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EXTRACELLULAR PROTEASE PRODUCED BY *BACILLUS SUBTILIS* ISOLATED FROM CONTAMINATED PARACETAMOL ORAL PREPARATION

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ABSTRACT

In a study to evaluate the microbiological safety of some paracetamol oral solutions sold in some Nigerian drug stores, 40.0% of the samples examined was contaminated with protease- producing *Bacillus subtilis*. The production of extracellular protease was induced by casein in the minimal medium and was found to be the major enzyme in the growth medium. The enzyme was partially purified by ammonium sulphate precipitation technique and the specific activity determined. The quantitative assay revealed that the produced enzyme exhibited proteolytic activity at different assay conditions. The level of enzyme production increased with increase in cell density. The enzyme was stable at the pH and temperature ranges 7.5-10 and 45-60°c, respectively, indicating proteolysis over a wide range of environmental conditions.

KEYWORDS: Microbiological safety, Paracetamol oral solutions, *Bacillus subtilis*, Extracellular protease, Proteolysis.

INTRODUCTION

Bacteria play a major role in biodeterioration of pharmaceutical preparations. Proteolytic bacteria elaborate proteases which exhibit their activity on the protein component of the drug molecule by attacking a wide variety of polypeptides and reducing them to simple assimilable forms. Paracetamol syrups are especially formulated with protein-based carriers such as albumen, milk and essential amino acids (British Pharmacopeoia,1988).These ingredients may provide suitable substrates for microbial growth and production of proteolytic enzyme and thus, facilitate drug spoilage.

In many cases the nature of proteases or the organisms involved are not known except the effect of their actions on the drugs. This effect can manifest as putrefaction, or change in colour and taste. Alkaline, neutral and acid proteinases from *Aspergillus* spp are known to play important role in the hydrolysis of protein to form oligo-peptides and amino acids (Fujimaki *et al.*, 1971).

Although *B. subtilis* proteases have been commercially exploited for the benefit of man (Aderibigbe et al., 1990; Odunfa and Oyewole, 1986), information seems to be scarce on the role of this enzyme on biodeterioration of liquid drugs. This study is, therefore, conducted to examine the effect of *B. subtilis* protease on microbiological stability of paracetamol oral preparations. It is hoped that findings from this work shall provide an index of evaluating drug spoilage and add to the present seemingly scanty information on the role of proteolytic enzyme in biodeterioration of pharmaceutical preparations.

MATERIALS AND METHODS

Source of organism

Bacillus subtilis was isolated from contaminated paracetamol oral solutions, purified by repeated sub-culturing and identified using the identification scheme of Holt *et al.* (1994). The organism was stored on nutrient agar slants at 4°c and resuscitated on casein medium before the extraction of the enzyme.

Screening of *B subtilis* for protease production

The ability of *B. subtilis* to produce protease was determined using the method of Aderibigbe (1997). Casein agar medium comprising per litre of distilled water, 1.0g, KCl; 0.12g,$MgSO_4.7H_2O$; 0.2mg,$MnCl_2.4H_2O$; 0.01g, CaCl; 0.3mg,$FeSO_4$; 0.3mg; K_2HPO_4 buffer (pH 7.0); 1.6g, nutrient broth base; 15.0g, agar agar (Biotech) and 0.7% (v/v) casein.

The composition, except casein was mixed and sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to 40°c. Casein was sterilized by membrane filtration and aseptically added to the pre-cooled agar medium. The medium was dispensed in 15 ml portions into sterile petri dishes and allowed to set. Thereafter, the solid agar surface was inoculated with the test organism by spread plate technique, and the plate incubated at 37°C for 24h. Protease production was indicated by the formation of halos around the discrete colonies on casein agar plate.

Preparation of inoculums

Colonies showing ability to produce protease were transferred to 15ml of fresh casein broth and incubated at 37°C without shaking. The inoculum was standardized using McFarland's nephelometry. This was done by comparing the turbidity of the broth culture of the test organism with that of McFarland turbidity standard (MTS), consisting of 0.6 ml of 1% (w/v) solution of barium chloride and 99.4 ml of 1% (v/v) solution of sulphuric acid (Cheesbraugh, 2000).

Incubation was stopped when the turbidity of the test suspension matched with that of the MTS, which was equivalent to an absorbance of 0.5 at 578 nm wavelength.

