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ORIGINAL ARTICLE

Optimization of exopolysaccharide production by probiotic yeast *Lipomyces starkeyi* VIT-MN03 using response surface methodology and its applications

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Abstract
In the present study, the cultural conditions for exopolysaccharide (EPS) production from probiotic yeast *Lipomyces starkeyi* VIT-MN03 were optimized using response surface methodology (RSM) to maximize the yield of EPS. Interactions among the various factors viz. sucrose concentration (1–3 g%), NaCl concentration (2–4 g%), pH (3–5), temperature (20–30 °C), and incubation period (20–40 days) during EPS production were studied using Box-Behnken design (BBD). The EPS was purified and characterized using various instrumental analyses. The properties like adhesion, antioxidant, biosurfactant, cholesterol removal, and binding ability to mutagens were also tested for EPS produced. Sixfold increase in EPS production (4.87 g L^{-1}) by *L. starkeyi* VIT-MN03 was noted under optimized condition. EPS showed a high viscosity (1.8 Pa S^{-1}) and good shear-thinning properties. Instrumental analysis showed that EPS was heteropolysaccharide composed of glucan, mannan, and rhamnan. *Lipomyces starkeyi* VIT-MN03 exhibited good self-adhesion (95%) and co-aggregation ability (93%). Adhesion efficiency for yeast inoculum containing 5.5×10^7 CFU mL^{-1} per 9.2 cm^2 of Caco-2 cell (colorectal adenocarcinoma) was noted. The probiotic EPS displayed strong antioxidant ability to scavenge hydroxyl radical and DPPH by 58% and 71% respectively. In addition, biosurfactant activity (86%) and cholesterol removal (90%) ability of probiotic EPS was also tested. EPS bound cells of *L. starkeyi* VIT-MN03 showed good binding ability to mutagens. These results support the effectiveness of using RSM for maximum EPS production. To the best of our knowledge, this is the first report on optimization of EPS production by probiotic yeast.

Keywords Exopolysaccharides · *Lipomyces starkeyi* VIT-MN03 · Optimization · Probiotic properties · Response surface methodology (RSM)

Introduction

Exopolysaccharides (EPS) are high molecular weight polymers secreted by microorganisms which can be used as bioadhesives, bioflocculants, biosorbents, gelling agents, stabilizers, and thickeners. There are reports on EPS-producing yeast genera viz. *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Lipomyces*, *Pichia*, *Pseudozyma*, *Rhodotorula*, *and Sporobolomyces* (Rusinova-Videva et al. 2010). The EPS produced by *Candida* yeast exhibited physicochemical and rheological properties which are useful in food, cosmetic, and pharmaceutical industries (Gientka et al. 2016). Probiotic yeasts viz. *Saccharomyces cerevisiae*, *Candida* sp., and *Pichia* sp. have also been reported for EPS production (Syal and Vohra 2013; Ragavan and Das 2017b). It was reported that colonization of probiotic can be enhanced as EPS is retained for longer period in the gastrointestinal tract (Looijesteijn et al. 2001).

Response surface methodology (RSM) is a powerful statistical tool, which is being used to predict the optimization of nutritional conditions in many analytical fields (Cui et al. 2010). Few attempts have been made to optimize the

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conditions for EPS production in macrofungi (Lung and Huang 2010; Cui and Zhang 2011). So far, no report is available on the application of RSM for optimization of EPS production by probiotic yeast.

The cellular aggregation is an important feature for probiotics as it is related to inter- and intraspecies microbial interaction as well as interaction with host epithelial cells. Probiotic strains with auto-aggregation can also prevent the pathogen colonization along the intestinal epithelial surfaces (Ray et al. 2017). Cell surface hydrophobicity (CSH) plays a crucial role in the attachment to, or detachment from the surfaces. The more hydrophobic cells adhere more strongly to hydrophobic surfaces (Krasowska and Sigler 2014). Probiotic strains of high adherence capacity effectively prevent diarrhea and alleviate inflammatory responses (Daliri and Lee 2015). In addition, EPS-producing probiotic bacteria *L. plantarum* was also identified as a potential source for adhering to Caco-2 cells lines (Wang et al. 2014).

Considerable attention has been focused to evaluate the biological functional activities of probiotic EPS viz. flocculating, emulsifying, solubility, antioxidant, antibacterial, and antitumor activities which have great potential in food, biomedicine, and pharmaceutics industries (Riaz Rajoka et al. 2018). Also, EPS can serve as gelling agents. Microbial EPS with their unique structural and functional properties attract the increasing interest of researchers for natural antioxidants (Yangfang et al. 2018).

In addition, the binding ability of probiotic EPS increases the inactivation of mutagens. EPS extracted from probiotic bacteria *L. plantarum* showed antimutagenic activity (Tsuda et al. 2008). So far no report is available on EPS-producing probiotic yeast showing antimutagenic activity.

Therefore, the aim of the present study includes (i) optimization of EPS production by probiotic yeast *L. starkeyi* VIT-MN03 using RSM (ii) characterization of EPS by instrumental analysis and (iii) evaluation of adhesive properties and antioxidant and antimutagenic activities of EPS.

Materials and methods

Probiotic yeast strain and culture condition

The yeast strain *Lipomyces starkeyi* VIT-MN03 was isolated from the gastrointestinal tract of goat and already reported as EPS-producing probiotic yeast strain in our previous study (Ragavan and Das 2017a-b). Stock culture was maintained at − 80 °C in YEPD (Merck, Germany) broth with 20% (*v*/*v*) glycerol. Then it was propagated twice in YEPD broth at 37 °C for 16–18 h, prior to the experiments. *Lipomyces starkeyi* VIT-MN03 was inoculated in 100 mL of basal medium (glucose 30 g L^{-1}, $(NH_4)2SO_4$ 2.5 g L^{-1}, KH_2PO_4 1 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, NaCl 0.1 g L^{-1}, and $CaCl_2.2H_2O$ 0.1 g L^{-1} at pH 4) and incubated on a rotary shaker (180 RPM) at 22 °C for 168 h for exopolysaccharide production (Ibrahim et al. 2012).

Statistical optimization of EPS production using RSM

The optimization of various parameters for maximum EPS production in *L. starkeyi* VIT-MN03 was done by RSM using Box-Behnken design (BBD). The software Design Expert (Version 11) was used to reveal the interactions of different factors on EPS production. A design of 46 experiments was formulated and experiments were carried out in a flask containing different concentrations of sucrose and sodium chloride at different pH, temperature, and incubation period. Five percent of inoculum per 100 mL was added to the flask. EPS production was used as the dependent variable (response) and the 3D contour plots were prepared to know the interactions of different factors and to evaluate the optimized conditions which influence the responses (Maran et al. 2013).

A set of 46 experiments were carried to evaluate the effects of the five variables viz. sucrose (%), sodium chloride (%), pH, temperature (°C), and incubation period (d) each with three different concentration levels of low (− 1), medium (0), and high (+ 1) on responses as weight of EPS (g L^{-1}). The ranges and levels of the three variables were selected (Table S1) and the weight of EPS was taken as a response.

Exopolysaccharide extraction and purification

Probiotic yeast *L. starkeyi* VIT-MN03 culture was centrifuged at 10,000 RPM for 20 min at 4 °C. To the supernatant, two volume of ice-cold isopropanol was added to precipitate the EPS overnight. The precipitate was centrifuged at 10,000 RPM for 30 min and 10 mL of supernatant was taken to dialysis through 10 kDa membrane against distilled water at 4 °C for 72 h with 2–4 changes per day to remove low molecular weight impurities and the remaining were lyophilized overnight (Wang et al. 2015).

