OPTIMIZATION AND BIOCHEMICAL CHARACTERIZATION OF THERMO-ALKALOTOLERANT CELLULASE ENZYME FROM *ASPERGILLUS TERREUS* PPCF

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The aim of this study was to boost the production rate of a novel thermo-alkalotolerant cellulase (FPase) enzyme from the fungal isolate *Aspergillus terreus* PPCF. Initially, the extracellular FPase activity was 0.166 ± 0.03 UmL^{-1} in the culture filtrate, which further increased up to 0.91 ± 0.13 UmL^{-1} under optimized conditions (3% w/v wheat bran and 0.5% w/v ammonium sulfate). Through response surface methodology, the FPase activity was enhanced to 10.78 UmL^{-1} under the cumulative effect of different factors. The zymogram analysis revealed only one activity band, with the molecular weight of ~110 KDa. The optimum pH was 7.0, but the enzyme was stable in a pH range of 4-10. The optimum temperature was 50 °C, but the stability range of the enzyme was 30-90 °C. The maximum effect of Mg^{2+} was observed on the FPase enzyme. The findings of the present study demonstrate the thermo- and alkalotolerance of the obtained cellulase enzyme, which will be beneficial to the biofuel and other industries.

Keywords: FPase enzyme, OVAT, RSM, zymogram analysis

INTRODUCTION

Enzymes are applied in various fields, including for technical use, in food manufacturing, cosmetics, medication, and as a tool for research and development. At present, almost 4000 enzymes are known, and of these, approximately 200 microbial original types are used commercially.[1] With improved understanding of the enzyme production biochemistry, fermentation processes, and recovery methods, an increasing number of industrial enzymes can be foreseeable and, among them, cellulase is becoming a crucial catalyst. Cellulases have attracted much interest due to their diverse application in textiles, detergent, leather, food, bioethanol and paper industries, and also have an important role in the carbon cycle globally by breaking down the insoluble cellulose to soluble sugar molecules.[2]

Our planet is threatened by the scarcity of fossil fuels because of the rising demand of fuel in the transportation sector and the environmental pollution caused by burning these fossil fuels. The enzymatically based renewable cellulosic ethanol production technology, as an alternative to depleting fossil fuels, was selected as a key area for biomass technology development.[3] The conversion of cellulose into valuable products is carried out by cellulose degrading enzymes, *i.e.*, the cellulase complex (endoglucanase, exoglucanase and β-glucosidase), into sugars, which are further fermented by yeast into bioethanol as a renewable and clean source of energy.[4] The mechanism of lignocellulosic biomass degradation through the cellulase enzyme complex is described in Figure 1. Various researches have been performed on cellulosic ethanol production, but the ineffectiveness (low activity) and high cost of cellulase enzyme is the main hurdle in its use in fermentation industries.[5]

For resolving these issues, the optimization method is utilized to minimize the cost, while increasing the enzymatic activity. Both OVAT (one-variable at a time) and statistical techniques are exploited for optimizing the cellulase enzyme production. The OVAT approach requires more time, while the interaction of medium components with each other is not studied. To overcome this problem, response surface methodology is being

widely used as it allows more experimental trials within a short time, with accuracy, and each medium component is considered in its interaction with the others.[5,6]

For improving the performance and function of the enzyme's catalytic activity, characterization is a vital step. The application of the cellulase enzyme in the biofuel industries demands the identification of a stable enzyme that can be active at high pH and increased temperature. To achieve this, the characterization of the enzyme (temperature, pH and metal ions) for analyzing its stability is an essential step in obtaining high plant biomass conversion with high sugar yield.[2]

Molecular characterization through the activity staining (zymogram) method is employed for the detection of the activity band of a particular enzyme by reacting with a suitable substrate under native-PAGE conditions, and reveals the molecular weight of the enzyme. In the context of the industrial demand for efficient enzymes, the present research has been carried out to optimize the production conditions of FPase (overall cellulase) enzyme from the fungal isolate *Aspergillus terreus* PPCF by utilizing both the OVAT and RSM techniques, and characterizing the temperature and pH optima, along with different metal ions of the enzyme. Zymogram analysis was also performed to estimate the molecular weight of the FPase enzyme through the activity band method.

