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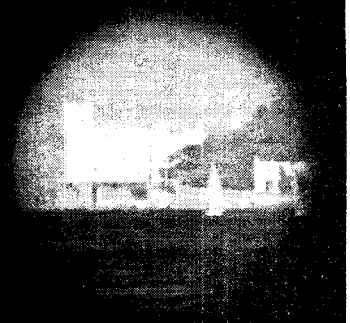

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ABSTRACT

Waste water from cassava mash fermented with pure strain of *Saccharomycees cerevisae* together with *Lactobacillus delbruckii* and *Lactobacillus coryneformis* (3days) was assayed for amylase activity. The result of the study indicated that the fermentation waste –water had amylase activity, the unit activity and the specific activity of the amylase in the waste water was 0.22μmole/min and 0.06μmole/min/mg, respectively. The amylase was partially purified using Gel filtration (Sephadex- G150). The partially purified enzyme was maximally active at pH 6.0 and 60°C temperature. It had is maximum stability between pH 6 – 7 for 4hr, and 30°C for 50mins. NaCl, NH_4Cl, $FeCl_3$, KCl, $NaNO_3$ activates the enzyme activity while $CUSO_4$ and $HgCl_2$ inhibit the activity of the amylase. It could be concluded that these amylases from the fermented cassava waste amylase were active at wide temperature and pH ranges, this quality could be explored in the industrial sector (most especially food industry) as a source of industrial amylase that requires a wide range of conditions (temperature and pH).

INTRODUCTION

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Similar to cellulose, starch molecules are glucose polymers linked together by the alpha-1,4 and alpha-1,6 glucosidic bonds, as opposed to the beta-1,4 glucosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylase, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units [1].

Since a wide variety of organisms, including humans, can digest starch, amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contains a large amount of amylase for starch digestion. The specificity of the bond attacked by amylases depends on the sources of the enzymes. Currently, two major classes of amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefing and sacccharifying. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called liquefaction, bacteria amylase usually does that. The final stages of depolarization are mainly the formation of mono-, di-, and tri-saccharides. This process is called saccharification, due to the formation of saccharides, saccharification is usually carried out by fungal amylase [1].

Two important wastes are generated during the processing of cassava for garri production, namely, the cassava peels and the liquid squeezed out of the mash. Cassava peels derived from garri processing are

normally discarded as wastes and allowed to rot. As a rough estimate, about 10 million tonnes of cassava are processed into garri annually in Nigeria alone [2]. Since these peels could make up to 10% of the wet weight of the roots, they constitute an important potential resource if properly harnessed by a bio-system [2]. The peels contain toxic cyanogenic glucosides [3], while the liquid contains a heavy load of microorganisms, lactic acid, lysine (from *L. coryneformis*), amylase (from *Saccharomycees spp*) and linamarase (from *L. delbruckii*) capable of hydrolyzing the glucosides [2,4,5]. The resulting products of fermentation of cassava peels with squeezed out liquid can be dried and used as animal feeds [2]. This study therefore aims at isolating and characterizing the amylases that may be present in the fermented cassava waste water.

MATERIALS AND METHOD

Materials

Cassava tubers were collected from the Research farm of the Federal University of Technology, Akure, Nigeria. The chemicals used were of analar grade, and glass distilled water was used.

Methods

Sample Preparation

Whole cassava tubers were peeled, washed, grated, after which 1 kg of the processed pulp was transferred into a desiccator. *Lactobacillus delbruckii, Lactobacillus coryneformis* and *Saccharomycees cerevisae* were inoculated into the pulp. The mash was allowed to ferment for three days. The pressed out waste water from the fermented cassava mash destined for cassava flour and gari production were collected and stored in the refrigerator for enzyme assay (Figure 1).

Determination of amylase activity of the waste water

Amylase activity was determined using a modification of the Alli *et. al.* [6] method. 1 ml of waste water was added to 1 ml of standard starch solution (containing 1% soluble starchand 0.006 M NaCl in 0.2 M phosphate buffer pH 6.9) and incubated at 30 °C for 30 minutes. Reducing sugars were determined by adding 2 ml of dinitrosalicylic acid (DNSA) reagent, boiled for 5 minutes and then cooled under running tap water. 20 ml of sterilized distilled water was then added and allowed to stabilize for about 5 mins. The absorbance of the resulting solution was determined at 540nm with Corning Colorimeter against a reagent blank. One unit of amylase activity was taken as the amount of enzyme in 1 ml of crude amylase that produced 1.0 mg of reducing sugars under the assay conditions.

