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REVIEW

Response surface methodology for optimization of microbial cellulase production

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Abstract

Contrary to conventional techniques, statistical tools like response surface methodology (RSM) have gained importance in past ten years for optimization of various process parameters. Use of RSM to enhance the cellulase production from different microorganisms is desirable due to its application in various industrial processes and to decipher a clue for selection of different factors affecting its activity. Amongst different statistical packages, Design-Expert has been preferred over Minitab for optimization of dependent and independent variables. Mostly, screening of multiple influencing factors utilizes first-order Placket-Burman designs. Further, the interactive effect of screened parameters involves second order central composite design (CCD) to analyze the quadratic response surface. Statistical optimization of microbial cellulases generally led to enhancement of their activities up to 20-folds. Combined effect of media ingredients such as carboxymethyl cellulose (CMC), yeast extract, peptone, $MnCl_2$ and K_2HPO_4 significantly influences the bacterial cellulase production. However, a need exists to delineate the production technologies which are economically feasible using less expensive carbon and nitrogen sources to obtain a greater cellulase yield at industrial-scale.

Keywords: Cellulase, Design-Expert, Minitab, Response surface methodology.

Introduction

Current scientific literature shows enzymatic hydrolysis of cellulosic material for industry and biorefinery to be among the most intensively studied topic. Research and development on these enzymes, their production, properties and applications is important if their actions are to be controlled and better utilized. Considerable progress has been made to enhance the cellulase production by optimization of best possible fermentation conditions for the development of economically feasible bioprocess owing to its various industrial applications. Worldwide, cellulases are considered as the third largest industrial enzyme used for finishing of cellulose-based materials in textile industry (KARMAKAR & RAY [1]). In addition, application of cellulase in paper and pulp industries has increased considerably during last few decades (MAI et al. [2]). Furthermore, hydrolysis of lignocellulosic biomass into fermentable sugars for bioethanol production is one of the major areas where cellulases are being applied. Over the years, characterization of cellulase producing bacteria (YIN et al. [3]; AKHTAR et al. [4]; SADHU et al. [5]) and fungi (SARAVANAN et al. [6]; MEKALA et al. [7]) has been investigated extensively, capable of hydrolyzing diverse cellulosic and hemicellulosic substrates.

