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Study of Novel Bacterial Soil Isolates for the Evaluation of Protease Production

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ABSTRACT

Proteases are one of the major causative agents used in the biological systems for the inter conversion of protenacious matter. This fact has led to the exploitation of especially bacterial proteases for their use in various industries and in such scenario, the present study explored the potential of the newly isolated soil bacterial isolates, B1, B3, B4 & G2 for the production of proteases using protease specific broth media at 30°C & 120rpm for five days by shake flask culturing method. In the results, the bacterial isolate B3 has emerged as the best protease producer strain with ~52U of enzyme activity by 48Hrs of cultivation and this B3 strain was identified as *Pseudomonas* sp. SPSU B3, the other bacterial strain B4 was found as species with an extended period (72hrs) of non-growth associated protease production (~45U) and this strain was identified as *Aneurinibacillus migulanus* sp. SPSU B4. The other two bacterial strains B1 & G2 were also found to be of comparable protease activity and hence deserve further studies on optimization and production of protease along with the strains of B3 & B4.

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1.Introduction

Proteases are one of the most important means of mass conversion, especially of proteins among the life forms; where proteins are considered to be the major constituents next to the water. Hence proteases are under constant study and exploration for better comprehension of life forms, and utilization of proteases for the mankind in their daily life, medicines, industrial process and as natural cleaning agents [1-5]. So, the basic property of amide bond cleavage by the proteases, in the protein peptide chain has been exploited to generate the desired amino acids that serve as building blocks in all the life forms; facilitating the inter conversion of biomass [6]. This same property is also exploited to clean the laundry stained by the protenacious dirt using proteases as the major ingredients in the detergents. Similarly, these proteases are being used in various process industries like, tenderization of meat, digestion of meat & its products to produce high value products like amino acids and organic acids; manufacturing of cheeses, milk coagulation, production of aspartame [7-11] etc.

All the life forms without exception contain proteases as part of their biological metabolism. So broadly, the proteases may be produced and derived from microbial organisms, animals and plants. However, the major production of proteases was sought from microbial organisms as they are amenable to experimentation, handling, production and yield [12-16]. In such scenario, the fungal organisms are being explored to produce and isolate acid proteases whereas bacterial organisms are being explored to produce and isolate the alkaline proteases that are extensively used in detergent and bioprocessing industries [1, 2, 19]. Out of the bacterial genera, *Bacillus* sp. has been the most fruitful and yielding species isolated, studied and used for the production of proteases [3, 5, 11, 15, 20-21]. In the similar lines the present study illustrates the protease potential of the newly isolated soil bacterial strains that may be of interest for bioprocess industries and academic research.

2. Materials & Methods

2.1. Soil Samples

Soil samples were collected from the outskirts of Guntur (Andhra Pradesh), Nagpur (Maharashtra) and Udaipur (Rajasthan), India and these soil samples were processed [22] in the Microbiology Laboratory, Department of Biotechnology, Sir Padampat Singhania University, Udaipur for the isolation of bacteria.

2.2. Isolation and selection of protease bacteria

The bacterial isolates from the soil samples were obtained using, serial dilution method [22] with the help of nutrient agar selective media. Selected bacterial isolates were inoculated separately on to protease agar plates of protease specific medium comprising (g/l) K_2HPO_4 2.0, glucose 1.0, peptone 5.0, gelatin 15.0, and agar 15. These prepared plates in duplicates were incubated at 30°C for 48hrs and then subsequently flooded with mercuric chloride solution. The resulting clear zone around the bacterial colonies were measured [17] and the best protease producing bacterial isolates were picked and named as B1, B3, B4 and G2. These protease isolates were sub cultured routinely and preserved in agar slants at refrigerated conditions.

2.3. Broth Culturing of Protease Bacterial Isolates

Sterile protease specific broth media (Glucose 5g/l; Peptone 7.5g/l; $MgSO_4.7H_2O$ 5g/l; KH_2PO_4 5g/l; $FeSO_4.7H_2O$ 0.1g/l; PH 7.0) of 100ml was prepared in five conical flasks and were inoculated with respective protease isolates of 0.1 ml (0.5 McFarland standard) inoculum separately except that of the control following aseptic procedures. These culture flasks were incubated in the shaker incubator at 120 rpm and 30°C for five days. Broth samples were withdrawn aseptically at 24hrs interval from each culture separately and used them for the preparation of the respective crude protease enzyme samples.

2.3.2. Preparation of Crude Protease Samples

Microbial broth of 2ml were withdrawn separately from the broth cultures of B1, B3, B4 & G2 isolates at an interval of 24hrs for five days in a sterile vial aseptically and centrifuged them at 10000g and 4°C for 10 min. in a cooling centrifuge. The

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supernatant was collected in a fresh vial from each sample and used them immediately as a protease crude enzyme [23] for agar well diffusion & spectrophotometric assay of protease.

