Chapter IV

Immobilization of β-galactosidase in k-carrageenan and its application in the hydrolysis of raffinose oligosaccharides

4.1. INTRODUCTION

Enzyme immobilization provides many advantages over use of enzymes in soluble form. The ability to make high cost enzymes reusable has meant that immobilized enzymes have attracted a great deal of attention. Entrapment of biocatalysts within a polymeric matrix is superior in its wide applicability for the immobilization of not only single enzyme but also several enzymes. In the gel entrapping method, the enzyme is physically entrapped in the matrices of gel network. From a more positive viewpoint, an entrapped enzyme is not subjected to bacterial attack (O’Driscoll, 1976).

The entrapment method most often used for the gels agar/agarose, k-carragenan, polyacrylamide, and alginate. k-Carrageenan favored by many workers mainly because of nontoxicity, mild, cheap and simple method of entrapment, which preserve the integrity of the immobilized biocatalysts (Audet et al., 1988). Among the technique for immobilizing living cells and enzyme, gel entrapment in natural polymers such as k-carrageenan is favoured by many scientists (Takata et al., 1977; Luong, 1985; Godia et al., 1987). k-Carrageenan gels have been used with lactic acid bacteria (Prevost et al.,
1985). The k-carrageenan method was chosen since it is more advantageous for industrial purpose than the polyelectrolyte and alginate method (Wada et al., 1979).

k-Carrageenan is one of the most widely used immobilization matrix. k-Carrageenan is a linear, sulfated polysaccharide extracted from marine red algae. The primary structure is made up of alternating $\alpha$(1,3)-D-galactose-4-sulphate and $\beta$(1,4)-3,6-anhydro-D-galactose residues. k-Carrageenan forms gels, undergoing a coil (disordered sol state) to helix (ordered state) transition, triggered by a reduction in temperature and/or through ionic interactions (De Ruiter and Rudolph, 1997).

Although $\alpha$-galactosidase is isolated from wide range of microorganisms, interestingly there are very few reports of $\alpha$-galactosidase immobilization. Therefore $\alpha$-galactosidase immobilization was pursued. A.oryzae with the GRAS status (generally regarded as safe) status was obvious choice as the source of enzyme. A.oryzae $\alpha$-galactosidase is used in food products and is relatively inexpensive compared to enzymes from other sources (FAO/WHO JECFA, 1988; Albaryrak and Yang, 2002).

$\alpha$-Galactosidase was immobilized in carrageenan and carrageenan: locust bean gum (2:1) ratio. The conditions for the immobilization of enzyme were optimized and an effort was made to reduce the raffinose family sugars in the legume-based products like soymilk and black gram milk. The immobilized enzyme was used in three modes i.e. batch, repeated batch and continuous. For continuous studies fluidized bed reactor was designed and flow rate was optimized to reduce the raffinose family sugars in soymilk and black gram milk.

4.2. MATERIALS AND METHODS
4.2.1. Chemicals

The chemicals used are of analytical grade and are listed in Table 4.1.

4.2.2. Collection of seed sample

Black gram cultivars and soybean cultivars were collected from Pulse Research Station, ARS, Gulbarga. Local varieties of soybean and black gram seeds were procured from local market, Gulbarga. All samples were cleaned from dust and foreign particles and stored in polythene bags.

4.2.3. Preparation of buffers

Buffers were prepared according to Gomori (1951). All the preparations were carried out using double distilled water and 0.5M solution of potassium hydrogen phthalate was used to standardize pH meter.

4.2.4. Fungal strain

The *Aspergillus oryzae* used for this study was isolated by Prashanth and Mulimani (2005). The fungi produced extracellular α-galactosidase. It was identified at Indian type culture collection, Indian Agricultural Research Institute (IARI) New Delhi. It was maintained on PDA (Potato-Dextrose-Agar) slants and stored at 4°C. The fungus was subcultured periodically.

4.2.5. Enzyme production

4.2.5A. Inoculum preparation

The spore suspension was prepared by scrapping the spores of *Aspergillus oryzae* from 7 day grown slants. The spore suspension was prepared by adding sterilized distilled water containing 0.01% Tween -80.

4.2.5B. Medium
The following medium was used. The composition of medium was as follows (g/l):

- $K_2HPO_4$: 3g
- $MgSO_4 \cdot 7H_2O$: 0.5 g;
- Yeast extract: 5g
- Guar gum: 20g.

The pH of the medium was adjusted to 5.5.

