

ORIGINAL ARTICLE

Storage temperature and stabilizers in relation to the activity of commercial liquid feed enzymes: a case study from Egypt

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Abstract

The present work was carried out in order to study the storage stability at different temperatures of commercial liquid enzymes imported to Egypt. In this respect, two samples containing the same enzymes (xylanase, amylase and cellulase) with the same initial activities and from the same biological origin were divided into four bottles. The first two bottles were labeled (A, D) having water as the carrying material and the second two bottles labeled (B, C) having glycerol as the carrying material. Samples A and B were stored at 4 °C, while samples C and D were stored at 30 °C, the study was conducted for 7.5 months. The results showed that samples stored at 4 °C whether carried in water or glycerol had better enzyme recovery percentages compared to those stored at 30 °C. Also samples having glycerol as a carrying material had better enzyme stability compared to those having water as a carrying material except for cellulase stored at 30 °C. The results obtained suggest that for shipment to tropical regions, suppliers must ensure enzyme storage at refrigerated conditions below 5 °C. It is also recommended that an enzyme stabilizer is used as it will lead to a more efficient enzyme and thus will increase the economic potential of existing enzymatic processes.

Key words: enzyme; storage; stability; glycerol; water

INTRODUCTION

Enzymes are catalytic proteins with their active site housed inside a 3D structure, making them sensitive to some physico-chemical conditions (Pérez-Portabella and Roura 2001) such as pH,

hydrothermal conditioning, frictional forces and to the heavy metals that are added to certain animal feeds (Steen 2001).

Many studies have shown that enzyme supplementation has potential for improvement of the nutritive value of feed stuffs (Pérez-Portabella and Roura 2001). The industrial enzyme business is steadily growing. In 2004, global demand for animal feed enzyme cost US\$240 million, and this is expected to increase to US\$375 million in 2012. The compound average annual growth rate will be 6%, being the highest average compared to technical enzymes (3.5%) and food enzymes (4%) (Padma and Ananthanarayan 2008).

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Recently, many liquid enzyme products have been imported into Egypt to be used as feed additives. Most of these enzymes are stored in locations not equipped with refrigerated containers and the enzymes might not contain stabilizing material. The temperature inside the local storage fluctuates between 30–35 °C. In general, liquid enzymes are inherently less storage stable than their granular counterparts. In liquid form, water activity is sufficiently high for the component enzymes to remain active, whereas in granular form the low water activity renders the component enzymes relatively inactive prior to ingestion by the animal. Commercially available enzymes are typically less than 100% pure and often contain side enzyme activities in addition to the desired main activity or activities. These side activities can include trace levels of proteases, which even at low activity can cause some degradation of the main activity (e.g. Xylanase). An aqueous environment also introduces a significant risk of microbial contamination and proliferation, which can cause a rapid decline in enzyme activity in liquid products. The problem of storage stability is increased further when different liquid enzymes are mixed to produce “multi-enzyme” liquid products and when liquid enzyme products are stored at ambient temperatures in different parts of the world (Steen 2001).

Enzyme stabilization has notable importance due to the increasing number of enzyme applications. Stabilization of enzymes is used in order to realize their full potential as catalysts. There are different approaches to enzyme stabilization. It can be studied from the point of view of the various denaturation reactions that occur, the kinetics involved therein and with respect to its intended industrial use and storage stability (Padma and Ananthanarayan 2008).

In order to enhance soluble enzyme stability, three major routes of research have been pursued (Padma and Ananthanarayan 2008): (a) screening for enzymes from extremophiles and their isolation, (b) production of stable enzymes in genetically manipulated mesophilic organisms and (c) stabilization of unstable enzymes by methods such as protein engineering, chemical modification, immobilization and medium engineering by using/employing additives.

Most of the reports on enzyme stabilization are focused on the effect of additives on protein stability showing that it has been the most popular method of enzyme stabilization. Using additives is a good approach towards increasing enzyme

storage stability. Among the various additives that can be employed for this purpose the addition of sugars and polyols to aqueous solutions of enzyme has been shown to strengthen the hydrophobic interactions among non-polar amino acid residues leading to protein rigidification and resistance to thermodeactivation (Padma and Ananthanarayan 2008).

The objectives of the present study are (a) to investigate the effect of storage temperature under the Egyptian stores conditions compared to the recommended conditions for enzymes storage and (b) to evaluate the effect of the presence/absence of a stabilizing material on liquid enzyme activity at different storage temperatures.

MATERIALS AND METHODS

Materials

Two commercial Indian liquid feed enzymes were used for the determination of enzyme stability by measuring their activities each 1.5 month for 7.5 month. The matrix of the first sample contained the following enzymes (xylanase, cellulase and α -amylase) and glycerol was the carrying material (sample 1). The second sample contained the same enzymes with water as the carrying material (sample 2). Enzymes in both products had the same recorded enzyme activity and the same biological origins (xylanase from *Sclerotium* sp., cellulase from *Trichoderma* sp. and α -amylase from *Bacillus* sp.).