Greater yield of cellulase with cost effective method can be achieved by optimization of process parameters. Optimization refers to improving the biochemical process to obtain a reliable product in less expensive ecofriendly manner. Designing an appropriate production media and conditions (temperature, pH and incubation time) is important to improve the productivity and efficiency of bioactive microbial metabolites via fermentation processes (HEGDE et al. [8]). Traditionally, optimization is carried out by monitoring the influence of one factor at a time (OFAT) on experimental responses, keeping other parameters constant. The major disadvantage using OFAT strategy counts for exclusion of interactive effects among the studied variables (LUNDSTEDT et al. [9]). Also, estimating effect of large number of variables on response, leads to wastage of time, manpower and reagent making the process expensive. Whereas, statistical methods can evaluate the interactive effect of many factors, minimizing the number of experiments and assay conditions for desirable response (AKHTAR et al. [10]). In this context, among most relevant multivariate technique, response surface methodology (RSM) has shown remarkable application in various process optimization such as ethanol production (DASGUPTA et al. [11]), bacteriocin production (KUMAR et al. [12]), dye degradation (DEMIREL & KAYAN [13]), spore production from *Coniothyrium minitans* (CHEM et al. [14]), production of chitinase from *Chitiolyticbacter meiyuanensis* SYBC-H1 (HAO et al. [15]), pyruvic acid from *Torulopsis glabrata* TP19 (ZHANG & GAO [16]), alkaline protease from *Bacillus* sp. (ADINARAYANA & ELLAIAH [17]), jiean-peptide from *Bacillus subtilis* (ZHANG et al [18]) and cellulase from bacteria (AHTAR et al. [10]; DEKA et al. [19]; LEE et al. [20]) and fungi (SHU et al. [21]; SONI et al. [22]; MEKALA et al. [7]; JABASINGH & NACHIAR [23]). Response surface methodology is a collection of mathematical and statistical techniques rest on the fit of a polynomial equation to the experimental data to describe the data behaviour with an objective of making statistical previsions to optimize response (BEZERRA et al. [24]). Since last five years, optimization of cellulase using RSM from bacteria (AKHTAR et al. [10]; SINGH & SHARMA [25]; DEKA et al. [19]; and fungi (SANDHU et al. [26]; JABASINGH, [27]; SARAVANAN et al. [6]) has been explored for optimum activity and specificity towards various cellulosic substrates. To attain the optimal conditions, linear model-derived generated surfaces can be used to indicate the direction in which the original design must be displaced (BEZERRA et al. [24]). Approximation of response functions of experimental data is conducted using quadratic RSM design where linear function can not be described. There are many types of quadratic RSM design being used for optimization purpose which includes central composite, Doehlert and Box-Behnken (SANDHU et al. [26]) design. These designs are symmetrical but differs from each another in selecting number of variables, runs and blocks and experimental points (BEZERRA et al. [24]). When it comes to microbial cellulase production, strain type, culture conditions, availability of substrates and nutrients are important players affecting cellulase yield. Media ingredients such as CMC, yeast extract, peptone, and $MnCl_2$ have been reported for enhanced production of cellulase (AKHTAR et al. [10]). The optimization of parameters for cellulase production by *B. subtilis* NA15 (AKHTAR et al. [10]; *B. subtilis* AS3 (DEKA et al. [19]); *B. pumilus* EWBCM1 (SHANKAR & ISAIARASU [28]); *Bacillus* sp. VITRKHB (SINGH et al. [29]); and *Bacillus amyloliquefaciens* UNPDV-22 (ZAMBARE & CHRISTOPHER [30]) have been successfully executed using RSM. Screening of large number of basal ingredients for newly isolated strains in optimization of fermentation processes, traditional techniques have shown redundancy in effectiveness making the whole process expensive and labour intensive. Therefore, design based on statistical approaches unlocked the platform for various optimization processes. The present paper represents various aspects of statistical designs of RSM viz. orthogonal designs for first order polynomial (Plackett-Burman and 2K design) and second order designs (central composite design, Box-Behnken design and Doehlert design) and their application in optimization of cellulase produced by microorganisms.

Response surface methodology

Response surface methodology is an approximation of the response function [$y = f(X_1, X_2,\ldots,X_q) + e$ (X_1, X_2 are independent variables, e is error)] and consists of techniques involved in mathematics and statistics which are based on the fit of empirical models to the data obtained in relevant experiment. First-order, second-order and three-level fractional factorial are the major RSM approximating functions, which can be numerically as well as graphically applied in process optimization. First- and second-order designs are most commonly used in optimization of cellulases produced by various microorganisms. In case of two or more responses, it is important to find out the compromised total optimal fit to the absolute function in both domains (OEHLERT [31]). The desirable property of the response surface designs are constant variance check, estimation of transformations, orthogonality and rotatibility, lack of fit detection, internal error estimation, suitability for blocking, construction of higher order designs and graphical analyses. Applications of different RSM designs viz. CCD, Placket-Burman design, Box-Behnken designs for optimized production of different microbial cellulases have been mentioned in Table 1.

Plackett-Burman design

Placket-Burman is a two-level factorial design used to screen a large number of factors which significantly affect the process. Many variables may affect the desirable response and it is practically not feasible to identify their individual contribution. Therefore, a need exists to select the significant variables with profound effect on optimization of cellulase. It allows two levels for each of the k control variables, similar to 2^k design, requiring less experimental runs (BOX & HUNTER [32]). The main effect of each variable was determined according to the following equation:

$$E_{xi} = (M_{i+} - M_{i-}) / N$$

where, E_{xi} is the variable main effect, M_{i+} and M_{i-} are the activity percentage in trials, in which the independent variable (xi) was present in high and low concentrations, respectively, and N is the half number of trials. For statistical optimization of cellulase from *Aspergillus niger* HN-1, Design-Expert software was used to employ Plackett-Burman design followed by CCD (SANDHU et al. [26]). The optimization of the fermentation process for *Saccharomyces cerevisiae* was also done using Placket-Burman design to study the effect in 11 different batches (MANWAR et al. [33]) to screen significant parameters affecting CMCase production. The screening and optimization of various ingredients for optimized cellulase production are represented in Table 2. Most of the bacterial isolates were screened for preferred carbon source as CMC, which was generally found in a range of 2-20 g/L, however, for yeast extract and peptone it was 0.5-9 and 0.25-8 g/L of the media, respectively.

Central composite design (CCD)

The CCD is a second-order design, suitable for parallel experimentation, which allows reasonable information to test the lack of fit without involving large number of design points (SOMAYAJULA et al. [34]). It is an appropriate design for fitting a quadratic surface, which works well to optimiza the process parameters (OZER et al. [35]), providing information on direct and pairwise-interaction effects and curvilinear variable effects. After screening of the significant parameters using linear model, the goal shifts towards product and process optimization of selected influencing factors. Study of interaction effect between two variables and efficiency with minimum number of runs makes CCD a unique and most widely used model for optimization of various processes. The major process variables in their units of measurement affecting the response are used for further optimization. However, while designing an experiment, coding of variables are done as X_1 and X_2 is done, which is centred at 0, and extend to +1 and -1 from the centre of the experimentatal region. The goal is to start somewhere using our best prior or current knowledge and search for the optimum spot of either maximized or minimized response. Four stages of optimization are mainly involved in CCD: (1) execution of statistically designed experiments as per plan (2) accurate prediction of the mathematical model, based on the experimental data and focus on statistics obtained from analysis of variance (ANOVA) (3) controlling the efficiency of the predicted model with diagnostic plots (4) estimation of response followed by model validation (ZHANG et al. [36]).

Model equation for second-order quadratic terms is written as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_{12} + \beta_{22} X_{22} + \varepsilon$$

where, Y is the predicted response, β_0 is the constant coefficient, β_1 is the linear coefficient, β_{12} is the coefficient for interaction effect, X_1 is the dimensionless coded value and ε is the error. Central composite design was used to optimize the process parameters (xylose, beef extract, NaCl concentration, pH and temperature range) that influence the cellulase production from marine *Bacillus* sp. VITRKHB (SINGH et al. [29]). Optimization of the conditions for endo-1,4 glucanase production by *A. nidulans* SU04 and *A. nidulans* MTCC344 under solid state fermentation involving a four-factor-five-level CCD was also employed, leading to maximum CMCase activity of 32.59 and 28.96 U/g from *A. nidulans* SU04 and *A. nidulans* MTCC344, respectively (JABASINGH [23]). Study carried out by ZAMBARE & CHRISTOPHER [30] to determine interactive effect of fermentation medium components employing CCD showed that wheat bran, soybean meal and malt dextrin had significant impact on production of cellulase, resulting in 70% increase in activity.

Box-Behnken design

Box-Behnken (BB) is a class of rotatable second-order design based on three-level incomplete factorial design, which can be used as an alternative to CCD (BOX et al. [32]). BB design has advantages of avoiding corner and the star extreme points in less number of experiments, however, CCD can better fit the model, giving better information about the function as it includes all the extreme points. Hence, it is recommended to choose suitable design according to the need of process optimization. The CCD may be considered as a ball like model in which all of the corner points lie on the surface, whereas, the ball in BB design is embedded inside the frame-designed box composed of the edges of the box. When two-level factorial design is combined with incomplete block design with complex confounding of interaction, BB design are constructed. However, the designs are economical and therefore particularly useful when it is expensive to perform the necessary experimental runs. Response surface methodology-based BB designs have been widely employed to optimize the physical parameters such as pH and incubation time in addition to media components. In BB design there is a fine adjustment between quadratic model and experimental statistics. To understand the relationship between response and other influencial parameters, statistics-based contour plots are generated to evaluate the changes in the response surface for prediction of response-factor relationship. The incubation temperature and time along with inducer concentration were optimized to enhance the cellulase activity from *Trichoderma reesei* RUT C30 using BB Design and highest FPase activity (25.6 U/g dry substrate) was reported (MEKALA et al. [7]).

