# THE SOLID SUBSTRATE FERMENTATION OF AFRICAN LOCUST BEANS (*Parkia filicoidea* Welw.)

by

© Marcel James Ikenebomeh

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Department of Microbiology
Macdonald Campus of McGill University
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# PARKIA FERMENTATION

Ikenebomeh

## ABSTRACT

Ph.D. Marcel James Ikenebomeh Microbiology

THE SOLID SUBSTRATE FERMENTATION OF

AFRICAN LOCUST BEANS, Parkia filicoidea Welw.

The fermentation of African locust beans was studied. These beans in the raw state are inedible but may be rendered palatable by fermentation. Both their texture and taste are thereby considerably improved.

The beans were first processed by boiling and dehulling. They were then fermented at 37 ± 2°C in stationary fermentor units. Physico-chemical, microbiological, biochemical and enzymological investigations were carried out on the substrate undergoing spontaneous fermentation and on radappertized (11 kGy) controls. Reductions in carbohydrate (52%), protein (12%) and energy (9%) contents, as obtained from the mass and energy balances, occurred during fermentation. The physico-chemical changes were due to the action of extracellular carbohydrases and proteases produced by a Bacillus sp. Pure culture inoculation with the Bacillus sp. gave the fermented product within 24 h in contrast to the normal 72 h fermentation period.

Both pH and titratable acidity increased during fermentation. Ammonia was produced as a result of proteolysis. Fermented African locust beans are rich in both protein and fat and feature in the diet of a large proportion of the 200 million people in West Africa.

RESUME

Ph.D. Marcel James Ikenebomeh Microbiologie

SUBSTRAT SOLIDE DE FERMENTATION

DES CAROUBES (fèves Africaines), Parkia filicoidea Welw.

Nous avons étudié le procédé de fermentation des caroubes. A l'état brut, ces fèves ne sont pas comestibles mais peuvent le devenir grâce à un processus de fermentation qui permet d'en améliorer considérablement le goût et la texture.

Les caroubes ont d'abord été bouillies et décortiquées. Elles ont ensuite été fermentées dans des unités stationnaires de fermentation à 37 ± 2°C. Des études physico-chimiques, microbiologiques, biochimiques et enzymatiques ont été effectuées sur le substrat en cours de fermentation spontanée de même que sur des substrats témoins scellés et irradiés (11 kGy). Les bilans de masse et d'énergie indiquent une diminution du contenu glucidique (52%), protidique (12%) et énergétique (9%) au cours de la fermentation. Ces changements sont dus à l'action des saccharases et des protéases extracellulaires de Bacillus sp. L'inoculation de Bacillus sp. dans une culture pure nous permet d'obtenir un produit fermenté après une période de 24 h comparativement à la durée normale de fermentation de 72 h.

Le pH ainsi que le titre acide ont augmenté durant la fermentation et la production d'ammoniaque resulte d'une protéolyse.

Les caroubes fermentées sont riches en protéines et en lipides. Elles occupent une place prépondérante dans le régime d'une large proportion des 200 millions d'habitants de l'Afrique occidentale.

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## CLAIM OF CONTRIBUTION TO KNOWLEDGE

Three principal aspects of the solid substrate fermentation of Parkia filicoidea Welw. were investigated in this thesis. The original contributions to knowledge which the author wishes to claim from this study are listed below:

1. Information on the physico-chemical aspects of the dawadawa fermentation with detailed data having been made available on:
   - Moisture contents
   - Fat contents
   - Protein contents
   - Ash contents
   - Total carbohydrate contents
   - Objective texture measurements
   - Density of whole beans
   - Mass balance during processing and fermentation
   - Energy balance during the fermentation.
2. Knowledge of the microbiological aspects of the African locust bean fermentation with detailed data on:
   - The changes in microbial population during processing and fermentation.
   - The obligatory presence of microorganisms for the process of bioconversion to occur.
   - The nature of the microorganisms responsible for bioconversion.
   - The organism's ability to bioconvert processed substrate to the fermented product.

- The simultaneous increases in pH and titratable acidity during the fermentation.
- The effects of various conditions on the progress of the fermentation e.g. effects of temperature, addition of salt, water activity, presence of oxygen and pure culture inoculation.

3. Information on some biochemical and enzymological aspects of the fermentation with detailed data on
   - The ability of the bioconverting microorganisms to secrete extracellular enzymes.
   - The production, during fermentation, of extracellular carbohydrases and proteases.
   - The absence of qualitatively detectable extracellular lipases during fermentation.
   - The production of ammonia at the advanced stage of fermentation.

The information reported above, in practical terms, may contribute to:

(i) the scale up of the process of the solid substrate fermentation of the African locust beans;

(ii) the consistency of the dawadawa product;

(iii) the elimination of possible food spoilage and health hazard microorganisms by the use of pure culture inoculations; and

(iv) the development of other industrial processes involving either the isolated microorganism or the extracellular enzymes produced by them.

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AECL | Atomic Energy of Canada Limited |
| Aw | Water activity |
| BioD | Biodeteriorated sample |
| BV | Biological value |
| BY | Bioyield |
| CFU | Colony forming units |
| DB | Dehulled beans |
| DPM | Drop plate method |
| DS | Dissolved solids |
| FB | Fermented beans |
| Fig | Figure |
| FPS | Fermenting processed substrate |
| g | Gram |
| G.E.C. | Gross energy content |
| h | Hour |
| kGy | kiloGray |
| kJ | kilojoule |
| kPa | kiloPascal (Unit of pressure) |
| LDFB | Lyophilized defatted fermented beans |
| LDPS | Lyophilized defatted processed substrate |
| LFB | Lyophilized fermented beans |
| LPFS | Lyophilized processed fermenting substrate |
| LPS | Lyophilized processed substrate |
| LRADB | Lyophilized radappertized beans |

| | |
|---|---|
| MDB | Mechanically dehulled beans |
| min | minutes |
| Mrad | Mega rad |
| MRT | Mechanically removed testa |
| N | Newton (Unit of force) |
| NPB | Non-processed beans |
| NPU | Net protein utilization |
| NS | Presumably 'sun dried' fermented beans from Nigeria |
| ø | Zero |
| O.D | Optical density |
| ρ | Density ($kg/m^3$) |
| PER | Protein efficiency ratio |
| PF | Peak force |
| PFS | Processed fermenting substrate |
| PS | Processed substrate |
| RADB | Radappertized beans |
| RB | Raw beans |
| SPC | Standard plate count method |
| SPM | Surface plate or surface spread method |
| T.A. | Titratable acidity |
| TACC | Total available carbohydrate contents |
| µm | micrometre ($10^{-6}$ m) |
| V/W | Volume for weight |
| WB | Whole beans |
| WFB | Wet fermented beans |
| WPFS | Wet processed fermenting substrate |

| | |
|---|---|
| WPS | Wet processed substrate |
| W/W | Weight for weight |

**Media**

| | |
|---|---|
| BHIB | Brain heart infusion broth |
| MRS | De Man Rogosa Sharpe medium |
| PCA | Plate count agar |
| mPCB | mPlate count broth |
| PDA | Potato dextrose agar |
| SMA | Standard method agar |
| SMCA | Standard method caseinate agar |
| TJA | Tomato juice agar |
| WA | Wort agar |

# CHAPTER 1

# INTRODUCTION

# 1. INTRODUCTION

A major plant protein source for humans and livestock is leguminous seeds. These are derived mostly from seasonal leguminous plants such as soybean, peanut and black eye bean. However, within the tropics of Cancer and Capricorn are found many perennial leguminous trees which, unlike their seasonal relations, are less known and in most cases do not enter into international trade. This latter group has for centuries been exploited by the people of the various locales in which they are found. They serve as sources of plant protein, medicine, timber and dyes. They also provide shelter and act as windbrake and recirculate soil nutrients to arid lands. A plant in this category is the African locust bean tree (Parkia filicoidea Welw. and Parkia biglobosa Benth.) found in West Africa.

The Parkiae belong to the Family Angiospermae, Division Lignosae, Order Leguminales, Suborder Mimoseae and Tribe Parkieae. They produce large, pendulous, edible fruits or pods containing a yellowish, dry, powdery, sweet pulp in which are embedded the seeds. The seeds may be dark brown or black. They may be oblong, convex or flattened, having mid point measurements of 11, 9 and 5 mm for their length, width and thickness respectively. They have a mean weight of 250 mg per seed. Since these seeds are extremely hard when raw, they are usually consumed after roasting like coffee beans or after processing followed by a solid substrate fermentation.

Solid substrate fermentation of agricultural products to obtain fermented foods is currently receiving some attention as a means of promoting organoleptic improvements, post harvest preservation and the nutritional improvement of diets made up mostly of carbohydrate materials. These foods, in addition to other fermented foods, will probably gain considerable popularity in the near future.

Despite the known advantages of fermentation, very few fermented foods have been scientifically studied (Hesseltine, 1965) and fewer still in processes involving solid substrate fermentation since measurements of parameters such as pH, oxygen level and others are much more difficult to make.

Many calls have been made by scientists and international organizations on the need for research into fermented foods, especially those from the developing countries. The understanding of the changes involved in these local fermented foods is often far from complete and there is still much to be learned. The United Nations workshop on 'World Hunger' (UN, 1979) confirmed that food fermentation could be used to considerably improve world preservation and conservation of food. It stated that the use of specific microorganisms with perishable foods such as black gum, cassava, corn, eggs, fish, meat, milk, rice, sorghum, soybeans, taro and wheat can result in bioconversion to products with better nutritive value, digestibility, appearance and shelf life than the original raw materials. It also suggested controlled fermentation of various agricultural waste products into palatable foods. The workshop recommended future research and more work with the fermentation of indigenous foods to permit their

higher consumption. Similar conclusions were reached by DaSilva and Doelle (1980). The International Foundation for Science (IFS) in its Triennial Report (1981) listed as one of its research priorities the area of indigenous food fermentation. The conditions necessary for scale up or industrialization of indigenous fermented foods were discussed recently by Steinkraus (1981). He proposed investigations into the basic questions common to each fermentation process such as the organisms involved and the optimum conditions and parameters for the processes.

The fermented product of African locust beans is known as "dawadawa". It features in the diet of a large fraction of the 200 million people in West Africa. This has given rise to a handful of chemical and nutritional investigations reports and two review articles. These still leave a lot of questions unanswered. It is not known what actually is taking place in terms of physico-chemical changes which occur during the fermentation. There are no known conditions to maximize product output and the agents responsible for fermentation have, until now, not been identified. There are no records of the microorganisms or enzymes involved in the bioconversion (Stanton and Wallbridge, 1969 and Beuchat, 1978a, b).

Notwithstanding the general agreement on the need for research into indigenous fermented foods, one may still need to know why the Parkia seeds should be fermented or studied at all. There are several reasons for this. The endosperm is rich both in protein and fat. The knowledge of the fermentation process will lead to production optimization, scale up,

a safer product which can serve as a meat substitute, influence the use of arid lands for agriculture, improve product homogeneity, open up new possibilities and fill the knowledge gap.

Presently the product is obtained largely by uncontrolled fermentation. In this the initial conditions are set to favor the naturally occurring microorganisms by "rule of thumb" substrate preparation. The solid substrate is then allowed to undergo spontaneous fermentation. This approach can be problematic should the substrate be contaminated with food poisoning or food spoilage microorganisms. It may not necessarily allow for the most rapid product turnover since no scientific data about the optimal conditions for rapid growth of the right type of microorganism and for bioconversion exist.

The present study was therefore undertaken. Three separate parts which were linked by microbial activities, were studied. In the first part the physico-chemical aspects of the fermentation were studied on a time sequenced basis on the substrate incubated at 37°C. The observable changes during the physico-chemical investigation were related to the presence of microorganisms. In the second part the microbiological aspects of the fermentation, the importance of microorganisms to the process and the conditions promoting their growth were studied. In the third part the biochemical and enzymological aspects of the fermentation were investigated.

It is shown from the physico-chemical results that changes do occur in the processed substrate during the fermentation of African locust beans. Reductions in carbohydrate, protein and energy contents were obtained from the mass balance of the fermentation system. Textural changes also occurred during fermentation.

The principal microorganisms responsible for the changes were found to be a Gram positive, sausage-shaped, catalase-positive and spore-forming Bacillus sp. associated with the processed substrate. The importance of this organism to the process was its ability to produce extracellular amylases and proteases through which the substrate is hydrolyzed and bioconverted.

Some variable but measurable parameters of the fermentation (pH and titratable acidity developed) may be used to characterize the product of fermentation at any given period during the process of bioconversion.

# CHAPTER 2

# LITERATURE REVIEW

## 2. LITERATURE REVIEW

Fermented foods are consumed in all the continents of the world. Their production is mediated by the action of microorganisms and/or their enzymes on suitable organic substrates which may be derived from either animal or plant sources. For example, milk serves as the substrate for the production of cheese and yoghurt while beer and bread are respectively obtained from the fermentations of barley and wheat flour. Soybeans can be used alone, as in the production of natto and tempeh, or in a mixture as in the production of miso and shoyu.

In West Africa an 'unusual' substrate from a perennial leguminous plant the African locust bean is exploited. The beans, when raw, are practically inedible. By processing and subsequently fermenting them an excellent product eaten as a condiment by the people of West Africa is obtained. In the following sections this product, known as 'Dawadawa', and some closely related products are reviewed.

## 2.1. FERMENTED AFRICAN LOCUST BEANS 'DAWADAWA'

Although fermented foods play an important role in the diet of the people of West Africa, relatively little is known from the literature about many of these. This is true particularly of 'Dawadawa' or 'Soumbara'. Dawadawa consists of the boiled, dehulled and fermented seeds of either Parkia filicoidea Welw. or Parkia biglobosa Benth. (Aykroyd and Doughty, 1964; Platt, 1964; Hesseltine, 1965 and Stanton and Wallbridge, 1969). Both these species are commonly referred to as the 'African locust bean tree'. Their seeds, which are borne in pods, are known as 'African locust bean seeds'. Dawadawa is a tasty product of high nutritive value being rich in both protein and fat. When dried it may contain up to 50% protein (Perissé, 1958; Aykroyd and Doughty, 1964 and Schery, 1972). These qualities make dawadawa a very useful addition to the West African diet.

Like dawadawa, many other fermented foods have been developed through native technology in different parts of the world (Platt, 1964 and Hesseltine, 1965). Some of these foods are discussed below.

## 2.2. WORLD TRADITIONAL FERMENTED FOODS

Traditional fermented foods have been produced for centuries in different parts of the world even when the microorganisms responsible for the bioconversion were not known. Hesseltine (1965) indexed more than 170 of these foods. Information on the form of the product, the substrate employed, the microorganisms involved and the geographical location of the production for many of these were reported upon by Hesseltine (1965), Hesseltine and Wang (1967), Whitaker (1978), Beuchat (1978a, b) and Wang and Hesseltine (1979).

For some of the products high protein substrates are used. For example, fish is the substrate for Katsuobushi, Bagoong and Nuoc-man; milk is the substrate for cheese and yoghurt (Whitaker, 1978). Some other foods are produced from grains, legumes and oilseeds (Beuchat, 1978b) and fruits and vegetables (Pederson, 1979). These include: Ang-kak, Beer, Bread, Dawadawa, Garri, Hamanatto, Idli, Injera, Lao-chao, Minchin, Miso, Nata, Natto, Ogi, Ontjom, Pickles, Ragi, Sauerkraut, Shoyu, Sufu, Tempeh and Wines (Hesseltine, 1965; Hesseltine and Wang, 1967; Beuchat, 1978b; Jay, 1978; Whitaker, 1978 and Pederson, 1979).

In many areas of the world fermentation is probably the only method available for vegetable preservation (Pederson, 1979). These fermentations include cabbage to sauerkraut, cucumbers to pickles, green olives to fermented olives and the preservation of carrots by lactic acid bacteria (Pederson and Albury, 1961, 1969; Niketic-Aleksic et al., 1973; Etchelles et al., 1975; Vaughn, 1954, 1975 and Pederson, 1979).

In table 1 are listed some fermented foods, their form, substrate, the active microorganism(s), changes brought about in the substrate and the areas of the world in which each is produced. Some of these foods are reviewed in the next section.

### 2.2.1. Description of Some Traditional Fermented Food Processes

Most of the information in the literature about fermented food processes, other than those to produce alcoholic beverages, sausages and milk products, has been published during the last three decades. Major contributions have been made by Yokotsuka (1960), Platt (1964), Steinkraus et al. (1960, 1961, 1965), Hesseltine (1965), Hesseltine and Wang (1967), Stanton and Wallbridge (1969), Yong and Wood (1974), Beuchat (1978b), Jay (1978), Whitaker (1978), Pederson (1979) and Wang and Hesseltine (1979). A large fraction of these reports have dealt with the fermentation of plant materials, particularly soybeans in the Orient.

The production of these foods may be complex, as exemplified by miso, shoyu and sufu or simple e.g. tempeh and natto. One common feature of all the processes is that the presence of microorganisms is a requirement. Below are presented the important steps in the preparation of some of these foods.

Ang-kak

Polished rice is the substrate for ang-kak. The rice is soaked in water

Table 1. Some World-Fermented Foods*.

| Product | Substrate | Microorganism(s) | Nature of Product | Change in Substrate | Geography |
|---|---|---|---|---|---|
| Animal: | | | | | |
| Cheeses | | | | | |
| Roquefort | Milk | *Penicillium roqueforti* | Solid | Flavor, color, texture | Europe, America |
| Camembert | Milk | *Penicillium camembert* | Solid | Flavor, texture | Europe, America |
| Cheddar | Milk | *Streptococcus lactis*, *S. cremoris* (sometimes) *S. durans*) | Solid | Flavor, texture | Europe, America |
| Cottage | Milk | *Lactobacillus lactis* | Solid | Texture | Europe, America |
| Yoghurt | Milk | *Streptococcus thermophilus* and *Lactics bulgaris* | Viscous liquid | Flavor, texture | Europe, America |
| Meat | Beef | *Thamnidium* spp. | Solid | Texture, flavor | - |
| Katsuobushi | Bonito (fish) | *Aspergillus*; possibly *A. glaucus* | Solid, hard dry | Flavor, texture | Orient |
| Bagoong | Fish | Not known | Paste | Flavor, texture | Philippines |
| Nuoc-mam | Fish | Halophilic bacteria | Sauce | Flavor, texture (salty, cheese-like) | South-east Asia |

Table 1 continued.

| Product | Substrate | Microorganism(s) | *Nature of Product | Change in Substrate | Geography |
|---|---|---|---|---|---|
| Miso | Rice and soybeans or rice and other cereals such as barley | Aspergillus oryzae, Lactobacillus bacteria, Saccharomyces rouxii | Paste | Texture, flavor | Japan, China |
| Natto | Soybeans | Bacillus subtilis | Solid | Flavor (strong, musty) | Japan |
| Ogi | Maize | Lactic bacteria, Cephalosporium, Fusarium, Aspergillus, Penicillium spp., Saccharomyces cerevisiae, Candida mycoderma | Paste | Texture, flavor | Nigeria West Africa |
| Ontjom | Peanuts | Neurospora sitophila | Solid | Flavor, color (pink). Mince meat or almond-like flavor | East Indies |
| Palm-wine | Palm sap | Acetobacter spp., Lactics, yeasts | Liquid¶ | Flavor¶ | Nigeria |
| Pickles | Cucumbers | Pediocuccus cerevisiae, Lactobacillus plantarum | Solid¶ | Flavor¶, texture | Worldwide |
| Sauerkraut | Cabbage | Leuconostoc mesenteroides, Lactobacillus plantarum | Solid¶ | Texture¶, flavor | Worldwide |
| Shoyu | Soybeans | Aspergillus oryzae, Lactobacillus, Hansenula and Saccharomyces | Liquid | Flavor, color, texture | Orient, America, Europe |

Table 1 continued.

| Product | Substrate | Microorganism(s) | Nature of Product | Change in Substrate | Geography |
|---|---|---|---|---|---|
| Sufu (Chinese cheese) | Soybean curd | Actinomucor elegans and Mucor spp. | Solid | Flavor | China, Formosa |
| Tao-si | Soybeans plus wheat flour | Aspergillus oryzae | Semi-solid | Flavor | Philippines |
| Tape | Cassava or rice | Saccharomyces cerevisiae, Hansenula anomala, Rhizopus oryzae, Chlamydomucor oryzae, Mucor spp., Endomycopsis fibuligera (Saccharomycopsis spp.) | Soft solid | Texture | Indonesia |
| Tempeh | Soybeans | Rhizopus spp. principally Rhizopus oligosporus | Solid | Texture, flavor | Indonesia, Surinam |
| Torani | Rice | Hansenula anomala, Candida quilliermondii, Candida tropicalis, Geotrichum candidum | Liquid | Texture, flavor | India |
| Wines | Grapes, other fruits | Saccharomyces ellipsoideus strains | Liquid | Flavor, texture | Worldwide |

* Adapted in part from Hesseltine (1965), Hesseltine and Wang (1967), Beuchat (1978b), Jay (1978) and Whitaker (1978).

# Product of interest.

† Current studies.

¶ Author.

for 24 h, drained of excess water and inoculated with the mold Monascus purpureus. The inoculated rice is allowed to ferment for about 3 weeks. During the fermentation period, a purplish red color develops on the substrate (Whitaker, 1978 and Wang and Hesseltine, 1979). After fermentation, the product is dried, ground and employed as a food coloring agent. According to Nishikawa (1932), the purplish red color is composed of two pigments. These are monascorubrin ($C_{22}H_{24}O_5$)-red and monascoflavin ($C_{17}H_{22}O_4$)-yellow. Both pigments were reported to be produced by the mold mentioned above (Nishikawa, 1932; Whitaker, 1978 and Wang and Hesseltine, 1979).

