Journal of Industrial Microbiology, 6 (1990) 129–134
Elsevier

SIM 00266

The use of bacterial alginates to prepare biocontrol formulations

Anthony J. DeLucca II[1], William J. Connick Jr.[1], Deborah R. Fravel[2], Jack A. Lewis[2] and John M. Bland[1]

[1]*Southern Regional Research Center, USDA-ARS, New Orleans, LA, and* [2]*Beltsville Agricultural Research Center, USDA-ARS, Beltsville, MD, U.S.A.*

(Received 21 June 1989; revised 2 November 1989; accepted 5 December 1989)

Key words: Bacterial alginate; Biological control; Plant pathogen; Kelp alginate; Biocontrol formulation

SUMMARY

Formulations which are economical and which can deliver a viable organism are critical to developing successful biocontrol products for plant pathogens. In the present study, alginates derived from commercial kelp and produced by *Azotobacter vinelandii* isolates ATCC 9104 and 12837 were compared in their ability to form stable, biodegradable granular formulations of the biocontrol fungi *Talaromyces flavus* and *Gliocladium virens*. Bacteria were grown in shake flask cultures (180 rpm) at 32°C for 104 h. The cultures were monitored for pH, dissolved oxygen, glucose concentration, dry cell weight, and alginate dry weight. Aqueous solutions of the bacterial alginates, as well as the kelp-derived alginate products, gelled readily in 0.25 M calcium chloride. Mannuronate (M) and guluronate (G) compositions of the alginate samples were determined by circular dichroism. M/G ratios for cultures of isolate 12837 averaged 0.98 ± 0.18; for isolate 9104, 1.59 ± 0.12; and for kelp, 1.54 ± 0.39. The viability of *T. flavus* in the kelp and bacterial alginate formulations were similar over 84 days. An exploratory experiment indicated good viability of *G. virens* using the same bacterial alginates. This study demonstrated a practical use for bacterial alginate as a potentially less costly substitute for kelp alginate in the preparation of biocontrol agent formulations.

INTRODUCTION

Alginate is a naturally-occurring polysaccharide that is obtained for commercial purposes from seaweed (kelp) and is widely used in the food, pharmaceutical, textile, and other industries. Alginates from kelp have been used in agriculture to encapsulate biocontrol agents [9,28] and rhizobia as inoculants for legumes [13]. Chemically, alginate is a block copolymer consisting of (1–4)-linked β-D-mannuronate (M), -L-guluronate (G), and alternating-sequence blocks (MG) [10]. Alginate is also produced as an extracellular polysaccharide by certain bacteria. These include *Azotobacter vinelandii* [4] and several pseudomonads [7,11,17]. There is considerable interest in producing this commercially important polysaccharide from potentially high-yielding bacteria rather than extracting it from seaweed where harvests and quality can vary [23]. Various aspects of commercial alginate production from bacteria have been reported [3,8]. Bacterial alginates have been the subject of several recent reviews [8,10,27].

The objectives of this research were to determine whether alginate obtained from *A. vinelandii* ATCC 9104 and 12837 could be used to prepare granular biological control formulations, and to show that bacterial alginate could be a substitute for kelp alginate.

MATERIALS AND METHODS

Freeze-dried cultures of *A. vinelandii* ATCC 9104 and 12837 were inoculated into a broth basal medium and incubated at 30°C overnight. The broth basal medium consisted of: K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 5.0 mg; soil extract, 100 ml; mannitol, 20.0 g; deionized water, 900 ml (American Type Culture Collection Media Handbook, American Type Culture Collection, Rockville, MD, 1984). The soil extract consisted of: African violet soil, 77.0 g; Na_2CO_3, 0.2 g; distilled water, 200 ml (Autoclaved for 1 h followed by filtration through paper before adding extract to medium). Afterwards, the broth cultures were streaked onto basal medium slants and incubated as before. A second medium, Medium A, was employed for the production of alginate. Medium A consisted of: D-glu-

Correspondence: Anthony DeLucca, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124, U.S.A.

cose, 20.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 200.0 mg; $FeSO_4 \cdot 7H_2O$, 50.0 mg; $NaMoO_4 \cdot 2H_2O$, 5.0 mg; $CaCl_2 \cdot 2H_2O$, 50.0 mg; NH_4OAc, 2.3 g; and deionized water, 1.0 liter. (From: Larsen, B. and A. Haug. Biosynthesis of Alginates. Carbohydr. Res. 17: 287–296, 1971). This medium, amended with 1.5% agar, was also used to maintain stock and working cultures.

