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Extracellular polysaccharide from *Weissella confusa* OF126: Production, optimization, and characterization

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ABSTRACT

The production, optimization, and characterization of exopolysaccharide (EPS) from *Weissella confusa* OF126 and the *in-vitro* probiotic potentials of this strain was investigated. The EPS produced on sucrose modified-MRS broth was characterized. The purified EPS had an average molecular weight of 1.1×10^6 Da. HPLC analysis revealed the presence of glucose monomers, indicating its homopolysaccharide nature. The structural characteristics of the EPS were investigated by FTIR, and NMR spectroscopy. FTIR spectroscopy revealed the presence of hydroxyl, carboxyl, N-acetyl and amine groups. NMR analysis confirmed that the EPS contained α-(1 → 6) linkage and α-(1 → 3) branched linkage. The EPS showed strong *in-vitro* antioxidant activity. Four significant factors were optimized using Central Composite Design (CCD) and Response Surface Methodology (RSM). The predicted optimum conditions for EPS production were cultivation time (48.50 h), sucrose concentration (24.00 g/L), pH (7.00) and yeast extract (2.50%).The EPS produced was predicted to be 3.10 g/L, while the experimental yield was 3.00 g/L. This strain was found to possess desirable probiotic attributes by its ability to survive at pH 2.0 and in the presence of bile salts (0.50% (*w*/*v*)) for 4 h. The results obtained from this study demonstrate *W. confusa* OF126 as a promising probiotic and the EPS produced can find useful applications in industries.

1. Introduction

Lactic acid bacteria (LAB) are beneficial microorganisms commonly isolated from various fermented foods [1, 2]. They are known to perform essential roles during the fermentation of many Nigerian indigenous fermented food products such as *gari*, *fufu*, *ogi*, *kunu*, *masa etc.* [3–5], the preparation of these indigenous fermented foods and beverages remains a household art. *Ogi* is a staple cereal food made from maize (*Zea mays*), sorghum (*Sorghum bicolor*), or millet (*Pennisetum glaucum*). *Ogi* is produced by soaking cereal grains in water for two to three days followed by wet milling and sieving through a screen mesh and allowed to settle down and ferment for 24 h, then marketed as wet cake. These indigenous foods have also been documented for their health benefits and probiotic potentials [6]. Probiotics are defined as live microorganisms that confer valuable health effect on the host when sufficient quantities are ingested [7]. On this basis, the selection of LAB species as probiotics are primarily considered based on the stability, safety and health benefits conferred, as their effects are strain specific [8].

Lactic acid bacteria possess traits that could give desirable attributes to fermented food products such as the production of EPS. The industrial application of EPS produced from LAB has greatly revealed the importance of these microbes; they have the ability to secrete EPS with useful industrial physicochemical properties and potential bioactivities, in which they find their wide applications in the food, chemical, pharmaceutical and other industries with huge commercial values [8, 9]. Furthermore, EPS produced by LAB have been reported to have unique physical and rheological properties that make them useful in the food industry as viscosifying, stabilizing, gelling, or emulsifying agents [9, 10]. They also confer beneficial physiological effects such as antitumor activity, immunomodulation bioactivity and anticarcinogenecity on human health [11].

Exopolysaccharide producing LAB with potential probiotic, functional and technological properties have been isolated from spontaneously fermented foods, which have the potential of replacing the costly hydrocolloids for improvement of sourdough rheology and bread texture [12]. Some *Weissella* species have been reported to be involved and impacted positively in the fermentation of a variety of traditional Asian and African foods [1, 13]. *Weissella* strains are also receiving research attention as potential probiotic. Application of probiotic *W. cibaria* strains in oral health and their ability to inhibit *Streptococcus mutans* biofilm formation, both *in vitro* and *in vivo* have been reported

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[14]. *W. confusa* and *W. cibaria* species have been extensively studied for their ability to produce EPS especially dextran which is very useful in the food and cosmetic industries [15–17]. They have also been reported for their ability to produce a high quantity of EPS [18], which indicates acid resistance, thus improving the probiotic capacity during passage through gastrointestinal tract [19].

Free radicals are atoms with unpaired electrons; they are formed as byproducts of many normal reactions in the body, which may lead to oxidative stress causing several diseases, including cancer and diabetes [20]. Certain EPSs have demonstrated important role as a dietary free-radical scavenger for the prevention of oxidative damage and are therefore regarded as profound therapeutic agents [21–23]. Synthetic antioxidants are strong radical scavengers but may display potential risks with adverse effects on the human liver and kidney when consumed [22, 24]. Therefore, natural compounds such as bacterial EPS with similar antioxidant effectiveness with the synthetic ones have been reported and recommended for usage [25, 26].

The total yield of EPS produced by LAB varies among different species and depends on several factors such as the composition of the medium, growth conditions like temperature, pH, and incubation period [27]. Therefore, the optimization of the fermentation process is necessary for maximum yield of EPS. This present work reported the production, structural characterization, and antioxidant activity of the EPS produced from *W. confusa* OF126 isolated from *ogi* (traditionally fermented cereal gruel consumed in Sub-Saharan Africa). The *in-vitro* probiotic potentials of this *Weissella* sp. were also evaluated. Central composite design (CCD) and response surface methodology (RSM) were used to optimize the culture condition and the media composition in order to obtain the highest EPS production.

2. Materials and methods

2.1. Bacterial strain

EPS producing bacterial strain OF126 previously isolated from *ogi* was used as the source of exopolysaccharide in this study [1]. The bacterial strain was identified as *W. confusa* according to its biochemical characteristics and the use of 16S rDNA gene analysis. The sequence was deposited in GenBank under the nucleotide accession number KU892398.

2.2. Reagents and chemicals

Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), bovine serum albumin (BSA), phenol, concentrated sulfuric acid, Folin-Ciocalteu reagent, methanol, ferric chloride, potassium ferricyanide, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), pyrogallol, ascorbic acid, D-xylose, D-galactose, D-glucose, D-fructose, L-rhamnose, D-mannose and D-glucuronic acid were all purchased from Sigma Chemical Company Ltd. (St. Louis, USA). Dialysis membranes (Mw cut-off 8000–14,000 Da) were purchased from Spectrum Laboratories Inc. (Himedia).

2.3. Isolation and purification of the EPS

A total of 900 mL modified MRS-sucrose broth was prepared into which 100 mL of a freshly grown culture of *W. confusa* OF126 was inoculated and incubated using an incubator shaker (170 rpm, at 30 °C) for 24 h. The EPS was purified according to the method described by Savadogo et al. [28] with some modifications. After 24 h incubation period, the culture medium was heated at 100 °C for 10 min to inactivate enzymes that can degrade the EPS. The cells were removed by centrifugation at 12,000 xg for 15 min at 4 °C, and the cell-free supernatant was precipitated by mixing in two volumes of absolute cold ethanol, shaken vigorously and the precipitated EPS was collected by centrifugation at 5000 ×*g* for 30 min at 4 °C. This step was repeated twice and the resulting EPS was dried at 50 °C.

The precipitates were dissolved in ultrapure water and were again precipitated with a double volume of chilled ethanol and were collected by centrifugation. This procedure was repeated thrice to remove cell debris. The fraction was dialyzed against distilled water using 10 kDa dialysis membranes for 48 h at 4 °C. The partially purified EPS was frozen at −20 °C and lyophilized for 48 h and was subsequently used for further characterization.

