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Production and Optimization Studies of Protease by Soil Isolate *Actinomycetes* Using Mixed Substrate of Pomegranate Peel Powder and Banana Peel Powder under Solid State Fermentation

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The *Actinomycetes* are gram-positive, unicellular bacteria that grow in a typical filamentous manner on solid substrates and may synthesis a wide range of secondary metabolites, including enzymes. Proteases are universally present enzymes that catalyze hydrolytic processes that break down protein molecules into peptides and amino acids. The protease enzyme is used in food and detergent industries. The goal of the current investigation is to identify and isolate a prospective isolate of *Actinomycetes* capable of solid state fermentation for the protease enzyme production. The soil samples yielded a total of 30 isolates. On starch casein nutrition agar medium, B5 isolate was observed to be a potential strain for production of protease enzyme. The optimum conditions for the maximum protease enzyme production were observed to be at 30°C with a pH of 7 when incubated for 5 days with a 6^{th} day old culture and 2mL of the inoculum volume for protease with substrate concentration (pomegranate and banana peel powder of each 2.5 g) of 5 g. The addition of Carbon source starch and Nitrogen source casein to the production medium has enhanced the enzyme activity to 442.8 U/gds.

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Keywords: Actinomycetes; protease; solid state fermentation; pomegranate peel powder and banana peel powder; isolation.

1. INTRODUCTION

Gram-positive, aerobic, spore-forming bacteria in the order Actinomycetales are known as "*actinomycetes*," and they can be identified by their tendency to produce aerial mycelium on a substrate. Studies that coupled DNA with rRNA and catalogued 16S ribosomal RNA revealed that it has a high (G+C) ratio in the DNA (> 55 mol%) and is phylogenetically connected. The *Actinomycetes* have the potential to create antibiotics and other substances with medicinal use. *Actinomycetes* produce a variety of bioactive secondary metabolites, including antibiotics, anticancer drugs, immunosuppressive substances, and enzymes [1]. The antibacterial, antifungal, antioxidant, neurotogenic, anti-cancer, anti-algal, anthelmintic, anti-malarial, and anti-inflammatory properties of these metabolites are well documented. They also play a significant part in the organic matter cycling in the soil ecosystem. Among the 18 primary lineages that are currently recognized as belonging to the domain bacteria, it constitutes one of the largest taxonomic units. They have been found in harsh habitats, particularly in cryophilic regions. Comparative research has shown that the population of *Actinomycete* bacteria is highest in surface level soils and gradually declines as soil depth rises. Individual *Actinomycete* strains are found in all soil levels [2,3].

Proteases are universally present enzymes that facilitate hydrolytic processes that break down protein molecules into peptides and amino acids. Proteases are enzymes that are found in all living things from prokaryotes to eukaryotes to viruses [4]. Microorganisms, as opposed to plants and animals, make for an appealing source of proteases because they can be produced in high numbers in a short amount of time using proven fermentation techniques, and they consistently and abundantly produce the needed output. Additionally, microbial proteins can be kept for longer periods of time without significantly losing their functionality when kept under optimal storage conditions. One of the three main classes of commercial enzymes, proteases account for around 60% of all enzyme sales globally [5,6].

Fermentation process that takes place without or almost without free water is known as solid-state fermentation (SSF). In most SSF processes, the carbon and energy source is a natural raw resource. SSF can also use an inert substance as a solid matrix; however this involves adding extra nutrients and a carbon source to the nutrition solution [7,8]. However, the solid substrate (matrix) needs to have enough moisture. Depending on the substrate, there may be one or more times as much water absorbed as there is dry weight. This causes relatively high water activity on the solid/gas interface, which facilitates faster rates of biological reaction [9-12].

Pomegranate peel contains high amount of polyphenols, condensed tannins, catechins, and prodelphinidins. The higher phenolic content of the peel yields extracts for use in dietary supplements and food preservatives [13,14]. The protein content in peel was reported to be approximately 3%.Banana peel is rich in proteins, fiber, potassium, essential amino acids, and unsaturated fatty acids. Banana peel extract of banana peel could be considered as a good antibacterial agent against different species of spoilage bacteria. Aqueous extracts of fresh yellow banana peels could be considered as a good antibacterial agent against both Gram positive and negative bacteria to replace the synthetic medicines in treatment of diseases caused by these bacteria. The protein content in the banana peel is rich source of crude protein which is approximately 9%.

