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Original Article

Augmented cellulase production by *Bacillus subtilis* strain MU S1 using different statistical experimental designs

C.P. Sreena, Denoj Sebastian *

Department of Life Sciences, University of Calicut, Malappuram, Kerala 673635, India

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ABSTRACT

The production of cellulase by *Bacillus subtilis* MU S1, a strain isolated from Eravikulam National Park, was optimized using one-factor-at-a-time (OFAT) and statistical methods. Physical parameters like incubation temperature and agitation speed were optimized using OFAT and found to be 40 °C and 150 rpm, respectively, whereas, medium was optimized by statistical tools. Plackett-Burman design (PBD) was employed to screen the significant variables that highly influence cellulase production. The design showed carboxymethyl cellulose (CMC), yeast extract, NaCl, pH, $MgSO_4$ and $NaNO_3$ as the most significant components that affect cellulase production. Among these CMC, yeast extract, NaCl and pH showed positive effect whereas $MgSO_4$ and $NaNO_3$ were found to be significant at their lower levels. The optimum levels of the components that positively affect enzyme production were determined using response surface methodology (RSM) based on central composite design (CCD). Three factors namely CMC, yeast extract and NaCl were studied at five levels whilst pH of the medium was kept constant at 7. The optimal levels of the components were CMC (13.46 g/l), yeast extract (8.38 g/l) and NaCl (6.31 g/l) at pH 7. The maximum cellulase activity in optimized medium was 566.66 U/ml which was close to the predicted activity of 541.05 U/ml. Optimization of physical parameters and medium components showed an overall 3.2-fold increase in activity compared to unoptimized condition (179.06 U/ml).

1. Introduction

Cellulose, the most abundant and renewable material on earth, is a biopolymer of D-glucose units connected by β-1, 4 glycosidic linkages. The complete hydrolysis of cellulose into glucose involves the enzyme cellulase. Cellulase is a multi-enzyme system comprising of endo-β-1, 4-glucanases [EC 3. 2. 1. 4], exo-β-1,4-glucanases or cellobiohydrolases [EC 3. 2. 1. 91] and β-1,4-glucosidases [EC 3. 2. 1. 21]. These enzymes have proven their potential application in wide variety of industries like food and animal feed, laundry and detergents, pulp and paper, textiles, brewing and wine making, and biofuel. Besides this they find application in medical/pharmaceutical industry, protoplast production, genetic engineering, pollution treatment and waste management [1]. Cellulases accounted for approximately 20% of world enzyme market between 2005 to 2010 [2] and its demand is thought to increase drastically due to its application in second generation bioethanol production.

Cellulases are produced by bacteria, fungi, protozoans, plants and animals [3]. However, enzymes of microbial origin are more widespread due to their broad biochemical diversity, feasibility of mass culture, and ease of genetic manipulation. Moreover, they possess high degree of stability under extreme conditions [4]. Currently most of the commercial cellulases are obtained from fungi mainly *Trichoderma, Humicola, Aspergillus,* and *Penicillium* [5]. However, bacterial cellulases are gaining attention because of their high natural diversity, higher growth rate, easier product recovery and ability to produce enzymes that withstand harsh environmental conditions [6,7]. The cellulolytic potentials of bacteria belonging to different genera such as *Acetivibrio, Bacillus, Bacteroides, Cellulomonas, Clostridium, Erwinia, Ruminococcus,* and *Thermomonospora* have been well studied. Among them *Bacillus* spp. are known to produce and secrete large quantities of extracellular enzymes and hence dominate the bacterial workhorses [8]. Moreover, the endospore forming ability and production of secondary metabolites give them an additional advantage over competitors under conditions of slow growth on cellulosic substrates [9]. The strains of *B. sphaericus* and *B. subtilis* are excellent cellulase producers [10,11].

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* Corresponding author.
E-mail addresses: sreenacp@rediffmail.com (C.P. Sreena), drds@uoc.ac.in (D. Sebastian).

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The cost of enzymes is one major factor determining the economics of a biocatalytic reaction and it can be reduced by finding the optimum conditions for their production, isolation of hyperproducers and production of efficient strains by genetic engineering [12]. Cost reduction by media optimization is the basic research for industrial application. Various factors are known to influence the extracellular enzyme production. Some of these are temperature, pH, aeration [13], and medium constituents [14]. The Plackett-Burman design is a two level fractional factorial design, which allows screening and selection of most significant variables from among large number of variables. However, it does not consider the interactions between the variables [15]. The selected variables can be further optimized by using statistical and mathematical optimization tools such as Response Surface Methodology [16]. This technique enables to evaluate the optimal level of each variable, their interactions with other variables and their effect on product yield [17]. Recently, PBD and RSM have been successfully used to optimize many fermentation media [6].

