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Hydrolysis of oligosaccharides in soybean products by *Debaryomyces hansenii* UFV-1 α-galactosidases

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Abstract

α-Galactosides are abundant sugars found in legumes such as soybean. Since humans and monogastric animals lack α-galactosidase in the digestive tract, they are unable to digest these sugars, which induce flatulence. The use of α-galactosidases is promising as a means to overcome this problem, and to increase the consumption of soy products. Immobilized α-galactosidase, derived from *Debaryomyces hansenii* UFV-1, exhibited an activity of 40 U per g of silica and an activity yield of 50%. The optimum pH of free and immobilized α-galactosidase was 5.0 and the optima temperatures were 60 and 80 °C, respectively. The soymilk stachyose was completely hydrolyzed by different enzyme forms after incubation for 4 h at 60 °C, while raffinose was reduced by 100%, 25% and 68% by free, immobilized enzymes and permeabilized cells, respectively. The soy molasses treatment with free enzyme for 6 h promoted reduction in stachyose and raffinose contents by 100% and 50%, respectively.

Keywords: *Debaryomyces hansenii* UFV-1; α-Galactosidases; Raffinose oligosaccharides; Immobilization; Cell permeabilization; Flatulence reduction

1. Introduction

α-Galactosidase (α-D-galactoside galactohydrolase, E.C 3.2.1.22) catalyzes the hydrolysis of terminal α-1.6-linked D-galactose residues present in galacto-oligosaccharides of sugars of the raffinose family and galactomannan polysaccharides (Ulezlo & Zapromelava, 1982). The potential use of α-galactosidase for processing legume-based food products has been demonstrated (Guimarães, de Rezende, Moreira, Barros, & Felix, 2001; Thippeswamy & Mulimani, 2002). The presence of raffinose oligosaccharides (RO), in particular raffinose and stachyose, in soybean seeds may cause gastric distress in humans since these sugars are metabolized with extensive gas production by the microbial flora of the lower intestinal tract (Puchart, Vrsanská, Bhat, & Biely, 2000). Soybeans typically contain 9.0–12.0% of total sugars, including 4.0–5.0% of sucrose, 1.0–2.0% of raffinose, 3.5–4.5% of stachyose, and melibiose and verbascose in smaller quantities (Greiner, 1990). The use of soybean is restricted due to certain antinutritional compounds such as RO, which not only depreciate its nutritive value, but also restrict its wider acceptance. As a result, the demand for a α-galactoside-free soybean product is strong. Of all techniques proposed to reduce the RO content in soy products, the enzymatic processing by α-galactosidases has proved most effective (Prashanth & Mulimani, 2005; Viana et al., 2005, 2006). Nevertheless, the use of enzymes with poor stability or of microbial origin without generally recognized as safe (GRAS) status has restricted the potential of this approach (Gote, Umalkar, Khan, & Khire, 2004; King et al., 2002).

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The α-galactosidases described in this study were isolated from *Debaryomyces hansenii* UFV-1. This is the most frequent yeast in traditional cheese and sausages, with an acknowledged contribution to the special flavours of these products (Saldanha-da-Gama, Malfeito-Ferreira, & Loureiro, 1997; Seiler & Busse, 1990). We described applications of free and immobilized extracellular α-galactosidase and permeabilized cells of *D. hansenii* UFV-1 containing intracellular α-galactosidase for soymilk and soy molasses treatment. The objective is to reduce the RO in these products.

2. Materials and methods

2.1. Microorganism

The yeast strain used in this study was isolated from a dairy environment in Minas Gerais, Brazil, and maintained in the culture collection of the Laboratory of Microorganism Physiology, BIOAGRO, Federal University of Viçosa (UFV), Brazil. The yeast was identified by the Institute of Yeasts Identification, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as *D. hansenii* (Zopf) Lodder & Kreger-van Rij var *fabryi* Nakase & Suzuki. In this study, it is designated as *D. hansenii* UFV-1.

