“Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides”

Authors: L. Fernandez-Arrojo, B. Rodriguez-Colinas, P. Gutierrez-Alonso, M. Fernandez-Lobato, M. Alcalde, A.O. Ballesteros and F.J. Plou

Published in:

Process Biochemistry 48, 677-682 **(2013)**

<http://dx.doi.org/10.1016/j.procbio.2013.02.015>

Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides

Lucia Fernandez-Arrojo^a, Barbara Rodriguez-Colinas^a, Patricia Gutierrez-Alonso^b, Maria Fernandez-Lobato^b, Miguel Alcalde^a,
Antonio O. Ballesteros^a and Francisco J. Plou^{a,*}

^a Instituto de Catálisis y Petroleoquímica, CSIC, 28049 Madrid, Spain

^b Centro de Biología Molecular Severo Ochoa, UAM-CSIC, 28049 Madrid, Spain

Corresponding author: Francisco J. Plou, Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Marie Curie 2, 28049 Madrid, Spain. Phone: +34-91-5854869. Fax: +34-91-5854760. E-mail: fplou@icp.csic.es. URL: <http://www.icp.csic.es/abgroup>

ABSTRACT

A modification of the classical calcium alginate enzyme entrapment technique is described aiming to overcome some of the limitations of the former gel-based biocatalysts. DALGEEs (Dried ALGinate Entrapped Enzymes) were obtained dehydrating calcium alginate gel beads containing entrapped enzymes. A fructosyltransferase from *Aspergillus aculeatus*, present in Pectinex Ultra SP-L₂, was entrapped using this technique. The resulting DALGEEs were successfully tested both operating batchwise and in a continuous fixed-bed reactor for fructooligosaccharide (FOS) synthesis from sucrose. Interestingly, DALGEEs did not re-swell upon incubation in concentrated (600 g/L) sucrose solutions, probably due to the lowered water activity (a_w) of such media. Confocal laser scanning microscopy of DALGEEs revealed that the enzyme molecules accumulated preferably in the shell of the particles. DALGEEs showed an approximately 30-fold higher volumetric activity (300 U/mL) compared with the calcium alginate gel beads. Moreover, a significant enhancement (40-fold) of the space-time-yield of fixed-bed bioreactors was observed when using DALGEEs as biocatalyst compared with gel beads (4030 g/day·L of FOS *vs.* 103 g/day·L). The operational stability of fixed-bed reactors packed with DALGEEs was extraordinary, providing a nearly constant FOS composition of the outlet during at least 700 h. It was also noticeable their resistance against microbial attack, even after long periods of storage at room temperature. The DALGEEs immobilization strategy may also be

1 useful for other biotransformations, in particular when they take place in low a_w
2 media.

3
4 **Keywords:** enzyme immobilisation; entrapment; hydrogels; glycosidases;
5 fructooligosaccharides; food oligosaccharides.

6

1. INTRODUCTION

Compared with covalent immobilization methods, enzyme entrapment in alginate beads is a low-cost approach that does not exert any chemical modification to the protein structure. Alginate is a highly negatively charged polysaccharide extracted from brown algae that forms rigid gel-like structures in presence of divalent cations. Enzyme entrapment in alginate is straightforwardly accomplished under mild conditions, providing outstanding activity recoveries and easy diffusion of substrates and products through the matrix pores [1-3]. Typically, alginate entrapment is widely applied to the immobilisation of living cells [4;5].

Alginate gel-based biocatalysts present several practical limitations such as a low volumetric activity and microbial contamination of the beads, even in the refrigerator, due to their high content of water and carbon [6;7]. Undesired enzyme leakage from the gel beads due to the size of the pores [6] is another typical disadvantage of alginate entrapment method. The diffusion out of the beads can be minimised by increasing alginate concentration and/or mannuronic (M)/guluronic (G) acid ratio, as well as decreasing the pH [8]. Among other strategies to avoid protein leakage, enzymes can be cross-linked with glutaraldehyde [9] or bound to a carrier such as activated coal [10] prior to the entrapment process. In another recent twist, the crosslinking of alginate with glutaraldehyde has been also explored [11;12]. Nevertheless, the complete removal

1 of toxic crosslinking chemicals is difficult and thus may be inappropriate for its
2 application in food processing industries [13].

