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Optimization and characterization of a polysaccharide produced by *Pseudomonas fluorescens* WR-1 and its antioxidant activity

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ABSTRACT

The extracellular polysaccharide produced by a newly isolated strain *Pseudomonas fluorescens* WR-1 was purified and characterized and its production was optimized using response surface methodology. The results showed that the strain WR-1 produced one kind of EPS that was composed of arabinose, glucose and uronic acid. The molecular weight of the EPS was determined to be 6.78×10^6 Da. The preferable culture conditions for EPS production were pH 7.0, temperature 28 °C for 72 h with peptone and maltose as best N and C sources, respectively. The model predicted that the maximum EPS production (39.6 g L^{-1}) was appeared with maltose 48.65 g L^{-1}, Mn^{2+} 1118 μM and Zn^{2+} 901 μM. The EPS also showed good H_2O_2 scavenging activity while moderate free radical scavenging activity and reductive ability were determined. The EPS from WR-1 may be a new source of natural antioxidants with potential value for health, food and industry.

1. Introduction

Natural polysaccharides are biologically produced polymeric materials, which possess interesting physico-chemical and rheological properties. They are ionic or non-ionic water-soluble polymers and the repeating units of these polymers are very regular, branched or unbranched and interconnected by glycosidic linkages. The polysaccharides have a wide range of industrial applications such as the production of textiles, pharmaceuticals, adhesives, detergents, cosmetics, food additives brewing, wastewater treatment and pharmacology (Sutherland, 1999; WanNgah, Teong, & Hanafiah, 2011). The polysaccharides also contribute to various physiological activities in human beings as anti-tumor, anti-viral and anti-inflammatory agents (Zhang, Zhang, Cheung, & Ooi, 2004). These qualities make polysaccharides a natural fit for sustainable development and research. Nowadays, most of the market is dominated by the polysaccharides produced by plants (e.g. gums or pectin), algae (e.g. alginate, carrageenan) and crustacean (e.g. chitin) and microbial polysaccharides (e.g. xanthan gum, succinoglycan and bacterial alginate) represent only a small part of biopolymer market (Canilha, Silva, Carvalho, & Mancilha, 2005). During the last years, there has been an increasing interest in isolating and identifying new microbial polysaccharides that may compete with traditional polysaccharides due to their biotechnological properties (Ge, Duan, Fang, Zhang, & Wang, 2009; Sutherland, 1999). In addition, microorganisms are better suited for the production of polysaccharide than plants or algae, because of their higher growth rates and being more amenable to manipulate the conditions for enhancing growth and production of polysaccharides (Raza, Makeen, Wang, Xu, & Shen, 2011; Urbani & Brant, 1989).

Members of genus *Pseudomonas* have frequently been reported to produce exo-polymers and have been suggested to be primary colonizers of surfaces in aqueous environments (Kives, Orgaz, & Sanjosé, 2006). The strains of genus *Pseudomonas*, *P. fluorescens* are ubiquitous, Gram-negative, motile, biofilm-forming bacteria commonly present in soil and water habitats. The organism plays an important role in food spoilage, drinking water quality, plant disease, and nosocomial infections. These strains have been reported to produce extracellular polysaccharides (EPS). The ability of EPS produced by *P. fluorescens* PF01 to remove the Cu^{2+} ions was investigated by using batch biosorption procedure (Mao et al., 2010). The polysaccharides associated with free (planktonic) and surface-attached (biofilm) cells from the cultures of *P. fluorescens* strain B52 were compared (Kives et al., 2006) and the role of EPS in surface adhesion of a number of isolates was investigated (Read & Costerton, 1987).

Until now, there is no report about the optimization of culture conditions for the production of EPS from *P. fluorescens* strains and their antioxidant potential has not been investigated yet. The production of EPS is not species specific and each strain of same species

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produces different kinds of EPS with different biotechnology properties. For that reason, maximum strains should be assessed to find out EPS with extraordinary properties. Considering these, a new strain of *P. fluorescens* was isolated from soil and its EPS was isolated and purified from culture broth and its chemical composition was determined. The antioxidation potential of EPS was also investigated and for the optimization of EPS production first, single factor experiments were carried out, later, considering the effective factors, response surface methodology (RSM) was employed which is a well-known method applied in the optimization of medium compositions and other critical variables responsible for the production of bio-molecules (Gharibzahedi, Mousavi, Hamedi, Khodaiyan, & Razavi, 2012; Raza, Wu, & Shen, 2010).

2. Material and methods

2.1. Isolation and identification of P. fluorescens strain

The strain of *P. fluorescens* was isolated from the healthy muskmelon rhizosphere soil in a field infected with *Fusarium oxysporum* f. sp. *melonis* by dilution plate technique as an antagonist against *Fusarium oxysporum* f. sp. *melonis* and designated as WR-1. It was cultured in nutrient broth with 50% glycerol and stored at −70 °C before use. For the identification, *rpoD* gene was amplified directly from the bacterial cells. The primers used for *rpoD* gene were PsEG30F ATYGAAATCGCCAARCG and PsEG790R CGGTTGATKTCCTTGA (Mulet, Bennasar, Lalucat, & Garcıĭ-Valde, 2009). The PCR product was purified, sequenced and blasted against the known sequences of Genetic sequence database at the National Center for Biotechnical Information (NCBI). Only results from the highest score queries were considered for phylotype identification, with 98% minimum similarity. The results identified the strain as *P. fluorescens* WR-1 (WR-1). The *rpoD* gene sequence of WR-1 was deposited in NCBI under the GeneBank accession No. JQ317786.

