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Research Article

Characterization of α -Amylase from Soursop (*Annona muricata* Linn.) Fruits Degraded by *Rhizopus stolonifer*

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Abstract

Rhizopus stolonifer is a fungus and one of the most common species of the genus *Rhizopus*. The organism has been a very important microbe used in the field of industrial microbiology. It has been used in the production of many hydrolytic and extracellular enzymes among which is the α -amylase. This enzyme has found various uses in the industry. Fruit juices are important sources of nutrients and they contain several important therapeutic properties that may reduce the risk of various diseases. An investigation on α -amylase extracted from soursop fruits deteriorated by *R. stolonifer* and the effect of the enzyme on soursop juice clarification was carried out in this study. The results obtained shows that the soursop juice with low concentration of extracted enzyme and less incubation time was more viscous and cloudy compared with the juice with high concentrations of amylase and higher incubation time which was clearer and less viscous. The results of this research will be very useful in soursop juice producing companies.

Key words: *R. stolonifer*, α -amylase, fruit juices, soursop juice

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Enzymes are substances produced by living organisms which act as a catalyst to bring about a specific biochemical reaction. Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable hydrolytic enzymes especially amylase (Dar *et al.*, 2014). Soursop fruit as it is commonly called is derived from the *Annona muricata* plant. Ajayi *et al.* (2015) reported that the fruit had been associated with preharvest and postharvest deterioration caused by microorganisms and this reduces the total production of the soursop fruit. The deterioration process was accompanied by the production of cell wall degrading enzymes which has the advantage of being used for industrial purposes. Alpha (α) amylase is a hydrolase that stands out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries (Adeniran and Abiose, 2009). Alpha-amylase is an important class of amylases applied industrially; others being β -amylase and amyloglucosidase. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. Soursop juice is highly encouraged (Nwachukwu and Ezeigbo, 2013). Soursop juice has many therapeutic properties which includes diuretic, antiurethritis, antihematuria, antiantibacterial, anticancerous, astringent, sedative and anti-aging (Ajayi *et al.*, 2015). Soursop fruit juice is rich in nutrients such as amino acids, vitamins, fibre, proteins, unsaturated fats and essential minerals (Rice *et al.*, 1990; Amusa *et al.*, 2003). Clarification of fruit juice is a process whereby the juice from fruits is filtered to remove the pulp from the fruit. It can be carried out by centrifugation or by enzyme treatment. Available reports on the clarification of soursop juice with microbial enzymes are scanty (Ajayi *et al.*, 2015).

MATERIALS AND METHODS

Organisms and culture conditions: The isolates of *Rhizopus stolonifer* used for this study were obtained using decaying bread as solid substrate. It was grown and subcultured at room temperature on potato dextrose agar plates. *Rhizopus stolonifer* was identified using its cultural and morphological characteristics under the microscope using lacto-phenol blue. The organism was stored in PDA slants. A 96 h old culture of the organism served as the inoculum.

Collection of sample: Fresh, unblemished matured soursop fruits were obtained from Oju ore market, Ogun state. The fruits were transported in clean paper bag to the laboratory for enzyme extraction.

Inoculation of soursop fruits: The fruits were surface sterilized using 10% (v/v) sodium hypochlorite for 15 min and rinsed with sterile distilled water 5 times to remove the residual of the sodium hypochlorite solution. Soursop fruit was cut in half and inoculated with *R. stolonifer* using sterile inoculating loop. The controls were treated with similar fashion but not inoculated with the organism. The fruits were then transferred into an anaerobic bell jar and observed for 5 days for deterioration.

Extraction of enzyme from soursop fruit: The enzyme was extracted after 5 days. This is because within the 5 days of inoculation the soursop fruit had collapsed extensively. The enzyme was extracted as described by Puri *et al.* (2013). The soursop fruits were decored and deseeded, the pulp was homogenized with 1 M citrate phosphate buffer (pH 4.5) in the ratio 1:3 (w/v) in a binatone blender at top speed. The homogenate was filtered through Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was then centrifuged at 4000 rpm for 20 min and the supernatant filtered again. The filtrate was then used as the extracted enzyme.

