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ORIGINAL ARTICLE

Production of Antioxidant Exopolysaccharide from *Pseudomonas aeruginosa* Utilizing Heavy Oil as a Solo Carbon Source

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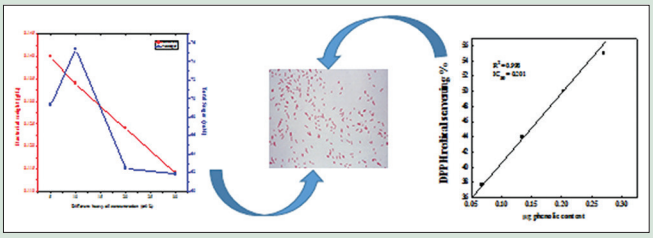
ABSTRACT

Aim/Background: *Pseudomonas aeruginosa* is capable of utilizing heavy oil hydrocarbons as a sole carbon source. *P. aeruginosa* produce exopolysaccharide (EPS) in an inorganic medium in the presence of crude oil. Several environmental factors affect the majority of EPS production. **Materials and Methods:** Strain of *P. aeruginosa* were managed under various media (maintenance medium, inoculum, and basal media). Different heavy petroleum oil concentrations (5, 10, 20, and 30 ml/L) were used as solo carbon source to the basal medium. Various conditions of bacterial growth were monitored. The growth of cells was estimated by measuring the absorbance of the mixture of 1 ml of the basal medium diluted with 1 ml of distilled water at 600 nm spectrophotometrically. *P. aeruginosa* was grown aerobically in a production medium at 37°C and 150 rpm on a rotary shaker. The culture broth was centrifuged to separate the cells. The precipitated polysaccharide was separated by centrifugation and washed with ethanol, acetone, and ether, and then dried under reduced pressure oven at 45°C. The DPPH test was carried out as described by Burits and Bucar to monitor the free radical scavenging activities of the extracts. **Results:** The preferable culture conditions for EPS production were at 10 ml/L heavy oil, with 0.5 g/L $NaNO_3$ as best N sources at pH 6.0 after 5 days incubation. The net weight of purified EPS production was 0.5 g/L. **Conclusion:** The obtained polysaccharide showed antioxidant activity that possesses DPPH radical scavenging activity, with an EC_{50} = 0.201.

Key words: Environmental factors, exopolysaccharides, heavy oil hydrocarbons, *Pseudomonas aeruginosa*

SUMMARY

Pseudomonas aeruginosa is capable of utilizing heavy oil hydrocarbons as a sole carbon source. *P. aeruginosa* produced exopolysaccharide (EPS) in an inorganic medium in the presence of crude oil. Several environmental factors affect the maximum EPS production. The preferable culture conditions for EPS production were at 10 ml/L heavy oil, 0.5 g/L $NaNO_3$ (as best N source) and at pH 6.0 after 5 days of incubation. The net weight of purified EPS production was 0.5 g/L. This polysaccharide shows antioxidant activity that possesses DPPH radical scavenging activity, with an EC_{50} = 0.201.



Abbreviations Used: DPPH: 2,2-diphenyl-l-picrylhydrazyl; EPS: Exopolysaccharide; GAE: Gallic acid equivalent; TPC: Total phenolic content; NaOH: Sodium hydroxide.

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INTRODUCTION

Raw petroleum is a standout among the essential vigorous assets on the planet. Both aliphatic and aromatic hydrocarbons are the major sources of carbon for micro-organisms in crude oil. Organic compounds include organic acids such as acetic, benzoic, butyric, formic, propanoic, and naphthenic acids reaching up to 100 mM produced from the biodegradation of crude oil.[1] About 22 genera of microscopic organisms including *Pseudomonas, Aeromonas, Bacillus, Flavobacterium, Corynebacterium, Micrococcus* are known to consume oil hydrocarbons. *Pseudomonas aeruginosa* is the most dynamic hydrocarbon utilizer in raw petroleum. Past perceptions have distinguished the Pseudomonas class most effectively among hydrocarbon-degrading micro-organisms.[2-4] *P. aeruginosa* can use natural oil, aliphatic, monoaromatic hydrocarbons and alcohols as the solo carbon source. *P. aeruginosa* is additionally ready to tolerate and develop in high fixations (up to half v/v) of unrefined petroleum.[5] Micro-organisms produce exopolysaccharide (EPS) to perform various functions, for instance, biofilm arrangement[6] resistance to hydrocarbons,[7] cryoprotectants,[8] shield against antimicrobials,[9] collection and biofouling,[10] and bioleaching of metals.[11] Conversely, other bacterial EPSs possess unique properties that can launch a range of new commercial opportunities (e.g., bacterial cellulose or levan).[6,7] A few types of microscopic organisms of the genera *Azotobacter* and *Pseudomonas* produce an EPS that looks to some extent like that of algal production. Biofilm belongs to the bacterium community which adhere to the biotic and abiotic surface and embed in a polymeric matrix composed mainly of polysaccharides, proteins, and nucleic acids.[12] As the creation of the alginate polymer finds a critical place in lung

