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EXTRACTION OF ALGINATE BIOPOLYMER FROM ISOLATED *AZOTOBACTER* SPECIES AND ITS APPLICATIONS

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ABSTRACT

Alginate is a biopolymer and composed of (1-4)-β-D mannuronic acid and (1-4)-α-L guluronic acid. Currently, commercial alginate, used for most applications, from marine algae through extraction process. In this work, we have isolated *Azotobacter* species for the extraction of the biopolymer-Alginate. Four different soil samples from various locations in Kalyan were used for the isolation of *Azotobacter* species. Four different isolates were obtained from four samples. Their Gram nature and biochemical characteristics were studied and they were identified as *Azotobacter* species. The biopolymer was extracted using centrifugation method by scraping out the growth of bacteria. Optimization of growth conditions for *Azotobacter* species was studied. The effect of time, temperature, pH, NaCl, carbon source and nitrogen source on the biopolymer production was observed. A sugarcane molasses fermentation media was designed to check for the production of biopolymer using isolated strains. Biopolymer alginate is used in various industrial applications because of its unique properties that include gelling, non-toxicity, biocompatibility, biodegradability, good stabilizer, thickening agent. The alginate produced was used for the production of fruit jam and lip balm.

KEYWORDS: *Azotobacter* isolates, Biopolymer-Alginate, Centrifugation, Fruit jam, Lip balm.

1. INTRODUCTION

The word alginate is derived from alginic acid or algin, they are extracellular polymer that either occurs naturally in certain brown seaweeds, known as alginophyter or is produced from the natural derivatives. Commercially alginates are extracted from brown seaweeds such as species of *Ascophyllum, Laminaria, Lessonia, Macrocystis, Sargassum*, and *Turbinaria* in the

form of calcium, sodium and magnesium salts of alginic acid. Two bacterial species have been shown to secrete alginate, *Pseudomonas* and *Azotobacter*. *Azotobacter* is a nonpathogenic and nitrogen fixing bacteria. Although seaweeds are the significant source of alginate, however, the bacterial alginate is considered of good quality than of the algal alginate. The bacterial alginate has the higher selling price due to its unique properties. The use of *Azotobacter* bacteria for alginate biosynthesis is more advantageous than the work with *Pseudomonas* bacteria. There are physical and chemical parameters used that affects bacterial growth and extraction of polymers. It shows that the amount of alginate decreases by the addition of phosphates, on the other hand addition of nitrogen sources increases synthesis. Alginates have many applications such as food additives, pharmaceuticals, cosmetics, textile printing, dental Impression, wound dressing, welding rods, animal feed, ceramic and paper industry.[1,2,3] In this work we have isolated *Azotobacter* from soil samples and extracted the alginate. The alginate obtained was used for various applications.

2. MATERIALS AND METHODS

All the chemicals and media were prepared in distilled water.

Collection of soil Sampling, Enrichment and Bacterial Isolation

Different soil samples were collected from different areas such as - Garden soil of B.K.Birla college (Soil sample1), - Residential soil (society) (Soil sample 2), Petrol pump (Kalyan) (Soil sample 3), KDMC Dumping ground (Kalyan) (Soil sample 4). One gm of the four different soil samples were individually inoculated in 100ml of sterile Ashby's Mannitol Broth (Mannitol, 10.0 g.; K_2HPO_4, 0.5 g.; $MgSO_4$. $7H_2O$, 0.2 g.; NaC1, 0.2 g.; $MnSO_4$, trace; $FeCl_3$, trace; distilled water, 1000 ml.) And incubated for 72-96 hours at room temperature. A loopful culture of each soil sample were streaked using 5-sided streaking technique on Ashby's mannitol agar plate and they were incubated at room temperature for 72hour.[4]

Identification of the bacterial isolates

Colony characters of the organisms were recorded by observing the colonies grown on sterile Nutrient agar plates. Gram staining was carried out to determine the Gram nature of the organisms. Biochemical tests were performed to identify the organisms. The tests included:

a) IMViC (Indole, Methyl Red, Vogues Proskeur, Citrate utilization)

b) Sugar fermentation test

c) Triple Sugar Iron agar test (TSI)
d) Catalase test
e) Motility test.

