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RESEARCH ARTICLE

Production and partial characterization of protease from *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from domestic waste dumpsite

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ABSTRACT

Background: Microorganisms are the leading source of industrial proteases. As a result of this, researchers are searching for new microbial strain with novel characteristic for protease production. **Methods:** In this study, some microbial strains were isolated from a soil sample obtained from domestic waste dumpsite. The isolate was screened for protease activity using casein as substrate. The proteolytic isolates were identified and used for protease production. The optimum pH and temperature as well as kinetic parameters of the enzyme were determined to predict their suitable industrial applications. **Results:** *Pseudomonas aeruginosa* and *Bacillus subtilis* were identified to be active proteases producers. The optimum temperature of the *Pseudomonas aeruginosa* and *Bacillus subtilis* were both recorded at 50^{O}C while the optimum pH of *Pseudomonas aeruginosa* and *Bacillus subtilis* were recorded at 8.0 and 9.0 respectively. The K_m and V_{max} of protease produced from *Pseudomonas aeruginosa* were 222.22U/ml and 0.625mg/ml respectively while the K_m and V_{max} were proteases produced from *Bacillus subtilis* were 135.13 U/ml and 0.402 mg/ml respectively. **Conclusion:** The results in this study suggest that domestic waste dumpsite could be a potential source of proteolytic isolates of novel characteristic**.**

Keywords: Domestic waste, Partial characterization, protease *Pseudomonas aeruginosa* and *Bacillus subtilis*

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1.0 Introduction

In recent years, new innovations are opening up new avenues in the areas of industrial biotechnology in which many chemical processes are replaced by enzymatic methods. Proteases are a group of enzymes with a wide range of applications and account for 40 – 60% of the total enzyme sales [1]. Proteases refer to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. They are ubiquitous in nature and perform a major role with respect to their applications in both physiological and commercial fields [2]. They are used extensively in the detergent, leather, pharmaceutical, and food

industries. Proteases of industrial-scale are mostly produced by microorganisms although there are some produced by plants and animals [3]. The total value of protease covers 60% of the total worldwide enzyme sales. This is because microbial properties exhibit advantageous properties that are useful for biotechnological processes such as their resilience under extreme temperature, pH and the presence of inhibitors [4].

The high demand of proteases in industries brings the attention of researchers to explore novel sources for proteases where isolation, screening and characterization of new promising strains are a continuous process [5]. Population increase, rapid urbanization, booming economy, and the rise in the standard of living in developing countries have greatly accelerated the rate, amount and quality of the municipal solid waste generation

A better yield of enzymes at the industrial scale in Nigeria can be achieved by isolating novel strains with an inherent capacity for enzyme production by process optimization or by improving the strains that are already being used [6]. Indigenous enzyme production will help in the creation of jobs by converting wastes to wealth and also conservation of foreign exchange. In order to curtail the challenge of enzyme production cost, the use of agro-waste materials as a source of carbon in place of expensive carbon sources such as molasses, corn starch and glucose could bring significant commercial success in biotechnological enzyme production [3]. These agricultural wastes can be used as substrates for enzyme production due to their availability and higher biomass [7]. Their proper use can also help in solving pollution problems which may be caused by their disposal.

Waste may be generated during the extraction of raw materials, the processing of raw materials into intermediate and final products, the consumption of final products from households, and other human activities [8]. When waste is dumped on land, microorganisms such as bacteria and fungi proliferate using the components of the waste materials as a source of nutrients for growth as well as degrading the organic materials in the waste [9]. These microorganisms degrade domestic wastes through their metabolic processes and this property of microbes has been exploited in bioprocess technology [10]. Hence, the aim of this study is to isolate, partially characterize and produce protease from domestic waste dumpsite isolates.

2.0 Materials and Methods

2.1 Sample Collection

The soil sample was collected in a sterile polythene bag from domestic wastes dumpsite at Zungeru, Niger State, Nigeria and immediately transferred to the laboratory for the isolation of bacteria and fungi.

