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# Studies On Commercially Important Alkaline Protease From Bacillus Lichniformis N-2 Isolated From Decaying Organic Soil

## [Çürümekte Olan Organik Topraktan İzole Edilen, Bacillus Lichniformis N-2 Tarafından Sentezlenen ve Ticari Önem Taşıyan Alkalen Proteaz Üzerine Çalışmalar]

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**ABSTRACT**

Eighty bacterial strains were isolated from upper layer (0-5 cm) of decaying organic soil sample. Thirty eight isolates (47.50 %) exhibited the prominent zones of clearance on skim milk agar medium at pH 10. These isolates were then characterized and identified. Thirty two (40 %) of the alkalophylic isolates belonged to the genus *Bacillus*. Maximum enzyme activity (123.29 ± 1.61 PU/ ml) was found in strain designated as *Bacillus lichniformis* N-2 at pH 10 and temperature 37 °C. Some fundamental properties like effects of different temperature, pH, metal ions and inhibitors on protease activity were also studied. Maximum activity was obtained at pH 11 at 60 °C. $Ca^{+2}$ and $Mg^{+2}$ ions were found to enhance the relative enzyme activity up to 132 and 118 % respectively. However, the activity of protease was completely inhibited by phenylmethylsulfonylflouride that showed its serine nature. The results indicated that enzyme produced by *B. lichniformis*-N2 is active within broad ranges of temperature and pH. These characteristics render its potential use in leather and detergent industries.

**Key Words:** Alkaliphiles *Bacillus* species, alkaline protease, decaying organic soil.

**ÖZET**

Çürümekte olan organik toprağın üst yüzeyinden (0–5 cm) seksen bakteriyel suş izole edilmiştir. Otuz altı izolat (% 47.50) az yağlı süt agarında (pH 10) belirgin berraklaşma alanı göstermiştir. Bu suşlar karakterize edilmiş ve tanımlanmıştır. Alkalophilic izolatlardan otuz iki tanesi (% 40) *Bacillus* genomuna ait olduğu saptanmıştır. *Bacillus lichniformis* N-2 olarak belirlenen suşdan elde edilen maksimum enzim aktivitesi pH 10 ve 37 °C'de 123.29 ± 1.61 PU/ml'dir. Farklı sıcaklıklar, pH, metal iyonları, inhibitörler ve proteaz aktivitesi gibi bazı temel özelliklerin enzim aktivitesine etkisi de çalışılmıştır. Maksimum aktivite pH 11, 60 °C'da elde edilmiştir. $Ca^{+2}$ ve $Mg^{+2}$ iyonlarının göreceli enzim aktivitesini % 132 ve 118 oranlarında artırdığı gözlenmiştir. Buna rağmen proteaz aktivitesinin fenilmetilsulfonilflorid ile tamamen inhibe edilmiş olması bu enzimin bir serine proteaz olduğunu göstermektedir. Sonuçlarımız *B. lichniformis*-N2 tarafından sentezlenen enzimin geniş ısı ve pH aralıklarında aktif olduğunu göstermiştir. Bu karakteristikler sebebi ile deri ve deterjan endüstrilerinde kullanıma uygundur.

**Anahtar Kelimeler:** Alkaliphiles *Bacillus* species alkalen proteaz, çürümekte olan organik toprak

## INTRODUCTION

Alkalophilic bacteria which grow well in pH range from 9 to 11 on the pH scale are of ecological, industrial and basic bioenergetics interests (1). Alkaliphiles are reported to be a rich sources of alkaline active enzymes e.g. proteases, amylases, cellulases and xylanases etc. which have numerous applications in various industrial processes including food, detergents, pharmaceutical, and tanneries sectors (2,3). The environments frequented for these alkaliphiles are different from standard laboratory conditions. Naturally occurring highly alkaline environments are minimal with most notable being soda lakes, alkaline springs, artificially occurring industrial-derived waters, desert soils and soil with decaying organics matters (4,5). Enzymes from microorganisms that can survive under extreme pH could be particularly useful for commercial applications under highly alkaline reaction conditions, e.g. in the production of detergents. Alkaline proteases produced by *Bacillus* species are of great importance in detergent industries due to their high thermal and pH stability (6,7). With increasing industrial demands for the biocatalysts that can cope with industrial processes at harsh conditions, the isolation and characterization of new promising strains is a recent approach to increase the yield of such enzymes (8). Despite the fact that to date more than 3000 different enzymes have been identified and many of these have found their ways into biotechnological and industrial applications. The present toolbox is still not sufficient to meet all the demands. A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. As a result, the characterizations of microorganisms that are able to thrive in extreme environments have received a great deal of attention. Microorganisms also account for a two-third share of commercial protease's production in the world (9).

