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# BIOCONVERSION OF AGRICULTURAL BY-PRODUCTS TO ALGINATE BY *AZOTOBACTER VINELANDII* AND PHYSICO-CHEMICAL OPTIMIZATION FOR HYPER-PRODUCTION

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## ABSTRACT

Alginate, a biopolymer of industrial interest is commercially extracted from marine brown algae. Due to variation in composition from different species, there is rising interest in bacterial alginate. The present study was designed to assess the feasibility of using different agro-industrial wastes like wheat bran, rice polishing and cane molasses for alginate production by *Azotobacter vinelandii*. Optimization of basal media composition and various physical parameters was also done. On fermentation of 7.5% (w/v) wheat bran maximum alginate production was reported at 48 hours of incubation time with 6% (v/v) inoculum size at pH 7.0, $30^0$C and agitation speed of 200 rpm. Addition of different optimum levels of ionic salts i.e. 1.5% $CaCl_2$ and 2% $MgSO_4$. $7H_2O$ gave significantly higher quantity of alginate whereas $KH_2PO_4$ and NaCl reduced the yield of alginate. Among different nitrogen sources tested, 2% corn steep liquor showed significantly higher yield of alginate (7.46 g/L). Alginate produced was found to be 98% pure by HPLC method. The present exploration depicted that alginate can be efficiently produced utilizing cheap agricultural wastes to save the foreign exchange.

**Keywords:** Alginate, *Azotobacter vinelandii*, Agricultural by-products, fermentation.

## INTRODUCTION

In pharmaceutical industry, the alginate is utilized in traditional wound dressing, in some formulations for preventing gastric reflux and as main component in dental impression material. It is also used in paper industries for surface sizing, coating material for welding rods, manufacturing of ceramics and water-treatment (Prompaphagorn, 2008).

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 from *Pseudomonas* and *Azotobacter* serve as the alternative and promising tool to fulfill the industrial needs. Keeping in view the potential hazards of pathogenicity and poor jellifying properties (due to lack of poly-guluronate blocks) associated with *Pseudomonas* alginate, makes *Azotobacter* the best microorganism for biopolymer production (Hay *et al*. 2013).

The cost of the substrate plays a significant role in total process costs of fermentation process. The media commonly used for alginate production contains glucose and sucrose as the substrate (Prompaphagorn, 2008). Pakistan, as an agricultural country has an annual production of agricultural waste around 50-60 million tons. Hence, considering the nutritional importance of agricultural wastes, commercially competitive production of alginate can be achieved by using these by-products as feedstock for microbial fermentation. This strategy will solve the pollution problem that may arise due to disposal of these residues and also provide financial assistance for the overall cost of fermentation (Amin *et al.* 2014).

Due to the growing demand of alginate, the present study was designed to assess the potential of agriculture by-products e.g: wheat bran, rice polishing and molasses for alginate production by *Azotobacter vinelandii* and optimization of several operating variables (substrate water ratio, incubation time, volume of inoculum, pH, temperature, agitation speed, effect of ions and nitrogen source) for hyper-production.

## MATERIALS AND METHODS

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

**Physico-Chemical optimization for hyper-production of alginate:** For inoculum preparation, one loop full of bacterial culture was transferred to 25 mL of Jarman medium contained in 250 mL conical flask. Then it was incubated in a rotary shaker at $30^o$ C and agitation speed of 200 rpm .Twenty four hours old culture, having OD of 0.6, at 660nm was used as an inoculum (Butt *et al.* 2011).

Different agricultural by-products like cane molasses, wheat bran and rice polishing (2.5, 5, 7.5 and 10%) were used for optimization of best carbon source at incubation time of 48 hours. Various time intervals (12, 24, 36, 48, 60 and 72 hours) and percentages of inoculum (2, 4, 6, 8 and 10%) were also optimized (Vermani *et al*. 1997). Different physical parameters like pH values (5, 6, 7, 8, 9 and 10), degree of temperature (25, 30, 35, 40, 45 and $50^0$C) and agitation intensities (120, 160, 200, 240 and 280 rpm) were tested to achieve high yield of alginic acid (Butt *et al*. 2011). Various concentrations of different ionic salts (0.5, 1, 1.5, 2 and 2.5%) like $MgSO_4.7H_2O$, $CaCl_2$, NaCl and $KH_2PO_4$ were used for optimization (Garcia *et al.* 2001). Corn steep liquor and Yeast sludge with varying concentrations (1, 1.5, 2, 2.5 and 3%) were used to select the best organic nitrogen source to achieve higher amount of alginate (Galal and Ouda, 2014). All the parameters were optimized in shake flask of 250 mL (Erlenmeyer flask) having 25 mL of total fermentation medium in triplicates.

