

Production of Thermostable Alkaline Protease from *Streptomyces* sp-A5⁴

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Abstract

Bacterial extracellular alkaline proteases have been found to have broad spectrum industrial applications because of their stability characteristics among the bacteria. The Actinomycetes are of enormous importance as they can be recovered easier than other bacteria after fermentation. Thus, the study was aimed at sourcing for potential protease producers among the Actinomycetes species and determining the cultural conditions for their optimal protease yields. Among 67% of the recovered soil isolates that demonstrated potentials for protease production on skimmed milk agar, only *Nocardia* sp-A2², *Micromonospora* sp-A2³, *Streptosporangium* A-5², and *Streptomyces* sp-A5⁴ produced protease with higher activity (18.5-20.5mm) than the others (8.5-16.5mm). Preliminary protease assay in submerged shake-flask fermentation confirmed *Streptomyces* sp-A5⁴ as the highest producer. Further studies on protease production by *Streptomyces* sp-A5⁴ showed that maximum protease yield was achieved within 72h at 0.4% inoculum concentration. All the test organic carbon and nitrogen substrates supported protease yield ($\geq 1.5/2.0$ u/ml) but soybean and sweet potato meal gave the highest support ($\geq 2.5/2.53$ u/ml). However, their protease yields were significantly ($p < 0.05$) less than that of refined glucose and peptone when substituted as the carbon and nitrogen sources. The produced crude enzyme demonstrated optimal activity at pH 8.0 and temperature of 50°C. Therefore, the ability of *Streptomyces* sp-A5⁴ to produce thermostable protease at a relatively low concentration indicated its potential as a good source for industrial applications.

Keywords-*Streptomyces*-spA5⁴, Protease-Fermentation, Optimization, Cultural Condition.

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Introduction

Proteases are enzymes that catalyze the hydrolysis of protein into amino-acid. Proteases, especially alkaline type constitute about 60-65% of the global industrial enzyme market (Gupta et al., 2010). Among different proteases, alkaline proteases produced by microorganisms especially Actinomycetes are of main interest from a biotechnological perspective due to their biochemical diversity and susceptibility to genetic manipulation (Giarrhizo et al., 2007). Their applications in several industrial sectors include food, tannery, pharmaceutical, meat tenderization, baking and brewing, peptide synthesis, medical diagnosis, infant formulae and detergent preparation. (Gupta et al., 2002).

Microbial alkaline proteases are produced mostly from *Streptomyces* spp and

Bacillus spp (George-Okafor and Odibo, 2011). Actinomycetes particularly *Streptomyces* are known to secrete multiple (greater) proteases in culture medium which is generally regarded as safe with food and drug administration (Puri et al., 2002). Actinomycetes are of enormous importance since they possess a capacity to produce and secrete a variety of extracellular hydrolytic enzymes (Tan et al., 2009). *Streptomyces* spp., that have been reported to produce proteases include *S. claviligerus*, *S. griseus*, *S. rimonus*, *S. thermoviolaceus* and *S. thermovulgaris* (Mukesh, 2014).

However, it was well documented that the differences in production character of each microbial strain mainly depend on fermentation, nutritional requirement, physiological and genetic nature (Gupta et al.,

2010). Since the composition of fermentation media plays an important role in the production of primary and secondary metabolites, designing an appropriate fermentation medium is of critical importance because medium composition, product concentration, yield and volumetric production

Materials and methods

Materials: The dehydrated Nutrient agar and broth (Oxoid) produced by LAB^M company were purchased from Nnaberg chemical shop located at Ogbete main market, but the skimmed milk agar and mineral salt medium were prepared according to specifications by George-Okafor et al. (2013).

