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Full Length Research Paper

Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) waste-water

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The processing of cassava tubers for the production of nutrient enriched cassava flour, gari and farinha madioca, is usually accompanied with the production of stinking wastewater which usually constitute nuisance to both terrestrial and aquatic life. Thus, this study sought to assess the potential utilization of the wastewater as a source of industrial amylase. Wastewater from cassava mash fermented with pure strains of *Saccharomyces cerevisae* together with *Lactobacillus delbruckii* and *Lactobacillus coryneformis* for 3 days was assayed for amylase activity. The result of the study indicated that the fermentation wastewater had amylase activity, the unit activity and the specific activity of the amylase in the wastewater was 0.22 µmole/min and 0.06 µmole/min/mg, respectively. The amylase was purified using gel filtration (Sephadex- G150). The purified enzyme was maximally active at pH 6.0 and 60°C temperature. It had is maximum stability between pH 6 – 7 for 4 h, and 30°C for 50 min. 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 therefore be concluded that these amylases from the fermented cassava waste water are 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 require a wide range of conditions (temperature and pH).

Key words: Cassava, fermentation, wastewater, amylase, industry.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial vegetatively propagated shrub cultivated throughout the lowland tropics for its starchy roots. It is of great economic importance to several tropical countries of Africa where its consumption (in terms of carbohydrate content) exceeds those of other crops. The advantages it has over other root crops include its propagation, high yield, pest and drought resistance. However, certain varieties contain a large amount of cyanogenic glucosides (linamarin and lotaustralin) which can be hydrolysed to hydrocyanic acid (HCN) by the endogenous enzyme (linamarase) when the plant tissue is damaged during harvesting, processing or other mechanical processes (Oboh and Akindahunsi, 2003a).

Two principal methods are available for increasing the protein content of fermented cassava products. The first method involves adding protein to the deficient food from external sources in such a way as not to alter significantly the organoleptic qualities of the original food. The second, through controlled fermentation, microflora could be made in large numbers in the mash (Rembault, 1998; Oboh et al., 2002; Oboh and Akindahunsi, 2003a). Solid media fermentation of cassava products has been reported in Canada where *Aspergillus fumigatus* was

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used, in Burundi and Nigeria where *Rhizopus oryzae* and *Aspergillus niger* were used in enriching cassava products with proteins (Akindahunsi et al., 1999; Oboh et. al., 2002). Recently, Oboh and Akindahunsi (2003a) reported that *S. cerevisae* (10.5%) could also be used for enriching cassava products through solid substrate fermentation.

This processing of cassava tubers into the various forms (gari, fufu, lafun, pupuru) in which it is popular consumed in Nigeria and farinha de madioca in South America is not without a lot of hazards both to the environment and the processors. The two major wastes generated during cassava processing namely the cassava peels and the effluents were reported to cause a lot of havoc to vegetation, houses and bring about infection of microbe and infestation. While the traditional processing method presently adopted in the country has lead to various pathology ranging from general body high temperature due to smoke from the frying pan to fatigue, ache and pains (Oboh, 2004).

Two important wastes are generated during the processing of cassava tubers, 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 (Okafor, 1998). 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 (Okafor, 1998; Oboh and Akindahunsi, 2003b; Oboh, 2005). The peels contain toxic cyanogenic glucosides (Oke, 1968), 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 (Raimbault, 1998; Akindahunsi et. al. 199). The resulting products of fermentation of cassava peels with squeezed out liquid can be dried and used as animal feeds (Okafor, 1998, Oboh and Akindahunsi, 2003b; Oboh, 2005). This study therefore aims at isolating and characterizing the amylases that may be present in the fermented cassava wastewater for possible industrial use.

MATERIALS AND METHOD

Materials

Cassava tubers were collected from the Research farm of the Federal University of Technology, Akure, Nigeria. The chemicals used were analytical grade, while the water was glass distilled. The microorganisms were collected from Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria.

