Journal of Bioprocessing & Biotechniques

Tavea et al., J Bioprocess Biotech 2016, 6:4
http://dx.doi.org/10.4172/2155-9821.1000279

Research Article Open Access

Production and Partial Characterization of an Extracellular Thermophile Alkaline Protease from a Selected Strain of *Bacillus* sp Isolated from Abattoir Soil in the North Region of Cameroon

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Abstract

The present study aims at screening and isolates thermostable alkaline protease producing bacteria from soil sample in the northern part of Cameroon, and characterizes their enzyme. Samples of soil were collected from four (04) North Cameroonian towns (Ngaoundere, Garoua, Figuil and Maroua). Six (06) isolates showed important proteolytic activity on skim milk agar. The strain N2.3.5 from Ngaoundere showed the largest hydrolysis halo (24 mm) and was selected for protease production. The partial identification of strain N2.3.5 through morphological and biochemical characterization enabled us to classify it as belonging to the genus *Bacillus* sp. The growth of the strain N2.3.5 and its enzyme production profile showed that the growth of the bacteria was a diauxic type, and that the protease production was not directly associated with the microbial growth. In a medium composed of soya flour, corn flour, yeast extract and at alkaline pH, following the monitoring of each of these parameters by a complete factorial experimental design, the pH value of 10 was found to be significant for an optimal growth. Meanwhile, the corn flour, the soya flour and the yeast extract did not have a consequent influence on the protease production by the bacteria, under the chosen experimental conditions. The extracellular protease produced by *Bacillus* sp N235 was partially purified using a precipitation with ammonium sulphate, a dialysis and a gel filtration on sephadex G-75. A specific activity of 1516.7 U/mg and a purification yield of 37.63% were obtained. The partially purified enzyme was able to maintain its activity after been heat-treated at 80°C for 30 min. The maximal activity was exhibited at temperature of 80°C and to a pH 12. The enzyme activity and stability was enhanced in presence of the Ca^{2+}.

Keywords: Microbial enzymes; Thermophile alkaline proteases; Bioprocessing; Fermentation

Introduction

Proteases are enzymes used in the hydrolysis of proteins into oligopeptides. These enzymes are very important in the world market where they account for about 59% [1]. They have numerous biotechnological applications in the food industry, pharmaceutical industry and in the manufacture of detergents [2,3]. Proteases like most enzymes may have a vegetable, animal, or microbial origin. Microbial proteases are advantageous in that they can be produced easily at lower cost and in large quantities in industrial scale. Genetic engineering techniques are readily applicable to microbial strains; they improve the efficiency of enzyme production [4].

Many studies on microbial proteases have described mesophilic enzymes with maximum activity around 30-40°C [5,6]. However, very few studies mention the thermostability of microbial proteases. Thermostability is a very important property of industrial enzymes, hence the need to seek a thermostable protease.

The thermal stability is advantageous in that it contributes to the reduction of microbial contaminants and cooling costs. Our previous studies [7] showed that soil of hot regions are favorable sites for growth of thermostable and thermophile enzyme producing microorganisms. Cameroon North Region, the study area has a climate characterized by very high temperatures within the day. In soil around slaughter houses of this region natural fermentations occur. Owing to the presence of cow meat wastes in such environment, natural fermentation involved proteolytic microorganisms. This justifies the choice of this site for the screening of thermophilic and thermostable proteases producing bacteria.

This study aims to isolate and identify a bacterial strain with high proteolytic activity that produces a thermophile and thermostable alkaline protease.

Material and Methods

Study area

This study was carried out in Northern part of Cameroon, the slaughter houses of four towns of the Northern region of Cameroon was investigated on the basis of the intensity of the degradation activities of cow and cattle meat wastes on the surface of the soil surrounding the abattoirs, and the harsh climatic conditions with extreme temperature variations (above 40°C during the day and less than 10°C in the night).