Sample Collection and Isolation of Organism for Screening: Soil samples within 2cm depth were randomly collected with the help of pointed iron rod from different locations in Gariki-Awkunanaw within Enugu metropolis Nigeria. The samples were serially (10^{-1} - 10^{-5}) diluted and 0.1ml of each sample was plated on Nutrient agar and on Starch-casein agar containing Amphotericin B, for isolation of pure cultures. The isolated Actinomycetes strains were further identified and characterized.

Identification of the Isolates: The isolated actinomycetes strains were identified based on morphological and biochemical characteristics (Tasie and George-Okafor, 1999) and were compared with the standard characteristics described by Sivar-kumar (2001).

Screening for Proteolytic Activity: The identified actinomycetes were plated onto skim milk agar plates and incubated at room temperature for 24-48h. A developed clear zone of hydrolysis on the skim milk agar around the colonies gave an indication of protease production. Four actinomycetes isolates *Streptomyces* sp-A5⁴, *Streptosporangium* sp-A5², *Micromonospora* sp-A2³ and *Nocardiasp*-A2² were selected for further studies.

Protease Production: Initial (72 h) shake flask fermentation (200 rpm) at room temperature was carried out with 1.6% inoculum in a fermentation medium containing (w/v): glucose (1%), yeast extract (0.05%), peptone (1%), K₂PO₄ (0.1%), NaCl₂ (0.05%), FeSO₄ (0.002%) and MgSO₄.7H₂O (0.02%). The fermentation broth was centrifuged at 1000revmin⁻¹ at 4°C for 30min and the recovered clear supernatant used as crude enzyme was concentrated to 10% for protease assay.

influence the product productivity (Giarrhizo et al., 2007). Hence, the study was aimed at selecting an Actinomycetes isolate with protease production potential and optimizing different fermentation parameters for obtaining higher protease yield.

Determination of Protease Activity:

The protease activity was determined by incubating equal volumes of the enzyme and 1% buffered casein (pH7.2) at 40°C for 30min. The buffered casein was prepared with 0.2M phosphate buffer. The reaction was stopped by addition of 2ml of 5% Trichloroacetic acid (TCA) with shaking. The reaction mixture was centrifuged, filtered with No 1 filter paper and the supernatant measured for protease activity (George-Okafor et al., 2013). The isolate (*Streptomyces* sp-A5⁴) which demonstrated the highest proteolytic activity was selected for further experiments. One unit (U) of the protease activity was defined as the amount of protease that released 1µg of tyrosine from casein per min under the applied condition.

Effect of Carbon Sources on Protease Production: The effect of different carbon sources on protease production was examined. Glucose (1%) in the mineral salt medium was substituted with 1% of potatoe, cassava, yam, corn and rice (powder) respectively. Thereafter, the fermentation was initiated and proteolytic activity determined as earlier described.

Effect of Nitrogen Sources on Protease Production: To test the effect of different organic nitrogen sources on protease production, peptone (1%) in the basal mineral salt medium was substituted with 1% (w/v) of various organic nitrogen sources: urea, bambara meal, groundnut cake, soybean meal and cowpea meal. Fermentation was conducted for 72 h at room temperature for protease production.

Effect of Inoculum Concentration and Fermentation Period on Protease Production: Varying concentrations of inoculum for optimum enzyme yield were determined by fermenting with inoculums ranging from 0.2to4.8%. Similarly, optimal production period for protease yield was assayed. The fermentation with *Streptomyces* sp-A5⁴ culture was conducted for 24-120h at room temperature. Samples were pooled aseptically every 24h and the protease yield determined as earlier described.

Enzyme characterization

pH Profile: The effect of pH on protease activity from *Streptomyces* sp-A5⁴ was determined by subjecting the crude enzyme to different ranges of pH (3-10). The pH (3-6) was prepared using citrate buffer, phosphate buffer for pH (6-8) and phosphate/sodium hydroxide for pH (9-10). Thereafter, equal volumes of crude enzyme and soluble casein were reacted and assayed as earlier described.

