



# Yields of alginates produced by fluorescent pseudomonads in batch culture

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## SUMMARY

Saprophytic and plant pathogenic fluorescent pseudomonads are possible sources of bacterial alginates to be used as substitutes for algal alginates for certain commercial applications. In this study, a total of 115 strains of fluorescent *Pseudomonas* species (*P. cichorii*, *P. fluorescens*, *P. syringae* and *P. viridiflava*) were tested for yields of alginates when grown in batch culture in a proprietary liquid medium (PLM). The PLM contained either fructose or glucose (both at 5%, w/v) as the primary carbon and energy source. For comparison, selected strains were also grown in a modified Vogel and Bonner medium (MVBM) containing gluconate (5%, w/v) and formulated to support maximal alginate production by the human pathogen *P. aeruginosa*. After five days of incubation at 24 °C with shaking (250–300 r.p.m.), alginates were harvested from the culture fluids by precipitation with three volumes of isopropanol. Maximum yields of alginates, based on assays for uronic acid content of precipitable material, were 5 g L<sup>-1</sup> for PLM with fructose, 3 g L<sup>-1</sup> for PLM with glucose and 9 g L<sup>-1</sup> for MVBM.

## INTRODUCTION

Alginates isolated from brown algae are used for a variety of food, industrial and pharmaceutical uses due to their gelling, water-holding, stabilizing and emulsifying properties [15]. Alginates are composed of D-mannuronate and L-guluronate which can be present both as homopolymeric and heteropolymeric sequences [15]. The amount of guluronate present is dependent on the algal species, age at harvest, geographic location of harvest and plant part extracted [9]. In the presence of calcium ions, stronger gels are formed by alginates containing homopolymeric blocks of guluronate [6].

Alginates are also produced as exopolysaccharides by bacteria in the genera *Pseudomonas* and *Azotobacter* [6]. The most studied alginate-producing fluorescent pseudomonad is *P. aeruginosa*, but this species is not suitable for commercial production due to its clinical significance. Recently, we demonstrated that in addition to *P. aeruginosa* several additional naturally-occurring fluorescent pseudomonads are capable of producing alginate as an exopolysaccharide [5]. Previous to our studies, alginate-producing variants were obtained in the laboratory for *P. fluorescens*, *P. putida* and *P. mendocina* [7]. Bacterial alginates differ from algal alginates in that they are acetylated and are usually lower in the amount of guluronate present. In addition, alginates produced by *Pseudomonas* spp. do not contain homopolymeric blocks of guluronate [6].

Bacterial alginates have been proposed as substitutes for algal alginates for certain applications [13], but have not yet been commercialized because of a lack of polyguluronate block structures in the pseudomonad alginates which significantly affects gelation properties, and because of the current high cost of production by fermentation due to inadequate polymer yields. The purpose of our study was to determine the yields of alginates produced by various fluorescent pseudomonads other than *P. aeruginosa* when grown as batch cultures in a proprietary liquid medium formulated to support bacterial exopolysaccharide production. As these bacteria are incapable of utilizing starch, the feedstocks fructose and glucose were compared as primary carbon and energy sources. For comparison, selected strains were also tested using a modified Vogel and Bonner medium (MVBM) [3] containing gluconate and formulated to support alginate production by *P. aeruginosa*.

## MATERIALS AND METHODS

### *Bacteria*

The 115 bacterial strains used in this study represented both plant pathogenic bacteria as well as saprophytic plant-associated bacteria. Short-term storage of the strains was on *Pseudomonas* agar F medium (Difco Laboratories, Detroit, MI, USA) at 4 °C. Long-term storage was at -80 °C in trypticase soy broth (BBL, Becton Dickinson, Cockeysville, MD, USA) amended with 15% (v/v) glycerol.

### *Culture conditions*

Seed cultures were grown overnight in nutrient broth (8 g L<sup>-1</sup>)–yeast extract (2 g L<sup>-1</sup>) (both from Difco) medium (NBY medium). Inoculum (0.5 ml) was added to 25 ml of

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liquid medium contained in 250-ml Erlenmeyer flasks. Two media were tested: a complex proprietary medium of Kelco, Division of Merck & Co. (San Diego, CA, USA), with D-fructose or D-glucose (5%, w/v), and a modified Vogel and Bonner medium (MVBM) with 3 mM Mg<sup>2+</sup> and 5% (w/v) of D-gluconate [3]. Alcalase 2.4 L (Novo Industri A/S, Copenhagen, Denmark) was added to each medium to a final concentration of 0.005% to inhibit any alginate depolymerase (alginase) activity. Cultures were incubated at 24 °C with shaking (250–300 r.p.m.) for 5 days. This period of incubation was sufficient to reach stationary phase for production of alginate.

