

Extraction And Optimization Of Extracellular Polysaccharide Production In Spirulina Platensis MK 343101

Nagananthini.G ,Rajapriya.S, Arivuvel amuthan P.S, Sivasankari.S, David Ravindran.A

Abstract: Spirulina is a blue-green alga belonging to Oscillatoriaceae family that typically grows in freshwater lakes, natural springs, and saltwater in subtropical and tropical climates. This blue-green alga is packed with nutrients. It has the outer layer made up of polysaccharide which is water-soluble known as extracellular polysaccharides (EPS), which possess various biological activities such as antitumor, antiviral, antioxidant and anticoagulant activity. Extraction of polysaccharides from Spirulina has been carried out by three different methods viz., hot water extraction, precipitation using ice-cold ethanol and combination of three different solvents methanol: ethanol: chloroform (2:2:2). Exopolysaccharide production was optimized by using Fractional Factorial Design.

Keyword: Antioxidant, Antitumor, Extracellular polysaccharides, Spirulina.

1.INTRODUCTION:

Arthrospira platensis, generally known as Spirulina platensis, is a blue green alga (cyanobacterium) characterized by the high levels of proteins, vitamins, minerals, poly unsaturated fatty acids, zeaxanthin and myxoxanthophylls [8]. Spirulina sp. secrete a large amount of extracellular polymeric substances (EPS) into the environment under stress conditions, these released EPS are polymers of 81 to 98 kDa [14]. The main biological role of EPS is anti-inflammatory, immuno modulatory, anti tumor, antiviral, anti parasitic, antioxidant, hypoglycemic and hypocholesterolemic activities. The potential application of EPS of S. platensis is in the cosmetic industry also widely used as adhesives, food and beverage , pharmaceuticals, oil, and metal recovery from ore industry. The important biological properties of sulphated polysaccharides is the ability to act as the anti-coagulants, to interfere with the absorption and penetration of enveloped viruses [16]. Spirulina exopolysaccharides are capable with antiviral activity against herpes simplex virus type 1, inhibitory effects on corneal neovascularisation, increased immuno stimulatory activity, and anticoagulant activity mediated by heparin cofactor II [2]. This study was undertaken to determine the optimal conditions for the production of EPS by S. platensis as influenced by various factors such as different NaCl concentrations ,pH , temperature ,different light intensities and media by Fractional Factorial Design.

MATERIALS AND METHODS:

The cyanobacteria S. platensis MK343101 culture was obtained from the Department of Biology, Gandhigram Rural Institute- Deemed to be University, Dindigul. The culture S. platensis was grown in standard Zarrouk medium [18] maintained at pH 9.5-10.5 at 37°C. The culture flasks were aerated with 1 ml/min of air provided by aeration pump and constantly irradiated with the light intensity of 2500 lux (Fig.3.a). The culture was subsequently transferred to fresh media to avoid deprivation of nutrients

and loss of cells. Using spectrophotometer, the growth of cell was measured at 650 nm [15].

2.1 Screening of Extra Cellular Polysaccharide (EPS) extraction methods:

In this study, we used three different types of extraction methods.

2.1.1 Hot water extraction method:

A volume of 100 ml culture broth was transferred in a sterile 250 ml Erlenmeyer flask. Place this in the sonicator where the ultrasonic waves were allowed to disrupt the cells and , and then the liquid mixture was centrifuged. The pellet was collected and used for the extraction of capsular polysaccharide. The resulted supernatant was concentrated under vacuum at 55°C in a rotary evaporator to 1/5 of the original volume. Three times the volume of 95% ethanol was added to the solution. The solution mixture was placed in a freezer overnight. The EPS was precipitated at the bottom of the flask. The precipitate was collected by centrifugation and then dried. The pellet was again centrifuged by using hot water. The absolute ethanol was added to this supernatant, the capsular polysaccharide was precipitated.

2.1.2 Extraction by using different solvents (methanol, ethanol, and chloroform):

A volume of 100 ml culture broth was crushed by using 0.1 g of acid wash sand. This solution was centrifuged and the pellet was discarded after collecting the supernatant. Add 2 ml equal volume of methanol, chloroform and ethanol (2:2:2) and allowed to the formation of methanol layer and added the same volume of ethanol and to leave it overnight at 4°C. Centrifuged the sample to collect the precipitated EPS and allowed it to dry and to obtain the crude EPS.