Purification of crude amylase from waste water using Sephadex-G150

The resin, Sephadex G-150 was washed (twice) with 0.2 M phosphate buffer pH 6.9. 10ml sample was applied, and eluted by 0.2 M phosphate buffer pH 6.9.

Physico-chemical Studies on the Amylase Preparations

Effects of Temperature on Amylase Activity

The effect of temperature was assayed at 20-80 ^{o}C, pH 6.9 for 30 minutes. After a 10-minute incubation, amylase activity was determined for each temperature regime as described earlier.

Effects of Temperature on Amylase Stability

The thermal stability of the enzyme was determined by incubating about 4 ml of the pooled enzyme fraction at various temperatures between 20 and 90 ^{o}C without the substrate for 1 hr. At 10 minutes intervals, aliquots of 0.5 ml of the incubated enzyme were assayed for activity.

Effect of pH on Amylase Activity

The effect of pH on amylase activity was determined on starch solutions (1% starch and 0.006M NaCl) at pH 3.0 – 9.0, 30 ^{o}C for 30mins. The amylase activity was determined as outlined earlier.

Effect of pH on Amylase Stability

The effect of pH on the stability of the enzymes was carried out according to the method of Eke and Oguntimehin [7]. The enzyme solution was incubated at room temperature for 6 hrs in a 10 ml buffer solutions of pH 4-8. At 1h interval, aliquots of 0.5 ml from each mixture were assayed for activity under standard assay conditions.

Effect of Substrate Concentrations on Amylase Activity

Amylase activities of the various crude amylase preparations were determined at various substrate concentrations of 1-9% starch solutions containing 0.006M NaCl in 0.2M phosphate buffer at pH 6.9 by the method described earlier.

Effect of some salts on amylase activity .

Effect of some salts (NaCl, KCl $CuSO_4$, $NaNO_3$, $HgCl_2$, $FeCl_3$ and NH_4Cl) on amylase was determined as described earlier.

RESULTS AND DISCUSSION

The squeezed out water from micro-fungi fermented cassava products was assessed as a source of industrial enzymes [2]. Here, the waste water from *Saccharomycees cerevisae* together with *L. coryneformis,* and *L. delbruckii* fermented cassava products was screened for amylase activity. The results indicate the presence of amylase activity in the waste water. The unit activity and the specific activity of the amylase in the waste water was 0.22µmole/min and 0.06µmole/min/mg, respectively. This specific activity was higher than the specific activity reported by Oboh and Ajele [8] on the activity of the crude ß-amylase from sweet potatoes. This indicates that the activity of this enzyme is appreciably higher when compared to some plant amylases.

Figure 2 shows the elution profile of the purified amylase from waste water from *Saccharomycees cerevisae* fermented cassava products. Three peaks [A (21 –30), B (31 –39), C (40 –47)] having amylase activity were obtained after partial purification on sephadex – G150 gel. The specific activity of the pooled peaks were as follows; peak A had 0.13µmole/min/mg, while peak B and C had 0.08µmole/min/mg respectively. This would possibly indicate that the amylase exists in three forms in the waste water. This assertion could be attributed to the fact that the fermented cassava waste water activity could have been from the cassava tuber itself, *Saccharomycees cerevisae*, *Lactobacillus coryneformis* and *Lactobacillus delbruckii* [2]. This results agreed with that of ungerminated and germinated rice seeds where five kinds of amylase were reported. Moreover, *Bacillus polymyxa* ß-amylase was also reported to show two kinds of amylase with only a slight difference in their isoelectric points [9].

The effect of pH on the enzyme activity (Figure 3) and stability (Figure 4) indicates that the micro-fungi fermented waste water amylase is active in the pH range 3 - 7. This would suggest that the enzyme will be useful in processes that are subject to wide range of pH change from acidic to neutral range and vice-versa. Most commonly described ß-amylase lacks significant activity at extreme pH (2-3) [7,10]. The waste water amylase had its optimal activity at pH 6. This result agreed with the optimal pH for certain fungi amylase reported by Alli *et. al.*[6]. The amylase from *A. flavus* and *M. pusillus* exhibited their peak amylase activity at pH 6.0, while ß-amylase from *Clostridium thermocellum* SS8 had an optimal pH of activity of 6.0 [11]. However, there was drastic decline in the activity of the enzyme at pH above 7, which indicates that the enzyme losses activity at alkaline region [7,11]. In agreement with Alli *et. al.* [6] reports on the pH activity of *A. flavus, A. niger, R.oryzae* and *M. pusillus,* the waste water showed some residual activity at pH 7 - 9.

