INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Volume: 3: Issue-1: Jan - Mar-2012 *ISSN 0976-4550*

Accepted: Oct-2011 *Research Article*

SMALL SCALE PRODUCTION AND CHARACTERIZATION OF ALGINATE FROM *AZOTOBACTER CHROOCOCCUM* USING DIFFERENT SUBSTRATES UNDER VARIOUS STRESS CONDITIONS

Gayathri Pandurangan [1], Jyothi Subbiah [1], Karunakaran Thiyagarajan [2], Joseph K. David[2]

[1]Department of Biotechnology, University of Madras, Chennai- 600 025, India
psgayathri@yahoo.com, jyothiranjani@gmail.com

[2]National Institute of Epidemiology (ICMR), TNHB, Ayyapakkam, Chennai- 600 077, India
thiyakaruna@gmail.com, drjosephkdavid@gmail.com

ABSTRACT: Alginates form an important family of biopolymers. These are linear polysaccharides composed of variable amounts of (1–4)-β-D-mannuronic acid and its epimer, α-L-guluronic acid. Currently, commercial alginates are extracted from marine brown algae. Considering the merits of bacterial alginates, optimal fermentation conditions aiming at the maximization of alginate using bacterium *Azotobacter chroococcum* from different substrates were examined. Whey, molasses, ammonium nitrate, starch, yeast extract, butanol, mannitol, and glucose have been used. The alginate obtained from whey (45.15%), ammonium nitrate (46.02%) and butanol (47.3%) varied. Among the physical stress conditions, the production of alginate was maximum at heat shock 50 ^{0}C for 30min (42.96%) followed by 41.29% on UV radiation for 10 min. At pH 7 and 8 the alginate produced was 44.63% and 46.64% respectively. Carbazole reagent was used to recognize alginate; it was lyophilized and quantitated by gas chromatography.

Key words : Alginate, biopolymer, *Azotobacter chroococcum*, exopolysaccharide,

INTRODUCTION

Alginates are linear copolymers of β-D-mannuronicacid (M) and it's C-5-epimer, α-L-guluronicacid (G) and it is a commercially important polysaccharide. Alginate immobilized cell systems are used as biocatalysts in several industrial processes such as ethanol production by yeast cell and in beer manufacture to enhance the foam. It is also used in water treatment procedures for increasing the aggregate sizes in the flocculation processes. In food industry it is used mainly in ice creams, frozen custards, cake mixtures and fruit drinks to assist the suspension of fruit pulp (Neidleman 1991). Its immunological use is mainly for the production of monoclonal antibodies from hybridoma cells (Crescenzi 1995), stimulating immune cells to secrete cytokines, such as tumor necrosis factor-α, interleukin-1 and interleukin-6 (Otterlei *et al*. 1991).

About 30,000 metric tons of sodium alginates per year are currently used in the food, pharmaceutical, textile and paper industries as thickening, stabilizing and jellifying agents. All alginates used for commercial purposes are currently being produced from brown seaweeds. However, considering the quality of bacterial alginate and the environmental impact associated with seaweed harvesting and processing, bacterial alginate is a better choice for commercial purpose (Dekwer and Hempel 1999). Another reason for the interest in bacterial alginate is the potential development of products with a wide range of molecular weights and properties compared to algal alginates.

However the technology has never been commercialized and is unlikely to ever be competitive against seaweed based alginate production. The genus *Azotobacter* seems to be the best candidate for the industrial production of alginate. It is a gram-negative soil bacterium, which under adverse environmental conditions forms resistant cysts. The mature cysts are surrounded by two capsule-like layers containing a high proportion of the exopolysaccharide alginate. These molecules characterized by a chemical composition, molecular mass and distribution suited to well-defined applications required in the biotechnological, biomedical and pharmaceutical fields (Clementi 1997).

MATERIALS AND METHODS

Initial culturing of organism

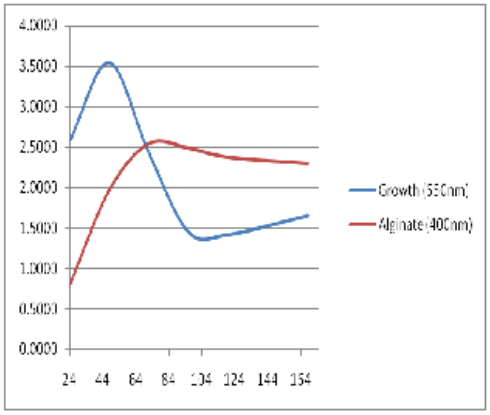
The bacterial isolates grown on selective medium (Mannitol agar) were identified and confirmed using standard biochemical tests. *Azotobacter chroococcum* were grown in Mannitol broth at 35^{0}C for 24h in rotary shaker maintained at 160 rpm

Growth conditions

1ml of culture was inoculated in100 ml-Erlenmeyer flask containing production medium (Winogradsky's medium). This culture was incubated on a rotary shaker at 300rpm and 35^{0}C for 48h. From the production medium kept in the incubator, optical density (OD) was taken at 650 nm, to ensure the growth of organism. Simultaneously, optical density at 400nm assay (CARY 50 scan UV/Vis spectrum spectrophotometer) was observed for alginate production at the regular intervals of 24h (fig. 1) (Schwartz and Bodie 1985).

