PECTINASE PRODUCTION FROM *SACCHAROMYCES CEREVISIAE* USING ORANGE PEELS AND MAIZE COBS AS SUBSTRATE FOR SOLID-STATE FERMENTATION

ABSTRACT

Background of the study: Degradation of waste especially fruit waste is important because these wastes accumulate in the environment. The fruit wastes can be used to biosynthesize pectinase enzyme at a cheaper rate due its numerous industrial applications. The ability of *Saccharomyces cerevisiae* to degrade these wastes to produce pectinase enzyme was exploited.

Aim: The aim is to isolate and characterize *Saccharomyces cerevisiae* and study the optimal conditions needed for pectinase production on the orange peel with maize cobs and study the effectiveness of the enzymes produced in the extraction of orange juice.

Place and Duration of Study: The study was conducted at the Microbiology Laboratory of Abubakar Tafawa Balewa University Bauchi, Bauchi State, between August to September 2021.

Methodology: Soil samples from the orange seller stand was collected, the serial dilution method was used to reduce the solution to a more usable concentration, and the sample was streaked based on Yeast Peptone Dextrose Agar (YPDA). The plates were incubated for 3 to 9 days at 28+- ^{o}C. The isolated yeast was identified based on the morphological and biochemical test, the isolates were inoculated on the prepared fermentation media under different optimal conditions. The crude enzyme were extracted, and the extracts were tested for pectinase activity using the dinitrosalicylic reagent (DNS) method for the presence of reducing sugar. The resulting pectinase was determined to effectively extract juice from orange mash.

Results: *Saccharomyces cerevisiae* showed relatively pectinase hydrolysis using orange peels and maize cobs as substrate by solid-state fermentation under predetermined optimum fermentation conditions. Optimization for the pectinase production was done by altering the conditions for the production medium. Pectinase activity of (2.81 $\pm$ 2.31 ug/ml) was observed at 72hr of incubation at 35^{o}c.

Conclusion: The study demonstrates that orange peel and maize cob can be successfully used to induce the production of pectinase using *Saccharomyces cerevisiae* under solid-state fermentation.

Keywords: Isolation, Orange Peels, Maize cobs, Pectinase, Orange Juice, *Saccharomyces cerevisiae*.

1. INTRODUCTION

Pectinase is an enzyme whose demand has now increased worldwide in industries [1]. Pectinases are enzymes that catalyze the breakdown of pectin-containing substances through de-polymerization and de-esterification in metallic reactions like hydrolysis reactions [2]. Pectin is a complex polysaccharide present in the middle lamella between the plant cell walls. It is composed of various units of D-Galacturonic acid linked by α (1, 4) glycosidic linkage. Pectinase occurs

naturally in the plant to improve the ripening of fruits and has major applications in food processing industries for the production and clarification of wine and juices. Pectinolytic enzymes are produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoans, and plants. The said enzymes have been reported in higher plants and microorganisms including bacteria and fungi [4]. However, microbial pectinases possess more advantages as it is involved in plant-microbial symbiosis and the breakdown of dead plant materials [2]. Microorganisms for enzyme production must not be pathogenic or poisonous, they must meet the standard of Generally Regarded as Safe (GRAS) status as opposed to bacteria due to their pathogenicity. Some of the commonly used organisms are *Aspergillus, Penicillium, Rhizopus, Saccharomyces, Zymomonas, Bacillus,* and others [6].

Fungi stand out as a major source of industrially applicable and stable enzymes [7] since they are organoheterotroph as they derived their growth requirements from degrading complex organic molecules into simpler monomers. This is accomplished through the secretion of extracellular enzymes onto the substrate. Hence fungi are good sources of extracellular enzymes, the enzymes are easily extracted without breaking the cells of the organism [8].