Preparation of crude protease

Sterile casein broth in 100ml Erlenmeyer flask was inoculated with 1.0ml of the inoculum and incubated at 37°C in a shaker water bath maintained at 120 rpm for 36 hours. At the end of incubation, the culture was centrifuged at 4000 x g in refrigerated centrifuge at 4° C for 15 minutes to remove bacterial cells. The resulting supernatant was used as crude protease for further studies.

Partial purification of the enzyme

This was done using the ammonium sulphate precipitation technique of Colowick and Kaplan (1984), as described by Ukoha (1998). The method involved the following sequential fractionation steps.

Five test tubes containing 3.0ml each of the crude enzymes was set up. The tubes were cooled to about 4°C in the refrigerator and increasing amounts of finely powdered solid ammonium sulphate was added slowly with stirring until visible precipitation of protein occurred. The amount of ammonium sulphate used to bring about this precipitation was noted and the suspension centrifuged at 4000 x g for 15

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minutes. The supernatant was decanted into separate tube and both the supernatant and precipitate (sediment) were assayed for enzyme activity and total protein content. If activity was observed in the supernatant, more solid ammonium sulphate was added until further precipitation occurred. This process was repeated until no more precipitation occurred.

The degree of purity of enzyme fraction was determined based on its specific activity per milligram of protein and was calculated as

$$\text{Specific activity} = \frac{\text{Units (i.e. enzyme activity)}}{\text{Mg protein}}$$

An increase in specific activity indicated higher purification. Total activity of the enzyme preparation was determined as

Total activity = (specific activity) x total mg protein in the preparation.

Assay of protease activity

Measurement of protease activity followed the casein hydrolysis procedure described by Brain and Schmitz (1980). A 2% casein (Sigma Chemical Co, St Louis) was prepared in distilled water; heat denatured at 100°C in a water bath for 10min and allowed to remain at 35°C for further 1 hour before use. To 0.5ml of the 2% casein solution in a tube was added 0.3ml of the enzyme sample and 0.1ml of 1M tris/HCl (pH 8.0). The preparation was thoroughly mixed and incubated at 35°C for 1 hour in a water bath. The reaction was stopped at appropriate time intervals (e.g. 10, 20, 40, and 60 minutes) by addition of 2.0ml of cold 7% perchloric acid. To the control tube, perchloric acid was added before the addition of casein. The unhydrolysed casein was allowed to coagulate by refrigeration at 4°C and the supernatant retained for analysis.

Protein determination in the supernatant was done following the procedure of Lowry *et al* (1951), using Bovine Serum Albumin as standard for the preparation of standard curve. All assays were done in triplicates. One unit of protease activity was defined as the amount of enzyme that would yield 10 μmol of non- precipitable protein.

EFFECTS OF pH, TEMPERATURE AND SUBSTRATE CONCENTRATION ON ACTIVITY AND STABILITY OF PROTEASE

The parameters studied were the effects of pH, temperature and substrate concentration on the activity and stability of the protease. The effect of pH on protease activity and stability followed the method of Aderibigbe (1997). For the effect of pH on enzyme stability, buffers with pH 2-10 were used. Buffers with pH 2,3 and 4 were prepared using citrate HCl; pH 5 and 6 were citrate NaOH. Equal volumes of each buffer and the enzyme preparation were mixed and incubated at 37°c for 30min. Samples were taken at 5-minute intervals and assayed for proteolytic activity. Results were compared with that of reference pH (8.0).

Thermal stability and activity studies were conducted using the method of Dass *et al.* (1995). The effect of substrate concentration[s], followed the procedure described by Eriksson and Pettersson (1982), using [s] values of 2.5mg/ml to 40mg/ml.

The percentage activity of the enzyme at different temperature conditions was calculated as units (i.e. enzyme activity)

$$\frac{\quad}{\text{mg protein}} \times 100 \text{ (Clark and Switzer, 1987)}$$

RESULTS

Protease assay

Figure 1 shows the time course of protease production in relation to cell growth. The highest level of

