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Effect of Storage Conditions on Antioxidant Enzymes and Polyphenol Oxidase Activity of African Star Apple Juice

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Abstract

This study investigated the effect of storage on both antioxidant enzymes and polyphenol oxidase activities of African star apple juice. African star apple (*Chrysophyllum albidum*) was sorted, washed, peeled and deseeded before milling the pulp to get fruit paste. The milled paste was then filtered to get a clear juice. The clear juice was packed in sterilized bottles before pasteurizing in a water bath at 71.2 ºC for 30 minutes; after which a part was stored under refrigeration condition (6±2 ºC), while the other part was stored under tropical ambient condition (28±2 ºC). Catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) all increased as storage period increased, with samples stored under refrigeration conditions recording higher values than those stored under tropical ambient conditions. On the other hand, the selenium-containing microsomal enzyme, glutathione peroxidase (GPx), registered an inverse relationship with storage period at both conditions. Polyphenol oxidase optimal thermal activity was observed at 80 ºC and that of pH was at 7, whereas the enzyme was more stable at 40 ºC at all stages and conditions of storage tested. The study also showed that polyphenol oxidase was more active when purified partially, compared to the crude enzyme. In conclusion, polyphenol oxidase can be isolated from African star apple juice stored under different conditions.

Keywords: *African star apple; stored juice; antioxidant enzymes; polyphenol oxidase*

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Introduction

African star apple (*Chrysophyllum albidum*) is a tropical ever green edible fruit tree. It belongs to the family of Sapotaceae and it is common throughout the tropical Central, East and West Africa regions for its sweet edible fruits and various ethno-medical uses. It is widely distributed in tropical West Africa and other African countries such as Ghana, Nigeria, Kenya, Sierra Leone, Sudan, and Uganda (Oguntimehin et al., 2022a). It is a forest plant but is usually planted as a compound tree in villages. The plant is a seasonal fruit which usually spans from December to March of every year. When ripe, it has an ovoid to sub-globose shape, pointed at the apex, and up to 5 cm diameter and length of 6 cm. The skin or peel, is orange to golden yellow when ripe and the pulp within the peel may be orange, pinkish, or light yellow. There are three to five seeds present within the pulp which are not usually eaten. The seed-coats are hard, bony, shiny, and dark brown, and when broken reveal white-coloured cotyledons. The fruit has immense economic potential, especially following the report that jams that could compete with raspberry jams and jellies could be made from it (Okoli and Okere, 2010).The fruit has been found to have the highest content of ascorbic acid per 100g of edible fruit or about 100 times that of oranges and 10 times that of guava or cashew (Okoli and Okere, 2010). It is reported as an excellent source of vitamins, iron, and flavours to diets (Oguntimehin et al, 2021). It contains not more than five (5) seeds in a single fruit. The colour is green when unripe and yellow when ripe. The pulp is consumed in its natural form by pressing hard and sucking the pulp. The taste is mildly acidic. The tree is about 8-36 m in height, the fruit is seasonal (December–April).

African Star Apple (*Chrysophyllum albidum*) fruits are often left unexploited and are allowed to waste due to their excess supply in their season of glut (Oguntimehin et al., 2022b). To prevent or reduce these losses to the barest minimum, processing into other valued products like juice is being researched into. The fleshy pulp of the fruits is vastly consumed by the local populations and the pulp can taste either very sweet or sour. Locally, the variation of the fruit exocarp color is said to be correlated with the pulp taste. The exocarps of the sweet fruits are yellow while those of the sour ones have a mixture of yellow and green colours when matured (Asare et al., 2015). Previous studies on *C. albidum* in western Africa reported the importance of the species for local community livelihood improvement and its potentiality for the food industries. The proximate composition of African star apple includes moisture content: 66.7%, crude fat: 9.38%, ash: 2.12%, protein: 5.66%, crude fibre: 4.5%, carbohydrate: 78.34%; ascorbic acid: 19.68%; total metabolisable energy: 420.42 kcal as reported by Christopher and Dosunmu (2011). Producing functional beverages from such nutrient-rich local biodiversity has been the focus of recent research efforts (Enujiugha, 2020). This study examined the effect of refrigerated and ambient storage conditions of the processed juice on both the antioxidant enzymes and polyphenol oxidase activity, with a view to ascertaining the stability during storage.

