Isolation of Amylase Producing Bacteria and Fungi from Kitchen Waste Water of Restaurants and Hostels in Ile-Ife, Osun State

ABSTRACT

Organisms of industrial use are often isolated directly from the environment, most times from the soil, air and water. Amylase producing bacteria and fungi were obtained and isolated from kitchen waste water samples gotten from different hostels and restaurant across Ile-Ife, Osun State, Nigeria. The samples were first screened using pour plate method on nutrient agar and potato dextrose agar, then the isolates were further screened using other differential medium like starch agar.

The isolates recovered were further characterized macroscopically, microscopically and biochemically to ascertain for amylase producing ability. Four bacterial and three fungal isolates were presumptively identified as; *Bacillus macerans, Bacillus circulans, Bacillus brevis* and *Aspergillus niger, Aspergillus flavus , Aspergillus fumigatus.*

Keywords: Amylase; kitchen waste; enzymes; industrial microorganism; waste water.

1. INTRODUCTION

Microorganisms are very minute organisms that cannot be viewed with the naked eye and can only be viewed with the help of microscope. These microorganisms are ubiquitous in nature and can be found in all environments including hot springs and ice glaciers [1].

Microorganisms are majorly classified as bacteria, protist, fungi, algae and virus. They exist in different kinds of environment including the terrestrial and aquatic habitat. They can also strive for existence in extreme weather conditions [2,3]. The focus of this research work is to isolate amylase one of the main enzymes used to industrially hydrolyse starch. Amylase can be obtained from variety of sources, including plants, animals as well as microorganisms [4]. A major advantage of amylase sourced from microbes is the ability for mass production, ability to produce enzymes of certain qualities as well being economical [5].

Waste water is also referred to as sewage which can be defined as the water by-product of a society after various uses. Waste water contains various microorganisms of domestic, foot and diary origins, kitchen waste water is a nutrient rich or eutrophic environment containing high levels of carbohydrates, lipids, proteins and other organic molecules which can support abundant populations of microorganism [6]. Examples of commonly found microbes in waste water include; faecal Streptococci, spore forming Bacilli, bacteria of the Genera Sphaerotilus, Genothrix, Rhodospirillum, protozoans such as Amoeba, Flagellates, stalked Ciliates, Saprophytic fungi and Helminths.

Based on the nature of the waste water; industrial, agricultural or domestic, the quantity and type of microorganisms present may differ and vary. Most micro biota of waste water adapt to differing conditions of the environment such as the oxygen levels, pH, nutrient level, temperature, salinity, hydrostatic and osmotic pressure. Microorganism adapt to these different conditions through stabilization of enzymes/ nucleic acid, nature of cell membrane, dormancy in spore forms, formation of biofilms, nature of cytoplasmic membrane, protein components and charges on enzymes. All these different physiology and molecular adaptations allow these microorganisms adapt to their environment.

Microorganisms have successfully proved their usefulness as rich sources of useful enzymes. Most common amylase producing bacteria include *Bacillus* sp, *Halobacillus* sp, *Chromohalobacter* sp, and fungi such as *Aspergillus* sp, *Penicillium* sp, *Thermomyceslanuginosus*[7]. Several enzymes including amylases, cellulases, lipases, proteases etc. are currently available for variety of industrial applications. Among these enzymes, amylase is one of the highly versatile enzymes in

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commercial industries owning to high abundance of starch on Earth. Starch is one of the most abundant carbohydrate reserves in plants; it is used by plants as source of carbon and energy [8]. It is the main reserve polysaccharide of cereal grains, roots, and tubers [9,10] and a main energy source in the human diet. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits [11,12].

Amylases are hydrolytic enzymes that catalyse the hydrolysis of starch into low molecular weight sugar molecules. They are used for a variety of applications in different industries include ng food, paper, detergent and textile industry; they also have potential use in pharmaceutical and fine chemical industries. Recently, the potential uses of microorganisms as biotechnological sources of industrially relevant enzymes have stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms. These enzymes from microbial sources generally meet industrial demand and are more stable than plant and animal enzymes that are cheaply obtained [13].

Amylase can be derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [14]. Amylase catalyses the hydrolysis of starch into sugars by cleaving the bond or linkages in the starch, there are majorly 3 isoforms of amylase; α-amylase, β-amylase, γ-amylase which act on different glycosidic bonds[15].