2.2. Culture conditions and EPS analysis

Tryptone medium (tryptone, 10; NaCl, 5 and sucrose, 10 g L^{-1}; pH 7.5) was used for the production of EPS from the strain WR-1. For the quantification, the EPS were precipitated from cell free liquid culture by adding two volumes of cold ethanol. The precipitates were collected by centrifugation, dissolved in distilled water and the EPS concentrations were determined by phenol-sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. One-factor-at-a-time experiments

One-factor-at-a-time method was used to investigate the factors affecting cell growth and EPS production by WR-1. The time course experiment was carried out in 1-L flask containing 250 mL of the culture medium up to seven days. The optimum initial pH for EPS production was determined by adjusting pH with 1 M HCl and 1 M NaOH before sterilization and the optimum temperature for EPS production was evaluated by incubating the liquid cultures at 24, 26, 28, 30, 32 and 34 °C, respectively. The medium composition was optimized by using different carbon and nitrogen sources (1%) to supplement the basal medium. The effect of different metal ions on EPS production was evaluated by supplementing the medium with different metal ions (Ca^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+} and Fe^{3+}) at different concentrations. Initial Ca^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and Pb^{2+} contents in tryptone broth determined by Spectra AA, 220 FS atomic absorbance spectrometer, were 115, 0.65, 3.1, 0.51, 97, 3.8, 11 and 0.76 μM, respectively. In all above experiments, the sterilized medium was inoculated with 100 μL of over night culture of WR-1 in tryptone broth.

2.4. Fractional factorial design (FFD)

Six factors were identified by one factor-at-a-time experiments to have effect on EPS production by WR-1. Later, FFD was used to screen the most significant factors. The parameters for six factors (maltose, peptone, Zn^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+}) were chosen as main variables and designated as x_1, x_2, x_3, x_4, x_5 and x_6, respectively. The low, middle and high levels of each variable were designated as −1, 0, and +1 and a total 19 experiments including three replications were conducted. FFD was based on the following first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where Y was the predicted response (EPS production), β_0 was the model intercept, β_i was the linear coefficient and x_i was the level of independent variable. The variables significant at 95% of confidence level ($P < 0.05$) were considered to have significant effects on EPS production by WR-1.

2.5. Path of steepest ascent experiment

Three variables were screened by FFD (maltose, Zn^{2+}, Mn^{2+}) and were further optimized by using steepest ascent design. The experiment was moved along the steepest ascent path in which the concentrations of maltose, Zn^{2+}, Mn^{2+} were increased according to their coefficients in the above mentioned first-order model until the response was not increased any more. This point would be near the optimal point and could be used as a center point to optimize the medium by central composite design.

2.6. Central composite design (CCD)

In the end, the three most significant variables (maltose, Zn^{2+}, Mn^{2+}) were optimized by CCD. These three independent variables were evaluated at five different levels (−1.68, −1, 0, +1, +1.68) and 20 experiments, containing six replications at the center point, were conducted in triplicates. The behavior of the system was explained by the following second-degree polynomial equation:

$$Y = B_0 + \sum_{i=1}^{n} Bixi + \sum_{i<j}^{n} Bijxixj + \sum_{j=1}^{n} Bjjx_j^2 \quad (2)$$

where Y was response; B_0 was a constant, B_i was the linear coefficient, B_{ij} was the second-order interaction and B_{jj} was the quadratic coefficient. The variables, x_i and x_j were the non-coded independent variable. In the present study, three variables were involved and hence n took the value 3. Thus, by substituting the value 3 for n, Eq. (2) became:

$$Y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2$$

where Y was the predicted response, and x_1, x_2 and x_3 were input variables. B_0 was a constant and B_1, B_2 and B_3 were linear coefficients. B_{12}, B_{13} and B_{23} were cross-product coefficients and B_{11}, B_{22} and B_{33} were quadratic coefficients.

2.7. Data analysis

Design Expert software of version 8.0.7.1 Trial (Stat-Ease Inc., Minneapolis, USA) was used for the experimental designs, analysis of variance (ANOVA) and regression analysis of the experimental

data. The quality of the polynomial model equation was judged by the determination of coefficient R^2, and its statistical significance was determined by *F*-test. Microsoft excel 2003 was used for the standard deviations and the whole experiment was repeated twice.

2.8. Purification of the EPS

For the isolation of EPS, the optimized tryptone medium containing maltose, $ZnSO_4$, and $MnSO_4$ was inoculated with 1 mL overnight culture of WR-1 and incubated in an incubator shaker (170 rpm, 30 °C). The EPS were purified from the culture broth by the method of Lee et al. (1997) with some modifications. The culture broth was diluted two times with distilled water and centrifuged. The cell bound EPS was removed with 0.25 N NaOH for 2–3 h and mixed with supernatant. The supernatant was mixed with two volumes of absolute ethanol and kept over night at 4 °C. The precipitated EPS were collected, washed with ethanol and dried in a vacuum drier. After that, the dried fraction was dissolved in 800 mL distilled water and precipitated with 10% cethyl trimethyl ammonium bromide (CTAB). This mixture was left over night at 37 °C. The precipitants were collected, washed with distilled water, redissolved in 0.5 M NaCl (500 mL) and precipitated again with an equal volume of ethanol. The precipitants were collected, washed with 70% ethanol and dissolved in 0.25 M NaCl solution (800 mL). The precipitants were again precipitated with 600 mL of ethanol, collected by centrifugation and dissolved in 300 mL of distilled water. The fraction was dialyzed against distilled water at 4 °C for 2 days and lyophilized. The purity of EPS was confirmed by chromatography. The EPS solution (5 mg mL^{-1}) was applied to DEAE-52 column (2.6 mL 30 cm) and stepwise eluted with distilled water, 0.1, 0.3 and 0.5 M NaCl at a flow rate of 1 mL min^{-1}. Elutes (10 mL $tube^{-1}$) were collected and the carbohydrates were determined by the phenol–sulfuric acid method.

