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Statistical medium optimization for enhancing high biomass production of *Lactobacillus reuteri* using response surface methodology

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Abstract

The high cell mass production of probiotic lactic acid bacteria using low-cost substrate is critical for successful large-scale production. *Lactobacillus reuteri* DSM20016^T is a heterofermentative lactic acid bacterium that is well recognized for its probiotic properties. Defining an optimal cultivation medium could enhance the yield and reduce the production cost as well. In this paper, seven media reported in the literature were reviewed and assessed for their capability to be used as a growth medium of *L. reuteri.* The most appropriate cultivation medium for *L. reuteri,* which has reached the best cell mass yield; annotated M5; was selected. The selected medium from the screening stage was optimized using response surface methodology (RSM). The optimized medium composition was (gL^{-1}): sucrose (55), yeast extract (44), sodium acetate (2.6), tri-ammonium citrate (0.2), KH_2PO_4 (0.2), Mg SO_4 (0.2), Mn SO_4 (0.05), and NaCl (0.01). The comparative study showed 28% increase was achieved in the optimized medium with the yield of 3.31 gL^{-1} compared to the cell mass production in the unoptimized medium that was 2.59 gL^{-1}. A significant cell mass growth rate accompanied the achieved increment. Finally, an optimum medium was proposed for enhanced cell mass production.

Keywords: *Lactobacillus reuteri*, Lactic acid bacteria, Medium optimization, Response surface methodology, Biomass production

1. Introduction

Probiotics are microbial organisms that their consumption as dietary supplements enhance gastrointestinal microbiota activities and minimize their potentially harmful effects [1]. Consumption of probiotics is associated with the enhancement of human health by the restoration of intestinal microbial balance. Lactic acid bacteria (LAB) are a large group of bacteria with probiotic properties that contains a diverse assemblage of species [2]. Prebiotics are non-digestible but fermentable dietary food ingredients such as oligosaccharides that benefit the host by selectively stimulating the growth or activity of probiotics in the colon [3]. *Lactobacillus reuteri* is a Gram-positive, rod-shaped, non-motile, non-spore-forming member of the LAB that can be isolated directly from the gastrointestinal ecosystem or feces of mammals and birds [4].

Helicobacter pylori are widely accepted as the major pathogenic cause of chronic gastritis and most peptic ulcers [5]. The bacterium overcomes the gastric defense system by its spiral shape allowing it to adhere to the gastric mucosa [6]. Several proteins mediate adherence of host cells to the gastrointestinal tract's outer membrane, namely adhesins assisting the bacteria to become a human stomach colonizer [5]. *L. reuteri* plays an essential role in treating *H. pylori* infection by producing a unique antimicrobial substance named reuterin [7]. The change in human's lifestyle and dietary pattern from the 1960s to the recent time has caused a significant decrease of *L. reuteri* contents in human's total gastrointestinal environment. Hence, supplementation of *L. reuteri* is an alternative way of favourably modulating the intestinal microbiota.

There are several media to support the good growth of *L. reuteri*. The fermentation medium could be of chemically defined composition or commercially obtainable standard ingredients, and the cultivation medium provides a suitable growth system for bacteria [8]. The addition of carbon sources can enrich growth media (e.g., glucose, sucrose, corn syrup, and molasses), nitrogen sources (e.g., peptone, yeast extract, beef extract, milk powder, soybean meal, and urea), nutrients (e.g., minerals, vitamins, and cofactors), and some concentrations of dissolved oxygen [9].

Response surface methodology (RSM) is a set of statistical and mathematical techniques widely used for optimizing complex processes [10]. RSM was used to determine the optimal condition for multivariate systems by studying the impact of various factors affecting the response [11]. Several studies have used RSM to optimize the cultivation medium [11,12]. The Plackett–Burman design (PBD) method allows the estimation of the critical variables with relatively fewer experiments for cell mass production without a substantial loss of information. On the other hand, Box–Behnken design (BDD) is a powerful tool to determine the optimal level of process parameters with less number of experiments when compared with other design of experiment models [13].

