

# Optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil

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## ABSTRACT

Alkaline proteases have high commercial value and find multiple applications in various industrial sectors. The present study intended to isolate a suitable bacterium for alkaline protease production. Protease producing bacteria were isolated from organic waste containing soil, screened for protease production on skim milk agar plates and confirmed the protease production through protease assay. The bacterial isolate showing highest alkaline protease production was selected and identified by microscopic, macroscopic, biochemical and 16 S RNA phylogenetic analyses as *Bacillus cereus* FT 1. Maximum enzyme production by the isolate was obtained at 35°C; pH, 9.5; 2% lactose as a carbon source and 3.5% casein as a nitrogen source after 48 h of incubation. Among the various surfactants tested, tween 20, tween 80 and poly ethylene glycol were found to be increasing the protease production by the isolate. Mn<sup>2+</sup>, among the metal ions tested tremendously increased the protease production. The best organic solvent for protease production was found to be petrol. With all the optimised cultural conditions, maximum enzyme activity was found to be 187 U/mL and the enzyme was a promising one for detergent industry as an additive enzyme.

## INTRODUCTION

Proteases are enzymes with many physiological roles in all living organisms including cell growth and differentiation (Barrett *et al.*, 2001; Burhan *et al.*, 2003). They are also recognized as industrially important and occupy almost 60% of the total enzyme market (Gupta *et al.*, 2005; Chu, 2007; Verma *et al.*, 2011). Among the proteases, alkaline proteases have the applications in industries like laundry detergents, pharmaceutical, leather, food processing and proteinaceous waste bioremediation (Bayoudh *et al.*, 2000). They are highly active and stable under alkaline conditions (Maurer, 2004; Saeki *et al.*, 2007).

Proteases produced by bacteria are most significant as their properties can be easily modified through genetic manipulations to suit their various applications (Najafi *et al.*, 2005). Most of the commercially important alkaline proteases are produced by *Bacillus* spp. The alkaline proteases derived

from *Bacillus* sp. are highly active and stable at different pH and temperature ranges, broad substrate specific, and can be easily purified with low cost (Maurer, 2004; Haddar *et al.*, 2009; Jellouli *et al.*, 2009). Detergent industry requires efficient, environmental friendly and economical strategies for unwanted protein degradation. Alkaline proteases in detergent formulations can act against proteinaceous stains like blood, food and grass stains (Hameed *et al.*, 1996; Smulders *et al.*, 2002; Huang *et al.*, 2003; Wang *et al.*, 2007; Kalpana Devi *et al.*, 2008).

The optimization of different fermentation parameters like nitrogen and carbon source, media pH, incubation temperature, agitation and incubation time can enhance the yield of industrially useful enzymes (Huang *et al.*, 2003; Tobe *et al.*, 2005; Boominadhan *et al.*, 2009; Aruna *et al.*, 2014; Lakshmi *et al.*, 2014). Although, industrially applicable protease enzymes have been identified from different sources, most of them could not resist drastic environmental changes and most of the sources are incapable to produce required quantities to fulfil industrial demands. So, new bacterial strains that can withstand harsh environmental conditions should be isolated for the enhanced production of such enzymes. The present study is aimed at the

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isolation of a proteolytic strain from soil biome and optimization of its cultural conditions for the enhanced enzyme production.

## MATERIALS AND METHODS

### Sample collection

Soil samples contaminated with organic kitchen wastes were collected in sterile glass bottles from five different locations of Vazhayoor Panchayat, Malappuram District, Kerala, India.

### Isolation and screening of alkaline protease producing bacteria

The soil samples were serially diluted using sterile distilled water, spread plated on skim milk agar plates and incubated at 30°C for 24 to 48 h (Anbu *et al.*, 2013). After incubation, the colonies showing clear zones of proteolysis were selected and inoculated to fresh skim milk agar plates by spotting method and incubated for 24 to 48 h to confirm protease production on the basis of clear zone development. A total of 30 isolates were selected and designated as PS 1 to PS 30. Protease assay was done to find out whether they produce acidic, neutral or alkaline protease (Jani *et al.*, 2016).

