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(RESEARCH ARTICLE)

Isolation, partial purification and immobilization of locally sourced tyrosinase on different fiber materials

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Abstract

Tyrosinase has diverse functions in biological systems including melanin synthesis for defense against harmful effects of ultraviolet light. It oxidizes tyrosine especiallyL-3, 4-dihydroxyphenylalanineto L-Dopaquinone. The objective is to isolate, partially purify and immobilize tyrosinase on coconut fiber and palm wood chips using gum Arabic as binder. Yam (*Dioscorea rotundata*) was used as source of tyrosinase. It was extracted by mashing yam with potassium phosphate buffer (P^H 7). The slurry was centrifuged, supernatant decanted and mixed with solid ammonium sulphate [$(NH_4)_2SO_4$] for partial purification of the enzyme. Its kinetic parameters were determined spectrophotometrically by measuring its activity at varying concentrations of substrate (L-DOPA). Line weaver-bulk double reciprocal plot was plotted to derive Michealis-Menten constant (K_m) and maximum velocity (V_{max}) while the optimum temperature and P^H was determined by varying temperature and P^H ranges (5.5-8.5) of reaction system. The immobilized tyrosinase activitywas compared with that of partially purified enzyme. Results showed that, crude enzyme activity was 7.2 micromoles/min, immobilized enzymes activity on coconut fiber was7.7micromoles/min and that on palm wood chip was 11.1micromoles/min. K_m and V_{max} of partially purified tyrosinase was 33.3mM and $0.016S^{-1}$, that of immobilized enzyme on coconut fiber was 28.5mM and $0.020s^{-1}$ while that on palm wood chips was 20.8mM and $0.033s^{-1}$ respectively. Optimum P^H and temperature for partially purified and immobilized enzymes was 6.5 and 55^0C. Immobilization of yam tyrosinase on palm wood chips increased the activity of partially purified enzyme by 15%. Conclusively, palm wood chip is considered a better support to coconut fiber for immobilization of yam tyrosinase.

Keywords: Yam; Tyrosinase; Immobilization; Palm wood chips; Coconut fiber

1. Introduction

Tyrosinase (polyphenol oxidase (EC 1.14.18.1)) are multi-copper containing biocatalyst that bind specific substrate such as tyrosine, particularly L-DOPA (L-3,4-dihydroxyphenylalanine) and convert it to L-dopaquinone. It is the enzyme that catalyzes the committed step in melanin biosynthesis. It is widely distributed in animals, plants and micro-organism [1] and has been isolated and purified from a number of plants and animal sources [2]. The rough endoplasmic reticulum in biological cells such as in man is the site for synthesizing tyrosinase and tyrosinase-like proteins. After synthesis, the nascent tyrosinase is transported via the golgi apparatus, and targeted to small vesicles that pass through the endosomal-lysosomal compartment and fuse with melanosomes [3].

Tyrosinases are exploited for a variety of biotechnological and environmental applications and thus have attracted various groups actively engaged in molecular characterization and bioengineering studies [4]. The application of tyrosinase in cereal processing has been well studied as it can catalyze oxidation of phenolic compounds present in cereal-proteins and polysaccharides by either producing linkages between polysaccharides or proteins themselves. In dairy products, cross-linking can be exploited for prevention of synergies to make a soft texture firmer. Hetero-cross-

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linking in cereal, milk and meat biopolymers provide a probable means to produce novel food products with precise functionalities and characteristics [5]**.** The enzyme is capable of oxidizing tyrosine residues in proteins to the corresponding quinones, which can further react with free sulfhydryl (thiol) and/or amino groups resulting in formation of tyrosine-cysteine and tyrosine-lysine cross links. Quinones have also been suggested to form tyrosine-tyrosine linkages by coupling together. More recently tyrosinase has been used to graft antioxidant phenolic substrates like caffeic acid and chlorogenic acid onto wool protein substrate. The efficiency of phenolic substrate enzymatic grafting and the antioxidant property of functionalized wool fibers are being assessed [6]. This is why the search to isolate, purify tyrosinase from other sources such as food components have intensified in recent times. The enzyme is also implicated in neurodegenerative abnormally, such as Parkinson's disease, facilitate nerve cells growth and also cause browning reactions which are of importance to the cosmetics and food industries [7].

