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Characterization of proteases produced from *Microsporum canis* (JNU-FGC#503)

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Abstract

The selected isolate of Microsporum canis JNU-FGC#503 was optimized for the production of proteases grows on casein containing basal salt medium. This exhibited the highest production on 6th day of incubation and optimum temperature for production was recorded at 45˚C whereas pH was 10. During the studies on the activity of proteases with metal ions at the concentration of 50 mM/ml of reaction mixture, Cu^{2+}, Mg^{2+} and Fe^{2+} ions enhanced the activity of enzyme whereas the concentration increased to 100 mM/ml, all the ions greatly inhibited the activity except Mg^{2+} and Fe^{2+}. Stronger inhibitory effect was observed in the presence of Hg^{2+}, Ag^{2+} and Zn^{2+}. The effect of solvent on the enzyme activity at the rate of 50 mM/ml of reaction mixture, aniline and acetaldehyde enhance the activity of enzyme but acetic acid (5.05 U/ml) and formic acid (6.65 U/ml) showed maximum inhibition followed by ethanol (9.2U/ml). As the concentration was increased at the rate of 100 mM/ml, all solvents except aniline greatly decreased the activity. Among inhibitors, β- mercaptoethanol (6.15 U/ml) and EDTA (4.05 U/ml) were greatly inhibiting the activity whereas SDS and sodium meta bisulphate not affected the enzyme activity at this concentration. When the concentration was increased to 100 mM/ml of mixture, all the inhibitors greatly inhibited the activity except sodium meta bisulphate

Key words: *Microsporum canis*, Protease, Enzyme activity

INTRODUCTION

The proteolytic enzymes from micro-organisms have many physiological functions, ranging from the generalized protein digestion to the more specific regulated processes [1, 2]. Proteases are one of industrially most important enzymes, which covers approximately 60% of all enzyme market, and are utilized extensively in a variety of industries, including detergents, meat tenderization, cheese-making, baking, brewery, the production of digestive aids, and the recovery

of silver from photographic film[3, 4]. In general, proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell. Although protease can be obtained from several sources such as plant, animals but enzyme from microbial sources especially fungi generally fulfill industrial requirement. In the present study an opportunistic pathogen *Microsporum canis* was selected and optimize for the protease production because this fungal keratinase (protease) enzyme may have develop as recent trend of ecofriendly technology and it replaced to chemical hydrolysis of protein used in various industries especially in leather treatment, bioremediation processes, and in pharmaceutical industries for preparation of medicines[5]. Therefore, the present study is important to optimize the production of proteases by using high protease producing selected fungal isolate *Microsporum canis* JNU-FGC#503.

MATERIALS AND METHODS

Fungal isolate:-
Microsporum canis JNU-FGC#503 was isolated from keratin rich soil samples collected from barber's shops of Jaipur (Rajasthan) by hair bait technique [6]. One set of pure culture of isolate was maintained on Sabouraud dextrose agar at 28˚C and other set was stored at 4˚C in refrigerator for further use**.**

Extraction of enzyme:-
Enzyme extraction was done as per Upadhyay *et al*, [7]. 15 ml of basal salt medium with 1% casein was autoclaved, seven days old *Microsporum canis* was inoculated and incubated at 28˚C. Crude enzyme extract was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 5 min at 4˚C. The crude extract was stored at -20˚C for further experiment [8]**.**

Enzyme activity:
Proteases activity was determine as per Riffel *et al.*, [9] with slightly modification. Crude enzyme extract (1.0 ml) was added to 1.0 ml of substrate solution (1.0 % casein in 0.1 M Sodium phosphate buffer, pH 7.4) and the mixture was incubated at 37˚C for 60 min. The reaction was terminated by the addition of 3.0 ml of 5 % trichloroacetic acid, and the mixture was kept at room temperature for 10 min followed by filtration through Whatman filter paper no.1. The absorbance of the filtrate was measure at 280 nm against the blank.

