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Optimization of Cultural conditions for Protease production by *Bacillus subtilis* using Banana peel waste

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Abstract

Enzymes are biocatalysts for most chemical reactions that occur inside living cells and are necessary for life. In the present research, the *Bacillus subtilis* in Tomato rhizosphere soil samples was isolated by Spread plate method and identified by Microscopic examination, Plating on Culture medium and Biochemical tests. Efficient *Bacillus subtilis* isolates was screened for its Proteolytic activity on Skimmed milk agar plates and the *Bacillus subtilis* isolate with the Highest clear zone was selected for the further studies. The effect of pH on the growth of *Bacillus subtilis* was studied at pH 6, pH 7, pH 8, pH 9 and pH 10. The growth of the *Bacillus subtilis* was maximum at pH 7. The effect of Substrate concentration (Banana peel waste) on the growth of *Bacillus subtilis* was studied at 1 %, 2 %, 3 %, 4 % and 5 %. The growth of the *Bacillus subtilis* was maximum at 3 %. The effect of Incubation period on the growth of *Bacillus subtilis* was determined at 1st Day, 2nd Day, 3rd Day, 4th Day and 5th Day. The growth of the *Bacillus subtilis* was maximum at Day 3. The effect of Carbon sources on the growth of the *Bacillus subtilis* was studied with Glucose, Mannose, Lactose, Fructose and Sucrose. The growth of the *Bacillus subtilis* was maximum at Fructose. The effect of Nitrogen sources on the growth of the *Bacillus subtilis* was tested with Ammonium chloride, Tryptone, Beef extract, Yeast extract

and Peptone. The growth of the *Bacillus subtilis* was maximum at Yeast extract. The Pectinase activity of *Bacillus subtilis* was determined by Spectrophotometer method and *Bacillus subtilis* exhibited the greater Protease activity (75 IU/ml).

Key words : Protease, *Bacillus subtilis*, Banana peel waste and Optimization of culture condition.

Proteases are the major class of enzymes used in numerous industries, including detergent, pharmaceutical, leather, photographic, culinary, food, and agricultural industries. In addition, it is utilized in cheese production, baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, some medicinal therapies for virulent wounds and inflammation, and sheepskin dehairing. Proteases are also playing a key Biotechnological tool for the Bioremediation procedures[10]. Comparatively, bacteria are effective in the production of Proteases than fungi. Additionally, several *Bacillus* species are known to produce extracellular Proteases among other bacteria[22]. Because of their excellent pH and heat stability, Alkaline proteases made by *Bacillus* species are the most important type of Proteases in various sectors[30]. Protease manufacturing for Industrial applications is a Continuous process that involves the isolation and characterization of new, promising strains using inexpensive carbon and nitrogen sources[13]. Protease was the dominant enzyme in the largest segment of the market[24]. These enzymes have been employed in a number of industrial processes to break down protein products[31].

One crucial class of industrial enzymes are Proteolytic enzymes[19]. Protease from bacteria, molds, yeasts, and mammalian tissues can be used in Industrial applications. Nowadays, *Bacillus* species are used to create a large percentage of the Alkaline proteases that are sold commercially. Physical parameters including temperature, pH, incubation duration, agitation, and inoculum density, as well as media components, particularly carbon and nitrogen sources, have a significant impact on the generation of Proteases by microorganisms[16]. Numerous methods have been developed to use agricultural and industrial waste as a starting point for the synthesis of bulk chemicals[27]. Agricultural and industrial waste can also be used in bioprocesses to provide alternative substrates and address environmental issues[21].

