Evaluation of the Effects of Nutritional and Environmental Parameters on Extracellular Protease Production by *Stenotrophomonas acidaminiphila* Strain BPE4

Philomena Effiom Edet[1], Atim David Asitok[1]✎, Maurice George Ekpenyong[1], Sylvester Peter Antai[1]

[1]Environmental Microbiology and Biotechnology Unit, Department of Microbiology, Faculty of Biological Sciences, University of Calabar, P.M.B.1115 Calabar, Nigeria

Abstract: An aerobic mesophilic Gram-negative rod-shaped bacterium isolated from fermenting bean processing effluent (BPE) and identified as *Stenotrophomonas acidaminiphila* strain BPE4, produced an extracellular protease on skimmed-milk minimal medium. Time-course of enzyme production revealed peak productivity at 12 h but enzyme concentration gradually increased till 36 h beyond which both concentration and productivity gradually decreased. Evaluation of the influences of major nutritional sources on enzyme concentration revealed significant ($P < 0.05$) effects of fermentation time and nutrient sources, with corn steep liquor and NH_4Cl emerging as best carbon and nitrogen sources respectively. The study also selected K_2HPO_4/KH_2PO_4 (2:1) and 10^8 cfu/mL as most appropriate phosphate combination and inoculum size respectively for maximum release of protease within the fermentation time of 36 h. One unit of proteolytic activity was defined as the amount of crude enzyme that digested 1 mg of azocasein in one minute under the assay conditions. Assessment of optimum conditions of temperature and pH for test enzyme activity revealed optimum enzyme activity of 158.83 U/mL (2647.70 nkatal) at 40°C and pH 9.0 suggesting protease of the alkaline kind. The enzyme was specific for casein as a protein substrate. *Stenotrophomonas acidaminiphila* strain BPE4 could be exploited for commercial production of extracellular protease on corn steep liquor as a waste management option and industrial applications.

Keywords: *Stenotrophomonas acidaminiphila*; Caseinolytic protease, Production, Corn steep liquor; Environmental factors

1. Introduction

Proteases, variously called peptidases or proteinases, are hydrolytic enzymes belonging to the group of hydrolases (EC 3.4.21-24) which catalyze the hydrolytic cleavage of peptide bonds in proteins and polypeptides releasing peptides of shorter lengths and monomeric amino acid units. They are synthesized by all life forms including plants (papain, bromelain and ficin), animals (chymosin, trypsin and pepsin) and microorganisms (subtilisin, bacillopeptidases and aspergillopepsin) [12, 15]. Proteases perform several physiological roles in producing organisms such as digestion of food proteins, blood clotting, protein turn-over, signal transduction, cell division as well as in the life cycle of organisms [6, 15]. Majority of these roles form the bases for exploitation of the enzyme in diverse biotechnological concerns including food, pharmaceutical, detergent, waste water treatment, organic synthesis, leather tanning and textile processing [3].

Proteases constitute 60% of the global enzyme market with current world sale put at 3 billion USD per annum [12] thus making proteases the most sought-after group of enzymes. Microbial proteases, in particular, control a large portion of the protease market owing to the high growth rate, ease of cultivation and propensity for genetic and metabolic manipulation of producing organisms designed for improvement in both quantity and quality [8]. Several microbial genera have been reported as sources of proteases with differing capacities for production. Among them, the genera *Bacillus, Pseudomonas, Microbacteria, Vibrio, Streptomyces, Xanthomonas, Aspergillus, Fusarium, Paecilimyces* and *Cephalosporium* are frequently reported [11, 12, 16].

✎ **Atim David Asitok (Correspondence)**
✉ atimasitok@yahoo.com
☎ +234-8037302627

The major drawback of large-scale production has been the nature of producing microorganism, availability of utilizable carbon substrates and other major nutritional elements. Environmental factors especially pH and temperature have been reported for their influences on protease production but more significantly on the activity of the various proteases reported **[10].**

In this study, we report the potential of *Stenotrophomonas acidaminiphila* strain BPE4 to elaborate protease and the nutritional and environmental conditions required for production and activity of the enzyme.

2. Materials and methods
2.1 The microorganism
The bacterium was isolated in pure culture on tryptic soy agar from a bean processing effluent left untended for 4 days by local bean ball processors in Calabar municipality, Nigeria. The organism was characterized in terms of cultural morphology, microscopy and biochemical characterizations **[9].** Molecular characterizations of the bacterium followed DNA extraction, amplification and sequencing as described in **[10]**

2.2 Screening procedures for protease production
2.2.1 Protease production on solid medium
The screening medium was skimmed milk-minimal agar medium containing (g/L) $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; Skimmed milk 1% (v/v); Agar-agar 15; Distilled water 1000 mL; pH was adjusted to 7.0 with 1M HCl/1M NaOH. Minimal medium was sterilized by autoclaving at 121°C for 15 min. Skimmed milk was selectively sterilized by filtration (0.45 μM-Millipore). Triplicate plates were inoculated by single-line streak technique and incubated at the ambient conditions of the laboratory, specifically room temperature (28 ± 2°C) for 36 h. *Vibrio fluvialis* OWPB63 UCCM 0004 obtained from the University of Calabar Collection of Microorganisms served as positive control. Development of a clear zone around the line of streak on the opaque (cloudy) milk-agar plate indicated protease production. Diameter of clear zone, measured by means of a meter rule and presented in millimeters, was compared with that of the control.