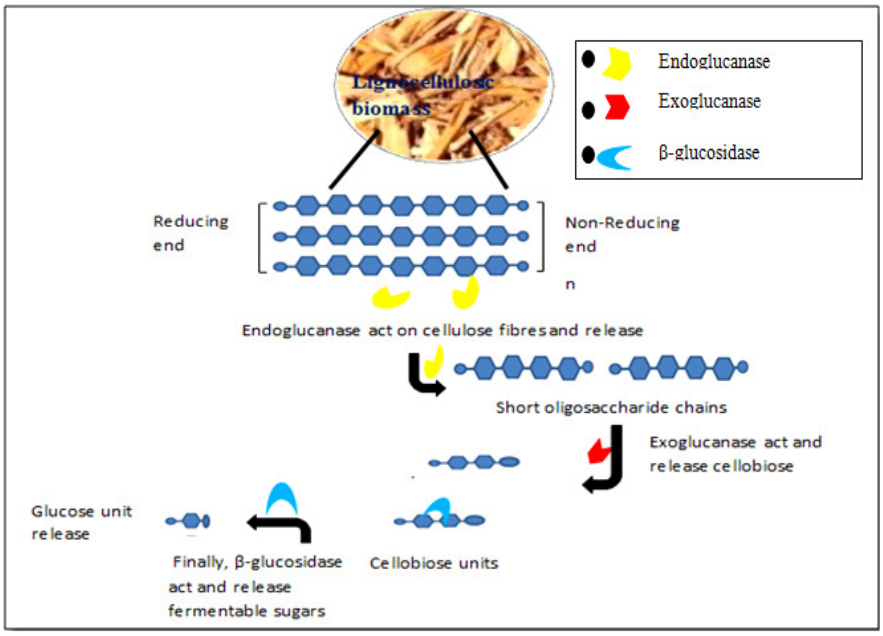

Figure 1: Catalysis of cellulase enzyme complex for glucose production

EXPERIMENTAL

Microbial culture

A cellulolytic fungal culture of *Aspergillus terreus* PPCF, isolated from paper waste contaminated soil, was screened for cellulase activity and used for the production of the extracellular FPase enzyme. The initial isolation and characterization of the native fungal culture was described in a previous paper.[7]

Enzyme (FPase) assay

Before enzyme production, *A. terreus* was maintained on CMC plates incubated at 28 ± 2 °C. Before inoculation, the spores were resuspended in sterile water to achieve a final concentration of 3.2 × 10^8 spores/mL. For enzyme production, a 1000 mL Erlenmeyer flask was filled with 300 mL of enzyme production medium (CMC broth) and inoculated with an inoculum size of 3% (v/V). The flasks were incubated under static and submerged conditions at 28 ± 2 °C for 168 h. After incubation, the cultures were centrifuged at 8000 rpm (4 °C) for 12 min. The cell free supernatant was collected and used as a crude enzyme for estimation of the overall cellulase (FPase) enzyme activity. The reaction mixture for estimating the FPase activity contained 0.5 mL of appropriately diluted crude enzyme in 1.0 mL of 0.05 M sodium acetate buffer (pH 5.5) with 50 mg of a Whatman No. 1 filter paper strip (1.0 × 6.0 cm) and then incubated at

50 °C for 60 min. After incubation, 3 mL of DNS reagent was added to the reaction tube and boiled for 5 min to measure the reducing sugars liberated during the reaction by the development of color. The boiled tubes were cooled immediately and absorbance was recorded at 540 nm. The amount of glucose liberated was quantified using the standard curve of glucose.

Optimization by OVAT

Enzyme production conditions were optimized using one variable at a time to raise the enzyme productivity from the cellulolytic fungal isolate *A. terreus* PPCF using different carbon sources (wheat bran, rice bran, rice husk, wheat straw, banana pulp and cellulose (1% g/100 mL)), concentrations of the optimized carbon source, varying from 0.5 to 3.0% (g/100 mL), nitrogen sources (urea, peptone, ammonium sulfate and ammonium nitrate (0.25%, w/v)) and concentrations of the selected nitrogen source (0.125 to 1.5% g/100 mL). The inoculated flasks were incubated for 120 h at 28 ± 2 °C and the FPase activity was assayed as described earlier. For the optimization of the carbon source, lignocellulosic agriculture residues were utilized to achieve cost effectiveness in the production process.

Optimization by response surface methodology

The analysis of the experimental design and the processing of data were performed by using the Design expert software version 10.0. The central composite rotatable design (CCRD) was used for the analysis of the fermentation parameters, including pH, liquid content (mL), incubation days (d) and incubation temperature (°C), with wheat bran (3% w/v) and ammonium sulfate (0.5% w/v) as previously optimized carbon and nitrogen source, respectively. Experiments were conducted with five coded levels (−2, −1, 0, +1, and +2) and their actual values were presented in Table 1. The significance of the model was related to a probability of less than 0.05. The coefficient of determination (R^2) and Fisher F-test was used to analyze the developed model and its statistical significance.

