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# STATISTICAL OPTIMIZATION OF CULTIVATION PARAMETERS FOR EXOPOLYSACCHARIDES PRODUCTION BY *Bifidobacterium pseudocatenulatum* ATCC 27919 IN GLUTINOUS RICE WATER MEDIUM

Hifa Nazirah Mohammed Yaziz[1], Husna Zulkipli[2], Mohd Ilham Adenan[3], Maslinda Musa[1], Norhafezah Kasmuri[4] and Khalilah Abdul Khalil[1*]

[1]*Department of Biomolecular Sciences,Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*
[2]*Faculty of Applied Sciences, Tapah Campus, Perak Branch, Universiti Teknologi MARA,35400 Tapah Road, Perak, Malaysia*
[3]*Pahang Branch, Bandar Tun Abdul Razak, Universiti Teknologi MARA, 26400 Jengka, Pahang, Malaysia*
[4]*Faculty of Civil Engineering, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*

*Corresponding Author: khali552@uitm.edu.my

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**Abstract**

Microbial exopolysaccharides (EPS) have received increased attention over recent decades due to their unique chemical structures and functions contributed by the variation of carbon sources in the cultivation medium. However, the low yield of EPS has hindered their industrial application. Optimization of low-cost carbon sources in cultivation medium is able to increase the production of EPS as well as cell biomass from Lactic Acid Bacteria (LAB) and directly lower the cost of production. In the present study, the potential of glutinous rice water (RW) as an alternate carbon source was ascertained for both cell growth and EPS production by *Bifidobacterium pseudocatenulatum* ATCC. Experimental use RW as a carbon source led to enhanced significant ($p$<0.5) EPS production approximately 5.391 ± 0.3684 mg/ mL in standing culture in comparison with commercial medium (MRS). The optimized cultivation parameters for the maximum EPS production were pH 6, incubation temperature was 29°C, and 26h of cultivation period generated by Central Composite Design-Response Surface Methodology (CCD-RSM). Under optimum cultivation parameters, the predicted EPS production from *B. pseudocatenulatum* ATCC27919 was 10.140 mg/mL, while the actual experimental value was 10.292 mg/ mL. The increment of 1.23 times of EPS production in an optimized medium as compared to the standard MRS medium was observed. During 2-L batch cultivation for the EPS production under optimized cultivation parameters, 16.285 mg/ mL of EPS production was obtained.

## INTRODUCTION

Bacterial exopolysaccharides (EPS) are long-chained polysaccharides accumulated on the cell wall; in the form of capsules or slime, or excreted into the extracellular environment [1]. Microbial EPS can be produced by bacteria, fungi, archaea but of all the EPS producers, EPS-producing Lactic Acid Bacteria (LAB) are known to provide a variety of health benefits for their hosts including anti-tumor, anti-inflammatory, antioxidant, cholesterol-lowering effect, stress-tolerant effects, and enhancement of immune system [2,3]. LAB is well known as an EPS producer and approximately 30 species of *Lactobacilli* have been reported to produce EPS such as *Lactobacillus*

*plantarum, Lactobacillus Rhamnosus, Lactobacillus acidophilus,* and more [4]. According to [5], EPS production is a relatively novel characteristic in the genus *Bifidobacterium*. However studies on EPS produced by *Bifidobacterium* are scarce, consequently, the information on the EPS structure and bioactivities of EPS produced by the genus *Bifidobacterium* is limited and insufficient. *Bifidobacteria* are predominant species colonized in the intestinal tract of healthy adults [5]. *Bifidobacteria* has been stated as "Generally Recognized as Safe" (GRAS) for human consumption and has been used as a probiotic [6].

The preferred features for microbial exopolysaccharides (EPS) as described previously are as follows: high EPS yield, high EPS productivity, capability to grow insufficiently low-cost substrates, and possess curative properties [7]. On an industrial scale, microbial EPS prefer from the other sources due to (a) fast cell growth and simple production that make the EPS yield produced higher in a short period [4,8], (b) the fermentation medium using cheap raw materials, for instance, rice bran and sucrose for the production of EPS by LAB makes the production process cost-effective and (c) the diversity of the structures of EPS influenced by the species of microorganism and culture condition [4]. According to [9], the most economical and valuable bioproducts produce from natural sources and industrial wastes. One of the limiting factors in the commercial success of EPS production schemes is the cost of the sugar substrate used for EPS formation. Attempts have been made to increase yields and improve the physical properties of commercial EPS from LAB by varied techniques [10].