#### Quantitation of alginate production

After incubation, any alginate produced was precipitated by the addition of cold isopropanol (3 vol) and the alginate fibers were collected on an 80-mesh wire screen. The crude alginate samples were then air-dried and weighed. The actual alginate content of selected crude samples was estimated by the colorimetric method for uronic acid of Blumenkrantz and Asboe-Hansen [1] with algal alginate (Sigma Chemical Co., St Louis, MO, USA) as standard.

#### RESULTS

A total of 115 strains of *P. cichorii*, *P. fluorescens*, *P. syringae* and *P. viridiflava* were examined for their ability to produce alginate in PLM medium with fructose or glucose (5%, w/v) as the primary carbon and energy source. The cultures were incubated at 24 °C, a temperature lower than that optimal for growth [12]. Incubation temperatures lower than those optimal for cell growth often favor bacterial exopolysaccharide production [17]. Of the strains tested for alginate production with fructose as the primary carbon source in PLM, 24 strains gave yields of precipitable material of 10 g L<sup>-1</sup> or higher up to a maximum of 17 g L<sup>-1</sup> (Fig. 1(A)). The culture fluids of all 24 strains ranged from highly viscous to gel-like. When inverted, the contents of cultures which we classified as gel-like descended only very slowly along the sides of the flasks. Based on uronic acid values, actual alginate yields were from 2 to 5 g L<sup>-1</sup> (Table 1).

With glucose substituted for fructose in PLM many of the cultures exhibited poor growth, most likely due to acidification of the medium by the formation of gluconic acid via the action of glucose dehydrogenase [8,10]. The culture fluids of only seven strains yielded 10 g L<sup>-1</sup> or more of precipitable material (Fig. 1(B)) and were either highly viscous or gel-like. Based on uronic acid values actual alginate yields for these strains were 2–3 g L<sup>-1</sup>. These seven strains (*P.s. pv. papulans* 5 and 24, *P.s. pv. savastanoi* 213, *P.s. pv. syringae* 1147(s), *P.s. pv. tabaci* 11528 and pt113, and *P.s. pv. tagetis* K26) also gave good yields when grown on fructose (Table 1).

For comparison, 61 of the strains, representative of the four species included in the study, were also tested for yields of alginates when grown in Vogel and Bonner medium as modified by Chan et al. [3] to support alginate production by *P. aeruginosa*. Yields of precipitable material and for alginate based on uronic acid determinations for the selected strains

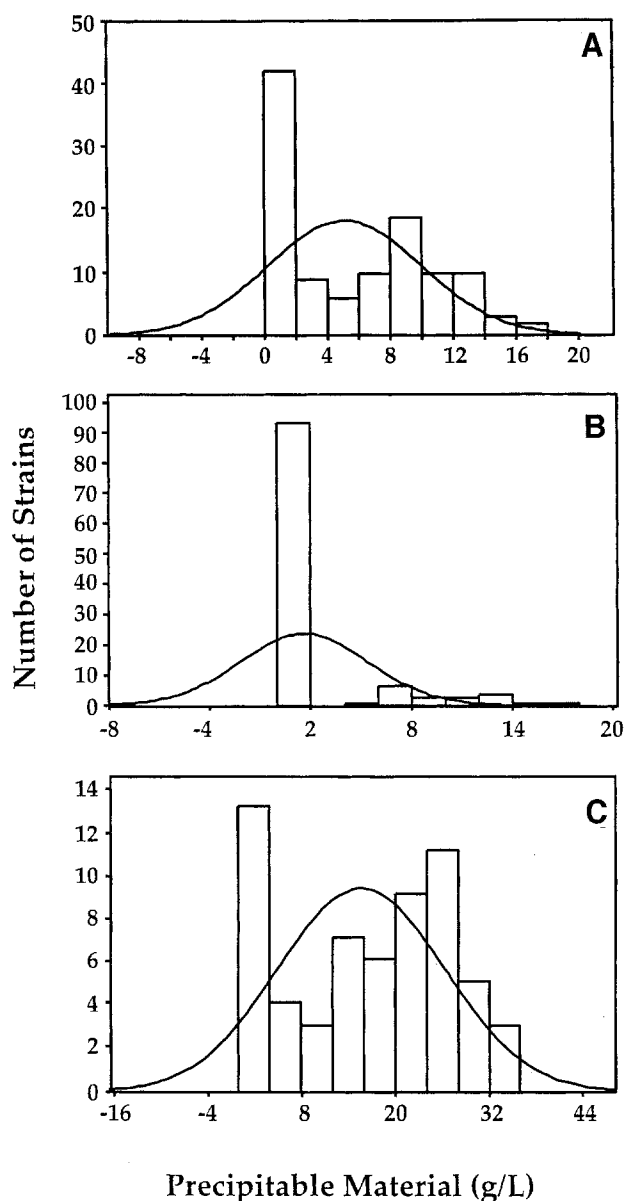


Fig. 1. Yields of extracellular isopropanol precipitable material when *Pseudomonas* strains were grown in a proprietary liquid medium with fructose (A) or glucose (B) or in a modified Vogel and Bonner medium with gluconate (C). Each carbon and energy source was present at 5%, w/v. Cultures were incubated at 24 °C with shaking (250–300 r.p.m.) for 5 days. The theoretical normal distribution is shown on each bar graph.

were generally higher than those obtained in the other media (Fig. 1(C)). The highest yielding strains in MVBM were *P. viridiflava* 15 and *P.s. pv. phaseolicola* R1 with alginate yields of 8 and 9 g L<sup>-1</sup>, respectively.