2.2.2 Protease production in submerged culture
Protease production ability of the bacterium was also assessed in shake flasks using skimmed milk-minimal broth medium containing (g/L) $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; Skimmed milk 1% (v/v); Distilled water 1000 mL; pH was adjusted to 7.0 with 1M HCl/1M NaOH. Minimal medium and skimmed milk were sterilized

as earlier described. An 18 h-old tryptic soy broth culture of the bacterium was inoculated into 250 mL Erlenmeyer flask containing 50 mL of medium at 2% (v/v) and flasks incubated at room temperature (28 ± 2°C) on a rotary shaker agitating at 150 rpm for 36 h. Protein was detected by the Bradford method as described in **[4]** bovine serum albumin (Research Organics, USA) as standard protein. A calibration curve was prepared using standard protein with concentrations ranging from 5 to100 μg/mL. Test protease concentration was determined from the regression equation derived from the standard curve.

2.3 Determination of bacterial productivity of protease
Time-course of protease production was monitored to determine the protease productivity of the bacterium and to also inform the duration of fermentation studies. Medium formulation, sterilization and inoculation were as described earlier. Incubation was at room temperature on a rotary shaker agitating at 150 rpm for 48 h with periodic triplicate protein determinations made every 4 h. Fermentation time was extended to 96 h with triplicate protein determinations made every 12 h. Analysis of variance of data was conducted with Excel 2007 software.

2.4 Biochemical and molecular characterizations of the bacterium
Cultural characterizations including consistency, elevation, form, margin and pigmentation; microscopic characterizations including cell shape, size and arrangement; physiological and biochemical characterizations as described in **[9]** were employed for bacterial identification. Identity of the isolate was confirmed by molecular methods using 16S rRNA gene amplification and sequencing.

The molecular methodology was based on PCR and Sanger sequencing analysis. DNA extraction and bio-informatic analysis of sequences were performed at the Molecular Laboratory Services Division of Teddy & Thaddeus Nig. Co., Akoka, Lagos, Nigeria. Sequencing analysis was done at Inqaba Biotechnology Pty, South Africa. DNA extraction and purification were done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps. Model D6005 (Zymo Research, California, USA) using universal degenerate primers 27F.1 Forward 5´AGRGTTTGATCMTGGCTCAG 3´ and 1492R reverse 5´ GGTTACCTTGTTACGACTT 3´ that amplifies the entire 16S variable region at annealing temperature of 58°C. The PCR cycle started with an initial denaturation step at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, an extension at 72°C for 5 min and cooling to 4°C. Sequence analysis from

resultant nucleotide base pairs was performed by BLAST analysis using MEGA5 software. The resultant top hits with minimum E-score for the BLAST showing species name was used to identify the specific organism **[10].**

2.5 Selection of appropriate major nutritional sources for protease production

2.5.1 Effect of carbon source on protease production

Six different carbon sources including cassava processing effluent, rice processing effluent, corn steep liquor, glucose, sucrose and fructose were separately screened for optimal protease production by isolate BPE4. Medium was skimmed milk-minimal broth containing (g/L) $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; Skimmed milk 1%; Distilled water 1000 mL; pH was adjusted to 7.0 with 1M HCl and 1M NaOH. Medium formulation, sterilization and inoculation were as described earlier. Incubation was at room temperature ($28 \pm 2°C$) on a rotary shaker agitating at 150 rpm for 36 h. Triplicate protein determinations were made every 3 h. *Vibrio fluvialis* OWPB63 UCCM 0004 served as positive control while protein reagent (Coomasie brilliant blue solution) was the negative control. A two-factor analysis of variance of data and separation of significant means were conducted with Excel 2007 software to determine the dual influences of carbon source and duration of fermentation on extracellular protease production.

2.5.2 Effect of nitrogen source on protease production

Five different nitrogen sources including ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate and sodium nitrate were separately screened for optimal protease production. Medium was skimmed milk-minimal broth containing (g/L) $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; Skimmed milk 1%; Filter-sterilized corn steep liquor 1000 mL; pH 7.0. The nitrogen sources were added at 1% (w/v) to media. Sterilizations, inoculation, incubation, determinations and analysis were as described in section 2.4.

2.5.3 Effect of phosphorus source on protease production

Four different phosphate combinations including K_2HPO_4/KH_2PO_4, Na_2HPO_4/NaH_2PO_4, Na_2HPO_4/KH_2PO_4 and K_2HPO_4/NaH_2PO_4 were separately screened for optimal protease production. Medium was skimmed milk-minimal broth containing (g/L) NH_4NO_3 1.0; $MgSO_4.7H_2O$ 0.5; Skimmed milk 1%; Filter-sterilized corn steep liquor 1000 mL; pH was adjusted to 7.0 with 1M HCl and 1M NaOH. The phosphate combinations (2:1) were added at 1% (w/v) to media. Sterilizations,

inoculation, incubation, determinations and analysis were as described in section 2.4.

2.5.4 Effect of inoculum size on protease production

Five different inoculum sizes including 10^5, 10^6, 10^7, 10^8 and 10^9 cells/mL were separately screened for optimal protease production. Inoculum sizes were standardized according to McFarland standards. Medium was skimmed milk-minimal broth containing (g/L) NH_4NO_3 1.0; K_2HPO_4/KH_2PO_4 (2:1) 1.0; $MgSO_4.7H_2O$ 0.5; Skimmed milk 1%; Filter-sterilized corn steep liquor 1000 mL; pH was adjusted to 7.0 with 1M HCl and 1M NaOH. The different inoculum sizes were added at 2% (v/v) to media. Sterilizations, inoculation, incubation, determinations and analysis were as described in section 2.4.

