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Assessment of process parameters for enhanced production of microbial alkaline protease

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Abstract

In the present study, alkaline protease producing bacterial strains were isolated from various soil samples. A total of 12 strains showed zone of clearance around colony on casein agar plate. Bacterial isolate 12 F shown maximum zone of clearance and also maximum alkaline protease activity under submerged fermentation. Culture conditions like pH, temperature, carbon sources and nitrogen sources were optimized. The Optimum conditions for protease production were found to be 30°C at pH 9 with Glucose as carbon source and Peptone as nitrogen source.

Keywords: Alkaline protease, Optimization, bacterial strain.

Introduction

Proteases (EC 3:4, 11-19, 20-24, 99) constitutes as a large and complex group of enzymes (Jisha, V. N., et al., 2013)**.** These are the group of enzyme which carry out proteolysis i.e. break down of proteins by hydrolysis of the peptide bond that exists between two amino acids of polypeptide chain (Singhal P., et al.**,** 2012). Proteases are differing in properties such as substrate specificity, active site, catalytic mechanism, pH and temperature optima (Sumantha, et al., 2005).
Proteases are widely distributed in each part of the biological source. They are ubiquitous, being found in the wide diversity of sources such as plants, animals and microorganisms (Rani K., et al., 2012). The inability of the plant and animal proteases to fulfill current world demands, microbial proteases has increased demand now a days (Rani K., et al., 2012). Alkaline proteases are the important industrial enzymes which are produced by the major bacterial genera including *Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Halomonas, Pseudomonas and Serratia* (Shafee, et al., 2005).

Alkaline proteases (EC.3.4.21-24, 99) are those proteases, which are active in a neutral to alkaline pH. They either have a serine centre (serine protease) or they are of metallo-type (metallo protease) (Gupta, et al., 2002b). Alkaline proteases are of considerable important by their activity and stability at alkaline pH (Singh, et al., 2001a). Commercially using alkaline proteases are produced by *Bacillus* species by their high pH and temperature stability (Priest, 1977; Joo et al., 2002; Gupta et al., 2002b). Alkaline proteases have commercial applications in detergent industry, food industry, leather industry, chemical industry, brewing industry, pharmaceutical industry, photographic industry, synthesis of peptide and degumming of silk. The present study, aimed was to isolate bacteria from different soil sample and to optimize the different parameters for maximum alkaline protease activity.

Materials and Methods

Materials

Nutrient agar (Hi-Media Laboratories, Mumbai), Casein (Sisco Research Laboratories, Mumbai), all carbon and nitrogen sources (High-Purity Laboratories, Mumbai), Follin phenol reagent (High-Purity Laboratories, Mumbai) and others chemicals (LobaChemie Pvt. Ltd., Mumbai) used were of analytical grade. All these reagents and media were prepared in distilled water.

Methods

Bacterial strains isolation and Screening

Various Bacterial strains were isolated from soil samples collected from the garden of KBS Nataraj College, Aarti Chemicals, Dairy Farm, Aarti garden in Vapi, Gujarat-396195, India. The soil samples were suspended in sterile distilled water and appropriate dilutions of soil solution were spreaded on the casein agar plate contained (gm/liter):10 casein; 10 peptone ; 5 glucose ; 5 KH_2PO_4; 20 NaCl ; 5 $MgSO_4.7H_2O$; 0.2 $FeSO_4.7H_2O$; 30 agar ,pH 9.0. The plates were incubated at 30°C for 24 hrs. After incubation period the casein agar plates were observed for colonies showing zone of clearance. The isolated culture was further screened for the protease production under submerged fermentation.

Enzyme production

Alkaline protease was produced in submerged fermentation, which was carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium for enzyme production.

Inoculum preparation

An isolated colony, from the preserved culture plate was transferred into 50 ml Erlenmeyer flask containing nutrient broth. The flask was incubated at 30°C for 24 hrs at 150 rpm. The freshly grown 24 hrs old culture with 1.0 O.D. at 600 nm is used as inoculum to inoculate in production medium.

