

THE ROLE OF LINAMARASE IN CASSAVA (MANIHOT  
ESCULENTA) DETOXIFICATION DURING FERMENTATION FOR  
GARI PRODUCTION

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BY

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## DEDICATION

Dedicated to my lather Mr. J.U. Onyike, to my Mother Mrs. H.U. onyike and to my brothers and sisters for their continued love.

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I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

Date .....

Signed by \_\_\_\_\_  
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### ABSTRACT

The detoxification of fermenting cassava through hydrolysis of the constituent cyanogenic glucosides was attributed to endogenous linamarase activity rather than to the hydrolytic activity of organic acids generated by the fermentative activity of micro-organisms; The addition of partially purified cassava cortex linamarase to fermenting grated cassava resulted in 8-fold detoxification; and the gari produced contain innocuous levels of cyanide. D-glucono (1→5) lactone reduced linamarase activity considerably, while the cyanide content still remained high\*

Microbial source of linamarase-like activity was sought. Some fungi;

Penicillium citrinum., Aspergillus zydowii Aspergillus terreus, Aspergillus Ochraceus Fusarium equiseti, Fusarium Moniliforme Penicillium steckii, have shown the ability to catalyze formation of p-nitrophenolate ion from para-nitrophenyl- $\alpha$ -D glucoside, an analogue of linamarin. They were also able to hydrolyze linamarin; linamarase assay indicated presence of cyanide.

Quality control tests on 13 gari samples from different localities in Nigeria were conducted. Factors considered were crude fiber, starch, acidity, swelling power, moisture content, cyanide level, appearance and foreign material., Based on these quality criteria including taste, the laboratory made gari scored very high.

## CHAPTER 1

### INTRODUCTION

About two-thirds of the developing worlds population live in countries where food output has been rising more slowly than effective demand for food. In the third world as a whole, the actual number of hungry persons have quite certainly increased (UN/FAO, 1975).

The inability to produce sufficient food is mainly because the crops that can grow best in these areas have not been given sufficient attention. The root crops and tubers that can grow very well in these areas have been neglected while a great deal of research effort has been put into developing cereal grains. High yielding varieties of cereal grains have been developed but these haven't solved world's food problem. At a symposium by the United Nations Research Institute for Social Development (UNRISD) on food and new agricultural technology, Ingrid Palmer (1972) pointed out that the African continent is in a stationary state of per capita food consumption and nutrition, and that the efforts that have gone into high yielding varieties for rice in West African countries and maize in Kenya do not appear to have done more than hold the line in aggregate per capita terms. He further described Africa's nutritional picture as characterized by physical environment generally unfavourable to delicate high

yielding varieties, and a colonial pattern of export oriented agriculture.

Although production statistics for cassava are notoriously unreliable, the best available evidence indicates that on a tonnage basis, annual world production is only exceeded by six other crops (UN/FAO 1971). Under experimental conditions yields of more than seventy tons per hectare have been obtained in a twelve-month period, inspite of the fact that cassava has received relatively little attention from agricultural scientists (Nartey 1978). Although there are major difficulties in bringing about yield and quality improvements in this species, the potential for yield improvements appear to be considerably greater than that of many plant species that have been subjected to intensive study for many years (Nartey 1978). Cassava productivity in terms of calories per unit land area per unit of time appears to be significantly higher than that of other staple food crops (de Vries et. al. 1967). Coursey and Haynes (1970) indicated that cassava can produce  $250 \times 10^3$  calories per hectare per day as compared to  $176 \times 10^3$  for rice,  $110 \times 10^3$  for wheat,  $200 \times 10^3$  for maize, and  $114 \times 10^3$  for sorghum. Cassava therefore is an important dietary staple and a cheap source of calories for 200 - 300 million people in the tropical developing world.

The growing use of cassava for human consumption and animal feed could be hampered by the presence of toxic cyanogens in its tubers (Ikediobi et. al. 1980)

These toxic cyanogens are present in cassava as cyanogenic glucosides, linamarin and lotaustralin. Like all cyanogenic glucosides, linamarin and lotaustralin possess a bitter taste and are readily soluble in cold water and dilute acids, in which they undergo slow decomposition. Their glycosidic bonds are readily cleaved by hot dilute acids, forming D-glucose and the corresponding  $\alpha$ -hydroxynitriles which readily dissociate into a carbonyl compound and HCN, a process catalyzed by bases. Their glycosidic bonds are stable in bases, which generally convert the glycosides to the acid derivatives and ammonia (Nartey 1978). Naturally occurring linamarin and lotaustralin have been shown to be stable to acid hydrolysis (Wood 1966).

The crushing or other wise destroying of the cellular integrity of cassava tissue leads to the release of the sugar moiety and the  $\alpha$ -cyanohydrin components of linamarin and lotaustralin. This reaction is catalyzed by a  $\beta$ -glycosidase in cassava tissue, so that on disruption of the roots both enzyme and substrate diffuse together from their cellular compartments, thus resulting in the hydrolysis of the substrates (Nartey 1978). Following the cleavage of the glycosidic bond, the  $\alpha$ -hydroxynitrile formed dissociates into a ketone and HCN. This dissociation is catalyzed enzymatically by an  $\alpha$ -hydroxynitrile lyase (Oxynitrilase) in sorghum and in linum (Conn and Butler 1969) and probably the enzyme is present in cassava. The  $\alpha$ -cyanohydrins are capable of non enzymatic dissociation depending on their concentration and pH value of the medium.

As far as is known, no non-cyanogenic cassava clones have yet been found (De Bruijn 1973). The use of its fresh tissues for food or feed leads to the evolution of hydrogen cyanide in humans and animals, resulting in various degrees of cyanide poisoning and sometimes death. Acute toxicity arises from the consumption of poorly prepared or completely unprocessed tubers (Bolhuis 1954) from which large amounts of HCN may be released. The situation may result in symptoms of acute cyanide poisoning such as abdominal pains, vomiting, mental confusion, muscular paralysis, and respiratory distress on account of the cytochrome oxidase systems, followed by coma (Montgomery 1969). Chronic cassava toxicity, which is probably more serious, arises from the use of cassava and its products as staple food, which results in exposure of millions of people in the developing countries to constant or prolonged low levels of HCN released probably from linamarin and lotaustralin present in cassava foodstuffs (Nartey, 1973). Cyanide is normally detoxified in animals by conversion to thiocyanate and cyanocobalamine in enzyme catalyzed reactions in which inorganic and organic sulphur compounds as well as hydroxo cobalamin act as substrates (Nartey 1973; Oke 1973). It has been shown that high diets of cassava products lead to increased levels of cyanide and thiocyanate, and decreased levels of sulphur amino acids and vitamin B<sub>12</sub> (Osuntokun, 1973, Ekpechi 1973, Delange *et. al.* 1973, Wilson 1973) in the blood of patients suffering from goitre, tropical ataxic neuropathy and vitamin B<sub>12</sub> deficiency.

Traditional methods of processing cassava for food do not remove all the cyanogenic glucosides present in the cassava. These traditional methods involve either simple boiling or drying in the sun or fermenting for 3 or 4 days before cooking, roasting or drying in the sun (Coursey 1973). During fermentation, hydrolysis of the cyanogenic glucosides in cassava are known to take place, but the precise mechanism of this hydrolysis is not yet clear. A thorough knowledge of the basis of cassava detoxification during fermentation would lead to the establishment of optimum conditions which could be utilized in an industry for producing cassava products with little or no cyanide (Ikediobi et. al. 1980).

## LITERATURE REVIEW

Cassava (Manihot esculenta Crantz) belong to the family of euphorbiaceae. It is a herbacious or semi-shrubby perennial whose botanical characteristics show tremendous variation within species, so indicating a large degree of hybridization; interspecific, intraspecific, and both (Magoon 1967).

Native of tropical America, cassava was brought into cultivation probably 4,000 years ago (Coursey et. al. 1970); it was later taken into cultivation to Africa, countries bordering the Indian ocean, and finally to the Pacific. In Africa, the Portuguese took it to their stations around the mouth of the Congo river, and to the island in the Bight of Benin during the last half of the 16th century, and it was probably introduced to the countries bordering the Indian Ocean, Ceylon, Malaya and Indonesia during the 18th century (Jones 1959).

### Plant Characteristics and features:

At its base, the plant consists of one or more stems two to three centimeters in diameter. Usually each stem divides into three branches and each branch in turn divides into three and so on. When the stem is cut, the sap that flows is white and looks much like milk. Inside the stem is the pith. The stem of cassava is not very hard: It is easily broken by a strong wind (U.N./F.A.O., 1977).

Cassava leaves have a long stalk and much divided leaf blade. The leaf veins are green and red. The flowers are pink, red, yellow or green. There are both male and female flowers

in the same cluster. The fruit is divided into three parts and each part contains a seed (U.N./F.A.O., 1977).

The farmer grows cassava chiefly for its roots. Some of the roots become large and fat by storing up reserves, other thinner roots continue to feed the plant (U.N./F.A.O., 1977). The mature root tuber possesses three distinct regions namely, the thin brown outer skin, the cortex or peel and flesh or parenchyma tissue. (U.N./F.A.O., 1977).

#### Distribution:

Cassava is a short day plant whose distribution in Africa is confined largely to areas lying between latitude  $15^{\circ}$  North and  $15^{\circ}$  South, where the annual rainfall expectation is 75mm, although it is also cultivated in the savannah regions where the annual rainfall is significantly less (Nartey 1978).

Two-thirds of the world's cassava comes from Brazil, Indonesia, Zaire, Nigeria and India. Other cassava producing areas are Mozambique, Uganda, Thailand, Paraguay, Burundi, Ghana, Angola, Tanzania, Madagascar, Togo, Colombia, Central African Republic, Cameroun, Dahomey, North Vietnam, Ivory Coast, Guinea, Peru and sixty-three other countries (Nartey, 1978).

In Nigeria cassava is produced mostly in the Southern part of the country. Production is largely in the hands of small scale farmers. These farmers are now moving out of the rural areas to get jobs in the urban areas. As a result, there is shortage of cassava, and cassava products are now expensive in our local markets (personal observation).

Currently two federal government funded research Institutes: Federal Institute of Industrial Research Oshodi and National Root Crops Research Institute Umudike, are looking into the cassava utilization problem.

#### Cultivation.

The plant is hardy and will thrive under the most diverse conditions of climate except frost. It thrives from sea level to elevations as high as 3500 feet and can withstand and survive dry season for six months (Oke 1968, Nartey 1978).

Propagation and cultivation of cassava is very simple; 25 centimeter cuttings of cassava stem from plants 10 - 12 months old are planted one metre apart on top of ridges so that buds point upwards (NRCRI advisory bulletin 1978). About two-thirds of the stick is buried in the soil in a slanting position ( $45^{\circ}$ ). Cassava can be grown as a sole crop or with other crops like maize or cow peas. As a sole crop it can be planted any time from April to October. If it is planted with maize, the best time to plant it is with the early rains of March - April. If cassava is to be inter-cropped with cow-peas, the best time to plant is in July/August (NRCRI advisory bulletin 1978). Cassava plant matures within 12 - 15 months after planting. It is best to harvest it when the soil is wet, especially when it is planted on hard soils, to avoid damage to tubers. It can be left in the ground for considerable periods without serious damage, and harvested whenever needed (Coursey and Haynes 1970).

When harvested, it is advisable to use tubers within 24 hours. After about 2 days, it becomes stale and discolouration of the flesh begins to take place and this continues inwards towards the centre of the tuber. The substances responsible for these discolourations have been extracted and analyzed at the Tropical Products Institute, London. They are 2-leuco-anthocyanin, leucocyanidin and leucodelphin which are colourless compounds located in the inner corky layer of the cortex, and turn bluish mauve under alkaline conditions (Oke, 1968). The discolouration reactions are due to internal bruises which render that part of cassava more susceptible to oxidation and decay. Cassava can be stored for about 4 - 5 days by burying in moist earth or about a week by keeping in an atmosphere of  $\text{CO}_2$  (Oke 1968), and up to 10 days at  $4^\circ\text{C}$ .

Cassava is known to be attacked by more than 30 diseases, but the two most important ones in Nigeria are, the cassava bacterial blight (CBB) whose symptoms are water-soaked angular spots, wilting of branches, gum exudate on stems or green branches and die back of aerial parts. These symptoms are most evident in the rainy season. The other disease, common in Nigeria is the cassava Mosaic disease (CMD) and the most obvious symptoms are chlorosis or discrete areas of the lamina distortion and reduction of size of affected leaves. Both diseases cause considerable reduction in yield. (NRCRI advisory bulletin 1978).

When a cassava field is flooded or when the field is in a water logged area, the roots get rotten. Rot is known to reduce yield. (NRCRI advisory bulletin 1978).

Nutritive value:

Fresh unprocessed cassava is very rich in ascorbic acid and calcium and appears to have nutritionally significant amounts of thiamine, riboflavin, niacin and iron (Oke 1968, 1967, Jones 1959). The protein content is very small and of low quality and lacks the sulphur-containing amino acids, cysteine and methionine. Fresh unprocessed cassava tubers also have very high amount of toxic cyanogens which exist largely as the aliphatic glucosides, linamarin and lotaustralin (Butler et. al. 1960, Butler and Conn 1964, Butler 1965).

Processed and unprocessed cassava tubers are rich in carbohydrates and so are a rich source of calories. Processed cassava, especially that which has undergone the process of fermentation has protein content of 1 - 5 percent (Jones 1959, Oke 1968). Values from different sources vary; they depend largely on the method of analysis. There is evidence that processing increases the protein content of gari (Strasser et. al. 1970), a popular cassava product.

Vitamin and mineral contents of cassava are reduced to insignificant amounts by processing. The amount of each vitamin and mineral that remains in the final products depends on the kind of treatment it has undergone. As food for human consumption, cassava products are a good source of calories (Coursey, 1973).

It is generally eaten for its calories rather than for nutritional purposes. Vegetable, meat and fish stews or soups are commonly eaten along with it (Oke, 1968). There have been several attempts to fortify cassava products with protein and/or vitamins from fungi or soya beans, but none has been quite satisfactory (Reade et. al. 1975, Joseph 1973).

#### International Research on Cassava.

As food products, the root crops of which cassava is one, have higher biological efficiency (Coursey and Haynes, 1970). Coursey and Haynes (1970) attribute this efficiency to structural engineering considerations since the edible part of the tuberous roots lies beneath the ground and does not have to be supported by a stem. Infact 60-85 percent of total dry weight of root crops may be edible, whereas in wheat, the figure is only up to 36 percent (Nestle 1970). In order to take advantage of the biological efficiency of cassava and to develop the crop, more efficient use for it have to be found.

Two major new international agricultural research centres, the International Centre for Tropical Agriculture (CIAT) in Colombia and the International Institute for Tropical Agriculture (IITA) in Ibadan, Nigeria, were created in the early 1970's to find ways of solving problems connected with root crops.

The CIAT board of trustees approved a full objective scale cassava programme in June 1971. The importance of cyanide toxicity does not form a major part of CIAT programme. They are

looking for low or zero cyanide or linamarin varieties of cassava to be used in their breeding program, so as to eliminate the problem of chronic toxicity entirely. They are neither searching for new or improved methods of detoxification nor attempting to investigate the mechanism (Cock, 1973).

IITA's main interest is in cassava utilization by humans and livestock. Selection for acyanogenesis by IITA is based on finding cassava lines that lack the glucosides, linamarin, and lotaustralin, the  $\beta$ -glucosidase (linamarase) or both glucoside and  $\beta$ -glucosidase (Sidki et. al. 1973).

