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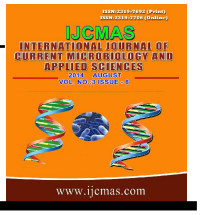

Original Research Article

Optimization Parameters for Alkaline protease Production using Bacterial isolates from different coastal regions of Tamil Nadu, India

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K e y w o r d s

Alkaline protease, Marine bacteria, Optimization, Enzyme activity, *Bacillus subtilis*

A B S T R A C T

Fifteen bacteria were isolates from the coastal region of Tamil Nadu, India and screened for their ability to produce protease. Out of fifteen isolates, five isolates showed maximum activity were taken for optimization parameters. The cultures were identified as *Pseudomonas sp* (MB7), *Bacillus subtilis* (MB10), *E. coli* (MB11), *Serratia sp* (MB13) and *Bacillus cereus* (MB15). The culture conditions such as pH, temperature, carbon source, nitrogen source and incubation period were analysed for the optimization. From the result, it was revealed that the pH 9 and temperature 50℃ optimum for protease production. Among the carbon sources, glucose showed maximum and for nitrogen source, yeast extract showed maximum. Among the isolates, the isolate *Bacillus subtilis* showed maximum protease activity of 497U/ml compared to other isolates.

Introduction

Protease enzyme catalyses the hydrolysis of proteins into peptides and amino acids and considered as one of the most useful enzyme groups (Gupta *et al*., 2002; Amit *et al*., 2011). Proteases account for approximately 60% of the total enzyme sales in the world. According to the market research report available on world enzymes published in 2007, the world market for enzymes is expected to grow 7.6% per year in 2011, (Shama and Hameed Abdul, 2011).

Proteolytic enzymes are ubiquitous in occurrence and found in all living organisms and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes mainly due to the recognition that these enzymes which not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al*., 2002). Proteases are produced commercially and used in detergent formulation, leather and dairy industries (Anwar and Saleemuddin, 1998; Dias *et al*., 2008). Several bacterial species, belonging to a variety of genera such as *Bacillus sp, Pseudomonas sp, Aeromonas sp, Staphylococcus* sp etc. are reported to produce alkaline protease having diverse industrial applications (Mukhtar and Haq, 2008; Saha *et al*., 2011; Habib *et al*.,

2012; Ahmad and Ansari, 2013). Among these *Bacillus subtilis* is the most important group of bacteria that are involved in the enzyme industries and also *Bacillus subtilis* produce a variety of extracellular and intracellular protease (Nisha and Divakaran 2014). The present study aims to isolate the protease producing microorganism from marine sample. The yield of extracellular enzyme is influenced by the physiochemical conditions.

Hence, physiochemical parameters are optimized for the maximum production of protease. This study presents effect of different cultural conditions on production of protease from Bacteria isolated from marine sample.

Materials and Methods

Screening and Isolation of Proteolytic bacteria

For isolation of marine bacteria from each water sample, 1.0 ml of the sample was mixed with 9.0 ml of distilled water and serial dilution was performed up to 10^{-5}. From this about 0.1ml of the aliquots was spread on the surface of the Marine agar (Hi-media 2216); Triplicates were maintained for each dilution. The inoculated plates were incubated at 37^{o}C for 7-10 days. Colonies developed after incubation were purified by repeated sub culturing on marine agar and finally, bacterial colonies with distinct characteristics such as pigmentation, size, opacity, elevation, margin and surface appearance (Yeon *et al.,* 2005) were chosen for further characterization.

