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# Influence of nutritional and environmental factors on polysaccharide production by *Azotobacter vinelandii* cultured on 4-hydroxybenzoic acid

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**The capacity of 4-hydroxybenzoic acid to support exopolysaccharide (EPS) biosynthesis was investigated. Carbon source concentration, nitrogen supplementation, and other nutritional and environmental factors were optimized to obtain maximal EPS recovery. Higher EPS yields were obtained in nitrogen-free media amended with 20–30 mM 4-hydroxybenzoic acid. In general, modifications in inorganic salt concentration did not alter EPS production, except in the case of magnesium ions. Increased levels of this cation were correlated to greater EPS yields. Production was strongly influenced by certain environmental factors. Optimal values of 34°C, 80 rpm and neutral or slightly basic conditions were selected. Under these conditions, more than 25% of the carbon source supplied was converted to EPS and the production was improved about 42% in comparison to that observed in the initial media.** *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 5–10.

**Keywords:** *Azotobacter*; polysaccharides; 4-hydroxybenzoic acid

## Introduction

Exopolysaccharide (EPS) production is a common feature among bacteria. These compounds have several applications in different industries, mainly food and textile, though in the last few years they have become much more important in other fields (i.e., the oil industry) [19,22,36]. Their use stems from their capacity for altering the rheological properties of aqueous solutions [18]. Alginate is a widely used EPS, obtained commercially from several brown algae [30]. This EPS is also produced by bacteria in two genera, *Pseudomonas* and *Azotobacter* [7,12,13,21]. In both cases, the polysaccharides are copolymers of $\beta$-D-mannuronic acid and its epimer, $\alpha$-L-guluronic acid, distributed in blocks of different lengths depending on the origin of the polymer. Alginate produced by *Pseudomonas* lacks blocks of guluronate, but as with the EPS obtained from *Azotobacter*, it is acetylated to a variable extent in the mannuronate residues [33].

Variability in monomer block structure and acetylation are the factors that most influence the physical, chemical and rheological properties of alginate [30] and in determining its commercial value. In this regard, bacterial alginates show a wider range of capabilities, although there are some disadvantages for their production in large-scale fermentations, mainly due to the cost of feedstocks. For other bacterial polysaccharides, some attempts have been made to investigate the utilization of cheaper substrates in batch fermentation [2,23,25].

The aim of the present work was to determine the suitability of a phenolic compound (4-hydroxybenzoic acid) to support EPS biosynthesis by *Azotobacter vinelandii* and to elucidate the optimum nutritional and environmental conditions under which the polymer is maximally produced. Phenolic acids are available from a variety of sources, including soil and residues of vegetal origin, from which they are slowly but constantly released. These kinds of compounds are also present in wastes derived from agricultural activity, and are produced abundantly in southeast Spain, where a huge amount of land is dedicated to intensive horticulture in greenhouses. Conversion of this phenolic acid to alginate could be taken as a useful model to go further in the possible reutilization of some agricultural waste fractions for microbial polysaccharide production.

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## Materials and methods

### Microorganism

*A. vinelandii* ATCC 12837 was used throughout this study. It was maintained on slants of nitrogen-free Burk's medium [37] and checked periodically for purity. 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

Media used for growth of *A. vinelandii* had the same salt composition as nitrogen-free Burk's medium [37]. Media were supplemented with 4-hydroxybenzoic acid at different concentrations (10 to 50 mM). The carbon source and the basal salt medium were sterilized separately to avoid precipitation. Variations in all medium components, including the addition of $NH_4NO_3$ when necessary, were made to optimize EPS recovery. All assay values are shown in Table 1.

