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Production of high-value added exopolysaccharide by biotherapeutic potential *Lactobacillus reuteri* strain

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ABSTRACT

Exopolysaccharides (EPS) are produced by various microbial groups including bacteria. EPS produced from *Lactobacilli* is gaining popularity as a new generation functional food to replace various chemically synthesized food additives. EPS production by strains of *L. reuteri* were reported previously in sourdough fermentation. The submerged cultivation of *L. reuteri* strain DSM 20016 ^{T} was optimised for crude EPS production. Initially, the factors controlling crude EPS generation were explored using one-at-a-time (OFAT). Among the investigated carbon sources, sucrose supplemented medium increased maximal crude EPS generation. Yeast extract was determined to be the greatest nitrogen source for increasing crude EPS and cell mass. Plackett-Burman experimental design (PBD) was employed to screen the important parameters impacting crude EPS production in the growing medium. Three important factors were identified: sucrose, yeast extract and sodium acetate. The RSM quadratic model for crude EPS generation was significant (R^2:0.9373) and correlated with the observed response at roughly 94%. About 3.4 g L^{-1} crude EPS was produced under optimal fermentation conditions of sucrose (106.0 g L^{-1}), yeast extract (30.0 g L^{-1}) and sodium acetate (7.0 g L^{-1}). This study shows the crude EPS synthesis of *L. reuteri* from sourdough can be used in submerged fermentation and biotechnological applications.

1. Introduction

As the 21st century moving towards its third decade, the modern lifestyle has leads to a significantly increased consumer demands in the field of food production. Consumers believe has raised towards production of "new generation foods" which are characterized by an improved functional quality and contribute directly to their health [1]. The raising demand on "new generation foods" can be explained by the increasing cost of healthcare, the life expectancy and the increasing desire of older people for improved quality of their later years [2–4]. Today's food on plates is mostly intended to satisfy hunger and lacking for necessary nutrients. Various synthetic food additives available in the market have lower nutrition value and is also negatively affect human body by causing health complications such as allergic, intolerances or

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cancer [5]. Growing consumer awareness has changed the global trend to a healthy lifestyle, which has urged scientists to search for natural food additives. The food additive market was predicted with an increased global demand by 5.6% between the year 2015–2020, to reach USD 52.2 x 10^9 [6]. To address the demand for food safety and consumer demand, the food manufacturers are exploring new food resources like microbial by-products, especially for polysaccharides [2].

In recent years, microbial exopolysaccharides (EPS) have attracted wide attention of researchers to extensively used in food industries [7, 8]. Some microbial EPS such as pullulan, gellan and xanthan are used directly as alternative for algae or plant-based polysaccharides [9–11]. Their structural diversity, versatile physio-chemical and biological as well as therapeutic properties of microbial EPS has attracted one of major trend change in food additive productions [12]. The microbial EPS are natural and biodegradable polymers, which are utilized in many areas besides food industry such as cosmetics, pharmaceutical, textiles, agriculture, bioremediation and medical application [10]. These industries have exploited the properties of microbial EPS into different inventions such as adhesives, absorbents, lubricants, rheology modifiers, viscosifiers, emulsifiers and chelating agents [13,14].

There is overwhelming reports on biotherapeutic potentials and immunomodulatory activities of microbial EPS [15,16]. For example, microbial EPS was found to stimulate growth of immune cells [17], induce nitric oxide production [16], secretion of proinflammatory and anti-inflammatory cytokinesis [14]. The microbial EPS also has beneficial effects for the human health such as cholesterol lowering properties [18], anti-microbial and anti-tumoural potentials [15]. These microbial EPS biomaterials are also intended to be served in drug delivery systems, encapsulation of bioactive molecules, anticancer and antimicrobial agents, as well as reducing and stabilizing agent in synthesis of metal nanoparticles [13].

Major criteria for selecting microbial EPS for both food and medical application is EPS have been reported to be generally recognized as safe (GRAS) compounds, meaning that they do not poses a health risk in their usage. Regarding this, genera of *Lactobacillus* represent the best producers of microbial EPS [19,20]. Beside many strains owning GRAS status, the genera of *Lactobacilli* are also efficient in preventing gastrointestinal diseases and maintains its normal microflora by preventing pathogen adhesion. The best EPS-producers of this genera are *Lactobacillus acidophilus* [21], *Lactobacillus delbrueckii* sp. *bulgaricus* [22], *Lactobacillus plantarum* [17], *Lactobacillus lactis* [23], *Lactobacillus rhamnosus* [24], *Lactobacillus casei* [25], *Lactobacillus curvatus* [19], *Lactobacillus helveticus* [16], *Lactobacillus kefiri* [26], *Lactobacillus fermentum* [27], *Lactobacillus sanfranciscensis* [28] and *Lactobacillus reuteri* [29]. Different species of bacteria in the same genus can synthesize structurally different types of EPS. *Lactobacilli* were proven with capabilities to produce both homopolymers and heteropolymers [7]. The heteropolysaccharides made up of multiple types of sugar residues which were synthesized by combined action of multiple glycosyltransferase systems [8]. Homopolysaccharides consist of sole substrates such as sucrose and it was synthesized single enzyme systems [20]. Various research has been conducted to explore the capability of different species of bacteria [2].

Besides from living host and dairy products, numerous strains of *L. reuteri* was also isolated from sourdough environment [30,31]. Sourdough is prerequisite to produce a high-quality gluten-free bread. These healthy breads are often characterized with formation of poor crumb and crust characteristics, also with formation of poor palate texture and flavor [32]. To acquire the acceptable crumb structures, various chemical additives like hydrocolloid hydroxypropyl-methyl cellulose are used [33]. It was demonstrated that EPS from sourdough origin *L. reuteri* strains had improved gluten-free breads with increased the specific volume and decreased baking loss as well as staling [34]. Sourdough fermentations enhanced production of EPS from *L. reuteri*, that improve aroma formation, leaving, acidification and this had replaced the application of such additives. Many findings had reported various homopolysaccharide production in strains of *L. reuteri* by sucrase-type enzymes [35]. Several sucrase-type EPS producing enzymes had been reviewed in *L. reuteri*, such as reutransucrase, levansucrase and dextransucrase that produce different EPS like levan, reutran and dextran [36].

