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Dextran synthesis by immobilized dextran sucrase.

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Résumé.

La production de dextransucrase (E.C. 2.4.1.5.) par fermentation de *Leuconostoc mesentéroïde* NRRL B-512 a été étudiée avec et sans addition continue de sucrose. La préparation enzymatique a été concentrée et purifiée. Son activité spécifique a été augmentée par addition de chlorure de calcium. Cette préparation a été immobilisée par liaison covalente sur un support de silice poreuse (Sphérosil) activé par du glutaraldéhyde.

Une activité de 830 unités par g de support a été obtenue avec des dérivés de dextransucrase immobilisés.

Summary.

Dextran sucrase has been produced by fermentation of Leuconostoc mesenteroides NRRL B-512, with and without continuous sucrose addition to improve enzyme production. The enzyme preparation has been concentrated from the fermentation broth by ultrafiltration and purified by gel permeation chromatography on Ultrogel. The specific activity of the dextran sucrase was greatly enhanced by calcium chloride addition to the purified enzyme. This enzyme preparation has been immobilized by covalent coupling onto an amino porous silica support (Spherosil) activated with glutaraldehyde. Immobilized dextran sucrase derivatives with an activity up to 830 dextran sucrase units per g. support could thus be obtained. The effect of the support specific area on coupling efficiency and reaction kinetics has been investigated, and the effect of intraparticular diffusion underlined. The molecular weight distribution of the dextran has been determined when varying several parameters.

Introduction.

Dextran sucrase produced from Leuconostoc mesenteroides NRRL-B 512 is a transglycosylase that catalyzes the polymerization of dextran from sucrose. The numerous industrial applications of this polysaccharide justifies the great number of publications dealing with the fermentation conditions for maximal dextran sucrase production [1-3].

The enzyme with a molecular weight of 280,000 [4] can be recovered from fermentation broth by alcohol [5] or ammonium sulphate [6] precipitation, or concentrated by ultrafiltration [7].

The reaction mechanism of enzyme action has been demonstrated by Robyt *et al.* [8-12] : the enzyme forms an active complex with glucose and dextran, and glucose units are inserted between the enzyme and the reducing end of the dextran chain, forming an α [1-6] glucosidic bond. Branching accounts for 5 per cent of dextran bonds.

The molecular weight of the dextran produced can be affected by several parameters ; the presence of sugars of low molecular weight, called acceptors, serve to terminate polymerization of dextran displacing the growing chain from the active site of the enzyme, therefore diminishing the molecular weight. Temperature as well as sucrose and enzyme concentration have also been reported as affecting the molecular weight of dextran [5].

In the present work we have studied the production of dextran sucrase in a batch system with continous feeding of sucrose, the only enzyme inductor known. We have purified the fermentation broth in order to have an enzyme preparation with a high specific activity that could be immo-

bilized on a solid support. Such a derivative could be used for the continuous polysaccharide production with a better control, particularly of the molecular weight of the dextran synthesized.

Materials and Methods.

Culturing of Leuconostoc mesenteroides. The enzyme was obtained by culturing Leuconostoc mesenteroides NRRL-B 512 in 3 l of medium containing sucrose (2 per cent, yeast extract (2 per cent), K_2HPO_4 (2 per cent) or $CaCl_2$ (0.005 per cent) and growth factors as has already been described [3] ; pH was regulated with 10 N NaOH and temperature at 30°C. In some experiments a solution of ~ 1 g/ml of sucrose was continuously fed at different rates.

Purification. Cells from fermentation broth were centrifuged in a Beckman J-21 centrifuge at 12,100 g and the supernatant immediatly ultrafiltered in a Sartorius filter unit with 4 membranes 16 × 16 cm, SM 12134 in parallel, to obtain a 10-12-fold concentration. The enzyme was then purified by gel permeation chromatography on Ultrogel AcA-34 in a 4 cm diameter, 1 m long column with citrate buffer (pH 5) as elution agent. Dextran sucrase is eluted in the void volume.