Procedures

Both sample 1 and sample 2 were homogenized and divided into two bottles; bottles of sample 1 were labeled B and C, while bottles of sample 2 were labeled A and D. The first group was stored at a temperature of 30 °C (bottle C and D), while the second was stored in the refrigerator at 4 °C (bottle A and B). The samples were stored for 7.5 months. Enzyme activity was measured each month and a half.

The activity of xylanase was measured according to the method of (Bailey and Poutanen 1989). Xylan was used as a substrate at 0.9% of incubation mixture. This assay was based on the measurement of reducing sugar (xylose) following a 5 min hydrolysis at 50 °C. One xylanase activity unit (XU) was defined as the amount of enzyme that liberates 1 μ mol of xylose in 1 min. under the conditions of the assay. Optical densities of samples were determined through measuring the absorbance spectrophotometrically at 540 nm.

Cellulase activity was measured following the method of Miller (1959). Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution plus 1.8 ml of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 37 °C in a shaking water bath (GFL, Germany) for 30 min. The reaction was terminated by adding 3 ml of DNS reagent. The colour was then developed by boiling the mixture for 5 min. Optical densities of samples were measured spectrophotometrically at 540 nm.

Total α -amylase activity was determined using Amylazyme tablets (Megazyme) as per the manufacturer's instructions (McCleary 1991). The α -amylase activity was measured by adding an Amylazyme tablet to an aliquot of the enzyme solution diluted in 100 mM (MOPS) buffer (pH 7.0) in test tubes. The tubes were incubated at 40 °C for 10 min. The reaction was terminated with a weak alkaline solution, the slurries were filtered and the absorbances of the filtrates were measured spectrophotometrically at 590 nm.

Samples were analysed for possible contamination by their biological source (*Sclerotium* sp., *Trichoderma* sp. and a *Bacillus* sp.) according to (NMKL 2003). Samples were also checked for the presence of protease using the

azocasein method (Brock et al. 1982), where 20 μ l of the crude enzymes were incubated at 37 °C in a mixture (400 μ l) containing 2% azocasein (230 μ l), and 0.2 M Ntris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer pH 7.0 (150 μ l) for 2 h. To terminate the reaction, 1.2 ml of 10% trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 min and the supernatant (ca 1.4 ml) was collected after centrifugation (10,000 rpm; 5 min). An equivalent volume of 1 M NaOH was then added and the solution was mixed thoroughly prior to measuring the absorbance at 440 nm.

All analyses were performed in replicates and the mean data were presented.

All chemicals used in the experiment were of analytical grade.

RESULTS

The xylanase, and amylase activities of the studied samples were 605.214 and 345.924 U ml⁻¹; respectively, whereas cellulase activity was 415.907 KU ml⁻¹.

Fig. 1 shows the effect of different storage temperatures on xylanase activity in sample 2, bottles A and D. The results showed that whether

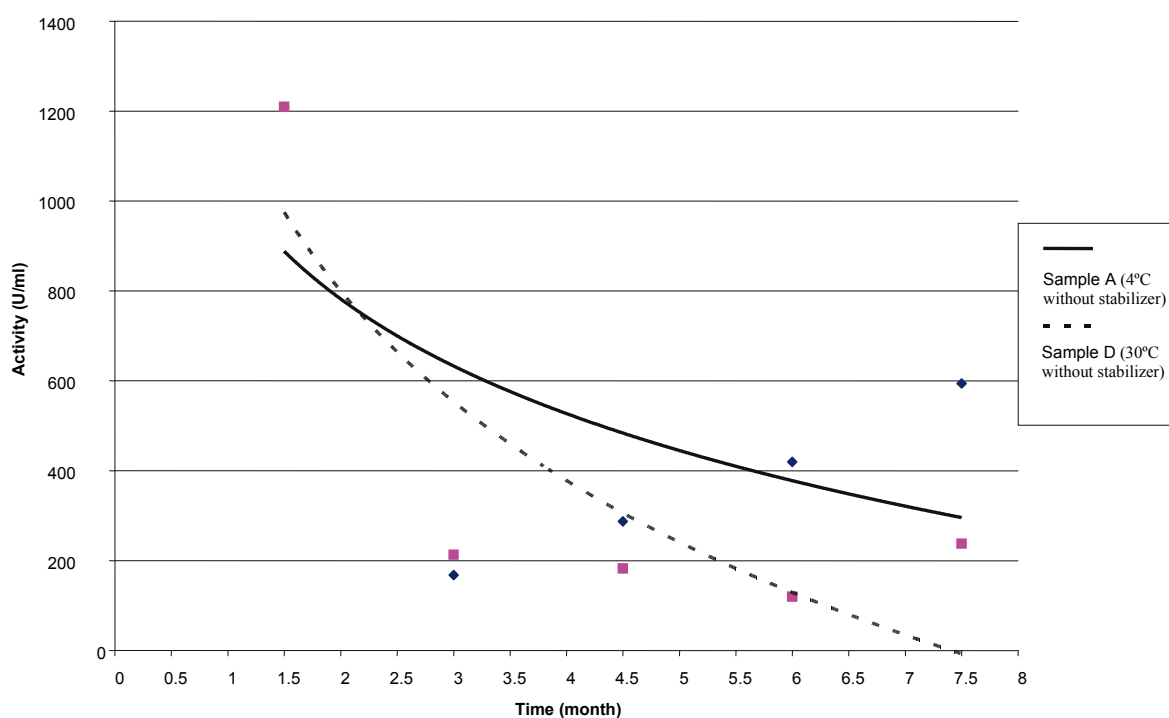


Fig. 1. Effect of different storage temperatures on xylanase shelf-life in commercial products having water as a carrier

