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Characterization of tannase-producing bacteria in cowpea *(Vigna unguiculata L.Walp)*

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Bacteria populations isolated from Cowpea were screened on tannic acid agar medium and determined by zones of hydrolysis after 48 h of incubation at 35°C with three bacteria isolates with isolate codes; DVN 5, DVN 8 and DVN 17a, identified as *Enterococcus faecalis, Enterococcus sp.* and *Staphylococcus sp.* respectively observed as having the highest zones of hydrolysis and subjected to further screening. Tannase production was carried out via submerged fermentation at 37°C and 120 rpm for 120 h. Tannase activity was measured using the Dinitrosalicyclic method and DVN 5 showed the greatest activity; its enzyme was labeled A1 and was subjected to further enzyme characterization. The optimum condition for tannase activity was determined using a number of parameters such as temperature, pH, effect of substrate concentration, effect of various cations and effect of cation concentration. Optimum conditions were therefore ascertained at a temperature of 60°C, a pH of 9.0, substrate concentration of 0.8 g and a Mg^{2+} concentration of 1 mM. The Michealis-Menten constant (Km) value for the hydrolysis of tannic acid was approximately 0.0037 mg/ml while the maximum velocity (Vmax) was approximately 9.1408. This study established that tanninolytic bacteria species can be isolated from cowpea and used to produce tannase in appreciable quantities.

Key words: Cowpea, bacteria, tannase, enzymatic activity, optimum conditions.

INTRODUCTION

Tannase (EC 3.1.1.20) or tannin-acly hydrolase is an inducible enzyme that catalyzes the hydrolysis of tannins to release gallic acid and glucose (Belur et al., 2011; Banerjee et al., 2012; Jana et al., 2013; Lekshimi et al., 2021). It is produced by plants, animals and microbes (mostly bacteria and fungi). Tannins are a group of naturally occurring astringent plant phenolic compound distributed in different parts of vascular plants (Belmares et al., 2004, Kwan et al., 2022). Tannins are the fourth most abundant plant constituents (Mansor et al., 2019) and provide antimicrobial protection to plants (Mohapatra et al., 2006; Raitanen et al., 2020). Cowpea (*Vigna unguiculata*) is a member of the *Leguminosae* family. It has relatively low-fat content and a total protein content that is two to four times greater than cereal and tuber crops. The protein in cowpea grain is rich in amino acids,

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lysine and tryptophan, compared to cereal grains.

Cowpea can be used at all stages of growth as vegetable crop and the leaves contain significant nutritional value (Nielson et al., 1993; Ahenkora et al., 1998; Kouam et al., 2018). The aim of this study was to diversify the potential use of cowpea in Nigeria for enzyme production rather than simply for consumption.

MATERIALS AND METHODS

Chemicals

The chemicals used throughout the study were of analytical reagent grade manufactured by Oxoid.

Sample collection

A total of fifty cowpea seeds were purchased from the Poka market in Epe, Lagos State. They were transported in nylon bag to the Department of Microbiology Laboratory of Augustine University, Ilara-Epe, Lagos.

Sample preparation

The Cowpea seeds were put in a plastic container and dampened by placing it under a tap in the sink of the laboratory with the tap being turned on just enough for drops of water to flow out. They were then kept in a drawer in the laboratory to allow for microbial growth and dampened again every 24 h to promote the growth of microorganisms. This was done for a week at room temperature of 27^0C.

Isolation of bacteria

The Cowpea seeds with the most observable microbial growth and spoilage were pounded using a mortar and pestle. The grounded seeds (3.68g) were dissolved in 10mls of deionized water to prepare the stock sample solution. The stock was serially diluted up to 10^{-2} and 10^{-4} and plated using both Plate Count Agar and Tryptone Soy Agar and appropriately labeled and incubated at 35^0C for 48 h. After incubation, samples were further sub-cultured into nutrient agar to produce pure bacteria isolates.