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Received April 15, 2016; **Accepted** April 25, 2016; **Published** April 29, 2016

Citation: Tavea F, Fossi BT, Fabrice NT, Ngoune LT, Ndjouenkeu R (2016) Production and Partial Characterization of an Extracellular Thermophile Alkaline Protease from a Selected Strain of *Bacillus* sp Isolated from Abattoir Soil in the North Region of Cameroon. J Bioprocess Biotech 6: 279. doi:10.4172/2155-9821.1000279

These soils were considered as potential niches for thermostable alkaline protease producing bacteria.

Isolation of and selection of protease producing bacteria

Samples of soil were collected around abattoir and put in polyethylene aseptic bags. Protease producing bacteria were isolated from soil samples using a serial dilution method described by Sjodahl et al. [8]. Samples were inoculated on skim milk agar plates containing peptone (0.1%), NaCl (0.5%) and skim milk (10%) medium prepared using sea water, and then incubated at 30°C for three days. Colonies having clear halo were considered as protease producing bacteria. The diameters of the clear zones were measured and was considered as and indicators of proteolytic activity.

Identification of the high yielding strain

Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals [9].

Microbial growth and protease production

In order to study the time course for microbial growth and protease production, the selected microorganism was propagated at 40°C for 70 h in 50 ml of a basal medium containing: powder milk, 1% (w/v); yeast extract, 0.5% (w/v) placed in 100 ml Erlenmeyer flask with shaking at 150 oscillations per min in an alternative shaker (Kotterman, Germany). The initial pH of the medium was adjusted to 6.5 (Alkaline) using 0.1 M HCl. The growth of the selected microbial strain was estimated each 10 h using plate counting method on nutrient agar. After removal of cells by centrifugation (8,000 × g, 30 min, 4°C) in centrifugator (Heraeus, Germany), the supernatant was considered as the crude enzyme solution and was used to measure protease activity.

Optimization of the enzyme production

A factorial design was used for the optimization of the enzyme production. The factors selected in this study were: corn flour (carbon source), soya bean flour (source of nitrogen), and yeast extract (source of vitamins and amino acid), the initial pH of the fermenting medium. Protease production by the selected microbial strain was taken as the response and was giving by the following equation:

$$Y = \alpha_o + \sum_i \alpha_i X_i + \sum_{ij} \alpha_{ij} X_i X_j$$

Y is the predicted response (protease activity), X_i and X_j are the independent variables, α_o is the mean value, α_i is the linear coefficient and α_{ij} is the interaction coefficient. The experiments were carried out in duplicate. The levels of significance of the model and equation parameter were evaluated by Fisher test. The experimental range and level of the variable are represented in Table 1.

Enzyme production

For the protease production, 1 ml of 24 h of inoculum from the selected bacterial strain was carried out in 250 ml Erlen meyer flask. Growth medium was composing of glucose, 1% (w/v); soybean meal, 1% (w/v); K_2HPO_4, 0.5% (w/v); $MgSO_4.7H_2O$, 0.05% (w/v); NaCl, 0.05% (w/v) and $CaCl_2.2H_2O$, 0.05% (w/v). Cell-free supernatant was used as crude enzyme solution.

Variable	Symbol coded	Range and level	
		-1	+1
Nitrogen source (soybean flour) (%w/v)	X_1	0.75	1.25
Carbon source (Corn flour) (%w/v)	X_2	0.75	1.25
Co-Nitrogen source (Yeast extract) (% w/v)	X_3	0.5	1.0
pH	X_4	8.0	10.0

