Journal of Biotechnology and Bioengineering
Volume 2, Issue 1, 2018, PP 1-7

Production of Bacterial Acid Protease by Submerged Fermentation using Aeromonascaviae from Dairy Effluent

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ABSTRACT

Proteases are proteolytic enzymes having wide range of applications in various industries such as pharmaceutical industry, textile, leather, food industry and medicine. Microorganisms are considered potentially to be the most suitable sources of acid protease. Bacterial acid proteases are characterized by their maximum activity atacidicpH4.7. As a commodity product, pressure on protease market is on prizer education and increasing performance. Hence our objective was to isolate a potent protease producing microorganism and production of acid protease using dairy effluent. Dairy effluent contain rich amount of organic and inorganic substances. In order to achieve the objective, a proteolytic bacterium was isolated from soil using milk agar medium and the bacteria was identified as Aeromonascaviae by morphological and biochemical characterization. Dairy industry effluent was then used as a medium for acid protease synthesis by the potent bacteria. The highest amount of acid protease synthesized by bacterium was 56.66U/ml when incubated for 80hat37°C and optimum enzyme titers were found atpH4.7 when incubated at 120rpm. Partial purification with ammonium sulphate and complete purification were showed 2-fold increase in the specific activity (104.66U/mg protein).Dairy industry effluent was thus found to be an effective medium for acid protease synthesis by using Aeromonascaviae.

Keywords: *Acid Protease, Milk Agar, Aeromonascaviae, Production Medium, Dairy Effluent;*

INTRODUCTION

Proteases are the single class of enzymes, which occupy apivotal position with respect to their application in both physical and commercial fields. They are degradative enzyme which catalyzes the total hydrolysis of proteins. Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots and processing and transport of secretary proteins across the membrane. The current estimated value of the worldwide sales of industrial enzymes is $1billion.The current estimated value of the worldwide sales of industrial enzymes is $1 billion [1] Kindly change citations to numbered citations. In the end God frey *et al.* should be placed at no.1 in the reference list. Proteolytic enzymes are them ost important industrial enzymes, representing worldwide sales of about 60% of the total enzyme market [2-4] and the industrial enzymes, 75% are hydrolytic.

Proteases execute a large variety of functions extending from the cellular level to the organ and organism level to produce systems such as home ostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal patho physiological condition. Their involvement in the life cycle of diseases causing organisms has led them to become a potential target for developing the rapeutic agents against fatal diseases such as cancer and AIDS. The vast diversity of proteases, in contrast to the specificity of their function, has attracted worldwide attention in attempts to exploit their physiological, biochemical and biotechnological applications (Foxetal1991, Kaliz1988, Morihara and Oda 1993, Rao et al 1983 and Poldermans 1990).

Acid proteinases having their proteolytic activities in acidic H regions have recently been called as partic proteinases since a pair of carboxyl groups of as partic acid residues has been shown to be involved in their catalytic function (Furuton1987). Most of the as partic proteases show maximal activity at low pH (pH3–4) and have iso electric points in range of

pH3–4.5. Their molecular masses are in the range of 30 to 45kDa. Some well-known acid proteases are pepsin, gastrin chymos in and catheps in D. Most of these enzymes share similar amino acids equences, three dimensional structures, active site structures and catalytic mechanisms (Tang1977, Kostka1985, Tang and Wong1987). Since these proteases contain two as partic acid residues in their catalytic sites, they are also called as partic proteases. The structural and functional relationships of as partic proteases are involved in diseases such as rennin hyper tension and acquired immune deficiency diseases (an acid proteases is associated with the maturation if the Human immune deficiency Virus) and the availability if high resolution crystal structures of several as partic proteases has made these enzymes attractive models for the study of structure function and relationship.

