

PRODUCTION OF PROTEASE FROM *Bacillus species* USING AGRICULTURAL WASTE BY SUBMERGED FERMENTATION

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Abstract :

The present investigation carried out on production of protease from *Bacillus species* using agricultural waste by submerged fermentation. 10 different soil sample was collected from Department of Microbiology, RTMNU, Nagpur. Out of 10 soil samples four soil samples were confirmed for the production of protease enzyme from *Bacillus species*. The present study describes the screening of two agricultural wastes i.e. gram husk and wheat husk to enhance the production of protease enzyme by *Bacillus species* by submerged fermentation. The present study on the optimization of protease production with respect to incubation time and it results that protease production increased with the use of agro-industrial waste like gram husk and wheat husk. The best result was obtained by *Bacillus megaterium* at incubation time of 4 days (96h) yielded 0.144mg/ml protease production by using wheat husk as a substrate (agrowaste). The environmental conditions of the fermentation play a vital role in the growth and metabolic production of microbial population. Optimization of different medial components can greatly affect the enzyme production cost.

Keywords : Protease, *Bacillus species*, Agricultural Waste, Submerged Fermentation

Introduction :

The agro wastes are being dumped into the domestic environment causing unhygienic to the residents, and foul odor is becoming a common problem. Most of the agricultural wastes are contributing solids wastes, which are becoming hazardous to the public. By utilize the agro wastes for the bioconversion process we can reduce the environmental pollution problems. Solid wastes from Indian cities contain high proportion of organic matter and also have high moisture content. The organic food content attracts flies and rodents that cause foul smell. Among the solid waste the agricultural wastes comprises 50-65 %. The agricultural waste from animal manual and crop residue . The principle sources of solid waste are domestic commercial, industrial and agricultural activities. Agricultural wastes comprise both crop residues and animal wastes such as manure and urine [1]. A protease is any enzyme that starts protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. They perform both degradative and synthetic functions. With respect to their physiological roles as well as their commercial applications proteolytic enzymes are considered one of the most useful of enzyme groups [2]. Proteolytic enzymes from microorganisms may be located within the cell (intracellular), cell wall associated (periplasmic), or excreted into the media (extracellular) [3]. Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch, and the digested products are transported into the cell where they are used as nutrients for growth [4]. Intracellular proteases



are important for different cellular and metabolic processes such as protein turnover, sporulation and differentiation, maturation of hormones and enzymes and maintenance of the cellular protein pool. Extracellular proteases are vital for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products [5]. They have also been commercially exploited to help protein degradation in industrial processes [6].

Conventionally, commercial production of proteases has been carried out using submerged fermentation (SmF). SmFs are usually carried out with a substrate, which is either dissolved or remains suspended in an aqueous medium. Almost all the large scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling. To meet the growing demands in the industry, it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production. Purification of protease is carried out using different technique which includes precipitation method, ion-exchange chromatography and electrophoretic method. Purification by precipitation can be carried out by inorganic salts, organic solvents and high molecular weight polymers. The most commonly used salt is ammonium sulphate. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method which is used to identify and monitors proteins during purification, to obtain the purified fraction and to determine their molecular weights. Single band in such a gel is a criterion of purity. The enzyme once isolated and purified, is characterized to determine the effect of pH, temperature, additives, inhibitors and chelators on its activity and stability [7]. The global demand of enzymes, for a wide variety of applications, is significant. Proteases have extensive application in food, detergent, leather and pharmaceutical industries. In addition, they are also involved in management of waste from domestic and industrial activities.

MATERIALS & METHODS:

Sample collection:

To study the proteolytic activity of various bacterial isolates, soil sample was collected from Department of Microbiology, RTMNU, Nagpur. The sample was collected in plastic bags and taken to the laboratory.

