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Optimizing Cellulase Production from a thermo stable bacterium *Rhodthermus* marinus

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Abstract: The high demand of a cheap and sustainable energy source are necessity for all nation all over the world. Cellulose fulfills these conditions and it is the most abundant energy source on earth. Conversion of cellulose into renewable biofuels such as ethanol or butanol requires digestion by cellulases, which are abundant in microbial world. Thermotolerant bacteria are good and promising producers of this enzyme. In this study, we optimized the growth condition of *R. marinus* to produce the highest activity of this enzyme. The Centeral Composite Design was used to optimize the physical factors (pH, temperature, and incubation period), and Placket-Burman experimental design was used to determine the best nitrogen and minerals sources. The enzyme activity was determined by the reducing sugar method, which showed that manganese sulphate (0.01g/L) was the best. While, yeast extract was the best for growth of the tested bacterium and the malt extract produced the highest amount of enzyme. In conclusion, the study showed that CMC, malt extract and yeast extract in presence of manganese sulphate *R. marinus* produced the highest concentration of thermotolerant cellulase needed for conversion of cellulose into biofuel.

keywords: *Rhodothermus marinus*, cellulase, optimization, Response surface methodology**.**

1.Introduction

One of the most abundant and renewable energy sources on the planet is cellulose that may be used to create valuable goods like sugar and biofuels.. Microbial cellulases (EC3.2.1.4) from fungi, mold, and bacteria, which are widely spread in nature, degrade celluloses [1]. Bacteria, a unicellular microbe, are the most preferred organism for fermentative production of cellulase due to fast growth, ease of handling and easy genetic manipulation and less energy utilization [2].

Cellulases are the enzyme systems that release glucose units in the cellulose polymer by hydrolyzing the β-1, 4 glycosidic bonds.[3]. It has remarkable a cellulose-degradings industries such as pharmaceutical industry, cattle feed, alternate energy, food nutrition, agriculture industry, and textile detergent [4].

Geothermal sources are where thermophiles mostly flourish since they offer the right kind of heated climate, which is what they prefer. [5]. Hot spring water's characteristics and chemical composition can vary in terms of its organic and inorganic chemical elements, pH, and nutrient content, which may create favourable conditions for microbial biodiversity. [6]. Under harsh conditions, some of the microorganisms that live in these hot springs develop and produce enzymes that have a high thermal stability. The phylum Bacteroidetes, which has three classes (Bacteroidetes, Flavobacteria, and Sphingobacteria), includes *Rhodothermus marinus* [7]. a thermophile and a halophile [8]In the cultivation supernatants of *R. marinus DSM 4253*, an exceptionally thermostable cellulase known as endo-b-1,4glucanase has been discovered. The enzyme was shown to break down carboxymethyl cellulose into glucose, cellobiose, cellotriose, and a combination of cellopentaose and bigger oligosaccharides. [9]

To increase enzyme production, various parameters can be manipulated, including physical ones like pH, incubation period, and temperature, as well as media elements like

carbon, nitrogen, and mineral supplies. These variables play a significant role in the cost of producing enzymes, which is frequently cited as the main bottleneck in biotechnological processes. Plackett-Burman Design is a factorial-based statistical technique that is used to assess the relevance of the important variables[10], while response surface methodology (RSM) is used to assess the interactions of the independent process variables. The goal of this study is to increase the activity of the cellulase produced by thermophilic bacteria's.

2. Materials and methods

Microbial Culture

Rhodothermus marinus, a local thermophilic bacterium isolated from Hammam Pharaon (South Sinai, Egypt, at latitude 29, 197112 and longitude32, 956179) and it was available at Bacteriology and Molecular Biology Laboratory, Faculty of Science, Mansoura University [11].This isolate was stored at -80ºC in 50 %glycerol and it was growen on zobelle media : yeast extract, 3.75g/l pepton, 9g/l NaCl, 2g/l $MgCl_2$ and .525 g/l KCl. pH was adjusted at 7 and incubated for seven days at 65ºC.

Measurements of cellulase activity

a.Qualitative assay

The cellulolytic activities of *R. marinus* strain (Qualitative and/or quantitative) were screened. The bacterium was grown on agar plates for 7 days at 65 ºC, then The 0.1 % Congo red solution was poured all over the plate., left for 20 minutes, washed several times with 1M NaCl solution [12].

