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Original Research Article

Detannification of *Ananas comosus* Rinds Juice with Tnnase Immobilized on the Beads of *Chrysophyllum albidum* Seeds

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ABSRTACT

Billion tons of agro wastes that constitute hazard to the environment are generated annually all over the world. How to curb this menace remains a major challenge in the field of biotechnology. In this study, a novel immobilization material for microbial tannase was developed from *Chrysophyllum albidum* seeds. Optimization studies for effective performance of the novel immobilization material were conducted. The characterized immobilized tannase was explored for the detannification of tannin present in *Ananas comosus* rinds juice. Activities of free and immobilized tannase from *R. mucilaginosa* CBS 316 were 724.49 and 497.77 U/g/min respectively. Optimal temperature and pH for tannase activity were 35ºC and 7 respectively. Optimum tannase load, bead size and bead number for immobilized tannase were 35%, 8mm and 20 respectively. Immobilized tannase was reusable for up to 3 times. $MgSO_4$, KCl, and $ZnSO_4$ enhanced tannase activity while $PbCl_2$, MgCl, $BaCl_2$ and EDTA inhibited the activity of tannase. Tannin content of the three varieties of *Ananas comosus* rinds juice from Bene, Cotonou and Local source were drastically reduced from 17.8, 16.3 and 5.9 mg/100g to 3.9, 2.9 and 1.8 mg/100g respectively when treated with immobilized tannase. This study established the detannification of *Ananas comosus* rinds juice with microbial tannase immobilized on the novel beads of *Chrysophyllum albidum* seeds.

Keywords: *Chrysophyllum albidum*, tannase, *Ananas comosus*, Tannin, *Rhodotorulla mucilaginosa*

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Introduction

Tannins are polyphenolic compounds produced by plants in order to protect themselves from invading microorganisms and herbivores. Tannin causes indigestion in herbivores when ingested; sometimes leading to death when ingested in more quantities. *Ananas comosus* rinds are known to contain high tannin content[1]. Microbes produce tannase enzyme as a strategy to protect itself from tannin. Tannase enzyme (Tannin acyl hydrolase EC 3.1.1.20) hydrolyzes ester bonds in tannin to produce glucose and gallic acid[2].

Tannase is widely used in industries such as food, chemical, pharmaceuticals, breweries, tannery effluent treatment and production of animal feed. Tannase is also widely used for the production of gallic acid, which is a key intermediate required for the synthesis of an antibiotic drug, trimethoprim and used to produce propyl gallate, which is mainly used as an antioxidant in fats, oils and beverages[3]. Gallic acid is also used in the fabrication of semiconductors, dyes and in photographic revelation[2]. Thus tannase finds its application in several industries.

Despite the several important applications of tannase in food, feed, chemical and pharmaceutical industries, high scale use of this enzyme is severally restricted due to high production costs. Thus tannase is considered a specialty enzyme.

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Therefore, there is a continuous search for new sources of tannase, as well as improved methods for production, recovery, and application of the enzyme. In order to overcome some limitations of free tannase, several attempts have been made to immobilize the enzyme on a suitable matrix[4].

Enzyme immobilization facilitates the efficient recovery and reuse of costly enzymes and enables their use in continuous, fixed-bed operation. Immobilized enzymes are easier to handle and to separate from the product, thereby minimizing or eliminating protein contamination of the product. Additionally, immobilized enzymes are often more stable than the free ones, allowing the repeated reuse of the biocatalyst[4].

A variety of materials of various origins can be used as supports for enzyme immobilization. This includes agarose beads, alginate beads, chitosan beads, k-carrageenan beads and nanogel beads. Some of these matrices are expensive[5]. However, in Nigeria, some natural polymers such as the seed cotyledons of *Chrysophyllum albidum* which is cheap, readily available and have comparable properties to conventional immobilization materials can equally be explored. Beads made from seed cotyledon of *Chrysophyllum albidum* has never been used for enzyme immobilization. Therefore this study investigates the removal of tannin content of *Ananas comosus* rinds juice using tannase immobilized on beads made from seed cotyledon of *Chrysophyllum albidum*.

Materials and Methods

Microorganism

Tannase producing *Rhodotorula mucilaginosa* CBS 316 was obtained from the culture bank of Federal University of Agriculture Abeokuta, Nigeria (7º13′59.8″N 3º26′12.0″E). The yeast isolate was sub cultured and young culture was screened for tannase activity.