Isolation of *Bacillus spp.* from soil sample by serial dilution:

1. One gram of soil sample was added in 100ml of sterile distilled water.
2. Further 1ml of the suspension is removed with a sterile pipette and transferred to 9ml distilled water blank. It was vortexed thoroughly and labelled.
3. This dilution step was repeated seven times, each time with 1ml of suspension and 9ml distilled water blank. Labelling of these tubes was done sequentially starting from 10^{-1} to 10^{-9} . This resulted in serial dilutions of 10^{-1} through 10^{-9} gram of soil per ml.

Spread plate technique for isolation:

1. To grow bacterial colonies, take three pre prepared HiChrome Bacillus Agar plates and label them as 10^{-5} , 10^{-6} and 10^{-7} .



2. Sample tubes 10^{-5} , 10^{-6} and 10^{-7} were vortexed and pipette 0.1ml onto each plate. This increases the dilution value, by a factor of ten.
3. Open the plate lid and with the help of a sterilized glass spreader, spread the drop of inoculum around the surface of the agar until traces of free liquid disappears. Replace the plate lid.
4. Re-flame the spreader and repeat the process with the next plate, working quickly so as not to contaminate the agar with airborne organisms.
5. The bacterial plates were incubated.
6. The plates were kept inverted during the incubation to prevent drops of moisture due to condensation from falling onto the agar surface.
7. Plates were incubated inverted position at 37°C for 24 hours.
8. After 24 hrs plates were observed for colonies

Identification of Bacteria

On the basis of morphological, biochemical and cultural characteristics the bacteria were identified and preserve the bacterial culture for the further process.

Production of enzyme using Solid State Fermentation:

A) Screening for best strain producing protease:

The isolated strain of *Bacillus spp.* was streaked on Skim Milk Agar. The bacteria produce clear zone when streaked on Skim Milk Agar after 24hrs incubation. This strain was selected for this investigation. The bacteria were subculture on nutrient agar plates before use as inoculum for production under SSF condition.

B) To produce enzyme in Submerged Fermentation (SmF):

1. In an attempt to choose a potential substrate for SmF which supports protease production, various agro residues like Gram husk (GH), Wheat husk (WH) were screened individually.
2. SSF was carried out by taking 5g dry substrate in a 250 ml Erlenmeyer flask to which 100ml distilled water was added to adjust the require moisture level.
3. The flask was containing 100ml of production medium (KH_2PO_4 - 0.1%, NaCl-1%, MgSO_4 - 0.01%, Yeast extract- 0.5% and peptone – 0.5%). The contents of the flasks were mixed and autoclaved at 121°C for 15min.
4. The flasks more cooled to room temperature and then inoculated with 1.0ml of 24hrs grown bacterial culture under sterile conditions and incubated at 35°C for 6 days [8].

C) Extraction of enzyme produced:

1. The enzyme from the fermented bacterial bran was squeezed through a muslin cloth.
2. Extracts were pooled and centrifuged at 4°C for 10 min at 4000rpm to separate small particles of different substrates, cells and spores.



- The brown, clear supernatant was used in enzyme assay. [9]

D) Determination of enzyme activity:

Protease activity was determined by using casein according to the method of Leighton *et al.* (1973). Principle behind this procedure is as follows:

The amount of protease produced was estimated and expressed in micrograms of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity was expressed in microgram of tyrosine released by 1ml of enzyme in 30min at 30°C on tyrosine equivalent.

Protocol:

Table No. 1 : Preparation of Tyrosine Standard

Sr. No.	Reagents	Blank	S1	S2	S3	S4	S5
1	Tyrosine solution	0	0.2	0.4	0.6	0.8	1
2	NaOH(0.2N)	2	1.8	1.6	1.4	1.2	1
3	Sodium Carbonate	5	5	5	5	5	5
Stand for 10min at room temperature							
4	Follin's Reagent	0.4	0.4	0.4	0.4	0.4	0.4

Stand for 30min at room temperature, read the color at 660nm.

