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Optimization of Physical Parameters of Fermentation Medium for Biosynthesis of Alginate by a Vinelandii Using Molasses as 'C' Source

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Abstract

Biopolymer have varied industrial applications. Alginate is one such polymer conventionally extracted from brown algae of marine waters having versatile applications. Due to its increased market demand alternative options are being investigated for its synthesis from microbes. The present study focuses on the optimization of various physical parameters at flask level. Fermentation medium consisted of pre-treated sugar cane molasses (2%) as carbon source and peptone as nitrogen source (0.5 g). The optimized parameters were found to be, temperature as 30°C, pH 7.0, incubation period 84 hours, agitation rate 150 rpm, inoculum age 36 hours and inoculum level 5%. Under optimized parameters the significant ($P<0.05$) alginate yield was found to be 5.71 g/L (PS) and 6.28 g/L (MS) with the corresponding dry cell mass (DM) as 6.28 g/L (PS) and 6.92 g/L (MS). The plate assay of the alginate lyase activity indicated that the improved strain has less activity (11 mm) as compared to the parent strain (14 mm). Alginate lyases influence the yield and the molecular weight of the product. Hence the zone of hydrolysis on the plates correlates with the maximum yield obtained. HPLC chromatogram indicated the peak of purified alginate with retention time of 3.332 minutes with purity of 81.22 %.

Keywords: Alginate, Alginate Lyase, *Azotobacter vinelandii*, Molasses.

Introduction

Alginates is one of the natural polysaccharides obtained from seaweeds and can also be biosynthesized by using bacteria. Unlike brown algae the biosynthesis from *Azotobacter* species have several advantages (1). Bacterial alginate differ in physicochemical properties they possess O-acetylation at C_2 and C_3 positions in mannuronic acid residues (2). This affects its viscosity, interaction with calcium ions and a reaction of mannuronan epimerase, and mannuronan lyase (3, 4). Bacterial synthesis under controlled laboratory conditions is advantageous for obtaining the polymer in a variety of physicochemical properties (5, 6). Alginate biosynthesis with *Azotobacter* genus is preferable as it is a non-pathogen (7). Alginates are used as stabilizers, thickeners, gelling or film-forming agent for various applications in the food, medical or pharmaceutical industries and (8-10) environmental.

They show excellent biocompatibility and biodegradability. Algal alginates show low consistency which limits their role in advanced biomedical applications. Microbial alginate production is lucrative due to its potential for synthesizing customized alginate molecules with stable characteristics. Economic concern is the major limiting factor for the commercialization of microbial alginates. However, carbon-rich wastes from dairy, sugar and biodiesel industries may serve as substitutes for pure carbon sources thus reducing the substrate costs. Fermentation parameter control may improve the production efficiency (11). In the present research work optimization of major physical parameters and some biological parameters (inoculum age, inoculum %) has been done to improve the yield using pre-treated molasses as a source of carbon.

Materials and Methods

Culture Isolation and Preservation

Azotobacter vinelandii (Parent Strain) was isolated from the soil. The isolate was maintained as pure culture on Burk's Nitrogen free agar

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medium slants (12). Burk's medium in g/l (K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.2g, NaCl 0.2g, $CaSO_4$ 0.1g, Fe-Mo Mixture – 0.1 ml, Sucrose 20.0g, H_3BO_3 100µg, $ZnSO_4.7H_2O$ 100µg, $MnSO_4.4H_2O$ 10.0µg, $CuSO_4.5H_2O$ 3µg, KI 1.0µg, D/W 100 ml, pH – 7.3); (Fe-Mo Mixture preparation - $FeCl_3.6H_2O$ – 1.45g, $Na_2MoO_4.2H_2O$ – 0.253g; D/W – 100 ml) incorporated with 0.5 g peptone was used for inoculum development and fermentation.

