Optimization of Temperature for Extraction of Protease from Coconut Shells

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Abstract

Proteases are the most important biomolecule in all organisms. It is present in all organisms. There are different types of protease in the range of microbes to macrobes. The main classes include Cysteine proteases, Aspartic acid proteases, Serine proteases, Metalloproteases, Glutamic acid proteases and Threonine proteases. They play both structural and functional roles and the types of proteases differ accordingly in different organisms. The key role of Protease is selective and specific as its cleavage is limited for a specific substrate. It also has potential therapeutic applications and with the help of this enzyme, many diseases are cured like thrombolysis, cardiovascular disease and the treatment of sepsis, cystic fibrosis, digestive disorders, inflammation and retinal disorders. Proteases are also used for neuromuscular treatment. Other than these vital roles, proteases are used in many industries like food, detergent, leather, etc., for different purposes. In the performed study, Protease was isolated from Coconut shell chips and Coconut shell powder at different temperatures such as 30°C, 50°C, 60°C, 80°C and 100°C. The optimum temperature for extraction of Protease from coconut shell was found by estimation of protein, determination of Protease activity and performance of plate assay. The extracts obtained were subjected to partial purification. Among the different temperatures used for extraction, Protease concentration was found to be grater at an extraction temperature of 100°C.

Keywords: Protease, protein, extraction temperature and Plate assay

INTRODUCTION:

Proteases are one of the three major groups of industrial enzymes that are used in the detergents, pharmaceuticals, leather industry and the food industry. Protease enzymes are essential in the digestion process because they hydrolyse the peptide bonds in protein foods, releasing the amino acids that the body requires. Plant proteases have been used in different industries, because of their high stability in harsh conditions, substrate specificity, good solubility and activity over a wide pH and temperature range.[1] Plant-derived proteases, one of the major groups of proteolytic enzymes, are involved in many regulatory processes in plants. Despite being the most numerous class of proteases, the regulatory roles and activities of plant proteases are not understood, because ,of lack of identification. The majority of the plant proteases that have been identified, isolated and are employed for different roles in the food industries.[2] As a result, the proteases are of less cost for industrial applications. Furthermore, the search of novel proteases from plant sources is ongoing, with the researchers hoping to understand the physiological roles of these enzymes in order to develop useful and cost-effective solutions for industry.[3]

The coconut or *Cocos nucifera* L. has been called the “tree of life”. Every component of coconut has its own importance. The coconut tree and all of its components have several health benefits. Researchers have recently identified that coconut has antimicrobial and anti-cancer properties. Intake of coconut reduced the death and disability associated with high cholesterol and heart disease.[4] One of the wastes and environmental concerns in the coconut-producing nation is coconut shells. Several agricultural wastes, such as coconut shells have been investigated as bio sorbents for wastewater treatment and the applications of various types of plant parts such as shell, fibre, coir and so on, have also been studied. According to the statistical analysis, 70% of the entire output was generated in India, Indonesia, and the Philippines.[5] A large volume of coconut shells is being discarded into the environment, which is contaminating soil and water supplies. The time taken for degradation of coconut shell is about a year. However, the usage of coconut wastes has provided a supply of resources for both the general public

and the processing sector. The discarded coconut shell is used for producing different products such as coir, charcoal, coco-diesel, etc.[6]

Materials and method:

Collection of plant material:

Plant Material (*Cocos nucifera L. Arecaceae*) endocarp was collected from Paduvampalli village in the Coimbatore district Latitude: 11.197˚ and Longitude: 77.13˚. The shells were collected and dried in sunlight. The outer fibres were scraped off, then crushed into small chips, were taken as one of the source for protease extraction. Coconut shells were pulverized to a fine powder and sieved using 80 mesh sieve to get uniform particle size which is used as another source for protease extraction. Coconut shell chips and Coconut shell powders were used for the enzyme extraction process

Preparation and enzyme extraction:

Coconut Shell chips Extract:

About 10 grams of coconut shell chips were weighed and soaked in 100 ml of hydro alcohol. For extraction five different temperatures were followed viz., 30℃, 50℃, 60℃, 80℃ and 100℃ in water bath. The samples were incubated at defined temperatures for 24hrs. After incubation, the extract was filtered using whatman no. 1 filter paper and stored at 4℃ for further use.

Coconut Shell Powder Extract:

About 10 grams of coconut shell powder was weighed and soaked in 100 ml of hydro alcohol. For extraction five different temperatures were followed viz., 30℃, 50℃, 60℃, 80℃ and 100℃ in a water bath. The samples were incubated at defined temperatures for 24hrs. After incubation, the extract was filtered using Whatman no. 1 filter paper and stored at 4℃.

Salt precipitation (Ammonium sulphate): The collected extract was subjected to partial purification. 30% and 60% ammonium sulfate salt precipitation method was used for partial purification. Ammonium sulfate precipitation was done at 4℃ overnight. After incubation, Ammonium sulfate precipated sample was collected by centrifugation at 10000rpm for 10 minutes. The pellet was dissolved in double distilled water.

