PRODUCTION AND CHARACTERIZATION OF CELLULASES FROM *ASPERGILLUS NIGER* UNDER STATIC FERMENTATION

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The ever-increasing uses of cellulase in various industries have made it popular among researchers. Annually, a large amount of fruit wastes go into vain, which is a great source of cellulases. The objective of this study is to use grape wastes as substrate for production of cellulases from *Aspergillus niger* using static fermentation. CMCase and FPase assays were performed to characterize the cellulases. The cellulases' CMCase and FPase activities and stabilities were analyzed for optimum temperature and pH. The effect of substrate concentration and kinetic constants K_m and V_{max}, along with thermodynamic analysis, were determined. The effects of several metals on the activity of the enzyme were observed. The optimal temperatures were found as 40 °C and 50 °C for CMCase and FPase activity, respectively. CMCase activity shows stability at 20 °C-60 °C, FPase shows low thermal stability as its activity starts to decrease after 50 °C. CMCase and FPase both show maximum activity at pH 6, and maintain their stability at pH 6-7. The values of K_m and V_{max} obtained from Lineweaver and Burk plot for CMCase are 0.648 mM and 12.953 mM/min, and for FPase are 0.975 mM and 41.493 mM/min. The Arrhenius plot was used to calculate activation energy (Ea) as -19 kJ/mol, and enthalpy of reaction (ΔH) as 16.4 kJ/mol, while entropy ΔS -16.4 kJ/mol was obtained from the plot of ln(Vmax/T) versus the inverse of temperature (1/T). Most metals induce enzyme activities, whereas EDTA inhibits enzyme activities. The findings suggest *A. niger* has remarkable cellulase production potential from grape wastes in static fermentation, at optimum temperature and pH levels for achieving enzyme activity and stability.

Keywords: *Aspergillus niger*, cellulase, characterization, CMCase, FPase

INTRODUCTION

Cellulose, a prominent naturally occurring nanostructured renewable material, constitutes a fundamental component in all plants, algae, and the tunics of marine organisms. Cellulases comprise multi-enzyme complexes primarily composed of 3 distinct components: endo-1,4-β-D-glucanases, exoglucanase, and β-glucosidase. They collaborate cooperatively for the hydrolyzation of cellulose polymer into its glucose monomers. Firstly, endo-1,4-β-D-glucanases initiate the process by acting randomly within cellulose fibers, creating both reducing and non-reducing ends. Subsequently, exo-cellobiohydrolase targets these ends, excreting cellobiose, which consists of glucose dimers linked by β-1,4 glycosidic bonds. Ultimately, it undergoes hydrolysis by β-glucosidase (BGL), creating glucose monomers, which are the final product. Different cellulase types are endoglucanases (EC 3.2.1.4), exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), β-glucosidase (BG) (EC 3.2.1.21), cellobiase, cellodextrin phosphorylase, and cellobiose epimerase.[1-3] Exoglucanase is commonly referred to as FPase (filter paper activity) and endoglucanase as CMCase (carboxymethyl cellulase). FPase assess enzyme's ability for catalization of cellulose hydrolysis into soluble sugars using filter paper as substrate, while CMCase measures the enzyme's capability to degrade into smaller and soluble fragments.[4]

Several organic wastes are used to extract cellulases. These organic wastes act as substrate for fermentation. Among these organic wastes, agricultural wastes are widely used. A few of the agricultural wastes used for cellulase production are sorghum hulls, sugarcane bagasse, soybean hulls, rice straw, groundnut husk, cassava peels, finger millet hulls, sawdust, banana peels, corn stalk and sheanut cake,[5] rice, sugarcane, and wheat,[6] and *Arachis hypogaea* shells.[7] Mainly fungi and bacteria are used for cellulase production. However, fungi are considered as a more potential candidate due to their better penetration and ability to work well with different substrates.[8] The ability to synthesize cellulase is generally recognized in microorganisms belonging to the genera *Trichoderma* and *Aspergillus*, and the enzymes produced by these species are sold commercially for usage in industrial settings.[7] Cellulolytic fungi have the significant benefit of using secretory routes in addition to the yields of high-quality protein produced. In addition, other fungi that are thought to be viable substitutes for *Trichoderma* include *Penicillium*, *Acremonium*, and *Chrysosporium.*[9] *Trichoderma reesei* and *Aspergillus niger* are the most prevalent microbes that produce cellulases.[10]