The pH stability studies of the micro-fungi fermented waste water amylase, as shown in Figure 4, indicate a general decline in the activity of the enzyme within one to four hours of incubation except at pH 6.0 in which the enzyme was stable within the first two hours of incubation. The enzymes were more stable

at pH 6 and 7 as indicated by their activity over a period of four hours. However, they exhibited rapid decline in activity (stability) at extreme pH (4,5 and 8) [7,10].

The different pH optima for activity and stability obtained for different amylases from different sources may be explained in terms of the rate of protonation and deprotonation of the enzyme. This view was supported by Zhang *et. al.* [12] and Riha *et.al.*[13]. They suggested that deamination of asparagine and glutamine, a protonation process which leads to the formation of aspartic acid and glutamic acid respectively, occurs at different rates depending on the source of the protonating agent (that is the enzyme). The protonation and deprotonation rates, however, could be due to the uniqueness of the amino acid constituent, that is, sequence and proportion in any particular enzyme from a given source. It has also been observed that exchange of disulphide bonds and destruction of cystine residues by ß-elimination have considerable effect on enzyme activity, especially in alkaline environment.

Effect of temperature on the activity and stability of *Saccharomycees cerevisae* fermented cassava waste water amylase is shown in Figure 5 & 6, the result indicated a gradual increase in the enzyme activity at 20 – 50°C, while there was a drastic increase in the activity of the enzyme at 50 - 60 °C. The enzyme has its maximum activity at 60°C, while there was a decline in its activity after 60 °C. However, the enzyme was active within a large temperatuere range of 20 - 80 °C. This attribute could be exploited in industrial activity that requires a wide temperature range (20 - 80 °C). The optimal temperature for maximum activity of the fermented cassava waste water amylase (60 °C) is the same with that of ß-amylase from *Clostridium thermocellum* SS8 [11], *Bacillus circulans* [14] and *Bacillus megaterium* [15]. The result also agrees with the temperature for optimal activity reported for *A. flavus* and *M. pusillus* [6]. However, there was a rapid decline in the activity of the enzyme at temperature above 60 °C, this agreed with Alli *et. al.* [6] to the extent that, apart from *A. niger* amylase that showed appreciable activity at 70 °C, *A. flavus, M. pusillus* and *R. oryzae* amylase activity rapidly declined.

The temperature stability study indicates that there was a general decrease in the stability of the enzyme with increase in time (0 – 50mins) at all the temperatures tested (30 - 70 °C), as indicated by decrease in activity of the enzyme. This decrease in stability increases with increase in temperature as shown in Figure 6, with the enzyme showing the highest stability at 30 °C and the least stability at 70 °C. This temperature inactivation of the enzyme can be attributed to the formation of incorrect conformation due to processes such as hydrolysis of the peptide chain, destruction of amino acid and aggregation [16, 17, 18].

As shown in Figure 7, there was a drastic increase in the enzyme activity with increase in starch concentration from 1.0 to 3.0%, a minimal increase from 3.0 to 6.0%, while there was a slight decline in the activity of the enzyme from 6 - 7% starch, and subsequently the activity remained constant. This result is in agreement with Alli *et. al.* [6] report on the effect of substrate concentration on some fungi (*A. flavus, A. niger,R. oryzae* and *M. pusillus*) amylase activity, which indicated that increase in substrate concentration

from 1% to 3% led to progressive increase in amylase activity (*A. flavus, A. niger,R. oryzae* and *M. pusillus*). This indicates that for optimal utilization of resources, the use of the amylase from this waste water amylase should be correlated with the starch to be hydrolyzed at 3% substrate concentration level.

Effects of some salts on the activity of the amylase are shown in Figure 8. As earlier stated, NaCl, NH_4Cl, $FeCl_3$, KCl and $NaNO_3$ activated the activity of the amylase, while $CuSO_4$ and $HgCl_2$ decreased the activity of the enzyme. The activation of the enzyme by various concentration of NH_4Cl, $FeCl_3$ and KCl except for NaCl which had no effect at 0 – 20 mM concentration of the salt, may probably be due to the presence of chloride ions. Previous studies reported by Vega-Villasante *et.al.* [19]; Oboh and Ajele [8] and Mohapatra *et. al.* [20] have shown that metallic chlorides are usually potent activators of amylases.

NH_4Cl gave the highest activation at virtually all the concentration tested (10 – 70 mM), while $NaNO_3$ (non-chloride) gave the least activation at all the concentrations tested (10 –70 mM). It could be reasoned that the chloride ion alone might not necessarily be responsible for the activation, since a non-chloride salt $NaNO_3$ also activates the enzyme. Moreover, if it is responsible alone, one would expect the same degree of activation to be the same for all the metallic chloride tested. This argument is in line with findings of Wakim *et. al.* [21] which showed that halides activate mammalian amylase activity, but that the halide ions are not mandatory for the activity of the enzyme. However, the activation of the enzyme could be a contribution from the chloride ion and the cations [8].

