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# From seed to feed: assessment and alleviation of Raffinose Family Oligosaccharides (RFOs) of seed- and sprout-flours of soybean [*Glycine max* (L.) Merr.] - a commercial aspect

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**Abstract**

The purpose of the present work was to propose a commercially viable method for the reduction of flatulence-inducing Raffinose Family Oligosaccharides (RFOs) in soybean mature seed- and sprout-flours. For the same, the industrial application of purified food-grade α-galactosidase (α-GAL) from *Aspergillus niger* was evaluated by calorimetric and high-performance liquid chromatography (HPLC) methods. From mature seed to sprout formation with ~80% germination at a pilot-scale, an inherent decline of 76-80% in total RFOs [with a respective decline of 84%, 79% and 64% in corresponding raffinose (RAF), stachyose (STA) and verbascose (VER) content] was observed. Following treatment with exogenous food-grade α-GAL at an optimised level, a significant reduction of 98-99% and 93-96% in total RFOs (with a respective decline of 95%, 99%, 100% and 84%, 99%, 80% in corresponding RAF, STA and VER content) was observed in mature seed- and sprout-flours, respectively. Herein we reported for the first time, a simple and sequential combination of two processing methods (sprouting followed by α-GAL hydrolysis) that could open up the commercial use of soybean flour to feed- and food-industries to take advantage of its functional and nutritional properties, without any anti-nutritional problems usually associated with it. The results from the present work could also be extended to other agronomical important legumes, thereby offering promising revenue for the large-scale production of nutritionally enriched and RFOs-free flours- and -products thereof.



## Introduction

Soybean [*Glycine max* (L.) Merr.], widely regarded as "miracle/wonder/gold bean", represents a leguminous seed crop of tremendous economic importance. Being a rich source of high quality protein with all eight essential amino acids, oil, saccharides, vitamins, fibre, essential fatty acids, phytochemicals and lecithins (Singh *et al*., 2008), soybean nutritional quality has been well recognised and appreciated globally. In the last decade, the world soybeans production has increased significantly from 200 million MT in 2005 to 324 million MT in 2016 (USDA, 2016). Soybean consumption is determined mainly by its oil (20%), protein (40%) and soluble carbohydrate content (15%) (Singh *et al*., 2008). Soy-based food products provide a range of health benefits to consumers and is highly recommended by nutritionists/medical doctors mainly due to their hypo-lipidemic, anti-cholestrolemic and anti-atherogenic properties as well as their ability to reduce allergenicity and reduced risk of osteoporosis, prostate/breast cancer, cardiovascular and most hormone-associated health disorders (Asif and Acharya, 2013; Ahmad *et al*., 2014; Sharma and Baluja, 2015). However, despite being rich in all the essential nutrients and health benefits, its limited human consumption is influenced in parts by the indigestible flatulence causing raffinose family oligosaccharides (RFOs), primarily raffinose (RAF), stachyose (STA) and verbascose (VER) (Calloway and Murphy, 1968; Cristofaro, Mottu and Wuhrmann, 1974; Rackis, 1981). RFOs, being major sugar components in different varieties of legume seeds, have also been the object of many studies, and gained considerable attention by biochemists and nutritionists alike (Cerning-Beroard and Filiatre, 1976; Silva *et al*., 1990; Muzquiz *et al*., 1999; Muehlbauer, 2002; Martínez-Villaluenga *et al*., 2005; Giannoccaro *et al*., 2006; Kotiguda *et al*., 2007; Xiaoli *et al*., 2008; Aguilera *et al*., 2009; Kumar *et al*., 2010).

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RFOs are considered anti-nutritional units mainly due to the lack of α-galactosidase or melibiase (α-D-galactoside galactohydrolase, EC 3.2.1.22) in the gut of mono-gastric animals (Kotiguda *et al*., 2007). α-galactosidase (α-GAL) hydrolyses the terminal non-reducing α-D-galactose residues from the α-D-galactosides including galactose oligosaccharides (melibiose and RFOs) and branched polysaccharides [galactomannans and galacto-(gluco-) mannans] in an exo-fashion, thereby liberating the simple sugars (Naumoff, 2004). The predominant and relative large molecules of RFOs i.e. RAF and STA belonging to a class of fibres called FODMAPs (Fermentable Oligo-, Di-, Mono-saccharides and Polyols) remain undigested, enter the large intestine wherein they are fermented by native microbial flora thereby producing gases ($CO_2$, $H_2$ and to a lesser extent $CH_4$), resulting in the characteristic features of flatulence namely bloating, pain, nausea, cramps, diarrhoea, abdominal rumbling, social discomfort associated with the ejection of rectal gas and further worsen the symptoms of irritable bowel syndrome (IBS), a common digestive disorder (Cristofaro *et al*., 1974; Messina, 1999; Tsangalis and Shah, 2004). As a prophylaxis measure, commercially available dietary supplements of α-GAL such as Beano (AkPharma Inc, Pleasantville, NJ), has been recommended to improve the digestion and reduce the flatulence caused by the consumption of legumes.