the samples were stored at 30 °C or at 4 °C, xylanase activities decreased. Yet the degradation in xylanase activity in sample D was much higher (20% recovery after 7.5 month Fig. 4) than that of sample A (50% recovery after 7.5 month Fig. 4).

Fig. 2 shows the effect of different storage temperatures on cellulase activity in sample 2 bottles A and D. Cellulase activity did not decrease

when the sample was stored at 4 °C for 7.5 month (sample A). The recovery of cellulase activity in sample A after 7.5 months was 105% (Fig. 4). On the other hand, degradation of cellulase activity occurred when the sample was stored at 30 °C (sample D). The recovery of cellulase activity in sample D after 7.5 month was 76.2% (Fig. 4).

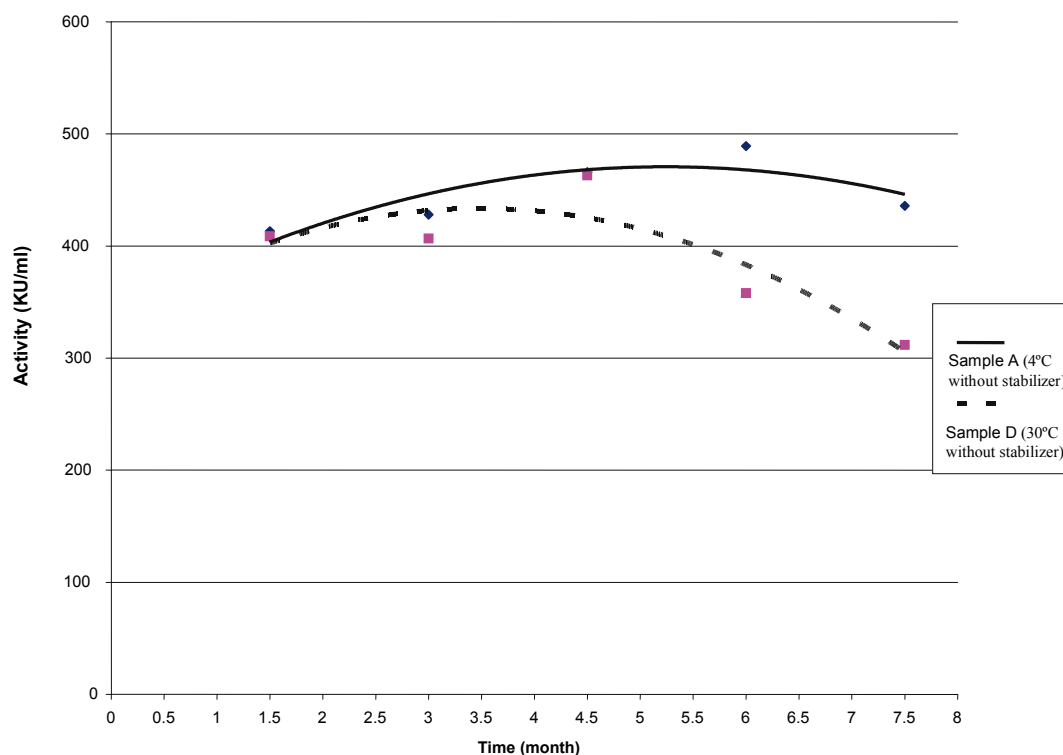


Fig. 2. Effect of different storage temperatures on cellulase shelf-life in commercial products having water as a carrier

Fig. 3 shows the effect of different storage temperatures on amylase activity in sample 2 bottle A and D. Amylase showed an increase in its activity for 5 months when the sample was stored at 4 °C, after which the activity started to decline, although recovery of amylase activity in sample A after 7.5 month was 117% (Fig. 4). On the other hand, degradation of amylase activity occurred when the sample was stored at 30 °C (sample D). The recovery of cellulase activity in sample D after 7.5 month was 30.4% (Fig. 4).

Fig. 5 shows the effect of different storage temperatures on xylanase activity in sample 1 bottle B and C. No degradation in xylanase activities resulted from storing the sample whether at 30 °C or at 4 °C. Xylanase recovering activity at 4 °C and 30 °C after 7.5 month was 108.3% and 99.5 %, respectively (Fig. 8).

Fig. 6 shows the effect of different storage temperatures on cellulase activity in sample 1 bottle B and C. A slight degradation in cellulase activity occurred when sample B was stored at 4 °C. Cellulase activity recovery in sample B after 7.5 month was about 86%. On the other hand, cellulase activity recovery in sample C after 7.5 month was 75.5% (Fig. 8).

