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Detection of Protease Production by Plate Assay, Submerged Culture and Solid State Fermentation: Statistical Comparison and Significance

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Present investigation describes isolation, screening and primary level characterization of 24 protease producing bacterial strains, obtained from diverged geographical locations like alkaline soil, hot spring, soda lake and dairy, solvent and sugar industry effluent. The protease production efficiencies of isolated strains were detected by skim milk agar plate assay. Each isolated strain was analyzed for protease production by broth assay after growing the bacterial strain by submerged culture method. Majority of strains produces large zone on skim milk agar were not imitate the protease yield in submerged culture (Correlation coefficient – 0.049). The protease production abilities were also evaluated by cultivating each strain by solid state fermentation. We found that zone of clearance due to proteolysis can be correlated with protease production by solid state fermentation (Correlation coefficient – 0.9726).

Key words: Protease, Submerged culture, Solid sate fermentation, Correlation coefficient.

Proteases are the class of enzyme which occupies a central position due their commercial exploitation in detergent, pharmaceutical, brewing, leather, food, diagnostic and fine chemical industries (Rao *et al.,* 1998). Proteases are found in all living organisms and are physiologically important for cell growth and differentiation. Although, protease production is an essential ability of all microorganisms, but only those organisms that produce substantial amounts of extra-cellular enzymes are of industrial significance (Gupta *et al.,* 2002). Microorganisms represent an excellent source of enzymes due to their broad biochemical diversity, their susceptibility to genetic manipulation and robust nature. Microbial proteases account for approximately 40 % of the total worldwide enzyme sales (Godfrey and West, 1996). In view of these aspects, attention to isolation and characterization of proteases from diverged location is important.

Primary screening approach for protease producer is rapid plate assay by using solid agar medium by incorporating proteinacious substrate like casein, gelatin or skim milk (Vermelho *et al.,* 1996). The rapid and sensitive techniques for the detec-tion and characterization of

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microbial proteases are highly desirable (Lantz and Ciborowski, 1994). The conventional practice to detect the protease producer is to grow a large number of strains on agar plate and to relate the enzyme production capability to the radius of zone of clearance (Kumar and Takagi, 1999). The zone of clearance is common parameter to decide the enzyme production ability. Screened strains are utilized for further optimization studies. It is common practice to discard the narrow proteolysis zone producer strains during screen program. Alkaline proteases are generally produced by submerged fermentation - SmF (Kumar and Takagi, 1999). In addition, solid state fermentation (SSF) processes have been exploited to a lesser extent for production of these enzymes (Chakraborty and Srinivasan, 1993; Malathi and Chakraborty, 1991; George *et al.,* 1995).

Solid state fermentation is defined as fermentation involving solids in the absence or near absence of free water. The substrate must possess sufficient moisture to support growth and metabolism of microorganisms (Pandey, 1992). In industrial production, alkaline protease was largely produced by SmF processes (Kumar and Takagi, 1999). However, SSF of alkaline protease would be more eco-friendly and economical than SmF because of less water consumed and lower cost of substrates and equipment (Dayanandan *et al.,* 2003; Pandey *et al.,* 2000).

The present investigation describes a qualitative method for detection of extracellular proteases on agar plates, the protease secretion ability of various newly isolated strains in submerged culture and by solid state fermentations. The study also emphasize on correlation with zones of clearance on skim milk agar plate and protease production in liquid and solid media with statistical comparison and significance.

MATERIAL AND METHODS

Micro organisms

Various sites were explored for the isolation of efficient protease-producing bacteria. Soil and water sample from Soda Lake, Lonar (Dist. Buldana, Maharashtra); alkaline soil, Sugar factory, Diary Plant Shirpur (Dist. Dhule, Maharashtra); solvent polluted industry effluent, GIDC Ankaleshwar (Gujarth); Hot Spring Unapdev Dist. Dhule, Maharashtra), Shirpur. Total 24 proteolytic strains were isolated, specific code was assigned to each strain.