Numerous conventional industrial processes over the years have used pectinases for the treatment of industrial wastewater comprising pectinaceous material, textile, plant fiber processing, tea and coffee industries, cleansing of viruses, and paper manufacturing and oil extraction [9]. The two fermentation techniques used for enzyme production are submerged fermentation (SMF) and solid-state fermentation (SSF) to increase demand for this enzyme. During solid-state fermentation, microorganisms are grown directly on the substrate in low water activity as against submerged fermentation, where they are grown under the liquid [10] and several advantages of solid-state fermentation have also been documented. Filamentous fungi are preferably grown on solid media and bacteria in the submerged fermentation process. Solid fermentation offers a near-to-natural condition for fungi to thrive and yield metabolites since their mycelia needed to be in direct interaction with the substrate, the low availability of water also helps limit bacterial contaminants.

Wastes and their disposal have become an environmental concern worldwide, the use of agro-industrial waste for the production of enzymes especially when these wastes are biodegradable to useful goods and services [11]. Orange peels belong to this group of valuable biomass wastes [12]. Orange production was predicted to approach 66.4 million tons by 2010, representing a 14% increase within 12 years [13]. About 40-60% of oranges are squeezed into liquid juices and the remainder containing peel, segment membranes, and other by-products are considered citrus processing waste [14]. Orange peel mainly consists of cellulose (13.6%), hemicellulose (10%) [15], as much as 25%–30% (dry weight) pectins [16], chlorophyll pigments and other low molecular weight compounds [17]. Literature abounds on the use of other substrates and bacteria for enzyme production but seems to be scarce on the use of orange peel and *Saccharomyces cerevisiae*. Pectinase is an enzyme relevant to many segments of life and substantial to the survival of the human race, though production is not yet localized and indigenous production industries import this enzyme with huge amounts of money. There is a need to exploit this waste for local enzyme production. Hence, this study is aimed at using orange peel and maize cob as native substrates for the production of pectinase through the activity of inhabitant fungi in solid-state fermentation.

2. MATERIALS AND METHODS

The pectinolytic microorganism was isolated by a simple isolation method. Soil sample from a fruit seller stand was brought from the market and Kofar Gombe area in Bauchi in a clean polyethylene bag and transported to the laboratory. The sample from the botanical garden of the

campus was used as a control. The sample was streaked on yeast peptone dextrose agar medium with 200 mg/ml of chloramphenicol to prevent bacterial contamination. The plates were incubated for 3 to 9 days at 28±2ºC. Purification of yeast colonies was done based on their morphology on the YPDA medium achieved by streaked methods. Selected yeast strains were identified based on their morphological growth pattern, characteristic features, and colour, stained by lactophenol cotton blue and investigated microscopically and through the biochemical test (the ability of yeast isolates to assimilate carbon sources) [18].

The orange peels and maize cobs were purchased at Muda Lawal market packed and transported to the laboratory for analysis. The substrates were pretreated with 1% (w/v) NaOH solution in the ratio of 1:10 (substrate: solution) for 1h to remove the lignin content and brought to neutral pH by washing thoroughly and sterilized at 121^{o}C for 1 hr in an autoclave.

Proximate substrate composition was determined according to the Association of Official Analytical Chemists; AOAC 952.08, 2016. Solid-state fermentation was carried out in 250ml Erlenmeyer flasks containing NaCl (2.25 g), NH_4SO_4 (10.5g), Na_3PO_4 (15.0 g), and Urea (2.25g) supplemented with 5 grams of maize cobs and orange peels. The pH was adjusted to different levels (3.5, 4.0, 4.5, 5.0, 5.5, and 6.0) and moisture content was adjusted to 1:3. The flask was covered and autoclaved at 121oC for 15 minutes after which the flasks were cooled at room temperature. Inoculation of substrates was carried out with 2 ml of *Saccharomyces cerevisiae* cell suspension and incubated at different temperatures (20, 25, 30, 35, and 40^{o}C) for 120hours.

