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Partial Structural Linkages and Physico-chemical Activities of an Extracellular Polysaccharide Produced by *Pseudomonas fluorescens* Strain WR-1

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Abstract

The extracellular polysaccharide produced by *Pseudomonas fluorescens* strain WR-1 was partially characterized structurally and its physico-chemical activities were determined. The results showed that the EPS consisted of (1→4)-linked-glucose backbone branched with (1→4, 6)-linked-glucose and (1→4)-linked-arabinose. The physico-chemical activity analyses showed that the EPS of strain WR-1 presented good flocculating, metal chelating and hydroxyl radical scavenging activities while moderate lipid emulsification, superoxide radical scavenging and lipid peroxidation inhibitory activities were observed. These results revealed that the EPS of WR-1 has great potential to replace synthetic chemicals in industry. © 2014 Friends Science Publishers

Keywords: Antioxidant activity; Extracellular polysaccharides; Glucose backbone; Structural linkages

Introduction

Microbes play a central role in biotechnology, not only as convenient tools but also as organisms that can be improved to serve a particular idea (Atawodi *et al*., 2013). Therefore, there is need to find more and more new microbial species to improve and meet the demands of increasing microbial products. Among microbes, the genus *Pseudomonas* has potentially been utilized in a number of different biotechnological applications. *Pseudomonas* strains have been shown to be of significance in bioremediation as a result of their wonderful ability for biodegradation (Kim *et al*., 2005). They also offer substantial role in agronomic applications, since many strains are bioactive, fast-growing, great colonizers of plant roots and are able to suppress the pathogenic microorganisms by producing antibiotics and hydrolytic enzymes (Walsh *et al*., 2001).

Among *Pseudomonas* species, *P. fluorescens* is rod-shaped, Gram-negative bacterium that has very versatile metabolism. These strains are obligate aerobes and can be found in soil and water. There are also some strains of *P. fluorescens* that can use nitrate in place of oxygen (Palleroni *et al*., 1984). Some *P. fluorescens* strains showed good biocontrol properties against plant pathogens (Haas and Keel, 2003). Extracellular polysaccharides (EPS) with good physiological and biological potentials were also produced by some of the *P. fluorescens* strains (Mao *et al*., 2010; Raza *et al*., 2012). The EPS with adhesive properties was isolated from *P. fluorescens* (Read and Costerton, 1987) and the ability of EPS produced by *P. fluorescens* PF01 to remove Cu^{2+} was investigated (Mao *et al*., 2010).

We isolated a new strain of *P. fluorescens* WR-1 (WR-1) and purified EPS produced by this strain and optimized its production. The polysaccharide was comprised of glucose, arabinose and glucuronic acid. The optimum production of this EPS in liquid culture was achieved with maltose, Zn^{2+} and Mn^{2+}. The EPS presented moderate reductive ability and free radical scavenging activity while good H_2O_2 scavenging activity was found (Raza *et al*., 2012). In this study, the EPS of WR-1 was further characterized for its partial structural linkages information and physico-chemical activities to better understand its biological and physiological potential. This will help to find out new polysaccharides with efficient biological properties to substitute synthetic chemicals.

Materials and Methods

Microbial Strain, Culture Medium and Protein Analysis

The WR-1 strain of *P. fluorescens* (Gene Bank accession No. JQ317786) was previously isolated from the rhizosphere of muskmelon. The strain was grown in tryptic soya agar plates and stored at 4ºC. Previously purified EPS was used in this experiment (Raza *et al*., 2012) and the protein concentration of EPS was also estimated (Lowry *et al*., 1951).

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Structural Characterization

The FT-IR spectrum of EPS produced by WR-1 was determined on a Tensor-27 FT-IR spectrometer (Bruker Optics, Wissembourg, France). The EPS sample was pulverized with KBr into pellet and in 4000–400 cm^{-1} frequency range spectrum was determined.

