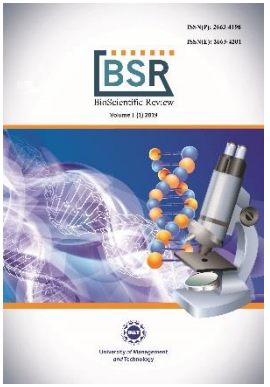

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Implementation of Response Surface Methodology (RSM) for the Enhanced Production of Endoglucanase by Thermophilic *Aspergillus fumigatus*

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Abstract

Enzymes are biocatalysts which play several key roles in the bodies of living organisms. Cellulose is a major source of plant biomass. Its β-1,4-glucosidic bonds are hydrolyzed by cellulases. These cellulases are produced by a variety of microorganisms including fungi, bacteria and actinomycetes and used in various industries. The current study was aimed to optimize the cultural conditions required for the maximum production of endoglucanase by *Aspergillus fumigatus* through the Solid State Fermentation (SSF) of sugarcane bagasse. Response Surface Methodology (RSM) was employed using the Central Composite Design (CCD) for the optimization of various growth parameters including pH, temperature, time period and inoculum size. Moreover, different nutritional parameters including glucose, fructose and $(NH_4)_2SO_4$ were also optimized. The effect of different metal ions on endoglucanase production was also monitored. It was partially purified by $(NH_4)_2SO_4$ precipitation and via gel filtration chromatography. Finally, endoglucanase was characterized for the optimum pH, temperature and kinetic parameters. Maximum enzyme activity was found to be 0.9 IU/mL/min in the presence of 6 g substrate, 3.5 mL inoculum, 4.5 pH, and at 40°C temperature at the incubation time of 84 hrs. After the 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 the addition of 0.42% of $CaCl_2$. Maximum purification was achieved at 50% saturation by ammonium sulphate $(NH_4)_2SO_4$. 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 produced by *Aspergillus fumigatus* may be suitable for various industries.

1. Introduction

Lignocellulose is a renewable natural source and a major structural component of all plants, substantially composed of hemicellulose, cellulose, lignin and a small amount of other materials, such as ash and pectin, in different degrees. Cellulose chains, being the most abundant, are coupled by hydrophobic interactions, hydrogen bonding, and Van der Waals interactions [1]. Through the biotransformation of lignocellulosic biomass including forestry residues, agricultural wastes, and paper wastes, various kinds of industrially important enzymes with a high yield are produced in a cost effective manner [2, 3]. In Brazil, the residues of sugarcane comprise one of the largest cellulosic agro-industry in the world, of which the bagasse portion comprises approximately 50% of cellulose and 25% of hemicellulose and lignin. Whereas, the straw portion consists of cellulose, hemicellulose and lignin with a 37.4%, 30% and 18.5% share, respectively [4].

Among various kinds of cellulolytic enzymes produced from different microbes used for the bioconversion of industrial and agricultural wastes, cellulases are regarded as more efficient in hydrolyzing cellulose into glucose and other useful components [5]. Cellulases contribute to 8% of the global industrial enzyme demand 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 including endoglucanase, exoglucanase and b-glucosidase act synergistically on cellulose chains, producing glucose as their 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 an end product by acting on the ends of cellulose chains, followed by the production of glucose by the action of β-glucosidase upon β-cellobiose disaccharides [6]. In cellulase chains, when endoglucanase cuts β-1, 4-bonds, it generates two ends. In most types of endoglucanase, catalytic modules have a grove shaped active site. This active site allows the respective endoglucanase to bind and cleave to the cellulose chain in order to generate glucose and other components [10-15].