Seed cultures were begun by inoculating six-10 ml tubes containing Medium A broth from a stock culture slant. The broth culture was inoculated overnight at 30°C, and the tubes were then aseptically added to 30 ml of Medium A broth in each of six 125-ml Erlenmeyer flasks and placed on a rotary shaker at 180 rpm overnight at 30°C. Each of six 500-ml Erlenmyer flasks containing 300 ml of Medium A broth were inoculated with a 30-ml shake culture and incubated as previously described. At regular intervals, duplicate samples (10 ml) from separate flasks were taken to measure pH and dissolved oxygen. The dissolved oxygen was measured using a dissolved oxygen meter (calibrated prior to use) integral in a Bioflow II benchtop fermentor (New Brunswick Scientific, Edison, NJ). Measurements of dissolved oxygen were made immediately after aliquots of culture fluid were removed from the culture flasks. Glucose concentrations were measured by the DNS method [20]. Duplicate plate counts were performed using Medium A agar. To obtain dry cell weights, duplicate aliquots (10 ml) of culture fluid were removed and centrifuged (1465 × *g* for 10 min). The supernatant was discarded and the cells were washed twice with saline and centrifuged. The wash was discarded and the cells were dried (24 h at 100°C) in a forced-air drier and weighed.

Cultures were centrifuged at 1465 × *g* for 15 min at 4°C to separate cells from the culture fluid which was then carefully decanted and placed in an ice bath. Absolute ethanol (2:1, v/v), previously chilled to 4°C, was slowly added without stirring to the culture fluid. Alginate quickly precipitated at the bottom of the fluid, and later rose to the surface where it was easily recovered by decanting. The crude alginate was freeze dried and weighed.

Ascopores of *Talaromyces flavus* were produced on potato dextrose agar for 4 weeks at 30°C in the dark [18]. An aqueous suspension of ascospores from this slant was prepared and stored at 4°C until needed.

Dried, finely minced bacterial alginate (from separate shake flask runs), 1% by weight, was added with stirring to a mixture of 10% Pyrax™ (pyrophyllite, hydrous ammonium silicate, R.T. Vanderbuilt Co., Norwalk, CT) in deionized water. After the alginate had dissolved, a 2% (final volume) aqueous suspension of *T. flavus* ascospores (10^5 to 10^8 spores/ml) was added to the mixture with stirring. The alginate-Pyrax-ascospore suspension was stirred continuously while it was dripped through Pasteur pipettes (with a 1.0 mm diameter orifice) into a solution of 0.25 M $CaCl_2$ as previously described [9]. Gel beads quickly formed upon addition of each droplet of the mixture to the $CaCl_2$ solution. After the mixture was added, the solution was gently stirred, decanted, and the gel beads were gently washed twice with distilled water. The beads were placed on brown wrapping paper and air dried overnight at room temperature in a laminar air-flow hood. The dried pellets that resulted were stored during the test period in screw-capped glass jars at room temperature. As a control, commercial kelp-derived sodium alginate (Fisher Scientific Company, Fair Lawn, NJ) was used to prepare similar granular preparations by the same process.

Viable populations of *T. flavus* were determined before exposing the organism to the $CaCl_2$ solution, immediately after bead formation, at 24 h (after drying), and at regular intervals thereafter for up to 84 days. The pellets were insoluble in water but disintegrated in a mixture of 8.7×10^{-2} M KH_2PO_4 and 3.0×10^{-2} M Na_2HPO_4 (pH 7.7). The concentration of viable spores was determined by dilution plating [8]. The assays were performed on a semi-selective agar developed for *T. flavus* [18]. The assay was conducted with alginates from four different lots each of ATCC 9104 and 12837.