Sample preparation

Whole tuberous roots of cassava were peeled, washed, grated, after which 1 kg of the processed pulp was spread in a tray (about 50 cm diameter) to an average layer thickness of 2 cm, a 10 g mixture of freshly sub-cultured pure strains of *L. delbruckii, L. coryneformis* and *S. cerevisae* (2: 1: 1) was carefully added to the solid matrix in order to obtain a well homogenized mixture. The mash was allowed to ferment for three days (as it is normally done in the production of nutrient enriched cassava flour and gari); the incubation temperature and the relative humidity of the air were 30ºC and 90-93%. After the fermentation the wastewater was pressed out and stored in the refrigerator for amylase analysis (Figure 1).

Determination of amylase activity of the waste water

Amylase activity was determined using a modification of Alli et al. (1998) method. 1 ml of waste water was added to 1 ml of standard starch solution (containing 1% soluble starch and 0.006 M NaCl in 0.2 M phosphate buffer pH 6.9) and incubated at 30°C for 30 min. Reducing sugars were determined by adding 2 ml of dinitrosalicylic acid (DNSA) reagent, boiled for 5 min and then cooled under running tap water. 20 ml of sterilized distilled water was then added and allowed to stabilize for about 5 min. The absorbance of the resulting solution was determined at 540 nm 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

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

Effects of temperature on amylase activity

The effect of temperature was assayed at 20-80°C, pH 6.9 for 30 min. After a 10 min incubation, amylase activity was determined for each temperature regime as earlier described .

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°C without the substrate for 1 h. At 10 min 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 0.006 M NaCl) at pH 3.0 – 9.0, 30°C for 30 min. The amylase activity was determined as earlier outlined.

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 (1992). The enzyme solution was incubated at room temperature for 6 h in a 10