Table 1: Experimental range and levels of the independent variables

Protease assay

The selected isolate was inoculated in 50 ml of protease specific medium broth containing (g/L) glucose, 5.0; peptone, 7.5; $MgSO_4.7H_2O$, 5.0; KH_2PO_4, 5.0; and $FeSO_4.7H_2O$, 0. 1, pH-7.0 and were cultured in a rotary shaker (180 rpm) at 30°C for 3 days. After 48 hours of fermentation, the whole fermenting broth was centrifuged at 10,000 × g at 4°C, and the clear supernatant was recovered. This Cell Free Supernatant (CFS) was taken as crude enzyme solution. The proteolytic activity was determined using the modified method of Drouin [10]. In this method, casein is used as substrate. The hydrolysis of casein releases tyrosine which is quantified with the aid of Folin-Ciocalteu reagent and sodium carbonate (20% w/v). 0.5 mL of casein solution was added to 4.5 mL of 0.1 M Tris HCL buffer, pH 9.0. Then 50 µL of the diluted enzyme solution was mixed with the substrate at pH 9.0 and incubated at 40°C for 30 min. The enzymatic reaction was stopped by the addition of 0.5 mL of 10% Trichloroacetic acid solution. The mixture was incubated at 37°C to allow the precipitation of non-hydrolyzed casein and the filtrate with the aid of Whatman paper No 1(Whatman International Ltd). Then 0.2 Folin-Ciocalteu reagents were added and the absorbance was measured at 578 nm using a Rayleigh Spectrophotometer. The unit of protease activity (U) was defined as the amount of enzyme that hydrolyses casein to give a staining corresponding to 1µmole of tyrosine per min at pH 8.2 and 37°C.

Protein assay

Total protein contents were determined according to the method of Lowry et al. [11], using bovine serum albumin as a standard.

Enzyme characterisation

Partial purification of the enzyme: Ammonium sulphate a different saturation level (40-80%) was added to the cell free supernatant to precipitate the protease. After each addition, the enzyme solution was stirred for 1 h at 4°C. The protein precipitated was collected by centrifugation at 12,000 × g for 20 min at 4°C and resuspended in minimum volume of 0.05 M Tris-HCl buffer, pH 8.0 to obtain the concentrated enzyme solution. The enzyme solution was dialyzed against the same buffer with 4-6 changes.

The concentrated enzyme solution was purified on sephadex G-75 (Pharmacia) column (1.5 cm × 30 cm) (Biologic LP, Bio-Rad, USA). The column was equilibrated with 0.05 M Tris-HCl buffer of pH 8.0. The dialyzed enzyme sample was loaded onto a Sephadex G-75 column and then eluted with the same buffer. Fractions of 4 ml were collected at a flow rate of 30 ml/h by fraction collector (Model 2110, Bio-Rad). Protein concentration was estimated in each fraction by the method of Lowry et al. [11] and the protease activity was also quantified as described above. The active fractions were freeze dried and stored at -20°C for further studies.

Effect of temperature on enzyme activity and stability

The effect of temperature on purified protease activity was determined by incubating reaction mixtures at different temperatures ranging from 30 to 80°C by using 1% (w/v) casein solution. Thermophily and thermostability was examined by incubating purified enzyme at different temperatures (30-80°C) for 30 min in a water bath (Eyela, Japan). Then the remaining activities were then measured as described above.

Effect of pH on enzyme activity

For the study of the effect of pH on protease activity, different buffers were prepared at concentration of 0.05 M: phosphate (pH 6-7), tris–HCl (pH 8-9) and glycine-NaOH (pH 10-12). The activity of protease was measured at pH values ranging from 6 to 12. The enzyme solution was pre-incubated at these various pH values at 30°C for 30 min and then the activities were measured as described above using 1% (w/v) casein solution as the substrate.

Effect of inhibitors and metal ions on protease activity

The effect of some metal ions (Ca^{2+}, Zn^{2+}, Pb^{2+}), ethylene diamine tetra acetic acid (EDTA) and Sodium Dodecyl Sulphate (SDS) (each at 5 mM) were tested. The purified enzyme solution was pre-incubated with the above effectors for 30 min at 80°C. Then the activity was measured as describe above.

Results and Discussion

Protease producing bacteria strain

Among the bacteria isolated from the soils, 86 isolates presented a clear halo on the isolation medium. The diameter of the hydrolysis halo were range between 2-28 mm. Six isolates showed important halo comprised between 10-19 mm. Table 2 shows the hydrolysis halo of the isolates that showed important protease activity. The isolate N235 presented the highest proteolytic activity and was selected for further characterizations. The proteolytic activity of this isolate is shown on Figure 1. Phenotypic characterization of this isolate showed that N235 is a strain of *Bacillus* sp. The characteristics are summarized in Table 3.

