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OPTIMIZATION AND CHARACTERIZATION OF PECTINASE PRODUCED BY *ASPERGILLUS FLAVUS* SS13 USING CITRUS PEEL

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ABSTRACT

Pectinases have multipurpose applications in both industrial and biotechnological sectors. But they are expensive when available. This study was carried out to isolate fungi, screen them for pectinase activity, identify selected fungi isolate, mutate, optimize, produce, and characterize the partially purified pectinase. *Aspergillus flavus* SS13 was isolated using standard technique, it exhibited the highest pectinase activity of 36.0±0.3 mm zone of hydrolysis and it was identified based on its macroscopic and microscopic characteristics. EMS mutated *A. flavus* SS13 had a pectinase activity of 40±0.2 U/mL, UV-mutated *A. flavus* SS13 exhibited 36.0±0.5 U/mL while the SA-mutated *A. flavus* SS13 showed pectinase activitiy of 35±0.4 U/mL, and unmutated A flavus SS13 had 28.0 ±0.2 U/mL at pH 3.0, temperature 40 °C and incubation period of 96 h utilizing orange peel as carbon. The partially purified pectinase of *A. flavus* SS13, *A. flavus* SS13-EMS, *A. flavus* SS13-UV, and *A. flavus* SS13-SA had between 1.3 to 1.5 fold purification and 26.0 to 30.0 % recovery. The partially purified pectinase of *A. flavus* SS13 wild type and the three mutants had improved activity in the presence of tween-80, while a significant pectinase activity was noted with cations such as Cu^{2+}, $Ca^{2+,}$ and K^{+} in all the three mutants' enzyme while the ions of ammonium, cobalt, and sodium repressed pectinase activity in the wild type *A. flavus* SS13. The stability of the pectinase produced by the mutants at acidic pH and elevated temperature could be an added advantage for its diverse applications in the food, detergent, and leather industries.

Keywords: *Aspergillus flavus* SS13, Pectinases, Mutagenesis, Optimization, Characterization

INTRODUCTION

Pectin is a high molecular and basic constituent found between the cell wall and lamella of higher plants. It consists of acidic heteropolysaccharides that are linked together by α-1, 4- glycosidic bond. Pectin along with cellulose and hemicellulose are responsible for the structural integrity of higher plants (Carrasco et al. 2019). To break down this complex biomaterial called pectin for industrial purposes, there is a need for the use of enzyme pectinases. Most industrial and biotechnological processes can hardly be successful without enzymes. The treatment of substrates with the aid of chemicals is fading away quickly due to their side effects and is being replaced by enzymes of microbial origin (Haile & Ayele, 2022).

Pectinases are made up of a complex group of enzymes that are capable of breaking down the bonds holding pectin units together. Among this group of enzymes are significant ones such as lyases of pectin and pectate, polygalacturonase, and pectin esterase (Haile & Ayele, 2022). Pectinases or pectinolytic enzymes have diverse industrial and biotechnological applications such as removal of cloudiness in fruit extract that is usually cloudy as a result of the presence of pectin, treatment of pulp as well as bleaching of papers, environmental treatment of wastes, breaking down of cellulose, hemicellulose, and lignin while producing bioethanol (Ezugwu et al. 2023).

Microorganisms notably fungi that belong to the genera *Aspergillus* as well as *Penicillium* have been mentioned to be good producers of pectinase. Madika et al. (2020) reported that all the fifteen *Aspergillus* species they isolated exhibited pectinase activity in their study. They are mostly used in pectinase production compared to bacteria and yeasts (Antier et al. 1993). The choice of microbial strains needed in producing industrial enzymes that include pectinases remains an important factor in the success of industrial processes (Haile & Ayele, 2022). Pectinases just like other industrial enzymes are still expensive and not available as expected due to patency and the use of refined substrates (Adeleke et al. 2012; Ajayi et al. 2021). Hence, there is a need to isolate and screen for indigenous strains with considerable pectinase activity. In addition to this, one of the ways by which the industrial production of pectinases can be increased is by strain modification through mutation (Antier et al. 1993). The exposure of filamentous fungi or their spores to different mutagens has been an acceptable technique by which mutants with a high yield of pectinases are generated. Mutation agents like dinitrogen oxide, diethylsulfate, as well as ethyl methanesulfonate have been mentioned to be effective mutagens (Premjet, 2012). Nigeria with large arable lands generates a lot of biomaterials from plants such as orange peel known to be rich in pectin and an inducer of pectinolytic enzymes can be utilized in pectinases production (Göğüş et al. 2014; Jekayinfa et al. 2020). Nigeria ranked high among citrus producers in the world with the peel wasting away annually. This citrus plant biomass could be used in the production of valuable pectinase that is needed in food and pharmaceutical industries. Hence, this work aimed to isolate, identify fungi, screen the fungi for pectinase activity, mutate the selected fungus as well as production using citrus peel as carbon and energy sources, optimization and characterization of pectinase produced by the wild and mutant strains.

MATERIALS AND METHODS

Samples collection

Samples consisting of sawdust, decaying wood, wastewater, and soil were collected from different points in Yola, Adamawa State, Nigeria. Samples collected were placed in sterile bottles and then conveyed to the Microbiology Department at Modibbo Adama University, Yola for laboratory work.

Agro-wastes such as the peels of banana, orange, and yam as well as rice bran, and oil palm fibre were sourced from local markets in Girei Local Government Area of Adamawa State and Otukpo Local Government Area of Benue State.

Isolation, screening, and identification of pectinase-producing fungi

To carry out isolation, one gram each of soil, decaying wood, and sawdust were mixed in nine millilitre (9 mL) sterilised water in different test tubes and diluted up to 10^{-7} using a ten-fold serial dilution technique. One millilitre of wastewater was also transferred into 9 millilitre of sterilised water and was diluted serially up to the seventh test tube using a tenfold dilution technique based on the description of Kamalambigeswari et al. (2018).

