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Isolation and identification of Exopolysaccharide producing Cronobacter species from root nodules of leguminous plants

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Abstract

Exopolysaccharides have various applications due to their unique rheological properties as in food, pharmaceutical and other industries. Exopolysaccharides also have some medical applications as anti-cancer, anti-viral and immune-modulator. In present study, 72 bacterial strains were isolated from root nodules of eight different leguminous plants and screened for exopolysaccharide production. Out of 72 isolates, 15 bacterial isolates showing good growth and exopolysaccharide production in primary screening were secondarily screened. Two isolates of YC7 and YC10 showing maximum exopolysaccharide production of 3.6g/L and 3.0g/L per dry weight, and 890µg/ml of exopolysaccharide were estimated by phenol sulphuric acid assay. Both strains identified by 16s rRNA YC7 were Cronobacter dublinensis subsp. dublinensis DES187(T), and YC10 was Cronobacter muytjensii ATCC 51329(T). The synthetic carbon sources were replaced with agro-industrial waste products. Cane molasses gave maximum exopolysaccharide production (4.25 g/l and 710µg/ml) at 5%, and rice bran showed a negligible effect. The effect of UV mutagenesis was examined (4.35 g/l and 630µg/ml), and showed positive effect on exopolysaccharide production.

Keywords: Exopolysaccharide, Cronobacter sp., Root nodules

Introduction

It was considered from long time that root nodule occupation was restricted to rhizobia, but later wide range of α-, β-, and γ-Proteobacteria have been isolated from root nodules of different leguminous plants as nodule associated bacteria or root nodulating bacteria. Non-symbiotic nodule endophytes have been poorly studied as compared to symbiotic bacteria (Saidi et al. 2013). Different genera of bacteria have been presented as endophytic bacteria in roots, and nodules of leguminous plants may increase the ability of plants to absorb nutrients from soil and solubile phosphorus as in peas, soybeans, chickpeas and cowpeas, and the generally occurring bacteria include Bacilus, Staphylococcus, Mycobacterium, Rhodopseudomonas, Enterobacteria etc. (Surjit & Gupta 2014; Ji et al. 2008). Iverson and forsythe (2003) hypothesized that the Cronobacter species might be of plant origin due to its physiological features such as gum like extracellular polysaccharide production, and its desiccation resistance (Kucerova et al. 2011). In (2009), schmid et al. found evidences for plant origin of Cronobacter, and investigated biochemical traits associated with plant microorganisms in Cronobacter species, and found that all strains were able to produce indole acetic acids, produce siderophores, and solubilize mineral phosphate. The strains were also able to colonize maize and tomato roots. The isolation of Cronobacter has been reported from wide spectrum of environmental sources including soil, water, vegetables, and herbs, and also from lemon root stocks, rice, and soybean plants (Schmid et al. 2009). Early patent for food thickener was reported for material extracted from Enterobacter sakazakii isolated from chinese tea (Kucerova et al. 2011; Scheepe et al. 1986).

Microbial cells generally contain three types of polysaccharides with different functions -i) intracellular polysaccharide which provides a mechanism for carbon and energy storage, ii) structural polysaccharides such as lipopolysaccharides and teichoic acids that are presented as integral components of cell walls; iii) extracellular polysaccharide or exopolysaccharides (EPS) which are water soluble gums with unique and novel properties (Ayer et al. 2005). Due to their structural and physical properties, exopolysaccharides are good emulsifying, gelling, thickening, stabilizing, suspending, film coating, coagulating and viscofying agents as an indication of their diverse capabilities in both industry and research (Patel and Patel 2011). Exopolysaccharides are used in various industries as in food, pharmaceutical, textile, cosmetic, metal mining, oil recovery and other industries (Kaur et al. 2013; Sivakumar et al. 2012). In medical fields exopolysaccharides are used as anti-tumor, anti-viral, immune-modulator agents for drug delivery devices, in vaccine preparations, wound healing (Morris and Harding 2009; Emmanuel et al. 2012).

