Optimization of Protease Enzyme Extraction from Calatropis Procera Using Aqueous Two-Phase Purification

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ABSTRACT

Though protease enzyme extraction has mainly been from microbes, plant proteases have been sought out as a supplement to further boost production and overcome the expensive and tedious nature of microbial protease extraction. At present, efficient and easily affordable processes for the partitioning and purification of enzymes that provide ample yield and high purity of the final products have been demanded by most industries. Thus, this study seeks to optimize conditions and experimental procedures to generate a pure, folded, and dimeric enzyme in its maximum yield from Calotropis procera. The aqueous two-phase purification system (ATPS) was used for the extraction process. The factors pH, NaCl, and temperature were confirmed as having a significant impact on the ATPS and therefore were selected for further optimization to maximize yield and purity. The study found that the maximum protease recovery for the leaves was 93%, 77%, and 89% at pH 6, 20°C, and a salt concentration of 0.0 M, respectively. The maximum protease recovery for the bark was 54%, 51%, and 47% at pH 6–8, 10–20°C, and a salt concentration of 0.0 M, respectively. Additionally, it showed that the leaves had the maximum protease purity at pH 8, temperature 10, and salt concentration of 0.2 M, respectively, while the bark had the highest protease purity at pH 8, temperature 30, and salt concentration of 0.2 M, respectively. Thus, the aqueous two-phase purification method is effectively optimized for maximum yield and purity of the protease enzyme extraction from the leaves and bark of the Calotropis procera plant at or around neutral pH, a temperature range of 10–30°C, and low salt concentrations of 0.0 M–0.1 M.

Keywords: Proteases, catalysts, *Calotropis Procera*, aqueous two-phase purification system(ATPS), coagulation

1.0 Introduction

Proteases are enzymes that hydrolyze proteins into relatively smaller peptides and amino acids. They can be found in almost all organisms and they constitute a famous group of biocatalysts with a wide range of applications with respect to their biochemical qualities and substrate specificity (Pant *et al.,* 2015). Organisms produce protease to degrade improperly folded and damaged proteins to maintain homeostasis. These proteases also serve as signaling molecules, facilitate protein-protein interactions and generate novel bioactive molecules within the organisms (Lopez-Otin and Bond, 2008). These proteolytic enzymes have ultimately turned out to be extremely valuable for industrial purposes. Proteases are often employed in brewing, leather processing, meat tenderization, cheese making, detergent manufacturing, baking, and digestive aid manufacturing (Gimenes *et al.,* 2021).

For many years, fermentation processes have been used to isolate these proteases from microbial sources. Protease production from Bacteria, Fungi, and Yeasts is estimated to account for more than 40% of total global enzyme sales

due to the ease with which these microbial proteases are secreted into the fermentation broth and the ease with which the microbes can be genetically manipulated (Chiberkujwo *et al.*, 2020; Gimenes *et al.,* 2021). However, recovering the target enzyme from the fermentation broth is expensive and cumbersome, accounting for 70-90% of the protease extraction expenditure (Gimenes *et al.,* 2021). Other problems associated with microbial protease production have been the difficulty in screening microbes from the environment, the expensive medium for culturing microbes, and the difficulty in identifying suitable growth conditions (Upadhyay *et al.,* 2010). Thus, researchers have sorted to isolate protease from various sources including plants. Popular proteolytic enzymes extracted from local plants include papain from *Carica papaya*, bromelain from pineapple, and ficin from *Ficus* (Obed *et al.,* 2021). Another major plant that has lately received scientific attention due to its potential to contain a prominent proteolytic enzyme is *Calotropis procera* (Obed *et al.,* 2021). *C. procera* is a tropical and equatorial plant that is well-known for its ability to create latex (Rawdkuen *et al.,* 2011). The latex extract from *C. procera* has been employed as a curdling agent in the manufacturing of a traditional cheese variety known as Wara cheese in Southwestern Nigeria over the past decade. The ability of the extract to coagulate milk at a high temperature (over 70°C) makes it effective for the production of Wara cheese and as a replacement for rennet enzyme in cheese manufacturing (Raheem *et al.,* 2007; Oseni and Ekperigin, 2013).

