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Full Length Research Paper

Effects of Lyophilization on the Catalytic Properties of Extracellular Fructosyltransferase from *Rhodotorula* sp. LEB-V10

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The lyophilization of fructosyltransferase (FTase) from *Rhodotorula* sp. LEB-V10 was carried out and its significant potential for synthesis of fructooligosaccharides (FOS) was evaluated and compared to the non-lyophilized enzyme. Twelve cryoprotectant additives were selected and analyzed both individually and together; the results generally showed that lyophilized enzymes have a higher enzymatic activity per gram although different levels of relative enzyme activity were observed, depending on the dilution of the initial solution. Lyophilization without additives of an enzymatic solution diluted in 1:2 (v/v) sodium acetate buffer (200 mM) pH 4.5 presented the greatest increase in enzymatic activity per gram, almost 6.5-fold higher after lyophilization and a weight reduction of almost 89%, but this result also indicated a loss of 26% of the initial enzymatic activity. Additives such as CMC at 1.25% (w/v), mannitol, ammonium sulfate, sorbitol and xylitol at 2.5% (w/v) increased enzymatic stability after 6 months from 3 to 37%. Effects of the additives alone were better than when mixed. Lyophilization also affected the biocatalytic activity of FTase, especially by increasing GF_4 (% composition) by almost 3-fold.

Keywords: Enzyme Lyophilization, Free Enzyme, Freeze Drying, Fructooligosaccharides Synthesis, FTase.

INTRODUCTION

Industrial biotechnology (white biotechnology) has generated much interest because it is frequently associated with reduced energy consumption, new food and biofuels sources, and is frequently able to present economic, political and social solutions (Tang and Zhao, 2009).

Studies carried out with extracellular fructosyltransferase (FTase, EC 2.4.1.9) partially purified (ethanol precipitation) from *Rhodotorula* sp. LEB-V10 (Aguiar-Oliveira and Maugeri, 2010; Alvarado-Huallanco and Maugeri, 2010; Hernalsteens and Maugeri, 2008; Maugeri and Hernalsteens, 2007), in free and immobilized forms, have demonstrated great industrial potential for the production of fructooligosaccharides (FOS) - a prebiotic sugar (Maiorano *et al*., 2008; Sangeetha *et al*., 2005) - by promoting conversions of approximately 58% of sucrose to FOS under optimized conditions with the enzyme immobilized on niobium (Aguiar-Oliveira *et al*., 2012). In traditional processes using fructosyltransferases purified from filamentous fungus, conversions vary from 50 to 70% with purified enzymes. The use of partially purified fructosyltransferase reduces the costs of FOS production, and its immobilization techniques (Aguiar-Oliveira and Maugeri, 2010) favor continuous industrial use; therefore, the studies presented in the present study regarding the lyophilization of fructosyltransferase from *Rhodotorula* sp. LEB-V10 are designed to solidify the feasibility of this enzyme in industrial applications. Kinetic studies with this same enzyme also immobilized in niobium (Alvarado-Huallanco and Maugeri, 2010) revealed that in either the purified or partially purified forms, sucrose conversions to FOS were practically the same, and this justifies application of the partially purified enzyme. The present

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study evaluated the lyophilization of fructosyltransferase from *Rhodotorula* sp. and made comparisons with the non-lyophilized enzyme in terms of activity, synthetic capacity and stability.

Lyophilization

Lyophilization (freeze drying) is one of the most well-known preservation processes, and it consists of removing water from proteins or cells in suspension by sublimation (Blanch and Clark, 1997; Cabral *et al*., 1994; Aehle, 1990; Chaplin and Bucke, 1990). This process is highly indicated for adding commercial value to the protein in question since it facilitates transport/distribution, storage, use, etc. (for both purposes pharmaceutical and alimentary) despite the fact that it is a high cost technology due to energy consumption and preparation time (Roy and Gupta, 2004; Tang and Pikal, 2004; Partridge *et al*., 1998; Carpenter *et al*., 1997; Fágáin, 1997). Conservation methods such as refrigerated storage in phosphate buffer (pH = 7.0) and ethanol solutions, or freezing in the presence of a reducing agent (*e.g.* glutathione) results in low shelf-life, moreover, these techniques are not industrially feasible (Kobayashi *et al*., 1984). Additionally, they can induce proteolysis, chemical degradation (*e.g.* deamidation or oxidation) and/or physical degradation (*e.g.* degradation, aggregation and precipitation) (Carpenter *et al*., 1997; Fágáin, 1997).

Various authors have observed different levels of activity and increased stability in lyophilized enzymes in a variety of methodologies: Ru *et al.* (2001) described a greater than 20,000 fold increase in the biocatalytic activity of lyophilized enzymes in the presence of salts; Morgan and Clark (2004) observed a 10 fold increase in activity of a lyophilized xanthine oxidase in the presence of salts; when lyophilizing a glucose dehydrogenase with sorbitol and especially with trehalose, Sode and Yasutake (1997) managed to increase thermal stability in relation to the non-lyophilized condition; Wang and Mei (2007) increased the conversion of lipase lyophilized with cyclodextrins from 9 to 14%. This activation and the preservation of the three-dimensional structure after lyophilization may occur due to the most varied mechanisms. Lee and Dordick (2002) and Ru *et al.* (2001) stated that the use of certain additives increase the solubility of stabilizer solutes around the protein, forming a protective layer. According to Partridge *et al.* (1998), an efficient enzyme "dehydration" process occurs when water molecules are removed, so that the protein molecules can maintain their three-dimensional structure as intact as possible. Fágáin (1997) stated that the more an enzyme is able to maintain an ionic balance, corresponding to the optimum pH level, the better the preservation of its biocatalytic functions.

In order to reduce the stress suffered by the protein, various cryo- or lipo-protectant compounds can be added (Kobayashi *et al*., 1984; Ru *et al*., 2001; Hirakura *et al*., 2004; Gibson and Woodward, 1993; Roser, 1990). After freezing, some buffers have their pH levels altered due to a change in pKa or selective crystallization and precipitation of a less soluble salt; the most inadequate buffers would be those of phosphate and pyrophosphate which present a reduction of 2.3 units in pH after freezing and the Tris buffer shows a pH level increase of the same magnitude (Carpenter *et al*., 1997; Hirakura *et al*., 2004; William-Smith *et al*., 1977). No references were found regarding the sodium acetate buffer defined for the fructosyltransferase from *Rhodotorula* sp. LEB-V10 (Hernalsteens and Maugeri, 2008), where the only reference found analyzed the effects induced by the sodium acetate salt in concentrations as high as 98% (w/v) (Ru *et al*., 2001; Borole and Davison, 2008). The activation mechanism promoted by high salt concentrations appears to involve several factors including changes in the polarity of the enzyme or the kosmotropicity of the activating salt (stabilizer capacity). Some authors suggest the use of buffers with higher salt concentrations such as 200 mM, since 98% (w/v) of a salt in a product formulation with applications in the food industry can interfere with the final product's composition (Roy and Gupta, 2004; Partridge *et al*., 1998; Ru *et al*., 2001; Morgan and Clark, 2004).

Pharmaceutical drugs and other lyophilized proteins often contain glucose and/or fructose (hexoses), sucrose (dissaccharide) or trehalose (non-reducing disaccharide) in their formulations (Carpenter *et al*., 1997; Kobayashi *et al*., 1984; Gibson and Woodward, 1993), and these compounds are capable of reducing the denaturation impact during lyophilization by increasing the medium density or by removing water molecules. Sucrose is the most recommended sugar due to its low cost compared to trehalose (Carpenter *et al*., 1997), however, for fructosyltransferase from *Rhodotorula* sp. LEB-V10 it would probably not be recommended to use sucrose, glucose or fructose formulations because they are substrates and by-products of the FOS synthesis, and at different concentrations, these three compounds can induce kinetic inhibition (Alvarado-Huallanco and Maugeri, 2010). Although Chaplin and Bucke (1990) supported the use of substrates in formulations for lyophilization, Carpenter *et al.,* (1997) argues that even if it is effective, these additives have the propensity to degrade proteins via Maillard reactions. Trehalose has been widely used due to its high stabilizing effect and ability to preserve the protein structure (Carpenter *et al*., 1997; Roser, 1990) at different concentrations including

100 mM (Carpenter *et al*., 1997), 300 mM (Sode and Yasutake, 1997) and from 0.05 to 20% (Roser, 1990).

