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Production Characteristics and Molecular Properties of Protease of *Pediococcus acidilactici* Isolated from Beef under Cold Storage

Fadahunsi Ilesanmi Festus[1*], Duyilemi Olubunmi Pauline[1] and Onilude Abiodun Anthony[1]

[1]Department of Microbiology, Faculty of Science, University of Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author FIF wrote the protocol, literature searches and wrote the first draft of the manuscript. Author DOP managed the analyses of the study and performed the statistical analysis. Author OAA designed the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To investigate the production characteristics and molecular properties of protease from *Pediococcus acidilactici* under cold storage temperature.
Study Design: Identification and re-identification of *Pediococcus acidilactis* and determination of optimal conditions for protease production and molecular properties.
Place and Duration of Study: All the work was carried out in the Department of Microbiology, Faculty of Science, University of Ibadan, Nigeria between March 2014- January, 2015.
Methodology: Identification of the organism was done using API kit. Determination of inoculums concentration was carried using the Neubaeur chamber. pH, incubation period, substrate concentration, influence of ions, were determined by varying this parameters using standard methods to optimise values. Purification of the enzyme was obtained by gel chromatography using sephadex beads G-100, G-50 and finally by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

*Corresponding author: E-mail: sanmifadahunsi@yahoo.com;

Results: The highest protease activity of 49.46 units ml^{-1} was recorded at pH 5.5, temperature of 20ºC, incubation period of 96 h, inoculum size of 0.1 ml (1.70 x 10^4) with 1% casein substrate concentration in the presence of 0.1 M manganese ion and 0.1M of Ammonium ions concentration. Separation of protease enzyme on SDS-PAGE showed that the molecular weights of the major peaks were found to be within the range of 45-55 kDa.

Applications: This study revealed that bacteria (*Pediococcus acidilactici*) could be induced for protease production and their applications in industrial spheres were confirmed.

Keywords: Pediococcus acidilactici; protease activity; cold-temperature-storage; production characteristics; molecular properties.

1. INTRODUCTION

Proteases are enzymes that speed up the degradation of protein compounds to peptide and also catalyze synthesis of peptide fragment in low aqueous organic solvent [1,2]. The production of protease is achieved optimally at commercial level by the use of microorganisms. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases [3] Microorganisms are easily manipulated genetically to produce new categories of enzymes with different biotechnological properties [4]. It is important to add that enzymes from microbial sources are better than enzyme from other sources because they exhibit nearly all the characteristics necessary for biotechnological applications [5]. Microorganisms such as bacteria, yeast and fungi have been studied for the production of protease using solid state and submerged fermentation techniques [6, 7]. [1] Reported the production of extracellular protease from high yielding strains of *Bacillus* spp, *Alcaligenes faecalis, Pseudomonas fluorescens* and *Aeromonas* using submerged fermentation technique [5]. The conditions required for optimum production of enzyme vary from one microorganism to the other [8, 9, and 10].

[3,11] Reported that pH of the production medium, incubation time, inoculums concentration, type of strain, media composition, the presence of metal ions, temperature and nutrient requirement, method used in the cultivation of the microorganism are important factors determining the production of protease from bacteria. In addition, microbial enzymes which are stable at extreme pH conditions are preferable in commercial applications [12-14]. Protease enzymes constitute a single largest enzyme of industrial significance and it accounts for about 60% of the total global sales of enzymes [15,16].

