Upsurge production of cellulase from maize stover under soildstate conditions mediated by Streptomyces enissocaesilis DQ026641

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ABSTRACT

Maize stover was explored as a sole substrate of carbon for the extra cellular production of cellulase under solid-state conditions by an actinobacterium, *Streptomyces enissocaesilis* DQ026641. The upsurge production of cellulase was optimized manually regulating important process variables such as substrate (particle size, moisture content), organism (inoculum size), physicochemical attributes (pH, temperature) and mineral salts (NaCl, $MgSO_4$, $FeSO_4$) following one factor at a time approach. The maximum production (71.10 IU/ml) of cellulase was obtained at particle size 2 mm, moisture content 1.25%, temperature 45°C, inoculum size 1×10^8 and NaCl 1.5%. Enhanced production (79 IU/ml) of cellulase was achieved by following Response Surface Methodology with Centre Composite Design, considering pH 6.5, temperature 45°C and sodium chloride 1.5% as critical process variables. The quadratic impact of pH, temperature and sodium chloride significantly contributed for the production of cellulase under solid-state conditions from maize stover mediated by *S. enissocaesilis* DQ026641.

1. INTRODUCTION

Annually, about 960 million tons of solid waste are generated as byproducts of manufacturing, urban, agricultural, and other processes, with 350 million tons of organic waste from agricultural sources alone [1]. Bajar *et al.* [2] proposed that these lignocellulosic squanders, especially farming buildups, are the best carbon hotspots for the creation of microbial chemicals. Crop residues rich in cellulose account for half of the dry weight of agricultural waste. Biodegradation of cellulose is an essential aspect of the biosphere's carbon cycle [3]. Because of their higher stability and activity over a wider range of temperatures and pH, various extremophiles such as thermophilic, alkalophilic, or acidophilic microorganisms are used as sources of thermostable and broad range of pH stable enzymes in the biotransformation processes [4]. Similarly, various researches were carried out to ascertain the suitability of agricultural biomass waste as alternative substrates for cellulase enzyme processing [5].

Since cellulose accounts for up to 60% of the mass of lignocellulose, cellulases are the most important enzymatic group involved in its degradation [6]. The synthesis of cellulase enzyme was studied using a variety of solid substrates, including rice straw [7], Saw dust [8], water hyacinth [9], egg shell waste [10], wheat bran [11] and potato peel [12].

Submerged fermentation or solid-state fermentation (SSF) are currently used to produce cellulases [13]. C.W. Hesseltine was the one, who for the first time published the scientific information on solid state bioprocess in 1977. For the manufacture of microbial cellulase, solid state bioprocessing has emerged as a promising technology [14,15].

Different microorganisms being used for cellulase production using solid state bioprocess viz. *Aspergillus niger* and *Penicillium decumbans* [16], *Trichoderma reesei* [17], *T. reesei RUT C30* [18]. Dasari *et al.* [14] and Yoon *et al.* [19] reported major microorganisms employed in cellulase production via solid state bioprocess viz., *Acidothermus cellulolyticus*, *Bacillus subtilis*, *Bacillus pumilus*, *Clostridium acetobutylicum*, *Clostridium thremocellum*, *Cellulomonas fimi*,

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Cellulomonas bioazotea, *Cellulomonas uda*, *Streptomyces drozdowiczii*, *Streptomyces lividans*, *Thermonospora fusca* and *Thermonospora curvata*.

Response surface methodology (RSM) is the most widely used and accepted technique for optimizing the process parameters that are driven by multiple independent variables [20]. RSM is used in the optimization of a variety of products, including ethanol, bacteriocin, dye degradation, and the synthesis of enzymes such as chitinase, alkaline protease, and cellulase [21].

In the present study, efforts were made to achieve the enhanced production of cellulase enzyme under solid-state bioprocess by employing a *Streptomyces enissocaesilis* isolated from extreme environment of limestone quarries. Maize stover is regionally available agro-waste rich in cellulose and hence, was explored for the first time as an economic solid substrate for cellulase production by *S. enissocaesilis*.

2. METHODOLOGY

2.1. Microorganism

Streptomyces enissocaesilis DQ026641 isolated from limestone quarries of Karnataka Province, India, was used in this study. The selected isolate was identified using morphological, biochemical and 16S rRNA sequencing methods. After Polymerase Chain Reaction (PCR) amplification, purified DNA molecule fragments containing 16S rRNA genes were processed at BioEra Pvt. Ltd., Pune, for 16S rRNA gene sequencing.