2.6 Determination of BPE4 protease activity

Activity of the test protease was investigated as described in [4] using the non-specific protein, azocasein, as substrate. One unit of protease activity was defined as the amount of crude enzyme required to digest 1 mg of azocasein in one minute under the assay conditions **[5].**

2.7 Selection of appropriate temperature for extracellular protease activity

The effect of temperature on the activity of protease was assessed by measuring the release of trichloroactic acid (TCA)-soluble peptides from the digestion of azocasein after incubation at temperatures ranging from 5 to 65°C for 10 min. Absorbance was measured at 440 nm in triplicates using a spectrophotometer. Protease activity was calculated using the formula

$$EA\,(U) = C_{azo} \times V^2/t \times v$$

where C_{azo} = concentration of azocasein derived from the standard curve regression equation C_{azo} (mg/mL) $= -0.5895 + 22.399 \times$ Absorbance; V= total volume of reaction mixture; t = duration of incubation; v = volume of crude enzyme.

2.8 Selection of appropriate pH for extracellular protease activity

The effect of pH on the activity of protease was assessed by measuring the release of trichloroactic acid (TCA)-soluble peptides from the digestion of azocasein after incubation at pH ranging from 3 to 12 for 10 min. For pH 3 to 6; sodium-phosphate buffer was used; Tris-HCl buffer was used for pH 7 to 8; potassium phosphate buffer for pH 9 to 10 and bicarbonate-phosphate (sodium) buffer for pH 11 to 12. Absorbance was measured at 440 nm in triplicates using a spectrophotometer.

2.9   Effect of substrate on protease activity

The activity of test protease on different protein substrates was conducted to establish the specificity, or otherwise, of the enzyme. Four protein substrates were used including casein, bovine serum albumin, gelatin and keratin. Activities were monitored at 40°C, pH 9 for 30 min. Triplicate determinations were made in all cases.

3.   Results

The study bacterium, isolated from fermenting bean processing effluent, produced a clear zone of diameter 37.0 mm on skimmed-milk minimal medium at room temperature within 36 h. The bacterium also elaborated 142.87 µg/mL of crude protease in shake flask experiments within the period.

Results of time course study designed to establish the productivity and possible duration of fermentation by the bacterium revealed that the bacterium produced maximum protease at 36 h. These results, presented in Table 1, also show that peak productivity of the bacterium occurred at 12 h when the bacterium amassed 32.6 µg/mL of protease within 4 h giving a peak productivity of 8.18 µg/mL/h. Productivity dropped to 4.55 by the 20th hour only to increase again to 4.66 µg/mL/h at 24 h. The table shows that protease productivity in the bacterium gradually decreased from this point to the end of the fermentation period of 96 h. Negative protease productivities commenced from 40 h till the end of fermentation. By 96 h, maximum recoverable protease from fermentation broth was 4.52 µg/mL at a productivity of -2.75 µg/mL/h. These results set duration of fermentation by this isolate at 36 h.

The sole responsibility of an enzyme is to catalyze a biochemical reaction hence the activity of the enzyme was studied at 30°C and pH 7 using the non-specific protease substrate, azocasein, as standard. One unit of proteolytic activity was defined as the amount of crude enzyme that digested 1 mg of azocasein in one minute under the assay conditions of 30°C and pH 7.0. The activity of the crude protease was found to be 100.83 U/mL after 30 min of incubation.

The test bacterium, isolate BPE4, was identified by cultural, biochemical, physiological, microscopic and molecular methods involving 16S rRNA gene amplification and sequencing. The results are presented in Table 2. The table shows that the bacterium was catalase and oxidase positive but could not reduce nitrate. Ornithine and lysine were decarboxylated but phenylalanine was not deaminated. A limited number of sugars were utilized including glucose, mannose, fructose and arabinose. The bacterium was defined as a light-yellow, motile, Gram-negative, aerobic, curved rod with 99.4%

sequence homology with *Stenotrophomonas acidaminiphila* strain AMX 19T and 98.8% sequence homology with *Stenotrophomonas maltophila* and *Stenotrophomonas nitritireducens.* The bacterium was deposited at the University of Calabar Collection of Microorganisms (UCCM).

Results of the evaluation of major medium variables for protease production are presented in Figures 1, 2, 3 and 4. Figure 1 shows that corn steep liquor was the best carbon substrate for protease production by *Stenotrophomonas acidaminiphila* strain BPE4. The substrate mediated the synthesis of 242.74 µg/mL within 36 h. Other substrates and their protease concentrations were, in descending order, cassava processing effluent (CPE) - 190.67 µg/mL > rice processing effluent (RPE) - 173.24 µg/mL > glucose - 172.56 µg/mL > fructose -168.43 µg/mL > sucrose - 161.80 µg/mL > control - 142.87 µg/mL. The control received no extraneous carbon source but skimmed milk served both as carbon and nitrogen sources. The two-factor analysis of variance revealed that both carbon source and fermentation time significantly (carbon source, $P = 3.06E-30$; fermentation time, $P = 7.41E-14 < 0.05$) influenced protease production.

Figure 2 presents the effect of nitrogen sources on protease production by the bacterium. The figure reveals that ammonium chloride (NH_4Cl) was best nitrogen source for the process. This nitrogen source accumulated 267.92 µg/mL of protease in 36 h. NH_4Cl was followed by $(NH_4)_2SO_4$ which directed the synthesis of 245.76 µg/mL > control - 242.74 µg/mL. The control received no nitrogen source other than the skimmed milk substrate. A two-factor analysis of variance showed significant effects of both nitrogen source and fermentation time (nitrogen source, $P = 4.29E-20$; fermentation time, $P = 3.79E-13 < 0.05$) on protease production. However, results of the *t*-Test for two samples assuming unequal variances revealed that no significant difference ($P > 0.05$) existed among the three nitrate-nitrogen sources including NH_4NO_3, KNO_3 and $NaNO_3$.

