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Utilization of Dairy Effluent for Food Grade Protease Production Using Bacillus *sp.*

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Abstract: Food grade proteases are proteyolytic enzymes having application in baking, food processing, protein modification etc. As a commodity product, pressure on protease market is on prize reduction and increasing performance. Hence our objective was to isolate a potent protease-producing microorganism and formulate a cost effective medium for neutral protease synthesis by the potent microbial culture. In order to achieve the objective, a proteolytic bacterium was isolated from soil using milk agar medium and the bacteria was identified as *Bacillus* sp. by morphological and biochemical characterization. Dairy industry effluent was then studied as a medium for neutral protease synthesis by the potent bacteria. Supplementation of mineral salt to the medium did not show profound influence of environmental factors such as medium pH, incubation temperature, agitation rate and incubation time on enzyme production. Optimum enzyme titers were found at pH7 when incubated at 37°C and 120 rpm 48 h. Dairy industry effluent was thus found to be a cost effective medium for neutral protease synthesis by *Bacillus* sp.

Keywords: Dairy Industry, Dairy Effluent, *Bacillus* sp., Neutral Protease, Milk Agar, Mineral Salt Medium

1. Introduction

Food grade proteases are proteyolytic enzymes having application in baking, food processing, protein modification etc. As a commodity product, pressure on protease market is on prize reduction and increasing performance. Industrial production of enzymes dates back to 1894 when 'fungal taka-diastase' was marketed for pharmaceutical use (Singh 1998). Enzymes have been produced commercially from plants, animals, and microbial source. However, microbial enzymes have the enormous advantage of being able to produce in large scale quantities by established fermentation techniques (Stanburg *et al*., 1995). At present, economically most important industrial enzymes are extracted from bacteria (*Bacillus* sp., *Staphylococcus* sp., (*Pseudomonas* sp.), fungi (*Aspergillus* sp., *Candida* sp., *Saccharomyces* sp.) and Actinomycetes (*Streptomyces* sp.). Over 300 tons of enzymes are being annually produced from *Bacillus* sp. and most of them are proteases (Kumar 1998). Solid as well as submerged fermentation has been widely used for the production of proteases (Pandy *et al.,* 2000a, Dunaevsky *et al*., 2000).

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. They are degradative enzymes, which catalyze 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 membranes. The current estimated value of the worldwide sales of industrial enzymes is $1 billion (Godfrey *et al*., 1996). Proteolytic enzymes are the most important industrial enzymes, representing worldwide sales of about 60% of the total enzyme market (Woods et al., 2001, Raju *et al.,* 1994, Mclntyre *et al*., 2000) and the industrial enzymes, 75% are of hydrolytic in nature.

The neutral proteases, which are active at neutral or weakly alkaline or weakly acidic pH include cysteine proteases, metalloproteases and some of the serine proteases. Thermolysin, a neutral protease, is the most thoroughly characterized member of metalloproteinase protease produced by *Bacillus sp*. Histidine residues from the

HEXXH motif serve as Zn ligands and Glu has a catalytic function (Weaver *et al*., 1977). Thermolysin produced by *B. stearothermophilus* is a single peptide without disulfide bridges and has a molecular mass of 34 kDa. It contains an essential Zn atom embedded in a cleft formed between two folded lobes of the protein and four Ca atoms, which impart thermo stability to the protein. Thermolysin is a very stable protease, with a half-life of 1 h at 80°C. Elastase produced by *Pseudomonas aeruginosa* is another important member of the neutral metalloprotease family. Recent studies showed that *Bacillus* species isolated from dairy effluent are used for the production of protease (T. M. Vijayalekshmi *et al.,* 2015).

Dairy industry waste water contain large amount of organic and inorganic substances organic matter fat, protein and carbohydrates and nutrients mainly nitrogen and phosphorus origination from the milk and the milk products. Micro organisms are considered potentially to be the most suitable sources of neutral protease for industrial application. Among the various bacteria *Bacillus* sp. was found to be the major group producing protease (Ferrero *et al*., 1996). Hence the present study also deals with the isolation of Protease producing *Bacillus* sp. from the dairy industry plant effluent. 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 also plays a major role in enzyme production (Afshan *et al.,* 2011, C. A, Mazzucotelli *et al.,* 2014). The optimization of media components and cultural parameters are the primary task in a biological process. So, that media components and cultural conditions are optimized by providing different incubation condition. Hence our objective was to isolate a potent protease-producing microorganism and formulate a cost effective medium for neutral protease synthesis by the potent microbial culture.

2. Materials

2.1. Sample Collection

Soil samples were collected in a sterile polythene bag from kitchen premises near the institution and immediately transferred to the laboratory for the isolation of bacteria.

