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Succinoglycan production by solid-state fermentation with *Agrobacterium tumefaciens*

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Abstract Succinoglycan was produced by cultivating *Agrobacterium tumefaciens* on various solid substrates, including agar medium, spent malt grains, ivory nut shavings, and grated carrots, impregnated with a nutrient solution. Fermentations were performed on a laboratory scale, both under static conditions and with agitation, using bottles and a prototype horizontal bioreactor. Several fermentation parameters were examined and optimized, including carbon and nitrogen composition, water content and layer thickness of the substrate. The yields and rheological properties of the polymers obtained under different fermentation conditions were compared. The highest succinoglycan yield was achieved in static cultivation, reaching 42 g/l of impregnating solution, corresponding to 30 g/kg of wet substrate. The polymer production in the horizontal bioreactor was faster, but the final yield was lower (29 g/l of impregnating solution).

Introduction

Succinoglycans are heteropolysaccharides synthesized by a variety of bacteria belonging to the family Rhizobiaceae (Rhizobium, Agrobacterium), as well as by other microorganisms like *Alcaligenes faecalis* and *Pseudomonas* spp. (Zevenhuizen 1997). They are acidic polysaccharides composed of octasaccharide repeating units in which galactose and glucose monomers are found in a molar ratio of 1 to 7. Succinate and pyruvate are commonly found as non-saccharide substituents, whereas acetate might or might not be present, depending on the source of polymer (Matulova et al. 1994). Natural and chemically modified succinoglycans exhibit a high stability even under drastic operational conditions, which makes them particularly suitable for use in tertiary and enhanced oil recovery (Dasinger and McArthur 1988).

Of the variety of succinoglycan-producing bacterial strains, only *Agrobacterium* species so far have been employed on an industrial scale, because they are non-pathogenic and produce an exopolysaccharide (EPS) of good quality in satisfactory yields. Several fermentation processes for succinoglycan production by *Agrobacterium* strains have been patented or reported (Knipper et al. 1993; Stredansky et al. 1998). Continuous processes have also been studied (Linton et al. 1987). In the course of fermentation, the excretion of EPS results in a highly viscous shear thinning broth. The rheological behaviour of the viscous fermentation broth causes serious problems to mixing, heat transfer and oxygen supply, thus limiting both the maximum polymer concentration achievable and product quality (Peters et al. 1989; Gibbs and Seviour 1998).

Several strategies have been devised in the attempt to overcome these problems. EPS production by immobilized cells did not produce satisfactory results (Lebrun et al. 1994), whereas xanthan fermentations in emulsions and water/oil dispersions resulted in high EPS yields in the water phase (Ju and Zhao 1993). But yields are much lower when the total volume of the culture fluid is taken into account; besides, the downstream processing is complex. More remarkable results were obtained from fermentors equipped with impeller systems of improved design (Galindo 1994), or from fed-batch processes (Wernau 1981; Stredansky et al. 1999a). Recently we reported on the feasibility of bacterial EPS production using solid-state fermentation (SSF) to overcome the problems connected to broth viscosity (Stredansky et al. 1999b).

In this work we evaluated SSF as an alternative strategy for the production of succinoglycan by *Agrobacterium tumefaciens*. The choice was based on the observation that solid substrates reproduce the natural

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habitat of this bacterium, commonly isolated from plant roots, plant galls and soil (Lippincott et al. 1981). Some aspects of the process are investigated and discussed, such as the effect of substrate composition, moisture, layer thickness and mixing on succinoglycan production.

Materials and methods

Microorganism, media and cultivation conditions

The succinoglycan-producing strain *Agrobacterium tumefaciens* ID 95-748 was obtained from Consorzio Environmental Polytech (Catania, Italy). It was maintained and subcultured monthly on YMB agar slants containing (g/l): yeast extract 0.4; mannitol 10; K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.1; agar 20.