Kinetic of growth and enzyme production

Microbial growth kinetic shows a lag phase during the first 5 hours, followed by a start-up phase of about 10 hours. The microbial population during this period shows a slight growth. The exponential phase begins from the 15th hour and lasts until the 40th hour. This phase is quite long; but advantageous in industrial scale production were bacteria maximize production. It may also be noted during this phase a plateau between the 20th and the 35th time; this allows us to think that the bacterium has diauxic phenomenon in its growth between 10 to 15 hours. The specific growth rate found was lower than that obtained by Drouin [10], which was 0.42 for *Bacillus licheniformis*.

Town	Isolate number	Hydrolysis Halo (mm)
Ngaoundere	N131	18.5
	N132	13
	N233	16.5
Garoua	N235	24.0
	G124	19.5
	G332	10.5

Table 2: Proteases overproducing strains and their diameter of hydrolysis on skim milk agar

Characteristics	Observation
Aspect of colony on agar plate	White circular colony
Microscopic features	Rod shaped
Gram staining	+
Catalase	+
Acidification of glucose	+
Oxidase	+
Indole test	+

Table 3: Phenotypic characteristic of the selected proteolytic bacterium (*Bacillus* sp N235)

Figure 1: Plate assay for proteolytic activity, isolate N235 exhibited the highest proteolytic activity

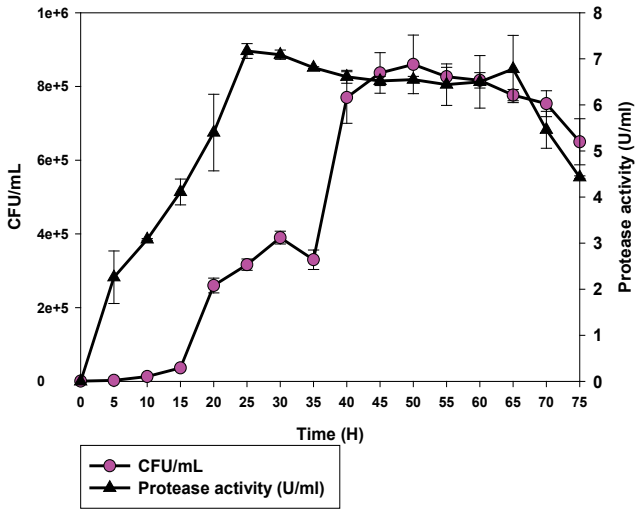

Figure 2: Time course of growth and protease production by *Bacillus* sp N235. The data shown are averages of triplicate assays. The error bars indicate the standard deviation (SD) within 10% of mean value.

The enzyme production curve (Figure 2) over time, shows that it is increasing to a maximum of 7.17 U/mL after 25 hours of fermentation. It then tends to decrease slightly to a value of 4.43 U/mL after 75 hours of experience. The production curve has the same profile as the growth of the microbial population curve, but considering that the maximum production was recorded in 25 hours, not 45 hours (maximum recorded for growth) and it then remained more or less constant, it can be concluded that the production of this enzyme follows the mixed metabolite production law (associated and non-associated microbial population growth) [12,13]. This profile can differ depending on culture conditions and substrates selected for the cultivation of the bacteria. But it nevertheless depends much more on genetic characteristics of the bacterial species selected [14].

The effect of different components of the culture medium on enzyme production was studied using a factorial design analysed by Design Expert 8.03 (State-Eease, Mineapolis, USA). The results obtained are shown in Table 4. The equation predicting the enzyme production as function of the various factors (parameters) studied is given by:

$$Y = 7.69 + 1.066X_1 - 0.738X_2 + 0.82X_3 + 2.19X_4 + 0.618X_1X_2 + 0.98X_1X_3 + 101.29X_1X_4 + 1.194X_2X_3 - 0.916X_1X_4 - 0.718X_3X_4$$

	Experimental parameters				Protease activity (U/mL)	
Run no	X_1(Soy bean)	X_2(Corn)	X_3	X_4(pH)	Observed	Predicted
1	-1	-1	-1	-1	4.295	5.61
2	+1	-1	-1	-1	3.78	4.34
3	-1	+1	-1	-1	3.855	2.34
4	+1	+1	-1	-1	3.91	7.09
5	-1	-1	+1	+1	5.57	4.34
6	+1	-1	+1	-1	7.635	6.99
7	-1	+1	+1	-1	4.41	5.85
8	+1	+1	+1	-1	10.52	10.97
9	-1	-1	-1	+1	14.105	13.06
10	+1	-1	-1	+1	13.02	12.19