Enzyme activity. Enzyme activity was mesured determining the initial rate of fructose production by the dinitrosalicylic acid method [12], under the conditions previously described [13]. The absence of invertase and levan sucrase contaminating activities was verified by HPLC analysis of the sugars produced during enzymic reaction on a μ-Bondapak NH_2 column (Waters Associates). Protein concentration was mesured by the Lowry method with bovine albumin as standard. One dextran sucrase unit (DSU) is defined as the amount of enzyme which converts 1 mg of sucrose in one hour to dextran under the conditions of assay (one unit reported to the liberation of 1 μmole of fructose per minute corresponds to 20.52 DSU).

Enzyme immobilization. The preparation of purified dextran sucrase was immobilized by covalent coupling onto an amino porous silica support (Spherosil, Rhône Poulenc), activated with 2 per cent glutaraldehyde in a pyrophosphate buffer at pH 8.6 [14-15]. Several specific areas were employed : 6, 24, 50, 122 and 445 m^2/g. The activated support (100-200 mg) was agitated during 24 hrs at 4°C with 10-20 ml of purified enzyme. After immobilization, the support was washed several times with citrate buffer and with 1.0 M NaCl, and the activity measured.

Dextran analysis. Molecular weight distributions of dextran were determined by gel permeation chromatography on Agarose Ultrogels A 2 (fractionation range 120,00 - 25,000,000) and A 6 (25,000 - 2,400,000) with 0.3 per cent as elution agent. Dextran was detected and measured by the anthrone method [17].

Experimental Results and Discussion.

Dextran sucrase production and purification. 3 l of a culture of Leuconostoc mesenteroides were produced in each experiment from a 3-5 per cent inoculum. Initial pH was adjusted to 6.9, agitation to 280 rpm and a mild aeration of 0.06 VVM provided. In 6-7 hours all the substrate was consumed and the stationary phase reached. Under such conditions dextran sucrase production follows cell growth and reaches 20-30 DSU/ml when

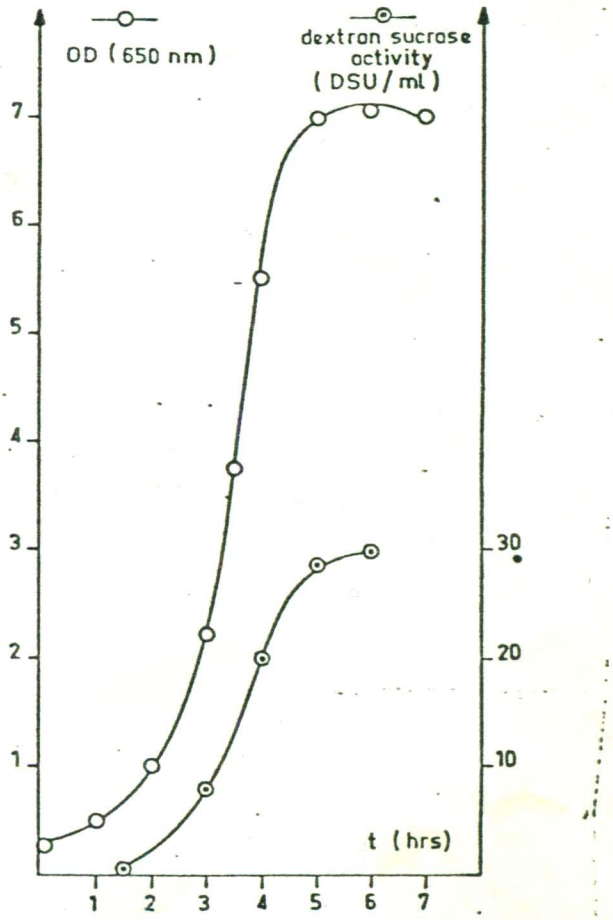

FIG. 1. — *Production of dextran sucrase in batch fermentation of* Leuconostoc mesenteroides.

the fermentation is finished. We have calculated a rate of substrate consumption of 10-11.2 g of sucrose/l.hr during the exponential growth and a specific growth rate of 0.8 hr^{-1} (figure 1).

In order to activate enzyme induction we fed a sucrose solution of 1 g/ml at different rates regulating the pH at 6.6-6.7 to minimize dextran production. Biomass production was almost doubled and enzyme production higly increased (figure 2). The addition was carried out during the logarithmic phase and stopped when the cells reach the stationary phase.

The effect of continuous sucrose addition on enzyme production is given in table I .

