PRIMARY ARTICLE

A Study On Secretion, Extraction & Purification Of Extracellular Polysaccharides By *Chlorococcus Spp*.from Coastal Regions Of Gujarat.

S. M. Sharma And H. V. Sukhadia

ABSTRACT

Exopolysaccharides (EPS) are characterized as secondary metabolites being produced predominantly during the stationary growth phase of the microorganism. The capacity of Cyanobacteria to produce extracellular polysaccharides has been related since the beginning of the 1950s. In stress conditions, a lot of Cyanobacteria produce a great amount of EPS, being in that way a metabolic strategy of these microorganisms for growth and development in unfavorable conditions. They have the purpose of protecting them against tensions of extreme habitats and harmful conditions. EPS are compounds whose chemical composition is complex and with peculiar properties. Those biopolymers present inhibition properties against several types of viruses as well as tumors. They also show physical-chemical properties with advantages for industrial use, being utilized in food, textiles, cosmetics and pharmaceutical industries. The full biotechnological use of EPS produced by Cyanobacteria is expected soon. This research field is wide, prosperous and of great scientific and lucrative potential.

KEYWORDS :
Cyanobateria, Chlorococcus.

INTRODUCTION

Cyanobacterial extracellular polymeric substances (EPS) are heteropolysaccharides that possess characteristics suitable for industrial applications, notably a high number of different monomers, strong anionic nature and high hydrophobicity. Chlorococcus spp. was used as model organism. Our results revealed that this strain is among the most efficient EPS producers, and that the amount of RPS (released polysaccharides) is mainly related to the number of cells, rather than to the amount produced by each cell. Light was the key parameter, with high light intensity enhancing significantly RPS production, especially in the presence of combined nitrogen!

Chlorococcus spp., *Anabaena spp., Gleocapsa spp.*, and *Chlorococcus spp.* were isolated from the different costal regions of Gujarat, India and cultivated in optimized conditions. Among all of these *Chlorococcus spp.* was selected for the secretion, extraction and purification of Extracellular polysaccharides.

MATERIAL AND METHODS:

For the preparation of inoculum, Inoculum at concentration of 10% was inoculated in BG-11(Modified) medium in 100ml of Erlenmeyer flask. Optimum growth conditions like 12/12 h light and dark cycles, 28° C are provided. Humidity was also maintained by regular spray of water over filter papers kept in incubator (at least twice/thrice per day). Many researchers recommended Cyclohaxemide to avoid the contamination of diatoms and higher algae. But due to unavailability of cyclohaxemide we have used chlorohexidine for the same purpose. Over and above antibiotics Ampicillin-Streptomycin were used at a concentration of 40µg/ml and

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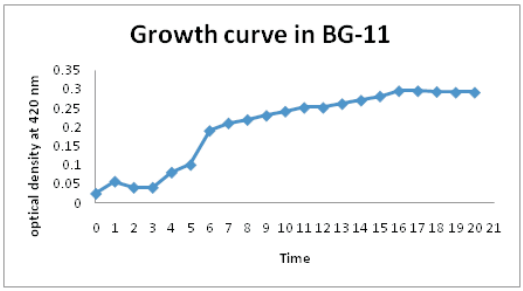
100µg/ml respectively to keep the medium free from bacterial contamination. Many times Floxacin and Chloremphenicol were also used as an antifungal in adequate amount and culture was incubated. Axenity of cultures was checked every 3rd day by streaking the culture on to BG-11(Modified) solid medium.

OPTIMIZED CULTURAL CONDITIONSPURE CULTURE OF *Chlorococcus spp*

For EPS production inoculum was transferred to Erlenmeyer flask containing 500ml of BG-11(Modified) medium with 100 ml of inoculum to make final concentration of cell to reach up to Optical Density 1.0 (nm). The culture was shaken twice a day to provide sufficient mass transfer of the nutrients or the metabolites secreted by the cells.[4][17].

TABLE: GRAPH – 1 Growth Curve Studies of Chlorococcus spp. In BG – 11 Medium

Day	Optical Density of *Chlorococcus* (BG-11 Medium)
day 0	0.024
day 1	0.036
day 2	0.04
day 3	0.04
day 4	0.08
day 5	0.1
day 6	0.19
day 7	0.21
day 8	0.219
day 9	0.23
day 10	0.241
day 11	0.251
day 12	0.251
day 13	0.261
day 14	0.27
day 15	0.281
day 16	0.295
day 17	0.295
day 18	0.293
day 19	0.291
day 20	0.291



Supernatant after centrifugation was used to detection of EPS production by estimating extracellular soluble carbohydrate content by various quantitative analytical methods.