Enzyme production medium

Production medium contained (g/l) glucose 0.5gm, peptone 0.15gm, $FeSO_4$ 0.1gm, KH_2PO_4 0.5gm, $MgSO_4$ 0.5gm. 10 ml of medium was taken in a 100 ml conical flask. The flasks were sterilized in autoclave at 121^{o} C for 15 min and after cooling the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37^{o}C in shaker incubator for 24 hr. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Protease assay

Protease was determined by the method of Folin Lowry method (Lowry *et al*., 1951). Protease activity is assayed by using 1 ml of 1% casein in 0.05 M Tris HCl buffer (pH 7.8) as substrate. Casein solution is incubated with 0.5 ml of enzyme at 50ºC for 30 min. After 30 min, the reaction is terminated by the addition of 2 ml of 10% TCA. Mixture is centrifuged and 1 ml of Supernatant was added to 5 ml alkaline reagent. This is preceded by the addition of 0.5 ml Folin Ciocalteau Reagent. After 25-30 min, the colour developed is read at 750 nm against a reagent blank prepared in the same manner. One unit of protease activity is defined as the amount of protease which liberates 1µg of tyrosine min-1 under experimental conditions

Process optimization for maximum protease production

Effect of pH on protease production

The optimum pH for protease production was determined by adjusting the production medium to different pH values, for which pre-autoclaved medium was prepared individually at pH 7, 8, 9, 10, 11 and 12 and inoculated with experimental bacterium at 37°C.

Effect of temperature on protease production

Production medium at pH9 was inoculated with overnight grown selected bacterial

strain. The broth was incubated at different temperatures from 30, 40, 50, 60, 70 and 80^{o}C for 24 hr. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source.

Effect of carbon sources on protease production

The effect of various carbon sources such as starch, glucose, maltose, lactose, xylose and fructose was examined in the production medium.

Effect of nitrogen sources on protease production

The different nitrogen sources like as yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulphate were examined for their effect on protease production.

Effect of incubation Period- Production medium at pH 7 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different time period from 24-72h. At the end of incubation period the cell free culture filtrate is obtained and used as enzyme source.

Results and Discussion

Protease producing bacteria were isolated from marine sample. Based on the morphological and biochemical characteristics the isolates were identified as *Pseudomonas sp, Bacillus subtilis, E.coli, Serratia sp* and *Bacillus cereus*

Effect of pH: All the five isolates were allowed to grow in media at different pH ranging from 7.0 to 12.0. Maximum enzyme activity was observed in medium of pH 9 for all thee cultures. Among the isolates, *Bacillus subtilis* showed maximum alkaline protease activity of 473.56U/ml followed by the isolates *Serratia sp* and *E.coli*. The pH 7 and 12 recorded minimum activity (Table. 1). However, all the organisms showed activity in all the pH level tested. However, majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline condition (Mukesh kumar *et al*., 2012).

Effect of temperature: Enzyme activity recorded at different temperatures revealed that the all the five isolates yielded maximum protease production at 50^{o}C (Table. 2). The temperature was found to influence extracellular enzyme secretion possibly by changing the physical properties of the cell membrane (Rahman, R N *et al*., 2005). El-Safey *et al*. (2004) reported the same findings in production, purification and characterization of protease enzyme from *Bacillus sp.* Related studies also reported that protease production by *Bacillus laterosporous* was best at 37^{o}C (Usharani B and M Muthuraj 2010).

Effect of Carbon source: Various sources of Carbon such as Glucose, Fructose, Lactose, Xylose and Sucrose were used to replace Glucose which was the original carbon source in growth media. Results obtained were showed that, Glucose brought the highest protease production compared to other carbon sources at 24 hr incubation in *Bacillus subtilis*. Fructose and sucrose also showed high protease production at 24 hr of incubation (Table 3). For commercial production, sugars like fructose, lactose, mannitol, sucrose will be prohibitive due to their cost. Similar findings were observed by Jedeja and Bhatiya (2010) in optimization of environmental and nutritional factors for alkaline protease production.

Effect of Nitrogen Source: The nitrogen sources like beef extract, yeast extract, ammonium sulphate, ammonium chloride, urea and peptone were used.