Tannase production medium
Cultivation of yeasts were carried out by submerged fermentation in 250mL Erlenmeyer flasks using the Czapek's Dox medium supplemented with sterilized tannic acid (BDH, England) at 1% concentration as sole source of carbon. This was autoclaved (Dixon portable autoclave DA 025T) at 121°C for 20min and inoculated (MINI75 Genilab, United Kingdom) with prepared inoculum (1%v/v) and incubated for 72hrs at room temperature (28±2ºC). Samples were withdrawn at regular intervals of 24hrs and observed for tannase activity.

Enzyme extraction
Tannase was extracted according to standard enzyme extraction method[6]. At the end of incubation, the cell-free filtrate was obtained by filtering through Whatman no. 1 filter paper. The filtrate was centrifuged (2-16KL, Sigma Germany) at 3400× g for 14 min at 4°C and this serves as a crude enzyme preparation. The crude tannase was stored for further analysis.

Tannase assay
Tannase activity produced by isolates was assayed using the Rhodanine method[7]. Solution containing 50µL of enzyme was incubated with 100µL of methyl gallate (Sigma) (0.01 M concentration) for 5min at 37°C. At the end of incubation time, the reaction was stopped by adding 300µL methanolic rhodanine (0.667 %) and the tubes were incubated for 3min at room temperature. To all tubes, 100 µL of 0.5 M KOH and water was added to a final volume of 2mL before taking reading at 520nm. Control tubes were incubated with same concentration of heat denatured enzyme along with substrate and methanolic rhodamine (BDH, England), blank was made without enzyme. Amount of gallic acid liberated was estimated using standard gallic acid calibration curve. One unit of tannase was taken as the amount of enzyme required for liberating 1µm of gallic acid per mL per min.

Protein determination
The total soluble protein was determined by measuring the optical density of developed color at 595nm[6] using spectrophotometer (T70 PG Instrument UV model). The µg of protein was estimated using µg standard of bovine serum albumin (BSA). The pure solution without any protein content was measured (BSA) and used as standard. Different dilutions of this standard were made. The optical density was measured for 1µg/µL and higher concentrations such as 2µg/µL and 4µg/µL. The sample with the unknown protein content was measured with the spectrophotometer.

Tannase immobilization
The crude enzyme was immobilized using *Chrysophyllum albidum* seeds. The seeds were blended into powder and defatted using a soxhlet extractor with ethanol as extracting solution. The seed powder (10% w/v) was cross linked with 2.5% glutaraldehyde solution at 30°C for 10 minutes. The known volume of the tannase in its pure form was added to the seed slurry and stirred continuously for 5min. Gel mixture was injected drop wise into ethanolic -formaldehyde solution (60:40% v/v) for 24hrs to produce immobilized beads. Defatted seeds were sterilized and dried to constant mass in the hot air oven and stored at 6°C.

Effects of different incubation temperatures
Effect of different incubation temperatures on tannase production was determined. The flasks containing the enzyme was supplemented with tannic acid (substrate solution) and were kept at temperatures ranging from 15°C to 65°C for 4 days. The tannase activity was measured as described above by Sharma et al.,[7].

Effect of different pH
The effect of initial pH on tannase production was studied by adjusting the production medium at various levels of pH by HCl and NaOH solutions ranging from 3.0 to 10. The tannase activity was measured as described above[7].

Effect of different metal ions
Effects of metal ions on tannase activity was determined by incubating purified tannase with metal ions such as $MgS0_4$, MgCl, $ZnSO_4$, $BaCl_2$, $CdCl_2$, $PbCl_2$, $HgCl_2$, and KCl for 30min at room temperature for three different concentrations (1, 5 and 10mM). After incubation residual tannase was collected and used for determination of tannase activity described above[7]. Purified tannase activity in the absence of any metal ions was taken as control.

Effect of chelator
Effect of additive on tannase activity was studied by incubating purified tannase with 1% Ethylene Diamine Tetraacetic Acid (EDTA) for 30min at room temperature. The residual tannase was collected for estimation of enzyme activity as described above[7]. Purified tannase activity in the absence of any metal ions was taken as control.
Optimum temperature for tannase
The enzyme was incubated with tannic acid at different temperature ranging from 15 to 65°C for 15mins. The activity was then determined as described above.