Characterization of probiotic EPS

High-performance liquid chromatography analysis

The compositional analysis was performed by high-performance liquid chromatography analysis (HPLC) (PerkinElmer, series 200, USA). The polysaccharides were first hydrolyzed with 4 M trifluoroacetic acid (TFA) at 121 °C for 2 h in a sealed hydrolysis bottle. After the excessive TFA was removed by evaporation under a stream of N_2, the EPS hydrolysate was dissolved in deionized water, filtered through 0.45 μm nylon filter, and then analyzed by HPLC. The column was eluted at a flowrate of 0.2 mL min^{-1} and the injection volume of sample was 20 μL (Shao et al. 2014).

Fourier transform infrared spectrum analysis

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared (FT-IR) spectrophotometer (Shimadzu, DR-800, Japan). The purified polysaccharide was ground with potassium bromide (KBr) powder and pressed into pellets for FT-IR measurement in the frequency range of 4000–400 cm^{-1}, at a resolution of 4 cm^{-1} (Shao et al. 2014).

Gas chromatography analysis

The composition of the EPS was analyzed through gas chromatography (GC) (JEOL GC MATEII). Briefly, hydrolyzation of the purified EPS (10 mg) was performed at 120 °C for 6 h with 2 mL of 2 moL^{-1} trifluoroacetic acid (TFA), and the remnant TFA in the hydrolysate was eliminated by evaporation. The dried hydrolysate was transformed to acetylated derivatives. GC analysis was performed on an instrument equipped with a flame ionization detector (FID) using an HP-5 capillary column (30 m × 0.32 mm; I. d 0.25 m) (Agilent Technologies Co., Ltd., USA). The following were the operation conditions: injection temperature 250 °C; injection volume 3 μL; detector temperature 250 °C; split ratio 3:1. Compared with the standard sugars (glucose, fructose, arabinose, galactose, rhamnose, and mannose), the composition of the EPS was identified according to the methods of Yang et al. (2015).

X-ray diffractive analysis

The crystallinity of EPS was determined by collecting X-ray diffraction diagrams using Bruker D8 Advance diffractometer (Netherlands) with Cu Ka radiation generated at 45 kV and 40 mA. The lyophilized EPS were pressed into flat pieces and mounted onto a quartz sampler holder. The data were generated in reflection mode and collected in the 2θ range 20°–80° at a scan rate of 1.0° min^{-1} at room temperature (Liu and Catchmark 2018).

Nuclear magnetic resonance spectroscopy analysis

The chemical structure of the exopolysaccharide produced by probiotic yeast *L. starkeyi* VIT-MN03 was investigated using ^{1}H proton nuclear magnetic resonance spectroscopy (NMR) and ^{13}C NMR spectroscopic analysis respectively using a Bruker Advance II 500 spectrometer (Bruker Co., Billerica, MA). About 20 mg of EPS sample was dissolved in 99.96% D_2O. The ^{1}H NMR spectrum and ^{13}CNMR spectrum were recorded using a Bruker Advance III, 400 MHz NMR spectrophotometer, at a probe temperature of 25 °C. Chemical shifts such as resonance signals (δ) were reported in parts per million (Saravanan and Shetty 2015).

Scanning electron microscopy analysis

The microstructure and surface morphology of the EPS was observed using scanning electron microscopy (SEM) at an acceleration voltage of 10.0 KV and under × 200, × 400, and × 1000 magnifications. The lyophilized EPS sample was fixed to the SEM stubs with conductive tape and coated with a layer of 10 nm Au before SEM observation (Yangfang et al. 2018).

Viscosity analysis

The purified EPS powder was mixed thoroughly and 2 g was taken. The viscosity behavior of EPS solution was analyzed at constant temperature 25 °C with (Oswald's viscometer, UK) under different shear rates 30, 60, 90, and 120 per second (Amer 2013). In order to evaluate the specific viscosity changes of the EPS, the viscosity values at the shear rate of 100 1/s were compared. Xanthan gum served as a positive control.

Adhesion properties

Auto-aggregation

Lipomyces starkeyi VIT-MN03 cell pellets were obtained after washing and resuspending the cells with PBS to obtain a final cell density of around 1×10^{9} CFU/mL at 600 nm (UV-2450, Shimadzu, Japan). Yeast suspension (2 mL) was transferred into four test tubes and 1% of EPS suspension was added. Absorbance was read at 600 nm against the blank solution at different time in travel (6, 12, and 24). The auto-aggregation ability (%) was calculated using the following formula (Lohith and Anu 2014).

$$\text{Auto}-\text{aggregation} = (1-\text{At}/\text{A0}) \times 100$$

where At is the absorbance readings at different time points ($t = 6$, $t = 12$, and $t = 24$) and A0 indicates absorbance readings were taken initially.

Co-aggregation

Co-aggregation ability of *L. starkeyi* VIT-MN03 with bacterial pathogens was evaluated following the method of Jankovic et al. (2012) with modifications. Bacterial pathogens viz. *Escherichia coli*, *Staphylococcus aureus*, *Salmonella sp.*, *and Klebsiella sp*. were obtained in log phase culture. The yeast and the pathogenic cell suspension were prepared with the final density of 1×10^{9} CFU/mL at 600 nm. Two milliliters of each pathogen and the yeast cells were dispensed into sterile tubes. The tubes were thoroughly mixed and incubated for 60 min. The absorbance was read at 600 nm. Control tubes for each of pathogens and the yeast cells were prepared and

absorbance was read individually. The percentage of co-aggregation was determined according to the formula

$$\text{Co-aggregation}\ (\%) = [(Ax + Ay)/2 - A\ (x + y)/(Ax + Ay/2)] \times 100$$

where Ax represents absorbance of EPS-producing yeast strain, Ay represents absorbance of the pathogen under study; A (x + y) represents absorbance of the mixture of both.

Adhesion to hydrophobic solvent

The cell surface hydrophobicity of *L. starkeyi* VIT-MN03 was measured by measuring microbial adhesion to hydrocarbons as described by Sica et al. (2012) with minor modifications. EPS-producing yeast suspension (4 mL) was added to 1 mL of n-hexadecane, and chloroform separately. The tubes were vortexed for 2 min to separate two phases. The aqueous phase was gently separated out and the OD was read at 600 nm. A decrease in the OD of the aqueous phase was taken as a measurement of cell surface hydrophobicity (H %) and the percentage of cells bound to the organic phase was calculated according to the formula as follows:

$$\text{Hydrophobicity}\ (\%) = (1 - \text{ODa}/\text{ODb}) \times 100$$

where ODb is an optical density of cell suspension before mixing and ODa is optical density after mixing.

Caco-2 cell adhesion

The Caco-2 cell adhesion assay was performed following the method of Piatek et al. (2012) with minor modifications. EPS-producing *L. starkeyi* VIT-MN03 (5×10^5, 5×10^6, and 5×10^7) were suspended in 1 mL of Dulbecco's modified Eagle's minimal essential medium (DMEM) and incubated with Caco-2 cells for 90 min under standard conditions (5% CO_2, 37 °C, 95% humidity). For adhesion assays, Caco-2 monolayers were prepared on cover glass placed in 24-well tissue culture plates. The Caco-2 monolayer was washed three times with PBS buffer to remove non-adhering yeast cell. To release attached yeast cells, the Caco-2 monolayer was treated with a solution of 1% Triton X-100 detergent mixed with PBS buffer. The lysis was carried out on ice for 10 min. Then the lysates were centrifuged at 4500*g* for 10 min. The supernatant was washed twice with PBS. Finally, the supernatant was suspended in 1 mL of 0.9% NaCl. The number of adhered yeast cells was quantified by pour plate method (10^{-5}–10^{-7}).