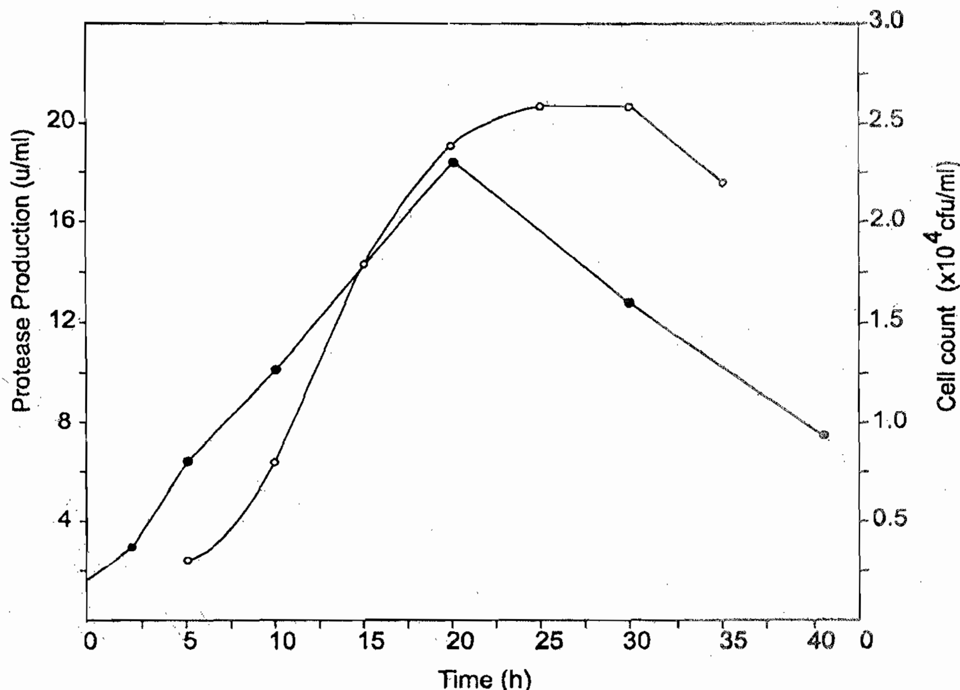

Fig. 1: Time course of growth and protease production in broth culture of B. subtilis