Thermal stability of tannase
The stability of the enzyme at high temperatures, the samples of tannase in test tubes of uniform size were incubated in water at different temperatures (30 °C, 40 °C, 50 °C, 60 °C, 75 °C,) and different times (0, 10, 20, 30, 40, 50 and 60min). After heating, the tubes were cooled in an ice bath, and the residual activity measurement was carried out at pH 5.0 and 30°C[7].

pH Stability
The stability of the crude and purified enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3 to 8 (0.1 M citrate phosphate buffer for pH 3–7 and tris HCl buffer for pH 8) for 12hrs at 30°C. Residual activity was estimated under standard conditions and expressed as percentage of the relative tannase activity[8].
Optimization of immobilized tannase
Effects of enzyme load on tannase activity
Various enzyme concentrations were dissolved in 15.52% (w/v) defatted powder of *Chrysophyllum albidum* in phosphate buffer solution (pH 7.0) containing 15, 25, 35, 45, and 60% (v/v) enzyme solution activated with 2.5% (v/v) glutaraldehyde solution. Beads were formed and activity was assayed as described above[7].

Effects of bead size on tannase activity
Optimum enzyme concentration was used to prepare the gel. Various bead sizes (5–9 mm) were achieved by dropping gel through laboratory dropper of various diameter sizes. Tannase activity was assayed as described above[7].

Effects of number of beads on tannase activity
At optimum enzyme load and bead size, various numbers of beads (5–30mm) were used to hydrolyze *Ananas comosus* rinds juice to study the effect on tannase activity. Tannase activity was assayed as described above[7].

Effects of reusability of immobilized tannase
Optimum numbers of beads were used in a repeated batch cycle to study the effect of reusability on *Ananas comosus* rinds juice, tannase activity was assayed as describe by Sharmal et al.,[9]. At the end of each batch, the beads were washed in phosphate buffer (pH 7.0), dried, and used for the next batch.

Determination of tannin content of Ananas comosus rinds juice
The *Ananas comosus* were properly washed. The rinds were pulverized and oven-dried at 50℃ for 48 h. Blender was used to blend the dry samples into 0.5 µm sizes. The blended samples were used for tannin determination as follows:
The tannin content was determined by Folin-ciocalteu method[10]. About 0.1mL of the sample extract of *Ananas comosus* rinds was added to a volumetric flask (10mL) containing 7.5mL of distilled water and 0.5mL of Folin-ciocalteu reagent. Exactly 1 mL of 35%

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Na_2CO_3 solution was diluted to 10mL with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100µg/mL) were prepared in the same manner as described earlier. Absorbance for the test and standard solutions were measured against the blank at 725nm with UV/visible spectrophotometer (T70 PG Instrument UV model). The tannin content was expressed in terms of mg of GAE/g of extract.

Ananas comosus rinds juice preparation
Five grams of *Ananas comosus* rinds was cut into small pieces and crushed in a mortar pestle. Juice was then extracted by adding 100mL of distilled water and homogenizing in a blender, followed by filtration through muslin cloth and the extracted juice was stored at 4℃ for further studies

Detannification of Ananas comosus rinds juice
Fifteen 15mL of *Ananas comosus* rinds juice was treated in a 100mL conical flask with beads of the immobilized tannase. Aliquots of 0.2mL from the treated juice of *Ananas comosus* rinds were taken out at 60min intervals for analysis of the tannin content as described by Mueller[10].

Statistical analysis
Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 26.0 for descriptive statistics such as mean and standard deviation. One-way Analysis of Variance (ANOVA) was used to determine the clear zones produce by isolates on Dimitri's agar and the data on enzyme activity observed under the different conditions. Means were separated by Duncan's multiple range tests. The level of significance was set at 5% probability level.

