Chapter VI
APPLICATIONS OF *STREPTOMYCES GRISEOLOALBUS*
α-GALACTOSIDASES

6.1. INTRODUCTION

What make an enzyme widely acceptable and attractive are its excellent industrial applications and also its characteristics that suit its field of application. The interest of scientific community in α-galactosidase research stems from its versatile biotechnological and medicinal applications in diverse fields. α-Galactosidases play a crucial role in improving the nutritional value of legume-based food. Legumes are widely grown throughout the world and their dietary and economic importance is globally appreciated and recognized. Dry legumes not only add variety to human diet, but also serve as an economical source of proteins, dietary fibers and a variety of micronutrients and phytochemicals (Messina 1999). Legumes also have the potential to lower cholesterol and serum glucose and quicken adjustment to high altitude induced stress (Mazur et al. 1998). Supplementing cereal-based diets with legumes improves overall nutritional status and is one of the best solutions to protein calorie malnutrition in the developing countries.

Soybean (*Glycine max*) is a legume, rich in proteins and having a well-balanced amino acid pattern (Smith & Circle 1972). It has received considerable attention for its potential role in the prevention and treatment of cancer and osteoporosis since it is a rich source of isoflavones (Messina 1999). Soymilk, the aqueous extract of soybeans is a nutritious beverage rich in high quality proteins and contains no cholesterol or lactose and only small quantities of saturated fatty acids. It is considered as a low-cost substitute for dairy milk in developing countries and as a nutritive supplement for the lactose-intolerant persons. Horse gram (*Dolichos biflorus*) and green gram or mung bean (*Vigna radiata* L.) are among the most important food legumes grown and consumed in India. In addition to proteins, horse gram is a rich source of iron and molybdenum. Green gram is an excellent source of carbohydrates,
proteins and minerals and its protein quality is similar to or better than other legumes such as chickpea, black gram, peas, pigeonpea, etc. (Jood et al. 1986). Horse gram and green gram are consumed as whole seeds or sprouts by a large population in rural areas of southern India. Despite these facts, the utilization of legume food crops is constrained due to the presence of large amount of anti-nutritive factors, mainly the raffinose-family oligosaccharides (RFO), which induce flatulence.

The production of flatulence is regarded as being due to the lack of ability of human intestinal tract to synthesize the enzyme α-galactosidase, which is necessary to hydrolyze oligosaccharides containing α-galactosidic linkages. The predominant RFO, raffinose and stachyose, are relatively large and are hence not resorbed by the intestinal wall. The intact oligosaccharides therefore enter the lower intestine where they are metabolized by the microflora into carbon dioxide, hydrogen and, to a lesser extent, methane by anaerobic fermentation. It is the production of these gases which leads to the characteristic features of flatulence, namely nausea, cramps, diarrhea, abdominal rumbling, and the social discomfort associated with the ejection of rectal gas (Cristofaro et al. 1974). RFOs make up a substantial part (40%) of the soluble sugars found in soybean seeds. It is highly desirable to decrease the oligosaccharide content of legumes if they are to be more effectively exploited as relatively cheap substitutes for good quality proteins.

Many methods are being practiced for the processing of legume seeds, such as soaking, cooking and sprouting (Mulimani & Devendra 1998; Viana et al. 2005), which may bring about changes in the levels of RFO. The newly released high-yielding cultivars may not only have different grain quality characteristics, but also may behave differently from existing cultivars after processing and cooking. Of all the techniques proposed, the enzymatic processing by α-galactosidase has proved most effective (Mansour & Khalil 1998; Kotwal et al. 1998; Scalabrini et al. 1998; Thippeswamy & Mulimani 2002; Viana et al. 2005).

Immobilization of enzymes is now a widely used approach for obtaining reusable derivatives of enzymes. It is generally carried out by adsorption, covalent coupling to solid matrices or entrapment in polymeric substances such as alginate, κ-
carrageenan etc. Often, immobilization also results in improvement of enzyme stability under process conditions. In addition immobilization offers the advantage of reusability of enzymes. Thus lower capital/energy costs and better logistics are associated with a process using an immobilized system. α-Galactosidase has been extensively studied in immobilized forms. Immobilization of α-galactosidase in different gel matrices such as calcium alginate (Prasanth & Mulimani 2005), κ-carrageenan (Girigowda & Mulimani 2006), gelatin (Naganagouda et al. 2007) etc. for treatment of soymilk has been well documented.