Several fermentation techniques are reported in the literature to obtain cellulases. Solid state fermentation (SSF) and submerged fermentation are widely used to extract enzymes from various agricultural and domestic wastes.[11-13] Static SSF under nonaseptic conditions has been used to successfully produce cellulases from *Trichoderma harzianum*.[14] Cellulases have applications in various industries, such as the food industry, textile industry, wine and brewery, pulp and paper, bioethanol industry, and in waste management, and therefore, their efficient production is important for the industrial sector.[8,15-17] The current study focuses on the production of cellulases by *A. niger* using grape waste as a carbon source under static fermentation, and on the characterization of the crude cellulase.

EXPERIMENTAL

Materials

All chemicals used in the current research were of analytical grade and were purchased from Sigma Plus, Lahore, Pakistan. Biological media were obtained from Scientific Research Labs, Pakistan.

The Department of Biotechnology, University of Sargodha, provided the fungal strain of *A. niger.* The PDA plates, prepared at room temperature for 3-5 days, were used to revive the strains.

Grape waste was collected from local fruit juice shops of the district Sargodha, Pakistan. A mortar and pestle was used to obtain the mashed grapes that were later used as substrate for CMCase and FPase production in further steps.

CMCase and FPase production

For enzyme production, static aseptic fermentation was used. Twenty grams of finely crushed grapes were kept in a plastic bottle along with 100 mL of distilled water. Inoculation with *A. niger* strain was done, followed by incubation for 96 h at room temperature. Centrifugation of fermented media was done for 15 min at 10,000 rpm. The supernatant of centrifugation was used as enzyme for FPase and CMCase assays.

CMCase assay

The DNS method was used to assay the target supernatant for cellulase activity by assessing the amount of reducing sugars. 0.5 mL of 1% carboxymethyl cellulose (CMC) was mixed with 0.5 mL of sample. Incubation was done at 50 °C for 30 min. Later on, 1.5 mL of DNS was added and the mixture was boiled for 10 min and then cooled down at room temperature. After that, the absorbance was checked at 540 nm using a spectrophotometer.[18,19]

FPase assay

The filter paper assay (FPase) was used to analyze cellulase activity. A cultured filtrate of 0.5 mL, along with 0.5 mL of 0.2 M sodium citrate buffer (pH 4-9.5), was incubated with 50 mg of dry Whatmann No. 1 filter paper strip for 30 min. The DNS method was used to measure released glucose. The quantity of enzyme needed to release one micromole of reducing sugar per milliliter under assay conditions in both cases is known as one unit of enzyme activity.[20]

Characterization of enzyme

Effect of temperature on enzyme activity and stability

The incubation of enzyme with the substrate was done at various temperatures, *i.e*., 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C. The cellulases activity was determined by Miller's method.[21] In a test tube having 0.5 mL of crude cellulases, 0.5 mL of 1% CMC prepared in 0.5 M citrate buffer with pH 4.0 was added to obtain a mixture. The incubation of mixtures at various temperatures, as mentioned above, was done for 30 min. The reaction was terminated by the addition of 1.5 mL of DNS solution, followed by placing the mixture in boiling water for 10 min. Later, the mixture was cooled down and measurements of absorbance were taken at 540 nm.[22,23]

To determine the thermal stability of cellulases, the incubation of crude enzyme at 20-80 °C for 2 h was done, without enzyme substrate. The relative enzyme activities for CMCase and FPase were measured

subsequently at different time intervals (20, 40, 60, 80, 100 and 120 min) using a standard assay method.[22]

Effect of pH on enzyme activity and stability

Three buffers, namely, 0.1M acetate buffer (pH 4-5), 0.1 M citrate phosphate (pH 6.0-7.0), and 0.1M Tris HCL buffer (pH 8.0-9.0), were used to determine the effect of pH (4-9) on enzyme activity by performing enzyme assays. In a test tube containing 0.5 mL of crude cellulases, 0.5 mL of 1% CMC prepared in 0.5 M of buffers with varying pH, in the range of 4.0-9.0, was added to obtain a mixture. The incubation of mixtures at 50 °C was done for 30 min. The addition of 1.5 mL of DNS solution was ensured to terminate the reaction, followed by placing the mixture in boiling water for 10 min. Later, the mixture was cooled down and measurements of absorbance were taken at 540 nm.[18]