2.2. Solid State Fermentation and Extraction of Crude Enzyme

A standard protocol recommended by Idris *et al*. [18] was used to analyse the solid-state bioprocess. The maize stover substrate powder (10 g) was placed in 250 ml Erlenmeyer flasks and moistened with sterile distilled water to achieve a moisture content of 65%. The contents were sterilised before being inoculated with a 1 ml inoculum of *S. enissocaesilis* test culture. The flasks were incubated at 35°C for 5 days. Using whole flasks, samples were taken every 24 hours for enzyme extraction by employing a simple contact process [22]. A total volume of 100 ml of citrate buffer (0.05 M, pH 4.8) was applied to the fermented substrate and mixed for 1 hour on a rotary shaker. The suspensions were filtered and centrifuged, and the supernatant was used as the crude enzyme preparation for enzyme activity assays.

2.3. Regulation of Process Variables Affecting Cellulase Production

Various parameters influence the cellulase production in SSF. To know the ideal conditions and impact of various parameters, the important process variables such as particle size, moisture, pH, inoculum size, temperature and nutritional variables were regulated in different range to achieve maximum cellulase production during bioprocess.

Particles with size 1, 2, 3, 4, and 5 mm were used in the fermentation. Using sterile distilled water, the moisture content of the substrates was adjusted to 59.08%–71.28% at four levels using a moisture analyser tool. pH range of 4.0 to 9.0 was selected and set of five conical flasks containing fermentation media were used. Dilute (0.1N) NaOH/HCl was used to achieve the desired pH level. The prepared flasks were then autoclaved and inoculated with *S. enissocaesilis* spore suspension (1×10^8/ml). In a static incubator, the flasks were incubated at 40°C. The amount of cellulase generated was measured every 24 hours for a total of 120 hours incubation period. The activity of cellulase was determined at every 24 hours. Temperature range of 30°C–50°C was also regulated. Important mineral salts such as sodium chloride, magnesium sulphate, and ferrous sulphate were also controlled at different concentrations of 0.01%, 0.05%, 0.1%, 0.15% and 0.2%. Mineral salts in various concentrations, such as NaCl-0.2%, $MgSO_4$-0.005%, and $FeSO_4$-0.001%, were prepared in sterile distilled water and added as moisture content.

2.4. Assay of Endoglucanase

The enzyme assay was performed by incubating a 1 ml assay mixture containing 0.5 ml crude enzyme extract and 0.5% appropriate substrate in citrate buffer (0.5 ml with pH-4.8) at 50°C for 30 minutes. Dinitro salicylic acid method was used to calculate the amount of reducing sugar formed [23], by spectrophotometrically measuring the absorbance at 540 nm and the enzyme activity was calculated.

2.5. Statistical Optimization of Cellulase Production by RSM with (Central Composite Design) CCD

The enhanced production of endoglucanase was carried out by RSM with CCD under solid state bioprocess system to optimize three critical process variables namely temperature (A), sodium chloride (B) and pH. The Design Expert Software, USA, was used to set the CCD of 20 runs (Version 11.0). All of the experiments were performed in duplicate, and the average amount of cellulase generated after 96 hours was used as the dependent variable or response (*Y*). The predicted response was determined using the second-degree polynomial equation, which took into account all of the terms.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where *Y* is the response vector, β_0 is the intercept coefficient, β_i is the linear effect coefficient, *ii* is the quadratic effect coefficient, and β_{ij} is the β_{ij}th interaction coefficient effect. $X_i X_j$ are input variables that have an effect on the response variable *Y*, with β_i being the β_ith linear coefficient [24]. The study of variance was used to conduct the statistical and numerical analysis of the model [Analysis of Variance (ANOVA)].

Fisher's *F*-test, its related probability *p* (*F*), correlation coefficient *R*, and determination coefficient R^2 were used to determine the statistical significance of the model, which describes the consistency of the polynomial model. For each variable, response surface curves were created and the quadratic models were represented as contour plots [three-dimensional (3D)].

3. RESULTS AND DISCUSSION

The maize stover (Fig. 1) was explored as solid-state substrate for the production of cellulase by *S. enissocaesilis*. Table 1 depicts the details of maize stover exploited for solid state bioprocess at different fermentation intervals. Maize stover was found to be an excellent natural substrate for cellulase production, which

Figure 1: 1: Maize stover for the production of cellulase by *S. enissocaesilis* DQ026641.

Table 1: Screening of maize stover for solid state bioprocess.

Fermentation period (hours)	Enzyme activity (IU) at different fermentation period
24	08.10
48	11.00
72	26.30
96	35.40
120	33.10

yielded 35.40 IU/10 g of enzyme before optimization in the present study. Sinjaroonsak *et al.* [25] exploited *Streptomyces thermocoprophilus* TC13W strain and produced 925 U/g using pretreated palm oil empty bunch. Ratnakomala *et al.* [26] exploited *Streptomyces* Bse 7-9 and produced 4.499 IU using agriculture sugarcane bagasse.