Optimization of Physical Parameters for Alginate Production

Preparation of inoculum - Inoculation of loop full of bacterial culture in 25 mL of Nitrogen-free sucrose medium contained in 250 mL conical flask with incubation on rotary shaker at 30° C. Twenty-four hour's old culture was used as inoculum with culture density set as 0.6 at 600 nm (12). Pre-optimized carbon and nitrogen sources i.e. molasses [2%] and peptone [0.5%] were added in the Burk's medium. Optimization of parameters was done in conical flasks of 250 mL (Erlenmeyer flask) having 50 mL of total fermentation medium (triplicates). Parameters included various time intervals [24, 48, 72, 96, 108 hours], percentages of inoculum [1-10%] (13), inoculum age [24, 36, 48, 60 hours], pH values [5, 6, 7, 8, 9], degree of temperature [25°C, 30°C, 35°C, 40°C, 45°C] and agitation rate [0, 100, 150, 200 rpm] were tested to achieve maximum yield of alginate.

Alginate Lyase Plate Assay

Crude clear supernatant was obtained after centrifugation of Burk's culture medium at 8000 rpm for 20 minutes, it was used as the source of alginate lyase. Plate assay was performed using plates containing 1% agarose and 0.1% commercially available sodium alginate. Alginate lyase activity was assessed by well diffusion method. 0.1 ml aliquots of crude enzyme from the wild type and mutant strains were poured in the wells and kept overnight for diffusion along with medium blank as a control. Plates were then flooded with Gram's iodine solution, zone of hydrolysis was observed (14, 15).

Alginate Extraction

1 mL of EDTA sodium salt solution (0.5M) and 0.5 mL of NaCl solution (5.0M), was added to the fermented broth, then centrifuged at 18000 rpm at 20°C for 30 minutes to separate the bacterial cell mass and substrate residues. Supernatant was then cooled by keeping in ice bath and three volumes of ice cold isopropanol wasadded. The mixture was left overnight at 4°C. Further it was centrifugation was done at 18000 rpm at 4°C for 30 min to precipitate alginate. The residue obtained was then dissolved in sterile distilled water, centrifuged, and finally the precipitates were dried at 80°C for 24 hours. Weights of the dried estimates were obtained gravimetrically (16). Precipitates of alginic acid were moistened with sterile distilled water and sodium carbonate was added to enhance the dissolution of alginate. Chilled ethanol was added to coagulate sodium alginate.

HPLC Analysis

The coagulated sample was further analyzed by HPLC method (17, 18). Sample was analyzed on Shimadzu Class – VPV 6.14 SP1 system at 30°C using Optimac C18 (150 x 4.6 mm) column. Mobile phase – 40 mM K_2HPO_4, pH was adjusted to 6.0 by NaOH. Selected detector wavelength – 200 nm, injection volume – 20 µl, alginate sample concentration – 1mg/ml (19).

Statistical Analysis

The data obtained in triplicates was analyzed using MS Excel and by One-Way ANOVA tool (20).

Results and Discussion

Optimization of temperature, pH, incubation duration (hours), agitation rate (rpm), inoculum age (hours), inoculum level (%) for production of alginate. Analysis of purified alginate by HPLC.

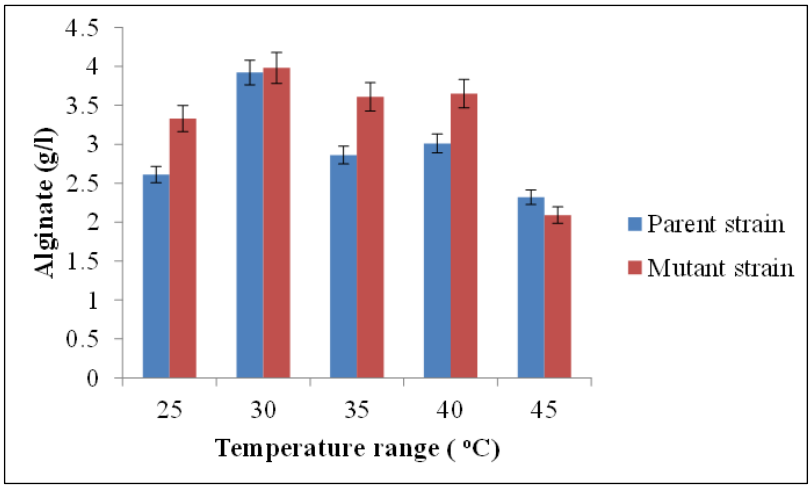

Figure (1a): Effect of Temperature on Production of Alginate (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

