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OPTIMIZATION OF BASAL MEDIUM, CARBON AND NITROGEN SOURCES FOR ALGINATE BIOSYNTHESIS BY A VINELANDII

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ABSTRACT

Alginate is a product obtained conventionally from brown algae growing in marine waters. It is having increased market requirement, hence the most sought after polysaccharide due to its widespread applications. It can be synthesized from microbes. This study focuses on optimization of basal medium, carbon and nitrogen sources at flask level. Maximum alginate yield was obtained in Burk's medium 2 was 15.4 % (A vinelandii PS), pre-treated sugar cane molasses and peptone were found to be good carbon and nitrogen sources respectively. Alginate yield in pre-treated sugar cane molasses was 18.57 % (A vinelandii PS) and 23.25 % (A vinelandii MS). The yield with peptone as nitrogen source was 19.42 % (A vinelandii PS) and 22.94 % (A vinelandii MS). The data was statistically analyzed by one way t-test and paired t-test and was found to be significant with $P<0.05$.

INTRODUCTION

Alginate is an anionic linear polysaccharide made up of ß-D-mannuronic acid and its C-5-epimer α-L-guluronic acid linked by ß-1,4 glycosidic bond. It can be produced by microbes such as Azotobacter species and Pseudomonas species (Usov et al., 1995; Jain & Ohman., 2005; Gimmestad et al., 2009). Biosynthesis of this polysaccharide in bacteria is under a stringent regulatory mechanism (Maunders & Welch., 2017; Wood & Ohman., 2012; Utruvia et al., 2017). O-acetylation at O-2/O- 3 position of mannuronate residues differentiates bacterial alginates from seaweed alginates (Baker et al., 2014; Skjåk-Bræk et al., 1989a). M and G residues are present as homopolymers or as randomly alternating units called as blocks. Block type and molecular weight influences gel forming ability and viscosity. Variable three-dimensional structures and its arrangement in the units affects the properties of alginate (Wong et al., 2000). G blocks form a "buckled" chain conformation and chelate divalent metal ions. This leads to the formation of strong but brittle gels, thus reducing the flexibility with the increasing G content (Skjåk-Bræk et al.,1989b). Acetyl groups affects its ion-binding and water-binding properties (Donati et al., 2015; Braccini & Pérez., 2001). Alginate formation was partially growth-associated, its synthesis occurs after the end of growth. Higher concentration of alginate yield was obtained when dissolved oxygen was not controlled (Parente et al., 2000; Deavin et al., 1977). Bacterial alginate are reported to posses more pseudoplasticity than algal, other parameters like gel formation capacity, thermostability, effect of pH, temperature and NaCl on viscosity were significantly similar. Alginates conjugated with fire-retardent polymers are being explored as a new generation flame retardant material(Kabir et al.,2021). Alginate production using glucose as the carbon source by Azotobacter vinelandii DSM 576 was optimized with respect of agitation speed, C/N ratio, sodium phosphate and acetate in a buffered medium, glucose concentration and temperature (Clementi et al., 1995). When bacterial alginate was added to Cu2+ and Zn2+ ions, it forms gel structures.A novel 6A1 strain of P. mandelii isolated from antarctic increases biofilm formation due to alginate overproduction at low temperatures. This might be the result of down regulation of MucA (alginate operon repressor) (Clementi F 1997). Bacterial alginate production process is costly due to the carbon source used. Molasses, a byproduct of sugar manufacturing can be used as a cheap carbon source. Azotobacter vinelandii ATCC 9046 strain was inoculated into the modified Burk's medium (Changing sugar source) for alginate production. To make the bacterial alginate cost-effective use of molasses resulted in high yield than maltose (Vásquez-Ponce et al., 2017). A higher conversion of sucrose to alginate and alginate-specific production rates were obtained under diazotrophy (nitrogen-fixing condition). Under diazotrophic conditions the specific productivity was 0.24 ± 0.03 g g^{-1} h^{-1}. A higher alginate molecular weight (725 ± 20 kDa) was produced under diazotrophic conditions (Contreras-Abara et al., 2023). Varying the source of peptone could alter the yield upto 30 % (Brivonese & Sutherland., 1989). Beef extract, casein, malt extract. peptone (Butt & Qadeer., 2014), whey, yeast extract and ammonium nitrate (Khanafari & Sepahei., 2007), corn steep liquor and yeast extract (Galal & Ouda., 2014; Saeed et al., 2016). In the present study the appropriate basal medium was selected for further optimization of carbon and nitrogen sources.

