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Evaluation of cultivation condition for enhanced production of exopolysaccharide by bacterial isolate P 11 under submerged culture condition

Prakash M Rawal, Poonam B Chauhan, Hiren Prajapati, Mayur Gahlout*

Department of Microbiology, KBS Commerce and Nataraj Professional Science College, Vapi. Dist. Valsad, Gujarat, India. PIN: 396195

Corresponding author; Dr. Mayur Gahlout

E-mail: *mayur_nu@yahoo.com*

Abstract

Exopolysaccharides (EPS) are environment friendly natural polymers secreted by microorganisms in the surrounding medium. Due to the presence of unique structural composition, EPS shows diverse applications such as in food formulations, pharmaceutical, and cement based construction industry, etc. In the present investigation, the bacteria producing higher exopolysaccharide was screened among bacteria isolated from indigenous soil samples. Maximum exopolysaccharide production was shown by isolate P 11. Further investigation was done to determine the optimal variables of nutritional and environmental conditions to get maximum EPS production from the isolated bacteria. Glucose and beef extract enhanced the EPS yield at pH 7 and 30°C incubation temperature.

Keywords: Exopolysaccharides, glucose, beef extract, temperature.

1. Introduction

Exopolysaccharides are eco-friendly natural polymers secreted by microorganisms in the surrounding medium. Due to the presence of unique structural composition, exopolysachharides shows diverse applications such as in food formulations, pharmaceutical, and cement industry and plastic industry. Different microorganisms such as bacteria, fungi and actinomycetes produces exopolysaccharides during their life cycle. For example, most bacteria produce capsular and slime type exopolysaccharides during different phase (Wingender *et .al*., 1999). Polysaccharides are the saccharides that are secreted out by the micro-organisms. Microorganism have the capacity to form solutions viscosity even at low exopolysacchaides concentrations. Polysaccharides are the carbon sources which are found in large amount in the earth (Cummins and Sutton., 2005).

The polysaccharides are playing important role to protect bacterial cells from desiccation, invade of toxic metals, antibiotic, phagocytosis, phage attack. Now days bacterial polysaccharides used as immunostimulatory, immunomodulatory, antitumor, antiviral, anti-inflammatory and antioxidant agents in various medical and pharmaceutical industries. Microbial exopolysaccharides such as dextrans, xanthan, gellan, pullulan, yeast glucans and bacterial alginates are used in many industries as food additive. Microorganisms are easily produce exopolysaccharides than microalgae and plant (Wang *et. al*., 2008).

On their position of polysaccharide secreted named as exopolysaccharides and capsular polysaccharides. Capsular polysaccharides forms a protective capsule and prevent the pathogenic micro-organism from immune system defences (Cummins and Sutton., 2005). Exopolysaccharides act as barrier in preventing the harmful intruders and capsular polysaccharides are avoid any antibody responses (Morris and Harding., 2009) .

Homopolysaccharides consist of fructose or glucose, and are usually produced in large amount from sugars by the action of glycansucroses e.g. dextran, levan, alternan, reuteran etc. Heteropolysaccharides are mostly composed of identical repeating more monosaccharides e.g. galactose, glucose, rhamnose and fructose and hetropolysaccharide; Kafiran which is produced by many *Lactobacillus* species is a water soluble heteropolysaccharide (Micheli *et. al.*, 1999).

2. Materials and Methods

2.1 Reagents and Media

Glucose , Yeast extract, Beef extract , Peptone (HPLC pvt LTD Mumbai India) , $MgSO_4$ (Loba chem, Mumbai, India), NaCl , K_2HPO_4 , KH_2PO_4, $(NH_4)_2SO_4$ (Rankem, Ankleshwar, India) and all the other chemicals and media used were of analytical grade. All the media and reagents were prepared in distilled water.

2.2 Isolation and screening of Exopolysaccharide producing bacteria

Samples were collected from different location of Vapi, Gujarat, India. Soil, curd sample, spoilage fruit sample and garbage water ample are collected. The environmental samples were serially diluted in sterile distilled water and appropriate dilution was plated by spread plate method on basal media containing (gm\lit) Glucose 20; Yeast extract 3.0; $MgSO_4$ 0.2; K_2HPO_4 5; Agar 30; and pH 7. The inoculated plates were incubated at 30°C for 24 hrs. Upon incubation plates were observed for isolated colonies producing mucous colonies, from the samples. The mucoied colonies were further purified by streaking on nutrient agar plates in pure form and store at 4°C temperature.

