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Physiology of exopolysaccharide production by *Azotobacter vinelandii* from 4-hydroxybenzoic acid

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The relationship between exopolysaccharide (EPS) production by *Azotobacter vinelandii* ATCC 12837 from 4-hydroxybenzoic acid as sole carbon source and other physiological parameters was investigated. In relation to growth, *Azotobacter* needed more time in 4-hydroxybenzoic acid to reach levels of biomass similar to those obtained when sugars were used, although the phenolic compound led to a more extensive exponential phase. The encystment process was initiated after cells had grown for 24 h, in which small amounts of EPS were synthesized and poly-β-hydroxybutyrate (PHB) accumulation began. Both polymers, EPS and PHB, showed a similar evolution with time, as well as the formation of cysts, which points out the existence of a relation between these parameters. This was corroborated by a statistical study, in which significant correlations ($P<0.05$) were observed when each parameter was compared to the two others.
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Keywords: *Azotobacter vinelandii*; exopolysaccharide production; 4-hydroxybenzoic acid; poly-β-hydroxybutyrate; cysts

Introduction

Polysaccharides obtained from several species of algae and higher plants are used in industry on the basis of their ability to alter the rheological properties of aqueous solutions [17]. Some bacterial genera such as *Pseudomonas* and *Azotobacter* also produce these polymers. *Azotobacter vinelandii* synthesizes several exopolysaccharides (EPS), alginate being the most important one [10,41]. Alginate is a linear polymer of nonrepetitive structure composed of β-D-mannuronic acid and its epimer, α-L-guluronic acid. Distribution and length of blocks depend on nutritional and environmental conditions and the producing microorganism [29]. Both culture conditions and microorganism also influence the presence of acetyl groups, which are present only in mannuronosyl residues of bacterial alginates [35].

Bacteria are not extensively used as commercial producers of EPS because of higher fermentation process costs. However, because of their structural diversity, bacterial EPS show better properties than those obtained from other sources [38] and research on this subject has become more important over the last few years. Efforts have been made to reduce costs, especially with regard to nutrient sources. In this sense, attempts have been made to study the ability of some cheaper substrates, such as whey [9], citrus wastes [1], molasses [8], and olive oil waste waters [19] to support growth and EPS production.

A. vinelandii shows wide metabolic activity, which allows it to degrade numerous substrates, even some highly recalcitrant compounds such as aromatic compounds [3,4,23,44,45]. These kinds of substrates are present in soils as a result of vegetal decomposition [11,16,43] and they can be used by *Azotobacter* and other diazotroph microorganisms as alternative carbon sources when readily assimilated substrates are unavailable [4,6]. Although their environmental concentration depends on the type of vegetation, season, and soil characteristics, levels of 10^{-4} μmol/g have usually been detected [30,36].

This work investigates some physiological aspects of EPS production by *A. vinelandii* in cultures amended with 4-hydroxybenzoic acid as sole carbon and energy source. This phenolic compound, frequently found in habitats in which degradation of plant material is accomplished [12], was selected in previous studies on the possibility of reutilization of some agricultural waste fractions for microbial polysaccharide production [24].

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Materials and methods

Microorganism

A. vinelandii ATCC 12837 was used in this study. Slants of nitrogen-free Burk's glucose medium [40] were used as a maintenance medium. Stock cultures were subcultured every 3 months, grown at 30°C for 48 h, and stored at 4°C. Working cultures were subcultured from the stock culture every 3 months and were themselves subcultured monthly.

Media, inocula, and culture conditions

Modified Burk's nitrogen-free medium, composed of KH_2PO_4 0.16 g; K_2HPO_4 0.64 g; $MgSO_4 \cdot 7H_2O$ 0.4 g; NaCl 0.2 g; $CaSO_4 \cdot 2H_2O$ 0.05; $FeSO_4 \cdot 7H_2O$ 0.0025 g; $Na_2MoO_4 \cdot 2H_2O$ 0.001 g in 1 l of distilled water, was used for the growth of *A. vinelandii*. 4-Hydroxybenzoic acid (25 mM) was added as carbon source [39]. The carbon source and the basal salt medium were sterilized separately to avoid precipitation.