Table 4: Experimental design showing observed and predicted protease activity

The Analysis of Variance (ANOVA) of this model (Table 5) showed that R^2 is 91.83% which means that the model could explain 91.83 % of the variability in protease production by the selected strain (*Bacillus* sp N235). The Model F-value of 23.62 implies that the model used for the production of protease by *Bacillus* sp N235 is significant. Values of "Prob>F" less than 0.0500 indicate that model terms are significant. X_1, X_4, X_1X_3, and X_2X_3 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 799.27 implies that the Lack of Fit is significant relative to the pure error. There is a 0.01% chance that a "Lack of Fit F-value" could occur due to noise. The R^2 value (multiple correlation coefficient) closer to 1 indicates better correlation between the observed and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by a high value of CV. In the present case a moderate CV of 16.08 shows that the experiments performed are reliable. The P-value denotes the significance of the coefficients and is also important in understanding the pattern of the mutual interactions between the variables.

The Pareto analysis (Figure 3) helps us to confirm the most significant factors for Protease production used in our study. Pareto analysis is a formal technique useful where many factors are competing for a process. This technique helps to identify the top portion of causes that need to be addressed to resolve the majority of problems. Pareto chart enables of identification of factors that produce positive or negative effect on protease production by *Bacillus* sp N235. The maximal effects are given in descending order from left to right. A factor that had a positive effect on the protease production is depicted as an orange bar, while a negative effect is given as a blue bar. The horizontal red line, which is located at the higher level, represents the Boneferroni limit whereas the black horizontal line represents the t-value limit. The Boneferroni limit and t-value limit are statistically based acceptance limits (similar to confidence level intervals) for each bar in Pareto chart [15,16].

The effect of each parameter on enzyme production and the interaction between parameters were determined and represented in Pareto chart (Figure 3). From this analysis; only corn flour showed a negative effect on protease production. This result was similar to that of Mabrouk et al. [17] who demonstrated that corn inhibits protease activity. The corn flour was not significant for the model used in this work (Figure 3). However when corn flour was combined with the yeast extract, the protease production increased. This could be explained by the fact that yeast extract plays the role of nitrogen source for protease synthesis. The combination of soybean flour and the yeast extract was the best substrate- combination for protease production by *Bacillus* sp N235. When the soybean flour and yeast extract were combined together in the fermenting medium, the protease production reached 14 U/mL, protease production increased by 1.8 fold compared to the production obtained in the original medium. This result is in accordance to that obtained by Chauchan et al. [18] during the production of protease by *Bacillus* sp RGR-14. Other authors such as Oskouie et al. [19] and Haddar et al. (2010) [20] reported values for protease production lower than that observed in this study.

Partial purification of the enzyme

The different stages of enzymes purification are shown in Table 6. The specific activity increases progressively and as purification progresses, until reaching a 1516.7 U/mg specific activity after purification step by gel filtration on Sephadex G-75. This high value of the final specific activity is explained by the fact that compared to the initial amount of protein, the amount of proteins present in the pure fraction is very low, a large amount of protein having no activity proteolytic were eliminated during the purification. The important difference between the volume of the crude extract of beginning and the pure fraction of the end is also responsible for this shift in part. The large quantity of proteins present in the crude extract therefore consisted of soluble proteins in the ammonium sulfate to 70% saturation and also a size of between 3000 and 80000 Da. The purification yield was 37.63%, which indicates that some proteases have been eliminated during the purification with other proteins, or a part of protease is complexed with other proteins removed during purification. But despite this, the yield is higher than that obtained by Yang et al. [21]. It is also virtually equal to that obtained by Manachini et al. [22] (38%) whose work yet used in addition, the ion exchange chromatography method.

