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Purification and Characterization of Protease from *Bacillus badius* Isolated from Cassava Effluent Dump Site

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Abstract

Protease enzyme was produced from a strain of *Bacillus badius* isolated from cassava processing waste dump site, purified and characterized. Sixty-one (61) strains of bacteria were isolated from cassava waste and screened for secretion of protease on the casein agar plate as substrate. Purification was achieved using ion exchange (DEAE Sephadex A-50 column) and gel filtration (Sephadex G-200) chromatography. The effects of pH, temperature, production time, substrate specificity and metal ions were investigated. Thermal and pH stabilities as well as K_m and V_{max} of the purified enzyme were determined. The molecular weight of the purified enzyme was also determined, with single protein band on SDS-PAGE suggesting that the enzyme was homogenous. One activity peak was observed in ion exchange chromatography; the enzyme yield was 9.22 while purification fold of 1.22 was achieved after the gel filtration. The estimated molecular weight of purified enzyme from SDS-PAGE was 34.17 kDa, while pH and temperature optima were 7.0 and 40 °C, respectively. The kinetic parameters V_{max} and K_m of the purified protease were 59.88 mmol/ml/nm and 1.98 mM, respectively. The protease activity was enhanced in the presence of Co^{2+}, Hg^{+}, Mn^{2+}, Cu^{2+} and Mg^{2+} in concentration-dependent manner, while it was slightly inhibited in the presence of Zn^{2+} and Na^{+}. Furthermore, ascorbic acid, beta-macaptoethanol, EDTA, and urea showed serious inhibition of the enzyme, while its activity was stimulated by cysteine. The results revealed that protease produced by *Bacillus badius* from cassava waste dump site can be exploited for potential industrial applications.

Keywords: *Cassava effluent, dump site, Bacillus badius, protease, purification, characterization.*

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Introduction

Generally, enzymes as biocatalysts that are produced by living cells, promote specific biochemical reactions which take place in the cell metabolic processes (Mohammad and Mastan, 2013; Esan *et al*., 2023; Enujiugha *et al*., 2024). Enzymes can be sourced from plants (Enujiugha *et al*., 2004; Esan *et al*., 2023), mcroorganisms (Sanni *et al*., 2019) or animals; and most often they are the secreted/released metabolites during fermentation processes (Adejobi *et al*., 2024a). Microbial enzymes are often preferred for industrial applications compared to those from plants and animal sources because they are cheaper in production, more predictable, controllable and reliable (Abu *et al*., 2014; Sanni *et al*., 2019); and also because of their more flexible biochemical diversity, feasibility of mass culture as well as ease of genetic manipulation (Oyeleke and Oduwole, 2009), with significant influence on fermentations and other reactions (Enujiugha *et al*., 2002; Oyedokun *et al*., 2016). Efforts are therefore being directed towards the choice of microorganisms that produce enzymes with new physiological properties and tolerance to extreme conditions of temperature and pH commonly employed in industrial processes (Leiola *et al*., 2001).

Proteases (EC 3.4.2124), which are responsible for the symmetrical cleavage of peptide bonds between the amino acid residues in polypeptides and proteins, are one of the excellent and important groups of industrial enzymes (Annamalai *et al*., 2014; Oludumila *et al*., 2015). Based on the mode of action of proteases, they are classified into four categories, namely alkaline, acid, thiol and metallo proteases (Rao *et al*., 1998). Among the different types of proteases, alkaline proteases have found wide applications in various industries such as detergent, leather, pharmaceutical, protein processing, foods, diagnostic reagents, soy processing, peptide synthesis industries, extraction of silver from used X-ray film and wastes treatment (Yildirim *et al*., 2017; Asha and Palaniswamy, 2018). Therefore, the demand for industrially important highly active alkaline proteases with high specificity and stability of pH, temperature, and organic solvents

continues to drive the search efforts for new enzyme sources (Vijayaraghavan and Vincent, 2012; Oludumila *et al*., 2015).
Cassava (*Manihot esculenta* Crantz) is a perennial crop that is vegetatively propagated and cultivated throughout the lowland areas in tropical and sub-tropical regions for its important starchy roots (Isaac-Bamgboye *et al*., 2020). The advantages it has over other root crops include its easier propagation, high yield, and pest and drought resistance. However, certain varieties contain a large amount of cyanogenic glucosides (linamarin and lotaustralin) which can be hydrolyzed to hydrocyanic acid (HCN) by the endogenous enzyme, linamarase, when the plant tissue is damaged during harvesting, processing or other mechanical processes (Isaac-Bamgboye *et al*., 2020; Oboh and Akindahunsi, 2003). Cassava wastes are highly problematic because they are quite often toxic and cause nuisance especially to aquatic environment (Cumbana *et al*., 2007). The two major wastes generated during processing of cassava (cassava peels and effluent) have been reported to cause a lot of problems to vegetation, houses and bring about infection by microbes and infestation. Considering the enormous interest recently generated on the safety implications of food processing (Osundahunsi *et al*., 2016; Enujiugha *et al*., 2023), it becomes highly imperative to seek valorization of cassava wastes via their conversion to useful products. Effluent generated from cassava processing sites and released into the environment is acidic in nature and harmful to all life-forms; however, a number of bacteria can survive the acidity of such contaminated sites. Among such microorganisms that can thrive on the cassava processing dump rite is *Bacillus badius*. The present study sought to produce, purify and determine physicochemical properties of protease from *Bacillus badius* isolated from cassava effluent contaminated dump site. The main objective of this study was to isolate and characterize bacteria from the dumpsite, generally screen the bacteria isolates for protease production, purify and characterize protease from high protease producing bacteria grown on cassava effluent-contaminated soil, and investigate if the proteases obtained are relatively stable at operating conditions. This was with a view to highlighting the industrial potentials of the purified enzyme for large-scale processes.

Materials and Methods

Sample collection

Soil samples were collected from a cassava processing effluent dump site at Ilara-mokin, Ondo State, Nigeria. The samples were aseptically collected into sterile polyethylene bags from the top soil and by digging the soil up to 2 cm and 5 cm deep. The samples then transported to the laboratory and were subsequently maintained at 4 ^{o}C prior to being used for analysis. All the chemicals and reagents used in the study were of analytical grade, while the water was glass distilled.

Isolation of *Bacillus badius*

Bacillus badius for this research work was isolated from soil samples collected from the cassava processing dump site. One gram (1 g) of soil sample was aseptically transferred to 9 mL sterile distilled water. The resulting solution was well mixed and serially diluted from each tube containing the soil extract; then 0.1 ml sample was taken and spread on freshly prepared nutrient agar (NA) plates before incubating them at 37 ^{o}C for 24 h.

Microbial Analysis and Identification

Populations of microorganisms in the cassava processing dump site (that is, from the fermenting medium) were assessed by standard pour plate technique according to Omemu *et al* (2018) with slight modifications, using nutrient agar. Tenfold serial dilution was made and 1 ml of desired dilution plated. Bacteria plates were incubated at 37 ^{o}C for 24 hours. Any visually distinct bacterial colony appearing on the plates was isolated and streaked onto the appropriate media until single colonies were obtained. The isolated bacterial colonies were characterized and identified using a series of biochemical tests and identification keys (Adejobi *et al.*, 2024b). Pure cultures of all isolates were maintained in the appropriate agar slants and stored in the refrigerator.

Isolation and characterization of protease producing bacteria

The bacteria were sub-cultured on casein agar and the isolates, which produced clear zones on casein agar after 24 h incubation, were maintained on nutrient agar plates. The potential isolate showed clear zone of inhibition than other strains on casein agar, and was retained for this study. The isolated bacterial strains were identified on the basis of their morphological, cultural and biochemical characteristics (Adejobi *et al*., 2024b). The obtained data were compared with standard description provided in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994).