Being the most important sources for enzyme production, the selection of suitable microorganism plays a key role in high yield of desirable enzyme. The present study reports isolation, characterization and identification of proteolytic bacteria isolated from decaying organic soil. A maximum producer of proteolytic enzyme has been studied in detail and some fundamental properties of the enzyme are also described for its commercial significance.

## MATERIALS AND METHOD

### *Isolation of alkaliphiles*

The sample was collected from the upper most 0-5 cm layer of soil characterized by compost containing dead animal's remnants from the vicinity of Kamalia, District T. T. Singh of Province Punjab. About 1.0 g of soil sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37 $^{o}$C for 15 minutes on water bath shaker (Eyela, Japan). The sample was then heated at 80 $^{o}$C for 15 minutes to destroy all the vegetative microbial cells. The soil suspension was then diluted in serial up to $10^{-7}$ dilutions. One ml of each dilution was poured into petri plates containing nutrient agar (Oxoid) medium of pH 10. The inoculated plates were then incubated at 37 $^{o}$C for 24 hours.

### *Screening of bacterial alkaliphiles*

Individual bacterial colonies were screened for proteolytic enzyme production on skim milk agar medium containing skim milk 1.0 %, peptone 0.1 %, sodium chloride 0.5 %, and agar 2.0 %. The pH of the medium was adjusted at 10 with 1 N HCl/ 1N NaOH before sterilization at 121 $^{o}$C for 15 minute. The inoculated plates were then incubated at 37 $^{o}$C for 48 hrs and observed for zones of clearance which indicate proteolytic activities.

### *Identification of the proteolytic isolates*

The bacterial isolates with prominent zones of clearance were processed for the determination of morphology, gram characteristics, motility, citrate utilization, oxidase, urease, gelatin liqufication, catalase, VP and indol tests, acid production from D-Glucose, D-Arabinose, D- Lactose, D-Mannitol, D-Galactose and D-Maltose. The isolates were also grown at different temperatures, pH and NaCl concentrations. These isolates were then identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology (10) and Diagnostic Microbiology (11). The identified strains were maintained on nutrient agar slants having pH 10 at 4.0 $^{o}$C.

### *Preparation of crude enzyme extracts*

Fifty ml of nutrient broth (Oxoid) having pH 10 was inoculated with each isolate in triplicates and incubated at 37 $^{0}$C for 48 hrs in a water bath shaker (Eyela, Japan) at 140 rpm. The inoculated broth was then centrifuged at 90000 x g for 10 minutes at 4.0 $^{0}$C. The supernatant was used to determine the protease activity.

### *Determination of proteolytic activity*

Proteases activity was determined by a slightly modified method of Yang and Huang (12). The reaction mixture containing 1 ml of 1.0 % casein solution in 0.05 M Glycine-NaOH buffer having pH 10 and 1 ml of a given enzyme solution were incubated at 40 $^{o}$C for 20 minutes and the reaction was then stopped with 3 ml of 10 % tri-chloroacetic acid (TCA). The absorbance of the liberated tyrosine in the filtrate was measured at 280 nm. One proteolytic unit was defined as the amount of the enzyme that released 1ug of tyrosine under the assay conditions.

### *Determination of biomass*

The biomass (dry weight) of the bacterial isolates were determined after centrifugation of the fermented broth at 90000 x g for 10 minutes at 4.0 $^{o}$C. The residual material in the form of pellet was dried at 105 $^{o}$C till consistant weight was achieved.

### *Total protein assay*

Total protein contents of the enzyme solution were measured according to the method described by Lowry (13); using bovine serum albumin (BSA) as a standard.

## PARTIAL CHARACTERIZATION OF PROTEASE

### *Effect of pH on protease activity*

The effect of pH on alkaline protease from *B. lichniformis* N-2 was determined by assaying the enzyme activity at different pH values ranging from 6.0 to 12.0, using the following buffer systems: phosphate (pH 6-7) tris–HCl (pH 8-9) and glycine-NaOH (pH 10-12). The concentration of each buffer was 0.05 M.