3 In this work, we have tackled some of the hurdles of calcium alginate
4 immobilization method by applying a drying process to the gel beads. It is well
5 reported that drying of alginate beads is widely applied in the delivery of drugs
6 and enzymes (esp. in the detergents industry), since the beads, in contact with
7 water, swell to their original size releasing the bioactive molecules [14;15]. Our
8 hypothesis was that dried-alginate beads should not re-swell in a medium with
9 low water activity (a_w) such as highly concentrated sugar solutions. In addition,
10 the strategy of drying the calcium alginate gel-based biocatalyst could lead to an
11 increased volumetric activity and thus a lower reactor volume to obtain a given
12 productivity.

13 For this purpose, we selected the fructosyltransferase from *Aspergillus*
14 *aculeatus* [16] and the resulting immobilized biocatalyst was characterized and
15 tested in batch and continuous reactors for the production of
16 fructooligosaccharides (FOS). FOS are fructose oligomers with a terminal glucose
17 group in which 2-4 fructofuranosyl moieties are linked by different bonds (Fig. 1)
18 [17-19]. Commercial FOS are mainly composed of 1-kestose, nystose and ¹F-
19 fructofuranosylnystose [20]. They are produced at multi-ton scale from
20 concentrated sucrose solutions using fungal transfructosylating enzymes such as
21 those from *Aspergillus niger*, *Aspergillus oryzae* and *Aureobasidium pullulans* [21;22].

1 FOS are non-cariogenic ingredients, have a sweetness of 40-60% relative to sucrose,
2 and exhibit prebiotic properties [23;24].

3 An effective immobilisation method of fructosyltransferases is highly
4 desired as it would allow a continuous FOS production process, the reuse of the
5 biocatalyst and the reduction of the total costs for industrial settings [25-27]. In
6 addition, immobilisation often protects enzymes from inactivation at extreme pH,
7 high temperatures or organic solvents [28;29]. In this work, the dried alginate
8 biocatalysts were studied characterised, and their advantages over the classical
9 alginate gel beads (volumetric activity, stability and space-time-yield) were
10 highlighted.

2. MATERIALS AND METHODS

2.1. Materials.

Pectinex Ultra SP-L (batch no. KRN05409), a preparation from *Aspergillus aculeatus* used in fruit juice processing that contains pectinolytic, cellulolytic and fructosyltransferase activities [16], was kindly donated by Novozymes A/S (Denmark). Sodium alginate SG300 was obtained from Degussa Texturant Systems, Spain. Sodium alginate Algogel 6021 and Algogel 3021 were kindly donated by Cargill Iberica, Spain. Sucrose, glucose and fructose were from Merck. 1-Kestose and nystose were from TCI Europe. 1^F-Fructosyl-nystose was from Megazyme (Ireland). All other reagents were of the highest available purity.

2.2. Preparation and characterisation of dried alginate-entrapped enzymes (DALGEEs).

The gel beads were prepared by ionotropic gelation as described elsewhere [1] with some variations. A 4% (w/v) sodium alginate solution was prepared in distilled water and stirred until a homogeneous clear solution was observed. The solution was let to settle for 2 hours in order to eliminate all air bubbles. The alginate solution was then gently mixed in a ratio 1:1 (w:w) with the enzyme solution (Pectinex Ultra SP-L diluted 1:2 v/v with 10 mM sodium acetate buffer, pH 5.6). The resulting enzyme-alginate mixture was dropped with the help of a peristaltic pump (P-1, GE Healthcare) onto a 0.2 M CaCl₂ solution in sodium acetate buffer (pH 5.6, 10 mM). A pipette tip was used at the outlet of the pump and placed 5 cm over the CaCl₂ solution. The drops instantly formed gel beads in

contact with the CaCl_2 solution, which was maintained under magnetic stirring at 100 rpm. The beads were hardened in the CaCl_2 solution for 20 min with mild agitation. The diameter of the beads obtained was around 3 mm. The gel beads were then separated from the solution and washed twice with 250 mL of sodium acetate (pH 5.6, 10 mM) for 15 min under mild agitation. The gel beads obtained as described above were dehydrated by air flow or controlled evaporation at 35°C until the size of the particles remained constant. The resulting beads were called DALGEEs (Dried ALGinate Entrapped Enzymes).

