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Studies on the Optimization of Protease Production by Thermophilic *Bacillus Subtilis* Isolated From Raw Milk Sample

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Abstract:
The present investigation produces more thermophilic protease enzymes and it to be applied for various applications. The milk samples were aseptically collected from different areas of Chidambaram in a sterile container. The proteolytic bacteria was isolated and further identified based on morphological and biochemical test indicate that the suspected organisms were Bacillus subtilis. Enrichment culture technique enabled the isolation of strains with proteolytic activity in skim milk agar plates. Among the 10 isolates two isolates Bacillus subtilis (B7) and Bacillus subtilis (B3) showed high proteolytic activity. These efficient two strains were selected for further studies. The efficient isolates of Bacillus subtilis (B7) and Bacillus subtilis (B3) were studied at different optimization parameters (pH, temperature, carbon source, nitrogen source) and incubation time for the protease production. The maximum protease production was recorded at 24 hrs of incubation (0.74 U/ml) by Bacillus subtilis (B7) isolates. In all the optimization studies Bacillus subtilis (B7) recorded maximum protease activity when compared to Bacillus subtilis (B3) isolates. The protease activity of purified enzyme was higher than of the crude enzyme isolate from the efficient Bacillus subtilis (B7).

Key Words: *Bacillus subtilis, Milk sample, Protease, Enrichment culture technique, Optimization studies.*

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I. Introduction

Proteases are a group of enzymes that have been found in several microorganisms like bacteria and fungi which are involved in breakdown of complex protein molecules into simple polypeptide chains (Absida, 1985). The induction of protease requires a substrate like peptone, casein and other proteins. The ammonia as final product of enzymatic reaction of substrate hydrolysis, responses enzyme synthesis by a well-known mechanism of catabolite repression. This extracellular protease has also been commercially exploited to assist protein degradation in various industrial processes (Srinubabu*et al*., 2007).

Extracellular protease high commercial value and multiple application in various industrial sectors, such as detergent, food, pharmaceutical, leather, diagnostic, waste management and silver recovery industries (Godfrey and West, 1996). Among proteases, alkaline proteases are defined as enzymes that are active from the neutral to the alkaline pH range (Gupta *et al*., 2002). These enzymes are generally active between pH 9.0 and 11.0 with the exception of a few higher pH values of about 12.0 and 13.0 (Kumar and Takagi, 1999).

Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments. *Bacillus* is a rod-shaped, gram positive, spore forming, aerobic, usually catalase positive, chemoorganotropic bacterium. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, and potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources (Akbalık, 2003).

Enzyme cost is also the most critical factor limiting wide use of alkaline proteases for different applications. A large part of this cost is accounted for the production cost of the enzyme which includes cost of media components as well as downstream processing. In submerged fermentation up to 40% of the total production cost of enzymes is due to the production on the growth substrate (Enshasy*et al*., 2010).

SSF is preferred over SMF since it exhibit advantages such as; reduced production cost, higher yield and less energy consumption (Pandey, 2003). Proteases are also envisaged as having extensive applications in development of eco-friendly technologies as well as in several bio-remediation processes (Wang *et al.*, 2008).

Most of the studies on microbial proteases are confined to characterization of enzymes with relatively fewer reports on optimization of enzyme production (Bajaj and Sharma *et al*., 2011).

Microbial proteases are among the most important hydrolytic enzymes and have been extensively since the advent of enzymology. They are essential constituents of all forms of life on earth. Prokaryotes, fungi, plants and animals. They can be cultured in large quantities in relatively short time by established fermentation methods and produce an abundant, regulate supply of the desired product. In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts. So, the present study was undertaken with the following objectives.

II. Materials And Methods

GENERAL METHODS

Cleaning of glassware

All the glassware's were soaked in cleaning solution (100g potassium dichromate was added to 100ml of distilled water followed by addition of 500ml of concentrated sulphuric acid) for about 12 hours and washed in tap water. They were thoroughly rinsed in tap water and dried. They were sterilized at 180ºC for 3 hours in hot air oven.

Sterilization

All the media were sterilized in an autoclave at 15lbs pressure for 20 minutes. The glass wares were sterilized at 180ºC for 3 hours in hot air oven.

Chemicals

All the chemicals used in the experiments were of analytical reagents (AG) grade and distilled water was used throughout the period of study.