Cultural, morphological and biochemical characteristics of isolates

The isolate was sub-cultured on nutrient agar medium. Colony characteristics such as size, shape, texture, consistency and transparency were noted down. Gram staining was carried out for the isolate. Biochemical tests such as; Indole test, Methyl red /Voges-Proskauer test, Motility tests were carried out on the isolates

Sugar fermentation tests

Selected bacteria isolates were analyzed for the ability to break down certain sugars, that is; glucose, sucrose, galactose, lactose, etc.

Isolation and screening of tannase producing bacteria

Screening of tannic acid bacteria was carried out according to the method of Nadaf and Ghosh (2011), whereby pure isolates were inoculated into a defined medium containing 0.1% tannic acid as the main carbon source. Isolates 5, 8 and 17 were chosen as the best and subjected to secondary tannase production.

Secondary screening

Secondary screening was done to estimate the enzyme activity. Submerged fermentation was performed on the 3 isolates in a screening sterilized media having following composition (g/L): Tannic acid 5.0; Peptic digests of animal tissue 5.0; Sodium chloride 5.0; Beef extract 1.50 and Yeast extract 1.50. The pH was adjusted to 7.5 (+/-0.2). The medium contained 0.5% (w/v) tannic acid as sole source of carbon and acted as substrate for tannase producing bacteria (Brahmbhatt and Modi, 2015). 48-h old cultures grown in nutrient broth were inoculated into the screening media at (2% (v/v)). Erlenmeyer flasks were used to incubate the isolates at 37^0C for 5 days and were kept in a rotatory shaker (SEARCHTECH INSTRUMENTS, ZHP-100) at 120 rpm.

Tannase production

The crude enzyme was collected after intervals of every 24 h, bringing a total of 15 crude enzyme solutions, 5 solutions for each of the 3 bacteria isolate chosen; isolate AUI 5 was labeled **A1-A5** corresponding to the number of days each enzyme solution had fermented before recovery, isolate AUI 17a was labeled **B1-B5** and AUI 8 was labeled **C1-C5**. The crude enzyme broth was aseptically removed from the flask and filtered through Whatman filter paper no.4. 1.5 ml of culture filtrate was collected and subjected to centrifugation using a refrigeration centrifuge (SEARCHTECH INSTRUMENTS, TGL-16R) at 12,000 rpm for 10 min at 4^0C. The supernatant was collected as crude enzyme and used for enzyme assays.

Tannase assay

Tannase activity was determined by estimating the reduced glucose liberated using 3, 5-dinitrosalicylic acid reagent (Miller, 1959; Dhiman et al., 2022). Standard curve was studied by preparing dilutions up to 1200 g/ml of glucose solution. The activity was determined by using the supernatant obtained after centrifugation and pre-incubating it with 0.1M acetate buffer (pH 5.0) containing 0.5% tannic acid as substrate. 1 ml of crude enzyme was reacted with the equal amount of substrate which was dissolved in the buffer and incubated for 30 min at 37°C followed by incubation in boiling water bath for another min to deactivate the enzyme-substrate activity. From this 2 ml system, 1 ml was withdrawn and reacted with 3,5-dinitrosalicylic acid reagent (DNSA) and finally the system was diluted by adding 10 ml distilled water and absorbance was measured at 540 nm on spectrophotometer (Model VIS-723N). The formula to calculate activity was: Enzyme activity (U/ml) = Microgram of glucose produced / V x T, where microgram of glucose can be obtained from the standard graph, V is the aliquot of enzyme sample and T is the time of hydrolysis (Brahmbhatt and Modi, 2015). Static as well as shaking conditions were studied by Miller method. One unit of tannase activity was defined as the amount of enzyme releasing 1µmol of glucose per minute under assay conditions.

