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Production and properties of a dextransucrase from *Leuconostoc mesenteroides* IBT-PQ isolated from '*pulque*', a traditional Aztec alcoholic beverage

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Dextransucrase was produced from a *Leuconostoc mesenteroides* isolated from *pulque*, a traditional Aztec alcoholic beverage produced from agave juice containing sucrose as the main carbon source. Almost all the dextransucrase activity (87%) was associated with the cells, and was unusually high (1.04 U mg^{-1} of cells). The culture medium composition was optimized through a Box-Behnken method resulting in a process yielding 2.2 U ml^{-1} of insoluble glucosyltransferase activity. The enzyme had a molecular weight of 166 kDa. Optimal temperature was 35°C with a half-life of 137 min at the same temperature. As with dextransucrase from the industrial strain *L. mesenteroides* NRRL B-512F, the enzyme showed Michaelis–Menten kinetic behavior with excess substrate inhibition (K_m and K_i values of 0.026 M and 1.23 M respectively); produced soluble linear dextran with glucose molecules linked mainly in α(1–6) with branching in α(1–3) in a proportion of 4:1 as shown by NMR studies; and produced a high yield of isomalto-oligosaccharides in the presence of maltose.

Keywords: dextransucrase; *Leuconostoc mesenteroides*; *pulque*; glucosyltransferase

Introduction

'*Pulque*' is an ancient Mexican alcoholic beverage that was mastered by the Aztecs in the XIIIth century. It is produced by fermentation of the juice of various agave species, but mainly *Agave atrovirens*. Although more than 30 microbial genera have been identified in *pulque*, three main fermentations take place in the process: an alcoholic fermentation (carried out by *Zymomonas mobilis* and various yeasts like *Saccharomyces cerevisiae*), a lactic fermentation and a 'viscous fermentation' in which *Leuconostoc mesenteroides* produces dextran, giving the beverage its 'gummy' texture [21]. Although some attempts have been made to produce *pulque* from pure cultures, its production is still carried out by spontaneous fermentation [4,19].

Dextran is a d-glucose polymer composed mainly of α(1–6) linkages in the linear chain and α(1–2), α(1–3) and/or α(1–4) branch linkages. Various species of the genera *Leuconostoc*, *Lactobacillus* and *Streptococcus* synthesize an extracellular and in some cases, cell-associated dextransucrase (EC. 2.4.1.5), a glucosyltransferase responsible for dextran synthesis from sucrose. The chemical structure of dextran is highly specific to the glucosyltransferase. For instance, dextran produced from *L. mesenteroides* NRRL B-512F contains 95% α(1–6) linkages and 5% α(1–3) [16] whereas the NRRL B-1355 strain produces two glucosyltransferases: one similar to the dextransucrase already described and a second enzyme known as alternansucrase, as the polymer alternates α(1–6) and α(1–3) linkages in its main chain [2]. A third example is the strain *L. mesenteroides* NRRL B-1299 from which a gene coding for dextransucrase producing a linear dextran has been cloned in *E. coli* [13]; however, the NRRL B-1299 strain is mainly known for its cell-associated dextransucrase activity responsible for α(1–2) linkages in the polymer structure [15]. Dextransucrases are also known for their oligosaccharide synthesis activity. In the presence of efficient acceptor sugars such as maltose, the reaction is shifted towards oligosaccharide synthesis.

The isolation of dextran-producing strains from *pulque* was undertaken both as a search for glucosyltransferases with properties different from those already described in dextransucrases, and also to describe and understand the properties of this traditional fermentation product. Among the isolated microorganisms, a *Leuconostoc mesenteroides* strain was identified producing large amounts of insoluble dextransucrase activity. In this investigation the production and properties of a dextransucrase from this strain are described.