Screening of protease producing bacteria (Qualitative plate assay)

The isolates were obtained by suspending 20 g/20 ml sample in 100 ml sterile saline and transferring a 5 ml aliquot to 100 ml medium comprising of (g/l): glucose 10, peptone 5, yeast extract 5, KH_2PO_4 1, and $MgSO_4.7H_2O$ 0.05, adjusted to pH 9.0 with sterile 0.4 % (w/v) Na_2CO_3. Each culture broth was incubated at 30^0C, with constant shaking at 150 rpm for 72 h. Each culture broth was subjected thrice to batch culturing in order to obtain pure culture. The ability of various bacterial strains to grow at various pH values (pH 7, 8, 9, 10, 11, 12) and temperatures (25, 30, 37, 40, 50 and 55 ^{0}C) studied by growing the bacterial culture on glucose peptone medium. The pH of medium was adjusted by separately sterilized 1% sodium carbonate. The optimum pH and temperature was determined for each strain.

The protease secretion capabilities of each strain were detected on skimmed milk agar medium (SMA), comprising of (g/l): skimmed milk 28, enzymatic casein hydrolysate 5, yeast extract 2.5, dextrose 1, agar 15 and respective optimum pH and temperature for 48 h. The zone of proteolysis was calculated by taking ratio of diameter of clarified zone to colony diameter; for this, average readings of five well isolated colonies were considered.

Enzyme production by submerged culture method

Each bacterial strain was grown by submerged culture method at respective optimum temperature with shaking for 48 h in 100ml (g/l): casamino acids, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.05, pH adjusted with sterile Na_2CO_3, in a 500 ml capacity Erlenmeyer flask by inoculating 2 ml aliquot of a 24 h old seed culture (0.5 OD at A540). Cultures were incubated at respective temperature with rotary shaking at 150 rpm for 48 h. The level of protease production was assayed from cell free supernatant obtained after centrifugation. Results reported in this study are averages of triplicate findings.

Enzyme production by solid state fermentation

Ten grams of wheat bran and 0.1% casamino acid as an inducer were mixed

thoroughly with 8 ml water in 250 ml Erlenmeyer flask and sterilized by autoclaving (121°C for 20 min. The pH adjustment of solid medium was achieved by adjusting the pH of water by employing Na_2CO_3. After cooling the flasks to room temperature, the flasks were inoculated with 2 ml aliquot of a 24 h old seed culture (0.5 OD at A540) broth under sterile conditions. The contents of the flasks were well mixed and incubated at predetermined temperature for 48 h. Moisture content of the solid medium was maintained by increasing the quantity of moisturizing medium and through mixing. After 48 h the bran was extracted with 10 ml of suitable buffer thoroughly mixed the suspension intermittently for 1 h. The suspension was filtered under vacuum and the filtrate was used as a crude enzyme extract. Results reported in this study are averages of triplicate findings.

Protease activity assay

Alkaline protease activity was assayed using casein as a substrate (Nakanishi *et al.,* 1974). Aliquot of protease (1.0 ml) was added to 1.0 ml casein prepared in buffer and the reaction mixture was incubated for 10 min. The optimum temperature of bacterial strain was maintained during enzyme assay, while respective optimum pH value 2 mM carbonate buffers were used for substrate (Casein) preparation. The reaction was stopped by the addition of chilled 3.0 ml TCA mixture (0.11 M trichloracetic acid, 0.22 M sodium acetate and 0.33 M acetic acid). The inactivated reaction mixture was maintained at room temperature for 30 min, filtered through Whatman filter paper No. 1 and absorbance was measured at 275 nm with tyrosine as a standard. One unit of alkaline protease activity was defined as that amount of enzyme required to produce peptides equivalent to 1.0 µg of tyrosine in the filtrate per minute per ml at respective pH and temperature.

