Statistical Optimization of Medium Components by Response Surface Methodology for Enhanced Production of Bacterial Cellulose by *Gluconacetobacter persimmonis*

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**Abstract**

Bacterial cellulose has been found to be attractive for novel applications due to its material properties. An optimization of the medium used for the production of bacterial cellulose using *Gluconacetobacter persimmonis* was carried out. Plackett-Burman (PB) Design for screening of the medium constituents and a Central Composite Design (CCD) for optimization of significant factors were employed. Glucose, yeast extract and peptone were estimated as significant factors from PB design. Bacterial cellulose concentration of 1.72% (w/v) was obtained in the medium optimized using CCD method as compared to un-optimized medium that yielded 0.318% (w/v). Hence a 6 fold increase was observed after the optimization of medium was carried out. The combined effects of medium components and their optimum regions are reported in this paper.

**Keywords:** *Gluconacetobacter persimmonis*; Bacterial Cellulose; Plackett Burman Design; Central Composite Design

**Introduction**

Cellulose is the most abundant earth biopolymer, recognized as the major component of plant biomass and a representative of microbial extracellular polymers. Efficient producers of cellulose are members of acetic acid bacterium *Gluconacetobacter* (earlier known as genus *Acetobacter*). Bacterial cellulose from *Acetobacter* strains displays unique physical, chemical and mechanical properties including high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility [1]. Although bacterial cellulose finds applications in several fields, productivity of cellulose production needs to be addressed so as to make it economically compatible. Hence it becomes necessary to optimize the yields of cellulose production by the use of process improvement strategies. Various workers have optimized medium constituents and process parameters for increased bacterial cellulose (BC) yield [2-6].

Optimization of processing parameters plays an important role in the development of any process owing to their impact on the economy and efficacy of the process. Designing an appropriate production medium and conditions is of crucial importance to improve the efficiency and productivity of bioactive microbial metabolites fermentation process, because it can significantly affect product concentration, yield, and the ease and cost of downstream product separation. Statistically based experimental designs have proved to be more efficient than one-at-a-time method, which is complicated and time-consuming, especially on multi-variables screening, and do not consider the complex interactions among different variables. On the other hand, statistical experimental designs provide a systematic and efficient plan for experimentation to achieve certain goals so that many factors can be simultaneously studied. Therefore, in recent years number of statistical designs were used to search the key factors rapidly from a multivariable system, such as Plackett–Burman design and response surface methodology [2,3,5,7-9] because statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components and their interactions.

Plackett-Burman design allows the evaluation of (n-1) variables by n experiments; n must be multiple of 4 ex: 4,8,12 etc. Any factor not assigned to a variable can be designated as dummy variable. The incorporation of dummy variables into experiments makes it possible to estimate the variances of an effect (experimental error). The effect of dummy variables is calculated in the same way as the experimental variables. If there is no interaction and no error in measuring the response, the effect shown by dummy variable should be zero. If the effect is not equal to zero, it is assumed to be a measure of analytical error in measuring the response. This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum value to use.

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses [5]. It also has important applications in design, development and formulation of new products as well as in improvement of existing product designs. Statistical experimental design minimizes the error in determining the effect of parameters and it shows the simultaneous, systematic, and efficient variation of all parameters. Response Surface Methodology (RSM) is an effective tool for optimizing the process condition that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations [2]. It usually involves an experimental design such as Central Composite Design (CCD) to fit a second-order polynomial by a least squares technique. An equation is used to describe the test variables, and describe the combined effect of all the test variables in the response.

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**Received** December 04, 2013; **Accepted** December 23, 2013; **Published** December 30, 2013


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The objectives of the present investigation include optimization of BC production from \textit{G. persimmonis} GH-2 by RSM and to study the interrelationship among the media ingredients on BC yield using response surface plots. The medium ingredients were screened by Plackett-Burman (PB) Design and optimization of significant factors was done applying a Central Composite Design (CCD). We observed a 6 fold increase in cellulose yield as an optimum response of the study.

**Materials and methods**

**Microorganism**

Cellulose producing \textit{Gluconacetobacter persimmonis} reported earlier was used in this study [10]. The culture was maintained on HS agar slants, transferred and stored at a temperature of 2oC -8oC in the refrigerator.