Mutagenesis is a source of all genetic variations. Strain improvement can be carried out by different techniques such as rational screening and genetic engineering, or by the traditional method of mutagenesis (Sreeju et al. 2011). Random mutagenesis, as a classical method of strain improvement, has been widely used to improve microbial strains (Tayo et al. 2017). Apart from screening, containing the biodiversity for selection of new isolates, UV induced mutagenesis for the improvement of the existing strains is a promising strategy. For a cost effective

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production, agro industrial wastes are used as substrates. Agro-industrial wastes could be a better source of carbon due to its higher sugar content.

As compared to the applications of exopolysaccharides in different fields, there are very few bacterial exopolysaccharides in the market, so the objective of this study is to isolate and screen-out efficient exopolysaccharides producing bacteria from root nodules of leguminous plants which could be industrially applicable

Materials and Method

Isolation of bacteria

Root nodules of eight different leguminous plants (Cicer arietinum, Glycin max, Cajanus cajan, Pisum sativum, Trigonella foenum-graecum, Arachis hypogeae, Vigna unguiculata, Vigna mungo, Indigofera duthiei, Phaseolus aureus) were collected from the farmers' fields from different regions of Ambajogai taluka, and brought into the laboratory. Pink, unbroken, healthy root nodules were detached from the roots of leguminous plants, and surface- sterilized with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes, then with 70% ethanol for 30 seconds, and washed multiple times with sterilized distilled water. Surface sterilized root nodules were crushed with sterilized glass rod, and the root nodule extract was serially diluted. Higher dilutions were spread on Yeast Extract Mannitol Agar (YEMA) plates, and incubated at 30^0 C for 2-3 days. The isolated colonies were purified by continuous streaking, and then stored (Nirmala et al. 2011; Balamurugan and Prakash 2012).

Primary screening of EPS production

The isolated bacterial strains were streaked on YEMA plates supplemented with Congo red dye (0.025 g/L). The bacterial isolates producing EPS forming large, circular, mucoid colonies appeared white in pink colored background. By taking the colony with the platinum loop, a long viscous filament was formed (Gharzauli et al. 2012).

Secondary screening

Colonies showing good EPS production in primary screening were secondarily screened in YEM broth supplemented with 1% mannitol as a carbon source. Firstly, loopful culture was transferred in 30 mL YEM broth, and incubated for 24 hrs for inoculum preparation, and after the incubation, 1mL inoculum was transferred in 50mL YEM fermentation broth, and incubated at 30^0 C for 66 hrs on a rotary shaker (Nirmala et al. 2011).

Extraction of exopolysaccharide

After incubation fermentation broth was centrifuged (at 10,000rpm for 15 minutes), the pellet was discarded, and the supernatant was mixed with three volumes of chilled ethanol, and left overnight at 4^0 C. The precipitated EPS was collected by centrifugation (at 10,000rpm for 15 minutes). The harvested EPS was dried at 60^0 C, and weighed to know which organism showed higher EPS production, the best strain was used for further studies (Balamurugan and Prakash 2012; Pawar et al. 2013; Razack et al. 2013).

Estimation of EPS

After the centrifugation, the cell free supernatant was used for estimation of EPS by phemol sulphuric acid method followed by Dubois et al. (1956). The reaction mixture in the test tube contained 1 mL EPS solution, and 1 mL (5%) aqueous phenol in which 5 mL concentrated sulphuric acid was added rapidly. After vigorous shaking for 20 minutes, OD was taken at 490nm. The amount of EPS was determined by using standard curve of glucose (Nirmala et al. 2011; Data and Bassu 1999).

Identification of isolates

Preliminary grams staining and some biochemical tests (Indol, citrate, catalase, oxidase test) were performed in laboratory for identification of the strains. After that, the biochemical test strains were identified by 16s rRNA sequencing in ARI, Pune as per was gone through the following procedure.