Effect of Temperature on Protease Activity: This was determined by subjecting

the reaction mixture (1ml of crude enzyme and 1ml of substrate) at different temperatures (30-80°C) for 30min in a water-bath. Finally, protease activity was measured as earlier described.

Statistical Analysis: The relative activity of each variable was expressed in 100%. Then, the software application (Statistical Package for Social Sciences (SPSS), Version 16.0, was utilized to analyze the data using paired t-test. A calculated p-value 0.05 was considered to be statistically significant.

Results and Discussion

Isolation and Screening for Protease Producing Organisms: Only 10 isolates were able to produce protease (table 1). This number represented 45.5% of the total isolates recovered from the sampled soils. *Streptomyces* sp-A5⁴ was found to have the highest potential for protease production (20.5mm). The result was similar to proteolytic activity (26.5mm) obtained from *Streptomyces pulveraceus* MTCC8374 but varied from protease production (45.5 mm) of *Bacillus licheniformis* (Jaysaree et al., 2009 and Folasade et al., 2005).

Preliminary Identification of the Isolate: The identified isolates were gram positive organisms with slightly different morphological features based on colonial appearances. The whitish colored isolate with highest proteolytic activity was identified as *Streptomyces* sp-A5⁴ (table 2). The identification was in agreement with the findings of Mukesh (2014), who identified

Table 1: Protease Potentials of the Actinomycetes Isolates

Recovered Isolates	Protease Potentials [zone of hydrolysis (mm)]
A1 ¹	16.5
A2 ²	19.0
A2 ³	19.0
A3 ³	12.0
A4 ²	8.5
A5 ¹	15.5
A5 ²	18.5
A5 ³	14.0
A5 ⁴	20.5
A6 ²	9.0

actinomycetes isolates as filamentous gram positive bacteria with different colonial appearances using morphological and biochemical methods. Jaysaree et al. (2009), had earlier reported the ability of the actinomycetes species mostly *Streptomyces* to produce mycelia with whitish colored colony and exhibit good hydrolytic properties.

Table 2: Identification of Actinomycetes Isolates

IC	GR	CA	CT	CU	SU	CAU	MR	GH	UA	GF	NR	VP	Probable-identity
A5 ⁴	+ Filamentous rod	Mycelia-whitish in colour	+	+	+	+	-	+	-	+	-	-	Streptomyces sp
A2 ²	+ Filamentous rod	Mycelia with rough edges and whitish Mycelia, creamy in colour	+	+	-	+	-	+	-	+	-	+	Nocardia sp
A2 ³	+ Filamentous rod	Mycelia, with rough peripheral edges elevated at the middle with-smooth peripheral edges	+	+	+	+	-	+	+	+	-	-	Micromonospora – sp
A5 ²	+ Filamentous rod	Mycelia, with-smooth peripheral edges	+	+	+	-	+	-	-	+	-	-	Streptosporangium -Sp

Legend: IC= Isolate Code, GR= Gram Reaction, CA= Colonial Appearance, CT= Catalase, CU= Citrate Utilization, SU= Starch Utilization, CAU= Casein Utilization, MR=Methyl-Red, GH= Gelatin hydrolysis, UA= Urease Activity, GF=Glucose fermentation, NR=Nitrate Reduction, VP=Vogues Proskauer.

Effect of Carbon and Nitrogen Sources: Among the assayed local carbon substrates, sweet potato supported the highest protease yield (2.50 u/ml) although the yield was lower than that of glucose (figure 1). The preference could be explained on the ground that glucose can be easily assimilated by *Streptomyces* sp-A5⁴ compared to other substrates. Many researchers had reported the increased yield of enzyme from refined carbon sources such as lactose, maltose and sucrose (Gupta et al., 2002, Gupta et al., 2010, Rathnaka and Chandrika, 1993). On the other hand, wheat bran, starch and rice bran were reported best for optimal production of alkaline protease by various *Bacillus* species (Naidu and Devi, 2005; Asokan and Jayanthi, 2010).