The purified isolates were maintained on nutrient agar slants and kept at 4°C. They were subcultured after every 2 weeks for maintenance.

Extraction of Biopolymer

Azotobacter culture was grown in 100ml of Ashby's Mannitol Broth. The culture was streaked on Ashby's Mannitol Agar plate and incubated at room temperature for 48 hrs. After incubation, growth was scraped out, and it was suspended in 2ml saline, centrifuged at 3000rpm for 5 mins. Supernatant was collected and 3 drops formalin was added in to the supernatant. 2 volumes of chilled ethanol were added in to the supernatant. Tubes were kept at RT for 30 mins. All centrifugation tubes kept for centrifugation at 3000 rpm for 10 mins. Supernatant was discarded and ethanol was evaporated in pellet. Pellets was dried and it was weighed on weighing machine.[5]

Weight of Biopolymer (Dry pellet of alginate)

Pellet was dried in hot air oven at 50℃. After drying, dry pellet containing alginate was weighed on weighing machine.[6]

Qualitative analysis of carbohydrate by Molisch's Test

Alginate is a naturally occurring polysaccharides. So, they are polymeric carbohydrates. To detect the presence of carbohydrates in Alginate; Molisch's test was performed. Carbohydrates when reacted with conc. H_2SO_4 gets dehydrated to form furfural these reacts with sulphonated α- naphthol to give a violet reddish ring colored complex.[7]

Effect of various physiological and chemical parameters on the growth of the bacterial isolates and amount of biopolymer produced

Various physical and chemical parameters were studied for Optimization of growth conditions for bacteria to extract alginate. The growth of bacteria investigated using different parameters such as time, temperature, pH, salt concentrations and substrate variation.[3]

Time

24-hour old culture suspensions of the bacterial isolates were grown on sterile Nutrient agar

slants and incubated for various time durations such as 2 weeks, 1 week, and 72 hours. The growth of the bacteria was checked and the amount of biopolymer produced was determined.

Temperature

24-hour old culture suspensions of the bacterial isolates were grown on sterile Nutrient agar slants at various temperatures, 10˚C, 28±2˚C, and 37˚℃. The inoculated tubes were kept at the respective temperatures for 24 hours. The growth of the bacteria was checked and the amount of biopolymer produced was determined.

pH

24-hour old culture suspensions of the bacterial isolates were grown on sterile Nutrient agar slants of various pH (5.0, 7.0, 9.0 and 12.0). The inoculated tubes were incubated at 28±2˚C for 24 hours. The pH containing nutrient broths were adjusted using 1 N NaOH and 1 N HCl according to their acidic and basic conditions. The growth of the bacteria was checked and the amount of biopolymer produced was determined.

Salt concentrations

24-hour old culture suspensions of the bacterial isolates were grown on sterile Nutrient agar slants with different salt (NaCl) concentration 1.5%, 2.5%, 3.5%, and 4.5%. The tubes were incubated at 28 ± 2 ˚C for 24 hours. The growth of the bacteria was checked and the amount of biopolymer produced was determined.

Various Substrates

Yeast extract is vitamins, minerals and digested nucleic acids source. 0.6% of Yeast Extract powder was added with nutrient agar. 24-hour old culture suspensions of the bacterial isolates were grown on sterile yeast extract agar slants. The tubes were incubated at 28±2℃ for 24 hours. The growth of the bacteria was checked and the amount of biopolymer produced was determined.

Malt extract is carbon, nutrient and protein source. 0.6% of Malt extract powder was added with nutrient agar. 24-hour old culture suspensions of the bacterial isolates were grown on sterile malt extract agar slants. The tubes were incubated at 28±2℃ for 24 hours.

Ammonium sulfate is a nitrogen source. 0.6% of Ammonium sulfate was added with nutrient agar. 24-hour old culture suspensions of the bacterial isolates were grown on sterile ammonium sulfate agar slants. The tubes were incubated at 28±2℃ for 24 hours.