Table 1. Morphological and biochemical characteristics of bacteria strain AUI 5 (*Enterococcus faecalis).*

Isolate	Colony morphology	Gram'S stainZ	MSA	MOT	IND	S.H	URE	TDA	CAT	VP	MR	CIT	SM
AUI 5	Cocci, single orientation, round colony shape, cream pigmentation, transluscent, smooth surface	Positive	+	-	-	-	-	-	+	+	-	+	-

+ = Positive result; **-** = negative result; MSA= Mannitol salt agar; G/+ve= Growth with mannitol production; CIT = Simmon citrate test; TDA= Tryptophan deaminase test; MOT= Motility test; M.R = Methyl Red; S.H = Starch hydrolysis; V.P = Vogues Proskauer; URE = Urease test; CAT = Catalase test; IND = Indole production; SM = Skimmed milk test.
Source: Authors

Protein estimation and specific enzyme activity

Protein estimation of the enzyme samples was carried out according to Lowry et al. (1951). Absorbance was taken at 750nm; 1ml of the isolate was used. Specific enzyme activity was calculated by dividing the total enzyme activity by the protein content. It denoted the purity of the enzyme solutions. Enzyme A1 from bacteria sample AUI 5 showed the greatest activity and was subjected to further characterization tests.

Tannase characterization

The effect of various physio-chemical factors like pH, temperature, substrate concentration, cations and cation concentration on tannase activity were assayed. For evaluating the effect of the above factors on tannase activity, the bacteria culture was grown on nutrient medium (Muslim et al., 2015; Dayana et al., 2021).

Effect of temperature, pH and Substrate (Tannic Acid) Concentration on tannase activity

The effect of temperature and pH on tannase activity was measured by standard assay as described by Muslim et al. (2015) with incubation temperatures ranging from 30^0C to 75^0C while pH ranged from 4.0 to 9.0 and substrate concentrations ranged from 0.2 to 2.0%

Effect of cations and cation concentration

The effect of various cations at 1mM concentrations were studied using their water soluble salts. The cations were Cu^{2+}, K^+, Mg^{2+} and Ca^{2+}. The soluble salts of each cation were Copper (II) Sulphate, Potassium Nitrate, Magnesium Sulphate heptahydrate and Calcium Chloride respectively. Tannase activity was measured by standard assay (Muslim et al., 2015). The cation with the highest positive impact on the tannase enzyme from the cation assay was used in the cation concentration test. Concentrations were varied from 2mM to 10mM, with 1 mM used as a relative value. Tannase activity was measured by standard assay (Muslim et al., 2015).

Kinetic determination

Initial reaction rates of tannase were determined at different substrate concentrations ranging from 0.2 to 2% tannic acid. The kinetic constant (Km) was estimated following the method of Lineweaver and Burk (1934) for tannase from isolate code AUI 5 and enzyme designation A1 (Ranaldi et al., 1999).

RESULTS AND DISCUSSION

Cultural, morphological and biochemical characteristics of isolate

The Cultural, morphological and biochemical characteristics of Bacteria strain AUI 5, identified as *Enterococcus faecalis* were ascertained and the results are represented in Tables 1 and 2.

Isolation and screening of tannase producing bacteria

16 morphologically different bacteria colonies were obtained from the degraded Cowpea sample used in this investigation. Out of the 16, isolates AUI 5, 8 and 17a exhibited the highest zones of tannic acid hydrolysis, with AUI 5 and 8 having the highest at 21 mm and 20 mm respectively (Figure 1), while 17a had 13 mm. They were thus selected for secondary tannase screening to determine their enzyme activity. The isolate AUI 5 its tannase enzyme, A1, showed a specific activity of 0.0043 U/mg.

Tannase assay

The result of this investigation revealed that bacteria isolates from Cowpea produced tannase. The investigation revealed tannase production after 120 h incubation from all three chosen isolates for secondary screening (Figure 2).

This is supported by Schons et al. (2012) who also reported tannase production after 120 h. Enzyme isolates

Table 2. Sugar fermentation test results of bacteria strain AUI 5.

Isolate	GLU	FRU	GAL	MAL	SUC	LAC	MANT	D-XYL	GLY	TRE	RAF	MAN	SAL	ARA
AUI 5	A	A	A	-	+	-	A+	A+	A+	A+	-	A+	A+	A+

GLU = Glucose - = neither acid nor gas produced; GAL = Galactose; A = Acid produced only; FRU = Fructose; A+ = Acid produced (durham tubes absent); MAL = Maltose; + = Acid and Gas Production; SUC = Sucrose; TRE = Trehalose; LAC = Lactose; RAF = D-Raffinose; MANT = Mannitol; M AN = D-Mannose; D-XYL = D(+) Xylose; SAL = Salicin; GLY = Glycerol; ARA = L-arabinose.
Source: Authors.