The transglycosylation property of α-galactosidases, in addition to the hydrolytic property, also makes them excellent candidates for industrial application. Until recently, oligosaccharides have been used in the food industry as a source of energy or as sweeteners. Nowadays knowledge of their biological function and their role in cell-surface interactions has opened a new field of glycotechnology (Gabius & Gabius 1997). Apart from their traditional use, oligosaccharides find new applications as immunostimulating agents or prebiotic compounds able to modulate the colonic microflora towards a healthy balance (Gibson & Roberfroid 1995; Murata & Usui 2006). α-Galactosidases have been used for the synthesis of α-galacto-oligosaccharides (Hashimoto et al. 1995; Weignerová et al. 2001; Tzortis et al. 2003; Yamashita et al. 2005) which are expected to have possible application as drug carriers in drug delivery systems (Kitahata et al. 1992; Hara et al. 1994; Koizumi et al. 1995). α-Galactooligosaccharides produced by transfer reaction of α-galactosidase can be used as a prebiotic in functional food (Rivero-Urgell & Santamaria-Orleans 2001).

This chapter describes the potential applications of *Streptomyces griseoloalbus* α-galactosidases in the processing of legumes as well as in galacto-oligosaccharide synthesis by transglycosylation reaction.

**6.2. MATERIALS AND METHODS**

**6.2.1. Production and extraction of crude α-galactosidase**

The production of α-galactosidase from *S. griseoloalbus* was done by solid-state fermentation and the crude enzyme was extracted as described in Chapter IV.
6.2.2. Partial purification of α-galactosidase

The proteins in the crude enzyme extract were concentrated by ammonium sulphate precipitation. The α-galactosidase active fractions (50-80% saturated fractions) were pooled and the precipitate obtained by centrifugation (10,000 rpm, 20 min) was dissolved in minimal amount of buffer (50 mM sodium phosphate buffer, pH 7.0), dialyzed against the same buffer overnight at 4 °C and used as partially purified enzyme preparation.

6.2.3. Characterization of crude and partially purified α-galactosidase

The optimum pH for α-galactosidase activity was determined in the assay mixture over a pH range of 4.0 to 9.0 and the relative activity was calculated. For pH stability determination, enzyme preparations were incubated at pH values in the range of 4.0 to 9.0 for 5 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

The optimum temperature for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 40 to 75 °C and the relative activity was calculated. For temperature stability determination enzyme preparations were incubated at temperatures in the range of 50 to 75 °C for 5 h at optimum pH and residual activity was determined as described previously.

6.2.4. α-Galactosidase assay

α-Galactosidase assay was carried out using the chromogenic substrate p-nitrophenyl α-D-galactopyranoside as described in Section 2.2.4 of Chapter II.

6.2.5. Processing of soymilk

6.2.5.1. Preparation of soymilk

Soybeans (Glycine max) (Fig. 6.1) purchased from the local market were used for the preparation of soymilk (Fig. 6.2) according to the method described by Thippeswamy & Mulimani (2002).
6.2.5.2. Immobilization of α-galactosidase in gelatin-alginate blended beads

Partially purified α-galactosidase was entrapped in gelatin-alginate blended beads according to the method of Tanriseven & Dogan (2002). The partially purified α-galactosidase preparation (80 U) was mixed thoroughly with a solution containing 0.5 g alginate, 1 g gelatin, 4 mL glycerol and 2 mL acetate buffer (0.1 M, pH 5.0). The mixture obtained was then extruded drop-wise through a syringe fitted with a luer-lock needle into 50 mL of 0.2 M CaCl₂ prepared in 0.1 M acetate buffer (pH 5.0) containing 5% (v/v) glutaraldehyde. The beads were allowed to harden for 2 h, washed with acetate buffer and then with double-distilled water. The beads were stored in the same buffer until use. Since citrate and phosphate are reported to have a dissolution effect on alginate matrices, the immobilization experiments were carried out using 0.1 M acetate buffer (pH 5.0) instead of the McIlvaine buffer (0.1 M, pH 5.0) used in all other experiments.
The activity yield (%) of α-galactosidase immobilized in gelatin-alginate beads was calculated as the ratio of enzyme activity of the immobilized enzyme to the total units of soluble enzyme used for immobilization. The activity of immobilized enzyme was calculated as the difference between the total amount of enzyme used for immobilization and the enzyme activity left in the curing solution and wash outs.

\[
\text{Activity yield} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100
\]  

Eq. (1)

6.2.5.3. Enzymatic treatment of soymilk

6.2.5.3.1. Batch experiments with free and immobilized α-galactosidase

Fifty milliliters each of soymilk was incubated separately with 50 U of free and immobilized α-galactosidase at 65 °C for 3 h. Two mL aliquots were removed at specific time intervals and kept in boiling water bath for 5 min to terminate the reaction. The proteins were precipitated by adding 0.2 mL of 0.3 M barium hydroxide and 0.2 mL of 0.18 M zinc sulphate. The sample was centrifuged to remove the precipitated proteins and the galactose liberated by enzymatic treatment was determined as reducing sugar by the method of Nelson (1944).

