Volume 13 Number 2 DECEMBER 2011

IFE JOURNAL OF SCIENCE

A Journal of the Faculty of Science
Obafemi Awolowo University, Ile-Ife, Nigeria

ISSN 0794-4896

Ife Journal of Science

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ISOLATION AND CHARACTERIZATION OF A BACTERIAL THERMOSTABLE PROTEASE FROM POULTRY DUNG

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Received: November 2010; Accepted: July 2011

ABSTRACT

Thermophilic bacteria identified as *Klebsiella species* was isolated from poultry dung using nutrient broth inoculated with pure colonies. The species were grown between temperatures of 45°C to 65°C and was found to be most thermotolerant at 62°C. Protease enzyme was isolated from *Klebsiella species* and was further purified and characterized through ammonium sulphate precipitation and gel filtration on Sephadex G-100 column chromatography. The specific activity obtained for the partially purified enzyme was 0.1 units/mg protein with 4.2 % protein recovery. The apparent molecular weight as estimated by gel filtration on Sephadex G-100 column was 21.5kDa. The enzyme has optimum activity at pH 9.0 and at temperature of 35°C. An apparent k_m for the hydrolysis of casein was found to be approximately 0.75 mg/ml with 0.6 % (w/v) casein.

Key words: Poultry dungs, Bacteria, Thermostable protease, Thermophiles, *Klebsiella species*

INTRODUCTION

The thermophilic microorganisms are the heat-loving microbes, which can grow from 45°C to 75°C with optima between 50°C and 60°C (Hatman *et al*., 1989; Panikov *et al*., 2003). Some of these are obligate, requiring high temperatures for growth; others only tolerate it and have comparatively low temperature optima (Pelczar *et al*., 1993). They attract much interest in connection with their biotechnological potentials and the significant ecological functions they perform in natural and disturbed ecosystem (Panikov *et al*., 2003). Thermophiles have been found in most samples of the soil, mud or water from regions of widely differing climate (Brock 1967; Hatman *et al*., 1989; Panikov *et al*., 2003). They also occur in compost, sewage, in deep ocean floor deposits and in hydrocarbon-containing rocks (Cowan, 1992). Although thermophiles are ubiquitous, they mostly occur in habitats that offer little or no scope for growth at high temperature (Kempner, 1963).

Few bacteria are obligate thermophiles, most are facultative such as the aerobic spore formers, *Bacillus coagulans* and *Bacillus circulans* (McKane and Kandel, 1996). Economically, the most important thermophile is *B. stearothermopilus*. It is particularly troublesome in the food industries because its spores are so resistant to heat that some strains will grow at 80°C 85°C (O'Brien and Campbell, 1975). Magnesium ions have been reported to play a part in enzyme stability, increasing the concentration of magnesium increase the resistance of some enzyme preparations to inactivation by heat (Brown *et al*., 1957). Friedman and Weinstein (1966), working with enzyme systems from *Bacillus stearothermophilus* and the mesophile *Escherichia coli*, also emphasized the importance of magnesium ions in stability of the enzyme and they further suggested that thermophiles might make their protein synthetic apparatus stable to heat by maintaining high concentration magnesium or other polyvalent cations.

Temperature is one of the most important environmental factor influencing the growth and survival of organisms (Brock, 1967). As temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rate and growth becomes faster. However, above a certain temperature, proteins, nucleic acids, and other cellular components become sensitive and may be irreversibly denatured. Thermophiles offer some major advantages for biotechnological processes, many of which run more rapidly and efficiently at high temperatures. Increasing temperatures increase the diffusion rate and solubility of non-gaseous compounds and tend to discourage microbial contamination. Microbial processes carried out at high temperatures also eliminate or greatly reduce cooling costs, which may be a significant part of the total cost of running a large-scale microbial process at lower temperatures. Several thermophilic strains rapidly and efficiently produce enzymes like endonucleases, amylases, proteases, restriction enzymes, lipases, reductases and other enzymes, whose metabolic pathways are optimized to make use of the available resources (Adams *et al*., 1995; Adams and Kelly, 1998). This work is therefore

aimed at characterizing a protease from a thermostable bacteria obtained from a poultry dung.