Biopolymer production using Molasses

Molasses, a byproduct of sugar manufacturing, an example of a cheaper source. Molasses contains mostly sugars like sucrose, glucose, and fructose comprising up to 50%. It was investigated as a carbon source for biopolymer production On the other hand, alginate production from molasses was comparably rare.[8]

3 gm of sugarcane bagasse added to 200 ml of empty conical flask. 100 ml of distilled water + 0.6 % $(NH_4)_2SO_4$was added in this flask. Solution was heated for10 mins. It was cooled and filtered through muslin cloth. 2 loopful bacterial isolates inoculated and incubated at 28±2 for 1week. After every alternate day, 20ml of aliquots were removed. The growth was suspended in saline, centrifuged at 3000 rpm for 15 mins. Alginate pellet was formed and weighed. Alginate extract was used for carbohydrate test by Molisch reagent.

1. APPLICATIONS

Fruit jam

Jam was produced according to the traditional method using orange as a raw material. The formula consisted of orange fruit pulp, lemon juice and sugar were used for preservation purposes. Alginate was used for thickening agent and this was extracted from MB 3 isolate.[9] In a small beaker 20ml of prepared Orange fruit pulp was added. This beaker was kept in the boiling water bath. On medium heat temperature 4ml of alginate was added in pulp, mixed well and after continuous stirring, pulp was thickened. 3gm sugar was added and the mixture was cooked under continuous stirring for 25-30 mins. 1teaspoon of lemon juice was added, mixed well. After cooling mixture was stored in refrigerator.

Lip balm

Easy homemade lip balm was prepared by the simple method using beeswax and various essential oils. Alginate was used as a thickening agent and that was extracted from MB 3 isolate.[10] 3gm of Beeswax + coconut oil was taken in a small beaker and placed in the boiling water over medium heat for 5-7 minutes. After stirring for 6 mins, 2ml of alginate was added, the heat was reduced to low temperature for stirring. 1 vitamin E capsule and 1 teaspoon of Vaseline were added, it was mixed until the oils combined with beeswax. Beaker was removed and 1-2 drops of essential oil was added immediately. Then it was cooled. It was poured into desired container and left out at Room temperature.

3. RESULTS AND DISCUSSION

Four *Azotobacter* stains were isolated from the obtained soil samples and they were designated as MB1, MB2, MB3, and MB4 (Fig 1). Colony characters of the isolated bacteria were determined and it was found that all species showed similarity in consistency and color of colony. Isolates were reported evenly in color and flowing consistency mucoid, viscous, gummy with dull to cream white as described by Upadhyay et al.[11] The isolates were able to grow in nutrient broth/agar. The Gram nature of the isolate was found to be Gram negative in nature. The isolates were subjected to various biochemical tests and were identified according to Bergey's manual of Systematic Bacteriology (Volume I and II).

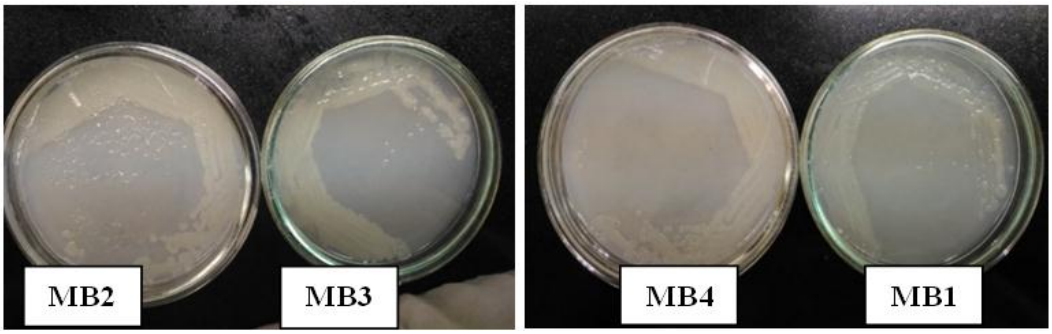

Figure 1: Pure bacterial isolates obtained after enrichment from.

