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Original Research Article

Tannase Production from Different Strains of *Rhodotorula species* using *Chrysophyllum albidum* Seed Shell Flour as Substrate

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ABSRTACT

Tannin is one of the major components of plants material. Tannase is an enzyme that breakdown tannin which is commonly implicated as an anti-nutritional factor in food and feed products. This study is aimed to determine the potential of *Chrysophyllum albidum* seed shell flour as substrate for microbial production of tannase. *Rhodotorula species* was isolated, screened and utilized for tannase production. A novel substrate, *Chrysophyllum albidum* seed shell flour, was unveiled for tannase production by solid state fermentation in this study. Six *Rhodotorula species* were isolated and characterized as *Rhodotorula graminis, Rhodotorula rubra, Rhodotorula mucilaginosa, Rhodotorula minuta, Rhodotorula toruloides* and *Rhodotorula glutinis* from thirteen soil samples obtained from different locations in Abeokuta, Ogun State, Nigeria. Only *Rhodotorula mucilaginosa* CBS 316, *Rhodotorula toruloides* strain bca-286 and *Rhodotorula glutanis* CBS20 were best tannase producers after screening and identification using molecular tools. Tannase activities from medium formulated with *C. albidum* seed shell flour were 542.45 U, 513.39 U and 489.50 U for *R. mucilaginosa* CBS 316, *R. toruloides* strain bca-286 and *R. glutanis* CBS20 respectively. Tannase activities were 610.75 U, 508.40 U and 485.85 U for *R. mucilaginosa* CBS 316, *R. toruloides* strain bca-286 and *R. glutanis* CBS20 respectively using medium formulated with wheat bran and sugar cane bagasse. This study indicated the potential of *Chrysophyllum albidum* seed shell flour as a novel substrate for tannase from strains of *Rhodotorula species*.

Keywords: *Persea Americana,* starch, disintegration, dissolution, tablet properties

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Introduction

Tannin acyl hydrolase commonly known as tannase (EC3.1.1.20) is an extracellular inducible enzyme that catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid, releasing gallic acid (3,4,5-trihydroxybenzoic acid) and glucose[1]. Hydrolysable tannins are found in most of the residues from higher plants. They are polyphenolic compounds formed by the association of sugar, gallic acid, and ellagic acid via ester linkages[2]. Tannic acid is a polyphenolic mixture of polyhydric alcohols that can easily bind and form insoluble complexes with virtually any protein. Tannins are considered as plant secondary metabolic product because they have less important role in the metabolism. Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins. The astringency from the tannins causes the dry and puckery feeling in the mouth following the consumption of red wine, strong tea, or an unripened fruit[3].
Tannase has numerous applications in skin care, pharmaceuticals, beverages, and in the food industry.

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The high tannin content in coffee-flavored beverages, fruit juices, wine, beer, and iced tea contributes to precipitation of other molecules in beverages[4]. Besides, tannase is also used in the enzymatic synthesis of propyl gallate (propyl 3,4,5-trihydroxybenzoate), which is primarily used as an antioxidant in fats, oils, and fat-containing food[4]. Tannase can be produced from plants, animals and microorganisms. Microbial enzymes are more preferred in the industries because of their ease of cultivation, biochemical diversity and amenability to genetic manipulation[5]. Among the microbial sources, production of tannase from yeast like *Rhodotorulla species* has been scarcely reported except in the study done by Darah et al[6].
Leftovers that are generated from agricultural and industrial activities mostly go waste, including those that are produced directly on the field during harvesting, and are, therefore, regarded as agro industrial residues[7]. Enzyme fermentation medium can utilize and convert different agro-lignocellulosic substrates – straws, husks and brans of cereals, bagasse, molasses, oil cakes, the peels and pulps of tubers, fruits and vegetables, paper pulp, etc., – into some of the industrial products[8]. The search for novel agro residue that can be explored as substrate with scarcely utilized microorganism for tannase production is very attractive, since many substrates are yet poorly known. Keeping this in view, this study revealed production of tannase from different strains of *Rhodotorula species* using *Chrysophyllum albidum* seed shell flour as substrate. To the best of our knowledge, this is the first report that explored *Chrysophyllum albidum* seed shell flour as substrate for tannase production by solid state fermentation.