2.3 Production of exopolysaccharide under submerged fermentation condition

2.3.1 Preparation for inoculum

Inoculum was prepared in a nutrient broth, containing 50 ml of nutrient broth in 100 ml conical flask. Take a loopful culture from preserved culture plate and inoculate it into nutrient medium and incubate it for 24 hrs in shaking incubator at 30°C, 150 rpm for 24 hrs. The freshly grown culture with 1 O.D. at 600 nm was used as inoculum for the submerged fermentation process.

2.3.2 Submerged fermentation

EPS production was carried out by submerged fermentation. For submerged fermentation, the production medium (Containing (gm/lit) Dextrose 20; beef extract 15; NaCl 5; K_2HPO_4 8; KH_2PO_4 2; $MgSO_4$ 0.5; pH 7) was inoculated with 1% inoculumn of 1 O.D.. The inoculated flask was incubated at 30°C, 150 rpm for 48 -72 hrs. A sample of 10 ml was withdrawn from the fermentation flask at regular time interval, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS.

2.3.3 Precipitation of EPS

Exopolysaccharide in the fermentation medium was precipitated using chilled ethanol. In the first step 10 ml sample were added to centrifuge tube and centrifuged at 5,000 rpm for 20 minutes, and the supernatant was put in another tube and pellets are discard. In the second step, an equal amount of chilled ethanol was added and incubated in refrigerator at 4°C for 30 min, the precipitated EPS was centrifuged for 20 minutes and the supernatants were discarded. The pellet obtained was dried in filter paper in oven at 60 - 70°C and dry weight of exopolysaccharides was taken to estimate the yield of EPS in fermentation flask.

2.4 Optimization of exopolysaccharide production

The present work involves optimization of different parameters governing exopolysaccharides production. The effects of various nitrogen sources, carbon source, incubation temperature, pH etc on exopolysaccharides production were examined by one factor at a time method.

2.4.1 Effect of incubation time on EPS production

Effect of incubation time on EPS production was performed by inoculating 1% of inoculum in fermentation flask. The inoculated flask was incubated at 30°C, 150 rpm for 60 hrs. A sample of 10 ml was withdrawn from the fermentation flask at regular time interval, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS.

2.4.2 Effect of inoculation size on EPS production

Effect of inoculation size on EPS production was performed by inoculating the fermentation flask with inoculum size in the range of 0.5% to 3%. The inoculated flasks were then incubated at 30°C, 150 rpm for 24 hrs. A sample of 10 ml was withdrawn from the fermentation flask after 24 hrs of incubation period, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS yield.

2.4.3 Effect of pH on EPS production

In present study, medium pH was adjusted in the pH range of 3 – 11 pH, to determine the effect of pH on bacterial growth and EPS production. The fermentation flask with respective pH was inoculated with 2% inoculum. The inoculated flasks were then incubated at 30°C, 150 rpm for 24 hrs. A sample of 10 ml was withdrawn from the fermentation flask after 24 hrs of incubation period, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS yield.

2.4.4 Effect of Temperature on EPS production

In present study the production flask was inoculated with 2% inoculum. The effect of temperature was studied by incubating the inoculated medium in a temperature range of 15°C - 60°C at 150 rpm for 24 hrs. A sample of 10 ml was withdrawn from the fermentation flask after 24 hrs of incubation period, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS yield.

2.4.5 Effect of carbon source on EPS production

Effect of carbon source on exopolysaccharide production was studied by taking different carbon sources i.e (dextrose, fructose, maltose, lactose, sucrose and mannitol) in the production medium. The fermentation flask with respective carbon source was inoculated with 2% inoculum. The inoculated flasks were then incubated at 30°C, 150 rpm for 24 hrs. A sample of 10 ml was withdrawn from the fermentation flask after 24 hrs of incubation period, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS yield.

2.4.6 Effect of nitrogen source on EPS production

Different nitrogen source (Yeast extract, beef extract, peptone, urea, ammonium sulphate, ammonium chloride and ammonium carbonate) were introduced into the production medium individually to determine the effect of nitrogen source on EPS production. The fermentation flask with respective nitrogen source was inoculated with 2% inoculum. The inoculated flasks were then incubated at 30°C, 150 rpm for 24 hrs. A sample of 10 ml was withdrawn from the fermentation flask after 24 hrs of incubation period, centrifuged at 5000 rpm for 20 min and supernatant was used to estimate EPS yield.