Enzyme characterization

Influence of temperature, pH and metal ion concentration on FPase activity

The impact of temperature on the FPase activity was observed by incubating the substrate and the enzyme mixture at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C) for 60 min, and the enzyme activity was determined as described earlier. For enzyme stability, the enzyme was pre-incubated at different temperatures (30, 40, 50, 60, 70, 80 and 90 °C) for 1/2 h and 1 h to analyze the stability of the FPase enzyme. The influence of pH on the FPase was also studied at pH values of 4, 5, 6, 7, 8, 9 and 10, by using buffers (4.0 to 5.0, sodium acetate; 6.0 to 8.0, phosphate buffer; 9.0, tris (hydroxymethyl) amino methane and 10.0, alkaline borate buffer), and the enzyme activity was measured. Enzyme stability was analyzed by incubating the enzyme with 1 mL of each buffer at 50 °C for 1/2 h and 1 h. After incubation, the enzyme reaction mixture was measured for FPase activity and relative activity (%) by considering the maximum as 100%.

Native PAGE and zymogram analysis

Zymogram analysis is an electrophoretic method for the detection of hydrolytic enzymes, based on the substrate reaction, such as CMC, which can be degraded by the FPase enzyme and thus the degradation product emits fluorescence or produces changes of color during the reaction period. The enzyme fraction was electrophorized by SDS and Native PAGE with 10% separating gel and 5% stacking gel. After electrophoresis, the gel slab was stained with 0.1% CBB-R250. For zymogram analysis of FPase enzyme, 0.1% (w/v), CMC was incorporated into the polyacrylamide during gel preparation. The electrophoresis was performed at 4 °C. The gel was then soaked in 100 mM phosphate buffer, pH 7.0 for 1 h prior to staining with 0.2% (w/v) Congo red. The gel was destained with 2M NaCl solution until the cellulase activity was visualized as a clear band against the red background.[8]

Table 1
Range of parameters (coded and actual levels) used for central composite rotatable design (CCRD)

Parameters	Coded and actual levels				
	-2	-1	0	+1	+2
pH	5	6	7	8	9
Liquid (mL)	3	5	7	9	11
Incubation period (days)	4	5	6	7	8
Temperature (°C)	26	28	30	32	34

Data analysis

All the experiments were conducted in triplicate and the standard deviation (SD) was calculated using MS Excel. Multiple comparison tests for each experimental treatment were carried out by using Duncan's Multiple Range Test (DMRT) at the 5% probability level (p = 0.05) in the SPSS program.

RESULTS AND DISCUSSION

Over many decades, cellulases have been used in various industrial applications, securing the third rank among enzymes in annual sales and expected to exceed the protease in the near future.[17] On the basis of quantitative assessments, the fungal culture of *A. terreus* PPCF showed maximum FPase enzyme activity on the 5th day, with 0.166 ± 0.03 UmL^{-1}, which started declining with further incubation (Table 2). Filamentous fungi have been reported to produce the cellulase enzyme complex with the ability to penetrate into cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles, which increases their cellulase hydrolysis capability in comparison with that of bacterial cellulase.[18]

The carbon source is the major factor affecting cellulase production, which is attributed to the fact that cellulases are inducible enzymes that are expressed by cells in response to different carbon sources present in the fermentation medium. For optimization of carbon sources, the fungal isolate *A. terreus* PPCF was grown in mineral salt medium supplemented with different carbon sources, such as wheat bran, rice bran, rice husk, wheat straw, banana pulp and cellulose (1% w/v). In the wheat bran supplemented medium, the FPase activity reached up to 0.631 UmL^{-1}, with the productivity of 5.26 $UL^{-1}h^{-1}$, as presented in Figure 2A. In general, wheat bran has been found to induce the production of a large variety of hydrolases by *Aspergillus* sp.[9] In the present study, wheat bran has been found as the best carbon source for enzyme production. Therefore, to find the optimum concentration of the substrate, the enzyme production was carried out using different concentrations, ranging from 0.5 to 3.0% (w/v) of wheat bran. Enzyme activity increased from 0.631 to 0.843 UmL^{-1} for FPase enzyme with 3% wheat bran concentration. In Figure 2B, the productivity (7.0 $UL^{-1}h^{-1}$) also enhanced as the substrate concentration was increased from 0.5 to 3.0% of wheat bran. The results undoubtedly indicated the inducible nature of the enzyme, as its expression increased with the increasing concentration of the substrate, achieving the maximum value at an optimum concentration (3%) of wheat bran. Various studies have described the utilization of lignocellulosic based substrates in cellulase production.