The effectiveness factor of the immobilized system was defined as the ratio of galactose liberated by the immobilized α-galactosidase to that of the free α-galactosidase.

\[
\text{Effectiveness factor} = \frac{G_{\text{imm}}}{G_{\text{free}}}
\]  

Eq. (2)

where \(G_{\text{imm}}\) is the amount of galactose liberated by immobilized enzyme and \(G_{\text{free}}\) is the amount of galactose liberated by free α-galactosidase.

6.2.5.3.2. Repeated batch experiments with immobilized α-galactosidase

Repeated batch experiments were carried out to study the operational efficiency of immobilized enzyme system. Fifty milliliters each of soymilk was treated
with 50 U of immobilized α-galactosidase in a rotary shaker at 50 and 65 °C for 3 h. After 3 h the increase in the amount of galactose liberated was estimated and the immobilized enzyme beads from the spent soymilk samples were transferred to fresh soymilk samples after washing with sterile distilled water and the catalytic cycle was continued for 3 h. Six successive catalytic cycles were performed and the operational efficiency of the immobilized enzyme system at each operating temperature (50 and 65 °C) was determined by the following equation:

\[
\text{Operational efficiency (\%)} = 100 \times \left( \frac{G_f}{G_1} \right)
\]

where \( G_1 \) is the amount of galactose liberated in the 1\textsuperscript{st} catalytic cycle and \( G_x \) is the amount of galactose liberated in the \( x \textsuperscript{th} \) catalytic cycle.

**6.2.5.4. Thin layer chromatographic analysis of soymilk**

For the preparation of samples for thin layer chromatography (TLC), 50 mL of soymilk was incubated with 50 U of free enzyme at 65 °C for 3 h with agitation. Twenty five mL aliquots were removed at specific time intervals and the reaction was terminated by boiling the reaction mixture for 20 min in a boiling water bath. The boiled reaction mixture was poured into 60 mL absolute ethanol to partially precipitate the soy proteins. Complete precipitation of the proteins was effected by adding 0.25 mL of 0.3 M barium hydroxide and 0.25 mL of 0.18 M zinc sulphate. The precipitated proteins were removed by filtration through Whatman No. 1 filter paper. The filtrate was then extracted with 40 mL of chloroform. The aqueous phase was concentrated to 2.5 mL under vacuum and analyzed using TLC.

TLC was performed on silica gel G plates (10 x 20 cm) (Merck Co. Ltd.) using \( n \)-propanol: ethyl acetate: water (6:1:3, v/v) as the solvent system (Tanaka et al. 1975). The sugars spots were located by keeping the plates in an oven at 140 °C for 5 min after spraying with 1\% (w/v) α-naphthol in absolute ethanol containing 10\% of orthophosphoric acid (Albon & Gross 1950).
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6.2.6. Processing of legume seed flour

Dry whole seeds of horse gram (*Dolichos biflorus*) and green gram or mung bean (*Vigna radiata* L.) (Fig. 6.3) purchased from the local market were used for the study.

![Image of horse gram and green gram seeds](image)

Fig. 6.3. (a) Horse gram and (b) green gram used for treatment with α-galactosidase

6.2.6.1. Conventional methods of legume processing

The conventional methods of legume processing such as soaking and cooking were done with dry whole seeds of horse gram and green gram. The seeds were soaked in distilled water (1:10, w/v) for 12 h at room temperature. After 12 h, the water was drained off and the soaked seeds were washed three times with distilled water. In cooking treatment the seeds were cooked in distilled water (1:10, w/v) on a hot plate for 60 min and rinsed three times with distilled water. The seeds were then dried and powdered before estimation of the oligosaccharide content.

6.2.6.2. α-Galactosidase treatment of legume seed flour

Five grams of horse gram and green gram seed flour, which passes through a 500 μm sieve, was treated with 40 U of α-galactosidase diluted in 50 mL of 0.1 M McIlvaine buffer (pH 5.0), in a rotary shaker at 120 rpm and 65 °C for 2 h. After incubation, the treated seed flour samples were filtered through a Whatman No.1 filter paper, dried and the oligosaccharide content was determined. For control, the volume of enzyme was replaced with equal volume of buffer.

