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ALGINATE PRODUCTION BY A MUTANT STRAIN OF *AZOTOBACTER VINELANDII* USING SHAKE FLASK FERMENTATION

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Abstract

The present study describes the improvement of parent strain of *Azotobacter vinelandii* NRRL-14641 for enhanced alginate production using shake flask fermentation. Results indicated that a mutant *A. vinelandii* EMS-45 obtained after EMS treatment gave 1.55 fold better alginate productions than that of the parent strain. Different cultural conditions were employed to optimize the polymer production in the fermentation broth. It was noted that maximum alginate production (6.17 g/l) was obtained with nitrogen rich phosphate limited medium (NRP) after 110 h of incubation period. The incubation temperature 30°C, pH 7.0, agitation intensity 200 rpm, carbon and nitrogen source were also optimized and sucrose and peptone were found as the best to support 1.55 fold higher alginate than that in un-optimized medium.

Introduction

Alginate is a linear copolymer of 1, 4-β-D-mannuronic acid and α-L-guluronic acid, linked *via* β -1, 4-glycosidic bond. It was first named algic acid or algin and only a few of its properties were known (Barrera *et al*. 2009). Over the next hundred years, more fascinating properties of the polymer were discovered. Alginate has negative charge on it, which enables it to form solutions with higher viscosity (gel-like). Commercially alginate is obtained from brown seaweeds such as *Laminaria digitata, L. hyperboreau* and *Macrocystis pyrifera* (Gorin & Spencer, 1966).

The alginate is widely used as thickening, stabilizing, gelling and emulsifying agent in industries like food, textile, paper and pharmaceutical (Remminghorst & Rehm, 2006). More than half of the total alginate produced world wide is used in food industries such as ice cream, icy custards, cream and cake mixtures, as well as to keep the contents in suspension in fruit juices (Saude *et al*., 2002).

Although, seaweeds are the major source of alginate for commercial use, however, the alginate produced by the bacteria is considered to be of good quality as compared to that obtained from the algae. The bacterial alginate has better qualitative properties, so it may be sold at higher price as compared to that from seaweed alginate (Ertesvag *et al*., 1998). Different bacterial strains such as species of *Azotobacter* and *Pseudomonas* have been reported since long to produce alginate. *A. vinelandii* is more suitable for the biosynthesis of alginate in view of its latent utilization as a food stabilizer (Funami *et al*., 2009). Moreover, the alginate obtained from *Pseudomonas* has poor jellifying ability (Brivonese & Sutherland, 1989). On the other hand considerable pathogenic ability associated with species of *Pseudomonas* has made *Azotobacter* as favorite genus for the alginate production (Moresi *et al*., 2009).

The aim of the present study is to obtain a stable mutant and optimize different cultural conditions for enhanced alginate production by *A. vinelandii* by using search technique-using one variable at a time approach.

Materials and Methods

Microorganism, maintenance and storage: The parent strain of *A. vinelandii* NRRL-14641 was taken from the culture bank of IIB, Government College University Lahore. The strain (in lyophilized form) was originally supplied by the Agricultural Research Service (A.R.S.) of United States department of agriculture (U.S.D.A). The culture was aseptically transferred to a 250 ml shake flask containing 50 ml of sterilized Jarman medium for maintenance (Jarman *et al*., 1978). The flask was placed in an incubator shaker (Gallenkamp, UK) at 30°C and 200 rpm for 24 h. After 24 h, 0.1 ml of this suspension was aseptically transferred to Burk's nitrogen free agar medium slants. The slants were placed in an incubator (Sanyo, Japan) at 30°C for 24-48 h. After the bacterial growth, the slants were stored at 4°C in a cool cabinet (Sanyo, Japan).

Mutagenesis: For strain improvement, the parent culture was exposed to physical i.e. UV irradiation for various time intervals (10-60 min) under a UV lamp (Mineral Light UVS.12, California, USA, λ =253 nm at cycles/S, 220 V). The distance between lamp screen and bacterial culture was attuned at 5 cm for each trial. The hyper producer strain was then subjected alternatively to nitrous acid (HNO_2, 0.1-0.4 M) and ethyl methane sulphonate (EMS, 25-100 µl/ml) treatment.

Inoculum preparation: A loopful of bacterial culture was aseptically transferred to 50 ml of sterilized Jarman medium in 250 ml conical flask. The flask was incubated at 30°C in a rotary shaker at 200 rpm for 24 h. This bacterial suspension was used as an inoculum for shake flask experiments. The density of bacterial cells (number of bacteria per unit volume) was measured with haemacytometer, Neubauer improved; precicdor HBG. Germany, (Tiefe depth profondeur 0.100 mm and 0.0025 mm^2 area). The counting was done according to the procedure of Sharma (1989).

Shake flasks studies: Fifty ml of NRP medium (pH, 7.0) was transferred to each of 250 ml cotton plugged conical flasks. The flasks were sterilized in an autoclave for 25 min (Temp. 121°C and Pressure, 15 lbs/in^2). After cooling at room temperature, the flasks were inoculated with 2 ml of the bacterial inoculum and kept at 30°C in a rotary shaking incubator at 200 rpm. The flasks were removed after specific time periods and the fermented broth was then used for the estimation of alginate and dry cell mass (DCM).