MATERIALS AND METHODS

1. Cultivation and maintenance of microorganisms: The parent strain of the Azotobacter vinelandii was isolated from the soil. The organism was revived and maintained on Burk's Nitrogen free agar medium slants (Butt & Qadeer., 2011).

2.Selection of the basal medium: The culture was grown on various medium for a period of 72 hours by adjusting the initial pH of the medium to 7.0, maintaining temperature 30ºC with the inoculum percentage and age as 10% and 48 hrs. Burk's Medium 1 (10 g glucose, 0.41 g KH_2PO_4, 0.52 g K_2HPO_4, 0.05 g Na_2SO_4, 0.2 g $CaCl_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.0025 g $Na_2MoO_4 \cdot 2H_2O$, 2.0 g agar) (Wilson & Knight., 1952; Park et al., 2005). The pH of the medium was adjusted to 7.0 ± 0.1 before autoclaving at 121ºC for 15 min. Burk's medium 2 in g/l (NCIM Catalogue for media formulations). Nitrogen free medium of Norris & Jensen, Nitrogen free medium containing sucrose.

3.Optimization of carbon and nitrogen sources for maximum production of alginate

Processing of sugarcane molasses

Sugarcane molasses was diluted with equal quantity of double distilled water and kept at 40ºC for 5 hrs. Centrifugation was done at 2000 rpm for 5 min and the supernatant was collected. Furhter the pH of the supernatant was adjusted to 7.0 with 0.1 N NaOH. 2% of tri-calcium phosphate was added to the solution and autoclaved at 105oC for 5 min. It was cooled down and centrifuged at 3000 rpm for 15 min (Kundu et al., 1984). The solution was passed through activated charcoal for decolourisation and adsorption of heavy metals. Further the pH of the liquor was adjusted to 2.0 by 0.1 N HCl and kept for 6 hours. Centrifugation was done at 3000 rpm for 20 min and the supernatant was used for preparing the fermentation medium by adjusting the required pH 7.0±0.2.

Processing of potato starch

Starch hydrolysis was done enzymatically as described by (Akerberg et al., 2000). To 450 gms of the potato pulp, 100 ml of

Optimization of carbon source

Burk's medium 2 was added with various carbon sources like potato starch, molasses, sucrose, glucose, lactose, maltose. Production was carried out under optimized parameters (Temperature 30 ºC, pH 7.0, incubation period 72 hours, agitation rate 150 rpm, inoculum age 36 hours, inoculum level 5%). Dry cell mass (DCM), viscosity, residual sugar and alginate yieldwas determined.

Optimization of nitrogen source

Burk's medium 2 was added with various nitrogen sources like ammonium sulphate, peptone, tryptone, yeast extract. Production was carried out under optimized parameters.

Viscosity - Measurements were done by using Brook'sfield viscometer (Devina et al., 2018).Determination of DCM - 10 ml of fermented broth after 72 hrs of incubation period was centrifuged at 8,000 rpm for 20 minutes. The pellet obtained was washed thrice with double distilled water and dried in the hot air oven at 50ºC. After complete drying the weight was measured (Hacking et al., 1983).Residual sugar estimation was done by the method described by (Miller, G.L., 1959).Alginate precipitation was obtained by treating with fermented broth as per the procedure described (Hacking et al., 1983; Van Der Berg et al., 1995).Alginate yield (%) was calculated by the formula. YA/S=Amount of product formed(g)/Amount of substrate utilized in grams x 100 (Permatasari et al., 2022).