1.1 Aim of the Study

The specific objectives of this research are to:

- To screen and isolate amylase producing microorganism obtained from different sample site
- To identify the isolated microorganism using their cultural microscopic and biochemical characteristics.

2.STARCH

Starch is the most abundant carbohydrate reservoir in plants and is found in leaves, flowers, fruits, seeds, different types of stems and roots. Starch is used by plants as source of carbon and energy [5]. Starch is formed in the chloroplasts of green leaves and amyloplasts, organelles responsible for the starch reserve synthesis of cereals and tubers [8,16].

Starch granule is a heterogeneous material; chemically, it contains both linear (amylose) and branched (amylopectin) structures; physically, it has both amorphous and crystalline region [17].

Amylose is composed of linear chains of alpha-1, 4 linked D-glucose residues hence it is extensively degraded by α-amylase; amylopectin is a highly branched polymer. Amylase forms complex with iodine to form intense blue colour and this forms the basis of a method for quantitative determination of amylase. The hydrolysis of starch may be carried out using either acid or enzyme as catalyst.

2.1 Enzymes

Enzymes are macromolecular biological catalysts that accelerate the rate of chemical reactions including most metabolic processes in the cell. An enzyme attracts the substrate to its active site, catalyzes a chemical reaction through which the product is formed. An enzyme will typically attract the substrate to form an enzyme/substrate complex; the substrate undergoes a change through the process of catalysis to form the enzyme/product complex, after the product is formed, it is released and the enzymes is ready to work on another substrate molecule.

Enzymes are known to catalyze more than 5000 biochemical reactions types. Most enzymes are proteins, although a few are catalytic RNA molecules. For the good activities and better productivity of enzymes, their optimal temperature and pH are required [18].

2.2 Sources of Enzyme

Enzymes can be obtained from various sources and these include;

- Plants e.g. Ficin, papain, bromelain, esterase
- Animals e.g. Chymosin (rennin), pepsin, trypsin
- Microorganisms e.g. Pectinase, glucoamylase, keratinase etc.

Classification of enzymes:

Based on the classification given by [19], enzymes can be classified as follows;

- Oxidoreductase: These catalyze oxidation-reduction reactions e.g. Oxidases, Reductases
- Transferases: These catalyze the transfer of functional groups from donor molecules to acceptor molecules e.g. Protein kinase

- Hydrolases: Catalyzes the hydrolysis of various bonds e.g. Proteases
- Lyases: Catalyzes the addition of groups to double bonds e.g. Galdoses
- Isomerases: Catalyzes isomerisation reaction within single molecules e.g. Epimerase
- Ligases: It catalyzes the joining of molecules with covalent bonds e.g. DNA ligase.

2.3 Amylase

Amylases are hydrolases that function by the breakdown or hydrolysis of starch into reducing fermentable sugars, mainly maltose and reducing non fermentable or slowly fermentable dextrins. Numerous microorganisms like *Saccharomycopsis capsularia, Bacillus coagulans, Bacillus* spp have been used for alpha amylase production by solid state fermentation using agro-industrial residues [20]. Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering several industrial processes [21,13,22].

2.4Development of Amylase Research

Amylases are group of important enzymes which are mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars [23,24,25]. Amylases accounts for about 30% of the world's enzyme production [26]. Due to wide range application of amylases in various sectors like confectionery, baking, paper, textile, detergent and pharmaceutical, many are gaining the attention of researchers [27,28].
Amylases are produced on a large scale normally by submerged fermentation (SmF) but in recent years, the attention towards solid state fermentation (SSF) is increasing due to advantages like lower capital expenditure, cheaper fermentation media, super productivity, reduced energy requirements and lack of rigorous control of fermentation parameters [29,30].

Due to ever increasing demand for this enzyme, people are still trying to increase productivity of amylases by a variety of approaches like selection of a high enzyme producing strain, process optimization, usage of cheaper substrates, effective downstream processing etc. [31]. Many researchers have done good amount of work on isolation of some of the thermo-tolerant strains [32]. Several reports have been published by many researchers showing amylase production with *Aspergillus sp* [29,33]. However, it would be economically competitive if the new isolated strains showed some advantages over existing products and every strain has its own unique microbial and biochemical properties [34,35]. Among the various types of amylases produced, commercially thermo-stable amylases are gaining much more advantages in comparison with other types [36]. A great deal of attention is now being given to thermophilic microorganisms and their enzymes [37,12].