3.1. Optimization of Solid-State Bioprocess

3.1.1. Effect of particle size

The test isolate with particle size ranging from 1–5 mm was grown in media at different fermentation period. The effect of particle size on cellulase production is shown in Figure 2. At 96 hours of fermentation period, a particle size of 2 mm produced the maximum cellulase with an enzyme activity (IU) of 43.20, whereas a particle size of 5 mm produced the least with an enzyme activity (IU) of 22.45.

3.1.2. Effect of moisture content

Enzyme activity was recorded at different moisture level ranging from 59.08% (1 ml)–71.28% (1.7 ml). The effect of moisture content on cellulase production is depicted in Figure 3. At 96 hours
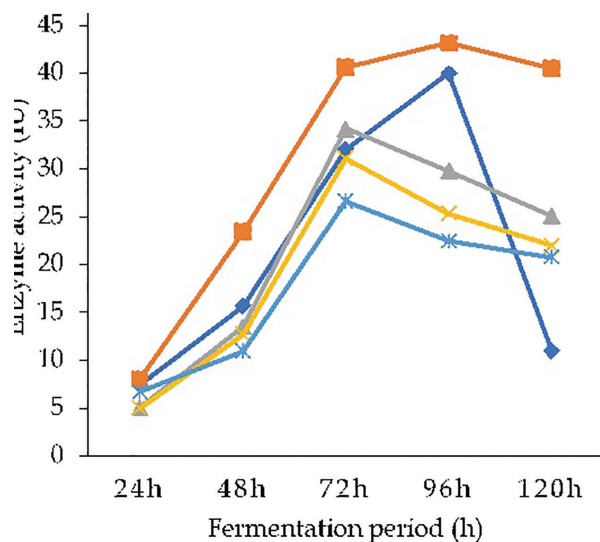
of fermentation, a moisture content of 64.61 % (w/v) was found to be optimal for the highest production of cellulase with enzyme activity (IU) of 48.15, and a moisture content of 2.00% was found to exhibit the least enzyme activity (IU) 30.80. Whereas, the species such as fungi *A. niger* ITBCC L74 showed the enzyme activity of 2.569, 1.606, and 1.302 U/ml, for rice straw, water hyacinth, and corn cobs respectively, at moisture content of 50%, 60%, 70%, and 80% and fermentation period of 2, 4, 6, 8 and 10 days [27]. Marraiki *et al.* [28] reported the activity of 54 ± 2.3 U/g using *Trichoderma hamatum* NGL1 with 70% moisture content.

3.1.3. Effect of pH

The test isolate was grown in media of different pH ranging from 5–7 at different fermentation period. The effect of pH on cellulase production is depicted in Figure 4. At 96 hours of fermentation, a pH of 6.5 was found to be optimal for the highest production of cellulase, with enzyme activity (IU) 57.20, and a pH of 7.0 was found to show the least enzyme activity (IU) 30.75. Similar results were observed by El-Nahrawy *et al.* [29]. He showed that at higher pH, cellulase production was greatly reduced and maximum cellulase production was observed at pH 6.0. According to Kshirsagar *et al.* [30], the highest CMCase activity was 2.38 U/g, at optimized conditions of 2.5 g of substrate, 75% (w/w) moisture content, initial medium pH 4.5, 1×10^6 spores/ml of inoculum, and incubation at ambient temperature (30°C) without additional carbon and nitrogen.

Figure 2: Effect of particle size on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.
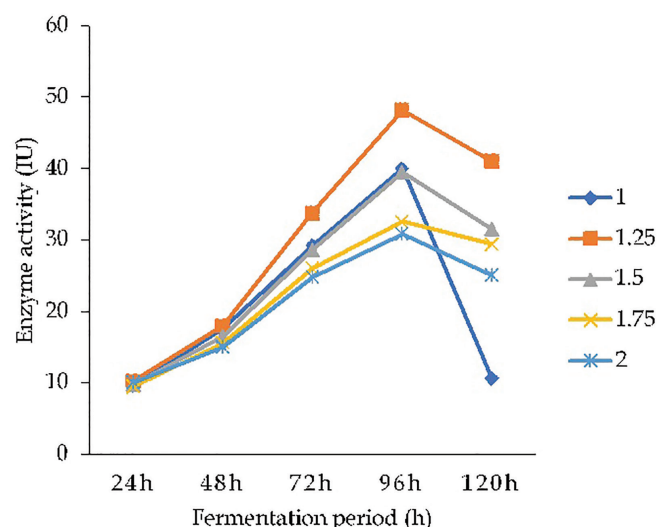