The heterofermentative *L. reuteri* harbors sucrase-type enzymes that synthesizing polysaccharides made up of either glucose residues (glucan) or fructose residues (fructan) [37,38]. Teixeira et al. [39] had evaluated a levansucrose on *L. reuteri* strain LTH5448 able to regulate raffinose metabolism and EPS production. Both levansucrase and sucrose phosphorylase enzymes possessed by *L. reuteri* were induced by sucrose [31]. The fructansucrases and glucansucrases in *L. reuteri* able to produce novel exopolysaccharides as an alternative to the sucrose metabolism [40]. Rühmkorf et al. [34] reported that *L. reuteri* synthesize EPS named dextran, with 8–9% branching at O-3 and 18–19% branching at O-4, . Dextransucrase and reutransucrase enzymes of *L. reuteri* were found to have great effects on bread volume and crumb hardness [33]. The role of glucansucrase enzyme in the EPS production has been evaluated in sourdough isolates of *L. reuteri*. The study evaluated the glucansucrase gene that responsible for glucan type EPS production in *L. reuteri* [32]. These studies have proven the role of sucrose in fermentation medium to induce various sucrase-type enzymes including fructansucrases and glucansucrases for crude EPS production in *L. reuteri* [32]. This glucan and fructan type EPS from *L. reuteri* can be used as viscosifying, stabilising, emulsifying, sweetening, gelling, or water-binding agents in food as well as in the non-food industry [32]. EPS produced from strains of *L. reuteri* were identified as alternative to chemical thickeners in different types of food and this could create a healthier consumer food. Emerging research efforts have investigated potential applications of EPS from *L. reuteri* in medicinal benefits [41–43].

Most of the EPS productions by *L. reuteri* were reported in sourdough fermentation [36]. The isolated and characterized *L. reuteri* EPS from sourdough showed low production of EPS. Extraction of EPS from sourdough was not only scarce but also difficult for industrial application and human welfare [11]. The industrial production of microbial EPS is depending on the cleaner production and efficiency of synthesis. In order to increase efficiency while incurring as little technological expenditures as feasible, optimization studies are essential in industrial and laboratory size manufacturing [4]. The present study is motivated by the importance of this subject for the industry. This investigation has been carried out in order to concentrate on the creation of a low-cost liquid culture medium for the enormous production of exopolysaccharides from *L. reuteri*, while taking into consideration the cleaner, economic and technological characteristics. The results obtained so far have indicated that the cultivation medium composition is a determinant to influence an efficient EPS production [24].

The aim of this study was to develop an optimal cultivation medium composition by using conventional and statistical platforms for effective synthesis of crude EPS from *L. reuteri* strain DSM20016^T.

2. Materials and methods

2.1. Microorganisms and cultivation medium

L. reuteri strain DSM20016^T used in this was obtained from DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with Genbank accession numbers (16 S rRNA gene: X76328). A cryoprotectant broth made of 10% (w/v) skim milk and 10% (v/v) glycerol was used to sustain the culture, which was kept at − 80 ºC. The De Man-Rogosa-Sharpe medium (MRS) was used to prepare the seed culture medium, which contained the following ingredients (g L^{-1}): 10.0 peptone, 10.0 meat extract, 5.0 yeast extract, 2.0 dipotassium phosphate, 2.0 ammonium citrate, 5.0 sodium acetate, 0.1 magnesium sulphate, 0.05 manganese sulphate and 1.0 Tween®80. Prior to sterilization at 121 °C for 15 min, the pH of the medium was adjusted to

6.5. Glucose was employed as the only carbon source with a concentration of 20.0 g L^{-1} and sterilized separately for 10 min at 110 °C to prevent caramelization.

Fermentation medium is slightly modified in this study compared to the commercial MRS medium [4]. The initial fermentation medium was composed of (g L^{-1}) 20.0 glucose; 10.0 peptone; 5.0 sodium acetate; 2.0 dipotassium phosphate; 0.1 magnesium sulphate; 0.05 manganese sulphate; 2.0 ammonium citrate and 1.0 Tween®80. Other components were omitted based on the previous work (unpublished) in order to reduce media cost [9]. Components of the fermentation medium was further optimized in this study by using statistical approach to increase crude EPS production.

2.2. Inoculum preparation

The stock cultures of *L. reuteri* strain DSM20016^T was transferred to sterile seed medium (MRS agar plates) for reactivation at 37 °C for 48 h. Then, grown colonies were inoculated into broth seed medium and 37 °C for 24 h. This step is repeated for two successive pre-cultures. The bacterial cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C aseptically. Then, the cell pellets were washed using sterile distilled water and resuspended as inoculum for all following studies. The absorbance of resuspension was adjusted to be more than 1.0 at 600 nm (approximately 1 $\times 10^7$ CFU/mL according to our preliminary study).

2.3. Production of crude EPS

All experiments were carried out in 50 mL Erlenmeyer flasks with 30 mL culture medium in order to create microaerophilic conditions. About 10% (v/v) of overnight grown cultures were inoculated into fresh medium. These inoculated flasks were incubated statically at 37°C for 48 h. All experiments were performed in triplicates.

2.4. Effect of different carbon and nitrogen sources on crude EPS production

To identify the *L. reuteri* strain DSM20016^T preferred carbon and nitrogen sources, the effects of each source on cell growth and crude EPS production were screened by OFAT approach. Initially, the carbon source in the fermentation medium was then substituted separately with equal concentration of various carbohydrates such as glucose, sucrose, fructose, lactose, isomaltose and xylose. Other components in the fermentation medium were maintained as stated compositions. Then, cells were harvested after 48 h incubation and analyzed for maximum cell mass and crude EPS production. After a suitable carbon source was selected, the nitrogen source in the fermentation medium was then screened with different types of other nitrogen sources including peptone, meat extract, urea and yeast extract. The amount of nitrogen sources was adjusted based on the percentage of total nitrogen like original fermentation medium composition. All flasks are cultivated under the same conditions.

2.5. Optimization of crude EPS production by statistical methods

In the next phase of the study, the fermentation medium was further optimized by statistical approach. Carbon and nitrogen sources in the MRS medium play's vital role in the growth of Lactobacillus species. In this study, traditional optimization approach was employed to select suitable carbon and nitrogen sources. Statistical optimization approach was employed to identify significant factors affecting the growth and crude EPS production.

2.5.1. Plackett-Burman design (PBD)

Lactobacillus strains were often cultured on MRS medium, which included a variety of different and complicated nutritional constituents. These components are not required to be used in the manner recommended and may be varied or eliminated depending on the strains used and the goal of the fermentation, among other considerations. When it comes to screening the most important elements for the growth and crude EPS production from *L. reuteri* strain DSM20016^T, the Plackett-Burman experimental design is invaluable in reducing the time it takes to find them. The experimental technique used in the PBD study was based on a first-order model with no interaction between the parameters investigated in Eq. 1.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i \quad (1)$$

where Y is the response [crude EPS produced (g L^{-1})] while β_0, β_i and X_i are represented as the level of model intercept, linear coefficient and independent variable. In this work, a total of eight components in the modified MRS medium were chosen for further investigation and evaluation. Each variable was represented at two levels, (−1) for the low level and (+1) for the high level, resulting in a total of 24 runs, as shown in Table 1.

For the purpose of the affirming the crucial factors for the growth and crude EPS production of *L. reuteri* strain DSM20016^T, variance analysis was applied and if the p value is below 0.05, the corresponding factors were crucial factor. Ultimately, the high and low levels were selected according to positive or negative significant effect. Rest of the non-significant factors were not omitted as all components are essential for the growth of Lactobacillus species.