Aliquots of 0.1 mL from 10^{-3} to 10^{-5} were spread on 10-15 mL sterile Sabouraud Dextrose Agar (SDA, Himedia) plates that have been seeded with antibiotic (Chloramphenicol 0.5 mg/L) to inhibit the proliferation of bacteria. The medium was incubated at ambient temperature. The culture medium was observed for between 3-5 days for fungal growth and the plates showing growth of fungi were sub-cultured onto fresh plates of SDA to get pure isolates. The pure cultures were identified using macroscopic and microscopic characteristics. The cultures obtained after sub-culturing were inoculated on SDA slants and kept at 4 ºC in a refrigerator until hen needed.

During the screening for pectinase-producers, spores of different fungi were cultured on modified pectin agar (g/L) Pectin 5.00, K_2HPO_4 0.50, $MgSO_4.7H_2O$ 0.10, NaCl 0.20, $CaCl_2.2H_2O$ 0.20, $FeCl_3.6H_2O$ 0.01, Yeast extract 1.00, Agar 20.0 and incubated for four days (Amilia et al. 2017). The clearance zone formed around colonies was determined by pouring in excess the plates with potassium iodide-iodine solution (5.0g KI and 1.0 g of iodine in 330 mL of distilled water). The zone of clearance was determined using the metre rule. Fungi with good pectinase activities according to the hydrolytic zone were picked for the next levels of the study.

To identify the fungi, the colony characteristics were considered. These characteristics are size, aerial view, arrangement, morphology of the conidiophores, texture (moist mycelium), and colony colour were observed.

Microscopic characteristics such as the type of mycelium is it septate or not, conidia, as well as the kind of reproductive structures were observed using the lactophenol blue staining method. The slide was viewed using the x40 objective eyepiece of a light microscope. The observed features of the fungi were juxtaposed with those earlier presented by Domsch et al. (1980).

Chemical mutagenesis

Ethyl methane sulfonate (EMS)

The spores of the selected fungal species were grown on SDA at room temperature (28 ºC – 30 ºC). They were collected, then dispersed in twenty millilitres of distilled water. The control consisted of one millilitre of sodium phosphate at pH 7 which served as the buffer added to nine millilitre of fungal spore suspension. To effect chemical mutagenesis, a mixture of ethyl methanesulfonate of 150 µg/mL and sodium phosphate buffer of pH 7 was made. Furthermore, one millilitre of this mixture was introduced into a nine-millilitre fungal spore suspension. Both the control and treated fungal spores were kept for an hour in a room. After incubation, the treated sample and the control were subjected to centrifugation for half an hour at 4500 rpm. It was then washed with water free from microbial contaminants. This was followed by re-suspending the samples in sodium phosphate buffer of pH 7. and centrifuging at 4500 rpm for 120 seconds. The pellets obtained were then suspended in a millilitre of sodium phosphate buffer at pH 7. The pellet suspensions were diluted, and a hundred microlitre of 10^{-6} dilution was placed on SDA followed by incubation at ambient temperature for two days to assess the status of the fungal isolate treatment with EMS and to retrieve mutants. The mutants were picked randomly based on their appearance on the SDA plate, stored on the SDA slant, and kept at 4 ºC in a fridge till it is required for further use. The stability of the mutant was checked by subculturing the mutant on the SDA plate (Kamalambigeswari et al. 2018).

Sodium azide

Spores of the selected fungi that were cultured at room temperature were harvested after 24 h in 5.0 mL decontamined water. Two millilitre each of the spore suspension was placed in two test tubes, one serving as the control and the other contained spore suspension and 0.5 mL of 0.1 % sodium azide. Both were incubated at ambient temperature and a varying period of 20, 40, and 60 min. This was by centrifugation at 3,000 rpm for 10 min, then washed in a phosphate buffer of pH 7.0. A one hundred microlitre of the washed spores was placed on Petri dishes of SDA, and incubated for 2 days at ambient temperature. The mutated colonies were picked randomly based on their appearance on SDA plates and kept on SDA slants in a fridge at 4 ºC until they were to be used for further study. The stability of the mutant was observed by sub-culturing the mutant on the SDA plate taking note of the appearance of the mutant (Kamalambigeswari et al. 2018).

Physical mutagenesis

Ultra violet (UV) irradiation

Physical mutagenesis was carried out with UV irradiation using the description by (Popa, Israel-Roming, & Cornea, 2017). 5.0 mL sterile water was spread on the surface of the 96 h culture of the selected fungi for mutagenesis to get spore suspension. A suspension of the spores was made out of which one millilitre was taken into two different test tubes, one to serve as the treated sample and the other to serve as the control. The two test tubes containing the spore suspensions were placed within the irradiation of wavelength 254 nm at a distance of 10 cm for 50 min. This was done in a vertical laminar flow cabinet in a dark room to prevent photo-reactivation. The Petri dishes were left open and mixed occasionally. After each UV treatment period, 1.5 mL of the UV-treated isolate was removed for study. The UV-treated spore suspension was diluted serially, and a hundred microlitre was dispensed onto SDA, spread evenly, and then incubated at room temperature for 2 days. This is to assess whether isolates have been mutated or not. The mutated isolates were identified by their phenotypic changes and then selected for further work.

The mutant obtained from the three different treatments were screened for pectinase production and activity and the strain with the highest pectinase activity was used for the production of pectinase. The mutants were preserved on SDA slants, and placed in a fridge at 4 ºC. The stability of the mutant was checked periodically by sub-culturing the mutant and morphological characteristics of the mutants on the SDA plate were noted.

Pectinase production

Pectinase was produced by submerged fermentation using a basal medium that contained (g/litre) 10 glucose, 1.4 g $(NH_4)SO_4$, 6 g K_2HPO_4, 2 g KH_2PO_4, 0.1 g $MgSO_4.7H_20$ and 1000 mL (Soares et al. 1999). Two hundred and fifty millilitre of the sterilised medium was inoculated with five millilitre of

the spore suspension of the wild-type isolate and the three mutants.
Fermentation was done in 250 mL of the sterilized production medium which was inoculated with 5.0 mL (4.39×10^6 spores) of inoculum each of wild type and the three mutants. Incubation took place at room temperature for 96 hours on a rotary shaker at 160 rpm. The fermented broth was then centrifuged at 10,000 rpm for 15 min. The supernatant was used to assess the pectinase activity. (Ahmed et al., 2015).

