Implementation of Response Surface Methodology for Enhanced Production of Endoglucanase by Thermophilic Aspergillus Fumigatus

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as

doi: https://doi.org/10.32350/BSR.0304.05

Abstract

Enzymes are biocatalysts which play key roles in the body of living organisms. Cellulose is major source of plant biomass. Its β-1,4-glucosidic bonds are hydrolyzed by cellulases. These cellulases can be produced by a variety of microorganisms including fungi, bacteria and actinomycetes which are used today for the industrial applications. The current study was aimed to optimize the cultural conditions for maximum production of endoglucanase by Aspergillus fumigatus through solid state fermentation of sugarcane bagasse. Response Surface Methodology (RSM) was employed under Central Composite Design (CCD) for the optimization of growth including pH, temperature, time period & inoculum size and nutritional parameters including glucose, fructose and (NH₄)₂SO₄. The effect of different metal ions on
endoglucanase production was also monitored. It was partially purified by (NH₄)₂SO₄ precipitation and gel filtration chromatography. Finally, endoglucanase was characterized for optimum pH, temperature and determination of kinetic parameters. Maximum enzyme activity was found as 0.9 IU/mL/min in the presence of 6 g substrate, 3.5 mL inoculum, 4.5 pH, 40 °C temperature at incubation time of 84 hrs. After addition of carbon and nitrogen sources enzyme activity increased to 1.4 IU/mL/min. It was further increased to 1.56 IU/mL/min with 0.42% of CaCl₂. Maximum purification was achieved at 50% saturation by ammonium sulphate (NH₄)₂SO₄. Optimum temperature and pH were 40 °C and 5 respectively, whereas the values for $K_m$ and $V_{max}$ were 5.37 mM and 696 uM/mL/min., respectively. These findings suggested that endoglucanase by Aspergillus fumigatus could be suitable for various industries.

**Key Words:** Aspergillus fumigatus, Solid state fermentation, Response surface methodology, endoglucanase, central composite design

## 1. INTRODUCTION

Lignocellulose is renewable natural source and major structural component of all plant substantial composed of hemicellulose, cellulose, lignin and a small amount of other material such as ash, pectin etc. in different degrees. Cellulose chains being most abundant are coupled by hydrophobic interactions, hydrogen bonding as well as van der waal’s interactions [1]. By biotransformation of lignocellulosic biomass including forestry residues, agricultural wastes, paper wastes etc. various kinds of industrially important enzymes are produced with high yield in a cost effective manner besides energy demands of today’s era can be overwhelmed [2,3]. In Brazil, the residues of sugarcane comprise one of the largest cellulosic agro-industry, of which the bagasse portion comprises approximately 50 % cellulose and 25 % hemicellulose and lignin whereas straw portion consists of cellulose, hemicellulose and lignin as 37.4 %, 30 % and 18.5 %, respectively [4].

Among various kinds of cellulolytic enzymes produced from different microbes for bioconversion of industrial and agricultural wastes, cellulases are regarded more efficient to hydrolyze cellulose into glucose and other useful components [5]. Cellulases contribute to 8% of global industrial enzyme demands and have been available commercially for more than 30 years [6, 7]. Cellulases are used in food and feed stock, pharmaceuticals, biofuel production, waste management, genetic engineering, pulp and paper industry, textile industry and protoplast induction [8, 9].

Three hydrolytic enzymes of cellulases i.e., endoglucanase, exoglucanase and b-glucosidase act synergistically on cellulose chains producing glucose as final product in a chain
of reactions. Endoglucanase haphazardly attacks internal O-glycosidic bonds and produces glucan chains of different lengths. Exoglucanase produces β-cellobiose as end product by acting on the ends of cellulose chains followed by the production of glucose by action of β-glucosidase upon β-cellobiose disaccharides [6]. In cellulase chains, when endoglucanases cuts β-1, 4-bonds, it generates two ends. In most endoglucanases, the catalytic modules have a grove shaped active site. This active site allows endoglucanase to bind and cleave cellulose chain in order to generate glucose and other components [10-15].

A number of microbes have cellulolytic abilities but fungus is considered most suitable for production of enzymes on industrial scale. The fungi kingdom has approximately 200 species of Aspergillus that are isolated from soil and used in production of variety of extracellular enzymes. The best known species having high commercial value are A. fumigatus, A. niger, A. flavus, A. oryzae, A. nidulans and A. versicolor [16]. Currently thermophilic enzymes are given more preference due to tolerance of wide range of pH variations and resistance to denaturing agents. A review of literature revealed that A. fumigatus strains were reported for maximum cellulase production under solid state fermentation [17]. Solid state fermentation process has more advantages than submerged state fermentation due to its easy handling and good control on environmental conditions. As the fungus has great potential to grow on solid substrate in absence of free liquid so fungi is regarded more effective in enzyme production under solid state conditions [18].