Results and Discussion

3.1 Isolation and screening of EPS producing microorganisms

Various types of garden soil sample, garbage soil, garbage water, spoilage fruits and curd sample are used to isolate the mucoied colony producing bacteria. The appropriate dilution was speeded of the samples were plated on basal agar medium plate and incubated at 30°C for 24 hrs. Upon incubation period the plates were observed for mucoied colony. A total of 11 mucoied colonies were isolate and purified by sub-culturing on nutrients agar plate. The capsule staining of the isolates show capsule around the bacterial cell, which is the primary indication of EPS production by bacterial cells.

All the isolated bacterial strains were further assessed for EPS production under submerged fermentation condition. Maximum EPS production was shown by isolate P11 (EPS yield 3.65 gm/lit) after 24 hrs of incubation at 150 rpm under submerged fermentation. Isolate P 9 shows EPS yield 2.1 gm/lit, isolate P 10 shows EPS yield of 1.6 gm/lit and isolate P 8 shows EPS yield of 1.3 gm/lit at 30°C, 150 rpm after 24 hrs of incubation. The EPS production by other bacterial isolate was shown in the range of 0.2 to 0.8 gm/lit (Table 3.1).

Table 3.1 Screening of EPS producing bacterial strains.

Sample & Isolate	Capsule staining	EPS yield (gm/lit)
KBS garden Soil P 1	Capsulated	0.59
P 2	Capsulated	0.37
Garbage soil P 3	Capsulated	0.20
P 4	Capsulated	0.20
Garbag water sample P 5	Capsulated	0.68
P 6	Capsulated	0.90
Curd sample P 7	Capsulated	0.87
P 8	Capsulated	1.3
Spoilage fruits sample P 9	Capsulated	2.1
P 10	Capsulated	1.6
P 11	Capsulated	3.65

3.2 Optimization of culture condition for EPS production

3.2.1 Time course study for the production of EPS by isolate P 11

The EPS production by isolate P 11 was studied at various incubation time i.e. 0 - 60 hrs. The results observed shows that as the incubation period increases EPS production was also increased till 24 hrs of incubation under determined fermentation condition. Maximum EPS production was achieved at 24 hrs of incubation with EPS yield of 3.44 gm/lit. However, further incubation results in decreased EPS yield along with the incubation time. The EPS prduction and growth profile as shown in fig 3.1, indicates that EPS production was maximum in mid log phase of the bacterial growth and stationary growth phase results in loss of EPS from fermentation flasks.

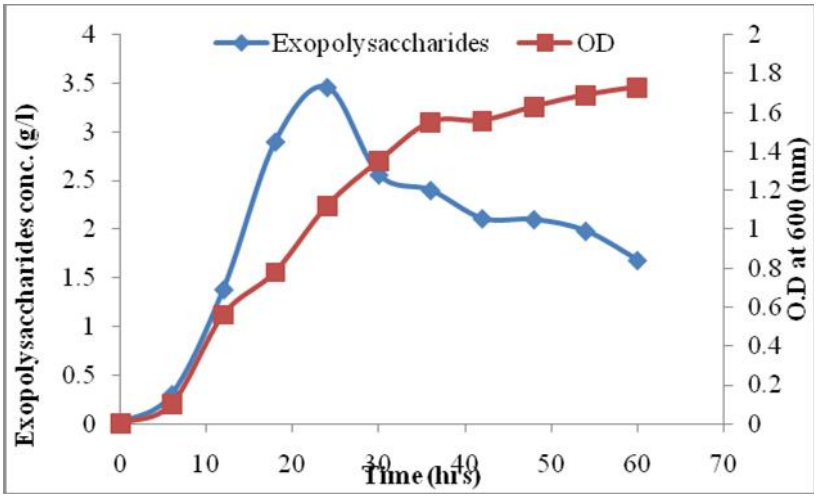

Fig 3:1 Time course study of isolate no 11 for the production of EPS.