2.3.3. Agar Well Diffusion Assay of Protease isolates

Gelatin agar media was prepared and autoclaved at 121°C, 15 psig for 15 min and then cooled to 45°C under laminar air flow where the media was poured in to the sterile glass petri plates aseptically. The agar media was let cool under laminar hood and closed the lid and kept inverted for 24hrs to obtain the sterile gelatin agar plates. These sterile gelatin agar plates were punched aseptically with sterile cork borer to obtain two 4mm dia. wells in the two halves of the plates with approximate separation distance of 30mm. These gelatin agar plates were loaded with freshly extracted crude protease enzyme of 50µl in each well separately for each extracted crude enzyme and incubated at 30°C in the incubator for 48 hrs. The developed clear zones around the wells were measured (mm).

2.3.4. Spectrophotometric Assay of Protease Activity

Protease activity of the crude enzyme was determined [24] using casein as a substrate where a mixture of 500µl of 1% (w/v) of casein in 50mM phosphate buffer, pH 7 and 200µl crude protease extract were incubated in a water bath at 40°C for 20 minutes and then the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA). This reaction mixture was kept at room temperature for 15 minutes and then centrifuged to separate the unreacted casein at 10,000g and 4°C for 10 minutes. The supernatant of 0.5 ml taken in a fresh vial was mixed with 2.5 ml of 0.4M Na_2CO_3 and then 1 ml of 3-fold diluted Follin Ciocalteus phenol reagent, was added. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue color developed was measured at 660 nm against a reagent blank of a tyrosine standard [25]. One unit of protease is defined as the amount of enzyme that releases 1µg of tyrosine per ml per minute under the standard conditions of supernatant solution [26].

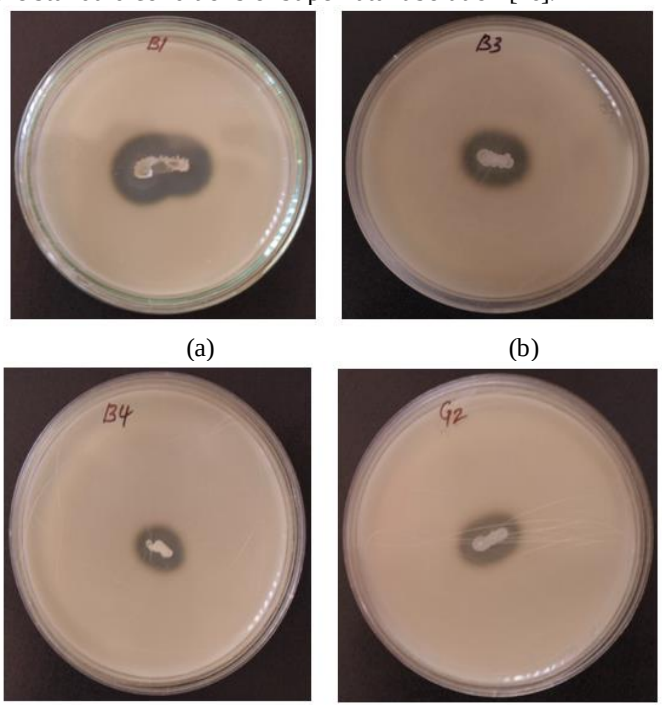

Figure 1. The protease agar plates of bacterial soil isolates exhibiting the protease activity through the clear zone formation around the colonies of isolates B1 (a), B3 (b), B4 (c) and G2 (d)

2.4. Morphological and Biochemical Characteristics of the Protease Producing Isolates

Morphological studies of the protease producing isolates B1, B3, B4 and G2 were studied under the light microscopy by Gram staining method and their biochemical characterization was performed as per the standard procedures [27].

Table.1 Agar well diffusion assay of crude protease extracted from the bacterial isolates B1, B3, B4 and G2 grown for 24hrs in the protease specific broth media at 120 rpm and 30°C.

Sl. No.	Bacterial Isolate	Zone of Clearance (mm)
1	B1	26
2	B3	23
3	B4	20
4	G2	24

Table.2 Physical and biochemical observations of the bacterial soil isolates B1, B3, B4 and G2

Physical / Biochemical Tests	Observations of Bacterial Soil isolates			
	B1	B3	B4	G2
Gram Staining	Positive	Negative	Positive	Positive
Morphology	Rod	Rod	Rod	Rod
Indole	Negative	Negative	Negative	Negative
Methyl Red (MR)	Negative	Negative	Negative	Negative
Voges Proskauer (VP)	Negative	Negative	Negative	Negative
Citrate	Positive	Negative	Negative	Negative
Urea	Negative	Negative	Negative	Negative
Gelatin Liquefaction	Negative	Negative	Negative	Negative
Phenol Red Dextrose Broth	Negative	Negative	Negative	Positive w/o gas
Phenol Red Maltose Broth	Negative	Negative	Negative	Negative
Phenol Red Mannitol Broth	Negative	Negative	Negative	Negative
Phenol Red Sorbitol Broth	Negative	Negative	Negative	Negative
Phenol Red Dulcitol Broth	Negative	Negative	Negative	Negative