Different environmental conditions can affect the enzyme production by microorganisms[18]. Different physical or chemical substances can greatly reduce or destroy the enzyme activity, which causes the enzymes to lose their ability to fulfil their activities. It is well recognized that microorganisms are essential to the technology used to produce intracellular and extracellular enzymes on a large scale[32]. Selected organisms were cultivated in fermenters under ideal circumstances to produce the highest yield possible, and the resulting products can be utilized to make cheese, bread, wine, and beer[29]. Extracellular proteases are mostly produced by *Bacillus* species, and *Bacillus subtilis* is widely used

in the industrial sector to manufacture a variety of enzymes[36]. The majority of enzymes derived from microbial sources are Proteases, of which only a small number are advised for use in industrial production[25]. *Bacillus subtilis* is mostly found in soil, include Grass *Bacillus* and Hay *Bacillus*. It is a rod-shaped organism that can withstand harsh environmental conditions and create a hard, protective Endospore[34]. Both free-living and pathogenic *Bacillus* species are obligate aerobes or facultative anaerobes[17,26]. A recent trend in waste conversion is the utilization of microbes and their enzymes to produce useable biomass from waste materials[4]. To address the increasing demand for this enzyme, new Protease producing bacteria and optimized fermentation technologies are required[11].

Approximately 60 % of all enzyme sales globally are made up of Proteases, one of the three major classes of Industrial enzymes[2, 9]. Peptide bonds between amino acid residues in proteins are broken by Proteases, which also include Proteinases, Peptidases, and Proteolytic enzymes[8]. They are categorized as Hydrolases because they do this using a Water[33]. Almost 60 % of all enzyme sales are made up of proteases, one of the most significant classes of industrial enzymes[5,14]. Each organism or strain has its own specific requirements for the greatest enzyme production. Aeration, pH, temperature during cultivation, shaking conditions, and shaking state are some of the physical elements that influence the general guidelines for optimizing the synthesis of Microbial proteases. To encourage, stimulate, improve, and maximize the synthesis of proteases, these elements are crucial[12]. Protein hydrolyzes are catalyzed by Proteases and as a result, are essential to many Industrial applications[1, 6].

Collection of Banana peel waste :

The Banana peel wastes (Poovan Banana variety) used in this present research was collected from the Tirupattur Vegetable Market, Tirupattur, Tirupattur district, Tamil Nadu, India. The collected Banana peel waste was shade dried, powdered and stored in the Sterile plastic container at Room temperature for the further use[3].

Collection of Rhizosphere soil :

For the isolation of the *Bacillus subtilis*, the Tomato (PLR variety) rhizosphere soil sample was collected from the Farmer's agricultural field in Tirupattur, Tamil Nadu, India. The soil sample was collected at a depth of 3 – 5 cm in a sterile container. The collected sample was sealed properly, labelled and transported to the laboratory. The sample was processed in the laboratory within 24 hours of collection[7].

Isolation of Bacillus subtilis from Soil :

The *Bacillus subtilis* Bacteria in rhizosphere Soil samples was isolated in the *Bacillus* Differentiation agar (Hi-media, India) by Serial Dilution Method (Spread plate method). The isolated Bacteria isolates were sub-cultured in the *Bacillus* Differentiation agar slants and stored in the Refrigerator at 4°C [15].

Identification of Protease enzyme producing Bacillus subtilis :

The bacteria isolated from the

rhizosphere soil sample will be identified by

a) Microscopic examination (Gram staining, Endospore staining and Motility test).
b) Plating on Nutrient agar, Selective medium (*Bacillus* Differentiation Agar).
c) Biochemical Tests (Carbohydrate fermentation test, Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Oxidase test and Urease test).

Screening of Bacillus subtilis for its Protease activity :

Efficient *Bacillus subtilis* isolates was identified and screened preliminary for its Proteolytic activity when inoculated on Skimmed milk agar plates. The *Bacillus subtilis* isolate with the Highest clear zone (halo zone) (measured in mm in dm) was selected for the further studies[20].

Optimization of Culture conditions for Protease production :

The ability of the selected *Bacillus subtilis* isolate to produce the Protease enzyme was optimized according to the determination of significant parameters influencing the process particularly the pH (pH 6, pH 7, pH 8, pH 9 and pH 10), Incubation time (1 day, 2 days, 3 days, 4 days and 5 days), Substrate concentration (1 %, 2 %, 3 %, 4 % and 5 %), Carbon source (Glucose, Sucrose, Fructose, Lactose and Mannose) and Nitrogen source (Peptone, Beef extract, Yeast extract, Tryptone and Ammonium chloride)[28,37].