Figure 3: Effect of moisture on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.
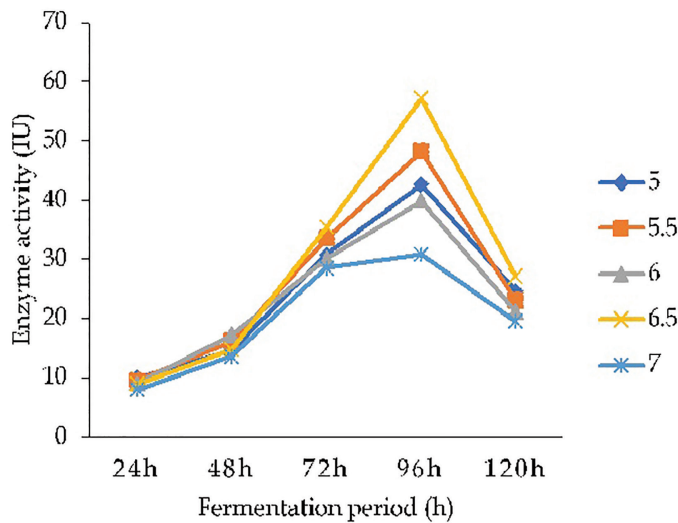
Figure 4: Effect of pH on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.

Figure 5: Effect of inoculum size on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.

3.1.4. Effect of inoculum size

The test isolate with inoculum size ranging from 1×10^5–1×10^9 was grown in media at different fermentation period. Figure 5 represents the effect of inoculum size on cellulase production. At 96 hours of fermentation, an inoculum size of 1×10^8 spores/ml was found to be optimal for the highest output of cellulase with enzyme activity (IU) 58.60, and an inoculum size of 1×10^5 spores/ml was found to show the lowest enzyme activity (IU) 38.00. This was in line with the findings of Kaur *et al.* [31], who used the fungus *Humicola fuscoatra* Microbial Type Culture Collection and Gene Bank (MTCC) 140 to produce maximum cellulase under solid state fermentation with an inoculum size of 1×10^7 spores/ml.

3.1.5. Effect of temperature

Enzyme activity was recorded at different temperatures ranging from 30°C–50°C. Figure 6 represents the effect of temperature on
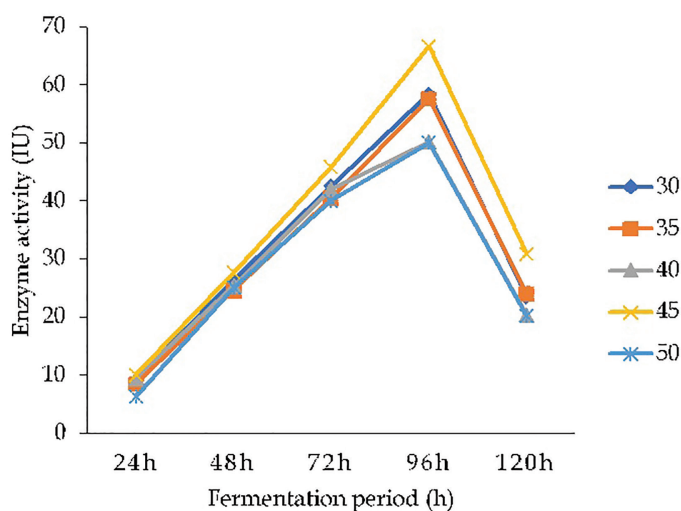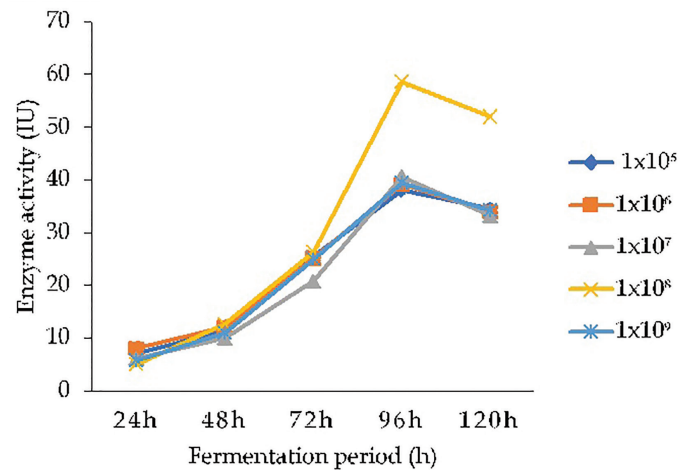
cellulase production. At 96 hours of fermentation, a temperature of 45°C was found to be optimal for the highest production of cellulase, with enzyme activity (IU) of 66.65 and the lowest enzyme activity (IU) of 50.00 was recorded with the temperature of 50°C at 96 hours. The requirement of high temperature for cellulase production may be attributed to the indigenous property of the strain as it was isolated from harsh environment. At 40°C, inoculum size 0.6/100 ml, and a 96-hour incubation time, Akurathi and Thoti [32] increased enzyme activity efficiency. Kshirsagar *et al.* [30] found that enzyme activity was 13.91 ± 0.89 at a temperature of 30°C and a pH of 6.5 using corn straw and the cellulase activity after 6 days decreased. This was also in the justification of the present study.

