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Research Paper

Isolation and screening of protease producing bacteria from different market soils

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Abstract

The objective of the present study was to isolate and screen protease producing bacteria from soil samples collected from local markets. Total sixteen bacterial colonies were isolated and streaked on gelatin agar plate and incubated at 37ºC for 48 hours, after which four isolates showed clear zone around the colony indicating protease activity. This was followed by morphological, biochemical test and MALDI-TOF-MS. Among the four isolates (T1, T2, T3, T4) T4 isolate produced highest protease activity, identified as *Bacillus subtilis*. The optimization parameters like pH, temperature and incubation time was observed and noted a maximum activity of 47.1 U/ml at pH 7 and the temperature for maximum activity (16.59 U/ml) of enzyme was found to be 40ºC in 24 hours. The unknown concentration of crude protease was determined using tyrosine standard curve. These parameters were carried out for rest of the 3 isolates and values recorded which showed similar trend as that of T4 isolate. Hence, a novel source for the production of protease from soil samples obtained from different market sources was approached at the end of this study.

Keywords: Protease, soil, pH, temperature, incubation time

Introduction

Microbes live in almost every place possible due to the presence of air, water or food source. Some of the familiar habitats are soil, water and food[1]. They are the most important source for enzyme production, isolation and characterization of newly promising strains using carbon and nitrogen source as a continuous process[2]. Microbes have become increasingly important as producer of industrial enzymes. However, about less than 50 species are actually used to produce the entire list of microbial enzymes of commercial importance. The potential obviously exists to search for the species producing enzymes with better properties and yield[3].

Microbial proteases are one of the most important groups of the enzymes, used in various industrial processes as food, pharmaceutical and detergent industries, as well as in the preparation of leather, textile and wool among others[4]. It also has promising application in medical usage and management of industrial and household waste. The use of microbial enzymes is the best alternative for generation of pollution free industries. Considering vital and unlimited application of microbial product, there is a need to investigate new microorganism because they are the major source of all commercially important enzymes[5].

This enzyme can be used therapeutically for the treatment of various diseases related to oxidative stress and is used in the manufacture of anti-wrinkle skin lotions[6].The demand for industrial enzymes,

particularly microbial origin, is ever increasing owing to their considerable industrial applications in a wide variety of process[7].

Materials and Methods

Source of sample collection

Different areas of Puliampatti, Erode district, Tamil Nadu was travelled to visit 4 different markets - local fruit market, local vegetable market, local fish market and local market selling corn. Handfull of soil was collected and packed in polythene bags and brought to the lab for further studies.

Isolation of protease producing bacteria

One gram of soil sample was weighed and serial dilution (10^{-2} to 10^{-6}) of each soil samples were carried out and spread on nutrient agar plate (37˚C, 24hr). After incubation the colonies were streaked on gelatin agar plate and incubated for 37˚C for 48 hr and flooded with Bromocresol green to observe the zone of hydrolysis for each sample. The colony showing highest zone of hydrolysis was selected for further study.

Identification of Bacteria

Morphological Test: The identification of bacteria was carried out by morphological studies.

Biochemical Test: Indole test, Methyl red, Voges-Proskauer, Citrate utilization, H_2S production, starch hydrolysis urease production and nitrate reduction was done.

MALDI-TOF-MS analysis: The organisms were further confirmed using MALDI-TOF-MS analysis.

Qualitative test for protein: To identify the crude sample is protein in nature, Ninhydrin test was done.

Quantitative assay of protein: The total protein content of the samples was determined by Lowry's method, using Bovine Serum Albumin (BSA) as a standard (1mg/ml).

Preparation of casein solution: Casein was used as substrate. It was prepared from alkali soluble casein which was dissolved in 10 ml distilled water (pH 8.0).

Crude enzyme preparation: The protease producing bacterial colony was inoculated in casein broth medium, incubated at 37˚C for 48 hrs. After filtration, the filtrate was subjected to centrifugation (10,000rpm for 10 minutes). The supernatant was used as crude enzyme preparation for further studies.

Protease activity assay: To study the proteoloytic activity, the supernatant was used as the enzyme source and the assay was carried out[8].