2. MATERIALS AND METHODS

Materials used: Trichloro Acetic Acid, L-Tyrosine, Sodium Carbonate, Sodium bicarbonate, EDTA (Ethylene Diamine Tetra Acetic acid), Sodium dihydrogen orthophosphate, Tween -80, Sodium hypochlorite, Fructose, D-Galactose, D-Glucose, Urea, Lactose, Maltose, Dextrose, Cellulose, Soluble Starch, Sucrose, Beef Extract, Peptone, Casein, Yeast Extract, Ammonium Chloride, Ammonium Nitrate, Ammonium Sulfate, Potassium Nitrate, Sodium Nitrate, Potassium dihydrogen Orthophosphate, Magnesium Sulphate, Bromocresol green indicator, Sodium Chloride, Agar- Agar, Gelatin, Sodium hydroxide, Ferrous sulphate, Calcium carbonate, Nutrient agar, Monobasic sodium phosphate, Dibasic sodium phosphate.

2.1 Substrate

The mixed substrate of ripened pomegranate peel and banana peel powder were used are obtained from reliable sources.

2.2 Sample Collection

The soil samples are collected from the different wastelands and garden habitats of the PG Block, Department of Chemical Engineering of AUCE, Andhra University, Visakhapatnam. Each collection was made at a depth of 15 to 25 cm in the soil, and it was crushed by hand and sieved for uniform size (4-5mm) after one week of air drying. *Actinomycetes* were then isolated from these sieved soils.

2.3 Isolation of *Actinomycetes* from Soil Sample

1 g of dirt was suspended in 100 mL of physiological water with 0.85 g of sodium chloride per lit, and then incubated at 28°C with 200 rpm for 30 mins. The incubated soil containing test organisms of 0.1 mL of each dilution from 10-1 to 10-5 was spread evenly over the surface of starch casein agar after mixtures were allowed to settle. Serial dilutions up to 10-5 were prepared using sterile physiological water and agitated at higher speed. Refracamin (2.5 mg/mL) and amphoteracin B (7.5 mg/mL) were added to the media to prevent contamination. Plates were observed after 48, 72, and 96 hours of recurrent streaking on incubators set at 28°C and 37°C starch casein agar medium plates were used to purify bacterial colonies that appeared to be *Actinomycetes.* The isolation strains are maintained for a longer amount of time for serial culturing at 4°C for two months.

2.4 Pour Plate Technique

From orbital shaker samples, the soil sample of 1mL was taken and utilized for serial dilution. 10 sterile test tubes were filled with 9mL of distilled water. In order to create a concentration of 10^{-1}, 1mL of liquid sample containing the test organism is added to the first test tube. Next, a small amount of liquid (1 mL) is moved from test tube to test tube (its conc will be 10^{-2}). In a similar manner, 1 mL of liquid containing an organism is systematically transferred to the following test tube until the concentration reaches 10^{-5} in the last test tube. SCA media in molten form was combined with 0.1 mL of serially diluted material and poured aseptically into petri plates, allowed to solidify and were then kept at 28°C for 2 weeks. *Actinomycetes* need two weeks to grow, after which the selected individual colonies were streaked on SCA plates to produce pure cultures.

2.5 Maintenance of Pure Culture

Actinomycetes that could be distinguished from one another by visual inspection were transferred to SCA petri plates and cultured at 28°C for two weeks. The isolates had liquid pigment, suspension of aerial mycelium reversal color, and elongated colony textures that were visible to the human eye. For enhanced sporulation, pure cultures were observed in a petri plate on SCA media. Slants were inoculated with the pure culture from petri dishes with SCA and then incubated for 7 to 10 days at 28°C. Good growth was not noticed below 4°C. Every four weeks, The Culture underwent a subculture.

2.6 Collection and Preparation of Pomegranate and Banana Peel

Ripened Pomegranate and Banana fruits were obtained from Rythu bazar, then the peels were separated from the rest of the fruit parts, and then the peels were sterilized with a solution of sodium hypochlorite at a concentration of (1%) free chlorine was then washed with sterile distilled water dried with a dry paper, and dried using an electric oven at a temperature of 50°C for 48 hours, then it was ground using an electric grinder until a fine powder was obtained and kept in plastic containers until use.