3.1.6. Effect of Mineral salts

Utilization of various mineral supplements namely Magnesium sulphate, Ferrous sulphate and Sodium chloride are used at
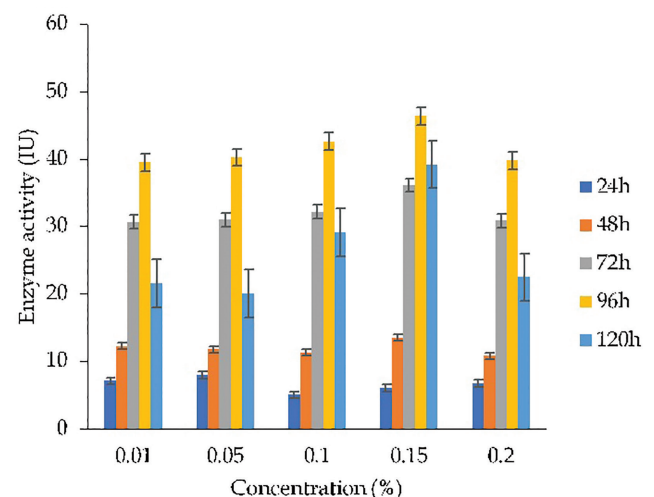

Figure 6: Effect of temperature on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.

Figure 7: Effect of Magnesium sulphate on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.
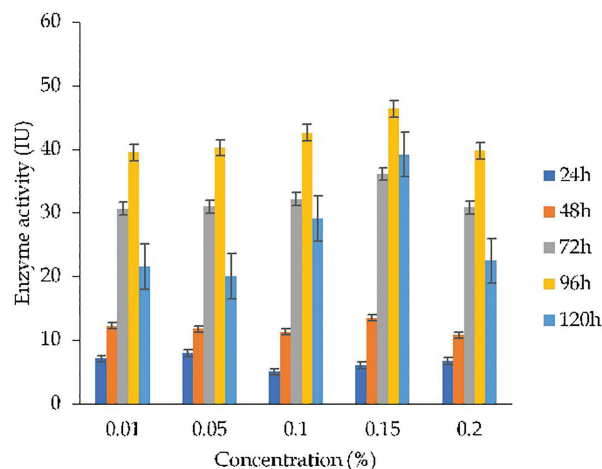
Figure 7: Effect of Magnesium sulphate on cellulase production at different fermentation periods *by S. enissocaesilis* DQ026641.
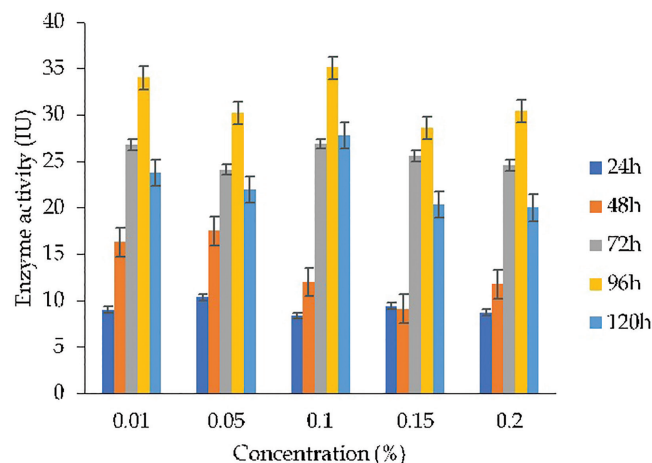

Figure 8: Effect of Ferrous sulphate on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.

different concentrations in Carboxy Methyl Cellulose Beef Extract Medium (CMC BeM) media for the production of cellulase and are illustrated from Figures 7 to 9.

At 96 hours of fermentation, concentrations of 0.15% were found to be optimal for the highest production of cellulase, with enzyme activity (IU) of 46.35 for Magnesium sulphate and 71.10 for Sodium chloride. At 96 hours of fermentation, a concentration of 0.10% was found to be optimal for the highest output of cellulase with enzyme activity (IU) 35.10 using Ferrous sulphate.