2.9. Determination of molecular weight

The molecular weight of EPS was determined using an Agilent 1100HPLC system equipped with a TSK-GEL G3000SWxl column (7.5 mm × 300 mm, Tosoh Corp., Tokyo, Japan) and a refractive index detector (RID). The column was eluted with 0.1 M Na_2SO_4 at a flow rate of 0.8 mL min^{-1}. Dextran-50, Dextran-100, Dextran-140 Dextran-500 Dextran-1000, Dextran-1500 and Dextran-2000 (Sigma–Aldrich, USA) were used as standards.

2.10. EPS chemical composition analysis

For the monosaccharide composition analysis, EPS (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol, reduced with $NaBH_4$ for 30 min at 20 °C and acetylated with acetic anhydride and pyridine at 100 °C for 20 min. The standard sugars were prepared in the same way. The alditol acetates of EPS were analyzed on Agilent Technologies 7890A GC equipped with flame ionization detector (FID) and a HP-5 fused silica capillary column (30m × 0.32 mm × 0.25 mm). The nitrogen gas was used as the carrier gas at a flow rate of 1 mL min^{-1}. The temperatures of injector and detector were set at 250 °C and 280 °C, respectively; the initial column temperature was 100 °C for 5 min, then at a rate of 5 °C min^{-1} to 150 °C and held for 5 min, subsequently at a rate of 5 °C min^{-1} to 240 °C and held for 10 min. The uronic acid was determined by the carbazole–sulfuric acid method by using glucuronic acid as standard (Li, Koki, Sverker, Mikael, & Goran, 2007). These experiments were repeated twice.

2.11. Antioxidant activity assays

For the evaluation of antioxidant activity of EPS produced by WR-1, assays for reductive ability, free radical scavenging activity and H_2O_2 scavenging activity were determined according to the methods of Oyaizu (1986), Yang et al. (2006) and Ruch, Cheng, and Klauning (1989), respectively.

3. Results and discussion

3.1. One-factor-at-a-time experiments

The result regarding the time course experiment revealed that the EPS production by WR-1 was growth (OD_{600}) dependent. The maximum EPS production was determined after 72 h (data not shown). Our results were not similar with the results of Conti, Flaibani, O'Regan, and Sutherl (1994) who reported maximum alginate type polysaccharides production by *P. fluorescens* after 50 h of growth. This difference might be because of different type of EPS production or different strain used. The results about the effect of temperature on EPS production by WR-1 revealed that the maximum growth and EPS production was determined at 28 °C (data not shown). These results were not in agreement with Bueno and Garcia-Cruz (2006) who reported the maximum EPS production at 30 °C by *P. fluorescens*. The initial pH of liquid culture is important as it may effect the cell growth, EPS production and uptake of different nutrients. The trend of EPS production and growth of WR-1 at different pH values was same as the effect of temperature on the EPS production and maximum growth and EPS production was determined at the pH value of 7.0 (data not shown). These results were consistent with the findings of Bueno and Garcia-Cruz (2006) who reported maximum EPS production by *Pseudomonas* species at pH 7.

Different C and N sources were evaluated for their effect on EPS production by WR-1, the results revealed that C source maltose gave maximum EPS production followed by starch while peptone as N source, gave maximum EPS production followed by proteose peptone (Table 1). Our findings were consistent with Wang et al. (2011) who reported that maltose and peptone gave maximum EPS production by *Bacillus thuringiensis* 27. Almost all C sources except xylose, galactose and cellulose, showed more growth of WR-1 than maltose so it seemed likely that an enhanced metabolic flux of the maltose, but a limited utilization for the cell growth, could stimulate the EPS production by maltose. Williams and Wimpenny (1977) reported that glucose, fructose, sucrose, maltose and lactose were good substrates for high polysaccharide yields by *Pseudomonas* sp., however; maximum EPS production was obtained with lactose as C source which was not in agreement with our results. Maltose is a cleavage product of starch and the biosynthesis pathway of EPS production by WR-1 might activate the maltose unitizing genes network which involves maltose phosphorylase and β-phosphoglucomutase as has been reported for lactic acid producing bacteria (Andersson & Rådström, 2002). Conti et al. (1994) reported the maximum EPS production by *P. fluorescens* using 0.1 g L^{-1} NH_4Cl as N source, however, we found peptone as best N source for EPS production by WR-1. The stimulatory effect of peptone might be due to its rich contents of protein, amino acids and vitamins. Additionally, it was found in the study that the organic nitrogen sources were much more suitable for the growth and EPS production by WR-1. This phenomenon has been reported earlier that organic nitrogen sources are absorbed by the cells easier than the inorganic ones (Kim et al., 2005).

The results about the effect of metal ions on EPS production revealed that all tested metal ions except Fe^{3+} promoted the EPS production by WR-1. The metal ions Zn^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+},

Table 1
The effect of different carbon (C) and nitrogen (N) compounds on the production of extracellular polysaccharides by *Pseudomonas fluorescens* WR-1. The data is presented as the mean value ± standard deviation of three replicates.