2.5.2. Response surface methodology (RSM) by Box-Behnken design (BBD)

A strategy based on RSM was used to further determine the optimal concentration of screened factors and to investigate the interaction of these variables on the crude EPS generation by *L. reuteri* strain DSM20016^T. With at least one key element selected from the PBD screening process, the experimental design was a BBD.

It was decided to conduct the experiment on three independent variables, each of which was set to one of three distinct levels [(−1); (0); (+1)] for the low, medium and high values. Using a multiple regression approach, the generation of crude EPS from the *L. reuteri* strain DSM20016^T was fitted to an equation of second order polynomial form. Using the most significant factors as inputs, an empirical model was developed. The following second order polynomial equation, which may be written as follows, explains the performance of this statistical optimization system in Eq. (2).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x^2 + \sum_{i=1}^{n} \sum_{j>i}^{n} \beta_{ij} x_j x_i \quad (2)$$

Where Y is the crude EPS produced in g L^{-1} (response), β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction; x_i and x_j are coded level of independent variables.

The preliminary research found that sodium acetate, yeast extract and sucrose were important factors in the formation of EPS. Because they had no apparent impact on the formation of EPS, the concentrations

Table 1
Experimental range and levels of factors influencing EPS production from *L. reuteri* strain DSM20016^T in a two-level Placket–Burman design.

Variable	Unit	Code	Low value (-)	High value (+)
Sucrose	g L^{-1}	**A**	20	100
Yeast extract	g L^{-1}	**B**	5	28
Ammonium citrate	g L^{-1}	**C**	1	2
Sodium acetate	g L^{-1}	**D**	1	5
Dipotassium phosphate	g L^{-1}	**E**	0.2	1
Magnesium sulphate	g L^{-1}	**F**	0.1	0.5
Manganese sulphate	g L^{-1}	**G**	0.05	0.1
Tween 80	g L^{-1}	**H**	0.5	1

of the other medium constituents were maintained throughout the research. The coefficient of determination R^2 can be used to assess the precision and applicability of the aforementioned polynomial model. The experimental data collected in each experimental design were subjected to analysis of variance (ANOVA) in order to determine the significance of the independent factors taken into account in the development of EPS. The primary (linear and quadratic) and interaction (linear by linear) effects were estimated and tests were run to see whether the various factor levels were equivalent. Replications at the centre point make it possible to estimate the pure error caused by repetitions. As a result, the sum of the square residuals (SSres) can be divided into the squares due to pure error (SSpe) and the squares due to lack of fit (SS_{lof}), allowing for a lack-of-fit test to determine whether the model is appropriate. By resolving the regression equation, the ideal quantities of sodium acetate, yeast extract and sucrose were discovered. This made it possible to create empirical models that included linear, quadratic and interaction factors to describe the relationships between operational and experimental variables. In order to anticipate the ideal experimental conditions for EPS production, the equations were derived from experimental data using multiple regressions and the least-squares method. Each run was performed in triplicates and Minitab® software (version 16) was employed for the experimental design. Statistical analysis was performed for regression and graphical analysis of data obtained.

2.6. SEM images of the dried EPS

Dried EPS prepared with *L. reuteri* DSM20016^T was dried and tested for their morphology in order to observe potential effect of in situ EPS production. The morphologies were observed by SEM analysis using a variable-pressure scanning electron microscope (VP-SEM) JEOL JSM-IT300LV Instrument. The dried samples of the EPS (5 mg) were fixed to the SEM stubs with double sided tape, then coated with a layer of platinum, ~10 nm thick. The EPS sample was observed at high resolution of 3.0 nm at 30 kV with a magnification level of 1000–2000 × .

2.7. Analytical methods

Growth was determined based on cell dry weight (CDW) by converting absorbance at 600 nm with pre-determined regression equation. The pH value of the fermentation broth was determined bench-top pH meter (Eutech™ pH 700 Meter) and a mean value of pH is obtained for every replicate. The crude EPS produced were determined by slightly modified methods from previous investigations [9]. Briefly, the cells were harvested by centrifugation at 8000 rpm for 20 min at 4 ℃, in order to remove bacterial cells. Then, about three volumes of pre-cold absolute ethanol were added to the centrifuged supernatants. Suspension was mixed thoroughly and kept 24 h at 4 ℃ for crude EPS precipitation. Finally, the crude EPS was recovered by centrifugation at 8000 rpm for 20 min at 4 ℃. Pellet formed were then dried at 80 ℃ in oven. The maximum crude EPS yielded in g L^{-1} were determined by measuring the final dry weights. The PBD and RSM experimental designs as well as their subsequent regression analysis of the obtained experimental data was performed by using Minitab® software (version 16). The same software was also used to obtain response surface curves and corresponding contour plots from the RSM experiments. Each experiment was performed in triplicates. All results were expressed as mean μ and standard deviation σ of the number of runs.

3. 3. Results and discussion

3.1. Effect of different carbon and nitrogen sources

In this study, *L. reuteri* strain DSM20016^T was cultures under microaerophilic conditions in 100 mL Erlenmeyer flasks for 48 h. The bacterium was grown glucose, sucrose, fructose, lactose, isomaltose and xylose as main carbon source. The growth and crude EPS production determined were presented in Fig. 1. *L. reuteri* is commonly isolated from gastrointestinal environment and the genome of the bacterium was adapted to utilize most of the carbohydrates [30]. Like other members of *Lactobacilli* genus, strains of *L. reuteri* are also saccharolytic and plays vital role in carbohydrate metabolism in the colon [44]. Several studies have highlighted on the carbohydrate utilization in *L. reuteri* strains [45].

L. reuteri strain DSM20016^T grown in a modified MRS medium were able to utilize all tested carbon sources. Lactose being the carbon source that enhanced highest growth. Lactose supplemented medium did not yield highest crude EPS. Growth in MRS-sucrose medium resulted in highest crude EPS productions of about 1.57 g L^{-1} compared to other carbon sources. This data is consistent with the previous work published by Årsköld et al. [35] which stated that sucrose was one of the most suitable carbon sources for crude EPS production. Sandra et al. [36] also suggested sourdough supplemented with sucrose had enhanced crude EPS production by *L. reuteri*. When sucrose was used, higher amounts of crude EPS were obtained than with glucose as carbon source. Crude EPS produced from MRS-glucose was about 0.90 g L^{-1}. Amount of crude EPS produced was almost the same amount in MRS-lactose, MRS-isomaltose and MRS-xylose which were 1.17 g L^{-1}, 1.40 g L^{-1} and 1.30 g L^{-1}. There are relatively little cell dry weight production (0.076 g L^{-1}) and less crude EPS formed (0.77 g L^{-1}) occurred in MRS-fructose medium. Sucrose is chosen as the suitable carbon source for growth and crude EPS production for *L. reuteri* strain DSM20016^T.

