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Co-immobilization of dextransucrase and dextranase in epoxy-agarose-tailoring oligosaccharides synthesis

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ABSTRACT

The best support reported for dextransucrase (DS) and dextranase (DN) immobilization was Eupergit C, but this carrier was discontinued and is no longer available in the market. Thus, epoxy-agarose support is suggested as an alternative to co-immobilize DS from *Leuconostoc mesenteroides* B-512F and DN from *Chaetomium erraticum*. The co-immobilization approach improved DS performance, compared to immobilized DS, due to the removal of the dextran layer accumulated on DS by DN, and enhanced both enzymes catalytic activities under a wide range of pH and temperature. Since DS and DN have the same optimum pH and temperature, the co-immobilization was done in a single step. The EPA-DS-DN0.5 (co-immobilized DS (26.16 UI) and DN (2.76 UI) in epoxy-agarose) showed the highest recovered activity (59.54%). The biocatalyst was stable at 4 °C, retaining above 70% of activity for 60 days and was efficient producing oligosaccharides, yielding 12.68 ± 0.09 g/L of oligosaccharides with a degree of polymerization up to 5.

1. Introduction

Dextransucrase (DS) from *Leuconostoc mesenteroides* is a glucosyltransferase that synthesizes dextran using sucrose as substrate releasing fructose as a byproduct. Dextran has many applications in food and nonfood industries such as texture modifier in foods and blood plasma substitute in medicine [1]. This enzyme can synthesize oligosaccharides in the presence of acceptors, such as maltose. DS polymerizes the glucosyl units by α-1,6 glucoside linkages, which makes the oligosaccharides synthesized by DS resistant to the digestive enzymes bringing benefits to the human body because beneficial bacteria consume them in the human colon [2–4].

Many studies evaluated the immobilization of enzymes aiming cost reduction of enzyme-based processes. Enzyme immobilization facilitates the catalyst recovery, increases the stability against adverse conditions (pH and temperature), and facilitates the reuse of the enzymes [5,6]. Several authors reported that DS immobilization is a difficult task because of its large size (a 180 kDa trimmer with three subunits of 60 kDa each) and due to its attached dextran chain, which covers its reactive group. The synthesis of oligosaccharides by DS follows the acceptor reaction mechanism, where glucosyl moieties are deviated from the dextran chain, minimizing dextran formation without avoiding it. Dextran chains grow at low rates while oligosaccharides are synthesized, but its formation rate does not halt. The acceptor reaction mechanism is well described elsewhere [18,19].

Alginate entrapment was considered an alternative to high immobilization yield, good operational stability, and production of prebiotic oligosaccharides at bench scale. The co-immobilization with dextranase (DN) minimizes the accumulation of dextran in the alginate capsule [12–17]. Even using DN, diffusional problems occur due to the entrapment technique, making it not suitable for processes involving high molecular weight substrates and products because of the mass transfer limit through the entrapment matrix. After prolonged use, the dextran formed during the synthesis of oligosaccharides accumulates in the alginate beads causing their rupture [7–11]. Furthermore, the lack of mechanical stability of the beads does not allow their employment in continuous reactors [9,20–22].

Covalent immobilization is another approach widely reported for enzyme immobilization. This technique is a good strategy to prevent enzyme leakage and to improve the stability of monomeric and multimeric enzymes, mainly due to multipoint attachments [23,24]. Covalent bonds can immobilize DS in alkylamine activated porous silica, porous silica, amino-spherosil, chitosan functionalized with glutaraldehyde, and Eupergit C [1,3,25,26]. However, due to dextran chain

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covalently attached to DS, those protocols, except for Eupergit C resulted in low immobilization yields [10,12,26,27]. Some authors studied the rupture of the dextranosyl-enzyme complex by dextranolytic action of dextranases, which makes the amino acids residues on enzyme surface more accessible, allowing immobilization by covalent bonds, which promote high rates of immobilization and operational stability [1,3,21,25,26].

The co-immobilization technique with DN has the advantage of synergic effect in oligosaccharides production. DS and DN have similar features that allow its co-immobilization onto the same support, such as optimum pH and temperature [1,13,28–30]. Therefore, epoxy-activated supports can be an interesting strategy to immobilize both enzymes because, among the previously reported supports for DS and DN immobilization, Eupergit C yielded the most successful covalent co-immobilization. Dextran-free DS was immobilized onto Eupergit C 250 L presenting 22% of recovered activity [25]. Parlak et al. [1] developed a bio-engineering study, that removed the dextran and fused the DS from *L. mesenteroides* B-512 F to glutathione S-transferase, truncated in both terminals and immobilized on Eupergit C 250 L, resulting in a recovered activity of 83.3%. This support also presented good results for DN with a 90% immobilization yield and the biocatalyst being able to synthesize isomaltooligosaccharides [15,28,30].

Eupergit C is an epoxy support with many epoxy groups where proteins might be immobilized. The Eupergit C reactive groups enable reactions with different nucleophilic groups on the protein surface (e.g., amino and carboxylic acids) to form extremely strong linkages (secondary amino bonds, ester, and ether bonds) with minimal chemical modification of the protein [31–34]. Eupergite C was reported as a suitable support for enzyme immobilization at any pH value, which allows the immobilization at pH of maximum activity or stability of the enzyme preventing its denaturation [15,25,29]. However, Eupergit C is no longer available in the market, because of the discontinuation of its production. Thus, new strategies to immobilize DS are necessary, as the other alternatives reported in the literature are less efficient than Eupergit C.

Epoxy-agarose based supports might offer similar characteristics of Eupergit C presenting good immobilization yields for different enzymes, whereas, being able to immobilize under mild conditions (e.g., low ionic strength and low temperatures) [31,35–37]. Agarose is an inert matrix, approved for food industry use and classified as Generally Recognized as Safe (GRAS), hydrophilic, resistant to mechanical stirring and available with a variety of pore size [38,39]. For industrial applications, an effective immobilization method is required to ensure continuous processing and biocatalyst reuse [40]. Activated supports with epoxy groups are suitable to develop secure protocols for DS and DN co-immobilization.

This study evaluated the co-immobilization of DS and DN onto epoxy-agarose tailoring oligosaccharides synthesis. Previously published studies did not report the separate activity of co-immobilized DN and DS. Usually, only the DS activity is reported, by measuring the amount of fructose released, despising the DN activity in the system. This work measured the activities of both enzyme by the amount of fructose (DS activity), and the amount of glucose (DN activity) released, aiming to design a biocatalyst containing co-immobilized DS and DN onto epoxy-agarose support and evaluated its use on oligosaccharides synthesis.

2. Materials and methods

2.1. Materials and chemicals

L. mesenteroides NRRL B-512 F DS was obtained in our laboratory as previously described [41]. All the chemicals and reagents were of analytical grade. Thin layer chromatography (TLC) silica gel aluminum sheets (plate number 105553) were obtained from Merck. DN from *Chaetomium erraticum* (D0443) was purchased from Sigma-Aldrich and agarose (Sepharose CL-6B) from GE Healthcare.