Experiments have also shown that immobilized tyrosinase is much more stable and reusable than the free tyrosinase in storage and that the immobilized tyrosinase could even retain about 78% of its original activity after repeated use of six times in a batch system. Nowadays, immobilized enzymes are preferred over their free counterpart due to their prolonged availability that curtails redundant downstream and purification processes [8].

Natural fibers offer an excellent environment for the growth of micro-organisms when conditions like moisture, oxygen and temperature are appropriate. Coating with collagen, a very useful biomaterial with bactericidal and fungicidal properties could be used to improve the properties of fiber materials, when applied in hygienically sensitive applications [9]. Tyrosinases when immobilized on fiber material, catalyzes the oxidation of tyrosine residues in fiber (e.g. wool) as model substrates, this is determined by UV-VIS spectroscopy [10].

The functional and mechanical properties of treated fiber such as wool when analyzed were shown to have no significant alterations [10]. The immobilization of tyrosinase on pulverized natural fiber calcium (egg shell powder) coated with polyethylene imine (PEI) showed that desorption of tyrosinases from this support medium under study showed negligible indication that the attachment to such a support is strong. The PEI imparts negative charge onto the egg shell powder as it is a polycationic reagent and tyrosinase coupled support was then cross linked by glutaraldehyde solution which makes the immobilization of tyrosinase to be strong [11]

The immobilization of tyrosinase of edible mushroom on to this pulverized natural fiber calcium (egg shell powder) coated with PEI, significantly minimize the leakage of the enzyme from the support. It helps to maximize the access of the substrate to the active site of the enzyme [12].

2. Material and methods

The list of materials, reagents and equipment used for the work includes: yam(source of tyrosinase), fiber materials (coconut fiber and palm wood chips), Gum Arabic, 50mM potassium phosphate buffer (P^H 7.0), Varying concentrations of L-DOPA, Ice, Mortar and pestle, Buchner Funnel, Cheese Cloth, centrifuge and UV-VIS Spectrophotometer.

2.1. Procurement of Materials and Reagent

Yam (*Discoreaspp*) the source of the enzyme was obtained from Gboko main market. Coconut fiber and palm wood chips were cut from the bush around Mkar village. Gum Arabic was purchased from foremost Daires limited, Lagos, Nigeria. L-DOPA was purchased from weyland nutrition, United States of America. All other reagents and equipment were obtained from the chemical science laboratory of University of Mkar, Mkar Benue State Nigeria.

2.2. Preparation of Phosphate Buffer

50mM potassium Phosphate buffer was prepared using potassium di-hydrogen phosphate, $KH_2PO_4$and di-potassium hydrogen phosphate, K_2HPO_4. 50mM phosphate buffer was prepared by dissolving 6.39g of KH_2PO_4 and 0.52g of K_2HPO_4 in 1000mls of distilled water.

2.3. Enzyme Extraction and Purification

100g of yam was placed in a mortar and 120mls of 50mM phosphate buffer (P^H 7.0) was added. The combination was crushed thoroughly, packed in cheese-cloth and filtered using the Buchner funnel. The filtrate was then centrifuge at 4000 rpm for 15minutes. The supernatant was decanted and placed on ice. The isolated tyrosinase was partially purified using ammonium sulphate, $[(NH_4)_2SO_4]$. Solid ammonium sulphate was added to the extract (placed on ice) and stirred to equilibrate for 45 minutes. The mixture was then centrifuged at 1,500 rpm for 15 minutes. The pellets in the tube

were collected and 60ml phosphate buffer (P^H 7.0) was added to it. The mixture was again spine at 1,500 rpm for 15 minutes and the supernatant collected in a beaker placed on ice. This mixture contained the enzyme.