Fig. 1 Growth of Azotobacter chroococcum with respect to alginate production

Time (h)	Growth (650nm)	Alginate (400nm)
24	2.5698	0.7875
48	3.5412	1.9620
72	2.4298	2.5412
96	1.4568	2.4875
120	1.4232	2.3754
168	1.6562	2.2984



Comparative evaluation of *Azotobacter chroococcum* with various substrates for optimal production of alginate

To the production medium (Winogradsky's medium) different substrates like Whey, Butanol, Molasses, Starch, Mannitol, Glucose, Ammonium nitrate and Yeast extract were added separately with 1% concentration in eight 250 ml conical flasks respectively.

Optimization

Production medium with varying pH values 5, 6,7,8,9 were prepared and inoculated with test organism. The effect of pH on the production of alginate was determined. The inoculated production medium was maintained at varying temperatures ranging from 28^{0}C to 40^{0}C and their productivity was analyzed to determine the effect of temperature. Similarly samples were analyzed on 24h basis for a week and the time of maximum production was determined. The organism was then subjected to u-v shock and temperature shock (50^{0}C) for 10 min and 30min respectively. The productivity was determined after 48 h.

Violamine staining

An air-dried and smear was treated with violamine stain for 45 seconds, washed and counter stained with dilute crystal violet (0.05%) for 1min. After rinsing with water the preparation was subjected to microscopic examination

Recovery of polysaccharide

Culture broth samples were centrifuged at 5000 rpm at 100C for 30 min by using a refrigerated super speed centrifuge, to settle bacteria and capsular material. The pellet containing bacteria and capsular material was suspended in 10mM Ethylenediamine tetra acetic acid with sodium salt for 2 min to solublise the cell-associated alginate, and finally centrifuged at 7500 rpm for 30min at 100C. The alginate free cell precipitate was washed twice with distilled water, centrifuged, dried at 1050C till constant weight to yield the biomass concentration. The supernatant was frozen at 200C until needed for chemical analyses or immediately used to recover exopolysaccharide by adding 3 volumes of 95% (v/v) ethanol. After centrifugation at 7500 rpm at 40C for 20 min, the alginate- based sediment was washed twice with 95% (v/v) ethanol, recentrifuged and freeze dried. The samples were freeze dried at -400C and 100Kpa vacuum in a lyophilizer. The obtained biomass, final pellet and alginate powder after lyophilization were weighed separately.

Carbazole reagent assay

Reaction of alginate sample with Carbazole reagent (Dekwer and Hempel, 1999) was studied by adding Carbazole reagent. It was mixed and heated at 55 ^{0}C for 30 min; colour was stable for 2 h at room temperature. Standard curves of mannuronic acid as well as guluronic acid were compared with the readings of alginate samples taken at 400nm (CARY 50 scan UV/Vis spectrum spectrophotometer).

Gas chromatography

Alginate assay by GC was done as per the method of Stephen *et al*., 2002. The alginate obtained was dissolved in methanol. The methylated compound containing D-mannuronicacid and L-guluronicacid were subjected to GC analysis. GC was equipped with a carbowax 20M 25m X 0.25mm, df = 0.25µm. The peak area ratios of the monomers and the internal standard allowed the determination of the polymer accumulated by the cells.

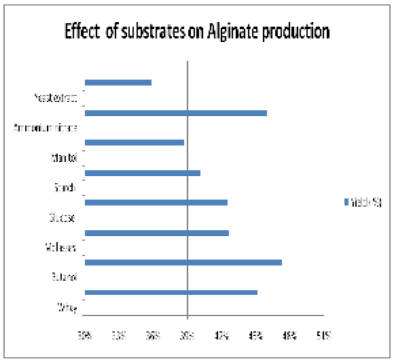
RESULTS AND DISCUSSION

Azotobacter chroococcum is well adapted to the laboratory conditions of growth and was able to produce significant amount of alginate at a variety of physical conditions when grown on the basic mannitol growth medium. There was insignificant growth observed up to 48h in Mannitol agar at 35^{0}C. The colonies were mucoid due to the secretion of exopolysaccharide (alginate). From the results obtained *Azotobacter chroococcum* is regarded as an efficient producer of alginate biopolymer when subjected to different fermentation substrates of carbon and nitrogen sources like whey, butanol, molasses, glucose, ammonium nitrate and yeast extract respectively. The biopolymer production was minimum in mannitol broth (38.74%), while maximum was obtained when butanol (47.13%), ammonium nitrate (46.02%) and whey (45.15%) were used as substrates. Increasing yeast extract and ammonium nitrate as organic and inorganic nitrogen sources showed production as high and least respectively (fig. 2).