2.2. Enzyme activity determination

DS activity was determined by quantification of the fructose released from sucrose in the enzyme reaction where a sucrose solution (10% w/v) dissolved in 20 mM sodium acetate buffer containing 0.05 g/L of $CaCl_2$ at pH 5.2 is the substrate. DN activity was determined by quantification of the glucose released due to the dextran hydrolysis. The substrate was dextran 1% (w/v) in the same buffer. The molar mass of dextran used was in the range 450–650 kDa. The enzymes were then incubated in their respective substrate at 30 ∘C for 10 min. The amount of fructose or glucose, corresponding DS and DN respectively, released was determined by quantifying the reducing sugar by the DNS method [42]. The results were expressed as IU/mL (free enzyme) or IU/g catalyst (immobilized enzyme). The international unit activity (IU) is defined as the amount of enzyme that releases 1 μmol of fructose (DS) or glucose (DN) per minute at the assay conditions. Protein determinations were carried out using the Bradford method with bovine serum albumin as the standard [43].

2.3. Epoxy-agarose support preparation

The epoxy-agarose (EPA) carrier was obtained as previously reported [44]. Agarose beads 6BCL (10 g) were washed with abundant distilled water. The water was removed using a vacuum filtration system. The agarose beads were kept under agitation in a solution containing 44 mL of water, 3.28 g of NaOH, 0.2 g of $NaBH_4$, 16 mL of acetone and 11 mL of epichlorohydrin. After 16 h, the suspension was washed with an excess of water using a vacuum filter system.

2.4. Determination of epoxy groups

The number of epoxy groups was determined as described in previous works [44]. The epoxy groups (1 g of support) were hydrolyzed by 10 mL of H_2SO_4 0.5 M for 2 h. The hydrolyzed support was oxidized with $NaIO_4$ [45]. Agarose beads 6BCL with the epoxy groups (non-derivatized) were used as a control. The number of epoxy groups was calculated by the difference of $NaIO_4$ consumption between the epoxy-agarose support and non-modified support (control), determined through titration with sodium thiosulfate.

2.5. Fourier Transform Infrared (FTIR) spectroscopy

Fourier-transform infrared spectroscopy was applied to investigate the functional groups in the supports (agarose, epoxy-agarose, Eupergit CM, and biocatalysts). The samples were lyophilized and analyzed using an FTIR CARY 630 (Agilent Technologies). The sample was placed directly over the reading cell because this equipment does not require any sample preparation. The IR spectra were collected at wavelengths from 900 to 4000 cm^{-1} at a spectral resolution of 1 cm^{-1}.

2.6. Co-immobilization of dextransucrase and dextranase in epoxy-agarose support

The enzyme solution was prepared by the addition of different amounts of DN: 0 (control, without DN), 0.5 μL (2.76 IU), 2.5 μL (13.80 IU), and 4.5 μL (24.84 IU), corresponding to the biocatalysts EPA-DS-DN0.5, EPA-DS-DN2.5, and EPA-DS-DN4.5 respectively, in 1 mL (26.16 IU/mL) of the DS enzyme. In this step, the enzymes were carefully mixed to promote the endogenous dextran hydrolysis by DN. Thus, it was added to 4 mL of sodium acetate buffer (20 mM) containing 0.05 g/L of $CaCl_2$ at pH 5.2. The co-immobilization was carried out adding 1 mL of the enzyme solution into the epoxy-agarose support. The amount of protein was 12.76 μg, 13.50 μg, 15.03 μg and 17.23 μg of protein for biocatalysts EPA-DS, EPA-DS-DN0.5, EPA-DS-DN2.5, and

EPA-DS-DN4.5, respectively. The mixture was incubated for 24 h at 4 °C with a tube rotating mixer. The immobilization yield, the immobilization efficiency, and the recovered activity were calculated according to the Eqs. (1)–(3) [46]. The immobilized activity was calculated from the difference between the initial and final enzyme activity (U/mL) in the supernatant (theoretical immobilized activity). The starting (or initial) activity was the enzyme activity offered to the support at the beginning of the immobilization process. The observed activity was the activity of the immobilized enzyme (experimentally determined in the immobilizate).

$$Immobilization\ yield\ (\%) = \frac{Immobilized\ activity}{Starting\ activity} x\ 100 \quad (1)$$

$$Immobilization\ efficiency\ (\%) = \frac{Observed\ activity}{Immobilized\ activity} x\ 100 \quad (2)$$

$$Recovered\ activity\ (\%) = \frac{Observed\ activity}{Starting\ activity} x\ 100 \quad (3)$$

2.7. Effect of enzyme load on the immobilized biocatalysts

The effect of enzyme load on the immobilized biocatalysts was investigated by incubating the enzyme solution, containing 13.50 μg of protein/mL (DS + DN). The immobilization was carried out in a single step with an enzyme solution containing DS (1 mL, 26.16 IU), DN (0.5 μL, 2.76 IU) mixed with 4 mL of sodium acetate buffer (20 mM with 0.05 g/L $CaCl_2$; pH 5.2). The enzyme solution was added to different amounts of epoxy support (100 mg, 200 mg, 300 mg, 400 mg, and 500 mg). Immobilization was carried out for 24 h under gentle agitation on a tube rotating mixer at 4 °C. After this period, the enzyme activity was performed as described in Section 2.2.

2.8. Temperature and pH effect on the enzyme activity

The temperature effect on the enzyme activity was measured in the range of 15 °C to 40 °C using acetate buffer (20 mM with 0.05 g/L $CaCl_2$; pH 5.2). The pH effect was evaluated ranging from 4.5 to 7.0 (sodium acetate buffer 20 mM from 4.5 to 5.5 and phosphate buffer 20 mM to 6.0 to 7.0, both containing of 0,05 g/L of $CaCl_2$) at 30 °C. Also, both pH and temperature were evaluated by the fructose and glucose released under different amounts of DN enzyme: 0.5 μL (2.76 IU), 2.5 μL (13.80 IU) and 4.5 μL (24.84 IU). The results were expressed as relative activity, calculated as the ratio between the activity at each temperature or pH and the maximum obtained. The fructose and glucose were quantified by TLC as described further on.

2.9. Thin layer chromatography analyses

2.9.1. Thin layer chromatography: simple sugars

The sugars obtained in the enzyme activity determination assay were quantified by TLC. This analysis was carried out using silica gel 60 plates irrigated for three ascents in a solvent mixture composed of acetonitrile: water (85:15, v/v). After each ascent, the plates were dried using a hairdresser. A suitable aliquot (2 to 7 μL) of the sugar mixture was sprayed on the plate by an automatic TLC Sampler (ATS 4, Camag) with band length 6.0 mm, with 15.0 mm of distance from left edge and 8.0 mm distance from the lower edge. The sugars (fructose and glucose) were visualized by dipping the plate into a solution containing 0.3% (w/v) of 1-naphthyl ethylenediamine dihydrochloride in methanol with 3% (v/v) sulfuric acid using the Chromatogram Dipping Device III (Camag). The plate was then dried at ambient temperature and heated at 95 °C for 10 min [47]. The sugar quantification was done by densitometry as further described.