3.2.2 Effect of inoculum size on EPS production

In present study the fermentation flask was inoculated with inoculum size in the range of 0 to 3.0% with 1.0 O.D at 600 nm. The result obtained shows (fig. 3.2) that as inoculum size was increased from 0.5 % to 2.0 %, to inoculate in fermentation flask, the amount of EPS production was increased. The optimum inoculum size for maximum EPS production (4.1 gm/lit) was found to be 2% inoculum. However, inoculum size greater than 2%, results in decreased EPS yield in production flask. Similar results have been shown by other researchers (Sivakumar *et al.*, 2012).

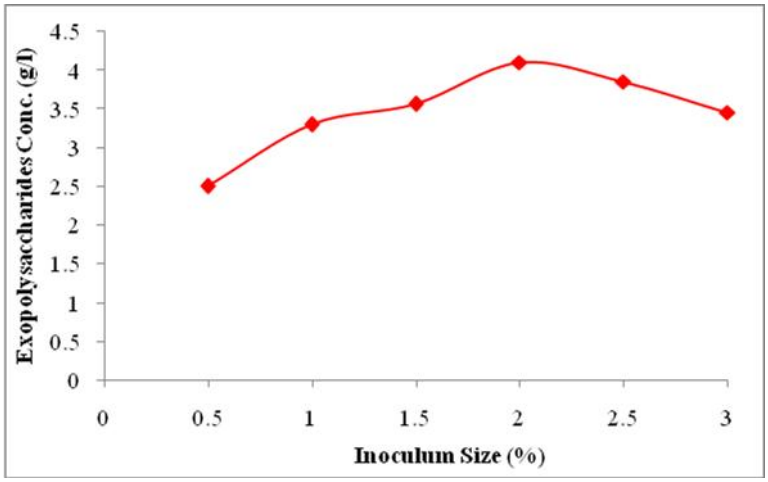

Fig 3.2 Effect of inoculum size on EPS production

3.2.3 Effect of incubation temperature on EPS production

In order to find optimum temperature for maximum EPS production, The 250 ml Erlenmeyer flask with 100 ml production medium was inoculated with 2% inoculum. The inoculated flask was incubated in the temperature range of 10 – 60°C at 150 rpm and EPS production was estimated after 24 hrs of incubation. The results obtained shoes that as the incubation temperature was increased EPS production was increased simultaneously till incubation temperature and maximum EPS production (4.5 gm/lit) was achieved at incubation temperature 30°C. However, further increase in incubation temperature results in decreased EPS production. Similar results had been shown by Sivakumar *et al.,* (2012) who reported that optimum temperature for EPS production was 30°C. Ko *et al.,* (2000) reported that maximum exopolysaccharide yield (9.19 g/l) was obtained during incubation temperature 20-25°C.

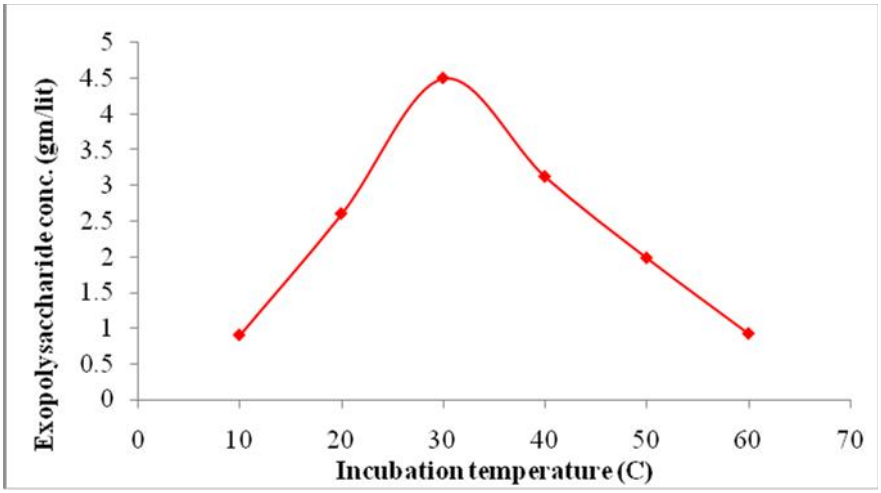

Fig 3.3 Effect of temperature on EPS production

3.2.4 Effect of pH on EPS production

In order to find out optimum pH for maximum EPS production, the fermentation flask was adjusted in the pH range of 3 to 11 by 1 N HCl and 1 N NaOH. The result obtained shows that EPS yield was lower in production flask with alkaline and acidic pH. The EPS production was higher in production flask near to netral pH. The EPS production was found to be in the range of 4.2 – 4.6 gm/lit, in the fermntation flask with in a pH range of 6-8 and optimum pH for maximum EPS production was found to be pH 7. Lower EPS yield in fermentation with alkaline and acidic pH was due to deacreased enzymetic activity required for growth and EPS production (Sivakumar *al el.,* 2012). Ko *et al.,* (2000) reported that EPS production was higher in pH range 6-8 pH and maximum EPS was obtained at netural pH (9.19 g/l).