2.1.3 Precipitation of EPS by using ice - cold ethanol:

A volume of 100 ml *S.platensis* culture broth was centrifuged at 15,000 × g for 20 min at 4°C. The supernatants were pressure-filtered through rotary vacuum evaporator and the EPS was precipitated from the concentrated filtrate by the addition of three volumes of ice-cold ethanol and the solution was kept at 4°C for overnight. Excess water was removed under vacuum evaporator before lyophilisation. The extracted EPS was lyophilized using a lyophilizer (Fig.3.b). The lyophilized exopolysaccharide was stored at room temperature until chemical and physical analysis was performed.



Fig.3a *Spirulina* cultivation

Fig.3.b EPS from *Spirulina*

2.2 Optimization of Exopolysaccharide

production in *S.platensis*

The statistical screening methods were used to analyse the five important factors that enhances the production of EPS in *S.platensis* such as temperature, pH, light intensity, media and NaCl concentration by fractional factorial design.

2.3 Measurement Of Growth Rate:

The value X_1 and X_2 is the optical density measured at absorbance 650 nm in spectrophotometer with the time interval of 9 days represented by t_1 and t_2 . The specific growth rate of algae calculated by the following formula:

$$\mu = \frac{X_2 - X_1}{t_2 - t_1}$$

2.4 FT-IR analysis:

The lyophilized powdered sample of exopolysaccharide was mixed with the dry potassium bromide pellet (KBr) and subjected to a pressure of about 5×10^6 pa in an evacuated die to produce a transparent disc of diameter 13mm and thickness of 1mm. The FT-IR spectra of EPS was recorded in the absorbance mode from 4,000-400 cm^{-1} in Fig.6

2.5 X ray Diffraction (XRD) analysis:

The crystalline size of the EPS from *S.platensis* was analyzed by the XRD technique, and the result was depicted in Fig.7.

2.6 SEM analysis:

The SEM microscopic image of EPS of *S.platensis* are provided in Fig.8. The SEM image was used to study the surface morphology of exopolysaccharide.

3.RESULT AND DISCUSSION:

Microalgae are the suitable energy resource because they are photoautotrophic organisms converting atmospheric carbon-dioxide into biomass. Microalgae are easy to culture; their growth requires light, carbon dioxide and a minimum amount of mineral salts. Based on these facts, the present work was designed to study the growth pattern of the microalgae *Spirulina* and to extract the exopolysaccharides from it.

Table. 3.1. Screening the effective method for EPS

No	Effective method for extraction of EPS	EPS obtained (g/100ml)
1.	Hot water extraction	10.4 ± 0.74
2.	Extraction by using different solvents (chloroform, ethanol and methanol) (2:2:2)	1.5 ± 0.32
3.	Extraction by using ice-cold ethanol	12.6 ± 0.62

Primary studies was done with different

extraction methods of *S.platensis* EPS viz. the hot water extraction method, extraction by using different solvents such as chloroform, methanol and ethanol and by ice-cold ethanol. Among these methods, the last one appeared to be the most attractive method to increase yield of EPS allowing the breakdown of the interactions between EPS and microalgae cell wall. The maximum yield of dry biomass of EPS about $12.6 \text{ g} \pm 0.62/100 \text{ ml}$ obtained in the ice-cold extraction method (Table 3.1.)

Media Optimization using FFD (Fractional Factorial Design)

The statistical screening methods were used to analyse the five important factors that enhances the production of EPS in *S.platensis* such as temperature, pH, light intensity, media and NaCl concentration by fractional factorial design. The FFD was used to determine which factor significantly affect the yield of EPS in *S.platensis* (Fig 3.c). Fractional factorial design for the 11 runs and the corresponding response for the production of EPS. In FFD R^2 value is always lies between 0% -100%. The value of the determination coefficient (R^2) for EPS production was 98.90% and the adjusted R^2 was 94.48% (table 3.2 a and b). The above statistical value is closer to 100%, it indicates that the experiment model is very significant. The half normal and normal plot (Fig 4.a and Fig 4.b) showed that the factors influenced the production of EPS. The factors like pH and NaCl concentration showed red spots in the plot it indicates they are significant and blue spots in the plots show that they are non significant. In half normal plot none of the factors lies below 0 it stands for no negative effects and factors above 0 denotes that all the factors affected the production of EPS. In pareto-chart, the length of the bar

(Fig.5) showed that the effects of independent factors and their interaction for the yield of EPS. In pareto-chart, A (pH) has highly influenced the yield of EPS and followed by B (NaCl concentration) is also influenced the yield of EPS.