The industrial application of protease enzyme is wide and well documented. It is employed in both physiological and commercial spheres [17,18]. In addition they are used in the production of bio insecticides, secondary metabolites and Pharmaceuticals [19,20]. Similarly the application of protease enzyme is seen in tendering of meat, infant formula preparation, baking, brewery and detergent industries [21]. There are several available documented studies on the production of protease from various sources. [22-24] reported the production of protease from *Pseudomonas* spp, while [25-28] described the optimization of protease from *Bacillus* species. The productions of protease from *Actinomyces and Alcaligenes* were discussed by [29,30]. *Aspergillus* spp were able to produce significant quantity of protease when tested [31-34]. However studies on the production of protease from lactic acid bacteria especially cold protease from *Pediococcus acidilactici* is very scanty despite their biotechnological applications ranging from food processing to cold-water laundry detergents [35]. Therefore this present study is intended to investigate the production characteristics and molecular properties of protease from *Pediococcus acidilactici* under cold storage temperature because the potential of an enzyme for industrial application is determined by these parameters.

2. MATERIALS AND METHODS

2.1 Identification Procedure

Pure cultures of *Pediococcus acidilactici* obtained from the culture collection of Professor A.A. Onilude of the Microbial Physiology and Biochemistry unit, Dept of Microbiology University of Ibadan, Nigeria and reidentified using the API 50 CH strips and API50CH medium (API system Montalieu Vercieu France).

2.2 Enzyme Production

2.2.1 Determination/ standardization of inoculum

One ml of cell suspension of *Pediococcus acidilactici was* transferred into 100 ml of sterilized MRS broth in 150 ml Erlenmeyer flask and incubated at 35°C for 24 h. The number of cells in 1 ml of the suspension was enumerated using the Neubauer chamber.

2.3 Protein Assay

This was carried out using the modified method of [36] while protein estimation was investigated by employing the methods of [37] One unit of protease activity was defined as the amount of protease enzyme that produced one µmol of amino acid under standard assay condition.

2.4 Determination of Optimum Inoculum Concentrations for Protease Activity

Determination of Optimum inoculum concentrations was studied using the modified method of [38] Four 250 ml Erlenmeyer flasks containing 200 mls sterile MRS broth were inoculated with different concentrations of *Pediococcus acidilactici* cells suspension, (0.05, 0.1, 0.15, 0.2 mls) and incubated (a J.P. selecta Sia 0338954 model incubator) at 35°C for 96 h. The enzyme (Protease) was harvested by ultracentrifugation using Damon/TEC B-20A model centrifuge set at 10,000 rpm for ten minutes at 4ºC. The crude protease was harvested as the supernatant.

2.5 Determination of Optimum pH for Protease Activity

Sterile MRS broth was prepared and adjusted to pH levels of 5.0 and 5.5 using 0.1 M hydrochloric acid (HCl). Ten mls of the MRS broth was differently dispensed into separate 25 ml screwcapped flasks and sterilized at 121°C for 15mins. The tubes were allowed to cool, inoculated with the optimum inoculum size for protease production (0.1 ml of cell suspension of *P. acidilactici* (1.70 x 10^4 cfu/ml) and incubated at 20°C for 24 h. The broth cultures were centrifuged at 10,000 r.p.m at 5°C for 30 mins and the supernatant obtained was used for enzyme assay.

2.6 Determination of Optimum Temperatures for Protease Activity

In order to determine optimum temperature twenty five mls of MRS broth was dispensed variously into screw-capped Erlenmeyer flasks and sterilized. After cooling, the flasks were inoculated with 24 h old culture of *P. acidilactici* using the optimum inoculum size and pH for protease production and incubated differently at 10°C, 20, 30 and 35°C for 1 hr. The broth cultures were centrifuged at 10,000 r.p.m at 5°C for 30min and the supernatant obtained was used for assay.

2.7 Determination of Optimum Incubation Period for Protease Activity

This was investigated by dispensing 50 ml of MRS broth in each 100 ml Erlenmeyer flask and sterilized at 121ºC for 15 mins. The flasks were inoculated with equal quantity of inoculums and incubated at 20ºC for 96 h. The culture filtrates were collected and used for enzyme activity. The experiment was performed in triplicate.