2.2. Methods

2.2.1. Isolation of Protease Producing Bacteria

The bacteriological studies were carried by serial dilutions followed by plating and later this culture was used for further studies based on their morphological and biochemical characteristics as out lined 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 10 times. Each tube was shaken vigoursly.0.1ml solution was spread to petri plates containing milk agar medium (pH 7) and incubated for 24h at 37°C. Colonies showing proteolytic activity was selected and further purified. The milk agar medium contains (g/L) Skimmed milk powder 100**,** Peptone waste water 5, Agar 15g. Here milk powder was autoclaved separately.

2.2.2. Submerged Fermentation for Enzyme Production

The proteolytic bacteria was inoculated into 40 ml seed culture media in a 250 ml conical flask and kept in an environmental shaker at 120 rpm and 37°C for 24 h.

2.2.3. The Composition of Seed Culture Medium (LB Media PH 7)

Tryptone - 10gm
Yeast extract - 5gm
Sodium chloride - 10gm
Distilled water - 1000ml

1ml of 24h old seed culture was inoculated into 40ml of production media (10, 20, 30, 40, 50 and 100% V/V) in a 250ml of conical flask and incubated in an environmental shaker at 120rpm and 37°C for 48 h. In this study, waste water from Dairy Industry supplemented with mineral salt was treated as production medium. Diary wastewater was collected in a sterile bottle from Manjor milks, Kottayam, Kerala.

The culture medium was then centrifuged at 4°C and 7500rpm for 15 minutes. The supernatant was collected in vials and estimated for neutral protease activity.

2.2.4. Composition of Mineral Salt Medium

Yeast extract - 1gm
$NH_4)_2$ SO4 - 2gm
K_2HPO_4 - 1gm
KH_2PO_4 - 1g
$MgSO_4.7H_2O$ - 0.4gm
$MnSO_4.H_2O$ - 0.01gm
$FeSO_4.7H_2O$ - 0.1gm
Distilled water - 1000ml

2.2.5. Neutral Protease Assay

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

i. Reagents

0.2 M Potassium phosphate buffer (1M), pH 7.5
Casein (1%)
TCA (10%)
0.44M Na_2CO_3
Folin's reagent (1:1)

ii. Procedure

To 0.2 ml of enzyme extract, 0.5 ml of casein (1%) and 0.3 ml of 0.2 M phosphate buffer pH 7 was added. The reaction mixture was then incubated at 60°C for 10 minutes and arrested by adding 1 ml of 10% TCA. Then reaction mixture was then centrifuged at 3000 rpm for 15 minutes and to the supernatant collected, 5 ml of 0.44M Na_2CO_3 and 1 ml of 2 fold diluted folin Ciocalteau reagent was added. The

resulting solution was then incubated for 30 minutes at 30°C and absorbance read at 660 nm (Keay et al., 1970). One unit of enzyme activity can be defined as the amount of enzyme that liberated 1µg of tyrosine min^{-1} under assay condition and reported in terms of unit per ml.

2.3. Identification of Proteolytic Bacteria Isolated from Soil

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

2.3.1. Morphological Characters

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

2.3.2. Motility

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

2.3.3. Gram Staining

Gram staining was done using smear preparations from 18 h old 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 30 sec and rinsing in water followed this. After drying, slides were flooded with saffranin, a counter stain for 1 minute, again washed with gentle stream of water blotted to dryness and observed under oil immersion.

2.3.4. Biochemical Tests

i. 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 15 lb 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 48 h and the biochemical changes were observed.

ii. Citrate Test

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

iii. 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.

iv. Methyl Red Test

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

v. 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.

vi. 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.

vii. H_2S Production

This test is 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 a SIM agar media and incubated at 37°C for 48h. The observations were noted after 48h.

viii. 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°C for 48h. The observations were noted.

2.4. Maintenance of the Microorganisms

The microorganisms were maintained on nutrient agar slant and incubated for 24 hrs at 37°C. The slants were stored at 4°C in refrigerator and sub cultured regularly at every 2-week interval.

2.5. Growth of Potent Culture in Seed Culture Medium

The growth of the potent culture was determined by measuring the turbidity of the seed culture medium at 660 nm at an interval of 2 h up to 30h.

2.6. Effect of Mineral Salt Supplementation in Dairy Industry Wastewater for Protease Production

The efficiency of mineral salt supplementation in dairy wastewater was studied by comparing neutral protease production in both wastewater medium and also mineral salt supplemented wastewater medium. Both the medium was then inoculated with the potent culture and incubated at 37°C and 120 rpm for 48h.