Table 3.2.a and b Coded Coefficients and analysis of variance

Term	Effect	Coef	SECoef	T-value	P-value	VIF
constant		10.14	0.537	18.8	0.003	
Temp	4.478	2.239	0.537	4.17	0.053	1.00
pH	8.453	4.226	0.537	7.86	0.016	1.00
Light	1.273	0.636	0.537	1.18	0.358	1.00
Media	3.388	1.694	0.537	3.15	0.088	1.00
NaCl	8.318	4.159	0.537	7.74	0.016	1.00
pH*light	2.723	1.361	0.537	2.53	0.127	1.00
pH*NaCl	3.288	1.644	0.537	3.06	0.092	1.00
CtPt		-3.71	1.03	-3.61	0.069	1.00

S 1.51990
 R-sq 98.90 %
 R-sq (adj) 94.48%
 P< 0.05 -significant



Fig.3.c Optimization of EPS production by using FFD

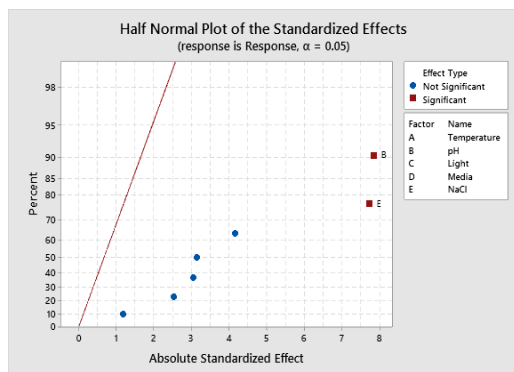


Fig.4 a. Half normal plot of the standardized effects

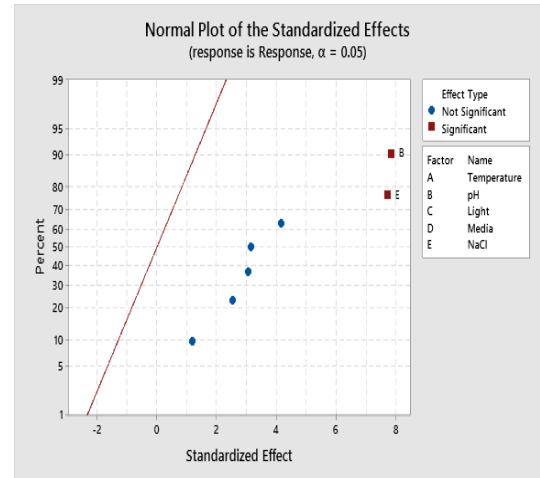


Fig .4.b Normal plot of a standardized effect

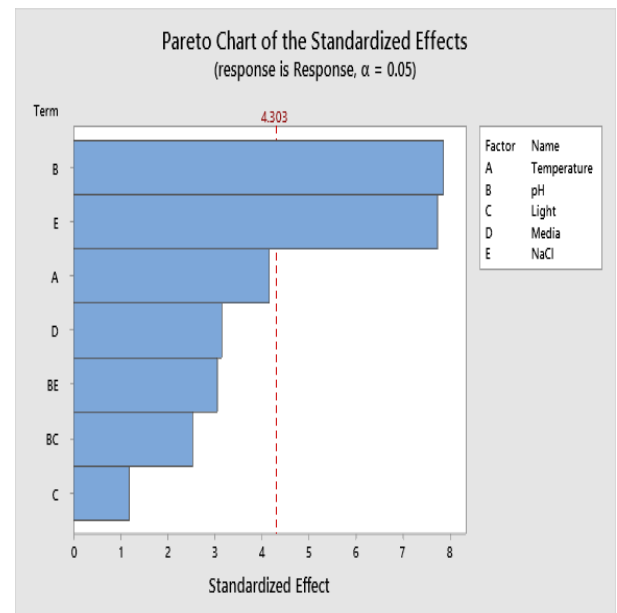


Fig.5 Pareto chart for standardized effect

Table.3.b

Source	DF	AdjSS	AdjMS	F-value	P-value
Model	8	414.026	51.753	22.40	0.043
Linear	5	347.536	69.507	30.09	0.032
Temperature	1	40.096	40.096	17.36	0.053
pH	1	142.890	142.890	61.85	0.016
Light	1	3.239	3.239	1.40	0.358
Media	1	22.950	22.950	9.93	0.088
NaCl	1	138.362	138.362	59.89	0.016
2-way interaction	2	36.439	18.220	7.89	0.113
pH*light	1	14.824	14.824	6.42	0.127
pH*NaCl	1	21.615	21.615	9.36	0.092
Curvature	1	30.051	30.051	13.01	0.069
Error	2	4.620	2.310		
Total	10	418.646			

Spirulina was cultivated in the laboratory in an open batch photo bioreactor using Zarrouk medium, Spirulina requires the optimum pH ranging from 9.5-10.5 for their growth. The bio reactors were illuminated under light intensity (1900

lux) lamps, CO₂ was sparged for every 3 mins for a period of 12 hours. The growth of Spirulina was determined by spectrophotometric method [13]. A two-stage culture method was followed for the production of polysaccharides from Spirulina platensis, the first stage increases the maximum biomass yield under 96 μmol photons m⁻² s⁻¹ light intensity and the second stage stimulates the production of polysaccharide by culturing the algae with a higher light intensity of 192 μmol