2.4. Enzyme activity assay

The enzyme was assayed spectrophotometrically by measuring the rate of conversion of 3, 4-dihydroxyphenylalanine (DOPA) to DOPA-chrome. The reaction mixture contained 1.0ml of 50mM L-DOPA as substrate, with 0.1ml of the enzyme extract. Final volume of reaction mixture was made to 5.0ml using phosphate buffer. The blank solution contained all the other components except the substrate. The enzyme activity was determined by measuring the absorbance at 475nm from 1-4 minutes.

Tyrosinase activity was computed by measuring the slope of the initial linear portion of the curve of absorbance against time. This value is equal to the rate of enzyme activity, V. The value obtained was then converted to M/min (i.e. change in concentration of DOPA-chrome produced per minute) using the Beer-Lambert Law ($A=\varepsilon cl$), and solving for C=concentration of DOPA-chrome produced. Where:

A= Absorbance Per minute,$\varepsilon = molar\ absorptivity\ of$ DOPA − chrome $at\ 475nm = 3600M^{-1}$cm^{-1}

l=path length =1cm.

The value was further converted to micromoles/minutes by multiplying the figure obtained by 1000,000.

2.5. Enzyme kinetics

The reaction rate of tyrosinase at a series of substrate concentration was carried out. 2.0mls of L-DOPA at varying concentrations (5, 10, 15 and 20mM) was taken with 1.0ml of phosphate buffer (P^H 7) immediately after the addition of enzyme extract and the absorbance readings were recorded. The reaction rate versus substrate concentration was plotted to fit the Michealis-Menten equation using line weaver bulk plot or double reciprocal plot as thus:

$1/v=K_m/V_{max} \times 1/[S] +1/Vmax.$

Where:

V= rate of enzyme activity, [S]=substrate concentration, plotting 1/v against 1/[S] gives a straight line as stated by line weaver bulk. With

Y intercept = $1/V_{max}$, X intercept =-1/km and gradient = Km /V_{max}.

The V_{max} was obtained as absorbance per second; it was then converted to absorbance per minute by multiplying the obtained figure by 60. The value obtained was converted to M/min using the Beer – Lambert Law.

2.6. Optimum P^H for Tyrosinase Activity

Effect of P^H on tyrosinase activity was investigated by measuring tyrosinase activity at room temperature over a P^H range of 5.5 to 8.5 in 50mM phosphate buffer and the results plotted. A graph of relative activity of tyrosinase was plotted against P^H to note the point of maximum activity, which was traced down the P^H axis to give the P^H optimum.

2.7. Optimum Temperature for Tyrosinase Activity

The optimum temperature of tyrosinase enzyme was determined by measuring the rate of enzyme activity over temperature range of 15^0c -65^0c. A graph of relative activity of tyrosinase was plotted against temperature and the point of maximum activity noted. This was then traced down to the temperature axis to get the value for optimum temperature.

2.8. Enzyme Immobilization

Tyrosinase enzyme was immobilized by gel entrapment into porous matrix, coconut fiber and palm wood chips as described by [13]. The tyrosinase and gum Arabic (binding agent) were mixed thoroughly before coconut fibers and palm wood chips were soaked in the mixture for 3 hours. The coconut fiber and palm wood chips were removed and left to dry at room temperature.

3. Results

100g of yam produced 79.4mls of partially purified tyrosinase. The extract was characteristically faint-brown in colour. The results from enzyme activity assay produced values that were found to be increasingly steeply with time, for about 2 minutes after which the activity started to decline as shown in Figure 1.0. This is due to the slow conversion of DOPA-chrome to other products which are precursors of melanin. The slope of the curve of absorbance versus time depicting tyrosinase activity was 0.026min^{-1} which is 0.00000722M of DOPA-chrome produced per minute (from Beer Lambert Law). This figure is equal to 7.22micromoles/min. During the course of the reaction as the product (DOPA-chrome) began to form, the reaction mixture started to turn orange. After about 24hrs, the reaction mixture turned black. This suggests that the reaction has proceeded to form melanin.

