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ORIGINAL ARTICLE

Submerged Alginate Biopolymer production by *Azotobacter chroococcum* Isolated from soil

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ABSTRACT
The purpose of this study was submerged production of alginate biopolymer by Azotobacter sp. which was isolated from soil and elevation of production by experiment design for optimization of culture conditions. Soil samples were cultured in Azotobacter broth medium enriched by 0.1% phenol as sole carbon source. Initial identification of Azotobacter sp. was done by morphological and biochemical properties and the isolated bacterium was exactly identified by PCR amplification of nifH-g1 region in Azotobacter genome. Production of alginate biopolymer was estimated by gravimetric method in culture supernatant after separation of Azotobacter cell biomass in Clementi medium. Also, the produced biopolymer was confirmed as alginate by biological method via immobilization of Vibrio fischeri ATCC 7744 cells in it and detection of bioluminescence production by immobilized cells. Then the factors affecting alginate biopolymer production by the isolated Azotobacter sp. including glucose, lactose and molasses as carbon sources (20 g L^{-1}); yeast extract, urea and ammonium sulfate as nitrogen sources (6 g L^{-1}); temperature (24, 25 and 26°C) and pH values (7.2, 7.4 and 7.6) were optimized in Clementi medium using Taguchi statistical method (Qualitek 4 software). The isolated bacterium was characterized as Azotobacter chroococcum after alignment of amplified genome region in Gene bank. The highest amount of alginate biopolymer obtained in optimal conditions using lactose and yeast extract as nutritional sources in 25°C and pH=7.6. The amount of polymer production was equal to 3.58 g L^{-1} in mentioned conditions. Also, the main effect was belonged to carbon source and the interaction between carbon and nitrogen sources was reported as the most effective interactions between analyzed factors.
Keywords: *alginate biopolymer, Azotobacter chroococcum, Taguchi statistical method*

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INTRODUCTION

The genus *Azotobacter* which depends to gamma-proteobacteria, is an obligate aerobe free-living, heterotrophic and Gram-negative soil bacterium that is non-spore forming and flagellated [1]. This bacterium is able to fix nitrogen by *nif* genes, the process which had been known in agricultural soils [2, 3] and is able to induce production of valuable products such as drugs by plants [4]. Bacterial species such as *Azotobacter vinelandii* and *Azotobacter chroccoccum* produce two biotechnological important polymers: alginate which is extracellular polysaccharide [5-7] and poly-β-hydroxybutyrate (PHB) which is intracellular polyester from polyhydroxyalkanoates (PHAs) family [8-9].

PHB is biodegradable, non-toxic polyester [8]. Alginate is a linear exopolysaccharide which consists of (1-4) linked β-D mannuronic acid and its C5 epimer α-L-guluronic acid. This biopolymer is found as homopolymer and heteropolymer according to building blocks [10]. Algae are world base for alginate production [11], which is especially secreted from marine brown algae (*Phaeophyta*) such as *Eclonia*, *Lossonia* and C*ystoseria*, as a structural component of cell walls and intracellular space [12]. Furthermore alginate is produced by bacteria including species of *Pseudomonas* and *Azotobacter* [5, 7]. It has been shown that formation of alginate play an important role in protecting *Azotobacter vinelandii* nitrogenase in phosphate-limited nitrogen-free culture, in addition to high rate of respiration [13]. Also, disadvantage role of this polymer has been reported in pulmonary infections in cystic fibrosis patients [7].

Alginate hydrogels are used for *in vivo* and *in vitro* cell immobilization, medicinal usages and for tissue engineering. In the food industry, alginate is vastly used as additive for improvement of viscosity, emulsifying and jell formation [10, 6, 14]. The aim of present study was optimization of culture conditions for elevation of alginate production by *Azotobacter* sp. which was isolated from soil.

MATERIALS AND METHODS

Sampling and isolation of bacteria: Soil samples obtained from the rhizosphere of agricultural crops in Iran (Isfahan, Falavarjan and Qahderijan regions). For enrichment of *Azotobacter* strains 1 g of each soil sample was inoculated into 100 ml Erlenmeyer flask containing 20 ml of *Azotobacter* broth (modified Clementi medium) with the composition of K_2HPO_4 0.8 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1},$FeSO_4.6H_2O$ 0.10 g L^{-1}, $CaCl_2.2H_2O$ 0.05 g L^{-1}, $NaMoO_4.2H_2O$ 0.05 g L^{-1} Ethylene glycol (1%) and 0.1% phenol as sole carbon source. pH adjusted to 7.4-7.6. All cultured media were incubated at 37°C for 2-5 days [15, 16, 1].