Fig. 7 shows the effect of different storage temperature on amylase activity in sample 1 bottle B and C. No degradation in amylase activities resulted from storing the sample whether at 30 °C or at 4 °C. Amylase recovering activity at 4 °C and 30 °C after 7.5 month was 114% and 103%, respectively (Fig. 8).

Regarding the effect of the carrying material (glycerol or water) on xylanase activity (Fig. 9), it was observed that at the same storage

temperature the samples carried on glycerol (B and C) showed better recovery percentages compared to the corresponding samples carried on water (A and D).

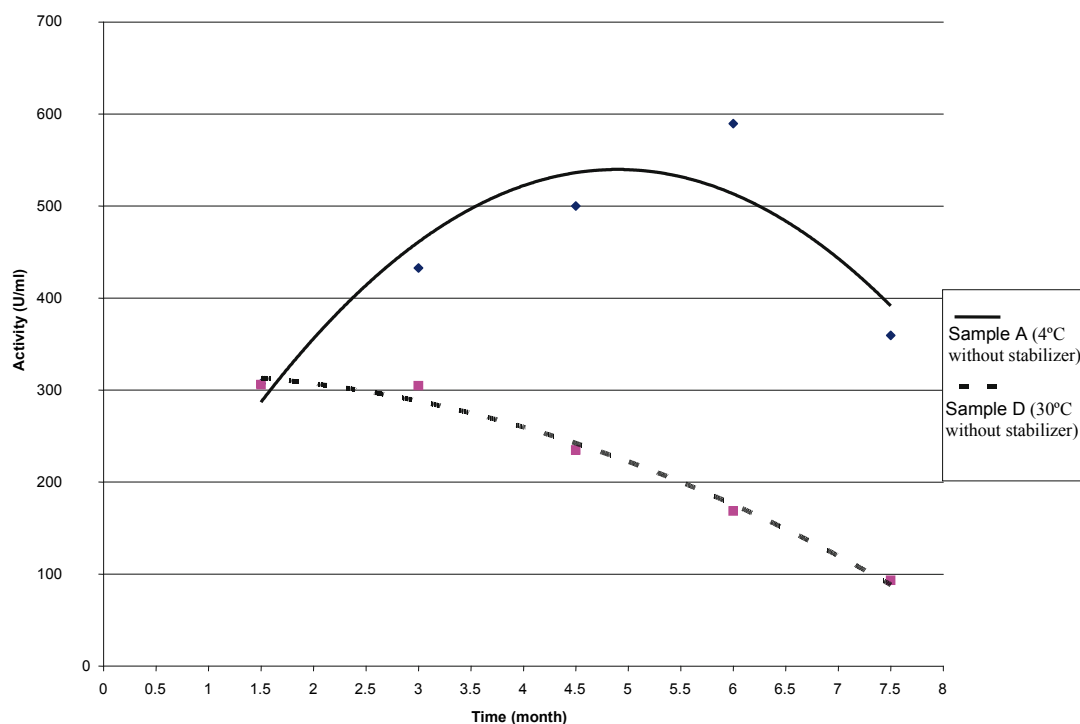


Fig. 3. Effect of different storage temperatures on amylase shelf-life in commercial products having water as a carrier.