Screening and Production of protease

Production of protease was done using the method of Keay *et al*. (1972), with some modifications. The medium component consists of 0.5% casein (w/v), 0.2% starch (w/v), 0.2% KH_2PO_4 (w/v), 0.2% KNO_3 (w/v), 0.5% NaCl (w/v), 0.005% $MgSO_4.7H_2O$ (w/v), 0.002% $CaCO_3$ (w/v), 0.001% $FeSO_4.7H_2O$ (w/v), with pH adjusted to 7.4. The fermentation medium was incubated and maintained at 37 °C for 36 h at 200 rpm using orbital shaker. The cultured fluid was centrifuged using refrigerated centrifuge at the speed of 10,000 rpm for 15 min. The supernatant obtained was stored as crude enzyme at 4 ^{o}C.

Preparation of Bacterial Inoculum

Bacterial inoculum was prepared using the method described by Gangadharan *et al.* (2006). Briefly, fifty millilitres (50 ml) of nutrient broth was inoculated with a loopful of cells from a 24 h old slant and kept at 37 °C in a rotary shaker (100 rpm). After 12 h of incubation, 5 ml of this nutrient broth culture of *Bacillus badius* was used as the inoculum for solid state fermentation.

Assay of protease activity

Extracellular proteolytic activity was determined according to the method of Naik *et al.* (2013), with slight modifications using casein as the substrate. The reaction mixture contained 1 mL of 1% (w/v) casein in 0.1 M phosphate buffer (pH 7.0) and 1 mL of culture supernatant. The mixture was incubated at 40 °C for 30 min. The enzyme reaction was terminated by addition of 6 mL 5% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 min and filtered through Whatman no. 4 filter paper. To 1 mL of filtrate, 3 mL of 0.5 M Na_2CO_3 solution and 1 mL of 3-fold-diluted Folin-Ciocalteu reagent (BDH Chemicals Ltd, Poole England) were added and mixed thoroughly. The colour developed after 30 min of incubation at 30 °C and was measured in a Unico UV-2102 PC spectrophotometer at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate one microgram (1 µg) of tyrosine from casein per minute at 40 °C under the assay conditions described above.

Determination of protein concentration

The protein concentration was determined routinely according to the method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein. The absorbance was measured at 595 nm

Enzyme Purification

Ammonium sulphate precipitation

The crude enzyme solution of 700 mL was brought to 40% and 80% ammonium sulphate saturation after appropriate amount of solid ammonium sulphate has been calculated using Encorbio tool. The solid ammonium sulphate was gradually added to the crude enzyme filtrate at 4 ^{o}C followed by continuous gentle stirring. It was thereafter left overnight in order to allow the enzyme to precipitate. The precipitate was centrifuged at 10,000 rpm and the pellet was dissolved in 10 mL 0.05M Tris/HCl buffer and stored at 4 ^{o}C. The enzyme solution was dialyzed against the same buffer with three changes of buffer for 72 hours. The dialysate was stored at 4 ^{o}C for further purification step

Ion-Exchange Chromatography

The dialyzed aliquot enzyme was loaded against a previously well-packed DEAE Sephacel resulting (column of 2.5 x 10 cm and equilibrated with 50 mM Tris/HCl buffer, pH 7.4 (Flow rate: 60 mL/h). After unbound protein have been collected, the bound protein was thereafter eluted using 1 M NaCl dissolved in 0.5 mM Tris/HCL buffer. The eluents obtained were investigated for the presence of protein at 280 nm and protease activity using standard assay procedures. The eluents that exhibited protease activity were pooled and concentrated using 4 M sucrose. The concentrated enzyme solution was kept at 4 ^{o}C for further purification (Xiong *et al*., 2012)

Gel-filtration chromatography

The concentrated eluent obtained after ion-exchange chromatography was loaded on Sephadex G-100 which had been previously equilibrated with 50 mM Tris-HCl buffer (pH 7) using gel-filtration chromatography column (2.5 x 70 cm). The fractions were collected at flow rate of 20 ml/h. After which the protein and protease activity present in the eluent were determined at 280 nm and standard assay procedures, respectively. The fractions that exhibited protease activity were pooled together and concentrated for subsequent determination of molecular weight to confirm homogeneity of the protein and investigation of physicochemical properties of the purified enzyme.

Molecular Weight Determination

Molecular weight of the purified enzyme was determined by employing sodium dodecyl sulphate –polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gel according to Laemmli (1970) with standard marker proteins of molecular weight 103.14, 81.35, 47.05, 34.17, 27.26, 17.67 kDa and staining of protein with Coomassie brilliant blue.

Preparation of Gel slab

A ten percent (10%) agarose solution was prepared and poured on a smooth plane surface before the two glass plates were placed vertically on it. This was allowed to polymerize thereby sealing the bottom of the two glass plates. The solution for the resolving gel was prepared and poured between the glass plates and allowed to polymerize, after which saturated butanol was added to level it and then the comb was placed. Stacking gel solution was prepared and then poured into the glass plates through the comb teeth and allowed to polymerize.

Sample Application and Electrophoresis

The purified enzyme solution (50 µL) was pipetted into an Eppendorf tube and mixed with 10 µL loading dye; this was boiled for 1 min at 100 ^{o}C and applied into the wells of the slab gel. The electrophoresis was run at 80 volts and 21 milliamps using the running buffer.

Gel-Staining and De-Staining

The gel was carefully removed from the plates and stained with Coomassie brilliant blue (1.25 g/L) for 24 h at 25 ^{o}C, after which it was de-stained using a de-staining solution. The de-staining solution was composed of 530 ml of distilled water, 400 mL of methanol and 70 mL of acetic acid. Protein bands were observed after destaining the gel.

Physicochemical properties of the purified protease

Effect of pH on the activity of the enzyme

The optimum pH of the protease was investigated by varying pH value from 2 to 12 using 100 mM glycine HCl (pH 2-3), Acetate and acetic acid (pH 4-5), phosphate buffer (pH 6-7), Tris/HCl (pH 8-10), and Tris/NaOH (pH 11-12). The effect of pH on protease activity was determined at 40 °C in the different buffers at 100 mM.

Effect of temperature on the activity of the enzyme

The optimum temperature was determined by estimating the protease activity at pH 7 and temperature ranging from 30 to 90 °C. The enzyme solution and the substrate (casein) in 100 mM Tris/HCl buffer (pH 7) were incubated at these aforementioned temperatures and the enzyme activity was determined according to the standard assay procedures described above.

Effects of pH on the stability of protease

The effect of pH on the stability of protease was determined by incubating 0.5 ml of the diluted enzyme solution and 0.5 mL of phosphate buffer ranging pH from 3 to 12, for 6 h at 40 ^{o}C. Aliquot enzyme solution was first withdrawn at 0 minute and subsequently after 30 min interval for 6 h while the enzyme activity was done according to the afore-described assay procedures.

Effect of temperature on protease stability

The purified protease was incubated between the temperature 30-90 ^{o}C at 10 ^{o}C interval for 2 h in 100 mM Tris /HCl buffer, pH 7. Aliquot 0.5 mL of the enzyme were withdrawn at 0 and then 30 min interval. The protease activity was determined according to the method described earlier.

Determination of kinetic parameters:

The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the protease for casein was determined at different substrate concentrations of 0-1% in Tris/HCl buffer, pH 7. They were evaluated by plotting the data on a Lineweaver-Burke double-reciprocal graph ($1/V_o$) versus (1/[S]) (Lineweaver and Burke, 1934).