Procedure for 16s rRNA

Total genomic DNA was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions, and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 Mm $MgCl_2$; 1x PCR buffer (Bangalore Genei, Banglore, India); 200µM each dCTP, dGTP, dATP, d TTP;2 pmol of each, forward and reverse primer; and 1U of Taq DNA polymerase (Bangalore Genei, Banglore, India) in a final volume of 20 µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene. The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94^0C for 5 min; 30 cycle of 94^0C, 60^0, and 72^0 for 1 min each; and the final extension at 72^0 C for 10 min, and the mixture was held at 4^0 C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice-using 70% ethanol, and dissolved in Tris-HCL (10 mM, pH 8.0).

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence nearly complete genes. The sequencing reaction and template preparation were performed, and purified in accordance with the directions of the manufacturer (Applied Biosystems). The samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the accompanying DNA sequence Analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology Information GenBank entries by using the BLAST algorithm.

Optimization using agro industrial sources

The effect of agro-industrial waste on EPS production was checked with YC 10 bacterial strain which showed maximum EPS production.

Cane molasses

To check the effect of agro industrial sources on EPS production, Cane molasses was obtained from a sugar factory. Cane molasses was diluted with distilled water containing sodium dihydrogen orthophosphate, at a concentration of 2 g/L at a ratio of 1:1. The

solution was autoclaved at 121°C for 15 min, and then kept overnight for settling. The clarified molasses was then diluted with sterile distilled water at different concentrations (1, 2, 3, 4, and 5%), and used as raw carbon source replacing synthetic carbon source for the production of EPS.

Rice bran

Rice bran was obtained from a local rice mill and pretreated by heating at 100°C for 30 min in a hot air oven, and stored in a moisture-free environment. This pretreated rice bran was later used as carbon source at varying concentrations of 1%, 2%, 5%, 7%, and 10%.

Effect of UV radiation on EPS yield

A loopful culture of isolated bacterial strains (YC 10) maintained on YEMA slants were inoculated in 50 ml of YEM broth, and incubated at 30°C for 24 hours. 1 ml from 24 hours old culture was serially diluted in 9 ml of sterile saline (0.85%) up to 10^{-9}. 100 µl from dilution 10^{-4} was spread-plated on YEMA plates. The petri plates were exposed to UV radiation at a distance of 30 cm for 0secs (control), 5secs, 10secs, 15, 30, 45, and 60secs. Each plate was taken in duplicate for accuracy. Immediately, after exposure plates were kept in dark (wrapped in a black paper) to avoid photo reactivation. Then the control and UV exposed plates were incubated for growth at 35°C for 24 hours (Patel & Goyal 2010).

Results

Isolation and primary screening

Total 72 bacterial strains were isolated from root nodules of eight different leguminous plants, and serially named YC1 to YC72. Out of 72 bacterial isolates, 15 bacterial isolates showing good growth and EPS production on the plates in primary screening (Figure 1) with large, circular, translucent, white colored colonies were selected for secondary screening in broth.

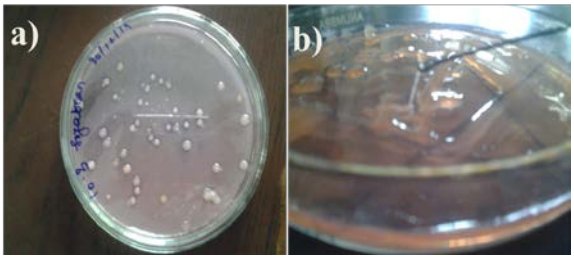

Fig. 1 a) Isolation on YEMA plate, b) Formation of long viscous filament.

Secondary screening

In primary screening, the colonies showing good EPS production were grown in YEM broth supplemented with 1% mannitol as carbon source. In secondary screening, out of 15 isolates only two isolates YC7 and YC10 showed maximum EPS production (3.6g/L and 3.0g/L) as per dry weight respectively, and (890µg/mL) of exopolysaccharide estimated by phenol sulphuric acid assay (Table 1).