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contaminations found in cystic fibrosis patients, the alginate generation of *P. aeruginosa* has been the subject of extreme investigation.[13,14] Subsequently, the use of bacterial strains could be stretched out to the creation of alginate, as has been feasible for different polysaccharides of financial significance, for example, gellan, hyaluronic corrosive, and xanthan, which are acquired through bacterial maturation.[15] Using antioxidants from microbial source has been as early as in the 1980s; however, the relationship between micro-organisms and antioxidants was established only in the beginning of this century.[16] About nine genera of bacteria, including both Gram-positive and Gram-negative, were found to produce the compound with antioxidant activity. The maximum activity was found in the methanol fractions of three *Pseudomonas* species.[17]

This work aims to produce EPSs by *P. aeruginosa* from the heavy crude oil hydrocarbon as a solo carbon source and investigate some environmental conditions for the optimization of EPSs and evaluating their antioxidant activity.

MATERIALS AND METHODS

Materials

The crude oil was obtained from King Abdulaziz University, Faculty of Engineering, Chemical Engineering Department, and the strain of *P. aeruginosa* was obtained from Al-Hada Military Hospital-Microbiology Department, Saudi Arabia.

Methodology

Media

Maintenance medium (g/L)

10 g of meat extract, 10 g of peptone, 5 g of NaCl, and 20 g of agar were dissolved in 1 L, at pH 7. The slants were inoculated with bacterial strain and then incubated at 37°C for 24 h. The slants were then maintained at 0°C–4°C in refrigerator. All chemicals were obtained from Sigma Aldrich, UK.

Inoculum medium (g/L)

10 g of meat extract, 10 g of peptone, 5 g of NaCl were dissolved in 1 liter, at pH 7. The medium was portioned into 500 ml. Each Erlenmeyer flask contained 100 ml of the medium and sterilized. The flasks were inoculated with a slant of *P. aeruginosa* and incubated at 37°C on a rotary shaker (150 rpm) for 24 h.

Basal medium

Unless stated otherwise, the bacterium will be grown in shake flasks in a basal medium of the following composition (g/L).[18]

1, KH_2PO_4; 2, K_2HPO_4; 1, $NaNO_3$; 1.5, yeast extract; 0.21, $MgSO_4$ $7H_2O$. The basal medium will be supplemented with 10 ml heavy crude oil, tween 80 3 ml, and xylene 20 ml. The pH of the medium will be set at pH 7.0. The basal medium was portioned into 250 ml. Conical flasks contained 50 ml of the medium and sterilized. The sterile medium was inoculated at 10% (v/v) level and incubated at 37°C on a rotary shaker (150 rpm) for 48 h. The samples were removed at intervals for the determination of total sugar and growth of cells.

Different heavy petroleum oil concentrations (5, 10, 20, and 30 ml/L) were added to the basal medium as a solo carbon source.

Effect of various nitrogen sources on polysaccharide production by Pseudomonas aeruginosa

The effect of different nitrogen sources on EPS production was studied by the addition of 0.5 g of $(NH_4)_2SO_4$, $NaNO_3$, NH_4CL, and NH_4NO_3 on basal medium. The control medium did not contain any nitrogen source. EPS were produced after 96 h of incubation at 37°C.

Effect of the best concentration of $NaNO_3$ on polysaccharide production by Pseudomonas aeruginosa

The effect of the best concentration of $NaNO_3$ on EPS production was studied by the addition of 0.5, 1, 1.5, and 2 g/L of sodium nitrates $NaNO_3$ on basal medium. Control medium did not contain any nitrogen source.

Effect of pH on polysaccharide production by Pseudomonas aeruginosa

Initial pH of the medium that could support maximal EPS production by *P. aeruginosa* was worked out. The basal medium was adjusted from 5.0, 6.0, 7.0, 8.0, to 9.0 with either 1 N NaOH or HCl.

Effect of different temperatures on polysaccharide production by Pseudomonas aeruginosa

The basal medium was portioned into three Erlenmeyer flasks (250 ml), each containing the same volume of the fermentation medium (50 ml) and different temperatures (30°C, 37°C, and 45°C) to investigate the effect of temperature on polysaccharide production by *P. aeruginosa*.