A number of microbes have cellulolytic abilities but fungus is considered as the most suitable for the production of enzymes on an industrial scale. The fungi kingdom has approximately 200 species of *Aspergillus*, isolated from the soil and used in the production of a variety of extracellular enzymes. The best known species with a 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 their tolerance of a wide

range of pH variations and their resistance to denaturing agents. A review of literature revealed that *A. fumigatus* strains were reported for maximum cellulase production under Solid State Fermentation (SSF) [17]. SSF /is more advantageous than Submerged State Fermentation due to its easy handling and good control on environmental conditions. As the fungus has a great potential to grow on solid substrate in the absence of free liquid, so fungi is regarded as more effective in enzyme production under solid state conditions [18].

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

2. Methodology

2.1. Fermentative Organism

Fungal strain of *A. fumigatus* was collected from the 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 future use. These plates were stored at -4°C.

2.2. Fungal Inoculum

Fungal inoculum was prepared in broth media (pH 5.5) for its subsequent use in SSF.

2.3. Substrate Preparation

Firstly, sugarcane bagasse was collected and dried in the shadow. After removing its 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 the SSF process, flasks (250 mL) were used containing substrate with 50% moisture. These flasks were moistened with distilled water and sterilized in autoclave at 121°C for 15 mins. After sterilization, they were placed in the laminar air flow. On their cooling down to room temperature, inoculum medium was added aseptically to each flask and incubated at conditions according to the experimental design (Table 1).

2.5. Extraction of Crude Enzyme

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

2.6. Response Surface Methodology (RSM)

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

Table 1. Experimental Design using RSM for the Optimization of Growth Parameters

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
28.	24	20	3	1	2

2.7. Optimization of Endoglucanase Production

During the 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 conducted at each level for the optimization of cultural and nutritional conditions, designed with the JMP software using 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

Table 2. Experimental Design using RSM for the Optimization of Nutritional Parameters

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
22.	0.3	0.3	0.3	0.3	0.3
23.	0.3	0.3	0.3	0.3	0.1
24.	0.5	0.3	0.3	0.3	0.3
25.	0.5	0.5	0.1	0.1	0.1
26.	0.3	0.3	0.1	0.3	0.3
27.	0.3	0.3	0.3	0.3	0.3
28.	0.1	0.5	0.5	0.5	0.5

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 the protocol described by Mahmood *et al.* (2013), using Carboxy Methylcellulose (CMC) as substrate and glucose as standard [20]. Standard factor was determined by taking the absorbance of different concentrations of glucose (0.5-4.0 µM / mL with a difference of 0.5) at 540 nm. Enzyme activity was calculated using the following formula.

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

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

where,

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

where,

dilution factor = 50 and standard factor = 3.108

2.9. Purification of Endoglucanase

Crude endoglucanase was partially purified by ammonium sulphate precipitation and via 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 with 40%, 50%, 60%, 70% and 80% ammonium sulphate. These tubes were placed at 4^0C for 4 hours, then centrifuged at 10,000 rpm for 15 mins. The pellet was suspended in 2 mL citrate buffer and assay was performed [22].

For gel filtration chromatography, 5% Sephadex solution was prepared in d.H_2O and left overnight. It was gently mixed and used to fill the column slowly to avoid any air bubbles. Then, 2 mL of crude enzyme was poured at the top of the column and elutions (91 mL) were collected from the bottom. A total of 24 elutions were collected at the flow rate of 30 mL/hr and the first 5 were discarded, while the other 19 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 at 6 different temperatures (35^0C to 60^0C) and with 5 different pH values (between 4-8), separately [20, 22].

To determine the kinetic parameters, that is, K_m and V_{max} of endoglucanase, 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 the Line-Weaver Burk graph between the inverse of substrate concentrations and activity. The equation obtained from the graph was used to calculate K_m and V_{max} [20, 24].

3. Results

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

3.1. Optimization of Growth Parameters for *A. fumigatus*

Cultural conditions were optimized using the experimental design given in Table 1.