Effects of Various parameters on the activity of proteases
Effects of incubation periods:
The enzyme activity was observed from 1 to 10 days of incubation. 1.0 ml of crude enzyme along with 1.0 ml substrate was incubated for 60 min at 37˚C as described above and protease activity was observed spectrophotometrically against the blank [7]**.**

Effect of pH and temperature:
The effect of pH and temperature on the activity of protease was determined as hydrolysis of casein at the pH range of 3 to 11 the optimum temperature of enzyme was determined by incubation of reaction mixtures at the following temperature viz; 10˚C, 15˚C, 20˚C, 25˚C, 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, 55˚C and 60˚C.

Effect of metal ions:
The effect of metal ions on the activity of enzyme was studied to determine nature of active site of enzyme. During this study following salts of ions were taken at the rate of 50 mM/ml and 100 mM/ml of reaction mixture. The ions used was Na^{+} (NaCl), K^{+} (KCl), Mg^{+2} ($MgCl_2$), Ca^{+2} ($CaCl_2$), Mn^{+2} ($MnCl_2$), Fe^{+2} ($FeCl_3$), Co^{+2} ($CoCl_2$), Cu^{+2} ($CuSO_4$), Zn^{+2} ($ZnSO_4$), Cd^{+2} ($CdCl_2$), Hg^{+2} ($HgCl_2$), and Ag^{+} ($AgNO_3$).

Effects of solvents:
The effects of various solvents on enzyme activity were studied in order to find some valuable information about catalytic site of enzyme. Following solvent were added with reaction mixture at the rate of 50 mM/ml and 100 mM/ml concentration. The solvent used were ethanol, formic acid, amyl alcohol, carbon tetra chloride, formaldehyde, toluene, aniline, benzene, chloroform, acetic acid and acetaldehyde.

Effects of inhibitors:
Enzyme inhibitors viz; EDTA, Sodium nitrite, DTT, SDS, Sodium meta bisulphate, Hydrogen peroxide, β-mercaptoethanol and Urea were added with reaction mixture at the rate of 50 mM and 100 mM per ml of mixture.

RESULTS AND DISCUSSION

The time course of protease secretion and degradation of casein by *Microsporum canis* in a basal salt medium was determined by batch culture. After complete incubation, extract was prepared from culture supernatant, which acts as crude extra cellular enzyme extract. The production of enzyme increased during cell growth and reached maximum value 44.95 U/ml at 6^{th} day of incubation thereafter decreases the enzyme activity as periodic increases. It was also reported from our experiment that if defatted hair was taken instead of casein the production of enzyme was started after 10^{th} day because keratins are hard substrate and it increases lag phase of cell growth, similar observation was recorded by Raju *et al* [10].

Effect of pH and temperature:
pH is the most important factor, which markedly influence enzyme activity. Extremely high and low pH values generally complete loss of activity for most of enzymes. From the fig (2) it was confined that the activity was also increased from 3 to 6.0, then decreased periodically but it was again increased the activity when pH reached at 10.0 to11. Two pH optima were recorded for this isolate. This alkaline pH plays a crucial role in the bioprocessing of used X-ray or photographic film for silver recovery [11]. The influence of temperature on protease production is related to the growth of the organisms. Hence the optimum temperature depends on whether the culture is mesophilic or thermophilic. Optimum temperature for proteases activity was recorded at 45˚C (Fig 3), which is thermophilic rang. The present result is supported by Letourneau *et al* [12] while studied keratinolytic activity of *Streptomyces* species.

Effect various metal ions, inhibitors and solvent:
The influence of various metal ions on enzyme activity was studied. As reported from studies on the activity of enzyme at the concentration of 50 mM/ml of mixture, metal ions affected the enzyme activity. Mg^{+2}, Fe^{+2} and Cu^{+2} enhanced the activity of enzyme whereas metal ions viz;

Na^{+}, Mn^{+2}, Co^{+2}, Ca^{+}, K^{+} and Cd^{+2} affected approximately 60-70% relatively activity , when the concentration was increased at 100 mM/ml of mixture all the ions greatly inhibited the activity except Mg^{+2} and Fe^{+2}. From the fig (4) a stronger inhibitory effect was observed in the presence of Hg^{+2} , Ag $^{+}$ and Zn^{+2} because these binds irreversible with protease enzyme molecules, inhibiting the catalytic activities permanently. The protease reaction stopped and not affected by an increase in substrate concentration [13].