L. reuteri inhibits the colonization of pathogenic microbes, enhances the host immune system, strengthens the intestinal barrier, and ameliorates inflammatory diseases. The change in diet and lifestyle dramatically affects the abundance and composition of diverse microbiota in the human gastrointestinal tract. In this study, the cell growth kinetics of *L. reuteri* is investigated, and different statistical designs are presented to optimize the cell mass growth.

2. Materials and methods

Cultivation of *L. reuteri* was carried out by selecting the most appropriate medium and enhancing the composition to achieve high cell mass concentrations. The used material and methodology for the experimental investigation and the strategies for analysis and design stages are discussed in the following.

2.1 Microorganism and cultivation medium

Lactobacillus reuteri DSM 20016^{T} obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) has been used as the reference strain in this study. Once the bacterial strain condensate of *L. reuteri* was obtained, it was activated in a deMan Rogosa Sharpe (MRS) broth medium based on the instructions of the strain manufacturer. Then the activated *L. reuteri* cells were streaked onto MRS agar plates to maintain the growth and purity of the strain. Subsequently, a sterile glycerol solution with a concentration of 20% v/v was added to the agar plate sub-culture to prevent the inactivation of bacterial cells. Finally, the suspension was immediately deep-frozen (at −80 °C) and preserved until used.

2.2 Preparation of the inoculum

In order to prepare a 10% v/v inoculum, the preserved master culture at −80 °C was used as the starter. A 5mL inoculum was inoculated into a 50 mL broth medium and then incubated at 37 °C in micro-aerophilic conditions in a screw-cap bottle. The bacterial strains grown in the broth medium were harvested at the exponential phase due to the higher concentration of viable cells accentuated in this phase [14]. In the next step, *L. reuteri* bacterial culture was spun down. Cell pellet and supernatant were used for high-performance liquid chromatography (HPLC) and cell mass assays. The exploited cell pellets were washed twice with sterile water, and the washed pellets were re-suspended and adjusted to OD_{600}. The 10^7 CFU/mL for cell biomass is equivalent to the absorbance of 1 OD_{600} in the spectrometric measurement.

2.3 Production media and cultivation conditions

The culture medium and inoculum composition are fundamental factors in enhancing the high growth rate of culture and industry-scale cell mass production [15]. Seven cultivation media were initially prepared, tested, and compared to provide the required data for the optimization process. Using different culture media with various compositions (e.g., different carbon sources, nitrogen sources, and other medium constituents) and concentrations is a practice that has been successfully employed in the media screening stage in a wide range of studies [12,16,17]. All cultivation media were prepared in a 100 mL conical flask at 50 mL working volume. The best cultivation medium was selected based on providing the highest cell mass among all tested media. Table 1 shows the selected media and their compositions from the literature review.

Table 1 The media selected from the literature review.

Components	Medium						
	M1	M2	M3	M4	M5	M6	M7
	MRS	PY+ Lactose (5%)	mMRS	SPY	YEM	M17+ Glucose 0.5%	TPY+ Glucose 5%
Ammonium citrate	2.00	-	2.00	-	-	-	-
Ammonium phosphate	-	-	-	-	-	-	-
Ascorbic acid	-	-	-	-	-	0.50	-
Beef extract	-	-	-	-	-	5.00	-
$CaCl_2$	-	-	-	-	-	-	0.15
Disodium-glycerophosphate	-	-	-	-	-	1.90	-
FeCl3	-	-	-	-	-	-	0.005
Glucose	20.0	10.0	11.0	25.0	45.0	-	-
"Lab-Lemco" powder	-	-	-	-	-	5.00	-
K_2HPO_4	2.00	-	-	-	-	-	2.00
KH_2PO_4	-	-	-	-	0.20	-	-
L-cysteine	-	-	-	-	-	-	0.50
Meat extract	8.00	-	-	-	-	-	-
$MgCl_2$	-	-	-	-	-	-	0.50
$MgSO_4$	0.40	-	0.20	-	0.20	0.25	-
$MnSO_4$	0.056	-	0.05	-	0.05	-	-
Na_2HPO_4	-	-	2.00	-	-	-	-
NaCl	-	-	-	-	0.01	-	-
Peptone	10.0	20.0	30.0	-	-	-	-
Phyton	-	-	-	-	-	-	5.00
Sodium acetate	5.00	-	8.29	-	0.50	-	-
Soy peptone	-	-	-	25.0	-	5.00	-
Tri ammonium citrate	-	-	-	-	0.20	-	-
Trypticase	-	-	-	-	-	-	10.0
Tryptone	-	-	-	-	-	5.00	-
Tween 80	1.00	-	1.00	-	-	-	1.00
Yeast extract	4.00	10.0	6.00	25.0	20.0	2.50	2.50
$ZnSO_4$	-	-	-	-	-	-	0.25
Reference	[20]	[21]	[22]	[23]	[18,19]	[24]	[25]