SAMPLE COLLECTION

The raw milk sample was aseptically collected in a sterile container from ten different places of Chidambaram. The isolation of protease producing microorganisms was carried out under laboratory condition.

ISOLATION OF *Bacillus subtilis*

Both dilution plate and enrichment method were used for isolation of *Bacillussubtilis* from milk sample. For the enrichment method, 1 ml of sample was subjected to heat treatment for 10 min at 80°C in a water bath in order to kill most of the vegetative cells and thus to eliminate non - spore forming bacteria (Mora *et al.,* 1998). After heat treatment, the samples were transferred into 100 ml of skim milk agarmedium. Incubation was performed in a rotary shaker at 50°C until turbidity obtained. Then, 500 µl of the broth was platted on skim milk agar medium. For the dilution plate method, 1g of sample was transferred in 9 ml of 0.85% saline water. After pasteurization at 80°C for 10 min, 1 ml aliquot from each of the samples was transferred in 9 ml of 0.85% saline water and 6 fold dilutions were prepared. One ml of dilutions was platted on skim milk agar plates and incubated for 48 - 72 hrs at 37°C. Single colonies with different morphologies were picked and purified using streak plate method. After incubation Isolate showing the largest zone of clearance on all plates was selected for further studies. Protease production optimized in 100 ml medium contains (g/l): Trisodium citrate 10, peptone 10, $Mgso_4$ 0.5, K_2 Hpo_4 2, KCl 0.3, pH 6, 9-7.

COLONY CHARACTERIZATION

The *Bacillus* isolates were observed under the microscope, the colony morphology was noted with respect to colour, size, shape and nature of colony.

Microscopic characterization

LightMicroscopy

Gram staining

A drop of sterilized distilled water was taken on the middle of the clear slide. Then, a loopful bacterial suspension (young culture) was transferred to the sterilized drop of water and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it over the gentle flame for two or three times. The slide was flooded with crystal violet solution and allowed to stand for 30 sec and then washed thoroughly with gentle stream of tap water. The slide was then immersed in iodine solution for 1 minute and washed thoroughly with 95% alcohol for 10 sec. Alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then covered with Safranin for 1 minute. After washing with tap water and blotted dry it and examined under microscope.

Motility demonstration (Hanging drop method)

Vaseline was applied on four corners of the cavity slide and a loopful of bacterial culture was placed over the cover slip. A clean glass slide was placed on the cavity slide and it was gently pressed to form a seal between slide and the cavity slide. The slide was gently inverted without disturbing the drop and it was observed under high power objective along the margin of the drop.

Spore staining

One drop of sterile saline water was taken on a clean glass slide for spore staining. A loopful bacterial old slant culture was taken in the drop and smear was made on the slide. The film was dried over flame gentle heating. The slide was then placed over a beaker and 5% malachite green was added drop wise on the slide. Boiling of the malachite green was avoided by adding more malachite green. Then slide was taken out of the stream and washed gently with tap water. The preparation was needed with safranin solution for 1 minute and washed with gentle stream of tap water, and placed under immersion lens with immersion oil.

Biochemical characterization

Indole test

Peptone broth was prepared and sterilized and it was dispensed into sterile test tubes and culture was inoculated and incubated. After 24 hours, a few drops of Kovac's reagent were added. Formation of red color ring at top of the broth indicates positive result and yellow color indicates negative result.

Methyl red test

MR-VP broth was prepared and distributed into test tubes and it was sterilized. The test cultures was inoculated and incubated. After 24 hrs, a few drops of methyl red indicator were added. Formation of red color indicates positive result and yellow color indicates negative result.

Voges Proskauer test

In a sterilized MR-VP medium, the test cultures was added and incubated. After 24 hrs, Barrits reagent A and B was added respectively. Red color indicates positive result and yellow color indicates negative result.

Citrate utilization test

Sterile Simmon's citrate agar medium was prepared and poured in test tubes and sterilized. After sterilization, slants were made. The test organisms were streaked with the cultures and incubated at 37^{o} C for 24 hours. After incubation, Color Change from green to blue indicated positive result. No color change indicated negative result.

Triple sugar iron agar test

TSI agar slants were prepared and test cultures were streaked along the slants and the tubes are incubated at 37˚ C for 24 hrs. After 24 hrs the tubes are taken and examine the result.

Nitrate reduction test

Nitrate broth was prepared and dispensed into test tubes. The test tubes were sterilized and one loop full of cultures were inoculated and incubated for 24 hrs. After incubation, few drops of alpha naphthalamine and sulphanilic acid were added. The positive test indicated by red color formation.

