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Present status on removal of raffinose family oligosaccharides – a Review

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Abstract: Raffinose family oligosaccharides (RFOs) are α-galactosyl derivatives of sucrose or glucose. They are found in a large variety of seeds from many different families such as beans, vegetables and whole grains. Due to absence of α-galactosidase in the digestive tract of humans and other monogastric animals, RFOs are responsible for intestinal disturbances (flatulence) following the ingestion of legume-derived products. Structural relationships of RFOs and their enzymatic degradation mechanism are described. Concentration and distribution from various seed sources are introduced. The present status on removal of the RFOs (such as soaking, cooking, germination, and addition of α-galactosidase) is summarized. At the meantime, α-galactosidases from botanic and microbial sources and their partial enzymatic properties are also presented in detail. Based on a comparison of various removal treatments, the microbial α-galactosidases are thought as the most optimum candidate for removing RFOs in legumes, and the ideal system for the RFO removal is proposed.

Keywords: alpha-galactosidase; autoclaving; cooking; germination; soaking; α-galactose oligosaccharides

Abbreviations: DCB – dormancy coffee bean; FSBM – fermented soybean meal; GCB – germination coffee bean; GOS – galactooligosaccharides; RFOs – raffinose family oligosaccharides; SB – soybean; SBM – soybean meal; SmF – submerged fermentation; SMO – soybean meal oligosaccharides; SSF – solid state fermentation

Raffinose family oligosaccharides, which are known as α-galactose oligosaccharides, are α-galactosyl derivatives of sucrose or glucose. They are almost ubiquitous in the plant kingdom (Blöchi *et al*. 2007), being found in appreciable amounts and in a large variety of seeds from many different families, such as beans, cabbage, Brussels sprouts, broccoli, asparagus, cottonseed, and whole grains. Among them the most common are raffinose, stachyose, and verbascose, which contain α-galactoside-glucose and α-galactoside-glucose bonds and are non-reducing. Ajugose, a higher oligosaccharide (DP 6), is found in trace quantities in some cultivars of seeds (Girigowda *et al*. 2005).

Structural relationships of RFOs

Melibiose is a reducing disaccharide formed by α-1,6 linkage between galactose and glucose. It differs from lactose in the chirality of carbon where the galactose ring is closed and the galactose is linked to a different point on the glucose moiety. Melibiose can be broken down into D-glucose and D-galactose by α-galactosidase (Linden 1982). Raffinose is a trisaccharide consisting of galactose, glucose, and fructose. It can be hydrolyzed to D-galactose and sucrose by α-galactosidase. In the presence of invertase, raffinose is disintegrated into fructose and melibiose (De Rezende & Felix 1999). Stachyose is a tetra-

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saccharide composed of two α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit sequentially linked as gal (α 1 → 6) gal (α 1 → 6) glc (α 1 ↔ 2 β) fru (Rehms & Barz 1995). A pentasaccharide verbascose is formed by three α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit (Rehms & Barz 1995). Structural relationships of RFOs are shown in Figure 1.

Concentration and distribution

Concentrations of the RFOs in plants are related to seed sources and their cultivars (Table 1). The primary oligosaccharides found in soybean (*SB*) and soybean meal (SBM) are galactooligosaccharides (GOS), and the highest concentration of the GOS is in stachyose, followed by raffinose and verbascose (Karr-Lilienthal *et al.* 2005). Soybean oligosaccharides contain approximately 5% of the soybean dry matter (*DM*). Dry *SB* seeds from 18 cultivars selected for high oil content collected in 1991 and 1992 contained similar stachyose and raffinose concentrations to those from 20 cultivars selected for high protein, and stachyose and raffinose concentrations in the seeds ranged from 30 to 56 mg/g and from 4.8 to 20.1 mg/g, respectively (Hartwig *et al.* 1997). When processed into SBM, the GOS are not removed or eliminated, therefore, GOS represent about 4–6% of soybean meal *DM*. In SBM produced from 10 commercial processing plants in the US, concentrations ranged between 41.0–57.2, 9.8–14.3, and 1.6–2.4 mg/g *DM* for stachyose, raffinose, and verbascose, respectively (Grieshop *et al.* 2003).

Potential hazards

When the oligosaccharides of the RFOs are ingested by humans, two enzymes (invertase and α-galactosidase) are required for complete hydrolysis. Humans and other monogastric animals (such as pigs and poultry) do not possess α-galactosidases to break down RFOs, these oligosaccharides pass through the stomach and upper intestine undigested (De Rezende & Felix 1999). Consequently, the presence of substantial amounts of these RFOs impedes the full nutritional utilization of bean. In the lower intestine, they are fermented by gas-producing bacteria that possess α-galactosidase and make short-chain fatty acids, carbon dioxide, methane or hydrogen (Bäckhed *et al.* 2005). According to Dibofori *et al.* (1994), the average flatus volume in humans caused by a 5-g portion of raffinose is equivalent to that produced by 100 g of cowpeas. When soybean meal oligosaccharides (SMO) were used in the diet of broiler chickens during the first 2 weeks post-hatch, *in vitro* trial results showed that SMO produced 245.7 ml of gas/g *DM*, 261.8 mg of acetic acid/g *DM*, 187.2 mg of propionic acid/g *DM* and 155.2 mg of butyric acid/g *DM* (Lan *et al.* 2007). These compounds greatly improve the osmotic pressure and permeability in the large intestine, which are responsible for flatulence. Besides flatulence, other symptoms that can appear are nausea, cramps, abdominal pain, headache, mental disturbance, etc. (Yamaguishi *et al.* 2013). Alpha-galactose also affects animal manure, mainly generating higher faecal volume and softer faecal texture (Félix *et al.* 2013). Thus, the reduction in the RFOs is highly desirable so that soy-based food