MB2- Residential area MB3- Petrol pump

MB4- Dumping area MB1- College garden

Extraction of Biopolymer

The extraction of the biopolymer was carried out and the alginate obtained was dried and weighed. The color of liquid alginate was off white. The consistency was sticky in nature (Fig 2). After drying the liquid alginate, dry alginate powder was obtained. The texture of dry alginate was smooth and lustrous (Fig 3). The detection of the extracted biopolymer was carried out using Molish test which shows a positive test as a reddish-purple ring at the interface (Fig 4).

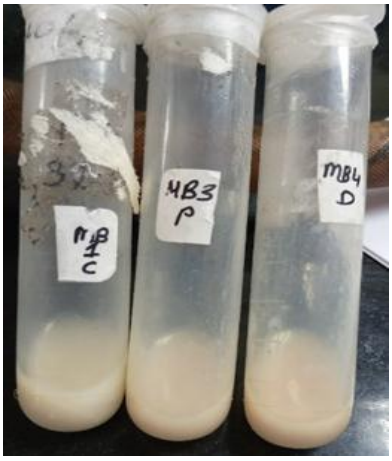

Figure 2: Alginate extract. **Figure 3: Dry alginate powder.**

Figure 4: Molish test.

Effect of various physiological and chemical parameters on the growth of the bacterial isolates and alginate production

The weight of Alginate obtained from the isolated *Azotobacter* strains was measured in gm/ml for each analysis.

Effect of Time

As the time of incubation increased, MB1 and MB3 isolates were capable of producing more amount of alginate. Incubation period is directly proportional to the growth of bacteria up to a certain extent and after that growth of bacteria start decreasing which can be attributed to the decrease in the supply of nutrients to microorganisms or may be accumulation of some toxic compounds in the broth.[3] It was seen in some isolates that the amount of alginate increased after 1st week of incubation but decreased when the incubation period was extended upto 2 weeks. This may be due to the nutrient getting exhausted (Fig 5). Isolate MB3 showed maximum production after one week *i.e.* 0.67 gms/ml.

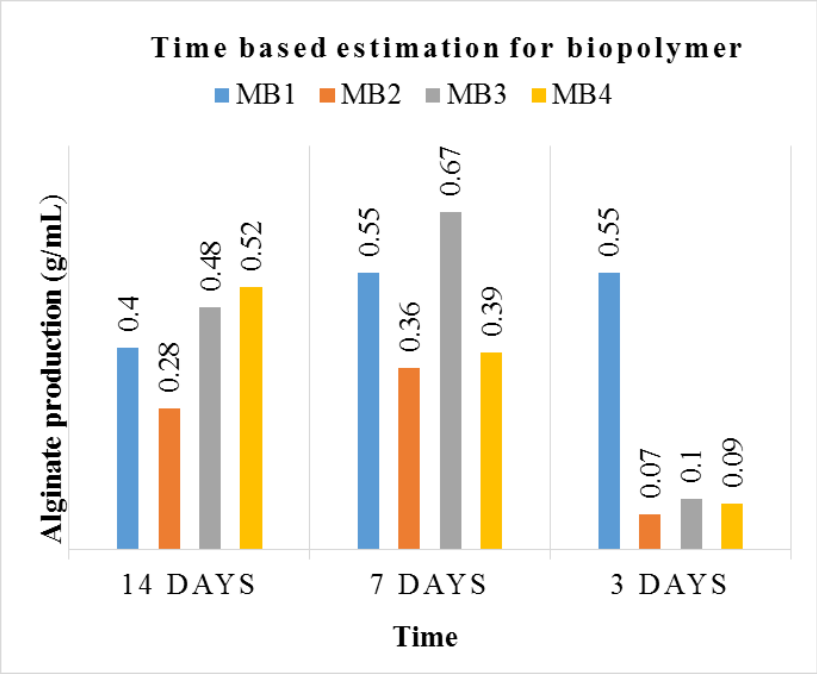

Figure 5: Effect of Time on Alginate production.