The critical factors that influenced the production of biopolymer EPS as reported in earlier studies are: (a). Medium compositions: Carbon and nitrogen compositions in the cultivation medium influence the production and concentration of EPS. Sugars such as glucose, lactose, sucrose, and mannose are utilized by LAB strains as the carbon sources for EPS production [11]; (b). Cultivation parameters such as temperature, pH, and incubation duration [12]; (c). Strain-dependent: metabolite EPS produced attributed to the species of LAB resulting in the diversity of EPS [13].

The current situation for the industrial application, cultivation of LAB for the growth and EPS production was conducted in a commercial medium; Man, Rogosa, and Sharpe (MRS) or modified MRS, which make the process non-economic. Therefore, a low-cost carbon source with nutritional values was rigorously studied as an alternative cultivation medium that provides a carbon source as this contributed to the biosynthesis of EPS by LAB. Researchers suggesting the use of alternative low-cost carbon sources especially from agricultural wastes such as molasses, coconut water, sugarcane juice, taro waste, and many more [14,15] as the main carbon source as a sugar substitute concurrently for high cell biomass and EPS yield by the LAB.

Rice water contains a significant level of starch, in addition to proteins, lipids, and various inorganic compounds [16]. Several authors reported that the high starch content in starchy crop plants can serve as a good source of nutrients for microbial cultivation [15] and the best carbon source for the production of EPS [7]. For centuries, rice water has been used traditionally for hair and skin treatment. Some finding has reported that rice water can be used for the treatment of the digestive problem by taken orally [27]. Up to date, there is no research has been reported on the uses of RW as a cultivation medium for lactic acid bacteria (LAB) cultivation. Thus, RW could be exploited for the sustainable production of EPS by the metabolism of carbohydrates. This study is the first effort made to determine the potential of glutinous rice water (RW), a by-product of rice processes that often discarded which contained high nutrient levels, as a low-cost carbon source for EPS production by *Bifidobacterium pseudocatenulatum* ATCC 27919.

The application of optimization is one of the main strategies suggested to obtain the maximum yield of microbial EPS. In order to obtain maximum EPS yields (mg/mL), a statistical and mathematical tool was applied using Response Surface Methodology (RSM). Optimization of cultivation parameters (pH, cultivation period, and incubation temperature) was carried out using Central Composite Design-Response Surface Methodology (CCD-RSM) approach for enhancing EPS productivity in RW medium. RSM has been widely used for designing experiments, estimating the effect of interactions between factors, and obtaining optimal conditions of factors for desirable response [17]. Many researchers have employed RSM especially for the optimization of culture conditions and medium compositions for fermentation processes [3]. Optimization of culture conditions and the fermenter design have proved most successful in the case of xanthan as reported by [10].

The aim of this study is to obtain optimal cultivation parameters (pH, cultivation period, and incubation temperature) for higher EPS production (mg/mL) by *B. pseudocatenulatum* ATCC 27919 using RSM. The CCD-RSM was employed to determine the effects of significant factors and their interactions to identify the optimum cultivation parameters that can be experimentally validated. Previous researches have focused on EPS production by LAB, especially *Lactobacillus* sp. cultivated in commercial MRS medium. While this study aims to evaluate the potential of *B. pseudocatenualtum* ATCC 27919 to produce novel EPS in RW medium and directly increase the EPS production by optimization of cultivation parameters. This finding will improve EPS yields by *Bifidobacteria* for various industrial applications.

## MATERIALS AND METHODS

### Plant Materials

Glutinous rice, Floral brand was bought from a market in Shah Alam, Selangor, Malaysia.

### Chemicals

deMan Rogosa Sharpe (MRS) and L-cystine were purchased from Merck. Beef extract was purchased from Oxoid. Pancreatic digest of casein was purchased from Sigma-Aldrich. Sodium chloride (NaCl) was purchased from R&M Chemicals and glucose was purchased from Bio Basic.