Assay methods

Cell biomass: The dry weight of biomass was determined by the method of Knutson & Jeanes, (1968). The EDTA sodium salt solution (1.0 ml of 0.5M solution) and sodium chloride salt solution (0.5ml of 5.0 M solution) were put in to a 25 ml of fermented broth for the isolation of the capsular alginate. These samples were centrifuged at 18000 rpm at 20°C for half an hour in order to separate the bacterial cells. The supernatant was separated for alginate determination. The cells were washed twice with distilled water, centrifuged again and then dried at 80°C for 24 h for the determination of DCM.

Alginate estimation: The separated supernatant was then cooled in an ice bath and 3-volumes of ice cold isopropanol were added to the supernatant. This was kept at 4°C over night. Precipitates of alginate were recovered by centrifugation at 18000 rpm at 4°C for 30

min. The precipitates were dissolved in water, precipitated again, centrifuged and finally dried at 80°C for 24 h. The alginate was estimated by weighing the dried precipitates.

Guluronic acid: mannuronic acid ratio: The guluronic to mannuronic acid ratio was measured at 546 nm through colorimetric reaction of carbazole after Knutson & Jeanes (1968). For the reactivity ratio of 50 µg of alginate, the calculations were made as:

$$R = \frac{A'_{mix}}{A_{mix}} = \frac{f_1 A'_1 + (1-f_1) A'_2}{f_1 A_1 + (1-f_1) A_2}$$

where

A'_{mix} was the absorbance of 50 µg alginate sample without borate at 55°C

A_{mix} was the absorbance of 50 µg alginate sample with borate at 55°C.

f_1 was the fraction of mannuronic acid in the alginate sample.

A_1 and A_2 were the absorbance of the mannuronic acid and guluronic acid (50µg). The reaction was carried out at 55°C without borate.

A'_1 and A'_2 were the absorbance of the mannuronic acid and guluronic acid (50µg). The reaction was carried out at 55°C with borate.

From the equation we have:

$$f_1 = \frac{A'_2 - A_2 R}{R(A_1 - A_2) - A'_1 + A'_2}$$

And thus

the % of the mannuronic acid = $f_1 \times 100$

the % of the guluronic acid = $f_2 =$ $(1-f_1) \times 100$

Reaction of alginate without Borate: One ml of concentrated sulphuric acid and 0.1 ml of 0.5 g/l of alginate solution sample were taken in the test tube. The test tube was cooled in the ice bath and the mixture was shaken and kept in the water bath at 55°C. After 30 min, the sample was cooled in ice bath and 30 µl of carbazole reagent (0.1% in ethanol) was added. The tube was allowed to stand for about 3h and then the absorbance was taken at 546 nm. The color was stable for 2 h.

Reaction of alginate with Borate: One ml of borate sulphuric acid reagent (10 mM H3BO3 in concentrated sulphuric acid) was mixed with 0.1 ml of 0.5 g/l of alginate solution in a test tube and then this mixture was cooled in an ice bath. Rest of the procedure was carried out as above.

Results and Discussion

Mutagenesis: The vegetative cells of parent strain of *Azotobacter vinelandii* (24 h old) were subjected to UV irradiation for different time intervals (15-60 min) for the isolation of mutants with enhanced alginate production. After 60 min, 100 % death rate was observed and all the survivors (UV treated) strains were picked up and examined for alginate production. Out of 98 survivors, 30 isolates gave negligible amount of polymer (Table 1). The strain designated UV-66 gave maximum polymer production (4.40 g/l) as compared to parent (3.35 g/l). This might be due to the formation of thymine-thymine dimmers which resulted in mutation (Roldan *et al*., 2003b., Chen *et al*., 1985).

Table 1. Screening of UV treated isolates of *A. vinelandii* NRRL- 14641 for the production of alginate in shake flasks.

UV isolates	Exposure time	Alginate (g/l)	Dry Cell Mass (g/l)
Parent strain	**(min)**	**3.35±0.34**	**4.60±0.28**
UV-1	15	2.56±0.18	3.05±0.20
UV-2		3.50±0.21	4.55±0.12
UV-3		3.20±0.09	4.20±0.32
UV-4		2.25±0.11	3.35±0.21
UV-5		3.30±0.30	2.50±0.15
UV-6		3.11±0.25	4.10±0.23
UV-7		2.34±0.14	3.36±0.25
UV-8		3.69±0.24	4.55±0.28
UV-9		3.05±0.40	4.10±0.15
UV-10		3.55±0.21	4.45±0.20
UV-11		2.13±0.25	3.15±0.19
UV-12		2.56±0.08	3.65±0.25
UV-13		3.28±0.32	4.58±0.28
UV-14		3.11±0.47	4.25±0.15
UV-15		2.80±0.23	3.50±0.29
UV-16		3.00±0.33	3.80±0.45
UV-17		2.90±0.25	3.55±0.20
UV-18		2.80±0.10	3.74±0.27
UV-19		3.12±0.16	4.25±0.27
UV-20		3.50±0.27	4.35±0.35
UV-21		3.27±0.11	4.35±0.40
UV-22		3.39±0.23	4.50±0.25
UV-23		3.00±0.36	4.10±0.19
UV-24		3.40±0.15	4.45±0.32
UV-25	30	3.40±0.18	4.50±0.25
UV-26		2.90±0.06	3.55±0.27
UV-27		2.98±0.03	3.30±0.15
UV-28		3.12±0.23	4.25±0.46
UV-29		3.23±0.22	4.30±0.36
UV-30		3.10±0.26	4.15±0.27
UV-31		2.76±0.18	3.65±0.18
VU-32		3.50±0.34	4.35±0.32
UV-33		2.45±0.13	3.50±0.36
UV-34		3.10±0.20	4.20±0.23
UV-35		2.60±0.19	3.55±0.18
UV-36		3.15±0.41	4.20±0.38
UV-37		3.10±0.28	4.23±0.33
UV-38		3.12±0.39	3.95±0.17
UV-39		3.20±0.28	4.55±0.25
UV-40		1.75±0.08	2.95±0.06
UV-41		2.00±0.16	3.35±0.19
UV-42		3.15±0.15	4.25±0.28
UV-43		1.50±0.05	1.95±0.14
UV-44		2.75±0.18	3.50±0.09
UV-45		3.70±0.28	4.55±0.12

Table 1. (Cont'd.).