Results and Discussion

Microorganism often secret varieties of enzymes to provide for their own metabolic needs under different physiological condition. Tannase is one such enzyme produced by microbes to protect themselves from tannin mediated toxicity and to generate glucose from tannin under scarcity of glucose in the surrounding environment[11]. Screening for tannase production from *Rhodotorula mucilaginosa* CBS 316 was found to have 34mm zone of tannic acid hydrolysis in 48hrs on agar plates fortified with tannic acid. This shows the ability of the yeast to utilize tannic acid as a source of carbon. Appearance of clear zone of hydrolysis around the colony on the agar plate indicates the ability of microorganism to produce enzyme[12]. Statistical analyses of Variance (ANOVA) showed that the tannnase activity from *R. mucilaginosa* CBS 316 was 724.49 U/g/min after 96hrs in submerged fermentation (Figure 1). Selwal and selwal[13] who produce microbial tannase using agro residue under submerged fermentation reported that this method is advantageous due to its better yield, process control and simple sterilization technique.
Effect of incubation temperature at different days showed that the activity of tannase production was highest at 35℃ for 72hrs. Thereafter, there was drastic reduction in tannase activities as the temperature was raised beyond 35ºC (Figure 2). Although Darah et al.,[14] reported maximal incubation temperature of 25ºC for tannase produced from *Rhodotorulla species*, nevertheless, higher temperature of above 30ºC is often preferred for industrial important enzymes. Effect of incubation pH was observed to be highest at pH 7 after which there was decreased tannase activity as shown in Figure 3. Tannase produced from *Rhodotorulla species* is maximal at pH 7.0[15].
Optimum temperature for tannase from *R. mucilaginosa* CBS 316 was 35℃ with 510.26 U/g/min tannase activities as shown in Figure 4. Further increase in temperature resulted in decrease in tannase activity which could be due enzyme denaturation at elevated temperature. Many of the microbial tannase exhibit an optimal temperature in the range between 30 and 40 °C[16]. Optimum activity was observed at pH 6 with the value of 506 U/g/min tannase activities as shown in Figure 5. This finding corroborated with work reported by Chong-Boon et al.,[15] who characterized tannase produced from red yeast, *Rhodotorulla glutinis* DB2. The authors explained that maximum tannase activity was observed at pH 6.0 with 100% activity, higher or lower than pH 6.0 conditions decreased the rate of enzyme reaction.
Metal ions and chelators affect tannase produced from *R. mucilaginosa* CBS 316 in different dimensions. $MgS0_4$, KCl, and $ZnS0_4$ increased the activity of tannase produced from *R. mucilaginosa* CBS 316. This was against the report of Mahmoud et al.,[17] who observed KCl as an inhibitor of the activity of tannase produced from *Pestalotiopsis rolfsii* and *Kluyveromyces marxianus*. $CdCl_2$ and $HgCl_2$ indicated little inducing effect while inhibitory effects on the enzyme activity were impacted by $PbCl_2$, MgCl, and $BaCl_2$, and EDTA as shown in Figure 6. This is in agreement with many findings for tannase produced from fungal species[18,19,20]. In addition, chelating action of EDTA can remove ions that are important for tannase activity[19].

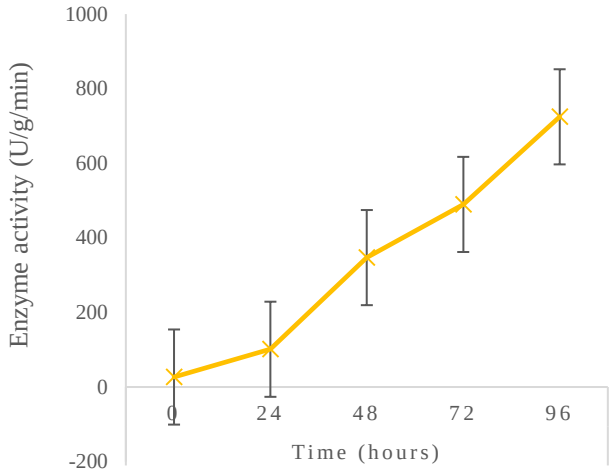

Figure 1: Submerged fermentation for tannase production by selected *Rhodotorula mucilaginosa* CBS316
Enzyme Activity at 0 Hours
Enzyme Activity at 24 Hours
Enzyme Activity at 48 Hours
Enzyme Activity at 72 Hours