Table 1.Chemical composition of CMC agar media [13]. .

Compound	Amount g/L
KH_2PO_4	1.0
$MgSO_4$	0.50
NaCl	10
FeSO4	0.01
MnSO4	0.01
NaNO3	1.0
C.M.C	10.0

b.Quantitative assay

Quantitative cellulase activity was determined in the culture supernatant by the standard methods [14, and 15]. *R. marinus* was in the CMC broth (g/L: 1 KH_2PO_4, 0.5 $MgSO_4$, 10 NaCl, 0.01 $FeSO_4$, 0.01 $MnSO_4$ 0.01, 1 $NaNO_3$, and 10 CMC) for 7 days at 65 ºC. 0.5 ml of the culture supernatant was added to 0.5 ml of CMC that has been dissolved in sodium phosphate buffer (0.1M, pH 7), and incubate for 1 hour at 65 °C., then 1 ml of the alkaline copper tartrate solution was added , incubated for 10 minutes in a water bath that is boiling, and then allow to cool. Then, for colour stabilisation, arseno-molybdate solution was added , and use a spectrophotometer (JENWAY 6395) to determine the optical density at OD 620 nm.One unit of enzyme equals the amount of 1.0 µg of glucose liberated in min/ml.

Optimization of physicochemical parameters

The best pH, temperature, and incubation period were optimized using the Central Composite Design (CCD). This is to show their effects on bacterial growth and cellulase production and activity [16]. Each variable in the CCD matrix has five levels, as stated in **Table 2. :** Variables and their levels used for the CCD experiment for pH, temperature and Incubation PeriodLevels

Variables	-2	-1	0	1	2
pH	5.31	6	7	8	8.68
Temperature	43.18	50	60	70	76.18
Incubation Period(days)	2.53	4	6	8	9.36

Screening for best nitrogen source

Plackett-Burman Design was used to determine the best nitrogen source for growth and cellulase activity following optimizing cultivation conditions by CCD in 100mL CMC broth at 60ºC for 6 days at pH7. Six different nitrogen sources were screened: beef extract, malt extract, ammonium sulfate, yeast extract, peptone, and ammonium chloride. The standard error was calculated by including a dummy variable, maximum (+) and minimum (-) values were used for each variable (Table 3).

Table 3. : The Six Different nitrogen sources and their levels

Variables	Unit	Minimum level (-)	Maximum level (+)
Yeast extract	g/l	0.5	2
Malt extract	g/l	0.5	2
Beef extract	g/l	0.5	2
Peptone	g/l	0.5	2
Ammonium sulfate	g/l	0.5	2
Ammonium chloride	g/l	0.5	2
Dummy	-	Bidistilled water	Distilled water

Responses were estimated in terms of growth (OD_{600nm}) and cellulase activity (U/ml) .

The experimental data were fitted in a linear regression equation

$$Y= \beta 0 + \Sigma \beta_i X_i(I =1,2,\ldots\ldots,k) \text{ (Eq1)}$$

Where Y is the calculated response, β0 is model intercept, βi is the regression coefficient for each variable, Xi is the corresponding variable and k is the number of variables [17]. Significance of variables was determined by probability with *p*-values less than 0.1.

Optimizing of the medium component using CCD

A subsequent CCD was done to detect the optimum levels of yeast extract, malt extract and CMC on cellulase activity. To optimise the possible medium component, 20 trials were run across 3 parameters and 5 levels (Table 4) [18].

Table 4. : Variables and their levels used in the CCD experiments for carbon and nitrogen sources optimization

Levels					
Variable(g/l)	**-1**	**-2**	**0**	**1**	**2**
Yeast extract	0.318	1	2	3	3.68
Malt extract	0.318	1	2	3	3.68
CMC	1.59	5	10	15	18.4

The low and high levels for yeast and malt extracts were 1 g/l and 3 g/l, respectively. While the levels for CMC (c-source) were 5 g/l and 15 g/l respectively.

