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Characterization of protease from *Alcaligens faecalis* and its antibacterial activity on fish pathogens

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Abstract

Alcaligens faecalis AU01 isolated from seafood industry effluent produced an alkaline protease. The optimum culture conditions for growth as well as enzyme production were 37°C and pH 8. The partially purified protease had specific activity of 9.66 with 17.77% recovery with the molecular weight of 33 kDa and it was active between 30-70°C and optimum being at 55°C and pH 9. The enzyme retains more than 85% activity at 70°C and 78% even at pH 10.The enzyme inhibited the growth of fish pathogens such as *Flavobacterium* sp., *Pseudomonas fluorescens, Vibrio harveyi*, *Proteus* sp. and *Vibrio parahaemolyticus*. From the present study it can be concluded that *Alcaligens faecalis* AU01 has the potential for aquaculture as probiotic agent and other several applications.

Key words

Alkaline protease, *Alcaligens faecalis,* Antibacterial, Seafood effluent, Fish pathogens

Introduction

Seafood industry generates large quantities of by products as waste that includes viscera, shell, scales, fins and frame bones. The wastewater from seafood processing plants contains large amounts of organic matter, small particles of flesh, breading, soluble proteins, and carbohydrates. Generally, head, shell and tail portions of shrimp are removed during processing and these account for approximately 50% of the raw materials. Increasing production of inedible parts such as heads, shells and tails, is causing environmental problems as a result of uncontrolled dumping. Visceral wastes alone contribute to the total of 300,000 ton and they are good source of proteins including enzymes and fats (Mahendrakar, 2000). Microflora of the digestive tract of fish may produce antibacterial materials preventing pathogenic bacteria from getting into an organism (Sugita *et al*., 1996). Gastrointestinal bacteria take part in the decomposition of nutrients and also provide the macro organisms with physiologically active materials like enzymes, amino acids and vitamins (Sugita *et al*., 1997). However, many proteolytic bacteria have been reported to be associated with marine food processing wastes (Bhaskar *et al.,* 2007).

Proteases include a group of enzymes, which hydrolyze peptide bonds in aqueous environment and synthesize peptide bonds in non-aqueous environment. Proteases also known as peptidyl-peptide hydrolysis constitute 60-65% of the global enzyme market (Banerjee *et al*., 1999; Genckel and Tari, 2006). The vast diversity of proteases, in contrast to the specificity of their action, has

attracted the attention of scientists in an attempt to exploit their biotechnological prospects (Rao *et al.,* 1998).

Microbial proteases are an important group of enzymes that can have application in various industries such as leather processing, food processing, pharmaceutical and bioremediation process and in textile industry to remove protein based stains (Gupta *et al*., 2002; Najafi *et al*., 2005). Microbial proteases are the most important group of secondary metabolites that are widely exploited (Ferrari *et al*., 1993). These bacteria also compete for space on surfaces displacing other bacteria if they are present in high numbers and it inhibits other bacteria from growing rapidly thereby exercising an inhibitory effect by utilizing nutrients (Moriarty, 1998). Hence, the present study was made to purify and characterize protease from *Alcaligenes faecalis* strain AU02 with special emphasis on antibacterial activity against fish pathogens.

Materials and Methods

Isolation and characterisation of protease: Bacteria isolated from the seafood industry effluent was screened for gelatinase and caseinase production, on gelatin agar and casein agar medium respectively. This strain was identified as *Alcaligenes faecalis* by employing the standard schemes of morphological, biochemical and 16S rRNA gene sequencing (Marmur, 1961) and designated as *Alcaligenes faecalis* AU01. Protease production was carried (Harrigan and Mc Cance, 1972) out in a medium containing: Casein 10 g, peptone 1 g, maltose 4 g, NaCl 5 g, $CaCl_2$ 0.05 g, $MgSO_4$ 0.05 g, water 1000 ml (pH 7.5). Cultures were incubated on a shaker (125 rpm) for 48 hr at 37°C. Cell growth and enzyme production was measured from the aliquots withdrawn every 6 hr. The culture broth was centrifuged at 3,000x*g* for 30 min at 4°C and the supernatant was used as crude enzyme.