4.2.5C. Submerged fermentation

For batch culture 50 ml of basal medium was taken in 250ml Erlenmeyer flask, and sterilized. After autoclaving, the flasks were inoculated with spores ($2 \times 10^6$) of $A. oryzae$. The flasks were incubated at 37°C for 5 days on an orbital shaker at 120 rpm. The mycelium was removed from culture broth by filtration through muslin cloth followed by Whatman No.1 filter paper and the clear supernatant phase was used as crude $\alpha$-galactosidase.

4.2.6. Ammonium sulphate precipitation of $\alpha$-galactosidase

To the crude $\alpha$-galactosidase, 80% ammonium sulphate was added and the mixture was allowed to stand for overnight at 4°C. The precipitate containing $\alpha$-galactosidase was recovered by centrifugation at 15,000 rpm for 20 min at 4°C. The precipitate thus obtained was dissolved in minimal amount of buffer (0.1 M acetate buffer, pH 4.8).

For convenience and clarity, processing of soymilk and black gram milk with free and immobilized $\alpha$-galactosidase is discussed under the following heads.

4.2.7. Use of soluble and immobilized $\alpha$-galactosidase from $A. oryzae$ in the processing of
a) Soybean milk
b) Black gram milk

4.2.8. α-Galactosidase was immobilized in k-carrageenan and k-carrageenan:locust bean gum (2:1). Therefore the above section was subdivided into the following headings:

i) k-Carrageenan immobilization and application of carrageenan immobilized α-galactosidase in soymilk treatment.

ii) k-Carrageenan:locust bean gum (2:1) immobilization and application of immobilized α-galactosidase in soymilk/black gram milk treatment

4.2.9. Use of soluble α-galactosidase from A. oryzae in the processing of soymilk and black gram milk.

This section is divided into following heads.

4.2.10. Preparation of soymilk

Soybean seeds were ground to flour. The soybean flour was defatted with hexane (1:1 W/V). Soymilk was prepared according to the method of Mulimani and Ramalingam (1995). The fat free soybean flour was suspended in 10 volume of distilled water and heated to boiling. Undissolved residue was separated from soymilk by centrifugation for 5 min at 5000 rpm. The supernatant containing soymilk was stored at 4°C till further use.

4.2.11. Preparation of black gram milk

Black gram milk was prepared from black gram in the same way as that of soymilk as described above.
4.2.12. Extraction of oligosaccharides in soymilk/black gram milk

Soymilk/black gram milk (15 ml) was poured into 35 ml of absolute ethyl alcohol and centrifuged at 6000 rpm for 15 min at 37°C. The centrifugate was concentrated and dissolved in 15 ml of distilled water. The amount of sucrose, raffinose, stachyose, verbascose and ajugose were estimated by the method of Tanaka et al., (1975) as described in chapter III, Page No. 62.

4.2.13. Sample preparation for HPLC.

Sample was prepared according to the method of Mulimani and Ramalingam (1995) 50 ml enzyme treated soymilk was taken and the enzymatic reaction was stopped by placing the mixture in a boiling water bath for 15 min, followed by the addition of 2 ml of 0.2 M barium hydroxide and 2 ml of 0.18 M zinc sulphate. The precipitated protein was removed by centrifugation and the supernatant thus obtained was subjected to HPLC.

4.2.14. Separation of oligosaccharides by TLC.

Oligosaccharides were separated by using 0.2 mm thick plates and estimated by Tanaka et al., (1975) as described in the chapter II, Page No.44.

4.2.15. Separation of oligosaccharides by HPLC

HPLC analysis was performed with Shimadzu (Shimadzu Corporation, Japan) equipment as described in the chapter II, Page No.45.

4.2.16. \( \alpha \) -Galactosidase assay

\( \alpha \) -Galactosidase assay was carried out according to the method of Dey and Pridham (1972) as described in the chapter III, Page No.66.
4.2.17. **Protein estimation**

Protein was estimated by the method of Lowry *et al.*, (1951) as described in the chapter II, Page No.37.

4.2.18. **Immobilization of α-galactosidase in k-carrageenan and k-carrageenan: locust bean gum and their application in the reduction of raffinose family sugars in soymilk/black gram milk.**

4.2.18A. **Immobilization of α-galactosidase in k-carrageenan**

Entrapment in k-carrageenan was carried out by the method of Tosa *et al.*, (1979). 32 ml of enzyme (100 mg) was dissolved in 32 ml of buffer containing 3.4 g k-carrageenan at 37°C. The mixture was cooled at around 10°C for 30 min. This solution was dropped into a solution containing cold 0.3 M KCl at a constant speed. Bead-type gels of 3 mm in diameter were obtained by this procedure. k-Carrageenan entrapped beads were treated for 3 min in 1% glutaraldehyde. The beads were filtered off, washed with sterile water and stored at 4°C until used.