UV isolates	Exposure time	Alginate (g/l)	Dry Cell Mass (g/l)
Parent strain	**(min)**	**3.35±0.34**	**4.60±0.28**
UV-46	45	2.65±0.15	3.45±0.22
UV-47		3.50±0.27	4.75±0.43
UV-48		3.16±0.36	4.20±0.37
UV-49		2.65±0.19	3.35±0.22
UV-50		2.95±0.23	3.99±0.18
UV-51		3.67±0.21	4.40±0.41
UV-52		3.17±0.39	4.35±0.34
UV-53		3.38±0.28	4.40±0.26
UV-54		2.18±0.17	3.20±0.19
UV-55		1.99±0.07	2.95±0.16
UV-56		3.19±0.23	4.30±0.36
UV-57		3.15±0.25	4.25±0.24
UV-58		3.33±0.24	4.35±0.23
UV-59		2.56±0.16	3.65±0.36
UV-60		3.10±0.31	4.10±0.35
UV-61		3.13±0.09	4.35±0.23
UV-62	60	4.17±0.17	5.20±0.47
UV-63		3.95±0.05	4.80±0.15
UV-64		4.32±0.38	5.55±0.18
UV-65		3.81±0.27	4.30±0.34
UV-66		**4.40±0.03**	**5.50±0.15**
UV-67		4.23±0.29	5.35±0.23
UV-68		3.85±0.15	4.95±0.17

Incubation 120 h, Temperature 37°C, pH 7.0

Each value is an average of three parallel replicates. ± indicate standard deviation

The strain *A. vinelandii* UV-66 developed by UV method was further treated with different concentrations of nitrous acid (0.1-0.4 M) for improvement of product yield. Fifty two strains were isolated after the nitrous acid treatment and evaluated for the alginate production (Table 2). The isolate no. NA-33 gave maximum alginate production (4.70 g/l). Nitrous acid brings about changes in DNA bases by replacing amino group with a hydroxyl group which may cause mutation and hence increase polymer production (Vazquez *et al*., 1999). The NA-33 strain was further treated with different concentrations (25-100 µl/ml) of ethyl methane sulphonate (EMS). Seventy five isolates were picked up (Table 3) and were then screened for alginate production. The isolate EMS-45 gave maximum alginate production (5.20 g/l). This might be due to the fact that EMS reacts with DNA and adds ethyl group to the bases and this results in mispairing of the affected base or its entire loss, leading to mutation (Chen *et al*., 1983).

Culture medium: Medium composition has great influence on the production of alginate and the growth of the microorganism. The components of the medium provide nutritional requirement for the optimal growth and polymer production by the bacteria (Clementi *et al.,* 1995., Garcia *et al.,* 2001). Six different media were tested for alginate production (Fig. 1). Of all the media examined, M_3 (NRP) gave maximum biopolymer production (5.30 g/l with 6.45 g/l of DCM). This might be due to the fact that M_3 medium has peptone and metal ion activators (Mg^{+2}, Ca^{+2} and K^{+}). The peptone is partially digested protein and has free amino acids. These amino acids are easily available to the bacteria for growth and polymer production.

Table 2. Screening of nitrous acid treated isolates of *A. vinelandii* UV-66 for the production of alginate in shake flasks.

Nitrous acid isolates	Concentration of NA (M)	Alginate g/l	Dry cell mass (DCM) g/l
UV-66 strain		**4.40±0.03**	**5.50±0.05**
NA-1	0.1	3.21±0.19	4.10±0.28
NA-2		3.45±0.14	4.55±0.26
NA-3		4.30±0.29	5.35±0.28
NA-4		4.10±0.18	5.20±0.26
NA-5		3.40±0.26	4.50±0.21
NA-6		3.85±0.25	4.80±0.28
NA-7		4.40±031	5.55±0.35
NA-8		4.40±0.26	5.50±0.37
NA-9		4.37±0.18	5.40±0.23
NA-10		3.10±0.27	4.15±0.23
NA-11		3.25±0.19	4.36±0.28
NA-12		2.59±0.28	3.50±0.21
NA-13		3.70±0.12	4.60±0.16
NA-14		4.42±0.24	5.50±0.28
NA-15		4.30±0.35	5.20±0.35
NA-16		2.50±0.16	3.60±0.16
NA-17		3.98±0.12	4.80±0.21
NA-18		2.98±0.15	4.00±0.15
NA-19		3.82±0.25	4.90±0.25
NA-20		4.30±0.37	5.35±0.18
NA-21		4.24±0.16	5.30±0.38
NA-22	0.2	4.27±0.23	5.35±0.29
NA-23		3.15±0.26	4.22±0.15
NA-24		4.10±0.25	5.17±0.16
NA-25		4.19±0.21	5.30±0.29
NA-26		3.50±0.31	4.45±0.26
NA-27		3.45±0.28	4.30±0.14
NA-28		4.60±0.34	5.40±0.27
NA-29		3.45±0.32	4.55±0.22
NA-30		2.60±0.17	3.50±0.15
NA-31		2.58±0.12	3.65±0.13
NA-32		3.85±0.21	4.95±0.17
NA-33		**4.70±0.26**	**5.80±0.26**
NA-34		4.55±0.22	5.50±0.13
NA-35		3.45±0.15	4.40±0.23
NA-36		3.75±0.13	4.80±0.28

Table 2. (Cont'd.).