**Estimation of Alginate:** The fermented broth, after addition of 1 mL of EDTA sodium salt solution (0.5M) and 0.5 mL of NaCl solution (5.0M), was centrifuged at 18000 rpm at $20^0$C for 30 minutes 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 $4^0$C for overnight. Then it was further subjected to centrifugation at 18000 rpm, $4^0$C for half an hour to precipitate alginate. The residue was then dissolved in water, centrifuged the finally the precipitates were dried at 80ºC for 24 hours. The alginate was gravimetrically estimated by weighing the dried precipitates (Knutson and Jeanes, 1968).

The dried precipitates of alginic acid were moistened with water and sodium carbonate was added to dissolve the alginate completely. Finally, ethanol was added to coagulate sodium alginate. The coagulated material was then used as sample for quantitative estimation by HPLC method (Awad and Aboul- Enein, 2013).

**Statistical analysis:** All the experiments were performed in triplicates. The data were analyzed using SPSS 13.0 software through One-Way ANOVA technique and multiple comparisons were made through Least Significant Difference test (Steel *et al*., 1997).

## RESULTS AND DISCUSSION

As Pakistan is an agricultural land and hundred thousand tons of agricultural by-products are produced annually that could be utilized for biotechnological production of useful products like alginate. Therefore, different agricultural by-products like wheat bran, rice polishing and molasses were tested to serve as an inexpensive source of carbon for the production of alginate with respect to time. Wheat bran at 7.5% concentration produced the highest alginate (4.23 g/L) at 48 hours followed by rice polishing 7.5% (3.74 g/L) and molasses at 10% yielded the least (2.8 g/L) under the same set of conditions as shown in fig. 1. Thus, the wheat bran gave the highest amount of alginate. Probably it is rich source of starch and insoluble carbohydrates along with minerals and vitamins which has enhanced the production of alginic acid. The highest proportion of soluble sugars is present in the form of starch. *Azotobacter vinelandii* can produce amylases and thus can utilize starch to meet its energy requirements.

The optimization of incubation period for production of alginate was investigated using 7.5% wheat bran as the substrate. A significantly higher ($P \leq 0.05$) alginate production was observed at 48 hours (4.29 g/L) as shown in fig. 2a. As the incubation time was further increased, the decrease in product formation was observed due to exhaustion of nutrients and accumulation of waste products. 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 hours 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) obtained maximum alginate (8.8 g/L) after six days on solid state fermentation of wheat bran,4 % Zahdi date extract and 0.75% baker yeast. But in the present study, *A.vinelandii* can efficiently utilize wheat bran as the substrate with increased product formation in less time.

In the present study, inoculum size, 6% was found to be optimum and it gave significantly ($P \leq 0.05$) higher yield of alginate (4.6 g/L) as shown in fig. 2b. Vermani *et al*. (1997) found 2% inoculum size to be optimum for biopolymer production (8.25 g/L) under optimum cultural conditions.

In the present study, pH 7.0 was found as optimum and produced significantly higher ($P \leq 0.05$) alginate (4.6 g/L) as shown in fig. 2c. The pH 7.0 has been reported to be optimum for exopolysaccharide productionby *Azotobacter vinelandii* in various studies (Vermani *et al*. 1997; Butt *et al*. 2011). Pandurangan *et al*. (2012) differed slightly from the present study and indicated pH 8.0 to be optimum pH for maximum alginate using *Azotobacter chroococcum* (46.64%). As the pH is increased beyond the optimum level, it results in the reduction of the energy available for biopolymer production and hence the yield of alginate was reduced.

The fermentation was carried out at different incubation temperatures ($25^0$C- $50^0$C).The optimum temperature 30ºC was found to be significant ($P \leq 0.05$) for maximum alginate production (4.6 g/L) as shown in fig. 2d. These results are supported by Vermani *et al*.

(1997) and Butt *et al*. (2011), as they also indicated the incubation temperature of $30^0$C as optimum for maximum biopolymer production. They reported the final amount of precipitated alginate to be 7.8 and 5.8 g/L respectively. In contrast, Pandurangan *et al*. (2012) reported $34^0$C to be the best temperature for increased exopolysaccharide yield percentage by *Azotobacter Chroococcum* (45.63%).

A significantly ($P \leq 0.05$) higher amount of alginate production was obtained at 200 rpm i.e. 5.21 g/L (fig. 2e).These results are in line with Butt *et al*. (2011), as they observed increased alginate production (5.78g/L) at 200 rpm in shake flask studies. Emtiazi *et al*. (2004) also reported 200 rpm to be the optimized speed for alginate production (7.5g /L).