2.8 Effect of Substrate Concentration on Protease Activity

Different concentrations of Casein (1% to 5%) was prepared and added separately to composed MRS broth without beef extract in several 100 ml Erlenmeyer flasks and sterilized at 121ºC for 15 mins.After cooling they were inoculated with 0.1 ml cell suspension of *P. acidilactici* and incubated for 96h at 20°C, the filtrate obtained was used for enzyme assay The experiment was carried out in triplicate [39].

2.9 Influence of Various Ions on Enzyme Activity

The method described by [39] was adopted. Five mM concentration of the various metal ions was added to the enzyme preparation and incubated for 30 minutes at 20°C. Relative protease activity was measured by adding the substrate and carrying out the enzyme assay under the optimum conditions. The experiment was carried out in triplicate.

2.10 Purification of Crude Protease

Two hundred ml of the crude protease was subjected to Ammonium sulphate precipitation using graded levels of the salt within 0-100%

saturation [40]. The obtained filtrate was on each occasion further separated by ion exchange Chromatography using sephadex beads G-100, G-50 and finally by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [41].

3. RESULTS AND DISCUSSION

3.1 Isolation Procedure

Studies were carried out on the production characteristics and molecular properties of protease of *Pediococcus acidilactici* isolated from beef under low temperature storage. The identity of the lactic acid bacteria associated with beef under refrigerated storage was confirmed as *Pediococcus acidilactici,* with the aid of API 50CH strips and API 50CH medium (API System Montalieu, Vercieu, France).

The presence of LAB under psychrophilic temperature had earlier been reported [42,43]. The survival of LAB at low temperature could be due to low activation energy, presence of unsaturated fatty acids in the cell membranes coupled with conformational changes in the ribosomal proteins and regulatory enzymes. The alteration in substrate uptake and cell permeability of the microorganisms also contributes to its ability to survive at refrigerated temperature [44-47].

3.2 Determination of Optimum Inoculum Size

This was investigated by inoculating the production medium (MRS) with different sizes of inoculums. The results obtained are shown in Table 1. The highest protease production of 49.46 unit/ml was seen at inoculums size of 0.1 ml and below this value protease activity decreased. This result indicates that inoculums sizes significantly affect protease activity and that protease activity increases with increase in inoculums sizes.

This observation was previously reported by [48] on a study carried out on the optimization of protease production by *Actinomycetes sp*. In this study, inoculums size of 1% stimulated the highest protease activity which is in conformity with the findings reported by [18]. However maximum protease activities were observed at inoculums sizes of 2%, 3% and 5% [31,49,50], while [51] explained that very large inoculums sizes are inhibitory in nature and increase in inoculums size causes decrease in protease activity.

Increase in protease activity with low level of inoculums size may be attributed to the higher ratio between the surface area and volume.

However, the low protease activity observed at higher concentration of inoculums might be linked to rapid utilization of the nutrient for growth and metabolism, thereby resulting in low protease activity and aggregation of cells arising from reduction of sugar and oxygen uptake rate might also be another contributing factor [49, 52].

3.3 Determination of Optimum pH for Protease Activity

The result of the determination of optimum pH for protease activity is presented in Table 1 and Table 2 In this investigation two pH ranges were selected by incubating the production media at 5.0 and 5.5 because LAB is reported to grow best at these pH ranges. The optimum protease activity of 49.46 unit/ml was observed at pH 5.5 closely followed by an activity of 30.91 unit/ml at pH 5.0. This result infers that protease from LAB is acidic in nature because its maximum activity was enhanced at acidic pH.

This observation is contrary to previously available reports. The widely reported findings are showing optimum activity at pH ranges above 6 indicating alkaline protease of mesophilic bacteria of *Bacillus* origin. Similarly, [53] observed that *B. cereus* SV1 and halo-alkaliphilic *Bacillus* sp. 17N-1 showed maximum activity at pH 7 and 8.0 respectively. However, [54] discovered that the maximum activity of protease from *Bacillus laterosporus*-AK1 was optimum at pH 9.