Inoculation of production medium

The sterilized production medium containing (/liter): 10g casein; 10g peptone ; 5g glucose ; 5g KH_2PO_4; 20g NaCl ; 5g $MgSO_4.7H_2O$; 0.2g $FeSO_4.7H_2O$, pH 9.0 was inoculated with 1% of the inoculums of bacterial strain and incubated at 30ºC for 48 hrs under shaking condition at 150 rpm. The samples were collected at regular intervals and centrifuged at 5000 rpm for 20 min. The cell-free supernatant was used as the source of crude alkaline protease enzyme and the pellet was used to measure the cell biomass.

Protease assay

According to Udandi Boominadhan and Rajendran Rajakumar (2009), the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate – Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 30°C. The reaction was terminated by adding 3ml of 10% ice-cold trichloroacetic acid (TCA). The tubes were incubated for 1 hr at room temperature. Precipitate was centrifuged at 5000rpm for 15 min and after then supernatant was collected. 5ml of 0.4 M Sodium carbonate and 0.5 ml of Follin phenol reagent were added to 1ml of supernatant, vortexed immediately, then incubated for 30 min at room temperature and OD was taken at 660 nm. Concentration of tyrosine in the supernatant was read from a standard curve for tyrosine already prepared. One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as units ml^{-1} enzyme.

Cell biomass

The pellet was washed with equal volume of distilled water and two times with normal saline and then mixed in equal volume of normal saline and then the bacterial cell biomass was determined by measuring the absorbance at 600 nm (Henroette, et al., 1993).

Optimization of Alkaline protease production medium

In present study, 12F bacterial strain with high alkaline protease activities were optimized with respect to time course, carbon source, nitrogen source, pH and temperature.

Effect of Time course on alkaline protease production

Effect of time course was performed with 1% of the inoculums of bacterial strain. The cultures were incubated for different time period (0, 6, 12, 18, 24, 30, 36 and 48 hrs) at 30°C with shaking at 150rpm. At the end of incubation period, the samples were

collected and centrifuge at 5000rpm for 20min. After then, the cell free supernatant is used as enzyme source.

Effect of Carbon source on alkaline protease production

Effect of carbon source was studied by replacing glucose in the production medium with various simple and complex carbon sources including fructose, maltose, lactose, sucrose, galactose and mannitol. A control is represented by production medium with glucose as carbon source was performed at the same time. Alkaline protease activity was determined using the cell-free supernatant.

Effect of Nitrogen source on alkaline protease production

Effect of different nitrogen sources including yeast extract, peptone, beef extract, urea, ammonium sulphate, ammonium chloride, sodium nitrate was studied by replacing peptone in the production medium. A control is represented with peptone and casein use as nitrogen source was also performed. The enzyme activity was monitored using the cell-free supernatant.

Effect of pH on alkaline protease production

Effect of pH was studied by adjusting the pH of the production medium in the range of 5 to 12 using 1 N NaOH and 1N HCl after sterilization. Protease activity was determined in the cell- free supernatant.

Effect of Temperature on alkaline protease production

Effect of temperature was studied by incubating the production medium with 1% of the inoculum at temperatures in the range of 15°C to 60°C, pH 9 for 24 hrs under shaking conditions at 150 rpm. Protease activity was determined individually after 24 hours.

Results and Discussion

Isolation and Screening of protease producing microorganisms

In present study, a total of 12 morphologically different bacterial strains were isolated from different soil samples and were identified as alkaline protease producers, which shows zone of clearance around the colonies on casein agar plates. In quantitative screening under submerged fermentation conditions, bacterial strains 12F showed highest production (41.5 U/ml/min) compared to other strains (Table 1).