2.9.2. Thin layer chromatography: oligosaccharides

The oligosaccharides were determinate by TLC. The analysis was carried out using silica gel 60 plates irrigated for two ascents in a solvent mixture composed of acetonitrile/ethyl acetate/1-propanol/water (85:20:50:90, v/v). After each ascent, the plates were dried using a hairdresser. A suitable aliquot (5 μL) of the sugar mixture was sprayed on the plate by an automatic TLC Sampler (ATS 4, Camag) with band length 6.0 mm, with 15.0 mm of distance from left edge and 8.0 mm distance from the lower edge. The oligosaccharides were visualized by dipping the plate into a solution containing 0.3% (w/v) of 1-naphthyl ethylenediamine dihydrochloride in methanol with 3% (v/v) sulfuric acid using the Chromatogram Dipping Device III (Camag). The plate was then dried at ambient temperature and heated at 95 °C for 10 min [47].

2.10. Densitometry analyses

The content of glucose, fructose and oligosaccharides were determined by densitometry analysis using a TLC scanner 4 (Camag) at a wavelength of 490 nm, slit dimension 5.00 × 0.30 using the peak area for quantification. Data was acquired and handling using the winCATS software (Camag). Calibration curves of glucose and fructose were built to quantify the sugars released. The oligosaccharides were quantified using the glucose as indirect standard [3,47].

2.11. Operational and storage stability of co-immobilized enzymes

The operational stability of EPA-DS-DN0,5 and Eupergit CM was assayed in subsequent cycles at 25 °C in a medium containing sucrose (10% w/v) and sodium acetate buffer (20 mM with 0.05 g/L CaCl2; pH 5.2). After 10 min of reaction, the immobilized enzymes were separated by centrifugation (4025 x *g*, 2 min, 25 °C), washed with sodium acetate buffer and then the substrate was replaced. The process was repeated before each new cycle. The reaction was conducted as described in Section 2.2.

The storage stability of the biocatalyst EPA-DS-DN0.5, containing both enzymes (13.50 μg of protein/mL related to DS 1 mL, 26.16 IU/mL and DN 0.5 μL, 2.76 IU) at pH 5.2 was investigated. For that, the co-immobilized enzymes were stored at 4 °C for 60 days, and the enzymatic activity was determined every seven days.

2.12. Production of oligosaccharides using free and co-immobilized enzymes

Oligosaccharides synthesis was carried out using DS and DN free and co-immobilized in epoxy-agarose (1 IU/mL, 0.9 IU/g). The amount of maltose (45 g/L) and sucrose (40 g/L) used was already selected as optimum conditions to produce oligosaccharides according to a previously published work [48]. The synthesis was carried out during 6 h, at 25 °C and pH 5.2. The synthesis was also carried at low temperature (4 °C) to evaluate the temperature effect on the enzyme selectivity as previously reported [49]. Afterward, the samples were analyzed by TLC plates.

2.13. Statistics

All assays were carried out in triplicate. Tukey's test was used to determine the significant differences among the values, when appropriate, at 95% of confidence level using STATISTICA software (Statsoft v 13.0). The results reported are the average ± standard deviation.

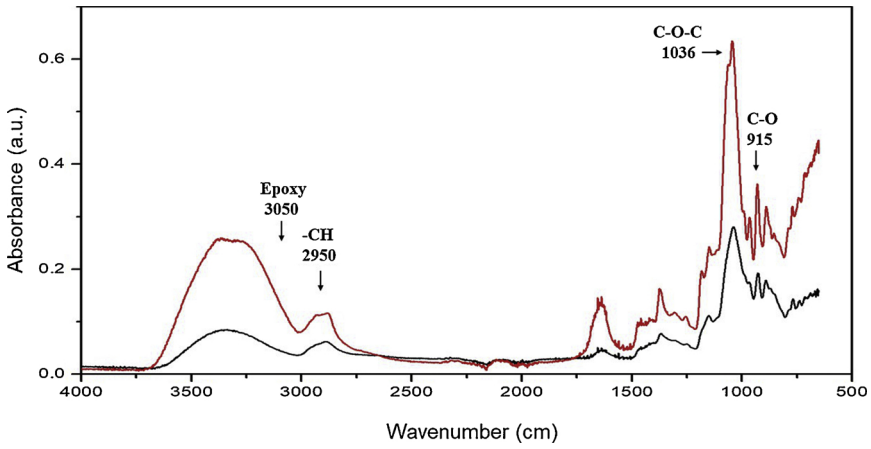

Fig. 1. Infrared spectrum of unmodified agarose () and epoxy-agarose support ().

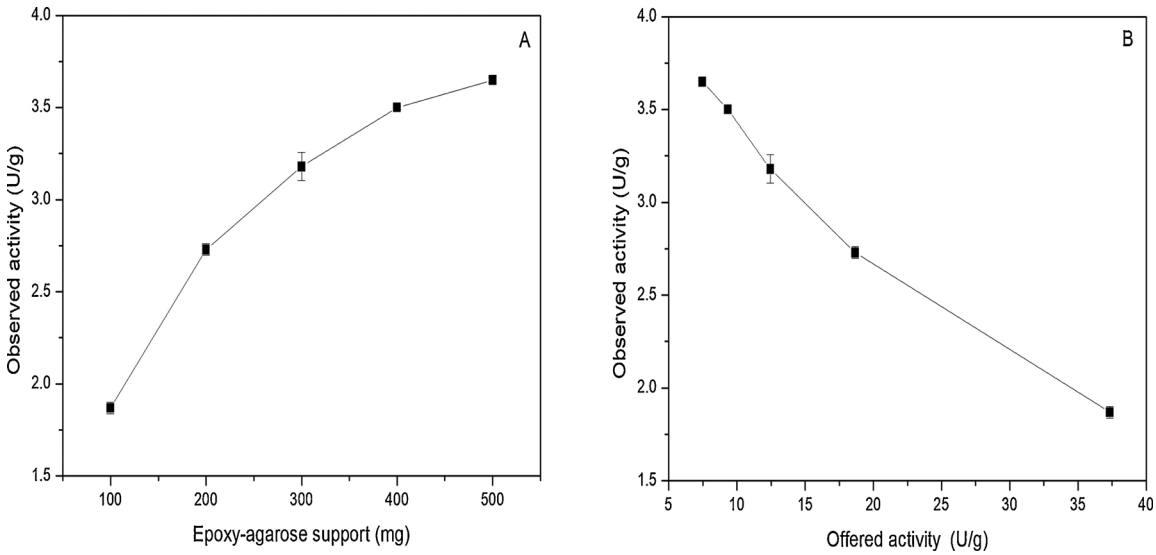

Fig. 2. Effect of the co-immobilization onto epoxy-agarose support on the dextransucrase observed activity (U/g) related to (A), amount of epoxy-agarose (mg) and (B) offered activity (U/g). Enzyme solution containing 13.50 μg of protein/mL (DS 26.16 IU/mL + DN 0.5 μL, 2.76 IU) was evaluated with different amounts of epoxy-agarose support (100–500 mg). Results are presented as mean ± SD (n = 3).

3. Results and discussion

3.1. Characterization of the epoxy-agarose support

3.1.1. Infrared spectrum of the epoxy-agarose support

Agarose has been used as a matrix in immobilization protocols due to its surface hydroxyl groups, which can be modified to generate reactive groups. The characterization of the support was carried out based on the infrared spectrum (FTIR) of agarose and epichlorohydrin modified agarose. The characteristic peaks of agarose are 3481 cm^{-1} (hydroxyls), 1078 cm^{-1} (glycosidic bonds) and 931 cm^{-1} (vibration of C—O—C bridge of 3,6-anhydro-L-galactopyranose) [50].