Determination of the ability of Bacillus subtilis for Protease production using the Banana peel waste :

Sterilization of the Banana peel waste :

The dried Banana peel was taken and cut with small size. The pieces were cleaned with tap water to remove the dust particles. Then, the Banana peel was washed with Distilled water and dried at 45 °C for 24 hours in Hot air over and then grind as fine powder. One gram of Banana peel powder was used for both test and control experiment[17].

Preparation of the Banana peel substrate:

The Banana peel powder was washed with the Distilled water and allowed to dry for 24 hours at 60 °C. The dried form of Banana peel powder was ground to fine powder and then placed in the flask.

Protease assay :

Protease activity was determined by Spectrophotometer method as reported by Penner and Ashton [21]. To 0.5 ml of *Bacillus subtilis* culture broth, 0.5 ml of substrate (1 % Banana peel powder) and 1.5 ml Sodium phosphate buffer pH 7.6 were added and incubated at 35 °C for one hour. After incubation, 2.0 ml of each sample was taken and 2.0 ml of 15 % Tricarboxylic acid was added and centrifuged for 5 min at 4000 rpm. To 1 ml aliquot, 4.0 ml 0.5 N NaOH and 1 ml Folin-phenol reagent (1:1) were added and then final volume was made up to 10 ml by adding 4.0 ml double distilled water. The absorbance was measured at 625 nm. One unit of Protease activity was defined as the amount of Protease required to catalyze the liberation of 1 µg of Tyrosine under the assay conditions. Protein

content was determined by the method of Lowry *et al*.[13].

Extraction of Protease crude enzyme :

Fifty ml of *Bacillus* Differentiation broth was inoculated with the *Bacillus subtilis* and incubated for 24 hours with the best Carbon source and Nitrogen source. After incubation, the *Bacillus subtilis* cultivated broth was centrifuged at 2000 rpm for 20 mins. Then, the pellet was discarded and the supernatant was stored. Double volume of Acetone was added along with the supernatant and kept it for overnight, again the supernatant was centrifuged at 300 rpm for 20 mins. Finally, the supernatant was discarded and the pellet was dissolved in the Phosphate buffer (Volume of Na_2HPO_4 + 1 ml of NaH_2PO_4). The filtrate was used as the Crude enzyme collection.

Fourier – Transform Infrared Spectroscopy (FT-IR) Analysis :

The Fourier-transform infrared spectroscopy (FT-IR) analysis of the Crude Protease sample was performed using a Perkin-Elmer 2000 spectrometer (Phoenix, USA). FT-IR spectrophotometer was used at the wavelength of 4000 - 400 cm^{-1} and the resolutions of 4 cm^{-1}. A mixture of 5.0 mg dried sample and 200 mg KBr was pressed into a water for the FT-IR measurement. It displays the extent of absorption of a single wavelength infrared light by a sample at each wavelength.

Identification of Bacillus subtilis from Tomato Rhizosphere Soil :

The bacteria isolated from the Rhizosphere soil was identified by Gram staining, Endospore staining, Motility test, Platting on Nutrient agar and Selective medium (Bacillus Differentiation Agar), and Biochemical tests. The isolated *Bacillus* sp. was identified as *Bacillus subtilis* and its characteristics was given in Table – 1.

Table-1. Characteristics of *Bacillus subtilis*

S. no	Test	Results
1	Gram staining	Gram positive, thick, 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	*Bacillus* Differe-ntiation Agar	Colour change from Violet to Yellow
9	Glucose fermen-tation	Acid produced
10	Mannitol fermen-tation	Acid produced
11	Sucrose fermen-tation	Not fermented
12	Dextrose fermen-tation	Not fermented
13	Indole	Negative
14	Methyl Red Test	Negative
15	Voges Proskauer Test	Positive
16	Citrate utilization	Positive
17	O-F test	Positive
18	Nitrate reduction	Positive
19	Gelatin hydrolysis	Positive
20	Starch hydrolysis	Positive
21	Urease	Negative

Screening of Bacillus subtilis for its Protease activity :