Materials and methods

Screening of polymer-producing strains

As dextransucrase is a sucrose-inducible enzyme, the screening process was designed to isolate strains producing polymers from sucrose. The 'gummy' colonies able to grow on sucrose were subcultured in glucose disregarding those producing polymers in both sugars. The culture media were similar to the one reported by Tsuchiya *et al* [21] containing (g L^{-1}): sucrose or glucose 20; yeast extract (Prolabo Distribution, Paris, France) 20; K_2HPO_4 20; $MgSO_4 \cdot H_2O$ 0.2; $CaCl_2 \cdot 2H_2O$ 0.05; NaCl 0.01; $MnSO_4 \cdot H_2O$ 0.01 and $FeSO_4 \cdot 7H_2O$ 0.01. Agar (2%) was included for solid media. The pH was adjusted to 6.9. Samples of *pulque* were collected in the cities of Apan and Singuilucan, in the state of Hidalgo, in Mexico; 10 ml of *pulque* were transferred to

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100 ml of sucrose medium. The flasks were incubated at 30°C at 200 rpm for 48 h. At the end of the incubation period the cell suspension was diluted 1/1000, plated on a solid sucrose medium and the plates were incubated at 30°C for 24 h. After incubation the viscous colonies were selected and propagated on the liquid sucrose medium at 30°C and 200 rpm for 24 h. From this culture the isolates were plated again on the solid media containing sucrose and glucose as described.

Identification of microorganism

The biochemical identification of isolates was carried out by means of the API 50 CH Kit (BioMerieux SA, Marcy l'Étoile, France). The experimental results were analyzed with the software package APILAB Plus V3.2.2 (Version B 01.93-D.09, BioMerieux SA). One of the isolated strains was identified as *Leuconostoc mesenteroides* and is referred to as *L. mesenteroides* IBT-PQ. It was maintained as a glycerol stock (30% v/v) in vials containing 5 ml at 4°C. All fermentations were started by inoculating 50 ml of culture with the contents of a glycerol vial.

Enzyme production

L. mesenteroides IBT-PQ was inoculated into 50 ml of sucrose medium in a 250-ml Erlenmeyer flask and incubated at 30°C and 200 rpm for about 8 h. The fermentation was followed by measuring the optical density (OD) at 650 nm after a 1/10 dilution. At the end of the fermentation the culture broth was centrifuged and the cells washed with acetate buffer (50 mM, pH 5.4). Both the supernatant phase and the cell suspension were assayed for enzyme activity. A calibration curve was prepared to correlate the optical density and the number of cells. Cells were counted in a Coulter Multisizer II, Coulter Electronics, Luton, UK.

Dextransucrase activity was measured by following the initial release of reducing sugars with the DNS method [20]. After dilution, the enzyme was incubated in a 12-ml reaction mixture containing 10% w/v sucrose in acetate buffer (50 mM, pH 5.4) at 30°C. Samples (500 μl) were withdrawn at 5-min intervals for 20 min and immediately poured into the DNS reagent for assay of reducing power (fructose). One unit of dextransucrase activity is defined as the amount of enzyme releasing 1 μmol of fructose per minute. Protein was measured as described by Lowry *et al* [11].

Experimental design for optimization of the culture medium

The concentration of sucrose (X_1 at 15, 20, 25 g L^{-1}), yeast extract (X_2 at 10, 20, 30 g L^{-1}) and K_2HPO_4 (X_3 at 10, 20, 30 g L^{-1}) were chosen as the critical variables in a 3^3 experimental design (Box-Behnken [1]), in order to optimize the production of biomass and dextransucrase. All experiments were conducted in duplicate.

Properties of Leuconostoc mesenteroides IBT-PQ dextransucrase associated with the cells

The optimum temperature was determined by measuring the activity of a known amount of cells at different temperatures at standard conditions. Thermal stability was evaluated by measuring the residual activity of cells stored at various temperatures in 50 mM acetate buffer (pH 5.4). All samples were assayed for activity at 30°C. The kinetic constants were evaluated by measuring the initial reaction rate at different sucrose concentrations.

Synthesis and characterization of oligosaccharides

Reactions were carried out using 2 U ml^{-1} of dextransucrase associated with *L. mesenteroides* IBT-PQ cells or soluble dextransucrase from *L. mesenteroides* NRRL B-512F [14] in 50 mM acetate buffer (pH 5.4) with 10% sucrose and 5% maltose. Samples were withdrawn at different times and the reaction was stopped by heating the mixture in boiling water for 10 min. The profile and the amount of oligosaccharides synthesized was determined by an HPLC equipped with a differential refractometer and using a μ-Bondapak C_{18} column (Waters-Millipore, Milford, MA, USA) with water as eluant (0.7 ml min^{-1}). The fructose and glucose released were also measured enzymatically (Boehringer Mannheim GmbH, Mannheim, Germany).

