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Isolation and Optimization of Protease Producing Bacteria from Marine Sediment

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ABSTRACT

In the present study investigation protease producing potent bacterial strain was isolated from the Marine sediment soil. Based on the morphology, biochemical and 16S rRNA sequencing, it was identified as *Oceanobacillus iheyensis.* The optimisation of protease production in submerged fermentation at various parameters like pH, temperature, carbon, nitrogen, metal ions, sodium chloride, surfactants, inoculums concentration, incubation time, static, shaking, aminoacids and buffers were tested. The isolated crude protease enzyme was partially purified by using Sephadex-A50 column chromatography.

Keywords: *Ocenobacillus iheyensis, Protease, 16S rRNA sequencing, optimization, Sephadex-A50 and surfactant*

INTRODUCTION

Enzymes are biological catalysts; they are highly specialized catalytic proteins with extra ordinary catalytic power and also have remarkable specificity. They are extracellular essential for all forms of life by catalyzing the chemical reactions in the cells (Anushu *et.al,* 2007). Proteases are necessary for life of living organisms being found in wide diversity, which performs both degradative and synthetic functions (Ferid abidi *et al.,* 2008). Proteases have been found in almost every micro-source such as bacteria, fungi and yeast (Phetcharat and Duangpaeng, 2012). Nowadays, with the increasing number of researches in bacteria isolated from fermented food, the proteolytic enzyme produced by these kinds of bacteria also comes to the researchers' sight. The proteolytic enzymes can be found almost from any sources such as bacteria and fungi, on the other hand, it was found in plant and animal (Costa *et al.*, 2010). The caseinase was studied in several different kinds of fungi (*Absidia cylindrospora, Aspergillus flavus, Aspergillus ochraceus, Aspergillus wentii, Penicillium canescens, Syncephalastrum* sp.*).* Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B.licheniformis; B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus and Bacillus subtilis* (Ellaiah *et al.,* 2002). Alkaliphiles are defined as organisms which exhibit optimum growth in an alkaline pH environment, particularly in excess of pH 8, and generally in the range between pH 9 and 10. Alkaliphiles may also be found in environments having a pH as high as 12. HPLC is a versatile, robust and widely used technique for the isolation of natural products. It is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture. (Martin and Guiochon, 2005). For several physiological processes the action of the proteolytic enzyme is essential, e.g. in digestion of food proteins, protein turnover, cell division, blood clotting cascade, signal transduction, processing of polypeptide hormones, apoptosis and also in the life cycle of disease - causing organisms including the replication of retrovirus (Delvin *et al.,* 2002). They are widely used as detergent, in food, pharmaceutical and leather tanning industries (Kalpana Devi *et al.,* 2008). Microbial proteases are increasingly used in treatment of various disorders namely cancer, inflammation, cardiovascular disorders, necrotic wounds etc. (Sabu

et al., 2003). In the present study protease producing marine bacteria was identified and enzyme was purified by using HPLC.

MATERIALS AND METHODS

Isolation and identification of proteolytic bacteria

The soil sample was collected from Rameswaram, Mandabam beach area, Ramanathapuram (Dist), Tamilnadu, India. The soil sample was serially diluted. 0.1ml of the diluted sample was plated on zobell marine agar plates. The plates were incubated at 37°C for 24 hours. Casein agar medium was sterilized at 121°C for 15 mins at 15lbs pressure. The isolates were streaked on the medium. The zone formed around the colonies due to the production of caseinase enzyme, which is used to hydrolyse the casein. Isolated bacterial colonies were identified by morphological, biochemical and molecular characters.

Enzymatic hydrolysis of casein

0.5ml of casein solution was incubated with 0.5ml of enzyme solution at 37^{0}C. After 10 mins, the reaction was terminated by addition of 1ml of 10% trichloro acetic acid. The mixture was centrifuged at 10,000 rpm for 5mins. Supernatant was collected 5ml of 0.44M Sodium carbonate and 1ml of two fold diluted Folin's reagent were added. After 30 mins incubation, blue colour developed was read at 660nm against a reagent blank prepared in the same manner without enzyme (Kunitz, 1947).

Media optimization for protease production

The crude protease enzyme obtained by cultivating the selected strains under the optimized conditions was investigated. The factors like pH, temperature, carbon, nitrogen, metal ions, Nacl, surfactants, inoculum size, incubation time, amino acids, buffers, RPM, static and shaking condition (EI-Enshasy, 2008).

PURIFICATION OF PROTEASE CARBINOL PRECIPITATION

In the present study, 48 hours fermented medium was extracted and centrifuged at 10,000rpm for 10 mins. Carbinol was added to the crude extract with constant stirring between 50% and 70% of saturation. The samples were stored at 4°C for overnight. The supernatant was removed and the precipitate was dissolved in buffer Glycine NaOH and pH 10 Maruthaiah *et al.,* (2014).