The prediction of the optimum medium composition for cellulase activity was generated by second-order polynomial model, as shown in Eq. 2 . :

$$Y = \beta 0 + \Sigma \beta i Xi + \Sigma \beta ii Xii + \Sigma bij Xij \text{ (Eq2)}$$

Where βi is the regression coefficient for each factor, βii is the regression coefficient for square effects and βij are the regression coefficients for interactions. Analysis of variance (ANOVA) was done by using Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA.

Screening of mineral sources

The effects of six different mineral salts (KH2PO4, MgSO4 .7H2O, NaCl, FeSO4 .7 H2 O, MnSO4. H2O, CaCl2) on the activity of the cellulase produced by *R. marinus* were tested. The standard errors of the experiments were calculated by inclusion of one dummy variable. Each variable was tested at a low (-) and a high (+) levels as shown in Table 5. The experiments were done in a 100ml medium (CMC 10g/l, yeast extract 2g/l, malt extract 2g/l) incubated at optimized condition 60ºC for 6 days at pH 7. Responses or cellulase activity (U/ml).were estimated.

Table 5. The Plackett-Burman Design of the six minerals salts and their levels on cellulase activity.

Variables	Unit	Minimum level (-)	Maximum level (+)
KH_2PO_4	g/l	0.5	2
$MgSO_4.7H_2O$	g/l	0.5	2
NaCl	g/l	5	20
$FeSO_4.7\ H_2O$	g/l	0	.02
$MnSO_4.\ H_2O$	g/l	0	.02
$CaCl_2$	g/l	0	.3
Dummy	-	Bidistilled water	Distilled water

3. Results and Discussion

Demonstration of cellulase activity

By spotting a pale orange to clear zone surrounding the expanding colonies against a red backdrop, a quick initial screening of the cellulase activity of the local *R. marinus* strain was conducted. (Fig 1). This suggests the ability of the local bacterium to secrete cellulase enzyme, which diffused to the surroundings and hydrolyzed the CMC. This is in accordance to the reports of several investigators such as [19] ,[20].

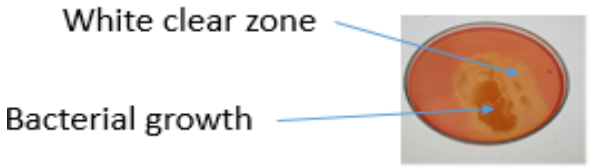


Fig. 1. *R. marinus* growth and cellulase production on CMC medium, arrow shows the clear zone and bacteria grwoth.

Central Composite Design and optimization of physico-chemical parameters

Optimizing growth and cellulase activity started with optimization of the physicochemical factors: pH, temperature and incubation period. Twenty different trials were performed, each with its unique integration, the responses were evaluated at the end of each experiment. The highest activity of cellulase was 76.6U/ml and maximum bacterial growth

were obtained in trials numbers: 2, 3, 7, 8, 14 and 20 as shown in Table 6 . A multi-way ANOVA analysis was performed on the data (Table 7), and it was observed that a cut-off P-value of 0.1 was utilised to indicate a factor's statistical significance at a 90 percent confidence level. Significantness was defined as a P-value of equal to or less than. Table provided a summary of the analysis of quadratic effects, the interactions between components, and the linear variance (7)

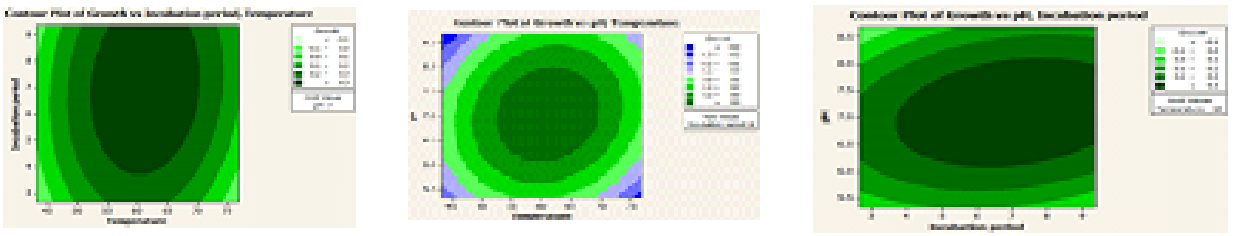
Table 6. : Matrix and responses for the CCD experiment for pH, Temperature and Incubation period.