2.4. In vitro evaluation of probiotic potentials

2.4.1. Tolerance to low pH

The ability of *W. confusa* OF126 strain to low pH was carried out as described by [29]. Freshly grown culture (18–24 h) was centrifuged at 5000 xg for 15 min at 4 °C, and the cell pellet obtained was washed two times with phosphate buffered saline (PBS) (g/L) (NaCl: 9; $Na_2HPO_4 \cdot 2H_2O$: 9; KH_2PO4:1.5; pH 7.0) and was adjusted to obtain about 8 log CFU/mL and was then re-suspended in MRS broth with pH adjusted to 2 and 2.5. Aliquots were taken at 0 h and after 4 h and were serially diluted in sterile saline solution (0.85% NaCl) and the viable cell population was determined by the spread plate method on MRS agar. The plates were then incubated at 37 °C for 48 h.

2.4.2. Bile resistance

The ability of *W. confusa* OF126 to survive in different concentration of bile salts was performed [29]. Freshly grown culture (18–24 h) was centrifuged at 5000 ×*g* for 15 min at 4 °C, and the cell pellet obtained was washed two times with PBS and was adjusted to obtain about 8 log CFU/mL and then re-suspended in MRS broth with different concentrations of bile salts (Oxgall) (0.15, 0.30 and 0.50% (*w*/*v*)). Aliquots were taken at 0 h and after 4 h and the viable cell population was determined by the spread plate method on MRS Agar. The plates were incubated at 37 °C for 48 h. The bacterial cell in MRS broth without bile salt was used as a control.

2.4.3. Tolerance to gastric acid

Tolerance of *W. confusa* OF126 strain to gastric acid was also determined [30]. A simulated gastric juice was prepared by dissolving 3 mg/mL of pepsin in 0.5% sterile saline and the pH was adjusted to 2.0 with concentrated HCl. A total of 1.5 mL of overnight culture was centrifuged at 9500 ×*g* for 5 min at 4 °C and washed twice in quarter strength Ringer's solution (QSRS). After this, 0.3 mL of the washed suspension was added to 1.5 mL simulated gastric juice (pH 2.0) in a 2.0 mL tube and vortexed for 60 s. Simulated gastric juice was replaced by 1.5 mL QSRS and used to determine the initial cell count. Aliquots of 100 μL were removed after 90 and 180 min at 37 °C and viable counts were determined by serial dilutions on MRS agar after incubation at 30 °C for 24–48 h.

2.4.4. Cell surface hydrophobicity (CSH) assay

Two hydrocarbons: n-hexadecane and xylene were used for the *in vitro* CSH assay according to the standard method [30]. Overnight grown test cultures were centrifuged at 10,000 ×*g* for 10 min, the supernatant was removed and the cells were washed twice to remove non adherent cells and then re-suspended in 1.2 mL phosphate urea magnesium sulfate (PUM) buffer (g/L) ($KH_2PO_4.3H_2O$: 22.20; KH_2PO_4:7.26; Urea: 1.80; $MgSO_4$:0.20; NaCl: 8.50; pH 7.10) and optical density (OD_{600nm}) of 1.0 absorbance (A_0) was maintained. The hydrocarbon (0.3 mL each) were added separately to 1.2 mL of *W. confusa* OF126 suspension (4:1), vortexed for 2 min and was allowed to stand by incubating at 37 °C for 30 min for the phases to be separated. The upper layer which contained the hydrocarbon was allowed to rise completely by keeping undisturbed. The lower aqueous phase was gently removed and transferred to 1 mL cuvette; the absorbance (A_0) OD_{450} was taken. The percentage of cell surface hydrophobicity (% Hp)

of the strain adhering to each n-hexadecane and xylene was calculated using the equation:

$$\%Hp = [(A_{initial} - A_{final})/A_{initial} \times 100].$$

2.5. Determination of molecular weight (Mw)

The molecular weight of the EPS was determined by high performance gel permeation chromatography (HPGPC) equipped with a TSK-GELG3000SWxl column (7.5 mm × 300 mm) (Tosoh Corp., Tokyo, Japan) column and a refractive index detector (Agilent RID-10A Series). The column was eluted with 0.1 M Na_2SO_4 solution at a flow rate of 0.8 mL min^{-1}. The molecular weight was estimated from the standard graph which was plotted using dextran standards (Mw 500, 150, 70, 40, and 10 kDa, Sigma Chemical Co., MO, USA).

2.6. Chemical composition analysis of the EPS

Total sugar and protein contents were determined by the phenol-sulfuric acid method and Folin-Lowry method [31, 32].

2.7. Analysis of monosaccharide composition of purified EPS

Monosaccharide composition of the purified EPS was analyzed by TLC and HPLC. Briefly, 5 mg of EPS sample was hydrolyzed with 0.5 mL of 6 N trifluoroacetic acid (TFA) at 100 °C for 2 h. TFA was then removed by evaporation under N_2 gas. Two microlitres (2 μL) hydrolyzate was spotted onto silica gel coated aluminum TLC plates. The mixed solvent system n-butanol, ethanol and water (50:30:20 (v/v/v)) were used for separation of carbohydrates, the plate was dried and developed by dipping it in anisaldehyde-sulfuric acid reagent, the fractions were visualized on the plate by heating the TLC plates in the oven at 110 °C for 10 min after dipping it in anisaldehyde-sulfuric acid reagent [33]. Hydrolyzed EPS was neutralized using 5 N NaOH and volume was made to 50 mL with Milli Q water. HPLC of hydrolyzed EPS was performed on an Agilent 1100 series HPLC (Agilent Technologies Palo, Alto, CA, USA). The mobile phase consisted of acetonitrile and water (80:20 (v/v)), the sample was eluted at a flow rate of 1.0 mL/min. Monosaccharide composition was carried out by comparing their retention times with those of standards under the same HPLC conditions. D-xylose, D-galactose, D-glucose, D-mannose and L-rhamnose were used as monosaccharide standards. Refractive Index Detector (RID) was used to detect monosaccharide content of the EPS sample.

2.8. Fourier transform infrared (FT-IR) analysis of the EPS

FT-IR was performed using potassium bromide (KBr) pellet method to determine the functional groups present in the purified EPS sample with spectrum range from 400 to 4000 cm^{-1} using Fourier transform-infrared spectrophotometer (FTIR, Thermo Nicolet, USA).

2.9. Nuclear magnetic resonance (NMR) spectroscopy analysis of the EPS

Twenty milligrams (20 mg) of purified EPS was dissolved in 1.0 mL of 99.96% D_2O and was analyzed. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX Advance400 MHz spectrometer.

2.10. Screening of culture condition and media composition

The culture conditions and media compositions for EPS production were screened. Carbon sources (sucrose, glucose, galactose and lactose (20 g/L)), organic nitrogen sources (yeast extract, beef extract, tryptone and peptone (25 g/L)), were supplemented each into the MRS broth and 24 h old culture were inoculated, keeping other components constant at different incubation time (12–96 h), initial pH of the media (6–8) and at different temperatures (20–45 °C). The EPS yield and viable cell count were then evaluated.

2.11. RSM optimization for EPS production

Based on the important factors such as cultivation time, sucrose concentration, yeast extract and pH, the statistical approach using CCD and RSM was performed towards obtaining the maximum EPS production. The statistical software package used was Design-Expert®, version 8.0.7.1 (Stat Ease Inc., Minneapolis, USA).

