Isolation of Proteolytic Bacteria and Characterization of their Proteolytic Activity

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Abstract

Proteases are necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Proteases are the key enzymes in industrial application. Microbial protease plays an important role in biotechnological process. In this work the focus of our study is isolation of proteolytic bacteria and characterization of their proteolytic activity. For this fifteen bacterial cultures were isolated from soil sample collected from KMF, Dharwad, India. Out of fifteen bacterial cultures twelve were protease producers, as they shown zone of clearance on skim milk agar plate. From the twelve cultures, P5 and P12 were selected as the best cultures for protease production under submerged fermentation studies. Both these cultures were characterized based on microscopic characterization and found that P5 & P12 were gram positive rod and gram positive cocci respectively. P5 and P12 produced proteases in both alkaline and Neutral pH, however the optimal pH was found to be 10 which was alkaline condition. The optimum incubation time for time protease production was found to be 48 hrs in both cultures at alkaline condition. The highest enzyme activity for P5 was found to be 184.8 U/ml at 48th hr of incubation, and that of P12 culture was 175.85 U/ml. Further enzyme activity was tested at 55oC and it was found that enzyme activity for P5 was 15.714 U/ml and that for P12 was 8.571 U/ml. These findings clearly explain that proteolytic activity was hindered by increase in temperature to 55oC and proteolytic activity was decreased to around 90% in both the cultures.

Key words: Microbial Proteases, gram positive rod, gram positive cocci, skim milk agar plate, submerged fermentation.

Introduction

Proteases constitute one of the most important groups of enzymes both industrially and academically. Proteases are the enzymes that hydrolyze proteins by addition of water across peptide bonds and catalyze peptide synthesis in organic solvents with low water content [24, 12]. Proteases are essential constituents of all forms of life on earth, including bacteria, fungi, actinomycetes, plants and animals. Proteases are classified according to their structure or the properties of the active site. There are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral, and alkaline [18]. Proteases are the most important industrial enzymes of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale [9, 6, 13]. They are generally used in detergents [4,15,21], food industries [15,5], [15,21,10], meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds [20,19]. They also have medical pharmaceutical applications [5]. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Microbial sources are the best choice for economic production of proteases. As they can be cultured in large scale in relatively short time by well established fermentation reactions and also able to produce abundant and regular supply of the enzyme. As per the forecast, the global demand for enzymes will rise 7% per annum and this increasing economic importance has directed the research community to isolate hyperactive strains for the production of novel proteases and subsequent optimization of the various fermentation parameters for maximizing enzyme production [31]. Although a wide range of microorganisms such as bacteria, fungi, actinomycetes and yeasts are known to date to produce proteases, a large potential of commercially available alkaline protease are derived from gram positive bacillus strains because of their ability to secret large amounts of alkaline protease having significant proteolytic activity and stability at considerable high pH and temperatures [30,8,14]. Bacillus is highly favorable bacterium for protease production because it is non-pathogenic and well explored for
producing various types of proteases. Among Bacillus strains, B licheniformis, B subtilis, B acidophilous and B lentus were important strains exploited industrially so far for protease production [32]. Bacillus species produce two types of proteases, alkaline and neutral. Bacterial neutral proteases are active in narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. This property is advantageous for controlling their activity during the production of food hydrolysis [33].

This paper reports the results of a study carried out to investigate the Isolation and characterization of proteolytic bacteria from dairy waste soil samples. And also characterization of the proteolytic activity of isolated bacterial strains by submerged fermentation and evaluating the parameters such as incubation period, pH for better protease production and optimum temperature for maximum enzyme activity relatively low thermo tolerance. This property is advantageous for controlling their activity during the production of food hydrolysis [33].

Materials and methods
All the media ingredients and chemicals required for isolation of bacteria were procured from Hi media Lab (Mumbai) Ltd.

Collection of sample: Soil sample rich in protein content were collected from surroundings of Karnataka Milk Federation (KMF) Dharwad, Karnataka, India.