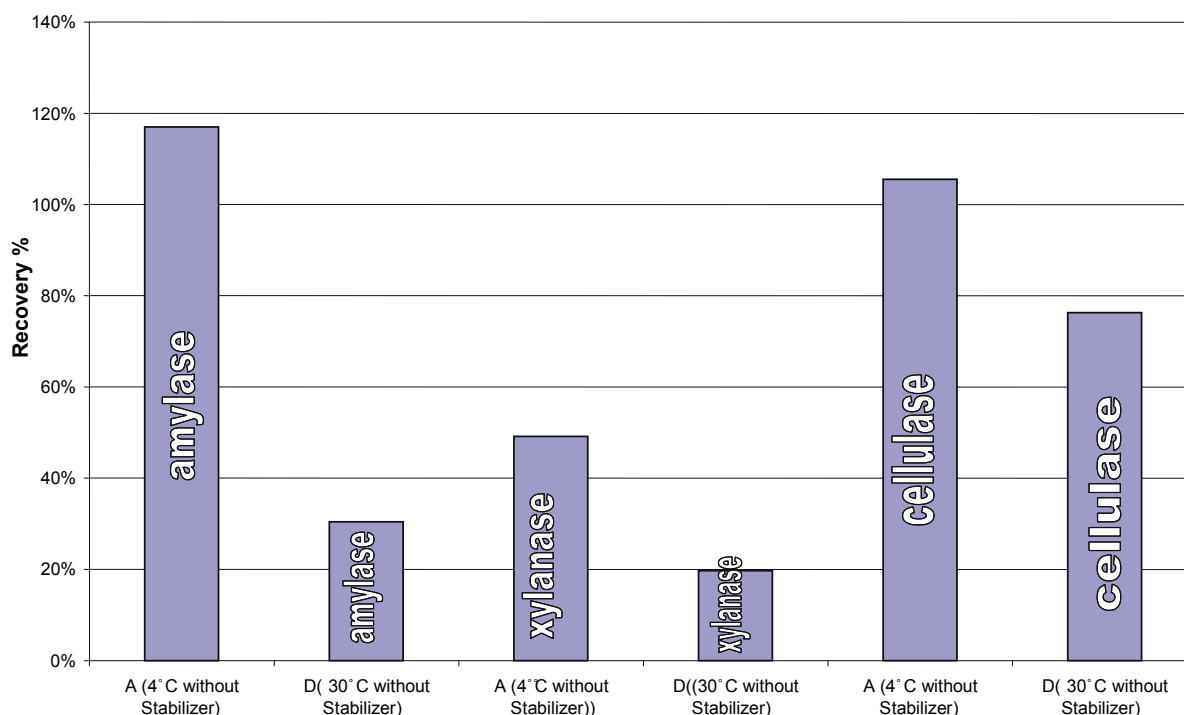


Fig. 4. Effect of storage temperature on the recovery of enzymes (without stabilizer)

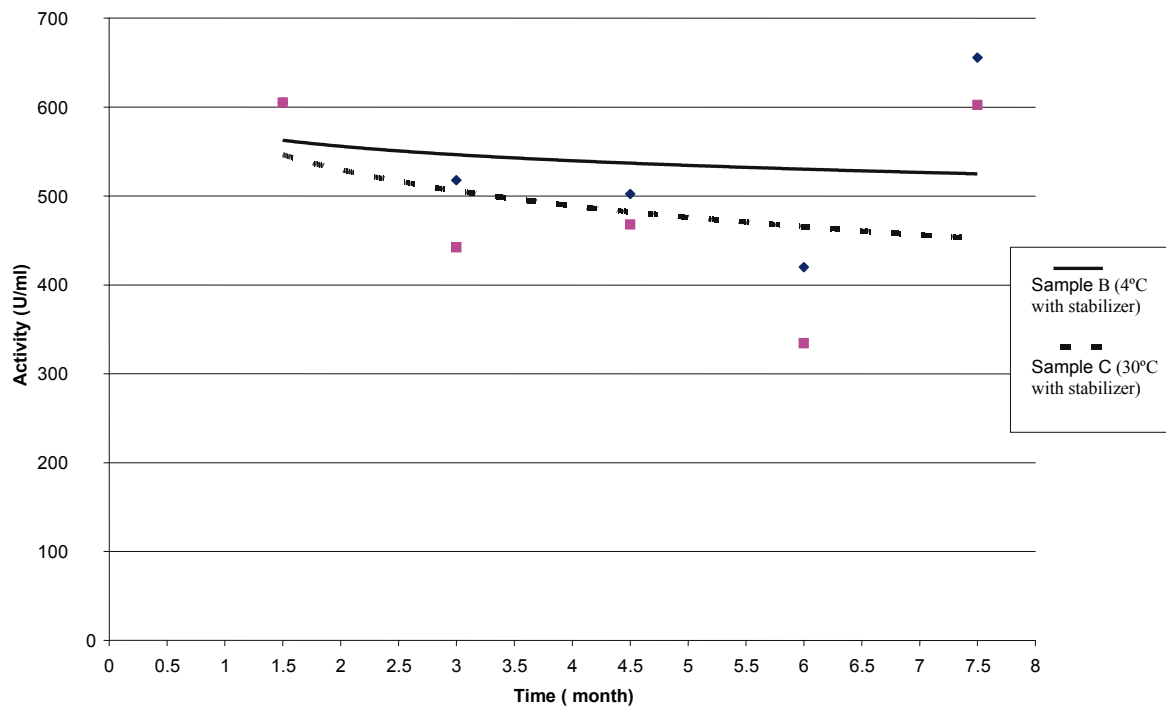


Fig. 5. Effect of different storage temperatures on xylanase shelf-life in commercial products having glycerol as a carrier

