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SOME PHYSICOCHEMICAL PROPERTIES OF ACID PROTEASE PRODUCED DURING GROWTH OF ASPERGILLUS NIGER (NRRL 1785)

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ABSTRACT

The growth of *Aspergillus niger* (NRRL 1785) was investigated and monitored over a five-day fermentation period. Acid protease synthesis by this fungus was also investigated during the period. The effect of growth of *Aspergillus niger* on acid protease synthesis was determined. Some of the physicochemical properties of the acid protease were examined. The growth curve obtained for *Aspergillus niger* revealed four phases of growth which are the lag phase, the exponential phase, the deceleration phase and the death phase. Acid protease synthesis by *Aspergillus niger* increased in direct proportion to growth up to the early hours of the death phase. Maximum enzyme synthesis was obtained at the 96th hour of fermentation, which was 12 hours into the death phase. The acid protease synthesized had optimum pH of 4.0, while the enzyme was stable (60% relative activity and above) over a wide range of pH 2.5 - 5.5. The optimum temperature for acid protease was determined to be 50°C. The enzyme was also stable up to this temperature (50°C) at pH 4.0 for 1 hour. Calcium ions had activating effect on the acid protease while sodium and mercury ions had inhibitory effects. The results obtained in this paper show a direct correlation between the growth of *Aspergillus niger* and acid protease synthesis, hence the particular phase of growth of *Aspergillus niger* determines the extent of enzyme yield by the fungus.

KEYWORDS: Proteolytic enzyme; Acid protease; microbes; *Aspergillus niger*

INTRODUCTION

Proteases are proteolytic enzymes derived from a wide range of sources and have widely differing pH and temperature optima and specificities (Kalisz, 1988). They are one of the most important groups of enzymes constituting two thirds of the total industrial enzymes marketed (Singh et al., 1994). Acid proteases have been isolated and characterized from mammals, plants, fungi, yeast, retrovirus and bacteria (Wu and Hang, 1998). Acid proteases play an important role in meat tenderisation and in the production of fermented foods by molds from soybean, rice and other cereals (Nout and Rombouts, 1990; Gerhartz, 1990).

Microbial acid protease are usually preferred to enzymes from plant and animal sources due to the rapid growth rate of microorganisms, large quantity of enzymes produced, the ease of purification and the potential of microbes to be engineered towards acquiring novel characteristics. Many factors are known to affect the production of the enzyme in a culture brew. Specifically, it has been reported that the biosynthesis or production of the proteases is often correlated with a particular phase of the microbial culture (Hagspiel *et al*, 1989). It has been reported that enzymes from different strains of microbes belonging to the same species do differ in their biochemical characteristics (Mchahon et. al., 1999).

In this paper, the growth of *Aspergillus niger* (NRRL 1785) was studied and some physicochemical properties of the acid protease produced during the growth were determined.

MATERIALS

Microorganism

Aspergillus niger (NRRL 1785) isolated from stale milk and maintained on Potato Dextrose Agar was obtained from the Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos, Nigeria.

Chemicals

Chemicals and Reagents were products of Sigma Chemical Company, St. Louis, USA and Merck, Darmstadt, Germany.

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METHODS

Preparation of Fermentation Medium For Growth of Culture

Aspergillus niger (NRRL 1785) constituted the parent culture. The method used in preparing the fermentation medium followed that of Sarath *et al* (1989) with modifications. The culture conditions and media for growth and synthesis of acid protease by *Aspergillus niger* were optimized to give maximum growth and enzyme production. The optimized medium was referred to as a basal medium. The basal medium contained 2% glucose, 1% mycological peptone, 0.1% KH_2PO_4 and 0.3% yeast extract. The pH of the medium was adjusted to 4.0 using 0.05M citrate buffer.

Spore inoculum of *Aspergillus niger* under study was prepared by adding 5ml sterile distilled water to a 5 day old slant culture of *Aspergillus niger* NRRL 1785 with vigorous shaking for 1 minute and poured aseptically into the basal medium. The resulting mixture was incubated at 30°C for 24 hours in a Gallenkamp Economy Incubator. Fermentation media in which the *Aspergillus niger* was grown and acid protease synthesis monitored were prepared in like manner with the basal medium to cover a 5-day fermentation period. 5ml inoculum was pipetted from the mixture of basal medium and spores into each fermentation medium. These were later incubated at 30°C on a Gallenkamp Rotary Shaker at 200 rev/min for the period ranging from 12 to 120 hours.