For gel chromatography on Sephadex G-75, the elution pattern is shown in Figure 4, peak of maximum proteolytic activity were observed in the 9th fraction, therefore fraction and the 8th to 10th fractions were collected as containing pure protease. It is also found that the amount of proteins corresponding to these peaks is not very high with up to 22.86 micrograms corresponding to the 10th fraction, while the maximum amount of protein is observed in the 53rd fraction 64, 07 micrograms.

The relative molecular weight of the enzyme

The relative molecular weight of the protease was calculated using

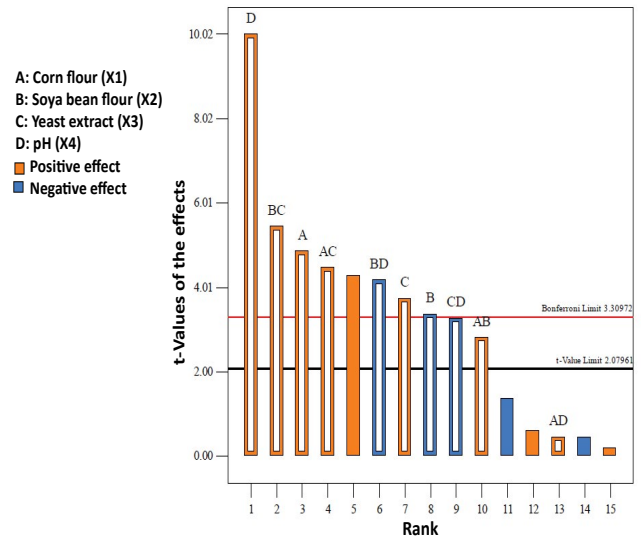

Figure 3: Pareto analysis for protease production by *Bacillus* sp. N235. BC, AC, BD, CD, AB and AD represent the interactions among factors.

Source	Sum of square	df	Mean square	F-value	P-value
Model	0.90	100	0.090	23.62	<0.000 Significant
A-Soya bean flour (X_1)	0,091	1	0,091	23,75	<0.0001
B-Corn flour (X_2)	0.044	1	0.044	11.42	0.0028
C-Yeast extract (X_3)	0.054	1	0.054	14.42	0.0028
D-pH	0.38	1	0.090	23.62	<0.0001
AB	0.031	1	0.031	8.01	0.0100
AC	0.077	1	0.077	20.09	0.0002
AD	0.0004	1	0.0004	0.21	0.6478
BC	0.11	1	0.11	29.84	<0.0001
BD	0.067	1	0.067	17.55	0.0004
CD	0.041	1	0.041	10.79	0.0035
Residual	0.080	21	0.0038		
Lack of Fit	0.080	5	0.016	799.27	<0.0001significant
Pur error	0.0003	16	0.00005		
Cor Total	0.98	31			

R2= 91.83%, CV= 16.08

Table 5: Analysis of Variance (ANOVA) of the model used for the production of protease by *Bacillus* sp N235

Fractions	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
F_0 (Supernatant)	100	1.9	6.4	190	642.8	0.3	100	1
F_1 (Precipitated and dialyzed fraction)	10	8.3	0.3	83.7	3.0	27.8	44.0	94.3
F_2 (chromatographed fraction)	3	23.8	0.01	71.5	0.04	1516.7	37.6	5131.6

Table 6: Stages of enzyme purification

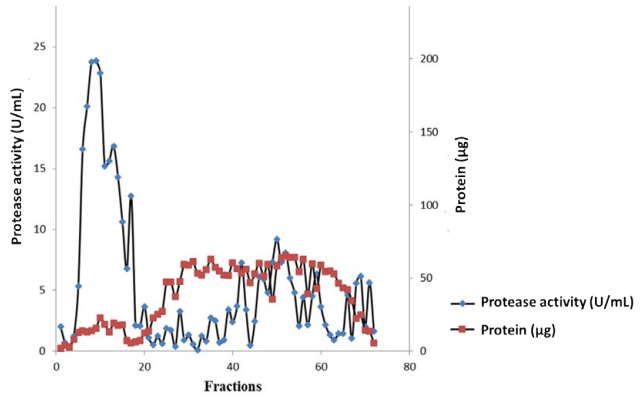

Figure 4: Sephadex G75 elution pattern.

the elution volume of the active fraction and the molecular makers. From Figure 5, the relative molecular weight of the protease is 180 478.7548 Da, compared to the molecular weight blue dextran (200,000 Da), one can see that they are close. This indicates that the protease is a high molecular weight protein complex and that the use of a Sephadex gel whose pore size is larger (G-100 or G-200 for a resolution of up to 300,000 Da) will make a separation for better resolution.

