IRJSE International Research Journal of Science & Engineering
Int. Res. J. of Science & Engineering, Volume 8 (4) 2020
ISSN: 2322-0015
SJIF Impact Factor 6.70
RESEARCH ARTICLE
OPEN ACCESS

Statistical Optimization of Cellulase production by haloalkaliphilic *Bacillus* sp. Isolated from Meteoritic Crater of Lonar Lake

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Manuscript Details
Received :12.05.2020
Revised : .01.07.2020
Accepted : 19.08.2020
Published 30.08. 2020

Available online on https://www.irjse.in
ISSN: 2322-0015

Editor: Dr. Arvind Chavhan

Cite this article as:
Umrikar AM and Hamde VS. Statistical Optimization of Cellulase production by haloalkaliphilic *Bacillus* sp. Isolated from Meteoritic Crater of Lonar Lake, *Int. Res. Journal of Science & Engineering*, 2020, Volume 8(4): 125-130.

Abstract

The enzyme cellulase has the ability to catalyze the carbohydrate which is a polymer of glucose linked with β-1, 4, glycosidic linkage. Such type of cellulase plays an important role in different industries like food, pharmaceutical, textile, leather, fuel etc. In the present investigation the potential haloalkaliphilic bacterial strains were used for the cellulase production. Because the present bacterial strain grows in two different extreme conditions such as high salt and high pH. The obtained cellulase after optimization may have some different chemical composition and improved activity as well. The six physicochemical parameters were used such as glucose, yeast extract, inoculums size, agitation, pH & temperature. Statistical model was designed on the basis of response surface methodology (RSM). In the obtained model among the 33 responses we got 7th run combination showed the 1.2 U/mg of cellulase activity. When we find the analysis of variance (ANOVA) it reveals below 0.05 p values it indicated the proposed model was significant. The obtained p value was 0.0482 it means a significant data for the cellulase production by the *Bacillus sp*.

Key words: Lonar Lake, Soil sample, Haloalkaliphilic strains, cellulase, RSM.

1. Introduction

The Lonar Crater Lake is a Lonar Soda Lake situated in the Buldhana District of the Maharashtra State of India. The formation of Lonar lake is the result of meteor impact.

The alkalinity of Lonar lake is in the range of 10-10.5 which is due to the presence of sodium carbonate [1]. Cellulase is one of the most important enzymes possessing the industrial importance [2, 3]. The diverse array of microorganism in the soda lake was also studied in reference to their industrial importance [4, 5]. The selection of isolates is based on their ability to tolerance of pH, salinity and enzyme production at optimum level [6, 7]. Halophilic bacteria possess the ability to produce cellulase which has broad range of application in certain cases the enzyme activity is shown to be affected by temperature [8]. The uniqueness of Lonar Lake is its salinity and alkalinity which harbors various unidentified, unique haloalkaliphilic bacterial species.

Chemoorganotrophs produce hydrolytic enzymes such as proteinases, cellulases, lipases and amylases in order to utilize the products of primary production. Alkaliphiles require pH 9 or more for their growth [9]. The genus *Bacillus* comprises a variety of industrially important species and has a history of safe use in both food and pharmaceutical industry. Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much interest because of their ability to produce extracellular metabolites that are active and stable at high pH [10]. Halophilic bacteria from marine environment are also better sources of enzymes of industrial importance that may have potential of pharmaceutical and biotechnological applications from Lonar Lake which could be further explored for its biotechnological potential.

2. METHODOLOGY

Haloalkaliphilic isolate:
The sediment sample was collected from Lonar Lake in sterile screw cap tubes and streaked over the Nutrient agar plates and CMC agar plates. All the plates were incubated at 35°C for 24 to 48 hrs. The medium Carboxy Methyl Cellulose [CMC] broth pH 9.5 was used for the enrichment of the cellulolytic bacteria. Among all the isolate the most potential haloalkaliphilic microorganism is used in the present investigation for optimization of higher cellulase production [11].