The nitrogen source is another one of the most important factors for the growth of micro-organisms and for metabolic production, as it is a constituent of amino acids, nucleic acids, nucleotides, and coenzymes of living cells. It is also involved in governing the productivity of the enzyme.[19] To identify the most suitable nitrogen source for FPase production from the fungal isolate *A. terreus* PPCF, the medium was amended with ammonium sulfate, ammonium nitrate, urea and peptone as nitrogen sources. It is evident from the results that the FPase activity was greatly influenced by the nitrogen source. The maximum activity for the FPase enzyme (0.851 UmL^{-1}) was recorded with ammonium sulfate as nitrogen source, while the lowest activity was recorded with urea as nitrogen source (0.240 U mL^{-1}) (Fig. 2C). When urea is used as nitrogen source, it starts increasing the pH of the medium up to 8.0 or 9.0, *i.e.* towards alkalinity, which is not acceptable in the case of fungal growth. After optimizing the nitrogen source, the concentration of ammonium sulfate was optimized by supplementing the production medium with varying concentrations (0.125 to 1.5%) of ammonium sulfate. The enzyme activity was significantly affected by the amount of ammonium sulfate in the culture medium, and maximum FPase (0.910 UmL^{-1}) activity was recorded with 0.5% (w/v) concentration, as presented in Figure 2D. The inducing effect of ammonium salts was due to the direct entry of ammonium in protein synthesis.

Table 2

Overall extracellular cellulase (FPase) activity of fungal isolate *Aspergillus terreus* PPCF in the culture* filtrate

Microbial isolate	FPase (UmL^{-1}) at different time intervals (days)						
	1	2	3	4	5	6	7
Aspergillus terreus PPCF	0.148 ±0.07^{b}	0.151± 0.01^{b}	0.154± 0.05^{b}	0.159 ±0.02^{b}	0.166 ±0.02^{b}	0.162 ±0.02^{b}	0.085 ±0.03^{a}

*The cultures were grown in mineral salt medium containing carboxymethyl cellulose (CMC) as sole carbon source (1% w/v) at 28 ± 2 °C for 7 days. Values with the same superscripts are significantly similar

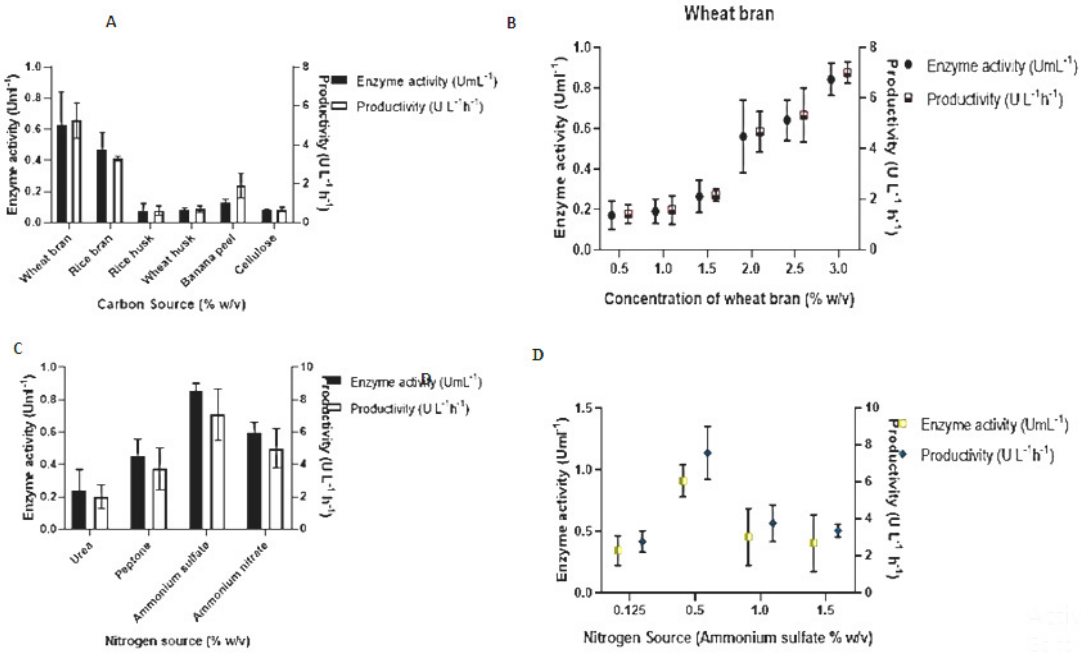

Figure 2: Effect of different carbon and nitrogen sources on enzyme activity and productivity of FPase enzyme from fungal isolate *Aspergillus terreus* PPCF (A: different agricultural residues used as carbon sources; B: concentration of optimized carbon source (wheat bran); C: various nitrogen sources; D: concentration of optimized nitrogen source (ammonium sulfate))

The use of ammonium salts is very common in cellulase production, as compared with other nitrogen sources. During the present research, wheat bran and ammonium sulphate have been found as optimum, as both showed the maximum effect on the productivity of the enzyme.

