Geo-Biochemical properties of peroxidase extracted from *Cola nitida* At the Ninth Year of Planting.

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Abstract

Background: This study reports the biochemical properties of plant peroxidase from white cultivars of *Cola nitida* for sustained biotechnological applications.
Methods: Peroxidase was extracted from *Cola nitida,* in 20 mM sodium phosphate buffer pH 7.0 containing 10% glycerol, 1% PEG, , 0.015% citric acid, 0.010% L-Cysteine and 0.010% ascorbic acid Buffer C). The extracted peroxide was subjected to a step wise purification stages which separated it into two peaks. The native molecular weight was determined for the major peak to be 21 ± 1.16 kDa while the subunit was estimated to be 15.5 ± 2.1 kDa.
Results: Optimum pH and temperature was studied to be 4.0 and 40 °C respectively. Further studies required investigating several methods of immobilization on the nine years harvested *Cola nitida* in order to enhance its biotechnological applications.
Conclusion: However, this study was able to establish that *Cola nitida* is a strong alternative to the commonly purchased horseradish peroxidase.

Propriétés géo-biochimiques de la peroxydase extraite de Cola nitida à la neuvième année de plantation

Résumé
Objectif : Cette étude rapporte les propriétés biochimiques de la peroxydase végétale provenant de cultivars blancs de Cola nitida pour des applications biotechnologiques soutenues.
Méthode: La peroxydase a été extraite de Cola nitida, dans un tampon phosphate de sodium 20 mM pH 7,0 contenant 10 % de glycérol, 1 % de PEG, 0,015 % d'acide citrique, 0,010 % de L-cystéine et 0,010 % de tampon d'acide ascorbique C). Le peroxyde extrait a été soumis à des étapes de purification par étapes qui l'ont séparé en deux pics. Le poids moléculaire natif a été déterminé pour le pic principal à 21 ± 1,16 kDa tandis que la sous-unité a été estimée à 15,5 ± 2,1 kDa.
Résultat: Le pH et la température optimaux ont été étudiés comme étant respectivement de 4,0 et 40 °C. Des études complémentaires ont nécessité l'étude de plusieurs méthodes d'immobilisation sur les neuf années de récolte de Cola nitida afin d'améliorer ses applications biotechnologiques.
Conclusion: Cependant, cette étude a pu établir que Cola nitida est une alternative solide à la peroxydase de raifort couramment achetée.
Mots-clés : Cola nitida, efficacité catalytique, applications biotechnologiques

INTRODUCTION

Peroxidases (PODs, E.C. 1.11.1.7) are haemoproteins that are known to catalyse the oxidation of large varieties of substrates through a reaction with hydrogen peroxide. Peroxidase are abundant in nature and have been found in all living organisms where they are involved in a variety of biological processes (Kalsoom, Bhatti & Asgher, 2015). It is a key enzyme that controls plant differentiation and development and has great functions in the construction, rigidification and eventual lignification of cell walls, in the protection of plant tissues from damage as well as infection by pathogenic microorganisms, and similarly in wound healing (Gorton, 1995; Shigeto and Tsutsumi, 2016). Peroxidases are used also in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals.

Presently, the major source of commercially available peroxidase is roots of horseradish (*Armoracia rusticana*) which are widely used in clinical biochemistry and enzyme immunoassays (Veillette.,Coutu, Richard,.,Batraville, Désormeaux. ,Roger & ,Finzi , 2014). Other applications of peroxidases suggested include treatment of waste water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from materials such as food stuffs and industrial wastes (Agostini, Medina, , Silvia, Forchetti, & Tigier 1997).

Peroxidase is reported to exist in both soluble and membrane-bound forms (Robinson, 1991a), and is involved in many plant functions such as hormone regulation defence mechanisms, indole acetic acid degradation during maturation senescence of fruits vegetables and lignin biosynthesis (Thongsook and Barrett, 2005). Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants (Estrada, Bernal, Dı´az, Pomar, & Merino 2000).