Moreover, purity assessment is known to enhance enzyme activity and functional applicability. This necessitates high precision and cost-effective separation process (Porto *et al.,* 2008). At present, efficient and easily affordable processes for the partitioning and purification of enzymes that provide ample yield and high purity of the final products have thus been demanded by most industries. On this premise, the aqueous two-phase purification process (ATPS) has been employed in this study for the purification of the protease enzyme (Sripokar *et al.,* 2017). ATPS is created by combining an aqueous solution of two immiscible hydrophobic polymers or a polymer and a salt at a certain concentration. Biomolecules segregate between the two aqueous phases in accordance with their partition coefficient (Sripokar *et al.,* 2017). The ATPS has several advantages, including quick processing time, low energy consumption, a high-capacity yield, ease of scaling up, biocompatibility, and non-toxicity (Sripokar *et al.,* 2017).

Furthermore, Proteases, like all other enzymes, operate efficiently in conditions that preserve their well-defined 3-D structure. Due to the lack of appropriate operating conditions, the present proteolytic enzyme from *C. Procera* has not been able to fully meet industrial expectations. In reality, little information is known about the optimal conditions and requirements for the ample yield of protease from *C. procera* and little study has been done to characterize the enzyme. Also, conventional extraction methods often disrupt the well-defined 3D structure of the enzyme. This often results in inconsistencies between reported and observed properties and/or activities of the enzyme as well as the inability to fully explore the enzyme's uses. The problems with the enzyme stability and lack of valuable information for the utmost yield of the enzyme have thus motivated researchers to optimize conditions and experimental procedures to generate a pure, folded, and dimeric enzyme in its maximum yield.

Therefore, the goal of this work is to optimize and characterize protease enzyme extraction from *Calotropis procera* leaves and bark utilizing an aqueous two-phase purification technique. Due to the increased demand for the enzyme, it is imperative to exploit some novel sources of proteases as well as revise some existing methods to increase the production of the enzyme to satisfy its high global demand. Again, the optimization of the extraction process will result in achieving pure and active enzymes, maximum enzyme yield and maximum activity thereby boosting its product yield and catalytic efficiency. All these will increase the recognition and patronization of plant protease as the best supplement or alternative to microbial protease to satisfy the growing demand for protease. This research also serves as an encouragement for more studies to be undertaken on the prospect of

obtaining proteases from other distinct sources for commercial usage and other applications.

2. 0 Materials And Methods

2.1 Raw material collection and Study site

Calotropis procera fresh leaves and stem bark were obtained from the matured plant growing in the slightly humid zone at Kotei community in the Kumasi Metropolis of Ghana. The plant's leaves were plugged by hand whiles the bark was removed with a kitchen knife. The leaves and the bark were subsequently conveyed directly to the Department of Food Science Laboratory of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

2.2 Preparation of crude enzyme extract

The defect-free leaves and bark were next chosen and washed thoroughly with distilled water to eliminate surface dirt and microbes. A mass of 100 g of each plant portion (leaves and stem bark) was individually measured with an electronic balance and then blended with a laboratory blender in the presence of 100 ml of 0.05 M sodium acetate buffer adjusted to a *pH* of 5.5. Each blended mixture was then filtered through a clean cheesecloth into two separately labeled sterile beakers leaving the suspended debris behind, which was then discarded.

The filtrates from the leaves and the bark were dispensed separately into smaller volumes in 50 ml falcon tubes and clearly labeled. The filtrates were subsequently subjected to centrifugation at 4000 rpm for 15 minutes. Following that, the supernatant (crude extract) for each plant part was poured into two separate sterile glass jars (one for crude enzyme extract prepared from the leaves and the other for crude enzyme extract prepared from the bark), covered, labeled, and kept at 4°C for subsequent analysis (Obed *et al.*, 2021).

2.3 Aqueous two-phase purification of the crude extract

An aqueous two-phase extraction process was conducted by combining 2.0 ml of 50% ethanol and 2.0 ml of 50% K_2HPO_4 solutions, together with a volume of 2.0 ml crude extract in 15 ml centrifuge tubes. A glass rod was then employed to stir the mixture gently for about half an hour and then centrifuged at 4000 rpm for approximately 15 minutes before being placed in a water bath for about 30 minutes for complete phase separation to occur (Raja *et al.,* 2013). The two phases formed were separated carefully to carry out estimations in both the top phase and bottom phases and then used for further analysis (Bai *et al.,* 2013). The procedure was repeated for each crude extract obtained from the leaves and the bark of the plant.