Various concentrations of salts were optimized to obtain higher yield of alginate under pre-optimized conditions. Significantly ($P \leq 0.05$) higher production of alginate (5.83 g/L) was observed at 1.5 % $CaCl_2$ and 2% $MgSO_4.7H_2O$ (6.08 g/L), while addition of NaCl and $KH_2PO_4.H_2O$ reduced the yield (fig. 3). The present results are supported by Garcia *et al*. (2001), who obtained maximum alginate production at 0.05g/L $CaCl_2$ and 0.4 g/L $MgSO_4.7H_2O$, while sodium ions resulted in decrease of alginate yield. A similar trend in data was reported by Vermani *et al.* (1997) using *A.vinelandii* MTCC 2460. The highest alginate yield was observed at 2g/L $CaCl_2$ and 1 g/L MgSO4.7$H_2O$ (4.25 g/L) while increasing concentration of sodium and phosphate ions resulted in lower yield. Galal and Ouda (2014) differed from the present results and reported the maximum production at 0.20% phosphate concentration by using *A. chroococum*. Thus, the effect of phosphate ions on exopolysaacharide production is controversially described in literature.

*A .vinelandii* is nitrogen fixing bacteria and thus can meet their protein requirements by fixing atmospheric nitrogen. In the present report, the effect of varying concentrations of nitrogen sources i.e. yeast sludge and corn steep liquor (1-3%) was studied. Corn steep liquor with 2% concentration yielded significantly ($p \leq 0.05$) higher alginate i.e. 7.46 g/L where as yeast sludge with 2.5% level resulted 6.55 g/L (fig. 4). The effect of nitrogen is controversially reported in the literature. The present results are in accordance with Butt *et al*. (2011) who reported that peptone yielded the best alginate production (6.08 g/L). These results are also in agreement with Galal and Ouda (2014), who reported yeast extract (0.6%) and corn steep liquor (0.8%) as the appropriate nitrogen source for biopolymer production by *A. chroococum*. Emtiazi *et al*. (2004) differed from the present results and stated that exopolysaccharide production by *Azotobacter* spp was not affected by the addition of vitamin and different nitrogen sources.

The quantitative estimation of alginate was done by HPLC using sodium alginate standard of Sigma – Aldrich. The HPLC chromatogram indicated the peak of pure alginate at retention time of 2.770 minutes with percentage purity of 98% as compared to standard as shown in fig. 5.

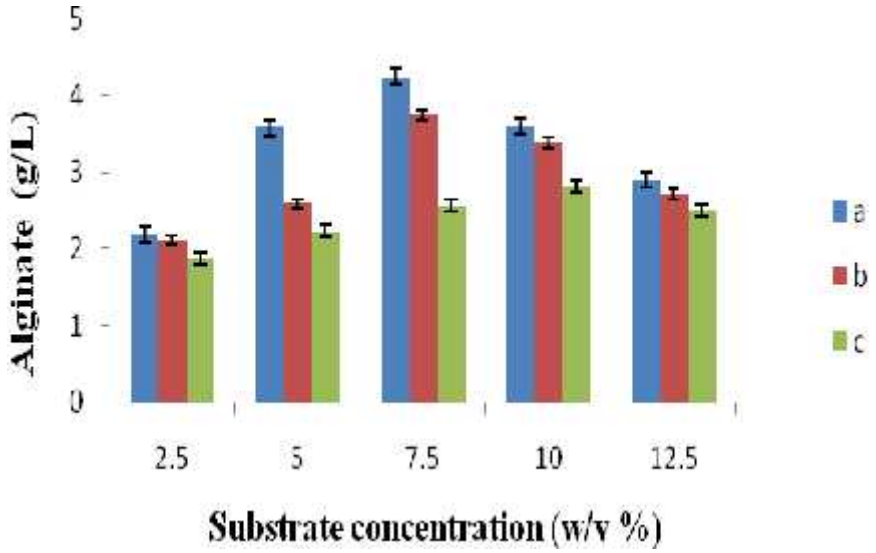


**Figure 1. Influence of different substrates (%) on Alginate production by *Azotobacter vinelandii.***
(a) Wheat bran (b) rice polishing (c) Molasses.