The EPS was methylated (Ciucanu and Kerek, 1984) and FT-IR spectrum analysis was performed again to confirm complete methylation. Then the permethylated EPS was hydrolyzed with $C_2HF_3O_2$ (60°C for 30 min), reduced with $NaBH_4$ and then acetylated with pyridine and acetic anhydride (Wack and Blaschek, 2006). The resulting residues were examined by GC–MS [Varian CP-3800 gas chromatograph-Saturn 2000 ion trap mass spectrometer (Walnut Creek, CA, USA)] using DB-5 capillary column (30 m×0.25 mm×0.25 mm). Helium was used as carrier gas. The initially oven temperature was 100°C, that was increased to 250°C @ 6°C min^{-1} and held for 5 min.

Physico-chemical Activities of EPS

Superoxide scavenging activity: For the detection of superoxide radical scavenging activity, the reaction mixture was contained of 78 µM NADH, 10 µM phenazin methosulfate, 50 µM nitroblue tetrazolium and sample solutions containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} EPS in 3 mL of Tris-HCl buffer (16 mM, pH 8.0). The absorbance of color reaction was monitoring at 560 nm. NADH was replaced with Tris-HCl buffer in control while ascorbic was used as positive control (Ponti *et al*., 1978).

Hydroxyl radical scavenging activity: For the detection of hydroxyl radical scavenging activity, the reaction mixture was contained of 0.15 mM $FeSO_4$-EDTA, 6 mM H_2O_2, 2 mM sodium salicylate and sample solutions containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} EPS in sodium phosphate buffer (150 mM, pH 7.4). In control, H_2O_2 was replaced with sodium phosphate buffer. The absorbance of solutions was determined at 510 nm after incubation at 37°C for 1 h. Ascorbic was used as positive control (Smirnoff and Cumbes, 1989).

Metal chelating activity: For the evaluation of metal ion chelating activity, the reaction mixture was contained of 12.5 µM ferrous sulfate and sample solutions containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} EPS in HEPES buffer (20 mM, pH 7.2). The reaction was initiated by ferrozine (75 µM), shaken vigorously and incubated at room temperature for 20 min. later, the absorbance was measured at 562 nm. Ascorbic acid was used as a positive control (Haro-Vicente *et al*., 2006).

Lipid peroxidation inhibitory activity: For measuring lipid peroxide inhibitory activity, 0.5 mL egg homogenate (10% v/v), 0.5 mL of EPS (0-1.1 mg mL^{-1}) containing solutions and 0.05 mL of $FeSO_4$ (0.07 M) were mixed and incubated for 30 min. Then, 1.5 mL $C_4H_4N_2O_2S$ (0.8% w/v) in 1.1% SDS and 1.5 mL 20% acetic acid (pH 3.5) were added and vortexed. Later, the mixture was heated at 95°C for 60 min and 5.0 mL of 1-butanol was added after cooling and centrifuged (3000×*g*) for 10 min. The organic layer (upper) was separated carefully and absorbance was measured at 532 nm. Inhibition of lipid peroxidation (%) was calculated by $[(1-E/C)\times100]$ where C is the absorbance value of control and E is the absorbance in the presence of EPS or ascorbic acid as positive control (Ruberto *et al*., 2000).

Flocculating ability: To test the flocculating ability of EPS of WR-1, a suspension was made by mixing well 10 mL of activated carbon (0.5%), 100 µL of $CaCl_2$ (1%) and 500 µL of 1 and 2 mg of EPS solutions. The suspension was monitored for three minutes during incubation at room temperature in comparison with control (Nakamura *et al*., 1976).

Lipid emulsifying ability: For the evaluation of lipid emulsifying ability of EPS produced by WR-1, a lipid emulsion was prepared by shaking equal volumes of olive oil and EPS solution (5%) in distilled water for 10 min at 150 rpm. Later, the emulsion was centrifuged (2000×*g*) for 5 min and the height of emulsified layers was measured and expressed as percentage of the height of whole layer (Yasumatsu, 1972).