Prior to analytical studies, the bacterial and kelp alginates (Fisher S-211, Fisher Chemical Co., and Kelgin HV, Kelco, San Diego, CA) were purified. An aqueous solution containing 0.1–0.3% (w/v) of an alginate sample was dialyzed (using 3500 mol. wt. cut-off tubing) against deionized water for 4 days at room temperature with frequent water changes. Following dialysis, the alginate solution was centrifuged (14500 × *g* for 20 min) and freeze dried. The alginate sample was dried in a vacuum oven at 62°C for 24 h over P_2O_5 and afterwards stored in a desiccator over P_2O_5 [14,21].

Alginate purity was determined quantitatively by the method of Kennedy and Bradshaw [14,15] which has been used for microbial alginate [2]. Purified Kelgin HV sodium alginate was used as the analytical standard. The reagent was Cosmocil CQ (ICI Americas, Inc), a 20% aqueous solution of polyhexamethylene biguanine hydrochloride. Ultraviolet (UV) absorbance was measured at 235 nm against deionized water using a Hewlett Packard

Model 8450A diode array spectrophotometer. A minimum of two replicates were averaged for each sample.

The ratio of mannuronate to guluronate (M/G) residues was estimated by the method of Morris et al. [21] using circular dichroism (c.d.) (peak height/trough depth ratio method). Circular dichroism spectra were recorded on a JASCO Model J-500A spectropolarimeter at 2 m°/cm sensitivity, 1 nm slit width, 16 s time constant, 10 mm/cm wavelength expansion, 0.2 cm/min chart speed, and using a 10-mm cell. Sample concentration was 0.10–0.30 mg/ml. Clarification of alginate solutions was performed by centrifugation prior to analysis. Concentration was adjusted to give a trough depth of 4–6 cm on the scan. Data from at least two replicates per sample were averaged.

Acetyl content of each alginate sample was determined by the method of McComb and McCready [19] with β-D-glucose pentaacetate (Aldrich Chemical Co., Milwaukee, WI) as the standard. Iron(III) perchlorate (11.3% Fe; Alfa, No. 22116, Danvers, MA) was used to make the stock solution (3.53 g $Fe(ClO_4)_3 \cdot H_2O$ per 100 ml of deionized water) needed for the acetyl analysis. Results of two or more determinations were averaged. Infrared (IR) analysis was used as an additional check for the presence of *O*-acetyl groups (1250 cm^{-1} and near 1730 cm^{-1}) on alginate [4,11,17,25]. IR spectra (KBr disc) of purified bacterial and commercial alginates were recorded on a Digilab FTS-40 Fourier transform instrument.

A regression of the viability data of each alginate formulation was performed. A log transformation was deemed necessary to construct the appropriate regression model. A Fisher test (*F*-test) was performed on this data to determine the statistical similarity of the three regression models.

RESULTS AND DISCUSSION

The shake flask cultural parameters of *A. vinelandii* ATCC 9104 and 12837 were measured during three separate runs and averaged. These measurements were performed in order to elucidate the cultural conditions associated with production of alginate having the desired properties. Table 1 contains the data for the growth of *A. vinelandii* ATCC 9104, and for *A. vinelandii* ATCC 12837.

The viable bacterial count peaked at 24 h for culture 9104; peak count did not occur until 48 h for culture 12837. The cellular dry weight was highest at 72 h for both cultures. Glucose was slowly, but steadily, utilized by both cultures. Culture 12837 reached minimum residual glucose levels (36% of original) at 96 h; culture 9104 reduced glucose level to 45% of the original amount by the same time. The cellular dry weight and glucose utilization data indicated that culture 12837 was more efficient than 9104 in converting glucose into metabolic products. However, when the amount of alginate produced was compared to the amount of glucose utilized, it was determined that culture 12837 was only marginally better than culture 9104 in alginate production.

