DETERMINATION OF PROTEOLYTIC POTENTIAL OF MICROORGANISMS ISOLATED FROM SELECTED DUMPSITE IN ILARO AND ITS ENVIRONMENT

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Abstract
Microbial enzymes, including proteolytic proteases, are notable contributors to the bio-economy world and have been the subject of comprehensive study in enzymology since its genesis. In this study, soil samples were collected from Oja Odan and Ilaro dumpsites in Ogun State, Nigeria, to determine the proteolytic potential of the microorganisms present. The pour plate method was employed to isolate the microorganisms, which were identified based on their colonial, microscopic, and biochemical features. In total, six (6) bacterial strains; *Pseudomonas* spp, *Staphylococcus* spp, *Escherichia coli*, *Salmonella* spp, *Shigella* spp, with *Coliform* bacteria were identified from both dumpsites. Additionally, two fungal strains were identified: yeast and yeast-like cells and *Fusarium oxysporum*. In the Ilaro soil sample, isolate IL-1 had the highest colony count, while isolate IL-8 had the lowest at 6×10^3. In the Oja Odan soil sample, isolate OO-1 had the highest colony count, and isolate OO-8 had the lowest at 6×10^3. All bacterial and fungal isolates tested positive for casein hydrolysis, showing their ability to produce the enzyme protease.

Keywords: colony, Proteolytic, protease, dumpsite,

Introduction

The use of chemicals has increased tremendously in the industrial world, affecting the health of the populace. Therefore, substituting these toxic and poisonous chemical substances with eco-friendly products for the improvement of healthy living around the globe has been the motive of the modern world today. Microorganisms form a considerable amount of the world's biodiversity, with the earth playing a crucial role in several ecological processes required for life maintenance. Microorganisms are widely dispersed almost in all biome (atmosphere, hydrosphere, and lithosphere) with the soil being the highest ranking habitat because it plays a depository role for diverse microorganisms (Ilesanmi *et al.*, 2023). Microbial enzymes including lipases, polymerases, amylases and proteases are notably contributors to the bio-economy world over. Protease-producing microorganisms are ubiquitous and can be isolated from soil (Parthasarathy & Gnanadoss, 2020), wastewater and sludge (Gormez, 2020), as well as undersea fumaroles (Iqbalsyah et al., 2019) and can be found in numerous species of microorganisms which includes bacteria, fungi, and yeast (Barrett and McDonald, 1986).

These multifunctional enzymes known as microbial proteases belong to hydrolase groups that catalyze the breakdown of various proteinaceous polymers into their peptides and amino acids constituents (Guleria et al., 2016; Nonso et al., 2020). During protein modification, these degradative enzymes exhibit high specificity and selectivity (Rao et al., 1998). Among industrial enzymes, protease contribution is approximately sixty (60) percent globally, which is primarily bacteria (*Bacillus* spp) source (Bouacem et al., 2015). Several proteases enzymes that are produced by *Bacillus* spp has numerous functions and, are readily available for commercial purpose (Nonso et al., 2020).

Proteases serves as one of the essential component of life on earth and can be classified according to (i) where they originate from (ii) their catalytic activity and (iii) what kind of reactive group they possess in their catalytic site. Based on their catalytic mechanism, proteases can be grouped into two according to their catalytic mechanism which includes; exopeptidases (which hydrolyze proteins from their terminal) and endopeptidases (hydrolyze proteins from the interior) (Gurumallesh et al., 2019). Gupta et al. (2002) classified proteases based on pH into acidic, neutral, and alkaline. Amidst them, alkaline proteases gained high potency for industrial purposes, which could be as a result of their capability to withstand higher pH conditions (Sakpal & Narayan, 2015).

Proteolytic proteases have extensively been studied since dawn of enzymology (Razzaq et al., 2019). They have gained attention in addition to being widely utilized in a variety of industries (Younes & Rinaudo, 2015). Microbial-derived proteases are the market leaders due to their high yield, efficient use of time, low spatial requirements, easily manipulable genetics, and affordability factors that perfected them for biotechnological applications (Ali et al., 2016). Proteases are also employed in detergent and food industries which make them the largest product segment for enzymes in the world industrial market (Acrofan, 2021; FOC Group, 2022). With the development of science and technology, there is an increase in the use of protease enzymes in several bioremediation processes and leather treatments (Research and Markets, 2021). Furthermore, the use of protease enzymes are functional in medicine production, due to the high potency of protease enzymes in treating multiple diseases which includes heart, lung, eye, skin ulcer, and digestive tract diseases and soreness (Shrivastava et al., 2019).

