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Research article

Production of co-immobilized dextransucrase and dextranase preparations and their application in isomalto-oligosaccharides synthesis

Barbara Sikora,* Celina Kubik, Stanisław Bielecki

Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences Lodz University of Technology, Stefanowskiego 4/10, 90-924 Lodz

*barbara.sikora@p.lodz.pl

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Abstract: *Dextransucrase (DS) from Leuconostoc mesenteroides and dextranase (DN) from Penicillium funiculosum were co-immobilized by entrapment in calcium alginate and used to produce isomaltooligosaccharides (IMOs) from sucrose. DS convert sucrose into dextran, which is the substrate for DN, so that IMOs are products of dextran hydrolysis. Before the co-immobilization DS was cross-linked with glutardialdehyde (GA), while DN was adsorbed on hydroxyapatite (HAp). Cross-linking was essential for the stability of DS and pre-immobilization of DN to prevent enzyme from leaking out of the alginate beads. Operational stability of co-immobilized preparations of DS and DN was estimated based on amounts of isomaltose and isomaltotriose formed during successive 24h processes of IMOs synthesis, carried out at 30°C, pH 5.4 and 200 rpm in 10% (w/v) sucrose solutions. Preparation characterized by the initial DS/DN activities ratio of 1/14 was found to maintain these activities at least 100 h of IMOs synthesis (5 repeated batch reaction).*

Keywords: *dextranase, dextransucrase, co-immobilization, hydroxyapatite, sodium alginate.*

Introduction

Immobilized preparations of enzymes and whole microbial cells have been used for years in biotechnological processes. Application of co-immobilized biocatalysts in multi-step processes offers the opportunity to produce a wider range of compounds from various, also alternative raw materials [1-5]. Theoretically, one bead or microcapsule of immobilized preparation may contain many different enzymes that can be also attached to the surface of one matrix under appropriate conditions of co-immobilization. Also conditions of application of such preparations must be suitable because different enzymes are usually optimally active under different conditions and require different cofactors, stabilizing agents etc.

Isomaltooligosaccharides (IMOs) are a group of nondigestible (NDS) oligosaccharides that are prebiotics, resistant to degradation by digestive enzymes acting in the gastrointestinal tract. Therefore, they enter the large intestine where they are metabolized by indigenous microflora, mainly by bacteria of genera *Bifidobacterium* and *Lactobacillus*. The growth and metabolic activity of these bacteria reduce the development of intestinal pathogens (strains of genera *Clostridium, Staphylococcus, Listeria, Escherichia, Salmonella, Shigella* and *Klebsiella*) [6-8].

IMOs molecules are built of α-D-glucopyranose residues that are linked by α-1,6-glycosidic bonds (linear, isomaltose-like IMOs) or by α-1,6 and α-1,4 linkages (branched, panose-like IMOs). Branched IMOs are produced from starch treated with amylases, pullulanase and α-glucosidase [9-11] or from sucrose and maltose (acting as an acceptor of glucosyl residues) in a process catalyzed by dextransucrase [12-14]. Linear IMOs are derived from (i) linear dextran subjected to digestion by dextranase [16], (ii) sucrose – through dextransucrase-catalyzed reaction involving glucose or isomaltose as acceptors [17-18] and (iii) sucrose – in a process catalyzed by two enzymes: dextransucrase and dextranase [3, 19-21]. In the latter process, both the synthesis and hydrolysis of dextran take place. Because ultimate concentrations of isomaltose and isomaltotriose among IMOs depend on initial concentration of sucrose and the ratio of DS and DN activities, the process can be directed to promote the synthesis of one of these two sugars. Our earlier work focused on IMOs synthesis by the latter method by using either soluble or immobilized DS [20-21].

An interesting approach to IMOs synthesis from sucrose is a process catalyzed by co-immobilized DS and DN. Co-immobilization of these two enzymes has been hitherto described in only two publications. DS and DN (the latter adsorbed on various solid carriers) were entrapped in calcium alginate beads by Erhardt et al. [3] but these preparations were found to be unstable in isomaltose synthesis from sucrose. Ölçer and Tanriseven [22] used calcium alginate to co-immobilize DS and DN attached to Eupergit C with the relatively high recovery of activity (64-71%). These preparations had the form of capsules, fibers and beads. The capsules of co-immobilized DS and DN, which were obtained in the presence of starch, maintained their activity for 60 h of IMOs synthesis while the fibers and beads were unstable both during their usage and storage at 4°C.