Figure 10 depicts the results of optimising the concentrations of all carbon sources used in the study for cellulase production. At optimised conditions, sodium chloride was found to be an excellent source for cellulase production, with enzyme activity (IU) 71.10, while ferrous sulphate had the least cellulase production with enzyme activity (IU) 35.10 at optimized conditions.

Sodium chloride was discovered to be an excellent source for cellulase production in this study showing enzyme activity (IU)

71.10 at the optimized conditions revealing the alkalophilic nature of *S. enissocaesilis* DQ026641 which needed high sodium chloride concentration level and high alkaline conditions for its growth. Similar observations were made by Stalin *et al*. [33]. In marine actinomycete, maximum enzyme activity was observed at high alkaline conditions of pH 8–10, temperature 40°C–60°C, and sodium chloride concentration 2%–4%. In *T. hamatum* NGL1, maltose induced cellulase production [28] and in *Aspergillus hortai*, lactose induced cellulase production [34].

3.1.7. Enhanced production using RSM

Identification of the optimized condition is critically needed for maximum cellulase production. Optimization of variables showed that, pH of 6.5, inoculum size of 1×10^8, temperature of 45°C, NaCl and MgSO$_4$ of concentration 0.15 and FeSO$_4$ of 0.10 concentration was found to be optimum. Considering the high amount of cellulase production, pH, temperature, and NaCl were identified as critical variables among all the optimised variables.
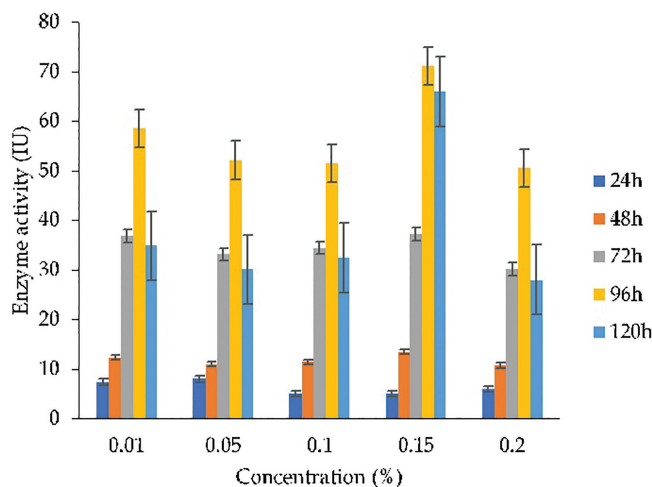

Figure 9: Effect of Sodium chloride on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.
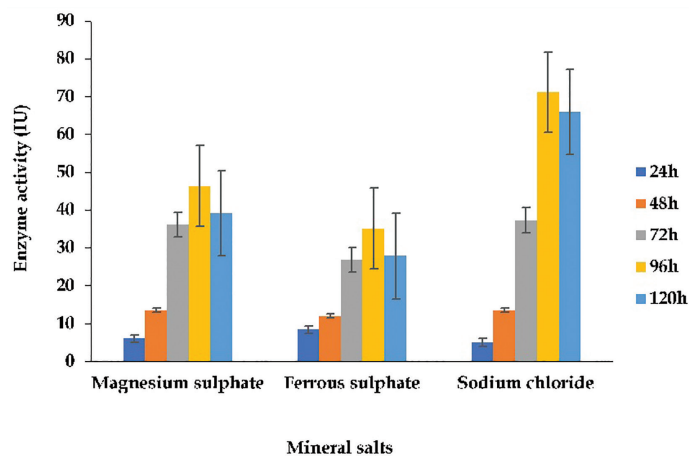