Waste disposal sites have been identified as rich sources of microorganisms with various industrial significances. Various garbage dumpsites, such as abattoirs, refuse trash dumpsites, and domestic waste dumpsites, contain sewage sludge (Badhe et al., 2016). The problem at hand is the disposal of waste on land, including waste generated during the extraction and conversion of raw materials and the consumption of final products in households and other human activities (Bharathiraja et al., 2017). Microorganisms like bacteria and fungi thrive by using waste materials as a source of nutrients for their growth and for breaking down organic material within the trash (Williams & Hakam, 2015), making them valuable in bioprocess technology (Varjani et al., 2017). Waste dumpsites contain a complex mix of materials from different sources. The dumpsite's proximity to residential areas, businesses, and markets significantly affects its composition (Bharathiraja et al., 2017). Chemically, these waste materials consist of proteins, lipids, cellulose, fats, and other organic matter. When microbes break down organic matter in the trash quickly due to moisture and salt content, it results in the production of unpleasant odors (Melikoglu & Lin, 2015). Recently, biological catalyst enzymes have been increasingly used as industrial catalyst agents. Proteases are produced by various plants and animals (Prakash & Banik, 2004).

Microorganisms capable of producing enzymes are widely acknowledged to exist on Earth. To discover new industrial enzymes, proteolytic enzymes need to be extracted and examined from soil samples. Dumpsites are known to harbor a wide variety of microorganisms, some of which remain unknown and have the ability to produce enzymes. The extraction and screening of enzymes from dumpsites are crucial for the progress of biotechnological processes and for supporting the national economy. This study aims at determining the proteolytic potential of isolated microorganisms from dumpsites in Ilaro and its environment.

MATERIALS AND METHODS

Materials

Nutrient agar, potato dextrose agar, skim milk agar, *Salmonella-Shigella* agar, Eosin Methylene Blue Agar (EMB), soil samples, Ziploc bags, distilled water, petri dishes, conical flask, measuring cylinder, beaker, inoculating loop/pin, microscope, weighing balance, foil paper, methylated spirit lamp, incubator, colony counter, Needle and syringe, Autoclave, Hand glove, Glass slide, cover slip, lactophenol cotton blue, Gram's stain, Lugol's iodine, McCartney bottle, paper tape, dropper, soil auger, and thermometer.

Methods

Samples Collection

Soil samples were aseptically collected from two dumpsites in Ilaro and its environs using a sterile soil auger from a depth of 0-20cm and stored in two separate Ziploc bags. The dumpsites were Iyana Egbo and Oja-odan dumpsites located in Yewa region, Ogun state (as shown in Figures 1 and 2). These samples were A and B, respectively. Soil temperature at the location was measured using a thermometer, and the average temperature range was recorded for each sampling site. The samples were then conveyed to the microbiology laboratory in the department of Science Laboratory Technology at the Federal Polytechnic, Ilaro, for microbiological analysis.