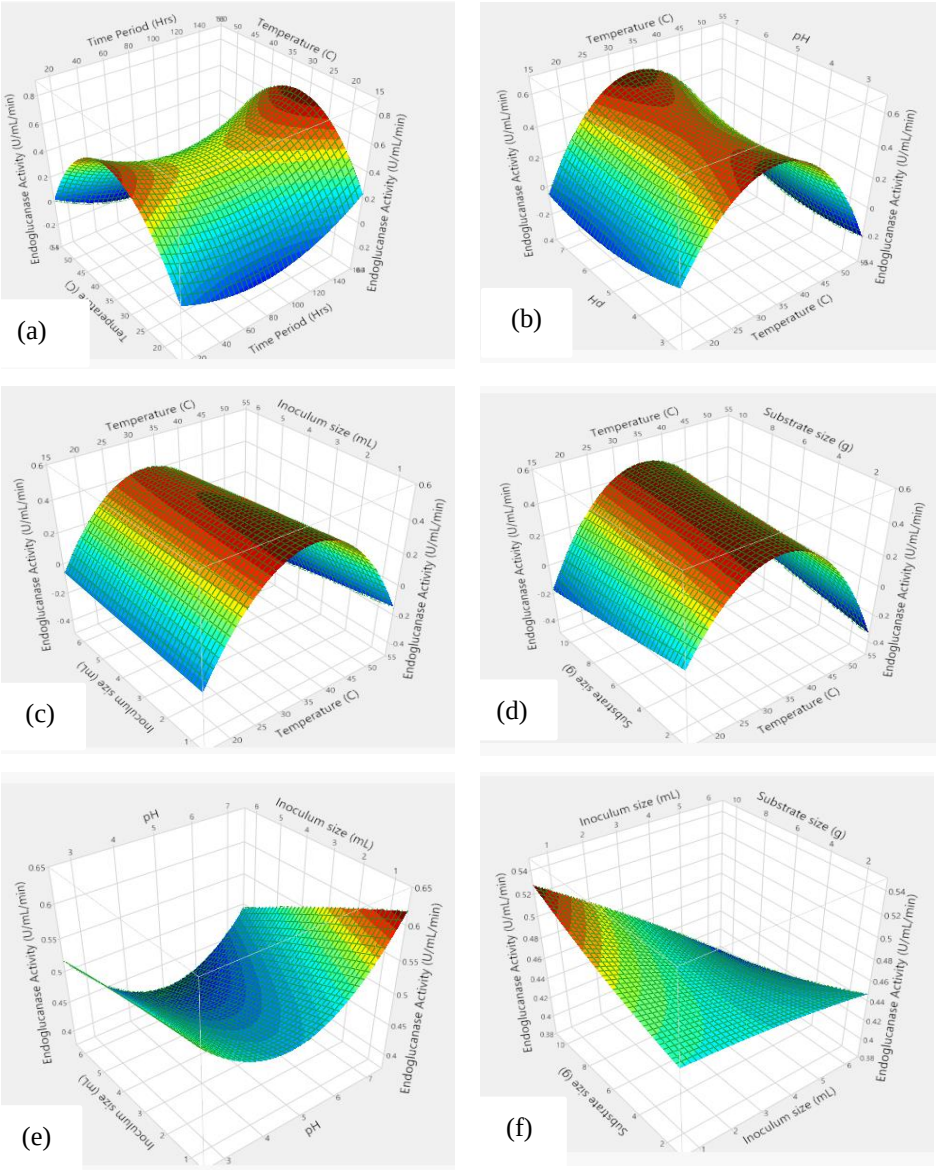

Figure 1. 3D response surface graphs showing interaction between (a) time period and temperature (b) temperature and pH (c) temperature and inoculum size (d) temperature and substrate size (e) pH and inoculums size (f) inoculum size and substrate size during the production of endoglucanase by *A. fumigatus*

3.2. Optimization of Nutritional Parameters for Endoglucanase Activity

Fungi produce a variety of enzymes depending upon the availability of nutrients in media. The results of experiments conducted for the optimization of nutritional parameters were also analysed by plotting 3D response surface graphs through the JMP software. Glucose is considered as a major source of carbon for most organisms. It is a monosaccharide and is also known 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 was charted. When a response surface graph was plotted keeping in view the interaction between glucose, fructose and enzyme activity, enzyme activity was found to be maximum (1.4 U/mL/min) at 0.12% glucose and 0.4% fructose (Fig. 2a).