2.2. α-Galactosidase production

A stock culture of *D. hansenii* UFV-1 was maintained at – 80 °C in glycerol and YPD medium (1% yeast extract, 2% peptone and 2% glucose). *D. hansenii* UFV-1 was streaked on a YPD agar surface (1.5% agar) and maintained in an incubation chamber at 30 °C for 36 h. The yeast was then activated in YPD liquid medium and incubated for 12–15 h, 200 rpm, at 30 °C. The cells obtained after centrifugation (4000*g* for 5 min at 4 °C) were inoculated in to a mineral medium containing 0.62 g/l of KH_2PO_4, 2.0 g/l of K_2HPO_4, 1.0 g/l of $(NH_4)_2SO_4$, 0.1 g/l of $MgSO_4 \cdot 7H_2O$ and 5.0 g/l of yeast extract with galactose as the carbon source. After incubation at 30 °C, 200 rpm for 31 h, the supernatant separated by centrifugation was used as a source of the extracellular enzyme and the biomass as a source of the intracellular enzyme.

2.3. Extracellular α-galactosidase purification

The supernatant was lyophilized and the α-galactosidase was purified according to Viana et al. (2006).

2.4. Permeabilization of D. hansenii UFV-1 cells

Samples of 1 ml yeast culture were centrifuged (25,900*g* for 5 min at 4 °C) and the precipitate was resuspended in 0.45 ml of 50% (v/v) ethanol solution. After agitation for 30 s, 100 μl fractions were removed to determine the intracellular α-galactosidase activity.

2.5. α-Galactosidase immobilization

The extracellular α-galactosidase was immobilized by the covalent linkage method in chemically modified silica gel with 3-aminopropyltriethoxysilane according to Ettalibi and Baratti (2001). Silanized silica beads (1.0 g), containing amino reactive groups, were mixed with 50 ml of 5% glutaraldehyde solution under agitation for 1 min. Glutaraldehyde was used as the cross-linking agent. After reaction, the silica was washed five times with distilled water and the enzyme solution (80 U) was added. The mixture was shaken for 10 min to permit the covalent linkage of enzyme with the aldehyde groups. The silica gel containing the immobilized α-galactosidase was washed five times with distilled water and stored in 0.1 M sodium phosphate buffer, pH 5.0, at 4 °C.

2.6. Enzyme assay

The standard assay for the α-galactosidase contained 650 μl of 0.1 M sodium acetate buffer, pH 5.0, 100 μl of enzyme solution (0.75 μg protein/mL) and 250 μl of 2 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal). In the case of immobilized α-galactosidase, the reaction system contained 2 ml of immobilized enzyme preparation and 3 ml of 2 mM *p*NPGal solution prepared in 0.1 M sodium acetate buffer (pH 5.0). The reactions were run for 15 min at 60 °C and brought to a close with the addition of 1 ml of 0.5 M sodium carbonate. The amount of *p*-nitrophenol (*p*NP) released was determined at 410 nm. One unit of enzyme activity (U) was defined as the amount of protein required to produce 1 μmol of *p*-nitrophenol per minute.

The activities against sucrose were assayed for 30 min at 60 °C, using a reaction mixture containing 750 μl of 0.1 M sodium acetate buffer, pH 5.0, 100 μl of enzyme solution (0.75 μg protein/ml) and 150 μl of a 0.1 M sucrose solution. The produced amount of reducing sugar was determined by adding 1 ml of 3.5-dinitrosalicylate reagent (DNS), according to Miller (1956).

2.7. Effects of pH and temperature

The influence of pH and temperature on α-galactosidase activities was determined using the standard assay, except that the pH was modified to a range of 4.0–8.0 (McIlvaine, 1921) and temperatures of 40–90°C, respectively.

Results of the analyses are presented as mean ± SD for three measurements.

2.8. Thermostability

To determine the thermal stability, samples of free α-galactosidase and immobilized enzyme were pre-incubated for several periods at 50, 60, 70 °C and 70, 80, 90 °C, respectively. The residual activity was determined using

the standard assay. Results of the analyses are presented as means ± SD for three measurements.