Response surface methodology (RSM) was carried out with 30 experimental runs in CCRD, having combinations of four most critical growth parameters, *viz*., pH, liquid (mL), incubation period (d) and temperature (°C), named as A, B, C and D, respectively. Maximum production of FPase enzyme was observed as 11.51 UmL^{-1} in run 16 against minimum FPase production of 1.44 UmL^{-1} (in run 26) (Table 3). Considerably higher enzyme activities obtained after the optimization by RSM, as compared to traditional methods, which clearly indicates that RSM is a better option for optimization with the combined effect of production conditions. Statistical analysis yielded a regression equation, which shows the empirical relationship of the response and the test variables. The predicted regression equation for FPase (overall cellulase) enzyme is as follows:

$$Y = 10.53-0.46A-0.0052B+0.24C-0.39D+ 0.54AB+1.07AC+0.14AD+0.16BC-0.63BD- -2.0CD-1.99A2-1.32B2-1.25C2-0.26D2 \quad (1)$$

The equation is useful in identifying the relative impacts of the factors (A, B, C, D) on the response. It demonstrated that the model is highly significant, as evident from the F-value of 4.30, with low probability P-values, as described in Table 4. The R^2 value was found to be 0.80 (FPase), which indicated that the model can explain 80.0% of total variations. The closer the value of R^2 to 1, the stronger is the model to predict the response.

The 3D response surface plots were drawn to illustrate the interactive effects of fermentation factors on the FPase (overall cellulase) activity. The 3D plots presented in Figure 3 clearly show that the maximum cellulolytic (FPase) activity should occur with moderate conditions of incubation temperature, incubation days and pH of the medium for the microbial growth to flourish. In a numerical optimization, the quadratic model predicted the maximum FPase activity of 10.13 UmL^{-1}, which can be achieved with optimal values of fermentation conditions *i.e*., pH 6.98, liquid content of 8.52 mL, 6 days of incubation and 28 °C incubation temperature. The validation of the final optimized media was done with the predicted optimum values of different parameters. The validated enzyme activity for the

optimized media was observed to be 10.78 UmL^{-1}, which is in excellent agreement with the experimental value of 11.51 UmL^{-1}. It indicated that the developed model was accurate and reliable for predicting the production of FPase enzyme by *Aspergillus terreus* PPCF.

Besides optimization, some other environmental factors also affect the enzyme activity, causing either an escalating or a declining response. Each enzyme is characterized by its specific pH and temperature optima for activity and stability; besides these, some co-factors also influence the enzyme activity. Hence, it is necessary to characterize the hydrolytic enzyme for efficient application at an industrial level. Considering the importance of temperature and pH for enzymatic activity and stability, the influence of temperature, pH and co-factors on the FPase enzyme from the fungal isolate *A. terreus* PPCF was studied over a wide range of temperature (30 to 90 °C) and pH (4 to 10). It is evident from Figure 4A that the optimum temperature for FPase activity was recorded at 50 °C. The enzyme activity, however, declined when the temperature was raised above this optimum.

Similarly, another report describes the optimum temperature (50 °C) for β-glucosidase enzyme activity from the fungus *Daldinia eschscholzii*.[10] The results depicted that the FPase enzyme retained high enzyme and relative activity of more than 80% up to 90 °C.

Table 3
Experimental response of the dependent variables (FPase activity) from the fungal isolate PPCF using central composite rotatable design (CCRD)

S. No.	Coded variables				Overall cellulase FPase activity (UmL^{-1})	
	A (pH)	B (Liquid, mL)	C (Days, d)	D (Temperature, °C)	Observed	Predicted
1	1	1	1	1	2.20	4.38
2	2	0	0	0	1.54	1.66
3	1	1	-1	1	3.17	5.44
4	0	0	0	0	11.35	10.53
5	0	2	0	0	8.26	5.24
6	1	-1	-1	1	6.46	5.94
7	1	-1	-1	-1	2.05	1.18
8	1	-1	1	1	6.35	4.25
9	0	0	0	2	10.66	8.71
10	1	-1	1	-1	6.44	7.49
11	-2	0	0	0	4.78	3.50
12	1	1	-1	-1	3.35	3.19
13	0	0	0	0	11.35	10.53
14	0	0	0	0	11.35	10.53
15	0	0	0	0	11.35	10.53
16	1	1	1	-1	11.51	10.13
17	-1	1	-1	-1	3.86	5.45
18	0	0	-2	0	5.86	5.03
19	-1	-1	1	-1	10.4	7.62
20	-1	1	-1	1	6.98	7.14
21	-1	-1	1	1	1.99	3.83
22	0	0	0	-2	9.48	10.26
23	-1	1	1	-1	5.91	8.11
24	0	-2	0	0	3.41	5.26
25	-1	-1	-1	1	8.93	9.80
26	-1	1	1	1	1.44	1.80
27	0	0	0	0	11.35	10.53
28	-1	-1	-1	-1	5.11	5.60
29	0	0	0	0	11.35	10.53
30	0	0	2	0	4.46	6.00