Dairy industry waste water contain large amount of organic and inorganic substances. Organic matters such as, fat, protein and carbohydrates. In organic nutrients are mainly nitrogen and phosphorus contains the milk and the milk products (Madhu2017). Microorganisms are considered potentially to be them ost suitable sources of acid protease for industrial application. Hence the present study also deals with the isolation of protease producing bacteria from soil samples. The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors, the cultural parameters like temperature, pH, and incubation time were plays a major role in enzyme production (Afshan *et al.*,2011, Mazzucotelli *et al.*,2014) and so the optimization of media components and cultural parameters is the primary task in a biological process. So, the media components and cultural conditions were optimized. The acid proteases were produced, extracted, purified and characterized. Hence the present study aim to isolate and identify a potent protease producing bacteria from soils ample and develop a fermentation medium for acid protease production through submerged fermentation.

MATERIALS

Sample Collection

Two different sources of soil samples were collected in a sterile polythene bag from kitchen premises and meat processing unit near the institution and immediately transferred to the laboratory for the isolation of bacteria.

Methods

Isolation of Protease Producing Bacteria

The bacteriological studies were carried out by serial dilutions followed by plating and later this culture were used for further studies based on their morphological and biochemical characteristics as outlined in the Bergey`s Manual of determinative bacteriology (Buchanan *et al.*,1972). 1gm of soil sample was mixed with 9ml of saline solution i.e. Master dilution and 1ml of solution was serially transferred to tubes containing 9ml saline each so that for each transfer the suspension was diluted 10times. Each tube was shaken vigoursly. 0.1ml solution was spread to petri plates containing milk agar medium (pH7) and incubated for 24hat37°C. Colonies showing proteolytic activity was selected and used for further studies. The milk agar medium contains (g/L) Skimmed milk powder100**,** Peptone waste water5, Agar 15g. Here milk powder was auto claved separately.

Sub merged Fermentation for Protease Production

The proteolytic bacteria was inoculated into 40ml seed culture mediaina 250ml conical flask and kept in an environmental shaker at 120rpm and 37°C for 24h. The composition of seed culture medium was explained in Madhu 2017. 1 ml of 24 hold seed culture was inoculated into 40ml of production mediaina 250ml flask and incubated in an orbital shaker at 120rpm at 30^{o}C for72h. Dairy waste water used as a medium for the protease production. The dairy waste water was thankfully provided from Manjoor Milks, Kottayam, Kerala and water samples were collected in sterile bottle. The culture medium was then centrifuged at 4°C and 7500rpm for 15minutes.The supernatant was collected in vial sand estimated for acid protease activity.

Acid Protease Assay

Enzyme assay involves the estimation of amount of tyrosine released during the hydrolysis of protein. The Folins Ciocalteaure agent reacts with tyrosine released to produce a blue coloured complex (Lowry *et al.*, 1951), which was read at 660nm.

Reagents used for the protease assay was 1). Sodium acetate (2M) Acetic acid (1M), 0.1M Acetate buffer (pH4.7), Sodium acetate (0.5M),

0.3MHCl, TCA10%, Substrate (Hemoglobin2%) and Folin's Ciocalteaure agent (Hananetal2012). One unit of enzyme activity can be defined as the amount of enzyme that liberated 1µg of tyrosinemin^{-1}under assay condition and reported in terms of Units/ml.

Identification of Proteolytic Bacteria Isolated from Soil

The selected potential strain was then identified by morphological and biochemical characteristics by using microbiology laboratory manual.

Morphological Characters

The cell shape, pigmentation and fluorescence of the isolated bacteria were studied using microscope.

Motility

Hanging drop slides were prepared from nutrient broth cultures (18hold) were observed under microscope.

Gram Staining

Gram staining was done using smear preparations from 18 hold cultures. The slides were heat fixed and treated with crystal violet for 1 minute. Rinsed gently in a stream of water, dried, blotted and flooded with grams iodine solution for 1 minute. Washing in 95% alcohol for 30sec and rinsing in water followed this. After drying, slides were flooded with saffranin, a counter stain for1minute,again washed with gentle stream of water blotted to dryness and observed under oil immersion.

Biochemical Tests

Make appropriate heading Carbohydrate fermentation four types of carbohydrates (glucose, fructose, lactose, sucrose) were used as substrate for fermentation test. Each carbohydrate was dissolved separately and pH was adjusted to 7. Medium was sterilized at 15lb for 15 minutes. When cooled they were inoculated with the bacterial strain. A control was kept and the tubes were incubated at 37°C for 48h and the biochemical changes were observed.