MATERIALS AND METHODS

Reagents of high analytical grade were obtained from different commercial sources. Bovine serum albumin, tyrosine, acrylamide, N-N-methylene-bis-acrylamide, (MBA) N, N, N, N tetramethyl ethylene diamine (TEMED) and Folin reagent were from Sigma Chemical Company St. Loiuse, USA. Ammonium persulphate, ammonium sulphate, bromophenol blue, methanol, glacial acetic acid, Coomassie Brilliant Blue R-250 were from BDH Chemical Limited, London. Sephadex G-100 and sodium dodecyl sulphate were from Pharmacia Chemicals Limited, Uppsala, Sweden.

The poultry dung was collected asceptically at 35°C from Obafemi Awolowo University Farm, Ile-Ife Osun, Nigeria.

Isolation, Screening and Identification of the most Thermophilic Bacteria

The poultry dung sample was dried under the sun and the stones, eggshells and other debris were picked out before weighing. About 20 g of the sample was measured and a suspension was made in 200 ml of sterile water, well-shaken and left to settle. The supernatant was filtered twice using absorbent wool on a funnel followed by using filter paper. The filtrate was collected and 0.2 ml of the filtrate was dispensed into a sterilized microtitre plate containing 0.2 ml of sterilized double strength nutrient broth and incubated in water bath at 37 °C for 24 hrs.

The temperature was kept constant at 37°C. The wells in the microtitre plate that showed turbidity were pooled together into a sterile bottle and 1 ml of the pooled sample was serially diluted up to 10^{-6} and plated out by pour plate method (technique). Each discreet colony was picked out on a sterile nutrient agar. The selected pure cultures were re-purified by re-streaking on a sterile nutrient agar before they were transferred to labeled agar slants as working stock cultures for further use.

Nutrient broth was prepared and dispends into labeled bijoux bottles and sterilized by autoclaving at 121°C and 15 mmHg for 15 minutes. After autoclaving, the labeled bijoux bottles were inoculated with the pure isolates from the labeled stock culture and incubated at 45°C 65°C in the water bath. The most thermotolerant bacteria that grew at 62 °C were identified.

Identification of the most Thermophilic Bacteria

The most thermophilic bacteria isolate was identified using standard microbiological techniques as directed by Cowan and Steel (1985) and Bergey's Manual of Determinative Bacteriology (Buchaman and Gibbons, 1975). The identification of the isolate was based on cultural, morphological and biochemical characteristics up to the genus level.

Cultural Characteristics on Media

The bacteria isolate was streaked on nutrient agar plates, incubated at 37°C for 18-24 hrs and its colonies observed for shape, elevation, surface, pigmentation, opacity, size, edge and consistency. The isolate was also cultured for 18-24 hrs in nutrient broth and observed for the formation of sediments, pellicle and surface growth. The choice of biochemical tests was based on the Gram staining reaction. The tests performed included Gram staining, motility, growth on mannitol salt agar (M.S.A), Triple sugar iron agar, catalase, citrate, sugar utilization, oxidation fermentation, Hydrogen sulphide production, methyl red, vogesproskaues, gelatin liquefaction test and nitrate reduction test.

Enzyme Extraction

The sterile nutrient broth was inoculated with the thermophilic bacteria that grew at the highest temperature. This was incubated at 37°C for 24 hrs. The cell suspension from this culture served as cell inocula. The inoculum was used to inoculate 50 ml portions of sterile nutrient broth in 250 ml conical flask. These were incubated for 36 hrs at room temperature on a shaker at 150 revolutions per minute. The cells were removed after incubation by centrifugation at 4,000 r.p.m for 1hr at 4°C. The clear supernatant obtained was used as the crude enzyme solution.