Higher yields of protease activity were observed with peptone (2.56u/ml) and soybean cake (2.50u/ml). The earlier reports by Gupta et al. (2010) also revealed the positive effect of soybean in protease production by *Bacillus* spp. However, many reports revealed the utilization of both organic and inorganic nitrogen sources by various microorganisms. For instance, Puri et al. (2002) reported that peptone, casein, skimmed milk and yeast extract favoured maximum protease production in *Streptomyces* spp, whereas Sangeetha et al. (2008) and El-Safety and Abdul-Raouf, (2004) reported maximum protease production by *Bacillus* sp with (NH₄)₂SO₄ as the nitrogen source.

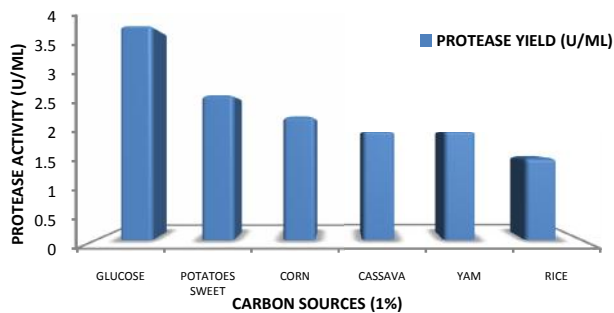


Figure 1: Influence of Different Carbon Sources on Protease Production by *Streptomyces* sp-A5⁴

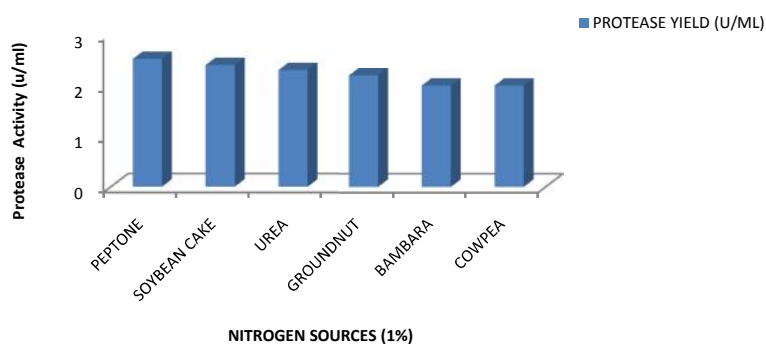


Figure 2. Influence of Different Nitrogen Sources on Protease Production by *Streptomyces* sp- A5⁴

Inoculum Concentration: Maximum protease activity (2.20u/ml) was observed at 0.4% inoculum (Figure 3). There was decrease in protease yield (0.75u/ml), when inoculum size was reduced to 0.2%. This may be attributed to lower inoculum for the fermentation. On the other hand, higher inoculum size with reduced protease yield can

be attributed to reduced dissolved oxygen and increased competition towards nutrients (Saadoun et al., 2007). The observed result differs from reports of Jayasree et al. (2009) and Gupta et al.(2010) where 3% of *Streptomyces pulverus* and 2% of *Bacillus* spp gave optimal enzyme production.

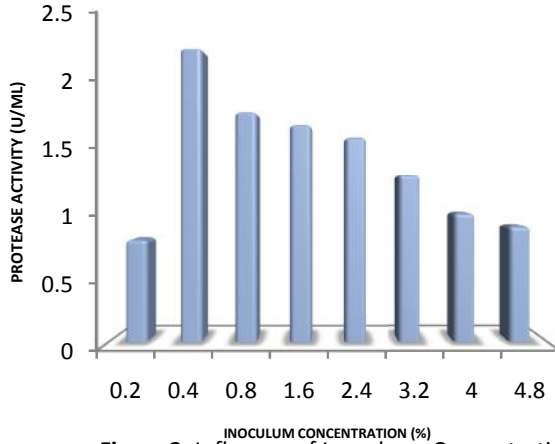


Figure 3: Influence of Inoculum Concentration on Protease Production by *Streptomyces* sp- A5⁴