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6.2.6.3. Determination of oligosaccharide content

The raffinose oligosaccharides were extracted by treating 5 g of seed flour sample with 50 mL of 70 % ethanol (v/v) in a rotary shaker at 120 rpm for 12 h. The alcoholic extract obtained after filtration through Whatman No.1 filter paper was concentrated under vacuum at 40 °C in a rotary evaporator. The concentrated sugar syrup was made up to 10 mL with distilled water. Ten microliters each of the sugar extract was applied to silica gel G plates (20 x 20 cm) (Merck Co. Ltd.) and developed by ascending thin layer chromatography and the sugar spots were visualized according to the method described above in Section 6.2.5.4. For quantitative determination, the area (2 x 2 cm) corresponding to each oligosaccharide spot was scraped from unsprayed duplicate plates and eluted with 3 mL distilled water for 12 h. The mixture was centrifuged to remove silica gel and 1 mL of the supernatant was used for the estimation of oligosaccharides by the method of Tanaka et al. (1975). One millilitre of the supernatant was treated with 1mL of 0.2 M thiobarbituric acid followed by 1 mL of concentrated HCl and boiled in a water bath for exactly 6 min. After cooling, the oligosaccharide contents were quantified spectrophotometrically at 432 nm. The oligosaccharide content of the samples was expressed on dry weight basis.

6.2.6.4. Determination of total soluble sugars and reducing sugars

Total soluble sugars in the concentrated sugar syrup were estimated by phenol-sulphuric acid method (Dubois et al. 1956). The reducing sugars were estimated by the method of Nelson (1944).

6.2.7. α-Galacto-oligosaccharide synthesis (Transglycosylation)

Transglycosylation reactions were carried out separately with the three purified α-galactosidases - α-Gal I, α-Gal II and α-Gal III - obtained by the purification steps described in Chapter V. Transgalactosylation reactions were carried out in a reaction mixture containing 50 μL of suitably diluted enzyme (1 U), 50 μL of galactosyl donor, 50 μL of acceptor sugar and 50 μL of McIlvaine buffer (0.1 M, pH 5.0). Reactions were carried out separately for 2 h at the temperature optimum for each α-galactosidase (α-
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Gal I, 65 °C; α-Gal II 50 °C and α-Gal III 55 °C). Aliquots were removed at suitable time intervals and heated in a boiling water bath for 5 min to terminate the reaction. Galactosyl donors used were stachyose, raffinose and melibiose, at final concentrations of 100 mM in the reaction mixture. Acceptor sugars used were galactose and glucose at a final concentration of 500 mM. Transfer products were detected by TLC on silica gel G plates (10 x 20 cm) (Merck Co. Ltd.) using n-propanol: acetic acid: water (1:1:0.1, v/v/v) as the solvent system. The sugar spots were visualized as described above in Section 6.2.5.4.

6.2.8. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the average of triplicate determinations and the standard deviation for all the values were <± 5%.

6.3. RESULTS AND DISCUSSION

6.3.1. Characterization of crude and partially purified α-galactosidase

Generally crude or partially purified α-galactosidase preparations are used for the processing of legume-based food. Hence characterization of α-galactosidase in its crude and partially purified state is essential to identify the operational conditions suitable for the enzyme. Characterization studies of the crude and partially purified enzyme preparation indicated that the optimum pH for highest α-galactosidase activity was 5.0 (Fig. 6.4). The enzyme was active over a wide range of pH (4.0-9.0) with more activity towards the acidic range than towards alkaline range. At pH 4.0 the crude and partially purified α-galactosidase showed respectively 90% and 85% of the activity obtained at pH 5.0. Only a negligible amount of activity was obtained at pH 9.0. The α-galactosidase activity of both crude and partially purified enzyme preparations remained stable for 5 h over a wide pH range (5.0 to 7.0) (Fig. 6.4). This is an appreciable character for application in the treatment of legume-based food. The pH of legume slurries meant for enzymatic treatment varies considerably. An enzyme with activity over a wide range of pH is advantageous, because it will eliminate the necessity
for pH adjustment, which may cause precipitation of proteins leaving a sour taste to such food (Gote et al. 2004). Mansour & Khalil (1998) have reported α-galactosidase activity in Aspergillus oryzae and Aspergillus niger that is stable in the pH range of 4.0 to 7.0 and Cladosporium cladosporioides α-galactosidase is stable in the pH range of 5.0 to 7.0.