Effect of Metal ions and Enzyme Inhibitors on Protease Activity

The effect of various metal concentrations on enzyme activity was investigated using $CoCl_2$, $HgCl_2$, $MnCl_2$, $ZnCl_2$, NaCl, $CuCl_2$, $MgCl_2$. The reaction mixture consisted of aliquot enzyme, casein and metal ion in Tris/HCl buffer (pH 7.4). Purified enzyme was incubated with ions for 30 min at 40 °C and then the remaining enzyme activity was estimated using casein as a substrate. The activity of the enzyme without metals was taken as the control.

Effect of Inhibitors on Protease Activity
The effect of various inhibitors on enzyme activity was investigated using EDTA, Sulfamoybenzamide (SBA), Sphingomyelin synthase (SMS), Ascorbic acid, Urea, Mercaptoethanol (B.methy) and Cystein. The reaction mixture consisted of aliquot enzyme, casein and inhibitors in Tris/HCl in pH 7.4. Purified enzyme was incubated with the inhibitors for 30 min at 40 °C and then the remaining enzyme activity was estimated using casein as a substrate.

Substrate Specificity of the Purified Protease
The ability of the purified protease to degrade various native proteins was evaluated with the following substrates (1% w/v): Casein, Ammonium sulphate, Agarose, Bovine serum albumin, Gelatin, Peptone and Urea. Protease activity was determined as described earlier.

Results and Discussion

Identification of Organisms

The results presented in Table 1 clearly show that cassava effluent processing dump site soil samples harbour different types of microorganisms, bacteria specifically. In spite of high acidic nature of the soil, the bacteria exhibited inherent ability to dwell in this site and thereby building resistance to the very low pH. The acidic nature, and even cyanide nature, of the dump site can easily affect conformation of amino acid at the active site of the various enzymes used for metabolism of different macromolecules and substrates. Hence, this confers usage of organism (bacterium) isolated from this site for production of different macromolecules such as amylase, protease, phytase and so on, because they are expected to possess exceptional and unique physicochemical properties which can be employed in different industrial processes (Enujiugha, 2020).

The morphological and biochemical characteristics presented in this work were based on Bergey's manual of determinative bacteriology as outlined by Adejobi *et al*. (2024b), and thus the phenotypic characteristics show that the bacteria isolates belong to the family of *Bacillus* spp. The naturally occurring environments provide excellent source of nutrients for microorganisms. Isolation and screening of bacteria from these natural environments can be useful for obtaining bacterial species with potential of producing protease enzyme. The isolated *Bacillus badius* had maximum protease production after 24 h, with protease production of 0.325 U/ml.

Screening of bacteria for Protease Production

Table 2 shows the casein hydrolysis capacity of isolated organisms from cassava fermentation dump site soil samples. The primary screening for protease producing bacteria strains was done in casein agar medium based on formation of zones of inhibition. The bacterial isolates which formed zones around the colonies were considered to be protease positive strains. The observation revealed that after 24 h of incubation, *Baciilus badius, Brevibacillus latesporus, Bacillus microflavus* and *Bacillus insolitus* showed zones of casein hydrolysis (positive); but among all of them, *Bacillus badius* had the highest zone of casein hydrolysis measuring 3.3 cm in diameter.

Extracellular Protease production from organism

Extracellular production of protease from isolated bacteria is presented on Figure 1. In this study, a total of four (4) bacteria were enumerated. Then, these isolates were screened for protease production. Following incubation, the plates were inoculated and left for 24 h and clear zones were observed around colonies indicating casein hydrolysis. The four bacterial isolates that showed protease activity, identified as *Bacillus badius, Brevibacillus latesporus, Bacillus microflavus* and *Bacillus insolitus*, were selected, with *Bacillus badius* having the highest zone of inhibition. *Bacillus badius* had maximum protease production at 24 h with protease production of 0.325 U/ml while at 36 h the production declined to 270 U/ml.

The production of protease and other related enzymes has been reported in *Bacillus cereus SU12 isolated from oyster Saccostrea cucullata (Umayaparvathi et al., 2013) and Aspergillus flavus under Solid State fermentation* (*Chinnasamy et al., 2011). Bacillus* strains usually enumerated included *B. amyloliquifaciens, B. subtilis* and *B. licheniformis*. Some other bacterial species which are also known for their protease production potential are of the genera *Staphylococcus, Pseudomonas, Serratia, Alcaligenes, Vibrio, Brevibacterium, Flavobacterium* and *Halobacterium* from kitchen waste (Gupta *et al.*, 2005). Others included *Corynebacterium alkanolyticum ATH3 isolated from fish gut* (Goutam *et al., 2015), Aspergillus niger isolated from yam peels (*Oludumila *et al., 2015), Bacillus* sp. obtained from abattoir soil sample (Mamoud *et al.*, 2018*), Bacillus subtilis AKAL7 and Exiguobacterium indicum AKAL11 by using organic municipal solid wastes and Bacillus subtilis Asasbt isolated from termite soil (*Sujatha and Subash, 2018*).*
Production time for the protease by *Bacillus badius* in this study was optimal at the 7th day (Figure 2). This result is in agreement with the report of Johnvesly and Naik (2002) and Improlsup *et al.*, (1981) who observed maximum protease enzyme production occurring during the 7th day of incubation using *Aspergillus flavus*. Thus, incubation period for the enzyme is directly related to production of enzyme and other metabolites, to some certain extents.

Fermentation and Cultivation periods play an important role in the extracellular alkaline protease production (Olajuyigbe and Ajele, 2005, 2011; Rahman *et al*., 2005). The results obtained in this study also revealed that appreciable production of protease enzyme started from 12 hours of incubation for *Bacillus badius* and enzyme was continuously produced up to 30 hours. Afterwards it was observed that as the incubation period increased, the proteolytic activity decreased. Thus, this can be traceable to the fact that there was reduced availability of nutrient, and an accompanying production of toxic metabolites (Romero *et al.*, 1998)

Table 1: Biochemical characterization and the occurrence of bacterial isolates

S/N	Gram Reaction	Glu	Lac	Fru	Man	Mal	Suc	Ara	Gal	Rib	Urea	H_2S	Citrate	Gas	Cat	Probable identity
1	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	*Brevibacillus latesporus*
2	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	*Bacillus Microflavus*
3	+	-	+	+	+	+	+	-	+	+	-	-	-	-	+	*Bacillus insolitus*
4	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	*Bacillus badius*

Table 2: Qualitative screening of isolated soil microorganisms for protease production

Test	Organism	Observation After 24 Hours
	Bacillus badius	+zone 3.3 cm
Casein +Agar	*Brevibacillus latesporus*	+zone 3.0 cm
	Bacillus microflavus	+zone 3.1 cm
	Bacillus insolitus	+zone 2.9 cm

