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OPTIMIZATION OF PHYSIOLOGICAL PARAMETERS FOR EXOPOLYSACCHARIDE PRODUCTION USING *Pseudomonas aeruginosa* MSSRFV42

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ABSTRAT: Exopolysaccharides (EPS) are long-chain polysaccharides containing branched, repeating units of sugars or sugar derivatives such as glucose, fructose, mannose and galactose etc, which are secreted into their surrounding environment during the bacterial growth. Due to their unique properties and vast array of application, isolation and study of new EPS producing microbes is highly concerned issue. In the present study potent exopolysaccharide producing AB4 bacteria (*Pseudomonas aeruginosa* MSSRFV42) which was previously isolated and screened from marine sample was used. To increase the yield of exopolysaccharide significant physiological parameter were determined Submerged fermentation at pH 10 and temperature of 30^0C gave maximum yield of EPS after 72 hrs incubation using 1% inoculums in media containing 3.5% NaCl. Outcome of this study will definitely contribute in the elucidation components of AB4 EPS in various industries .

Keywords: EPS, *Pseudomonas aeruginosa*, Optimization

INTRODUCTION :

Exopolysaccharide (EPSs) are high molecular weight, biodegradable polymers biosynthesized by a wide range of bacteria . The Earths surface consists of 70% water, which inhabited by 80% of all the life forms and consequently aquatic life have a greater diversity than their terrestrial counterparts. EPS are high molecular weight compounds and are sticky in nature (Passow *et al*., 1994) In the natural environment, Exopolysaccharide (EPSs) is generally heteropolymeric (made of different monomeric units), non-sugar components like uronic acid, methyl esters, sulphates, pyruvates, proteins, nucleic acids and lipids. EPS also contain divalent metal cations that act as ionic bridges linking adjacent polysaccharide chains(Shanker *et al*,2014) bacteria produce capsular form of EPS during the exponential growth phase and slime type EPS during the stationary growth phase (Wingender *et al*., 1999). The microbial EPS possesses superior and unique properties that enable the development of new commercial entities (Freitas *et al.*, 2011). So, in recent years, there has been an increasing demand for the isolation and identification of new microbial polysaccharides that can compete with traditional polymers because of their improved chemical and physical properties, higher flocculating and emulsifying activities, biological activity and resistance to solvents (Kumar *et al*., 2007). The exopolysaccharide production is one of the emerging fields of research to demonstrate its potential for various medicinal and industrial applications. Environmental factors and specific culture conditions such as pH, temperature, carbon-tonitrogen(C/N) ratio, oxygenation rate, and carbon sources can impact EPS production(Ozlem , 2015).
Many researchers have optimized the production conditions for exopolysaccharide (EPS) in submerged culture by *Fomes fomentarius* (Chen *et al*. 2008), Pholiota squarrosa (Wang *et al*. 2004), *Agrocybe cylindracea* (Kim *et al*. 2005), *Cordyceps militaris* (Kim *et al*. 2003), *Aureobasidium pullulans* (Moubasher and Wahsh 2014). Production of EPS by bacterial species is greatly determined by a number of factors, such as phases of growth, nutritional condition and the environmental status. The nature and concentration of nutrients in particular, are necessary components for stimulation of growth and synthesis of EPS (Laws and Marshall, 2001; Pal and Paul, 2008). Further, the fermentation conditions play important role in determining the conformation, molecular mass, monosaccharide ratios and functional properties of the EPS (Sutherland, 1994; Finore *et al*., 2014).
In this study effect of inoculums size ,pH of the medium, time of incubation, temperature of incubation, ,agitation speed on the exopolysaccharide production by AB4 marine *Pseudomonas aeruginosa* was studied.