2.7 Solid State Fermentation (SSF)

Solid state fermentation and culture conditions were maintained by taking, five gram of pomegranate peel and banana peel powder was taken in a 250 mL Erlenmeyer flask, moistened with 10 mL of the salt solution [composition (%w/v) (g/100 mL): Ammonium nitrate 0.5, Potassium dihydrogen orthophosphate 0.2, Sodium chloride 0.1 and Magnesium sulphate 0.1]. The flasks were sterilized for 15 min, at 121.5ºC (15 lb), cooled, aseptically inoculated with 1mL of fungal spore suspension (106 spores/mL) and incubated at 35ºC for 7 days.

2.8 Extraction of Crude Enzyme

The substrate was homogenized on a rotary shaker at 180 rpm for one hour. A solution of TWEEN-80 (0.1%) was added to the 100 mL of distilled water, and 25 mL of this water was added to the 5g of cultured substrate. Centrifuging the homogenate at 8000 rpm for 15 minutes at 4ºc removed these particles, and the clear, supernatant that was left over was

employed as a crude enzyme in further experiments.

2.9 Assay for Protease

A test tube containing 500µl of casein (1%) and 300µl of 0.2 mol/l phosphate buffer (pH 7) was then filled with 200µl of crude enzyme extract. After 10 minutes at 60℃, the reaction mixture was stopped by adding 1 mL of 10% trichloroacetic acid. The reaction mixture was centrifuged at 8000 rpm for 15 minutes at 40°C, and the supernatant was added along with 5 mL of 0.4 mol/l $Na_2 CO_3$ and 1 mL of the 3-fold diluted Folin-phenol Ciocalteau's reagent. After 30 minutes of incubation at room temperature, the solution was tested for concentration using a tyrosine standard curve and a visible spectrophotometer to measure the absorbance of the blue colour that had developed at 660 nm [15].

One unit of enzyme activity was defined as the amount of enzyme that liberated 1µg of tyrosine from substrate (casein) per min under assay conditions. Enzyme yield was expressed as the activity of protease per gram dry substrate (U/gds).

3. RESULTS AND DISCUSSION

Among 30 isolates B5 isolate showed highest protease activity. For the production of neutral protease, *Actinomycetes* (Isolate B5) is grown in natural medium of mixed substrate pomegranate peel powder and banana peel powder under solid state fermentation and optimization of process parameters was carried out.

3.1 Effect of Fermentation Time

Gradual increase in the enzyme activity was observed from 1st day to 5th day. Maximum Protease activity showed on 5th day with activity 358.5 U/gds. The decrease of activity observed since then due to decrease in the fermentation medium. The maximum enzyme activity was observed is 358.5 U/gds.

Debananda et al. [16] reported that for culture media that was evaluated on skim milk agar, the maximum protease activity was obtained after 120 hours of incubation.

3.2 Effect of Fermentation Temperature

The temperature range of 20°C to 45°C for enzyme production. According to Fig. 2, 30°C was discovered to be the ideal incubation temperature for the production of protease 367.5 U/gds. The activity gradually decreased as the temperature rose because high temperatures can denature extracellular enzymes. As a result, 30°C is thought to be the ideal temperature for protease action.

The maximum protease activity was reportedly produced by Actiomycetes sp. under solid state fermentation at the optimum temperature of 30°C, according to Alissara Reungsang et al. [17].

3.3 Effect of pH

By observing the fermentation at several pH levels of medium ranging from 3 to 9 pH, the optimum pH for protease production was observed. The pH 7 produced the highest amount of enzyme protease 389.3 U/gds, as indicated in Fig. 3. The pH controls the production and proliferation of extracellular enzymes as well as the movement of different substances across cell membranes, supporting the development of new cells and their products.

Similar outcomes for the synthesis of neutral protease utilizing rice mill waste as substrate were reported by Paranthaman et al. [18].

3.4 Effect of Inoculum Age

Different inoculum ages were used during the fermentation to study the effect of inoculums age on protease production. According to Fig. 4, the culture that was six days old produced the most protease, 394.8 U/gds. This is due to the well growth of the extensive mycelia on the 6th day.

Similar results were observed by Suhad A. Ahmed et al. [19] for protease production.