+Zone indicate positive region of casein hydrolysis

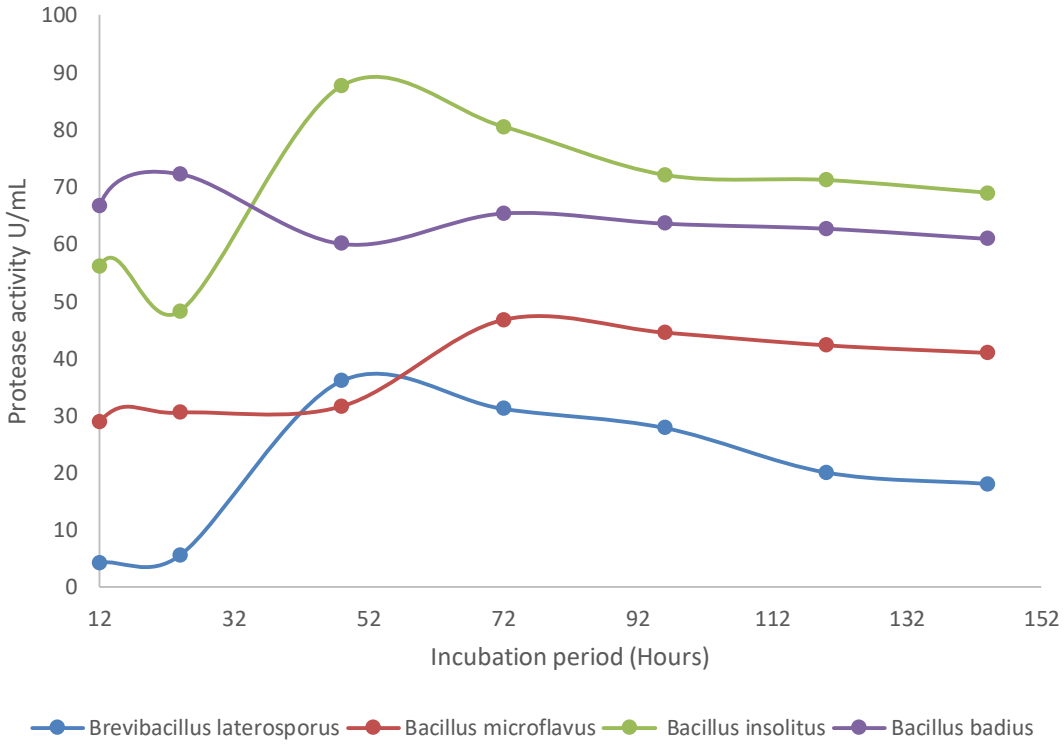

Figure 1: Protease activity for production of enzyme from bacteria in culture medium containing 2% casein

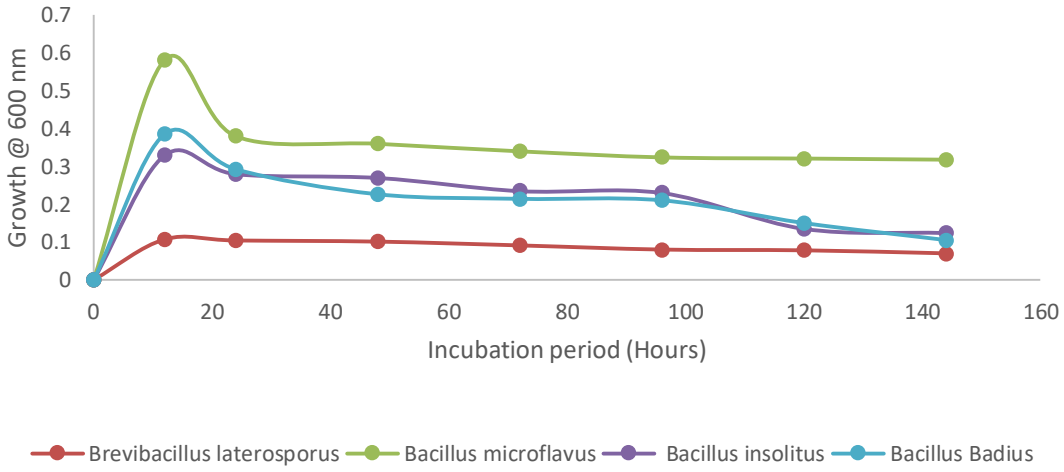

Figure 2: Biomass accumulation for production of enzyme from bacteria in culture medium containing 2% casein

Purification table for protease production

The activity of crude enzyme from *Bacillus badius* observed was 82.5 U/ml while protein concentration and specific activity were found to be 94.54 mg/ml and 0.87 U/ml, respectively. When the dialysate was loaded on DEAE sephacel, three sharp activity peaks were observed as shown in Figure 3 due to stepwise NaCl elution used. Tube 62-65 pool A, 69-80 pool B and 81-94 are labelled pool C, respectively. The peak with the highest activity was then loaded on the Sephadex G–100 resin. The main protease activity peaks were observed during the ion exchange chromatography; the fraction between tubes 69-80 has the highest peak and these were pooled together and labelled. The pooled fractions were subjected to Sephadex A-100 column (2.5× 75cm) (Figure 3), with an enzyme yield of 2.96% for Pool A, 3.15% for Pool B and 1.29% for Pool C, respectively. Purification fold was 1.58, 1.51 and 1.48 for Pools A, B and C, respectively

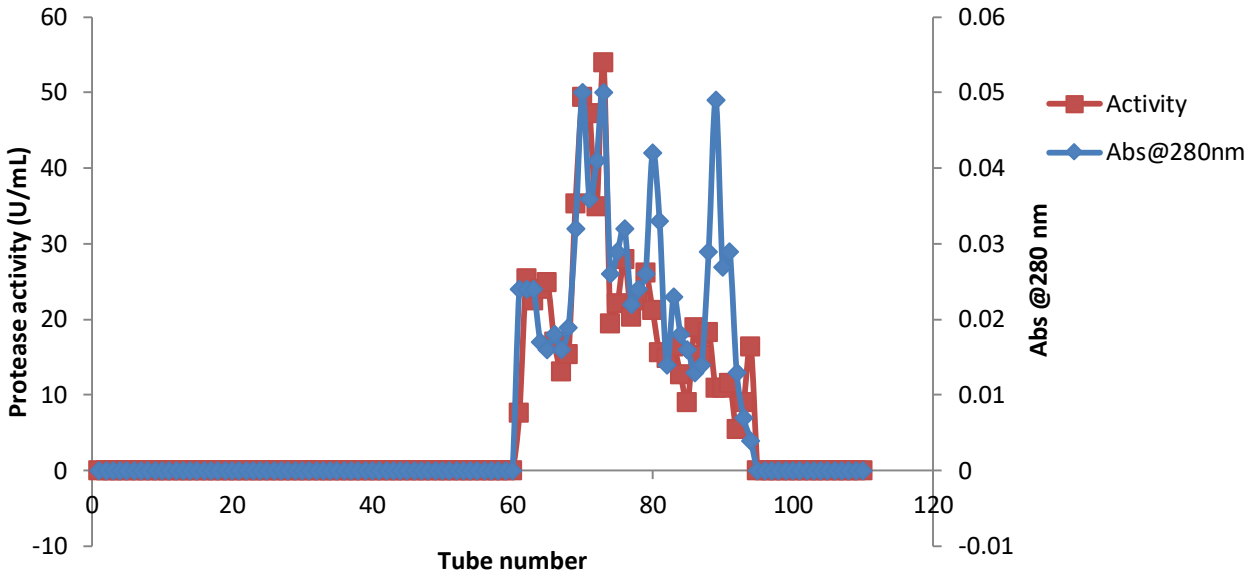

Figure 3: Chromatogram of *Bacillus badius* protease on ion exchange (2.5 × 3.5 *cm*) column of DEAE Sephadex A -50 using CM- cellulose anionic exchange.