2.2.1. Confocal microscopy.

Protein distribution in DALGEE particles was studied using confocal fluorescence microscopy. Before immobilisation, proteins present in Pectinex-Ultra SP-L were labelled with fluorescein isothiocyanate (FITC), a fluorophore that chemically attaches to amino groups in proteins [30]. Pectinex Ultra SP-L was diluted 1:2 (v/v) in 0.1 M Na_2CO_3 . Then, FITC dissolved in N,N-dimethylformamide was added to the protein solution in a ratio of 5 µg of FITC per milligram of protein [31]. The unbound FITC was removed using a pre-packed PD-10 column (Amersham Biosciences). The FITC-labelled protein sample was diluted 1:2, 1:100 and 1:1000 (v/v) with 10 mM acetate buffer (pH 5.6) before the immobilisation in calcium alginate and further drying. The resultant DALGEEs were analysed with a confocal laser scanning microscope (Leica, model TCS SP2-AOBS). An oil immersion objective (40.0 × 1.25) was used for all measurements and the pinhole aperture was set to 1.50 Airy (122 µm). The excitation wavelength was 488 nm and

the emission was collected at 500-550 nm, with the corresponding filters. Micrographs were taken at different plane depths. The exposure of the micrographs was adjusted using the software for image processing.

2.2.2 Porosimetry.

The specific surface area (S_{BET}) of the DALGEEs was determined from analysis of nitrogen adsorption isotherms at -196°C using a Micromeritics ASAP 2010 equipment. The samples were previously degassed at 100°C for 12 h to a residual vacuum of 5×10^{-3} Torr in order to remove any loosely held adsorbed species.

2.2.3. Water content.

A volumetric Karl-Fisher titrator (Mettler Toledo, model DL31) was used for measuring the water content of DALGEEs, employing Hydranal composite 5 as reagent.

2.2.4. Scanning electron microscopy

The DALGEEs were mounted on aluminium SEM stubs and sputter-coated with a thin layer of gold at completed Torr vacuum. Samples were examined by scanning electron microscopy using an XL3 microscope (Philips) at an acceleration potential of 20 kV.

2.3. Activity assays

The enzymatic activity towards sucrose was determined at 60°C in 0.2 M sodium acetate buffer (pH 5.6) by measuring the release of reducing sugars from 100 g/L sucrose solution using the dinitrosalicylic acid (DNS) method [32]. The assay was adapted to 96-well microplates as described in our previous work [25]. One unit (U) of activity was defined as that catalyzing the formation of 1 µmol of reducing sugars per minute under the above conditions. The activity of the immobilised biocatalysts was determined incubating in an Eppendorf tube approx. 50 mg of alginate beads with 0.45 mL of 100 g/L sucrose solution in 0.2 M sodium acetate buffer (pH 5.6). The mixture was maintained at 60°C and 600 rpm for 20 min in a Vortemp 56 incubator (Labnet). A 200 µL sample of the supernatant was withdrawn and submerged for 10 min in a water bath at 95°C, in order to inactivate the possible lixiviated enzyme. Then, 50 µL of this sample were transferred to one of the wells of the microplate, and the concentration of reducing sugars was measured as described above.

2.4. Operational stability of DALGEEs in batch reactor

DALGEEs (approx. 10 mg) were incubated with 250 µL of 600 g/L sucrose solution at 35°C and 900 rpm in a Vortemp 56 incubator (Labnet). After 20 minutes, the DALGEEs were separated from the reaction medium. The supernatant was incubated at 95°C for 10 min in a water bath (in order to inactivate any possible lixiviated enzyme) and the content of reducing sugars was determined using the DNS method. The DALGEEs were washed twice with a cooled (4°C) sucrose solution using high vortex agitation for 30 seconds each wash. A total of 13

1 reaction cycles, with washes in between, were performed to evaluate the
2 operational stability of DALGEEs.

3 4 **2.5. Operational stability of DALGEEs in a continuous fixed-bed reactor**

5 DALGEEs containing the entrapped fructosyltransferase were packed in a
6 HiTrap™ column with 1 mL of total volume (7 x 25 mm, GE Healthcare). The inlet
7 of the column was connected to an isocratic pump with dual reciprocating pistons
8 (model 515, Waters), in order to precisely control the flow of the feeding solution
9 to the bioreactor (600 g/L sucrose in 0.2 M sodium acetate buffer, pH 5.6). Both the
10 bioreactor and the feeding solution were maintained at 35°C in an Ovan chamber
11 incubator (Lovango). At different times, samples were taken at the outlet stream of
12 the bioreactor and analysed by HPLC. When using gel beads, a column XK 16/20
13 (GE Healthcare) with 30 mL of total volume was used.