After 120 hours, crude enzyme extracts were prepared from the solid fermented media by adding 100ml of acetate buffer (pH 5.0) to each flask, agitated for 5 minutes using the Fischer Scientific mini vortexes, and filtered using filter paper after which the culture filtrate was centrifuged at 4000 rpm for 20 minutes. The enzyme precipitate was dissolved in an acetate buffer solution (pH 5.0) and stored in 4^{o}c for further use.

Pectinase activity determination was carried out using a method for pectinase activity assay. This assay is based on the hydrolysis of pectin and the resulting galacturonic acid determined spectrophotometrically at 570 nm. One unit of pectinase activity is said to cause an increase of 0.010 of absorbance per minute under the conditions of the assay. A unit of the enzyme was defined as the amount of enzyme required to release one μL of reducing sugar (galacturonic acid) per minute under the assay condition.

The absorbance (sample-extraction solvent) will be read over 8 minutes. A graph will be plotted of time versus absorbance on the y-axis.

Enzyme activity can be calculated using the following formula

$$\text{Enzyme activity (μ/ml)} = \frac{mg\ of\ galacturonic\ acid}{194.1 * v * t}$$

Where "v" is the enzyme volume used in the assay, "194.1" is the molecular weight of galacturonic acid, and t is the reaction time in minute.

Various time (15, 30, 45, 60, and 75 minutes) was used to determine the optimum reaction time for extraction of juice (measured in ml), orange juice was washed under running water, peeled cut, and mashed to increase the surface area while making more areas accessible to the enzyme to breakdown pectin in plant cell walls thereby releasing more juice. Two hundred grams of mashed oranges were separately weighed into a labelled beaker and 1ml of the concentrated protein (10 mg/ml) with the best pectinase was added, stirred, and covered with plastic wraps, which were left to stand for various reaction times. At the end of each reaction time the content will be filtered over 6-minute period using a funnel and filter paper, the volume of juice obtained was compared to the orange mash with no enzyme.

3. RESULTS AND DISCUSSION

3.1 Microscopic Identification of Yeast Isolates

In this study, the yeast colonies appeared as round or oval, moist, raised, smooth cells that were creamy in colour. The organism was identified as *Saccharomyces cerevisiae*. The isolates were subjected to further streaking until completely purified. All selected yeast isolates MK, KG and BG assimilated glucose and maltose and were unable to assimilate lactose as a sole carbon source. The table shows the number of yeast cells grown on yeast peptone dextrose agar medium (YPDA). From the result, it can be seen that MK has the highest number of isolates with 13 while KG has 6 and BG with 3.

Table 1: Distribution of *Saccharomyces cerevisiae* isolated from soil sample in this study

Sample location	Number of Yeast Isolated (n = 22)	Percentage (%)
Market	13	59.1
Kofar Gombe	06	27.7
Botanical Garden	03	13.2

Key: MK= Market, KG= Kofar Gomber, BG=Botanical Garden

Samples from MK had the highest number of isolates, which could be a result of the environment where the sample was taken which contains a higher concentration of yeast than those in KG and BG. Samples KG contained an average high population of cellulolytic organisms because there are various samples while BG is used as a control for research purposes because there are samples planted in the garden and no orange or fruit waste was grown. The difference in percentage frequency might be from the sugar content of mucilaginous and pulp waste, the orange peel species, or the nature of the dumping system that allows more microbial colonization for carbohydrate degradation. The obtained results from the morphological characteristics of the isolated colonies together with the biochemical tests were highly significant in yeast identification.

3.2 Effect of temperature on pectinase activity

Based on the results shown in Figure 1, the optimum pectinase activity was found to be at 35^{o}c after which there was a decline. The reduction in enzyme activity beyond 35^{o}c during fermentation at elevated temperatures may be due to unfavourable heat stress encountered by the yeast cells as reported in a study of the metabolic response of *Saccharomyces cerevisiae* to continuous heat stress thereby discouraging the growth of *Saccharomyces cerevisiae* [20]. Extreme temperatures or excess heat effect possibly changed the physical properties of the organism's cell membrane thereby affecting protein secretion and uncoiling some of the secreted proteins into random configurations due to heat stress, leading to a decline in pectinase activity.

