REVIEW

α-Galactosidases: characteristics, production and immobilization

PATRÍCIA CRISTINE SCUSSIATO DE ANDRADE TABORDA – LÍGIA ALVES DA COSTA CARDOSO – SUSAN GRACE KARP

Summary
The enzyme α-D-galactoside galactohydrolase (E.C. 3.2.1.22), known as α-galactosidase or α-gal, catalyses the hydrolysis of oligosaccharides present in legumes, in particular soybean. This enzyme is not present in the gastrointestinal tract of humans, thus restricting the consumption and nutritional benefits of legumes due to gastrointestinal discomfort, flatulence and diarrhea caused by no metabolization of oligosaccharides such as melibiose, raffinose or stachyose. One of the options to solve these problems is the enzymatic treatment of these food ingredients, during preparation or inside the organism, through ingestion of the enzyme. The objective of this review is to present the main biochemical and technological characteristics of α-galactosidases, and also to describe aspects related to their production, purification, immobilization and applications in food products.

Keywords
α-galactosidase; non-digestible oligosaccharides; immobilization; submerged fermentation

The human consumption of certain legumes such as soybeans, beans and peas has been limited by the presence of non-digestible oligosaccharides, particularly melibiose, raffinose and stachyose. The main reason for this restriction is that the human being, as well as all monogastric animals, does not produce the enzyme α-D-galactoside galactohydrolase (EC 3.2.1.22), known as α-galactosidase, in the gastrointestinal tract. This enzyme is necessary for the hydrolysis of α-1,6-galactosidic bonds found in these oligosaccharides, releasing α-D-galactose. Since the oligosaccharides are not metabolized, they are available to be fermented by gas-producing microorganisms present in the intestine, causing flatulence, which can be accompanied by diarrhea, headaches, dyspepsia, confusion and decreased concentration at work [1, 2].

The benefits of soybean for human health are clearly an important point to be considered for its promotion as a food ingredient. Besides the quality of its protein, studies showed that soybean can be used preventively and therapeutically in the treatment of cardiovascular diseases, in the prevention of osteoporosis and for reducing the symptoms of menopause. Besides that, it presents anticancer effects, protective effect against obesity and also lowers cholesterol [3, 4]. Thus, the solutions to reduce the discomfort associated with the consumption of this legume can be very beneficial for health. In addition to the direct intake of α-galactosidase enzyme during the meals, the pre-treatment of food-ingredients with α-galactosidase is an alternative [5].

For direct action in the gastrointestinal tract, the enzyme must resist the stomach acid pH. However, for the purpose of food pre-treatment, the enzyme thermotolerance is interesting. The immobilization of enzymes represents an alternative for increasing the stability of the biocatalyst in relation to pH changes, temperature and composition of the reaction medium. Moreover, this procedure allows repeated use of the biocatalyst for more than one cycle and also its use in a continuous process [6].

In 1990, Alan Kligerman from AkPharma (Edison, New Jersey, USA) developed the product Beano, after nine years of research on flatulence-causing vegetables. This patent is owned by Glaxo-SmithKline (Brentford, United Kingdom), which purchased it in 2001 from Block Drug (Jersey City,

Patrícia Cristine Scussiato De Andrade Taborda, Lígia Alves Da Costa Cardoso, Susan Grace Karp, Industrial Biotechnology Program, Positivo University, Professor Pedro Viriato Parigot de Souza Street 5300, 81280-330 Campo Comprido, Curitiba, Brazil.

Correspondence author:
Patrícia Cristine Scussiato De Andrade Taborda, e-mail: julipaty@terra.com.br

New Jersey, USA) [7]. In 2012, GlaxoSmithKline sold Beano and 16 other brands to Prestige Brands (Brentford, United Kingdom) [8]. In addition to the ingredient α-galactosidase, obtained from the microorganism *Aspergillus niger,* Beano tablets also contain a second enzyme, which can be used therapeutically, invertase. The US patent (5445957) was received on 29 August 1995 [9]. In April 2014, the product Beano + Dairy Defense (Prestige Brands) was introduced, containing the enzymes α-galactosidase and lactase. This product is not commercialized in Brazil. In accordance to the manufacturer's information, Beano should be stored at a temperature below 25 °C (77 °F), because higher temperatures can inactivate the enzyme. Therefore, this product should not be used for food pre-treatment during cooking [7].

The Code of Federal Regulations Title 21 (21CFR) established by the Food and Drug Administration (FDA) permits the use of α-galactosidase derived from *Mortierella vinaceae* var. *raffinoseutilizer* as food additive for direct use in foods for human consumption [10]. In Brazil, three species of microorganisms are listed as safe sources of α-galactosidase for human consumption, according to the Collegiate Board Resolution (RDC) of the National Health Surveillance Agency (ANVISA) No 53 of October 7, 2014: the fungi *Aspergillus niger, Mortierella vinacea* and *Saccharomyces carlsbergensis* [11]. This restriction limits the possibilities to obtain enzymes with properties suitable for use in conditions requiring, for example, temperature and pH stability. The problems with stability as well as difficulties in production and purification can make these enzymes extremely expensive for food applications, which has motivated studies on enzyme immobilization [12, 13]. The immobilization of enzymes in solid or semi-solid supports is an alternative that can, in addition to increasing the stability, allow their repeated use in enzyme catalysis processes, making them more economical.