2.7. Effect of PH on Protease Production

The medium pH was adjusted using 0.1N HCl or 0.1N NaOH. The influence of medium pH was studied at 4, 5, 6, 7, 8, and 9. The experiment was conducted in waste water medium.

3. Result and Discussion

3.1. Isolation of Proteolytic Bacteria

Table 1 showed the zone of hydrolysis produced by the

proteolytic bacteria. 9 proteolytic bacterial colonies were obtained in milk agar medium. Bacterial culture Sample 3 (S3) showed maximum hydrolytic zone (diameter 10 mm) was selected for further study. Figure 1 proteolytic activity observed with the potent culture on milk agar. Usually a plate assay 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 *et al*., 1987, Adesh *et al*., 2002).

Table 1. *Zone of hydrolysis by proteolytic bacteria.*

Bacterial cultures	Zone of hydrolysis (mm)
S1	0
S2	9
S3	10
S4	7
S5	6
S6	5
S7	5
S8	6
S9	8

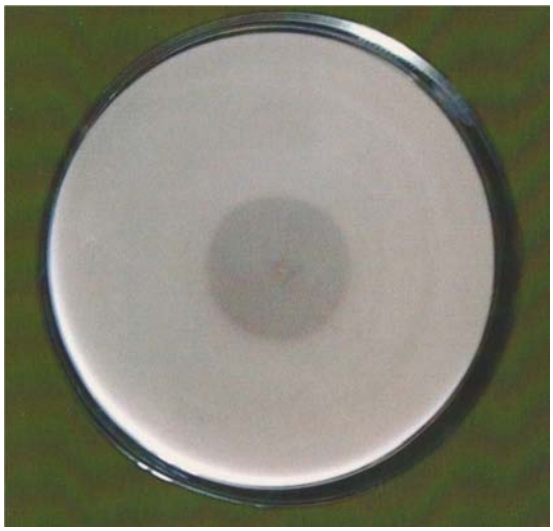

Figure 1. *Zone of hydrolysis.*

3.1.1. Production of Neutral Protease by Submerged Fermentation

Synthesis and secretion of extra cellular proteases by microorganisms was reported to be influenced by inducers and its concentration in the medium. Casein was used as an inducer for protease synthesis by *Aspergillus* sp (Nehara *et al*., 2004). The potent proteolytic bacterium obtained was then studied for neutral protease production by submerged fermentation. The potent culture was then inoculated into different concentrations of production medium and incubated for 48h at 37°C and 120 rpm. The effect of different concentrations of the medium on neutral protease synthesis is presented in figure 2. Optimum neutral protease synthesis was observed with 100% medium. Figure 3, A, B, and C were showed different stages of protein synthesis, A-production of crude enzyme, B – control dairy effluent alone C – purified enzyme by centrifugation respectively. Casein content in the waste water might be the inducer. This shows that mineral salt supplemented waste medium is an effective neutral protease producing medium.

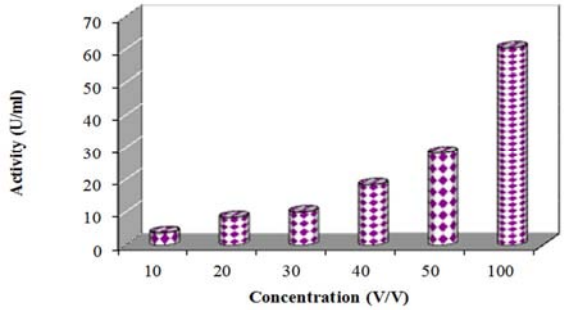

Figure 2. *Effect of different medium concentrations on neutral protease synthesis.*

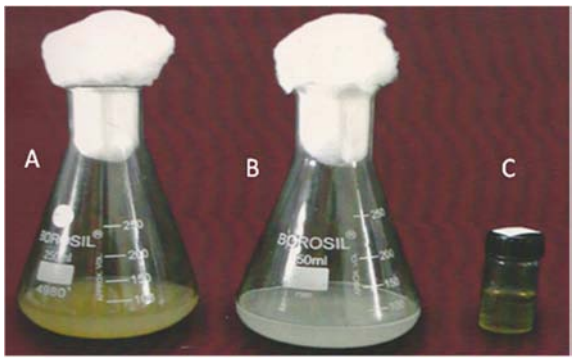

Figure 3. *(a) After production, (b) Control (c) Purified enzyme*

3.1.2. Identification of Proteolytic Bacteria

The result of the study is depicted in table 2.