Statistical analysis: All the experiments were performed in triplicates. Statistical analysis was done by t-test.

RESULTS AND DISCUSSION

1. Selection of basal medium for alginate production

Production of alginate in various basal medium incorporated with sucrose as carbon source was studied. Maximum yield of alginate (16 %), dry cell mass (3.25 g/l), residual sugar (9.45 %) and high viscosity (90 cps) was observed with Burk's medium 2 as shown in figure 1a and 1b. One sample t-test revealed that resultsfor the yield of alginate is significant (P<0.05).

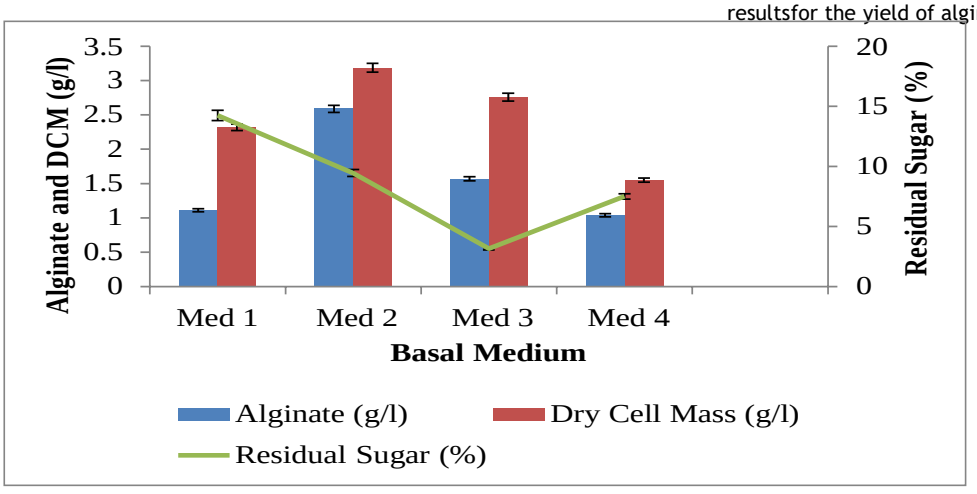
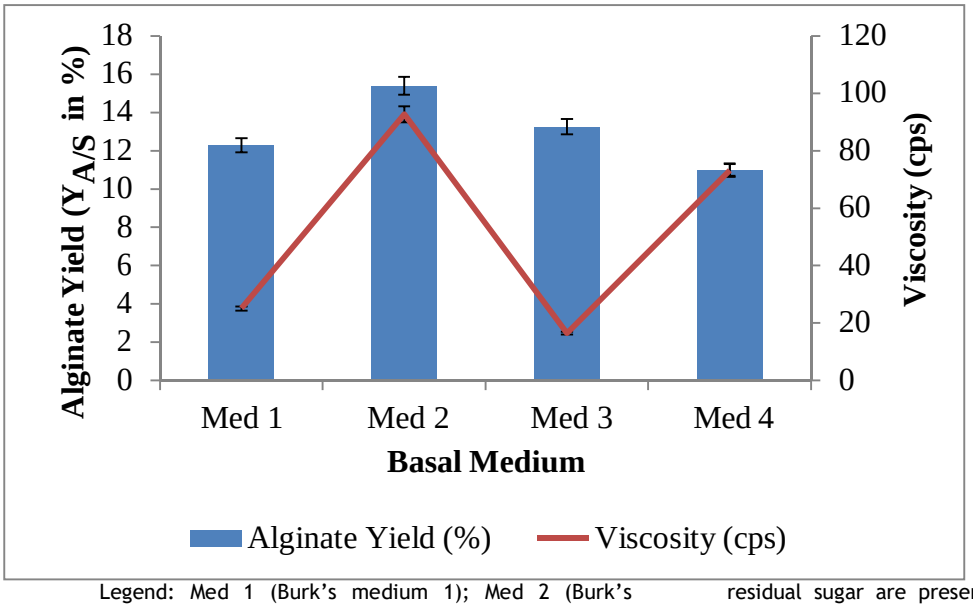


Figure 1a: Determination of Alginate (g/l), Dry Cell Mass (g/l) and Residual Sugar (%) in various basal medium.