Gene sequencing: A molecular identification of the isolate was achieved by 16S rRNA gene sequencing. The extraction and purification of DNA was carried out by the phenol chloroform method (Kocabiyik and Caba, 1972; Marmur, 1961). The primer sequences were chosen from the conserved regions as reported earlier for the bacterial 16S rRNA (Marchesi *et al*., 1998). Sequencing was done using the forward primer (5'-AGGCCTAACACATGCAAGTC-3') and reverse primer (5'-GGGCGGTGTGTACAAGGC- 3'). PCR reactions were performed with the following program for the 16S rDNA gene: 30 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by a final extension step of 5 min at 72°C. The 16S rRNA sequence was analyzed by an automated DNA sequencer (Megabace, GE). The sequence was also analyzed for homology using CLUSTAR X software. A phylogenetic tree was constructed by using the neighbor joining DNA distance algorithm.

Protease assay: Protease activity was assayed by using casein as a substrate (Kunitz, 1947). The reaction mixture containing 100 µl Tris-HCl buffer (100 mM, pH 8.0), 100 µl of 1% casein solution and 200 µl of suitably diluted enzyme solution were incubated at 40°C for 30 min. The reaction was terminated by addition of 400 µl of 10% trichloroacetic acid solution and the non-hydrolyzed casein was precipitated by centrifugation at 3,000 rpm for 15 min. Peptide concentration of the supernatant was determined by measuring absorbance at 280 nm using tyrosine as a standard. One unit of protease activity was defined as the amount of the enzyme required to liberate 1 µmol of tyrosine per minute.

To the culture supernatant, solid ammonium sulphate was added to 60% saturation and allowed to stand overnight at 4°C. The precipitate was harvested by centrifugation at 6,000x*g* for 15 min, dissolved in 100 mM Tris–HCl buffer (pH-8.0) and dialyzed against the same buffer overnight (4°C). The dialyzed enzyme was loaded at a flow rate of 0.5 ml min^{-1} onto a DEAE-Cellulose column (5 x 20 cm) previously equilibrated with Tris–HCl buffer (pH-8.0). The column was washed with 100 mM Tris-HCl (pH 8.0) buffer and bound proteins were eluted with linear gradients of NaCl ranging from 0 to 1M at a flow rate of 0.5 ml min^{-1} (Wang *et al*., 2007). Fractions (3.0 ml) were collected and assayed for protease activity by using casein as a substrate. Fractions were pooled together, dialyzed overnight against 100 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was assayed for protease activity and protein content. Protein content in the crude and purified enzyme was determined according to the method described by Lowry *et al.* (1947) using bovine serum albumin (Hi-Media chemical laboratories, India) as a standard.

Native PAGE was performed by the method of Davis *et al*. (1964) in a 7.5% (w/v) polyacrylamide gel with Tris/glycine buffer (pH 8.3). SDS-PAGE was performed to estimate the molecular weight of the purified protein using 5% stacking gel and 12% resolving gel according to method of Laemmli (1970) and electrophoresis was performed with 15 mA fixed current. Molecular weight was estimated by comparing the relative mobility of proteins of different molecular size using a standard molecular weight marker (205, 97.4, 66, 43 and 29 kDa) (Genie; Bangalore, India).

Effect of pH and temperature on activity and stability of protease: The optimum pH for the activity of protease was studied in the range of 3.0-13.0 with casein 1% (w/v) as substrate and the pH stability of the protease was determined by incubating enzyme preparation in buffers of different pH in the range of 3.0-13.0 for 1 hr. Aliquots were withdrawn and proteolytic activity was determined by the standard assay (Kunitz, 1947). The following buffer systems were used: Sodium acetate buffer of pH 3.0 -5.0, potassium phosphate buffer of pH 6.0-8.0; Tris-HCl buffer (100 mM) of pH 8.0-9.0 and glycine–NaOH buffer of pH 9.0-12.0.

The effect of temperature on the enzyme activity was examined by incubating the reaction mixture (enzyme+substrate) at various temperatures (20-80°C) at pH 9.0. To determine thermal stability, the enzyme was preincubated at different temperatures (20-95°C) for 1 hr and then the residual activity (%) was assayed under standard assay conditions (Kunitz, 1947).

Aliquots were withdrawn at desired time intervals to test the remaining activity. The non-heated enzyme was considered as control (100%).

