

Isolation of Alkaline Protease from Fruit Waste and its Application as Detergent

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ABSTRACT

The modernization of the society has made our environment face a series of changes. One among them is the enzymatic method produced by microorganisms from any waste. Considering the fruit waste globally several industrial uses have been fulfilled by the fruit waste. The most abundant enzyme now a days is protease from papaya waste isolated from *Bacillus spp.* Alkaline protease has been chosen for the detergent industries widely since it has pH range over 7.5. It performs more effectively in decomposing proteins. Since, it is stable over wide temperature and pH range.

The aim of the study was to isolate alkaline protease enzyme from fruit waste and its applications as detergent against three different stains such as blood stain, banana stain and tea stain. Papaya fruit waste was used to extract protease enzyme to create a useful detergent for stain removal. The enzyme's activity was tested at different pH and temperature, and the best results were obtained from three samples. The study suggests that alkaline protease enzyme from papaya waste is effective in removing protein stains and could help manage solid waste while being produced at lower temperature.

KEY WORDS: ALKALINE PROTEASE, BACILLUS SPECIES,

INTRODUCTION

Fruit and vegetable waste is the ever growing global question. Several techniques have been developed to facilitate the waste into possible sources of energy and helps in reducing the environmental pollution. Globally, the overall production of fruits and vegetables is about 675 million metric tons annually and out of the which 1.3 billion ton wastage is produced. India alone produces 86.602 million metric tons of fruits and vegetables and constitute about 5.6 million ton of waste annually. Many of the garbage are removed in ecologically unsustainable manner or the minimum quantity of waste was enzymatically decomposed with microorganisms due to their ability to secrete enzymes (Elvira C et al. 1998 Chao et al. 2017). Papaya is a popular fruit consumed worldwide and its well known for food and nutritional values. It is used in food industries, detergent industries, chewing gum industries etc. As a result, the industries generate huge amounts of papaya peel (PP) and seeds as by-products, which are typically considered a waste, and thus discarded, but that can be converted into many value-added products (Pathak et al. 2019).

Now a days enzymes play a key role in industrial areas. The two major enzymes from papaya include lipase and protease. Commercial proteases account for nearly 60% of the total industrial enzyme market (Katsuhisa et al., 2007). *Bacillus* sp is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively (Patel 1985). Although many other classes of enzymes are currently in industrial use, the focus of this paper is on alkaline proteases from bacterial species. This is because the organisms producing enzymes capable of catalysing the reactions at the extremes of pH above neutrality (Arunachalamand & Saritha 2009). The genus *Bacillus* is vital for commercially important alkaline protease which is active at alkaline pH ranging between 9 and 11. The detergent industry consumes alkaline proteases most abundantly (Moon, & Parulekar 1991). The largest application of protease is in laundry detergents and leather industry, where they remove protein based stains from clothes and dehairing purpose, respectively. As the modern world focuses on ecofriendly products and product output, subsequently more chemical processes are being replaced by enzymatic methods (Manavalan et al., 2020 Bektas et al 2023).

Protease is one of the important enzymes used in textile industry. The presently available proteases are not sufficient to meet industrial demands. Hence; there is continuous search

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for new proteases with novel characteristics for industrial application from diverse bacteria isolates. Microbes from varied habitats have been examined by many researchers to obtain the industrially suitable proteases. This present study was to discuss about the isolation of alkaline protease from the fruit waste papaya peel and discuss the stain removal (detergent activity) against the different stains as made.

MATERIAL AND METHODS

Isolation and characterization of microorganism:

Protease producing isolates were analysed from the papaya peel using a skim milk agar plates after incubation. Among the isolates, *Bacillus* species exhibited a prominent clear zone. Pure culture was obtained for the best three isolates of clear zone after the incubation period of 24-48 hrs at 37°C. This was selected for the study of protease production. This was maintained at 4°C in skim milk agar plates^[10]. The morphological and physiological properties of the isolate were investigated according to Bergey's manual of determinative bacteriology (Towatana 1999 & Becerra et al., 2016).