Protease production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn promotes the cell growth and enzyme synthesis [55]. It has been reported that pH could significantly influence enzyme activity in many perspectives or dimensions. [56] Explained that factors such as ionization of groups in the enzyme's active site, ionization of groups of substrate, or by altering the conformation of either the enzyme or the substrate, which could result in changes in enzyme activity. This present

result is in contrary with the findings obtained for the optimum pH for enzymatic activity of some reported *Bacillus species*. Previous investigation revealed that *B. cereus* KCTC 3674 and *B. licheniformis* Lbbl-11 showed optimum activities at pH 8.0. [57,58] while [59] reported that *Bacillus sp. isolated* from fermented soybean showed optimum protease activity at pH6.

3.4 Determination of Optimum Temperature for Protease Activity

This was achieved by incubating the production media at different temperatures keeping other conditions constant. The maximum activity was seen at temperature of 20°C (Tables 1 and 2) after which further increase in temperature resulted in decrease in activity. Enzyme activity is affected by variation in temperature because at high temperatures the rate of enzymatic reaction is accelerated while low temperature retards enzyme reaction [60]. The decrease in enzyme activity at high temperature could be attributed probably to the destruction of enzyme or changes in its tertiary structure.

Several studies revealed that temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane. [18,61,62] Reported that temperature could regulate the synthesis and secretion of extra cellular proteases by microorganisms. The low optimal temperature for protease activities observed in this study makes the enzyme suitable for detergent formulations for washing at normal temperatures [63]. High activity of enzymes at low temperatures is a characteristics that makes suitable for industrial applications, especially in certain food processing operations that require low temperatures. Higher temperature have been reported to produce some adverse effects on metabolic activities of microorganism [64] due to inhibition of the growth of the microorganism and denaturation of the enzyme resulting from loss of its catalytic properties due to stretching and breaking of weak hydrogen bonds within enzyme structure [65]. However the result obtained for optimum temperature for protease activity in this study is at variance with the submissions of earlier researchers. [66] Reported that optimum protease activity was at 30°C for *Bacillus* specie while *B. licheriformis* and *B. licheniformis* VSG1 exhibited maximum activity at 45°C and *B. subtilis* and *B. subtilis* SHS-04S showed optimum protease activity at temperature of 50°C [61,67].

3.5 Determination of Incubation Time for Optimum Protease Activity

It was determined by monitoring the enzyme activity at various time intervals. The results obtained are represented on Tables 1 and 2 indicating the optimum incubation time for protease activity at 96h with an activity of 49.46 unit ml^{-1} followed by an activity of 30.91 units ml^{-1} recorded at 72h incubation and beyond this period enzyme activity decreased .This observation is in conformity with the earlier report of [31] that noticed maximum protease production by *Aspergillus tamarii* at an incubation period of 96 h in both solid state and broth culture. However contrary reports to our findings in this study on incubation periods are available [68] reported that optimum protease activities for *Pseudomonas species, Bacillus cereus* 146 [25], *Bacillus subtilis* [69] *Bacillus amovivorus* [70] *Pseudomonas* aeruginosa [22] were recorded at 48 h of incubation.

Similarly, optimum incubation period of 72 h was reported for *Bacillus* species, SVN12, *Bacillus* species HPE10 and *B.cereus* [18,50]. The decrease in activity after optimum incubation period could have been caused by the denaturation or decomposition of the protease [71]. It is important to note that the growth rate of the microbial strain and its enzyme production pattern are important factors in determining the optimum incubation period [71]. [72,73] Observed that incubation period for optimum production of enzyme is relatively shorter for bacteria because of their fast generation time. Prolonged incubation period has been observed to cause auto digestion of the proteases and proteolytic attack by other proteases resulting in decrease in enzyme activity [73,74].