2.5 Source of Amylase

Alpha amylase can be derived from several sources such as plants, animals and microbes [13]. Alpha amylases have been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Amylases from plant and microbial sources are employed for centuries as food additives [38].

Barley amylases are used in brewing industry; fungal amylases are widely used in preparation of oriental foods [36]. Fungal and bacterial amylases are mainly used for industrial applications due to their cost effectiveness, consistency, less time and space requirement for production and ease of process optimization and modification [29].

Among bacteria *Bacillus* sp. is widely used for the production of amylases, species like *B.subtilis, B. stearothermophilus, B.licheniformis,* and *B. amyloliquefacien*s are known to be good producers of alpha amylase. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylases [39].

Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of alpha amylase. Starch in cereal, root and tuber crops has been extensively characterized. Apples, like many other fruit crops, accumulate starch at early stages of maturation and progressively degrade starch to increase sweetness during ripening [40].

2.6 Types of Amylases

Amylase can be classified into three types: α-amylase, β-amylase, and γ-amylase. The three types differ in how they hydrolyze the polysaccharide bonds [15].

2.7 Industrial Applications of Amylase

Amylase, a starch degrading enzyme have gained importance in various industrial process like pharmaceutical, food, brewing, paper, textile and chemicals. It is extensively used in pharmaceutical industries in digestive tonics, for hydrolysis of starch to produce different sugars like glucose and maltose which have several applications. The most wide spread applications of α-amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups [41].

1. Removal of starch sizer from textiles (Desizing): In textiles weaving, starch paste is applied for warping. This gives strength to textiles during weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of the string due to laid down wrap. After weaving the cloth, the starch is removed and the cloth goes to scouring and dyeing. The starch on cloth is usually removed by application of alpha amylase [42].

Textile industries are extensively using alpha amylases to hydrolyse and solubilise the starch, which then wash out of the cloth for increasing the stiffness of the finished products. Alpha amylase is used as desizing agent for removing starch from the grey cloth before its further processing in bleaching and dyeing. Many garments especially the ubiquitous 'Jean' are desized after mashing. The desired fabrics are finally laundered and rinsed [43].

2.Bread and bakery industry: Amylases play important role in bakery products. For decades, enzymes such as malt and fungal alpha-amylases have been used in bread-making. Amylases can degrade starch and produce small dextrins for the yeast to act upon. The alpha-amylases degrade the damaged starch in wheat flour into small dextrins, which allows yeast to work continuously during dough fermentation, proofing and the early stage of baking. The result is improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavour [44].

3.Direct starch fermentation to ethanol: The amylolytic activity rate and amount of starch utilization and ethanol yields increase in several folds in co-cultures [26]. Moulds amylases are used in alcohol production and brewing industries. The advantages of such systems are uniform enzyme action in mashes, increase rate of saccharification, alcohol yield and yeast growth [30].

Alpha amylases convert starch into fermentable sugars. Starch such as grain, potatoes etc. are used as a raw material that helps to manufacture ethyl alcohol. In the presence of amylases, the starch is first converted into fermentable sugars. The use of bacterial enzyme partly replaces malt in brewing industry, thus making the process more economically significant. Alpha amylase can also carry out the reactions of alcoholysis by using methanol as a substrate [45].

4.Paper industry: Starch paste when used as a mounting adhesive modified with additives such as protein glue or alum frequently, causes damage to paper as a result of its embrittlement. Starch digesting enzymes; alpha amylase, in immersion or as a gel poultice are applied to facilitate its removal. Alpha amylase hydrolyses the raw starch that is used for sizing and coating the paper instead of expensive chemically modified starches. So, starch is extensively used for some paper size press publications [46].

5. Detergent industry: In detergent industries, the enzyme alpha amylase plays a vital role. It is widely used for improvement of detergency of laundry bleach composition and bleaching without colour darkening [47]. The addition of enzyme stabilizes the bleach agent and preserves effectiveness of the bleach in laundry detergent bar composition [48,49].

6. Bio fuel production: Fossil fuels cause a lot of pollution and cause a lot of harm than benefits. Over the last few decades due the environmental concern and high prices of the fuels, bio fuels have generated so much interest. Bio fuels mainly include ethanol fuel.

Ethanol can be derived from renewable resources such as waste generated from the agriculture crops and by products. Enzymes such as alpha amylase and others like glucoamylase and cellulose are important to produce fermentable sugars to produce ethanol [50].

