Optimization of medium composition to induce cellulase production of *Bacillus sp.* by Response Surface Methodology

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ABSTRACT:

In the past, optimization in biological analyses has been performed by measuring influence of one factor at a time. The disadvantage of this technique is not presence any interactive effects of factors studies and it needs a large number of experiments. Consequently, the Response Surface Methodology (RSM) is the most popular choice for optimization. It used the effects of mathematics and statistics to study optimization with minimum experimental trials and therefore interactive variables may be involved. This study has selected and screened seven Bacillus strain in Vietnam Type Culture Collection which can product cellulase in the medium with CMC as an inducer. VTCC-B-0497 presented the best result. Then, only 15 experiments of design matrix by Box-Behnken were necessary to determine optimal concentrations of three variables including the concentration of Carboxymethylcellulose, Yeast extract and Meat extract to product cellulase. The model could be employed to predict the response. The maximum of cellulase activity was 2.48 U at the concentration of Carboxymethylcellulose 0.25% w/v, yeast extract 0.7% w/v and meat extract 0.2% w/v.

Key works: *Fermentation, Bacillus sp., Cellulase, Optimization.*

1. INTRODUCTION

Cellulose is a major polymeric component of plant matter and is the most abundant polysaccharide on the earth. It is composed of repeating cellobiose units linked by β-1,4-glucosidic bonds and forms a crystalline structure [1]. It is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere (~100 billion dry tons/year). Cellulose biodegradation by cellulases and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO_2, is very important in several agricultural and waste treatment processes and could be widely used to produce sustainable biobased products and bioenergy to replace depleting fossil fuels. Cellulose is useful as an energy and chemical resource; it is not useful in its polysaccharide form. It must be converted into smaller molecules (for example glucose, ethanol,

and other biochemical) [2]. In this context, this energy demand other thinks to enhance its value. Cellulolytic microbes are proving useful for these types of conversions, as they produce a variety of different cellulose-degrading enzymes.

Cellulases, enzyme catalyze hydrolyze cellulose, belong to the large family of glycosyl hydrolases and are produced by a variety of bacteria, fungi, and animals. Enzymatic hydrolysis of cellulose is attractive because its conversion can be achieved without environmental pollution. Thus, this provides a potentially renewable energy resource and improves the technology of bioconversion of plant biomass into useful products [2]. The cellulases and related enzymes that are in wide use today in agriculture and other fields (i.e., food, brewing and winemaking, animal feeds, textiles and laundry, pulp and paper industries, etc.) are mostly of fungal origin. The demand for these enzymes increases rapidly. However, the activity of cellulase is usually unstable. It is influenced by the source of cellulose, environment stress such as high temperature and organism [3]. Compared with fungal cellulases, cellulase derived from bacteria has many benefits such as: short generation, easy growth on very high cell density using inexpensive carbon and nitrogen sources. Bacteria are good sources of secreted enzymes. The expression system and manipulation of bacteria is much more convenient; therefore, high-level expression of endogenous cellulase is more easily achieved in bacteria than it is in fungi [2].

Bacteria belonging to the genus *Bacillus* have a long and distinguished history in the realms of biotechnology. *Bacillus* is not identical and they are adaptability to the environment because these bacteria can product many kinds of enzymes that allow transformation of complex organic molecules into simple material for growth and development of microorganisms. These important different enzymes are protease, lipase, cellulase, xylase, etc. They can also convert into spores under difficult condition or stress of environment. *Bacilllus* is rod-shaped, Gram positive, facultative aerobes. *Bacillus subtilis* is considered a good strain because it does not have the characteristics pathogenic for humans, animals and plants. The potential risks only relate to the use of these bacteria in fermentation process. *Bacilli* is also a rich source of antibiotics, the purine nucleoside, surface-active substances and many other products [4]. In particular, environmental factors affect the nature of the process of metabolism and enzyme production. Composition and concentration of fermentation medium influents production of intracellular and extracellular enzymes. Optimization of culture medium makes the most desirable products.

2. MATERIALS AND METHODS

2.1. Microorganism

Cellulase-producing bacterial strains were isolated from water, soil samples. The strains were deposited in the Vietnam Type Culture Collection (VTCC), Vietnam. Their codes in VTCC are A-2434, VTCC-B-0497, VTCC-B-0490, VTCC-B-0499, VTCC-B-0486, VTCC-B-1013, AT-186 and were renewed in this study such as No1, No2, No3, No4, No5, No6 and No7, respectively. Those strains were maintained on Nutrient Broth (HiMedia Laboratories Pvt, India) and Agar (20g/L) slant at 4^{o}C and sub cultured every two months.