Table 4
ANOVA for overall cellulase (FPase) enzyme activity based on central composite design

Source	Sum of squares	df	Mean square	F Value	p-Value Prob > F
Model	264.06	14	18.86	4.30	0.0041
A	5.08	1	5.08	1.16	0.2992
B	6.510E-004	1	6.510E-004	1.483E-004	0.9904
C	1.41	1	1.41	0.32	0.5788
D	3.58	1	3.58	0.82	0.3808
AB	4.64	1	4.64	1.06	0.3201
AC	18.36	1	18.36	4.18	0.0588
AD	0.31	1	0.31	0.071	0.7934
BC	0.40	1	0.40	0.091	0.7675
BD	6.30	1	6.30	1.44	0.2495
CD	63.94	1	63.94	14.56	0.0017
A^2	108.31	1	108.31	24.67	0.0002
B^2	47.70	1	47.70	10.86	0.0049
C^2	42.95	1	42.95	9.78	0.0069
D^2	1.85	1	1.85	0.42	0.5266
Residual	65.87	15	4.39		
Lack of Fit	58.56	10	5.86	4.01	0.0694
R^2			0.80		
Adj R^2			0.78		

*A, pH; B, liquid (mL); C, incubation days (d); D, temperature (°C)

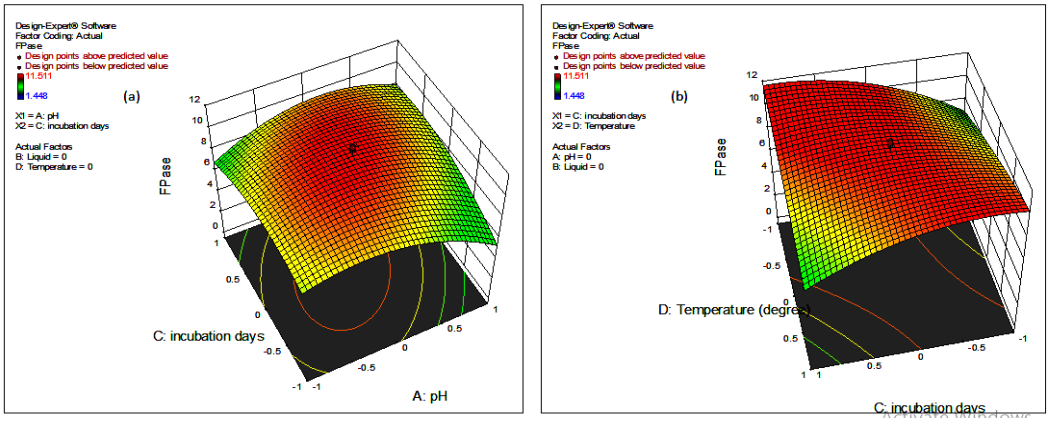

Figure 3: 3D plots showing the interactive effects of variables on FPase activity from the fungal isolate PPCF; A: Effect of incubation days and pH; B: Effect of temperature and incubation days

It is also clear from the results that the cellulase enzyme from the fungal isolate has a wide temperature tolerance (30 to 90 °C), which describes the thermotolerant nature of the enzyme. It is evident from the data given in Figure 4C that the relative activity (%) for FPase enzyme varied from 87% (at 90 °C) to 93% (at 30 °C), while showing 100% relative activity at 50 °C for 1 h. In spite of the thermostable nature of cellulase, the retention of ~50% activity at higher temperature has been reported by earlier workers, while the cellulase enzymes obtained from the fungal isolate in the present study showed higher tolerance, retaining more than ~80% relative activity.[11] The high thermotolerance of the FPase enzyme from the fungal isolate *A. terreus* PPCF indicates the possibility of its wide industrial application. It is questionable how a mesophilic fungus has evolved thermostable enzymes, maybe by a strategy enabling energy conservation through decreased need for enzyme synthesis due to higher enzymatic stability. Another explanation for such a modification may be the horizontal transfer of the enzyme encoding gene from some

thermophilic organism to a mesophilic one.[20] Most enzymatic hydrolysis processes are carried out at high temperature and therefore, they require preferentially thermostable enzymes. This study reports on a highly thermostable cellulase enzyme from the fungal isolate *A. terreus* PPCF, which is worth considering for efficient hydrolysis of lignocellulosic biomass for proficient bioethanol production.