2.9. Soymilk preparation

Soymilk was prepared from dry seeds (50 g). The seeds were chopped, homogenized in 400 ml distilled water at 80 °C, incubated at 85 °C for 10 min, and filtered through cheese-cloth.

2.10. Soymilk and soy molasses treatment with α-galactosidases

Soymilk samples (5 ml) were incubated with either water or 10.5 U of free α-galactosidase (2 ml) for 0, 2, 4 and 6 h under shaking (100 rpm) at 60 °C. In the case of permeabilized cells, 2 ml of this suspension, containing 10.5 U of intracellular α-galactosidase, were incubated with 5 ml of soymilk under the same conditions and then centrifuged (25,900*g* for 30 min at 4 °C). For the soymilk treatment with immobilized α-galactosidase, 1.0 g of silica, containing 40 U of immobilized enzyme, was incubated with 25 ml of soymilk under the same conditions for 0, 2, 4, 6, 8 and 10 h.

Initially, samples of heavy and light soy molasses were mixed with distilled water (1:5 w/v). The preparations (5 ml) were incubated with either distilled water or 10.5 U of free α-galactosidase (2 ml) under the same conditions as described for 0, 2, 4 and 6 h.

Each reaction mixture or supernatant was lyophilized. The soluble sugars were extracted from 20 to 30 mg of dried powder with 80% of aqueous-ethanol (v/v) according to Guimarães et al. (2001). The solvent was evaporated at 50 °C and the sugars resuspended in 1 ml of 80% (v/v) ethanol and analyzed by HPLC.

2.11. HPLC analysis of soluble sugars

The sugars were analyzed by HPLC on a Shimadzu series 10A chromatograph. For this purpose, an analytical column [aminopropyl (–NH_2)] was used, eluted with an acetonitrile-water isocratic mixture (80:20 v/v) at 35°C at a flow rate of 1 ml/min, according to Guimarães et al. (2001). The individual sugars were automatically identified and quantified by comparison with the retention times and standard sugar concentrations. Gentiobiose was used as an internal standard since it does not interfere with other sugars and is not found in soybean seeds.

3. Results and discussion

The yeast *D. hansenii* UFV-1 produced intracellular and extracellular α-galactosidases when grown in a medium containing galactose as carbon source. The chemical immobilization of purified extracellular α-galactosidase in modified silica resulted in an activity yield of 50%. This activity yield was calculated by dividing the activity of immobilized enzyme by the activity of enzyme contacted with the support. The α-galactosidase activity in the immobilized preparation was 40 U per g of silica. Onal and Telefoncu (2003) showed that the α-galactosidase from watermelon covalently immobilized on chitin exhibited an activity yield of 67%.

The pH influence on free and immobilized α-galactosidase from *D. hansenii* UFV-1 is shown in Fig. 1. The optimum pH for the maximal free α-galactosidase activity was 5.0, which is in agreement with the value reported by Kotwal, Gote, Sainkar, Khan, and Khire (1998) for the α-galactosidase from *Humicola* sp. The maximal activity of the immobilized α-galactosidase was also observed at pH 5.0. However, the immobilized α-galactosidase activity, in a pH range above 5.0, was higher when compared with free enzyme. The immobilized α-galactosidase maintained 62% of its maximal activity at pH 7.0 while, at the same pH value, the free enzyme activity was completely lost (Fig. 1). For the watermelon α-galactosidase, the optimum pH (6.0) for activity was not affected by immobilization (Onal & Telefoncu, 2003). According to Thippeswamy and Mulimani (2002), the optimum pH of *Gibberella fujikuroi* α-galactosidase was 5.8, whereas the optimum pH for immobilized enzyme in polyacrylamide gel was in the range 5.2–5.6.