METHODS USED FOR PRODUCTION, ISOLATION AND PURIFICATION OF EXTRA CELLULAR POLYSACCHARIDES:

EPS was produced in a 1ltr Erlenmeyer flask containing modified BG – 11 medium supplemented with glucose (30 g/L) at 28°C. The culture medium was inoculated at 10% (v/v) with a bacterial suspension in the exponential growth phase. The pH was adjusted and maintained at 7.2 by automatic addition of NaOH. The medium was oxygenated at 30 L/h with an agitation rate of 200 to 1100 rpm. After 25th to 30th day of incubation, bacterial cells were removed from the culture medium by centrifugation (15,000 rpm for 20 min). The supernatant containing the excreted EPS was then purified by filtration through a cellulose membrane (0.7 µm) and then by ultra-filtration (100 kDa) before being freeze-dried and stored at room temperature away from light and moisture.

EPS EXTRACTION & ESTIMATION:

For extraction of the Cyanobacterial EPS, 100 ml of the culture was subjected to centrifugation (3000 rpm) and after separating the settled biomass, cell-free culture containing the EPS was taken. It was concentrated half of its volume by using a rotary vacuum evaporator at a temperature of 35oC for further use in the experiment. In a similar way another set was used for quantitative estimation of EPS produced by the Cyanobacteria at different NaCl concentration (S1= 55 mM, S2= 110 mM Na+).

After concentrating the EPS containing cell-free culture tenfold by evaporation at 40°C, it was precipitated with isopropanol. The precipitates were washed with isopropanol 2-3 times to remove any contaminants, dried at 370C and hydrolysed with acid (2 M HCl) at 100°C for 2 hr. The hydrolysate was analyzed for glucose (Seifter, 1959) for all the treatments.

REMOVAL OF SODIUM BY EPS:

In order to see the Na removal efficiency of the EPS, to 100 ml of the cell free extract taken in Erlenmeyer flask 55 and 110 mM of Na+ as NaCl was added, shaken on the illuminated orbital shaker with fluorescent light at 120 rpm at 250C for 12 hrfollowing Freire-Nordi et al. (2005) and Na+concentration in the aqueous medium was estimated on flame photometer (Allen et al., 1986).

POLYSACCHARIDES EXTRACTION & PURIFICATION:

The polysaccharides extracts were isolated by method suggested by Yoon et al. (2003) with some modification until purified polysaccharides obtained. In brief, samples were suspended and refluxed in methanol at 700 C and thereafter the suspensions were filtered to remove the methanol-soluble materials such as colored materials, phenolic compounds and lipids. The filtrates were collected, suspended and refluxed in sterile water. Clear supernatants were collected after low speed centrifugation. Afterwards, the protein contaminations were removed by Sevag method. The polysaccharides in the concentrated supernatants were precipitated with absolute ethanol, subsequently re-suspended in sterile water, and dialyzed by using 10kDa cut off membrane. The purified solutions were finally lyophilized (15).

POLYSACCHARIDES CHARACTERIZATION BY COLORIMETRIC (PHENOL-SULFURIC ACID) METHOD:

The content of polysaccharides in the extracts was determined by Phenol-Sulfuric acid method. Two milliliters of standard grade sugar solutions (ranging from 10 and 100 µg/ml concentrations of sugar) and 2 ml of purified polysaccharides from grown cultures of Cyanobacterial spp at 100 µg/ml concentrations were pipetted into a test tube, and 1 ml of 5% phenol solutions were added. Then 5 ml of concentrated sulfuric acids were added rapidly. The tubes were shaken and placed in water bath at 30oC before readings procedure was taken.The colorimetric analysis of all solutions was carried out using a UV–Vis spectrophotometer. The absorbance of the characteristic yellow-orange color was measured at 490 nm for hexose monosaccharide and 480 nm for pentose monosaccharide and uronic acid. Blanks were prepared by substituting distilled water for the sugar solution. The

amount of polysaccharides in fungal extracts were determined and expressed as amounts of hexose and pentose sugars by using constructed standard curves of each standard sugar (Dubois et al., 1956).