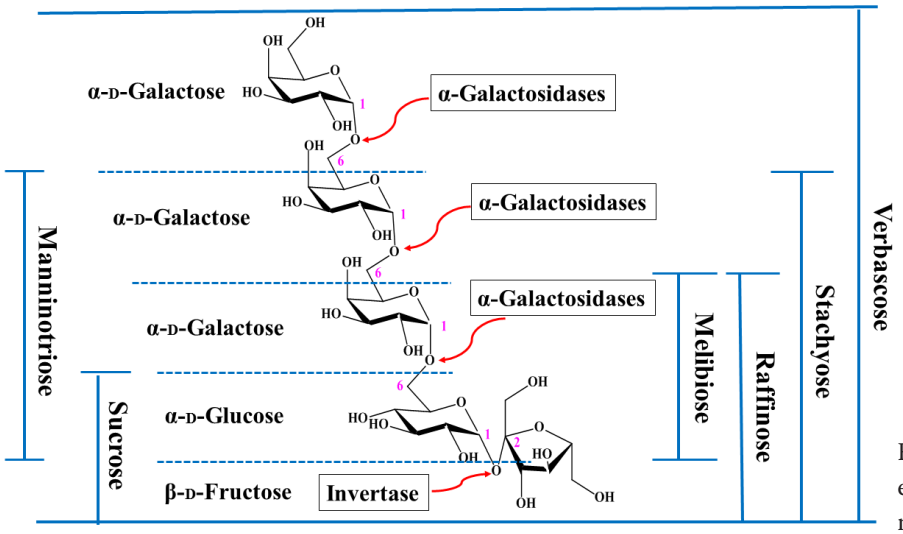

Figure 1. RFO structure and their enzymatic degradation mechanism

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Table 1. Concentration of the RFOs present in various seed sources (g/kg *DM*)

Seed	Raffinose	Stachyose	Verbascose	Reference
Benth.	3	11.6	–	Fialho *et al.* (2008)
Black gram	2.7–7.6	2.5–8.0	11.2–33.2	Girigowda *et al.* (2005)
Chickpea flour	19.0–28.0	9.0–17.0	–	Mulimani & Ramalingam (1997)
Coffee seed	0.23–0.45	0.35–0.70	–	Shadaksharaswamy (1968)
Cotton seed	5.0–10.0	0.5–2.0	–	Shiroya (1963)
Cowpea	7.7	30.0	3.0	Abdel-Gawad (1993)
Cowpea meal	20.3–29.7	31.4–47.8	–	Somiari & Balogh (1995)
Faba bean	5.2	14.1	18.5	Abdel-Gawad (1993)
Green gram	16.5	27.5	–	Anisha & Prema (2008)
Horse gram	6.8	19.4	–	
Jack bean	6.8–7.9	7.8–8.7	35.1–38.7	Pugalenthi *et al.* (2006)
Kidney bean	5.0–14.0	32.0–47.0	–	Trugo *et al.* (1990)
Lentil	4.5	16.5	6.2	Abdel-Gawad (1993)
Lima beans	2.77–2.97	28.3–31.6	1.94–2.46	Oboh *et al.* (2000)
Pigeon peas	4.23–6.20	8.57	10.7–15.6	
Red gram	5.2–9.2	7.4–12.0	36–60	Mulimani & Devendra (1998)
Soybean	4.0–18.0	12.0–30.0	trace	Hymowitz & Collins (1974)
Sword bean	7.2–16.0	7.5–26.0	37.0–66.5	Pugalenthi *et al.* (2006)
Yambean				
Black	6.0	21.7	1.5	Azeke *et al.* (2007)
Marble	8.2	24.6	1.1	
White	2.9	18.1	1.5	

products can be exploited maximally for serving the human population. It could be a better alternative of milk for the lactose intolerant population (Iacono *et al.* 1998).

REMOVAL APPROACHES OF RFOs

Many studies have been carried out investigating the effectiveness of different processing techniques for the removal of RFOs from beans. The following is a summary on the recent situation of the methods used and their effectiveness in removing RFOs.

Soaking, cooking and autoclaving

Several processing techniques, such as dehulling, soaking, cooking, steam blanching, and autoclaving (Trugo *et al.* 1990; Abdel-Gawad 1993; Vijayakumari *et al.* 1996; Pugalenthi *et al.* 2006; Shimelis & Rakshit 2007) have been used to reduce or eliminate anti-nutritional factors and toxicants.

Soaking styles. Once soaking in distilled water does not result in a significant reduction in the levels of raffinose family oligosaccharides, however, in the same soaking time decanting of soaking water at a time interval can improve removal efficiency (Mulimani *et al.* 1997). Ibrahim *et al.* (2002) found that soaking in alkaline water (0.03% $NaHCO_3$ solution) reduced the RFOs of cowpea seeds according to the time of soaking, after 16 h of soaking, complete removal of raffinose and more than 80% of stachyose. Vijayakumari *et al.* (1996) found that a relatively low elimination efficiency of RFOs in Tamil Nadu germplasm was obtained by only once soaking at a ratio of seed to distilled water being 1 g per 10 ml. A similar result in *Dolichos lablab* was also observed by Revilleza *et al.* (1990). However, at the same condition (bean to water ratio, 1:10), soaking soybean seeds up to 16 h resulted in a mean decrease of 44.8% for stachyose and 80.3% for raffinose when the soybean (*Glycine max*) was soaked in water and the soaking water was decanted at 4 h intervals (Mulimani *et al.* 1997). Song & Chang (2006) found that soaking of pinto beans resulted in a 9.8% reduction in RFOs,

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while soaking followed by boiling for 90 min resulted in a 57.6% reduction in RFOs.

