

**PRODUCTION OF ALKALINE PROTEASE ENZYME FROM *BACILLUS SUBTILIS* 168 ISOLATED FROM SOIL SAMPLES COLLECTED FROM A DAIRY FARM****Anitha, D. and A. Kumaraswamy\***P.G. and Research Centre, Department of Zoology, S.T. Hindu College, Nagercoil 629 002,  
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**ABSTRACT**

Screening and isolation of protease producing strains of bacteria were carried out from a dairy farm located in Nagercoil, Tamilnadu. The isolates were positive on skim milk agar (1%) and thus are selected as protease producing strain. The organisms were tested for various biochemical tests, which lead to their identification as *Bacillus subtilis* producing protease enzyme. These *Bacillus subtilis* could grow up to 40°C and pH range 6-9 with optimal growth temperature and pH at 37°C and 8.0 respectively. It was also optimized for carbon test and nitrogen test with optimal growth in dextrose and peptone respectively. Enzyme production was carried in 1 litre of optimized media in the fermenter at 37°C for 48 hours at pH 8.0. This result showed that *Bacillus subtilis* under study is a good producer of extra cellular protease, which can be beneficial for industries.

**Keywords:** *Bacillus subtilis*, cellular protease, identification.

**1. INTRODUCTION**

Proteases, one among the three largest groups of industrial enzymes, accounts for about 60% of the total worldwide sale of enzymes from biological sources since they possess almost all characteristics desired for their biotechnological applications (Adinarayana *et al.*, 2003). Proteases constitute a class of industrial enzymes, which alone form approximately 60% of the total world-wide enzyme production (Chu 2007). Among the various proteases, microbial proteases play an important role in biotechnological processes. Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather (Saeki *et al.*, 2007, Dias *et al.*, 2008). In recent years a number of studies have been conducted to characterize alkaline protease from different microorganisms. However, many of the alkaline proteases applied to industrial purposes face some limitations such as low stability towards surfactants and production cost of the enzymes arisen from growth medium (Joo and Chang, 2005). Using of cost effective growth medium for the production of alkaline proteases from an alkalophilic *Bacillus* sp. is especially important (Joo *et al.* 2002). Therefore, there is a need to the search of new strains of bacteria that produce proteolytic enzymes with novel properties and the development of low cost media.

**2. MATERIALS AND METHODS****2.1. Collection of samples**

Protease producing organisms were isolated from soil samples collected from a dairy farm located in Nagercoil, Tamilnadu. Samples were serially diluted using distilled water and spread plated on the surface of casein agar plates (nutrient agar with 1% casein) and incubated at 30°C for 48hrs (Naidu and Devi, 2005).

**2.2. Screening of protease producers**

The collected samples were serially diluted and streaked on skin milk agar plates. The plates were incubated for 48hrs at 37°C and protease producers were selected by observation of zone of hydrolysis around the colonies (Genka *et al.*, 2006).

**2.3. Characterization of protease enzyme**

The total protein contents of the samples were determined according to the method described by Lowry's method using Bovine Serum Albumin (BSA) as standard. Enzyme activity was determined using culture supernatant collected by centrifuging culture broth at 10,000 rpm for 15min. Protease activity was measured by standard assay procedure proposed by Akcan and Uyar, 2011. About 0.5ml of 0.5% casein and 1.25ml of Tris buffer (pH-8.0 to 14.0) was added into 0.2ml of each of the culture supernatant separately. Mixture was

incubated for 30 min at 37°C. About 3 ml of trichloroacetic acid was added and incubated at 40°C for 10 min to form precipitate. The mixture was centrifuged at 10,000 rpm for 15 min and 0.5 ml of supernatant was collected.

Reagent containing sodium carbonate, copper sulphate, sodium potassium tartarate was mixed with 1 ml of Folin phenol reagent. The mixture was incubated at dark for 30 minutes to form blue colour. The absorbance was read at 660 nm to determine the optical density of each sample. The obtained OD was extrapolated in the standard graph. The standard curve was obtained for series of known concentrations of bovine serum albumin. From the graph, the amount of protein liberated due to the action of enzyme protease in the supernatant was determined. One unit of protease activity was defined as the amount of enzyme required to liberate 1 g/ml tyrosine under the experimental conditions. Enzyme activity = OD value X amount of protein released (g) / concentration of substrate X time of incubation X weight of the sample.