The basic liquid medium had the following composition (g/l): C-source (type and concentration varied as specified in the Results section), sodium glutamate 1.5, KH_2PO_4 0.2, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, yeast extract 0.1; trace elements: $CaCl_2 \cdot 2H_2O$ 0.04; $FeCl_3 \cdot 6H_2O$ 0.0025; $MnCl_2 \cdot H_2O$ 0.001; $NaMoO_4 \cdot 2H_2O$ 1×10^{-5}; $ZnSO_4 \cdot 7H_2O$ 1×10^{-5}; $CuSO_4 \cdot 7H_2O$ 1×10^{-5}; H_3BO_3 1×10^{-5}; $CoCl_2 \cdot 6H_2O$ 1×10^{-5}. The same medium composition was used for cultivation on agar plates with the addition of 2% agar, and for the preparation of the nutrient solution impregnating the solid support in solid-state cultivations. N compounds were each supplied at a concentration in which N was equimolar to 0.15% sodium glutamate.

Cultivations on agar surface were performed using 100 mm diameter Petri dishes containing 25 ml of 2% agar medium incubated in a water saturated atmosphere at 30 °C for either 4 or 8 days.

Solid-state cultivations were carried out in 250-ml Erlenmeyer flasks containing 10 g of spent malt grains impregnated with 30 ml of liquid medium. The effect of substrate moisture was studied using 10–50 ml of the liquid base to impregnate the substrate. Spent malt grains, obtained as a residue from the wet milling process of wort preparation from a local brewery (Moretti, S. Giorgio di Nogaro, Italy), were washed twice with tap water and dried in an oven before use.

The solid substrates containing carrot were prepared by mixing 20 g of fresh grated carrots (maximum size 3 mm, dry weight 10% of wet weight) with 8 g of spent malt grains in 250-ml Erlenmeyer flasks. The substrate was soaked for 20 min in 12 ml of a solution containing sodium glutamate and salts as in the basic medium and 40 g/l of sucrose.

All substrates and media were sterilized by autoclave at 121 °C for 15 min and all cultivations were done at least in triplicate. Solid substrates were inoculated with 5% (v/v) preculture (Stredansky et al. 1999b), relative to the volume of impregnating liquid and cultivated at 30 °C in a humidified atmosphere for 6 days.

Rotating bottle and bioreactor cultivations

Bottles (500 ml; 75 mm diameter × 165 mm length) were filled with 20 g of spent malt grains impregnated with 60 ml of liquid medium. Shavings of ivory nuts (a waste product from industrial button manufacture) were used as an inert solid support instead of spent malt grains in some experiments. Cultivations were performed on a roller culture apparatus (Wheaton Instruments, New Jersey, USA). The rotating bottles were incubated at 30 °C for a period of 3–6 days, at a rotation speed of 0.5 rpm.

Bioreactor experiments were carried out in a prototype 2 l aerated rotating horizontal bioreactor (Fig. 1). Dry spent malt grains (100 g) were soaked in 300 ml of liquid medium. The bioreactor with substrate was sterilized by autoclave at 121 °C for 25 min. After inoculation with 15 ml of a 2-day-old preculture, the substrate was incubated at 30 °C at a rotation speed of 1 rpm.

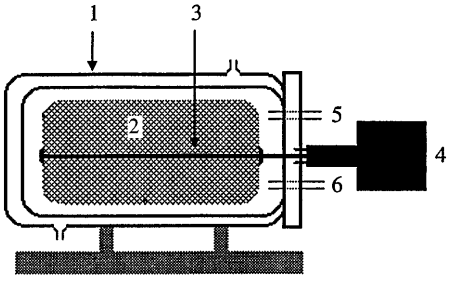

Fig. 1 Bioreactor for solid state fermentation (SSF): *1* jacketed glass vessel, *2* stainless steel basket, *3* shaft, *4* electric motor, *5* air inlet, *6* air outlet

During cultivation forced sterile humidified air was supplied at a constant rate of 50 ml/min.

Biomass and EPS determination

In agar plate cultures the slimy growth was scraped off the agar surface with a spatula and weighed. After dilution with distilled water, the polysaccharide was separated from the biomass by ultracentrifugation (25 000 *g*, 20 min). The cell pellet was washed twice with distilled water, dried to a constant weight at 70 °C and determined gravimetrically. The polysaccharide was precipitated from the pre-cooled cell-free supernatant after addition of 1% KCl, with 2 volumes of cold acetone. The precipitated EPS was dissolved in 1% KCl, precipitated again with acetone, dried at 70 °C and weighed.