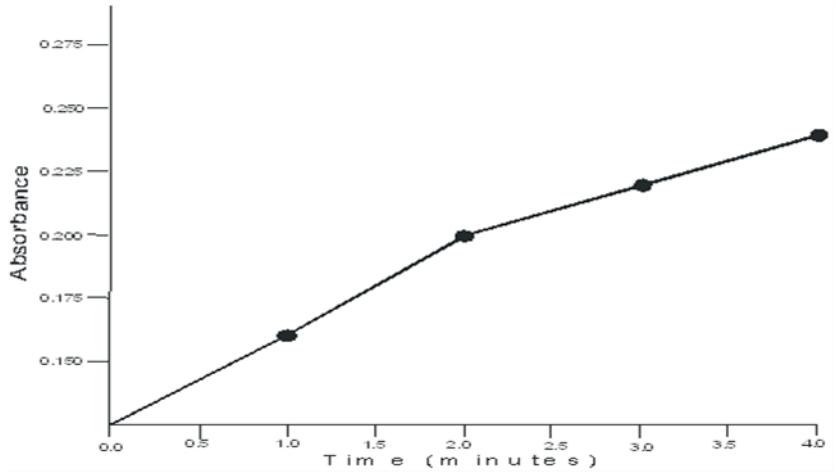

Figure 1 Change in absorbance with time

Table 1 Effect of Varying Substrate Concentration on Rate of Tyrosinase Activity.

Concentration of substrate (L-DOPA)	Change in absorbance/minute	change in absorbance/sec (absorbance/min/60)
5mM	0.124	0.0020
10mM	0.258	0.0043
15mM	0.294	0.0049
20mM	0.338	0.0056

The result from the table shows the effect of substrate concentration on tyrosinase activity. An increase in activity was observed with increasing substrate concentration as indicated by an increase in the strength of absorption (i.e. absorption per minute) due to an increase in product formation.

3.1. Michealis-MentenConstant (K_m) for Yam Tyrosinase

The Km for yam tyrosinase was found to be 33.3mM from the x-intercept of the plot.

The x-intercept was -0.03Mm. But from line-weaver-bulk plot, we have that;

X-intercept =$-1/k_m$

This follows that, $-1/k_m = -0.03$

Making Km the subject of the above equation, we have $K_m = -1/-0.03$

Thus K_m =33.3mM. By dividing the Km value by 1000 gives 0.0333M as Km.

3.2. Maximum Velocity (V_{max}) for Yam Tyrosinase

Vmax as determined from the y-intercept of the Line-weaver-bulk plot was $0.016s^{-1}$.

. Y-intercept was found to be $60s^{-1}$.

Recall that, y intercept $=1/V_{max}$ (from Line-Weaver-Bulk plot)

This implies that, $1/V_{max} = 60s^{-1}$

By making $V_{max}$the subject of the equation, $V_{max} = 1/60$.

Thus, $V_{max} = 0.016s^{-1}$.

The value above is equivalent to 266micromoles/minute; this clearly shows that at maximum reaction rate of the enzyme (yam tyrosinase), 266micromoles of DOPA-chrome are formed per minute.