Moulis et al. [23] demonstrated that isomaltooligosaccharides from DP 2 to 27 can be produced with a 58% yield by acceptor reaction with glucose using engineered *Leuconostoc mesenteroides* NRRL B-512B dextransucrase variant.

This study aimed at production of co-immobilized preparations of DS and DN and estimation of their operational stability during IMOs synthesis processes from sucrose.

Experimental

Materials

The enzymes, dextransucrase by *Leuconostoc. mesenteroides* L and dextranase by *Penicillium funiculosum* 72, were synthesized in Institute of Technical Biochemistry (Lodz University of Technology). The strain of *L. mesenteroides* L was purchased from KZF Polfa (Kutno, Poland), where it was employed for clinical dextran production. *P. funiculosum* 72 was from pure culture collection of Institute of Technical Biochemistry (Lodz University of Technology).

L. mesenteroides L was grown under stationary conditions for about 16h at 25ºC in a standard culture medium containing: sucrose (20 g l^{-1}), yeast extract (20 g l^{-1}), K_2HPO_4 (20 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g l^{-1}), NaCl (0.01 g l^{-1}), $MnSO_4 \cdot H_2O$ (0.01 g l^{-1}) and $CaCl_2$ (0.01 g l^{-1}) [24]. pH of the culture medium was adjusted to 6.8 prior to autoclaving (121ºC, 15 min). On completion of the culture the cells were harvested by centrifugation (10 000 × g, 20 min, 4ºC).

For dextranase production *P.funiculosum 72* was cultivated aerobically at 30ºC for 6 days in a modified Hultin and Nordstrom medium containing: native dextran (20 g l^{-1}), $(NH_4)_2SO_4$ (0.75 g l^{-1}), $NaNO_3$ (1.6 g l^{-1}), KH_2PO_4 (3.75 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (0.075 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.25 g l^{-1}), $MnSO_4 \cdot 4H_2O$ (0.045 g l^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.044 g l^{-1}) and $CuSO_4 \cdot 5H_2O$ (0.00125 g l^{-1}) [25]. For obtaining extracellular dextranase, mycelial pellets were filtrated. All chemicals and reagents are commercially available and were purchased from POCh (Gliwice, Poland) or Sigma - Aldrich (St. Louis, USA) or BD (San Jose, USA).

Synthetic hydroxyapatite (HAp) was produced in the Department of Technology of Ceramics and Fireproof Materials at Mining and Metallurgy Academy in Cracow (Poland), sodium alginate M (with medium viscosity) from *Macrocystis purifera* was purchased from Sigma-Aldrich (St. Louis, USA) Glutardialdehyde was procured from Merck (Darmstadt, Germany) and sucrose was bought in a local store.

Methods

Analytical methods

DS activity was determined by measuring the initial rate of fructose production using the basic dinitrosalicylic acid solution [26]. The reaction conditions were as follows: temperature of 30ºC, pH 5.4 (0.05 M sodium acetate buffer), 0.02 g l^{-1} $CaCl_2$ and 75 g l^{-1}sucrose.

One unit of DS activity denotes 1 micromol of fructose liberated per 1 min under the above reaction conditions.

DN activity assay was based on an increase in concentration of reducing sugars released from dextran 110 (16.7 g l^{-1}) dissolved in 0.05 M sodium acetate buffer solution, pH 5.4. The reducing sugars were quantified according to Somogyi-Nelson [27-28].

One DN activity unit denotes 1 micromole of isomaltose released from dextran 110 in 1 minute at 30°C and pH 5.4.
Protein concentration was determined according to Lowry et al. [29] by using BSA as a standard.

Cross-linking of DS

DS was partially purified by ultrafiltration through membrane with a cut-off of 10 000 Da and cross-linked with GA (its concentration was 5-fold lower than total protein concentration in the solution) at 4°C for 68h.

Adsorption of DN on hydroxyapatite

DN was partially purified by ultrafiltration through membrane with a cut-off of 10 000 Da. Adsorption of DN on HAp was carried out using 5 ml aliquots of DN solutions containing various amounts of protein (1-10 mg) and 100-500 mg portions of HAp. The process was conducted at 15°C, pH 5.4 (0.05 M sodium acetate buffer solution) and 150 rpm for 24 h. The immobilized DN preparation was separated on sintered glass funnel under reduced pressure and rinsed twice with sodium acetate buffer solution (20 ml, 0.05 M, pH 5.4). All the adsorption processes and the assays were conducted in at least triplicate.