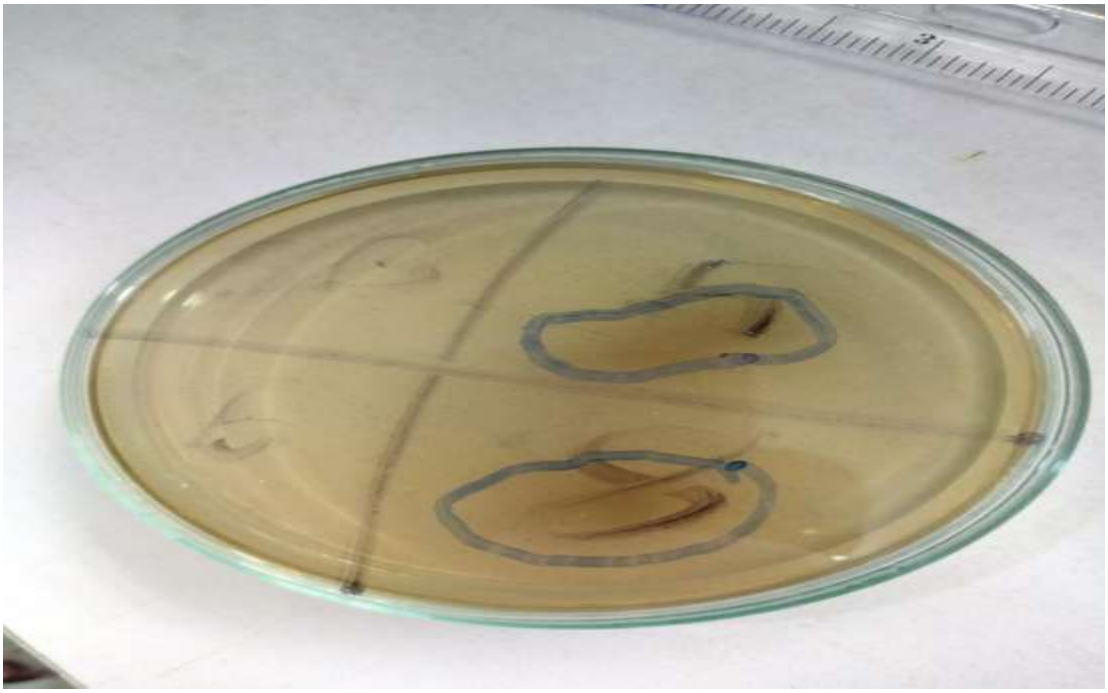

Figure 1. Marked zones of tannic acid hydrolysis for AUI 5 (above) and AUI 8.
Source: Authors.

'A' and 'C' showed maximum tannase activity within 24-72 h whereas 'B' showed maximum activity at 24 and 120 h. Brahmbhatt et al. (2015) reported maximum tannase production within 72-96 h. Of all 15 enzyme solutions from A1 to C5, A1 produced the highest total activity (32.194 U/ml) and highest protein estimate (7402 µg/ml) (Table 3), and thus, had the highest specific activity. It was thus chosen for further tannase characterization tests.

Effect of temperature, pH and substrate (tannic acid) concentration on tannase activity

The optimum temperature for tannase in this study was at 60^0C (Figure 3). Previous reports on temperature optima for tannase were between 20 to 60^0C depending on the microbial source (Sharma et al., 1999; Mondal et al., 2001; Kaziecka-Burnecka et al., 2007; de Lima et al., 2022). Brahmbhatt et al. (2015) and Selwal et al. (2010) both reported tannase optima at 37°C. Kumar et al. (1999) reported maximum tannase activity at 30^0C. Mondal et al. (2001) reported an optimum tannase activity at 40^0C. Sabu et al. (2006) reported maximum tannase activity at 30°C. Das Mohapatra et al. (2006) reported maximum tannase activity at 35°C. de Lima et al. (2022) reported optimum activity at 30°C and 80% activity at 40 to 60°C. Amitabh et al. (2018) reported an optimal temperature range of 30 to 70°C. Liu et al. (2018) reported an optimum temperature of 40^0C.