The aim of the present study was to increase the cellulase production by optimizing fermentation conditions. Initially the physical parameters were optimized using OFAT method. Later, Plackett-Burman Design was used for selecting relevant medium components which were further optimized by central composite design of RSM and the model was verified.

2. Materials and methods

2.1. Microorganism and culture condition

A cellulolytic bacterium *Bacillus subtilis* MU S1 (accession No. KT715518) isolated from Eravikulam National Park was used for the study [18]. The culture was maintained on nutrient agar slant at 4 °C and subcultured regularly. The seed culture was produced by inoculating a loopful of the culture into 50 ml unoptimized medium [18] containing following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and yeast extract (1.0). The pH of the medium was adjusted to 7.0. The culture was grown at 37 °C in shaking incubator. One percent of the overnight grown culture (adjusted to a McFarland standard of 1.0) was used as seed for extracellular cellulase production using the unoptimized medium and conditions described above. After 24 h incubation, the culture was centrifuged at 10,000*g* for 10 min and the supernatant was used to analyze enzyme activity.

2.2. Cellulase assay

Cellulase assay was performed according to Ghose et al. [19] using CMC as the substrate. One milliliter of the enzyme was incubated with same volume of substrate in 0.05 M sodium citrate buffer (pH 5.0) for 30 min at 40 °C. The reducing sugar released was estimated using DNS method [20]. All experiments were performed in duplicates and the enzyme activity was determined using calibration curve of glucose. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 µmol of glucose per minute under the assay conditions.

2.3. Optimization of physical parameters by OFAT design

The temperature and agitation speed for cellulase production were optimized by OFAT method. For optimizing temperature the unoptimized medium was inoculated with seed culture and incubated at varying temperatures (30 °C, 40 °C and 50 °C) in shaking incubator. After 24 h the culture was centrifuged and the cell-free supernatant was used as crude enzyme for cellulase assay. The optimal agitation speed was determined by inoculating and incubating medium at different agitation speeds (50, 100, 150 and 200 rpm) at optimized temperature. Cellulase assay was performed in duplicate with crude enzyme obtained from 24 h culture. The medium components were optimized by growing the organism under the optimized physical conditions.

2.4. Plackett-Burman design for screening of significant medium components

The most important variables that significantly influence cellulase production were selected by PBD using the statistical software package MINITAB (Release 16, PA, USA). A total of ten parameters were screened and each parameter was examined at its low level (−1) and high level (+1) (Table 1). PBD is based on the first order polynomial model:

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

where Y is the response (cellulase activity), β_0 is the models intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. This design does not consider the interaction among variables and a linear approach is considered to be sufficient for screening. The main effects of such a design are calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (−1) [15].

The ten variables were screened in 20 experimental runs. Averages of cellulase activity obtained from duplicate experiments were taken as the response (Table 2). Regression analysis was performed to determine the factors that influence enzyme production. The factors which were significant at or above 95% level ($p < 0.05$) were selected and later optimized by central composite design.

2.5. Central composite design

After identifying the significant variables for cellulase production by PBD, response surface methodology using CCD was employed to determine the optimal levels of these variables. A 3-factor-5-level design was used and five coded levels ($-\alpha$, −1, 0, +1, $+\alpha$) were assigned to each factor (Table 3). Alpha is the extended level with value of $(2)^{3/4} = 1.682$. A 2^3 full-factorial CCD experimental design containing three significant medium components (CMC, yeast extract and NaCl) at five coded levels was generated using the statistical software package "Design Expert 7" (Stat Ease Inc., Minneapolis, USA). The experimental design comprised of 20 runs ($=2^k + 2k + n_0$), where '*k*' is the number of independent variables and n_0 is the number of replicate runs at center point of the variables. All experiments were carried out in duplicate and the averages of the cellulase activity were taken as the response (Table 4).

Table 1
Medium components and their variables used in Plackett- Burman design.

Nutrient code	Nutrients (g/l)	Low (−1)	High (+1)
A	CMC	2	18
B	Yeast extract	0.5	10.5
C	NaCl	2	14
D	$(NH_4)_2SO_4$	0.5	2.5
E	KH_2PO_4	0.05	2.05
F	K_2HPO_4	0.05	0.45
G	$MgSO_4$	0.01	0.21
H	$CaCl_2$	0.005	0.405
I	$NaNO_3$	0.005	0.805
J	pH	5	7

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