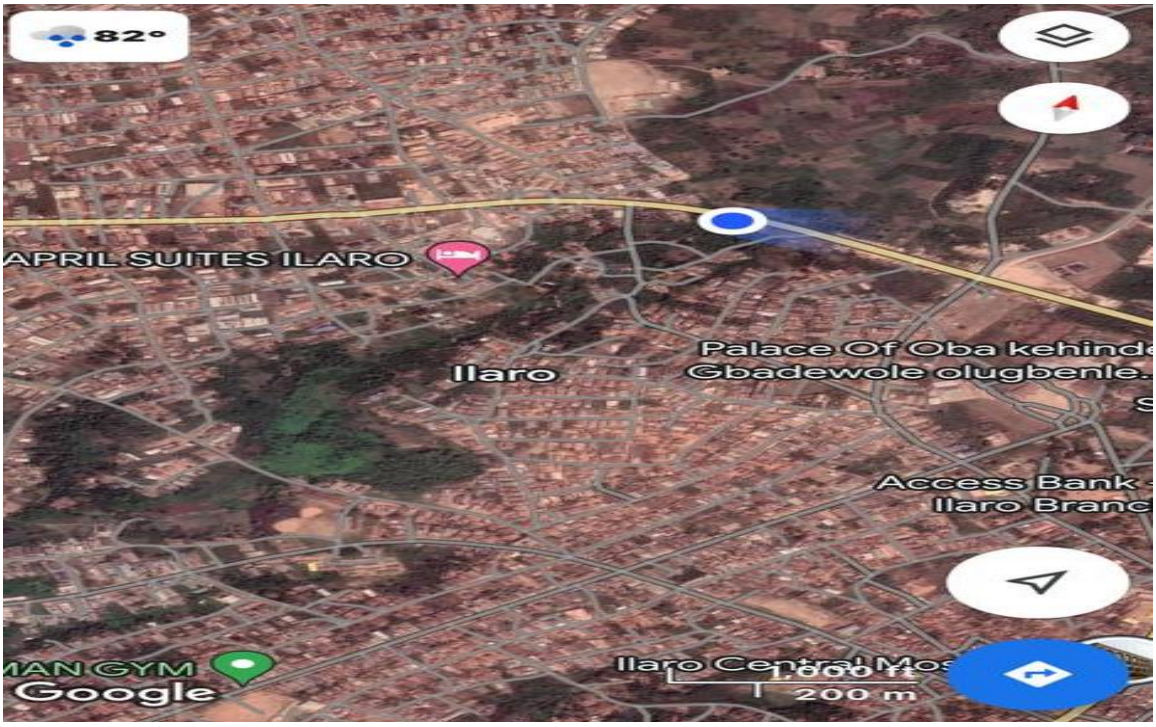

Figure 1: Ilaro Dumpsite Map

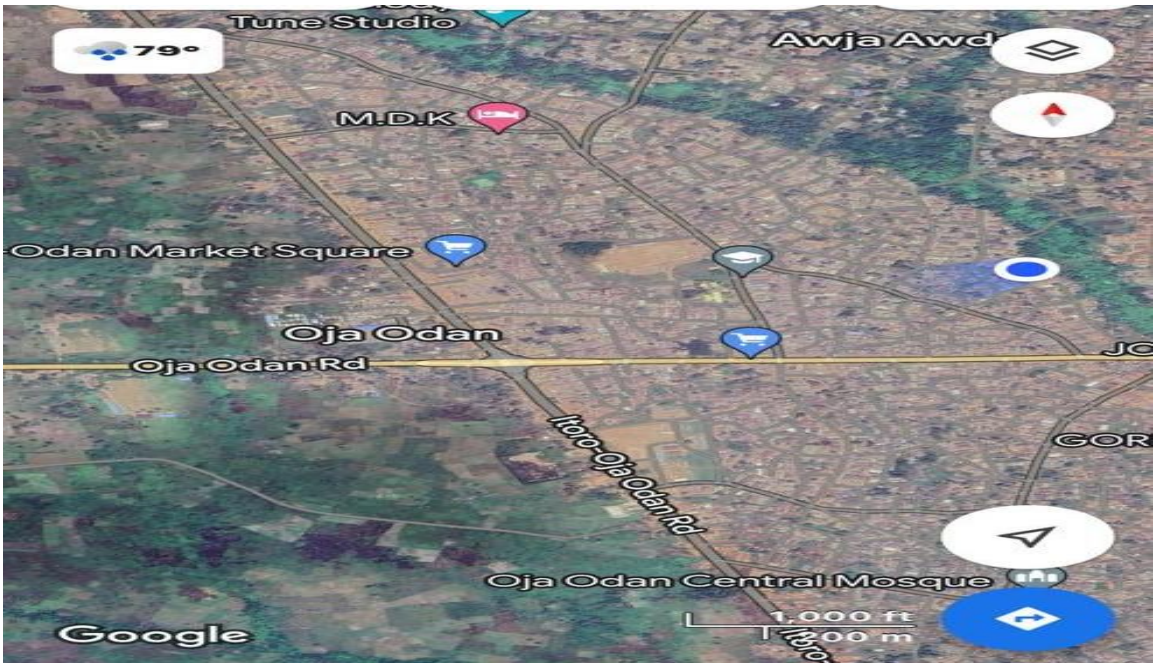

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Figure 2: Oja-Odan Dumpsite Map

Sample Preparation
Upon receiving the soil samples in the laboratory, a serial dilution was carried out on samples A and B until a 10^{-6}fold dilution was achieved. This procedure aimed to decrease the microbial concentration in the soil sample. Dilution factors of 10^{-4} and 10^{-6} were employed for microbial culture.

Preparation of Media
The culture media employed include nutrient agar, potato dextrose agar (PDA), Eosin Methylene Blue Agar (EMB), *Salmonella-Shigella* Agar (SSA) and Skim milk agar, all prepared as per the manufacture's guidelines. The PDA was enhanced with chloramphenicol (50mg/L). Conical flasks used for preparation were sealed with cotton plugs sterilized at 121^0C for 15minutes, followed by cooling before dispensing into Petri dishes.