The synthesis and activity of enzymes from various organisms are greatly affected by the pH of their environment. The most favorable pH is the value at which the enzyme is most active, which is known as the "optimum pH". The effect of pH on the activity and stability of the FPase enzyme was analyzed, considering different pH values ranging from 4 to 10, using different buffers during the present investigation. It is clear from Figure 4B that the maximum activity for the FPase enzyme was recorded at pH 7.0, with 70% relative activity in the broader pH range from 4 to 10. Contrary to certain previous reports, the overall cellulase (FPase) obtained from the fungal isolate during the present study showed considerable stability up to an alkaline pH of 10. The novelty of the results consists in the FPase enzyme obtained from the fungal isolate being more alkaline tolerant and retaining more than 70% activity up to pH 10, which is generally not shown by fungal systems.

As known for the activity of each enzyme, there is also a pH optimum for enzyme stability. During the present study, the stability of the FPase enzyme was determined in a wide pH range from 4 to 10 for 1 h and 1/2 h of incubation; the enzyme showed better pH stability at 1/2 h of incubation compared to 1 h. In Figure 4D, the fungal FPase enzyme showed a very high degree of pH stability as it exhibited more than 95% relative activity when pre-incubated for 1/2 h in the pH range from 4 to 10. At 1 h of pre-incubation at different pH values, the stability declined, but was still maintained over a level of 80%.

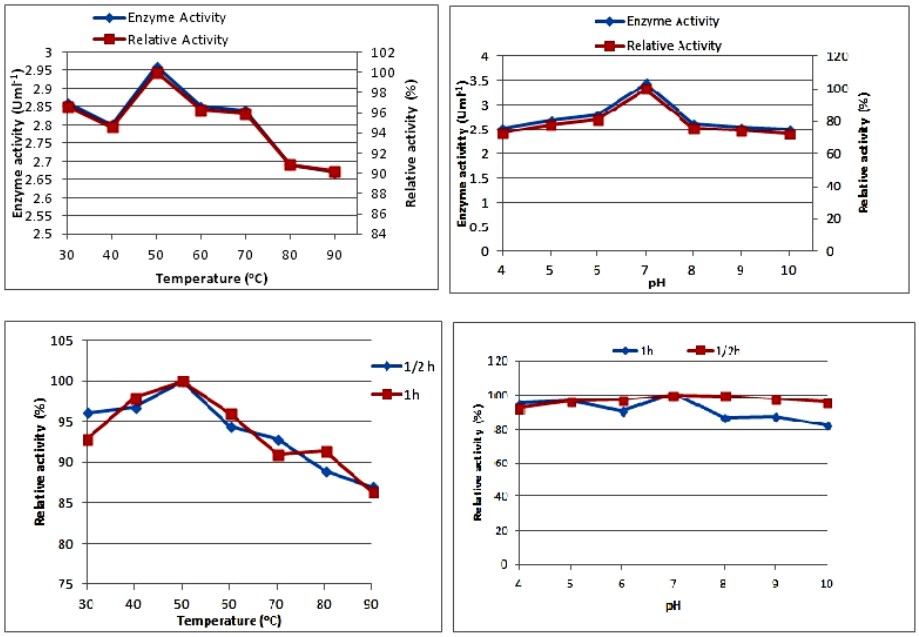

Figure 4: Temperature and pH optima and stability of the enzyme FPase; A: Temperature optima; B: pH optima; C: Temperature stability; D: pH stability

It is apparent from the results that the fungal FPase enzyme from the present study showed a high degree of stability in a wide pH range, indicating its both acidic and alkaline nature, and the overall cellulase enzyme from the fungus – as a versatile enzyme, tolerating acidic/alkaline pH conditions, which is highly beneficial for industrial purposes. Earlier reports indicated only the acidic nature of the cellulase enzyme, as its activity decreased at alkaline pH values.[12] This feature, *i.e.* its acidic/alkaline pH tolerance, is beneficial for the saccharification of alkali pre-treated lignocellulosic substrates, which usually retain alkalinity after the pre-treatment and inhibit

the catalysis process. Extremely high or low pH values generally result in complete loss of activity for most of the enzymes. The pH profile is dependent on a number of factors. As the pH changes, the ionization of groups both at the enzyme's active site and on the substrate can alter, influencing the rate of binding of the substrate to the active site.[21]