### Enzyme assay

The selected isolate was inoculated in 20 ml of protease production media (Composition [g/L]: lactose 10 g; casein 10 g;  $\text{KH}_2\text{PO}_4$  2 g;  $\text{K}_2\text{HPO}_4$  2 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1 g) and incubated for 48 h on a rotary shaker (200 rpm) at room temperature (Sharma and Aruna, 2012; Saraswathy *et al.*, 2013; Tambekar and Tambekar, 2013). After incubation, the culture media were centrifuged at 5000 rpm for 20 min. at 4°C and the cell free supernatants were collected and used as the crude enzyme for protease assay. Protease assay was conducted by a modified method of protease activity assay as described by Cupp-Enyard (2008). Casein dissolved in different pH buffers [acidic (pH: 4), neutral (pH: 7) and alkaline (pH: 9)] were used as the substrate for the assay. The reaction mixture containing casein and the enzyme solution was incubated for 10 min at 37°C, reaction was stopped by adding 3 ml of 20% ice cold Tri chloro acetic acid. Precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was mixed with 2.5 ml of 0.5 M  $\text{Na}_2\text{CO}_3$  and kept for 20 min. at room temperature. Finally, added the appropriately diluted Folin's phenol reagent to the mixture, kept for 10 min. and absorbance was measured at 660 nm against the blank sample. The amount of enzyme required to liberate 1  $\mu\text{g}$  tyrosine per ml per minute under the standard conditions defined one unit of protease activity (Hameed *et al.*, 1999).

### Identification of the isolate

The bacterial isolate showing maximum alkaline protease activity and maximum proteolytic zone size was selected for further studies. The isolate was identified based on the methods described in Bergey's manual of determinative bacteriology and diagnostic microbiology based on its morphological, cultural and biochemical characteristics (Bergey *et al.*, 1974) and further confirmed based on the 16S rRNA sequence analysis and BLAST identification.

## Optimization of cultural conditions for protease production

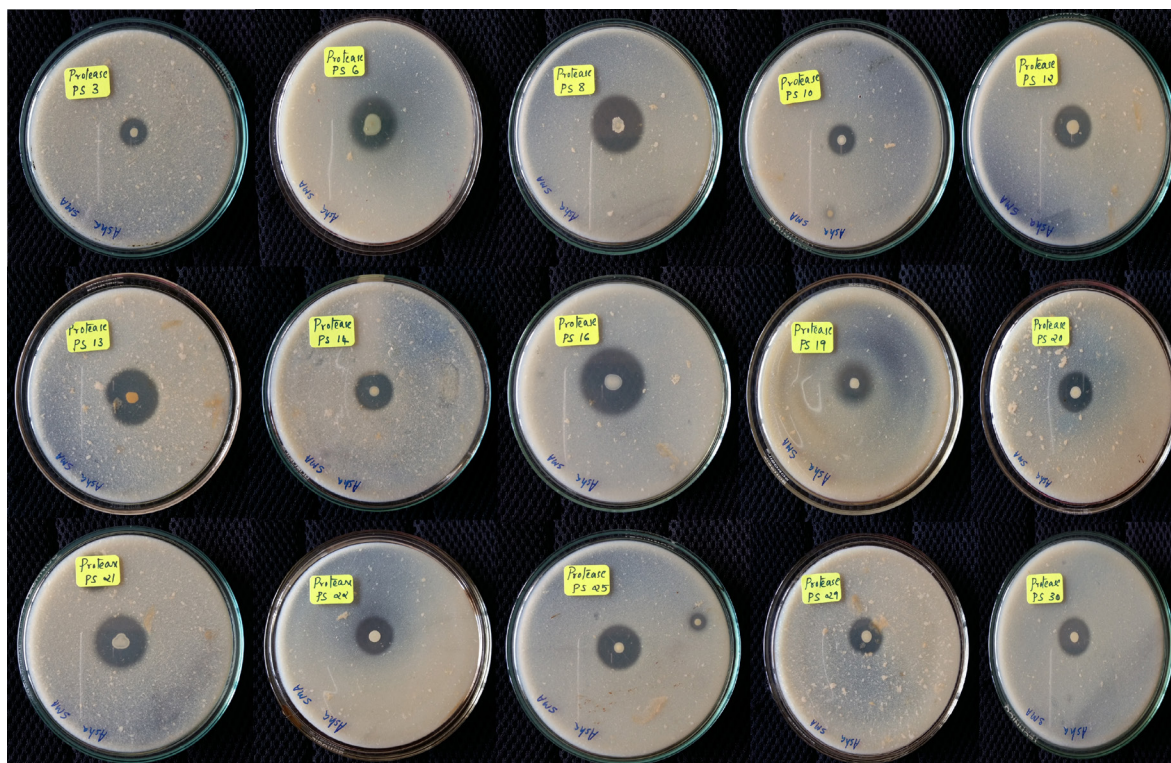
Different cultural parameters including incubation temperature, broth pH, incubation period, carbon and nitrogen sources and their concentrations were optimised for maximum protease production based on 'One parameter at a time approach'. Protease activity was determined for different ranges of each parameters tested by the above mentioned protease assay procedure using only the alkaline buffer. Optimum temperature for protease production was checked in the range of 10°C to 65°C. Ranges of the broth pH checked include 3 to 11. Protease production was determined at various incubation time intervals such as 12, 24, 36, 48, 60 and 72 h. Effect of different carbon sources on protease production was checked by adding 1% w/v of glucose, lactose, fructose, maltose, sucrose, starch, galactose and glycerol. The optimised carbon source was again tested for its optimum concentration in a range of 0.25% to 4%. Different organic and inorganic nitrogen sources at a concentration of 1% were checked for maximum protease production including casein, soya bean meal, yeast extract, peptone, beef extract, meat extract, skim milk powder, ammonium acetate, sodium nitrate, ammonium chloride and potassium nitrate. The optimised nitrogen source was again tested for its optimum concentration in a range of 0.25% to 4% (Aruna *et al.*, 2014).

