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Studies on The Optimization of Protease Production by Thermophilic *Bacillus subtilis* Isolated from Raw Milk Sample

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ABSTARCT
The milk samples were aseptically collected. 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 (B3) were studied at different optimization parameters viz., pH, Temperatures, carbon source, nitrogen source and incubation time for the protease production. 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 effiecient Bacillus subtilis (B7).
KEYWORDS: *Isolation, Bacillus subtilis, parameters, Protease assay and Skim milk*

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INTRODUCTION

Proteases are a group of enzymes that have been found in several microorganism like bacteria and fungi which are involved in breakdown of complex protein molecules into simple polypeptide chains [1].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 reaction synthesis by a well known mechanism of catabolite repression .This extracellular protease has also commercially exploited to assist degradation in various industrial processes [10].

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 [4]. Microbial protease are ubiquitous ,physiological and regulatory functions .Microbial proteases ,especially from Bacillus sp. Have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulation [2].

Protease are among the most valuable catalysts used in food pharmaceutical and detergent industries because they hydrolyse peptide bonds in aqueous environment and synthesize peptide bonds in microaqueous environments [6].Microbial proteases dominate the commercial applications with large market share taken by subtilize proteases from Bacillus sp .The thermostable proteases are advantageous in some application ,due to employing higher processing temperatures ,thus yielding faster solubility of nongaseous reactants and products and reducing incidence of microbial contamination by mesophilic organisms. Proteases secreted from thermophilic bacteria are unique and have become increasingly useful in wide range of commercial applications [2].

The potential use of thermostable enzyme in range of biotechnological application is widely acknowledged .Thermostable proteases are advantageous in some application because higher processing temperature can be employed ,resulting faster reaction rates due to s decrease in viscosity and increase in diffusion co-efficient of substrates .Microbial proteases are among the most important hydrolytic enzymes and have been extensively since the advent of enzymology [7].

MATERIAL AND METHODS

ISOLATION OF BACILLUS SUBITILS

For the enrichment method ,1 ml of sample was subjected to heat treatment for 10 min at 80^0C 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 transformed into 100 ml of skim milk agar medium .Incubation was performed in a rotatory shaker at 50^0C 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 80^0C 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 hours at 37^0C. 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_2Hpo_4 - 2,KCL 0.3, pH -6,9-7.

COLONY CHARACTERIZATION

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

MICROSCOPIC CHARACTERIZATION

Light Microscopy

***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 od water and a very thin film was prepared on the slide 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 od red color indicates positive result and yellow color indicates negative result.

Voges Proskauer test : In sterilized MR-VP medium, the test cultures was added and incubated. After 24 hours , 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 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 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 37^0C for 24 hours . After 24 hours 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 hours. 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 hours . 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 methyl paraphenylene diamine dihydrochloride. Purple color 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 37^0C and observe the results of sugsr 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 center of the plates and the plates were inverted and incubated at 37^0C for 24 hours. 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 37^0C for 24 hours. 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 PROTESE ACTIVITY

All the isolates were subjected to screen the protease activity using skim milk agar. After inoculation of the *Bacillus subtilis* isolates in skim milk agar medium , the plates were incubated for 3 to 4 days at 50^0C. Opaque halos around the colonies were taken as the indication of protease activity.

PROTEASE ASSAY

The culture broth was incubated at 50^0C in a rotary shaker operated at 200 pm for 24 hours. Afterwards the bacterial cell cultures were centrifuged at 10,000 rpm for 10 mins. The reaction mixtures containing and assayed for protease activity. The reaction mixtures containing 1 ml enzyme solution and 1.5 % casein were incubated in a water bath at 50^0C for 10 min. The supernatant was obtained by centrifugation at 10,000 rpm for 10 mins . Next , 0.4 M NA_2CO_3 and Folin's reagent were added to terminate the reaction, and the raction 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 1U/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/l): 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 121^0C for 20 min. Solid state fermentation was carried at 30^0C with substrate initial moisture content of 64% for 72 hours using 2 ml spore suspension as inoculums. Studies were also performed at evaluate the influence of supplementations 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 – 5, pH – 6, pH – 7, pH – 8 and pH – 9 for production media using 1 N NaOH or N HCl. Protease production using solid state fermentation in different production vessels: enzyme production was studied in 250 ml conical flask.