### *Effect of temperature on protease activity*

The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (pH 11) for 20 minutes at different temperature ranging from 30 $^{o}$C to 80 $^{o}$C. The activity of the protease was then measured as per assay procedure.

### *Effect of inhibitors and metal ions on protease activity*

The effect of various inhibitors such as phenyl methyl sulphonyl fluoride (PMSF), di-isopropyl fluorophosphate (DFP), cysteine inhibitors p-chloromercuric benzoate (pCMB), ethylene diamine tetra acetic acid (EDTA) and metal ions ($Zn^{+2}$, $Mg^{+2}$, $Na^{+2}$, $Ca^{+2}$, $Al^{+3}$, $Cu^{+2}$, and $Hg^{+2}$) on protease activity were investigated to further characterize the enzyme. The crude alkaline protease was pre-incubated with the above-mentioned chemicals for 30 minutes at 40 $^{o}$C; afterwards the residual activity (%) was measured by standard protease assay. The final concentration of each inhibitors and metal ions was 5mM at the time of pre-incubation.

## RESULTS AND DISCUSSION

In this study 80 bacterial strains were isolated form decaying organic soil (DOS) sample suspension processed on nutrient agar medium. Our great concern was to isolate and identify alkalophilic *Bacillus* sp. having a vital tendency to secrete extra-cellular proteolytic enzymes. Out of 80 isolates 47.5 % exhibited vivid zones of clearance on 1.0 % skim milk agar medium at pH 10 as shown by *Bacillus lichniformis* N-2 (Figure 1). These isolates were identified and 40.0 % were represented by the genus *Bacillus*; namely *Bacillus subtilis* (8 strains), *Bacillus lichniformis* (6 strains), *Bacillus megatarium* (4 strains), *Bacillus polymyxa* (3 strains), *Bacillus pumilis* (2 strains), *Bacillus brevis* (2 strains), *Bacillus macerans* (5 stains) and *Bacilluis coagulans* (2 strains) as shown in Table 1. This shows that *Bacillus* sp. are ubiquitous in humus soil and endures the extreme conditions of heat and desiccation in environment by forming endospores. Many workers reported isolation of diverse species of *Bacillus* from natural soil. For instance, 42 different species of genus *Bacillus* were identified from grassland soil samples of which five species were recognized as novel species of *Bacillus* and designated as *B. novalis* sp. nov., *B. vireti* sp. nov., *B. soli* sp. nov., *B. bataveinsis* sp. nov., and *B. drentensis* sp. nov. (14). In another study, it was observed that 27 bacteria out of 40 isolates from soil samples belonged to the genus *Bacillus* (15). It is known that proteolytic bacteria are more abundant in topsoil as compared to a subsoil sample (16). All these findings indicate that *Bacillus* species are widely distributed in soil and other natural environments characterized with wide range of different physiological conditions.

Most of the commercially important alkaline proteases are derived from Bacillus species. In fact these bacteria are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (17,18). In the present study, the proteolytic activities of the isolated Bacillus species were tested under extreme alkaline conditions to find out the best alkaline protease producer. It was found that B. lichniformis N-2 produced maximum proteolytic enzyme (123.29 ± 1.61 PU/ml) followed by Bacillus subtilis N-5 with the enzymatic activity 111.45 ± 1.34 PU/ml (Table 2).

Figure 2 gives the effect of temperature on protease activity. An initial increase in temperature up to 60 $^{o}$C

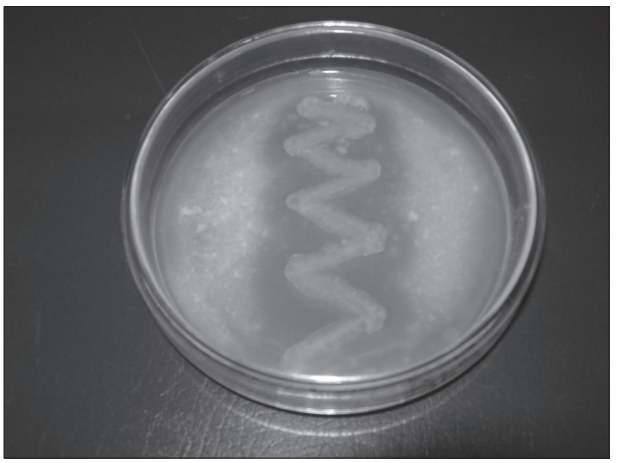

**Figure 1.** Proteolytic activity of Bacillus lichniformis N-2 on skim milk agar medium (pH 10) incubated for 48 hrs at 37 $^{o}$C