The three bacterial isolates were inoculated separately to three conical flasks containing 100 ml nutrient broth and incubated over 2-3 days period at 37°C in a rotary shaker. Nutrient broth without bacterial inoculation kept as control. The contents of the flasks were collected in a centrifuge tube and it was centrifuged at 10000 rpm for 10 minutes at 4°C in order to get the cell free supernatant containing crude enzyme.

Quantitative analysis of protease enzyme activity:

Protease activity in the culture supernatant was determined by using casein as a substrate. A mixture of 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 7 and 200 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000 rpm for 5 minutes. The supernatant mixed with 2.5 ml of 0.44M Na₂CO₃. 1 ml of 3-fold diluted FollinCiocalteus phenol reagent was added (Lu et al., 2018).

The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue colour developed was measured at 660 nm against a reagent blank using a tyrosine standard (Sony & Potty 2016). This was modified as Lowry's method (Lowry et al., 1951). One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution.

Enzyme activity was calculated by the formula

$$\text{Units/mL enzyme} = \frac{\text{Umol tyrosine equivalents released} \times \text{total volume of assay}}{\text{Vol. of enz used} \times \text{length of assay} \times \text{vol. used in calorimetric determination}}$$

Effect of pH on enzyme activity: The culture was incubated at 37°C for 48hrs and the pH was adjusted using different buffers ranging from 3-9. The OD values of enzyme activities were taken in spectrophotometer at 600nm (Badhe et al., 2016).

Table 1. Activity performed with different dilutions against different stains

Stains	Tap water (C)	Broth 10 ⁻⁵	Broth 10 ⁻⁷	Broth 10 ⁻⁹	Detergent + Broth	Detergent
Blood stain	20ml	20ml	20ml	20ml	0.2g in 20ml of tap water, 10 ⁻⁵ , 10 ⁻⁷ , 10 ⁻⁹	0.2g in 20ml tap water
Banana stain	20ml	20ml	20ml	20ml	0.2g in 20ml of tap water, 10 ⁻⁵ , 10 ⁻⁷ , 10 ⁻⁹	0.2g in 20ml tap water
Tea stain	20ml	20ml	20ml	20ml	0.2g in 20ml of tap water, 10 ⁻⁵ , 10 ⁻⁷ , 10 ⁻⁹	0.2g in 20ml tap water

Effect of Temperature on enzyme activity: To study the optimum temperature where an enzyme shows its maximum activity were exposed to different temperature ranging from 4°C – 100°C. After incubation the OD values were taken in spectrophotometer at 600nm (Olajuyigbe et al., 1980).

Stain removal activity with crude enzyme: The three sample broths were taken for the washing activity.

Application of protease enzyme by isolated organism as a detergent additive was studied (Kalapana et al., 2008). The washing test is performed with the three prepared broth samples for three different stains.

Partial Purification Method: Based on this washing test the best stain removal test sample medium 10⁻⁵ was identified and done partial purification for the sample by

saturated Ammonium sulphate method (Mark et al., 2002).

Characterization of Proteins Using SDS-PAGE analysis: Electrophoresis is the process of migration of charged molecules in response to an electric field. Proteins have a

net charge at any pH other than their isoelectric point (pI), thus when placed in an electric field, proteins will migrate towards the electrode of the opposite charge. This principle is used to separate molecules of differing charges (Sanbrook et al., 1989).

