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Short Communication

Hyperproduction of Alginate by Mutated Strain of *Azotobacter vinelandii* Through Submerged Fermentation

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Authors' Contribution

SS, ASH and IUH conceived and designed the study. SS, MT and AAA carried out the experimental work. ARA and SS analyzed the data. SS and SF wrote the article.

Key words

***Azotobacter vinelandii*, mutated strain, hyper-production of alginate.**

ABSTRACT

The present study was designed to improve the parent strain of *Azotobacter vinelandii* NRRL-14641 for hyper-production of alginate by random mutagenesis. The mutant (EtBr-02) obtained after ethidium bromide treatment gave 2.11 fold higher alginate production than the parent strain. The maximum concentration of alginate (15.61 g/L) was obtained by utilizing 10% (w/v) wheat bran, 8% (v/v) inoculum size at 48 h of incubation period, pH 7.0, 30°C and agitation speed of 200 rpm. Inclusion of 2.5% corn steep liquor raised the alginate concentration to 15.8 g/L. These optimized conditions can be executed for the mass production of alginic acid on pilot scale.

Alginate, the biopolymer of β-D- mannuronic acid its C-5 epimer α-L-guluronic acid, has wide range of industrial and pharmaceutical applications (Butt *et al*., 2011). The annual consumption of alginate by different industrial sectors is 30,000 metric tons, particularly in food sector as a viscosifier, stabilizer, thickener, emulsifier, gelling and water binding agent (Lakshmipriyad *et al*., 2013). In pharmaceutical industry, it is utilized in traditional wound dressing, in some formulations for preventing gastric reflux (Gaviscon, Bisodol, and Asilone) and also serve as the main component in dental impression material (Awad and Aboul- Enein, 2013). Alginate immobilized cells have been used in cell transplantation where it acts as the barrier between transplant (immobilized cell) and host immune system. Different cells are immobilized to fulfill the requirement like adrenal chromaffin cells, parathyroid cells and insulin-producing cells (Langerhans islets) for the treatment of Type I diabetes (Then *et al*., 2012). Alginic acid is also used in paper industries for surface sizing, coating material for welding rods, manufacturing of ceramics and water-treatment (Steinbuchel *et al*., 2001).

The alginate is commercially obtained from farmed brown seaweeds such as *Laminaria hyperborea*, *Laminaria digitata*, *Macrocystis pyrifera* and *Ascophyllum nodosum* (Auhim and Hassan, 2013). Due to difference in chemical composition and product quality, only few species of seaweed are considered appropriate for extraction (Moresi *et al*., 2009). Therefore bacterial alginates, isolated from *Pseudomonas* and *Azotobacter*, serve as the alternative and promising tool to fulfill the requirements. Due to the potential hazards of pathogenicity and poor jellifying properties associated with *Pseudomonas* alginate, makes *A. vinelandii* the best microorganism for biopolymer production (Hay *et al*., 2013). The alginate production by microbial fermentation is considered to be better strategy to obtain the product of known composition, unaffected by tides and the place of production can be set anywhere to utilize cheap substrate.

The cost of the substrate plays a significant role in total process costs of fermentation process (Nadeem *et al*., 2014). The media commonly used for alginate production contains glucose and sucrose as the substrate (Belder, 1993). It is desirable to use cheap and economic substrate like agricultural waste to meet the requirements (Amin *et al*., 2014). Due to the growing demand of alginate, the present study was designed to obtain the stable mutant and optimize different cultural conditions for hyper-production of the exopolysaccharide.

Materials and methods

The parent strain of the *A. vinelandii* NRRL-14641 was supplied by the Agricultural Research Service (A.R.S.) of United States Department of Agriculture

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(U.S.D.A) in lyophilized form. The organism was revived and maintained on Burk's Nitrogen free agar medium slants (Butt *et al.,* 2011).

The above strain was subjected to various physical and chemical mutagens to improve the yield of alginate. The details of the treatments applied are as under:

(i) UV irradiation: For strain improvement, freshly prepared inoculum (1 mL) of *A. vinelandii* was transferred to agar plates and subjected to UV irradiation (Mineral Light UVS.12, California, USA, λ =253 nm at cycles/S, 220 V) for various time intervals (05-60 min). The distance between the lamp screen and culture was adjusted at 8.0 cm to get more than 90% kill rate. UV exposed cells were kept in dark to prevent photoactivation and then further subjected to mutant selection media. Auxotrophs were then screened for their ability to produce alginate (Butt *et al.,* 2011).