Protease Assay

The casein digestion method of Kunitz (1946) was used to determine the enzyme activity of the protease. Soluble casein (1%; w/v) dissolved in 0.1 M citrate phosphate buffer (pH 7.0) and heat denatured at 100°C for 15 minutes in a water bath and allowed to cool before use. The reaction mixture for the assay consisted of 1ml of casein (1%) as substrate and 0.2 ml of enzyme solution and incubated 10 minutes at 37°C. The

reaction was terminated by introducing 3ml of trichloroacetic acid (TCA). The tube was allowed to stand for about 30 minutes and the content filtered into a clean dry test tube with Whatman No.1 filter paper. The amount of digested casein in the filtrate was determined by the methods of Lowry *et al.* (1951). The control or the blank was prepared by the same procedure as described above except that the TCA was added to the casein before enzyme was added so no reaction took place in the control tube.

The protein concentration was routinely determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard.

Enzyme Purification

The crude extract was brought to 80% ammonium sulphate saturation with the addition of solid ammonium sulphate with gentle stirring and the solution was left to stand at 10°C overnight. The precipitates was collected after centrifugation at 4000 rpm for 1hr at 4°C and dissolved in 10 ml of 0.1 M citrate-phosphate buffer (pH 7.0). This was later dialyzed for 6 hrs in 0.1 M citrate-phosphate buffer, pH 7.0.

Gel Filtration Sephadex G-100

Prepared Sephadex G-100 gel was packed into the column (1.6 x 40 cm) and was equilibrated with 0.1 M citrate-phosphate buffer, pH 7.0. 3 ml of enzymes solution from the preceding step was carefully layered on the column and the enzyme fractions were eluted with the same buffer (0.1 M citrate-phosphate buffer, pH 7.0). Fractions of 3 ml were collected at a flow rate of 18 ml per hr. Protein concentration of each fraction was read at 280 nm, while the enzyme activity was determined by the method of Kunitz (1946). Fractions with activity were pooled and further concentrated by dialysis against 50% glycerol in the citrate phosphate buffer.

Effect of pH on Enzyme Activity

The effect of pH on protease activity was determined by carrying out the assay of the enzyme using 1.0% solution of casein prepared in 0.1 M of the following buffers: pH 3-7.5 (citrate-phosphate buffer); pH 8-9 (Tris-HCL buffer); pH 10-12 (Glycine-NaOH buffer).

Effect of Temperature on Enzyme Activity

The effect of temperature on the rate of casein hydrolysis was carried out at temperatures between 30°C and 65°C using the standard assay procedure.

Effect of Substrate Concentration on Enzyme Activity

Effect of different concentrations of casein between 0.01% and 0.2% dissolved in 0.1M citrate phosphate buffer, pH 7 for 10 minutes at 37°C was determined.

Molecular Weight Estimation of the Enzyme by Gel Filteration

The apparent molecular weight of the enzyme was estimated on a calibrated Sephadex G-100 column (40 x 1.6cm) using the following protein markers (Bovine serum albumin; 68,000 dalton, Ovalbumin; 43,000 dalton, chymotrypsinogen A; 25,000 dalton and catalase; 58,000 dalton). 3 mg/ml of the marker proteins were use in the calibration of the marker proteins. The column was eluted at a flow rate of 12 ml/h with a 0.1M citrate phosphate buffer, pH 7.0. Fractions of 3ml were collected and monitored for protein at 280 nm. The void volume (V_o) of the column was determined by the elution volume of Blue Dextran.

TABLE 1: BIOCHEMICAL CHARACTERISTICS OF THE BACTERIAL ISOLATE P-11

I	Gr	Ce	Gl	Su	M	Ma	L	In	Mo	Ci	G	N	Of	Ca	Gr	V	Me	L	T
P-11	-	MLR	Y	Y	NC	G	+	-	-	-	+	+	0^{++}/F+	+	Y NC NC	+	-	+	*K*

Legend

I=isolate code, G=gram reaction, Ce=cell description, Gl=glucose Su=sucrose, M=maltose, Ma=manitol, In=indole production, Mo=motility, Ci=citrate utilization, G=gelatin liquefaction, N=nitrate reduction, Of=oxidative fermentation text, Ca=catalase, Gr= growth on T.S.I, V=voges proskauer, Me=methyl red, L=lactose, T=tentative identification, K= *Klebsiella species,* MLR= medium long rods, Y= acid production, + = positive, NC= no change, - = Negative, O = Oxidative, G= Growth, F= Fermentative