**Culture media and growth conditions**

Standard Hestrin-Schramm (HS) medium containing 2.0% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.15% citrate and 0.3% disodium phosphate (pH 5.5-6.0) was used in the study [11]. A volume (100 ml) of medium in 250 ml conical flask was inoculated with bacteria and incubated at room temperature for 14 days under stationary conditions to observe the cellulose pellets that would form at air-liquid interface.

**Quantification of bacterial cellulose**

Quantification of cellulose was carried-out as reported in our earlier study [10]. The cellulose pellets obtained after 14 days of incubation were subjected to filtration to remove excess media and the pellets were retained in flasks. A solution of 2% NaOH was added to the pellets and boiled for 15 min. The mixture was then filtered and dried in hot air oven at 75oC for 6h and dry weight of pellicle was taken to find out the yield.

**Results and Discussion**

**Screening of the independent variables- Plackett-Burman Design**

The concentrations of medium components like glucose, yeast extract, peptone, citric acid and disodium phosphate were varied from high to low concentrations (Table 1) and 16 experimental trials were conducted in two batches their effects on BC production was from high to low concentrations (Table 1) and 16 experimental trials extract, peptone, citric acid and disodium phosphate were varied.

**Optimization of the independent variables- Response surface methodology**

To optimize carbon source (glucose), nitrogen source (yeast extract and peptone), a Central Composite Design (CCD), consisting of a set of 20 experiments with replicates at central point was conducted. A three factor 5 level CCD with 20 experiments was used. Trial for 20 sets of media with different concentrations of media components were inoculated with 2 ml of \textit{Gluconacetobacter persimmonis} and were incubated at room temperature for 14 days under static condition of growth.

The following second-order polynomial equation was adopted to study the effects of variables to the response.

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3
\]

Where $Y$ is the response (Cellulose yield, g/l), $\beta_0$ is the constant term, $\beta_1, \beta_2, \beta_3$, the coefficients of linear terms and $\beta_{11}, \beta_{22}, \beta_{33}$ are the coefficients of quadratic terms and $\beta_{12}, \beta_{13}, \beta_{23}$ are the coefficients of cross product terms, $X_1, X_2$ and $X_3$ represent the factors glucose, yeast extract and peptone respectively.

**Table 1:** Components of HS medium and their high and low concentrations.
Optimization of the independent variables: Response surface methodology

To optimize carbon (glucose) and nitrogen (yeast extract and peptone) sources, a Central Composite Design consisting of a set of 20 experiments with six replicates at central point was conducted. Table 3 shows variables and their levels for central composite design (CCD). The CCD matrix of the independent variables in coded units along with predicted and experimental values of yield is given in Table 4. All the experiments were performed in 250 ml Erlenmeyer flask containing 100 ml of media. The quadratic model response is given in Table 4. The R² is the proportion of variability in response values explained or attributed to the independent variable. In this study, R² = 0.824 suggested that the model is a good fit though not accurate as the lack of fit was not significant. The value of coefficient of determination (R²) was found to be significant (p < 0.05) and the more significant is the corresponding coefficient. This means that quadratic main effects of glucose, yeast extract and peptone are more significant. The response obtained under different combinations of variables and defined experimental design was analyzed using the Analysis of Variance (ANOVA) appropriate to the experimental design Table 4 which indicated that the sum of squares due to regression (first and second-order terms) was found to be significant (p < 0.05) and lack of fit was not significant.

The estimated coefficients for glucose (%), yeast extract (%), and peptone (%) in the model are presented in Table 6. The R² value for the model was 0.824 and the adjusted R² value was 0.783. The model was statistically significant with a low p-value (0.000) indicating that the model is significant. The model has a high adjusted R² value of 0.783, indicating that the model can explain 78.3% of the variability in the response. The lack of fit test also supports the adequacy of the model, as the lack of fit is not significant (p-value > 0.05).

The lack of fit test (p-value > 0.05) is significant at the 0.05 level, indicating that the model is adequate for the purpose of regression. The analysis of variance (ANOVA) showed that all the terms in the model are significant except for the quadratic term for glucose and peptone. The p-values for these terms are greater than 0.05, indicating that they are not statistically significant.