### Cassava toxicity

The first reference to toxicity of cassava was made by Clusius in 1605 and the association of cassava toxicity with bitter principle was first recognized in 1830 by Richard Medianna who isolated Manihotoxin from cassava tubers (Nartey 1978). The association of the bitter and toxic Manihotoxin with hydrocyanic was subsequently made by Henry in 1834 and Henry and Bortron-Charland in 1836, while the occurrence of HCN in bound form as cyanogenic glycoside was first recognized by Henry in 1836 (Nartey 1978). In 1906, Dunstan, Henry and Auld isolated and identified in cassava tubers a compound which proved to be identical with phaseolunatin and which occurs in phaseolus lunatis and Linum usitatissimum and for which Jorrison and Hairs had adopted the name linamarin in 1891. Linamarin is structurally 2-(B-D-glucopyranosyloxy) isobutyronitrile; under enzymic or acidic hydrolysis it liberates free HCN, together with acetone and glucose.

Linamarin and Lotaustralin occur together in a wide variety of higher plant species belonging to the families of Euphorbiaceae, Compositae, Fabaceae, Linaceae, and Papaveraceae. Among the Euphorbiaceae, the cyanogenic glucosides occur in Manihot esculenta in the ratio linamarin: lotaustralin of 93:7 (Nartey 1968). Small variations have been noted in the concentrations of lotaustralin in cassava plants from various regions, but the general trend is that this glucoside forms a

very small proportion of the total cyanogenic material in the species (Pisset *et. al.* 1969; De Bruijn 1973).

Lotaustralin hydrolyses under similar conditions as linamarin to yield HCN, methylethylketone and glucose. The figure below shows the structures and hydrolytic products of Linamarin and lotaustralin.

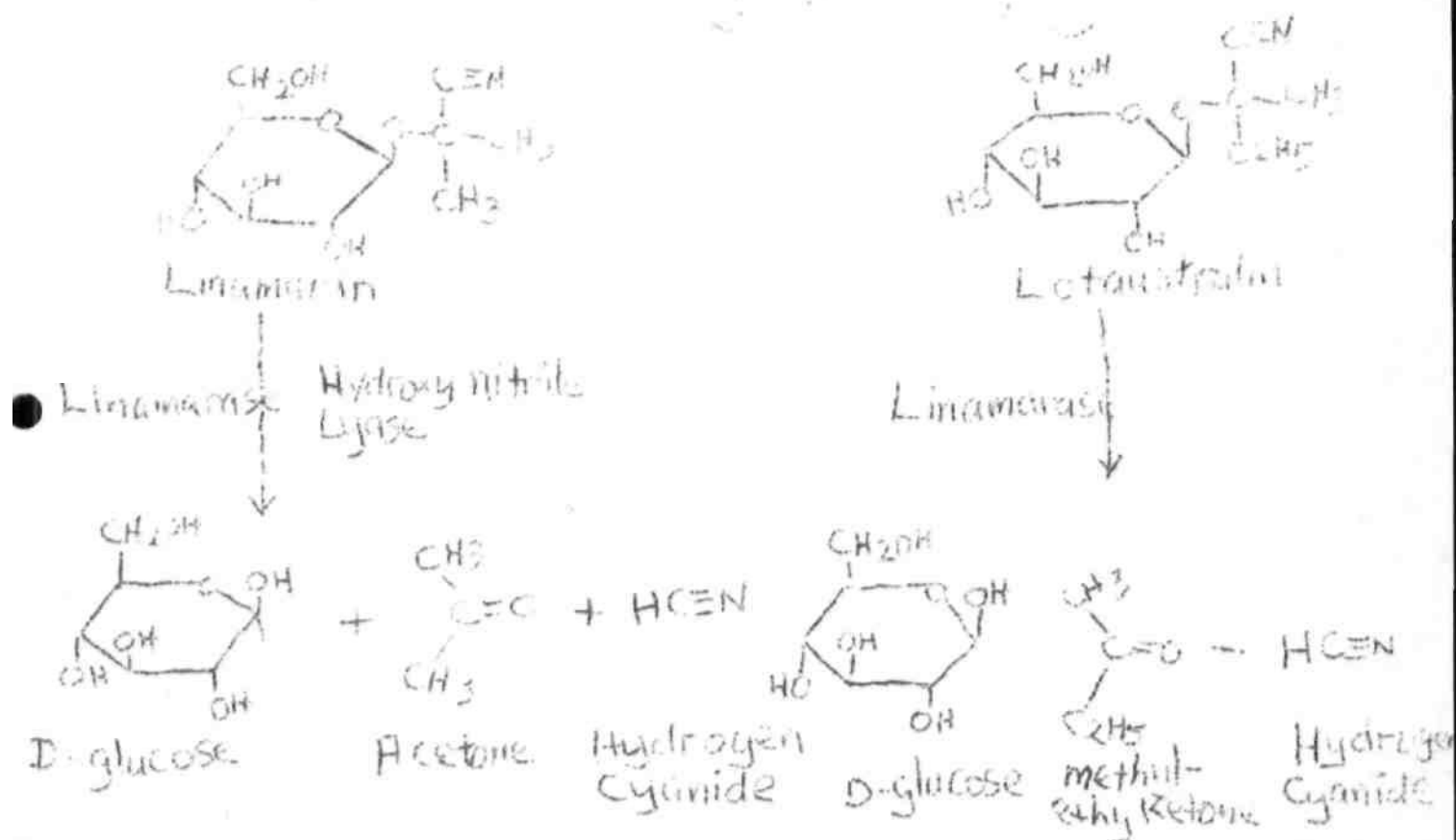


Fig. 1L: Hydrolysis of linamarin and lotaustralin.

The toxicity of cassava, therefore, arises from the presence of these cyanogenic glucosides which may hydrolyze readily as shown above to free HCN, which is a potent inhibitor of the terminal cytochrome oxidase in humans and animals. Cassava is often described as "bitter" or "sweet" according to the amount of cyanide present, but these are at best only approximate terms, and to attempt to associate cyanide levels with particular botanical taxa is quite incorrect (Bolhuis 1954). In general, bitter cassava has a high cyanide content while sweet cassava tends to have lower values.

The cyanogenic glucosides of cassava are accompanied in the plant tissue by a hydrolytic enzyme linamarase, similar but not identical to emulsin (Armstrong and Horton 1910; Wood 1965b, 1966). In active, healthy tissue of the growing plant, enzyme and substrate do not come into contact, but contact occurs when the tissues are mechanically damaged or when loss of physiological integrity occurs as result of post-harvest deterioration of tubers, or wilting of the leaves; hydrolysis then takes place, liberating HCN.

For most animals, especially rats and humans predominantly cassava diets containing finite levels of cyanide results in chronic or acute toxicity. Variations in biological susceptibilities, the way the cassava is prepared and eaten, species of cassava, and other food items and supplements especially animal protein that goes with it, determines the extent of toxicity. (Coursey, 1973).

In patients with a syndrome referred to as tropical ataxic neuropathy (Osuntokun 1973), it was observed that plasma levels of cyanide and thiocyanate and also urinary thiocyanate were high. Epidemiological studies of the same disease showed correlation of the disease with intensity of cassava cultivation, frequency of cassava meals and plasma thiocyanate levels.

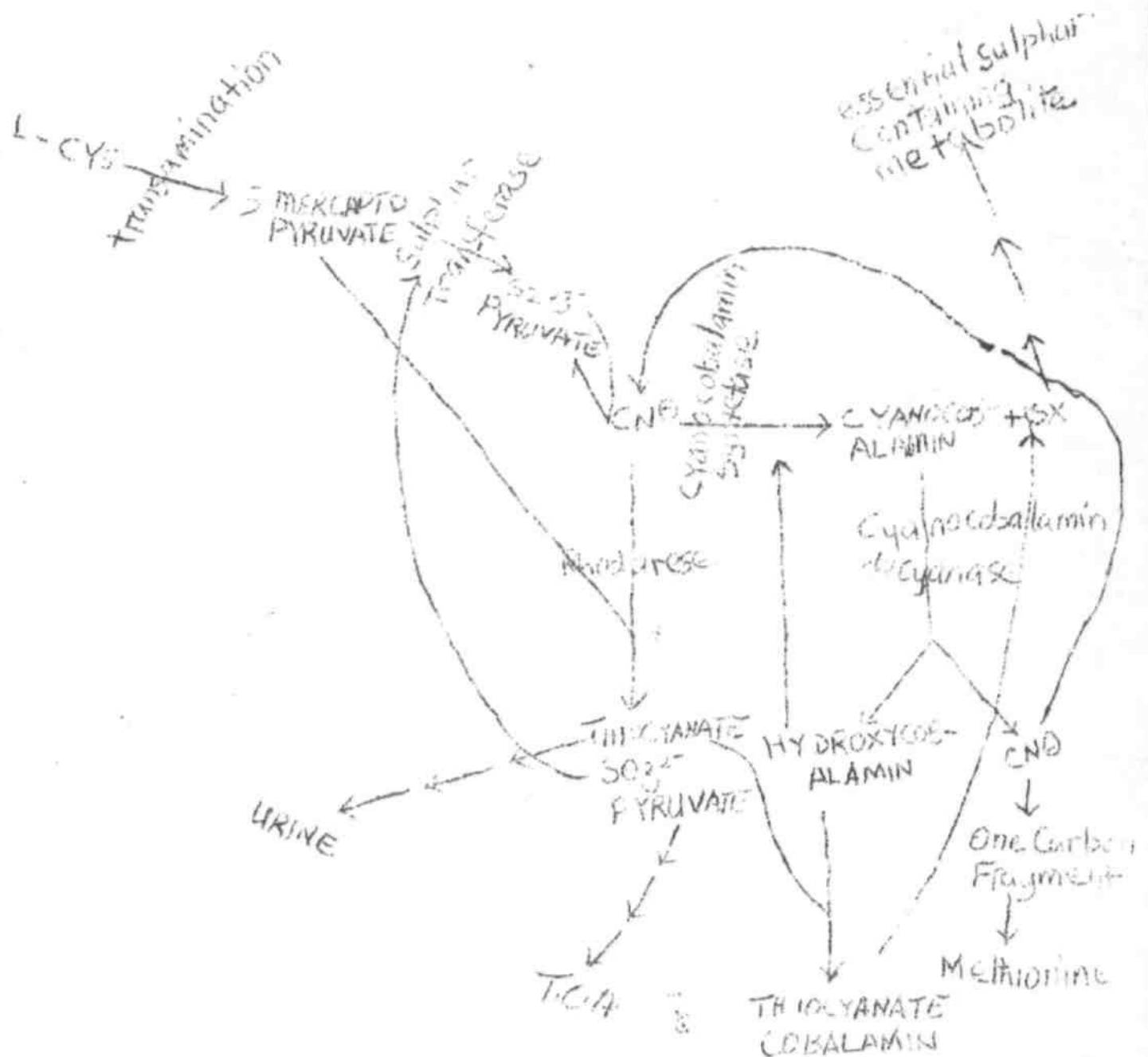
Comparison of rats on standard diet and rat on 100 percent cassava diet, showed higher thyroid weight and higher thyroid uptake of radioactive iodine at 24 hours (Ekpechi 1973). A pure iodine deficient diet did not significantly change the thyroid weight after 206 days. The increased uptake of radioactive iodine in cassava-fed animals could have been due to presence of a low concentration goitrogenic substances in cassava. (Ekpechi, 1973).

In a research undertaken in order to detect an antithyroid activity in men and rats, in the food eaten in Idjwi Island, Republic of Zaire (Delange 1973), the results obtained agreed with the view that antithyroid action of cassava is related to the endogenous production of thiocyanate from cyanide released by the cyanogenic glucosides present in cassava.

In the human body, there are essentially five ways in which the minute amounts of cyanide formed in the body during metabolism or minute amounts taken in food is detoxified. When HCN is converted to thiocyanic acid, there is a 200 fold reduction in toxicity (Oke, 1973). Most of the detoxifications due to conversion of HCN to thiocyanic acid are due to action of rhodanese (Lang 1933a,b, Saunders and Himwich (1950) ).

The enzyme, rhodanese, through combination with thiosulfate or colloidal sulfur, forms a polysulfide chain that can react with cyanide to release sulphur in a suitable form to give thiosulphate (Saunders and Himwich 1950). Cyanocobalamin (vitamin B<sub>12</sub>) occurs in the liver to some extent as hydroxocobalamin (vitamin B<sub>12</sub>a) which is capable of reacting with cyanide to give cyanocobalamin, providing another independent pathway for cyanide detoxification (Mushet et. al. 1952). 3-Mercaptopyruvate, arising from cysteine by transamination or deamination can provide sulphur as rapidly as thiosulphate for cyanide detoxification (Meister 1950, Wood and Fielder 1953). Cysteine can react with cyanide to form cysteine and S-thiocyanoalanine which tautomerizes to 2-aminothiazolidine-4-carboxylic acid or its equivalent, 2-imino-4-thiazolidine carboxylic acid which is excreted (Voegthine et. al. 1926, Wood and Cooley 1952). Finally the thyroid gland shows some detoxicating effect (Hunt 1905 - 1906); In the presence of powdered sheep thyroid the lethal dose of acetonitrile for mice was 1.4mg/g whereas it was 0.32mg/g for those not fed the powdered sheep thyroid (Bauman et. al. 1933). The figure shown below is a summary of all the reactions involved in the detoxification of cyanide in the body.

Fig. 2L: Reactions associated with the detoxification of cyanide in the body.



Utilization of Cassava for food.

In practice, most traditional food preparation techniques appear to be designed to bring together enzyme and substrate by cell rupture, followed by elimination of the liberated HCN by either volatilization or solution in water (Coursey 1973). In some processes the initial hydrolysis of the linamarin is assisted by the use of fermentation processes (Coursey 1973).

In the Congo (Zaire) the most common way to process cassava is to soak it in a pool or border of a lake/stream or swamp for 3 - 4 days (Jones, 1959). When the roots become soft by fermentation, they are taken out from the water and prepared in several ways:

Chickwangue: Chickwangue (Jones, 1959) is the common form in which cassava is consumed in Zaire. Here the fermented roots are washed, peeled and pounded into paste, the fibrous parts are removed, a handful is wrapped in banana leaves and bound firmly. It is then cooked and pounded. The fermented roots can also be allowed to dry in the sun and pounded into flour with mortar and pestle. Then sifted through a fine sieve for smoother flour.

Konkonte: Konkonte (Jones 1959), common in Ghana, comprises dried split roots of cassava from which flour can be made.

Starch and Tapioca: These are common in Togo. To make either starch or tapioca (Jones, 1959) cassava roots are grated and soaked in water then pressed and mixed to free the starch.

Fufu, alibo and lafun (Oke, 1968) are products of cassava common in Nigeria.

Fufu: Peeled cassava roots are soaked in water to ferment for about 2 days. The soft flesh is marcerated with hand, sieved while washing through with water and the filtrate allowed to settle. The residue on the filter is usually discarded. The sedimented material (mainly starch) is then transferred to a bag to allow excess moisture to drain off. The product is stored in an earthen and layered vessel with little clean water to keep it fresh. This layer of water is changed daily to maintain freshness. To prepare a fufu meal, the processed product is mixed and made into balls that are boiled in water at 100°C for 30 minutes, and pounded, then boiled again for 30 minutes before pounding to a rubbery paste (Ekpechi, 1973).