Isolation and screening of proteolytic bacteria: Fifteen bacterial cultures were isolated by spread plate method on nutrient agar medium (0.5 % Peptone, 0.3 % yeast extract, 1.5 % agar, 0.5% NaCl) from soil sample collected. They were identified on the basis of morphological, cultural and microscopic characteristics. All the fifteen bacterial cultures were screened for their ability of protease production on skim milk agar plate (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skim milk powder 2.8% and agar 1.5%). In this test all the bacterial cultures were separately plated on skim milk agar plate and incubated at room temperature and after 24 hrs the zone of clearance was recorded. On the basis of zone of clearance two best producers P5 and P12 were selected for submerged fermentation studies.

Protease production by Submerged Fermentation: The selected proteolytic bacteria were tested for yield of protease production by submerged fermentation.

Fermentation Medium used: Nutrient broth supplemented with 2.8% skim milk powder used as a Production medium.

Inoculum preparation: Loopful of culture was inoculated into 5ml of production medium and incubated at room temperature (27º ± 2º c) in a rotary shaker with continuous shaking at 200 rpm for 24hrs. After this 250μl of the incubated medium was transferred to 5ml of fresh production medium and was kept in rotary shaker at 200 rpm for 6 hrs at room temperature (27º ± 2º c). This was used as an inoculum for submerged fermentation process.

Submerged fermentation protocol: 1ml of the inoculum was inoculated into 50ml of the production medium. This inoculated medium was then incubated up to 72 hrs at room temperature agitating at 200 rpm in rotary shaker. The fermented broth was centrifuged at 10,000 rpm for 20 min; the supernatant was separated and used as crude enzyme source for the assay of protease produced.

Analytical methods
Protease enzyme assay by casenolytic activity: Protease activity was determined according to the method with little modification as mentioned in Arima K et al. 0.5ml of the enzyme was taken in the test tube, to this 0.5ml of phosphate buffer (pH 7.0) was added. After this 2ml of 1% (w/v) casein solution prepared in phosphate buffer was added and kept for incubation at 37º C in water bath for 10 min and the reaction was terminated by adding 2.5ml of 5% TCA. The precipitate formed was removed by filtration through whatman filter paper. To the 1 ml of the filtrate collected, 2.5ml of 0.4M sodium carbonate was added and immediately after that 1ml of 2N Folin Ciocalteau reagent was added and was incubated for 20 min at 37ºC for colour development. The optical density at 660 nm was observed in the spectrophotometer and concentration of tyrosine liberated was determined by using standard graph. One unit enzyme activity was defined as the amount of enzyme that releases 1μg of tyrosine per ml per min under the above assay conditions. The effect of various factors like incubation time, pH and incubation temperature on production of protease was studied.
For the determination of enzyme activity at pH 10(alkaline pH) Glycine-NaoH buffer of pH 10 was used in place of phosphate buffer for caseinolytic activity.

**Results and Discussion**

**Isolation of bacteria**

The naturally occurring environments provide good protein source for microorganisms. Isolations and screening of bacteria from these natural environments can be supposed to be useful for obtaining bacterial species with potential of producing protease enzyme. So in this study isolation of protease producing bacteria was aimed from soil source contaminated with dairy waste which acts as a protein source. The soil sample for this purpose was collected from from surroundings of Karnataka Milk Federation (KMF) Dharwad, Karnataka, India. The bacterial species were isolated by culturing on Nutrient agar. Fifteen bacterial colonies appeared on the plate which was further streaked on nutrient agar plate to get pure culture. All the bacterial isolates were further processed for proteolytic activity.

**Screening for proteolytic activity by skim milk Agar plate assay**

All the isolated bacterial colonies were screened on skim milk agar plate for their proteolytic activity. For this a loopful of each bacterial isolates were separately inoculated on skim milk agar plate and incubated at room temperature (27°± 2° C) for about 24 hrs. After incubation we found that twelve isolates out of fifteen isolates showed considerable proteolytic activity by distinctly forming clear zone around the colonies in skim milk agar plate (fig.1). Later two best protease producers based on the diameter of zone of clearance were selected for protease production by submerged fermentation. Of all the cultures P12 showed highest zone of clearance of 20mm diameter (fig.3), followed by P5 which showed 15mm diameter (fig.2). These two cultures (P12 and P5) were selected for protease production by submerged fermentation.
Microscopic identification and characterization of bacterial isolates