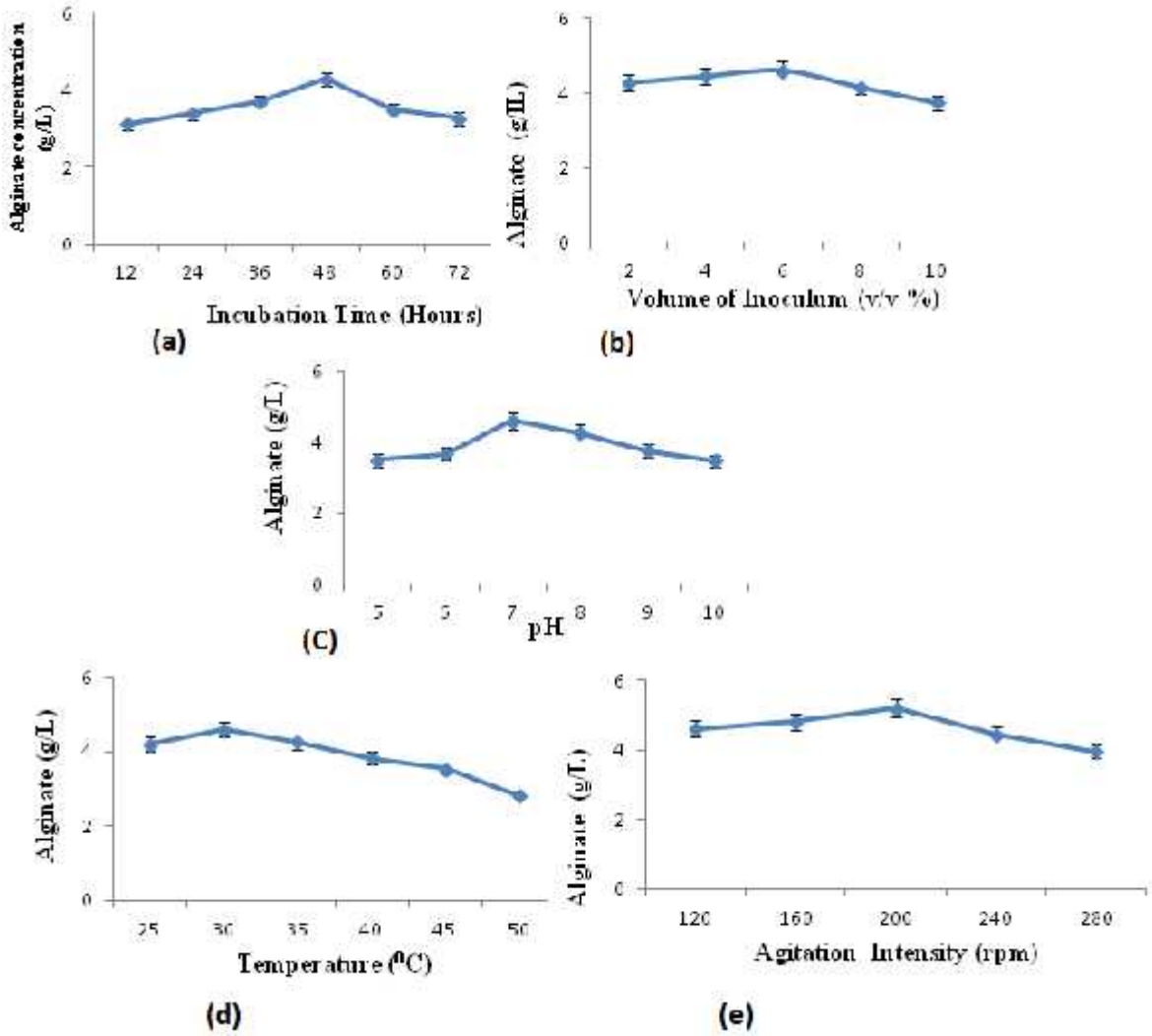


**Figure 2. Effect of physical parameters on alginate synthesis by *Azotobacter vinelandii.***
(a) Incubation time (b) Inoculum size (c) pH (d) Temperature (e)Agitation speed