4.2.18B. **Immobilization of α-galactosidase in carrageenan:locust bean gum**

Ammonium sulphate precipitated α -galactosidase was entrapped in k-carrageenan. Entrapment in k-carrageenan was carried out by the modified method of Audet *et al.*, (1988). 10ml (6.2U) enzyme and 2:1 g carrageenan:k-carrageenan was dissolved in 60 ml at 4°C. This solution was dropped into a solution containing cold 0.3 M KCl at a constant speed.

4.2.19. **Activity yield**

The activity yield of α -galactosidase immobilized in k-carrageenan and k-carrageenan:locust bean gum was calculated. The activity yield (%) was defined by
Activity of immobilized enzyme
AY = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100

4.2.20. General properties of free and immobilized $\alpha$-galactosidase

4.2.20A. Optimum pH

In order to determine optimum pH, 100 µl of suitably diluted (in 0.2 M acetate buffer of pH 4.8) ammonium sulphate precipitated enzyme was incubated with 100 µl 2.0 mM pNPG in different buffers (800 µl) having pH range from 3.6 to 6.2 for 15 min at 37°C. The following range of buffers was used: pH 3.6-5.6 (acetate), pH 5.8–6.2 (citrate-phosphate). For immobilized enzyme 10 mg of beads were used instead of free enzyme.

4.2.20B. Optimum temperature

Optimum temperature was determined by incubating 100 µl of suitably diluted enzyme (10 mg beads for immobilized enzyme) + 800 µl of buffer (0.2M, pH 4.8)+ 100 µl of pNPG (2mM) for 15min at different temperatures ranging from 5° to 65°C.

4.2.20C. Thermostability

For determination of thermal stability the enzyme (10 mg of immobilized beads) was incubated with the substrate without any stabilizers at 50°C for different incubation periods. Residual activity in each sample was calculated by doing assay against enzyme control sample at pH 4.8 and 50°C.

4.2.21. Treatment of soymilk by soluble and immobilized enzyme

4.2.21A. Batch reaction

The batch reactions were performed for both soluble and immobilized enzymes at different incubation periods. For the reaction involving soluble enzyme, enzyme (with known units of activity) was added to 60 ml of soymilk in Erlenmeyer flaks (250 ml). For
the reaction involving immobilized enzyme, appropriate amount of weighed immobilized enzyme was added to 60 ml of soymilk. The hydrolysis reaction was carried out at 50°C in an incubator shaker (200 rpm) at different incubation periods i.e. 2, 4, 8, and 12h. After the incubation period an aliquot of the reaction mixture was taken out and kept in boiling water bath for 10 minutes to arrest the enzyme reaction. Afterwards the sample was analyzed for degradation of oligosaccharides. The control experiments were performed in the same manner with 0.2M acetate buffer (pH 4.8) replacing the enzyme.

4.2.21B. Repeated batch

Soymilk (60 ml) and suitable amount of immobilized enzyme beads were taken in 250 ml Erlenmeyer flasks and were kept in an incubator shaker (200 rpm) maintained at 50°C. After every incubation period i.e. 4 h an aliquot of soymilk sample was taken and its oligosaccharide concentration was determined. The beads were separated by filtration on the sinter, washed with sterile distilled water and transferred into fresh batch of soymilk (60 ml) for another 4h incubation.

4.2.21C. Continuous reaction

Design of fluidized reactor

For the two matrices i.e. k-carrageenan, k-carrageenan:locust bean gum, fluidized bed reactor studies were carried out. The experimental set up was similar for the two matrices.

Fluidized bed reactor studies were carried out in jacketed glass column of 75 cm in length and 1.5 cm in diameter, with bed volume of 130 ml. The jacket temperature was maintained at one constant temperature depending upon the bead strength, the jacket temperature was maintained with the help of water bath from Julabo, Germany. The feed
solution either soymilk or black gram milk preheated in a water bath was introduced from the bottom of the column through a peristaltic pump (Amersham Pharmacia Biotech, Sweden) and the product was withdrawn from the top of column. The immobilized beads containing α-galactosidase from A. oryzae were packed in the column. The upward substrate stream fluidized the beads filled up in the column. Different flow rates (ml.h⁻¹) were used for the degradation of oligosaccharides in soymilk/black gram milk. The outlet stream was continuously collected from the frontal end of the column in a container. The effluents were analyzed for the degradation of oligosaccharides.
4.3. RESULTS AND DISCUSSION