Antimicrobial activity assay: The antimicrobial activity was determined on typtic soya agar plates for *Bacillus subtilis* and *Escherichia coli* and on potato dextrose agar plates for *Fusarium oxysporum* by agar diffusion assay.

Statistical Analysis

All experiment had three replicates in completely randomized design and the significance of data was assessed with one-way ANOVA. Duncan's multiple-range test was applied when one-way ANOVA revealed significant differences ($P<0.05$). All statistical analysis was performed with SPSS version 11.5 statistical software (SPSS, Chicago, USA).

Results

Structural Characterization of EPS from WR-1

The results of FT-IR spectrum are presented in Fig. 1. The IR spectrum of the EPS displayed a broad band around 3281 cm^{-1} that represents O-H stretching absorption and a minor band at 2920 cm^{-1} shows C-H stretching. An asymmetrical stretching band and a weak symmetrical stretching band were also found at 1636 cm^{-1} and 1410 cm^{-1}, respectively. Some other bands that presented C–H deformation vibration were found in the region of 1500 and 1200 cm^{-1}. A strong absorption band was determined at 1023 cm^{-1} that was a signal of C-O stretching.

The results of GC–MS analysis of the partially methylated alditol acetates showed that the EPS consisted of five methylated fragments with different molar ratios

(Table 1). The major derivative for EPS was 2, 3, 6-tri-methylated glucose, indicating that (1→4)-linked-glucose was the largest amount residue of the EPS structure followed by 2, 3-di-methylated glucose and 2, 3-di-methylated arabinose. The presence of 2, 3, 4, 6-tetra-methylated glucose and 2, 3, 4-tri-methylated-arabinose indicated that the glucose and arabinose may be found at the non-reducing ends as terminal sugars. These results showed that the polysaccharide has a (1→4)-linked-glucose back bone having branched structure with (1→4, 6)-linked-glucose and (1→4)-linked-arabinose and the side chains are terminated with glucose and arabinose.

Physico-chemical Activities of EPS from WR-1

Superoxide radical scavenging activity: The superoxide radical scavenging activity of the EPS produced by WR-1 was good and increased with the increase in EPS concentration. A maximum superoxide radical scavenging activity was determined at 1 mg mL^{-1} concentration of EPS; however it was 13.7% less than the ascorbic acid at the same concentration (Fig. 2A).

Fe^{2+} chelating activity: The EPS produced by WR-1 showed excellent Fe^{2+} chelating activity, which was slightly higher than that of ascorbic acid at the same concentrations, increased in a concentration dependant manner and reached up to 52% at 1 mg EPS concentration (Fig. 2B).

Hydroxyl radical scavenging activity: The results showed that the EPS from WR-1 has good potential for the hydroxyl radical scavenging activity. The EPS from WR-1 increased the hydroxyl radical scavenging activity in a concentration dependent manner and reached to 68% at 1 mg concentration of EPS which was 23.6% higher that the ascorbic acid at the same concentration (Fig. 2C).

Lipid peroxidation inhibitory activity: The results revealed that the lipid peroxidation inhibitory activity of EPS from WR-1 was increased with the increase of EPS concentration and at 1 mg mL^{-1} EPS level, 58% lipid peroxidation inhibition activity was determined while ascorbic acid showed at the same concentration level, 90% inhibition of lipid peroxidation activity (Fig. 2D).

Flocculating test: The EPS of WR-1 showed excellent flocculating activity against activated carbon-water suspension. The EPS concentration of 1 and 2 mg showed good flocculation activity after 3 min of incubation (data not shown).

Lipid emulsification activity: The lipid emulsification activity was determined by using olive oil and 0.5% EPS solution in equal amounts. The EPS of WR-1 exhibited 78% lipid emulsification activity which showed good potential of EPS of WR-1 in lipid processing industry.