Antioxidant activity

Total antioxidant activity

Total antioxidant activity of probiotic EPS was measured using Arun et al. (2017) method with minor modifications. Briefly, the tubes containing polysaccharides at various concentration (1–3 mg mL^{-1}) and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. Ascorbic acid was used as standard. The antioxidant capacity was expressed as ascorbic acid equivalent.

DPPH scavenging activity

The DPPH radical-scavenging capacities of probiotic EPS was determined as described by Saleh et al. (2010). A volume of 500 μL of EPS sample at different concentrations (1–3 mg mL^{-1}) was added to 375 μL of 99% ethanol and 125 μL of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken and then incubated for 60 min in a dark room at room temperature. Scavenging capacity was measured spectrophotometrically (UV mini 1240, SHIMDZU, China) by monitoring the decrease in absorbance at 517 nm. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. Ascorbic acid was used as positive control. The scavenging activity is determined using the formula:

$$\text{Scavenging activity} = [(A0 - A1)/A0] \times 100$$

where A0 represent the absorbance of the control and A1 represent the absorbance of the sample, respectively.

Hydroxyl radical scavenging activity

The scavenging activity of hydroxyl radical by probiotic EPS was assayed by deoxyribose method as the same described by Nagai et al. (2002) as follows: in clean test tubes, 0.45 mL of sodium phosphate buffer solution (0.2 M, pH 7.0), 0.15 mL of 2-deoxyribose solution (10 mM), 0.15 mL of FeSO-EDTA solution (10 mM FeSO, 10 mM EDTA), 0.15 mL of hydrogen peroxide solution (10 mM), and EPS suspension (50–100 μl) were added. The solutions were completed to a final volume (1.5 mL) with DW then incubation at 37 °C for 4 h. After incubation, the reaction was stopped by adding 0.75 m L of trichloroacetic acid solution (2.8%, *w*/*v*) and 0.75 mL of thiobarbituric acid solution (1% in 50 mM NaOH solution) then the solutions were boiled for 10 min and cooled in water. The absorbance of the solutions was measured at 520 nm. Control

was prepared by the same procedure without EPS suspension. Ascorbic acid solution (0.03%) was used as positive control. Inhibition of deoxyribose degradation (I %) represents hydroxyl radical scavenging activity and it was calculated using the following equation:

$$I\% = [(A0-A1)/A0] \times 100\%$$

Determination of reducing power

The reducing power of probiotic EPS was determined by the method Mathew and Abraham (2006) as follows: in clean test tubes, a serial of known volumes (1–3 mg mL^{-1}) of EPS were added. The solutions were completed to 1.0 mL with DW. 2.5 mL of phosphate buffer solution (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%, *w/v*) were added to each tube then mixed well. The mixtures were incubated at 50 °C for 20 min. After incubation, 2.5 mL of trichloroacetic acid solution (10%) were added to each mixture then centrifuged at 5000*g* for 10 min. A known volume (2.5 mL) of each clear solution obtained after centrifugation (supernatant) was taken in another clean test tube then 2.5 mL of DW and 0.5 mL of ferric chloride solution (0.1%) were added and mixed well. The absorbance was measured at 700 nm. Control was prepared by the same procedure without EPS suspension. Ascorbic acid used as standard.

Biosurfactant activity

The biosurfactant from the EPS suspension was estimated using orcinol assay method (Tuleva et al. 2002). Various concentrations of EPS (1–3 mg mL^{-1}) were used to determine the maximum biosurfactant activity. EPS solution from each concentration (100 μL) was added to 900 μL of a solution containing 0.19% orcinol (in 53% H_2SO_4). Samples were heated for 30 min at 80 °C and cooled at room temperature. The solution absorbance was measured at 421 nm. Xanthan gum was used as a positive control.

Cholesterol removal

Lipomyces starkeyi VIT-MN03 was inoculated on basal media containing bile salt (1%) and water-soluble cholesterol (100 μg) adjusted to pH − 2, incubated at 37 °C for a different time in travels, 4, 8, 12, 24, and 48 h. Five milliliters of culture was taken in each in travel and centrifuged at 5000 RPM for 5 min (Ragavan and Das 2017b). Cholesterol removal rate was measured at 600 nm and following formula was used to calculate the removal rate.

$$(\text{Cholesterol Conc.in control}-\text{Cholesterol Conc.in sample})/(\text{Cholesterol Conc.in control}) \times 100$$

Binding of mutagen

The binding ability of probiotic EPS was determined by the method of Sreekumar and Hosono with minor modifications. Amino acid pyrolysates such as 2-amino-6-methyldipyrido imidazole (Glu-P-1) and 2-amino-3,4-dimethyl-imidazo quinoline (MeIQ) (Sigma Aldrich, USA) were used to investigate binding properties of EPS. EPS suspension (0.1 mL) were added to 0.9 mL of mutagen and incubated at 37 °C for 30 min and filtered. Mutagens in the filtrate were quantified with a reverse-phase HPLC system (Shimadzu, Japan). A mobile phase of 0.1 M citrate, 0.2 M disodium hydrogen phosphate (pH 3.0), acetonitrile, and triethylamine (60:40:0.05) was used, and the absorbance was measured at 254 nm. Mixtures in which phosphate buffer was substituted for suspensions were run as positive controls. Percentage binding was calculated with the following equation (Tsuda et al. 2008):

$$\text{Binding ability}\ (\%) = [1-(\text{peak area of samples with mutagen}/\text{peak area of positive control})] \times 100$$

Statistical analysis

Statistical analysis of the model was performed to calculate the analysis of variance (ANOVA). The experimental designs and regression analysis was done by Design Expert software (Version 11). The superiority of polynomial model equation was judged by determination of coefficient *R* and its statistical significance was identified by F-test. All experiments were performed in triplicate. The obtained results were expressed as the average of three biological replicates ± standard deviation (SD).

Results

Process optimization of EPS production using response surface methodology

The statistical design of Box-Behnken model was applied to optimize EPS production from probiotic yeast *L. starkeyi* VIT-MN03 by varying the parameters sucrose (A), sodium chloride (B), pH (C), temperature

(D), and incubation period (E) at different concentration range. Predicted values and experimental responses were computed by ANOVA to check whether the polynomial expression is able to predict the responses. Parameters were optimized by Box-Behnken design with three central points and the response of EPS yield was studied. Second-order polynomial equation for EPS production is given below:

$$\begin{aligned}\text{EPS production} = {} & +4.87 \\ & + 0.5908*A - 0.4421*B - 0.6162*C \\ & + 0.1733*D - 0.3762*E - 0.4406*AB \\ & + 0.6225*AC - 0.5719*AD \\ & + 0.7400*AE \\ & + 0.5900*BC - 0.5683*BD \\ & + 0.2175*BE \\ & + 0.4375*CD \\ & + 0.1550*CE - 0.0175*DE - 1.72*A2 - 1.14 \\ & \quad *B2 - 0.8770*C2 - 0.6392*D2 - 0.7186*E2\end{aligned}$$

The optimum levels of each variable for maximum EPS production, three-dimensional response surface plots were made (Fig. 1). The results showed a significant influence of variables on EPS production either individually or interaction with each other ($p < 0.0001$). Interactive effect of variables, AB (sucrose vs sodium chloride), AC (sucrose vs pH), AD (sucrose vs temperature), and AE (sucrose vs incubation period) had a most significant positive impact on EPS dry weight (Fig. 1a and d) as compared to BC (sodium chloride vs pH) and other interactions (Fig. 1e–j). The predicted values of EPS production were calculated using regression analysis and related to experimental data which were well agreed with the predicted response values (Fig. 1k). The actual EPS production (4.86 g L^{-1}) was close to the predicted value (4.87 g L^{-1}) indicating the validity of the model (Table S2).

