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Isolation and Characterization of Neutral Proteases Producing Soil fungus *Cladosporium* sp PAB2014 Strain FGCC/BLS2: Process Optimization for Improved Enzyme Production

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Proteases account for nearly 60% of the industrial enzyme market and have wide applications. Present study has been designed to isolate and screen protease producing fungi from various soil samples of Jaipur region and optimum physico-cultural conditions for enhanced protease production from selected isolate was also investigated. Isolate fungi were screened by growing them onto skim milk agar plates and the zone of proteolysis was noted. Isolate FGCC/BLS2 showed maximum hydrolysis capacity when compared to wild type positive reference strain *Aspergillus flavus*. Molecular Characterization of the FGCC/BLS 2 isolate confirmed it as *Cladosporium* sp. PAB 2014 strain FGCC/BLS 2 (Submitted to Gen Bank: Accession Number KU 752193). The highest enzyme activity was obtained with production media at 120 hour (60.9±2.17 U/mg protein) with 2% inoculums (63.19±0.59 U/mg protein), pH 7 (63.77±2.45 U/mg protein), dextrose as the carbon source (63.9±1.63U/mg protein) and tryptone as a nitrogen source (66.9±2.34 U/mg protein). The optimum conditions for protease assay was found to be 40^{o}C temperature, 1.5% substrate concentration and at pH 7.0 respectively.

Keywords: Protease, *Cladosporium* sp, Optimization, Submerged Condition

Introduction

The new potential of using microbes as biotechnological sources of industrially relevant enzymes have stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms[1,2]. Different microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. Selection of the right organism plays a key role in high yield of desirable enzymes[3]. Proteases account for nearly 60% of the industrial enzyme market and have wide applications in many industries[4]. Proteases execute a large variety of functions and have important biotechnological applications in detergents, leather, food, pharmaceutical industries and bioremediation processes[5,6]. Fungal proteases magnetized the interest of researches due to high diversity, broad substrate specificity, and stability under extreme conditions; also it offers an advantage of separation of mycelium by simple filtration[7,8]. Optimization of culture conditions is of utmost importance for better enzyme production because change in pH, substrate concentration affects properties of enzymes[9]. Considering the industrial importance of proteases, fungi were isolated from soil of Jaipur region and screened for protease production. The studies revealed that isolate FGCC/BLS2 is potent protease producer when compared to *Aspergillus flavus* (Wild type reference strain) in plate assay. Furthermore, study was undertaken for the assessment of culture conditions on protease production from FGCC/BLS2 fungal isolate by submerged fermentation and to evaluate the effect of temperature, pH and substrate concentration on protease activity.

Materials and methods

Sample collection and isolation of fungi

Soil samples from different environmental sources like farm soil, uphill soil and garden soil of Jaipur region were collected aseptically from 6 to 10 cm depth in sterile zip lock plastic bags. One gram of each soil sample was serially diluted in sterile conditions. From diluted suspension 100 µl was spread on potato dextrose agar (PDA) medium,

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incubated at 28°C for 3-4 days, and was monitored every day for the growth of fungal colony. Further these fungi were sub-cultured on PDA to obtain the pure strain. The fungal isolates were preserved in slants covered with parafilm at low temperature for future studies.

Primary screening for proteolytic fungi by casein hydrolysis plate assay

Preliminary screening was done by inoculating the fungal isolates (n=30) on skim milk agar medium containing casein as protein source at 28°C for 4 days. After incubation, the plates were observed for the clear zone of hydrolysis surrounding the colony. Extracellular secretion of proteases was exhibited by a zone of proteolysis, which was demonstrated by clear area surrounding the fungal growth. The hydrolysis capacity was calculated by measuring hydrolysis zone and total fungal radial growth zone and expressed as hydrolysis capacity [Ratio of hydrolysis zone (HZ) / Total fungal radial growth zone (GZ)][10].