Enzyme assay
The method described by Okafor et al. (2010) was used to evaluate pectinase activity using pectin as the substrate. One millilitre of pectin (1.0 %) prepared in sodium acetate buffer of pH 5.5 was combined with one millilitre of the crude enzyme and incubated at 50 °C for 30 min. Furthermore, a dinitrosalicylic acid solution was prepared (DNSA 1.0 g, 2M NaOH 20 mL, sodium tartrate 30 g, and distilled water 50 mL) from which 1 mL was taken and dispensed into a mixture of incubated pectin and crude enzyme. The mixture was allowed to stand in boiling water for 10 min, after which it was cooled to ambient temperature. A similar mixture was prepared but without the crude enzyme to serve as the control. The method described by Miller, (1959) was used to assess the amount of reducing sugars produced. Three millilitre of glucose was prepared and one millilitre of DNSA was introduced, the mixture was placed in a boiling water for 5 min. The solution was then cooled under running water to room temperature. The strength of the colour of the solution was determined using a spectrophotometer at 540 nm. The pectinase activity was said to be the quantity of the enzyme that could liberate one micromole (1.0 µmol) of D-galacturonic acid per min.

Optimization of pectinase Production condition of wild-type and mutant
The influence of different factors that could affect pectinase production was investigated using the description of Thakur et al. (2010). These are nutritional and environmental factors such as carbon and nitrogen sources, incubation period, temperature, and pH.

Effect of different carbon sources on pectinase production
To assess the influence of carbon sources on pectinase production and activity, cheap and readily available agro-wastes such as the peels of banana, orange, and yam as well as rice bran, and palm oil fibres were used. The agro residues were dried at room temperature, then milled to fine particles and stored in airtight containers at ambient temperature till when they were used. These powdered agro-wastes along with glucose, sucrose, and maltose were used to assess the effect of carbon sources on pectinase production. Each of the carbon sources was used to substitute the carbon source in the basal medium used for crude production (g/litre) 10 glucose, 1.4 g $(NH_4)SO_4$, 6 g K_2HPO_4, 2 g KH_2PO_4, 0.1 g $MgSO_4$. $7H_20$ and 1000 mL (Soares et al. 1999). Incubation and extraction of the crude pectinase were as described in the production. The pectinase activity was determined using culture supernatant.

Effects of different nitrogen sources on pectinase production
The influence of different nitrogen sources on pectinase production was assessed by incorporating peptone, urea, ammonium nitrate, and yeast extract in the basal medium in place of ammonium sulphate. Incubation was done at room temperature after filtration and centrifugation at 10000 rpm for 15 min were carried out to obtain a clean supernatant that contained the crude pectinase. The obtained crude enzyme was used for pectinase activity.

Effect of different incubation times on the production of pectinase
Different incubation times 24, 48, 72, 96, 120, 144, and 168 h were used during the production of pectinase. The pH medium was adjusted to pH 5 using acid/base while the ambient temperature was used. At the end of each incubation time, extraction of the crude pectinase was carried out using filter paper (Whatman No1). The crude enzyme was extracted by filtration using Whatman No 1 filter paper and the filtrate was centrifuged at 10,000 rpm for 15 min. The culture supernatant was used to determine pectinase activity.

Effect of temperature on the production of pectinase
The impact of temperature on pectinase production was carried out by incubating the production medium at different temperatures of 25, 30, 35, 40, 45, 50, and 55 °C for four days at a constant pH of 5. The fermented broth was subsequently filtered with Whatman No 1 filter paper and then for 15 min, centrifugation was carried at 10,000 rpm. The collected supernatant was employed in determining pectinase activity.

Effect of pH on production of pectinase
The influence of pH was done by adjusting the medium pH to different pH values. These values are 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using 0.1 M buffers of different buffering capacity; namely sodium acetate buffer (4.0, 4.5, 5.0 and 5.5) and phosphate buffer (6.0-8.0). After a fermentation period of 4 days, filtration and centrifugation were done to obtain crude pectinase. The crude pectinase was used to ascertain the activity of pectinase.

Partial purification of crude enzyme
Ammonium sulphate precipitation of the crude enzyme was carried out by adding 32.8 g of ammonium sulphate (30 %). The suspension was centrifuged for 10 min at 6000 rpm. The pellet obtained was suspended in a small quantity of sodium phosphate buffer of pH 7. Furthermore, 103. 2 g of ammonium sulphate was slowly added at 4 °C. The mixture was kept overnight in a refrigerator at 4 °C. This was followed by centrifuging the suspension at 6000 rate per minute (6000 rpm) for 15 min. The obtained pellets were dissolved in sodium phosphate buffer pH 7.

Dialysis
Dialysis was done following the technique of Carmona et al. (1998). Dialysis was carried out in a dialysis tube which was treated by boiling the tube with sodium bicarbonate (2 percent) for 10 min. The boiling eliminates sulphides from the tube. The tube was further washed in using distilled water. To remove heavy metals from the tube, the tube was boiled for 15 min in 10 mM EDTA solution. The dialysis tube was washed in sterile distilled water and placed at 4 °C in the refrigerator. To use the dialysis tube, one end of the dialysis tube was clamped tight to prevent leakage. The obtained cell-free crude pectinase was suspended in a minimal amount of 50 mM Na-acetate buffer of pH 7 at room temperature for 24 h. The suspended crude enzyme was dialyzed for 120 min in a refrigerator of 4 °C. Then the buffer was changed and dialysis was carried out at 4 °C overnight against millimolar. The partially purified enzyme obtained was then used to determine pectinase activity.

Characterization of Pectinase activity

The partially purified pectinase obtained from dialysis was used for the characterization based on the technique of Okonji et al. (2019).