MRS: De Man Rogosa and Sharpe; PY: peptone yeast; mMRS: modified De Man Rogosa and Sharpe; SPY: soy peptone yeast; YEM: yeast extract medium; TPY: trypticase phytone yeast.

In order to reduce the number of the contributing factors, the optimization process is carried out by neglecting the factors with less significant impact. The remaining components are optimized using the response surface method (RSM) described by Kepli et al. [12], Eyahmalay et al. [26], and Hamid et al. [27]. The cultivation was carried out on all selected media microaerophilically at 37 °C for 48 h. The initial pH of all media was adjusted to 6.25 using NaOH and HCl (3 M). Before inoculation, the carbon source was separately autoclaved at 121 °C for 20 min.

2.4 Plackett Burman design (PBD)

Optimization procedure involving factorial designs is a key subject in optimal factor assignment to enable building statistical models with a small number of runs [28]. Factorial designs need 2^N experiments where N is the number of the factors to be investigated. In the selected medium, the carbon source was switched from glucose

to sucrose to reach the maximal level of cell mass. The medium contained eight components, including nutritional supplementation and minerals. Based on the composition and the significance of effect upon the growth capacity, four process variables of sucrose; yeast extract; sodium acetate ($C_2H_3NaO_2$), and potassium dihydrogen phosphate (KH_2PO_4) were included in the factorial design to investigate the effects of nitrogen and carbon sources and mineral constituents on cell growth.

Initially, implementing a complete factorial design requires several runs to include all possible combinations of experiments that can be expensive and time-consuming. Therefore, fractional factorial designs were chosen as an efficient alternative. Higher-order interactions are excluded in fractional factorial designs assuming that they are negligible. The Plackett–Burman design (PBD) method allows establishing the estimation of the critical variables with relatively fewer experiments for cell mass production without a substantial loss of information. The PBD experiment provides a polynomial mathematical model incorporating the different interactions of nutrition and mineral supplements. The high and low values selected for the PBD include sucrose (10 and 100 g L^{-1}); yeast extract (8 and 80 g L^{-1}); sodium acetate (0.2 and 5 g L^{-1}); potassium dihydrogen phosphate (0.1 and 1.5 g L^{-1}). The first-order model of the PBD statistical design is given as the following equation:

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

where Y is the cell dry weight (CDW) in g L^{-1}, β_0, βi, and X_i intercepts the model, estimates of variable, and independent variables, respectively. All assays were implemented in triplicate form, and mean values of triplicate runs of the produced cell biomass were taken as responses. To determine whether a factor had a significant effect on CDW, a 95% confidence level was considered for each incorporated factor.

2.5 Box-Behnken design (BBD)

In order to further analyze and estimate the factors and levels, the results of PBD were used for further optimization in BBD. A three-level BBD was applied to evaluate the significant effect of sucrose, yeast extract, and sodium acetate to study both individual and mutual interaction effects between those medium components on enhancing *L. reuteri* cell biomass. Selected factors were studied at three different concentration levels of high, middle, and low, which have been coded as −1, 0, and 1, respectively [11].

Analysis of variance (ANOVA) was carried out for each independent variable to analyze and identify the main factors' significance and interactions and determine validity. Surface plots were generated to investigate the effect of process factors and their interactions. Moreover, the plots were used to determine the optimum medium concentrations, which significantly affect the response. All PBD and Box-Behnken design (BBD) experimental designs and variance analysis were performed in Minitab®17 software.