2.12. Assay of antioxidant activities

2.12.1. DPPH free radical scavenging assay

The DPPH free radical scavenging activity was measured as described by Rai et al. [34]. Briefly, 2.0 mL DPPH radical solution (0.16 mM) was added to 1.0 mL sample solution (0.5–4 mg/mL) and then 2.0 mL of deionized water was added. The mixture was mixed vigorously and incubated at 37 °C in the dark for 30 min. The absorbance was measured at 517 nm against the blank. Methanol with DPPH served as positive control. The sample blank was prepared by replacing DPPH

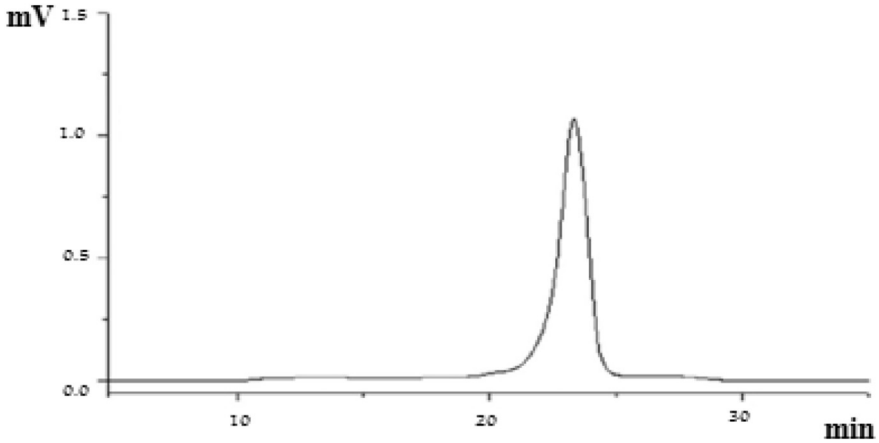

Fig. 1. HPGPC chromatogram of EPS from *Weissella confusa* OF126 on a TSK-GEL3000SWxL column.

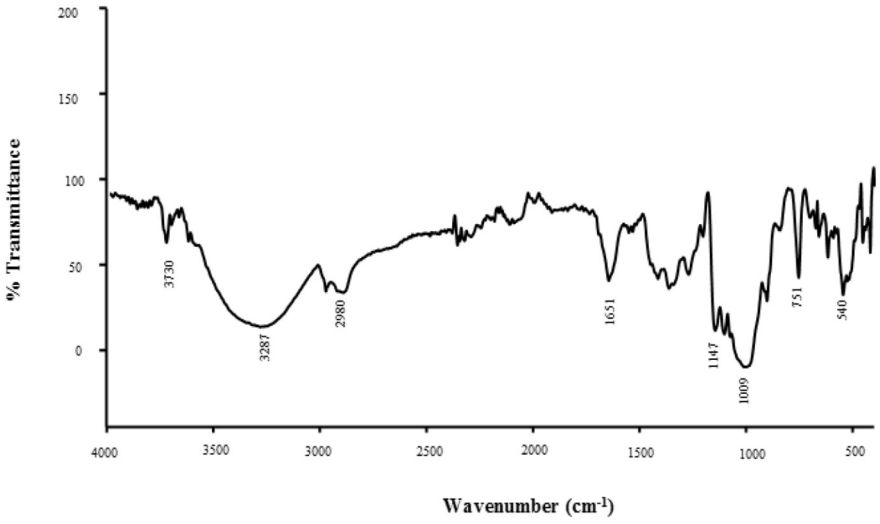

Fig. 2. FTIR spectrum of EPS from *Weissella confusa* OF126.

with methanol.

$$\text{The scavenging activity on DPPH radical } (\%) = 1 - \frac{[\text{Asample-Ablank}]}{\text{Acontrol}} \times 100$$

2.12.2. Reducing power potential

The reducing power potential of the EPS sample at different concentration was measured as described by Balakrishnan et al. [35]. To 100 μL of the EPS sample, 900 μL of phosphate buffer (0.2 M, pH 6.6) and 900 μL of 1% potassium ferric cyanide were added. The solution thoroughly

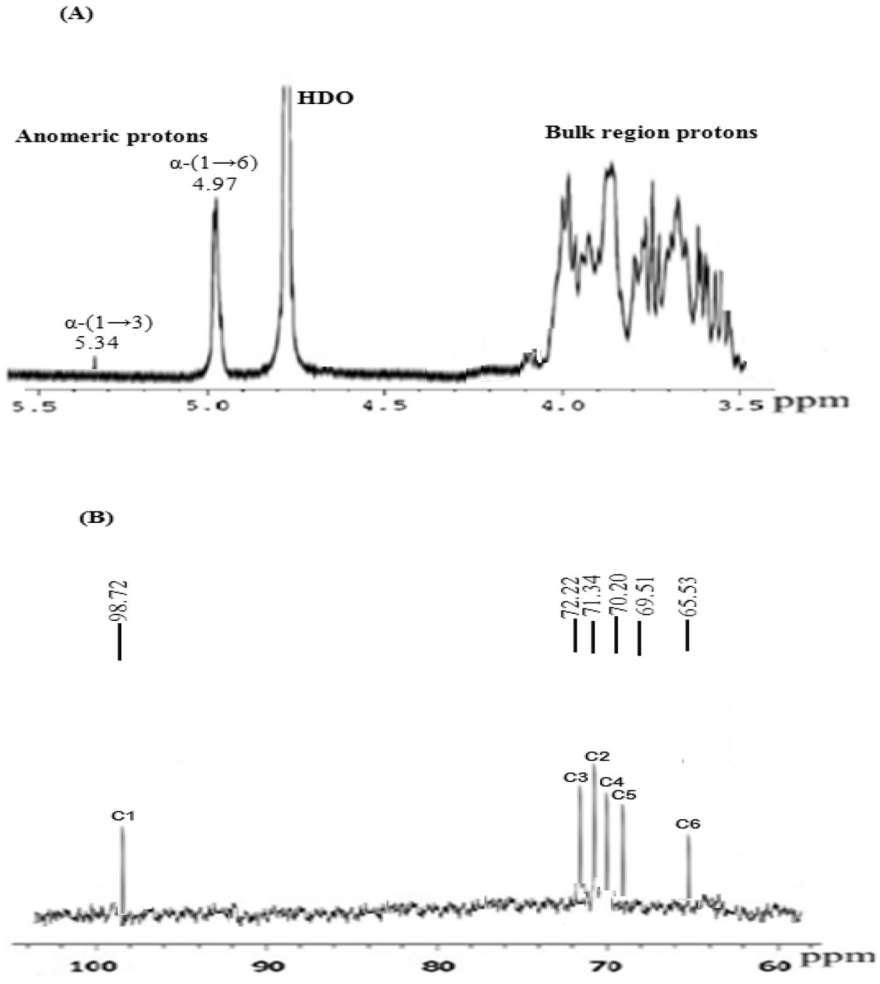

Fig. 3. ^{1}H (A) and ^{13}C (B) NMR spectra of EPS from *Weissella confusa* OF126.

Table 1
Effects of carbon and nitrogen sources on cell growth and EPS production by *W. confusa* OF126.

