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SCREENING AND PARTIAL PURIFICATION OF PROTEASE PRODUCED BY BACTERIA ISOLATED FROM TERMITE SOIL

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Abstract – Soil is the richest source of microorganisms. Soil microorganisms act as a source for natural products like antibiotics, enzymes and other bioactive compounds. Microbial enzymes are applicable in many industrial processes and new applications are being identified continuously. These enzymes are alternative to chemicals, since they improve the properties and characteristics of the products. In the present study, bacteria were isolated from the termite soil and its morphology and characteristics were identified by staining and biochemical tests. The results showed that the isolated bacteria was Gram positive, rod shaped belonging to *Bacillus* species. They were also screened for the synthesis of cellulase, amylase, lipase and protease by qualitative and quantitative methods. Qualitative screening result showed that the isolated bacteria have the ability to produce all the four enzymes and quantitative screening proved that the isolated bacteria have the ability to synthesize more of protease than other enzymes. Therefore, protease enzyme was selected for purification by ammonium sulphate precipitation. Among various fractions, 60-80% fraction showed better precipitation, which was further purified by dialysis. The specific activity and purification fold were increased after dialysis, indicating the purity of the isolated enzyme.

INTRODUCTION

Enzymes are a type of protein molecule which is necessary for all form of organisms. They are biocatalyst, which catalyzes the specific biochemical reactions depending on its nature. Biotechnological industries have been synthesizing eco-friendly products from natural resources. Replacing chemical with biological products such as enzymes reduces the effluent formation, thereby decreasing the environmental pollution. Microorganisms play a vital role in the production of different types of enzymes which have high demands in industries for various purposes. The microorganisms which synthesize economically important enzymes are bacteria, fungi and yeast. The enzymes which are commonly isolated from microorganisms are cellulase, amylase, lipase and protease.

Cellulose is a crystalline insoluble structure, homopolysaccharide made of glucose units linked by α-1, 4 glycosidic bonds in linear manner and is degraded by the enzyme called cellulase. The conversion of lignocellulosic plant waste materials into useful products like biofuels depends on the activity of cellulase enzyme. Major cellulase producing bacteria are *Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp., (Patagundi *et al*., 2014). Amylase is a type of hydrolytic enzyme having starch as its substrate. The food industry uses amylase in large scale for various processes like making different types of syrups, to improve shelf-life of fruit juices (Karnwal and Nigam, 2013). The other wide range use of amylase includes pharmaceuticals, paper industries, clinical research, starch analytical chemistry and medical chemistry. Lipase hydrolyses the glycerol esters present in acylglycerols to liberate fatty acids and glycerol. It converts long chain triglycerides into diglycerides, monoglycerides, fatty acids and glycerol (Veerapagu *et al*., 2013). Lipases have wide range of applications in food, cosmetic, pharmaceutical industries and also in both synthesis and degradation of esters in glycerol and fatty acids. Proteases are the type of hydrolase enzyme which breaks the polypeptide chain that connects the aminoacids present in the proteins. Proteases have important position in the

commercial industries (Chudasama *et al*., 2015). The commercially available protease is of microbial origin than the plants and animals because of high productivity, require limited space for cultivation and genetic manipulation can be done easily (Singhal *et al*., 2012). The predominant producer of protease are bacteria especially *Bacillus* species, which excretes alkaline and neutral protease (Shahzad *et al*., 2015). Among these enzymes, proteases are more important because of its wide biotechnological applications (Vikramathithan and Dhandapani, 2014) like detergent, pharmaceutical, dehairing process in leather industry, brewing and cheese making, textiles, laundry and waste processing industries. The medicinal application of protease is that it can be used as healing agent in skin ulcer management (Asker *et al*., 2013).

The industrially important microbial enzymes can be isolated from termite soil. Termites are the type of polymorphic group of social insects that live in the specially built nest called termataria or termite mounds. The important functions of these termite mounds are to protect the insects and to provide optimum environment to the insects. Termite mounds are made of soil particles along with different types of minerals mixed with saliva or the faeces of the termites depending on its species. The composition of termite soil varies with the surrounding soil and its composition depends on type of soil used to build nest and type of termite species. Termite soil is slightly acidic with more amount of total organic matter. The minerals present in termite soil are magnesium, chloride, phosphate and iron in higher amount and calcium, potassium and aluminium in lower concentration than the surrounding soil (Kamalu and Okolie, 2013). Therefore, the termite soil can be used to isolate microorganisms which produce industrially important enzymes.