Antimicrobial activity: The EPS produced by WR-1 was unable to show any antimicrobial activity against tested Gram positive strain *B. subtilis*, Gram negative strain *E. coli* and fungal strain *F. oxysporum*.

Table 1: Results of methylation analysis of extracellular polysaccharide produced by *Pseudomonas fluorescens* WR-1

Methylated sugars	Molar ratio	MS data of main fragments (m/z)	Linkage types
2, 3, 6-Tri-Me-Glc	25	45, 59, 73, 88, 101, 116, 133, 146, 159, 232	1,4
2, 3-Di-Me-Glc	5.70	45, 59, 73, 88, 101, 116, 133, 146, 174, 232	1,4,6
2, 3, 4, 6-Tetra-Me-Glc	0.92	45, 71, 87, 101, 117,129, 145, 161, 205	1
2, 3, 4-Tri-Me-Ara	1.00	43, 71, 87, 101, 117, 129, 161	1
2, 3-Di-Me-Ara	12.5	43, 59, 71, 87, 102, 118, 129, 162, 189	1,4

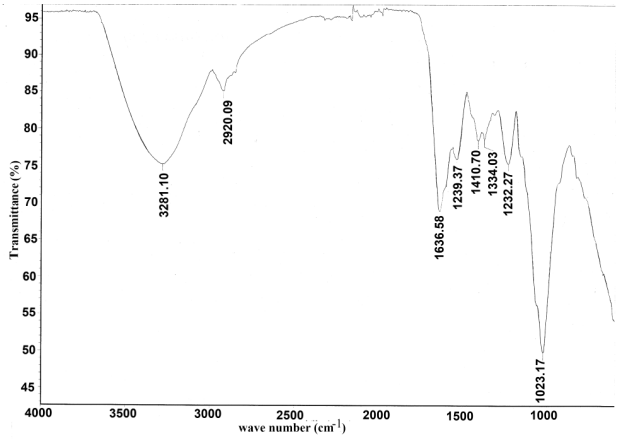

Fig. 1: The infrared spectra of extracellular polysaccharide produced by *Pseudomonas fluorescens* WR-1

Discussion

Previously, the EPS from WR-1 was purified and its molecular weight was determined to be 6.78 $\times 10^6$ Da. The EPS consisted of uronic acid, arabinose and glucose in a molar ratio (%) of 66:31:3, respectively (Raza *et al*., 2012). The EPS was also consisted of 0.8% (w/w) protein. In this study, the structure of EPS was further characterized by FT-IR spectroscopy and methylation analysis. The FT-IR spectrum showed eight bands. Among those, a weak band at 1410 cm^{-1} was the indication of uronate in this EPS as this band represents the presence of carboxylate ion. The bands in the region of 1500 and 1200 cm^{-1} were assigned to C–H deformation vibration. Another band at 1636 cm^{-1} showed the presence of bound water. Moreover, a signal of C-O stretching was obtained by a strong absorption band at 1023 cm^{-1}, which indicated the presence of a methoxyl group which would be expected to have an effect on the functional properties of the polysaccharide such as gel formation (Lapasin and Pricl, 1995).

The partial linkages analysis results showed that the polysaccharide has a (1→4)-linked-glucose back bone having branched structure with (1→4, 6)-linked-glucose