Run	**Variables**			**Responses**	
	pH Temperature Incubation°C Period			**Optical CellulaseDensity Activity OD($_{600}$) (U/ml)**	
1	8	70	8	0.316	2 4.00
2	7	60	6	0.390	76.3
3	7	60	6	0.400	76.53
4	8	70	4	0.150	37.95
5	8.68179	60	6	0.103	54.79
6	8	50	4	0.050	3.00
7	7	60	6	0.392	76.60
8	7	60	6	0.400	76.60
9	7	60	2.63641	0.200	1.50
10	6	70	8	0.205	27.29
/11	8	50	8	0.134	32.20
12	6	50	8	0.159	10.95
13	6	70	4	0.166	4.87
14	7	60	6	0.410	76.50
15	5.31821	60	6	0.050	2.50
16	7	60	9.36359	0.300	12.70
17	7	43.1821	6	0.032	3.20
18	7	76.8179	6	0.003	0.06
19	6	50	4	0.147	14.93
20	7	60	6	0.400	76.60

Table 7. Analysis of variance (ANOVA) of the CCD experiment for pH, temperature and Incubation Period optimization.

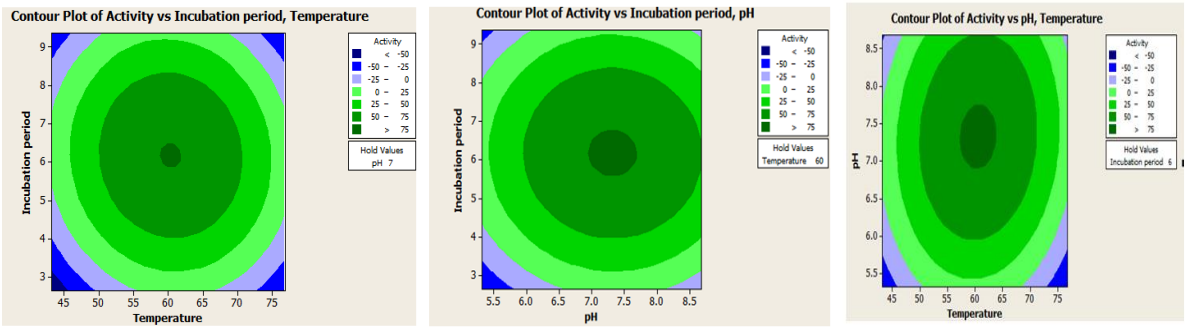
Variable	**Responses**					
	Optical density (OD600)			**Cellulase Activity (U/mL)**		
	Sum of squares	F value	P value	Sum of squares	F value	P value
A:pH	0.000283	0.10	0.758	1183.6	8.18	0.017
B:Temperature	0.006512	2.31	0.159	56.0	0.39	0.548
C:Incubation Period	0.016119	5.72	0.038	202.7	1.40	0.264
A^2	0.099986	47.40	0.000	1533.8	20.57	0.001
B^2	0.188066	70.16	0.000	6878.0	56.99	0.000
C^2	0.017593	6.24	0.032	6967.3	48.15	0.000
AB	0.005886	2.09	0.179	52.8	0.37	0.559
AC	0.004950	1.76	0.215	1.3	0.01	0.925
BC	0.001485	0.53	0.485	34.7	0.24	0.635

The R^2 for cellulase activity value is 92.12%. and R^2 for growth (OD_{600}) is 92.36%



(A) (B) (C)

Fig 2. : Contour plot showing the interaction between the tested variables for maximum bacterial growth vs A . incubation period, and temperature, B. temperature and pH, and C. incubation period and pH.



(A) (B) (C)

Fig 3. : Contour Plot showing the optimum interaction between testd variables on maximum cellulase activity. vs A. incubation period, and temperature, B. incubation period and pH., and C. temperature and pH.