MATERIALS AND METHODS

Collection of sample

The termite soil formed on the surface of the dried leaves was collected from the local area near to Coimbatore, Tamil Nadu. The collected soil sample was stored in clean dry polythene bag, brought to the laboratory and kept at 4°C until used.

Isolation and maintenance of bacteria

The bacteria were isolated by serial dilution technique. The sample was dissolved in sterile distilled water to make soil suspension. It was diluted to ten-folds and 0.1 mL from 10^{-4} dilution was plated directly on nutrient agar and the plates were incubated at 37 °C for 48 hours. The individual colonies obtained were sub cultured and stored at 4°C for further characterization.

Identification of the isolates

The identification of the isolates was made based upon the morphological and biochemical characterization of the colony. This involved culturing the isolate in nutrient agar plates for studying the appearance of the colonies following Gram's staining to identify the morphological characteristics (Sundarajan, 1995) and biochemical tests were performed to identify the characteristics of the isolated bacteria (Kannan, 1996).

Qualitative screening of enzymes

The bacterial culture was screened for four different types of enzymes. The presence of cellulase, amylase, lipase and protease producing bacteria was identified by the methods given by Basavaraj *et al.,* (2014); Rao *et al.,* (2013); Ranjitha *et al*., (2009) and Sayali *et al*., (2013) respectively.

Preparation of crude enzyme extract

The screened bacterial isolates were inoculated into the medium containing glucose, peptone, yeast extract, $CaCl_2$, K_2HPO_4 and $MgSO_4$ and incubated at 37°C for 24 hours in shaking incubator for the production of large amount of crude enzyme. The crude enzyme obtained was collected in the form of clear supernatant by centrifuging at 4000 rpm for 20 minutes (Dam *et al.,* 2013).

Qualitative determination of enzyme activity

The crude enzyme extract obtained from the above procedure was used to determine the activity of different enzymes. The activity of cellulase, amylase, lipase and protease were determined based on the methods of Denison and Koehn (1977); Bernfeld (1955); Selvam *et al.,* (2011) and Dam *et al.,* (2013) respectively.

Purification of protease

The enzyme extracted from the bacterial culture having the highest activity was partially purified by two methods:

Ammonium sulphate precipitation

The crude extract was primarily purified by

ammonium sulphate precipitation method, also known as salting out method. In this, the crude extract was subjected to precipitating with different concentrations of ammonium sulphate such as 0-20, 20-40, 40-60, 60-80 and 80-100 percent (Simpson, 2004). The percent of ammonium sulphate which shows highest activity of the enzyme was used for further purification.

Dialysis

The precipitate showing highest enzyme activity obtained from ammonium sulphate precipitation method was further purified by dialysis in order to remove salts or other small molecules which is present along with protease enzyme (Roe, 2001).

Purification profile

Protease activity

The activity of the protease was determined in the crude and samples purified by ammonium sulphate precipitation and dialysis using casein as standard (Dam *et al.*, 2013).

Estimation of protein content

The amount of protein present in the crude extract, ammonium sulphate precipitated and dialyzed protease samples were calculated by Lowry *et al.*, (1951) using Bovine Serum Albumin as standard.

Specific activity

The specific activity of the crude extract, ammonium sulphate precipitated and dialyzed sample was calculated using the formula as follows:

$$\text{Specific activity (U/mg)} = \frac{\text{Total activity (Units/mL)}}{\text{Total protein (mg/mL)}}$$

Recovery percentage

Recovery percentage was calculated from the percentage of the total protease activity of the sample and the crude extract using the formula:

$$\text{Recovery percentage} = \frac{\text{Protease activity of the sample}}{\text{Protease activity of the crude extract}} \times 100$$

Purification Fold

Purification fold was calculated for ammonium sulphate precipitated sample and dialyzed sample using specific activity of the sample and the crude extract. The formula used as follows:

$$\text{Purification fold} = \frac{\text{Specific activity of the sample}}{\text{Specific activity of the crude extract}}$$

RESULTS AND DISCUSSION

Isolation and identification of bacteria

In the present study, the bacteria was isolated from the termite soil by serial dilution method and cultured in nutrient agar medium by streak plate method. The colonies formed were noticed and the clearly formed colony was selected for the further use. The selected colony was sub cultured and stored at 4 ^{o}C.