The percent dissolved oxygen (DO) quickly fell in both cultures. Within 1 h after inoculation, the DO dropped to

TABLE 1

Cultural parameters of *Azotobacter vinelandii* ATCC 9104 and 12837 in shake flasks[a]

Time (h)	Plate count[b] (cfu × 10^7/ml)		Cellular dry wt. (mg/ml)		Glucose (mg/ml)		Alginate dry conc. (mg/ml)		Dissolved oxygen (%)		pH	
	9104	12837	9104	12837	9104	12837	9104	12837	9104	12837	9104	12827
0	0.18[c]	0.03	ND[d]	ND	20.00	20.00	ND	ND	28.00	28.00	6.77	6.78
24	6.10	0.12	0.30	0.60	17.05	16.60	0.09	0.18	0.00	0.25	7.70	7.75
48	1.60	3.40	0.30	0.60	13.23	11.38	0.16	0.26	0.00	0.25	7.77	7.63
72	1.90	1.70	0.60	0.65	12.03	9.30	0.25	0.30	0.00	0.25	7.27	7.48
96	1.80	1.90	0.40	0.57	8.90	7.14	0.23	0.37	0.00	0.00	6.83	7.10

[a] Averages of three separate runs.
[b] Average inoculum plate count was 1.8 × 10^6 cfu/m for 9104, and 3.3 × 10^5 cfu/ml for 12837.
[c] Inoculum plate count.
[d] ND, not determined.

TABLE 2

Chemical analyses[a] of *Azotobacter vinelandii* ATCC 9104 and 12837 and commercial kelp alginates

Sample	Alginate recovery (%)[b]	Alginate purity (%)[c]	M/G ratio	Acetyl content (%)
A. vinelandii ATCC 9104	63.0	82.3	1.59 ± 0.12	0.09
A. vinelandii ATCC 12837	66.7	85.3	0.98 ± 0.18	0.08
Fisher S-211	89.0	105.0	1.54 ± 0.39	0.03
Kelgin HV	84.0	100.0	1.30 ± 0.48	0.05

[a] Values for the bacterial alginates are each derived from three separate production runs, with each of these being analyzed at least twice.

[b] After purification: (weight of purified alginate divided by weight of crude alginate) × 100.

[c] After the purification procedure.

zero for isolate 9104; that for isolate 12837 was reduced to 0.25. A rapid drop in DO was necessary for production of alginate having the proper gelling characteristics for bead formation (unpublished data). The rise and fall of pH in both cultures were similar and roughly paralleled the bacterial plate counts throughout the study.

Results of the chemical analysis of bacterial alginates from *A. vinelandii* ATCC 9104 and 12837 and two commercial (kelp) alginates are reported in Table 2. Strain 12837 gave a slightly higher recovery and purity after the purification procedure than strain 9104. The commercial kelp alginate, Kelgin HV, was used as the analytical standard (100%) for purity determination after being purified, utilizing the same procedure used with the bacterial alginates. The Fisher S-211 kelp-derived alginate, which was used in the viability study as the control gelling agent, gave a value of 105% purity under these conditions.

The preparation of granular formulations of biocontrol agents by the alginate process [5,9,16] depends upon the gelation of an alginate solution with Ca^{2+}. Gelling properties depend upon the chemical composition of the alginate, especially the relative amounts of D-mannuronate and L-guluronate (M/G ratio), block structure, and the extent of acetylation [24,25,26]. Because Ca^{2+} binds more strongly with the guluronate, lower M/G ratios favor stronger gel formation [25]. The M/G ratios of the bacterial alginate averaged 0.98 ± 0.18 and 1.59 ± 0.12 for strains 12837 and 9104, respectively. This suggests that compositional differences can occur due to bacterial species diversity. Compositional differences can also result from fermentation conditions [1,12,22]. A parallel exists in kelp-derived alginate where the uronic acid composition can also differ substantially, depending on species and the part of the plant from which the alginate was obtained [12].