Table No. 2 : Determination of enzyme activity

Sr. No.	Reagents	Blank	E1 (GH)	E2 (WH)
1	Substrate Casein	1	1	1
2	Phosphate buffer (0.1M) pH=7.5	1	1	1
3	Enzyme solution	1	1	1
4	Distilled water	1	1	1
Incubate for 30min at 37°C				
5	Ice cold TCA (5%)	3	3	3
Filter				
6	Filterate	1	1	1
7	NaOH(0.2%)	1	1	1



Stand for 10min				
8	Sodium Carbonate	5	5	5
9	Follin's Reagent	0.4	0.4	0.4

Mix and stand for 30min at room temperature, read the color at 660nm.

RESULTS AND DISCUSSION

Isolation of *Bacillus species* from soil sample:

For the production of protease from *Bacillus species* are used. *Bacillus species* is predominant in soil, hence soil sample is used for the isolation of bacillus. Inoculate the Hichrome media by spread plate technique from serially diluted soil sample and by streaking method from master tube.

Identification of *Bacillus species* on the basis of morphology, cultural and biochemical characteristics:

Isolated colonies obtained from serial dilution and spread plate techniques are subcultured on hichrome media. After incubation, identification of these bacterial isolate i.e. B1 (*Bacillus subtilis*) and B2 (*Bacillus megaterium*) was done on the basis of morphology, biochemical and cultural characteristics.

Identification of bacterial isolates on the basis of morphology:

Table No. 3 : Morphological characteristics of bacterial isolates

Bacterial isolates	Gram Staining	Motility	Endospore staining
B1	Short rod Gram positive	Motile	Spore forming
B2	Short rod Gram positive	Motile	Spore forming

Identification of bacterial isolates on the basis of cultural characteristics:

Table no. 4 : Cultural characteristics of bacterial isolates

Bacterial isolates	Characteristics on Hichrome Bacillus Agar
B1	Green coloured colonies are observed
B2	Yellow coloured, mucoid colonies are observed



Identification of bacterial isolates on the basis of biochemical characteristics:

Table No. 5 : IMViC test

Bacterial isolates	Indole	MR	VP	Citrate
B1	-	-	-	-
B2	+	-	-	+

Table No. 21: Sugar fermentation test

Bacterial isolates	Glucose		Lactose		Sucrose		Mannitol	
	A	G	A	G	A	G	A	G
B1	+	-	+	-	+	-	+	-
B2	+	+	+	+	+	+	+	+

Table No. 6 : Other Biochemical tests

Bacterial Isolates	Urease	TSI			Catalase	Oxidase
		A	G	H ₂ S		
B1	-	+	-	-	+	+
B2	-	+	-	-	+	+



Fig 1: Colonies of Bacillus species on Hichrome Bacillus Agar by spread plate method

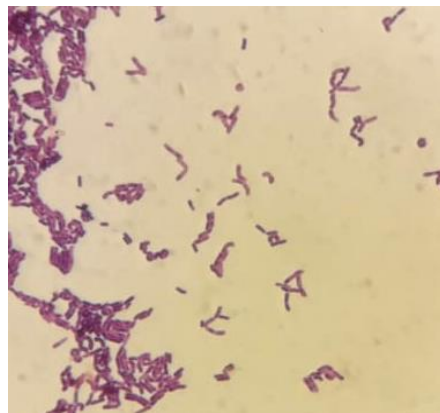


Fig 2: Gram staining of Bacillus species



Fig 3: Biochemical characteristics of *Bacillus subtilis*

Screening Test:

Skim Milk Agar plates assay determines the quantitative determination of protease activity. *Bacillus subtilis* and *Bacillus megaterium* produce zone of hydrolysis on Skim Milk Agar plates, this indicate they have the potential to produce protease enzyme. Hence, *Bacillus subtilis* and *Bacillus megaterium* were selected. While, *Bacillus cereus* and *Bacillus thureingensis* were not selected as they do not produce clear zone of hydrolysis on Skim Milk Agar. *Bacillus subtilis* and *Bacillus megaterium* were separately inoculated in the production media.

