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Research Paper

Optimization of *Cronobacter dublinensis subsp. dublinensis DES187(T)* isolated from root nodules of Soybean for exopolysaccharide production

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Abstract: In present study conditions were optimized by one-factor-at a time method to enhance EPS production by *Cronobacter dublinensis subsp. dublinensis DES187(T)* isolated from root nodules of Soybean (*Glycin max*). Effect of different parameters were examined on EPS production and maximum EPS production was observed with 2% D-Glucose as carbon source (750µg/ml), 0.2% beef extract as nitrogen source (770µg/ml) and with 300µg/ml of MgSo4.7H_2O as metal ion (820µg/ml) after 66 hours of incubation period (880µg/ml). EPS was harvested with ethanol and estimated by phenol sulphuric acid assay. With all this optimized conditions *Cronobacter dublinensis subsp. dublinensis DES187(T)* produced 4.0 g/l exopolysaccharide as per dry weight and 920µg/ml of exopolysaccharide estimated by phenol sulphuric acid assay.

Keywards: *Cronobacter dublinensis*, exopolysaccharide, optimization, phenol, sulphuric acid assay

INTRODUCTION

Microorganisms produces wide spectrum of multifunctional polysacchrides such as polysaccharids, structural polysaccharides, and extracellular polysaccharides (Razack et al. 2013, Freitas et al. 2011). Microbial exopolysacchride (EPS) has generated greater attention of researchers due to their physical and structural properties diversity. EPS are extensively used in different fields such as in medical field EPS used as immune-modulator, antitumor, antiviral, and antiulcer and antioxidant, and in drug delivery as coating agent (Razack et al. 2013, Sivakumar et al. 2012). EPS used as stabilizing, emulsifying, gelling, thicking agent in different fields such as in food, pharmaceutical, cosmetic, concrete additives, in enhanced oil recovery, metal recovery and waste water treatment (Duta et al. 2006, Hui et al.2013 and Qiang et al. 2013). Bacterial EPS help in the cell adherence to surfaces, in biofilm formation, also involved in symbiosis, pathogenesis, protecting against osmotic shock, toxic stress and antibacterial compounds

microbial aggregation, cell protection (Razack et al. 2013, Naseem H and Bano A, 2014).

The structure, composition and viscosity of microbial polysaccharide depends upon several factors such as composition of culture medium, specially carbon and nitrogen sources, mineral salts, trace elements, type of strain and fermentation conditions (such as pH, temperature, oxygen concentration, agitation). Optimized fermentation conditions particularly associated to physical and chemical parameters is of primary and great importance for development of any process (Duta et al. 2006). Several researchers studies have reported that the yield and quality of microbial EPS are greatly affected by environmental and nutritional condition. Previous studies also suggested that increase in EPS production is possible by manipulating its cultural conditions. Optimization of growth conditions is important to get maximum EPS production (Qiang et al. 2013, Pawar et al, 2013).

Now a day a lot of efforts is put in selection of new microbial strains and optimization of cultural conditions to achieve maximum yields of those EPSs already commercially successful. Furthermore there is a considerable interest in finding new EPSs that have potential industrial applications, either by using new bacterial strains or by applying different cultural conditions (Prathima et al. 2014). The objective of this study was to optimize cultural conditions to get maximum EPS yield from isolated bacterial strain.

MATERIAL AND METHODS

Total 100 bacterial strains were isolated from root nodules of different leguminous plants collected from different regions of Ambajogai taluka, India. For isolation healthy, pink colored, unbroken root nodules were selected, surface sterilized, crushed, and serially diluted. Higher dilutions were spread on Yeast Extract Mannitol Agar and incubated at 30^0C for 3 to 5 days (Balamurugan G and prakash S. 2012). After isolation isolated bacterial colonies were purified and stored. All the isolates serially named YC1 to YC100 were screened for EPS production in Yeast Extract Mannitol broth supplemented with 1% Mannitol as carbon source. Strain YC7 showing maximum EPS production were identified by grams staining, some biochemical characters and by 16s rRNA sequencing as gram negative *Cronobacter dublinensis subsp. dublinensis DES187(T)*. Strain YC7 showing maximum production were used for further study and effect of different parameters on EPS production by *Cronobacter dublinensis subsp. dublinensis DES187(T)* were examined. All experiments were conducted in triplicates.

Optimization for EPS production

Effect of incubation time on EPS production: Loopful culture of *Cronobacter dublinensis subsp. dublinensis DES187(T)* was inoculate in 30ml of Yeast Extract Mannitol (YEM) broth and incubated for 24 hours at 30^0C. After incubation 1% 24 hours old culture was used as inoculum for fermentation (Nirmala et al. 2011).

For optimization of incubation period, *Cronobacter dublinensis subsp. dublinensis DES187(T)* was grown in YEM broth supplemented with 1% mannitol as carbon source, production medium were incubated at different incubation time (24, 48, 66, and 96 hrs) to determine effect of incubation time on EPS production.

Effect of carbon sources on EPS production: Eight different carbon sources (D-Mannitol, D-glucose, Sucrose, maltose, Arabinose, Xylose, Fructose, and Lactose) were added to the basal medium (YEM broth) replacing Mannitol with varying

concentrations from (0.5% to 5%). The medium was inoculated with *Cronobacter dublinensis subsp. dublinensis DES187(T)* and incubated at 30^0C for 72 hours. Then after fermentation EPS was isolated and estimated by assay method.

Effect of Nitrogen source on EPS production: Effect of different nitrogen sources (yeast extract, beef extract, peptone, ammonium sulphate, ammonium chloride, Ammonium nitrate, urea, tryptone, glycine) on EPS production were examined by introducing different nitrogen sources in production medium (with varying concentrations from 0.1% to 2%) with most suitable carbon source.