14 15 **2.6. HPLC analysis**

16 The analysis and quantification of the different carbohydrates present in the
17 transfructosylation reactions was carried out by HPLC with a quaternary pump
18 (Delta 600, Waters) coupled to a 4.6 x 250 mm Luna-NH₂ column (5 µm, 100 Å)
19 from Phenomenex. Detection was performed using an evaporative light scattering
20 detector DDL-31 (Eurosep) equilibrated at 85°C. Acetonitrile/water 75:25 (v/v),
21 degassed with helium, was used as mobile phase at 0.9 mL/min for 3 min. Then, a
22 gradient from this eluent to acetonitrile/water 70:30 (v/v) was performed in 1
23 min, and held for 2 min. A new gradient to acetonitrile/water 60:40 (v/v) was

1 performed in 2 min and held for 6 min. Total analysis time was 14 min. The
2 column temperature was kept constant at 25°C. The data obtained were analysed
3 using the Millennium Software.

4 **2.7. Water activity**

6 Water activity of sugar solutions was determined using a humidity and
7 temperature digital indicator Thermoconstanter TH200 (Novasina, Switzerland).

8 The humidity sensor was calibrated with control saturated salts solutions of
9 different a_w values (LiCl, 0.11; potassium acetate, 0.22; NaBr, 0.57; NaCl, 0.75;
10 $K_2Cr_2O_7$, 0.98) at 25°C.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterisation of dried alginate-entrapped enzymes

(DALGEEs)

Fructosyltransferase activity from *A. aculeatus*, present in Pectinex Ultra SP-L [16;33], was entrapped in calcium alginate beads using different commercial sodium alginates (SG300, Algogel 6021 and Algogel 3021) and their immobilisation yields were compared (Table 1). The immobilized enzymatic activity of the alginate beads was determined indirectly by measuring the initial activity of the solution before gelation and the activity present in the CaCl₂ and washing solutions. The value calculated was considered as the theoretical immobilised activity. The main contribution to the loss of activity occurs while the drop is not fully gelled to form the bead [34]. Table 1 shows that sodium alginate SG300 was the most adequate for our purpose, probably due to an optimal mannuronic/guluronic acid ratio, with an immobilisation yield close to 50%. This value was also in accordance with the total protein immobilisation yield (47%) measured by the Bradford assay. Initial protein concentration in Pectinex preparation was approx. 17 mg/ml and fructosyltransferase accounted for only 0.4% of total protein [16]. Theoretical immobilisation yields in the range 40-80% are usually reported [1;9;11;13] and, therefore, the SG300 alginate was selected for further experiments.

The so-called apparent activity (experimental) of the immobilised biocatalysts, which takes into account mass transfer and diffusional restrictions

[35], was measured using 3 gel-beads (approx. 50 mg) and 100 g/L sucrose. The volumetric apparent activity of the SG300 gel-based biocatalyst was 10 U/mL, which is nearly 4-fold lower than the theoretical value. The difference between theoretical and apparent activities seems to be related with the mass transfer of substrates and products within the alginate matrix.

We performed the drying process of the calcium alginate gel beads and the resulting biocatalysts, with a crystalline appearance, were called DALGEEs (Dried ALGinate Entrapped Enzymes). The size of the alginate beads was significantly reduced, from 3 mm to less than 1 mm upon drying, which accounted for a volume reduction of approx. 96% (considering the beads as perfect spheres). The images obtained by scanning electron microscopy of the DALGEE particles are shown in Fig. 2. Beads were nearly spherical and quite homogeneous in size, showing a rough surface. Santagapita et al. reported that roughness or smoothness is affected both by the beads composition and drying method [36].

A crucial point for the applicability of DALGEEs was to analyse if they rehydrated when they were submerged in the sugar solution, as swelling may be accompanied by enzyme leakage. Fig. 3 shows that DALGEEs rehydrate in buffer solution. In contrast, DALGEEs beads maintain its initial size after 1 day incubation in 600 g/L sucrose. This fact seems to be related with the decrease of water activity (a_w) caused by sucrose [37]. This property is important since many chemical, enzymatic and microbiological processes are dependent on the availability of water [38;39]. The DALGEEs swell very slightly when they are in

1 contact with a concentrated sucrose solution; this smooth swelling may facilitate
2 the contact between the enzyme molecules and the substrates. We observed that a
3 600 g/L sucrose solution lowers the a_w of water from 1.0 to 0.97; this small change
4 is enough to prevent DALGEEs from recovering their initial size. When increasing
5 sucrose concentration up to 950 g/L, the a_w diminishes to a value of 0.87.
6 However, the swelling process may be also influenced by the M/G ratio of the
7 alginate and/or the drying method.