#### DISCUSSION

In this study maximum conversion rates of the industrially acceptable substrates glucose and fructose of 6–10% were obtained. The theoretical yield with glucose as feedstock is approximately 50% due to the metabolism of glucose primarily

TABLE 1

*Pseudomonas* strains which gave the highest yields of alginate when grown in a proprietary liquid medium in the presence of fructose (5%, w/v)

Bacterium	Strain	Source	Alginate <sup>a</sup> (g L <sup>-1</sup> )
<i>P. cichorii</i>	P14	A.R. Chase	3
	P52	A.R. Chase	3
<i>P. syringae</i>	2664	U. Mazzuchi	3
	NCPBP 2159	NCPBP <sup>b</sup>	4
	A-29-2	W. Fett	5
	J3-17-2	W. Fett	5
	J3-20-4A	W. Fett	4
	R6	N.T. Keen	5
<i>pv. morsprunorum</i>	486	F.L. Lukezic	3
	5	T.J. Burr	3
<i>pv. papulans</i>	17	T.J. Burr	4
	24	T.J. Burr	3
	31	T.J. Burr	3
	Race 2	D.M. Webster	3
<i>pv. pisi</i>	Race 3	D.M. Webster	5
	213	G. Surico	5
<i>pv. savastanoi</i>	GTG24	G. Surico	4
	1147(s)	R. Samson	3
<i>pv. syringae</i>	Y30	D.M. Webster	3
	11528	R.D. Durbin	2
<i>pv. tabaci</i>	pt113	R.D. Durbin	4
	K26	R.D. Durbin	2
<i>pv. tagetis</i>	83-36	R.D. Gitaitis	3
	PT4	J.B. Jones	3

<sup>a</sup> Alginate yields based on the uronic acid content of isopropanol precipitable material in the culture fluids.

<sup>b</sup> NCPBP, National Collection of Plant Pathogenic Bacteria, Harpenden, UK.

through the Entner–Doudoroff pathway before a triose is utilized for alginate biosynthesis, while that for fructose is approximately 90% because of direct conversion to fructose-6-phosphate [6]. The conversion rates obtained in our study may be low due to the use of high initial levels of glucose and fructose in the medium. Main et al. [11] reported that a mucoid strain of *P. aeruginosa* grown as shake flask cultures in a glucose (2%)–yeast extract–mineral salts liquid medium resulted in the production of almost 10 g L<sup>-1</sup> of precipitable extracellular material. Piggott et al. [14] reported alginate yields of 7.5–11.5 g L<sup>-1</sup> for mucoid strains of *P. aeruginosa* grown in shake flasks containing a yeast extract medium with 2% gluconate. However, in neither study was the purity of the alginate preparations assessed. Sengha et al. [16] reported that in pH-controlled batch culture highly mucoid strains of *P. mendocina* produced up to 20 g L<sup>-1</sup> of alginate from media containing a high level of glucose (50 g L<sup>-1</sup>), but it is not clear if alginate determinations were done gravimetrically or by

uronic acid analyses. Conversion values of up to 25–50% for sucrose in batch or continuous culture by *A. vinelandii* have been reported [4,8,17], but this bacterium has a very high oxidation rate which makes it less than ideal for large-scale reactors [6,8]. Also, at low oxygen concentrations this bacterium produces a significant amount of polyhydroxybutyrate at the expense of alginate production [2]. Similar to our results for high yielding strains, when alginate yields reached 7.5 g L<sup>-1</sup> for *A. vinelandii* in batch culture the cultures gelled [2]. Sucrose is not a viable feedstock for alginate fermentation by many fluorescent pseudomonads due to either their inability to utilize sucrose or their ability to synthesize the polyfructan levan by the action of the extracellular enzyme levansucrase [12]. In addition, none of the fluorescent pseudomonads can utilize starch [12].

Increased yields of alginate during culture of the fluorescent pseudomonads tested in this study in fermentation vessels may be achievable by maintaining a highly dissolved oxygen concentration. Also, if pseudomonad alginates of low viscosity are the preferred product, exclusion of protease from the medium may lead to increased yields due to a reduction in culture fluid viscosity by the action of alginases.

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