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Statistical Optimization Approaches for High Cell Biomass Production of *Lactobacillus casei*

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Probiotic bacteria are known to treat and prevent diseases and hence promote physical and mental wellness due to their significant brain-gut relationship. The main challenge involved in probiotic commercialization is the bio processing limitation to produce high cell mass, especially with the cultivation of lactic acid bacteria which produces lactic acid as a by product. Synthesis of lactic acid by lactic acid bacteria inhibits bacterial growth, and in turn disrupts high cell mass production. Current work presents the findings for *Lactobacillus casei* medium optimization by response surface methodology in shake flask level. A simple medium using 4 components: lactose, soybean meal, yeast extract and magnesium sulphate has been identified to produce high cell mass than generic mediaused for probiotic cultivation, such as the MRS medium. Secondly, response surface methodology using Box-Behken Design was employed as an optimization strategy. After optimization process, the production of *Lactobacillus casei* biomass increased by about 164.6% recording 6.51g.L^{-1} compared to cell biomass obtained using initial un-optimized medium (2.46g.L^{-1}).

Keywords: *Lactobacillus casei*, High cell biomass, Statistical optimization, Medium optimization, Box-Behnken design.

Introduction

The growth of probiotic industries increases annually, with Asian and European regions as the main consumers, with a forecast of 48 billion dollars in the next five years[1-3]. *Lactobacillus* belongs to lactic acid bacteria, which can produce lactic acid as a main end-product of carbohydrates fermentation[4,5]. This genus includes a large number of GRAS species (Generally Recognized As Safe) among LAB too[6-8]. *L. casei* is a homo fermentative that can efficiently produce L(+)-lactic acid, making it the desired industrial microorganism for the production of lactic acid[9]. In addition to improving gut health, *L. casei* possesses some antimicrobial, anti-diarrheal, anti-mutagenic and cholesterol lowering activities, and can also enhance the immune system and lower blood glucose level[7].Increasing the biomass through high cell density cultivation reduces fermentation time and wastewater volume and allows faster downstream processing time along with reduced cost and facilitated recovery process[10]. Furthermore, the production yield can be improved[11-13]. Therefore, in this study, high cell mass cultivation of *L. casei* was investigated through the optimization of cultivation medium using Response Surface Methodology (RSM), which helps in predicting product properties throughout the tested region[14].

Materials and Methods

Microorganism

Lactobacillus casei strain number NRRL 1917, obtained from Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA) was used throughout this study. Cells obtained from the master cell bank were used to produce a working cell bank through preservation of cells in glycerol culture and stored at -80°C.

Cell propagation

Inoculum is prepared by inoculating 250mL Erlenmeyer flask containing 50mL of MRS liquid

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medium (Difco Laboratories, MI, USA) with 1 cryogenic vial of frozen glycerol stock of working cell culture. The flasks inoculated with *L. casei* were incubated at 37ºC for 24h at 200rpm. Then, 1mL of the inoculum was used to inoculate 250mL flasks, which were incubated for 24 h in a rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200rpm, 37ºC for biomass production.

Production medium and cultivation conditions

5 mL of inoculum was used to inoculate 250mL Erlenmeyer flasks containing 50mL of different cultivation media (Table 1). 6 types of media from previous literature were selected for the screening experiment to choose the most optimal medium allowing highest cell mass production. The initial pH of each medium was adjusted to pH 6.5 for all experiments. The media were then incubated on rotary shaker at 200 rpm and 37ºC for 48 h to obtain biomass production.For media containing soybean meal, the meal was grinded and sieved to pass through a fine mesh strainer of 0.6mm aperture size.