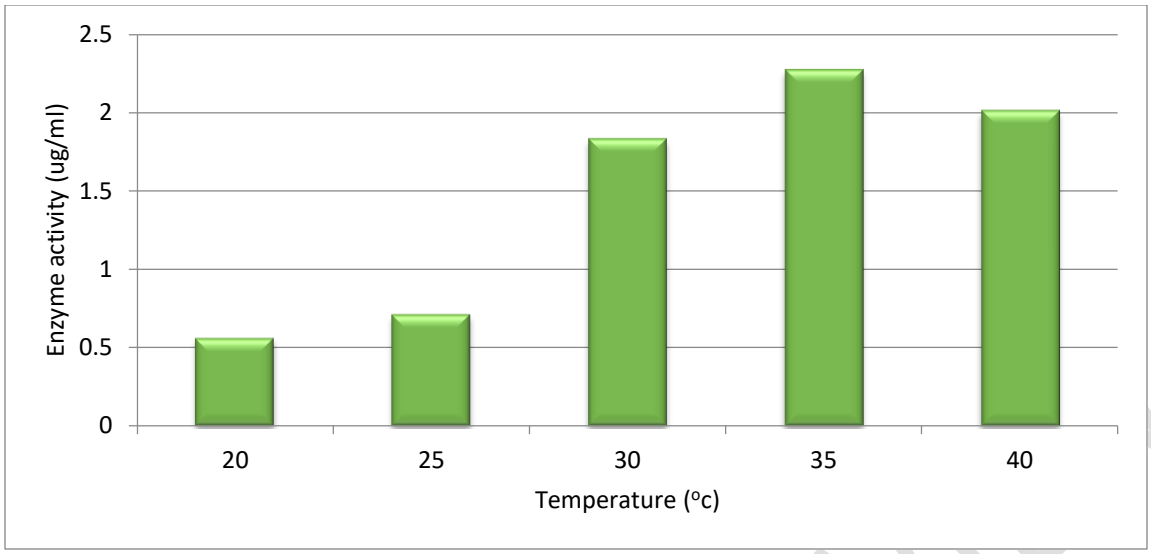

Figure 1: Effect of Temperature on Pectinase production

3.3 Effect of pH on pectinase activity

pH alters enzyme conformation, recognition site, active site, and substrate conformation, hence determining pH for maximum pectinase activity is important. Optimum pectinase activity is observed at pH 4.5 (Figure 2). The decline in activity beyond pH of 4.5 may be due to denaturation of the enzyme which is a common phenomenon during fermentation due to the release of various by-products in the media [23] or instability of enzyme me at extreme pH value since they are proteins that are generally denatured at extreme pH values [24].