Citrate Test

The Simmons citrate agar medium was prepared, transferred to culture tubes and sterilized at 15l b for 15 minutes. The bacteria were streaked in the slants with sterile loop and incubated for 48hrs at 37°C. Then the biochemical changes are observed.

Indole Test

Tryptone broth was prepared in the test tubes and sterilized. The medium was incubated with a loop full of culture and incubated at 37°C for 48h. 0.3ml of Kovac's reagent was added to 5ml of broth culture. The tubes were shaken and as allowed to stand for 5 minutes. The observations were noted.

Methyl Red Test

The glucose phosphate broth was sterilized and inoculated with bacterial culture and incubated at room temperature for 48h. A few drops of methyl red indicator were added and observations were noted.

Voges-Proskauer Test

The tubes of glucose phosphate broth were inoculated with a loop full of bacterial cultures. The tubes were then incubated at room temperature for 48hrs. Then 3ml of 40%KOH solution was added. The observation was then noted.

Catalase Test

A few drop of H_2O_2 was kept on a clean glass slide. A loop full of isolated bacterial culture was placed into the drop and the observations were noted.

H2SProduction

This test was used to differentiate the ability of the microorganism to produce hydrogen sulphide gas from substrate such as sulphur containing amino acid or inorganic sulphur complex. The bacteria was streaked on SIM agar media and incubated at 37°C for 48h.The observations were noted after 48h.

Urease Test

Inoculate loop full of bacteria into a tube containing urea broth and streak on a urea agar. Incubate the tubes and plates at 37°Cfor 48h. The observations were noted.

Maintenance of the Microorganisms

The microorganisms were maintained on nutrient agar slant and incubated for 24 hrs at37°C.The slants were stored at 4°Cin refrigerator and sub cultured regularly at every 2-week interval.

Effect of Substrate Concentration on Enzyme Production

To maximize enzyme production by the potent bacterial culture, different concentration of

casein (0.5, 1, 1.5, and 2%w/v) were employed in SmF medium.

Influence of Incubation Time in Enzyme Synthesis

Different incubation time (0-112h) with 8h intervals was studied to determine its influence on acid protease production by the potent bacterial strain.

Partial Purification of Enzyme Extract by Ammonium Sulfate

For the purification of enzyme, 75ml enzyme was taken and 36.6g ammonium sulfate was added to attain 80% saturation. The precipitate was collected by centrifugation and dissolved in 3mlof0.5M sodium acetate buffer at pH4.7.it was dialyzed against0.005M sodium acetate buffer pH4.7.

Result and Discussion

Isolation of Proteolytic Bacteria

Table1 showed the zone of hydrolysis produced by the proteolytic culture, 8proteolytic bacteria were obtained in milk agar medium (Figure1). Bacterial culture showing maximum hydrolytic zone (diameter7mm) were selected for further study.

Table1. *Zone of hydrolysis (mm) by proteolytic bacteria.*

No	Bacterial cultures	Zone of hydrolysis(mm)
1	M1	1
2	M2	5
3	M3	2
4	M4	5
5	K1	7
6	K2	3
7	K3	5
8	K4	3