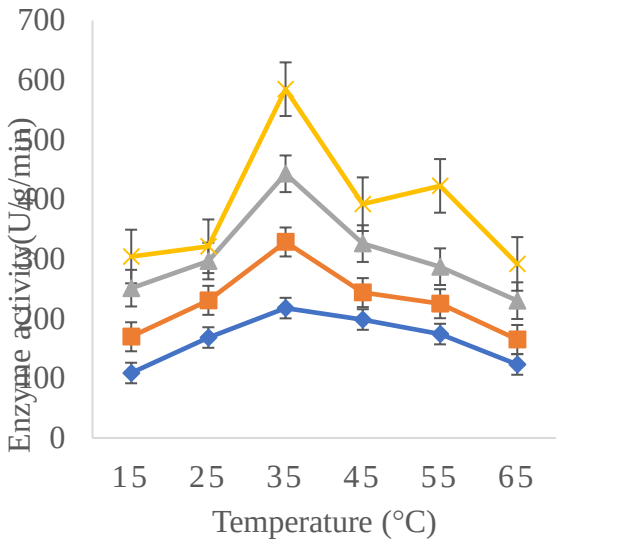

Figure 2: Effect of different incubation temperature on tannase activity from *Rhodotorula mucilaginosa* CBS316
Enzyme Activity at 0 Hours
Enzyme Activity at 24 Hours
Enzyme Activity at 48 Hours
Enzyme Activity at 72 Hours

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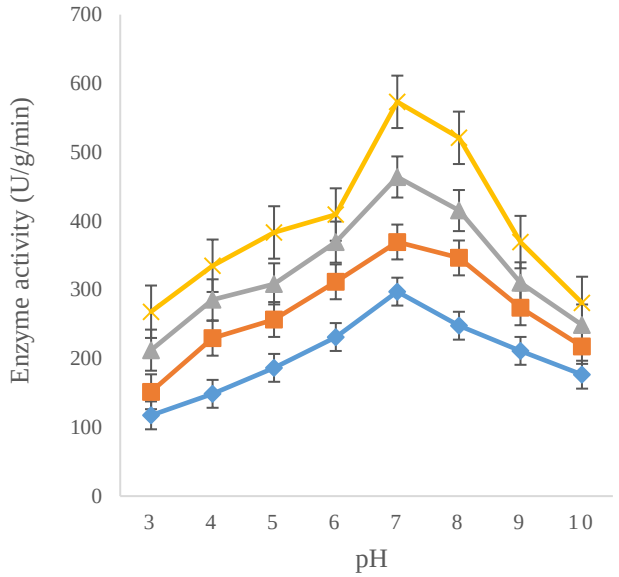

Figure 3: Effect of incubation pH on tannase activity produced from *Rhodotorula mucilaginosa* CBS 316.

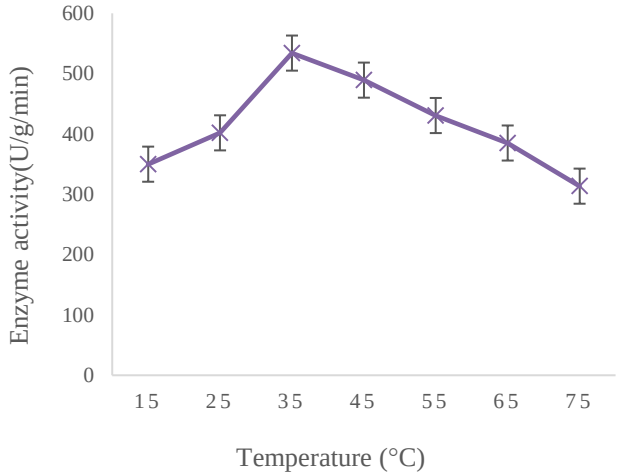

Figure 4: Optimum temperature for tannase produced from *Rhodotorula mucilaginosa* CBS 316.

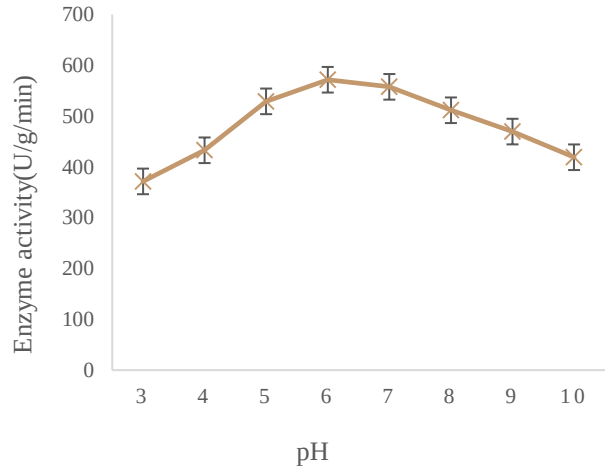

Figure 5: Effects of pH on tannase activity by selected *Rhodotorula mucilaginosa* CBS 316.