Effect of phosphate combinations are presented in Figure 3. The figure shows that K_2HPO_4/KH_2PO_4 (2:1) combination, which also served as the control combination, was the best possible phosphate combination for protease production by the bacterium. All other phosphate combinations reduced final protease concentration. Analysis of variance showed that phosphate combinations significantly (phosphate combination, $P = 2.02E-10$, fermentation time, $P = 1.59E-19 < 0.05$) influenced protease production by the test bacterium.

Results of the influence of inoculum size on protease production are presented as Figure 4. The figure

reveals that a starting inoculum size of 10^8 cells/mL led to the production of highest amount of protease of 293.67 µg/mL within 36 h followed by inoculum size of 10^7 cells/mL which produced 279.12 µg/mL protease.

Table 1 Time course and protease productivity of BPE4

Time (h)	Mean crude protease concentration ± Standard deviation (µg/mL)	Productivity (µg/mL/h)
0	0 ± 0.00	0
4	8.45 ± 0.82	2.11
8	26.52 ± 1.34	4.52
12	59.22 ±2.84	8.18
16	77.68 ±4.32	4.62
20	95.88 ± 4.18	4.55
24	114.53 ±3.29	4.66
28	128.36 ±4.15	3.46
32	139.52 ±7.37	2.79
36	142.87 ± 8.85	0.84
40	132.45 ± 9.56	-2.61
44	121.65 ± 6.76	-2.70
48	110.05 ± 6.62	-2.90
60	92.64 ± 6.72	-1.45
72	68.53 ± 4.32	-2.01
84	37.36 ± 2.23	-2.60
96	4.52 ± 4.52	-2.75

Table 2 Biochemical characterization and molecular identification of isolate BPE4

S/N	Colonial characteristics	
1	Pigmentation	Light-yellow
2	Consistency	Butyrous
3	Margin	Entire
4	Elevation	Raised
5	Form	Round
	Microscopic morphology	
6	Gram reaction	Negative
7	Cell shape	Curved rods
8	Cell size	Less than 0.5 µM
9	Cell arrangement	Singles
10	Motility	Positive
	Biochemical characteristics	
11	Oxidase	Positive
12	Catalase	Positive
13	Hydrogen sulfide production	Positive
14	Phenylalanine deamination	Negative
15	Ornithine decarboxylation	Positive
16	Lysine decarboxylation	Positive
17	Nitrate reduction	Negative
18	Citrate utilization	Negative
	Sugar utilization	
19	D-glucose	Positive
20	D-mannose	Positive
21	D-fructose	Positive
22	Lactose	Negative

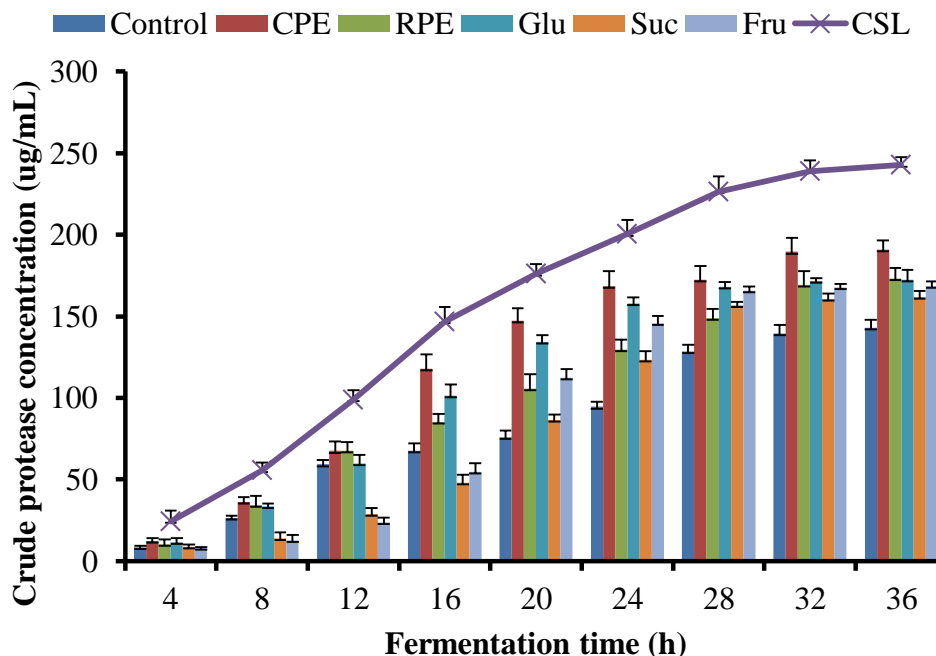| 23	Arabinose	Positive
24	Adonitol	Negative
25	Sorbitol	Negative
26	16S rRNA gene amplification and sequencing	99.4% similarity with *Stenotrophomonas acidaminiphila*

Fig.1 Selection of appropriate carbon source for protease production by *Stenotrophomonas acidaminiphila* Strain BPE4.
CPE-cassava processing effluent; RPE-rice processing effluent; Glu-glucose; Suc-Sucrose; Fru-fructose; CSL-corn steep liquor

Other inoculum sizes produced protease concentration lower than 267.92 µg/mL of the control. Analysis of variance revealed that inoculum sizes and fermentation time significantly (inoculum size, $P = 0.012$; fermentation time, $P = 1.45E-14 < 0.05$) influenced final protease concentration.