Table 1- Secondary screening of Bacterial isolates for EPS production

Bacterial Strains	EPS Dry Wt. (g/L)	EPS estimated (µg/mL)
YC1	0.4	200
YC3	0.7	350
YC5	1.3	600
YC7	3.6	890
YC9	0.8	430
YC10	3.0	890
YC12	0.8	550
YC23	0.7	500
YC28	0.6	300
YC36	0.4	320
YC43	1.0	290
YC46	1.8	800
YC59	0.54	250
YC61	0.7	280
YC69	0.5	320

Identification of bacterial isolates

YC7 and YC 10 showing maximum exopolysaccharide production in secondary screening were gram negative rods. YC7 and YC 10 were identified by some biochemical test (Table: 2) and by 16s rRNA Cronobacter dublinensis subsp. dublinensis DES187(T) and Cronobacter muytjensii ATCC 51329(T), respectively.

Table 2- Biochemical characteristics of YC7 and YC10 strains

characteristics	YC7	YC10
Indol	+	+
citrate	+	+
catalase	+	+
oxidase	-	-

+= Positive, -=Negative test.

CCGTGNGNCATATTGCATAATGGGCGCTAGCCTGATGCAG
CCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGT
ACTTTCAGCGAGGAGGAAGGGGTTAAGGTTAATAACCTTN
GCCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGT
TAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTG
CATTTGAAACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
GGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACT
GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGG
AGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT
TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGNTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGATGCACCGCGAA

Fig. 2 Nucleotide sequence of YC 7 strain

Table 3- Maximum identity table of YC7 strain

Strain Designation	Closest phylogenetic affiliation	Max ident
YC7	Cronobacter dublinensis subsp. dublinensis DES187(T) 16S ribosomal RNA gene partial sequence (EF059892)	98.38%

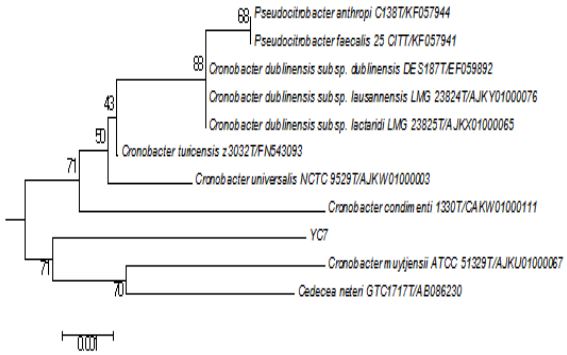

Fig. 3 Phylogenetic tree for YC7 strain

Identification of YC10 strain

GGAANCCATGCGNCATTTGCNTAATGGGCGCTAGCCTGATG
CAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAA
GTACTTTCAGCGAGGAGGAAGGGGTTAAGGTTAATAACCTT
GGCCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAA
GTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT
TGAAACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTC
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGC
CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACC
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAANAACCNTTACCTGGTCTTGACATCCAGA
GAATCCTGCAAA

Fig. 4 Nucleotide sequence of YC 10 strain

Table 4- Maximum identity table of YC10 strain

Strain Designation	Closest phylogenetic affiliation	Max ident
YC10	Cronobacter muytjensii ATCC 51329(T) 16S ribosomal RNA gene partial sequence (AJKU01000067)	97.88%

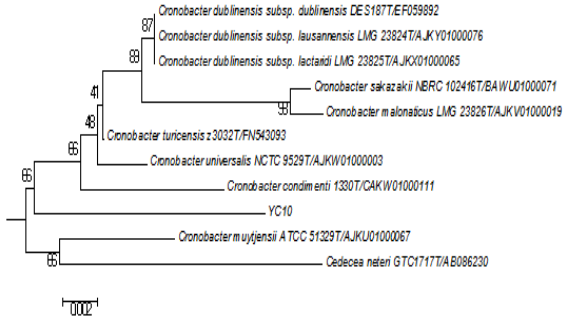

Fig. 5 Phylogenetic tree for YC10 strain

Effect of agri-cultural waste product on EPS production

Cane molasses as a carbon source replacing synthetic carbon sources gave maximum EPS production at 5% (4.25g/l) (Fig. 3).