•——•: Protease production

○——○: Cell growth

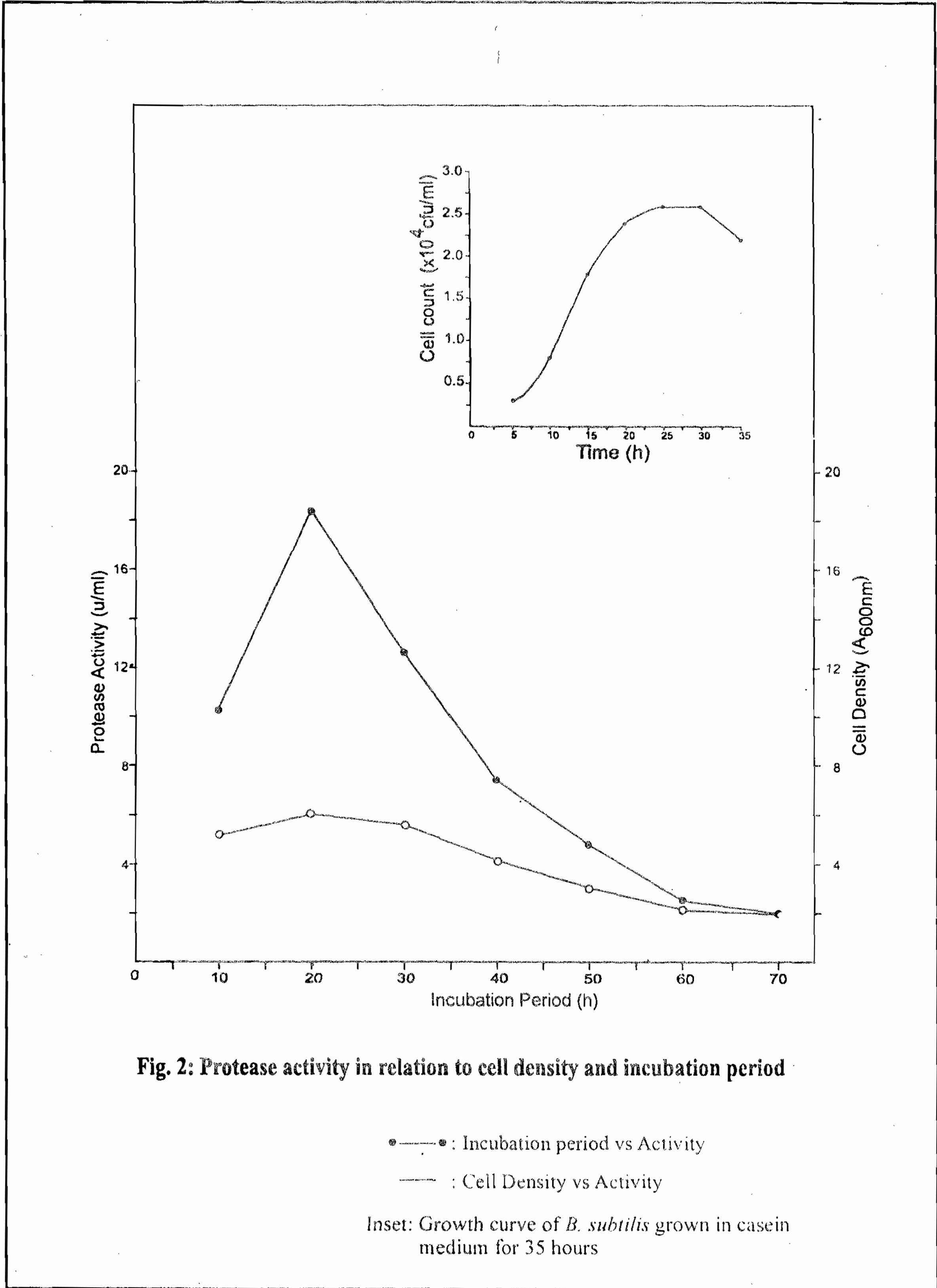

Fig. 2: Protease activity in relation to cell density and incubation period

production (18.34 u/ml) was reached at 20 hours, followed by a decline in production as the reaction time increased to 30 hours and above. The enzyme activity in relation to cell density is presented in Figure 2. The enzyme activity increased during log growth phase. The highest level of specific activity was achieved at late exponential phase of growth. As the cell density increased the activity increased. The inset of figure 2 shows the growth curve of *B. subtilis* grown in casein broth for 35h. Protease activity was related to cell population. The activity increased during the exponential growth phase. At stationary growth phase the activity began to decline.