### Microorganism and Culture Conditions

*Bifidobacterium pseudocatenulatum* ATCC 27919 was purchased from American Type Culture Collection (ATCC) and was stored at -80°C. The strain was revived in MRSC (deMan Rogosa Sharpe supplemented with 0.05% L-cystine) (Merck Darmstadt, German) broth and incubated in anaerobic condition for 24 h at 37°C. The cells were harvested by centrifugation at 10,000 x g, 15 min at 4 °C and were wash twice with peptone water to minimize carryover of previous medium during fermentation process.

Briefly, free-carbon-basal medium was prepared; beef extract (1.0% w/v), pancreatic digest of casein (1.0% w/v) and natrium chloride (0.5% w/v) and sterilized. Each culture media was supplemented with 0.05% (w/v) L-cysteine hydrochloride, (Merck Darmstadt, German). RW medium was prepared according to [18], 3.65% (v/v) of rice water and 5.52% (w/v) glucose were added aseptically into sterile basal media before inoculation. The strain was cultivated for 24 h at 37°C in anaerobic condition.

### Glutinous Rice Water Preparation

RW was prepared as described previously with some modifications [19]. 50 g of glutinous rice was boiled in 1100 mL distilled water for 6 minutes and stirred continuously. When cooled, the rice congee was sieved and discarded. RW was collected and lyophilized. RW was stored at -20°C until use.

### Extraction and Purification of EPS

The EPS produced in RW medium was extracted and purified followed by [20] with some modifications. Initially, cultured medium was heated at 100 °C for 30 min After cooling, the supernatant was retrieved by centrifugation at 9,000 x *g* for 20 min at 4° C. Then, trichloroacetic acid (TCA) was added and kept at 4°C for 3h followed by centrifugation at 25,000 x *g* for 20 minutes at 4°C and precipitate was removed. EPS was precipitated by the addition of equal ratio of absolute ethanol (100% v/v) to the supernatant. After precipitation at 4°C (overnight) the sample was recovered by centrifugation at 25,000 x *g* for 30 min at 4°C, and the crude EPS was collected. The purified EPS was dialyzed at 4°C for every 24h for two days. The lyophilized samples were stored at -20°C until further analysis.

### Quantification of EPS Production

The EPS concentration was determined by the phenol-sulphuric acid method using glucose as standard [21]. Initially, 1 mL of purified EPS were mixed with 1 mL of 5 % phenol aqueous solution. Next, 5 mL of sulphuric acid was added quickly and stirred. The samples were incubated for 10 min for color development. Subsequently, the absorbance of the samples was measured at wavelength 490 nm using spectrophotometer. All analysis was performed in three independent replicates.

### Screening the Ability to Produce EPS in RW

Preliminary screening to observe the ability of *B. pseudocatenulatum* ATCC 27919 to produced EPS in RW medium was observed. Free-carbon-basal medium was prepared; beef extract (1.0% w/v), pancreatic digest of casein (1.0% w/v) and natrium chloride (0.5% w/v) supplemented with 0.05% (w/v) L-cysteine hydrochloride, (Merck Darmstadt, German). While, RW medium was prepared according to [18], 3.65% (v/v) of RW and 5.52% (w/v) glucose were added aseptically into sterile basal media before inoculation. The strain was cultivated for 24 h at 37°C anaerobically.

### Optimization using CCD-RSM

Based on previous research, pH, cultivation time, and incubation temperature plays an important role for the EPS production [3]. In order to determine optimal cultivation parameters for enhancing EPS production and to observe the interaction between these parameters, a central composite design (CCD) was employed as described by [22]. The experiment design, with coded values is shown in Table 1. A quadratic model was generated consists of 14 factorial runs and five center runs to allocate treatment combinations in this experiment, with the EPS productions (mg/mL) as response. Each run was performed in triplicate. The design was generated by Design Expert Software (Version 12.0.7.0, Stat-Ease, Minneapolis, MN, USA). The experiment consisted of three independent parameters: pH, cultivation time (h), and incubation temperature (°C). The quadratic polynomial model for optimal points prediction was expressed according to Eq. (1);

$$y = \beta_0 + \Sigma \beta_i \chi_i + \Sigma \beta_{ii} \chi_i^2 + \Sigma \beta_{ij} \chi_i \chi_j \qquad (1)$$

where *Y* is the predicted response; *β*o represented the intercept term; *xi* and *xj* are the level of independent parameters; *βi* was the linear coefficient of *xi; βii* was the quadratic coefficient of *xii; βij* was the interaction coefficient.