#### 2.4. Optimization of conditions for enhanced enzyme production (Das and Prasad, 2010)

Standard methods were adopted to optimize the parameters like cultural conditions, carbon, nitrogen, temperature, pH, inoculum size and substrates.

#### 2.5. Mass production of alkaline protease

The fermentation was carried out in a sterile Stirred Bed Reactor (SBR). The vessel was maintained at optimized temperature, pH and other & incubated for 48 h in a shaking incubator. At the end of fermentation period, the whole culture both was centrifuged at 10,000 rpm for 15 minutes, to remove the cellular debris and the clear supernatant was used for enzyme analysis.

#### 2.6. Characterization of partially purified alkaline protease

The culture filtrate (crude protease) was collected aseptically after upstream production in a SBR under controlled conditions. The required volume of the spent media was centrifuged at 10,000 rpm for 15 min at 4°C in order to obtain a cell free filtrate. About 200 ml of the cell free filtrate containing protease were collected and their proteolytic activity was determined. Protease enzyme was purified by ammonium sulfate fractionation the concentration of ammonium sulphate required for precipitation varies from protein to protein and should be determined

empirically. The two milliliter of the crude protease enzyme was first brought to 20% (w/v) saturation with solid ammonium sulfate (enzyme grade) and 100% saturated dialysis against distilled water in a dialysis bag (cut off 30) for 3 h, followed by dialysis against phosphate buffer at pH 7.0. The obtained protease enzyme preparation was concentrated against crystals of sucrose and kept in the refrigerator at 4°C. The enzyme activity and protein content was determined for salted out dialyzed enzyme fractions. The enzyme activity of the purified fractions of the alkaline protease after harvesting, ammonium sulfate precipitation and dialysis was determined by the method of Gomori (1955). Separation and size determination of enzyme was performed by SDS-PAGE (Joo *et al.*, 2002).

### 3. RESULTS AND DISCUSSION

#### 3.1. Density of protease producers

In the present study soil samples collected from a dairy farm located in Nagercoil were plated on casein agar medium and the microbial density was found to be in the range of  $3.22 \times 10^3$  to  $1.6 \times 10^4$  CFU/g (Fig. 1).

#### 3.2. Screening for proteolytic activity

From casein agar plates 151 strains of varying morphology were selected and screened for proteolytic activity adopting well assay method. The zone of clearance was measured and found to be in the range of 4 mm-15 mm. As most of the strains showed activity with 4 mm range, 5 potential strains alone were selected for the further study. Among the five, the one with 15 mm of zone was selected for protease production (Fig. 3).

#### 3.3 Identification of strains

The potential strains were identified using biochemical methods according to Bergy's manual of determinative bacteriology and identified as *B. cereus*, *E. coli*, *B. subtilis*, *B. pumilis* and *P. aeruginosa* and were designated with their strain number as *B. cereus* DF 101, *E. coli* DF 52, *B. subtilis* DF 49, *P. aeruginosa* DF 11 and *B. pumilis* DF 78.

#### 3.4. Inoculum concentration

When the log phase culture of *B. cereus* DF 101 was tested for the suitable inoculum concentration in the range of 0.5 – 3%, 1% inoculum resulted in the maximum OD value of 1.202. On further increase in concentration of inoculum decrease in growth of the culture in shake flask was noted. Likewise the protease production also found to be the maximum at this inoculum concentration

(1284U/ml/min.). Surprisingly at 3% inoculum concentration growth was reduced to 0.88 OD at which enzyme production was found to be only 4U/ml/min. (Fig. 4 and 5).

### 3.5. Static and shaking conditions

The effect of agitation was tested at the range of 50 – 200rpm. At 50rpm the OD value was found to be 0.6 which was in increasing trend on further increase up to 150rpm, where OD value of 1.202 was observed. Further increase in agitation reduced the growth and value of enzyme activity (i.e) 0.89 and 800 U/ml/min. observed at 200 rpm. However least growth and enzyme activity were found when incubation was done in static condition. When cultures were kept static growth attained the level of 0.332 OD, which resulted in only 250 U/ml/min. of enzyme activity (Fig. 6 and 7).