Fig. 1 shows the bands related to epoxy groups, such as the band at 3050 cm^{-1} (C–H epoxy ring). Despite being very close to the strong absorption band of the hydroxyl group (≈ 3500 cm^{-1}), this band is an indication of the presence of epoxy groups in the molecule. Epoxy peaks were also observed at 915 cm^{-1} (C–O of the epoxy) and 1036 cm^{-1} (C–O-C) [51]. The peak at 2950 cm^{-1} that corresponds to carbonic chains has also increased after epoxidation indicating the formation of glyceryl groups during the activation process of the epoxy-agarose support [44].

3.1.2. Quantification of the activated epoxy groups

The number of activated epoxy groups was determined because the number of epoxy groups on the surface of the support may affect the stabilization and immobilization of the enzyme [52]. The epoxy-agarose support had 45.5 ± 3.5 μmol of epoxy groups/g. The literature reports that the number of epoxy groups varies in the range of 10–60 μmol of epoxy groups/g of support depending on the amount of cross-linked agarose and on the reactions conditions, such as alkaline pH and epichlorohydrin content [31,53].

3.1.3. Effect of enzyme load on the immobilized enzyme activity

To define the amount of epoxy-agarose support, the enzyme solution containing 13.50 μg of protein/mL (DS 26.16 IU/mL + DN 0.5 μL, 2.76 IU) was added to different amounts of epoxy-agarose (100–500 mg). The immobilization process was carried out at pH 5.2 because under this pH the covalent reaction, by the nucleophilic attack, was oriented through the region rich in carboxylic acids to form ester bonds [25,30,54]. DS from *L. mesenteroides* B-512 F has a significant number of asparagine and glutamate amino acids leading to 216 carboxylic groups/mol of protein, which allows the formation of ester bonds [55].

The enzyme solution offered to the support contained a mixture of DS and DN as described earlier. The enzyme activity load was constant

because the dextran layer removal by DN affects the DS activity. Thus, the same enzyme solution was offered to different amounts of support. This strategy was applied before for the immobilization of DS and DN [1,28,56]. The observed DS activity increased with the amount of the support offered (Fig. 2A). At low support amounts, the reactive groups onto the support are quickly saturated, becoming unavailable to interact with the enzyme. The increase in the amount of support provides more reactive groups favoring the interaction enzyme-support and, allowing a better conformation arrangement due to a higher superficial area. The observed activity (3.65 U/g) using 500 mg of support was only 14% higher than the activity (3.18 U/g) obtained using 300 mg of support. Thus, 300 mg of epoxy support was chosen for further experiments, which corresponds to an offered activity of 12.45 U/g. Fig. 2B shows the observed activity as a function of the offered activity (U/g of support). The observed activity decreased with the increase in the enzyme load (U/g of support). The saturation phenomena pronounced with the increase in the offered activity per mass of support. The results presented in Fig. 2B show that the highest observed activities were obtained using 12.5 U/g, which corresponds to 300 mg of support.

3.2. Characterization of the biocatalyst

The enzyme activity measured by the 3,5-dinitrosalicylic acid method and reported as total reducing sugars (glucose and fructose) released is a usual procedure, even for co-immobilized DN and DS. However, this methodology overestimates the DS activity because fructose is released only when DS breaks the sucrose linkage while the glucose moieties are polymerized into dextran [53]. Dextransucrase also presents a hydrolytic activity which might result in some glucose release [54]. On the other hand, DN breaks the dextran chain releasing glucose from the polymer. In this study, the activity of dextransucrase and dextranase was individually determined by densitometry, allowing the quantification of carbohydrates in the nanogram scale after TLC separation. Sucrose was the substrate because the objective was to evaluate the DN activity on the dextran formed during the activity assay. The amount of fructose released was higher than glucose. The DS activity was higher than the DN activity because of the release of fructose due to the sucrose cleavage by the DS activity. This result was expected because of the higher amounts of DS offered to the support for immobilization.

3.2.1. Co-immobilization parameters

Table 1 shows the immobilization parameters obtained offering different amounts of DN and a fixed amount of DS to the epoxy-agarose support. The results were statically significant ($p < 0.05$) for all parameters, except for the immobilization yield of EPA-DS-DN0.5 and EPA-DS-DN2.5. The DS immobilized without DN (EPA-DS) presented the highest immobilization yield (21.50%) but the recovered activity (39.62%) and the immobilization efficiency (184.44%) were the lowest among the studied biocatalysts. Enzyme denaturation during the immobilization process and the presence of a dextran layer that covers the enzyme and blocks the active site explains this result [1].

DS co-immobilized with DN in epoxy-agarose resulted in immobilization efficiencies higher than 100%. The DN action on the dextran layer increased the compatibility between the enzyme and the support due to the greater access to the active site of the enzyme (Table 1). The enzyme immobilization can increase the enzyme activity by changing the protein conformation for a more reactive or more stable form. The immobilization may also improve the enzyme rigidity enhancing its stability. The rigidification in some protein areas causes distortions that may change the enzyme properties related to enzyme activity, selectivity, and specificity [6, 23 54]. In the present study, immobilization efficiency higher than 100% were obtained, which means that the enzyme was hyperactivated in the derivate. The DS catalytic activity also increased because of the addition of Ca^{2+} ion to the buffer solution, which when associated with the active center of the DS from *L. mesenteroides* B512 F increases its catalytic activity [3,57]. The presence of calcium stabilizes the 3D structure of the enzyme, which may have generated this high efficiency in the biocatalysts in synergy with the dextran removal by DN [58,59]. Dextran-free DS immobilized in activated-chitosan resulted in an immobilization efficiency of 11%. However, this latter study did not use calcium ion in the immobilization buffer [21].

This study demonstrated that the use of DN improved DS immobilization (Table 1). Immobilized DS (EPA-DS) presented the lower recovered activity (39.62%) while co-immobilized presented the best result with 59.54% for EPA-DS-DN0.5. The increase of DN negatively affected the recovered activity of the co-immobilized enzymes. EPA-DS recovered activity was 59.54% whereas EPA-DS-DN4.5, with a nine-fold higher amount of DN, was 50.65%. Despite the use of DN is necessary to remove the dextran layer allowing the DS linkage with the reactive support groups; some dextran is necessary to stabilize DS [9,15]. Besides, the increase of the amount of DN offered to the support might have increased the competition for the binding sites, decreasing the amount of DS immobilized in the matrix. Another concern on the higher amount of DN is its hydrolytic effect, which can lower the oligosaccharide yields during the synthesis.

In previous works, dextran removal enabled DS immobilization yielding a biocatalyst with suitable properties to produce dextran and oligosaccharides [3,21,25]. Although dextran can be removed before the co-immobilization protocol, a new chain is formed during the oligosaccharide synthesis. The co-immobilization using DN allows continuous removal of native dextran and improves the oligosaccharides synthesis because it breaks dextran chains into oligosaccharides. DS co-immobilized by entrapment in alginate capsules with previous immobilization of DN onto Eupergit C produced oligosaccharides through the acceptor reaction mechanism [28]. Co-immobilization of enzymes by entrapment in alginate produced more oligosaccharides in orange juice than the immobilized DS in the same matrix [17].