TABLE 2: PURIFICATION OF PROTEASE FROM KLEBSIELLA SPECIES

Step	Volume (ml)	Total protein (mg)	Total Enzyme Activity (units. ml^{-1})	Enzyme Specific Activity(unit.ml^{-1}.mg $protein^{-1}$)	Degree of Purification	% Recovery
Crude extract	500	630	10	0.0159	1	100
Ammonium sulphate (0.080%) concentration	28	36.88	1.4	0.038	2.39	14
Sephadex G - 100 Gel filtration	14	4.20	0.42	0.10	6.29	4.2

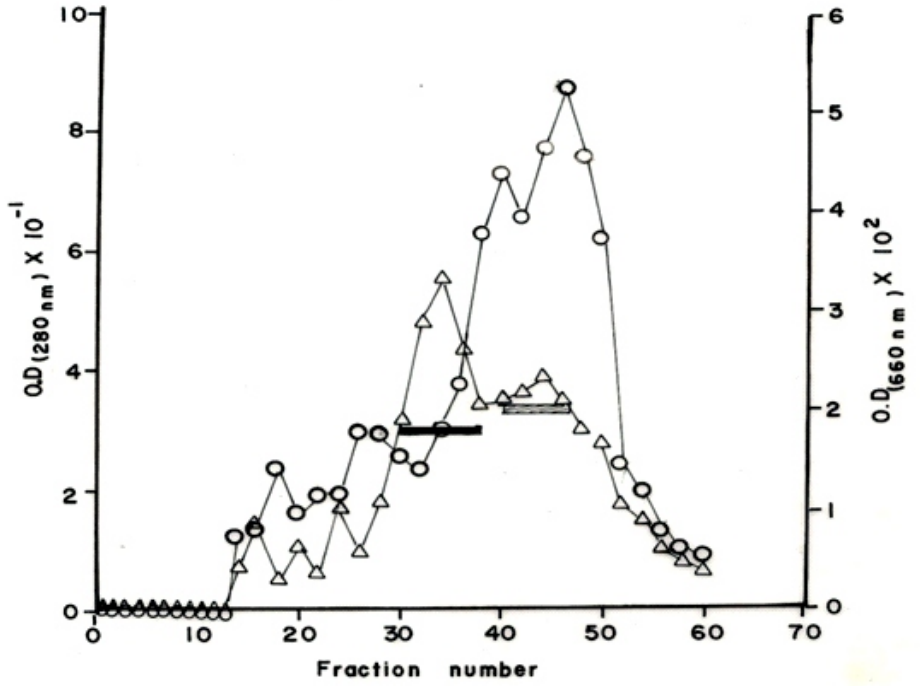

Figure 1**:** The elution profile of *Klebsiella species* protease after Sephadex G-100 gel chromatography. Peak I ( ), Peak II ( )