Dialysis: Ammonium sulfate precipitate collected was dialysed. Dialysis was carried out by using cellulose acteate membrane pore size of 5-10nm. Ammonium sulfate precipitated protein was dialysed against PBS (Phosphate Buffer Saline) buffer for 12 hrs at 4℃ by changing PBS buffer at an interval of 2 hrs.

Quantitative assay of protein: The total protein content of the samples was determined by Lowry's method. Bovine Serum Albumin (BSA) at a concentration of 1mg/ml was used as standard. Protein estimation was done for coconut shell chips extract and coconut shell powder extract.[8]

Determination of Proteolytic activity:

The protease activity of the extract was estimated by taking 1mL of the substrate (1% casein dissolved in 0.1 M phosphate buffer, pH 7 and incubating it at 45°C for 15 minutes, with 1 milliliter of the crude enzyme extract. The reaction mixture was incubated at 45°C for 20 minutes. The reaction was stopped by adding 2 mL of 0.4 M Trichloroacetic acid (TCA) and then it was incubated at room temperature for 20minutes. For the blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as described above. One milliliter of the filtrate obtained after TCA precipitation was added to 5 mL of 0.4M sodium carbonate solution and 1 mL of Folin's reagent and incubated at 37°C for 20 minutes for color development and absorbance was read at 660 nm. A standard curve was plotted using 0.1–1.0 mg/mL tyrosine solutions. The proteolytic activity was expressed as units of µg tyrosine per milliliter.[9]

$$\text{Enzyme activity (U/ml)} = \frac{\mu \text{ mol tyrosine equivalent releases} * \text{Total volume of assay}}{\text{Volume of enzyme taken} * \text{Incubation time}}$$

Protease estimation was done for coconut shell chip extract and coconut shell powder extract.

Observation of qualitative parameters to define optimization:

SDS PAGE:

Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 12% resolving and 5% stacking gels for separating proteins. Laemmli's method was followed for gel electrophoresis. The samples were mixed with an equal volume of gel loading buffer and heated at 95°C in a water bath for 2 mins. The samples were electrophoresed at 90 V for the first 10 mins and then run at 150 V with Biorad mini protean gel electrophoresis system. After a complete run, the gel was stained with Coomassie Brilliant Blue and observed the result.

Qualitative Analysis of Protease by Plate Assay Method: The extracted enzyme was further confirmed on a casein agar plate for the presence of protease. A 0.5% casein agar with 0.0015% BCG dye was prepared and wells were made (40 mm). To the wells, 0.5U of the purified extract and standard Proteinase K were added. Sterile distilled water was used as negative control. After 24 h of incubation at 37°C, the clear zone confirmed the presence of protease enzyme. The zone diameter was measured to define the optimum extraction methodology.

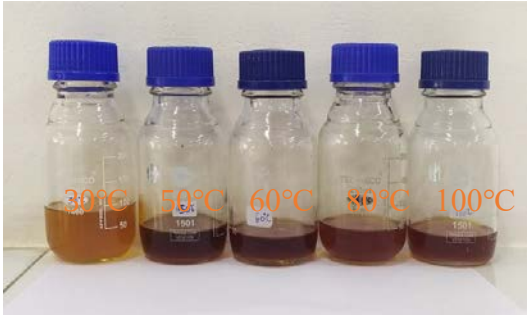
RESULTS AND DISCUSSION:

The study was carried out on the *Cocos nucifera* shells, and it revealed the presence of Protease. Protease was isolated from Coconut shell chips (figure 1a) and Coconut shell powder (figure 1b) at different extraction temperatures (30℃, 50℃, 60℃, 80℃ and 100℃) and it was partially purified through ammonium sulphate precipitation. After partial purification, using 0-30% and 30-60% of the protein concentration, protease concentration were estimated for all the extracts of different temperatures (30℃, 50℃, 60℃, 80℃ and 100℃). The effective extraction temperature for maximum yield of protein was identified.

Fig: 1(a) Coconut shell Chips

Fig: 1(b) Coconut shell Powder

Protease was extracted from coconut shell at different temperature like 30℃, 50℃, 60℃, 80℃ and 100℃. According to Sharmin *et al.*, 2007, the sample was heated at 80℃ and the thermostable protease producing bacteria was isolated, similarly the coconut shell sample was heated for protease extraction in the study. The extracts of coconut shell chips and coconut shell powder of different temperatures were shown in the figure 2(a) and 2(b).



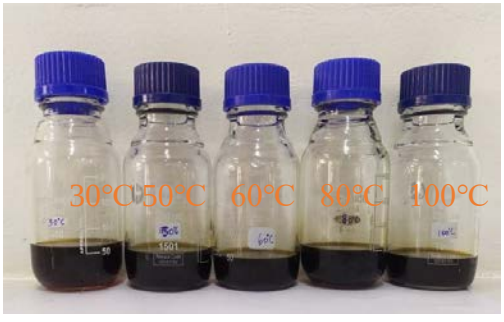


Fig: 2(a) Extracts of coconut shell chips

Fig: 2(b) Extract of Coconut shell powder

Optimization of extraction process by quantitative parameters

Protein Estimation:

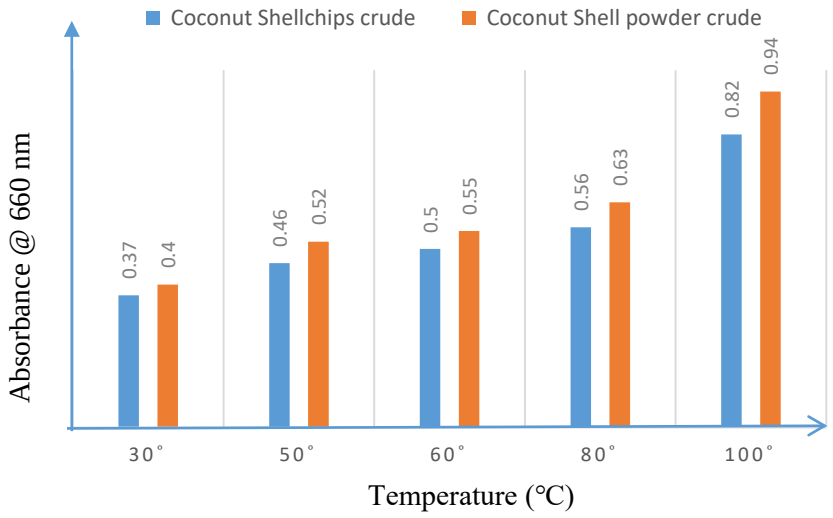


Figure: 3 Protein Estimation of Crude extract of Coconut shell chips and Coconut shell

Temperature (℃)	Protein Concentration of Crude Enzyme (mg/ml)	
	Coconut shell chips	Coconut shell powder
30℃	0.10	0.32
50℃	0.42	0.43
60℃	0.44	0.45
80℃	0.49	0.48
100℃	**0.8**	**0.96**

Table 1: Protein concentration of Crude enzyme extracted at differen temperatures from coconut shell chips and coconut shell powder

The Protein concentration of crude protease enzyme at different temperature from coconut shell chips and coconut shell powder were shown in figure 3. In figure 3, the protein concentration was found to be more in 100℃ than other temperatures in both the crude enzyme extracts. On comparing the 100℃ extract of coconut shell chips and coconut shell powder, coconut shell powder extract showed more protein concentration than crude coconut shell chip extract. The protein concentration of the extracts was shown in Table1. According to Swarnali Banik *et al.* 2018, the protein concentration from moringa leaf extract of crude protease was0.56 mg/ml, similar to the study of Swarnali Banik *et al.*, 2018, the current study on protein concentration of Crude protease of Coconut shell chips and Coconut shell powder showed 0.8 and 0.96 mg/ml. This shows a significant yield of

protease at an extraction temperature of 100℃ from Coconut shells compared to the previous work from other plant sources.

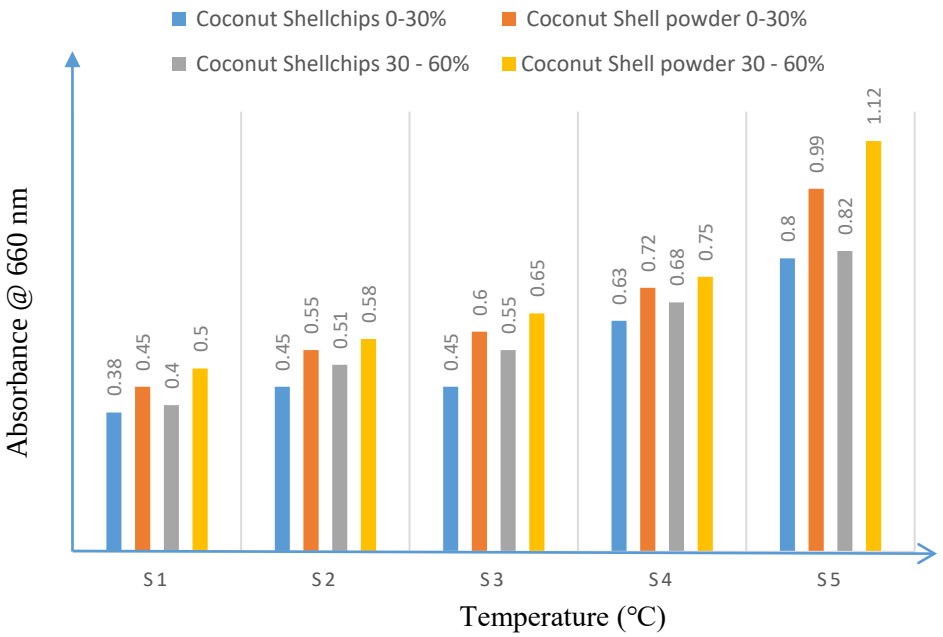


Figure 4: Protein Estimation of partially purified enzyme of Coconut shell chips and Coconut shell extracts (0-30% and 30-60%) at different temperatures