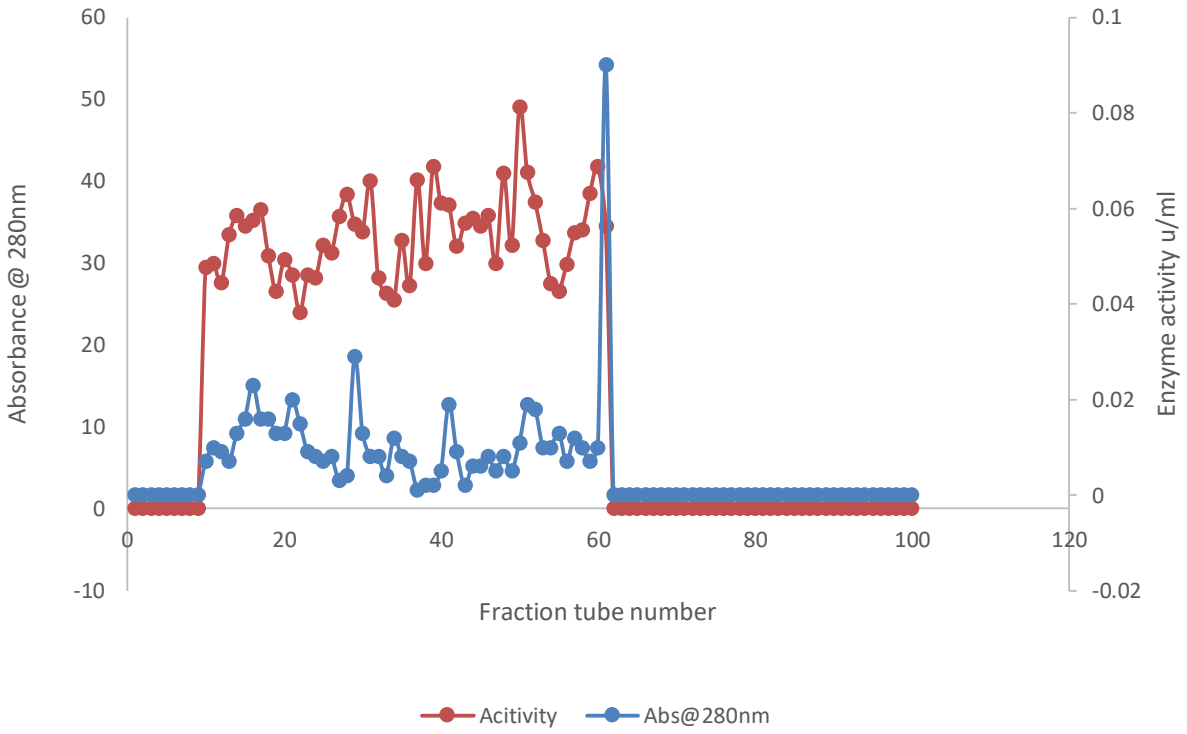

Figure 4: Elution profile of protease produced from *Bacillus badius* on Sephadex G-100 column chromatography.

B. badius protease showed a specific activity of 5.28 U/mg with 3% recovery and 6-fold purification. The result obtained in this study is lower than that reported by Srividya *et al*. (2020) for cre recombinase with 85% yield. Suberu *et al*. (2019) reported that serine alkaline protease from *Bacillus subtilis* RD7 had 606.73 mg/ml, 98.29 mg/ml and 6.24 U/mg for total activity, protein concentration, and specific activity, respectively. Hence, increase in the purification fold is directly proportional to the increase in the purity of the partially purified enzymes as compared with the crude enzyme, while increase in the specific activity of enzymes is a good suggestive ability of inactive protein during purification (Bajpai, 2014: Cheng *et al*, 2016)

Table 3: Purification table of protease production from *Bacillus badius*

Purification Step	Volume (mL)	Activity (U/ml	Protein (mg/ml	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	% recovery	Purification fold
Crude Enzyme	700	82.5	94.45	57750	66178	0.87	100	1
$(NH_4)_2SO_4$ ppt	49	108.75	101.81	5328.75	4989	1.07	9.22	1.22
DEAE Sephadex	25	120.37	59.09	3009.25	1477	2.04	5.21	2.34
Sephadex G-100 (A)	15	125.4	42.72	1881	641	2.94	3.26	3.37
B	15	121.5	38.81	1822.5	582	3.13	3.15	3.59
C	15	120	22.72	1800	341	5.28	3.12	6.06

Determination of the molecular weight

The enzyme was purified to homogeneity as revealed by a single protein band as observed for, on the PAGE as showed in Figure 4. The molecular weight of protease from the three pool was estimated to be 34.17 kDa.

The molecular weights of the purified protease isoforms were 34.17 kDa. Thus several work on *Bacillus* had showed different molecular weight with 30.9 kDa thermophilic *Bacillus* stain HS08 (Huang *et al*., 2006); 75.0 kDa *Bacillus megaterium* (Reungsang *et al*., 2006) and 15.0 kDa for *Bacillus subtillis* protease. This observation revealed that the enzyme is homogeneous due to the protein band which is single and can be monomeric but contains isoforms.

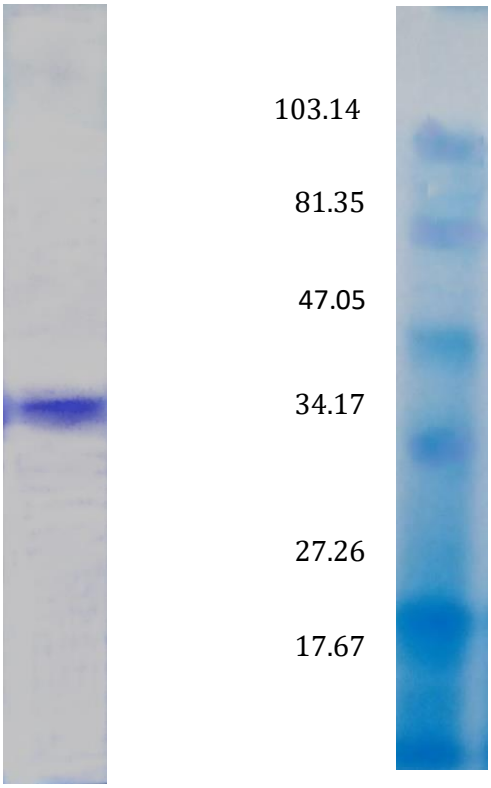

Figure 5: Molecular weight (kDa)
KEY: (34.17 KDa)

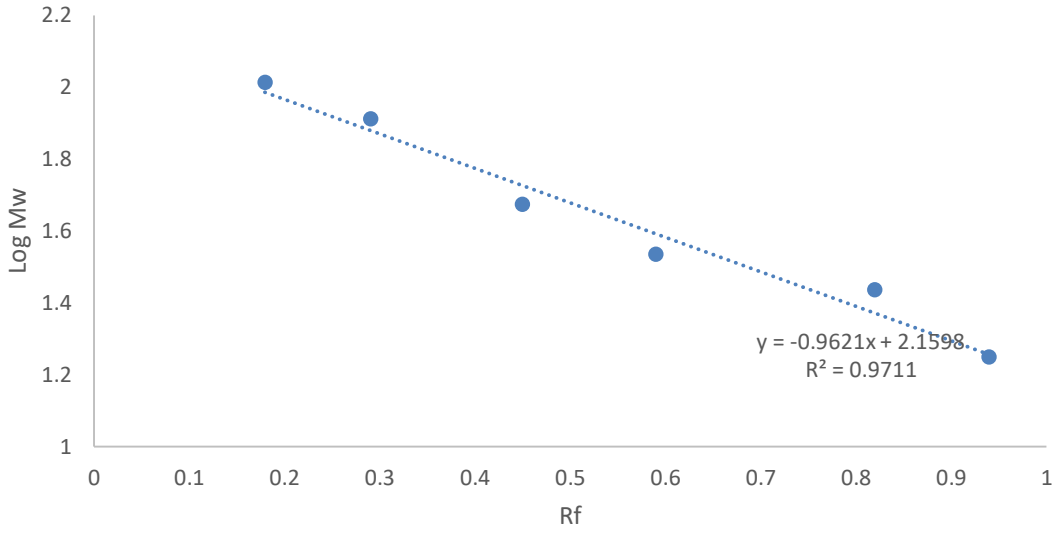

Figure 6: Molecular weight of the purified enzyme from *Bacillus badius* showing the Log Mw and R**f**