### 3.6. pH

When a pH range of pH 6 to pH 11 was tested pH 10 resulted in higher OD as well as higher enzyme activity. A maximum of 1249 U/ml/min. was observed at pH 10 at 36 hrs where it was 802 U/ml/min. at pH 8, 1031 U/ml/min. at pH 9 and 981 U/ml/min. at pH 11. However it was only 561 U/ml/min. at pH 7 at 36 hrs. of incubation (Fig. 8 and 9).

### 3.7. Temperature

At 35°C maximum OD value of 1.05 was obtained in which the protease activity observed was 1057/ml/min. At the end of 42 hrs protease production reduced and the activity was found to be 997U/ml min. with a growth of 0.95 OD. Lower growth and enzyme activity were observed at both extremes (i.e.) 25°C and 45°C (Fig. 10 and 11).

### 3.8. NaCl concentration

When NaCl concentration of 0 to 2% was tested at an interval of 0.5%, the maximum growth and enzyme activity were observed at 0.5% NaCl. Maximum OD value of 1.14 with an enzyme activity of 1092 was obtained at 36 hrs at that concentration. The minimum was observed at 2% NaCl with an OD value of 0.78 at 36 hrs, at which only 523 U/ml/min. of enzyme activity was observed. Irrespective of concentration after 36 hrs both OD value and enzyme production were decreased (Fig. 12 and 13).

### 3.9. Carbon sources

In the present study, to select a potential carbon source, glucose, maltose, fructose, sucrose and starch were incorporated in separate flasks at

1% concentration. Among them glucose favoured the maximum growth and protease production respectively with 1.3 OD and 1012U/ml/min. The minimum growth (0.8 OD) and enzyme production (587U/ml/min.) were observed when starch was used as the sole carbon source (Fig. 14 and 15).

### 3.10. Concentration of carbon source

The ideal carbon source glucose was tested from 0.5% to 2.5% in which 1% resulted in maximum growth as well as the maximum enzyme activity. At 1% glucose concentration the OD value was found to be 1.392 at which maximum enzyme activity of 996U/ml/min. was noted. At 2.5% of glucose growth was reduced to 0.98 OD with corresponding enzyme activity of 759U/ml/min. (Fig. 16 and 17).

### 3.11. Nitrogen sources

When yeast extract, beef extract and peptone were selected as organic nitrogen sources and ammonium nitrate, ammonium sulphate and potassium nitrate were selected as inorganic nitrogen sources, organic nitrogen sources resulted in more growth as well as enzyme production compared to the inorganic forms. Yeast extract showed a maximum of 1.056 OD of growth and 995U/ml/min. of enzyme production, whereas with potassium nitrate a minimum of 0.509 OD and 412U/ml/min. of enzyme production were observed (Fig. 18).

### 3.12. Concentration of nitrogen source

The concentration of ideal nitrogen source (i.e.) yeast extract was tested at 0.1 – 1% level, 0.5% favoured the growth of the organism resulting in 1.056 OD at the end of 36 hrs of incubation. At this concentration, the maximum enzyme activity of 878U/ml/min. was observed. When yeast extract concentration was further increased, correspondingly enzyme activity decreased, recording the lowest of 522U/ml/min. at 1%. Even 0.1% resulted in slightly higher enzyme activity (579U/ml/min.).

*Bacillus* species are attractive industrial organisms for a variety of reasons, including their higher growth rates leading to shorter fermentation cycles, their capacity to secrete proteins as extracellular into the medium, and the GRAS (generally regarded as safe) status with the Food and Drug Administration for most of its species, such as *B. subtilis*, *Bacillus licheniformis* etc., The present study was on protease production by a *B. cereus* DF 101 strain isolated from a dairy farm soil of

Nagerkoil District. Microbial proteases are produced from bacteria, fungi and yeast using many processes like solid-state fermentation as well as submerged fermentation (Anwar and Saleemuddin, 1998; Kumar and Takagi, 1999 and Haki and Rakshit, 2003). In the present research work, submerged fermentation technique was used. Even fungi like *Aspergillus flavus*, *A. mellens*, *A.niger*, *Chrysosporium keratinophilum*, *Fusarium graminearum*, *Pencillium griseofulin*, *Scedosporium apiosermum* etc., were reported to produce protease.