Effect of different surfactants like tween 20, tween 80, Poly Ethylene Glycol (PEG), Sodium Dodecyl Sulphate (SDS) and triton X 100 on protease production was studied by incorporating them in to the culture medium and determining protease activity. They were added separately at 0.1% concentration. Effect of different trace elements or metal ions including barium chloride, copper sulphate, potassium chloride, magnesium chloride, zinc sulphate, ferric chloride, manganese sulphate, mercuric chloride and calcium sulphate were checked by incorporating 0.1% of each metal ion separately in culture media and protease activity determination. Protease production in presence of organic solvents like petrol, kerosene, methanol, chloroform, hexane and DMSO was also checked by individually adding different solvents at a concentration of 2% to the culture medium. Protease production media with no added surfactants, metal ions or organic solvents was also kept as control (Ananthan, 2014).

## RESULTS AND DISCUSSION

### Isolation and screening of protease producing bacteria

A total of 30 bacterial isolates showing zone of proteolysis were selected from skim milk agar plates inoculated with the soil sample and all of them were confirmed for protease production on further screening by spotting on skim milk agar plates. The isolates were designated as PS1 to PS30. Colony morphology and Gram stain properties of the 30 isolates were studied. Among the 30 isolates obtained, 15 isolates were showing maximum protease activity in presence of alkaline buffer, and hence were proved to be producing an alkaline protease. The proteolytic zone size of each isolate was measured (Table 1). Figure 1 shows the 15 isolates on skim milk agar plates. Among the rest of the isolates, 8 showed maximum protease activity in presence of acidic buffer and hence were confirmed to be producing acidic protease and 7 showed maximum protease activity in presence of neutral buffer and were confirmed to be producing neutral protease.



**Fig. 1:** Alkaline protease producing bacterial isolates on skim milk agar plates showing zone of proteolysis.

The optimum pH range of alkaline proteases is generally 9 to 11 (Singh *et al.*, 2001; Joshi *et al.*, 2007; Maal *et al.*, 2009), and some of them even showed optimum activity at a range of pH 11-13 (Takami *et al.*, 1989; Takami *et al.*, 1990; Fujiwara *et al.*, 1993; Gessesse and Gashe, 1997).