Lafun: Unpeeled roots are soaked in big pots of water for about 3 - 4 days after which the peels are removed. The flesh is washed and spread out in the sun, on rocks, dry slabs, or mats to dry. This takes about 4 days. It is then pounded in wooden mortar and sieved. To prepare lafun meal the product is stirred in boiling water until it thickens to a brown mass. Alibo is another name for lafun.

Gari: This is perhaps the most common and popular Cassava product in West Africa. The method for preparing it was learnt from repatriated Brazilian slaves who settled in Dahomey Coast around 1790. The missionaries who came from Sierra Leone too were

supposed to have this knowledge and might have taught the natives also (Jones, 1959, Oke 1968). The roots are harvested and processed on the same day. First, they are washed and peeled, then grated on a sheet of iron or steel that has been roughened on one side by making holes on it with a nail or a board with nails projected through it. When a sufficient quantity has been grated, the mash is put into a large cloth bag and set in the sun to drain and to ferment under pressure (maintained by placing huge pieces of stone on top of bag) for 3 or 4 days. It is then fried with without oil (Jones 1959, Oke, 1968, personal observation). Below is a list of tentative classification of traditional cassava processing (Coursey, 1973).

1.        No special detoxification applied
  - 1.1        Totally unprocessed (i.e. eaten raw)
  - 1.2        Simple cooking techniques only.
    - 1.21       Boiling, stewing etc.
    - 1.22       Roasting
    - 1.23       Frying
  - 1.3        Sundrying
    - 1.31       Sundrying without subsequent processing
    - 1.32       Sundrying with subsequent processing
      - 1.321      Different type of milling
  - 1.4        Kiln or Hot air drying
 

(subdivide as for 1.3)

When evaluated, the bread made with 20 percent cassava in place of wheat proved satisfactory.

As food for animals, cassava has been proved a good substitute for some cereal or grain meals for pig, poultry and cattle (Jones 1959); for sheep and goats cassava peel is even fed (Oyenuga et. al. 1957). It has also been proved adequate for all types of livestock (Coursey and Haliday 1974). The optimum levels of dry cassava that can be used in animal diet is being investigated by some workers (Nestle and Graham 1977).

Starch production from cassava has received much attention in recent years, both in developing countries and the technologically advanced countries. While the cassava starch is used mainly for food in developing countries until very recently, the technologically advanced countries use it in the textile, adhesive and paper industries. It is usually exported by the cassava **producing** countries especially Thailand and Brazil, to the E.E.C. countries and the United States (Thanh and Lohan 1978).

The use of cassava as food is limited by the following factors (Oke, 1968): the presence of toxic cyanogens in both processed and unprocessed tubers, the lack of essential vitamins and minerals in the processed tubers and the presence of low and poor quality protein in the tubers.

Biosynthesis of cyanogenic glucosides in Cassava.

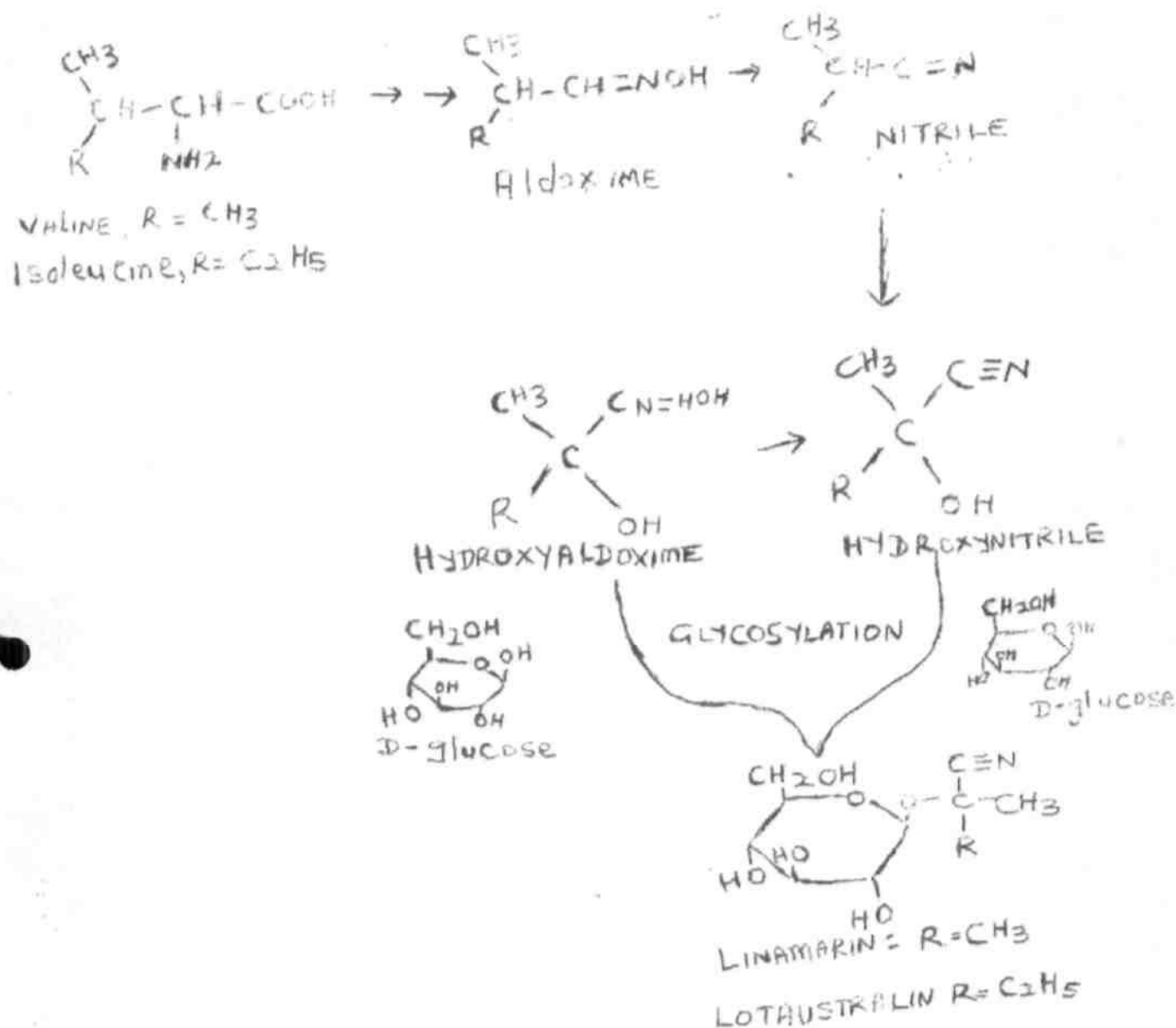
A common biochemical feature of the cassava plant is that it synthesizes and accumulates cyanogenic material in its vegetative tissues, especially the edible leaves and tubers. Tissues of all cassava cultivars so far examined contain cyanogenic glucosides, although in varying concentrations (Nartey, 1978). Variations in the HCN concentrations in tubers, as well as the morphological characteristics of the plants, form basis of a taxonomic differentiation between the bitter (high HCN) and the sweet (low HCN) cultivars (Rogers 1965).

Linamarin and lotaustralin are the cyanogenic glucosides known to occur in the cassava plant (Nartey 1968, Butler 1965).

Research in several laboratories (Conn 1969, 1973, Nartey 1968), has demonstrated that the aglycone moieties of cyanogenic glucosides may be synthesized from structurally related amino acids. These motivated radioactive labelling studies on the effectiveness of valine and isoleucine as precursors of the aglycone moieties of linamarin and lotaustralin, respectively.

Results showed that both sweet and bitter varieties of cassava seeds contained only trace amounts of valine and isoleucine. However, during germination, large amounts of these amino acids are accumulated through the degradative action of proteolytic enzymes on seed storage proteins. Concurrently, the concentration of linamarin and lotaustralin increase sharply. Shown below is the pathway for biosynthesis of linamarin and lotaustralin in the cassava plant.

Fig. 3L: The biosynthesis of linamarin and Lotaustralin.



The path way incorporates data from Conn and Butler 1969, Nartey 1962 and Tapper et. al. 1972. Their experiments suggest that the biosynthetic pathway involves an initial decarboxylation of the amino acid, followed by a four-electron oxidation of  $\alpha$ -carbon to a nitrile function in which the  $\alpha$ -amino nitrogen is retained. Subsequently, a two electron oxidation of the  $\beta$ -carbon leads to its hydroxylation followed by a glycosylation to yield linamarin.

Since most plants containing linamarin also synthesize lotaustralin, with probable exception of Hevea brasiliensis (Butler 1965), it may be assumed that the pathway for biosynthesis of lotaustralin proceeds from L-Isoleucine via

hydroxy- $\beta$ -methylbutyrylonitrile, followed by glycosylation to form the cyanogenic compound.

#### Role of cyanogenic glucosides in the cassava plant:

Cyanogenic glucosides have been considered as nitrogen reserves and precursors for protein synthesis. They are considered also as inert excretory waste products of nitrogen metabolism (Robinson 1930) and as compounds involved with the protection of plants which contain them. Since the major fraction of cyanogenic glucosides and linamarase of cassava tubers seem to be localized within the phelloderm, tubers can be considered as possessing a chemical defence mechanism whereby the HCN produced on bruising functions as a repellent to rodent and insect attack (Nartey 1978). In the cassava plant as a whole it is very probable that cyanogenesis is related to resistance to incidence of disease such as cassava mosaic which is transmitted by white fly Bermish spp (Jones 1972).

#### Respiration of cassava tissues:

The mitochondria isolated from cassava root tubers possess a cyanide-insensitive electron transport pathway (Passan 1976). This alternative oxidase system branches from the cytochrome chain on the substrate side of the antimycin inhibition site.

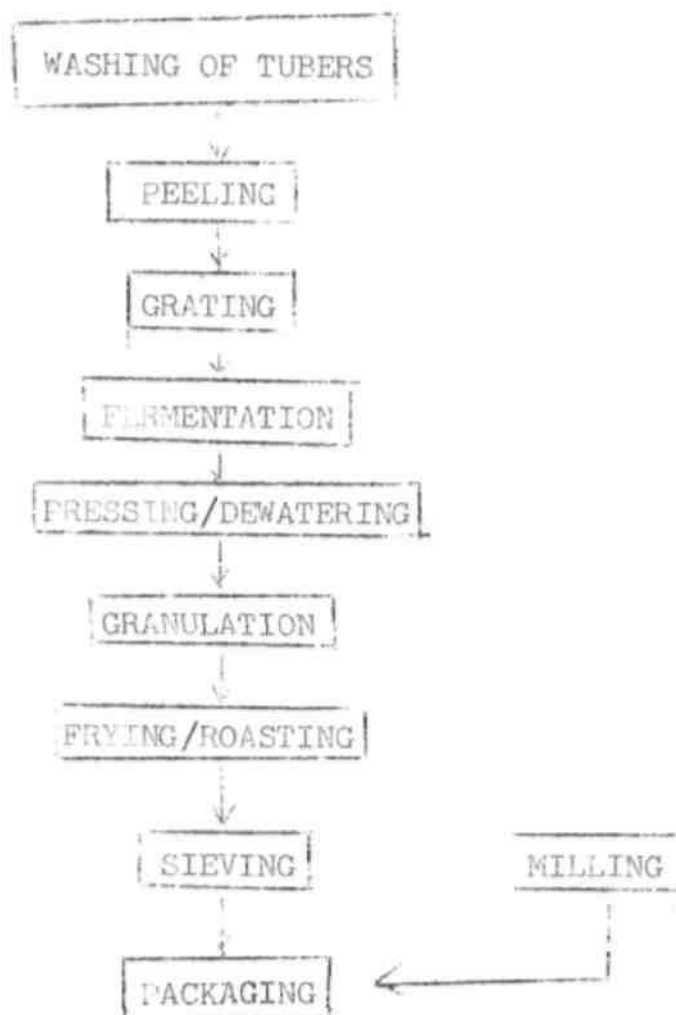
It is sensitive to salicylhydroxamic acid and therefore similar to that described for other plant mitochondria (Bendal and Bonner 1971).

Cassava mitochondria are considerably more resistant to potassium cyanide than are mitochondria from other root crops. This suggests that either the activity or concentration of the alternative oxidase is higher in cassava.

Mitochondria isolated from cultivars having higher endogenous cyanide content are about 50 percent less sensitive to potassium cyanide or salicylhydroxamic acid when added separately than are mitochondria isolated from cultivars with low cyanide content (Passam 1976).

Detoxification of cassava for Human food:

Processing of cassava (M. esculanta Crantz) into gari consists of the following steps shown below



The most important of these unit operations is fermentation. During fermentation the cyanide in cassava is detoxified. The nature of the biochemical reactions taking place during this process has been considered (Akinrele 1964, Collard and Levi 1959, Wood 1965, 1966, Clapp *et al.* 1966).

Collard and Levi obtained partially purified cyanogenic glucoside from pressed juice of cassava roots, by centrifugation followed by filtration. They assumed the juice was protein-free.

On analysis, they found the glucoside content was of the same order of magnitude as that of bitter cassava variety, and undergoes spontaneous hydrolysis at pH 7. They then purified the glucoside by crystallization from alcohol, and the pure glucoside hydrolyzed spontaneously after acidification, liberating hydrocyanic-acid and glucose. They consistently isolated 2 organisms from grated cassava during fermentation. The organisms they isolated were members of genus corynebacterium and the fungus geotricum candida. Corynebacterium consistently increased in the first 48 hours and was replaced by Geotricum, which on the third and fourth day was predominant. This study encouraged Collard (1963) to study the microbiology of cassava fermentation. He attributed the detoxification to the spontaneous hydrolysis of the cyanogenic glucosides principally linamarin, brought about by the organic acids produced during starch degradation by fermentative action of corynebacterium manihot.

That fermentation of grated cassava is accompanied by evolution of heat is well established (Akinrele 1964). Effect of sunlight was observed only after 48 hours of normal fermentation; it tends to promote acidification of mash and coincides with onset of second stage of fermentation, during which the fungus proliferates and produces aldehydes and esters (Akinrele 1964).

This could mean that light may have a stimulating effect on the growth of fungus whose optimum pH for growth is known to be about 3. Aeration was noticed to slow down the rate of acidification and it was assumed inhibitory. Akinrele and co-workers also observed that optimum temperature for fermentation is  $35^{\circ}\text{C}$ , that fermentation can take place satisfactorily at  $45^{\circ}\text{C}$  but at  $50^{\circ}\text{C}$  a marked retardation is noticed which results in suppression of the second stage. Two organic acids, lactic and formic acids have been identified (Akinrele 1964) as products of fermentation of cassava, although only lactic acid is found in gari.

Acidity seems to play no part in hydrolysis of linamarin, the major cyanogenic glucoside of cassava, although Collard and Levi concluded that it did in their pioneer work on cassava fermentation. No appreciable amount of HCN was liberated by dilute acids at  $100^{\circ}\text{C}$  (Wood 1966).

In his experiments, Okafor (1977) noticed that over a 6-day period, the predominant micro-organism associated with cassava fermentation for gari production was Leuconostic spp. and their number increased progressively throughout the 6-day study. Yeasts were present in increasing numbers too. The explanation for the abundance of lactic acid bacteria is the availability of free fermentable sugars. Collard and Levi acknowledged the possibility of the presence of lactic acid in the fermenting pulp but did not isolate any lactic acid bacteria because nutrient agar was the only medium they used.