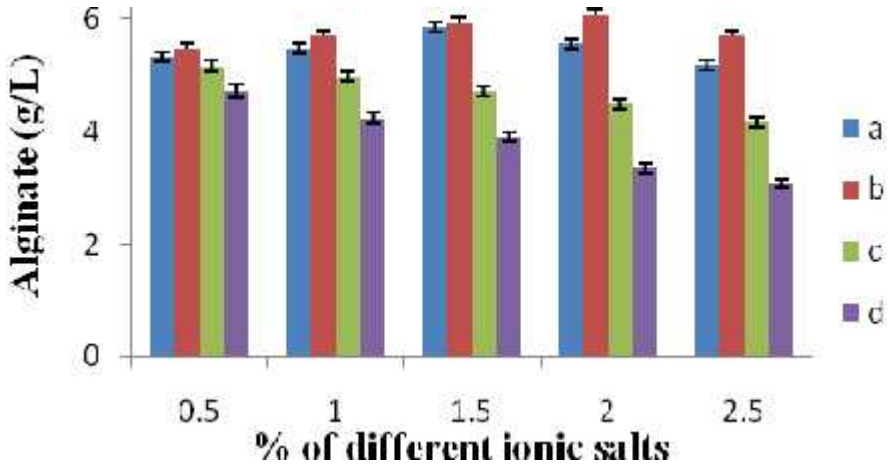


**Figure 3. Effect of ionic salts on alginate production by *Azotobacter vinelandii.***
(a) $CaCl_2$ (b) $MgSO_4$. $7H_2O$ (c) NaCl (d) $KH_2PO_4.H_2O$.Each bar represents mean value of three replicates. Y error bar denotes standard error of mean.

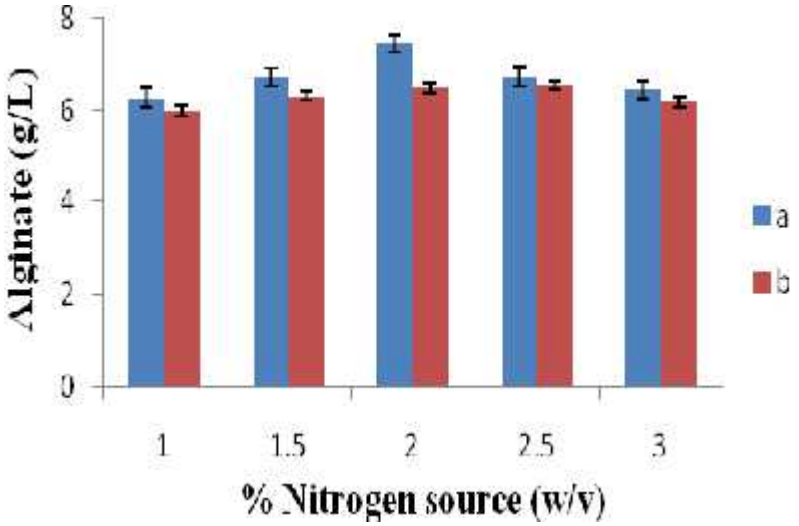


**Figure 4. Effect of organic nitrogen sources (%) on alginate production by *Azotobacter vinelandii.***
(a) Corn steep liquor (b) Yeast sludge. Each bar represents mean value of three replicates.

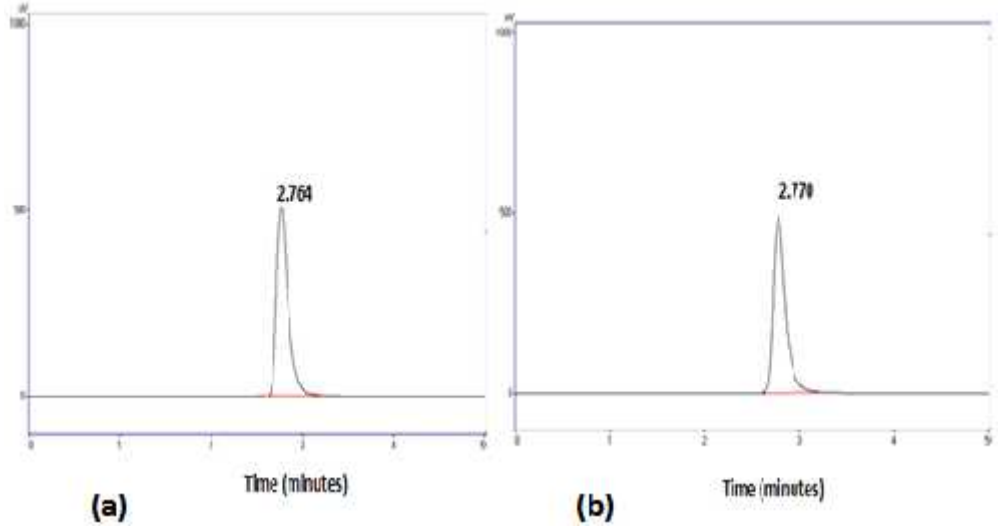


**Figure 5. Chromatogram of sodium alginate.**
(a) standard (b) sample

**Conclusion:** From different agricultural by-products tested, wheat bran was found to be the best carbon source for alginate production. In this research, a simple and cheap method for alginate production was developed which can be exploited on commercial scale.

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