Int J Pharma Res Health Sci. 2017; 5 (1): 1581-1585
DOI:10.21276/ijprhs.2017.01.12
Harish *et al.*
CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Isolation, Characterization of Protease Producing Microbes from Soil of Agriculture Land and Purifcation of Protease

Harish R *, Jyoti Bala Chauhan

Department of Biotechnology, Microbiology & Biochemistry, Pooja Bhagavat Memorial, Mahajana Post Graduate Centre, wing of SBRR Mahajana Education, Mysore, Karnataka, India

ARTICLE INFO

Received: 08 Feb 2017
Accepted: 21 Feb 2017

ABSTRACT

The objective of this experiment was to isolate protease producing microorganisms from soil samples collected from fertile agricultural land. Totally seven isolates were isolated from the soil samples by serial dilution technique, among them isolate 1 was selected for the experiment since it showed the maximum activity. The activity of protease was estimated by measuring the liberated amino acids from the protease by titration method in liquid broth of skimmed broth, also by visualizing the zone of degradation of casein from the plated skimmed agar plates. Further the isolate was studied for its culture characteristics, staining for its identification and purification of the enzyme was also done. Based on the biochemical and colony morphological study it was identified as gram positive cocci bacteria. This isolate was further studied with different parameters such as, effect of temperature, pH, nitrogen and metal ions. The optimum conditions of various parameters mentioned above were estimated.

Key word: Bacterial protease, purification, industrial importance of protease

1. INTRODUCTION

Protease is an important industrial enzyme, generally obtained from microbes, animal and plant sources. This enzyme breaks the complex polypeptides into simples chains of aminoacids. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% total worldwide sale of enzymes[1]. Several microbial and fungal strains viz., *Aspergillus flavus, Aspergillu smiller, Aspergillus niger, Penicillium griseofulvin, Bacillus licheniformis* and, *Bacillus firmus*,) have reported to produce

Corresponding author *
Dr Harish R
Department of Biotechnology, Mahajana
Post Graduate Centre, wing of SBRR Mahajana Education,
Mysore, Karnataka, India
E Mail: harishrh@vahoo.com

IIIIIIII© International Journal of Pharma Research and Health Sciences. All rights reserved

proteases. Among these, *Bacillus* genes gained importance at industrial scale[2].

Though proteases are present in different sources. The best source used for the industrial application is microbes, since it's easy to cultivate and rapid growth rate with minimal space. Also using genetic engineering several nonprotease producers microbes have been developed to protease producers by insertion of specific genes with thermostable for its various applications in industries[3]. Production of proteases from microbial sources depends on several factors such as media composition, temperature, pH, incubation, moisture[4].

Application of proteases is several in physiological and pharmaceutical aspects in humans, it plays vital role in immune system of humans system in defending against pathogens by lysing them[5]. They also play an important role in proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms[6].

Alkaline protease has been used in different industries such as detergent where they help to remove the protein stain, leather industry as dehairing agent[7]. In textile industry used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved softness[8]. Protease is also used in pharmaceutical products like contact lens proteins cleaner. It also helps in natural healing process of skin by removal of necrotic tissue.

In this paper we have focused in isolating proteases producing microbes, from soil and purify enzyme which could be beneficial for several industries and to study the factors affecting their activity to present potential application of the proteases for industrial applications

2. MATERIALS AND METHODS

2.1 Screening and isolation of proteolytic microorganisms

Proteolytic microbes were isolated from soil collected from agricultural land, following enrichment media and the isolates obtained were plated by repeated streak plate method to get pure culture. The isolation was screened for protease producing ability by skimmed milk hydrolysis method such as skimmed milk agar media (Skimmed milk powder 1%, Agar1.5% in distilled water) and skimmed milk broth (skimmed milk powder 1% in distilled water).

2.2 Characterization and identification of isolate

The isolate I was identified based on cellular morphology, growth, staining and different biochemical, physiological characteristic and these were compared for standard description of the Bergey's Manual Determinative Bacteriology[2].

2.3 Determination of protease activity

The protease activity was measured by measuring the protein (casein) accordingly to walker's method[9]. The higher the level of casein is less activity and visversa. After required incubation period of inoculum, reaction was stopped by adding formalin to the media which, blocks amine groups in proteins and reveals activity of all proteins free carboxyl groups, effecting increase of milk acidity. This level of increased acidity is the base to measure amount of protein present in media by titrating against 0.1 M NaoH using phenolphthalein indicator till pale pink color appears. This is index of measuring the amount of protein degraded by proteases. The amount of protein content is calculated according to the equation

$X = a \times 1.47$

Where, a=Amount of 0.1M NaoH used to titrate after formalin addition.