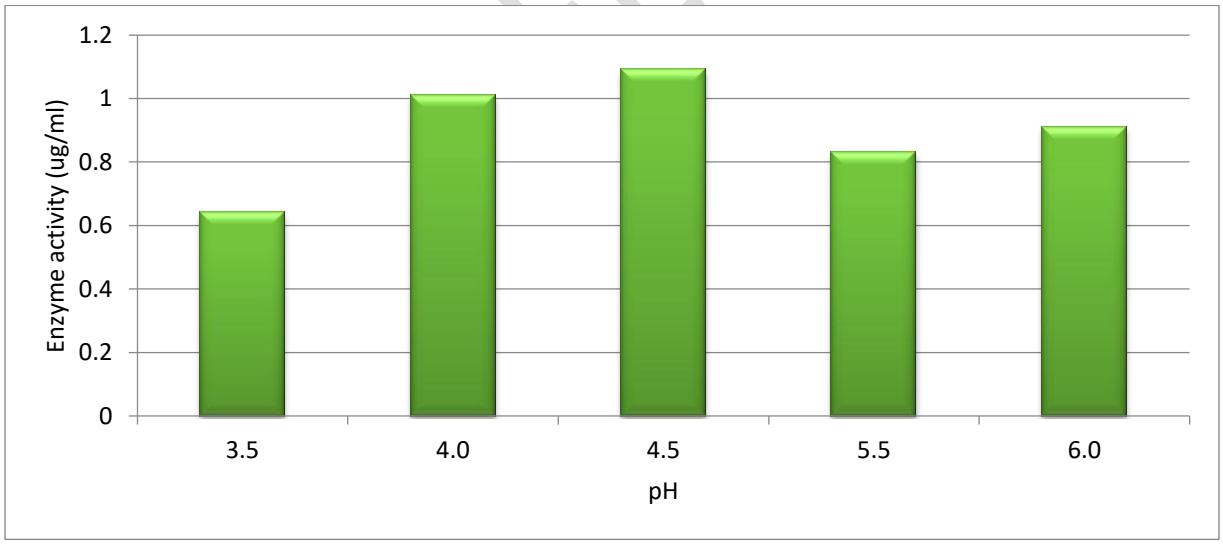

Figure 2: Effect of pH on Pectinase production

3.4 Effect of fermentation time on pectinase activity

The effect of the duration of fermentation on pectinase activity with orange peels and maize cobs as the substrate is illustrated in figure 3. Maximum pectinase enzyme activity (2.81 $\pm$ 2.31 u/ml) was obtained on the 3rd day of fermentation at 35°c and pH 4.5.

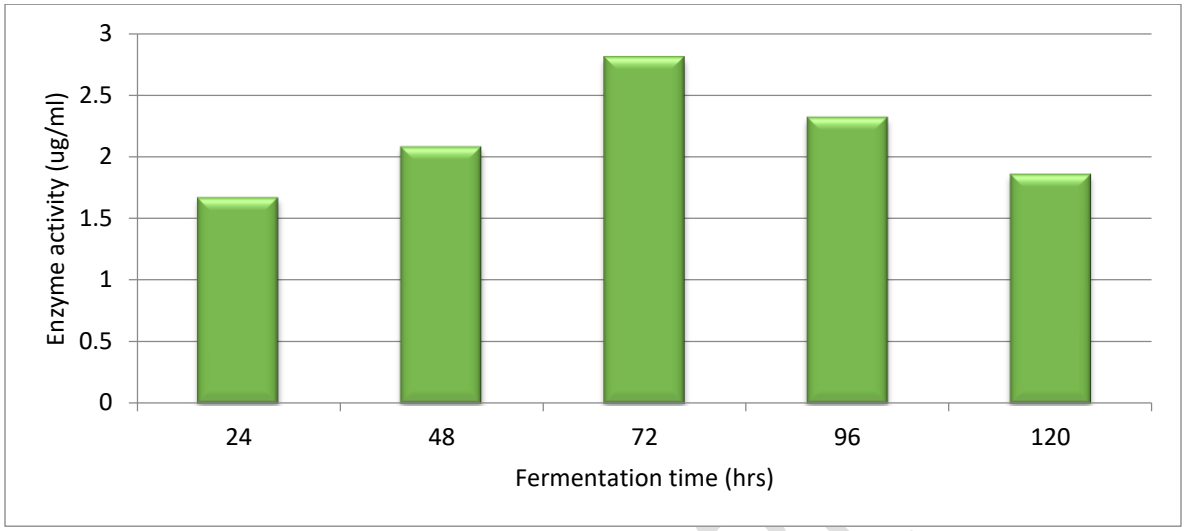

Figure 3: Effect of Fermentation time on Pectinase production

The increase in the enzyme activity as temperature increases may be a result of a change in the enzyme conformation which brings the essential residues to proximity for catalysis. The decrease in the activity on the 4th day could be a result of thermal denaturation at high temperatures, the enzyme synthesis caused by low-temperature values.

This, therefore, indicates other proteins aside from pectinases such as xylanases [26] and cellulases [27] could be present in the crude extract since the cell wall of plants is composed primarily of polysaccharides (such as cellulose, hemicellulose and pectin substances) [28].