The inhibition of the enzyme activity by $CuSO_4$ and $HgCl_2$ could be due to Cu^{2+} and Hg^{2+}. Babu *et.al.* [22] and Chang *et.al.* [23] had both reported inhibition of ß- amylase by $CuSO_4$ and $HgCl_2$. Swamy *et. al.* [11] had earlier reported that high molecular weight metal ions such as Ag^+, Fe^{2+}, Zn^{2+} Cu^{2+} and Hg^+ inhibited the activity of ß- amylase from *Clostridium thermocellum* SS8.

The results obtained from this assessment of the waste water micro-fungi fermented cassava products indicate that the waste water had amylolytic activity (amylase). The physico-chemical properties indicate that the amylases in the waste water could be of three types. Furthermore, the result of the physico-chemical studies compared more favourably with fungi amylase than either bacteria or plant amylase, which indicate that the domineering amylase in the waste water could be fungi amylase from *Saccharomycees cerevisae.* The fact that the amylases were active at wide temperature and pH ranges could be explored in the industrial sector as a source of industrial amylase that require a wide range of conditions (temperature and pH).

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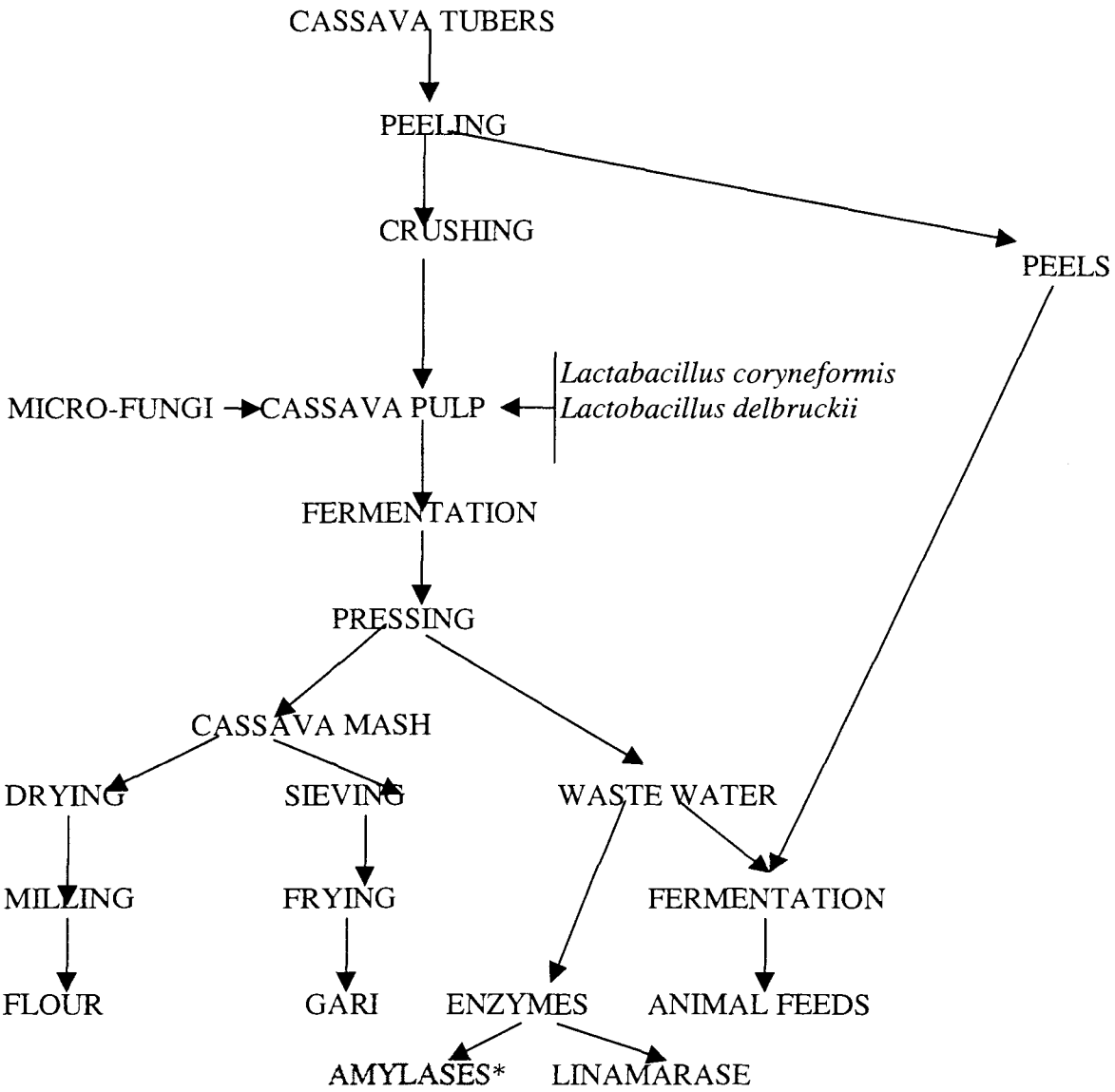