Effect of temperature on Pectinase Activity and stability

To determine the optimal temperature for the partially purified pectinase, the enzyme was pre-incubated in sodium carbonate buffer of pH 7 at different temperatures of 30, 40, 45, 50 55, 60, and 65 °C. Periodically an aliquot of the enzyme was taken at 30, 60, 90, and 120 min, and used to assess the residual pectinase activity while pectin was used as substrate. Pectinase thermal stability was done employing the technique by Tari et al. (2008). The crude enzyme was pre-incubated in sodium carbonate of pH 7 at a temperature of 40 °C, 45 °C, and 50 °C. An aliquot of the enzyme was removed at 15, 30, 45, 60, 75, 90, 105, and 120 min to determine the residual pectinase activity using pectin as substrate.

Effect of Salts on Pectinase Activity

To determine the effects of different salts on the activity of enzyme pectinase, different salts NaCl, KCl, NH_4Cl, $CaCl_2$, $CoCl_2$, $CuCl_2$, and $MgCl_2$ of 5 mM concentration were used. This was carried out by pre-incubating for 10 min at 40 °C the partially purified pectinase in the presence of the salt and pectin. The remnant pectinase activity was then carried out as previously described. The partially purified enzyme sample without the salt solution was used as the control with 100 % activity.

Effect of pH on pectinase activity and stability

The effect of pH on pectinase optimal activity was evaluated by incubating the partially purified pectinase in varied buffer's pH of pH 3.0, 4.0, 5.0, 6.0, 7.0, and pH 8.0 for 30 min which was then followed by adding pectin as substrate. Buffers such as sodium acetate buffer (pH 3.0 to 5.5), sodium phosphate buffer (pH 6.0 to 7.5), and sodium carbonate buffer (pH 8.0 to 9.0) were used in this section. The residual pectinase activity was assessed as earlier described.

The stability of the pectinase at different pH was assessed when the partially purified pectinase was pre-incubated for an hour at pH 5.0, 5.5 as well as 6.0. The remnant pectinase activity was evaluated by comparing the activities of both the treated and untreated enzyme which was assumed to be 100 % (control). The percentage residual activity was calculated using:

$$\text{Relative activity (\%)} = \frac{\text{Residual activity} \times 100}{\text{Control activity}}$$

Effect of inhibitory substances on pectinase activity

The influence inhibitors on the partially purified pectinase were studied using the following chemical inhibitors; ethylenediaminetetraacetic acid (EDTA), Triton X-100, cetrimide, and Tween-80. Pre-incubation of the partially purified pectinase was done in 1 % (v/v) of each inhibitor for 30 min at room temperature before initiating a reaction with pectin (substrate). The pectinase activity was assessed to ascertain its residual. Pectinase that was not pre-incubated with inhibitory substances served as a control with 100 % activity.

Statistical analysis

Data analyzed in this study were collected in duplicates and subjected to mean standard deviation (Mean ± SD). The results were presumed to be significantly different at P values ≤ 0.05.

RESULTS AND DISCUSSION

Forty-eight fungal isolates were obtained from soil, decaying wood, sawdust, and wastewater used for the study (Table 1). These isolates were identified to be species of *Aspergillus*, *Rhizopus*, *Penicillium*, and *Fusarium* based on their macroscopic and microscopic characteristics (Plate 1A and Plate 1B). However, only eleven showed notable pectinase activity that ranged between 10 to 36 mm in diameter (Fig. 1). Isolate SS13 identified to be *Aspergillus flavus* exhibited most pectinolytic activity with hydrolytic zone of hydrolysis of 36.0±0.5 mm. Hence, it was selected for mutation as well as for pectinase production.

Fungi generally have been mentioned to be widely distributed in soil and the environment. They can survive on most nutrients and moisture in the environment (Ketipally & Ram, 2018). Similar genera were observed to be present in soil samples used for isolation by Sudeep et al. (2020). The genus *Aspergillus* has been identified as a good producer of pectinolytic enzymes which supports our findings in this study (Sudeep et al. 2020).

Table 1: Macroscopic and microscopic characteristics of isolated fungi

No. of isolates	Macroscopic	Microscopic	Probable identity
10	Yellow to a green colony with a golden to red-brown reverse and has a rapid growth pattern.	Conidia are typically spherical to subspherical, conspicuously spiny, variable, and 3-6 um in diameter, conidiophores are heavy-walled, hyaline, coarsely rough.	*Aspergillus flavus*
15	Black colony with a white to yellow reverse, moderate growth with submerged mycelium	Finely wrinkled conidia with 3-5 um diameter with a globular/ ellipsoidal shape 20-73 um diameter of vesicle and warty ornamentation.	*Aspergillus brasiliensis*
6	Bright green with white margin colony with a dull yellow or greenish brown reverse, has restricted growth; larger, plane, and centrally umbonate growth pattern.	Conidiophores are hyaline, erect, developed from aerial hyphae, branched penicillate-ly at the apexes, and have verticillate conidia at each phialide and catenulate conidia by the phialide, having a cylindrical columnar greyish-green conidial heads.	*Penicillium* sp
4	Fluffy white texture with a variable color typically violet to purple with a magenta red turning violet reverse with	Conidiophores are hyaline and short, or not that differentiated from hyphae, having spore masses at the end. Canoe-shaped, multicelled macroconidia. Macroconidia	*Fusarium* sp

	concentric zone of dark and light reddish coloration.	are borne in banana-like clusters which dislodge easily.	
7	White to grey aerial mycelium with an extremely fluffy texture. At maturity, it turns black colour with colourless reverse.	Sporangiosphores are long, unbranched, and clustered at nodes opposite rhizoids that form along the stolon. Sporangia are dark-walled, spherical, and filled with round hyaline spores. Columellae are light brown, subspherical, and umbrella-shaped when dehisced.	*Rhizopus* sp.
6	Yellowish green, raised colony with a greenish reverse. Has a white margin and a rough surface scattered	Conidiophores are erect, simple, and rough on the surface, with foot cells basally, swollen at the apex to form globose vesicles, having radiate conidial heads made up of centulate conidia on uniseriate or hardly biseriate phialides: conidial heads are yellowish- green in colour, and with radiate, columnar.	*Aspergillus* sp.