Antibacterial activity against fish pathogens: Antibacterial activity of protease produced by *Alcaligenes faecalis* AU01 was assayed by disc diffusion method. For evaluating the effect of the partially purified protease on different fish pathogens, *Streptococcus* sp.*, Flavobacterium* sp., *Pseudomonas fluorescens, Vibrio harveyi*, *Proteus* sp. and *Vibrio parahaemolyticus* were taken as the indicator organisms. These organisms were grown individually in nutrient broth for 24 hr at 37°C. The culture broth at 1% level was added into warm, but unset plate count agar, mixed uniformly and poured into sterile petri plates. Appropriate number of discs impregnate with aliquots (5, 10 and 15 µl) of the partially purified protease, with known protein content, were placed on the plates. The plates were pre-incubated at 4°C for 3 hr to allow the test material to diffuse into the agar, after which the plates were incubated at 37°C for 24 hr. After incubation, the plates were examined for zone of clearance around the individual discs. The diameter of the zone of clearance was measured and expressed as arbitrary units per ml (AU/ml).

Results and Discussion

***Alcaligenes faecalis*:** AU01 is a Gram-negative, rod-shaped, motile, non-nitrate reducing, oxidase positive, catalase positive, and citrate positive obligate aerobe that is commonly found in the environment. The bacterium degrades urea, creating ammonia which increases the pH of the environment. Although *A. faecalis* AU01 is considered to be alkaline tolerant, it maintains a neutral pH in its cytosol to prevent the damaging or denaturing of its charged species and macromolecules. The 16S rRNA gene sequencing was done and the phylogenetic tree (Fig. 1) constructed by the neighbor-joining method and the sequence was deposited in Gene bank with the accession number-HM145896.

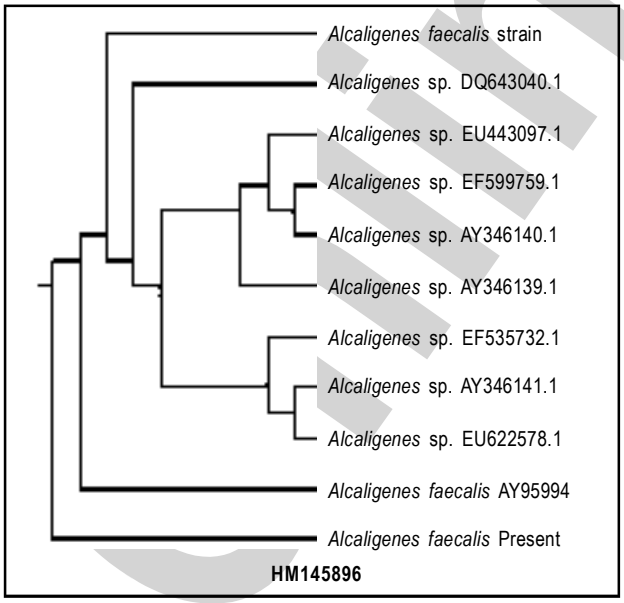


Fig. 1: Phylogenic tree showing the taxonomic position of the *Alcaligenes faecalis* AU01 with accession number HM 145896

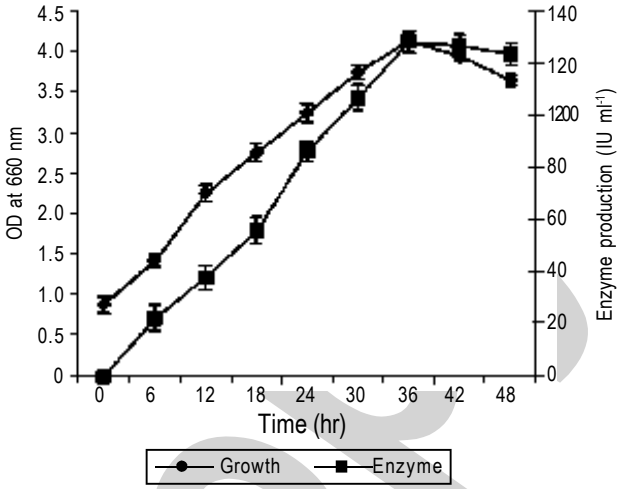


Fig. 2: Growth as protease production during growth and stationary phase with accession number. HM145896