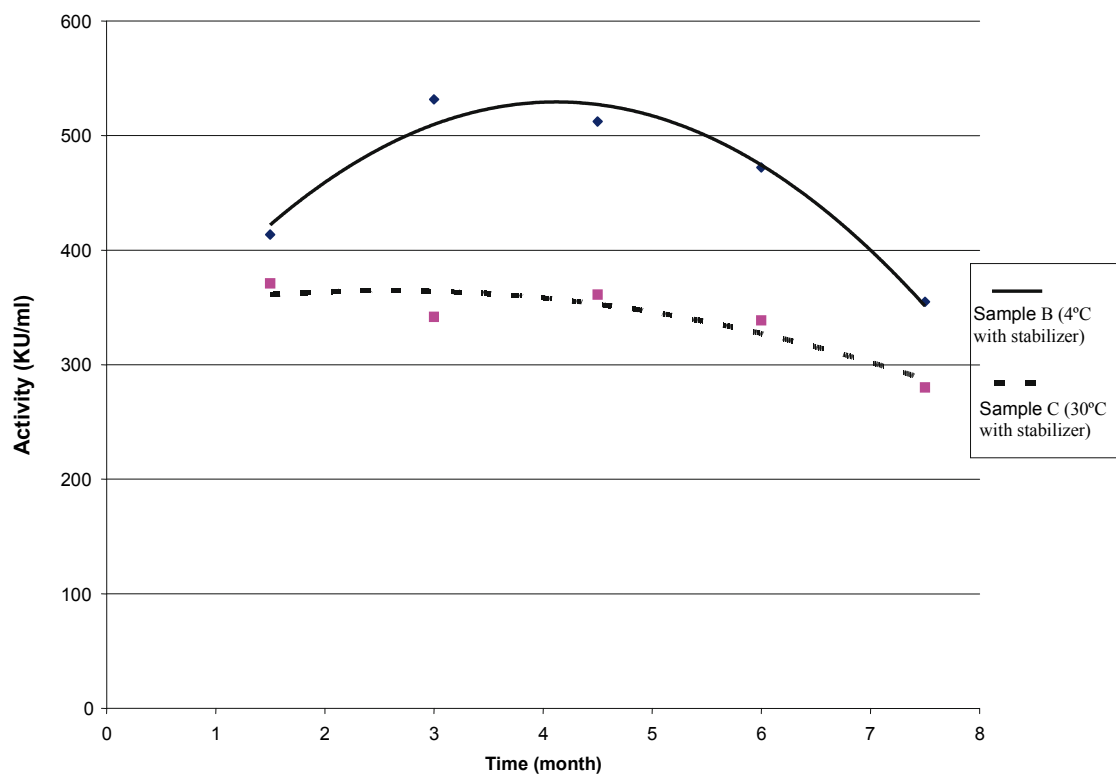


Fig. 6. Effect of different storage temperatures on cellulase shelf-life in commercial products having glycerol as a carrier

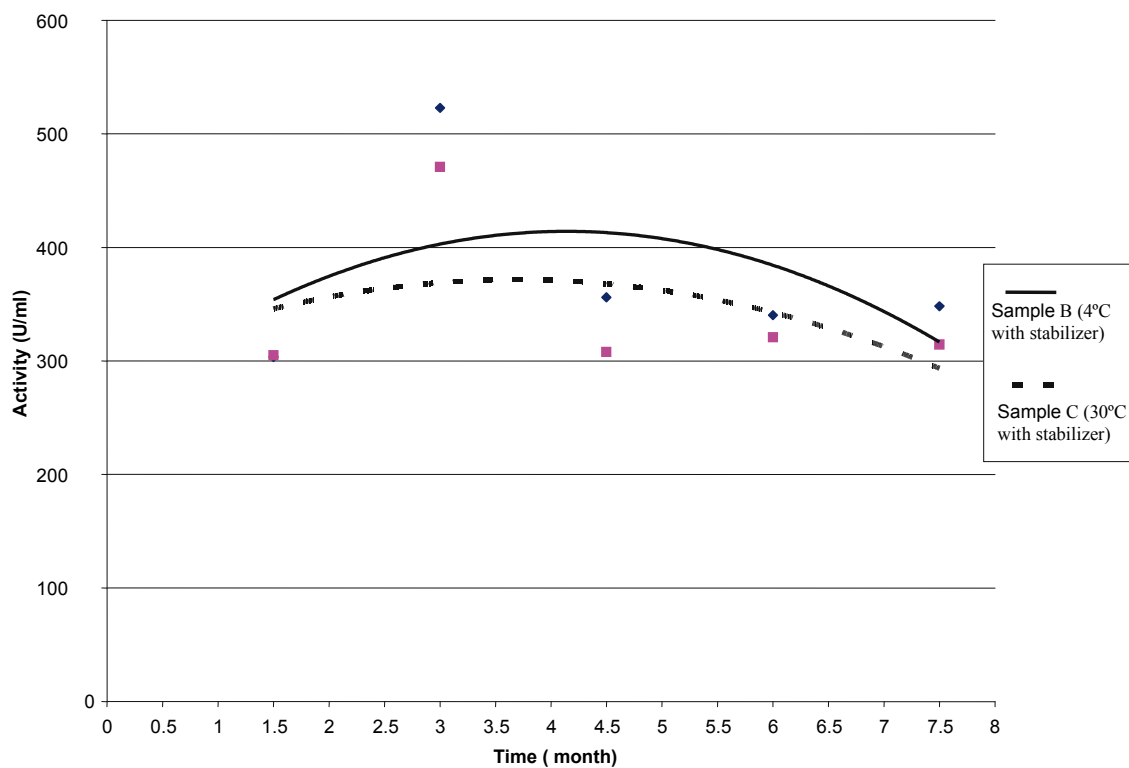


Fig. 7. Effect of different storage temperatures on amylase shelf-life in commercial products having glycerol as a carrier