Nitrous acid isolates	Concentration of NA (M)	Alginate g/l	Dry cell mass (DCM) g/l
UV-66 strain		**4.40±0.03**	**5.50±0.05**
NA-37	0.3	3.45±0.14	4.55±0.23
NA-38		3.77±0.21	3.75±0.14
NA-39		3.95±0.25	4.80±0.29
NA-40		3.48±0.25	4.50±0.37
NA-41		3.08±0.31	4.15±0.23
NA-42		3.12±0.25	4.10±0.27
NA-43		3.57±0.29	4.80±0.25
NA-44		3.75±0.37	4.80±0.17
NA-45		2.15±0.09	3.25±0.06
NA-46		2.45±0.25	3.55±0.25
NA-47	0.4	2.54±0.27	3.65±0.15
NA-48		1.70±0.33	2.85±0.25
NA-49		2.25±0.12	3.10±0.17
NA-50		1.90±0.26	3.00±0.09
NA-51		2.50±0.16	3.35±0.32
NA-52		2.30±0.19	3.40±0.28

Incubation time 120 h, Temperature 37°C, pH 7.0

Each value is an average of three parallel replicates. ± indicate standard deviation

Physical Parameters

Incubation period and temperature: The flasks were incubated at different temperatures (25-45°C) for different time intervals (10-120 h). The optimal alginate production (5.63 g/l with 6.80 g/l of DCM) was obtained after 110 h at 30°C (Figs. 2 & 3). This might be due to the fact that bacterial metabolic activities are very sensitive to the temperature and metabolism might be disturbed at high temperature. The bacteria produce some alginate in the last tropo phase and most of the amount is produced in the idio phase. The bacteria produce the cyst under unfavorable conditions and the alginate is the major component of the cyst (Nunez *et al*., 1999; Page & Sadoff, 1975).

pH: pH of the growth medium is an important parameter for bacterial growth and alginate production. The effect of different pH values of growth media was also investigated and it was noted that at pH 7.0 the maximum alginate (5.71 g/l with 6.83 g/l of DCM) was produced (Fig. 4). Increase or decrease in the medium pH resulted in the decreased alginate amount. This might be due to the fact that bacterial growth is very sensitive to the pH and so the alginate production (Rehm & Valla, 1997. Lange *et al*., 2002).

Agitation speed: A proper agitation speed is important for appropriate air supply and proper mixing of medium components. The degree of agitation depends on the organism and the composition of the fermentation medium. In the present study, the utmost amount of alginate (5.78 g/l with 6.82 g/l of DCM) was obtained when agitation intensity was kept at 200 rpm (Fig. 5). Further increase in the agitation intensity decreased the alginate production and this might be due to the breakage of the bacterial cell at high agitation speed. At low agitation the lesser amount of alginate is obtained and it might be due to improper mixing of the medium (Jimenez *et al*., 2005, Pena *et al*., 2008).

Table 3. Screening of ethyl methane sulphonate (EMS) treated isolates of *A. vinelandii* NA-33 for the production of alginate in shake flasks.

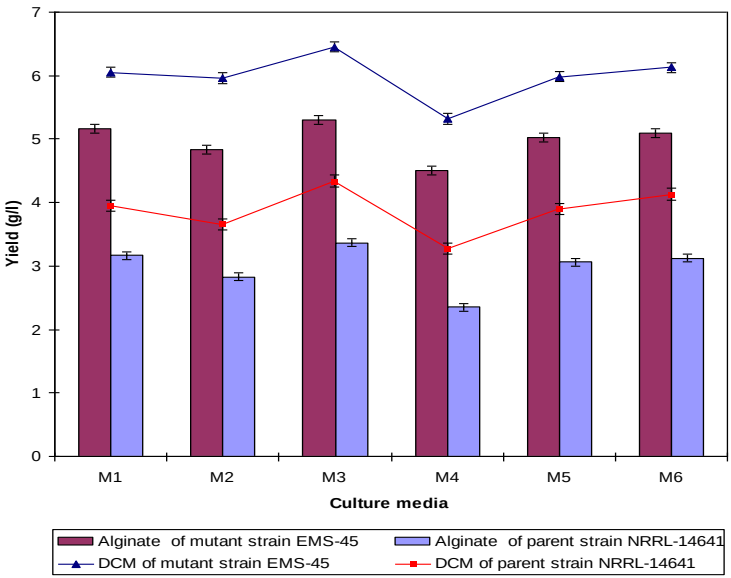
EMS isolates	Concentration of EMS (µl)	Alginate g/l	Dry cell mass (DCM) g/l
NA-33 strain		**4.70±0.26**	**5.80±0.26**
EMS-1	25	3.50±0.19	4.10±0.28
EMS-2		2.45±0.34	3.55±0.16
EMS-3		4.00±0.21	5.50±0.38
EMS-4		2.10±0.28	3.20±0.26
EMS-5		3.68±0.36	4.70±0.29
EMS-6		3.90±0.25	4.80±0.28
EMS-7		4.35±0.36	5.50±0.35
EMS-8		4.40±0.26	5.50±0.37
EMS-9		3.37±0.18	4.50±0.23
EMS-10		2.00±0.27	3.10±0.23
EMS-11		3.75±0.19	4.86±0.28
EMS-12		3.59±0.18	4.50±0.26
EMS-13		2.70±0.22	3.60±0.16
EMS-14		3.60±0.14	4.50±0.28
EMS-15		4.60±0.25	5.80±0.35
EMS-16		3.50±0.16	4.60±0.33
EMS-17		2.98±0.12	3.50±0.24
EMS-18		3.68±0.15	4.00±0.35
EMS-19		3.75±0.17	4.50±0.25
EMS-20		3.90±0.22	4.20±0.18
EMS-21		4.20±0.16	5.50±0.38
EMS-22		3.28±0.32	4.30±0.29
EMS-23		4.05±0.36	5.20±0.35
EMS-24		4.70±0.35	5.85±0.36
EMS-25		4.09±0.25	5.10±0.35
EMS-26		4.48±0.21	5.40±0.26
EMS-27		2.05±0.18	3.30±0.15
EMS-28		4.65±0.24	5.60±0.29
EMS-29		3.80±0.17	4.70±0.28
EMS-30		3.60±0.15	4.50±0.25
EMS-31		1.58±0.08	2.40±0.13