Table.1 Effect of pH on the proteolytic activity

	Enzyme activity (U/ml)				
P^H	*Pseudomonas sp*	*Bacillus subtilis*	*E.coli*	*Serratiasp*	*Bacillus cereus*
pH7	155.39	158.45	150.09	153.21	148.35
pH8	241.45	265.15	244.13	238.91	235.15
pH9	400.53	423.56	409.07	410.12	401.56
pH10	363.72	381.61	369.34	361.56	340.61
pH11	250.79	262.12	247.23	246.11	222.12
pH12	128.09	132.12	123.82	121.31	114.33

Table.2 Effect of temperature on the proteolytic activity

	Enzyme activity (U/ml)				
Temperature	*Pseudomonas sp*	*Bacillus subtilis*	*E.coli*	*Serratiasp*	*Bacillus sp*
30°C	150.66	188.01	157.07	157.09	156.45
40°C	205.73	251.12	220.12	199.13	225.15
50°C	400.54	471.34	445.15	449.37	438.56
60°C	261.74	353.11	337.92	339.34	351.61
70°C	243.65	267.67	222.56	247.32	242.12
80°C	123.76	112.21	91.09	93.89	92.42

Table.3 Effect of different carbon source on the proteolytic activity .

	Enzyme activity (U/ml)				
Carbon source	*Pseudomonas sp*	*Bacillus subtilis*	*E.coli*	*Serratiasp*	*Bacillus sp*
Lactose	87.17	100.43	96.90	94.54	97.35
Fructose	223.51	230.23	221.31	209.71	201.60
Glucose	448.47	494.78	446.70	433.21	434.31
Sucrose	268.74	298.45	267.12	286.90	262.53
Xylose	85.12	93.52	88.33	84.67	72.58

Table.4 Effect of Nitrogen Source on the proteolytic activity

Nitrogen source	*Enzyme activity*(U/ml)				
	Pseudomonas sp	*Bacillus subtilis*	*E.coli*	*Serratiasp*	*Bacillus sp*
Peptone	128.89	235.90	150.65	166.75	151.09
Yeast extract	412.32	498.12	434.00	444.16	425.78
Ammonium chloride	139.34	189.34	162.87	160.84	167.16
Ammonium sulphate	111.19	127.15	118.23	102.51	102.02
Sodium nitrate	88.13	109.31	96.01	80.45	78.12
Urea	73.78	92.24	59.13	79.09	50.12
Beef extract	418.42	481.72	439.07	444.09	454.5

The result revealed that the best nitrogen sources were beef extract and yeast extract for *Bacillus subtilis* (Table. 4) respectively which showed highest level of protease activity compared to other sources of organic nitrogen. Among the inorganic nitrogen studied ammonium chloride was found to be the best for the *Bacillus subtilis* isolates. The result of our present study are in the line with the findings of shafee *et al*., 2005, who reported that beef extract among the different organic nitrogen sources ammonium chloride among the inorganic nitrogen sources lead to a high proteolytic activity by *Bacillus sp.,* at 48hrs of incubation period.

Effect of Incubation Period: Enzyme synthesis is related to cell growth and therefore there is a co-relation between incubation period and enzyme production (Kaur, M *et al*., 1998). The results revealed that the protease production proceeded at a slower rate after which it increased sharply reaching a maximum value at 48 hours for all the isolates (Table.5). Under most growth conditions, *Bacillus* species produce extracellular protease during the post exponential growth phase (Dawson, P.S.S. *et al*., 1969). Further incubation resulted in a sharp decline in the enzyme production. This decline might be due to cessation of enzyme synthesis together with auto proteolysis. Similar findings were also reported by some workers (Olajuiyigbe, F.M *et al*., 2005) in which maximum enzyme production were observed at 48 hours of growth.

The present study concluded that the bacterial isolate produce protease at alkaline culture conditions and different factors greatly regulates the growth and production of proteases. The results in this study on different factors will be useful during further production of protease by these microorganisms. Of all the isolates evaluated, the highest protease activity was obtained from *Bacillus subtilis*. Proteases produced by *Bacillus* species are by far the most important group of enzymes being industrially exploited. The results presented here are in agreement with the literature, as several *Bacillus subtilis* are known to be good alkaline protease producers and have been widely used in the detergent industry.

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