In addition to the digestive action, α-galactosidases play an important role in the metabolism of glycosphingolipids in lysosomes. Their lack or absence, resulting from mutations in the gene encoding for α-galactosidase A in lysosomes, linked to X chromosome, is known as Fabry disease. This deficiency results in globotriaosylceramide accumulation in the vascular endothelium and visceral tissues, mainly affecting the skin, heart, kidney and central nervous system. One form of treatment for this disease is intravenous enzyme replacement. For this purpose, human α-galactosidases produced in animal cells are used, such as the algasidase beta (Fabrazyme, Genzyme, Cambridge, Massachusetts, USA), obtained by recombinant therapy in hamster ovary and approved by FDA [14], by European Medicines Agency and by National Health Surveillance Agency in Brazil [11]. Another option is algasidase alpha (Replagal; Transkaryotic Therapies, Cambridge, Massachusetts, USA) [15], produced by human fibroblast culture in the presence of active transcription promoters [16]. Marketing authorization of the latter product was granted by European Commission and validated throughout European Union on 3 August 2001 [15, 17]. This product is also approved by National Health Surveillance Agency in Brazil [18].

Structure and characteristics of α-galactosidases

In 1895, Bau, Fischer and Lindner isolated, from a yeast strain, an enzyme able to hydrolyse the disaccharide melibiose, melibiase. Later, the name was changed to melibiase-galactosidase by Weidenhagen, who studied the specific action of the enzyme, using a variety of saccharides with non-reducing α-D-galactosyl terminus [19]. According to Enzyme Comission (EC), α-D-galactoside galactohydrolase is classified as EC 3.2.1.22. The number 3 indicates a hydrolase, the sub-class 3.2 of glycosylases comprises enzymes that hydrolyse glycoside bonds, and 3.2.1 are glycosidases responsible for hydrolysing O- and S-glycosyl compounds [20]. There are two types of galactosidases, α-galactosidases and β-galactosidases, depending on the configuration of the anomeric carbon atom of galactose in the substrate molecule upon which they act [19]. α-Galactosidase catalyses the reaction presented in Fig. 1.

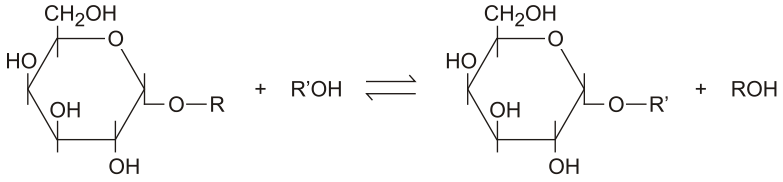

Fig. 1. Reaction catalysed by the enzyme α-galactosidase [21].

The hydroxyl acceptor molecule, R'OH, is generally water, although R and R' can be aliphatic or aromatic groups. This means that the enzyme can hydrolyse a single molecule of α-D-galactoside as well as complex molecules such as oligosaccharides or polysaccharides [21].

α-Galactosidases are widely distributed in microorganisms, plants and animals. Among these, microorganisms are capable of producing α-galactosidases with high yield [2]. Since the classification of enzymes based on the type of catalysed reaction and specificity towards the substrate does not take into account evolutionary events or sequence similarity and structure, and since the complete sequence and the N-terminal amino acid of many α-galactosidases obtained from plants, animals and microorganisms have been known, HENRISSAT [22] proposed and classified glycoside hydrolases in families based on amino acid sequence similarities. Based on sequence and structural similarity, α-galactosidases from eukaryotic and prokaryotic cells have been placed in families 27 and 36 for the classification of glycoside hydrolases, respectively [19, 22].

Bacterial α-galactosidases have the most complex structure and their molecular weight varies considerably between 45 kDa and 400 kDa, while most of the yeast α-galactosidases have an average molecular size of 50 kDa. The multimolecular forms of this enzyme are predominantly found in plants, although they were reported in several microorganisms, including *Aspergillus niger* and *Mortierella vinacea.* For *A. niger*, the molecular forms I, II, III and IV with isoelectric points of 4.15, 4.5, 4.7 and 4.8 and molecular masses of 350 kDa, 117 kDa, 117 kDa and 117 kDa, respectively, were reported by ADEMARK et al. [23]. For *M. vinacea*, the molecular forms reported by CIVAS et al. [24] were I and II with their respective isoelectric points of 5.4 and 8.5, and molecular masses of 240 kDa and 60 kDa. These multimolecular forms appear due to proteolytic cleavage or due to differential glycosylation of proteins [19].

The international unit of enzyme activity is represented by U, and according to Enzyme Comission, one unit (U) of activity is the amount of enzyme that catalyses the consumption of 1 μmol substrate or the formation of 1 μmol product per minute, at specific conditions of temperature, pH and substrate concentration [25]. An α-galactosidase activity unit can be defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl α-D-galactopyranoside (PNPG) per minute under the conditions of the standard assay [26].

Various substances may cause a reduction in the rate of the reactions catalysed by α-galactosidases. Some of these are protein-denaturing agents that act non-specifically. Others generally operate in a very specific way, either reversibly or irreversibly, and are known as inhibitors. Divalent ions such as Hg^{2+}, Ag^{2+} and Cu^{2+} are potent inhibitors of α-galactosidases [19].

Production of α-galactosidases by fermentation

There are two processes for the production of microbial enzymes, submerged fermentation and solid-state fermentation. Submerged fermentation is traditionally used for this purpose, because it allows better control of some important process parameters, such as pH and cell multiplication, and the recovery of extracellular enzymes and biomass is facilitated [27]. The selection of the process should be based on considerations about the microorganisms and the products to be obtained. For solid-state fermentation, the most commonly used microorganisms are filamentous fungi, for their better growth capacity at limited water activity [28]. One of the known differences between the submerged fermentation and the solid fermentation is fungal sporulation, since it is easier to obtain spores in the solid fermentation [27]. However, for the production and recovery of enzymes from the fermented medium, the presence of spores can be an additional obstacle for product purification. No report on α-galactosidase production by solid-state fermentation on an industrial scale has been published yet.