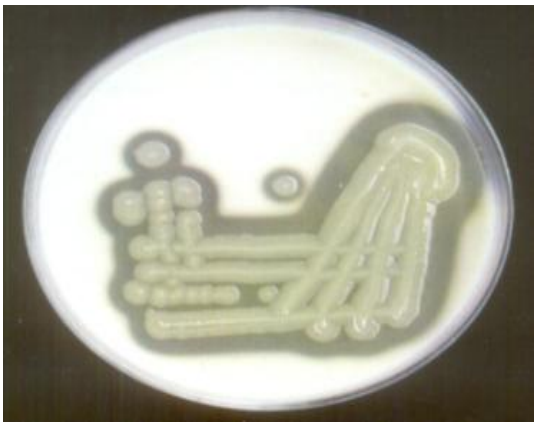

Figure1. *Proteolytic activity observed with the potent culture on milk agar.*

Screening of Bacterial Culture for Protease Production

Screening was done by two steps, primary screening and secondary screening. Primary screening, usually plate as say on milk agar medium will be carried out to isolate protease producing organisms, by measuring the clear zone of hydrolysis formed on milk agar (Ellaiah et al 2002, Rajamani and Hilda1987, Adesh et al (2002).Among the bacterial colonies studied, eight colonies exhibited significant proteolytic activity. And result represented in the table1. From the result it was evident that colonies M2, M4, and K3 showed similar zone of hydrolysis. Moreover, it has been reported that *Bacillus licheniformis* produces narrow zone of hydrolysis on case in a gard espite being very good producers in submerged culture. Figure 2 showed proteolytic activity observed with potent culture on milk agar.

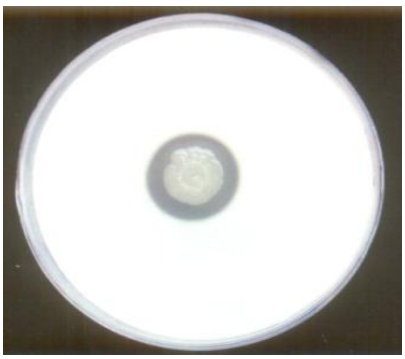

Figure2. Zone of hydrolysis in milk agar

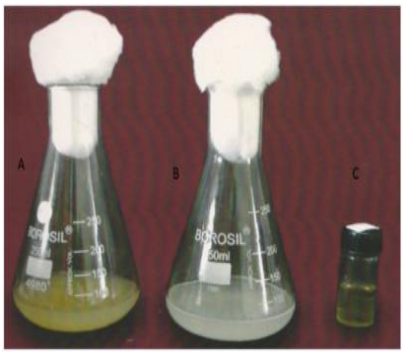

Figure3. *Showed A. after fermentation B before fermentation C. crude enzyme.*

Secondary screening were carried out by determining protease production by the eight colonies in submerged fermentation (SmF) medium and the most potent protease producer was selected. The fermentation media was prepared as in materials and methods. Data on acid and neutral protease production showed by M2 is 8.5U/m land neutral protease is 2.71U/ml. Table2 showed the comparison of acid and

neutral protease production by isolated bacterial strains. Thus it was evident that culture M2 was a potent acid protease producer. Figure 3 showed flask with fermentation media before and after the fermentation and vial containing crude enzyme extract.

Table2. *Comparison of acid and neutral protease production.*

No.	Sample	Acid Protease Activity (U/ml)	Neutral Protease Activity (U/ml)
1	M1	0.11	0.40
2	M2	8.5	2.71
3	M3	0.00	0.60
4	M4	0.00	0.6
5	K1	0.48	0.74
6	K2	0.00	0.60
7	K3	0.00	0.00
8	K4	0.16	0.0

Identification of Bacteria

Experiments on morphological and biochemical characterization of the selected bacterial strain (M2) showed it was a gram negative, rod shaped motile organism. The result obtained from biochemical results were depicted in the Table3.

The potent organism was identified as *Aeromonascaviae*, by cell morphology, gram staining and biochemical test results and also by Microbial Identification System (MIS) at NIO (National Institute of Oceanography) Kochi (Figure4). This was done by Microbial identification of DNA finger printing method (fatty acid profile studies) using MIS. The harvested and extracted cells were loaded to Sherlock MIS, which compared the fatty acid profile of the loaded strain to the built in library of the strains in the software. *A.caviae* was found to synthesize both intra cellular and extra cellular proteases with the latter account for major portion of the total activity (Karunakaran and Davi 1995). Figure 4 showed the gram staining of *A.caviae*. It was also reported that *A.caviae* produces atleast two proteases, a 34-kD a metallo proteases (AP34) and a19–k D a protease (Tomaetal1999).

