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Production and Optimization of Alginate by *Azotobacter species* Isolated from Soil

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A B S T R A C T

Alginates are biocompatible, biodegradable, and safe biopolymers that are employed in a variety of pharmaceutical and medical applications as an anti-inflammatory, immobilization agents, radioactive suppression agents, and wound healing agents. In the present study, bacteria *Azotobacter* were isolated from rhizospheric soil, identification was carried out using selective media and biochemical methods. The screening for the production of alginate was carried out. The screened potential strain was checked for its ability to produce alginate using an alginate production assay. Characterization of the extracted compound was done by Fourier-Transform Infrared Spectroscopy (FTIR). Optimization of parameters like Carbon sources, Organic and Inorganic nitrogen sources, pH was carried out using Plackett Burman Design of Experiment, the production of alginate biopolymer from *Azotobacter* species was studied by statistical method and the optimization study concludes that *Azotobacter* (S1J) strain gives the highest production of alginate. In the present study the production of alginate biopolymer by optimizing medium from diazotrophic bacteria *Azotobacter* species successfully studied and found increased. The findings support commercial synthesis of alginate from bacterial sources, as it is of higher quality than algal alginate.

***Key words*:** Alginate production, *Azotobacter*, Optimization, Soil, FTIR

Alginates are a form of biopolymer with a wide range of applications in industry and technology. They are employed as a key component in dental impression material in the pharmaceutical industry, as well as in traditional wound dressings and various formulations for treating gastric reflux. Surface sizing, welding rod coating, ceramics manufacture, and water treatment are all applications in the paper business [1]. Alginates are widely utilised in the food, textile, and paper industries as thickeners, stabilisers, gelling agents, and emulsifiers [2-3]. Alginates are often used as a microencapsulating agent for probiotics and as food additives to improve their viability throughout the manufacturing process. Alginate has been used in the food industry to coat fruits and vegetables, as a microbiological and virus protection product, as a gelling, thickening, stabilising, or emulsifying agent, and as a gelling, thickening, stabilising, or emulsifying agent [4-5]. Alginates are widely employed in pharmaceutical and medical applications as anti-inflammatory, radiation suppression, and wound healing agents as they are biocompatible, biodegradable, and safe biopolymers [6-7], Alginate is a biopolymer with unique physical and chemical properties that makes it functionally an ideal material for attachment with proteins. Immobilization of enzymes on alginate is well known to show altered catalytic functions and improved operational stability with no or minimal drawbacks [8].

Based on chemical structure of alginate, with an abundance of free carboxyl and hydroxyl groups along the polymer chain backbone, various modifications of these two active groups were conducted. This improved their chemical and physical properties for further applications [9]. Due to their wide range of applications, global annual industrial production of alginates was predicted to exceed 30,000 metric tonnes in 2009 [10]. In 2016, the global alginate market was worth USD 624.0 million. Consumption of frozen desserts, ice creams, beer, and yoghurt will increase demand for alginates in the food industry, resulting in a significant market rise of alginate value and use [11].

Apart from algae, several bacterial strains, mostly belonging to the Azotobacter sp. and Pseudomonas sp. families, are now commonly used in the industrial manufacture of Alginic acid. During the late exponential development phase, these bacteria can generate alginates as extracellular polysaccharides. Azotobacter species are the most recommended Alginate producing strains for large-scale

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manufacturing as according to the FDA's GRAS (Generally Recognized as Safe) status, it is safe, and alginates generated from this type of microbe have contiguous sequences of L-guluronic acid residues similar to those of algal Alginates. Bacterial alginate is less expensive than its algal counterparts [12-13]. This study was undertaken to screen alginate production by different isolates of *Azotobacter* species isolated from Botanical soil samples under investigation and to standardize optimized production conditions for increased production of Alginate.