The use of bulking agents such as mannitol or sorbitol (hexahydric sugar alcohol), and xylitol (pentahydric sugar alcohol) are recommended at varying concentrations, from 2 to 10%, 50 mM or in the proportions of 1.1 or 1:6 (protein:sugar) (Tang and Pikal, 2004; Carpenter *et al*., 1997; Kobayashi *et al*., 1984; Ru *et al*., 2001; Sode and Yasutake, 1997; Gibson and Woodward, 1993). It is known that glycerol is often employed in pharmaceutical formulations due to its viscosity, and ice-crystal formation at sub subzero temperatures (Chaplin and Bucke, 1990), and as well as ethylene glycol, sorbitol and xylitol, have a protective effect from direct interaction (whether specific or not) with enzyme polypeptides, but glycerol has the disadvantage of being a good bacterial substrate (Fágáin, 1997). Ethylene glycol is used as a protection group for the carbonyl groups in organic synthesis, and it is known for its desiccant properties (Soares *et al*., 2002).

Poly(ethylene glycol) or PEG (amphipathic polymer and lipo-protectant) is often applied in lyophilized protein formulations at 49% combined with KCl (Ru *et al*., 2001) or sucrose (Mosharraf *et al*., 2007), ranging from 155 to 323 mM (Mine *et al*., 2001) or in a 1:2 ratio of enzyme:PEG (Borole and Davison, 2008) and is also indicated as a good molecular imprinter (Lee and Dordick, 2002). Carboxymethyl cellulose (CMC) is known as a viscosity modifier or thickener, as well as a good emulsifing agent in various products of the food industry. Sode and Yasutake (Sode and Yasutake, 1997) investigated the addition of ammonium sulfate at 50 mM, among other compounds.

According to Carpenter *et al.* (Carpenter *et al*., 1997), increased enzymatic concentration heightens the resistance to degradation during freezing. It is believed that damage could be caused by the ice-water interface during the freezing process, and for this reason it is important to evaluate the optimal concentration or dilution rate for each system studied.

MATERIAL AND METHODS

Partial purification of the extracellular fructosyltransferase and the cultivation of *Rhodotorula* sp. LEB-V10 was carried out according to Aguiar-Oliveira and Maugeri (Aguiar-Oliveira and Maugeri, 2010), as well as the methodologies for determination of enzymatic activity from sugars released using the Somogyi-Nelson methodology and Glucose-oxidase enzymatic kit, and FOS by ion-exchange chromatography (HPLC-PAD).

Additives Selection

The additives evaluated in this study were: ammonium sulfate, carboxymetyl cellulose (CMC), ethylene glycol, glycerol, inositol, mannitol, polyethylen glycol (PEG 6000), sorbitol, trehalose and xylitol added to sodium acetate buffer (pH 4.5) at concentrations of 100 and 200 mM. These compounds were acquired from trusted and available companies.

In the first step, each additive was individually evaluated at the concentration of 2.5% (w/v or v/v). Later, some additives were chosen for use in the formulations: CMC, PEG 6000, mannitol, xylitol, trehalose, and ammonium sulfate, all at 1.25% (w/v), with the dispersion and dilution solution being sodium acetate buffer at 200 mM and pH 4.5.

Sample preparation and analysis

In the first stage of this study, the individual effect of each additive was studied; all lyophilizations were performed in duplicate in which each had a starting volume of 3.0 mL. An enzymatic solution with transfructosylation activity of 134.06 ± 13.68 U_{TF}/mL (ρ = 0.718 ± 0.0050 g/mL) was used without dilution and diluted to 1:6 (v/v) in sodium acetate buffer (50 mM), pH 4.5; each additive (listed in Table 1) was assessed as part of both enzymatic solutions at 2.5% (w/v), where only CMC was also evaluated at 1.25% (w/v).

After selecting the best additives according to individual performances, the enzyme solutions used for the second stage of this study (formulations of additives) were diluted to 1:2 and 1:6 (v/v) in 200 mM sodium acetate buffer, pH 4.5, and the following experiments were carried out: three formulations containing two additives were chosen for the dilution of 1:2 (v/v) and three formulations containing three additives for the dilution of 1:6 (v/v), as presented in Table 3. In this second stage, sample preparation was similar to the first stage, always in duplicate, and the concentration of each additive in the formulation was 1.25% (w/v).

Based on results from a previous work (Aguiar-Oliveira and Maugeri, 2011), fructosyltransferase was pre-activated (performed by 15 min of incubation at 52 or 60°C followed by submersion in an ice bath) before lyophilization.

Lyophilization of the free fructosyltransferase

All samples were previously frozen at -60°C in an ultra-freezer for about 18 h prior to lyophilization. These samples were then lyophilized for 24 h in a Terroni®

Table 1. Characterization of fructosyltransferase from *Rhodotorula* sp. LEB-V10, lyophilized in the presence and absence of stabilizing additives. The characterization parameters were as follow: residual enzyme activity [R_o^{Lyoph}], thermal residual enzyme activity [$R^{Lyoph.65°C}$], specific enzyme activity [${}^{*}U_{ptn}^{Lyoph} = U_{ptn}^{Lyoph} / mg$], FOS yield [Y_{FOS}] and specific FOS productivity after 24 h [Pr_{FOS} = mg/U_{TF}.h]. Syntheses were carried out with 0.02 g of the lyophilized powder in 5 mL of 50% (w/v) of sucrose in 50 mM sodium acetate buffer, pH 4.5.

Initial Enzyme Solution	**U_{TF}°/g**	**$R^{65°C}$ ($U_{TF}^{65°C}/U_{TF}^{o}$)**	***U_{ptn} (U_{TF}^{o}/mg)**	**Y_{FOS} (24h) (Eq.04)**	**Pr_{FOS} (24h) (Eq.05)**
	186.65 ± 19.04	0.37 ± 3.8E^{-2}	5.59 ± 0.16	0.45	17.63
Lyophilized enzyme from the initial undiluted enzyme solution					
Lyophilized enzyme without addictive	**R_o^{Lyoph}** (Eq. 01)	**$R^{Lyoph.65°C}$** (Eq. 02)	***U_{ptn}^{Lyoph}** (Eq.03)	**Y_{FOS}** (24h) (Eq.04)	**Pr_{FOS}** (24h) (Eq.05)
	4.11 ± 0.35	0.58 ± 1.4E^{-2}	5.16 ± 0.15	0.42	2.89
Lyophilized enzyme with additives	**R_o^{Lyoph}** (Eq. 01)	**$R^{Lyoph.65°C}$** (Eq. 02)	***U_{ptn}^{Lyoph}** (Eq.03)	**Y_{FOS}** (24h) (Eq.04)	**Pr_{FOS}** (24h) (Eq.05)
Ammonium sulfate - 2.5% (w/v)	3.15 ± 0.34	0.50 ± 5.2E^{-2}	4.55 ± 0.10	0.50	4.61
CMC - 2.5% (w/v)	4.46 ± 0.40	0.38 ± 1.7E^{-2}	6.27 ± 0.08	0.50	4.64
Ethylene glycol - 2.5% (v/v)	2.99 ± 0.14	0.25 ± 2.3E^{-2}	2.96 ± 0.14	0.52	4.41
Glycerol - 2.5% (w/v)	2.54 ± 0.37	0.43 ± 3.5E^{-2}	3.36 ± 0.15	0.49	4.34
Inositol - 2.5% (w/v)	3.47 ± 0.41	0.41 ± 7.9E^{-3}	4.59 ± 0.02	0.41	3.84
Mannitol - 2.5% (w/v)	3.30 ± 0.25	0.35 ± 2.2E^{-2}	4.37 ± 0.06	0.50	5.54
PEG 6000 - 2.5% (w/v)	4.91 ± 0.19	0.32 ± 8.6E^{-3}	6.42 ± 0.21	0.59	3.33
Sorbitol - 2.5% (w/v)	3.04 ± 0.37	0.28 ± 7.8E^{-2}	4.01 ± 0.13	0.30	2.40
Trehalose - 2.5% (w/v)	3.38 ± 0.30	0.35 ± 1.7E^{-2}	4.09 ± 0.07	0.47	3.65
Xylitol - 2.5% (w/v)	3.00 ± 0.29	0.57 ± 3.5E^{-3}	3.95 ± 0.12	0.33	2.74
Lyophilized enzyme from the initial enzyme solution diluted at 1:6 (v/v) in sodium acetate buffer, 50 mM and pH 4.5.					
Lyophilized enzyme without additive	**R_o^{Lyoph}** (Eq. 01)	**$R^{Lyoph.65°C}$** (Eq. 02)	***U_{ptn}^{Lyoph}** (Eq.03)	**Y_{FOS}** (24h) (Eq.04)	**Pr_{FOS}** (24h) (Eq.05)
	3.32 ± 0.28	0.63 ± 3.6E^{-2}	4.65 ± 0.02	0.44	3.75
Lyophilized enzyme with additives	**R_o^{Lyoph}** (Eq. 01)	**$R^{Lyoph.65°C}$** (Eq. 02)	***U_{ptn}^{Lyoph}** (Eq.03)	**Y_{FOS}** (24h) (Eq.04)	**Pr_{FOS}** (24h) (Eq.05)
Ammonium sulfate - 2.5% (w/v)	2.14 ± 0.23	0.26 ± 3.4E^{-3}	3.62 ± 0.11	0.37	4.04
CMC - 2.5% (w/v)	1.85 ± 0.15	0.76 ± 4.2E^{-2}	2.83 ± 0.01	0.44	5.32
CMC - 1.25% w/v	2.99 ± 0.28	0.57 ± 9.8E^{-3}	4.22 ± 0.08	0.45	10.89
Ethylene glycol - 2.5% (v/v)	1.16 ± 0.10	0.58 ± 4.3E^{-2}	1.56 ± 0.03	0.17	2.45
Glycerol - 2.5% (w/v)	2.09 ± 0.25	0.28 ± 1.8E^{-2}	3.94 ± 0.03	0.37	4.89
Inositol - 2.5% (w/v)	2.44 ± 0.31	0.31 ± 6.2E^{-3}	5.35 ± 0.08	0.33	4.79
Mannitol - 2.5% (w/v)	1.95 ± 0.19	0.27 ± 2.3E^{-2}	3.95 ± 0.03	0.38	5.06
PEG 6000 - 2.5% (w/v)	3.57 ± 0.28	0.71 ± 1.4E^{-2}	4.80 ± 0.08	0.44	3.43
Sorbitol - 2.5% (w/v)	2.44 ± 0.31	0.26 ± 8.7E^{-3}	4.99 ± 0.03	0.31	3.58
Trehalose - 2.5% (w/v)	1.90 ± 0.25	0.63 ± 7.2E^{-2}	3.77 ± 0.08	0.47	3.68
Xylitol - 2.5% (w/v)	1.93 ± 0.13	0.61 ± 8.1E^{-2}	3.89 ± 0.05	0.34	5.01
Lyophilized enzyme from the initial enzyme solution diluted at 1:6 (v/v) in sodium acetate buffer at pH 4.5					
Buffer concentration	**R_o^{Lyoph}** (Eq. 01)	**$R^{Lyoph.65°C}$** (Eq. 02)	***U_{ptn}^{Lyoph}** (Eq.03)	**Y_{FOS}** (24h) (Eq.04)	**Pr_{FOS}** (24h) (Eq.05)
100 mM	3.62 ± 0.42	0.52 ± 4.4E^{-2}	4.97 ± 0.08	0.39	3.00
200 mM	3.65 ± 0.45	0.55 ± 7.1E^{-3}	5.04 ± 0.06	0.41	3.12