Catalase Test

In a clean glass slide, a drop of bacterial suspension was placed on it. A drop of hydrogen peroxide was added to the culture. Evolution of bubbles indicates positive result. No change, negative result.

Urease Test

Urea agar was prepared and sterilized and poured into test tubes and slants were made. The test culture was streaked with the slants and incubated at 24 hrs. Color change from yellow to red indicates positive result. No color change indicates negative result.

Oxidase Test

The cultures were rubbed over the filter paper containing a reagent N-N tetra methyl paraphenylene diamine dihydrochloride. Purple colour indicated positive result.

Sugar fermentation

Nutrient broth was prepared with following sugar such as glucose, sucrose, lactose, maltose and mannitol. All these are prepared with indicator (phenol red). The broth was distributed in test tubes and Durham's tube were introduced and sterilized. The organism were inoculate in sugar tubes and incubate the culture for 24 - 48 hours at 37°C and Observe the results of sugar fermentation have recorded the color changes in broth and gas production, yellow color indicates positive and red color remains means negative.

Starch hydrolysis test

Starch agar medium was prepared and poured in sterile Petri plates. After solidification, the cultures were streaked in the centre of the plates and the plates were inverted and incubated at 37˚C for 24 hrs. After incubation, the plates were flooded with iodine solution for 30 sec. Blue black color was seen around the streak region indicates positive result. No blue black color indicates negative result.

Gelatin hydrolysis test

Gelatin medium was prepared and plated in a sterile Petri plates. After solidification, the test bacterial cultures were streaked in centre of plate and the inoculated plated were incubated at 37˚C for 24 hrs. After incubation, the hydrolyzing activity was tested by using mercuric chloride solution which was flooded on the gelatin agar surface. Formation of clear zone around the line of streak after the addition of mercuric chloride indicates positive result. No clear zone indicates negative result.

SCREENING OF PROTEASE ACTIVITY

The skim milk agar medium was used for the protease screening. After inoculation of the *Bacillussubtilis* isolates in skim milk agar medium, the plates were incubated for 3 to 4 days at 50°C. Opaque halos around the colonies were taken as the indication of protease activity.

PROTEASE ASSAY

The culture broth was incubated at 50˚ C in a rotary shaker operated at 200 rpm for 24 hrs. Afterwards the bacterial cell cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was collected and assayed for protease activity. The reaction mixtures containing 1ml enzyme solution and 1.5% casein were incubated in a water bath at 50˚C for 10 min. The supernatant was obtained by centrifugation at 10,000 rpm for 10 min. Next, 0.4M NA_2CO_3 and Folin's reagent were added to terminate the reaction, and the reaction mixture left to stand at room temperature for 10 min. Protease activity was determined spectrophotometrically at 660 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 U/ml of tyrosine per min at 660 nm under control conditions. Specific activity was expressed as units per mg of protein of the enzyme extract.

SOLID STATE FERMENTATION

Five gram of substrate (casein) was taken into a 250 ml (flask) Erlenmeyer flask and to this a protease production medium containing (g/1): trisodium citrate 10, peptone 10, $MgSO_4$ 0.5, K_2HPO_4, KCl 0.3, pH, 9-7. 1-2 drops tween 80 as emulsifier was added to adjust the require moisture level. The contents of the flasks were mixed thoroughly and autoclaved at 121˚C for 20 min.

Solid state fermentation was carried at 30˚C with substrate initial moisture content of 64% for 72 hrs using 2 ml spore suspension as inoculums. Studies were also performed to evaluate the influence of supplementation of substrate with different carbon sources such as Glucose, Maltose, Sucrose, Lactose, Galactose, Fructose (3% w/v) and nitrogen source such as Peptone, Ammonium chloride, Yeast extract, Meat extract, ammonium citrate (3%w/v).