Table 1 — General composition of 6 different media for *L. casei* cultivation

Component	Type of medium (concentration, $g.L^{-1}$)					
	1	2	3	4	5	6
Glucose	44	30	-	-	50	23.9
Molasses	-	-	40	10	-	-
Corn steep powder	-	-	-	-	-	17.5
Meat extract	-	-	-	10	-	-
Yeast extract	60	6	5	5	20	12.7
Tryptone	60	-	30	-	40	-
Peptone	-	6	-	10	-	-
$MgSO_4$	-	-	0.2	-	-	-
Tween 80	1.1	1	-	1	-	-
K_2HPO_4	2.7	-	-	2	0.2	-
KH_2PO_4	-	1.5	-	-	0.2	-
$MgSO_4.7H_2O$	0.2	0.4	-	0.1	0.3	-
$MnSO_4.H_20$	0.05	-	-	0.05	-	-
$MnSO_4.4H_20$	-	-	-	0.05	-	-
Sodium acetate	-	1	-	5	-	-
Citric acid	-	0.5	-	-	-	-
Ammonium citrate dibasic	-	1	-	-	2	-
Diammonium hydrogen citrate	-	-	-	2	2	-
Diammonium sulphate	-	-	-	-	0.5	-

Statistical medium optimization

RSM has been used in various fields such as engineering and science to produce appreciable results[15]. The effect of different carbon and nitrogen sources as well as basal salts and ions on the growth of *L. casei* were studied by using a full factorial design, which is the Plackett-Burman design. Plackett-Burman design aids to identify important factors to be selected in the initial phase of the experiment. RSM experiment will be done using significant factors screened from Plackett-Burman experiments to identify the optimum concentration of each factor for high cell mass production. Box-Behnken design will be applied using the MINITAB 16 software to optimize the concentration of each factor. After obtaining the cell biomass from experiments, an analysis of variance (ANOVA) test was done to validate the obtained results.

Determination of cell dry weight and pH

10 mL samples were taken at different time intervals, for OD measurements using spectrophotometer (Model DR/2500, Hach Company, Loveland, CO, USA) at 600 nm after series of dilutions. The cultivated broth was diluted to give values less than (1 OD_{600}) for better accuracy. The OD of the culture was converted to dry cell mass through a linear correlation standard curve. 1 OD_{600} was approximately equivalent to 0.3 $g.L^{-1}$.The pH of medium was measured using a benchtop pH meter (Mettler Toledo, USA).

Results and Discussion

Effect of medium composition on cell growth and pH

The first part of the work was designed to obtain the most optimal medium composition between different cultivation media reported earlier. As seen in Figure 1, among the tested 6 different media, medium No. 3, which contained ($g.L^{-1}$) sugar cane molasses, 40.0; peptone, 30.0; yeast extract, 5; and $MgSO_4$, 0.2, produced the highest cell biomass of about $2.7g.L^{-1}$, and recorded a final pH of 3.7. Compared to the other media, medium No. 3 and 4 consisted only of 4 components, suggesting that it is not necessary to include complex components such as mineral salts and vitamins to produce high cell biomass of *L. casei*. Furthermore, Shahravy *et al.*[16] reported that K_2HPO_4, Tween 80, $MnSO_4$ and sodium acetate were non-significant factors contributing to *L. casei* biomass production, while carbon source, nitrogen source, yeast extract and $MgSO_4$ were identified as the

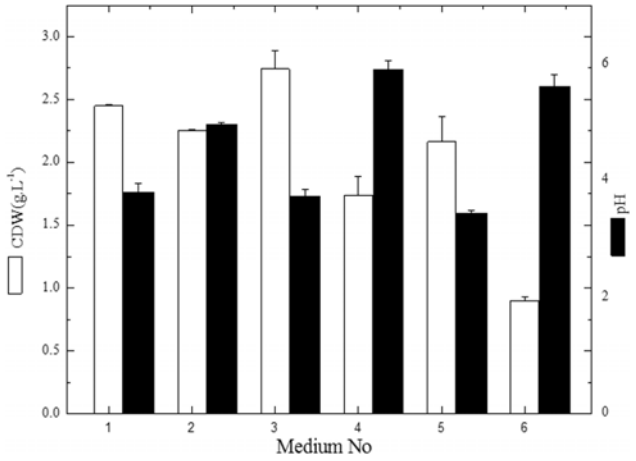

Fig. 1 — Cell dry weight of *L. casei* and final pH obtained using different cultivation media