3.2.2. pH effect

One of the advantages of enzyme immobilization is the increase in stability against adverse conditions when compared to the free enzyme. Thus, the effect of pH on enzyme activity was evaluated, and the results are presented in Fig. 3A for DS and Fig. 3B for DN. EPA-DS (control) retained around 100% of relative activity in the range of pH from 5.5 to 6.5. The results reported herein are consistent with previous studies

Table 1
Parameters of dextransucrase and dextranase co-immobilization in epoxy-agarose support.

Biocatalyst	Immobilization yields (%)	Immobilization efficiency (%)	Recovered activity (%)	Activity of immobilized biocatalyst (U/g of dried support)
EPA-DS	21.50 ± 0.49[a]	184.44 ± 5.10[a]	39.62 ± 1.10[a]	4.20 ± 0.12[a]
EPA-DS-DN0.5	11.16 ± 0.42[b]	534.17 ± 8.11[b]	59.54 ± 0.91[b]	4.93 ± 0.07[b]
EPA-DS-DN2.5	12.51 ± 1.13[b]	435.72 ± 1.83[c]	54.25 ± 0.23[c]	5.66 ± 0.02[c]
EPA-DS-DN 4.5	16.65 ± 2.62[a]	333.54 ± 1.57[d]	50.65 ± 2.08[d]	5.95 ± 0.04[d]

EPA-DS (control, DS 26.16 IU/mL), EPA-DS-DN0.5 (DS 26.16 IU/mL + DN 0.5μL, 2.76 IU), EPA-DS-DN2.5 (DS 26.16 IU/mL + DN 2.5μL, 13.80 IU) and EPA-DS-DN4.5 (DS 26.16 IU/mL + DN 4.5μL, 24.84 IU). Enzyme solution (1 mL) was added to 300 mg of epoxy-agarose support. All the values are related to dextransucrase activity. Means in the same column sharing the same letter are not statistically different according to the Tukey test ($p > 0.05$).

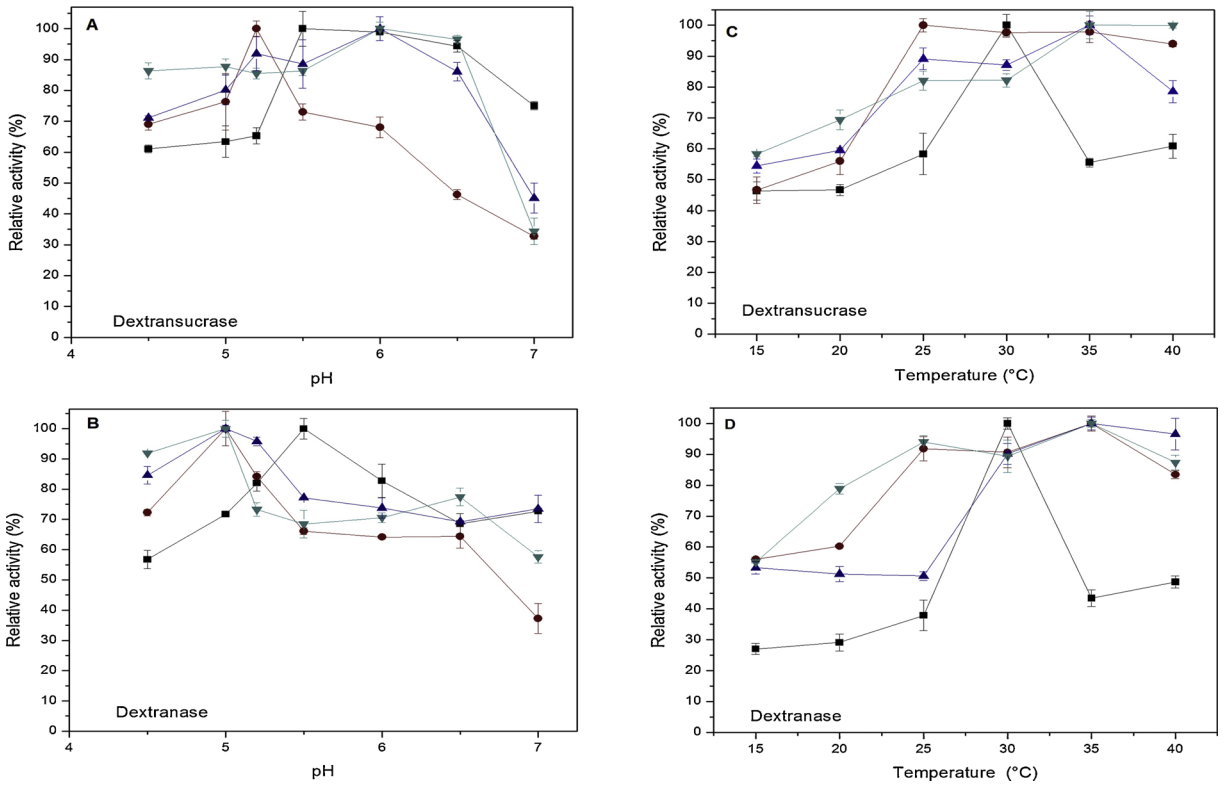

Fig. 3. Dextransucrase relative activity (A), determined as fructose released, and dextranase relative activity (B), determined as glucose released, as a function of pH at 30 °C for biocatalysts. Dextransucrase relative activity (C), determined as fructose released, and dextranase relative activity (D), determined as glucose released, as a function of temperature at pH 5.2. EPA-DS (■), EPA-DS-DN0.5 (●), EPA-DS-DN2.5 (▲) and EPA-DS-DN4.5 (▼). Results are presented as mean ± SD (n = 3).

that showed that the optimum pH value for DS immobilized in alginate capsules and Eupergit C 250 L was in the range of 5 to 6 [17,25]. The co-immobilized biocatalysts presented similar behavior to the immobilized DS, with higher relative activity at pH below 5.2. This behavior is interesting since free DS denatures quickly at pH below 5.0. EPA-DS0.5, on the other hand, showed a bell-shaped pH profile with a maximum activity at 5.2. The co-immobilized enzymes presented higher activity in the pH range of 4.5 to 5.2 compared to the immobilized DS, showing that the co-immobilization enhanced enzyme catalytic activity at low pH values. It is important to mention that, high enzyme activity at low pH values is interesting because prebiotic beverages using fruit juices are usually acidic [56], and the immobilized enzyme could be applied to synthesize oligosaccharides directly in the fruit juice.

The DN relative activity, in the co-immobilized biocatalysts, showed an optimal pH at 5.0 (Fig. 3B). DS also presents hydrolytic activity with a maximum at pH 5.5. The relative hydrolytic activity of the co-immobilized enzymes remained above 60% up to pH 7. Several studies reported the benefits of the co-immobilization of DS and DN due to the synergic effect for the production of oligosaccharides due to dextran hydrolysis, which can be used as a prebiotic food ingredient [13,56,60]. The amount of DN affects the DS activity and the hydrolytic activity of the catalyst. These features can be used to modulate the synthesis products towards the desired ones using DN to hydrolyze dextran to produce oligosaccharides [15].