**Table 1** Effect of nutritional and environmental culture conditions

| Compound | Nutritional modifications | | | | | |
|---|---|---|---|---|---|---|
| 4-Hydroxybenzoic acid (mM) | – | 10 | 20 | 30 | 40 | 50 |
| $NH_4NO_3$ (mM) | Free | 10 | 20 | 30 | 40 | 50 |
| $KH_2PO_4$ (g/l) | Absent | 0.06 | 0.12 | 0.16 | 0.24 | 0.32 |
| $K_2HPO_4$ (g/l) | Absent | 0.24 | 0.48 | 0.64 | 0.96 | 1.28 |
| $MgSO_4 \cdot 7H_2O$ (g/l) | Absent | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 |
| NaCl (g/l) | Absent | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 |
| $CaSO_4 \cdot 2H_2O$ (g/l) | Absent | 0.01 | 0.03 | 0.05 | 0.08 | 0.1 |
| $FeSO_4 \cdot 7H_2O$ (g/l) | Absent | 00005 | 0.0015 | 0.0025 | 0.004 | 0.005 |
| $Na_2MoO_4 \cdot 2H_2O$ (g/l) | Absent | 0.00025 | 0.0005 | 0.0001 | 0.0015 | 0.002 |
| Factor | Environmental variations | | | | | |
| Temperature (°C) | – | 26 | 30 | 34 | 38 | |
| Agitation (rpm) | – | 80 | 120 | 160 | 200 | |
| pH | 5 | 6 | 7 | 8 | 9 | |

Microorganisms from maintenance slants were cultured in nitrogen-free glucose liquid Burk's medium, and then transferred to nitrogen-free Burk's liquid medium amended with 25 mmol $l^{-1}$ 4-hydroxybenzoic acid. After three consecutive transfers, inocula were prepared from this medium. *A. vinelandii* cells were incubated at 30°C with continuous agitation (120 rpm) for 48 h, harvested by centrifugation and washed twice in sterile phosphate buffer. One-milliliter volumes of the suspensions (OD 0.5 at 540 nm) were inoculated into 250-ml Erlenmeyer flasks containing 50 ml of medium supplemented with 25 mmol $l^{-1}$ 4-hydroxybenzoic acid. Optical density measurements were made with a Shimadzu UV-160A spectrophotometer. All media, once inoculated, were incubated at 30°C on a rotary shaker (120 rpm). pH was monitored and adjusted to 7.3 as needed. Modifications of temperature, agitation and pH were also made (Table 1) to study the influence of culture conditions on polysaccharide production. Experiments were performed in triplicate.

### Chemicals

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

### Analytical procedures

Culture media samples were removed at intervals and each sample was divided into subsamples for the determination of biomass and EPS concentration.

For biomass estimation, 1,0-ml volumes of culture media were harvested by centrifugation, the cells were washed twice with sterile distilled water and evaporated to dryness in preweighed vials for 3 h at 105°C.

EPSs were extracted with three volumes of isopropanol from the culture supernatant by vigorous shaking, according to the method of Jarman *et al* [18]. After 10 min, precipitated EPSs were filtered through predried and preweighed GF/A Whatman filter discs (Whatman International, Springfield Mill, Kent, England), and washed with 100 ml of isopropanol/water (3:1, v/v). The filter disc plus precipitate was dried under vacuum at 45°C for 24 h. Filters were reweighed and the concentration of EPSs in the culture broth was calculated.

## Results

The influence of nutritional and environmental conditions on EPS production by *A. vinelandii* grown on 4-hydroxybenzoic acid as sole carbon source was tested following a three-step experimental design. Assays were carried out sequentially; those conditions that resulted in maximal EPS recovery were applied in the next experimental phase.

### Effect of carbon and nitrogen concentration

*A. vinelandii* ATCC 12837 was grown in Burk's medium supplemented with five different concentrations (10, 20, 30, 40 and 50 mM) of 4-hydroxybenzoic acid. Biomass and EPS production were detected for every concentration investigated, although some differences were observed (Figure 1). Growth levels between 0.9 and 1.2 g $l^{-1}$ were obtained in most cases, with the exception of media amended with the lowest substrate concentration (10 mM), in which only 0.5 g $l^{-1}$ of biomass production was reached. Similar results were obtained when the influence of nitrogen concentration was investigated (Figure 1). Generally, biomass production was slightly lower in the absence of added nitrogen.