Figure 1:Production chart for micro-fungi fermented cassava products

* present study

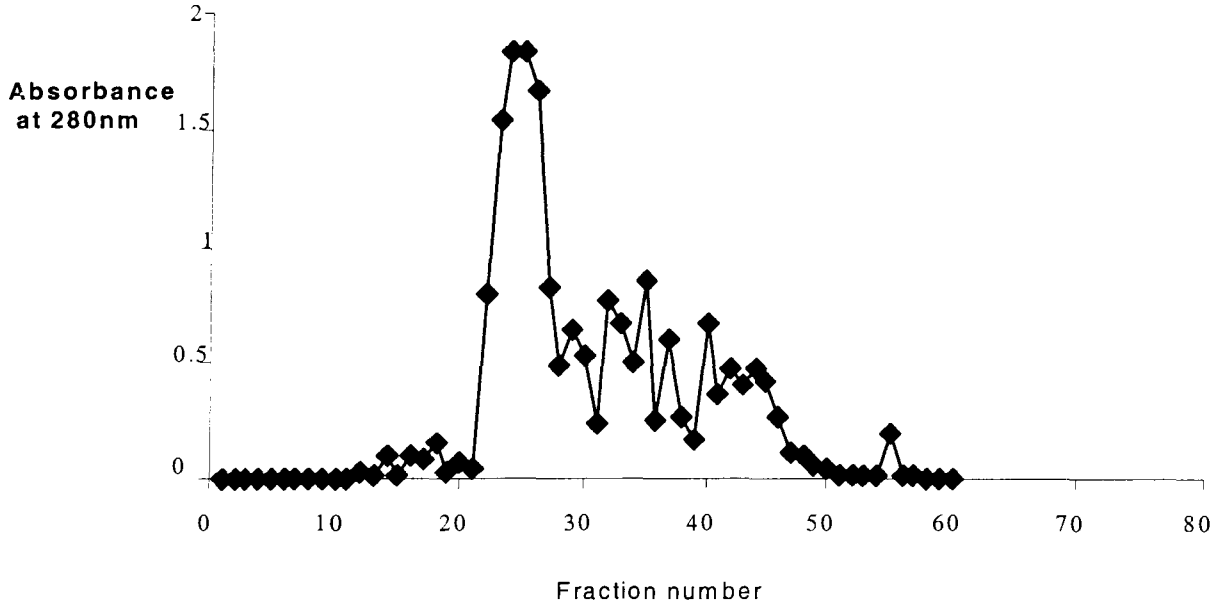

Figure 2: Elution profile of amylase from micro-fungi fermented cassava waste water using Sephadex G150

Table 1: Purification summary of amylase from fermented cassava waste water.

Sample	Vol. (ml)	Protein Conc. (mg/ml)	Unit activity (µmol./min)	Specific activity (µmol./min/mg)
Crude extract	250	3.80	2.2	0.6
Acetone Precipitation	50	5.00	7.6	1.5
Sephadex-G150 Purification				
A	50	6.00	7.8	1.3
B	45	4.30	3.3	0.8
C	40	3.50	2.7	0.8

Legends: A, pooled fractions 21-30; B, pooled fractions 31-39; C, pooled fractions 40 –47.