7. Treatment of starch processing waste water (SPW): Starch is also present in waste produced from food processing plants. Starch waste causes pollution problems. Biotechnological treatment of food processing waste water can produce valuable products such

as microbial biomass protein and also purifies the effluent [51,52,53,54].

3. MATERIALS AND METHODS

3.1 Materials

The materials used in during the course of this research work include various apparatus, equipment, glassware, media and reagents listed as follows Autoclave, weighing balance, incubator, Bunsen burner, Light microscope, hot plate, gas cylinder, refrigerator, spatula, aluminium foil paper, pH meter, cotton wool, inoculating loop and needle, marker, hand gloves, test tube rack, glassware, distilled water,

Media: HiMedia M001- 500G Nutrient agar, HiMedia M096-500G Potato Dextrose agar, starch agar.

Reagents: Aldon 95% Ethanol, Aldon Gram's iodine, Aldon Safranin, Distilled water, immersion oil.

3.2 Methods

Sample collection: The kitchen waste water sample was collected from different kitchen in hostels and restaurant around Ile-Ife, Osun state. The isolates used in this study were obtained from kitchen waste water samples collected from 5 different sites in Ile-Ife;

1. Ajike hostel in maintenance (WSA) 7.4988N, 4.5202E
2. Awolowo hall (WSB)7.520767N,4.530315E
3. Larry-ronk hostel(WSC) 7.4667N, 4.5667E
4. Fine touch hostel, Ede-road (WSD)7.490462N,4.552127E
5. New-Buka, OAU, Ile-Ife (WSE) 7.520767N,4.53031E.

The samples were collected aseptically in sterile McCartney bottle and transported to the laboratory for analysis within 24 hours.

Sterilization and disinfection: All media unless if otherwise stated (some sugars) were sterilized by autoclaving at 121°C for 15 minutes. All processes were carried out under aseptic conditions close to a lighted Bunsen burner flame. The sterile distilled water to be used for serial dilution is prepared in test tubes and it is sterilized in the autoclave. All glasswares were washed with liquid soap and rinsed with water before use.

Preparation of media: According to the manufacturer's instruction, specific grams of the agar powder is measured on a foil paper and weighed using a weighing balance. The agar powder is then added to appropriate volume of water. The mixture is further homogenized by placing the content (in a conical flask) in a water bath with little amount of water to boil, sealed using cotton wool. It is then autoclaved to sterilize the agar which is done at 121°C for 15mins**.** The agar is afterwards left to cool to a lower temperature, the workbench is swabbed with ethanol to disinfect the area, after which Petri dishes are arranged for agar to be poured into. This process is carried out aseptically (i.e. it is carried out beside a Bunsen burner on the work bench). The agar is the poured carefully into Petri dishes and allowed to solidify.

Serial dilution of sample: Ten-fold serial dilution was carried out using exactly 9mL of sterile distilled water in 9 test tubes starting with the original sample in the McCartney bottle. The sample is serially diluted by measuring 1mL of stock solution (sample) into 9mL of sterile distilled water in test tube labelled 10^{-1}, this is then repeated for all 9 test tubes using 9 different pipettes labelled according to each dilution. The process is done in order to reduce the population of the microorganism.

Isolation procedure: Using pour plate method, 0.1mL of 10^{-3}, 10^{-6}, 10^{-9} dilution factor was inoculated into labelled respective Petri dishes containing solidified agar medium aseptically. This inoculation is done on both nutrient agar and potato dextrose agar for isolation of bacteria and fungi respectively. The process is carried out in duplicates so that for each sample collected, twelve plates were inoculated, six for nutrient agar and the other six for potato dextrose agar, it is then incubated appropriately, 37°C for 24 hours for bacteria and at 25°C for 3-5days for fungi.

Enumeration of isolates from water samples: Each sterile Petri dish is labelled with; Sample name/code, Date of collection/incubation and Name of media.

Plating and plate count: The plates were allowed to set and incubated at appropriate temperatures. Sample on nutrient agar were incubated 37°C for 24 hours for bacteria and at 25°C for 3-5days for fungi. Fifty milligram per litre of streptomycin was added to the potato dextrose agar in order to prevent bacterial growth. The nutrient agar plates were inverted during incubation while the PDA plates were not inverted (to allow for spore formation). Plates were examined after the appropriate hours of incubation, the colony forming units were enumerated and the isolates obtained were sub-

cultured on fresh agar plates using the streaking method.