Determination of Growth of *Aspergillus niger* (NRRL 1785)

Growth of *Aspergillus niger* (NRRL 1785) and acid protease synthesis were monitored at intervals of 12 hours. At the end of each fermentation period, the fermentation broth was filtered through pre-desiccated and pre-weighed filter paper. The residue, which was the mycelium, was dried in an airtight Gallenkamp oven at 105°C for 3 hours to obtain the dry weight of the mycelium. The dry weight of the mycelium was recorded as biomass concentration in mg/100ml of broth and used to measure the growth of *Aspergillus niger*. The clear filtrate was the source of the acid protease.

Assay of Acid Protease activity

The synthesis of acid protease by *Aspergillus niger* under study was measured through the assay of acid protease activity in the fermentation broth. The assay procedure was that of Sarath *et al* (1989), which comprised setting up the test, blank and standard. The test contained reaction mixture of lml of 1% w/v casein (substrate), 0.4ml of 0.05M citrate buffer, pH 4.0 and 1.0ml enzyme solution. One millilitre (1.0ml) of buffer replaced enzyme solution in the blank while the standard was prepared with tyrosine (100µg/ml). The test and the blank were incubated at 50°C for 15minutes; the reaction was stopped by adding 3.0ml of 5% trichloroacetic acid to precipitate the enzyme. The mixtures were later centrifuged at 5000rpm for 15minutes and the supernatant was collected. Four millilitre (4.0ml) of 0.4M sodium carbonate were added to 1.0ml of supernatant with thorough mixing. Five hundred microlitres (0.5ml) of Folin - Ciocalteau reagent were added to the mixture and this was left for 10minutes to allow for colour development. The absorbance at 660nm was determined in a Biochrom UV/Visible spectrophotometer (Pharmacia LKB). One unit of acid protease activity was defined as micrograms of tyrosine equivalents per minute under the standard assay conditions.

Some Physicochemical Properties of Acid Protease Synthesized by *Aspergillus niger* (NRRL 1785)

Effect of pH on acid protease activity

Citrate buffer (0.05M) of different pH (2.5 - 6.5) were prepared. The acid protease activity was determined at varying pH. The citrate buffer of the pH under study replaced the citrate buffer of pH 4.0 in the standard assay procedure above.

Effect of pH on the stability of acid protease

Enzyme solution (1.0 ml) was incubated with buffer of test (or the pH under study) for 24 hours at room temperature, after which the pH of the enzyme solution was adjusted to 4.0 with 0.05molL^{-1} citrate buffer, 1ml of substrate solution (1% casein) was then added to the enzyme buffer solution and the acid protease activity was assayed at 50°C as earlier outlined. The activity obtained was termed residual activity.

Effect of temperature on acid protease activity

Water baths were set at temperatures ranging from 30°C - 80°C. The assay reaction mixture was incubated at the different temperatures for 15 minutes after which the acid protease activity was determined.

Effect of temperature on stability of acid protease

Enzyme solution (1.0ml) was incubated at different temperatures (30°C - 80°C) for one

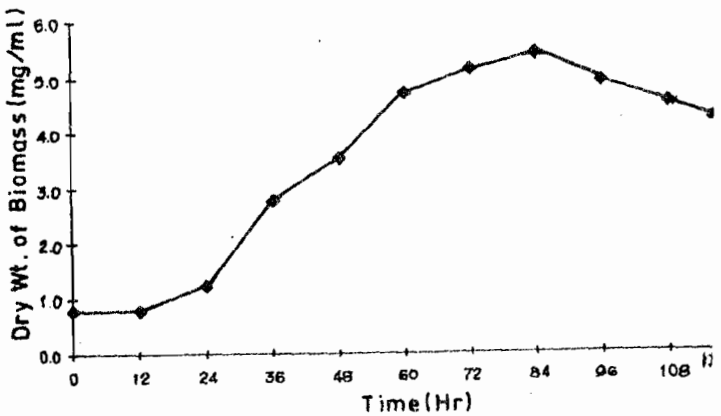

Figure 1: Fermentation Profile of *Aspergillus niger* NRRL 1785 showing the dry weight of Biomass (growth) against time.