The current study is focused on production of endoglucanase by A. fumigatus under solid state fermentation using sugarcane bagasse as substrate and optimizing its production by different cultural and nutritional parameters. The enzyme is then purified and characterized for further analysis.

2. Methodology

2.1 Fermentative Organism
Fungal strain of Aspergillus fumigatus was collected from Industrial and Environmental Biotechnology Laboratory, Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi. Culture plates and slants of fungi using PDA media (pH 5.5) were prepared for its preservation and for future use, which were stored at -4 °C.

2.2 Fungal Inoculum
Fungal inoculum was prepared in broth media (pH 5.5) for its subsequent use in solid state fermentation process (SSF).

2.3 Substrate Preparation
Sugarcane bagasse was collected and dried in shadow first. After removing moisture content completely, it was ground to powder having 40 mm mesh size and stored in air tight plastic jars.

2.4 Fermentation Process
To carry out Solid State Fermentation process, flasks (250 mL) were used containing substrate with 50% moisture level. These flasks were moistened with distilled water and were sterilized in autoclave at 121 °C for 15 mins. After sterilization, they were placed in laminar air flow. On cooling to room temperature, inoculum medium was added aseptically to each flask and incubated at conditions according to experimental design (Table 1).

2.5 Extraction of Crude Enzyme
After incubation at specific conditions, flasks were taken out and 50 mL distilled water was added in each of these flasks. Flasks were then placed in shaking incubator at 150 rpm for 60 mins so that extracellular enzymes dissolved in water. Later the mixture was filtered and centrifuged at 10,000 rpm for 15 mins at 4 °C. The supernatant was stored at -4 °C as crude enzyme [19-20].

2.6 Response Surface Methodology (RSM)
The optimization of different parameters and study of effects of these parameters on production of endoglucanase was studied by Response Surface Methodology (RSM). It is a statistical tool used to study when there is one dependent variable, influenced by many independent variables. The experiments were designed on JMP software under Central Composite Design (CCD). It helped to find effect of each of the parameter as well as interaction between these parameters on the process under study.

2.7 Optimization of Endoglucanase Production
During current study, various cultural and nutritional parameters were optimized for better endoglucanase production by *A. fumigatus*. Cultural parameters included temperature, time period, pH, inoculum size and substrate size (Table 1) whereas in nutritional parameters, various carbon (glucose, fructose and sucrose) and nitrogen sources (Ammonium sulphate and urea) were optimized (Table 2). There were 28 experiments at each level for optimization of cultural and nutritional conditions, designed with JMP software under CCD. Moreover, some metal ions (Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$) were also added in their soluble form to check the effect of these ions on the production of endoglucanase. One percent solution of each metal ion was
prepared and various concentrations of each such as 0.14 %, 0.28 %, 0.42 %, 0.57 % and 0.71 % were used in experiments [21].

2.8 Endoglucanase Assay

Endoglucanase assay was performed according to protocol described by Mahmood et al., 2013 by using Carboxy Methyl Celluloase (CMC) as substrate and glucose as standard [20]. Standard factor was determined by taking absorbance of different conc. of glucose (0.5-4.0 µM / mL with difference of 0.5) at 540 nm and using equation II. Enzyme activity was calculated by following formula.

\[
\text{Enzyme activity (IU/mL/min)} = \frac{\text{Absorbance of enzyme soln.} \times \text{Standard factor} \times \text{Dilution factor}}{\text{Time of incubation (min)}}
\]

Where,

\[
\text{Standard Factor} = \frac{\text{Concentration of standard (µM / mL)}}{\text{Absorbance of standard at 540 nm}}
\]

Where: dilution factor = 50 & standard factor = 3.108.

One unit of enzyme activity is the amount of enzyme which released 1 µmol of the product per minute.

2.9 Purification of Endoglucanase

Crude endoglucanase was partially purified by Ammonium Sulphate Precipitation and Gel Filtration Chromatography using Sephadex G-100 column.

For ammonium sulphate precipitation 10 mL of crude enzyme was taken in different falcon tubes and saturated separately for 40 %, 50 %, 60 %, 70 % & 80 % with ammonium sulphate. The tubes were place at 4 °C for 4 hours than centrifuge at 10,000 rpm for 15 min., pellet was suspended in 2 mL citrate buffer and assay was performed [22].