Finally, Okafor speculated that the breakdown of chemically stable linamarin was due to action of endogenous enzyme linamarase, which is released during the grating of the root, and suggested further research on the 2-stage fermentation process, and factors that influence the organisms encountered and role if any, these organisms may have in glucoside breakdown and flavour production.

The effect of acidity or decrease in pH of grated fermenting cassava has recently been clarified (Ikediobi and Onyike 1981). Subjection of a crude mixture of linamarin and lotaustralin (boiled cassava) to different acid treatments did not result in hydrolysis sufficient to account for the amount of detoxification observed in grated fermenting cassava, instead it was found that endogenous linamarase activity was responsible for the hydrolysis of these cyanogenic glucosides.

#### Methods for assay of cyanide in Cassava and Cassava products

Most research data are reported in terms of potential cyanide yield rather than the glycoside content itself because of its medical significance and relative ease of its detection and determination (Zitnak 1973). The assays are based on the determination of HCN liberated by acid or enzyme hydrolysis. One of the inconveniences of this assay is the volatility of HCN (b.p  $26^{\circ}\text{C}$ ) which necessitates working in a closed analytical train.

Early work on cyanogens stimulated by acute and sometimes lethal toxicity cases (Montgomery 1969) placed fewer demands on cyanide detection limits since it was sufficient to determine potentially toxic cyanide levels. Recent concern about chronic toxicity through continuous uptake of low cyanide foods and forages and the necessity to study the physiological effect of such intake stimulated the development of procedures which would allow detection and quantitation of trace amounts of cyanide. There are 3 distinct steps in the HCN assays:

- (1) Liberation of cyanide through acid or enzyme hydrolysis
- (2) Extraction and isolation of cyanide from substrates
- (3) Determination of the isolated cyanide.

Most of the early methods relied on acid hydrolysis or spontaneous autolysis by enzyme contained in ruptured plant tissues neither of which appears to give "a total potential yield of cyanide" (Wrinkler 1951). With acid hydrolysis, discrepancies occur due to formation of amides and ammonia while in spontaneous autolysis, the activity of the enzyme is no doubt influenced by conditions prevailing in the heterogeneous plant substrate, the accompanying secondary reactions and accumulation of products of hydrolysis. Pulss (1962) attributed the incomplete recovery of cyanide from flax and clover to secondary binding of cyanide in digest, especially when older necrotic tissues were assayed.

Wood (1966) claimed recovery of at least 87 percent of cyanide present in the glucoside of cassava extract when extraneous enzyme was used for hydrolysis. On the other hand Montgomery (1964) found a 3-hour acid hydrolysis at 100° satisfactory for tropical pulse, and de Bruijn (1971) doubted the necessity of using an enzyme preparation and employed a 24-hour spontaneous hydrolysis in cassava macerated to liberate the cyanide.

Cooke (1978) has introduced a rapid enzymatic assay for determining total cyanide of cassava. This involves a 15-minute incubation of extract with exogenous enzyme which causes hydrolysis of bound cyanide prior to cyanide estimation. The short incubation period minimizes any side reactions that may hinder the release of cyanide. Both rhodanese and cyanoalanine synthetase have been detected in cassava and could cause such interference (Nartey 1973a): rhodanese and cyanoalanine synthetase lead to detoxification and assimilation of hydrogen cyanide by the cassava plant. Cooke evaluated his assay method by measuring the extent of added linamarin recovery and observed that recovery depended on added enzyme. He obtained 95 percent recovery. With his assay method, Cooke could determine the distribution of cyanide in cassava of 8 different cultivars. It also made it possible to determine ratio of free to glycosidic cyanide in cassava.

Linamarase has been studied by several workers in different laboratories. The enzyme is thermostable (Coop 1940; Ikediobi et. al. 1980). The pH optimum has been shown to be between 5 and 6 (Wood 1966, Cooke et. al. 1978), and it is highly specific for linamarin and lotaustralin. The configuration of these glucosides had been investigated by infra-red studies and found to be  $\beta$ -linked (Butler et. al. 1965). A survey of  $\beta$ -glucosidase activity in unpurified extracts from different plants shows that although the extracts from different plants were all rich in  $\beta$ -glucosidase activity, the ability to hydrolyze linamarin and lotaustralin was very limited. Linseed and white clover extracts were able to hydrolyze linamarin and lotaustralin (Butler et. al. 1965). All species of plants known not to contain cyanogenic glucosides yielded extracts which did not hydrolyze these glucosides. Extracts from fungus and protozoa hydrolyzed all the glucosides (Butler et. al. 1965). Extracts from bacterial species, leuconostoc mesenteriodes, have **also** been found to hydrolyze these glucosides.

After hydrolysis of the cyanogenic glucoside the cyanide released is trapped in an alkaline solution. This is then quantitated. Numerous quantitative methods differing in sensitivity and detection limits have been employed in determining the isolated glucosidic cyanide (Zitnak, 1973). The two generally used quantitative methods are colorimetric. The first is Cooke's modified version of Epsteins method (1947).

It involves the use of chloramine-T and bispyrazolone (3,3-dimethyl-1,1'-diphenyl-(4,4'-bis-2-Pyrazoline)-,5'-dione) and 3-methyl-1-phenyl-5-pyrazolone and pyridine. The presence of cyanide leads to formation of a soluble blue complex.

The alkaline picrate test is based on the formation of a soluble orange - red complex by reaction of cyanide and alkaline picrate. Its use was attributed to Guignard (1906). A variation of this technique (Gilchrist et. al. 1967) designed to minimize interference from other compounds, was to suspend a filter paper strip impregnated with alkaline picrate above the cyanide source in a closed vessel. The liberated cyanide reacts with a constant size of saturated alkaline picrate paper and reaction products are measured colorimetrically at 515nm. Results obtained by Gilchrist et. al. (1967) and William (1980) show that the picrate system is not simple.

A detailed examination of the system by Williams and Edward (1980) showed that for the cyanide - picrate system, absorbance is governed by (i) total picrate present (ii) the pH of the picrate and reaction medium. (iii) the time of incubation and (iv) the temperature of the reaction. For reaction of cyanide with picrate in free solution, the picrate content is much more easily controlled.

Studies on the nature of the standard curves and the effect of temperature time and pH on absorbance by the red complex at 510nm suggests the reaction of cyanide with picrate proceeds irreversibly to give the red complex which then undergoes further reversible reaction to give a compound which absorbs little or not at all at 510nm (Williams and Edwards 1980).

A rapid and inexpensive assay for total cyanide has recently been described by Ikediobi et. al. (1980). The assay involved, the use of exogenous enzyme of known activity to obtain complete hydrolysis of the endogenous cyanogens in a diluted extract of fresh cassava parenchymal tissue. The cyanide released after hydrolysis was allowed to react with constant amount of alkaline picrate in free solution.

There was no real loss in absorbance after 3 days at room temperature or one week at refrigerated storage. Results of sensitivity tests using progressively decreasing concentrations of KCN as source of cyanide, indicated that this modified alkaline picrate method was capable of detecting cyanide down to concentrations of  $0.16 \mu\text{g}/\text{cm}^3$  (detection limit) which compares favourably with Cooke's method which has detection limit of  $0.01 - 1.0 \mu\text{g}/\text{cm}^3$ . Precision as indicated by coefficient of variation was 3 percent. The method was in addition found to be rapid simple, inexpensive and ideally suited for the analysis of large number of cassava tissues and products such as may be encountered in cassava agronomy, breeding work or in an industrial quality control laboratory.

Using this method, cyanide content of gari and other cassava products were determined. Their results showed that substantial amount of cyanide still remain in processed cassava products. It thus appears that despite various detoxification methods employed in cassava processing in different parts of the world, products containing appreciable traces of cyanide are still obtained. It is therefore, necessary to re-examine the mechanism involved in these detoxifications and improve them.

The present study is designed to evaluate precisely, the relative contributions of acid and linamarase to cassava detoxification during fermentation. This will clarify the mechanisms of cassava detoxification. The information obtained will help in the design of a mechanized gari processing plant capable of producing large quantities of high quality gari with negligible or no cyanide.

MATERIALS AND METHODSMaterials:

Freshly harvested cassava tubers of about 12 - 24 months old were obtained as needed from a local farm in Zaria.

Gari samples were obtained from markets in the following parts of the country: Agbor, Ngwa, Oji, Oshodi (F.I.R.O.), Ekwuoma (Umunede), Lagos, Ndi-oru, Ife, Onitsha, Owerri, Enugu (National Root Crops Production Company Ltd.) Umudike (National Root Crops Research Institute), and Umuahia.

Potatoes were purchased locally. The fungi and their authorities listed below, were obtained from the Plant Pathology Department, Institute for Agricultural Research, Zaria.

|                    |                      |                                          |
|--------------------|----------------------|------------------------------------------|
| <u>Aspergillus</u> | <u>Ochraceus</u>     | Wilhem                                   |
| <u>Aspergillus</u> | <u>Zydwii</u>        | (Banier and sartory)<br>Thom and church. |
| <u>Aspergillus</u> | <u>Terreus</u>       | Thom                                     |
| <u>Aspergillus</u> | ( <u>Micheli</u> )   | Gorda                                    |
| <u>Aspergillus</u> | <u>Amstelodeni</u>   | Thom and Church                          |
| <u>Aspergillus</u> | <u>Carneus</u>       | Van Tieghem                              |
| <u>Penicillin</u>  | <u>Variable</u>      | Sopp                                     |
| <u>Penicillin</u>  | <u>Spiculisporum</u> | Lehman                                   |
| <u>Penicillin</u>  | <u>funiculosum</u>   | Thom                                     |
| <u>Penicillin</u>  | <u>brefeldianum</u>  | Dodge                                    |
| <u>Penicillin</u>  | <u>Citrinum</u>      | Tom                                      |
| <u>Penicillin</u>  | <u>Steckii</u>       | Zaleski                                  |

|                 |                     |                    |
|-----------------|---------------------|--------------------|
| <u>Rhizopus</u> | <u>Ehrenberg</u>    |                    |
| <u>Fusarium</u> | <u>Oxysporum</u>    | Schlechtendahl     |
| <u>Fusarium</u> | <u>acuminatum</u>   | Ellis & Everhart   |
| <u>Fusarium</u> | <u>Moniliiforme</u> | Sheldon            |
| <u>Fusarium</u> | <u>Semitectum</u>   | Barkeley & Ravenel |
| <u>Fusarium</u> | <u>equiseti</u>     | (corda) Saccado    |

#### Chemicals:

Anhydrous picric acid and anhydrous sodium carbonate for cyanide assays, boric acid and acetic acid for preparing buffers were purchased from BDI Chemical Co.; Poole England, while p-nitrophenyl- $\beta$ -D glucoside (PNP-glucoside) and linamarin used for assaying linamarase activity was bought from Sigma Chemical Company, St. Louis, Mo. U.S.A. D- glucono (1 $\rightarrow$ 5) lactone, an inhibitor of linamarase, was purchased from Sigma. Sodium hydroxide and hydrochloric acid were purchased from May and Baker Ltd. Dagenham England. Dextrose and Agar powder for preparing culture media and potassium hydroxide were purchased from Hopkin and Williams Chemical Co., Chadwells Heath Essex England. All other routine chemicals and solvents used **were of analytical grade.**

#### Preparation of Linamarase (Cooke et. al. 1978).

The thin brown outer skin of freshly harvested cassava tubers was carefully removed with a knife revealing the underlying cortex. The latter was removed and sliced into small pieces.

Approximately 100g of the slices were homogenized in 300ml of prechilled 0.01M acetate buffer pH 5.50. The homogenate was collected in prechilled container and filtered by suction through a 1cm-layer of kieselguhr. The filtrate was used to extract a second 100g batch of sliced cassava cortex as described above. The filtrate from the second extraction was similarly used to extract a third 100g batch. This final extract was kept overnight at 3 - 5°C.

To this extract were added 2.3 volumes of cold acetone and the resulting solution swirled occasionally for 5 minutes within a 2-hour period. A pale green to brown precipitate was removed upon decantation. This precipitate was subsequently extracted thrice with 10ml portions of cold 0.1M acetate buffer pH 5.50. The combined extract was centrifuged at 500 x g for 5 minutes at 4°C and the resulting supernatant was dialyzed either at room temperature or in the cold room against the 0.10M acetate buffer to remove any residual linamarin. The extract obtained after dialysis was assayed for activity and subsequently used for cyanide assays.

Assay of Linamarase activity. (Cooke et. al. 1978)

The partially purified cassava cortex linamarase was assayed for activity using p-nitrophenyl-B-D-glucoside as the substrate. One millilitre of a solution containing 21mg of the PNP-glucoside in 0.010M sodium phosphate buffer of pH 6.8 was incubated for one hour at room temperature ( $25 \pm 2^\circ\text{C}$ ) with 0.10ml of the enzyme preparation.

At the end of the incubation, the reaction was stopped by the addition of 2ml of 0.20M borate buffer of pH 9.8. The reaction solution was then brought up to 4ml with distilled water and the absorbance of the resulting solution read at 400 nm in a Cecil model CE.505 high performance scanning spectrophotometer, or Corning Model 253 colorimeter. One unit of linamarase activity was defined as the amount of enzyme which produced one micromole of p-nitro-phenolate/minute (molar extinction coefficient of p-nitrophenolate at 400 nm is  $1.8 \times 10^4$  liter. (mole.cm)<sup>-1</sup>).

Preparation of alkaline picrate solution:

Twenty five grammes of anhydrous sodium carbonate and 5g of anhydrous picric acid were added to a 1-liter volumetric flask. The mixture was dissolved in a minimal amount of water at 40°C and the solution was made up to mark with chilled distilled water.

Quantitative determination of Cyanide:

Total cyanide was reliably assayed by the enzymatic method of Ikediobi et. al. (1980) which involves incubating linamarin-containing extract with a linamarase preparation to release free cyanide which forms soluble orange-red complex with added alkaline picrate.

About 0.1 - 0.5ml of an aqueous cassava or cassava product (e.g. gari) extract was incubated with an amount of linamarase preparation containing 0.04 - 0.06 units of enzyme activity for 15 minutes at room temperature in a tall tightly stoppered test tube. The volume of the incubation mixture was made up to 2ml with 0.20M sodium phosphate buffer of pH 6.80 or distilled water. At the end of the incubation period, 4.5 - 5ml of alkaline picrate solution were added and the resulting solution was incubated in a waterbath at 90 - 95°C for 5 minutes. Upon cooling to room temperature, the absorbance of the orange-red colour formed was read in a Corning Model 253 colorimeter at 490nm. The cyanide concentration was extrapolated from a standard curve previously prepared with potassium cyanide as standard. Extracts incubated with and without enzyme preparation assayed total and free cyanide, respectively. The difference between the two cyanide levels gave the so-called bound cyanide or unhydrolyzed cyanogenic glucosides.

#### Fermentation of Cassava:

Freshly harvested cassava tubers were washed, peeled to remove the skin and cortex and the exposed parenchymatous tissue was grated with regular kitchen hand grater. Fermentation of the grated mash was carried out at room temperature in 500ml beakers (covered with aluminium foil) and 1.1 liter Vacco Thermos flasks respectively.

The pH and temperature of the fermenting mash were monitored at regular intervals to assure that fermentation was proceeding normally. To measure pH, an aliquot of the fermenting cassava was removed, hand-squeezed in a cheese cloth and the pH of the expressed juice determined with a pH meter. Samples were similarly taken at regular intervals, extracts of them made with distilled water and then assayed for linamarase activity, total and free cyanide.