The removal of soybean RFOs reduces the flatulence considerably (Suarez *et al*., 1999) and also increases the metabolisable energy of the diet (Coon *et al*., 1990; Sebastian *et al*., 2000). Conventional domestic processing methods such as soaking, boiling, cooking, roasting, toasting, parching, frying, steaming, gamma-radiation, ultrasonic, high hydrostatic pressure, fermentation and sprouting have been adopted, depending upon tradition and taste preferences, to reduce the RFOs levels in legumes. Soaking is the easiest, but also most ineffective way of reducing the RFOs (33.3% and 46.6% reduction in RAF and STA, respectively) in soybean (Han and Baik, 2006). Ultrasound (47 MHz) and high hydrostatic pressure (621 MPa) applications were reported to be relatively more effective (with 55.7%, 33.9% and 28.6%, 7.4% reduction in corresponding RAF and STA, respectively) by promoting the enhanced leaching of RFOs (Han and Baik, 2006). Cooking and autoclaving of pre-soaked soybean resulted in losses of 13%, 8%, 78% and 12%, 11%, 81% in RAF, STA and VER contents, respectively (Ramadan, 2012). By combination of soaking, de-hulling, washing and cooking, >50% of total RFOs can be removed (Egounlety and Aworh, 2003). Fermentation with *Rhizopus oligosporus* resulted in >50% and >80% reduction in soybean RAF and STA content, respectively (Egounlety and Aworh, 2003). In raw, cooked and roasted soybean, fermentation with *Lactobacillus plantarum* resulted in a respective losses of RAF by 28%, 58%, 68% and STA by 30%, 72%, 76% (Adeyemo and Onilude, 2014). Low RFOs meal from genetically modified soybeans also represents another way of reduction of RFOs in the diet (Parsons, Zhang and Araba, 2000). Among all these methods, soybean germination represent a cost-effective way of reducing RAF and STA content by 75% and 87%, respectively (Silva *et al*., 1990). However, so far none of the aforementioned methods are commercially viable as well as full-proof enough to completely eliminate RFOs levels in soybean and other legumes. α-GAL from various other sources such as bacteria, fungi, plants and animals (Keller and Pharr, 1996; Matsuura *et al*., 1998; Marraccini *et al*., 2005; Cao *et al*., 2007; Cao *et al*., 2010) draws a lot of interest in the scientific community around the world by offering a promising solution in the elimination of RFOs from legume flours (Somiari and Balogh, 1993; 1995). Nevertheless aside from circumstantial evidence reported only under laboratory conditions, a practical utilisation of crude α-GAL in legumes is very scarce. Notably, Matella *et al* (2005) proposed a commercial removal of STA and VER from legume (*Phaseolus vulgaris*) flours. However, their method relies on the cumbersome extraction of soluble sugars from beans, followed by α-GAL treatment and addition of reduced RFOs sugars back to the bean slurry prior to drying and milling, without taking care of improvements in other nutritional parameters. With an intent of viable commercial perspective, herein we described the evaluation and enzymatic (food-grade α-GAL from *A. niger*) reduction of RFOs components i.e. RAF, STA and VER in both soybean mature seed- and sprout-flours at a pilot-scale. To the best of our knowledge, this novel information (sprouting followed by α-GAL hydrolysis) could also stimulate the application of these inexpensive and easy methods for industrial-scale production of nutritionally enriched and RFOs-free flours from other legumes.

## Materials and methods

### *Chemicals*

α-GAL (10,000 GAL units/g) in industrial quantity was purchased from Alferm Biotec, Bengaluru, India. The activity of this product was maintained by the vendor on a periodic lot-to-lot basis by performing a standard α-GAL assay.

One GAL unit is defined as the amount of enzyme required to liberate p-nitrophenol from synthetic substrate p-nitrophenyl-α-D-galactopyranoside (PNPG) at the rate of 1.0 µmol/min at pH 6.5 at 37°C under the standard assay conditions. Raffinose/ Sucrose/D-Glucose assay kit (Catalog#K-RAFGL) was procured from Megazyme International Ltd., Ireland (Wicklow, Ireland). HPLC-grade sugar standards: D-(+)-glucose (Catalog#G8270), sucrose (Catalog#S7903), D-(+)-raffinose (Catalog#R0514), stachyose (Catalog#S4001) and verbascose (Catalog#56217) were procured from Sigma-Aldrich (Sigma Chemical Co., St. Louis, USA). Sugar-pak I chromatographic column, 10 µm, 6.5 × 300 mm (Part No. WAT085188) was purchased from Waters Corporation (Waters India Pvt. Ltd.). Ethylenediaminetetraacetic acid calcium disodium salt (Catalog#ED2SC) and other analytical reagents (AR) grade chemicals used in the present work were obtained from Sigma-Aldrich (St. Louis, MO, USA).