Materials and Methods

Sample Collection

Fresh fruits of African star apple (*Chrysophyllum albidum*) were obtained from a local farm in Owena, Osun state. Plastic bottles and muslin cloth were purchased at Oja Oba in Akure. All reagents and chemicals used in the study were of analytical grade.

Production of African Star Apple Juice

The fruits were sorted, washed thoroughly with clean water to remove any adhering substances, peeled and its seeds were removed. The flesh was sliced into small pieces using sharp stainless-steel knife and blended until it became pure juice. A mesh cloth was used to remove solid materials from the juice.

The juice extracted was then filled into sterilized glass bottles and then pasteurized in a water bath at 71.2 °C for 30 min. The juice was divided into two equal parts. The first part was stored under ambient temperature (28±2 ºC) while the other was stored at refrigerated temperature (6±2 ºC) for further analysis and use.

Determination of Antioxidant Enzymes

Determination of catalase activity of African star apple juice

Catalase activity was determined by using the method of Sinha (1972) as described by Bobadoye et al. (2016). About 1 ml of the sample will be mixed with 9 ml distilled water. The assay mixture containing H_2O_2 solutions and 5 ml of phosphate buffer in a 10 ml flat bottom flask. Properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. This reaction will be carried out at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromatic or acetic acid reagent at one minute intervals. The hydrogen peroxide contents of the withdrawn sample was determined by this method stated above. The catalase activity of the reaction was calculated with this formular: Catalase activity (K) = H_2O_2 consumed / mg Protein

Determination of superoxide dismutase (SOD) activity

The method of Badejo et al. (2016) was adopted. About 1 ml of the sample was diluted in 9 ml of distilled water. An aliquot of the sample was added to 2.5 ml of 0.05 M carbonate buffer (PH 10.2) to equilibrate in the spectrophotometer. The reaction started with the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. Increase in absorbance at 480 nm was monitored at 30 seconds intervals for 150 seconds. SOD activity = Increase in absorbance per minute / 2.5

Determination of reduced glutathione (GSH) level

GSH level will be estimated as described by Beutler et al. (1963). Exactly 0.2 ml of the sample was added to 1.8 ml of diluted water and 3 ml of the precipitating solution was mixed with the sample. The mixture was allowed to stand for about 5 minutes before it was filtered. 1 ml of the filtrate was added to 4 ml of 0.1 M phosphate buffer before adding 0.5 ml of the Ellman reagent. A blank was prepared with 4 ml of the 0.1 M phosphate buffer, 1 ml of the diluted precipitating solution and 0.5 ml of the Ellman reagent. The optical density was measured at 412 nm.

Assay of glutathione peroxidise (GPx) activity

GPx activity in the sample was determined according to the method adopted by Rotruck et al. (1973). The reaction mixture containing 500 µl phosphate buffer, 100 µl of sodium azide, 200 µl GSH, 100 µl H_2O_2 were added to 500 µl of the sample, after which 600 µl of distilled water was added and mixed thoroughly. The whole reaction mixture was incubated at 37 ºC for 3 minutes after which 0.5 ml of TCA was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 1 ml of each of the supernatants, 2 ml of K_2HPO_4 and 1 ml of DNTB was added and the absorbance was read at 412nm against a blank.

Preparation of Crude Extract

African star apple juice was thoroughly homogenized in 450 ml of ice cold 25 mM phosphate buffer (pH 6.8) containing 10 mM ascorbic acid using a warring blender for 3 min with 60 seconds resting period to avoid local elevation in temperature. The mixture was filtered using four layers of cheese cloth. The filtrate obtained was centrifuged in a refrigerated centrifuge at 6,000 rpm for 30 minutes at 4 °C. The supernatant obtained was stored in a refrigerator and used as crude extracts for further studies.