2.6 Spectrophotometric quantitative analysis

The cell growth of the *L. reuteri* strain in the medium was obtained by measuring the absorbance. Optical density (OD) is proportional to the number of microorganisms in the culture medium determined at 600 nm wavelength within appropriate intervals. The culture OD was correlated to cell mass production by a liner correlation standard curve, in which 0.3 g L^{-1} is approximately equivalent to 1 OD_{600}.

3. Results and discussion

The results of L *reuteri* cultivation in different broth media and selection of the best composition to achieve high mass production are presented. Moreover, the result and discussion of the statistical design and analysis and the related experimental investigation to reach optimal cell-mass production are provided in the following.

3.1 Effect of cultivation media

The cell growth of *L. reuteri* in seven different media is presented in Figure 1. It was observed that all screened media could support high cell growth at different levels. Growth medium M5 [18,19] was the best medium to achieve the highest biomass yield (2.59±0.07 g L^{-1}). It is characterized by the highest sugar feeding in the original medium (glucose 45.0 g/L), having no peptone or meat extract compared to all tested media leading to the low cost of inputs. Several papers have reported that MRS and mMRS media has shown better performance for cell growth and production of *L. reuteri* than other media [29–32]. However, in this study the yeast extract medium (YEM) broth with the composition of (g L^{-1}): glucose (45); yeast extract (20); NaCl (0.01); sodium acetate (0.5); tri-ammonium citrate (0.2); KH_2PO_4 (0.2); $MgSO_4$ .7 H_2O (0.2); $MnSO_4.7H_2O$ (0.05) was found to be the best medium for *L. reuteri* production. M5 broth resulted in 10% and 15% times more cell dry weight than M2 and

M3 media. All readings except those annotated in the text were carried out on a triplicate basis, and the average and standard deviation values were reported.

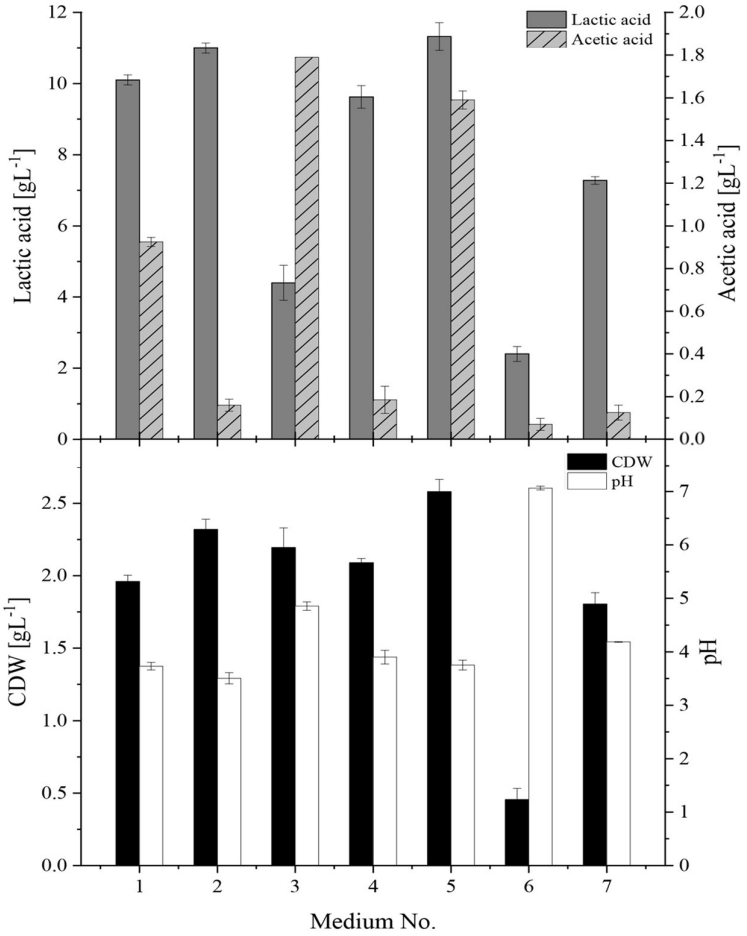

Figure 1 Media screening for cell mass production of *L. reuteri*. Error bar represents the standard deviation of triplicate experiments (n=3).