The temperature effect on the activity of *D. hansenii* UFV-1 α-galactosidase was studied in a range of 40–90 °C (Fig. 2). Temperature rises promoted increases in the immobilized enzyme activity and gave maximal activity at 80 °C. The maximal activity of free α-galactosidase occurred at 60 °C. At 90 °C, the immobilized α-galactosidase maintained 94% of its maximal activity. The immobilized enzyme was more stable than free α-galactosidase. Approximately 85% of the immobilized enzyme activity was maintained at 70 °C, while the free enzyme lost 90% of its activity at the same temperature (Fig. 2). The silica immobilization system was able to protect the enzyme from thermal effects, allowing activity even at 80 °C. This is convenient, since high temperatures diminish microbial

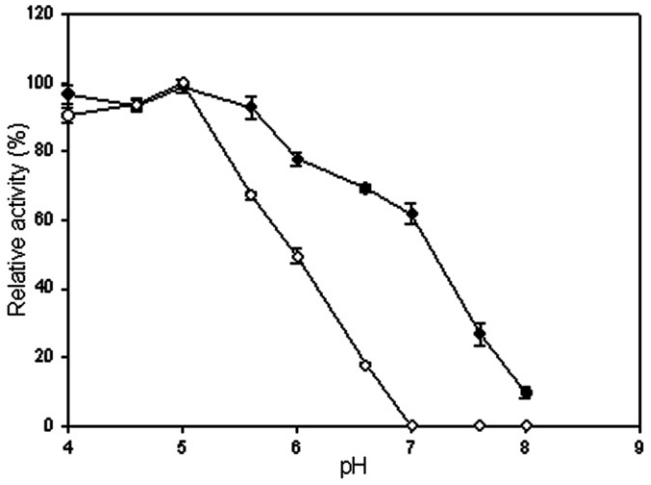

Fig. 1. Effect of pH on: (○) free and (●) immobilized α-galactosidase from *Debaryomyces hansenii* UFV-1.

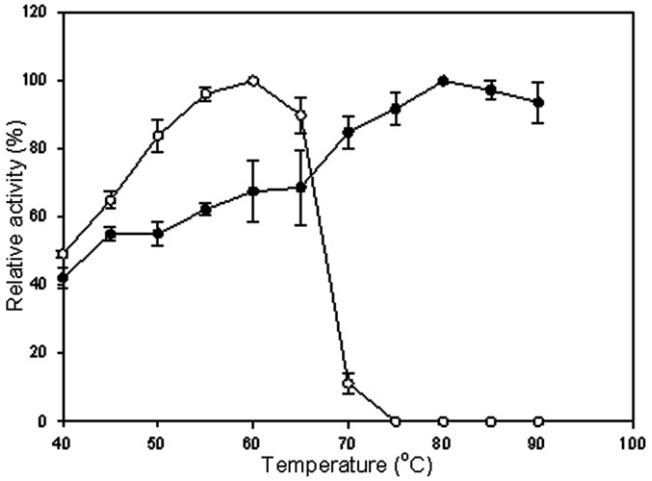

Fig. 2. Temperature effect on: (○) free and (●) immobilized α-galactosidase from *Debaryomyces hansenii* UFV-1.

contamination during the treatment of soybean products. For *Aspergillus oryzae* α-galactosidase, the maximum activity of alginate-entrapped enzyme was obtained at 57 °C compared to 50° for the soluble enzyme (Prashanth & Mulimani, 2005).

Thermal stability experiments were performed with free and immobilized α-galactosidase, which were pre-incubated at 50, 60, 70 °C and 70, 80, 90 °C, respectively. At 70 °C, the immobilized α-galactosidase maintained 94% and 70% of its original activity after pre-incubation for 16 and 37 h, respectively (Fig. 3B). On the other hand, when pre-incubated at 70 °C, the free enzyme maintained 58% of its original activity after 15 min and was totally inactive after 2 h (Fig. 3A). The immobilized enzyme maintained 94% of its original activity when pre-incubated at 80 °C during 90 min. At 90 °C, the immobilized enzyme maintained 11% of its activity for 30 min. In agreement, Ettalibi and Baratti (2001) showed that the thermal stability of inulinases from *Aspergillus ficuum* increased upon immobilization in modified silica. The stability of free and immobilized α-galactosidase from *D. hansenii* UFV-1 was further determined, incubating the enzymes at 4 °C during a three-month period. The immobilized enzyme maintained full activity during this period. The free enzyme maintained 94% and 78% of the original activity after one and three months, respectively. Enhanced thermal stability and a wide pH range were observed after enzyme immobilization in silica for α-galactosidase. The high thermostability of α-galactosidase would be a great advantage for industrial application, especially when removing RO from soymilk and other soy-based products.

