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Isolation of Protease Producing Bacteria and Optimization of Protease Production and Enzyme Activity Parameters

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Abstract

Proteases are the class of enzymes which catalyse the hydrolysis of peptide bond from protein molecule. The present study deals with isolation of protease producing bacteria from different soil samples. Total 30 isolates obtained on skim milk agar plate by spread plate method from three different soil samples. Among them 18 isolates were screened out on the basis of size of zone of casein hydrolysis and then 4 isolates namely T4, T6, N2 and N4 were screened out by performing protease activity assay on the basis of their enzyme activity. Optimization of protease production was performed for these selected isolates where parameters like pH, temperature, inoculum size, incubation period and media ingredients were studied. Effect of temperature and pH on protease activity was studied. While optimizing the parameters for enzyme activity T4 produced 1.59 U/ml protease at pH 8 (at 50◦c), T6 produced 1.70 U/ml protease at 9 pH (at 50◦c), N_2 produced 1.57 U/ml protease at 7 pH (at 37◦c).N4 produced 1.71 U/ml at 8 pH (at 50◦c).While optimizing the parameters for protease production after 72 hours of incubation T4 produced 1.75 U/ml protease at 8 pH (at 37!), T6 produced 1.82 U/ml protease at 9 pH (at 50!), N2 produced 1.69 U/ml protease at 7 pH (at 37!) and N4 produced 1.72 U/ml at 8 pH (at 50!).

Keywords: Protease Activity, Soil, Skim milk agar medium, Tyrosine standard curve

Introduction

A protease enzyme hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming a protein molecule. Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. Proteases possess considerable industrial potential due to their biochemical diversity and wide applications in tannery, food industries, medicinal formulations, detergents, and processes like waste treatment, silver recovery, and resolution of amino acid mixtures. The largest application of proteases is in laundry detergents where they help

removing protein based stains from clothing. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved lusture and softness. Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners. The most extensively studied alkaline protease producers among bacteria are the genera of *Bacillus*, (Kalisz 1988; Gupta *et al* 2002; Kumar and Takagi 1999) and *Pseudomonas* (Bayoudh*et al* 2000; Ogino*et al* 1999), within actinomycetes is *Streptomyces* species (Petinate et al 1999) among Fungi the genera of *Conidiobolus*, (Bhosale et al 1995) *Rhizopus* (Banerjee and Bhattacharya, 1993) and *Aspergillus* (Chakrabarti et al 2000), and among Yeasts is the genus *Candida* (Poza *et al* 2001). But the survey of literature conclusively indicates that the *Bacillus* sp. is by far the most popular source of commercial alkaline proteases to date, with Novozymes, Denmark being the most established manufacturer worldwide. Alkaline proteases from both neutral and alkalophilic microorganisms are of commercial application (Horikoshi 1999a).The most extensively studied alkaline proteases, the subtilisins BPN' and Carlsberg are produced by neutral strains of *Bacillus Subtilis* (Horikoshi, 1999a).

Materials and Methods

Sample collection

Three soil samples were collected from: (1) Garden of Dept. Of Microbiology and Biotechnology(GU), (2) Garden of Dept. of Chemistry and(3) Poultry farm, Juhapura, Ahmedabad.

Isolation of protease producing bacteria

The techniques used for isolation of bacteria were serial dilution and spread plate method. 1 g of soil sample was weighed and serial dilution ($10^{-1}$to 10^{-3})of each soil sample was carried out. 0.1 ml aliquot of each dilution was spread on skim milk agar plate (96%v/v Nutrient agar + 4%v/v skim milk) and incubated at 37! for 24 hour. The zone of hydrolysis was noted for each sample.

Screening of isolates

Primary screening was done on the basis of diameter of zone of casein hydrolysis while secondary screening was carried out by protease activity assay. Protease assay was performed by modified method of Tsuchida*et al.*, 1986 in which casein was used as a substrate.