The pH stability of the enzyme was determined in acidic, basic, and neutral conditions in the pH range of 4-9. Crude enzyme, which was the supernatant of CMCase and FPase, was taken in 2 mL of respective buffer with pH 4-9. The incubation of the mixtures was done at room temperature for 2 h. Later, enzyme assays were done at 50 °C to analyze its activity.[18]

Effect of metal ions

To determine the effect of metal ions (NaCl, $CaCl_2$, KCl, $ZnCl_2$, $CuSO_4$, $MgCl_2$, $FeCl_3$, and $MnCl_2$) and chelating agent (EDTA) on cellulase activity, concentrations of 1 mM, 3 mM, and 5 mM of each salt were taken and further activities were performed as per the assays protocol.[24]

Enzyme kinetics

CMC and FPase are the two substrates, which were used to test substrate specificity of CMCase and FPase. Once optimum pH was determined, the Michalis-Menten model was used for determining K_m and V_{max} values using GraphPad Prism 8.0 software, by investigating varying substrate concentrations (0.5-3%), and assays were conducted for cellulases.[25]

Thermodynamic analysis

The Arrhenius method was employed to calculate the activation energy (Ea) of cellulases, it was further used to calculate thermodynamic parameters, enthalpy change (ΔH) and entropy (ΔS). The equations for these parameters are as below:

$$E_a = -\mathrm{m} * \mathrm{R} \quad (1)$$

where m is slope and R is Boltzmann constant, whose value is 8.314 $J/K^{-1}.mol^{-1}$.

$$\Delta H = E_a - (\mathrm{R} * \mathrm{T}) \quad (2)$$

$$\ln(V_{max}/\mathrm{T}) = \ln\left(\frac{K_b}{h}\right) + \frac{\Delta S}{R} - \left(\frac{\Delta H}{R} * 1/T\right) \quad (3)$$

where K_b is the gas constant with the value of 1.38*10–23 $J.K^{-1}$, h is Planck's constant whose value is 6063*10-34 J.s, and T is absolute temperature.[26]

RESULTS AND DISCUSSION

Effect of temperature and thermal stability

The optimum temperature for the CMCase activity of the enzyme was observed to be at 40 °C, as shown in Figure 1. At the beginning, the CMCase activity keeps increasing with the increase in temperature, and once it reaches its peak value, further increase in temperature leads to a decrease in the CMCase activity of the enzyme. Ain and coworkers noticed optimum temperature around 40 °C using rice husk and sawdust as substrates for CMCase assay.[27] Coral *et al*. also observed optimum temperature for CMCase activity to be around 40 °C, while varying temperature between 30 °C to 90 °C.[28]

Figure 1 shows optimum temperature for FPase activity, which is 50 °C. Initially, the activity of the enzyme keeps increasing with an increase in temperature, and once it reaches its peak value, further increase in temperature decreases the enzyme activity. Rawat and colleagues observed a similar trend for FPase activity while using rice straw and *A. niger*. By testing enzyme activity at 5 different temperature levels from 50 °C to 80 °C, 55 °C was found to be the optimum temperature. Enzyme activity was decreased on both sides of the optimum.[29] The 5 °C difference in optimum temperature can be accounted for by the difference in substrates, techniques, and environmental factors.

Figure 2 shows the thermostability of CMCase activity in the temperature range of 20-70 °C. CMCase maintained its maximum activity (approx. >> 20 IU/mL) in the temperature range from 20 °C to 60 °C. At 70 °C, the activity starts to decrease and falls in the range of 10-14 IU/mL. From the start to 80 min, CMCase activity remains thermally stable in the temperature range 20-60 °C, and starts to decrease at 100 min. A study conducted on thermostability observed a similar trend, while using *A. fumigatus* SK1 on oil palm trunk under SSF. The study reveals that, when temperature is varied from 30 °C to 80 °C at 0, 10, 20, 40, 60, 80, 100 and 120 min, CMCase retains its maximum activity during the first 100 min of incubation at a temperature ranging from 30 to 60 °C. Later on, at 70 °C and 80 °C, its starts to decrease.[22]