2.2. Medium

Cultivation:Bacillus strains were incubated two times in 5ml and 10ml Nutrient Broth before fermentation

Culture for screening contains Nutrient Broth, 0.5% CMC (Carboxymethylcellulose) and Agar.

Culture for optimization: After the screening, one of seven *Bacillus* strains was chosen for

optimization. The culture grown at 37ºC with 150 rpm for 32 h. 5% of the culture inoculums was transferred to 250 mL flask containing 50 mL of cellulolytic medium described by Kwong-Yu CHAN with the modification, contain : CMC, g/L; yeast extract, g/L; meat extract, g/L are the variable of optimization and the factors fixed in the medium are K_2HPO_4 ,1mM; $MgSO_4.7H_2O$,0.3 mM; $FeSO_4.7H_2O$, 0.4 mM; $MnCl_2.4H2O$, 1µM [5]. The medium was steriled at 121ºC, 15min.

2.3. Analyze cellulase productivity for screening:

For screening, The Carboxylmethyl cellulose clear zone method was used. After 24h of incubation, 50µl supernatant was used for testing the presence of cellulase in the broth. Those Petri dishes were placed at 37ºC in 24h. Production of cellulase of seven strains were estimated the by the diameter of clear zone (cm) with Lugol's solution 1% as an indicator. All the experiments were performed in triplicate.

2.4. Analyze enzyme activity

The culture broth after 36h of incubation was centrifuged at 10,000g for 10 min to separate the cells. The crude enzyme solution was analyzed for enzyme activity. The cellulase activity was calculated by estimation of reducing sugars liberated from carboxymethylcellulose. The reducing sugar was estimated by DNS method. A standard curve was prepared with glucose [6]. One unit (U) of cellulase activity is defined as the amount of enzyme that liberates 1µmole of reducing sugar (glucose) at 50ºC in 30min in 0.05M sodium citrate buffer, pH 6.5.

2.5. Response Surface Methodology and statistical analysis

Response Surface Methodology (RSM) using the Box–Behnken design (create by Box and Behnken in1960) of experiments was used to develop a mathematical correlation between three independent variables on production of endo-β -1,4-glucanase. Three independent variables were: CMC concentration (X_1) , yeast extract concentration (X_2), meat extract concentration (X_3) and the response variable (Y) was enzyme activity like endo-β-1,4-glucanase. Low, middle and high concentration levels of each were designated as -1, 0 and 1, respectively, was generated by NemrodW software. This study had 15 runs and three the replicate runs at center point of the variables. The relationships and interrelationships of the variables were determined by fitting the second order polynomial equation to data obtained from 15 experiments.

Table 1. Experimental variables used for optimization of cellulase production

Process variables	**Range and level**		
	-1	0	1
CMC concentration (X_1) (% w/v)	0.1	0.5	0.9
Yeast extract concentration (X_2) (% w/v)	0.1	0.4	0.7
Meat extract concentration (X_3) (% w/v)	0.05	0.2	0.35

Table 2. Three factorial Box-Behnken for production of celluluase from *Bacillus sp.* and their activities.

No	CMC	Yeast extract	Meat extract	Endo-β-1,4-glucanase activity		
	X_1	X_2	X_3	Measured	Predicted	Residual
1	-1	-1	0	1.160	1.0439	0.1161
2	1	-1	0	2.143	2.1176	0.0254
3	-1	1	0	2.134	2.1594	-0.0254
4	1	1	0	2.311	2.4271	-0.1161
5	-1	0	-1	1.239	1.2855	-0.0465
6	1	0	-1	1.974	1.9298	0.0442
7	-1	0	1	1.558	1.6023	-0.0443
8	1	0	1	2.346	2.2995	0.0465
9	0	-1	-1	1.071	1.1406	-0.0696
10	0	-1	-1	2.222	2.1501	0.0719
11	0	1	1	1.709	1.7809	-0.0719
12	0	1	1	2.266	2.1964	0.0696
13	0	0	0	2.220	2.2243	-0.0043
14	0	0	0	2.240	2.2243	0.0157
15	0	0	0	2.213	2.2243	-0.0113

To evaluate the form of the true response, a second degree model is used. This model is given by the equation 1, where Y is the response, b_0 is the intercept of the y axis, b_i, b_{ii},..., b_{ij} are the various coefficients of the model (linear and quadratic), X_i and X_j are the independent variables (factors) and ε is the error of model with ∀ i, and $\varepsilon \sim N(0, \sigma^2)$ (normal distribution).

$$Y = b_0 + \sum_{i=1}^{3}(b_i X_i) + \sum_{i=1}^{3}(b_{ii} X_i^2) + \sum_{i<j}\sum(b_{ij} X_i X_j) + \varepsilon \quad (eq.1)$$

2.6. Interpretation and data analysis

The results of the experimental design were analysed and interpreted using the NemrodW, statistical software of LPRAI Company (France). This software offers easy to use tools to generate experimental designs, to perform statistical analysis of the experiments and to use models for prediction.