**Table 1.** Coded levels of the cultivation parameters for optimization using FCCD-RSM

| Independent parameters | Unit | Symbol | Range and levels | | |
|---|---|---|---|---|---|
| | | | -1 a | 0 | +1 a |
| pH | | A | 4 | 6 | 8 |
| Incubation temperature | °C | B | 27 | 37 | 47 |
| Cultivation time | h | C | 6 | 21 | 36 |

[a] -1 and +1 are coded levels.

**Validation Experiment by the Model**

Verification experiment was performed to observe EPS yields produced by *B. pseudocatenulatum* ATCC 27919 under optimized culture conditions; pH6, 29°C of incubation temperature and 26h of cultivation period. In this condition, the EPS yields production was 10.140 mg/mL as predicted by FCCD-RSM using phenol-sulphuric acid methods.

**2-L Batch Fermenter Analysis**

Further, batch fermentation was conducted according to [22] with slight modification. The stirred-batch culture was conducted in a 2-L BIOSTAT® B double-walled, round-bottom glass bioreactor (B. Braun Biotech International, Melsungen, Germany) with a heated water jacket. Initially, the medium components were sterilized in situ and 10% (v/v) of inoculum was inoculated aseptically. The culture was incubated in anaerobic condition under previous optimal cultivation parameters; temperature at 29°C, pH was maintained at 6.0 for 26h of incubation time. The culture was stirred at 200 rpm and aerated with $N_2$ gas at the rate of 1.67 vvm for 26h. The EPS was extracted after fermentation has completed. EPS yield (mg/mL) was calculated as total carbohydrate content by using phenol-sulphuric acid method.

**Microbiology Analysis**

To determine the number of viable cells (cfu), serial 10-fold dilutions of sample were done using sterile saline solution and was plated onto MRS agar plates in three replicates. The plate was incubated anaerobic condition at 37°C for 48h before enumeration. Each colony was derived from a single viable cell or a colony forming unit. All the data are presented are the average of triplicate.

**Total Carbohydrate Analysis**

Carbohydrate quantification was determined by using phenol-sulphuric acid method using glucose as a standard [21].

**Statistical Analysis**

All experiments were performed in triplicate, and mean values are presented. Data were expressed as mean ± standard deviations [23] by three biological replicates for each experimental data. Data of all replications were analyzed using statistical tools by MINITAB 16 statistical software package (Minitab Inc., Pennsylvania, USA).

**RESULTS AND DISCUSSION**

**EPS Productions in RW Medium**

RW medium was evaluated for their efficiency to produce EPS by *B. pseudocatenulatum* ATCC 27919. *B. pseudocatenulatum* ATCC 27919 was able to utilize a carbon source in the RW medium for cell growth and is capable of synthesis EPS biopolymer as the product of metabolism. EPS production from *B. pseudocatenulatum* ATCC 27919 in RW medium obtained was 5.391 mg/mL. The results in Table 2 revealed the EPS produced significantly difference ($p< 0.5$) in comparison with the commercial medium, MRS. Previous studies reported the EPS production by *Lactobacillus* sp. ranges from 0.06 to 0.15 mg/mL for *L. delbrueckii* subsp. *bulgaricus* and 0.05 to 0.06 mg/mL for *L. casei* in supplemented MRS media [11]. While, a study by [24] reported that the highest EPS production obtained were 7.276 ± 0.115 mg/ mL and 2.753 mg/mL by *L. reuteri* and *B. longum*, respectively in commercial MRS medium. In this study, although EPS produced in commercial MRS medium higher than RW

medium, the studied strain showed a relatively average level of EPS production in comparison to the reported level of EPS production among different LAB strains. This result clearly shows that RW was capable to support the growth of *B. pseudocatenulatum* ATCC 27919 by stimulating probiotic growth and EPS biosynthesis. This screening results showed the potential of glutinous rice water (RW) as the low-cost carbon source for both cell growth and EPS production by *B. pseudocatenulatum* ATCC and therefore, RW medium can be an alternate culture medium to substitute commercial MRS medium for the growth of LAB to produce EPS.