Isolation of microorganisms from Soil sample
Following serial dilution, 1ml of the 10^{-4} and 10^{-6} dilution was inoculated onto sterile nutrient agar plates and potato dextrose agar plates in duplicates using the pour plate method. The nutrient agar plates were them incubated at 30 ± 2^0C for 24 - 48 hours, whereas the PDA plates were incubated at 28 ± 2^0c for 4 - 8 days.
After the specified incubation periods, the plates were examined for microbial growth, and the number of colonies was enumerated to determine the viable count of microorganisms in the sample. Representative colonies were subsequently subcultures of the isolates. Identification of bacteria and fungi was performed based on their colonial morphology, microscopic characteristics, and biochemical tests. The identified strains were preserved on PDA and nutrient agar slants at 4^0C (Guatam et al., 2012).

Identification and Characterization of microorganisms

Biochemical characterization
Biochemical tests were performed to characterize the isolates, encompassing the catalase test, citrate test, indole test, urease test, oxidase test, methyl red test, gelatinase test, casein hydrolysis test, and sugar fermentation test. The interpretation of these tests aligned with the standard reference from Bergey's Manual of Determinative Bacteriology.

Microscopic examination of fungal cells
Each isolated fungus was sub-cultured on potato dextrose agar (PDA) and subjected to detailed characterization. Observations focused on spore head morphology, mycelial color, and hyphal characteristics. Sections of young mycelium from the culture periphery were carefully excised using a sterile razor blade and transferred onto clean glass slides. The agar section was briefly flamed to liquefy the agar and then stained with lactophenol cotton blue. Cover slips were applied to each slide, which were subsequently examined under a microscope using the x40 objective lens (Cheesbrough, 2009).

Determination of Proteolytic potential of microorganisms
The proteolytic activity of all isolated strains was evaluated using casein agar medium. The milk agar medium was prepared with slight modifications, autoclaved, and poured into petri plates (20 mL/Plate) (Sivanandhini et al., 2015). After the medium solidified completely, strains were streaked onto the surface of the agar. Additionally, separate milk agar plates were prepared with wells of 5mm diameter (4 wells per plate), created under sterile conditions. These wells were then inoculated with 10UL of nutrient broth culture containing 24 hour growth of selected strains.
The plates were then incubated at 37^0C for 24 hours and protease production was assessed by observing the zone of clearance in the opaque milk protein. The diameter of the halo or clear zone surrounding each well was measured to quantify the protease enzyme activity (Mazzucotelli *et al.*, 2013). High-yielding protease strains were identified based on the measurement of the hydrolytic zone on skim milk agar.

Results and discussion

Table 1: Total viable counts of microorganisms isolated from Ilaro dumpsite soil sample

Isolate Code	Isolated Organisms	Number of Colonies	Microbial Count (Cfu/g)
IL-1	Total Aerobic microbial viable counts	410	
IL-2	*Pseudomonas* spp.	4	
IL-3	Total yeast and yeast like cells	40	
IL-4	Total mould counts	70	
IL-5	Total coliform bacteria	8	
IL-6	*Escherichia coli*	70	
IL-7	*Salmonella* spp.	7	
IL-8	*Staphylococcus* spp.	6	6
IL-9	*Shigella* spp.	150	

KEY: IL - Ilaro

Table 2: Total viable counts of microorganisms isolated from Oja-odan dumpsite soil sample

Isolate Code	Isolated Organisms	Number of Colonies	Microbial Count (Cfu/g)
OO-1	Total Aerobic microbial viable counts	340	
OO-2	*Pseudomonas* spp.	6	
OO-3	Total yeast and yeast like cells	190	
OO-4	Total mould counts	70	
OO-5	Total coliform bacteria	50	
OO-6	*Escherichia coli*	7	
OO-7	*Salmonella* spp.	90	
OO-8	*Staphylococcus* spp.	5	
OO-9	*Shigella* spp.	15	

KEY: OO-Oja Odan

Table 3: Biochemical characterization of bacterial isolates

Catalase	Citrate	Indole	Urease	Oxidase	Methyl red	Gelatinase	Possible Identity
+	-	+	-	-	+	-	*Escherichia coli*
+	+	-	-	+	-	+	*Pseudomonas* spp.
+	+	-	-	-	+	+	*Salmonella* spp.
+	-	-	+	-	-	+	*Staphylococcus* spp.
+	-	-	-	-	+	-	*Shigella* spp.
+	+	+	-	-	+	-	Coliform Bacteria

Table 4: Colonial and microscopic characteristics of fungal isolates

Fungal Isolates	Colonial Characteristics	Microscopy (Morphological Characteristics)
Yeast and yeast like cells	Creamy, moisty colonies and oval in shape.	Pseudohyphae,smooth, transparent and single-celled.
Fusarium oxyporum	Oval-shaped, fluffy, white growth and paleyellow colonies.	Septate hyphae, hyaline (Colourless), macroconidia, chlamydospores and branched.

