

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, [Wwaste water](#)

INTRODUCTION

Comment [Nr1]: Introduction should be elaborate focusing the needs and impacts of performing this research

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].

~~Water covers seven tenth of the Earth's surface and serves as habitat for a wide variety of animals, plants and microorganisms. Life in the aquatic environment (freshwater and marine) has numerous potential advantages over terrestrial existence. These include physical support, accessibility of three-dimensional space, passive movements by water currents, dispersal of motile elements (gametes) in liquid medium, lower extremes of temperature and solar radiation and availability of soluble organic and inorganic nutrients. Water microorganisms are capable of flourishing in different water habitats, from several kilometres below seafloor to the surface of lakes and streams. Three major domains compose water microorganisms; eukaryota, archaea and bacteria as well as viruses [4].~~

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 [5].???

Comment [Nr2]: List out the most abundantly found microorganisms in such samples

Comment [Nr3]: Explain the adaptive mechanisms of microorgisms living in the kitchen environment

Comment [Nr4]: Mention a few bacterial and fungal genera or species that are highly significant in the production of amylase

Microorganisms have successfully proved their usefulness as rich sources of useful enzymes.

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 commercial industries owing 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 [6]. It is the main reserve polysaccharide of cereal grains, roots, and tubers [7] [8] 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 [9] [10].

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 including 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 [11].

Amylase can be derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [12].

Comment [Nr5]: Comment on the isoforms of amylase and which can be commonly purified from microorganisms

2 LITERATURE REVIEW

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2.1 Starch

~~Before describing the action pattern and properties of amylolytic enzymes, it is essential to discuss the features of the natural substrate, 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 (Smith, 2001). Starch is formed in the chloroplasts of green leaves and amyloplasts, organelles responsible for the starch reserve synthesis of cereals and tubers [6] [13].

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Starch granule is a heterogeneous material; chemically, it contains both linear (amylose) and branched (amylopectin) structures; physically, it has both amorphous and crystalline region [14].

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.

2.2 Enzymes

Enzymes are macromolecular biological catalysts that accelerate the rate of chemical reactions including most metabolic processes in the cell. 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 [15].

Comment [Nr8]: Bring out the general working principle of enzymes

2.2.1 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.

2.2.2 Classification of enzymes

Based on the classification given by [16], 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 dextrans. Numerous microorganisms like *Saccharomyces cerevisiae*, *Bacillus coagulans*, *Bacillus* spp have been used for alpha amylase production by solid state fermentation using agro-industrial residues [17]. Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering several industrial processes [18][11][19].

2.4 Development 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 [20][21][22]. Amylases accounts for about 30% of the world's enzyme production [23]. 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 [24][25].

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 [26][27].

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. [28]. Many researchers have done good amount of work on isolation of some of the thermo-tolerant strains [29]. Several reports have been published by many researchers showing amylase production with *Aspergillus sp*[26][30]. 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 [31][32]. Among the various types of amylases produced, commercially thermo-stable amylases are gaining much more advantages in comparison with other types [33]. A

great deal of attention is now being given to thermophilic microorganisms and their enzymes [34] [10].

2.5 Source of amylase

Alpha amylase can be derived from several sources such as plants, animals and microbes [11]. 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 [35].

Barley amylases are used in brewing industry; fungal amylases are widely used in preparation of oriental foods [33].

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 [26].

Among bacteria *Bacillus* sp. is widely used for the production of amylases, species like *B.subtilis*, *B. stearothermophilus*, *B.licheniformis*, and *B.amyloliquefaciens* 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 alpha amylases [36].

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 [37].

2.6 Types of Amylase

Amylase can be classified into three types: alpha-amylase, beta-amylase, and gamma-amylase. The three types differ in how they hydrolyze the polysaccharide bonds. Neither beta nor gamma-amylase is found in animal tissue.

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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 [38].

2.7.1 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 [39].

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 [40].

2.7.2 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 [41].

2.7.3 Direct starch fermentation to ethanol

The amylolytic activity rate and amount of starch utilization and ethanol yields increase in several folds in co-cultures [23]. 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 [27].

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 [42].

2.7.4 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 [43].

2.7.5 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 [44]. The addition of enzyme stabilizes the bleach agent and preserves effectiveness of the bleach in laundry detergent bar composition [45] [46].

2.7.6 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 [47].

2.7.8 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 [48][49][50][51].

2.7.9 Other applications

Amylases, especially alkaline amylases are used in detergents. To some extent amylases are also used as digestive aids to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients.

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2.8 Objectives 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.