Doehlert design

Doehlert design comprises of another class of experimental designs which study different factors at different numbers of levels (DOEHLERT & KLEE [37]). A circular domain was described for two variables, spherical for three variables and hyperspherical for more than three variables, which leads to the uniformity of the analyzed experimental variables (BEZERRA et al. [24]. It is used when some of the experimental factors deserve more attention as compared to others (FERREIRA et al. [38]), therefore this design has been less employed for microbial cellulase optimization.

Taguchi design

Taguchi design is being used over a diverse scientific field, including optimization of processes parameters involved in manufacturing of raw materials and industrial products which are ready for market entrance. It can also be applied to the processes involved in engineering designs, computer-aided designs and banking and service sectors. TAGUCHI [45] proposed two types of input variables for an experiment (1) easily controlled factors and
(2) noise factors. The noise factors are the main cause of variations in a production process and are difficult to control.

Table 1. Statistical methods for optimization of microbial cellulase production.

Microbial species	Software used	Factorial design	No. of factors screened	No. of runs	Optimized activity	Fold increase	Reference
Bacillus subtilis NA15	Design-Expert	PBD, CCD	11, 4	12	0.47 U/mL	7	AKHTAR et al. [10]
B. subtilis AS3	Minitab	PBD, CCD	7, 3	20, 20	0.43U/mL	6	DEKA et al. [19]
B. amyloliquefaciens UNPDV-22	Design-Expert	CCD	3	20	---	1.7	ZAMBARE & CHRISTOPHER [30]
B. pumilus EWBCM1	Design-Expert	CCD	3	20	0.5751 IU/mL	---	SHANKAR & ISAIARASU [28]
Bacillus sp. JS14	Design-Expert	CCD	4	30	---	20	SINGH & SHARMA [25]
Bacillus sp. BVITRKH	Design-Expert	CCD	5	31	---		SINGH et al. [29]
Bacillus sp. JS14	Design-Expert	CCD	4	30	2040 IU/ L	---	SINGH & KAUR [39]
Cellulomonas fimi NCIM-5015	Minitab, Design-Expert	PBD, CCD	12, 4	20, 30	0.58 IU/mL	---	ALI et al. [40]
Trametes hirsute	Design-Expert	CCD	4	30	---	---	JEYA et al. [41]
Aspergillus niger HN-1	Design-Expert	PBD, CCD	11, 4	12, 30	---	2-3	SANDHU et al. [26]
Aspergillus nidulans SU04	Design-Expert	CCD	4	30	28.94 U/g	---	JABASINGH, [27]
Aspergillus nidulans MTCC344	Design-Expert	CCD	4	30	32.57 U/g	---	JABASINGH, [27]

Trichoderma reesei NCIM 1186	Design-Expert	BBD	5	46	---	1.243	SARAVANAN et al. [6]
Trichoderma reesei and *Aspergillus oryzae*	Design-Expert	CCD	3	20	---	---	BRIJWANI et al. [42]
Trichoderma reesei RUT C30	Design-Expert	BBD	3	17	0.71 U/mL	5	MEKALA et al. [7]
Aspergillus oryzae	---	OFAT, BBD	4	27	0.92 ± 0.09 FPU/g	---	HOA & HUNG [43]
Myceliophthora sp. IMI 387099	Minitab	BBD	3	17	8.66 U/g	4.23	BADHAN et al. [44]
Aspergillus fumigatus AMA	Minitab	BBD	3	17	9.73 U/g	2.88	SONI et al. [22]
Aspergillus nidulans	Design-Expert	PBD, CCD	7 , 4	8, 30	39.56 U/mL	8.05	JABASINGH & NACHIYAR [23]
Trichoderma reesei HY07	Design-Expert	BBD	3	15	406.42 U/g	1.45	SHU et al. [21]