Cellulase production medium:
The cellulase producing liquid medium containing [%] $NaNO_3$- 0.1, K_2HPO_4-0.1, KCl-0.1, $MgSO_4$-0.1, Yeast Extract-1.5, glucose-1, NaCl-5, pH-9.5. The medium was sterilized in autoclave at 121°C for 15-20 min. After sterilization medium were cooled and 3% active *Bacillus* sp. culture was inoculated in the production medium. Incubate the medium at 35°C for 48 h in shaker incubator having 130 rpm. After incubation the culture medium was centrifuged at 8000 rpm having for 15 min at 4°C in high speed centrifuge [Remi make]. After centrifuge collect the supernatant as a crude enzyme source for to study the cellulase production rate [7].

Cellulase assay:
Cellulase activity was measured by mixing 20µl of crude enzyme and 200 µl of 0.5% CMC in 25mM Tris-HCl buffer [pH-9]. The reaction mixture was incubated at 37°C for 30 min in shaker water bath. The released reducing was measured at 540nm in UV-Vis Spectrophotometer.

Table 1: Experimental Design layout of physicochemical compounds using central composite design [CCD].

Variables	Unit	Lower [-1]	Higher [+1]	Alpha -	Alpha +
Glucose	%	**1**	**3**	0.434915	3.56508
Yeast extract	%	**1**	**5**	-0.130169	6.13017
Agitation	RPM	**100**	**200**	71.7458	228.254
pH	pH	**8**	**11**	7.15237	11.8476
Temperature	°C	**25**	**40**	20.7619	44.2381
Inoculum size	%	**1**	**5**	-0.130169	6.13017

Protein content was determined by Lowery's method using BSA as a standard protein. One unit [U] of cellulase activity was defined as the amount of crude enzyme that catalyzes 1 µmol of reducing sugar as glucose per minute under assay conditions and expressed as U/ml. All the experiments were performed in triplicate [7].

Optimization of Physicochemical parameters for cellulase production with Response surface methodology [RSM]:

The following physicochemical parameters were selected for the present investigation (Table: 1). The statistical software package Design-Expert 8.0 [Stat-Ease Inc., Minneapolis, USA] was used to analyze the experimental design. A factorial central composite experimental design, with six factors, leading to a set of 33 experiments, was achieved to optimize the production of cellulase from *Bacillus sp*. The minimum and maximum ranges of variables investigated [12].

3. RESULTS AND DISCUSSION

The cellulase production was analyzed by using different physical and chemical parameters it includes Temperature, Agitation rate, Incubation time, pH, Carbon source, Nitrogen source, etc. in the present investigation response surface methodology (RSM) was used to find the central composite design (CCD).

Table 2: Central composite design [CCD] for six variables with observed response [Cellulase activity].

Std	Run	Factor 1 A:Glucose %	Factor 2 B:Yeast extr... %	Factor 3 C:Agitation RPM	Factor 4 D:pH pH	Factor 5 E:Temperature °C	Factor 6 F:Inoculum si... %	Response 1 R1 U/mg
8	1	3.00	5.00	100.00	8.00	40.00	5.00	0.7
11	2	1.00	5.00	100.00	8.00	25.00	5.00	0.51
28	3	2.00	3.00	150.00	9.50	32.50	6.13	0.63
20	4	2.00	6.13	150.00	9.50	32.50	3.00	0.86
33	5	2.00	3.00	150.00	9.50	32.50	3.00	0.85
2	6	3.00	5.00	200.00	8.00	40.00	1.00	0.61
32	7	1.00	1.50	130.00	9.50	35.00	3.00	1.2
10	8	1.00	1.00	200.00	8.00	25.00	5.00	0.63
21	9	2.00	3.00	71.75	9.50	32.50	3.00	0.41
23	10	2.00	3.00	150.00	7.15	32.50	3.00	0.31
7	11	1.00	5.00	200.00	8.00	25.00	1.00	0.34
6	12	3.00	1.00	200.00	11.00	25.00	5.00	0.68
18	13	3.57	3.00	150.00	9.50	32.50	3.00	0.93
14	14	1.00	1.00	200.00	11.00	40.00	5.00	0.83
13	15	1.00	1.00	100.00	11.00	40.00	1.00	0.8
12	16	3.00	1.00	100.00	8.00	40.00	1.00	0.69
19	17	2.00	0.00	150.00	9.50	32.50	3.00	0.23
30	18	2.00	3.00	150.00	9.50	32.50	3.00	1.01
25	19	2.00	3.00	150.00	9.50	20.76	3.00	0.73
9	20	3.00	1.00	100.00	11.00	25.00	1.00	0.84
5	21	1.00	5.00	100.00	11.00	40.00	5.00	0.73
26	22	2.00	3.00	150.00	9.50	44.24	3.00	0.35
17	23	0.43	3.00	150.00	9.50	32.50	3.00	0.21
4	24	3.00	1.00	200.00	8.00	40.00	5.00	0.63
31	25	2.00	3.00	150.00	9.50	32.50	3.00	1.09
1	26	3.00	5.00	200.00	11.00	25.00	1.00	0.96
27	27	2.00	3.00	150.00	9.50	32.50	0.00	0
3	28	3.00	5.00	100.00	11.00	25.00	5.00	0.67
29	29	2.00	3.00	150.00	9.50	32.50	3.00	1.12
24	30	2.00	3.00	150.00	11.85	32.50	3.00	0.29
15	31	1.00	5.00	200.00	11.00	40.00	1.00	0.39
16	32	1.00	1.00	100.00	8.00	25.00	1.00	0.26
22	33	2.00	3.00	228.25	9.50	32.50	3.00	0.53