Enzyme assay: Alpha amylase was determined using the method described by Adejuwon *et al.* (2013). The reaction mixture consisted of 2 mL of 0.2% (w/v) starch in citrate phosphate buffer at pH 4.5 as substrate and 0.5 mL of enzyme. The control experiments consisted of only 2 mL of the prepared substrate. The content of the experimental and control test tubes were incubated at 35°C for 20 min. The reaction in each test tube was terminated with 3 mL of 1N HCl. The enzyme (0.5 mL) was then added to the control tube. Two milliliters of the mixture from each of the set of experimental and control was transferred into new set of clean test tube. Three millimeters of 0.1N HCl was added into the content of each new test tubes after which 0.1 mL of iodine solution was added. The optical density reading was taken at 540 nm.

Properties of the extracted enzyme: The influence of some parameters on the activity of the crude enzyme was examined. The reaction mixture for the enzyme is described above under enzyme extraction unless otherwise stated.

Effect of temperature: The influence of temperature on the activity of the enzyme was investigated. The reaction mixture consisted of 1 mL of enzyme solution and 1 mL of 1% of soluble starch in citrate phosphate buffer at pH 4.5 as the substrate with temperature ranging from 25-45 °C for 30 min, to the control only the substrate was incubated. The incubation of the reaction mixture was stopped with 3 mL of 3, 5 dinitrosalicylic acid reagent. The test tubes were heated for 15 min in a water bath and cooled to room temperature. Two milliliter of the buffer was used as reference blank and absorbance was taken at 540 nm using a double-beam UV-VIS spectrophotometer (Perkin-Elmer Lambda).

Effect of pH: The influence of pH in the activity of the enzyme was examined. Substrate with pH ranging from 3.0-5.0 was prepared. The reaction mixture consisted of 1 mL of enzyme solution and 1 mL of 1% soluble starch in citrate phosphate buffer at different pH. The reaction mixture was incubated at 35 °C for 30 min and the incubation of the reaction mixture was stopped with 3 mL of 3, 5 dinitrosalicylic acid reagent. The test tubes were heated for 15 min in a water bath and cooled to room temperature. The absorbance was taken at 540 nm using a double-beam UV-VIS spectrophotometer (Perkin-Elmer Lambda).

Effect of substrate concentration: The effect of various concentration of the substrate on the enzyme activity was determined. Concentration of starch from 0.2, 0.5, 1.0, 1.5 and 2.0% starch, respectively was prepared and their effect tested on the enzyme activity.

Clarification of soursop juice: The soursop fruit was blended with sterile distilled water and strained using a sieve to obtain the juice. Five milliliters of juice was clarified using 10, 20, 30, 40 and 50% of 5 mL of the enzyme concentration in a test tube. The optical density of the mixture was taken at different incubation time between 3-6 h at 640 nm using a double-beam UV-VIS spectrophotometer (Perkin-Elmer Lambda 25).

RESULTS

Deterioration of soursop fruits: The experimental soursop was deteriorated extensively after 5 days of incubation but the control soursop fruit became harder after the 5 days. The infected fruits exhibited appreciable amylase activity while the uninfected soursop fruit only possessed traces of enzyme activity. The uninfected soursop got dehydrated after it was cut open.

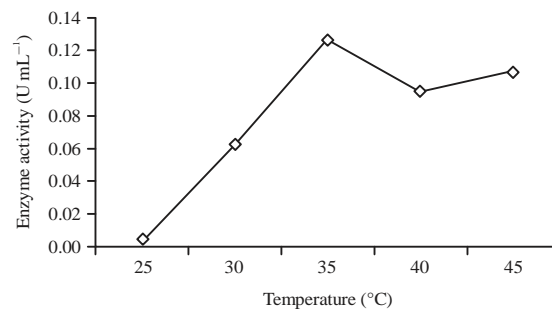


Fig. 1: Effect of temperature on the activity of α -amylase obtained from *Rhizopus stolonifer* deteriorated by soursop

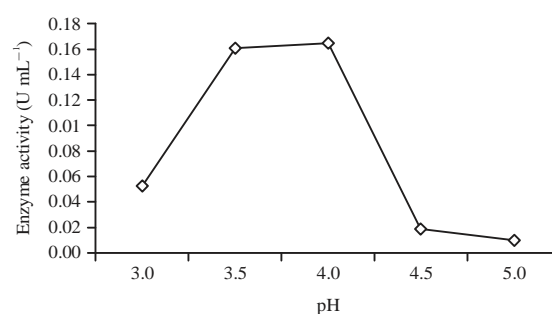


Fig. 2: Effect of the pH on the activity of α -amylase obtained from *Rhizopus stolonifer* deteriorated soursop

Production of α -amylase: After the complete deterioration of the soursop fruits, the extracts tested showed favorable amylase activity. The extract from the uninoculated soursop fruit showed only traces of enzyme activity showing that the enzyme produced were of the fungal organism.