**Table 2.** The EPS production (mg/mL) produced by *B. pseudocatenulatum* ATCC 27919 in RW medium and commercial media (MRS medium) using Phenol-Sulphuric Acid Method

| Cultivation Media | EPS Concentration (mg/mL) |
|---|---|
| EPS-RW | 5.391 ± 0.368[a] |
| EPS-MRS | 8.403 ± 0.811[b] |

[abc]Value in the same column with different letters were significantly different ($P < 0.05$).

**Optimization Culture Parameters for Higher EPS Yields**

The cultivation parameters were selected based on previous studies [3, 22] and were varied according to the design provided, and EPS was extracted and the fit was determined. A CCD-RSM is one of the efficient central composite design to explore the optimal condition for the highest response [17]. Based on regression analysis, Table 3 shows considerable variation in the EPS yield depending upon the interaction of various levels of three independent parameters of cultivation parameters. EPS yield varied significantly between 1.979 ± 0.127 mg/mL and 9.314 ± 0.185 mg/mL (Table 3). This significant difference confirmed the indispensability of optimizing the medium for maximum EPS yield. The experimental results were modeled with the following first-order polynomial equation to explain the correlation between the observed parameters and EPS yield by *B. pseudocatenulatum* ATCC 27919;

$$y=4.59 + 0.05967A - 1.50B + 0.4607C + 0.8315AB - 1.02AC - 0.0708BC - 1.21A2 + 0.4818B2 + 3.39C2$$

Where y is the EPS yields (mg/mL), and the predicted response parameters; A is pH, B is incubation temperature and C is cultivation time.

**Table 3.** Experimental design and responses of CCD-RSM

| Run | Factor A | Factor B | Factor C | Response[a] |
|---|---|---|---|---|
| | pH | Temperature (°C) | Time (h) | EPS production (mg/mL) |
| 1 | 6 | 37 | 6 | 6.891 ± 0.020 |
| 2 | 8 | 47 | 6 | 9.314 ± 0.185 |
| 3 | 6 | 27 | 21 | 7.483 ± 0.224 |
| 4 | 8 | 37 | 21 | 3.373 ± 0.262 |
| 5 | 6 | 37 | 21 | 4.225 ± 1.148 |
| 6 | 4 | 37 | 21 | 2.506 ± 0.152 |
| 7 | 4 | 47 | 36 | 7.012 ± 0.240 |
| 8 | 6 | 37 | 21 | 5.130 ± 1.148 |
| 9 | 8 | 27 | 6 | 7.711 ± 0.038 |
| 10 | 6 | 47 | 21 | 1.979 ± 0.127 |
| 11 | 8 | 27 | 36 | 8.750 ± 0.200 |
| 12 | 6 | 37 | 21 | 6.958 ± 1.148 |
| 13 | 6 | 37 | 21 | 6.802 ± 1.148 |
| 14 | 4 | 47 | 6 | 2.159 ± 0.078 |
| 15 | 8 | 47 | 36 | 5.733 ± 0.068 |
| 16 | 4 | 27 | 36 | 9.018 ± 0.378 |
| 17 | 6 | 37 | 21 | 5.789 ± 1.148 |
| 18 | 4 | 27 | 6 | 8.219 ± 0.057 |
| 19 | 6 | 37 | 36 | 8.388 ± 0.306 |

[a] EPS concentrations produced by *B. pseudocatenulatum* ATCC 27919 are in average of three replicates.

According to CCD-RSM, factor B (temperature) was the only significant model term of all the factors but interactions of other factors were also found equally important for EPS production. Incubation temperature significantly influenced EPS yield compared to pH and cultivation time but for further optimization process, all three factors were taken into account for CCD-RSM due to pH, incubation temperature and time are the critical parameters for EPS production other than carbon and nitrogen sources [3, 11]. Therefore, all factors were selected prior optimization process.