The carbon source used in microbial enzyme production may be the most important factor in determining the viability of the process. For the production of α-galactosidase, glucose, galactose, lactose, melibiose, raffinose and stachyose can be used both on laboratory scale or on commercial scale. Agricultural residues such as wheat bran or flour, flour or soy meal, sorghum, maize, among others, can also be used for the production of this enzyme. Optionally, raffinose or melibiose can be added in the medium to induce the expression of α-galactosidase [19]. It is important that the most specific inducers of α-galactosidase activity, which are the carbohydrates melibiose, raffinose and stachyose, and also the monosaccharide galactose, are not cheap and easily available resources. Thus, the use of agroindustrial residues derived from soybean, beans and other natural sources of these carbohydrates can be an important aspect in the development of an economical industrial process. As an example, the study with soybean vinasse by SANADA et al. [2] can be mentioned.

The main physical and chemical parameters that affect the production of α-galactosidase by

submerged fermentation are pH, temperature, aeration and agitation. The pH is a critical parameter in microbial growth and thereby in the production of the enzyme. α-Galactosidases obtained from yeasts, fungi and plant seeds showed a broad range of optimum pH, between 3 and 6. More acidic forms of α-galactosidase, active at pH 2.5–4.5, were observed in fungi, including *A. niger* [19].

In order to achieve optimum yield, the fermentation should be carried out at a constant temperature. The optimum temperature for α-galactosidase production is between 30 °C and 37 °C [25, 29]. The rate of aeration and agitation are also factors affecting the success of the fermentation process. Aeration may be beneficial for growth and performance of the microbial cells by improving the mass transfer features related to the substrate, product and oxygen, as well as agitation is an important parameter for adequate mixing and heat transfer. Few reports were published that describe the best conditions of agitation and aeration to produce α-galactosidase. Gote [19] reported an aeration of 0.8 l of air·per 1 l of medium per minute and agitation of 6.67 Hz for the maximum production of α-galactosidase from *Bacillus stearothermophilus,* and also 0.5 l of air·per 1 l of medium per minute and stirring of 4 Hz for improved production of α-galactosidase by *Circenella musae.*

Microorganisms used for the production of α-galactosidase

Filamentous fungi have been used for centuries as producers of many metabolites and enzymes, and are used in biotechnological processes for the production of pharmaceuticals and food ingredients [30]. Many fungi naturally secrete proteins and have been exploited commercially as "factories" of enzymes, either of fungal or heterologous origin [31].

Among the filamentous fungi of major industrial and biotechnological interest is the species *A. niger*, widely used for the production of organic acids and extracellular enzymes. It is one of the most common species of the genus *Aspergillus,* belonging to the class of Ascomycetes [32–34]. Fermentations with *A. niger*, both submerged or in solid state, were developed at laboratory and industrial scales, and this fungus is the most important source of α-galactosidase for human consumption in Brazil.

The filamentous fungus *Mortierella vinacea* is a Zygomycete, a terrestrial fungus presenting hyphae with chitinous cell walls. Fungi of the genus *Mortierella* can grow in yeast extract, fish meal or other media rich in nutrients. The cultivation and growth of *Mortierella* fungi are affected by the carbon source and the addition of mineral nitrogen source. Monosaccharides are most commonly used for growth. Yeast extract and soy flour are widely used as sources of nitrogen. In particular, the balance between carbon and nitrogen in the medium influences the fungal culture [34]. Cultivation of *M. vinacea* in submerged fermentation is not a simple process, especially because the fungal biomass does not grow homogeneously in the liquid medium. In order to standardize the initial biomass concentration, a suspension of spores can be prepared from a solid culture, which can be then transferred to a pre-culture to form vegetative cells to be used as inoculum. Besides this initial preparation, fermentation with *M. vinacea* usually takes many days, which demands significant energy and other inputs, and increases the chance of contamination. Moreover, a great portion of α-galactosidases produced by *M. vinacea* is intracellular, which requires a step of cell lysis for recovery.

Yeasts are used to obtain high concentrations of enzymes economically through genetic manipulation and adjustment of culture conditions, screening of producing microorganisms, short fermentation cycles and use of low cost fermentation media. Yeasts of the genus *Saccharomyces*, which are Ascomycetes, are the most employed industrially [35]. *Saccharomyces carlsbergensis* is a yeast species known to present an unusual genomic structure. One genomic assembly is structurally similar to *Saccharomyces cerevisiae,* while the other is similar to *Saccharomyces monacensis* [33]. The taxonomic name *Saccharomyces pastorianus* is also attributed to this species, which has been traditionally used in the production of lager beer. Industrial fermentations with yeasts of the genus *Saccharomyces* are well established and easy to operate, which represents an advantage for its use at enzyme production.

Tab. 1 shows the enzymatic activity values obtained by three microorganisms, *Aspergillus niger, Mortierella vinacea* and *Saccharomyces carlsbergensis*, in certain conditions, according to the literature. The highest enzymatic activity values found in literature for α-galactosidases were obtained from strains not approved for food applications, such as *Neosartorya fischeri* with 310.42 $U \cdot ml^{-1}$ in the crude extract [39]. The enzymes obtained from this microorganism showed different characteristics such as stability at temperatures between 60 °C and 70 °C. Chen et al. [40] reported heterologous expression of α-galactosidase using an enzyme gene (designated as *RmgalB*) cloned from the thermophilic fungus *Rhizomucor*

Tab. 1. Values of α-galactosidase activity for *Aspergillus niger*, *Mortierella vinacea* and *Saccharomyces carlsbergensis* on different carbon sources.