Effect of temperature and pH on enzyme activity and stability

After studying the effect of temperature on the protease, the curve of Figure 6 has been established. It can be seen that after exposure of the protease at temperatures ranging from 20°C to 80°C for 30 min at pH 9, the optimum temperature for proteolytic activity was 80°C, and furthermore the enzyme was stable at this temperature. This shows that the alkaline protease from the selected bacterium *Bacillus* sp N235 is thermophile and thermostable. These findings are closed to those of Rahman et al. [23], whose work has focused on the bacterium of the genus *Bacillus stearothermophilus* F1, the optimal temperature was 85°C, with a similar profile to the effect of temperature on its protease. The presence of Ca^{2+} in the medium may explain the protease stability at such temperatures as shown by Rahman et al. [23] and Haddar et al. [20]. They reported that the presence of Ca^{2+} 2 mM and 5 mM respectively in the reaction mixture contributed to stabilize the protease produced by *Bacillus stearothermophilus* F1 and *Bacillus mojavensis* A21 respectively.

The study of the effect of pH showed that enzyme exhibited maximal pH between 11 and 12 (Figure 6). This high value pH joined the work done by Haddar et al. [20] on alkaline protease from *Bacillus mojavensis* A21 which showed an optimum activity at pH 11. This optimum pH is higher than that found by Rai et al. [24] for the alkaline protease *Bacillus lichniformis* NH1. It was also noted that the presence of Ca^{2+} helps in stabilizing the protease activity as demonstrated by Rahman et al. [23].

Effect of some metal ions, EDTA and SDS on enzyme activity

The study of the effect of some metal ions, EDTA, SDS is shown in Figure 7. Zn^{2+} ions showed the highest effect on the activity of protease, or 12.5298 U/ml, followed very closely the effect of EDTA and the effect of Ca^{2+} ions, the effects were respectively 12.2768 U/ml and 12.5049 U/ml. The SDS is found with an activity worth 7.4030 U/ml. The presence of Ca^{2+} as second stabilizer effector confirms its status as demonstrated in the work of Rahman et al. [23]. The effect of ions Zn^{2+} and Ca^{2+} ions on protease can reveal that belong to the class of metallo proteases, including Zn^{2+} ions are necessary for the activity and Ca^{2+} for stabilizer [9]. This again provides clarification on the likely membership of the bacterial strain producing protease, indeed metalloproteinases are

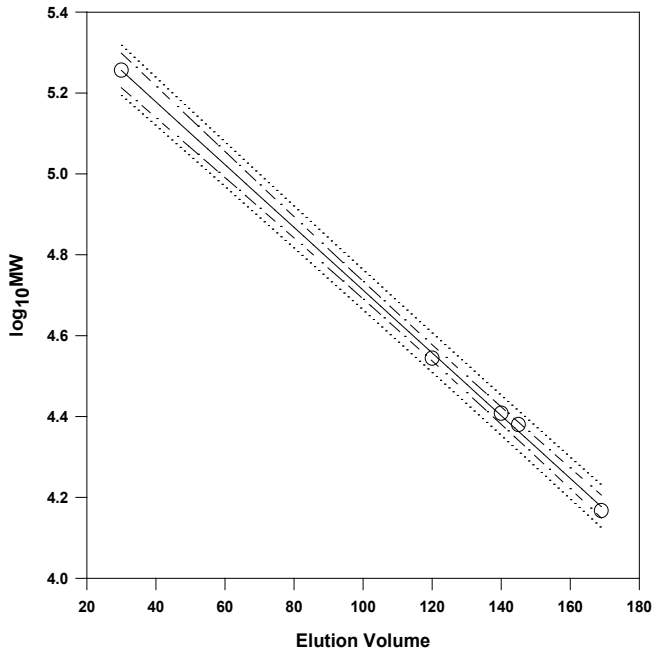

Figure 5: Determination of Molecular weight of protease produced by *Bacillus* sp N235.