Production Optimization

Production was carried out on medium used by Singhal *et al* 2012 containing(g/L); casein 5.0, glucose 10.0, yeast extract 5.0, KH_2PO_4 1.0, Na_2CO_3 1.5.

Physiological conditions that were evaluated: (i) Incubation period viz. 24, 48, 72, and 96 hours (ii) Inoculum size of 1, 2, 3, 4 and 5% v/v. (iii) Different pH viz. 7, 8, 9 and 12. (iv) Incubation temperature viz. 15, 28, 37, 50 and 65

°C.

Optimization of enzyme activity

For maximum protease activity, assays were performed under various environmental conditions as follows: pH viz. 2, 5, 7, 8, 9 and 12, Temperature viz. 4, 15, 28, 37, 50, 80 and 100.

Protease assay

Tyrosine standard curve was plotted to measure the enzyme. Protease activity was assayed by a modified method of Tsuchida*et al.*, 1986 by using casein as substrate. 100 of enzyme solution was added to 900 of substrate solution (2 mg/ml casein in 10mMTris-Cl buffer, pH 8.0). The mixture was incubated at 50 for 10 min. Reaction was terminated by the addition of an equal volume of 10% chilled tri-chloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4, the acid soluble product in the supernatant was neutralized with 5ml of 0.5M solution. The color developed after adding 0.5 ml of 3 fold diluted Folin-Ciocalteau reagent was measured at 660 nm. All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1 of tyrosine per ml per minute under the above assay conditions.

Results and Discussion

Total 30 isolates were obtained on skim milk agar plate. Out of these 30 isolates, 18 isolates were screened out on the basis of size of zone of hydrolysis and among them 4 best isolates were selected for production optimization and optimization of enzyme activity by performing protease assay on the basis of their enzyme activity.

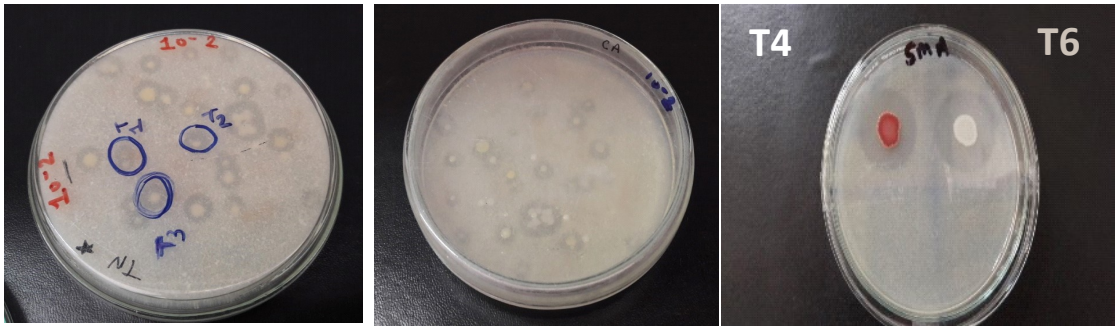


Fig. 3: Zone of casein hydrolysis of T4 and T6

Table:1 Colony characteristics and Gram's staining of the bacterial isolates which shows clear zone on Skim milk agar plates.

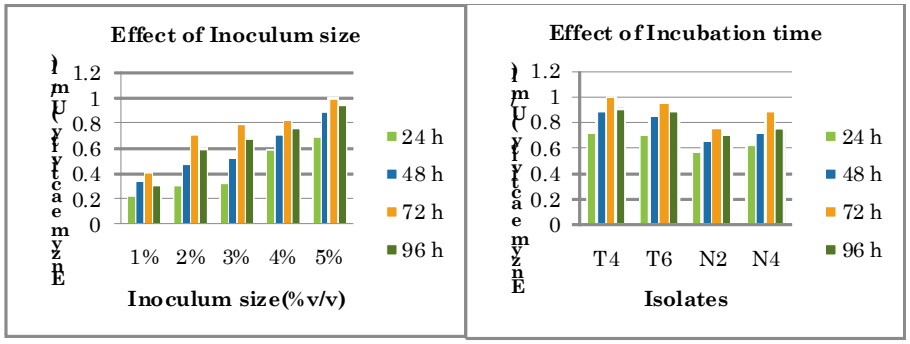
Isolates	Size	Shape	Elevation	Texture	Margin	Opacity	Pigmentation	Gram's Reaction
T4	Small	Round	Slightly Raised	Smooth	Uneven	Opaque	Reddish orange	Gram negative rods
T6	Large	Irregular	Flat	Rough	Uneven	Opaque	Nil	Gram positive Bacilli
N2	Small	Round	Flat	Smooth	Even	Opaque	Nil	Gram positive cocci
N4	Small	Round	Flat	Smooth	Uneven	Opaque	Nil	Gram positive Bacilli