Genome of several strains of *L. reuteri* has been studied previously and intestinal isolates of *L. reuteri* displayed numerous carbohydrate-active enzymes [45]. Sucrose was presented better EPS yield than glucose in this study. This is suggesting sucrose can be considered as carbon source formulation of fermentation medium for higher crude EPS production in *L. reuteri*. This is also suggested by several previous reports which utilized sucrose for different types of EPS productions in *L. reuteri* [32].

Sucrose also utilized by other species of *Lactobacillus* for EPS production. Study conducted by Ale et al. [27] has used sucrose in a semi-defined medium for EPS production from *L. fermentum*. Seitter et al. [28] has reported that *L. sanfranciscensis* utilized sucrose for production of EPS in wheat dough fermentation process. Other carbon sources also utilized for EPS productions in several *Lactobacillus* strains. *L. lactis* isolated from milk sources yielded highest EPS in maltose supplemented MRS medium [23]. Similarly, isolates from naturally fermented pickles including *L. plantarum*; *Lactobacillus namurensis* and *Pediococcus ethanolidurans* utilized glucose as main carbon source in MRS medium for EPS production [46].

The carbon source in the fermentation medium is responsible for EPS production, while the nitrogen source will enhance cell mass. The role of nitrogen sources may contribute indirectly to the production of EPS which is a postbiotic by the enhancement of the microbial cell development [27]. In this study, the *L. reuteri* strain DSM20016^T was cultivated with MRS-sucrose supplemented with different types of nitrogen sources including peptone, meat extract, yeast extract and urea as shown in Fig. 2. It was found that nitrogen-free formulation production lower cell dry weight (0.28 g L^{-1}) and EPS (0.10 g L^{-1}). The MRS-sucrose supplemented with yeast extract as sole nitrogen source, has highest cell dry weight and crude EPS obtained were 2.83 g L^{-1} and 1.50 g L^{-1}, follow up by medium with peptone yielded 0.93 g L^{-1} and meat extract yielded 0.63 g L^{-1} crude EPS. Cell dry weight obtained in all cultivation medium had no significant difference, where growth obtained in medium supplemented with yeast extract, peptone and meat extract were 2.67 g L^{-1}, 2.59 g L^{-1} and 2.57 g L^{-1}.

The strains of *L. reuteri* were fast-growing *Lactobacilli* which required MRS medium supplemented with complex nitrogen sources such as peptone, meat, yeast extract or liver digests [47]. These complex substances supported the growth and allowed for speedy recovery of Lactobacilli culture [48]. The initial fermentation medium used in this

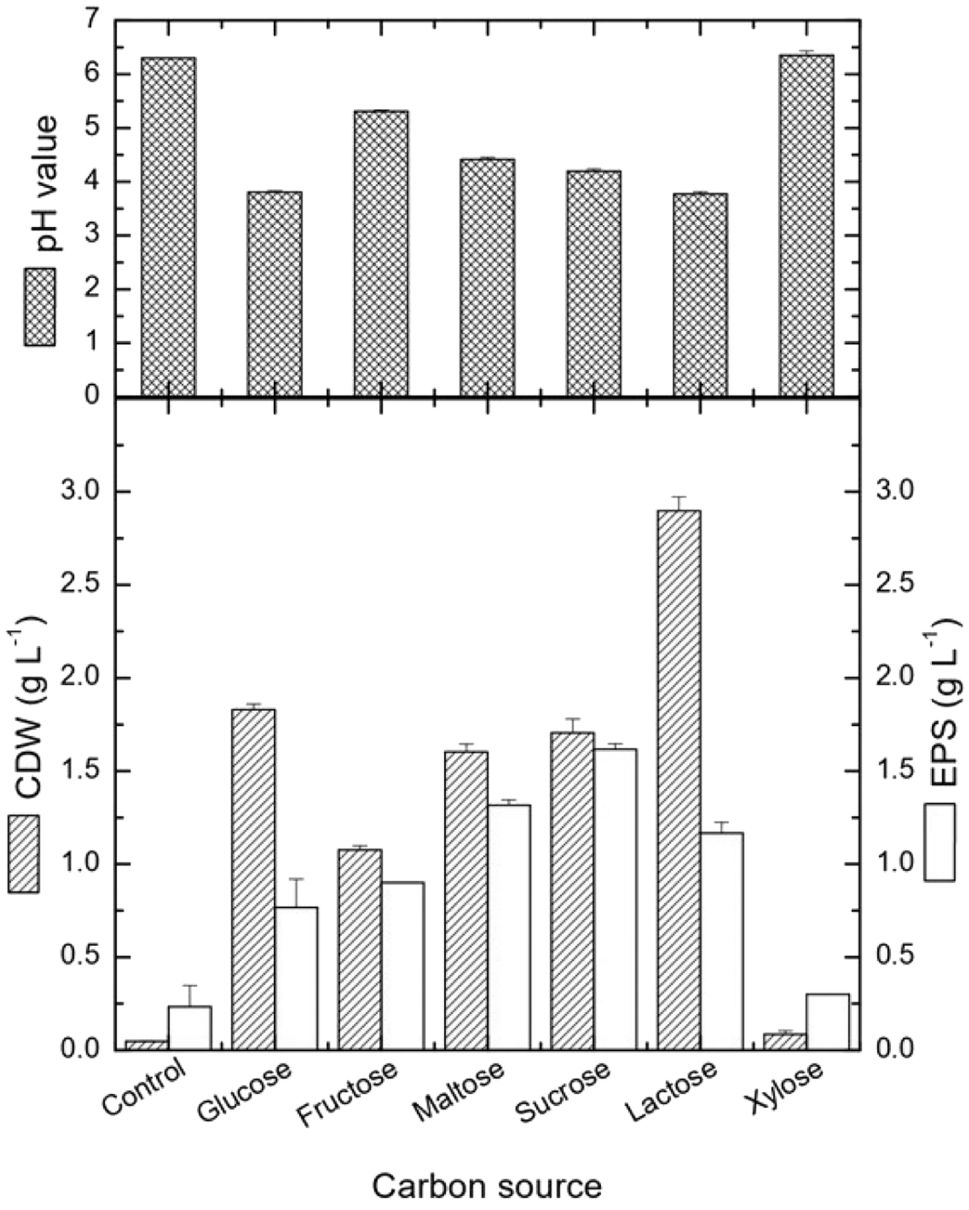

Fig. 1. Effect of carbon sources on growth and EPS production from *L. reuteri* strain DSM20016^T. CDW: Cell dried weight (g/L).

study was composed of peptone which enhance growth of *L. reuteri*. Peptone was widely used for cultivation of various EPS producing Lactobacillus strains [46]. Highest crude EPS was yielded MRS-sucrose supplemented with yeast extract medium. Yeast extract contains diverse vitamins and trace elements which could be essential the EPS biosynthesis in the *Lactobacillus* system [27]. Soumya et al. [49] had formulated cassava-hydrolysate medium with 40 g L^{-1} yeast extract to enhance EPS production in *L. plantarum*. Yeast extract supplemented medium had produced a maximum of 6.5 $\pm$ 0.5 g L^{-1} of crude EPS from static flask cultivation [49]. In addition, Chen et al. [33] also reported that yeast extract is more suitable to enhance growth a cell development for secondary metabolism in *L. reuteri* strains.