Compared to CMCase, the thermal stability of FPase is less, as its activity starts to decrease after 50 °C, as shown in Figure 3. From the start to 80 min, FPase activity mostly keeps on increasing (above 40 IU/mL) for different temperature

ranges (20-50 °C). However, at 60 °C FPase activity begins at 35 IU/mL, which further decreases after 60 min. For 70 °C, activity begins at around 25 IU/mL, and reaches minimum value at 60 min. Rawat and team noticed FPase to be less thermally stable, as it preserved 70% of its activity at a temperature below 50 °C, and further activity decreases at 60 °C, 70 °C, and 80 °C by 120 min.[29]

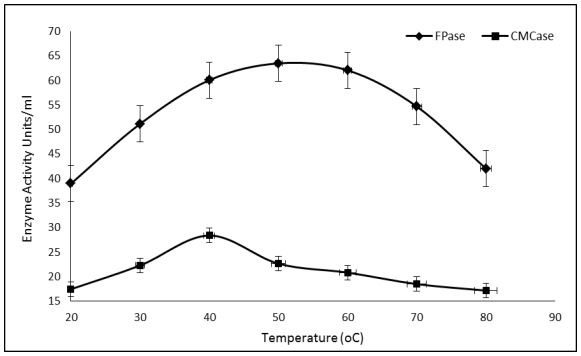

Figure 1: Effect of temperature on CMCase and FPase activity produced by *A. niger* in static fermentation

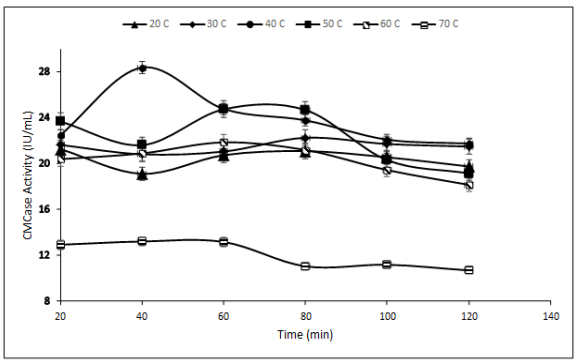

Figure 2: Effect of temperature on the stability of CMCase produced by *A. niger* in static fermentation at 20-70 °C

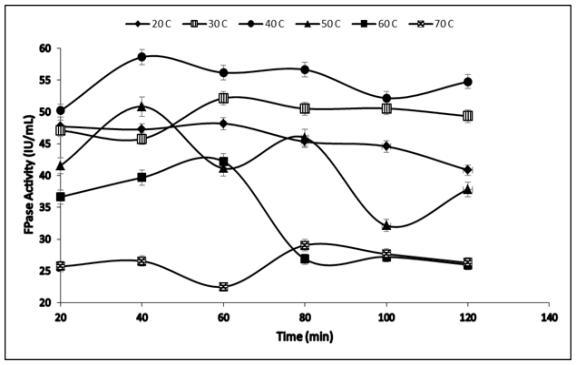

Figure 3: Effect of temperature on the stability of FPase produced by *A. niger* in static fermentation at 20-70 °C

Effect of pH on enzyme activity and stability

The enzyme shows maximum activity in the CMCase assay at pH 6, as shown in Figure 4. Initially, when the pH starts increasing, the activity of the enzyme increases as well, but once it reached its peak value, the activity starts decreasing. The minimum enzyme activity was noted at pH 9. A study using *A. niger* on rice husk and sawdust was conducted and its results showed maximum enzyme activity at pH 5.[27] Another study reveals maximum production at pH 4 and 5 for rice straw and wheat straw substrates, respectively, by *A. niger.* The findings further suggest a decrease in production after pH 7.[30] Coral and coworkers observed two peaks for enzyme production *viz.*, pH 4.5 and 7.5 on *A. niger* Z10.[28] Studies suggest that the effect of pH on cellulase CMCase activity is influenced by substrates, as well as by other factors.