MATERIALS AND METHODS

Microorganisms

Azotobacter was isolated from the soil samples collected from the botanical garden of K. N. Bhise College, Kurduwadi. District Solapur, using Ashby's Nitrogen free mannitol agar. After collection, a portion of each sample was immediately transferred to the laboratory and stored at 4°C for microbial analysis. Collected samples were thoroughly mixed on a piece of clean cloth; lumps were broken using wooden pestle- mortar and were air-dried. The air-dried samples were sieved using in 10 mesh diameters sieve, stored in glass bottles and labeled for analysis.

Screening of alginate producing Azotobacter

1 g of the soil samples was mixed with 10 ml sterilized saline solution and Kept on Shaker incubator at 30°C, after 24 hours, the supernatant fluid was filtered and cultured on agar medium with Composition Sucrose 20g/l, Yeast extract 5 g/l, (NH_3) SO_4 2g/l, $MgSO_4$ 0.2 g/l, $CaSO_4$ 1 g/l, Na_2MoO_4 0.002g/l, Tri ammonium citrate.05g/l, pH 7 The selection criterion of the colonies was rapid growth (in 24 h). A colony with the fastest growth rate on the Ashby's N_2 free Mannitol agar was selected for further studies, to investigate various factors significant in Alginate production.

Identification of Azotobacter

Identification of all 11 isolates of *Azotobacter* was carried out using morphological characteristics and biochemical characteristics described in Bergey's manual of determinative bacteriology [14].

Alginate production

Screening of isolate for production of alginate

The total 11 isolates of *Azotobacter* were taken in Erlenmeyer flasks containing 50 ml alginate production medium (20 g sucrose, 0.6 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, and 6 g yeast extract, 2 g $CaCO_3$ per liter of distilled water at pH 7.2) were inoculated with ~10^4 CFU/ml of each colony of Azotobacter and were incubated at 28°C at 180 rpm for 96 h [15].

Alginate purification

Extraction and Partial purification of Alginate was done by modified Method of Clementii [16]. We used 50 ml of fermented broth and an equal volume of ice cold 99% ethanol was added and contents were kept in the refrigerator for one hour. Then centrifuged for 30 minutes at 6000 rpm and the pellet was washed with distilled water and dried in a hot air oven at 105°C for 1 hour.

Optimization studies

The typical 'one-factor at a time' approach to multivariable system optimization is not only time demanding, but it also frequently overlooks alternative effects between components [17]. In addition, this procedure necessitates a series of studies to identify the optimal levels. These flaws in the single factor optimization procedure can be overcome by employing a statistical approach and Plackett and Burman design of experiments to optimize all of the influencing parameters simultaneously. The Plackett–Burman experimental design is a two-factorial design that screens n variables in n + 1 experiments to identify the crucial physicochemical parameters required for enhanced alginate Dry wt. production [18].

The following chart was obtained as in (Table 1). In the present study, 12 experiments were run as indicated by the Placket Burman Design of experiment. Dry weight of Alginate produced in each of the experiments was noted (Table 1) and data was analyzed with the help of Minitab statistical software.

Table 1 Plackett Burman Design

S. No.	Sucrose (A)	Yeast extract (B)	$(NH_4)_2SO_4$ (C)	$MgSO_4$ (D)	$CaCO_3$ (F)	$FeSO_4$ (G)	Na_2MoO_4 (H)	Time (I)	Volume (J)	pH (K)	Dry wt. g/l
1.	40	10	5	0.1	5	0.10	0.01	96	50	6	10.05
2.	10	10	5	2.0	1	0.10	0.10	48	150	6	4.56
3.	40	10	1	2.0	1	0.01	0.01	96	150	8	8.44
4.	10	10	5	0.1	5	0.01	0.01	48	150	8	7.80
5.	40	5	1	0.1	5	0.10	0.10	48	150	8	3.68
6.	10	5	5	2.0	5	0.01	0.10	96	50	8	9.44
7.	10	5	1	2.0	5	0.10	0.01	96	150	6	3.09
8.	40	10	1	2.0	5	0.01	0.10	48	50	6	5.06
9.	10	10	1	0.1	1	0.10	0.10	96	50	8	6.10
10.	40	5	5	0.1	1	0.01	0.10	96	150	6	12.86
11.	10	5	1	0.1	1	0.01	0.01	48	50	6	3.70
12.	40	5	5	2.0	1	0.10	0.01	48	50	8	3.94