Table 3. (Cont'd.).

EMS isolates	Concentration of EMS (µl)	Alginate g/l	Dry cell mass (DCM) g/l
NA-33 strain		**4.70±0.26**	**5.80±0.26**
EMS-32	50	2.95±0.13	3.50±0.17
EMS-33		3.80±0.18	5.30±0.26
EMS-34		3.75±0.22	4.50±0.13
EMS-35		3.65±0.15	5.00±0.23
EMS-36		4.95±0.23	5.90±0.28
EMS-37		3.90±0.14	5.05±0.23
EMS-38		2.77±0.15	3.60±0.14
EMS-39		3.90±0.21	5.10±0.19
EMS-40		4.08±0.25	5.10±0.31
EMS-41		5.08±0.36	5.90±0.23
EMS-42		4.20±0.25	5.50±0.37
EMS-43		4.60±0.29	5.80±0.25
EMS-44		3.90±0.27	4.80±0.27
EMS-45		**5.20±0.09**	**6.25±0.16**
EMS-46		3.80±0.25	4.80±0.25
EMS-47		3.78±0.21	4.75±0.21
EMS-48		4.70±0.33	5.65±0.23
EMS-49		5.04±0.12	6.10±0.17
EMS-50		5.06±0.36	6.10±0.09
EMS-51		3.65±0.16	4.75±0.22
EMS-52		2.80±0.11	3.90±0.18
EMS-53	75	2.90±0.28	4.05±0.35
EMS-54		3.60±0.15	4.70±0.26
EMS-55		3.19±0.27	4.25±0.29
EMS-56		3.60±0.16	4.50±0.36
EMS-57		2.50±0.15	3.60±0.29
EMS-58		1.50±0.08	2.60±0.30
EMS-59		3.80±0.17	4.90±0.25
EMS-60		3.80±0.15	4.75±0.38
EMS-61		2.90±0.16	3.80±0.23
EMS-62		3.50±0.17	4.60±0.29
EMS-63		5.00±0.28	6.10±0.35
EMS-64		3.90±0.23	5.10±0.35
EMS-65		2.75±0.19	3.85±0.23
EMS-66		3.33±0.17	4.50±0.23
EMS-67	100	3.69±0.26	4.70±0.29
EMS-68		3.72±0.28	4.70±0.37
EMS-69		3.45±0.27	4.50±0.27
EMS-70		2.87±0.18	3.90±0.29
EMS-71		3.66±0.26	4.59±0.33
EMS-72		3.50±0.37	4.60±0.37
EMS-73		3.60±0.18	4.50±0.33
EMS-74		3.85±0.17	4.60±0.28
EMS-75		3.50±0.15	4.60±0.22

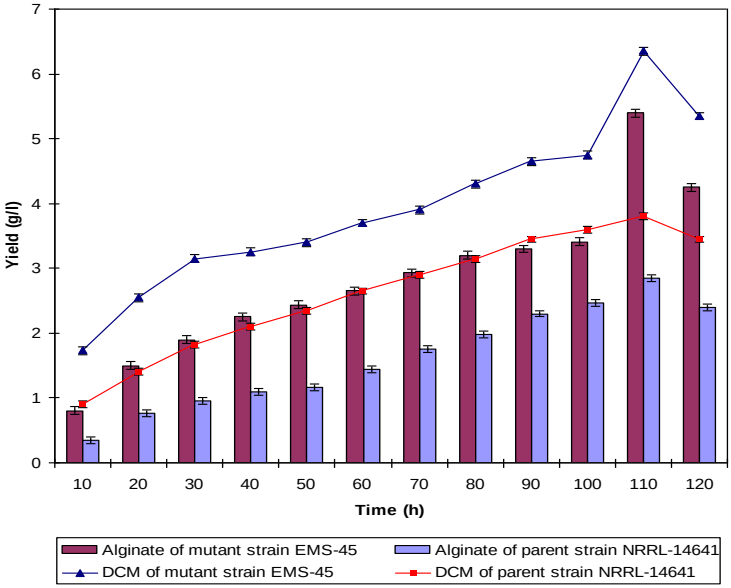
Incubation 120 h, Temperature 37°C, pH 7.0

Each value is an average of three parallel replicates. ± indicate standard deviation.