Table No.7 : Screening test of bacterial isolates

Bacterial isolates	Skim Milk Agar
B1	Clear zone of hydrolysis
B2	Clear zone of hydrolysis



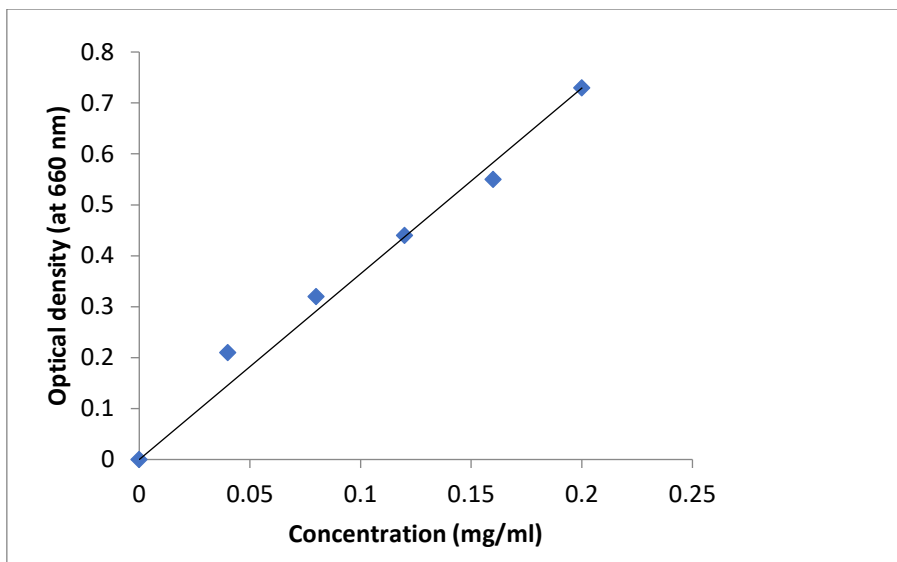
Fig 4 : Screening of Bacillus megaterium on Skim Milk Agar

Production of protease:

Optimization was taken place on the basis of incubation time so results were taken on the basis of effect of different incubation times and agro-waste substrate i.e. gram husk and wheat husk.

Table No. 8 : Observation table of standardization of protease activity

Test tube	Concentration (mg/ml)	Optical Density (at 660nm)
Blank	0	0.00
Standard 1	0.04	0.21
Standard 2	0.08	0.32
Standard 3	0.12	0.44
Standard 4	0.16	0.55
Standard 5	0.20	0.73



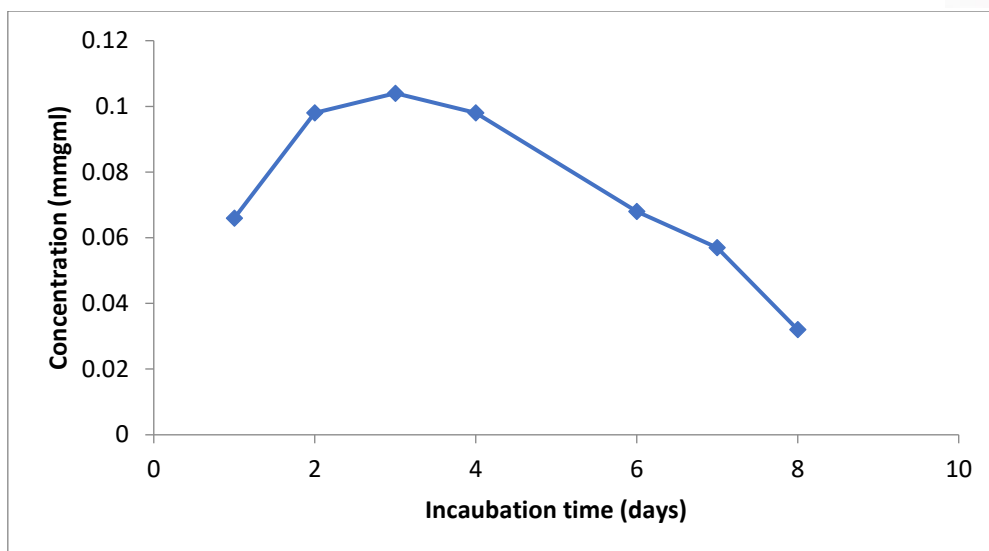
Graph 1: Standard graph with respect to tyrosine as a constant

By plotting the concentration of tyrosine on X-axis in mg/ml and optical density on Y-axis, we got the graph of standard solution.