In solid substrate cultures, the EPS was extracted from the fermented mass with 7 volumes of water by shaking at 250 rpm for 2 h. Solid support particles were removed by filtration through a fine nylon mesh. Smaller particles were separated by centrifugation at 4000 *g* for 10 min, followed by ultracentrifugation at 25 000 *g* for 20 min. The EPS was precipitated from the particle-free supernatant as described above.

Determination of sucrose

Sucrose was determined by a sucrose-specific composite biosensor utilizing the procedure described by Svorc et al. (1997). Glucose oxidase, mutarotase and invertase (Sigma) were co-immobilized on the surface of the composite transducer containing ferrocene as a redox mediator. Amperometric measurements were carried out with a potentiostat Amel 559 (Amel Instruments, Milan, Italy) at 300 mV against a saturated calomel electrode.

Results

Agar surface fermentation

Cultivation of *A. tumefaciens* on agar medium was used to predict its potential exopolysaccharide productivity by SSF. The slime formed on the agar surface was collected and used for polymer and cell yield quantification. Succinoglycan yields obtained from agar plates with increasing sucrose concentrations and at different incubation times are reported in Table 1. It can be seen from the results expressed as yield per plate that, while cell growth is almost complete after 4 days of incubation, polymer production continues after cell growth has stopped, in particular at high sucrose concentration in

Table 1 Production of succinoglycan on agar plates (*DCW* dry cell weight)

Initial sucrose (g/l)	Cultivation time (days)	Slime per plate (g)	DCW per plate (mg)	Succinoglycan per plate (mg)	DCW in slime (g/kg)	Succinoglycan in slime (g/kg)
25	4	5.75	23.9	132	4.16	22.9
50	4	5.93	25.7	139	4.33	23.5
75	4	5.79	27.3	131	4.72	22.7
100	4	5.23	25.1	111	4.80	21.3
150	4	3.55	28.2	71	7.94	20.1
25	8	7.73	26.7	182	3.46	23.5
50	8	7.45	24.4	168	3.28	22.5
75	8	7.53	24.9	165	3.30	21.9
100	8	7.46	27.0	159	3.62	21.3
150	8	5.19	27.0	105	5.21	20.3

the substrate. An inhibitory effect of high sugar concentration on both cell growth and polymer production is also apparent. Interestingly, the concentration of succinoglycan in the slime is practically constant, ranging from 2% to 2.3% (w/w), independent of cultivation time and sugar concentration in the medium.

Solid substrate composition

Carbon and nitrogen compounds supplied as nutrients to bacterial cultures are known to affect EPS productivity, depending on their composition and relative amounts. Succinoglycan production in SSF was initially studied using an inert matrix (spent malt grains) as a solid support, impregnated with a nutrient solution containing appropriate amounts of C and N sources. Of the various sugars tested (Fig. 2), sucrose and mannitol proved to be the most suitable C sources for succinoglycan production. Cultures grown in the presence of sucrose generally produced superior yields, thus sucrose was chosen for further studies under SSF conditions. The effect of sucrose concentration on succinoglycan production is illustrated in Fig. 3. The EPS yield rose with increasing concentration of sugar in the substrate, but conversion of the initial sucrose to succinoglycan dropped when more than 40 g/l of sucrose was added to the impregnating nutrient solution. The highest EPS yield of 38.5 g/l (calculated as grams per litre of the impregnating solution and corresponding to 28.8 g/kg of the wet solid fermented mass) was obtained with 80 g/l of sucrose. All experiments were performed utilizing 1.5 g/l of sodium glutamate as the sole N source.

The effect of various N sources is shown in Fig. 4. Sodium glutamate allowed the highest yield with no significant influence of its concentration in the range 1.5–3 g/l. At higher nitrogen levels the EPS yield dropped. Of the other tested N sources, only nitrate gave satisfactory results. No acidification of the substrate was observed during fermentation on solid substrates containing both sodium glutamate and nitrates. In fact, the aqueous extract from the fermented mass had pH values ranging from 6.9 to 7.7, whereas in the presence of other N sources pH dropped to 5.1–6.2, resulting in a lower succinoglycan productivity. Although combining the buffering N sources with others having no buffering capacity also gave good results (data not shown), sodium glutamate was selected from the N sources for further experiments. All experiments were performed utilizing 30 g/l of sucrose in the substrate as the sole C source.