Table 2. Activity performed with effective broth dilution against different stains

Stains	Tap water	Broth 10 ⁻⁵	Detergent + Broth	Detergent
Blood stains	20ml	20ml	0.2g in 10ml tap water and 10ml broth	0.2g in 20ml tap water
Banana stain	20ml	20ml	0.2g in 10ml tap water and 10ml broth	0.2g in 20ml tap water
Tea stain	20ml	20ml	0.2g in 10ml tap water and 10ml broth	0.2g in 20ml tap water

Stain removal capability after partial purification:

The washing test activity is done again after the partial purification method to identify the capacity of the enzyme in stain removal ability. The three different stains were tested against the sample 10-5 medium. The stain removal of the enzyme was studied along with the detergent powder. The stain removal was studied under the following ways in table 2

RESULTS AND DISCUSSION

Isolation and characterization of microorganism: Out of 86 isolates screened the best hydrolysis zone were obtained for three isolates after 48hrs of incubation at 37°C. The zone of isolation of three isolates were named as S1, S2, S3. The zone of hydrolysis on skim milk agar is shown in Fig.1 Their morphological and physiological study for casein hydrolyse study as shown in table 2 and 3.

Estimation of Protease activity: The maximum enzyme activity was given by the sample test 1 which showed its higher activity at 0.45nm. The enzyme activity gradually increased as the substrate concentration also increased and also there was a gradual decrease in the test 2 and 3 with its substrate concentration. Table 4 shows the test samples enzyme activity.

Optimization of growth using Temperature and pH: The enzyme showed the best activity between temperature range 25°C and 45°C with maximum activity at 37°C. As the graph shown in (Fig 2) the enzyme was constant and stable at 37°C. At 45°C and above it started to decrease its activity and get completely inactivated by 120 min at the further temperatures. And in the same way the enzyme showed the reasonable activity between the pH 8 and 11 with maximum activity at 10 as shown in (Fig 3) which shows enzyme was stable at 10. At above pH 11 the enzyme started to reduce its activity after few minutes and get inactivated by 90min at further pH.

Fig 1(a): Growth of microbes in 10-5dilution



Fig 1(b): Growth of microbes in 10-7dilution



Fig 1(c): Growth of microbes in 10-9dilution



Table 3. Shows the results of Morphological study

Sl.No	Gram staining	Negative staining	Spore staining
S1	Positive	Short chains	Endo spores
S2	Positive	Short chains	Endo spores
S3	Positive	Short chains	Endo spores

Stain removal activity with crude enzyme: A good detergent additive protease enzyme must have a capable to remove the protein stains by cleaving the protein bond breakage. Therefore, the effects of various oxidizing agents

and surfactants on the protease enzyme were studied. stability of enzyme towards these stains were also analysed with the incubation period of 60min and 120mins (Kumar; Takagi 1999 & Joo 2005). protein stability has commercially wide applications in detergent industry has been reported in many studies (Kumar & Bhalla 2004) Oxidizing agents are major ingredients of modern bleach based detergents. Alkaline protease from bacillus shows extreme stability towards oxidizing agents (Nadeem et al., 2013).

of 2hrs .The best results were obtained from the sample 1 isolate as shown in (Fig 2,3 and 4).

Partial purification and molecular weight determination: To obtain proteins from cell free culture filtrate the sample 1 isolate were partially purified with ammonium sulphate at 75%(w/v)saturation by adding powdered Ammonium sulphate slowly with continuous stirring . The yield of protein was 2.30mg/mL, with a total activity of 415.26U/mL per min. This yield of partially purified protein was undergone for molecular weight determination by SDS -PAGE analysis and the result was shown in the(Fig 5)

Table 4. Shows the results of Physiological study : Casein hydrolyse test:

Sample	Growth	Zone of inhibition(mm)
S1	Yes	15mm
S2	Yes	11mm
S3	Yes	9mm

Washing test with partially purified enzyme: The enzyme with extreme stability towards oxidizing agent is commercially significant as the peroxidise are common ingredients of bleach based detergents. The enzymes prepared are mostly stored under low temperatures which will prevent its denaturation if the enzymes can withstand room temperature it is very useful in lifetime of a detergent product. So by considering these issues the partially purified enzyme sample 1 isolate has withstand its room temperature and also removed the blood stain, banana stain, tea stain. Thus this reveals its use in detergent industry. The washing test performed is shown in (Fig 6,7, and 8.)