Ammonium sulphate is an inorganic salt with the formula $(NH_4)_2SO_4$. It contains 21% nitrogen and 24% sulphur [27]. Fig. 2b shows a 3D response surface graph which depicts the interaction between glucose and ammonium sulphate as a slight up rise curve. This curve shows the peak of both factors. The enzyme showed 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 was 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 the current study, the interaction between fructose and sucrose was observed by plotting a 3D graph, which showed that enzyme activity was high with low quantities of sucrose and fructose. Enzyme activity was found to be 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, when the quantity of fructose and ammonium sulphate was smaller endoglucanase activity started to reduce, as shown by the 3D response surface graph.

The interaction between ammonium sulphate and urea was observed by plotting a graph which showed an umbrella like curve, indicating a positive interaction between these factors. The activity of enzyme was increased by increasing these nutritional parameters. Enzyme activity was 0.55 IU/mL/min with 0.2% urea and 0.15% ammonium sulphate (2f). Its activity was very low below this percentage of ammonium sulphate, as well as that of urea. So, the analysis of the results through the JMP software concluded that nutritional factors are also significant for any enzyme to work. The selection of suitable conditions is very important to ensure maximum enzyme production. Central Composite Design (CCD) is suitable for such kind of experiments.

3.3. Effect of Metal Ions on Endoglucanase Activity

Different metal ion solutions were used in their specific concentrations. Firstly, calcium chloride ($CaCl_2$) was used to check the effect of Ca^{2+} ion. Five concentrations of $CaCl_2$ were prepared as described above and enzyme activity was calculated. Graph