2.2 Media, Chemical and Reagent

Nutrient agar (NA), glucose, 0.87% sodium saline, 1% $CaCO_3$, 70% and 95% alcohol, crystal violet (0.1g), gram's iodine (0.18g), saffranine (0.2g), glycerine, 3% KOH, Malachite green (0.5g), carbolfuchsin stain, hydrochloride acid (conc. 3ml), methylene blue chloride (0.3g) beef extract (0.3%), zinc chloride (1g), potassium iodine (0.1g), powdered zinc metal, yeast extract (0.5g), $MgSO_4$ (0.02g), K2HPO_4 (0.1g), NaCl (0.5g), methyl red (0.008g), tryptone (1g), potassium phosphate (0.5%), sodium citrate (0.2g), agar (1.5g), bromomethyl blue (0.08g), (NH4)H_2PO_4 (0.1g).

2.3 Isolation of microorganism

One gram (1gm) of soil sample was mixed with 9ml of saline solution (Master dilution) and 1ml of the solution was serially transferred to tubes containing 9 ml saline each so that for each transfer the suspension was diluted 10times. Each tube was shaken vigorously. 0.1ml solution was spread to Petri plates containing sterilized nutrient agar and saboroud dextrose agar for bacterial and fungal isolation. The pure isolates were stored in bottles for further studies.

2.4 Screening for proteolytic activity

The isolates obtained from the domestic waste dump site were spread on Petri plates containing milk agar medium (pH 7) and incubated for 24h at 37°C and 5 days at 25°C for bacterial and fungal isolates respectively. A clear zone of skim milk hydrolysis indicated protease producing organisms. Colonies showing proteolytic activity were selected for protease enzyme production [11].

2.5 Identification of Proteolytic Bacteria Isolated from Soil

The selected potential strain was then identified by morphological and biochemical characteristics by using a microbiology laboratory manual [12]

2.6 Production of Protease Enzyme by Submerged Fermentation

Protease production was carried out by inoculating protease producing isolate into a basal medium (NH_4Cl-0.5%, NaCl-0.5%, $CaCl_2$-0.2%, $MgCl_2.6H_2O$-0.2%, K_2HPO_4-0.4%, KH_2PO_4 0.3%) containing 0.7% peptone and 0.5 % as nitrogen and carbon source respectively. The mixture was adjusted to pH 7.5 and maintained at 37°C on a shaker at 250 revs/min for 96 hours. Samples were withdrawn and centrifuged every 12 hours and the supernatant was regarded as a crude protease enzyme [13].

2.7 Determination of Protease Enzyme Activity

The activity of protease was assessed in triplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.5% (w/v) casein in Tris-HCl (pH 9.0) at 60◦C for 10 min. The 1mL reaction was terminated by adding 0.5mL of 10% trichloroacetic acid. It was left for 15 min and then centrifuged at 14000 g for 10 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μg of tyrosine/min under standard conditions [14].

2.8 Determination of Kinetic Parameters of Protease Enzyme

Factors affecting proteases activities such as pH, temperature and substrate concentration were determined

2.9 Effect of pH on protease activity

The effect of pH on enzyme activity was carried out by incubating the reaction mixture at 40°C over a pH range of 4-9. This was achieved using various buffers at different pH ranges; 0.05M sodium citrate buffer (pH 4-6) and 0.05M Tris-HCl (pH 7-9). Then the enzyme activity was determined by the standard enzyme assay.

2.10 Effect of temperature on protease activity

The effect of temperature on enzyme stability was carried out by incubating the reaction mixture over a varied temperature of 30 to 80°C at a predetermined pH. Then the enzyme activity was determined by the standard enzyme assay.

2.11 Effect of substrate concentration on protease activity

Effect of substrate concentration on protease activity was determined in reaction mixtures containing varied concentrations of casein solution (mg/ml); 2.5, 5.0, 10.0, 25.0, 30.0, 35.5. Michaelis-Menten constant (Km) and maximum velocity (Vmax) of protease were calculated from the plotted graph of $1/V_o$ against [1/S.

3.0 Results

3.1 Identification of the microorganisms

A total of 11 isolates obtained from the domestic waste dump site were screened on skim milk agar plates to have proteolytic activity and all of them were confirmed for protease production on further screening by submerged fermentation. The isolates code DDS3A and DDS2B were selected and identified to be *P. aeruginosa* and *B. subtilis* respectively (Table 1).