High Performance of Liquid Chromatography (HPLC) analysis

The crude extract of *Oceanobacillus iheyensis KB7* was analyzed for protein component (enzyme) study through HPLC. The instrumentation was performed using waters 600 pump and waters 2487 dual detector set to 270 nm. The separation was carried out with an isocratic elution program (60% CH_3CN,40% H_20), waters Nova-Pack C18 column (4 Km, 3.9 × 20mm) adapted to flow rate of 1ml/min. Acetonitrile (CH_3CN) was HPLC grade from Merck (Darmstadt, Germany); Distilled water (H_2O) was purified by a Milli-Q system (Millipore, m Bedford, MA,USA). The analyses were carried out at room temperature (25◦C) and with volume of 20µl and three injections. Peaks were identified by comparision of their retention times. The presence of enzyme components was analyzed through the instrument and they were plotted in the graphical format by appearing peaks.

RESULTS

Identification of protease producing bacteria

The selected strain was identified by various morphological, biochemical analysis and molecular Characterisation. The protease producing bacteria was identified as *Oceanobacillus* sp. by following the standard keys of Bergeys manual (Table 1). The phylogenetic analysis of the 16S rRNA sequencing of the bacteria *Oceanobacillus iheyensis* has 94% similarity with the nearest match in the genbank.

Table 1: Morphological and biochemical characteristics of *Oceanobacillus* sp.

Cultural Characteristics	Observation
Gram's staining	Gram positive, short rod.
Motility	Non-motile.
Biochemical characteristics	
Indole production test	-
Methyl red test	+
Voges-Proskauer test	+
Citrate utilization test	-
Oxidase	-
Urease test	+
TSI	-
Catalase	+
Carbohydrate fermentation tests	
Glucose	+
Arabinose	-

Mannitol	+
Maltose	-
Lactose	+
Galactose	+
Starch hydrolysis	+
Casein hydrolysis	+

Screening of protease producing bacteria

The isolated bacteria was screened for protease enzyme producing ability of casein agar which form a clear zone to hydrolysis of casein showed in fig. 1.

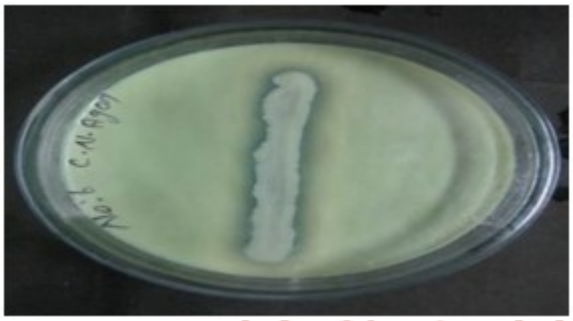

Fig. 1: Protease activity of *Oceanobacillus iheyensis* on casein agar plate

Optimization of cultural conditions for protease production

Effect of pH on protease production

Maximum protease production for *Oceanobacillus iheyensis KB7* was recorded at pH 8 (total activity 1.455 ± 0.003 IU/ml). Minimum protease production for *Oceanobacillus iheyensis KB7* was recorded at pH 2.0 (0.628 ±0.003 IU/ml) (Fig. 3)

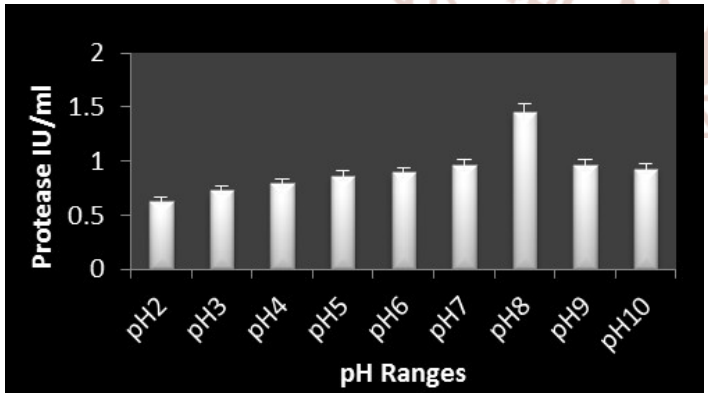

Fig. 3 Effect of pH on protease production

Effect of temperature on protease production

The protease production was assayed under various temperatures like 10, 20, 30, 40 and 50ºC at 72hrs incubation. Maximum protease production for *Oceanobacillus iheyensis KB7* was recorded at 40ºC (total activity 1.325 ± 0.003 IU/ml). Minimum protease production for *Oceanobacillus iheyensis KB7* was recorded at 10ºC (total activity 0.362 ± 0.114 IU/ml) (fig. 4).

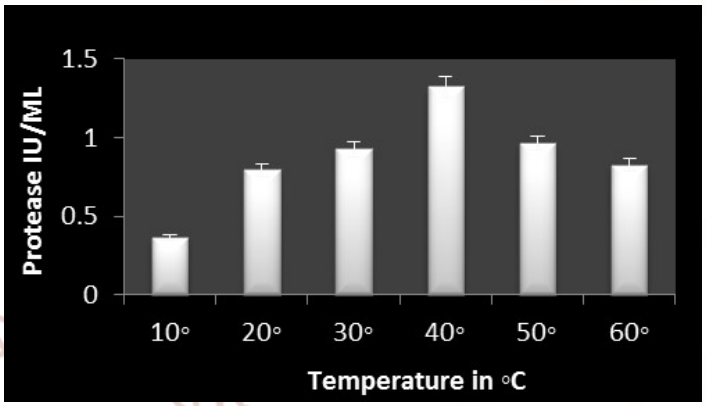

Fig. 4 Effect of temperature on protease production

Effect of carbon sources on protease production

Here the maximum protease production was recorded in lactose (total activity 1.163 ± 0.003 IU/ml).The minimum protease production was recorded in sucrose (total activity 0.596 ± 0.002 IU/ml) (fig. 5).