Results for the selection of appropriate temperature and pH for protease activity are presented in Figures 5 and 6 respectively. Figure5 shows that the most appropriate temperature for enzyme activity was 40°C, where the enzyme demonstrated highest activity of 122.89U/mL at 30 min. This was followed by 111.07 U/mL and 100.83 U/mL for 45°C and 30°C respectively. A two-factor analysis of variance of data showed that temperature and duration of exposure significantly (Temperature, $P = 1.31E-07$; Time, $P = 3.1E-05 < 0.05$) influenced proteolytic activity. Figure 6 shows that activity of the protease increased with increasing pH until it peaked at pH 9 from where it declined again.

The activity of an enzyme is intrinsically linked to its substrate and so different protein substrates were screened for activity. The results are presented as Figure 7. The figure shows that casein had the highest relative activity (%) at 40°C and pH 9 for 30 min. None of the remaining three substrates could drive up to 50% relative activity of the protease.

4. Discussion

The test bacterium, strain BPE4, identified by cultural, biochemical, physiological, microscopic and molecular methods involving 16S rRNA gene amplification and sequencing as *Stenotrophomonas acidaminiphila*, was defined as a motile, light-yellow, catalase-positive, oxidase-positive, Gram-negative, aerobic, curved rod with 99.4% sequence homology with *Stenotrophomonas acidaminiphila* strain AMX 19T. The bacterium could neither reduce nitrate nor deaminate phenylalanine but could decarboxylate both ornithine and lysine. A limited number of sugars were utilized including glucose, mannose, fructose and arabinose. To the best of our knowledge, this is

the first report on the potential of this bacterium for protease production.

Productivity; defined, in this study, as the amount of protease released per unit time; was found to reach its peak of 8.18μg/mL/h in the study bacterium at 4 h. This implies that the protease is a primary metabolite required for cellular metabolism, primarily for growth and reproduction. The negative values for productivity beyond 36 h indicate protein turn-over where the enzyme is denatured and possibly degraded into the amino acid building blocks after completing its catalytic role. In a batch mode fermentation for protease production reported by [13], incubation time for protease production was prolonged for 72 h suggesting the protease to be a secondary metabolite. If the protease was to catalyze the breakdown of a proteolytic substrate to generate simple amino products which would serve as nitrogen source, then cellular metabolism would have been delayed in the organism except there was a readily available inorganic nitrogen source in the medium. On the other hand, the present finding is in agreement with [16] who reported an incubation period of 24 h for

their bacterial strains confirming the primary metabolite (trophophasic) status of the product.

Proteases act on a variety of substrates depending on their nature. The gold standard substrate for evaluation of proteolytic activity has been reported as azocasein owing to its non-specific nature [5]. In this study, one unit of proteolytic activity was defined as the amount of crude enzyme that digested 1 mg of azocasein in one minute under the assay conditions of 30°C and pH 7.0. The activity of the crude protease in this study without recourse to the carbon, nitrogen and phosphorus sources was 100.83 U/mL or 1680.84 nkat (nanokatal) after 30 min of incubation. The katal is defined as the amount of enzyme that converts one mole of a substrate in one second. It was recommended by the General Conference on Weights and Measures in 1978 as the standard unit representing enzyme activity because 'minute' is not the SI unit for time. The recommendation was adopted officially in 1999 and the conversion of enzyme activity in U to the katal followed the equation below [18];
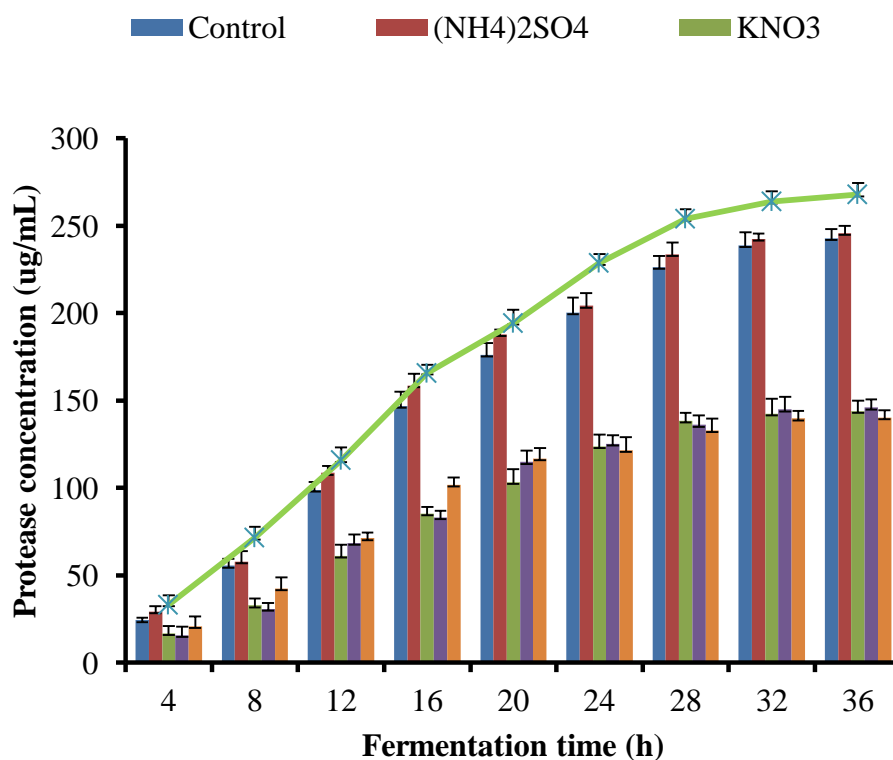