Temperatures and Optimization: The effect of temperatures values of was carried out to determine the optimum temperatures value for protases productivities by *Bacillus subtilis* .Protease production was studied un 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 *Bacillus subtilis*.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 *Bacillus subtilis* .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 55^0C on shaker for 24 hours . 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 hours. 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% 0^0C on the mixture was incubated overnight at 4^0C. Precipitated protein was then collected by centrifugation at 10,000 x g for 20 mins , 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.

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 shown 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 sample. Among them, two isolates *Bacillus subtilis* (B7) (16 mm) and *Bacillus subtilis* (B3)(15mm) Table-2. Showed maximum proteolytic activity and these two strains were taken for further studies.

Protease assay: Protease activity was determined at 50^0C by using casein as asubstrate and the result 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)(0.59U/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 viz., pH-5, pH-6, pH-7, pH-8 and pH-9, the results were presented in table- 4. Among the two *Bacillus subtilis* , maximum protease activity was observed by the *Bacillus subtilis*(B 7) followed by *Bacillus subtilis*(B 30). Maximum protease production was noticed by the *Bacillus subtilis*(B 7) 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(0.57U/ml).

Effect of Temperature*:* The effect isolated *Bacillus subtilis*(B3 and B7) for the protease enzyme production were determined at different temperatures viz., 30^0C, 40^0C, 50^0C, 60^0C, 70^0C and 80^0C, the result 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(0.78U/ml) followed by 50^0C(0.74U/ml), 40^0C(0.58U/ml), 30^0C(0.47U/ml) 70^0C(0.50 U/ml) and 80^0C(0.45U/ml).The enzyme temperature 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 viz. Glucose, Maltose, Lactose, Galactose, Fructose and Sucrose and the results were presents in the table -6.Among the two *Bacillus subtilis* isolates ,maximum activity was recovered by *Bacillus subtilis*(B7) followed *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 (0.28U /ml).

Effect of Nitrogen source*:* The isolated bacillus subtilis(B3 and B7) were estimated for its protease enzyme production against the different nitrogen source viz., Peptone, Ammonium chloride, Meat extract, Yeast Extract , Tryptone and Ammonium citrate and the results were showed in Table-7. *Bacillus subtilis* isolates (B7) exhibit maximum protease production than the *Bacillus subtilis*(B3) isolate. Maximum

protease production were exhibited by the isolates (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 (0.55U/ml), Yeast extract (0.52 U/ml), and Meat extract(0.43 U/ml).

Effect of Incubation*:* The effect of isolated *Bacillus subtilis* (B3 and B7) for the protease enzyme production were studied at different incubation hours viz., 12,24, 48,72 and 96 and 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 production was observed by the *Bacillus subtilis* (B7) at 24 hours (0.74 U/ ml) followed by 48 hours (0.68 U /ml), 72 hours (0.60 U/ml), 12 hours (0.42 U/ ml) and 96hours(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*(B 3) (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

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
	B10	6

TABLE – 3: Protease assay

S. No	Isolates	Protease activity U/ml
1.	*Bacillus subtilis*	0.68
2.	*Bacillus subtilis*	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 (^{0}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 source on protease production by *Bacillus subtilis* isolates

Different carbon source	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 source on protease production by *Bacillus subtilis* isolates

Different nitrogen source	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 nitrogen source 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

DISCUSSION

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 micro organisms , emphasis is on the micro organisms 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. Santong and Warangkana [9] isolated *Bacillus* species from raw milk samples using LB supplemented with 2% skimmed milk as a selective media. They isolated a 41 isolates with a clear zone. Among the 41 isolates they selected 10 isolates that exhibit a maximum zone of inhibition more than 10mm were selected and evaluated for the presence of protease activity.