Microorganism	Enzymatic activity [U·ml^{-1}]	Carbon source	Fermentation time [h]	Reference
A. niger	3.42	Locust bean gum	168	[23]
A. niger	4.07	Guar gum	168	[23]
M. vinacea	0.95	Lactose 1%, D-glucose 1% and steep liquor 1%	72	[36]
S. carlsbergensis	1.79	Extracted biomass used in the production of beer	–	[37]
S. carlsbergensis	0.37	Melibiose	15	[38]
S. carlsbergensis	0.40	Raffinose	15	[38]
S. carlsbergensis	1.05	Stachyose	15	[38]

miehei and expressed in *Pichia pastoris.* The gene belongs to the family 36 of glycoside hydrolases, has an open reading frame (ORF) of 2241 bp encoding 746 amino acids with two introns. The recombinant α-galactosidase (RmgalB) was secreted at high level, 1953.9 U·ml^{-1}, which is the highest yield achieved for an α-galactosidase. The enzyme, purified as a tetramer, showed a single band corresponding to a molecular mass of 83.1 kDa in denaturing polyacrylamide gel electrophoresis. The enzyme exhibited a specific activity of 505.5 U·mg^{-1} and also showed specificity for raffinose and stachyose. The optimum temperature for the activity of RmgalB was 55 °C and the optimum pH was 5.5.

Recovery and purification of α-galactosidase

After the fermentation, recovery and purification steps are chosen according to the enzyme origin (intracellular or extracellular) and to its physico-chemical and biological properties, inherent to each enzyme. The level of purification of an enzyme depends primarily on the use for which it is intended, e.g. high purity is required for therapeutic applications. In general, purification depends on the number of steps employed in the process because, at each stage, loss of activity takes place. So in order to increase the yield, a minimum number of steps should be performed [28].

The first step in the enzyme purification is to isolate it from the cultured cells. The enzyme α-galactosidase is predominantly extracellular, being secreted into the fermentation medium [1, 28]. In this case, the isolation can be done by centrifugation or filtration [15]. However, a number of α-galactosidases of fungal and bacterial origin are reported to be intracellular and, in such cases, the recovery was done either by French press, cell homogenizer, by grinding with an abrasive or by sonication. Moreover, in a few cases, organic solvents were used for the extraction of intracellular α-galactosidase [19]. Normally, α-galactosidases occur in the cell in association with several other glycosidases, so several isolation techniques are required to obtain a pure enzyme. One of the purification techniques consists in precipitation of proteins using ammonium sulphate, a method based on solubility differences that proteins exhibit depending on ionic strength of the solution. At low concentrations, neutral salts increase the solubility of many proteins, a phenomenon called "salting-in". However, when the salt concentration is increased, generally the maximum point of the protein solubility is reached, and further increase will result in less water available to solubilize the proteins. Finally, the protein begins to precipitate when there are no sufficient water molecules to interact with protein molecules, and this phenomenon is called "salting-out" [41]. Dialysis is performed subsequently to remove ammonium sulfate from the enzymatic solution [42].

Chromatography is another important method for protein purification, especially the ion-exchange chromatography, which is a separation process based on the affinity of sample components with ionic groups to a solid matrix. The stationary phase, electrically charged, is capable of retaining solutes of opposite sign charges, which are present in the mobile phase. Ion exchangers covalently bound to the matrix are classified into anionic and cationic [43].

Considering the high costs of the purification procedures, and also the losses of activity associated with them, obtaining of a crude extract with considerable enzymatic activity is the first step in the development of a feasible industrial process. In this context, most of the values of enzymatic activity reported in Tab. 1 would not be economical for a scale-up. This fact, together with the restrictions of species approved for food applications, represents an obstacle for promoting the production and use of α-galactosidases. The

development of strategies to overcome these difficulties could be directed either towards strain and process improvement, e.g. through directed evolution or metabolic engineering, or towards optimized application, e.g. through enzyme immobilization.

Immobilization of α-galactosidase

Although enzymes present excellent catalytic properties, the enzymatic properties usually have to be improved before application on an industrial scale [44]. Immobilization of enzymes offers a simple solution to the challenges of applying them on a large scale. In general, immobilized enzymes showed certain advantages over soluble enzymes, such as higher thermal stability, reusability, wide range of optimum pH, storage stability increase and easy separation from the reaction mixture, which leads to significant reductions in cost [45, 46].

The immobilization mechanisms can be classified essentially to gel entrapment, physical adsorption or covalent binding [47]. Several methods have been developed, including entrapment in calcium alginate and cellulose acetate fibres, covalent binding to chitosan, polyurethane foam, gelatin or bone powder. However, use of some of these methods resulted in low immobilization productivity or continuous leaking of the enzyme. For example, entrapment in calcium alginate, despite being a simple and widely used method, exhibited poor stability and a high porosity gel, resulting in loss of the enzyme [29, 47]. Some reports were published on α-galactosidase immobilization in calcium alginate and polyacrylamide gel with application to reduce soymilk oligosaccharides, but a polyacrylamide support should not be used in food processing due to its toxicity [29].

Alginate is a linear copolymer derived from seaweed, consisting of α-L-guluronic and β-D-manuronic acid units. It is widely used due to its thickening, stabilizing and gelling properties. Its gel structure is relatively stable at acidic pH and easily disintegrated under alkaline conditions [48]. Alginate is widely used in the food, pharmaceutical, cosmetics, textile and paper industries due to its non-toxicity, biodegradability and biocompatibility [26, 39]. Alginate supports are usually prepared by cross-linking between the carboxylic group of α-L-guluronic acid and a solution containing cationic binders, such as calcium chloride. Although the gel of calcium alginate does not show toxicity against cells, it may be chemically unstable in the presence of calcium chelators such as phosphate, lactate or citrate, and cations such as sodium or magnesium, which are able to remove calcium atoms and liquefy the system [49, 50].