Use of Linamarase in gari production:

Five equal samples of grated cassava were simultaneously fermented at room temperature for 5 days. Four of the samples received gradually varying quantities of exogenous linamarase added at the beginning of each day in progressively increasing amounts. The first sample received enzyme addition on the first day only, while the second sample received on the first and second day. The third and fourth samples received enzyme on three consecutive days only and on all the days, respectively. The fifth sample did not receive any enzyme and was used as control. Table 1 presents the sequence of linamarase additions in this fermentation experiment.

"Garification" of fermented cassava:

After 5 days of fermentation, the fermented mash was placed in a cheese cloth and dewatered or pressed by hand-squeezing as much as possible. The dewatered material was then garified in a flat frying pan by controlled frying on a hot plate for 30-45 minutes. This usually gave gari of approximately 9 percent moisture content.

Determination of Starch in gari (Akinrele 1962):

Three grammes of gari were treated with 50ml of cold water for one hour, stirring frequently. The residue was collected on a filter paper and washed with sufficient water to make a total volume of 25ml. The filtrate contains the soluble carbohydrates. The undissolved residue was heated with 2.5 percent hydrochloric acid in a flask equipped with a reflux condenser, allowed to boil for 2 hours and then left to cool to room temperature. The contents of the flask were neutralized with sodium hydroxide solution and made up to 250ml. The resulting solution was filtered and the dextrose content determined by the method of Munson and Walker (A.O.A.C. (1975) ) as follows:-

- (1) Asbestos: asbestos (Amphibole variety) was digested with 10 percent HCL for 2 - 3 days, then washed acid free and digested for 2 - 3 days with 10 percent NaOH solution. The asbestos was further digested for several hours with 1 volume nitric acid mixed with 3 volumes of water.
- (2) Alkaline tartrate solution: 173 grammes Sodium potassium tartrate (Rochelle salt) and 50 grammes of NaOH pellets were dissolved in water, and diluted to 500ml. The solution was allowed to stand for 2 days and then filtered through freshly prepared asbestos.

The digested asbestos was washed again to free it from acid and shaken to a fine pulp with water. A film of asbestos 6mm thick was made in a prepared gooch and treated with hot alkaline tartrate solution.

- (3) Copper sulphate solution: 34.639 grammes of  $\text{Cu} \cdot \text{SO}_4 \cdot 5\text{H}_2\text{O}$  was dissolved in water. The solution was diluted to 500ml and filtered through glass wool or Whatman filter paper.
- (4) Invert sugar standard solution: 9.5 grammes pure sucrose was dissolved in a little water. 5ml of HCL was added and the solution was made up to 100ml. The solution was stored at room temperature for 3 days. It was made up to 1 litre, and aliquots neutralized with 1N NaOH or diluted to desired concentration immediately before use. The solution was also clarified by neutralizing with lead acetate solution.

Determination of starch:

25ml each of copper sulphate solution and alkaline tartrate solution was mixed in 400ml beaker made of alkali resistant glass. 50ml of reducing sugar solution was added. (If smaller volume of sugar solution was used, water was added to make final volume 100ml). The beaker was covered with a watch glass and heated on asbestos gauze over a bunsen burner. The bunsen flame was regulated so that boiling began in 4 minutes, and continued for 2 more minutes.

The hot solution was filtered at once through asbestos mat in porcelain gooch using suction. The precipitated cuprous oxide was thoroughly washed with water at  $60^{\circ}\text{C}$ . The precipitate was further washed with 10ml alcohol and 10ml ether, then dried at  $100^{\circ}\text{C}$  for 30 minutes, cooled in a dessicator and weighed. The weight of reduced sugar solution or inverted sugar solution, equivalent to weight of cuprous oxide is obtained from a standard table or from a standard curve.  $\text{Wt of glucose} \times 0.90 = \text{wt of starch}$ .

Determination of total acidity of gari (Akinrele 1962, A.O.A.C., 1975).

Eighteen grammes of gari were weighed out and transferred to a 500ml conical flask and 200ml of  $\text{CO}_2$ -free water was added. The flask was loosely stoppered and kept for an hour in a water bath at a temperature of  $40^{\circ}\text{C}$  shaking occasionally. The contents of the flask were filtered and the filtrate titrated with 0.20N NaOH using phenolphthalein as indicator.

Determination of crude fibre (Akinrele 1962, A.O.A.C., 1975).

Two grammes of gari were weighed out and transferred to a 1 liter flask. 200ml of 1.25 percent sulphuric acid which had been heated to boiling was added and the contents were made to boil within one minute. The contents of the flask were refluxed for 30 minutes. The contents of the flask were poured at once into the shallow layer of hot water remaining in a Buchner funnel (the experiment was discarded if the bulk of 200ml took more than 10 minutes to filter).

The residue was washed free of acid and washed back into the flask with 200ml of 1.25 percent sodium hydroxide which had been heated to boiling point. The contents of the flask were boiled for 30 minutes as before and filtered immediately through filter paper containing boiling water. The residue was washed with HCL and again with boiling water until free of acid. It was washed twice with 95 percent alcohol and three times with ether. The residue was then transferred to a dried and weighed filter paper and dried to constant weight at  $100^{\circ}\text{C}$  in an oven. After weighing the residue, it was transferred to a porcelain dish and ignited for 30 minutes at  $600 \pm 15^{\circ}\text{C}$ . It was allowed to cool in a dessicator and weighed.

Crude fibre = loss in weight on ignition.

Determination of Crude Protein (Pearson, 1976).

0.5g sample was weighed out and transferred into a 100ml digestion flask and digested using 0.8g of an anhydrous catalyst mixture (96 percent anhydrous  $\text{NaSO}_4$ , 3.5 percent  $\text{Cu}\cdot\text{SO}_4\cdot 5\text{H}_2\text{O}$  and 0.5 percent selenium dioxide) and 5ml of concentrated sulphuric acid. The digest was diluted to 100ml. 10ml of the diluted digest was transferred to a steamed out micro Kjeldahl distillation apparatus and made alkaline with concentrated aqueous alkali (at least 15ml of 40 percent sodium hydroxide), and ammonia steam distilled for 5 - 10 minutes into 10ml of a 2 percent boric acid solution containing 3 - 4 drops of screened methyl red (0.016 percent methyl red and 0.083 percent bromocresol green in alcohol). After lowering receiving flask clear of the condenser, the apparatus was steamed out for further 5 minutes. The distillate was then titrated with 0.01N HCL.

Percent crude protein =

$$\frac{\text{Sample titre} - \text{Blank titre} \times \text{Na} \times 0.014 \times 6.25}{\text{wt of sample (g)}}$$

where Na = Normality of HCL

#### Determination of Moisture content:

A known weight of given gari sample was left in an oven for 24 hours at 100°C. It was then allowed to cool in a dessicator and then reweighed.

$$\text{Moisture content} = \frac{\text{Initial weight} - \text{final weight} \times 100}{\text{Initial weight}}$$

#### Determination of swelling power (Akinrele, 1962):

The sample of gari was poured into a 100ml cylinder to reach the 25ml mark (initial volume). Water was added to reach the 100ml mark. The cylinder was inverted a number of times, (5 - 10 times). Then the mixture was allowed to settle. The new volume of the gari was taken after 15 minutes. This was the final volume.

$$\text{Swelling power} = \frac{\text{final volume}}{\text{Initial volume}}$$

#### Determination of foreign matter:

A sample of gari of known weight (W) was spread on a flat surface. All materials that were not gari were carefully removed and weighed (w)

$$\text{Percent foreign matter} = \frac{w}{W} \times 100$$

Media for culturing fungi:

Peeled potatoes were boiled for 5 minutes in distilled water (200g potatoes per liter of water). The extract was filtered using Whatman number 2 filter paper. Dextrose and agar (20 grammes each per liter filtrate) were added and stirred continuously until a clear solution appeared. The solution was sterilized at  $120^{\circ}$  in an autoclave for 20 minutes at 15 psi. The solution was allowed to cool to about  $50^{\circ}\text{C}$ . Streptomycin sulphate solution was added (0.05g streptomycin sulphate per liter of solution). The solution was poured into sterilized petri dishes in a sterilized room. They were allowed to cool to room temperature and used or stored at  $4^{\circ}\text{C}$ , but used within 2 weeks of preparation.

Growth of fungi:

The fungi were cultured for 10 days at  $27^{\circ}\text{C}$  on PDAS (see media above). They grew on top of the media, although some of the fungi did not grow very well.

Enzyme extraction from fungal mycelia:

The fungi were scraped avoiding as much of the agar as possible from the petri dishes into 100ml conical flask. Twenty milliliter of 0.10M acetate buffer was added and the flask was placed in a shaker for 30 minutes (100 rpm) at room temperature to extract the extracellular enzymes.

The mixture was later filtered using a Whatman No. 2 filter paper.

The filtrate containing most extracellular fungal enzymes was assayed for linamarase activity using P-nitrophenol- $\beta$ -D-glucoside and linamarin as substrates.

Assay of linamarase:

Assay was as already described above except that 1.00ml of enzyme extract was used instead of 0.10ml. Assay with PNP-glucoside was as described before. To assay with linamarin, 1.0ml of the enzyme extract was incubated with 10  $\mu$ moles of linamarin in 1.00ml of 0.01M acetate buffer to release free cyanide which forms soluble orange-red complex with added alkaline picrate solutions. The incubation was at room temperature in a tall stoppered test tube. At the end of a twenty-minute incubation time, 4.5ml of alkaline picrate solution was added and the resulting solution was incubated in a water bath at 95°C for 5 minutes. Upon cooling to room temperature, the absorbance of orange-red colour formed was read at 490 nm in a Corning Model 253 colorimeter.

Fermentation of cassava in the presence of D-glucono (1  $\rightarrow$  5) lactone

Three equal samples of grated cassava (approximately 500g each) were simultaneously fermented at room temperature for 5 days in 1 liter beakers covered with aluminium foils. 50ml each of 0.20M and 0.40M D-glucono (1  $\rightarrow$  5) lactone was added to two of the beakers with grated cassava, and thoroughly mixed or worked into the mash before fermentation started. No D-glucono (1  $\rightarrow$  5) lactone was added to the third sample which was used as control.

## Chapter 3

RESULTSFermentation of Cassava:

Natural fermentation of grated cassava conducted in beakers and Thermos flasks resembled fermentation usually carried out in jute sacks and drums in gari manufacturing factories in Nigeria. Evidence that fermentation occurred was obtained by monitoring the pH which usually became more acid with fermentation time and temperature which increased steadily as a result of the exergonic nature of the fermentation reactions. The presence of typical gari flavour upon frying the fermented dewatered mash provided yet additional proof of normal fermentation. Figure 1 shows the observed increase in temperature and decrease in pH accompanying cassava fermentation.

Endogenous linamarase activity and development of acid pH during cassava fermentation:

Figures 2A and 2B represent results of periodic determinations of endogenous linamarase activity and cyanide content of the fermenting cassava mash. It was found that the linamarase activity (Fig. 2A) and total cyanide (Fig. 2B) in both fermentation system (TF and BK) steadily decreased with duration of fermentation for the first three days after which they levelled off somewhat. The level of free cyanide which is indicative of the activity of endogenous linamarase gradually decreased within the period of fermentation.

### Chapter 3

#### RESULTS

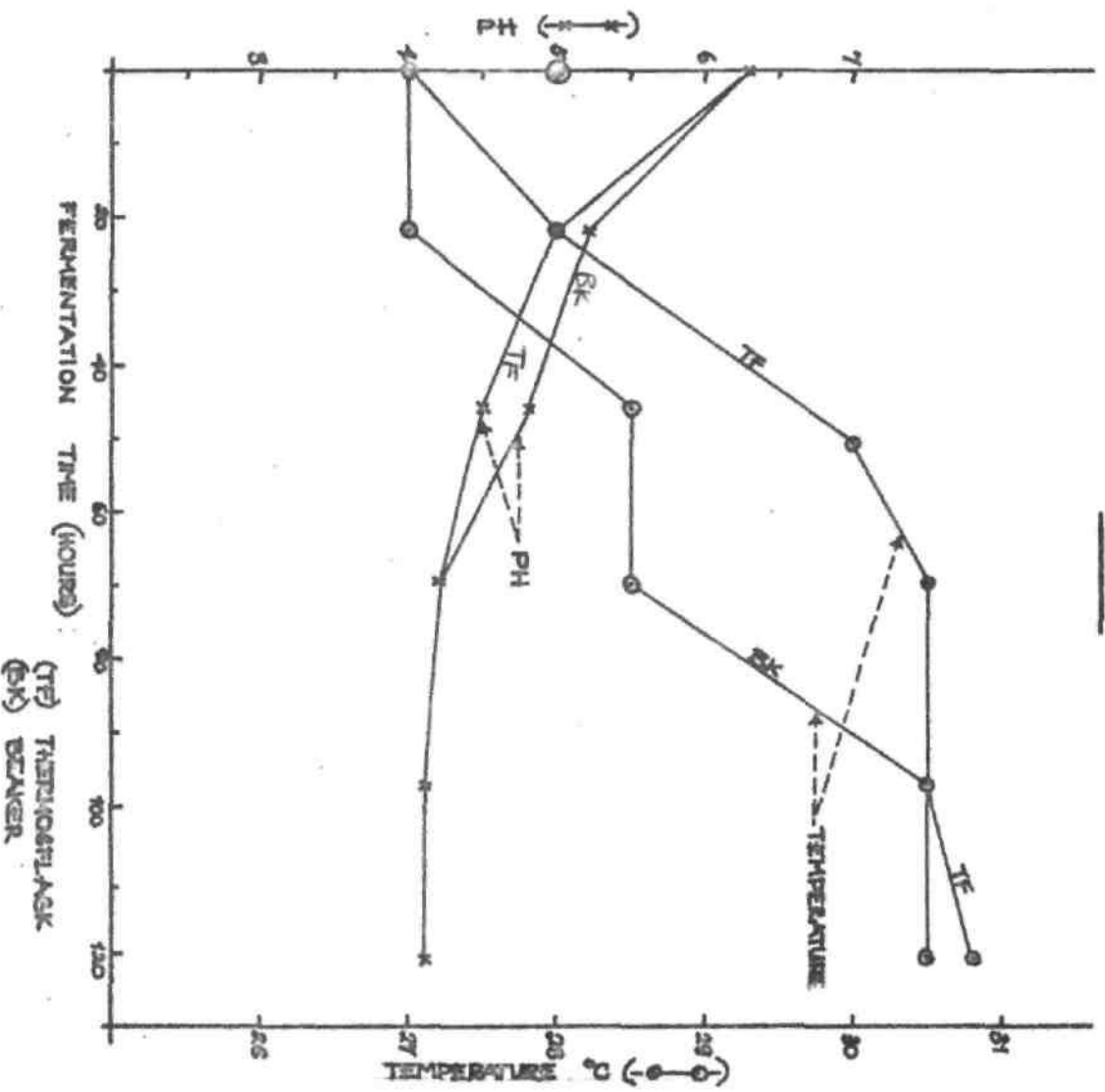
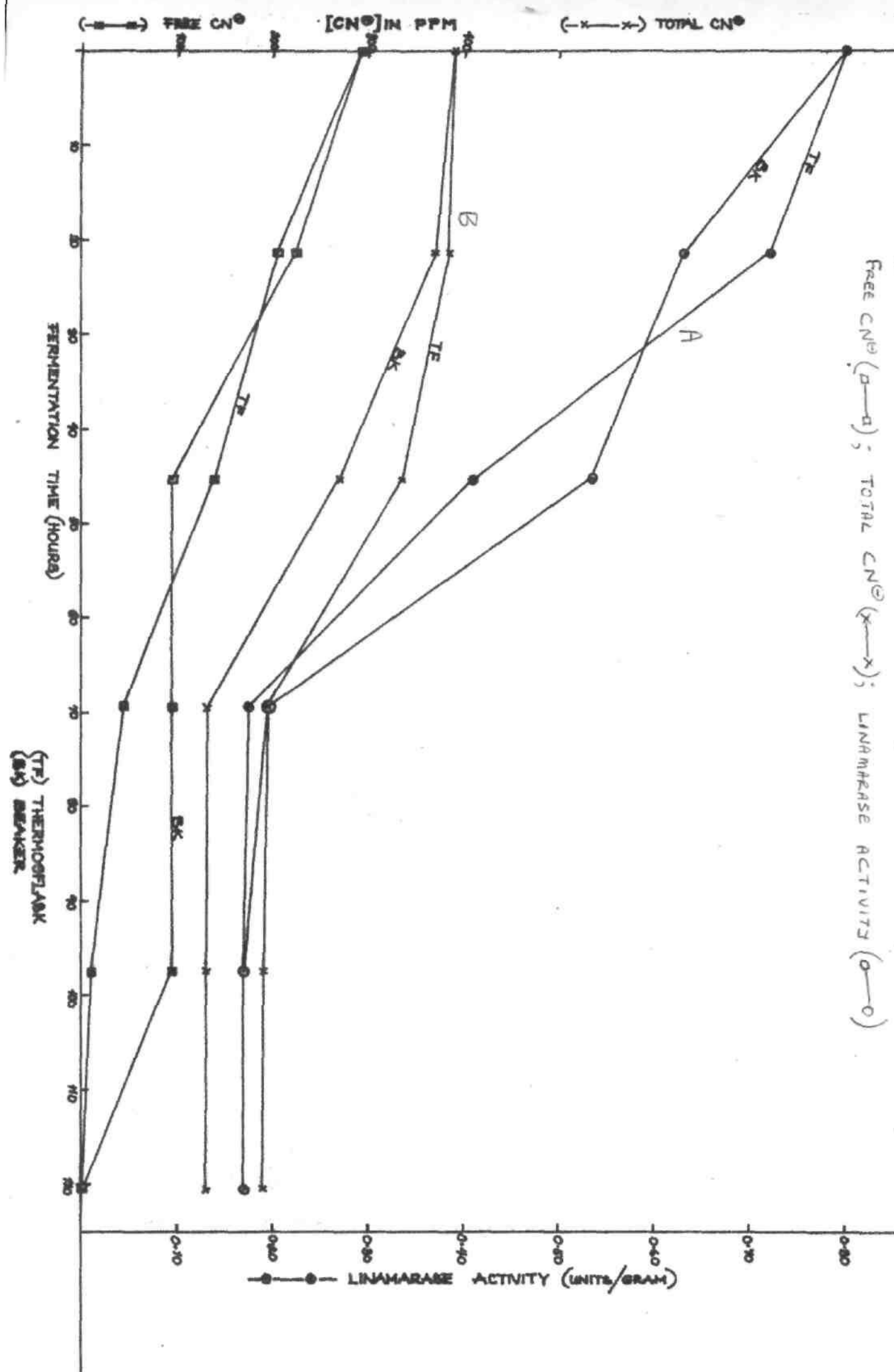