Determination of activity of DN adsorbed on HAp

Activity of DN immobilized on HAp was assayed like the activity of free enzyme using 4 ml of substrate solution (dextran 110, 16.7 g l^{-1}) and approximately 0.02 g of the immobilized DN preparation. Reaction mixtures were incubated for 10 minutes at 30°C and 200 rpm. Then concentration of reducing sugars released from the substrate was determined.

Co-entrapment of DS and DN in calcium alginate

6% (w/v) aqueous solution of sodium alginate was mixed (in a proportion of 1:1, v/v) with a suspension of cross-linked DS and DN adsorbed on HAp. These blends were added dropwise (through a syringe) into 0.2 M $CaCl_2$ in 0.05 M sodium acetate buffer solution, pH 5.4. Resulting beads were hardened (with mixing) for 2 h and used in IMOs synthesis as co-immobilized preparations of DS and DN.

Operational stability of co-immobilized preparations of DS and DN

Operational stability of co-immobilized preparations of DS and DN was estimated based on amounts of isomaltose and isomaltotriose generated during successive 24h processes of IMOs synthesis from 10% (w/v) sucrose solutions with pH 5.4, conducted at 30°C and 200 rpm. The initial DS activity in the co-immobilized preparations was 0.7 U per 1 ml of substrate solution.

Quantification of sugars by HPLC

HPLC analyses of sugars were conducted by using a system Gold from Beckman (USA). The sugars were separated at 25°C on amino column (LiChrospher from LichroCART). The rate of mobile phase flow (a mixture of acetonitrile and water, 70:30, v/v) was kept at 0.7 ml/min and the sugars were quantified by using a refractometric detector working in a continuous mode.

Results and discussion

The synthesis of IMOs from sucrose using the co-immobilized DS and DN in alginate are economically advantageous. Such preparations can be easily recovered and reused. Enzyme/enzyme systems of co-immobilization are usually not entrapped within alginate, because this matrix retains only few enzymes [30]. Dextransucrase can be entrapped with very high efficiencies, since the dextran layer covering the protein surface prevents leakage of the enzyme through this pores of the matrix. Dextranase quickly released from alginate carrier and therefore has to be adsorbed onto a fine dispersed carrier, e.g. bentonite or starch derivatives, before it can be co-entrapped with DS. In addition, the immobilization of DN prior to co-entrappment restrict the access of this enzyme to DS by compartmentalization, so that DS denaturation is hindered [3].

In this study, hydroxyapatite, inorganic and water insoluble mineral belonging to the family of calcium phosphates was used as a support for immobilization of dextranase.

DN adsorption on HAp

Adsorption was conducted using 5 ml aliquots of DN solutions containing 1-10 mg protein (166 to 1660 U of DN activity) and various amounts of HAp (100-500 mg).

Table 1. Adsorption of dextranase on hydroxyapatite

HAp	166 U DN (1 mg protein)			830 U DN (5 mg protein)			1245 U DN (7.5 mg protein)			1660 U DN (10 mg protein)		
[mg]	ΣU[a]	U/g[b]	%[c]	ΣU[a]	U/g[b]	%[c]	ΣU[a]	U/g[b]	%[c]	ΣU[a]	U/g[b]	%[c]
100	42	271	25.3	68	442	8.2	92	596	7.4	98	615	5.9
200	57	192	34.3	134	422	16.2	174	572	14.0	180	586	10.8
300	68	144	41.0	207	394	25.0	265	536	21.3	270	560	16.3
400	69	98	41.5	228	356	27.5	321	504	25.8	337	545	20.3
500	70	81	42.0	250	275	30.0	370	446	29.7	410	502	24.7

[a]- The sum of DN activity units linked to the carrier

[b]- The activity of preparation expressed in U per 1 g of wet carrier

[c]- The yield of immobilization expressed as the recovery of initial DN activity

The analysis of the data from Table 1 shows that the amount of DN adsorbed on the support surface per 1 g of the HAp matrix depended on both the amount of this adsorbent and the initial activity of DN.