Kola nut occupies a unique place among West Africans as it is widely consumed by Africans. It is of particular importance in the social life and religious customs of people in the tropics of West Africa. Kolanut belongs to the family Sterculiaceae, having about 125 species of trees native to the tropical rainforests of Africa. Of these species, the most common in Nigeria are *Cola nitida* (Goro) with two phenotypic varieties: the white and red cultivars, *Cola acuminata* (Abata) and *Garcina cola* (Orogbo) and *Buchholzia coriacea* popularly known as wonderful kola, but this study focused solely on *Cola nitida* (white cultivars only). Nigeria alone accounts for about 70% of the total world production of kolanuts (Adebisi, 2004). About 90% of the kola produced in Nigeria is consumed within the country while 10% is exported (Adebisi, 2004).

Rationale / Statement of Research Problem: The unaffordable cost of horseradish peroxidase and its effect on our economy necessitated the need to search for alternative source of peroxidase which is equally local to our environment.

Specific Objectives

- Extraction of peroxidase levels activities in *Cola nitida*
- Estimating peroxidase activity in Cola nitida
- Estimating protein concentration
- Purification of peroxidase
- Native and subunit molecular weight determination
- Effect of pH and temperature studies

MATERIALS AND METHODS

Materials

Kolanut (*Cola nitida*) white and red cultivar was purchased from a local farmer at Ilesa, Osun State.

Chemicals: Bovine serum albumin (BSA), o-dianisidine, hydrogen peroxidase, Coomassie brilliant blue R-250, trizma base, trizma acid, HCL were purchased from Sigma chemical company. St. Loius, USA. Disodium hydrogen phosphate, monosodium dihydrogen phosphate, methanol, ethanol, acetic acid were obtained from BDH chemicals Ltd. Poole, England. Sephadex G-100 was purchased from GE Healthcare Bio-sciences, Uppsala, Sweden. Molecular weight standard for SDS-PAGE was obtained from Carl Roth GmbH Karlsruhe, Germany. Other chemicals were of analytical grade and were obtained from reputable chemical suppliers.

Methods

Extraction of peroxidase: 50g of neatly cut and washed *Cola nitida* (White & Red cultivars) was spinned at 15000 rpm for 20 mins and peroxidase (clear supernatant) was extracted using three different buffers following the procedure established by Adewale and Adekunle, 2018. The first buffer contained only 10 mM phosphate buffer at pH 6.0 (Buffer A), while the second buffer was 20 mM Tris-HCl pH 7.0 containing 10% glycerol (Buffer B) and third was Tris-HCl

buffer pH 7.0 containing 10% glycerol, 1% PEG, 0.015% citric acid, 0.010% L-Cysteine and 0.010% ascorbic acid (Buffer C). Extraction of peroxidase from kolanut with buffer C, gave the highest amount of peroxidase and was purified as stated.

Peroxidase activity assay: Peroxidase activity in crude, partially purified and purified enzyme was routinely determined using the method of Kay *et al*., 1967. The standard assay mixture contained 1mM hydrogen peroxide in final concentration, 0.25mM o-dianisidine, 0.1M of sodium phosphate buffer pH 6.5 and an aliquot of the enzyme gives a change in absorbance of 0.02-0.07 per minute at 460 nm due to the oxidation of o-dianisidine in the presence of hydrogen peroxide.

One unit of activity is defined as the amount of enzyme that oxidizes 1 µmol o-dianisidine/min. (ε_{460} = 11.3 mM^{-1} cm^{-1}). Peroxidase activity was calculated using the formula below:

$$\text{Activity (µmole/min)} = \frac{\triangle OD \times V \times d.f}{\varepsilon \times v}$$

Determination of the protein concentration: The protein concentration in the crude homogenate and purified peroxidase was determined following the method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein.

Enzyme purification

Enzyme purification: Two peaks of peroxidase activity were observed on gel filtration with a recovery percentage of 46 and 3% respectively and a purification fold of 2.

Purification of the crude homogenate by Aqueous Two-Phase Partitioning (ATPs): The crude peroxidase supernatant was subjected to aqueous two-phase partitioning (ATP) in which fifty seven millilitres (57 ml) in the supernatant of *Cola nitida* equivalent to 10 mg of protein was used to dissolve poly ethyleneglycol 6000 (PEG) (24%, w:v), ammonium sulphate (7.5%, w:v) and NaCl (2%, w:v). The mixture was incubated on ice for 3 hr to achieve phase separation according to Adewale and Adekunle, 2018. The upper phase and the lower phase were collected in separate tubes and each was assayed for peroxidase activity and protein concentration. The salt rich lower phase has higher percentage of enzyme activity and was therefore dialyzed against buffer C for 6 hr at 4 °C to remove salts.