The data presented for all α-galactosidase activity determinations are mean values of triplicate assays in which the standard deviations were always smaller than 10%.

The enzymatic hydrolysis of raffinose family sugars in soymilk over different periods, by free and immobilized α-galactosidase from *D. hansenii* UFV-1 and by permeabilized cells of *D. hansenii* UFV-1 containing the intracellular α-galactosidase, is shown in Table 1. During the incubation period of 4 h, the raffinose family sugars were completely hydrolyzed by free α-galactosidase. A reduction of 100% was detected, in the amount of stachyose, by immobilized α-galactosidase and by permeabilized cells containing intracellular enzyme. However, in the same incubation period, raffinose was hydrolyzed to an extent of 25% and 68% by immobilized enzyme and permeabilized cells, respectively. After an incubation of 6 h, permeabilized cells of *D. hansenii* UFV-1 had hydrolyzed 71% of raffinose and after 10 h of incubation, 63% of raffinose had been hydrolyzed by immobilized α-galactosidase. Although satisfactory hydrolysis is obtained by such processing, a single usage of free enzyme seems uneconomical and the processed soymilk possibly contains foreign proteins (Prashanth & Mulimani, 2005). A literature survey identified few reports on oligosaccharide reduction in soymilk by α-galactosidase immobilization (Thananunkul, Tanaka, Chichester, & Lee, 1976; Thippeswamy & Mulimani,

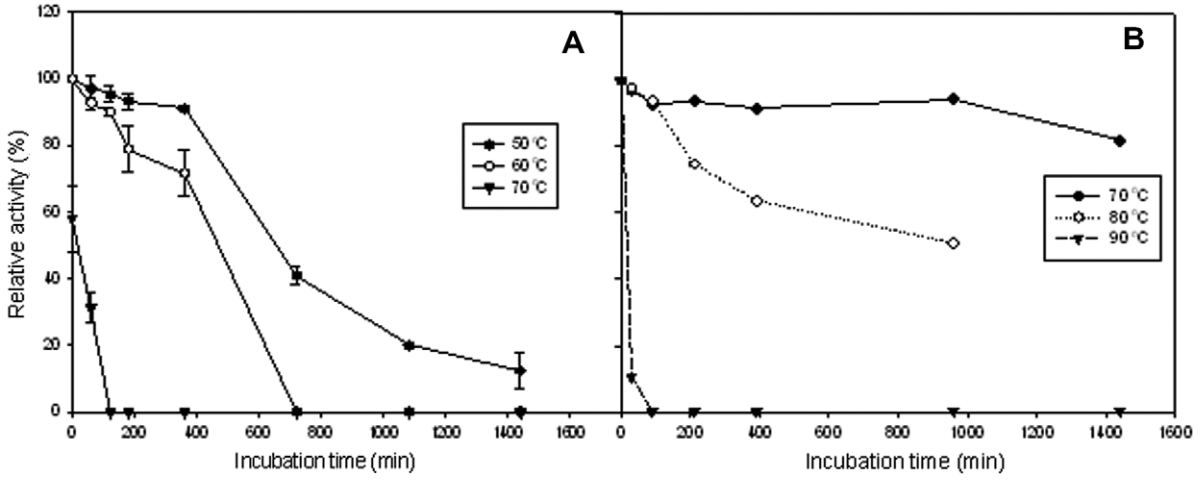

Fig. 3. Temperature influence on the stability of α-galactosidases of *Debaryomyces hansenii* UFV-1 on: (A) free, (●) 50 °C; (○) 60 °C; (▼) 70 °C and (B) immobilized enzyme, (●) 70 °C; (○) 80 °C; (▼) 90 °C, for up to 26 h.