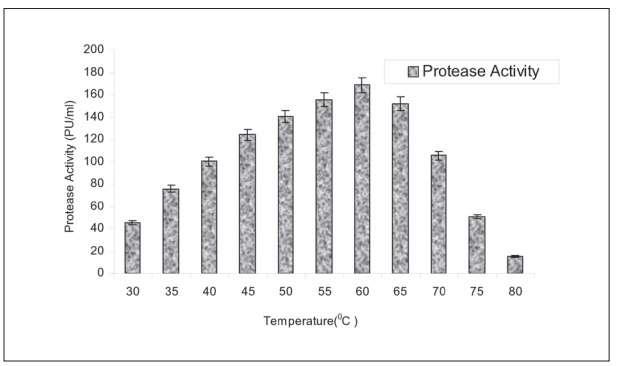


**Figure 2.** Effect of different temperature on protease activity freoillus lichniformis N-2. Bars represent S.D.

**Table 1.** Phenotypic and biochemical comparison of proteolytic Bacillus species isolated from decaying organic soil

| Tests | | Bacterial isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Bacillus subtilis* | *Bacillus lichniformis* | *Bacillus megatarium* | *Bacillus polymyxa* | *Bacillus pumilis* | *Bacillus brevis* | *Bacillus macerans* | *Baccilus coagulans* |
| | | 8 | 6 | 4 | 3 | 2 | 2 | 5 | 2 |
| Morphology | | Rods | Rods | Rods | Rods | Rods | Rods | Rods | Rods |
| Gram staining | | + | + | + | + | + | + | + | + |
| Motility | | + | + | + | + | + | + | + | + |
| Growth at $^{o}$C | 5 | - | - | - | - | - | - | - | - |
| | 30 | + | + | + | + | + | + | + | + |
| | 40 | + | + | + | + | + | + | + | + |
| | 50 | + | + | W | W | + | W | - | W |
| | 55 | W | W | - | - | - | - | - | - |
| Growth at pH | 8 | + | + | + | + | + | + | + | + |
| | 9 | + | + | + | + | + | + | + | + |
| | 10 | + | + | + | + | + | + | W | W |
| | 11 | + | ++ | W | W | W | W | - | - |
| | 12 | W | W | - | - | - | - | - | - |
| Growth in NaCl | 2.% | + | + | + | + | + | + | + | + |
| | 5 % | + | + | + | + | + | + | + | + |
| | 7 % | + | + | + | + | + | + | + | + |
| | 10 % | + | + | W | W | + | W | W | - |
| | 15 % | - | W | - | - | - | - | - | - |
| Growth in Carbohydrates | Glucose | + | + | + | + | + | + | + | + |
| | Arabinose | + | + | + | + | + | - | + | - |
| | Maltose | - | + | - | + | + | - | - | + |
| | Galactose | - | + | - | + | + | + | - | + |
| | Mannitol | + | + | + | + | + | - | + | - |
| | Xylose | - | - | + | + | + | + | - | + |
| | Sucrose | + | + | + | + | + | - | + | + |
| | Lactose | + | - | - | + | - | - | + | + |
| Hydrolysis of | Casein | + | + | + | + | + | + | + | + |
| | Gelatin | + | + | + | + | + | + | + | + |
| Biochemical tests | Citrate utilization | + | + | + | + | + | - | - | - |
| | Urease | - | - | - | - | - | - | - | - |
| | Catalase | + | + | + | + | + | + | + | + |
| | Indol | - | - | - | - | - | - | - | - |
| | VP test | + | + | - | + | + | - | - | + |
| | Oxidase | + | - | + | + | | + | - | + |
| | Growth on MacCkony agar | + | + | + | + | + | - | - | + |

+, Positive; -, Negative; W, Weak Growth.

increased the rate of enzyme's catalyzed reaction which resulted in increase of proteolytic activity. However, the enzyme denatured rapidly and thus loosed its activity. Maximum proteolytic activity of *Bacillus* strains HR-08 and KR-8102 isolated from the soil of west and north parts of the Iran have been recorded at 65 $^{o}$C and 50 $^{o}$C respectively (19). These findings show that strains isolated from different habitats or localities express extra-cellular products of varying characteristics. Similarly, pH also plays an important role in the activity of enzyme. The enzyme produced by *B. lichniformis* N-2 was found to yield maximum activity at pH 11 (Figure 3). This aspect is in good agreement with earlier findings describing maximum enzyme activity at a temperature range of 60-65 $^{o}$C and pH 11 by *Bacillus clausii* 1-52 (20).