The optimum temperature was 60 °C, the optimum pH range was 7 and incubation period range 6 days which agreed with what has been reported [21]. Transporting of a number of chemical products and enzymes across the cell membrane strongly influenced by growth medium pH so it affect many enzymatic reactions [22,23]. The highest activity of the cellulase produced by tested strain was obtained at pH 7 that was agreed [24] who reported that *Streptomyces sp. B-PNG23* produce far more cellulase enzyme at a pH value of 7, which was found to be ideal. According to earlier studies, *Bacillus sp*. produced the highest cellulase when the starting medium pH was between 7.0 and 7.2 in submerged fermentation [25,26]. But [27] discovered that cellulase production by bacteria isolated from cow dung and municipal solid waste was optimum at beginning medium pH of 6.0 and incubation temperature of 40 °C. In another study [28] an initial medium pH of 6.5 was optimized through central composite design of response surface methodology for cellulase production by *Brevibacillus parabrevis (MTCC 2208)*.[29] showed that the pH 9.0 was the optimal level for *Clostridium acetobutylium* to produce cellulase. Temperature altered extracellular enzyme secretion, potentially by altering the physical characteristics of the cell membrane. [30]. In the present study, the optimum temperature for the activity of the cellulase produced by *Rhodothermus marinus* was 60°C. This result was in agreement with [31] who found that the optimal temperature for *Bacillus subtilis* strain *LFS3* to produce cellulase was 60 °C. [32] reported that the best pH and temperature for the crude CMCase enzyme produced from *B. subtilis K-18* during submerged fermentation were 7.0 and 50 °C. According to a another investigation, *Bacillus sp.* generated cellulase enzyme with ideal pH and temperature ranges of 6 and 50 °C. [33]. According to [34], the ideal pH and temperature for *Bacillus subtilis* cellulase were7 and 50 °C. [35]. It was discovered that *Pseudomanas sp*. and *Bacillus sp*. both produce cellulase best at temperatures of 40 and 50 ^{o}C, respectively.

Screening for best nitrogen source

The most significant nitrogen source for bacterial growth and activity of cellulase were determined by Plackett-Burman screening experiments design, The best combination was then found using a Response Surface Methodology, and a mathematical model that could be applied in the prediction process was built. The values of the targets to be optimised (growth and cellulase activity) were computed for a total of 12 trials, each with a distinct combination of the examined factors as showen in table 8.

The experimental responses and parameter estimations were analysed using an ANOVA; the results are described in Tables 9 and 10. At a 90% level of confidence, statistical analyses of the data using Minitab 16 identifies the most important variables that affect bacterial growth and cellulase activity. A negative significant variable's effect indicates that the variable has had effect on the response with low

concentration. A significant variable's positive effect indicates that the variable has an influence on the response with high concentration

Table 8: six Different material studied by their levels on bacterial growth and cellulase enzyme production by *Rhodothermus marinus* using Plackett-Burman design.

Run	Variables							Responses	
	Yeast extract	Peptone	Malt extract	Beef extract	Ammonium chloride	Ammonium sulfate	Dummy	OD_{600}	activity (U/ml)
1	2.0	0.5	2.0	0.5	0.5	0.5	Distilled water	1.360	18.880
2	0.5	2.0	0.5	0.5	0.5	2.0	Distilled water	0.555	30.414
3	0.5	2.0	2.0	0.5	2.0	0.5	Bidistilled water	0.455	14.600
4	2.0	2.0	2.0	0.5	2.0	2.0	Bidistilled water	0.331	17.280
5	2.0	2.0	0.5	2.0	2.0	0.5	Distilled water	1.630	0.020
6	0.5	2.0	2.0	2.0	0.5	2.0	Distilled water	0.343	10.260
7	2.0	0.5	2.0	2.0	0.5	2.0	Bidistilled water	0.461	22.680
8	0.5	0.5	0.5	2.0	2.0	2.0	Bidistilled water	0.800	0.020
9	0.5	0.5	2.0	2.0	2.0	0.5	Distilled water	0.472	15.170
10	2.0	2.0	0.5	2.0	0.5	0.5	Bidistilled water	1.400	0.020
11	0.5	0.5	0.5	0.5	0.5	0.5	Bidistilled water	0.677	0.020
12	2.0	0.5	0.5	0.5	2.0	2.0	Distilled water	1.230	2.840

Table 9. : Statistical analysis of Plackett-Burman experiment showing the effect, regression coefficient, *T*-value and *P*-value for each variable on cellulase activity.