(ii) Nitrous acid treatment: The inoculum of parent strain with the final density of $3x\ 10^8$ cells per mL was centrifuged and cell pellet thus obtained was washed twice with phosphate buffer (0.2 M of pH 7.0). Finally 0.9 mL of acetate buffer (0.1 M, pH 6.8) and 0.1 mL sodium nitrite of different concentrations (1, 2, 3, 4, 5, 6, 7 and 8 M) was added to generate the mutagenic agent called nitrous acid. The cells were incubated in the mixture at 37°C for 30 min. Then the cells were centrifuged and the cell pellet was washed with phosphate buffer to remove the traces of mutagen. The cells were resuspended in 1mL phosphate buffer and after serial dilution spread on the nutrient agar plates. The plates were incubated for 48 h and the survival curve generated for the applied treatment (Mishra *et al.,* 2014).

(iii) Ethidium bromide treatment: The parent strain of *A. vinelandii* was grown to the late logarithmic phase of growth in Burk's nitrogen free medium. The cells were harvested by centrifugation at 10,000 rpm for five minutes and resuspended in 2 mL of normal saline solution. The cell suspension was then subjected to mutagenesis by adding 1 mL of varying concentrations of ethidium bromide (2.5, 5, 7.5, 10, 12.5 and 15 mg/mL). Then the mixture was placed in shaking incubator at 200 rpm, 30ºC for 1 h. Thereafter the cells were separated by centrifugation at 10,000 rpm for 5 min, resuspended in 0.2 M phosphate buffer of pH 7.0, serially diluted and spread on the nutrient agar plates. The plates were then incubated at 30ºC for 48 h. Thereafter that plate was selected for alginate production which showed more than 90% kill rate (Sudi *et al.,* 2008).

After the applied treatments, the colonies were transferred to minimal medium (Burk's Nitrogen Free agar medium) and complete medium *i.e.* Nutrient agar through replica plating method (Saued *et al.,* 2013). The colonies which showed growth on complete medium (containing all the essential amino acids) but not on minimal medium were considered as auxotrophic (mutants).The mutants were then screened for hyper-production of alginate against the parent strain by using the pre-optimized conditions for wild culture.

The loop full of refreshed culture of *A. vinelandii* from nutrient agar plate was inoculated in Erlenmeyer flask (250 mL) containing 25 mL of autoclaved nutrient broth. The flask was kept in orbital shaker at 30°C and 200 rpm for 24 h. The culture having the optical density of 0.6 at 600 nm was used as an inoculum for further study (Butt *et al.,* 2011).

In order to obtain higher yield of alginate, the mutants were grown under the pre-optimized conditions of wild *A. vinelandii* for a period of 48 h to select the best mutant. The fermentation medium was prepared by using the pre-optimized concentrations such as wheat bran (7.5%), $MgSO_4.7H_2O$ (2%), $CaCl_2$ (1.5%), and Corn steep liquor (2%). The pH was adjusted at 7 after sterilization, freshly prepared inoculum was added. The shake flask studies were carried out on orbital shaker at 30ºC, 200 rpm for 48 h. Then the hyper-producer strain was further used to optimize all the physic-chemical conditions to increase alginate biosynthesis.

Different concentrations of wheat bran (2.5, 5, 7.5, 10 and 12.5%) were used to optimize the alginate production by mutant *A. vinelandii*. Various time intervals (12, 24, 36, 48, 60 and 72 h) and concentrations of inocula (2, 4, 6, 8 and 10%) were also optimized (Vermani *et al*., 1997). The effect of different pH values (5, 6, 7, 8, 9 and 10) was tested to find the optimum level of pH at which maximum biopolymer was synthesized. Various degrees of temperature (25, 30, 35, 40, 45 and 50ºC) and agitation intensities (120, 160, 200, 240 and 280 rpm) were optimized to achieve high production of exopolysaccharide (Butt *et al*., 2011). Several percentages of corn steep liquor (1, 1.5, 2, 2.5 and 3%) were tested to select optimum nitrogen concentration for increased alginate synthesis by the mutant strain (Galal and Ouda, 2014).