Calculation:

$$\text{Amount of tyrosine} = \frac{\text{conc. of std. tyrosine}}{\text{OD value of std.}} * \text{unknown OD}$$

$$\text{Enzyme activity} = \frac{\text{micromole of tyrosine equivalent released} * \text{total assay volume (ml)}}{\text{vol.of enzyme assay (ml)} * \text{time of assay (min)} * \text{vol. in calorimeter(ml)}}$$

Specific activity is the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins. The specific activity U/mg was calculated using formula,

$$\text{Specific activity} = \frac{\text{Enzyme activity U/ml}}{\text{Total protein content in mg/ml}}$$

Effect of temperature and incubation time on enzyme activity: The following temperatures were chosen for optimization 30˚C, 40˚C and 50˚C and incubated for 24, 48 and 72 hrs. The contents were then centrifuged at 10,000 rpm at 4˚C for 10 min. Enzyme activities were determined by standard enzyme assay.

Effect of pH on enzyme activity: This was observed using different pH ranging from 7 to 9. It was incubated at 37˚C for 24hr.The contents were centrifuged at 10,000 rpm at 4˚C for 10 min. Enzyme activities was determined by standard enzyme assay.

Results and Discussion

Isolation and screening of bacteria: Among the 16 isolates, 4 isolates were showed maximum zone of hydrolysis around the streaked lines on gelatin agar plate. Those 4 bacterial isolates were selected for protease production in fermentation broth. Zone of hydrolysis was obtained on gelatin agar plate.

Bacterial identification and characterization:

Morphological test and Biochemical Test: The following morphological and biochemical tests were carried out to confirm the species which is elaborately shown in the Table 1.

Table 1: Morphological test and Biochemical Test

S. No.	Indole	Methyl Red	Voges-Proskauer	Citrate utilization	H_2S	Motility	Gram staining	Shape	Organism
T1	N	N	N	P	N	P	N	Rod	*Pseudomonas lundensis*
T2	N	N	P	P	N	P	N	Rod	*Serratia marcescens*
T3	N	N	N	P	N	P	N	Rod	*Pseudomonas syringae*
T4	N	N	P	P	N	P	P	Rod	*Bacillus subtilis*

N = Negative P = Positive

Further these isolates were confirmed by using the emerging technique, MALDI–TOF–MS which confirmed the bacterial genus and species with their identification numbers.

Qualitative test for protein: The present study showed that all the 4 samples were protein in nature since purple colored product was obtained on Ninhydrin test. This test gives colour reactions on the basis of amino acid present in the samples were proved by Dalal[9].

Quantitative assay for protein: The amount of protein was determined by using BSA as a standard and to estimate the amount of protein present in the crude samples of 4 isolates by Lowry's method. Ashwini[10] used this method to prove the quantity of protein in the samples.

Enzymatic Assay: Production of protease by bacteria and to optimize parameters such as pH, temperature and incubation time.

Protease production by bacterial isolates: Protease activity in the crude enzyme extract was determined according to the method of Carrie cupp Enyard[8] by using casein as a substrate. Specific activity is defined as the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg),a standard curve using tyrosine was plotted.

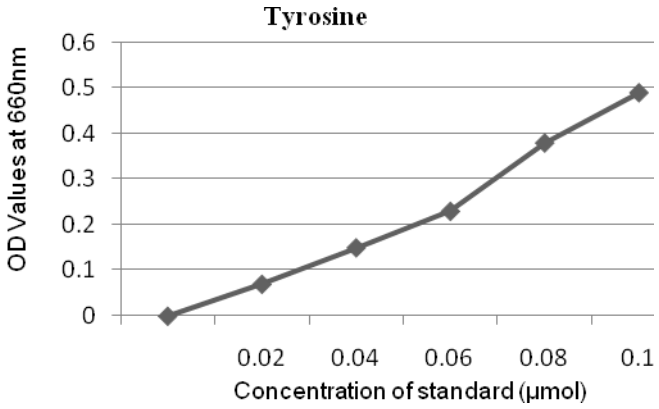

Figure 1: Tyrosine standard curve:

Effect of temperature on incubation time

To optimize the temperature ranges from 30˚C to 60˚C was chosen. It was incubated for 24, 48 and 72 hours of duration. The activity is shown from Graph 1 and 2. From four different isolate *Bacillus subtilis* has maximum activity. The similar trend was observed in the result of *Bacillus species*[11]. The activity of the enzyme was reduced to half at the third day of incubation (72 hr). It was evidentin the results of *Aspergillusniger*that as the incubation time increased, the enzyme activity was reduced[12]. The maximum activity of enzyme was found at 40˚C in 24 hrs as shown in the Graph 1 to 2.