The analysis of variance (ANOVA) for the obtained model was tabulated in Table 1. The Model F-value of 52.17 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. In this case, A, B, C, D, E, AB, AC, AD, AE, BC, BD, CD, A^2, B^2, C^2, D^2, and E^2 are significant model terms. The lack of fit F value of 1.36 implies the lack of fit is not significant relative to the pure error. Significant impact on the production of EPS occurs due to p value less than 0.05. The total determination of the coefficient ($R^2 = 0.9766$), showed a realistic fit of the model to the experimental data. The adjusted coefficient value (adj $R^2 = 0.9579$) also proved that the model was highly significant with the coefficient of the variation (C.V) (7.59%) (Table 1).

Characterization of probiotic yeast EPS

HPLC analysis

Purified probiotic EPS were analyzed with high-performance liquid chromatography (HPLC) system. The composition of *L. starkeyi* VIT-MN03 was identified by sugar standards of the same retention time. HPLC peaks exhibited the compounds viz. arabinose, ribose, galactose, glucose, xylose, rhamnose, and mannose (Fig. S1a) and characterized as a heteropolysaccharide.

FT-IR analysis

Probiotic EPS showed maximum polysaccharide peaks (Fig. S1b). A characteristic absorption band appeared at 1658.04 cm^{-1} attributes the stretch of C=O bond (carbonyl group), 2737.01 cm^{-1} attributes the stretching vibration of methylene group (hexose), 2681.02 cm^{-1} attributes –CHO in aldehydes, 1056.52 cm^{-1} and 1030.18 cm^{-1} attributes the C–O stretch vibration (carbohydrates), and 1017.59 cm^{-1} indicating the presence of carbon ring compounds. A band at 879.28 cm^{-1} showed the presence of ß-glycoside bond which makes the linkage between sugar monomers. The absorption bands at 1437.46 cm^{-1}, 1405.57 cm^{-1}, 684.17 cm^{-1}, 600.75 cm^{-1}, and 576.12 cm^{-1} containing carboxylic group indicated the presence of carboxylic acids. Peaks at 1160.01 $cm^{-1}$and 1114.56 cm^{-1} indicate the presence of thiocarbonyl groups.