Purification by gel filtration chromatography on Sephadex G-100: The dialyzed peroxidase protein (partially purified) from aqueous two phase partitioning was further subjected to gel filtration chromatography on Sephadex-G-100 column (1cm x 50cm) previously equilibrated with 20 mM phosphate buffer, pH 7.0 containing 10% glycerol. Elution was done with equilibration buffer at a flow rate of 15 ml/hr. Fractions containing peroxidase activity were pooled and dialyzed for characterization.

Determination of native and subunit molecular weight: Native molecular weight was determined on Sephadex G-100. The column was calibrated with Sephadex G-100

The void volume (V_o) of (1 × 50 cm column) was determined using blue dextran (2 mg/ml). Total volume (V_t) of the column was calculated Bovine Serum Albumin (67 kDa), ovalbumin (45 kDa), peroxidase (40 kDa), chymotrypsinogen A(25 kDa) and lysozyme (14.4 kDa). The native molecular weight of peroxidase from *Cola nitida* was obtained by interpolation of K_{AV} value on the standard curve. Subunit molecular weight was determined on SDS-polyacrylamide gel of 12% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel, according to the method of Laemmli, 1970. The subunit molecular weights of the purified peroxidase were obtained by interpolation of the R_m value on the standard curve.

RESULTS

Levels of peroxidase activities in different kolanut species

Figure 1-4 is a summary of the levels of peroxidase activity in crude extract of *Cola nitida* (White cultivars) harvested at different years with their respective specific activities. Highest peroxidase concentration was found in *Cola nitida* (white cultivar) harvested at the seventh year of planting when extracted with 20 mM Tris-HCl buffer pH 7.0 containing 10% glycerol, 1% PEG, 0.015% citric acid, 0.01% L-Cysteine and 0.010% ascorbic acid (Buffer C). Figure two however is the profile of the expressed levels of peroxidase activity in the crude homogenate of *Cola nitida* (Red cultivars) with a reduced specific activity when compared with white cultivars. The peroxidase activity of *Cola acuminata* is shown in figure 3 with great reduction in the expressed peroxidase activity at the same year of harvesting. It was observed that for all species of kolanut studied, highest amount of peroxidase was obtained at the ninth year of planting, beyond which there was a great reduction in the amount of peroxidase.. Figure 4

is the profile showing the relative abundance of all species of kolanut at their different obtained specific activity.

Native and subunit molecular weight determination

Polyacrylamide gel electrophoresis of peroxidase from *C.nitida* (being the kolanut with highest amount of peroxidase activity) in the presence of SDS gave a very faint single band equivalent to 15.5 ± 2.1 kDa. The molecular weight of the native enzyme was estimated to be 21 ± 1.16 kDa

Native Molecular Weight Determination by Gel-Filtration on Sephadex G-100

The native molecular weight for purified white *C. nitida* peroxidase was 37.11 ±2.5 kDa.. This value was obtained through a plot of log of molecular weight against K_{av} (Fig 4.3.2a).

Effect of Temperature on the activity of peroxidase

The optimum temperature for white *C. nitida* peroxidase and red *C. nitida* isoenzyme A and B was 37 °C and the estimated activation energy for the purified Cola nitida peroxidase is 1.9 KCal/mol,

Effect of pH on the Activity of Purified Peroxidase from *C. nitida* Nuts

The optimum pH for the peroxidase isoenzymes is shown in Fig 4. Optimum pH for peroxidase from *C. nitida* was 4.5.

DISCUSSION

Studies on years of planting of the three species of kolanut proofed that maximum quantity/ amount of peroxidase was obtained at the ninth year of planting. The implication of this is that kolanut harvested after the ninth year of planting is more favourable in terms of peroxidase quantity.

The extraction of peroxidase from *Cola nitida* gave higher amount of peroxidase in comparism with other species and the white cultivars produced more peroxidase when compared with the red cultivars. The obtained quantity is about seven fold increment and this is in line with the result of Cheng *et al*., 2006.