3.2.3. Temperature effect

Fig. 3 presents the effect of temperature on DS (Fig. 3C) and DN activities (Fig. 3D) for all assays. EPA-DS presented a bell-shaped profile, with maximal activity at 30 °C (Fig. 3C). Similar behavior for free and immobilized DS on porous silica and alginate beads are reported in the literature [61,62]. The co-immobilized enzymes presented a broader optimum temperature compared to EPA-DS since the biocatalysts retained 75–100% of its activity from 25 °C to 40 °C. This behavior may be explained by the synergic effect promoted by the co-immobilization where DN improved the stability of DS by removing the dextran layer, changing the enzyme conformation and stabilizing DS at high temperatures. These results are in agreement with other studies that used different immobilization materials, which reported activity at 30 °C [25] and 35 °C [56].

DN activity presented similar behavior, showing an increase in stability against temperature for the co-immobilized enzymes (Fig. 3D). Again, the immobilized DS presented optimal hydrolytic activity at 30 °C. The co-immobilized enzymes presented maximal DN activity at 35 °C. The oligosaccharide synthesis can be controlled by the concentration of substrates (sucrose and acceptor) along with a moderate amount of dextranase, which contributes to the oligosaccharide formation by breaking native dextran into smaller molecules. The dextran synthesis cannot be avoided in DS oligosaccharides synthesis. Thus, the presence of DN in the biocatalyst limits the growth of the dextran chain in a controlled way because the amount of DN offered to the support was 10 times smaller than the amount of the dextransucrase. The glucose released in the medium increased in the co-immobilized treatments when compared to the immobilized DS. Endodextranases hydrolyze the α-(1–6) linkages in dextran chain releasing glucose, which can be attached to a dextran or oligosaccharides chain by the DS acceptor reaction [13].

3.2.4. Infrared spectrum of the biocatalyst

The biocatalyst EPA-DS-DN0.5 was evaluated by infrared spectroscopy (Fig. 4). The absorption of the spectrum wavenumbers characteristics of epoxy groups (3050 cm^{-1}, 1036 cm^{-1}, and 915 cm^{-1}) decreased after immobilization indicating that the enzyme was covalently attached to the epoxy-agarose support. The analysis of the spectra indicated that the enzyme also interacts with hydrocarbon double bond (C═C) and glyceryl group (O−CH_2) given the steep decrease in the

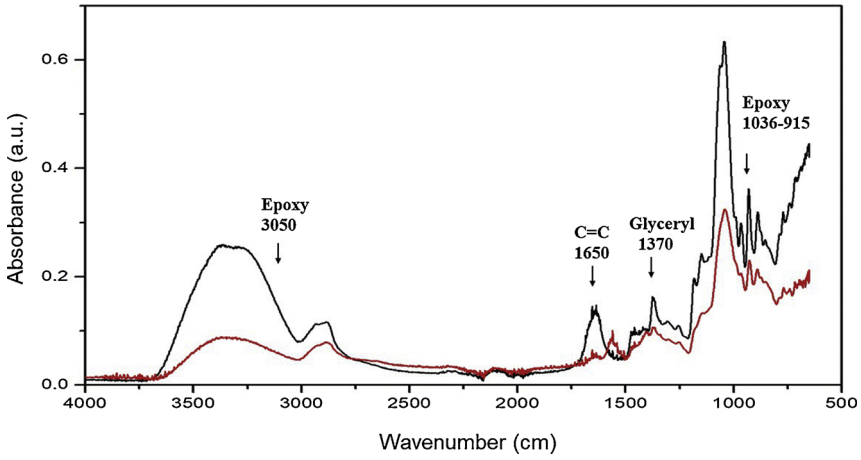

Fig. 4. Infrared spectrum of epoxy-agarose support () and biocatalyst EPA-DS-DN0.5 ().

absorption of these bands at 1650 cm^{-1} and 1370 cm^{-1} respectively [63]. The bands referring to primary amines (1650-1500 cm^{-1}) and secondary amines (1580-1490 cm^{-1}) are an indication of the presence of enzymes in the support.

3.2.5. Storage stability of co-immobilized enzymes

Fig. 5 presents the storage stability at 4 °C, and pH 5.2 of the biocatalyst EPA-DS-DN0.5 (DS 26.16 IU/mL + DN 0.5 µL, 2.76 IU). The co-immobilized enzymes retained more than 70% of its initial activity for at least 60 days. Other authors [21,56] reported that the co-immobilized enzymes (DS + DN) retained 90% of their initial activity for at least 30 days when immobilized in alginate beads and activated-chitosan.

3.2.6. Oligosaccharides synthesis

The synthesis of oligosaccharides was carried out using the free enzymes (DS + DN) and the co-immobilized DS and DN (EPA-DS-DN0.5) for 6 h, at 4 °C and 25 °C at pH 5.2 to evaluate the catalyst efficiency. In Fig. 6, the TLC plate shows the isomaltooligosaccharides formed along with their degree of polymerization (DP) for free and co-immobilized enzymes. The results obtained with the co-immobilized and free enzymes were similar. Co-immobilized enzymes (B2) had similar behavior of the free enzymes (A2) at 25 °C presenting oligosaccharides with DP up to 5. Co-immobilized DS and DN in alginate capsules synthesized oligosaccharides with DP up to 8 after 9 h of

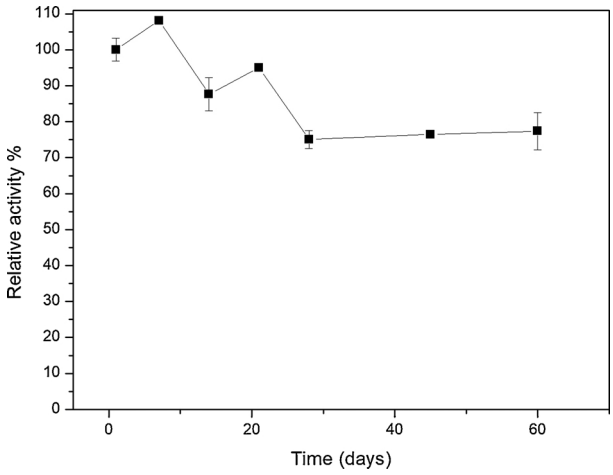

Fig. 5. Storage stability of the biocatalyst EPA-DS-DN0.5 enzyme solution containing 13.50 µg of protein/mL related to dextransucrase (1 mL, 26.16 IU) and dextranase (0.5 µL, 2.76 IU) co-immobilized onto epoxy-agarose support.