CMCase by *A. niger* maintains its stability around pH 6-7. Before and after these pH values, the cellulase shows relatively unstable activity, which increases before pH 6 and decreases later on. Zang and co-workers observed the enzyme stability in the range of pH 4.4 to 6 by *A. niger.*[31] Another study revealed the stability of cellulases around pH 4-9 by *A. fumigatus* JCM 10253.[18]

Maximum enzyme activity for FPase (44.58 IU/mL) was observed at pH 6, as shown in Figure 5. At pH 7, it slightly decreases to 44.52 IU/mL. Before reaching the maximum peak of FPase activity at pH 6, enzyme activity increases with increasing pH. A study conducted using *A. niger* for cellulase production from agricultural wastes observed maximum FPase activity at pH 4.2.[9] Mrudula and Murugammal noticed two peaks of pH for FPase activity at 6 and 7.5, while using *A. niger* under SmF.[32]

FPase by *A. niger* maintains its maximum stability around pH 7-8. Bansal and team observed the stability of cellulases under SSF by A. *niger* NS-2 using wheat bran as substrate, in the range of pH 3-9 for 24 hours. FPase maintained its stability around pH 3-4.[33] The stability range differences possibly occur due to differences in fermentation, substrate, and duration.

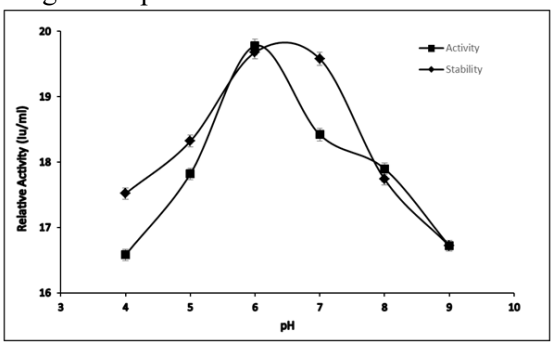

Figure 4: Effect of pH on CMCase activity and stability produced by *A. niger* in static fermentation

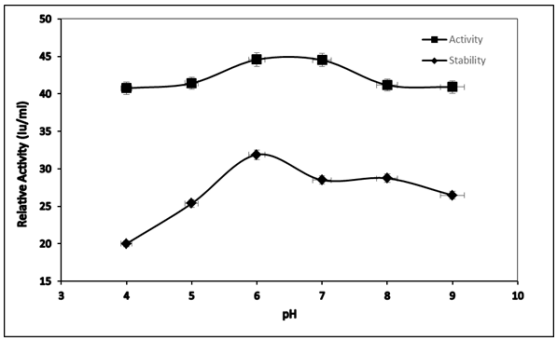

Figure 5: Effect of pH on FPase activity and stability produced by *A. niger* in static fermentation

Effect of metal ions

The maximum CMCase activity for 1 mM concentration of metals was observed for Mn ion (102%), whereas minimum activity was observed for Mg ion (87%) and EDTA (86.7%). Mn ion shows maximum activity (102%) of cellulases for 3 mM concentration, meanwhile K ion (92%) and EDTA (85%) lead to minimum activity. The trend continues when concentration moves up to 5 mM, here again Mn ion shows the highest enzyme activity (104%) and the least is noted for Mg ion (87%) and EDTA (86.7%) – all results shown in Figure 6.

A study showed that most divalent cations as Cu and Zn induced and monovalent like Na inhibited enzyme activity at 1 mM concentration of salts using *Streptomyces macrosporeus* BB 32 for cellulases production.[34] Current results are in accordance with the literature, as Na and K inhibit and divalent cations Mn, Ca, Fe, Cu, and Zn induce enzyme activity, with the exception of Mg. Mg diversion from the trend possibly occurs due to differences in the substrate, strain, fungi and other environmental conditions.

Saroj and co-workers observed that enzyme activity declines at 2 mM and 5 mM concentrations of K, Na and Zn ions, and it enhances with Mn ion using *A. fumigatus* JCM 10253. Furthermore, Saroj *et al*. suggest Mn^{2+} acts as cofactor in enhancing cellulase activity.[18] The results in Figure 6 show Mn ion inducing maximum enzyme activity for 1 mM, 3 mM, and 5 mM, hence acting as a cofactor in inducing the activity of the enzyme. A decline in enzyme activity is observed by Na and K ions for 5 mM. Zn and Cu ions decrease enzyme activity at 5 mM when using *A. niger* HQ-1.[31] Cu ion leads to a significant decline in enzyme activity (97.81%) at 5 mM. EDTA shows no significant impact on enzyme activity at 1 mM, 3 mM, and 5 mM.[18,22]