8 The apparent enzymatic activity of DALGEEs containing
9 fructosyltransferase from *A. aculeatus* was 300 U/mL, which is 30-fold higher than
10 that measured for the gel beads. The textural studies revealed the low porosity of
11 this material, with a BET area of 7.6 m²/g and a total pore volume of 0.072 cm³/g,
12 measured from the adsorption nitrogen isotherms. The water content of
13 DALGEEs, determined by the Karl Fisher assay, was of only 1.5 % (w/w).

14 The distribution of immobilised FITC-labelled proteins in DALGEEs was
15 studied by fluorescence confocal microscopy. Although fluorescence was found
16 throughout the whole volume of the beads, a clear preferential accumulation of the
17 enzyme in the shell of the particle was observed when lowering the exposure of
18 the emission signal (Fig 4A). This result is in accordance with the expected
19 hypothetical drying process, as it occurs from the outer to the inner layers thus
20 enriching the beads with protein in the external layers (Fig. 4B) [40].

22 3.2. FOS synthesis in batch reactor with fructosyltransferase in DALGEEs

The reuse of DALGEEs was studied in a batch reactor measuring the activity of the beads in a sequence of reaction cycles. Fig. 5 illustrates the operational stability of the DALGEEs in 13 successive reaction cycles of 20 min each. As it is shown, the operational stability of DALGEE biocatalysts was very satisfactory. This behaviour was probably related with the fact that DALGEEs do not swell in concentrated sucrose solution and thus the enzyme leakage is minimised.

3.3. FOS synthesis in continuous fixed-bed bioreactors with fructosyltransferase in DALGEEs

A fixed-bead reactor of small volume (1 mL) was packed with the DALGEEs. The operation of the continuous reactor for FOS synthesis was assayed at 0.01 mL/min and 35°C. The bioreactor was operated continuously for 700 h during which samples were taken and analysed by HPLC. As shown in Fig. 6, the composition of the outlet was nearly constant at least during 700 h, with an average FOS concentration of 275 g/L, which indicated an optimal operational stability of the DALGEEs biocatalysts. The composition of the outlet was approximately as follows: 175 g/L glucose, 148 g/L sucrose, 140 g/L 1-kestose, 96 g/L nystose, 30 g/L 1^F-fructosyl-nystose and 11 g/L 1^F-fructosyl-fructosyl-nystose. Considering the volume of the bioreactor and the mean concentration of total FOS measured at the outlet, the space-time yield was approx. 4030 g FOS/day · L.

1 The above space-time yield was compared with that of a 25 mL fixed-bed
2 reactor packed with the calcium alginate gel beads, which was also operated in
3 continuous mode, at 35°C and with a flow rate of 0.01 mL/min. Samples at the
4 outlet of the column were analysed by HPLC. The sucrose conversion reached
5 with this system was 67%, and the space-time yield was 103 g FOS/ day · L, which
6 is about 40-fold lower than that determined for a reactor packed with DALGEEs.

7 The fructooligosaccharide composition obtained from enzymatic
8 transfructosylation is highly dependent on the reaction time [25], which is
9 proportional to the residence time in a continuous reactor. The FOS composition
10 was therefore studied operating the reactor at different flow rates in order to
11 control the residence time. The results of this study (Fig. 7) showed that high
12 residence times led to a final FOS product enriched in tetra- and
13 pentaoligosaccharides, whereas low residence times yielded to a trisaccharide-
14 enriched FOS mixture. The specific effects of prebiotic oligosaccharides depend,
15 among other factors, on their degree of polymerization [18;41;42], so it is
16 interesting to control the FOS distribution varying the flow rate of the fixed-bed
17 reactor. The study also revealed that an increase of the residence time was
18 translated into higher sucrose conversion values, achieving a maximum value of
19 91%. Elsewhere, the extraordinary operational stability of DALGEEs biocatalysts
20 in continuous fixed-bed reactors ensures a product with a constant FOS
21 composition.

22 This immobilization method may also be suitable for other biotechnological
23 processes, in particular those involving the transformation of carbohydrates.

1 Indeed, we have also applied the DALGEEs technology to obtain glucose/fructose
2 syrups using a β -fructofuranosidase from *Rhodotorula dairensis* [43], to convert
3 inulin into fermentable sugars using an inulinase, and for galactooligosaccharides
4 synthesis using a β -galactosidase (data not shown).

6 CONCLUSIONS

7 The methodological simplicity for the preparation of DALGEEs and the low
8 cost of the required materials make this process attractive for its application in
9 large-scale FOS synthesis. The drying process of the gel beads resulted in particles
10 with crystalline appearance and increased volumetric activity (30-fold) compared
11 with the corresponding gel-based biocatalysts. In addition, its storage stability
12 (absence of microbial growth compared with the hydrated beads), operational
13 stability and no swelling when used in low a_w media (e.g. concentrated sucrose
14 solutions) offer excellent perspectives for applications in biotransformations.