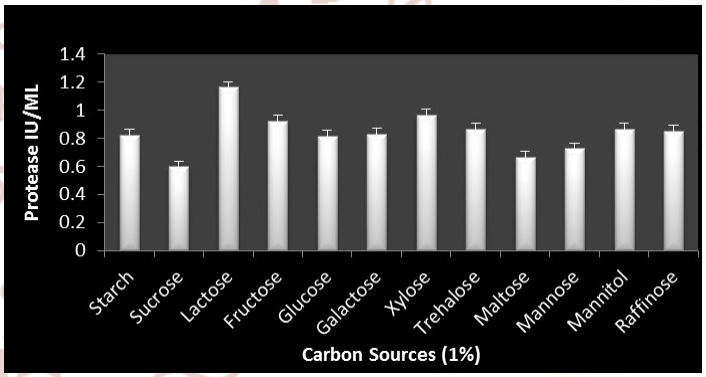

Fig. 5 Effect of carbon sources on protease production

Effect of nitrogen sources on protease production

After 72 hrs of incubation period at 40ºC showed maximum amount of enzyme production in peptone (total activity 0.895 ± 0.002 IU/ml) and the minimum amount of protease production in ammonium sulfate (total activity 0.366 ± 0.002 IU/ml) (fig. 6) was observed.

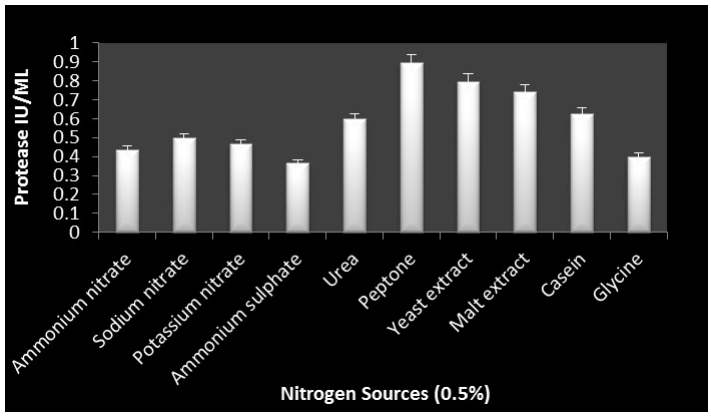

Fig. 6 Effect of nitrogen sources on protease production

Effect of metal ions on protease production

Maximum amount of enzyme production in calcium chloride (total activity 0.864 ± 0.003 U/ml) and minimum amount of protease production in mercuric oxide (total activity 0.524 ± 0.003 IU/ml) (fig. 7) was observed.