The model F-value = 3.97 and low *p*-value ($p>$ F, 0.0413) implies the good fit of the model. *p*-values less than 0.05 ($p< 0.5$) indicate model terms are significant. The correlation coefficient ($R^2$) was calculated as 0.836 for EPS production (mg/ mL) by *B. pseudocatenulatum* ATCC 27919 indicating that the model can explain 84% of variability in the response. The statistically insignificant lack of fit value of ($p> F$) = 0.179 shows that the model is adequate for the prediction of the EPS production in the range of the parameters employed. From all these data, this model can predict the quantity of the EPS satisfactorily. Models are used for prediction in order to generate response surface graphs and contour plots.

Figure 1 are three-dimensional response surface plots represented the interactions between the incubation temperature and cultivation time; pH and cultivation time; pH and incubation temperature on EPS production by *B. pseudocatenulatum* ATCC 27919. Figure 1 (a) surface plot shows the EPS yield was higher at 27°C in combination

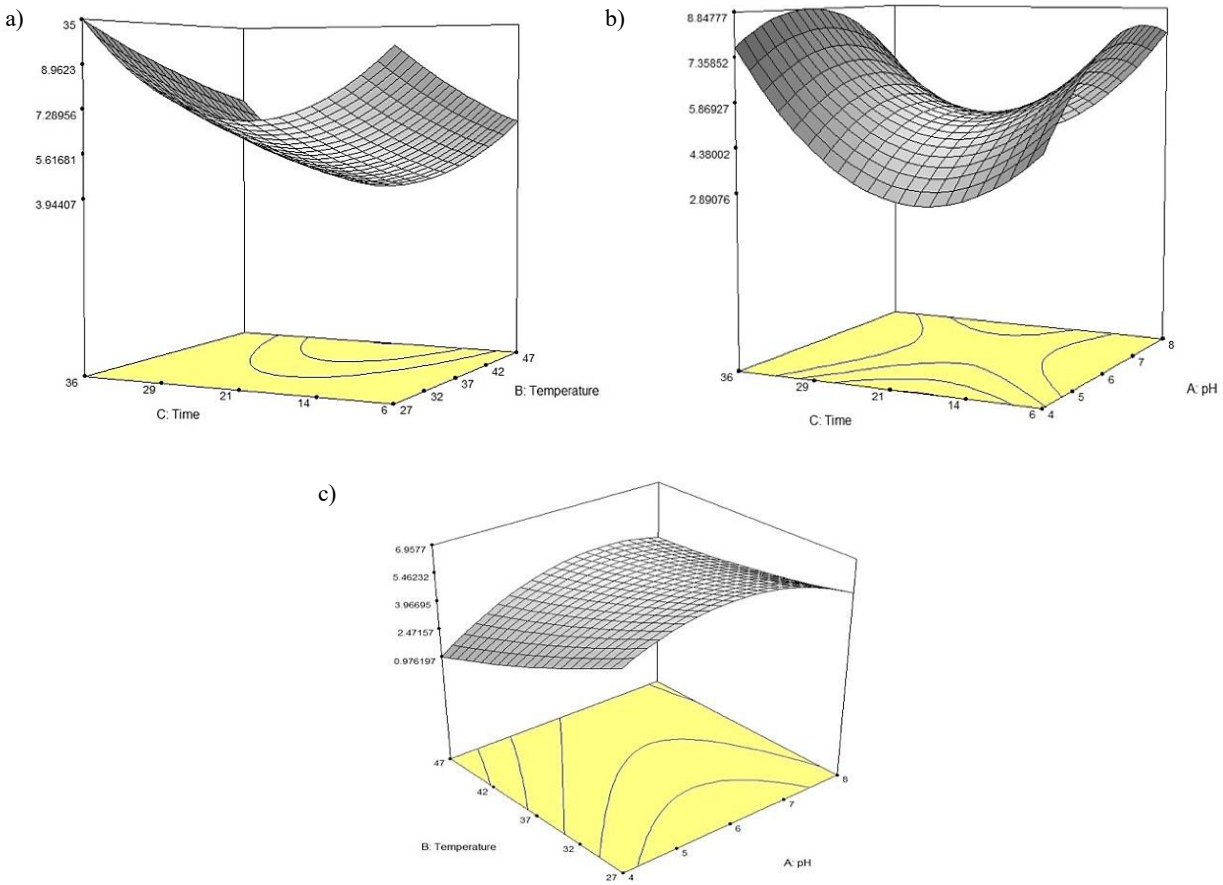


**Figure 1.** Three dimensional surface plot of the effect of three parameters on EPS production by *B. pseudocatenulatum* ATCC 27919 for a) temperature and time, b) pH and time, c) pH and temperature