Cells from nitrogen-free liquid Burk's glucose medium were transferred to 250-ml Erlenmeyer flasks containing 50 ml of modified nitrogen-free Burk's medium supplemented with 4-hydroxybenzoic acid and incubated at 30°C under continuous

agitation (120 rpm) for 48 h. After three serial transfers, cells were harvested by centrifugation and washed twice in sterile distilled water. Cellular suspensions were adjusted to an optical density of 0.5 (540 nm). Volumes (0.5 ml) of this cellular suspension were used to inoculate cultures. All media were incubated at 30°C with continuous agitation (120 rpm) for 168 h. The pH of 7.5 was adjusted as needed. Experiments were performed in triplicate.

Chemicals

4-Hydroxybenzoic acid was obtained from Aldrich Chemical (Milwaukee, WI, USA).

Analytical procedures

Cells from 1.0 ml of culture broth were harvested by centrifugation (14,000×*g*, 10 min, 4°C), washed twice with sterile distilled water, transferred to preweighed vials, and evaporated to dryness at 105°C.

Intracellular proteins were quantified according to Lowry *et al* [20], following modifications by Herbert *et al* [14]. Pellets from 1 ml of centrifuged culture (14,000×*g*, 10 min, 4°C) were washed twice with sterile phosphate buffer and resuspended in 1 ml of 0.5 N NaOH. Tubes were placed in a boiling water bath for 5 min. After cooling the tubes in cold water, 2.5 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate and 5% Na_2CO_3 in a 25:1 proportion were added and tubes were allowed to stand at room temperature for 10 min. Finally, 0.5 ml of 50% Folin–Ciocalteu reagent was added and left standing for 30 min to allow full colour development. Distilled water was used as blank and bovine serum albumin (Sigma, St. Louis, MO, USA) as standard.

Nitrogen-free Burk's agar plates amended with glucose 0.5% were used for viable cell counts.

Poly-β-hydroxybutyrate (PHB) was quantified in 10–20 mg of freeze-dried cells according to the method of Law and Slepecky [18]. Samples were measured at 235 nm and poly-β-hydroxybutyric acid (Sigma) was used as standard.

The remaining 4-hydroxybenzoic acid concentration was measured by a modification of the method described by Marambe and Ando [22]. Culture supernatant (0.5 ml) was diluted with 7 ml of distilled water, and 0.5 ml of Folin–Ciocalteu reagent was added, with vigorous shaking. After the solution had stood for 3 min, 1 ml of 20% Na_2CO_3 was added and solution was made to reach 10 ml with distilled water. Absorbance was measured at 725 nm after 1 h.

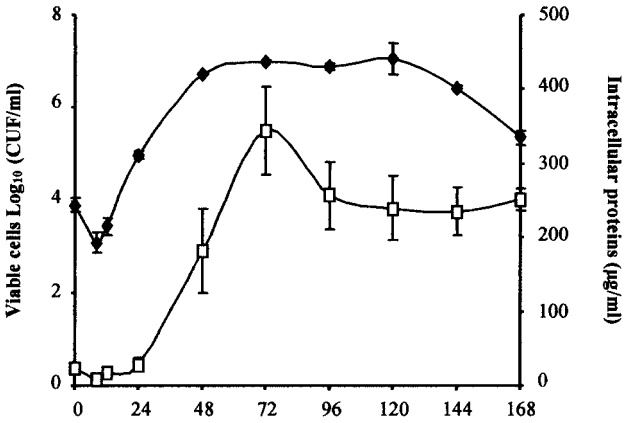

Figure 2 Growth of *A. vinelandii* ATCC 12837 on 4-hydroxybenzoic acid. (□) Viable cells; (◆) intracellular proteins.