4.3.1. Raffinose family oligosaccharide content in soybean cultivars

Oligosaccharide content of the raw soybean seeds is presented in the table 4.2. The TLC photograph showing oligosaccharide separation is shown in figure 4.1. The data from the raw samples showed that among the oligosaccharides, concentration of stachyose was the highest among all soybean cultivars. The cultivar implies that the local variety and Monetta showed highest concentration of stachyose and MACS-58 cultivar had the least concentration of stachyose. The raffinose concentration of the local variety was highest and the JS-335 variety had the least amount of sucrose. It is evident from the table 4.2. that the local cultivars had the highest concentration of stachyose, raffinose and sucrose compared to the other five cultivars analyzed. The relative levels of raffinose and stachyose found in our study were comparable with those presented by other workers (Hymowitz et al., 1972; Tansui et al., 1972) while the levels of sucrose were lower, compared to the values reported for sucrose in soybean by other investigators (Tanaka et al., 1975; Kawamura, 1954; Hymowitz et al., 1972). The lower level of sucrose could be attributed to (1) difference in the cultivars and (2) specific methodology used for the estimation of oligosaccharides separated from the concentrated sugar syrup.
PART-A

k-Carrageenan

4.3.2. Immobilization of α-galactosidase in k-carrageenan

Carrageenans are naturally occurring hydrocolloids consisting of high molecular weight linear-sulfated polysaccharides. k-Carrageenan is a polysaccharide prepared commercially by extraction from red algae seaweeds and are widely used in the food and cosmetic industries as a gelling, thickening and stabilizing agent. The main sources of carrageenans are species of Chondrus, Gigartina, Eucheuma, Iredeae and Hypnea. There are three principal types of natural carrageenans, i.e., kappa (κ), iota (ι), and lambda (λ), only the kappa and iota are suitable as a support for biocatalyst immobilization; the k-form is considered to be most suitable for immobilization. The molecular structure of k-carrageenan is shown in figure. 4.2. Carrageenans are mainly composed of 3, 6-anhydro-α-D-galactose and their ester sulfate derivatives. Since it is used as a food additive, it is suitable for the immobilization of enzymes, which have applications in food processing industries (Tosa et al., 1979). Its molecular weight ranges from 100000-800000. k-Carrageenan may be brought to gel consistency by cooling. Gelation may also occur by contact with a solution containing gel-inducing reagents such as K⁺, NH₄⁺, Ca²⁺, Cu²⁺, Mg²⁺, Fe³⁺, amines and water-miscible organic solvents. Both procedures, cooling and/or contact with an aqueous solution containing K⁺ or NH₄⁺ are very easy to carry out.

Carrageenans, which can form hydrocolloidal gels, are doing so because of double-helix formation (Figure. 4.3). At temperatures over the melting point of the gel thermal agitation overcomes the tendency to form helices, and the polymer exists in solution as a random coil. On cooling, a three-dimensional polymer network builds up
with double helices forming junction zones between the polymer chains (gel I). Further cooling leads to aggregation of these junction zones (gel II).

In k-carrageenan, the pore size of the gel matrix is small enough to prevent higher molecular weight compounds such as enzymes from leaking out from gel lattice, although the lower molecular weight substrates and products can pass through the gel. The k-carrageenan method is more advantageous for industrial purposes than the polyacrylamide and/or alginate methods. (Wada et al., 1979).

4.3.3. General properties of free and k-carrageenan immobilized α-galactosidase

4.3.3A. Effect of pH on free and k-carrageenan immobilized α-galactosidase

The pH dependence of the relative activity of the immobilized α-galactosidase was compared with that of native enzyme. The effect of pH on soluble and immobilized enzyme is depicted in figure. 4.4. The optimum pH value of soluble α-galactosidase was 4.8. Cruz and Park (1982) made similar observation that the optimum pH of 4.8 for the soluble α-galactosidase from Aspergillus oryzae. In k-carrageenan immobilization, the optimum pH for the immobilized α-galactosidase was similar to that of the soluble enzyme. Mansour and Dawoud (2003) reported that invertase from Saccharomyces cerevisiae showed optimum activity at pH 4.6, with no shift in optimum pH for the immobilized enzyme. Godbole et al., 1990; Arruda and Vitolo (1999) reported that the invertase activity was found to vary with the pH values. The optimum pH for the immobilized invertase activities was 4.6 with no shift from the optimum pH of the soluble enzyme.
4.3.3B. Effect of temperature on free and k-carrageenan immobilized $\alpha$-galactosidase