Miso

The miso fermentation is a two step process (Hesseltine, 1965). The first step involves the fermentation of the primary substrate, rice, by the mold Aspergillus oryzae. The mold produces the enzymes required for the second step of the process. Usually the primary fermentation is carried out at 40°C or below for 48-50 h. The molded rice is harvested before the formation of spores.

In the second stage of the process a mixture of the molded rice, soybeans, salt and inoculum is fermented. The inoculum is usually derived from a previous miso fermentation. The molded rice serves as a source of amylolytic enzymes which further hydrolyze the rice starch to simple sugars. These sugars are metabolized for energy by yeasts and other microorganisms present during the second stage of fermentation (Hesseltine, 1965). This stage is initially carried out at a temperature of 28°C for

7 days. The temperature is then raised to 35°C and the fermentation is allowed to continue for several months. The whole process may take two years to complete.

Mixed cultures are used during the second stage. Hesseltine (1965) has pointed out that the use of mixed cultures has obvious disadvantages in this type of fermentation. Some of these include: (i) propagation of contaminants from one batch to the next, (ii) loss of vigor by the desired microorganisms due to competition and (iii) prolonged fermentation due to loss of vigor.

Hesseltine (1965) carried out the process with pure culture isolates obtained from the fermentation brew. He reported that Saccharomyces rouxii Bontroux was the principal organism involved in the second stage and concluded that the use of a pure culture reduced the time required for fermentation with no loss in organoleptic properties of the product.

Natto

Natto production involves the fermentation of whole soybeans with Bacillus subtilis (Bacillus natto). Hesseltine (1965) has stated that detailed studies of natto fermentation initially were carried out by Hayashi (1959-1960) and Sakurai (1960).

According to Hesseltine (1965), cleansed soybeans are soaked in water for 12 to 20 h until their weight is approximately doubled. The beans are steamed for about 15 min. to soften, cooled and then inoculated with Bacillus subtilis (Bacillus natto). The inoculated beans are subsequently

wrapped in leaves and allowed to ferment for 18 to 20 h at 40-43°C. Aeration may be required during the fermentation to prevent overheating.

Ontjom

Ontjom is made from peanuts whose oil has been extracted. The residue after oil extraction is called 'peanut press cake'. The cake is soaked in water for 24 h. During this period the remaining oil floats to the surface and is then poured off with excess water. The defatted peanuts are washed several times with water, steamed and inoculated with Neurospora sitophila (Hesseltine and Wang, 1967 and Beuchat, 1976).

Hesseltine and Wang (1967) have observed that the product of fermentation was pink as a result of N. sitophila conidia formation on the cake. The organism is an active producer of lipases and proteases. The enzymatic hydrolysis of peanut constituents greatly enhances the edibility and, presumably, the digestibility of the product (Beuchat, 1976).

Shoyu

Shoyu, also known as soy sauce, traditionally is produced in a multi-stage batch process. Three types of microorganisms - molds, bacteria and yeasts have been reported to be involved in the fermentation (Whitaker, 1978). Beuchat (1978b) has indicated that the major organisms involved were Aspergillus oryzae or A. soyae, Lactobacillus bacteria and Saccharomyces rouxii (Zygosaccharomyces spp). Outlines of the shoyu process have been published by Yokotsuka (1960), Hesseltine (1965), Hesseltine and Wang (1967), Yong and Wood (1974) and most recently by Wang and Hesseltine (1979).

According to Yong and Wood (1974), the soy sauce fermentation is quite complex. In its preparation, soybeans and wheat flour are mixed and inoculated with a mold - Aspergillus oryzae or A. soyae. The mold is allowed to grow for three days on the mixed substrate. After this period luxurious mold growth has usually occurred. The mold covered substrate is mixed with a brine wherein an anaerobic fermentation takes place. Lactobacillus bacteria and the yeast Saccharomyces rouxii are the organisms active in this step. The liquid obtained after the anaerobic fermentation is the shoyu or soy sauce of commerce (Yong and Wood, 1974).

Wang and Hesseltine (1979) have reported that Japan leads the world in soy sauce production as a result of the implementation of advanced technology. The Japanese have extended their operations into North America by building a plant for soy-sauce production at Walworth, Wisconsin in 1972 (Wang and Hesseltine, 1979). Ebine (1976) has reported that 14,000 metric tons of whole soybeans and 176,000 metric tons of defatted soybean products were used in Japan in 1974 to produce more than one million kilo litre (kl) of soy sauce. These figures show the importance of soy sauce in commerce.

Sufu

Sufu, a traditional Chinese food, is a soft cream cheese type product made from cubes of soybean curd (tofu) by the action of a mold. According to Wang and Hesseltine (1979), to prepare sufu, soybeans are washed, soaked and ground with water. A water to dry bean ratio of 10:1 is reported to be commonly used. The ground mass is then boiled or steamed

for about 20 min. to facilitate filtering, to inactivate growth inhibitors and to reduce the bean flavor. The hot mass is strained through a cloth bag, a double layered cheese cloth or a fine metal screen to separate the liquid fraction (soybean milk) from the solid residue. A coagulant such as calcium sulfate, magnesium sulfate or acetic acid, is then added to the milk to coagulate proteins. The curd is transferred into a cloth-lined wooden box and pressed with a weight on top to remove the whey. A soft, but firm, cakelike curd (tofu) forms. Typically, tofu used for sufu fermentation contains 83% water, 10% protein and 4% lipids (Wang and Hesseltine, 1979).

The tofu is cooled and then inoculated with Actinomucor elegans. Other organisms reported by Beuchat (1978b) to be involved in the sufu fermentation include Mucor hiemalis, M. silvaticus and M. subtilissimus. Wai (1968) has confirmed the presence of all three organisms in the fermentation of sufu and concluded that the molds involved did not develop any disagreeable odor, astringent taste or mycotoxins.

After inoculation the curd is placed in an incubator at a temperature of 20°C or below for a period of 3 to 7 days. The duration of incubation depends upon the culture employed (Hesseltine, 1965).

The molded curd is cut into cubes and these are salted by putting them into a tub in alternate layers with salt for a day. The cubes are rinsed with water and packed into glass or earthenware containers (Wang and Hesseltine, 1979). Brine solution, made up of varying amounts of distilled liquor, salt and flavoring agents, is added to the packed cubes.

The sufu is allowed to age for 1-3 months and then is ready for market (Wang and Hesseltine, 1979).

During the brining and ageing process, the enzymes released by the molds act on their respective substrates and yield various hydrolytic products (Wang and Hesseltine, 1979). For example, soybean proteins are converted to peptides and amino acids (Hesseltine, 1965). Wang (1967) has reported that enzymes released by the molds are normally extracellular but usually loosely bound to the mycelium, possibly by ionic linkages. These enzymes can be eluted by sodium chloride or other ionic solutions (Wang, 1967). Once eluted from the mycelium, these proteases penetrate the molded cubes to act upon the proteins. The brining process is therefore essential to the fermentation of sufu. Wang (1967) and Wang and Hesseltine (1979) have reported that the minimal salt concentration for efficient release of mycelium bound protease is 3%.

Tempeh

Tempeh, judged from the number of publications, is one of the most studied fermented food products. Hesseltine et al. (1963), Martinelli and Hesseltine (1964), Steinkraus et al. (1960, 1961, 1965) and Wang and Hesseltine (1966, 1979) have all published reports on tempeh production, the processes involved and the possible scale up of these processes to an industrial size.

Tempeh fermentation involves the use of soybeans which have been soaked overnight in water at 25°C. The beans' seed coats are first removed and they are boiled for about half an hour at atmospheric pressure and drained

of excess water. The dehulled, boiled beans are cooled and inoculated with spores of an appropriate Rhizopus strain (Hesseltine, 1965).

Stahel (1946) and van Veen and Schaefer (1950) have stated that the mold responsible for tempeh fermentation is Rhizopus oryzae. However, Hesseltine et al. (1963) employed a strain of Rhizopus identified as R. oligosporus saito NRRL 2710. This strain is believed to be the principal species involved in the fermentation of Indonesian tempeh. Wang et al. (1975) have found that an inoculum level of $10^6$ spores of R. oligosporus per 100 g of cooked substrate to be adequate for tempeh production. These spores could be mass produced for the industrial fermentation of tempeh (Wang et al., 1975).

After inoculation of the prepared beans the fermentation is reported to take a short time, between 20 to 24 h, at 30 to 31°C (Whitaker, 1978). During the fermentation, the mold binds the loose beans together. Tempeh is frequently consumed after being deep fried.

## 2.3. THE SUBSTRATE: AFRICAN LOCUST BEANS AND PARKIA SPECIES

The seeds of the Parkia species found in West Africa are the substrate for dawadawa production. They are borne in large, pendulous, edible pods produced by two closely related leguminous tree species - Parkia filicoidea and Parkia biglobosa (Bailey, 1963; Schery, 1972 and Anon, 1979). The species found in South East Asia, P. speciosa Hassk and P. javanica Merr., also produce pods and seeds used for food (Hutchinson, 1964).

Typical legume seed pods of the African species have been reported to arise from clavate or depressed globose heads (Hutchinson, 1964). The pods, during maturation, continually change in color, from green to light brown and finally to black. Each pod holds about 12 to 22 seeds (Dalziel, 1937) and the seeds, when raw, are very hard (Aykroyd and Doughty, 1964). They can, however, be fermented into a cheese-like product (Aykroyd and Doughty, 1964; Busson, 1965; Stanton and Wallbridge, 1969; Schery, 1972; Campbell-Platt, 1980 and Felker, 1981).

The composition (by weight) of Parkia pods was reported by (a) Dalziel (1937) and (b) Campbell-Platt (1980):

| | (a) | (b) |
|---|---|---|
| Pod Case (Husk) | 41.2% | 42% |
| Pulp | 33.4% | 36% |
| Seed | 25.4% | 22% |

Varied estimates of the yield of pods per tree have been given. Anon (1922) estimated 52 kg while Busson (1965) reported 25 kg. Stanton et al. (1966) have observed that a yield of 350-500 kg pods/ha could be expected if the trees are planted as an orchard. More recently, Rachie and Roberts (1974) have reported that an average productivity of 350 to 500 kg of seeds/ha is possible. They did, however, not report on the number of trees per hectare.

Usually, the Parkia spp. are not planted nor cultivated. Most of their seeds, therefore, are collected from the wild (Schery, 1972) by individuals (Campbell-Platt, 1980). It is therefore not surprising that accurate estimates of annual production are widely different from each other, for example, in one report it was stated that: "although this tree, Parkia filicoidea Welw., is not normally planted, the seeds gathered annually in Nigeria must probably be many thousands of kilograms" (Stanton et al., 1966). In contrast, a recent estimate by Cobley and Steele (1976) of the amount of beans collected every year in Northern Nigeria was '200,000 tons'. The actual figure, it is presumed, probably lies between these two extremes.

Internationally, the Food and Agriculture Organization (FAO) at present does not report annual production statistics for African locust beans although the importance of the Parkia bean in nutrition has been recognized, as indicated by some of the publications in which this crop is mentioned (Aylward, 1961; Aykroyd and Doughty, 1964; Stanton et al., 1966 and FAO, 1970).

In most West African countries there exists an active trade in both the dry African locust bean seeds and the fermented product, dawadawa. For example, Parkia seeds have been reported to be carried across the borders of Dahomey (presently Republic of Benin) in West Africa. Some of the bean seeds gathered in Northern Nigeria are carried to other West African countries whereas others are transported to Southern areas of Nigeria in bags weighing 100 or 500 kg. These bean seeds are employed mainly for dawadawa production in Nigeria.

The perennial, leguminous, Parkia species have been reported to have a special advantage over the conventional, seasonal legumes in that they can survive on lands generally considered as unproductive for normal agriculture (Schery, 1972 and Felker, 1981). This attribute implies that the supply of the substrate will probably not be a problem when dawadawa production will be changed from the present day home processing operation to an industrial scale. The botany of the plant is discussed next.

### 2.3.1. Botany of Parkia Species

Park (1799), a Scottish surgeon (Campbell-Platt, 1980), who explored the source of the River Niger in West Africa, was the first man to describe the Parkia plant. The genus was named after him (Henderson, 1890; Bailey, 1963 and Campbell-Platt, 1980).

Hutchinson (1964) classified the Parkiae as follows:

Family: Angiospermae

Division 1: Lignosae

Order 7: Leguminales

Sub-order: Mimoseae

Tribe 2: Parkieae

According to Hutchinson (1964) the type species, P. filicoidea Welw., was described by Welwitsch in 1862. Heywood (1971) in his classification described the Parkiae as belonging to the family Leguminosae, sub-family Mimosoideae and order Leguminales. He stated that the taxonomic divisions of the Leguminosae are yet to be generally agreed upon; however, there is no disagreement as to what is meant when the name 'legume' is used. Basically, a legume is a dehiscent fruit that develops from a single carpel and splits into two valves that surround the fruit (Heywood, 1971).

Sorsa (1969) and Heywood (1971) reported that Parkia species have 24 or 26 chromosomes. The African species, according to Henderson (1890), is a large tree which bears bi-globular heads of scarlet flowers at the ends of long stalks. It is possible that P. biglobosa, an African Parkia species, might have obtained its name from the bi-globular heads of the flower. Hutchinson (1964) in his description of the tribe Parkieae has noted that Parkia species have spikes which are condensed into a dense, globose or clavate head with no stamens.

More extensive descriptions of the species have been given by Hutchinson and Dalziel (1927), Bailey (1963) and Hutchinson (1964). They stated that Parkiae are unarmed trees, having bi-pinnate leaves with numerous

pinnae and leaflets. The flowers have been observed to be very numerous in large clavate or depressed globose heads. Ten stamens and staminodes are said to be present with a cylindrical calyx. The ovary has been noted to be sessile or stipitate with numerous ovules and a terminal stigma. The fruit, according to Hutchinson (1964), can be oblong or elongated, straight or curved, compressed, rather woody or fleshy. He has also stated that the seeds may be transverse, thick, ovoid or compressed.

Heywood (1971) has noted that the Leguminosae are known to exhibit entomophilous pollination mechanisms. It is not clear from this statement whether Parkia species exhibit this type of mechanism or not. On the other hand, there have been reports in the literature that pollination in some Parkia species is mediated by bats (Baker and Harris, 1957 and Campbell-Platt, 1980).

The African species, P. biglobosa, according to Hutchinson and Dalziel (1927), flowers in December and produces fruit in March.

Rachie and Roberts (1974) noted that Parkia species will tolerate and thrive within a wide range of climatic conditions. Their global distribution is discussed in the next section.

### 2.3.2. World Distribution of Parkia Species

The Parkiae are a small but widely spread genus of Leguminosae found in Africa, Brazil, India, Java and Surinam (Henderson, 1890). They have

been found in the tropics of both hemispheres (Bailey, 1963 and Hutchinson, 1964).

Hutchinson (1964) stated that about sixty (60) different Parkia species are known to exist in the tropical zones of the world. Fifty-eight (58) species were listed by Hooker and Jackson (1895) while Bailey (1963) indicated that about ten (10) species could be found in the tropics of the two hemispheres. In a revision of the genus Parkia found in Africa, Hagos (1962) stated that the number of species could be less than the fifty-eight earlier reported. There is no doubt that some species might have been named more than once.

Parkia species have been found in Northern South America (e.g. Brazil), Central, East and West Africa, Malaysia and Indonesia. Uphof (1959) and Hutchinson (1964) reported upon the presence of two species in South East Asia. These are Parkia speciosa, 25 m tall, and P. javanica also called P. roxburghii, 27 m tall. The latter was found in Assam and Burma (Hooker, 1879), in Bangladesh (Brandis, 1906) and in India, Indonesia and Malaysia (Hooker, 1879; Ridley, 1922; Burkill, 1935; Corner, 1940 and Bor, 1953).

One species, Parkia timoriana Merr., has been reported by Bailey (1963) to have been imported by the United States Department of Agriculture (USDA) who had offered it for distribution in the United States. The plant was described as very large with pendulous pods, each of about 450 mm long.

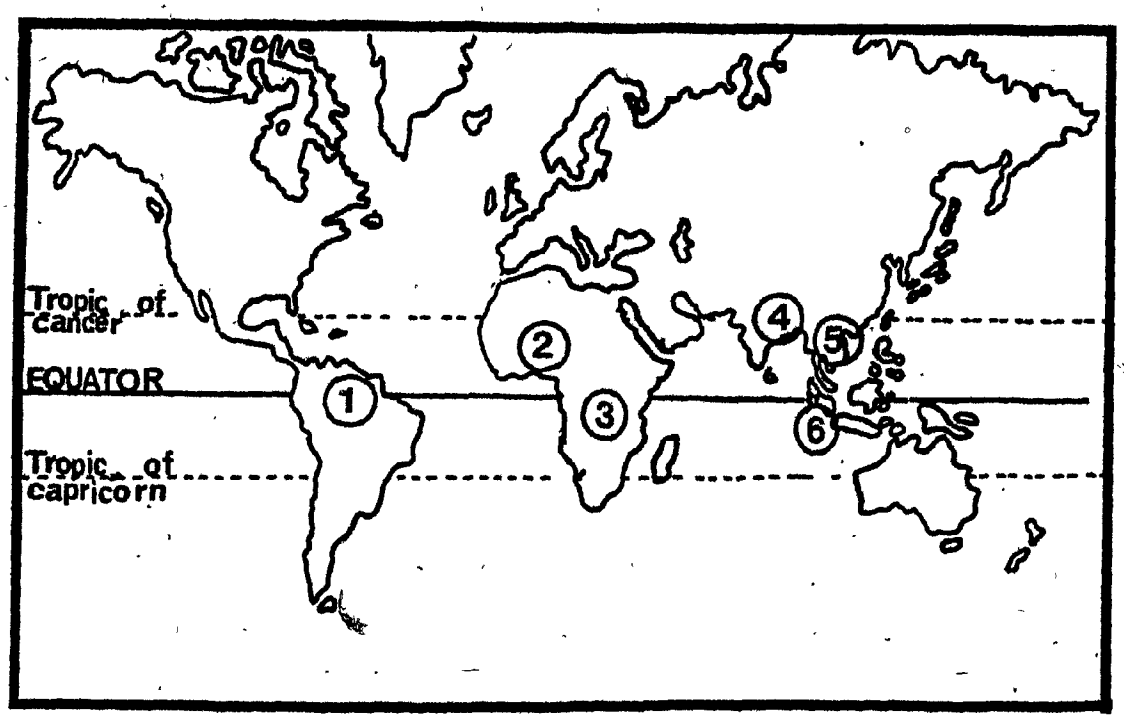
Felker (1981) has suggested that the capability of the legume trees to deal with the nitrogen deficiency of the desert might have led them to co-evolve complementary drought resistance mechanisms. This, he noted, could probably explain why leguminous trees should have evolved to dominate the arid and semi-arid ecosystems. Proof of the existence of the above suggested mechanism is yet to appear in the literature. It is known, however, that some Parkia species are found in large numbers in the Savannah regions of the world. In Fig. 1 is shown the global distribution of Parkia species. The species found in West Africa are reviewed next.

### 2.3.3. Species Found in West Africa

Only a few of the estimated sixty Parkia species have been reported to grow in Africa and fewer still in West Africa. The most detailed information about these was published by Dalziel (1937). He described three species from this area: Parkia bicolor A. Chev., Parkia biglobosa (Jacq.) Benth. and Parkia filicoidea Welw. Busson (1965), in addition to these three, has listed a fourth: Parkia clappertoniana Keay. This is, however, believed to be the same species as P. filicoidea. The three listed by Dalziel (1937) have been noted to have synonyms such as Parkia agboensis for P. bicolor (Busson, 1965), Parkia africana R. Br. for P. biglobosa (Uphof, 1959) and Parkia clappertoniana Keay for P. filicoidea (Keay et al., 1964).

Fig. 1: World distribution of *Parkia* species.

Figure 1




World distribution of Parkia species.

1. South America-Brazil
2. West Africa
3. Central and East Africa
4. India
5. Burma
6. Indonesia & Malaysia

P. bicolor, according to Dalziel (1937), has been found in French Guinea (Guinea), Sierra-Leone, Liberia, Ivory Coast, Gold Coast (Ghana) and Southern Nigeria. P. biglobosa has been found in Senegal, Guinea, Portuguese Guinea (Guinea Bissau), Gambia, Sierra-Leone, Ivory Coast and Ghana (Dalziel, 1937), but rarely in Nigeria. Dalziel (1937) observed that P. filicoidea Welw. is common in Northern Nigeria. The plant is believed to extend to about 14°N latitude, beyond the Northern borders of Nigeria. The species was claimed to be the most typical and, in some areas, the dominant tree of the Park Savannah. Areas in West Africa in which P. filicoidea has been found include Ivory Coast, Ghana, Togo and Northern and Southern Nigeria.