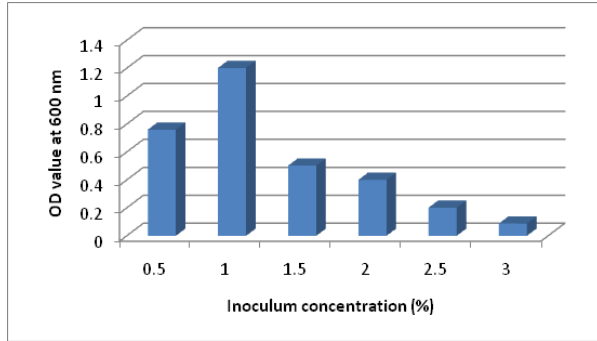


Fig. 1. Effect of inoculum concentration on growth of *B. cereus* DF 101

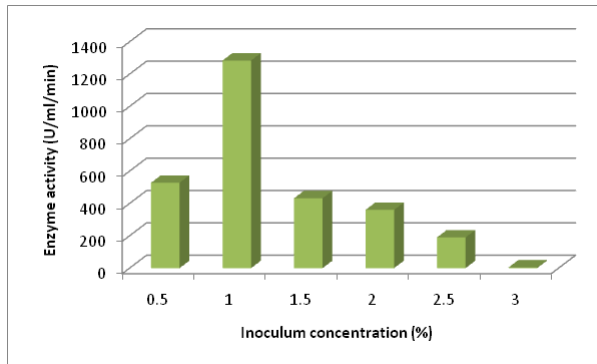


Fig. 2. Effect of inoculum concentration on protease production by *B. cereus* DF 101