Interprise 1 bench-type lyophilizer, where they were cooled to ≈ -55°C by a hermetic compressor with forced ventilation (air) and using a vacuum pump with capacity of 5/370 (CFM/W). The lyophilized cakes were gently

milled with a glass baguette until turning to dust. Lyophilized enzymes were then stored in standard glass flasks (5 mL), without vacuum seal, in a freezer (-20°C) until all analyses were performed; analyses were performed within one week of lyophilization (t = 0) for the initial characterization, and 6 months after refrigerated storage (t = 6 m) for the shelf-life analysis.

Characterization of the lyophilized enzyme

According to Carpenter *et al.* (1997), the final analysis of a lyophilized proteic formulation should consider factors such as: protein stability during and after lyophilization, final application of the lyophilized material, formulation tonicity, cake structure and other physicochemical properties.

Enzymatic activity of lyophilized free fructosyltransferase was determined from a solution containing 0.02 g of lyophilized powder dissolved in 10 mL of 50 mM acetate buffer, pH 4.5. Standard FOS syntheses were performed at 50°C using 0.02 g of the powder in 5 mL of 50% (w/v) of sucrose in sodium acetate buffer (50 mM and pH 4.5); FOS synthesis under optimized conditions (Aguiar-Oliveira *et al*., 2012) was basically carried out the same way except for the following conditions: 48°C, pH 6.0 and 10 U_{TF}/mL.

All lyophilized samples were analyzed and compared to a non-lyophilized standard condition without any additives. The lyophilized enzymes were characterized mainly according to residual activity of the lyophilized enzyme (Eq. 01), thermal residual activity of the lyophilized enzyme (Eq. 02), specific activity of the lyophilized enzyme (Eq.03), FOS yield (Eq.04), specific productivity of FOS (Eq.05) and 6 months shelf-life.

Residual Enzyme Activity (R_o^{Lyoph})

Expressed by Equation 01 according to the lyophilized free enzymatic activity (U_{TF}^{Lyoph}) and the non-lyophilized free enzymatic activity (U_{TF}^{o}).

$$R_o^{Lyoph} = \frac{U_{TF}^{Lyoph}}{U_{TF}^{o}} \quad (01)$$

Thermal Residual Enzyme Activity ($R^{Lyoph.65°C}$)

Expressed by Equation 02 according to the lyophilized free enzyme activity after incubation at 65°C for 15 minutes followed by submersion in an ice bath [$U_{TF}^{Lyoph.65°C}$], and the lyophilized free enzymatic activity of the same sample before this thermal treatment [U_{TF}^{Lyoph}].

$$R^{Lyoph.65°C} = \left(\frac{U_{TF}^{Lyoph.65°C}}{U_{TF}^{Lyoph}} \right) \quad (02)$$

Specific Enzyme Activity (U_{TF}^{Lyoph})

Expressed by Equation 03 according to the lyophilized free enzyme activity [U_{TF}^{Lyoph}] and the protein value (mg) determined per gram of lyophilized enzyme. Lowry's methodology (Lowry *et al*., 1951) was used to determine the total protein in the lyophilized enzyme formulation.

$$^{*}U_{ptn}^{Lyoph} = \frac{U_{TF}^{Lyoph}}{mg} \quad (03)$$

FOS yield (Y_{FOS})

Expressed by Equation 04 according to the total FOS concentration (g/L) and the initial sucrose concentration (500 g/L). 200 µL samples were collected from the synthesis product during different periods, and analyzed by ion-exchange chromatography (HPLC-PAD) according to the methodology described in a previous work (Aguiar-Oliveira and Maugeri, 2010).

$$Y_{FOS} = \frac{[FOS]}{[sucrose]} = \frac{[FOS]}{500g/L} \quad (04)$$

FOS Specific Productivity (Pr_{FOS} = mg/U_{TF}.h)

Ratio expressed by Equation 05 according to the total FOS concentration (mg/mL) and the concentration of free enzyme activity used in the synthesis (U_{TF}/mL), as a function of the synthesis elapsed time (h).

$$\mathrm{Pr}_{FOS} = \frac{[FOS]}{(U_{TF})(h)} \quad (05)$$

Water Activity (Aw)

The lyophilized enzymes without additives had their water activity (Aw) determined in triplicate in an AQUALAB

Figure 1. Standard lyophilized material of fructosyltransferase from *Rhodotorula* sp. LEB-V10: **a)** without dilution (lyophilized cake with Aw = 0.187 ± 0.001 and with greater resistance to crushing and dissolution), **b)** diluted to 1:2 (v/v) (lyophilized with Aw = 0.192 ± 0.004, fine powder aspect and fast dissolution) and **c)** diluted to 1:6 (v/v) (lyophilized with Aw = 0.276 ± 0.001, lighter coloration, very fine uniform powder, easy dissolution). The dilutions were achieved in sodium acetate buffer (50 mM and pH 4.5).

Series 3TE device, Decagon Devices Inc. (USA), at 25°C after lyophilization (time zero) and after 6 months of refrigerated storage.

Factor for Enzyme Activity Reduction after Lyophilization ($F_{U_{TF}}$)

The total reduction of enzymatic activity after lyophilization was determined according to Equation 06 for the lyophilized free enzyme without additives, using the enzymatic activities per gram before (U_{TF}/g) and after lyophilization (U_{TF}^{Lyoph}/g) and the weight of the samples before (6 g) and after lyophilization.

$$F_{U_{TF}} = \left(\frac{U_{TF}^{Lyoph.}}{g}\right) * (g)^{lyophilize\ d} \Big/ \left(\frac{U_{TF}}{g}\right) * (6g)^{solution} \quad (06)$$

RESULTS AND DISCUSSION

According to Oetjen and Haseley (2004), freezing of complex organic solutions is often difficult to predict and the growth rate of ice crystals is crucial because it is dependent on the temperature of initial freezing and the viscosity of the solution, which increases significantly with the increasing solution concentration. The water not properly frozen can forms highly viscous occlusions between the crystals, and by adding excipients the crystallization can again be delayed because of the viscosity of the initial solution.