Oxygen levels were determined using a Hansatech DW1 oxygen electrode coupled to a Hansatech CB1-D control box. Aliquots (0.5 ml) were placed into the reaction chamber and oxygen consumption was measured for a period of 30 s. Calibration was performed with oxygen-saturated water and sodium dithionite for 100% and 0% of O_2, respectively.

An Olympus BX-40 phase contrast microscope was used to determine the relative proportion of vegetative cells and cysts at different incubation times.

EPS was determined by precipitating alcohol nonsoluble material in the culture supernatant by adding of 2 vol of cold isopropanol [17]. The precipitated EPS was filtered onto predried and preweighed GF/A Whatman filter discs (Whatman International, Springfield Mill, Kent, UK), washed with 50 ml of isopropanol/water (3:1 vol/vol), and dried at 60°C.

Alginate lyase activity was determined according to the method described by Weissbach and Hurtwitz [42]. Aliquots (100 µl) of culture broth were added to 125 µl of 0.025 M periodic acid in 0.125 M sulphuric acid and the mixture was shaken vigorously. Samples were allowed to stand for 20 min and then 250 µl of 2% (wt/vol) arsenic acid (sodium salt) in 0.5 N hydrochloric acid was added to the tubes, which were left at room temperature for 2 min.

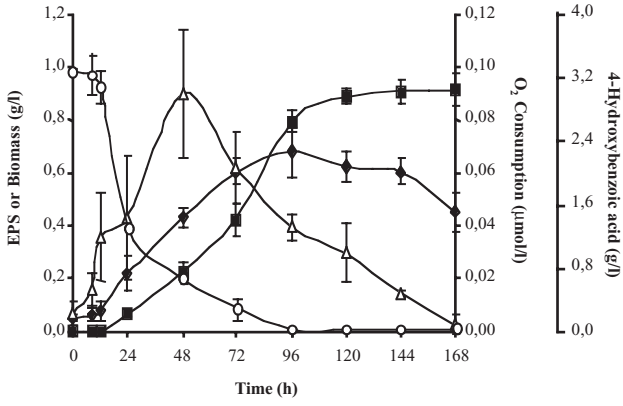

Figure 1 Effect of incubation time on production of bacterial EPS. (■) EPS; (◆) dry cell weight; (○) residual 4-hydroxybenzoic acid in culture supernatant; (△) O_2 consumption.

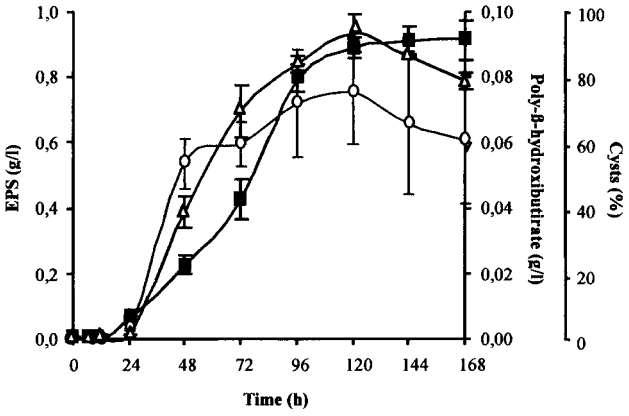

Figure 3 Production of EPS by *A. vinelandii* and relation to cyst formation and PHB accumulation. (■) EPS; (○) cysts percent; (△) PHB.