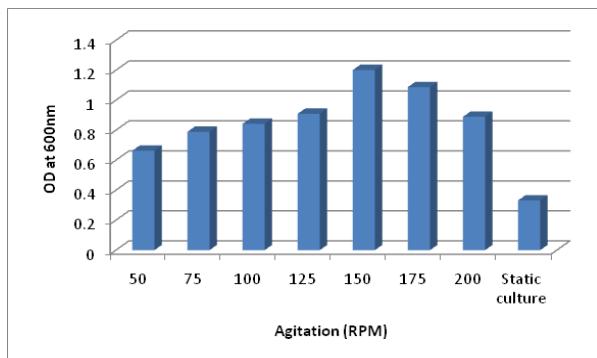


Fig. 3. Effect of agitation on growth of *B. cereus* DF 101

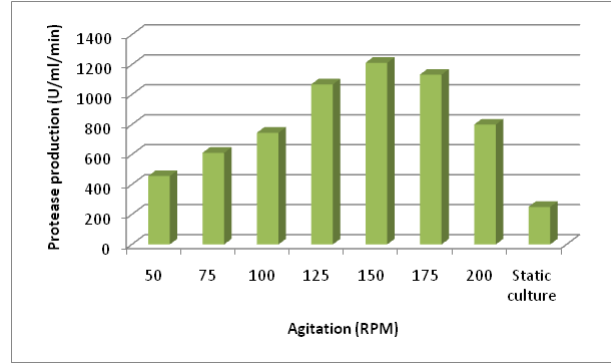


Fig. 4. Effect of agitation on protease production of *B. cereus* DF 101

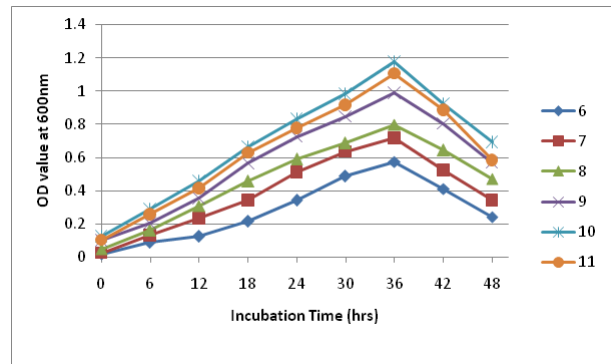


Fig. 5. Effect of pH on growth of *B. cereus* DF 101

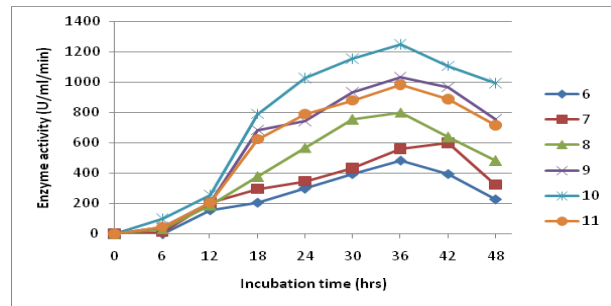


Fig. 6. Effect of pH on protease production of *B. cereus* DF 101

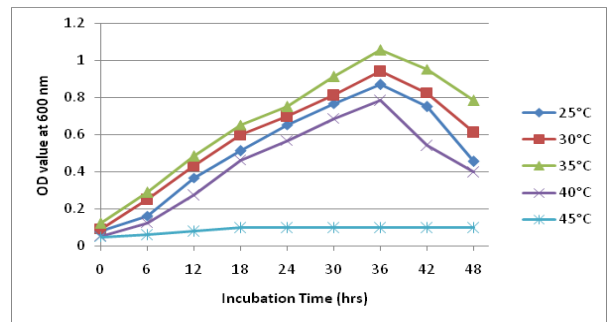
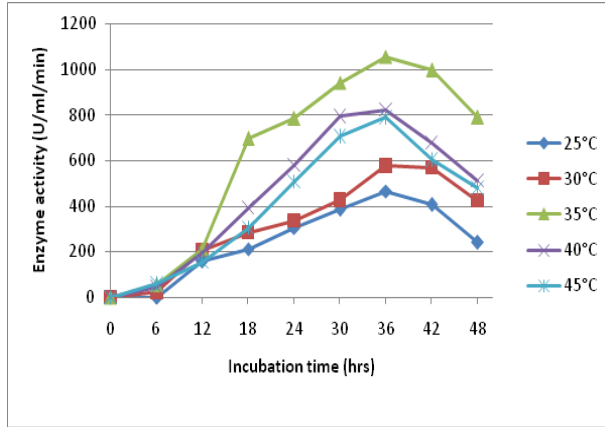
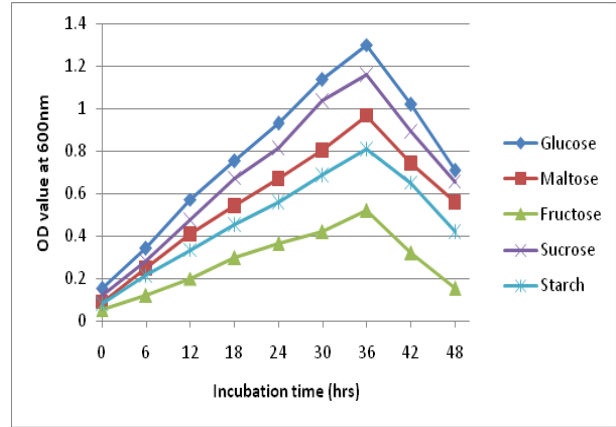


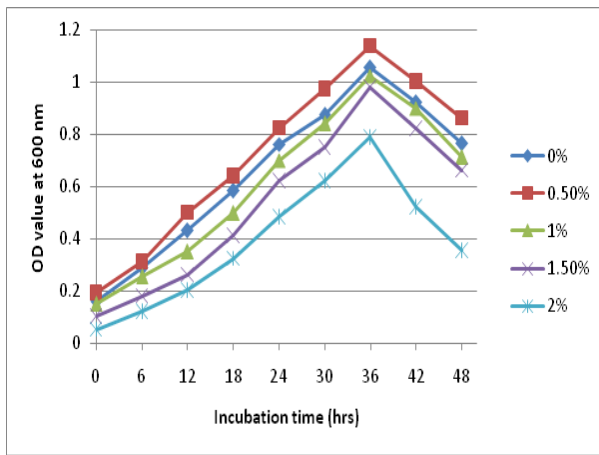
Fig. 7. Effect of temperature on growth of *B. cereus* DF 101