![Graph](image)

**Fig. 6.4. Effect of pH on activity and stability of crude (■, △) and partially purified (□, ▲) α-galactosidase**

The optimum temperatures of the crude and partially purified α-galactosidase preparations were determined at pH 5.0. The crude and partially purified α-galactosidase preparations were most active at 65 °C and exhibited 83% and 74% activity respectively at 70 °C (Fig. 6.5). Thermostability studies showed that both crude and partially purified enzyme preparations were completely stable up to 65 and 55 °C respectively for 5 h (Fig. 6.6). The half-life ($t_{1/2}$) of inactivation of partially purified α-galactosidase at 65 °C was 3.5 h. At 70 and 75 °C the enzyme activity decreased rapidly. The high thermostability of the enzyme is advantageous for industrial application, especially when removing RFO from soymilk and other soy-based products, as high temperature (usually 65–70 °C) used during the pasteurization step following the soybean processing leads to the denaturation of thermolabile enzymes.
6.3.2. Enzymatic treatment of soymilk

Analysis of enzyme treated soymilk by TLC (Fig. 6.7) indicated the complete disappearance of stachyose from the soymilk samples within 30 min of incubation and raffinose within 1 h of incubation. Unlike the reports of Gote et al. (2004) on the hydrolysis of soymilk by *Bacillus stearothermophilus* α-galactosidase, no melibiose was
found to accumulate in the soymilk samples after 1 h of incubation, which indicated the complete hydrolysis of the soy oligosaccharides to the respective end products. Sucrose, the hydrolysis product of raffinose, was not found to appear after enzyme treatment of soymilk indicating the presence of invertase activity in the culture supernatant of *S. griseoloalbus*. These results could be advantageous as the treatment of soymilk with *S. griseoloalbus* α-galactosidase would render the soymilk free of all the oligosaccharides. The results suggest that α-galactosidase from *S. griseoloalbus* has a great potential in development of soymilk-based food products.

![Thin layer chromatogram of soymilk samples](Fig. 6.7. Thin layer chromatogram of soymilk samples. Lanes: (1) Standard sugars, (2) Soymilk before treatment, (3) Soymilk after 30 min, (4) 1 h, (5) 2 h and (6) 3 h incubation with free α-galactosidase.)

### 6.3.2.1. Comparison of free and immobilized α-galactosidase

A time course of the hydrolysis of oligosaccharides in soymilk by free α-galactosidase and α-galactosidase immobilized in gelatin-alginate blended beads showed an increase in the amount of reducing sugars liberated with increase in incubation time (Fig. 6.8). However, free α-galactosidase liberated more galactose compared to immobilized α-galactosidase. This could be due to the diffusional limitations in the immobilized system which causes resistance to the substrate to diffuse into the immobilized matrix and to the products to diffuse out (Abdel-Naby et al. 1999; Erginer et al. 2000). On the contrary, free α-galactosidase has easy access to the RFO in soymilk.
The partially purified α-galactosidase was physically entrapped in gelatin-alginate blended beads (Fig. 6.9). The activity yield obtained after immobilization was 69.7%. Generally calcium alginate beads used for the immobilization of enzymes have the disadvantage of large pore sizes leading to enzyme leakage. Immobilization of enzymes in gelatin requires additional processes such as freezing and thawing which denatures the enzyme and lowers the immobilization yield. In the present study the advantages of gelatin and alginate are combined. Gelatin is a protein used for the immobilization of cells and enzymes using glutaraldehyde as a cross-linking agent. Gelatin is cross-linked with glutaraldehyde under mild conditions and the reaction between gelatin and glutaraldehyde, accompanied by a colour change due to the formation of the aldimine linkage, involves only the lysine residue of the protein (Tanriseven & Dogan 2002). To improve the immobilization system, sodium alginate and gelatin were used along with glycerol. The polyol or glycerol imparts a stronger resistance and confers better characteristics with respect to smoothing and rehydration.
effects (Casas et al. 1990), probably because these polyols provide more hydroxyl groups and these polyhydroxylic additives have a positive effect on enzyme stability. Similar results were reported by Naganagouda & Mulimani (2006) for immobilization of α-galactosidase and Tanriseven & Dogan (2002) for immobilization of β-galactosidase in alginate-gelatin fibers. An inherent problem in enzyme immobilization is the leakage of enzyme from beads, even at higher concentrations, which gradually leads to loss of enzyme activity. In order to avoid leakage 5% glutaraldehyde solution was used as a hardener. Ates & Mehmetoglu (1997) have reported that on treatment with glutaraldehyde solution, the activity of β-galactosidase in the alginate beads was stable. Glutaraldehyde cross-links enzyme and gelatin forming an insoluble structure. Glutaraldehyde treatment also stabilizes the alginate gel, helping in the prevention of enzyme leakage (Aetes & Mehmetoglu 1997; Tanriseven & Dogan 2002).