2.4 Calotropin enzyme characterization

2.4.1 Protein Concentration Determination

Bradford method was used to estimate the protein concentrations while bovine serum albumin (BSA) served as a reference. A mass of 0.1 g BSA was dispensed in 100 ml distilled water in a conical flask and stirred gently to give the BSA solution.

Different dilutions were obtained by pipetting different volumes (10 µl, 20 µl. 40 µl, 60 µl, 80 µl, 100 µl and blank) of the prepared solution into sterile centrifuge tubes, clearly labeled and topped up with distilled water to 100 µl. The absorbance of the various BSA solutions was measured at 595 nm and their respective absorbance and concentrations were used to generate a conventional curve from which protein concentration in unknown samples was estimated (Kielkopf et *al.*, 2020). A pipette was then used to transfer a volume of 100 µl of each enzyme extract (i.e., the crude enzyme prepared from the leaves and the crude enzyme prepared from the bark) into separately labeled clean tubes followed by the addition of 5 ml of Bradford reagent to each, and the absorbance of each enzyme solution measured with a spectrophotometer at 595 nm. The absorbance of a particular enzyme solution was used to trace its concentration from the standard curve (Obed *et al.*, 2021).

2.4.2 Calculations of partition parameters

The partitioning of the enzyme between the two phases was further quantified by partition coefficients (K), phase volume ratio (R), and efficiency of extraction (E) defined as

$$K = \frac{C_t}{C_b} \qquad R = \frac{V_t}{V_b} \qquad E = \frac{C_t V_t}{C_t V_t + C_b V_b}$$

Where; C_t =concentration of enzyme in the top phase; C_b=concentration of enzyme in the bottom phase; V_t =volume of enzyme in the top phase; V_b =volume of enzyme in the bottom phase (Bai *et al*., 2013)

2.4.3. Analysis of calotropin activity (Gelatin hydrolysis method)

A gelatin degradation procedure was used to assess the extracts for protease activity. A volume of 1.0% gelatin solution in 0.05 M citrate phosphate buffer at a *pH* of 7.5 was heated in a water bath at 100°C for 15 minutes, chilled, and used as a substrate. A volume of 1.0 ml of each enzyme extract was then added to 1.0 ml substrate in the buffer while 1.0 ml of the substrate and citrate buffer without an enzyme extract served as a control. The reaction was terminated with 3.0 ml of cool 10% trichloroacetic acid at the end of 60 minutes of incubation at 35°C (Obed *et al*., 2021). After that, the tube was refrigerated for about an hour at 2°C to cause the precipitation of non-hydrolyzed gelatin and then centrifuged at about 3000 rpm for 15 minutes.

Next, 1.0 ml Folin Ciocalteu reagent was added to each sample to combine with liberated tyrosine to produce a blue-colored product within the supernatant. A spectrophotometer operating at 660 nm was used to measure the absorbance of the free supernatant as well as the absorbance of different standard tyrosine concentrations, while the blank served as a control. Different tyrosine concentrations (20-220 µl) were plotted against their respective absorbance to form a standard tyrosine curve from which tyrosine concentrations in the various supernatants were estimated (Upadhyay *et al*., 2010). The quantity of the enzyme that liberates 1µmol of tyrosine per minute under the conventional assay conditions is equivalent to one unit of protease activity (Rajagopalan *et al*., 2019). This procedure was then repeated for all enzyme extracts from the bark and leave of the plant under various experimental settings.

2.4.4 Calculation of the specific activity and percentage yield of calotropin enzyme

The specific activity and the percentage recovery after the purification step of an enzyme were determined by the formulas below;

- Specific Activity = **Enzyme Activity/protein Concentration**
- Recovery / % yield = **(total activity of purified enzyme/ total activity of crude extract) *100** (Rathnasamy and Kumaresan, 2014).

2.5 Determination of the influence of different extraction process parameters on the activity and percentage yield of the calotropin enzyme.

The aqueous two-phase system was used to purify the crude enzyme extract under different *pH* values (2 to 10), different temperature conditions (0 to 40) °C, and different salt concentrations (0.0 M to 1.0 M). The concentration, activity, percentage yield, and other partitioning parameters of protease were determined for each enzyme extracted under various conditions, using the fore-mentioned procedure.