significant factors. Furthermore, Mondragón-Parada *et al.*[17] has used a simple whey-based media supplemented with only ammonium salt and low level of yeast extract. Moreover, Aguirre-Ezkauriatza *et al.*[7] has solely used un-supplemented ultra-filtration de-proteinized whey to produce *L. casei* biomass. Medium No. 3 was further modified by substituting its carbon and nitrogen sources with the sources which produces higher biomass via preliminary study. Then, a new medium which contained lactose, soybean meal, yeast extract and $MgSO_4$ was finalized and used through statistical optimization steps. Cells of *L. casei* preferably utilize lactose and glucose sugars for their carbon and energy requirement. Strong galactosidase and glycosidases help cells to hydrolyse glucose polysugars (sucrose, raffinose, and stachyose) during fermentation[18]. Oligosaccharides such as raffinose and stachyose are regarded as prebiotics, which promotes the growth of probiotics such as *L. casei.* Furthermore, soybean meal contains significant amount of a *L. casei* growth promoting-factor known as streptogine, which were significantly increased cell mass upon replacing the nitrogen source of medium No.3 with soybean meal.

Full factorial design of medium composition

The statistical medium optimization approach was carried out with full factorial Plackett Burman design for obtaining the significant components using the MINITAB 16 software. Pareto chart results obtained from Plackett Burman design (data not shown) revealed that all bars representing the four medium components has crossed the reference line at 2.09. The length of each bar in the pare to chart corresponded to the value of the predicted effect or regression coefficient[19]. This means that all the evaluated factors are significant and therefore, were selected for further optimization process. In order to optimize the concentration of each medium component, Box-Behnken design was used. Since all four medium components selected were significant variables, therefore each component was investigated at three different levels coded as +1.0, 0.0 and -1.0during RSM design. Experimental results for the interaction between each variable are shown in the form of surface plots (Figure2). The interaction effect between soybean meal and lactose, $MgSO_4$ and lactose, and yeast extract with soybean meal (Figure 2) tend to produce converging graphs, whilst the other interactions produced non-converging graphs. The optimized medium compositions were ($g.L^{-1}$): lactose, 76.57; soybean meal, 72.63; yeast extract, 2; $MgSO_4$,0.7. These results were then analyzed using multiple regression method. The estimated regression coefficients for *L. casei* cell mass production using Box-Behnken design are shown in Table 2. The quality of fit was evaluated by determining the regression coefficient (R^2) value. The R^2 recorded is 0.9847 for cell dry weight, which means that the model fits well for the sets of observation and there is only 1.53% discrepancy predicted in the model. The adjusted and predicted coefficients of the model were in good agreement with each other that were 0.979 and 0.9697, respectively. This indicates a high significance of the model.

The second order polynomial model obtained can be represented as:

Cell mass = $-1.3028+0.0352*A+0.2509*B-0.2809*C+1.0169*D-0.0003*A^2$ $-0.0018*B^2 +0.0309*C^2 -1.7502*D^2 + 0.0001*AB-0.0004*AC$

$+0.0115*AD - 0.0025*BC+0.0062*BD+0.1009*CD.$

The results of ANOVA analysis showed that the quadratic model is significant with a P-value of less than 0.05 and an F-value of 179.49. The good fitting of the model was evaluated. Parameters with P-values of less than 0.05 indicate that these model terms significantly affect design response. Lactose, soybean meal and yeast extract recorded a significant P-value, while $MgSO_4$ recorded a P-value of 0.254. Furthermore, from the F-value in ANOVA table, it can be seen that soybean meal is the most dominating factor among all factors