EPS production was also investigated using different concentrations of carbon and nitrogen sources. Preliminary experiments were performed in order to determine the incubation time for optimum recovery of EPSs in *A. vinelandii* cultures. Samples were removed at intervals and quantitative extraction of EPS was carried out. According to these results (data not shown), maximum EPS recovery was obtained in 5-day-old cultures, so this incubation period was selected for EPS extraction and quantification in further experiments. As indicated in Figure 1, little polymer was obtained when the carbon concentration was 10 mM. An increase in 4-hydroxybenzoic acid concentration to 20–30 mM produced the best results (0.6 g $l^{-1}$). Further increases in carbon concentration did not result in rise in EPS recovery; on the contrary, lower production was observed. EPS production was also strongly influenced by the nitrogen concentration present in culture media (Figure 1). Generally, EPS recovery was higher as nitrogen concentration decreased. Thus, higher levels of EPS were obtained in nitrogen-free media.

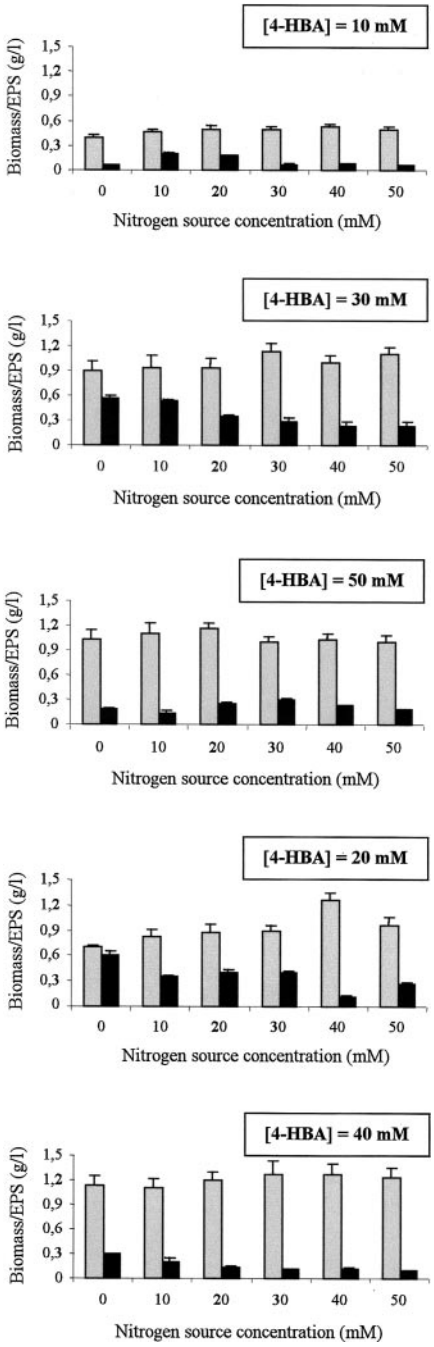


**Figure 1** Effect of carbon and nitrogen concentration on growth and exopolysaccharide production by *Azotobacter vinelandii* ATCC 12837. ■: Biomass and ■: EPS production (figure shows nitrogen results grouped by 4-hydroxybenzoic acid concentration).

On the basis of these results, Burk's nitrogen-free medium amended with 4-hydroxybenzoic acid (25 mM) was optimal for EPS recovery and consequently these carbon and nitrogen concentrations were used in further experiments.