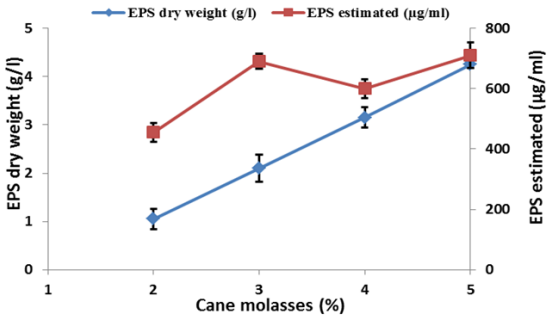

Fig. 6 Effect of cane molasses on EPS production

Effect of UV radiations on EPS production

UV exposed strains (Fig 4) showed enhanced EPS production (4.35g/l) then unexposed strains (3.3g/l) (Table.2).

Table 5- Effect of UV mutagenesis

Bacterial strain	EPS Dry weight (g/l)	EPS estimated(µg/ml)
Control	3.3	595
UVM 1	4.35	630
UVM 2	4.15	540
UVM 3	3.9	545

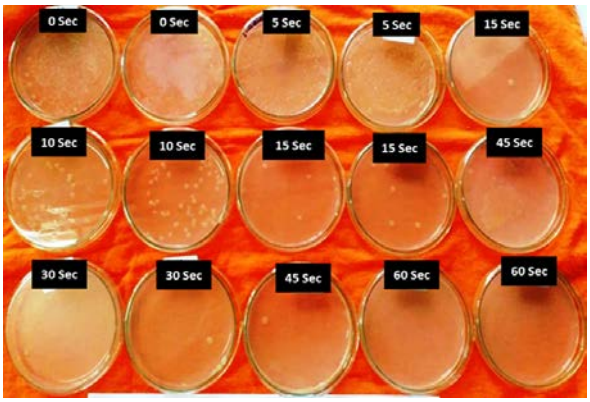

Fig. 7 Growth on UV exposed plates

Discussion

In this study, 72 bacterial strains isolated and screened for EPS production, out of which only two YC7 and YC10 showed maximum EPS production in secondary screening. YC7 and YC10 isolates were identified as Cronobacter dublinensis subsp. dublinensis DES187(T) and Cronobacter muytjensii ATCC 51329(T), respectively by 16s rRNA. Large, circular, mucoid colonies appeared white on pink colored background, and formed long viscous filament when taken with a loop which indicated EPS production.

The isolation of 24 non-rhizobial taxa was reported from nodules of different leguminous plants (Muresu et al. 2008). The bacteria isolated from legume tissues included Agrobacterium, Bacillus,

Enterobacter, Pseudomonas, and others. 15 endophytic non-rhizobial bacterial strains were isolated from surface sterilized root nodules of alfalfa legume, out of 15 bacterial strains, 5 strains were gram positive, three strains LR1k, 4148pk, and SNji were identified as Bacillus megaterium, and two strains 251s, 236 were closely related to Brevibacillus chosinensis and Microbacterium trichothecenolyticum showed some plant growth promoting potential (Olivera et al. 2009). 115 endophytic bacteria were isolated from root nodules of wild legume Sphaerophysa salsula. Out of 115 bacterial strains, 65 strains were non-symbiotic bacteria related to 17 species in genera Paracoccus, Sphingomonas, Inquilinus, Pseudomonas, Serratia, Mycobacterium, Nocardia, Streptomuces, Paenibacillus, Brevibacillus, and Bacillus which were universally coexistent with symbiotic bacteria in the nodules (Zhen et al. 2011).