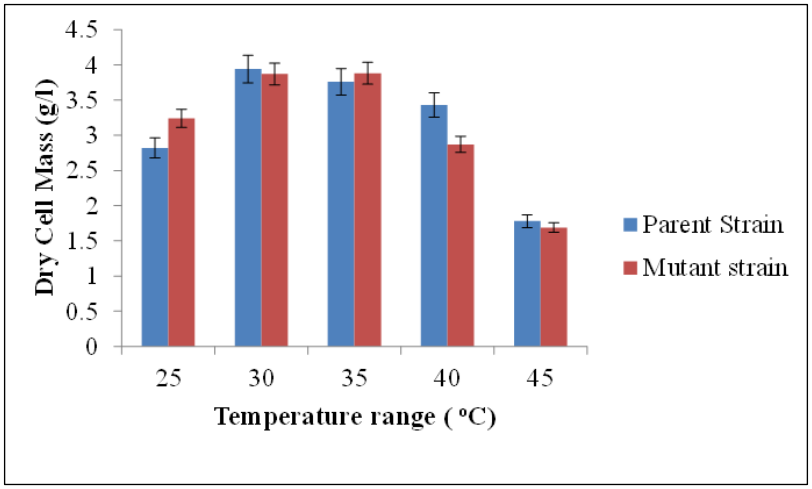

Figure (1b): Effect of Temperature on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

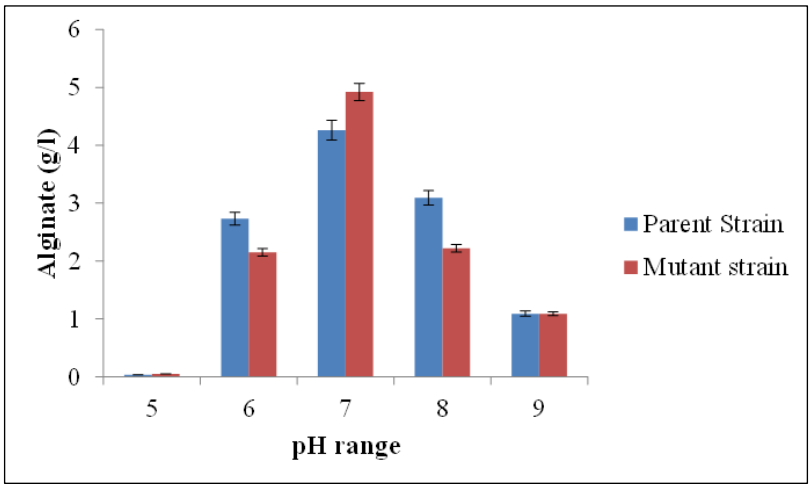

Figure (2a): Effect of pH on Production of Alginate (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

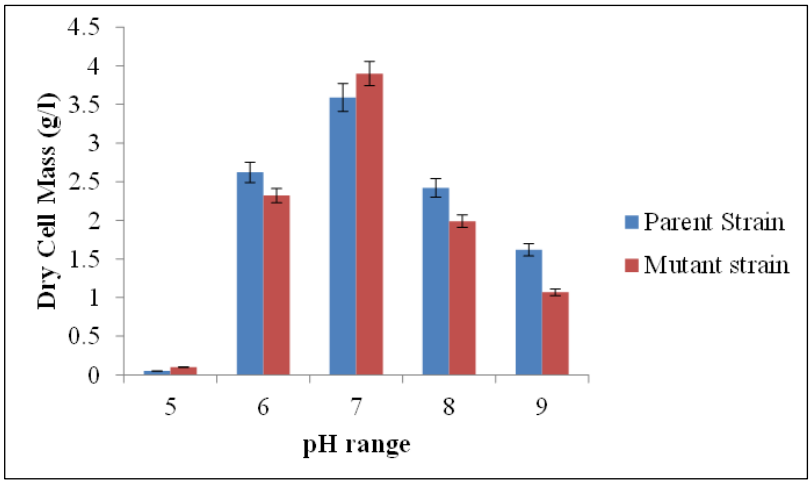

Figure (2b): Effect of pH on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