Effect of incubation period on protease production

In present study protease assay was performed after 24, 48, 72 and 96 hours of incubation. Themaximum protease production observed in T4, T6, N2 and N4 was 0.95, 1.0, 0.76 and 0.89 U/ml protease respectively after 72 hours of incubation. It was reported that there was an equal protease production in *Bacillus* species and *Micrococcus luteus* at the 24 and 48 hours of incubation which was 1.21065 U/ml/min (Odu N.N., Akujobi C.O. *et al* 2012).

Effect of inoculum size on protease production

Different inoculum size (1, 2, 3, 4 and 5%v/v) were used for protease production and maximum protease production observed was 1.0 U/ml protease at 5% of inoculum size after 72 hrs of incubation.It is well documented that an inoculum size of 2% to 5% is optimum for protease production (Mabrouk et al, 1999; Kanekar et al, 2002). Moreover in the reports of Sinha and Satyanarayan (1991) range of 1% to 8% inoculums was the optimum**.** Itwas reported that maximumprotease production observed in *Micrococcus luteus* was 1.17282 U/ml/min at 1% while *Bacillus* species gave 1.16021 U/ml/min at 2% of inoculum concentration (Odu N.N., Akujobi C.O. *et al* 2012).

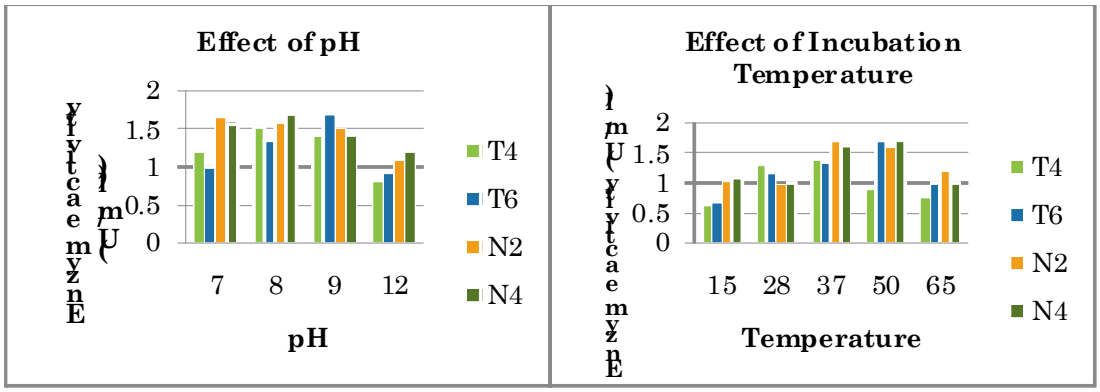


Effect of pH on protease production

To study effect of pH culture media pH was adjusted using 1N NaOH and different pH (7, 8, 9 and 12) were used in optimization. The optimum pH for protease production in cultures T4 and N4 was 8 (1.53U/ml and 1.68 U/ml respectively) while in culture T6 it was 9 (1.70 U/ml) and in culture N2 it was7 (1.65 U/ml).Maximum alkaline protease production has been reported in different pH ranges (i.e. 7-11) for different *Bacillus* species. It was reported that maximum protease production observed in *Micrococcus luteus* was 1.23586 U/ml/min at pH 7 while it was 1.19804 U/ml/min in *Bacillus* species at pH 9 (Odu N.N., Akujobi C.O. *et al* 2012).