Partial Purification of Polyphenol Oxidase from African star apple juice

The supernatant obtained from each species was brought to 80% Ammonium sulphate ($(NH_4)_2SO_4$) saturation with solid Ammonium sulphate. The precipitated enzyme (polyphenol oxidase) was separated by centrifugation at 6,000 rpm for 30 minutes. The precipitate was dissolved in small amount of 0.1 M phosphate buffer (pH 6.8) and dialyzed at 4 °C overnight with three changes of buffer.

Determination of Polyphenol Oxidase Activity

Polyphenol oxidase activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 475 nm (Coseteng and Lee, 1987). One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per minute (Dabesor et al., 2022). The sample cuvette contained 0.7 ml of 10 mM 3,4-dihydrophenylalanine solution in 0.1 M phosphate buffer (pH 6.8) and 0.3 ml of the enzyme solution while the blank contained only 0.7 ml of 10 mM 3,4-dihydrophenylalanine and 0.3 ml of 0.1 M buffer solution.

Effect of pH on Polyphenol Oxidase Activity and stability

The effect of pH on polyphenol oxidase was investigated using pH range of pH 4.0–9.0, 0.1 M acetate buffer (pH 4.0– 5.0), 0.1 M phosphate buffer (pH 6.0–7.0) and 0.1 M Tris/HCl buffer (pH 8.0–9.0). Each of the buffer solution was used to prepare the substrate used in determining the activity of the enzyme at a particular pH. Enzymatic activity was measured according to the standard assay procedures.

The effect of pH on the stability of the enzyme was carried out by incubating the enzyme in a buffer solution at different pH in room temperature. Four fold dilution of the enzyme was prepared using the buffer solution. Enzymatic activity was determined at 0 min and at 1 hour interval for six hours period using the standard assay procedure.

Effect of Temperature on Polyphenol Oxidase Activity and stability

The assay mixture was incubated at different temperatures varying from 30 to 80 °C for 10 min in a regulated water bath. The activity assay was determined at 10 °C temperature interval while the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached.

Thermostability of polyphenol oxidase was investigated using the enzyme solution incubated for 1 hour in regulated Gallenkamp water bath at a particular temperature within the temperature range of 30 to 80 °C at 10 °C interval. Aliquot was withdrawn and cooled for the determination of enzyme activity at 10 min interval.

Statistical Analysis

The experimental design used was completely randomized design, and data collected were analyzed using one-way analysis of variance. Means were separated by Duncan's new multiple range test, and the level of significance was accepted at ($p<0.05$). Microsoft Excel 2016 was employed in the plotting of all the graphs and charts.

Results and Discussion

Antioxidant Enzymes Activities of African Star Apple Juice during Storage

Antioxidant enzymes are one of the primary defense mechanisms that protect cells from ROS damage by converting them to non-toxic stable molecules (Badejo et al., 2016). This alleviates oxidative stress and maintain redox homeostasis to sustain their lives and also to serve heterotrophic organisms, man inclusive (Saez and Estan-Capell, 2014).

Catalase (H_2O_2 oxidase, EC 1.11.1.6; CAT) is an oxidoreductase enzyme and one of the primary antioxidant enzymes in plants. It catalyzes the dismutation of H_2O_2 into H_2O and O_2. This enzyme plays an important role in the plant metabolism and also in signal perception (Anjum et al., 2016). The CAT of the stored African star apple juice increased as storage time increased; this can be associated with the increase of non-enzymatic antioxidants and phytochemical as storage time increases.

Superoxide dismutase activity like CAT also increased as storage time increased with the one stored in refrigeration condition higher than that stored at ambient temperature. SOD is a metalloenzyme that catalyzes the dismutation of superoxide anion to oxygen and H_2O_2 thus, protecting plant tissues from oxidation of lipids and DNA damages (Badejo et al., 2016). It is the first defense mechanism that converts free oxygen radicals O_2 to H_2O_2 to scavenge other free radicals (Li et al., 2018). The observed increase as storage period increased is in conjunction with the findings of Razavi et al. (2018) in stored peach fruits at 1 ºC for 2-4 weeks.