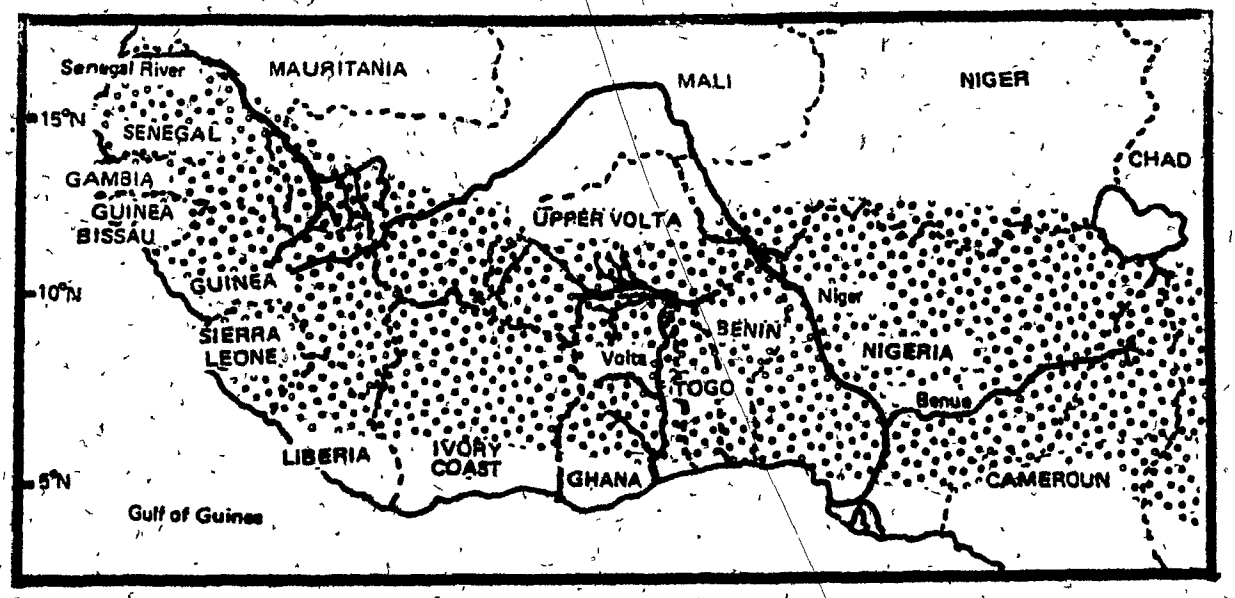
In Fig. 2a is shown the political map of West Africa and the distribution of Parkia species within this region. The existence of one, two or the three species in many, if not all, parts of West Africa is supported by a large number of native names given to these plants. Dalziel (1937) has listed and compiled an index of such names.

In Nigeria (Fig. 2b), Parkia species were reported to be found in the mixed Savannah areas of the country (Perkins and Stembridge, 1975). Of the three species reportedly found in West Africa, only two have been mentioned in Nigeria. According to Keay et al. (1964) and Purseglove (1968) these are Parkia filicoidea Welw. and Parkia bicolor. The former is said to be the more abundant species and is widely distributed throughout Nigeria with the larger concentration in the Northern part of the country (Purseglove, 1968).

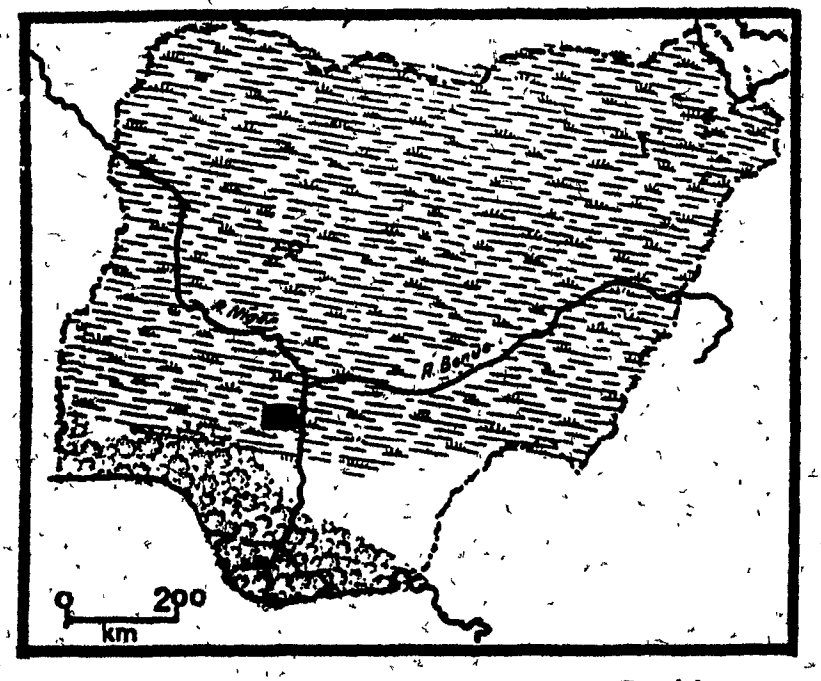
Fig. 2a: Political Map of West Africa and *Parkia* species Distribution.

Fig. 2b: Map of Nigeria and the two *Parkia* species found therein.

Figures 2a and 2b




Political Map of West Africa and Parkia species (⁘) Distribution.




Map of Nigeria and the two Parkia species found therein.

≡: P. filicoidea Welw. (P. clappertoniana)

⁘: P. bicolor

■: Fermentation substrate source (Uzairue)

The two types are differentiated by the size and arrangements of their leaflets. P. filicoidea leaflets usually alternate, measuring between 13.0 to 19.1 mm in length while those of P. bicolor are always opposite and shorter, measuring 6.4 to 12.7 mm in length. For this study seeds of P. filicoidea Welw. were obtained from Uzairue, Bendel State of Nigeria.

In Fig. 2b is shown the location of the substrate source. The chemical composition of the seed and dawadawa are reviewed next.

### 2.3.4. Chemical Composition of Some Parkia Species

The first report on this topic was probably that published in 1922 by the Imperial Institute in Britain (Anon, 1922). Subsequently, Platt (1962), Busson (1965), Oyenuga (1968) and FAO (1970) have investigated this subject. During the last decade additional studies have been carried out on Parkia species and reports have been published by: Girgis and Turner (1972), Fetuga et al. (1973a, b and 1974), Oke and Umoh (1978) and Eka (1980).

In most of these publications, the protein content of the seed of Parkia has been reported as being 30% or greater while a value of about 20% or greater has been claimed for the fat content. In interpreting the literature, a certain amount of caution must be used since not all authors have analyzed exactly the same material. For example, protein and fat contents for the whole beans, including the cotyledons and testa

are reported in some instances, whereas other authors have reported upon these properties of the cotyledons alone.

Fetuga et al. (1973a) reported on the composition of ground whole beans. This material was prepared by boiling the beans for 3 h, sun drying and grinding them (cotyledons and testa) so that the material passed through a 30 mesh sieve. Oke and Umoh (1978), however, analyzed beans whose testa had been removed. They prepared samples by boiling the beans for 5 h, drying them overnight at 90°C and grinding them.

Fetuga et al. (1974) carried out more detailed studies. They investigated the composition of the whole bean (cotyledons and testa), the raw beans (cotyledons with testa and adhering pulp), the cooked whole beans, the cooked whole beans with testa removed, the testa and dawadawa.

Eka (1980) employed a more complex method to obtain cooked cotyledons and dawadawa from the whole beans and analyzed both materials.

Duke (1981) has also reported on the composition of seeds of Parkia species. It is not, however, clear whether the seed referred to is the combination of the cotyledon and testa or the cotyledon alone. Although the materials analyzed by the various authors varied to a certain extent, they are presented together in tables 2a and 2b to allow a comparison to be made.

From the values reported by Platt (1962), Leung et al. (1968), Fetuga et al. (1974) and Eka (1980), shown in table 2b below, it is obvious that both removal of the testa and fermentation increase the crude

Table 2a. Proximate Composition of Some *Parkia* Species Seeds (g/100 g Dry Matter).

| Species | *P. filicoidea* Welw. | | | *Parkia* species |
|---|---|---|---|---|
| Reference | Fetuga *et al.* 1973a | Fetuga *et al.* 1974 | Eka 1980 | Duke 1981 |
| Crude protein (g) | 30.0 | 30.3 | 30.8 | 34.6 |
| Fat (Ether extract) (g) | 19.6 | 19.6 | 15.2 | 21.1 |
| Carbohydrate (g) | 26.0 | 31.2 | 49.1 | 40.1 |
| Ash (g) | 5.4 | 6.8 | 5.1 | 4.4 |
| Gross Energy (kJ/g)* | 20.1 | - | - | - |
| Others (g) | 19.0 | 12.1 | - | - |

* Units recalculated (Author).

Table 2b. Proximate Composition of *Parkia* Seed, Seed Components and Dawadawa (g/100 g sample).

| SAMPLE ANALYSED | Raw seed | Cooked seed with testa removed | Raw seed with testa + pulp | Cooked seed with testa | Cooked seed without testa | Seed testa | Dawadawa | | |
|---|---|---|---|---|---|---|---|---|---|
| REFERENCE | Fetuga *et al.* (1974) | Eka (1980) | | Fetuga *et al.* (1974) | | | Platt (1962) | Leung # *et al.* (1968) | Eka (1980) |
| Crude Protein (g) | 30.3 | 30.8 | 22.7 | 29.9 | 42.5 | 10.2 | 47.4 | 39.7 [10] # 30.7 - 49.2 | 38.5 |
| Fat (Ether Extract) (g) | 19.6 | 15.2 | 12.1 | 19.8 | 24.6 | 1.6 | 32.7 | 31.4 [9] 11.1 - 39.8 | 31.2 |
| Carbohydrate (g) | 31.2 | 49.1 | 48.6 | 32.1 | 22.1 | 54.2 | 15.4 | 16.0 | 23.6 |
| Ash (g) | 6.8 | 5.1 | 7.6 | 6.6 | 7.2 | 5.9 | - | 5.2 [9] 3.1 - 6.1 | 6.8 |
| Others (g) | 12.1 | - | 9.0 | 11.6 | 3.6 | 28.1 | - | 7.7 | - |
| Gross Energy (kJ/g)* | - | - | - | 20.3 | 23.6 | - | 4.5 | - | - |
| Moisture (g/100 g wet wt.)† | - | 12.7 | - | - | - | - | 22 ¶ 70 ¶ | 13.4 [10] 6.0 - 22.4 | - |

*Units recalculated (Author).

†For information only; determination on wet wt. basis.

¶Two values provided in the literature.

#Number of samples analysed in square brackets. Range of values below square brackets.

protein and fat contents of the product. The nutritional significance of processing and fermentation of the substrate are discussed next.

### 2.3.5. Nutritional Significance of the Substrate and Dawadawa

The nutritional importance of the West African Parkia species and their fermented product has been acknowledged (Aykroyd and Doughty, 1964; Busson, 1965 and Schery, 1972). In an earlier study Anon (1922) stated that neither the seeds nor the adhering pulp of the West African Parkia species, contain alkaloids or cyanogenetic glucosides. These are poisonous substances whose presence has been reported in the bitter species of cassava tubers. However, like other legumes, Parkia beans have been found to be deficient in sulfur containing amino acids such as cystine and methionine. They are also low in histidine but high in lysine (Oyenuga, 1968; Fetuga et al., 1973a and Campbell-Platt, 1980).

FAO (1970) reported that Parkia beans (preparation not specified) contain 6.5 g lysine/16 g Nitrogen. In a similar study, Fetuga et al. (1973a) have reported lysine contents of 6.7 g/16 g and 6.2 g/16 g N respectively for the whole beans of Parkia filicoidea Welw. and dawadawa. These values compare favorably with those of whole egg which has 7.0 g/16 g Nitrogen and those of soya bean meal which contains 6.10 g lysine/16 g Nitrogen (FAO, 1970 and Fetuga et al., 1973a).

Most cereals lack the amino acid lysine (Fetuga et al., 1973a). It is one of the limiting amino acids in the Savannah regions of West Africa

where dawadawa is eaten (Annegers, 1974). Dawadawa complements, therefore, the other foods which are eaten in conjunction with it (Campbell-Platt, 1980 and Anon, 1981).

In table 3 below are presented the amino acid contents of locust beans and dawadawa as determined by different authors. The reported values should be interpreted with caution as a result of the possible differences in the material analyzed.

Busson (1965) and Oyenuga (1968) have reported on the amino acids of the seed of P. filicoidea Welw.; however, they did not specify the type of treatment given to the seeds. In the analysis carried out by Fetuga et al. (1974) the material analyzed was interchangeably referred to as African locust bean seed and African locust bean meal. Oke and Umoh (1978) reported on samples whose testa had been removed, as did Eka (1980). The results of all the investigators are pooled in table 3 to allow for possible comparison between the seed, dawadawa and whole egg.

A study of the vitamin content of raw Parkia beans and dawadawa showed significant differences between the two samples (Campbell-Platt, 1980). Platt (1962, 1964) and Leung et al. (1968) reported that during fermentation the riboflavin content of dawadawa tripled while the thiamine and niacin contents, respectively, decreased to 17 and 66 percent of their original levels. According to Eka (1980) the vitamin $B_1$ and $B_2$ contents of dawadawa respectively were double and triple that of the amounts found in the cotyledons.

Table 3. Amino Acid Composition (mg/g protein) of the African Locust Bean Seed, Dawadawa and Whole Hen's Egg.

| MATERIAL ANALYSED | *P. filicoidea* Welw. seed | | | | Dawadawa | Whole Egg |
|---|---|---|---|---|---|---|
| REFERENCE | Busson (1965) Oyenuga (1968)* | Fetuga *et al.* (1974) | Oke and Umoh (1978)* | Eka (1980)* | Eka (1980)* | FAO (1970) |
| AMINO ACIDS | Amount (mg/g protein)† | | | | | |
| | | | | ¶ | ¶ | |
| Arginine | 45 | 67 | 73 | 46 | 37 | 61 |
| Histidine | 18 | 30 | 30 | 26 | 21 | 24 |
| Isoleucine | 31 | 36 | 48 | 14 | 5 | 63 |
| Leucine | 54 | 69 | 85 | 23 | 10 | 88 |
| Lysine | 52 | 67 | 73 | 68 | 83 | 70 |
| Methionine | 8 | 6 | 4 | 2 | 3 | 37 |
| Cystine | 18 | 19 | 7 | 19 | 26 | 24 |
| Phenylalanine | 35 | 44 | 58 | 5 | 2 | 56 |
| Tyrosine | 27 | 36 | 44 | 18 | 10 | 42 |
| Threonine | 27 | 33 | 38 | 61 | 55 | 51 |
| Tryptophan | - | 9 | - | 2 | 3 | 16 |
| Valine | 41 | 42 | 56 | - | - | 69 |
| Aspartic acid | 231 | 89 | 98 | 136 | 148 | 90 |
| Glutamic acid | 159 | 174 | 195 | 258 | 242 | 127 |
| Alanine | 49 | 45 | 54 | 90 | 97 | 59 |
| Glycine | 37 | 45 | 50 | 110 | 114 | 33 |
| Proline | 46 | 55 | 50 | 68 | 76 | 42 |
| Serine | 40 | 50 | 52 | 52 | 70 | 77 |
| Ammonia | - | - | - | 52 | 61 | - |

* Units recalculated (Author).

† Protein = N x 6.25

¶ Quantities were recalculated. See note below.

NOTE: The quantities in Eka's (1980) Table 3, 'Data on Amino Acids' were questionable since reported amino acid levels were unreasonably low. The units used by Eka were: mg amino acid/100 g sample. The amino acid values were recalculated, based on two assumptions:
(1) the quantities had really been determined as mg/g of sample; and
(2) the quantities reported had been divided by 6.25.

When these two assumptions were used the protein contents of the unfermented and the fermented beans were 39% and 51% respectively.

Fetuga et al. (1973b), in rat feeding experiments, have reported values of true digestibility of 86% for the fermented Parkia beans, 89% for cooked beans without testa, 82% for whole beans without testa and 76% when whole beans with testa were employed in the feed. A meal made from the whole beans and testa gave a negative protein efficiency ratio (PER) in the range -3.1 to -2.54, a low net protein utilization (NPU) with a range of 19.8 to 20.8 and a biological value (BV) of 22.3 to 23.7 (Fetuga et al., 1973a). In a similar study Umoh and Oke (1974) employed beans whose testa had been removed and then fermented for 3 days. When the product was fed to rats, higher values of PER, NPU and BV were obtained. A true digestibility of 93 to 99% was found and positive PER values, in the range 0.88 to 1.20, were obtained. NPU determinations showed values of 60 to 65 and an improved BV between 59 and 65 was reported. Oke and Umoh (1975), in the in vitro estimation of the true digestibility by the pepsin/trypsin and pepsin/pancreatic hydrolysis method found a range of 94 to 98% when fermented seeds of Parkia filicoidea were analyzed.

Oyenuga (1968), from the amino acid analysis profile, concluded that Parkia filicoidea Welw. seeds should form a useful source of protein concentrate for man and livestock, especially in Northern Nigeria where the seeds become available in fairly large quantities between March and September. Eka (1980) concluded from his studies that fermentation improved the nutritional quality of locust beans. It has been suggested (Anon, 1981) that after ground nuts, locust beans and dawadawa are probably the cheapest sources of protein in Nigeria. The economic roles of Parkia species are discussed next.

### 2.3.6. The Economic Significance of *Parkia* Species

Economically, the *Parkia* species are important in many of the areas of the world where they are found. Their usefulness, as judged from some of the available literature (Burkill, 1935; Dalziel, 1937; Aykroyd and Doughty, 1964; Hutchinson, 1964; Busson, 1965; Campbell-Platt, 1980; Anon, 1981 and Felker, 1981), can best be considered under three major headings of food, medicine and miscellaneous (Dalziel, 1937). These are discussed in turn, below.

2.3.6.1. *Food*: Fruits of *Parkiae* are usually large pods whose contents, pulp and seeds, have been exploited to varying degrees in different tropical locales of the world (Schery, 1972). In the African Sahel and associated Savannah, *Parkia* probably provides more food consumed directly by humans than any other tree legume genus (Felker, 1981). The dry, yellow, powdery pulp, which surrounds the seeds in the pods, when mixed with cereals, meat or soup, is a valuable food. Since the material is rich in sweet carbohydrates (Fetuga *et al.*, 1974), it may be made into candies or drinks or pressed into cakes for preservation until required (Dalziel, 1937 and Felker, 1981).

The seeds, employed in fermentation, are probably the most important portion of the pod. They can be processed and fermented (Dalziel, 1937; Platt, 1964; Busson, 1965 and Anon, 1981). The seeds, when roasted, produce what is known as 'Cafe du Soudan' (Dalziel, 1937; Hutchinson, 1964 and Schery, 1972).

P. speciosa and P. javanica reportedly found in South East Asia are similarly exploited for food. Their fruits are eaten raw, cooked as vegetable side dishes or used as a flavoring material (Uphof, 1959; Hutchinson, 1964 and Anon, 1981). The use of Parkia in local medicine is discussed next.

2.3.6.2. Medicine: Almost all the Parkia species have at one time or another been believed to provide relief from various ailments varying from stomach upset to leprosy (Pobeguin, 1912; Dalziel, 1937; Covi, 1971; Lewis and Elwin-Lewis, 1977; Campbell-Platt, 1980 and Anon, 1981). In Gambia, a lotion prepared from the bark and leaves of P. bicolor is reported to be effective in the cure of sore eyes. Similar cures have been associated with another lotion prepared from the roots and leaves of P. biglobosa (Dalziel, 1937 and Anon, 1981). The bark of P. bicolor, when dried and pulverized, is used as a dressing for wounds in Guinea (Dalziel, 1937).

A diuretic drink is prepared from the pulp of P. biglobosa. A decoction of macerated bark of this plant is used as a bath and, when hot, as a mouthwash to steam the mouth and throat and to relieve toothaches (Dalziel, 1937 and Uphof, 1959). The use of P. biglobosa Benth. in this manner is common in Gambia and Guinea.

An infusion obtained from the bark of P. filicoidea Welw. is regarded as a tonic while a decoction of the husks, usually taken cold, is believed to be an astringent for diarrhoea. Dalziel (1937) has noted that the young, unexpanded buds of P. filicoidea are used as preventive medicine

for leprosy. The seeds of this species are also noted as being capable of rendering foul water potable (Dalziel, 1937).

In Burma the gum from the bark of P. insignis is reported as being used against diarrhoea (Lewis and Elwin-Lewis, 1977). An infusion obtained from boiled and pounded seeds of P. speciosa is used to alleviate stomach pain in Singapore and Malaysia (Ridley, 1922; Burkill, 1935 and Campbell-Platt, 1980). The same infusion may be mixed with other spices and the mixture used as traditional medicine in Indonesia (Campbell-Platt, 1980). The Malays have been reported to treat hypertension with seeds of P. speciosa (Anon, 1981).

Other non-food, non-medicinal uses mentioned in the literature are discussed further.

2.3.6.3. Miscellaneous Uses of Parkia Species: Dalziel (1937) has noted that P. bicolor is used in Ivory Coast and Liberia as a source of timber and for canoe building. In Senegal the burnt husks of P. biglobosa Benth. are mixed with either tobacco to increase its pungency or with indigo to improve the lustre of dyed fabric (Dalziel, 1937). The bark of this tree is used for tanning or, when mixed with husks, may be used to incapacitate fish to facilitate their catching (Chipp, 1913; Thonner, 1915; Holland, 1922; Dalziel, 1937; Hutchinson, 1964 and Campbell-Platt, 1980). The branches of P. filicoidea Welw. are used as firewood and the resulting ash used in soap making. The bark of this plant may contain 12-14% tannin, a red coloring matter used for hides and skins (Dalziel, 1937).