Although *Azotobacter chroococcum* could produce biopolymer at 25^{0}C incubation, maximum yields were obtained from fermentation done at 36^{0}C. The formation of exopolysaccharide found to be maximum at temperature 36^0 C (47.14%) (fig. 4) . At pH 7-8 the percentage of alginate production ranges from 43.63%-46.64% (fig. 3).

Fig.2 Effect of substrates on Alginate production

Substrate (g)	Biomass (g)	Pellet weight (g)	Dry weight (g)	Yield (%)
Whey	3.5371	3.4170	2.9120	45.15%
Butanol	3.9562	3.9123	3.5621	47.3%
Molasses	3.5721	3.2481	2.6481	42.57%
Glucose	3.3213	3.1769	2.4560	42.51%
Starch	3.4630	3.2034	2.3179	40.09%
Mannitol	3.1272	2.4564	1.9780	38.74%
Ammonium nitrate	3.8268	3.4851	3.2631	46.02%
Yeast extract	3.6285	3.2952	2.0255	35.82%



This strain was able to produce biopolymer under uncontrolled pH condition also. Maximum production was seen in fermentations where the pH was maintained at 7.5. When fermentations were done under aerated condition, there was improvement in polymer production. Experimental results show that, alginate production was decreased at pH 5 (21.57%). The pH, time & temperature affects both bacterial growth and biopolymer production. When the bacterial cells are exposed to pH beyond their optimum range, maintenance energy is used for pH control. This reduces the energy available for biopolymer production, thus the bacterial ability to produce the biopolymer is reduced. The media pH also affects the permeability of the bacterial cell membrane thus affecting the biochemical activities of the cell required for biopolymer production. The optimized time period showing high production of alginate is 72hr of incubation (fig 5) with gradual decrease in the production. Under UV stress condition and temperature shock (50^0C) alginate production was found to be 41.29% and 42.96% respectively (fig 6). After optimizing environmental factors such as pH, incubation time, temperature, UV stress treatment and temperature shock (50^0C). *Azotobacter chroococcum* was able to produce alginate greater than 3mg/ml.

Fig.3 Effect of pH on alginate production

pH	Biomass (g)	Pellet weight (g)	Dry weight (g)	Yield (%)
5	0.9762	0.6319	0.2685	21.57%
6	1.3259	0.7172	0.4134	23.76%
7	3.4351	3.1254	2.7691	44.63%
8	3.5268	3.4322	3.0832	46.64%
9	2.8330	2.5002	2.0349	41.80%

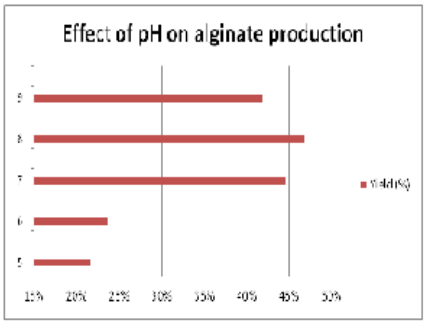


Fig.4 Effect of temperature on Alginate production

Temperature (^{0}C)	Biomass (g)	Pellet weight (g)	Dry weight (g)	Yield (%)
28	3.0289	1.9021	1.3631	31.03%
30	3.2891	2.0775	1.7658	34.93%
32	3.3963	3.1796	2.8402	45.54%
34	3.3305	3.0153	2.7953	45.63%
36	3.5634	3.3725	3.1790	47.14%
38	3.4683	3.2681	2.9021	45.5%
40	3.3821	3.1832	2.8632	45.84%

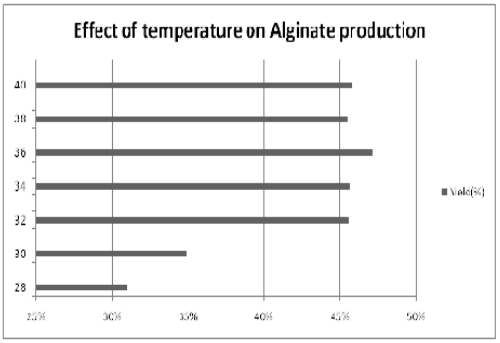


Fig.5 Effect of time on alginate production

Time (h)	Biomass (g)	Pellet weight (g)	Dry weight (g)	Yield (%)
24	2.5634	1.4058	0.5718	18.23%
48	3.2261	2.5395	2.3190	41.82%
72	3.3412	2.8897	2.5757	42.95%
96	3.3351	2.8062	2.5098	42.93%
120	3.3293	2.7658	2.4919	42.80%
144	3.3106	2.6604	2.3984	42.01%
168	3.2891	2.0775	1.7658	34.93%