### *Effect of inorganic salts concentration*

As shown in Figure 2, $CaSO_4$ and $FeSO_4$ seem essential for growth of *A. vinelandii*, since the absence of either prevented good growth of the microorganism. However, supplementation of media with increasing concentrations of calcium and iron produced different results. Thus, $CaSO_4$ concentrations up to 0.05 g/l resulted in an increase in biomass production. A decrease was observed at higher levels. On the contrary, in the range assayed, growth was enhanced as $FeSO_4$ concentration increased. With regard to EPS recovery, maximal production was obtained at 0.05 g/l $CaSO_4$ and 2.50 mg/l $FeSO_4$. Beyond these concentrations, further increases led to gradual reductions in EPS production. This effect was more drastically observed in the case of ferrous ions. NaCl and $Na_2MoO_4$ had no effect on EPS recovery and growth, although biomass values showed a slight upward tendency as concentration of both nutrients was increased. Both EPS and biomass production were adversely affected by the absence of $PO_4^{2-}$ in the medium, although neither was completely inhibited. Supplementation of media with concentrations up to 0.80 g/l of $PO_4^{-2}$ stimulated EPS synthesis, whereas further increases resulted in a fall in polysaccharide production. $MgSO_4$ had the strongest influence on EPS production, since higher concentrations correlated with increasing EPS levels, although differences were more remarkable at lower concentrations.

Only magnesium ions demonstrated a direct effect on EPS production, since higher levels of this nutrient stimulated EPS recovery (Figure 2). Thus, no modifications were made in basal Burk's medium, except in magnesium salt concentration, which was set at 0.4 g/l.

### *Effect of culture conditions*

Both bacterial growth and EPS production were strongly affected by the environmental conditions tested (Figure 3). Extreme values of pH resulted in low levels of biomass and EPS recovery, especially at acid pH values. Neutral or slightly basic pH enhanced both parameters.

In regard to aeration, changes in agitation led to a different response for both growth and EPS production. Thus, biomass was generally higher as aeration increased, while there was no improvement in EPS yields. However, lower results for EPS recovery and bacterial growth were obtained when maximal agitation (in the range tested) was applied.

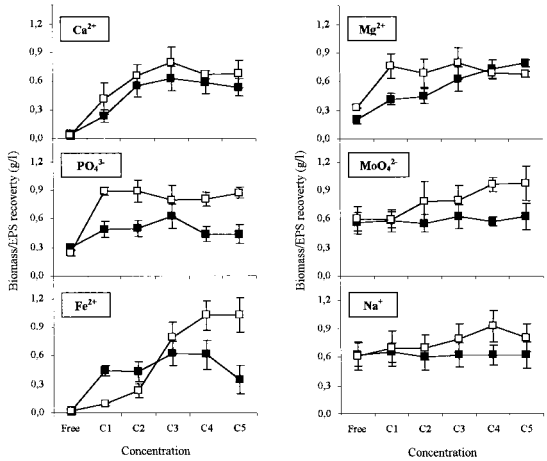


**Figure 2** Influence of nutrients concentration on growth and exopolysaccharide production by *Azotobacter vinelandii* ATCC 12837. □: Biomass and ■: EPS production (Concentrations are the same as in Table 1).

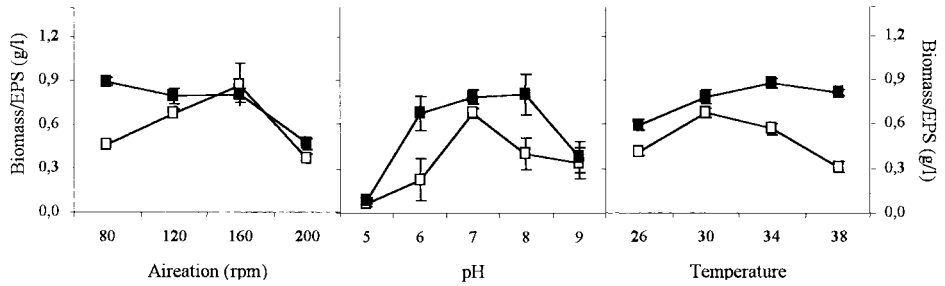


**Figure 3** Influence of environmental conditions on growth and exopolysaccharide production by *Azotobacter vinelandii* ATCC 12837. □: Biomass and ■: EPS production.

The optimum temperature for growth and EPS synthesis was found to be different. A temperature of 30°C was best for growth, while 34°C was optimum for EPS recovery. On the basis of the results obtained, EPS production was enhanced in media with slightly basic pH values (7–7.5), and incubated at 34°C under low aeration rates (80 rpm).