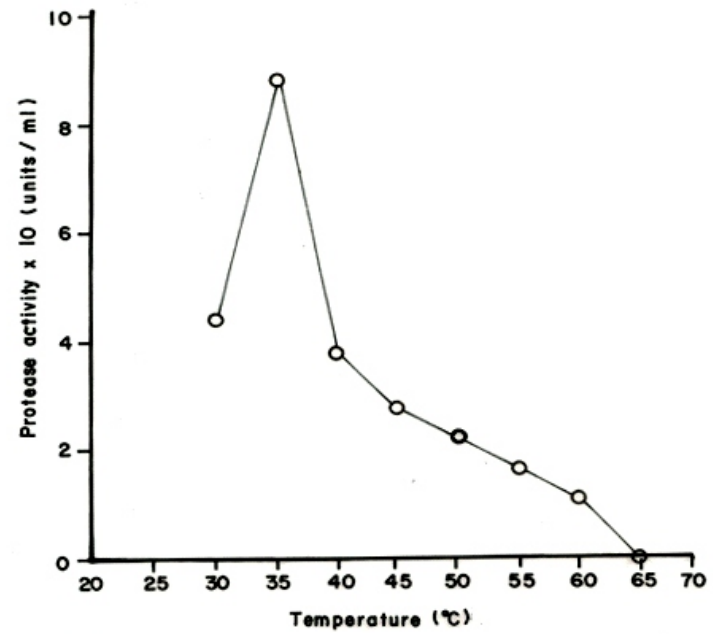

Figure 2: Effect of temperature on the activity of Klebsiella species protease.

RESULTS

Table 1 shows the list of bacteria isolates, obtained from poultry dung sample (Isolates P-1 to P-32). After incubation at a temperature of 45°C, 24 isolates were able to survive. At a temperature of 50°C, 8 isolates were able to grow, while at 55°C and 60°C only isolate P-11 survived. The cultural and biochemical characteristic of the isolate P-11 were examined and the results are summarized in Table 1. The isolate was Gram negative, non-motile, liquefies gelatin and ferments glucose. Based on Bergey's manual of determinative bacteriology, the isolate was tentatively assigned to the *Klebsiella* species.

Partial Purification of *Klebsiella sp*. protease

A summary of the purification procedures is show in Table 2. The elution profile of the fractionation of the dialysate from a column of Sephadex G-100 is present in figure 1. Two protein peaks were obtained, with peak 1 having the highest proteolytic activity. About 6-fold protease purification of peak 1 was achieved with a final recovery of about 4.2%.

Effect of temperature and pH on the partially purified protease

At pH 7.0, the activity of the enzyme increased with an increase in temperature and reached a maximum at 35°C. The enzyme still retained activity up to 60°C but at 65°C, there was no detectable protease activity (Figure 2). The enzyme was found to be active over the pH range 3.5 11 with maximum at pH 9.0 (see Figure 3).

Effect of increasing substrate concentration

The results for the effect of different concentrations of casein are shown in Figure 4. Maximum enzyme activity was recorded against 0.6% (w/v). Figure 5 shows the Woolf plot for the determination of apparent k_m for the hydrolysis of casein, and was found to be approximately 0.75 mg/ml.

Molecular Weight estimation by Sephadex G 100 Chromatography

The molecular weight of the purified protease was estimated by Sephadex G 100 column and was estimated to be about 21,500 dalton (Figure 6).