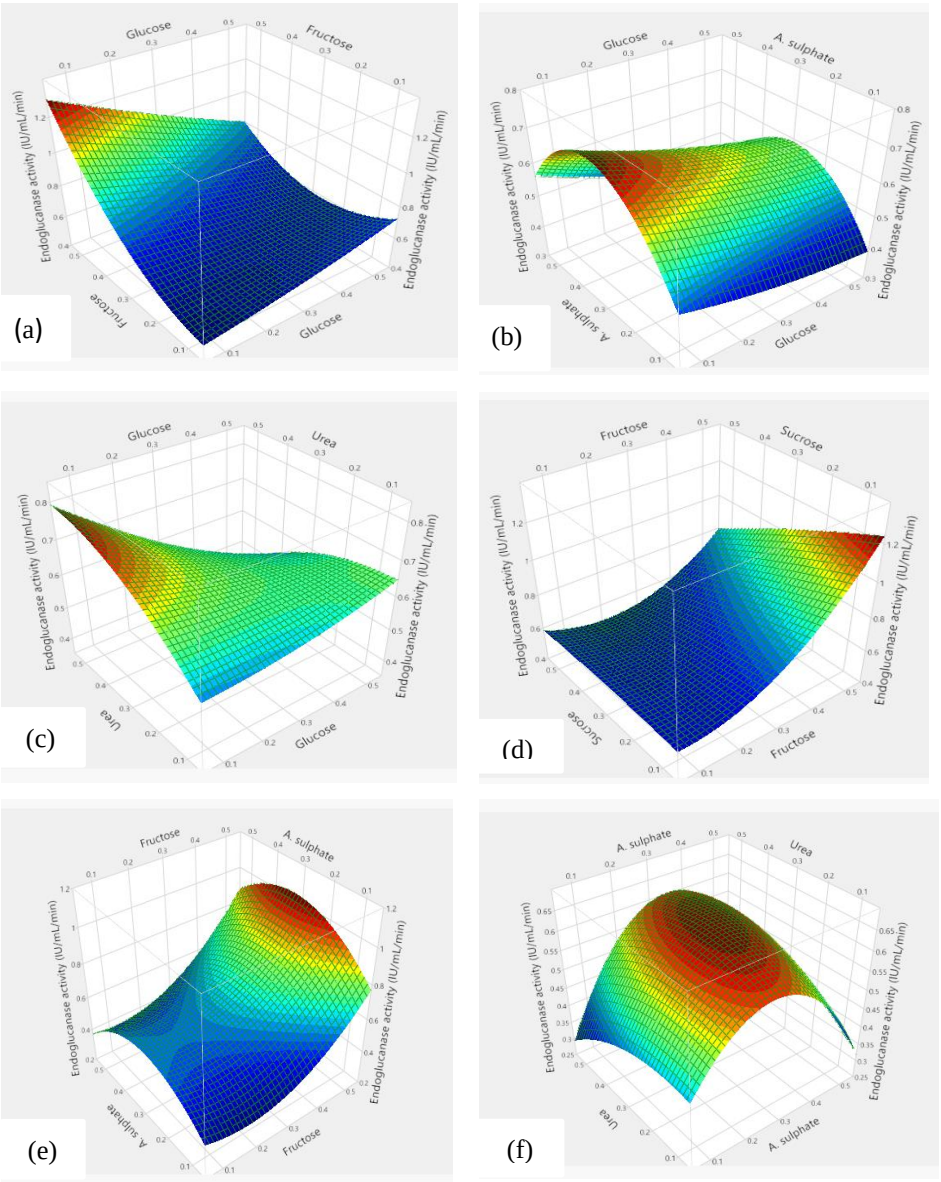

Figure 2. 3D response surface graphs showing interaction between various nutritionalparameters (a) glucose and fructose (b) glucose and Ammonium sulphate (c) glucose and urea (d) fructose and sucrose (e) fructose and ammonium sulphate (f) ammonium sulphate and urea during the production of endoglucanase by *A. fumigatus*

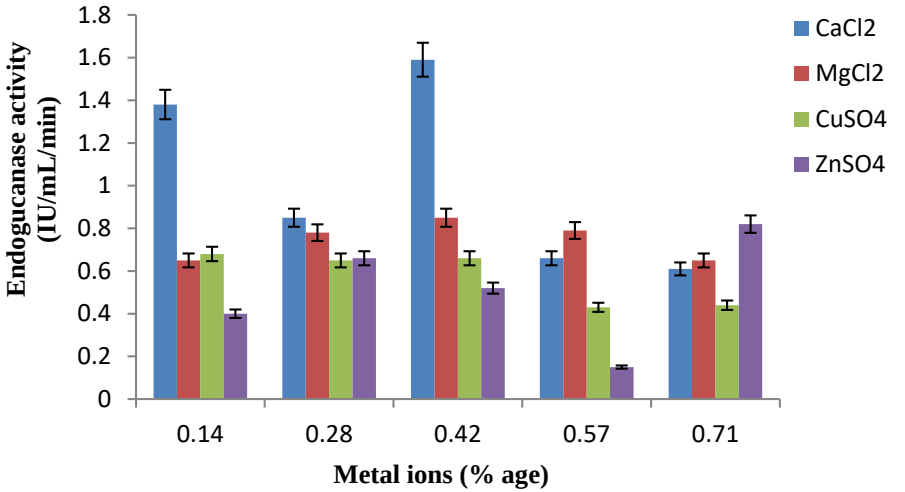

Figure. 3. Effect of various metallic ions on endoglucanase activity

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

was plotted between the concentrations of $CaCl_2$ and the calculated enzyme activity. There was maximum enzyme activity (1.6 IU/mL/min) at 0.42% of $CaCl_2$ (Fig. 3). In case of $MgCl_2$, $CuSO_4$ and $ZnSO_4$, enzyme activity did not increase further (Fig. 3). So, it was concluded from the results that calcium chloride has a positive effect on endoglucanase activity, whereas other metal ions have no effect on its activity.