To overcome some of the limitations of the use of alginate, such as high porosity, enzyme leakage and low mechanical strength, the use of chitosan, a natural cationic polymer produced by *N*-deacetylation of chitin, has been reported, as a coating material for the alginate spheres to improve their stability and resistance [39, 49, 51]. Chitin is the second most abundant natural polymer in nature after cellulose, and is found e.g. in exoskeletons of marine crustaceans. Chitosan has many advantages such as biodegradability and biocompatibility, non-toxicity, antibacterial properties, hydrophilicity and affinity for proteins [45]. However, it is not sufficiently soluble at neutral pH, but only in acidic media such as acetic acid or hydrochloric acid solutions. Chitosan can be used as an outer coating of alginate microcapsules, or by ionic complexation with alginate, which presents negative charge [39, 50].

Immobilization of enzymes in hydrocolloid matrix can be increased with the use of glutaraldehyde. In addition to activating the microspheres, glutaraldehyde allows connection at multiple points, since it promotes the reaction between free amino acids of the matrix and the enzyme, thus forming covalent bonds. In this way, the formed polymer network allows greater flexibility to conformational changes required for enzyme activity [52, 53]. The use of glutaraldehyde for treating alginate microspheres, to promote covalent bonds between the matrix and the enzyme, was presented as an alternative to avoid the loss of enzyme from the microspheres [47]. However, due to toxicity of this cross-linking agent, the formed hydrogels should be washed and cleansed in order to remove unreacted agents, as these are harmful to health [54].

Another possibility for enzyme recovery, which also avoids food contamination by enzyme subunits, is chemical crosslinking without a carrier. The presence of a carrier reduces the enzyme activity per mass or volume, considering that the carrier has no catalytic function. Thus, the use of cross-linked enzyme crystals (CLEC) or cross-linked enzyme aggregates (CLEA), which are produced by enzyme crystallization and enzyme precipitation, respectively, followed by cross-linking, can also be a promising alternative [55]. Bhattacharya et al. [56] obtained high hydrolysis efficiency when using combined cross-linked enzyme aggregates (combi-CLAEs) of lignocellulolytic enzymes and accessory enzymes, including α-galactosidases. Studies on the application of α-galactosidase-CLAEs for food applications are, however, very scarce.

The use of permeabilized cells containing intracellular α-galactosidase, instead of free or immobilized enzymes, is another alternative for reducing galacto-oligosaccharides in food products. VIANA et al. [57] evaluated the use of *Debaryomyces hansenii*, a generally recognized as safe (GRAS) yeast, for the treatment of soy milk. Permeabilization of *D. hansenii* cells was achieved by incubation of the cell suspension in the presence of a 50% (v/v) ethanol solution, which reduced the permeability barrier of the cell envelope to substrates and products, allowing free diffusion. The permeabilized cells, containing intracellular α-galactosidase, hydrolysed 70% and 100% of raffinose and stachyose, respectively, after incubation for 6 h, while immobilized α-galactosidase hydrolysed only 47% of raffinose in the same period.

CONCLUSION

Soybeans and other legumes such as beans and peas present many benefits for human health, however, their consumption is limited by the presence of non-digestible oligosaccharides such as melibiose, raffinose or stachyose. The use of the enzyme α-galactosidase, by direct ingestion or to pre-treat the food ingredients, is an alternative to reduce the discomfort associated with the consumption of these food products. This enzyme can be obtained from various sources, among which microorganisms are able to produce it with high yield and productivity. In Brazil, only the microorganisms *Aspergillus niger, Mortierella vinacea* and *Saccharomyces carlsbergensis* are listed as safe sources for obtaining α-galactosidase, which restricts the possibilities of obtaining an enzyme with adequate properties for use in conditions that require temperature stability and pH, for example. Immobilization of the enzymes in hydrocolloids such as alginate and chitosan, crosslinking without a carrier or the use of permeabilized cells are alternatives to increase stability and to allow repeated use of the enzyme, thus enhancing the economical feasibility of the enzymatic process.

REFERENCES

1. Goés, S. P. E. – Ribeiro, M. L. L.: α-Galactosidase: aspectos gerais e sua aplicação em produtos a base de soja. (α-Galactosidase: general aspects and its application on soybean oligosaccharides.) Semina : Ciencias Agrárias, *13*, 2002, pp. 111–119. DOI: 10.5433/1679-0359.2002v23n1p111. In Portuguese.
2. Sanada, C. T. N. – Karp, S. G. – Spier, M. R. – Portella, A. C. – Gouvęa, P. M. – Yamaguishi, C. T. – Vandenberghe, L. P. S. – Pandey, A. – Soccol, C. R.:Utilization of soybean vinasse for α-galactosidase production. Food Research International, *42*, 2009, pp. 476–483. DOI: 10.1016/j.Foodres.2009.01.017.
3. Behrens, J. H. – Da Silva, M. A. A. P.: Atitude do consumidor em relação à soja e produtos derivados. (Consumer attitude towards soybean and related products.) Ciencia e Tecnologia de Alimentos, *24*, 2004, pp. 431–439. DOI: 10.1590/S0101-20612004000300023. In Portuguese.
4. Fenko, A. – Backhaus, B. W. – van Hoof, J. J.: The influence of product- and person-related factors on consumer hedonic responses to soy products. Food Quality and Preference, *41*, 2015, pp. 30–40. DOI: 10.1016/j.foodqual.2014.11.009.
5. King, M. R. – White, B. A. – Blaschek, H. P. – Chassy, B. M. – Mackie, R. I. – Cann, I. K.: Purification and characterization of a thermostable α-galactosidase from *Thermoanaerobacterium polysaccharolyticum*. Journal of Agricultural and Food Chemistry, *50*, 2002, pp. 5676–5682. DOI: 10.1021/jf0202281.
6. Canilha, L. – Carvalho, W. – Silva, J. B. A.: Uso de células e enzimas imobilizadas em processos biotecnológicos. (Use of immobilized enzymes and cells in biotechnology processes.) Biotecnologia Ciencia e Desenvolvimento, *9*, 2006, No 36, pp. 48–57. ISSN: 1414-6347. <http://www.biotecnologia.com.br/revista/bio36/bio_36.pdf> In Portuguese.
7. Block Drug Company, Inc. Compositions and method for reducing gastro-intestinal distress due to alpha-d-galactoside-linked/containing sugars. Inventor: Kligerman, A. E. CPC A23L29/06, A61K38/47, C12Y302/01022. United States patent US 6344196 B1. 05 February 2002.
8. Block Drug Company, Inc. Composition and method for reducing gastro-intestinal distress due to alpha-D-galactoside-linked/containing sugars. Inventor: Kligerman, A. E. CPC C12Y302/01022, A61K38/47, A23L29/06. United States patent US 5989544 A. 23 November 1999.
9. Triarco Industries, Inc. Enzyme food supplement composition containing beta-fructofuranosidase, cellulase and hemicellulase. Inventors: Rohde Jr., R. R. – Schuler, E. F. – Handel, R. A. IPC A61K38/54. United States patent US 5445957 A. 29 August 1995.
10. 21CFR173.145. Alpha-galactosidase derived from *Mortierella vinaceae* var. *raffinoseutilizer*. In: FDA – U.S. Food and Drug Administration [online]. Maryland : US Food and Drug Administration, revised 1 April 2015 [cit. 21 August 2015]. <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=173.145&SearchTerm=alpha%20galactosidase>
11. Resolução da Diretoria Colegiada - RDC no 53 de 07/10/2014 dispõe sobre a lista de enzimas, aditivos alimentares e veículos autorizados em preparações enzimáticas para uso na produção de alimentos em geral. (Resolution of the Collegiate Directorate - RDC No 53 of 10/07/2014 Provides

