
<td>301±1.4</td>
<td>147±3.8d</td>
<td>360±5.6</td>
<td>7.2±1.1e</td>
</tr>
<tr>
<td>20</td>
<td>R7-9</td>
<td>33.1±0.5b</td>
<td>26.9±0.9d</td>
<td>15.2±2.0c</td>
<td>47.9±1.4bc</td>
<td>201±5.6c</td>
<td>265±1.8</td>
<td>129±4.9c</td>
<td>325±5.5</td>
<td>5.6±1.2c</td>
</tr>
<tr>
<td>15</td>
<td>R10-12</td>
<td>38.5±1.2c</td>
<td>29.3±0.8d</td>
<td>19.5±1.2e</td>
<td>42.6±0.5a</td>
<td>147±4.9b</td>
<td>209±3.1</td>
<td>129±1.6bc</td>
<td>277±4.4</td>
<td>4.1±1.4d</td>
</tr>
<tr>
<td>30(C)</td>
<td>1-3</td>
<td>36.7±0.5c</td>
<td>29.2±1.6b</td>
<td>21.5±0.5g</td>
<td>49.3±2.6c</td>
<td>240±6.1g</td>
<td>311±1.8</td>
<td>114±4.7a</td>
<td>376±3.6</td>
<td>7.1±0.7e</td>
</tr>
<tr>
<td>25(C)</td>
<td>4-6</td>
<td>38.4±1.1d</td>
<td>22.2±1.4a</td>
<td>20.7±0.9f</td>
<td>47.4±2.1c</td>
<td>216±4.9e</td>
<td>284±1.3</td>
<td>115±2.5ab</td>
<td>345±4.1</td>
<td>6.4±0.5d</td>
</tr>
<tr>
<td>20(C)</td>
<td>7-9</td>
<td>36.2±1.3c</td>
<td>31.5±1.3bc</td>
<td>17.6±1.4d</td>
<td>45.2±1.6b</td>
<td>203±3.4d</td>
<td>266±1.7</td>
<td>126±5.5c</td>
<td>334±3.9</td>
<td>4.5±1.2b</td>
</tr>
<tr>
<td>15(C)</td>
<td>10-12</td>
<td>37.9±0.6d</td>
<td>38.7±1.7cd</td>
<td>12.4±1.8a</td>
<td>41.5±1.1a</td>
<td>184±4.6b</td>
<td>238±2.6</td>
<td>124±4.8c</td>
<td>315±5.5</td>
<td>3.9±0.7a</td>
</tr>
</tbody>
</table>

† Total higher alcohols = sum of I-Propanol, Isobutanol and Amyl alcohols (mg/L)
‡ Total volatiles = sum of Acetaldehyde, Ethyl acetate and Total higher alcohols, excluding Methanol.

(C) = Control, fermentation batches with free yeast cells; (R) = Repeated batch fermentations with biocatalyst.

Values not sharing a common superscript letter differ significantly at p ≤ 0.01 Duncan’s Multiple Range Test (DMRT)
The major compounds defining the overall volatile effects on wine aroma are acetaldehyde, ethyl acetate and higher alcohols such as 1-propanol, isobutyl alcohol, and amyl alcohols. The effect of temperature and the immobilization technique on the concentrations of these compounds in the produced mango wines are summarized in Table 5.3.

5.4.3.1 Higher alcohols: Higher alcohols or fusel alcohols are the largest group of aroma compounds in alcoholic beverages and are secondary products of alcoholic fermentation. Fusel alcohols have a strong pungent smell and taste. Although they exhibit a harsh, unpleasant aroma at the concentrations generally found in wine, below 350 mg/L they usually contribute to the desirable complexity of wine. The principal higher alcohols produced by yeast are the aliphatic alcohols such as n-propanol, isobutanol (2-methyl-1-propanol), active amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-methyl-1-butanol) commonly account for about 50% of the aromatic constituents of wine.