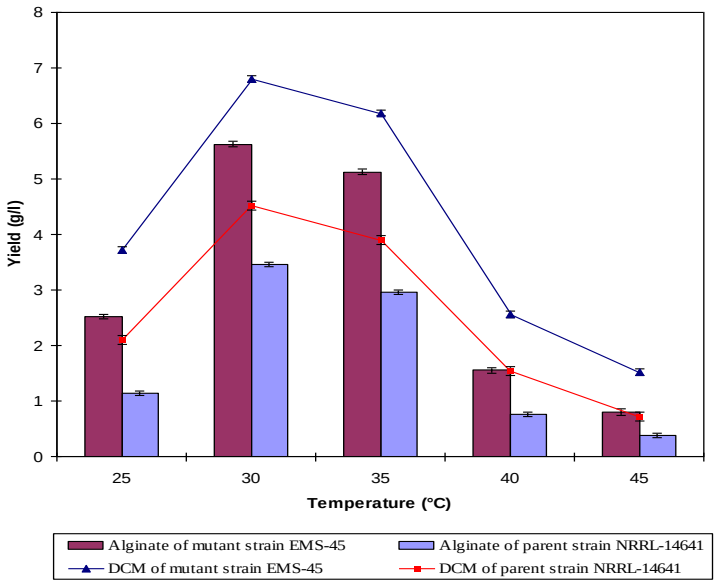
- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 120 h, pH 7.0, agitation rate 200 rpm

Fig. 1. Screening of culture media for the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.



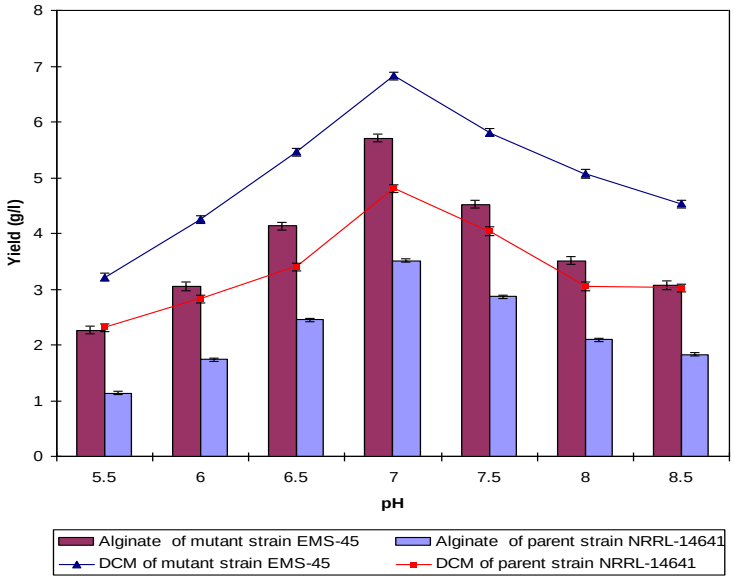
- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 120 h, pH 7.0, agitation rate 200 rpm

Fig. 2. Rate of fermentation for the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.



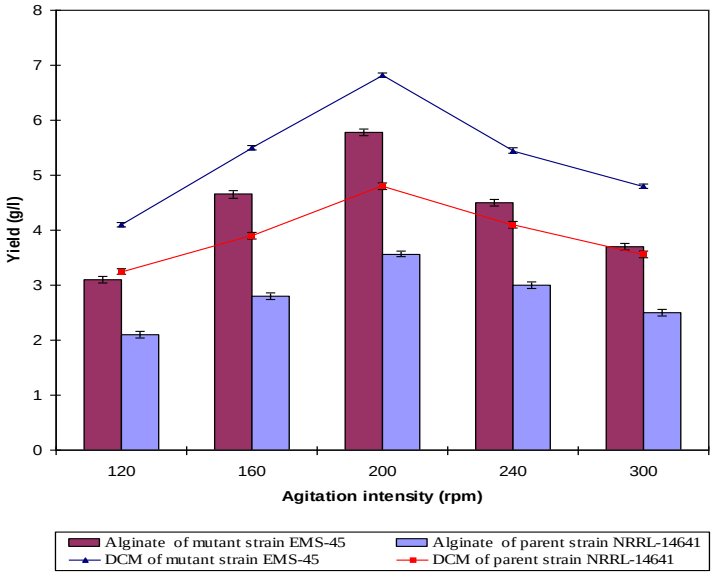
- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 110 h, pH 7.0, agitation rate 200 rpm

Fig. 3. Effect of incubation temperature on the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.



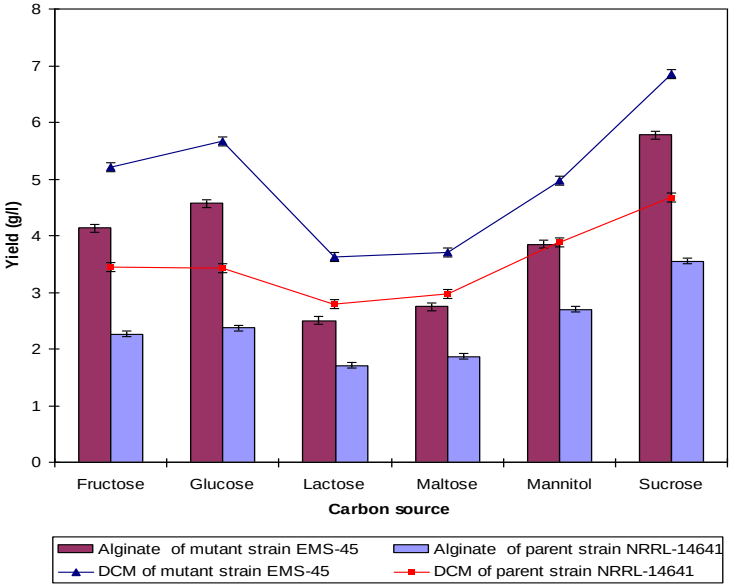
- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 110 h, temp 30 °C, agitation rate 200 rpm

Fig. 4. Effect of initial pH of medium on the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.