Microscopic identification of fungal isolate FGCC/BLS2

The isolate (FGCC/BLS2) in primary screening shows maximum casein hydrolysis capacity. The isolate was identified on the basis of cultural/macroscopic and microscopic characteristics using lacto phenol cotton blue (LCB) staining method and confirmed as per standard manuals, reference slides and available literature[11]. Appropriate references were made using mycological identification keys and taxonomic descriptions.

Molecular characterization of fungal isolate FGCC/BLS2

The molecular characterization of fungal isolate was carried out by the gene sequence analysis of D2 region of LSU gene and construction of phylogenetic tree based on evolutionary relationship of taxa by neighbor-joining method at Xcleries genomics lab. For this firstly, DNA was isolated from the culture 14 day old culture of fungal isolate. Quality was evaluated on 1.2% agarose gel. Fragment of D2 region of 28S rDNA gene was amplified by PCR from the isolated genomic DNA. The PCR amplicon was purified and further process for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The D2 region of 28S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score fifteen sequences were selected and the phylogenetic Tree was constructed using MEGA 5.

Extracellular protease production

Fungal strain FGCC/BLS2 was subjected to protease production medium [KH_2PO_4 (0.1%), K_2HPO4 (0.1%), $MgSO_4$ (0.02%), Glucose (2.0%), Yeast extract (1%), Casein (1%), pH 7.0, autoclaved) and incubated in a shaking incubator for 4 days at 120 rpm and 28°C.

Extraction of enzyme from fungi (recovery of protease)

The isolate was grown at 28°C for 4days in 50 ml of protease production medium in 100 ml Erlenmeyer flasks and placed in a shaker incubator operated at 120 rpm. After incubation period, biomass was filtered through whatman filter paper. The culture filtrate was centrifuged at 4°C, 7000 rpm for 10 minutes and the supernatant was used as crude enzyme source for enzyme assays in subsequent experiments.

Protease activity assay

Protease activity was determined as described by Kamath *et al* (2010) with modifications[4]. The protease activity of crude enzyme was determined using 1% casein as substrate, prepared in sodium phosphate buffer (0.1 M, pH 6.0). The reaction mixture containing 0.5 ml of enzyme and 0.5ml of substrate (1% casein) along with the appropriate blank, were incubated at 30°C for 30 minutes. When the protease under testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin Ciocatleu Phenol or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer at 660 nm. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in microgram of tyrosine equivalents released from casein per minute under assay conditions.

Estimation of total protein content

The total protein content in cell free filtrate (extracted from flask) was determined by Lowry's method of protein estimation in which crude enzyme extract was reacted with Lowry's reagent and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein bovine serum albumin (1mg/ml) with Lowry's reagent. Then a graph was plotted between concentration of standard protein on X axis and absorbance at 750 nm at Y axis. Enzyme activity is expressed as specific activity, which is represented as U/mg of protein. The experiments were carried out in duplicates and were statistically analyzed[12].

Table 1 — Primary screening of fungal isolates for proteolytic activity by casein-agar plate technique

S.No	Isolate No.	Hydrolysis Capacity (Ratio-HZ/GZ)
1	FGCC/BLS1	1.1
2	FGCC/BLS2	2
3	FGCC/BLS3	1.06
4	FGCC/BLS4	1.05
5	FGCC/BLS5	1.03
6	FGCC/BLS6	1.08
7	FGCC/BL7	1.12
8	FGCC/BLS8	1.1
9	FGCC/BLS9	1.06
10	FGCC/BLS10	1.12
11	FGCC/BLS11	1.11
12	FGCC/BLS12	1.73
13	FGCC/BLS13	1.16
14	FGCC/BLS14	1.14
15	FGCC/BLS15	1.25
16	FGCC/BLS16	1.06
17	FGCC/BLS17	1.22
18	FGCC/BLS18	1.28
19	FGCC/BLS19	1.26
20	FGCC/BL20	1.07
21	FGCC/BLS21	1.40
22	FGCC/BLS22	1.08
23	FGCC/BLS23	1.33
24	FGCC/BLS24	1.33
25	FGCC/BLS25	1.36
26	FGCC/BLS26	1.13
27	FGCC/BLS27	1.28
28	FGCC/BLS28	1.16
29	FGCC/BLS29	1.26
30	FGCC/BLS30	1.26
31	Positive Control	1.6

