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`Production and Characterization of Metalloenzyme Protease from Bacillus Sp.

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Abstract:
The bacterium was Gram positive, aerobic, motile, sporulating and rod-shaped bacterium and optimal growth temperature was found to be 37°C. The cells were able to grow well at a pH range of 8-10 which indicate that *Bacillus* sp DS2 was alkalophilic. The protease exhibited its optimal activity at 90°C and optimum pH 10.5. The enzyme activity was enhanced by Ethanol, Methanol, Chloroform, Benzene, Hexane, Propanol, Acetone, Xylene and Acetic acid. However, the protease activity was inhibited by Ethyl acetate. The optimum protease activity was enhanced in presence Chloroform (208.07%). The enzyme activity has enhanced by $FeCl_3$, $BaCl_2$and $CaCl_2$and optimum enhanced by $MgCl_2$ indicating it was a metalloenzyme with a potential to be a candidate for the biotechnological potential.
(**Key words:** Lonar Lake, Alkaliphiles, *Bacillus*, Protease)

INTRODUCTION

Generally the most concentrated and widespread occurrence of the microorganism is observed in moderate environments. It has been belived that there are extreme environment on the earth which was thought to be the controversial about the existence of life. In this habitat, environmental condition such as pH, temperature and salinity concentration are extremely high or low. Extreme environments are a populated groups of organisms that are specifically adapted to these extreme conditions and these types of microorganism are usually referred as alkaliphiles, halophiles and thermophiles which are reflected the extreme environment (Horikoshii and Akiba, 1982).

The high alkaline condition which accesses naturally or artificially on the basis of extreme condition is referred to as alkaline environments and bacteria survive in this environment is called as alkaliphiles. In the naturally occurring alkaline environments, soda Lake are the most stable with pH values generally higher than 10 and occasionally reaching at 12 (Jones *et al.,* 1998). These alkaline environments are naturally made by a combination of geological, geographical and climatic conditions (Grant and Tindall, 1980). At the time of alkalinity formation, other salts also accumulate, giving rise to haloalkaliphilic environment in which the native microflora is subjected to a number of extreme ecological pressure (Joshi *et al.,* 2007; Wani *et al.,* 2006).

The microbial diversity of saline lakes has been studied primararily acting on the isolation and characterization of organisms with biotechnological potential (Horikoshi 1999; Jones et al. 1998). Martin et al. (2001) were found both alkali tolerant and obligate alkaliphiles were found and identified by phylogenetic analysis as the microbial species found in Ethiopian soda lake microbial population and known for being good enzyme producers. As far as Indian soda lakes are concerned, a culture-dependent approach has not been yet applied to analyze bacterial diversity and alkaliphilic protease producing microorganisms isolated from Lonar Lake (Joshi et al. 2007). In view of the above facts, the present study focused on the optimization of alkaline protease production from a haloalkaliphilic bacterial strain isolated from Indian Soda Lake.

MATERIALS AND METHODS

Isolation of Alkaliphiles:

The 1.0 g of soil sample was transferred to 100 ml sterilized distilled water in 250 ml conical flask and agitated (200 rpm) at 37°C for 15 min in shaker. The suspension was then diluted to 10^{-7} dilutions. One ml of each diluted sample was spread by spread plate technique into petri plates containing Horikoshii medium and nutrient agar medium (A, B, C and D) and inoculated at 37°C for 24 hrs (Tambekar and Dhundale, 2012)

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Screening of bacterial alkaliphiles:

Individual bacterial colonies were screened for proteolytic activities on Skim milk agar medium. The pH of the medium was adjusted to pH 10 with 1N NaOH before and after sterilization. The inoculated plates were incubated at 37°C for 48 hrs and observed for zones of clearance, indicating proteolytic activities.

Identification of the proteolytic isolates:

The bacterial isolates with prominent zones of clearance on casein agar medium were processed for identifications based on morphology, Gram characteristics, motility, oxidase, catalase tests and acid production from dextrose, fructose, sucrose, xylose, arabinose, maltose and mannitol. The isolates were also tested for their growth at different temperatures and pH. These isolates were identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology and Diagnostic Microbiology. The identified strains were maintained on nutrient agar slants having pH 10 at 4°C.