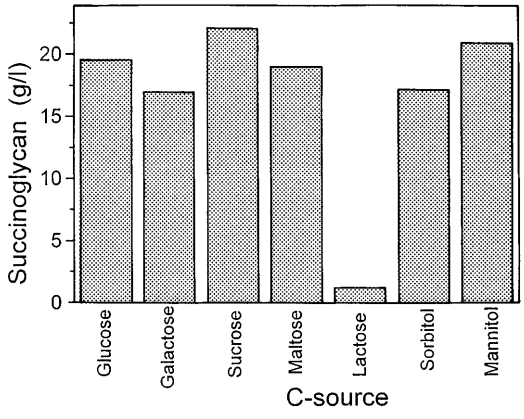

Fig. 2 Effect of various carbon sources on succinoglycan production in SSF

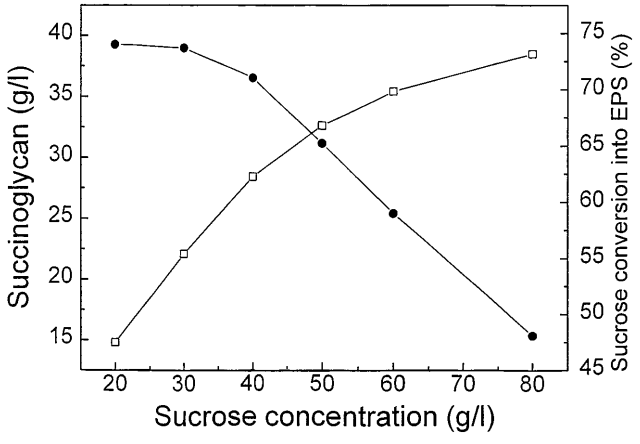

Fig. 3 Effect of sucrose concentration on succinoglycan production in SSF: □ succinoglycan, ● percentage of sucrose conversion into succinoglycan

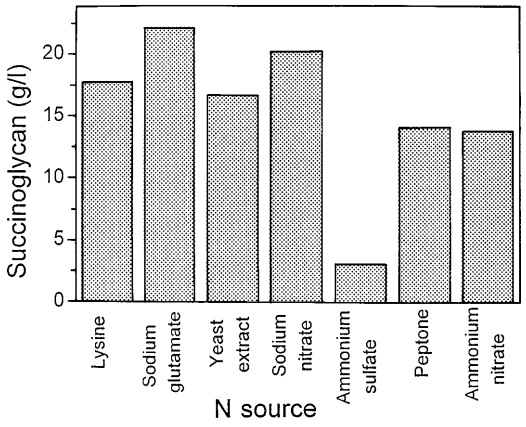

Fig. 4 Effect of various nitrogen sources on succinoglycan production in SSF

The profiles of EPS production and sucrose consumption are illustrated in Fig. 5. Succinoglycan production started soon after inoculation and was proportional to sucrose utilization. The highest EPS production rate of 0.38 g/l per hour was observed on the 2nd day.

To increase succinoglycan yields in the presence of high sucrose concentration, the effect of non-ionic surfactants (Brij 76, Tergitol NPX) and organic acids (acetate, tartrate) was investigated. These compounds had been found to stimulate succinoglycan production in submerged fermentation (Stredansky et al. 1998). However, they showed no significant effect (data not shown).

Cultivation on the solid substrate prepared with carrots, which were selected among natural cheap substrates for the high sugars content and low acidity, resulted in the highest EPS yield presented here (42 g/l corresponding to 30 g/kg wet solid fermented mass). On the other hand, the use of an inert support such as spent malt grains, allowed the solid material to be recycled after polymer extraction, for at least four cycles with no substantial decrease in succinoglycan production (data not shown).

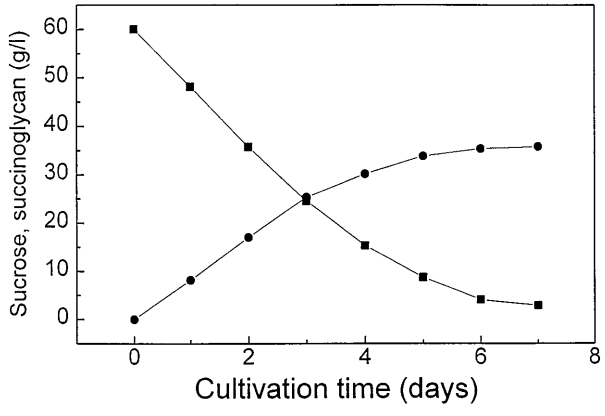

Fig. 5 Time course of fermentation of *Agrobacterium tumefaciens* in SSF: ● succinoglycan, ■ residual sucrose