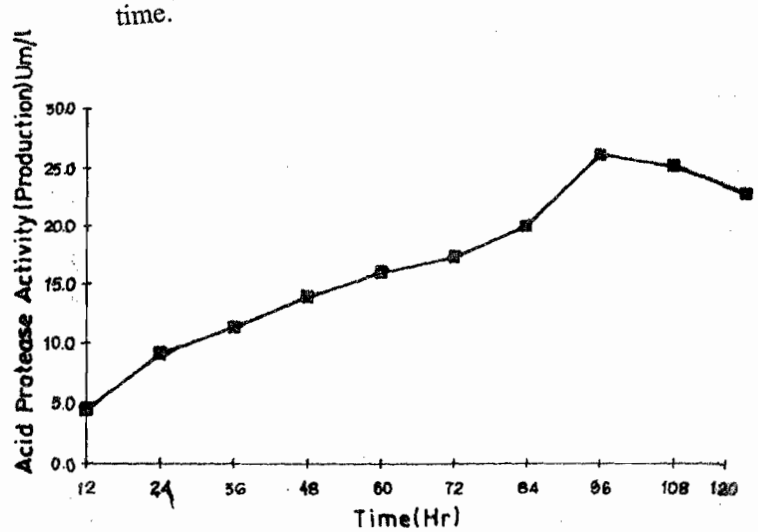

Figure 2: Fermentation Profile of *Aspergillus niger* NRRL 1785 showing the acid protease activity (protease production) against time

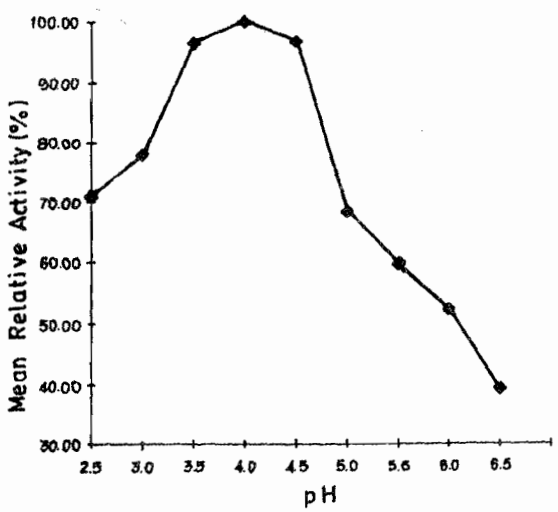

Figure 3: Effect of pH on acid protease activity

hour after which the acid protease activity was determined following the assay procedure.

Effect of metal ions on acid protease activity

Six different salts (Calcium chloride, Sodium chloride, Magnesium sulphate, Mercury chloride, Copper sulphate and Lead acetate) were tested for their effect on acid protease activity at a concentration of 50mM. Enzyme solution (1.0ml) was incubated with 0.4ml of each salt solution at room temperature for 30 minutes after which the acid protease activity was assayed.

RESULTS

Growth of *Aspergillus niger* (NRRL 1785)

The pattern of growth of *Aspergillus niger* is illustrated in Fig. 1. It consists of four phases of growth: the lag phase (0 - 12 hrs), exponential phase (12hrs - 60hrs), deceleration phase (60hrs - 84hrs) and the death phase (84hrs - 120hrs).

Synthesis of Acid Protease

There was a lag period of about 12 hours before the commencement of synthesis (Fig. 2) after which enzyme activity began to appear in the medium. Synthesis of acid protease continued to increase until the 96th hr when a maximum activity of 26.05Uml^{-1}was obtained.

Physicochemical Properties

Effect of pH on acid protease activity

The effect of pH on acid protease activity is shown in Fig. 3. Acid protease activity increased with pH from pH 2.5 to pH 4.0 after which it began to decrease. The optimum pH was 4.0. Relative activity was expressed as a percentage of the activity at pH 4.0, which was taken as 100%.