The greatest amount of DN (615 U) per gram of the HAp was immobilized from the DN solution containing 10 mg of protein (1660 U DN) and 100 mg HAp. The lowest DN concentration on HAp matrix (81 U g^{-1}) was obtained when adsorption was performed on 500 mg HAp from the DN solution containing 1mg of protein (166 U of DN).

The yield of the process is not correlated with the amount of immobilized enzyme. Adding the same amounts of HAp to a series of DN solutions containing increasing amount of protein caused a significant reduction in the efficiency of enzyme adsorption process. This decrease is due to the fact that adsorbent has

a fixed number of active sites and at higher concentration of proteins, the active sites become saturated. In this study, the highest yield of 42% was obtained for adsorption from the enzyme solution containing 1 mg of protein and 500 mg HAp. The lowest yield of about 6% was obtained for the process performed from the enzyme solution containing 10 mg of protein and 100 mg of HAp.

Co-entrapment of DS and DN adsorbed on HAp

According to Erhardt et al. [3] certain solid carriers (bentonite, hydroxyapatite, starch derivative) inactivated DS when used for immobilization of this enzyme. The weakest inactivating effect was exerted by hydroxyapatite, although at its high content (92 mg HAp g^{-1} Alg) DS activity was approximately halved after 48 h contact. Our experiments revealed that the applied HAp preparation was completely inert for DS activity, even in the amount of 2 g HAp g^{-1} Alg.

Co-immobilized preparations were obtained by mixing suspensions of cross-linked DS (with activity of 1.4 U ml^{-1}) and DN adsorbed on HAp (preparation activity of 394 U DN g^{-1} HAp) at the ratio of DS/DN activities ranging between 1/4 and 1/16. Then these blends were entrapped in 3% (w/v) calcium alginate. Resulting preparations were used in IMOs synthesis in 10% sucrose solutions. Data presented in Fig. 2 demonstrate that when DN activity in the co-immobilized preparation increased, isomaltose content in the mixture of IMOs synthesis products also rose while isomaltotriose concentration was decreased. The sum of these two sugars was kept at almost constant level.

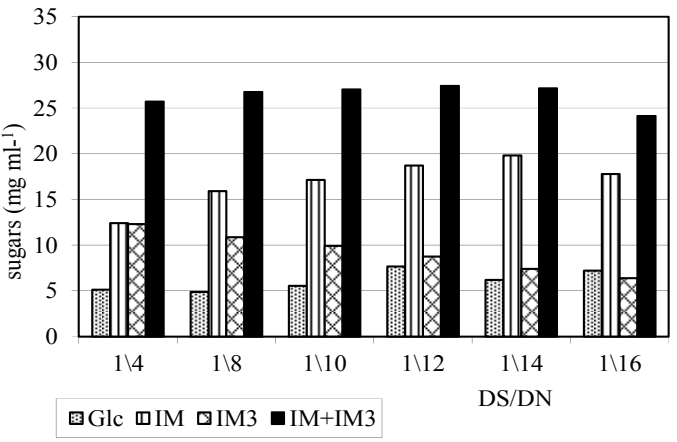

Figure 2. Concentrations of sugars (the average of triplicate samples) in mixtures of IMOs synthesis products obtained in the first batch in 10% (w/v) sucrose solutions (processes were catalyzed by co-immobilized DS and DN adsorbed on HAp, DS activity was kept constant while DN activity was changed)

All produced co-immobilized DS and DN preparations were used in IMOs synthesis processes. Results for the most stable of these preparations (with DS/DN = 1/14) are presented in Fig. 3. The mixtures of reaction products contained 20-16 mg ml^{-1} isomaltose and 9-7 mg ml^{-1} isomaltotriose. The degree of sucrose conversion reached 73-70%.