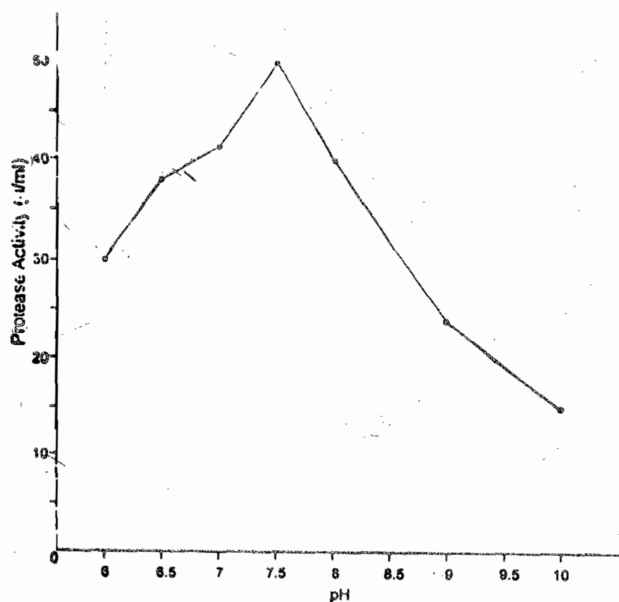

Fig. 3: Effect of pH on activity of protease of *B. subtilis*

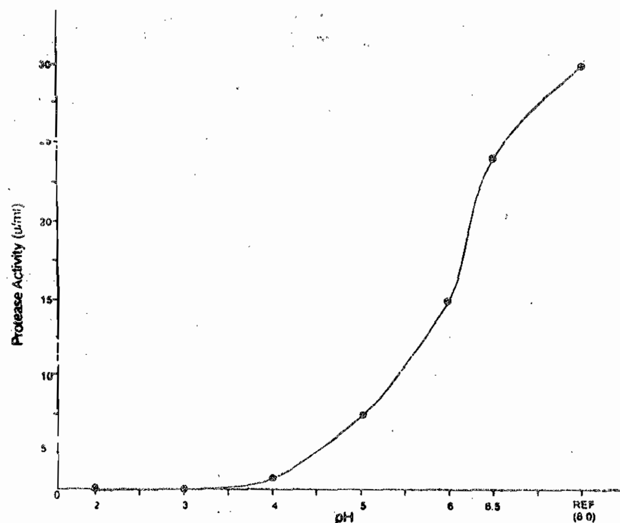

Fig. 4: Effect of pH on stability of *B. subtilis* protease

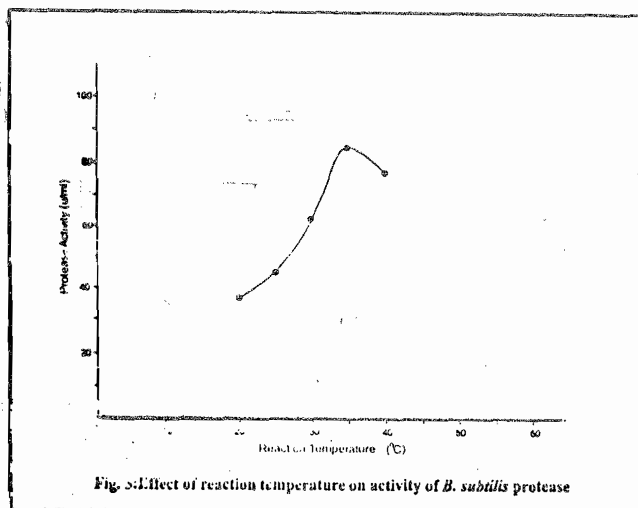

Fig. 5: Effect of reaction temperature on activity of *B. subtilis* protease

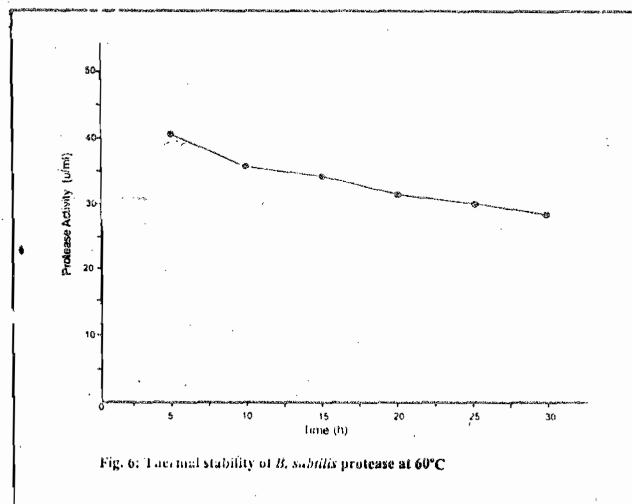

Fig. 6: Thermal stability of *B. subtilis* protease at 60°C

Characterization of the extracellular protease

The enzyme exhibited optimal activity at the pH 7.5 (Figure 3). However the protease was stable at the pH range 6.5-10. (Figure 4). About 83% of total activity was detected after 20 min at pH 6.5 when compared with the reference standard (pH 8.0). At pH 5, 75% of enzyme activity was lost within 15 minutes while only 6.2% of activity was detected at pH 4 in 15 minutes. There was a complete lost of activity at pH 2 and 3 within 5 minutes.

The result on the effect of reaction temperature on activity of the enzyme preparation is presented in Figure 5, while Figure 6 shows the thermal stability of the enzyme at 60°C. The optimum temperature for the activity was 35°C (at pH 7.5). The enzyme was relatively stable at the temperature regimes of 40, 50, and 60°C with 95, 90 and 80% activity respectively, after 30 minutes of incubation. The protease activity was completely lost at 70° C within 30minutes.

Figure 7 represents the effect of substrate (casein) concentration on proteolytic activity of the *B. subtilis*. Proteolytic activity increased with increase in casein concentration up to 30.0mg/ml. At this concentration the optimum activity was 1.30 x 10^2 units/ml.

DISCUSSION

In this study, *B. subtilis* isolated from contaminated paediatric paracetamol syrup have been found to liberate extracellular protease during their log phases of growth. The concentration of extracellular protease in the culture filtrate and the rate of production were highest when cells were at the exponential growth phase. This is in agreement with the report of Aderibigbe (1997) on characterization of extracellular proteinases from strains of *B. subtilis*. The protease concentration declined with increase in incubation period probably due to autolysis of protein. The gradual decrease in the enzyme production could also be due to the decrease in metabolic activities as cells enter stationary and death phases of growth. As also observed by Sakellaris (1991), when cells enter death phase of growth, they become metabolically inert and this may have accounted for a decline in protease production. The role of microbial enzymes on biodeterioration of drugs has been reviewed by Hugo and Russell (1992).