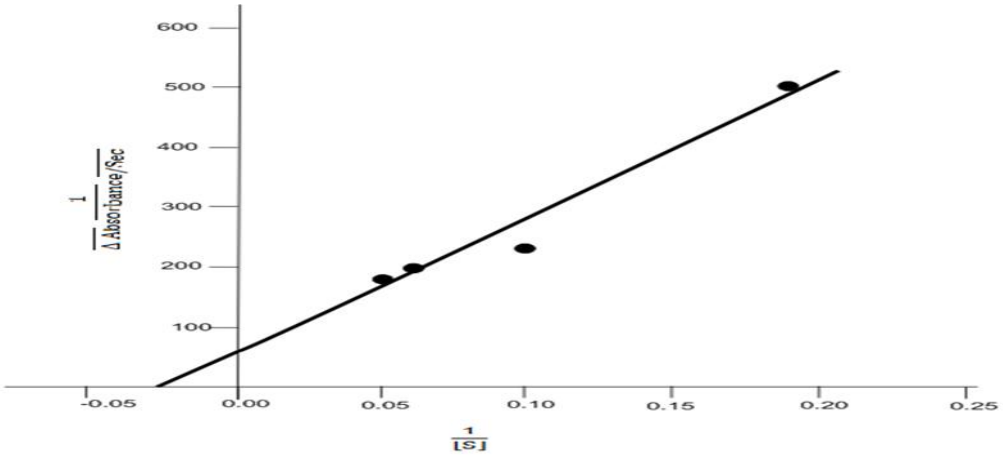

Figure 2 Line Weaver-Bulk double reciprocal plot

3.3. Optimum PH for Yam Tyrosinase Activity

Among the seven different selected P^H values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) of the extraction buffer, the yam tyrosinase showed optimal activity at P^H of 6.5. The variation of enzyme activity due to various buffer P^H values followed a bell-shaped curve as shown in figure 3.0.

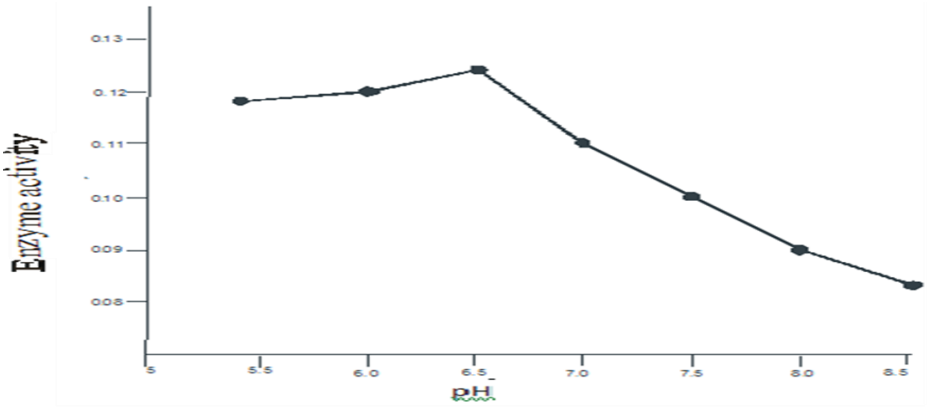

Figure 3 Optimum pH for Tyrosinase Activity

3.4. Optimum Temperature for Yam Tyrosinase Activity

Varying temperatures of 15°, 25°, 35°, 45°, 55° and 65°C, tyrosinase activity was found to be increasing steadily with increasing temperature. The activity peaked at 55°C after which it started to decline and diminishes at temperatures greater than, 75°C. Optimal temperature of yam tyrosinase was found to be 55°C.