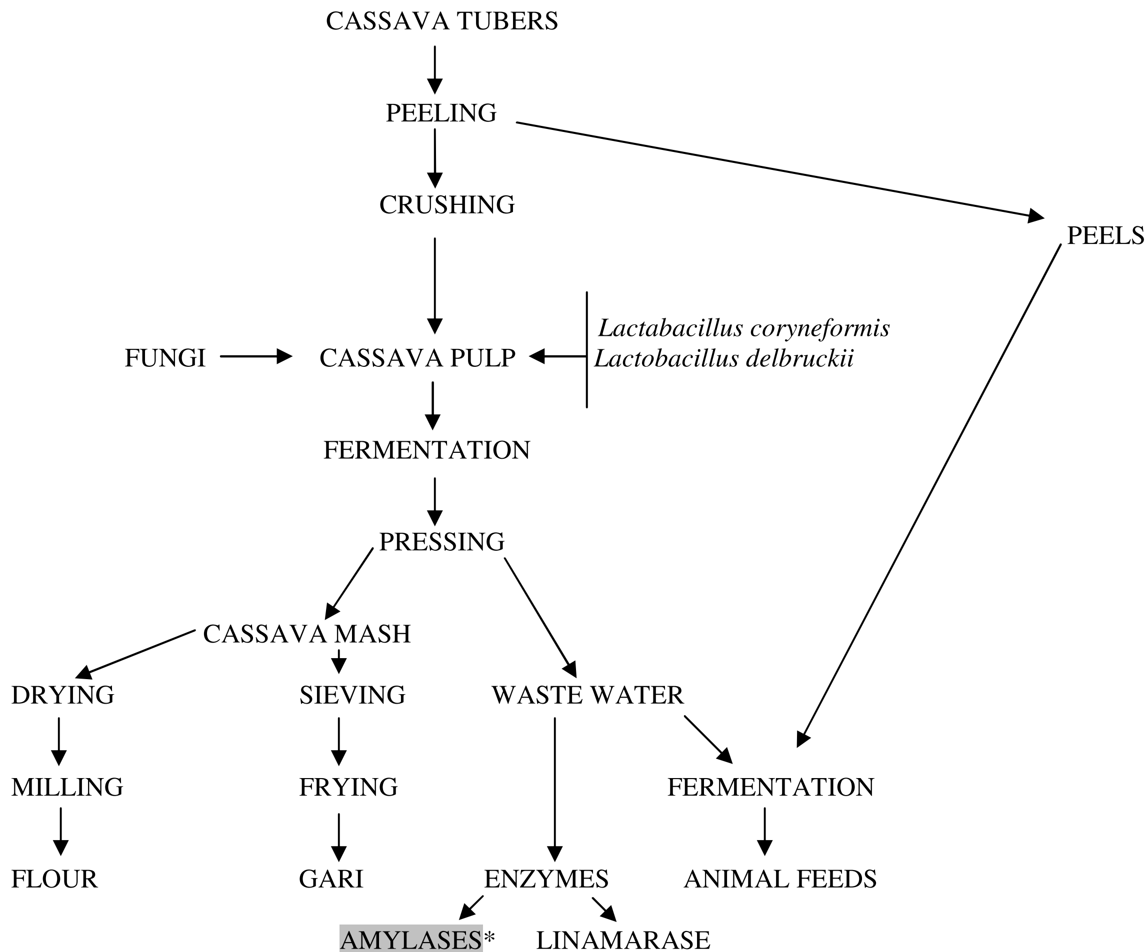

Figure 1. Production chart for micro-fungi fermented cassava products.
*present study

ml buffer solution 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 0.006 M NaCl in 0.2 M phosphate buffer at pH 6.9 by the method earlier described.

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 earlier described.

RESULTS AND DISCUSSION

The squeezed out liquid from the fermented cassava pulp was assessed as a source of industrial enzymes (Okafor, 1998).The result of the study indicates the presence of amylase activity in the waste water. The activity and the specific activity of the amylase in the wastewater were 0.22 µmole/min and 0.06 µmole/min/mg, respectively.This specific activity was higher than the specific activity reported by Oboh and Ajele (1997) on the activity of the crude β--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 wastewater from the fermented cassava pulp. Three peaks [A (21–30), B (31–39), C (40–47)] having amylase activity were obtained after purification on sephadex-G150 gel (Table 1). The specific activities of the pooled peaks were 0.13, 0.08 and 0.08 for peaks A, B and C, respectively. This would possibly indicate that the amylase exists in three forms in the wastewater. This assertion could be attributed to the fact that the fermented cassava wastewater amylase activity could be from the cassava tuber itself and from the fermenting organisms; *S. cerevisae*, L. coryneformis and L. delbruckii (Okafor, 1998). These results agreed