The presence of different types of bacteria and the production of carboxy methyl cellulose in the termite soil was reported by Sreeremya *et al.*, (2016). The other studies which prove the presence of bacteria in the termite soil were reported by Manjula *et al.*, (2014).

Characteristics of the isolated bacteria

The morphology and characteristics of the selected colony was identified by staining and biochemical tests. Staining result showed that the isolated bacterium was rod shape and appeared purple in colour. This indicates the isolated bacterium was gram positive in nature. The biochemical tests performed to characterize the isolated bacteria was given in Table 1.

Table 1. Biochemical characteristics of the isolated bacteria

Biochemical Test	Result
Indole	Negative
Methyl Red	Negative
Voges Proskauer	Negative
Citrate Utilization	Positive
Starch Hydrolysis	Positive
Triple Sugar Iron	Positive
Catalase	Positive
Urease	Positive

From Table 1, it was clear that the isolated bacteria had the following characteristics; It has the ability to use citrate as carbon source and produce acetate and alkaline carbonate and the synthesis of catalase, urease and amylase by isolated bacteria. The negative result for the indole test indicates that the isolated bacteria did not have the ability to convert tryptophan to indole due to the absence of tryptophanase. Result of methyl red test indicates that the bacterial culture produce less amount of acid during glucose fermentation, and also it does not produce acetyl methyl carbinol during glucose

metabolism. Triple sugar iron agar test indicates that the bacteria undergo glucose, lactose or sucrose fermentation with hydrogen sulphide formation. Based on the results obtained from staining and biochemical tests, the isolated bacterium has been identified as *Bacillus* sp. It is similar to the results reported by Duza and Mastan, (2013) and Vikramathithan and Dhandapani, (2014).

Qualitative screening for enzymes

The isolated bacterial culture was screened for the presence of enzymes cellulase, amylase, lipase and protease. The ability of the isolated bacterial culture to produce cellulase enzyme was confirmed by qualitative screening using carboxy methyl cellulose agar as the medium. The presence of zone of inhibition around the bacterial culture in Figure 1(a) indicates the synthesis of cellulase. The presence of cellulase producing bacteria in the termite soil was also reported by Saptarini and Indriyati, (2014). The production of amylase by the bacteria isolated from termite soil was identified using starch agar as the medium. The zone of inhibition formed in the agar shows the production of amylase and the zone was clearly viewed using iodine solution in Figure 1(b). The amylase isolated from agricultural soil also shows clear zone of inhibition as reported by Madhav *et al.,* (2011) and Karnwal and Nigam, (2013). Rhodamine B and olive oil were used to identify the lipolytic activity of the isolated bacteria. Orange fluorescence in Figure 1(c) confirms the synthesis of lipase by the bacteria isolated from termite soil. Similar result was observed for the bacteria isolated from soil by Rabbani *et al.,* (2015) and from waste vegetable oil contaminated soil by Lechuga *et al.,* (2016) and Thomas and Kavitha, (2015) from slaughter house soil. The isolated bacteria inoculated on the peptone gelatin agar showed the clear zone of inhibition which indicates the production of protease. The zone formed was displayed on the Figure 1(d). The bacterium isolated from the soil also shows the similar zone of inhibition (Bhavani *et al.,* 2013 and Josphine *et al*., 2012).