### *Optimal medium*

An experiment was performed to check the effect of the combined optimal modifications. As shown in Figure 4, once the optimization process was completed an increase of about 42% over the initial EPS production was obtained. This increase was correlated with lower growth, with the final biomass 10% below the initial value.

## Discussion

4-Hydroxybenzoic acid was investigated with regard to its ability to support bacterial growth and EPS production by *A. vinelandii*. This phenolic acid allowed microbial growth at every concentration tested. It has been reported that concentrations greater than 10 mM 4-hydroxybenzoic acid prevented growth of the closely related *Azotobacter chroococcum* when the microorganism was cultured in nitrogen-free media. This adverse effect was ascribed to an inhibition of the nitrogenase activity by the phenolic compound [1]. However, utilization of 4-hydroxybenzoic acid in the range of 10–50 mM by *A. vinelandii* cultured in nitrogen-free media, has been described previously [24,26,27]. Utilization of phenolic acids by *Azotobacter* in nitrogen-free media is extremely dependent on the oxygen concentration. Since these compounds are metabolized through dioxygenases, molecular oxygen does not act simply as a terminal electron acceptor, but it is also a specific substrate for a number of step reactions [6,14]. On the other hand, under nitrogen-fixing conditions, high respiratory activities are needed to protect nitrogenase against damage by oxygen. Thus, inappropriate aeration rates, not high enough to support both the activity of dioxygenases and the high respiratory activity required to protect nitrogenase, can lead to inhibition of microbial growth.

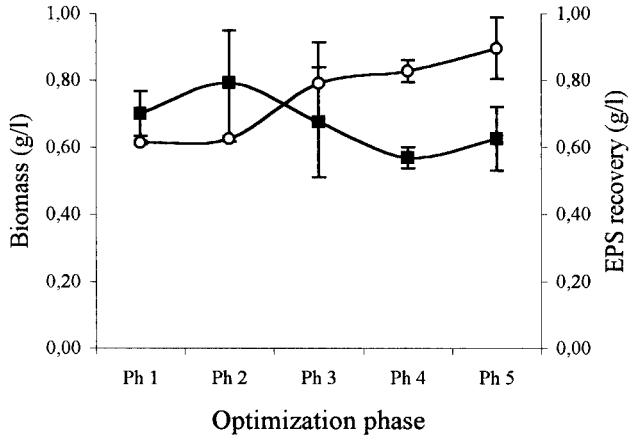


**Figure 4** Variations on biomass and EPS recovery at different optimization phases. Ph 1: Start conditions; Ph 2: C and N optimization; Ph 3: Inorganic salts optimization; Ph 4: Culture conditions optimization; Ph 5: Final optimization. ■: Biomass and ○: EPS recovery.

The best results on EPS production were obtained when a carbon concentration of 20–30 mM was used. Thus, higher levels of polymer were not correlated with maximum levels of biomass (Figure 1). In this sense, it has been shown that some conditions can increase cell efficacy in synthesizing EPS [36]. In this case, the special nature of the carbon substrate could contribute to this effect due to the complex energy and material balances that control its availability for EPS production [30].

With regard to bacterial growth in nitrogen-amended media, it was observed that biomass levels were higher as nitrogen concentration increased. Similar results have been reported by Vermani *et al* [38]. Under these conditions, EPS synthesis showed an opposite pattern to that observed for growth. Generally, EPS production was higher at lower nitrogen concentration. Thus, maximum levels were reached in nitrogen-free medium. Although it has been reported that supplementation with small amounts of combined nitrogen stimulates EPS yield [4,39], the 4-hydroxybenzoic acid concentration used in these experiments is high enough to support a nitrogenase activity which allows suitable growth and produces a high C/N ratio, conditions that promote EPS synthesis [18,31].