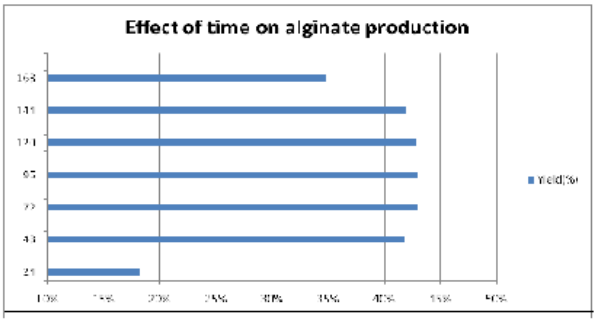
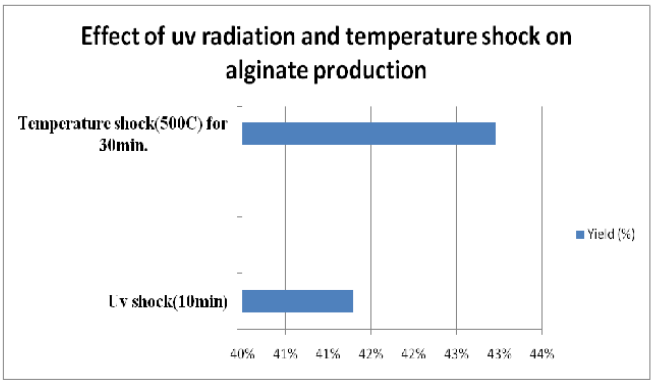


Fig.6 Effect of UV radiation and temperature shock on alginate production

Stress condition	Biomass (g)	Pellet weight (g)	Dry weight (g)	Yield (%)
UV shock (10min)	2.2261	1.9210	1.5658	41.29%
Temperature shock (50^0C) for 30min.	2.5176	2.0424	1.7461	42.96%



The colour was produced by reaction of alginate samples with carbazole reagent and was stable for 2h. The white powder that was obtained under optimized conditions of temperature, time, pH and different substrates by *Azotobacter chroococcum* was quantitated using gas chromatography. Therefore the production of biopolymer with commercial importance by different substrates utilization under different stress conditions ensures that not only value added products are obtained, but the products such as whey, molasses, starch can also be fruitfully utilized.

REFERENCES

Clementi, F., (1997). Alginate production by *Azotobacter vinelandii*. Crit. Rev. Biotechnology, **17** (4), 327-61.

Crescenzi, V., (1995). Microbial polysaccharides of applied intrest. Ongoing research activities in Europe. Biotech. Prog., **11**, 251-259.

Dekwer, D. Hempel, D.C., (1999). Microaerophilic production of alginate by *Azotobacter vinelandii*. Von der Gemeinsamen Naturwissenscha ftlichen, Fakultat der Technischen UN. Carolo-Wilhelmina zu Braunschweig, Edited by Wael Sabra, aus Alexandria, Agypten, 37-54.

Emtiazi, G. Ethemadifara, Z.; Habibib, M.H., (2004). Production of extra-cellular polymer in *Azotobacter* and biosorption of metal by exopolymer. African J. Biotech., **3** (6), 330-333.

Grasdalen, H., (1983). High field, 1H-n.m.r. spectroscopy of alginate: sequential structure and linkage conformations. Carbohyd. Res., 118, 255-260.

Neidleman, S.L., (1991). Microbioal production of biochemical. The genetic engineer and biotchnologist. Biopaper J., 20-22.

Otterlei, M.; Ostgaard, K.; Skjak-Braek, G.; Smidsrod, G., (1991). Induction of cytokine production from human monocytes stimulated with alginate. J. Immonother., 10, 286-291.
Sabra, A.; Zeng, P.; Lonsdorf, H.; Deckwer, W.D., (2000). Effect of oxygen on formation and structure of Axotobacter vinelandii alginate and its role in protecting nitregenase. Appl. Environ. Microbiol., 66, 4037-4044.
Schwartz, R.D.; Bodie, E.A., (1985). Production of high viscousity whey broths by lactose utilizing Xanthomonas campestris strain. Appl. Environ. Microbial., 50, 1483-1485.
Stephen M Alistair ., Glyn O. Phillips, Peter A. Williams (2002). Food Polysaccharides and Their Applications, Marcel Dekker, Inc., New York, pp. 441-462.