In the present study, the protease producing bacterial strains were isolated from raw milk sample and identified as *Bacillus subtilis.* Enrichment culture techniques enable the isolation of stains with proteolytic activity in skim milk agar plates. Totally 10 isolates were isolated from the raw milk samples. Among them, two isolates *Bacillus subtilis* B7(16mm) showed high proteolytic activity followed by *Bacillus subtilis* B5(mm).Maximum protease production was obtained in the medium supplemented with 1% skim, 1% starch and 0.6% MgSO4.7H2O, initial pH 8.0 at 35^0C. The best enzyme production was obtained during the stationary phase in which the cell density reached to 1.8x 10^8 cells/ml. The level of protease was found to be low in the presence of inorganic nitrogen sources [4].

The effect of isolated *Bacillus subtilis* isolates for the presence enzyme production was studied at different pH *viz.,* pH-5, pH-6, pH-7, pH-8 and pH-9. 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 isolate *Bacillus subtilis* (B7) at pH-8(0.80 U/ml) followed by pH-5(0.57 U/ml), pH-6 (0.66 U/ml), pH-7(0.76 U/ml) and pH-9 (0.54 U/ml). The organisms were able to grow in the pH range 6-10. However, alkaline protease secretion was best at pH 8 [8, 9].The effect of isolated *Bacillus subtilis* isolates for the protease enzyme production was determined at different temperatures *viz.,* 30^0C,40^0C,50^0C,60^0C,70^0C and 80^0C. Among the two *Bacillus subtilis* isolates, maximum protease activity was observed by the *Bacillus subtilis* (B7). Followed by the *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), 70^0C(0.50 U/ml), 30^0C(0.47U/ml) ,80^0C(0.45 U/ml) .The enzyme activity was decreased in the temperature of 70^0C and 80^0C.

The effect of isolated *Bacillus subtilis* isolates for the protease enzyme production was determined at different carbon source *viz.,*Glucose ,Sucrose , Lactose , Maltose ,Galactose and Fructose. Among the two *Bacillus subtilis* isolates , maximum protease activity was observed by the *Bacillus subtilis* isolates , maximum protease activity was observed by the *Bacillus subtilis*(B7) followed by the *Bacillus subtilis*(B3). Maximum protease production was observed by the *Bacillus subtilis*(B7) in the presence of Lactose (0.81 U/ml) followed by Glucose (0.49 U/ml), Sucrose (0.52 U/ml), Maltose (0.46 U/ml), Galactose (0.27 U/ml), Fructose (0.28 U/ml). Lactose was the bestcarbon source that induced the production of protease by the *Bacillus subtilis.* [3].

The isolated *Bacillus subtilis* isolates (B3 and B7) were estimated for its protease enzyme production against the different nitrogen sources *viz.,* Peptone, Ammonium chloride , Meat extract , Yeast extract , Tryptone and Ammonium citrate *Bacillus subtilis* isolates (B7) exhibit 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 (0.68U/ml), Tryptone (0.61 U/ml),Peptone (0.55 U/ml), Yeast extract (0.52 U/ml), and Meat extract (0.43 U /ml).The different incubation hours used for present study *viz.,* 12,24,48,72 and 96 . The maximum protease activity was observed by *Bacillus subtilis* (B7) followed by *Bacillus subtilis* (B3). Maximum protease production was observed by the *Bacillus subtilis*(B7) at 24 hrs (0.74 U/ml) followed by 48 hrs (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) maximum production of proteases with 48 to 72 h if incubation by bacteria..

CONCLUSION

Protease constitutes one of the most important groups of commercial enzymes accounting for approximately 60-65% of global enzyme market. Proteases can be produced from a wide range of organisms such as Bacteria, Yeast, Molds, Plants and animals. Thermophilic protease are of particular interest 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 wit casein as substrate in the pH – 8, temperature 600C, lactose as carbon source , Ammonium

citrate as the nitrogen source. The present investigation produces more thermophilic protease enzymes and it to be applied for various applications.

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