3. RESULTS AND DISCUSSION

In many study, there are many organisms, bacteria and fungi especially, capable to produce cellulase. Most of cellulase studies were due to fungi e.g. *Tricoderma reesei*, *Trichoderma citrinoviride* EB-104, *Acremonium cellulolyticus*… However, bacteria have a great extent in different effects. So, this work was focus of the production of cellulase by *Bacillus* strains.

3.1. Screening of Bacillus strains

The screening cellulase activity of the cell supernatant of *Bacillus* was cultured on the culture of screening at 37±1ºC with shaking at 150 rpm for 24 h. Fresh bacterial inoculums was prepared and inoculated in the plat which was prepared such as assay of cellulase productivity for screening. Bacteria strains was spread and cultured on the agar plate supplemented with 0.5% Carboxymethylcellulose (CMC). The zone

of clearance as qualitative measure of extracellular cellulase activity was screened after flooding plates with Lugol's iodine solution.

Table 3. Descriptive statistic to screening of *Bacillus* strains

	N	Min	Max	Mean		S.D	Var
	Stat	Stat	Stat	Stat	S. E	Stat	Stat
Cellulase No1	3	2.3	2.4	2.333	.0333	.0577	.003
Cellulase No2	3	**2.4**	**2.5**	**2.467**	**.0333**	**.0577**	**.003**
Cellulase No3	3	2.3	2.3	2.300	.0000	.0000	.000
Cellulase No4	3	2.2	2.2	2.200	.0000	.0000	.000
Cellulase No5	3	2.2	2.4	2.300	.0577	.1000	.010
Cellulase No6	3	2.3	2.3	2.300	.0000	.0000	.000
Cellulase No7	3	2.2	2.2	2.200	.0000	.0000	.000

2.1. Optimization component of culture

The effect of CMC concentration (X_1), yeast extract concentration (X_2), meat extract concentration (X_3) and the response variable was enzyme activities like endo-β-1,4-glucanase. When the relationship between the variables and the response has been established by the modeling, predictions can be made. The global predicted capacity of a mathematical model is generally explained by the coefficient of determination R^2 which measures the variability explained by the factors and their interactions in the observed responses. In this research, it is 0.98 for the model, from which it can be concluded that 98% of the endo-β-1,4-glucanase activity is attributed to three independent variables and only 2% of the total variability is not explained by the mode. The decisive factor, R^2 and $R^2_{Adj,}$ are greater than 0.9; Thus, the model explains the global variability; it is globally predictive [7].

Table 4. Estimates and statistical coefficients

Standard deviation of the answer		0.0140	
R^2		0.980	
R^2_{Adj}		0.944	
R^2 pred		0.683	
Press		0.905	
Degre of freedom		2	
Nom	Coefficient	T-exp	p-value
b0	2.2243	274.96	<0.01
b1	0.3354	67.70	0.0210
b2	0.3562	71.91	0.0195
b3	0.1716	34.64	0.0546
b11	-0.1625	-22.29	0.123
b22	-0.1248	-17.11	0.212
b33	-0.2825	-38.75	0.0455
b12	-0.2015	-28.76	0.0755
b13	0.0132	1.89	20.0
b23	-0.1485	-21.20	0.136

Coefficients of the Eq. (1) are values coded b_0-b_{23}. T-exp shows the values estimated by t-distribution. The p-value is probability of obtaining a test statistic when they make a judgment a hypothesis statistics about the capability of coefficient equal to zero. We start with the null hypothesis (H_0) which is a claim of "no difference". The opposing hypothesis is the alternative hypothesis (H_1). The alternative hypothesis is a claim of "a difference in the population". p-value greater than α significant ilustrates that the existence of coefficient is not significant and it can be confident that $1-\alpha$ % capacity of this coefficient equal to zero [7]. The p-value indicates a high significance X_1 of the corresponding coefficient. The results revealed that Yeast extract concentration (X_2) had the largest coefficient followed by CMC concentration (X_1) and meat extract concentration (X_3). If coefficient is negative observed for these three factors indicates that a decrease in their levels can increase cellulase production but in this study, three coefficients are positive that indicate an increase in three factors level can flow up cellulase production.