Effect of pH on acid protease stability

The effect of pH on acid protease stability is shown in Fig. 4. The optimum pH for enzyme stability was pH 3.5. The residual activity was expressed relative to the activity at pH 3.5. The activity at the optimum pH was taken as 100%. The enzyme was said to be stable at any pH at which relative residual acid protease activity was greater than 60%. From the figure below, the enzyme was stable over the pH range of 2.5 - 5.5.

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Acid protease activity increased with increase in temperature up to 50°C, where the

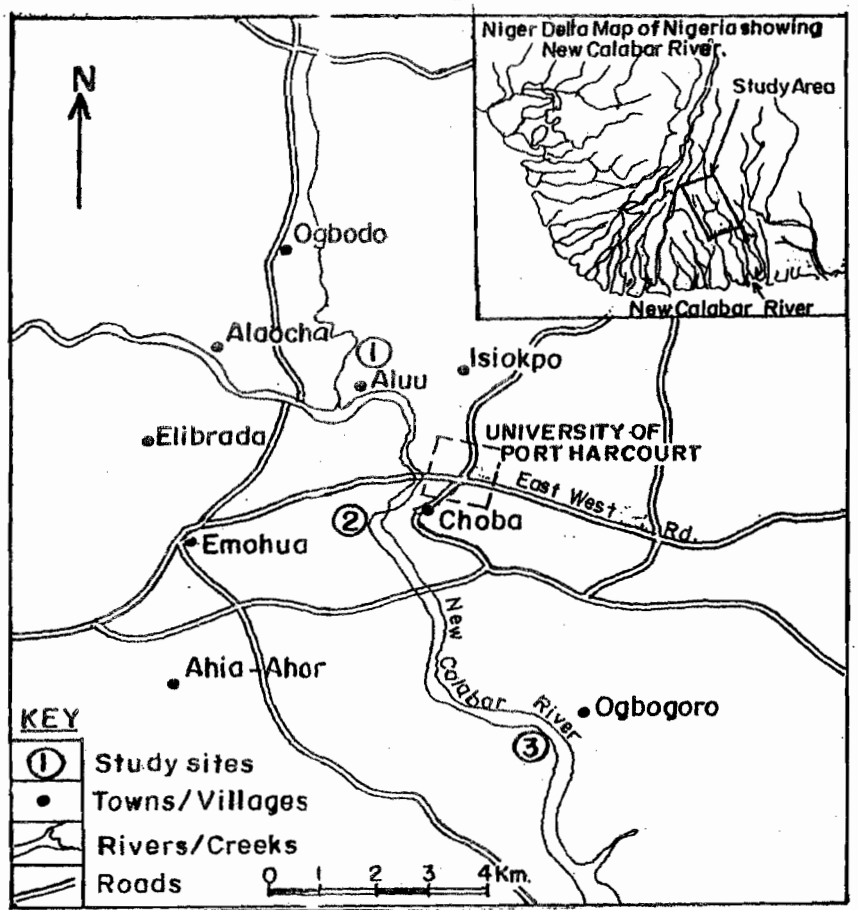


Fig 1: A map showing the three sampling sites (1-3) of the New Calabar River.

3)

(Laddaga and Silver, 1985), biosynthesis of metallothineins and other proteins that trap metals (Higham *et al.*, 1984), precipitation (Aiking et al., 1985), and transformation to volatile compounds (Meissner and Falkinham, 1984).

Although many different chemical methods have been employed to remove metals from wastewater, for example hydroxide and sulphide precipitation, ion exchange, membrane processes (Metzner, 1977) addition of natural and synthetic polymers and acid treatment (Wheatland *et al.*, 1975). The technique of selective bioaccumulation of heavy metals by microbial systems offers the best possible approach for the remediation of contaminated water bodies.

This study was thus conducted to investigate seasonal changes in the distribution of heavy metal resistant bacteria and the probable contribution of inputs of heavy metals by industrial effluent discharges into the New Calabar River water and sediment.