The optimum temperature was 50°C for native enzyme. k-Carrageenan entrapped enzyme showed the optimum temperature at 53°C (Figure. 4.5). Thananunkul et al., (1976) made a similar observation that $\alpha$-galactosidase from *M. vinacea* had an optimal activity at 50°C and upon immobilization in polyacrylamide its optimum temperature increased to 55°C. Prashanth and Mulimani (2005) have reported that $\alpha$-galactosidase from *Aspergillus oryzae* had an maximum activity at 50°C, whereas Ca-alginate immobilized enzyme had an optimum activity at 57°C. Arica et al., (1996) speculated that hydrophobic interactions and other secondary interactions of the immobilized enzyme might impair conformational flexibility necessitating higher temperatures for the enzyme molecules to reorganize and attain a proper conformation for its functioning and binding of the substrate. Farag and Hassan (2004) showed that keratinase from *Aspergillus oryzae* had an optimum temperature 50°C upon immobilization, exhibited optimum activity at 60°C, the increase in the optimum temperature upon immobilization indicate some change in the physical properties of the enzyme molecule. k-Carrageenan immobilization system could protect the enzyme of thermal effects allowing activity at 53°C. This is suitable since high temperature optimum diminishes microbial contamination during treatment of soymilk.

4.3.3C. Thermostability of free and k-carrageenan immobilized $\alpha$-galactosidase

Figure. 4.6 displays the effect of thermostability on $\alpha$-galactosidase. Thermal stability experiments were carried out with free and immobilized $\alpha$-galactosidase, which were incubated without any stabilizers at 50°C. After 24h, 44% activity was retained by
free enzyme, whereas k-carrageenan immobilized enzyme retained 67% activity. Batsavola et al., (1987) have similarly reported that β-galactosidase immobilized in PVA was considerably more stable than soluble enzyme. Enhanced thermostability was reported, naringinase is immobilized in Ca-alginate gel (Puri et al., 1996) and tyrosinase is entrapped Cu-alginate (Munjal and Sawney, 2002). Schnapp and Shalitin (1976) have attributed the greater stability of immobilized enzymes to the stabilizing effects of immobilization.

4.3.4. Use of soluble and immobilized α-galactosidase from A. oryzae in the processing of soymilk and black gram milk.

α-Galactosidase immobilized in k-carrageenan was tested for its ability to reduce the raffinose family sugars in legume based product soymilk.

4.3.5. Treatment of Soymilk

4.3.5A. Batch reaction

The identity of sucrose, raffinose, and stachyose in soymilk was further confirmed by HPLC, comparing the retention time of standards with the retention times of peaks in the sample. On the system used, raffinose eluted at 20.5 min and stachyose eluted at 31.0 min. Soymilk sample initially contained 583 mg of total oligosaccharide per 100 ml, out of which 467 mg stachyose and 122 mg raffinose were the main constituents. The hydrolysis of raffinose family sugars by free and immobilized α-galactosidase from A. oryzae is shown in figure.4.7. In the batch experiments, the free and immobilized enzyme was incubated with soymilk at different incubation periods i.e. 2, 4, 8 and 12 h. HPLC analysis of enzyme treated soymilk for 4 h indicated the hydrolysis of raffinose family sugars (Figure.4.8). The 2 h incubation with free and immobilized enzyme resulted in
88% and 75% hydrolysis respectively. After 8 and 12 h incubation free α-galactosidase led to 91% and 95% degradation whereas immobilized α-galactosidase resulted in 82% and 84% reduction in raffinose family oligosaccharides in soymilk. In the batch, more than 80% oligosaccharide hydrolysis occurred within 2 h incubation for free enzyme suggested that prolonged incubation does not significantly increase the percent hydrolysis (Figure 4.7). Further it was observed that, for free enzyme incubation beyond 12 h did not significantly increase the extent of hydrolysis. The soluble enzyme showed better degradation. The immobilized enzyme showed lesser degradation, which could be due to diffusional limitation (i.e. resistance of substrate to diffuse into the immobilization matrix and resistance of the products to diffuse out) (Abdel-Naby et al., 1999). Hydrolysis of 76% of raffinose family sugars in soymilk by immobilized enzyme for 12h is the highest reduction so far among the other reports on polyacrylamide immobilization of α-galactosidase (Thananunkul et al., 1976; Thippeswamy and Mulimani, 2002). Higher oligosaccharide hydrolysis by free enzyme may occur because of better access of substrate to the free enzyme. Thananunkul et al., (1976) have reported 50% hydrolysis whereas Thippeswamy and Mulimani (2002) have reported 71% hydrolysis of RFOs after 12 h incubation of soymilk with α-galactosidase immobilized in polyacrylamide. Almost complete removal of oligosaccharides has been reported by using purified α-galactosidase from A. saitoi (Sugimoto and Van Buren, 1970), crude α-galactosidase from A. oryzae (Cruz and Park, 1982) and G. fujikuroi (Mulimani and Ramalingam, 1995). But these free enzyme preparations were not reusable, which made the process economically unviable.
4.3.5B. Repeated batch reaction