Physiological and biochemical characteristics: In order to initial identification of *Azotobacter* spp., morphology of colony and its solubility in water, Gram-staining, lipid granules (Sudan Black staining), capsule formation, catalase, oxidase, nitrate reduction and fermentation of different sugars were performed [17, 1, 18].

Molecular identification: Molecular identification was performed by PCR [19] using *nifH-g1* region from *Azotobacter* (Gen Bank accession numbers M11579, M20568) with primer pairs: fD1 (5′TTCCATCAGCAGCTCTTCGA-3′) and rP1 (5′GGCAAAGGTGGTATCGGTAA-3′). Also, DNA extracted from *Azotobacter* sp. ATCC 21748 was used as positive control for PCR.

Sequencing of amplified fragments: The amplified final products were sequenced by AB13730XL system (Bioneer Corporation, Korea) and aligned with the current sequences in the BLAST database.

Alginate production: The medium for alginate production by *Azotobacter chroccoccum* contained 20 g L^{-1} sucrose, 0.6 g L^{-1} $(NH_4)_2SO_4$, 2 g L^{-1} Na_2HPO_4, 0.3 g L^{-1} $MgSO_4.7H2O$, and 6 g L^{-1} yeast extract at pH=7.2. every 100 ml alginate production medium were inoculated with ~4×10^8 CFU ml^{-1} of each isolate of *Azotobacter* and were incubated as submerged culture at 28°C at 180 rpm for 170 h. The amounts of cell biomass and alginate production were determined and recorded every 24 h [15, 20, 5].

Separation of cell biomass: Five ml for each sample was centrifuged at 8400 rpm at 15°C for 30 min in pre weighed tubes. The supernatant was removed and pellet suspended in NaCl (5M) and Na_4EDTA (0.5M). Centrifugation was done as in previous step. The harvested biomass washed with deionized water several times and then dried at 60°C for 24 h to measure biomass weight [20, 15].

Exopolymer determination: Exopolymer was measured by gravimetric method as the following procedure: The supernatant of previous step was removed and equal volume of ice-cold 95% ethanol was added, stirring slowly. The mixture was centrifuged at 12000 rpm at 4°C for 20 min. The supernatant was carefully removed. The tubes were dried at 105°C for 24 h and dried exopolymer was weighted. Also the growth curve of isolated bacterium was drown by biomass assessment every 24 h in Clementi medium at 25°C and the time of most biopolymer production was determined [15, 21].

Alginate biopolymer was confirmed by biological method: 100 ml of 3% (w/v) exopolymer solution was prepared in distilled water. Then 100 ml of cultured *Vibrio fischeri* ATCC 7744 (1.5×10^8 CFU ml^{-1}) was added and the suspension was shaken gently. Calcium chloride solution 2% (w/v) was prepared separately. The suspension of exopolymer and bacterial cells was dropped in calcium chloride solution with 10-ml syringe and then granulated gels washed with distilled water. Calcium chloride solution according to the above condition was considered as negative control [22]. Bioluminescence production of immobilized cells was determined by luminometer (GloMax® Discover Multimode Detection System).

Optimization of alginate biopolymer production: According to other reports which were done by one factor at a time optimization [2, 5, 6], approximate ranges of factors affecting alginate production by *Azotobacter* were initially determined. So, using Taguchi statistical method (Qualitek 4 software), the factors affecting alginate biopolymer production by the isolated *Azotobacter* sp. including glucose, lactose and molasses carbon sources (20 g L^{-1}); nitrogen sources including yeast extract, urea and ammonium sulfate (6 g L^{-1}); temperatures (24, 25 and 26°C) and pH values (7.2, 7.4 and 7.6) were optimized in Clementi medium using L-9 orthogonal array in 4 repeats for every experiment. The results were reported as the amount of biopolymer production after 96 h incubation.

RESULTS

Morphology of the isolated bacterium: Colorless, viscous, transparent, shiny, mucoid colonies with irregular borders and negative in Gram-staining were selected in phenol ethylene glycol agar. Colonies was dissolvable in distilled water on glass slide (figure 1).

Figure 1: Colonies of the isolated bacterium were soluble in distilled water.