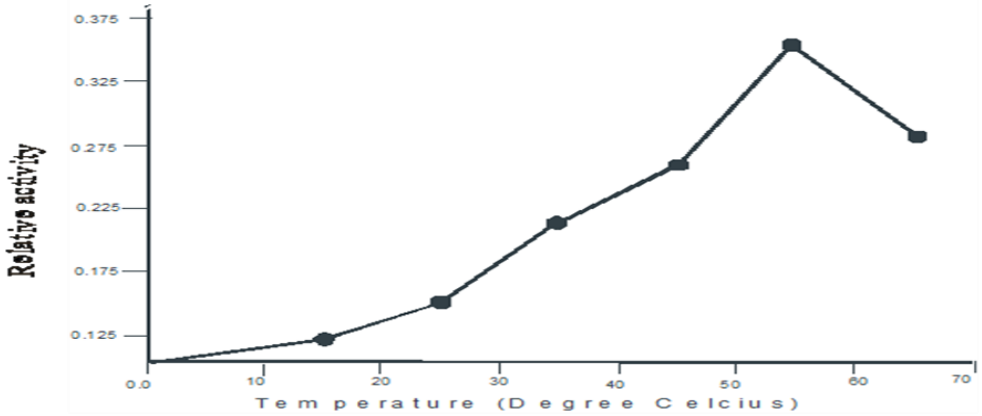

Figure 4 Optimum temperature for yam tyrosinase activity

3.5. Activity of Immobilized Tyrosinase on Coconut Fiber and Palm Wood Chips

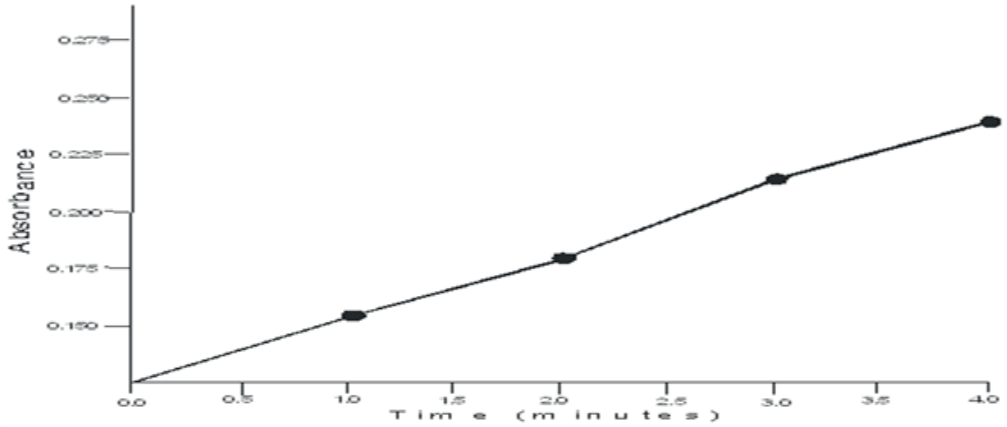

Figure 5 Immobilized Tyrosinase on Coconut Fiber.

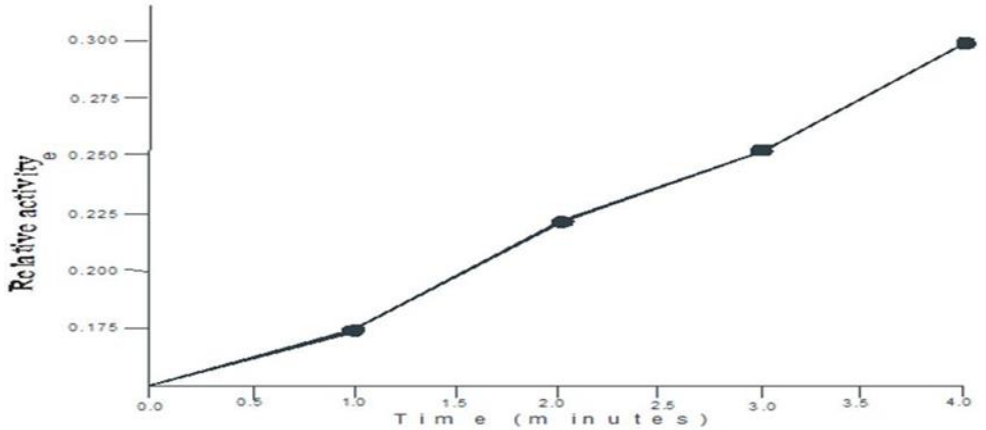

Figure 6 Immobilized tyrosinase on palm wood chips

Figure 5.0 and figure 6.0 shows the activity of yam tyrosinase immobilized on coconut fiber and palm wood chips respectively using L-DOPA as substrate. Yam tyrosinase immobilized on palm wood chips had an activity of 0.04/min while that on coconut fiber is 0.028/min (58.6% compared to 41.4%).

Table 2.0 Comparison of Partially Purified and Immobilized Yam Tyrosinase.

