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Optimizing Medium Components to Enhance High Cell Mass Production of Biotherapeutic Strain *Lactobacillus reuteri* DSM 20016^T by Statistical Method

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Probiotics referred to a group of living microorganisms which highly influence the human health. A number of studies have highlighted on the bio-therapeutic potential of *Lactobacillus reuteri* strains, especially in treating eradication of *Helicobacter pylori*. Therefore, in present investigation, statistical methods were applied to optimize medium composition for high cell mass production of *L. reuteri* strain DSM 20016^T. Most influencing medium components were screened by using Plackett-Burman and optimized using Box-Bhenken experimental design. The concentration of lactose, yeast extract and phosphate in cultivation medium has shown significant effect on the cell mass production. The highest cell mass obtained after 48h incubation was 3.96 ± 0.02 gL^{-1} in RSM-optimized medium compared to 1.76 ± 0.17 gL^{-1} in un-optimized medium.

Keywords: Probiotic cultivation, High cell mass production, Medium optimization

Introduction

Nowadays, probiotics are widely used with increased demand for many human, animal, and plant application to improve human life to reduce the extensive use of chemicals and unsustainable practices.[1–6] Growing cells also increases the acidification of the fermentation medium which could suppress the cell viability.[3,7,8] In recent studies, *Lactobacillus reuteri* exhibited high efficiency against *Helicobacter pylori* load in humans and also help to control gastric inflammation.[9–13] Therefore, the present work focuses on the development of a suitable and low cost production medium for high cell mass production using the standard strain *L. reuteri* in submerged cultivation system.

Materials and Methods

Bacterial Strain and Medium

Lactobacillus reuteri (DSM 20016^T; ATCC 23272) is a type-strain which was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig Germany). The lyophilized culture was re-vitalized as instructed by the strain collector. Suspended cells were inoculated into DSMZ-Medium 11, which is a modified Rogosa's Medium (MRS).[5,7] The cultivation medium was composed of (gL^{-1}) glucose, 20.0; meat extract, 10.0; tryptone, 10.0; yeast extract 5.0; sodium acetate, 5.0; ammonium citrate, 2.0; dihydrogen phosphate, 2.0; magnesium sulphate, 0.5; manganese sulphate, 0.05; Tween®80, 1.0 and 20.0 agar. The initial pH of the medium was adjusted at 6.5, and the basal components of media were autoclaved at 121°C for 20 min.

Inoculum Preparation

The stab cultures were used starter culture to prepare 10% v/v inoculum for all experiments. Inoculums were prepared in 100 mL screw-cap bottle with 100mL broth of MRS medium 11 (DSMZ) to create micro-aerophilic conditions and incubated at 37 ± 1°C. The overnight grown cells were harvested at exponential phase (within 18h). Cultures were spinned down in Hettich® ROTINA 380 R benchtop centrifuge at 8000 rpm for 10 minutes at 4°C. Supernatants were discarded and the cells were washed two times by using sterile distilled water. The

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cells were re-suspended again sterile distilled water and adjusted to absorbance of 1 OD_{600} as an OD_{600} measurement of 1 is equivalent to 10^7 cfu/mL.

Production Medium and Cultivation Conditions

The culture media evaluated in this work were prepared as similar composition to MRS medium 11 (DSMZ) but the meat extract and tryptone were omitted. This modification was aimed to developed low cost cultivation medium with sole carbon and nitrogen sources. The other components were optimized by statistical methods as described below.[3,14] All cultivation medium was prepared in 50 mL Erlenmeyer flask at 30 mL working volume and incubated statistically under micro-aerophilic condition. The pH of all media was adjusted to 6.5 and carbon source was autoclaved separately before adding to the medium directly before inoculation. Inoculated flasks were incubated at 37 ± 1°C statistically for 48h. All experiments were performed in triplicates.