All the parameters were optimized in shake flask of 250 mL (Erlenmeyer flask) having 25 mL total fermentation medium.

The fermented broth, after addition of 1 mL of (0.5M) EDTA sodium salt solution and 0.5 mL of (5.0M) NaCl solution, was centrifuged at 18000 rpm at 20°C for 30 min to separate the biomass and substrate residue. The supernatant was then cooled in an ice bath and three volumes of ice cold isopropanol added. The mixture was left at 4°C for overnight. Then it was further subjected to centrifugation at 18000 rpm, 4°C for half an hour to precipitate alginate. The residue was then suspended in water, centrifuged then finally the precipitates were dried

at 80ºC for 24 h. The alginate was gravimetrically estimated by weighing the dried precipitates (Knutson and Jeanes, 1968).

All the experiments were performed in triplicates. The data was analyzed on SPSS 13.0 software, by comparing mean through One-Way ANOVA and multiple comparison was made through LSD and Descriptive analysis (Irshad *et al*., 2015).

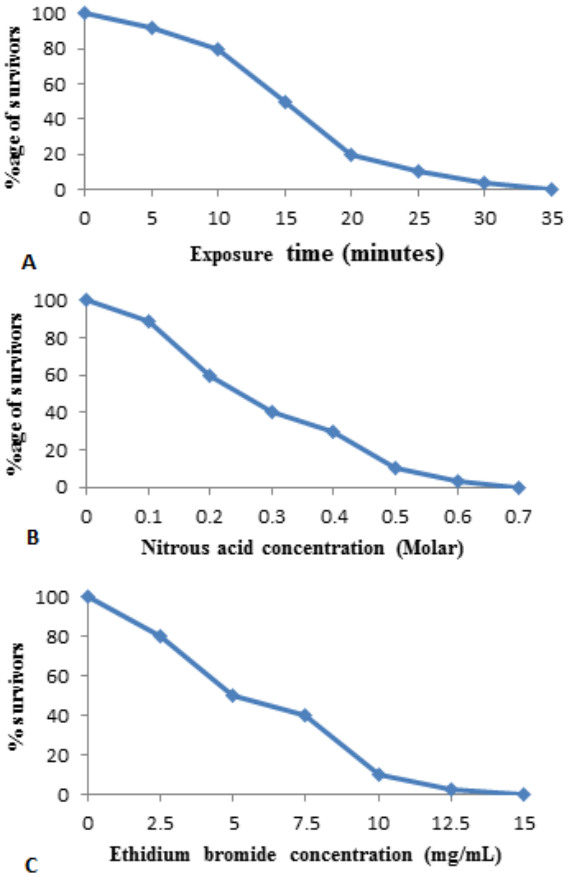

Fig. 1. Survival/ kill curve of *A. vinelandii* after treatment with physical and chemical mutagens. A, UV irradiation; B, nitrous acid; C, Ethidium bromide.

Results and discussion

A. vinelandii (wild) was exposed to physical mutagen *i.e.* UV rays for different time intervals (Fig. 1A) and to chemical mutagens (Nitrous acid and Ethidium bromide) at different concentrations (Figs. 1B, 1C) to obtain more than 90% kill. The survivors were tested for mutation on Minimal Medium (Burks's Nitrogen free medium) and Complete medium by replica plating method (Saued *et al.*, 2013). Ten auxotrophs obtained were screened for alginate production in comparison to wild *A. vinelandii* under pre-optimized conditions in order to select the hyper-producer strain. EtBr-02 gave the highest production of alginate (13.8 g/L) in comparison to wild and the other mutants of *A. vinelandii* (Fig. 2).