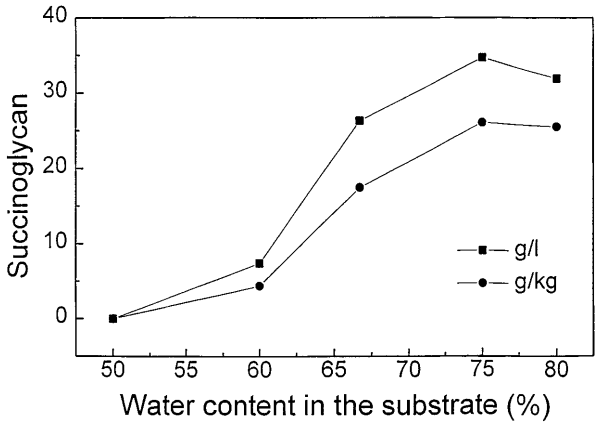

Fig. 6 Effect of the water content in the substrate on succinoglycan production: ■ succinoglycan (g/l of impregnating liquid), ● succinoglycan (g/kg of wet solid substrate)

Effect of substrate moisture

The water content of the solid substrate is an important parameter which affects microbial growth and productivity. As can be seen in Fig. 6, a high moisture requirement proved critical for succinoglycan production with *A. tumefaciens*. The highest productivity was observed with 75% water content in the substrate, whereas it was barely detectable with 60% water content.

Cultivation in rotating vessels

Agitation facilitates the maintenance of homogenous conditions, particularly in the gaseous environment, within the SSF bioreactor. It can easily be achieved on a laboratory scale by the rotating bottle. Results from rotating bottle cultivations and static cultivations are compared in Table 2. Although EPS yields are slightly lower in the rotating cultures, agitation results in a substantially higher viscosity of the aqueous EPS extracts.

The same behaviour was also observed in the 2-l aerated rotating horizontal bioreactor illustrated in Fig. 1. Relatively gentle mixing of the substrate and the application of forced air allowed a good homogeneity of the solid and gaseous phases, resulting in a quick biomass growth and succinoglycan production. In fact the cultivation time necessary for sugar consumption and

Table 2 Succinoglycan production in rotating bottles and in static cultivation (*SMG* spent malt grains, *INS* ivory nut shavings)

Substrate	Rotation	Succinoglycan (g/l)	Viscosity[a] (Pa.s)
SMG	−	21.8	0.09
SMG	+	20.5	0.19
INS	−	20.2	0.08
INS	+	18.8	0.17

[a] Viscosity measurements were performed on the aqueous extract obtained from the fermented mass (see Materials and methods). Shear rate 10 s^{-1}

EPS formation was reduced by 20–25% with respect to the corresponding static flask cultures. The maximum succinoglycan yield of 29.2 g/l was achieved with an initial sucrose concentration of 45 g/l (relative to the liquid volume impregnating the substrate). This proved to be the optimal sucrose amount in these experiments. At higher sugar concentrations in the substrate the extra polysaccharide formed was not absorbed completely into the solid support particles. Instead, a slimy layer appeared on the particle surface, which caused the substrate particles aggregate, when they were subjected to rotation. As a result oxygen diffusion was hindered, thus diminishing the performance of the fermentation process.

Effect of substrate layer thickness

To investigate the effect of substrate layer thickness, which is one of the most important parameters in the static SSF process, experiments were performed in wide cylindrical vessels (Ø 12 cm) containing various amounts of solid substrate. Results are shown in Fig. 7. A substrate layer thickness exceeding 6 cm negatively affected polymer production, probably due to insufficient oxygen availability.

Discussion

SSF has been known for many centuries in the East and in African countries, where SSF processes have been used for the production of fermented food and soy sauce. Although submerged cultivation is commonly applied in the large scale preparation of most biotechnology products, there are indications that in some cases SSF could be fully competitive with submerged cultivation (SC) (Pandey 1992; Besson et al. 1997).