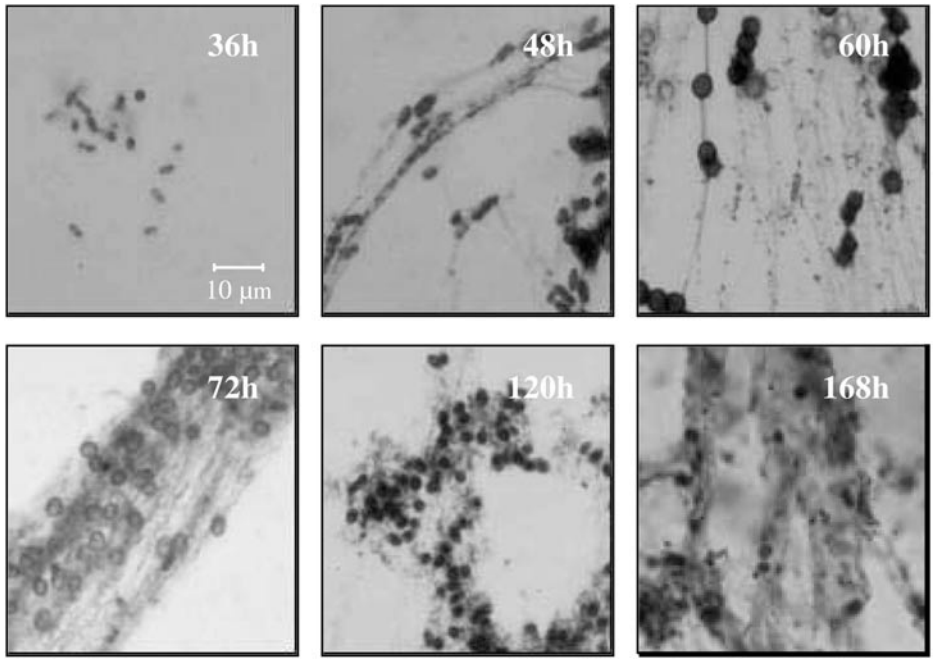

Figure 4 Methylene blue stains of cells from a broth culture of *A. vinelandii* grown on 4-hydroxybenzoic acid. Vegetative cells and cysts are trapped in a reticulated structure.

Then 1 ml of 2-thiobarbituric acid was added and the tubes were placed in a boiling water bath for 10 min. Samples were cooled slowly to avoid precipitation and the resultant red colour was measured at 549 nm.

Statgraphics Plus Version 4.0 was used to obtain a matrix of Pearson product–moment correlations between pairs of variables at the 95% confidence level.

Results

The growth of *A. vinelandii* on Burk's nitrogen-free medium supplemented with 25 mM 4-hydroxybenzoic acid is shown in Figure 1. Maximal growth increase was obtained between 24 and 72 h, although higher biomass levels were reached at 96 h (about 0.7 g/l). Longer incubation times did not lead to higher levels of biomass. Similar results were observed for intracellular protein concentration and viable cells (Figure 2). Increasing levels of intracellular protein were obtained until 72 h. At that time, there was a significant drop in protein concentration and lower values were maintained until the end of the incubation period (Figure 2).

Exponential growth was correlated with higher O_2 consumption levels (Figure 1). Thus, between 8 and 48 h, there was a strong increase in the required amounts of O_2. Thereafter, O_2 consumption decreased until the end of the culture period. 4-Hydroxybenzoic acid was totally exhausted after 96 h of incubation, although in 48-h cultures, less than 20% of carbon supplied remained in the medium.

EPS biosynthesis began after 24 h. Maximum yield (0.9 g/l) was observed at 120 h, although production at 96 h only was slightly lower. The same pattern was observed for PHB accumulation and cyst formation (Figure 3). Very small values for these parameters were detected at 24 h and a strong increase was obtained at 48 h. This increase continued until 96–120 h, after which a small drop in both PHB accumulation and cyst formation was observed.

Parallel to EPS determination, photomicrographs of methylene blue-stained samples were made (Figure 4). Images showed the presence of a reticular-like structure when EPS synthesis

Table 1 Correlation between different physiological parameters of *A. vinelandii* growth on 4-hydroxybenzoic acid (significant correlations, $P<0.05$, in **bold** type)