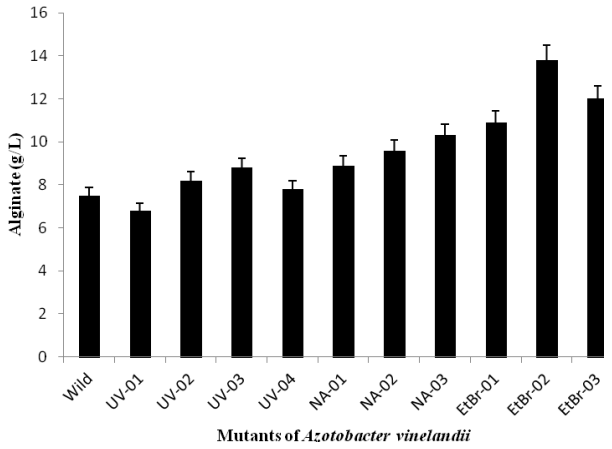

Fig. 2. Screening of mutants for hyper production of alginate.

The physico-chemical parameters were optimized for hyper-producer mutant strain (EtBr-02) to increase the yield of alginate. The maximum significant ($P<0.05$) alginate concentration by *A. vinelandii*, EtBr-02 was observed at 10% wheat bran and incubation period of 48 hours (15.17g/L) as shown in Figure 3A and 4A, whereas the wild strain gave the highest production (7.48 g/L) at 7.5% wheat bran concentration at 48 hours of incubation.. The results presented are in agreement with Butt *et al.* (2011) as they reported the same optimum incubation period for both wild and mutant strain of *A. vinelandii i.e.* 110 h for maximum product formation. In contrast, Khanafari and Sepahei (2007) reported the alginate yield of greater than 5 mg/mL by *Azotobacter chroococum* 1723 when lactose (whey) was used as carbon source at 24 h of incubation time whereas the highest concentration of alginate (7.5 mg/mL) was obtained by Emtiazi *et al.* (2004) in fermentation medium containing sucrose (1%) and beet molasses (2%) as the carbon source by *Azotobacter* AC2 after incubation period of four days. Ali *et al*. (2005) used wheat bran as the supporting medium for the production of alginic acid in solid state fermentation. The maximum alginate (8.8 g/L) was observed with the addition of 4% Zahdi date extract and 0.75% baker yeast as the nitrogen source in the ratio 5:1 (mL:g wheat bran) at six days of incubation period. In a similar study Chen *et al.* (1985) used 2% sucrose and

observed the maximum concentration at 110 h of incubation period by mutant strain of *A. vinelandii*, C-14. Thus it is concluded that *A. vinelandii*, EtBr-02 has greater production efficiency in less time while using wheat bran as the substrate.

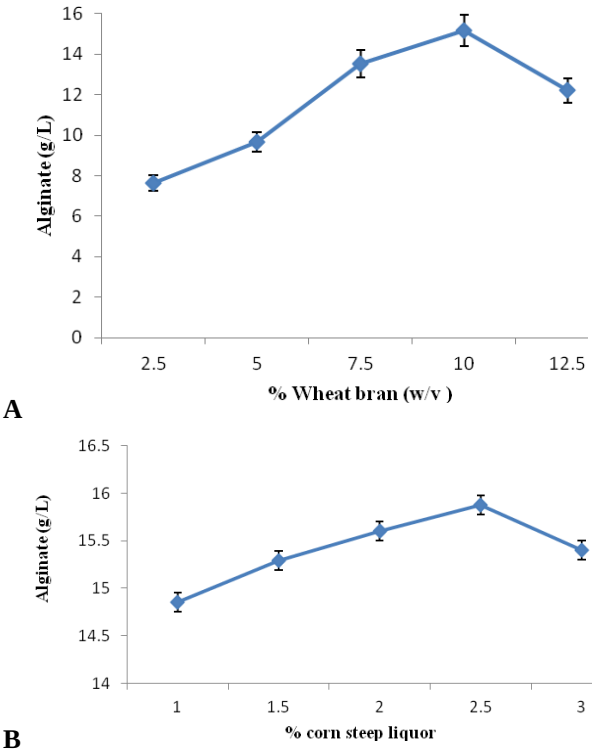

Fig. 3. Optimization of % wheat bran and corn steep liquor for maximum alginate production by mutant *A. vinelandii*, EtBr-02 at 48 h of incubation period.

The maximum significant (P< 0.05) yield was observed at 8% inoculum size (15.61 g/L) as shown in Fig. 4B, while for wild strain the maximum yield was indicated at 6%. Vermani *et al.*, (1997) suggested that 2% inoculum size gave the best concentration (8.25g/L) using *A.vinelandii* MTCC 2460.