Figure 1 showed that there was an increase in reduced GSH level as storage time increases, with ASA juice stored at refrigeration temperature being higher than that stored under ambient temperature. At week 0, reduced GSH level was 39.94 µ/ml and increase was observed with the activity of GSH level being 56.34 µ/ml on the 4th week of storage under ambient temperature and 60.94 µ/ml for refrigeration temperature. This can be as a result of increase in the phytochemical and scavenging ability observed during the storage of the juice. This suggests that consumption of African star apple juice would boost immune system by activating and strengthening natural body defenses, in addition to reduction of oxidative stress. The selenium-containing microsomal enzyme, GPx, catalyses the degradation of hydrogen peroxide to water and reduces organic peroxides to alcohols, providing another route for eliminating toxic oxidants (Bobadoye et al., 2016). Studies have shown an inverse association between GPx and storage period. As shown on Figure 2 there was a decline in GPx activity with the highest activity level obtained in the freshly

extracted juice. GPx play an important role in reduction of lipid and hydrogen peroxides.

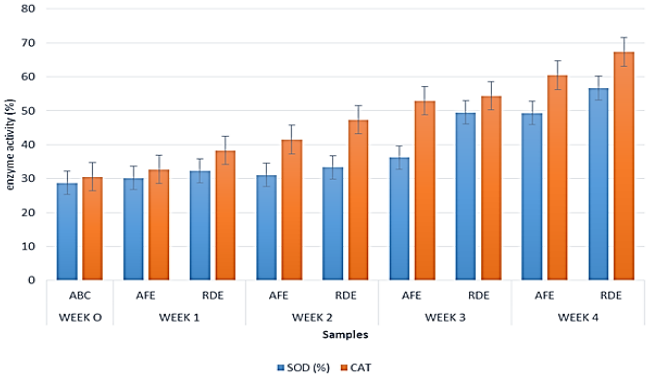

Figure 1: Evaluation of SOD and CAT activities of African star apple juice during storage.
ABC- Freshly extracted African Star apple Juice; AFE -African Star apple juice stored at ambient temperature; RDE - African Star apple juice stored at refrigeration temperature.

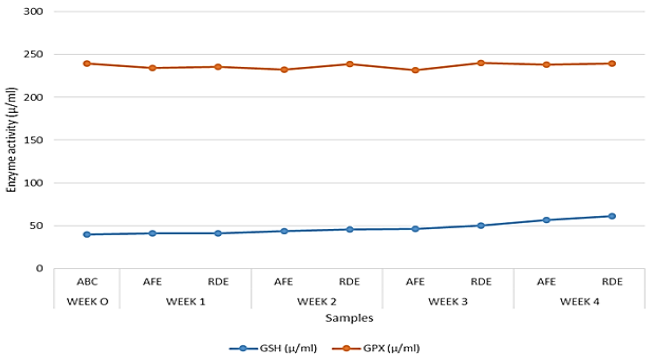

Figure 2: Evaluation of GSH and GPx activities of African star apple juice during storage.
ABC- Freshly extracted African Star apple Juice; AFE -African Star apple juice stored at ambient temperature; RDE - African Star apple juice stored at refrigeration temperature.

Protein Concentration of Polyphenol Oxidase of African Star Apple

Most browning reactions in fruits are assumed to be a direct consequence of polyphenol oxidase (PPO) action on phenolic compounds. This reaction produces quinones, highly reactive compounds that can polymerize spontaneously to form brown pigments, which are responsible for the loss of quality of freshcut fruit and vegetable products (Derardja *et al*., 2017). Many studies have shown that enzymatic browning is an undesirable phenomenon during storage and processing of plants food and is mainly initiated by the action of PPO (Munoz-Pina *et al*., 2018). In this study, partial purification of polyphenol oxidase had higher protein concentration than crude purification (Fig. 3). The concentration of protein increased as storage time increased notwithstanding the various storage temperatures. Also, protein concentration of ASA juice stored under refrigeration temperature was lower than those stored under ambient temperature for both partial and crude purification of polyphenol oxidase. However, it was observed that crude enzyme without purification had lower protein content compared to that of partial purification and this may be due to the differences in the purification mechanisms.