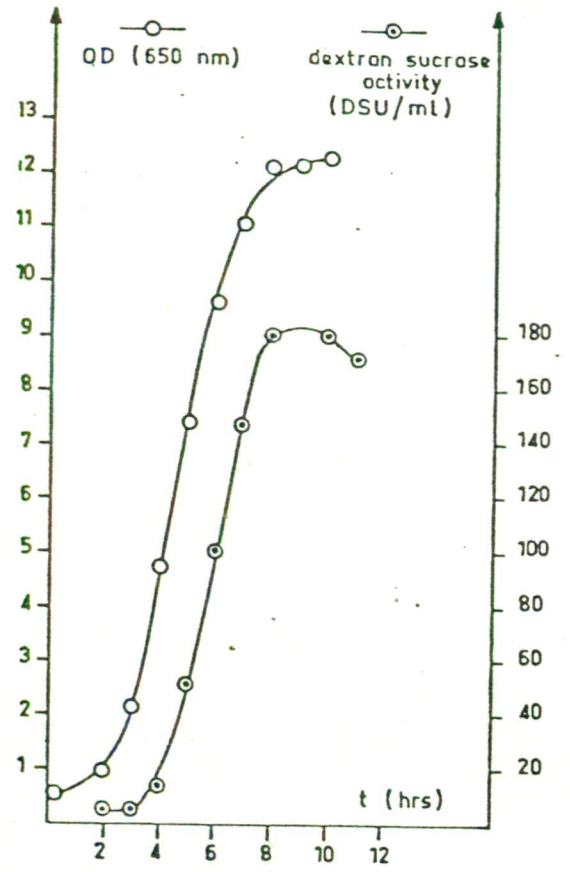

FIG. 2. — *Production of dextran sucrase in batch fermentation with continuous addition of sucrose.*

Sucrose (1 mg/ml) was added continuously (20 $g.l^{-1}.h^{-1}$) to the fermentation medium.

Initially we tried to recuperate dextran sucrase from the fermentation broth by ethanol precipitation ; although a concentrated enzyme solution can be obtained, it forms insoluble active agglomerates that prevent from any further purification. The contact of this solution with solid supports accelerates the agglomeration process. We therefore concentrated dextran sucrase by ultrafiltration followed by purification by GPC on Ultrogel AcA-34. The enzyme is eluted in the void volume. Robyt and Walseth [10] added dextranase to hydrolyze dextran from the enzyme-dextranosyl complex in order to separate dextran sucrase from invertase and levan sucrase contaminating activities, but the dextran sucrase then looses stability and only 33 per cent of initial activity (before GPC) is recovered. As HPLC analysis dit not show any significant amount of contaminating activities in the purified solution, we purified the enzyme without any pretreatment. In figure 3 the diagram of elution shows the clear separation between dextran sucrase and the rest of the proteins in the fermentation medium.

TABLE I.

Enzyme production by Leuconostoc mesenteroides. Effect of sucrose added during exponential growth.

Rates of sucrose addition (g/l.hr)	Enzyme activity in fermentation broth DSU/ml
—	30
4.4	41
4.4 — 18 (b)	120
20	180
20 (a)	100
0.4 (a)	65

(a) 0.005 per cent of $CaCl_2$ was used (K_2HPO_4 omitted).
(b) the rate was increased during the addition.

Enzyme production is enhanced by sucrose addition to the fermentation broth but an important amount of dextran is also produced ; as concentrating this solution would increase the medium viscosity, the enzyme was purified by GPC without ultrafiltration. We have summarized in table II the purification results of several experiments. The presence of dextran increases enzyme stability during the purification procedure as can be observed from the percent of activity recovered.

The presence of 0.05 per cent of $CaCl_2$ in the enzyme solution has been shown to stabilize dextran sucrase [15]. The fermentation broth prepared with 0.005 per cent $CaCl_2$ does not present any modification in activity by further 0.05 per cent $CaCl_2$ addition ; but the purified enzyme activity is very strongly affected. For instance a purified solution increases from 12.6 DSU/ml to 33.9 DSU/ml on 0.05 per cent $CaCl_2$ addition, so that 86 per cent of initial activity is recovered instead of 33 per cent calculated without $CaCl_2$ addition.