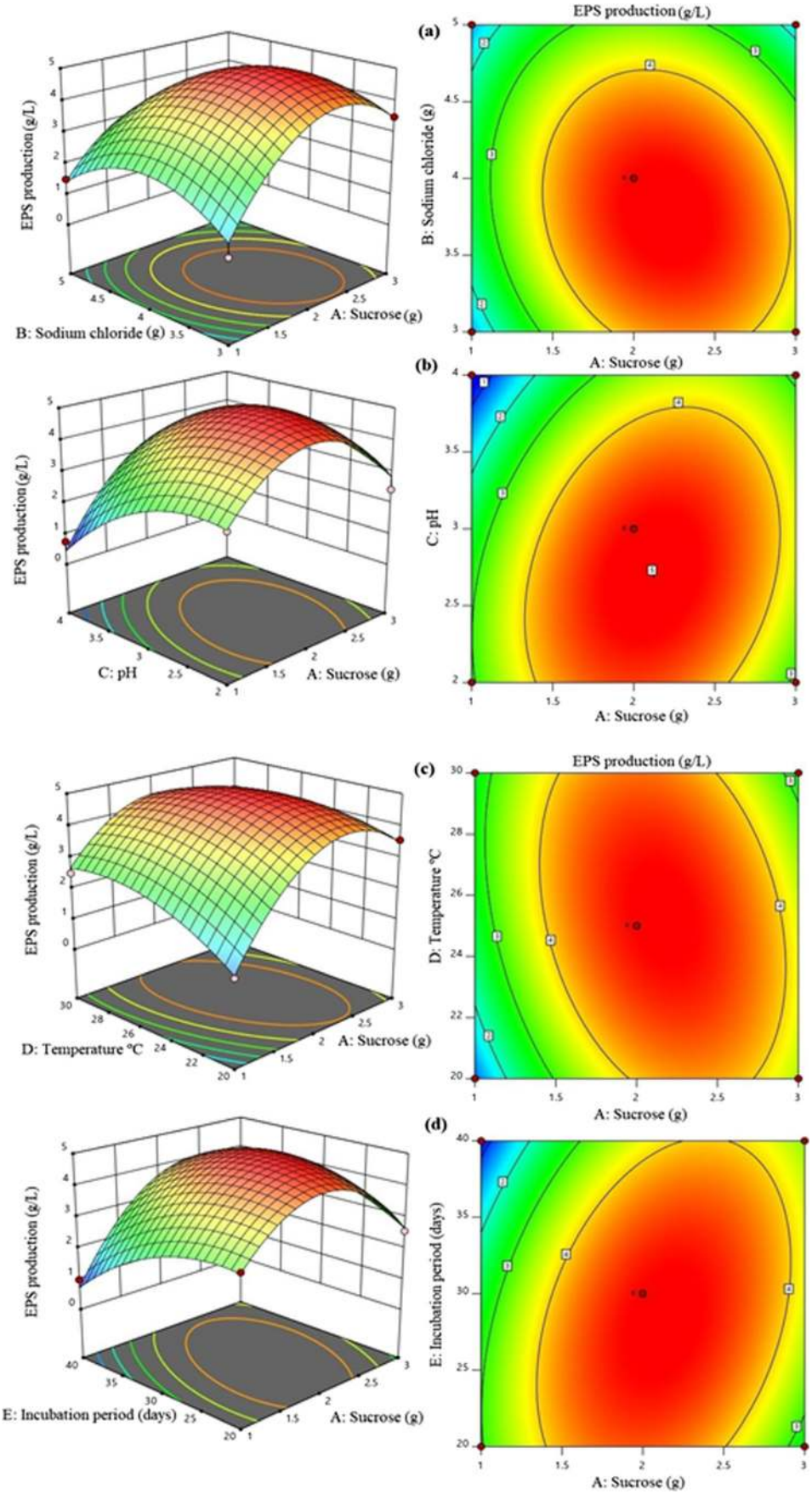
GC analysis

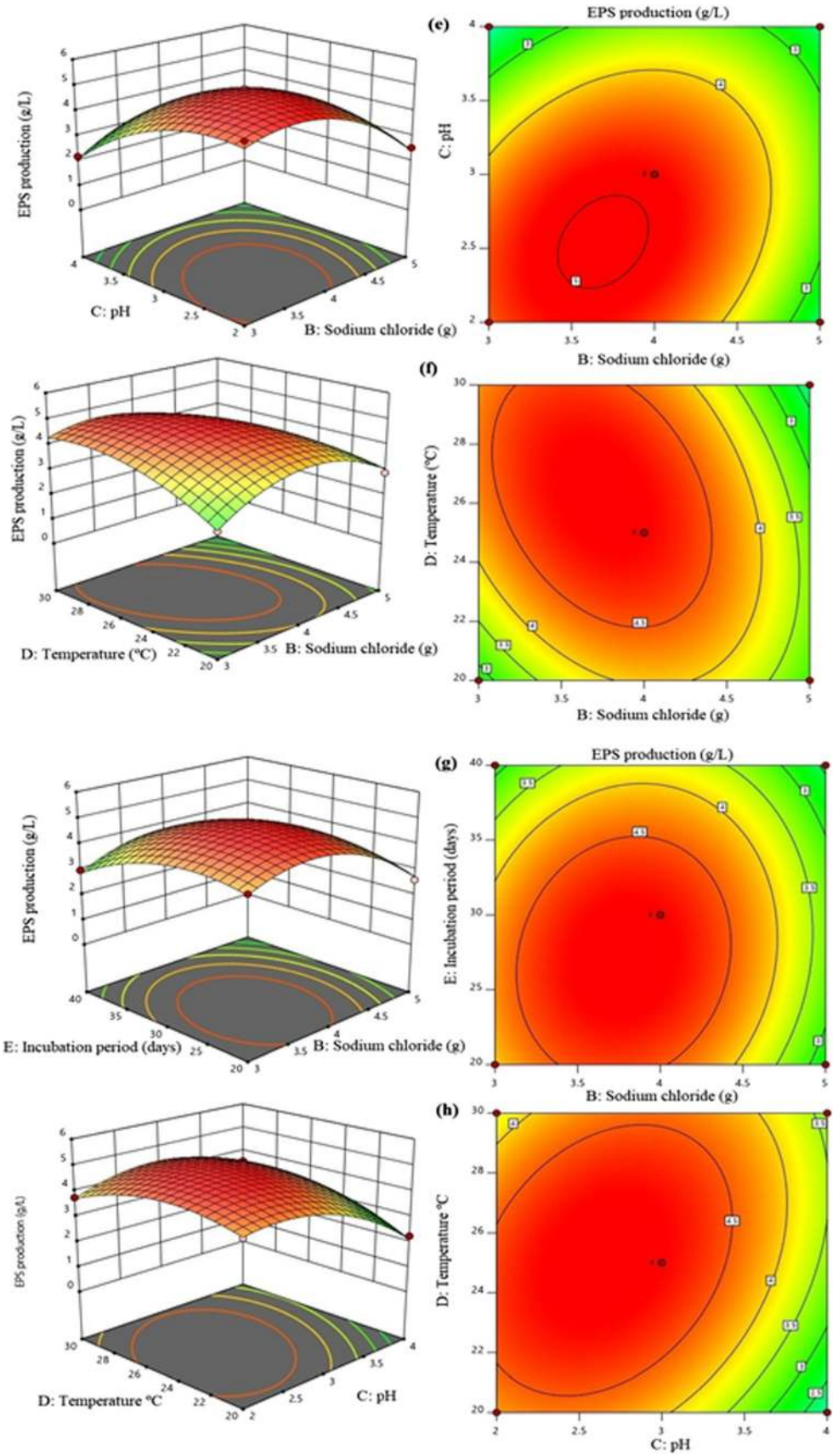
GC-MS analysis of EPS revealed that it was heteropolysaccharide composed of rhamnose, ribose, fucose, D galactose, mannose, and glucose respectively (Fig. S1c).

XRD analysis

X-ray diffractive (XRD) analysis was carried out to predict the nature of EPS whether amorphous or crystalline. Powder XRD spectra revealed (Fig. S1d) the distinguishing diffraction peaks at 28 °C, 30.7 °C, 35.4 °C, 40 °C, and 43.2 °C with inter-planar spacing (d-spacing) 3.186567 Å, 2.90937 Å, 2.532792 Å, 2.247331 Å, and 2.092394 Å respectively. The ratio between sharp thin diffraction peaks and a wide-ranging

Fig. 1 3D and 2D interaction between the different components of the media that were optimized to increase the production of EPS in *L. starkeyi* VIT-MN03, where (a) represents sucrose (A) vs sodium chloride (B), (b) sucrose (A) vs pH (C), (c) sucrose (A) vs temperature (D), (d) sucrose (A) vs incubation period (E), (e) sodium chloride (B) vs pH (C), (f) sodium chloride (B) vs temperature (D), (g) sodium chloride (B) vs incubation period (E), (h) pH (C)vs temperature (D), (i) pH (C) vs incubation period (E), (j) temperature (D) vs incubation period (E), (k) normal plot of residuals and predicted vs actual


EPS production (g/L)
(a)
EPS production (g/L)
B: Sodium chloride (g)
A: Sucrose (g)
(b)
EPS production (g/L)
C: pH
A: Sucrose (g)
(c)
EPS production (g/L)
D: Temperature °C
A: Sucrose (g)
(d)
EPS production (g/L)
E: Incubation period (days)
A: Sucrose (g)



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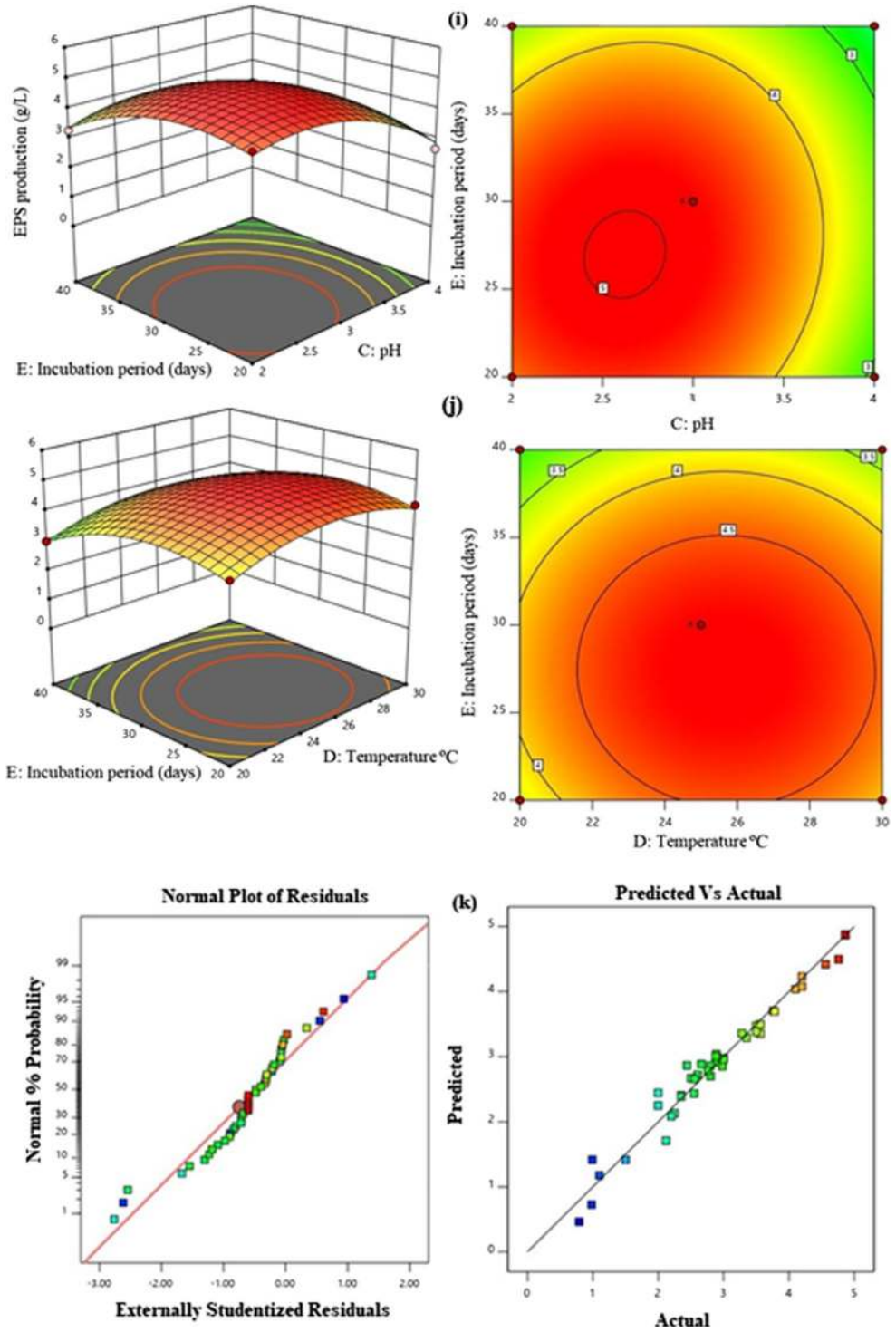

Fig. 1 continued.

peak was used to determine the quantity of crystallinity in the EPS. From XRD pattern, it was found that crystalline peaks were obtained in the amorphous phase of the EPS inferring a partial crystalline (67.4%) CIxrd = 0.674. XRD analysis revealed that *L. starkeyi* VIT-MN03 EPS are partially crystalline.