MATERIALS AND METHODS

In the present study bacterial strains of *Psudomonas aeroginosa* (AB4) previously isolated from marine water samples and characterized by using Bergey's Manual of Determinative Bacteriology was used . (Chudiwal A.B. *et al.*, 2019)Strains were preserved in glycerol and also maintained on Zubella marine agar or Kings agar slant at 4ºC and stored at refrigerator for further use. For inoculum production, cultures were prepared in the marine broth and incubated for 24h at room temperature with shaking condition 120rpm .The bacterial growth was adjusted to 1 unit Optical Density at 600 nm and 1% volume of this suspension was used to inoculate the liquid medium for exopolysaccharide production. Cultures were prepared in Erlenmeyer flasks (100 mL) containing 25 mL of Zubell Marine Broth (Himedia). The initial pH was adjusted to 7.5 .

Optimization of production medium

Nutrient Broth and marine broth supplemented with 1% glucose, Sutherland medium , and YMG broth , Clementi Medium, Production Medium were inoculated with laboratory isolated *Pseudomonas aeruginosa* and incubated in shaking condition at 30˚C for 72h and after incubation bacterial growth and Exopolysaccharide production was

determined separately to know best medium for EPS production.

Optimization of physiological parameters Exopolysaccharide production

Optimization of inoculum size

Pseudomonas aeruginosa AB4 has inoculated in yeast extract malt broth (YMG Broth) for 24 hrs. The 1OD inoculum (10^7 cfu/ml) added in 50ml of YMG broth at 0.5 % to 5% inoculum size of strain *Pseudomonas aeruginosa AB4* and incubated at 30˚ C for 24 Hrs. After incubation period ,growth at 600 nm ,pH of supernatant and yield of exopolysaccharide g/l was determined after ethanol precipitation .

Optimization of incubation time

The 1 OD inoculum of optimized inoculum size inoculated in each flask containing 50 ml of YMG Broth. The flask was incubated for 12,24,48,72,96 hrs. and checked their growth at 600 nm, pH of supernatant and yield of exopolysaccharide (g/l).

Optimization of pH

The pH provides an environment for the microorganism to grow abundantly. To study the effect of different pH on exopolysaccharide production YMG broth was used adjusted to different pH ranging from 3, 4, 5, 6, 7, 7.5, 8, 9, 10 to 11 . So for the optimization of the pH, the selected AB4 bacteria was inoculated in YMG broth having different pH (3-11) for 72 hrs. at 30˚ C temperature .After incubation ,the exopolysaccharide was extracted by alcohol precipitation and optical density was measured at 540 nm to find the yield of the exopolysaccharide in g/L.

Optimization of incubation temperature

This Experiment was carried out to determine the optimum growth temperature for maximum Exopolysaccharide production .The flask contained YMG broth inoculated with 1OD inoculum at 1%(v/v) and incubated at temperature ranging from 20 ˚C,30˚C,37˚C,40˚C,50˚C. After incubation growth at 600 nm ,pH of supernatant and yield of exopolysaccharide (g/l) was determined

Optimization of Nacl Concentration

The effect of salt concentrations on EPS production was checked by adding NaCl at varying concentrations from 0.5% to 5.0% supplemented in the basal YMG broth medium and the flask was inoculated and incubated and assessed under the same above conditions .

Effect of Agitation on EPS production

Agitation intensity was also essential a critical factor influencing on both the cell biomass and EPS production .To study the effect of agitation on EPS production Basal YMG broth was inoculated and incubated on shaker viz. 0, 25, 50, 75, 100, 150 and 200 rpm. After incubation growth at 600 nm ,pH of supernatant and yield of exopolysaccharide (g/l) was determined .

RESULT AND DISCUSSION :

Microorganism used: The organism under study was Gram negative, motile small rods identified as *Pseudomonas aeruginosa* by biochemical and16s rRNA sequencing approach (data not mentioned). It produces blackish green colonies with smooth surface and mucoid and glistening colonies on Zubell marine agar (fig. No.2)

Optimization of physiological parameters Exopolysaccharide production

Optimization of medium

The ability of selected strain of *Pseudomonas aeroginosa* to produce exopolysaccharide in different media was assessed and it was found that the maximum yield of exopolysaccharide (3.2g/l)obtained in yeast extract malt glucose broth (YMG Broth Medium III). Pawar *et al*. (2013) was optimized the exopolysaccharide production under different environmental conditions. EPS production was maximum in Nutrient Broth, contains 2% sucrose. They found that EPS production was highest in 3 days incubation period (5.2 g/l).