Note: S1 - 30℃, S2 - 50℃, S3 - 60℃, S4 - 80℃, S5 - 100℃

The Protein concentration of partially purified enzyme at two different Ammonium sulfate precipitation 0-30% and 30-60% at different extraction temperatures from coconut shell chips and coconut shell powder were shown infigure 4. In figure 4, the protein concentration of partially purified enzyme at 0-30% Ammonium sulfate precipitation was found to be more in 100℃ than other temperatures in the shell chips and shell powder. Similarly, in 30-60% Ammonium sulfate precipitation, of all the other temperatures, extract at 100℃ showed more protein concentration. On comparing both the precipitation (0-30% and 30-60%), 30-60% Ammonium sulfate precipitation showed higher protein concentration. Maximum protein concentration observed was 1.1mg/ml at 30-60% Ammonium sulfate precipitation at an extraction temperature of 100℃. Ammonium sulfate precipitation at 30-60%, in both, coconut shell chips and coconut shell powder shows similar concentration of protein till 60℃ and when the extraction temperature was increased from 60℃ to 80℃ and 100℃, the protein concentration significantly varied between coconut shell chips and coconut shell powder. Coconut shell powder showed maximum yield of protein than the coconut shell chips. The protein concentrations of the extracts were shown in the Table 2

Temperature (℃)	Partially Purified enzyme (0-30%) (mg/ml)		Partially Purified enzyme (30-60%) (mg/ml)	
	Coconut shell chips	Coconut shell powder	Coconut shell chips	Coconut shell powder
30℃	0.1	0.45	0.32	0.38
50℃	0.45	0.48	0.43	0.45
60℃	0.48	0.48	0.48	0.48

80°C	0.48	0.64	0.63	0.64
100°C	0.64	**0.96**	0.8	**1.1**

Protease Estimation:

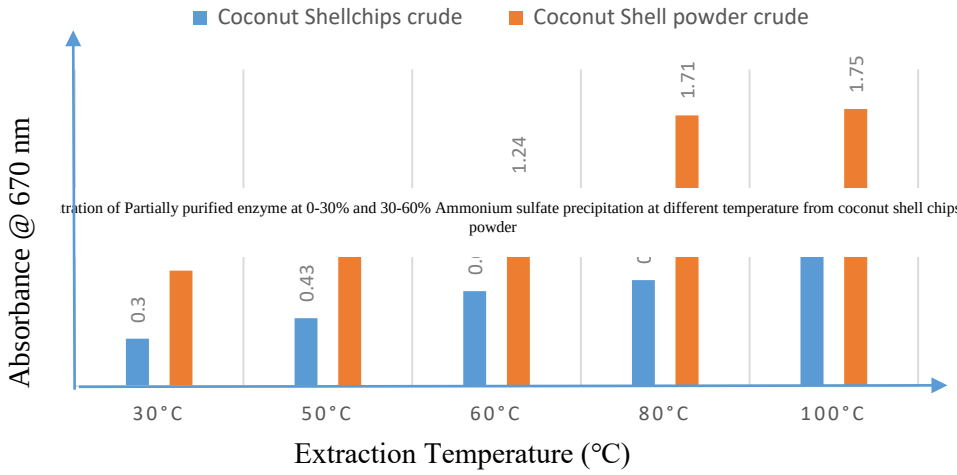


Table 2: Pr tration of Partially purified enzyme at 0-30% and 30-60% Ammonium sulfate precipitation at different temperature from coconut shell chips and coconut shell powder

Figure :5 Protease Estimation of Crude extract of Coconut shell chips and Coconut shell extracts at different temperatures

Protease assay was performed by Folin Lowry method, and the proteolytic activity was determined for all samples at different temperatures of extraction. The Proteolytic activity of crude enzyme extracted at different temperature from coconut shell chips and coconut shell powder were shown in figure 5. In Figure 5, the proteolytic activity was found to be greater in 100°C extract than other temperature extracts in both the samples. On comparing 100°C extract of coconut shell chips and a coconut shell powder, crude enzyme of coconut shell powder extracted at 100°C showed more activity than the crude enzyme of coconut shell chips extracted at 100°C. The Protease enzyme concentration in units were shown in the table 3.

Temperature (°C)	Crude Enzyme (U/ml)	
	Coconut shell chips	Coconut shell powder
30°C	0.30	0.73
50°C	0.43	0.84
60°C	0.60	1.24
80°C	0.67	1.71
100°C	**0.91**	**1.75**

Table 3: Crude enzyme concentration extracted at different temperature from coconut shell chips and coconut shell powder