**Fig. 6.9. α-Galactosidase physically entrapped in gelatin-alginate blended beads**

### 6.3.2.2. Operational efficiency of immobilized α-galactosidase

The operational stability of the immobilized α-galactosidase was evaluated in a repeated batch process at 50 and 65 °C. The results indicated that the immobilized enzyme system was 100% efficient up to 4 catalytic cycles at 50 °C and up to 2 catalytic cycles at 65 °C. Even after six cycles of usage the immobilized enzyme system could maintain 77% operational efficiency at 50 °C. At 65 °C the operational stability of immobilized enzyme dropped considerably after two cycles of usage and
after six cycles the operational efficiency was only 36%. Though the α-galactosidase is thermostable, the immobilization matrix used in the present study could not withstand the operating temperature as high as 65 °C as a result of which enzyme leakage increased correspondingly. This could be the reason for the rapid decrease in operational efficiency at 65 °C. The immobilized enzyme system was operationally more efficient at 50 °C. Naganagouda & Mulimani (2006) and Tanriseven & Dogan (2002) also reported 50 °C as the most suitable temperature for α-galactosidase and β-galactosidase immobilized in alginate-gelatin fibers. In the present study the immobilized enzyme system working at 55 °C could liberate only less amount of galactose from soymilk as compared to the free α-galactosidase working at 65 °C, which is the optimum temperature. The lower hydrolysis efficiency of the immobilized enzyme can, however, be compensated by re-using the system, whereas the free enzyme is lost after single use. The glutaraldehyde treatment rendered high stability to the gelatin-alginate blended beads and prevented the leakage of enzyme. Naganagouda & Mulimani (2006) reported the use of α-galactosidase immobilized in alginate-gelatin fibers for the removal of RFO from soymilk, where the immobilized fibers maintained 60% operational efficiency after five runs. Ates & Mehmotoglu (1997) found that after treatment with glutaraldehyde Co-alginate immobilized β-galactosidase could be used eight times with high activity.

![Graph showing operational efficiency](image)

**Fig. 6.10.** Operational efficiency of gelatin-alginate-entrapped α-galactosidase in liberating galactose from soymilk at 50 and 65 °C over repeated catalytic cycles. The duration of a single catalytic cycle was 3 h.
6.3.3. Treatment of legume seed flour

The levels of RFO in raw horse gram and green gram flour samples are presented in Table 6.1. The results showed that green gram contained more RFO than horse gram and the concentration of stachyose was highest in both horse gram and green gram. The relative levels of raffinose and stachyose obtained in our study were in confirmation with those presented by other workers (Rathbone 1980; Adsule et al. 1986).

Table 6.1. Oligosaccharide content of raw horse gram and green gram

<table>
<thead>
<tr>
<th>Seed flour sample</th>
<th>Total soluble sugars (g/kg DM*)</th>
<th>Raffinose (g/kg DM*)</th>
<th>Stachyose (g/kg DM*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse gram</td>
<td>28.9</td>
<td>6.8</td>
<td>19.4</td>
</tr>
<tr>
<td>Green gram</td>
<td>59.2</td>
<td>16.5</td>
<td>27.5</td>
</tr>
</tbody>
</table>