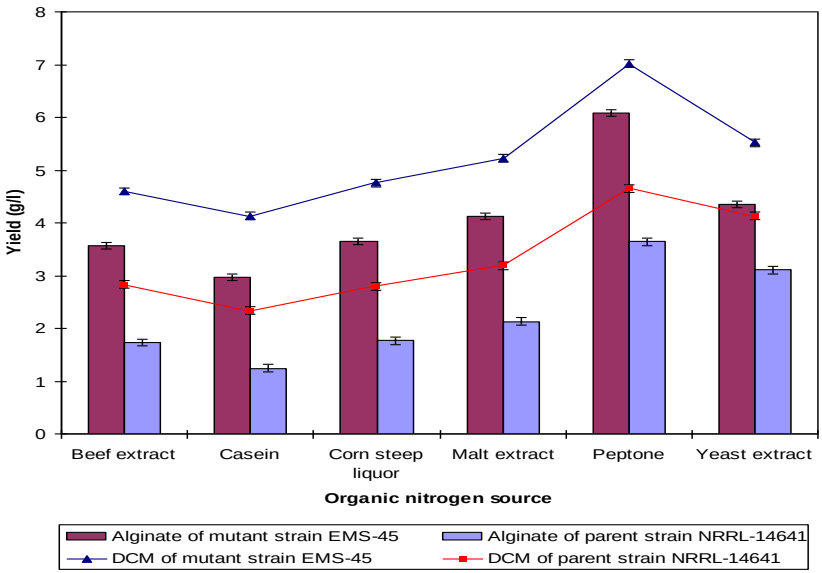
- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 110 h, temp 30°C

Fig. 5. Effect of agitation intensity on the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.



- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 110 h, pH 7.0, temp 30°C, agitation rate 200 rpm

Fig. 6. Effect of carbon source on the production of alginate by *A. vinelandii* NRRL 14641 and its isolate *A. vinelandii* EMS-45.



- Each bar represent mean value based on three replicates and Y error bars denotes standard error of mean.
- Incubation time 110 h, pH 7.0, temp 30°C, agitation rate 200 rpm

Fig. 7. Effect of organic nitrogen source on the production of alginate by *A. vinelandii* NRRL-14641 and its isolate *A. vinelandii* EMS-45.

Carbon source: Carbon source had a dual effect as the building block and the energy requirement for the organism. Suitable and readily absorbable sugars can be the best choice for bacterial culture to gain good production (Okabe *et al*., 1981., Moreno *et al*., 1999., Clementi *et al*., 1999). In the present study, different carbon sources (fructose, glucose, lactose, maltose, mannitol or sucrose) were examined for alginate production (Fig. 6). Sucrose proved to be the best carbon source and maximum alginate (5.78 g/l with 6.85 g/l of DCM) was obtained when sucrose was used as carbon source. It might be due to the reason that sucrose is readily metabolized and easily employed by the bacteria (Asami *et al*., 2004). Other carbon sources might be slowly metabolized by the bacteria.

Organic nitrogen source: Different organic nitrogen sources (Beef extract, Casein, Corn steep liquor, Malt extract, Yeast extract or Peptone) were also evaluated for alginate production (Fig. 7). Of all the nitrogen sources tested peptone gave the better yield of alginate (6.08 g/l with 6.92g/l of DCM). It might be due to the fact that peptone provides better available nitrogen for the bacterial growth and alginate production (Savalgi & Savalgi, 1992; Garcia *et al*., 2001., Khanafari & Sepahei, 2007).

References

Asami, K., T. Aritomi, Y.S. Tan and K. Ohtaguchi. 2004. Biosynthesis of polysaccharide alginate by *Azotobacter vinelandii* in a bubble column. *J. Chemical Eng. Jpn*., 37(8): 1050-1055.

Barrera, A.D., P. Silva, R. Avalos and F. Acevedo. 2009. Alginate molecular mass produced by *Azotobacter vinelandii* in response to changes of the O_2 transfer rate in chemostat cultures. *Biotechnol. Lett*., 31: 825-829.

Brivonese, A.C. and I.W. Sutherland. 1989. Polymer production by a mucoid stain of *Azotobacter vinelandii* in batch culture. *Appl. Microbiol. Biotechnol*., 30: 97-102.

Chen, W.P., J.Y. Chen and C.L. Su. 1983. Production of bacterial alginate by a mutant of *Azotobacter vinelandii*. *Natl. Sci. Counc. Mon*., 11: 1197-1207.

Chen, W.P., J.Y. Chen, S.C. Chang and C.L. Su. 1985. Bacterial Alginate Produced by a Mutant of *Azotobacter vinelandii. Appl. Env. Microbiol*., 543-546.

Clementi, F., M.A. Crudele, E. Parente, M. Mancini and M. Moresi. 1999. Production and characterization of alginate from *Azotobacter vinelandii*. *J. Sci. Food Agric*., 79(4): 602-610.