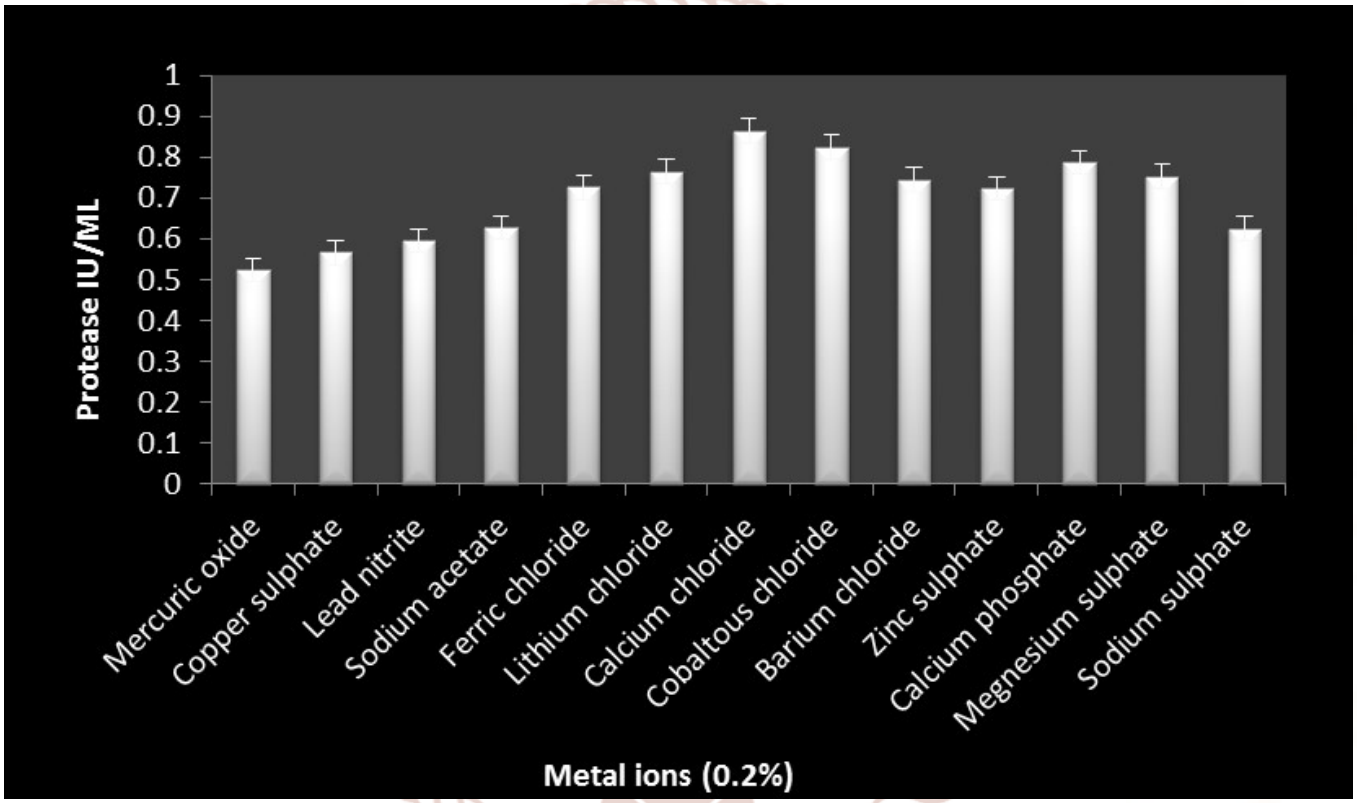

Fig. 7 Effect of metal ions on protease production

Effect of NaCl concentration on protease production

The effect of different NaCl concentration such as 1, 1.5, 2, 2.5 upto 5% for protease production after 72 hrs of incubation period at 40ºC showed maximum amount of enzyme production in 2.% NaCl (total activity 0.925 ± 0.002 IU/ml) and minimum amount of protease production in 5% NaCl (total activity 0.333 ± 0.003 IU/ml) (fig. 8).

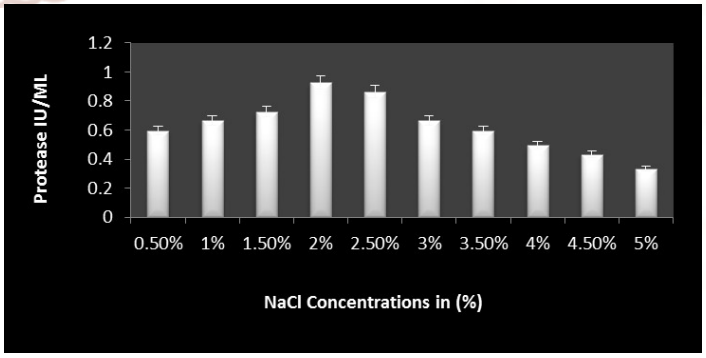

Fig.8 Effect of NaCl concentration on protease production

Effect of surfactants protease production

Maximum amount of enzyme production in Tween 20 (total activity 0.926 ± 0.002 IU/ml) and minimum amount of protease production in SDS (total activity 0.335 ± 0.004 IU/ml) was observed (fig. 9).

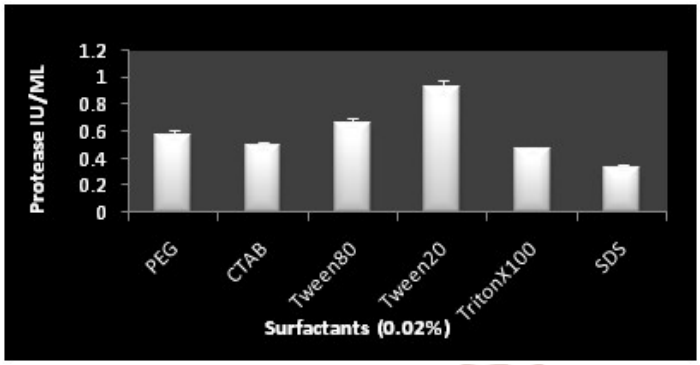

Fig. 9 Effect of surfactants protease production

Effect of inoculum concentration on protease production

Maximum amount of enzyme production in 4% inoculum (total activity 1.176 ± 0.002 IU/ml) and minimum amount of protease production in 0.5% inoculum (total activity 0.296 ± 0.002 IU/ml) was observed (fig. 10).