A study about diversity and ubiquity of Cronobacter genus has been done, and it was found that Cronobacter strains might be of plant origin, and the rhizosphere might act as a reservoir of the bacterium (Kucerova et al. 2011). The evidences found that two strains of Cronobacter sakazakii were able to colonise plant roots of tomato and maize, and were able to produce indole acetic acid and siderophores, and also able to solubilize mineral phosphate (Schmid et al. 2009). Some physiological features, such as yellow pigment production, the formation of a gum-like extracellular polysaccharide, and the ability to persist in a desiccation, suggested an environmental niche for these organisms (Kucerova et al. 2011; Schmid et al. 2009). However, in the present study, 72 bacterial strains were isolated from root nodules of eight different leguminous plants, and screened for exopolysaccharide production. Out of 72 bacterial strains, 2 strains of YC7 and YC10 were identified as Cronobacter dublinensis subsp. dublinensis DES187(T) and Cronobacter muytjensii ATCC 51329(T) respectively by 16s rRNA. Cronobacter species as gram negative, non-spore forming, rod shaped, motile, facultative anaerobe, belongs to Enterobacteriaceae family and is catalase positive and oxidase negative (putthana et al. 2012). The organism was able to produce heteropolysaccharide capsule composed of glucuronic acid, D-glucose, D-galactose, D-fucose, and D-mannose. The strains from NICU outbreaks produced so much polysaccharide on milk agar plates which have been patented as thickening agent in food (Scheepe et al. 1986).

Total 10 bacterial strains from root nodules of Vigna mungo were isolated and designated vm1 to vm10, and screened for EPS production in YEM broth. Among 10 isolates, vm6 showed maximum EPS production (1680µg/ml) (Nirmala et al. 2011). Five bacterial strains from the root nodules of Tephrosia purpurea were isolated and checked for their antibacterial activity against human pathogens. Out of five isolates, two isolates having good antibacterial activity were used for further study, and identified as Bacillus licheniformis and Klebsiella pneumonia. These two strains showed good EPS production, Klebsiella pneumonia showed 210mg/ml before dialysis and 12.3mg/ml after dialysis, and Bacillus licheniformis showed 70mg/ml before dialysis and 10.97mg/ml after dialysis (Balamurugan & Prakash 2012). However, in the present study YC7 and YC10 showed maximum exopolysaccharide production as 3.6g/l and 3.0 g/l as per dry weight, and 890 µg/ml of exopolysaccharide was estimated by phenol sulphuric acid assay, respectively.

In the present study, different agricultural waste products were used as carbon sources instead of synthetic carbon sources. The effects of these different agricultural waste products on EPS production was examined. Clarified cane molasses and finely powdered rice bran were used with different concentrations in the medium. Cane molasses as a carbon source replacing synthetic carbon sources gave maximum EPS production at 5% (4.25g/l as per dry weight and 710µg/ml estimated by assay method). The effect of different raw agricultural wastes as a carbon source for EPS production was tested. 2% cane molasses produced maximum EPS yielded 4.86 g/l, and rice bran produced maximum EPS 2.14 g/l at 5% concentration (Sirajunnisa et al. 2013). Cane Molasses has significant growth stimulatory effects, as it possesses high vitamin and mineral contents, and also has a significant growth stimulatory effect (Liu et al. 2011). Due to its many advantageous properties like ready availability, ease of storage, high sucrose and other nutrient contents, and the low cost, molasses has been used as a substrate for fermentation production of commercial polysaccharides like curdlan, xanthan, dextran, scleroglucan, and gellan (Mao et al. 2011). B. cereus B-11 produced biopolymers in a medium containing molasses waste-water, replacing glucose as a carbon source yielding 500 mg/L EPS. The same experiments with rice bran instead of cane molasses as carbon substrate were repeated in which rice bran gave inefficient production. This was clearly indicated that cane molasses gave maximum EPS production due to the high sucrose content.

UV radiation showed positive effects on EPS production upon exposure to UV radiation after regular time intervals at a distance of 30 cm. The bacterial strains exposed under UV for 10 seconds, 15 seconds and 30 seconds were selected for EPS production showed enhanced EPS production (4.35g/l dry weight) than unexposed strain (3.3g/l EPS dry weight). The effect of UV radiations on growth and EPS synthesis was negative, decreasing the growth rate and quantity of EPS products (Sirajunnisa et al. 2013).

Further study should be carried out on optimization of different parameters for maximum exopolysaccharide production and characterization of exopolysaccharide.

Conclusion

It could be concluded from the present work that efficient exopolysaccharide producing bacterial strains were successfully isolated from root nodules of leguminous plants. Cane molasses would be a better carbon source compared to the other synthetic carbon sources. The effect of UV mutagenesis was positive on exopolysaccharide production.

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