Inorganic salts usually show a strong influence on EPS synthesis. Thus, some mineral nutrient limitations enhanced EPS production by *A. vinelandii* [17,18], mainly due to their influence on the cyst-formation process [28], in which EPS production is necessary to be successfully completed [4]. Nevertheless, the process depends on every nutrient and its concentration.

Six different mineral nutrients were investigated for their capacity to promote EPS production. Variations in sodium and molybdate concentrations did not alter EPS production, even in unamended media, although growth was slightly higher as molybdate concentration increased. Similar results have been reported by Vermani *et al* [39], and a correlation has been observed between sodium concentration and EPS yield in

**Table 2** Effect of inorganic salts concentration on biomass and EPS recovery[a]

| Concentration (g/l) | $CaSO_4$ | | Concentration (g/l) | $MgSO_4$ | | Concentration (g/l) | NaCl | |
|---|---|---|---|---|---|---|---|---|
| | Biomass | EPS | | Biomass | EPS | | Biomass | EPS |
| 0.00 | 0.035 | 0.047 | 0.00 | 0.324 | 0.201 | 0.00 | 0.617 | 0.627 |
| 0.01 | 0.418 | 0.231 | 0.05 | 0.763 | 0.417 | 0.05 | 0.698 | 0.653 |
| 0.03 | 0.662 | 0.550 | 0.10 | 0.688 | 0.448 | 0.10 | 0.704 | 0.604 |
| 0.05 | 0.791 | 0.625 | 0.20 | 0.791 | 0.625 | 0.20 | 0.791 | 0.625 |
| 0.08 | 0.667 | 0.588 | 0.30 | 0.691 | 0.731 | 0.30 | 0.929 | 0.627 |
| 0.10 | 0.676 | 0.532 | 0.40 | 0.674 | 0.790 | 0.40 | 0.801 | 0.625 |
| Concentration (g/l) | $PO_4^{2-}$[b] | | Concentration (mg/l) | $FeSO_4$ | | Concentration (mg/l) | $Na_2MoO_4$ | |
| | Biomass | EPS | | Biomass | EPS | | Biomass | EPS |
| 0.00 | 0.245 | 0.292 | 0.00 | 0.017 | 0.025 | 0.00 | 0.603 | 0.558 |
| 0.30 | 0.894 | 0.489 | 0.50 | 0.093 | 0.447 | 0.25 | 0.591 | 0.585 |
| 0.60 | 0.895 | 0.496 | 1.50 | 0.233 | 0.433 | 0.50 | 0.781 | 0.556 |
| 0.80 | 0.791 | 0.625 | 2.50 | 0.791 | 0.625 | 1.00 | 0.791 | 0.625 |
| 1.20 | 0.806 | 0.437 | 4.00 | 1.025 | 0.611 | 1.50 | 0.962 | 0.575 |
| 1.60 | 0.872 | 0.440 | 5.00 | 1.028 | 0.349 | 2.00 | 0.977 | 0.623 |

[a]Results in grams per liter.
[b]Concentration expressed as total $PO_4^{2-}$ ($KH_2PO_4 + K_2HPO_4$).

*Pseudomonas aeruginosa* [10], another alginate-producing bacterium. Small increases in growth caused by higher molybdenum concentrations could be due to the role that this element plays on the nitrogen-fixation process [11], although the ability to accumulate molybdenum by *Azotobacter* has been reported [29].

Iron-deficient media yielded very little growth, and supplementation with low levels of this element stimulated biomass production. As previously said for molybdenum, these results could also be explained by the influence that iron exerts on nitrogenase synthesis [3]. EPS yield increased until intermediate iron concentrations, and a fall in polysaccharide synthesis was observed beyond this point, as other authors have reported [39].

The influence of phosphate on EPS production has been investigated exhaustively [15,18]. According to these studies, increased phosphate concentrations enhance biomass production and reduce EPS synthesis. In our results, media with no phosphate added gave minimum levels of both growth and EPS; in amended media, there was a slight reduction in biomass as phosphate concentration increased, while maximal EPS production was obtained at an intermediate concentration. Low biomass values obtained in phosphate-deficient media could be due to the influence that phosphate plays in the energy metabolism [8], and the lower EPS yields observed at higher phosphate concentrations could be ascribed to the decrease in the activity of some specific enzymes (phosphomannose isomerase, GDPmannose pyrophosphorylase and GDPmannose dehydrogenase) catalysing the polymer synthesis, as demonstrated by Horan *et al* [15].