1.47=Counting factor for casein.

2.4 Effect of temperature on enzyme activity

The skimmed milk media of broth and agar plates was inoculated and incubated at different temperature (4^0C, 27^0C, 37^0C, 40^0C & 50^0C) for 24-48hr, to get optimum enzyme production. For this reason, equal quantity of inoculum was added in each conical flask containing 50ml of media for broth and 20ml for SKP plates. Plates were observed for degradation of casein by observing zone formation and broth culture was titrated using walker's method to quantify the enzyme activity.

2.5 Effect of pH on enzyme activity

The effect of pH on the enzyme activity was performed both in broth and agar plates. To 50 ml of 1% skimmed milk broth different pH concentration was set (3, 5.7,8 & 9) and skimmed milk agar plates were also prepared similarly. It was then inoculated and incubated at 37^0C for 24-48hr. Plates were observed for degradation of protein and broth culture was titrated using walker's method to quantify the enzyme activity.

2.6 Effect on nitrogen source on enzyme activity

Yeast extract, beef extract, sodium nitrite, casein, tryptone, ammonium sulphate, urea and peptone (0.1%) was selected as sole nitrogen source which was supplemented with 1% SKP medium in respective broth flask and agar plates respectively. Further, the inoculated broth and plates were kept for incubation for 24-48 hr and enzyme activity was performed.

2.7 Effect of metal ions on enzyme activity

Magnesium chloride, EDTA, manganese sulphate, zinc sulphate and ferrous sulphate (0.01%) was selected as sole metal ions which were supplemented to SKP media in respective broth and agar plates. The flask and plates were sterilized, and medium was inoculated with inoculums and kept for incubation at 37^0C. After 24 hours the enzyme was assayed to check the maximum activity.

2.8 Enzyme purification

The protease purification was performed according to the method described by Mckevitt, A I.; Bajaksouzian [10]. Purification of enzyme involves following steps.

1) The cocci bacteria were allowed to grow in the SKP broth at 37^0c for 24 to 48 hr. Further the crude enzyme was prepared by lysing the bacteria by sonicator and centrifuged whole SKP broth at 4000 rpm at 40c for 10 min. The filtrate

IIIIIIII© International Journal of Pharma Research and Health Sciences. All rights reserved

obtained containing the protease was collected and its proteolytic activity was performed.

2) The filtrate was subjected to ammonium sulphate precipitation according to the method mentioned by Dioxon and Webb [11]. Initially the filtrate was brought to 20% (w/v) saturation and the precipitated protein was collected by centrifuging at 8000 rpm for 15min. Further the precipitate obtained was dissolved in 0.2mM phosphate buffer pH7.0. Similarly the left out supernatant was subjected to different saturation of 40, 60 80 and 100% (w/v) using ammonium sulfate and enzyme activity was determined for each fraction.

3) The fractions (20-100%) obtained was done dialysis, by filling the each fractions to dialysis bag respectively and kept to dialysis against distilled water to remove all the salt content and to concentrate the enzyme. The dialysis was kept for 24 hrs with a change of distilled water three times at interval of 7 hr. The obtained enzyme after dialysis was stored in a vial at 4^0c for further purification.

4) Column chromatographic technique was done to purify the enzyme, preparation and fraction procedures was determined as described by Ammar et al [12]. Ready prefilled Sephadex G-200 column with size of 3x7 cm was used. The column was stabilized with 0.2 M phosphate buffer pH 6.2 for 1 d at room temperature. Sodium azide (0.02%) was added to prevent any microbial growth. To determine the void volume, a mixture of blue dextran 2000 and bromophenol blue was eluted. One ml of the enzyme sample was applied carefully to the top of the bed column. It was allowed to pass into the gel by running with column buffer without disturbing the bed surface and eluted with the elution buffer. Fifty fractions were collected (each of 5 ml-1).Each fractions collected was assayed for proteolytic activity, activity guided fractions were pooled, concentrated and purified which was confirmed by SDS PAGE electrophoresis

3. RESULTS AND DISCUSSION

3.1 Screening and isolation of proteolytic microbes on skimmed milk agar plate and broth:

The soil samples obtained was serially diluted and plated on skimmed agar plates and screened for proteolytic activity by observing zone of hydrolysis of casein in the inoculated plates. Totally five isolates showed clear zone of inhibition on skimmed milk agar plates, indicating that isolates have tendency to produce proteases to degrade milk protein (casein). The isolates showing inhibition zone are shown in Fig.1. Similarly in SKP broth also performed, among five isolates, isolate I showed maximum activity. Further, isolate I was selected for studying various parameters and to purify and characterization.