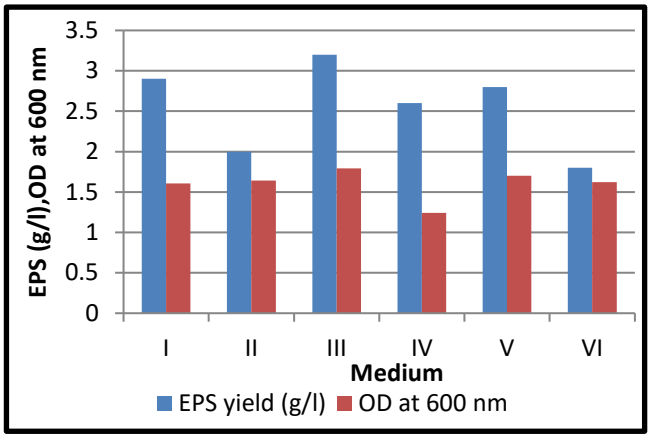

Fig. 1Effect of different types of media on EPS production

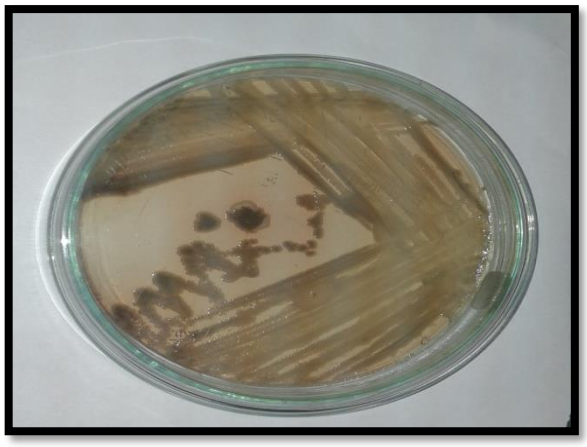

Fig.2 AB4 marine *Pseudomonas aeruginosa*

Optimization of inoculum size

The maximum exopolysaccharide production obtained with 1.56 g /l at 1 %(v/v) inoculam and minimum is 0.6g/l at 3.5%(v/v). The data is recorded in figure 3 .The similar result of effect of 1% inoculam size was reported by Vuyst *et al* .,1998 while 3%(v/v)inoculum was preferred for exopolysaccharide production of *pseudomonas fluroscence* by Zhao *et al* .,2012 .