Parkia trees play a vital role in nutrient recycling from the sub-soil to the surface through their roots and subsequent leaf fall. They hold the soil together and provide a canopy and shade (Stanton et al., 1966; Ashton, 1976 and Campbell-Platt, 1980). They have the ability to fix nitrogen and consequently increase the fertility of the soil (Felker, 1981). Their pods also provide incidental sustenance to animal life.

From the foregoing, the economic importance of Parkia species appears to be considerable and diverse. However, probably their most important use is in the production of dawadawa by the solid substrate fermentation of the beans. In the next section fermentation is defined and its probable future role discussed.

## 2.4. FERMENTATION AND FOOD FERMENTATION DEFINED AND CHARACTERIZED

'Fermentation' is believed to have probably been first used to signify a gently bubbling or boiling condition associated with the production of wine. For example, in an active ethyl alcohol fermentation, as in the production of wine or cider, carbon dioxide is liberated in the form of gas bubbles which, at the height of the reaction, may cause a marked agitation or movement of the liquid medium, especially in a large vat or tank, sufficient to give it the appearance of a boiling liquid (Prescott and Dunn, 1949 and 1959). The boiling definition sufficed until Gay-Lussac studied the process and modified the term to signify the breakdown of sugar into alcohol and carbon dioxide. As a result of Pasteur's researches into the cause of change in the nature of the material fermenting, the definition was readjusted again and fermentation became associated with microorganisms and later with enzymes, which are the biologically produced catalysts by which microorganisms act on the substrate (Prescott and Dunn, 1949 and 1959).

Although fermentation is frequently or even generally associated with the evolution of gas, caused by the action of living cells, neither gas evolution nor the visible presence of living cells is today regarded as an essential criterion of fermentation (Prescott and Dunn, 1959). It is known that in certain fermentations, for example in some of the lactic fermentations, no gas is liberated and also that the process may result, though infrequently, from the use of cell free extracts which may catalyze the essential reactions (Prescott and Dunn, 1959).

The modern definition of fermentation is based on a molecular concept of the metabolic process in which carbohydrates and related compounds are oxidized with the net release of energy in the form of ATP, in the absence of any external electron acceptors. In this form of metabolism the final electron acceptors are organic compounds produced directly from the partial breakdown of the carbohydrate (Jay, 1978). Only a small amount of energy is released since the Tricarboxylic acid (TCA) cycle or Krebs cycle is inoperative. According to Jay (1978), a good example of fermentation is found in the lactic acid bacteria which lack functional heme-linked electron transport systems or cytochromes and consequently can only obtain their energy through substrate level phosphorylation while oxidizing carbohydrates. In this molecular definition it is usual to associate the process of fermentation with anaerobic growth of microorganisms. This may be due to Pasteur's description of fermentation as "la vie sans air", although many aerobic industrial processes are also referred to as 'fermentations' (Sokatch, 1969). The pathway for the molecular utilization of glucose during fermentative metabolism is shown in Fig. 3.

Glucose is converted to ethanol and carbon dioxide by yeasts. Bacteria have been reported to differ from yeasts in their metabolism of glucose in that the majority of bacteria give rise to a diversity of products by the further metabolism of pyruvate (Mandelstam and McQuillen, 1973). Mandelstam and McQuillen (1973) have outlined some of the products resulting from the bacterial transformation of pyruvate and the various organisms involved in these processes. Products formed from pyruvate

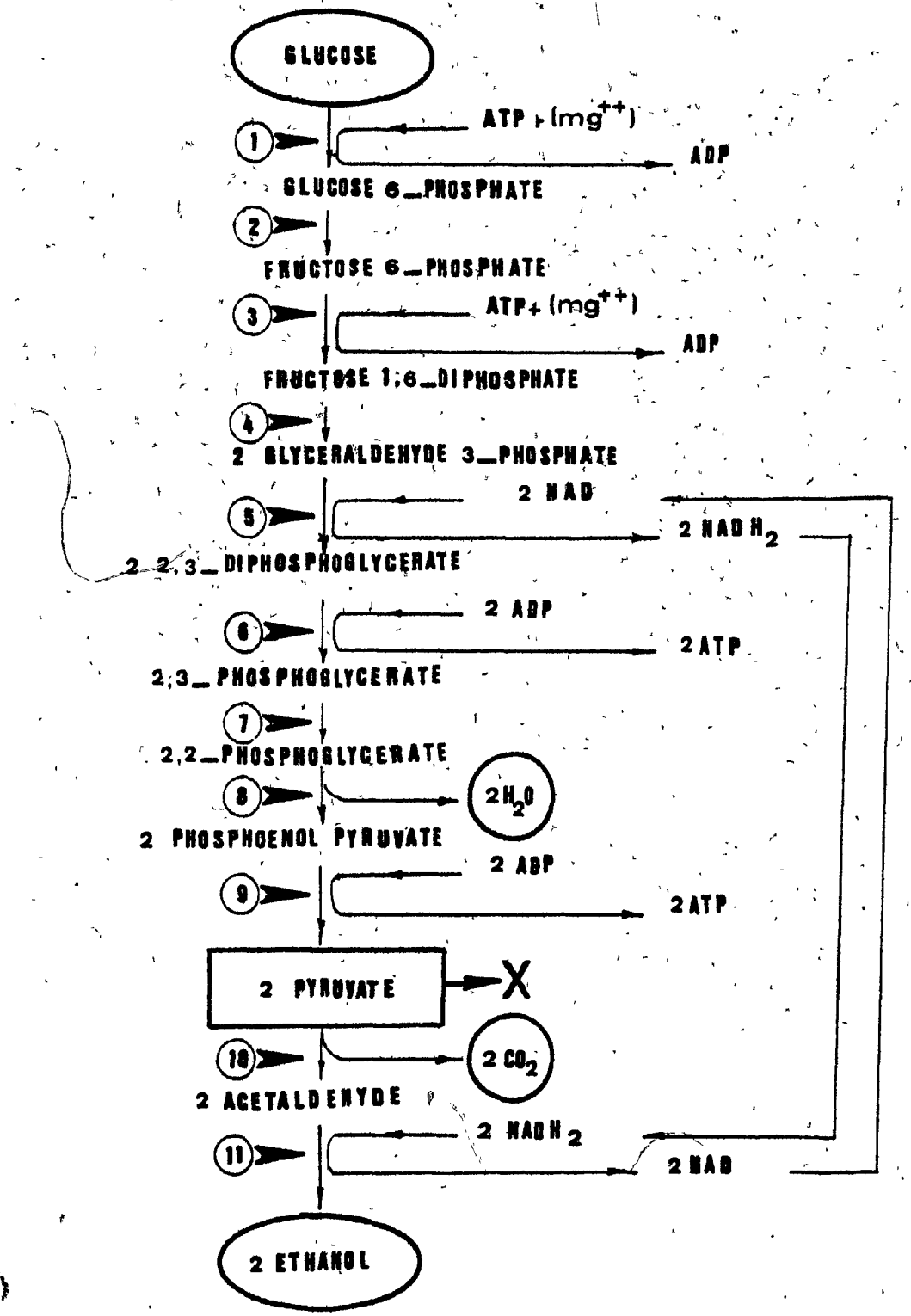
Fig. 3: Fermentation of Glucose to Ethanol and Carbon Dioxide by Yeasts.

Pyruvate can be metabolised to a wide range of products by different species of bacteria (Mandelstam and McQuillen, 1973 and Gottschalk, 1979).

ENZYME INVOLVED:

| | |
|---|---|
| 1 | Hexokinase |
| 2 | Phosphohexose isomerase |
| 3 | Phosphofructokinase |
| 4 | Aldolase |
| 5 | Glyceraldehyde 3-P dehydrogenase |
| 6 | 3 phosphoglycerate kinase |
| 7 | Phosphoglycerate mutase |
| 8 | Enolase |
| 9 | Pyruvate kinase |
| 10 | Pyruvate decarboxylase |
| 11 | Alcohol dehydrogenase |
| X | Bacterial metabolism products |

Figure 3


GLUCOSE
ATP + (mg++)
1
ADP
GLUCOSE 6_PHOSPHATE
2
FRUCTOSE 6_PHOSPHATE
3
ATP+ (mg++)
ADP
FRUCTOSE 1;6_DIPHOSPHATE
4
2 GLYCERALDEHYDE 3_PHOSPHATE
2 NAD
5
2 NADH2
2 2,3_DIPHOSPHOGLYCERATE
2 ADP
6
2ATP
2;3_PHOSPHOGLYCERATE
7
2,2_PHOSPHOGLYCERATE
8
2H2O
2 PHOSPHOENOL PYRUVATE
2 ADP
9
2ATP
2 PYRUVATE
X
10
2 CO2
2 ACETALDEHYDE
2 NADH2
11
2 NAD
2 ETHANOL

include acetate, lactate, succinate, propionate, acetone, iso-propanol, butanol, butyrate, ethanol, 2,3-butanediol, acetoin, formate, hydrogen and carbon dioxide.

Although the molecular definition of fermentation truly represents the biochemical changes taking place during the process, the definition of the term has been broadened, especially with respect to food and industrial microbiological processes carried out under aerobic conditions (Sokatch, 1969 and Jay, 1978). Prescott and Dunn (1959) have stated that fermentations are so varied in character that any broad definition which covers the whole class of biochemical processes must be couched in very general terms. Fermentation, therefore, in the broad sense in which the term is now used may be defined as a process in which chemical changes are brought about in a substrate through the action of enzymes elaborated by microorganisms (Prescott and Dunn, 1959; Doelle, 1975 and Jay, 1978).

Carbohydrates, according to Prescott and Dunn (1959), have been often regarded as being essential materials for fermentation. However, other materials such as organic acids, including amino acids and their salts, proteins and their derivatives, fats, sterols, alcohols, esters, and other organic compounds are fermentable under certain conditions by selected microorganisms.

The above broad definition of 'fermentation' has been found acceptable to industrial microbiologists, mycologists and technologists working with microorganisms. By these various groups, 'fermentation' has also often been taken to mean 'a process for upgrading a cheap carbon source

to produce a high valued product through the agency of microorganisms and/or their enzymes' (Demain, 1981 - Personal communication). It is in the context of this broadened industrial microbiological definition that the word is employed in this study.

Pederson (1979) has defined food fermentations as 'complex chemical transformations of organic substances brought about by the catalytic action of enzymes either native to the substance or elaborated by some of the many species of microorganisms present in and/or on the food'. Fermented foods, according to Pederson (1979), are frequently prepared by means of processes that were developed in the home centuries ago. He has noted that relatively few individuals know why foods ferment, how they acquire their qualities and how they remain safe for consumption, even though fermentation is one of the oldest methods of food preservation known to mankind and antedates recorded history.

Hesseltine (1981) has predicted that foods prepared by fermentation will probably increase in production and consumption and that their increased use will spread to all parts of the world, including Western developed countries. Conditions which will promote future acceptance of these foods world-wide have been discussed by Hesseltine (1981). His projection was influenced by what he termed 'several positive factors' which suggest a greater role for fermented foods. The two main fermentation systems are discussed next.

## 2.5. TYPES OF FERMENTATION SYSTEMS

Hesseltine (1977a) and Cannel and Moo-Young (1980a) have stated that, in general, two types of fermentation systems can be considered. The two types are: liquid or semi-liquid and solid or semi-solid substrate.

### 2.5.1. Liquid or Semi-Liquid Type System

This type characterizes many bacterial fermentations in which liquid media or semi-liquid substrates such as milk are used (Hesseltine, 1977a). This system is often used in the Western world. It is employed in the conventional stirred tank submerged liquid or dilute slurry fermentations of beer, various alcoholic drinks, milk and pharmaceutical products. The liquid substrate provides the bacteria or yeast with sufficient nutrient and free water which the organisms must have for their biological activities (Hesseltine, 1977a). An important advantage of the system is the oxygen transfer rate which can be varied by controlling the agitator speed (Blanch and Bhavaraju, 1976; Bailey and Ollis, 1977 and Cannel and Moo-Young, 1980a). Other advantages include ease of measurement of parameters such as pH, oxygen and carbon dioxide. Some of its reported disadvantages include high energy requirements and waste disposal problems (Cannel and Moo-Young, 1980a).

### 2.5.2. Solid State (Substrate) Fermentation Systems (SSF)

This type of fermentation is usually employed in Asia and Africa to produce such foods as Ang-Kak, Dawadawa, Garri, Idli, Miso, Natto and Tempeh. Hesseltine (1977a) and Cannel and Moo-Young (1980a) have defined solid state fermentation (SSF) as a term which refers to the growth of microorganisms on solid materials in the absence of free liquid. However, since microorganisms require moisture for their growth, bio-conversion and metabolic activities, the solid substrate, therefore must provide the organisms with enough biologically available water (Hesseltine, 1977a and Cannel and Moo-Young, 1980a). Thus, the required moisture must be present within the solid matrix. Cannel and Moo-Young (1980a) have provided further clarification of the term SSF by stating that SSF differs significantly from the fermentations of solids in liquid media and does not refer to either the fermentation of slurries or of liquids with high levels of insoluble solids. The upper moisture limit for a particular substrate employed in SSF has been stated as being due to the absorbancy of that particular substrate. For most solid substrates it has been defined as a maximum of 80% moisture content (g $H_2O$/g solid). After this upper limit, free moisture from the solid material becomes apparent in most solid substrates (Cannel and Moo-Young, 1980a).

Golueke (1977) and Cannel and Moo-Young (1980a) have specified the minimum level of moisture for the operation of the system to be 12%. Most biological activities cease below this moisture level.

Some of the advantages of the SSF system which have been reported include: low space requirement for both the process and storage of the product, less liquid waste for disposal, elimination of filtration, lower solvent requirement for extraction e.g. in the extraction of the enzymes from the solid substrate, reduction of contamination since most SSF operations are carried out at moisture levels at which bacterial proliferation is discouraged. In addition, continuous aeration is not required since this is achieved in most cases by diffusion through the interparticulate air spaces. This, in conjunction with occasional stirring, is usually adequate. SSF is also easily adaptable to either the continuous or batch process with no great complexity in the equipment being required (Hesseltine, 1977b and Cannel and Moo-Young, 1980a). In contrast to the advantages, the difficulty in the measurement of the fermentation parameters such as moisture, pH, oxygen and carbon dioxide levels in the system has been noted as a major disadvantage (Cannel and Moo-Young, 1980a).

Hesseltine (1977a, b) has stated that solid state fermentations are frequently used in the production of mold enzymes. The method is particularly used in Japan to produce both the amylolytic and proteolytic enzymes used in shoyu and sake fermentations. The system is also of interest in the production of secondary metabolites such as aflatoxin (Hesseltine, 1977a, b). According to Cannel and Moo-Young (1980a) three traditionally developed SSF systems are presently in use. These are: Oriental food fermentations, mold ripened cheese and composting.

In carrying out the bioconversion of the solid substrate three different methods are available (Hesseltine, 1977a). These are:

(i) stationary fermentation in trays, petri-dishes or flasks;

(ii) periodic agitation involving occasional turning over of the substrate, and

(iii) continuous agitation in which kernels or grains may be the substrate.

The agitation in (iii) above frequently prevents unwanted sporulation of the organism on the substrate. The process may be carried out in a drum or cement mixer type of fermenter. Anderson et al. (1980 - Personal communication) employed the third method in their fermentation studies on straw.

Solid state fermentation, as discussed by Hesseltine (1977a) and Cannel and Moo-Young (1980a), offers the greatest possibilities when fungi are used. These organisms, in nature, have the ability to grow on diverse solid substrates such as wood, seeds, roots, leaves, and drier parts of animals such as skin, bones and faecal material that is low in moisture. A fungus, Eurotium halophilicum, has been reported to grow and reproduce on wheat which has a moisture content of only 13 to 14% (Christensen et al., 1959 and Hesseltine, 1977a). Microorganisms usually employed in fermentation are discussed next.

## 2.6. MICROORGANISMS EMPLOYED IN FERMENTATION

Microorganisms used in fermentation include bacteria, molds and yeasts. They have been reported to differ widely in morphology, size, reaction to free oxygen, manner of reproduction, growth requirements, ability to assimilate or ferment organic substrates and in other ways. They are, however, similar in that during fermentation they all produce enzymes by which they catalyze the processes ascribed to them (Prescott and Dunn, 1949 and 1959 and Pederson, 1979).

Pederson (1979) has stated that these microorganisms are very numerous and 'most valuable to mankind'. The ability of man to control their activities has given him 'unlimited food and drink'. Some of these microorganisms are very specific in function while others are broader in their adaptation to the environment. Specificity is related to the type of microorganisms, the substrate it acts upon and the enzyme(s) present.

The importance of microorganisms in fermentation has been noted by Hesseltine and Haynes (1973), who have stated that microorganisms are the key to the success or failure of any fermentation whether complex or simple. The microorganisms provide the catalysts that make the fermentations work. The attributes of these organisms are discussed next.

### 2.6.1. Attributes Required of Fermentation Microorganisms

Ideally, microorganisms employed in food, industrial, pharmaceutical and related fermentation processes should possess several attributes in order to be useful in the processes which they are known to affect (Prescott and Dunn, 1949 and 1959 and Hesseltine and Haynes, 1973). These attributes have been listed to include the following:

(1) Rapid growth in or on suitable substrate.

(2) Easily cultivated in large quantity.

(3) Maintain physiological constancy under the conditions of growth and readily produce in large amounts the necessary enzymes that are required for the desired biochemical changes in the substrate.

(4) It should be able to carry out its activities notwithstanding moderate modifications of environmental conditions e.g. slight changes in pH of the medium or in oxygen levels. Since the reaction is usually exothermic (metabolic activities) it should be able to operate with little input of external energy for heating.

(5) The organism must be genetically stable to ensure continuous uniformity of the product it generates.

(6) It should readily produce reproductive units such as vegetative cells, mycelia or spores for future propagation.

(7) It should grow vigorously in seed tanks employed in the scale up of inoculum prior to fermentation.

(8) It must be pure, free of micro and macro contaminants and also be free of phages.

(9) It should produce the required product within a short period of time, and according to Hesseltine and Haynes (1973), in three days or less.

(IO) The desired product should be produced, to the exclusion of all toxic substances.

(11) The organism should be easy to maintain for long periods.

(12) It should be responsive to mutagenic treatment in order to promote higher yields or better product.

(13) The organism must give a predictable amount of product in a given fermentation time.

Microorganisms that meet the above requirements can be isolated from nature or obtained from culture collections. The ultimate sources of cultures for fermentations are: soil, water, living plants and animals, sewage, fresh and spoiled food, insect droppings, air and other microbial habitats (Hesseltine and Haynes, 1973). With the probable exception of single cell protein such as food yeast, microorganisms are mostly important to fermentative processes because of their enzymes. In the next section some important microorganisms of fermentation are reviewed.

### 2.6.2. Some Important Microorganisms Involved in Fermentation

Microorganisms have been linked directly or indirectly to the desirable organoleptic properties of the product such as texture, color, taste and flavor (Beuchat, 1978b; Jay, 1978; Whitaker, 1978 and Margalith,

1981). They may be added to the substrate as pure or mixed cultures or in some instances the natural microflora may be adequate, if they are of the right type and present in sufficient numbers in or on the substrate (Smith and Palumbo, 1981).

Haas (1976) has stated that "even though the types of foods obtained by fermentation are many, only a few genera of microorganisms are involved in many of these foods. These are: Saccharomyces, Aspergillus and various lactic organisms". Prescott and Dunn (1949, 1959), Peppler (1967), Sellars (1967), Hesseltine and Haynes (1973), Haas (1976), Frazier and Westhoff (1978), Jay (1978) and Rose (1979) have discussed some important microorganisms involved in the production of various fermented products. Alphabetical listings of some of these organisms and the products they affect have been compiled by most of the above authors. In table 4 below are shown some products together with the type and genus of microorganisms involved. Important microorganisms of fermentation are usually preserved. Methods for their preservation and areas of the world from which they may be obtained have been published (Wickerham and Flickinger, 1946; Haynes et al., 1955; Carmicheal, 1956; Hwang, 1960 and 1966 and Hesseltine and Haynes, 1973).

In the next section the biochemical changes which occur during fermentation of some products are reviewed.