The isolated *Bacillus subtilis* was screened for its Protease activity because Banana peel wastes are Protein in nature and Enzymatic activity is very essential for the Protease production. In Skimmed milk agar, the *Bacillus subtilis* isolates showed the zone of clearance and confirms the Proteolysis activity. According to the previous reports, the isolated bacterial species can produce the Proteolytic enzymes like Protease. Gitishree and Prasad[20] found that the *Bacillus subtilis* isolated from the soil showed the zone of clearance on 1 % Skim milk agar and were chosen as the strain for the Protease production. Sharma and Aruna [33] screened for the *Bacillus subtilis* for the Protease production in the first instance by looking at the clearance zone on Gelatin and Skim milk agar plates. Ozgur and Nilufer[17] found the production of Protease in Fifteen different bacteria that were isolated from soil samples and choosed the bacteria with the highest Protease activity.

Optimization of Culture conditions for production of Protease :

Effect of pH *on the growth of Protease producing Bacillus subtilis :*

The effect of pH on the growth of Protease producing bacteria (*Bacillus subtilis*) was studied in this present research at pH 6, pH 7, pH 8, pH 9 and pH 10, and the results were furnished in the Table–2. The growth of the *Bacillus subtilis* was very good, luxuriant and maximum at pH 7 (Optical Density – 1.03 at 560 nm) followed by pH 8 (Optical Density – 1.00 at 560 nm), pH 6 (Optical Density – 0.88 at 560 nm) and pH 10 (Optical Density – 0.24 at 560 nm). *Bacillus subtilis* exhibited minimum Turbidity at pH 9 (Optical Density – 0.04 at 560 nm). Geethanjali and Anitha[7] selected the top *Bacillus subtilis* strains that produced Proteases. Their study's conclusions showed that the best pH for optimizing the *Bacillus subtilis* for the Protease production was pH 7.0. Sharma and Aruna[33] screened the *Bacillus subtilis* for Protease production and reported pH 6 as an Optimum condition.

Effect of substrate concentration on the growth of Bacillus subtilis :

The effect of substrate concentration (Banana peel waste) on the growth of the Protease producing bacteria *Bacillus subtilis* was studied at various concentrations *viz*., 1 %, 2 %, 3 %, 4 % and 5 % and the findings were given in the Table – 3. The growth of the *Bacillus subtilis* was very good, luxuriant and maximum at 3 % (Optical Density – 1.69 at 560 nm) followed by 2 % (Optical Density – 1.30 at 560 nm), 4 % (Optical Density – 1.22 at 560 nm) and 5 % (Optical Density – 0.82 at 560 nm). *Bacillus subtilis* exhibited minimum Turbidity at 1 % (Optical Density – 0.18 at 560 nm). Randa *et al*.[25] isolated thermostable organic solvent-tolerant Protease producer and optimized the culture conditions. For the optimal culture conditions, inoculation with 3 % (v/v) of Inoculum size in a culture medium produced the largest amount of Protease (4042.4 U/mg and 444.7 U/ml). The impact of pH and Substrate concentration on Protease production by Submerged Batch Fermentation under Optimal condition was investigated by Sugumaran *et al*.[24]. The Protease enzyme functioned at its peak at pH

7 and 5 % Substrate concentration.

Effect of Incubation period on the growth of Bacillus subtilis :

The effect of Incubation period on the growth of *Bacillus subtilis* was determined at 1st Day, 2nd Day, 3rd Day, 4th Day and 5th Day, and the results were presented in the Table – 4. The growth of the *Bacillus subtilis* was very good, luxuriant and maximum at Day 3 (Optical Density – 1.75 at 560 nm) followed by Day 4 (Optical Density – 1.25 at 560 nm), Day 2 (Optical Density – 0.67 at 560 nm) and Day 1 (Optical Density – 0.57 at 560 nm). *Bacillus subtilis* exhibited minimum Turbidity at Day 5 (Optical Density – 0.55 at 560 nm). Georage *et al*.[27] isolated *Bacillus subtilis* and showed the highest level of Protease activity under the Shake-flask fermentation process at 180 rpm. Under ideal culture conditions of pH 9.0, 45 °C, and 5 % inoculum density, with soy meal (1 %) and sugar cane bagasse (1 %) serving as the Nitrogen and Carbon sources of the fermentation medium, the maximum Protease output (2.697+0.19 IU mc^{-1}) was reached after 3 days of fermentation.