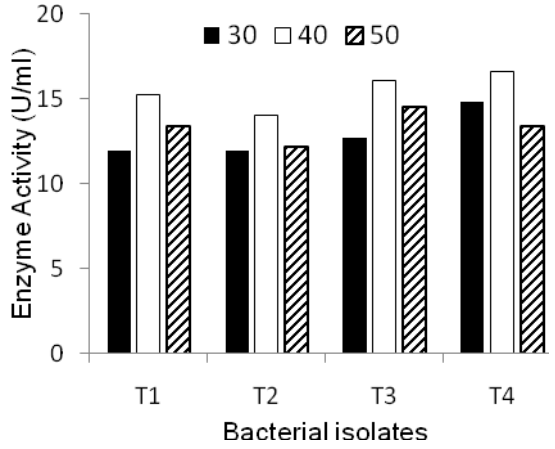

Figure 1: Effect of temperature

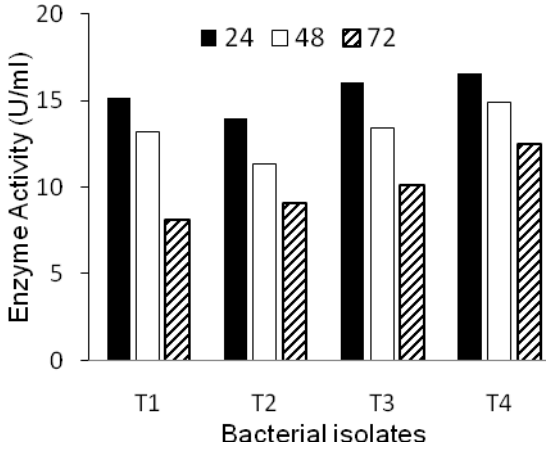

Figure 2: Effect of incubation period

T1 – isolate 1, T2 – isolate 2, T3 – isolate 3 and T4 – isolate 4

Among the 4 different isolates, bacillus species showed maximum activity in this study based on temperature and incubation time. Similar results were derived by Hanan[13] and Singh[14] observed that isolates such as *Pseudomonas lundensis, Pseudomonassyringae*and*Serratiamarcescens* also has activity at temperature 40˚C in 24 hrs.

Effect of pH

To optimize the pH, the ranges from 7 to 9 were chosen as shown in Graph 3among which T4 isolate shows maximum activity. The activity gradually decreased in the range of pH.Among the 4 isolates *Bacillus subtilis*showed maximum activity at the pH 7. The value was concordant with a study conducted by Dalal[9] where the *Bacillus species* shows maximum activity at pH 7.4. Another observation showed that the Bacillus species PCSIR EA – 3 shows maximum pH at 6.5[15].

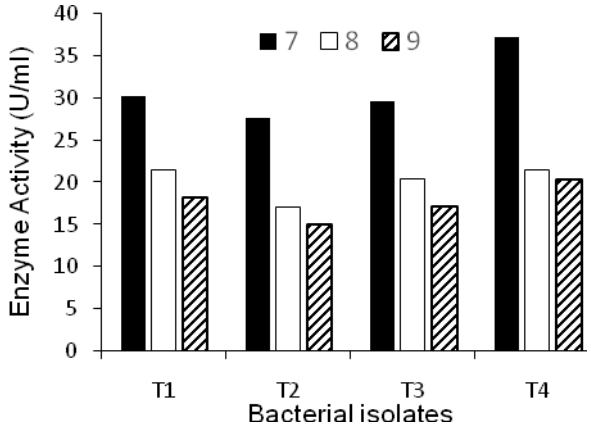

Figure 3: Effect of pH

T1 – isolate 1, T2 – isolate 2, T3 – isolate 3 and T4 – isolate 4

Conclusion

The microorganisms isolated from different local market soils showed the potential for the production of industrially useful enzyme namely protease and was optimized with varying condition of pH, temperature, incubation time and substrate concentration to enhance the production of protease which showed that the maximum protease activity was observed in *Bacillus subtilis* at 40ºC in 24 hours at pH 7. The conditions for *B. subtilis* proved to be a benchmark for the rest of the isolates as rest of the 3 isolates also responded in a similar way with the above optimized conditions. Therefore, the current study is a cutting edge in the industrial arena for the production of protease production with phenomenal applications in the biotechnology industry.Hence, these isolates can be used as an alternative source in the production of protease.

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