Table 4. Some Fermentation Products and the Genus and the Type of Microorganisms Associated with Their Production*.

| Product | Genus | Type of Microorgansim |
|---|---|---|
| AMINO ACIDS | | |
| Aspartic acid | Pseudomonas | Bacteria |
| Glutamic acid | Bacillus | Bacteria |
| (monosodium glutamate) | Brevibacterium | Bacteria |
| | Micrococcus | Bacteria |
| Isoleucine | Pseudomonas | Bacteria |
| Lysine | Micrococcus | Bacteria |
| Phenylalanine | Micrococcus | Bacteria |
| Threonine | Bacillus | Bacteria |
| Valine | Micrococcus | Bacteria |
| ANTIBIOTICS | | |
| | Aspergillus | Mold |
| | Bacillus | Bacteria |
| | Cephalosporium | Mold |
| | Fusidium | Mold |
| | Micromonospora | Actinomycetes |
| | Nocardia | Actinomycetes |
| | Penicillium | Mold |
| | Streptomyces | Actinomycetes |
| BEVERAGES | | |
| Beer | Saccharomyces | Yeast |
| Distilled Spirits | Saccharomyces | Yeast |
| Sake | Aspergillus | Mold |
| | Saccharomyces | Yeast |
| Wine | Saccharomyces | Yeast |
| ENZYMES | | |
| -Amylase | Aspergillus | Mold |
| | Bacillus | Bacteria |
| | Endomycopsis | Yeast |
| | Rhizopus | Mold |

Table 4 continued.

| Product | Genus | Type of Microorganism |
|---|---|---|
| (Amyloglucosidase or Glucoamylase) | | |
| Asparaginase | Erwinia | Bacteria |
| | Escherichia | Bacteria |
| Catalase | Aspergillus | Mold |
| | Penicillium | Mold |
| Cellulase | Aspergillus | Mold |
| | Myrothecium | Mold |
| | Trichoderma | Mold |
| Dextranase | Penicillium | Mold |
| -Fructofuranosidase | Saccharomyces | Yeast |
| -Galactosidase | Saccharomyces | Yeast |
| Glucoamylase | Aspergillus | Mold |
| | Endomycopsis | Yeast |
| | Rhizopus | Mold |
| (Glucose isomerase or Xylose isomerase) | | |
| Glucose oxidase | Aspergillus | Mold |
| | Penicillium | Mold |
| -Glucosidase | Aspergillus | Mold |
| -Glucosidase | Aspergillus | Mold |
| (Hemicellulase also Xylanase) | | |
| (Invertase also -Fructofuranosidase) | | |
| (Lactase also -Galactosidase) | | |
| (Laundry enzymes also proteases, alkaline | | |
| Lipase | Aspergillus | Mold |
| | Candida | Yeast |
| | Mucor | Mold |
| (Milk-clotting enzymes also Rennin) | | |
| (Pectinase also Polygalacturonase) | | |
| Penicillinase | Bacillus | Bacteria |
| Penicillin amidase | Many | |
| (Pentosanase also Xylanase) | | |
| Plasmin | Streptococcus | Bacteria |
| Polygalacturonase | Aspergillus | Mold |

Table 4 continued.

| Product | Genus | Type of Microorganism |
|---|---|---|
| Proteases | Aspergillus | Mold |
| | Conidiobolus | Mold |
| | Mucor | Mold |
| | Streptomyces | Actinomycetes |
| Proteases, alkaline | Bacillus | Bacteria |
| Rennin | Endothia | Mold |
| | Mucor | Mold |
| (Streptokinase also Plasmin) | | |
| Xylanase | Many | |
| Xylos isomerase | Streptomyces | Actinomycetes |
| FOODS | | |
| Ang-kak | Monascus | Mold |
| Bacterial starters | Lactobacillus | Bacteria |
| (Fermented dairy products | Leuconostoc | Bacteria |
| and sausage) | Pediococcus | Bacteria |
| | Propionibacterium | Bacteria |
| | Streptococcus | Bacteria |
| Bantu beer | Lactobacillus | Bacteria |
| | Saccharomyces | Yeast |
| Blue cheese flavor | Penicillium | Mold |
| Bread and Bakers' yeast | Saccharomyces | Yeast |
| Cheese and fermented | Lactobacillus | Bacteria |
| dairy products | Penicillium | Mold |
| | Propionibacterium | Bacteria |
| | Streptococcus | Bacteria |
| Chinese cheese | Chlamydomucor | Mold |
| | Hansenula | Yeast |
| | Rhizopus | Mold |
| | Saccharomyces | Yeast |
| Fermented Fish | Aspergillus | Mold |
| | Bacillus | Bacteria |
| | Lactobacillus | Bacteria |
| Hamanatto | Aspergillus | Mold |
| Koji | Aspergillus | Mold |
| | Rhizopus | Mold |
| Magou | Lactobacillus | Bacteria |
| Miso | Aspergillus | Mold |
| | Saccharomyces | Yeast |
| Nata | Acetobacter | Bacteria |
| Natto | Bacillus | Bacteria |

Table 4 continued.

| Product | Genus | Type of Microorganism |
|---|---|---|
| Ontjom | Neurospora | Mold |
| Pickles and Sauerkraut | Lactobacillus | Bacteria |
| | Streptococcus | Bacteria |
| Shoyu | Aspergillus | Mold |
| | Saccharomyces | Yeast |
| | Torulopsis | Yeast |
| Sufu | Actinomucor | Mold |
| | Mucor | Mold |
| Tempeh | Rhizopus | Mold |
| Yeast | Candida | Yeast |
| | Saccharomyces | Yeast |
| **INDUSTRIAL SOLVENTS** | | |
| Acetone | Clostridium | Bacteria |
| Butanol | Clostridium | Bacteria |
| 2,3- Butanediol | Aerobacter | Bacteria |
| | Bacillus | Bacteria |
| Dihydroxy acetone | Acetobacter | Bacteria |
| Ethanol | Clostridium | Bacteria |
| | Saccharomyces | Yeast |
| Glycerol | Saccharomyces | Yeast |
| | Torulopsis | Yeast |
| **MISCELLANEOUS** | | |
| Alkaloids | Claviceps | Mold |
| Bioinsecticides | Bacillus | Bacteria |
| Dextran | Leuconostoc | Bacteria |
| Gibberellin | Giberella (Fusarium) | Mold |
| Inosinic acid | Bacillus | Bacteria |
| | (Many) | Yeast |
| Nucleotides | Brevibacterium | Bacteria |
| | Candida | Yeast |
| Rhizobium culture | Rhizobium | Bacteria |
| Silage | Lactobacillus | Bacteria |
| Sorbose | Acetobacter | Bacteria |
| Steroid transformations | Aspergillus | Mold |
| | Corynebacterium | Bacteria |
| | Curvularia | Mold |
| | Rhizopus | Mold |
| | Streptomyces | Actinomycetes |

Table 4 continued.

| Product | Genus | Type of Microorganism |
|---|---|---|
| ORGANIC ACIDS | | |
| Acetic | Acetobacter | Bacteria |
| Citric | Aspergillus | Mold |
| Erythorbic | Penicillium | Mold |
| Fumaric | Rhizopus | Mold |
| Gluconic | Aspergillus | Mold |
| Itaconic | Aspergillus | Mold |
| Itatartaric | Aspergillus | Mold |
| 2-Ketogluconic | Pseudomonas | Bacteria |
| 5-Ketogluconic | Acetobacter | Bacteria |
| -Ketoglutaric | Pseudomonas | Bacteria |
| Kojic | Aspergillus | Mold |
| Lactic | Lactobacillus | Bacteria |
| PROTEIN (SCP) | | |
| | Chlorella | Algae |
| | Saccharomyces | Yeast |
| | Torula | Yeast |
| VITAMINS | | |
| Ascorbic acid (Vitamin C in part) | Acetobacter | Bacteria |
| ($B_{12}$) or Cyanocobalamin | Bacillus | Bacteria |
| | Propionibacterium | Bacteria |
| | Streptomyces | Actinomycetes |
| -Carotene | Blakeslea | Mold |
| Riboflavin | Ashbya | Yeast |
| | Eremothecium | Yeast |

* Compiled in part from Prescott and Dunn (1949, 1959), Peppler (1967), Hesseltine and Haynes (1973)#, Miller and Litsky (1976) and Jay (1978).

# Major source.

## 2.7. BIOCHEMICAL CHANGES DURING FERMENTATION

Products of fermentation are numerous and only a few of these can be covered in this review. Yong and Wood (1974) and Whitaker (1978) have discussed the biochemical changes occurring during the fermentation of some foods. They pointed out the lack of data on many of these. It is known, however, that fermented products differ from the raw materials from which they are obtained. These differences are due to the biochemical changes brought about by the microorganisms. Some of these changes have been reported upon (Patton, 1950; Girolami and Knight, 1955; Saisithi et al., 1966 and Whitaker, 1978) and attempts to explain the nature of these have been made by Yokotsuka (1960), Wagenknecht et al. (1961), Walker (1961), Schormuller (1968), Sekine et al. (1970) and Yong and Wood (1974). More recently, Margalith (1981) has discussed the role of these changes in the enhancement of flavor in some fermentation products.

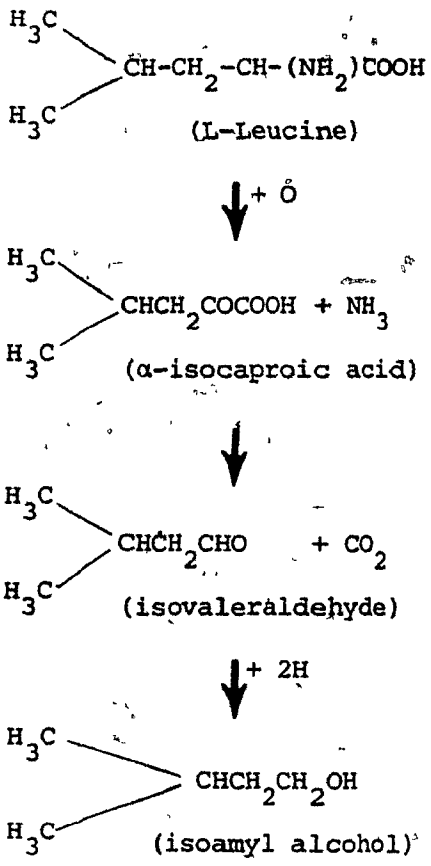
One of the first fermentation processes known to man, most probably, was brewing. The process utilizes cereals, grains and fruits as raw substrate. Haas (1976) has given an overall formula for the conversion of these substrates to alcohol in the form:

$$C_{12}H_{22}O_{11}\ H_2O \longrightarrow 4\ C_2H_6O + 4\ CO_2$$

Other minor compounds found in beer during fermentation, aside from ethanol, include: alcohols, organic acids, sulfur compounds, esters,

carbonyl compounds, hydrocarbons, amino acids and sugars. Wilkinson and Rose (1963) reported that during alcoholic fermentation, by strains of Saccharomyces cerevisiae, small amounts of higher alcohols, known as fusel oils may be formed. The most abundant of these oils is iso-amyl alcohol (3-methyl-1-butanol). Their formation is due to the exogenous nitrogenous substances added to the brew. However, Ingraham and Guymon (1960), Guymon et al. (1961) and Ingraham et al. (1961) have shown that higher alcohols can be produced, by yeasts, from the intermediates in the synthesis of leucine, isoleucine and valine and, therefore, independently of exogenous amino acids.

These higher molecular weight aliphatic alcohols, fusel oils, play an important role in the organoleptic qualities of some alcoholic beverages. For example, during the ageing of whiskey they become esterified by fatty acids. The esterified products are significant factors in whiskey flavor developments (Wilkinson and Rose, 1963). The amyl alcohols are formed by the deamination and decarboxylation of amino acids. Intermediates such as oxo-acids are also formed during their synthesis (Wilkinson and Rose, 1963). A scheme proposed by Neubauer and Fromherz in 1910, for the conversion of L-leucine to iso-amyl alcohol, is shown below (Wilkinson and Rose, 1963):




In the scheme shown above, the amino acid L-leucine is oxidatively deaminated to form an oxo-acid which is then decarboxylated and reduced to yield a higher alcohol (Isoamyl alcohol).

Vinegar is produced by acetic acid bacteria (Acetobacter). The major reactions involved according to Haas (1976) are as follows:

$$2\ CH_3CH_2OH + O_2 \rightarrow 2\ CH_3CHO + 2\ H_2O$$

$$2\ CH_3CHO + O_2 \rightarrow 2\ CH_3COOH$$

Specific organisms responsible for the reaction are: Acetobacter xylinoides, A. orleanse and A. acetigenum. An initial alcohol level of 10-12% is frequently used at a temperature of 26-31°C. The organisms employed are usually alcohol tolerant.

Numerous studies have been carried out on dairy products, probably because of the nutritional importance of milk and the products obtained from it. Milk, as a raw material, has a relatively short shelf life. Extension of this is achieved by fermentation to products such as cheese (Jay, 1978).

Studies on hard Roquefort and Cheddar cheeses have shown that several biochemical changes take place during the bioconversion of milk to these products. In the preparation of hard cheese, proteolytic enzymes derived from rennin or from Mucor pusillus or M. miehi are added to sweet milk together with lactic acid bacteria (Whitaker, 1978). The proteolytic enzymes, according to Ernstrom and Wong (1972), split the peptide bonds between phenylalanyl and methionyl residues in K-casein. This splitting destabilizes the casein micelle and causes it to aggregate and precipitate as curd. Secondary proteolysis may follow which may affect the texture and taste (including bitterness) of the final product. The precipitated curd is inoculated with appropriate microorganisms which, during ripening, also secrete enzymes to further modify the product.

Roquefort cheese is made by inoculating the casein curd with Penicillium roqueforti (Scott, 1968 and Gray, 1970). Two major enzyme groups, proteases and lipases, are involved in the ripening of Roquefort cheese.

The addition of these enzymes in powder form to the curd shortens the ripening period by several months (Scott, 1968 and Gray, 1970). The lipases of P. roqueforti hydrolyze the lipids present in the curd and produce compounds such as: acetic, butyric, capric, caprylic and caproic acids (Currie, 1914). Some of these acids, capric and caproic, are believed to be responsible for the burning, peppery taste of the Roquefort cheese.

The unique flavor of Roquefort cheese is due to methyl ketones produced by the β-oxidation of the fatty acids (Whitaker, 1978). Patton (1950) and Girolami and Knight (1955) have isolated acetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone and 2-nonanone from this Roquefort cheese. The 2-heptanone is believed to play a major role in the flavor developed (Whitaker, 1978).

Cheddar cheese, ripened by bacteria, is very popular in North America (Whitaker, 1978). To prepare Cheddar cheese, rennet, a source of protease enzyme, and lactic acid bacteria (Streptococcus lactis and S. cremoris or S. durans - a more heat stable species) are added to whole milk to coagulate the casein. The added bacteria are primarily responsible for the ripening, which occurs uniformly throughout the curd, and the flavor development (Whitaker, 1978). During ageing, which may last for a year, the pH initially drops to between 4.95 to 5.3 and later rises to between 5.3 to 5.5. The carbohydrates, proteins and lipids present in the curd are all modified by the carbohydrases, proteases and lipases derived from the starter cultures and rennin.

The proteolysis of the milk proteins, the extent of which is a function of the microorganisms employed and the duration of ageing, results in free amino acids and peptides of various sizes in the product (Schormuller, 1968 and Whitaker, 1978). Both transamination and decarboxylation also occur during ripening of Cheddar cheese. A considerable amount of tyramine is produced, in addition to smaller quantities of agmatine, cadaverine, histamine, putrescine, tryptamine and α-amino butyric acid, during Cheddar cheese fermentation (Dacre, 1955; Schormuller, 1968 and Whitaker, 1978).

The lipases, contributed by the microorganisms, rennet and the milk, hydrolyze the lipids to fatty acids. Forss and Patton (1966) have stated that fatty acids from butyric to linoleic are important in cheese flavor. The β-oxidation of these fatty acids results in the production of neutral carbonyl compounds. Those whose production has been observed in Cheddar cheese include: formaldehyde, acetaldehyde, acetone, 2-butanone, 2-pentanone, 3-methyl butanol, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, 2-pentadecanone, diacetyl, n-decanol, n-dodecanol and acetoin (Wong, 1972).

There is no agreement on what compounds actually constitute the major Cheddar cheese flavor (Whitaker, 1978). Wong (1972) has stated that dimethyl sulfide, diacetyl and 3-hydroxy butanone may be the most significant flavor compounds. Walker (1961) believed that the Cheddar cheese flavor is due to a blend of methyl ketones, fatty acids and hydrogen sulfide. Kristoffersen and Gould (1960) have suggested that ammonia, free amino acids, free fatty acids and hydrogen sulfide may

be the important components. Whitaker (1978) has noted that it is likely that the unique flavors of cheeses are the result of the relative amounts and kinds of many different compounds. McGugan and Howsam (1973) have reported the presence of phenol and p-cresol in Cheddar cheese. Anisole, 4-methyl anisole and 2,4-dimethyl anisole have also been found in Camembert cheeses (Margalith, 1981). All these compounds contribute to individual product flavor profiles.

Ang-kak is a fermented product obtained by inoculating rice with the mold Monascus purpureus. One of the major changes observed in ang-kak is the color development.

The color, according to Nishikawa (1932) is due to two pigments. These are:

(i) Monascorubrin (Red)

$C_{22}H_{24}O_5$

(ii) Monascoflavin (Yellow)

$C_{17}H_{22}O_4$

Due to their poor solubility the two pigments accumulate in the microorganisms.

Soy sauce is made from soybeans, wheat and salt with a mixture of molds, yeasts and bacteria. During soy sauce production, proteins, carbohydrates and other constituents of soybeans and wheat are hydrolyzed to peptides, amino acids, sugars, alcohols, organic acids and other low molecular weight compounds by the enzymes of the microorganisms employed (Wang and Hesseltine, 1979).

The steps involved in the fermentation and the possible biochemical events occurring in the substrate during the process have been reviewed by Yokotsuka (1960) and Yong and Wood (1974). Yokotsuka (1960) has reported that of the total nitrogen present in soy sauce, 40-50% is amino acid nitrogen, 40-50% is peptide and peptone nitrogen, 10-15% is ammonia nitrogen and less than 1% is protein. Seventeen common amino acids are said to be present with glutamic acid and its salts constituting the principal soy sauce flavor. Organic bases such as adenine, hypoxanthine, xanthine, guanine, cytosine and uracil, believed to be hydrolyzed products of nucleic acids, have also been reported. Sugars which have been found in soy sauce include glucose, arabinose, xylose, maltose, galactose and two sugar alcohols of glycerol and mannitol (Yokotsuka, 1960). The presence of organic acids such as lactic, succinic, acetic and pyroglutamic has been reported in soy sauce; the product's color is believed to be due to non-enzymatic browning (Wang and Hesseltine, 1979).

The compounds that decisively characterize soy sauce are many. More than a hundred have already been reported as being responsible for the flavor. It has, however, been proposed that the guaiacyl compounds seem to have an overall important effect on the flavor of Japanese soy sauce (Wang and Hesseltine, 1979).

Both proteases and amylases are produced by the mold Aspergillus oryzae and the organism secretes two types of amylases. The first, α-amylase or Taka-amylase A, is a starch liquefying enzyme (Akabori et al., 1954

and Sawasaki, 1960a). The second is a β-amylase or Taka-amylase B which is a saccharogenic enzyme (Okazaki, 1955a, b and Sawasaki, 1960b). The endosplitting α-amylase converts the starch to smaller fragments i.e. dextrins. The exo-splitting β-amylase acts on the dextrin to produce maltose and a few larger fragments containing 1,6-glucosidic linkages.

Whitaker (1978) has noted that A. oryzae proteases are complex and that at least three types have been found during soy sauce fermentation. Hayashi et al. (1967) have reported on one of these: alkaline protease. The other two, neutral and acid proteases, were said to have been reported upon by Tsujita in 1967 (Whitaker, 1978). The sequence of action by these proteases is yet to be elucidated (Whitaker, 1978).

Soybean proteins are hydrolyzed during tempeh fermentation. Consequently the amount of soluble nitrogen compounds increases from an initial value of 0.5 to 2.0%. A similar increase, of up to 85 times, in the free amino acid content of the product has also been reported (van Veen and Schaefer, 1950; Steinkraus et al., 1960, 1965; Stillings and Hackler, 1965; Murata et al., 1967 and van Buren et al., 1972).

The soybean lipids are also hydrolyzed. The composition of ether extractable material changed during fermentation (van Veen and Schaefer, 1950; Wagenknecht et al., 1961; Murata et al., 1967 and van Buren et al., 1972). Van Buren et al. (1972) have, for example, reported that fatty acids contributed 2.2% of the total fat (ethanol-chloroform extractable material) before the fermentation, they formed 29.4% of this material after 72 h of fermentation.

Both reducing compounds and hemicellulose have been reported to decrease in tempeh (van Veen and Schaefer, 1950 and Steinkraus et al., 1960) as a result of their utilization by the mold. The fibre content increases from 3.7 to 5.8%, possibly because of the mold mycelium formation in the product (van Veen and Schaefer, 1950; Steinkraus et al., 1960; Murata et al., 1967 and van Buren et al., 1972).

The organism responsible for the above biochemical changes in tempeh is Rhizopus oligosporus. During fermentation, the beany flavor and the flatulence and antinutritive constituents of soybeans are removed. Wagenknecht et al. (1961) have reported that R. oligosporus produces large amounts of lipases and proteases. However, very little amylase and pectinase are produced (Hesseltine, 1965). Whitaker (1978) has stated that some of the results of the tempeh fermentation are probably due to the proteolysis of the soybean proteins. For example, the compounds responsible for the beany flavor are bound to the proteins. The hydrolysis of the proteins results in their release.

A scheme for the biochemical changes that may occur during the fermentation of high protein foods has been proposed. In Fig. 4 below are shown the possible or probable pathways of such a scheme (Whitaker, 1978). Bioconversion is made possible by the bio-catalysts. In table 5 below are shown some enzymes which have been reported to be produced in large amounts and the microorganisms responsible for their secretion. In the next section some physical changes occurring during fermentations are discussed.

Fig. 4: Hydrolysis of Proteins and Decomposition of Amino Acids (Whitaker, 1978).

ENZYMES INVOLVED:

| | |
|---|---|
| 1 | Primarily endoproteases |
| 2 | Primary exoproteases (carboxypeptidases, aminopeptidases, di- and tri-peptidases) |
| 3 | Decarboxylases |
| 4 | Deaminases |
| 5 | Transaminases |
| 6 | Cysteinase (substrate is cysteine) |
| *7 | Deamination enzyme |
| 8 | Probably through β-elimination of methionine by enzyme similar to cysteinase |

* Author.