Effect of Temperature

Optimum temperature for the growth of *Azotobacter* has been reported as 30°C but some species can grow at optimum temperature of 34°C [12]. MB2 and MB3 showed maximum alginate production at 28 ±2°C while MB1 and MB4 showed at 37°C (Fig 6). A little temperature rise increases the growth rate because the velocity of enzyme-catalyzed reaction also increases with it and as the rate of increases; microorganism grows faster.[13]

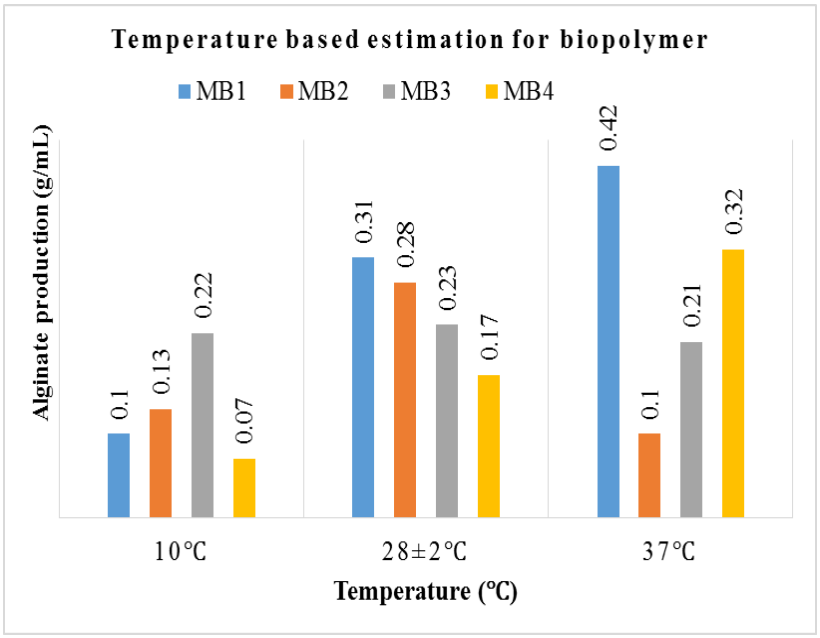

Figure 6: Effect of Temperature on Alginate production.

Effect of pH

Azotobacter grows at a wide range of pH[14] but their growth is more at a neutral pH. It was observed that all the isolates could grown and produce alginate at all the pH values it was subjected to grow (Fig 7).

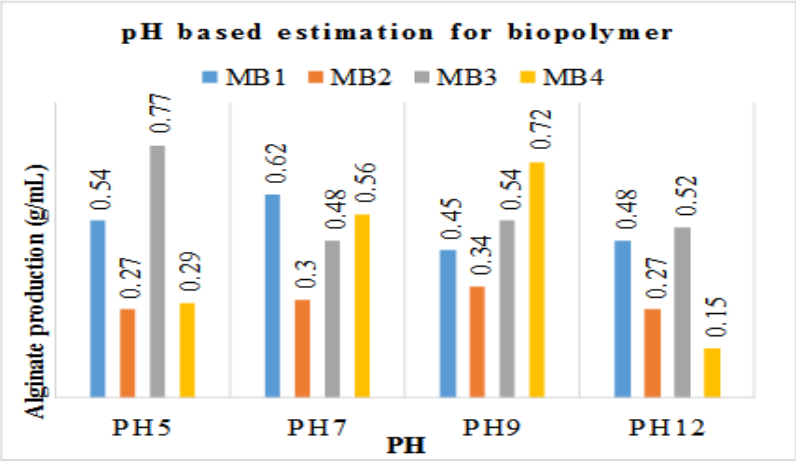

Figure 7: Effect of pH on Alginate production.

Effect of NaCl concentration

MB1 isolates showed maximum amount of alginate production in 1.5% and in 3.5% NaCl concentration. In 2.5% NaCl, MB1 and in 4.5% MB4 isolates gave the maximum amount of alginate (Fig 8).