Culture conditions	Cell growth (log cfu/ml)	EPS (g/L)
Carbon sources (20 g/L)		
Glucose	8.25 ± 0.01	1.86 ± 0.02
Sucrose	8.01 ± 0.02	2.05 ± 0.03
Lactose	7.66 ± 0.01	1.81 ± 0.14
Galactose	7.98 ± 0.01	1.94 ± 0.17
Sucrose conc. (g/L)		
10	7.97 ± 0.02	2.02 ± 0.12
20	8.10 ± 0.04	2.10 ± 0.08
30	7.91 ± 0.01	1.94 ± 0.06
40	7.88 ± 0.01	1.87 ± 0.04
Nitrogen sources (25 g/L)		
Yeast extract	7.85 ± 0.03	2.00 ± 0.02
Beef extract	7.56 ± 0.02	1.91 ± 0.04
Tryptone	6.78 ± 0.01	1.98 ± 0.06
Peptone	7.02 ± 0.04	1.87 ± 0.10
Yeast extract conc. (%, *w/v*)		
1.0	7.64 ± 0.02	1.61 ± 0.11
1.5	7.70 ± 0.02	1.75 ± 0.08
2.0	7.83 ± 0.02	1.79 ± 0.01
2.5	8.09 ± 0.03	1.92 ± 0.11
3.0	8.21 ± 0.01	1.90 ± 0.06

Data are means ± SD of triplicates.

mixed and incubated at 50 °C for 20 min. After this, 900 μL of 10% TCA was added, mixed and centrifuged at 5000 ×*g* for 15 min and 900 μL of the supernatant solution was mixed with 900 μL of distilled water and 900 μL of 0.1% ferric chloride. The solution was mixed and the absorbance was taken at 700 nm.

2.12.3. Superoxide radical scavenging activity

Superoxide scavenging property of the EPS sample was measured as described by Balakrishnan et al. [35]. To 0.3 mL of the EPS sample at different concentration (0.5–4 mg/mL), 2.6 mL of 50 mM phosphate buffer (pH 8.2) was added and to this 90 μL of freshly prepared 3 mM pyrogallol dissolved in 10 mM HCl was added. Then the absorbance was measured from 0 to 10 min at 325 nm. To 0.3 mL of distilled water, 2.6 mL phosphate buffer (50 Mm, pH 8.2) was added and this served as blank.

$$\text{Scavenging rate } (\%) = 1 - \frac{[A_{10} - A_0]}{[C_{10} - C_0]} \times 100$$

A_0 and A_{10} are the absorbances of the EPS sample at 0 and 10 min.
C_0 and C_{10} are the absorbances of the control at 0 and 10 min.

2.12.4. Assay of hydroxyl radical scavenging activity

The hydroxyl radical scavenging effect of the EPS sample was measured with the Fenton reaction. Briefly, the reaction mixture contained 1.0 mL of brilliant green (0.435 mM), 2.0 mL of $FeSO_4$ (0.5 mM), 1.5 mL of H_2O_2 (3.0%, *w/v*). To this mixture was added 1 mL of the sample at various concentrations (0.5–4 mg/mL) was added and was incubated

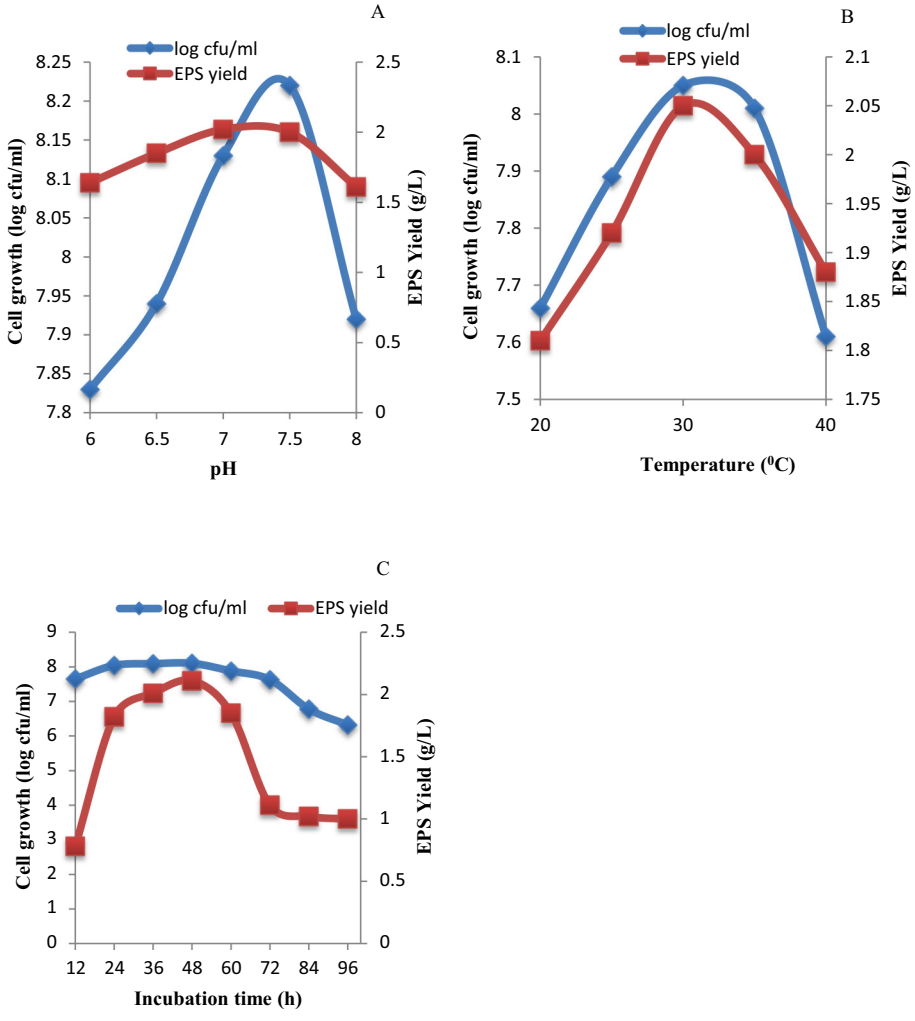

Fig. 4. Effects of (A) initial pH (B) temperature and (C) incubation time on cell growth and EPS production by *Weissella confusa* OF126. Data are means ± SD of triplicates.

at room temperature for 20 min, and the absorbance was measured at 624 nm.

$$\text{The hydroxyl radical scavenging activity } (\%) = \frac{[A_S\text{-}A_0]}{[A\text{-}A_0]} \times 100$$

A_S = Absorbance of the sample.
A_0 = Absorbance of the control.
A = Absorbance of deionized water without the sample and Fenton reaction.

2.13. Statistical analysis

All experiments were performed in triplicates and the results represented by their mean ± SD (standard deviation). Tests of significant differences were determined by Duncan's Multiple Range Test at ($P < .05$).

3. Results and discussion

3.1. In vitro evaluation of probiotic potentials

The tolerance of LAB strains to acid and bile conditions are commonly used as preliminary selection criteria for potential probiotic candidates. The growth of LAB lowers the pH of the food due to lactic acid being produced. LAB ferment sugars to acid in indigenous fermented foods and thus improving the texture of the products. Bacteria that can tolerate low pH in stomach acidic condition can be considered as probiotic [36]. *W. confusa* OF126 strain was able to survive at pH 2 and 2.5 after 4 h with 7.21 ± 0.02 and 7.98 ± 0.08 log CFU/mL. Leite et al. [37] characterized LAB with excellent properties from kefir grains using tolerance to acidic conditions. Similarly, Liong and Shah, [38] reported that the survival of LAB at pH 3.0 for 2 h was one of the requirements for bacteria to be considered as probiotic. It is also important to evaluate the ability of a potential probiotic to survive in different concentrations of bile salt. Tolerance to bile salt is a prerequisite for colonization and metabolic activity of probiotic bacteria in the small intestine of the host [39]. Although the bile concentration of human gastrointestinal tract varies, the mean bile concentration of intestine was reported to be 0.30% and approximate staying time of about 4 h [36]. In this study, tolerance to bile salts at different concentrations; 0.15, 0.30 and 0.50% after 4 h was 8.02 ± 0.13, 7.96 ± 0.11, 7.87 ± 0.05 log CFU/mL respectively. Tolerance to gastric acidity enables the probiotic bacteria to survive during passage through the gastrointestinal tract [40]. After exposure to pepsin containing simulated gastric juice (pH 2.0), the viable counts of *W. confusa* OF126 strain at the end of 180 min was 6.06 ± 0.02 log CFU/mL. Hydrophobicity contributes to the adhesion of bacterial cells to host tissue. It is an essential characteristic which helps the probiotics to colonize and modulate host immune system [41]. The degree of hydrophobicity with xylene and n-hexadecane were 34% and 27% respectively. The results obtained from the probiotic characterization studies confirmed the probiotic nature of *W. confusa* OF126 strain. Similar reports have been previously documented [37, 42–44].