Figure 4

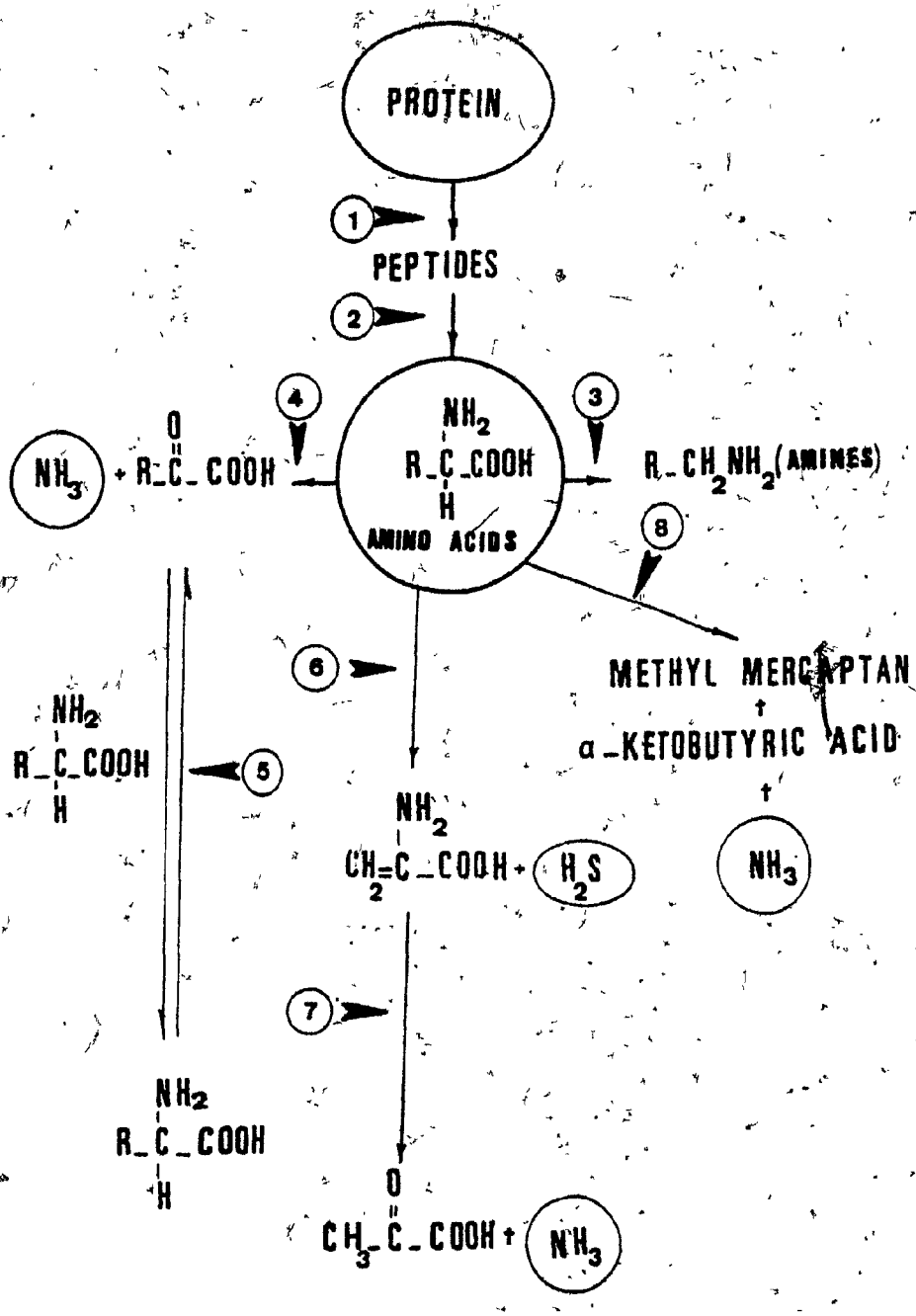

PROTEIN
1
PEPTIDES
2
NH2
R-C-COOH
H
AMINO ACIDS
3
R-CH2NH2 (AMINES)
4
NH3 + R-C-COOH
O
8
METHYL MERCAPTAN
α-KETOBUTYRIC ACID
NH3
6
NH2
CH2=C-COOH + H2S
5
NH2
R-C-COOH
H
7
NH2
R-C-COOH
H
O
CH3-C-COOH + NH3

Table 5. Some Microorganisms and Enzymes Produced in Large Amounts by Them*.

| Source | Enzymes | Specific Microorganisms |
|---|---|---|
| Bacteria | Amylases and Proteases | Bacillus subtilis |
| | Streptokinase | Streptococcus hemolyticus |
| Fungi | Amylases and Amyloglucosidase | Aspergillus oryzae |
| | | Aspergillus niger |
| | Proteases | Aspergillus oryzae |
| | Pectinases | Aspergillus niger |
| | Glucose oxidase and Catalase | Aspergillus niger |
| | Lipase | Aspergillus niger |
| Yeast | Invertase | Saccharomyces cerevisiae |

* Reference Sources: Beckhorn (1967), Fogarty et al. (1974a, b) and Kelly and Fogarty (1976).

## 2.8. PHYSICO-CHEMICAL CHANGES

Preservation, texture, color, flavor, aroma, solubilization, digestibility, nutritional improvement, less cooking and removal of toxic substances are some of the reasons which have been given for fermenting foods. Some antinutritional substances which have been reported to be found in soybean include: protease inhibitors, phytohaemaglutinins, goitrogens, phytic acid, amylase inhibitors, estrogens, flatulents, allergic substances, phenolic compounds, sterols and saponins (Strong, 1973; Rackis, 1974 and Whitaker, 1978).

Processing and fermentation eliminate some of these from the soybean. In the tempeh fermentation, the organoleptic quality of soybeans has also been found to be improved (Wagenknecht et al., 1961; Steinkraus et al., 1960, 1965; Murata et al., 1967 and Robinson and Kao, 1977). The process is also accompanied by weight losses (Smith et al., 1964 and Whitaker, 1978) and textural changes (Steinkraus et al., 1960).

Jurus and Sundberg (1976) have indicated that soybean cotyledons are penetrated by mold hyphae during tempeh production. They speculated that mycelial infiltration might partially explain the rapid physical and chemical changes which occur during the process. Wang and Hesseltine (1979) have noted that the hyphae may mechanically push the bean cells apart prior to, or in conjunction with enzymatic digestion. The beans would become soft in this manner. Likewise, the penetration of enzymatic activity could also be enhanced, since the distance over which the diffusion of the enzymes must occur is greatly reduced. Some microbiological growth parameters are discussed next.

## 2.9. MICROBIOLOGICAL GROWTH PARAMETERS

Microorganisms play a central role in bioconversion (Hesseltine and Haynes, 1973) and therefore, their growth is important to the process. This may occur in sequence so that various species of microorganisms succeed one another on the substrate. According to Pederson (1979) there is usually an initiating species which provides a suitable environment for the succeeding ones.

In order to have growth, conditions must be suitable for metabolic activities. Jay (1978) has discussed the intrinsic and extrinsic parameters which affect the growth of these organisms. Moisture, water activity, acidity, pH, salt, oxygen, temperature, nutrient availability, antimicrobial constituents and biological structure of the substrate employed have all been reported to be important parameters in the growth of microorganisms (Frazier and Westhoff, 1978; Jay, 1978 and Pederson, 1979). Consequently they strongly affect the fermentation process. The extent to which each parameter affects growth depends on the substrate under consideration and the type of microorganism that will bring about its bioconversion.

# CHAPTER 3

# PHYSICO CHEMICAL ASPECTS

# OF THE FERMENTATION

# 3. THE PHYSICO CHEMICAL ASPECTS OF THE FERMENTATION

In this chapter are presented the results of the experimental investigation on the physico chemical aspects of the raw substrate, the processed substrate and the fermented product. Time sequenced analyses were carried out on the fermenting substrate and the results obtained during these are also reported.

The parameters studied include: moisture, fat, protein, amino acids, total carbohydrate, available carbohydrate, ash, gross energy, texture, density and specific gravity. The process mass balance is also presented.

Each investigated parameter is reported as a complete module of the methods employed, observations and discussion.

## 3.1. SOURCE OF SUBSTRATE

The substrate employed throughout this study, Parkia filicoidea Welw., was obtained from Uzairue, Bendel State of Nigeria. Upon receipt, the beans appeared to be sun dried. They were covered with a thin layer of a pulpy plant material.

## 3.2. SUBSTRATE PREPARATION AND FERMENTATION

The substrate was cleaned of gross dirt and pulpy material by soaking 1 kg of the sun dried beans in 4 l of tap water overnight (12 h) to soften the pulp. The excess water was poured off and the pulp was removed by rubbing the beans between the palms and by washing with water. The cleaned, depulped beans were drained of excess water and then of the adhering water by spreading them out on a laboratory bench at room temperature (about 20°C) for 48 h. The cleaned, dark brown or black beans were then stored in 100 g lots in polyethylene bags at room temperature (about 20°C) and employed as required. For identification purposes the cleaned beans described above are referred to as 'whole beans' while the beans with the adhering pulpy material are referred to as 'raw beans'.

The whole beans were processed to obtain the substrate for the fermentation in the manner described below. Tap water was added to whole beans in a flask to give a bean:water ratio of 1:5 (W/W). The flask, with its contents and a cover, was placed on a hot plate operated at its 'Hi' temperature dial setting (Fisher Thermix, Fisher Scientific Co.). The heating was carried out at atmospheric pressure for 8 h. Water was added every 2 h to replace that lost during boiling so as to keep the whole beans covered. During the heating period, the hard, leathery, black testa softened and parts of them could be observed to be broken off and carried up in the foam produced at this stage.

After the 8 h heating period the flask and contents were allowed to cool at room temperature for about 2 h. The excess water was drained off and the beans' softened testa were removed by rubbing the beans between the palms and washing with water. The beans, free of testa, are referred to as 'dehulled beans'. These are usually light yellow in color. The dehulled beans were returned to the flask for a second boiling. Tap water was added in the ratio 1:5, dehulled beans to water (W/W). The flask was again covered, placed on the hot plate and brought to a boil. Boiling was continued for 30 min. The flask was then removed, and allowed to cool for 1 h at room temperature (about 20°C). The water and beans were separated by straining. The beans having been soaked, cleaned, heated, dehulled, boiled and drained of excess water as described, formed the solid substrate employed for fermentation. This material is referred to throughout this thesis as 'processed substrate'.

It is here noted that this 'processed substrate' is different from 'mechanically dehulled beans'. To produce this latter material, the hard, leathery testa were removed from the whole beans with a pair of pliers. Mechanically dehulled beans and mechanically removed testa were required for some of the moisture content studies.

To obtain the fermented product, the processed substrate was dispensed into a sterile petri plate (90 mm diameter) containing sterile 90 mm filter paper. The beans were covered with another sterile filter paper and the lid put on. The plate, the lid, the two filter papers and the processed substrate formed the fermentation unit. The unit was placed in an incubator at 37°C for 72 h. After this period the processed

substrate had invariably undergone spontaneous fermentation. The light yellowish-gray product is referred to as 'fermented beans'.

Control samples were obtained by irradiating processed substrate with gamma rays (1.1 Mrad = 11 kGy; cobalt 60 in a unit supplied by Atomic Energy of Canada Limited). These beans are referred to as 'Radappertized beans' (RADB).

For the analysis of moisture the samples employed were: raw beans, whole beans, mechanically dehulled beans, mechanically removed testa, processed substrate, fermented beans and radappertized beans. Each sample was ground with a mortar and pestle. The ground tissue was stored in hermetically sealed, labelled, glass containers (Mason Jar with standard Mason Snap lid - Mason Jar Canada Co.). The samples were then placed inside polyethylene bags and kept in the deep freezer at -20°C. Samples were retrieved and used when needed.

For the other analyses, except where stated otherwise, the samples employed were treated in the following manner: they were first frozen at -20°C in a freezer. After freezing, they were quickly transferred into a freeze dryer (Labconco, Freeze Dry 5). The samples were then freeze dried under a vacuum of 10 um for 48 h without input of heat. A condenser temperature of -50°C was maintained during all sample preparation involving freeze drying. Each frozen and lyophilized sample was ground in a power driven mill (Arthur H. Thomas Co., Phila., PA, U.S.A.) to pass a USA standard sieve No. 60 (nominal wire diameter: 0.180 mm). The samples were then stored in labelled, hermetically sealed, glass containers. The labelled containers holding the samples were put in polyethylene bags and stored at -20°C until they were needed.

## 3.3. MOISTURE

Water is the principal constituent of many raw and processed foods. Therefore, moisture determination is an important aspect of any scientific investigation dealing with food (Karmas, 1980).

Karel (1973, 1975) and Karmas (1980) have stated that water in food exists in two forms: free and bound. The free water is not strongly attached to food components and it is sometimes called 'unbound' or 'freezable' water. The bound water is strongly complexed to individual sites in the food and it is sometimes called 'unfreezable' water. Therefore, in any food material, the total water present can be represented as:

Total water = (Bound + Free) water

Karmas (1980) has listed nineteen different methods for the quantitative determination of moisture in foods. All of these fall within two broad classes of either direct or indirect methods. The method employed in this study is the direct vacuum oven method. The objectives behind this experiment are set out below.

### 3.3.1. Objectives - Moisture

The objectives of this study were:

(1) To determine the minimum time required to obtain moisture contents

of the various sample materials using the vacuum oven method (VOM).

(2) To determine the moisture contents of the raw beans, whole beans, processed substrate, fermented beans, mechanically dehulled beans and mechanically removed testa.

(3) To determine the changes in moisture content of processed substrate during fermentation and to compare these changes with those of the radappertized beans.

### 3.3.2. Principles - Moisture (VOM)

Some food products may decompose and some of their constituents lost if a temperature greater than 100°C is used. These types of foods can be dried at lower temperatures under reduced pressure (i.e. 13 kPa). The effectiveness of the method depends on the maintenance of a low pressure by means of the quick removal of the water vapor liberated.

### 3.3.3. Materials and Methods - Moisture (VOM)

Cleaned, dry, flat base aluminium dishes, each of 50 mm internal diameter and 25 mm high, with close fitting lids were employed. The dishes and their lids were dried for 6 h in a vacuum oven (National Appliance Co., Portland, Oregon, U.S.A.) at 95°C, at a pressure of 13 kPa (AOAC, 1975). The dishes and their lids were cooled in a desiccator, containing freshly dehydrated $CaCl_2$, for 15 min and then weighed, to the nearest mg, with

a chemical balance (Fisher Scientific Co.). Each sample investigated was mixed thoroughly before adding to the marked, weighed aluminium dish. The weight of the dish containing the added sample was determined to the nearest mg and the weight recorded. The dishes and the samples were transferred into the vacuum oven with the lids partially open. The samples were dried under vacuum for the required length of time. After drying, the vacuum was broken and the dishes covered and transferred to the dessicator. After cooling, the aluminium dishes and their contents were each weighed again to the nearest milligram.

3.3.3.1. Methods: Minimum VOM Time: This experiment was carried out in order to determine the standard time required for the drying process using the VOM. The samples employed were: whole beans, mechanically dehulled beans and mechanically removed testa. Each sample was dried for periods of 6, 12, 24, 36, 48, 60 and 72 h.

3.3.3.2. Methods: Moisture Contents: In this experiment, the minimum time required for drying (VOM) was used to determine the moisture contents of the raw beans, whole beans, processed substrate, fermented beans, mechanically dehulled beans and mechanically removed testa.

3.3.3.3. Methods: Time Sequenced Moisture Contents: The changes in the moisture contents, of the processed substrate, during fermentation, were investigated. These changes were compared with those of the radappertized beans, also maintained at 37°C. Samples for analyses were taken at 0, 12, 24, 36, 48, 60 and 72 h.

### 3.3.4. Observations - Moisture

The observations are given below in subsequent sub, subsections corresponding to the sub, sub sections of 'materials and methods'. This approach is followed throughout in this thesis.

3.3.4.1. Observations: Minimum VOM Time: In Fig. 5 are shown the weight lost by the whole beans, the mechanically dehulled beans and the mechanically removed testa at different drying times. Detailed data are given in appendix A, Table A1.

3.3.4.2. Observations: Moisture Contents: The moisture contents of the raw beans, whole beans, processed substrate, fermented beans, mechanically dehulled beans and mechanically removed testa are shown as histograms in Fig. 6. Detailed results for these are given in appendix A, Table A2.

3.3.4.3. Observations: Time Sequenced Moisture Contents: In Fig. 7 are shown the curves of time sequenced moisture determinations for the processed substrate undergoing spontaneous fermentation and the radappertized beans. Detailed numerical data for these are given in appendix A, Tables A3a and A3b.

### 3.3.5. Discussion - Moisture

In Fig. 5 were shown curves which stabilized at 24 h. This is an indication that constant weight has been achieved i.e. that probably all the moisture had

Fig. 5: Percentage Weight Lost at Varying Drying Times of Whole Beans (WB), Mechanically Dehulled Beans (MDB), and Mechanically Removed Testa (MRT).

Figure 5

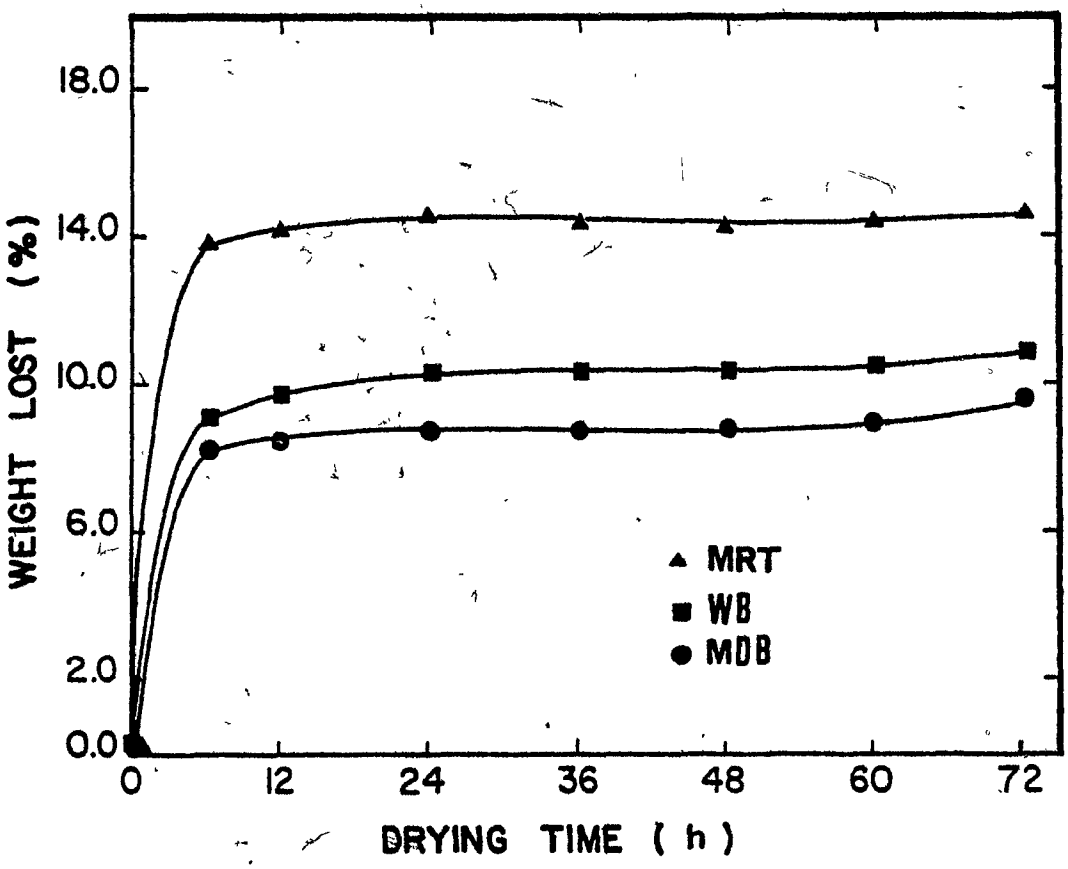

18.0
14.0
10.0
6.0
2.0
0.0
WEIGHT LOST (%)
0
12
24
36
48
60
72
DRYING TIME ( h )
MRT
WB
MDB

Fig. 6: Percentage Moisture Content ($g\ H_2O/100\ g$ Sample).