Chemical analyses

Growth estimation

Growth of cells was estimated by measuring the absorbance of the mixture of 1 ml of the basal medium diluted with 1 ml of distilled water at 600 nm spectrophotometrically. 100 ml of standard culture medium after fermentation was measured at 600 nm spectrophotometrically. The cultured medium was centrifuged; the precipitated cells were washed several times in petroleum ether (40°C–60°C) to remove the oil residue. The cells were transferred in a Petri dish and then transferred to an oven at 100°C until constant weight. The Optical Density (OD) of the test medium was compared with that of the standard medium; the dry weight of cells was calculated. The blank contained no inoculated fermentation medium.[19]

Determination of total sugars

Total hydrolysable carbohydrates were determined by phenol sulfuric acid method according to DuBois *et al*.[20]

Isolation and purification of exopolysaccharide

P. aeruginosa were grown aerobically in a production medium at 37°C and 150 rpm on a rotary shaker. The culture broth was centrifuged to separate the cells. The cell-free supernatant was subjected to protein denaturation by the addition of trichloroacetic acid (TCA) at a final concentration of 10%. The supernatant was neutralized with NaOH (Freitas *et al*., 2009). Then, the solution was centrifuged, and the precipitate protein was discarded. The supernatant was dialyzed in a dialysis tube against running tap water for 48 h and then distilled water for 24 h. The dialyzed solution was concentrated under reduced pressure at 40°C. Ethanol was added to reach a concentration of ethanol:supernatant 3:1 v/v, and the mixture was left overnight in the refrigerator. The precipitated polysaccharide was separated by centrifugation and washed with ethanol, acetone, and ether and then dried in a reduced-pressure oven at 45°C.

The molecular weight of Molecular Weight of Polypeptides (MEPS) was determined on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a Refractive Index Detector and FPl gel particle size (5 μm), 3 columns of pore type (100, 104, 105A°) on series, length 7.5 mm × 300 mm (1000–5000,000) were used for DMF solvent Styrogel HR-DMF, 3 μm (7.8 mm × 300 mm) Water Company Ireland. One column (5000–600,000) for water solvent (polyethylene oxide/glycol standard) PL aquagel-OH 7.5 mm and 30 um pore type 8 um particle size. PL aqua gel-OH 7.5 mm, 50 μm pore type, 8 μm particle size, in series Mw from 100 to 1250000 g/moL. The sample (0.01 g) was dissolved in 2 mL of the solvent and then filtered by siring filter 0.45 then the sample but in GPC

device (Waldbronn, Germany). The polydispersity index was calculated from the Mw/Mn ratio.

Total phenolic content assay

The total phenolic contents were determined spectrophotometrically according to Folin–Ciocalteu's (FC) as indicated by Kähkönen *et al.*[21] Gallic acid was used as standard unit for total phenolic content determination because it covers a wide spectrum of phenolic compounds.

Determination of free radical scavenging activities of the extracts

The 2,2-diphenyl-l-picrylhydrazyl (DPPH) test was carried out as described by Burits and Bucar.[22] 1 mL of fermented date seed extract at different concentration was mixed with 1 mL of DPPH reagent (0.002% [w/v]/methanol water solution). After an incubation period (30 min), the absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The results were expressed in percentage of radical scavenging activity, calculated using the following formula:

$$\%\,\text{radical scavenging activity} = \frac{(\text{A Blank} - \text{A Sample})}{\text{A Blank}} \times 100\%$$

A blank refers to the absorbance of the blank control, whereas a sample is the absorbance of the tested samples.

RESULTS AND DISCUSSION

Effect of different heavy oil concentrations on exopolysaccharide production by *Pseudomonas aeruginosa*

Different concentrations of heavy oil (5, 10, 20, and 30) were added to the fermentation medium. Results presented in [Figure 1] indicate that the growth of cells was found to be optimum at 5 mL heavy oil/L (0.14 g/L) and then the growth of cells decreases gradually as the concentration of oil increases. The output of total sugars (EPS) increased with increasing heavy oil concentrations reaching its maximum (75 mM) at 10 ml heavy oil/L. Numerous examinations show the influence of the type of carbon source on EPS production.[23,24] Similar results were found by Sonawdekar and Gupte[25] who cited that maximum amount of EPS was obtained by using 0.5% sodium nitrate at pH 7 in 48 h, in the presence of engine oil as a single carbon source. Sivakumar *et al.*[26] observed that the hydrocarbon neem oil produced the maximum amount of EPS.

Effect of various nitrogen sources on the production of exopolysaccharide by *Pseudomonas aeruginosa*

Among the nitrogen sources tested [Figure 2], $NaNO_3$ supported maximum total sugars (236 mM), whereas $(NH_4)_2SO_4$ showed minimum EPS production (26 mM). On the other hand, NH_4CL produced the best bacterial cells (0.49 g/L) although our results indicated that $NaNO_3$ produced maximum EPS. Conti *et al.*[27] referred the maximum EPS production by *P. fluorescens* using NH_4Cl as nitrogen source.