*Seed material and sprouting conditions*

Seeds of soybean (*Glycine max* (L.) Merr.) 'JS9560' (a popular commercial variety in Central India), procured from ICAR-Indian Institute of Soybean Research (IISR), Indore (Madhya Pradesh) were used in the present work. Soybean seeds were cleaned thoroughly to make them free from dust, dirt, stubbles and foreign matters. Damaged and immature/broken seeds with cracked hull were discarded mechanically. Cleaned and mechanically-sorted seeds were surface-sterilised with 0.5% (w/v) sodium hypochlorite (NaClO) solution for 10 min, and rinsed thoroughly with running distilled water to remove any traces of NaClO. Approximately 5 kg of cleaned and surface-sterilised seeds per batch were soaked in 25 L potable water for 4 h under constant shaking at 10 rpm in a customised motor seed dressing drum (GMW, Ambala, India), followed by drainage of water and rinsing with distilled water. The seeds were subsequently distributed evenly on filter paper in a single layer in sterile germination trays. Each germination tray was wrapped with a muslin clothes (to allow entry of oxygen for the germinating seed while minimising the contamination during the test-period), and placed in the customised seed germinator [ACM-78093-S, Acmas technologies Pvt. Ltd., India] at 30°C with 90% relative humidity (RH) for 72 h (Agrahar-Murugkar and Jha, 2009). Germination trays were watered daily according to its requirement with distilled water during the course of germination. Physiological germination in terms of visible radical protrusion of at least 2 mm (ISTA, 2012) was assessed each day over a test period of 3 d. The experiment was performed in three replicates.

*Soybean flour preparation*

Sprouts obtained after germination test period were subjected to drying in an hot air oven incubator (Inlab, Chennai, India; 230 volt, 5.4A) at 55°C to a final moisture content of 6-8%, a level recommended for the production of soyflour (Gandhi, 2008). Mature seeds and dried sprouts were milled to a fine powder using analytical grinder mill, passed through a 0.6 mm sieve to obtain flour of 500 µm particle size. The obtained fine flours were stored as a powder in tightly closed containers at room temperature till further use.

*α-GAL treatment*

Exogenous application of α-GAL was performed concurrently in both mature seed- and sprout-flours. Approximately, 1,000 g of mature seed- and sprout-flours were treated with different concentrations of α-GAL (0, 50, 100, 200 and 300 GAL units/mL) in a final volume of 3,000 mL distilled water, pH 6-7 (flour:water = 1:3), with a continuous shaking at 50 rpm in a rotary shaker at 50°C for different time points (0, 30, 60, 120 and 180 min). Untreated control was treated with distilled water only. Following incubation at an indicated time-point, contents of each tube were removed and filtered through a Whatman No. 1 filter paper. Samples were dried under vacuum at 40°C for 4 h, grounded to a fine powder to produce α-GAL-treated soybean flour, and quantified for RFOs estimation.

*Calorimetric estimation of RFOs*

The soluble carbohydrate concentrations of mature soybean seed- and sprout-flours were determined using an enzyme based Raffinose/Sucrose/D-Glucose assay kit (Megazyme) as per manufacturer's recommendation as described earlier (Kumar *et al*., 2010). It consisted of α-GAL (from *A. niger*), invertase (from yeast) and glucose determination reagent i.e. glucose oxidase peroxidase (GOPOD; glucose oxidase + peroxidase) for colorimetric estimation of sucrose and RFO content. The kit is based upon the principle to stepwise hydrolyse complex soluble carbohydrates to glucose followed by its colorimetric measurement. Soluble sugars such as sucrose and RFOs were hydrolysed with α-GAL and invertase into D-glucose, D-galactose and D-fructose. D-glucose concentration was determined using GOPOD reagent. The concentration of RAF, STA, VER and other higher homologues of the RFOs in flour samples were measured as a group, because α-GAL hydrolyses all members of the RFO family. Since 1 mol of each of the RFO contains 1 mol of D-glucose, the RFO concentrations were presented on a molar basis. Briefly, finely ground flour (0.5 ±

0.01g) of each sample was treated with 95% ethanol (to digest the endogenous enzymes completely) at 85°C for 20 min, and the final volume was made up to 50 mL using sodium acetate buffer (50 mM, pH 4.5). Digested mixture so obtained was incubated at the room temperature for 20 min and vortexed to obtain uniform slurry. Subsequently, 2 mL chloroform was added to 5 mL slurry obtained, and vortexed for 15 s followed by centrifugation at 1,000 g for 10 min. A volume of 0.2 mL from the aqueous phase of the supernatant so obtained was taken in three tubes (namely, A, B, and C). A volume of 0.2 mL sodium acetate buffer (50 mM, pH 4.5), 0.2 mL of invertase (8.3 U/mL) and a mixture of invertase + α-GAL (invertase 8 U/mL and α-GAL 40 U/mL) was added into tubes A, B, and C, respectively. All three tubes were incubated at 50°C for 20 min. Reagent blank (0.4 mL sodium acetate buffer) and glucose control (0.1 mL standard glucose solution, which contained 0.556 µmol of glucose + 0.3 mL sodium acetate buffer) were also taken simultaneously. Subsequently, 3 mL of GOPOD reagent was added in all of the tubes and incubated again at 50°C for 20 min. The glucose concentration for tubes A, B, and C and glucose control was determined by measuring the change in absorbance of quinoneimine dye at 510 nm against the reagent blank using a UV/Vis Microplate and Cuvette spectrophotometer (Thermo Scientific™ Multiskan™ GO). Glucose, sucrose and RFOs concentrations were shown in mmol/100 g flour. The concentrations of glucose, sucrose and RFOs were calculated as follows:

$$\text{Glucose (mmol/100 g)} = \Delta A \times F \times 50$$
$$\text{Sucrose (mmol/100 g)} = (\Delta B - \Delta A) \times F \times 50$$
$$\text{RFOs (mmol/100 g)} = (\Delta C - \Delta B) \times F \times 50$$

where ΔA, ΔB and ΔC were the absorbance of sample plus sodium acetate buffer, sample plus invertase and sample plus invertase and α-GAL enzyme solution, respectively.