Legend: Med 1 (Burk's medium 1); Med 2 (Burk's medium 2); Med 3 (Nitrogen-free medium of Norris & Jensen); Med 4 (Nitrogen-free sucrose medium).

Figure 1b: Measurement of Alginate Yield (%) and Viscosity (cps) invarious basal medium.

2. Effect of Carbon sources

Carbon sources usually affects the composition of the monomers or the molecular weight of exopolysaccharide production (Wachenheim et al., 1992). Glucose, sucrose, maltose, sugar cane molasses and hydrolyzed potato starch were used for studying the optimal carbon source. The effect of carbon source on alginate production,viscosity,dry cell mass (DCM) and residual sugar are presented in Figure 2a and 2b. As per the performed paired t-test significant ($P<0.05$) results were obtained for PS and MS strains i.e. 18.57 % and 23.25 % respectively. Viscosity, dry cell mass, reducing sugar concentration for PS was 116.9 cps, 4.86 g/l, 2.31 % and MS was 117.2 %, 4.35 g/l, 3.15 % respectively. Pre-treatment of molasses is essential as it removes toxic metal ions and thus contributes to maximum yield. The data for effects of carbon sources (2%) on alginate production showed that molasses gave significantly high production as compared to the other carbon sources (Prompahagorn A et al., 2004). Maximum yield with wheat bran was reported by investigators (Saeed et al., 2016).

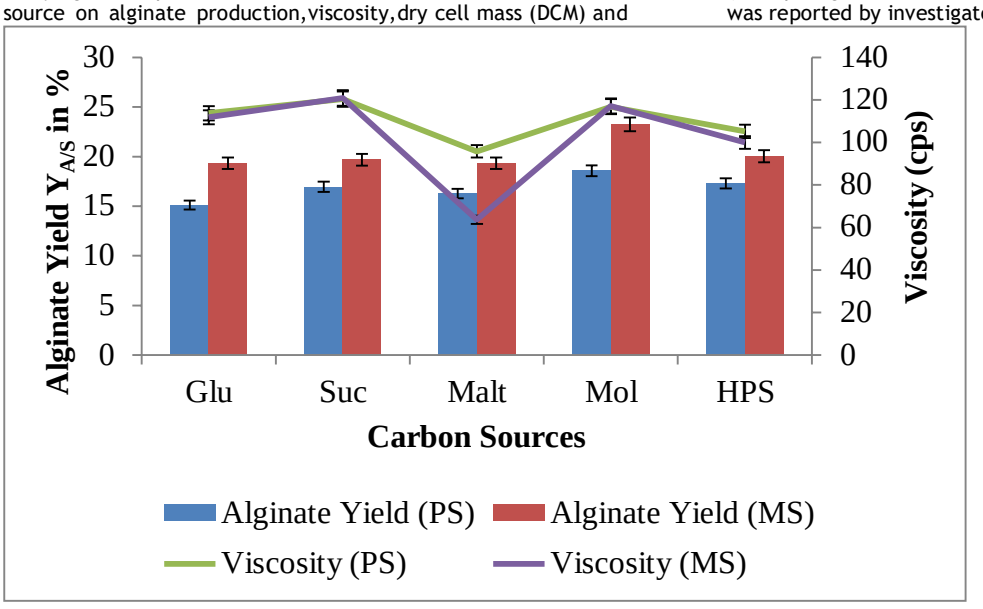
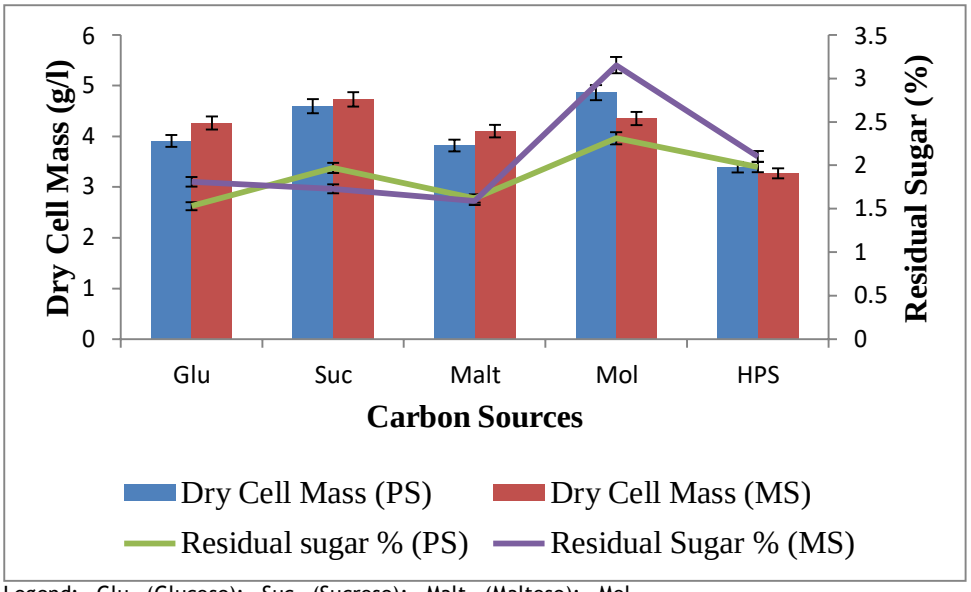