RESULTS AND DISCUSSION

Among the fifty isolates, 24 isolate showed good growth and protease production. Specific designation was given to each strain. To screen out efficient alkalophile, thermophile bacteria the optimum pH and optimum temperature were determined for each isolated strain. All the isolated strains were categorised as Alkalotoerant or alkalophile; based on their optimum pH requirement and thermophile or mesophile; based on their optimum temperature requirement. Table 1: summarizes strain designation, optimum pH and optimum temperature for respective strains and protease production (U/ml) by SmF and SSF. The correlation between the zone of clearance on agar plate and protease activity of liquid and solid culture was determined by calculating the Pearson correlation coefficient (Christian, 2005). The protease activity (U/ml) of submerged culture and zone of hydrolysis are not correlated; the

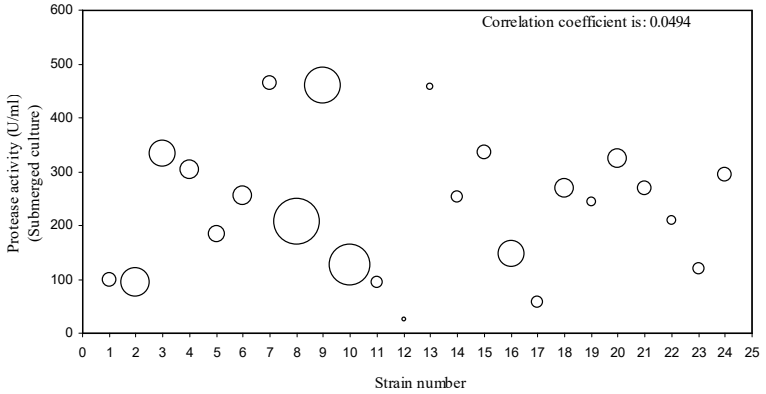

Fig. 1. Correlation between protease activity (U/ml) obtained by submerged culture and zone of clearance on skim milk agar plate.

Table 1. Details of newly isolated protease producing strains under investigation

Strain No.	Strain Designations	Optimum pH	Optimum Temperature (^{0}C)	Zones of hydrolysis (mm)[#]	SmF Enzyme activity(U/ml)	SSF Enzyme activity (U/ml)
1.	UA-1	10 (Alkalophile)	50 (Thermphile)	5.8 ± 0.5	99.0 ± 4.6	93.2 ± 6.6
2.	UA-2	9 (Alkalotolerant)	30 (Mesophile)	12. 1 ± 1.8	93.8 ± 6.4	219.1 ± 25.4
3.	UA-3	8 (Alkalotolerant)	37 (Mesophile)	11.1 ± 1.3	333.9 ± 9.1	161.6 ± 17.1
4.	UA-4	8 (Alkalotolerant)	30 (Mesophile)	8.1 ± 0.7	303.2 ± 4.1	147.4 ± 12.3
5.	UB-1	7 (Alkalotolerant)	27 (Mesophile)	7.6 ± 1.7	184.1 ± 28.8	121.7 ± 8.3
6.	UB-2	11 (Alkalophile)	55 (Thermphile)	7.7 ± 0.5	255.6 ± 15.3	133.9 ± 15.7
7.	UB-3	7 (Alkalotolerant)	25 (Mesophile)	6.1 ± 0.7	464.3 ± 5.7	127.3 ± 12.0
8.	UC-1	8 (Alkalotolerant)	30 (Mesophile)	18.2 ± 1.6	205.8 ± 5.7	305.6 ± 10.8
9.	UC-2	8 (Alkalotolerant)	30 (Mesophile)	14.2 ± 1.1	459.8 ± 12.2	248.6 ± 8.2
10.	UC-3	8 (Alkalotolerant)	30 (Mesophile)	16.1 ± 1.7	126.5 ± 6.1	280.7 ± 11.1
11.	UC-4	10 (Alkalophile)	55 (Thermphile)	5.9 ± 0.4	94.2 ± 11.6	100.6 ± 7.5
12.	UC-5	7 (Alkalotolerant)	30 (Mesophile)	2.6 ± 0.8	25.5 ± 5.7	58.2 ± 7.5
13.	UC-6	11 (Alkalophile)	55 (Thermphile)	4.0 ± 0.6	458.6 ± 14.5	55.2 ± 16.3
14.	UD-1	8 (Alkalotolerant)	30 (Mesophile)	4.8 ± 0.7	253.2 ± 11.4	126.9 ± 6.6
15.	UD-2	7 (Alkalotolerant)	30 (Mesophile)	6.5 ± 0.9	334.9 ± 29.4	138.6 ± 16.6
16.	UD-3	7 (Alkalotolerant)	30 (Mesophile)	10.4 ± 0.9	148.0 ± 6.6	189.6 ± 5.6
17.	UD-4	7 (Alkalotolerant)	30 (Mesophile)	5.0 ± 0.3	56.4 ± 4.4	93.4 ± 6.3
18.	UD-5	8 (Alkalotolerant)	40 (Mesophile)	8.2 ± 0.4	269.5 ± 10.0	132.7 ± 9.3
19.	UE-1	8 (Alkalotolerant)	30 (Mesophile)	4.0 ± 0.0	244.6 ± 28.7	94.2 ± 7.0
20.	UE-2	7 (Alkalotolerant)	30 (Mesophile)	7.2 ± 1.3	325.1 ± 12.5	138.2 ± 17.2
21.	UE-3	7 (Alkalotolerant)	30 (Mesophile)	6.0 ± 1.2	268.7 ± 3.6	101.2 ± 9.1
22.	UE-4	10 (Alkalophile)	50 (Thermphile)	4.5 ± 0.2	210.2 ± 10.0	101.2 ± 12.8
23.	UF-1	11 (Alkalophile)	37 (Mesophile)	5.9 ± 0.3	119.7 ± 8.8	119.5 ± 7.5
24.	UG-1	11 (Alkalophile)	37 (Mesophile)	6.0 ± 0.5	295.2± 17.5	129.7 ± 10.2