3.2. Determination of molecular weight (Mw)

The HPGPC result (Fig. 1) showed that the elution peak was a single peak. Based on the calibration curve of the elution times of dextran standards (Fig. S1, Supplementary material), the molecular weight of EPS was estimated to be 1.1×10^6 Da.

3.3. Chemical composition analysis of the EPS

The sugar and protein contents obtained were 80.4 and 1.51% respectively. The EPS displayed a significant amount of total sugar content with lesser protein impurity. High carbohydrate content of about 92.35% was reported from EPS isolated from *Lactobacillus plantarum* YW11 [45]. Liu et al. [46] reported 2.3% protein from EPS obtained from the fermentation of kefir grains in soya milk.

Table 2a
The matrix of the central composite design experiment showing corresponding predicted and actual values of exopolysaccharides produced from *Weissella confusa* OF126.

Run	A	B	C	D	EPS Yield (g/L)	
	Cultivation time (h)	Sucrose concentration (g)	Yeast extract (%)	pH	Actual	Predicted
1	45.00	16.00	2.00	8.00	3.00	2.98
2	45.02	16.00	2.50	6.50	3.01	3.00
3	44.00	20.05	3.00	6.00	2.98	3.05
4	47.01	21.00	2.80	7.50	3.00	3.00
5	46.01	18.00	1.60	6.00	2.67	2.70
6	46.05	17.50	3.00	8.00	3.01	2.99
7	48.50	24.00	2.50	7.00	3.00	3.10
8	48.09	23.50	2.93	7.50	2.98	3.00
9	44.12	22.00	1.95	6.00	2.56	2.55
10	45.34	16.05	1.90	6.60	2.60	2.65
11	48.01	18.00	1.68	7.55	2.45	2.50
12	48.17	20.00	2.48	8.00	2.85	3.05
13	49.00	22.00	2.90	8.05	2.95	3.90
14	49.00	23.05	2.70	7.50	3.02	3.05
15	46.00	18.50	3.46	6.07	2.55	2.70
16	46.09	16.00	2.47	7.50	2.88	2.95
17	48.86	17.50	2.58	6.50	2.95	3.00
18	47.90	22.01	2.75	6.45	2.65	2.60
19	45.98	24.00	2.38	7.02	2.95	3.00
20	48.17	18.70	1.59	6.30	2.10	2.20
21	48.00	16.00	2.70	7.05	2.94	3.00
22	48.00	16.00	1.60	6.55	2.15	2.20
23	44.31	17.50	2.60	7.14	2.05	2.00
24	45.24	18.00	2.65	6.57	2.70	2.85
25	44.73	20.22	4.46	6.63	2.76	2.88
26	48.04	21.50	1.75	6.80	2.65	2.50
27	48.13	19.90	2.20	7.32	3.00	2.90
28	48.17	16.55	1.60	6.57	2.60	2.50
29	44.25	17.50	2.75	7.05	2.99	2.92
30	43.11	20.05	2.05	6.50	2.15	2.20

3.4. Analysis of monosaccharide composition of purified EPS

The analysis of the monosaccharide composition of the EPS on TLC plate revealed one sugar spot with the retention force (Rf) of 0.56 and

Table 2b
Analysis of Variance (ANOVA) of quadratic model for production of exopolysaccharide in *Weissella confusa* OF126.

Source	Sum of squares	Df	Mean Square	F value	p-Value Prob > F	
Model	2.84	14	1.06	6.59	0.0004	Significant
A-Cultivation time	5.104	1	5.104	0.17	0.6895	
B-Sucrose conc.	3.037	1	3.037	0.099	0.7577	
C-Yeast extract	0.48	1	0.48	15.75	0.0012	
D-pH	0.035	1	0.035	1.12	0.3064	
AB	0.026	1	0.026	0.86	0.3689	
AC	0.066	1	0.066	2.16	0.1627	
AD	0.028	1	0.028	0.91	0.3547	
BC	2.256	1	2.256	0.073	0.7902	
BD	6.250	1	6.250	2.031	0.9888	
CD	4.556	1	4.556	0.15	0.7058	
A2	9.967	1	9.967	0.32	0.5777	
B2	6.696	1	6.696	2.177	0.9634	
C2	2.05	1	2.05	66.66	<0.0001	
D2	3.218	1	3.218	0.11	0.7485	
Residual	0.46	15	0.46			
Lack of fit	0.41	10	0.41	4.20	0.0635	not significant
Pure error	0.049	5	0.018			
Cor total	3.30	29				

R-Squared = 0.8602; Adequate Precision = 11.960.

was compared with the monosaccharide standards showing that the EPS contained glucose (Fig. S2a, Supplementary material). HPLC was also carried out to confirm the TLC result. The analysis of the EPS monosaccharide composition showed that glucose as the most abundant monomer, indicating that the EPS is a homopolysaccharide. The HPLC chromatograms are presented in (Fig. S2b, Supplementary material). Similarly, Baruah et al. [16] reported that EPS from *W. cibaria* RBA12 isolated from pummelo produces a dextran composed of only glucose as sugar unit. Other researchers have also reported the use of TLC and HPLC analysis for detection of monomers composition in EPS samples [18, 47].

3.5. FT-IR analysis of the EPS

Exopolysaccharides from LAB are complex polysaccharides containing different functional groups [47, 48]. The FT-IR spectrum shows the major functional groups and chemical bonds present in EPS sample.