The data are mean of three independent samples with triplicate determinations

* Dry matter

6.3.3.1. Effect of soaking

The reduction of RFO in dry whole seeds of horse gram and green gram by various treatments is given in Fig. 6.11a and 6.11b respectively. Soaking of dry whole seeds of horse gram in distilled water for 12 h resulted in a mean reduction of raffinose content by 23.8 % and stachyose content by 12.3 %. For green gram flour samples, the reduction of raffinose content was by 19% and that of stachyose was by 10%. The reduction of raffinose content was higher compared to that of stachyose content in both the cases. Mulimani et al. (1997) have reported that soaking of whole soybean seeds led to a mean decrease of 80.3% for raffinose and 44.8% for stachyose. Reduction in raffinose and stachyose content of red gram flour by 54.6% and 55.4% respectively was reported by Mulimani & Devendra (1998). Reduction of RFO in cow pea seeds by soaking is reported by Somiari & Balogh (1993). Leaching could be one of the reasons for the reduction of raffinose family of sugars during soaking (Price et al. 1988). Upadhyay & Garcia (1988) have demonstrated that the differential solubility of individual sugars and their diffusion rates are the two factors that influence the sugar
losses during soaking. The extent of reduction in level of oligosaccharides can be enhanced by increasing the soaking time and employing different soaking media (Pugalenthithi et al. 2006). But the presence of off-odour in flours obtained from the legume seeds after soaking would affect the acceptability of such products (Somiar & Balogh 1993).

![Graph showing RFO content (g/kg DM) for raw, soaked, cooked, and enzyme treated samples](image)

**Fig. 6.11. Raffinose and stachyose contents of raw, soaked, cooked and α-galactosidase treated (a) horse gram flour and (b) green gram flour**

### 6.3.3.2. Effect of cooking

Cooking brought about a greater reduction in raffinose family sugars than soaking (Fig. 6.11a and 6.11b). Cooking of horse gram and green gram seeds for 60 min resulted in a mean decrease of 49.6% and 46.3% respectively for raffinose and 24.3% and 20.1% respectively for stachyose. Mulimani et al. (1997) reported 52.3% removal of raffinose and 20.7% removal of stachyose from soybean seeds after cooking. Somiari & Balogh (1993) reported that cooking of cow pea for 50 min reduced the raffinose content by 44% and stachyose by 28.6%. Onigbinde & Akinyele (1983) have proposed that decrease in the levels of raffinose and stachyose during cooking might be attributed to heat hydrolysis to disaccharides and monosaccharides or due to the formation of other compounds. In contrast, Rao & Belavady (1978) reported an increase in the level of oligosaccharides after cooking of pulses.
Though cooking resulted in a decrease in the level of RFO, it affected the
colour, texture and aroma of the seed flours. It is also reported that legumes such as
horse gram require prolonged cooking to obtain products of acceptable nature (Kadam & Salunkhe 1985). Price et al. (1988) have reported that treatments such as soaking
and cooking could change the physicochemical properties of legumes. Moreover,
soaking and cooking alone will not be sufficient to bring about any significant reduction
in the flatulence-inducing activity of legumes (Price et al. 1988).

6.3.3.3. Effect of crude α-galactosidase treatment

Horse gram flour when treated with α-galactosidase resulted in a reduction of
raffinose content by 97.5% and stachyose content by 93.2% (Fig. 6.11a and 6.11b). The enzyme treatment of green gram samples resulted in 96.3% and 91.8% reduction
of raffinose and stachyose respectively. On the other hand, no reduction of RFO was
observed in the control. The reduction in RFO by crude α-galactosidase treatment was
due to the conversion of oligosaccharides into di- and monosaccharides by the
hydrolysis of α-galactosidic linkages between the sugar molecules. The crude α-
galactosidase extracts from S. griseoloalbus remarkably reduced the levels of raffinose
and stachyose in horse gram and green gram flours.

There are several reports available in the literature of the use of α-
galactosidase from fungal and plant sources for the removal of the RFO from soymilk
and legume flours. Somiari & Balogh (1993) have used crude preparations of α-
galactosidase from Aspergillus niger for the removal of raffinose and stachyose present
in cowpea flours. Mansour & Khalil (1998) have reported 100% reduction of raffinose
oligosaccharide content in chickpea flours by crude fungal α-galactosidase treatment.
Mulimani et al. (1997) have used crude α-galactosidase from germinating guar seeds
for the hydrolysis of galactooligosaccharides in soybean flour and have reported 90.4%
reduction of raffinose and 91.9% reduction of stachyose.