Determination of optimum conditions for enzyme production

The protocol adopted for optimization of process parameters aimed to evaluate the effect of an individual/one parameter at a time and to incorporate it at the standard level before optimizing the next parameter. Various process parameters influencing protease production like fermentation time (studied up to 144 hrs with 24 hrs. sampling), initial pH (5-9), medium supplements based on carbon (dextrose, sucrose, maltose, fructose, starch and lactose), organic nitrogen (beef extract, peptone, tryptone, and yeast extract) and inorganic nitrogen (ammonium nitrate, potassium nitrate and sodium nitrate) sources on enzyme production was studied by incorporating the constituent/parameter individually in the production medium[13].

Determination of optimum conditions for enzyme activity

The optimum substrate concentration for maximum enzyme activity was determined by varying concentrations of casein i.e., 0.5%-2% in sodium phosphate buffer incubated at 40°C with enzyme for 30 minutes. Effect of pH on protease activity was determined by incubating the reaction mixtures with 1% substrate at 40°C with pH value ranging from 5 to 9 using 1M citrate buffer and 1M phosphate buffer. For determining the optimum temperature of the enzyme activity, the enzyme was incubated with 1% substrate for 30 minutes at various temperatures ranging from 30°C to 80°C[13].

Statistical analysis

Experiments were run in triplicate and repeated at least three times. Each time three readings were taken, their mean, and standard error of the mean were calculated.

Results and Discussion

Isolation and screening of fungal isolates

Thirty soil fungal isolates were isolated from various ecological niches (farm, garden and uphill soil) of Jaipur region and screened for extracellular protease production. Among all the isolates FGCC/BLS2 showed maximum hydrolysis capacity (2) (Table 1) when compared to wild type reference strain *A. flavus* (1.6) and therefore selected for optimization studies. Moreover, the FGCC/BLS2 isolate when inoculated on skim milk agar medium with pH 5 (Figure 1a), 7 (Figure 1b) and 8 (Figure 1c) separately; it was observed that clear zone around colony was exhibited only on medium

with pH 7 which shows secretion of extracellular neutral proteases. Previous studies have also reported that *Aspergillus flavus* and *Aspergillus oryzae* NCIM 649produce protease extracellularly at neutral pH[14].

Microscopic and molecular characterization of FGCC/BLS 2

The fungal isolate was identified up to genus level based on their morphological (macroscopic and microscopic features). Morphology revealed that colonies are grayish to black with greenish black bottom, Conidiophores are branched or branched with conidia produced in branched acropetal chains (Figure 1d).The conidia closest to the conidiophore, and where the chains branch, are usually "shield-shaped" as observed during lacto phenol cotton blue staining. Molecular characterization of isolate FGCC-BLS2 was performed by using D2 region of LSU (Large Sub Unit: 28S rDNA) and gene sequence analysis. A single discrete PCR amplicon band of 700bp was observed when resolved on agarose gel (Figure 1e). The isolate was showing similarity to *Cladosporium sp.* PAB-2014 (Accession Number: KM099505.1) based on nucleotide homology and phylogenetic analysis. Evolutionary relationship of taxa based on above analysis was shown in Figure 1f.