Tannase was immobilized with beads made from seed cotyledons of *Chrysophyllum albidum*. This is the first time that tannase immobilized on beads made from seed cotyledons of *Chrysophyllum albidum* will be reported. The specific activity of immobilized tannase was 633.16 U/g/min as shown in Table 1. A variety of materials of various origins can be used as supports for enzyme immobilization. These materials may, in general, be organic, inorganic and hybrid or composite. The support should protect the enzyme structure against harsh reaction conditions and thus, helps the immobilized enzyme to retain high catalytic activity[21]. Immobilized tannase was observed to be thermal stable at 35℃ with enzyme activity of 100.11U/g/min as shown in Figure 7. This is against the report of Bagga et al.[22] who reported that optimum temperature of tannase produced from *Aspergillus aculaetus* tannase was stable up to 50 °C.

Optimal tannase load on the beads made from seed cotyledons of *Chrysophyllum albidum* was 35% at 550.65 U/g/min enzyme activities as shown in Figure 8. Effect of bead size on immobilized tannase activity showed that 8mm bead size had the highest enzyme activity of 565.53 U/g/min as shown in Figure 9. This result did not corroborate with report of Kareem at al.,[23] who stated that the bead size of 5.0 mm was most effective for fungal specie. The number of immobilized beads that were most effective was 20 beads with 552.38 U/g/min enzyme activities (Figure 10). Effect of reusability of the immobilized enzyme was also studied, the enzyme was actively reuseable for up to 3 times and there was decrease in enzyme activity after 3 times of usage as shown in Figure 11. Immobilized tannase was reusable up to six successful reuses, thereafter, loss of activity from immobilized beads is assumed to be due to loss of enzyme protein[24].

The results of the tannin contents of Benin, Cotonu and local source rinds juices of *Ananas comosus* before treatment with immobilized tannase were 17.805 mg/100g, 16.305mg/100g and 5.845mg/100g, respectively as shown in Figure 12. Arsyada et al.[1] reported that *Ananas comosus* extract contains chemical compounds namely: flavonoids, saponins, tannins, enzymes bromelain and flavonoids in their highest concentration of chemical compounds. When the *Ananas comosus* ring juices were treated with immobilized tannase, the tannin contents were 3.73mg/100g, 2.92mg/100g and 1.86mg/100g, respectively (Figure 13). This reduction in the tannin content after treatment implied that the immobilized tannase was able to detannify great amount of the tannins in the *Ananas comosus* rinds juice. Cavalcanti et al.,[25] described that tannase from *A. niger* and *P. variable* tannases showed degradation of 45% and 36% of tannins after 48 h, respectively. However, this is the first report on removal of tannin content of *Ananas comosus* rind juice using immobilized tannase produced from *R. mucilaginosa* CBS 316.

Conclusion

The menace generated from agro wastes cannot be overemphasized. Wastes from *Chrysophyllum albidum* and *Ananas comosus* cannot be exempted in this category. Beads made from seed cotyledon of Chrysophyllum albidum were efficiently explored to immobilize tannase from *Rhodotorula mucilaginosa* CBS 316. The immobilized tannase drastically removed the tannin content of *Ananas comosus* rinds juice. This study shows the immobilization potential of *Chrysophyllum albidum* seed in enzyme technology for utilization of agro wastes. This can be explored as cost effective and eco-friendly substitute compare to imported immobilization materials.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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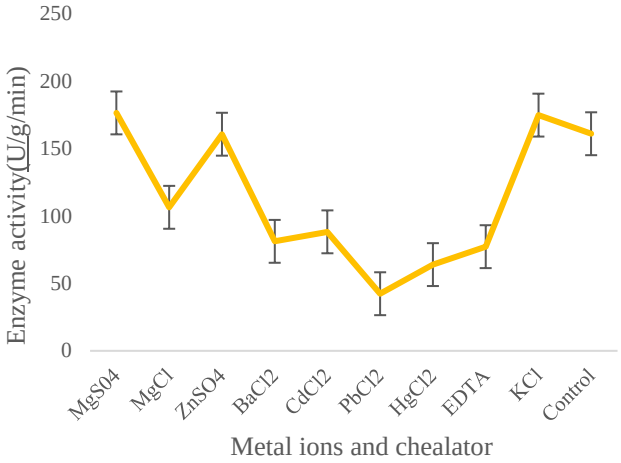