3.4. Estimation of Proteins

Total protein estimation was made by making different dilutions of standard protein, that is, Bovine Serum Albumin (BSA). Dilutions were prepared according to the concentrations given in Table 3. The absorbance of these dilutions was measured on spectrophotometer at 660 nm (Table 3).

3.5. Purification of Crude Enzyme

During ammonium sulphate precipitation of endoglucanase, maximum enzyme activity was shown by 50% saturation of $(NH_4)_2SO_4$) (Fig. 4a). Column gel chromatography showed 22nd eluted sample as the best purified (Fig. 4b).

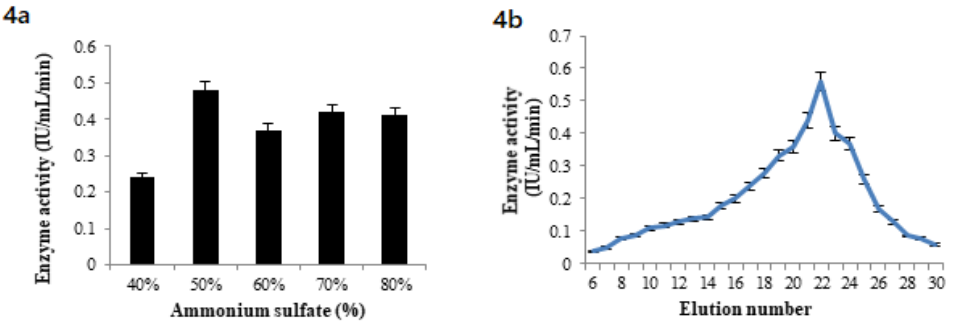

Figure. 4. Endoglucanase activity after (a) - ammonium sulphate precipitation and (b) - gel filtration chromatography

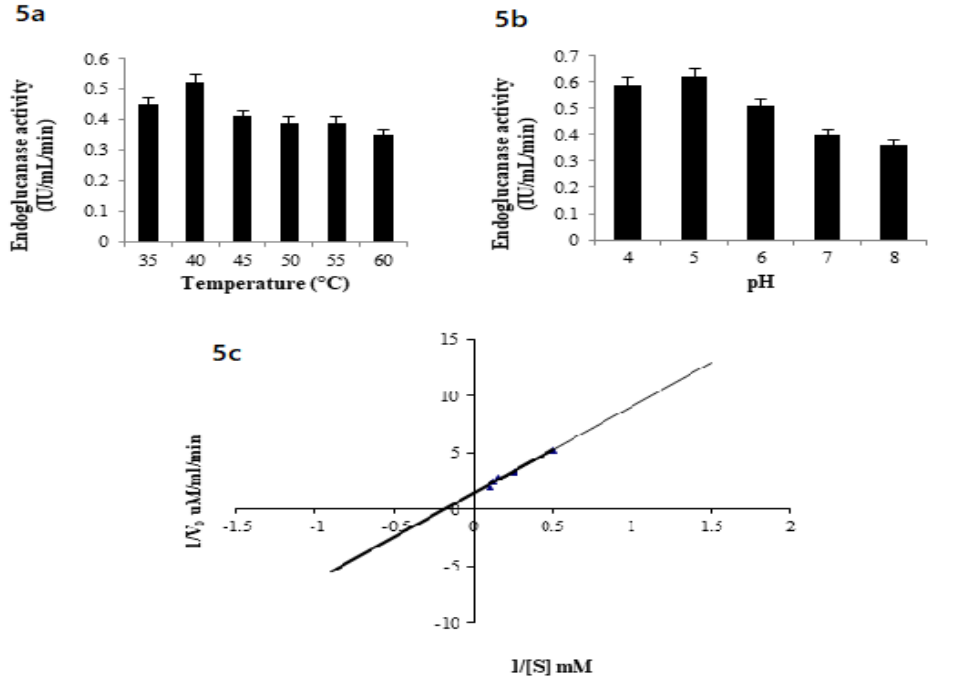

Figure 5. Characterization of endoglucanase for the determination of (a)- Optimum temperature (b)- Optimum pH (c)- Double reciprocal plot for the determination of K_m and V_{max}