6.3.4. α-Galacto-oligosaccharide synthesis by transglycosylation

α-Galactosidases have been generally known to catalyze hydrolytic reactions,
however some of the α-galactosidases also found to have galactosyl transfer activity

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(Hara et al. 1994; Hashimoto et al. 1995a, b; Eneyskaya et al. 1998; van Laere et al. 1999; Spangenberg et al. 2000; Yamashita et al. 2005) especially when incubated at very high substrate concentration. In the present study the three α-galactosidases- α-Gal I, α-Gal II and α-Gal III- purified from the crude enzyme extract of S. griseolobalus were used to catalyze transglycosylation reactions. The results showed that among the three α-galactosidases, only α-Gal II possessed transglycosylation property which can be exploited for the synthesis of novel α-galacto-oligosaccharides. When raffinose and galactose were incubated with α-Gal II, a transfer product with chromatographic mobility similar to that of stachyose was observed by TLC analysis (Fig.6.12). Two transfer products with chromatographic mobility similar to melibiose and stachyose were obtained when raffinose and glucose were incubated with α-Gal II. Incubation of stachyose and galactose with α-Gal II resulted in a new transfer product which was not similar to the chromatographic mobility of any of the standard sugars tested. When stachyose and glucose were incubated with α-Gal II two transfer products were obtained, one of which showed similarity to melibiose in chromatographic mobility and the other product was a new product observed as in the case of incubation with galactose.

Generally, α-galactosidases have been known to preferentially transfer galactosyl residues to the primary alcoholic groups of acceptor sugars (Dey & Pridham 1972; Mitsutomi & Ohtakara 1988). Since transfer of galactose to stachyose can yield a sugar which is structurally similar to the tetrasaccharide verbascose of the RFO, the new product obtained in the present investigation by incubation with stachyose and galactose/glucose was tentatively identified as verbascose. Pycnoporus cinnabarinus (Mitsutomi & Ohtakara 1988) α-galactosidase also produced mainly RFO consisting of stachyose, verbascose and ajugose by transglycosylation reaction. Tzortis et al. (2003) reported the yield of a trisaccharide from melibiose and a tetrasaccharide from raffinose with Lactobacillus reuteri α-galactosidase. Melibiose was not a good acceptor for the transglycosylation reaction of α-Gal II, since only the hydrolytic products were obtained when melibiose and galactose/glucose was incubated with α-Gal II (Fig. 6.12, Lanes 2...
Among the various sugars tested, raffinose was the only saccharide which acted both as a galactosyl donor as well as acceptor.

Fig. 6.12. Thin layer chromatogram showing the transfer products produced by transglycosylation reaction of α-Gal II from S. griseoloalbus. Lanes: (1) Standard sugars, (2) Melibiose + Galactose, (3) Melibiose + Glucose, (4) Raffinose + Galactose, (5) Raffinose + Glucose, (6) Stachyose + Galactose, (7) Stachyose + Glucose

6.4. CONCLUSION

The studies on the potential applications of thermostable α-galactosidases from S. griseoloalbus indicated the scope for their utilization in the processing of legume food. The crude and partially purified α-galactosidase preparations showed highest activity at pH 5.0 and 65 °C and were stable at pH 5.0 to 7.0 and up to 65 and 55 °C respectively for 5 h. The t₁/₂ of the partially purified α-galactosidase at 65 °C was 3.5 h. Usually legume food processing requires a high operating temperature which necessitates the involvement of thermostable α-galactosidases.

The hydrolysis of flatulence causing oligosaccharides in soymilk by the free and immobilized α-galactosidase was investigated. Thin layer chromatographic analysis of enzyme treated soymilk samples showed the complete hydrolysis of soy oligosaccharides liberating galactose, the final product. Though the immobilized system was less efficient compared to the free α-galactosidase, it had the advantage of
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reusability. The immobilized enzyme system was 77% operationally efficient at 50 °C even after six successive cycles of reuse. The investigations on legume seed flour treatment showed that the crude α-galactosidase extract from *S. griseoloalbus* was clearly most effective in reducing the levels of raffinose and stachyose than the traditional methods like soaking and cooking. The raffinose content in horse gram flour was reduced by 97.6% and stachyose content by 93.2%. The reduction in the raffinose content of green gram flour was 96.3% and that for stachyose was 91.8%. The information obtained from the present investigation is advantageous for the large-scale production of horse gram flour and green gram flour free from flatulence-causing oligosaccharides.

The studies on α-galacto-oligosaccharide synthesis by purified α-galactosidases via transglycosylation reaction showed that among the three α-galactosidases, only α-Gal II had the potential for transglycosylation. Incubation of raffinose/stachyose and galactose/glucose with α-Gal II resulted in the formation of transfer products. Raffinose and stachyose were good galactosyl donors, whereas melibiose was not. Incubation of melibiose and galactose/glucose with α-Gal II yielded only the hydrolytic products. Among the sugars tested, raffinose acted both as galactosyl donor and acceptor. The transglycosylation potential of α-Gal II can be made use of in synthesizing α-galacto-oligosaccharides which are having excellent probiotic as well as medicinal applications.