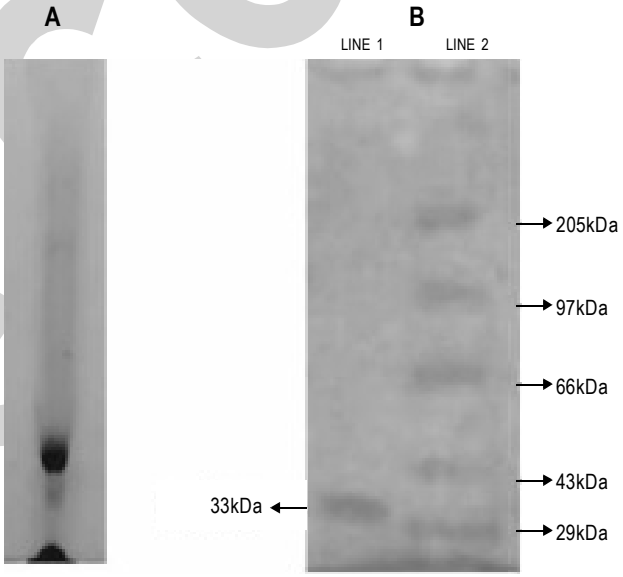


Fig. 3: (A) Native- PAGE, (B) SDS-PAGE of protease from *Alcaligenes faecalis* AU01. Lane. 1. Purified protein, Lane 2. Molecular markers

Protease production and purification: The growth kinetics and the enzyme production were measured at different time intervals. The maximum production was found during early stationary phase (36th hr) itself (Fig. 2). Generally, synthesis and secretion of the protease is initiated during the exponential growth phase, with a substantial increase near the end of the growth phase and maximum protease production in the stationary growth phase (Ferrero *et al.*, 1996). Anustrup (1980) reported that maximum extracellular protease activity was at the stationary phase and the optimum pH for protease

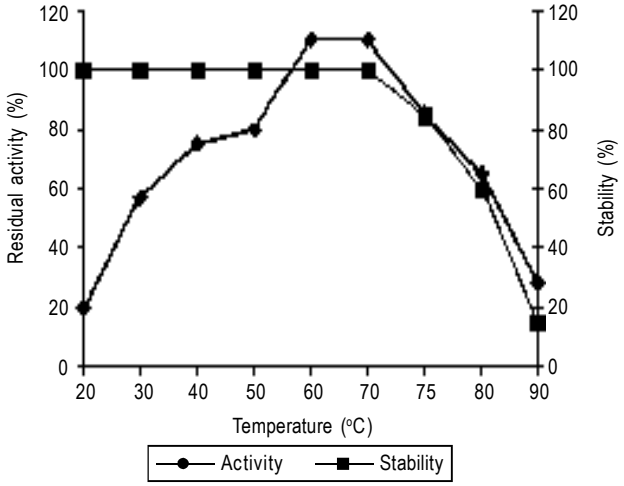


Fig. 4: Effect of temperature on the activity and stability of protease from *Alcaligenes faecalis* AU01

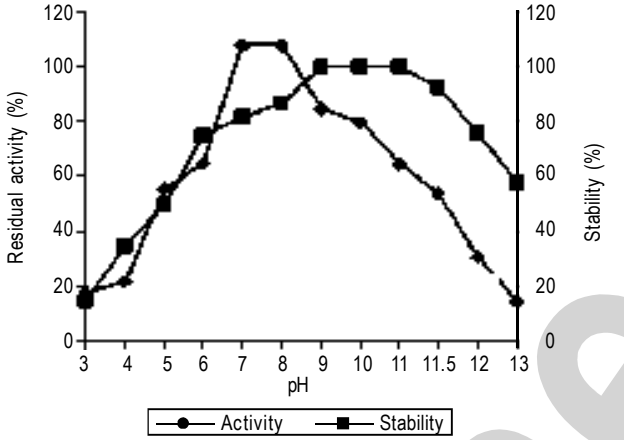


Fig. 5: Effect of pH on the activity and stability of protease from *Alcaligenes faecalis* AU01

production was higher than that for optimum growth in the alkalophiles, hence, the pH of the medium must be maintained above 8.0 throughout the fermentation period.