For gel filtration chromatography 5 % Sephadex solution was prepare in d.H2O and left overnight. It was gently mixed and used to fill the column slowly to avoid any air bubble. 2 mL of crude enzyme was poured at the top of column and elutions 91 mL) were collected from the bottom. Total 24 elutions were collected at flow rate of 30 mL/hr and first 5 were discarded while oother 18 were subject to endoglucanase assay [20, 23].

2.10 Characterization of Endoglucanase

Endoglucanase was characterized to determine its optimum temperature and pH by performing enzyme assay as discussed above at 6 different temperature (35 °C to 60 °C) and 5 different pH values between 4-8, separately [20, 22].

To determine the kinetic parameters i.e. $K_m$ and $V_{max}$ of endoglucanase the enzyme assays were performed with 2 mM, 4 mM, 6 mM, 8 mM and 10 mM solutions of CMC. The results were
used to draw Line-Weaver Burk graph between inverse of substrate conc. and activity. The
equation obtained from graph was used to calculate $K_m$ and $V_{max}$ [20, 24].

3. RESULTS

The major experiments were designed on JMP software by Response Surface Methodology
(RSM). Response Surface Methodology is a statistical approach that can be used to maximize
the production of special substance by streamlining the operational factors. It was first
introduced by K. B. Wilson and George E. P. Box in 1951. This methodology finds the
relationship between one or more response variables and several explanatory variables. A
sequence of designed experiments is used to get an optimal response in RSM [25].

3.1 Optimization of Growth Parameters for *A. fumigatus*

Cultural conditions were optimized according to the experimental design given in table 1. The
interaction between all parameters and effect of these parameters on endoglucanase production
was observed by plotting 3D response surface graphs through JMP software. The results
showed that there was positive interaction between time period and temperature which results
into better fungal growth with good endoglucanase activity. The incubation period and
temperature plays a vital role in the production of enzyme as well as the activity of enzyme.
By increasing the temperature, kinetic energy is increased and the interaction between substrate
and enzyme is stabilized. There is a point where kinetic energy is maximum and further
increase in temperature results in denaturing of enzymes [26]. So, enzyme activity increased
with an increase in temperature. Same effect was observed in case of time period.
Endoglucanase showed its maximum activity (0.9 IU/mL/min) after 130 hrs and at 34°C (Fig.
1a). Any further change in values results in the decrease of activity.

Similarly, every enzyme has its optimum pH, below or above this pH enzymatic activity
decline. Changing pH from optimum value damages the active sites. Moreover with increase
in temperature, frequency of collisions between molecules is increased and as a result, reaction
does not proceed to completion and activity decreased. During the study of interaction between
temperature and pH it was observed that maximum endoglucanase activity was (0.74
IU/mL/min) at 35 °C and pH 5.5 (Fig 1b). The graph shows that enzyme remains active for
broader pH range but smaller temperature range. In case of increasing inoculum size, numbers
of fungal spores are increased. Due to which a positive interaction was also observed between
inoculum size, enzyme activity and temp. (Fig.1c). Endoglucanase activity was 0.69 U/mL/min with 2 mL inoculum size at 34 °C.

Maximum endoglucanase activity (0.74 IU/mL/min) was observed at 37 °C and presence of 6 g of substrate in growth media (Fig. 1d). At temperature around 37 °C, there was high activity at broader concentration of substrate. There was 0.63 IU/mL/min activity at pH 6 and inoculum size 1 mL (Fig. 1e). Any change in values of these two parameters results in observing a negative interaction which leads to decrease in endoglucanase activity. In next figure (Fig.1f), substrate and inoculum size interact positively and gave maximum activity i.e., 0.55 U/mL/min at 8 g of sugarcane bagasse and 1.5 mL of inoculum size. Any change from these values disturb the availability of substrate to fungal spores, which decrease the enzyme production and activity (Fig. 1f). The analysis of results through JMP software concluded that the physical parameters have significant effect on endoglucanase activity.

3.2 Optimization of Nutritional Parameters for Endoglucanase Activity
Fungi produce a variety of enzymes depending upon the availability of nutrients in media. The result of experiments for optimization of nutritional parameters was also analysed by plotting 3D response surface graphs through JMP software. Glucose is considered as a major source of carbon for most of the organisms. Glucose is a monosaccharide and it is also called as blood sugar or grape sugar. Both glucose and fructose were provided as carbon sources to observe their effect on enzyme activity and the interaction between them. When a response surface graph was plotted between glucose, fructose and enzyme activity, enzyme activity was found maximum 1.4 U/mL/min at 0.12 % glucose and 0.4 % fructose (Fig. 2a).