In the first stage of this study, an enzymatic solution without dilution and an activity of 134.06 ± 13.68 U_{TF}/mL was used, whose corrected activity based on density (ρ = 0.718 ± 0.050 g/mL) was equivalent to 186.65 ± 19.04 U_{TF}/g. Lyophilization of this solution (without additives) resulted in a material with enzymatic activity of 763.64 ± 16.71 U_{TF}^{Lyoph}/g, a 4-fold increase in enzymatic activity per weight. Weight reduction after lyophilization was about 85%: 1g of the enzymatic solution resulted in 0.1522 ± 0.026 g of lyophilized powder. Concerning the enzymatic activity loss during the process, the factor F_{UTF} (Eq. 06) was 0.62, *i.e.* about 38% of enzyme activity was lost during the process with the non-diluted enzymatic solution. Water activity of this lyophilized enzyme at time zero was Aw = 0.187 ± 0.001.

The same concentrated enzymatic solution mentioned above was diluted by 1:2 (v/v) with sodium acetate buffer at 50 mM and pH 4.5, whose density was 0.993 ± 0.016 g/mL. Weight reduction after lyophilization was about 89%, indicating that 1g of the diluted solution produced 0.1129 ± 0.014 g of lyophilized powder. The F_{UTF} factor for enzymatic activity loss was of 0.73, signifying that about 26% of the enzymatic activity was lost (this was the smallest F_{UTF} among the three conditions analyzed). Water activity of this lyophilized enzyme at time zero was Aw = 0.192 ± 0.004.

Finally, a dilution of 1:6 (v/v) with the same initial enzymatic solution led to a density of ρ = 0.996 ± 0.010 g/mL. Weight loss after lyophilization was approximately 96%, meaning that 1g of the diluted solution resulted in 0.0258 ± 0.009 g of lyophilized powder. The F_{UTF} factor for reduction of enzymatic activity was 0.20, *i.e.,* about 80% of the initial enzymatic activity was lost during lyophilization; this condition resulted in the highest activity loss among the three analyzed conditions. Water activity of this lyophilized enzyme at time zero was Aw = 0.276 ± 0.001.

Effects of single additives were evaluated using the enzymatic solution without dilution and that diluted at 1:6 (v/v), as shown in Table 1. Afterwards, enzymatic solutions at the 1:2 and 1:6 (v/v) dilution ratios were used in a second step with formulations of the best additives

previously selected (Table 3).

Visual analysis of the lyophilized cakes (light brown color) indicated the differences between the concentrated solution and its dilutions. The lyophilization of solutions without dilution resulted in a harder cake, resistant to crushing, even forming larger and irregular granules (Figure 1a) which were dissolved slowly (or re-suspended) and requiring longer stirring times to achieved the total dissolution; the lyophilized powder from diluted solutions resulted in a softer cake which was easier to crush, with fine granules and easy dissolution, especially for the dilution at 1:6 (v/v) (Figure 1.c).

According to several authors, even after lyophilization an enzyme preparation still contains water which remains strongly linked and this plays a crucial role for the final product because water often acts as a catalyst for enzymes by increasing the internal flexibility of its molecules or by activating deteriorative reactions. In general, instability is increased when the final lyophilized product contains water above or below the ideal limit of the water layer *(Mo)* (Cabral *et al*., 1994; Fágáin, 1997; Mosharraf *et al*., 2007; Costatino *et al*., 1998). It should also be considered that during or immediately after lyophilization (freeze-drying) and/or reconstitution (rehydration) it is possible to form irreversible aggregates, a phenomenon commonly associated with a poorly designed or prepared lyophilized protein (Carpenter *et al*., 1997; Hirakura *et al*., 2004), resulting in low activity.

Individual effect of additives

Table 1 shows the results from the first stage of the study evaluating effects of individual additives.

It can be seen that all lyophilized enzyme products containing additives showed an increase in enzymatic activity after lyophilization ($R_o^{Lyoph.}$ > 1.0) compared to the non-lyophilized standard condition. According to Table 1, lyophilization of the undiluted enzymatic solution resulted in a greater than 4 times increase in enzymatic activity per weight; lyophilization of the diluted solution at 1:6 (v/v) resulted in an increase of more than 3 times. The small difference in $R_o^{Lyoph.}$ between the lyophilized conditions without additives reflects the fact that very concentrated solutions induce the formation of protein aggregates that jeopardize the final effective enzymatic activity. The undiluted enzymatic solution containing PEG 6000 and CMC both at 2.5% (w/v), the solution diluted in sodium acetate buffer at 200 and 100 mM and that diluted containing PEG 6000 and inositol at 2.5% (w/v) presented, in descending order, the highest lyophilized enzymatic activity (3.50 < $R_o^{Lyoph.}$ < 5.0). Literature reports that the addition of sorbitol, mannitol and sodium acetate, all at 1 mol/L, led to remaining enzyme activities of 75.3, 67.3 and 81.2%, respectively, after lactate dehydrogenase freezing and thawing (Oetjen and Haseley, 2004).

The thermal residual activity ($R^{Lyoph.65°C}$) revealed that lyophilization of the enzymatic solution increased thermal stability compared to the non-lyophilized solution (Table 1); for the standard lyophilized enzyme, a dilution of 1:6 (v/v) increased thermal stability by 9 %. For lyophilization of the undiluted enzymatic solution, none of the additives were capable of increasing thermal stability; when compared to the lyophilized standard condition ($R^{Lyoph.65°C}$ = 0.58), only the addition of xylitol resulted in a similar value ($R^{Lyoph.65°C}$ = 0.57). Lyophilization of the diluted enzymatic solution (1:6 v/v) presented an increased thermal stability with the addition of the PEG 6000 ($R^{Lyoph.65°C}$ = 0.71) and CMC ($R^{Lyoph.65°C}$ = 0.76), both at 2.5% (w/v), when compared to the standard lyophilized enzyme.

The specific lyophilized activity ($^*U_{ptn}^{Lyoph}$) should be analyzed with caution, since it is a partially purified enzyme solution and unidentified proteins are also precipitated along with the fructosyltransferase. Therefore, $^*U_{ptn}^{Lyoph}$ basically reflects the lyophilized enzyme activity, as shown by Equation 03. After the lyophilization procedures, the standard lyophilized enzyme from the undiluted solution showed a specific activity similar to the non-lyophilized solution; however, dilution and lyophilization resulted in a specific activity 10% lower than in the standard undiluted condition. The highest value was obtained with the lyophilized enzyme from the undiluted solutions with the addition of PEG 6000 (Table 1).

FOS synthesis performed with both the lyophilized enzyme without additives, and with the non-lyophilized solution showed similar conversions (0.42 < Y_{FOS} < 0.45). Moreover, for the lyophilized enzymes with additives, most Y_{FOS} values were near or higher than those for the standard undiluted lyophilized enzymes (Table 1). The highest conversions of sucrose (Y_{FOS} > 0.45) were obtained with the lyophilized enzymes (from the undiluted solution) supplemented with: PEG 6000, CMC, ethylene glycol, mannitol, glycerol, trehalose, and ammonium sulfate, all at 2.5% (w/v). Regarding the lyophilized enzyme from a diluted solution, only the addition of trehalose led to an increased Y_{FOS}. Hernalsteens and Maugeri (2008) reported that with free fructosyltransferase under the same synthesis conditions, and with 3 U_{TF}/mL, Y_{FOS} values reached 0.45 after 24 h of reaction. Concerning the FOS specific productivity (Pr_{FOS}), after 24 h of synthesis the lyophilized enzyme

Table 2. Analysis of the residual activity of the stored lyophilized enzyme (R_{6m}^{Lyoph}) after 6 months of storage at -20°C when compared with the enzyme activity at time zero (Table 1) for the lyophilized free fructosyltransferase from *Rhodotorula* sp. LEB-V10. The lyophilized enzymatic activities for the standards without additives after 6 months were 507.72 U_{TF}^{Lyoph} / g for the two conditions: diluted and without dilution.