Variables	Activity of cellulase (U/ml)			
	Effect	Coefficient	*T*-value	*P*-value
Malt	5.468	2.734	2.08	**.083**
Beef	-2.992	-1.496	-1.14	.299
Ammonium chloride	-2.696	-1.348	-1.03	.345
Ammonium sulfate	2.902	1.451	1.10	.312

Table 10. : Statistical analysis of Plackett-Burman experiment showing the effect, regression coefficient, T-value and P-value for each variable on bacterial growth.

Variables	Optical density OD_{600}			
	Effect	Coefficient	*T*-value	*P*-value
Malt	-.4783	-.2392	-3.25	.032
Beef	.0830	.0415	.56	.603
Ammonium chloride	.0203	.0102	.14	.897
Ammonium sulfate	-.3790	-.1895	-2.57	.062
Yeast	.5183	.2592	3.52	.025
Pepton	-.0477	-.0238	-.32	.763

Malt extract and ammonium sulphate were shown to have a statistically significant negative effect on bacterial growth with P-values< 0.1, however yeast extract was found to have a statistically positive significant influence on bacterial growth with P-values <0.1(0.025). (0.032for malt extract, 0.062 for ammonium sulfate). Malt extract was discovered to have a considerable positive impact on cellulase activity, with a P-value of less than 0.1to be 0.083, as indicated in the table 9

that agreed with[36] who stated that organic nitrogen was used to enhance cellulase production.[37] also stated that organic nitrogen sources were preferable to inorganic nitrogen sources for maximising the cellulase production by *B. subtilis and B. circulans*. [38] found that malt extract showed an increase in cellulase production among other nitrogen sources. In another study presence of peptone, yeast extract, and MgSO4 were discovered to be significant for cellulase synthesis in submerged fermentation by *Bacillus cereus* [39].However [40] ammonium sulphate and

ammonium hydrogen carbonate were found to be the optimum nitrogen sources for *Bacillus licheniformis APS2 MSU* and *Bacillus altitudinis APSMSU's* as it increase ability to produce cellulase.In another study *Bacillus aquimari*s, which was isolated from the intestine of a Labeo rohita, was shown to be the greatest nitrogen source for producing endoglucanase in submerged fermentation when using ammonium sulphate . [41]. Since nitrogen is a primary component of protoplasm and a primary building block of proteins, it has an impact on bacterial growth and cellulase synthesis . [42]

.**Optimizing of the medium component using CCD**

After identifying the most significant factors that affect bacterial growth and cellulase activity that was yeast extract for bacterial growth and malt extract for cellulase activity, As sources of nitrogen, yeast extract and malt extract were chosen and CMC as a carbon source. CCD matrix was used to detect the optimum medium composition for optimizing cellulase activity and bacterial growth and determine the interaction between tested variables. The responses and matrix were summarized in Table 11.

Twenty different trials were performed, each with its unique combination, in addition to a 6 replicates. The results were exposed to ANOVA analysis (Table 12). The optimum medium was malt extract 2 g/L, yeast extract 2g/L, CMC 10 g/L. [43] revealed that by maximising a variety of medium components using response surface methodology, the optimal concentration of yeast extract was2.00 g/L for cellulase production utilizing by *Bacillus tequilensis S28* in submerged fermentation.. [44] tested several medium components for the generation of cellulase and reported that yeast extract 2.14 g/Lwas using *Cellulomonas fimiNCIM-5015* in submerged fermentation employing response surface methods. That was agreed with obtained result. The best concentraction of CMC positively induced activity of cellulase

was10 g/L that was close to [45] which claimed that 15 g/L of CMC caused *Enhydrobacter sp. ACCA2* to exhibit its highest cellulolytic activity, [46] observed a sharp rise in cellulase activity in *Bacillus subtilis AS3* at higher concentrations (18 g/L) of CMC, while [47] found that *Bacillus amyloliquefaciens SS35* required 19.05 g/L of CMC to generate the highest levels of CMCase release.