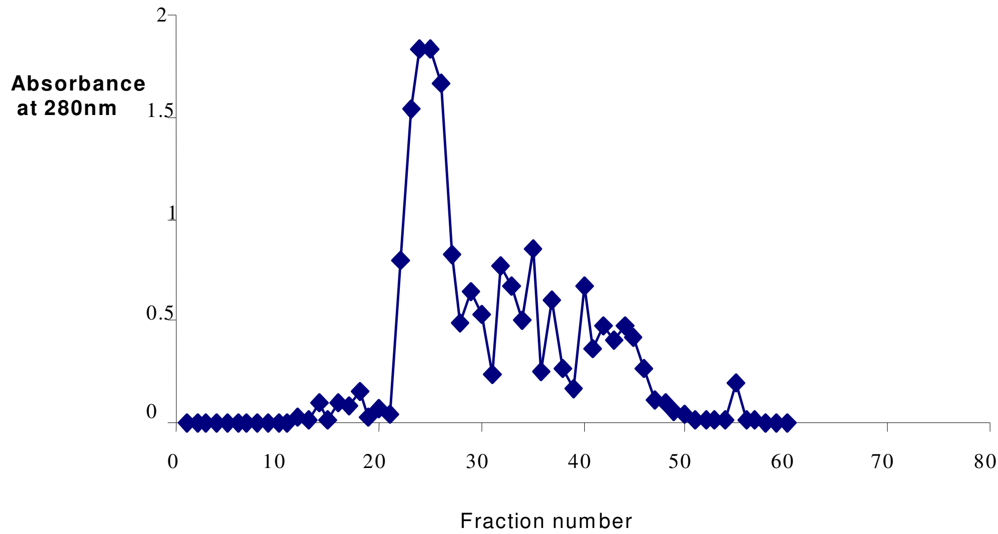

Figure 2. Elution profile of amylase from micro-fungi fermented cassava waste water using Sephadex G150

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

Sample activities (µ mol/min/mg)	Vol (ml)	Protein Conc. (mg/ml)	Unit activity (µmol/min)	Specific activity (µ mol/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.

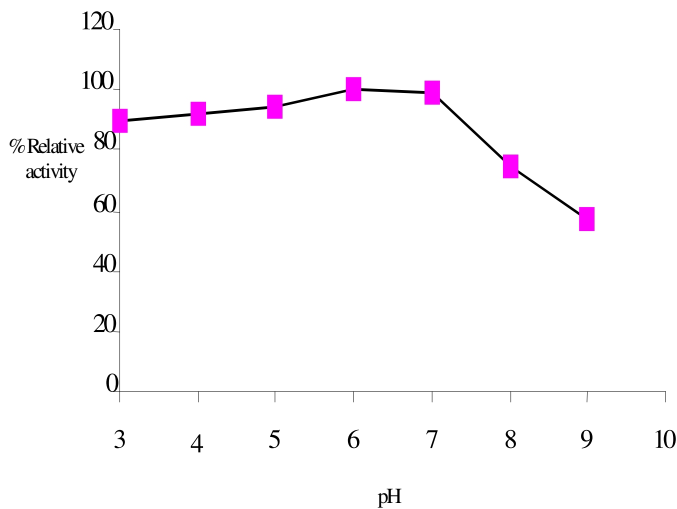

Figure 3. Effect of pH on the activity of amylase from fermented cassava waste-water.

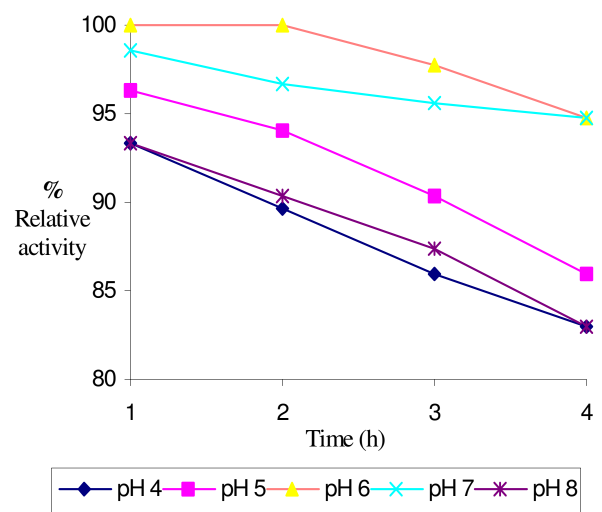

Figure 4. pH stability of amylase from fermented cassava waste water.

with that of ungerminated and germinated rice seeds where five kinds of amylase were reported. Moreover, *Bacillus polymyxa* β-amylase was also reported to show two kinds of amylase with only a slight difference in their isoelectric points (Eke and Oguntimehin, 1992).