Han & Baik (2006) investigated the effect of applying ultrasound or high hydrostatic pressure during soaking of various legumes on the amount of RFOs removed. Soaking soybeans for 3 h removed 33.3% of raffinose and 46.6% of stachyose. The application of ultrasound during the 3-h soaking period increased the amount of removed raffinose to 55.7% but reduced the amount of stachyose removed to 28.6%. Soaking is the easiest, but also the most ineffective way to reduce oligosaccharides in whole grains with around 25% reduction. By the combination of soaking, dehulling, washing, and cooking, more than half of the total oligosaccharides can be removed (Egounlety & Aworh 2003).

Soaking medium and temperature. Removal efficiency of α-galactose oligosaccharides is improved by extension of soaking time, alteration of soaking medium, and increase of processing temperature. In general, a longer soaking time results in greater removal efficiency of RFOs. Oligosaccharide reduction was higher with salt solution soaking than with distilled water soaking (Vijayakumari *et al*. 1996; Shimelis & Rakshit 2007). This phenomenon may be attributed to the dispersion effect of salts which improves the oligosaccharide solubility in water.

The loss percentage of oligosaccharides with autoclaving was higher than with cooking. When subjected to cooking (100°C) for 3 h, Kerala and Tamil Nadu germplasm seed materials of *Mucuna (T.) monosperma* showed a reduction in contents of raffinose (29 and 32%), stachyose (41 and 47%), and verbascose (23 and 29%), respectively. However, when treated by autoclaving (121°C) for 90 min, these seed materials exhibited a greater loss in raffinose (45 and 51%), stachyose (68 and 69%), and verbascose (52 and 46%), respectively (Vijayakumari *et al*. 1996). Moreover, Ibrahim *et al*. (2002) investigated that ordinary cooking (100°C/45 min) of pre-soaked cowpea seeds did not increase the removal percentage of stachyose, where pressure cooking (120°C/20 min, 1 kg/cm^2) of pre-soaked cowpea seeds relatively increased a stachyose loss from 59.8% to 66.1%. This is ascribed to the thermal hydrolysis of oligosaccharides to simple disaccharides and monosaccharides. The loss of RFOs during extrusion cooking may be due to the Maillard reaction (Borejszo & Khan 1992). Revilleza *et al*. (1990) found that dry roasting hyacinth beans for 2 min resulted in the complete removal of RFOs. The reduction of RFOs is thought to be caused by a non-enzymatic browning reaction, oxidation of sugars, or pyrolysis. However, this type of treatment will result in denaturation of a large proportion of bean proteins, which may not be desirable for some applications. Fasina *et al*. (2001) found that heating of various legume seeds to a surface temperature of 140°C via infrared heating followed by soaking for 24 h reduced raffinose concentration by 75.7–84.4% compared to reductions of 20.8–67.1% for soaking alone. The same treatment reduced stachyose concentration by 62.3–80.7% compared to reductions of 13.9–54.6% for soaking alone. They also reported small increases in the rate of water absorption for the infrared treated seeds and attributed this and the higher leaching rates of RFOs and other solutes to the cracking of seeds facilitating the access of water to the inner regions of the seeds.

As for soaking, it is usually carried out at room temperature, therefore, it does not require any additional energy consumption and can maintain the original flavour of the material. However, soaking also has some disadvantages. For example, it hardly eliminates trypsin inhibitors (Rehms & Barz 1995). Furthermore, soaking requires higher time consumption compared to cooking and autoclaving under the identical removal efficiency of RFOs (Vijayakumari *et al*. 1996). Obelesu & Bhagya (2006) suggested that the rate at which RFOs leach out of legume seeds during soaking is significantly affected by the temperature of soaking water. When the soaking water reached a sufficiently high temperature, the membranes and cell walls of the beans began to break down, which eased the access of water into the cells and therefore increased the rate of leaching.

Cultivar sources and hull hardness. Removal efficiency of α-galactose oligosaccharides is related to the cultivar sources and to the hardness of their hulls. Ibrahim *et al*. (2002) reported 100% reduction in RFOs of cowpea seeds after cooking seeds for 45 min, while Abdel-Gawad (1993) documented reductions of 41.5–47.2% for faba beans, lentils and common beans after cooking seeds for 60 minutes. A lower reduction of 8.5–25.9% in RFOs was observed for African yambean seeds after boiling seeds for 4 h owing to their hard hulls (Azeke *et al*. 2007).

Germination

Germination has been reported by many researchers as an effective way to eliminate the raffinose family oligosaccharides. Dibofori *et al*. (1994) investigated

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the effect of germination on the oligosaccharide and cyanide content of lima beans. Two days of germination were sufficient to degrade raffinose to a safe level, however, at this stage the cyanide level was high enough to cause chronic toxicity. They suggested that lima bean seeds (*Phaseolus lunatus*) should be germinated at room temperature for 5 days. Trugo *et al*. (1990) described the effect of cooking and germination on α-galactose contents of *Phaseolus vulgaris* in Brazil. Cooking for 60 min was sufficient to inactivate more than 90% of trypsin inhibitors but it reduced only about 15% of α-galactoside. Germination resulted in a progressive decrease of α-galactosides with 77% loss after the third day. It can be seen that germination is a more favourable way to remove α-galactosides than heat-moisture treatment.