OPTIMIZATION OF CULTURAL CONDITIONS FOR MAXIMUM PROTEASE PRODUCTION

pH Optimization

The effect of pH values was carried out to determine the optimum pH value for protease productivities by *Bacillussubtilis*. The pH was adjusted at pH – 5, pH – 6, pH - 7 pH – 8, and pH – 9 for the production media using 1 N NaOH or 1 N HCl. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical fl

Temperature Optimization

The effect of temperature values of was carried out to determine the optimum temperature value for protease productivities by *Bacillussubtilis*. Protease production was studied by incubating the production medium at 30^0C, 40^0C, 50^0C, 60^0C, 70^0C and 80^0C temperature. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Carbon sources Optimization

The effect of different carbon sources was studied for protease production by *Bacillussubtilis*. Four different carbon sources *viz*., Glucose, Maltose, Galactose, Lactose, fructose and Sucrose were selected for this present study. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Nitrogen sources Optimization

The effect of different nitrogen sources was studied for protease production by *Bacillussubtilis*. Four different nitrogen sources *viz*., Peptone, Ammonium chloride, tryptone, meat extract, Yeast extract and Ammonium citrate were selected for this present study. The protease production by replacing 1% trisodium citrate in the production medium with 1% and carbon source. The flasks were incubated at 55˚C on shaker for 24 hrs. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Incubation Optimization

The optimization of incubation time required for protease productivities by *Bacillus subtilis* was studied at 12, 24, 48, 72 and 96 hrs. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Purification of protease

Solid ammonium sulphate was added to the concentrated supernatant to a level of 80% 0˚C on the mixture was incubated overnight at 4˚C. Precipitated protein was then collected by centrifugation at 10,000×g for 20 min, the resulting pellet dissolved in 50 mM tris-HCL, pH 9.0, containing 2 mM $CaCl_2$ and dialyzed exhaustively against

the same buffer to remove residual Ammonium sulphate. The recovered solution was then used as the crude extract for further purification of the enzyme.

III. Result

CHARACTERIZATION OF *Bacillus subtilis* ISOLATED FROM RAW MILK SAMPLE

The proteolytic bacteria was isolated and further identified, characterized by their features as Gram positive, rod shaped motile organisms. The characteristics of the identified Bacterial isolates were showed in Table-1. Finally, the morphological and biochemical test tentatively indicated that the isolated organisms were *Bacillus subtilis*.

SCREENING OF *Bacillus subtilis* FOR ITS PROTEASE PRODUCTION

The culture techniques enable the isolation of strains with proteolytic activity on Skim milk agar plates. In total 10 isolates were isolated from the raw milk samples. Among them, two isolates *Bacillus subtilis* (B7) (16 mm) and *Bacillus subtilis* (B3) (15 mm) Table - 2. Showed maximum proteolytic activity and these two strains were taken for further studies.

PROTEASE ASSAY

Protease activity was determined at 50˚C by using casein as a substrate and the results were showed in Table - 3. The enzyme activity was higher in the isolate *Bacillus subtilis* (B7) (0.68 U/ml) when compared to *Bacillus subtilis* (B3) (0.59 U/ml).

OPTIMIZATION PROCESS FOR PROTEASE PRODUCTION

Effect of pH

The effect of isolated *Bacillus subtilis* isolates (B3 and B7) for the protease enzyme production were studied at different pH(pH – 5, pH – 6, pH – 7, pH – 8, and pH – 9) the results were presented in Table – 4. Among the two *Bacillus subtilis* isolates, maximum protease activity was observed by the *Bacillus subtilis* (B7) followed by *Bacillus subtilis* (B3). Maximum protease production was noticed by the *Bacillus subtilis* (B7) at pH – 8 (0.80 U/ml) followed by pH – 9 (0.54 U/ml), pH – 7 (0.76 U/ml), pH – 6 (0.66 U/ml), pH – 5 (0.57 U/ml).

Effect of temperature

The effect of isolated *Bacillus subtilis* isolates (B3 and B7) for the protease enzyme production were determined at different temperatures (30^0C, 40^0C, 50^0C, 60^0C, 70^0C and 80^0C) the results were given in Table – 5. Among the two *Bacillus subtilis* isolates, maximum protease activity was observed by the *Bacillus subtilis* (B7) followed by *Bacillus subtilis* (B3). Maximum protease production was observed by the *Bacillus subtilis* (B7) at 60^0C (0.78 U/ml) followed by 50^0C (0.74 U/ml), 40^0C (0.58 U/ml), 30^0C (0.47 U/ml), 70^0C (0.50 U/ml) and 80^0C (0.45 U/ml). The enzyme activity was decreased in the temperature of 70^0C and 80^0C.