3. Results

The bacterial soil isolates tested for the protease activity yielded four strains (Figure-1) namely B1, B3, B4 & G2 and they were further confirmed with the agar well diffusion assay as shown in the Table-1, with the minimum of 20mm clearance zone produced by isolate B4 and maximum of 26 mm produced by isolate B1. Further evaluation of crude protease from these bacterial isolates through broth culturing for five days as observed from the Figures 2 & 3 indicate that there had been the opportunistic growth of microbial organisms on the readily available glucose and other nutrients for the initial 24hrs of culturing and simultaneously produced, protease as growth associated product. So till 24hrs of cultivation, the bacterial soil isolate B1 produced the maximum protease of 49.3325U, and that must have driven its higher secondary growth profile in the later period. However by 48hrs of culturing, B3, B4 & G2 have recorded nearly the same protease activity in the range of (47-52)U, where B3 produced the maximum protease activity of 51.684U; similarly the isolate G2had recorded mostly the same profile of protease as that of B3. On the other hand, the bacterial soil isolate B4 recorded on an average 44U of protease activity between 24 & 96hrs of cultivation and further it had the decelerating protease activity up to 34.5U by 120hrs of cultivation.

4. Discussion

The bacterial soil isolate G2 recorded highest growth of 6.6 mg and produced the moderate protease concentration of ~39U by 24hrs of cultivation but later the growth got declined and reduced to an average of 4mg; which may be due to its instability in the production of protease after 24hrs of cultivation. On the other hand B1, though produced lower growth till 24hr, yielded higher protease of 49.332U, that could

produce enough substrates or nutrients from the protenacious substrate like peptone which in turn helped B1 to produce the highest growth profile in the later periods, but then as shown in Fig-2, the decrease in protease production may be due to diminishing protein content in the broth. Similar is the case of isolate G2 with an exception in decreased growth. In case of isolate B4, although there was an oscillation in the growth phase, it has recorded a constant & steady protease production of about 45U during the cultivation period of 24-96hrs; which may be symbolic to non-growth associated protease production. Conversely, the isolate B3 produced the maximum protease production ~ 52U by 48hrs of cultivation and then the production has dropped which may be due to drop in the protease inducer and increase in the growth nutrients. The physical and biochemical observations as shown in Table-2 revealed the bacterial soil isolates most likely to be of the rod shaped *Bacillus* sp. However the 16S rDNA sequencing analysis of B3 and B4 revealed them as *Pseudomonas* sp. SPSU B3 and *Aneurinibacillus migulanus* sp. SPSU B4 respectively [28].

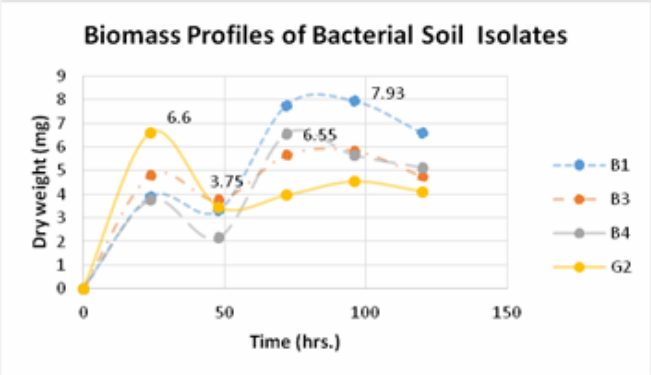

Figure 2. Biomass profiles of bacterial soil isolates B1, B3, B4 and G2 subjected to cultivation with protease specific broth media at 30°C and 120rpm.

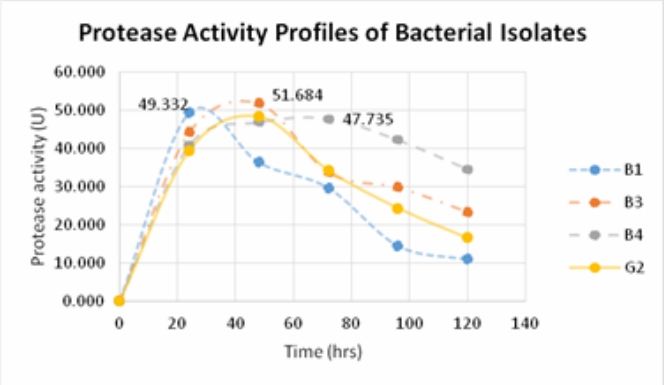

Figure 3. Protease activity profiles of bacterial soil isolates B1, B3, B4 and G2 subjected to cultivation with protease specific broth media at 30°C and 120rpm.

5. Conclusion

This study has brought out the bacterial soil isolate B3 as the best protease producing strain that was identified as *Pseudomonas* sp. SPSU B3. The next best strain was B1 with its maximum protease production of 49.33U and it was found to be opportunistic and of diauxic growth capabilities depending on the available nutrients in the culture media. The other bacterial strain B4, was found to be of the special strain with an average 45U of protease production for extended period of production with an average growth profile; and this strain was identified as *Aneurinibacillus migulanus* sp. SPSU B4. Further studies on these strain may produce the better results that may cater to the needs of industry and academia.

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