The operational stability of the immobilized $\alpha$-galactosidase was evaluated in a repeated batch process. The immobilized beads were used four times repeatedly at an interval of 4 h incubation period. Thus immobilized enzyme retained its activity well and the leakage of entrapped enzyme was evidently not serious. A reduction of RFOs for 77, 72, 68 and 61% was obtained after each successive incubation period (4 h) i.e. four consecutive cycles. It was observed that even after triple use, immobilized $\alpha$-galactosidase hydrolyzed 61% of oligosaccharides in soymilk.

There is only one report of repeated use of immobilized $\alpha$-galactosidase. Thananunkul et al., (1976) repeatedly used undisrupted mycelium of $M$. vinacea as source of $\alpha$-galactosidase to treat soymilk. Thananunkul et al., (1976) have reported 65.8, 61.8 and 57.2% degradation for the first three cycles, which is less, compared to the percent degradation obtained in the present study. They reported that after triple use, $\alpha$-galactosidase in the mycelium hydrolyzed 47.2% of the oligosaccharides in the soymilk, whereas the immobilized $\alpha$-galactosidase in the present study hydrolyzed only 42% of oligosaccharide over the same incubation period.

4.3.5C. Continuous reaction

The use of an immobilized enzyme makes it economically feasible to operate an enzyme process in a continuous mode. A fluidized bed reactor was constructed to explore the practicability of using the k-carrageenan entrapped enzyme in continuous system. The continuous degradation of oligosaccharides in soymilk was carried out in a fluidized bed reactor employing $\alpha$-galactosidase immobilized in k-carrageenan. The photograph of fluidized bed reactor is shown in figure 4.9. At 50 °C, oligosaccharide reduction in
soymilk was 92, 85, 68 and 55% for 25, 50, 75 and 100 ml h\(^{-1}\) flow rates respectively. The results suggested that a low flow rate (25 ml h\(^{-1}\)), i.e. a higher retention time gave a higher percent degradation. Optimal flow rate is one of the important parameter for the effective operation of fluidized column reactor (Sahasrabudhe et al., 1988).

Thananunkul et al., (1976) reported that fluidized bed containing α-galactosidase-acrylamide gel complex hydrolyzed 60% of the raffinose and stachyose in soymilk but at a flow rate of 30 ml h\(^{-1}\). Thippeswamy and Mulimani (2002) reported that oligosaccharide degradation of 84% at a flow rate of 25 ml h\(^{-1}\). Prashanth and Mulimani (2005) reported that oligosaccharide degradation of 90% at a flow rate of 40 ml h\(^{-1}\). Immobilized α-galactosidase from A. oryzae shows high optimum temperature and showed better operational stability by being stable even after being used 4 times. α-Galactosidase has been previously immobilized in polyacrylamide for the hydrolysis of oligosaccharide in soymilk (Thananunkul et al., 1976; Thippeswamy and Mulimani, 2002). But the usage of polyacrylamide in the process raises considerable apprehension because of the toxicity.
PART-B

k-Carrageenan:locust bean gum

4.3.6. Immobilization of $\alpha$-galactosidase in k-carrageenan:locust bean gum

Among the techniques for immobilizing living cells and enzymes, gel entapment in natural polymers such as alginites and k-carrageenan is favored by many workers. This is mainly due to the non-toxicity of these mild, cheap and simple methods of entrapment, which preserve the integrity of the immobilized biocatalysts. Carrageenan consists of sodium, potassium, magnesium and calcium sulfate esters of galactose and 3,6-anhydrogalactose units. It is a wholly natural ingredient, which is obtained from certain species of the red seaweed, class Rhodophycead. Popular sources for carrageenan are the *Chondrus erispus*, *Eucheuma cottonii* and *Euchema spinosum* species.