with 6h of cultivation time due to significant level of glucose as carbon source crucial for their growth at the beginning of log phase. Study by [25] revealed that some LAB strains favors low temperature for the production of EPS production. Subsequently, the EPS production start to decrease from 6h until 21h of cultivation time as the result of the uncoupling of cell growth and acid productivity which might cause the degradation of EPS [3]. However, after 21h we can observe the continuous increment of EPS production up to the 36h of cultivation period (maximum point). This result is in line with the finding by [27] which reported the highest EPS yield in the cultures produced at

the late stationary phase which makes the EPS is growth-associated because its reaches maximum towards the end of the stationary growth phase. Throughout the fermentation process, it can be observed that the optimum pH for high EPS yield was located around pH 6. According to [3], the high EPS production occurred between pH 5 to pH 6.

The optimization of the cultivation parameter in RW medium was successful with significantly enhanced EPS yield. As aforementioned, the EPS production is strain-dependent and EPS concentration were influenced by medium compositions and cultivation conditions used for microbial growth [26].

### Verification Experiment

To validate the adequacy of the model equations, a total of four replications of the verification experiment were carried out. EPS productions were validated using the quadratic model proposed by the CCD-RSM. Based on the result, the predicted data for the maximal EPS production under optimal cultivation parameters (pH6, incubation temperature was set to 29°C and 26h of cultivation period) showed no significant difference ($p< 0.5$) with a low error percentage (1.49%). The results obtained from the experiment were similar to the model predicted and therefore, the model is validated. The predicted response was 10.140 mg/mL, whereas the actual response was 10.292 mg/mL. This study successfully improves EPS yield by *Bifidobacterium* sp. 3.74 times higher when compared with EPS obtained from *B. longum* in unoptimized MRS medium [24].

### Batch Fermenter Analysis

Further, the performance of the optimal cultivation parameters for the production of EPS in the batch fermenter was conducted. 1-L culture medium was withdrawn after 26h and EPS was extracted. The concentration of EPS production was determined by phenol-sulphuric acid method [21]. Results showed the maximum production of EPS was found to be 16.285 ± 0.021 mg/mL. While the weight of the freeze-dried, purified EPS was 0.311g. The viable cells (cfu) was determined up to 11.524 ± 0.046 $\log_{10}$ cfu/mL. It was suggested that EPS produced by *B. pseudocatenulatum* ATCC 27919 in RW medium might be a primary metabolite since EPS biosynthesis shows growth-associated. This study successfully improved EPS production 27.278 times higher in comparison with EPS produced by *L. acidophilus* MTCC 10307 in MRS medium as reported by [22] under optimized parameters (pH, cultivation time and incubation temperature) with the same agreement that the EPS was enhanced in the fermenter than using shake-flask method. As conclusion, large number of EPS can be produced within a short period under controlled conditions in the fermenter [19].

## CONCLUSION

In the present study, optimization of culture parameters was achieved as a strategy to influence EPS production by *B. pseudocatenulatum* ATCC 27919. The data showed that glutinous rice water (RW) was a good alternative carbon source and was able to be utilized by *B. pseudocatenulatum* ATCC 27919 for the cell growth coupling with the production of metabolite EPS. As a result, EPS production was increased up to 10.292 mg/ mL in standing culture and 16.285 mg/mL in a 2-L batch fermenter under optimized culture parameters. The increment of 1.9 and 3.0 times, respectively. The higher EPS yield was achieved throughout this study and we can conclude that the EPS production was enhanced in the batch fermenter when compared with the standing culture experiment. Accordingly, future research is currently underway in our laboratories to investigate the effect of EPS on colorectal cancer cells via autophagic cell death mechanisms.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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