3.6 Effect of Substrate Concentration

The Effect of Substrate Concentration on Protease activity was investigated by the addition of different concentrations of Casein (1% to 5%) to composed MRS broth without beef extract and incubated for 96 h at 20°C. The result is displayed in Fig. 1, showing the highest activity of 32.0 unit ml^{-1} at 1% casein concentration while the lowest activity of 20.0 units ml^{-1} was noted with 5% casein concentration. This result is in accordance with the reported protease production in presence of different substrates concentrations [55] this observation might be attributed to increased level of substrates

concentration which could decrease the aeration and porosity of the medium, hence retarding the growth of the organism resulting in decreased enzyme activity. In addition [75] reported that substrate level inhibition of enzymes, might have emanated from high concentration of substrate.

3.7 The Influence of Various Ions on Protease Activity

In order to study the effect of various ions on protease activity, different concentration of the ions were added to the enzyme preparation and incubated for 30 minutes at 20°C under optimum conditions. The results obtained are displayed in Figs. 2 and 3. All the cations and anions used showed effect on enzyme activity. The best cation that stimulated the highest protease activity of 49.04 units ml^{-1} was 0.1 M manganese (Mn^{2+}) ions (Fig. 2) while the variations in anions concentration revealed that the highest protease activity of 37.36 units/mol^{-1} was recorded at 0.1 M of Ammonium ions (NH_4^+) In this study, it was observed that the highest activity of protease was supported by 0.1 M concentration of the cation ion and after this concentration a decrease in activity was noted.

However, the trend in the addition of anions showed that enzyme activity increased beyond 0.1M concentration especially with $S04^{2-}$ and NO_3^-. The observed increase in protease activity in the presence of Mn ion is in conformity with the earlier submissions of [25,76]. There are previously documented reports on the positive effects of metal cations on protease production [71,77,78]. [79,80] Reported, that some metal ions played vital role as cofactors in the synthesis of protease by microorganisms. The ability of ions to increase protease activity might be caused by their stabilizing effect and compatibility [81]. In addition they possess the ability to protect enzymes against denaturation and in maintaining the active conformation of the enzyme at high temperatures [82]. The type and concentration of the metal ion used in the growth medium determine stability of proteases [83].Variation in the concentration of the ion will reveal its ability as an inducer or activator [84].

