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CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM VIBRIO METSCHNIKOVII STRAIN XMB 057

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Abstract

From various natural sources on skim milk agar plates 51protease producer bacteria wereisolated by primary screening. Secondary screening was done by zone of clearanceformed by cell free broth on gelatin agar plates. From diameter of zone of clearance potent protease best protease producers Vibrio metschnikovii strain Xmb 057 was isolated. It was biochemically characterized by performing some common biochemical tests. Effect of temperature and pH on protease by Vibrio metschnikovii strain Xmb 057 was studied. Optimum temperature and pH was 50^0C and 9 respectively make it suitable to be used in leather and detergent industry. Protease was tolerant to Ca^{++} and Mg++ which suggest its activity in detergents used even in hard water.

Keywords: *Proteases, Vibrio metschnikovii, alkaline protease.*

Introduction:

All the metabolic reactions in the cell are catalyzed by enzymes. Enzymes speed up the rate of biochemical reactions without changing the rate of other biochemical reactions(Neitzel,2010).Proteases are the group of enzymes including Chymotrypsin, trypsin, thrombin, cysteine proteases like papain, metal peptidases like aminopeptidase and elastase. All are protein in nature still capable of degrading proteins, peptides and amides(Bender, 1965). Depending on chemical composition protease are categorized as serine proteases and metalloproteases. Serine proteases are having serine amino acid in the active site attached to aspartic acid and histidine. Due to their stability at broad range of pH, temperature, toxic metals and resistance to denaturation by detergent they are widely used in detergent industry (Sundus,2016).Proteases are among the top three enzymes having wide applications in

industries like food, pharmaceuticals, leather, etc. These enzymes play important role in digestion of protein containing food(Naidu,2011).Keratin rich waste of poultry and leather industries are also treated by alkaline protease as an ecofriendly method rather than hydrolysis by chemical and mechanical methods(Kudrya,1994).

Proteases can be obtained from various sources like plant, animals and microorganisms. Production of plant proteases require land for growing plants and it is time consuming. While production of proteases depends on available livestockand Government legislations. Microorganisms can synthesize broad variety of proteases and capable for genetic modifications therefore as compared to plants and animals, microorganisms are preferred source for production of proteases(Pandey,2008).Different proteases vary with respect to their stability and optimum pH and temperature. These properties decide the use of particular protease in the industry (Sumantha,2005).

Although many microorganisms can synthesize proteases many are either pathogenic or may produce toxic metabolites therefore only few are generally regarded as safe (GRAS) and can be used in commercial production in industries (Gupta,2002). One microorganism can synthesize many types of proteases. Type of proteases produced is also influenced by composition of nutrient medium, environmental conditions during production (Lee,1991). Protease are also involved in pathogenesis of certain organisms like *Pseudomonas aeruginosa* (Sokol,1979).

In the present study protease synthesizing bacteria was isolated and protease was characterized for optimum temperature, pH and tolerance of Ca^{++} and Mg^{++}.

2 Materials and Methods

2.1. Collection of sample

Water or soil samples were collected from fish market, slaughter house waste, marine sediments, Lonar lake in sterilized containers and kept in ice box during transportation from collection site to the laboratory. After appropriate dilution, diluted samples were used for screening of protease producers.

2.2 Primary screening of protease producer organisms

Primary screening was done on sterile skim milk agar plates containing peptone-0.1%, NaCl-0.5%, skim milk-10%, agar-2.5%, pH-7. Dilution of the sample was done to obtain well isolated colonies after incubation at 30^0C for 48hrs. Protease producing cultures showed zone of clearance around the colony. Based on morphological characters protease producer

colonies showing different morphological colony characters were selected and maintained on Nutrient agar slants for further screening.(Alnahdi, 2012,Siddaligeshwara,2010)

2.3 Secondary screening of protease producer organisms

For secondary screening protease producer cultures selected in primary screening were grown in sterile 5 ml Nutrient gelatin broth at 30^0C for 24 hrs. After incubation broth was centrifuged at 10000 rpm for 15 minutes at 5^0C. 50µl supernatant was added in 5mm diameter well prepared in gelatin agar plates. Plates were incubated at 30^0C for 16 hrs. On incubation plated were flooded with acidified $HgCl_2$. Zone of clearance around the colonies were recorded. Colonies showing greater zone of clearance were selected for protease production (El-Safey,2002).

2.4 Characterization of protease producers

Colony morphology like size, shape, color, margin, elevation, opacity, consistency, Gram character and motility of the potent protease was recorded.

2.5Batch production of protease

Protease production was done by inoculating culture in a medium containing Galactose-1%, Casein-0.5%, peptone- 0.5%, KH_2PO_4-0.2%, Na_2CO_3-1%, $MgSO_4$-0.2%, pH-8 in a 500ml flask. Incubated in shaker incubator at 30^0C for 24hrs. at 100rpm.