3.5 Effect of Inoculum Volume

Different volumes of inoculum concentrations 1-6 mL were taken and effect of protease production was studied. Maximum production of protease is 402.8 U/gds was observed at 4mL as shown in the Fig. 5. The production of enzymes gradually declined as inoculum volume was increased further because spore overcrowding from a significant increase in inoculum volume affected enzyme activity due to nutritional depletion.

B. Lakshmi et al. [20] reported the maximum protease production with 2 mL volume inoculum.

3.6 Effect of Substrate Concentration on Protease Production

The production medium of different concentrations 1, 2, 3, 4, 5, 6 grams were prepared at pH 7.0 in 250 mL flasks and each flask was inoculated. 5g of mixed substrate concentrarion observed to be optimum for the production of protease enzyme of 404 U/gds as shown in Fig. 6.

3.7 Effect of Carbon Source

Different carbon sources, including fructose, glucose, galactose, starch, dextrose, maltose, and sucrose, were supplied at concentrations of 2% w/w to the production medium and incubated at 30℃ for 7 days to study the effect of carbon source on protease production. The extracts protease activity was evaluated after 7 days. Starch was a good carbon source among these several carbon sources utilized for enrichment, giving the highest protease activity 412.95 U/gds as shown in Fig. 7.

Similar results on the production of proteases by *Actinomycetes* strain PS-18A isolated from a shrimp pond were reported by G. Vonothini et al. [21].

3.8 Effect of Nitrogen Source

Both organic and inorganic sources of nitrogen are significant boosting or limiting factors for the generation of microorganisms. The various nitrogen sources of 2% (w/w) are examined, including peptone, yeast extract, casein, ammonium nitrate, beef extract, and gelatin. Among these, casein was identified as a source for the enzyme's highest production rate (438 U/gds), as seen in Fig. 8.

Casein is the optimum organic nitrogen supplement, according to Alagarasami Sumantha et al. [22] for optimizing neutral protease synthesis with Rhizopus microsporus.

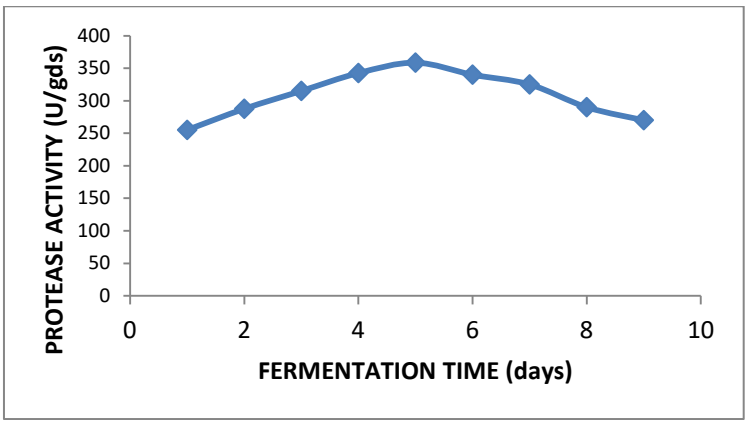

Fig. 1. Effect of fermentation time on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

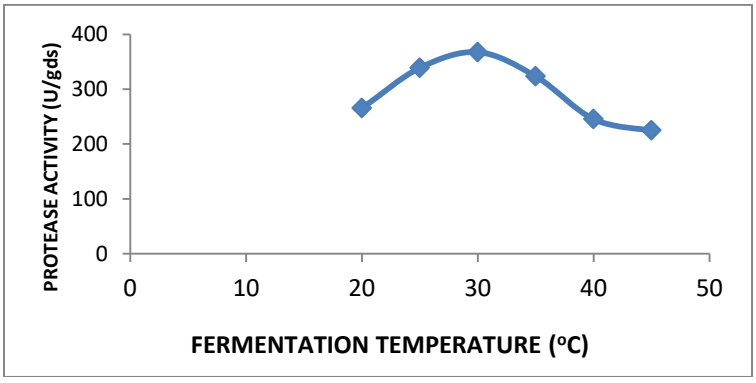

Fig. 2. Effect of fermentation temperature on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