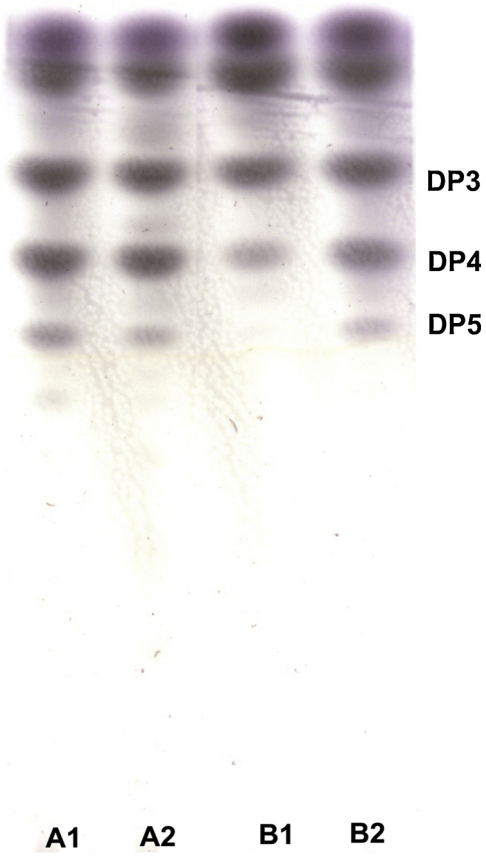

Fig. 6. TLC analyses of Degree of Polymerization (DP) of oligosaccharides synthesized by free (1IU/mL) and co-immobilized DS and DN (0.9 IU/g of support) at pH 5.2. A1 and A2 free enzymes at 4 °C and 25 °C, respectively. B1 and B2 co-immobilized enzymes at 4 °C and 25 °C.

synthesis in calcium acetate buffer (25 mM, pH 5.4) [15].

The oligosaccharides were quantified by densitometry analysis (Table 2). Despite the same degree of polymerization, the oligosaccharides yield was significantly different in all the synthesis ($p < 0.05$). The synthesis using the free enzymes presented a similar behavior at 4 °C and 25 °C with a total oligosaccharides synthesis of 15.73 ± 0.22 g/L and 17.15 ± 0.73 g/L, respectively whereas co-immobilized enzymes presented a lower yield at low temperature (4 °C) 7.21 ± 0.28 g/L compared to room temperature (25 °C) 12.68 ± 0.09 g/L. Daum and Buchholz [49] reported that the acceptor

Table 2
Oligosaccharides synthetized by free and co-immobilized DS and at pH 5.2. A1 and A2 free enzymes at 4 °C and 25 °C, respectively. B1 and B2 co-immobilized enzymes at 4 °C and 25 °C. DP represents the degree of polymerization of oligosaccharides produced.

Polymerization Degree g/L	A1 (4°C)	A2 (25 °C)	B1 (4 °C)	B2 (25 °C)
DP3	6.24 ± 0.15^{aA}	7.62 ± 0.13^{aB}	5.63 ± 0.22^{aC}	6.45 ± 0.03^{aD}
DP4	5.93 ± 0.05^{aA}	7.06 ± 0.18^{aB}	1.31 ± 0.05^{bC}	5.00 ± 0.07^{bD}
DP5	3.57 ± 0.33^{bA}	2.73 ± 0.19^{bB}	0.20 ± 0.01^{cD}	1.24 ± 0.01^{cD}
Total	15.73 ± 0.22^{A}	17.15 ± 0.73^{B}	7.21 ± 0.28^{C}	12.68 ± 0.09^{D}

Means in the same column sharing the same small letter are not statistically different according to the Tukey test ($p > 0.05$). Means in the same row sharing the same capital letter are not statistically different.

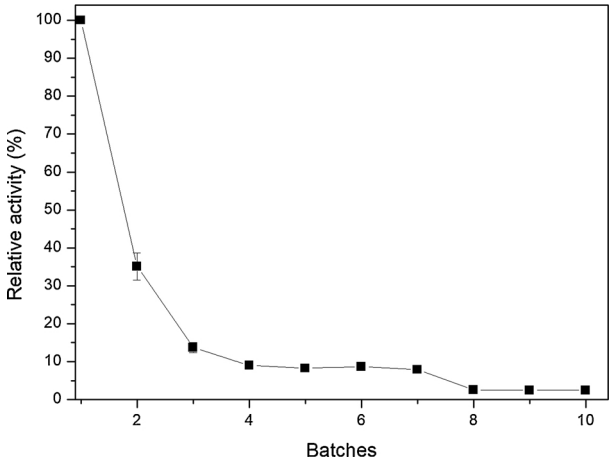

Fig. 7. Operational stability of the biocatalyst EPA-DS-DN0.5 enzyme solution containing 13.50 μg of protein/mL related to dextransucrase (1 mL, 26.16 IU) and dextranase (0.5 μL, 2.76 IU) co-immobilized onto epoxy-agarose support. Results are presented as mean ± SD (n = 3).

reaction catalyzed by DS raised the specificity and yield with decreasing reaction temperature. The previously reported advantage of low temperature on oligosaccharide chain elongation was not evident in the present study. The oligosaccharide yield obtained in the synthesis with free enzymes were close for both temperatures (4 and 25 °C), with a slightly higher amount of DP5 at 4 °C. Regarding the immobilized enzymes, the yield of DP4 and DP5 at 4 °C was much lower than at 25 °C. The presence of DN can hydrolyze dextran decreasing the degree of polymerization avoiding the elongation of the oligosaccharide chain.

3.2.7. Operational stability of co-immobilized enzymes

Enzyme immobilization is commonly applied to reuse enzymes and to improve its stability. Fig. 7 shows the operational stability of the co-immobilized enzymes (EPA-DS-DN0.5). The biocatalyst lost 65% of its initial activity in the first batch, maintaining around 10% of activity until the seventh batch. A possible explanation for this loss in activity is the leakage of the enzyme from the support during the repeated cycles. Although covalent bond formation was desired, noncovalently enzyme molecules may be adsorbed on the support, which was lixiviated by the solvent in the first reaction cycle. The truncated DS from *L. mesenteroides* NRRL B-512 F, covalently immobilized onto Eupergit C retained more than 90% of its activity after 15 batches [1]. Moreover, DS immobilized onto epoxy activated TiO_2 also showed poor operational stability retaining only 15% of the enzyme activity [64].

3.2.8. Infrared spectra of biocatalyst immobilized on epoxy-agarose after repeated cycles

Fig. 8 shows the IR spectrum of the epoxy-agarose support (EPA) and the EPA-DS-DN0.5 biocatalyst before and after the stability test. The biocatalyst EPA-DS-DN0.5 partially lost its enzymes during reuse as indicated by the lower absorbance of the peaks representatives of the immobilized biocatalyst. The reduction at the peak 3050 cm^{-1} is assigned to the C–H stretch of the methylene group from the epoxy ring, which is an indicator of the presence of epoxy groups. The characteristic peaks of primary amines (1650–1500 cm^{-1}) and secondary amines (1580–1490 cm^{-1}) exhibited a lower absorbance, which was near the values found for the non-epoxylated agarose, indicating the desorption of the enzymes. This behavior confirms the results shown in Fig. 7 and indicates that the enzyme-support bonds were not strong enough to promote the reuse of this biocatalyst and that the enzymes have lixiviated during the process. Desorption and dissociation of the subunits involved in the immobilization are one of the main problems in enzyme stabilization through immobilization process [31,54,65].