By considering these facts the washing test were performed for the blood stain, banana stain,tea stain with crude enzyme of three isolates were washed after the incubation period

Table 5. Estimation of protein by Lowry's method

Test tube	Tyrosine Vol. Conc.	Alkali ne reagent	Cond.	Dis. water	Folins phenol	Cond.	OD at 660nm
B	- -	2	Incubation	2.00	0.2	Keep	0.00
S1	0.05 5	2	in	1.95	0.2	in	0.25
S2	0.10 10	2	dark	1.90	0.2	dark	0.33
S3	0.15 15	2	room	1.85	0.2	room	0.40
S4	0.20 20	2	for	1.80	0.2	for	0.53
S5	0.25 25	2	10 mins	1.75	0.2	30mins	0.60
T1	1.00	2		1.00	0.2		0.45
T2	1.00	2		1.00	0.2		0.37
T3	1.00	2		1.00.	0.2		0.33

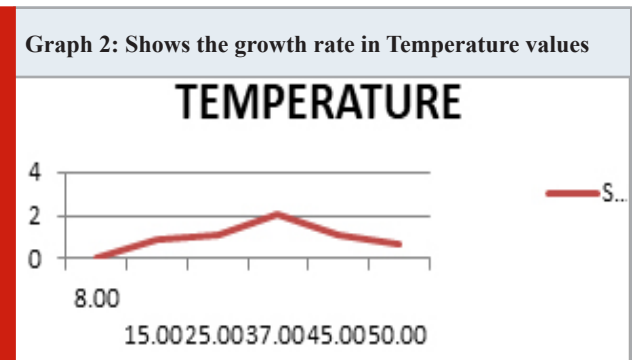
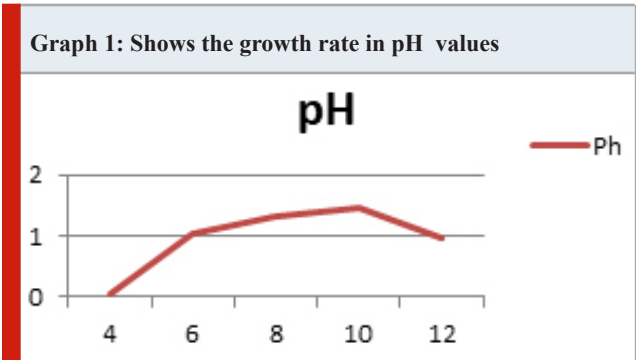


Figure 2(a) : Stained blood clothes in S1 sample.



Figure 2(b) : After 2 hrs the washing activity shows best

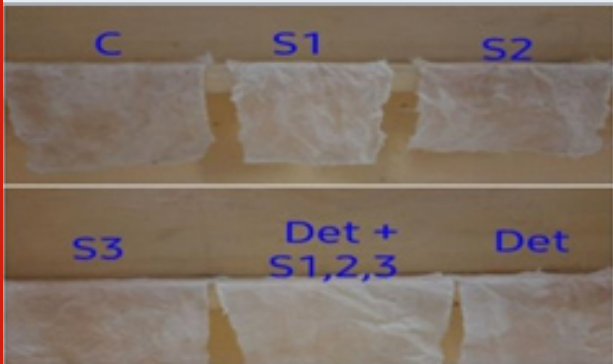


Figure 3(a): Stained banana clothes in S1 sample.

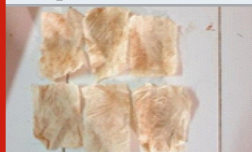
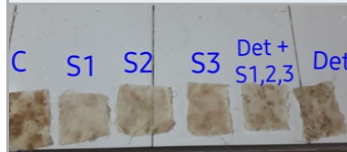


Figure 3(b) : After 2 hrs the washing activity shows best



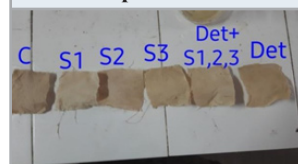
Isolation and characterization:

Growth of Bacillus sp., in different dilutions

Figure 4(a) : Stained tea clothes

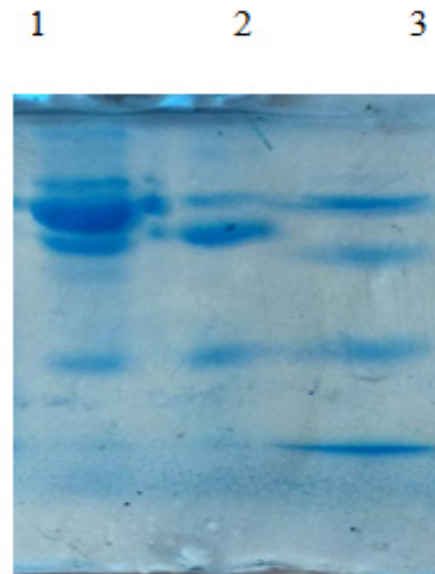


Fig 4(b) : After 2 hrs the washing activity shows best in S1 sample



Partial purification and molecular weight determination:

Figure (5) : SDS-PAGE analysis of isolated protease from papaya peel waste.



Lane -1 : Marker, Lane -2 : Crude lane, Lane -3 : Partial Purified lane Marker : 10kda , 50kda, 75kda, 100kda

Lane -1 : Marker, Lane -2 : Crude lane, Lane -3 : Partial Purified lane Marker : 10kda , 50kda, 75kda, 100kda

Molecular weight of crude proteins:

1st band – 82kda, 2nd band- 68kda, 3rd band- 12kda

Molecular weight of partially purified proteins:

1stband -81kda, 2nd band- 47kda, 3rd band – 10kda

Washing test with partially purified enzyme:

Banana stain:

Figure (6a) Blood stained clothes purified protein

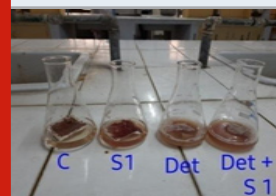


Figure 6(b) : Washing activity after 2hrs proved best partially results in 10⁻⁵

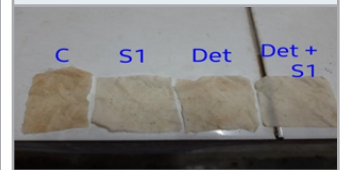


Fig (7a) Banana stain clothes in partially purified protein

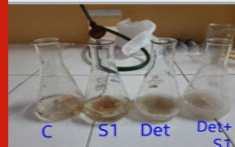


Fig: (7b) Washing activity after 2hrs proved best results in 10⁻⁵

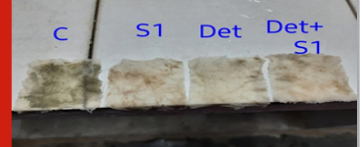


Fig (8a):Banana stain clothes in partially purified protein



Tea stain:

CONCLUSION

Papaya fruit waste was used to extract protease enzyme to create a useful detergent for stain removal. The enzyme's activity was tested at different pH and temperature, and the best results were obtained from three samples. Washing activity was performed with three different stains, and the best sample was identified as 10 - 5 broth, showing the best stain removal activity after 2 hours. The sample was partially purified and tested again, confirming the presence of protein by SDS - PAGE analysis with a specific molecular weight. The study suggests that alkaline protease enzyme from papaya waste is effective in removing protein stains and could help manage solid waste while being produced at lower temperature.

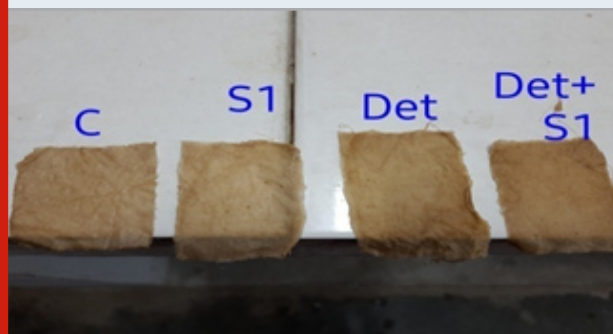
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Fig (8b): Washing activity after 2hrs proved best results in 10-5



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