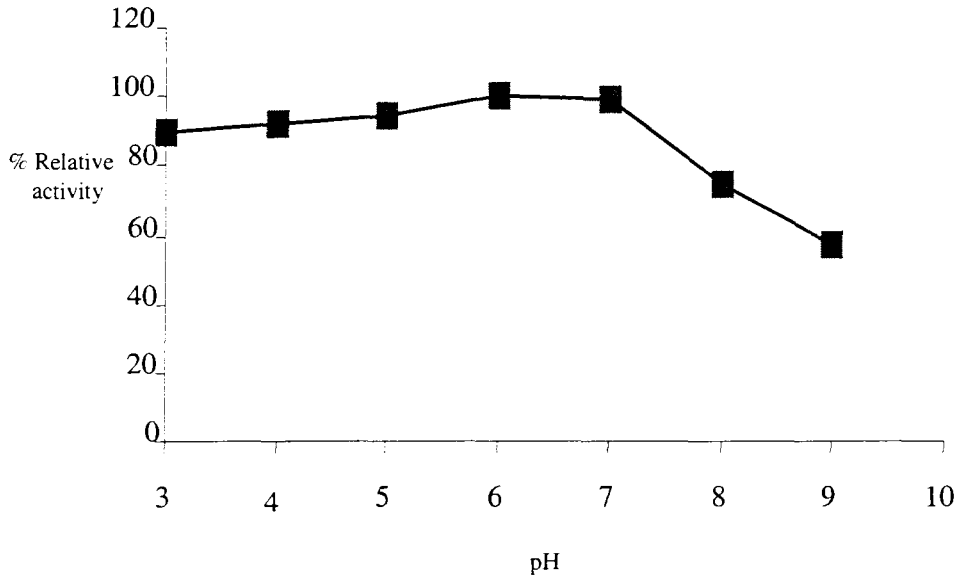

Figure 3: Effect of pH on the activity of amylase from fermented cassava waste-water