Preparation of crude enzyme extracts:

The 100 ml Casein nutrient broth (Casein 1gm, Peptone 0.5 gm, Yeast Extract 0.15 gm, Beef extract 0.15 gm, Sodium Chloride 0.5 gm, pH 10) was prepared. The sterile broth was inoculated with culture and incubated for 48 hrs at 37°C. After 48 hrs incubation, centrifugation of the broth at 5000 rpm for 15 min was carried out. The supernatant served as crude enzyme source.

Partial characterization of protease:

Partial characterization of protease from *Bacillus* species was carried out by effect of pH, temperature, substrate, enzyme, metal ions and organic solvents on alkaline protease activity was then measured as per assay procedure (Joo *et al*, 2002).

Effect of pH on alkaline protease activity:

The effect of pH on alkaline protease was determined by assaying the enzyme activity at different pH values ranging from 7.0 to 12.0 using the phosphate (PO_4) buffer systems with concentration of buffer as 0.2 M. The activity of the protease was then measured as per assay procedure.

Effect of temperature on alkaline protease activity:

The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (pH 10) for 10 min at different temperature ranging from 35°C to 100°C.The activity of the protease was then measured as per assay procedure.

Effect of substrate concentration on alkaline protease activity:

The effect of substrate concentration on alkaline protease activity was determined by incubating the reaction mixture for 10 minutes with different substrate concentration, ranging from 0.5 mg/ml to 5 mg/ml. The activity of the protease was then measured as per assay procedure.

Effect of enzyme concentration on alkaline protease activity:

The effect of enzyme concentration on alkaline protease activity was determined by incubating the reaction mixture (pH 10) for 10 minutes at different enzyme concentration ranging from 0.5 ml to 5 ml. The activity of the protease was then measured as per assay procedure.

ffect of different metal ions on protease activity:

The effect of different metal ions on alkaline protease activity was determined. The enzyme assay was performed in the reaction mixture as described above in the presence of various metal ions at a final concentration of 1 mM. The activity of the enzyme without any additives was taken as 100 %. The influence of various metal ions such as Potassium chloride, Sodium chloride, Ferric chloride, Barium chloride, Cupric chloride and Magnesium chloride (1 mM each) on protease activity was studied by pre-incubating the enzyme with the compounds for 15 min at 37°C. Then, the remaining activity was measured under the enzyme assay conditions.

Organic solvent stability of the protease:

Stability of the protease enzyme was determined in the presence of various organic solvents such as Ethanol, Methanol, Chloroform, Benzene, Hexane, Propanol, Acetone, Ethyl acetate, Xylene and Acetic acid (10 mM each). 0.25 ml of organic solvent was added to 1 ml of the protease solution in a 1.5 ml micro centrifuge tube with a screw cap. The mixture was shaken at 150 rpm for 100 min at 37 °C. Then, the remaining activity was measured under the enzyme assay conditions.

RESULTS AND DISCUSSION:

Screening of bacterial isolates, i**dentifications and characterization of bacterial cultures:**

In the present study, total one hundred and fourteen isolates obtained in the isolation exercise from different sediment samples and water samples of Lonar Lake. Out of one hundred and fourteen, thirty two bacterial cultures were found with proteolytic activity. All the bacterial strains were found alkaliphilic (7-12). The selected

isolates were studied morphologically and biochemically. According to Bergey's Manual of Systematic Bacteriology, the isolates were identified as *Bacillus* sp. All the bacterial cultures were found gram positive and spore forming bacilli. Out of these thirty two, one bacterial culture was selected for the further production and characterizations on the basis of their maximum proteolytic activity of *Bacillus* sp. DS-2.