C compounds			N compounds		
C sources	OD_{600}	EPS ($g\,L^{-1}$)	N sources	OD_{600}	EPS ($g\,L^{-1}$)
Glucose	1.07 ± 0.14	5.31 ± 0.53	Tryptone	1.64 ± 0.18	4.66 ± 0.58
Sucrose	1.59 ± 0.11	5.41 ± 0.67	Yeast extract	1.92 ± 0.16	5.01 ± 0.38
Fructose	1.44 ± 0.10	5.47 ± 0.68	Soya peptone	2.01 ± 0.21	3.64 ± 0.64
Galactose	0.89 ± 0.12	4.69 ± 0.61	Beef extract	1.72 ± 0.09	3.31 ± 0.28
Maltose	1.05 ± 0.07	11.50 ± 1.24	Peptone	1.70 ± 0.16	6.85 ± 0.39
Lactose	1.14 ± 0.07	7.93 ± 0.98	Proteose peptone	1.51 ± 0.19	5.38 ± 0.68
Glycerol	1.55 ± 0.14	1.31 ± 0.38	NH_4NO_3	0.34 ± 0.13	0.03 ± 0.01
Starch	1.16 ± 0.09	9.02 ± 0.97	$(NH_4)_2SO_4$	0.34 ± 0.07	0.02 ± 0.01
Xylose	0.54 ± 0.09	5.08 ± 0.76	Glutamic acid	0.58 ± 0.14	0.06 ± 0.00
Milk sugar	1.15 ± 0.12	8.39 ± 0.76	Asparagine	0.34 ± 0.15	0.01 ± 0.00
Cellulose	0.98 ± 0.06	4.19 ± 0.38	Glycine	0.36 ± 0.15	0.07 ± 0.01

Table 2
Effect of different metal ions on the growth (OD_{600}) and extracellular polysaccharide production by *Pseudomonas fluorescens* WR-1. The data is presented as the mean value ± standard deviation of three replicates.

Conc. (μM)	OD_{600}	EPS ($g\,L^{-1}$)	Conc. (μM)	OD_{600}	EPS ($g\,L^{-1}$)
Ca^{2+}			Ni^{2+}		
Ck	0.85 ± 0.08	5.35 ± 0.28	Ck	0.85 ± 0.05	5.35 ± 0.18
200	1.01 ± 0.11	6.50 ± 0.16	400	0.91 ± 0.04	6.82 ± 0.25
300	0.85 ± 0.07	6.85 ± 0.28	500	1.25 ± 0.06	8.26 ± 0.17
400	1.19 ± 0.09	7.80 ± 0.25	600	1.65 ± 0.07	11.14 ± 0.31
500	1.26 ± 0.08	8.32 ± 0.15	700	1.76 ± 0.06	11.97 ± 0.26
Fe^{3+}			Mg^{2+}		
Ck	0.93 ± 0.03	5.83 ± 0.24	Ck	0.58 ± 0.04	5.42 ± 0.14
300	0.91 ± 0.05	5.83 ± 0.19	500	0.65 ± 0.07	5.90 ± 0.16
400	0.81 ± 0.02	5.08 ± 0.24	600	0.45 ± 0.04	6.19 ± 0.24
500	0.79 ± 0.08	4.94 ± 0.15	700	0.57 ± 0.03	6.34 ± 0.21
600	0.91 ± 0.04	5.78 ± 0.17	800	0.73 ± 0.02	6.49 ± 0.19
Mn^{2+}			Pb^{2+}		
Ck	0.582 ± 0.08	5.42 ± 0.13	Ck	0.928 ± 0.05	5.93 ± 0.11
400	1.193 ± 0.06	8.25 ± 0.16	500	0.981 ± 0.07	6.31 ± 0.13
500	1.312 ± 0.05	8.71 ± 0.08	600	1.013 ± 0.08	6.55 ± 0.11
600	1.025 ± 0.07	8.93 ± 0.09	700	1.014 ± 0.03	6.55 ± 0.16
700	1.414 ± 0.09	9.45 ± 0.12	800	0.966 ± 0.08	6.21 ± 0.08
Zn^{2+}			Cu^{2+}		
Ck	0.928 ± 0.06	5.93 ± 0.08	Ck	0.928 ± 0.08	5.83 ± 0.10
200	1.478 ± 0.08	9.62 ± 0.13	500	1.024 ± 0.05	6.63 ± 0.13
300	1.436 ± 0.04	9.91 ± 0.10	600	1.066 ± 0.06	6.93 ± 0.15
400	1.44 ± 0.03	10.14 ± 0.12	700	1.15 ± 0.06	7.54 ± 0.14
500	1.531 ± 0.02	10.30 ± 0.12	800	1.229 ± 0.02	7.69 ± 0.15

Ck: control treatment.

Mg^{2+}, Pb^{2+} and Cu^{2+} increased the EPS production by 73.7, 74.4, 55.5, 123.7, 19.7, 10.5 and 31.9%, respectively, up to certain concentrations while higher concentrations were inhibitory to EPS production by WR-1 (Table 2). Cations are known to influence EPS synthesis both qualitatively and quantitatively. Similar to our results, the synthesis of the EPS in *P. aeruginosa* was stimulated by Mg^{2+}, Mn^{2+} and Ca^{2+} (Martins, Brito, & Sa-Correia, 1990) and by Cu^{2+} in a subset of *P. syringae* strains (Kidambi, Sundin, Palmer, Chakrabarty, & Bender, 1995). The positive effect of Ni^{2+} on EPS production by *Paenibacillus polymyxa* SQR-21 was reported by Raza et al. (2010). Yang et al. (2007) suggested that high Fe^{3+} in the medium suppressed the structural biofilm development that is directly related to the production of EPS in *P. aeruginosa*. Raza, Wu, Xu, and Shen (2011) reported that the inclusion of Pb^{2+} appeared to enhance the production of either extracellular proteins or carbohydrates at low concentration. According to the results, four metal ions (Zn^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+}) showing maximum positive effect on EPS production by WR-1 were selected for fractional factorial design.

These apparent controversial results showed that the culture conditions are very crucial for EPS-producing strains for efficient EPS production. The single factor method is tedious and ignores the interactions among different variables involved (Raza, Makeen, et al., 2011). Alternatively, RSM is a powerful technique that explores the relationships and interactions between multiple variables by reducing experimental trails. Until now, RSM has been successfully applied in the optimization of medium compositions for EPS production (Gharibzahedi et al., 2012; Raza, Makeen, et al., 2011).