3.5 Effect of enzyme reaction time on the volume of juice extraction

Reaction time was undertaken to determine the optimum time for enzyme interaction with orange mash substrate for effective clarification and extraction of juice. From (figure 4) 60 minutes gave the optimum reaction time at which the highest volume of free run juice was obtained beyond which no significant increase in juice volume extracted was obtained free run juice here was simply the juice produced at the end of the reaction and filtered with filter paper for six minutes with no external applied pressure [29]. Thus, 75 minutes reaction was adequate for the enzyme to fully interact with the orange mash substrate.

The clarification experiments observed that as the concentration of enzyme increased, the yield of the juice, the total soluble solids, absorbance, and transmittance also increased. At 1% concentration, the yield of the orange juice, total soluble solids, absorbance, and transmittance was found to be the highest, which indicates its potential application in juice processing industries. Several studies reported a rise in enzyme concentration markedly improves juice clarification.

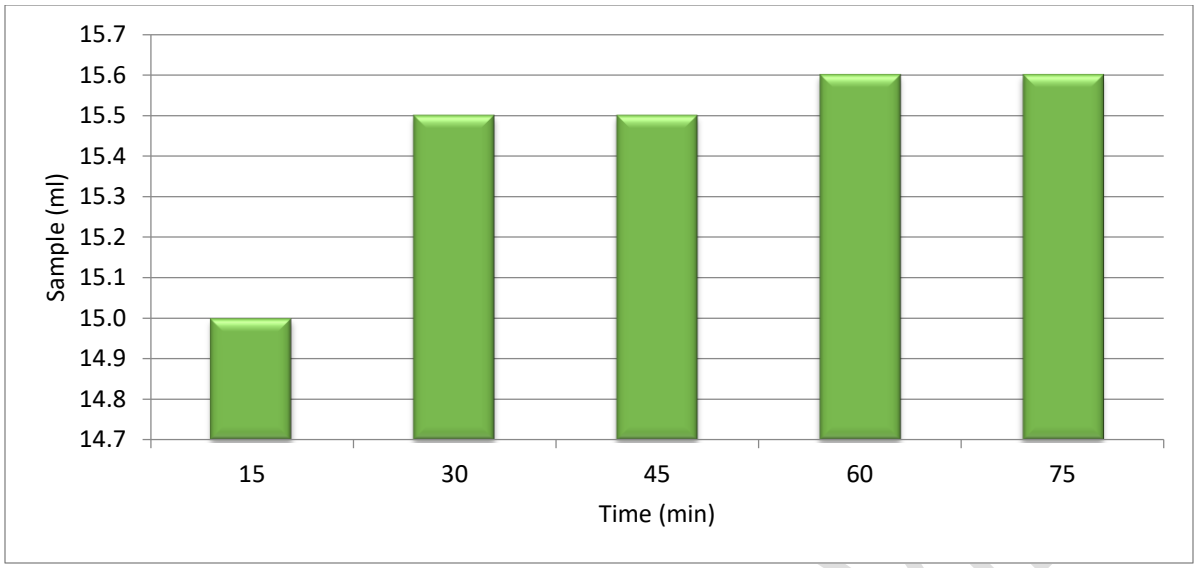

Figure4; Crude enzyme efficacy change in volume with time (ml)

4. CONCLUSION

Based on the result of this study, it can be concluded that *Saccharomyces cerevisiae* was identified as the notable organism for the production of pectinase enzyme via solid-state fermentation. Pectinase production by *Saccharomyces cerevisiae* occurs best at an optimized temperature of 35^0c, pH 4.5, and incubation period of 72hrs. An overall yield of pectinase produced by the isolate under optimum conditions was observed as (2.81±2.31ug/ml). The produced pectinase enzyme in this study showed proven ability in juice clarification, indicating a potential use in food processing industries. This research work therefore suggests the use of orange peels and maize cobs which is readily available in our environment for the production of pectinase by the yeast isolates studied in this work.

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