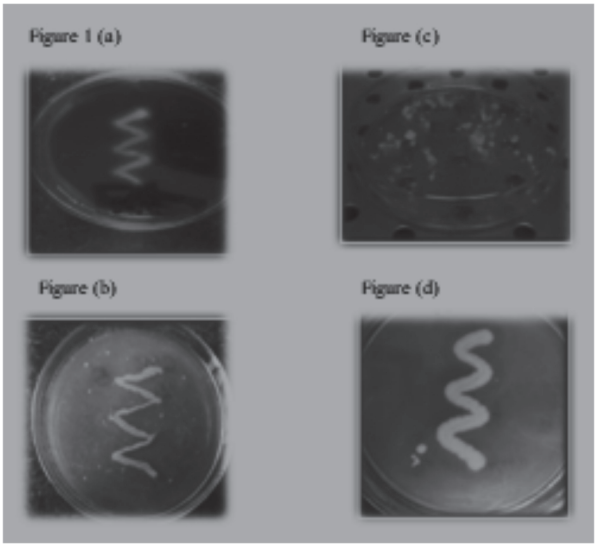

Fig. 1. (a) (b) (c) and (d): Qualitative screening of enzymes

Quantitative assay of enzymes

The crude enzyme was extracted from the isolated bacterial culture and used as enzyme source to determine the quantitative activity of cellulase, amylase, lipase and protease. The total activity of the four enzymes was shown in Figure 2.

The cellulase activity in the crude extract was determined spectrophotometrically using glucose as the standard. The total activity was calculated by constructing the standard graph and the value found was 2.82 U/mL. The protein content was also determined for the crude extract and the value obtained for protein content (2.58 mg/mL) was used to calculate specific activity. It showed 1.09 U/mg as its specific activity. According to Shanmugapriya *et al.,* (2012) the cellulase isolated from the cow dung shows the highest activity of about 4.32 U/mg. The amount of amylase in the crude extract was quantitatively estimated using maltose as its standard. The crude extract has total amylase activity of 2.71 U/mL and the specific activity calculated for the amylase was 1.05 U/mg. Kaur *et al.,* (2012) reported that the total amylase activity in *Bacillus amyloliquefaciens* isolated from soil in the potato field was found to be 0.0053 U/mL. The lipolytic activity in the crude extract was determined titrimetrically again sodium hydroxide. Total lipase activity was found to be 1 U/mL and specific activity

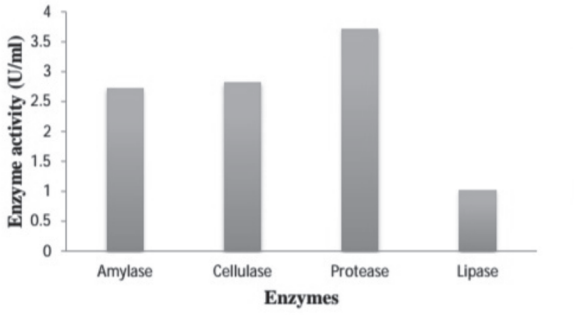

Fig. 2. Activity of different enzymes in crude extract

to be 0.38 U/mg in the crude extract. The bacteria isolated from the termite soil shows low lipase activity where as the bacteria from hill soil shows high activity of 4.280 U/mL (Rajeshkumar *et al.*, 2013). The protease activity was estimated using casein as substrate and L-tyrosine as standard. In the crude sample, the protease activity was found to be 3.70 U/mL and its specific activity was 1.43 U/mg. The protease isolated from different sources shows variation in total activity as follows - protease isolated from alkaline hot spring shows 1.2 U/mL (Wilson and Remigio, 2012).

From Figure 2, it was clear that the isolated bacterial culture has the ability to produce different enzymes at varying concentration. It produces protease in higher amount and lipase in very minute quantity. The activity of protease was found to be higher in the crude extract and it has been used for the further purification.

Isolation and purification of protease

Isolation of crude protease

The isolated bacterial culture having higher proteolytic activity was further increased by inoculating on the specific growth medium. The cell free extract was obtained from the growth medium by centrifugation and has been used as crude protease. The crude protease showed total activity of 3.70 U/mL and 1.43 U/mg specific activity. According to the results reported by Aqel (2012), the *Bacillus* strain HUTBS71 showed enzyme activity of 3.2 U/mL. To increase the specific activity of protease, the crude extract was subjected to purification by ammonium sulphate precipitation and dialysis.