16 ACKNOWLEDGEMENTS

17 We thank Fernando Gonzalez (Centro de Investigaciones Biologicas, CSIC)
18 for confocal microscopy support. We thank Ramiro Martínez (Novozymes A/S,
19 Madrid, Spain) for supplying Pectinex and for useful suggestions. Projects
20 BIO2010-20508-C04-01 and BIO2010-20508-C04-04 from Spanish Ministry of
21 Science and Innovation supported this research. We thank Fundacion Ramon
22 Areces by the institutional Grant to the Centro de Biología Molecular Severo

1 Ochoa. B.R.C was supported by a fellowship from the Spanish Ministry of Science
2 and Innovation (FPI program).
3

REFERENCES

- [1] Gomez de Segura A, Alcalde M, Bernabe M, Ballesteros A, Plou FJ. Synthesis of methyl α -D-glucooligosaccharides by entrapped dextranucrase from *Leuconostoc mesenteroides* B-1299. J Biotechnol 2006;124:439-445.
- [2] Busto MD, Ortega N, Perez-Mateos M. Characterization of microbial endo-beta-glucanase immobilized in alginate beads. Acta Biotechnol 1998;18:189-200.
- [3] Lu Y, Zhang H, Wang L, Guo X, Yao H. A novel method for the continuous production of gamma-aminobutyric acid using immobilized rice bran. Biocatal Biotransfor 2010;28:215-221.
- [4] Panesar R, Panesar PS, Singh RS, Kennedy JF. Hydrolysis of milk lactose in a packed bed reactor system using immobilized yeast cells. J Chem Technol Biotechnol 2011;86:42-46.
- [5] Chien CS, Lee WC, Lin TJ. Immobilization of *Aspergillus japonicus* by entrapping cells in gluten for production of fructooligosaccharides. Enz Microb Technol 2001;29:252-257.
- [6] Smidsrod O, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol. 1990;8:71-78.
- [7] Gomez de Segura A, Alcalde M, Plou FJ, Remaud-Simeon M, Monsan P, Ballesteros A. Encapsulation in LentiKats of dextranucrase from *Leuconostoc mesenteroides* NRRL B-1299, and its effect on product selectivity. Biocatal Biotransfor 2003;21:325-331.

- [8] Martinsen A, Storro I, Skjak-Braek G. Alginate as immobilization material: III. Diffusional properties. *Biotechnol Bioeng* 1992;39:186-194.
- [9] Shankar SK, Praveen Kumar SK, Mulimani VH. Calcium alginate entrapped preparation of α -galactosidase: Its stability and application in hydrolysis of soymilk galactooligosaccharides. *J Ind Microbiol Biot* 2011;38:1399-1405.
- [10] Richetti A, Munaretto CB, Lerin LA, Batistella L, Vladimir Oliveira J, Dallago RM, Astolfi V, Luccio MD, Mazutti MA, De Oliveira D, Treichel H. Immobilization of inulinase from *Kluyveromyces marxianus* NRRL Y-7571 using modified sodium alginate beads. *Bioproc Biosyst Eng* 2012;35:383-388.
- [11] Ortega N, Perez-Mateos M, Pilar MC, Busto MD. Neutralse immobilization on alginate-glutaraldehyde beads by covalent attachment. *J Agric Food Chem* 2009;57:109-115.
- [12] Naganagouda K, Mulimani VH. Gelatin blends with alginate: Gel fibers for alpha-galactosidase immobilization and its application in reduction of non-digestible oligosaccharides in soymilk. *Process Biochem* 2006;41:1903-1907.
- [13] Rajan A, Nair GR. Production of soya milk containing low flatulence-causing oligosaccharides in a packed bed reactor using immobilised α -galactosidase. *Int J Food Sci Technol* 2010;45:2023-2031.
- [14] Santagapita PR, Mazzobre MF, Buera MP. Formulation and drying of alginate beads for controlled release and stabilization of invertase. *Biomacromolecules* 2011;12:3147-3155.