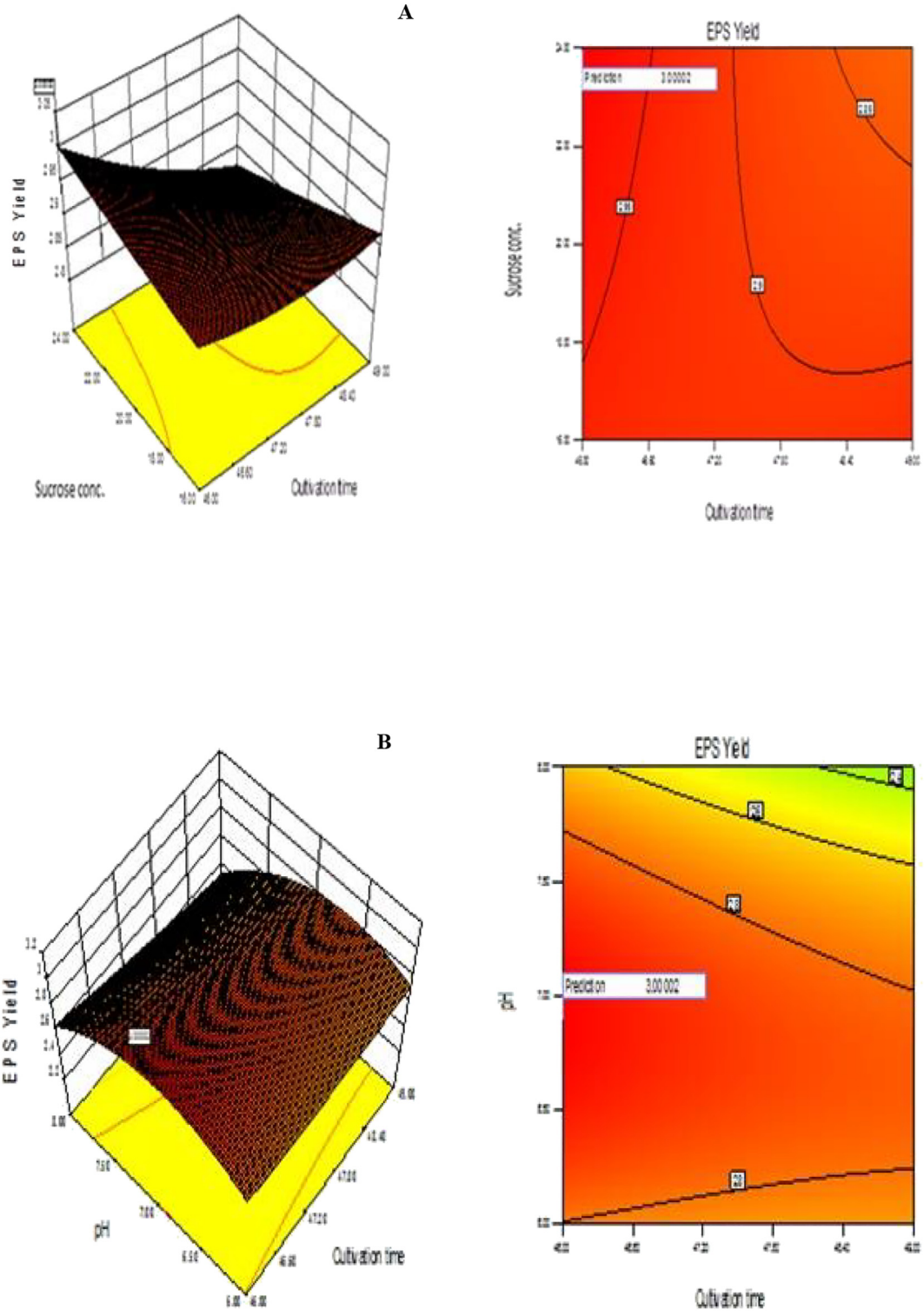

Fig. 5. Three dimensional plots and the contour plots of the four variables on *W. confusa* OF126 EPS yield (A) Sucrose concentration and cultivation time (B) pH and cultivation time (C) Yeast extract and cultivation time (D) pH and sucrose concentration (E) Yeast extract and sucrose concentration (F) Yeast extract and pH.

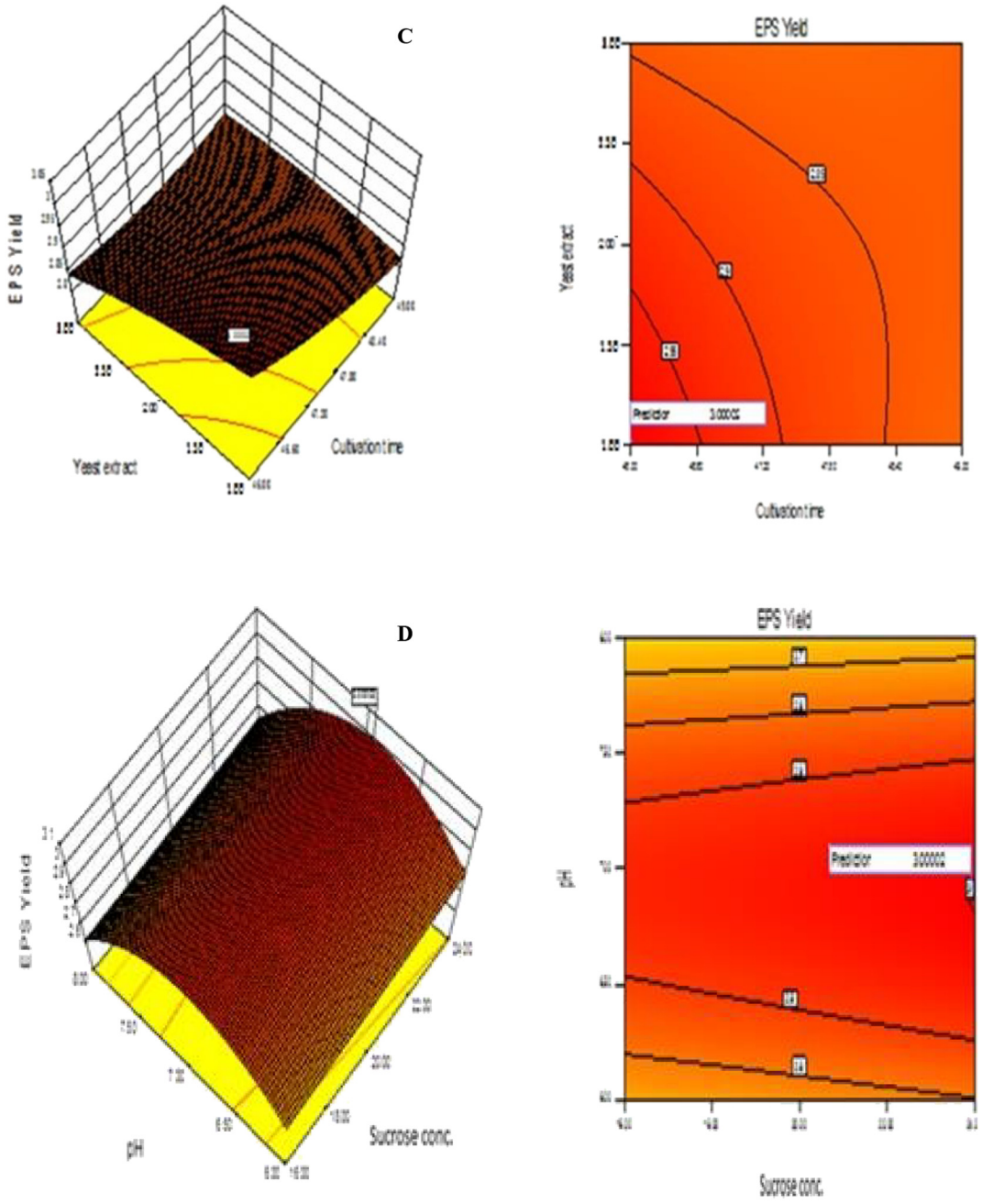

Fig. 5 (*continued*).

The FT-IR spectrum of the purified EPS as shown in (Fig. 2) revealed many peaks from 3730 to 540 cm^{-1}. The EPS contain a large number of hydroxyl groups (O–H), which displayed broad absorption peak around 3287 cm^{-1} [49]. The absorption peak exhibited in this region showed that the EPS is a typical carbohydrate [50]. The band at 2980 cm^{-1} was due to the stretching vibration of C–H and at 1651 cm^{-1} of C═O and carboxyl group [23]. The absorption around 1009 cm^{-1} was attributed to C—O—C bond stretching vibration. The FT-IR spectrum analysis revealed that the EPS has a complex polysaccharide structure containing different functional groups [41, 51–52].