Shadaksharaswamy & Ramachandra (1968) noticed the change in the contents of raffinose and stachyose from different varieties of coffee seeds during germination. Seventy-two hours of germination in a dark and sterile chamber resulted in complete disappearance of raffinose and stachyose in two varieties of coffee seeds (*C. arabica*), while in the species *C. robusta* these sugars continued to be present in small quantity. They believed that an effective mechanism of galactose utilization by the α-galactosidase is present during germination. Guimarães *et al*. (2001) observed that the content of raffinose family oligosaccharides changed in soybean seeds during germination. Germination for 60 h resulted in 100% loss of raffinose and stachyose, at the same time α-galactosidase activity was up to a maximum value. The above phenomena were also revealed by Fialho *et al*. (2008). They reported that the RFO content in germinating *Tachigali (T.) multijuga* seeds exhibited a declining tendency, on the other hand, α-galactosidase activity increased with the germination of *T. multijuga* seeds and reached a maximum value at 108 h after seed imbibition. Also, Ibrahim *et al*. (2002) found that the flatus-producing oligosaccharides (raffinose and stachyose) of cowpea seeds were entirely removed by germination after 24–48 hours.

Blöchl *et al*. (2007) stated that RFOs may be an essential source of rapidly metabolizable carbon as early germination events. The galactose liberated by RFO degradation may have functions in providing easily available energy for growth and building blocks for growth. Alpha-galactose may also be an important component of the sugar signalling pathway in the period of germination, and affects germination at the level of gene induction.

Owing to the extreme thermostability of galactooligosaccharides, traditional processing approaches such as soaking, cooking, autoclaving, and chemical treatment are unclear to eliminate them completely (Singh 1988; Chi & Cho 2016). Germination is an effective way to remove RFOs, though it requires higher time consumption. Enzyme treatment was more effective for removing α-galactosides than soaking and cooking (Somiari & Balogh 1993; Girigowda *et al*. 2005). Considering the commercial value improvement, practices of α-galactosidases from botanic and microbial sources remain much more economical and beneficial than the above-mentioned methods.

α-Galactosidases

Alpha-galactosidase (EC 3.2.1.22) is an exoglycosidase that catalyzes the degradation of terminal α-1, 6-linked-*D*-galactose residues from simple galactose-containing oligosaccharides such as melibiose, raffinose, stachyose, verbascose and ajugose, as well as more complex polysaccharides including galactomannans (Kapnoor & Mulimani 2010). A characteristic α-galactosidic bond between sucrose and galactose is very important as humans do not possess the α-galactosidase enzyme that is necessary for hydrolysing the bond typical of these oligosaccharides. Thus, they cannot be digested when consumed. Intact oligosaccharides reach the colon, where they are fermented by microorganisms that contain α-galactosidase. In plants, α-galactosidases are mainly distributed in seeds, fruits and leaves (Anisha & Prema 2008). Alpha-galactosidases had a great potential in a number of food-processing applications (Somiari & Balogh 1995; Ibrahim *et al*. 2002). In processing of beet sugar, the enzyme aided raffinose degradation can be utilized to avoid raffinose inhibition of normal crystallization (Ganter *et al*. 1988). In the pulp and paper industry, pulp biobleaching can be improved by addition of α-galactosidase in combination with xylanase and mannanase (Clarke *et al*. 2000). Another application area of α-galactosidase is seen in processing of leguminous food and feed, where they are utilized to hydrolyse raffinose and stachyose (Gote *et al*. 2004). The decrease in RFOs after treatment with α-galactosidase was due to the breakdown of these oligosaccharides into mono- and disaccharides. Through the hydrolysis of α-galactosidic linkages between the sugar molecules, sucrose content in-

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creased. Furthermore, conversion of the cheap guar gum to a more functional and more expensive locust bean gum can be achieved by partial removal of some galactoside units from guar gum (Bulpin *et al*. 1990; Shankar & Mulimani 2007). Ferreira *et al*. (2011) reported that some forms of alpha-galactosidase exhibited galactose inhibition. This phenomenon occurs when galactose produced as a product of the hydrolysis of stachyose and raffinose binds to the active site of the enzyme, preventing the access of substrates and thereby inhibiting the rate at which the substrates are hydrolysed. In these situations the further hydrolysis of the substrate progresses the higher the concentration of galactose in the system and the greater the inhibition effect.

Alpha-galactosidase has a wide variety of sources from plants, animals, and microorganisms. Seeds of legumes and whole grains, such as soybean seeds (Porter *et al*. 1990; De Fatima Viana *et al*. 2005), pea seeds (Blöchl *et al*. 2007), cowpeas *(Vigna unguiculata)*, guar seeds (McCleary 1983; Mulimani *et al*. 1997), coffee seeds (Shen *et al*. 2008), *Tachigali multijuga* Benth. seeds (Fialho *et al*. 2008), *Cucumis melo* plants (Gao & Schaffer 1999; Chrost & Schmitz 2000), *Phaseolus coccineus* seeds (Du *et al*. 2013), *Vicia faba* seeds (Dey *et al*. 1982), etc., are able to produce α-galactosidase during germination.