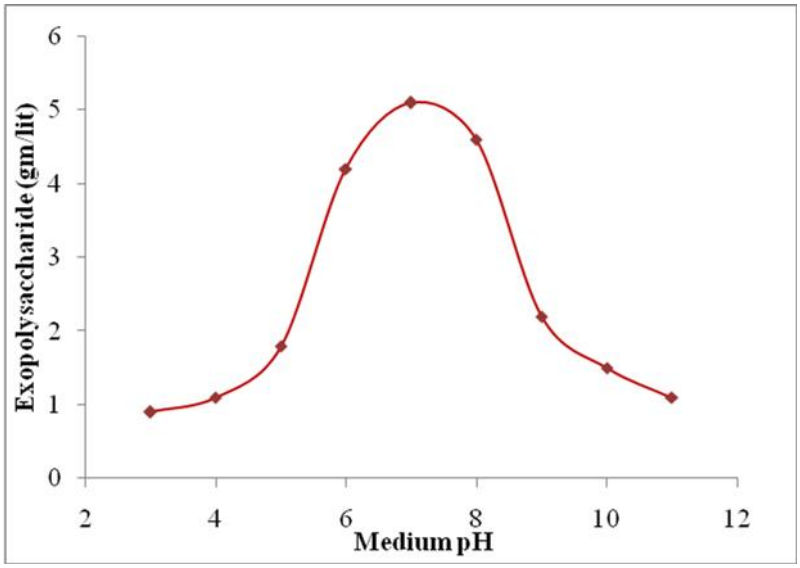

Fig 3.4 Effect of pH on EPS production

3.2.5 Effect of carbon source on EPS production

The effect of carbon source on exopolysaccharide production by isolate P 11 was studied by taking different carbon source i.e. glucose, sucrose, maltose, fructose, mannitol, lactose, galactose and xylose in the production flask. The fermentation flask with respective carbon source was then inoculated with 2% inoculum and EPS yield was observed after 24 hrs of incubation.