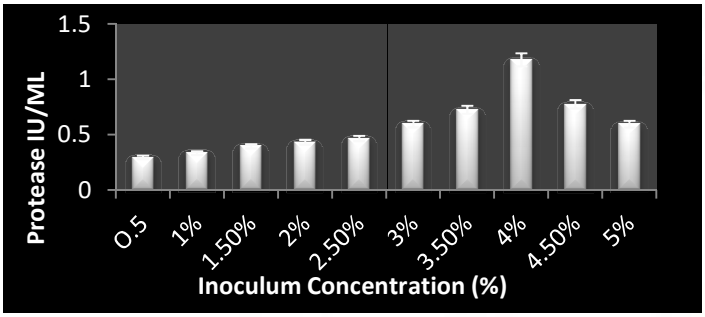

Fig. 10 Effect of inoculum concentration on protease production

Effect of incubation time on protease production

Maximum Protease production for *Oceanobacillus iheyensis KB7* was recorded at 72 hrs (total activity 1.226 ± 0.002 IU/ml). Minimum Protease production for *Oceanobacillus iheyensis KB7* was recorded at 24 hrs (0.334 ± 0.003 U/ml) (fig. 11).

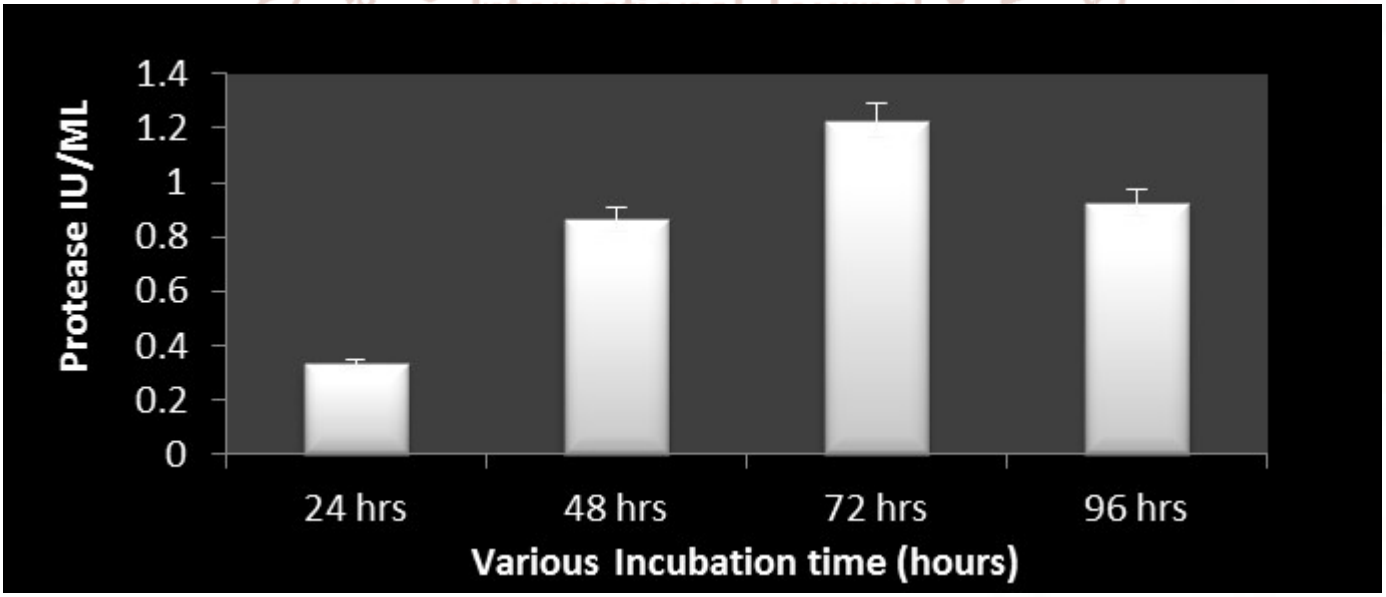

Fig. 11 Effect of incubation time on protease production

Effect of aminoacids on protease production

Maximum amount of enzyme production in cystein monohydrochloride (total activity 1.394 ± 0.003 IU/ml) and minimum amount of protease production in n-ornithine (total activity 0.334 ± 0.003 IU/ml) (fig. 13)