The statistical analysis reveals the 33 different run for to analyze the production rate of cellulase by *Bacillus* sp. According to Bhusare & *et al* the conventional method of optimization involves variation of one parameter at a time and keeping the others constant. This is a extremely time consuming and expensive method when a large number of variables are considered and also does not often bring up the effect of interaction of various parameters as compared to factorial design. These limitations of a single factor optimization process were eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM). Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria [12].

In order to search for the optimum formulation of the medium, central composite design (CCD) with six factors, this indicated that 33 experiments [run] were required for this procedure. The obtained result showed optimum parameters for cellulase production. (Table 2).

As per above obtained results the 33 variables showed different combinations of physicochemical parameters such as glucose (%), yeast extract (%), agitation[RPM], pH, temperature (°C) and inoculums size (%). In the above table 7, 18, 25 & 29 showed the maximum cellulase activity in term of U/mg. Among them the 7th run reveals optimum physicochemical parameters such as glucose 1%, yeast extract 1.5%, agitation rate 130 rpm, pH 9.5, temperature 35 °C and 3% active inoculums for cellulase production. After using this concentration we got 1.2 U/mg of cellulase activity. On the basis of the above obtained response it was summarized as below (Table:3).

Table 3 Summary selected parameters in CCD:

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared
Mean	< 0.0001			
Linear	0.6767	0.0675	-0.0665	-0.1776
2FI	0.9820	0.0265	-0.7770	-6.1017
Quadratic	0.9609	0.0059	-2.1422	-1198.8948
Cubic	0.0059		0.8296	

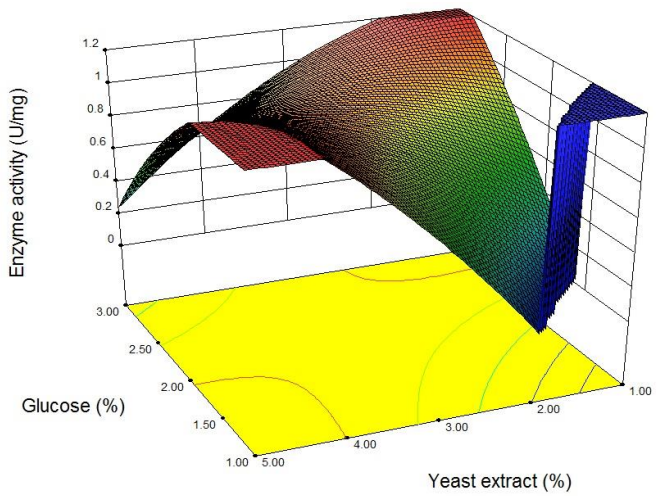

Fig: 1. Response surface plot of cellulase activity showing the interaction between glucose [%] and yeast extract [%] at the constant values of all other parameters.