**Table 1:** Proteolytic zone size and protease activity of selected 15 alkaline protease producing bacterial isolates.

Bacterial isolate	Zone size of proteolysis (mm)	Protease activity (U/mL)
PS 3	10	50
PS 6	19	90
PS 8	22	127.5
PS 10	12	50
PS 12	17	82.5
PS 13	23	142.5
PS 14	15	42.5
PS 16	26	152
PS 19	12	80
PS 20	16	107.5
PS 21	20	100
PS 22	15	87.5
PS 25	17	82.5
PS 29	16	75
PS 30	15	67.5

\*The 15 isolates shown in the table were showing maximum protease activity at alkaline pH (pH 9).

Among the 15 selected alkaline protease producing isolates, the isolate PS16 showing highest zone size and highest protease activity was selected for further optimization studies.

#### Identification of the isolate

The isolate PS 16 formed circular, smooth, opaque and white colonies on nutrient agar (Figure 2). Microscopic studies revealed Gram positive rods (Figure 3) which form endospores. The organism was motile. The isolate PS 16 was identified based on the methods described in Bergey's manual of systematic bacteriology as it belongs to *Bacillus* sp. depending upon its morphological, cultural and biochemical characteristics. The 16S rRNA gene sequencing of the isolate revealed its identity as *Bacillus cereus* strain FT1 (Accession number-KP729612.1) when compared with the reference of bacterial species in genomic database banks using the NCBI BLAST.



**Fig. 2:** PS 16 on Nutrient agar.



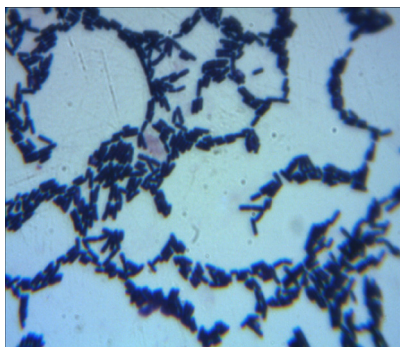


Fig. 3: Gram positive rods of PS 16.

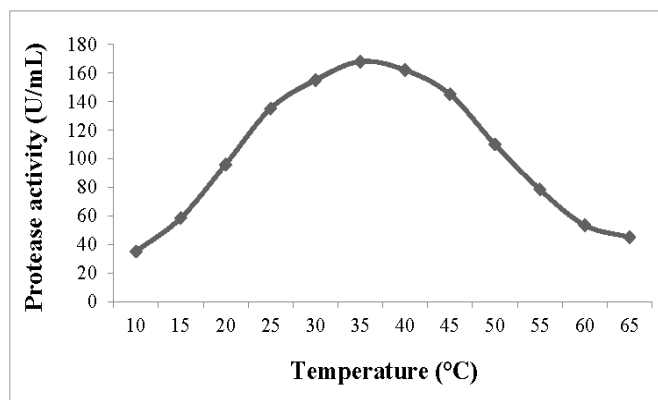


Fig. 4: Effect of temperature on protease production.

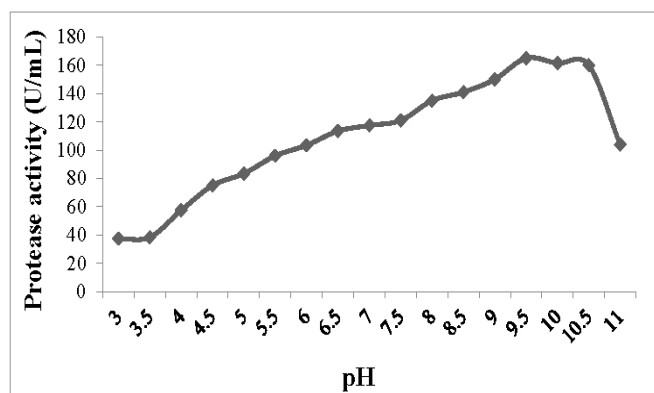


Fig. 5: Effect of pH on protease production.

Many researchers described Indian soil as a rich source of microorganisms which can produce proteases. Sharma *et al.*, (2014) reported a bacterial strain, *Bacillus aryabhathi*, isolated from Kurukshetra, Haryana, producing protease enzyme and Kumar *et al.*, (2014) reported two alkaline proteases producing *Bacillus* strains, *Bacillus subtilis* EN3 and *Bacillus megaterium* EN2 isolated from the agricultural soil of C R C Pantnagar. Other proteases producing *Bacillus* strains were also reported to be isolated from different localities of the country (Asokan and Jayanthi, 2010; Mala and Srividhya, 2010; Naik *et al.*, 2013).

