Chapter-V

Summary and Conclusions
5.1 SUMMARY

The process of making alcohol has been around since virtually as long as man has been on this earth, though it has been immensely refined and upgraded in recent years leading to much improved efficiency. The production process is carried out in sequence. Before the sequences or manufacturing steps can be carried out, a feed stock selection must be determined. Sugar content is one of the primary factors that are considered during selection stage. Since the demand of ethanol is ever increasing and many new potential uses await availability of ethanol at lower cost, the industry is always on the lookout for a cheap, economic, efficient and newer process technology.

A number of substrates have been evaluated by many researchers for the production of ethanol (Table 2.1 and 2.2). Cashew apple juice is one of the alternative substrates for the production of ethanol at economical cost and also it had good keeping quality and does not pose the problem of pretreatment.

The present studies established the potential usage of cashew apple juice as a cheaper and economical substrate. The biochemical data of the cashew apple juice were, 77.2 % moisture, 0.16 % protein, 0.20 % fat, 14.5 % total sugars, 4.9 % reducing sugars, 1.91 % tannin and 1.13 % ascorbic acid. The collected data may stimulate industrial exploitation by submerged fermentation technique using the novel substrate cashew apple juice for the production of ethanol.

A number of strains of Saccharomyces cerevisiae were subjected to screening for their ability to produce ethanol. Out of which 4 strains were found positive for ethanol production, S. cerevisiae NCIM 3090 gave the highest conversion of total sugars present in the medium. It is believed that the use of selected Saccharomyces cerevisiae NCIM-3090 is indispensable for the production of ethanol.
The production of ethanol in cashew apple juice with varying levels of substrate concentration (70 to 145 g/l) indicated that the optimum substrate concentration was 145 g/l (which was highest concentration of sugars present in the prepared cashew apple juice) and ethanol concentration of 19.79 g/l. These results proved the ethanol production from cashew apple juice.

Medium pH is one of the critical factors for successful ethanol fermentation. In the present investigation the mold yielded a maximum of 20.71 g/l of ethanol at pH 5.0. As the pH ranged from 3.0 to 5.0, the ethanol production was increased.

During the experimental study conducted for optimum temperature, the growth of the organism and ethanol production increased linearly between 25° to 30°C and gradually decreased after 30°C, with no product formation or growth at 50°C. The maximum production of 22.35 g/l of ethanol was produced at 30°C. Agitation at 150 rpm was optimized for maximum ethanol (23.86 g/l) production.

Seed culture is usually the best way of starting fermentation. Maximum ethanol production of 20.42 g/l was obtained at 4% (v/v) of inoculum level. The rate of sugar consumed and ethanol production were significantly affected as the inoculum size was increased from an initial level of 2 to 10% (v/v). Maximum amount of 21.96 g/l of ethanol was produced with cell suspension prepared from 48 h old slants. As the age of the slant increased the cell viability and germination properties change.

Enrichment of the medium with 2.0 g/l ammonium sulphate and 2.0 g/l of ammonium chloride proved promising and gave ethanol production of 36.92 g/l and 37.17 g/l respectively. 2.0 g/l of urea, 3.0 g/l of yeast extract, 4.0 g/l of peptone, 3.0 g/l of glycine and 4.0 g/l of casein hydrolysate were also equally good and ethanol production were 43.61 g/l, 42.22 g/l, 39.81 g/l, 36.23 g/l and 32.64 g/l respectively.
The effect of phosphate sources on ethanol production was studied by adding KH$_2$PO$_4$, K$_2$HPO$_4$, NaH$_2$PO$_4$ and Na$_2$HPO$_4$ and ethanol concentration observed were 31.14 g/l at 0.3 g/l, 32.42 g/l at 0.3 g/l, 30.48 g/l at 0.5 g/l and 31.98 g/l at 0.5 g/l respectively. Out of these four phosphate sources K$_2$HPO$_4$ gave highest ethanol yield by the end of 48 h of fermentation.

Trace metals are known to have a profound effect on the growth of Saccharomyces species, particularly in the production of organic acids. Mg$^{2+}$ added at a concentration of 0.06 g/l increased the production of ethanol from 21.81 g/l to 31.42 g/l, when compared to that without any added Mg$^{2+}$. Ca$^{2+}$ at a concentration of 0.04 g/l increased the ethanol from 21.92 g/l to 31.21 g/l; Fe$^{2+}$ added at a concentration of 0.06 g/l increased the ethanol from 20.89 g/l to 27.44 g/l, when compared to that without any added Ca$^{2+}$ and Fe$^{2+}$ respectively. The production decreased when 0.1 g/l of Ca$^{2+}$ and Fe$^{2+}$ were added to the medium. Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Al$^{3+}$ and Cu$^{2+}$ at a concentrations of 0.04 g/l, 0.03 g/l, 0.004 g/l, 0.004 g/l, 0.006 g/l and 0.004 g/l respectively increased the yields of ethanol upto 26.56, 26.39, 26.11, 26.92, 27.61 and 26.93 g/l respectively.