F = Factor to convert from absorbance to µmol of glucose

0.556 (µmol of glucose) / GOPOD absorbance for 0.556 µmol of glucose

250 = conversion to 50 mL of extract, 200 = conversion from 0.5 to 100 g of sample and 1/1000 = conversion from µmol to mmol.

All enzymatic assays were performed in three technical replicates ($n$ = 3) for each sample.

*High Performance Liquid Chromatography (HPLC) based estimation of RFOs*

*Sample preparation*

A method for the quantitative extraction of soluble sugars from mature seed- and sprout-flours and their subsequent recovery from the 80% (v/v) ethanol solvent was adopted as outlined earlier (Tahir *et al*., 2011; Gangola *et al*., 2014; Raja *et al*., 2015), with certain modifications. Approximately, 150 mg fine grounded flour of each sample was extracted twice with 40 mL 80% ethanol-water in a hot water bath at 55-60°C with a magnetic stirrer for 45 min. The samples were centrifuged for 30 min at 10,000 rpm, and the supernatant was collected. The extraction step was repeated, and the recovered supernatants were pooled. The pooled extract was reduced in volume by using a rotary vacuum evaporator at 70°C to evaporate the ethanol. The concentrated sugar syrup was re-dissolved in 10 mL distilled water, and filtered through a 0.45µm Millipore membrane (Millipore, Bedford, MA) into a 1.5 mL HPLC vial with a rubber slit septum. The samples were then ready for injection into HPLC.

*HPLC conditions and Instrumentation*

A HPLC system equipped with an auto-sampler, a gradient programmer, a solvent pump and a refractive index detector (Agilent 1200) was used. The chromatographic column used was a Waters sugar-pak I column (Part No. WAT085188) with an internal dimensions of 6.5 × 300mm, filled with micro-particulate size (10 µm) of cation-exchange gel in calcium form. The mobile phase consisted of 50 mg/mL solution of calcium disodium salt of ethylenediaminetetraacetic acid ($CaNa_2EDTA$). Operating conditions with a flow rate of 0.2 mL/min at an ambient temperature were maintained. Aliquots (50 µL) of filtered samples were injected into the mobile phase of HPLC via an auto-sampler to record chromatograms. The detection was done by measuring the change in refractive index of the column effluent passing through the flow-cell. All chromatograms were re-ordered on Agilent chemstation software. Authentic commercially available sugar standards: glucose, sucrose, RAF, STA and VER were dissolved at 5 mg/mL in water, immediately prior to HPLC analysis and subjected to HPLC in a concentration range of 0-100 µg/mL. A 50 µL aliquots of these standard solutions were injected into the chromatographic system, and the resulting peak areas were plotted against concentration for the linear calibration curve. Retention times of the standards were used to identify the corresponding

peaks on the HPLC chromatograms of flour samples. Peak area was quantified by Chemstation software (Agilent). The relative concentration of individual sugar was calculated after superimposing the chromatogram of the sample on their corresponding standard curve. Individual sugar concentration was expressed as mmol per 100 g on a dry weight basis. Concentrations of RAF, STA and VER were summed to compute the total RFOs concentration.

*Data analysis*

The results were expressed as means ± S.D. One-way Analysis of Variance (ANOVA) was used to analyse the level of statistical significance between groups. $p < 0.05$ was considered statistically significant.

## Results and discussion

Recent changes in cost of commodity-based sources of metabolisable energy (ME) inputs has put a tremendous demand on soybean feed- and food-products to deliver both protein and ME in diet. Being a rich source of total RFOs (Hagely *et al*., 2013), soybean also represents an ideal model system for the evaluation and reduction of RFOs in other legume flours. In the present work, an attempt was made to improve the soybean and its products consumption thereof, by lowering their RFOs levels within a permissible limit at commercial level.