Table 5: Colonial and microscopic characteristics of Bacterial isolates

Bacterial Isolates	Colonial Characteristics	Microscopy (Morphological Characteristics)
Escherichia coli	Colonies typically measure 2-3mm in diameter, round colonies, grayish, flat, smooth and moist surface.	Rod shaped (Bacilli), Gram negative, single rods.
Salmonella spp.	Colourless colonies with black centre, smooth surface and flat.	Rod shaped (Bacilli), Gram negative, single rods.
Staphylococcus spp.	1-3mm in diameter, round with edge, creamy-white on NA.	Cocci, cluster and gram positive.
Shigella spp.	Colourless colonies, round with smooth edge, typically low convex	Rod-shaped (Bacilli), single rods, Gram positive.
Pseudomonas spp.	1-5mm in diameter, round with irregular edges, smooth, flat, and moist	Bacilli, single rods, Gram negative.
Coliform Bacteria	Typically, rod-shaped (Bacilli), pink appearance on EMB Agar.	Gram negative, Single rods and motile.

Table 6: Casein hydrolysis test

Isolated Organisms	Casein hydrolysis test
Escherichia coli	+
Pseudomonas spp.	+
Salmonella spp.	+
Staphylococcus spp.	+
Shigella spp.	+
Coliform bacteria	+
Fusarium oxysporum	+
Yeast and yeast like cells	+

Discussion

Microbial proteases constitute approximately 60% of global enzyme sales. According to a 2007 market research report on world enzymes, the global enzymes market was projected to grow by 7.6% annually, reaching 6 million by 2011 (Shama & Hameed Abdul, 2011). In this research, six (6) bacterial strains and two (2) fungal strains tested positive for the casein hydrolysis test, indicating that bacteria produce more amylase compared to fungi. Many bacteria in the *Bacillus* genus are key producers of industrial and research enzymes as reported by Widsten & Kandelbaver, (2008).

These microorganisms are highly valued for their rapid growth rate, short fermentation times, ability to secrete proteins extra-cellularly, and their GRAS (generally recognized as safe) status, exemplified by *Bacillus subtilis* and *Bacillus licheniformis* (Parrado *et al*., 2014), and the current research aligns with this finding.

Bacteria, particularly *Bacillus* spp., are a significant source of industrial enzymes, including proteases, owing to their diversity and other merits (Bajaj and Sharma, 2011). *Bacillus* spp strains are prominent in the industrial enzymes market, accounting for thirty-five (35) percent of microbial enzyme sales (Jayakumar *et al*., 2012). These strains produce enzymes that perform well under industrial conditions, exhibiting tolerance to extreme temperatures, pH, solvents, detergents, and other

inhibitors (Joshi and Satyanarayana, 2013). This report has a significant similarity with the present research because most enzyme-producing microorganisms are thermally stable in nature, and an increment in temperatures at both locations speeds up their proliferation.

In a study by Obire *et al.*, (2020), fungi such as *Aspergillus*, *Fusarium*, Mucor, *Penicillium*, *Rhizopus*, and *Saccharomyces* were isolated from waste dumpsite. Additionally, the species *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus*, and various yeast species were identified in dumpsite leachates in research conducted by Michaela *et al.* (2018). The current study also demonstrated strains of *Fusarium oxysporum*, yeast, and yeast-like cells from both sites, which have a positive result for the casein hydrolysis test, showing a crucial similarity to these past reports.

Conclusion

Since the existence of enzymology, varieties of industries, including food, textiles, detergent, leather and pharmaceuticals have used enzymes. The use of inexpensive raw materials have been employed which led to increase in enzyme usage across the globe. This study indicates that numerous organisms capable of producing the enzyme protease were identified in soil samples collected from the Ilaro and Oja-Odan dumpsites in Yewa region, Ogun State, Nigeria. The study also highlights that dump site soil serves as a repository for a diverse range of microorganisms with enzymatic activity.

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