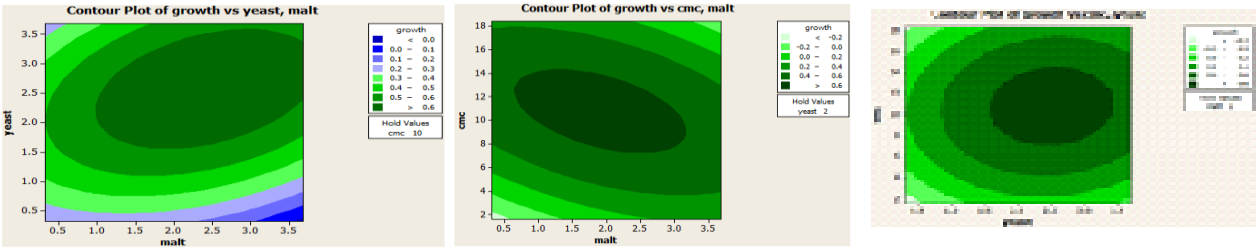
Table11. : Matrix and responses of CCD applied for carbon and significant nitrogen sources optimization.

Run	**Variables**			**Responses**	
	Malt extract	Yeast extract	CMC	OD_{600}	Activity (U/ml)
1	3.0	3.0	5.0	0.430	31.170
2	3.0	1.0	5.0	0.250	13.800
3	3.6	2.0	10.0	0.562	27.750
4	2.0	2.0	10.0	0.671	85.213
5	2.0	2.0	10.0	0.653	85.120
6	2.0	2.0	10.0	0.644	85.305
7	1.0	1.0	5.0	0.083	0.500
8	0.3	2.0	10.0	0.525	19.660
9	2.0	2.0	10.0	0.655	85.000
10	2.0	0.31	10.0	0.231	16.160
11	2.0	2.0	10.0	0.671	85.500
12	2.0	2.0	1.59	0.201	0.030
13	2.0	2.0	18.4	0.240	20.500
14	3.0	1.0	15.0	0.180	58.660
15	1.0	3.0	5.0	0.247	38.240
16	2.0	2.0	10.0	0.671	85.000
17	1.0	1.0	15.0	0.483	38.000
18	3.0	3.0	15.0	0.546	58.590
19	1.0	3.0	15.0	0.46 5	18.070
20	2.0	3.6	10.0	0.567	15.213

Table 12. : ANOVA for the CCD experiment of medium components.

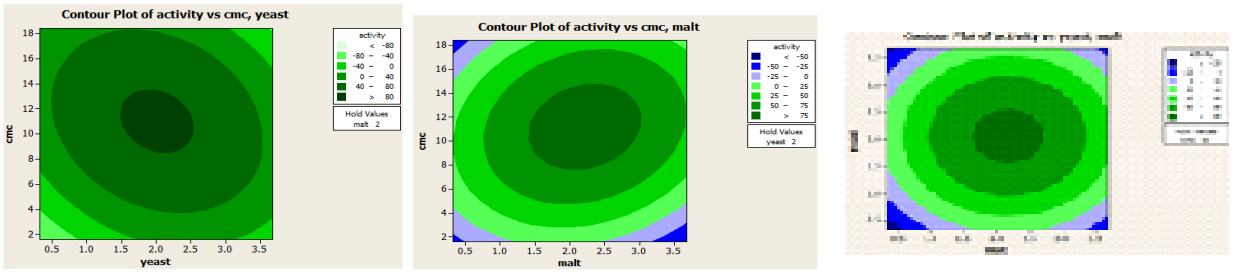
Variables	Optical density (OD600nm)			Cellulase Activity (U/mL)		
	Sum of squares	***F* value**	***P* value**	**Sum of squares**	***F* value**	***P* value**
A: Malt extract	0.002650	0.68	0.430	480.6	2.78	0.126
B: Yeast extract	0.115712	29.49	0.000	82.3	0.48	0.506
C: CMC	0.038977	9.93	0.010	1126.5	6.53	0.029
A^2	0.009330	8.82	0.014	2759.4	25.78	0.000
B^2	0.102404	36.79	0.000	4816.2	34.77	0.000
C^2	0.383801	97.82	0.000	7182.2	41.61	0.000
AB	0.020000	5.10	0.048	0.0	0.00	0.989
AC	0.040898	10.42	0.009	377.4	2.19	0.170
BC	0.000002	0.00	0.982	705.2	4.09	0.071

The R2 for cellulase activity value is (91.04%) and R2 for Optical density (OD600) (growth) is (94.79%).



(A) (B) (C)

Fig. 6. : Contour plot showing the interaction among the tested variables on the bacterial growth vs A. malt extract andyeast extract ,B. malt extract and cmc. C. CMC,yeast extract



(A) (B) (C)

Fig 7. : Contour plot showing the interaction among the tested variables on the activity of cellulase vs A. cmc and yeast extract , B. cmc and malt extract, C. yeast extract and malt extract.