Addition of EDTA of 0.6 g/l, sodium potassium tartrate of 0.8 g/l, diethylenetriamine penta acetic acid of 0.6 g/l and nitrilotriacetic acid of 0.4 g/l to fermentation medium increased the ethanol concentrations to 29.26 g/l, 28.69 g/l, 27.82 g/l and 26.51 g/l respectively, these chelators formed insoluble complexes and acted as metal ion buffers, releasing the metal ions at low concentrations commensurate with the requirements of the microorganisms used for fermentation. 4 ppm of biotin added to the medium increased ethanol concentration from 25.57 to 31.71 g/l.
Medium-I was formulated after standardization of physico-chemical and nutritional parameters. This was used for enhancement of ethanol production at shake flasks level and 5 l. bioreactor. The ethanol produced was 61.50 g/l equal to 82.98 % of its theoretical yield at shake flasks level and 62.61 g/l equal to 84.48 % of its theoretical yield in 5 l. bioreactor. The ethanol yield in the bioreactor is more when compared to the shake flasks. Agitation had critical influence on the rates of consumption of sugar and ethanol production at shake flasks level as well as bioreactor level. It was 150 rpm at shake flasks level and 200 rpm at bioreactor level.

Experimental design using response surface methodology (RSM) was used in the present investigation to optimize the media constituents and conditions of fermentation. The derived optimum values of media components are pH of 4.9, temperature at 30.26°C, urea of 2.2012 g/l, K$_2$HPO$_4$ of 0.3126 g/l, seed culture volume of 4.35 % (v/v), time of fermentation of 50.73 h, EDTA of 0.6408 g/l, magnesium of 0.6408 g/l, agitation of 206.5 rpm, biotin of 4.2829 ppm, glycine of 3.1258 g/l and seed culture age of 47.19 h. Medium II was designed by incorporating the statistically optimized medium components and conditions of fermentation on medium- I. Statistically optimized cashew apple juice gave the best results from an economic point of view, leading to the production of 70.31 g/l of ethanol at 48 h of fermentation utilizing 94.87 % of sugars initially present in the medium. Comparison of yields with those reported from other substrates indicated that conversions with cashew apple juice were equally good (Table 2.2).

Growth kinetics were evaluated and in general followed the Monod relation. A maximum specific growth rate of 0.25 h$^{-1}$ was observed.
The experimental results have indicated that maximum yield cannot be attained with single control variable. However with an accurate RSM measurement, the yield was enhanced and then results were improved. The production of ethanol was successfully optimized by physico-chemical and nutritional parameters using multivariable RSM.

The studies indicate that higher quality alcohol can be produced from cashew apple juice. It is an economical and novel substrate for industrial production of ethanol by *Saccharomyces cerevisiae* NCIM-3090. The high yields of ethanol achieved in conjunction with well accepted economic advantages of this substrate may also fulfill the need for reduction in the cost of ethanol.
5.2 CONCLUSIONS

- Cashew apple juice was chosen as a substrate for the production of ethanol. It is a cheaper and novel substrate and it is also interesting to note that the cashew apple juice can serve in ethanol production since it contains all the essential nutrients.

- The biochemical data evaluated indicates that the substrate can be directly used for ethanol production without any pretreatment.

- *Saccharomyces cerevisiae*-3090 strain was selected for the present study based on high ethanol production efficiency at shake flask level.

- The physical parameters for the fermentation of cashew apple juice such as substrate concentration, pH, temperature, agitation, fermentation time, seed culture volume and seed culture age were found to play a prominent role in ethanol production.

- The chemical parameters for the fermentation of cashew apple juice such as nitrogen source, phosphate source, trace elements, metal chelating agents and vitamins were also found to play a prominent role in the production of ethanol. Addition of nitrogen, phosphate and vitamins resulted in a considerable increase in ethanol production. When the medium was enriched with above elements, a profound increase of ethanol production and biomass were observed.
Summary and conclusions

• When the optimized medium-I was used for ethanol production in shake flasks and bioreactor, the production of ethanol in bioreactor was observed to be more and the reducing sugars consumed by the yeast were 84.48%. The final ethanol concentration of 61.50 g/l at shake flasks level and 62.61 g/l in bioreactor studies in 48 h of fermentation were obtained.

• Taking the optimized values of shake flasks experiments, a central composite design was formulated with 20 experiments. The media constituents and conditions were optimized with the above factorial design and formulated medium-II. The system was able to utilize total sugars completely in cashew apple juice, after 48 h of fermentation with an ethanol concentration of 70.31 g/l equal to 94.87% of its theoretical yield.

• The central composite design was used in order to determine the co-optimum level of the factors, as well as to provide an insight into the interactions among these factors during production of ethanol.

• The results were compared with RSM studies, which was suitable for higher production of ethanol.

• RSM is a powerful technique to optimize a response of media constituents and conditions for ethanol production.

• Growth kinetics were evaluated and the results followed Monod kinetics.

• The work done on ethanol production from cashew apple juice was encouraging and paved a way for industrial exploitation of submerged fermentation process.