Lyophilized enzyme from an initial solution without dilution	
Lyophilized Enzyme	R_{6m}^{Lyoph} [a]
Lyophilized without additive	0.66
Ammonium Sulfate (2.5% w/v)	1.37
CMC (2.5% w/v)	0.58
Mannitol (2.5% w/v)	1.03
PEG 6000 (2.5% w/v)	0.45
Sorbitol (2.5% w/v)	1.10
Trehalose (2.5% w/v)	0.80
Xylitol (2.5% w/v)	1.26
Lyophilized enzyme from an initial solution diluted at 1:6 (v/v) in sodium acetate buffer 50 mM at pH 4.5	
Lyophilized Enzyme	R_{6m}^{Lyoph} [a]
Lyophilized without additive	0.82
CMC (2.5% w/v)	0.80
CMC (1.25% w/v)	1.07
Inositol (2.5% w/v)	0.92
PEG 6000 (2.5% w/v)	0.67
Sorbitol (2.5% w/v)	0.79
Lyophilized from an initial enzymatic solution diluted at 1:6 (v/v) in sodium acetate buffer at pH 4.5	
Buffer concentration	R_{6m}^{Lyoph} [a]
100 mM	0.68
200 mM	0.84

$R_{6m}^{Lyoph} = U_{TF}^{Lyoph(t=6m)} / U_{TF}^{Lyoph(t=0)}$

from a diluted solution generally showed higher specific productivities than the lyophilized enzyme from an undiluted solution. Additionally, the additives used with the latter enzyme resulted in higher Pr_{FOS} than the standard undiluted lyophilized enzyme, except for xylitol (Pr_{FOS} = 2.74 $mg/U_{TF}^{Lyoph}.h$) and sorbitol (Pr_{FOS} = 2.40 $mg/U_{TF}^{Lyoph}.h$). Considering the enzymes obtained from a 1:6 diluted solution, the buffer concentration factor (100 and 200 mM) and the addition of PEG 6000, ethylene glycol, sorbitol and trehalose all led to lower Pr_{FOS} when compared to the lyophilized enzymes without additives.

Shelf-life after 6 months

According to the results in Table 1, conditions were selected for 6 month shelf-life evaluations of lyophilized enzymes that presented at least 70% of the total enzymatic activity of the standard lyophilized enzymes,

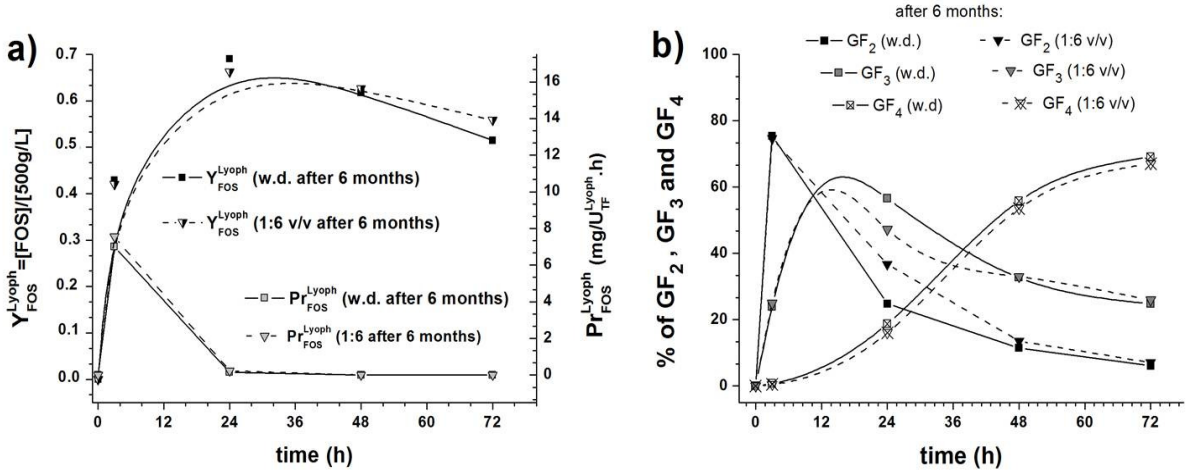

Figure 2a. FOS yield (Y_{FOS}) and FOS specific productivity (Pr_{FOS}), and **b)** percent composition of FOS from syntheses with lyophilized fructosyltransferase without dilution (w.d) (squares and filled lines) and diluted in sodium acetate buffer (50 mM), at a 1:6 (v/v) dilution factor. Syntheses were performed under the following conditions: 50% (w/v) sucrose in 50 mM sodium acetate buffer, pH 4.5, 50°C and 10 U_{TF}^{Lyoph}/mL.

without additives (with and without dilution). The results are shown in Table 2 and evaluated on the basis of residual enzymatic activity, *i.e.,* the maintenance of enzymatic activity compared to its initial activity ($R_{6m}^{Lyoph} = U_{TF}^{Lyoph(t=6m)}/U_{TF}^{Lyoph(t=0)}$), based on Equation 1. Enzymatic activities of the standard lyophilized enzyme without additives (with and without dilution) after 6 months showed the same values of 507.72 U_{TF}^{Lyoph}/g (Table 2), which represent enzymatic activity retentions of 66 and 82%, respectively. Among the other conditions evaluated, the two highest values of lyophilized enzymatic activity were observed with the following conditions: ammonium sulfate (802.11 U_{TF}^{Lyoph}/g) and xylitol (700.98 U_{TF}^{Lyoph}/g) both at 2.5% and without dilution, representing an increase of approximately 37 and 26%, respectively, in enzymatic activity after 6 months. This could be explained by possible water reabsorption during storage, associated with the type of additive that may somehow have given back some flexibility to the molecules, thus increasing activity.

It can be observed in Table 2 that the best results for maintenance of enzymatic activity after 6 months of storage, expressed by the residual storage lyophilized enzymatic activity (R_{6m}^{Lyoph} > 0.9), were obtained mostly using the lyophilized enzyme from an undiluted solution with the additives of ammonium sulfate, xylitol, mannitol and inositol (at 2.5% w/v) and CMC (at 1.25% w/v). The standard conditions showed loss of enzymatic activity after 6 months of storage; the lyophilized enzyme from a diluted solution showed a reduction of 18% in the initial enzymatic activity, and the undiluted lyophilized enzyme had a reduction of 34%.

FOS syntheses were performed with only the two standard lyophilized enzymes (with and without dilution) stored for 6 months in order to separately evaluate the effects of lyophilization and storage on transfructosylation activity. Figure 2a shows the values of FOS yield (Y_{FOS}) and specific productivity of FOS (Pr_{FOS}) for these two conditions. The results were very similar and the enzymatic concentrations used in both syntheses were the same: 10 U_{TF}^{Lyoph}/mL. The two syntheses presented decreasing Y_{FOS} values after 24 h; for the lyophilized undiluted enzyme, at 24 h, Y_{FOS} was 0.69 and with the lyophilized diluted enzyme at 1:6 (v/v), Y_{FOS} was 0.66. Specific productivity of FOS (Pr_{FOS}) showed maximum values in 3 hours for both conditions (around 7.0 $mg/U_{TF}^{Lyoph}.h$). Figure 2 shows that after 6 months of refrigerated storage, the lyophilized enzyme without additives maintained its transfructosylation activity. In both cases large quantities of 1-kestose (GF_2) were hydrolyzed reaching 6 to 7% of total FOS after 72 h; on the other hand, 1^F-fructofuranosyl-nystose (GF_4) showed higher compositions, reaching values up to almost 70% (~182.49 g/L) after 72 h of synthesis for both lyophilized enzymes (Figure 2b). This result has never been previously observed with the fructosyltransferase from *Rhodotorula* sp. LEB-V10 and may be technologically interesting since the larger the FOS molecule, the greater the benefits to the body (Stewart *et al.*, 2008). Therefore, lyophilization of fructosyltransferase from *Rhodotorula* sp. LEB-V10 led to a change in its biocatalytic specificity, in such a way that a significantly change in FOS composition was observed, especially with GF_4. Under optimum conditions with the non-lyophilized

Table 3. Characterization of the lyophilized fructosyltransferase from *Rhodotorula* sp. LEB-V10 with additive formulations. The characterization parameters for 1g of solution and lyophilized powder were: residual lyophilized enzyme activity [R_o^{Lyoph}], thermal residual activity of the lyophilized enzyme [$R^{Lyoph.65°C}$], specific lyophilized enzyme activity [${}^{*}U_{ptn}^{Lyoph}$], FOS yield [Y_{FOS}] and FOS specific productivity [Pr_{FOS}; mg/U_{TF} h], at 72 h of synthesis. FOS syntheses were carried out with 0.02g of the lyophilized enzyme in 5 mL of 50% (w/v) sucrose in 50 mM acetate buffer at pH 4.5. Analyses were performed in duplicate.