Table 1. Morphological and cultural characteristics of Different bacterial strains

Sr. no	Bacterial Strains	Colony characteristic	Gram's staining	Motility	Capsule staining	Endospore staining
1	7A	Large, round, irregular, mucoid, opaque	*Gram positive thin and thick rod*	Motile	Non-capsulated	Sporulated
2	11F	Medium, round, entire, smooth, translucent	*Gram positive thick rod*	Motile	Capsulated	Non-sporulated
3	12F	Medium, round, entire, smooth, moist, opaque	*Gram positive short thick rod*	Non-motile	Non-capsulated	Sporulated
4	10F	Small, round, entire, smooth, opaque	*Gram positive thick rod*	Motile	Capsulated	Non-sporulated
5	9F	Medium, circular, entire, smooth, moist, opaque	*Gram positive thin and thick rod*	Non-motile	Non-capsulated	Sporulated
6	2G	Large, round, irregular, smooth, opaque	*Gram positive slender rod with rounded ends*	Motile	Capsulated	Non-sporulated
7	1G	Small, round, entire, smooth, moist, opaque	*Gram positive slender rod*	Motile	Capsulated	Non-sporulated
8	5K	Large, irregular, smooth, butyrous, opaque	*Gram positive thin and thick rod*	Non-motile	Non-capsulated	Sporulated

9	7K	Medium, round, irregular , smooth, translucent	*Gram positive short thin rod*	Motile	Capsulated	Non-sporulated
10	8G	Large, round, entire, smooth, mucoid, opaque	*Gram positive slender rod with rounded ends*	Motile	Capsulated	Non-sporulated
11	1A	Medium, round, smooth, butyrous, opaque	*Gram positive thick sprout rod*	Non-motile	Non-capsulated	Sporulated
12	1K	Medium, irregular, smooth, butyrous, opaque	*Gram positive short rod*	Non-motile	Non-capsulated	Sporulated

Thus, 12F strain was selected for further study as it exhibited highest alkaline protease activity. Fig.1 shows zone of clearance around the colony of the bacterial strain 12F on casein agar plates. The bacterial isolate 12F was found to be Gram-positive, rod shaped bacteria, non-motile, non-capsulated, and sporulated bacteria .Alkaline protease production by *Bacillus* species has been previously reported by Aoyama et al. (2000); Feng et al. (2001); Adinarayana et al. (2003); Al-Shehri et al. (2004) and Schallmey et al. (2004).

Table 2 Screening and Protease activity of Different bacterial strains

Sr.no.	Bacterial Strains	Zone of clearance (mm)	Protease activity (U/ml/min)
1	7A	*17*	22.2
2	11F	9	8.4
3	12F	23	41.5
4	10F	15	21.4
5	9F	20	34.2
6	2G	6	4.8
7	1G	*13*	18.8
8	5K	19	23.4
9	7K	11	15
10	8G	3	3.6
11	1A	9	8.4
12	1K	12	16.2

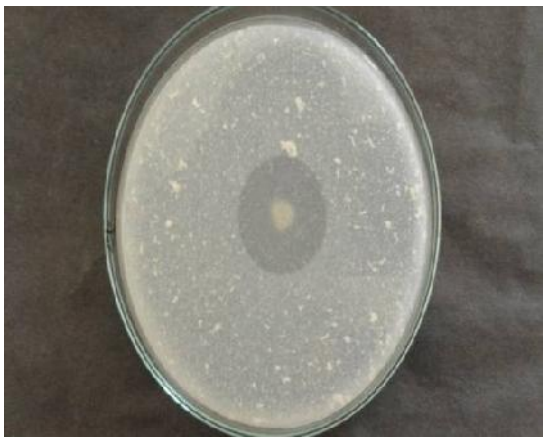

Fig.1 Zone of clearance around colony of 12F bacterial strain on casein agar plate

Optimization of Alkaline protease production medium

Effect of Time course on alkaline protease production

According to the results observed at different time intervals, it was noted that a high protease activity (43.9 U/ml/min) obtained at 24 hrs of incubation time. And also high biomass content (1.462) was recorded at 48 hrs of the incubation period as shown in Fig.2. 12F has ability to produce maximum alkaline protease in the period of 24–30 hrs. This finding is comparable with the findings of Suganthi C., et al., (2013) who reported high proteolytic activity in a halotolerant *Bacillus licheniformis* at an incubation time of 24 hrs.