Characterization of alginate by Fourier-Transform Infrared Spectroscopy (FTIR)

The Nicolet 6700 FT-IR spectrometer was used to perform FTIR measurements at room temperature. A cell with KRS-5 windows and a Teflon spacer was utilized to record spectra; the optical path length was 1 cm. Each spectra was subjected to 40 scans at a resolution of 2 cm^{-1}.

RESULTS AND DISCUSSION

Isolation and identification of Azotobacter

Total of 11 isolates named as S1J, S2J, S3J, S4J, S5J, J1K, J2K, J3K, J4K, J5K, S1K, were selected after enrichment, isolation, and screening from Botanical Garden soil sample. The identification of isolates was completed according to biochemical characteristics from Bergey's Manual of

Determinative Bacteriology. The isolate was identified as *Azotobacter* species.

Screening of isolate for production of alginate

Among the 11 isolates tested isolate coded as S1J was found to give the maximum Alginate production in terms of dry weight. This was selected for further optimization studies and other isolates were eliminated. The optimization of fermentation medium and conditions was analyzed by statistical method.

Optimization studies

12 experiments were run to see how selected variables affected the outcome. (Table 2) shows the findings of the Plackett-Burman design screening studies. Regression analysis was performed and a first-order polynomial model was produced. Following regression equation was obtained. By using following regression model, we can predict values of Dry weight for different Media components and conditions.

Regression Equation

Dry weight = -0.685 + 0.11806 A + 0.1770 B + 0.7746 C- 0.8482 D - 0.0196 E - 29.39 F + 8.65 G+ 0.03247 H+ 0.00355 I + 0.0058 J

Here A, B, C, D, E, F G, H, I and J corresponds to levels of Sucrose, Yeast extract, $(NH_4)_2SO_4$, $MgSO_4$, $CaCO_3$, $FeSO_4$, Na_2MoO_4, Time, Volume and pH respectively.

The above model satisfied all the necessary statistical tests for its acceptance. The P values" was less than 0.05, for three of the terms and brought out their importance and they are Sucrose, $(NH_4)_2SO_4$ e and caCO3

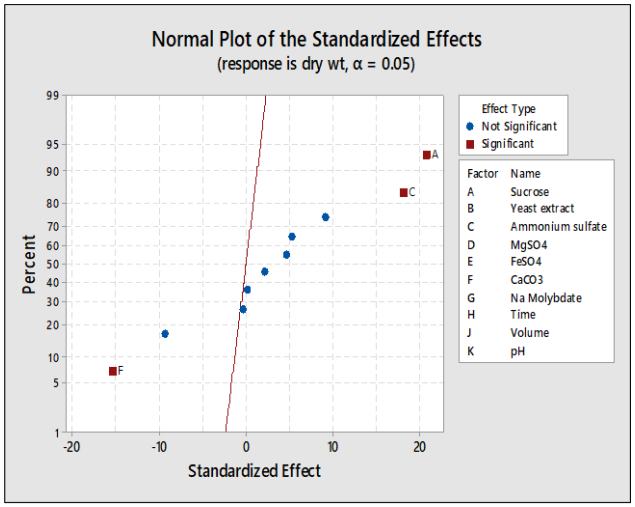
This model has a R^2 value of 99.92 percent, a predicted R^2 value of 88.12 percent, and a R^2 adjusted value of 99.09 percent. The S Value was found to be 0.297335, indicating that the standard error is low and the model is well-fitted. Sucrose, $CaCO_3$, and $(NH_4)_2SO_4$ are the components studied that exhibited a significant effect on Alginate formation, with P values less than 0.05.