The effect of pH on the enzyme activity (Figure 3) and stability (Figure 4) indicates that the wastewater amylase is active in the pH range 3 - 7. This suggests that the enzyme would be useful in processes that require wide range of pH change from acidic to neutral range and vice-versa. Most commonly described β-amylase lacks significant activity at extreme pH (2-3) (Eke and Oguntimehin, 1992; Hyun and Zeikus, 1985). The wastewater amylase had its optimal activity at pH 6. This result agreed with the optimal pH for certain fungal (*Aspergillus flavus* and *M. pusillus*) amylase activities reported by Alli et al. (1998). The β-amylase from *Clostridium thermocellum* SS8 also have an optimal activity at pH 6.0 (Swamy et al., 1994). However, there was drastic decline in the activity of the enzyme at pH above 7, which indicates that the enzyme looses activity at alkaline region (Eke and Oguntimehin, 1992; Hyun and Zeikus, 1985). However, the wastewater amylase showed some residual activity at pH 7 – 9 which is in agreement with Alli et al. (1998) report on the pH activity of amylase from *A. flavus, Aspergillus niger, Rhizopus oryzae* and *M. pusillus,*

The pH stability studies of the 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) (Eke and Oguntimehin, 1992; Hyun and Zeikus, 1985).

Effect of temperature on the activity and stability of the wastewater amylase is shown in Figures 5 and 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. The optimal temperature for maximum activity of the fermented cassava waste water amylase (60°C) is the same with that of β-amylase from *C. thermocellum* SS8 (Swamy et al., 1994), *Bacillus circulans* (Kwan et al., 1993) and *Bacillus megaterium* (Ray et al., 1995). The result also agrees with the temperature for optimal activity reported for *A. flavus* and *M. pusillus* (Alli et al., 1998). However, there was a rapid decline in the activity of the enzyme at temperature above 60°C, which agrees with Alli et al. (1998) reports to the extent that, apart from *A. niger* amylase that showed appreciable activity at 70°C, *A.*

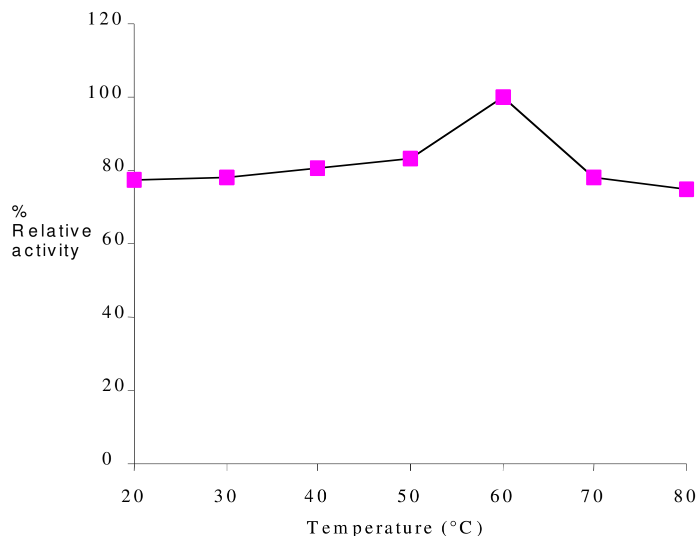

Figure 5. Effect of temperature on the activity of amylase from fermented cassava waste-water.

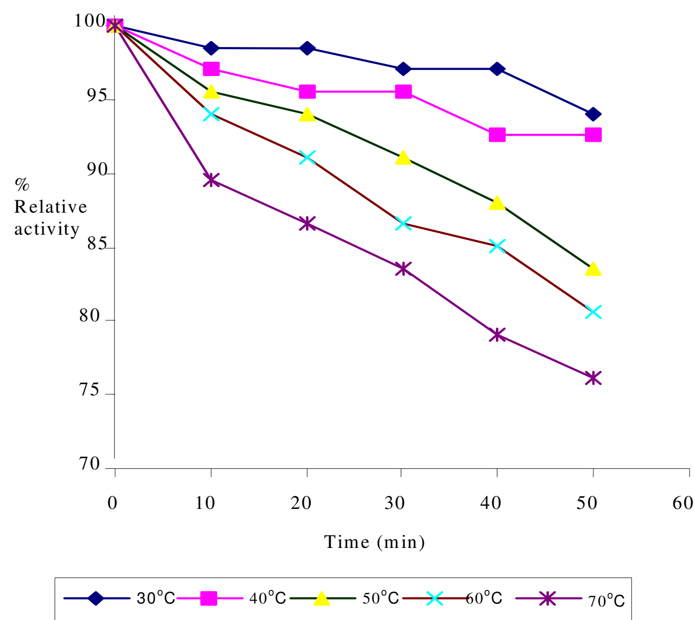

Figure 6. Temperature stability of amylase from fermented cassava waste water.