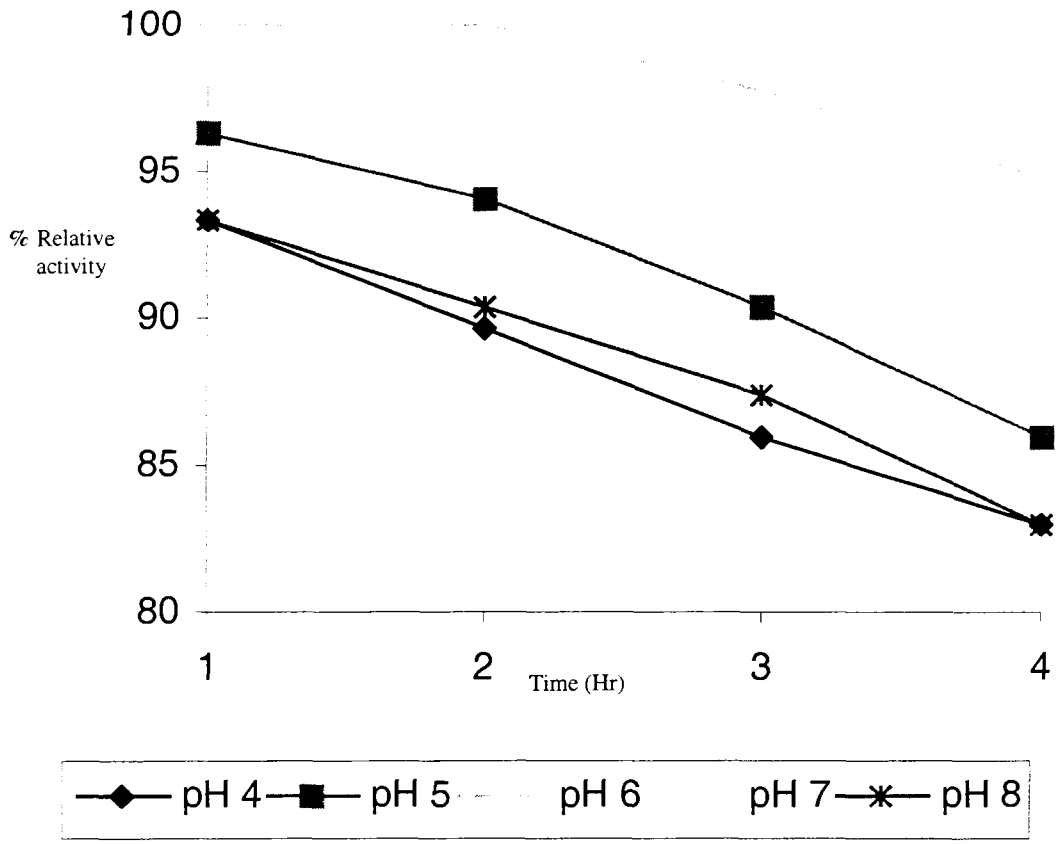

Figure 4: pH stability of amylase from fermented cassava waste water

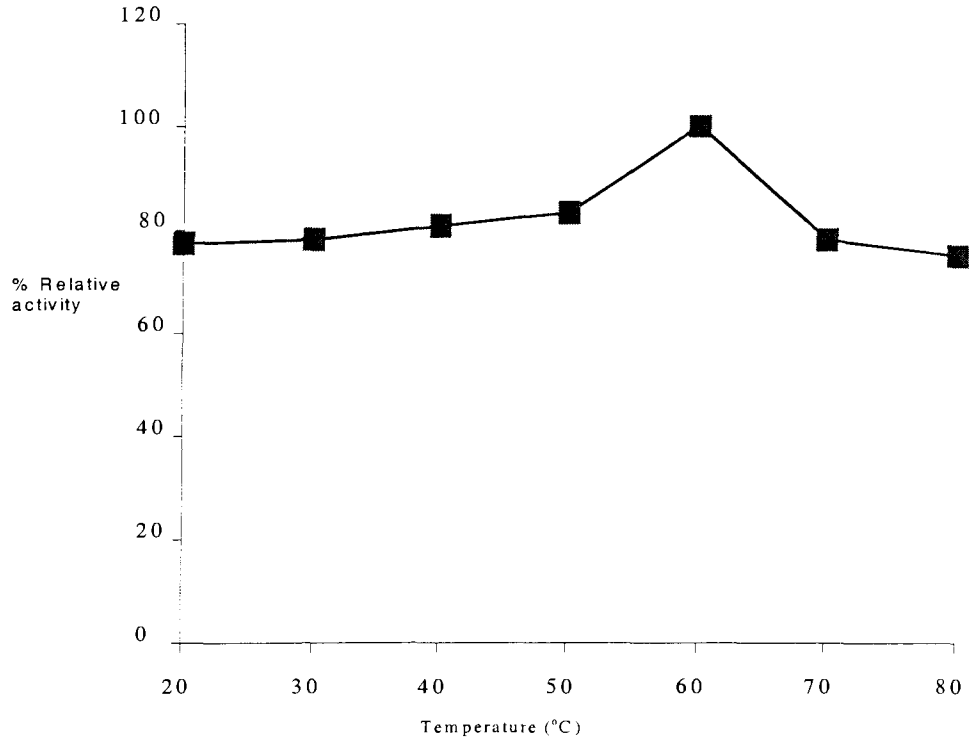

Figure 5: Effect of temperature on the activity of amylase from fermented cassava waste-water