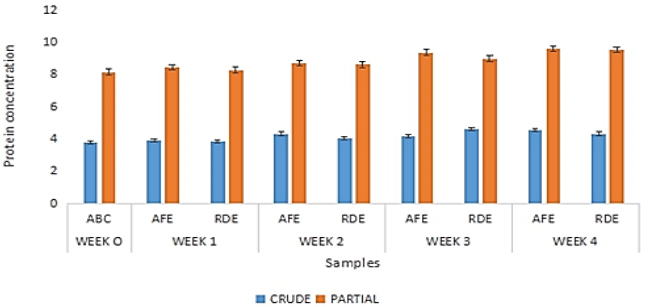

Figure 3: Protein concentration of polyphenol oxidase of African star apple
ABC- Freshly extracted African Star apple Juice; **AFE** -African Star apple juice stored at ambient temperature; **RDE** - African Star apple juice stored at refrigeration temperature.

Effect of Temperature and thermal stability on Polyphenol Oxidase Activities

Temperature is a very important factor that significantly influence the catalytic activity of polyphenol oxidase, as a decrease in temperature decreases kinetic energy and hence a low reaction rate (Sanni, 2016). Also, integrity of the delicate three-dimensional structure of the enzyme is subjected to disruption and denaturation at high temperatures (Yoruk and Marshall 2003). As shown on Figure 4, there was a steady rise in the activity of polyphenol oxidase in all storage conditions and maximum temperature was at 70 ºC then a decline sets in at 80 ºC. This revealed that complete inactivation of enzyme activity requires temperature above 80 ºC. This is in agreement with the results of Amiour and Hambaba (2016) who reported that a temperature above 80 ºC is needed to ensure PPO inactivation in processed horticultural products, such as juices, canned fruits, vegetables, etc. Temperature optima as low as 20 °C was observed for bartlet pear (Sidddiq and Cash, 2000) and as high as 60 °C for *Irvingia* spp has been observed (Sanni, 2016). An optima activity of 80 °C observed in ASA juice is high compared to the temperature optima of between 20 °C - 60 °C generally observed for various plants using different substrates by Yoruk and Marshall (2003) in their review of plant polyphenol oxidase. Yoruk and Marshall (2003) however noted that optimum temperature of polyphenol oxidase varies in different plant sources and that nature of the substrate influences the optimum temperature.

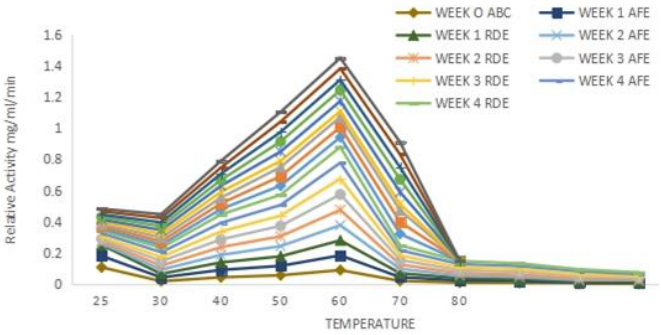

Figure 4: Effect of temperature on polyphenol oxidase activities
ABC- Freshly extracted African Star apple Juice; **AFE** -African Star apple juice stored at ambient temperature; **RDE** - African Star apple juice stored at refrigeration temperature.

The activity of PPO activity reduced as the time increased as shown on Figure 5. Highest stability of the enzymes is at 10 minutes with steady increase as the storage period increased for all storage conditions and the highest activity observed at the 4th week (0.8 mg/ml/min) and it experiences a huge decline at 20 minutes for all of the samples while decline becomes steady as from 30 minutes. The polyphenol oxidase of the ASA juice showed lower resistance to heat. Polyphenol oxidases from different sources exhibit different heat resistance (Sanni, 2016). The enzyme from two species of *Irvingia* were thermally stable at 25 °C, 30 °C and 40 °C while over 80% residual activity was observed at 50 °C and less than 15% residual activity at 80 °C (Sanni, 2016). Wakayama (1995) also observed about 1.4 minutes to 2.4 minutes were required for 90% inhibition of the polyphenol oxidase from the core of six Japanese apple varieties.