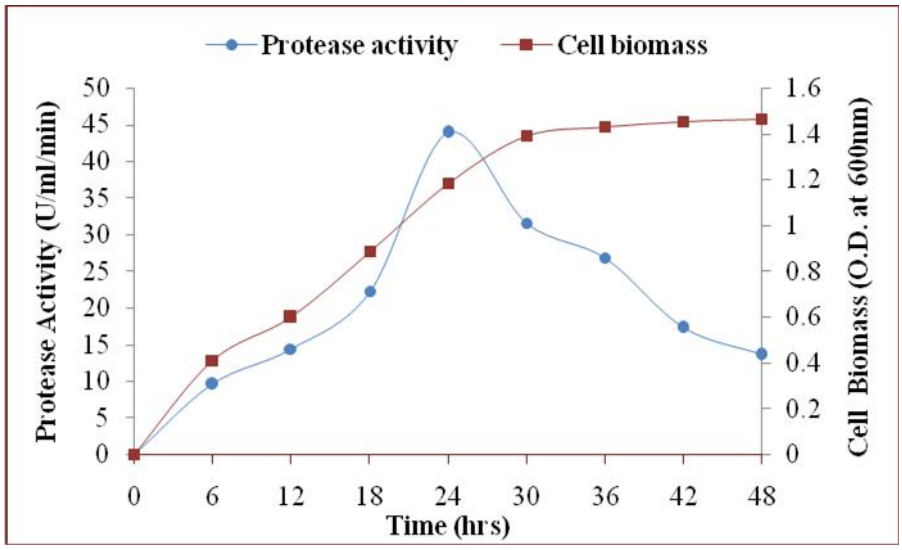

Fig. 2. Effect of Time period on alkaline protease production

Effect of Carbon source on alkaline protease production

Production of alkaline protease is highly dependent on the both carbon and nitrogen source available in the medium. The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Sudharshan et al., 2007). Maximum protease activity (49.8U/ml/min) was attained in the medium supplemented with glucose (5g/l) which was represented as positive control. Also, both fructose (35.2U/ml/min) and sucrose (30U/ml/min) has shown good protease activity near to positive control with compare to negative control (10.8U/ml/min). Less production of enzyme was recorded in the medium containing maltose (10.4U/ml/min) as shown in Fig. 3.

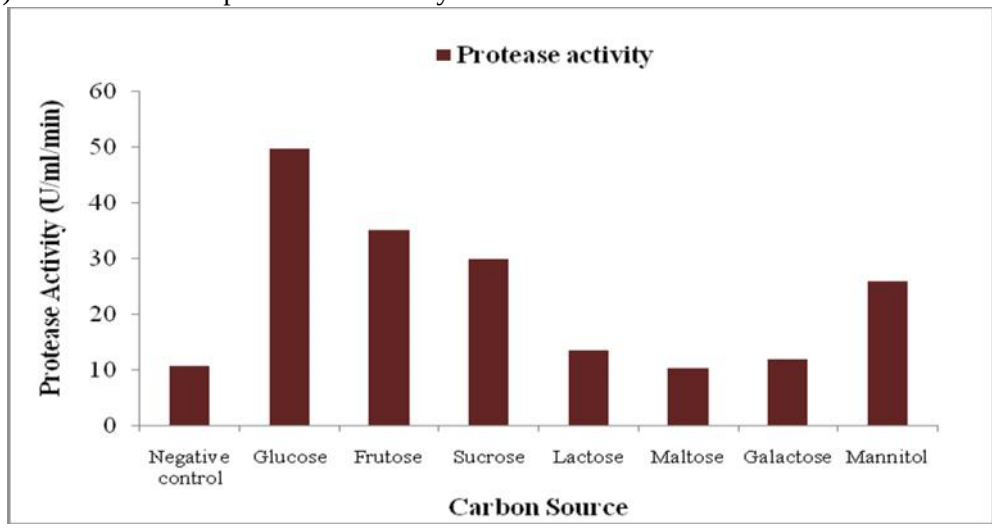

Fig. 3. Effect of Carbon source on alkaline protease production

This finding is comparable with reports from literature on preferred/optimized carbon sources for various *Bacillus* bacteria include glucose for *B. mojavensis* (Beg et al., 2002) and *B. subtilis* (Verma O. P., et al., 2011), *B. subtilis* NS (Nisha N. S., Divakarna J., 2014).