Effect of carbon sources

The effect of isolated *Bacillus subtilis* isolates (B3 and B7) for the protease enzyme production were evaluated on the presence of different carbon sources(Glucose, Maltose, Lactose, Galactose, Fructose and Sucrose)the results were presented in Table – 6. Among the two *Bacillus subtilis* isolates, maximum protease activity was recorded by the *Bacillus subtilis* (B7) followed by *Bacillus subtilis* (B3). Maximum protease production was observed by the *Bacillus subtilis* (B7) in the presence of lactose (0.81 U/ml) followed by Sucrose (0.52 U/ml), Glucose (0.49 U/ml), Maltose (0.46 U/ml), Galactose (0.27 U/ml) and Fructose (0.28 U/ml).

Effect of nitrogen sources

The isolated *Bacillus subtilis* isolates (B3 and B7) were estimated for its protease enzyme production against the different nitrogen sources(Peptone, Ammonium chloride, Meat extract, Yeast extract, tryptone and Ammonium citrate) the results were showed in Table – 7. *Bacillus subtilis* isolate (B7) exhibit maximum protease production than the *Bacillus subtilis* (B3) isolate. Maximum protease production were exhibited by the isolate (B7) in the presence of ammonium citrate as nitrogen source (0.74 U/ml) followed by Ammonium chloride (0.68 U/ml), Tryptone (0.61 U/ml), peptone (0.55 U/ml), Yeast extract (0.52 U/ml), and Meat extract (0.43 U/ml).

Effect of Incubation

The effect of isolated *Bacillus subtilis* isolates (B3 and B7) for the protease enzyme production were studied at different incubation hours (12, 24, 48, 72 and 96)the results were

presented in Table – 8. Among the two *Bacillus subtilis* isolates, maximum protease activity was recorded by the *Bacillus subtilis* (B7) followed by *Bacillus subtilis* (B3). Maximum protease production was observed by the *Bacillus subtilis* (B7) at 24hrs (0.74 U/ml) followed by 48hrs (0.68 U/ml), 72 hrs (0.60 U/ml), 12 hrs (0.42 U/ml), and 96 hrs (0.51 U/ml).

PARTIAL PURIFICATION OF PROTEASE

The protease enzyme was partially purified by APS and the results were presented in Table – 9. Purified enzyme exhibited the maximum protease activity in *Bacillus subtilis* (B7) (1.66 U/ml) followed by *Bacillus subtilis* (B3) (1.25 U/ml).

Table: 1 Characterization of *Bacillus subtilis*

S.No.	Test	Results
1.	Gram staining	Gram positive, thick, short rods.
2.	Endospore	Central spores present
3.	Motility	Non-motile
4.	Catalase	Positive
5.	Oxidase	Negative
6.	Nutrient agar	Large, circular, white, adherent, colonies, with membraneous growth
7.	MacConkey agar	Non-lactose fermenting colonies
8.	Glucose fermentation	Acid produced
9.	Mannitol fermentation	Acid produced
10.	Sucrose fermentation	Not fermented
11.	Dextrose fermentation	Not-fermented
12.	Indole	Negative
13.	Methyl Red Test	Negative
14.	Voges Proskauer Test	Positive
15.	Citrate utilization	Positive
16.	Oxidative Fermentative Test	Positive
17.	Nitrate reduction	Positive
18.	Gelatin hydrolysis	Positive
19.	Starch hydrolysis	Positive
20.	Urease	Negative

(+ Positive, -Negative)

Table – 2: Screening of *Bacillus subtilis* for its protease production

Sample	Isolates	Zone of inhibition (mm in dm)
Raw milk sample	B1	9
	B2	8
	B3	15
	B4	9
	B5	9
	B6	7
	B7	16
	B8	9
	B9	8
	10	6

Table – 3: Protease assay

S. No	Isolates	Protease activity U/ml
1	*Bacillus subtilis* (B7)	0.68
2	*Bacillus subtilis* (B3)	0.59

Table – 4: Different pH on protease production by *Bacillus subtilis* isolates

Different pH	Enzyme production U/ml	
	Substrate (casein)	
	***Bacillus subtilis* (B3)**	***Bacillus subtilis* (B7)**
5	0.52	0.57
6	0.62	0.66
7	0.72	0.76
8	0.76	0.80
9	0.51	0.54

Table – 5: Different temperature on protease production by *Bacillus subtilis* isolates

Different temperature	

(˚C)	Enzyme production U/ml	
	Substrate (casein)	
	***Bacillus subtilis* (B3)**	***Bacillus subtilis* (B7)**
30	0.43	0.47
40	0.54	0.58
50	0.71	0.74
60	0.74	0.78
70	0.47	0.50
80	0.42	0.45