the list of enzymes, food additives and vehicles authorized in enzymatic preparations for use in food production in general.) In: ANVISA – Agęncia Nacional de Vigilância Sanitária [online]. Brasília : Agęncia Nacional de Vigilância Sanitária, revised 8 October 2014 [cit. 25 May 2015]. <http://portal.anvisa.gov.br/legislacao#/visualizar/29252> In Portuguese.

12. Netto, C. G. C. M. – Toma, H. E. – Andrade, L. H.: Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes. Journal of Molecular Catalysis B: Enzymatic, *85–86*, 2013, pp. 71–92. DOI: 10.1016/j.molcatb.2012.08.010.
13. Liu, Y. – Sun, Y. – Li, Y. – Xu, S. – Tang, J. – Ding, J. – Xu, Y.: Preparation and characterization of α-galactosidase-loaded chitosan nanoparticles for use in foods. Carbohydrate Polymers, *83*, 2011, pp. 1162–1168. DOI: 10.1016/j.carbpol.2010.09.050.
14. Fabrazyme Approval Letter 4/24/03. In: FDA – U.S. Food and Drug Administration [online]. Maryland : U.S. Food and Drug Administration, revised 2 April 2009 [cit. 25 May 2015]. <http://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/therapeuticbiologicapplications/ucm128159.htm>
15. EMA/458725/2015. Replagal (agalsidase alfa). In: EMA – European Medicines Agency [online]. London : European Medicines Agency, revised 29 July 2015 [cit. 25 May 2015].<http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000369/WC500053606.pdf>
16. Boggio, P. – Luna, P. C. – Abad, M. E. – Larralde, M.: Doença de Fabry. (Fabry disease.) Anais Brasileiros de Dermatologia, *84*, 2009, pp. 367–376. DOI: 10.1590/S0365-05962009000400008. In Portuguese.
17. EMA/65766/2013. Fabrazyme (agalsidase beta). In: EMA – European Medicines Agency [online]. London : European Medicines Agency, revised 19 March 2013 [cit. 25 May 2015]. <http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000370/WC500020541.pdf>
18. Replagal (alfagalsidase). In: ANVISA – Agęncia Nacional de Vigilância Sanitária [online]. Brasília : Agęncia Nacional de Vigilância Sanitária, revised 20 July 2009 [cit. 06 January 2016]. <http://www.anvisa.gov.br/datavisa/fila_bula/frmVisualizarBula.asp?pNuTransacao=9853932013&pIdAnexo=1874428> In Portuguese.
19. Gote, M. – Umalkar, H. – Khan, I. – Khire, J.: Thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) and its application in the removal of flatulence causing factors from soymilk. Process Biochemistry, *39*, 2004, pp. 1723–1729. DOI: 10.1016/j.procbio.2003.07.008.
20. Nelson, D. L. – Cox, M. M. (Ed.): Princípios de Bioquímica de Lehninger. (Biochemistry principles of Lehninger.) 5th edition. Porto Alegre : Artmed, 2011. ISBN: 9788536324180. In Portuguese.
21. Dey, P. M – Pridham, J. B.: Biochemistry of α-galactosidases. In: Meister, A. (Ed.): Advances in enzymology and related areas of molecular biology, vol. *36.* Hoboken : John Wiley & Sons, 1972, pp. 91–130. ISBN: 9780471591719. DOI: 10.1002/9780470122815.ch3.
22. Henrissat, B. A.: Classification of glycosyl hydrolases based on amino-acid similarities. Biochemical Journal, *280*, 1991, pp. 309–316. DOI: 10.1042/bj2800309.
23. Ademark, P. – Larsson, M. – Tjerneld, F. – Stålbrand, H.: Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificity. Enzyme and Microbial Technology, *29*, 2001, pp. 441–448. DOI: 10.1016/S0141-0229(01)00415-X.
24. Civas, A. – Eberhard, R. – Le Dizet P. – Petek, F.: Glycosidases induced in *Aspergillus tamarii*. Mycelial α-D-galactosidases. Biochemical Journal, *219*, 1984, pp. 849–855. DOI: 10.1042/bj2190849.
25. Shivam, K. – Mishra, S. K.: Purification and characterization of a thermostable α-galactosidase with transglycosylation activity from *Aspergillus parasiticus* MTCC-2796. Process Biochemistry, *45*, 2010, pp. 1088–1093. DOI: 10.1016/j.procbio.2010.03.027.
26. Prashanth, S. J. – Mulimani, V. H.: Soymilk oligosaccharide hydrolysis by *Aspergillus oryzae* α-galactosidase immobilized in calcium alginate. Process Biochemistry, *40*, 2005 , pp. 1199–1205. DOI: 10.1016/j.procbio.2004.04.011.
27. Oliveira, A. C. D – Watanabe, F. M. F. – Vargas, J. V. C. – Mariano, A. B. – Rodrigues, M. L. F.: Comparação entre tręs bioprocessos para a produção de enzimas proteolíticas utilizando resíduos agroindustriais. (Comparison between three processes for proteolytic enzymes production using agroindustrial waste.) Revista Brasileira de Tecnologia Agroindustrial, *6*, 2012, pp. 822–831. DOI: 10.3895/S1981-36862012000200008. In Portuguese.
28. Lima, U. A. – Aquarone, E. – Borzani, W. – Schimidell, W.: Biotecnologia industrial: processos fermentativos e enzimáticos. (Industrial biotechnology: fermentation and enzymatic processes.) São Paulo : Edgard Blücher, 2001. ISBN: 9788521202806. In Portuguese.
29. Naganagouda, K. – Mulimani, V. H.: Gelatin blends with alginate: Gel fibers for α-galactosidase immobilization and its application in reduction of non-digestible olisaccharides in soymilk. Process Biochemistry, *41*, 2006, pp. 1903–1907. DOI: 10.1016/j.procbio.2006.03.040.
30. Hu, H. L – Brink, V. D. – Gruben, B. S. – Wösten, J. – Gu, D. – Vries, R. P.: Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi. International Biodeterioration and Biodegradation, *65*, 2011, pp. 248–252. DOI: 10.1016/j.ibiod.2010.11.008.
31. Bon, E. P. S. – Corvo, M. L. – Ferrara, M. A. – Vermelho, A. B. – Paiva, C. L. A. – Alencastro, R. B. – Coelho, R. R. R.: Enzimas em biotecnologia: produção, aplicaçőes e mercado. (Enzymes in biotechnology: production, applications and market.) Rio de Janeiro : Intercięncia, 2008. ISBN: 9788571931893. In Portuguese.