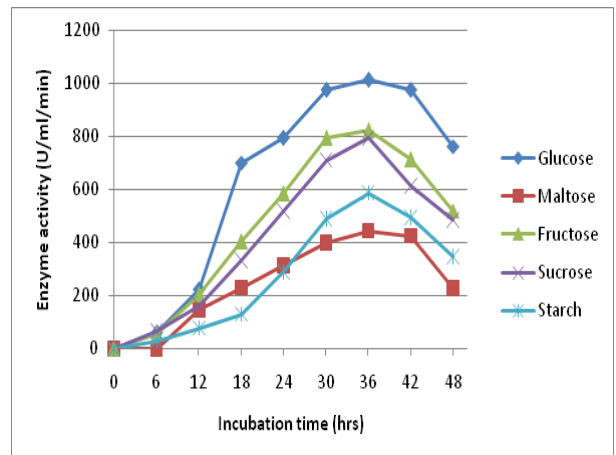
**Fig. 8. Effect of temperature on protease production of *B. cereus* DF 101**



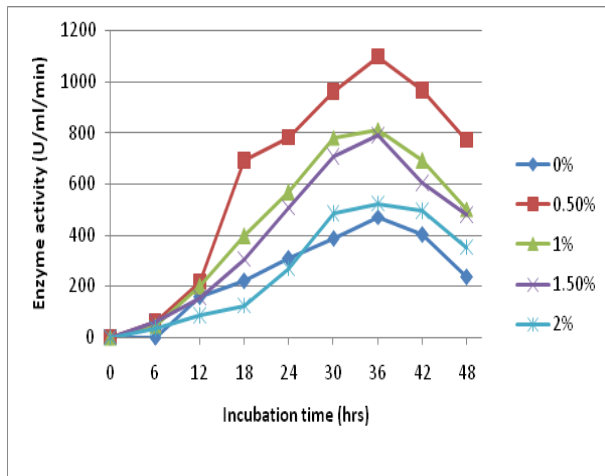
**Fig. 11. Effect of carbon source on growth of *B. cereus* DF 101**



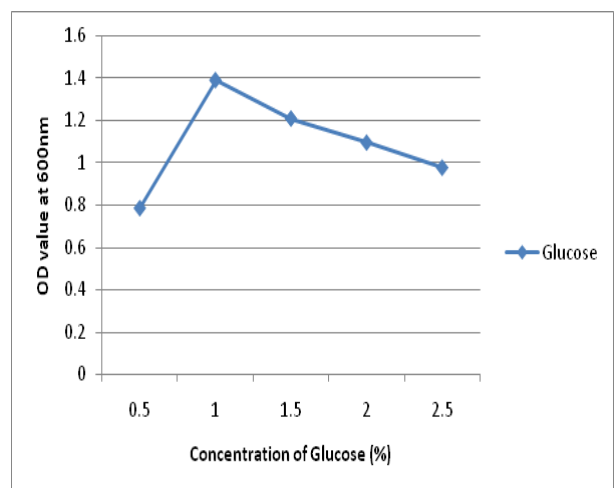
**Fig. 9. Effect of NaCl concentration on growth of *B. cereus* DF 101**



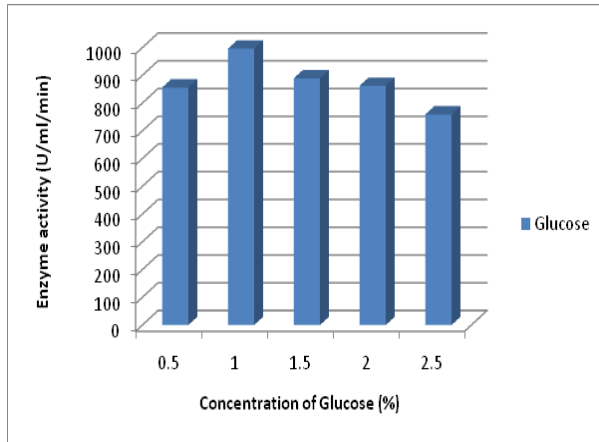
**Fig. 12. Effect of carbon source on protease production of *B. cereus* DF 101**



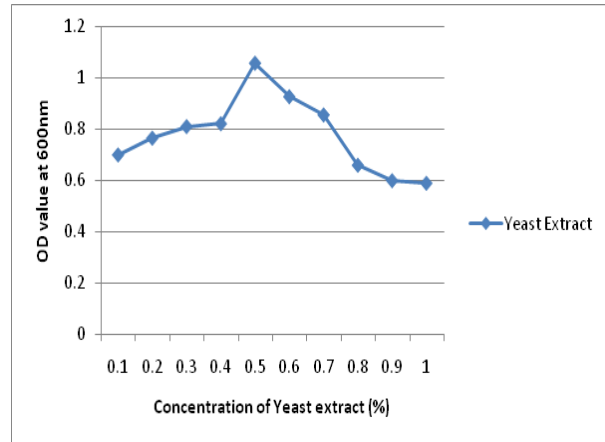
**Fig. 10. Effect of NaCl concentration on protease production of *B. cereus* DF 101**