The effects of different carbon sources on the fermentation process were investigated using eight distinct reconstructed YEM media with varying carbon sources, including glucose, lactose, fructose, sucrose, mannitol, glycerol, tryptone, and maltose. Of the eight carbon sources, the maximum cell mass yield was obtained by sucrose, followed by glucose and maltose, respectively. The influence of different nitrogen sources on the *L. reuteri* cell growth was investigated by reconstructing YEM media with a range of organic and inorganic nitrogen sources, including peptone; trypticase, urea, meat extract, soybean meat, ammonium chloride, ammonium sulfate, ammonium citrate, ammonium nitrate, ammonium acetate, and ammonium phosphate. The highest cell growth was achieved in the broth medium supplemented with yeast extract. Yeast extract provides a relatively larger proportion of free amino acids, short peptides, and other growth factors [33,34].

Bacterial cell cultures were controlled by agar plate test in every stage of the experimental assay to ensure viability and purity of the cultivations. Accordingly, the selected medium was used for the rest of the experiment as the basis for statistical optimization design and analysis.

3.2 Plackett–Burman design screening

A fractional factorial design was used to screen different medium components and to identify the most significant factors affecting CDW of *L. reuteri*. The result of PBD is shown in Table 2. The performed experiments (in total 36 runs with triplicates) revealed that the produced cell mass varies considerably in the 12 PBD experiment runs in the range of 1.16±0.01 g L^{-1} to 3.23±0.04 g L^{-1}. It was observed that the highest CDW is achieved using medium containing (g/L): sucrose, 100; yeast extract, 80; sodium acetate, 5; KH_2PO_4, 0.1. Experimental data fitted well to the regression model, and the determination coefficient value suitably explained the test results. The correlation coefficient (R^2) was obtained via the linear regression model showing a value of

0.9742, which is in good fitness of the model. It is pointed out that the linear regression model could not explain less than 3% of the variations. The obtained R^2 coefficient agrees with the acceptable range defined in applied statistics and the literature. A correlation coefficient higher than 0.75 is acceptable in high cell biomass production of probiotics [35].

Table 2 Cell dry weight obtained for Placket–Burman design (PBD) experiments.

No.	Sucrose (g/L)	Yeast extract (g/L)	Sodium acetate (g/L)	KH_2PO_4 (g/L)	Average cell dry weight (g/L)
1	100	8	5	0.1	2.55±0.09
2	100	80	0.2	1.5	2.93±0.05
3	10	80	5	0.1	1.81±0.10
4	100	8	5	1.5	2.55±0.06
5	100	80	0.2	1.5	2.90±0.09
6	100	80	5	0.1	3.23±0.04
7	10	80	5	1.5	1.81±0.02
8	10	8	5	1.5	1.35±0.02
9	10	8	0.2	1.5	1.20±0.07
10	100	8	0.2	0.1	2.05±0.06
11	10	80	0.2	0.1	1.76±0.03
12	10	8	0.2	0.1	1.16±0.01

Plackett-Burman's design-based Pareto chart for the effect of several media components on CDW response is shown in Figure 2. Based on these results, we concluded that the first three investigated medium components of sucrose, yeast extract, and sodium acetate were the most significant factors affecting CDW content. The significant effect of sucrose and yeast extract agrees with the finding of Hamid et al. [27]. Accordingly, the standardized value lower than the limit of 5% significance level shown with a red vertical line had an insignificant effect on enhancing *L. reuteri* cell mass production in the selected medium; hence KH_2PO_4 factor was excluded from the statistical analysis. In the fermentation process, KH_2PO_4 was used as the phosphorous source by preferential phosphorus (PO_4^{3-}) for microorganism growth [36]. During the experimental optimization studies, it was observed that KH_2PO_4 does not significantly affect biomass yield and growth. Although microorganisms need a minimum quantity of phosphate for biological growth, few reports indicated that phosphate could act as a limiting factor in the growth of bacteria [37].