3.2. Screening of key factors in fermentation medium by using Plackett-Burman experimental design

For the development of lactic acid bacteria, it was discovered that MRS medium included the most complementary medium components. Carbon and complicated nitrogen sources are grouped together by these meticulous bacteria to meet their development requirements [9]. The effect of other medium components on the growth of these bacteria also has been reported in the literature [46,49]. In this study, the effects of medium component on the growth and crude EPS production of *L. reuteri* strain DSM20016^T were investigated and screened using a population-based design (PBD) technique. Each of the eight components of the modified MRS medium, including sucrose as a carbon source and yeast extract as a nitrogen source, was used in this investigation.

Fermentation medium plays key role in the production of both cell mass and desired microbial secondary products. In this study, PBD optimization approach employed the role of the eight variables in cell growth and crude EPS production. Among the eight medium components, five components (sucrose, yeast extract, dipotassium phosphate, manganese sulphate and ammonium citrate) has positive effect on cell mass production. For crude EPS production, only three components (sucrose, yeast extract and sodium acetate) had positive effect. In both comparisons, the significant variables have shown larger magnitude of the *t*-value and a smaller *p*-value ($p < 0.05$). Sucrose and yeast extract have maximum standardized effect and higher F-value for both cell mass and crude EPS production of *L. reuteri* strain DSM20016^T.

Beside carbon and nitrogen sources, other components such as

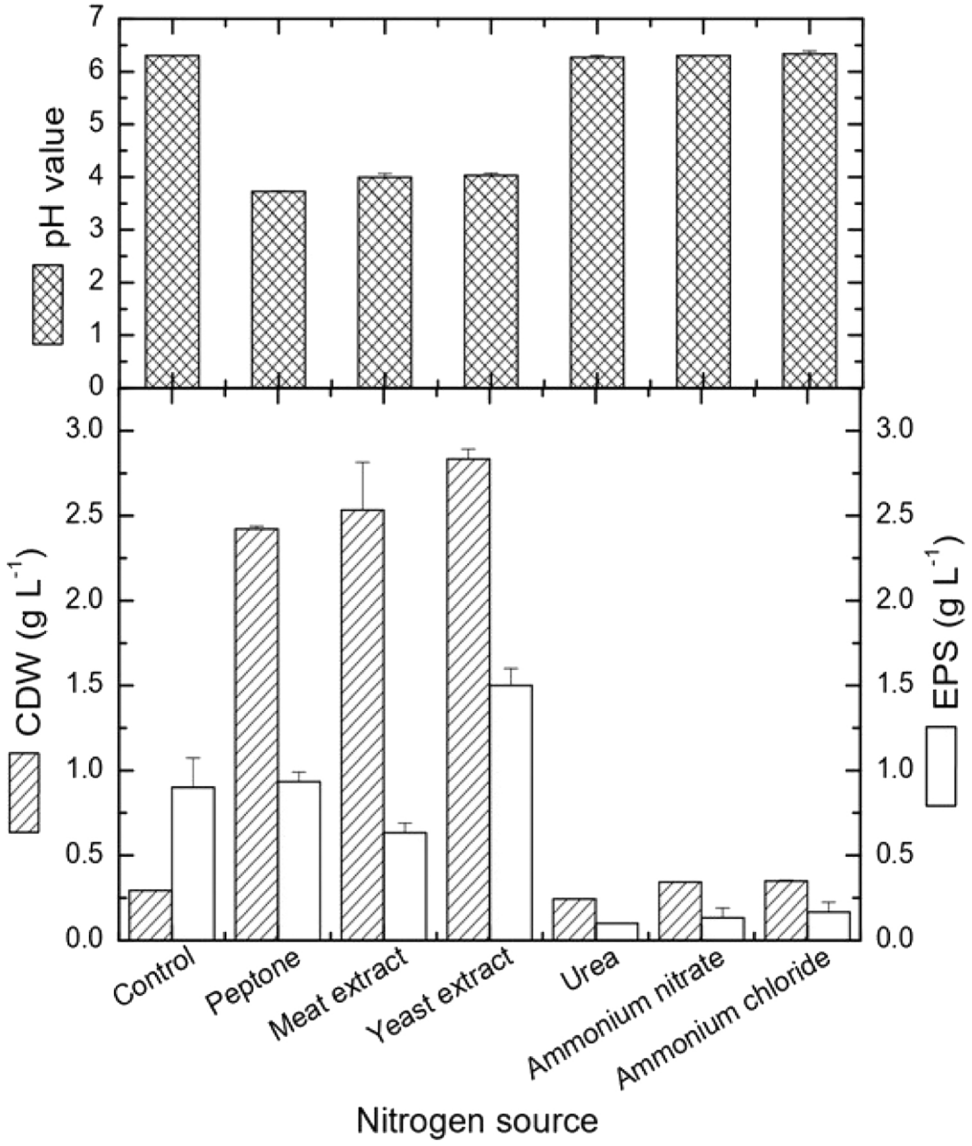

Fig. 2. Effect of different nitrogen sources on growth and EPS production from *L. reuteri* strain DSM20016^T. CDW: Cell dried weight (g/L).

dipotassium phosphate could act as buffering agent in the fermentation medium to maintain the pH value for a more extended span [50]. Presence of mineral ions such as manganese sulphate have reported to stimulate growth of lactic acid bacteria. The manganese ion also proven to enhance biofilm formation and acting as co-enzyme for vital enzymes such as lactate dehydrogenase in *Lactobacillus* [51]. Ammonium citrate selectively allows growth of Lactobacillus species. Studies also proven it is utilized by additional nitrogen source that could enhance cell proliferation in *Lactobacillus* cultivation [52,53].

The statistical analysis has showed the factors with significant effect on crude EPS production from *L. reuteri* strain DSM20016^T were variable B (yeast extract; $p < 0.0001$), A (sucrose; $p = 0.001$) and D (sodium acetate; $p = 0.015$). The level of sucrose as carbon source is important for crude EPS biosynthesis and yeast extract supports the sustained growth and crude EPS synthesis. Sodium acetate plays two roles in the MRS components: (1) it acts as buffering agent like dipotassium phosphate [50] and (2) certain *Lactobacillus* could utilize acetate as carbon source [54]. Wei et al. [55] had reported that both glucose and sodium acetate present in the fermentation medium affects the C/N ratio which could increase crude EPS production. Similarly, the sodium acetate used in this study could also increase carbon percentage in C/N ratio and enhanced crude EPS production.