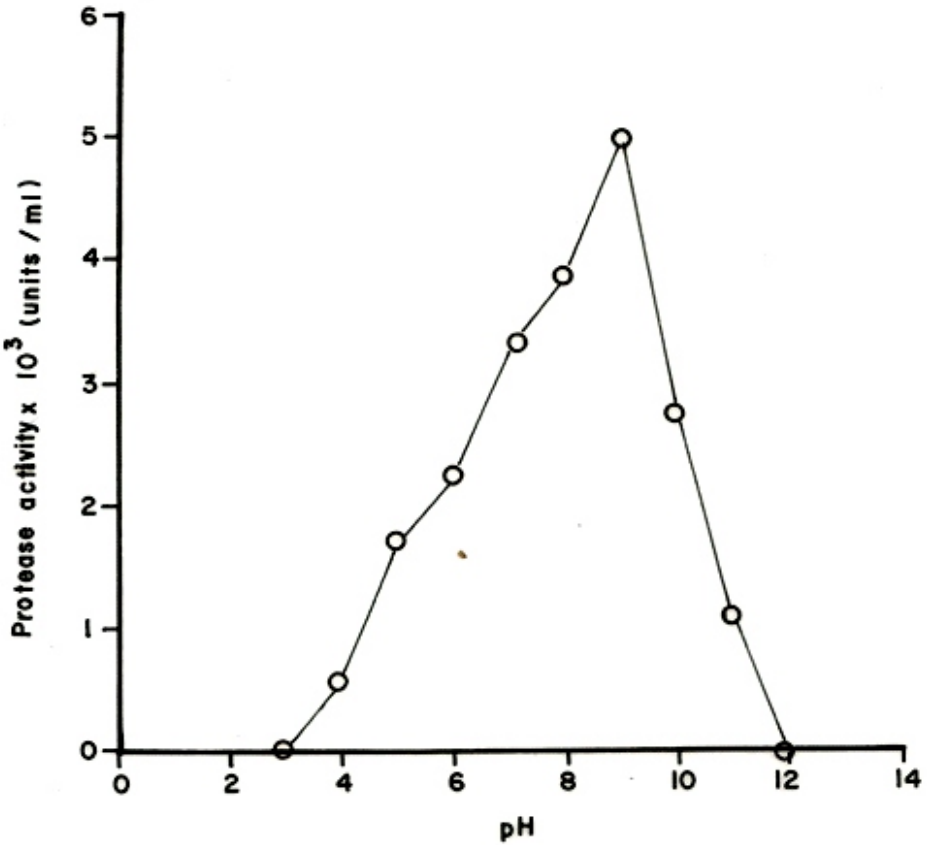

Figure 3: Effect of pH on the activity of *Klebsiella species* protease.

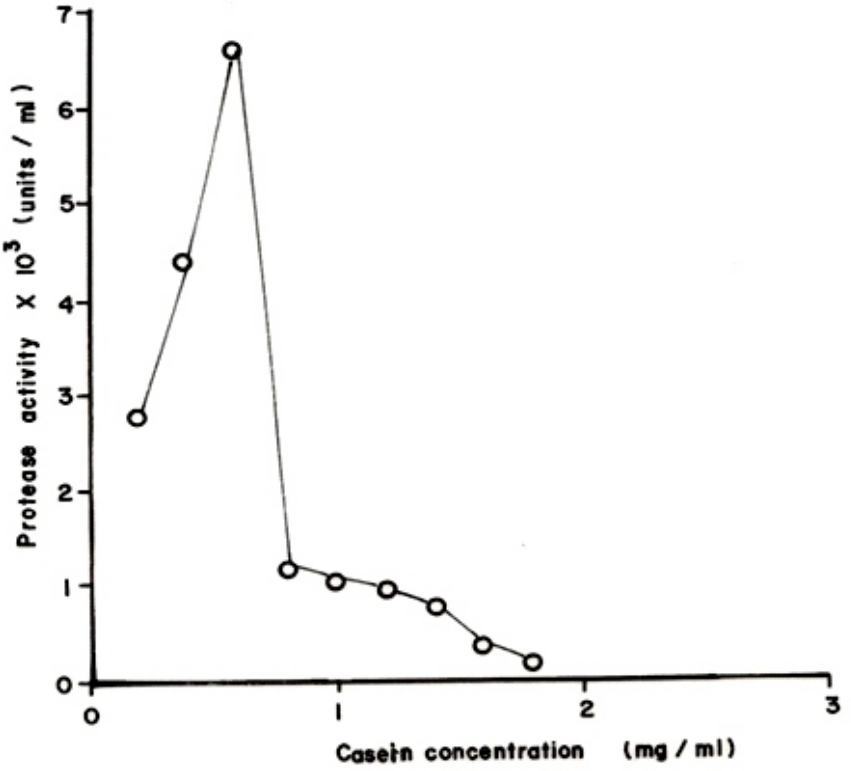

Figure 4: Effect of different concentrations of casein on the activity of *Klebsiella species* protease.