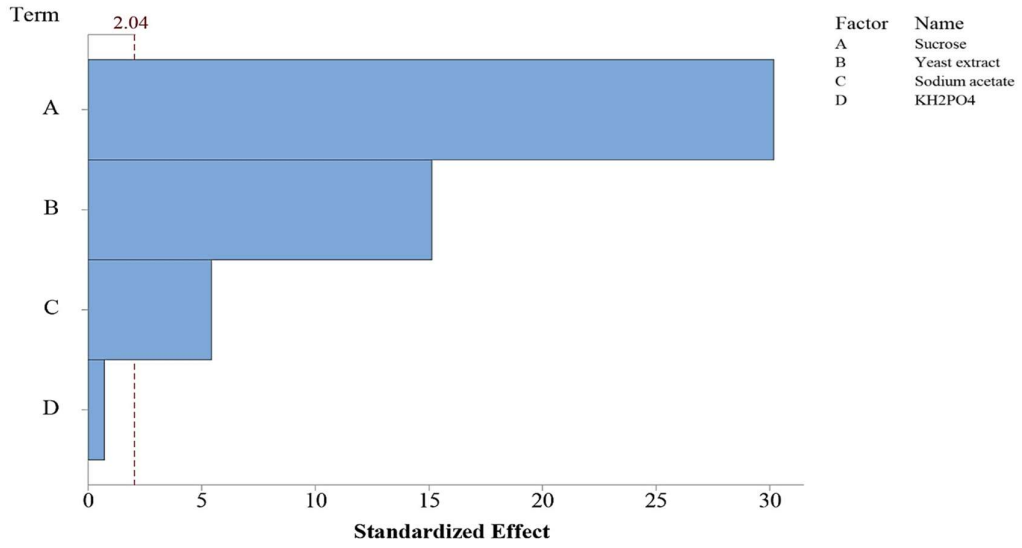

Figure 2 Pareto chart to estimate the influence of four factors on CDW response, α=0.05.

The predicted and adjusted R^2 values for response surface curves of the nutritional components were 0.9652 and 0.9709, respectively, which were in excellent agreement, in validating the fitness of the developed models with the experimental values. ANOVA was used for screening of the variables that have statistically significant effects on cell biomass. From the regression analysis, the significant factors conforming to $p< 0.05$ had a greater impact on high cell mass production. As a concluding remark for the analysis, sucrose, yeast extract, and sodium acetate were statistically significant factors; therefore, they were used for further optimization via BBD. Changes in the pH of the seven different fermentation media used in this study were monitored throughout the PBD

experimental design. It was observed that the pH in the fermentation medium dropped from 6.25 to 4.02 during the process of the *L. reuteri* strain indicating lactic and acetic acid release.

3.3 Box-Behnken optimization

BBD was carried out for further medium optimization of the significant factors screened in the PBD. According to the results of the single-factor test, three levels of sucrose, yeast extract, and sodium acetate factors were designed in BBD to get the response data. 15 sets of experiments (45 runs considering triplicate measurements) were performed. The three −1, 0; and 1 level for the significant factors were as follows (gL^{-1}): sucrose: 10, 55, 100; yeast extract: 8, 44, 80; sodium acetate: 0.2, 2.6 and 5. The highest cell biomass was achieved using the middle levels of sucrose, yeast extract, and sodium acetate as follows (g/L): sucrose, 55; yeast extract, 44; and sodium acetate, 2.6. The results obtained by carrying out BBD were then analyzed by standard ANOVA that provides the quadratic regression equation to predict the cell mass. The full quadratic model application suggests that the quadratic influence of sucrose and yeast extract concentrations ($p<0.0001$) was the most influential factor in the model. The sodium acetate's linear effects also play an important role in this model. Accordingly, the fitted experimental results of the high cell mass production (Y) are shown in the form of a second-order polynomial model equation. Fisher's statistical test for ANOVA was performed on the experimental values, while the test model was valid for above 84% of significance levels. Closer fitness of R^2 to 1.0 means a good correlation of the model [38].

$$Y = 3.043 + 0.8800A + 0.3392\,B + 0.0258\,C - 0.722\,A^2 - 0.230\,B^2 - 0.219\,C^2 - 0.031\,AB + 0.008\,AC - 0.008\,BC$$

Moreover, the model fitness was further checked by evaluating the predicted R^2 and adjusted R^2 coefficients. The values for the predicted and adjusted R^2 were 79.92% and 78.97, and the difference between predicted and adjusted R^2 was found to be less than 1 for the model. Based on the obtained results, the response equation of the provided model was well-suited to the BBD experiment. The 3D response surface plot of the model is presented in Figure 3 (A, B, and C).