Among the higher alcohols, propanol and isobutanol were significantly decreased with decrease in temperature (Table 5.3). Formation of higher alcohols was decreased with the decrease in temperature and the products formed with low concentrations of higher alcohols are of good quality (Mallouchos et al. 2003). The results from this study are comparable with earlier immobilization studies using watermelon pieces, quince fruit and pear pieces (Reddy et al., 2008; Kourkoutas et al., 2001). The higher alcohol formation varies during fermentations and is mainly dependent on yeast strain and fermentation conditions.

The concentration of amyl alcohols decreased significantly with the decrease in temperature, which is a positive factor in wine quality, as they are considered as off-flavours (Mallios et al., 2004). In general, low temperature greatly reduced the amount of higher alcohols. These results show that the product is of improved quality because of low concentrations of higher alcohols. These observations are in agreement with the results of Kourkoutas et al., (2001) in wines from apple pieces as immobilizing agent.
5.4.3.2 Ethyl acetate: Ethyl acetate is one of the important volatile compounds and its presence imparts a significant effect on the organoleptic characteristics of the wine. It is the most important and abundant ester in wines and is considered that at low concentrations of ethyl acetate (50-80 mg/L) it contributes to wine olfactory complexity having a positive impact on wine quality, and only at concentrations >120 mg/L may spoil the bouquet with an unpleasant, pungent tang. And in addition, it was found that any factor that decreases the speed of fermentation like temperature, pH, and low oxygen conditions simultaneously increases the amount of ethyl ester and acetaldehyde (Kandylis et al., 2008). In the present study also the ethyl acetate concentration increased with decrease in temperature. Ethyl acetate concentrations in wine produced with apple pieces as immobilizing agent was relatively high up to 150mg/L (Kourkoutas et al., 2001). However in the present study, the concentration of ethyl acetate was <50 mg/L, and there was no indication of vinegar odour in the final product; on the contrary, it had a fruity aroma and a fine taste.

5.4.3.3 Other components: Acetaldehyde is one of the most important carbonyl compounds formed during degradation of sugars by yeasts and constitutes more than 90% of the total aldehyde content in wine. At low levels, it gives a pleasant fruity aroma, but at high concentrations (>100 mg/L) it possesses a pungent irritating odour, which is undesirable for table wines and were poorly appreciated by the wine tasters. The acetaldehyde concentration in wines usually ranges from 13 to 40 mg/L, low acetaldehyde concentrations were detected in the present study with a maximum of 38 mg/L (Table 5.3). However, it may reach 75 mg/L (Mallios et al., 2004) or up to 115 mg/L (Kandylis et al., 2008) in some batches, this could be due to either incomplete fermentation, or the presence of SO₂ in the grape must used may reveal the relatively high amounts of acetaldehyde in finished wines (Romano et al., 1994). The differences in acetaldehyde content in wines could also be attributed to the effect of temperature and immobilization on the activity of pyruvate decarboxylase and alcohol dehydrogenase, which are implicated in the biosynthesis of acetaldehyde by yeasts (Tsakiris et al., 2010).
Glycerol is the major fermentation product after ethanol and carbon dioxide in wines. Glycerol is a non-volatile and has no direct impact on the aromatic characteristics of wine. However it has a favourable effect on wine quality by contributing sweetness, fullness and smoothness to the wine. Glycerol is naturally found in wines and its concentrations in wines vary between 1 and 10 g/L. Glycerol production is influenced by many factors like yeast strain, fermentation temperature, sulphur dioxide concentration, agitation time and pH levels. In the present study the glycerol concentrations in batches with immobilized cells ranged from 4.4 to 8.9 g/L, however, it was low in batches with free cells, and ranged from 3.9 to 7.19 g/L (Table 5.3). It was observed that the glycerol concentration in all the fermentation batches with immobilized cells on mango peel was decreased with decrease in temperature, showing that the fermentation temperature plays an important role in glycerol formation. However the glycerol concentration obtained in the present study with mango wine was lower when compared to the glycerol concentration with grape wine for immobilized cells (11.9-14.9 g/L) and free cells (10.2-12.8 g/L) (Balli et al., 2003) but was higher when compared to mango wine with glycerol concentrations about 6.94 g/L (Kumar et al., 2009). The increased glycerol concentration in the mango wines produced by immobilized yeast on mango peel could be attributed to the nature of the supports, immobilization and yeast strain.