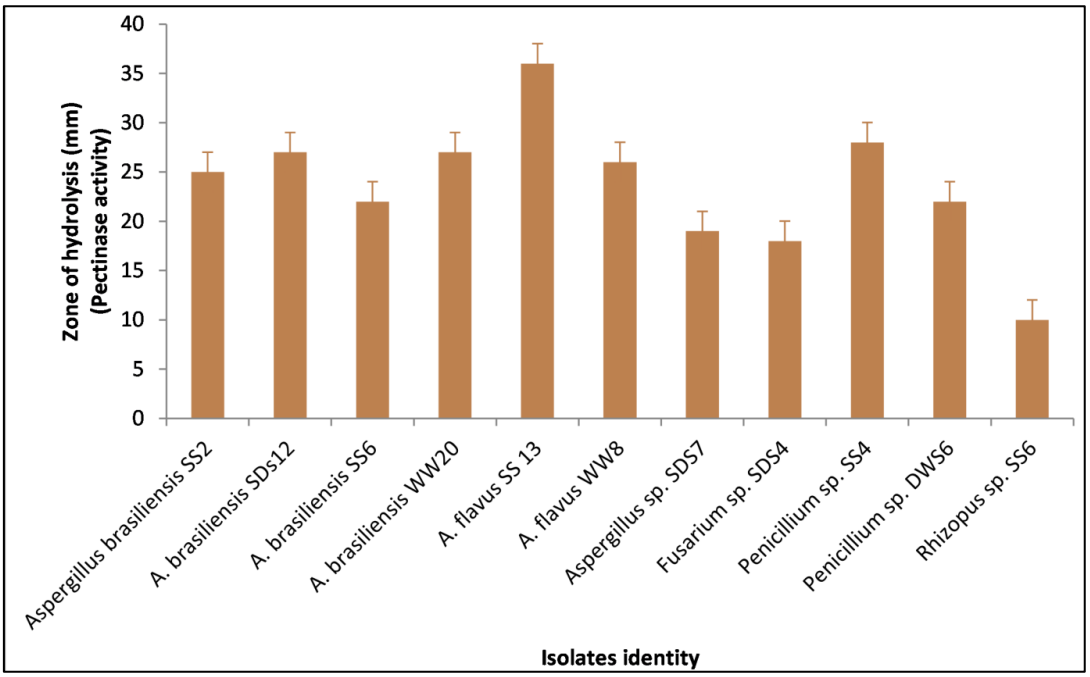

Figure 1: Pectinase Activity of Isolated Fungi
Key: SDS= Saw dust sample, DWS= Decaying wood sample, sample SS = Soil sample, WWS, waste water sample

Plate 1A

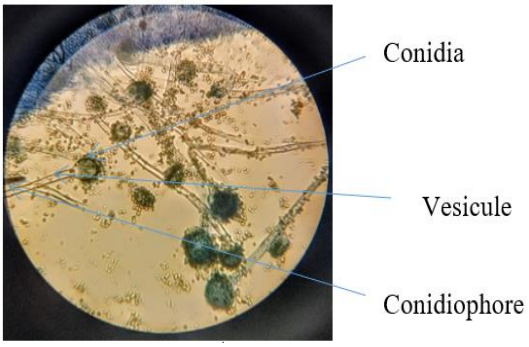

Plate 1B

Plate 1: A Colony characteristics of *Aspergillus flavus* SS13 and Plate B Microscopy of *A. flavus* SS13

Improvement of strains through mutation for commercial production of enzymes has been noted as an important means by which higher production of enzymes could be achieved. UV and EMS are two strong most commonly used mutagens in the improvement of enzyme production by microorganisms, and in other groups of organisms including mammals. UV and EMS have high mutation frequencies and low chromosomal aberrations, without killing the cells. Similarly, Sodium azide has been used to mutate filamentous fungi including *Aspergillus niger*, which greatly improved the synthesise of both primary and secondary metabolites. In this study, it was observed that the three mutants generated from

Aspergillus flavus SS13 showed an improved pectinase activity in comparison to the wild type (Fig. 2). The activity was 37.0±0.2 mm, 38.0±0.6 mm and 40.0±0.5 mm for Sodium azide, Ultraviolet, and Ethyl Methane Sulphonate treated respectively while the wild activity was 36.0±0.5 mm. Kamalambigeswari et al. (2018) and Ezugwu et al. (2023) who worked with different genera of fungi had reported improvement in pectinase activity as a result of either physical or chemical mutation. Mutants are known to have better performance as well as improved adaptation to the fermentation environment (Singh et al. 2015). The random changes due to deletion, insertion, duplication, and translocation in the copies of some of the genes or the whole genome could favourably expose the mutant to higher productivity (Singh et al. 2015).