Effect of Nitrogen source on alkaline protease production

It is well documented in the literature that nitrogen is metabolized to produce primarily amino acid, nucleic acid, protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme. The alkaline protease production was better with organic nitrogen sources than with the inorganic nitrogen sources. Peptone (7.5g/l) which was represented as positive control stimulated the highest protease yield (76U/ml/min) followed by urea (71U/ml/min) as nitrogen source. Less production of enzyme was recorded in the medium containing beef extract (41U/ml/min) as shown in Fig.4. This finding were agreement with findings of Wang and Hsu (2005) who found out that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicolo*.

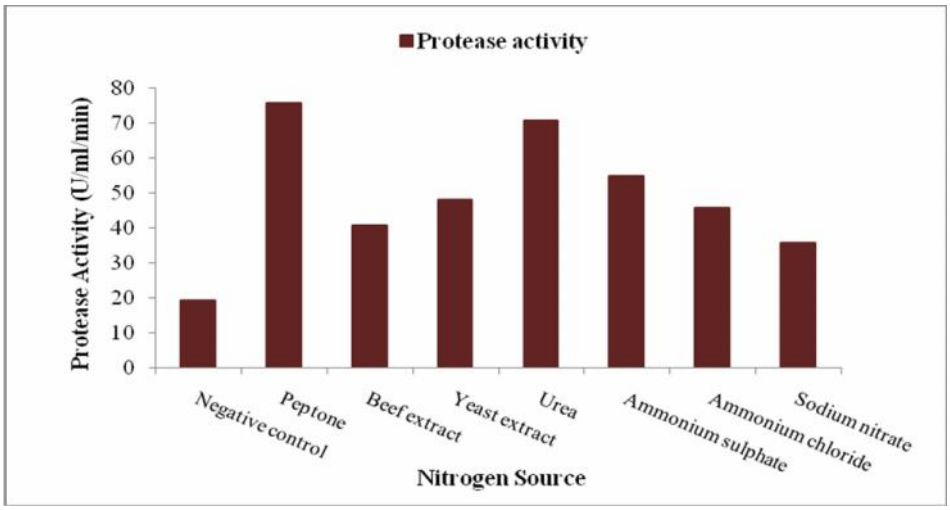

Fig. 4. Effect of Nitrogen source on alkaline protease production

Effect of pH on alkaline protease production

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum protease production was achieved at pH 9 (156.6U/ml/min) followed by pH 10(112U/ml/min). And less production of enzyme was recorded in the medium of pH 5 (27.4U/ml/min) as shown in Fig.5. The production of protease increased as pH of the medium was increased reaching maximum at pH 9. After pH 9, there was a decrease in enzyme production. Results suggest that there is a stimulation of enzyme production at alkaline pH. This could be indicative of the alkalophilic nature of the microorganism. This finding is comparable with the finding of Smita G. Sai, et al., (2012) for *Serratia liquifaciens*; Imran Shah, et al., (2014) for *Bacillus* sp.; Kalaiarasi and Sunitha (2009) for *Pseudomonas fluorescens*.