- [15] Elnashar MM, Yassin MA, Moneim AEA, Bary EMA. Surprising performance of alginate beads for the release of low-molecular-weight drugs. *J Appl Polym Sci* 2010;116:3021-3026.
- [16] Ghazi I, Fernandez-Arrojo L, Garcia-Arellano H, Ferrer M, Ballesteros A, Plou FJ. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. *J Biotechnol* 2007;128:204-211.
- [17] Alvaro-Benito M, de Abreu M, Fernandez-Arrojo L, Plou FJ, Jimenez-Barbero J, Ballesteros A, Polaina J, Fernandez-Lobato M. Characterization of a β -fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. *J Biotechnol* 2007;132:75-81.
- [18] Kelly G. Inulin-type prebiotics - A review: Part 1. *Altern Med Rev* 2008;13:315-329.
- [19] Rodriguez-Alegria ME, Enciso-Rodriguez A, Ortiz-Soto ME, Cassani J, Olvera C, Munguia AL. Fructooligosaccharide production by a truncated *Leuconostoc citreum* inulosucrase mutant. *Biocatal Biotransfor* 2010;28:51-59.
- [20] Tungland BC. Fructooligosaccharides and other fructans: Structures and occurrence, production, regulatory aspects, food applications, and nutritional health significance. In: Eggleston G, Cote GL editors. *Oligosaccharides in Food and Agriculture*. Washington: American Chemical Society; 2003. 135-152.

- [21] Sangeetha PT, Ramesh MN, Prapulla SG. Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202. *Process Biochem* 2005;40:1085-1088.
- [22] Plou FJ, Martin MT, de Segura AG, Alcalde M, Ballesteros A. Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides. *Can J Chem* 2002;80:743-752.
- [23] Saulnier DM, Kolida S, Gibson GR. Microbiology of the human intestinal tract and approaches for its dietary modulation. *Curr Pharm Design* 2009;15:1403-1414.
- [24] Linde D, Macias I, Fernandez-Arrojo L, Plou FJ, Fernandez-Lobato M. Molecular and biochemical characterization of a beta-fructofuranosidase from *Xanthophyllomyces dendrorhous*. *Appl Environ Microbiol* 2009;75:1065-1073.
- [25] Ghazi I, Gomez de Segura A, Fernandez-Arrojo L, Alcalde M, Yates M, Rojas-Cervantes ML, Plou FJ, Ballesteros A. Immobilisation of fructosyltransferase from *Aspergillus aculeatus* on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides. *J Mol Catal B Enz* 2005;35:19-27.
- [26] Csanadi Z, Sisak C. Immobilization of Pectinex Ultra SP-L pectinase and its application to production of fructooligosaccharides. *Acta Aliment* 2006;35:205-212.
- [27] Smaali I, Soussi A, Bouallagui H, Chaira N, Hamdi M, Marzouki MN. Production of high-fructose syrup from date by-products in a packed bed bioreactor using a

novel thermostable invertase from *Aspergillus awamori*. *Biocatal Biotransfor*
2011;29:253-261.

[28] Kunamneni A, Ghazi I, Camarero S, Ballesteros A, Plou FJ, Alcalde M.
Decolorization of synthetic dyes by laccase immobilized on epoxy-activated
carriers. *Process Biochem* 2008;43:169-178.

[29] Torres-Salas P, Del Monte-Martinez A, Cutiño-Avila B, Rodriguez-Colinas B,
Alcalde M, Ballesteros AO, Plou FJ. Immobilized biocatalysts: Novel approaches
and tools for binding enzymes to supports. *Adv Mater* 2011;23:5275-5282.

[30] Malmsten M, Xing KZ, Ljunglof A. Confocal microscopy studies of trypsin
immobilization on porous glycidyl methacrylate beads. *J Colloid Interf Sci*
1999;220:436-442.

[31] Torres P, Datla A, Rajasekar VW, Zambre S, Ashar T, Yates M, Rojas-Cervantes
ML, Calero-Rueda O, Barba V, Martinez MJ, Ballesteros A, Plou FJ.
Characterization and application of a sterol esterase immobilized on polyacrylate
epoxy-activated carriers (Dilbeads). *Catal Commun* 2007;9:539-545.

[32] Sumner JB, Howell SF. A method for determination of saccharase activity. *J Biol*
Chem 1935;108:51-54.

[33] Tanriseven A, Aslan Y. Immobilization of Pectinex Ultra SP-L to produce
fructooligosaccharides. *Enzyme Microb Tech* 2005;36:550-554.

[34] Betigeri SS, Neau SH. Immobilization of lipase using hydrophilic polymers in the
form of hydrogel beads. *Biomaterials* 2002;23:II.