Purification by ammonium sulphate precipitation

The crude protease was primarily purified by ammonium sulphate precipitation method. Different concentration of ammonium sulphate ranging from 0-20, 20-40, 40-60, 60-80 and 80-100 percent were used and among these various concentrations, 60-80 percent concentration showed increased total activity and specific activity when compared with the crude extract. The other fractions did not show significant enzyme activity. Aqel (2012) reported that protease isolated from the *Bacillus* sp., have maximum activity at 75-80 percent ammonium sulphate saturation. Protease isolated from *Penicillium janthinellum* and *Neurospora crassa* also show highest total protease activity at 70 percent ammonium sulphate concentration (Abirami *et al.*, 2011). The purification profile for the protease purified by ammonium sulphate precipitation at different concentration was summarized in the Table 2.

Purification by dialysis

The precipitate obtained from 60-80 percent ammonium sulphate concentration showed highest specific activity (2.10 U/mg) was used for further purification by dialysis. After dialysis, the specific activity of the enzyme increased to 2.87 U/mg with

Table 2. Purification profile of crude and ammonium sulphate precipitated protease

Sample	Total Activity (U/mL)	Protein Content (mg/mL)	Specific Activity (U/mg)	Recovery Percentage (%)	Purification Fold
Crude	3.70	2.58	1.43	100	1.00
0-20%	0.33	1.78	0.19	8.92	0.13
20-40%	0.34	1.31	0.26	9.19	0.18
40-60%	0.56	0.92	0.60	15.14	0.42
60-80%	0.78	0.37	2.10	21.08	1.47
80-100%	0.30	0.35	0.86	8.11	0.60

Table 3. Summary of purification profile

Sample	Totalprotease activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Recovery percentage (%)	Purification Fold
Crude extract	3.70	2.58	1.43	100	1.00
Ammonium sulphateprecipitation(60-80%)	0.78	0.37	2.10	27.90	1.92
Dialysis	0.66	0.23	2.87	17.84	2.01

2.01 purification fold. Increase in specific activity proved that purification of protease by dialysis method efficiently purifies the enzyme by removing impurities present in the precipitate obtained from ammonium sulphate precipitation. Table 3, summarizes the purification profile of the isolated protease. The similar result was reported for the protease isolated from *Bacillus cereus* having purification fold of 3.05 after dialysis (Umayaparvathi *et al.,* 2013). Muthulakshmi *et al.,* (2011) reported the protease isolated from *Aspergillus flavus* with purification fold of 2.53.

CONCLUSION

The present study was carried out with the isolation and identification of bacteria present in the termite soil, to determine the various enzymes produced by the bacteria and the isolation and purification of protease produced by the isolated bacteria. The bacterium was isolated from the termite soil and its morphology and characteristics were identified by gram staining and biochemical tests. From this, it has been found that the isolated bacteria was gram positive, rod shaped bacteria belonging to *Bacillus* species. The isolated bacteria was screened for the synthesis of four types of enzymes namely cellulase, amylase, lipase and protease by qualitative and quantitative methods. In qualitative screening, it has been identified that the isolated bacteria have the ability to produce all the four enzymes. To estimate the amount of each enzyme produced by the bacteria, the quantitative assay was performed. Based on the results, it was clear that the isolated bacteria have the ability to synthesize protease enzyme in the larger amount. Therefore, protease enzyme was selected for further purification studies.

The isolated protease was purified by ammonium sulphate precipitation and dialysis. Out of five fractions used, 60-80 percent ammonium sulphate precipitates the large amount of protease from the crude extract and it has been used for further purification by dialysis. After dialysis, the specific activity and purification fold was increased to that of ammonium sulphate precipitates and crude extract. Increase in specific activity and purification fold indicates that the dialyzed sample was purified to better extent from ammonium sulphate precipitation and crude extract. Thus the result of the present study concluded that the bacteria belongs to the *Bacillus* species was present in the termite soil and it has the ability to produce protease in larger quantity than the other enzymes. Purification by ammonium sulphate precipitation and dialysis increased the purity of enzyme by increasing the specific activity.

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