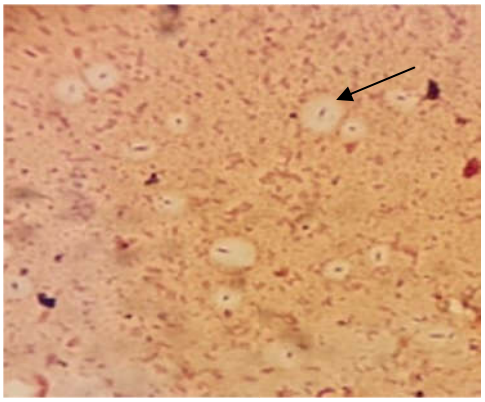
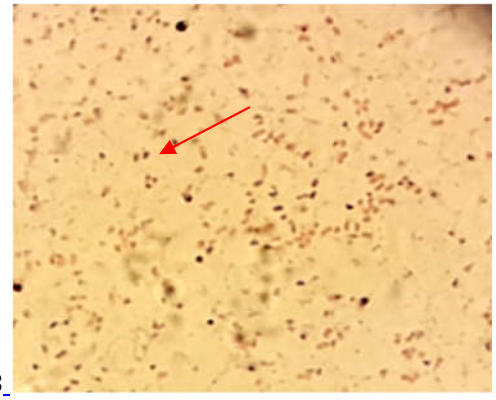
A  B 

Figure 2: Capsule (A) and lipid granules (B) formation by the isolated bacterium which showed with black and red arrows respectively.

The selected bacterium was Gram-negative rod with some polymorphisms in microscopic morphology. The cells surrounded with capsules with capsules and lipid granules detected in the cells by Sudan black staining (figure 2).

Identification by biochemical analysis: The results which obtained from biochemical analysis are shown in table 1.

Table 1: The results obtained from biochemical analysis of the isolated bacterium

Test	Result
Extra cellular catalase production	+
Oxidase production	-
Nitrate reduction	+
Fermentation of:	
glucose	+
sucrose	+
lactose	-
xylose	+
mannose	+
maltose	-
Identified isolate	*Azotobacter* sp.

Molecular identification: Amplification of the sequence from *nifH-g1* resulted the fragment with 700 bp length (figure 3). Alignment of the fragment in Gene bank confirmed an isolate of *Azotobacter chroococcum* with 99% similarity. The phylogenic tree is shown in figure 4.

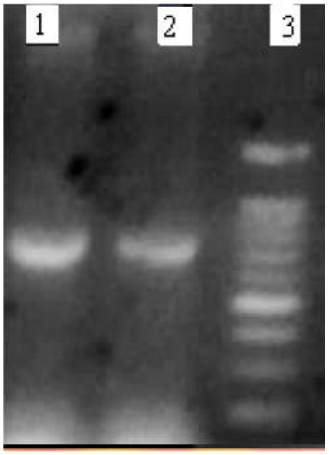


Figure 3: The 700 bp band resulting from amplification of *nifH*-g1 fragment (lane 1) from the isolated *Azotobacter* sp. and *Azotobacter* sp. ATCC 21748 (lane 2) according to 100 bp DNA marker (lane 3).