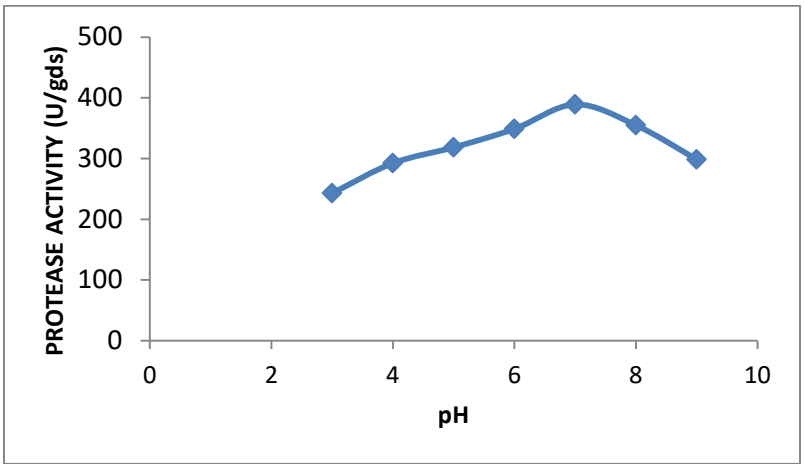

Fig. 3. Effect of pH on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

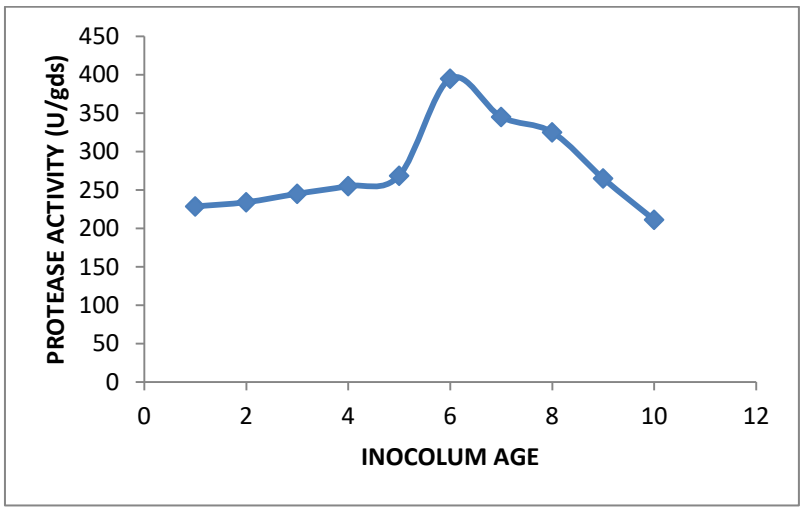

Fig. 4. Effect of inoculum age on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

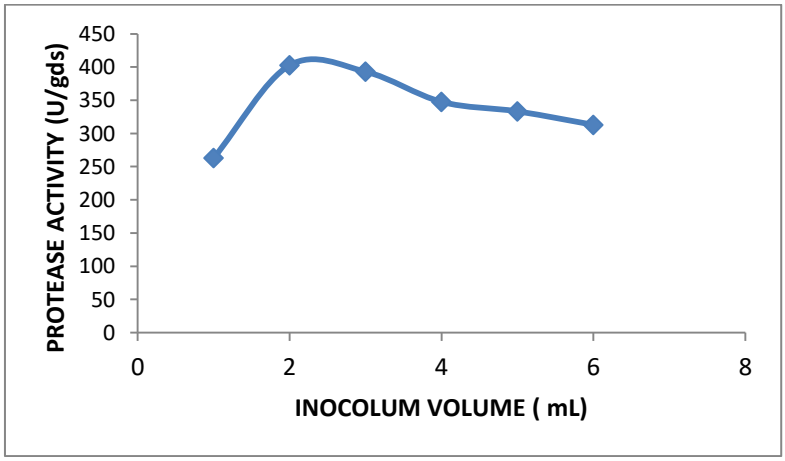

Fig. 5. Effect of inoculum volume on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