(1) Raw Beans

(2) Whole Beans

(3) Processed Substrate

(4) Fermented Beans

(5) Mechanically Dehulled Beans

(6) Mechanically Removed Testa

Figure 6

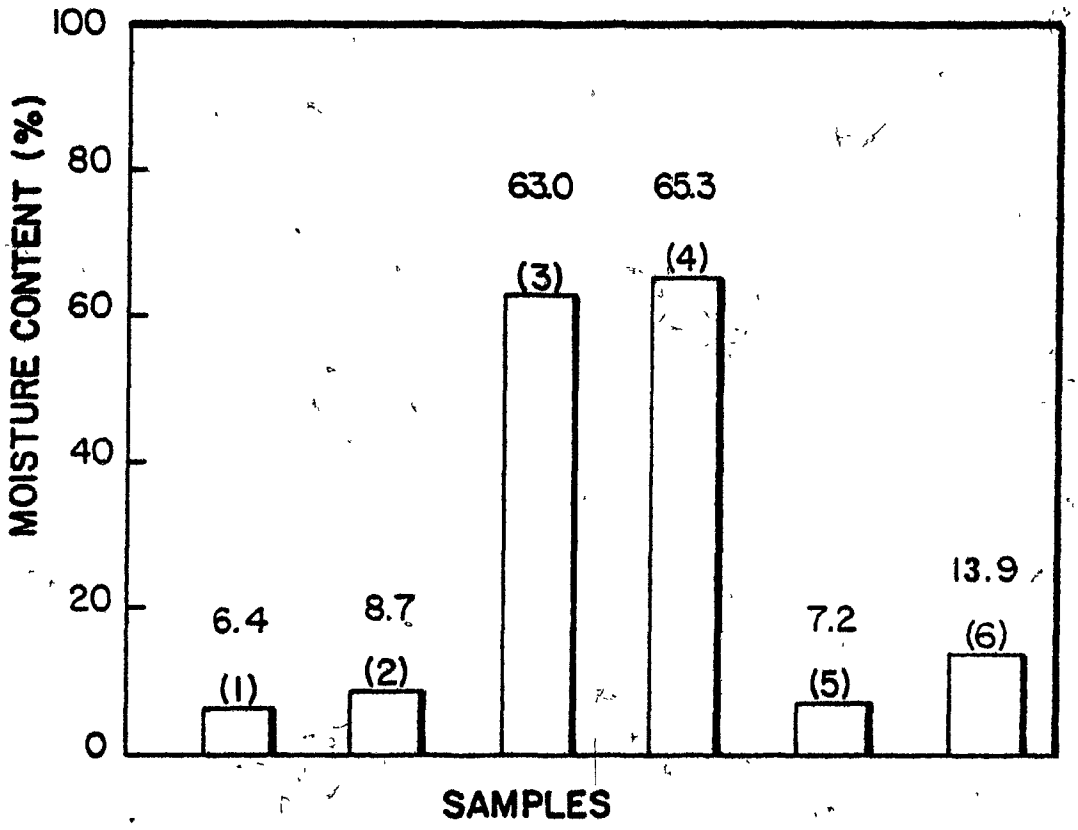
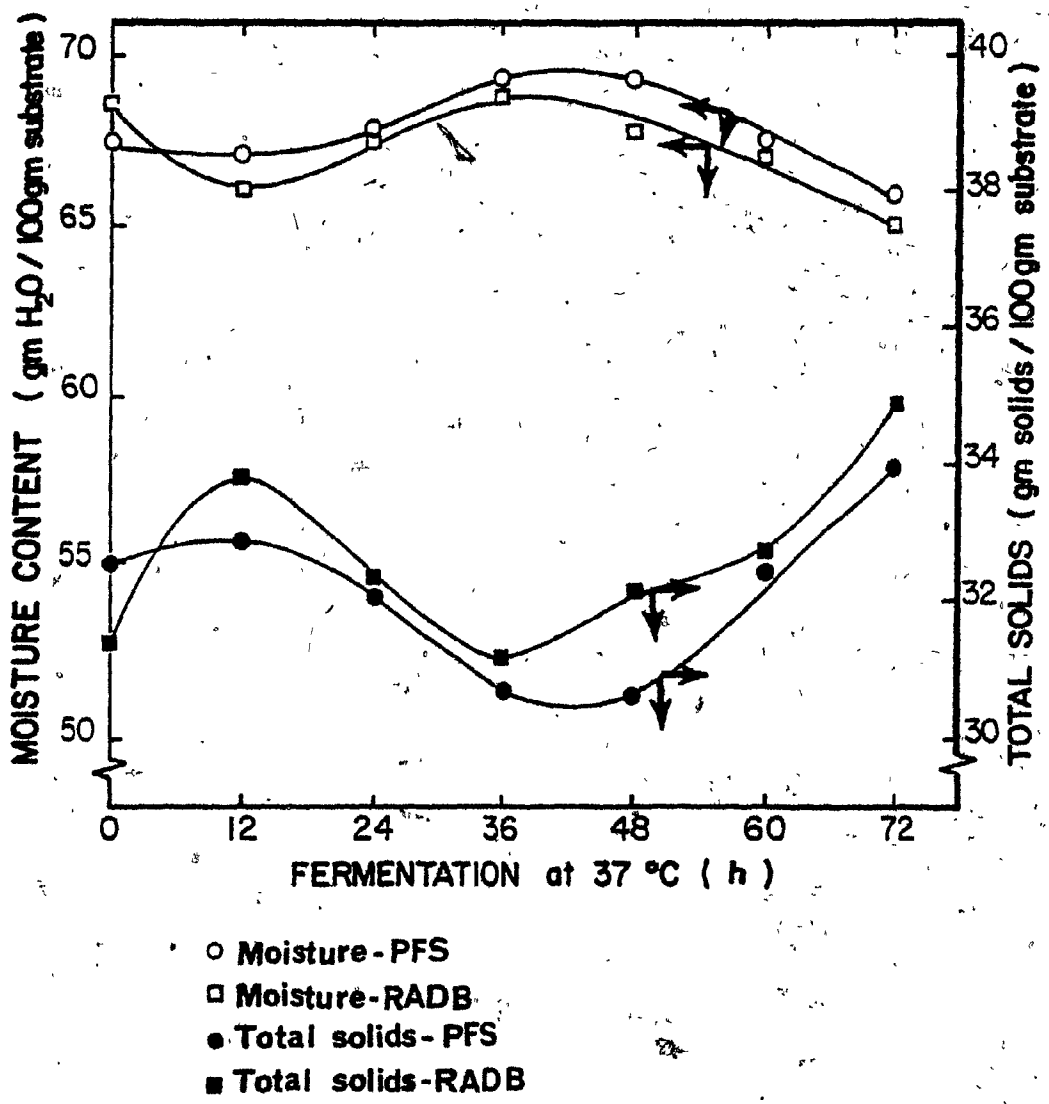

100
80
60
40
20
0
MOISTURE CONTENT (%)
6.4
(1)
8.7
(2)
63.0
(3)
65.3
(4)
7.2
(5)
13.9
(6)
SAMPLES

Fig. 7: Changes in Moisture Content and Total Solids with Fermentation Time of Processed Fermenting Substrate (PFS) and Radappertized Beans (RADB).

Figure 7


MOISTURE CONTENT ( gm $H_2O$ / 100gm substrate )
TOTAL SOLIDS ( gm solids / 100gm substrate )
70
65
60
55
50
40
38
36
34
32
30
0
12
24
36
48
60
72
FERMENTATION at 37 °C ( h )
○ Moisture - PFS
□ Moisture - RADB
● Total solids - PFS
■ Total solids - RADB

been removed. A 24 h period was therefore used for all other VOM moisture experiments.

By comparing the moisture contents of the raw beans and the whole beans (Fig. 6), it is evident that moisture migrated into the beans during the removal of the adhering pulp. Presumably, most of this moisture was absorbed by the testa since mechanically removed testa had a considerably higher moisture content than did either the raw beans or the mechanically dehulled beans. Due to this, the whole bean may be more susceptible to microbial attack, especially by fungi, than the raw beans. During processing, the beans gained moisture, the whole beans having a moisture content of 8.7%, the processed substrate having a moisture content of 63%. Such a high moisture content provides water required for microbial activity. During the 72 h fermentation a slight moisture content increase occurred. This may have been due to a loss of solids or be a result of microbial metabolism or moisture exchange with the atmosphere.

The raw data obtained for the moisture contents and total solids contents of the different samples used are presented in appendix A, Table A2. Analysis of variance was performed on the raw data using the statistical computer programme GLM (General Linear Model) from the statistical library of McGill University. The programme used is presented in appendix B, Fig. B1. This programme was used for all the statistical analysis. The resulting outputs are shown in tables 6a, 6b, 6c and 6d. Both the moisture contents and total solids contents were significantly affected by the treatment at 0.0001 level. The coefficient of correlation was 0.9999 and coefficient of variation (CV) was less than 1%. The means

Table 6a. Analysis of Variance (ANOVA) for the Moisture Contents using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 53 | 36708.73 | | | |
| T | 5 | 36706.30 | 7341.26 | 146825.2 | 0.0001 |
| R | 8 | 0.24 | 0.03 | 0.6 | 0.8161 |
| ERROR | 40 | 2.19 | 0.05 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9999

CV = 0.8547%

Table 6b. Analysis of Variance (ANOVA) for the Total Solids Contents using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 53 | 36708.73 | | | |
| T | 5 | 36706.30 | 7341.26 | 146825.2 | 0.0001 |
| R | 8 | 0.24 | 0.03 | 0.6 | 0.8161 |
| ERROR | 40 | 2.19 | 0.05 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9999

CV = 0.3225%

Table 6c. Moisture Contents as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 9 | 6.40 | F |
| 2 | 9 | 8.70 | D |
| 3 | 9 | 63.00 | B |
| 4 | 9 | 65.30 | A |
| 5 | 9 | 7.20 | E |
| 6 | 9 | 13.90 | C |

*Means with the same letter (Grouping) are not significantly different at alpha level 0.05.

Table 6d. Total Solid Contents as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 9 | 93.60 | A |
| 2 | 9 | 91.30 | C |
| 3 | 9 | 37.00 | E |
| 4 | 9 | 34.70 | F |
| 5 | 9 | 92.80 | B |
| 6 | 9 | 86.10 | D |

* Means with the same letter (Grouping) are not significantly different at alpha level 0.05.

were significantly different at alpha level 0.05 as indicated by the grouping (Tables 6c and 6d).

In Fig. 7 were shown the time sequenced moisture determinations and corresponding total solids. The moisture contents of the fermenting processed substrate and radappertized beans varied somewhat. During the course of the fermentation a slight loss in water content was observed. This differs from the results obtained in the previous set of moisture content analyses. A slightly higher moisture content value was also found for the processed substrate compared to the previous result. The variations observed may be due to moisture exchange between the beans and the air. The difference in water loss pattern may have been due to small differences in air moisture content, flow rate, convection pattern, etc. The difference in initial moisture contents, i.e. moisture contents of processed substrate may have been due to small variations in batch processing. It was precisely because of the variations in the moisture content value that lyophilized samples were employed for all other analytical studies discussed below.

## 3.4. FAT

Fats and oils are characterized by their extreme insolubility in water and very slight solubility in alcohols. They are, however, readily dissolved in ethyl ether, petroleum spirit, carbon disulfide and carbon tetrachloride. Other non-fat food constituents, except for small amounts of waxes, resins, and fat soluble pigments such as chlorophylls and carotenoids, are not appreciably soluble in solvents commonly employed for fat extraction. Joslyn (1950) has pointed out that long established custom has led to the use of dry ethyl ether for the determination of fat. He has stated that, since small amounts of substances other than fat may be included in the ether extract, the result is generally designated as "crude fat" or "ether extract".

It is usual to perform extractions of food materials with either anhydrous ethyl ether (Boiling point 34.6°C) or with petroleum ether (Boiling point 35-45°C). Ethyl ether has an advantage in that it is a better fat solvent and most of the existing standards for food analysis are based on the use of this solvent (Joslyn, 1950, 1970). It must, however, be free from both water and alcohol since moist ether will dissolve sugar and other materials which should not be included in the true ether extract.

In setting up the fat determinations two major objectives were aimed at in this study. These are outlined below.

### 3.4.1. Objectives - Fat

(1) To determine the amounts of fat in the raw beans, whole beans, processed substrate, fermented beans, mechanically dehulled beans and mechanically removed testa using the indirect and direct methods.

(2) To determine the changes in the fat content of the processed substrate during fermentation and to simultaneously determine this same parameter in radappertized beans.

### 3.4.2. Principle - Fat

The fat is extracted with ether from the prepared lyophilized sample. The solvent is removed from the extract by evaporation and the residue of fat is weighed (Osborne and Voogt, 1978). This is the 'direct' method (AOAC, 1975). Alternatively the food sample may be weighed before and after extraction in a previously dried, Alundum crucible. The fat content is then calculated from the weight loss. This is the 'indirect' method (AOAC, 1975).

### 3.4.3. Materials and Methods - Fat

Lyophilized, ground samples were employed in the fat determinations. The samples were prepared as reported under 'sample preparation' (Section 3.2.). Two types of extractor units were employed. The first was a large Soxhlet unit (Crampton laboratory modification) in which 20 samples were

simultaneously extracted. The second was a unitary Soxhlet apparatus (Pyrex brand laboratory glassware). Both were electrically heated. Also in both, extraction was carried out for 16 h at a condensation rate of 2-3 drops per second (AOAC, 1975: 7.044 - 7.046). The solvent used was purified anhydrous ethyl ether, suitable for fat extraction. It contained approximately 1 ppm butylated hydroxytoluene as a preservative (J.T. Baker Chemical Co.). With the large Soxhlet unit the indirect method was used; the smaller unit was operated as a direct method apparatus. The two methods, and their respective calculations, as applied to this study, are discussed below.

3.4.3.1. Methods: Fat Contents: The fat contents of raw beans, whole beans, processed substrate, fermented beans, mechanically dehulled beans and mechanically removed testa were determined. Both the direct and indirect methods were employed for these samples. In the direct method, ground sample (2 to 3 g) was measured into a preweighed Alundum crucible. The sample was extracted for 16 h with 150 ml of purified anhydrous ethyl ether contained in a 250 ml flask. After the extraction, the flask was placed in an oven for 30 min to drive off the ether. The oven was operated at atmospheric pressure and 100°C (AOAC, 1975). The flask and contents were cooled in a dessicator containing freshly dehydrated $CaCl_2$ and then weighed. The fat content was determined as follows:

If weight of sample (g) = $W_1$

weight of dry flask (g) = $W_2$

weight of flask + fat (g) = $W_3$

Then fat (% by weight) = $[(W_3 - W_2)/W_1]100$

In the indirect method, dried and preweighed alundum crucibles were used. Approximately 3 g of sample was added to each crucible and the weight recorded as 'crucible plus dry sample'. Twenty crucibles with their respective samples were held in a wire basket and extracted for 16 h with commercially obtained purified, anhydrous ethyl ether (J.T. Baker Chemical Co.). After the extraction the basket with crucibles was placed in a fume hood for 6 h and then dried overnight for 12 h in a vacuum oven. The oven was operated at 13 kPa and 95°C. The crucibles were cooled and weighed. The loss in weight was the ether extract of fat (AOAC, 1975).

If Dry sample (g) = $W_1$

Loss in weight after extraction (g) = $W_2$

Then % fat by weight = $[W_2/W_1 \times 100]$

3.4.3.2. Methods: Time Sequenced Fat Contents: In this experiment time sequenced changes in the fat contents of processed fermenting substrate and radappertized substrate were determined at 0, 12, 24, 36, 48, 60 and 72 h of incubation at 37°C. The direct method of determination was used throughout this experiment.

3.4.4.1. Observations: Fat Contents: In table 7 are shown the fat content data for raw beans (RB), whole beans (WB), processed substrate (PS), fermented beans (FB), mechanically dehulled beans (MDB) and mechanically removed testa (MRT). The indirect method data were slightly higher than corresponding values obtained with the direct method.

Table 7. Fat Contents.

| Sample | Fat (g/100g Sample)* | |
|---|---|---|
| | I† | II¶ |
| Raw Beans (RB) | 14.81 ± 0.12 | 15.78 ± 0.26 |
| Whole Beans (WB) | 15.37 ± 0.16 | 16.38 ± 0.11 |
| Processed Substrate (PS) | 22.29 ± 0.21 | 23.16 ± 0.51 |
| Fermented Beans (FB) | 35.10 ± 0.10 | 35.80 ± 0.12 |
| Mechanically Dehulled Beans (MDB) | 21.69 ± 0.38 | 22.32 ± 0.17 |
| Mechanically Removed Testa (MRT) | 0.49 ± 0.00 | 0.51 ± 0.02 |

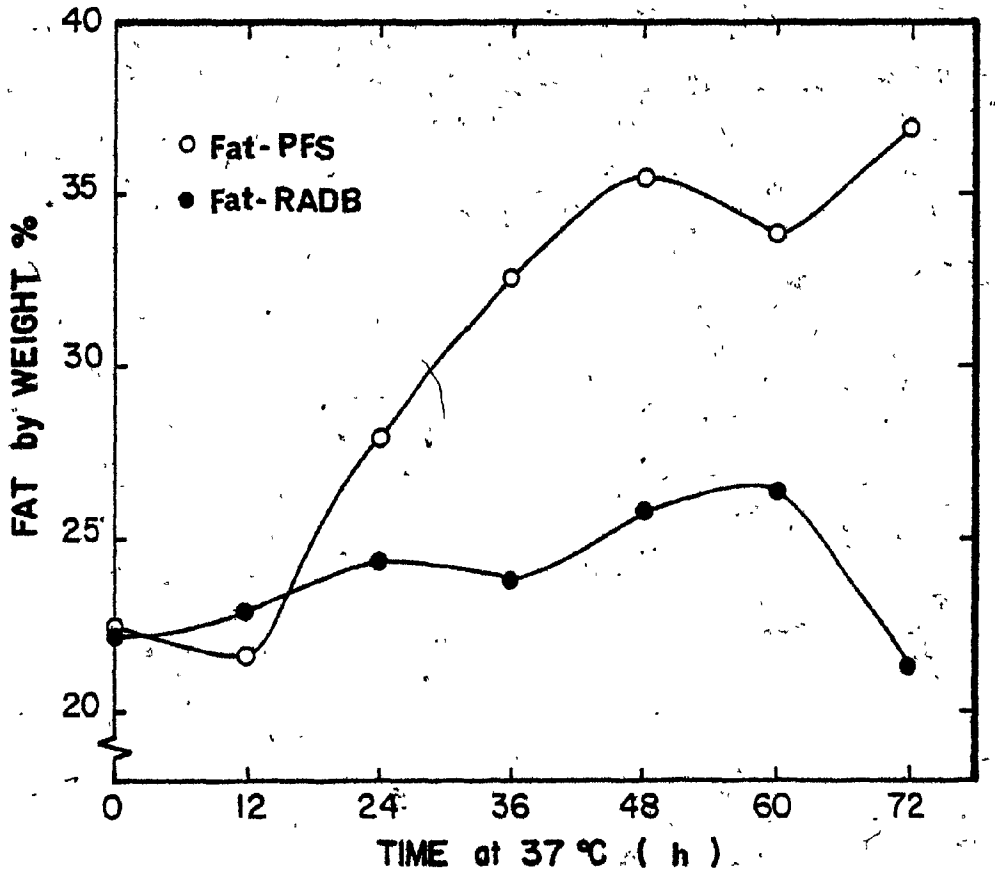
* Each result is a mean of 4 determinations ± standard deviation.

† Direct method analysis data.

¶ Indirect method analysis data.

Fig. 8: Time Sequenced Fat Contents of Processed Fermenting Substrate (PFS) and Radappertized Beans (RADB).

Figure 8


40
35
30
25
20
FAT by WEIGHT %
Fat-PFS
Fat-RADB
0
12
24
36
48
60
72
TIME at 37 °C ( h )

3.4.4.2. Observations: Time Sequenced Fat Contents: Fat contents of processed substrate and radappertized beans incubated at 37°C are shown in Fig. 8. Detailed data for these are given in appendix A, Table A4.

### 3.4.5. Discussion - Fat

In table 7 were shown the fat contents of the different samples analyzed. The mechanically removed testa had a fat content of only 0.5%. The raw beans, whole beans, processed substrate and mechanically dehulled beans had fat contents in the range 15-23%. Processing and fermentation resulted in an increase in the fat content of the beans. Slightly higher values were obtained for all the samples with the indirect method. Pitman (1932) has reported similar results when different methods were used to determine the amount of oil in olives.

The analysis of variance (ANOVA) was performed on the fat raw data presented in table 7 as previously discussed (section 3.3.5.). The resulting outputs are shown in tables 7a, 7b, 7c and 7d. The fat contents were significantly affected by the treatment at 0.0001 level (Tables 7a and 7d). The coefficient of correlation ($R^2$) was 0.9998 and coefficient of variation (CV) was less than 2%. The means were significantly different at alpha level 0.05 as indicated by the grouping (Tables 7c and 7d).

The fat contents of the fermenting processed substrate (Fig. 8) increased with fermentation time. The increase may be due to the utilization of other constituents of the processed substrate by the microorganisms

Table 7a. Analysis of Variance (ANOVA) for Direct Method Fat Contents using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 23 | 2590.8794 | | | |
| T | 5 | 2590.1562 | 518.0312 | 13455.36 | 0.0001 |
| R | 3 | 0.1450 | 0.0483 | 1.25 | 0.3256 |
| ERROR | 15 | 0.5782 | 0.0385 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9998

CV = 1.0733%

Table 7b. Analysis of Variance (ANOVA) for Indirect Method Fat Contents using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 23 | 2679.0815 | | | |
| T | 5 | 2677.9235 | 535.5847 | 6973.76 | 0.0001 |
| R | 3 | 0.0064 | 0.0021 | 0.03 | 0.9935 |
| ERROR | 15 | 1.1516 | 0.0768 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9996

CV = 1.4591%

Table 7c. Direct Method Fat Contents as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 4 | 14.81 | E |
| 2 | 4 | 15.37 | D |
| 3 | 4 | 22.29 | B |
| 4 | 4 | 35.10 | A |
| 5 | 4 | 21.69 | C |
| 6 | 4 | 0.49 | F |

* Means with the same letter are not significantly different at the 0.05 level according to DMRT.

Table 7d. Indirect Method Fat Contents as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 4 | 15.78 | E |
| 2 | 4 | 16.38 | D |
| 3 | 4 | 23.16 | B |
| 4 | 4 | 35.80 | A |
| 5 | 4 | 22.32 | C |
| 6 | 4 | 0.51 | F |

* Means with the same letter are not significantly different at the 0.05 level according to DMRT.

involved in the fermentation. The possible selective utilization of other constituents will, presumably, cause an increase in the fat content during fermentation. Slight increases in fat contents were also found for the radappertized beans, except at 72 h when a slight drop was obtained. The increases recorded for radappertized beans were very much less than those obtained for processed substrate undergoing fermentation. The fat content may be used as an index of the extent of fermentation of the processed locust beans.