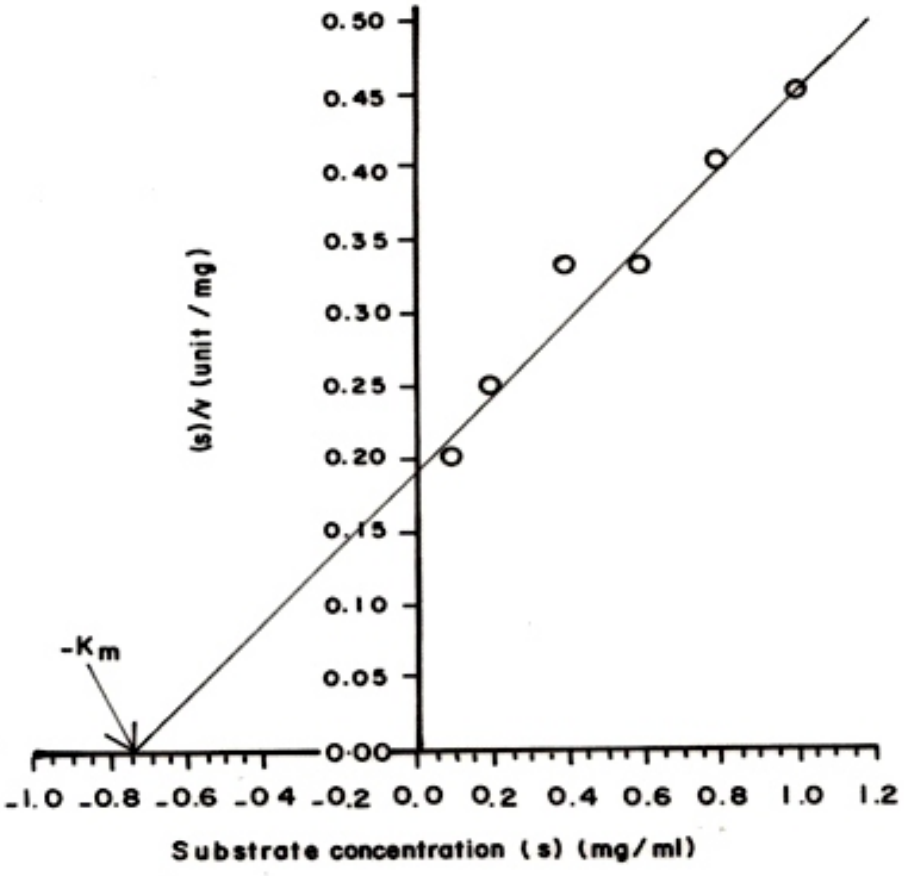

Figure 5: Lineweaver-Bulk plots showing the effect of varying concentration of casein.

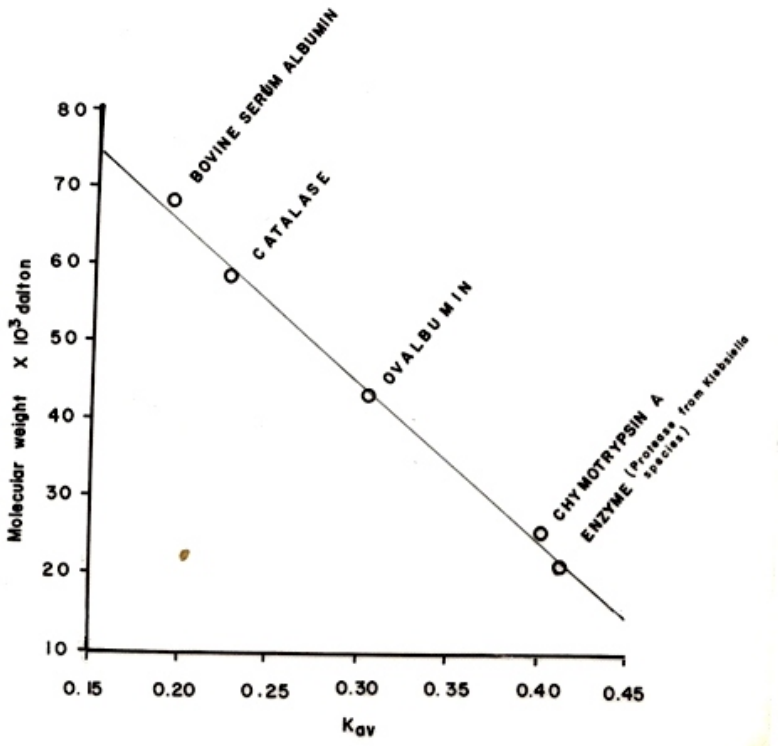

Figure 6: The apparent molecular weight of the *Klebsiella species* protease.