The optimum pH for tannase in this study was at 9.0 (Figure 4) showing a preference for alkaline environment, Belur et al. (2010) reported optimum tannase activity at pH 8.9, while Iwamoto et al. (2008) reported optimum

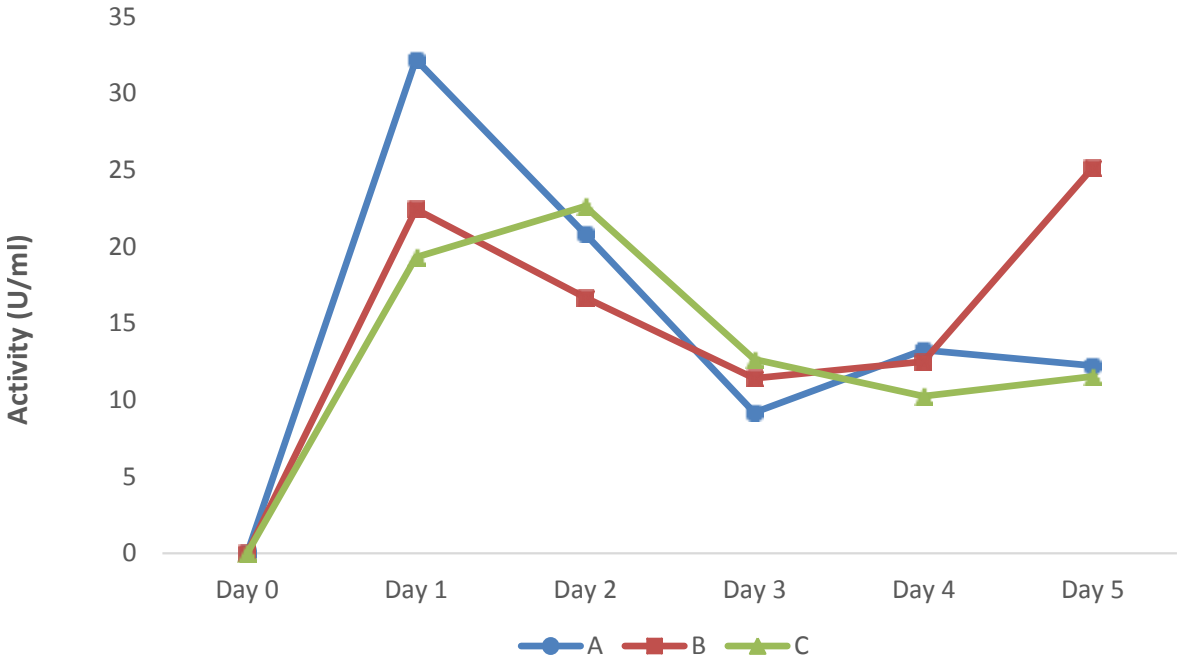

Figure 2. Total activity of tannase solutions in five days using submerged fermentation.
Source: Authors.

Table 3. Tannase assay results.

Enzyme code	Total activity (U/ml)	Protein (µg/ml)	Specific activity (U/mg protein)
A1	32.194	7402	0.0043

Source: Authors.

tannase activity at 8.0, however, generally, tannase exhibits optimum activity at acidic pH (Yao, et al., 2013), and several investigations have found tannase to be optimum at acidic pH range (Sharma et al., 1999; Mondal et al., 2000; Van de Lagemaat and Pyle, 2005; Sharma et al., 2011; Abdel-Nabey et al., 2011). Selwal et al. (2010) reported optimum pH of 5.5. Mondal et al. (2001) reported an optimum pH of 5.0. Ayed and Hamdi (2002) reported optimum pH at 6.0. Belur et al. (2010) reported optimum pH at 6.0. de Lima et al. (2022) reported optimum pH at 7.0. Amitabh et al. (2018) documented an optimal pH range of 3-10. Liu et al, (2018) reported an optimum pH at 7.0