3.6. NMR spectroscopy analysis

In the ^{1}H NMR spectrum (Fig. 3A), the anomeric proton signal at δ 4.97 and δ 5.34 ppm corresponds to the α-(1,6) linkages and α-(1,3) linkages, while the other signals obtained between δ 4.12 and δ 3.50 ppm were poorly resolved due to overlapping chemical shifts of bulk region protons. The ^{13}C NMR spectrum showed six signals in the region between 100 and 60 ppm (Fig. 3B), the signals appearing at δ 98.72 ppm and δ 65.53 ppm corresponds to C-1 and C-6, which are involved in the α-(1,6) linkages. The C-1 signal was assigned to an α-D-glucose α-hexopyranosyl residue, the remaining signals at δ 70.20, δ 69.51, δ 72.21, and δ 71.34 ppm corresponds to C-4, C-5, C-3 and C-2 respectively. Similar spectra pattern were reported in many lactic acid bacteria isolated from sour dough [53]. The ^{1}H NMR and ^{13}C NMR spectral analysis showed that the EPS has a structure consisting of a linear homopolymer of α-(1 → 6)-linked D-glucopyranosyl units with α-(1 → 3)-linked branches. These results are consistent with the previous spectral analysis data for the EPS produced by *W. confusa* strains [53–55].

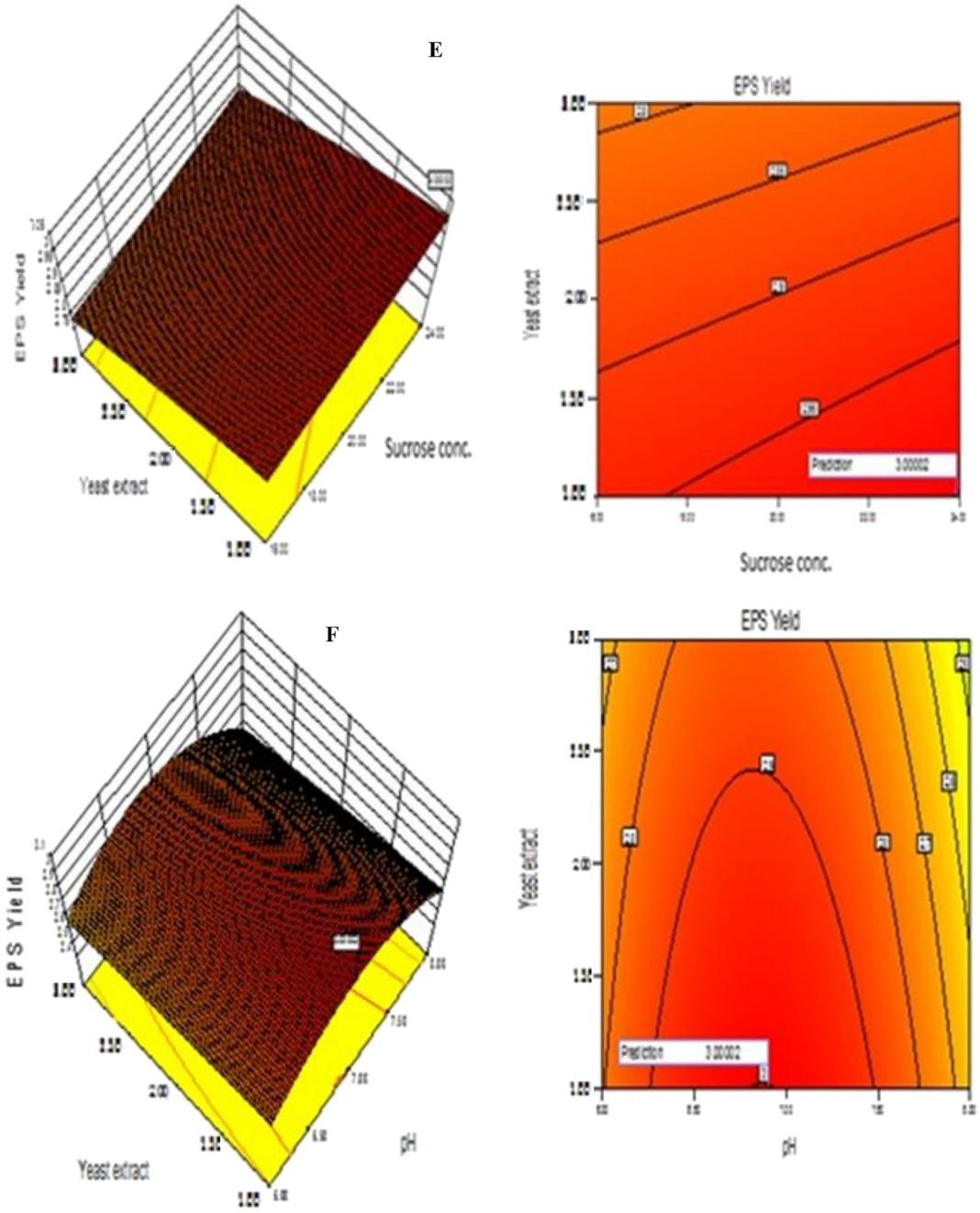

Fig. 5 (*continued*).

3.7. Screening of culture condition and media composition

It has been reported that the carbon sources had influence on the cell growth and EPS production [56]. Sucrose was found as the best carbon source for cell growth and EPS synthesis with the yield of 2.05 ± 0.03 g/L (8.01 log CFU/mL). At different concentrations of sucrose (10, 20, 30, 40 and 50 g), the optimum EPS yield and maximum cell growth of 2.10 ± 0.08 g/L (8.10 log CFU/mL) (Table 1) were observed at 20 g/L sucrose concentration. At 30–50 g/L sucrose concentration, decrease in the cell growth and EPS yield were observed. The influence of sucrose concentration on the cell growth was similar to its effect on EPS production. The effect of different organic nitrogen (yeast extract, beef extract, tryptone and peptone (25 g/L) on EPS production by *W. confusa* OF126 is shown in Table 1. At various concentration of yeast extract, maximum EPS was produced at 2.5% (1.92 ± 0.11 g/L). Several researchers have reported yeast extract as the most efficient nitrogen source during EPS production [48, 57]. The pH, temperature and cultivation time can also influence growth and production of EPS by LAB [50]. The EPS production was investigated in the modified MRS sucrose medium with initial sucrose concentration of 20 g/L at pH 6, 6.5, 7.5 and 8, different temperatures (20, 25, 30, 37 and 40 °C) and different cultivation time (12, 24, 36, 48, 60, 72, 84 and 96 h). The optimal pH, temperature and cultivation time were found to be 7.0, 30 °C and 48 h with the EPS yield of 2.05, 2.02, 2.11 (OD = 8.13, 8.05, 8.10 log CFU/mL) (Fig. 4).

3.8. RSM optimization for EPS production

Four significant factors (cultivation time, sucrose concentration, yeast extract and pH) influencing the EPS yield were selected for further optimization by CCD of RSM. A total of thirty experimental runs were generated by the software as shown in Table 2a. The experimental data were statistically analyzed using Fischer's statistical test for

analysis of variance (ANOVA) (Table 2b). The model F-value of 6.59 was significant. The values obtained for the coefficient of determination (R^2 = 0.8602) indicate good agreement between the experimental and predicted values of EPS and further signify the significance of the model. Adequate precision measures the signal to noise ratio and a ratio >4 is desirable. The ratio of 11.960 obtained in this study indicates an adequate signal. The value of lack-of-fit term was >0.05 and this is not significant relative to the pure error. This indicates that the model equation was adequate for predicting the EPS yield under any combination of values of the variables.