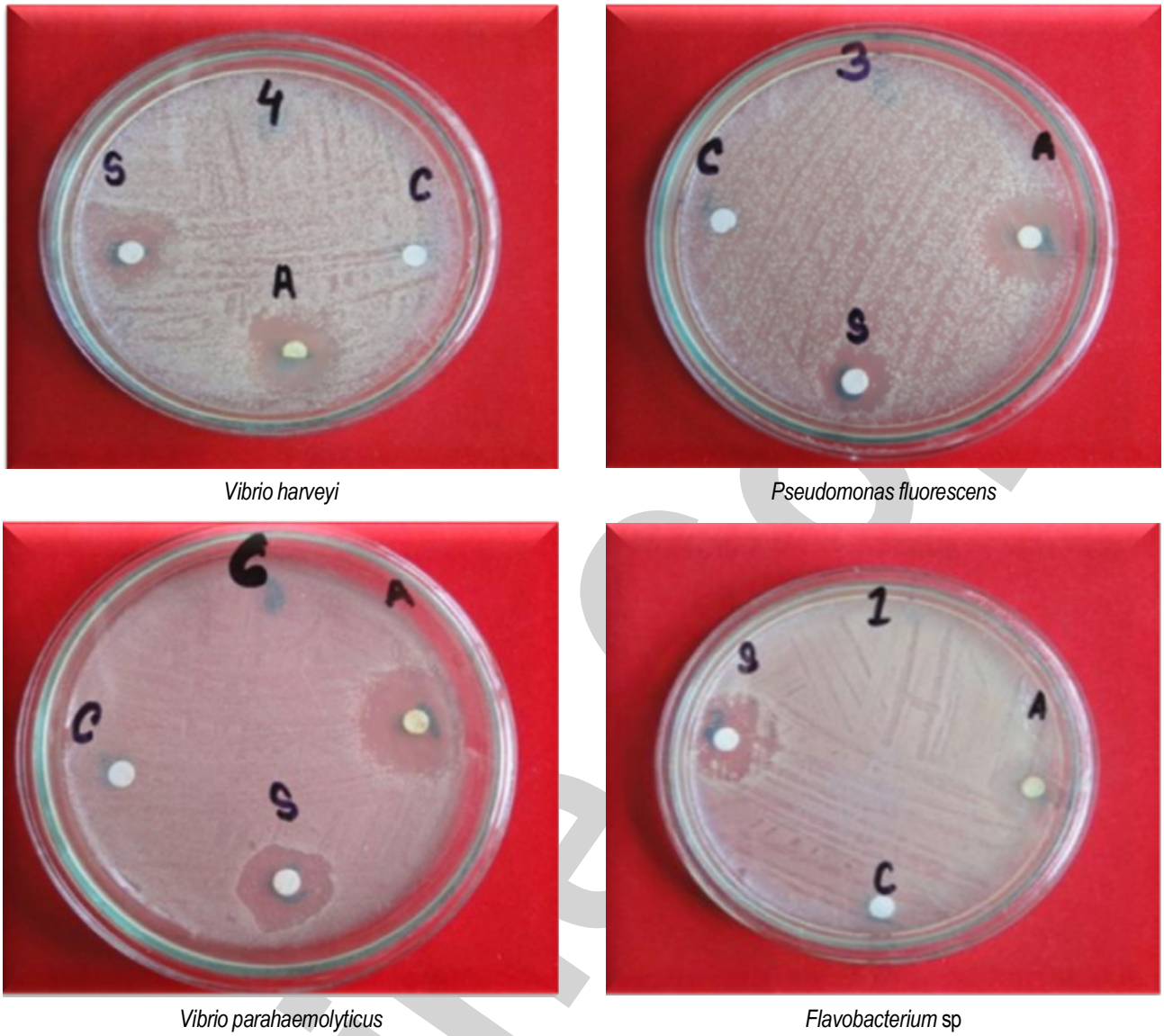
In purification step, with ammonium sulphate precipitation this protease attained 4.06 fold purification and 17.77% recovery and finally the purified enzyme attained 9.66 specific activities with 36.4% recovery. The results are in agreement with the previously reported proteases from *Vibrio fluvialis* and *Aspergillus parasiticus* (Tunga *et al.,* 2003; Venugopal and Saramma, 2006). Purified protease migrated as a single band in SDS-PAGE under reducing conditions, suggesting that the purified protease was homogeneous. The apparent molecular weight of the purified protease as revealed by SDS-PAGE is about 33 kDa (Fig. 3).The molecular weight of this protease is comparatively lower than the other proteases reported elsewhere (Miyoshi *et al.,* 2002; Karadzic *et al.,* 2004; Bhaskar *et al*., 2007).