Effect of temperature on protease production

The production flask was incubated at different temperature (15, 28, 37, 50 and 65°C) and protease assay was performed. The optimum temperature observed for protease production was 50°C for cultures T6 (1.69 U/ml) and N4 (1.70 U/ml) while it was 37 for cultures T4 (1.39 U/ml) and N2(1.68 U/ml).37 has been reported as optimum temperature for production in Bacillus subtilis (Chang *et al*., 1998; Hameed *et al*., 1996) while 55 temperature has been reported as optimum temperature in two *Bacillus* species, *Bacillus coagulans* (Gajju et al, 1996) and *Bacillus Stearothermophilus*(Dhandapani and Vijayaragavan, 1994). It was reported that maximum protease production was observed in *Bacillus* species which was 1.249 U/ml/min at 47 (Odu N.N., Akujobi C.O. *et al*(2012).

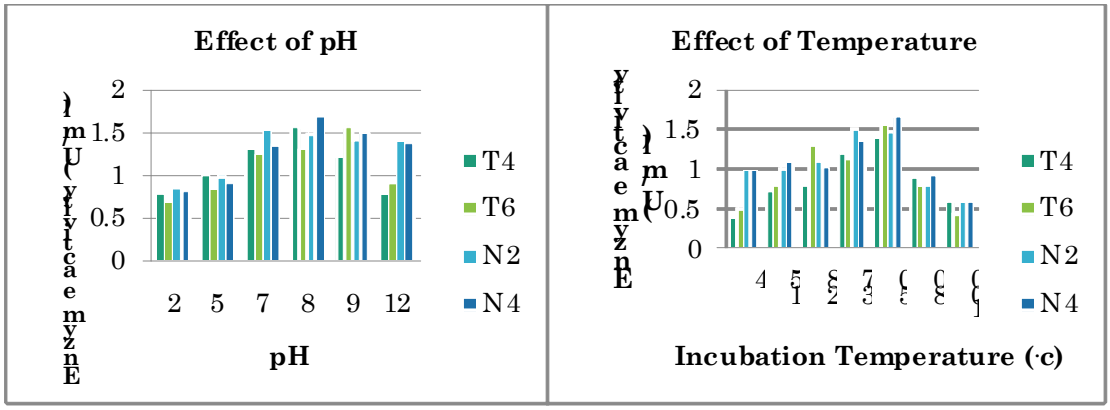


Effect of pH on protease activity

Different pH (2, 5, 7, 8, 9 and 12) were adjusted using 1N HCl and 1N NaOH. Protease assay was performed and maximum enzyme activity was observed in T4 (1.57 U/ml) and N4 (1.69 U/ml) at pH 8 while in T6 (1.58 U/ml) at pH 9 and in N2(1.30 U/ml) at pH 7.

Effect of temperature on protease activity

To study effect of temperature on protease activity different temperature were used (4, 15, 28, 37, 50, 80 and 100°C). The maximum enzyme activity was observed in T6 (1.56 U/ml) and T4(1.38 U/ml) at 50°C. While in N2 (1.50 U/ml) at 37



Conclusion

The maximum production of protease was observed in culture T6 which was 1.82 U/ml at pH 9 and temperature 50°C after 72 hours of incubation.

After optimization studies T6 produced 1.82 U/ml of proteasecompared to 0.95 U/ml which is 1.92 fold higher than normal production conditions.

After optimization studies T4 produced 1.75 U/ml protease compared to 1.0 U/ml which is 1.75 fold higher than normal production conditions.

After optimization studies N4 produced 1.72 U/ml of protease compared to 0.89 U/ml which is 1.9 fold higher than normal production conditions.

After optimization studies N2 produced 1.69 U/ml of protease compared to 0.9 U/ml which is 1.87 fold higher than normal production conditions.

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