Effect of Different Incubation Time:

Table No. 9 : Production of protease by *Bacillus subtilis* against different incubation time using gram husk as a substrate.

Incubation time (days)	Optical density (at 660nm)	Concentration (mg/ml)
Day 1	0.24	0.066
Day 2	0.36	0.098
Day 3	0.38	0.104
Day 4	0.36	0.098
Day 6	0.25	0.068
Day 7	0.21	0.057
Day 8	0.12	0.032

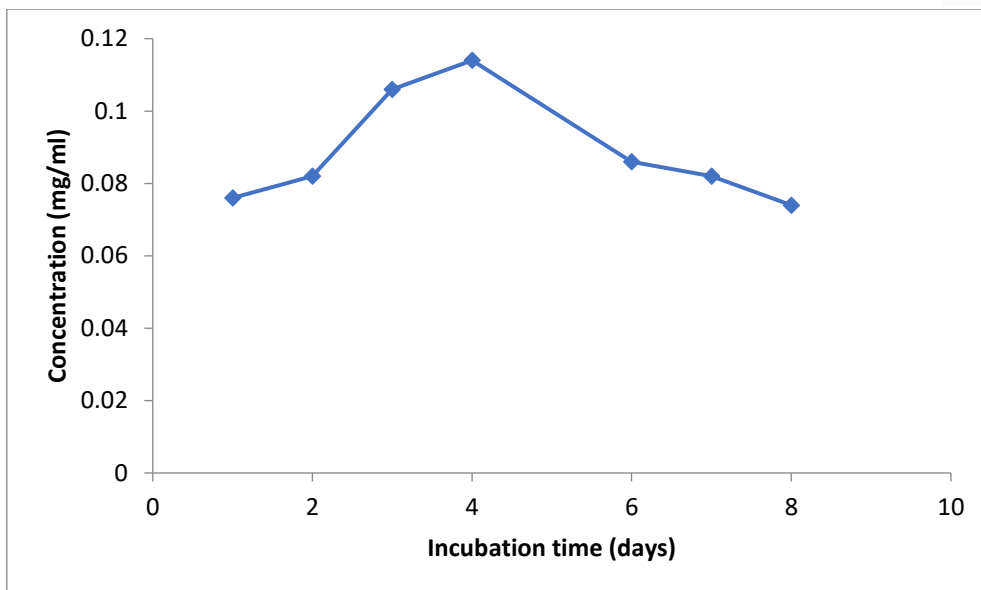


Graph 2: Production of protease by *Bacillus subtilis* against different incubation time using gram husk as a substrate.

The maximum production of protease by *Bacillus subtilis* using gram husk as a substrate was found 0.104 mg/ml in 3 days (72 hours) of incubation time.

Table No. 10 : Production of protease by *Bacillus subtilis* against different incubation time using wheat husk as a substrate.

Incubation time (days)	Optical density (at 660nm)	Concentration (mg/ml)
Day 1	0.28	0.076
Day 2	0.30	0.082
Day 3	0.39	0.106
Day 4	0.42	0.114
Day 6	0.32	0.086
Day 7	0.30	0.082
Day 8	0.27	0.074