Methanol is not a major constituent in wines and has no direct sensory effect. The amount of methanol found in wine is primarily generated from the enzymatic breakdown of pectins. The methanol content in the present study ranged from 113.4 to 154.6 mg/L; however, in traditional grape wine fermentations the usual range of methanol content is less than 100 mg/L. Unlike most fruits, grapes are low in pectin. As a result, grape wine generally has the lowest methanol content of any fermented beverage. In the first 4 batch fermentations, the methanol concentrations in mango wines produced by immobilized cells (141.64-154.67 mg/L) were higher than those of free cells (114.24-126.32 mg/L) as expected. This could be attributed to the fact that the mango peel contained pectin substances, which after enzyme hydrolysis might release methanol. After that, a reduction in methanol concentration was observed and the
methanol content of the mango wines produced from 5th batch fermentations of must by immobilized cells remained at low levels similar to those of free cells. The methanol concentration, in general, was not affected by a reduction in incubation temperature (Table 5.3). Similarly, the formation of methanol was not affected by immobilization of cells as its formation was not due to metabolic activity of the yeast.

5.4.4 Sensory evaluation: After the chemical analyses, the beverage was subjected to sensory analysis to assess its acceptance among the consumers. Table 5.4 presents notes attributed to the beverage by 15 trained tasters, designated in the Hedonic scale of nine points (1 = dislike extremely; 9 = like extremely). The average values were recorded for the four evaluated attributes whereas the aroma is the one with slightly higher value, followed by taste, appearance and overall acceptance, with respective notes of 7.9, 7.7, 7.6 and 7.5. Tests indicated some improvement in aroma and taste of the mango wines produced by using cells immobilized on mango peels, particularly at low temperatures, when compared to mango wines produced from free cells (Table 5.4). This can be attributed to the reduction of amyl alcohols, which are off-flavor compounds, at lower temperatures and, therefore, an increase in the percentage of other aroma compounds on total volatiles. Mallouchos et al. (2003) reported that wines produced by immobilized cells on grape skins have better fruity aroma.

Table 5.4. Effect of the use of immobilized yeast on sensory characteristics.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Wine from free yeast cells</th>
<th>Wine from immobilized yeast on mango peels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>5.6±0.82</td>
<td>7.6±0.54 (P&lt;0.0243)</td>
</tr>
<tr>
<td>Aroma</td>
<td>6.1±0.25</td>
<td>7.9±0.73 (P&lt;0.0156)</td>
</tr>
<tr>
<td>Taste</td>
<td>6.9±0.81</td>
<td>7.7±0.24 (P&lt;0.1763)</td>
</tr>
<tr>
<td>General acceptance</td>
<td>6.7±0.67</td>
<td>7.5±0.61 (P&lt;0.2009)</td>
</tr>
</tbody>
</table>

Similar results were also reported by Garcia-Romero et al., (1999) that there was a considerable improvement in the wine sensory profile when fermentations were carried out in contact with skins of Airen white wine grapes because of the transfer of the precursors of volatile compounds like esters, aldehydes and alcohols in to wine. The
mango wines produced by immobilized yeast biocatalyst had a fine clarity at the end of fermentation with low free cell concentrations as well as characteristic pleasant soft aroma and fruity taste.

5.5 CONCLUSIONS

Yeast-mango-peel immobilized biocatalyst can be a good and effective system for mango wine fermentation at both low and room temperatures, as the mango wines produced by this had a potentially better aroma than that obtained from free-cell fermentation. The biocatalyst is economical, food grade and does not need special pretreatment before its use. Mango peel, which otherwise may pollute the environment can beneficially be used as an alternative cell immobilization support. This first study on the use of mango peel as an immobilization support for yeast during mango wine-making, showed the potentialities of this process. These results open possibilities to apply this process on other fermented beverages as well.