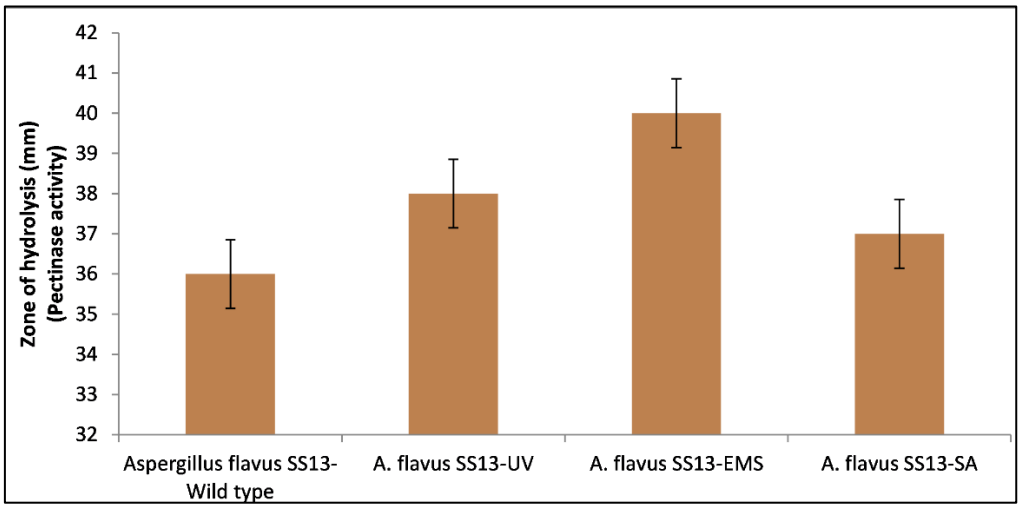

Figure 2: Pectinase activity of Wild type and Mutated *A. flavus* SS13

Key

A. flavus SS13-Ultraviolet

A. flavus SS13-EMS- Ethyl methane sulfonate

A. flavus SS13SS-Sodium azide

Different plant biomaterials that can be used as carbon sources such as yam peel, oil palm fibre, rice bran, orange peel banana peel as well as inorganic carbon sources like sucrose, maltose, and glucose were assessed in this work. It was noted that orange peel gave considerable pectinase activity with all the mutants of *A. flavus* SS13 with the highest pectinase activity (32.2±0.4 U/mL) observed in UV irradiation-treated *A. flavus* SS13 (Fig 3). A close result has been mentioned previously (Kaur, Singh et al. 2016) who reported the highest activity of pectinase at 88.0 U/mL with a bacterium *Bacillus pumilus*. The difference observed could be attributed to the fermentation condition, medium content as well as the strain of the microorganisms used for production. The wild type of *A. flavus* SS13 exhibited the most pectinase activity (22.44±0.2 U/mL) with yam peel. Orange peel is known to be endowed with pectin and it is a good inducer of pectinolytic enzymes. Besides these, it is a cheaper agricultural waste suitable for the production of industrial pectinases (Göğüş et al. 2014).

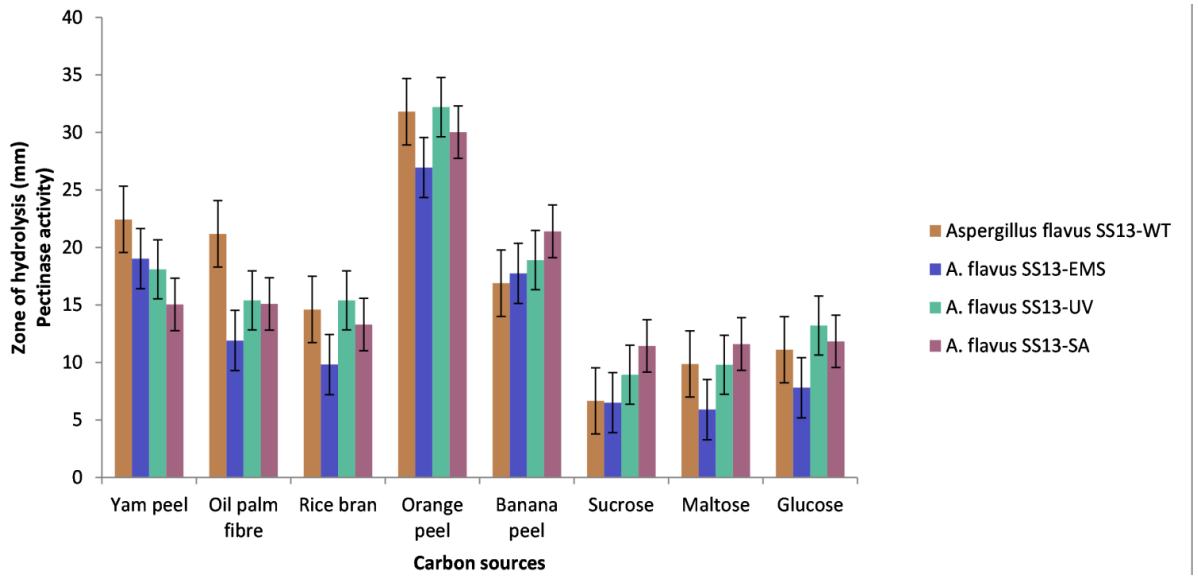

Figure 3: Influence of carbon sources on pectinase production

The impact of nitrogen sources on the production of pectinase was evaluated in this study. Highest pectinase activities were observed when the wild-type *A. flavus* SS13 and the three mutants *A. flavus* SS13-EMS, *A. flavus* SS13-UV, and *A. flavus* SS13-SA were cultivated in the production medium that contained ammonium sulphate (Fig 4). Inorganic salts such as ammonium phosphate and ammonium sulphate have been mentioned to improve pectinolytic enzymes (Patil & Dayanand, 2006). Our finding in this study where ammonium sulphate was found to enhance pectinase production has been corroborated by the previous report of Banu et al. (2010). However, Ketipally & Ram, (2018) reported enhanced activity of pectinolytic enzymes when organic nitrogen sources such as malt extract and yeast extract served as nitrogen sources. The differences observed could be attributed to different fermentation conditions like the pH, temperature, and the ability of the production strains to utilize these nitrogen sources.