**Table 2.** Protease production by different alkalophilic Bacillus species incubated at 37 °C for 48 hrs in nutrient broth medium at pH 10

| Strains | Dry cell mass ( g/L) ± S. D* | Total Protein (g/L) ± S. D* | Enzymes Activity (PU/ ml) ± S. D* |
|---|---|---|---|
| Bacillus subtilis N-1 | 0.55 ± 0.03 | 1.22 ± 0.05 | 80.65 ± 1.25 |
| Bacillus subtilisN-2 | 0.38 ± 0.02 | 1.10 ± 0.03 | 50.33 ± 1.05 |
| Bacillus subtilis N-3 | 0.85 ± 0.05 | 1.25 ± 0.15 | 43.14 ± 1.17 |
| Bacillus subtilis N-4 | 0.46 ± 0.07 | 1.36 ± 0.08 | 56.10 ± 1.22 |
| Bacillus subtilis N-5 | 0.95 ± 0.02 | 1.22 ± 0.01 | 111.45 ± 1.34 |
| Bacillus subtilis N-6 | 0.88 ± 0.05 | 2.01 ± 0.04 | 10.78 ± 0.67 |
| Bacillus subtilis N-7 | 1.02 ± 0.06 | 1.34 ± 0.8 | 73.00 ± 0.78 |
| Bacillus subtilis N-8 | 0.56 ± 0.04 | 1.11 ± 0.06 | 64.90 ± 1.08 |
| Bacillus lichniformis N-1 | 0.92 ± 0.04 | 1.98 ± 0.08 | 102.43 ± 1.30 |
| Bacillus lichniformis N-2 | 1.10 ± 0.06 | 1.11 ± 0.05 | 123.29 ± 1.61 |
| Bacillus lichniformis N-3 | 0.77 ± 0.03 | 1.88 ± 0.03 | 89.11 ± 1.44 |
| Bacillus lichniformis N-4 | 1.10 ± 0.05 | 2.66 ± 0.07 | 108.56 ± 1.83 |
| Bacillus lichniformis N-5 | 0.90 ± 0.02 | 2.06 ± 0.02 | 101.19 ± 1.26 |
| Bacillus lichniformis N-6 | 1.12 ± 0.08 | 1.90 ± 0.06 | 89.30 ± 1.12 |
| Bacillus megatarium N-1 | 0.43 ± 0.02 | 1.78 ± 0.07 | 101.08 ± 1.10 |
| Bacillus megatarium N-3 | 0.33 ± 0.01 | 2.67 ± 0.09 | 87.40 ± 0.88 |
| Bacillus megatarium N-3 | 0.55 ± 0.03 | 1.57 ± 0.02 | 76.21 ± 1.45 |
| Bacillus megatarium N-4 | 0.38 ± 0.03 | 1.35 ± 0.01 | 100.78 ± 2.08 |
| Bacillus polymyxa N-1 | 0.57 ± 0.04 | 1.89 ± 0.11 | 88.08 ± 1.07 |
| *Bacillus polymyxa* N-2 | 0.66 ± 0.03 | 2.98 ± 0.13 | 86.32 ± 1.31 |
| Bacillus polymyxa N-3 | 0.78 ± 0.05 | 1.45 ± 0.12 | 104.58 ± 1.12 |
| Bacillus pumilis N-1 | 0.65 ± 0.04 | 1.65 ± 0.11 | 108.31 ± 0.94 |
| Bacillus pumilis N-2 | 0.57 ± 0.02 | 1.11 ± 0.23 | 105.05 ± 1.40 |
| Bacillus brevis N-1 | 0.99 ± 0.05 | 2.34 ± 0.07 | 53.57 ± 1.25 |
| Bacillus brevis N-2 | 1.03 ± 0.07 | 1.89 ± 0.05 | 18.45 ± 1.21 |
| *Bacillus macerans* N-1 | 0.76 ± 0.05 | 2.33 ± 0.13 | 101.71 ± 1.22 |
| Bacillus macerans N-2 | 0.64 ± 0.03 | 2.09 ± 0.06 | 27.11 ± 1.03 |
| Bacillus macerans N3 | 0.83 ± 0.04 | 2.56 ± 0.11 | 95.52 ± 0.98 |
| Bacillus macerans N-4 | 0.67 ± 0.02 | 1.87 ± 0.34 | 55.36 ± 1.10 |
| Bacillus macerans N-5 | 0.77 ± 0.05 | 2.02 ± 0.08 | 67.66 ± 1.41 |
| Bacillus coagulans N-1 | 0.42 ± 0.02 | 1.83 ± 0.07 | 49.91 ± 0.70 |
| Bacillus coagulans N-2 | 0.35 ± 0.03 | 1.97 ± 0.02 | 97.23 ± 1.52 |