Locust bean gum is extracted from the seed of the carob tree (*Ceratonia siliqua*). Locust bean gum consisting of a (1-4) linked $\beta$-D-mannopyranose backbone with branchpoints from their 6-positions linked to $\alpha$-D-galactose (i.e.1-6-linked $\alpha$-D-galactopyranose)(Figure. 4.10). There are about 3.5 (2.8 – 4.9) mannose residues for every galactose residue. k-Carrageenan is a polysaccharide and its enrichment with locust bean gum significantly modifies the mechanical properties of the gel especially for the ratio k-carrageenan:LBG (2:1) (Ridout and Brownsey, 1985; Takata *et al.*, 1977).

4.3.7. General properties of free and k-carrageenan: locust bean gum immobilized $\alpha$-galactosidase

4.3.7A. Effect of pH on free and k-carrageenan: locust bean gum immobilized $\alpha$-galactosidase

The effect of pH on soluble and immobilized enzyme is depicted in figure.4.11. The optimum pH value of the soluble and k-carrageenan immobilized enzyme was 4.8.
Mansour and Dawoud (2003) reported that invertase from *Saccharomyces cerevisiae* showed optimum activity at pH 4.6, with no shift in optimum pH for the immobilized enzyme. Godbole *et al.*, 1990; Arruda and Vitolo (1999) reported that the invertase activity was found to vary with the pH values. The optimum pH for the immobilized invertase activities was 4.6 with no shift from the optimum pH of the soluble enzyme.

4.3.7B. Effect of temperature on free and k-carrageenan: locust bean gum (2:1) immobilized α-galactosidase

The temperature optima of the soluble and alginate entrapped enzyme preparation was determined by assaying the enzyme activity at indicated temperatures. Optimum temperature for the activity of k-carrageenan:locust bean gum entrapped enzyme was shifted to higher value than that of soluble enzyme (Figure.4.12). The maximum activity of entrapped enzyme was obtained at 55°C as compared to 50°C for the soluble enzyme. Arica *et al.*, (1996) speculated that hydrophobic interactions and other secondary interactions of the immobilized enzyme might impair conformational flexibility necessitating higher temperatures for the enzyme molecules to reorganize and attain a proper conformation for its functioning and binding of substrate. The k-carrageenan:locust bean gum immobilization system could protect the enzyme of thermal effects allowing activity at even 55 °C and this is suitable since high temperatures diminishes microbial contamination during treatment of soymilk.

4.3.7C. Thermostability of free and k-carrageenan: locust bean gum immobilized α-galactosidase.

Thermal stability of the soluble and immobilized α-galactosidase was examined by incubating these preparations without any stabilizers at 50 °C. Figure.4.13 depicts the
stability of α-galactosidase at 50°C. After 24 h, immobilized enzyme retained 76% activity. Batsavola et al., (1987) have similarly reported that immobilized enzyme preparation was considerably more stable than soluble enzyme. The greater stability of immobilized enzyme may be due to the stabilizing effects of immobilization (Schnapp and Shalitin, 1976).

4.3.8. Treatment of Soymilk

4.3.8A. Batch reaction

The hydrolysis of raffinose family sugars by soluble and immobilized α -galactosidase by batch experiment is displayed in Figure.4.14. In the batch experiments the soluble and immobilized enzyme was incubated with soymilk at different incubation periods i.e. 2, 4, 8, and 12h. HPLC analysis of enzyme treated soymilk for 4 h indicated the hydrolysis of raffinose family sugars (Figure.4.15). The 2 and 4h incubation with soluble enzyme resulted in 85% and 88% and immobilized enzyme resulted in 73% and 76% hydrolysis respectively. After 8 and 12h incubation soluble α-galactosidase led to 90% and 92% degradation, whereas immobilized α-galactosidase resulted in 80% and 83% reduction in raffinose family oligosaccharides in soymilk. The soluble enzyme showed a better percent of degradation. The immobilized enzyme showed lesser percent degradation, which could be due to diffusional limitation (i.e. resistance of substrate to diffuse into the immobilization matrix and resistance of the products to diffuse out)(Abdel-Naby et al., 1999). 92% hydrolysis of raffinose family sugars in soymilk by immobilized enzyme for the incubation period of 12h is the highest reduction so far among the other reports on immobilization of α-galactosidase (Thananunkul et al., 1976; Thippeswamy and Mulimani, 2002). But interestingly 85% oligosaccharide hydrolysis
occurred within 2h incubation for soluble enzyme implied that prolonged incubation does not significantly increase the percent hydrolysis. Hydrolysis after 2 h was very slow probably due to substrate depletion; it could also be due to product inhibition (Sugimoto and Van Buren, 1970). Thananunkul et al., (1976) have reported the 50% hydrolysis whereas Thippeswamy and Mulimani (2002) have reported 71% hydrolysis after 12h incubation of soymilk with α-galactosidase immobilized in polyacrylamide.