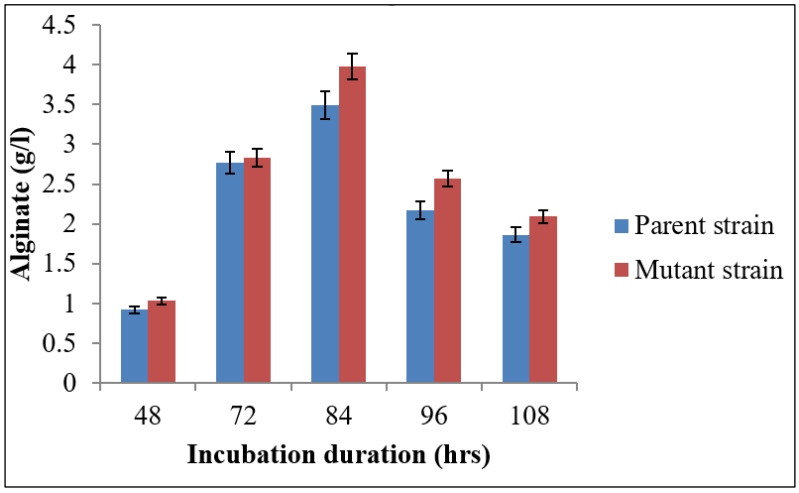

Figure (3a): Effect of Incubation duration on Production of Alginate (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

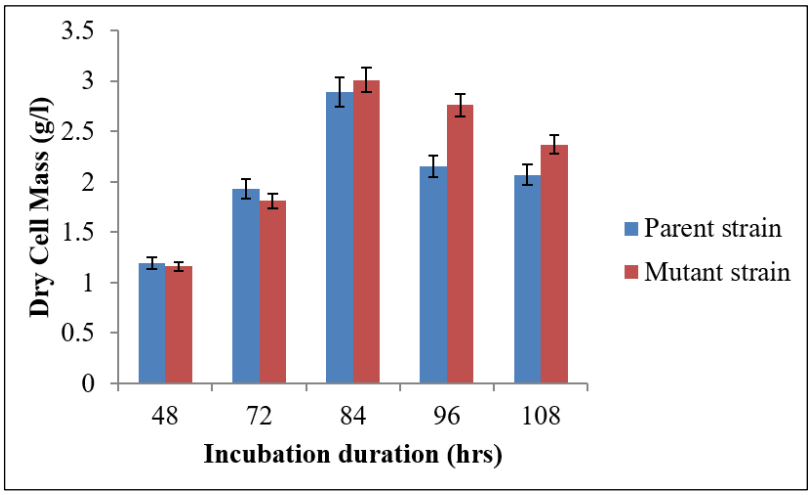

Figure (3b): Effect of Incubation duration on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

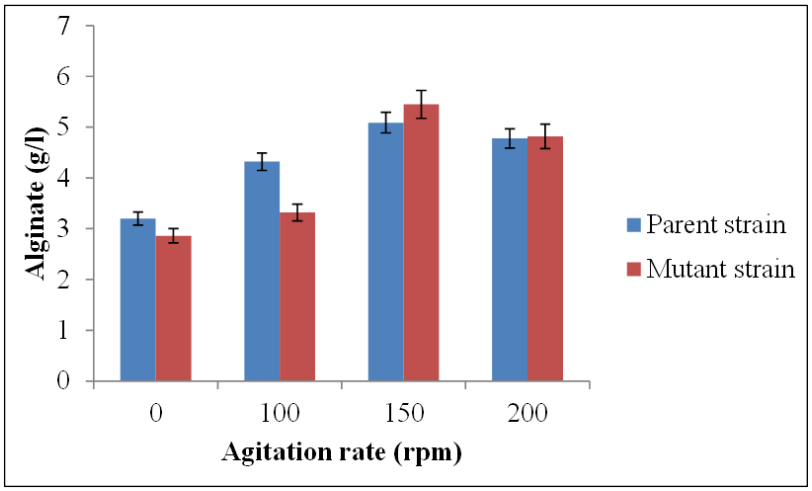

Figure (4a): Effect of Agitation Rate (Rpm) on Production of Alginate (G/L). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