NMR analysis

The ^{1}H NMR spectra of EPS produced by *L. starkeyi* VIT-MN03 was shown in Fig. S3a. Most of the signals in the spectra lie in the anomeric region (δ_H 4.5 to 5.5). The chemical shift at δ4.52, δ4.86 ppm corresponds to

Table 1 ANOVA for quadratic model (response: EPS production)

Source	Sum of squares	df	Mean square	F-value	*p* value
Model	56.60	20	2.83	52.17	0.0001***
A-Sucrose	5.72	1	5.72	105.49	0.0001***
B-Sodium chloride	3.03	1	3.03	55.83	0.0001***
C-pH	6.08	1	6.08	112.02	0.0001***
D-Temperature	0.4655	1	0.4655	8.58	0.0071**
E-Incubation period	2.27	1	2.27	41.76	0.0001***
AB	0.8592	1	0.8592	15.84	0.0005**
AC	1.55	1	1.55	28.58	0.0001***
AD	1.45	1	1.45	26.69	0.0001***
AE	2.19	1	2.19	40.38	0.0001***
BC	1.39	1	1.39	25.67	0.0001***
BD	1.14	1	1.14	21.07	0.0001**
BE	0.1892	1	0.1892	3.49	0.0736
CD	0.7656	1	0.7656	14.12	0.0009**
CE	0.0961	1	0.0961	1.77	0.1952
DE	0.0012	1	0.0012	0.0226	0.8817
A^2	26.18	1	26.18	482.63	0.0001***
B^2	11.56	1	11.56	213.04	0.0001***
C^2	6.66	1	6.66	122.85	0.0001***
D^2	3.61	1	3.61	66.49	0.0001***
E^2	4.47	1	4.47	82.49	0.000***
Residual	1.36	25	0.0542		
Lack of fit	1.36	20	0.0678		
Pure error	0.0000	5	0.0000		
Cor total	57.95	45			
Std. dev.	0.2329				
Mean	3.07				
C.V. %	7.59				
R^2	0.9766				
Adjusted R^2	0.9579				
Predicted R^2	0.9042				
Adeq precision	28.1463				

*** Highly significant, ** significant

the β-anomeric protons and δ5.28 ppm corresponds to anomeric protons with (1 → 3) glyosidic linkages. The signal at δ5.34 ppm confirms the presence of rhamnose with α-(1 → 2) linkage. The presence of mannose was observed at δ5.28 with α-(1 → 6) linkage. The ^{13}C NMR spectra of EPS produced by *L. starkeyi* VIT-MN03 is shown in Fig. S3b. Most of the signals in the spectra lie in the anomeric region approximately at δ16 to δ113 ppm. The chemical shift at δ 16.23, δ72.85 to δ103.06 and δ100.55 showed α-(1 → 3) glyosidic linkage which indicated the presence of rhamnose, mannose, and glucose respectively. Another signals at δ107 and δ109 ppm correspond to glucose with β-(1 → 3) glyosidic linkage.

SEM analysis

SEM analysis showed that probiotic yeast produced EPS surrounding the cell surface (Fig. 2a). EPS surface was found to be smooth having a consistent polymeric matrix which indicated the structural reliability essential for bio-based film formation (Fig. 2b). At × 5000 magnifications, smooth, consistent polymeric matrix of *L. starkeyi* VIT-MN03 indicated the structural reliability which is essential for bio-based films formation.

Viscosity analysis

The viscosity of the probiotic yeast EPS was tested. The relationship between the EPS solution and different shear rates

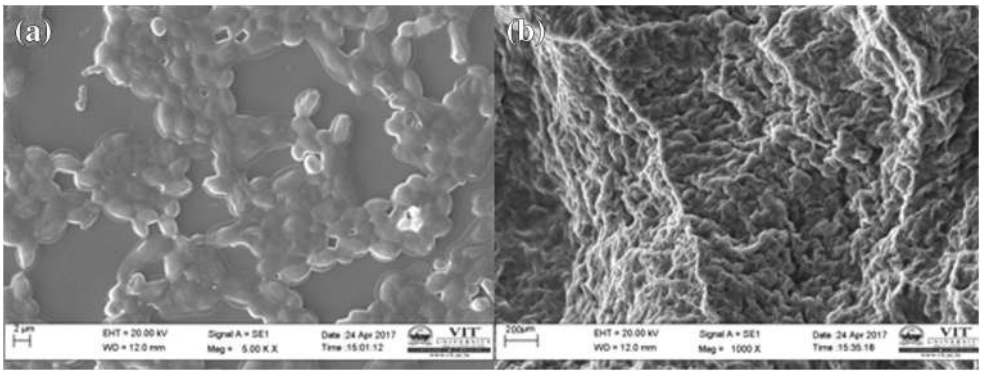

Fig. 2 SEM analysis of *L. starkeyi* VIT-MN03 (a) yeast biomass (b) EPS

was noted which was increased with different concentration indicating typical non-Newtonian behavior. Maximum viscosity was found to be 1.8 Pa S^{-1} at 1% EPS concentration. The apparent viscosity of EPS solution was slightly changed upon changing the pH ranging from five to nine (Fig. S2).

Adhesion properties

EPS-producing probiotic yeast *L. starkeyi* VIT-MN03 showed 95% auto-aggregation ability (Fig. 3a). The highest co-aggregation ability was noted in probiotic yeast associated with *Salmonella* sp. (93%) followed by *Escherichia coli* (85%), *Klebsiella* sp. (72%), and *Staphylococcus aureus* (69%) (Fig. 3b). Maximum hydrophobicity was noted in chloroform (80%) compared to n-hexadecane (76%) (Fig. 3c). Auto-aggregation ability will improve hydrophobicity as well as adhesion abilities of probiotic strains. Maximum co-aggregation was noted in *L. starkeyi* VIT-MN03 with *Salmonella* sp.

The greatest efficiency of adhesion was observed for yeast inoculum containing 5.5 × 107 CFU mL^{-1}per 9.2 cm^2 of Caco-2 cell. The dose of probiotic yeast was 160 cells per one Caco-2 cell. Approximately 88% of yeast cells were found to adhere to one Caco-2 cell (Table 2).

Antioxidant activity

In the present study, antioxidant property of probiotic yeast EPS was assessed based on its free radical scavenging activity. Probiotic EPS showed total antioxidant activity (84%), DPPH scavenging activity (71%), and hydroxyl radical scavenging activity (58%) which was substantially higher than ascorbic acid, a common antioxidant capable of scavenging radicals. Similarly, probiotic EPS showed significant reducing power (88%) as shown in Fig. 4a.