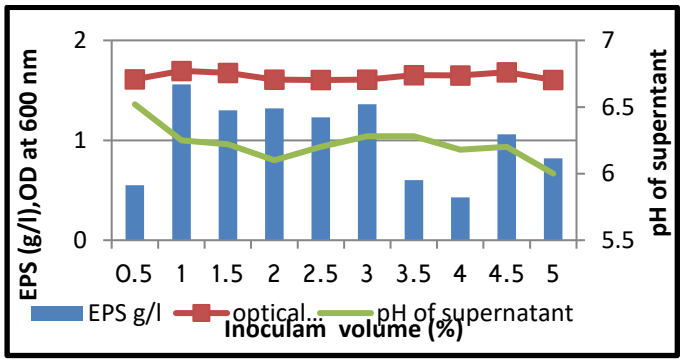

Fig.3 Effect of inoculum volume on EPS production

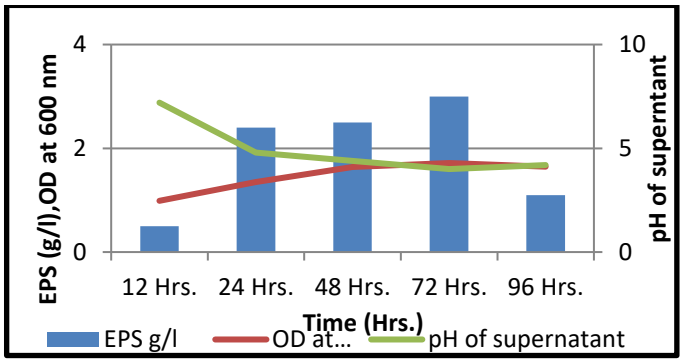

Fig.4 Effect of time on EPS production

Optimization of incubation time

To determine the effect of incubation time, the bacteria were grown at 30C in liquid basal YMG medium and the samples were removed at regular time intervals. Exopolysaccharide production increases from 12 to 72 h, maximum

production was observed after 72 h of incubation (3.0 g/l). Further increase in incubation time does not show positive effect on exopolysaccharide production. Sharma *et al*., 2017 reported that the maximum EPS production was 32.64 mg/ml observed after 24 h incubation .However, EPS production reached a maximum of 13.6 g/L crude EPS after 5 days (Asker *et al*., 2014)

Optimization of pH

The pH plays a vital role in maintaining the acidic or basic condition in the medium. The pH provides an environment for the microorganism to grow luxuriantly. Effect of different pH viz. 2,3,4, 5, 6, 6.5, 7, 7.5, 8, 9, and 10,11 was tested on production of exopolysaccharide . After incubation exopolysaccharide was extracted by ethanol precipitation method and exopolysaccharide yield was estimated in g/l. The maximum yield of exopolysaccharide in g /l (1.86), was obtained in the YMG broth with pH 10 when inoculated with AB4. Aayona *et al*.(2006) reported that alkaline pH was suitable for biomass as well as exopolysaccharide production Many of the exopolysaccharide-producers require a constant pH for maximum production of exopolysaccharide (Morin, 1998). Other microorganisms like *Neisseria meningilidis* produce more exopolysaccharide at acidic pH values (Morin, 1998)

Optimization of incubation temperature

To determine the effect of different temperatures on Exopolysaccharide production, theproduction medium was incubated at different temperature in the range of 20˚C , 28˚C, 30˚C, 37˚C,40˚C and 50˚C. After incubation for 72 h with pH 10 and constant shaking on rotary shaker, it was found that maximum production of exopolysaccharide was observed at 30˚C (1.08 g/l). This result indicates that optimum incubation temperature required to produce maximum exopolysaccharide is 30˚C and increase in temperature above it did not show further improvement in yield. Congregado *et al.*, 1985 reviewed that EPS production by *Pseudomonas* sp. strain was increased under conditions of high C/N ratios at the 25°C .Yang and Liau 1998 proved that a range between 30˚C and 33˚ C was found to be suitable for exopolysaccharide production by *Gonoderma lucidum*.

Optimization of Nacl Concentration

Sodium chloride could alter the osmolarity of the cell membrane of bacterium which favored the more extrusion of exopolysaccharide from cell to media . The concentrations of Nacl in the media are very important for cell growth and optimal exopolysaccharide production . As shown in Figure no.7 ,The highest yield of EPS (4.7 g/l) was obtained when a Nacl concentration of 3.5 % was used.