Culture purification and preservation on agar slants: Pure cultures were obtained by carefully picking a well isolated colony that gave distinct characteristics on agar plate and then transferred onto agar slants which was incubated at 37°C for 24 hours. A pure culture is very crucial for accurate and correct identification. Pure culture slants are stored in the fridge under 4°C prior to characterization so as to preserve the organisms for further experiment. They were sub-cultured every two weeks to maintain the viability of the organisms.

3.3 Morphological Characterization of Bacterial Isolates

Gram's staining: To start with, the heat fixed smears of pure bacteria culture taken aseptically from agar slant were prepared individually on clean glass slides, then flooded with crystal violet and led to stand for 30 seconds after which is rinsed with water for 5 seconds. Then the slides were covered with Gram's iodine mordant and allowed to stand for 1 minute, after that the slides were rinsed with water for 5 seconds and then decolorized with 95% ethanol for 15 to 30 seconds. Then the slides were rinsed with water for 5 seconds and counter stained with Safranin for about 60 to 80 seconds. Finally the slides were rinsed with water, air dried and examined under a microscope at 1000x magnification.

3.4 Biochemical Characterization/Test for Bacterial Isolates

1. Indole's test: This test is used to determine the ability of an organism to split tryptophan to form the compound indole. For that, the test strains were inoculated individually into nutrient broth and incubated at 35°C for 24 hours. After that, 0.5mL (about10 drops) of Kovac's reagent was added and the mixture is shaken gently. A deep red colour will develop due to hydrolyzing the tryptophan which indicates the presence of indole. Negative reactions remain colourless or light yellow. This test is based on the production of indole from tryptophan by the organism [55].

2. Methyl Red test: The methyl red test is a quantitative test for acid production (lactic, acetic and formic) from glucose through mixed acid fermentation pathways using only the initial phase of incubation. Prolonged incubation overcomes the pH of the medium and develops a red colour with the methyl red indicator. Red colour indicates positive reaction and yellow colour indicates negative reaction. The organisms were inoculated into MRVP broth and incubated at 35°C for 5 days. After incubation, 2.5mL of broth suspension was transferred to another tube. Then 0.5mL of Methyl Red reagent was added and the colour change was observed.
3. Voges-Proskauer test: The organisms were individually inoculated in to the MRVP broth and then incubated at 35°C for 5 days. After incubation 2.5mL of the broth suspension was transferred into another tube. Then about 0.5mL of VP reagent (alpha - napthol) was added and the tube was gently shaken and observed for colour change in form of a red ring.

4. Citrate utilization test: This test is used to determine the ability of an organism to use sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source. Bacteria that can grow in this medium turn the bromothymol blue indicator from green to blue. For this, the individual strain was streaked onto Simmons citrate agar slants and incubated for 24 to 48 hours at 35ºC. After incubation, slant cultures were observed for the positive or negative reactions. The development of a deep blue colour is confirmed as positive test.

5. Catalase test: This test detects the presence or absence of catalase in the isolates. Catalase is an enzyme found in most bacteria and is known to catalyze the breakdown of hydrogen peroxide to liberate molecular oxygen (O_2) and water H_20

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

To test for catalase, loop of test bacterial cultures was placed on glass slides individually. The cells were then mixed in a drop of 3% hydrogen peroxide and an immediate bubbling indicated a positive catalase test [55].

6. Oxidase test: To perform this test, Whatman's filter paper was placed in a sterile Petri dish and soaked with some drops of Oxidase reagent (N, N-dimethyl-P-phenylenediamine) which is a redox indicator. A loop was then used to pick 18-24 hours old culture of the organism and this is placed on the socked filter paper. Strains which produce oxidase will cause the disc colour change to purple within 15 to 30 seconds. Strain is regarded as delayed oxidase positive, when the colour changes to purple within 2 to 3 minutes. Strain is regarded as oxidase negative if the colour remains unchanged [55].

7. Nitrate reduction test: The nitrate reduction test is performed by growing bacteria in test tube with a nitrate broth medium containing 1% peptone water, 1g of KNO_3, 0.1% NaCl. This test is carried out to be able to determine if the organisms are capable of reducing nitrate to nitrite or even to hydroxylamine, ammonia, or nitrogen to produce Nitrogen gas. Tubes containing the peptone water, KNO_3 and NaCl with inverted Durham tubes are inoculated with the organisms to be tested and incubated for 2-3 days at 35°C. Add 5 drops of R1 (Griess Ilosuay's reagent), followed by R2 (Griess Ilosuay's reagent). A change in colour from yellow to red is a positive result and a yellow coloration is a negative result. Blank tube with no inoculum served as the control.