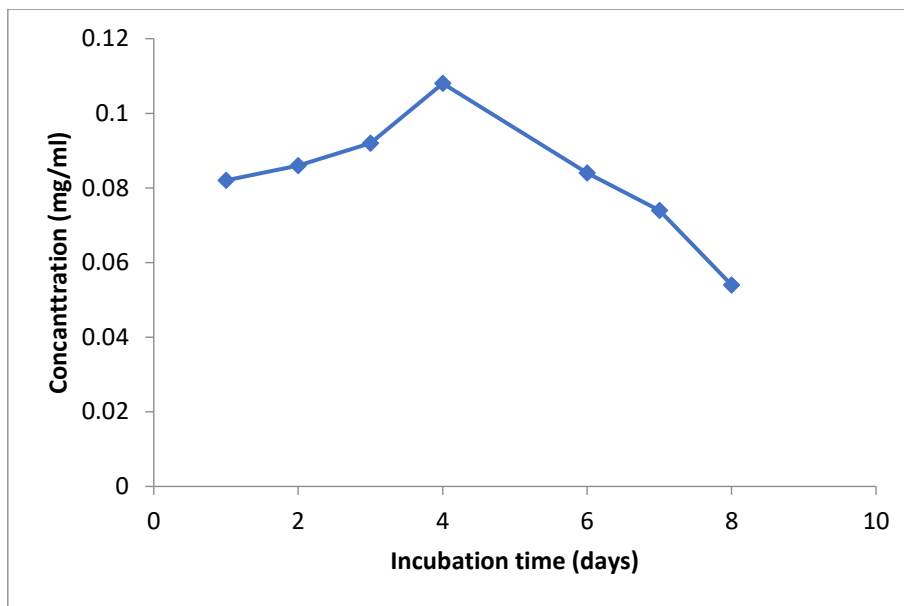


Graph 3: Production of protease by *Bacillus subtilis* against different incubation time using wheat husk as a substrate.

The maximum production of protease by *Bacillus subtilis* using wheat husk as a substrate was found 0.114 mg/ml in 4 days (96 hours) of incubation time.

Table No 11 : Production of protease by *Bacillus megaterium* against different incubation time using gram husk as a substrate.

Incubation time (days)	Optical density (at 660nm)	Concentration (mg/ml)
Day 1	0.30	0.082
Day 2	0.32	0.086
Day 3	0.34	0.092
Day 4	0.40	0.108
Day 6	0.31	0.084
Day 7	0.27	0.074
Day 8	0.20	0.054

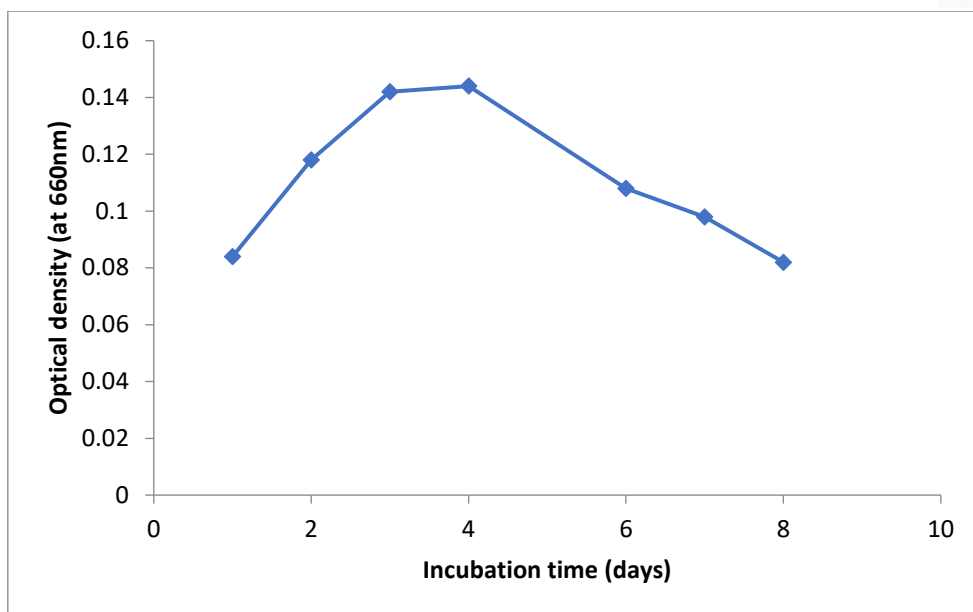


Graph 4: Production of protease by *Bacillus megaterium* against different incubation time using gram husk as a substrate.