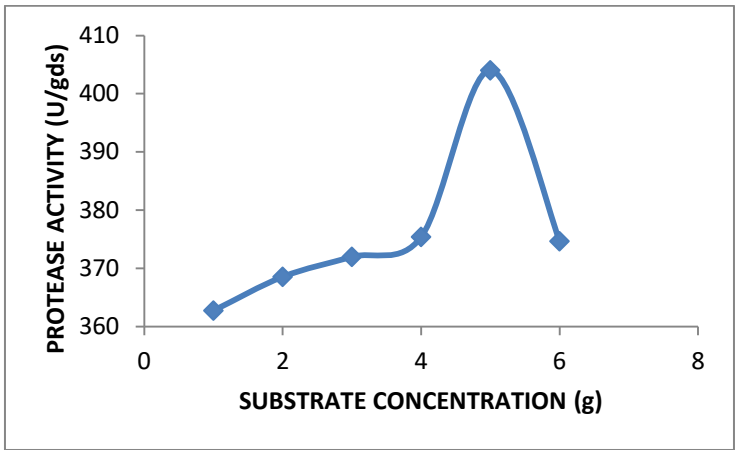

Fig. 6. Effect of substrate concentration on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

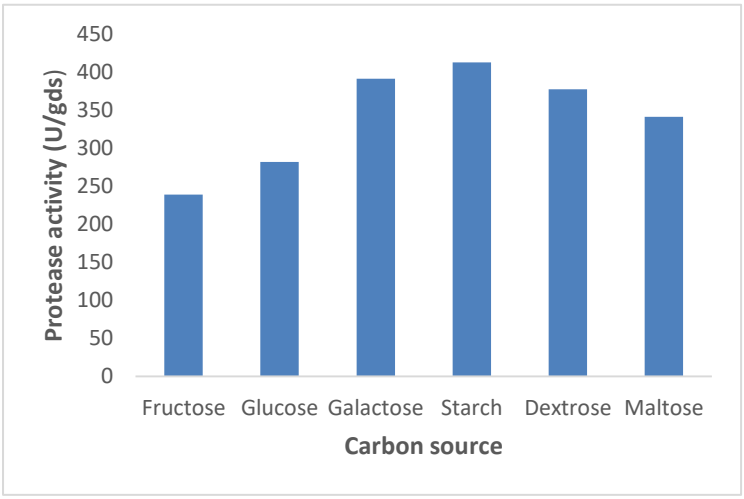

Fig. 7. Effect of carbon source on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

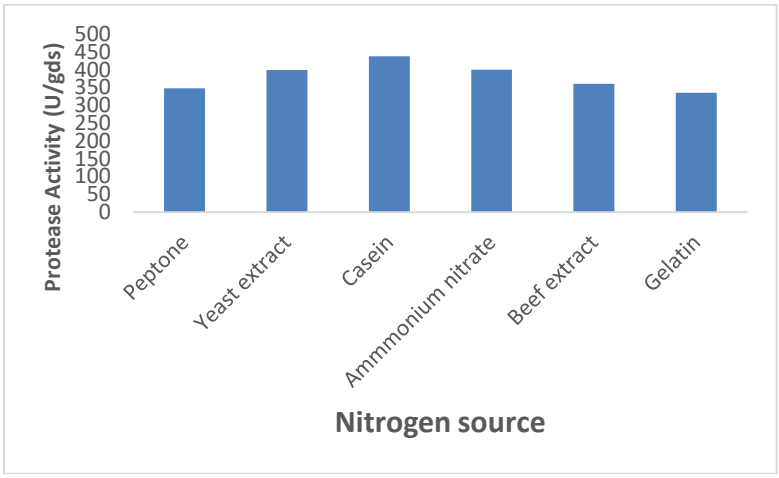

Fig. 8. Effect of nitrogen source on protease production by *Actinomycetes* using mixed substrate of pomegranate and banana peel

4. CONCLUSION

The present study reveals that soil environment is excellent source for isolation of *Actinomycetes* which can produce various enzymes. The increasing demand in microbial enzyme production has lead to produce protease from *Actinomycetes* under solid state fermentation. The protease enzyme is majorly used in detergent and textile industry. The results obtained in the study revealed that the isolate B5 had been more effective for the production of protease using solid state fermentation. The optimum conditions for the maximum production of protease were found to be at 30^{o}c with a pH of 7 when incubated for 5 days with 6th day old culture and 2mL of the inoculum volume for protease and the addition of casein as nitrogen source to the production medium has enhanced the enzyme activity to 442.8 U/gds.

From this studies it is observed that the *Actinomycetes* isolated from soil sample is producing a potential protease enzyme using Pomegranate peel powder and Banana peel powder as mixed substrate under solid state fermentation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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