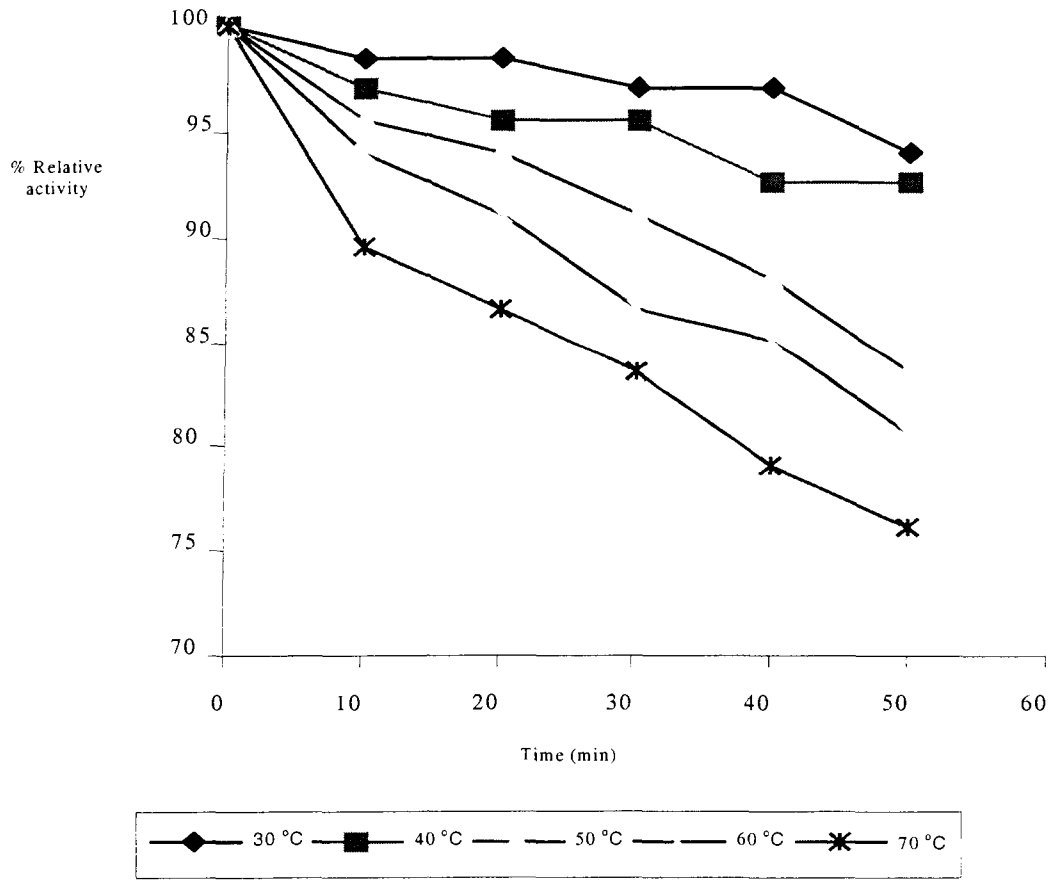

Figure 6: Temperature stability of amylase from fermented cassava waste water

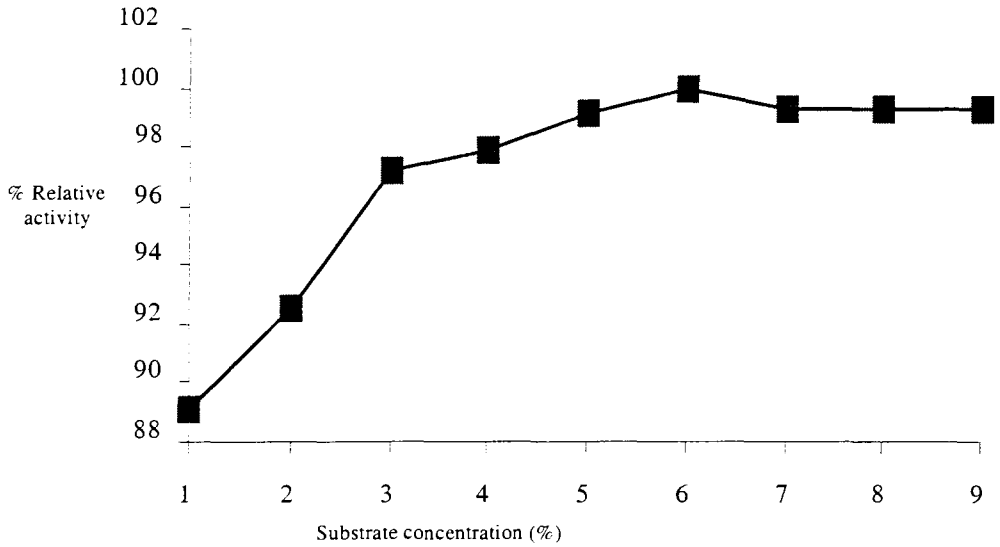

Figure 7: Effect of substrate concentration on the activity of amylase from fermented cassava waste-water

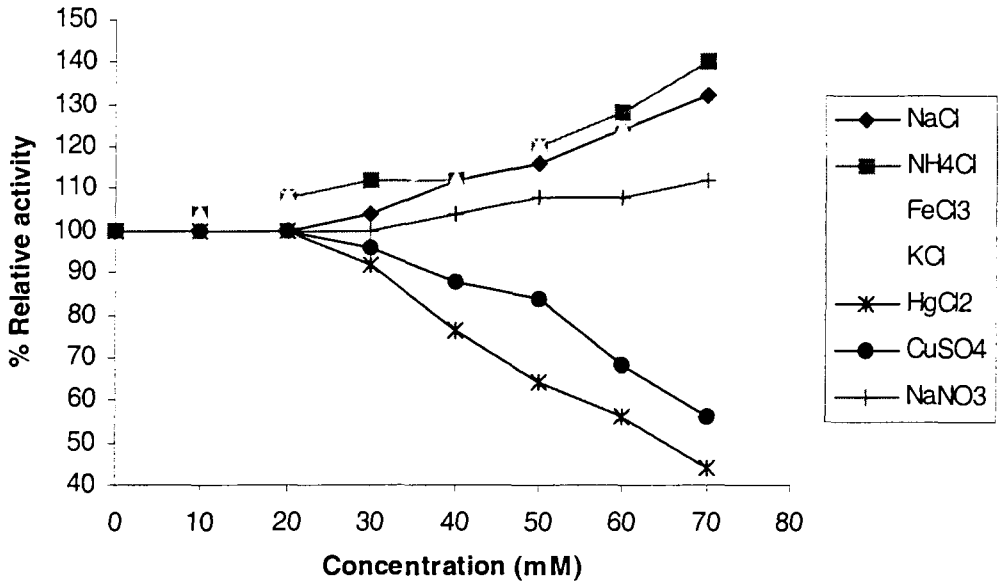

Figure 8: Effect of some salts on the activity of amylase from fermented cassava waste water.