The regression equation for crude EPS production was discovered using the PBD experimental platform and the coefficient of R^2 for this process was 0.8584 for crude EPS production. This suggested that just 14.16% of the variances could not be explained by the model under consideration. In the present work, the model equation for the crude EPS production from *L. reuteri* strain DSM20016^T is shown in Eq. (3).

$$Y = -0{\cdot}707 + 0.00990\,A + 0{\cdot}07065B - 0.192\,C + 0{\cdot}1396D - 0{\cdot}052E + 0.646\,F + 3{\cdot}50\,G - 0.083\,H \quad (3)$$

Where Y is the crude EPS produced (g L^{-1}), A, B, C, D, E, F, G and H are represented by sucrose, yeast extract, ammonium citrate, sodium acetate, dipotassium phosphate, magnesium sulphate, manganese sulphate and Tween®80. The high value of the adjusted determination coefficient (adj. $R^2 = 0.7828$) advocates for a high significance of the model. These results indicated that the response equation provided a suitable model for this PBD platform.

The positive variable for crude EPS production from *L. reuteri* strain DSM20016^T was chosen to be optimised further using the RSM technique. The negative variables of crude EPS production including the non-significant variables were not omitted from the medium formulation. These variables were maintained in its composition (in g L^{-1}).

Inclusion of these medium components are vital for the growth of *L. reuteri*. The inorganic salts, metal ions and trace elements are usually regarded as the important factors for the growth [56,57] and metabolism of the bacteria [50]. The composition of the fermentation medium is maintained as eight and only the concentration of sucrose, yeast extract and sodium acetate were optimized in RSM platform.

3.3. RSM optimization for enhancing the crude EPS production

For optimization, conventional OFAT and PBD approaches had limitation especially both platforms lack of interpretation about interactions among the significant factors. In fermentation technology, RSM is a most effective, economic, reasonable and adequately applied experimental design for integrated analysis and mathematical modeling with suggested values of probable optimum conditions [49]. Box-Behnken (BBD) experimental design was used to optimize the concentrations of sucrose, yeast extract and sodium acetate based on the findings of OFAT and PBD trials as shown in Table 2. The BBD allows for the calculation of variables and the related reaction at transitional levels that have not been defined by experimental studies [50]. The observed results revealed that the amount of crude EPS produced varied significantly depending on the fermentation medium used. In the replicated centre point settings, the highest possible crude EPS output can be observed. Following a multiple regression analysis, it was possible to derive the second order polynomial equation that accounts for crude EPS generation from *L. reuteri* strain DSM20016^T. The results were as Eq. (4).

$$Y = -0.016 + 0.020284\ A + 0{\cdot}088563B - 0.130917\ C - 0{\cdot}00089A^2 - 0.003400\ B^2 + 0.018519\ C^2 - 0{\cdot}000119AB + 0{\cdot}000299AC - 0.001190\ BCE \quad (4)$$

Where Y represents the amount of crude EPS generated (in g L^{-1}), A represents the sucrose concentration, B represents the yeast extract concentration and C represents the sodium acetate concentration.

A regression analysis was carried out in order to determine the degree to which the RSM design corresponded to the experimental outcomes. As the F value is 58.14, the model is significantly acceptable. The adj R^2 value was 0.9212, indicating that there was a significant degree of correlation between the experimental and predicted values, as evidenced by the results. The generated second order polynomial prediction equation was able to account for about 92% of the variances in the response data. In this model, the F value for lack of fit was 0.254, which was not statistically significant. This means that the pure error was not significant and that the model's fitness was good.

The most significant factors of this model were A, B and A^2, where their p values were less than 0.01 and followed by C^2. This means, the linear effect of sucrose concentration and yeast extract concentration were significant. For quadratic effect, the concentration of sucrose and concentration of sodium acetate were significant. Furthermore, the interaction between variables to produce crude EPS from *L. reuteri* strain DSM20016^T were also analyzed. There was no significant interaction demonstrated in this model. This could explain that crude EPS production is solely dependent on the sucrose concentration.

Following the results of the second-order polynomial model, a three-dimensional response surface and count plots were created with the help of the Minitab® statistical software as shown in Fig. 3. This representation would provide a better understanding of the link between factors and responses [58–61] in order to produce crude EPS. The 3D plots allowed us to see the interactions between factors and quickly establish the optimum values of each variable for maximal crude EPS generation by visualizing the interactions. In this study, the optimum parameters were determined for the generation of crude EPS from *L. reuteri* strain DSM20016^T using the second-order polynomial model that was developed using regression analysis. Exopolysaccharide synthesis by *L. reuteri* strain DSM20016^T was tested in a medium that included (g L^{-1}) 106.0 sucrose, 30.0 yeast extract, 7.0 sodium acetate, 2.0 dipotassium phosphate, 2.0 ammonium citrate, 0.1 magnesium sulphate, 0.05 manganese sulphate and 1.0 Tween®80. With multiple tests, the greatest experimental crude EPS produced under this projected optimal medium formulation was 3.00 g L^{-1} with this anticipated optimal medium formulation. While this was going on, the estimated crude EPS production at optimum medium formulation was 3.07 g L^{-1}. When the measured reaction was compared to the expected value, the findings were extremely close. In this study, no statistically significant differences were found between experimental and anticipated values. As a result, the models were appropriate and accurate for this investigation.

Table 2
Experimental range and levels of factors influencing EPS production from *L. reuteri* strain DSM20016^T in a three-level Box-Behnken design.

Variable	Unit	Code	Low value (-)	Centre value (0)	High value (+)
Sucrose	g L^{-1}	A	20	85	150
Yeast extract	g L^{-1}	B	2	16	30
Sodium acetate	g L^{-1}	C	1	4	7

3.4. Fermentation kinetics of unoptimized and optimized medium

The RSM-optimized medium composition was used to cultivate *L. reuteri* strain DSM20016^T to understand fermentation kinetics for EPS production. The fermentation was performed for 48 h where samples collected to determine growth and EPS production at every 2 h intervals. The growth and EPS production profiles for RSM-optimized and unoptimized medium were plotted in Fig. 4. The growth profile clearly shown that the newly optimized medium has improved the cell biomass production. The cell growth rate under optimized medium condition reached about 0.32 g L^{-1} h^{-1} while under un-optimized medium condition reached about 0.15 g L^{-1} h^{-1}. The cell growth rate under optimized medium condition is about 2.13 folds the cell growth rate under un-optimized condition. After 12 h, it can be observed that for both un-optimized and optimized medium cultivations started to obtain a constant growth which achieved 1.768 g L^{-1} and 3.059 g L^{-1}. Afterwards, cell mass remained constant until the end of the cultivation.

A maximal cell mass of 3.541 g L^{-1} is reached at 24 h for optimized cultivation while for un-optimized cultivation is at 28 h with 2.194 g L^{-1}. Accordingly, cell mass started to decrease after 30 h for optimized cultivation and reached 2.617 g L^{-1} by the end of cultivation. For un-optimized cultivation, cells started to decrease at 30 h and reached 1.986 g L^{-1} at the end of cultivation. This decrease can contribute to the consumption of medium components and depletion of growth limiting components. After inoculation, the bacterial cells grow exponentially for the first 10 h for both cultivation medium. This enhanced EPS production as the number of cells increases in both optimized and un-optimized cultivations. Maximal EPS (1.8 g L^{-1}) is achieved at 36 h for un-optimized cultivations while maximal EPS (3.15 g L^{-1}) is achieved at 26 h for optimized cultivations.