Purification scheme of Adewale and Adekunle, 2018 was followed because it combined purification and concentration processess. So also, the possibility that the method employed for purification have removed the polyphenols present in *Cola nitida* by Poly ethylene glycol (PEG) as explained by Cheng *et al*., 2006. The purification scheme adopted was able to purify all homogenates of *Cola nitida* in good yield to a condition of homogeneity. Native molecular weight obtained for kolanut peroxidase is 21 ± 1.16 kDa while the subunit molecular weight is 15.5 ± 2.1 kDa. Further studies would be to characterize this kolanut peroxidase, identify their kinetic properties and also immobilization characteristics.

The native molecular weights of purified peroxidase from *C. nitida* estimated using calibrated Sephadex G-100 gel filtration chromatography was 37.11 ±2.5 kDa. The subunit molecular weight of white *C. nitida* peroxidase was estimated to be 14 kDa and 17 kDa. On comparison, this reveals that peroxidase from *C. nitida* is an heterodimeric protein. The native molecular weight of peroxidase from *Caralluma umbellata* is 42 kDa (Achar *et al*., 2016). The differences in the molecular weights may be due to differences in tissue distribution and cellular location.

The performance of enzyme is generally affected by temperature, the more reason for the study of effect of temperature on the *C.nitida* peroxidase. With an initial increase in temperature, peroxidase specific activity increases just because the enzyme active site assumes its more favourable structure with increase in the kinetic energy of the molecules thus leading to enhanced interaction between enzyme active sites and substrate molecules, until an optimum temperature (37°C) is reached at which the enzyme shows a maximum activity.

The activity of peroxidase is significantly affected by conformation of enzyme active site because enzymes initiate their catalytic actions by physically and chemically attaching the substrate at their active sites, and the shape of active site are, therefore, of vital importance. Any fluctuation in pH is expected to cause changes in the shape of enzyme active site by changing ionic state of amino acids and thus leading to enzyme activity reduction (Campbell *et al*., 2008). Peroxidase from *Cola nitida* has an optimum pH of 4.5.

CONCLUSION

Cola nitida (White cultivars) harvested at the ninth year of planting as studied contain highest amount of peroxidase and could serve as a cost effective source of this enzyme. Further studies on characterization and immobilization will enhance the biotechnological uses of this enzyme.

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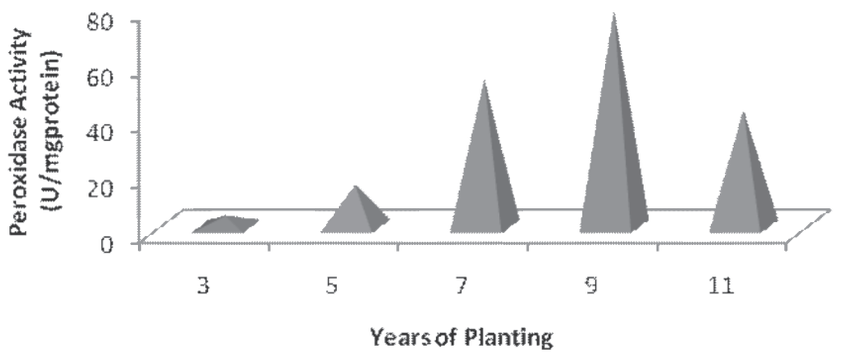


Fig 1: Relative amount of peroxidase in *C. nitida* (White cultivar) with respect to their years of planting

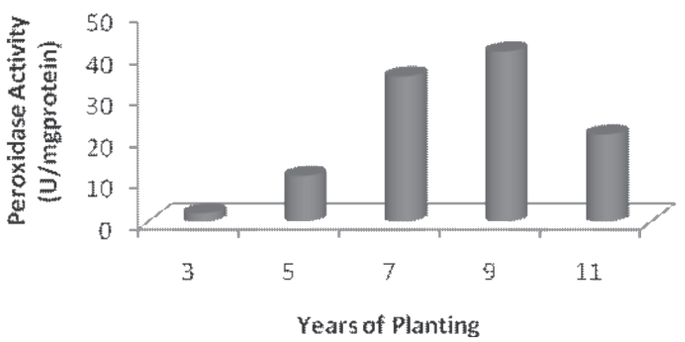


Fig 2: Relative amount of peroxidase in *C. nitida* (Red cultivar) with respect to their years of planting