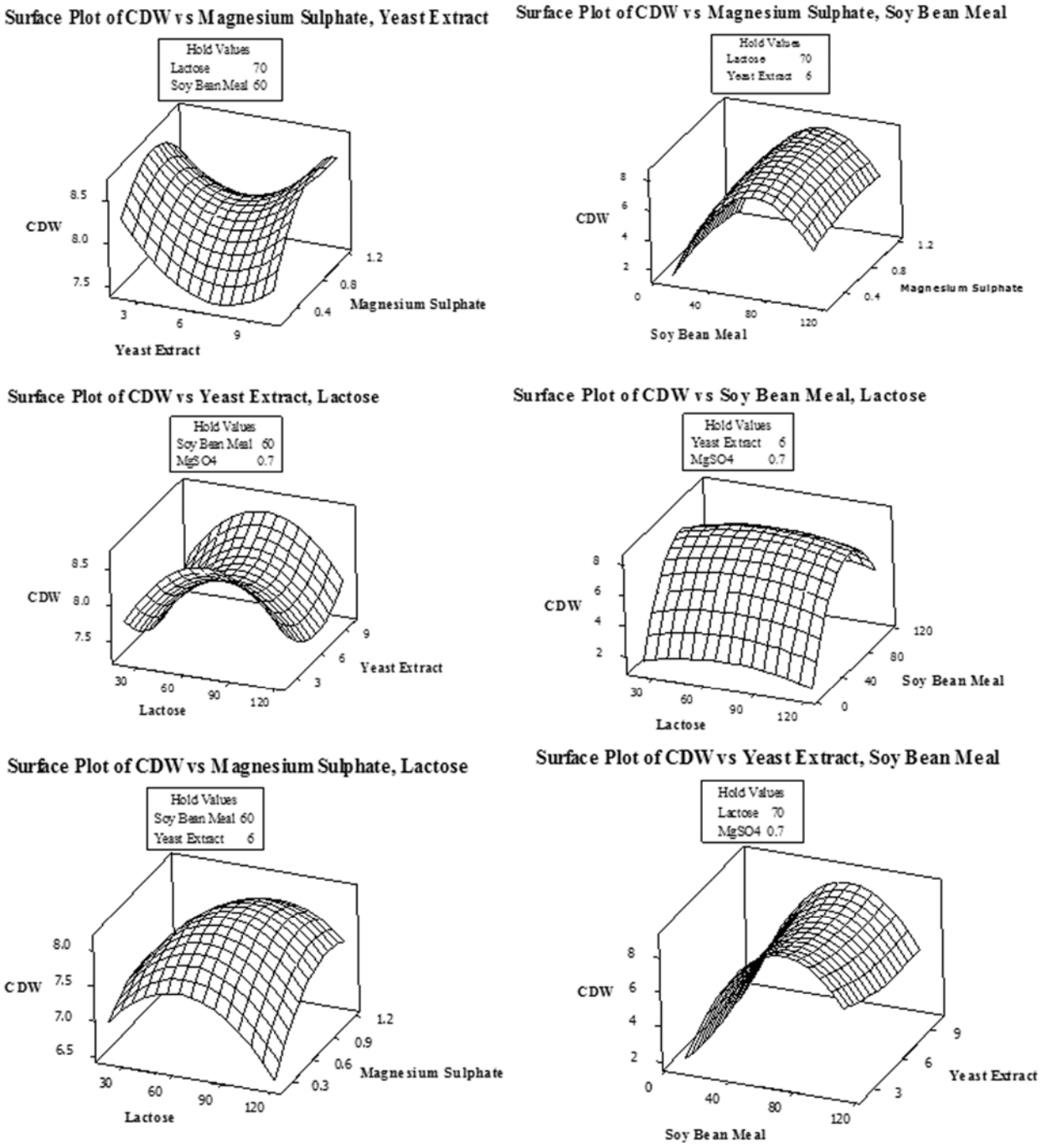

Fig. 2 — Response Surface 3-D plots of cell dry weight production of *L. casei* showing the interactions among lactose, soybean meal, yeast extract and $MgSO_4$.

contributing to higher cell mass production with a highest F-value of 893.91.

Comparison of cell growth kinetics under optimized and un-optimized medium compositions

Both un-optimized and optimized media were compared in 48h shake flask cultivations to evaluate cell growth and pH reduction throughout the cultivation. Samples were taken every 3 hours to determine cell biomass and pH level. Figure 3 represents growth kinetics obtained for both media. It can be seen that cell biomass production in the optimized medium improved significantly compared to the un-optimized medium. The maximum cell mass obtained for the RSM optimized medium recorded

Table 2 — Regression co-efficient for cell mass production using Box-Behnken design