photons m⁻² /s at 38°C for three days or in a medium containing 0.75 mol/ Litre NaCl for 2 days. NaCl concentration can simultaneously influence the growth of S. platensis and stimulate polysaccharide production. When 0.75 mol L⁻¹ NaCl was incorporated in the medium for 48–72 h, S. platensis produced the maximum level of polysaccharides to overcome environmental stresses, however the biomass was lowered. [3]. Under salt stress, Cyanobacteria produced maximum amounts of EPS which improves salt forbearance and carbohydrate metabolism [4]. EPS production increased only in the stationary phase of the organisms growth cycle because a nutrient limitation is necessary for the activation of EPS production [11]. High concentrations of NaCl damaged the D1 protein, decelerated the photosynthetic electron transport processes, and decelerated the sequential photosystem II processes. In these circumstances the exopolysaccharides provide as a cushion to alleviate the impact of high NaCl concentrations [9]. NaCl concentration strongly enhances the production of EPS in different cyanobacteria particularly in Spirulina platensis the augmentation of EPS used to avoid oxidative stress in cells and to protect membranes from desiccation [6]. Spirulina platensis was cultivated in the standard Zarrouk medium adjusted with different NaCl concentration levels in this study. The growth of the algae was monitored for a period of 9 days and the cell density was measured every 24 hrs. The highest growth rate was found in cultures maintained at 5g + medium in 1st day and the least growth rate was observed at 9th day in the same medium. The exopolysaccharide on extraction using ice-cold ethanol recorded highest amount of EPS with 5 g NaCl. The growth of the algae was monitored for a period of 9 days and the cell density was measured every 24 hrs. The uppermost growth rate was found in cultures maintained at pH 10. The maximum amount of

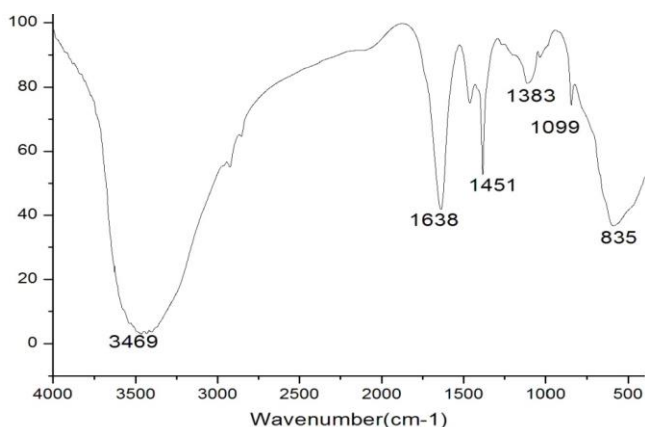


Fig.6 IR Spectra for EPS from *S.platensis*

EPS was obtained at pH 11.