Physiochemical properties of the purified protease
Effect of pH on the activity of protease from *Bacillus badius*

The optimum pH observed for the enzyme was pH 8.0. The enzyme showed a gradual increase in enzyme activity as the pH increased until the optimum pH of 8.0. However, the enzyme exhibited steady decrease at alkaline region

The purified protease showed a unique wide range of pH with optimum activity obtained at alkaline pH 8.0 while high relative activities of 62-76% and 73-95% were recorded at pH 2-7 and 9-11 respectively. The optimum pH obtained for *Bacillus badius* was similar to *Bacillus subtillis* reported by Yandri *et al*. (2008), and *Bacillus* sp reported by El Hadj-Ali *et al*. (2007). Susanti and Febriana (2017) reported protease optimum activity at 7.0 to 11. Sulthoiyah and Nursyam (2015) obtained optimum pH 7.5 for *L. plantarium* B10 and ph 7.5 while Sanatan *et al*. (2013) observed *Peripplaneta americana* proteases to be optimum pH 8.0. The pH of the culture medium is strongly in*fl*uences by many enzymatic processes and the transport of various components across the cell membranes (Ellaiah *et al*., 2002). The pH thermal stability was stable at pH 7.0. Greiner (2009) describe the stability of most plant enzymes as decreasing dramatically at pH values below 4.0 and 7.5, whereas, majority of the corresponding microbial enzyme are rather stable at pH 7.0.

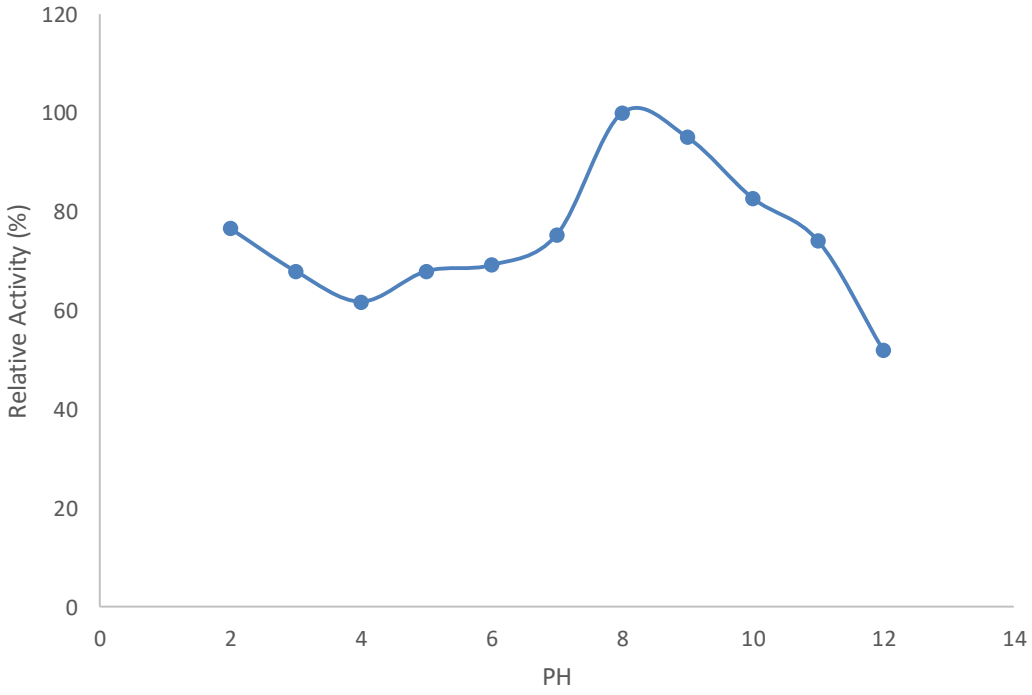

Figure 7: The effect of pH on protease activity.

Effect of pH on the stability of protease from *Bacillus badius*

Initial activity was measured at 0 minute while the residual activity of the enzyme was calculated as percentage of the initial activity taken as 100% with the pH range from 5-10. The influence of pH on the stability of protease is presented in Figure 8. The percentage residual activity of the enzyme was measured to be about 68% after 2 hours incubation at pH 6.0 while about 42-34% activity were retained at pH, 7 and 8 with a decline in residual activity at pH 9.0-11.0 respectively after 2 hours incubation period.

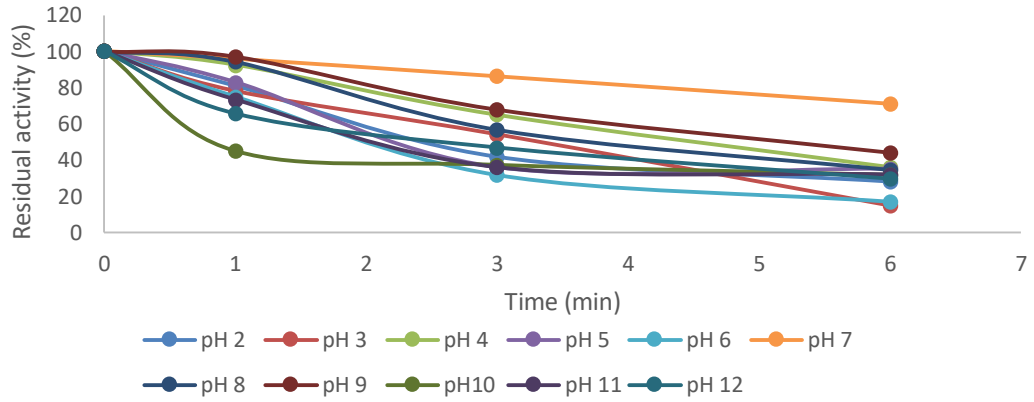

Figure 8: Effect of pH on the stability of purified protease. Maximum activity was expressed as 100% with pH ranges from 3.0- 9.0.in Glycine –HCL buffer (pH 3.0-4.0), Sodium acetate buffer (pH 5.0-6.0), Tris-HCl buffer (7.0-8.0) and Glycine – NaOH buffer (pH 9.0).

Effect of Temperature on the Activity of protease from *Bacillus badius*

The effect of temperature on the activity is presented in Figure 9 The optimum temperature was observed at 40 ºC, meanwhile the enzymes exhibited high relative activity at all temperature investigated with 86% relative activity obtain at 30 ºC were determined, while 97-85% relative activity were recorded between 50-90 ºC. The temperature ranging from 30-90 ºC with 10 ºC interval were determined.

Maximum protease activity was observed at 40 ºC. The work was in agreement with the findings of Vijah-Anand *et al.* (2010). The thermal stability was 30 ºC and relative minimum level at 60 ºC for 30 min of incubation. Hence, high temperature is found to have negative effect on microorganism and metabolic activity (Tunga, 1995) and cause inhibition of the growth of bacteria. Sequel to this, high temperature due to its denaturing ability can cause enzyme loosing ability and catalytic properties due to stretching and breaking of weak hydrogen bonds within enzymes structure (Conn *et al.*, 1987). Similar result was observed for *Bacillus lateroporous* at 40 ºC (Usharani and Muthuraj, 2010). Likewise, the optimum temperature for protease production varies from one species to another species, and it was reported that *B. licheniformis* and *Bacillus* sp. SMIA-2 produced maximum level of protease at 50 and 60 ºC, respectively (Al-Shehri *et al*., 2004; Nascimento and Martins, 2004).