*Each value corresponds to mean of three replicates ± standard deviation

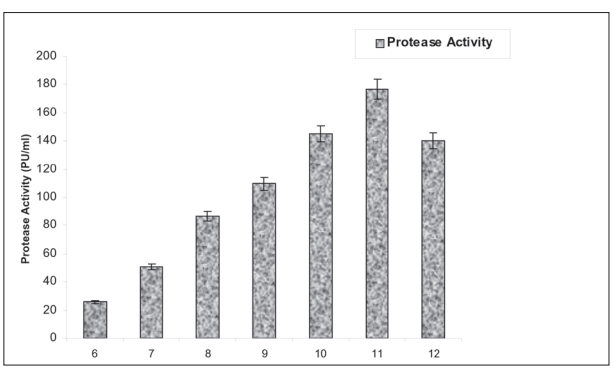


**Figure 3.** Effect of pH on protease activity from Bacillus lichniformis. Bars represent S.D.

Inhibition studies primarily give insight about the nature of the enzymes, its cofactor requirements and the active center of the enzyme (21). Effect of different inhibitors at 5 mM concentration was studied. It appeared that PMSF inhibited protease completely, while DFP exhibited 94 % inhibition. PMSF is a serine protease inhibitor which results in complete loss of the enzyme activity after inhibition (22,23). The protease produced by *B. lichniformis* N-2 was completely inhibited by PMSF that indicated serine protease. Other inhibitors slightly inhibited the protease activity. Effects of some metal ions on the protease activity were also studied and observed that $Ca^{+2}$ and $Mg^{+2}$ ions increase the relative

enzyme activity up to 132 and 118 % respectively (Table 3). Similarly, $Mn^{+2}$, $Ca^{+2}$ and $Mg^{+2}$ ions have been described to increase the relative protease activity produced by *Bacillus megatarium* isolated from thai fish sauce (24). These cations also have been reported to increase the activity and thermo stability of *Bacillus* alkaline proteases (25). It has been suggested that concerned metal ions apparently protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzymes at higher temperatures (26). Identification of proper ions and their suitable concentrations for rendering thermostability to the enzymes are very important for their applications at commercial levels.

## CONCLUSION

The aim of this research work was to isolate *Bacillus* species from local habitat and to investigate the capability of these strains to secrete proteolytic enzymes under alkaline condition (pH 10). *Bacillus lichniformis* N-2 was produced maximum yield of alkaline protease (123.29 ± 1.61PU/ml) and it was selected as a potent strain for further studies. The optimum temperature and pH were determined as 60 °C and 11 for the alkaline protease produced by *B. licnifromis*-N2. Effects of metal ions and inhibitors indicated that the alkaline protease belongs to the family of serine proteases. These properties indicate the potentional use of the enzyme in detergent and leather industries.


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**Table 3.** Effect of inhibitors and activator on the relative activity protease produced by B. lichniformis N-2 .

| Inhibitors/ Activator* | Relative Activity (%) |
|---|---|
| Control | 100 |
| PMSF | 00 |
| DFP | 09 |
| PCMB | 91 |
| EDTA | 96 |
| $Zn^{+2}$ ($ZnCl_2$) | 94 |
| $Mg^{+2}$ ($MgCl_2$) | 118 |
| $Na^{+2}$ ($NaCl_2$) | 98 |
| $Ca^{+2}$ ($CaCl_2$) | 132 |
| $Al^{+3}$ ($AlCl_3$) | 95 |
| $Cu^{+2}$ ($CuCl_2$) | 96 |
| $Hg^{+2}$ ($HgCl_2$) | 90 |

*PMSF = Phenylmethyl sulphonyl fluoride; DFP= di-Isopropyl fluorophosphate;

pCMB= p- Chloromercuric benzoate; EDTA= Ethylene diamine tetra acetic acid. The concentration of all inhibitors and metal ions was 5 mM.

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