Time Profile for *Streptomyces* sp-A5⁴:
 The protease production of *Streptomyces* sp-A5⁴ started increasing from 24h and reached maximum level after 72h of cultivation (Table 3). The protease production was observed to be growth related and directly proportional to the biomass yield as it occurred during active biomass production. This result is comparable to that of Petinate et al. (1999) and Yang and Wang (1999) who reported increase in protease production by *Streptomyces cyanens* and *S. rimosus* during 72h log phase.

Table 3: Time Profile for Protease Production.

Fermentation Period (h)	Protease Activity (U/ml)	Biomass Concentration (g/ml)
24	0.20 ±0.01	2.00 ± 0.10
48	0.24 ±0.01	2.10 ±0.10
72	0.27 ±0.02	2.30 ±0.16
96	0.19 ±0.01	1.36 ±0.03
120	0.17 ±0.01	1.25 ±0.01

pH Activity Profile: The optimum enzyme activity was at pH 8.0 (figure 4). The pH activity profile is the same with protease of *Bacillus licheniformis* which also demonstrated

maximum activity at pH 8.0 (Asokan and Jayanthi, 2010) and closely resembles protease of *Streptomyces pulveraceus* MTCC8374 which showed maximum protease activity at pH 9.0 (Folasade et al., 2005).

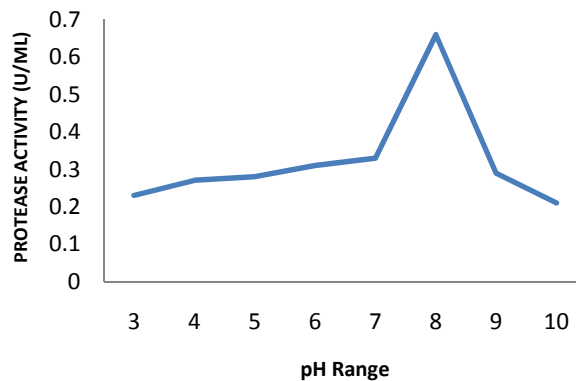


Figure 4: pH Profile for the Crude Enzyme Activity

Temperature Profile Activity: The protease demonstrated optimum activity at 50°C (figure 5). The optimal activity was higher than protease from *Streptomyces pulveraceus* MTCC8374 which demonstrated optimal activity at 33°C but slightly lower than

protease from *Bacillus* sp.N-40 and *Bacillus macerans* IKBM-11 which had their maximum protease activity at 65°C and 55°C respectively (Folasade et al., 2005; Nihan and Elif, 2011). However various actinomycetes isolates from

soil were reported to produce proteases at different temperatures (Sharmin et al., 2005).

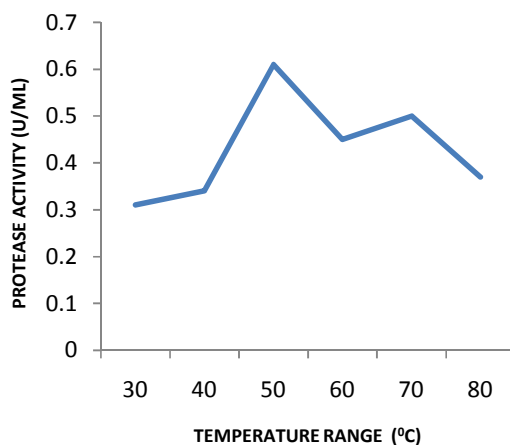


Figure 5: Temperature Profile of the Crude Enzyme Activity

Conclusion

The isolated *Streptomyces* sp-A5⁴ was able to produce thermostable alkaline protease from local substrates with relatively minimal inoculum concentration. This gave a strong indication of its suitability for industrial utilization.

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