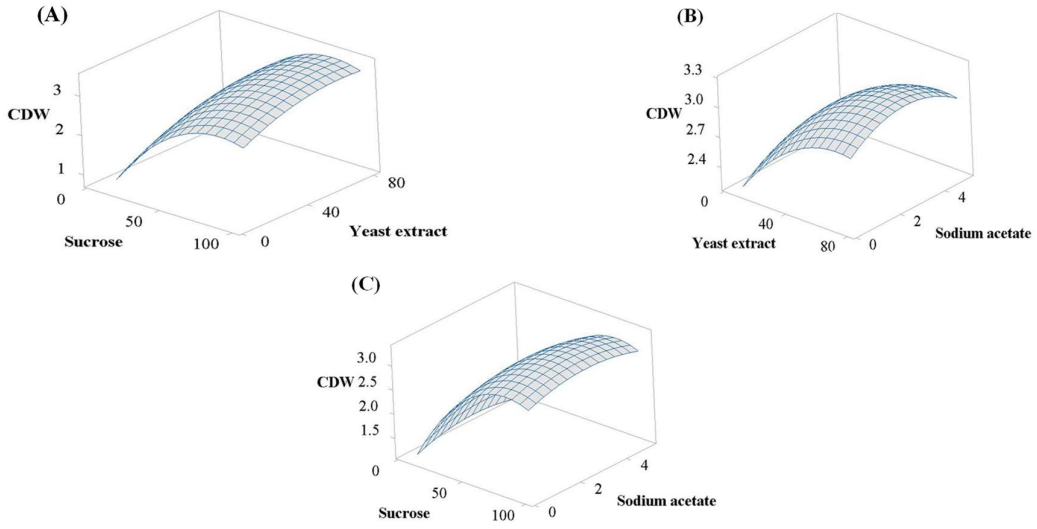

Figure 3 The 3D response surface plots of sucrose, yeast extract, and sodium acetate interactions in the production of *L. reuteri* cell dry weight (A) having variable sucrose and yeast extract with sodium acetate in the hold (B) having yeast extract and sodium acetate with sucrose in the hold (C) having sucrose and sodium acetate with yeast extract in the hold

Figure 3 (A, B, and C) shows the response surface plots of cell mass response with respect to the intercorrelating components. The correlations between different concentrations of sucrose (10 and 100 g L^{-1}) and yeast extract (8 and 80 g L^{-1}) at a constant concentration of sodium acetate (2.6 g L^{-1}) are shown in Figure 3(A). The interaction between the different concentrations of yeast extract (8 and 80 g L^{-1}) and sodium acetate (0.2 and 5 g L^{-1}) at a constant concentration of sucrose (55 g L^{-1}) is shown in Figure 3(B). Finally, the effect of different concentrations of sucrose (10 and 100 g L^{-1}) and sodium acetate (0.2 and 5 g L^{-1}) at a constant concentration of

yeast extract (44 g L^{-1}) is shown in Figure 3(C). Hence, the highest yield of cell mass (3.31± 0.02 g L^{-1}) was achieved at 55.0 and 44 g L^{-1} of sucrose and yeast extract concentrations, respectively.

3.4 Optimized and unoptimized medium compositions

The graph for growth kinetics of *L. reuteri* bacterial cells grown in optimized and unoptimized media is exhibited in Figure 4. It was observed that the pH level decreased while the concentration of lactic acid increased during the fermentation within 48 hours. The pH of the unoptimized and optimized media was reduced from 6.25 to 3.76 and 4.38, respectively, after 12 h from the initiation of the fermentation while maximum cell growth was observed. The highest recorded cell dry weight in the unoptimized medium was 2.59±0.07 g L^{-1} obtained after 48h incubation while it yielded the value of 3.31± 0.02 g L^{-1} by using RMS optimized medium composition. Cell growth and cell mass production are two indicators of cultivation performance. A comparison is carried out between the cultivation performance of the optimized and unoptimized medium composition. However significant increase was achieved in the rate and production yields of the optimized medium.