Properties of the crude enzyme

Effect of temperature: The change in temperature greatly affected the activity of the extracted enzyme. Enzyme activity increased with an increase in temperature of incubation until an optimum temperature was reached. Subsequent increase in temperature beyond the optimum leads to a fall in the activity of the enzyme (Fig. 1).

Effect of pH: The change in pH affected the enzyme activity. Enzyme activity increased with increase in pH until its maximum was reached, then the activity began to reduce after the optimum level (Fig. 2).

Effect of substrate concentration: The activity of the enzyme increased with an increase in the substrate concentration until a maximum was reached. Above this concentration there was no further increase in enzyme activity (Fig. 3 and 4).

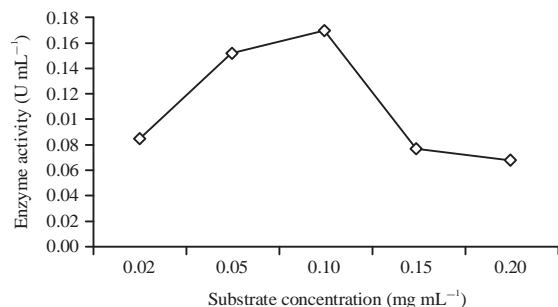


Fig. 3: Effect of the substrate concentration on the activity of α -amylase obtained from *Rhizopus stolonifer* deteriorated soursop

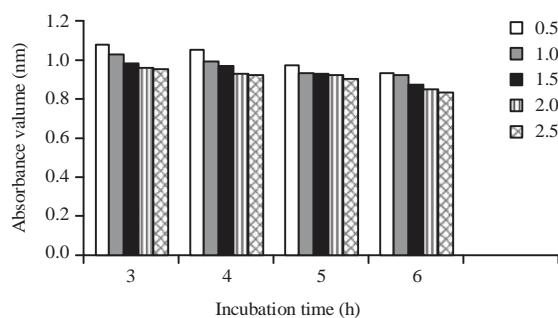


Fig. 4: Effect of incubation time and enzyme concentration on the clarification of soursop juice

DISCUSSION

In this investigation, *R. stolonifer* was inoculated into freshly ripe soursop fruits for the production of amylase. The result showed that the appreciable amylase activity that occurred in the extracts obtained from the soursop fruits infected by *R. stolonifer* was of fungal origin. This agrees with the work of Akpan *et al.* (1999) that amylase is produced by *R. stolonifer*. There have been previous reports on the extraction of enzymes from the soursop fruit using buffers (Bora *et al.*, 2004), this investigation however gives a report on the use of an organism in the production of amylase from deteriorated soursop fruits.

Rhizopus stolonifer caused the deterioration of the ripe soursop fruits within the five days of incubation. However, the uninoculated fruits became dehydrated, thereby becoming hard in the five day of incubation. This may be as a result of the hard bark of the soursop fruits and probably the absence of microorganisms.

The optimum temperature for the extracted enzyme is shown to be 35°C. This agrees with the work of Puri *et al.* (2013) and Oboh (2005). The pH and substrate concentration

optimum were pH 4.0 and 0.1 mg mL⁻¹, respectively showing similar characteristic with amylase from other fungal origins. The enzyme showed appreciable result when employed in the clarification of soursop juice. The α -amylase broke down the starch molecules, leaving a clear supernatant of the juice. This agrees with the study of Yusof and Ibrahim (1994). The clarity depended on the enzyme concentration, it showed a significant interaction effect between time and enzyme concentration. It was clear that the absorbance value decreased with the increase in enzyme concentration. Clarity showed the lowest absorbance value at highest enzyme concentration. Lower absorbance values indicated a clearer juice. Increase in enzyme concentration may increase the rate of clarification. It was also observed that the absorbance values decreased with increasing incubation time at fixed temperature.

CONCLUSION

This treatment will help in overcoming the turbidity that is usually associated with the production of fruit juices. The production of the soursop juice will help to make available the rich freshness of the soursop in and out of season so that its many uses can be enjoyed all year round.

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