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3. MATERIALS AND METHODS

3.1 MATERIALS

Comment [Nr12]: Bring all the chemicals, reagents, and instruments used in a single paragraph

~~The materials used in during the course of this research work include various apparatus, equipment, glassware, media and reagents. Below is the list of materials used:~~

3.1.1 Equipment

~~Autoclave, weighing balance, incubator, Bunsen burner, light microscope, hot plate, gas cylinder, refrigerator, spatula, aluminium foil, pH meter, cotton wool, inoculating loop and needle, marker, hand gloves, test tube rack, distilled water.~~

3.1.2 Glassware

~~Measuring cylinder, test tubes, conical flasks, microscopic slides, McCartney bottles, glass spreader.~~

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3.1.3 Media

~~Nutrient agar, potato dextrose agar, starch agar.~~

3.1.4 Reagents

~~Ethanol, Gram's iodine, Safranin, Distilled water, immersion oil.~~

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3.1.5 Sample

~~Kitchen waste water or kitchen sewage from hostels and restaurants.~~

3.2 METHODS

~~The methods and processes used during this experiment include~~

3.2.1 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 was obtained from kitchen waste water samples collected from 5 different sites in Ile-Ife; ~~Ajike hostel in maintenance (WSA), Awolowo hall (WSB), Larry-ronk hostel(WSC), Fine touch hostel, Ede-road (WSD), New-Buka, OAU, Ile-Ife~~

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

Comment [Nr14]: Mention the latitude and the longitude specifications

3.2.2 Sterilization and Disinfection

~~This process is carried out to ensure sterility of the materials prior to the usage. Inoculating loops and needles were flamed red hot and allowed to cool before and after each use. The work bench is disinfected using 70% alcohol before and after use.~~ 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 glassware swear washed with liquid soap and rinsed with water before use.

3.2.3 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 weighed agar powder is then added to appropriate volume of water (depending on the calculation done using the manufacturer's instruction) measured using a measuring cylinder in a conical flask, mix to form a homogeneous mixture. The mixture is then further homogenized by placing the content (which is in a conical flask) in a water bath with little amount of water to boil, it is sealed using cotton wool. It is then autoclaved to sterilize the agar which is done at 121°C for 15mins. The agar is then 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. using procedures or steps to minimize risk of contamination (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.

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3.2.4 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.

3.2.5 Isolation procedure

~~Petri dishes containing agar labelled according to each dilution factor is arranged a disinfected work bench.~~ 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.

Comment [Nr16]: Specify the conditions

~~3.2.6 Enumeration of isolates from water samples~~

~~3.2.6 The workbench and work area is always swabbed with disinfectant so as to avoid contamination. Sterile Petri is labelled with permanent maker for easy identification and detailed recording. Each sterile Petri dish is labelled with; Sample name/code, Date of collection/incubation and Name of media.~~

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3.2.7 Plating and plate count

The plates were allowed to set and incubated at appropriate temperatures. Samples 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. ~~The pure isolates were then stored on their agar slants and kept under a temperature of 4°C in the refrigerator after incubation at 37°C for 24 hours.~~

3.2.8 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 work. ~~They were~~ It was sub-cultured every two weeks to maintain the viability of the organisms.

3.3. MORPHOLOGICAL CHARACTERIZATION ~~OFFOR~~ BACTERIAL ISOLATES

3.3.1 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.

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3.3.2 Biochemical characterization for bacterial isolates

- 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 (about 10 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 [52].

- 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.

- 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 - naphthol) was added and the tube was gently shaken and observed for colour change in form of a red ring.

- 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 35°C. After incubation, slant cultures were observed for the positive or negative reactions. The development of a deep blue colour is confirmed as positive test.

- 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 (O₂) and water (H₂O).

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To test for catalase, loop of test bacterial cultures were 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 [52].

- 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 slide was then used to pick 18-24 hours old culture of the organism and this is placed on the soaked 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 [52].

- 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.

3.3.3 Proteolytic test for bacterial isolates

- 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

Comment [Nr18]: loop or needle?

starch primarily to glucose. To testFor this, the test strains were individually streaked on to the starch agar plates and incubated for 24 hours at 37°C. 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.

- Casein hydrolysis test

For casein hydrolysis, individual strains were streaked on to skim milk agar plates and incubated for 24 hours at 37°C. After incubation, a clear zone around the colonies resulted for hydrolysis of casein by extra cellular protease.

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- 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 37°C 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.

- 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 medium from purple to yellow.

3.3.4 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.