Screening of minerals sources

Placket-Burman Design (PBD) was used to screen six minral sources for their effect on the activity of the cellulase produced by *Rhodothermus marinus.* Twelve different trials, each with its unique combination of the tested variables, were performed and the values of the targets to be optimized (cellulase activity) were calculated as shown in Table 13 and it was found that $MnSO_4$ is statistically significant with a negative effect on cellulase activity.it means that activity of cellulase increase in presence of low concentration of Mnso4 that was agreed with [48]that reported the lower activities of the enzyme at higher concentrations of the $MnSO_4$. 4H2O may be due to the oxidation of Manganese to MnO2 by *Aspergillus niger AH3*. In another study it was found that metal ions CO_{2+} and Mn_{2+} activated cellulases of *Cellulomonas sp.ASN2* [49]. Another authors [50] statistically optimized medium for cellulase production and reported that importance of MgSO4 for maximum cellulase production by *Bacillus amyloliquefaciens MBAA3*.[51] observed that the presence of $CaC_{l2}.6H_2O$ significantly improved the generation of cellulase by *Bacillus licheniformis*.

.**Table 13.** : Six Different minerals studied by their levels on activityof a cellulase enzyme produced by *Rhodothermus Marinus* using Plackett-Burman design.

Run	Variables							Responses
	KH_2PO_4	$MgSO_4$	NaCl	$FeSO_4$	$Mnso_4$	$CaCl_2$	dummy	activity (U/ml)
1	2.00	2.00	20.0	0.00	0.02	0.30	Distilled water	50.22
2	0.50	2.00	20.0	0.02	0.00	0.30	Bi-distilled water	77.40
3	1.25	1.25	12.5	0.01	0.01	0.15	Distilled water	64.80
4	2.00	0.50	20.0	0.00	0.00	0.00	Bi-distilled water	67.50
5	0.50	2.00	20.0	0.00	0.02	0.00	Distilled water	70.83
6	2.00	2.00	5.0	0.02	0.00	0.00	Distilled water	78.39
7	2.00	0.50	5.0	0.00	0.02	0.30	Bi-distilled water	68.67
8	0.50	0.50	20.0	0.02	0.02	0.00	Bi-distilled water	60.57
9	2.00	0.50	20.0	0.02	0.00	0.30	Distilled water	85.59
10	0.50	0.50	5.0	0.00	0.00	0.00	Distilled water	63.90
11	0.50	2.00	5.0	0.00	0.00	0.30	Bi-distilled water	74.70
12	0.50	0.50	5.0	0.02	0.02	0.30	Distilled water	56.43
13	1.25	1.25	12.5	0.01	0.01	0.15	Bi-distilled water	62.46
14	2	2	5	0.02	0.02	0	Bi-distilled water	34.20

The experimental responses and parameter estimations were analysed using ANOVA; the results are listed in Table 14. With a 90% level of confidence and $\alpha = 0.1$, a statistical analysis of the responses was conducted in the Minitab 16 environment to identify the most important variables influencing the activity of cellulase. The non-significant factors (P-value > 0.1) were those with a *P*-value greater than or equal to.

Table 14. : Statistical analysis of design of experiment showing the effect, regression coefficient, T value, and p value for each variable on activity of cellulase produced by *Rhodothermus Marinus.*

Variables	Activity of cellulase (U/ml)			
	Effect	**Coefficient**	***T*-value**	***P*-value**
KH_2PO_4	-3.210	-1.605	-0.42	0.695
$MgSO_4$	-2.820	-1.410	-0.37	0.730
NaCl	5.970	2.985	0.77	0.474
$FeSO_4$	-0.540	-0.270	-0.07	0.947
$Mnso_4$	-17.760	-8.880	-2.30	0.070
$CaCl_2$	6.270	3.135	0.81	0.453

The analysis of the results showed that $MnSO_4$ is statistically significant with a negative effect on cellulase activity that shown in Table 14. *P*-values <0.1 (0.07) indicated that Mnso4 had a statistically significant negative impact on cellulase activity.

4 . References

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