In published reports [6,26] the bacterial alginate from *A. vinelandii* was described as being *O*-acetylated. However, Cohen and Johnstone [4] reported that *A. vinelandii* ATCC 12837 produced alginate free of acetyl groups. We found no appreciable *O*-acetyl content (≤0.09%) in any of the bacterial alginates we obtained (Table 4) under the preparation and purification methods used, while the kelp-derived alginate had even lower values (≤0.05%), as expected [18]. IR spectra of purified alginate from *A. vinelandii* strains 9104 and 12837 are shown in Fig. 1 compared with a commercial, kelp-derived alginate. The spectra are nearly identical. The absence of significant absorbance at 1250 cm^{-1} and 1730 cm^{-1} shows that the alginates are not significantly *O*-acetylated. The amount of acetyl is influenced by aeration rate. Annison and Couperwhite [1] reported that increasing the aeration rate resulted in increased acetylation. Our results suggest that, by allowing the dissolved oxygen level to quickly drop to 0.00–0.25, the degree of acetylation in the *Azotobacter* alginates can be kept to a minimum in shake flask cultures.

Table 3 shows viable propagule counts of the biocontrol agent, *Talaromyces flavus*, found during the study. Little difference could be seen in the counts from pellets formed with the two bacterial and the kelp alginates on a per assay basis, except on day 84 where strain 12837 counts were approximately 14.3% and 9.7%, respectively, of those of strain 9104 and Fisher alginate bead viable count. Even so, the viable propagule count up to 84 days after pellet production was not less than 2.0×10^4 cfu/g of pellets. These results indicated that the viability of the

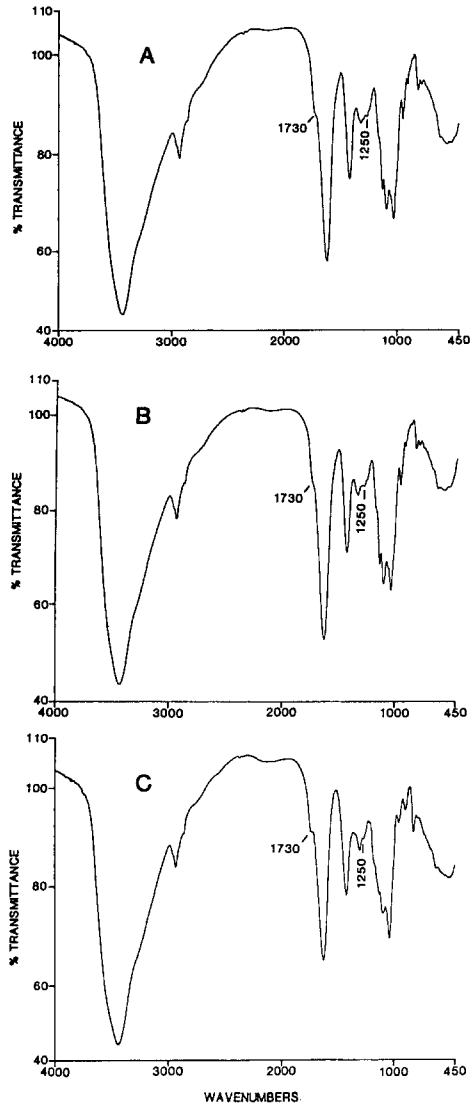

Fig. 1. IR spectra from purified bacterial and kelp alginates. (A) From *Azotobacter vinelandii* ATCC 9104. (B) From *A. vinelandii* ATCC 12837. (C) From Fisher S-211 (kelp-derived).

biocontrol agent, *T. flavus*, in *Azotobacter*-derived alginate is acceptable, especially if one assumes that only a few viable propagules of this agent would be required to begin the biocontrol process in the field [9].