3.5 Proteolytic Test for Bacterial Isolates

1. Starch hydrolysis test: To assimilate starch for energy and catabolic reactions, it must be degraded into basic glucose units by amylase. These enzymes are secreted by the microorganisms into the medium, which degrade starch primarily to glucose. To test for this, the test strains were individually streaked on to the starch agar plates and incubated for 24 hours at 37ºC. Then the plates were flooded with Gram's iodine solution and excess Gram's iodine was discarded. A clear zone around the colonies is recorded as positive for starch hydrolysis**.**

2. Casein hydrolysis test: For casein hydrolysis, individual strains were streaked on to skim milk agar plates and incubated for 24 hours at 37ºC. After incubation, a clear zone around the colonies resulted for hydrolysis of casein by extra cellular protease. The absence of membrane in gram-positive bacteria creates avenue for direct secretion of proteins by amylase producing microorganisms as seen in *Aspergillusawamori* which shows both amylase and protease activity under wide range of pH [56].

3. Gelatin hydrolysis test: This test is used to determine the ability of an organism to produce proteolytic enzymes (gelatinases) that liquefy gelatin. The medium used was nutrient broth to which 10% gelatin was added, converting it into a semisolid medium. Then the test strains were inoculated individually at aseptic condition into sterile bottles containing the gelatin medium and incubated at 37ºC for 24 hours. Then the bottles were placed into a refrigerator for 1 hour. Refrigeration causes undigested gelatin to resolidify. If gelatinase is present, the liquid medium will fail to solidify upon refrigeration.

4. Carbohydrate fermentation test for bacterial isolates: Carbohydrate fermentation test can also be used to detect the ability of an organism to produce acid and gas from different sugars. Basal broth was prepared and distributed into tubes with bromocresol purple acting as the indicator. Inverted glass vials (Durham's tube) were inserted into each tube without bubbles. After sterilization of the medium, 1% sugar solution was added. Carbohydrates such as glucose, fructose, lactose, sucrose, xylose, mannitol, dulcitol were selected for this test. The test organism was inoculated without disturbing the Durham's tube; it is then incubated at 35°C for about 3-5 days. The Durham's tubes were observed for the accumulation of gas and the acid production was observed by the colour change of the media from purple to yellow.

3.6 Physiological Studies for Bacterial Isolates

Growth in alkaline and acidic medium: This test is carried out to be able to determine if the strains can grow in acidic or alkaline medium. For acidic medium, nutrient agar and HCl to achieve a pH of about 3.9 and for alkaline medium, nutrient broth and NaOH or KOH is used to achieve a pH of about 9.4. Using a 18-24 hours old culture, incubation is carried out for 24 hours at 35°C. A positive test will show turbidity and growth while negative will produce no turbidity and no growth.

Growth at different temperature 25°C and 70°C: This test is carried out to determine if the strains are psychrophiles, mesophiles or thermophiles. Using 18-24 hours old culture of the bacterial isolates, the organism was streaked on nutrient agar plates and then incubated at high (70°C) and low (25°C) temperature. The test was done to determine the temperature that favors the growth of the bacteria and metabolism as indicated by growth on the agar. While carrying out the test un-inoculated plates were used as control.

Growth in 6.5% NaCl: This test is carried out to determine if the strains are halophilic in nature or not. It involves using 1% peptone water and 6.5g of NaCl in 100ml of distilled water. Then 18-24 hours old culture is inoculated and incubated at 37°C for 24 hours. A positive test shows turbidity and growth while negative shows no growth or turbidity.

3.7Identification of Fungi

The cultures were identified at genus level on the basis of macroscopic characteristics; colonial morphology, color, texture, shape and appearance of morphology.

Starch Hydrolysis Test (Screening of Potential Amylase Producing Fungi): The selected fungal isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The selected fungal isolates were streaked on the starch agar plate and incubated at 35°C for 2-3 days. After incubation and growth, iodine solution was flooded with dropper for 30 seconds on the starch agar plate. Presence of blue color around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The isolates that produced clear zones of hydrolysis were considered as probable amylase producers.