*Effect of sprouting on soybean RFOs levels at pilot-scale*

Soybean seeds of commercial variety, 'JS9560' were sprouted under controlled environmental conditions at pilot-scale, with a germination rate of ~80% (Fig. 1). High quality dried flours was made from both mature seeds and sprouts (Fig. 2A inset), as per the recommendation for the production of soyflour (Gandhi, 2008). The RFOs levels in soybean mature seed- and sprout-flours was evaluated and presented in Fig. 2. Our calorimetric and HPLC results demonstrated that soybean sprouting at a pilot-scale resulted in an inherent decline of 76-80% in total RFOs in sprout-flour (1.7-2.1 mmol/100 g dm) in contrast to their corresponding seed counterpart (8.41-8.68 mmol/100 g dm). Within RFOs, an individual and respective decline of 84% (from 1.98 to 0.32 mmol/100 g dm), 79% (from 6.3 to 1.34 mmol/100 g dm) and 64% (from 0.14 to 0.05 mmol /100 g dm) in corresponding RAF, STA and VER content was observed in sprout-flour (Fig. 2B). The data for a typical chromatographic separation of sugar standards and corresponding calibration curve of each sugar standard is not shown. Notably, total RFOs estimation by calorimetric and HPLC methods were largely in accord with each other, with a perfect positive correlation ($r = 1$) between these two methods. Additionally, a coherent decline of 96-98% in sucrose content of sprout-flours in comparison to its seed counterpart (from 4.16-5.75 to 0.1-0.26 mmol/100 g dm) was also observed (Supplementary Fig. S2). The observed decrease in RFOs as well as sucrose content in soybean sprouts was mainly due to the autolysis caused by the activation of endogenous α-GAL and invertase (β-D-fructofuranosidase, EC3.2.1.26), respectively during germination process (Kasai, 1976; Kuo, Doehlert and Crawford, 1990). Following germination, these endogenous hyper-active α-GAL and invertase resulted in a potent hydrolysis of their respective substrates, α-D-galacto-oligosaccharides and β-D-fructofuranoside into di- and/ mono-saccharides, which could be readily used as an energy or carbon source for plant growth (Kasai and Suzuki, 1980). Notably, apart from reducing the anti-nutritional factors, soybean germination has been also reported to significantly improve its nutritional, physico-chemical and biological properties (Bau *et al*., 1997, 2000; Dikshit and Ghadle, 2003; Agrahar-Murugkar and Jha, 2009). Thus, soybean germination at an industrial-scale could provide an exciting prospect of meeting up the soy-food market expectation, with a considerable low RFOs content along with a concomitant high nutritional value. Of note, there was no 100% removal of total RFOs during soybean sprouting at pilot-scale, and the residual RFOs levels in sprouts still raise a serious concern about its consumption, which cannot be ignored.

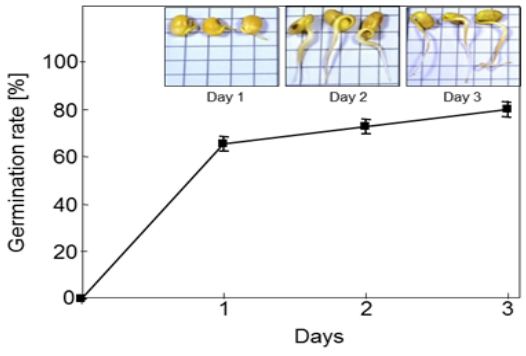


Fig. 1. Germination of soybean at pilot-scale. Soybean seed germination rate following four hours inbibition in water. Results are shown as a means ± SDs from three independent experiments (*n = 3)*, with 50 seeds per measurements. Inset depicts the representative images of temporal sprouts formation during the course of soybean germination test.