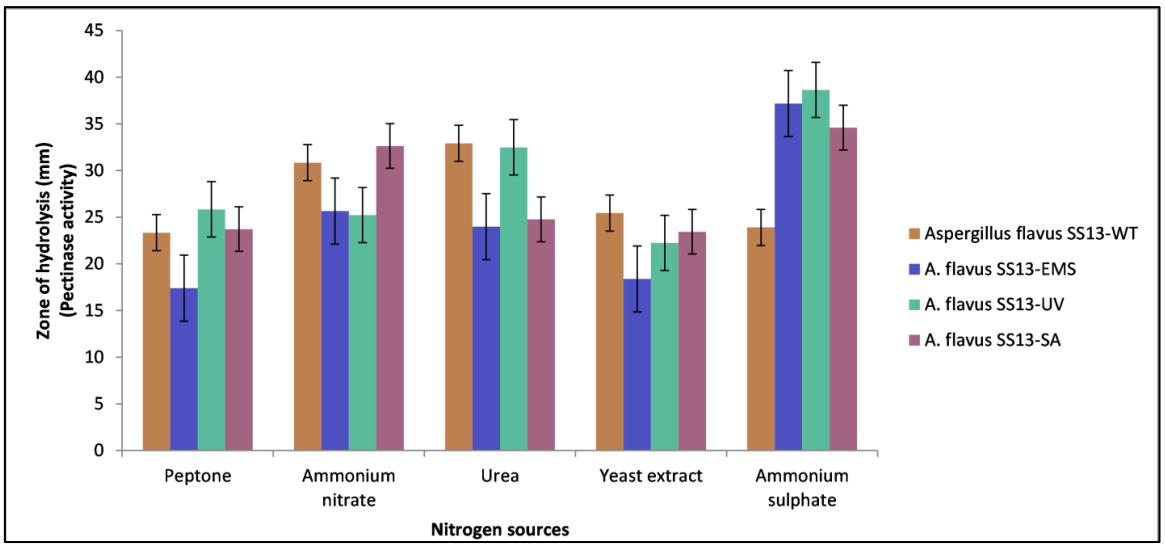

Figure 4: Influence of different nitrogen sources on pectinase production

The impacts of the incubation time on the pectinase production by the wild type and the three mutants of *A. flavus* SS13 as presented in Fig.5 indicated that pectinase was produced optimally by 96 h of fermentation. There was a noticeable reduction in pectinase activity after 96 h of fermentation, which could be as a result of growth rate, exhaustion of materials for growth, and accumulation of hazardous materials in the production broth (Phutela et al. 2005).

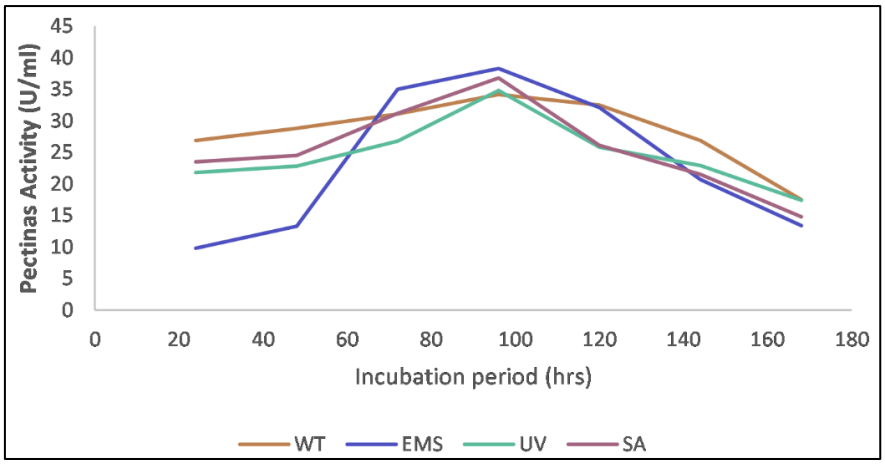

Figure 5: Impact of incubation period on pectinase production
Key:
WT= wild type *Aspergillus flavus* SS13
EMS= Ethyl methyl sulphonate mutant of *Aspergillus flavus* SS13
UV= Ultra violet ray mutant of *Aspergillus flavus* SS13
S.A= Sodium azide mutant of *Aspergillus flavus* SS13

Acidic pH has been mentioned to be favourable to pectinase production from fungal strains (Shin et al. 1983). The pH could be between 4.0 and 6.5 (Phutela et al., 2005). This study showed that an acidic pH of 3.0 optimally supported pectinase production by the wild type and three mutant strains used (Fig. 6). Sundeep et al. (2020) mentioned the pectinase activity that was optimum at pH at 3.2 and possesses activity up to 9.0. Pectinases that are acidic are biotechnologically relevant in the partial saccharification of sugars, protoplast formation as well as understanding phytopathogen invasion of plants ((Kadija Tul Kubra & Manam Walait, 2018).

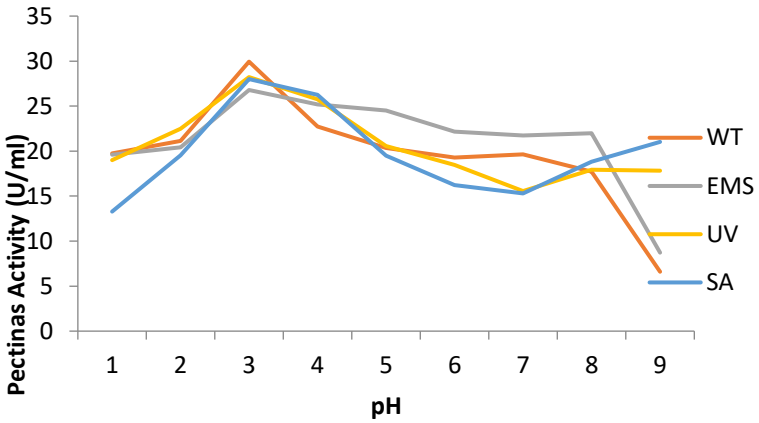

Figure 6: Influence of pH on pectinase production
Key:
WT= wild type *Aspergillus flavus* SS13
EMS= Ethyl methyl sulphonate mutant of *Aspergillus flavus* SS13
UV= Ultra violet ray mutant of *Aspergillus flavus* SS13
S.A= Sodium azide mutant of *Aspergillus flavus* SS13

The four strains *A. flavus* SS13-Wild type, *A. flavus* SS13-EMS, *A. flavus* SS 13-UV, and *A. flavus* SS 13-SA used in this study exhibited optimal pectinase activity at a temperature of 40 °C (Fig. 7). The result obtained in this study is close to the previous report of Adedayo et al. (2021). Temperature is known to influence the increase in the number of cells as well as the synthesise of metabolites by microbes. Temperature below or above the optimum usually lowers enzyme activity. Temperature values between 30 °C and 40 °C have been reported to give optimum pectinase activity produced by *Aspergillus* species (Khatri et al. 2015).