The analysis of variance (ANOVA) results showed that the glucose term has the highest p-value, indicating that it is not significant. The peptone term also has a high p-value, indicating that it is not significant. The yeast extract term has a p-value of 0.014, indicating that it is significant. The quadratic terms for glucose and peptone have high p-values, indicating that they are not significant. The linear terms for glucose and peptone have low p-values, indicating that they are significant. The interaction terms for glucose and yeast extract, glucose and peptone, and yeast extract and peptone have low p-values, indicating that they are significant.
accounted for by the model. The value of $R^2$ is always in between 0.0 and 1.0 $R^2$ value close to 1.0 implies that the model is accurate and predicts better response. However, model with higher $R^2$ value always does not mean that model is accurate. Large $R^2$ value also is resulted by addition of non-significant extra variables in the model. Thus, it may be possible of a model having higher $R^2$ value with poor prediction of response. So the term adjusted $R^2$ has been introduced which arranges the $R^2$ values for the sample size and for the number of variables in the model. Addition of insignificant model term in the model leads to decrease in adjusted $R^2$ value. So, the value of $R^2$ should be as close as that of adjusted $R^2$.

Parity plot (Figure 3) showed the distribution of experimental and model predicted values where data points are localized close to the diagonal line suggesting the model is adequate enough to explain cellulose production. The 3D plot (Figure 4) and their respective 2D contour plots (Figure 5) provide a visual interpretation of the interaction between two factors. In the response surface plot, glucose is held at an intermediate level and levels of peptone and yeast extract are varied from -1.5 to +1.5. It can be seen that the cellulose yield increases with increase in concentration of peptone and with decrease in concentration of yeast extract. The corresponding Contour plots indicate different regions of yields based on different colours. The maximum yield falls in the range 2-2.5 g/100ml as indicated by dark shaded region in the plot. Hence to optimize the levels of peptone and yeast extract, it is necessary to carry out experiments in this region of higher yield. So the levels of peptone has to be varied between 1.4 to 1.618 and yeast extract has to be varied from -1.5 to -0.5 to optimize BC production. The graphs also show that the yield of cellulose increases with increase in concentrations of peptone and glucose. The yield is found to decrease with decrease in concentrations of glucose and peptone. The Contour plot shows different ranges of yields. To get optimum conditions the experiments have to be done in the region of maximum yield and the levels of glucose and peptone have to be varied from 1 to 1.6 and 1.5 to 1.68 respectively.

From Figure 5, it can be observed that with increase in concentration of yeast extract and with decrease in concentration of glucose, the yield of cellulose increases considerably. The contour plot indicates the levels of yeast extract and glucose that have to be used to get optimum conditions as 1 to 1.6 for yeast extract and -1.5 to -0.5 for glucose.

In the previous studies for optimization for BC production, Embusacdo et. al., [2] used five-level, four factor central composite design. They found all four factors affected cellulose yield significantly from G. xylinus. This is in line with present investigation. However, Rani et. al., [5] optimized cultural conditions for BC production from G. hansenii by central composite design. They used coffee cherry husk and corn steep liquor as less expensive sources of carbon and nitrogen sources respectively. Casarica et. al., [6] brought about improvement in BC yield using poor quality horticulture substrates using Taguchi method. In the study carried-out by Bae and Shoda [3], culture conditions in a jar fermenter for BC production were optimized using Box-Behnken design, Response surface methodology was used to predict the levels of various factors.

**Conclusion**

This work has demonstrated the use of Central composite design by determining the conditions which are required to get optimum yield of cellulose production from G. persimmonis. This methodology could therefore be successfully employed to process development where an analysis of effects and interactions of many experimental factors are required. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Response curves are very helpful in visualizing the main effects and interaction of factors. Thus smaller and less time consuming experimental designs could generally suffice for the optimization of many processes. From the above Main effects plot and Pareto chart of standardized effects, it can be seen that...
nitrogen sources like peptone and yeast extract and carbon source glucose have significant effect on BC production when compared to disodium phosphate and citric acid. Hence to optimize BC production it is necessary to optimize the concentrations of yeast extract, glucose and peptone using different concentrations whereas other components of the medium can be kept constant. Hence statistical experimental designs are powerful tools for the rapid search of key factors from a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner.

Acknowledgement

The authors acknowledge Dr. Ashok Shettar, Principal, BVBCET for providing the facilities. The authors also acknowledge Dr. B.B Kotturshettar and Dr. V.N Gaitonde for their technical help during the studies.

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