The temperature stability profile of the purified protease suggested that the enzyme was thermostable. However, this enzyme could find potential commercial application as a food processing agent since most food industries such as brewing, baking, etc. that utilize proteases require their processes to be carried out at temperatures around 40, 50 and 60 ºC.

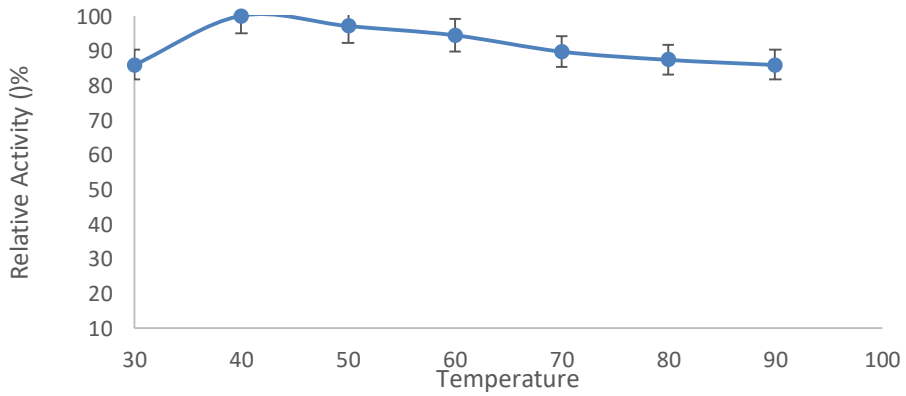

Figure 9: Effect of temperature on the activity of purified protease. Effect of temperature on the assay mixture was incubated at 30-80 ºC and the protease activity was measured using assay standard procedure.

Effect of Temperature on the Stability of Protease from *Bacillus badius*

The effect of temperature on the stability of protease from *B. badius* is presented in Figure 10. The influence of temperature on the stability of protease was studied by incubating the enzyme solution at different temperature (30 to 90 ºC) for 120 min, as represented in Figure 10. The percentage residual activity of the enzyme was found to be about 55% after 2 hours of incubation at 40 ºC. The maximum residual activity of 85-95% were obtained at 30-7 ºC while 73 and 63% were recorded at 80 and 90 ºC after 30min of incubation. After 1hr incubation over 80% residual activity was observed at 30-60 ºC and over 60% at 70-90 ºC. Meanwhile remaining activity of 59, 50, 45 and 35% were recorded at 40 ºC, 30 ºC, 50-60 ºC, and 70-90 ºC, respectively after 2 h incubation.

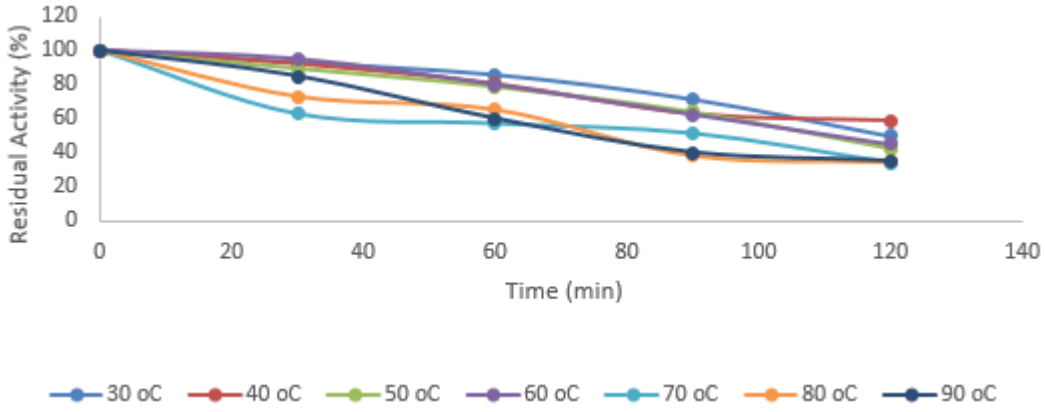

Figure 10: Effect of temperature on the stability of purified protease

The enzyme solution and Tris-Hcl, buffer, pH 7 was pre –incubated while initial activity was observed at 0minutes and at 30minutes interval thereafter for 2 hr. The enzyme activity was determined according to assay procedure,

Effect of metal ions on protease activity

Effects of metal ions on protease activity is presented in Table 4. The activity of protease was enhanced in the presence of Co^{2+}, Hg^{+}, $Mn2^{+}$, Cu^{2+} and Mg^{2+}, in a concentration –based dependent manner while it was slightly inhibited in the presence of Zn^{2+} and Na^{+}

Metallic ions have either inhibitory or stimulating effect on the activity of enzymes depending on the concentration of the metallic salt solution. The presence of metallic ions is important because their presence or absence regulate enzymes activity. The presence of metallic ions alongside with waste from food can inhibit or enhance protease activity, hence inhibitors and metal ions can cause reduction in substrate hydrolysis by proteolytic enzyme. From the study of the effect of metal ions and inhibitors on protease enzymes from *Bacillus badius* revealed that the activity of protease was enhanced in the presence of Co^{2+}, Hg^{+}, Mn^{2+}, Cu^{2+} and Mg^{2+} in a concentration based dependent manner while it was slightly inhibited in the presence of Zn^{2+} and Na^{+}. Also, the effect of inhibitors was assayed and the activity shown some significant value in their stimulating ability and inhibitory ability respectively. The strong activation of the protease in the presence of Mg2+ suggests that the protease could be protected from thermal denaturation due to their probable presence in the catalytic domain of the enzyme. The result obtained for *B. subtillis* and E. *indicum* also showed similar stimulating and inhibitory effect on Mg^{2+}, Ca^{2+} and K^{+} significantly increased the activities of protease from the both isolates. In the presence of Mg^{2+}, Ca^{2+} and K^{+}, In contrast, protease from the both isolates was strongly inhibited by Fe^{3+}, Zn^{2+}, Hg^{+} and Co^{2+}. It is reported that the activity of alkaline protease from *Bacillus sp.* and *Bacillus megaterium* RRM2 is increased by Ca^{2+} (Gupta *et al.*, 2005; Rajkumar *et al*., 2011) and decreased by Hg^{2+}, Zn^{2+} and Co^{2+} (Venugopal and Saramma, 2007; Olajuyigbe and Falade, 2014). Suberu *et al*. (2019) expressed the metal particle fe^{2+} and mg^{2+} expanded the relative action up into 114.77%, 103.65% and 107.89%, 98.70% at 5 mM and 10 mM for serine protease from *Bacillus*.

Table 4: Effect of metal ions on protease activity.

Metal Ions concentration	**1mM**	**5mM**	**10mM**
None	100.00±0.00	100.00±0.00	100.00±0.00
$CoCl_2$	125.13±0.55^{b}	132.38±0.40^{a}	133.56±0.63^{a}
$HgCl_2$	130.71±0.86^{a}	132.42±0.82^{a}	121.78±1.10^{b}
$MnCl_2$	149.32±0.72^{c}	156.67±0.63^{b}	161.39±0.63^{a}
$ZnCl_2$	94.16±0.63^{a}	92.05±0.29^{b}	91.20±0.54^{b}
NaCl	89.00±0.16^{b}	93.71±0.15^{a}	85.70±0.27^{a}
$CuCl_2$	114.41±0.58^{c}	122.33±0.32^{a}	120.68±0.19^{b}
$MgCl_2$	165.12±0.29^{b}	167.23±0.64^{a}	167.58±0.64^{a}

Results are mean ± SD of triplicate data. Mean with different superscripts within the same column are significantly different ($p < 0.05$).