Materials and methods

Sample Collection

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Soil samples from fruits waste dumping sites were collected from Olugbo, Osiele, Fadage and Obantoko in Odeda Local Government Area (7º18′45.0″N 3º32′42.5″E); Kobape, Siun, Asu and Ita-Osu in Obafemi-Owode Local Government Area (6º57′13.7″N 3º30′19.1″E); Sabo, Elega, Ilugun, Oke-Ata and Akomoje in Abeokuta North Local Government Area (7º13′04.1″N 3º20′59.6″E) in Ogun-State, Nigeria. *Chrysophyllum albidum* seed were equally obtained; the shells were removed, dried and milled into flour up to 300 millimicron particle size using Hammer mill.

Potato Dextrose Agar (PDA)
PDA (Oxoid) was prepared by weighing 39.0 g agar powder in 1L of distilled water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the medium. This was sterilized at 15lbs (121 °C) for 15 minutes using autoclave (NANBEI, NB-XD50D). The medium was allowed to cool to 45 to 50 °C, before it was poured aseptically into the sterile petri dishes. Agar plates were allowed to set then incubated in the incubator at 37 °C for 24hours. When no growth was observed, the plates were assumed to be sterile and used for further studies.

Sabouraud Dextrose Agar (SDA)
SDA (Oxoid) was prepared by weighing 65.0 g agar powder in 1L of distilled water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the medium. This was autoclaved at 121° C for 15 minutes and allowed to cool to 45 to 50°C, before it was poured aseptically into the sterile petri dishes. Agar plates were allowed to set and then incubated (MEMMERT, E07086) in the incubator at 37 °C for 24 hours. When no growth was observed, the plates were assumed to be sterile and used for further studies.

Rose Bengal Chloramphenicol Agar (RBCA)
RBCA was prepared by weighing 32.2 g in 1L of distilled water. This was heated with frequent agitation and boiled for 1 minute to completely dissolve the medium and autoclaved at 121° C for 15 minutes. The medium was allowed to cool to 45 to 50°C, before it was poured aseptically into the sterile petri dishes. The appearance of the prepared medium was pink to red. It was stored in the dark; the shelf life of plates was approximately 1 week and in bottles approximately 2 months.

Yeast Dextrose Peptone Agar (YEDPA)
Yeast dextrose peptone agar (YEDPA) was prepared by weighing 65.0 g in 1L of distilled water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the medium. This was autoclaved at 121° C for 15 minutes and allowed to cool to 45 to 50°C, before it was poured aseptically into the sterile petri dishes. These agar plates were allowed to set and then incubated in the incubator at 37 °C for 24 hours. When no growth was observed, the plates were assumed to be sterile and used for further studies.

Isolation of microorganisms from soil samples
Ten grams (10.0g) of each soil sample were separately homogenized in 90 mL sterile peptone water for 5 minutes. The homogenized samples were used for ten folds serial dilutions. One ml from serial dilutions 10^{-3} and 10^{-5} were spread on the prepared RBCA, YEDPA, PDA and SDA containing 50 mg/L chloramphenicol and 50 mg/L chlortetracycline to inhibit bacterial growth. The plates were incubated at 30 °C for 5 days. Colonies were then counted and expressed as colony forming units (cfu) per gram of the samples[9].

Maintenance of cultures
The isolated yeasts were maintained by sub culturing on Yeast Maintenance Media slants, incubated for 48 hours at 30 °C, and thereafter, stored in the refrigerator at –4 °C for future use[10].

Identification of Isolated Yeasts
The purified yeast colonies were identified by ID 32 C V2.0 API kit and standard tests classification schemes as described by Barnett et al[11]. The tests included those for colony and cell morphology, sporulation, fermentation tests and pseudomycelium formation.