Effect of different sodium nitrate ($NaNO_3$) concentrations on the exopolysaccharide production by *Pseudomonas aeruginosa*

Results presented in Figure 3 indicate that at the concentration of 0.5 g/L from $NaNO_3$, EPS output was increased to the maximum (28.17 mM). The further increase in $NaNO_3$ leads to decrease in the EPS production. On the other hand, at concentration of 1 g/L, $NaNO_3$ gave optimum cell growth at 0.306 g/L. The same results were reported by Sonawdekar and Gupte[25] who cited that maximum amount of EPS using 0.5% sodium nitrate from engine oil as a solo carbon source.

Effect of yeast extraction concentration on polysaccharide production by *Pseudomonas aeruginosa*

Different concentrations of yeast extract of 0, 0.5, 1, 1.5, and 2 g/L were added to the fermentation medium. The results presented in [Figure 4] indicate that the bacterial cells increased gradually as the concentration of yeast extract increased. At 2 g/L yeast extract, optimum bacterial cell growth (0.16 g/L) were detected. The output of total sugars (EPS) increased with increasing yeast extract concentrations reaching its maximum (82.1 mM) at 1.5 g/L yeast extract. Tanaka *et al.*[28] found that addition of natural nutrient, such, as yeast extract and meat extract was essential for polysaccharide formation by *Pseudomonas sp*. Raza *et al.*[17]

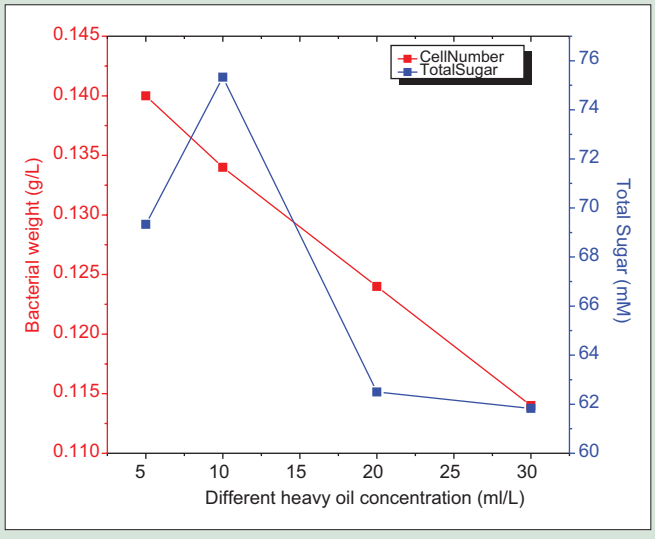

Figure 1: Effect of different heavy oil concentration on exopolysaccharide production by *Pseudomonas aeruginosa*

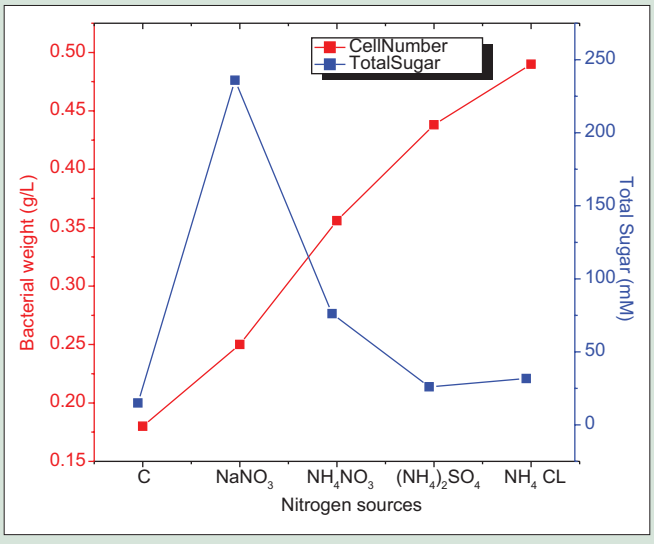

Figure 2: Effect of various nitrogen sources on the production of exopolysaccharide by *Pseudomonas aeruginosa*