Effect of pH on activity of enzyme protease:

The effect of pH on protease activity of *Bacillus* sp. DS-2 was determined by incubating the enzyme in different pH buffers ranging from 7.0-12.0 for 10 minutes at 37° C. The optimum pH of *Bacillus* sp. DS-2 protease was found to be 10.5. The enzyme was active between pH ranging from 10-12. Protease activity was relatively low at pH 7, 8 and 9; the enzyme has relative activities found to be 58.82%, 64.71% and 76.47% respectively. The optimum activity of this enzyme was at pH 10.5 with 2.83 Units/ml, which considered as 100%. Earlier studies have shown that proteases were active up to the pH of 5-10. It is comparatively less and the enzyme of *Bacillus* sp. DS-2 strain has still wider ranges of tolerances than the previously reported for proteases.

Effect of temperature on activity of enzyme protease:

Influence of temperature on *Bacillus* sp. DS-2 protease activity was observed by incubating the enzyme at different temperature ranging from 35-100°C and residual activity were determined under enzyme assay condition. The optimum activity of enzyme was taken as 100%. The temperature profile of protease activity of *Bacillus* sp. DS-2 were showed maximal enzymatic activity of 1 Unit/ml (100%) at 90°C, which indicated that the enzyme was thermostable at high temperature. The protease retained more than 75% of the highest activity between 70-80°C. Subsequently, the enzyme activity progressively decreased at 100°C. While the proteolytic activity of the crude supernatant was thermo sensitive at 80°C and high activity was detected at 90°C.

Effect of substrate concentration on activity of enzyme protease:

The influence of different concentrations of substrate was assayed ranging from 0.5-9 ml under constant assay conditions. The activity at 7.5 ml of substrate concentration was 2.33 Units/ml and it was considered as 100%. However, the activity was retained more than 75% with the substrate concentration from 4.5-7 ml. There was very less activity at 0.5 ml of substrate concentration (0.25 Units/ml).

Effect of enzyme concentration on activity of enzyme protease:

The effects of different enzyme concentrations ranging from 0.5-9 ml was carried out under assay conditions. The enzyme shows maximum enzymatic activity (6.25 Units/ml) at 7.5 ml of enzyme concentration and it was considered as 100%. The activity of protease decreases as the enzyme concentration increases more than 8 ml. There was very less activity at 0.5 ml of enzyme concentration (0.17 Units/ml).

Table 1: Composition of Skim milk Agar

Nutrient	Quantity
Casein	1.0 gm
Peptone	0.5 gm
Yeast extract	0.15 gm
Beef extract Sodium chloride	0.15 gm 0.5 gm
Agar – agar	2.0 gm
D.W.	100ml
pH	10

Influence of different metal ions on activity of enzyme protease:

Table 2 : Effect of different metal ions on activity of enzyme protease

Metal ions	Relative activity (%)
Control	100
KCl	183.33
NaCl	175
$FeCl_3$	125
$BaCl_2$	275
$CaCl_2$	133.33
$MgCl_2$	341.67

The influence of different metal ions on activity of *Bacillus* sp. DS-2 protease was carried out under the assay conditions. Metal ions have different effects on activity of protease. The enzyme activity without any additives was taken as 100%. The enzyme activity was enhanced by KCl, NaCl, $FeCl_3$, $BaCl_2$, $CaCl_2$ and $MgCl_2$. The optimum protease activity (341.67%) was enhanced in presence of $MgCl_2$. The enzyme activity has enhanced by KCL, NaCl, $FeCl_3$, $BaCl_2$ and $CaCl_2$, these activity indicating it was a metalloenzyme. The dissimiller results obtained from bacterium L21, protease enzyme retained 93% of its activity after 1 h of incubation with EDTA and was completely inhibited by 0.01 M PMSF (Genckal and Tari, 2006).

Influence

of various organic solvents on activity of enzyme protease:

Table 3: Effect of various organic solvents on activity of enzyme protease	
Organic solvents	Relative activity (%)
Control	100
Ethanol	175.81
Methanol	127.42
Chloroform	208.07
Benzene	188.71
Hexane	198.39
Propanol	101.61
Acetone	106.45
Ethyl acetate	95.16
Xylene	125.81
Acetic acid	109.68

The effect of organic solvents on the activity of *Bacillus* sp. DS-2protease was determined. Organic solvents have different effects on activity of protease. The enzyme activity without any additives was taken as 100%. The enzyme activity was enhanced by Ethanol, Methanol, Chloroform, Benzene, Hexane, Propanol, Acetone, Xylene and Acetic acid. However, the protease activity was inhibited by Ethyl acetate. The optimum protease activity (208.07%) was enhanced in presence Chloroform.