2.7 Analysis of Statistics

The research findings were calculated by averaging triplicate values and expressed as mean values while the data collected were analyzed using the SPSS software. The significant variations in average values of proteolytic activity and protein concentration of each sample were explored with one-way ANOVA, and all outcomes were displayed statistically using an MS Excel program (Obed *et al*., 2021)

3.0 Results

3.1 Purification and Characterization of Protease from Leaves and Bark Extract

Table 1: Characterization of crude extracts from leaves and bark of the plant

	Leaves	**Bark**
Absorbance by the enzyme at 595nm	0.42059 ± 0.02	0.75208 ± 0.02
Protein concentration (mg/ml)	0.805 ± 0.02	1.445 ± 0.02
Absorbance by the supernatant at 660nm	0.64191 ± 0.02	0.840 ± 0.02
Protease activity (µmol/ml/min)	0.056 ± 0.02	0.074 ± 0.02
Specific activity = Activity/Protein concentration	0.070 ± 0.02	0.051 ± 0.02
Percentage Yield = (Total activity after purification/ total activity of crude extract) *100	100%	100%
Fold purification=Specific activity of purified enzyme/Specific activity of crude extract	1	1

Table 2: Characterization of purified extracts from leaves and bark after purification

	PURIFIED ENZYME EXTRACT	
	LEAVES	**BARK**
Absorbance by the enzyme at 595nm	0.182 ± 0.02	0.091 ± 0.02
Protein concentration (mg/ml)	0.343 ± 0.02	0.167 ± 0.02
Absorbance by the supernatant at 660nm	0.864 ± 0.02	0.776 ± 0.02
Protease activity (µmol/ml/min)	0.076 ± 0.02	0.068 ± 0.02
Specific activity = Activity/Protein concentration	0.223 ± 0.02	0.410 ± 0.02
Percentage Yield = (Total activity after purification/ total activity of crude extract) *100	89%	46%
Fold purification = Specific activity of purified enzyme/Specific activity of the crude extract	3.185	8.035