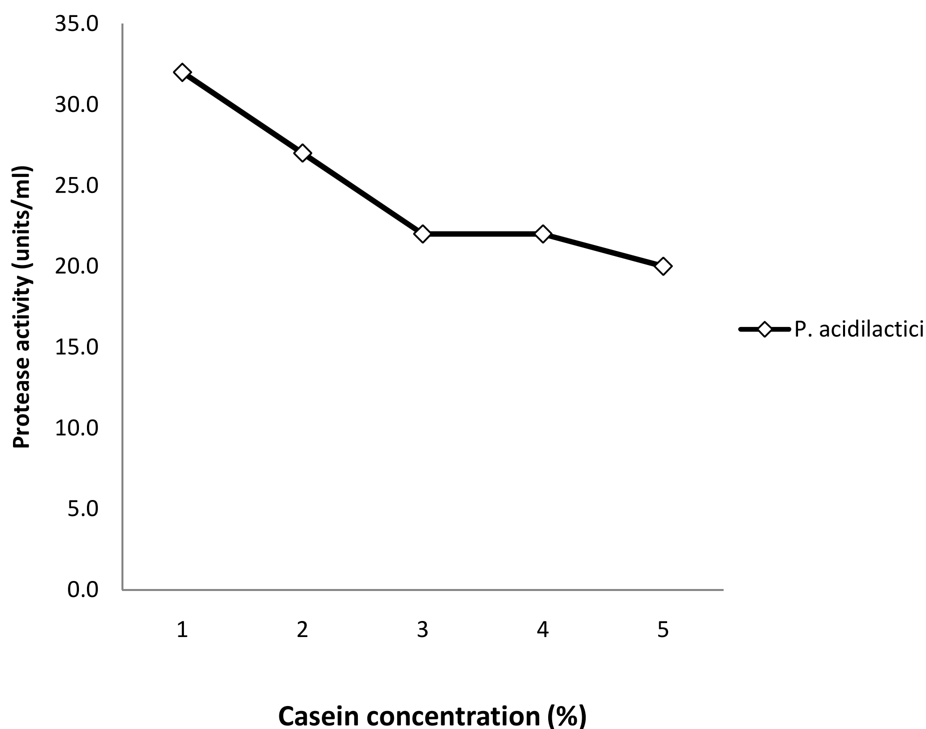

Fig. 1. Effect of substrate concentration on protease activity of *Pediococcus acidilactici*

Table 1. Protease activity (units/ml) of *Pediococcus acidilactici* isolates from stored meat cultivated in MRS broth at pH 5.5

Inoculums size (ml)	Temperature (ºC)/Incubation time (h)/Protease activity (units/ml)															
	10				20				30				35			
	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
0.05	*30.91	37.10	40.19	30.91	27.80	37.10	43.28	37.10	27.80	30.91	40.19	40.19	27.80	40.19	40.19	34.00
0.1	37.10	37.10	40.19	40.19	40.19	37.10	40.19	49.46	30.91	37.10	40.19	40.19	37.10	37.10	40.19	37.10
0.15	30.91	40.19	37.10	40.19	37.10	30.91	40.19	46.37	37.10	37.10	40.19	43.38	37.10	37.10	40.19	40.19
0.2	30.91	37.10	34.00	30.91	30.91	30.91	30.91	43.38	27.80	24.73	37.10	30.91	30.91	30.91	37.10	49.46

Each value is a mean of duplicate determinations

Table 2. Protease activity (units/ml) of *Pediococcus acidilactici* isolates from stored meat cultivated in MRS broth at pH 5.0

Inoculums size (ml)	Temperature (ºC)/Incubation time (h)/Protease activity (units/ml)															
	10				20				30				35			
	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
0.05	*21.64	18.55	15.46	24.73	15.46	24.73	27.80	27.80	24.73	18.55	30.91	24.73	27.80	15.46	18.55	24.80
0.1	21.64	21.64	21.64	24.73	15.46	21.64	21.64	24.73	24.73	21.64	21.64	27.80	21.64	24.73	18.55	24.73
0.15	18.55	24.73	24.73	18.55	18.55	15.46	21.64	18.55	21.64	21.64	15.46	24.73	15.46	21.64	15.46	24.73
0.2	15.46	21.64	21.64	15.46	12.37	15.46	15.46	15.46	18.55	15.46	12.37	21.64	15.46	18.55	12.37	21.64

Each value is a mean of duplicate determination

Some microorganisms require a single ion or their combination for optimum protease activity [85]. According to [79,80] metal ions contribute to increasing protease activity by forming salt or ion bridges between two adjacent amino acid residues while [86,87] reported that the observed increase in enzyme activity due to the supplementation of metal ions could be linked to membrane permeability through interaction with phospholipids or by binding to protein nucleic acid.