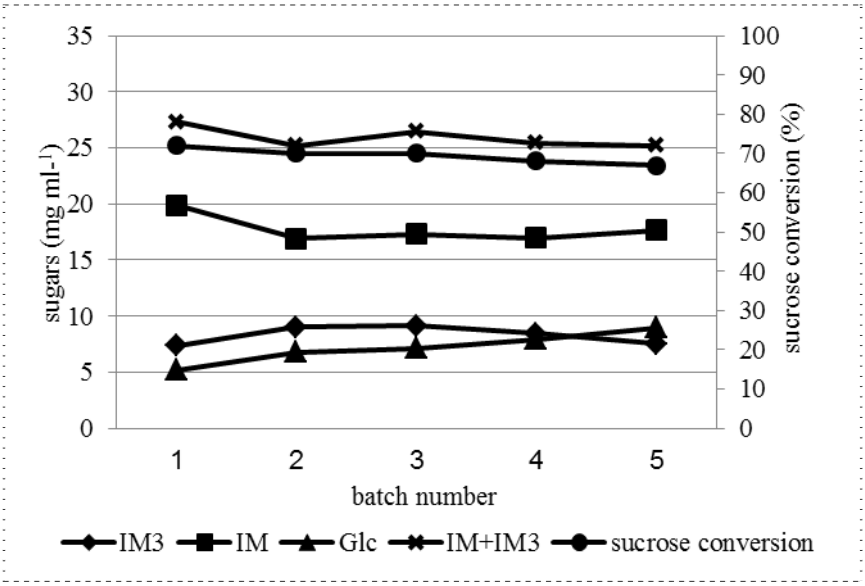

Figure 3. Concentrations of sugars (the average of triplicate samples) in mixtures of products of IMOs synthesis in 10% (w/v) sucrose solutions catalyzed by co-immobilized DS and DN (at DS/DN of 1/14, DS activity before co-entrapment of 1.4 U ml^{-1}, DN preparation with activity of 394 U g^{-1} HAp, co-immobilized preparation contained 0.83 g HAp g^{-1} AlgNa)

Co-immobilized preparations displaying the same DS and DN activities as aforementioned ones (DS/DN = 1/14) but containing different amounts of HAp used to adsorb DN (100, 300 and 500 mg, corresponding to 0.74 g HAp g^{-1} AlgNa, 1.12 g HAp g^{-1} AlgNa, and 1.99 g HAp g^{-1} AlgNa, respectively) were also used in IMOs synthesis processes.

Results obtained in successive experiments, which are shown in Fig. 4, indicate that different contents of HAp in the co-immobilized preparations had only a slight effect on the yield of IM and IM_3 synthesis and slightly higher amounts of these sugars were generated when HAp content in calcium alginate was higher. Presumably, higher content of HAp caused that DN adsorbed on this matrix was better dispersed in calcium alginate matrix and therefore the dextran chains synthesized by DS had easier access to the hydrolase.