Biosurfactant activity

The biosurfactant activity was recorded at different concentrations of EPS extracted from probiotic yeast showed 86% surfactant activity, which is 6% greater than the positive control (xanthan gum) (Fig. 4b). The maximum biosurfactant production reduces the chances towards colonization of pathogenic microbes in the gut which may be helpful for EPS application in biomedical field as well as in food industry.

Cholesterol removal

Cholesterol removal was investigated for EPS-producing probiotic yeast *L. starkeyi* VIT-MN03 at a different time interval (4, 8, 12, and 24 h). The removal rate was found to be maximum on 12th h (Fig. 4c).

Binding of mutagens

The mutagen binding ability of EPS-producing probiotic yeast *L. starkeyi* VIT-MN03 was noted in mutagen Glu-P-1 (82%) followed by mutagen MeIQ (66%) over a period of 60 min (Fig. 4d).

Discussion

The yield, composition, and structure of the EPS produced by the yeast *L. starkeyi* VIT-MN03 were significantly influenced by the culture conditions such as concentration of sucrose and sodium chloride, pH, temperature, and incubation time. The maximum EPS production was found to be 4.87 g L^{-1} under optimized condition (sucrose 2%, sodium chloride 3%, pH − 4, temperature 25 °C, and incubation period 30 days) which indicated sixfold increase compared to EPS cultivation on minimal media (0.79 g L^{-1}). EPS production was reported to be increased along with the increase in sucrose concentration (Cho et al. 2001; Kaditzky and Vogel 2008; Ryan et al. 2015) and salt concentration (Mishra and Jha 2009). A relationship between EPS production and tolerance to low pH were reported in case of probiotic bacteria *Bifidobacterium* spp. (Alp and Aslim 2010). The effect of temperature increases the viscosity to obtain maximum EPS production. There was a report on

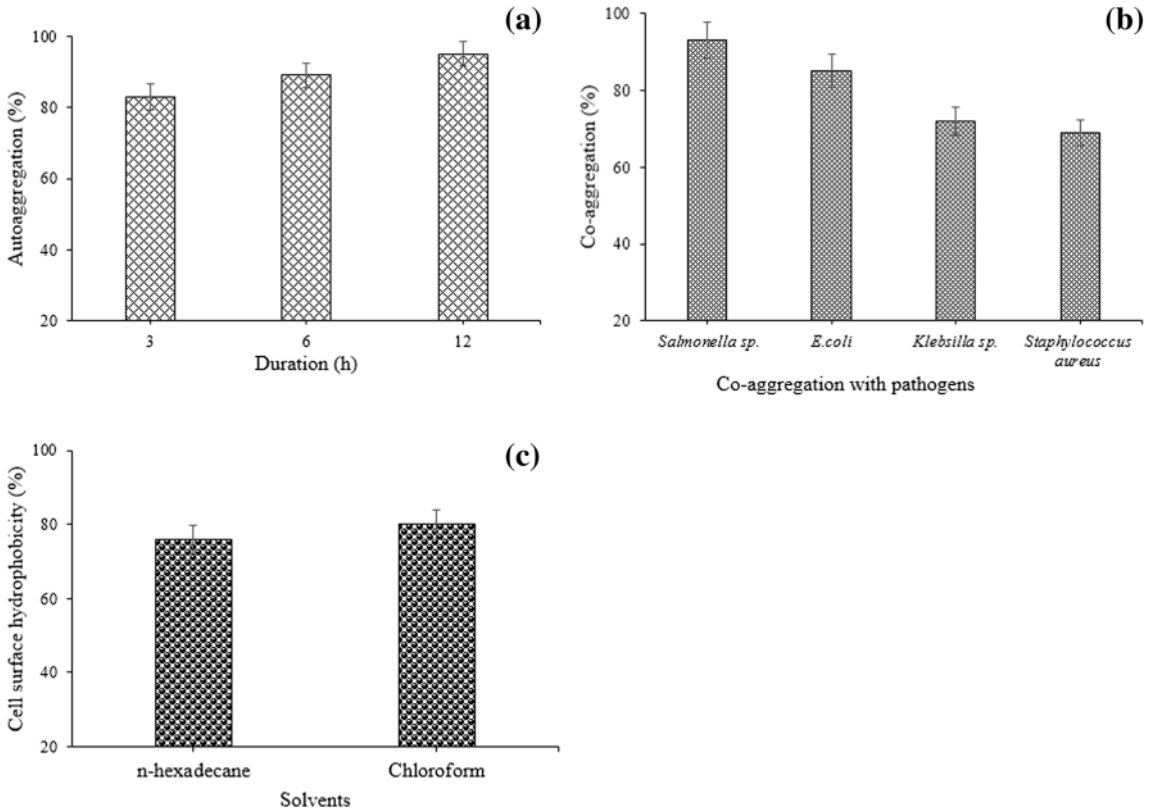

Fig. 3 Adhesion ability of EPS producing probiotic yeast *L. starkeyi* VIT-MN03 (a) auto-aggregation, (b) co-aggregation, and (c) cell surface hydrophobicity

L. mesenteroides and L. plantarum for maximum EPS production at 25 °C compared to 40 °C (Sanni et al. 2002). High amount of EPS production from *L. plantarum* ATCC 8014 was reported under optimized conditions using statistical experimental design of Box-Behnken (Othman et al. 2018). Similar results were noted in case of *Leuconostoc lactis* KC117496 and arctic marine bacterium *Polaribacter* sp. SM11 (Saravanan and Shetty 2015; Sun et al. 2015).

FT-IR spectra, HPLC, and GC exhibited a variety of typical absorption peaks of polysaccharides. Moreover, it revealed the presence of fucose which is having potential application in the medical field towards prevention of tumor cell colonization in the lung (anticancer effect), controlling the formation of white blood cells (antiinflammatory effect), treatment of rheumatoid arthritis, synthesis of antigens for antibody production (rational immunization), and in cosmeceuticals as skin moisturizing agent (Vanhooren and Vandammel 1999). Similar reports were found in EPS produced by *Rhodotorula glutinins* which was composed of L-fucose and D-galactose (Singh et al. 2012). Similar results were found in *Bacillus tequilensis* PS21 (Wu et al. 2007; Luang-In et al. 2018). XRD analysis showed partial crystalline nature of probiotic EPS. The same pattern was noted in EPS-producing *Bacillus licherniformis* (Flemming and Wingender 2010).

In the ^{1}H NMR spectrum, the anomeric region (4.5–5.5 ppm) signals were often used to differentiate the anomeric protons of sugar residues in polysaccharides. The present study confirmed the presence of three sugars viz. glucose, mannose, and rhamnose at the anomeric region with α-(1 →3), β-(1 → 3), α-(1 →2), and α-(1 → 6) glyosidic linkages (He et al. 2007; Hallack et al. 2009). Similar results were reported in EPS produced from *Leuconostoc* strains (Bounaix et al. 2009) and *Lactobacillus plantarum* MTTCC 9510. All the sugars are having pyranose ring configuration (Ismail and Nampoothiri 2010).