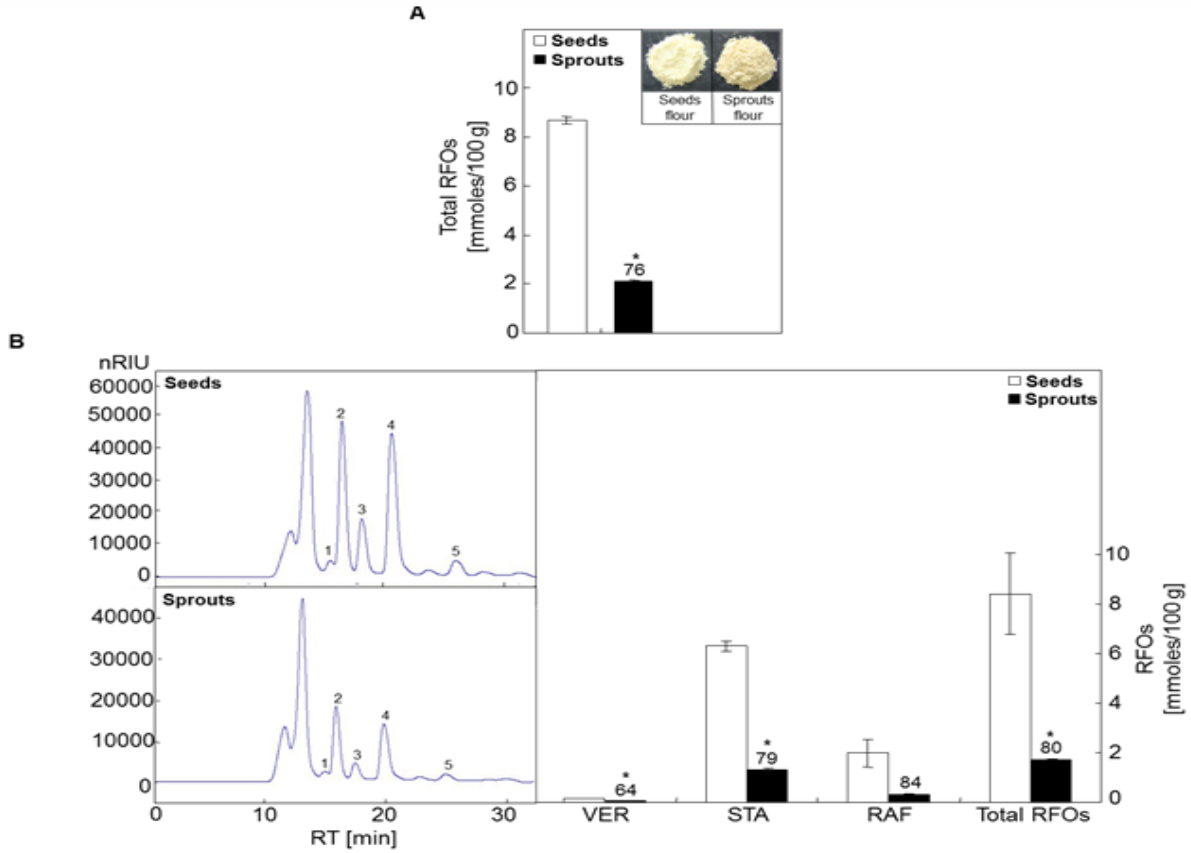


Fig. 2. Effect of germination on total and individual RFOs components of soybean.

A. Calorimetric- and B. HPLC-based estimation of total and individual RFOs components (RAF, STA, VER) in soybean mature seed- and sprout-flours following three days after germination. Inset shows the soybean flours prepared from the mature seeds and sprouts following three days after germination. Representative chromatogram shows the separation of ethanol soluble sugar extracts from mature seed- (top) and sprout-flours (bottom). Each sugar was evaluated by peak identification with overlapping retention times (in min) of corresponding standard sugar. Data are expressed in terms of mmoles per 100 g on a dry weight basis and plotted as bar graph. Numbers over the each bar indicate the percent reduction in respective sugar of sugar in sprout-flour relative to its corresponding seed counterpart. Each data represent means ± SDs from three independent experiments ($n$ = 3). Asterisks indicate the significant difference in RFOs levels of soybean sprout-flour at $p < 0.05$, when compared with their seed counterpart. RAF, Raffinose; STA, Stachyose and VER, Verbascose.

*Effect of exogenous α-GAL treatment on RFOs levels of soybean seed- and sprout-flours*

Recently, partially purified extracellular α-GAL prepared from *A. niger* has been reported to be effective in reducing the RFOs levels in seeds of all cultivars of red gram (*Cajanus cajan* L; Devindra and Aruna, 2016). In the present work, exogenous application of purified α-GAL from *A. niger* under optimum assay conditions (50°C for 3 h at pH 6-7) resulted in a significant reduction of up to 98-99% (from 8.41-8.68 to 0.14-0.17 mmol/100 g dm) and up to 93-96% (from 1.7-2.1 to 0.13-0.07 mmol/100 g dm) in total RFOs, with an individual and respective decline of 95% (from 1.98 to 0.11 mmol/100 g dm), 99% (from 6.3 to 0.06 mmol/100 g dm), 100% (from 0.14 to 0 mmol/100 g dm) and 84% (from 0.32 to 0.05 mmol/100 g dm), 99%, (from 1.34 to 0.01 mmol/100 g dm), 100% (from 0.05 to 0 mmol/100 g dm) in RAF, STA and VER contents of mature seed- and sprout-flours, respectively (Fig. 3A and B). The sucrose levels observed in each of α-GAL treated sample was relatively lower, while that of glucose was relatively higher at each corresponding time points as compared to their untreated enzyme control counterparts (without α-GAL addition; data not shown). The observed RFOs reduction with concomitant increase in glucose content by exogenous α-GAL addition was mainly due to the hydrolysis of α-galactosidic linkages of α-D-galacto-oligosaccharides into mono- or di-saccharides. The notable and unexpected decline in sucrose concentration during α-GAL treatment, despite the fact that it was also a by-product of RFOs hydrolysis can be explained by the fact that α-GAL has also been reported to possess intrinsic invertase activity and could cause the hydrolysis of sucrose at a site other than its active galactosidase site,