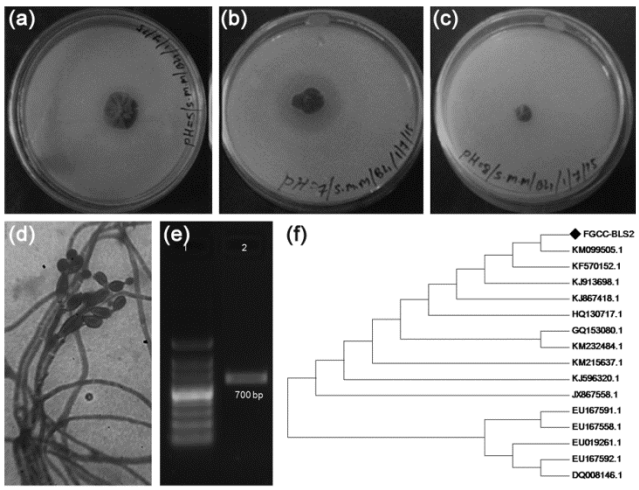

Fig. 1 — Screening of BL2 isolate for presence of acidic/neutral or alkaline proteases by plate assay on Skim Milk Agar medium with pH 5 (A), pH 7 (B) and pH 8 (C). Lactophenol cotton blue staining of the isolate BL2 (D). 0.8% Agarose gel showing 700bp amplicon (D2 region) of 28S rDNA. Lane 1: 1 Kb DNA Ladder and Lane 2: 700bp amplicon (D2 region) of 28S rDNA (E). Evolutionary relationship among listed organism (F).

Optimum growth conditions for enzyme production

The effect of following parameters of production medium on the protease production by FGCC/BLS2 isolate was assessed during enzyme assays with enzyme specific activity (U/mg).

Effect of incubation period

The protease production by microorganisms is greatly influenced by incubation period. The protease activity was found to be 36.2U/ mg protein at 24 h, this was followed by an increase in activity at 120 h (60.9 U/ mg protein), which is maximum protease activity. Further increase in time period led to decline in enzyme activity at 144 h (56.8 U/ mg protein) (Figure 2a). This can be explained in terms of feedback inhibition where complete consumption of nutrients and production of other byproducts inhibit enzyme activity[15]. Our result is in agreement with Oseni, O.A (2011)[16].

Effect of percent inoculum at different temperature

Inoculum concentration is one of the important factors that determine the growth as well as production of enzyme. Inappropriate inoculum concentration results in poor microbial growth and reduction in enzyme production. Fungi were grown using different inoculums percentage and protease production was evaluated. At 2% inoculums maximum protease activity was observed (63.19 U/ mg protein) which was followed by decrease in enzyme production at 6% inoculum (Figure 2b). These results suggest that the activity of enzyme is relatively higher in alkaline pH. The influence of percentage inoculums was also studied by Papagianni *et al* (2002)[17].

Effect of various pH on protease production

pH is the most important factor, which markedly influence growth of microorganism and enzyme activity. The effect of pH (4, 5, 6, 7 and 8) on activity of protease produced by *Cladosporium sp PAB 2014* was studied. At pH 4 protease activity was found to be 36.6 U/ mg protein, which increased upto pH 7 (63.7 U/ mg protein) (Figure. 2c). Further increase in pH led to decrease in protease activity. Result suggests that the activity of enzyme is relatively higher in neutral pH. These data are inconformity to the findings of Ikram-Ul-Haq *et al* (2006), who have also found similar results at pH 7 with *Penicillium chrysogenum* isolated from soil. It might be due to that change in pH cause denaturation of enzyme resulting in the loss of catalytic activity.

Effect of various carbon sources on proteases production

The production media was supplemented with various carbon sources i.e., dextrose, lactose, starch, sucrose, maltose, fructose at 2% level and then effect of various carbon sources on protease production was monitored. Dextrose (63.9 U/ mg protein) was found to be most suitable carbon source followed by sucrose (54 U/ mg protein) and dextrose (52.7 U/ mg protein) (Figure 2d).

Effect of various nitrogen sources on protease production

Furthermore, effect of various nitrogen sources i.e., tryptone, peptone, yeast extract, beef extract, potassium nitrate, ammonium nitrate, sodium nitrate at 1% level was evaluated for protease production. The highest protease activity was found using tryptone (66.9 U/ mg protein) as nitrogen source followed by peptone, yeast extract, beef extract, potassium nitrate, ammonium nitrate & sodium nitrate respectively (Figure 2e).