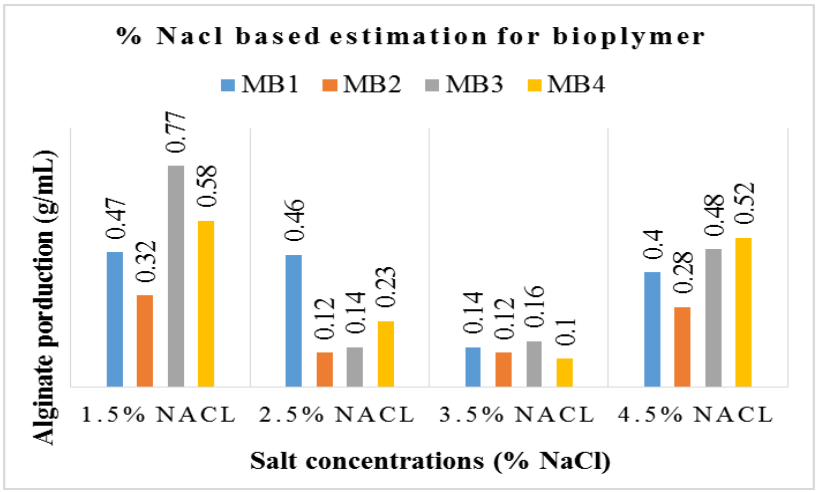

Figure 8: Effect of NaCl concentrations on Alginate production.

Effect of Various Substrate

Carbon is an essential component of growth medium but an excess of carbon source can cause undesirable metabolic waste products to accumulate into the microorganism while

deficiency of carbon source cause lesser growth as well.[13] The alginate obtained after the use of malt extract as a course of carbon was quite less. When yeast extract and ammonium sulfate were used, MB3 showed high amount of alginate production – 0.9 gm and 1.02 gms resp (Fig 9). *A. chroococcum* has all the oxidative enzymes for degrading a great variety of organic carbon compounds *via* the tricarboxylic acid cycle, as well as its alcohol derivates such as mannitol.[15,16] Ammonium sulphate used as best nitrogen source and have reported in the presence of ammonium sulphate, *Azotobacter* growth was increased.[17]

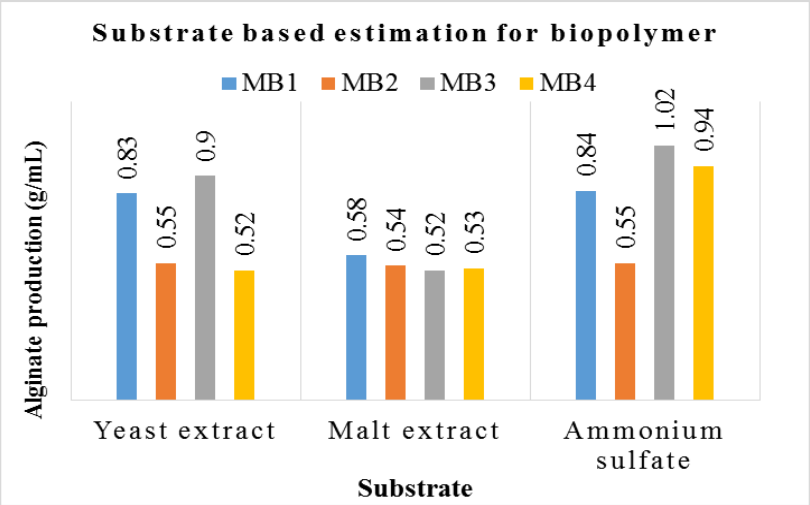

Figure 9: Effect of Substrate on Alginate production.

Biopolymer production using Molasses

Sugar cane molasses was used to design a fermentation media for the low-cost production of alginate (Fig 10). The experiment was set up for a week and the alginate extraction was carried out at specific intervals. On the 6th day, the production of alginate increased (Fig 11). These results corresponded with our earlier finding. Though the production was less, further studies can be carried out to increase the production.

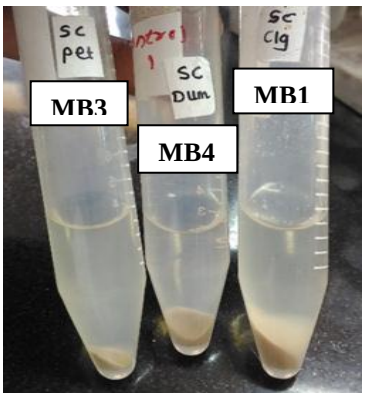

Figure 10: Alginate extraction using sugarcane molasses.