Screening of Isolated Yeasts for tannase
The isolated yeasts were screened for tannase production using Dimitri's agar medium containing (g/L) tannic acid (10.0), sodium nitrate (3.0), potassium phosphate (1.0), hydrated magnesium sulphate (0.5), hydrated Iron sulphate (0.01) and agar (30.0). The plates were point inoculated with the inoculums after which incubation was done at 32 °C for 72 hrs. The zones of hydrolysis which determine the potential of organism in tannase production were noted[12].

Molecular Identification of Yeast Isolates
DNA extraction
Genomic DNA extraction was carried out on the isolates using the Zymo Fungal DNA extraction kit according to manufacturer's instructions.

PCR Amplification of the Internal Transcribed Spacer (ITS) gene
Polymerase chain reaction was carried out to amplify the internal transcribed spacer (ITS) gene of the yeast using the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µL of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25 pMol of each primer (StabVida, Protugal), 2 unit of Hot FIREPol DNA polymerase (Biodyne); however, additional Taq DNA polymerase was incorporated into the reaction mixture to make a final concentration of 2.5 units of Taq DNA polymerase, proof reading enzyme, 2 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling
Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95 °C for 15 minutes, followed by 35 amplification cycles of 30 seconds at 95 °C: 1 minute at 58 °C and 1 minute 30 Seconds at 72 °C. This was followed by a final extension step of 10 minutes at 72 °C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out by transmulator ultraviolet light (ES-13090UV04) at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. After which 100 bp DNA ladder was used as DNA molecular weight standard. Finally, sequencing of genes was performed.

Production of Tannase using sugar cane bagasse and wheat bran
Five grams of mixed substrate of wheat bran powder and sugarcane bagasse powder (1:1 ratio) was taken in 250 mL Erlenmeyer flask and moistened with 5 mL of salt solution. The composition of the salt's solution was NH_4NO_3 0.5 %, NaCl 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 % and tannic acid 4% at pH 5.5. The contents were sterilized by autoclaving at 121°C, 15lbps for 20 minutes. The cooled sterilized solid substrate were inoculated with 1.0 mL of the spore inoculums, mixed properly and incubated at 30 °C for 96 hours.

Production of Tannase using Chrysophyllum albidum seed shells flour
Also, five-grams of mixed substrate of *C. albidum* seed shell flour with sugarcane bagasse powder (1:1 ratio) were taken in 250-mL Erlenmeyer flask and moistened with 5 mL of salt solutions (NH_4NO_3-0.5 %, NaCl-0.1 %, $MgSO_4 \cdot 7H_2O$-0.1 % and tannic acid 4% at pH 5.5. The contents were sterilized by autoclaving at 121 °C, 15lbps for 20 minutes. The cooled sterilized solid substrate was inoculated with 1.0 mL of the spore inoculums, mixed properly, and incubated at 30 °C for 96 hours.

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Tannase extraction

Tannase was extracted according to standard enzyme extraction method[13]. At the end of incubation, the cell-free filtrate was obtained by filtering through Whatman no. 1 filter paper. The filtrate was centrifuged at 3400× g for 14 minutes at 4 °C and this serves as a crude enzyme preparation. The crude enzyme was stored at -20 °C for further analysis.

Tannase assay

Tannase activity produced by isolates was assayed using the Rhodanine method[14]. Solution containing 50 µL of enzyme was incubated with 100 µL of methyl gallate (0.01 M concentration) for 5 minutes at 37 °C. At the end of incubation time, the reaction was stopped by adding 300 µL methanolic rhodanine (0.667 %) and the tubes were incubated for 3 minutes at room temperature. To all tubes, 100 µL of 0.5 M KOH and water was added to a final volume of 2 mL before taking reading at 520 nm. Control tubes were incubated with same concentration of heat denatured enzyme along with substrate and methanolic rhodanine, 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 1 µm of gallic acid per mL per minutes.