$$Y= 2.2243 + 0.3354X_1 + 0.3562X_2 + 0.1716X_3 - 0.1625X_1^2 - 0.1248X_2^2 - 0.2825X_3^2 + 0.2015X_1X_2 + 0.0132X_1X_3 - 0.1485X_2X_3 \quad \text{(Eq 2)}$$

Where Y is the predicted cellulase activity for critical medium components. The statistical significance of Eq. (2) was controlled by the t-statistic and p-values (Table 4). After that, Eq. (2) became Eq. (3) which has significatif coefficients.

$$Y= 2.2243+ 0.3354X_1+0.3562X_2+0.1716X_3 - 0.2825X_3^2 + 0.2015X_1X_2 \quad \text{(Eq 3)}$$

The surfaces generated by linear models can be used to indicate the direction in which the original design must be displaced in order to attain the optimal conditions. For quadratic models, the critical point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the critical point through the first

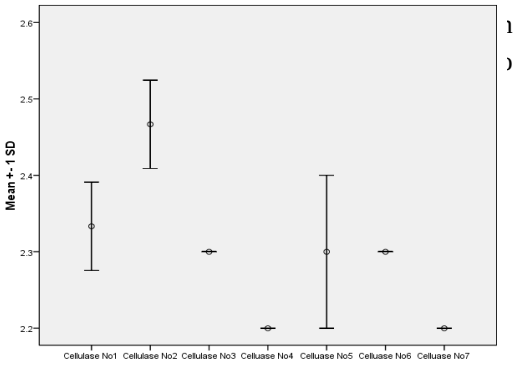

Figure 1. Comparison mean, Standard Deviation of the cellulase activity as zone of clearance on the agar plate

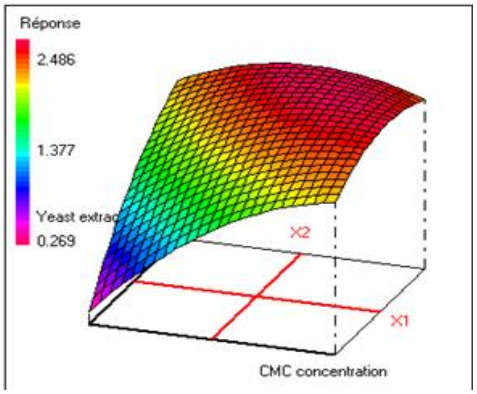

Figure 2. Response Surface plots showing effects of CMC concentration and yeast extract concentration wih the meat extract concentration fixed at 0.2% w/v

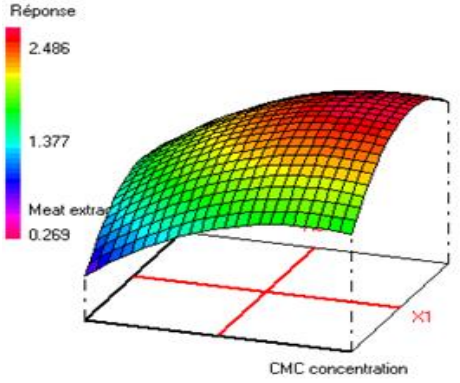

Figure 3. Response Surface plots showing effects of CMC concentration and meat extract concentration with the yeast extract concentration fixed at 0.4%w/v