Fig.2 Selection of appropriate nitrogen source for protease production by *Stenotrophomonas acidaminiphila* strain BPE4
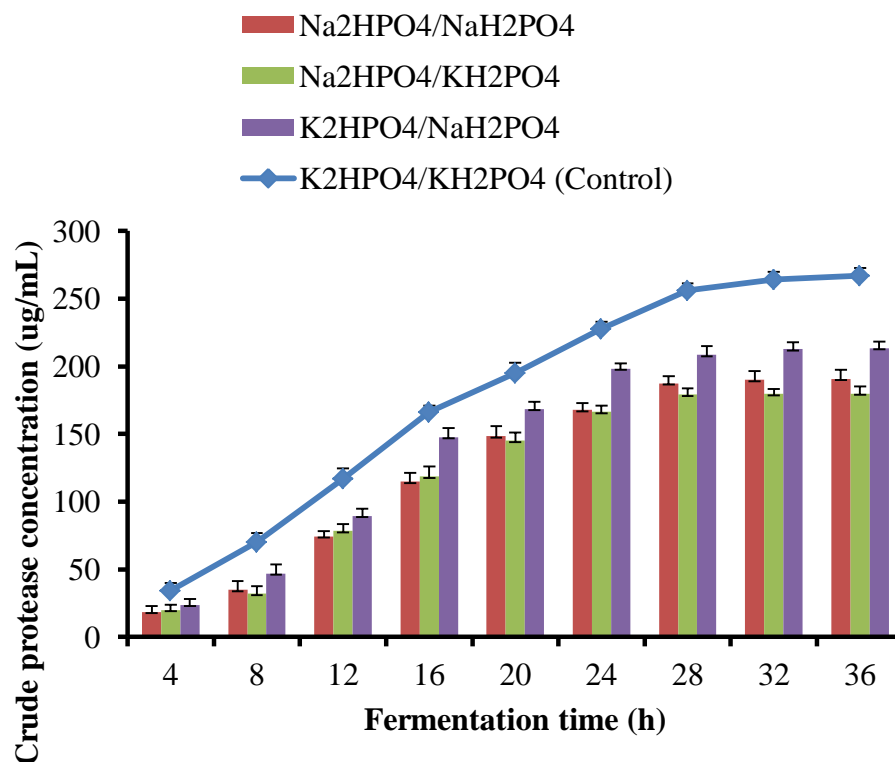
Fig.3   Selection of appropriate phosphate combination for protease production by *Stenotrophomonas acidaminiphila* strain BPE4
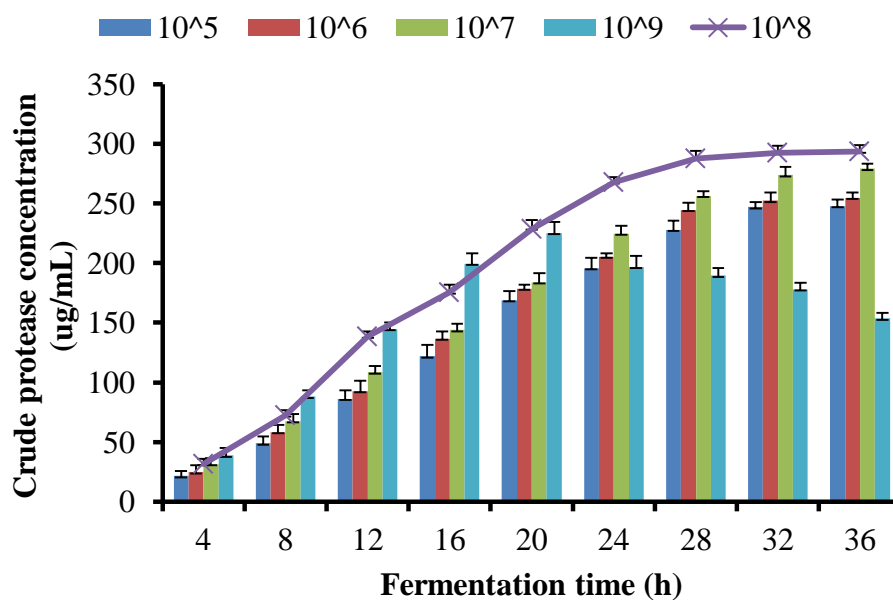