Figure 2b : Determination of Alginate Yield (%) and viscosity (cps) in various carbon sources.



Legend: Glu (Glucose); Suc (Sucrose); Malt (Maltose); Mol (Molasses); HPS (Hydrolyzed potato starch); PS (Parent Strain); MS (Mutant Strain).

Figure 2b: Determination of Dry cell mass (g/l) and viscosity (%) invarious carbon sources.

3. Effect of Nitrogen sources

The effect of various nitrogen sources, organic or inorganic (Ammonium sulphate, Yeast Extract, Peptone, Tryptone) were examined. Fig. 5 and 6 demonstrated that the incorporation of nitrogenous compounds into medium affected the alginate yield and viscosity. Maximum yield of alginate was obtained with peptone as nitrogenous source with 19.42 % and 22.49 % for PS and MS strains respectively and the results were found to be significant ($P<0.05$) by the performed t-test. Viscosity, Dry cell mass, residual sugar concentration for the PS strain was 109.5 cps, 4.77 g/l, 9.65 % and for MS strain was 110.2 cps, 4.16 g/l, 8.9%respectively.Maximum yield was reported with yeast extract, corn steep liquor (Ali et al.,2005; Galal & Ouda.,2014) peptone (Butt et al., 2011). Ammonium sulphate was found to be a least contributor as in the literature it is mentioned that inhibition of nitrogenase activity occurs by addition of NH^{4+} in whole cells of *Azotobacter* species (Hardy et al., 1968).