Term	Coefficient	Standard Error	T	P
Constant	-1.30280	0.778166	-1.674	0.102
Lactose, A	0.03521	0.008794	4.004	0.00
Soybean Meal, B	0.25088	0.008400	29.868	0.00
Yeast Extract, C	-0.28094	0.112579	-2.495	0.017
$MgSO_4$,D	1.01693	0.879392	1.156	0.255
A^2	-0.00031	-0.00031	-6.925	0.00
B^2	-0.00175	0.000044	-39.591	0.00
C^2	0.03093	0.006910	4.477	0.00
D^2	-1.75021	0.442222	-3.958	0.00
AB	0.00005	0.000051	1.072	0.290
AC	-0.00040	0.000638	-0.623	0.537
AD	0.01152	0.005106	2.256	0.030
BC	-0.00250	0.000638	-3.912	0.000
BD	0.00622	0.005106	1.219	0.230
CD	0.10094	0.063829	1.581	0.122

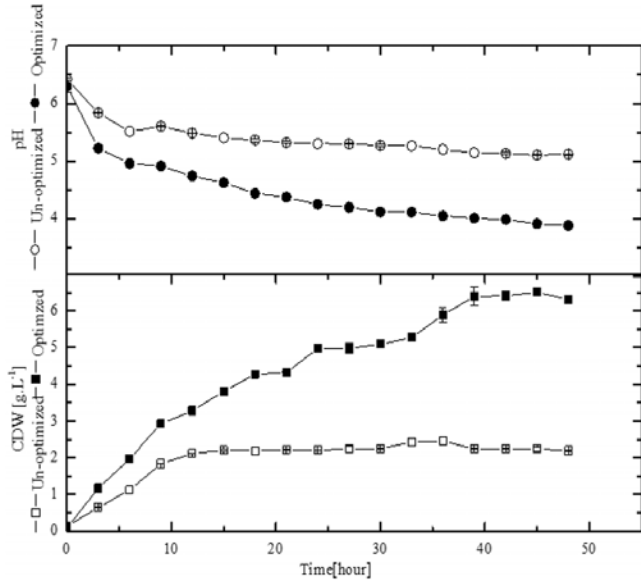

Fig. 3 — Cell dry weight and pH changes during shake flask cultures of *L. casei* using optimized and un-optimized media.

6.51 $g.L^{-1}$, which was higher than that obtained in the un-optimized medium by about 3-folds (2.46 $g.L^{-1}$). The specific growth rate (μ) for RSM optimized media was also calculated (0.146 h^{-1}), which was almost 2.7-folds higher that obtained for un-optimized medium (0.055h^{-1}). For the pH changes during cultivation, both un-optimized and optimized media showed a decreasing profile in pH over the 48 hours. Both media demonstrated an inverse correlation between cell biomass and pH, where the higher the cell biomass, the lower the pH. Moreover, optimized medium clearly showed a sharp pH decline as there was a remarkable pH change from the initial 6.5 to 3.87 after 48 hours. The changes in pH showed that growing *L. casei* cells were able to ferment medium components, and organic acids, mostly lactic acid, were produced as fermentation by-products. Table 3 summarizes different growth kinetic parameters of *L. casei* cultivated in un-optimized and optimized medium.

Table 3 — Growth kinetic of *L. casei* cultivated in un-optimized and optimized medium

Parameters	Un-optimized medium	Optimized medium
Cell mass, X ($g.L^{-1}$)	2.46	6.51
Maximum cell mass per hour, $X_{max}h^{-1}$ ($g.L^{-1}.h^{-1}$)	0.218	0.39
Specific growth rate, μ (h^{-1})	0.055	0.1460

Conclusion

Production of high cell mass of probiotic bacterium *L. casei* was achieved by statistical optimization using RSM method and Box-Behnken design with four factor-level. The medium optimized by the RSM method has improved cell biomass production of *L. casei* by about 164.6%, and it consisted of ($g.L^{-1}$): lactose, 76; soybean meal, 72; yeast extract, 2; $MgSO_4$, 0.7. Results of this study provides a new efficient medium for high cell mass cultivation of *L. casei* that potentially can be used in the industry as a cost effective and efficient cultivation medium for *L. casei* cell biomass production.

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