Alpha-galactosidases from microorganisms (bacteria and fungi) have the advantages of high production yields over those from plants. The typical bacteria for α-galactosidase production are *Bacillus (Bac.) stearothermophilus* (Gote *et al*. 2006), *Bac. amyloliquefaciens* (Chi & Cho 2016) and lactic acid bacteria (Garro *et al*. 1996; Yoon & Hwang 2008; Alazzeh *et al*. 2009). Among fungi yeasts and moulds are usually used to produce α-galactosidases. Those fungi, such as yeasts (Naumova *et al*. 2003; Tudor *et al*. 2013), *Aspergillus (A.) niger* (Somiari & Balogh 1992; Manzanares *et al*. 1998), *A. oryzae* (Cruz & Park 1982; Prashanth & Mulimani 2005), *A. parasiticus* (Shivam & Mishra 2010), *A. fumigatus* (De Rezende *et al*. 2005), *A. flavipes* (Ozsoy & Berkkan 2003), *A. terreus* (Falkoski *et al*. 2006), *Cladosporium (Cl.) cladosporides* (Mansour & Khalil 1998), *Gibberella (Gib.) fujikuroi* (Thippeswamy & Mulimani 2002), *Grifola (Gri.) frondosa* (Yang *et al*. 2015), *Mortierella (Mo.) vinacea* (Shibuya *et al*. 1997), *Penicillium (Pen.) purpurogenum* (Shibuya *et al*. 1995), *Pen. canescens* NCIM-5146 (Sinitsyna *et al*. 2008), *Pen. griseoroseum* (Falkoski *et al*. 2006), *Rhizomucor miehei* (Katrolia *et al*. 2012), *Rhizopus (Rh.) oligosporus* (Azeke *et al*. 2007), *Streptomyces* (Anisha & Prema 2008), *Thermomyces (Ther.) lanuginosus* (Puchart *et al*. 2000; Rezessy-Szabo *et al*. 2007), etc., have a potential to produce large quantities of α-galactosidases.

Production. Production procedures of crude enzyme preparation from plants usually contain such steps as preparation of pre-germinated seeds, powder, homogenization, resuspension, filtration, and centrifugation, etc. De Fátima Viana *et al*. (2005) described the method of crude α-galactosidase preparation from pre-germinated soybean seeds and observed that the α-galactosidase activity reached its peak after 48–60 h of germination. This finding was similar to the report of McCleary (1983), who reported that the level of α-galactosidase reached the optimum value when the guar seeds were germinated for 2 days. Isolation and purification of crude enzyme preparation from plants are similar to those from microbial sources.

There are two production approaches of alpha galactosidases derived from microbes which contain submerged fermentation and solid state fermentation (Gote *et al*. 2004; Shankar & Mulimani 2007). Submerged fermentation (SmF) is a fermentation process in which free flowing liquid (e.g., corn steep liquor, molasses and nutrient broths) is used as substrates to produce enzymes and bioactive compounds (Subramaniyam & Vimada 2012). During the fermentation process the substrates are utilized quite rapidly and hence need to be constantly supplied with nutrients (Subramaniyam & Vimada 2012). Submerged fermentation is the most suitable for microorganisms such as bacteria that require high moisture (Subramaniyam & Vimada 2012). Gote *et al*. (2004) investigated the effects of various culture conditions on the production of α-galactosidase from *Bac. stearothermophilus* (NCIM 5146) under submerged fermentation. The results from their study suggested that the maximum α-galactosidase activity (2.0 U/ml) was observed when *Bac. stearothermophilus* (NCIM 5146) grew on media based on soybean meal as a carbon source along with yeast extract and ammonium sulphate as a nitrogen source at 60°C.

The culture (or growth) media exert a remarkable impact on induction of enzyme production (Alazzeh *et al*. 2009; Kapnoor & Mulimani 2010). Generally speaking, α-galactosidases are usually secreted in the culture media which contain galactooligosaccharides. For example, Alazzeh *et al*. (2009) reported that *Lactobacillus* (*Lb.*) *reuteri* grown on raffinose

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displayed the highest α-galactosidase activity. While among carbohydrate sources including dextrose, galactose, lactose, melibiose, raffinose and sucrose, *Lb. reuteri* expressed the highest β-galactosidase activity on lactose. Yeast extract performed as the best protein source to produce both α- and β-galactosidases compared to other protein sources (beef extract, tryptic soy, and tryptone). These findings are consistent with the literature described by Gote *et al.* (2004). They also revealed that yeast extract was the best protein source for α-galactosidase from *Bac. stearothermophilus*.

Solid state fermentation (SSF) is defined as a fermentation process in which microorganisms grow on solid or semisolid materials and employ natural media as substrate (Krishna 2005). SSF has received further attention for the production of microbial enzymes over conventional submerged fermentation owing to its economic advantages. It can make use of cheap and abundant agro-industrial waste as substrate (Pandey *et al.* 2000) and the crude fermented product may be applied directly as the enzyme source (Krishna 2005; Kapnoor & Mulimani 2010). Among the various groups of microorganisms used in SSF, the filamentous fungi are most exploited for industrial applications mainly because they are able to grow on complete solid substrate, have broad stability profiles, and produce a wide range of extracellular enzymes (Manzanares *et al.* 1998; Kapnoor & Mulimani 2010).

Somiari & Balogh (1995) reported the characteristics of α-galactosidase formed by a strain of *A. niger* on wheat or rice bran and defatted soybean extract-based media. The highest activity of the crude preparation from wheat bran was 0.125 U per ml, whereas that from rice bran had a lower activity (0.08 U/ml). Kapnoor & Mulimani (2010) documented that the best substrate for *α*-galactosidase production by *A. oryzae* was the medium containing red gram plant waste-wheat bran (1:1, w/w) supplied with soybean flour (20%) in solid state fermentation (SSF). Rice flour, corn flour and wheat flour were less effective for the enzyme production. The maximum α-galactosidase activity occurred on 4th day of incubation at 35°C. Chen *et al.* (2010) reported that α-galactoside linkage oligosaccharides decreased dramatically in SBM fermented with *A. oryzae* owing to the secretion of α-galactosidases.