PBD: Plackett-Burman design; BBD: Box-Behnken design; CCD: Central composite design; OFAT: One factor at a time

The method primarily uses engineering concepts to decide optimal factor levels for multi-responses (PHADKE et al. [46]), resulting in an increase in uncertainty during the decision-making process. Worldwide, Taguchi method is generally utilized for the optimization of a single quality characteristics. The usefulness of Taguchi method for optimization of culture condition having five factors, designed for the production of xylanase by *Trichoderma longibrachiatum* was investigated and 41.9% enhancement was observed (AZIN et al. [47]). Xylanase production by a newly isolated *Aspergillus terreus* MTCC 8661 was also optimized in solid state fermentation for optimization of process parameters such as incubation temperature, moisture content, medium pH, particle size, incubation time, inoculum, xylose and sodium nitrate concentrations and their individual and interactive effects were studied (LAKSHMIA et al. [48]). Till date, Taguchi designs have not been applied in optimization of naturally occurring microbial cellulase production, however, it could be tested for optimization of fermentation parameters for other industrial products.

Validation of statistical models

The evaluation of model's fitness and accurate prediction of each studied variable requires model validation, both numerically and graphically. The way of result interpretation plays an important role for concluding the effect of each variable as well as their combination. Validating design is based on common mathematical and statistical tests like F-test, ANOVA and P-value to study the significance of the facts. The mathematical model found after fitting function to data may conflict or even erroneous conclusions to describe the experimental domain. Application of ANOVA is the most reliable way to evaluate the quality of model fitness, comparing the variation due to treatment and random errors inherent to the measurements of the obtained responses (VIEIRA & HOFFMAN [49]). Thereby leading to evaluation of regression significance to foresee responses, considering sources of experimental variance. The significance of regression can be calculated by the ratio of the media of the square of regression (MS_{reg}) and the media of the square of residuals (MS_{res}) and comparing these variation sources using the Fisher distribution test (F-test):

$$(MS_{reg} / MS_{res}) = F_{vreg,vres}$$

A large value of $F_{vreg,vres}$ indicates that the difference in the output caused by this source of variation is greater than the difference caused by noise (i.e. this source affects the output when statistically significant value of $F_{vreg,vres}$ is higher than the tabulated value for F). This indicates that the mathematical model is fitting well to the experimental data. The statistical significance of the second-order model equation for cellulase production from agricultural waste was validated by F-test (MUTHUVELAYUDHAM & VIRUTHAGIRI [50]). P-value is the probability of obtaining an effect at least as extreme as the one that was actually observed, assuming that the null hypothesis is true (GOODMAN [51]). If the p-value is less than alpha (confidence level: 95%), this source of variation is considered to have a significant effect on the output. Analysis of variance for CMCase production (U/g) indicated the 'F-value' to be 69.49, which implied the model to be significant. Model terms having values of 'Prob > F' less than 0.05 are considered significant, whereas, greater than 0.10 are insignificant (SHU et al. [21]). Lack of fit test is another way to evaluate the model significance. MS_{lof} (media of square lack of fit) should reflect only the random errors inherent to the system and MS_{pe} (Media of square of pure error) estimation of these random errors, key idea behind this test is that MS_{lof} and MS_{pe} are not statistically different.

$MS_{lof} / MS_{pe} \approx F_{vlof,\ vpe}$

If this ratio is higher than the tabulated value of F, it is concluded that there is evidence of a lack of fit and the model needs further improvization.