Preparation of crude dextransucrase

In order to characterize the enzyme, 100 ml of culture broth were centrifuged and the recovered cells were washed twice with distilled water. The cell-associated dextransucrase was extracted by freezing the cells in liquid nitrogen, grinding them with a pestle and mortar and suspending them in 2 ml of Tris-HCl buffer (0.125 M, pH 8). This preparation was used for gel electrophoresis without further purification.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis was prepared and run at 4°C according to the method of Laemmli [9] using 0.75-mm thick slabs of 6% T, 2.7 C; where T denotes the total weight of monomer (acrylamide plus *N*,*N*-methylene bisacrylamide) per 100 ml of solution and C denotes the weight of *N*,*N*-methylene bisacrylamide expressed as a percentage of total weight of monomer. The gel was stained with Coomassie blue R-250. For dextransucrase detection, the sample was prepared without mercapto-ethanol. After running the gel, it was treated with Triton X-100 and calcium chloride, and after washing the gel, it was incubated overnight in a solution containing 10% sucrose and 0.1% sodium azide in 50 mM acetate buffer (pH 5.4) at room temperature. Enzyme activity was visualized by the formation of an opaque band of dextran on the gel [8].

Preparation of dextran

Dextrans were prepared using the enzyme directly as a cell suspension (2 U ml^{-1}) in 10% sucrose at pH 5.4. The reaction was carried out overnight at room temperature until all the sucrose was converted to dextran. The product was recovered and purified by repeated cycles of ethanol precipitation, dialysis and finally stored after freeze-drying. The structure was studied by proton and ^{13}C Nuclear Magnetic Resonance (NMR).

Results and discussion

Of four isolates able to produce viscosity in the presence of sucrose but which grew as simple colonies on glucose,

one strain was identified as *L. mesenteroides* with 99.9% confidence. A second strain, identified as *Lactobacillus plantarum* with 80.3% confidence was later characterized as a levansucrase producer (levan is also produced in *pulque* by levansucrase from *Zymomonas mobilis*). The strain designated as *Leuconostoc mesenteroides* IBT-PQ was grown under the usual batch fermentation conditions employed for other dextransucrase-producing strains [21]. At the end of the fermentation 2 g L^{-1} of cells and insoluble material were produced. From an activity balance it was found that from a total dextransucrase activity of 2.4 U ml^{-1}, 87% was associated with the insoluble part of the culture, while 13% of the activity remained in soluble form.

Because of the difficulties in measuring the weight of cells due to the production of dextran during the fermentation process, the number of cells was determined by direct counting as described in Materials and Methods. A good correlation was found between optical density at 650 nm, the number of cells and the enzyme activity, so that it was possible to relate a given OD reading with both cell concentration and an enzyme activity. The usual cell counts at the end of fermentation were 4×10^8 cells ml^{-1} and an average specific activity value of 0.046×10^{-7} U per cell was calculated.

As the specific activity (U $cell^{-1}$) was constant, the fermentation was optimized by looking for reaction conditions resulting in the highest concentration of cells (Y = number of cells ml^{-1}). The data were fitted by a polynomial model of the form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3$$

The variable response was estimated solving the above equation using the software DESIGN-EXPERT (State-Ease Inc, Minneapolis, MN, USA). An analysis of variance was also carried out. The results indicate a close agreement between the experimental and calculated values for the final biomass concentration with a correlation coefficient (r^2) of 0.99. Optimal conditions were derived from contour plots, and were: sucrose, 30 g L^{-1}; yeast extract, 20 g L^{-1}; K_2HPO_4, 25 g L^{-1}; while the trace elements were kept constant at the levels already described. The experimental and calculated cell concentration values at the optimal culture conditions were 4.7×10^8 and 4.2×10^8 cells ml^{-1}, respectively. Based on the above results, it was also possible to conclude that yeast extract had less influence than sucrose and K_2HPO_4 on the production of *L. mesenteroides* cells. Dols *et al* [3] also reported a slight effect of yeast extract between 20–40 g L^{-1} in the production of dextransucrase from *L. mesenteroides* NRRL B-1299. Nevertheless, in many cases, significant effects on dextransucrase production have been found with yeast extract from different companies (results not shown). A final fermentation to verify these results was carried out in a 1-L Fernback flask with a working volume of 500 ml using the optimized culture medium. Within 4 h of inoculation with a 50-ml culture, the fermentation reached the stationary phase with a specific growth rate of 0.52 h^{-1}. At the end of fermentation the cell concentration reached 4.7×10^8 cells ml^{-1} with a total insoluble enzyme activity of 2.2 U ml^{-1} in the culture. The specific activity was constant (0.047×10^{-7} U $cell^{-1}$) when compared with previous results.