Effect of inhibitors on the activity of protease

Effect of inhibitors on the activity of protease is presented in Table 5. The enzyme activity was only stimulated in the presence of cysteine at all concentration investigated in a concentration dependent manner: Meanwhile ascorbic and β-mecaptoethanol, EDTA, and urea showed serious inhibition of enzyme in a concentration dependent manner

The enzyme activity was only stimulated in the presence of cysteine at all concentration investigated in a concentration dependent manner: Meanwhile ascorbic and β-mercaptoethanol, EDTA, and urea showed serious inhibition of enzyme in a concentration dependent manner. The activity of the protease in the presence of the EDTA is attributed to its useful and application of detergent as additive.

Table 5: Effect of inhibitors on protease activity

Inhibitors			
Concentrations	1mM	5mM	10mM
EDTA	43.29 ± 0.11^{c}	44.42 ± 0.03^{a}	44.03 ± 0.07^{b}
Sulfamoybenzamide	50.51 ± 0.03^{c}	55.86 ± 0.07^{b}	69.27 ± 0.37^{a}
Sphingomyelin	48.48 ± 0.38^{ab}	49.19±0.38a	47.88 ± 0.20^{c}
Ascorbic Acid	76.41 ± 0.42^{a}	77.02 ± 0.06^{a}	75.19 ± 0.11^{b}
Urea	50.48 ± 0.08^{b}	50.14 ± 0.05^{c}	50.80 ± 0.13^{a}
Betamcaptoethanol	77.62 ± 0.72^{b}	80.14 ± 0.20^{a}	78.35 ± 0.72^{ab}
Cystein	112.24 ± 0.43^{b}	$115.97\pm0.22^{a}1$	15.99 ± 0.70^{a}

Results are mean ± SD of triplicate data. Mean with different superscripts within the same column are significantly different ($p < 0.05$).

Substrate specificity

Protease showed optimum activity towards casein while ammonium sulphate and agarose displayed high relative activity of 61 and 70% respectively but the enzyme activity was strongly inhibited in the presence of bovine serum albumin, gelatin and peptone

One of the most importance of protease enzymes is their ability to distinguish between competing substrate and utility of these enzymes which often depends on their substrate specificity (Shankar *et al.*, 2011). The purified enzyme was capable of degrading natural substrate like ammonium sulphate, agarose, bovine serum albumin, gelatin, peptone, casein and urea to some extends. The activity obtained was higher in casein more than the remaining substrate, and thus, this indicate that the substrate specificity profile of the purified enzyme has a wide range of hydrolytic activity on the different protein substrate which currently of great importance and useful in biotechnology. Yusuf, (2018) reported CLP-IIA-IA5 has a structure that may be related to its ability to degrade casein better. In the food sector, alkaline protease has been employ and used in the preparation of protein hydrolysate with nutritional value.

Table 6: Substrate specificity of purified protease

Substrate	Protease Activity
Ammonia sulphate	61.53
Agarose	70.32
Bovine serum albumin	13.18
Gelatin	25.27
Casein	100
Peptone	24.17
Urea	47.25

Kinetic Analysis

The kinetic parameters of *B. badius* protease is presented in Figure 11. The K_m and V_{max} were estimated to be 1.98 mM and 59.88 µmol/ml/min, respectively.

The apparent K_m and V_{max} values for protease enzyme from *Bacillus badius* were determined from line Weaver Burks plots. The kinetic parameter (K_m and V_{max}) values were 1.98Mm and 59.88 mmol/ml/mm respectively. Michealis-menten constant, K_m can provide adequate information on the physiological and biochemical information about an enzyme (Ranaldi, 1999). Thus, the evaluation of kinetic constant is a central point in enzyme research and also help in industrial application because lower km value allows for easier and faster industrial processes (Silva *et al.*, 2005). The values obtained in this work signifies that the purified protease exhibit high affinity, degrading ability and catalytic efficiency for the substrate which is useful for industrial purpose that requires hydrolysis of polysaccharide. Devi *et al*. (2008) gave a lower K_m value of 0.8Mm using casein as substrate.

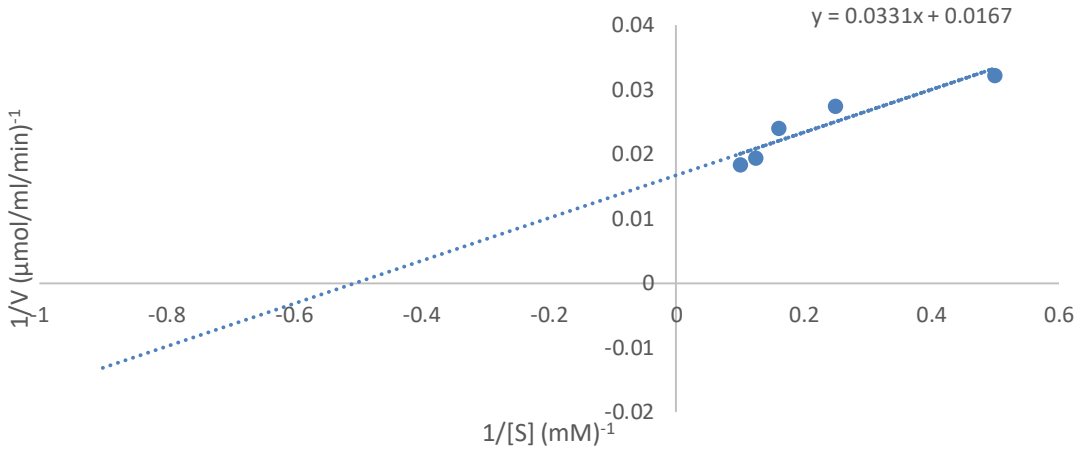

Figure 11: Line weaver Burk plot of purified protease activity

Conclusion

This work describes the isolation, purification and characterization of *Bacillus badius* strain from protease.one activity peak was determined for the ion exchange chromatography, after the gel filtration the % recover was 5.4 and fold was 2.34, hence, the three recovery and fold was 3.26, 3.15, 3.12 and 3.37, 3.59 and 6.06 which is the highest purification fold from the gel filtration for pool A, B and C respectively. The estimated molecular weight of purified protease from SDS-PAGE was 34.17 kDa. The fraction revealed optimum pH of 7.0 and optimum temperature of 40^{o}C.The kinetic parameters K_m and V_{max} of the purified protease were 1.98 Mm and 59.88 mmol/ml/mim. The activity of protease enhanced in the presence of Co^{2+}, Hg^{+}, Mn^{2+}, Cu^{2+} and Mg^{2+} in concentration based dependent manner while it was slightly inhibited in the presence of Zn^{2+} and Na^{+}, Furthermore, the enzyme activities was stimulated in the presence of cysteine at all concentration investigating in all concentration dependence manner, meanwhile ascorbic acid and β-mercaptoethanol, EDTA, and urea showed serious inhibition of enzyme in concentration dependent manner. Also, protease showed the optimum activity towards casein while ammonium sulphate and agarose displayed high relative activity of 61 and 70% respectively. but it was strongly inhibited in the presence of serum albumin, gelatin and peptone. Thus, the study has also standardized the growth parameter of bacteria for the maximum production, which can be effectively used in the large scale production of protease for industrial purpose such as food, pharmaceutical and various form of biotechnological application

Authorship contribution statement

J.O. Olorunnusi: conceptualization; methodology; formal analysis; Writing- Original draft. P.J. Agbi: provided resources; writing- review and editing. V.N. Enujiugha: conceptualization; methodology; project administration; supervision; writing- Original draft, review and editing. D.M. Sanni: methodology; formal analysis; Writing- review and editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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