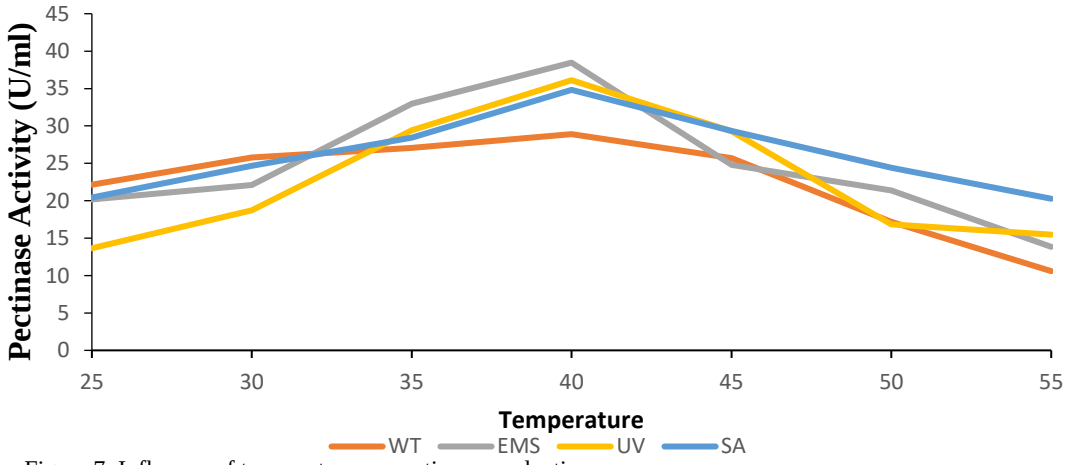

Figure 7: Influence of temperature on pectinase production
Key:
WT= wild type *Aspergillus flavus* SS13
EMS= Ethyl methyl sulphonate mutant of *Aspergillus flavus* SS13
UV= Ultra violet ray mutant of *Aspergillus flavus* SS13
S.A= Sodium azide mutant of *Aspergillus flavus* SS13

A two-step purification method was used to purify pectinases produced by the wild type *A. flavus* SS13 and the three generated mutants *A. flavus* SS13-EMS, *A. flavus* SS13-UV, and *A. flavus* SS13-SA. The best pectinase activity was recorded in *A. flavus* SS13-EMS with 9.70±0.3 U/mL, followed by *A. flavus* SS13-UV which exhibited 8.70±0.5 U/mL and the least activity of pectinase was 5.25±0.3 U/mL was recorded in *A. flavus* SS13-SA. All the mutants except *A. flavus* SS13-SA had higher pectinase activity compared to wild-type *A. flavus* SS13, 5.94±0.4 U/mL. A previous report by Akbar et al. (2015) that worked with UV-mutated *A. tamarii* and EMS-treated *A. brasiliensis* aligned with the outcome of this study that *Aspergillus* species when exposed to UV and EMS showed improved pectinase activity compared to the wild type of the species. A lower pectinase activity was observed with sodium azide-treated *A. brasiliensis* compared to the wild type.

The characterization of the partially purified enzyme produced by the wild type *A. flavus* SS13 and the three mutants *A. flavus* SS13-EMS, *A. flavus* SS 13-UV, and *A. flavus* SS 13-SA indicated that some cations such as Na^+, $Ca^{2+,}$ and NH_4^+ enhanced pectinase activity in UV treated *A. flavus* SS13 (Zhang et al. 2019). The partially purified enzyme produced by the wild-type *A. flavus* SS13 was slightly repressed by sodium and cobalt ions. Metallic ions are known to either enhance or repress enzyme activities (Alqahtani et al.

2022). They serve as cofactors to enhance enzyme catalysis, especially in hydrolytic reactions, either by acting as an inhibitor, an activator, or as stabilizers at certain concentrations. In this study, Na^{+}, $Co^{2+,}$ and NH^{4+} were observed to have repressed the pectinase activity produced by the wild-type *A. flavus* SS13.

Furthermore, the influence of different surfactants on the activity of partially purified pectinase showed that Tween-80 among the four surfactants used enhanced the pectinase activity of the enzyme produced by the three mutants of *A. flavus* SS13. The surface interaction during enzyme activity is essential for positive outcomes. Cetrimide was observed to slightly repress the partially purified enzyme activity produced by the wild type and UV-mutated *A. flavus* SS 13.

The partially purified pectinase produced by the three mutants was observed to be stable between 40 °C to 50 °C with good activities that ranged between 8.25±0.3 U/mL and 14.70±0.5 U/mL while the optimum pectinase activity of 13.46±0.6 U/mL was recorded at 40 °C in the wild type *A. flavus* SS13. This result aligned with the report of Okonji et al. (2019) who worked with *A. fumigatus* obtained from decomposing plant materials. The pectinase activity generally reduced below 40 °C and above 50 °C. The partially purified pectinase produced the wild type and the three mutants of *A. flavus* SS13 exhibited stability and high enzyme activity within the pH 4.5 and 5.0. A pH range of 3.0 to 6.0 was reported by Ezugwu et al. (2023). It was noted in this study that pH below 4.5 and 5.0 significantly reduced the pectinase activity.

CONCLUSION

A filamentous fungus *Aspergillus flavus* SS13 was isolated from the soil and identified appropriately. *A. flavus* SS13 is a good producer of pectinase. Hence, it was mutated using physical (UV) and chemical (EMS and SA) mutagens to improve enzyme yield. Pectinase was optimally produced at a pH of 3.0, a temperature of 40 °C, and an incubation period of 96 h with orange peel and ammonium sulphate as carbon and nitrogen sources respectively. Mutants of *A. flavus* SS13 generated with EMS and UV exhibited improved pectinase activity in comparison to the pectinase activity of the wild type of *A. flavus* SS13. The partially purified pectinase showed improved activity in the presence of tween-80 and some cations and it was stable at pH 4.5 to 5.0 and temperature 45 °C to 50 °C. Production of pectinase using *A. flavus* SS13 can be achieved using cheap and readily available agricultural biomass, orange peel. The pectinase is acidic and active at elevated temperatures giving it industrial and biotechnological advantages.

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