## Optimization of cultural conditions for protease production by *Bacillus cereus* FT 1

### Effect of temperature on protease production

The isolate showed a gradual increase in the protease production up to a temperature of 35°C and a gradual decrease thereafter. The highest enzyme production range was between 25°C and 45°C and the maximum recorded enzyme activity by the isolate was 168 U/mL when incubated at 35°C (Figure 4). At 40°C of incubation, 96% of the enzyme production was maintained and at 45°C of incubation, 86% of enzyme production was maintained. At a temperature of 65°C, the enzyme production was decreased to 27%. This reveals that the isolate is mesophilic. Studies revealed a link between enzyme synthesis and energy metabolism in bacteria which was controlled by the temperature (Frankena *et al.*, 1986). Similar result of the present study was reported for a *Bacillus cereus* strain isolated from soil (Ahamed *et al.*, 2016) and for *Bacillus licheniformis* strain 018 isolated from poultry farm with optimum protease production at 35°C. Other mesophilic *Bacillus* strains are also reported with protease production by Dorcas and Pindi (2016) and Kalpana *et al.*, (2016).

### Effect of pH on protease production

The enzymatic processes and nutrient transport across the cell membrane were strongly affected by the culture pH (Moon and Parulekar, 1991). *Bacillus cereus* FT 1 was producing considerable amount of protease enzyme at a pH range of 7.5 to 10.5 (Figure 5). The maximum enzyme production occurred at pH 9.5 with a protease activity of 165 U/mL, indicating the alkaliphilic nature of the isolate. An alkaline pH of the medium must be maintained for maximum protease yield from alkaliphiles (Aunstrup, 1980). Kumar *et al.*, (1999), Pastor *et al.*, (2001) and Khusro, (2015) demonstrated the alkaline pH optima of *Bacillus* sp. for protease production. Olajuyigbe and Ehiosun, (2013) and Sharmin *et al.*, (2005) reported maximum protease yield by *Bacillus coagulans* PSB-07 and *Bacillus amovivorus* at pH 8 and 8.5, respectively. A maximum yield of protease at pH 9 was reported by some strains of *Bacilli* such as *Bacillus proteolyticus* CFR3001, by Bhaskar *et al.*, (2007), *Bacillus cereus* by Uyar *et al.*, (2011) and *Bacillus flexus* by Verma *et al.*, (2013).

### Effect of incubation period on protease production

The isolate showed protease production in considerable amount at 48 h of incubation (Figure 6). Protease activity at 48 h was 148 U/mL. At 60 and 72 h of incubation, 89% and 82% of protease activity was maintained respectively. Similar results were reported by many scientists. Hoshino *et al.*, (1995) and Shumi *et al.*, (2004) reported maximum protease production by bacteria at 48 to 72 h. Aruna *et al.*, (2014) isolated *Bacillus tequilensis* strain SCSGAB0139 with optimum incubation period of 48 h for maximum protease production. Optimisation studies of *Bacillus coagulans* PSB-07 by Olajuyigbe and Ehiosun (2013), *Bacillus pumilus* D-6 by Bajaj and Jamwal (2013) and *Bacillus firmus* by Vadlamani and Parcha (2012) also reported a 48 h of optimum incubation period for maximum protease production.

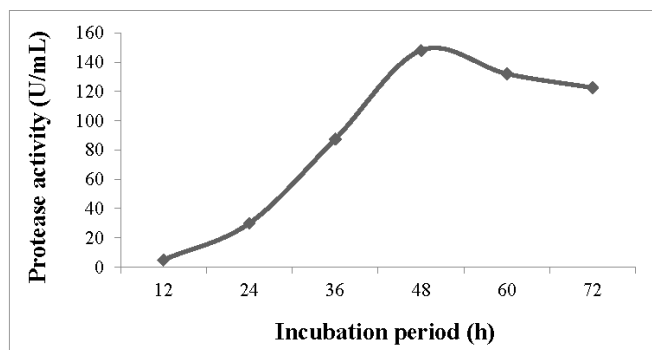


Fig. 6: Effect of incubation period on Protease production.