Ammonium sulphate is inorganic salt with formula (NH₄)₂SO₄. It contains 21% nitrogen and 24 % sulphur [27]. Fig. 2b shows a 3D response surface graph which shows interaction between glucose and ammonium sulphate as a slight up rise curve which shows the peak of both factors. Enzyme shows its maximum activity of 0.7 IU/mL/min with 0.14 % glucose and 0.24 % ammonium sulphate. Urea is a source of nitrogen and a significant nutritional factor. The graph between glucose and urea shows that enzyme activity is nearly 0.7 IU/mL/min with 0.07 % glucose and 0.38 % urea as shown in Fig 2c.

Fructose is a monosaccharide whereas sucrose is a disaccharide. Sucrose can also hydrolyze into glucose and fructose if it is heated or treated with an acid [28]. In current study, the interaction between fructose and sucrose was observed by plotting a 3D graph in which enzyme activity was high with low quantity of sucrose and fructose. Enzyme activity was found about 1.2 IU/mL/min with 0.13 % sucrose and 0.42 % fructose (Fig 2d). In case of fructose and Ammonium sulphate, enzyme activity was 1.18 U/mL/min with 0.42% fructose and 0.25 %
ammonium sulphate (Fig. 2e). Whereas in less quantity of fructose and ammonium sulphate, endoglucanase activity starts to fall as shown by 3D response surface graph. The interaction between ammonium sulphate and urea was observed by plotting graph showing an umbrella like curve indicating a positive interaction between these factors. By increasing these nutritional parameters, the activity of enzyme is also increased. Enzyme activity was 0.55 IU/mL/min with 0.2 % urea and 0.15 % ammonium sulphate (2f). Endoglucanase activity was very low below this percentage of ammonium sulphate as well as of urea. So analysis of results through JMP software concluded that nutritional factors are also momentous for any enzyme to work. Selection of suitable conditions are very important to set maximum enzyme production. The Central Composite Design (CCD) is suitable for such kind of experiments.

3.3 Effect of Metal Ions on Endoglucanase Activity
Different metal ions solutions were used in their specific concentrations. First of all, Calcium chloride (CaCl₂) was used to check the effect of Ca²⁺ ion. Five concentrations of CaCl₂ were prepared as described above and enzyme activity was calculated. Graph was plotted between concentrations of CaCl₂ and calculated enzyme activity. There was maximum endoglucanase activity (1.6 IU/mL/min) at 0.42 % of CaCl₂ (Fig. 3). In case of MgCl₂, CuSO₄ and ZnSO₄ endoglucanase activity did not increase further (Fig. 3). So, it is concluded from the results that calcium chloride has a positive effect on endoglucanase activity whereas the other metal ions have no effect on activity.

3.4 Estimation of Proteins
The total protein estimation was done by making different dilutions of standard protein i.e., Bovine Serum Albumin (BSA). The dilutions were prepared according to the concentration given in table 3. Absorbance of these dilutions was measured on spectrophotometer at 660 nm (Table 3).

3.5 Purification of Crude Enzyme
In Ammonium Sulphate Partial Purification of endoglucanase, maximum enzyme activity was shown by 50 % saturation of (NH₄)₂SO₄) (Fig. 4a). Column gel chromatography showed 22nd eluted sample as best purified (Fig. 4b).

3.6 Characterization of Endoglucanase
The characterization of endoglucanase revealed that it has optimum temperature 40 °C (Fig. 5a) and optimum pH 5 (Fig. 5b). The results showed that it remain active up to 60 °C with high activity, indicating thermophilic nature of enzyme with wide industrial applicability. The endoglucanase was also studied for the determination kinetic parameters including $K_m$ and $V_{max}$. These help to find the efficacy of enzyme and its affinity towards substrate. The results obtained
after performing assay with different molar concentration of substrate (CMC) were used to plot Line-Weaver Burk double reciprocal graph. The values of kinetic parameters were calculated from linear regression equation obtained from this graph by taking value of $x=0$ for $V_{max}$ and value of $y=0$ for calculating $k_m$ (Fig.5c). The endoglucanase of *Aspergillus fumigatus* has $V_{max}$ equal to 696 uM/mL/min whereas the $K_m$ equal to 5.37 mM. These values suggested that it is active enzyme with good affinity towards CMC and could be a good choice for various industrial applications.