Figure 10: Effect of optimized concentrations of all mineral salts on cellulase production at different fermentation periods by *S. enissocaesilis* DQ026641.
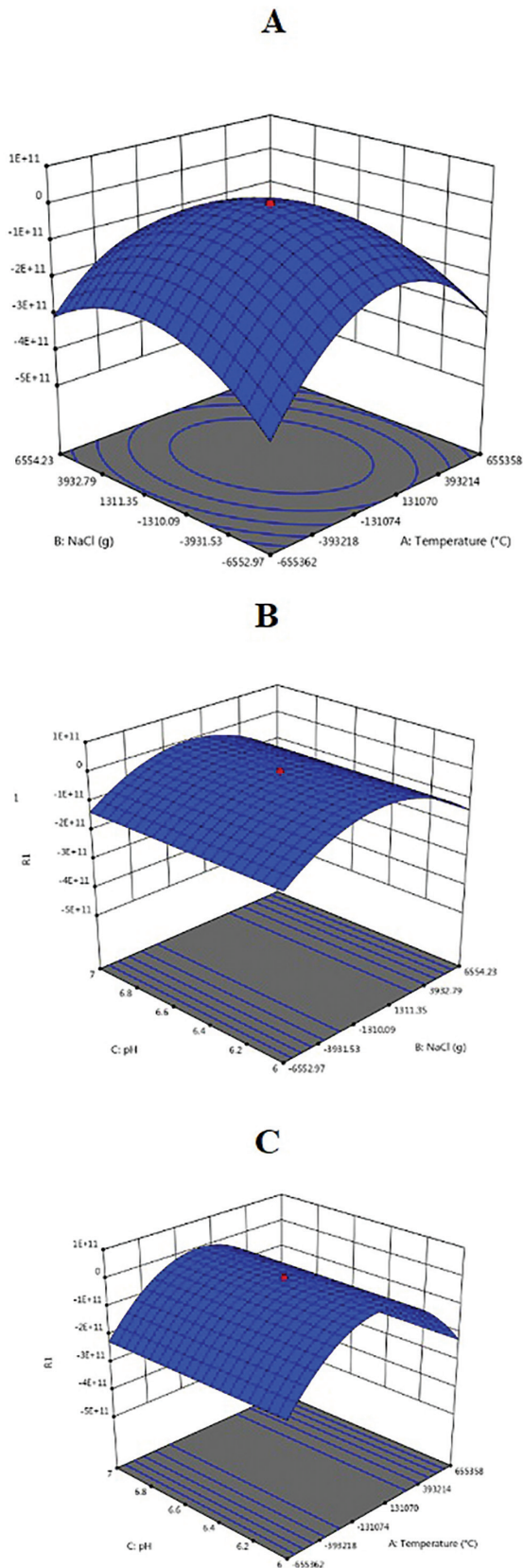
A

B

C

Figure 11: Response surface plots of the interactive effects: A, interaction between NaCl and temperature; B, interaction between pH and NaCl; C, interaction between pH and temperature.

The combined effect of physicochemical and nutritional variables on cellulase production was studied using RSM and CCD for the purpose of developing a suitable bioprocess. Table 2 depicts the details of combined effect of critical process variables for the production of cellulase showing the actual and predicted responses for different combinations of the critical parameters. In the second run, the highest enzyme activity was 79 IU/ml (Table 2), confirming RSM's prediction of cellulase activity of 74.61 IU/ml.

The interaction effects of critical variables such as pH, temperature, and NaCl for producing the highest cellulase activity were plotted using response surface curves. The interactive effects of pH, temperature, and NaCl on cellulase production by *S. enissocaesilis* DQ026641 are depicted in 3D response surface plots in Figure 11(A–C).

Table 3 depicts the ANOVA of the quadratic model showing the quantitative effect of individual variables as well as their interactions affecting the activity of cellulase. The model's higher *F*-value is important since the *p*-value is 0.0001, which is much lower than 0.5. The important model terms in this case are *A*, *B*, *C*, A^2 and B^2. The *F*-value of 284.04 indicates that the lack of fit is significant. There's just a 0.01% risk that a significant lack of fit *F*-value is caused by noise. The quadratic effect of temperature,

Table 2: Combined effect of critical process variables for the production of cellulase.

| Run	Critical process variables			Cellulase activity (IU)	
	Temperature °C	NaCl g/l	pH	Actual value	Predicted value
1	40	2.0	6	55	58.51
2	45	1.5	7.34	79	74.61
3	36.59	1.5	6.5	48	46.97
4	45	2.3	6.5	66	54.54
5	40	2.0	7.0	60	66.31
6	45	1.5	6.5	70	70.55
7	50	2.0	7.0	35	41.33
8	45	1.5	5.65	58	57.30
9	40	1.0	6.0.	42	39.27
10	45	0.7	6.5	36	42.36
11	45	1.5	6.5	71	70.55
12	45	1.5	6.5	70.05	70.55
13	45	1.5	6.5	70.5	70.55
14	50	1.0	7.0	46	46.09
15	45	1.5	6.5	71	70.55
16	50	2.0	6.0	35	41.03
17	40	1.0	7.0	62	59.57
18	50	1.0	6.0	36	33.29
19	45	1.5	6.5	69.9	70.55
20	53.40	1.5	6.5	25	20.94
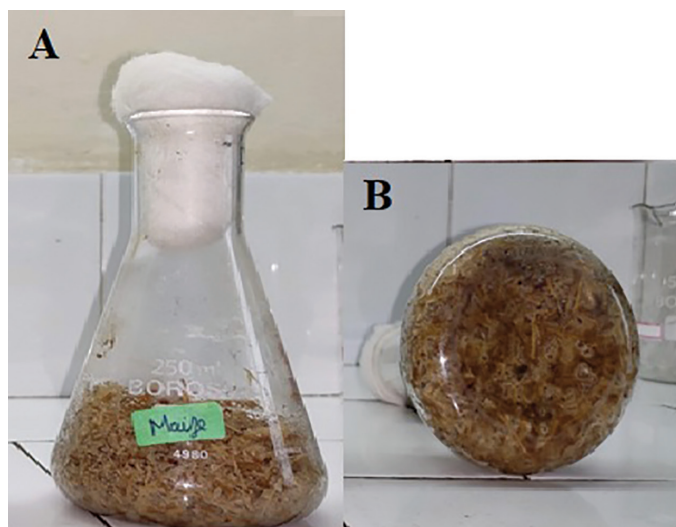
Figure 12: Production of cellulase from maize stover by solid state bioprocess: A, Sideview of the flask; B, Bottom view of the flask.