Table 2 Factorial Regression: Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	107.059	10.7059	121.10	0.071
Linear	10	107.059	10.7059	121.10	0.071
Sucrose	1	37.630	37.6302	425.64	0.031
Yeast extract	1	2.350	2.3497	26.58	0.122
$(NH_4)_2SO_4$	1	28.799	28.7990	325.75	0.035
$MgSO_4$	1	7.792	7.7924	88.14	0.068
FeSO4	1	0.018	0.0184	0.21	0.727
$CaCO_3$	1	20.988	20.9881	237.40	0.041
Na_2MoO_4	1	1.817	1.8174	20.56	0.138
Time	1	7.285	7.2852	82.40	0.070
Volume	1	0.378	0.3781	4.28	0.287
pH	1	0.000	0.0004	0.00	0.957
Error	1	0.088	0.0884		
Total	11	107.147			

Table 3 Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		6.5608	0.0858	76.44	0.008	
Sucrose	3.5417	1.7708	0.0858	20.63	0.031	1.00
Yeast extract	0.8850	0.4425	0.0858	5.16	0.122	1.00
$(NH_4)_2SO_4$	3.0983	1.5492	0.0858	18.05	0.035	1.00
$MgSO_4$	-1.6117	-0.8058	0.0858	-9.39	0.068	1.00
$FeSO_4$	-0.0783	-0.0392	0.0858	-0.46	0.727	1.00
$CaCO_3$	-2.6450	-1.3225	0.0858	-15.41	0.041	1.00
Na_2MoO_4	0.7783	0.3892	0.0858	4.53	0.138	1.00
Time	1.5583	0.7792	0.0858	9.08	0.070	1.00
Volume	0.3550	0.1775	0.0858	2.07	0.287	1.00
pH	0.0117	0.0058	0.0858	0.07	0.957	1.00



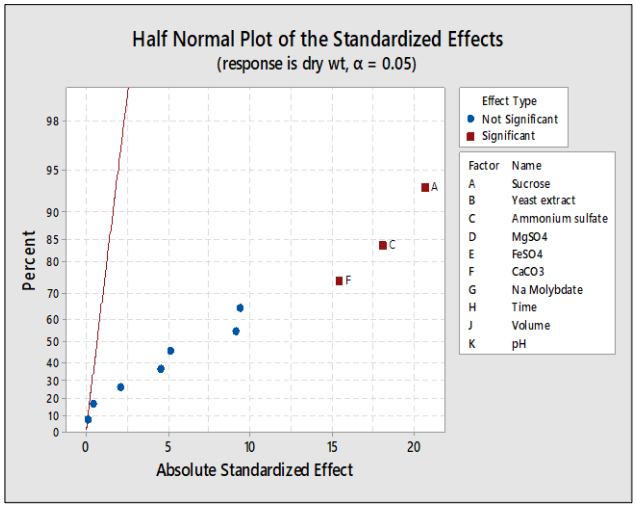


Fig 1 Standardized effects plots

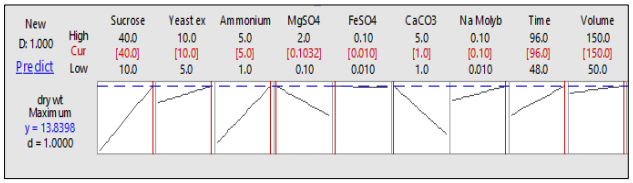
In the study, the optimization of the medium was carried out with the help of statistical data obtained. The following effective combination was obtained.

Table 4 Multiple response prediction

Variable	Setting g/l
Sucrose	40
Yeast extract	10
$(NH_4)_2SO_4$	5
$MgSO_4$	0.1
$FeSO_4$	0.01
$CaCO_3$	1
Na_2MoO_4	0.1
Time	96
Volume	150
pH	8

Response	Fit	SE Fit	95% CI	95% PI
Dry weight	13.843	0.285	(10.225, 17.460)	(8.612, 19.073)

From Pareto chart we can say that sucrose, $(NH_4)_2SO_4$ and $CaCO_3$ are most significant factors for fitted regression model under study.