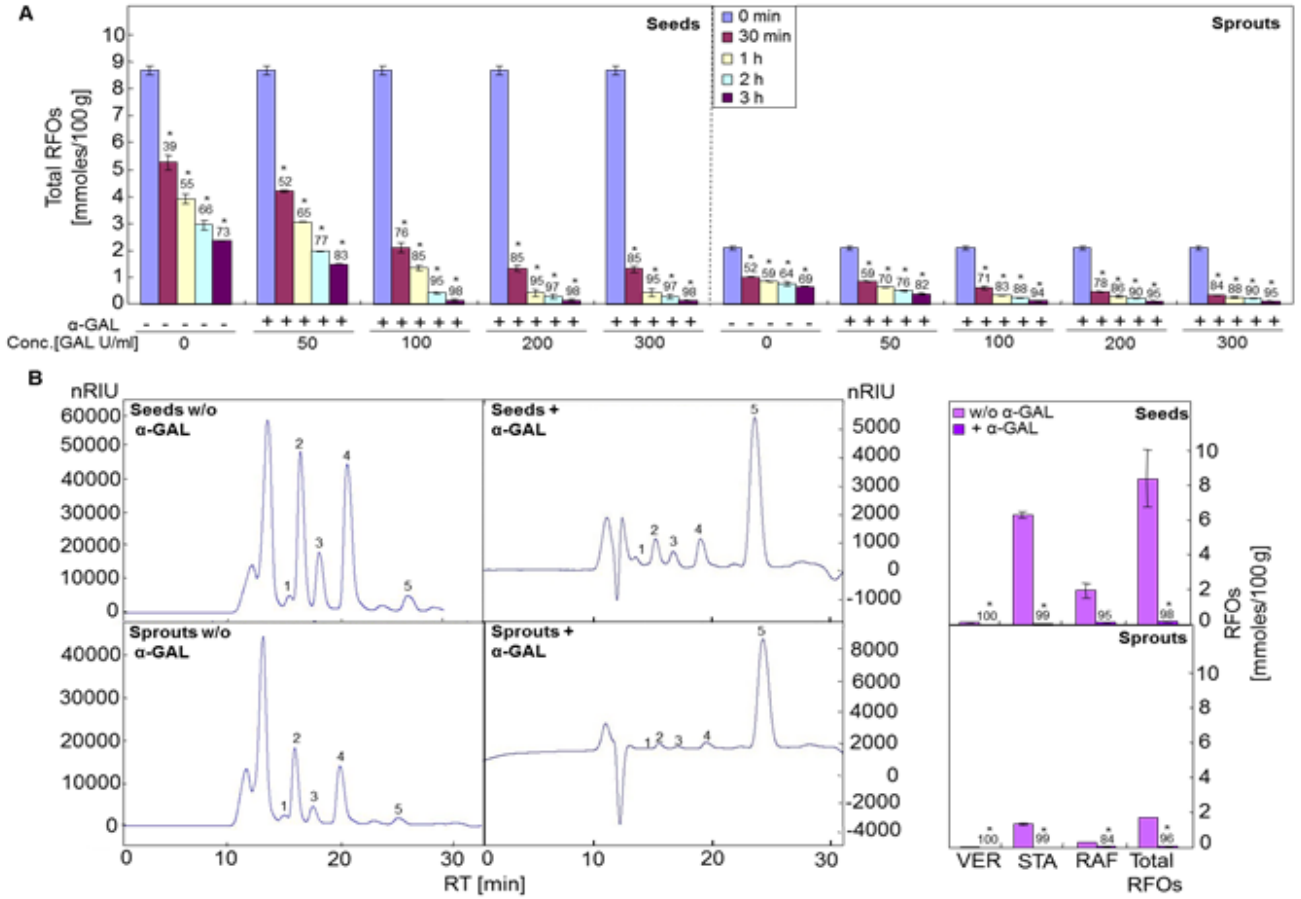


Fig. 3. Effect of α-GAL concentration and incubation time on total and individual RFOs components of soybean.

A. Calorimetric estimation of total RFOs contents in soybean mature seed- (left) and sprout-flours (right) over a range of α-GAL concentration (0-300 GAL units/mL) and incubation time (0-3 h) tested.

B. HPLC-based evaluation and validation of total RFOs and its individual components (RAF, STA, VER) at an optimised α-GAL concentration (100 GAL units/mL) and incubation time (3 h) in mature seed- (top) and sprout-flours (bottom). Each sugar was evaluated by peak identification with overlapping retention times (in min) of corresponding standard sugar. Data are expressed in terms of mmoles per 100 g on a dry weight basis and plotted as bar graph. Numbers over the each bar indicate the percent reduction in respective sugar of each flour at an indicated time point, relative to their corresponding t = 0 counterpart. Each data represent means ± SDs from three independent experiments (n = 3). Asterisks indicate the significant difference in RFOs levels soybean mature seed- and sprout-flours at $p < 0.05$, when compared with their corresponding t = 0 counterpart. RAF, Raffinose; STA, Stachyose and VER, Verbascose.

without any inhibitory effect on hydrolysis rate of its substrates, RAF and STA (Slominski, 1994; Brain, 2013). Notably during the complete assay period, a decline of up to 73% (from 8.68 to 2.37 mmol/100 g dm), 69% (from 2.1 to 0.65 mmol/100 g dm) in total RFOs and up to 67% (from 5.75 to 1.89 mmol/100 g dm), 54% (from 0.26 to 0.12 mmol/100 g dm) in sucrose content, with a concomitant increment of up to 481% (from 0.27 to 1.57 mmol/100 g dm) and 465% (from 0.23 to 1.3 mmol/100 g dm) in glucose content was also observed in untreated enzyme controls (without α-GAL addition) of both mature seed- and sprout-flours, respectively (Fig. 3). A similar reduction in total RFOs levels with an increased duration of soaking was also reported in a previous study (Mulimani and Devendra, 1998). The possibility of leaching out of RFOs and sucrose and their breakdown by endogenous α-GAL/invertase activation during soaking (that usually happens during seed germination) cannot be ruled out.

It is important to note that there may be certain other components of soybean (e.g. soluble fibre) that also contribute to flatulence, thus the flatulence response to α-GAL treated soybean flours should be investigated by *in vitro* as well as *in vivo* studies. In this context, it is worth mentioning that our future research is focused on sensory- and safety-evaluation to measure the acceptability, palatability, functionality, storage properties and other nutritional aspects of α-GAL treated soybean seed- and sprout-flours and -products thereof.