A significantly higher production of alginate (P<0.05) was observed at pH 7 and 30^{0}C (15.61 g/L) as shown in Figure 4C.The same conditions were also found to be optimum for alginate production by wild strain of *A.vinelandii*. Butt *et al.* (2011) also reported the similar conditions to be optimum for maximum alginate production by both the parent as well as mutant strain (EMS-45) of *A.vinelandii*. Pandurangan *et al.* (2012) reported pH 8.0, 34°C to be optimum pH and temperature for maximum alginate yield percentage using *Azotobacter chroococcum* (yield %= 46.64). Chen *et al.* (1983) also differed from the present results and they observed the highest yield of alginate at pH 6.5 and 28°C using mutant strain, C-14 of *A. vinelandii*.

The higher amount of alginate was observed at 200 rpm (15.61g/L) and then it decreased thereafter (Fig. 4E). This might be due to breakage of bacterial cells at high speed. The same agitation intensity was found to be optimum for the wild strain. These findings are in agreement with Butt *et al.* (2014) as they observed the intensity of 200rpm to be optimum for enhanced production using both the wild and mutant strains of *A.vinelandii*. Emtiazi *et al.* (2004) also reported 200 rpm to be the optimized speed for alginate production (7.5g /L).

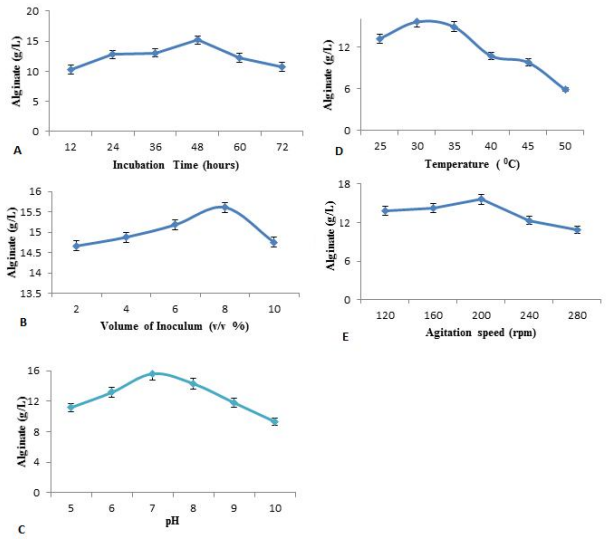

Fig. 4. Effect of physical parameters on alginate synthesis by mutant *A. vinelandii*, EtBr-02. A, incubation time; B, inoculum size; C, pH; D, temperature; E, agitation speed.

A significant (P<0.05) increase in concentration of alginate *i.e.*, 15.88 g/L was observed at 2.5% corn steep liquor (Fig. 3) as against 7.46 g/L at 2% corn steep liquor concentration for the wild strain. Similar results were obtained by Butt *et al.* (2011) who investigated the effect of various organic nitrogen sources (beef extract, casein, corn steep liquor, malt extract, yeast extract and Peptone) for alginate production. Of all the nitrogen sources tested, peptone gave the best yield of alginate by both wild and mutant strain (6.08 g/L). The present data is also supported by Pandurangan *et al.* (2012), as they noticed that increasing yeast extract and ammonium nitrate as organic and inorganic nitrogen sources showed production as high and lower respectively. The results obtained in the present study are also in agreement with

Galal and Ouda (2014), as they investigated the effect of variable organic nitrogen sources (Yeast extract and Corn steep liquor at concentrations of (0.2, 0.4, 0.6, 0.8 and 1.0%) on alginate production. The maximum alginate concentration was obtained by using yeast extract at 0.6% and corn steep liquor at 0.8% concentration by *A. chroococum* isolates n.1 and n.8, respectively.

Conclusion

The higher concentration of alginate was produced in shake flask studies (15.88 g/L), after optimization of different physico-chemical parameters by mutated *A. vinelandii*, as compared to the parent strain. In this research, a simple and cheap method for alginate production was developed which can be further exploited on commercial scale.

Statement of conflict of interest

Authors have declared no conflict of interest.

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