The maximum production of protease by *Bacillus megaterium* using gram husk as a substrate was found 0.108 mg/ml in 4 days (96 hours) of incubation time.

Table No. 12 : Production of protease by *Bacillus megaterium* against different incubation time using wheat husk as a substrate.

Incubation time (days)	Optical density (at 660nm)	Concentration (mg/ml)
Day 1	0.31	0.084
Day 2	0.43	0.118
Day 3	0.52	0.142
Day 4	0.53	0.144
Day 6	0.40	0.108
Day 7	0.36	0.098
Day 8	0.30	0.082



Graph 5: Production of protease by *Bacillus megaterium* against different incubation time using wheat husk as a substrate.

The maximum production of protease by *Bacillus megaterium* using wheat husk was found 0.144 mg/ml in 4 days (96 hours) of incubation time.

DISCUSSION

Present investigation is done on the production of protease from *Bacillus species* (*Bacillus subtilis* and *Bacillus megaterium*) by using agricultural wastes (gram husk and wheat husk) to enhance the production of protease by submerged or liquid fermentation method. Protease activity is determined by Lowery's method using tyrosine (0.2 mg/ml) as a standard.

In the present study, 4 independent *Bacillus* species were isolated from the soil sample and were screened for proteolytic activity on skim milk agar plates. Out of 4 bacterial isolates 2 isolates showed proteolytic activity by forming the clear zone of hydrolysis around the colonies. Two bacterial isolates, B1 and B2 were selected based on the zone of hydrolysis on skim milk agar plates for the production of protease enzyme.

The two bacterial isolates (B1 and B2) were found to be Gram positive, rod shaped, motile, spore forming and were tentatively identified as *Bacillus subtilis* and *Bacillus megaterium* based on their morphological, cultural and biochemical characteristics. *Bacillus subtilis* form green coloured colonies (Figure 5). While *Bacillus megaterium* form yellow coloured mucoid colonies on HiChrome *Bacillus* Agar plate (Figure 6).

Bacterial isolate B1 (*Bacillus subtilis*) found to be negative for all IMViC tests, produce acid but no gas in all sugars (Glucose, lactose, sucrose and mannitol) and found positive for urease, oxidase, catalase. Bacterial isolate B1 produces acid but no gas and H₂S production in TSI slant (Figure 7).

Bacterial isolate B2 (*Bacillus megaterium*) found to be positive for indole and citrate utilization tests but negative for MR and VP tests. It produces both acid and gas in all sugars (glucose, lactose, sucrose and mannitol). Bacterial isolate B2 produces acid but no gas and H₂S production in TSI slant (Figure 8).

The selected two isolates were further screened for the quantitative production of the enzyme in production medium. Protease enzyme was produced by *Bacillus species* (*Bacillus subtilis* and *Bacillus megaterium*). The enzyme production was optimized under the parameter (incubation time). Enzyme production began and increased gradually to a maximum level; thereafter, enzyme production decreased.

The effect of incubation time on protease production by *Bacillus subtilis* using gram husk as a substrate was studied over incubation period of 1-8 days (24-192 h) at 37°C. Result of this study showed that the highest enzyme production was recorded in the incubation period of 3 days i.e. 72 h and the concentration was found to be 0.104 mg/ml (Table 9).

The effect of incubation time on protease production by *Bacillus subtilis* using wheat husk as a substrate was studied over incubation period of 1- 8 days (24-192 h) at 37°C. Result of this study showed that the highest enzyme production was recorded in the incubation period of 4 days i.e. 96 h and the concentration was found to be 0.114 mg/ml (Table 10).

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The protease production by *Bacillus subtilis* using wheat husk as a substrate gave best result than that of using gram husk. Similarly, using wheat husk as a substrate *Bacillus megaterium* gave higher protease production than using gram husk. The present study also revealed that the best result of protease production was obtained by *Bacillus megaterium* using wheat husk as a substrate with respect to incubation time and the concentration was 0.144 mg/ml.

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