2.6Assay of protease enzyme

After incubation the whole broth was centrifuged at 10000 rpm for 10min. at 5^0C to separate cells. Cell free supernatant was used as a crude enzyme source for protease assay. Casein was used as a substrate. 100µl of crude enzyme was added in 4ml of 1% casein incubated for 30min. After incubation 5ml of 110 mM.Trichloroacetic acid was added to terminate the enzymatic reaction by precipitation of protein. Precipitate is separated by centrifugation at 4000rpm for 10 min. 1ml of centrifuged supernatant was added in 5ml of 0.4M Na_2CO_3. To this 0.5 ml of FolinCiocalteau reagent was added incubated for 30min. by taking absorbance at 660nm against reagent blank using UV Visible spectrophotometer amount of Tyrosine was calculated from standard curve of Tyrosine. One unit of protease was expressed as the amount of protease that liberate 1µmol of enzyme/ml/min(Anson,1938).

2.7 Effect of Environmental factor on enzyme production

2.7.1Effect of Temperature

To study effect of temperature on protease activity standard assay procedure was doneby incubating enzyme substrate mixtures at various temperatures (20 to 60^0C with increments of 10^0C.). After incubation enzyme activity was determined(El-Safey, 2004).

2.7.2Effect of pH

To study effect of pH on protease activity was by performing standard assay procedure with substrate- casein dissolved in buffers of different pH. For 5,6 pH- Citrate phosphate, 7pH-Sodium Phosphate, 8pH-TrisHCl and for 9 pH- Glycine NaOH is used. After incubation enzyme activity was determined(Nascimento,2004).

2.8 Activity of protease in presence of Ca^{++} and Mg^{++}

In order to check activity of protease in hard water containing more Ca^{++} and Mg^{++} salts were added in 50, 100,150 and 200ppm.

RESULTS

Screening of protease producer

As shown in figure 1 colonies showing zone of clearance on skim milk agar indicate production of protease. 51protease producer colonies were selected and used for further secondary screening by adding cell free broth in wells ingelatin agar. On addition of acidified $HgCl_2$ largest clearance zone showing culture was finally selected for protease production.

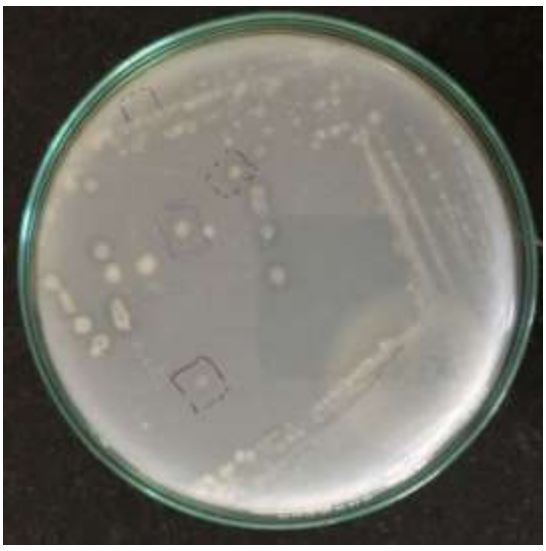

FIGURE 1: Colonies showing zone of clearance on skim milk agar.

Characterization of protease producers

Colony and biochemical characteristics of the given isolate was observed as shown in Table 1.

Sr. No.	Characteristics	Result
	Size, shape, margin, elevation, consistency, color, opacity of colony	2mm, circular, regular, convex, smooth, colorless, transparent.
	Gram character	Gram negative rods
	Motility	Motile
	Endospore	Absent
	Amylase production	+
	Gelatinase production	+
	Indole production	+
	MR test	+
	VP test	-
	Citrate utilization	-
	Oxidase	-
	Catalase	+
	Glucose utilization	+
	Lactose utilization	+
	Sucrose utilization	+
	Salt tolerance (% NaCl)	Up to 7%

TABLE 1: Colony and biochemical characteristics of *Vibrio metschnikovii* strain XMB 057.

Effect of temperature on protease activity.

With increase in temperature activity of protease increases up to 50^0C. After 50^0C protease activity decreases with increase in temperature as shown in figure 3. 50^0C is the optimum temperature for protease of *Vibrio metschnikovii* strain XMB 057.

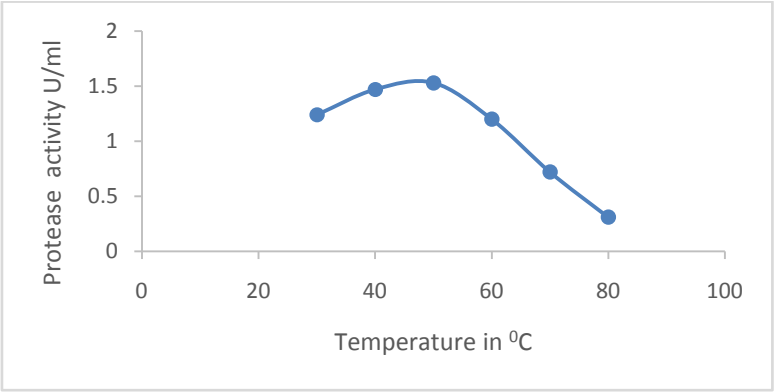

FIGURE 2: Graph of **e**ffect of temperature on protease activity.