4. SEM analysis

The ability of EPS from lactic acid bacteria to be used in many applications, including in situ food applications, can be determined by their microstructural characteristics. The glucan-type EPS' microscopic structure was extensively revealed by the SEM imaging, which is consistent with its potential application in enhancing the physical and chemical properties of foods [62]. SEM was used in this situation to investigate the morphological and microstructural properties of the crude EPS. The crude EPS produced by *L. reuteri* DSM20016^T emerged as uniform spheres with compact shape, as shown in Fig. 5 (A-C). According to earlier research, EPS from many sources, including glucan, have a compact structure [63]. To the best of our knowledge, irregular sphere like structure in a EPS was not reported previously. Additional

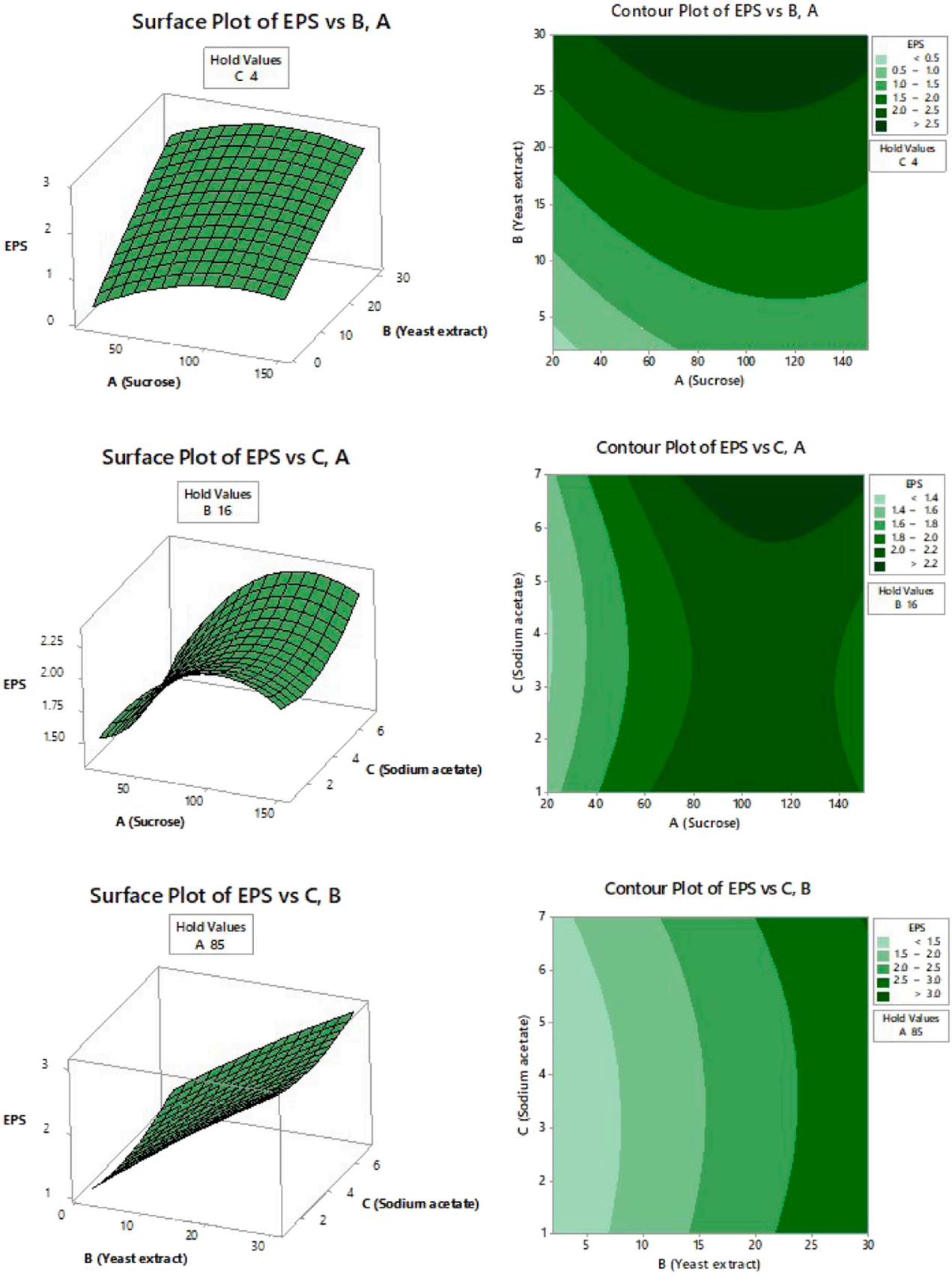

Fig. 3. The 3D plots illustrating the interactions between variables to determine the optimum values for EPS production.