The optimum tannic acid concentration for the enzyme in this study was 0.8% (w/v) (Figure 5). Abdel-Nabey et al. (2011) reported optimum tannase concentration to be at 2.0% (w/v). Seth and Chand (2000) reported maximum tannase activity at 3.5% (w/v). Ayed and Hamdi (2002) reported optimum tannase activity at 1.5% (w/v). Kanpiengjai et al. (2019) reported tannase activity at 1.0% (w/v).

Effect of cations and cation concentration

The effects of 4 cations ; Mg^{2+}, K^{+}, Cu^{2+} and Ca^{2+} using their soluble salts ($MgSO_4.7H_2O$, KNO_3, $CuSO_4$ and $CaCl_2$ respectively) (Figure 6) on the crude enzyme were estimated, Mg^{2+} was shown to promote the tannase activity the most, and thus was subjected to the cation concentration test.

K^{+} also promoted its activity whereas Ca^{2+}, Cu^{2+} inhibited it. Ca^{2+} has been reported as a tannase stimulator, which contradicts the investigation (Beniwal et al., 2010), Calcium Chloride (CaCl2) which was the soluble salt used in this study has been reported an a tannase inhibitor alongside Cu^{2+} which supports this study (Sabu et al., 2005; Wright 2005, Liu et al., 2018), which might explain why the enzyme was inhibited despite the presence of the calcium cation. K^{+} has been reported to stimulate tannase activity (Sabu et al., 2005; Wright, 2005). Magnesium was shown to have the maximum effect at 1 mM concentration in this investigation (Figure 7).

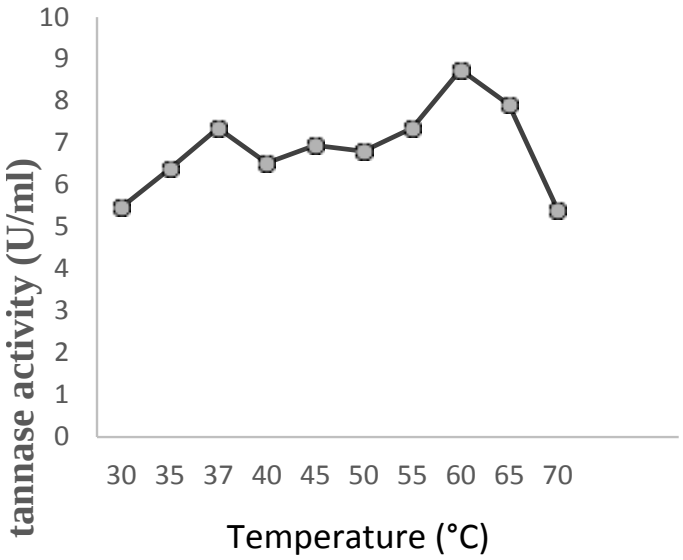

Figure 3. Effect of temperature on tannase activity.
Source: Authors.

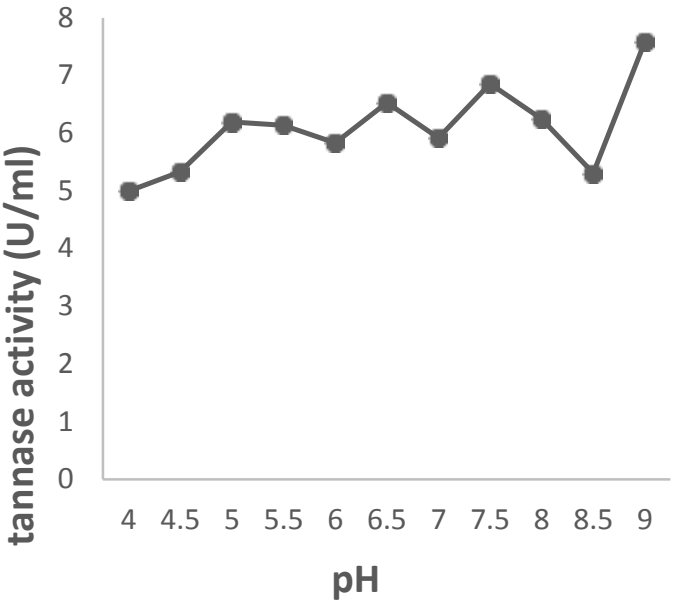

Figure 4. Effect of pH on tannase activity.
Source: Authors.