Effect of different chemical and physical parameters on the production of alginate biopolymer by Azotobactor species (a) Carbon sources, (b) Organic nitrogen sources, (c) inorganic nitrogen sources, (d) pH, (e) temperature, (f) Volume
Fig 3 Graphical representation of optimization of Ashby's medium for maximum alginate production

Validation of statistical optimization

With this setting obtained in (Table 2), we run a laboratory scale experiment. And we were able to achieve 13.05 g/l alginate production which is near to predicted value that is 13.843g/l.

Characterization of extracted alginate by IR

The optimization plots obtained further clarify the picture of different media components and fermentation conditions on the effect of alginate production. We can say that When Sucrose concentration increases alginate production increases, Yeast extract has a moderate effect. $(NH_4)_2SO_4$ directly affects the alginate production While the Concentrations of $MgSO_4$ and $CaCO_3$ tested Negatively affects the alginate production. All other factors tested have no Significant effect on Alginate production.

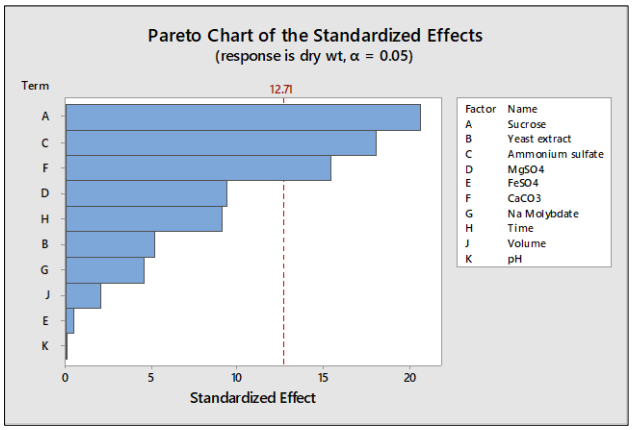


Fig 2 Pareto chart of standardized effects

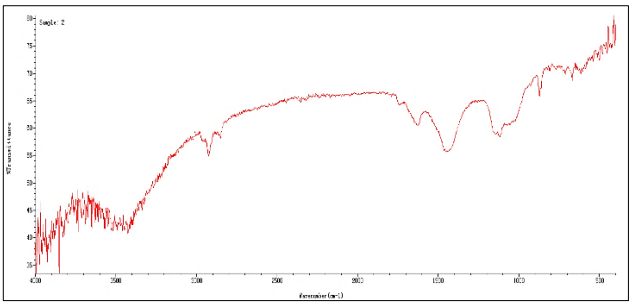


Fig 4 Characterization isolated alginate fraction is confirmed by the IR spectrum

The presence of sodium alginate in the isolated alginate fraction is confirmed by the IR spectrum. For alginate confirmation, we can see strong peaks in (Fig 4). The prominent peaks are very similar to those found in normal sodium alginate. The band at ~3700 cm^{-1} is due to H-O-H stretching vibrations, The band at ~2950 cm^{-1} is due to -O-H in acid(-COOH) while the band at ~1700 cm^{-1} is due to asymmetric stretching of C-O-

C carboxylate. C-OH deformation vibration with C-O-C symmetric stretching of the carboxylate group may be responsible for the band at ~1250 cm^{-1} [19-20].

CONCLUSION

The isolated strain was tested for its ability to generate alginate. The extraction of alginate was validated by FTIR findings, confirming that the isolated chemical is alginate. The highest alginate production was determined by comparing the different alginate production modified media. It can be determined that modified medium containing sucrose produces the greatest outcomes. The medium was used to investigate the impact of various physical and chemical variables on alginate synthesis. Optimization study concludes that Azotobacter (S1J) strain gives the highest production of alginate.

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