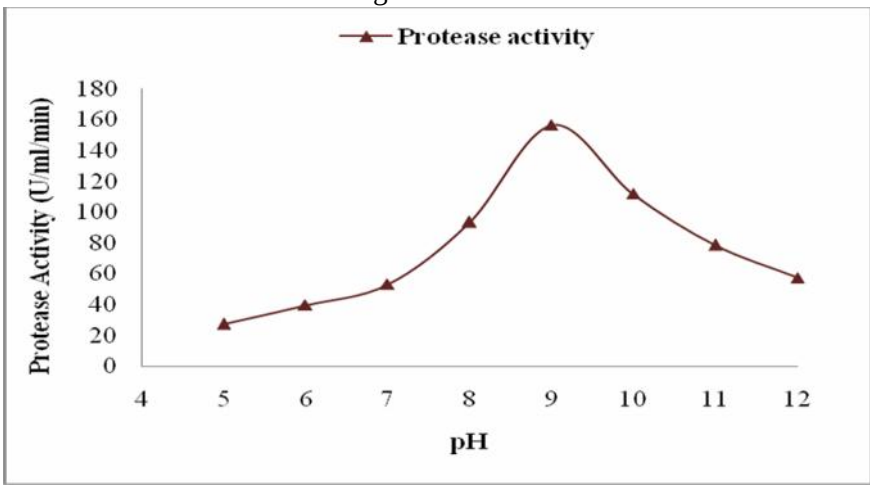

Fig. 5. Effect of pH on alkaline protease production

Effect of Temperature on alkaline protease production

The temperature found to influence extracellular enzyme secretion; possibly by changing the physical properties of the cell membrane (Rahman, et al., 2005). The optimum temperature for protease production was found to be 30°C (161.8U/ml/min) as shown in Fig.6. And also good protease production was found at 35°C (120U/ml/min). This finding is comparable with the finding of Singh J., et al, (2001a) for *B. sphaericus* who reported 30°C as optimum temperature for protease production.

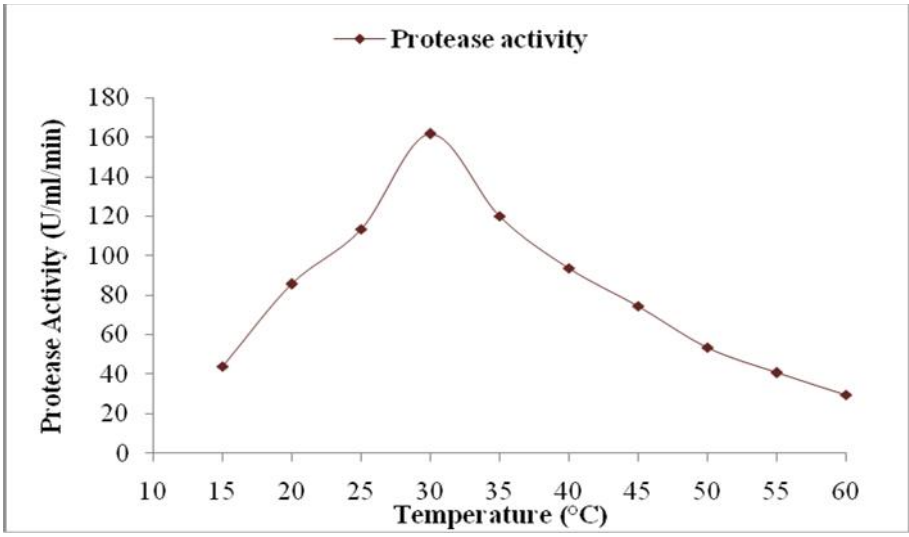

Fig. 6. Effect of Temperature on alkaline protease production

Summary and Conclusion

In this present study, 12 bacterial strains shows zone of clearance around colony on casein agar plate. Bacterial isolate 12F shows large zone of clearance. And 12F were characterized as Gram-positive, rod shaped bacteria, non-motile, non-capsulated and sporulated bacteria. Optimization of various process parameters shows that glucose, peptone, pH 9 and 30°C considered as optimum conditions for maximum alkaline protease activity by 12F strain. All the above results regarding the optimization of condition in fermentation process as well as activity of the proteolytic enzymes indicate the potential use of the 12F bacterial strain as biotechnological tools for various industrial activities.

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