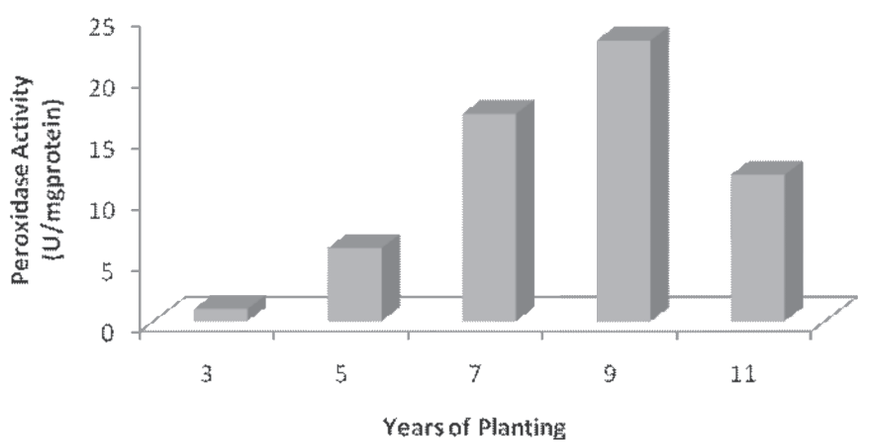


Fig 3: Relative amount of peroxidase in *C. acuminata* with respect to their years of planting

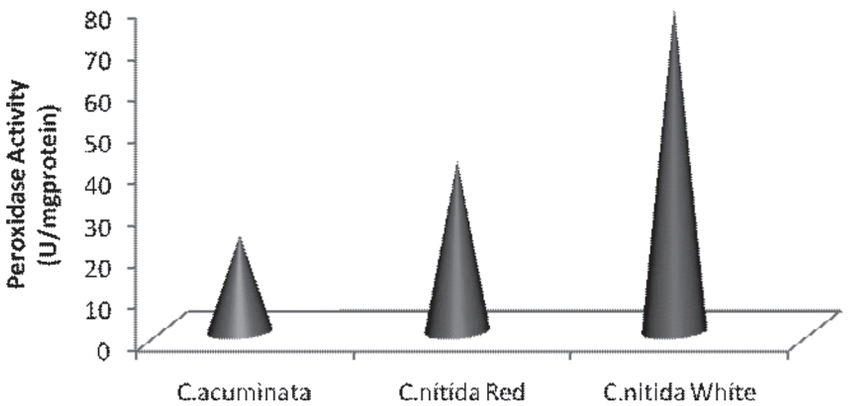
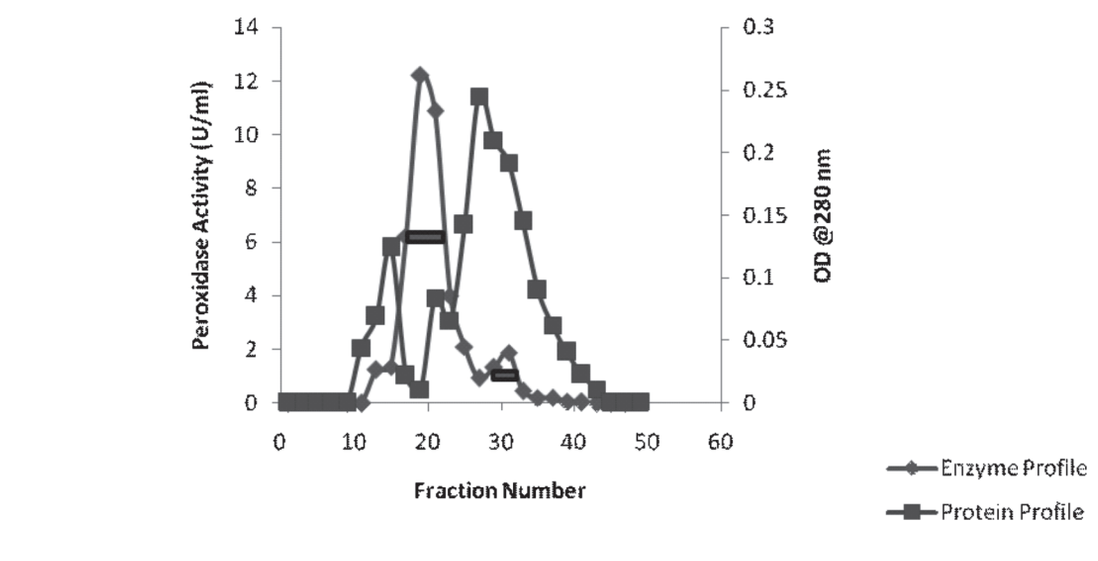