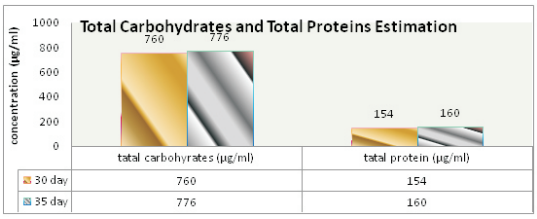
SEPARATION & PURIFICATION OF EXTRACELLULAR POLYSACCHARIDES:

After 30-35 day of incubation culture was used for the extraction of Extracellular polysaccharides. Cells were separated from the growth medium by centrifugation at 15,000 rpm for 40 min. at 5oC. The supernatant obtained was concentrated to 1/4th of total volume was obtained by keeping supernatant at 60°C for 10-12 hours. The polysaccharide from this concentrated supernatant was assessed to many organic solvents like Methanol, Ethyl alcohol, Isopropyl alcohol, and acetone and dimethyl sulfoxide assessed to precipitate the EPS. But among all above Isopropyl alcohol was best suited for precipitation, giving maximum yield with floating precipitate on the surface precipitated by gradual addition of equal amount of cold Isopropyl alcohol or Ammonium sulfate to the supernatant and kept at 4oC overnight. The precipitates were redissolved in buffer or in Double distillated water. This process of precipitation and redissolution was repeated to purify the polysaccharides. [21][4]

Total carbohydrate and protein content of the EPS fraction was measured by Duboi's method and Folin-Lowry's method. The EPSs were hydrolyzed with 2M TCA (Trichloroacid) for estimation of total carbohydrates. The process of hydrolysis was carried out at 100° C for 90 min.[4]

After acid hydrolysis of the obtained EPSs, four constituent of monosaccharide were found by performing Thin Layer Chromatography (TLC) and on the basis of references we conclude that these sugars were, Glucose, Mannose, Xylose and Ribose. Total sugar and proteins content of the EPS were calculated by Duboi's method and Folin-Lowery's method respectively.

TABLE: GRAPH – 2 Total Carbohydrates & Proteins of Chlorococcus spp. by Duboi's Method &Folin-Lowry's Method.



RESULT & DISCUSSION:

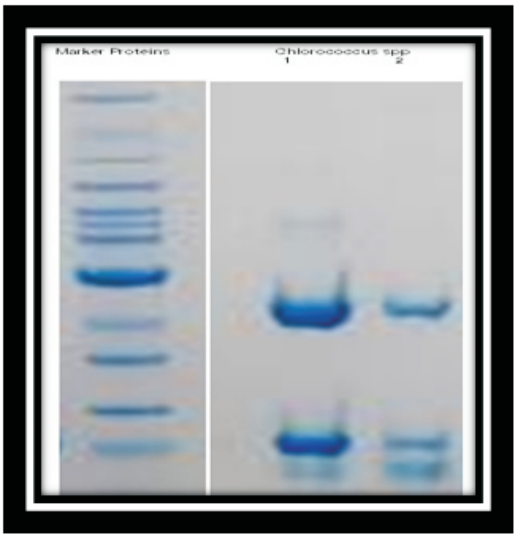
There is no one direct measurement of polysaccharides since there are mixed complex and combination of variety of monosaccharaides. Phenol sulfuric acid method is a colorimetric method widely used to determine the total concentration of carbohydrates. Monosaccharaides, oligosaccharides and polysaccharides rearrange themselves to furfural derivatives by the action of sulfuric acid at elevated temperature and furfural derivatives then react with phenol to give colored compounds. The absorbance of the characteristic color was measured at 490 nm for hexose monosaccharide and 480 nm for pentose monosaccharide and uronic acid (17).

The UV-Vis spectrograms showed the reactant reagents (5% phenol and conc. sulfuric acid) absorbed wavelength at 296 nm (Figure 4). Hexoses (mannose and glucose) absorbed wavelength at 490 nm and xylose absorbed wavelength at 480 nm. The results confirmed the Phenol-Sulfuric acid method to determine the amount of hexoses at 490 nm and pentoses at 480 nm. 760 µg/ml purified polysaccharides extracts from Chlorococcus spp. absorbed maximum visible light wavelength at 487.5 nm rather than 480 or 490 (representing pentose and hexoses), indicating the mixture between hexoses and pentoses in the extracts with more hexoses than pentoses because the shift of maximal absorption wavelength towards 490 nm.

Further, Total Carbohydrates and Proteins content of the EPS were calculated by using Duboi's method and Folin-Lowrey's method respectively. By performing its qualitative analysis of EPS sample by on the basis of different tests we found reducing and non-reducing sugar present in the EPS sample. And on the basis of different references we conclude that these were sugars like, Glucose, Mannose, xylose and ribose

present.

The protein so obtained was subjected to SDS-PAGE and the result obtained was photographed as mentioned below.



SDS – PAGEASSEMBLY FOR SDS - PAGE

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