Table3. *Identification of the potent bacteria.*

Morphological characters		
Motility	Motile	
Shape	Rod	
Gram staining	Gram Negative	
Pigmentation	-ve	
Florescence	-ve	
Biochemical characters		
Carbohydrate Fermentation		
Lactose	+ve	
Glucose	+ve	
Fructose	+ve Sucrose	+ve
IMVICTEST		
In dole	-ve	
Methyl red	+ve	
Vogue sp roskauer	-ve	
Citrate utilization	-ve	
Catalase		
H2Sproduction	-ve	
Urease test	+ve	

Effect of Substrate Concentration on Enzyme Production

Synthesis and secretion of extra cellular proteases by microorganisms reported to be influenced by inducers and its concentration in the medium. Case in was used as an inducer for protease synthesis by *Aspergillus sp.* (Nehraetal2004). Acid protease production by potent culture in Sm F media containing different concentration of case in varying from 0.5% to 2% indicated that among them, medium containing 1% case in shows highest enzyme activity (8.5U/ml).

Influence of Incubation Tine on Enzyme Synthesis

The time course experiment indicated highest production of enzyme at 82h. The effect of incubation period on acid protease production was evident in Figure 5. In *Teredinobacter turnire* cell, protease production started to increase after about 6 hand reached a maximum activity at 48 h (Beshay 2003) by *Streptomyces sp*. However, maximum protease production was obtained after 12h incubation (DeAzeredo et al 2004).

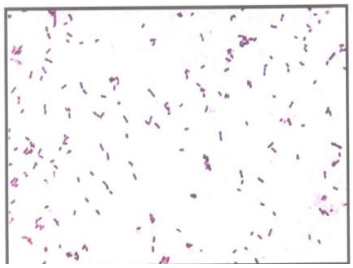

Figure4. *Identification of potent producer.*

Partial Purification

The proteins precipitated with 80% $(NH_4)^2SO_4$ was dissolved in 3ml of 0.5M sodium acetate buffer pH4.7. The dissolved precipitate was then dialyzed against 0.005M sodium acetate buffer (pH4.7). Data on specific activity showed a2-fold increase in specific activity (56.66 U/mg protein). Table5 shows activity specific activity and total protein content of crude and dialyzed enzyme. During the purification steps for *B.psudofirmus* AL-89 protease 60% ammonium sulphate saturation gave a purification of 1.5 fold where as in the case of *Nesternkonia sp.* AL-20 protease, 70% ammonium sulphate saturation gave 10.7 fold purification (Gessesse et al 2003).

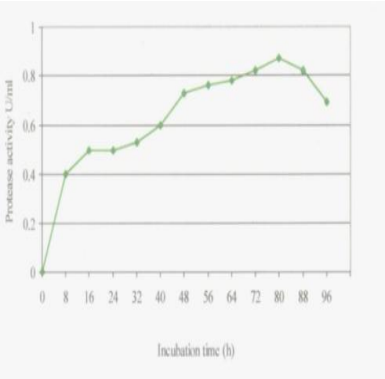

Figure5. *Growth curve of the potent organism.*

Table4. *Partial purification of enzyme extract.*

Enzyme	Activity(U/ml)	Total Protein Content(mg/ml)	Specific activity(U/mg protein)
Crude	8.5	0.150	56.66
Dialyzed	4.3	0.041	104.87

CONCLUSION

In the present study, a potent protease producing bacteria were isolated from soil samples using milk agar medium. Two types of screening were carried out to select the potent protease producer. Primary screening was done by measuring the zone of hydrolysis and secondary screening in sub merged fermentation. Eight bacterial colonies were selected for secondary screening. Among these bacterial colonies M2 showed highest acid protease activity 82 U/ml in SmF medium incubated for 72h at 37°C. Morphological, biochemical characterization and MIS test indicated that the potent bacterial culture was *Aeromonas*c aviae. The time course experiment indicated that this bacterium synthesized highest amount of acid protease of 104.66U/ml, when incubated for 80h at 37°C. 1%(w/v) of case in was found to be the most substrate concentration to induce protease production. In, particular purification of crude enzyme extracts employing ammonium suphate 80%. From the result, it was evident that *Aeromonascaviae* was a potent acid protease producer and a suitable candidate for protein waste degradation. The result was also confirmed that dairy effluent was suitable medium for the production of acid protease. Further studies on factors influencing enzyme production in the medium will be helpful in large-scale production of acid protease.

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