MATERIALS AND METHODS

STUDY AREA

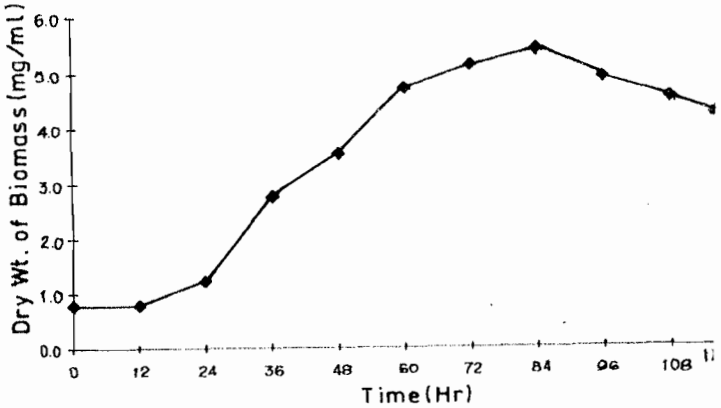
The New Calabar River is found in the lower Niger Delta, near Port Harcourt, Nigeria (Figure 1), with its source at Elele Alimini Umeozoro. It is freshwater from source to Aluu and brackish beyond this point. The river water is soft and acidic with pH 5.5-6.5 (Odokuma and Okpokwasili 1993a; Odokuma and Okpokwasili 1993b; Odokuma and Okpokwasili, 1997).

SAMPLE COLLECTION AND PRESERVATION

Three sampling points were chosen for this study. At Choba (site 2), an oil servicing company (WB), a fibre processing industry (HF) and a market are located. At Ogbogoro (site 3), a construction company (SPC), and a mini-market are present. Aluu (site 1) has no industrial activity.

Samples were collected using 2-litre sterile water containers. Containers were pre-washed with detergents, then with distilled water, soaked with 95% alcohol and allowed to dry. At points of collection, containers were rinsed severally with the river water and then used to collect samples from the surface (0-15cm) and sub-surface (15-30cm) of the river. Eckman grab sampler was used for collection of sediments samples.

A total of nine sampling trips were



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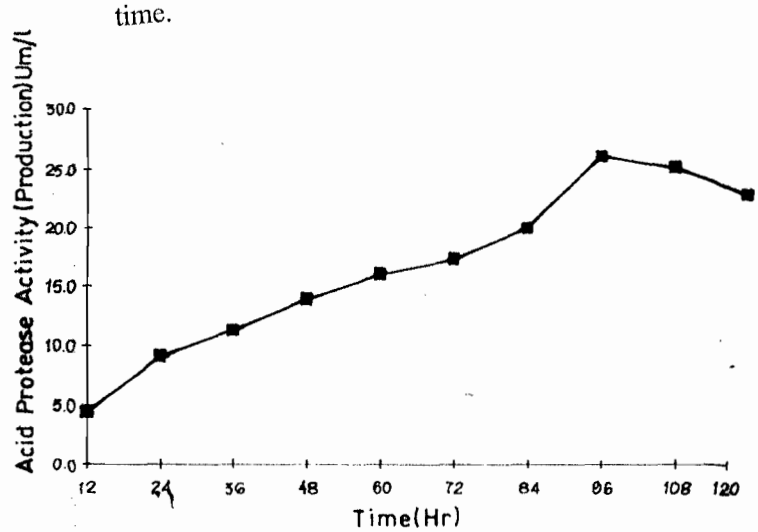

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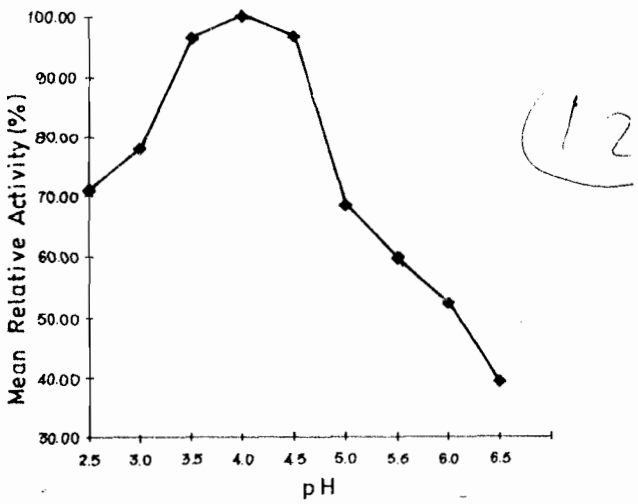