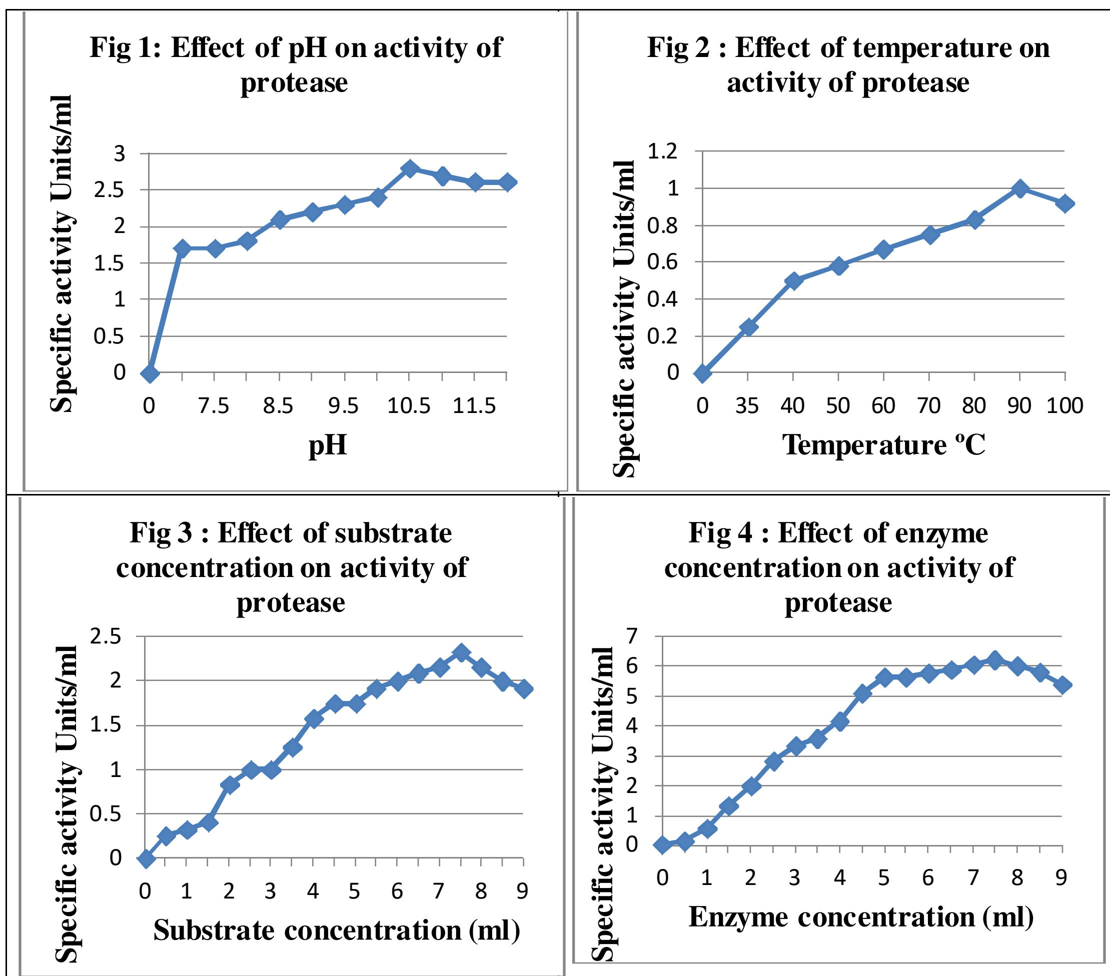

Fig 1: Effect of pH on activity of protease

Fig 2 : Effect of temperature on activity of protease

Fig 3 : Effect of substrate concentration on activity of protease

Fig 4 : Effect of enzyme concentration on activity of protease

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