4. DISCUSSION

Enzymes are the biocatalysts having applications in different industries. Microbes are the major source of industrially important enzymes [29]. It is essential for exploring the microbes producing efficient enzymes with industrial importance. The process of production of thermophilic endoglucases enzyme was studied during this research. Results are quite significant with higher production of efficient endoglucanase at optimized conditions.

The classical Response Surface Methodology is suitable technique for the optimization of processes depend on multiple factors. It was successfully implemented during current project to find suitable conditions for maximum enzyme production. The maximum thermophilic endoglucanase activity around 1 IU/mL/min was observed near 40 0C after 84 hrs of incubation time. Youseef et al., 2011 [30], worked on the enzymes production by thermophilic *Aspergillus* specie and found 40 0C as suitable temperature with pH range from 4-8. He concluded that wide working pH range enhance the industrial applicability of the enzyme and its demand. Sherief et al., (2010) [31], reported the cellulases production potential of *Asprgillus fumigatus* by using wheat bran and rice straw as substrate. They also reported acidic pH suitable for fungal cellulases with 40 0C as optimum.

The results showed that the enzyme production dependent on the availability of microbial spores and amount of substrate. Higher spores could yield more enzymes but substrate concentration may act as limiting factor, which can be avoided by increasing substrate. Further, with more substrate and higher spores the generation of toxic waste stop or decrease enzyme activity after certain time [32]. Abo-State et al., 2010) [33] performed similar studies on various fungal species with variety of substrates to find most suitable substrate and fungi. Their findings suggest, fungi are the microbes capable of more efficient substrate utilization and less effect of substrate depletion or waste accumulation.
There was almost 40% increase in endoglucase activity (0.94 IU/mL/min to 1.40 IU/mL/min) after the addition of additional carbon and nitrogen sources. Shoban and Maheshwari, 2013 [34], studied mesophilic Aspergillus fumigatus and reported the cellulase production by using various carbon and nitrogen sources. The production was enhanced with additional nutrients in the acidic environment at room temperature. There was 60% increase in the activity after the ammonium sulfate precipitation compared to initial activity. Farinas et al., 2011 [35], reported the 61-65% recovery of the endoglucase after the ammonium sulfate precipitation that cause its purification and enhance its application. Bagewadi and Ninnekar, 2015 [36], work on the kinetic of purified enzymes and also studied the effect of various metals ions, the results during the current project are very similar to them. There was positive effect of Ca ion and higher Vmax with lower km value.

5. Conclusions

It can be concluded that RSM is suitable technique for the optimization and production of enzymes through solid state fermentation. There was 0.94 IU/mL/min endoglucases activity observed after 84 hrs at 40 °C with 3.5 ml fungal inoculum. The maximum activity is the combinatorial effect of all the parameters under the study. The activity enhance to almost 40% by the addition of glucose, fructose and ammonium sulfate additionally in the media. The activity was further 20% increased when 0.42% Ca ions were used. At 50% ammonium sulfate saturation maximum precipitation of endoglucanase was observed with Vmax equal to 696 uM/mL/min and Km equal to 5.37 mM.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1: Experimental design using RSM for the optimization of growth parameter