Fig.4   Selection of appropriate inoculum size for protease production by *Stenotrophomonas acidaminiphila* strain BPE4
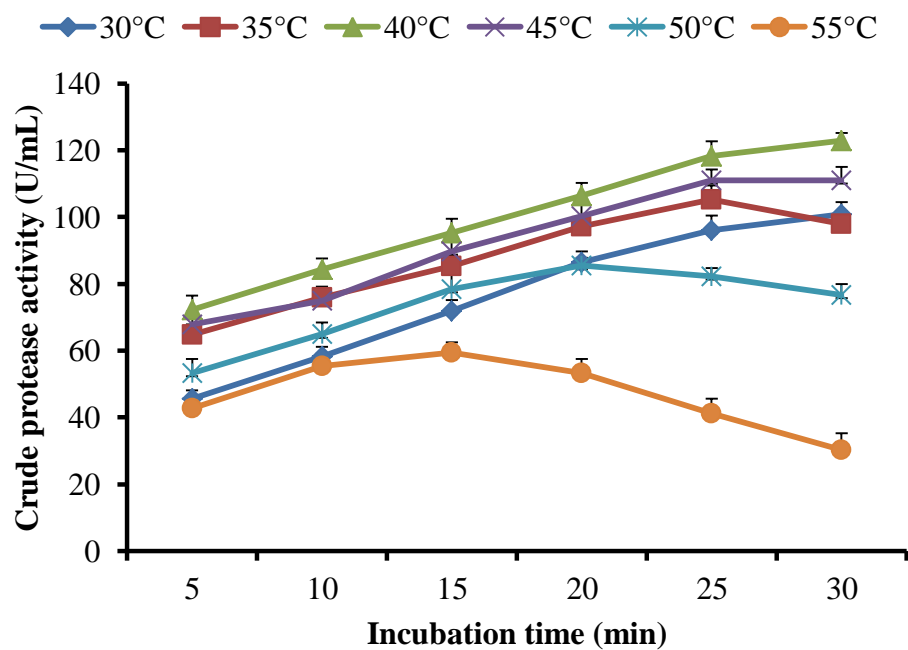
Fig.5   Selection of appropriate temperature for *Stenotrophomonas acidaminiphila* strain BPE4 protease activity
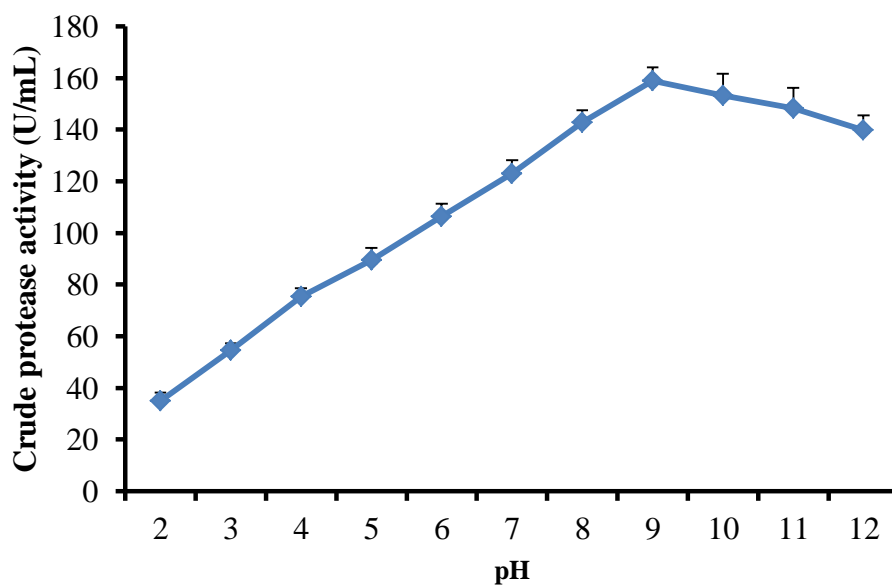

Fig.6   Selection of appropriate pH for *Stenotrophomonas acidaminiphila* strain BPE4 protease activity
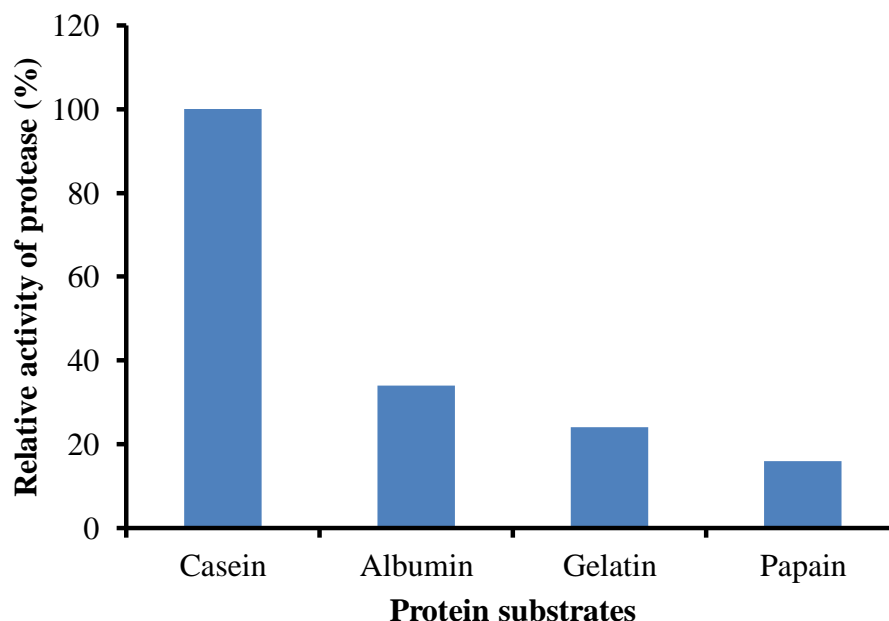
Fig. 7   Effect of substrate on protease activity of *Stenotrophomonas acidaminiphila* strain BPE4

$$U = \frac{1}{1000000} \div 60 \times 1katal = \frac{1}{60}\mu katal = 16.67\ nkatal$$

Corn steep liquor is a liquid waste obtained from wet corn milling used to produce numerous corn based products that are subsequently used as biofuel, ingredients in food and for livestock feed. These products include starch, high fructose corn syrup, oil, ethanol, bran, gluten feed and meal. Corn steep liquor is one of the by-products of wet corn milling directed to the production of animal feed. It is a mixture of soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins and minerals. Microbial biotechnologists employ it as a nutrient for microorganisms in the production of enzymes, antibiotics and other fermentation products. It could serve both as a source of carbon and that of nitrogen. In this study, it was employed as a source of carbon among others, and was selected as best carbon source for protease production by the test bacterium. When soluble starch, glucose, fructose or maltose were separately combined with corn steep liquor (data not shown), protease production by the bacterium was grossly repressed. In Figure 1, protease concentration, mediated by corn steep liquor, is presented as a line made to fly above concentrations mediated by other sources indicating significant preference of the carbon substrate for protease production by the test bacterium. Perhaps extraction of carbon atoms from corn steep substrate for cellular metabolism and energy generation required the expenditure of minimal maintenance energy by the bacterium when compared to other sources of carbon. Its preference could also be attributed its dual role as carbon and nitrogen source.