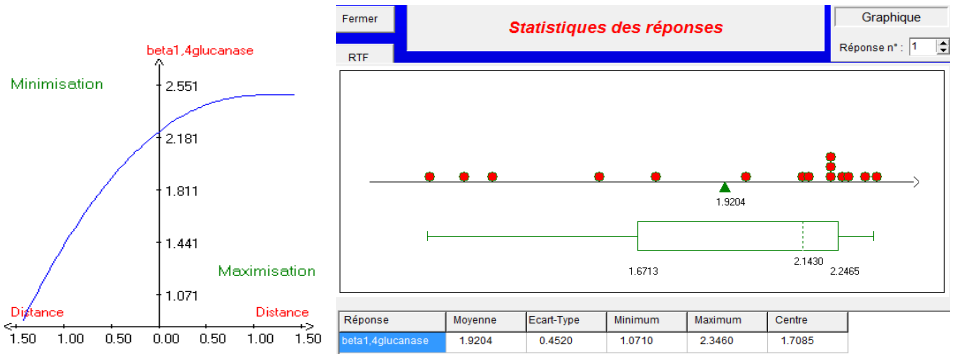

Figure 4. Study the optimum answer

The cellulase activity of *Bacillus subtilis AS3* was enhanced significantly by optimizing the medium composition by statistical methods. The famer was increased around 6 fold from 0.07 to 0.43 U/mL by the medium containing CMC (1.8%), peptone (0.8%), and yeast extract (0.479%) [8]. In another study, Carboxymethyl Cellulase from *Bacillus sp.* isolated from a paddy field found to be optimal at pH 6.5, 37°C and 150 rpm of shaking, the crude enzyme was found to have the highest activity on CMC (16.5 U/mg protein) [9]. The production of cellulase in *Bacillus amyloliquefaciens* UNPDV-22 was optimized using response surface methodology to study the interactive effect of fermentation medium components (wheat bran, soybean meal, and malt dextrin) on cellulase activity. Use of RSM resulted in a 70% increase in the cellulase activity over the control of non-optimized basal medium. Optimum cellulase production was found 11.23 U/mL in a fermentation medium containing wheat bran (1.03%, w/v), soybean meal (2.43%, w/v), and malt dextrin (2.95%, w/v) [10]. A research worked in Egypt, *Bacillus subtilis* KO strain was tested by two methods (CMC clear zone and DNS techniques). After 24h of fermentation, the best production cellulase activity isolated. It was 35 I.U by CMC method and 420 µg/ml broth medium by DNS method with cellulose and trypton inclused [11].

3. CONCLUSION

Production of cellulase enzymes using *Bacillus sp.* on medium synthesis was fitted to quadratic model. Endo-β-1,4-glucanase activity production showed significant dependence on CMC concentration, source nitrogen such as yeast extract and meat extract concentration. CMC played the role of Substrates Inducer which was very important in the production enzyme. If the absent of CMC can cause the absent enzyme cellulase and CMC concentration was influenced this enzyme activity. Additionally, several studies were verify that *Bacillus* were the bacteria used nitrogen source as very important energy source for many pathway in cell. In this work, those factors such as yeast extract and meat extract presented their effects to enzyme activity. The interaction effects of two independent variables were significant. It was interaction of CMC and yeast extract concentration. The optimum of the medium of fermentation from *Bacillus sp.* obtained by the statistical method that was successfully established to maximise the enzyme production in study's condition. The maximum of cellulase activity was 2.48 U at the concentration of Carboxymethylcellulose 0.25% w/v, yeast extract 0.7% w/v and meat extract 0.2% w/v.

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Tối ưu hóa thành phần môi trường lên men để sản xuất Enzyme Cellulase từ vi khuẩn Bacillus Sp. bằng phương pháp đáp ứng bề mặt

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TÓM TẮT

Trước đây, tối ưu hóa trong các thí nghiệm sinh học thường được thực hiện bằng cách phân tích ảnh hưởng của từng yếu tố tại một thời điểm nhất định. Nhược điểm của phương pháp này là không đưa ra được tương tác giữa các yếu tố nghiên cứu và số lượng các thí nghiệm lớn. Tuy nhiên, phương pháp đáp ứng bề mặt (Response Surface Methodology-RSM) đã được sử dụng rộng rãi cho mục đích tối ưu hóa đã giải quyết được hai vấn đề trên. Phương pháp này sử dụng toán học và thống kê để nghiên cứu tối ưu hóa với số lượng các thí nghiệm ít nhất. Trong nghiên cứu này, trước tiên, bằng những thí nghiệm sàng lọc, chúng tôi đã lựa chọn được một trong bảy chủng vi khuẩn Bacillus trong Vietnam Type Culture Collection (VTCC) có thể sản xuất cellulase với CMC là cơ chất cảm ứng. Trong số bảy chủng Bacillus, chủng VTCC-B-0497 cho kết quả tốt nhất. Sau đó, chỉ với 15 thí nghiệm được thiết kế theo dạng ma trận Box-Behnken đã xây dựng hàm mô tả bề mặt đáp ứng phụ thuộc vào thông số đầu vào có thể dùng để dự đoán. Hoạt độ cellulase tối ưu là 2.48 U tương ứng với nồng độ của ba yếu tố môi trường tối ưu lần lượt là Carboxymethylcellulose 0.25 % w/v, cao nấm men 0.7 % w/v và cao thịt 0.2 % w/v.

Từ khóa: *Lên men, Bacillus sp., Cellulase, Tối ưu hóa*

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