Figure 6: Effects of metal ions and chelator on activities of tannase produced from *Rhodotorula mucilaginosa* CBS 316

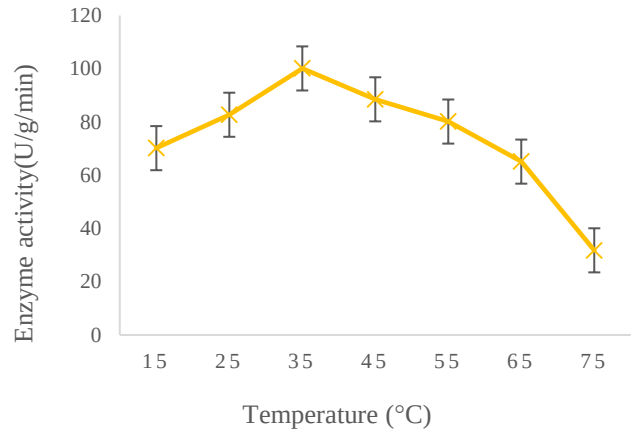

Figure 7: Thermal stability of immobilized tannase produced from *Rhodotorula mucilaginosa* CBS 316

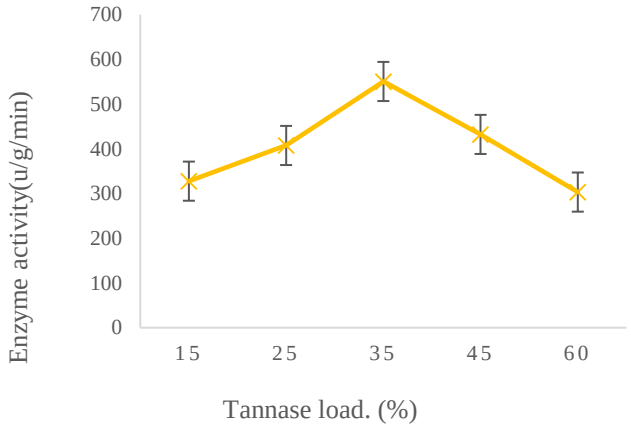

Figure 8: Effects of tannase load on beads using *Ananas comosus* rinds juice as substrate

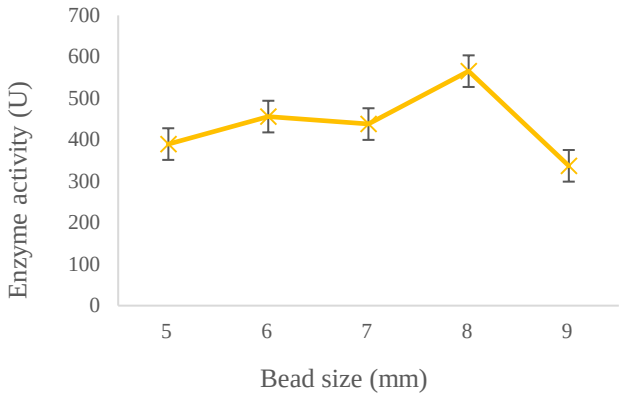

Figure 9: Effects of bead size on tannase produced from *Rhodotorula mucilaginosa* CBS 316 using *Ananas comosus* rinds juice as substrate