Table -6 Different carbon sources on protease production by *Bacillus subtilis* isolates

Different carbon sources	Enzyme production U/ml	
	Substrate (casein)	
	***Bacillus subtilis* (B3)**	***Bacillus subtilis* (B7)**
Fructose	0.25	0.28
Galactose	0.26	0.27
Maltose	0.41	0.46
Glucose	0.46	0.49
Sucrose	0.49	0.52
lactose	0.76	0.81

Table -7 Different nitrogen sources on protease production by *Bacillus subtilis* isolates

Different nitrogen sources	Enzyme production U/ml	
	Substrate (casein)	
	***Bacillus subtilis* (B3)**	***Bacillus subtilis* (B7)**
Ammonium chloride	0.65	0.68
Meat extract	0.37	0.43
Yeast extract	0.47	0.52
Peptone	0.51	0.55
Tryptone	0.58	0.61
Ammonium citrate	0.70	0.74

Table - 8: Different incubation on protease production by *Bacillus subtilis* isolates

Different incubation time (hrs)	Enzyme production U/ml	
	Substrate (casein)	
	***Bacillus subtilis* (B3)**	***Bacillus subtilis* (B7)**
12	0.37	0.42
24	0.71	0.74
48	0.64	0.68
72	0.56	0.60
96	0.47	0.51

Table -9 Purified Enzyme Activity (APS)

S.No	Samples	Protease activity U/ml
1	*Bacillus subtilis*	1.66
2	*Bacillus subtilis*	1.25

PLATE – 1: RAW MILK SAMPLES

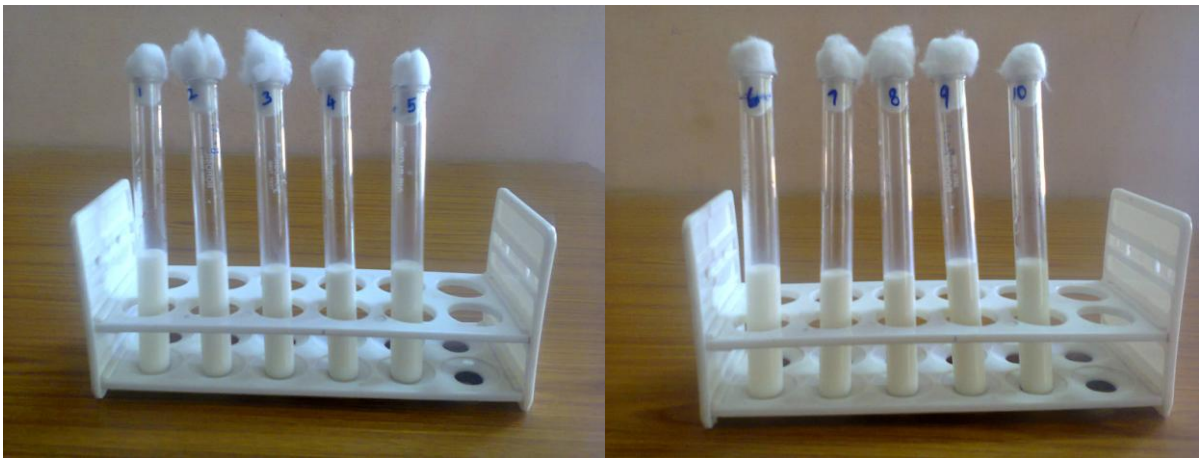
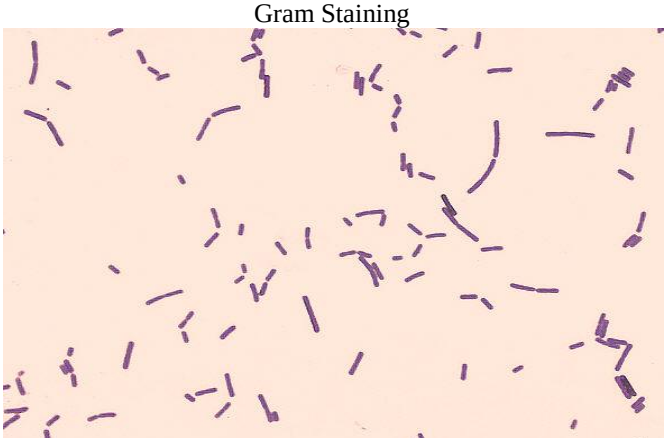