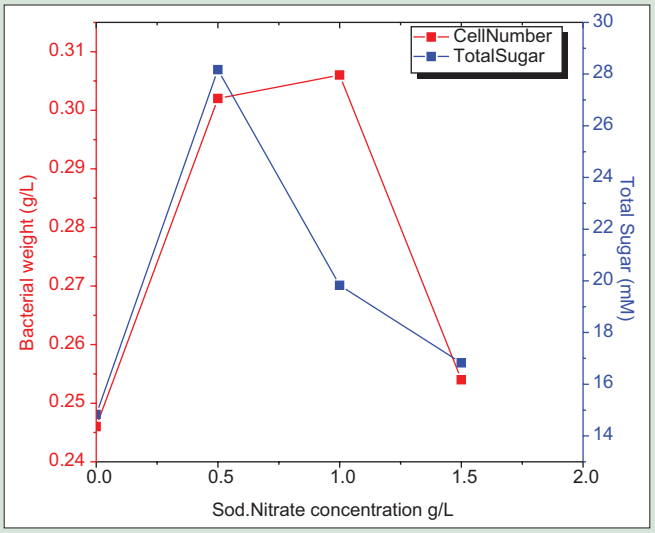

Figure 3: Effect of various concentrations of sodium nitrates on the exopolysaccharide production by *Pseudomonas aeruginosa*

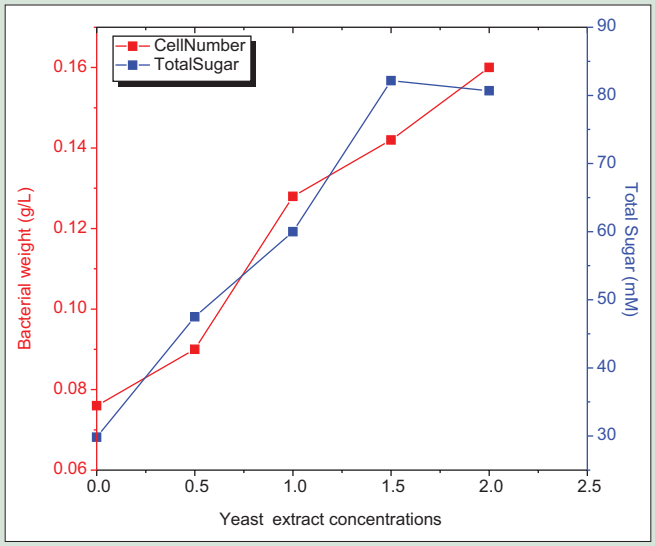

Figure 4: Effect of yeast extract concentration on polysaccharide production by *Pseudomonas aeruginosa*

reported that *P. polymyxa* SQR-21 produced one type of EPS using yeast extract and galactose as the best N and C sources, respectively. Their EPS showed good superoxide scavenging and moderate inhibition of lipid peroxidation and reducing activities.

Effect of pH on exopolysaccharide production by *Pseudomonas aeruginosa*

The results obtained [Figure 5] showed little increase in the final pH values of the fermentation medium. The bacterial cells were diminished at pH (5 and 9) to about 0.176 and 0.013 g/L, respectively. Optimum cell growth was obtained at pH 6 (0.27 g/L). Maximal EPS production (as total sugars) was also recorded at an initial pH of 6.0 (70.83 mM). At acidic pH, the total sugars (44.33 mM) were more than that found in alkaline pH. The initial pH of liquid culture is important as it may affect the cell growth, EPS production, and uptake of different nutrients.[29] Similar results were reported by Sivakumar *et al.*[26] who observed that the maximum EPS production was at pH 6.0. On the other hand, Bueno and Garcia-Cruz[30] reported that the maximum EPS production by *Pseudomonas* sp. at pH 7.

Effect of temperature on polysaccharide production by *Pseudomonas aeruginosa*

The fermentation medium (50 ml) was incubated at different temperatures (30°C, 37°C, and 45°C). Results in [Figure 6] indicate that the bacterial cells were found to be optimum at 37°C (0.088 g/L) and then the growth of cells decreased gradually as the temperature increased. The output of total sugars (EPS) was at its maximum production at 30°C (67.2 mM). Vijayabaskar *et al.*[31] and Abdul Razack[32] referred that 37°C was the optimum temperature for EPS, which is not consistent with our study. Similar results were obtained by Gao *et al.*,[33] who observed that the optimum temperature parameter for the EPS production was 25°C.

Effect of different fermentation periods on the production of exopolysaccharide by *Pseudomonas aeruginosa*

Results documented in [Figure 7] clearly testify that the microbial biomass approximately increased in the first 2 days late logarithmic phase and beginning of stationary phase (0.17 g/L). The bacterial mass remains stable after that and finally decreases to 0.12 g/L. Further, the results presented in Figure 7 also suggest that the optimum EPS (total sugars) production output was found after 5 days (100.33 mM) and then the polysaccharide decreased to 79.5 mM after 6 days. Our results were in accordance with the results of a study[34] in which the EPS was produced after 4 days. On the other hand, Raza *et al.*[29] and Conti *et al.*[27] produced EPS after 72 h and 50 h, respectively.