Under nitrogen starvation condition Spirulina sp., produced more amount of EPS as reported by [10], indeed nitrogen limitation contributing to the increased C:N ratio and thus promoting the assimilation of carbon into EPS. [17], interpreted that under nitrogen-deficient conditions algal cells mount up the carbon metabolites as carbohydrate.

FT-IR analysis:

The exopolysaccharide extracted from Spirulina had peaks recorded in FT-IR spectra appeared as 2,800–3,200 cm⁻¹ region corresponds to hydroxyl group present in the polysaccharides, peaks appeared in 1,300–1,450 cm⁻¹ was due to C-H bending vibration, C=O absorption of uronic acid occurred at

1,650cm⁻¹[1].

The FT-IR analysis of Spirulina EPS represents the following functional groups: alcohols at 3469 cm⁻¹ (O-H stretching) and alkene group at 1638 cm⁻¹ (C=C stretching), peak at a wavelength 1451 cm⁻¹ identified as C-H bond as an alkane, peaks at the wavelength of 1383 cm⁻¹ indicates the presence of sulfate group (S=O stretching), at a wavelength of 1099 cm⁻¹ attributed to C-N stretching of the amine group and wavelength of 835 cm⁻¹ attributed to C-O-S stretching of the β-type glycosidic linkage (Fig.6.).

X ray diffraction analysis:

The crystallite size of the EPS was calculated by Scherrer equation.

$$\beta(2\theta) = K\lambda / L \cos\theta$$

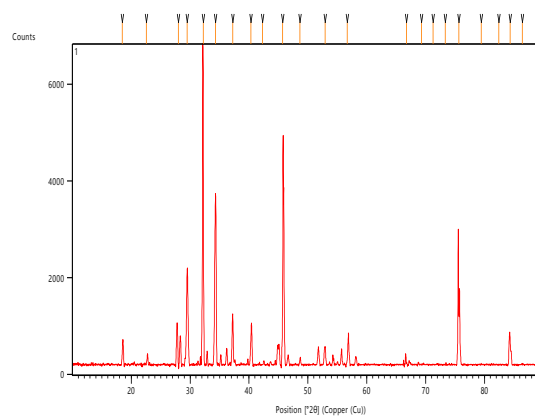


Fig .7 XRD image of *Spirulina platensis* EPS

The crystalline size of the EPS is 18nm and amorphous in nature. The SEM image of EPS from S.platensis was observed under magnification (Fig.8). Structural irregularities showed that the EPS extracted from S.platensis was an amorphous solid.

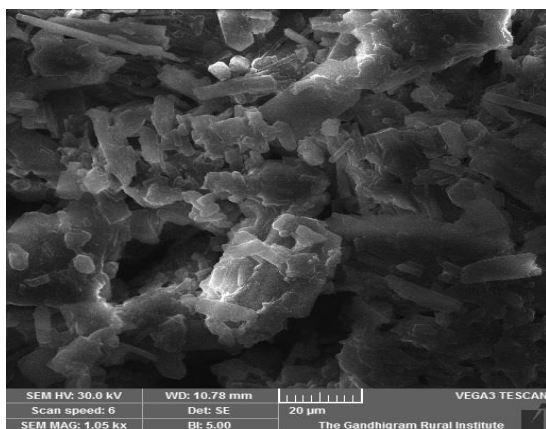


Fig.8 SEM image of *S.platensis* EPS

CONCLUSION:

Water soluble extracellular

polysaccharide was obtained from the microalgae *Spirulina platensis*. The three extraction methods were studied and screened for the effective method. The production of EPS was optimized using five factors viz., NaCl, pH, temperature, different light intensities and media. The IR spectra of EPS contains alkene group, amine group and sulphate groups. The XRD and SEM images showed that the crystallite size and surface morphology of the extracted EPS. The presence of sulphate group in EPS imparts antiviral and anticoagulant activities.

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