The kinetic characterization of the purified enzyme was carried out. In table III the initial rates of fructose production are reported as a function of substrate concentration. The Km for the free enzyme was calculated applying the Eadie-Hofstee model and was found to be 0.018 M.

Dextran sucrase immobilization.

We have immobilized dextran sucrase on porous silica (Spherosil) of different porosities. Results are shown in figure 4, reported as the activity per gram of silica for 5 different specific areas ; for each case 100 mg of silica were contacted with 15 ml of a purified enzymic preparation containing 130 DSU/ml. It is clear from these results that a support with a low specific area is required. Subsequent experiments were carried out with silica of 6 and 24 m^2/g. In table IV some results are shown as well as the effect of maltose addition to the enzyme-support mixture during immobilization

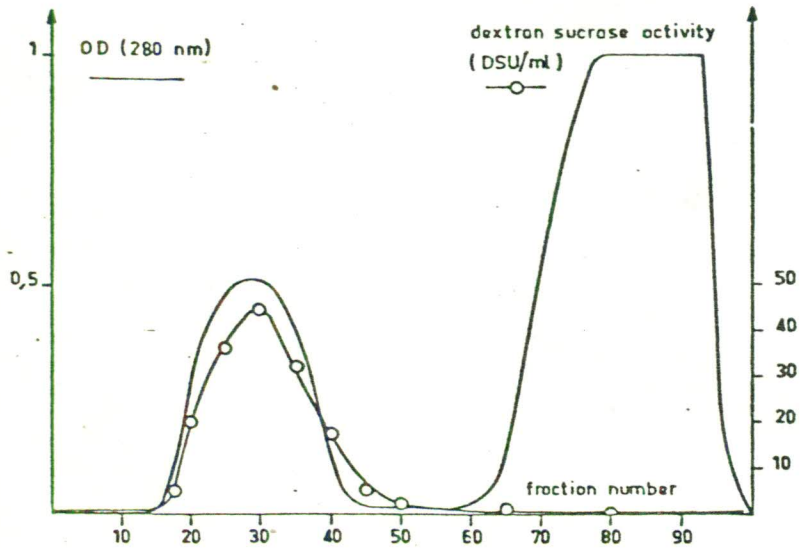

FIG. 3. — *Elution diagram of dextran sucrase on Ultrogel AcA 34.*

Dextran sucrase can be immobilized when pores are wide enough to allow not only the enzyme fixation but also the diffusion of dextran chains and sucrose. The best results were obtained for 6 m^2/g which corresponds to an average pore diameter of 550 nm. For 24 m^2/g two different rates

TABLE II.

Dextran sucrase purification.

		Volume (ml)	Activity DSU/ml	Specific activity DSU/mg	Per cent recovery (a)
Batch fermentation	Supernatant	2,500	24.5	8.63	—
	UF solution	185	93	23.8	51
	150ml ultrogel	189	18.4	613	25
Batch fermentation with 0.005 per cent $CaCl_2$	Supernatant	2,830	12.3	3.4	—
	UF solution	270	87.4	16.5	68
	175ml ultrogel	234	37.4	720	57
Batch fermentation with 0.005 per cent $CaCl_2$ 2 per cent sucrose added 0.4 $gl^{-1}h^{-1}$	Supernatant	2,500	26	4.33	—
	UF solution	230	193	26.2	68
	200ml ultrogel	72	54.3	862	
		89	130	1340	74
Batch fermentation with 0.005 per cent $CaCl_2$ 100 per cent sucrose added 10 $gl^{-1}h^{-1}$	Supernatant	2,500	100	20.8	—
	150 ml ultrogel	175	70.2	2400	82

(a) for all activity measurements 0.05 per cent $CaCl_2$ was added to the purified enzyme preparation.

were observed (figure 7) : an initial velocity which may corresponds to the action of enzyme on the surface and in the pores, and after approxi-

TABLE III.

Initial rates of reaction as a function of sucrose concentration.

Sucrose concentration $g l^{-1}$	Initial rate of reaction g fructose/l.hr
1	0.48
2.5	1.33
5	2.34
7.5	2.86
10	3.47
20	4.36
40	5.24
50	5.35
65	5.11
70	5.39

(Activity of dextran sucrase solution : 28.5 DSU/ml).