4. RESULTS

A total of fifteen (15) bacteria and six (6) fungi were isolated during the course of this study. The isolates were screened for amylolytic activity by starch hydrolysis on starch agar plate, out of fifteen bacteria isolates, four (4) were positive for starch hydrolysis while for the fungi, three (3) out of six (6) were positive.

After preliminary screening using starch hydrolysis, other tests were carried out, such as biochemical test, Gram staining and other proteolytic tests like casein hydrolysis and gelatin hydrolysis. The probable isolates were identified using Bergey's Manual of Descriptive Bacteriology [57].

This article contains a concise summary of results of the experiment carried out during the course of this research. The morphological characteristics of the bacterial isolates describing the shape, size, colour and appearance, edges and elevation is highlighted in Table 1, while results for all biochemical tests, Gram staining, proteolytic test and physiological studies for the bacterial isolates are shown in Table 2.

The mean microbial load of all five samples on nutrient and potato dextrose agar plates is enumerated in Table 3 while Table 4 shows the zones of hydrolysis of isolates (bacteria and fungi) on starch agar. The macroscopic and colony morphology of the fungal isolates are highlighted in Table 5.

5. DISCUSSION

Microbial amylase is one of the most important enzymes used in various industries today; in the brewing, detergent, paper, textile, bakery and food industries. Amylase production however depends on the inherent property of the microorganism, only microbes that can produce substantial amount of extracellular amylase are of industrial importance and can be exploited commercially. In this study, four (4) bacteria and three (3) fungal strains with suspected amylolytic activity were identified based on their ability to hydrolyse starch.

In this research, enzyme activity was related to the extent of starch hydrolysis, the starch hydrolysis shows the level of each isolate's amylase activity as shown in Table 4, for the bacterial isolates, highest activity was recorded in *Bacillus brevis* (0.7cm) and *Bacillus macerans* (0.7cm)which was closely followed by *Bacillus cirulans* (0.6cm), the least activity was shown by *Bacillus subtilis* (0.5cm). In the fungal strain, the highest enzyme activity was observed in *Aspergillus flavus* (0.9cm) followed by *Aspergillus niger* (0.8cm)and the least activity was shown by *Aspergillus fumigatus* (0.6cm).

Amylases are produced at wide ranges of temperature (35°C -80°C) by various *Bacillus* sp [58,59]. In this study, the bacterial enzyme production was carried out at different temperatures (25°C and 70°C) and maximum production was obtained at 25°C. Research by [60] states that *Bacillus* sp is capable of amylase activity from 30°C-70°C, which corroborates this study where optimum growth for bacterial growth was at 25°C.

The study in [5] also reports the influence of temperature on amylase production where optimum temperature for *Bacillus* sp and *Aspergillus* sp in the study was at 40°C thereby, validating the lack of growth and amylase production at 70°C as seen in this study and agrees with [61,12] who reported that amylase activity can be inhibited by heat.

It can then be discussed that the fungal and bacterial isolates in optimum conditions possess the ability to produce amylase. Evidences of amylase in yeast, moulds and bacteria have been reported and their properties documented [62,12]. Studies on bacteria amylase especially in developing countries have concentrated mainly on *Bacillus* sp probably because of the simple nature and nutritional requirements of this organisms [63,37,12]. *Bacillus* sp produce a large variety of extracellular amylase enzyme, which makes them have significant importance

[64]. Amylase producing ability of *Bacillus* sp and *Aspergillus* sp have been reported from several other studies [5]and can be exploited for large scale productions [65-68].

Table 1. Morphological Characteristics of Bacteria Isolate

Isolate Code	Shape	Size	Edges	Elevation	Colour and Appearance	Pigmentattion
BWSA1	Circular	medium	entire	Small	pale yellow	-
BWSD1	Circular	medium	lobate	low convex	creamy white	-
BWSA2	Irregular	medium	lobate	Flat	creamy white	-
BWSC1	Circular	Small	entire	low convex	creamy mucoid	-

Table 2.Proteolytic, gramstaining, physiological and biochemical characteristics of bacteria isolate