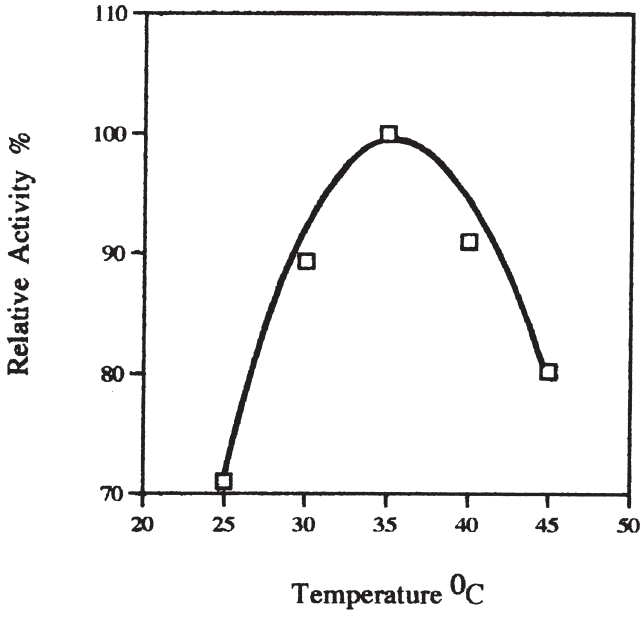

Figure 1 Effect of temperature on *L. mesenteroides* IBT-PQ dextransucrase activity associated with cells. Reactions were carried out with 2 U ml^{-1} in 10% sucrose solution, acetate buffer 50 mM, pH 5.4.

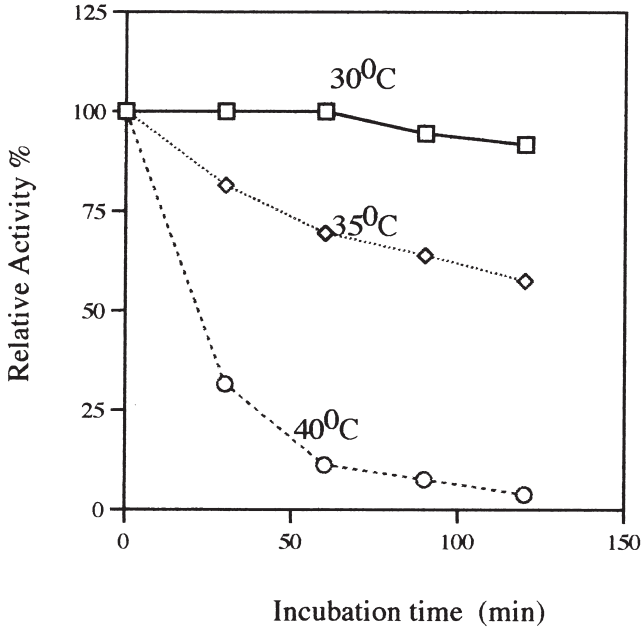

Figure 2 Effect of temperature on dextransucrase stability. *L. mesenteroides* IBT-PQ cells were stored at the indicated temperature and activity was measured at 30°C. Reactions were carried out with 2 U ml^{-1} in 10% sucrose solution, acetate buffer 50 mM, pH 5.4.