A similar pattern was observed for growth and EPS production when the influence of calcium concentration was investigated. As shown in Figure 2, $Ca^{2+}$ was essential for EPS biosynthesis as well as for growth, as demonstrated by Horan *et al* [16] whose experiments on calcium limitation gave similar results. Media supplementation enhanced both growth and EPS recovery due to the important role of this nutrient on metabolic processes and polymer synthesis [20]. Inhibition of calcium uptake when this element was present in high concentrations [40] could be responsible for slight decreases in biomass and EPS yields at the maximal levels tested.

The influence of magnesium on EPS synthesis has been reported previously [31,39]. EPS recovery increased in proportion to the magnesium concentration. Thus, polymer production with higher levels was fourfold higher than that obtained in nonsupplemented media and threefold with respect to minimal media. This positive effect could be due to the role that this nutrient plays in activation of enzymes implicated in EPS biosynthesis [10].

Although some studies have indicated that environmental conditions do not affect EPS biosynthesis [18], others had opposite results [4,5,39]. Temperature, pH and agitation have been described as the most influential factors on EPS yields [36]. According to our results, the optimum temperature for EPS biosynthesis was higher than the optimum for maximal growth. Similar results have been reported for *A. vinelandii* grown on sucrose [38], and for other EPS-producing bacteria [32].

Maximal EPS recovery was reached at pH close to neutrality. Extreme values of pH resulted in lower EPS yields, especially in the acid range. Similar results have been obtained in other studies [39], and confirmed the necessity to control pH to get optimal substrate uptake and optimal EPS synthesis [35].

**Table 3** Effect of environmental variations on biomass and EPS recovery[a]

| pH | | | Agitation (rpm) | | | Temperature (°C) | | |
|---|---|---|---|---|---|---|---|---|
| | Biomass | EPS | | Biomass | EPS | | Biomass | EPS |
| 5 | 0.069 | 0.088 | 80 | 0.463 | 0.891 | 26 | 0.415 | 0.598 |
| 6 | 0.227 | 0.679 | 120 | 0.674 | 0.790 | 30 | 0.674 | 0.790 |
| 7 | 0.674 | 0.790 | 160 | 0.871 | 0.797 | 34 | 0.568 | 0.880 |
| 8 | 0.406 | 0.807 | 200 | 0.365 | 0.467 | 38 | 0.311 | 0.814 |
| 9 | 0.343 | 0.383 | | | | | | |

[a]Results in grams per liter.

Oxygen supply is essential for growth of *A. vinelandii* in nitrogen-free media, mainly due to the respiratory protection of nitrogenase. In addition, aromatic compounds such as 4-hydroxybenzoic acid are degraded through an oxygen-dependent metabolic pathway [34]. As shown in Figure 3, opposite results were obtained for growth and EPS recovery. Thus, growth was higher as agitation was increased and EPS production was optimal at lower agitation. These results disagree with those described by others [9,16]. Probably, the differences could be explained on the basis of the different nature of the carbon source employed.

Optimal EPS production by *A. vinelandii* grown on 4-hydroxybenzoic acid was obtained in a modified Burk's nitrogen-free medium supplemented with a carbon source concentration of 25 mM, with a magnesium ($MgSO_4 \cdot 7H_2O$) concentration of 0.4 g $l^{-1}$, pH 7.5 and incubated at 34°C and 80 rpm. Under these culture conditions, EPS recovery was improved approximately 42% with respect to the initial conditions (Figure 4). More than 25% of the carbon source was converted to EPS by *A. vinelandii*. This conversion rate is similar to that reported in other studies, in which sugars were used as carbon source [5,9,38].

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