Fig. 4: Relative amount of peroxidase activity in different species of kolanut



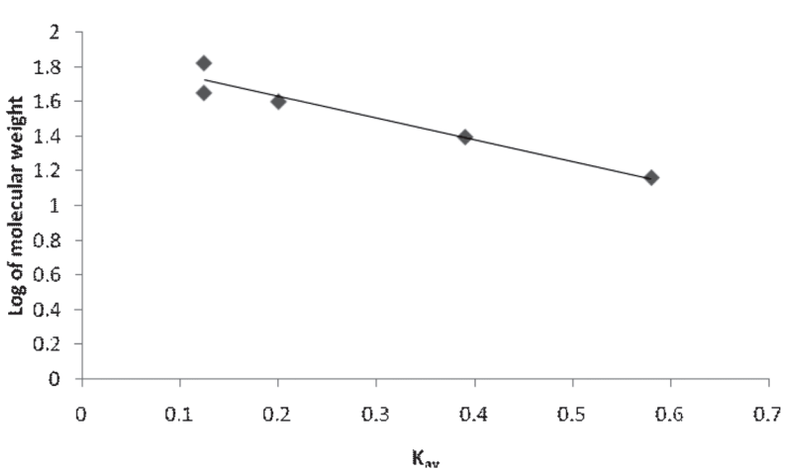


Figure 2: Plot of Log of molecular weight against K_{AV}

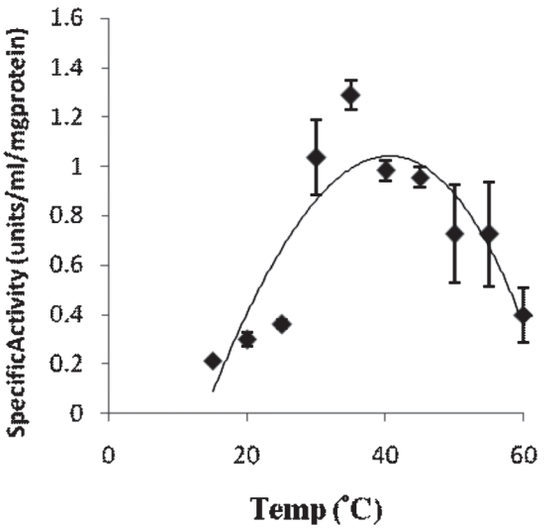


Figure 3: Plot of specific activity against temperature

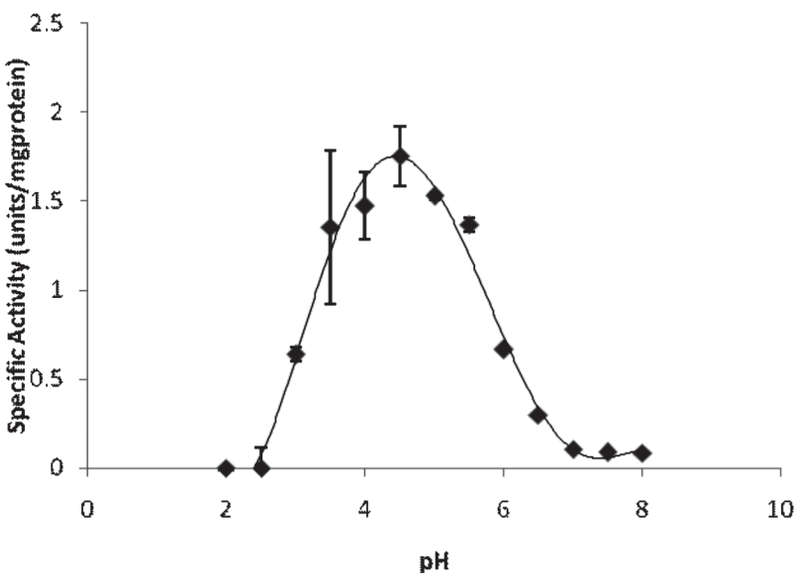


Figure 4: Plot of specific activity against pH