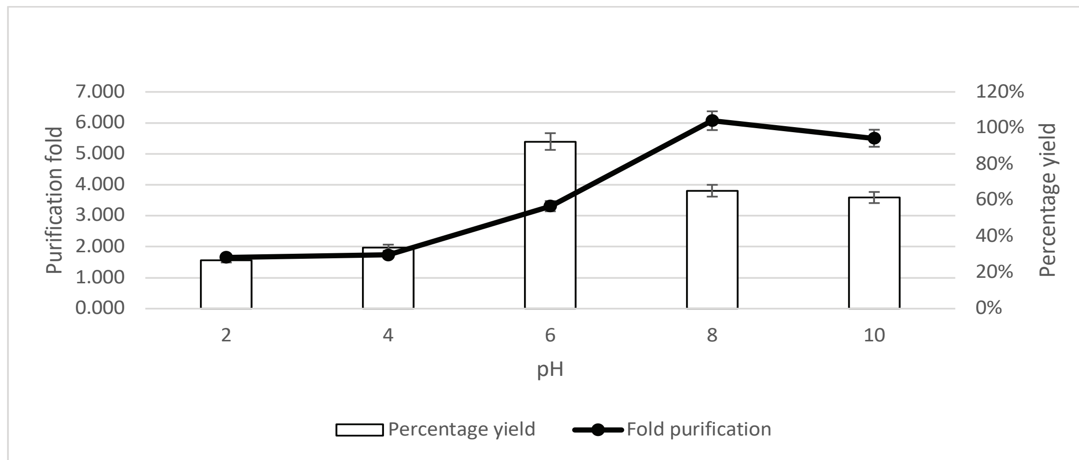

Figure 1: Effect of pH on purified extracts from leaves after the ATP system

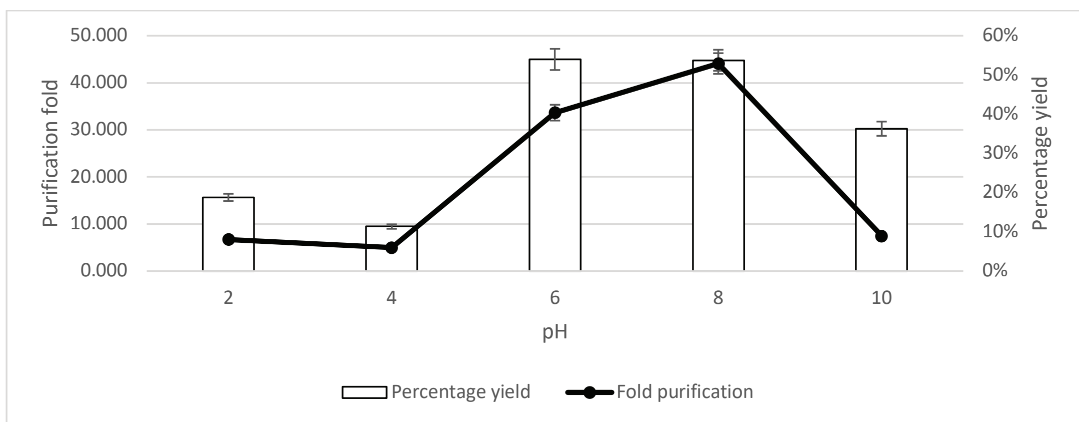

Figure 2: Effect of pH on purified extracts from the bark after the ATP system

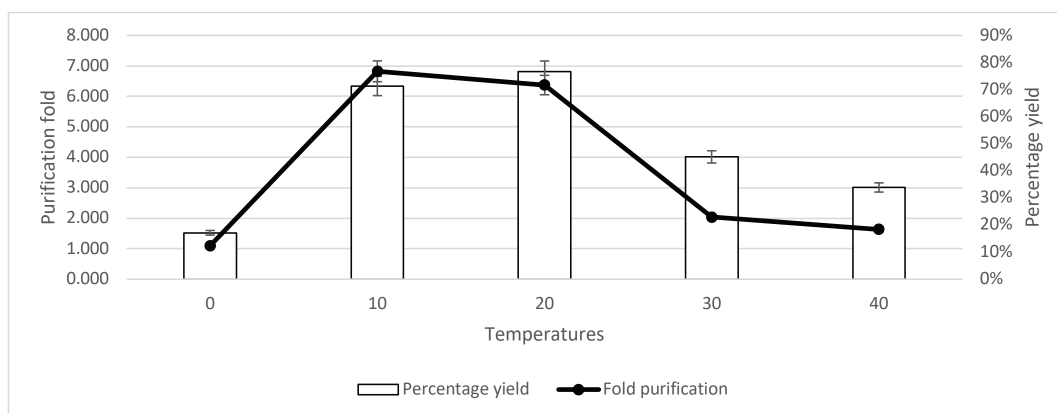

Figure 3: Effect of temperature on purified extracts from leaves after the ATP system

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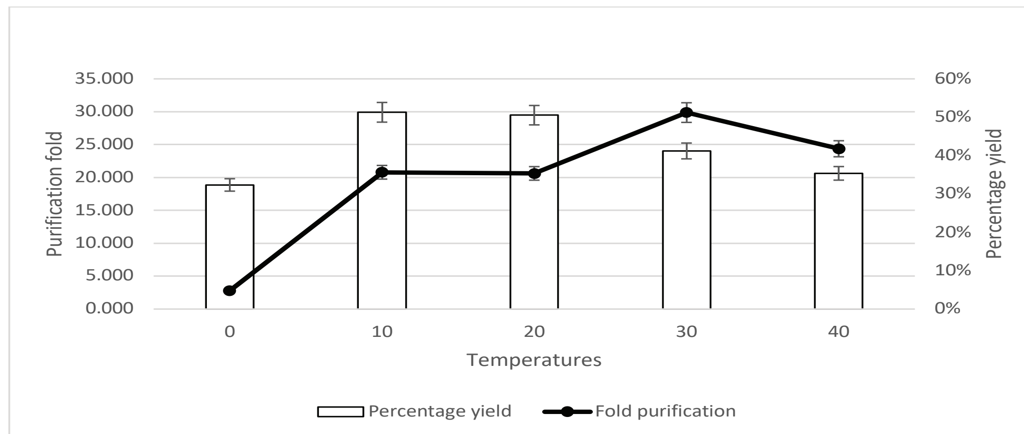

Figure 4: Effect of temperature on purified extracts from the bark after the ATP system