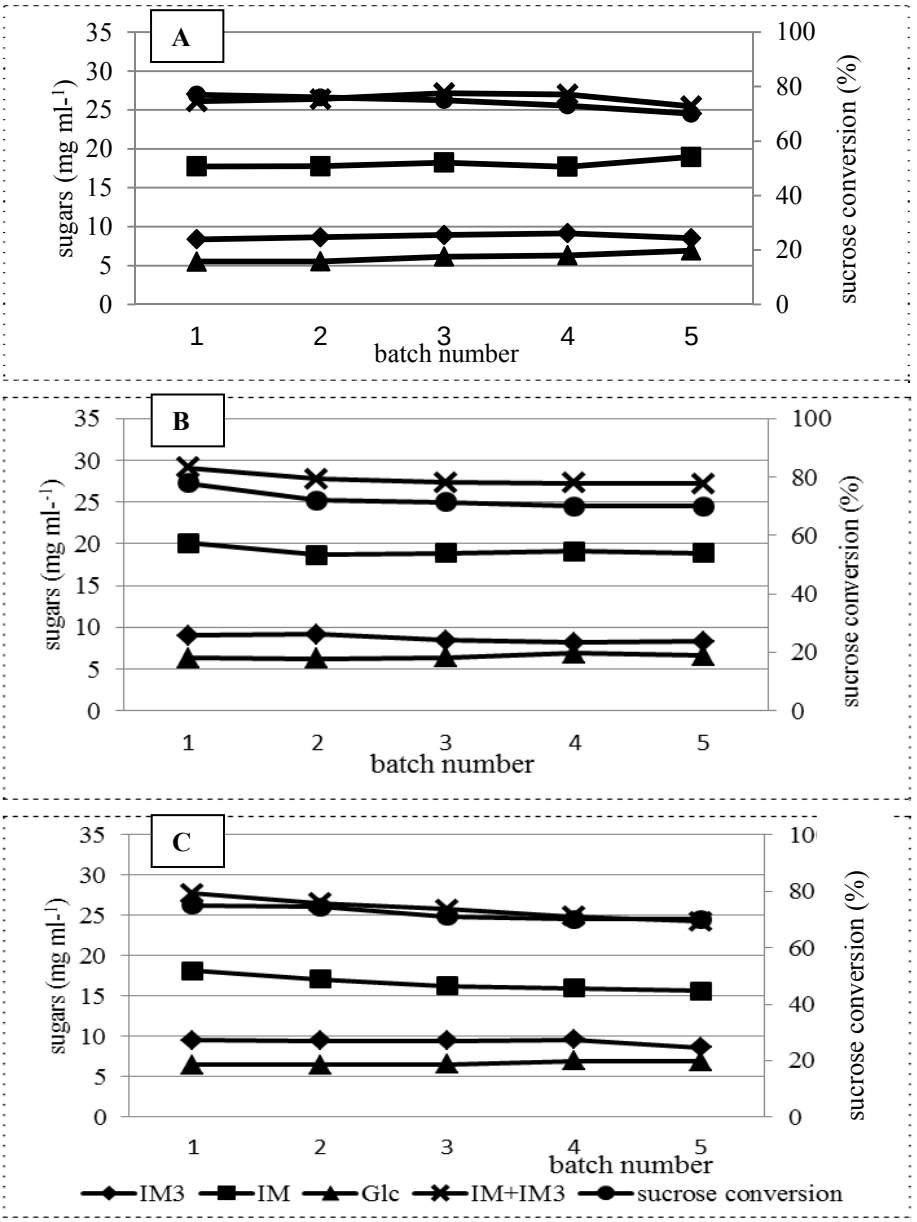

Figure 4. Concentrations of sugars (the average of triplicate samples) in mixtures of products of IMOs synthesis in 10% (w/v) sucrose solutions catalyzed by co-immobilized DS and DN preparations (at DS/DN = 1/14, DS activity before co-entrapment of 1.4 U ml^{-1}) containing various amounts of HAp with adsorbed DN:
A: 1.99 g HAp g^{-1} AlgNa; **B**: 1.12 g HAp g^{-1} AlgNa; **C:** 0.74 g HAp g^{-1} Alg

Erhardt et al. [3] who synthesized IMOs using DS and DN (adsorbed on HAp) co-entrapped in calcium alginate, found that the highest amounts of isomaltose were formed when the initial ratio of DS/DN activities in the preparation was of 1/0.3 (the authors did not cross-link the DS). At higher DN activities in the co-immobilized preparations, the leakage of DS from alginate matrix was observed, which in turn caused a decrease in IM synthesis yield. In our study, the highest amounts of isomaltose were generated when synthesis processes were catalyzed by the preparation with the initial ratio of DS/DN activities of 1/14. This high DN activity could be mixed with DS because the first enzyme was adsorbed on HAp and the second was cross-linked before the co-immobilization. This in turn caused the spatial separation of both the enzymes.

Preparations containing more HAp with adsorbed DN were slightly more stable (Fig. 4A and 4B) than these, which contained less adsorbent. Concentrations of IM and IM_3 synthesized by co-immobilized DS and DN preparations in five successive batches was similar (approximately18.5 mg ml^{-1} and 8.5 mg ml^{-1}, respectively; the sum of around 27 mg/ml) and the degree of sucrose conversion was kept at around 70%. The results demonstrate the high operational stability of preparations of DS and DN co-immobilized in calcium alginate, which may be used at least 100 h of IMOs synthesis from sucrose.

Co-immobilized preparations of DS and DN (alginate beads and fibers) obtained by Ölçer and Tanriseven [22] were unstable and lost approximately 80 and 30% activity, respectively, after 15 h of operation. Only when DS and DN were immobilized in the presence of starch in alginate capsules, the preparations were stable and maintained their whole initial activity after 60 h application.

The our procedure involves cross-linking of dextransucrase and adsorption of dextranase to hydroxyapatite surfaces prior co-entrapment in calcium alginate was very satisfying.

Conclusion

IMOS present prebiotic properties and can be produced by dextransucrase and dextranase from sucrose in dual enzyme systems, In this study, we used the co-immobilized enzymes to synthesis IMOs from sucrose. Co-immobilized preparations were obtained by mixing suspensions of DS cross-linked with glutardialdehyde and DN adsorbed on HAp at the ratio of DS/DN activities ranging between 1/4 and 1/16 and then these blends were entrapped in calcium alginate. The result of our study are stable preparations of DS and DN (the most stable with DS/DN = 1/14) which may be used at least 100 h of IMOs synthesis from sucrose.

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