Clementi, F., P. Fantozzi, F. Mancini and M. Moresi. 1995. Optimal conditions for alginate production by *Azotobacter vinelandii. Enz. Microbial Technol*., 17: 983-988.

Ertesvag, H., F. Erlien, G.S. Braek, B.H.A. Rehm and S. Valla. 1998. Biochemical properties and substrate specificities of a recombinantly produced *Azotobacter vinelandii* alginate lyase. *J. Bacteriol*., 180: 3779-3784.

Funami, T., Y. Fang, S. Noda, S. Ishihara, M. Nakauma, K. I. Draget, K. Nishinari, G. O. Phillips. 2009. Rheological properties of sodium alginate in an aqueous system during gelation in relation to supermolecular structures and Ca^{2+} binding. *Food Hydrocolloids*, 23: 1746-1755.

Garcia, M.C.V., M.J. Lopez, M.A. Elorrieta, F. Suarez and J. Moreno. 2001. Influence of nutritional and environmental factors on polysaccharide production by *Azotobacter vinelandii* cultured on 4-hydroxybenzoic acid. *J. Ind. Microbiol. Biotechnol*., 27: 5-10.

Gorin, P.A.J. and J.F.T. Spencer. 1966. Exocellular alginic acid from *Azotobacter vinelandii*. *Can. J. Chem*., 44: 993-998.

Jarman, T.R., L. Deavin, S. Slocombe and R.C. Righelato. 1978. Investigation of the effect of environmental conditions on the rate of exopolysaccharides synthesis in *Azotobacter vinelandii. J. Gen. Microbiol*., 107: 59-64.

Jimenez, R.P., C. Pena, O.T. Ramirez and E. Galindo. 2005. Specific growth rate determines the molecular mass of the alginate produced by *Azotobacter vinelandii. Biochem. Eng. J*., 25: 187-193.

Khanafari, A. and A.A. Sepahei. 2007. Alginate biopolymer production by *Azotobacter chroococcum* from whey degradation. *Int. J. Env. Sci. Technol*., 4(4): 427-432.

Knutson, C.A. and A. Jeanes. 1968. A new modification of the carbazole analysis: tion to heteropolysaccharides. *Anal.Biochem*., 24: 470-481.

Lange, H.C., D. Beunard, P. Dhulster, D. Guillochon, A.M. Caze, M. Morcellet, N. Saude and G.A. Junter. 2002. Production of microbial alginate in a membrane bioreactor. *Enz. Microbial Technol*., 30: 656-661.

Moreno, J., C.V. Garcia, M.J. Lopez and G.S. Serrano. 1999. Growth and exopolysaccharide production by *Azotobacter vinelandii* in media containing phenolic acids. *J. Appl. Microbiol*., 86: 439-445.

Moresi, M., I. Sebastiani and D.E. Wiley. 2009. Experimental strategy to assess the main engineering parameters characterizing sodium alginate recovery from model solutions by ceramic tubular ultrafiltration membrane modules. *J. Membrane Sci*., 26: 441-452.

Nunez, C., S. Moreno, G.S. Chavez and G. Espin. 1999. The *Azotobacter vinelandii* response regulator AlgR is essential for cyst formation. *J. Bacteriol*., 181(1): 141-148.

Okabe, E., M. Nokajima, H. Marooka and K. Nisizawa. 1981. Investigation of carbon and phosphorus sources in cultural media of a selected strain of alginate –producing *Azotobacter vinelandii*. *J. Ferment. Technol*., 59: 1-7.

Page, W.J. and H. L. Sadoff. 1975. Relationship between calcium and uronic acids in the encystment of *Azotobacter vinelandii. J. Bactriol*., 145-151.

Pena, C., M. Millan and E. Galindo. 2008. Production of alginate by *Azotobacter vinelandii* in a stirred fermentor simulating the evolution of power input observed in shake flasks. *Process Biochem*., 43: 775-778.

Rehm, B. H. A. and S. Valla. 1997. Bacterial alginates: biosynthesis and cations. *Applied Microbiology and Biotechnology*, 48: 281-288.

Remminghorst, U. and B.H.A. Rehm. 2006. Bacterial alginates: from biosynthesis to applications. *Biotechnol. Lett*., 28: 1701-1712.

Roldan, M.A.T., S. Moreno, D. Segura, E. Galindo and G. Espin. 2003b. Alginate production by an *Azotobacter vinelandii* mutant unable to produce alginate lyase. *Appl. Microbiol. Biotechnol*., 60: 733-737.

Saude, N., H.C. Lange, D. Beunard, P. Dhulster, D. Guillochon, A.M. Caze, M. Morcellet and G.A. Junter. 2002. Alginate production by *Azotobacter vinelandii* in a membrane bioreactor. *Process Biochem*., 38: 273-278.

Savalgi, V. and V. Savalgi. 1992. Alginate production by *Azotobacter vinelandii* in batch culture. *J. Gen. Appl. Microbiol*., 38: 641-645.

Sharma, P.D. 1989. Methods in Microbiology, Microbiology and Plant Pathology, Ed. 1st Rastogi and Company Meerut, India. pp. 33-35.

Vazquez, A.P., S. Moreno, J. Guzman, A. Alvarado and G. Espin. 1999. Transcriptional organization of the *Azotobacter vinelandii algGXLVIFA* genes: characterization of *algF* mutants. *Gene*, 232: 217-222.

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