Figure 1: Changes in pH and temperature of grated cassava during its fermentation at room temperature.

Figure 2A & B: Changes in endogenous linamarase activity and total and free cyanide in fermenting grated cassava as a function of fermentation period.



### Effect of acidity on the hydrolysis of linamarin:

Extracts of boiled cassava tubers containing known amounts of free and bound cyanide (crude linamarin) were incubated with HCL of different pHs for two hours in tightly stoppered test tubes and the cyanide released determined as described before. Figure 3 shows that the percent hydrolysis of the linamarin in the crude extracts was low but increased with an increase in pH. Although the tubers had been boiled for one hour prior to marceration and extraction, extracts obtained still showed some, though low, linamarase activity as indicated in Figure 3.

### Exogenous additions of linamarase to fermenting cassava for gari production:

Table 1 is an experimental set-up showing the sequence of linamarase additions to fermenting cassava during gari production on a laboratory scale. Figure 4 shows that there was considerable improvement in extent of detoxification as a result of exogenous addition of linamarase to fermenting cassava. The same figure also shows that after twenty-four hours, further linamarase additions did not result in significantly improved detoxification.

### Addition of D-glucono-(1 $\rightarrow$ 5) lactone to fermenting cassava:

Figures 5A and 5B show that after 24 hours there was 4 fold and 6 fold decrease in linamarase activity respectively, as a result of the presence of D-glucono (1  $\rightarrow$  5) lactone in the fermenting mash.

Figure 3: Effect of acidity on linamarin hydrolysis.

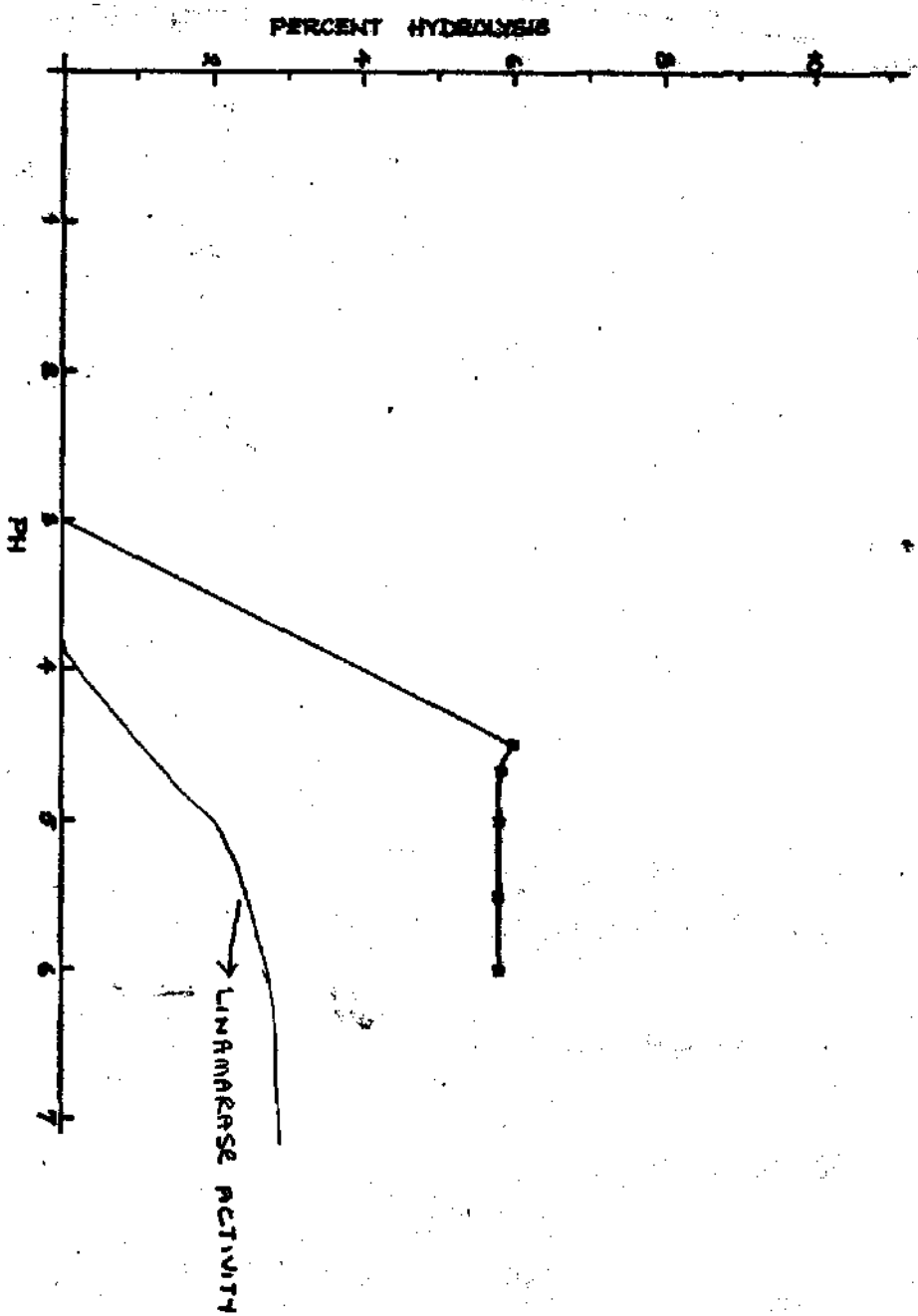


Figure 4: Effect of exogenous linamarase additions on the cyanide content of fermenting grated cassava.

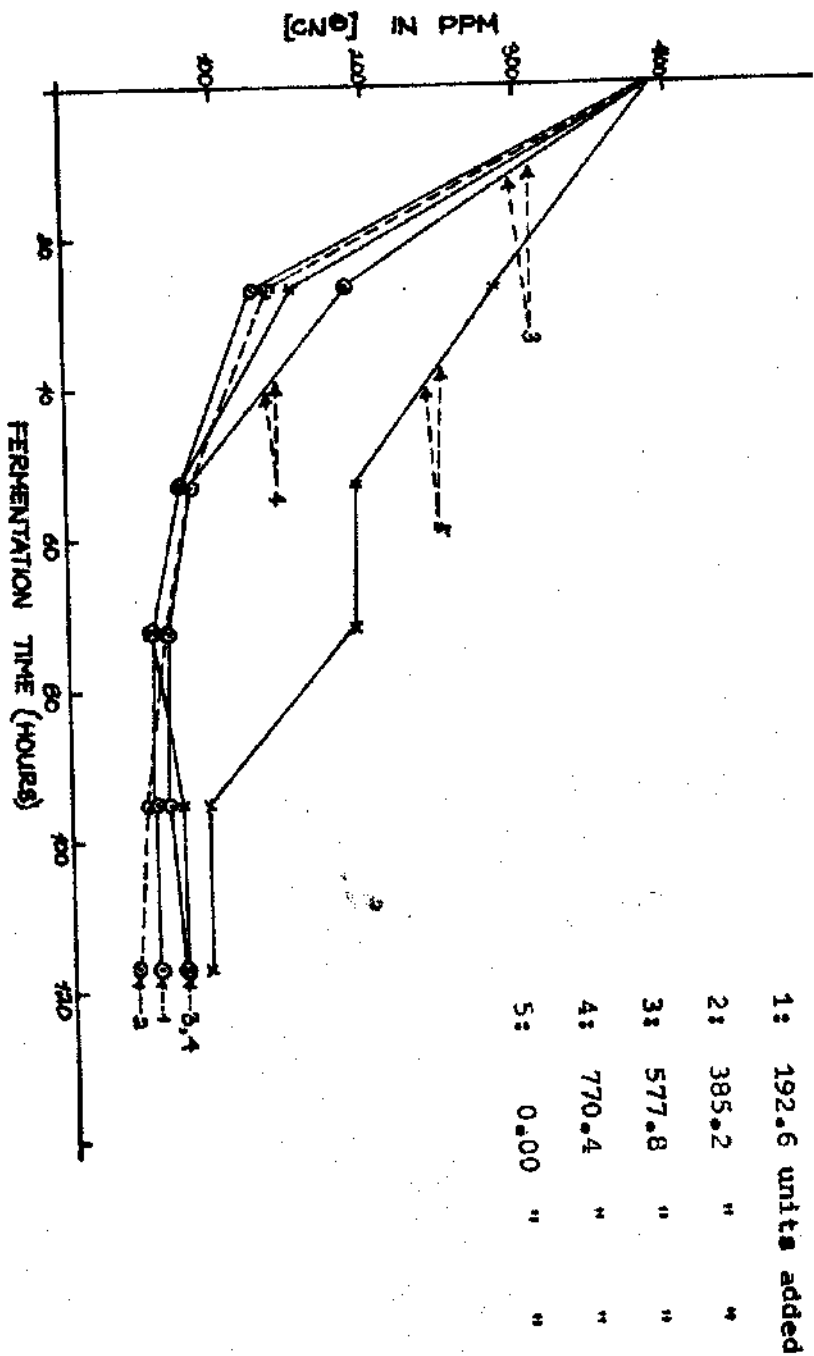


**Table 1:** Experimental setup for the 5 day fermentation of Cassava in the presence of exogenously added partially purified linamarase enzyme.

| Fermentation<br>time (day)                  | Amount of Linamarase enzyme added each time (units) |                           |                           |                           |                           |
|---------------------------------------------|-----------------------------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                                             | Fermenting<br>Sample<br>1                           | Fermenting<br>Sample<br>2 | Fermenting<br>Sample<br>3 | Fermenting<br>Sample<br>4 | Fermenting<br>Sample<br>5 |
| 1                                           | 192.6                                               | 192.6                     | 192.6                     | 192.6                     | -                         |
| 2                                           | -                                                   | 192.6                     | 192.6                     | 192.6                     | -                         |
| 3                                           | -                                                   | -                         | 192.6                     | 192.6                     | -                         |
| 4                                           | -                                                   | -                         | -                         | 192.6                     | -                         |
| 5                                           |                                                     |                           |                           |                           |                           |
| Total linamarase<br>activity added:         | 192.6                                               | 385                       | 577.8                     | 770.4                     | 0.00                      |
| Cyanide content of<br>resulting gari (ppm): | 33                                                  | 29                        | 22                        | 20                        | 62                        |

Figures 5A and 5B show that cyanide content of the fermenting mash did not decrease sufficiently to account for the loss in activity of linamarase. In the control (Figure 5C) linamarase activity decreased with the cyanide concentration.

Screening fungal Isolates for Linamarase activity:

Table 2A is the list of fungi screened for linamarase activity with P-nitrophenol  $\beta$ -D glucoside. Those that indicated some linamarase activity were cultured again and the extract assayed for activity with linamarin (Table 2b).

Quality Control tests on gari samples obtained from different localities in Nigeria:

Table 3 shows the results of the analysis of thirteen gari samples collected from different parts of the country. Gari obtained from Oshodi (Federal Institute of Industrial Research), Owerri and that made in the laboratory with linamarase, contained the least amount of cyanide. The swelling power of the laboratory gari was found to be low. There was no correlation between the starch content and swelling power of all the gari samples analyzed (Table 4 and Fig. 6).

Figure 5A, B & C: Effect of adding 50 ml of 0.20M (A) 0.40M(B)

D-glucono (1 $\rightarrow$ 5) lactone to fermenting grated cassava during gari production.

C: Control: NO D-glucono (1 $\rightarrow$ 5) lactone added.