In order to improve the nutritional value of soybean products, Chi & Cho (2016) evaluated the nutritional influence of several microorganisms on soybean meal by the solid state fermentation process. They performed a qualitative analysis of raffinose and stachyose in soybean meal (SBM) and fermented soybean meal (FSBM) by thin layer chromatography. The levels of both raffinose and stachyose decreased drastically and no observation of glucose and sucrose in FSBM with *Saccharomyces (S.) cerevisiae* CJ1697 was made owing to their secretion of α-galactosidase and other carbohydrases. Tudor *et al.* (2013) reported that the strain *S. cerevisiae* PJ69-4 could degrade RFOs (raffinose and stachyose) in soybean meal to below detectable levels after submerged fermentation. Naumova *et al.* (2003) believed that *Saccharomyces* yeasts hydrolysed and utilized α-galactosides owing to polymeric MEL genes coding for α-galactosidases. In addition, *S. cerevisiae* strains could possess up to 5 unlinked genes (SUC1 to SUC5) coding for invertases which have the potential to utilize sucrose and raffinose as sole sources of carbon and energy (Tudor *et al.* 2013).

Isolation and purification. Ammonium sulphate precipitation (or precipitation with organic solvents under a lower temperature), dialysis, column chromatography, etc., are usually utilized in laboratory to isolate and purify enzymes, and ultrafiltration is a conventional method for enzymatic concentration (Garro *et al.* 1996; Gote *et al.* 2006; Shen *et al.* 2008; Shivam & Mishra 2010).

Several studies have described the purification procedures of α-galactosidase derived from plant seeds. Guimarães *et al.* (2001) reported that the α-galactosidase from soybean was purified by separation in an aqueous two-phase system based on polyethyleneglycol (PEG 1500) and sodium phosphate, followed by ion-exchange and affinity chromatography. Shen *et al.* (2008) described the isolation of α-galactosidases from coffee beans (*C. arabica*) in germination (GCB) and dormancy (DCB) by partitioning steps of ammonium sulphate precipitation, acetone precipitation, and DEAE Sepharose chromatography. After separation and purification, GCB was thought as a better alternative to DCB in commercial α-galactosidase production.

For the isolation of microbial α-galactosidases, Shivam & Mishra (2010) reported that α-galactosidase from *A. parasiticus* MTCC-2796 was purified 16.59-fold by acetone precipitation, dialysis, followed by sequential column chromatography with DEAE Sephadex A-50 and Sephadex G-100. Garro *et al.* (1996) documented the purification steps of α-galactosidase from *Lb. fermenturn* by preparation of cell-free extract, ammonium sulphate precipitation, dialysis, followed

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by gel filtration in a Sephadex G-200 column, and fraction in ion exchange chromatography with DEAE-Sepharose. The purified enzyme had a specific activity of 422.68 U/mg protein with 21.03-fold purification. Gote *et al*. (2006) described the purification procedure of α-galactosidase from *Bac. stearothermophilus* (NCIM-5146) by ultrafiltration, ethanol fractionation, hydrophobic chromatography on phenyl Sepharose CL-4B. 388-fold enzymatic purification was achieved by a relatively simple and highly reproducible single-step chromatographic procedure which omitted tedious and time-consuming multistep purification procedures.

Characterization of α-galactosidases. Alpha-galactosidases from numerous sources can be divided into two groups, acid and alkaline, based on their activity response to pH. The majority of α-galactosidases are acidic proteases, however, Gao & Schaffer (1999) described an alkaline α-galactosidase from melon (*Cucumis melo*) fruit tissue with a substrate preference for raffinose. Their finding was similar to Chrost & Schmitz (2000). Partial enzymatic properties of α-galactosidases from various sources are listed in Table 2.

Some α-galactosidases from fungal and bacterial sources, such as *A. terreus, Bac. stearothermophilus, Mo. vinacea, Neosartorya (N.) fischeri* P1*, Talaromyces (Tal.) leycettanus* JCM12802*, Tal. emersonii, Ther. lanuginosus, Rhizomucor miehei,* etc., display stability with a broad pH range and their highest activity is usually above 60°C (Shibuya *et al*. 1997; Gote *et al*. 2006; Simila *et al*. 2010; Shankar *et al*. 2011; Katrolia *et al*. 2012; Wang *et al*. 2015, 2016). Other α-galactosidases, such as *A. niger, A. oryzoe, Cl. cladosporides, Lb. fermenturn,* etc., have lower thermostability compared to the above-mentioned microbes (Garro *et al*. 1996; Mansour & Khalil 1998). Alpha-galactosidases from botanic sources (e.g., soybean, melon fruit, and benth seeds) exhibit a narrow pH range, and their thermostability is usually below 50°C. In general, α-galactosidases from microbial sources have a significant advantage in thermal stability over those from botanic sources (Shibuya *et al*. 1997; Guimaraes *et al*. 2001; Grieshop *et al*. 2003; Gote *et al*. 2006).

Enzymatic treatment of RFOs. As for α-galactosyl derivatives of sucrose or glucose, the raffinose family oligosaccharides (RFOs), especially raffinose and stachyose, are considered the major factors responsible for flatulence after ingestion of soybean or other legumes (Yamaguishi *et al*. 2013). Song & Chang (2006) found that two hours of incubation with α-galactosidase at 55–60°C removed 100% of RFOs in pinto beans. This is a significantly greater reduction than was achieved by soaking followed by boiling for 90 min (54.2%). Matella *et al*. (2005) found that incubation of black, red and navy beans with α-galactosidase for 1 h at 23°C was more effective (30–50% reduction) in the reduction of RFOs than soaking of beans for 5 h at 23°C (1–35% reduction). Therefore, the enzymatic hydrolysis of RFOs may be of biotechnological interest. The degradation of RFOs may be accomplished by α-galactosidase or invertase or both. In the presence of the invertases which are responsible for hydrolysis of the α-1,2 linkage RFOs are disintegrated into fructose and α-galactosyl derivatives of glucose (e.g., melibiose, manninotriose, etc.) (Rehms & Barz 1995). While the α-galactosidase cleaves the α-1,6 linkage, joining the galactosyl residue to sucrose, yielding galactose and sucrose. Hence, flatulence-causing oligosaccharides can be ultimately removed by α-galactosidases (Linden 1982; Rehms & Barz 1995). The hydrolysis mechanism of RFOs by enzymes is presented in Figure 1.