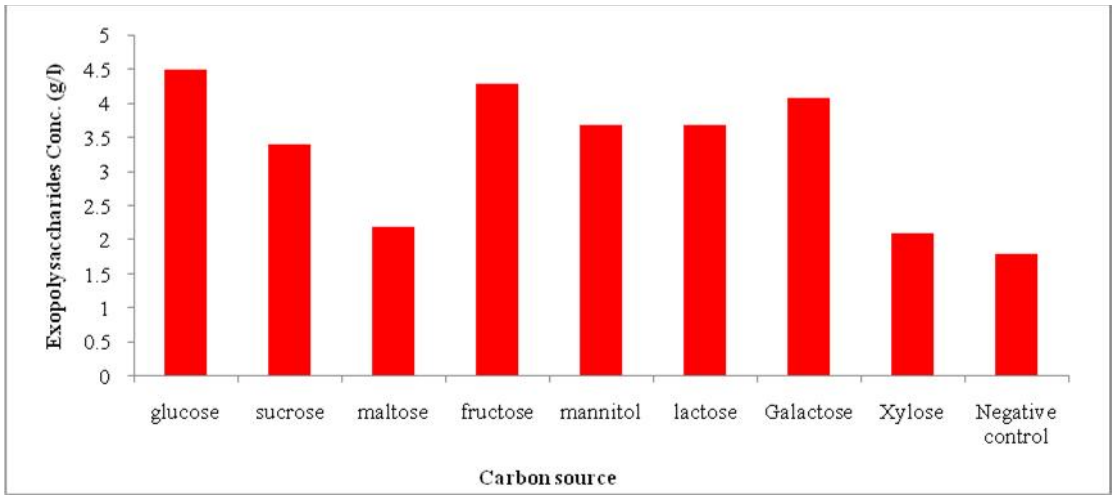

Fig 3.5 Effect of carbon source on EPS production

The result observed shows that maximum EPS production was observed in fermentation flask supplemented with glucose (4.5 g/l) supplemented medium. Production flask supplemented with fructose and galactose showed EPS yield in the range of 4.0 – 4.5 gm/lit after 24 hrs of incubation. Production flask with maltose and xylose shows decreased EPS yield after 24 hrs of incubation and production flask without any carbon source does not show significant EPS yield (1.8 gm/lit) in the fermentation flask. Cerning *et al*., (1994) reported sucrose as best carbon source for higher EPS yield using *Lactobacillus casei* strain.

3.3.7 Effect of nitrogen source on EPS production

The effect of nitrogen source on exopolysaccharides production by isolate P 11 was studied by taking different nitrogen source like yeast extract, peptone, beef extract, ammonium sulphate, urea and sodium nitrate in production flask. The production flask with respective nitrogen source was the inoculated with 2% inoculum and incubated at 30°C, 150 rpm. The EPS yield in each fermentation flask was estimated after 24 hrs of incubation.

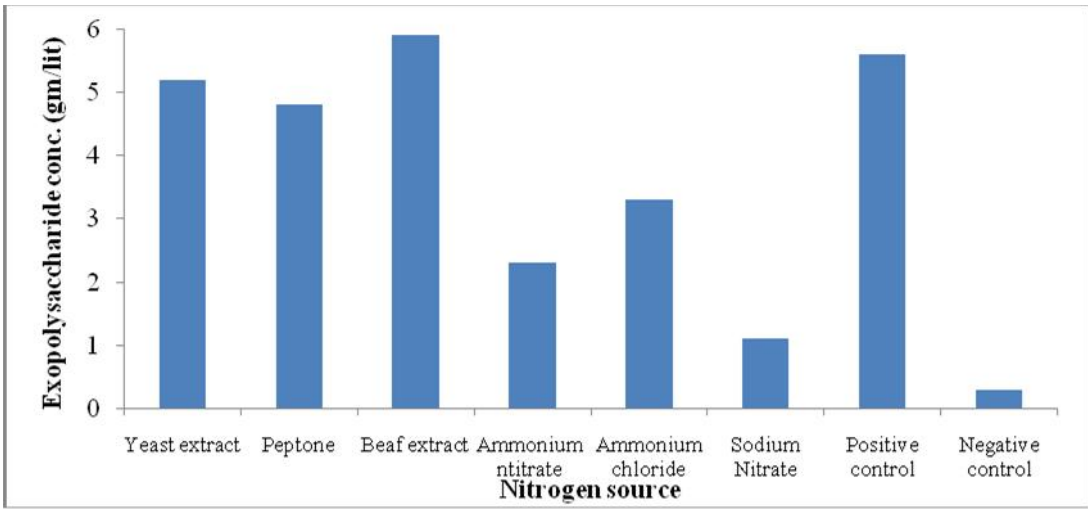

Fig 3.6 Effect of nitrogen source on EPS production

The result observed shows that fermentation flask supplemented with crude organic source showed higher EPS yield as compare to inorganic nitrogen source at 24 hrs of incubation period. Maximum EPS yield was observed in fermentation flask supplemented with beef extract (5.9 gm/lit). Fermentation flask supplemented with yeast extract and peptone shows EPS yield in the range of 4.5 – 5.5 gm/lit. However, fermentation flask without any nitrogen source shows negligible amount of EPS yield (0.3 gm/lit) at 24 hrs of incubation. Kumar *et al.*, (2012) shows maximum EPS yield in the presence of tryptone and urea as the sole nitrogen source in fermentation flask at 30°C after 72 hrs of incubation. Razack *et al*., (2012) shows yeast extract as the best nitrogen source for maximum exopolysaccharides yield (1.38 g/l) after 48 hrs of incubation period.

4. Conclusion

In present study, isolate P 11 was found to be the highest EPS producer i.e. 3.65 (gm/lit), amongst various isolate tested. pH 7 and temperature 30°C was found to be optimum pH and temperature for maximum EPS production. The best substrate for EPS production was found to be glucose as carbon source with exopolysaccharides production 4.5 (g/l) and beef extract 5.9 (g/l) as the best nitrogen source among different organic and inorganic nitrogen sources tested.

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