Dextransucrase properties

The effect of temperature on the dextransucrase activity of *L. mesenteroides* IBT-PQ cells is shown in Figure 1. The optimum temperature was found to be 35°C. Although it has been reported that the intracellular dextransucrases

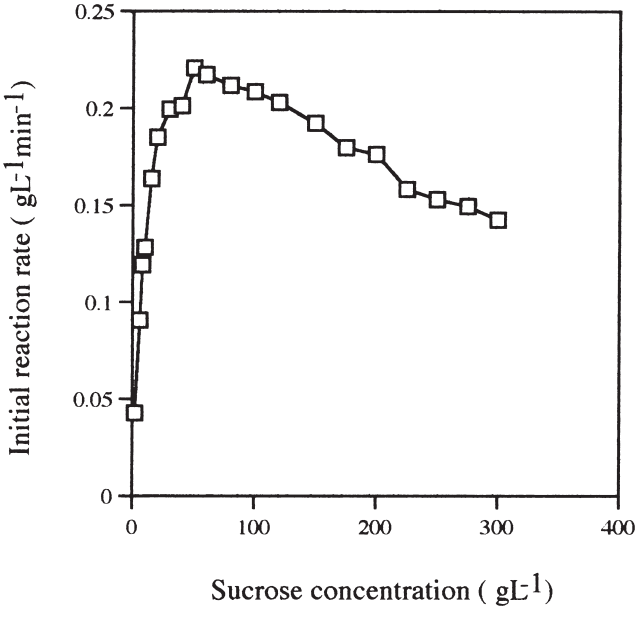

Figure 3 Effect of substrate concentration on the initial reaction rate of dextransucrase associated with *L. mesenteroides* IBT-PQ cells. Reactions were carried out with 1 U ml^{-1} in acetate buffer 50 mM, pH 5.4 and 30°C.

from *L. mesenteroides* NRRL B-1299 show optimum temperatures of 45°C and 35–40°C for enzymes I and II respectively [7], most dextransucrases exhibit an optimal temperature of 30–35°C. The reaction temperature is usually 30°C where the stability is higher. This is the case for dextransucrase from *L. mesenteroides* NRRL B-512F and alternansucrase from *L. mesenteroides* NRRL B-1355 [10].

The effect of temperature on stability is reported in Figure 2. The first order inactivation constants (k_i) were 0.001, 0.005 and 0.027 min^{-1} at 30, 35 and 40°C corresponding to half-lives of 639, 137 and 26 min respectively. The enzyme was stable at 30°C but deactivated rapidly at 40°C and retained only 11% of the original activity after 1 h. The thermal stability of dextransucrase also depends on the presence of dextran in the enzyme solution. Results at 35°C are similar to those reported by Willemot [22] who studied the stability of dextransucrase of *L. mesenteroides* NRRL N-512F in the presence and absence of dextran. The half-life of the enzyme with dextran was 3.33 times higher than the dextran-free enzyme.

Kinetic constants were obtained by measuring the initial reaction rate in a wide range of sucrose concentrations. The enzyme exhibited Michaelis–Menten type kinetic behavior with inhibition at high substrate concentrations as shown in Figure 3. The K_m and K_i values were 0.026 M and 1.23 M respectively. This behavior is similar to that reported for dextransucrase from *L. mesenteroides* NRRL B-512F with values of K_m 0.015 M and K_i 1.26 M of sucrose [12].

The soluble dextran produced by the cells was analyzed by ^{1}H and ^{13}C-NMR comparing the spectra with those reported by Seymour *et al* [17,18]. From the ^{1}H-NMR spectrum it was estimated that the proportion of $\alpha(1–6)$ to $\alpha(1–3)$ linkages is 4:1, while the distance between signals at 99.6:99.1 and 81.8:81.2 ppm in the ^{13}C-NMR suggests that two or three glucose residues are involved in each branching chain.

Acceptor reactions were carried out in the presence of maltose and the products analyzed by HPLC. It was found that this enzyme has similar properties to dextransucrase from *L. mesenteroides* NRRL B-512F in terms of the chemical structure of the resulting oligosaccharides. However there are some differences reported in Table 1, which refer to the efficiency of the acceptor reaction. Remaud *et al* [15], reported that soluble dextransucrase from *L. mesenteroides* NRRL B-1299 produced a higher yield of oligosaccharides and a lower yield of dextran than the insoluble enzyme. This was probably due to a diffusional effect. The insoluble form of dextransucrase in the strain IBT-PQ was more efficient than the soluble enzyme from the strain B-512F as it produced considerably less dextran in the presence of maltose.