3.2.9. Infrared spectra of biocatalyst immobilized on Eupergit CM

A test was carried out using Eupergit CM, which we had in stock. The assay was carried out only for comparison purposes because Eupergit C and Eupergit CM supports were both discontinued by the manufacturer. Fig. 9 shows the infrared spectrum of the epoxy-agarose support and the commercial carrier, Eupergit CM. This analysis aimed

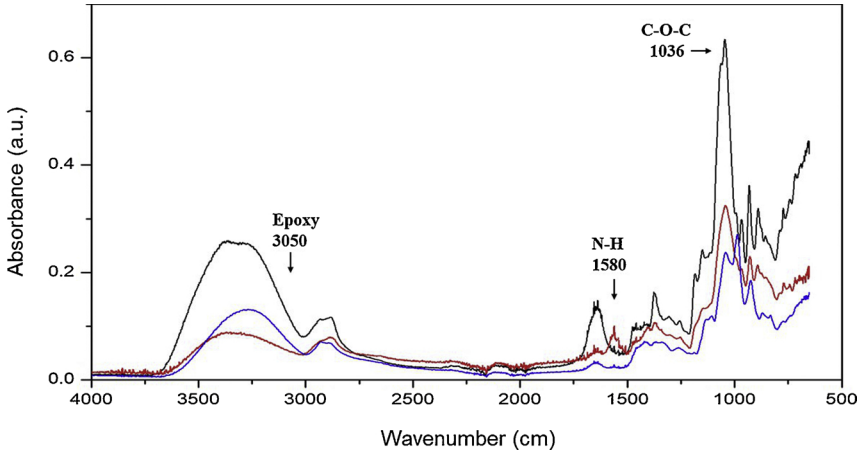

Fig. 8. Infrared spectrum of epoxy-agarose support (), biocatalyst EPA-DS-DN0.5 () and biocatalyst EPA-DS-DN0.5 after reuse ().

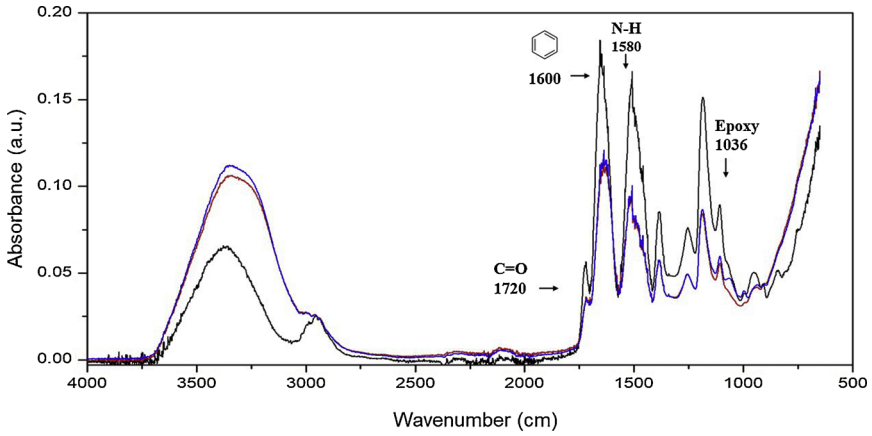

Fig. 9. Infrared spectrum of Eupergit CM support (), biocatalyst Eupergit CM-DS-DN0.5. () and biocatalyst Eupergit CM-DS-DN0.5 after reuse ().

to identify the groups present in both supports. The epoxy-agarose support presents some bands similar to those of Eupergit CM. However, there are differences in the region of wavenumbers 1600 cm^{-1}–1580 cm^{-1}, which are related to the presence of aromatic rings and amide groups [66]. These groups, especially the amide groups, can bind to the enzyme by hydrophobic interactions and covalent bonds. The FTIR spectra demonstrate the presence of other functional groups, which might be related to the DS bonded to the Eupergit C support [67]. Up to now, it was assumed that the DS immobilization was mostly due to the epoxy groups on the surface of the support. Some authors described the DS immobilization onto Eupergit C as a two-step binding mechanism. First an immobilization by adsorption, due to the hydrophobic matrix of epoxy supports, such as Eupergit C, followed by a nucleophilic attack to covalent binding [31,64,68].

Eupergit CM, the commercial support, was subjected to the same conditions of immobilization and operational test of the biocatalyst immobilized on epoxy-agarose. Fig. 9 presents the IR spectrum showing that the enzymes were co-immobilized on Eupergit CM. The regions related to epoxy groups (915–1036 cm^{-1}) and N—H bending vibration of amide groups (1580 cm^{-1}) presented lower absorbance than the Eupergit CM support [51], indicating that enzyme bonds to the epoxy and amide groups.

After reuse, the enzymes remained attached to the Eupergit CM support. No desorption of enzymes was detected during the reuse cycles, as confirmed by the IR profile of the new and used biocatalyst, which presented the same IR spectra. This result confirms the ability of this support to immobilize enzymes covalently, due to the high density of epoxy groups [65]. However, other peaks altered during immobilization, such as 1600 cm^{-1} (aromatic rings) and 1720 cm^{-1} (C═O symmetric stretching vibration of amide groups) [66]. Therefore, the enzyme may also be attached to other functional groups of Eupergit CM, in addition to their bond to epoxy groups. The absence of those reactive groups in epoxy-agarose led to the lower stability reported herein despite the good ability to co-immobilize DS and DN. To our knowledge, this is the first study where the groups involved in the DS immobilization were determined by FTIR spectroscopy and this approach allowed to verify the functional groups involved in the DS immobilization.

The superior performance of Eupergit CM has no practical application nowadays because this material is no longer available for purchase. However, the comparison between the epoxy-agarose and Eupercit CM IR spectra allowed to understand the functional groups involved in the enzyme's linkage. This comprehension will support strategies for further investigations on DS immobilization for the epoxy-agarose and also for other materials containing the functional groups reported herein.

4. Conclusion

In this study, an epoxy-agarose support was developed for co-immobilization of DS and DN. Among all biocatalyst tested, EPA-DS-DN0.5 showed the best values for co-immobilization parameters with a recovered activity 20% higher than the immobilized DS and an efficiency yield over 100%. The co-immobilized enzymes retained the activity in a wide range of temperature and pH, whereas the immobilized DS was unstable. The co-immobilized enzymes produced 12.68 ± 0.09 g/L of oligosaccharides with DP up to 5 close to the result obtained by free enzymes (17.15 ± 0.73 g/L) at 25 °C. Furthermore, the biocatalyst retained an activity over 70% for 60 days, results not reported in the literature for such a long time. However, the operational stability of this biocatalyst was inefficient, because the enzymes desorbed during the reuse cycles. The infrared spectrum of Eupergit CM confirmed the presence of others functional groups that are absent in epoxy-agarose, and that allows greater interaction between the enzyme and the support and that prevents the desorption of the enzyme during reuse batches. This is the first study where Eupergit C active groups were investigated regarding DS immobilization because previous studies did not report the importance of these other groups in the Eupergit C, just reported that epoxy groups were the responsible for covalent immobilization. Despite the low operational stability, the co-immobilized enzymes onto epoxy-agarose were suitable for the production of prebiotic oligosaccharides. Meanwhile, further studies are necessary to functionalize agarose with other reactive groups, besides epoxy groups, which might allow a stronger interaction between enzyme and support improving its stability.

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