Results obtained in this work clearly show the feasibility of succinoglycan production using solid-state cultivation techniques.

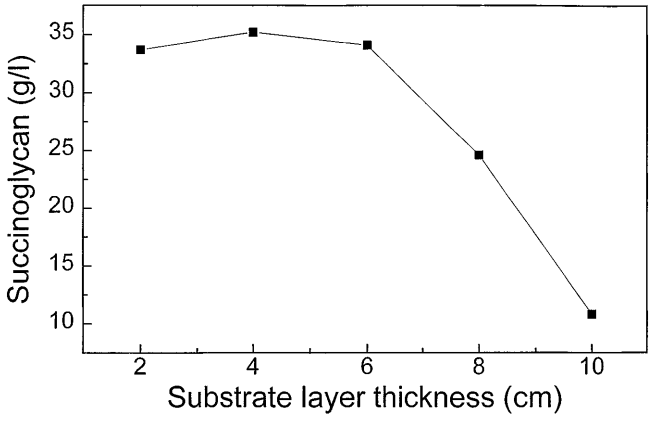

Fig. 7 Effect of substrate layer thickness on succinoglycan production

Succinoglycan production was initially studied in agar surface fermentation (ASF), which resembles the SSF system, as growth takes place on a solid surface. Interestingly, the concentration of succinoglycan in the slime grown on the agar surface was practically constant, and independent of the initial sugar concentration in the medium. The highest concentration recorded was 23 g succinoglycan/kg slime, corresponding to 24 g/l, relative to the volume. This might indicate a saturation condition, where the free arrangement of succinoglycan molecules within the viscous slime is restricted to concentrations not exceeding 23 g/kg (or 24 g/l). However, this limit was overcome in SSF, where succinoglycan concentrations up to 42 g/l, relative to the impregnating liquid volume, were obtained. This suggests a better arrangement of polymer molecules within the porous solid particles, probably due to adsorption effects. The medium acidification observed in submerged cultures of *A. tumefaciens* (Stredansky et al. 1998) was lower in agar plate cultures (final pH in the range from 6.2 to 6.9), which might prove advantageous in SSF, where pH control cannot be easily achieved.

As compared to SC, the increased succinoglycan production in ASF and SSF systems could be partially accounted for by a higher tolerance of the microorganism to high sugar concentration in the case of solid state cultivation. In fact, increasing the initial sucrose concentration above 15 g/l in submerged culture did not result in a higher succinoglycan yield, which suggests high sugar concentration has an inhibitory effect on polymer synthesis (Stredansky et al. 1998, 1999a). The microorganism proved more tolerant to higher sucrose concentrations in ASF, and even more in SSF, where the conversion efficiency of sucrose into succinoglycan significantly decreased only when more than 40 g/l of sucrose were supplied. Furthermore, a constant increase of succinoglycan production was recorded in the presence of increasing amounts of sucrose up to 80 g/l (Fig. 4). Favela-Torres et al. (1998) also found a much higher tolerance of the fungus *Aspergillus niger* to high sugar concentration in SSF than in SC, resulting in substantially higher specific growth and glucose uptake rates. Some authors suggest that the enhanced metabolic activity observed in micro-organisms growing in SSF might be stimulated by the formation of concentration gradients of sugar and nutrients within the fermenting mass. This would cause a local drop in substrate concentration, which significantly minimizes catabolite repression (Ramesh and Lonsane 1991; Solis-Pereira et al. 1993).

Substrate layer thickness affects the fermentation performance in static SSF in tray or packed bed bioreactors. Succinoglycan production in non-aerated cylindrical vessels was satisfactory with a substrate layer thickness not exceeding 6 cm (Fig. 7). However, the use of a bioreactor with a perforated bottom and forced aeration could allow an increase in the substrate layer thickness without diminishing the fermentation process performance.

Substrate agitation also needs to be considered in scaling up the SSF process (Lonsane et al. 1992). The results achieved in agitated culture (Table 2) indicate that fermentation in stirred bioreactors may prove a successful strategy in succinoglycan production, as compared to the rotating bioreactor used in this work. Nevertheless, the lower succinoglycan yield obtained in the rotating horizontal bioreactor was balanced by a reduced cultivation time and by the better rheological properties of the EPS produced, suggesting a higher degree of polymerization in the EPS formed (Table 2).

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