3.2. Fractional factorial design (FFD)

After one factor-at-a-time experiment, incubation time 72 h, pH 7, temperature 28 °C, maltose and peptone at a rate of 10 $g\,L^{-1}$ and metal ions like Zn^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+} were selected for the optimal production of EPS by WR-1. For the FFD experiment, incubation time, temperature and pH levels were kept constant and maltose (x_1), peptone (x_2), Zn^{2+} (x_3), Mn^{2+} (x_4), Ca^{2+} (x_5) and Ni^{2+} (x_6) were considered as main variables with three levels (−1, 0, +1). The results showed that the response (EPS) varied from 8.10 to 21.32 $g\,L^{-1}$, which reflected the importance of medium optimization for the maximum production of EPS (Table 3). The regression analysis showed that except Ca^{2+}, all variables showed positive effect on EPS production by WR-1, however, the effect of three variables maltose ($P < 0.0001$), Zn^{2+} ($P = 0.004$) and Mn^{2+} ($P < 0.0379$) was significant while other two variables (Ni^{2+} and

Table 3
Fractional factorial design arrangement and responses.

Runs	Real levels (coded levels)						EPS ($g L^{-1}$)	
	Maltose ($g L^{-1}$)	Peptone ($g L^{-1}$)	Zn^{2+} (μM)	Mn^{2+} (μM)	Ca^{2+} (μM)	Ni^{2+} (μM)	Actual	Predicted
1	10(−1)	10(−1)	500(−1)	700(−1)	500(−1)	700(−1)	8.10	8.36
2	20(+1)	10(−1)	500(−1)	700(−1)	700(+1)	700(−1)	15.53	16.48
3	10(−1)	20(+1)	500(−1)	700(−1)	700(+1)	900(+1)	9.63	9.22
4	20(+1)	20(+1)	500(−1)	700(−1)	500(−1)	900(+1)	18.63	17.46
5	10(−1)	10(−1)	700(+1)	700(−1)	700(+1)	900(+1)	10.50	10.82
6	20(+1)	10(−1)	700(+1)	700(−1)	500(−1)	900(+1)	18.01	19.07
7	10(−1)	20(+1)	700(+1)	700(−1)	500(−1)	700(−1)	10.36	10.71
8	20(+1)	20(+1)	700(+1)	700(−1)	700(+1)	700(−1)	20.18	18.84
9	10(−1)	10(−1)	500(−1)	900(+1)	500(−1)	900(+1)	10.58	10.22
10	20(+1)	10(−1)	500(−1)	900(+1)	700(+1)	900(+1)	18.96	18.34
11	10(−1)	20(+1)	500(−1)	900(+1)	700(+1)	700(−1)	10.01	9.99
12	20(+1)	20(+1)	500(−1)	900(+1)	500(−1)	700(−1)	16.83	18.23
13	10(−1)	10(−1)	700(+1)	900(+1)	700(+1)	700(−1)	11.70	11.59
14	20(+1)	10(−1)	700(+1)	900(+1)	500(−1)	700(−1)	21.32	19.84
15	10(−1)	20(+1)	700(+1)	900(+1)	500(−1)	900(+1)	12.61	12.57
16	20(+1)	20(+1)	700(+1)	900(+1)	700(+1)	900(+1)	19.46	20.70
17	15(0)	15(0)	600(0)	800(0)	600(0)	800(0)	15.76	15.53
18	15(0)	15(0)	600(0)	800(0)	600(0)	800(0)	16.50	15.53
19	15(0)	15(0)	600(0)	800(0)	600(0)	800(0)	14.99	15.53

Table 4
Experimental design and results of steepest ascent path experiment.

Runs	Maltose ($g L^{-1}$)	Mn^{2+} (μM)	Zn^{2+} (μM)	EPS ($g L^{-1}$)
1	25	900	700	20.37
2	30	950	750	23.81
3	35	1000	800	22.25
4	40	1050	850	29.56
5	45	1100	900	34.05
6	50	1150	950	26.70
7	55	1200	1000	25.72
8	60	1250	1050	24.34
9	65	1300	1100	22.21
10	70	1350	1150	19.08

peptone) showed nonsignificant effect at 95% confidence interval. By applying multiple regression analysis to the experimental data of EPS production by WR-1, the response and test variables were found to be related by the following second-order polynomial equation:

$$Y = 14.73 + 4.09X_1 + 0.19X_2 + 0.99X_3 + 0.66X_4 - 0.03X_5 + 0.27X_6$$

where Y was the predicted response (EPS production), X_1, X_2, X_3, X_4, X_5 and X_6 were coded values of maltose, peptone, Zn^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+}, respectively.

The statistical significance of the model was evaluated by F-test and P-value of <0.0001 revealed that the model was highly significant. The determination coefficient R^2 of the model was 0.945, indicating that 94.5% of the variability in the response could be explained by the model.

3.3. Path of steepest ascent experiment

The optimum center point was outside the domain of our experiment. Thus, the steepest ascent experiment was performed to reach the optimum domain of the maximum response. The path of steepest ascent experiment was started from the highest point of FFD and the concentrations of maltose, Mn^{2+} and Zn^{2+} were increased. The results showed that the maximum EPS production was determined at run 5 (Table 4) which indicated that the maximum EPS production would be near this point. Therefore, the combination of run 5 was selected for CCD experiment.

3.4. Central composite design (CCD)

Three variables, maltose, Mn^{2+} and Zn^{2+}, were selected based on the results of FFD and steepest ascent path experiments for the CCD experiments. The values of the response (EPS) obtained under different experimental conditions are given in Table 5.