32. Castellá, G. – Alborch, L. – Bragulat, M. R. – Cabañes, F. J.: Real time quantitative expression study of a polyketide synthase gene related to ochratoxin a biosynthesis in *Aspergillus niger*. Food Control, *53*, 2015, pp. 147–150. DOI: 10.1016/j.foodcont.2015.01.026.
33. Fujii, T. – Yoshimoto, H. – Nagasawai, N. – Bogaki, T. – Tamai, Y. – Hamachit, M.: Nucleotide sequences of alcohol acetyltransferase genes from lager brewing yeast, *Saccharomyces carlsbergensis*. Yeast, *12*, 1996, pp. 593–598. DOI: 10.1002/(SICI)1097-0061(199605)12:6<593::AID-YEA593>3.0.CO;2-B.
34. Moore-Landecker, E. (Ed.): Fundamentals of the fungi. 4th edition. New Jersey: Prentice-Hall, 1996. ISBN: 0133768643.
35. Ferrão-Gonzales, A. D. – Vital, A. V. D. – Lima, J. M. – Rodrigues, M. B. S.: Desenvolvimento sustentável para o resgate da cultura do cacau baseado no aproveitamento de resíduos. (Sustainable development for the recovery of cocoa cultivation based on waste utilization.) Interfaces Científicas – Saúde e Ambiente, *1*, 2013, pp. 41–52. DOI: 10.17564/2316-3798.2013v1n2p41-52. In Portuguese.
36. Suzuki, H. – Li, S.-C. – Teh, Y.-T.: α-Galactosidase from *Mortierella vinacea* : Crystallization and properties. Journal of Biological Chemistry, *245*, 1970, pp. 781–786. ISSN: 1083-351X (online). <http://www.jbc.org/content/245/4/781.full.pdf+html>
37. Durance, T. D. – Skura, B. J.: Isolation and characterization of the α-galactosidase of waste lager yeast (*Saccharomyces carlsbergensis*). Canadian Institute of Food Science and Technology Journal, *18*, 1985, pp. 306–310. DOI: 10.1016/S0315-5463(85)71963-3.
38. Flórez, I. G. – Lazo, P. S. – Ochoa, A. G. – Gascón, S.: The specificity of induction of α-galactosidase from *Saccharomyces carlsbergensis*. Biochimica et Biophysica Acta, *674*, 1981, pp. 71–77. DOI: 10.1016/0304-4165(81)90348-2.
39. Wang, Y. – Chen, H. – Wang, J. – Xing, L.: Preparation of active corn peptides from zein through double enzymes immobilized with calcium alginate-chitosan beads. Process Biochemistry, *49*, 2014, pp. 1682–1690. DOI: 10.1016/j.procbio.2014.07.002.
40. Chen, Z. – Yan, Q. – Jiang, Z. – Liu, Y. – Li, Y.: High-level expression of a novel α-galactosidase gene from *Rhizomucor miehei* in *Pichia pastoris* and characterization of the recombinant enyzme. Protein Expression and Purification, *110*, 2015, pp. 107–114. DOI: 10.1016/j.pep.2015.02.015.
41. Singh, R. – Kumar, V. – Kapoor, V.: Partial purification and characterization of a heat stable α-amylase from a thermophilic Actinobacteria, *Streptomyces* sp. MSC702. Enzyme Research, 2014, Article ID 106363, 8 pages. DOI: 10.1155/2014/106363.
42. Perone, C. A. S. – Queiroz, A. S. – Dalosso V. M. – Moreira, M. E. M.: Purificação parcial e caracterização cinética da enzima Polifenol oxidase de banana nanica (*Musa acuminata*). (Partial purification and kinetics characterization of enzyme Polyphenol oxidase of stunded banana (*Musa acuminata*).) Revista Instituto Ciencia e Saúde, *25*, 2007, pp. 239–246. ISSN: 0104-1894. <http://200.136.76.129/comunicacao/publicacoes/ics/edicoes/2007/03_jul_set/V25_N3_2007_p239-246.pdf> In Portuguese.
43. Asenjo, J. A. (Ed.): Separation process in biotechnology. NewYork : Marcel Dekker, 1990. ISBN: 0824782704.
44. Bayraktar, H. – Serilmez, M. – Karkas, T. – Çelem, E. B. – Önal, S.: Immobilization and stabilization α-galactosidase on Sepabeads EC-EA and EC-HA. International Journal of Biological Macromolecules, *49*, 2011, pp. 855–860. DOI: 10.1016/j.ijbiomac.2011.08.009.
45. Singh, N. – Kayastha, A. M.: Cicer α-galactosidase immobilization onto chitosan and Amberlite MB-150: optimization, characterization, and its applications. Carbohydrate Research, *358*, 2012, pp. 61–66. DOI: 10.1016/j.carres.2012.06.013.
46. Wu, J. C. Y. – Hutchings, C. H. – Lindsay, M. J. – Werner, C. J. – Bundy, B. C.: Enhanced enzyme stability through site-directed covalent immobilization. Journal of Biotechnology, *193*, 2015, pp. 83–90. DOI: 10.1016/j.jbiotec.2014.10.039.
47. Ateş, S. – Mehmetoğlu, Ü.: A new method for immobilization of β-galactosidase and its utilization in a plug flow reactor. Process Biochemistry, *32*, 1997, pp. 433–436. DOI: 10.1016/S0032-9592(96)00101-X.
48. Estevinho, B. N. – Damas, A. M. – Martins, P. – Rocha, F.: Microencapsulation of β-galactosidase with different biopolymers by a spray-drying process. Food Research International, *64*, 2014, pp. 134–140. DOI: 10.1016/j.foodres.2014.05.057.
49. Trabelsi, I. – Ayadi, D. – Bejar, W. – Bejar, S. – Chouayekh, H. – Salah, R. B.: Effects of *Lactobacillus plantarum* immobilization in alginate coated with chitosan and gelatin on antibacterial activity. International Journal of Biological Macromolecules, *64*, 2014, pp. 84–89. DOI: 10.1016/j.ijbiomac.2013.11.031.
50. Taqieddin. E. – Amiji, M.: Enzyme immobilization in novel alginate-chitosan core-shell microcapsules. Biomaterials, *25*, 2004, pp. 1937–1945. DOI: 10.1016/j.biomaterials.2003.08.034.
51. Kamalian, N. – Mirhosseini, H. – Mustafa, S. – Manap, M. Y. A.: Effect of alginate and chitosan on viability and release behavior of *Bifidobacterium pseudocatenulatum* G4 in simulated gastrointestinal fluid. Carbohydrate Polymers, *111*, 2014, pp. 700–706. DOI: 10.1016/j.carbpol.2014.05.014.
52. Tripathi, P. – Kumari, A. – Rath, P. – Kayastha, A. M.: Immobilization of α-amylase from mung beans (*Vigna radiata*) on Amberlite MB 150 and chitosan beads: A comparative study. Journal of Molecular Catalysis B: Enzymatic, *49*, 2007, pp. 69–74. DOI: 10.1016/j.molcatb.2007.08.011.
53. Singh, N. – Srivastava, G. – Talat, M. – Raghubanshi, H. – Srivastava, O. N. – Kayastha, A. M.: Cicer α-galactosidase immobilization onto functionalized graphene nanosheets using response surface method and its applications. Food Chemistry, *142*, 2014, pp. 430–438. DOI: 10.1016/j.foodchem.2013.07.079.
54. Berger, J. – Reist, M. – Mayer, J. M. – Felt, O. – Gurny, R.: Structure and interactions in chitosan