Table 2. Different media ingredients influencing activity of extra-cellular cellulase from microorganism

Microorganisms	CMC (g/L)	Yeast Extract (g/L)	Peptone (g/L)	$MgSO_4$ (g/L)	$(NH_4)_2SO_4$ (g/L)	Nacl (g/L)	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)	$CaCl_2$ (g/L)	$FeSO_4$ (g/L)	$MnCl_2$ (g/L)	Urea (g/L)	Reference
Bacillus subtilis NA15	2-18	1-9	2-8	0.05-1	---	0.02-0.5	---	0.5-2.0	---	0.05-1	0.01-1	---	AKHTAR et al. [10]
B. subtilis AS3	2-8	1-9	1-8	0.05-0.45	---	---	---	0.5- 2	---	0.05-0.45	0.01 - 0.1	---	DEKA et al. [19]
Bacillus sp. JS14	10	1	1	0.2	---	---	2.5	0.1	---	---	---	---	SINGH & SHARMA [25]
Bacillus sp. VITRKHB	20	20	---	0.82	---	1	3	1.25	0.005	0.01	0.00001	---	SINGH et al. [29]
Bacillus sp. C1	10	---	---	0.5	1	Traces	---	1	---	---	---	---	SADHU et al. [5]
Bacillus sp. 1139	---	0.5-3.5	0.25-1.25	0.3 - 0.5	---	---	1-5	2.0- 4.0	---	0.005 -0.1	---	---	ALI et al. [40]
B. amyloliquefaciens DL-3	10	5	5	2	---	5	1	---	0.1	---	---	---	LEE et al. [52]
Aspergillus niger HN-1	---	0.10-0.30	---	---	1-2	---	1 - 3	---	---	0.002-0.01	---	0.10 -0.30	SANDHU et al. [26]
A. nidulans	0.2-2	---	0.10-5.0	---	---	---	---	---	---	---	---	0.3 - 0.5	JABASINGH & NACHIYAR [23]
Trichoderma reesei NCIM 1186	---	---	10	0.3	7	---	10	---	1.5	---	1.56	1.5	SARAVANAN et al. [6]
T. reesei RUT C30	---	---	5	21	---	5	5	---	1	0.005	0.001	2	MEKALA et al. [7]

However, if the value is lower than that of tabulated, the model fitness can be considered satisfactory. Fit of model is also analysed with the help of different values like R-square (R^2) which is the percentage of total difference that is attributable to the factors under consideration. R^2 value obtained for cellulose produced uder solid-state fermentation by *T. reesei* RUT C30 was 0.8579, indicated that 85.79% of the sample variance is attributed to the factors and only 14.21% occurred due to chance (MEKALA et al. [7]). Higher value of R^2 signifies to better fit. Adjusted R^2 is the value that is adjusted for the number of parameters in the given model. Predicted R^2 is a measure of how finely the model predicts the observations, larger the value, the more accurate the model's predictions are likely to be. Regression coefficient of the term, represents the contribution of the term to the variation in the response as term X_1 having higher regression coefficient, indicates greater significance as compared to term X_2 with lower regression value. Low and high confidence are the lower and upper confidence bounds on the regression coefficient.

Conclusion

Response surface methodology helps in identification of casual relationship between different factors and response(s) using a mathematical model that represents the system that is demonstrated to be robust and reliable. Currently, application of RSM in optimization of cellulase has been widely preferred over classical OFAT method, due to generation of large number of information at a time from less number of experiments. The possibility of evaluating the interaction effect among the variables is another advantage of optimization strategy using RSM. An adequate experimental design is required to employ RSM-based experimental optimization in order to evaluate the quality of the fitted model and its accuracy to anticipate the relation to the experimental data obtained. Widely used symmetrical second-order experimental design for microbial cellulase production is CCD. However, Box-Behnken design presents more efficient matrices and has shown successful implication resulting in to number of published works in recent years. Optimization of physical parameters and media ingredients led to enhancement of the cellulase production and the strategy may be successfully implemented in a biorefinery proceess. Further optimization of process parameters affecting cellulase produced from *Bacillus* spp., *Trichoderma* spp. and *Aspergillis* spp. need to be studied for large-scale industrial production and establishment of a concrete bioprocess technology.

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