Statistical Screening of Nutritional Parameter by using Plackett-Burman Design

The modified MRS medium (omit meat extract and tryptone) used in this study had seven different components, including carbon and nitrogen sources. Thus, the first optimization step was to identify medium components which have a significant effect on enhancing biomass production of *L. reuteri* strain DSM 20016^T. Factorial designs are the useful experimental design tool to identify most significantly affecting factors (nutrients) and interaction between two or more factors in a relatively fewer number of experiments.[14,15] Commonly, in factorial design which require 2^N experiments, the ^{N} factors have to be investigated. In this first step to identify significant medium components from total number of seven variables, factorial design was designed to 128 experiments, which was a large number of runs.[3,15] Therefore, fractional factorial method was utilized in this study since the higher-order interactions were expected to be negligible. PBD was used to identify critical variables for cell biomass production by screening seven variables in N+1 (12) experiments at 2 levels (low and high concentrations) of each one. The variables chosen for the study were lactose (10 and 50 gL^{-1}); yeast extract (10 and 50 gL^{-1}); K_2HPO_4 (0 and 5 gL^{-1}); sodium acetate (2 and 5 gL^{-1}); ammonium citrate (1 and 3 gL^{-1}); $MgSO_4$ (0.1 and 0.5 gL^{-1}) and $MnSO_4$ (0.02 and 0.05 gL^{-1}). The PB experimental design was based on the first order model as given in the following equation:

$$Y(cell\ dry\ weight\ in\ gL^{-1}) = \beta_0 + \sum \beta_i X_i$$

Where, β_0 is the model intercept, β_i is variable estimates and X_i are independent variables.[15] All experiments were set in triplicate runs and the average of cell biomass produced were taken as responses. Based on a regression analysis of the variables, a confidence level of 95% ($p \leq 0.05$) for each factor was considered to have a significant effect on the high cell biomass production of *L. reuteri* strain DSM 20016^T.

Optimization of Nutritional Parameters by Response Surface Methodology via Box-Behnken Design

The results of PB experimental design were used for further optimization in BBD to estimate factors and levels. Lactose, yeast extract and dihydrogen phosphate were the three influence variables and their concentrations with three level's response surface analysis were formulated in BBD.[14] A total of 45 runs including triplicate sets were performed to obtain the response data. To identify the significance of the main effects and interactions, analysis of variance (ANOVA) was performed for each parameter. A p value ≤ 0.05 was considered statistically significant. A second order polynomial equation was used for the analysis of b-carotene production, and the data were fitted in the equation by multiple regression procedure. This resulted in an empirical model. Contour plots were generated to understand the interaction of various factors and then used to determine the optimum concentration of the medium majorly affecting the response.

Quantitative Analysis of the Experiments

Cell growth was determined by absorbance and cell biomass produced was obtained from a pre-determined calibration curve of absorbance versus cell dry weight. The optical density (OD) of the sample collected at appropriate intervals were determined at 600 nm by using spectrophotometer (SPECTRONIC® 200E, Thermo Fisher Scientific). The cell dry weight (gL^{-1}) were obtained through a liner correlation standard curve where 1 OD_{600} is equivalent to 0.3 gL^{-1}. All experimental designs including PBD and BBD as well as their analysis of variances were performed in Minitab®16 software.

Results and Discussion

Statistical Screening of Nutritional Factors using Plackett–Burman Design

Statistical optimization has been used as very useful tool for improvement of bioprocesses both upstream and downstream.[16–19] The PB experimental design results (Table 1) indicated that there was a variation of cell mass production in the triplicates of 12 experiment runs in the range from 0.30 to 2.88 gL^{-1}. Among the 7 variables tested, lactose, yeast extract, and K_2HPO_4 had shown positive signs of the effect on the cell mass production in modified MRS medium. All other components showed negative sign of the effect. The fitting of the experimental data to the regression model was checked and suitably explained by the value of determination coefficient. The coefficient of correlation, R^2 was found to be 0.8423 showing good fitness of the model. Though this value would be considered low in applied statistics, it can be accepted and be attributed to the high cell mass production of probiotics.[7,20] In their work, Le Man *et al.*[21] have emphasized any model with R^2 more than 0.75 are acceptable. In this study, about 15.77% of the total variations could not be explained by this model. The predicted R^2 was 0.7393 which is in reasonable agreement as the adjusted R^2 was 0.8028. The adequacy of the model was calculated and the variables exhibiting statistically significant effects were screened using ANOVA. Factors with $p < 0.05$ were considered to have significant effects on the production of high cell mass. Lactose, yeast extract, and phosphate were considered as the most significant factors and were therefore selected for further optimization studies using BBD. During cultivation of *L. reuteri* strain DSM 20016^T in 12 different media of PB experimental design, the pH of the fermentation culture drops from 6.5 to 3.9. This indicates the production of organic acids including lactic acids. *Lactobacillus* strains are well-known lactic acid producing microbial group.[3,5,7,22]