Table 1
Hydrolysis of oligosaccharides present in soymilk by α-galactosidases derived from *Debaryomyces hansenii* UFV-1

Incubation time (h)	Free α-galaclosidase		Immobized α-galactosidase		Permeabilzed cells	
	Content (%) ± SD					
	Raffinose	Stachyose	Raffinose	Stachyose	Raffinose	Stachyose
0	1.36 ± 0.02	3.58 ± 0.01	1.30 ±0.01	3.61 ± 0.01	1.33 ± 0.02	3.55 ± 0.01
2	1.07 ± 0.01	0.0	1.83 ± 0.01	0.43 ± 0.01	0.49 ± 0.01	0.0
4	0.0	0.0	0.98 ± 0.02	0.0	0.43 ± 0.01	0.0
6	0.0	0.0	0.69 ± 0.02	0.0	0.39 ± 0.02	0.0
10	nd	nd	0.48 ± 0.01	0.0	nd	nd

nd, not determined.

2002). In this study, immobilized α-galactosidase was less effective for RO hydrolysis than was the free enzyme. A factor which explains the lower RO hydrolysis level in soymilk in the immobilized system is the restricted substrate diffusion through the enzyme complex (Erginer, Toppare, Alkan, & Bakir, 2000). The lower hydrolysis efficiency of the immobilized enzyme can, however, be compensated by re-using the system, while the free enzyme, in contrast, is lost after use. Several studies show the advantages of procedures involving immobilized enzymes (Shchipunov, Karpenko, Bakunina, Burtseva, & Zvyagintseva, 2004), although the continuous use of immobilized enzymes was not tested in this study. The mean raffinose and stachyose contents in dried soymilk were 1.33% and 3.58%, respectively, but the stachyose in soymilk was more hydrolyzed than was the raffinose. This difference in hydrolysis might have been caused by raffinose accumulation, which is formed after stachyose hydrolysis. In addition, the enzyme presents a higher affinity for stachyose than for raffinose, as shown by K_m values, which were 9.66 and 16.0 mM, respectively (Viana et al., 2006). Similar results were observed by Thippeswamy and Mulimani (2002), who used free and immobilized α-galactosidase from *G. fujikuroi* for RO degradation in soymilk.

The most effective RO hydrolysis in the different treatments was achieved in the first 4 h of incubation, during which 100% of stachyose was hydrolyzed. This indicates that an initial incubation, period is preferable to a prolonged incubation with free or immobilized enzymes or with permeabilized cells. This result can be explained by the thermostability of free and immobilized α-galactosidase, which maintained, respectively, 75% and 100% of their original activities after incubation at 60 °C for 4 h.

Although the free and immobilized α-galactosidase extracted from *D. hansenii* UFV-1 attained maximal activity at pH 5.0, the soymilk pH was between 6.2 and 6.4. The immobilization process was particularly interesting since it enhanced the pH range of the enzyme activity.