3.2 Characterization and identification of isolate I

Based on the biochemical characterization shown in Table I the isolate I which showed highest activity was found to be gram-negative cocci shaped bacteria.

3.3 Effect of temperature on isolate I

The data shown in Fig.2. clearly indicates the influence of incubation temperature on protease production. Isolate showed maximum activity in 37^0 C, with increase in temperature the activity decreased. However, the activity declined to 50 % at 42^0C and 30% at 50^0C, still the isolate is important as industrial purpose due to withstanding more than the optimum temperature.

3.4 Effect of pH on isolate 1:

Isolate 1 showed optimum activity at pH 5, with increase in pH, the activity decreased. The results showed that the isolate is moderately tolerant to acidic condition, which is a good sign to with stand high acid concentration during fermentation. Since many of the organisms fails to replicate at high pH, this is an additional feature of the isolate.

3.5 Effect of nitrogen source and metal ions on isolate I:

An external nitrogen source is essential for increased utilization of substrate and reduction in fermentation time. The order of maximum activity was observed in sodium nitrite and urea. Also Addition of metal ions is essential for utilization of substrate. The order of maximum activity of isolate 1 was shown in zinc sulphate

3.6 Enzyme purification

The lysed culture broth supernatant was concentrated by ammonium sulphate precipitation. The optimum activity was obtained at 60 % (w/v) saturation with 6 fold increase in the specific activity compared to the other. Further, the fraction was purified using Sephadex G200 filtration. The purified fraction obtained after filtration was dialyzedagainst sucrose resulted in specific activity 3185.4 (units/mg prot/ml-1) and purification folds 4.5 times (Table II). Crude enzyme resulted in specific activity of 725.2 (units/mg prot/ml) with purification folds 1 times. Further, the purified enzyme was subjected to SDS PAGE electrophoresis to confirm the molecular wt of the enzyme. It was observed the Mw. Was 43 kDa.

Although the estimation of remaining casein was estimated after 48 hr incubation period, the best yield for the production of protease was observed on fifth day of incubation, these results is coping with that of johnvesly et al [13]. The incubation time plays a major role in the production of protease, once there is sufficient enzyme produced the microbes make use the casein for its metabolism and gradually after the declining of casein the enzyme level also decreases. During the growth there is also other secondary metabolites produced which may also hinder in the production of protease[14].

The production of enzyme was obtained at 37^0c since the growth declined followed by the enzyme activity at temperature 42-50^0 C. Though the enzyme activity was also observed at 42^0 and 50^0c. But their activity was reduced to 50% compared to optimum temperature. The optimum temperature and pH was used for the enzyme purification.

IIIIIIII© International Journal of Pharma Research and Health Sciences. All rights reserved

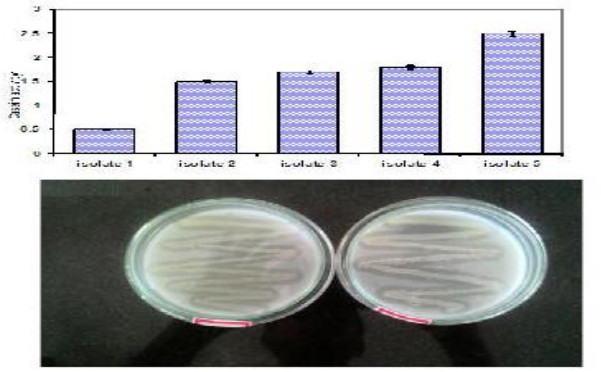

Fig 1: Protease activity of isolates (I-V) and petriplate showing degradation of casein of isolate1