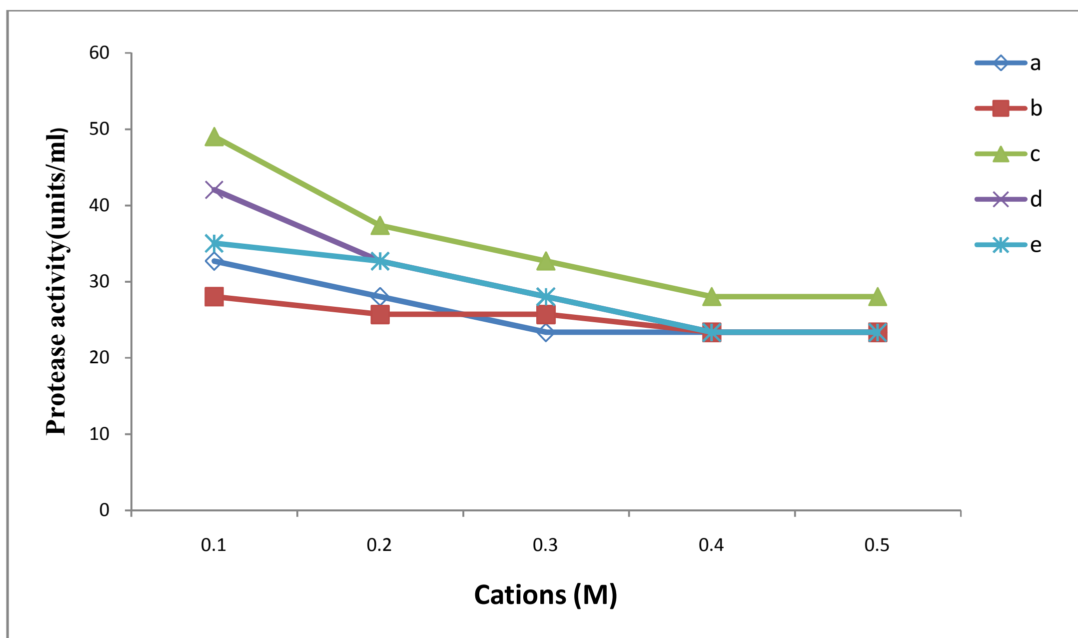

Fig. 2. Effect of cations on protease activity of *Pediococcus acidilactici*
Alphabets on legend represents: a = KNO_3 b=$CaCl_2$ c= $MnSO_4$ d=$MgSO_4$ e= NaCl

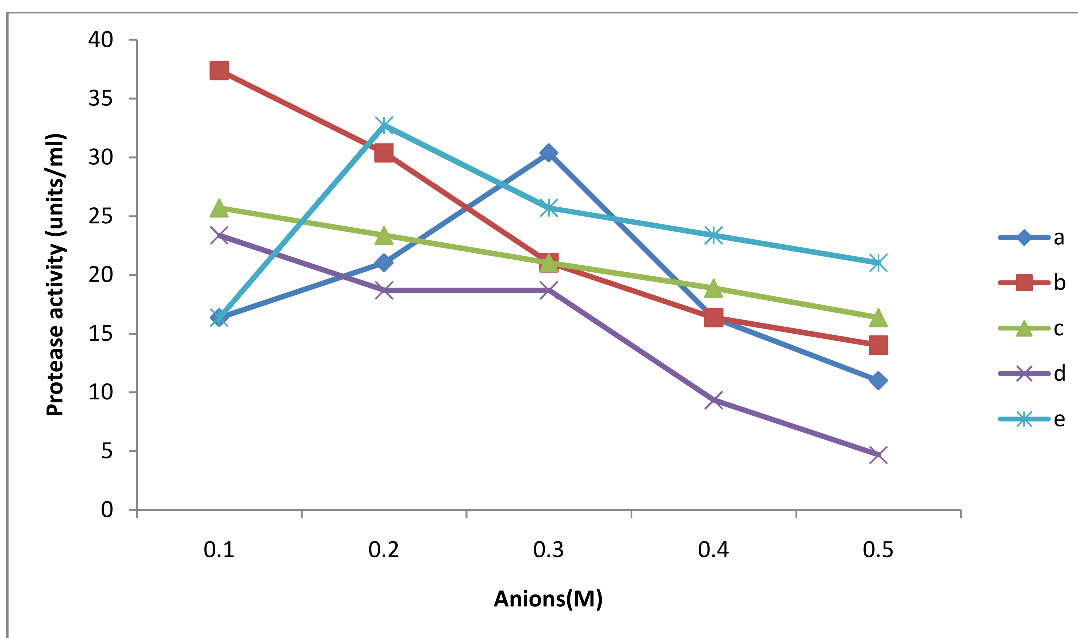

Fig. 3. Effect of anions on protease activity of *Pediococcus acidilactici*
Alphabets on legend represent: a= NH_4Cl b=NH_4NO_3 c=$FeSO_4$ d= $FeCl_2$ e= $NaNO_3$