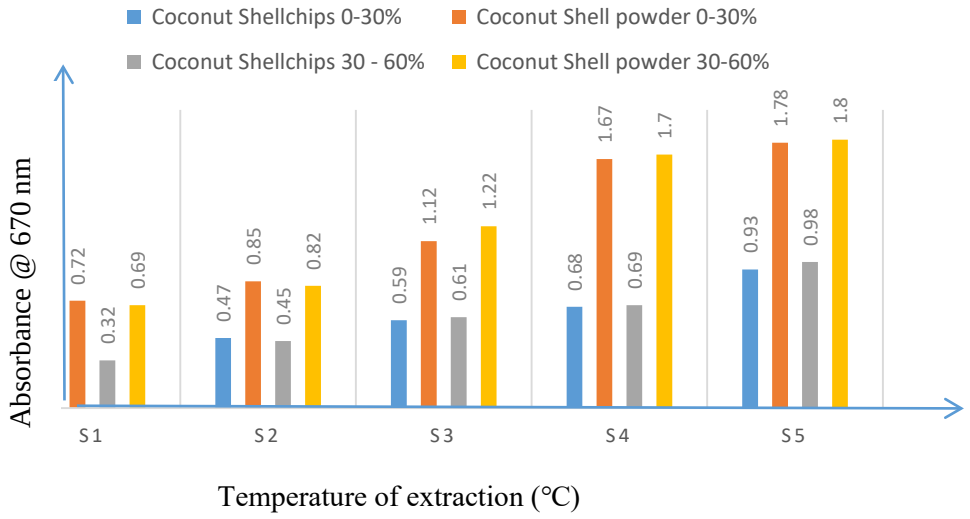


Figure 6: Protease Estimation of partially purified enzyme from Coconut shell chips and Coconut shell extracts (0-30% and 30-60%) Ammonium Sulfate precipitation at different temperatures

Note: S1 - 30°C, S2 - 50°C, S3 - 60°C, S4 - 80°C, S5 - 100°C

The Proteolytic activity of the partially purified enzyme extract at 0-30% and 30-60% Ammonium sulfate precipitation at different extraction temperatures from coconut shell chips and coconut shell powder were shown in figure 6. In figure 6, the proteolytic activity of partially purified enzyme extract at 0-30% Ammonium sulfate precipitation was found to be more at 100°C than other temperatures in the shell chips and shell powder. Similarly, in 30-60% Ammonium sulfate precipitation, extraction at 100°C shows more enzyme activity than other temperatures. In both the precipitation, 0-30% and 30-60%, results indicated that 100°C extraction temperature showed highest enzyme activity, and the highest concentration of the enzyme extracted was 1.80U/ml at 30-60% Ammonium Sulfate precipitation. The enzyme activity (U/ml) were shown in the table 4. Though the protein concentration was more in 30 - 60%, protease activity of both the Ammonium sulfate precipitation was found to be similar. This indicates that at 30 - 60% proteins other than protease also got precipitated.

Temperature(°C)	Partially Purified enzyme (0-30%) (U/ml)		Partially Purified enzyme (30-60%) (U/ml)	
	Coconut shell chips	Coconut shell powder	Coconut shell chips	Coconut shell powder
30°C	0.35	0.72	0.32	0.69
50°C	0.47	0.85	0.45	0.82
60°C	0.59	1.12	0.61	1.22
80°C	0.68	1.67	0.69	1.70
100°C	0.93	**1.78**	0.98	**1.80**

Table 4: Partially purified enzyme 0-30% and 30-60% of different temperature from coconut shell chips and coconut shell powder

SDS PAGE:

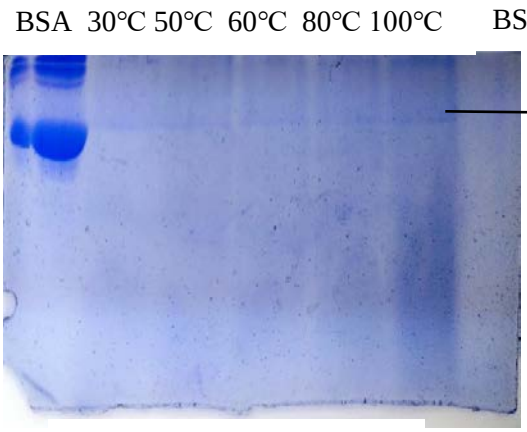


Fig: 7(a) SDS PAGE of 0-30% partially purified enzymes of coconut shell chips

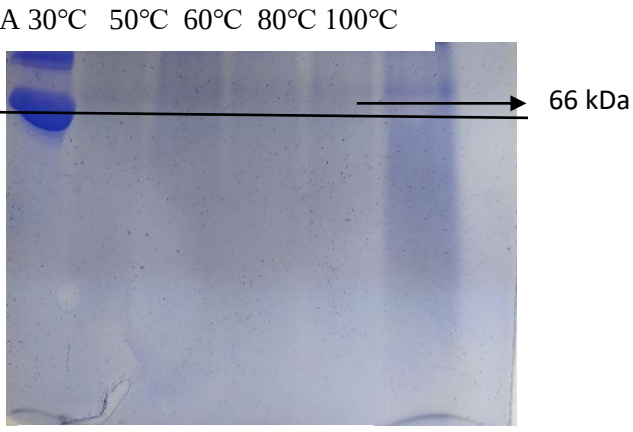


Fig: 7(b) SDS PAGE of 0-30% partially purified enzymes of coconut shell powder

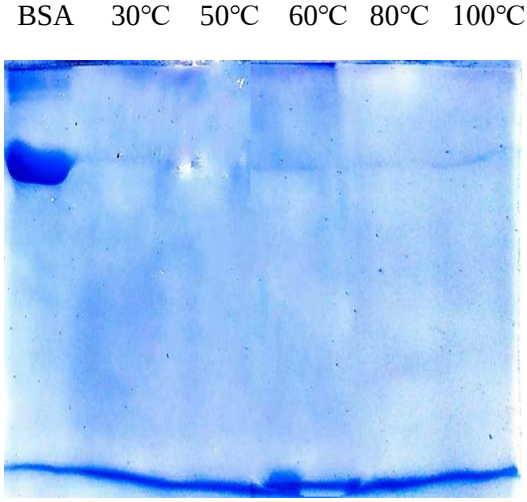


Fig: 7(c) SDS PAGE of 30-60% partially purified enzymes of coconut shell chips

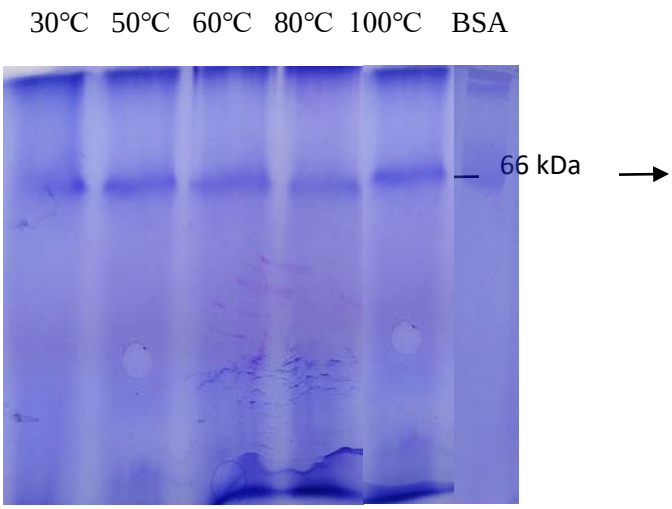


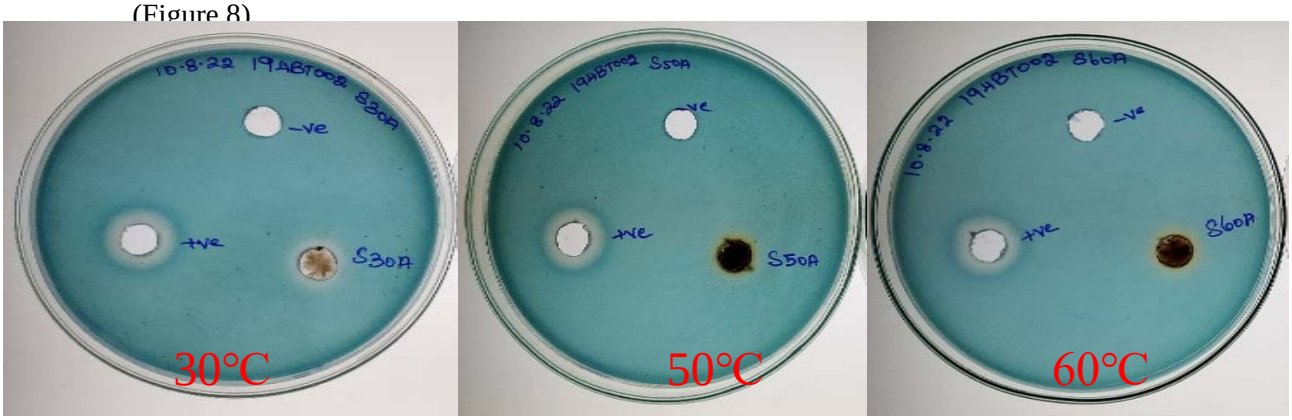
Fig: 7(d) SDS PAGE of 30-60% partially purified enzymes of coconut shell powder

Molecular characterization of the extract was done using SDS PAGE. The molecular weight of the protein in the SDS PAGE was found to be approximately 60kDa. Figure 7(a) represents the 0-30% partially purified extract of Coconut shell chips, Figure 7(b) represents the 0-30% partially purified extract of Coconut shell powder, Figure 7(c) represents the 30-60% partially purified extract of Coconut shell chips, and Figure 7(d) represents the 30-60% partially purified extract of Coconut shell powder. Of all the 4 gels, 30-60% partially purified coconut shell powder shows good band intensity than other partially purified extracts. Earlier studies showed that protease from different plant sources shows well-defined protease activity. Mainly the cysteine protease is found in plants ex: *Salvadora persica* and *Ficus microcarpa*[11, 12]. A single band in the SDS PAGE of partially purified extracts indicated that samples were purified to satisfaction to show only a single band.

Plate assay for protease activity determination

Protease activity was assayed qualitatively using Casein agar plate assay for the characterization of enzymes. The casein was degraded by the partially purified enzyme extract at 0-30% and 30-60% Ammonium sulphate precipitation at different temperatures

of extraction from Coconut shell chips and Coconut shell powder. Degradation of milk casein was observed after 24 hrs incubation at room temperature. Partially purified enzymes of all the temperatures had shown a clear zone, but the 100℃ extract of Coconut shell chips and Coconut shell powder showed better zones when compared to other temperatures. The zone of hydrolysis were found to be similar in both the precipitations, but on comparing 100℃ extract of Coconut shell chips and Coconut shell powder, the zone of hydrolysis of coconut shell powder shows highest zone of hydrolysis than 100℃ extract of Coconut shell chips, where 0-30% Ammonium sulphate precipitated extract of Coconut shell powder showed 12mm of hydrolysis and 30-60% Ammonium sulphate precipitation of Coconut shell powder showed 14mm of hydrolysis which is found equal to the zone of hydrolysis of positive control. Henceforth, the study proved 100℃ extract of coconut shell powder showed the best results. Zone of hydrolysis in millimeter were shown in the table 5. Clear zone indicating the caseinolytic ability of the extracts and thus the presence of protease enzyme activity in the samples were observed (Figure 8)