[#] Average of five readings of well isolated colonies (ratio of diameter of clarified zone: colony diameter)

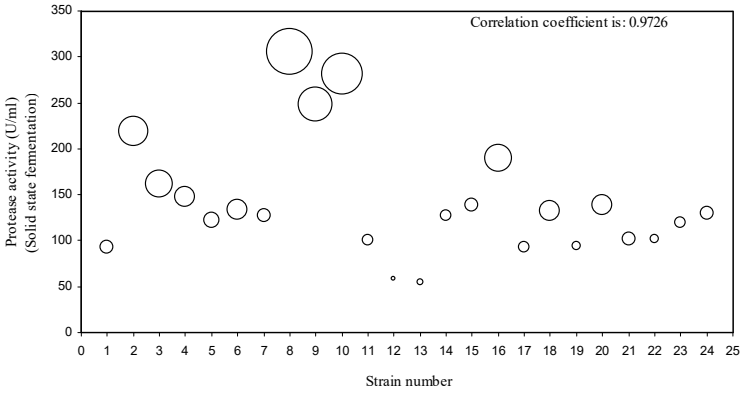

Fig. 2. Correlation between protease activity (U/ml) obtained by solid state fermentations and zone of clearance on skim milk agar plate.

correlation coefficient is: 0.0494 closer to zero indicates complete independence of these both variables (Fig: 1.); as majority of culture produces narrow zones despite being good protease activity in submerged culture. Similar result was reported, the *Bacillus licheniformis* produces narrow zones of hydrolysis on casein agar but produces good protease yield in submerged cultures (Mao *et al.,* 1992). The protease activity (U/ml) of crude enzyme extract obtained after solid state fermentation and zone of hydrolysis are correlated; the correlation coefficient is: 0.9726 (Fig: 2.).

Prakasham *et al.*, emphasized that alkaline protease production by isolated strain under solid-state fermentation was influenced particle size of the solid matrix was found to be important to achieve maximum enzyme production yields [Prakasham *et al.,* 2006]. The agar media provides solid matrix during plate assay might responsible to achieve reproducibility of assay results in protease production by SSF. This is first investigation which proofed statistically the relation between bacterial protease productions with plate assay results. .

CONCLUSION

Production of enzyme by solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc. (Pandey *et al.,* 2000). Although bacteria were always used to submerged fermentation and there were few reports of solid-state fermentation production of alkaline protease. The poor protease producers detected by plate assay might keep potential to give good yield of protease by solid state fermentation.

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