The cellulase FPase activity (results shown in Fig. 7) enhances with 1 mM of monovalent cation as K (130%), whereas Cu and EDTA behave as inhibitors with 97% and 85% activity, respectively. Most divalent cations contribute to an increase in enzyme activity. For 3 mM concentration, Mn ion shows maximum activity (109%), while Cu and EDTA lead to minimum activity, *viz*., 97% and 87%, respectively. At 5 mM concentration, K ion induces a level of activity of 141% and EDTA inhibits activity with 13%. Danmek and coworkers used *A. melleus* for cellulase production and observed similar results for ions of Mg, Ca, Mn, Zn and Na-EDTA, while Fe and Cu show a decrease in enzyme activity.[35] Differences in fungi and concentrations of salts, along with substrate and other environmental conditions, may cause the deviation in results.

Kinetic constants K_m and V_{max}

The values for K_m and V_{max} obtained from the Lineweaver and Burk plot shown in Figure 8 (A) for CMCase are 0.648 mM and 12.953 mM/min. K_m has a low value, which means the enzyme and CMC have strong affinity, it further shows that the calculation for V_{max} was done at a low concentration of substrate. Imran and coworkers reported values of 0.54 mM and 19 mM/min for K_m and V_{max}, respectively, using *A. niger* IMMIS1.[36] Another study found values of 25 µM

and 45.5 U/mL for K_m and V_{max}, respectively, for cellulase from *A. niger*.[37]

The values for K_m and V_{max} obtained from the Lineweaver and Burk plot shown in Figure 8 (B) for FPase are 0.975 mM and 41.493 mM/min, respectively. K_m has a low value, which means the enzyme and FPase have strong affinity, it further shows that the calculation for V_{max} was done at low concentration of substrate. Danmek *et al*. observed K_m and V_{max} values of 0.09 and 0.003, respectively, for FPase using *A. melleus* by solid state fermentation.[35] The difference in V_{max} values can be attributed to differences in fungi, substrates, fermentation techniques and other environmental factors.

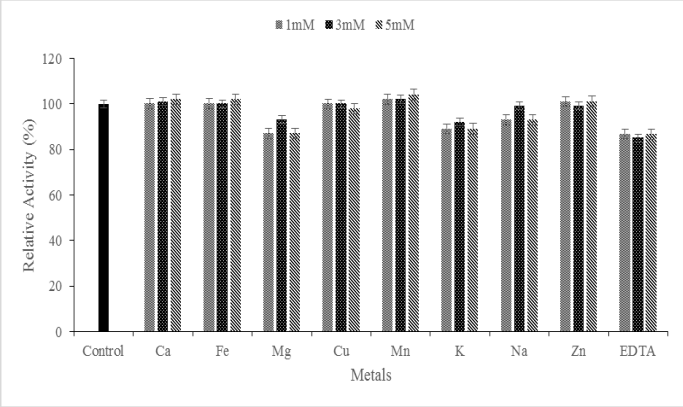

Figure 6: Effects of metals on the relative CMCase activity produced by *A. niger* in static fermentation

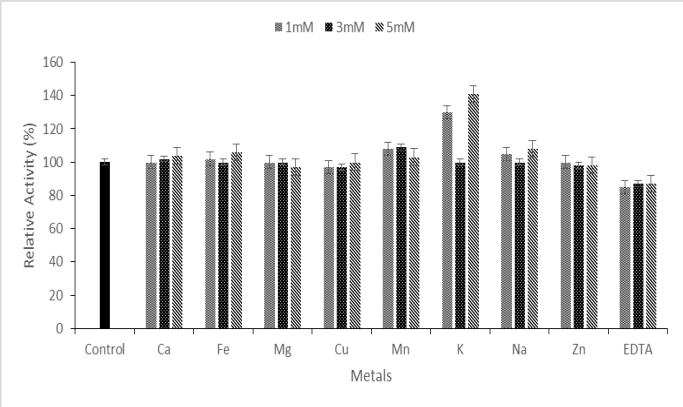

Figure 7: Effects of metals on the relative FPase activity produced by *A. niger* in static fermentation