The crude enzyme from the cells could not be extracted by sonication and/or lysozyme treatment, as these processes resulted in almost a total loss of activity. Therefore the cells were ground in a pestle and mortar and the proteins extracted with buffer. The recovered proteins were studied by SDS-PAGE gel electrophoresis without further purification and the enzyme identified by an activity reaction in the gel as described in Materials and Methods. As shown in Figure 4, the proteins associated to *L. mesenteroides* IBT-PQ cells showed a single band of enzyme activity corresponding to a molecular weight band of 166 kDa. In earlier studies single proteins have also been reported for the intracellular dextransucrase from *L. mesenteroides* NRRL B-1299 [7] and *Streptococcus mutans* [5]. However, the extracellular dextransucrase from *L. mesenteroides* NRRL B-512F exists in multiple forms with a molecu-

Table 1 Yield of maltose acceptor reaction products synthesized with dextransucrases associated with *L. mesenteroides* strains IBT-PQ and NRRL B-512F

	Dextransucrase of IBT-PQ	Dextransucrase of B-512F
Percent of fructose production (%)	88.5	87.9
Oligosaccharide yield (%)[a]	82	76
Hydrolysis yield (%)[b]	4	2
Dextran yield (%)[c]	14	22

Reactions were carried out with 100 g L^{-1} sucrose, 50 g L^{-1} maltose, 2 U ml^{-1} enzyme.
[a]Fructose released by oligosaccharide synthesis (g L^{-1})/total fructose released (g L^{-1}).
[b]Total glucose released (g L^{-1})/total fructose released by the reaction (g L^{-1}).
[c]Fructose released by oligosaccharide synthesis (g L^{-1}) – total glucose released (g L^{-1})/total fructose released (g L^{-1}).

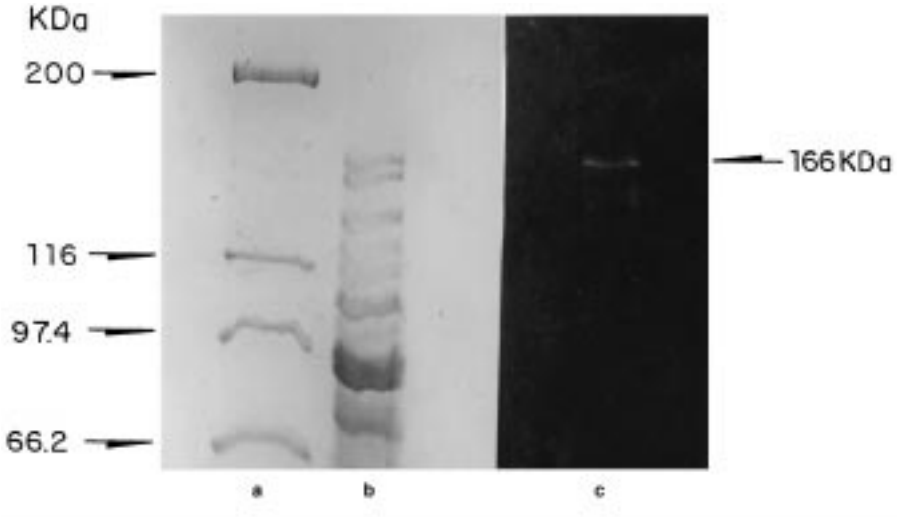

Figure 4 SDS-PAGE of dextransucrase of *L. mesenteroides* IBT-PQ. (a) Molecular weight markers. (b) Protein bands of the cell extract stained with Coomassie blue. (c) Enzyme activity developed after reactivation of the enzyme (see Materials and Methods).

lar weight of 65 kDa [8]. Kim and Robyt [6] observed that the enzymes from *L. mesenteroides* B-742, and constitutive mutants B-742C and B-742CA grown on glucose showed only one activity band at 184 kDa, but *L. mesenteroides* B-742 CB grown on sucrose showed two activity bands, at 184 and 240 kDa, with only one activity band at 184 kDa when cells were grown on glucose medium. The 240-kDa protein may represent a multimer of dextransucrase subunits.

It may be concluded that dextransucrase from *L. mesenteroides* IBT-PQ shows a very similar behavior to the enzyme from the industrial strain *L. mesenteroides* NRRL B512F. However, the enzyme produced by strain IBT-PQ is cell-associated so that it may be easily incorporated as an immobilised biocatalyst for linear isomalto-oligosaccharide production.

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