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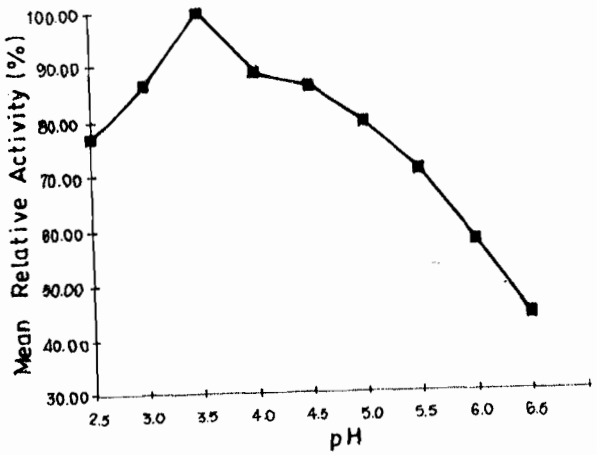

Figure 4: Effect of pH on acid protease stability

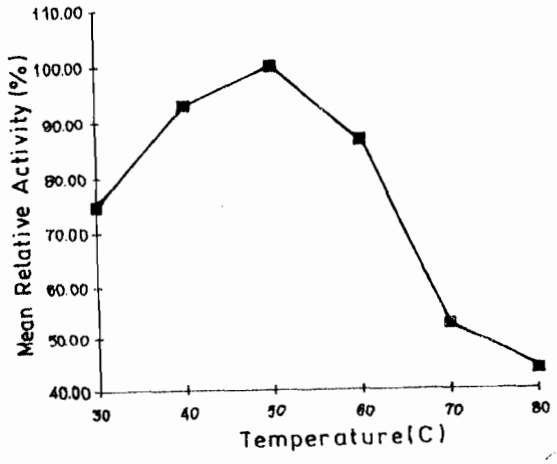

Figure 5: Effect of temperature on acid protease activity

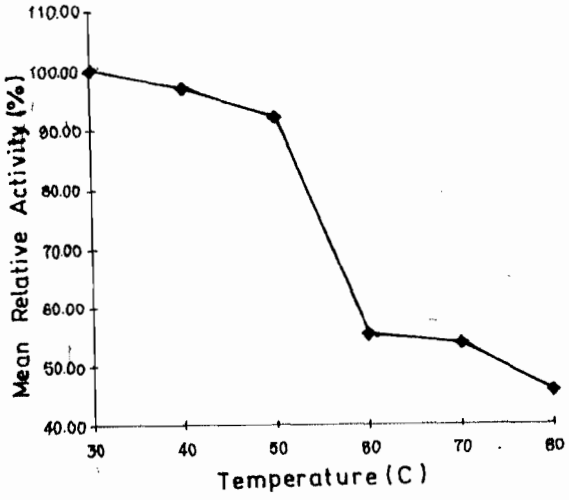

Figure 6: Effect of temperature on acid protease stability

optimum/maximum activity was obtained (Fig. 5). After 50°C there was loss of activity. Exposure to temperature of 60°C resulted in a loss of about 13% of the maximum activity of acid protease. Activity of the enzyme however reduced drastically to about 50% of the maximum activity on exposure to temperature of 70°C. Relative activity was expressed as a percentage of the activity at 50°C which was taken as 100%.

Effect of temperature on acid protease stability

The acid protease was optimally stable at 30°C as shown in Fig. 6. The relative residual activity was expressed as a percentage of the activity observed at 30°C. The enzyme was said to be stable at any temperature at which activity was greater than 60%. Acid protease from *Aspergillus niger* (NRRL 1785) was stable from 30°C up to 50°C, but lost about 45% of its maximum activity at 60°C after 1 hour incubation at pH 4.0.

Effect of metal ions

Table I shows the result of the influence of metal ions on acid protease activity. The enzyme was slightly activated in the presence of calcium and magnesium ions. Copper ion and lead ion had no effect on the activity of acid protease. Acid protease activity was inhibited in the presence of sodium and mercury ions.