Table 1: Biochemical profile of the isolated organism

Isolate Code	**DDS3A**	**DDS2B**
Gram reaction	_	+
Shape	Rods	Rods
Catalase	_	+
Coagulase	_	_
Starch hydrolysis	_	+
Oxidase	+	_
Mannitol salt agar	_	_
Citrate test	+	+
Urease test	_	_
Methyl Red	+	_
Vogue Proskauer	_	+
Indole	_	_
Isolates	*P. aeruginosa*	*B. subtilis*

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3.2 Optimization of protease production

The optimum temperature values of proteases produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* were both recorded at 50 °C (figure 1). The optimum pH values of proteases produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* were both recorded at 8.0 and 9.0 respectively (figure 2). The V_{max} and K_m of protease produced from *Pseudomonas aeruginosa* were 222.22U/ml and 0.625mg/ml respectively (figure 3) while the V_{max} and K_m of protease produced from *B. subtilis* were 135.13 U/ml and 0.402 mg/ml respectively (figure 4).

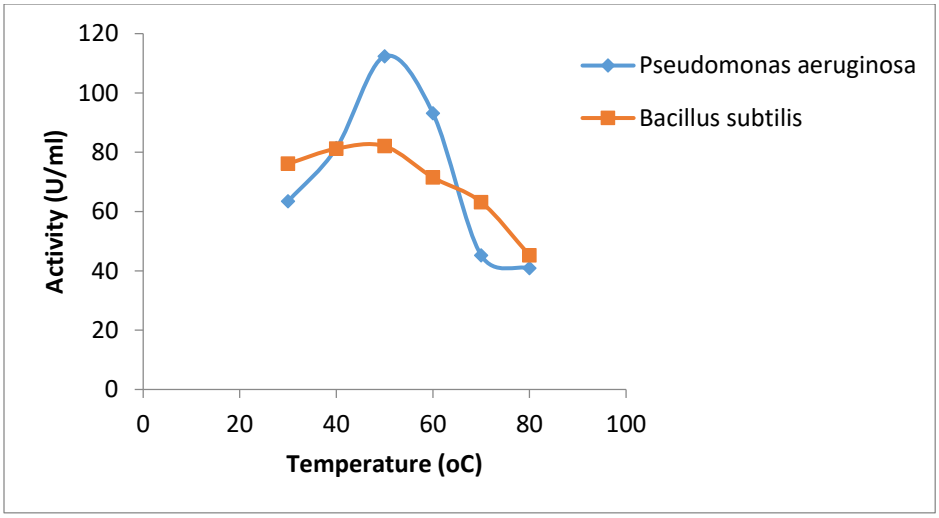

Figure 1: Temperature profile of proteases produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from domestic waste dumpsite

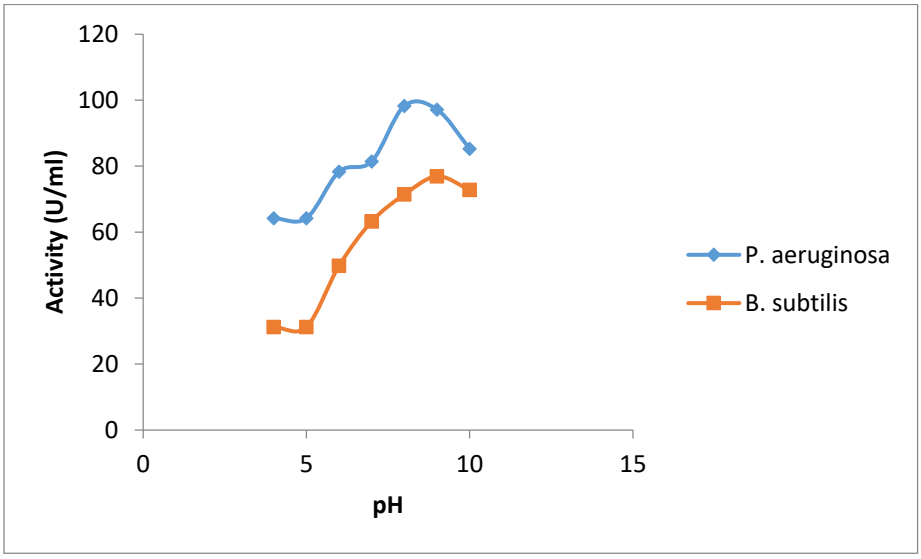

Figure 2: pH profile of protease produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from domestic waste dumpsite