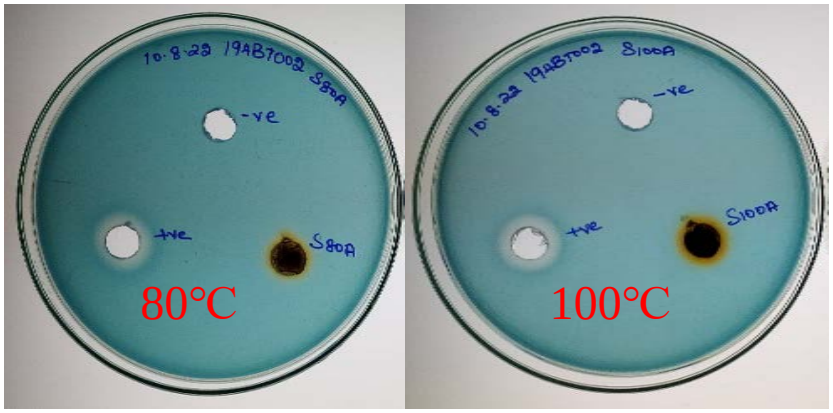
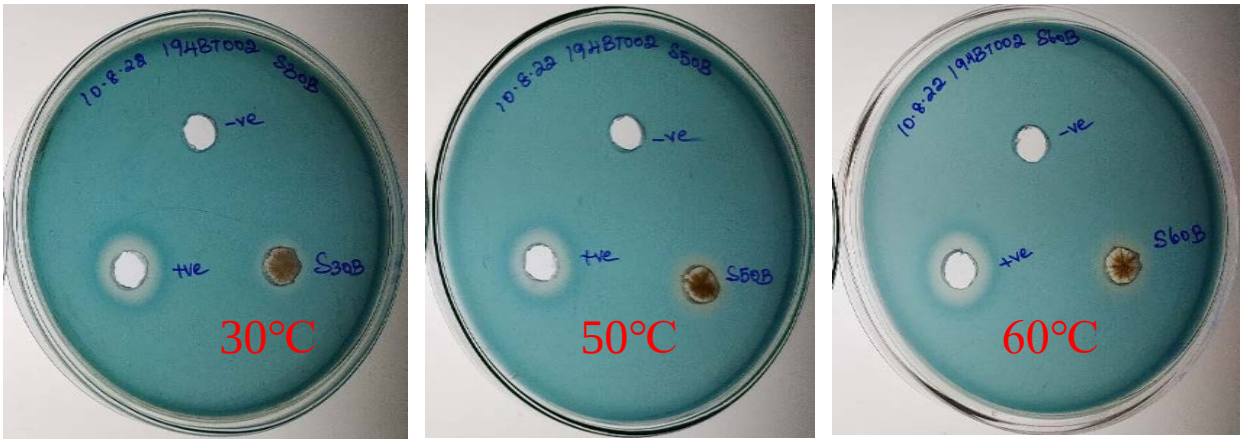


Fig: 8(a) Casein plate assay of coconut shell chips extract at different temperatures (0 - 30% precipitation)



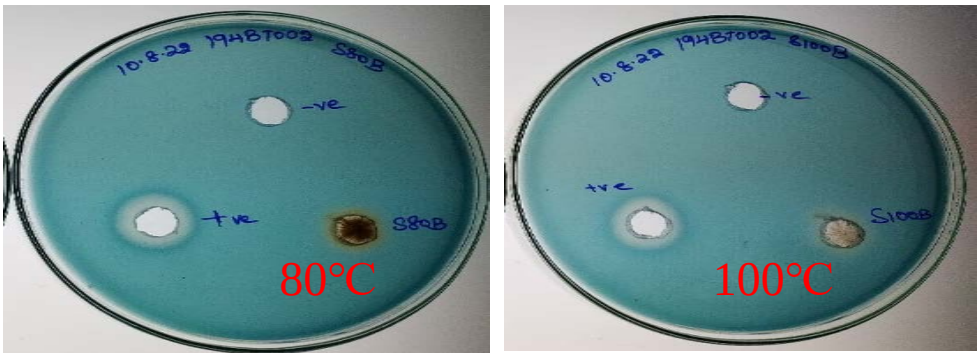


Fig: 8(b) Casein plate assay of coconut shell chips extract at different temperatures (30 - 60% precipitation)