Table–2. Effect of pH on the growth of Protease producing *Bacillus subtilis*

S.no	pH	Growth of *Bacillus subtilis* at 560 nm
1	6	0.88
2	7	1.03
3	8	1.00
4	9	0.04
5	10	0.24

Table–3. Effect of substrate concentration on the growth of Protease producing *Bacillus subtilis*

S. no	Substrate–Concentration Banana peel waste (%)	Growth of *Bacillus subtilis* at 560 nm
1	1 %	0.18
2	2 %	1.30
3	3 %	1.69
4	4 %	1.22
5	5 %	0.82

Table–4. Effect of Incubation Time on the growth of Protease producing *Bacillus subtilis*

S. no	Incubation Time (Days)	Growth of *Bacillus subtilis* at 560 nm
1	Day 1	0.65
2	Day 2	0.67
3	Day 3	1.75
4	Day 4	1.25
5	Day 5	0.55

Effect of Carbon source on the growth of Bacillus subtilis :

The effect of Carbon sources on the growth of the Protease producing *Bacillus subtilis* was studied with Glucose, Mannose, Lactose, Fructose and Sucrose and the results were presented in the Table – 5. The growth of the *Bacillus subtilis* was very good, luxuriant and maximum at Fructose (Optical Density – 1.69 at 560 nm) followed by Glucose (Optical Density – 1.30 at 560 nm), Sucrose (Optical Density – 1.22 at 560 nm) and Mannose (Optical Density – 0.82 at 560 nm). *Bacillus subtilis* exhibited minimum Turbidity

at Lactose (Optical Density – 0.18 at 560 nm). Nihan and Elif Demirkan[14] estimated the Protease synthesis from *Bacillus subtilis* and the studied the impact of carbon sources on the Protease production. Among the tested Carbon sources, Fructose demonstrated the greatest production potential of all the evaluated Carbon sources. Ashok *et al*.[3] found that the optimal Carbon source for *Bacillus subtilis* to produce Proteases was Glucose, yet it is also occasionally shown that elevated Glucose levels limit the formation of Protease. Raga *et al*.[22] studied the impact of various Carbon sources on the production of Protease and reported that the Protease production was enhanced by Fructose.

Effect of Nitrogen source on the growth of Bacillus subtilis :

The effect of Nitrogen sources on the growth of the Protease producing *Bacillus subtilis* was tested with Ammonium chloride, Tryptone, Beef extract, Yeast extract and Peptone and the findings were furnished in the Table – 6. The growth of the *Bacillus subtilis* was very good, luxuriant and maximum at Yeast extract (Optical Density – 1.89 at 560 nm) followed by Peptone (Optical Density – 1.29 at 560 nm), Ammonium chloride (Optical Density – 1.09 at 560 nm) and Beef extract (Optical Density – 1.07 at 560 nm). *Bacillus subtilis* exhibited minimum Turbidity at Tryptone (Optical Density – 0.68 at 560 nm). Nihan and Elif Demirkan[14] evaluated the Protease synthesis from *Bacillus subtilis* and the studied the impact of nitrogen sources on the Protease production. Among the tested Nitrogen sources, Skim milk exhibited the good production potential among the evaluated Nitrogen sources. Ashok *et al*.[3] found the optimal Nitrogen source for *Bacillus subtilis* to produce the Protease was Urea which was followed by Tryptone, Yeast extract, Organic nitrogen, Ammonium nitrate, Ammonium sulphate, and Potassium nitrate. Raga *et al*.[22] studied the impact of various nitrogen sources and showed that that the production of Protease was enhanced by the Soy bean meal. Annamalai *et al*.[2] maximized the Protease production by *Bacillus subtilis* using the Peptone as a Nitrogen source.