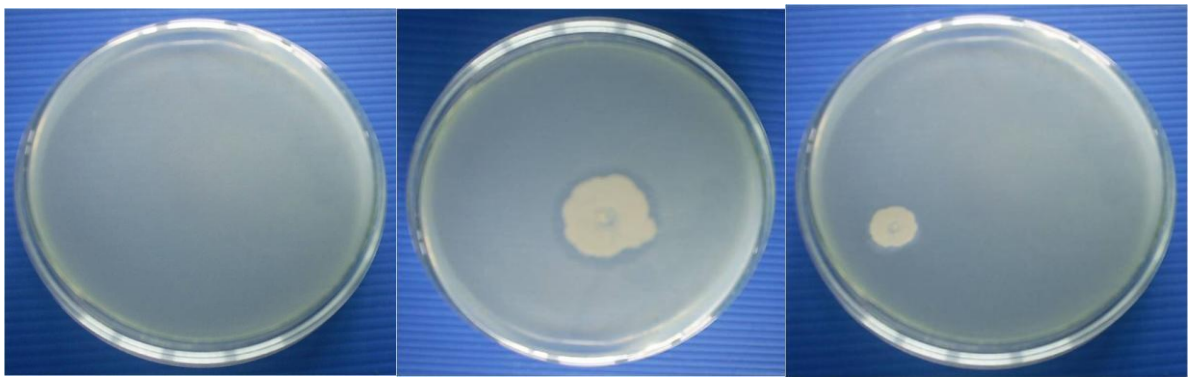
PLATE - 2: PHENOTYPIC CHARACTERIZATION

Gram Staining



Gram (+ve) Rod -*Bacillus* sp.

PLATE – 3: *Bacillus subtilis* IN SKIM MILK AGAR



Control*Bacillus subtilis* B7 ISOLATE*Bacillus subtilis* B3 ISOLATE

PLATE – 4: EFFECT OF pH ON PROTEASE PRODUCTION BY *Bacillus subtilis*

PLATE – 5: EFFECT OF TEMPERATURE ON PROTEASE PRODUCTION BY *Bacillus subtilis*

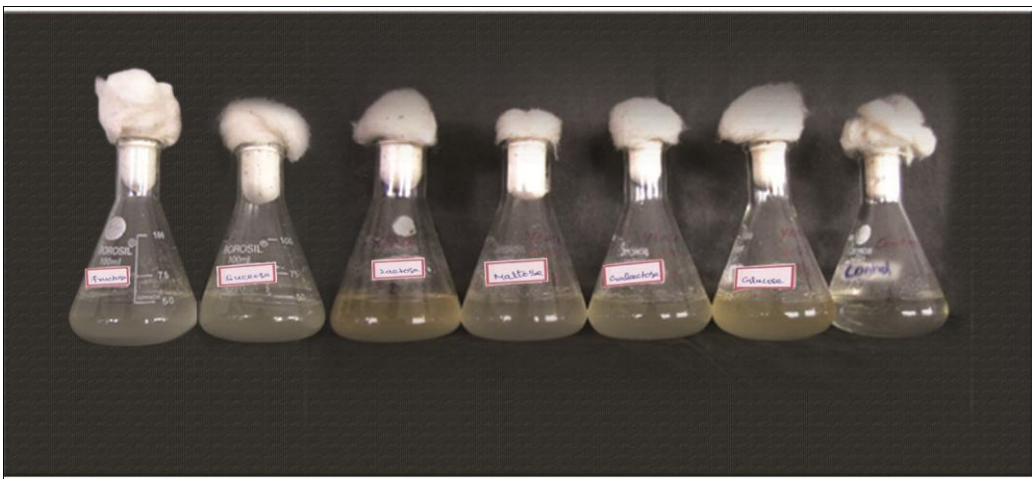


PLATE – 6: EFFECT OF CARBON SOURCES ON PROTEASE PRODUCTION BY *Bacillus subtilis*

PLATE – 7: EFFECT OF NITROGEN SOURCES ON PROTEASE PRODUCTION BY *Bacillus subtilis*

PLATE – 8: EFFECT OF DIFFERENT INCUBATION PERIOD ON PROTEASE PRODUCTION BY *Bacillus subtilis*

IV. Discussion

Proteases are enzymes occurring everywhere in nature be it inside or on the surface of living organisms such as plants, animals and microbes. Proteolytic enzymes (proteases) are ubiquitous being found in all living organisms and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various sectors. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases which account for the total worldwide enzymes sale (Beg *et al.*, 2003; Ellaiah*et al*., 2003; Nascimento and Martins, 2004).