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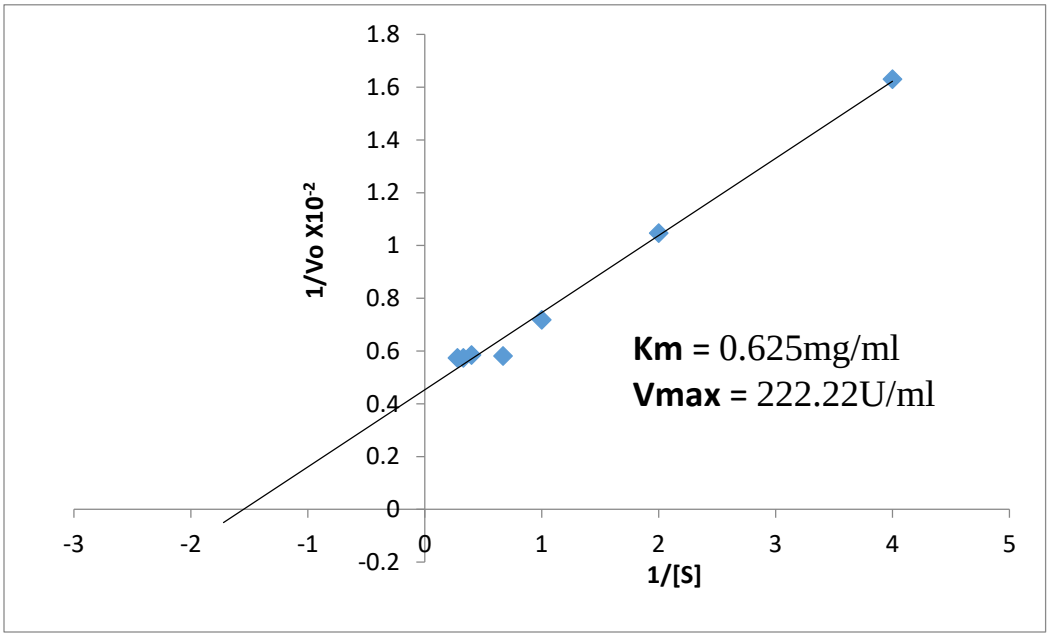

Figure 3: Effect of substrate concentration on the activity of protease produced from *Pseudomonas aeruginosa* isolated from domestic waste dump site