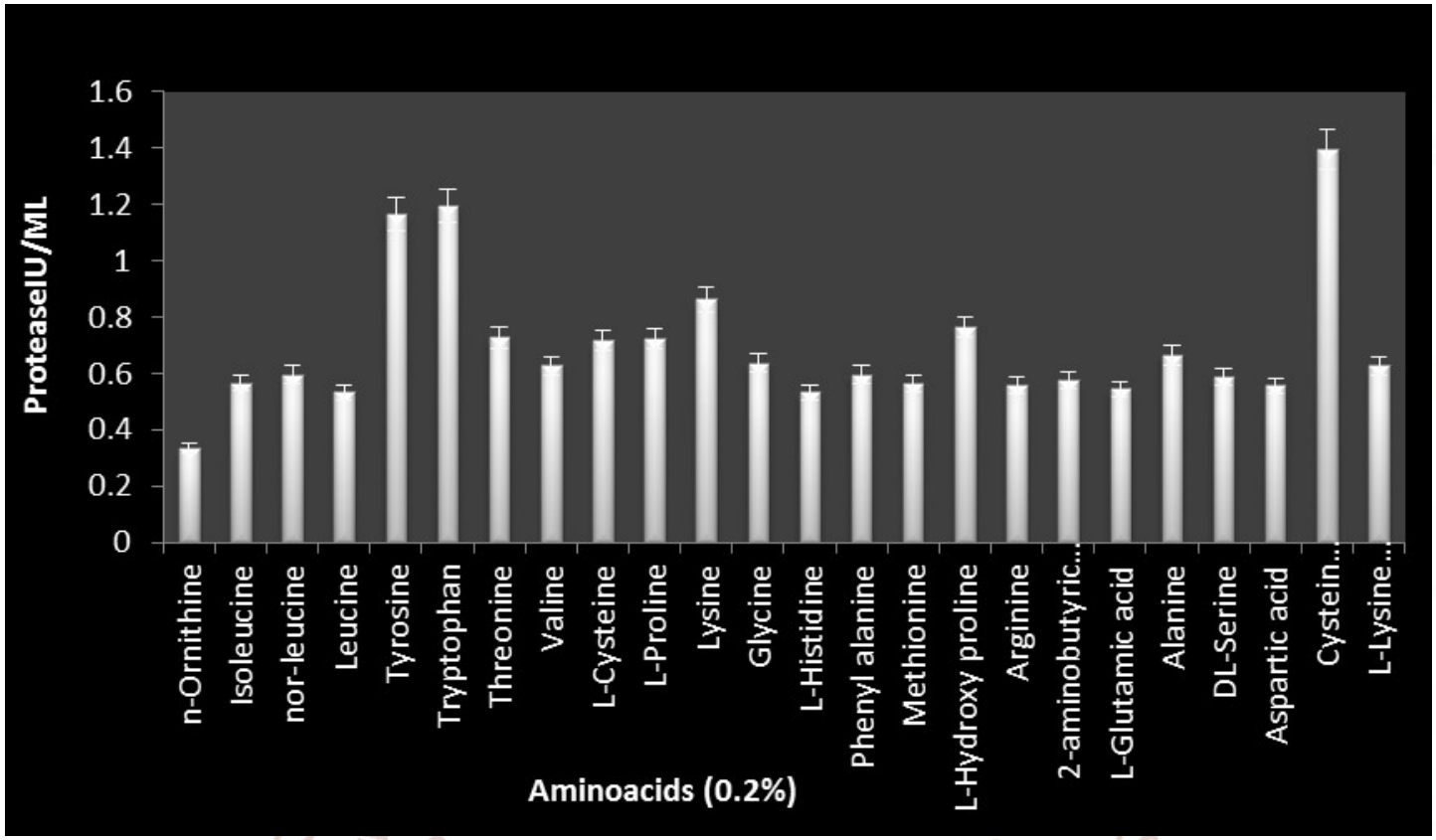

Fig. 13 Effect of aminoacids on protease production

Effect of buffers on protease production

Maximum amount of enzyme production in glycine NaOH (total activity 1.163 ± 0.003 IU/ml) and minimum amount of protease production in tris Hcl (total activity 0.826 ± 0.002 IU/ml) was observed in fig. 14.

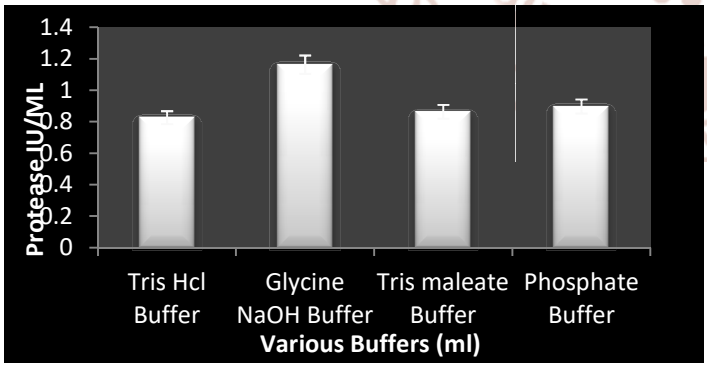

Fig. 14 Effect of buffers on protease production

Effect of RPM

The effect of various RPM condition was checked for protease production after incubation period at 40ºC for 72 hrs showed maximum amount of enzyme production in 100 RPM (total activity 1.099 ± 0.002 IU/ml) and minimum amount of protease production in 50 RPM(total activity 0.745 ± 0.002 IU/ml) (fig. 16)

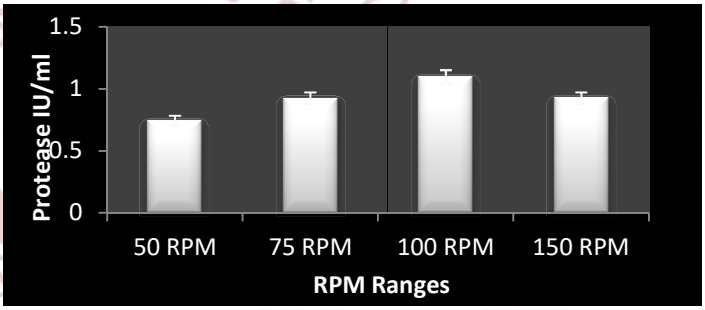

Fig. 16 Effect of RPM

Effect of static and shaking condition

The effect of static and shaking condition of for protease production after incubation period at 40ºC for 72 hrs showed maximum amount of enzyme production in shaking condition (total activity 1.427 ± 0.002 IU/ml) and minimum amount of protease production in static condition (total activity 0.435 ± 0.002 IU/ml (fig. 12).