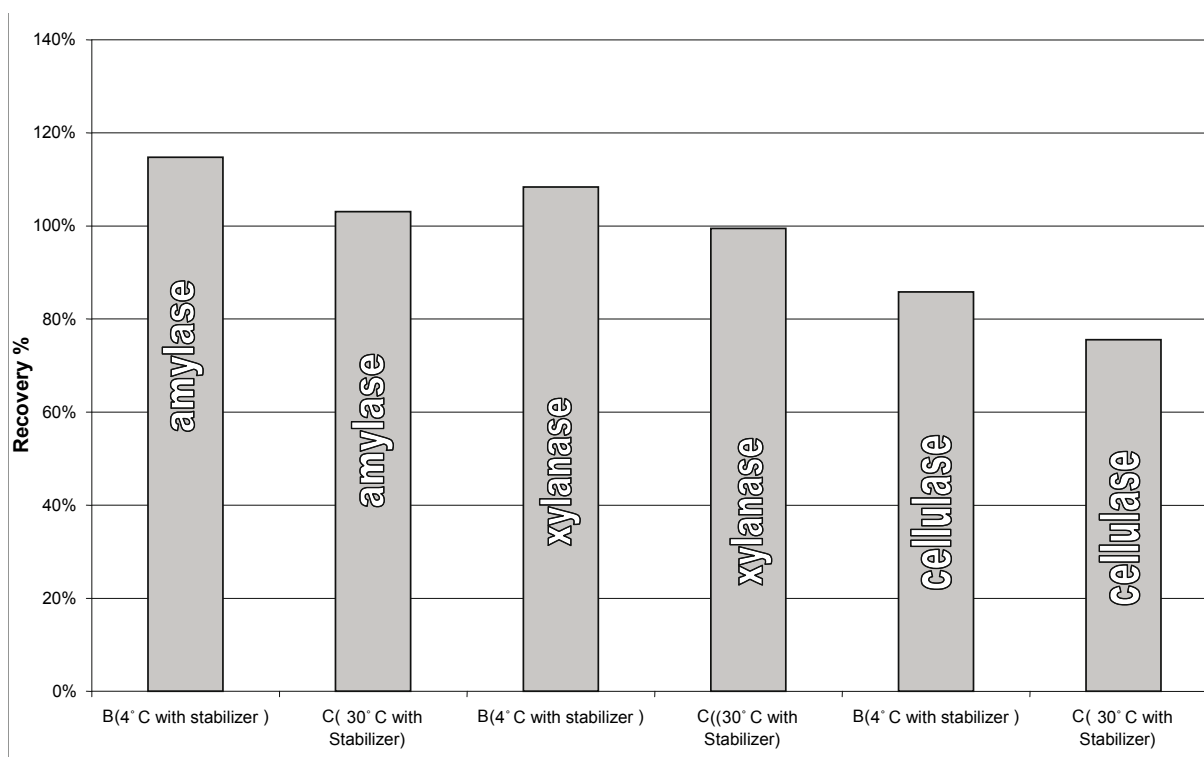


Fig. 8. Effect of storage temperature on the recovery of enzymes (with stabilizer)