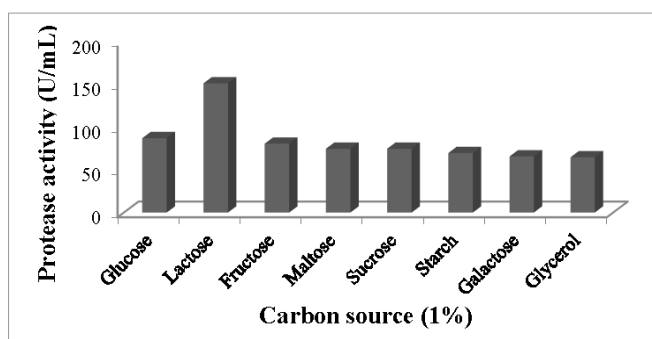


Fig. 7: Effect of carbon source on protease production.

The synthesis of protease enzyme in *Bacillus* species was controlled by mechanisms operative during the transition state between exponential and the stationary growth phases (Priest, 1977; Strauch and Hoch, 1993). It occurred while the culture was metabolically active (Kanchana and Padmavathy, 2010). However, Gupta *et al.*, (2002) reported the extracellular protease production as a manifestation of nutrient scarcity in the beginning of stationary phase. According to Ward (1995), more protease production by *Bacillus* sp. occurred during late exponential phase and was correlated with the high rate of protein turnover during endospores formation.

#### Effect of carbon source on protease production

Among the various carbon sources (1%) tested, lactose was found to be highly influencing the enzyme production with a protease activity of 151 U/mL whereas, all the other sources used resulted in only 50% enzyme yielding when compared to lactose (Figure 7). Concentration of lactose in the production medium was also optimised by conducting experiments with increasing the concentrations of lactose in the medium (Figure 8). The maximum protease activity was found to be 161 U/mL at 2% lactose and the minimum protease activity was 85 U/mL at 0.25% lactose. Above 2% lactose, protease production decreased slightly and at 4%, the activity was reduced to 87% of the maximum activity. Carbon source influenced the protease production considerably and different bacteria utilized different carbon source for their growth and metabolism. Tambekar and Tambekar, (2013) reported

a halophilic bacterium, *Bacillus odyseeyi*, which utilized lactose as a carbon source for maximum protease production compared to fructose, maltose or starch. A strain of *Vibrio GA CAS2*, as reported by Ananthan (2014) utilized lactose as the suitable carbon source for maximum protease production. Other important carbon sources for protease production as reported by previous studies included glucose (Badhe *et al.*, 2016; Dorcas and Pindi, 2016), maltose (Saraswathy *et al.*, 2013; Vanitha *et al.*, 2014) and galactose (Pant *et al.*, 2015).

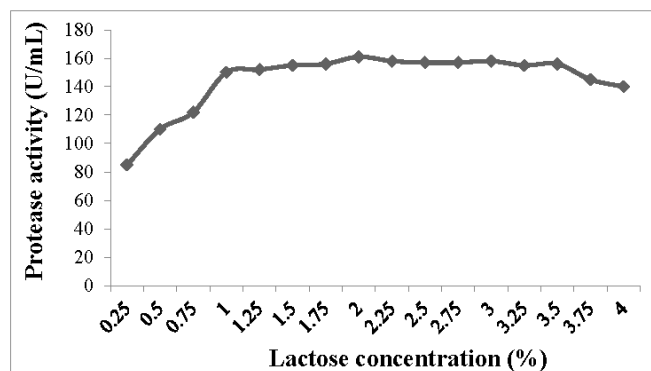


Fig. 8: Effect of lactose concentration on protease production.

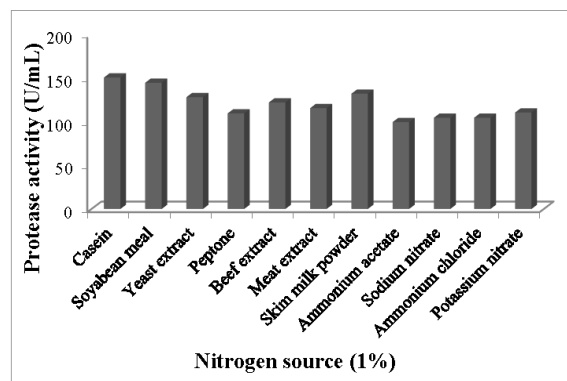


Fig. 9: Effect of nitrogen source on protease production.