Statistical analysis

Data were analyzed using Statistical Package for 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 produced by isolates and data on tannase activity observed under the different conditions. Means were separated by Duncan's multiple tests. The level of significant was set at 5% probability level.

Result and discussion

Soil samples were obtained from thirteen (13) different fruits waste dumping sites in Abeokuta Ogun state metropolis in Nigeria. Yeast counts from these soil samples on Rose Bengal Chloramphenicol (RBC) Agar, Yeast Dextrose Peptone Agar (YEDPA), Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) were shown in Table 1. These isolates were first identified using biochemical test as indicated in Table 2. A total number of six *Rhodotorula species* were isolated and characterized as *Rhodotorula graminis, Rhodotorula rubra, Rhodotorula mucilaginosa, Rhodotorula minuta, Rhodotorula toruloides* and *Rhodotorula glutinis.* Most associated microorganisms, especially yeasts, exhibited specific characteristics in terms of tannin tolerance and tannase production[15]. Although the qualitative screening for tannase using agar assay technique revealed that four (4) of these *Rhodotorula species* showed clear zones of tannin hydrolysis around their colonies as shown in Table 3. Appearance of clear zone of hydrolysis around the colony on the agar plate indicates the ability of microorganism to produce enzyme[16].

The three best tannase producing yeast isolates were characterized using molecular tools and identified as *Rhodotorula mucilaginosa* CBS 316, *Rhodotorula toruloides* strain bca-286 and *Rhodotorula glutanis* CBS20. Kanpiengjai et al[17], who assessed 82 yeasts for tannase production reported that a total of 12 strains exhibited positive results for tannase activity. The authors further stated that yeast tannin-tolerant ability had species variability. *Rhodotorula mucilaginosa* CBS 316 had total nucleic acid of 620 nucleotide bases long with Sequence ID: NR_073296.1, *Rhodotorula toruloides* strain bca-286 had total nucleic acid of 589 nucleotide bases long with Sequence ID: MN128864.1 and *Rhodotorula glutinis* CBS 20 had total nucleic acid of 610 nucleotide bases long with Sequence ID: NR_073294.1. Phylogeny tree showing evolution of the *species* and the branching representing speciation of the identified organisms were shown in Figure 1. The three *Rhodotorula* isolates with higher zones of tannic acid hydrolysis were further used to produce the tannase enzyme.

Tannase activities by the *Rhodotorula species* were 610.75 U, 508.40 U and 485.85 U for *R. mucilaginosa* CBS 316, *R. toruloides* strain bca-286 and *R. glutanis* CBS20 respectively using solid state fermentation formulated with wheat bran and sugar cane bagasse as carbon source at 96 hours as shown in Figure 2. Tannase produced from *R. mucilaginosa* CBS 316 had the highest. Zepf and Jin[18], who examined various agro residues including sugar cane bagasse and wheat bran for enzymes production explained that the substrate does not only serve as a source of nutrients but also acts as solid support on which the microorganisms thrive. These authors further explained that enzyme production is often simple, when agro residues like wheat bran, sugar cane bagasse, rice bran or wheat straw are used as substrate. Hence, enzyme activity is usually very high.

Studies on previous research indicated that Chrysophyllum albidum seed shell flour has never reported for utilization as carbon source for tannase production. Tannase activities for the solid state fermentation formulated with *C. albidum* seed shell flour were 542.45 U, 513.39 U and 489.50 U for *R. mucilaginosa* CBS 316, *R. toruloides* strain bca-286 and *R. glutanis* CBS20 at 96 hrs respectively as shown in Figure 3. This is the first study that utilized *C. albidum* seed shell flour for tannase production by solid- state fermentation. *C. albidum* revealed the presence of flavonoids, phenols, glycosides, terpenoids, saponins, steroids and alkaloids[19]. MacDonald et al[20], also revealed the presence of alkaloids, flavonoids, saponins and tannins in *C. albidum*. These can be utilized as carbon source for tannase production. Findings from this study revealed that the tannase activity increased progressively as the incubation period was increased. After 96hrs, increased incubation period did not resulted in further increase in tannase activity. Famotemi *et al*[5].