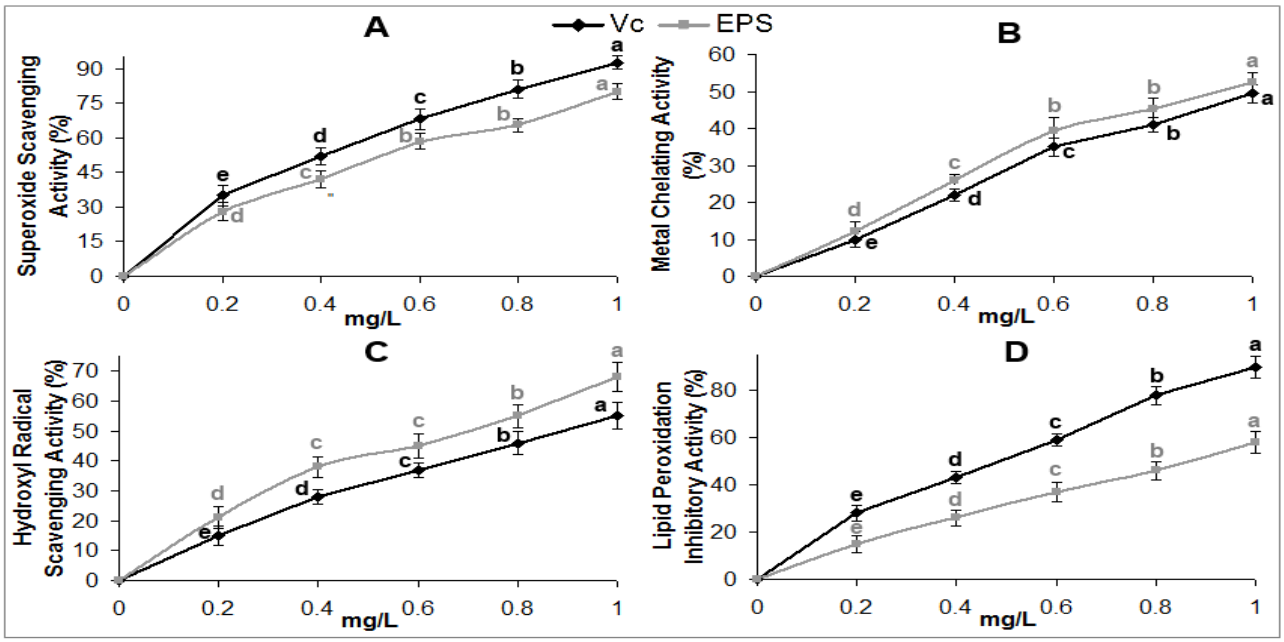

Fig. 2: The superoxide radical scavenging (A), metal chelating (B), hydroxyl radical scavenging (C) and lipid peroxidation inhibition (D) activities of a extracellular polysaccharide produced by *Pseudomonas fluorescens* WR-1. Bars indicate the standard deviations of the three replicates and different letters show the significant differences among means. Vc = Ascorbic acid; EPS = Extracellular polysaccharides

and (1→4)-linked-arabinose and the side chains are terminated with glucose and arabinose. The uronic acid was present in low quantity so it might be as the non-reducing ends. Many *P. florescence* strains have been reported to produce EPS which were characterized for their sugar and uronic acid compositions (Read and Costerton, 1987; Chin-Chang *et al*., 2005) but their monosaccharide linkage and functional groups information was not reported. The only report about the structural characterization of any EPS from *P. fluorescens* strain was from Osman *et al*. (1997) who reported that the EPS from *P. fluorescens* strain H13 was composed of D-glucose, 2-acetamido-2-deoxy-D-glucose and 4-O-acetyl-2-acetamido-2-deoxy-D-mannuronic acid. The structural dissimilarities define the differences of biological and physiological properties of EPS produced by same species.

The physico-chemical activity analysis is important to access the biotechnological potential of EPS. The EPS of WR-1 presented good flocculating, metal chelating and hydroxyl radical scavenging activities, while moderate superoxide radical scavenging, lipid peroxidation inhibition and lipid emulsification activities were determined. Superoxide anions are precursor of active free radicals that not only induce tissue damage but also participate in the formation of other reactive oxygen species, which enhance oxidative damage in different bio-molecules like DNA (Wickens, 2001). The EPS of WR-1 showed moderate superoxide radical scavenging activity. The possible mechanism of action for this activity of EPS is the combination of superoxide radicals with EPS that demolish the radical chain reactions (Halliwell and Gutteridge, 1984). Metal chelating capacity is important because of its role in the reduction of catalyzing transition metal ions in lipid peroxidation. The EPS of WR-1 showed excellent metal chelation. Different functional groups (–NR_2, –COOH, –OH, –PO_3H_2, –SH, –O–, C=O and –S–) play important role in metal chelating activity of different polysaccharides if their structures contain two or more of these functional groups in a favorable function–structure pattern (Shon *et al*., 2003). The FT-IR results showed that the structure of EPS of WR-1 contains some of these functional groups. Hydroxyl radicals are highly reactive and can induce severe damage to adjacent molecules like DNA, lipids and proteins (Chance *et al*., 1979). A good hydroxyl radical scavenging activity of EPS of WR-1 suggests that EPS has potentials of being used as alternative to synthetic antioxidants in arresting oxidative activity of hydroxyl ion.