By applying multiple regression analysis to the experimental data of EPS production, the response and test variables were found to be related by the following second-order polynomial equation:

$$Y = 38.00 + 4.35x_1 + 1.38x_2 - 0.31x_3 + 0.69x_1x_2 + 0.46x_1x_3 + 0.16x_2x_3 - 3.02x_1^2 - 2.68x_2^2 - -2.12x_3^2 \quad (3)$$

The correlation measure for testing the goodness of fit of regression equation is the adjusted determination coefficient R^2. The values of R^2, 0.9399 and AdjR^2, 0.8859 for Eq. (3) indicate a high degree of correlation between the observed and predicted values for the production of EPS by WR-1. Statistical testing of the model was done as analysis of variance (ANOVA). The regression model demonstrates that the model was highly significant, as is evident from the calculated F-values of 17.39 and a very low probability values ($P < 0.0001$). The model also showed statistically non-significant ($P > 0.05$) lack of fit for the production of EPS as is evident from greater computed F-values than the tabulated F-values ($F_{0.05}$ (9, 5) = 4.77) (Table 6). The model was, therefore, found to be adequate for prediction within the range of variables employed.

The coefficient values of Eq. (3), calculated and tested for significance indicated that the linear coefficients (x_1 and x_2) and quadratic term coefficients (x_1^2, x_2^2 and x_3^2) were significant and the other term coefficients (x_3, x_1x_2, x_1x_3 and x_1x_2) were not significant. The graphical representations of regression Eq. (3), termed as the 3D plots are given in Fig. 1. These 3D response surface plots provide a visual interpretation of the interactions between two variables and ease the location of optimum experimental conditions while the concentration of third variable is fixed at the 0 level. The model predicted that the maximum EPS production (39.6 $g L^{-1}$) was appeared with maltose 48.65 $g L^{-1}$, Mn^{2+} 1118 μM and Zn^{2+} 901 μM. To validate the adequacy of the model equation, five additional experiments in shake flasks under the optimum medium compositions were performed. The mean value of EPS production was 39.48 $g L^{-1}$, which was in good agreement with the predicted value. The yield of EPS

Table 5
Central composite design arrangements and responses.

Runs	Real levels (coded levels)			EPS ($g L^{-1}$)	
	Maltose ($g L^{-1}$)	Mn^{2+} (μM)	Zn^{2+} (μM)	Actual	Predicted
1	40(−1)	1050(−1)	850(−1)	25.37	26.07
2	50(+1)	1050(−1)	850(−1)	32.98	32.46
3	40(−1)	1150(+1)	850(−1)	25.05	27.15
4	50(+1)	1150(+1)	850(−1)	35.56	36.29
5	40(−1)	1050(−1)	950(+1)	23.52	24.22
6	50(+1)	1050(−1)	950(+1)	33.13	32.46
7	40(−1)	1150(+1)	950(+1)	23.98	25.92
8	50(+1)	1150(+1)	950(+1)	36.20	36.92
9	36.59(−1.68)	1100(0)	900(0)	24.70	22.16
10	53.41(+1.68)	1100(0)	900(0)	36.25	36.78
11	45(0)	1015.91(−1.68)	900(0)	27.54	28.10
12	45(0)	1184.09(+1.68)	900(0)	35.34	32.76
13	45(0)	1100(0)	815.91(−1.68)	33.62	32.52
14	45(0)	1100(0)	984.09(+1.68)	32.40	31.49
15	45(0)	1100(0)	900(0)	39.00	38.00
16	45(0)	1100(0)	900(0)	36.97	38.00
17	45(0)	1100(0)	900(0)	39.62	38.00
18	45(0)	1100(0)	900(0)	38.21	38.00
19	45(0)	1100(0)	900(0)	37.60	38.00
20	45(0)	1100(0)	900(0)	36.24	38.00

Table 6
Analysis of variance of predictive equation for the extracellular polysaccharides production by *Pseudomonas fluorescens* WR-1.

Source	Sum of squares	df	Mean square	*F*-Values	Prob>F
Model	542.34	9	60.26	17.39	<0.0001**
x_1 (Maltose)	258.08	1	258.08	74.47	<0.0001**
x_2 (Mn^{2+})	26.19	1	26.19	7.56	0.0205*
x_3 (Zn^{2+})	1.29	1	1.29	0.37	0.556NS
x_1x_2	3.81	1	3.81	1.10	0.319NS
x_1x_3	1.72	1	1.72	0.50	0.496NS
x_2x_3	0.20	1	0.20	0.06	0.816NS
x_1^2	131.03	1	131.03	37.81	0.0001**
x_2^2	103.18	1	103.18	29.77	0.0003**
x_3^2	64.76	1	64.76	18.69	0.0015**
Residual	34.65	10	3.47		
Lack of fit	26.69	5	5.34	3.35	0.105NS
Pure error	7.96	5	1.59		
Cor total	576.99	19			

*<0.05; **<0.01; NS: nonsignificant.

was about 7.32 times more than the original tryptone medium ($5.41 g L^{-1}$).

It is first report on the optimization of EPS produced by any *P. fluorescens* strain. There are only few reports available about the optimization of EPS from other *Pseudomonas* strains like Muller and Alegre (2007) reported the use of RSM to optimize the alginate type polysaccharide production by *P. mendocina* considering the temperature and agitation as main variables. Our results clearly demonstrated that maltose, Mn^{2+} and Zn^{2+} significantly effected the production of EPS by WR-1 while nitrogen source did not affect significantly. The significance of C source and metal ions to promote the production of EPS by other microbial strains has been reported earlier (Raza, Makeen, et al., 2011). Similar to our results were reported by Pilz, Auling, Stephan, Rau, and Wagner (1991) when Zn^{2+} stimulated the production of glucan in *Sclerotium rolfsii*, however, Reeslev and Jensen (1995) reported the reduction in EPS production by *Aureobasidium pullulans* in the presences of Zn^{2+}. The synthesis of the EPS in *P. aeruginosa* was stimulated by Mn^{2+} (Martins et al., 1990) and Williams and Wimpenny (1977) reported the maltose as good substrate for high polysaccharide yields by *Pseudomonas* sp.