A total eight inhibitors were studied with crude enzyme extract at the concentration of 50 mM/ml and 100 mM/ml of reaction mixture. It was reported from at EDTA (4.05 U/ml) and β-mercaptoethanol (6.15 U/ml) is greatly inhibited the activity of enzyme whereas Sodium meta bisulphate and SDS did not affected the activity and other showed only 65% relative activity at 50 mM concentration. When the concentration was increased at 100 mM all the inhibitors except sodium meta bisulphate inhibited the activity (Fig 6).

Amongst solvent at the concentration of 50 mM acetic acid, formic acid and ethanol greatly inhibit the enzyme activity whereas at the same concentration toluene, benzene and amyl alcohol partially inhibit activity, as the concentration was increased 100 mM/ml of mixture all solvent except aniline decreased the activity Fig (5). The present study on protease produced by *Microsporum canis* shows that fungi are proficient producer of protease in batch culture, which are active at pH 10.0 which are suitable for making enzyme based detergent and implicated in hydrolysis of keratin containing waste as well as leather industry. The optimum temperature was at 45˚C. This thermostable protease are advantageous in some applications because higher processing temperature can be employed, resulting in faster reaction rates , increases in solubility of nongaseous reaction and products and reduced incidence of microbial contamination by mesophilic organisms. It was activity by Mg, Fe and Cu and inhibited by EDTA and β-mercaptoethanol. It concluded that the protease may have useful for industrial exploitation.

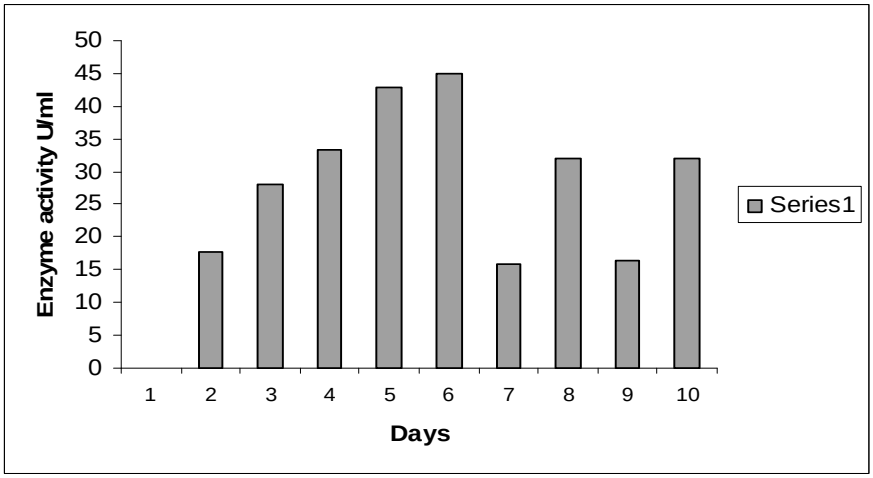

Fig 1. Effect of various incubation periods on protease production of *Microsporum canis* JNU-FGC#503