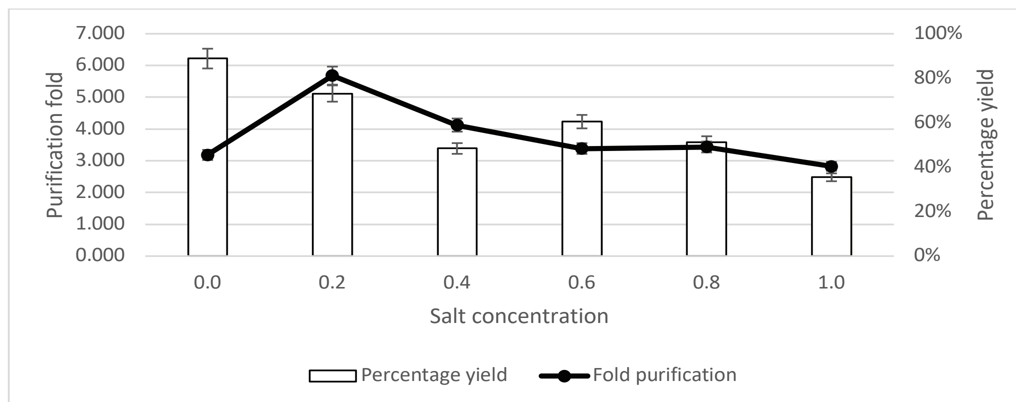

Figure 5: Effect of salt concentrations on purified extracts from leaves after the ATP system

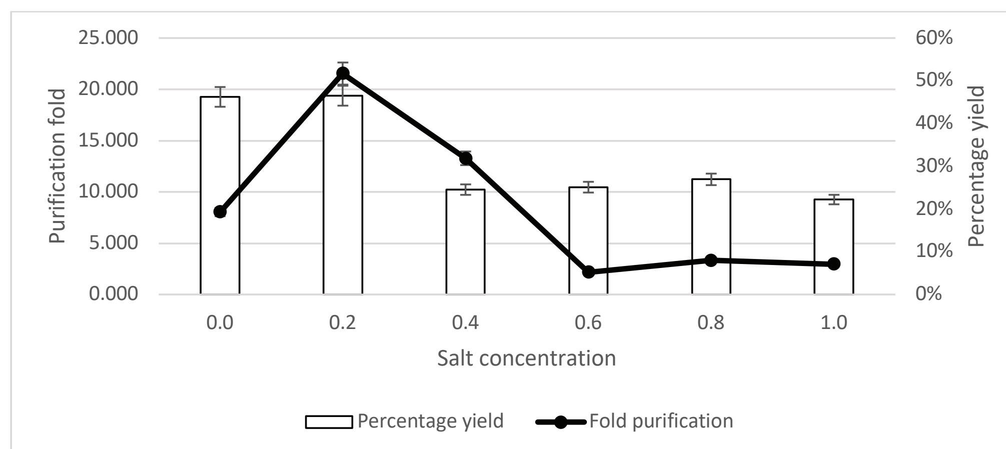

Figure 6: Effect of salt concentrations on purified extracts from leaves after the ATP system

4.0 Discussion

This work attempted to improve the extraction of protease from the crude source by developing enzyme partitioning towards one of the phases of the ATPS. The findings revealed that the ATPS tested had partition coefficient (Ke) > 1 and volume ratio (Rv) > 1 for the protease enzyme from both the leaves and bark, indicating that the enzyme was primarily distributed in the top ethanol-rich phase. The effect of various parameters on a specific activity for ATPS was studied in the search for the utmost enzyme separation. Two terms, fold Purification and percentage yield, were used to plot graphs to depict the influence of various parameters on the extraction process. Fold purification is an indication of the number of times the enzyme has been enriched following purification. Percentage yield on the other hand is an indication of the quantity of enzyme that remains after the purification step. (Saravanan *et al.*, 2008).

From the results of the study, there was a significant difference ($P < 0.05$) in terms of protein concentration between the crude and purified enzyme extract in the two plant parts. In terms of the plant part, crude enzyme extract from the stem bark showed a higher protein concentration of 1.445 mg/ml, whiles 0.805 mg/ml of proteins were found in the crude enzyme extract from the leaves. Thus, stem bark has a higher protein concentration as compared to the leaves (Table 2). In both the leaves and the stern bark, the crude enzyme extracts recorded higher protein concentrations (i.e., 0.805 mg/ml and 1.445 mg/ml respectively) than the purified extracts (i.e., 0.343 mg/ml and 0.167 mg/ml respectively). This suggests that the concentration of protein is always dependent on the purification step as a significant decrease in the protein concentration was observed after crude extracts were purified. The removal of unwanted proteins from a protein solution is ensured by the purification processes, which accounts for the observed drop in protein concentrations (Obed *et al.*, 2021; Harrison *et al.*, 1997).