Twelve of the proteolytic bacterial isolates were further characterized by microscopic observation based on gram’s nature (fig.4 &5), motility and endospore staining. (Table.1)

Table. 1. The details of microscopic characterization studies

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Gram nature</th>
<th>Shape</th>
<th>motility</th>
<th>Endospore staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>+</td>
<td>Rods</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P4</td>
<td>-</td>
<td>Rods</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P5</td>
<td>+</td>
<td>Rods</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P6</td>
<td>+</td>
<td>Cocci</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P7</td>
<td>+</td>
<td>Rods</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P8</td>
<td>+</td>
<td>Long rods</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P10</td>
<td>-</td>
<td>Rods</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P11</td>
<td>-</td>
<td>Rods</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P12</td>
<td>+</td>
<td>Cocci</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P13</td>
<td>+</td>
<td>Cocci</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P16</td>
<td>-</td>
<td>Coccobacillus</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Gram staining of P5 and P12 culture

Fig. 4. P5 culture (gram positive rod)

Fig. 5. P12 culture (gram positive cocci)

Quantification of proteolytic activity by submerged fermentation

The proteolytic activity of P5 and P12 bacterial strains was further quantified by caseinolytic assay as described by Arima et al. with little modifications. For enzyme production submerged fermentation was set up using nutrient broth supplemented with skim milk powder as media. The crude enzyme extracted from the media is then assayed for caseinolytic assay.

Effect of pH and incubation time on enzyme activity of P5 & P12 bacterial strain

Effect of pH is one of the major factors for the growth of microbes in the medium that affects the productivity of microbes [11].

In this study much of the stress was given to determine the protease activity at both alkaline and neutral pH. This helps in the evaluation of protease synthesized and to optimize the pH condition for higher productivity.

Table 2. The enzyme activity of P5 culture at alkaline and neutral pH

<table>
<thead>
<tr>
<th>Incubation time (hrs)</th>
<th>Enzyme activity (U/ml) at pH 10</th>
<th>Enzyme activity (U/ml) at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>48.57</td>
<td>17.14</td>
</tr>
<tr>
<td>48</td>
<td>184.8 *</td>
<td>66.85</td>
</tr>
<tr>
<td>72</td>
<td>58.42</td>
<td>108.85 *</td>
</tr>
</tbody>
</table>
It was found that the P5 and P12 showed maximum enzyme activity in alkaline condition at 48h i.e the maximum protease activity of P5 culture was found to be 184.8 U/ml and that of P12 culture was found to be 175.85 U/ml (fig.6).

Similarly both cultures shown maximum enzyme activity at neutral pH condition at 72h i.e the maximum protease activity of P5 culture was found to be 108.85 U/ml and that of P12 culture was found to be 186.28 U/ml (fig.7).

Effect of temperature on protease activity of P5 and P12 bacterial strains
Temperature is one of the prominent factors affecting growth of microbes; it also affects the productivity of enzymes as most of the enzymes are heat labile [12].
Table. 4. The enzyme activity of P5 & P12 at 27°C & 55°C

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Enzyme activity (U/ml) for P5</th>
<th>Enzyme activity (U/ml) for P12</th>
</tr>
</thead>
<tbody>
<tr>
<td>27°C</td>
<td>184.8*</td>
<td>175.85*</td>
</tr>
<tr>
<td>55°C</td>
<td>15.714</td>
<td>8.571</td>
</tr>
</tbody>
</table>

Fig. 8. The enzyme activity of P5 & P12 at 27°C & 55°C

Here in this study the enzyme activity of the selected cultures was at 27°C and 55°C. As compared to enzyme activity at room temperature (27°C± 2°C) the enzyme activity at 55°C was drastically decreased to 10 fold claiming the resultant proteases are Mesophilic in their nature (fig.8).

Conclusion

The proteases are the most important industrial enzymes, accounting a major volume of total worldwide enzyme sales. In this study two best strains out of twelve proteolytic bacteria were characterized for their proteolytic efficiency. Both P5 and P12 strains shown high protease production in alkaline pH at 27°C for 48hrs under submerged fermentation. Further these two strains can be exploited for large scale protease production.

References

[12] Ishitaq ahmed et al., Optimization of media and environmental conditions for alkaline protease