Optimizing the Significant Variables by using Response Surface Methodology

The effect of three independent variables; namely lactose, yeast extract and dihydrogen phosphate on the significant high cell mass production was studied. The results obtained from BBD were then analysed by standard analysis of variance (ANOVA) and the quadratic regression equation was applied to for prediction of high cell mass of *L. reuteri* strain DSM 20016^T. Based on the full quadratic model application, the quadratic effect for lactose concentration (p value < 0.0001) should be most significant on the model. The linear effects of yeast extract and K_2HPO_4 were still important factors in this model (p value < 0.05). The fitted second-order polynomial model equation for high cell mass production (Y) could be written as:

$$Y = 0.116200A + 0.144728C - 0.001029A^2 - 0.012799C^2 + 0.000554AB - 0.002084AC$$

On the basis of the experimental values, the statistical testing was carried out using Fisher's test for ANOVA (Table 2). The test model was statistically significant at the 99% level of significance.

Table 1 — Placket–Burman design variables with high cell mass production as response

Run	Variables (gL^{-1})							Average Cell dry weight (gL^{-1})
	Lactose	Yeast extract	K_2HPO_4	sodium acetate	ammonium citrate	$MgSO_4$	$MnSO_4$	
1	10	50	0	2	1	0.5	0.05	1.32 ± 0.08
2	50	50	6	2	3	0.5	0.02	2.48 ± 0.05
3	50	10	6	2	1	0.1	0.05	0.86 ± 0.02
4	50	50	0	5	3	0.1	0.05	1.35 ± 0.04
5	50	50	6	2	3	0.5	0.02	2.88 ± 0.11
6	10	10	0	2	1	0.1	0.02	2.81 ± 0.29
7	10	50	0	2	1	0.5	0.05	0.72 ± 0.13
8	50	10	6	5	1	0.5	0.02	0.30 ± 0.07
9	10	10	0	5	3	0.5	0.02	0.38 ± 0.00
10	50	50	0	5	1	0.1	0.02	1.18 ± 0.01
11	10	50	6	2	3	0.1	0.02	1.97 ± 0.08
12	10	10	0	2	1	0.1	0.02	1.25 ± 0.09

Table 2 — Analysis of variance (ANOVA) for cell mass production of *L. reuteri* strain DSM20016^T using Box-Behnken design

Sources	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	52.6241	52.6241	5.8471	110.15	0.000
Linear	3	32.3818	17.6927	5.8976	111.10	0.000
Square	3	17.6317	17.6317	5.8772	110.72	0.000
Interaction	3	2.6106	2.6106	0.8702	16.39	0.000
Residual Error	35	1.8579	1.8579	0.0531	—	—
Lack-of-fit	3	0.7324	0.7324	0.2441	6.94	0.001
Pure Error	32	1.1255	1.1255	0.0352	—	—
Total	44	54.4820	—	—	—	—