There are several studies available about the utilization of α-galactosidase derived from plants for the removal of raffinose family sugars in soymilk and legume flours. Mulimani *et al*. (1997) used 40 ml of the crude α-galactosidase from guar seeds (1.6 U/ml) to degrade RFOs in soybean flour (5 g) at 50°C for 4 h and observed the reduction of stachyose and raffinose by 91.9 and 90.4%, respectively. De Fátima Viana *et al*. (2005) used the partially purified α-galactosidase from germinating soybean to hydrolyse RFOs in soybean flour and found the reduction of stachyose and raffinose by 72.3 and 89.2%, respectively, after 6 h incubation at 40°C. Singh & Kayastha (2013) stated that enzymatic treatment for 6 h reduced raffinose and stachyose components of soybean flour by 80.0 and 85.0%, respectively. This can be utilized effectively for improving the nutritional quality of soy-based foods on a large scale, which could be one of the best alternatives for the lactose intolerant population for fulfilling their protein requirement.

A variety of successful attempts have been made on RFO enzymatic degradation by using α-galactosidases derived from microorganisms. Yoon & Hwang (2008) reported that *Leuconostoc mesenteroides* JK55 and *Lb. curvatus* R08 could completely degrade the non-digestive oligosaccharides in soymilk after fermentation for 18–24 hours. Somiari & Balogh (1992) used crude preparations of α-galactosidase from *A. niger* to degrade the RFOs present in cowpea flours and found the reduction of raffinose and stachyose

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by 95 and 82%, respectively. Cruz & Park (1982) demonstrated significant oligosaccharide removal by pretreatment of soymilk with crude extracts of α-galactosidase from *A. orvzae* culture. Ramalingam *et al*. (2010) used 10 ml of a partially purified enzyme (24 U) from *Pen. purpurogenum* to treat 100 ml of soymilk and observed that flatulence-causing raffinose and stachyose completely disappeared after 2 hours. Anisha & Prema (2008) reported over 90% reduction of the raffinose oligosaccharide content in horse gram and green gram flours by treatment with crude fungal α-galactosidase extracts from *Streptomyces griseoloalbus.* Enzymatic degradation by immobilized α-galactosidase can also be used to reduce RFOs (Kotiguda *et al*. 2007). Thippeswamy & Mulimani (2002) found that the raffinose family oligosaccharides were reduced to 79 and 66% by treatment of soymilk with free and immobilized α-galactosidase from *G. fujikuroi*, respectively. The enzymatic hydrolysis of RFOs by α-galactosidase is also an effective mechanism for the decrease of RFO content in navy bean flour. An advantage in the use of α-galactosidase to hydrolyse RFOs is that there is no loss in soluble solids, and the RFOs are simply converted into digestible sugars. This means that the vitamins and minerals that confer nutritional quality to the navy beans remain in the final product (Brain 2013).

Chi & Cho (2016) reported that the levels of both raffinose and stachyose in soybean meal drastically decreased by solid state fermentation with *S. cerevisiae*. During solid state fermentation of cottonseed meal, Zhang *et al*. (2016) successfully removed a variety of anti-nutritional factors including raffinose and stachyose by synergistic fermentation of three strains (*A. niger, A. oryzae* and *S. cerevisiae*).

Comparison of different treatments for RFO removal

Although a variety of traditional processing practices such as soaking, cooking, autoclaving, roasting,

Table 2. Partial enzymatic properties of α-galactosidases from various sources

Sources	Optimal pH/pH stability	Optimum *t*/thermostability (°C)	Stability time (h)	Reference
A. fumigatus	4.5/4–5	55/50	1.5	De Rezende *et al.* (2005)
A. oryzoe	4.5/4.0–8.0	50/50	> 0.5	Mansour & Khalil (1998)
A. niger	5.0/4.0–8.0	50/50	> 0.5	
A. terreus	5/–	65/65	0.5	Shankar *et al.* (2011)
Bac. stearothermophilus	6.5–7/3–9	65/70	0.5	Gote *et al.* (2006)
Benth. seed	5.0–5.5/3.5–6.5	50/40	12	Grieshop *et al.* (2003)
Cl. Cladosporides	5.0/5.0–7.0	40/60	> 0.5	Mansour & Khalil (1998)
Cucumis melo				
Acid form	5.5–6.0/4–8.0	50/–		
Alkaline form (1)	7.5/6.5–8.5	35–40/–		Gao & Schaffer (1999)
Alkaline form (2)	7.5/6.5–8.0	35–40/–		
Lb. fermenturn	5.8/5.0–6.5	45/50	0.5	Garro *et al*. (1996)
Mo. Vinacea (in 0.01% BSA)	3–4/2.0–7.0	60/50	1	Shibuya *et al.* (1997)
N. fischeri P1	4.0/3–11	75/70	1	Wang *et al.* (2015)
Pen. Purpurogenum	4.5/4.0–6.0	55/ < 40		Shibuya *et al.* (1995)
Pen. canescens	4–5/3–6	55/50	3	Sinitsyna *et al.* (2008)
Rhizomucor miehei	4.5/4.5–10	60/60	0.5	Katrolia *et al.* (2012)
Soybean (P1)	5.0/3–6	50/40	2	Guimarães *et al.* (2001)
Soybean (P2)	5.5/3–6	45/40	2	
Ther. Lanuginosus	5–5.5/3–11	65/55	24	Rezessy–Szabó *et al.* (2007)
Tal. Leycettanus	4.0/3–11	70/65	1	Wang *et al.* (2016)
Tal. emersonii	4.5/–	70/50	10 days	Simila *et al.* (2010)