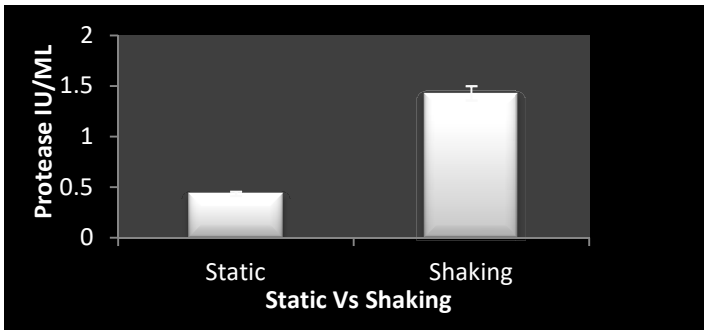

Fig. 12 Effect of static and shaking condition

HPLC analysis for purified enzyme

The purified enzyme of *Oceanobacillus iheyensis* were subjected to the analysis of HPLC chromatogram. The highest peak value of *Oceanobacillus iheyensis* in methanol extract i.e 2.143(mV) and lowest peak value was 2.707(mV).The results for HPLC of purified enzyme were shown in table:

Table: HPLC analysis of *Oceanobacillus iheyensis KB7* purified enzyme.

S.No	Retention time (min)	Area (%)	Height (%)	WO5(min)
1	2.143	33.1	70.6	0.11
2	2.707	19.0	16.2	0.49
3	2.870	47.8	13.2	1.03

FIG: HPLC chromatogram for *Oceanobacillus iheyensis KB7*

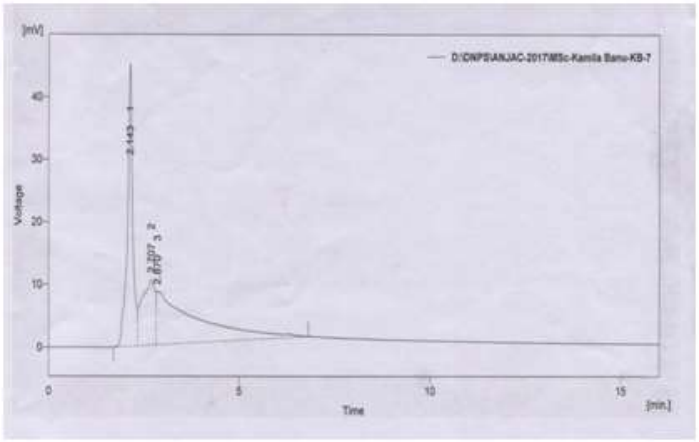

FIG: HPLC chromatogram for *Oceanobacillus iheyensis KB7*

DISCUSSION

Bacterial strains were isolated on alkaline agar medium from various soil fields and identified on the basis of morphological cultural and biochemical characteristics (Cheesbrough, 2000). In the present study, the proteolytic activity for *Oceanobacillus iheyensis* was detected in casein agar medium with the zone of clearance 38mm. Likewise *Bacillus subtilis* isolated were then characterized for protease production using casein agar. *Bacillus subtilis* was found to produce protease on basis of clear zone around the bacterial colonies on 1% casein agar plates at 37˚C after 24 hrs. The clear zones were due to hydrolysis of different substrates (Lin, 1996).

In the present study the pH of the medium seemed to be one of the important factors for the growth of the organisms. It was found that the maximum production was at pH 8.0 (1.455 ± 0.003 IU/ml) and the minimum activity was at pH 2 (0.628 ± 0.003 IU/ml). Maximum enzyme production was obtained at pH range from 8.0-9.0 whereas Kumar *et al.,* (2012) isolated two strains namely *Bacillus* species strain S_4 and *Pseudomonas* species strains S_{22} and reported that protease production was maximum at pH 7 and pH 9 for respectively.

Temperature has profound influence on production of protease by microorganisms. Yossan *et al.,* (2006) reported the optimum temperature of 50˚C for *Bacillus megaterium* protease which retained the activity at 30-40˚C with resulting relative activity of higher than 80%. In the present study effect of temperature on protease production was maximum (1.325 ± 0.003 IU/ml) at 40˚C and minimum (0.362 ± 0.0144 IU/ml) protease production recorded at 10˚C.

Carbon source is a primary energy source, which have important role in the improvement of growth of the organisms. In the present study, the experiments on the effect of various carbon sources on protease production showed that lactose maximize protease production was reported by several workers who used different sugars such as lactose, maltose, sucrose and fructose (Malathi and Chakraborthy, 1991). The results on the study of assimilation of carbon sources by *P. aeruginosa* showed that maximum growth was in sucrose for the parent strain whereas, for the mutant strain in presence of lactose and glucose (Mabrouck *et al.,* 1999). The maximum (1.163 ± 0.003 IU/ml) protease production was recorded in lactose supplemented medium. Minimum protease production was recorded in sucrose (0.596 ± 0.003IU/ml) added medium.