#### Effect of nitrogen source on protease production

Casein was found to be the best nitrogen source by the present isolate to produce maximum protease enzyme compared to other organic and inorganic nitrogen sources (1%) tested. A protease activity of 151 U/mL was obtained when the media was supplied with casein as the nitrogen source (Figure 9). Concentration of casein in the production medium was also optimised by conducting experiments with increasing concentrations of the casein in the medium. The maximum protease activity was found to be 182.5 U/mL at 3.5% casein and the minimum protease activity was 128.5 U/mL at 0.25% casein (Figure 10).

Enzyme production was influenced by nitrogen source (Ward, 1995; Mehta *et al.*, 2006) and different bacterial strains utilize different substrates as nitrogen sources for effective protease production (Gupta and Khare, 2007). Casein was reported as a major nitrogen source for many microorganisms for maximum protease production. Jayasree *et*

*al.*, (2009) reported 1% casein as the main source of nitrogen for alkaline protease production by *Streptomyces pulveraceus*. *Streptomyces halstedii* Salh-12 and *Streptomyces endus* Salh-40, two most potent proteolytic halotolerant thermophilic and mesophilic organisms respectively utilized 1% casein as a major nitrogen source in protease production medium as reported by El Zawahry *et al.* (2007). Many other organic and inorganic nitrogen sources were also found to be enhancing the production of the protease enzyme. In the present study, when yeast extract was used as a nitrogen source, it showed 85% protease production compared to casein. In many studies, yeast extract results in higher protease production than casein (Vanitha *et al.*, 2014; Badhe *et al.*, 2016; Khusru, 2016).

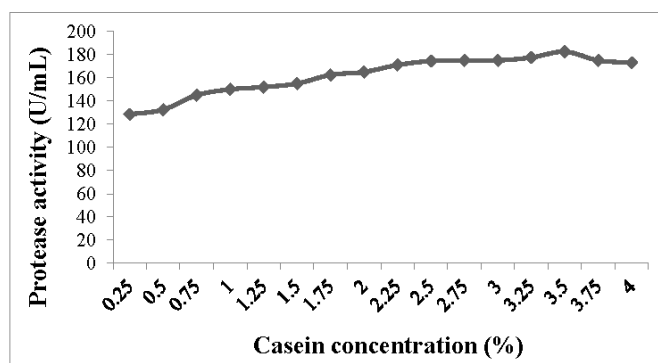


Fig. 10: Effect of casein concentration on protease production.

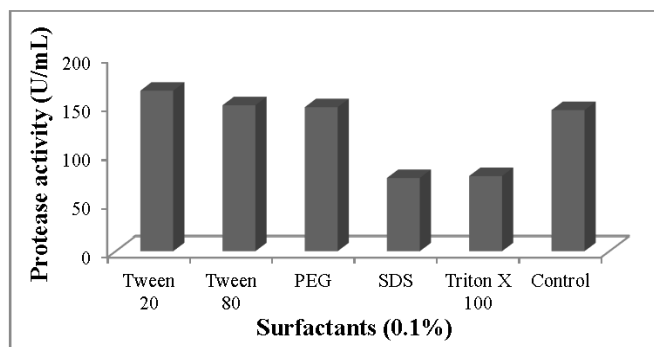


Fig. 11: Effect of surfactants on protease production.

With all the optimized cultural conditions like incubation period, temperature, pH and concentration of nitrogen and carbon sources, the protease enzyme produced by *Bacillus cereus* FT1 showed an enzyme activity of 187 U/mL.

#### Effect of surfactants on protease production

Protease production by *Bacillus cereus* FT 1 was higher with surfactants tween 20 (165 U/mL), tween 80 (150 U/mL) and poly ethylene glycol (148 U/mL) than the control (145 U/mL). Protease production was less in the presence of SDS (75 U/mL) and triton X 100 (77 U/mL) (Figure 11). Surfactants were reported to be encouraging the extra cellular enzyme production by bacteria including *Bacillus* sp. (Reddy *et al.*, 1999; Nascimento and Martins, 2006). Esakkiraj *et al.*, (2011) reported increased

protease production by *Serratia proteamaculans* in presence of triton X 100 and tween 80 and Ananthan, (2014), reported an increased protease production in the presence of tween 80 by a *Vibrio* sp. isolated from the ascidian, *Polyclinum glabrum*.