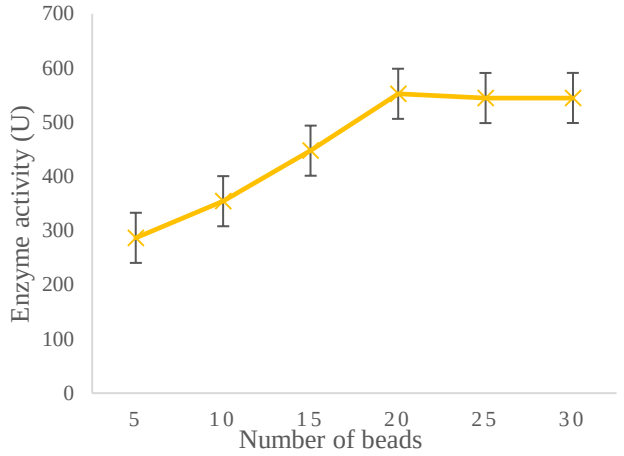

Figure 10: Effects of number of beads on tannase produced from *Rhodotorula mucilaginosa* CBS 316 using *Ananas comosus* rinds juice as substrate

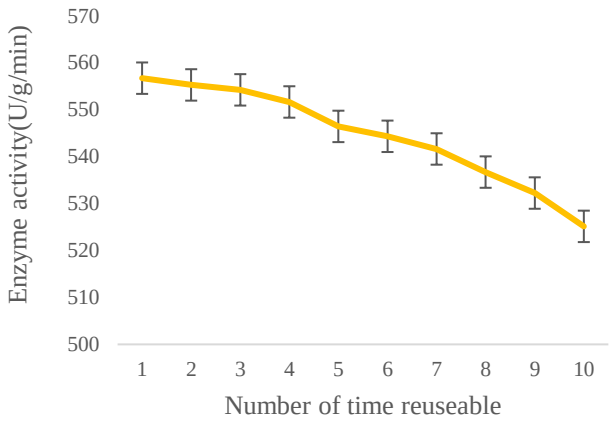

Figure 11: Effect of reusability of the immobilized tannase produced from *Rhodotorula mucilaginosa* CBS 316 using *Ananas comosus* rinds juice as substrate

Table 1: Immobilized Tannase Activity

Enzyme	Activity (U)	Protein Conc. (U/g/mim)	Specific activity (U/ml)
Tannase	497.77 ± 1.10^{b}	0.79 ± 0.03^{a}	633.16 ± 19.01^{c}

Mean±SD with different superscripts across the rows are significantly different ($p < 0.05$).

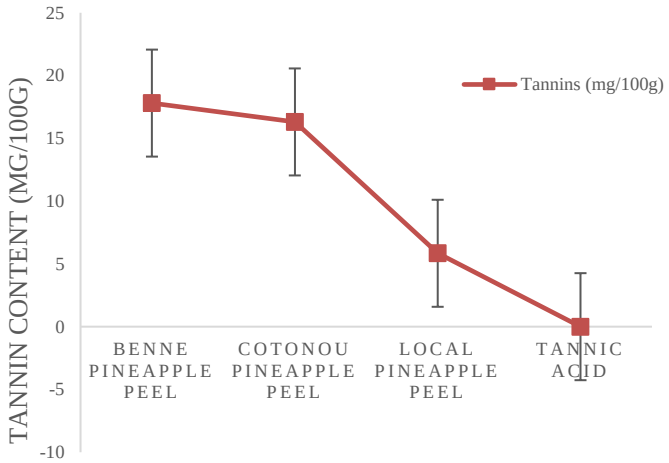

Figure 12: Tannin content of *Ananas comosus* rinds juice before treatment with tannase

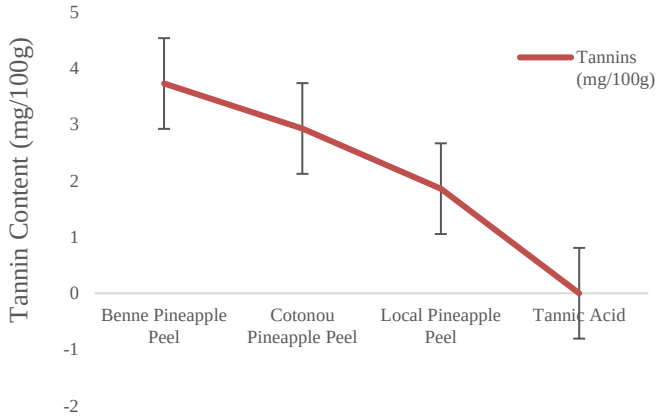

Figure 13: Tannin contents of *Ananas comosus* rinds juice after treatment with immobilized tannase produced by *Rhodotorula mucilaginosa* CBS 316

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