(Kumar and Tagaki, 1999) reported that effect of specific nitrogen supplement on protease production

differs from organism to organism. In present study the effect of various organic nitrogen sources was tested. The result showed that peptone gave their maximum influence on protease production. The maximum (0.895 ± 0.002 IU/ml) amount of enzyme production was observed in peptone supplemented medium.

Tolerance of enzyme upto 5M of NaCl over 24 hrs without losing original activity was reported by Jana *et al.,*(1997). Considering the importance of Sodium chloride, the present study was conducted and the result showed that the test organism can utilize the salt content within the range between 0.5-5 % for maximizing the protease production and it was remarkably high in 2% sodium chloride (0.925 ± 0.002) supplemented medium.

Joo and Chang, (2005) reported that the protease production from *Bacillus clausii* and *Bacillus spp*. which retained their activity with different surfactants such as Triton X 100, Tween-20 and SDS. The protease production was high (0.926 ± 0.002 IU/ml) at Tween-20 supplemented medium. Enzyme production was markedly reduced (0.335 ± 0.004 IU/ml) at SDS supplemented medium.

Metal ions are often required by bacterium for its growth and physiological activities. In the present study the maximum (0.864 ± 0.003 IU/ml) protease production was recorded in $CaCl_2$ supplemented medium. Minimum protease production was recorded in HgO_2 (0.524 ± 0.003 IU/ml) added medium. Najafi *et al.,*(2005) reported that protease production by *Aspergillus sp.* showed maximum activity with metal ions as Ca^{2+}, Mg^{2+} and inhibited by EDTA. Folasade *et al.,* (2004) reported that the supplementation of the culture medium with a solution of metal ions improved substantially the growth of *Bacillus sp* and also the enzyme production. Rahman *et al.,* (2006) reported protease production by *P. aeruginosa* was increased with metal ions such as Ca^{2+}, Mg^{2+} and K^{+}.

The incubation period of 30hrs gave the maximum production for the *Bacillus subtilis* PE-II cultures in protease production broth Johnvesly *et al.,* (2007). The protease production was high (1.226 ± 0.002 IU/ml) at 72 hrs of incubation. The nature of inoculums as well as its size may affect the microbial process to evaluate the effect of inoculums level on alkaline protease production. Protease enzyme production was high at 4% of inoculum size while using cotton seed as a fermenting medium. The maximum (1.176 ± 0.002 IU/ml) amount of enzyme production was observed in 4% inoculums added medium.

Buffers are often required by bacterium for its growth and physiological activities. In the present study the maximum (1.163 ± 0.003 IU/ml) protease production was recorded in Glycine - NaOH supplemented medium. Minimum protease production was recorded in Tris Hcl buffer (0.826 ± 0.002 IU/ml) added medium. Najafi *et al.,* (2005) reported that protease production by *Aspergillus sp.* showed maximum activity with buffers as sodium acetate buffer. Folasade *et al.,* (2004) reported that the supplementation of the culture medium with a solution of buffer improved substantially the growth of *Bacillus sp* and also the enzyme production. Rahman *et al.,* (2006) reported protease production by *P. aeruginosa* was increased with buffer in alkaline condition.

The RPM condition of 30 hrs gave the maximum production for the *Bacillus subtilis* PE-II cultures in protease production broth (Kumar and Tagaki, 1999). Similarly in the present study shaking condition of protease production was high (1.095 ± 0.003 IU/ml) at 100 rpm, 72 hrs of incubation. Enzyme production was markedly reduced in 50 rpm (0.744 ± 0.002 IU/ml) at 72 hrs of incubation. Atalo *et al.,* (1993) observed maximum production at 48 hrs.

The static and shaking condition of 30hrs gave the maximum production for the *Bacillus subtilis* PE-II cultures in protease production broth Johnvesly *et al.,* (2007). In shaking condition of protease production was high (1.427 ± 0.002 IU/ml) at 72 hrs of incubation. Enzyme production was markedly reduced in static condition (0.432 ± 0.003 IU/ml) at 72 hrs of incubation. Atalo *et al.,* (1993) observed maximum production at 48 hrs.

In the present study, first step of purification of the enzymes was carried out by precipitation of protein from the cell free dialysate with carbinol at the saturation level of 60%. This resulted in 3 folds of purification with yields of 91.45 and specific activity of (1.673 ± 0.002 IU/ml). However, at 60% saturation most of the protease enzymes were precipitate out. The salt content was removed by performing the ion-exchange chromatography.

The HPLC analysis of *Oceanobacillus iheyensis KB7* was result the peak formation they conclude the information about the amino acids and alkyl groups in

the extract. Similar studies have been done that the fractions purified by reversed phase HPLC and yielded six purified compounds, namely acacetin 8-*C*-(α-1- rhamnopyranosyl – (152) –β- d-glucopyranoside), 2"-*O*-α rhamnopyranosyl - vitexin, quercetin and kaempferol was observed. In this current study, 3 peaks were obtained from the HPLC analysis of the *Oceanobacillus iheyensis KB7* purified enzyme extracts.

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