Purified *Pseudomonas aeruginosa* polysaccharide

The culture broth of *P. aeruginosa* was centrifuged to separate the cells. The supernatant was treated with trichloroacetic acid to separate the protein and then the supernatant was neutralized with NaOH. Then, the solution was centrifuged, and the precipitate protein was discarded. The supernatant was dialyzed, and the dialyzed solution was concentrated at a reduced pressure at 40°C. Ethanol was added to the mixture and was left overnight in a refrigerator. The precipitated polysaccharide was separated by centrifugation and washed with ethanol, acetone, and ether and then dried in a reduced-pressure oven at 45°C. The net weight of polysaccharide was 0.5 g/L.

Total phenolic content

The total phenolic contents were determined in purified EPS solution produced by *P. aeruginosa* that growing in culture medium containing heavy oil. It was found that the total phenol compounds 0.203 µg/150 µg total phenol compounds were recorded in EPS solution that were measured by FC reagent in terms of gallic acid equivalence.

DPPH radical scavenging activity

The *in-vitro* antioxidant activity of the isolated EPS was determined by DPPH free radical scavenging ability. DPPH is one of the compounds that has a proton-free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers.[35] Furthermore, it is well accepted that the DPPH free radical scavenging activity by antioxidants is due to their hydrogen-donating ability. The findings of the present study showed that EPS isolated from *P. aeruginosa* had a noticeable DPPH free radical scavenging activity [Figure 8]. It was also observed that the DPPH scavenging activity increased in a dose-dependent manner (0–135 µg/mL). It was assumed that the

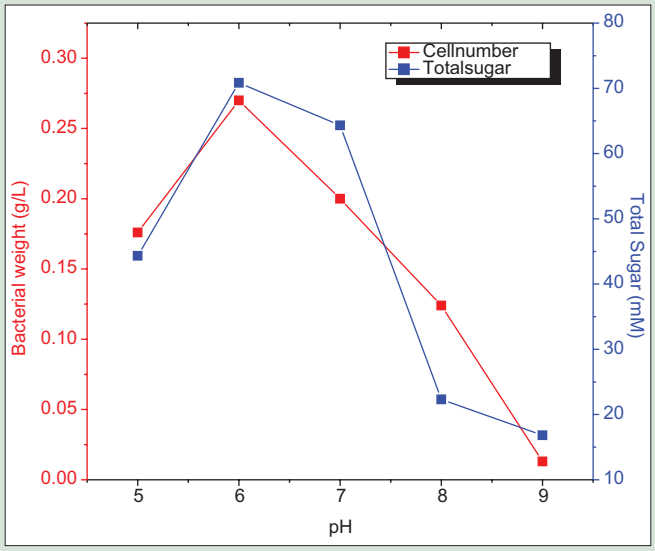

Figure 5: Effect of pH on exopolysaccharide production by *Pseudomonas aeruginosa*

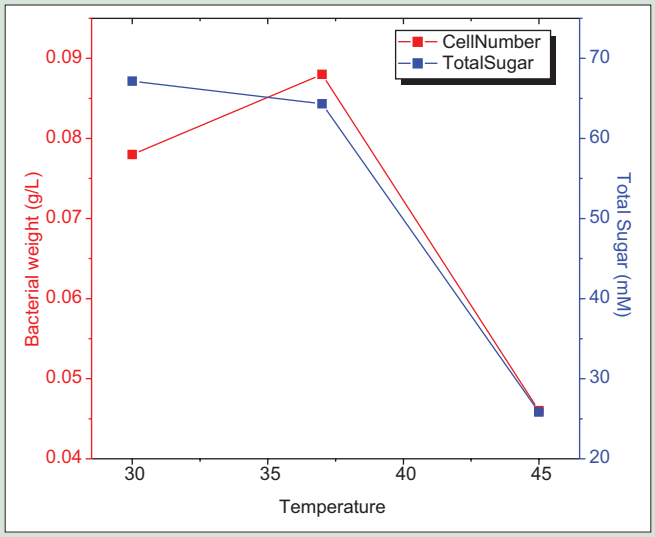

Figure 6: Effect of temperature on polysaccharide production by *Pseudomonas aeruginosa*

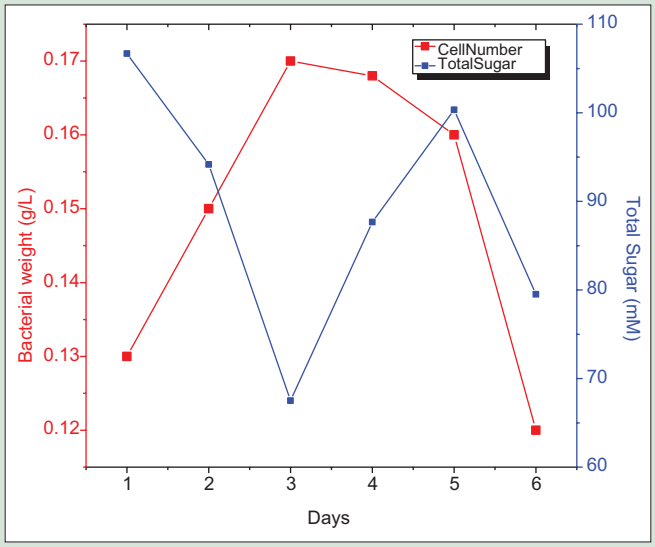

Figure 7: Effect of fermentation periods on exopolysaccharide production by *Pseudomonas aeruginosa*