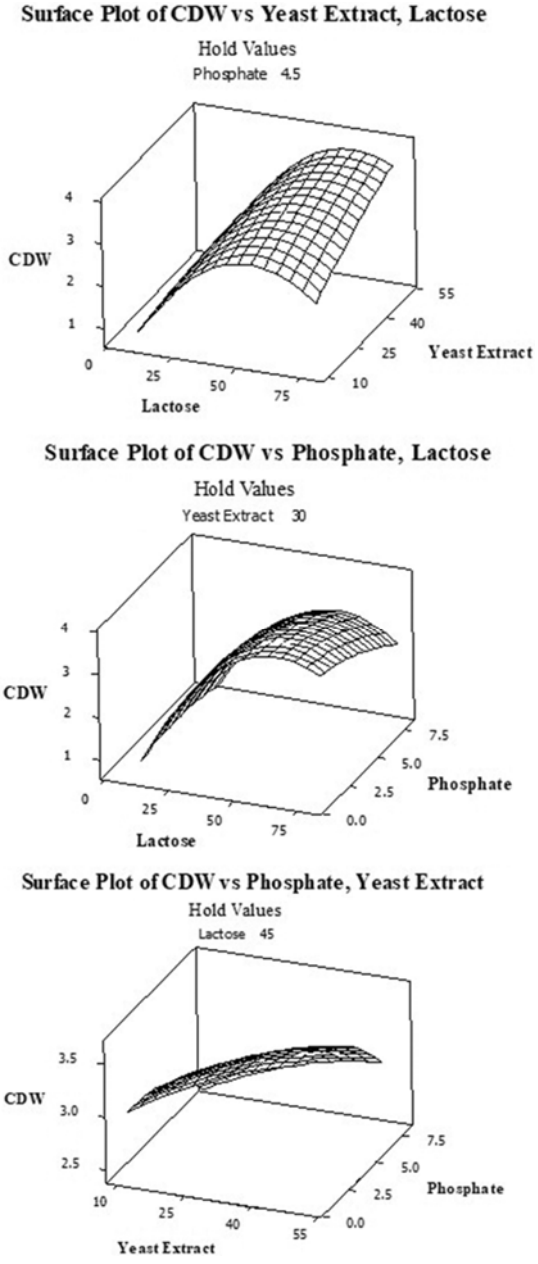

Fig. 1 — The three dimensional response surface plots of cell dry weight production of *L. reuteri* strain DSM20016^T showing the interaction between lactose, yeast extract and phosphate

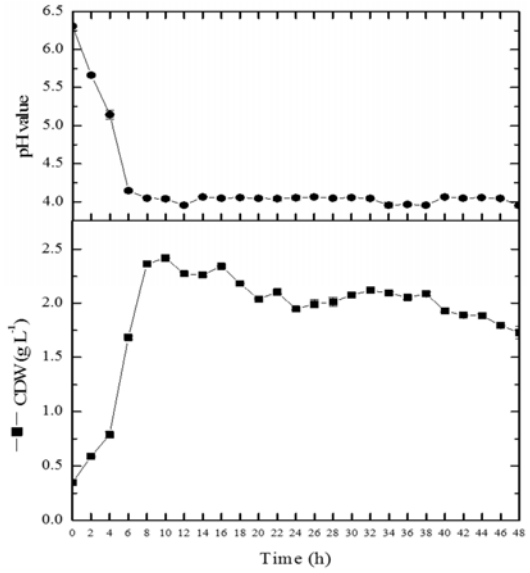

Fig. 2 — The growth kinetics and change of pH in static cultures for cell dry weight production of *L. reuteri* strain DSM20016^T using un-optimized medium

The quality of fit of the quadratic regression model equation was expressed by R^2, which equalled to 0.9959, indicating only about 0.41% of the variability in the response could not be explained by the model. The value of adjusted R^2 (0.9571) was also very high to advocate for a high significance of the model. These results indicated that the response equation provided a suitable model for the BBD experiment. The three-dimensional graph for the response surface model was shown in Fig. 1.

Growth Kinetic Study

The growth kinetics of *L. reuteri* strain DSM20016^T growing at static cultures using un-optimized medium is shown in Fig. 2. The pH of fermentation medium was lowered from 6.5 to 3.9 within 8h – 10h of incubations, where the maximum cell growth was observed. The cell mass obtained after 48h incubation was 1.76 ± 0.17 gL^{-1}. In comparison, the RSM optimized medium composition

Table 3 — Comparison between medium composition and cell dry weight produced before and after optimization studies

Nutrients		Medium (Components in gL^{-1})	
		Un-optimized medium	RSM Optimized medium
Lactose		20	68.6869
Yeast extract		5	50
K_2HPO_4		1	1
Sodium acetate		2	2
Ammonium citrate		2	2
$MgSO_4$		0.1	0.1
$MnSO_4$		0.05	0.05
Biomass production			
Maximum cell dry weight obtained (gL^{-1})	Actual	1.76 ± 0.17	3.96 ± 0.02
	Predicted	—	4.2473

has yield a maximum cell mass of 3.96 ± 0.02 gL^{-1} than un-optimized medium (Table 3). Hence, the optimized medium gave significant increase of cell mass and faster cell growth with approximately 3 folds of increment compared to the original medium.

Conclusions

Current study highlights the optimization of cultivation medium to produce high cell mass of bio-therapeutic potential *L. reuteri* strain DSM20016^T using statistical methods. Statistically optimized medium was successfully yields significant high cells mass compared to un-optimized medium. The optimized medium composed of (gL^{-1}): lactose, 68.687; yeast extract, 50.0; KH_2PO_4, 1.0; sodium acetate, 2.0; Triammonium citrate, 2.0; $MgSO_4.7H_2O$, 0.1; and $MnSO_4.5H_2O$, 0.05. The Box-Behnken experimental design provided a satisfactory description on developing optimum conditions for high cell mass production of probiotic strain.[3,7]

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