DISCUSSION

The growth curve of *Aspergillus niger* (fig. 1) in the basal medium consisted of the lag phase, exponential phase, deceleration phase and the death phase. The lag phase (a period of no visible growth) was followed by the exponential phase that was recorded between the 12 and 60 hours of fermentation. The exponential phase witnessed the initial growth following spore germination when the total mycelia/ length/or biomass was increasing exponentially and growth was not affected by nutrient limitation or inhibitory effects of secondary metabolites. This agrees with the report of Neustroev and Fisrov (1990) on the growth kinetics of *Aspergillus awamoris.* The declining growth rate during the deceleration phase (60 – 84 hours) might be due to exhaustion of some nutrients in the media that became limiting or by the accumulation of waste products in the medium (Griffin, 1981). The death phase (84^{th} – 120^{th} hr) might have resulted from the complete exhaustion of available nutrients most especially the carbon

Table 1: Effect of metal ions on acid protease activity

Metal ions (50mM)	Relative activity (%)
Control (0.00)	100.00
Ca^{2+}	114.45
Na^{+}	67.57
Mg^{2+}	108.79
Hg^{2+}	77.99
Cu^{2+}	100.44
Pb^{2+}	98.35

source which provided the maintenance energy (Tan-Wilson *et al*, 1996).

The remarkable increase in the rate of acid protease synthesis which was highest between the 84th and 96th hour of fermentation (12 hours into the death phase) (fig.2) could have been due to the lytic processes occurring in the mycelium leading to the release of more acid protease into the fermentation medium, which already contained extracellular acid protease. During autolysis, lytic enzymes could have been activated while enzymes involved in synthesis were inhibited. Kritzmah *et al* (1978) reported a correlation between synthetic and lytic enzymes and hyphae formation in *Sclerotium rolfsic*. A similar report was obtained with proteinases of *Eremothecium ashbyii* (Koltun *et al*, 1986).

Acid protease of *Aspergillus niger* NRRL 1785 was most active at pH 4.0 (Fig. 3) Optimum pH range for acid protease activity from *Aspergillus saitoi* (3.0 - 4.5), *Aspergillus niger* (3.0 - 4.0), have been reported, (Gerhartz, 1990, Singh *et al.*, 1994). Similar results were reported for acid protease from other fungi (Nizharadze *et al.* 1991; Chrzanowska *et al.* 1995; Ikasari and Mitchell, 1996; Wu and Hang, 1998). The enzyme was stable (60% and above) over a pH range of 2.5 -5.5 (Fig.4). Similar results were observed for acid protease from *Aspergillus saitoi* and *penicillium varioti* (Gerhartz, 1990), *Aspergillus niger* (Singh *et. al.*, 1994) and *Neosartorya fischeri* var. spinosa (Wu and Hang, 1998). Reports have shown that most acid proteases are characterized by maximum activity and stability at pH 2.5 -5.0 (Boing, 1982).

The optimum temperature for acid protease activity from *Aspergillus niger* NRRL 1785 was 50°C (Fig. 5). Acid protease from *Aspergillus saitoi* and *Rhizopus Oligosporus* were reported to have optimum temperature of 30°C and 40°C respectively (Kalisz, 1988; Farley and Ikasari, 1992) while that of *Neosatorya Fischeri* var. spinosa was 50°C (Wu and Hang, 1998). Acid protease of *Aspergillus niger* NRRL 1785, was stable up to 50°C (60% relative activity) at pH 4.0 for 1 hour (Fig. 6). Similar results were obtained for acid protease form *Aspergillus saitoi* and *Neosartorya fischeri* var spinosa (Tanaka et. al., 1977; Wu and Hang, 1998). Acid protease from *Penicillin expansium* was stable up to 45°C only (Abdel – Fattah and Amr, 1987).

The activity of acid protease in this study was affected by metal ions. Though the enzyme was active in the absence of metal ions, calcium ion had an activating effect on the acid protease activity while mercury, and sodium ions had inhibitory effects. The result obtained with mercury ion contradicts reports by Boing (1982), Gerhartz (1990), Singh et al (1994) and Wu and Hang (1998) that acid protease is insensitive to heavy metals.

CONCLUSION

This study has shown the synthesis of acid protease by *Aspergillus niger* (NRRL 1785) to be dependent on phase of growth of the organism. The synthesis was maximal at the inception of the death phase of the organism. The acid protease synthesized had characteristics reminiscent of typical acid proteases in literature except influence on it by metal ions.

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