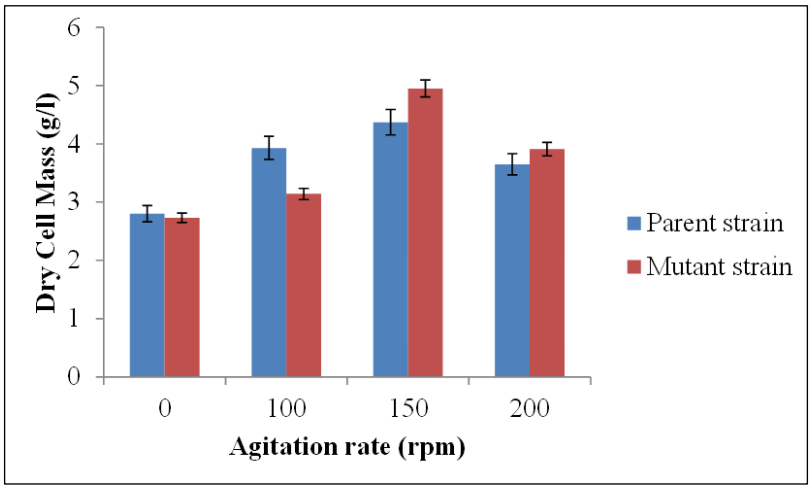

Figure (4b): Effect of Agitation Rate (rpm) on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

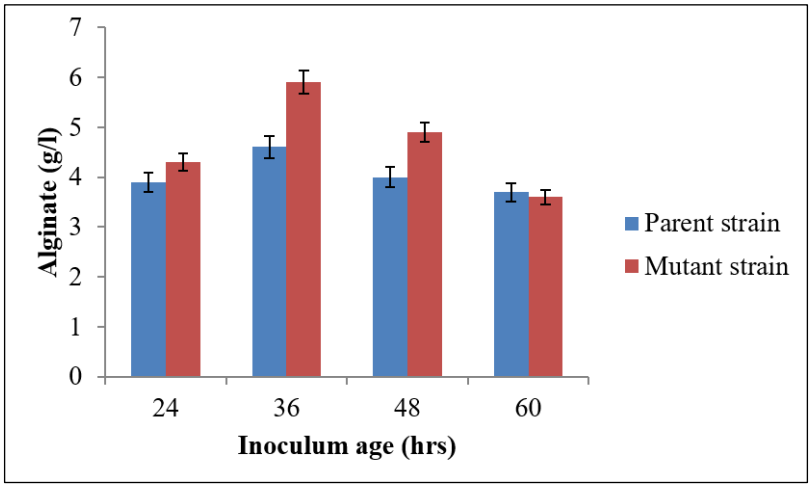

Figure (5a): Effect of Inoculum Age on Production of Alginate (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

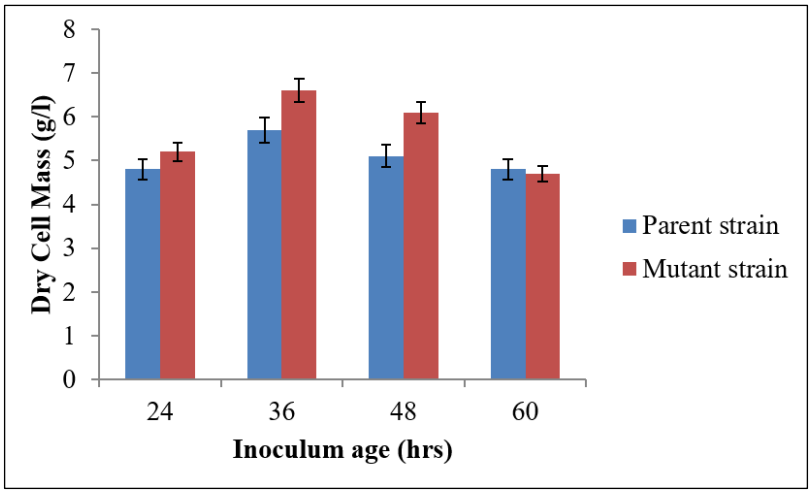

Figure (5b): Effect of Inoculum Age (Hrs) on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