Kinetic Parameters	Partially Purified tyrosinase	Immobilized Tyrosinase on coconut	Immobilized Tyrosinase on palm wood chips
Temperature (^oC)	55	55	55
P^H	6.5	6.5	6.5
K_m(mM)	33.3	28.5	20.8
V_{max}	0.016	0.020	0.033

4. Discussions

The tyrosinase activity measured in this study using L-DOPA as substrate was diphenolase activity. Generally, enzymes catalyzed reactions show a hyperbolic relationship between the rate of reaction and the concentration of substrate. Usually at low substrate concentration, there is a steep rise in the rate of reaction with increasing substrate concentration. The catalytic site of the enzyme is empty, waiting for the substrate to bind and the rate at which products can be formed is limited by the concentration of substrate available [14]. As the concentration of substrate increases, the enzyme becomes saturated with substrate. The rate of formation of products now depends on the activity of the enzyme itself, and adding more substrate will not affect the rate of reaction to any significant extent [15]. Maximum reaction velocity (V_{max}) is reached when all the enzymes active sites are saturated with substrate. The Michealis-Menten constant (k_m) is the substrate concentration at which the reaction rate is half of its maximum value. The K_m and V_{max} was different from those of tyrosinases gotten from other sources. This difference may be due to the nature of the source of enzyme, the substrate used, the type of buffer and the purity of the enzyme [16].

The relatively high K_m value for yam tyrosinase in this research suggests that under assay conditions (P^H 7.0), the enzyme possess low affinity for the substrate, which however, may be to some degree dependent on the P^H used for the assay. Most enzymes are active only within a narrow P^H range, usually between 5.0 and 9.0. The optimum P^H of yam tyrosinase determined in this research coincides with the P^H value of 6.5 for banana tyrosinase when dopamine was used as substrate [17, 18].

Temperature is another factor that affects enzyme activity. If the temperature is too low, there can be no noticeable reaction since collision between enzyme and substrate is low. Increasing the temperature of the system will increase the number of collisions of enzyme and substrate per unit time. If the temperature at which the enzyme is operating is well above 100^0C, then thermal deactivation and loss of enzyme activity can occur. It is stated that optimum temperature for tyrosinase are quite special and substrate dependent [19]. Enzymes of human origin have an optimum temperature of 37^0C.

Previous research studies have shown optimal temperature values for tyrosinase activity of 30^0C for mango pulp [20]; 40^0C for tomato tyrosinase and *Bacillius megaterium* [21], 55^0C for mushroom tyrosinase. The optimum temperature for yam tyrosinase obtained is 55^0C, thus, similar with previous observation.

Tyrosinase activity from yam was found to be increased upon immobilization on palm wood chips and coconut fiber. The activity of partially purified tyrosinase enzyme was found to be 0.026M/min while that of immobilized enzyme on coconut fiber and palm wood chips was found to be 0.028M/min and 0.040M/min respectively. The percentage increase in activity was 27.65% for partially purified enzyme, 29.78% and 42.55% for immobilized enzyme on coconut fiber and palm wood chips.

5. Conclusion

The demand for tyrosinase in industries is high as it constitutes one of the most important group of enzymes utilize in cereal, dairy and meat processing among others. It has wide application in cosmetic, pharmaceuticals and food industry. Despite the wide applicability of tyrosinase for commercial purposes its exploitation has being limited to micro-organisms, fruits and vegetables. This research work has shown that there is presence of tyrosinase in yam and as such

the kinetic parameters revealed a V_{max} of 0.016s^{-1} and Km value of 33.3mM for partially purified enzyme while that of the immobilized enzyme showed a V_{max} of 0.020s^{-1} and K_m value of 28.5mM on coconut fiber; and a V_{max} of 0.033s^{-1} and Km value 20.8mM on palm wood chips. Yam tyrosinase activity was found to be increased upon immobilization on palm wood chips and coconut fiber. Immobilized tyrosinase enzyme on coconut fiber showed a negligible increase in activity of 2% (29.78% compared to 27.65% of the partially purified enzyme) while that of palm wood chips showed an increase in activity of 15% (42.55% compared to 27.65% of the partially purified enzyme). Based on these findings, palm wood chips may be considered as the cellulose fiber of choice for the immobilization of yam tyrosinase.

Recommendation

We wish to recommend that purified form of tyrosinase from yam should be immobilized on the palm chips and the results compare with these findings.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no competing interests exist.

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