The regression model equations describing the relationship between the EPS yield (Y) and the coded values of independent factors (A = cultivation time; B = sucrose concentration; C = pH and D = yeast extract) and their interaction is shown below:

$$Y = +2.86 - 0.015A - 0.011B - 0.14C - 0.038D - 0.041AB - 0.064AC + 0.042AD - 0.012BC - 06.250BD - 0.017CD + 0.019A^2 + 1.563B^2 - 0.27C^2 - 0.011D^2$$

where Y = EPS yield (g/L).

The three-dimensional response surface and contour plots showing the interaction between the selected variables on EPS yield (Fig. 5A–F). The curvatures' nature of 3D surfaces gave good interaction between sucrose concentration and cultivation time, pH and cultivation time, yeast extract and cultivation time, pH and sucrose concentration, yeast extract and sucrose concentration, yeast extract and pH. Response surface plots obtained from the data clearly showed the significance of mutual interactions between the variables. The optimal values of the independent factors selected for the production of EPS were obtained by solving the regression equation using the Expert Design 8.1.7 software package. The optimal values of the tested variables were; cultivation time of 48.50 h; sucrose concentration of 24.00 g/L; yeast extract of 2.50% and pH of 7.00. Under these conditions, the maximum predicted yield of EPS was 3.10 g/L and its experimental yield was 3.00 g/L. The experimental values were found to be close to the predicted values and hence, the model was successfully validated. However, earlier reports showed varied amount of EPS produced by *Weissella* sp.: 4.80 g/L by *Weissella cibaria* GA44 [15], 17.2 g/L by *Weissella confusa* KR780676 [52], 5.1 g/L by *Weissella confusa* AJ53 [58], and 8.6 g/L by Weissella sp. [59].

3.9. Antioxidant activity tests

The ability of a compound to suppress radical scavenging activity is very important due to the damaging role of free radicals in biological systems during oxidative stress. Exopolysaccharide produced from

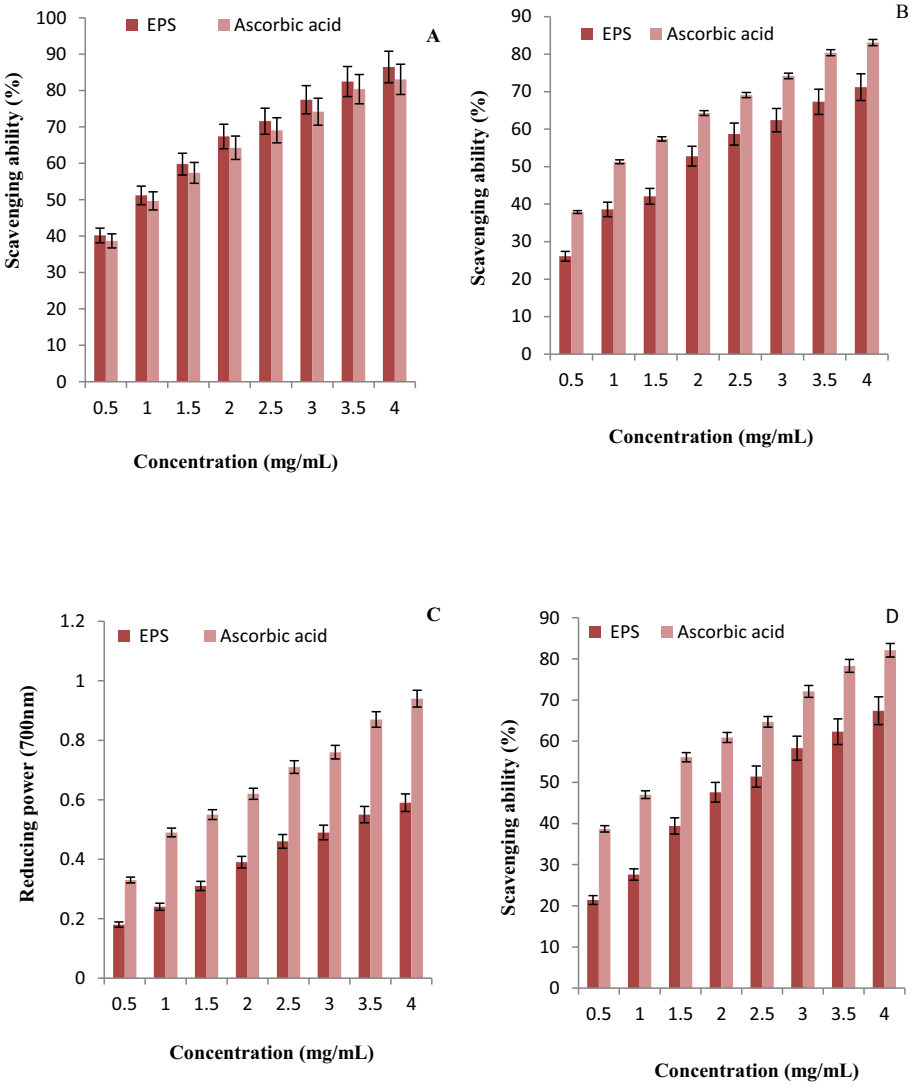

Fig. 6. Scavenging activities of *W. confusa* OF126 EPS (A) hydroxyl radical (B) superoxide radical (C) reducing capacity and (D) DPPH free radicals. Data are presented as means ± SD of triplicates.

W. confusa OF126 was studied for the hydroxyl radical scavenging activity, superoxide radical scavenging activity, reducing power potentials and DPPH free radical scavenging activity.

3.9.1. The hydroxyl radical scavenging activity of the EPS

Hydroxyl radicals are highly powerful oxidants, which can react with biomolecules in living cells and cause severe damage [60]. Fig. 6A shows that *W. confusa* EPS and ascorbic acid both exhibited hydroxyl radical scavenging activity. It can be seen that the scavenging activity of the EPS was 86.5 ± 0.02 at a dose of 4 mg/mL which was remarkably higher than that of ascorbic acid (83.1 ± 0.09).

3.9.2. Superoxide radical scavenging activity

Superoxide anions play important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [61]. At different concentrations ranging from 0.5–4 mg/mL, the EPS and ascorbic acid displayed varying degrees of antioxidant activity in a dose-dependent manner with ascorbic acid having stronger scavenging action (Fig. 6B).

3.9.3. Reducing power

The reducing power capacity of the EPS and ascorbic acid increased with increase in concentrations. The reducing power capacity of the EPS was lower than that of the ascorbic acid (Fig. 6C).

3.9.4. The DPPH free radical scavenging activity

The DPPH free radical is a stable free radical and has been widely used for estimating the free radical scavenging activities of antioxidants [62]. DPPH radical scavenging activity of the EPS was lower than that of ascorbic acid (Fig. 6D). The scavenging effect of the EPS increases with an increase in its concentration. The DPPH radical scavenging activities were 82.1 and 67.4% for ascorbic acid and the EPS respectively, at the concentration of 4 mg/mL.

4. Conclusion

Weissella confusa OF126 strain isolated from *ogi* displayed the ability to survive in acid and bile conditions, thus is an EPS-producing LAB strain with potent probiotic properties. The EPS produced by *Weissella confusa* OF126 is composed of only glucose with an average molecular weight of 1.1×10^6 Da. The EPS is a homopolymeric polysaccharide with α-(1 → 6) and α-(1 → 3) linked glucan. This study also evidenced that *W. confusa* EPS showed strong reducing power capacity, hydroxyl radical, DPPH and superoxide radical scavenging ability *in vitro*. The probiotic potential of this *Weissella* sp. from food origin and strong antioxidant properties displayed by the EPS produced can find wide application in food industries and can also serve as an adjunct culture in fermented food products.

Conflict of interest

Authors declare that there are no conflicts of interest whatsoever.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.01.060.

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