| Sr. No. | Time period (Hrs.) | Temp (°C) | pH | Inoculum size (mL) | Substrate size (g) |
|---------|--------------------|-----------|----|--------------------|-------------------|
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 1. | 24 | 20 | 7 | 5 | 2 |
| 2. | 144 | 40 | 7 | 5 | 2 |
| 3. | 84 | 30 | 4.5 | 1 | 6 |
| 4. | 84 | 30 | 4.5 | 3.5 | 6 |
| 5. | 24 | 20 | 3 | 5 | 10 |
| 6. | 24 | 40 | 7 | 1 | 2 |
| 7. | 144 | 20 | 7 | 1 | 2 |
| 8. | 24 | 40 | 3 | 5 | 2 |
| 9. | 144 | 40 | 3 | 5 | 10 |
|10. | 24 | 20 | 7 | 1 | 10 |
|11. | 84 | 30 | 3 | 3.5 | 6 |
|12. | 84 | 30 | 4.5 | 3.5 | 2 |
|13. | 84 | 30 | 7 | 3.5 | 6 |
|14. | 144 | 20 | 3 | 1 | 10 |
|15. | 144 | 20 | 7 | 5 | 10 |
|16. | 84 | 20 | 4.5 | 3.5 | 6 |
|17. | 24 | 40 | 3 | 1 | 10 |
|18. | 84 | 40 | 4.5 | 3.5 | 6 |
|19. | 144 | 30 | 4.6 | 3.5 | 6 |
|20. | 144 | 40 | 7 | 1 | 10 |
|21. | 24 | 40 | 7 | 6 | 10 |
|22. | 84 | 30 | 4.5 | 3.5 | 6 |
|23. | 24 | 30 | 4.5 | 3.5 | 6 |
|24. | 84 | 30 | 4.5 | 5 | 6 |
|25. | 144 | 20 | 3 | 5 | 2 |
|26. | 144 | 40 | 3 | 1 | 2 |
|27. | 84 | 30 | 4.5 | 3.5 | 10 |
| Sr. No. | Glucose (%) | Fructose (%) | Sucrose (%) | A. sulphate (%) | Urea (%) |
|---------|-------------|--------------|-------------|-----------------|---------|
| 1.      | 0.1         | 0.1          | 0.5         | 0.5             | 0.1     |
| 2.      | 0.5         | 0.1          | 0.1         | 0.1             | 0.5     |
| 3.      | 0.1         | 0.1          | 0.5         | 0.1             | 0.5     |
| 4.      | 0.3         | 0.3          | 0.3         | 0.5             | 0.3     |
| 5.      | 0.1         | 0.1          | 0.1         | 0.1             | 0.1     |
| 6.      | 0.1         | 0.1          | 0.1         | 0.5             | 0.5     |
| 7.      | 0.1         | 0.3          | 0.3         | 0.3             | 0.3     |
| 8.      | 0.3         | 0.3          | 0.5         | 0.3             | 0.3     |
| 9.      | 0.5         | 0.5          | 0.5         | 0.1             | 0.5     |
| 10.     | 0.5         | 0.5          | 0.1         | 0.5             | 0.5     |
| 11.     | 0.3         | 0.5          | 0.3         | 0.3             | 0.3     |
| 12.     | 0.5         | 0.1          | 0.5         | 0.5             | 0.5     |
| 13.     | 0.5         | 0.5          | 0.5         | 0.5             | 0.1     |
| 14.     | 0.3         | 0.1          | 0.3         | 0.3             | 0.3     |
| 15.     | 0.1         | 0.5          | 0.1         | 0.1             | 0.5     |
| 16.     | 0.1         | 0.5          | 0.5         | 0.1             | 0.1     |
| 17.     | 0.5         | 0.1          | 0.5         | 0.1             | 0.1     |
| 18.     | 0.3         | 0.3          | 0.3         | 0.3             | 0.5     |
| 19.     | 0.3         | 0.3          | 0.3         | 0.1             | 0.3     |
| 20.     | 0.5         | 0.1          | 0.1         | 0.5             | 0.1     |
| 21.     | 0.1         | 0.5          | 0.1         | 0.5             | 0.1     |

Table 2: Experimental design using RSM for the optimization of Nutritional Parameters
## Table 3: BSA standard dilutions and their absorbance at 660 nm

| S. No. | Concentration of BSA (mg/mL) | Absorbance at 660 nm |
|--------|------------------------------|----------------------|
| 1      | 0.05                         | 0.206                |
| 2      | 0.1                          | 0.245                |
| 3      | 0.2                          | 0.369                |
| 4      | 0.4                          | 0.55                 |
| 5      | 0.6                          | 0.745                |
| 6      | 0.8                          | 0.965                |
| 7      | 1                            | 1.13                 |
Fig. 1: 3D response surface graphs showing interaction between (a) time period and temperature (b) temperature and pH (c) temperature and inoculum size (d) temperature and substrate size (e) pH and inoculums size (f) inoculum size and substrate size during the production of endoglucanase by *A. fumigatus*
(a) glucose and fructose (b) glucose and Ammonium sulphate (c) glucose and urea (d) fructose and sucrose (e) fructose and ammonium sulphate (f) ammonium sulphate and urea during the production of endoglucanase by *A. fumigatus*

**Fig. 3:** Effect of various metallic ions on Endoglucanase activity.

**Fig. 5:** Characterization of endoglucanase for the determination of (a)- Optimum temperature (b)- Optimum pH (c)- Double reciprocal plot for the determination of $K_m$ and $V_{max}$
Fig. 4: Endoglucanase activity after (a) - ammonium sulphate precipitation and (b) - Gel Filtration Chromatography.
Fig. 5a

Fig. 5b

Fig. 5c