Table 2. *Biochemical characterization of bacteria.*

Morphological characters	
Motility	Motile
Shape	Rod
Gram staining	+ ve
Pigmentation	-
Florescence	-
Biochemical characters Carbohydrate Fermentation	
Lactose	- ve
Glucose	- ve
Fructose	- ve
Sucrose	- ve
Mannitol	- ve
IMVIC TEST	
Indole	- ve
Methyl red	- ve
Vogues proskauer	- ve
Citrate utilization	- ve
Catalase	+ ve
H_2S production	- ve
Urease test	- ve

Experiments on morphological characters of the selected bacterial strain (S3) showed it was a Gram positive, rod shaped, motile organism. The organism was identified as *Bacillus cereus* by biochemical characterization. Maintenance of the potent culture on nutrient agar slants is shown in the figure 4.

Figure 4. *Stock Culture in agar slant.*

3.1.3. Growth of Potent Culture in Seed Culture Medium

The growth of the potent culture was determined by measuring turbidity of the medium at an interval of 2h. Figure 5 depicts the growth curve of the organism. 26h old culture showing exponential growth was selected for further study.

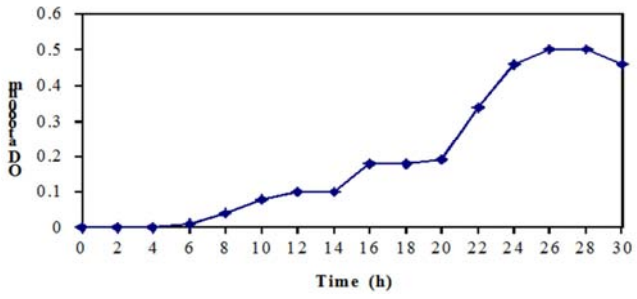

Figure 5. *Growth curve of the potent bacteria*

3.1.4. Comparison of Neutral Protease Synthesis in Mineral Salt Enriched Wastewater Medium and Waste Water

A comparative study on neutral protease production in both waste water and mineral enriched waste water medium showed increases the titers with waste water medium. The result of the study is given in figure 6. This showed that Dairy Industry waste water alone can be an effective medium for neutral protease production.

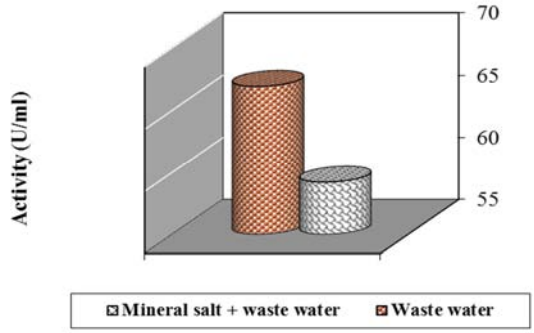

Figure 6. *Comparison of mineral salt supplemented and waste water medium.*

3.1.5. Effect of Medium PH on Neutral Protease Production

The study was carried out by changing medium pH. The result obtained is as shown in figure 7. From the result, it was clear that optimum neutral protease synthesis is at pH 7. The culture pH was reported to strongly affect many enzymatic processes and transport of various components across the cell membrane (Moon *et al*., 1991). Protease production was maximum when *Bacillus horikoshii* was grown in a medium of pH 9 while 6.5-7.5 was reported optimum pH for neutral protease production by *P. fluorescens* and *B. megaterium*.

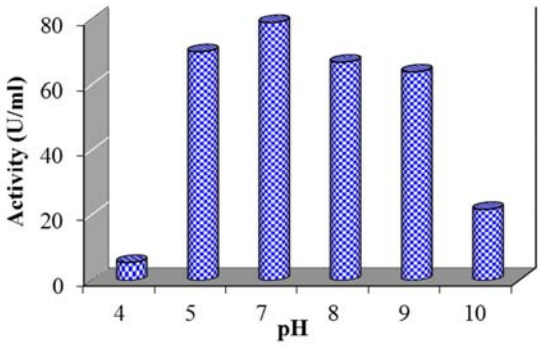

Figure 7. *Effect of medium pH on neutral protease synthesis.*

4. Conclusion

In the present study, a potent proteolytic bacteria was isolated from soil sample using milk agar medium. Dairy industry wastewater was found to be an effective production medium for neutral protease synthesis. The potent organism was identified as *Bacillus cereus* by morphological and biochemical characterization. A comparative study on effect of mineral salt supplemented wastewater and wastewater without mineral salt showed increased production with waste water medium. Medium pH was found to have a profound influence on neutral protease production by the potent organism. The optimum pH was found to be 7. From the

results it can be concluded that *B. cereus* is a potent bacteria and Dairy Industry waste water an effective medium for neutral protease synthesis. Detailed study require for the large scale production food grade protease.

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