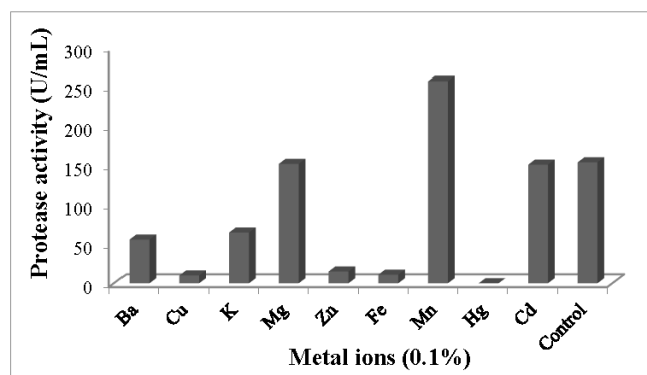


Fig. 12: Effect of metal ions on protease production.

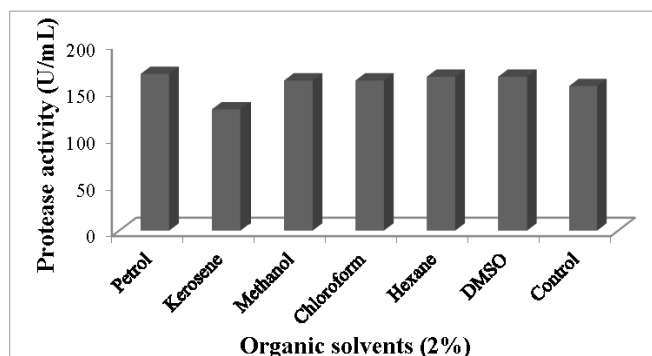


Fig. 13: Effect of organic solvents in protease production.

#### Effect of metal ions on protease production

The highest level of protease production was observed in the presence of  $Mn^{2+}$  for the isolate (258 U/mL) (Figure 12), which was far higher than the control value (155 U/mL).  $Mg^{2+}$  and  $Cd^{2+}$  did not affect the enzyme production much and the other metal ions tested resulted in a decrease in protease production with a complete absence of it in the presence of  $Hg^{2+}$ . The results suggested that the isolate required appropriate metal ion for protease production. Tambekar and Tambekar, (2013) worked on three *Bacillus* species, *Bacillus pseudofirmus*, *Cohnella thermotolerans* and *Bacillus odyssey* reported the effect of  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Mn^{2+}$  on improving the protease production by the isolates.

#### Effect of organic solvents on protease production

Six different organic solvents were screened for protease production in the present study. Among which, five of them expressed maximum protease production compared to the control. Petrol showed maximum protease production (168 U/mL) followed by hexane and DMSO (165 U/mL) and methanol and chloroform (161 U/mL) (Figure 13). Kerosene decreased protease production compared to the control. The ability of

microorganisms to tolerate organic solvents determines their protease production capacity (Ananthan, 2014). Many bacterial species have reported to be influenced by organic solvents in their extracellular enzyme production. *Bacillus sphaericus* DS11 showed enhanced production of protease enzyme with an activity of 1182.68 U/mL in the presence of optimised concentrations of glycerol (Liu *et al.*, 2010) and *Vibrio* sp. GA CAS2 showed a highest protease production (808.69 U/mL) in the presence of chloroform (Ananthan, 2014).

## CONCLUSION

Alkaline proteases have many applications in different industries and environmental bioremediations. Maximum enzyme production is aimed while selecting an organism for the enzyme production for commercial uses. The present study reported the increased alkaline protease production by the soil isolated *Bacillus cereus* FT 1 under optimised cultural conditions. The enzyme production by the isolate was even more enhanced in the presence of surfactants, metal ions and organic solvents. The results suggest the industrial usefulness of bacterial isolate and further studies are in progress in order to purify and characterize the enzyme for commercial applications.

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