3.5. Purification and molecular weight determination of EPS

The optimized tryptone medium was used for the maximum production of EPS by WR-1 and the purification of EPS was performed by ethanol and CTAB precipitation. The purity of EPS was confirmed by chromatography as a single peak and the lyophilized fraction was used as the purified polysaccharide for its characterization. The results indicated that the strain WR-1 produced only one kind of EPS (data not shown). Similar results were reported by Read and Costerton (1987) and Kives et al. (2006). The molecular weight of EPS produced by WR-1, detected by size exclusion chromatography by comparing with the elution times of standards, was estimated to be 6.78×10^6 Da.

3.6. Chemical composition

The chemical composition analysis of EPS showed that it contained monosaccharides and uronic acid in a ratio of 36.9:1, respectively. This result was not consistent with Chin-Chang, Santschi, and Gillow (2005) who reported that 70% of polysaccharide was consisted of uronic acid by *P. fluorescens* biovar II. The

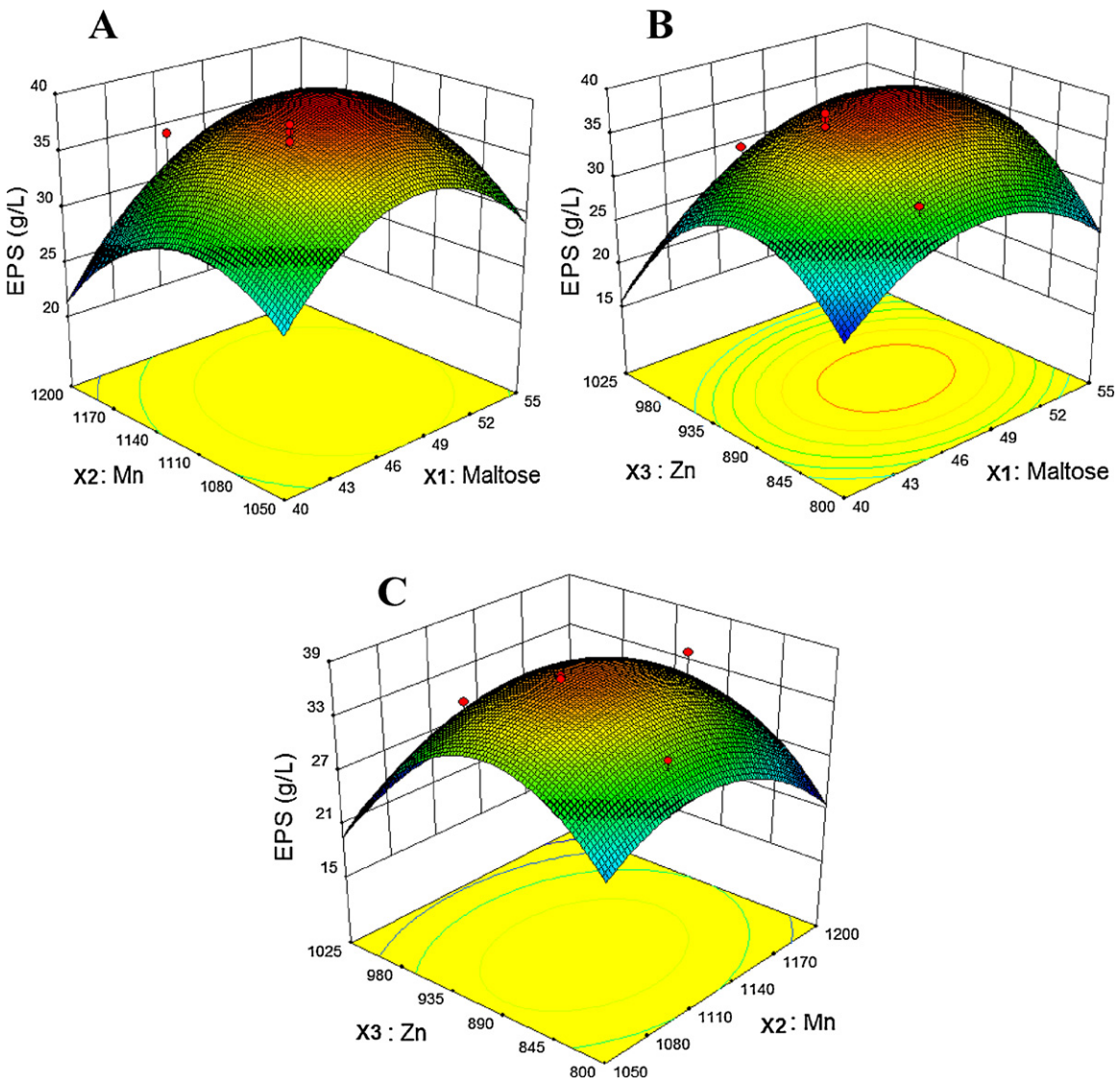

Fig. 1. Response surface 3D plots of the effect of Maltose and Mn^{2+} (A) Maltose and Zn^{2+} (B) and Mn^{2+} and Zn^{2+} (C) and their mutual interactions on the extracellular polysaccharides production by *Pseudomonas fluorescens* ***WR-1***.

results about the monosaccharide composition of EPS revealed that the EPS from WR-1 was comprised of glucose and arabinose in a ratio of 2.1:1, respectively (data not shown). There is a great variety of EPS produced by *P. fluorescens* strains depending on the type strains, culture conditions and medium composition. The EPS produced by *P. fluorescens* was composed of glucose, galactose, and pyruvate (Read & Costerton, 1987). The *P. fluorescens* strain OSU 64 was shown to produce only a mannan regardless of the type of carbon source employed as the nutrient (Eagon, 1956). The *P. fluorescens* strain B52 produced rhamnose, glucose and glucosamine containing polysaccharides (Kives et al., 2006). These dissimilarities reflect the species specific production and biotechnological potential of polysaccharides.