Production of industrial enzymes in particular and biotechnological products of microbial origin in general, using renewable sources is currently being explored and exploited at different levels all over the world with very commendable results. [17] produced commercial quantities of an alkaline protease from a silk degumming solution; a waste solution from the silk degumming process; using four different strains of *Aspergillus flavus* and *Bacillus licheniformis* strain TISTR 1010. In a production optimization experiment, [13] recorded highest predicted alkaline protease activity of 205.2 U/mL on molasses; a waste substrate from the sugar refining process using *Bacillus cereus* strain S8. The protease from *Stenotrophomonas acidaminiphila* strain BPE4 in the present study, however, could demonstrate highest activity of 158.83 U/mL (2647.70 nkatal) when corn steep liquor served as carbon source. [14] reported successful production of thermostable alkaline protease from *Kocuria varians* using various agricultural wastes like cow blood meal, fermented cassava wastes, potato, yam and plantain peels, fish meal and corn waste with remarkable success. Exploitation of agro-industrial wastes for production of various value-added microbial metabolites is strongly recommended.

Nitrogen is required as a major nutrient by microorganisms for the synthesis of amino acids, nitrogenous compounds, nucleic acids and the energy currency, ATP to name a few. These compounds are the major drivers of cellular metabolism leading to biomass accumulation. Oftentimes, in a biotechnological production venture, the nitrogen source adopted for cellular metabolism by an organism differs from the one required for secondary metabolite synthesis [1, 7]. In the present study, the simple readily available, readily assimilable NH_4Cl was selected for protease production by *Stenotrophomonas acidaminiphila* strain BPE4. This implies that the initial number of cells needed to elaborate substantial amount of protease for catalytic breakdown of casein was inadequate and the system had to turn to the incorporable form of nitrogen (ammonia-NH_3) to fulfill this requirement. Once again, the trophophasic status of the product is confirmed. The non-significant difference among the nitrate-N_2 sources investigated in the study on protease production by *Stenotrophomonas acidaminiphila* strain BPE4 is attributable to the inability of the bacterium to reduce the nitrate-nitrogen sources from the oxidation state of +5 to the incorporable (assimilable) oxidation state of -3 in ammonia. The nitrate-N_2 sources were therefore repressive to the synthesis of protease by the bacterium.

Phosphorus plays a dual role in microbial biotechnological productions serving first as a source of phosphorus required for the synthesis of nucleic acids and ATP. Phosphorus sources secondarily serve as buffering systems in fermentation, resisting drastic changes in the pH of the system thus allowing its continuous operation. A phosphate combination should therefore be employed optimally to fulfill these two requirements. In this investigation, K_2HPO_4/KH_2PO_4 (2:1) combination, which also served as the control combination, was the best possible phosphate combination for protease production by the bacterium. All other phosphate combinations were repressive to protease biosynthesis suggesting that the enzyme was produced in the trophophase of bacterial growth.

The driving reactants in microbial fermentations are the microorganisms themselves and their number by volume is an important control point in fermentation. Only a handful of researches report the number of microbial cells involved in a production study. [7] reported that 10^8 cells/mL were required to drive biosurfactant production by *Pseudomonas aeruginosa* strain IKW1 to completion. [14] reported 10^3 cells/mL as the inoculum size required for protease production in *Kocuria varians* using various

agricultural wastes. [2] used an inoculum size that produced an optical density of 0.5 at 660 nm wavelength for the production of thermostable organic-solvent tolerant protease by a halotolerant *Bacillus subtilis* strain Rand. In this study, an inoculum size of 10^8 cells/mL was selected for protease production. [7] argued that selection of this optimal inoculum size suggests that too little an initial cell population would delay metabolite production until such a time that its density reached the desired quorum and that too large an initial cell population might arrest production too early by reason of ineffective collisions between the bacterial cells (catalysts) and the substrates for want of space in a saturated system.

The influence of environmental factors like temperature and pH on enzyme activity has been copiously documented. It should be understood that optimum temperature for production is very different from that for activity of the enzyme. In this study, optimum temperature for protease activity was 40°C as against the room temperature adopted for production. This casts doubts on reports of enzyme activities obtained during the production or fermentation process. There would be better agreement in literature if enzyme activity reports are results of activities after finding optimum conditions and specific substrates. At the optimum temperature, the activity of the enzyme will not be at its peak until the pH is equally at its optimum. In this study, at the optimum temperature of 40°C, protease activity was 122.89 U/mL (2048.58 nkatal) at pH 7.0, but at the optimum pH of 9.0 selected, protease activity increased to 158.83 U/mL (2647.70 nkatal) at the optimum temperature. When substrates other than casein were employed in the assay, relative activity of the enzyme decreased tremendously (Figure 7) suggesting that the enzyme is a caseinolytic protease.

Conclusion
An unusual protease producing bacterium *Stenotrophomonas acidaminiphila* isolated from fermenting bean effluent was reported in this study. The bacterium produced protease copiously using corn steep liquor as carbon source in the trophophase of growth. Optimal conditions of temperature and pH were set at 40°C and 9.0 respectively. The protease activity was maximal when casein served as the protein substrate.

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