The enzyme activities and kinetic values were determined by using pPNGal as the substrate

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frying and chemical treatment have been proposed by several investigators to reduce the level of galactosyl oligosaccharides in legumes, unfortunately lower removal or elimination efficiency are observed (Vijayakumari *et al*. 1996; Song & Chang 2006). Germination is an effective way to eliminate RFOs, but it requires higher time consumption and results in a reduction of dry material (such as vitamins and minerals). Utilization of α-galactosidase is a promising solution for the degradation of these undesirable sugars in terms of time and cost (Singh & Kayastha 2013). The advantages of α-galactosidase application over conventional processing methods are mild reaction conditions, low energy consumption, high removal efficiency, and a relatively shorter reaction period (Rehms & Barz 1995; Song & Chang 2006; Singh & Kayastha 2013).

Mansour & Khalil (1998) reported that crude fungal α-galactosidases (e.g., *Cl. cladosporides, A. oryzae* and *A. niger*) were 100% effective in eliminating the levels of raffinose and stachyose in chickpea flour, followed by germination (reduction in the levels of raffinose and stachyose by 68.9 and 75.1%, respectively). While other traditional techniques, such as soaking, cooking, autoclaving, dry heat, presoaking-cooking, presoaking-autoclaving, presoaking-dry heat, were less effective in removing the contents of raffinose and stachyose.

Alpha-galactosidases from plant sources such as soybean seeds are uniquely suitable for degradation of RFOs in soy meal slurry because they do not present any restriction regarding safety for use in food processing (Porter *et al*. 1990). However, the amount of α-galactosidase in soybean seeds or other plants is rather low, and the purification procedures are quite difficult. Furthermore, α-galactosidases from soybean germinating seeds are inhibited by galactose, which results in incomplete hydrolysis for RFOs. Guimarães *et al*. (2001) investigated the hydrolysis of raffinose and stachyose in soybean milk by purified α-galactosidases (the P2 enzyme) from germinating soybean seed and found that only small parts of flatulence-causing oligosaccharides were hydrolysed at 30°C for 8 hours. Through the trials of 3 α-galactosidases to remove galactooligosaccharides in soybean flour and soy molasses, Falkoski *et al*. (2006) believed that the microbial enzymes (e.g., *A. terreus and Pen. griseoroseum*) were more efficient than the soybean enzyme. Among 3 α-galactosidases, only the soybean enzyme was inhibited by galactose.

On the basis of Singh & Kayastha (2013), it is clear that in comparison with soaking and cooking α-galactosidase of *Cicer* seeds was more efficient for RFO reduction. In general, most of the enzymes suggested for this purpose are of microbial origin and therefore they do not have the GRAS (Generally Regarded as Safe) status. The purified α-galactosidase from inexpensive *Cicer* seeds was used for the hydrolysis of oligosaccharides present in soy flour. *Cicer* α-galactosidase might be a better choice for improving the nutritional value of soybean flour. However, microbial α-galactosidases have the advantages in high productivity over those from plant sources. Due to having been approved for the GRAS standard, some microbial α-galactosidases, such as lactic acid bacteria, *A. niger, A. oryzae*, *S. cerevisiae*, can be directly utilized to hydrolyse raffinose and stachyose in the processing of leguminous food and feed through submerged fermentation or solid state fermentation. However, large quantities of α-galactosidases, which were newly found from microorganisms (e.g. *A. parasiticus, A. fumigatus, Thermomyces lanuginosus,* etc.), require to be further approved for the GRAS standard prior to technological applications. The ideal system for the RFO removal would be such that the concentration gradient between the leaching water and the bean flour remained at a maximum, but that required a minimal mass of leaching water and did not require the loss of all the soluble solids. To that end it is proposed that a combination of leaching and enzymatic hydrolysis be employed. The presence of α-galactosidase in the leaching water would hydrolyse the RFOs as they were leached out of the bean flour, effectively keeping the concentration of RFOs in the leaching water at zero. Enzyme processing at low moisture contents was also demonstrated. This processing method has not been reported elsewhere and offers significant advantages for the production of reduced RFO low moisture powders for use in extrusion or for cost-effective drying and later use.

CONCLUSIONS

Among various treatments of galactooligosaccharides, utilization of microbial α-galactosidase seems to be a promising solution for the degradation of RFOs. To further meet commercial requirements, on the one hand, the identification of source microorganisms which are safe for human or animal use and capable of producing high levels of α-galactosidase has become more and more urgent. Once safety allowance for food additives is confirmed, these microorganisms

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will be directly utilized to remove or eliminate the raffinose family oligosaccharides in legume-derived products without numerous purification steps. Furthermore, the use of these crude enzymatic preparations from active microbes in the gastrointestinal tract has a variety of advantages in thermal stability and storability over the purified enzymes. On the other hand, the genes encoding those high-yielding and thermostable α-galactosidases should be cloned and overexpressed in suitable organisms to produce the enzymes at low cost so that more legume-derived products be provided for human or animal use in a way with relatively lower or absence of RFOs.

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