Kinetic determination

The apparent Km and Vmax value for the hydrolysis of tannic acid by tannase produced from enzyme A1 were 0.0037 and 9.1408 respectively. The values were calculated using the Lineweaver-Burk plot (Figure 8) based on the method of Lineweaver and Burk (1934).

Conclusion

Tannic acid media was used to isolate the bacteria having natural ability to degrade hydrolysable tannins by producing tannase. 16 bacteria strains were isolated from various tannin rich soil samples. Among them, three strains; AUI 5, AUI 8, and AUI 17a (*Enterococcus*

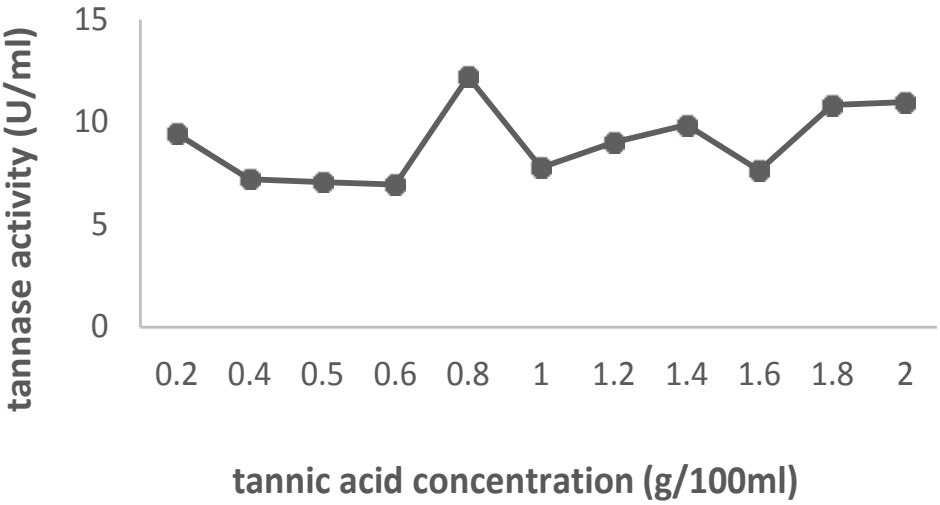

Figure 5. Effect of substrate concentration on tannase.
Source: Authors.

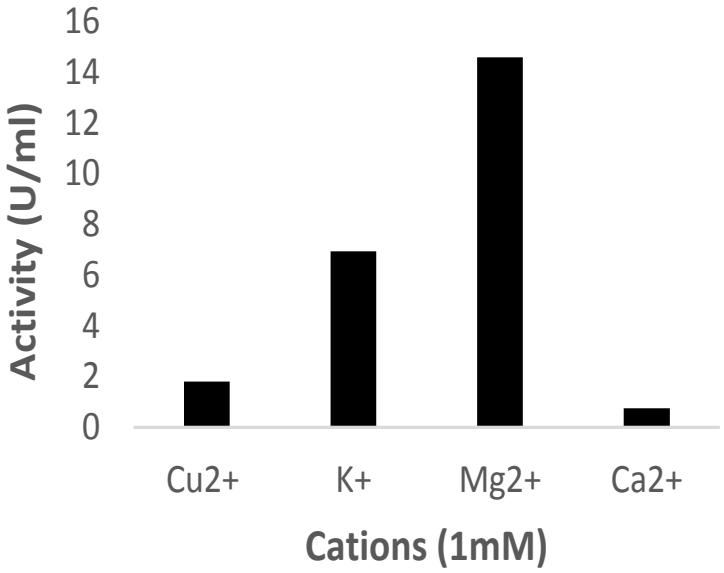

Figure 6. Effect of different cations on tannase activity (tannic acid).
Source: Authors.