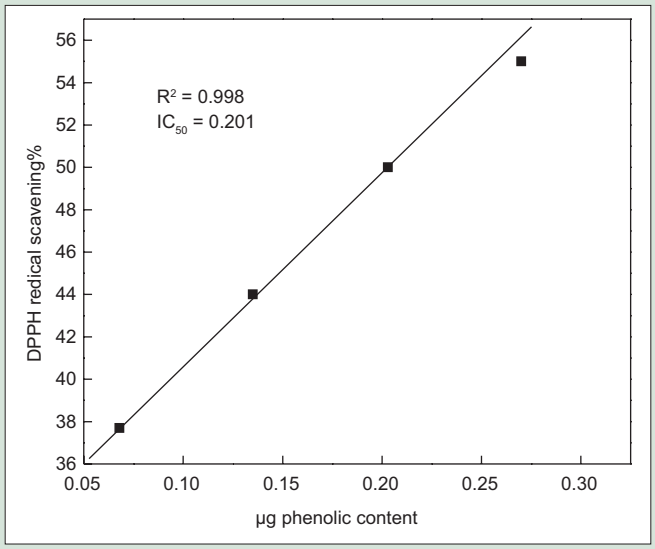

Figure 8: 2,2-Diphenyl-l-picrylhydrazyl free radical scavenging ability of *Pseudomonas aeruginosa* exopolysaccharide produced from heavy hydrocarbon oil

isolated EPS somehow donates hydrogen ions to react with the DPPH radical. The greatest scavenging rate of *P. aeruginosa* EPS was 55%, which was lower than the 72% observed for *Serratia ureilytica* TKU013[36] and 77% for *Paenibacillus* sp. TKU023.[35] The half-maximal effective concentration of *P. aeruginosa* EPS (IC_{50} = 0.201) is shown in Figure 8. TKU032 EPS was a potent and natural antioxidant that could be used as an alternative to synthetic antioxidants.

CONCLUSION

The present work dealt with the production of EPS from *P. aeruginosa* and optimization of environmental parameters for its production. Our study reported that for the production of 0.5 g/L yield of purified EPS, 10 ml/L heavy oil and 0.5 g/L $NaNO_3$ (as best N source) are required at pH 6.0 after 5 days of incubation period. This polysaccharide showed antioxidant activity that possesses DPPH radical scavenging activity, with an EC_{50} = 0.201.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Wolicka A, Borkowski A. Introduction for Enhanced Oil Recovery. Rijeka, Croatia: InTech Europe; 2012.
2. Lal B, Khanna S. Degradation of crude oil by *Acinetobacter calcoaceticus* and