Effect of pH on protease activity.

Proteaseof *Vibrio metschnikovii* strain XMB 057 was found to be more active in alkaline pH and shows optimum activity at 9pH with enzyme activity 1.61U/ml.

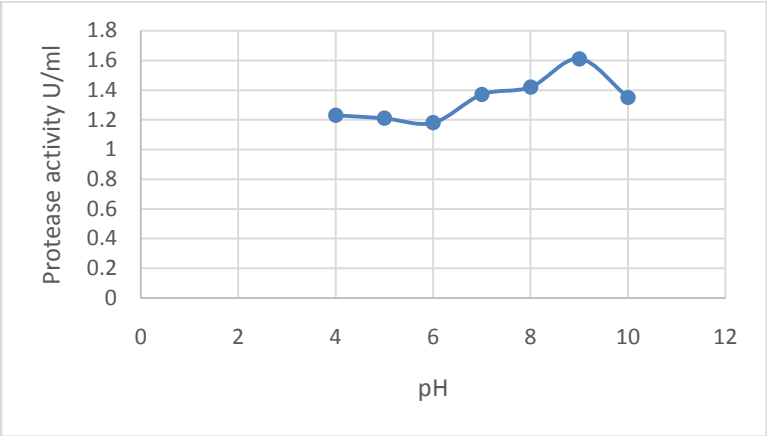

FIGURE 3: Graph ofEffect of pH on protease activity.

Protease Activity in presence of Ca^{++} and Mg^{++}

Activity of protease activity is stable with increase in Ca^{++}upto 150 ppm after this enzyme activity decreases. For Mg^{++} activity is stable upto100ppm after it slight decrease is observed as shown in Table 2.

Sr. no.	Cation present	Concentration in ppm	Protease activity U/ml
1	Ca^{++}	50	1.15
		100	1.11
		150	1.06
		200	0.32
2	Mg^{++}	50	1.12
		100	1.02
		150	0.69
		200	0.23

Table 2Protease Activity in presence of Ca^{++} and Mg^{++}

4 DISCUSSION

Bacterial strain capable of producing extracellular protease identified as *Vibrio metschnikovii* strain XMB 057 was isolated from sodic and alkaline water of Lonar Lake from the district of Buldhana, Mahrashtra. Bacterial strain showed proteolytic activity in both solid medium and liquid fermentation medium. Jellouli et al. 2009 reported the serine protease from *Vibrio metschnikovii* J1 isolated from alkaline sope water of detergent industry. Similar study pertaining to proteolytic enzymes from *Vibrio s*pp. has been carried out by Shinoda and Miyoshi, 1996, Mizuno et al. 2014.

Protease synthesized by*Vibrio metschnikovii* strain XMB 057hadoptimum temperature of 50^{o}C and pH 9. Since bacteria of *Vibrio sp*. genus are normal inhabitants of aquatic environments and mostly marine ecosystems (Shinoda and Miyoshi, 2011), the proteases produced by this genus are stable to alkaline pH. The results obtained in the present studies are in agreement with those reported by Jellouli et al. 2009 for protease characterized from

Vibrio metschnikovii. The protease had wide range of activity from 9-12 with optimum value at 11 and temperature of maximum activity was 60^{o}C which is greater than that observed in the present studies. Ponnambalam et al. 2012 also reported a protease from *Vibrio sp* whose optimum pH was 9 while temperature optimum was 55^{o}C. Thermostability of protease from *Vibrio metschnikovii* strain XMB 057 could be attributed to the presence of Ca++ ions as could be observed from the increase the proteolytic activity upon increase in the concentration of these ions in the reaction system. The calcium ions are one of the known agents that contribute to the thermal stability of the enzymes (Voordouw and Roche, 1976; Dahlquist et al, 1976). However, calcium ions at much higher concentration leads to the loss of the enzyme activity for a reason that could not explained.

In recent years, the application of proteases as industrial catalyst has increased tremendously due to their versatile nature and capability of carrying out hydrolysis of variety of protein substrates (Anwar, A. and M. Saleemuddin, 1998; Li and Yi, 2013). Particularly, the industries where these proteases have been the candidates of special interest are detergent industry and leather industry as a destaining agent and dehairing agent respectively (Valls et al. 2011; Sharma et al. 2017). Since processes in these industries are carried out at alkaline pH and elevated temperatures, the potential proteases need to be stable at given conditions, From the results obtained in the present studies and features of protease from *Vibrio metschnikovii* strain XMB 057 makes this particular protease suitable for use in detergents.As reported by Aftab et al.,2006 during dehairing of leathers pH is in between 8 to 10. As this protease shows pH optima of 9 and retains 83% activity even at pH 10. It suggests the possible application of this protease in leather industry. Activity of protease in presence of 100 and 50ppm of Ca^{++} and Mg^{++} respectively make suitable for use in detergents even in hard waters which contain more Ca^{++} and Mg^{++}.Recently proteases are also used in waste water containing organic matter. The protease from *Vibrio metschnikovii* strain XMB 057 thus presents potential scope to be explored as the agent in the biotechnological industry.

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