P-values for the regression models were significant. The *P*-values for 9104, 12837, and the Fischer alginate beads were 0.0171, 0.0097 and 0.0284, respectively. Regression models for each data set was analyzed by an *F*-test. In this pair-wise analysis of regression models, the models were found to be statistically similar (Table 4).

TABLE 3

Viable populations of *Talaromyces flavus* in bacterial and kelp alginate-based pellets[a]

Alignate source	Propagule viability (days)[b]					
	1	7	21[c]	35[c]	56[a]	84[c]
Azotobacter vinelandii ATCC 9104	4.7	4.3	6.2	2.5	1.6	1.4
Azotobacter vinelandii ATCC 12837	4.6	4.6	7.3	3.9	1.6	0.2
Fisher S-211	4.4	4.1	8.4	9.8	2.0	2.3

[a] Average of 3 separate runs.
[b] cfu $\times 10^5$/g pellets at indicated days.
[c] Average of 2 separate runs.

In an exploratory experiment using *Gliocladium virens*, a biocontrol agent of *Rhizoctonia solani*, the bacterial alginates could also be substituted for kelp-derived alginates. The pellets were produced as described earlier, except that IG350 alginate (Sinoport International, NY) was used as the control (kelp alginate). Viability was checked after a two-day storage, at room temperature, by homogenizing 0.1 g pellets in 10 ml sterile tap water for 30 s. Replicate plates, medium, and incubation time were the same as described in a published report [16]. Propagule counts were, for 9104, 0.9×10^7 cfu/g; for 12837, 1.9×10^7 cfu/g; and for IG-350, 0.6×10^7 cfu/g.

The results with *T. flavus* demonstrate that unpurified alginate from *A. vinelandii* ATCC 9104 and 12837 could be substituted for kelp alginate in a biocontrol formulation process while maintaining gelling quality as well as viable

TABLE 4

Fisher's test (*F*-test) for equality of regression models[a] for pairing of *Talaromyces flavus* viability for each test alginate formulation

Pairing	*P*-value[b]
A. vinelandii 9104 vs. *A. vinelandii* 12837 alginates	0.576
A. vinelandii 9104 vs. Fisher alginates	0.140
A. vinelandii 12837 vs. Fisher alginates	0.369

[a] Regression model: log (cfu − 1) = 13.04 − 0.027 (time).
[b] *P*-values are results of *F*-tests of the null hypothesis (regression models are the same).

propagules over a lengthy storage period. We believe that this is the first report of a practical use of bacterial alginates.

ACKNOWLEDGEMENTS

We thank J.E. Curry for excellent technical assistance, N.M. Morris and S.A. Lomenzo for IR spectra, and Brian Vinyard for statistical analysis.