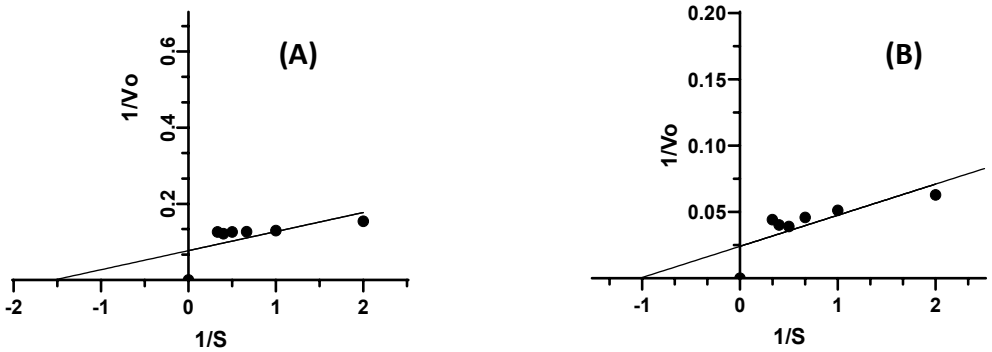

Figure 8: Lineweaver Burk plots for the estimation of K_m and V_{max} of CMCase (A) and FPase (B) produced by *A. niger* in static fermentation

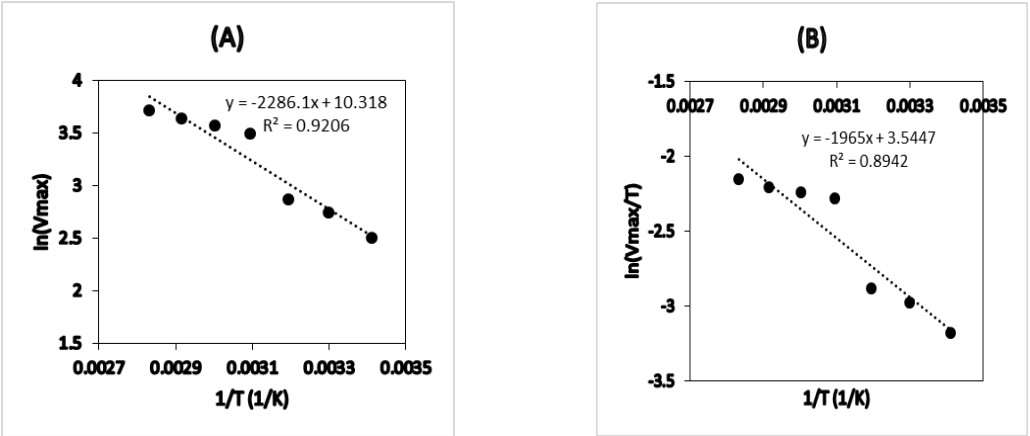

Figure 9: Arrhenius plots for the estimation of Ea and ΔH on the enzyme (A), and for determining entropy change (ΔS) on the enzyme produced by *A. niger* in static fermentation

Thermodynamic analysis

The value of enzyme activation energy Ea -19 kJ/mol was calculated using the Arrhenius plot (Fig. 9 (A)) and the enthalpy of reaction (ΔH) is 16.4 kJ/mol. Plotting $\ln(V_{max}/T)$ *versus* the inverse of temperature (1/T) (Fig. 9 (B)), value for entropy ΔS -16.4 kJ/mol is obtained. A previous study observed values for activation energy of -44.55, -50.02 kJ/mol and for enthalpy of reaction of 42.20; 47.70 kJ/mol for endoglucanases and betaglucosidases, respectively. The entropy was -5.1 and -5.7 kJ/mol for both the enzymes. The study used *Thermomyces dupontii* for cellulase production and further CMC hydrolysis was performed.[26] The difference in fungi and in hydrolysis plays a vital role in the differences in the values of the three parameters above.

CONCLUSION

In conclusion, the study highlights the potential of cellulase production from *A. niger* when utilizing grape wastes in static fermentation. The characterization of cellulases shows optimum results for temperature, pH, effects of metals, enzyme kinetics, and thermodynamics. The findings suggest the promising prospects of utilizing *A. niger* for efficient cellulase production from agricultural residues, contributing to the advancement of sustainable bioconversion processes and the utilization of agricultural by-products.

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