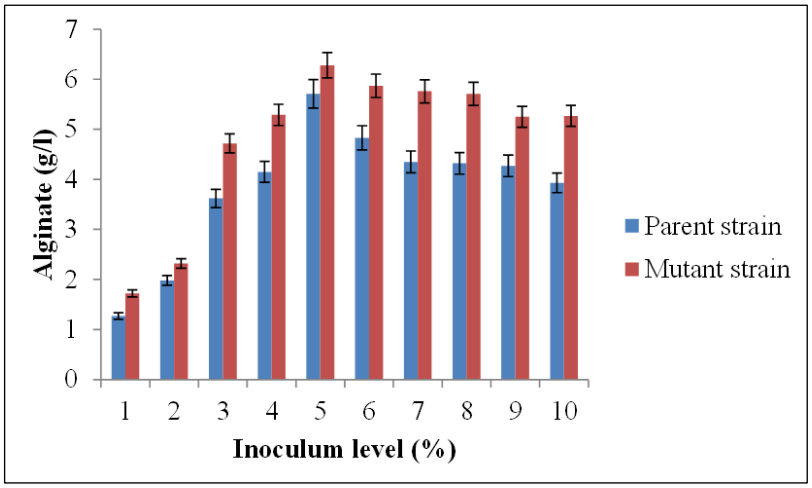

Figure (6a): Effect of Inoculum Level (%) on Production of Alginate (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

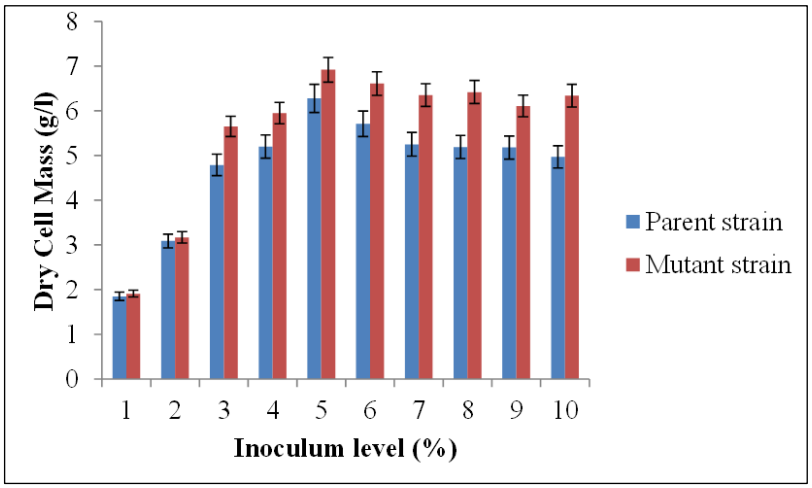

Figure (6b): Effect of Inoculum Level (%) on Dry Cell Mass (g/l). Each Bar Denotes Mean Value Based on Three Replicates and Y Error Bars are for Standard Error of Mean