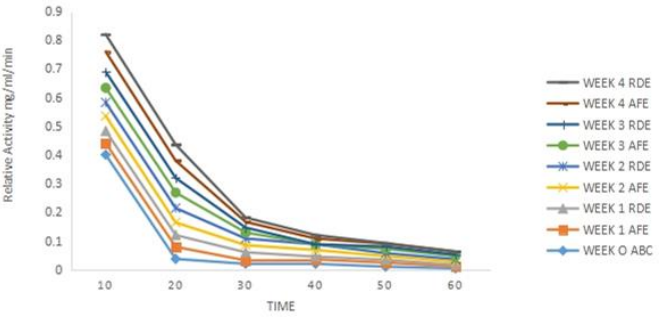

Figure 5: Effect of thermal stability on polyphenol oxidase activity
ABC-Freshly extracted African Star apple Juice; **AFE** -African Star apple juice stored at ambient temperature; **RDE** - African Star apple juice stored at refrigeration temperature.

Effect of pH of Polyphenol Oxidase Activity and its stability during Storage

Enzymes are extremely sensitive to pH, which would affect PPO's surface charge, its solubility, conformation and binding-ability with different substrates or inhibitors (Peng et al., 2019). The samples had their highest

activity at the pH of 7 as shown on Figure 6. There is a decline in the activity after attaining the optimal pH. Also activity at pH 3 for all the samples are higher than that of 4, there was a decline in the activity as pH increases to 4, then the activity increases steadily till it got to pH 7 which is the optimal pH before decline occurs at pH 8. This result showed that polyphenol oxidase activity is more inhibited in alkaline mediums than in the acidic medium, thus providing a method of controlling enzymatic darkening. The loss in activity as pH increases could be attributed to the denaturation of enzymes by ionization. The result revealed an optimum pH of 7 for the ASA juice at various storage conditions which indicates higher enzyme activity at a neutral pH. pH is important for PPO to achieve maximum activity. The results obtained in this study correspond correctly with those found in African bush mango (*Irvingia gabonensis*) and snake tomato (*Trichosanthes cucumerina*) juice (Sanni, 2010; Dabesor et al., 2022). Dabesor et al. (2022) reported that differences in optimum pH for PPO activity depended on the plant sources, extraction methods, enzyme purity, buffers, and substrates. However, the enzyme showed activity in all the pH investigated (3-12), which may prove that pH may not be effective in the inactivation of the activity of the enzyme at the levels tested as the storage period increased. The pH optimum for PPO is found to be dependent on the enzyme source, substrate and extraction methods used (Sener et al., 2011).

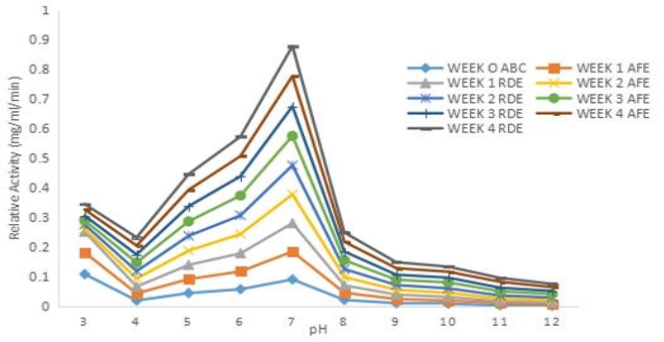

Figure 6: Effect of pH of polyphenol oxidase activity during storage
ABC- Freshly extracted African Star apple Juice; **AFE** -African Star apple juice stored at ambient temperature; **RDE** - African Star apple juice stored at refrigeration temperature.