Effect of temperature and pH on activity and stability of the protease: The effect of temperature on the activity of purified enzyme showed quite good activity over a wide range of temperatures between 30 and 70°C, the optimum being at 55°C. The enzyme exhibited more than 85% activity at 45 and 75°C and about 60% activity retained between 30 and 40°C (Fig. 4). (Activity is expressed in enzyme units and stability is expressed in % of retained activity). Very few reports are available on proteases which are active in the wide range of temperature variation (Joo *et al.,* 2002; Venugopal and Saramma, 2006; Wang *et al.,* 2007). Regarding thermostability, protease was 100% stable up to 60°C for 1 hr and it retained more than 85% at 70°C for 30 min (Fig. 4). At 90°C, it lost more than 80% activity and it retained only 13%. Protease from this *Alcaligenes faecalis* was highly stable compared to other proteases (Joo *et al.,* 2002; Wang *et al.,* 2007; Bhaskar *et al*., 2007).

Effect of pH on the activity and stability of the protease: The enzyme was found to be active over a wide ranges of pH between 5.0-11.0 and optimum pH for enzyme activity was 9.0 (Fig.5). The enzyme exhibited more than 87% activity at pH-11.0 and 50% at pH-5.0. This is comparatively high with the protease from *Vibrio fluvialis* VM 10 (Venugopal and Saramma, 2006) with an optimum pH at 8.0, *Vibrio fluvialis* TKU005 (Wang *et al.,* 2007) active at 7.5 and *Aspergillus* (Tunga *et al.* 2003) protease which was active at pH 8. Regarding stability, the enzyme was highly stable between pH 5 and 11 and it retained over more than 50% of activity. It was 100% stable at pH-9; it retained 87% at pH-8 and 78% at pH-10, respectively (Fig. 5). It is similar to the *Bacillus* protease (Bhaskar *et al*., 2007) and higher than the proteases reported earlier (Kumar *et al.,* 1999; Ghorbel *et al.,* 2003).

Antibacterial activity of protease from *Alcaligenes faecalis* AU01: The alkaline protease from *Alcaligenes faecalis* AU01 isolated from sea food industry effluent inhibited the growth of *Pseudomonas fluorescens, Vibrio harveyi, Vibrio parahaemolyticus* and it was not effective against *Flavobacterium* sp. at both 5 and 15 µl concentration. The diameters of inhibition zone of various aliquots of the dialyzed protease are presented in Fig. 6. The inhibition of pathogens indicated the antibacterial activities of this alkaline protease and it may cause some morphological changes (Moriarty, 1998).

Several microorganisms which produce proteolytic enzymes have been reported and are commercially exploited for their application in production of fish protein hydrolysis (Rebecca *et al*., 1991), beef liver protein hydrolysate (Sephton *et al*., 1996). Further, they are often used commercially in bioremediation mixes, or as probiotic agent in aquaculture (Moriarty, 1998) and also beneficial bacteria could be introduced by incorporating them into compound fish/shrimp diets (Ochoa and Olmos, 2006), in commercial aquaculture as a probiotics, especially in the larval stages, when the enzyme system is not efficient (Sangbrita *et al*., 2006). These clearly indicated that *Alcaligens faecalis* AU01 could have the potential for utilization in producing alkaline protease that could possess several applications.



Vibrio harveyi *Pseudomonas fluorescens*

Vibrio parahaemolyticus *Flavobacterium* sp

Fig. 6: Antibacterial activity of alkaline protease of *Alcaligenes faecalis* AU01against fish pathogens

Alcaligenes faecalis AU01 being a protease producer that has potential application in aquaculture as probiotic agent owing to its proteolytic activity apart from its antagonistic properties towards fish pathogens.

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