Microbial proteases are one of the important groups of industrially and commercially produced enzymes contributing approximately 2/3 of all enzyme sales. Through proteases are produced by many microorganisms, emphasis is on the microorganisms producing proteases with desired characters. As demand for novel proteases is increasing day by day the initial screening methods and assays for protease detection are of utmost importance (Ramesh *et al*., 2011). The present study was carried out to evaluate the protease activity of the proteolytic bacteria *Bacillus subtilis* isolated from raw milk samples. To understand the biochemistry of protease degrading bacteria *Bacillus subtilis*, it is needed to optimize under various physical and chemical parameters.

Maximum protease production was obtained in the medium supplemented with 1% skim milk, 1% starch and 0.6% MgSO4.7H2O, initial pH 8.0 at 35°C. The best enzyme production was obtained during the

stationary phase in which the cell density reached to $1.8x10^8$ cells/ml. The level of protease was found to be low in the presence of inorganic nitrogen *sources* (Fikret*et al*., (2011).Protease production is often related to the sporulation stage in many *Bacilli*, such as *Bacillus subtilis* (O'Hara and Hageman, 1990) and *B. licheniformis* (Hanlon and Hodges, 1981).

The optimum pH of this enzyme is comparable to several proteases reported that have an optimal pH close to 10 (Seifzadeh*et al*., 2008; Patel *et al*., 2006). Optimum pH ranges were reported to be suitable for maximum protease production including pH 8 (Gupta and Beg, 2003), pH 8.5 (Sanchez-Porro*et al.*, 2003), pH 10.5 (Banik and Parakash, 2004), pH 11 (Beg and Gupta, 2003) and pH 12 (Olivera *et al*., 2006). The enzyme was stable over a broad pH range of 6.0 to 8.6 at 80ºC, indicating that it is neutral enzyme.The organisms were able to grow in the pH range 6-10. However, alkaline protease secretion was best at pH 8. Similar results were recorded by Lee and Chang (1990) .Alkaline proteases from *Bacillus subtilis* were shown to be optimally active at 50˚C; similar to *Bacillus* thermophilic alkaline proteases reported earlier (Hawumba*et al*., 2002). This was supported by(El-Safey and Abdul- Raouf 2004). Madzak*et al.* (2000) recorded that the sucrose is good substrate for production extracellular proteases. Glucose was found to be the optimum carbon source for protease activity by all the four *Bacillus* isolates followed by sucrose, fructose, maltose, starch and cellulose (Boominadhan and Rajakumar, 2009). The best nitrogen source for protease production was beef extract for *Bacillus* sp. K-30, while yeast extract and tryptone were comparable (Naidu and Devi, 2005). Maximum protease production was observed by the *Bacillus subtilis* (B7) at 24hrs (0.74 U/ml) followed by 48hrs (0.68 U/ml), 72 hrs (0.60 U/ml), 12 hrs (0.42 U/ml), and 96 hrs (0.51 U/ml). Naidu and Devi (2005) reported that Maximum production of proteases with 48 to 72 h of incubation by bacteria.

The purification of proteases is important from the perspective of developing a better understanding of the functioning of the enzyme (Tsai *et al*., 1988). Precipitation is the most commonly used method for the isolation and recover of proteins from crude biological mixtures (Bell *et al*., 1983). It also performs both purification and concentration steps. Precipitation by ammonium sulfate is used in acidic and neutral pH solutions (Aunstrup, 1980). In our study also protease enzyme was partially purified by using Ammonium Sulphate Precipitation method.

V. Conclusion

Protease constitutes one of the most important groups of commercial enzymes accounting for approximately 60-65% of the global enzyme market. Proteases can be produced from a wide range of organisms such as Bacteria, yeast, molds, plants and animals. However, bacretial proteases represent an excellent source of protease enzymes in comparison with others. Thermophilic protease are of particular intrest for Bioengineering and Biotechnological applications. Proteolytic bacteria isolated from the milk samples, these isolates were studied in respect of enzyme activity, purification. The best results are obtained with casein as substrate in the pH-8, temperature 60˚C, lactose as carbon source, Ammonium citrate as the nitrogen source. The present investigation produce more thermophilic protease enzymes and it to be applied for various applications.

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