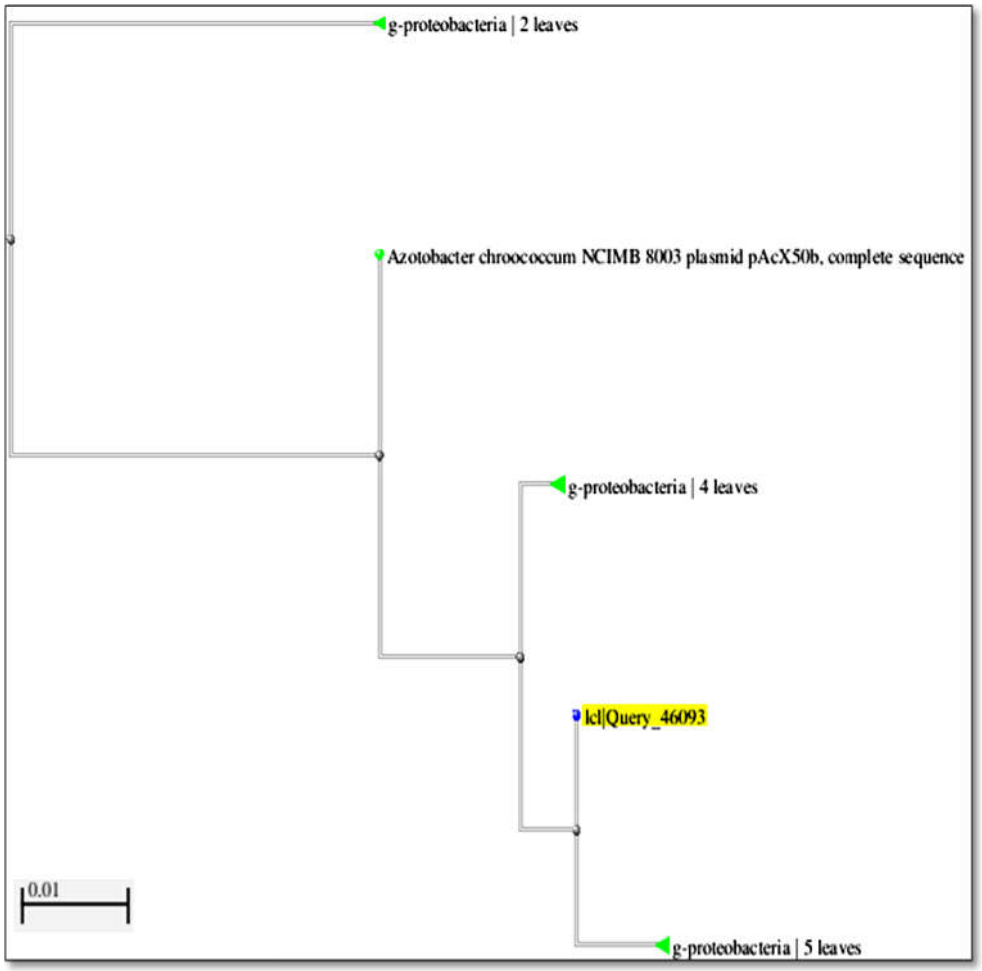


Figure 4: Alignment of the amplified fragment showed an isolate of *Azotobacter chroococcum* which illustrated in phylogenic tree with yellow highlighted.

Alginate biopolymer confirmation by biological method: Bioluminescence production was conserved after immobilization of *Vibrio fischeri* ATCC 7744 in exopolymer which was produced by the isolated *Azotobacter* with intensity of 8 units per second (figure 5).

Figure 5: Bioluminescence production by *Vibrio fischeri* ATCC 7744 after immobilization in exopolymer which has been produced by the isolated *Azotobacter*.

The most biopolymer production in isolated bacterium growth stages: As shown in figure 6, the isolated bacterium produce highest amount of biopolymer (0.19 g L^{-1}) after 130 h incubation in Clementi medium (at the beginning of the logarithmic phase) at 25°C.

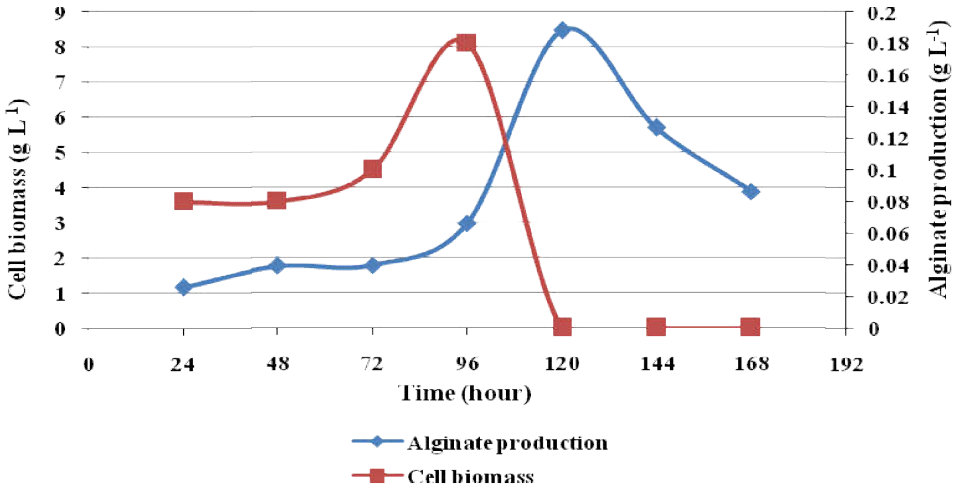


Figure 6: The most biopolymer production by the isolated bacterium according to growth curve stages in clement medium.

Optimization of alginate biopolymer production: As shown in table 2, The most production of alginate (3.58 g L^{-1}) obtained in the forth experiment condition using lactose (20 g L^{-1}) as carbon source, yeast extract (6 g L^{-1}) as nitrogen source, temperature of 25 °C and pH=7.6.

Table 2. The results obtained from 9 experiments which designed by Taguchi method (Quailitek 4) of alginate biopolymer production by the isolated *Azotobacter chroococcum*.