In ^{13}C NMR spectrum the anomeric region (16 to 113 ppm), signals were used to predict the aliphatic groups

Table 2 Adhesion ability of *L. starkeyi* VIT-MN03 on Caco-2 cell

Dose of yeast CFU ml^{-1}	Dose of yeast CFU Caco-2 cell^{-1}	Number of adhering yeast	Adhesion %
5.2×10^{5}	1.6	$(2.1\pm02)\times10^{5}$	40 ± 03
5.2×10^{6}	16	$(3.5\pm02)\times10^{6}$	67 ± 02
5.2×10^{7}	160	$(4.6\pm01)\times10^{7}$	88 ± 04

Average values (SD±) from three independent repetitions are presented

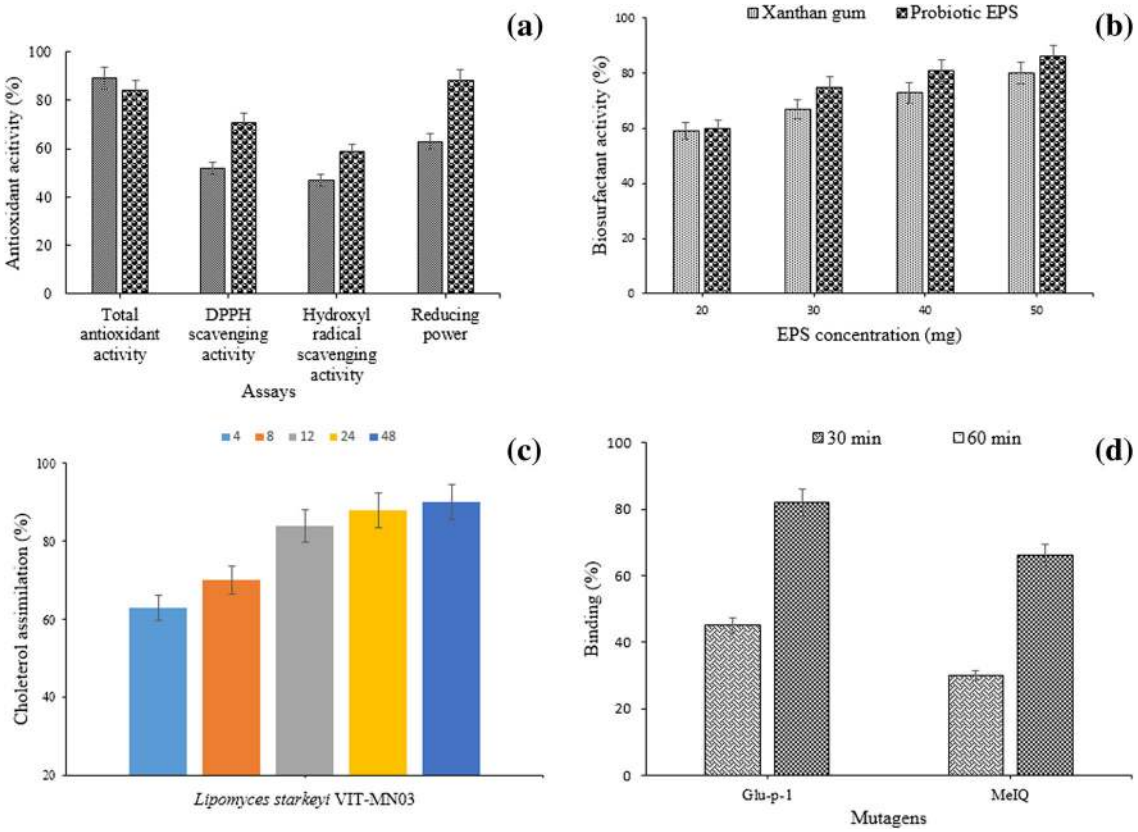

Fig. 4 EPS producing yeast *L. starkeyi* VIT-MN03 showing (a) antioxidant activity, (b) biosurfactant activity, (c) cholesterol removal, and (d) binding ability against mutagens

(C-H). In this study, six signals were observed in the anomeric region and confirmed the presence of three sugars with α and β linkages. The C-1 signal at δ103.06 ppm could be assigned to an α-D-mannopyranosyl residue. The signal at δ16.23 ppm indicates the presence of α-L-rhamnopyranosyl residue. These results indicated the presence of two types of glucopyrance residues in the probiotic EPS. Similar results were reported in EPS polymer of *Saccharomyces cerevisiae* (Amer 2013) and *Leuconostoc lactis* KC 117496 (Saravanan and Shetty 2015).

SEM images confirmed the presence of slimy layer around the cell wall of the probiotic yeast. This biofilm helps the colonization of microflora on the biotic surface in the intestine may impart various health benefits (Velasco et al. 2009). Moreover, rheological studies on the aqueous EPS showed that it had a high viscosity and good shear-thinning properties which may have potential advantages in food processing industry as a thickener and mixing agent (Yuksekdag et al. 2014).

Probiotic EPS exhibited strong co-aggregation ability with *Salmonella* sp. A number of bacterial strains namely *L. acidophilus* BAZ36, *L. delbrueckii* ssp. *Delbrueckii* BAZ32, and *L. salivarius* exhibited good co-aggregation ability with *Salmonella* sp. (Rodrigues and Teixeira 2010) which increases the protection against pathogen colonization in the gut.

In the present study, probiotic yeast *L. starkeyi* VIT-MN03 can be considered as highly adhesive strain as the level of adhesion exceeded 40 cells per one epithelial cell (Candela et al. 2008). A similar adhesive capacity of probiotic lactic acid bacterial strain to Caco-2 cell was reported (Dertli et al. 2015; Živković et al. 2016). These results suggest that EPS might play an important role in yeast aggregation and interaction with intestinal epithelial cells.

Reactive oxygen species, such as hydroxyl and superoxide radicals, are highly related to human health. They may cause aging, cancer, inflammation, and other diseases (Wang et al. 2012). *Lipomyces starkeyi* VIT-MN03 exhibited strong antioxidant activity. Similar results were reported in *Bifidobacterium animalis* RH (Xu et al. 2011), *Paenibacillus polymyxa* EJS-3 (Liu et al. 2012), and *L. brevis* D7 (Lai et al. 2014). The reducing power was also demonstrated in EPS-producing bacteria *L. paracasei* NTU 101 and *L. plantarum* NTU 102 (Liu and Pan 2010).

Additionally, there is a report for biosurfactant activity in probiotic bacteria *Lactococcus lactis* 53 (Rodrigues et al. 2006) cholesterol removal from the medium using EPS-producing strains (Patel and Prajapati 2013). Binding ability of probiotic bacteria *Bifidobacterium longum* showed high mutagen binding capacity for many other mutagens except Glu-P-1 (Sreekumar and Hosono 1998). In case of probiotic EPS from *L. starkeyi* VIT-MN03 showed maximum binding ability to mutagen Glu-P-1.

Conclusion

This study was conducted to enhance the EPS production in probiotic yeast *L. starkeyi* VIT-MN03 using various parameters. The maximum EPS production (4.86 g L^{-1}) was achieved under optimized conditions. EPS was characterized as heteropolysaccharide polymers composed of common sugars. SEM analysis confirmed the potential of EPS to have physical stability and smooth surface for film formation. Two-dimensional NMR spectroscopic technique was adopted to determine heteropolysaccharide composition of EPS constituted by α-(1 → 3)-(1 → 2)-linked rhamnan, α-(1 → 6)-linked mannan, and α and β (1 → 3)-linked glucan. The EPS showed good binding ability against Caco-2 cells and mutagen Glu-P-1 which indicated that probiotic EPS can play a significant role for controlling the anticancer and antimutagenic activity. In addition, probiotic EPS exhibited strong antioxidant and biosurfactant activity along with cholesterol lowering effects which may suggest its potential use as a natural source to be used for the production of nutraceuticals and functional foods.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals (if applicable) This study does not require a statement under this section.

Informed consent Informed consent statement is not applicable.

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