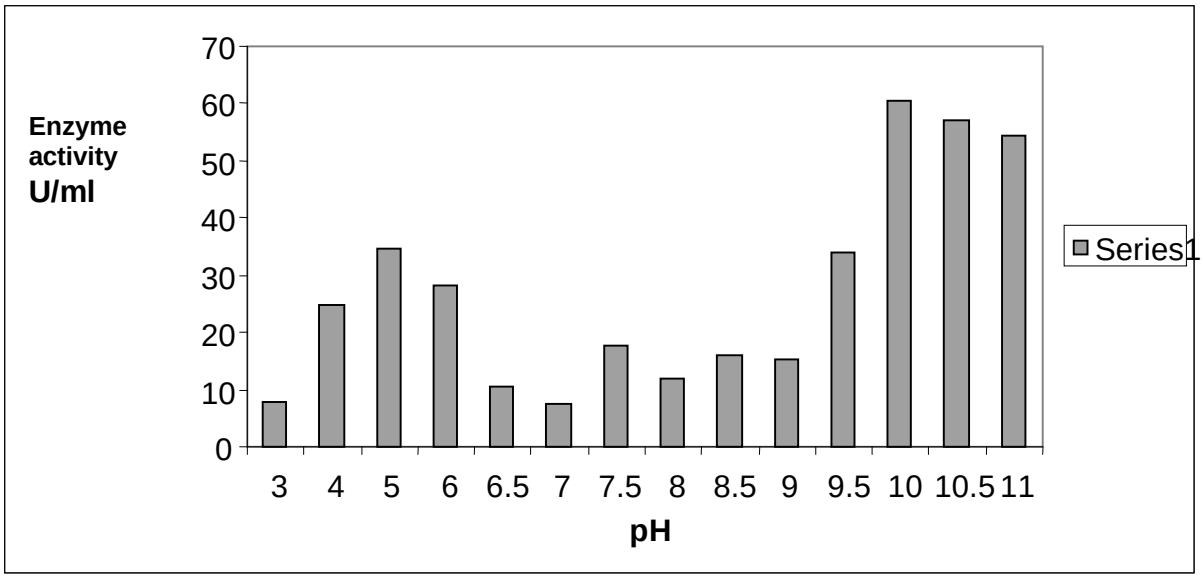

Fig 2. Effect of pH on protease activity of *Microsporum canis* JNU-FGC#503

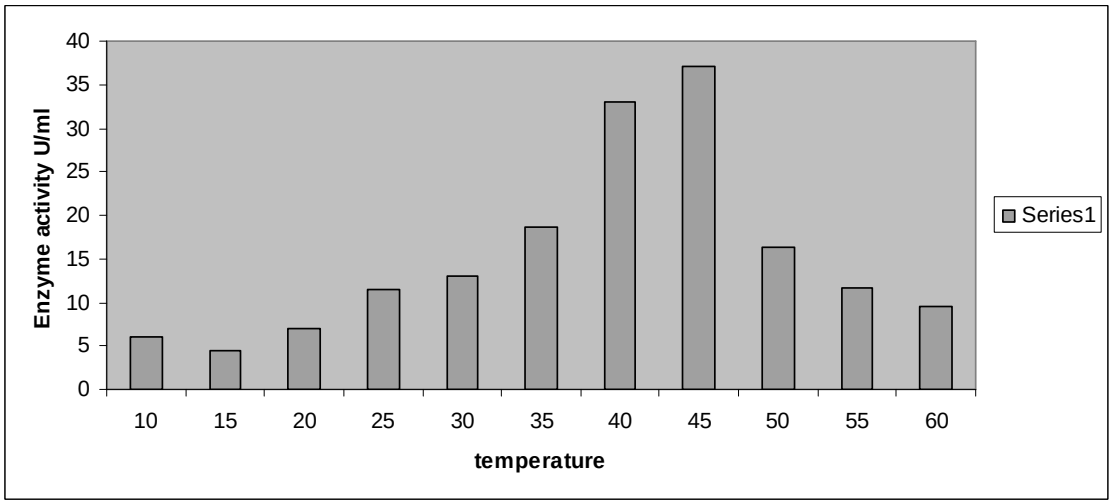

Fig 3. Effect of Temperatures (˚C) on protease activity of *Microsporum canis* JNU-FGC#503