- [35] Worsfold PJ. Classification and chemical characteristics of immobilized enzymes
- Technical report. Pure Appl Chem 1995;67:597-600.
- [36] Santagapita PR, Mazzobre MF, Buera, MP. Invertase stability in alginate beads:
Effect of trehalose and chitosan inclusion and of drying methods. Food Res Int
2012;47:321-330
- [37] Velezmoro CE, Oliveira AL, Cabral FA, Meirelles AJA. Prediction of water
activity in sugar solutions using models of group contribution and equation of
state. J Chem Eng Jpn 2000;33:645-653.
- [38] Gosling A, Stevens GW, Barber AR, Kentish SE, Gras SL. Effect of the substrate
concentration and water activity on the yield and rate of the transfer reaction of
 β -galactosidase from *Bacillus circulans*. J Agric Food Chem 2011;59:3366-3372.
- [39] Laroche C, Fine F, Gervais P. Water activity affects heat resistance of
microorganisms in food powders. Int J Food Microbiol 2005;97:307-315.
- [40] Magnin D, Dumitriu S, Magny P, Chornet E. Lipase immobilization into porous
chitoxan beads: Activities in aqueous and organic media and lipase localization.
Biotechnol Prog 2001;17:734-737.
- [41] Roberfroid M. Prebiotics: The concept revisited. J Nutr 2007;137:830S-837S.
- [42] Rodriguez-Colinas B, De Abreu MA, Fernandez-Arrojo L, De Beer R, Poveda A,
Jimenez-Barbero J, Haltrich D, Ballesteros Olmo AO, Fernandez-Lobato M, Plou
FJ. Production of galacto-oligosaccharides by the β -galactosidase from

1 Kluyveromyces lactis: Comparative analysis of permeabilized cells versus
2 soluble enzyme. J Agric Food Chem 2011;59:10477-10484.

3 [43] Fernandez-Arrojo L, Plou FJ, Alcalde M, Ballesteros AO, Fernandez-Lobato M,
4 Gutierrez-Alonso P. Alginate-based immobilized biocatalyst of high operational
5 stability for the transformation of carbohydrates in continuous reactors
6 (DALGEE's biocatalyst) . Patent PCT/ES2010/070104. 2010.

Figure legends

Fig. 1. Molecular structure of fructooligosaccharides (FOS) synthesized in this work.

Fig. 2. SEM micrographs of the DALGEE particles. Magnifications: upper picture, 45x; bottom picture, 1000x.

Fig. 3. Swelling of DALGEEs obtained with Algogel 3021: (1) Alginate gel particle; (2) DALGEE particle; (3) DALGEE particle after 24 h incubation in buffer; (4) DALGEE particle after 24 h incubation in 600 g/L sucrose.

Fig. 4. (A) Confocal image of FITC-labeled proteins, from Pectinex Ultra SP-L, entrapped in DALGEE particles. The images belong to a DALGEE particle in which the FITC-labelled protein solution was diluted 1:100 (v/v) prior to the gel-entrapment procedure. (B) Proposed mechanism of protein concentration on the shell of a DALGEE particle upon the drying process.

Fig. 5. Reuse assay of DALGEEs, containing the fructosyltransferase from *A. aculeatus*, in successive batch reactions. After each reaction cycle (20 min), the DALGEEs were separated from the reaction medium and washed. Reaction conditions: 600 g/L sucrose, 35°C, 900 rpm.

Fig. 6. Operational stability of the fixed-bed bioreactor packed with DALGEEs containing the fructosyltransferase from *A. aculeatus*. Reactor conditions: Feed, 600 g/L sucrose; Flow rate, 0.01 mL/min; Temperature, 35°C.

Fig. 7. Effect of the residence time on the sucrose conversion and FOS composition in the fixed-bed bioreactor packed with DALGEEs containing the fructosyltransferase from *A. aculeatus*. Operational conditions: Feed, 600 g/L sucrose; Temperature, 35°C.

Table 1. Effect of the source of alginate on the immobilisation by entrapment of *A. aculeatus* fructosyltransferase.

Alginate	Total activity before immobilisation (U)	Immobilised activity ^a (U)	Volume of gel beads ^b (mL)	Volumetric activity ^c (U/mL)	Immobilisation yield ^c (%)
SG300	274.0	138.8	3.4	40.7	50.7
Algogel 6021	193.6	34.9	2.4	14.5	18.1
Algogel 3021	180.9	46.4	2.3	20.6	25.6

^a Determined by subtracting the total initial activity in the solution prior to its gelation and the activity in the remaining CaCl₂ and washing solutions.

^b The gel-based biocatalyst volume was estimated considering a gel density of 1 g/mL.

^c Theoretical values.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7