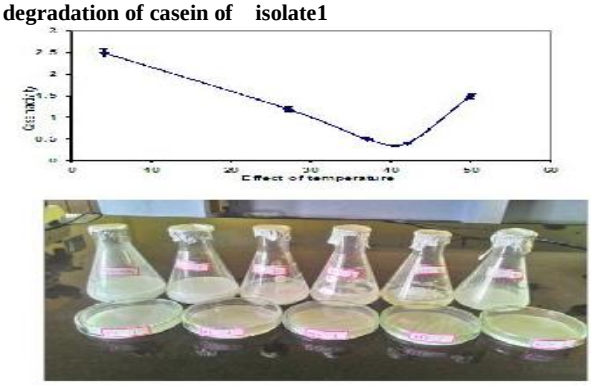

Fig 2: Protease activity of isolate I on effect of temperature in liquid and solid media

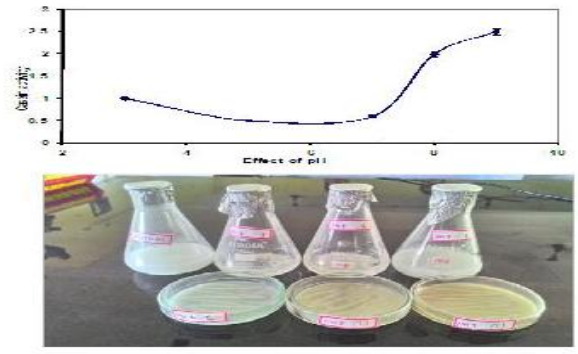

Fig 3: Protease activity of isolate I on effect of pH in liquid and solid media

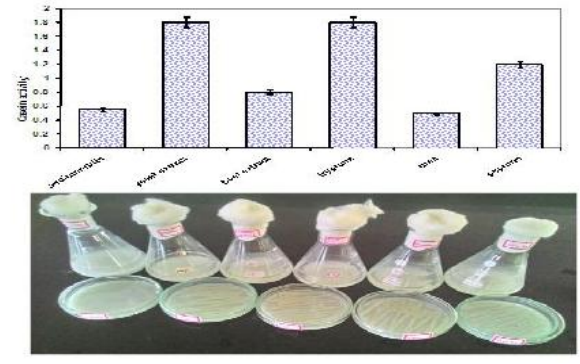

Fig 4: Protease activity of isolate I on effect of nitrogen source in liquid and solid media

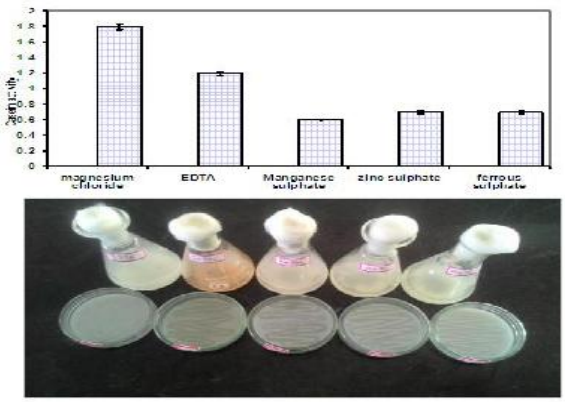

Fig 5: Protease activity of isolate I on effect of metal ions in liquid and solid media

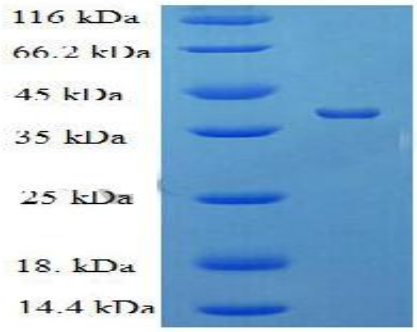

Fig 6: SDS PAGE of purified protease

4. CONCLUSION

The five isolates isolated from the soil showed protease producing activity. Among them isolate 1 showed the maximum activity; hence isolate 1 was selected for further assays. Isolate 1 was subjected to optimization parameters with temperature, pH, carbon sources, nitrogen sources and metal ions. Overall to conclude the isolate 1 showed the highest activity. Based on the biochemical characterization isolate 1 was identified as gram-negative cocci shaped, microbe. Further enzyme was purified.

The purified protease could be used for the various purposes like antibacterial activity of this alkaline protease against clinical pathogens also enzymes that degrade slime and bio films and as the result allowing the bacterium to penetrate slime layers around gram negative bacteria. It can also often used commercially in bioremediation process or as probiotic agent in aquaculture. Also, beneficial bacteria could be used as probiotics by incorporating them into fish/shrimp diet.