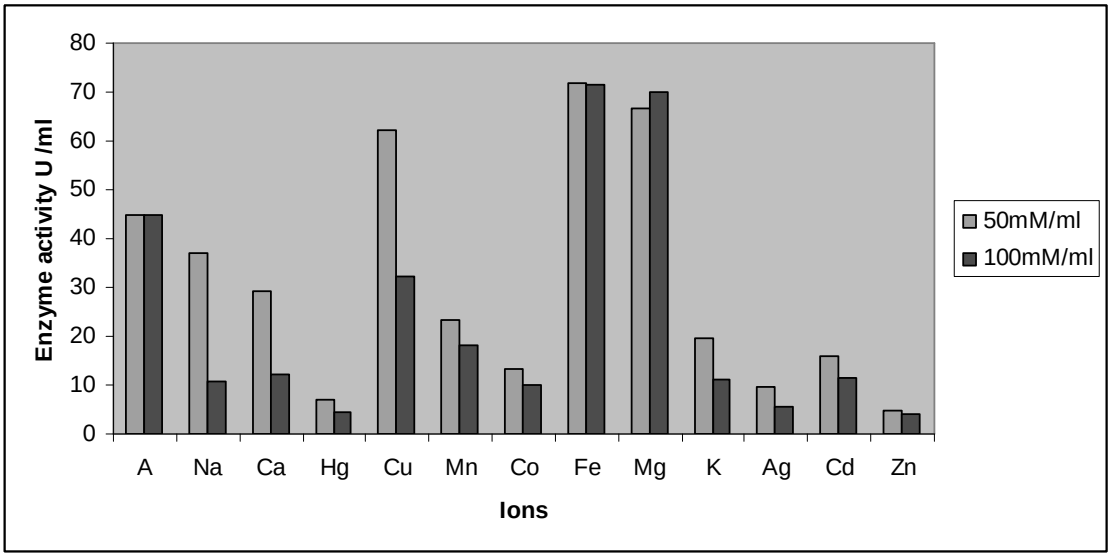

Fig 4. Effect of ions on protease activity of *Microsporum canis* JNU-FGC#503

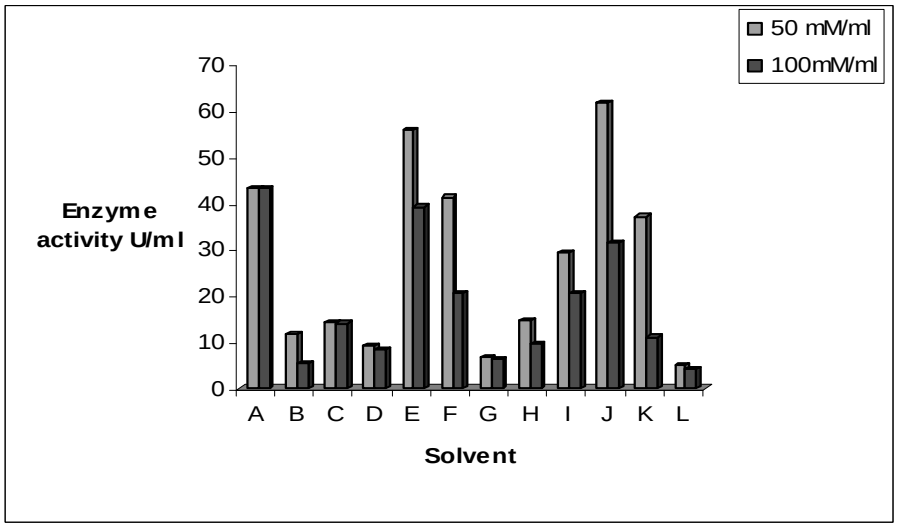

Fig 5. Effect of solvents on protease activity of *Microsporium canis* JNU-FGC#503 Control (A), Carbon tetra chloride(B), Formaldehyde(C), Ethanol(D), Acetaldehyde(E), Benzene(f), Formic(G), Chloroform(H), Amyl alcohol(I), Aniline(J), Toluene(K), Acetic acid(L)

Fig 6. Effect of inhibitors on protease activity of *Microsporium canis* JNU-FGC#503 Control (A), EDTA(B), Sodium nitrite (C), DTT(D), SDS(E), Na meta bisulphate(F), Hydrogen peroxide (G), β- mercaptoethanol(H), Urea(I)

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