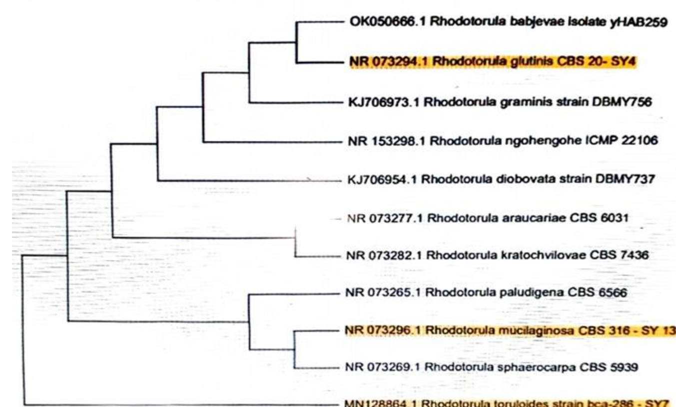

Figure 1: Phylogenetic tree of the identified *Rhodotorula species*

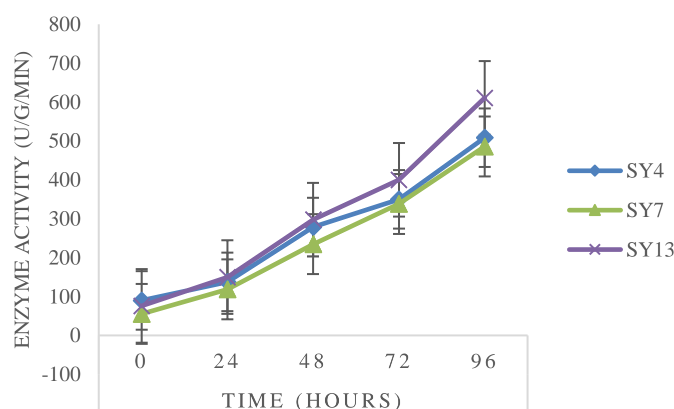

Figure 2: Solid state fermentation (conventional method) for tannase production by selected *Rhodotorula* isolates

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Note:
SY4: *Rhodotorula glutinis*
SY7: *Rhodotorula toruloides*
SY13: *Rhodotorula mucilaginosa*

Table 1: Yeast counts from thirteen soil samples collected from different fruits waste dump sites (cfu/g)

Sample code	RBC Agar (S.D ±)	YEDPA (S.D ±)	PDA (S.D ±)	SDA (S.D ±)
CPSS1 (Olugbo)	47.50±2.12	60.00±2.83	51.00±9.90	27.00±4.24
CPSS 2(Osiele)	113.50±1.34	56.50±3.54	75.50±4.95	37.00±1.41
CPSS 3(Fadage)	42.50±3.54	59.50±4.95	52.50±3.54	40.00±2.83
CPSS 4(Kobape)	70.00±2.83	43.00±2.83	63.00±4.24	45.00±4.24
CPSS 5 (Asu)	56.50±3.54	72.00±2.83	59.00±5.66	37.50±2.12
CPSS 6 (Obantoko)	62.00±2.83	64.00±2.83	72.00±1.41	60.00±2.83
CPSS 7 (Ita-Osu)	67.00±9.90	52.50±4.95	62.00±5.66	66.50±3.54
CPSS 8 (Siun)	64.00±5.66	57.50±2.12	71.50±2.12	50.50±2.12
CPSS 9 (Sabo)	57.00±1.41	47.50±2.12	71.00±7.07	53.00±1.41
CPSS 10 (Elega)	69.50±2.12	36.00±2.83	54.50±3.54	40.50±3.54
CPSS 11 (Ilugun)	61.50±2.12	42.00±2.83	45.00±4.24	51.50±2.12
CPSS 12 (Oke-Ata)	42.00±2.83	56.00±2.83	46.00±4.24	48.00±2.83
CPSS 13 (Akomoje)	73.00±4.24	55.00±1.41	47.50±2.12	67.50±3.54