The use of permeabilized cells of *D. hansenii* UFV-1 containing the intracellular α-galactosidase for RO hydrolysis in soymilk has advantages over the process of purified enzymes, since the steps of extraction and purification of intracellular enzyme can be spared. On the other hand, there are only few reports on the application of permeabilized cells for bioconversion (Liu et al., 1999; Liu, Fujita, Kondo, & Fukuda, 2000), partly due to the origin of the microorganisms, which lack generally recognized as safe (GRAS) status. *D. hansenii* UFV-1 is the most frequent yeast species in protein-rich fermented products, such as sausages and cheeses (Saldanha-da-Gama et al., 1997). There should be no restriction regarding safeness for the use of this microorganism in food processing. The cells permeabilized with 50% of ethanol, containing the intracellular α-galactosidase activity, hydrolyzed 70% and 100% of raffinose and stachyose, respectively, after incubation for 6 h. The high activity of permeabilized cells is attributable to the reduced permeability barrier of the cell envelope to the substrates and products. According to Jordão, Brandi, and Passos (2000), the alteration in plasma membrane permeability of cells permits the free substrate diffusion. This condition can easily be established by treating the cells with a simple organic solvent such as ethanol. Flocculent yeast *Saccharomyces cerevisiae* YF234 cells were also permeabilized with ethanol under various conditions and are very effective as whole cell biocatalysts (Kondo et al., 2000). Alterations in the conditions of use of permeabilized cells and an optimization of the immobilization process are being studied.

Molasses, a by-product in the soy processing industry, is used as an inexpensive animal feed, but the processing and use of soy molasses as functional food has been suggested (Najafpour & Shan, 2003). King, Yernool, Eveleigh, and Chassy (1998, 2002) isolated and characterized α-galactosidases from *Thermatoga neapolitana* and *Thermoanaerobacterium polysaccharolyticum* to determinate their potential for RO hydrolysis in soy molasses. In this study, *D. hansenii* UFV-1 free α-galactosidase was used to hydrolyze the raffinose and stachyose present in heavy and light soy molasses (Table 2). Commercially, these soy by-products are obtained after decanting soy molasses for 3–4 days, and separating the bottom fraction (heavy molasses) from the upper fraction (light molasses). The amounts of raffinose and stachyose present in the preparation of heavy molasses were 3.8% and 4.46%, respectively, and 4.83% and 5.15% in the light molasses, respectively.

The incubation of soy molasses with the free α-galactosidase from *D. hansenii* UFV-1 for a period of 2 h reduced the stachyose amount to zero, but the raffinose content increased in the same period, probably due to raffinose accumulation, which occurs after stachyose hydrolysis.

Table 2
Hydrolysis of oligosaccharides present in soy molasses by free α-galactosidase of *Debaryomyces hansenii* UFV-1

Incubation time (h)	Content (%) ± SD			
	Heavy molasses		Light molasses	
	Raffinose	Stachyose	Raffinose	Stachyose
0	3.80 ± 0.02	4.46 ± 0.01	4.83 ± 0.01	5.15 ± 0.02
2	5.49 ± 0.02	0.0	5.21 ± 0.01	0.0
4	3.43 ± 0.01	0.0	4.17 ± 0.02	0.0
6	1.91 ± 0.01	0.0	2.28 ± 0.01	0.0

Nevertheless, the enzyme hydrolyzed 50% and 53% of the initial raffinose content in the heavy and light molasses, respectively, after incubation for 6 h. As the enzyme preparation showed no invertase activity, the results indicate that the *D. hansenii* UFV-1 α-galactosidase acts on the oligosaccharides present in the soy products.

In this study, it was shown that the characteristics of α-galactosidases from *D. hansenii* UFV-1 are compatible with the requirements for the development of a RO reduction procedure in soy products.

4. Conclusions

In the present work, we compared the properties of the free α-galactosidase secreted by the *D. hansenii* UFV-1 yeast with immobilized enzyme in modified silica. The immobilization of α-galactosidase enhanced the thermal stability and broadened the pH range, enhancing the applicability for hydrolysis of soy products. The hydrolysis of indigestible oligosaccharides found in soymilk was tested using these enzyme forms, as well as permeabillized cells of the *D. hansenii* UFV-1 containing the intracellular α-galactosidase. The possibility of using permeabilized *D. hansenii* UFV-1 cells for RO hydrolysis is interesting, because this microorganism is found in several food products, and there should be no restriction, regarding safety, for its use in food processing. All studied forms of the α-galactosidase were effective for RO reduction in soymilk. The results presently reported indicate that the *D. hansenii* UFV-1 α-galactosidases may be used for the establishment of a process to improve the nutritional value of soy products.

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