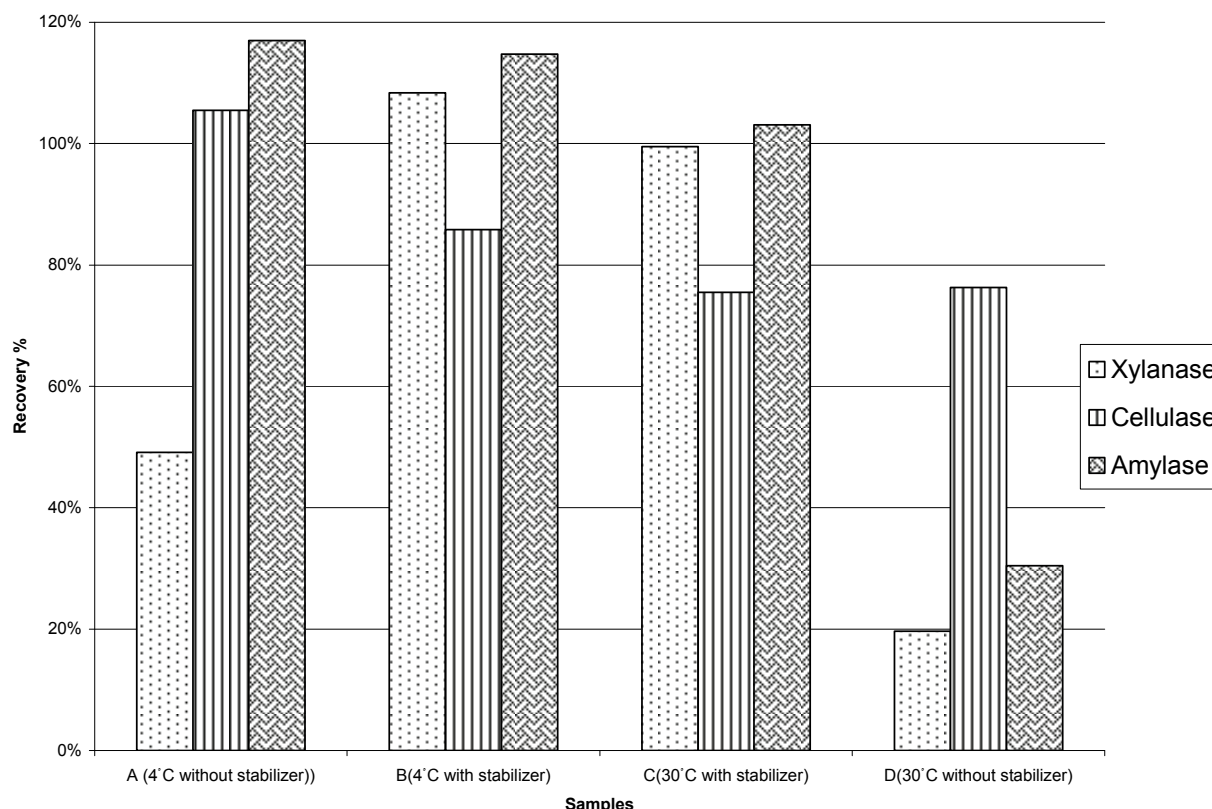


Fig. 9. Effect of stabilizer under different storage temperatures on xylanase, cellulase and amylase activities

Cellulase and amylase showed a similar trend (Fig. 9) when stored at 4 °C; sample (A) showed better recovery percentage comparing to sample (B). When the samples were stored at 30 °C the recovery percentage of amylase was better in sample (C) which was carried in glycerol compared to sample (D) carried in water, whereas cellulase gave nearly the same recoveries in both samples (Fig. 9).

Generally, samples stored at 4 °C whether carried in glycerol or water showed a better enzyme recovery percentage than those stored at 30 °C.

When the samples were analysed to see whether they were contaminated with their biological source or were contaminated by other enzymes, we found that sample 1 stored at 4 °C contained 2.8×10^3 CFU g⁻¹ of viable *Bacillus* sp. Also it was found that sample 1 contains a proteolytic activity; where sample 1 stored at 4 °C has a protease activity equal to 1321 U ml⁻¹ while that stored at 30 °C has protease activity equal to 3213 U ml⁻¹.

DISCUSSION

Liquid enzymes are inherently less storage stable than their granular counterparts. In this study, enzymes in sample 1 differed in their trend, where there was sharp decline in xylanase activity whether the sample was stored at 30 °C or 4 °C, while cellulase activity declined when the sample was stored at 30 °C. Amylase activity increased for 5 months then started to decline, yet its recovery was still high. When the sample was analysed for biological sources, it was found that sample 1 stored at 4 °C contained 2.8×10^3 CFU g⁻¹ of viable *Bacillus* sp.

In this product *Bacillus* sp. was the strain that was used in the production of amylase. This explains why amylase activity has increased at 4 °C. When proteolytic activity in sample 1 was measured, it was found that at 4 °C protease activity was 1321 U ml⁻¹ while at 30 °C it was 3213 U ml⁻¹. This proteolytic activity might have affected the xylanase activity. This is in agreement with Steen (2001); who stated that

commercially available enzymes are typically less than 100% pure and often contain side enzyme activities in addition to the desired main activities. These activities can include trace levels of proteases, which even at low activity can cause some degradation of the main enzyme (e.g. xylanase).

In general, enzymes are proteins and undergo essentially irreversible denaturation at temperatures above those to which they are ordinarily exposed in the environment. The actual loss of activity may be due to covalent changes such as the deamination of asparagine residue causing covalent changes such as the rearrangement of the protein chain (Anonymous 1990).

Even though enzymes in sample 1 and sample 2 had the same initial activities and were from the same biological source, each sample showed different trends regarding enzyme recovery across the period of storage in this study. This difference might be referred to the sample carrier, where in sample 1 water is the carrying material while glycerol is the carrying material in sample 2. According to (Steen 2001), in liquid form, water activity is sufficiently high for the component enzymes to remain active, whereas in granular form the low water activity renders the component enzymes relatively inactive prior to ingestion by the animal. It has been shown that the addition of sugars and polyols to aqueous solutions of enzymes strengthens the hydrophobic interactions among non-polar amino acid residues leading to protein rigidification and resistance to thermal deactivation (Padma and Ananthanarayan 2008). According to (George et al. 2001), glycerol has been found to stabilize extremophilic enzymes like xylanase.

Cellulase showed good stability compared to the other two enzymes when stored at 4 °C or at 30 °C whether carried in water or glycerol. This might be referred to the cellulosic enzyme resulting from *Trichoderma* sp. which is thermo stable and can handle the storage at different temperatures, although cellulase acts here in a way that may differ if the matrix is changed or even if the biological source is changed.

CONCLUSION

For shipment and storage of enzymes to tropical countries where the ambient temperature exceeds 22 °C, suppliers should ship and store enzymes in refrigerated containers that maintain the product

at a constant temperature below 5 °C prior to delivery to feed mill.

On the other hand, optimizing stabilizing parameters will not only lead to a more efficient enzyme but also will increase the economic potential in existing enzymatic processes and in novel areas where enzymes have not been used till now because of their instability.

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