Table 3: ANOVA for quadratic model.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	4,565.54	9	507.28	14.10	0.0001
A-temperature	817.80	1	817.80	22.73	0.0008
B-NaCl	179.08	1	179.08	4.98	0.0497
C-pH	362.06	1	362.06	10.06	0.0099
AB	66.13	1	66.13	1.84	0.2050
AC	28.13	1	28.13	0.7818	0.3973
BC	78.13	1	78.13	2.17	0.1713
A^2	2,412.99	1	2,412.99	67.08	<0.0001
B^2	879.76	1	879.76	24.46	0.0006
C^2	38.10	1	38.10	1.06	0.3276
Residual	359.74	10	35.97		
Lack of fit	358.48	5	71.70	284.04	<0.0001
Pure error	1.26	5	0.2524		
Cor total	4,925.28	19			

NaCl and pH was significantly contributing to cellulase production in *S. enissocaesilis* DQ026641.

Results showed that *S. enissocaesilis* is viable and highest cellulase producer at thermophilic and alkalophilc conditions compared to other organisms under extreme conditions by using RSM [35]. Using an optimization technique, Tai *et al.* [36] increased CMCase production from 2.55 to 124.5 U/g. Other researchers also enhanced cellulase production using RSM prediction [37,38].

3.1.8. Mechanism of action of S. enissocaesilis DQ026641

Herbivores lack the enzymatic capacity needed to degrade plant polysaccharides, particularly cellulose and instead depend on microorganisms which have this ability. The cellulose fermenting microbes survive by degrading insoluble polymers in highly competitive and nitrogen poor environment [39]. Several studies have examined the degradation of cellulose using various cellulolytic microorganisms [40].

In the present study, maize stover was used as a model feedstock to test the efficacy of *Streptomyces* cellulase for hydrolysis of lignocellulose. Figure 12 visual observations of cellulase production under solid-state bioprocess by *S. enissocaesilis* DQ026641. *Streptomyces enissocaesilis* uses *sec* secretion system. Generally, three types of glycosyl hydrolases namely Endoglucanases (EG; EC 3.2.1.4), Cellobiohydrolases (CBH, EC 3.2.1.91) and β-glucosidases (BG, EC 3.2.1.21) are responsible for the conversion of cellulose into glucose. Endoglucanases randomly break the internal bonds of the amorphous structure of cellulose. Cellobiohydrolases acts primarily on the crystalline portion and catalyses the release of glucose or cellobiose from the ends of the cellulose fibre. β-glucosidases act on oligosaccharides and disaccharides, catalyses the release of glucose [41].

The method of action and substrate specificities of different cellulases varies [42]. In actinomycete, cellulase are free and secreted extracellularly using specific secretion pathway.

Actinomycetes release extracellular cellulases by one or both of the common bacterial systems for extracellular protein secretion, namely the sec general secretion system and the sec independent twin arginine translocation systems. [43].

The investigation described above suggests that, in controlled laboratory conditions the cellulose is completely hydrolysed using *S. enissocaesilis*. Being thermoalkalophilic, it produces cellulase enzyme which is withstand high comparatively temperatures and alkaline conditions with respect to the cellulase produced by other cellulolytic microorganisms [30,32].

4. CONCLUSIONS

In the present study, efforts were made to increase the enzyme production at thermoalkalophilic conditions of SSF medium. The results revealed that cellulase activity of 79 IU/ml under optimised conditions by *S. enissocaesilis* DQ026641 is better than other reported strains. Herein, we report for the first time to our knowledge the increased production of cellulase by thermoalkalophilic *Streptomyces* sp. isolated from harsh environment of limestone quarries. this thermotolerant species will have more important biotechnology applications due to its ability to produce thermostable cellulase and even reduces the risk of contamination by mesophilic microorganisms.

5. ACKNOWLWDGEMENTS

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and

agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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