Effect of metal ions on EPS production

Different metal ions (mercuric chloride, magnesium sulphate, magnous sulphate ferric chloride, zinc sulphate, and zinc chloride) with varying concentrations (100µg/100ml to 1000µg/100ml) were introduced in production medium to examine their effects on EPS production with most suitable carbon and nitrogen sources.

Harvesting of EPS : After 72 hours of incubation fermentation broth was centrifuged at 10,000 rpm for 15 minutes, pellet were discarded and supernatant was mixed with three volumes of ice cold ethanol to precipitate the EPS and kept in refrigerator for overnight incubation. After overnight incubation EPS precipitate harvested by centrifugation dried in oven at 60^0C and weighed (Gharzauli et al. 2012).

EPS estimation assay

The cell free supernatant was used for EPS estimation by Phenol-sulphuric acid assay followed by Dubois et al., using glucose as standard (Dubois et al. 1956, Data C. and Bassu P. S. 1999)

RESULT

Effect of incubation time on EPS production: Fermentation broth incubated with *Cronobacter dublinensis subsp. dublinensis DES187(T)* were incubated at different incubation time. Maximum EPS production was observed after 66 hours of incubation time (Table: 1).

Table 1. Effect of incubation period on EPS production.

Time (hrs)	EPS Production (µg/mL)
24	350
48	700
66	880
96	680

Effect of carbon sources on EPS production: Effect of different carbon sources on EPS production was checked replacing mannitol in YEM broth. Maximum EPS production was observed with 2% D-glucose followed by Mannitol, and Sucrose (Table: 2).

Table 2. Effect of different carbon sources on EPS production.

Carbon sources (2%)	EPS production (µg/mL)
control	82
D-Mannitol	690
D-Glucose	750
Sucrose	670
Maltose	510
Lactose	390
Xylose	250
Arabinose	190

The control was devoid of any additional carbon source.

Effect of nitrogen sources on EPS production: Among the different nitrogen sources examined beef extract given maximum EPS production followed by Peptone and yeast extract (Table: 3).

Table 3. Effect of different nitrogen sources on EPS production.

Nitrogen sources (0.2%)	EPS production (µg/mL)
control	120
Yeast extract	680
Beef extract	770
Peptone	700
Ammonium chloride	550
Ammonium sulphate	510
Ammonium nitrate	480
Glycine	610
urea	540
tryptone	400

The control was devoid of any additional nitrogen source

Effect of metal ions on EPS production: Among the tested metal ions, maximum EPS production was observed in $MgSo_4$,followed by this FeSo4 showed good EPS production (Table: 4).

Table 4. Effect of different metal ions on EPS production.

Metal ions (300µg/100ml)	EPS production (µg/mL)
$Feso_4$	660
$Mnso_4$	380
$Zncl_2$	250
$ZnSo_4$	370
$Hgcl_2$	550
$MgSo_4$	820

DISCUSSION

In present study conditions were optimized for EPS production from *Cronobacter dublinensis subsp. dublinensis DES187(T)* isolated from root nodules of soybean plant. Maximum EPS production (4.0g/l as per dry weight and 920µg/ml estimated by phenol sulphuric acid assay) was observed after 66 hours of incubation period with 2% D-Glucose as carbon source, 0.2% beef extract as nitrogen source and 300µg/100ml $MgSo4.7H_2O$ as metal ion.

Bhatt P V and Vyas B R M (2015) isolated rhizobacteria *Cronobacter malonaticus* BR1 having multiple Plant Growth Promoting activities. Conditions were optimized with *Cronobacter malonaticus* to enhance EPS production and *Cronobacter malonaticus* showed maximum EPS yield in presence of sucrose (294µg/100ml) after 72 hours of incubation period.

Beshay U et al (2009) optimized the conditions for EPS production by *Streptomyces nasri* and found maximum EPS production with xylose followed by glucose (4.5g/I and 3.8g/l respectively). The maximum EPS production 5.15g/l was observed with 2.7g/l tryptone as nitrogen source.

Razack S A et al (2013) found maximum EPS production 2.66g/l by *Bacillus subtilis* with 2% sucrose as carbon source followed by that glucose and lactose were also able to yield good EPS 1.8g/l. And among the nitrogen sources tested maximum EPS production 1.38g/l was observed with 0.5% yeast extract. Gandhi et al (1997) reported that the ratio of carbon and nitrogen sources play very important role in EPS production and cellular growth. Degeest et al (1999) reported that higher amount of carbon and limited nitrogen concentration in the medium could yield maximum EPS production.

Baojing yuan et al (2012) found maximum EPS production 0.90g/l with glucose at 70g/l concentration the concentration of carbon sources in media are very important for cell growth and optimum metabolite production.
Pawar S T et al (2013) isolated five different organisms from saline soil. Conditions were optimized for isolate no. 4 and maximum EPS production was found with 4% sucrose (5.3g/l EPS) as carbon source, and after 3 days of incubation period (5.2g/l EPS).
Sivkumar T. et al (2012) optimized the conditions for EPS production by Frateuria aurentia and the optimal culture media were determined as follows after 72 hours of incubation, with 1% jaggery as carbon source and 0.5% tryptone as nitrogen source.
Sayyed R Z et al 2015 isolated the heavy metal resistant bacteria from field soil and identified as *Enterobacter* species RXS5. Threshold level of 50µM for Fe 2+ yield maximum EPS (3.22g/l), the concentration of 60uM of Ca^{2+} yielded maximum EPS (2.82g/l) and among the different concentrations of Mg^{2+} used60uM yielded optimum EPS (2.82g/l). Among the carbon sources used glucose gave maximum EPS yield (2.96g/l) and peptone as nitrogen source gave maximum EPS production (3.5g/l).

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