3.4. IDENTIFICATION OF FUNGI

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

3.5. SCREENING OF POTENTIAL AMYLASE PRODUCING FUNGI BY STARCH HYDROLYSIS TEST

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. RESULT

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. This [article chapter](#) 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.

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Comment [Nr22]: Include standard errors of the mean or standard deviations if available as you have performed the experiment in duplicates. See to it if the values are statistically significant.

TABLE 1: MORPHOLOGICAL CHARACTERISTICS OF BACTERIA ISOLATE

ISOLATE CODE	SHAPE	SIZE	EDGES	ELEVATION	COLOUR AND APPEARANCE	PIGMENTATION
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°C	Growth at 70°C	Alkaline medium	Acidic medium	Glucose	Fructose	Lactose	Mannitol	Xylose	Dulcitol	Sucrose	Growth in 6.5% NaCl	Probable identity
BWSA1	+	ROD	+	-	-	-	-	-	-	-	-	+	-	-	-	+G	+G	+G	-	(+)	-	-	(+)	<i>Bacillus macerans</i>
BWSD1	+	ROD	+	-	+	-	(+)	+	(+)	-	+	+	-	+	-	+	(+G)	+G	-	+G	-	+	(+)	<i>Bacillus circulans</i>
BWSA2	+	ROD	(+)	-	-	(+)	-	+	++	-	+	+	-	+	(+)	(+)	(+)	+	-	+G	-	-	+	<i>Bacillus subtilis</i>
BWSC1	+	ROD	+	+	-	-	(+)	+	++	-	-	+	-	(+)	-	+	+	(+)	(+)	-	-	(+)	+	<i>Bacillus brevis</i>

TABLE 3: MEAN MICROBIAL LOAD OF SAMPLE ON NUTRIENT AGAR AND POTATO DEXTROSE AGAR

SAMPLE No	SAMPLE LOCATION	TBC (cCfu/ml)	TFC (sSfu/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

| cCfu/ml- Colony forming unit per millilitre

| sSfu/ml- Spore forming unit per millilitre

TMTC- Too many to count

TABLE 4: ZONE OF HYDROLYSIS OF ISOLATES

	ISOLATE CODE	ZONE OF HYDROLYSIS (<u>cm</u>)
FUNGAL ISOLATES	FWSA	0.8
	FWSC	0.9
	FWSE	0.6
BACTERIAL ISOLATES	BWSA1	0.7
	BWSD1	0.6
	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	<i>Aspergillus niger</i>
FWSC	Rhizoid	Low convex	Brown	Yellow-green to brown filamentous colony	<i>Aspergillus flavus</i>
FWSE	Rhizoid	Low convex	–	Green filamentous colony with white edges	<i>Aspergillus fumigatus</i>

KEY

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

5. DISCUSSION

Microbial amylase is one of the most important enzymes used in various industries today. 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 amyolytic activity were identified based on their ability to hydrolyse starch.

In this research study, enzyme activity was related to the extent of starch hydrolysis. For the bacterial isolates, highest activity was recorded in *Bacillus brevis* and *Bacillus macerans* which was closely followed by *Bacillus cirulans*, the least activity was shown by *Bacillus subtilis*. For the fungal strain, the highest enzyme activity was observed in *Aspergillus flavus*, followed by *Aspergillus niger* and the least activity was shown by *Aspergillus fumigatus*.

It can then be discussed that the fungal and bacterial isolates showed on an average, equal ability to produce amylase. Evidences of amylase in yeast, moulds and bacteria have been reported and their properties documented [53] [10]. Studies on bacterial amylase especially in developing countries have concentrated mainly on *Bacillus* spp., probably because of the simple nature and nutritional requirements of this organisms [54][34][10]. *Bacillus* species produce a large variety of extracellular amylase enzyme, which makes them have significant importance [55].

Comment [Nr23]: List a few industries or industrial sectors

Comment [Nr24]: Discuss why the bacterial isolate in your study exhibited maximum and minimum qualitative amylase activities

Comment [Nr25]: Add

Comment [Nr26]: Discuss some of the quantitative amylase activities in the same microorganisms

Comment [Nr27]: Add similar discussion relative to other's experiments

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.

All these advantages makes microbial enzymes to be preferred to enzymes from other sources.

Comment [Nr28]: Conclude by stating the quantification of amylase activities could potentially help in finding out novel amylase producing microorganisms accordingly as a future perspective

Comment [Nr29]: Justify

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 these 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.

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Comment [Nr30]: Proof-read and correct the reference style for some references according to the journal's guidelines

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