Lipid peroxidation is a process related to free radicals that cause oxidative stress and damage living cells (Cai *et al*., 2011), for example it plays a vital role during trauma of central nervous system as a result of neurotoxicity (Koedel *et al.,* 2002). The less inhibition of lipid peroxidation by EPS of WR-1 proposed the contribution of metal ions and oxygen induced production of liberated radicals. As EPS of WR-1 presented excellent metal chelation, an arrangement might be connecting metal ions with EPS that obstruct the liberated radical chain reactions; however, this arrangement might be low or at least not very high. The EPS of WR-1

also presented excellent flocculating ability. The flocculation ability of EPS depends on its concentration, charge and hydrophobicity and is important for its use in fermentation industries and wastewater treatments to replace environment unfriendly artificial polymers (Lal and Tabacchioni, 2009). Microorganisms produce bio-emulsifiers for survival and desorption from hydrophobic substrates to increase the bioavailability of insoluble compounds. These bio-emulsifiers have ability to lessen the tensions on surfaces and among two different phases in mixtures containing water and hydrocarbons so are potential nominees for improving oil recovery (Jagtap *et al*., 2010). However, the EPS of strain WR-1 showed moderate lipid emulsification activity. The EPS of WR-1 did not show any antimicrobial activity.

In conclusion, The EPS from WR-1 exhibited good antioxidant activity on hydroxyl radicals while it was moderate on superoxide radicals. The excellent flocculation and metal chelation abilities and moderate lipid emulsification and lipid peroxidation inhibition abilities were also determined. These abilities make EPS of WR-1 a potential candidate for use as natural antioxidant in health and food industry. In addition, it also has potential to be used in biomineral processing, wastewater treatment and biological response modifiers. However, the toxicity test is recommended to perform before usage in any applications.

References

Atawodi, S.E., M.L. Liman and E.O. Onyike, 2013. Antioxidant effects of methanolic extracts of *Tamarindus indica* parts following acute and chronic carbon tetrachloride induced liver injury. *Int. J. Agric. Biol.*, 15: 410–418

Cai, F., L.J. Mei, X.L. An, S. Gao, L. Tang and F. Chen, 2011. Lipid peroxidation and antioxidant responses during seed germination of *Jatropha curcas*. *Int. J. Agric. Biol*., 13: 25–30

Chance, B., H. Sies and A. Boveris, 1979. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.*, 59: 527–605

Chin–Chang, H., P.H. Santschi and J.B. Gillow, 2005. Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* biovar II. *Carboh. Polym.*, 61: 141–147

Ciucanu, I. and F. Kerek, 1984. A simple and rapid method for the permethylation of carbohydrates. *Carboh. Res.*, 131: 209–217

Haas, D. and C. Keel, 2003. Regulation of antibiotic production in root–colonizing *Pseudomonas spp*. and relevance for biological control of plant disease. *Annu. Rev. Phytopathol*. 41: 117–153

Halliwell, B. and J.M.C. Gutteridge, 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219: 1–14

Haro–Vicente, J.F., C. Martinez–Gracia and G. Ros, 2006. Optimization of *in vitro* measurement of available iron from different fortificants in citric fruit juices. *Food Chem*., 98: 639–648