The proteolytic activities of the various samples for the bark and leaves (i.e., crude extracts and purified extracts by ATPS) are shown in Tables 1 and 2. Both plant portions displayed quite significant proteolytic activity. which was disclosed by the potential of the extracts to hydrolyze casein, demonstrating that calotropin enzymes are widely distributed throughout the plant's parts. There was a significant difference ($P < 0.05$) between the proteolytic activities of the crude extracts and the activities after purification by ATPS for both plant parts. Comparing proteolytic activity for stem bark and leaves, it was observed that the various samples for stem bark showed higher activity (0.074 and 0.068 μmol / min/ml) than the samples for the leaves (0.056 and 0.077 μmol / min/ml). According to Oseni & Ekperigin, (2013) who tested different *Calotropis procera* parts for protease activity, the latex of the plant exhibits the highest proteolytic activity, followed by the root, stem, leaf, and pods. The result in this study, with stem bark having a higher proteolytic activity, supports their findings.

The aqueous two-phase purification techniques, however, provided an improved value of specific activity at 0.223 U/mg with a purification fold of 3.185 and 89% yield for the leaves and an improved value of specific activity at 0.410 with a purification fold of 8.037 U/mg and 46% yield for the bark. This shows that the ATPS provided more highly purified enzymes in the bark than it did in the leaves, leading to a lower protein concentration free of impurities and an increase in proteolytic activity in the bark.

4.1: Effect of pH

Figure 1 and Figure 2 describe the influence of *pH* on the specific activity of the enzyme and hence the fold purification and percentage yield of the enzyme purified by the ATPS from the leaves and bark respectively. The results showed that the yield and purity of the enzyme in both samples increased with an increase in *pH* from 2 to 7; then it immediately declined at a *pH* above 8 (Li *et al.,* 2013). The graph in Figure 1 above shows that the highest enzyme yield of about 93% and the highest fold purification of about 6.07 were obtained for the leaves at *pH* values of 6 and 8 respectively. In Figure 2, similar observations of the highest percentage yield of

about 54% and the maximum fold purification of around 44.093 were made for the enzyme from the bark of the plant at *pH* values of 6 and 8 respectively. These findings demonstrate that the enzyme was obtained in the highest quantity and purity at *pH* values which are very close to the neutral *pH* for both samples. Raja and Murty's (2013) study indicates that the isoelectric point of the enzyme protein can be used to explain the observation regarding the percentage yield. Since plant-derived protease must be composed of soluble and globular proteins, it can have net charges above and below a *pH* of 6. Because of this, the charged protein molecules interact with both ethanol and salt, causing distribution in both the top and bottom phases. However, at/around the isoelectronic *pH*, where the enzyme protein's net charge is zero, the enzyme has no charges to interact with the salt, instead, it only interacts via hydrogen bonding with the ethanol, making it more soluble in the ethanol phase, causing partitioning of the enzyme primarily in the ethanol rich phase. This was manifested in the highest percentage yield of the enzyme in the top phase around neutral *pH* from both the leaves and the bark of the plant.

The findings regarding fold purification are explained by how *pH* affects the activity of the enzyme. Gale and Epps (1942) asserted that the number of electrostatic charges in an enzyme's active site determines its catalytic activity. The structure and hence the function of the enzyme might be impacted by *pH* changes since alterations in *pH* can change crucial ionization states and potentially break crucial bonds. The *pH* range of 6 to 8 may contain the protease enzyme's *pl* since it has the most specific enzyme activity and, hence, the highest fold purification (around *pH* ~7.0). The small increase in *pH* values above 7 improves the specific activity and hence fold purification value. The fold purification showed maximum values from *pH* of 6 to 8 for both samples, indicating that the protease present in *C. Procera* is most likely a neutral protease. The strength of the acidic or alkaline solution that influences the enzyme property is illustrated by a dramatic decline in the fold purification and thus in specific enzyme activity values (Pereira and Coutinho, 2020). Thus, the enzyme's optimal *pH* for activity and partitioning in an aqueous two-phase system is in the range of 6 to 8 (roughly the neutral *pH*) in both the plant's leaves and bark (Goja *et al.*, 2013).