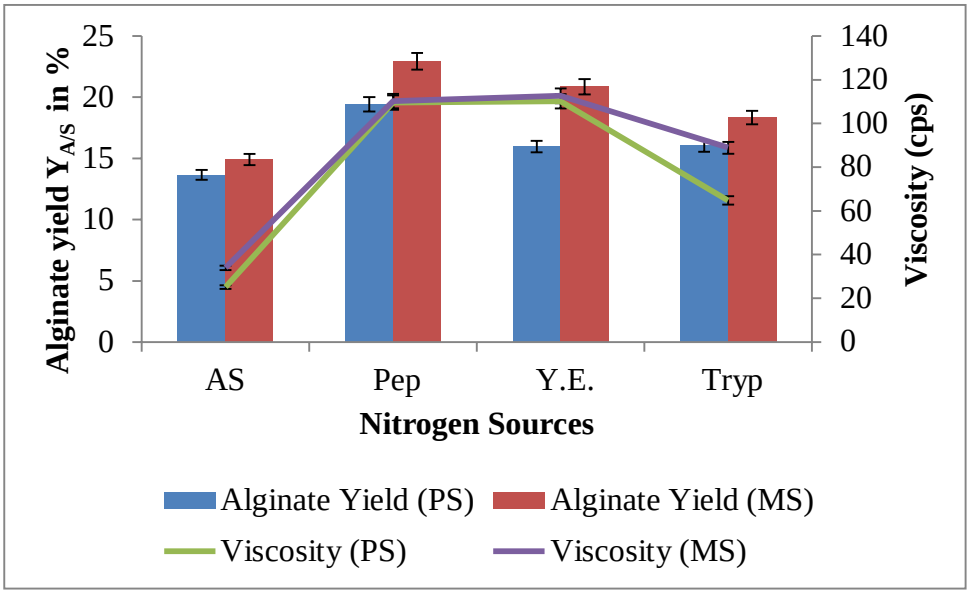
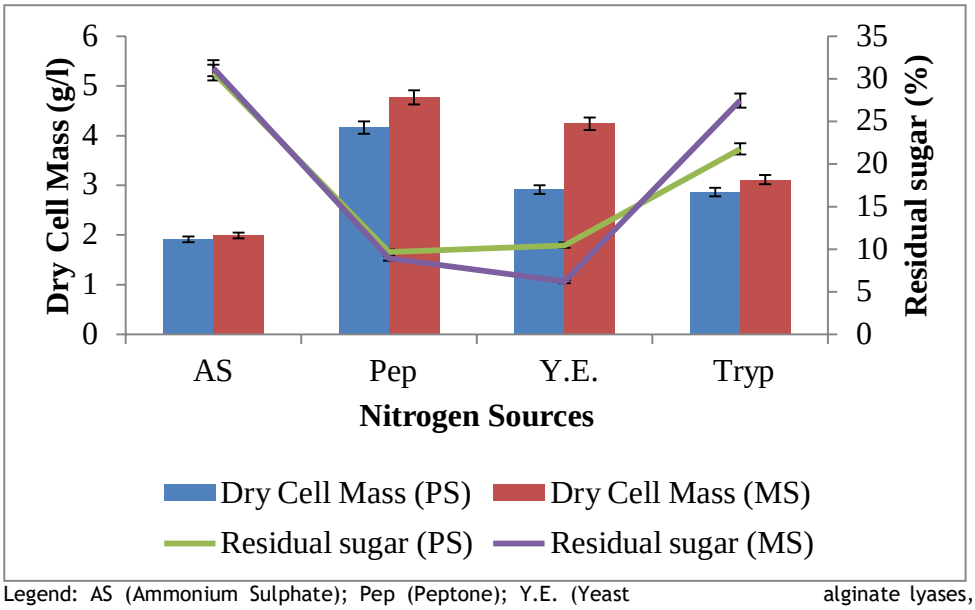


Figure 3a: Determination of Alginate (%) and Viscosity (cps) in various nitrogen sources.



Legend: AS (Ammonium Sulphate); Pep (Peptone); Y.E. (Yeast Extract); Tryp (Tryptone)

Figure 3b: Determination of Dry cell mass (g/l) and viscosity (%) invarious nitrogen sources.

CONCLUSION

Burk's medium 2 was found to be suitable for alginate production by this strain which indicates that the proportion of components was satisfactory enough to contribute to the enhanced yield. Sugar cane molasses being a cheap carbon source is suitable for the production and gave maximum yield which is found to be statistically significant. But it needs pre-treatment for the removal of toxic components as well as the colour, clear solution is preferred as it will not impart any colour to the end product. Treated cane molasses contains less concentration of sugars as compared to the crude, hence proper adjustment of sugar concentration is essential during medium formulation. Peptone was found to be significantly contributing in the yield. In the study of effect of carbon and nitrogen sources both parent and mutant strains were used in which mutant was found to be giving more yield.

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AUTHOR CONTRIBUTIONS

Zarina Shaikh conceptualized the study, collected the literature and methodology, performed the experiments, 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

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