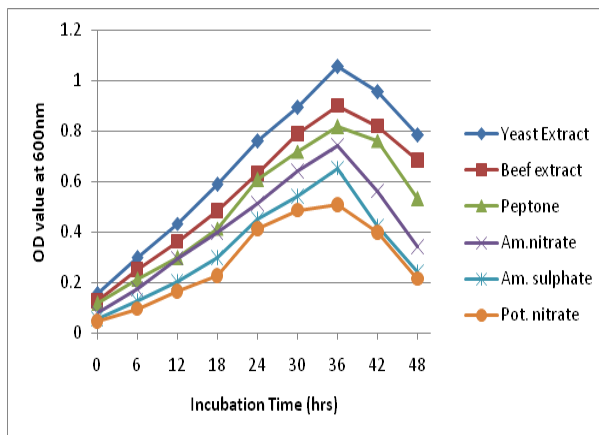
**Fig. 13. Effect of glucose concentration on growth of *B. cereus* DF 101 at 36hrs of incubation**



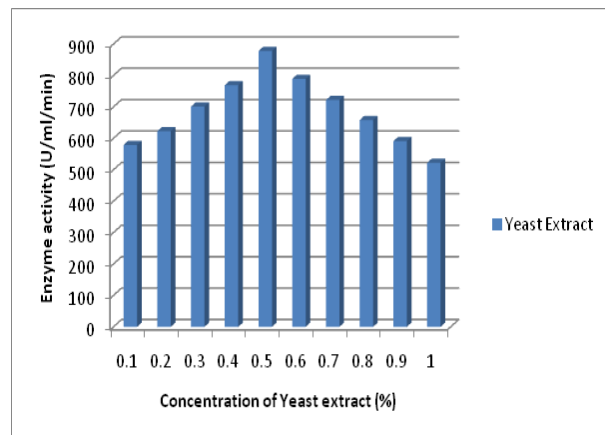
**Fig. 14.** Effect of glucose concentration on protease production in *B. cereus* DF 101 at 36hrs of incubation



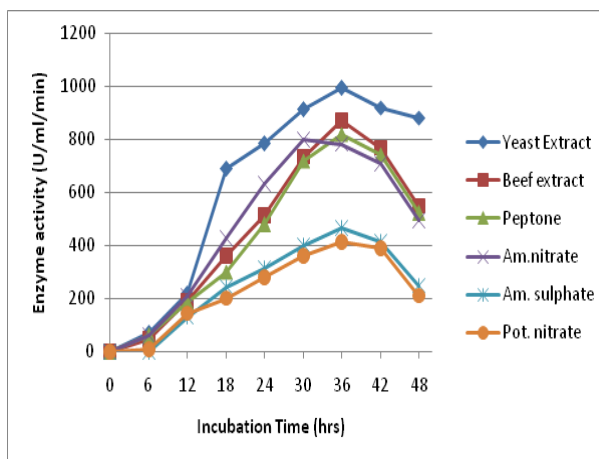
**Fig. 17.** Effect of yeast extract concentration on growth of *B. cereus* DF 101 at 36hrs of incubation.



**Fig. 15.** Effect of nitrogen source on growth of *B. cereus* DF 101



**Fig. 18.** Effect of yeast extract concentration on protease production in *B. cereus* DF 101 at 36hrs of incubation



**Fig. 16.** Effect of nitrogen source on protease production of *B. cereus* DF 101

*Bacillus* species are considered as major workhorse industrial microorganisms with roles in applied microbiology, which date back more than a thousand years, since the production of natto by solid-state fermentation of soybeans using *Bacillus subtilis* (natto) which was first practiced in Japan (Hara and Ueda, 1982).

#### 4. CONCLUSION

Thus in the present study a dairy farm soil originated *B.cereus* DF 101 strain was found to be an ideal producer of alkaline protease and the study also revealed the potential for the industrial scale production using this strain. The abundance of protease producers in dairy farm soil sample indicated them as a new source for the search of alkaline proteases.

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