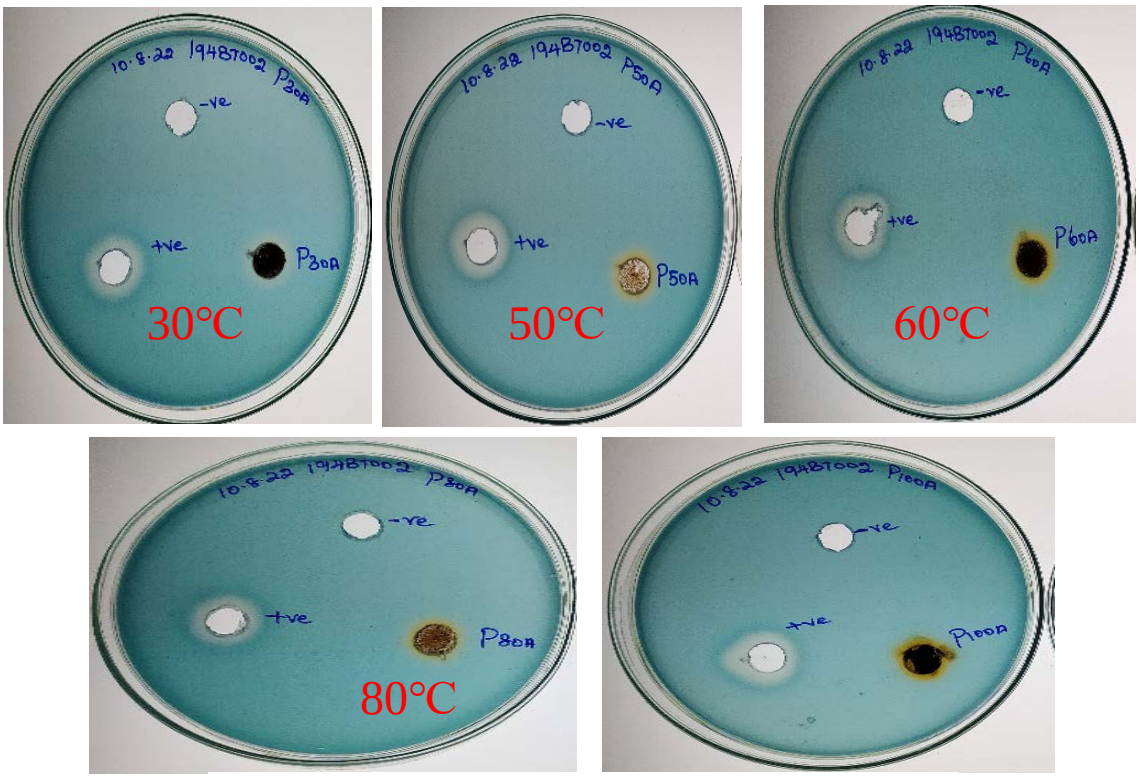


Fig: 4(c) Casein plate assay of coconut shell powder extract at different temperatures (0 - 30% precipitation)

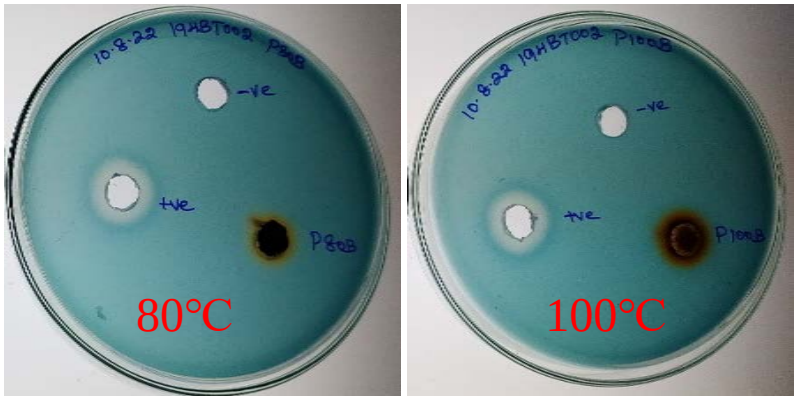


Fig: 8(d) Casein plate assay of coconut shell powder extract at different temperatures (30 - 60% precipitation)

Temperature (°C)	Zone of Hydrolysis(mm)					
	Positive control	Negative control	Coconut shell chips (0-30%)	Coconut shell powder (0-30%)	Coconut shell chips (30-60%)	Coconut shell powder (30-60%)
30°C	15	-	8	10	12	12
50°C	15	-	10	13	12	11
60°C	15	-	9	9	10	10
80°C	15	-	11	11	11	12
100°C	15	-	10	12	11	**14**

Table 5: Zone of hydrolysis of 0-30% and 30-60% precipitation of coconut shell chips and coconut shell powder extracted at different temperatures

Conclusion:

From this study, it is clear that the Coconut shell can be used as a source of protease enzyme. After analysing all the parameters, the best extraction temperature for maximum yield was found to be 100°C in both shell chips and shell powder. On comparing Shell chips and shell powder, Coconut shell powder is more effective as raw material for the extraction of protease enzyme.

Very limited work has been done on enzyme production using Coconut shells which calls for more detailed research in the future. A large amount of Coconut shells are disposed into the environment and it is considered as a contaminant, or waste thrown into the environment. From this study, an industrially important product is obtained from the waste, which proves a method of managing solid waste in the environment. By using this

water pollution is prevented.

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