Test number	Carbon source (20 g L^{-1})	Nitrogen source (6 g L^{-1})	Temperature (°C)	pH	Alginate biopolymer production (g L^{-1})
1	Glucose	Yeast extract	24	7.2	0.45
2	Glucose	Urea	25	7.4	2.58
3	Glucose	Ammonium sulfate	26	7.6	0.92
4	Lactose	Yeast extract	25	7.6	3.58
5	Lactose	Urea	26	7.2	2.41
6	Lactose	Ammonium sulfate	24	7.4	2.06
7	Molasses	Yeast extract	26	7.4	1.92
8	Molasses	Urea	24	7.6	1.64
9	Molasses	Ammonium sulfate	25	7.2	1.41

ANOVA analysis showed that carbon source with the percentage off 33.21 has the most effect on optimization of alginate production (table 3). Also, interactions between different factors are shown in table 4. The highest engagement between actions was related to the interaction between carbon and nitrogen sources (56.97%) and the least was related to temperature and pH factors (3.16%).

Table 3. Analysis of variance by ANOVA statistics which shows the most effective factor on alginate production by *Azotobacter chroococcum*.

Factors	Dof	Sums of squares	Variance	F-Ratio	Pure Sum	Percent (%)
Carbon source	2	11.11	5.55	50.34	10.88	33.21
Nitrogen source	2	4.27	2.13	19.33	4.05	12.34
Temperature	2	9.63	4.82	43.66	9.41	28.71
pH	2	4.80	2.40	21.74	4.58	13.95
Other/Error	27	2.97	0.11			11.78
Total	35	32.79				100

Table 4. Interactions between every two factors and the effects of them on biopolymer production.

Interacting factor pairs (order based on SI)	SI (%)	Col	opt
Carbon source × Nitrogen source	56.97	3	[1,1]
Carbon source ×pH	43.89	5	[1,1]
Nitrogen source × Temperature	30.9	1	[1,1]
Nitrogen source ×pH	16.71	6	[1,1]
Carbon source × Temperature	14.42	2	[1,1]
Temperature ×pH	3.16	7	[1,1]

DISCUSSION AND CONCLUSION

Alginate is a long chain linear polysaccharide polymer of alginic acid with negative charge that interacts with calcium ions resulting in the formation of health and non-toxic gel network. This polymer is proposed for its worldwide possible applications as coating material, controlled release of drug in systems such as microspheres, beads, pellets, gels, fibers and membranes [22, 24]. Commercially important types of highly viscous alginate have a molecular weight of 150 kD [6]. *Azotobacter* is an alginate producing bacterium which in contrast to some other alginate producing bacteria such as *Pseudomonas aeruginosa*, is non-pathogenic bacterium [7]. Soil is the best environment for the growth of *Azotobacter* species due to organic phosphates enrichment [25, 20].

Gaytán *et al*. [28] reported fourfold increase of specific alginate production by mutation which resulted in Na^+-NQR lack mutant of *Azotobacter vinelandii*. The results of our present study showed that we have been able to optimize the production of alginate biopolymer by *Azotobacter chroococcum* using Taguchi method with about 19 times improvement in contrast to traditional Clementi medium, without genetically modification. The best conditions for production of alginate extracellular biopolymer by *Azotobacter chroococcum* isolated from soil obtained by using lactose as carbon source, yeast extract as nitrogen source, temperature of 25°C and pH=7.6. In this condition the amount of alginate production was 3.58 g L^{-1} comparing to 0.19 g L^{-1} in Clementi medium with growth conditions of 28°C and pH=7.2. In other studies different maximum amounts of alginate was reported including 3 g L^{-1} by *Azotobacter vinelandii* [6], 3 g L^{-1} by *Azotobacter chroococcum* [5] and greater amounts using high concentration of sugars such as sucrose in two stage fermentation [27]. Most of previous studies were conducted using one factor at a time optimization. The comparison of the results obtain in our study with others indicates the importance of experimental design method. By using Taguchi experimental design and selection of best conditions, the alginate production by *Azotobacter chroococcum* was considerably elevated.

The genus *Azotobacter* is able to utilize a variety of carbon sources ([26]. This fact facilitates the optimization of biopolymer production by the bacterium. Also, Taguchi is a powerful, simple and effective method for experimental design to optimize different productions. Key factor in selection of Taguchi method is achievement of high quality without increasing cost. Another advantage of this method is the ability to perform parallel tests by one basic engineering design. Also, the results obtained from experiments can be statistically analyzed by the program using analysis of variance [23].

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