3.7. Antioxidant properties assays

3.7.1. Assay of reductive ability

The EPS produced by the strain WR-1 showed the reducing capacity that was increased in a concentration dependent manner. However, the reducing capacity of ascorbic acid was 45% higher than the EPS (Fig. 2A). The reducing capacity of a compound is a significant indicator of its potential antioxidant activity. The EPS from WR-1 has been found to consist of arabinose and glucose, which are actually potent reductive agents and might have a hidden aldehyde moiety (polyhydroxyaldehydes) as has been reported by Liu, Wang, Xu, & Wang (2007). The reductive ability of EPS from WR-1 is apparently based on the reductive nature of these monosaccharides. Similar results were reported by Raza, Makeen, et al. (2011).

3.7.2. Free radical scavenging activity

The data regarding the free radical scavenging activity showed that the EPS produced by the strain WR-1 increased the free radical scavenging activity in a concentration dependant manner, however, it was 27% lower than the free radical scavenging activity of ascorbic acid (Fig. 2B). Polysaccharide extracts from mushroom (Liu, Ooi, & Chang, 1997) and *Keissleriella* sp. YS 4108 exo-polysaccharide (Sun, Wang, Fang, Gao, & Tan, 2004) were also reported to have free-radical scavenging effects which were related to their affinity to the radical in the specific site. However, the

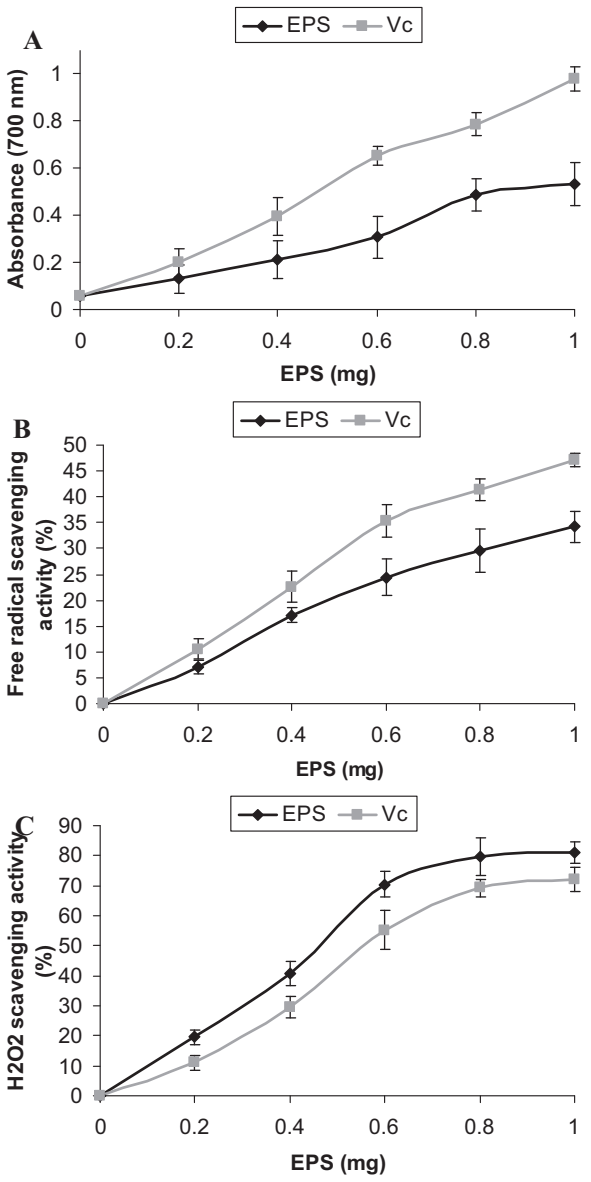

Fig. 2. Antioxidant potential assay of extracellular polysaccharides produced by *Pseudomonas fluorescens* WR-1 (A) Reductive ability, (B) Free radical scavenging activity, (C) H_2O_2 scavenging activity. Bars indicate the standard deviation of the three replicates.

mechanism of free-radical scavenging of polysaccharides is still not fully understood.

3.7.3. H_2O_2 scavenging activity

The results revealed that the EPS produced by the strain WR-1 showed good concentration dependent H_2O_2 scavenging activity which was 11% more than the ascorbic acid (Fig. 2C). H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radicals in the cells. The structural requirements for efficient quenching of hydrogen peroxide are more complicated than those established for radical scavenging. However, the character of substituents (carboxyl or acetyl group) and their position in relation to the hydroxyl groups might influence the H_2O_2 scavenging activity of EPS produced by WR-1 as has been reported by Sroka and Cisowski (2005).

4. Conclusion

The EPS produced by the strain WR-1 was composed of arabinose, glucose and uronic acid and maltose, Zn^{2+} and Mn^{2+} were found important factors for its optimum production. The EPS also showed good H_2O_2 scavenging activity while moderate free radical scavenging activity and reductive ability. The EPS demonstrated different chemical composition with other reported polysaccharides from different *P. fluorescens* strains that reflected the species specific production and biotechnological potential of polysaccharides. Maximum microbial strains should be evaluated to find out physiologically and biotechnologically efficient polysaccharides to replace synthetic chemicals. The EPS from WR-1 may be a new source of natural antioxidants with potential value for health, food and therapeutics.

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