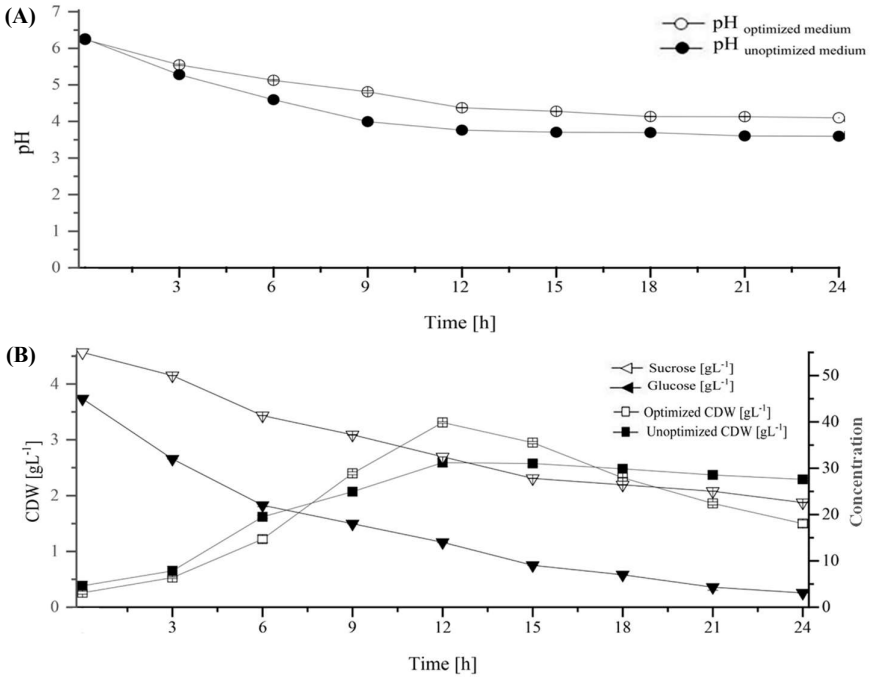

Figure 4 (A) Cell dry weight, pH variation, and (B) glucose and sucrose consumption of the *L. reuteri*. Closed and open symbols show the results in unoptimized and optimized media, respectively.

The increment in cell mass production in optimized medium cultivation was equal to about 28 % compared to the unoptimized medium. The exponential phase cells growth was from hours 3 to 12 in both media with glucose and sucrose concentration depletion. Specific growth rate, μ for the optimized and unoptimized medium, was observed 0.16 $h^{-1}$and 0.14 h^{-1}. Moreover, the cell yield $Y_{x/s}$ using sucrose and glucose in the optimized and unoptimized medium was 0.16 gg^{-1} and 0.11 gg^{-1}, respectively. The growth kinetic parameters of *L. reuteri* for the optimized and un-optimized medium are summarized in Table 3. In general, the obtained results are in agreement with those reported earlier [12,39]. The literature on LAB bacteria shows that the maximum biomass cell production is usually achieved at the exponential phase, which agrees with our results [40]. Statistical optimization has shown great potential in efficient and cost-effective cell mass exploitation [11].

Table 3 Growth kinetic parameters of *L. reuteri* for optimized and un-optimized medium.

Parameters*	Medium	
	Unoptimized	Optimized
X_{max} (g L^{-1})	2.59	3.31
D\dx/dt (g L^{-1} h^{-1})	0.22	0.28
μ (h^{-1})	0.14	0.16
Qs (g L^{-1} h^{-1})	2.00	1.95
$Y_{X/S}$ (g g $^{-1}$)	0.11	0.16

*X_{max}: maximal biomass production; dx/dt: growth rate; μ: specific growth rate; Qs: carbon source consumption rate; $Y_{X/S}$: Yield coefficient [g cells/g substrate].

4. Conclusion

The present study outlines the medium composition's effect in improving the cell biomass production of *L. reuteri* DSM20016^T using statistical optimization designs. In the first step, seven different growth media were reviewed, and the best potential medium was selected based on applicability on *L. reuteri* to yield high cell mass production. The optimized concentrations of sucrose, yeast extract sodium acetate using Box-Behnken statistical design were 55, 44, and 2.6 g L^{-1}, respectively. It was shown that the *L. reuteri* cell mass yield in the optimized medium was higher than the yield in the unoptimized medium.

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