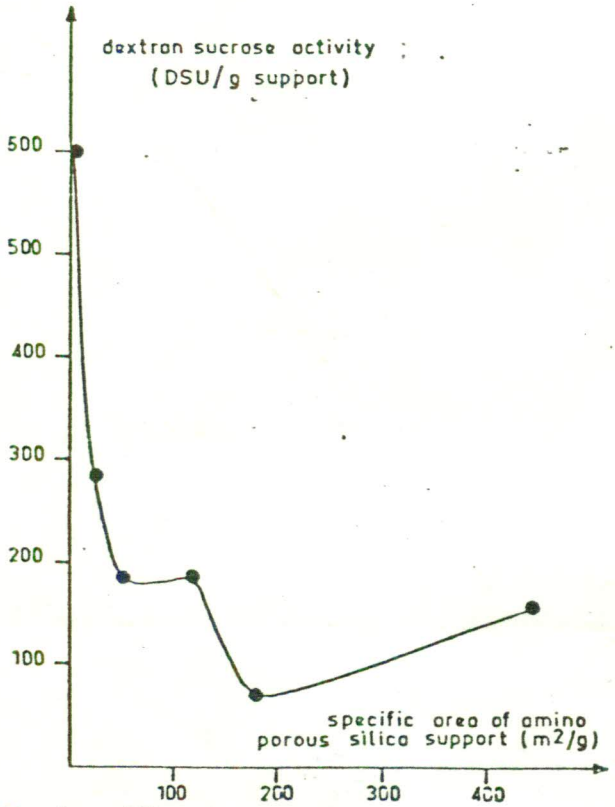

FIG. 4. — *Effect of specific area on activity of immobilized dextran sucrase.*

matively 30 min a reduction in reaction rate due to blocked pores by the dextran produced, leaving only the enzyme on the surface active.

As we increased the specific area we reduced the size of pores and, although the enzyme can be immobilized in this zone, as shown by the loss of activity in the solution in contact with the sup-

TABLE IV.

Dextran sucrase immobilization onto glutaraldehyde activated porous silica.

Specific area of silica support (m^2/g)	Amount of support (mg)	Per cent of activity recovered	Activity of immob. dextran sucrase DSU/g silica
6	100	3.1	605
6	100(*)	4.3	830
24	100	1.8	287
24	100(*)	3.25	634

(15 ml) of enzyme solution of 130 DSU/ml ; specific activity $= 1340 \frac{\text{DSU}}{\text{mg}}$.

(*) 200 mg of maltose added during enzyme immobilization.

port, only the enzyme on the surface remains active. For high areas (445 m^2/g) pores are so small (average diameter of 7 nm) that it is impossible even for the enzyme to penetrate.

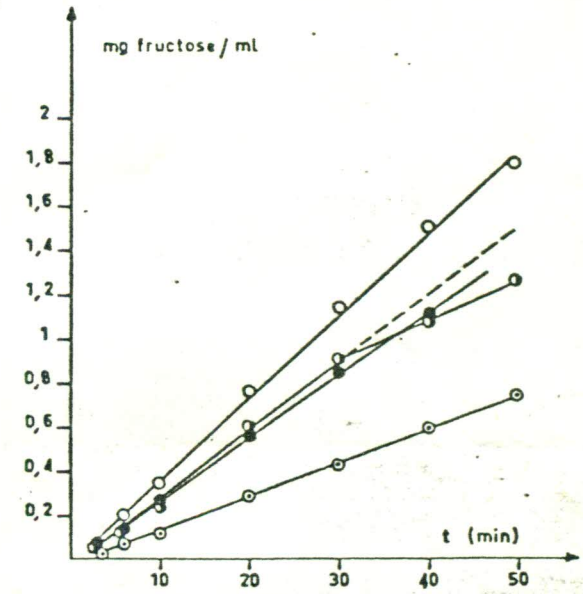

FIG. 5. — *Initial rate of fructose production.* (● 6 m^2/g, 605 DSU/g ; ○ 6 m^2/g, 830 DSU/g ; ⊙ 24 m^2/g, 287 DSU/g ; ◑ 24 m^2/g, 634 DSU/g).