Jagtap, S., S. Yavankar, K. Pardesi and B. d Chopade, 2010. Production of bioemulsifier by *Acinetobacter* species isolated from healthy human skin. *Ind. J. Exp. Biol.*, 48: 70–76

Kim, D.J., J.W. Choi, N.C. Choi, B. Mahendran and C.E. Lee, 2006. Modeling of growth kinetics for *Pseudomonas* spp. during benzene degradation. *Appl. Microbiol. Biotechnol.*, 69: 456–62

Koedel, U., F. Winkler, B. Angele, A. Fontana and H.W. Pfister, 2002. Meningitis associated central nervous system complications are mediated by the activation of poly (ADP–ribose) polymerase. *J. Cereb. Blood Flow Metab.*, 22: 39–49

Lal, S. and S. Tabacchioni, 2009. Ecology and biotechnological potential of *Paenibacillus polymyxa*: a mini review. *Indian J. Microbiol*., 49: 2–10

Lapasin, R. and S. Pricl, 1995. *Rheology of industrial polysaccharides, theory and applications.* Blackie, London, UK

Lowry, O.H., N.J. Rosebrough, A.L. Garr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem*., 193: 256–275

Mao, Y.L., R.Q. Liu, S.T. Luo, P.X. Xu, L. Du and K. Zhang, 2010. Biosorption characteristics of a novel extracellular biopolymer produced from *Pseudomonas fluorescens* and its use for copper removal. *Adv. Mat. Res.*, 113–116: 1755–1760

Nakamura, J., S. Miyashiro and Y. Hirose, 1976. Screening, isolation and some properties of microbial cell flocculants. *Agric. Biol. Chem*., 40: 377–383

Osman, S.F., W.F. Fett, P. Irwin, P. Cescutti, J.N. Brouillette and J.V. O'Connor, 1997. The structure of the exopolysaccharide of *Pseudomonas fluorescens* strain H13. *Carbohydrate Res.*, 300: 323–327

Palleroni, N.J., 1984. *In*: *Bergey's Manual of Systematic Bacteriology*. Pp: 141–199. Krieg, N.R. and J.G. Holt, (Eds). The Williams and Wilkins Co, Baltimore, Maryland, USA

Ponti, V., M.V. Dianzani and K.J. Cheeseman, 1978. Studies on the reduction of nitroblue tetrazolium chloride mediated through the action of NADH and phenazine methosulate. *Chem. Biol. Interact.* 23: 281–285

Raza, W., W. Yang, Y. Jun, F. Shakoor, Q. Huang and Q Shen, 2012. Optimization and characterization of a polysaccharide produced by *Pseudomonas fluorescens* WR–1 and its antioxidant Activity. *Carbohy. Polym.*, 90: 921– 929

Read, R.R. and J.W. Costerton, 1987. Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas flurescens*. *Can. J. Microbiol.*, 33: 1080–1090

Ruberto, G., M.T. Baratta, S.G. Deans and H.J.D. Dorman, 2000. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Med.*., 66: 687–693

Shon, D.H., Y.C. Kim, S.H. Oh, E.J. Park, X. Li and B.H. Lee, 2003. Hepatoprotective and free radical scavenging effects of *Nelumbo nucifera*. *Phytomedicine*, 10: 165–169

Smirnoff, N. and Q.J. Cumbes, 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, 28: 1057–1060

Wack, M. and W. Blaschek, 2006. Determination of the structure and degree of polymerisation of fructans from Echinacea purpurea roots. *Carboh. Res*., 341: 1147–1153

Walsh, U.F., J.P. Morrissey and F. O'Gara, 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.*, 12: 289–95

Wickens, A.P., 2001. Aging and the free radical theory. *Respir. Physiol.* 128: 379–391

Yasumatsu, K., 1972. Whipping and emulsifying properties of soybean products. *Agric. Biol. Chem.*, 36: 719–729

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