4.3.8B. Repeated batch reaction

The operational stability of the immobilized α-galactosidase was evaluated in a repeated batch process. The results indicated that the catalytic activity of the immobilized enzyme was durable under repeated use. Thus immobilized enzyme was able to maintain a good oligosaccharide reduction percentage with as much as 52 % even after 5 runs. Percent hydrolysis was determined to be 76% for immobilized beads on incubation for 4h. On subsequent uses i.e. after 2nd, 3rd and 4th cycles the percent hydrolysis was found to be 74%, 71%, 67% and 64% respectively. There was no drastic decrease in percent hydrolysis even after 5 uses, which could be due to glutaraldehyde treatment of beads, which prevented the leakage of enzyme. Ates and Mehmetoglu (1997) found that after treatment with glutaraldehyde the Cu-alginate immobilized enzyme could be used 8 times with high activity. β-Galactosidase immobilized in PVA was used 30 times for the hydrolysis of lactose in acetate buffer and 8 times for the hydrolysis of lactose in whey (Batsavola et al., 1987).

4.3.8C. Continuous reaction

The use of an immobilized enzyme makes it economically feasible to operate an enzyme process in a continuous mode. A fluidized bed reactor was constructed to explore
the practicability of using the k-carrageenan:locust bean gum entrapped enzyme in continuous system. Bodalo et al., (1991) suggested that the final choice of derivative for use in fluidized bed reactors should be based not only on the enzyme activity but also on the hydrodynamic behavior of the support. For the operation in fluidized reactor, small sized beads used help to minimize the mass transfer resistance. The continuous degradation of oligosaccharides in soymilk was carried out in a fluidized bed reactor. At 50°C, oligosaccharide reduction in soymilk was 90%, 77%, 73%, 51% and 37% for 40, 80, 120, 160 and 200 ml h⁻¹ flow rates respectively. These results suggest that a low flow rate (40 ml h⁻¹) i.e. a higher retention time leads to higher percent degradation (90%). Optimal flow rate is one of the important parameter for the effective operation of fluidized column reactor. Thananunkul et al., (1976) reported that fluidized bed containing α-galactosidase-polyacrylamide gel complex hydrolyzed 60% of the raffinose and stachyose in soymilk but at a flow rate of 30ml h⁻¹. Thippeswamy and Mulimani (2002) reported that oligosaccharide degradation of raffinose family oligosaccharides for 84% at a flow rate of 25 ml h⁻¹. Thus immobilized α-galactosidase from A. oryzae shows high optimum temperature and showed better operational stability by being stable even after being used 5 times.

4.3.9. Reduction of raffinose family sugars in black gram milk by k-carrageenan:locust bean gum immobilized α-galactosidase

Since α-galactosidase immobilized in k-carrageenan:locust bean gum showed promising results in the processing of soymilk, it was tempting to apply the same immobilized beads to reduce the raffinose family sugars in black gram milk. In an effort
to reduce the raffinose family sugars in black gram milk continuous studies were carried out.

4.3.10. Continuous studies

The use of an immobilized enzyme makes it economically feasible to operate an enzyme process in a continuous mode. Since fluidized bed reactor consisting of k-carrageenan:LBG beads gave better results compared to other systems, it was used to reduce raffinose family sugars present in black gram milk. At 50°C, oligosaccharide reduction in black gram milk was 90, 77, 53 and 35% for 25, 50, 100, and 200 mlh⁻¹ flow rates respectively. The results (Figure.4.16) suggested that 40 ml h⁻¹ which was the lowest flow rate obviously resulted in higher percent oligosaccharide degradation (90%).

*A. oryzae* used is considered as an excellent host for the safe production of harmless products (Barbesgaard *et al.*, 1992) The immobilization of *A. oryzae* α-galactosidase in k-carrageenan:locust bean gum and its subsequent use is comparatively safe, simple and cheap with durable enzyme activity.
IMPORTANCE OF WORK

- Legume based foods soymilk and black gram milk were processed successfully by using soluble as well as immobilized $\alpha$-galactosidase.

- k-Carrageenan and locust bean gum are widely used in food and pharmaceutical industries, and are highly compatible with living organisms. Therefore two matrices k-Carrageenan and k-carrageenan:locust bean gum can be used safely in food industry.

- Legumes rich in raffinose family oligosaccharides can be effectively hydrolyzed using fluidized bed reactors.

- $\alpha$-Galactosidase from Aspergillus has a GRAS status, the application of enzyme in the processing of legumes poses no problem.