Alcaligenes odorans. J Appl Bacteriol 1996;81:355-62.
3. Banat IM, Makkar RS, Cameotra SS. Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 2000;53:495-508.
4. Saadoun I. Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. J Basic Microbiol 2002;42:420-8.
5. Prakash B, Irfan M. *Pseudomonas aeruginosa* is present in crude oil contaminated sites of Barmer region (India). J Bioremed Biodegrad 2011;2:129. doi:10.4172/2155-6199.1000129.
6. Kreft JU, Wimpenny JW. Effect of EPS on biofilm structure and function as revealed by an individual-based model of biofilm growth. Water Sci Technol 2001;43:135-41.
7. Aizawa T, Neilan BA, Couperwhite I, Urai M, Anzai H, Iwabuchi N, *et al*. Relationship between extracellular polysaccharide and benzene tolerance of *Rhodococcus* sp 33. Actinomycetologica 2005;19:1-6.
8. Kim SJ, Yim JH. Cryoprotective properties of exopolysaccharide (P-21653) produced by the *Antarctic* bacterium, *Pseudoalteromonas arctica* KOPRI 21653. J Microbiol 2007;45:510-4.
9. Kumon H, Tomochika K, Matunaga T, Ogawa M, Ohmori H. A sandwich cup method for the penetration assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. Microbiol Immunol 1994;38:615-9.
10. Adav SS, Lee DJ. Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. J Hazard Mater 2008;154:1120-6.
11. Michel C, Bény C, Delorme F, Poirier L, Spolaore P, Morin D, *et al*. New protocol for the rapid quantification of exopolysaccharides in continuous culture systems of acidophilic bioleaching bacteria. Appl Microbiol Biotechnol 2009;82:371-8.
12. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623-33.
13. Gacesa P, Goldberg JB. Heterologous expression of an alginate lyase gene in mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. J Gen Microbiol 1992;138 Pt 8:1665-70.
14. Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, Green CG, *et al*. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. JAMA 2005;293:581-8.
15. Martins LO, Sá-Correia I. Alginate biosynthesis in mucoid recombinants of *Pseudomonas aeruginosa* overproducing GDP-mannose dehydrogenase. Enzyme Microb Technol 1991;13:385-9.
16. Janardhanan S, Mahendra J, Girija AS, Mahendra L, Priyadharsini V. Antimicrobial effects of *Garcinia mangostana* on cariogenic microorganisms. J Clin Diagn Res 2017;11:ZC19-22.
17. Raza W, Makeen K, Wang Y, Xu Y, Qirong S. Optimization, purification, characterization and antioxidant activity of an extracellular polysaccharide produced by *Paenibacillus* polymyxa SQR-21. Bioresour Technol 2011;102:6095-103.
18. Kita Y, Nakanishi I, Isono M. Isolation and characterization of biologically active polysaccharides produced by *Serratia piscatorum*. Agric Biol Chem 1974;38:423-31.
19. Hoischen C, Krämer R. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium* glutamicum. J Bacteriol 1990;172:3409-16.
20. DuBois KM, Gilles A, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.
21. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, *et al*. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 1999;47:3954-62.
22. Burits M, Bucar F. Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 2000;14:323-8.
23. Miqueleto AP, Dolosic CC, Pozzi E, Foresti E, Zaiat M. Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. Bioresour Technol 2010;101:1324-30.
24. Wang X, Xu P, Yuan Y, Liu C, Zhang D, Yang Z, *et al*. Modeling for gellan gum production by *Sphingomonas paucimobilis* ATCC 31461 in a simplified medium. Appl Environ Microbiol 2006;72:3367-74.
25. Sonawdekar S, Gupte A. Production and characterization of exopolysaccharide produced by oil emulsifying bacteria. Int J Curr Microbiol App Sci 2016;5:254-62.
26. Sivakumar T, Sivasankara Narayani S, Shankar T, Vijayabaskar P. Optimization of cultural conditions for exopolysaccharides production by *Frateuria aurentia*. Int J Appl Biol Pharm Technol 2012;3:133-43.
27. Conti E, Flaibani A, O'regan M, Sutherland IW. Alginate from *Pseudomonas fluorescens* and *P. putida*: Production and properties. Microbiology 1994;140:1125-32.
28. Tanaka H, Okuda T, Asai K. On Fuzzy-Mathematical Programming. J of Cybernetics 1974;3:37-46.
29. Raza W, Yang W, Jun Y, Shakoor F, Huang Q, Shen Q, *et al*. Optimization and characterization of a polysaccharide produced by *Pseudomonas fluorescens* WR-1 and its antioxidant activity. Carbohydr Polym 2012;90:921-9.
30. Bueno SM, Garcia-Cruz CH. Optimization of polysaccharides production by bacteria isolated from soil. Braz J Microbiol 2006;37:296-301.
31. Vijayabaskar P, Babinastarlin S, Shankar T, Sivakumar T, Anandapandian KTK. Quantification and characterization of exopolysaccharides from *Bacillus subtilis* (MTCC 121). Adv Biol Res 2011;5:71-6.
32. Razack SA, Velayutham V, Thangavelu V. Influence of various parameters on exopolysaccharide production from *Bacillus subtilis*. Int J ChemTech Res 2013;5:2221-8.
33. Gao J, Gu F, Abdella NH, Ruan H, He G. Optimisation of exopolysaccharide production by *Gomphidius rutilus* and its antioxidant activities *in vitro*. Carbohydr Polym 2012;87:2299-305.
34. Hereher F, ElFallal A, Abou-Dobara M, Toson E. Cultural optimization of a new exopolysaccharide producer "*Micrococcus roseus*". Beni-Suef University J Basic Appl Sci 2018;7:632-9.
35. Wang Z, Sheng J, Tian X, Wu T, Liu W, Shen L. Optimization of the production of exopolysaccharide by *Bacillus thuringiensis* 27 in sand biological soil crusts and its bioflocculant activity. Afr J Microbiol Rse 2011;5:2359-66.
36. San-Lang W, Kao-Cheng L, Tzu-Wen L, Yao-Haur K, Chen-Yu W. *In vitro* antioxidant activity of liquor and semi-purified fractions from fermented squid pen biowaste by *Serratia ureilytica* TKU013. Food Chem 2010;119:1380-5.