hydrogels formed by complexation or aggregation for biomedical applications. European Journal of Pharmaceutics and Biopharmaceutics, *57*, 2004, pp. 35–52. DOI: 10.1016/S0939-6411(03)00160-7.
55. Cao, L. – van Langen, L. – Sheldon, R. A.: Immobilised enzymes: carrier-bound or carrier-free? Current Opinion in Biotechnology, *14*, 2003, pp. 387–394. DOI: 10.1016/S0958-1669(03)00096-X.
56. Bhattacharya, A. – Pletschke, B. I.: Strategic optimization of xylanase-mannanase combi-CLEAs for synergistic and efficient hydrolysis of complex lignocellulosic substrates. Journal of Molecular Catalysis B: Enzymatic, *115*, 2015, pp. 140–150. DOI: 10.1016/j.molcatb.2015.02.013.
57. Viana, P. A. – Rezende, S. T. – Falkoski, D. L. – Leite, T. A. – José, I. C. – Moreira, M. A. – Guimarães, V. M. Hydrolysis of oligosaccharides in soybean products by *Debaryomyces hansenii* UFV-1 α-galactosidases. Food Chemistry, *103*, 2007, pp. 331–337. DOI: 10.1016/j.foodchem.2006.07.055.

Received 7 January 2016; 1st revised 20 August 2016; accepted 30 August 2016; published online 8 September 2016.