Parameters	Correlation matrix Pearson coefficient (r^2)								
	Time	Biomass	Viables	Proteins	Cysts %	EPS	O_2	p-HB	PHB
Time	1.00	**0.79**	0.37	**0.78**	**0.85**	**0.97**	−0.20	**−0.85**	**0.91**
Biomass	**0.79**	1.00	**0.79**	0.89	**0.97**	**0.86**	0.28	**−0.95**	**0.95**
Viables	0.37	**0.79**	1.00	**0.71**	**0.75**	0.51	0.49	**−0.64**	**0.69**
Proteins	**0.78**	**0.89**	**0.71**	1.00	**0.90**	**0.77**	0.26	**−0.87**	**0.89**
Cysts %	**0.85**	**0.97**	**0.75**	**0.90**	1.00	**0.90**	0.22	**−0.93**	**0.97**
EPS	**0.97**	**0.86**	0.51	**0.77**	**0.90**	1.00	−0.18	**−0.86**	**0.96**
O_2 consumption	−0.20	0.28	0.49	0.26	0.22	−0.18	1.00	−0.27	0.02
PHB consumption	**−0.85**	**−0.95**	**−0.64**	**−0.87**	**−0.93**	**−0.86**	−0.27	1.00	**−0.90**
PHB	**0.91**	**0.95**	**0.69**	**0.89**	**0.97**	**0.96**	0.02	**−0.90**	1.00

started and it became increasingly complex as EPS production increased. *Azotobacter* cells, mainly cysts, were attached to this structure.

Since some microorganisms can use EPS as a carbon and energy source under carbon-deficient conditions, samples were also investigated for the presence of alginate-degrading activity (alginate lyase). However, this enzyme was not detected at any time.

Finally, Table 1 shows a correlation matrix among parameters investigated. It can be seen that growth parameters and polymer production are closely related to carbon consumption.

Discussion

Growth of this bacterium in a medium supplemented with the phenolic acid was different from the results obtained when sugars were the carbon source. The most obvious difference was the longer time *Azotobacter* needed to reach exponential growth. With sucrose or glucose, less than 24 h was enough to reach this phase [2,7,15], but 48 h was necessary to obtain a similar result when 4-hydroxybenzoic acid was used (Figures 1 and 2). Since the enzymes that degrade the phenolic compounds are induced [16], the delay observed is probably the time *Azotobacter* requires to synthesize them. On the contrary, this phase of active growth lasted until 72–96 h, at time which growth had ceased when those sugars were in the media. However, major activity was observed between 48 and 72 h, the period in which oxygen consumption was maximal (Figure 1). After this time, growth decelerated. This cessation can be due to carbon exhaustion and probably limited oxygen diffusion attributed to higher viscosity of the medium, as a consequence of EPS production [32]. *Azotobacter* is one of the most oxygen-demanding organisms [21,26,34]. For the metabolism of 4-hydroxybenzoic acid, oxygen is needed not just as the final electron acceptor, but in several intermediate oxidative reactions in the β-ketoadipate pathway, by which the phenolic compound is degraded [6,12,23], which uses this element for enzymatic activity [13,28].

Production of EPS began between 12 and 24 h, but it was after this time when the greatest yield was obtained. Thus, EPS biosynthesis was correlated with growth of the microorganism, although its maximum level was reached in the stationary phase, in which active cellular division had ended, as reported in other papers [5,41]. In any case, the culture conditions and the type of bacterium affect the physiology of EPS production [7]; thus, modifications of these factors can alter the growth phase at which EPS is preponderantly synthesized.

The conversion of the nutritional source into the polymer PHB is an undesirable factor because it reduces the carbon available to produce EPS. However, accumulation of PHB is needed for cyst formation [2,37] and EPS secretion seems to be associated with this cellular stage [25,27], since the compact EPS capsule that surrounds the cyst (Figure 4) contributes to its resistance [32].

Our results confirm the interdependence between both polymers and cyst formation (Figure 3 and Table 1). However, PHB production by *A. vinelandii* from 4-hydroxybenzoic, at a maximal concentration of 0.15 g/g PHB, was lower than that obtained from sugars (ranging between 0.4 and 1.5 g/g) [31,46]. This may be a consequence of the high oxygen requirements of the metabolic pathway and the inhibition this element exerts on PHB synthesis [2,33].

EPS production is a complex process that is influenced by several factors. The use of 4-hydroxybenzoic acid as sole carbon source reduces PHB production without affecting encystment and, therefore, EPS production.

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