Optimum conditions for enzyme assays

Protease activity was optimized by varying temperature 25°C-80°C, casein concentration 0.5-2% and pH from 5-9 in assay buffer under optimum production condition. Maximum protease activity was obtained at 40°C temperature of reaction mixture (63.3±1.0 U/ mg protein) (Figure 3a) with 1.5 % casein (64.8±3.2 U/ mg protein) (Figure 3b), pH 7 (63±2.3 U/ mg protein) and mixture (Figure 3c).

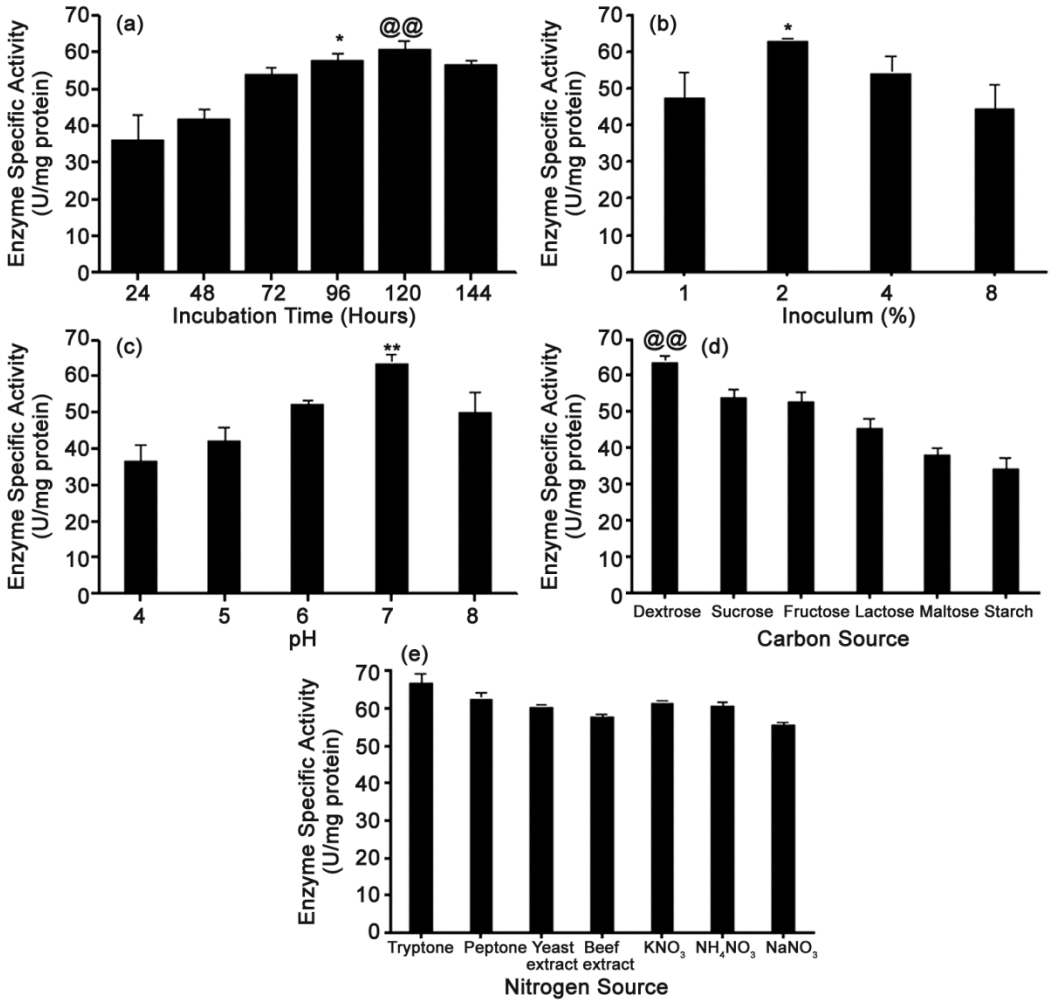

Fig. 2 — Effect of incubation time (A), percent inoculum (B), pH (C), carbon source (D) and nitrogen source (E) on the extracellular production of protease. The data is a representation of 3 independent experiments, and have been expressed as Mean± SEM. (*p<0.05 for 96 hours Vs 24 hours, @@p<0.01 for 120 hours Vs 24 hours, *p<0.05 for 2% inoculum Vs 1% inoculum, **p<0.01 for pH7 Vs pH4, ***p<0.001 for dextrose Vs starch, $^{@@}$p<0.01 for dextrose Vs maltose).

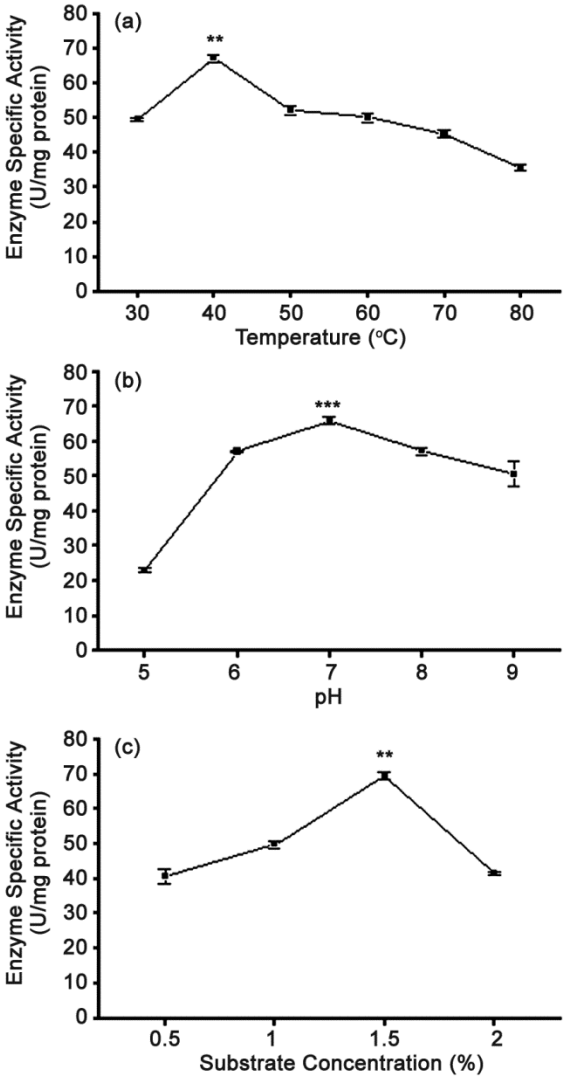

Fig. 3 — Effect of temperature (A), pH (B) and substrate concentration (C) on extracellular amylase activity. The data is a representation of 3 independent experiments, and have been expressed as Mean ± SEM. (**p<0.01 for 40°C Vs 30°C, ***p<0.001 for pH7 Vs pH5, **p<0.01 for 1.5% Vs 0.5%)

Conclusion

Large number of bioprocesses in industrial, environmental and food biotechnology make use of enzymes which can be procured from microbial sources[18]. The present study highlights the fact that soil harbors a diverse group of fungi that can be used as a source of industrially important protease enzyme. The *Cladosporium* sp PAB2014 strain FGCC/BLS 2 showed maximum protease production after 120 hours of incubation, 2 % inoculums, at pH 7 with dextrose as carbon source and tryptone as nitrogen source. Best protease activity was obtained at temperature 40°C in 1.5% casein as substrate in assay buffer with pH7. It can be concluded that this strain FGCC/BLS 2 is potential producer of extracellular neutral proteases which could find applications in industry.

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