DISCUSSION

In this study, 32 pure bacteria isolates were obtained at 37°C from the poultry dung at Obafemi Awolowo Teaching and Research Farm. After subsequent subjection of these isolates to different temperatures ranging from 37°C - 65°C, a thermophilic bacterium that was able to grow at 62°C was isolated. Although a lot of information is available on the characterization of protease from thermophiles (Taguchi *et al.*, 1983), only a few extracellular enzymes produced from Gram-negative thermophiles such as *Klebsiella* have been reported. This study indicates the secretion of an extracellular, thermostable protease by a thermophilic bacterium that was characterized as *Klebsiella species* using Standard morphological and biochemical tests described by Buchaman and Gibbons (1975). This *Klebsiella species* is a Gram-negative and non-motile organism. Some other important properties are given in Table 1. Based on these properties, this *Klebsiella species* exhibited some similarities to *Klebsiella ozena* and *Klebsiella rhinoscleromatis*, but differ from these two species with respect to growth at high temperature (62°C) and gelatin hydrolysis. Kenneth (1999) stated that the thermophiles are adapted to temperatures above 60°C in a variety of ways. Thermophiles have been reported to have high G+C content in their DNA.

Environmental influence or conditions can be the cause of the growth at high temperature and protease production by the *Klebsiella species*. Liu *et al.* (1994) in their work on the origin of deep subsurface bacteria found that some of the bacteria isolated from drilling mud exhibited a combination of physiological traits resulting from complementary interactions among members of the community of the microorganisms in the environment. They went further to state that the combinations of physiological characteristics help organisms to survive in their environments. It is therefore possible to assume that poultry dung would have had abundant microbial communities which interact with each other either by conjugation, transduction, or by transformation. Cruickshank (1969) stated that the characters that a bacterium exhibits at a given time (i.e. its phenotype) are determined both by its genetic constitution (genotype) and by environmental conditions which influence the contemporary expression of the genetic potentialities; it is likely that this *Klebsiella species* picked up those unique properties from its environment.

The partial purification procedures involved ammonium sulphate precipitation and gel filtration on Sephadex G-100. The Sephadex G-100 gel filtration gave two peaks with peak 1 having higher greater activity. The enzyme was purified six folds with a percentage recovery of 4.2%. The molecular weight value of protease from *Klebsiella species* as determined by gel filtration was 21,500 dalton. This molecular weight value compares very well with the molecular weight

values for other bacteria in enterobacteriacea family. *Serratia species* was reported by Ryden *et al.* (1968) to have a molecular weight of 30,000 dalton. Protease from *E. coli* strain BL21 was also found to have a molecular weight of 25,000 dalton (Vaslyeva *et al.*, 2000), while protease from *Methanococcus* was found to be 29,000 dalton (Michels and Clark, 1997).

Further characterisation of the protease show that the enzyme had maximum activity at pH 9.0 which agrees with results reported for most thermophilic bacteria using casein as substrate (McQuade *et al.*, 1969: *Serratia marcescens* protease). Bacteria such as *Bacillus species*, *Flavobacterium species*, and *Streptomyces* have been reported to produce alkaline proteases (Boguslawski *et al.*, 1983). The protease from *Klebsiella species* with a pH of 9.0 could therefore be called an alkaline protease. The enzyme showed optimum activity at 35°C. A value as high as 70°C have been reported for protease from *Bacillus stearothsrmophilus* from sewage sludge compost (Ohta *et al.*, 1995). Kubo and Hasumi (1998) isolated a thermostable *Bacillus circulan* that could degrade soybean protein at 50°C.

The kinetic study using casein as substrate showed maximum activity at concentration corresponding to 0.6 g (w/v) of casein, beyond this concentration; there was decrease in the activity of the enzyme. The low K_m of 0.75 mg/ml with 0.6 % (w/v) casein value obtained from *Klebsiella species* protease indicated that the *Klebsiella species* protease has a high affinity for casein.

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