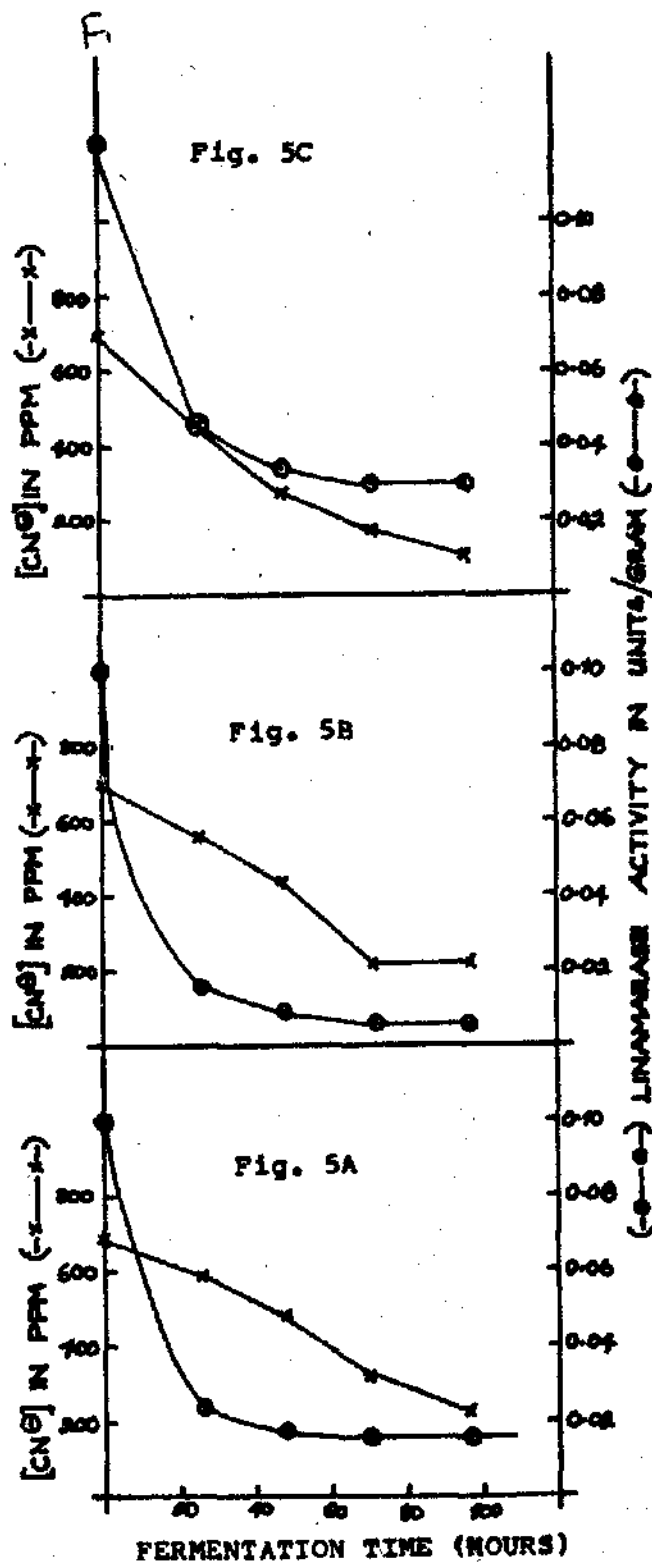


Figure 6: Correlation graph of 13 garl samples by percent starch and by swelling power.

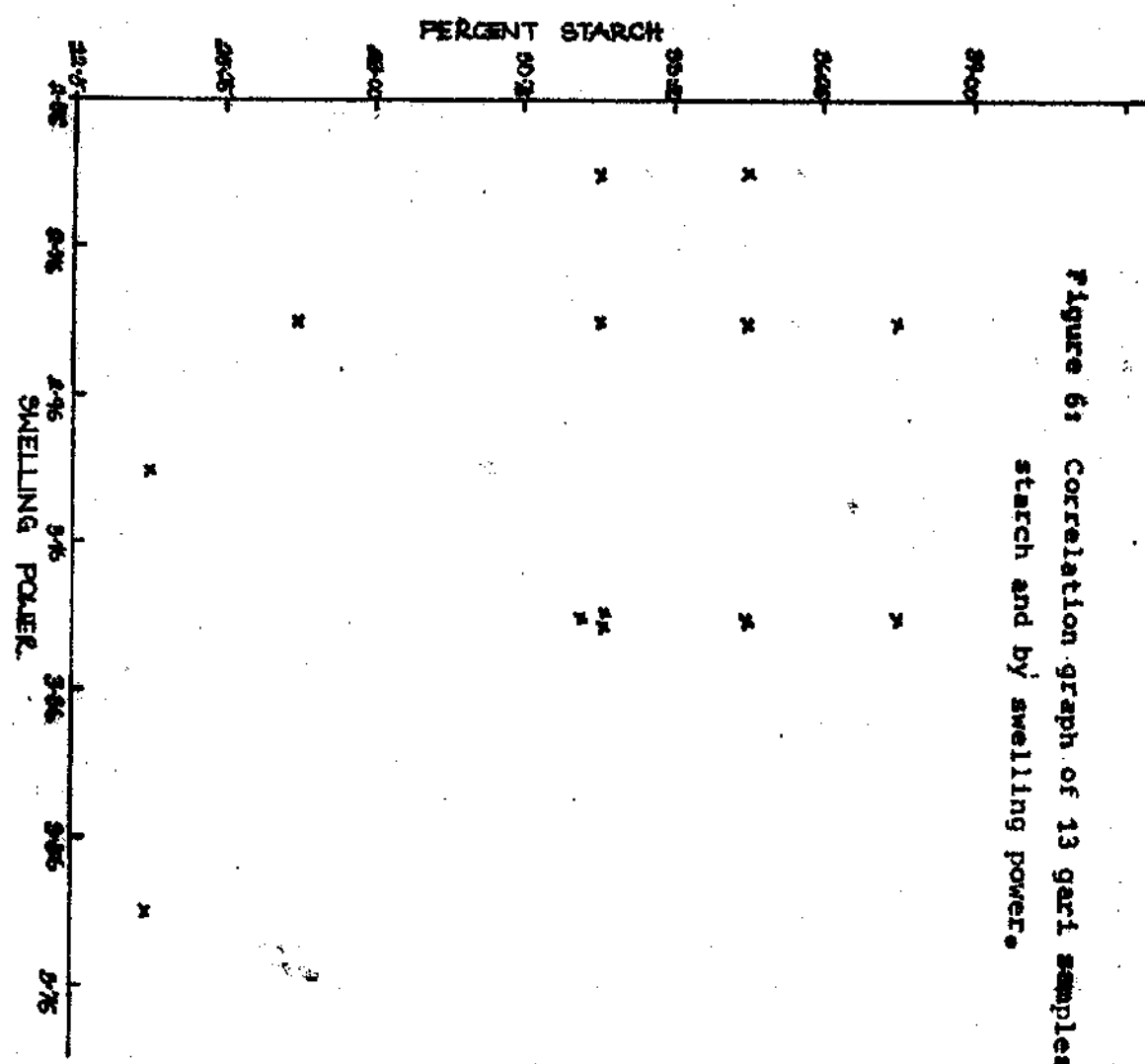


Table 2A: Screening fungal isolates for the presence of  
B-glucosidase activity using P-nitrophenol B-D glucoside  
as substrate.

|             |               |      |
|-------------|---------------|------|
| Rhizopus    | species       | --   |
| Penicillium | citrinum      | --   |
| Penicillium | steckii       | ++++ |
| Penicillium | funiculosum   | --   |
| Penicillium | brefeldianum  | --   |
| Penicillium | spiculisporum | --   |
| Aspergillus | terreus       | ++   |
| Aspergillus | carneus       | ++   |
| Aspergillus | species       | ++   |
| Aspergillus | ochraceus     | ++   |
| Aspergillus | amstelodani   | --   |
| Aspergillus | zydowii       | ++++ |
| Fusarium    | oxysporum     | --   |
| Fusarium    | accumination  | --   |
| Fusarium    | moniliforme   | ±    |
| Fusarium    | semitectum    | --   |
| Fusarium    | equiseti      | ++   |

+ shows positive test for linamarase activity.

-- shows no reaction with PNP - glucoside

± slight enzyme activity.

Table 2B: Assay of fungal extracts for Linamarase activity with  
Linamarin as substrate.

|             |           |      |
|-------------|-----------|------|
| Aspergillus | terreus   | ++   |
| Aspergillus | zydowii   | ++++ |
| Penicillium | steckii   | ++++ |
| Aspergillus | ochraceus | ++   |
| Fusarium    | equiseti  | ++   |

Table 3: Quality control tests on gari samples.

|              | %<br>Moisture | SP   | Percent<br>starch | Percent<br>fibre | Percent<br>protein | Percent<br>lactic<br>acid | CNO<br>PPM | Percent<br>foreign<br>matter |
|--------------|---------------|------|-------------------|------------------|--------------------|---------------------------|------------|------------------------------|
| Agbor        | 6.289         | 3.28 | 33.60             | 1.8              | 3.675              | 0.294                     | 127        | 0                            |
| *Nqwa        | 5.936         | 3.28 | 31.60             | 2                | 2.888              | 0.533                     | 116        | Negligible                   |
| Oji          | 4.77          | 3.76 | 24                | 1.20             | 3.675              | 0.38                      | +348       | Negligible                   |
| FIIR-O       | 4.749         | 3.2  | 32.25             | 1.37             | 4.2                | 1.48                      | 26         | 0                            |
| *Umunede     | 7.561         | 2.56 | 33.75             | 0.75             | 2.8                | 0.06                      | +549       | Negligible                   |
| *Lagos       | 10.829        | 2.88 | 31.50             | 1.4              | 4.2                | 0.12                      | 216        | Negligible                   |
| Ndi-oru      | 6.130         | 2.88 | 26.25             | 1.2              | 4.55               | 0.824                     | 43         | "                            |
| *Ife         | 6.043         | 2.92 | 37.50             | 1.6              | 2.88               | 0.08                      | 87         | "                            |
| *Onitsha     | 15.775        | 3.36 | 31.50             | 1.6              | 4.55               | 0.08                      | 128        | "                            |
| Owerri       | 6.765         | 3.32 | 39.00             | 1.9              | 4.55               | 0.85                      | 27         | "                            |
| NRCF         | 3.523         | 2.8  | 36                | 1.95             | 3.675              | 0.987                     | 33         | 0                            |
| NRCRI        | 3.977         | 3.08 | 22.50             | 1.1              | 3.15               | 0.213                     | 94         | 0                            |
| Lab.<br>gari | 3.078         | 2.6  | 33                | 1.4              | 3.675              | 0.46                      | 20         | 0                            |

\* Appearance changed from normal creamy yellow to grayish colour due to spoilage caused by mould formation in the gari.

+ The extremely high cyanide level obtained for gari samples from Oji and Umunede was probably due to some contaminant in the gari sample which probably increased the intensity of the soluble red complex.

Table 4: Correlation table of 13 gari samples by percent starch and by swelling power.

| Percent starch | <u>Swelling power</u> |                |                |                |                |                | total |
|----------------|-----------------------|----------------|----------------|----------------|----------------|----------------|-------|
|                | 2.56 -<br>2.76        | 2.76 -<br>2.96 | 2.96 -<br>3.16 | 3.16 -<br>3.36 | 3.36 -<br>3.56 | 3.56 -<br>3.76 |       |
| 22.5 - 25.25   | -                     | -              | 1              | -              | -              | 1              | 2     |
| 25.25- 28.00   | -                     | 1              | -              | -              | -              | -              | 1     |
| 28.00- 30.75   | -                     | -              | -              | -              | -              | -              | -     |
| 30.75- 33.50   | 1                     | 1              | -              | 3              | -              | -              | 5     |
| 33.50- 36.25   | 1                     | 1              | -              | 1              | -              | -              | 3     |
| 36.25- 39.00   | -                     | 1              | -              | 1              | -              | -              | 2     |
| total          | 2                     | 4              | 1              | 5              | -              | 1              | 13    |

CHAPTER 4DISCUSSION

The endogenous (E.C.3.2.1.21; Linamarin  $\beta$ -D-glucoside glucohydrolase) linamarase in cassava plays a crucial role in the hydrolysis of the cyanogenic glucosides, linamarin and lotaustralin in fermenting grated cassava during gari production.

The unavailability of commercial linamarase and the dearth of literature information on its purification have for many years hindered the use of an enzymatic approach to cyanide analysis until very recently when Cooke *et. al.* (1978) published the purification of cassava linamarase and introduced the use of this enzyme in estimating total cyanide in cassava tubers and leaves (Cooke, 1978).

The incorporation of an enzymatic step in the assay for total cyanide made it possible to determine the ratio of glycosidic to free cyanide in cassava extract. Glycosidic and free cyanide in cassava respond differently to cassava processing and storage, and their toxicities differ.

The well known alkaline picrate test for cyanide which has been improved by incorporating an enzymatic step to make the assay more specific and quantitative was used in the present investigation.



The hydrolysis of the cyanogenous glucosides, linamarin and lotaustralin, which occurs during the fermentation of grated cassava has generally been known as a detoxification process, but the precise mechanism of this detoxification process is still unclear.

If acid hydrolysis is the basis of this fermentation - associated detoxification as speculated in the literature (Collard 1963, Collard and Levi 1959, Akinrole 1962, 1963, 1964) it would have been possible to obtain considerable hydrolysis of crude linamarin (boiled cassava extract) when the latter was incubated with dilute acid. Figure 3 shows that little or no hydrolysis occurred at the very acid pH of 1 - 3 while up to 6 percent hydrolysis was obtained at the much less acid pH of 4 - 6. Although the cassava from which the crude linamarin was prepared had been boiled ostensibly to destroy all linamarase activity prior to extraction, enzyme assay performed later on these extracts, revealed evidence of residual linamarase activity. Obviously this residual linamarase activity was responsible for hydrolysis observed between pH 4 and 6. Infact, the pH optimum of linamarase has been shown to be around pH 5 and 6 (Wood 1966, Cooke 1978) and the enzyme is known to have negligible or no activity below pH 3. This explains the observations depicted in Figure 3 and shows that detoxification of fermenting cassava via hydrolysis of the cyanogenic glucosides must be attributed to factor(s) other than sheer acidity or decrease of pH brought about by the fermentative actions of micro-organisms. Wood (1966) working

with pure linamarin demonstrated rather convincingly that the latter is stable to acid when after heating it with N-hydrochloric or N-sulphuric acid at  $100^{\circ}\text{C}$  for 30 minutes, they failed to detect any hydrogen cyanide release. That the little hydrolysis observed in the present study (Fig. 3) was due solely to surviving linamarase activity is further supported by the reported unusual thermostability of linamarase (Coop 1940, Ikediobi et. al. 1980). Wood (1965) has even reported linamarase activity in gari and roasted cassava flour thus establishing the insufficiency of boiling, particularly of the tuber, as a means of eliminating the enzyme entirely.

The general decline in the activity of linamarase with increase in fermentation time (Fig. 2A) is in line with the simultaneous shift in the pH of the fermenting cassava mash away from around 6, which is about the pH optimum of this enzyme, to a pH of 4 at which the enzyme has considerably reduced activity (Cooke 1978, Wood 1965). The near cessation of further hydrolysis of the cyanogenic glucosides after 72 hours of fermentation (Fig. 2B) coincided with the levelling off of linamarase activity and attainment of an unfavourable acid pH of 4.2 in the fermenting medium thus demonstrating that hydrolysis of linamarin was infact associated with enzyme rather than acid catalysis. The effect of fermentation was thus virtually accomplished after 3 days. It should be

mentioned at this point that most gari producers whether at the village or industrial level ferment their cassava for only 3 - 4 days. It would seem, however, that, if the mechanism of detoxification is solely enzymatic as this work appears to suggest, it should be possible to hasten detoxification as well as increase extent of detoxification by adding exogenous crude or partially purified linamarase to the fermenting cassava mash. The 8-fold detoxification achieved with exogenous linamarase additions as opposed to slightly over 2 without any enzyme addition (Fig. 4), is further indicative of the enzymatic origin of the detoxification process. Two additional points of interest are obvious in this enzyme addition experiment. Firstly, higher fold detoxification was achieved after 24 hours of fermentation in the presence of added linamarase, (Fig. 4). Secondly very little additional detoxification was achieved by adding more linamarase to the fermenting mash after 24 hours (Fig. 4, Table 1).