Enzyme solution obtained by dilution in sodium acetate buffer 50 mM and pH 4.5 (partially purified FTase)					
Standards non-lyophilized	U_{TF} %/g	$R^{65℃}$ ($U_{TF}^{65°C}/U_{TF}^{o}$)	${}^{*}U_{ptn}$ ($U_{TF}^{o}/mg\ of\ ptn$)	Y_{FOS} - 72h (Eq. 04)	Pr_{FOS} - 72h (Eq. 05)
1:2 (v/v) dilution	179.47 ± 5.98	0.21 ± 0.01	4.77 ± 0.16	0.41 ± 0.01	3.94 ± 0.14
1:6 (v/v) dilution	135.02 ± 4.34	0.18 ± 0.01	5.02 ± 0.05	0.59 ± 0.04	15.95 ± 1.03
Lyophilized enzyme from an initial solution diluted at 1:2 (v/v) in sodium acetate buffer, 200 mM and pH 4.5.					
Lyophilized without additives (standard 1:2)	R_o^{Lyoph} (Eq. 01)	$R^{Lyoph.65℃}$ (Eq. 02)	${}^{*}U_{ptn}^{Lyoph}$ (Eq. 03)	Y_{FOS} (72h) (Eq. 04)	Pr_{FOS} (72h) (Eq. 05)
	6.46 ± 0.15	0.36 ± 4.1E^{-3}	7.54 ± 0.07	0.62 ± 0.02	0.93 ± 0.03
Formulates (1:2) composition (% w/v)	R_o^{Lyoph} (Eq. 01)	$R^{Lyoph.65℃}$ (Eq. 02)	${}^{*}U_{ptn}^{Lyoph}$ (Eq. 03)	Y_{FOS} (72h) (Eq. 04)	Pr_{FOS} (72h) (Eq. 05)
PEG 6000 (1.25%) + ammonium Sulfate (1.25 %) + mannitol (1.25 %)	4.26 ± 0.18	0.33 ± 0.01	4.98 ± 0.37	0.63 ± 0.01	1.43 ± 0.03
PEG 6000 (1.25%) + ammonium sulfate (1.25%) + xylitol (1.25%)	3.19 ± 0.21	0.54 ± 0.10	3.73 ± 0.37	0.55 ± 0.01	1.66 ± 0.04
PEG 6000 (1.25%) + ammonium sulfate (1.25%) + trehalose (1.25%)	3.14 ± 0.36	0.41 ± 0.01	3.65 ± 0.29	0.60 ± 0.06	1.85 ± 0.18
Lyophilized enzyme from an initial solution diluted at 1:6 (v/v) in sodium acetate buffer 200 mM and pH 4.5.					
Lyophilized without additives (standard 1:6)	R_o^{Lyoph} (Eq. 01)	$R^{Lyoph.65℃}$ (Eq. 02)	${}^{*}U_{ptn}^{Lyoph}$ (Eq. 03)	Y_{FOS} (72h) (Eq. 04)	Pr_{FOS} (72h) (Eq. 05)
	3.72 ± 0.07	0.61 ± 0.03	3.64 ± 0.04	0.56 ± 0.01	1.94 ± 0.04
Formulates (1:6) composition (% w/v)	R_o^{Lyoph} (Eq. 01)	$R^{Lyoph.65℃}$ (Eq. 02)	${}^{*}U_{ptn}^{Lyoph}$ (Eq. 03)	Y_{FOS} (72h) (Eq. 04)	Pr_{FOS} (72h) (Eq. 05)
CMC (1.25%) + mannitol (1.25%)	2.32 ± 0.26	0.67 ± 0.14	2.27 ± 0.29	0.56 ± 0.01	3.14 ± 0.02
CMC (1.25%) + xylitol (1.25%)	1.80 ± 0.29	0.92 ± 0.05	1.75 ± 0.20	0.57 ± 4.0.E^{-3}	3.61 ± 0.28
CMC (1.25%) + trehalose (1.25%)	4.76 ± 0.28	0.55 ± 0.04	4.66 ± 0.11	0.57 ± 0.01	4.09 ± 2.0E^{-3}

and immobilized fructosyltransferase, the relative GF_4 fraction was 4% (11.90 g/L) (Aguiar-Oliveira *et al.*, 2012).

Formulations

Formulations with selected additives and new lyophilization conditions were chosen, as shown in Table 3, taking into account the best previous results of residual activities (R_o^{Lyoph}, $R^{Lyoph.65°C}$ and $R_{6\ months}^{Lyoph}$) and FOS yield (Y_{FOS}).

For this set of experiments the 1:2 dilution was chosen for practical reasons, since the undiluted lyophilized cake is hard to dilute, and the 200 mM sodium acetate buffer was selected since it provided one of the highest thermal residual activity and improved shelf-life. Several works reported successful uses of highly concentrated buffers (Roy and Gupta, 2004; Ru *et al.*, 2001; Morgan and Clark, 2004).

CMC and PEG 6000 resulted in good residual lyophilized activities and thermal residual activities, therefore they were selected as bulking agents. Ammonium sulfate was selected since it permitted enhanced thermal residual activity and FOS yield (Table

Table 4. Analysis of residual activity for the stored lyophilized enzyme (R_{6m}^{Lyoph}) after 6 months stored at -20°C, compared with enzyme activity at time zero (Table 3) for the lyophilized fructosyltransferase from *Rhodotorula* sp. LEB-V10 in formulations. The enzyme activity of the standard lyophilized enzyme was 757.14 ± 31.18 U_{TF}^{Lyoph} / g (dilution 1:2) and 431.03 ± 44.36 U_{TF}^{Lyoph} / g (dilution 1:6) after 6 months. The concentration of each additive in all formulations was 1.25 % (w/v).

Lyophilized enzyme from an initial solution diluted at 1:2 (v/v) in sodium acetate buffer 200 mM and pH 4.5.	
Lyophilized Enzymes	R_{6m}^{Lyoph} [a]
Lyophilized without additives	0.65 ± 0.03
PEG + ammonium sulfate + mannitol	0.52 ± 0.02
PEG + ammonium sulfate + xylitol	0.64 ± 0.14
PEG + ammonium sulfate + trehalose	0.77 ± 0.04
Lyophilized from an initial enzymatic solution diluted at 1:6 (v/v) in sodium acetate buffer 200 mM and pH 4.5.	
Lyophilized Enzymes	R_{6m}^{Lyoph} [a]
Lyophilized without additives	0.86 ± 0.08
CMC + mannitol	0.67 ± 0.16
CMC + xylitol	0.90 ± 0.33
CMC + trehalose	0.23 ± 0.02

$R_{6m}^{Lyoph} = U_{TF}^{Lyoph(t=6m)} / U_{TF}^{Lyoph(t=0)}$

1). Mannitol, xylitol and trehalose were also selected because of the positive effects they have on thermal stability, and also because they are reliable cryo-protectants agents and effective in controlling osmotic pressure. Other conditions analyzed are summarized in Table 3. The dilution 1:2 (v/v) in the 200 mM buffer resulted in a residual lyophilized enzyme activity (R_o^{Lyoph}) about six times higher than the non-lyophilized enzymatic solution. This result confirms that highly concentrated protein solutions may promote the formation of reversible agglomerates, resulting in a lower apparent enzymatic activity. None of the lyophilized formulated conditions decreased enzymatic activity in comparison to the standard non-lyophilized solution ($R^{Lyoph65°C}$ < 1.0); on the other hand, the lyophilized enzyme without additives showed lower residual activity (Table 3).

Thermal residual lyophilized activity ($R^{Lyoph65°C}$) showed that the 1:6 (v/v) dilution in 200 mM buffer was more stable than the 1:2 (v/v) dilution in the same buffer (Table 3), and was also more stable than the lyophilized enzyme without dilution (Table 1); this reinforces the need for knowing the best initial dilution rate of the enzyme solution to avoid formation of aggregates. The formulation (CMC + xylitol) at the dilution of 1:6 (v/v) presented the highest thermal stability among all tested conditions ($R^{Lyoph65°C}$ = 0.92), proving that these two additives are, as the literature review affirms, good stabilizers of protein solutions.