4.2 Effect of temperature

The impact of temperature on the enzyme activity in the ATP system is depicted in Figure 3 and Figure 4. The endothermic nature of the process, in which greater values of temperature influence the protease partition, was disclosed as the enzyme activity and, consequently, recovery, rose as the temperature of the ATP system increased. The protease exhibits the greatest enzyme extraction for the leaves at 20°C and for the bark at 10–20°C, with the highest enzyme recovery values of 77% and 51%, respectively. Increasing temperature, according to Li *et al.* (2013), causes a reduction in fluid viscosity and an increase in the kinetic energy of biomolecules that are partitioning towards a particular phase, which causes an increase in the number of enzyme molecules moving to the top phase. As a result, the protease enzyme's extraction efficiency should have improved from 15°C to 55°C. But the extraction efficiency started to drop at temperatures exceeding 30°C. This was attributed to denaturation, which made the protease enzyme unstable at relatively high temperatures. At 40°C, more enzyme molecules were broken down, and the extraction efficiency for the leaves and bark, respectively, fell to 35% and 34%. Thus, a further increase in temperature over the optimal value impacts the enzyme activity due to denaturation (Li *et al.*, 2013).

The temperature also indicated a significant positive effect on the purification fold. The increase in temperature not only alters the structure of biomolecules but also changes the specific activity. From Figure 3, upon extracting protease from the leaves under different temperature conditions ranging from 0°C to 40°C, the highest specific activity of 0.478, and subsequently the highest fold purification value of 6.822 was observed at a temperature of 10°C. From Figure 4, the highest specific activity was 1.524, and a fold purification value of 29.877 for protease extracted from the bark was observed at 30°. Thus, at a temperature range of 10-30, the process yields more purified enzymes than at any other temperature.

5.3 Effect of salt

The addition of NaCl in the ATPS had a considerable impact. For the NaCl concentrations studied in this research (from 0.0 M to 1.0 M), it is observed from both samples that the addition of salt decreased the partitioning towards the top ethanol-rich phase. The extraction yield decreased from 89% to 35% for enzyme extraction from the leaves and decreased from 46% to 22% for the enzyme extraction from the bark (Figure 5 and Figure 6). This is because changes in the electrostatic potential difference of the enzyme result in an increase in the interaction of biomolecules with the salt-rich bottom phase. The affinity charge of the protease enzyme improves as the NaCl concentration increases which positively directs the partition coefficient of the system toward the bottom salt-rich phase. Further addition of salt concentration above the optimized value increases the anion content and resists the protease separation towards the bottom phase which manifested itself with a gradual rise in enzyme partitioning towards the ethanol-rich top phase at the 0.6-0.8 M salt concentration for both samples (Li *et al.,* 2013). However, a high concentration of neutral salts may cause denaturation of proteins existing in the system, thus low concentration range from 0.0 to 1.0 M is preferred but it favors partition towards the salt-rich phase rather than the ethanol-rich phase. (Goja *et al*., 2013).

Different salt concentrations (from 0.0 to 1.0 M) raised the enzyme's fold purification to a peak at 0.2 M, after which the enzyme activity again declined, indicating that salt enhanced the enzyme purity at low doses. However, the addition of higher concentrations of neutral salts (above 0.2 M) may denature proteins present in the system, resulting in a reduction in the specific enzyme activity and, consequently, the fold purification values for the enzymes extracted from both the plant's leaves and bark (Li *et al*., 2013).

5.0 Conclusion

This current study conducted revealed high protein concentration and proteolytic activity of calotropin enzyme from the leaves and stem bark of *the Calotropis procera* plant. Thus, protease assays can be used with various *Calotropis procera* components. Moreover, the aqueous two-phase purification process has proven to be very quick and efficient in the purification of the enzyme as compared to standard purification methods. The factors *pH*, NaCl, and temperature were confirmed as having a significant impact on the ATPS. The studies revealed that the aqueous two-phase purification process is efficiently optimized for maximum yield and purity of the protease enzyme extraction from the leaves and the bark of *Calotropis procera* plant at/around neutral *pH*, temperature range of 10-30°C and at low salt concentrations of 0.0 M-0.1 M.

Recommendation

In this work, the extraction process was optimized using a one-factor-at-a-time technique, in which the influence of one variable is studied at a time, leaving all other parameters constant and ignoring the interactions between two or more variables. Iqbal *et al.,* (2016) stated that optimization without considering the interactions between the variables could result in poor and false optimal conditions. Therefore, it is hereby advised that a multivariate statistical technique known as "Design of Experiments (DoE)" be utilized for the optimization of ATPS in subsequent similar research to remove uncertainty and assure flawless optimization and characterization. In DoE experiments are run at different combinations of the variables. Without a doubt, such a factorial design may provide very accurate results by accounting for potential variables interactions, but the number of experiments will increase.

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