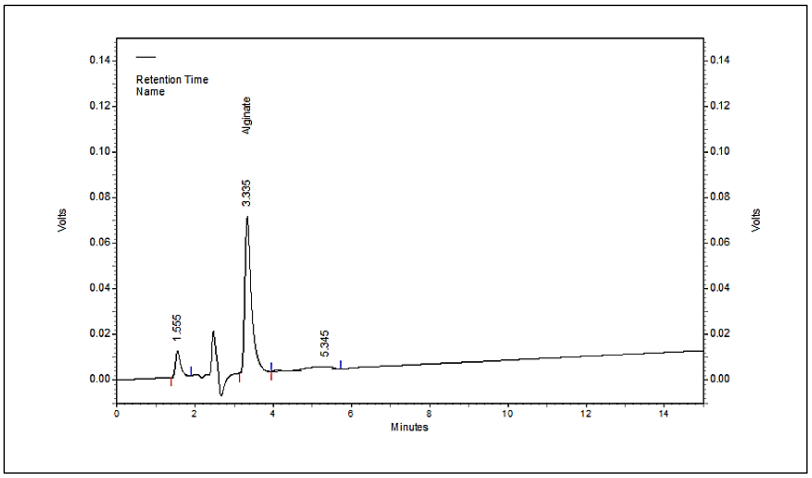

Figure 7: HPLC Chromatogram

Industrial by-products can be utilized for production of alginate (18). In the present study the optimum fermentation temperature 30°C was found to be suitable for maximum alginate production 3.92 g/L (PS) and 3.98g/L (MS) with the corresponding dry cell mass (DCM) as 3.76 g/L (PS) and 3.88 g/L (MS)as shown in Figure 1a and 1b. These results are supported by investigators (12, 13), as they also reported the same optimum incubation temperature for maximum biopolymer production. pH 7.0 was found as optimum with the production of alginate as 4.26 g/L (PS) and 4.92 g/L (MS) with the corresponding dry cell mass (DCM) as 3.59 g/L (PS) and 3.90 g/L (MS) as shown in Figure 2a and 2b. Same pH has been reported as optimum for exo-polysaccharide production by *Azotobacter vinelandii* in various studies (12, 13). As the pH increased beyond the optimum level, it resulted in the reduction of available energy for biopolymer synthesis thus decreasing the yield of alginate (21). The optimization of incubation period for alginate production was investigated. Higher concentration of alginate was observed at 84 hours, 3.92 g/L (PS) and 3.98 g/L (MS) with the corresponding dry cell mass (DCM) as 3.76 g/L (PS) and 3.88 g/L (MS) as shown in Figure 3a and 3b. With further increase in time, reduction in product formation was observed due to exhaustion of nutrients. Optimum agitation rate was at 150 rpm, with quantity of alginate 5.09 g/L (PS) and 5.45 g/L (MS) with the corresponding dry cell mass (DCM) as 4.37 g/L (PS) and 4.95 g/L (MS)(Figure 4a and 4b). These results gets correlated with the observations of other researchers (12, 22), they observed increased alginate synthesis at 200 rpm in shake flask studies. The suitable inoculum age was found to be 36 hours with quantity of alginate as 4.6 g/L (PS) and 5.9 g/L (MS) with the corresponding dry cell mass (DCM) as 5.7 g/L (PS) and 6.6 g/L (MS) (Figure 5a and 5b). In the present study, medium containing molasses (2%) and peptone (0.5%) under various optimized parameters and the inoculum size 5% was found to give significantly ($P \leq 0.05$) higher yield of alginate 5.71 g/L (PS) and 6.28 g/L (MS) with the corresponding dry cell mass (DCM) as 6.28 g/L (PS) and 6.92 g/L (MS) as shown in Figure 6a and 6b. 2% inoculum size was reported to be optimum (13). Another researcher reported maximum yield with carbon sources sucrose (1%) and beet molasses (2%) (22). *A. vinelandii* can efficiently utilize molasses as the substrate and release the product in shorter time duration. Zone of hydrolysis by plate assay for alginate lyase activity was found to be 14 mm and 11 mm for the parent strain and the improved strain respectively, which contributes in enhanced yield of the product (23-25). The quantitative estimation of alginate was done by HPLC using sodium alginate standard of Sigma – Aldrich. The HPLC chromatogram indicated the peak of purified alginate at retention time of 3.335 minutes with percentage purity of 81.22% as compared to standard as shown in Figure 7. Confirmation by HPLC is one of the best method and the results of extracted alginate in comparison to the standard seaweed alginate are in accordance with other investigators (26-28).

Conclusion

In this research work pre-treated sugar cane molasses as a cheap carbon source was used which can reduce the cost of biosynthesis of alginate and can be economically feasible. Organic nitrogen source like peptone favors the growth rate of the

microorganism and the yield of the product. The optimized parameters contributes significantly in the yield obtained. Screening for alginate lyases during fermentation must be an essential step in such research work, either a plate assay or spectrophotometric assay of enzymatic reaction. Purification of the product at the onset of secretion of enzymes or inhibition of alginate lyases is highly recommended. It is suggested to monitor DOT as it has been reported by the researchers to be contributing in obtaining the maximum yield of alginate with high molecular weight.

Abbreviations

PS: Parent strain, MS: Mutant strain, DCM: Dry Cell Mass, DOT: Dissolved Oxygen Tension, HPLC: High Performance Liquid chromatography, *A. vinelandii*: *Azotobacter vinelandii*.

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Author Contributions

Zarina Shaikh: Conceptualized the study, collected the literature and methodology, performed the experiments, and wrote the manuscript, Mohd. Shakir: provided valuable guidance and edited the manuscript.

Conflict of Interest

The authors hereby declare no conflicts of interest regarding the publication of the manuscript.

Ethics Approval

Not applicable.

Funding

Not applicable.

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