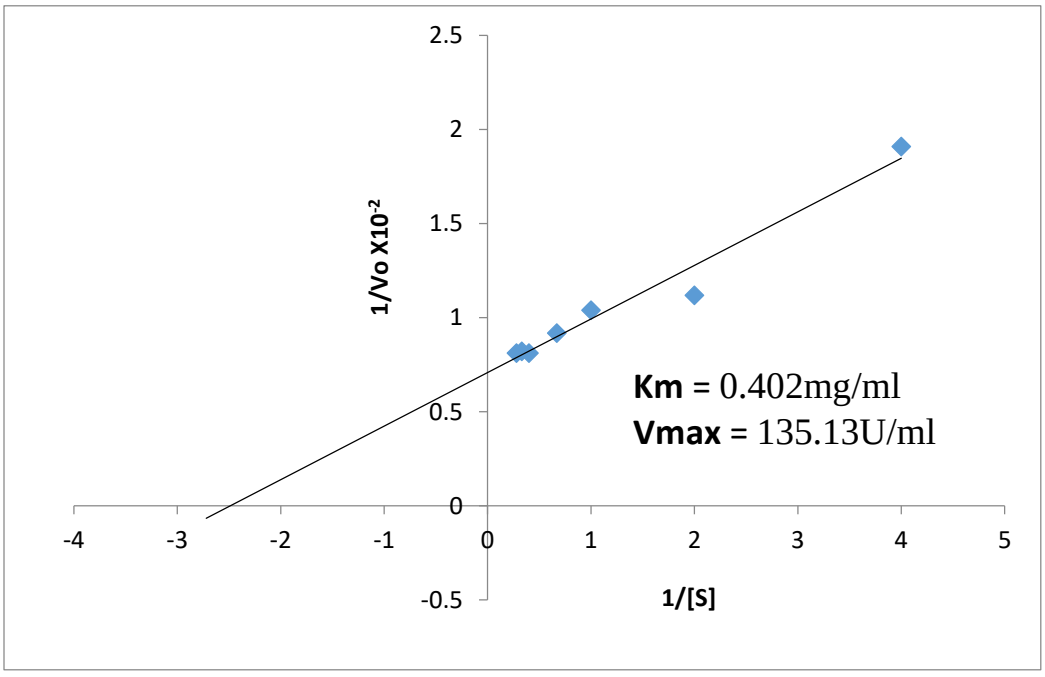

Figure 4: Effect of substrate concentration on the activity of protease produced from *B. subtilis* isolated from domestic waste dumpsite.

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4.0 Discussion

Among the isolates obtained from the domestic dumpsite, isolates DDS3A and DDS2B were found to have a significant positive effect in the degradation of the protein (skimmed milk) and thus indicating good potential for the production of protease enzyme. These isolates were identified to be *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively and were selected for protease production. The presence of these proteolytic isolates in domestic waste dumpsites depends greatly on the protein waste components arising from kitchen or food remnants. These bacteria are actively involved in the degradation of protein constituents of domestic waste, thereby making the dumpsite a natural habitat. They used the waste protein as their major source of nitrogen for extracellular protease production necessary for further protein degradation [15]. Proteolytic bacteria were also isolated by other researchers from landfills and biodegradable portions of domestic waste [16,17]. The findings of the present study thus show that domestic waste dumpsite could be considered a good source of proteolytic bacteria.

Enzyme characterization is important for industrial applications of the enzyme. The optimum temperature values of proteases produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* were both recorded at 50 ^{o}C (figure 1). A decrease in enzyme activity at a temperature beyond this point could be due to enzyme denaturation. Haddar *et al*. [18] reported that the constant and highest protease enzyme activity was observed at a temperature between 25-60°C when using casein as substrate. Most reported protease enzymes presented their maximum rates in the range of 30-85°C [19]

The optimum pH values of proteases produced from *Pseudomonas aeruginosa* and *Bacillus subtilis* were both recorded at 8.0 and 9.0 respectively (figure 2). These results suggest domestic dumpsite as a potential source of alkaline protease for industrial application. The decline in enzyme activity could be a result of conformational change in protein three-dimensional structures at extreme pH values. The optimum catalytic activity from different proteases reported is in the range of pH 7-11 [11,20,21].

The V_{max} and K_m of protease produced from *Pseudomonas aeruginosa* were 222.22U/ml and 0.625mg/ml respectively (figure 3) while the V_{max} and K_m of protease produced from *B. subtilis* were 135.13 U/ml and 0.402 mg/ml respectively (figure 4). The relationship between the rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate expressed as Km of protease enzyme [22]. Ahmed *et al*. [19] reported that the catalytic properties, Km and Vmax values of alkaline protease from *Bacillus subtilis* were 58 µM and 148 U/mL, respectively. An enzyme with low Km has a greater affinity for its substrate. Alkaline protease is highly substrate-specific and exhibits maximum activity towards casein as substrate.

5.0 Conclusion

From the present study, it is concluded that the identified species, *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from domestic waste dumpsite possess good protease activity. This domestic waste dumpsite is suggested to be a good source of proteolytic isolates. The alkaline proteases produced in this study may be used for various purposes in detergent industries, food industries and pharmaceutical industries owing to their high optimum temperature and alkaline pH values along with their high V_{max} and substrate specificity.

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