Table 2: Biochemical tests for yeast isolates identification

Isolate code	Color	Cellular morphology	Motility Test	Catalase	$N0_3$ Reduction	Ascospoe formation	Pseudomycellium Prodction	Glucose	Xylose	Raffinose	Sucrose	Fructose	lactose	Maltose	Mannitol	Melibiose	Trehalose	Arabinose	Galactose	Probable Identity
SY1	Red	Elongated	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	*Rhodotorula Graminis*
SY2	Red/ Pink	Cylindrical	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	*Rhodotorula Rubra*
SY3	Red	Elongated	+	+	-	-	-	+	+	+	+	-	-	+	-	-	+	+	+	*Rhodotorula mucilaginosa*
SY4	Red/ Pink	Elongated	+	+	-	-	-	+	+	+	+	-	-	+	-	-	+	+	+	*Rhodotorula mucilaginosa*
SY5	Red/ Pink	Cylindrical	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	*Rhodotorula Rubra*
SY6	Pink	Elongated	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	+	-	*Rhodotorula Minuta*
SY7	Red	Elongated	+	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	*Rhodotorula toruloides*
SY8	Red/ Pink	Cylindrical	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	*Rhodotorula Rubra*

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SY9	Red/ Pink	Elongated	+	+	-	-	-	+	-	+	+	-	-	+	+	-	+	+	+	*Rhodotorula Glutinis*
SY10	Red/ Pink	Elongated	+	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-	*Rhodotorula toruloides*
SY11	Red	Elongated	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	*Rhodotorula Graminis*
SY12	Red	Elongated	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	*Rhodotorula graminis*
SY13	Pink	Elongated	+	+	-	-	-	+	-	+	+	-	-	+	+	-	+	+	+	*Rhodotorula glutinis*

Table 3: Clearance zones (mm) exhibited by various yeast for Tannase screening

Isolate	24hrs (mm)	48hrs (mm)	72hrs (mm)
SY4	18[b]	28[b]	43[b]
SY6	10[c]	10[c]	10[c]
SY7	20[b]	33[a]	64[a]
SY13	22[a]	34[a]	64[a]

[abc] mean value with the same superscript alphabet in a column are not significantly different ($p < 0.05$)

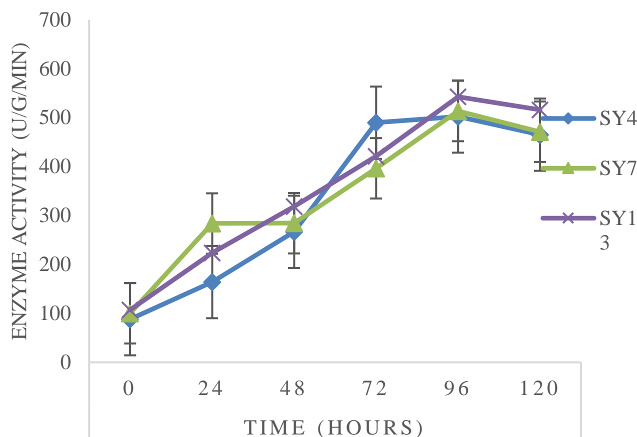

Figure 3: Solid state fermentation (modified method) with *chrysophylum albidum* seed shell by selected *Rhodotorula* isolates

Note:
SY4: *Rhodotorula glutinis*
SY7: *Rhodotorula toruloides*
SY13: *Rhodotorula mucilaginosa*

Conclusion

This study showed the prospect of *Chrysophyllum albidum* seed flour for use as substrate for microbial tannase production. *Rhodotorula species* has been underutilized for tannase production. To this end, utilization of *Rhodotorula species* can be recommended for converting *Chrysophyllum albidum* seed flour to tannase for products of industrial important.

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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