3.6. Characterization of Endoglucanase

The characterization of endoglucanase revealed that it has an optimum temperature of 40°C (Fig. 5a) and an optimum pH 5 (Fig. 5b). The results showed that it remained highly active up to 60°C, indicating the thermophilic nature of this

enzyme with a wide industrial applicability. Endoglucanase was also studied for the determination of kinetic parameters including *Km* and *Vmax*. These help to determine the efficacy of this enzyme and its affinity towards the substrate. The results obtained after performing assay with different molar concentrations of CMC as substrate were used to plot Line-Weaver Burk double reciprocal graph. The values of kinetic parameters were calculated using the linear regression equation obtained through this graph by taking the value of x=0 for *Vmax* and y=0 for calculating *Km* (Fig. 5c). The endoglucanase of *A. fumigatus* has *Vmax* equal to 696 uM/mL/min and *Km* equal to 5.37 mM. These values suggest that it is an active enzyme with a good affinity towards CMC and can be a good choice for various industrial applications.

4. Discussion

Enzymes are biocatalysts with applications in different industries. Microbes are the major source of industrially important enzymes [29]. It is essential to explore the microbes, which produce industrially important enzymes. The process of the production of thermophilic endoglucanase enzyme was studied in this research. The results are quite significant for the higher production of efficient endoglucanase at optimized conditions.

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

The results showed that enzyme production is dependent on the availability of microbial spores and the amount of substrate. Higher spores can yield more enzymes butsubstrate concentration may act as a limiing factor, which can be avoided by inreasing substrate saturation. Further, withmore substrate and higher spores, the generation of toxic waste stops or decreases enzyme activity after a certain time [32]. Abo-State et al. (2010) [33] performed similar studies on various fungal species with a variety of substrates to find the most suitable substrate and fungi. Their findings suggested that fungi are the suitable microbes capable of more efficient substrate utilization and bear less effect of substrate depletion or waste accumulation.

There was almost 40% increase in endoglucanase 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 *A. fumigatus* and reported cellulase production using various carbon and nitrogen sources. Production was enhanced with additional nutrients in an acidic environment at room temperature.

There was 60% increase in enzyme activity after ammonium sulphate precipitation as compared to the initial activity. Farinas et al. (2011) [35] reported 61-65% recovery of endoglucanase after ammonium sulphate precipitation that caused its purification and enhanced its application. Bagewadi and Ninnekar (2015) [36] worked on the kinetic energy of purified enzymes and also studied the effect of various metal ions. The results obtained during the current project are very similar to theirs. There was a positive effect of Ca ion and higher *Vmax* with a lower *Km* value.

5. Conclusion

It can be concluded that RSM is a suitable technique for the optimization and production of enzymes through Solid State Fermentation (SSF). There was 0.94 IU/mL/min endoglucanase activity observed after 84 hrs at 40^0C with 3.5 ml fungal inoculum. The maximum activity was the combinatorial effect of all the parameters under study. It was enhanced up to (almost) 40% by the addition of glucose, fructose and ammonium sulphate in the media. It was further increased (up to 20%) when 0.42% Ca ions were used. At 50% ammonium sulphate saturation, the maximum precipitation of endoglucanase was observed with V_{max} equal to 696 uM/mL/min and K_m equal to 5.37 mM.

Conflict of Interest

The authors declare no conflict of interest.

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