*Advantages and commercial aspect of α-GAL treatment*

An advantage of the use of α-GAL to hydrolyse RFOs in flour is that there is no loss of soluble solids (vitamins and minerals), wherein RFOs are converted to simple digestible sugars, unlike traditional method of soaking and boiling of seeds. In literature, there are various reports of beneficial nutritional implications by reducing the RFOs content in legume flour blends upon exogenous supplementation of crude α-GAL from either plant, bacterial or fungal sources. Addition of α-GAL to lentil, peas, cowpea (from *A. niger*) and chickpea (from *Gibberrella fujikuroi*) caused a decrease in RAF by 61-68%, 41-48%, 93.3%, 88-92% and STA by 80-85%, 67-91%, 82%, 82-86%, respectively (Somiari and Balogh, 1993; Mulimani *et al*., 1997; Frias *et al*., 2003). The use of crude α-GAL (from *Cladosporium cladosporides*, *A. oryzae* and *A. terreus*) in complete removal of RAF and STA in chickpea flours has also been reported (Mansour and Khalil, 1998). Crude α-GAL treatment (from *Streptomyces griseoloalbus*) reduced RAF by 97.5%, 96.3% and STA by 93.2%, 91.8% in horse and green gram flours, respectively (Anisha and Prema, 2008). In soybean flour, crude α-GAL from germinating guar, *Cyamopsis tetragonolobus*, *Cicer arietinum* and germinating *G. max* caused a respective reduction in RAF content by 90%, 80% and 89.2%, respectively, while a corresponding reduction of 92%, 85% and 72.3% in STA content was observed (Mulimani *et al*., 1997; de Fatima Viana *et al*., 2005; Singh and Kayastha, 2013). Fungal α-GAL (from *A. saitoi*, *Mortierella vinacea*, *Cyamopsis tetragonolobus*, *G. fujikuroi*, *A. oryzae*, *A. terreus* and *Cladosporium cladosporioides*) has also been reported for RFOs hydrolysis in soymilk and soya (Sugimoto and Buren, 1970; Thananunkul *et al*., 1976; Cruz *et al*., 1981; Cruz and Park, 1982; Shivanna *et al*., 1989; Mulimani, 1995; Shankar *et al*., 2006; Kotiguda *et al*., 2007; Ferreira *et al*., 2011). Notably, all of these findings use the laboratory-scale preparation of crude α-GAL in seed flours only, which were either time consuming, expensive, not full-proof enough in complete removal of total RFOs or not economically viable for their consideration as a commercial commodity. Moreover, authenticity of this preparation for their consumption in terms of Generally Recogniszed as Safe (GRAS) also raises a question mark over their practical utility in daily life.

α-GAL used in this study was produced by controlled fermentation of *A. niger* which complied with FCC and FAO/WHO JECFA recommended specifications for food-grade enzymes. This product is commercially available with a strict recommendation as a dietary supplement only. It is standardised to 30,000 GAL units/g and can be customised to strength from 1,000-30,000 GAL units/g. It is supplied in industrial quantity of 20-25 kg pails, with a shelf-life of 18 months at 30°C under dark storage that can be further extended by storing at <4°C. Being available as a dried powder and readily soluble in water, it also offers the possibility of blending with other legume flours, thereby allowing the RFOs hydrolysis to take place upon addition of water during subsequent processing steps. In the present work, >95% of total RFOs in both soybean mature seed- and sprout-flours was reduced by the aforementioned α-GAL, which can be procured at a current cost of approximately $185/kg at concentration of 10,000 GAL unit/g. At an optimum dose of α-GAL (100 GAL unit/mL) in a leaching water of three times the volume required to saturate the soybean flour (1 kg flour : 3 L water), the amount of enzyme required is 30,00,00 GAL unit/kg flour, equating the α-GAL cost of $5.55/kg of soybean flour. Considering the health benefits and value added feed- and food-products that can be made

from this RFOs-free soy flour, the incurred α-GAL cost is affordable and adds only a marginal cost to the soybean processing at a commercial level. Thus, α-GAL used in the present work has the potential commercial application in feed- and food-industries for the production of RFOs-free flours from soybean as well as other legumes.

## Conclusions

The present work demonstrated that soybean sprouting (~80%) at a pilot-scale resulted in a considerable decline of up to 76-80% in total RFOs levels. With a prospect of commercial viability, exogenous addition of purified food-grade α-GAL at 100 GAL unit/mL at 50°C for 3 h (pH 6-7) removes >95% of total RFOs in both soybean mature seed- and sprout-flours. Henceforth, it is concluded that sprouting followed by exogenous supplementation with food-safe and commercially viable α-GAL represents an efficient, effective and economical means of reducing the anti-nutritive values with concomitant increase in the nutritive value of soy-flour and -products thereof.

## Conflict of interest

All authors have read and approved the final manuscript. The authors declare that there are no conflicts of interest. The content of this manuscript does not necessarily reflect the views or policies of the LSTC-ITC.

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## Abbreviations:

ANFs, Anti-Nutritional Factors
FODMAPs, Fermentable Oligo-, Di-, Mono-saccharides and Polyols
HPLC, High-Performance Liquid Chromatography
RFOs, Raffinose Family Oligosaccharides

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