faecalis, Enterococcus sp. and *Staphylococcus sp.* respectively) could hydrolyze tannin and were subjected to secondary tannase screening. The bacteria isolates were identified by morphological and biochemical characteristics. Strain AUI 5 with enzyme AI was selected as the most effective. In this investigation, it showed a tannase activity of 0.0043 U/mg protein after 24 h of incubation. The effect of various environmental factors on enzyme activity was carried out by growing the organism in varying conditions. This research work has established the fact that Cowpea (*Vigna unguiculata* L. Walp) is host to a number of tannase-producing bacteria from which the enzyme can be produced commercially. The optimum conditions from the tannase solution chosen has been determined as; 0.8%, 6°C, pH 9.0, 1mM Mg2+ for substrate concentration, temperature. It had a Km value of 0.0037 and a Vmax value of 9.1408. The low KM value indicates a high affinity by the enzyme for tannic acid.

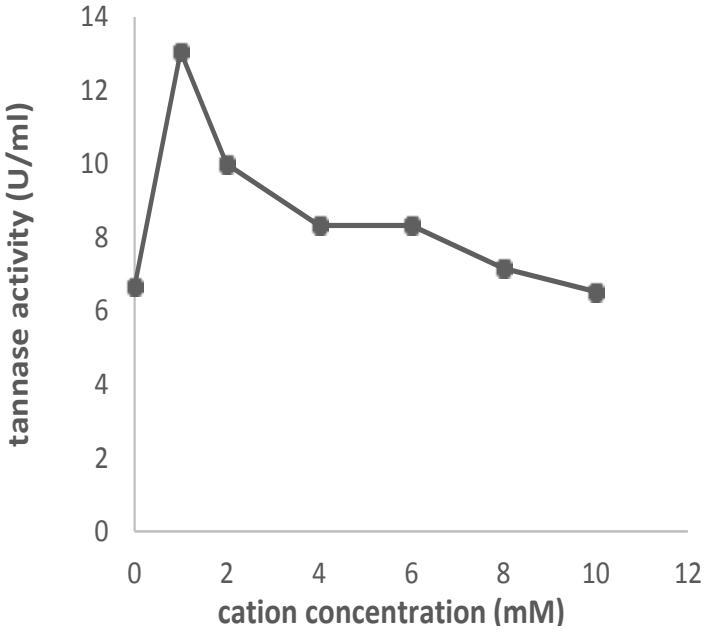

Figure 7. Effect on Mg^{2+} concentration on tannase.
Source: Authors.

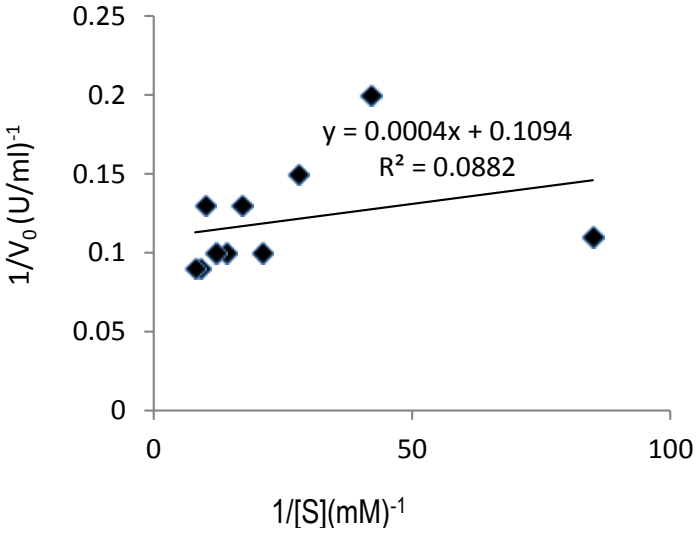

Figure 8. Lineweaver-burk plot for tannic acid hydrolysis activity.
Source: Authors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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