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 – 50 min) 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

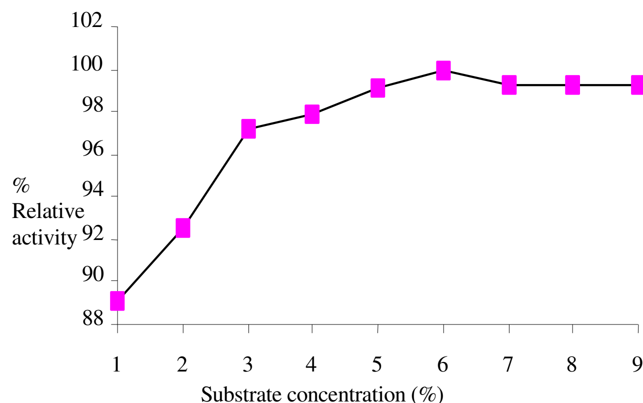

Figure 7. Effect of substrate concentration on the activity of amylase from fermented cassava waste-water.

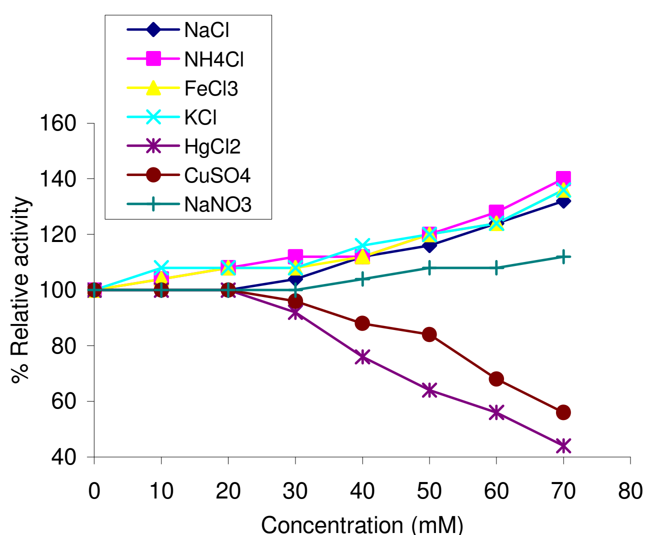

Figure 8. Effect of some salts on the activity of amylase from fermented cassava waste water.

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 (Schokker and Van Boekel, 1999).

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. (1998) report on the effect of substrate concentration on some fungal (*A. flavus, A. niger, R. oryzae* and *M. pusillus*) amylase activities, 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 wastewater 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 concentrations 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. (1993), Oboh and Ajele (1997) and Mohapatra et al. (1998) 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 degree of activation to be the same for all the metallic chloride tested. This argument is in line with the findings of Wakim et al. (1969) 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 (Oboh and Ajele, 1997).

The inhibition of the enzyme activity by $CuSO_4$ and $HgCl_2$ could be due to Cu^{2+} and Hg^{2+}. Chang et al. (1996) had both reported inhibition of β-amylase by $CuSO_4$ and $HgCl_2$. Swamy et al. (1994) had earlier reported that high molecular weight metal ions such as Ag^+, Fe^{2+}, Zn^{2+} Cu^{2+} and Hg^+ inhibited the activity of β-amylase from *C. thermocellum* SS8.

The results obtained from this assessment of the waste water fungal 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 fungal amylases than plant amylases, which indicate that the domineering amylase in the waste water could be fungal amylase from *S. 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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