The specific lyophilized enzyme activity ($^{*}U_{ptn}^{Lyoph}$) increased by almost 1.6 times with the lyophilization of an enzymatic solution diluted at 1:2 (v/v) with a buffer concentration 4 times greater (200 mM) than the standard buffer (50 mM). Lyophilization of the formulations with enzymatic dilution of 1:6 (v/v) generally displayed the lowest values of $^{*}U_{ptn}^{Lyoph}$.

FOS synthesis with the lyophilized formulates revealed an increase in the conversion of sucrose (Y_{FOS}) from 0.41 to 0.62 for the lyophilized enzymatic solution diluted at 1:2 (v/v), and a reduction in conversion from 0.59 to 0.56 when using the lyophilized enzymatic solution diluted at 1:6 (v/v). With the dilution of 1:2 (v/v), the formulation [PEG + ammonium sulfate + mannitol] exhibited the highest conversion (Y_{FOS} = 0.63) among all conditions analyzed, with a total FOS concentration of 314.16 ± 6.89 g/L after 72 h of synthesis. With the dilution of 1:6 (v/v), the lyophilized formulates from dilutions of

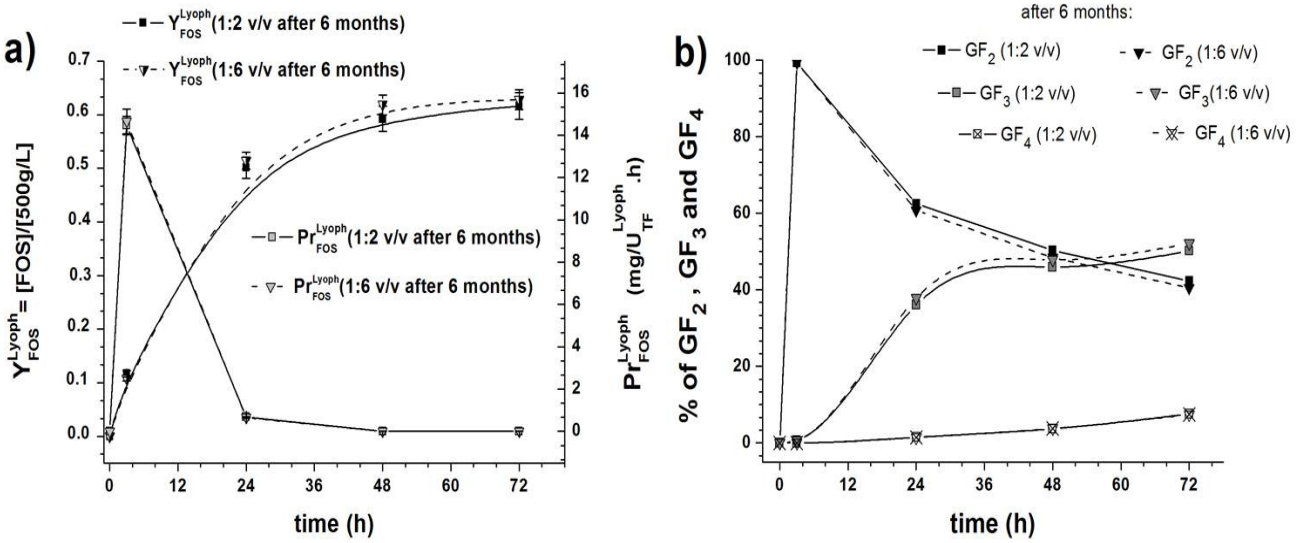

Figure 3a) FOS yield (Y_{FOS}) and FOS specific productivities (Pr_{FOS}), and **b)** FOS percent compositions from syntheses carried out with the lyophilized fructosyltransferase from *Rhodotorula* sp. LEB-V10 at dilution of 1:2 v/v (squares and filed lines) and 1:6 v/v (inverted triangles and dotted lines) in 200 mM sodium acetate buffer at pH 4.5. The lyophilized enzymes were stored at -20°C for 6 months and then FOS syntheses were carried out under at 50% (w/v) of sucrose, in 50 mM sodium acetate buffer, pH 4.5, 50°C and 10 U^{Lyoph}_{TF}/mL.

1:6 (v/v) revealed similar conversions of about 0.57.

Despite the increase in Y_{FOS}, the specific productivity of FOS (Pr_{FOS}) was reduced after lyophilization by about 4 times with the enzymatic solution diluted at 1:2 (v/v) and 8 times at a dilution of 1:6 (v/v). Formulations prepared with a dilution of 1:2 (v/v) displayed Pr_{FOS} values 2 times greater than with the 1:2 dilution of the standard lyophilized enzyme; the same was observed with formulations at a dilution of 1:6. In comparison with the FOS specific productivities with the individual addition of compounds (Table 1), the formulations resulted in lower values (Table 3). As reported by several authors, these results suggest that lyophilization without any additive and in higher dilutions favor the vulnerability of the enzyme, and it may induce changes in its structure that decrease its conversion rate, just like the excessive number of additives in a formulation may equally affect the enzyme. For a better understanding of the effects of lyophilization and formulations, it would be necessary to perform specific studies regarding the impact of lyophilization on the enzymatic molecular conformation, as mentioned by Oetjen and Haseley (2004). According to Kaplan and Taralp (1997), some physicochemical studies show that sometimes the structure after lyophilization can be very similar to that in solution, or can present some limited but reversible conformational changes; however, in several studies it is stated that the majority of enzymes retain their native structure after lyophilization in organic solvents.

Shelf-life after 6 months

The analyses of the lyophilized formulations after 6 months of storage at -20°C followed the same procedures used for the single additives. All lyophilized formulations shown in Table 3 were evaluated according to their residual storage activity (R^{Lyoph}_{6m} **)**, as presented in Table 4. The standard lyophilized material without additives, diluted in 200 mM acetate buffer at 1:2 and 1:6 (v/v), showed a reduction in enzymatic activity of about 35 and 14%, respectively. Considering the 1:6 dilution in acetate buffer at 50 mM (Table 2) and 200 mM (Table 2 and 5), it can be observed that the residual activity of the stored lyophilized enzyme increased by about 5% after 6 months. Among the formulations with the enzyme diluted at 1:2, only the formulation containing [PEG + ammonium sulfate + trehalose] showed a slightly smaller loss of activity (R^{Lyoph}_{6m} = 0.77) compared to the lyophilized enzyme without additives and diluted at 1:2; among the formulations with the enzyme diluted at 1:6 (v/v), that containing [CMC + xylitol] maintained the highest

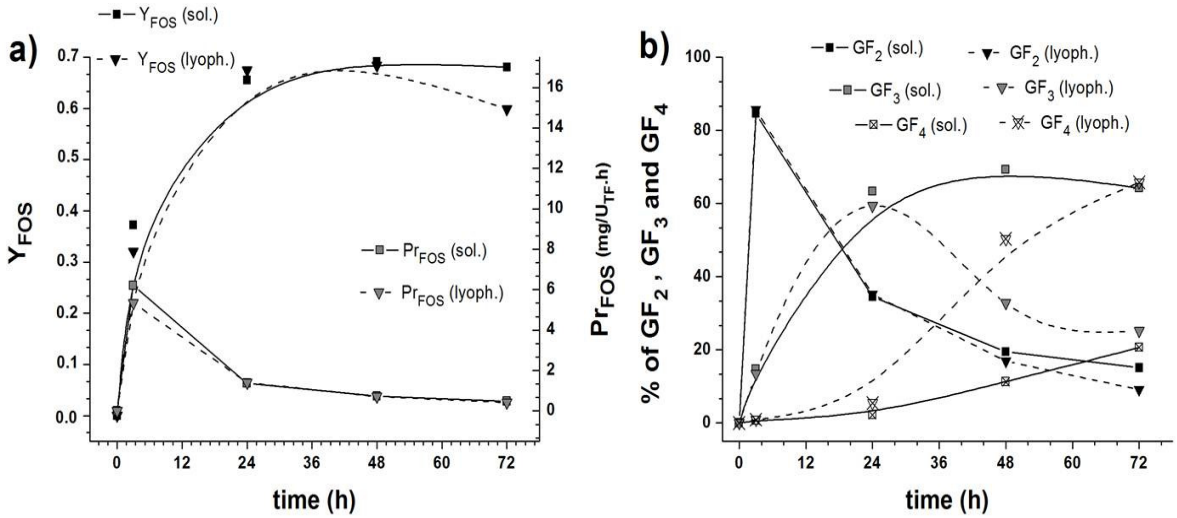

Figure 4. FOS syntheses as a function of reaction time: **a)** FOS yield (Y_{FOS}) and specific productivity of FOS (Pr_{FOS}), and **b)** percent compositions of FOS with non-lyophilized fructosyltransferase from *Rhodotorula* sp. LEB-V10 (squares and filed lines) and lyophilized at a 1:2 dilution ratio, in 200 mM sodium acetate buffer, pH 4.5 (inverted triangles and dotted lines). The syntheses were performed under optimized conditions: 48°C, 10 U_{TF}/ml, 50% (w/v) of sucrose in 50 mM sodium acetate buffer, pH 6.0.

enzymatic activity (R_{6m}^{Lyoph} = 0.90).