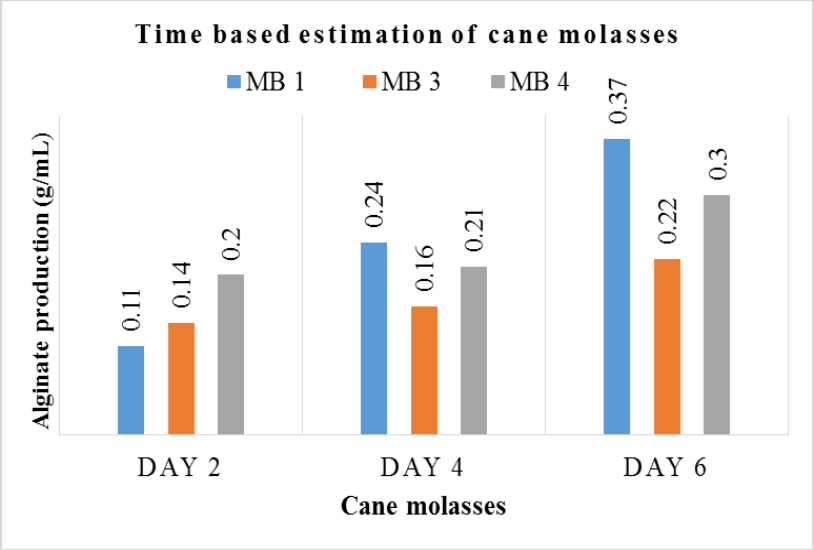

Figure 11: Effect of incubation period using cane molasses for alginate production.

Applications

Alginate is used in cosmetics area with several applications as it functions as a thickener and moisture retainer. Alginate helps to retain the color of lipstick on lip surface by forming gel-network. It is used as a thickening agent for jams and jellies. Fruit jam and a lip balm were made to which the extracted alginate was added. The jam thickened after addition of the alginate and refrigerating it for a few hours. The lip balm also retained the red tint when applied. The balm thickened after the addition of the extracted alginate (Fig 12).

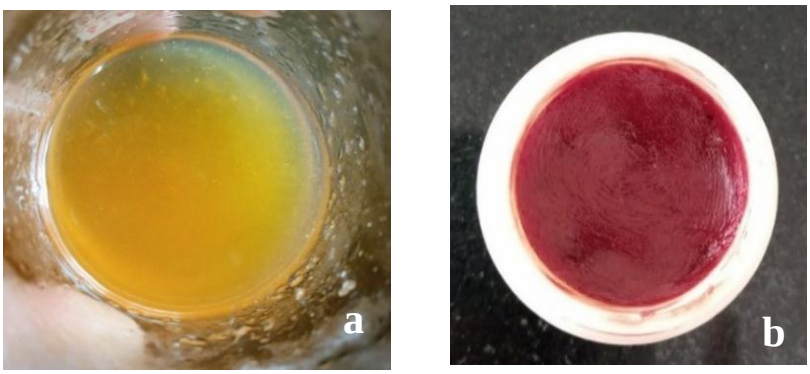

Figure 12: (a) Fruit jam and (b) Lip Balm.

4. CONCLUSION

Four *Azotobacter* species were isolated which were studied for their colony characters and biochemical characters. In order to reduce the cost of bacterial alginate production; molasses, maltose, mannitol was utilized as alternative low-cost carbon sources in this study. It was concluded that, the optimization of cultural and nutrition parameters resulted in enhancement in the growth of *Azotobacter* species and ultimately in the production of alginate. Sugarcane

molasses also can be utilized by the bacteria to produce more amount of biopolymer, but further studies have to be carried out to increase the production of alginate. The molasses can be supplemented with other essential nutrients to increase the production. Furthermore, the Alginate obtained from the *Azotobacter* species was used as a thickening agent in fruit juice and lip balm. Alginate is important in various biotechnological and biomedical applications, e.g. for immobilizing cells in the pharmaceutical or as a stabilizing, thickening and gelling agent in food production.[18] Hence it has a lot of scope for further studies which includes developing the cultures for maximum yield of product under optimized conditions or maintaining conditions to produce, molecular weight and viscosity. Hence its synthesis using cheap substrates and a totally nonpathogenic bacteria would be advantageous to fulfill the demands.

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