Table–5. Effect of Carbon sources on the growth of Protease producing *Bacillus subtilis*

S. no	Carbon source	Growth of *Bacillus subtilis* at 560 nm
1	Glucose	0.27
2	Fructose	0.46
3	Mannose	0.20
4	Lactose	0.19
5	Sucrose	0.22

Table–6. Effect of Nitrogen sources on the growth of Protease producing *Bacillus subtilis*

S. no	Nitrogen source	Growth of *Bacillus subtilis* at 560 nm
1	Ammonium	1.09
	chloride	
2	Tryptone	0.68
3	Beef extract	1.07
4	Yeast extract	1.89
5	Peptone	1.29

Protease assay :

The Protease activity of *Bacillus*

subtilis using Banana peel waste as a Substrate was determined by Spectrophotometer method and the findings were furnished in the Figure - 1. In Optimal fermentation medium, the *Bacillus subtilis* exhibited the greater Protease activity (75 IU/ml). Sathyaguru *et al*.[35] demonstrated that all the *Bacillus subtilis* could be able to produce the highest amount of Protease at 40 °C (8.666 to 10.666 IU/ml). Annamalai *et al*.[2] observed that the Protease production was found to be highly equate and experimentally coincident with the expected value. Using the optimization fermentation process, Protease was enhanced from 298.34 U/ml to 982.68 U/ml. Sathiya[28] demonstrated the *Bacillus subtilis* as the powerful source of the Protease enzyme. The protein concentration has increased as a result of the purifying procedures, which went ahead without any significant problems.

Fourier – Transform Infrared Spectroscopy (FT-IR) Analysis :

The Fourier-transform infrared spectroscopy (FT-IR) analysis of the Crude Protease sample was performed using a Perkin-Elmer 2000 spectrometer (Phoenix, USA). The FT-IR spectroscopy monitors the vibration modes of functional groups present in proteins, lipids, polysaccharides, and nucleic acids. Shifts in peak positions, changes in bandwidths, intensities, and band area values of the infrared bands are used to obtain valuable structural and functional information about the system of interest and the intensity and more accurately the area of the absorption bands is directly related to the concentration of the molecules[32]. The spectra of all groups are shown in Figure-2. The tentative vibrational frequency assignments of absorption spectra

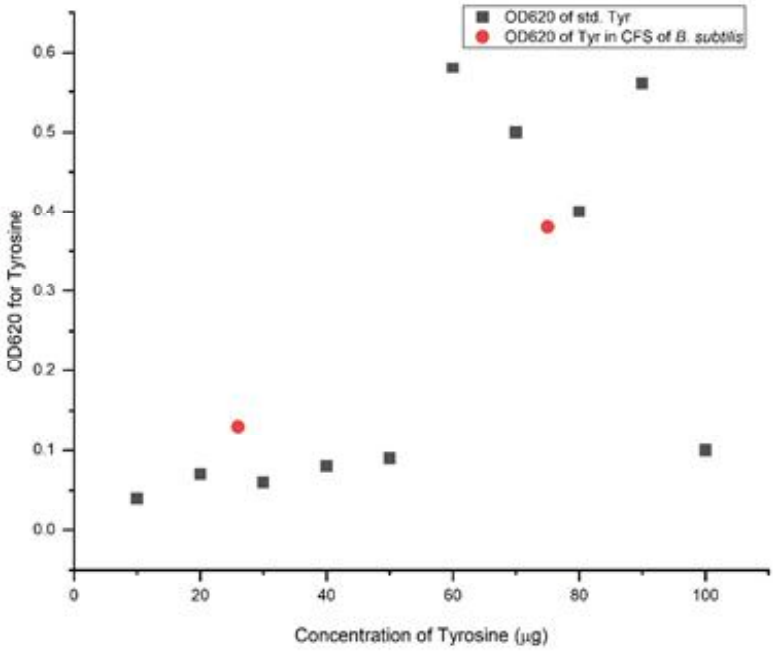

Figure - 1. Protease assay

are presented in Table-7. As shown in Table–7, the bands in this region arise from N–H and O–H stretching modes of proteins, polysaccharides and intermolecular hydrogen bonding[5]. The CH_2 asymmetric band at 2,928 cm^{-1} and CH_2 symmetric band at 2,856 cm^{-1} was caused by stretching vibration. The peak observed at 1,734 cm^{-1} indicates C=O stretching of protease. In the present study, the amide I is formed at 1,652 cm^{-1} in Protease. The position of this absorption is responsive to protein conformation. The amino acid side chain from peptides and proteins at 1,461 cm^{-1} is associated with the asymmetric CH_2 bending vibrations.