The presence of maltose greatly increases enzyme fixation. As has been shown [10], maltose acts as an acceptor of dextran chains, liberating

them from the enzyme-dextranosyl complex. It therefore increases the possibilities of enzyme-support contact eliminating the steric hindrance of dextran.

It was thus possible to obtain an immobilized dextran sucrase derivative with 830 DSU/g support. In previous studies on dextran sucrase immobilization, activities ranging from 10.4 DSU/g of porous silica [16] to 300 DSU/g of Bio-Gel P2 [8], had been obtained.

Characterization of synthesized dextran.

In the present work we compared the molecular weight distribution (MWD) of the dextran produced by free and immobilized dextran sucrase. All samples were eluted in the void volume of an Ultrogel A 6, which means that their molecular weight is well over 10^6. In figure 6 we compare the MWD of dextran synthesized by purified dextran sucrase, with dextran synthesized by the immobilized enzyme (GPC on Ultrogel A 2). Both dextran synthesis were carried out at 30°C, with 10 per cent sucrose, at the same enzyme activity level.

We found a clear increase in molecular weight of dextran produced by the immobilized enzyme. This phenomenon can be readily explained by diffusional limitations that prevent the acceptor molecules to approach the enzyme-dextranosyl complex, favouring the growth of dextran chains. The viscosity of the reaction medium is increased as dextran is synthesized.

Conclusion.

It was possible, by continuous addition of a concentrated sucrose solution to a Leuconostoc mesenteroides culture, to increase 6-fold the dextran sucrase production. The supernatant of the fermentation broth could thus be directly purified by GPC on Ultrogel AcA 34. This may constitute, for the obtention of a dextran sucrase preparation suitable for immobilization, an alternative route to enzyme concentration by ultrafiltration followed by GPC purification.

Dextran sucrase coupling onto a glutaraldehyde activated amino silica, yields a highly active derivative when using a low specific area support (6 m^2/g). This may be attributed to (i) the absence of any steric hindrance phenomenon (found for higher specific areas) at the enzyme level and (ii) the less important internal diffusional limitations, as shown by the kinetic results obtained for a 24 m^2/g specific area support. Dextran sucrase coupling efficiency was greatly increased by maltose addition during immobilization, as maltose releases dextran for dextran enzyme complexes.

The presence of diffusional limitations may also account for the increase of the molecular weight distribution of the dextran produced with the immobilized enzyme. Anyway, the application of dextran sucrase covalently bound to porous silica to continuous dextran synthesis, seems to be more suitable for low molecular weight polysaccharides production, when considering the high viscosity of high molecular weight dextran solutions.

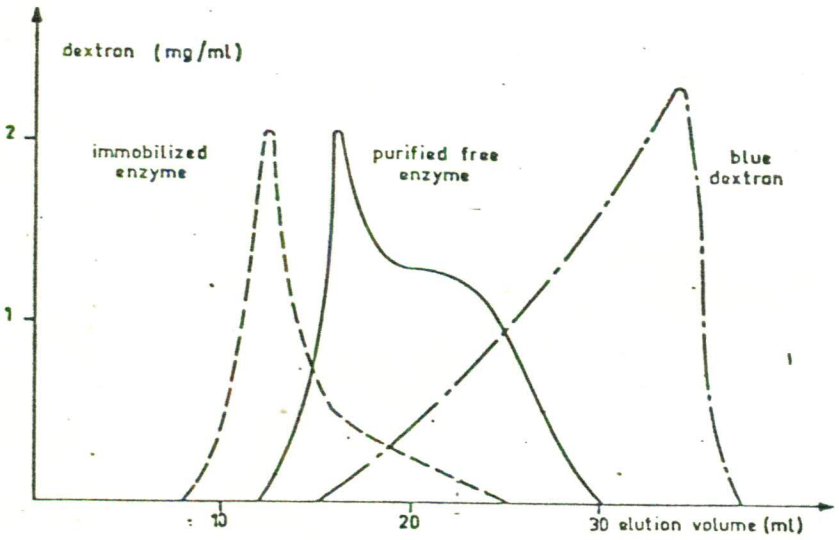

FIG. 6. — *Elution diagram of dextran produced by free and immobilized dextran sucrase.*

The conditions of dextran synthesis (enzyme activity, substrate concentration, pH, temperature) were the same for both free and immobilized dextran sucrase preparations.

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