5. ACKNOWLEDGEMENT

The authors acknowledge the encouragement given by, Director of the Institute, and his keen interest in this study. The authors also thank, President of the institute, for his constant support.

IIIIIIII© International Journal of Pharma Research and Health Sciences. All rights reserved

Table I: Biochemical Characterization of isolate I

Isolate	Grams Staining	H2S test	Catalase test	IMVIC Test				Sugar Fermentation test			Casein hydrolysis	Starch hydrolysis	Shape	TSI test	Urease test
				Indole	Methyl red	Voges Proskauer	Citrate	Sucrose	Lactose	Dextrose					
Isolate1	-ve	-ve	+ve	-ve	+ve	-ve	-ve	A	-	A	+ve	-ve	cocci, Irregular, raised & white colonies	+ve	+ve

Table 2: Enzyme activity and purification fold of proteases

Sl.No	Enzyme Activity	Specific activity (U/mg)	Purification fold	Total protein(mg)
1.	Crude enzyme	725	1.0	220
2.	Ammonium sulphate fractionation (60%)	3285	4.5	135

6. REFERENCES

1. Nurullah Akcan and Fikret Uyar.Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation.EuAsian J. Biosci,2011;(5):64-7
2. Sneath HAP, Halt GJ, eds. Bergey's Manual of Systematic Bacteriology. 1986. Vol 2 Baltimore, MD: Williams and Wilkins.
3. Tuhina Verma and Vandana Baiswar. Isolation and Characterization of Extracellular Thermoalkaline Protease *Bacillus cereus* isolated from Tannery Effluent. Inter J.Enginee & Sci.2013; 2 (7):23-29
4. Mukesh Kumar DJ, Premavathi Venkatachalam, Govindarajan N, Balakumaran MD, Kalaichelvan PT. Production and purification of alkaline protease from Bacillus sp. MPTK 712 isolated from dairy sludge. Global Veterinaria. 2012; 8(5): 433 439
5. Raju K, Jaya R, Ayyanna C. Hydrolysis of casein by Bajara protease importance. Biotechnol. Coming Decadea, 1994; 181: 55–70
6. Haq IU, Mukhtar H, Umber H. Production of protease by *Penicillium chrysogenum* through optimization of environmental conditions. J of Agri Social Sci. 2006, 2(1): 23–25.
7. Barindra S, Debashish G, Malay S, Joydeep M. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma *Proteobacterium* isolated from the marine environment of the Sundarbans. Proc Biochem, 2006; 41: 208–215
8. Sharma OP, Sharma KD, Nath K. Production of proteolytic enzyme by fungi. *Rev*. Roum. Biochem.1980; 17: 209–215.
9. FH. McDowall and AKR. McDowell.. The determination of casein by formol titration after precipitation with acid**.** Analyst**,** 1936; 61**:**387-390
10. Mckevitt AI, Bajaksouzian S, Klinger JD, Woods, D. E. Purification and characterization of an extracellular protease from *Pseudomonascepacia*. Infection and Immunity, 1989;57 (3): 771-778
11. Dixon M. and Webb, E. G. (1964). Enzyme, 2nd Edit. Academic Press. Inc. NewYork
12. Ammar M S, El-Ssaway, M Yassin, M, Sherif, Y M. (1998). Hydrolytic Enzymes of fungi isolated from Certain Egyptian Antiquities objects while utilizing the Industrial Wastes of Sugar and Integrated Industries Company (SIIC), Egypt. J. Biotechnol. 1998; (3): 60-90
13. Johnvesly B, Manjunath BR, Naik GR**.** Pigeon pea waste as a novel, inexpensive, substrate for production of a thermostable alkaline protease from thermoalkalophilic *Bacillus* sp. JB-99. Bioresour Technol.2002; 82(1):61-4.
14. SrinivasNaik L, Aruna K, SreevennelaP, VenkataRamanaDevi.2013.Isolati on and biochemical characterization of protease isolated from *Bacillus* sp SVN12.International J. rese in Pure and Appl Microbio. 2013; 3(3):94-101.

Conflict of Interest: None

Source of Funding: Nil

IIIIIIII© International Journal of Pharma Research and Health Sciences. All rights reserved