At various storage conditions, the polyphenol oxidase activity was stable in pH for 10 to 20 minutes with a gradual decrease for the next 10 minutes (Fig. 7). Maximum pH stability of the enzyme was at 40 minutes for all the samples. The PPOs stability at this point might be as a result of the formation of a structural conformation at the pH that favors interaction with other components (including other proteins), resulting in inhibition by aggregation and precipitation (Bojer Ramusssen et al., 2021). However, a major decline occurred in the activity of PPO in all the other samples at 50 minutes. PPO from banana pulp is also stable over a broad pH range (Yang et al., 2000), while PPO from waste potato peel (*Solanum tuberosum*) is not (Niphadkar et al., 2015).

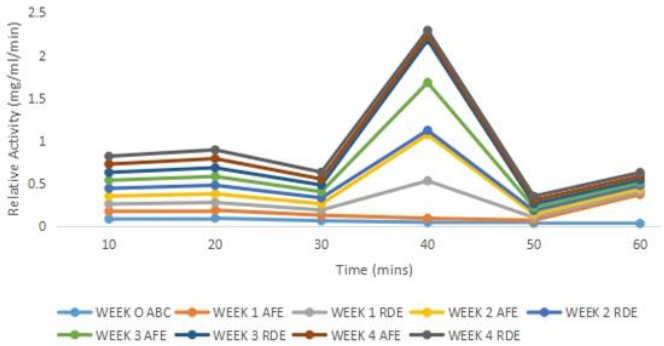

Figure 7: Effect of pH stability on polyphenol oxidase activity
ABC- Freshly extracted African Star apple Juice; AFE -African Star apple juice stored at ambient temperature; RDE - African Star apple juice stored at refrigeration temperature.

Activity of Crude and Partial Purified Polyphenol Oxidase

According to Fang et al. (2007), there are two main problems found in the determination of the extraction conditions of PPO: the difficulty in obtaining full solubilization of the membrane-bound PPO, and avoiding phenolic oxidation during and after extraction. The level of polyphenol oxidase activity of a particular plant species is inextricably connected to physiological needs of the plant. It has been reported that plants which possess relatively high levels of polyphenol oxidase activity are less susceptible to fungi and bacteria infections. This is obviously connected to the bacteriostatic properties of the brown products or pigments (melanin) of the enzyme action (Martinez and Whitaker, 1995). In the present study, activity of crude purification was lower than that of partial purification in all of the samples (Fig. 8), with the highest purification levels being attained on the 3rd and 4th weeks of storage. This revealed that partial purification brings about increased purification of polyphenol oxidase activity compared to the process of crude purification.

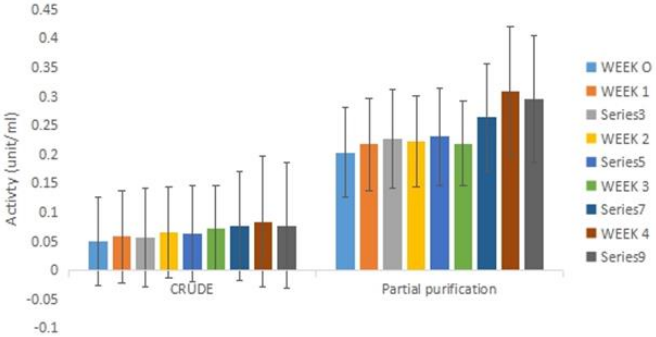

Figure 8: Activity of crude and partial purified polyphenol oxidase.
ABC- Freshly extracted African Star apple Juice; **AFE** -African Star apple juice stored at ambient temperature; **RDE** - African Star apple juice stored at refrigeration temperature.

Conclusion

This study has shown that thermostable polyphenol oxidase can be isolated from African star apple juice under viable conditions. Higher activities of polyphenol oxidase and antioxidant enzymes like SOD, CAT and GSH level were observed in the juice at week 4 stored under refrigerated temperature. The study also provided a database on the effect of temperature, pH and thermal stability on polyphenol oxidase activities in juice extracted from African star apple. Therefore, this indicates that African star apple can be used as an excellent raw material for isolation of these enzymes industrially. The contributions of both biological and chemical preservatives, in conjunction with different packaging materials on polyphenol oxidase activities, should be further investigated.

Declarations

Competing Interest

The authors declare no competing interest.

Authors' Contributions

All listed authors contributed equally to the literature writing, review, research process and editing of this article.

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