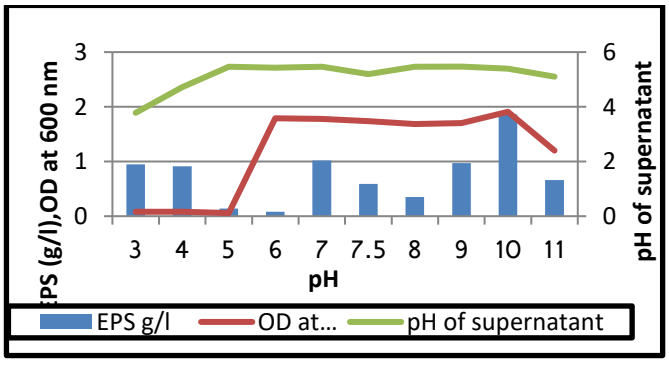

Fig.5 Effect of pH on EPS production

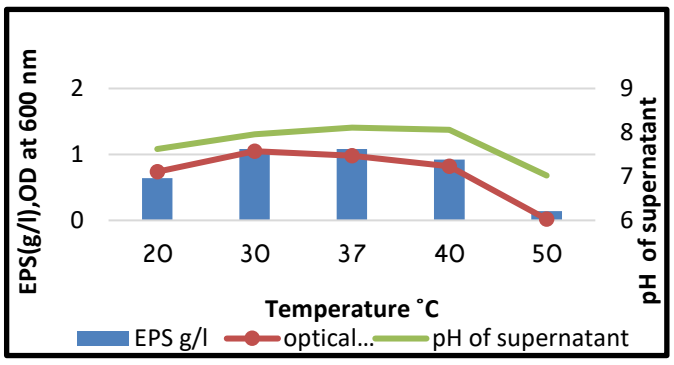

Fig.6 Effect of temperature on EPS production

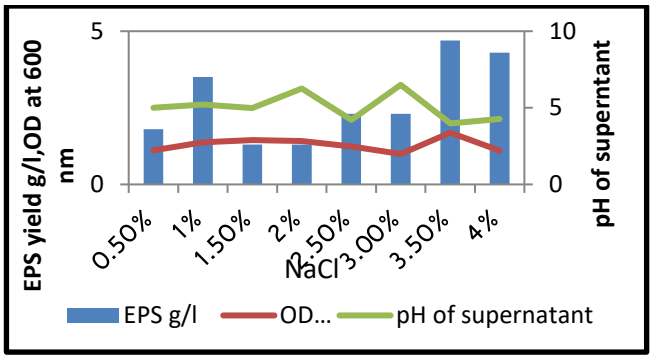

Fig. 7 Effect of NaCl (%) on EPS production

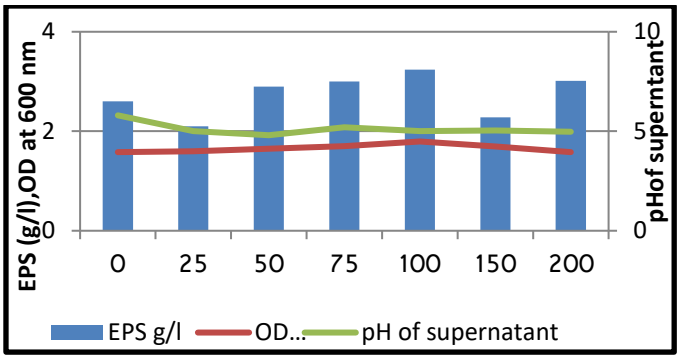

Fig. 8 Effect of agitation on EPS production

Effect of Agitation on EPS production

Effect of speed of agitation (viz. 0, 25, 50, 75, 100, 150 and 200 rpm) on production of exopolysaccharide was determined by incubating inoculated YMG broth at various rpm. After production yield of EPS was estimated. The maximum yield (3.24 g/L) of exopolysaccharide was obtained in flask agitated at 100 rpm when inoculated with AB4. With the increase in the speed of the agitation above 100 rpm, a decrease in the yield of exopolysaccharide was observed.

Conclusion:

In the present study it was concluded that yeast extract malt medium was a good medium for exopolysaccharide production. Laboratory isolated selected strain of *Pseudomonas aeruginosa* MSSRFV42 was found as potent exopolysaccharide producer when inoculated in the medium with pH 10, time of incubation 72hr. and temperature of incubation 30ºC. It was also found that agitation of the medium can significantly affect production process. The study also reveals that isolated strain was haloalkalotolerant which can be used in various industries .

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