Table-7. FT-IR vibrational assignment for Protease

Wavenumber (cm^{-1})	Definition of the Spectral assignment
3443	Amide A: mainly N–H stretching of proteins with the little contribution from O–H stretching
2928	CH_2 asymmetric stretch
2856	CH_2 symmetric stretching
1734	C=O stretch
1640	Amide I: C=O stretching of proteins
1461	CH_2 Bending: with the little contribution from proteins
1132	CO–O–C asymmetric stretching

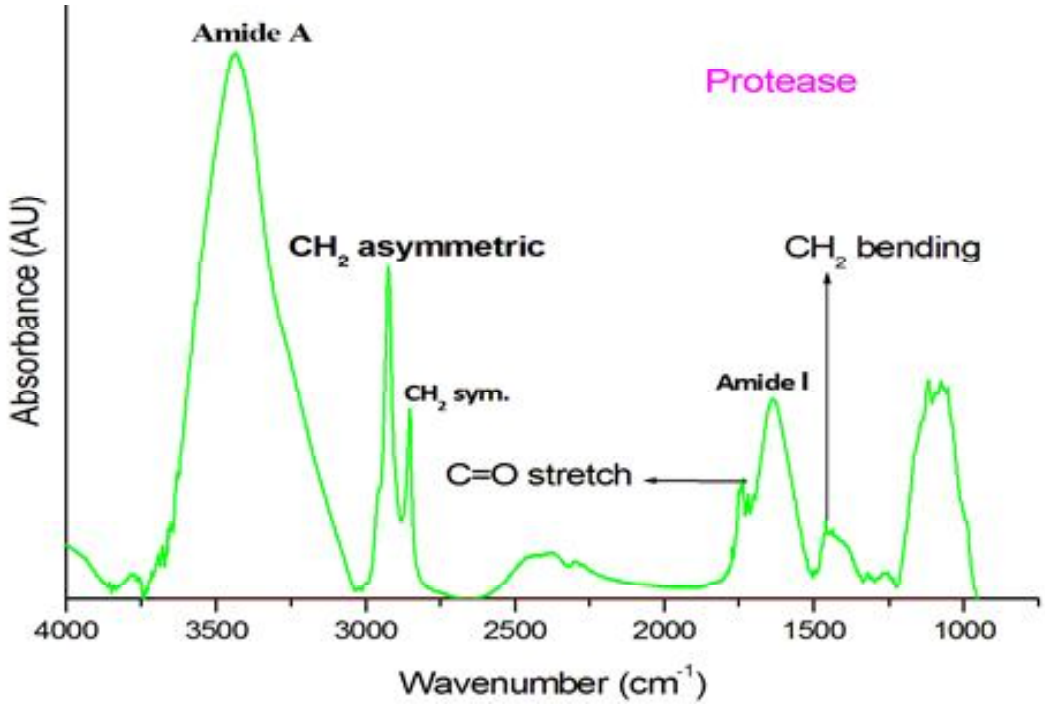

Figure – 2. FT-IR (Fourier Transform Infrared Spectroscopy) analysis

In conclusion, employing leftover Banana peel waste as a substrate to optimize the culture conditions for *Bacillus subtilis* manufacture of Proteases offers a viable path toward the sustainable synthesis of enzymes. Maximal protease yield can be attained by carefully adjusting the parameters including pH, Substrate concentration, Incubation period, Carbon source, and Nitrogen source. This eco-friendly method offers the possible financial advantages in addition to making use of a readily available waste product. Additional investigation into increasing production levels and investigating potential Downstream uses for the generated protease could have a substantial impact on the fields of Biotechnology and the environment. Finally, the best Optimal condition for the Production of Protease using the *Bacillus subtilis* is pH - 7, Substrate concentration (Banana peel waste) – 3 %, Incubation period – 3 Days, Carbon source – Fructose and Nitrogen source – Yeast extract.

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