In nature, biomass-degrading microbial enzymes (*e.g*., cellulases and hemicellulases) are most likely to be in contact with various metal ions (including redox-active ones). Metal ions could be thus effectors, since they exist universally in microbial habitats (Ca, Mg, Al), are adsorbed in the soil or dissolved in water (Fe, Mn) from anaerobe respiration and anoxygenic photosynthesis, and often serve as structural/functional co-factors (Ca, Zn, Fe, Mn, and Cu) of many enzymes, including cellulases.[13] Most enzymes require some metal ion as co-factor for their catalytic activity; therefore, the effect of metal ions on the activity of the FPase enzyme was studied at 5 mM concentration. It was observed that the FPase activity was significantly increased in the presence of all the metal ions, but Mg++ ions led to a maximum increase in the enzyme activity (3.78 ± 0.10 UmL^{-1}), followed by that in the presence of $ZnSO_4$ (3.65 ± 0.11 UmL^{-1}) and NaCl (3.07 ± 0.06 UmL^{-1}), being significantly higher than those recorded in the absence of metal ions. The results indicate clearly that $MgSO_4$ acts as a most suitable co-factor for the catalytic activity of the FPase enzyme from the fungal isolate, as described in Table 5. Some other scientists have also investigated the effect of metal ions ($CaCl_2$, $MgSO_4$, $FeSO_4$, $MnSO_4$ and $ZnSO_4$) on cellulase enzyme activity from *Bacillus altitudinis* and *Bacillus licheniformis*, and also reported the maximum cellulase activity (2.82 ± 0.42 UmL^{-1}) in magnesium sulfate supplemented medium.[13,14]

For molecular characterization, the purified enzyme filtrates (after gel filtration; Sephadex G-100) were analysed by SDS/Native PAGE. During electrophoresis, several bands with different intensities were visualized. The molecular weight of various extracellular proteins, as determined by Native and SDS PAGE analysis, varied from ~25 to 245 kDa, as presented in Figure 5.

Table 5
Effect of different metal ions on FPase activity from the fungal isolate A. terreus PPCF

Metal ions	Enzyme activity (UmL^{-1})	Relative activity (%)
	FPase	FPase
KCl	2.63±0.17[b]	70.07
NaCl	3.07±0.06 [c]	81.10
$MgSO_4$	3.78±0.10 [d]	100
$ZnSO_4$	3.65±0.11 [d]	95.53
$CuSO_4$	2.78±0.19 [b]	74.54
Control (without metal ions)	0.166±0.03 [a]	4.35

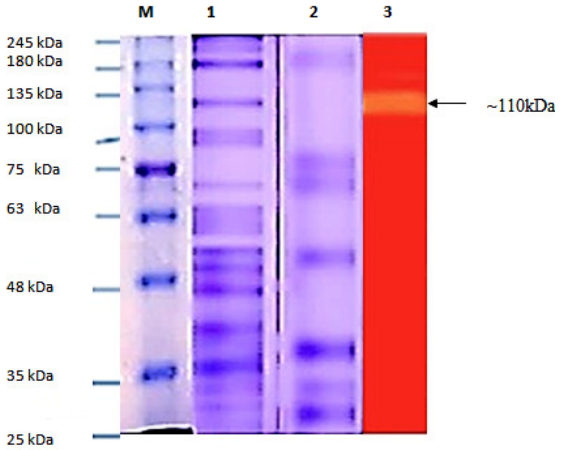

Figure 5: Extracellular protein profiling of selected cellulase producing fungal isolate PPCF by SDS and Native PAGE analysis; Lane M: Molecular marker, Lane 1: PPCF isolate SDS profiling, Lane 2: PPCF isolate native-PAGE profiling, Lane 3: activity staining of enzyme using CMC as substrate

The molecular weight of the cellulase enzyme from different sources has been reported in the range of 35 to 240 kDa.[15] The different intensities of various protein bands indicated differential expression of genes encoding different proteins present in the culture filtrate by PAGE. The enzyme fraction was analyzed by activity staining during Native PAGE for the activity bands, and only one thick band of yellow color was observed on the gel slab against the red background, with the molecular weight of ~110 kDa. This indicated the cellulose degrading ability of the enzyme, forming the hydrolysis zone. The presence of one band clearly indicated the production of only one isoform of the FPase enzyme by the fungal isolate. As observed in the present study, high molecular weight cellulase was produced from *Fomes* sp. EUM1 in SSF.[16] Zymogram analysis was done to determine the activity of the enzyme reacting with specific substrates and to identify the isoforms of the enzyme. CMC is considered as a standard cellulosic source commonly utilized in depicting overall cellulase (FPase) activity.

CONCLUSION

For industrial application, catalytic enzymes should exhibit high activity and stability over high pH, temperature and other stress conditions. Focusing on this issue, the FPase (overall cellulase) enzyme was optimized through conventional and statistical methods, and an increase in activity was observed by the RSM based CCRD method. The cumulative effect of the production factors on the FPase enzyme resulted in enhancing the activity from 0.910 U mL^{-1} to 10.78 U mL^{-1}. The FPase enzyme was found as highly stable over wide ranges of pH and temperature, as well as at different metal ion concentrations. As regards the fungal system, the study revealed a novel thermo- and alkalotolerant cellulase enzyme that can be utilized in various industries due to its high thermal and alkali stability.

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