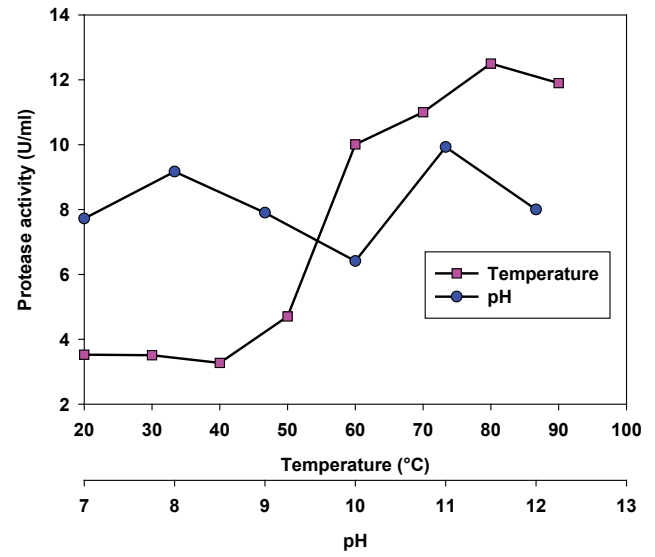

Figure 6: Effect of the temperature and pH on enzyme activity.

generally produced by bacteria of the genus *Bacillus,* such as *B. subtilis*, *B. cereus*, *B. stearothermophilus* [10]. The protease was found to be stable in the presence of EDTA, H_2O_2 and SDS, as its activity is not too affected by their presence in the environment; this gives the possibility to be incorporated into a detergent.

Conclusion

The selected isolate *Bacillus* sp N235 tested in this study showed very great potential to produce an extracellular thermophile alkaline protease (active at high temperature and in alkaline pH). This soil isolate is a confirmation that soil is an eco-niche for potential industrial

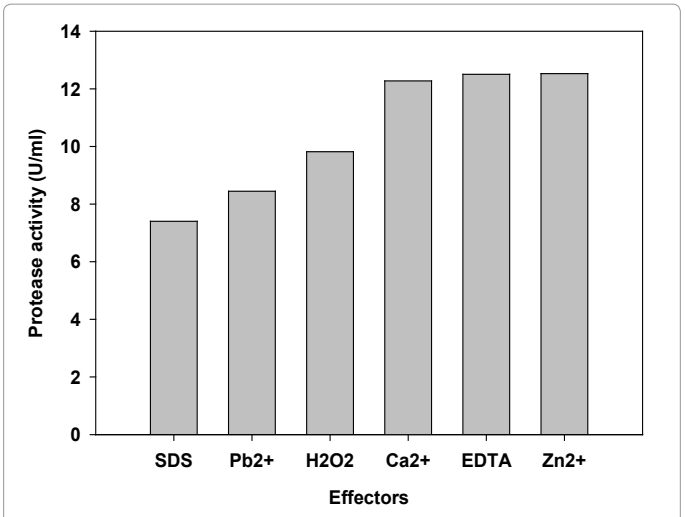

Figure 7: The effect of some ions, EDTA and SDS on protease production by *Bacillus* sp.

microorganisms. Furthermore this strain was isolated in a region where the climate presents periodically very high temperature within the day. The soils of such area are sources of thermophilic microorganisms producing thermophile and heat stable enzymes. The protease produced by *Bacillus* sp N235 could be used in many industrial processing such as the making of detergents.

Acknowledgment

The authors would like to acknowledge the Laboratory of Microbiology of the National School of Agro-Industrial Science of the University of Ngaoundere, Cameroon for providing facilities for research.

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Citation: Tavea F, Fossi BT, Fabrice NT, Ngoune LT, Ndjouenkeu R (2016) Production and Partial Characterization of an Extracellular Thermophile Alkaline Protease from a Selected Strain of *Bacillus* sp Isolated from Abattoir Soil in the North Region of Cameroon. J Bioprocess Biotech 6: 279. doi:10.4172/2155-9821.1000279