REFERENCES

1 Annison, G. and I. Couperwhite. 1986. Effect of limiting substrate concentrations, growth rate, and aeration on alginate composition and production by *Azotobacter vinelandii* in continuous culture. Food Hydrocoll. 1: 101–111.
2 Brivonese, A.C. 1985. Alginate biosynthesis in *Azotobacter vinelandii*. Ph.D. thesis, University of Edinburgh. (available as D 81070 from the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire, U.K. LS23 7BQ.)
3 Bucke, C., L. Deavin, C.J., Lawson and D.F. Pindar. 1975. The production of industrially important bacterial polysaccharides. Biochem. Soc. Trans. (558th Meeting, Edinburgh) 3: 844–847.
4 Cohen, G.H. and D.B. Johnstone. 1964. Extracellular polysaccharides of *Azotobacter vinelandii*. J. Bacteriol. 88: 329–338.
5 Connick, W.J., Jr. 1988. Formulation of living biological control agents with alginate. In: Pesticide Formulations: Innovations and Developments (Cross B. and H.B. Scher, eds.), pp. 241–250, ACS Symposium Series No. 371, American Chemical Society, Washington, D.C.
6 Davidson, I.W., I.W. Sutherland and C.J. Lawson. 1977. Localization of *O*-acetyl groups of bacterial alginate. J. Gen. Microbiol. 98: 603–606.
7 Fett, W.F., S.F. Osman, M.L. Fishman and T.S. Siebles III. 1986. Alginate production by plant-pathogenic pseudomonads. Appl. Environ. Microbiol. 52: 466–473.
8 Fyfe, J.A.M. and J.R.W. Govan. 1982. Synthesis, regulation, and biological function of bacterial alginate. In: Progress in Industrial Microbiology. Vol. 18, Microbial Polysaccharides (M.E. Bushell, ed.), pp. 45–83, Elsevier, New York.
9 Fravel, D.R., J.J. Marois, R.D. Lumsden and W.J. Connick, Jr. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75: 774–777.
10 Gacesa, P. 1988. Alginates. Carbohydr. Polymers 8: 161–182.
11 Gross, M. and K. Rudolph. 1987. Studies on the extracellular polysaccharide (EPS) produced in vitro by *Pseudomonas phaseolicola*. J. Phytopathol. 119: 206–215.
12 Haug, A., B. Larsen and O. Smidsrød. 1974. Uronic acid sequence in alginate from different sources. Carbohydr. Res. 32: 217–225.
13 Jung, G., J. Mugnier, H.G. Diem and Y.R. Dommergues. 1982. Polymer-entrapped rhizobium as an inoculant for legumes. Plant Soil 65: 219–231.
14 Kennedy, J.F. and I.J. Bradshaw. 1984. A rapid method for the assay of alginates in solution using polyhexamethylene-biguanidinium chloride. Br. Polym. J. 16: 95–101.
15 Kennedy, J.F. and I.J. Bradshaw. 1987. The rapid quantitative determination of alginates by poly(hexamethylene-biguanidinium chloride) complexation in industrial liquors extracted from brown seaweed. Carbohydr. Polym. 7: 35–50.
16 Lewis, J.A. and G.C. Papavizas. 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. Plant Pathol. 34: 571–577.
17 Linker, A. and R.S. Jones. 1966. A new polysaccharide resembling alginic acid isolated from pseudomonads. J. Biol. Chem. 241: 3845–3851.
18 Marois, J.J., D.R. Fravel and G.C. Papavizas. 1984. Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae*. Soil Biol. Biochem. 16: 387–390.
19 McComb, E.A. and R.M. McCready. 1957. Determination of acetyl in pectin and in actylated carbohydrate polymers. Anal. Chem. 29: 819–821.
20 Miller, G.L. 1989. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426–428.
21 Morris, E.R., D.A. Rees and D. Thom. 1980. Characterization of alginate composition and block-structure by circular dichroism. Carbohydr. Res. 81: 305–314.
22 Osman, S.F., W.F. Fett and M.L. Fishman. 1986. Exopolysaccharides of the phytopathogen *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 166: 66–71.
23 Paul, F., A. Morin and P. Monsan. 1986. Microbial polysaccharide with actual potential industrial applications. Biotechnol. Adv. 4: 245–259.
24 Penman, A. and G.R. Sanderson. 1972. A method for the determination of uronic acid sequences in alginates. Carbohydr. Res. 25: 273–282.
25 Sherbrock-Cox, V., N.J. Russell and P. Gacesa. 1984. The purification and chemical characterization of the alginate present in extracellular material produced by mucoid strains of *Pseudomonas aeruginosa*. Carbohydr. Res. 135: 147–154.
26 Skjåk-Bræk, G., B. Larsen and H. Grasdalen. 1985. The role of O-acetyl groups in the biosynthesis of alginate by *Azotobacter vinelandii*. Carbohydr. Res. 145: 169–174.
27 Sutherland, I.W. 1982. Biosynthesis of Microbial Exopolysaccharides. In: Advances in Microbial Physiology. Vol. 23 (Rose, A.H. and J.G. Morris, eds.), pp. 80–150, Academic Press, New York.
28 Walker, H.L. and W.J. Connick, Jr. 1983. Sodium alginate for production and formulation of mycoherbicides. Weed Sci. 31: 333–338.