Usually in the first 24 hours the pH of the fermenting cassava mash lay between 5 and 6 (Fig. 1) which range contains the pH optimum for linamarase. Thus the enzyme would be expected to be fairly active between pH 5 and 6 and catalyze the hydrolysis of linamarin releasing free HCN. The levelling off of free cyanide assay after 72 hours was thus more indicative of considerably less active linamarase enzyme than to the attainment of a steady state concentration by free cyanide.

The effect of D-glucono (1  $\rightarrow$  5) lactone), a potent inhibitor of linamarase (Wood 1966) is a further proof of the detoxifying role of linamarase. After 24 hours fermentation, the fermenting mash that has been treated with D-glucono (1  $\rightarrow$  5) lactone (Fig. 5A, B, C) had a considerable drop in linamarase activity with very little decrease in total cyanide content. The pH of the fermenting mash also dropped in 24 hours to about 4.5. In the fermenting mash without D-glucono (1  $\rightarrow$  5) lactone linamarase decreased with a concomitant drop in total cyanide after 24 hours. The pH of the control stood around 5.2 after twenty-four hours.

The much more acidic nature of the D-glucono (1  $\rightarrow$  5) lactone - treated cassava mash is probably due to hydrolysis of the lactone to gluconic acid which may contribute to acidity of the fermenting medium.

These experiments (Fig. 3, 4, Table 1) suggest that linamarase is the crucial factor in cassava detoxification. Gari produced with exogenous linamarase will therefore have very little or no cyanide in it. The 3 - 4 days used for fermentation of grated cassava will be cut down to about 24 - 30 hours.

The characteristic gari flavour, is partly due to Lactic and formic acids that are produced within the first 48 hours of fermentation (Akinrele 1963). In the third to fourth day of fermentation, further acidification of the fermenting medium

has been attributed to a mould, Geotricum candida (Akinrele 1963). Okafor (1977) has shown that the predominant micro-organisms isolated from dewatered cassava pulp fermented for gari production were mainly leuconostoc; (lactic acid forming bacteria). The micro-organisms were isolated in increasing numbers over a six day period from the fermenting mash.

Acidification of fermentation medium due to increasing concentration of lactic acid, and probably other flavour - forming compounds which give characteristic aroma of gari may not be achieved completely within 24 hours, but they can be easily supplemented.

Another problem that will hinder the commercial use of linamarase is the unavailability of large quantities of the enzyme. The enzyme, linamarase, is concentrated in the peel, in order to obtain linamarase that will be sufficient for a sizable quantity of gari several tons of cassava will be needed. To ferment 200 grams of grated cassava a minimum of 500 units of linamarase activity will be needed. 2.5 kilogrammes of fresh cassava will give about 350 grams of cassava peel which will in turn yield about 400 units of linamarase. The extraction of this enzyme is expensive and laborious and would make obtaining it from cassava tubers, leaves or any other plant source very expensive. Another source of linamarase is therefore needed.

Micro-organisms were considered a better source of linamarase. They grow very fast, so are capable of producing large quantities of the enzyme in a very short time period. Micro-organisms can be grown all the year round and can be isolated fairly easily, especially if the enzyme is extra-cellular. This will lead to a steady supply of the enzyme in large quantities and so continuous production of gari will be achieved. Several fungi known to produce amyloglucosidase (Wiserman 1979) were screened (Table 2A). Seven of the fungi screened (Table 2A) exhibited some  $\beta$ -glucosidase activity, penicillium steckii and aspergillus zydowii produced extracts with the greatest activity. Those fungi that exhibited  $\beta$ -glucosidase activity were cultured again and assayed for linamarase activity with linamarin (Table 2B). All the five fungi assayed released cyanide from linamarin.

The fungi screened here, do not seem to grow very well on potato dextrose agar-streptomycin (PDAS) medium. A medium that will allow these micro-organisms to grow maximally would lead to production of more of the enzyme. Since the enzymes were produced extracellularly, it should be possible to inoculate grated cassava with any of these linamarase-producing fungi, but there will be a need to find out if these fungi produce other enzyme or toxic substances that will make gari undesirable before using the fungal extract or inoculating with it.

To do this effectively a large number of cultures need to be screened and tested and a great deal of effort should be invested in standardising the procedures in order to obtain quantitatively reproducible results.

In most technologically advanced countries, new products, especially food and drugs have to meet some national established standards and secondly have to be acceptable to consumers. These established standards are a series of quality criteria for assessing the food/drug item. It differs for different foodstuffs. If an additive is to be used in food processing, this additive may not be used to deceive the consumer: the additive should not disguise the natural attributes or quality of the product.

Presently in Nigeria, most locally produced food items do not go through any established quality criteria. The consumer or cost of the food item determines whether it is going to be sold. There is therefore a need to establish quality criteria for foodstuff produced locally. Some quality criteria are very important in the production of good gari (Table 3). These are moisture content, swelling power, starch content, fibre, protein, acidity, cyanide content and foreign matter. The gari produced with linamarase was as good as any of the other gari samples. Its cyanide content was the lowest and the flavour, taste and aroma were normal. 

will be feasible to use linamarase, for commercial scale gari production. The present critical situation in the world food supplies especially Africa, demands that all our agricultural resources be utilized to the fullest. In order to achieve this it is essential that more applied and basic research be directed towards cassava production and utilization. The present study has provided information about:

- (1) a new method for the manufacture of gari which will remove the perennial fear of cyanide toxicity.
- (2) opened up a new area of research into cassava utilization, particularly in the area of mechanized gari production.

Suggestions for further work:

- (1) Basic research on the mechanism of formation of this enzyme and environmental factors influencing linamarase, formation and secretion by fungi.
- (2) Screening more micro-organisms for linamarase activity in order to obtain the ones that can grow readily and are safe for use in producing food destined for human consumption.
- (3) A good knowledge of the primary and 3-D structure of linamarase might in the long run improve its use. The use of linamarase in the immobilized form should be investigated. Although the technique is still new, one of its advantages will be that the enzyme may be recovered from the reaction mixture and reused. To do this effectively

the primary structure and the 3-dimensional structure of linamerase must be known to some extent.

- (4) A thorough knowledge of the inhibitors of linam~~er~~ase and the physical conditions favourable to them will throw more light on the natural conditions best suited for fermenting cassava and help in the design of an improved cassava processing plant for gari production.

APPENDICESAppendix 1: Calculations

## A. Cyanide concentration in parts per million (PPM)

Let Dry weight of sample = W(g)  
 and Volume of Extract = V(ml)  
 Vol. of Extract assayed = 0.1ml  
 Reaction volume = 5.5ml

A<sub>490</sub> = 0.2 = 1.44 ug/ml from standard curve

Cyanide in Reaction volume = 1.44 ug/ml x 5.5ml = 7.92 ug

Cyanide in the Extract =

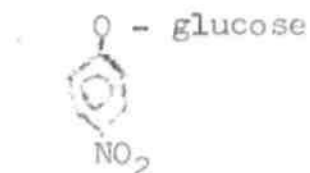
$$\left\{ \frac{7.92}{0.1\text{ml}} \right\} V(\text{ml}) \mu\text{g}$$

Cyanide concentration in PPM

$$\left\{ \frac{7.92}{0.1} \right\} V \mu\text{g}/W(\text{g})$$

## B. Activity:

1 unit of linamarase activity is the amount of linamarase which will catalyze the formation of  $\mu\text{mole}$  of p-nitrophenolate per minute from p-nitrophenyl  $\beta$ -D glucoside, i.e.



p-nitrophenyl  $\beta$ -D glucoside



p-nitrophenolate ion

$$E = (\text{Molar extinction coefficient}) = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

$$A_{400} = E c l.$$

$$\text{Enzyme units} = \frac{A_{400}}{60} \times 10^6 \mu\text{mole/liter/min}$$

$$= \frac{A_{400}}{60} \times 10^6 \times \frac{4^*}{10^3} \mu\text{mole/ml}$$

$$* \text{ Reaction volume} = 4\text{ml.}$$

$$= \frac{A_{400}}{60} \times \frac{1}{1.8 \times 10^4} \times 10^3 \times 4$$

$$= \frac{A_{400}}{270}$$

Let total vol of Extr.

$$= 40 \text{ (ml)}$$

Vol. assayed

$$= 0.1 \text{ (ml)}$$

Dry wt

$$= 15 \text{ grammes}$$

Concn. of Extract

$$= \frac{15}{10} \frac{\text{g}}{\text{ml}} = .375$$

amount assayed in grams

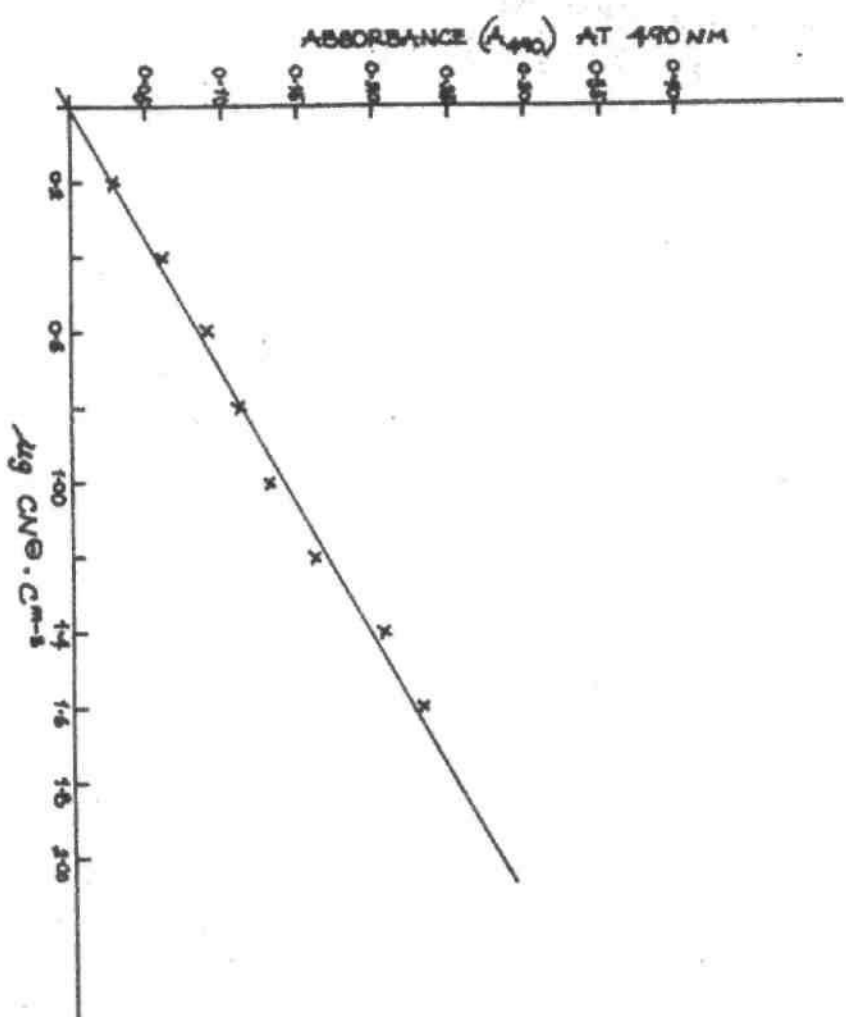
$$= 0.1 \times 0.375$$

Enzyme units/gram

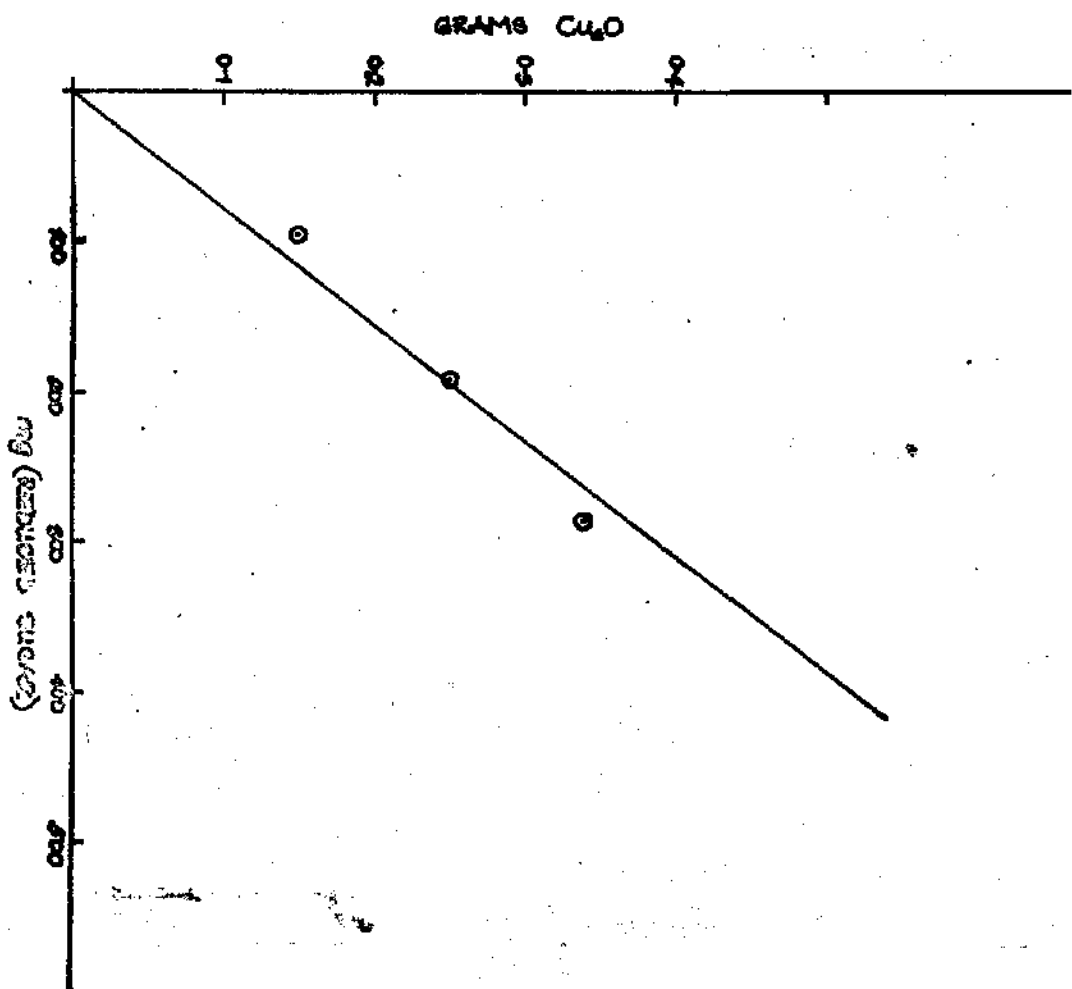
$$= \frac{A_{400}}{270} / 0.0375$$

APPENDIX 2

A: Standard Curve for cyanide estimation.



B: Standard Curve for sugar determination.



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