Isolate Code	Gram staining	Cellular Morphology	Catalase	Indole	MR	VP	Oxidase	Citrate	Casein hydrolysis	Gelatin Hydrolysis	Nitrate Reduction	Growth at 25^0C	Growth at 70^0C	Alkaline medium	Acidic medium	Glucose	Fructose	Lactose	Mannitol	Xylose	Dulcitol	Sucrose	Growth in 6.5% Nacl	Probable identity
BWSA1	+	ROD	+	-	-	-	-	-	-	-	-	+	-	-	-	+G	+G	+G	-	(+)	-	-	(+)	*Bacillus macerans*
BWSD1	+	ROD	+	-	+	-	(+)	+	(+)	-	+	+	-	+	-	+	(+)G	+G	-	+G	-	+	(+)	*Bacillus circulans*
BWSA2	+	ROD	(+)	-	-	(+)	-	+	++	-	+	+	-	+	(+)	(+)	(+)	+	-	+G	-	-	+	*Bacillus subtilis*
BWSC1	+	ROD	+	+	-	-	(+)	+	++	-	-	+	-	(+)	-	+	+	(+)	(+)	-	-	(+)	+	*Bacillus brevis*

Table 3. Mean microbial load of sample on nutrient agar and potato dextrose agar

Sample No	Sample Location	TBC (cfu/ml)	TFC (sfu/ml)
WSA	Ajike hostel, maintenace	3.173×10^6	6.730×10^8
WSB	Awolowo hall, oau campus	1.074×10^7	6.760×10^8
WSC	Larry-ronk hostel, parakin	5.262×10^6	4.004×10^8
WSD	Fine-touch hostel, ede road	2.459×10^6	6.69×10^7
WSE	New- buka, oau campus	6.792×10^8	TMTC

KEY
TBC- Total Bacterial Count
TFC-Total Fungal Count
cfu/ml- Colony forming unit per millilitre
sfu/ml- Spore forming unit per millilitre
TMTC- Too many to count

Table 4. Zone of hydrolysis of isolates

	Isolate Code	Zone of Hydrolysis (cm)
Fungal Isolates	FWSA	0.8
	FWSC	0.9
	FWSE	0.6
	BWSA1	0.7
	BWSD1	0.6
Bacterial Isolates	BWSA2	0.5
	BWSC1	0.7

Table 5. Macroscopic and colony morphology of fungal isolate

Isolate Code	Edges	Elevation	Pigmentation	Morphological Characteristics	Probable Identity
FWSA	Rhizoid	Low convex	_	Black filamentous colony	*Aspergillus niger*
FWSC	Rhizoid	Low convex	Brown	Yellow-green to brown filamentous colony	*Aspergillus flavus*
FWSE	Rhizoid	Low convex	_	Greenfilamentous colony with white edges	*Aspergillus fumigatus*

KEY

WSA – Water Sample A	*BWSA- Bacteria isolate from Water Sample A*
WSB- Water Sample B	*BWSB- Bacteria isolate from Water Sample B*
WSC- Water Sample C	*BWSC- Bacteria isolate from Water Sample C*
WSD- Water Sample D	*BWSD- Bacteria isolate from Water Sample D*
WSE- Water Sample E	*BWSE- Bacteria isolate from Water Sample E*
- Negative	*FWSA- Fungal isolate from Water Sample A*
+ Positive	*FWSB- Fungal isolate from Water Sample B*
++ Strongly positive	*FWSC- Fungal isolate from Water Sample C*
(+) Weakly positive	*FWSD- Fungal isolate from Water Sample D*
G- Gas production	*FWSE- Fungal isolate from Water Sample E*

6. CONCLUSION

According to the results obtained in this research, it can be concluded that both the fungal and the bacterial isolates serve as good sources of amylase enzyme. Therefore, providing evidence that microorganisms are excellent sources of enzymes for both natural and industrial bioconversion processes. In addition, isolation and recovery of extracellular products is easier in fungi and also in *Bacillus* species that produce a large variety of extracellular amylase enzyme. In conclusion, this study shows that microorganisms from kitchen waste water are suitable for large scale amylase production.

Furthermore, the quantification of amylase activity can help in the discovery of new amylase producing microorganism through optimal conditions of temperature, incubation, nutrients, moisture (salt concentration),carbon source, pH and nitrogen source through which novel microorganisms can be ascertained. All these advantages make microbial enzymes to be preferred to enzymes from other sources.

7. RECOMMENDATION

More effort should be put into microbial enzyme research, so as to gain more insight on the various ways enzymes can be modified and put to use. More studies on discovering novel and useful microorganisms will provide valuable tools for manoeuvring and enhancing enzyme production. Genetic engineering coupled with protein engineering and direct evolution can lead to development of a more stable enzyme, this research also illuminates the need to ensure judicious wastewater disposal due to presence of both deleterious and benign microorganisms isolated from the specimen.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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