3.8 Purification of Crude Protease

Fig. 4 shows the result of molecular mass of the purified protease enzyme determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of the major peaks was found to be within 45-55 kDa range. This result is in conformity with the findings of [88] that reported molecular weight of protease from *Penncilium jantinellum* and *Neurospora crassa* to be 45 kDa respectively. This observation is at variance with the submission of [89] that reported protease isolated from *Aspergillus orayzae* to possess a molecular weight of 33 kDa. However, the earlier reports of [90,91] showed that the molecular mass of approximately 48 kDa was recorded for protease from *Aspegillus* species. This value falls within the range of molecular mass of 45-55 kDa which was obtained in this study. In addition, the submissions of [92-94] confirmed the results obtained in this study.

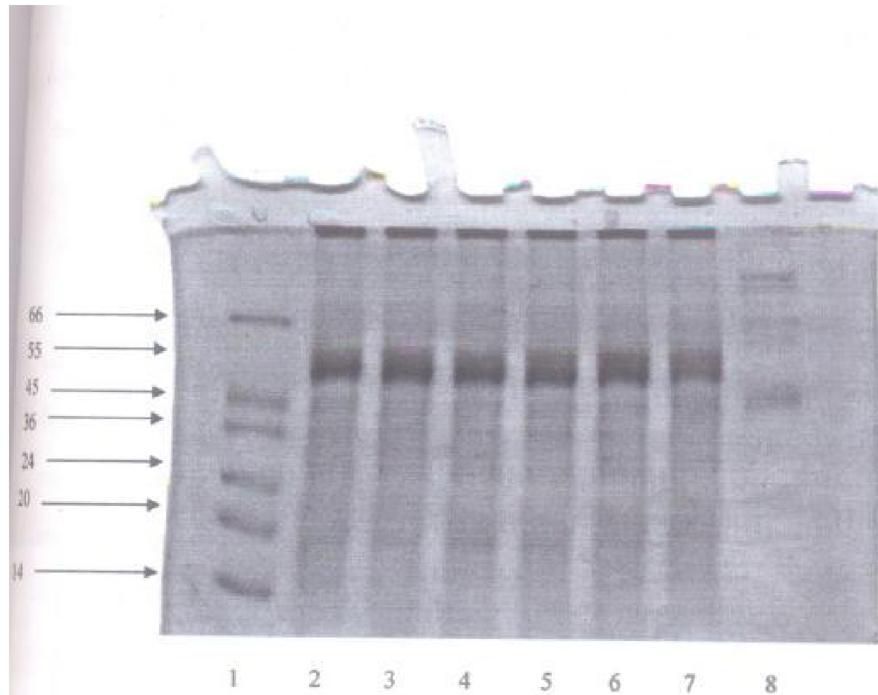

Figure 25 SDS-PAGE OF PROTEASE FROM *Lactobacillus plantarum* and *Pediococcus acidilactici.*

Lane 1 and 8 represents molecular weight marker protein standards.

Bovine serum albumin (66KDa)

Glutanic Dehydrogenese bovine liver (55KDa)

Ovalbumin, chicken egg (45KDa)

Glyceraldehyde - 3-phosphate Dehydrogenese rabbit muscle (36KDa)

Trypsinogen, bovine pancreas (24KDa)

Trypsin inhibitor, soyabean (20KDa)

α – Lactalbumin, bovine milk (14KDa)

Lane 2 to 7 represents purified protease

Fig. 4. Molecular mass of the purified protease enzyme determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4. CONCLUSIONS

This work revealed that Production characterization of protease from *Pediococcus acidilacti* could help to determine its biotechnological applications.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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