The three-dimensional response surface and contour presentations were plotted to study the interaction among various physical parameter formulation factors used and to find out the optimum level of each factor for maximum cellulase production from *Bacillus sp.*

However, to understand the interaction behaviors of physical parameters, the response surfaces were investigated for glucose and yeast extract of variables, keeping the other factors constant (Fig:1)

Table 4. Analysis of variance [ANOVA] for response surface quadratic model obtained from experimental designs.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.40	27	0.052	0.19	0.0482	significant
A-Glucose	*0.097*	*1*	*0.097*	*0.36*	*0.5742*	
B-Yeast extra	*0.077*	*1*	*0.077*	*0.29*	*0.6160*	
C-Agitation	*4.703E-006*	*1*	*4.703E-006*	*1.744E-005*	*0.9968*	
D-pH	*7.614E-003*	*1*	*7.614E-003*	*0.028*	*0.8732*	
E-Temperatu	*0.039*	*1*	*0.039*	*0.14*	*0.7192*	
F-Inoculum s	*0.27*	*1*	*0.27*	*1.01*	*0.3601*	
AB	*0.065*	*1*	*0.065*	*0.24*	*0.6435*	
AC	*5.021E-003*	*1*	*5.021E-003*	*0.019*	*0.8968*	
AD	*0.054*	*1*	*0.054*	*0.20*	*0.6735*	
AE	*0.080*	*1*	*0.080*	*0.30*	*0.6092*	
AF	*0.11*	*1*	*0.11*	*0.41*	*0.5502*	
BC	*0.18*	*1*	*0.18*	*0.65*	*0.4561*	
BD	*7.656E-003*	*1*	*7.656E-003*	*0.028*	*0.8728*	
BE	*0.032*	*1*	*0.032*	*0.12*	*0.7460*	
BF	*1.950E-004*	*1*	*1.950E-004*	*7.231E-004*	*0.9796*	
CD	*3.306E-003*	*1*	*3.306E-003*	*0.012*	*0.9161*	
CE	*0.046*	*1*	*0.046*	*0.17*	*0.6979*	
CF	*0.088*	*1*	*0.088*	*0.33*	*0.5922*	
DE	*0.011*	*1*	*0.011*	*0.040*	*0.8489*	
DF	*0.026*	*1*	*0.026*	*0.098*	*0.7670*	
EF	*6.006E-003*	*1*	*6.006E-003*	*0.022*	*0.8872*	
A^2	*0.036*	*1*	*0.036*	*0.13*	*0.7303*	
B^2	*0.011*	*1*	*0.011*	*0.041*	*0.8479*	
C^2	*6.808E-003*	*1*	*6.808E-003*	*0.025*	*0.8800*	
D^2	*0.13*	*1*	*0.13*	*0.48*	*0.5198*	
E^2	*2.028E-004*	*1*	*2.028E-004*	*7.517E-004*	*0.9792*	
F^2	*0.12*	*1*	*0.12*	*0.44*	*0.5375*	
Residual	1.35	5	0.27			
Lack of Fit	*1.30*	*2*	*0.65*	*44.60*	*0.0059*	*significant*
Pure Error	*0.044*	*3*	*0.015*			
Cor Total	2.75	32				

The p values showed that the all variables had significant effect on cellulase activity [P<0.05] from haloalkaliphilic *Bacillus* strain. The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used and cited in Table 4. The ANOVA of the quadratic regression model indicates the model is significant. Statistical analysis of the RSM demonstrates that the Model F-value implies the model is significant [table 4].

The present experimental model showed the overall designed statistical model is significant showed 0.0482 of its p-value and lack of fit was 0.0059. This means that the present model we can use for the production of cellulase by Bacillus strain. Hence the statistical model proves the combination of all the parameters are significant for the cellulase production in submerged conditions.

4. CONCLUSION

The present investigation showed the cellulase production in terms of cellulase activity was determined by response surface methodology [RSM] of its central composite design [CCD]. This statistical model proves the variations of the physicochemical parameters are essential to optimize the cellulase production by *Bacillus* sp. Because in the experimental table lower and higher values are gives us the variable runs having various concentrations.

Conflict of interest

No conflict of interest influenced in this research.

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