The standard lyophilized enzyme (without additives) shown in Table 4, diluted in sodium acetate buffer (200 mM and pH 4.5) at 1:2 and 1:6 (v/v), were used in FOS syntheses to evaluate their biocatalytic activity after 6 months of storage: the enzyme activities were 10 $U_{TF}^{Lyoph.}/mL$. Figure 3 shows the results for Y_{FOS} and Pr_{FOS} along with the percent compositions of each FOS. Again, the two conditions of lyophilized enzymes showed very similar Y_{FOS} and Pr_{FOS} profiles over time; the Y_{FOS} values increased over time, reaching 0.62 (dilution 1:2) and 0.63 (dilution 1:6) at the end of 72 h, and the Pr_{FOS} reached its maximum at 3 h (~14 mg/U_{TF}.h). The specific productivity of the lyophilized enzyme diluted in 50 mM buffer (Figure 2.a) and 200 mM buffer (Figure 3a) revealed that with a higher concentration of the buffer Pr_{FOS} values doubled in the first hours of synthesis; sucrose conversion values (Y_{FOS}) did not reach a maximum value as shown in Figure 2. Syntheses carried out with the standard lyophilized enzyme diluted at 1:2 and 1:6 (v/v) showed much lower GF_4 compositions (less than 8% after 72 h in both cases), in which nystose (GF_3) was the predominant FOS component, consisting of nearly 50% of the global composition (Figure 3b).

Effect of a pre-activation of the enzyme prior to lyophilization

In a previous study, Aguiar-Oliveira and Maugeri (2011) reported that when the immobilized fructosyltransferase was incubated at pH 4.5 and 6.0 for 15 min at 52°C and 60°C, and when the free fructosyltransferase was incubated at pH 4.5 and 52°C followed by a 5 min ice bath, different levels of enzymatic activation were obtained. Thus, pre-incubation of the free enzyme at both temperatures (52 and 60°C) prior to lyophilization was performed in order to determine the effect of such a treatment on the lyophilized material. An enzymatic solution of 107.06 ± 4.00 U_{TF}/g in acetate buffer, 50 mM and pH 4.5, was used for lyophilization without pre-incubation (standard condition) and with pre-incubation at 52 and 60°C.

The results showed that pre-incubation at 52°C reduced the residual activity of the lyophilized enzyme (R_o^{Lyoph}) to 0.56 ± 0.09, and interestingly at 60°C this value increased to 1.43 ± 0.18, which is a peculiar result since the 60°C treatment led to the activation of only the immobilized form (Aguiar-Oliveira and Maugeri, 2011). However, with respect to thermal residual activity of the lyophilized enzyme ($R^{Lyoph.65°C}$) and FOS yield (Y_{FOS}), the results confirmed that pre-activation followed by lyophilization of the enzyme is not a good methodology: $R^{Lyoph.65°C}$ was 0.17 at 52°C and 0.06 at 60°C, and Y_{FOS}

values were 0.39 and 0.02, respectively. The pre-activation previously observed (Aguiar-Oliveira and Maugeri, 2011), suggests a momentary alteration on the conformational structure of the molecule and/or on its energetic levels.

Effect of Lyophilization on FOS Synthesis

In previous studies, (Aguiar-Oliveira *et al.*, 2012) optimized FOS synthesis with the immobilized fructosyltransferase, whose optimal conditions are: 48°C, 20 U_i/mL, 24 h and 50% of sucrose in 50 mM sodium acetate buffer and pH 6.0. The performance of free fructosyltransferase was also evaluated under these same conditions, except for the enzymatic concentration which was 10 U_{TF}/mL. Therefore, in this work FOS was produced under the conditions mentioned above with non-lyophilized enzymes, the latter at a 1:2 dilution ratio in 200 mM sodium acetate buffer at pH 4.5. The results are shown in Figure 4.

In Figure 4.a it can be observed that the lyophilized enzyme led to Y_{FOS} values of 0.68-0.69 for reaction times between 24 and 48 h, followed by gradual decreases; for the non-lyophilized enzyme similar Y_{FOS} values were obtained over a longer reaction time, between 48 and 72 h. However, the specific productivities are quite similar in both cases.

Regarding the percent composition of FOS (Figure 4.b) there is a significant difference, since with the exception of GF_2 contents, the compositions changed at the end of the process more drastically for GF_4, where the lyophilized enzyme led to about 67% of the total FOS, nearly 3 times more than the non-lyophilized enzyme.

Studies conducted with a fructosyltransferase from *Bacillus macerans* EG 6 (Taralp and Kaplan, 1998) demonstrated that this enzyme was able to produce only the GF_4 from 500 g/L of sucrose, reaching a maximum of 45.2% of GF_4 after 48 h of synthesis. Borole and Davison (2008) observed a 6 to 7-fold increase in the initial rate of conversion with a salt-lyophilized lipase used in an organic medium. Moreover, Wang and Wei (2007) also obtained the same results with a lyophilized lipase in the presence of certain additives, showing increases ranging from 1.2 to 1.7 times the standard condition.

CONCLUSIONS

Lyophilization of the free fructosyltransferase partially purified from *Rhodotorula* sp. LEB-V10 proved to be an appropriate methodology for acquiring a stable enzyme with good transfructosylation activity and increased enzymatic activity and thermal stability. The density of the initial enzyme solution proved to be an important factor to take into consideration, since the proposed biotechnological application of this enzyme is in a partially purified form, thus the ideal dilution of the starting enzymatic solution is important in order to avoid hard enzyme agglomerates after lyophilization. The more liquid and uniform the appearance of the starting enzymatic solution, the better the characteristics of the lyophilized cake, making it easier to obtain a homogeneous powder which facilitates reconstitution. Many authors suggest that the formulations for lyophilization should be kept as simple as possible, and that the addition of single cryoprotectant compounds allow the maintenance of enzymatic activity for up to 6 months, better than in formulations. Changing the concentration of the buffer usually employed with fructosyltransferase from *Rhodotorula* sp. LEB-V10 to a value 4 times higher also proved to be favorable since more concentrated buffers are commonly utilized in protein lyophilization.

Lyophilization of the free fructosyltransferase did not affect its transfructosylation activity or production of fructooligosaccharides, where this technique positively contributed to the industrial applications of the enzyme by facilitating its transport and storage, without affecting its functionality. Although a reduction in enzyme activity after lyophilization was observed, the final lyophilized powder still presents advantages including increased enzyme activity per weight and reduced volume. Additionally, there was a significant change after lyophilization in which there was an increase in 1^F-fructofuranosyl-nystose (GF_4) composition with only the lyophilized enzyme of between 67 and 69% of the total FOS compared to the typical compositions of 10 to 20% with the non-lyophilized enzyme. The best results of FOS production were observed with the additives: PEG 6000, ethylene glycol, ammonium sulfate, CMC and mannitol; for the lyophilized enzyme with additive formulations, the best result was obtained with a mixture of PEG, ammonuim sulfate and mannitol.

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Nomenclature

Aw	Water Activity
CMC	Carboxymethil cellulose
F_{UTF}	Activity loss factor
FOS	Fructooligosaccharides
[FOS]	FOS Concentration (g/L)
FTase	Fructosyltransferase
M_o	Water Layer
PEG	Poly(ethylene glycol)
R_{6m}^{Lyoph}	Residual Activity of the Stored Lyophilized Enzyme after 6 months
R_o^{Lyoph}	Residual Activity of the Lyophilized Enzyme
$R^{Lyoph.65°C}$	Thermal Residual Activity of the Lyophilized Enzyme
$U_i^{Lyoph.65°C}$	Immobilized Activity of the Lyophilized Enzyme after 15 min at 65°C
U_i^{Lyoph}	Activity of the Lyophilized Enzyme (µmol/min)
U_i^o, U_i	Initial or Non-Lyophilized Enzyme Activity (µmol/min)
${}^*U_{ptn}^{Lyoph}$	Specific Activity of the Lyophilized Enzyme (U/mg)
Y_{FOS}	FOS yield
ρ	Density (g/mL)