Out of 40 samples of paracetamol syrups examined, 16(40%) were contaminated with protease-producing *B. subtilis*. However, only 5% of the samples contaminated with protease producing *B. subtilis* produced off-odour and bitter taste. It is not known whether both undesirable product characteristics are as a result of the proteolytic activity of *B. subtills* enzyme alone or there may be intrinsic factors leading to autodegradation of drugs under poor storage conditions, hence the bitter taste. The high contamination rate of *B. subtilis* shows manufacture in unhygienic environment and possible non-decontamination of the manufacturing environment.

The optimum pH and temperature for the extracellular protease activity were 7.5 and 35°C respectively. The enzyme preparation was stable at a pH range of 6.5-10 and temperature range of 40-60°C. At low (acidic) pH and high temperature conditions the activity was completely lost probably due to the denaturation of the enzyme. The stability of the *B. subtilis* protease at the pH range 6.5-10 and the

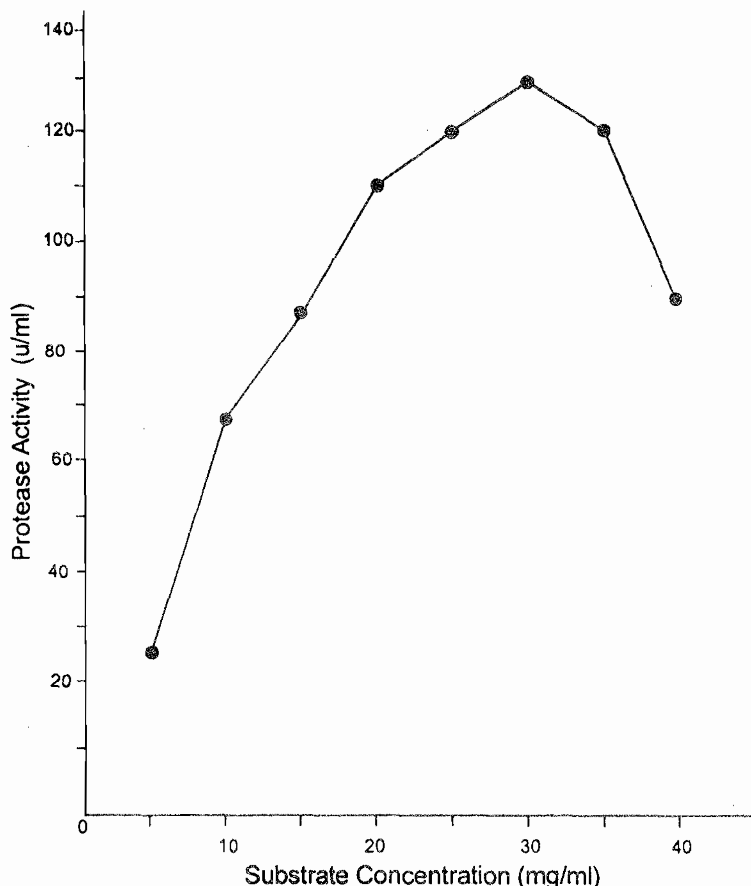

Fig. 7: Effect of substrate concentration [S] on protease activity

temperature range of 40-60°C suggested that throughout the storage period, the enzyme is likely to be active hence proteolysis could take place under different conditions in which the drugs could be stored. Hence, proteolysis is suspected to be one of the mechanisms of spoilage of liquid drugs during storage.

The effect of substrate concentration on protease activity showed that activity increased with increase in casein concentration up to 30mg/ml followed by a decline in activity with further increase in casein concentration. This decline in activity was expected because at higher substrate concentration[s], the active sites of the enzymes get saturated and the velocity of reaction decreases (Ukoha 1998).

This study has demonstrated that *B. subtilis* which caused contamination of paracetamol syrup produced extracellular protease. This enzyme could alter the nature of active ingredient of the drug and make it less efficacious, unstable and microbiologically unacceptable. Such extensive contamination by the air-borne proteolytic spore-former could be reduced or avoided by using treated air in the manufacturing environment, adopting good manufacturing practice (GMP) and maintaining high personal hygiene. The preparation should be stored at temperatures below the optimum for enzyme activity (35°C according to this study), thereby slowing down the rate of drug degradation by proteolysis.

The study also reveals that the extracellular protease produced by *B. subtilis* isolated from contaminated paracetamol syrup was an alkaline protease. More so, the level of enzyme production increased with increase in cell density. Therefore, a change in the pH of the formulation by the manufacturers to slightly acidic or neutral may discourage the production and activity of this enzyme as the producing organism may not be able to grow and build up biomass in the system.

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