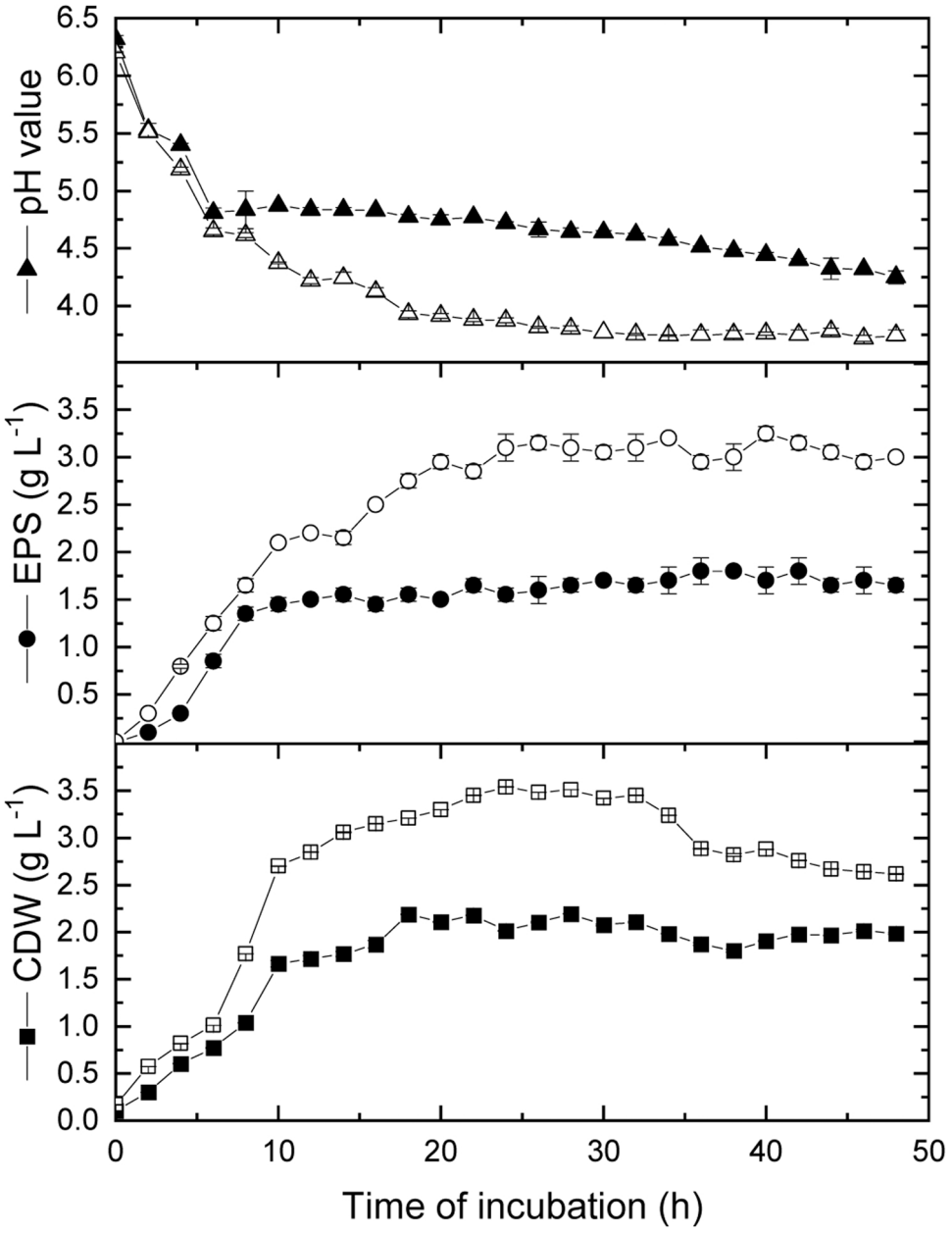

Fig. 4. The growth and EPS production kinetics for *L. reuteri* strain DSM20016^T cultivated in RSM optimized (empty bullets) and un-optimized medium (black bullets).

research is needed to understand the factors that lead to the development of this morphology. The EPS compact, smooth shape was also linked to their beneficial effects on the technological aspects of food goods and this EPS can also be evaluated for use in food applications.

5. Conclusion

In this study, conventional and statistical methods were employed to optimize crude EPS production from *L. reuteri* strain DSM20016^T using modified MRS (mMRS) medium. The carbon and nitrogen sources were determined by using OFAT approach. The PBD platform was used to screen significantly affecting parameters in mMRS medium. The crude EPS production from *L. reuteri* strain DSM20016^T was found to be significantly affected by sucrose, yeast extract and sodium acetate. Then, the concentration of these significant parameters was optimized by RSM using BBD platform. The optimized medium contained (g L^{-1}) 106.0 sucrose; 2.0 dipotassium phosphate; 7.0 sodium acetate; 2.0 ammonium citrate; 30.0 yeast extract; 0.1 magnesium sulphate; 1.0 Tween®80 and 0.05 manganese sulphat. The determination coefficient, $R^2 = 0.9212$, for crude EPS production demonstrated that the model is well-fitting and statistically significant. With triple trials, the greatest experimental crude EPS produced under this anticipated optimum medium formulation was 3.2 g L^{-1}, confirming the prediction. This could suggest the precision and reliability of the model for optimization of crude EPS production studies and further demonstrates the employability of the predicted optimum fermentation conditions for commercial and cost-effective crude EPS production for various industrial applications. Further study can be conducted to determine high purity of crude EPS produced using phenol-sulfuric acid method. The molecular weights and functional groups of the crude EPS produced can be analyzed and compared to other Lactobacillus strains to provide fundamental understanding of the crude EPS produced mainly by *L. reuteri* strain DSM20016^T.

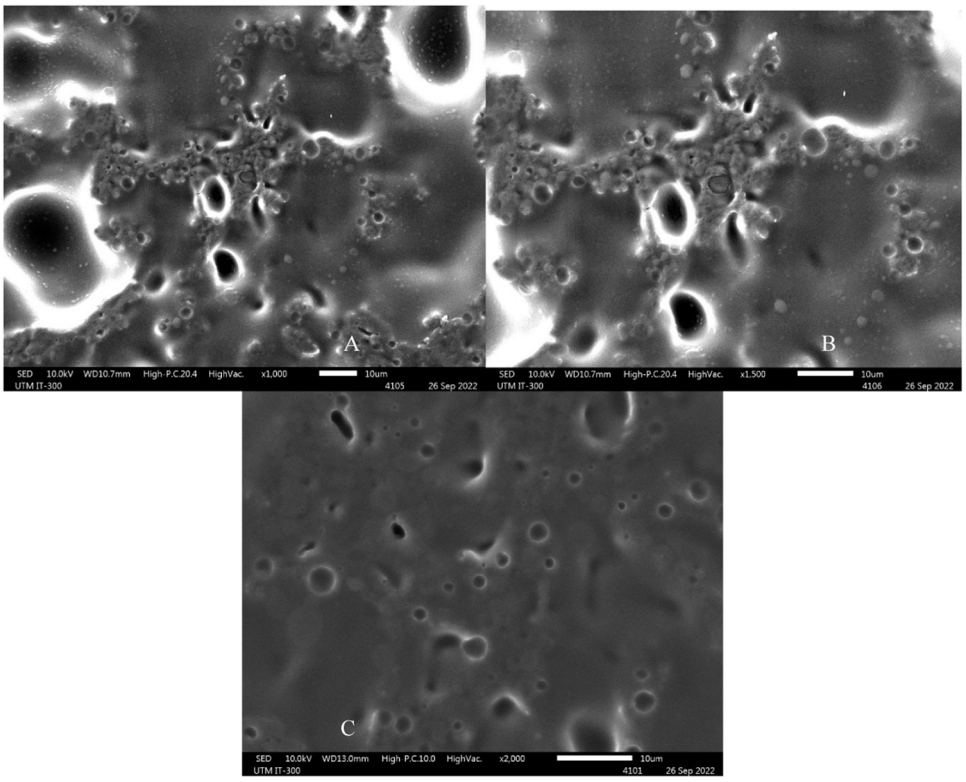

Fig. 5. SEM images of the EPS produced by *Lactobacillus reuteri* DSM20016^T at different magnification levels 1000 × (A), 1500 × (B), 2000 × (C), demonstrating its irregular sphere characteristics.

CRediT authorship contribution statement

Daniel Joe Dailin: Project administration, Conceptualization, Writing – original draft. **Shanmugaprakasham Selvamani**: Data curation, Writing – review & editing. **Khaw Michelle**: Formal analysis, Writing – review & editing. **Yanti Maslina Mohd Jusoh**: Writing – review & editing. **Lai Fatt Chuah**: Project administration, Methodology, Writing – review & editing. **Awais Bokhari**: Validation, Project administration, Writing – review & editing. **Hesham Ali El-Enshasy**: Methodology, Visualization, Writing – review & editing. **Muhammad Mubashir**: Software, Investigation, Writing – review & editing. **Pau Loke Show**: Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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