## 3.5. PROTEIN

The problem of providing adequate protein for an expanding world population is acute (Scrimshaw and Young, 1977) and it is second only to the overall food shortage (Pomeranz and Meloan, 1978). Therefore, there is a need to obtain data on unusual sources of protein which could be used to supplement the already known and exploited plant and animal sources. Apart from their well known contribution to adequate nutrition, proteins also contribute to flavor of foods. For example, during processing (heating, boiling, baking, roasting) the amino acid side chains are degraded or they may interact with other food components (i.e. lysine and reducing sugars) to give flavors (Danehy and Pigman, 1951 and Pomeranz and Meloan, 1978).

It is usual to determine total protein content of foods by determining a specific element or group in the protein and using thereafter an experimentally established multiplying factor. The most commonly used assay for protein in foods is the determination of nitrogen. In this procedure it is usually assumed that nonprotein nitrogen compounds are generally present in small quantities compared to the protein content. Therefore the protein content of a sample may be found by determining the nitrogen content and multiplying by the factor 6.25 (Pomeranz and Meloan, 1978). This is the basis of the Kjeldahl and Dumas methods.

Other methods include the "Biuret" method in which two or more peptide bonds form a purple complex with copper salts in alkaline solution. The biuret method, though simple, rapid and inexpensive is not an

absolute method and the color developed must be standardized against another method such as Kjeldahl (Pomeranz and Meloan, 1978). Another method widely used for protein in solutions is the phenol-reagent method. It is based on the interactions of phenol reagent and copper under alkaline conditions. Details of this method have been described by Wu (1922), Folin and Ciocalteau (1927) and Lowry et al. (1951). Although highly sensitive, the Lowry method, as it is now commonly known, is more time consuming than the other methods. It requires multiple operations on each sample, incubation between the addition of reagents and is sensitive to high concentrations of sucrose (Pomeranz and Meloan, 1978). Other methods that have been reported include: direct spectrophotometric methods (Zeleny, 1941 and Zeleny et al., 1942), dye-binding methods (Udy, 1954, 1956) and some others (Pomeranz and Meloan, 1978). In this study the Kjeldahl methods (micro and macro) were used. The objectives of the experiment are outlined below.

### 3.5.1. Objectives - Protein

The objectives were:

(1) To quantify the protein contents of whole beans, processed substrate, fermented beans and mechanically removed testa and to determine the effect on the protein content when each of the samples was defatted before protein determination.

(2) To determine whether there is a significant change in the protein content of the processed substrate undergoing fermentation and to compare this, if any, with that of the radappertized beans on a time sequenced basis.

### 3.5.2. Principle - Protein

Total nitrogen in the sample was determined using either the macro or micro Kjeldahl methods. Crude protein was calculated by multiplying total nitrogen by 6.25 (AOAC, 1975).

### 3.5.3. Materials and Methods - Protein

Lyophilized, ground samples were employed for all determinations. Defatted samples were obtained by defatting as described above in fat analysis. In both the macro and micro Kjeldahl total nitrogen determinations the procedure can be divided into four steps as:

(A) Digestion of the sample with concentrated acid during which organic nitrogen is converted to ammonium ions.

(B) Distillation - alkali is added to the digest and ammonia is liberated into standard acid.

(C) Titration of any unused acid which has not been neutralized by the liberated ammonia.

(D) Calculation to obtain total nitrogen and then crude protein.

Detailed accounts of Kjeldahl methods have been given (AOAC, 1975). The materials and reagents used and their preparations are given in appendix C, Fig. C1.

3.5.3.1. Methods: Protein Contents: The protein contents of whole beans, processed substrate, fermented beans, mechanically removed testa

and their corresponding defatted samples were determined using the macro Kjeldahl method. Between 0.7 and 1.0 g of sample was accurately weighed, digested, distilled and titrated. The total nitrogen and crude protein contents were then calculated. Blank determinations (duplicates) were also carried out and values obtained subtracted from sample results. Detailed procedures for macro Kjeldahl have been documented (AOAC, 1975; Osborne and Voogt, 1978 and Pomeranz and Meloan, 1978). The method employed in this experiment was that of the AOAC (1975).

3.5.3.2. Methods: Time Sequenced Protein Contents: The micro Kjeldahl method was used. Accurately weighed 0.7 to 1.0 g samples were digested in a 100 ml Kjeldahl flask. The digest was made up to volume with water and thoroughly mixed. Ten millilitres of the digest was distilled and titrated. Total nitrogen and crude protein were then calculated. Blanks were also run and their values subtracted from samples values. Detailed methodology of the micro Kjeldahl process is given by Lillevik (1970). Samples analyzed were obtained from processed substrate and radappertized beans. Both were incubated at 37°C. Analyses were carried out on these after they had been incubated for 0, 12, 24, 36, 48, 60 and 72 h. Lyophilized and ground samples, prepared as previously described, were used.

3.5.4.1. Observations: Protein Contents: In table 8 are shown the protein contents of whole beans (WB), processed substrate (PS), fermented beans (FB) and mechanically removed testa (MRT). Also shown are corresponding values for the defatted samples.

Table 8. Protein Contents.

| Sample | Protein (g/100 g Sample)* | |
|---|---|---|
| | I† | II¶ |
| Whole Bean (WB) | 28.8 ± 0.2 | 35.4 ± 1.0 |
| Processed Substrate (PS) | 38.5 ± 0.0 | 52.1 ± 0.0 |
| Fermented Beans (FB) (72 h) | 39.4 ± 0.3 | 54.3 ± 0.3 |
| Mechanically Removed Testa (MRT) | 3.1 ± 0.0 | 3.1 ± 0.1 |

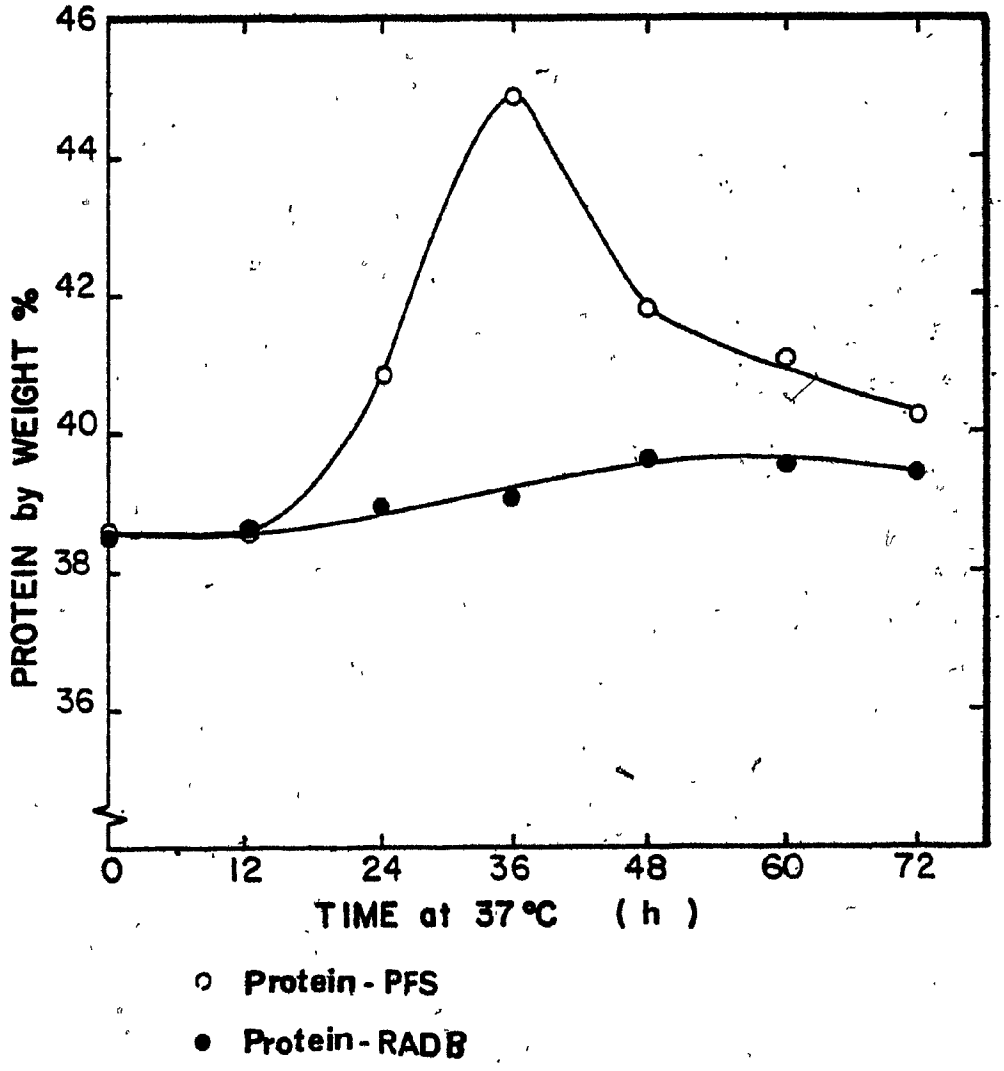
* Mean of 4 determinations ± standard deviation.

† Samples not defatted.

¶ Samples defatted.

Fig. 9: Time Sequenced Protein Contents of Processed Fermenting Substrate (PFS) and Radappertized Beans (RADB).

Figure 9


46
44
42
40
38
36
PROTEIN by WEIGHT %
0
12
24
36
48
60
72
TIME at 37 °C ( h )
Protein - PFS
Protein - RADB

3.5.4.2. Observations: Time Sequenced Protein Contents: In Fig. 9 are shown the time sequenced protein contents of fermenting processed substrate and radappertized beans incubated at 37°C. Detailed data for these are given in appendix A, Table A5.

### 3.5.5. Discussion - Protein

The protein content of MRT was 3%. This value was not affected when MRT was defatted before analysis, indicating that the fat content of this material was very low. This result complemented the earlier fat value obtained for MRT (0.5%). The WB, PS and FB had 28.8, 38.5 and 39.4% protein contents, respectively. These values indicated that removal of testa by processing increased the protein content of the beans. Slightly higher protein values were obtained for the fermented beans as compared to the processed beans. The defatted samples of WB, PS and FB gave significantly higher values of protein contents than corresponding nondefatted sample.

The analysis of variance (ANOVA) was performed on the protein raw data shown in table 8 as discussed under section 3.3.5. The resulting output are presented in tables 8a, 8b, 8c and 8d. The protein contents were significantly affected by the treatment at 0.0001 level (Tables 8a and 8b). The coefficient of correlation ($R^2$) were $\geq$ 0.9998 and the coefficient of variation (CV) were $\leq$ 1.1649%. The means were significantly different at alpha level 0.05 as indicated by the grouping (Tables 8c and 8d).

Table 8a. Analysis of Variance (ANOVA) for Protein Contents of Non-Defatted Samples using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 15 | 3432.9231 | | | |
| T | 3 | 3432.6724 | 1144.2241 | 41457.40 | 0.0001 |
| R | 3 | 0.0023 | 0.0008 | 0.03 | 0.9934 |
| ERROR | 9 | 0.2484 | 0.0276 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9999

CV = 0.6053%

Table 8b. Analysis of Variance (ANOVA) for Protein Contents of Defatted Samples using General Linear Model (GLM) Procedure.

| SOURCE | DF | SS | MS | F | PR > F |
|---|---|---|---|---|---|
| TOTAL | 15 | 6719.0746 | | | |
| T | 3 | 6717.0902 | 2239.0301 | 12578.82 | 0.0001 |
| R | 3 | 0.3826 | 0.1275 | 0.72 | 0.5666 |
| ERROR | 9 | 1.6018 | 0.1780 | | |

T = Treatment

R = Replicate

$R^2$ = Coefficient of correlation = 0.9998

CV = 1.1649%

Table 8c. Protein Contents of Non-Defatted Samples as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 4 | 28.80 | C |
| 2 | 4 | 38.53 | B |
| 3 | 4 | 39.35 | A |
| 4 | 4 | 3.12 | D |

* Means with the same letter are not significantly different at the 0.05 level according to DMRT.

Table 8d. Protein Contents of Defatted Samples as Affected by the Treatment using Duncan's Multiple Range Test (DMRT) and GLM Procedure.

| Treatment | Number of Replicate | Mean | Grouping* |
|---|---|---|---|
| 1 | 4 | 35.40 | C |
| 2 | 4 | 52.10 | B |
| 3 | 4 | 54.30 | A |
| 4 | 4 | 3.10 | D |

* Means with the same letter are not significantly different at the 0.05 level according to DMRT.

Defatted processed substrate and defatted fermented product may be used as nutritionally improved foods. The extracted lipids might also serve as a food fat source. There was no difference in the protein contents of processed fermenting substrate (PFS) and radappertized beans (RADB) between 0 and 12 h (Fig. 9). From 12 to 36 h there were progressive increases in the protein contents of PFS over RADB. This may have been due to microbial utilization of carbohydrate material resulting in relative increases in the other major constituents of the PFS. Between 36 to 72 h, the protein content of PFS progressively decreased, possibly due to proteolysis and deamination by microorganisms present on the PFS. In some of the fermentations the characteristic smell of ammonia was perceived. Very slight variations were obtained for the RB throughout the 0 to 72 h incubation period (Fig. 9). It is important to note (Fig. 9) that the protein contents may be a useful tool in deciding when to stop the process of fermentation. If the sole reason for fermentation was to increase the protein content, then 36 h would appear to be a logical point to stop the process.

## 3.6. AMINO ACIDS

The primary nutritional importance of proteins in foods is as a source of the twenty-four amino acids generally present in food materials. Eight of these amino acids (lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine) are designated as essential for physical and mental health (Osborne and Voogt, 1978 and Pomeranz and Meloan, 1978). The biological value to be attached to a food material depends on the relative amounts of these essential amino acids (EAA) present in it.

### 3.6.1. Objectives - Amino Acids

The objective was to determine the amino acid contents of (a) lyophilized, defatted processed substrate (LDPS), (b) lyophilized, defatted fermented (36 h) beans (LDFB), (c) lyophilized fermented (72 h) beans (LFB), (d) lyophilized radappertized beans (LRADB) and (e) presumably 'sun dried' fermented beans from Nigeria (NS).

### 3.6.2. Principle - Amino Acids

The sample containing the protein or proteins of interest is treated to release the amino acids (hydrolysis). The amino acids released are chromatographically determined (Spackman, Stein and Moore, 1958 and

Osborne and Voogt, 1978). The amount of each amino acid in the sample is determined by the comparison of each peak area of the unknown sample amino acids with that of the area corresponding to a known amount of incorporated internal standard (norleucine).

### 3.6.3. Materials and Methods - Amino Acids

The samples employed were as specified under section 3.6.1. The NS was a market sample of fermented locust beans obtained from Nigeria. The sample was reported to have been sun dried for 48 h after purchase before packaging. The NS was analyzed as received. Samples to be hydrolyzed were accurately weighed (10-15 mg). Each weighed sample was transferred into a separate 13 x 100 mm test tube. One millilitre of constant boiling hydrochloric acid was added, followed by 4 drops of 5% phenol. The phenol acts as an oxygen scavenger. The contents of the test tube were frozen in liquid air and flushed three times with nitrogen. The test tube was sealed and placed in a rotary oven at 110°C for 22 h to hydrolyze the proteins. After hydrolysis the tube was opened and evaporated in a vacuum dessicator containing NaOH to remove the HCl. The sample was dissolved in 5 ml 0.2 N sodium citrate buffer (pH 2.2), filtered through Whatman paper No. 1 and frozen prior to analysis. The chromatographic analysis was carried out in a Beckman Model 120 C amino acid analyzer modified to model 119. It had a single column - Amenex A-9 resin (330 mm height, 6 mm internal diameter). The column temperature was 55°C and 3 buffers in stepwise elution were used. The color reagent was ninhydrin.

### 3.6.4. Observations - Amino Acids

The amino acid contents of LDPS, LDFB, LFB, LRADB and NS are shown in table 9.

### 3.6.5. Discussion - Amino Acids

The actual chromatographic analysis was carried out by a technician of the Department of Agricultural Physics and Chemistry, Macdonald College. The results reported in table 9 are the final values as provided by the technician. There are possible limitations to the results as follows:

(i) They were based on a single chromatographic run per sample. Duplicates could not be analyzed as a result of the high costs involved.

(ii) Tryptophan is not reported, because of a limitation of the chromatographic method used.

On the basis of the above limitations, the values reported in table 9 should be interpreted with caution. These values were tabulated to allow for a possible comparison among different samples and with values reported by FAO (1970) for Parkia seeds. The glutamic acid content was high for all samples and particularly so for the LDFB sample. Glutamate is an important flavor component in fermented Oriental foods. The high levels of glutamic acid in all samples may be an indication of a similar role in African locust beans.

Table 9. Amino Acids Contents.

| AMINO ACIDS | g Amino Acid/100 g Sample | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | FAO† (1970) |
| | 1 | 2 | 3 | 4 | 5* | |
| Aspartic acid | 5.32 | 8.30 | 3.06 | 2.41 | 5.76 | 3.98 |
| Threonine | 1.58 | 1.40 | 2.51 | 0.76 | 1.60 | 1.01 |
| Serine | 3.19 | 5.09 | 1.83 | 0.79 | 1.03 | 1.49 |
| Glutamic acid | 8.70 | 10.99 | 5.16 | 4.74 | 5.92 | 5.94 |
| Proline | 1.80 | 1.74 | 1.78 | 1.59 | 9.71 | 1.52 |
| Glycine | 2.55 | 1.49 | 1.29 | 0.98 | 0.76 | 1.38 |
| Alanine | 2.57 | 2.51 | 0.98 | 0.84 | 0.91 | 1.60 |
| Valine | 6.17 | 4.31 | 2.04 | 1.46 | 3.43 | 1.54 |
| Cysteine | - | 1.27 | 0.33 | 0.19 | - | 0.38 |
| Methionine | 1.80 | 0.81 | 0.41 | 0.54 | 1.83 | 0.31 |
| Isoleucine | 4.01 | 0.23 | 3.14 | 4.48 | 6.81 | 1.29 |
| Leucine | 3.41 | 10.63 | 3.08 | 2.45 | 5.16 | 2.22 |
| Tyrosine | 6.54 | 4.19 | 3.35 | 2.46 | 4.31 | 1.17 |
| Phenylalanine | 5.33 | 6.48 | 2.35 | 2.00 | 4.67 | 1.54 |
| Lysine | 3.93 | 3.84 | 3.78 | 2.44 | 6.17 | 2.09 |
| Histidine | 0.62 | 3.39 | 2.58 | 0.68 | 1.11 | 0.87 |
| Arginine | 4.23 | 5.98 | 3.00 | 0.68 | 1.63 | 2.03 |
| TOTAL | 61.75 | 72.65 | 40.67 | 29.30 | 60.81 | 30.36 |

1. Lyophilized, defatted, processed substrate (LDPS).
2. Lyophilized, defatted, fermented beans (36 h) (LDFB).
3. 72 hour lyophilized, fermented beans (LFB).
4. Lyophilized radappertized beans (LRADB).
5*. Market purchased fermented beans from Nigeria (NS). Exact mode of preparation and fermentation not known.

† Parkia seeds - Preparation not specified (FAO, 1970).

## 3.7. ASH

When foods and food products are heated to temperatures of 500 to 600°C, water and other volatile constituents are evolved as gases. The organic constituents are combusted in the presence of oxygen to oxides of hydrogen, carbon, nitrogen and sulfur which are eliminated. The inorganic residue constitutes the ash of the food. According to Joslyn (1970) ash constituents include larger amounts of potassium, sodium and calcium and smaller quantities of aluminium, copper, iron, manganese and zinc. Also present, in trace amounts, are arsenic, fluorine, iodine and other elements. Ashing may be done by incineration over an open flame, in a muffle furnace, in a closed system in the presence of oxygen, by wet combustion with sulfuric, nitric or perchloric acid or with a mixture of these. For some foods of very low ash content the use of conductometric ashing has been proposed (Gillet, 1949). The ash contents of foods, on a dry weight basis, vary between 0.0% in whole egg, refined white sugar, oils and fats and 30.9% in sauerkraut (Watt and Merrill, 1963 and Joslyn, 1970). Ash content is of immense value as an index of quality in such foods as flour. The more bran is removed from the wheat, the lesser the amount of ash in the flour. Ash content measurement is used in the detection of adulteration in some foods such as marmalade. It is also useful for the determination of minerals or metallic salts present as constituents of foods or as contaminants.

### 3.7.1. Objectives - Ash

The objective of this experiment was to determine the ash content of raw beans (RB), whole beans (WB), processed substrate (PS), fermented beans (FB) and mechanically removed testa (MRT).

### 3.7.2. Principle - Ash

Organic matter in the sample is combusted and the inorganic material remaining, the ash, is cooled and weighed. Heating is carried out in stages, first to drive off the water, then to char the product and finally to ash at 550°C in a muffle furnace.

### 3.7.3. Materials and Methods - Ash

A muffle furnace (Thermolyne Furnatrol II Sybron Furnace Model F-A1730, Thermolyne Sybron Corp., Iowa, U.S.A.), thermostatically controlled at 550°C and dessicators with freshly prepared $CaCl_2$ dessicant were used. The method employed was that of AOAC (1975, 14.006). Porcelain dishes, 80 mm diameter and 25 mm deep were placed in the muffle furnace at 550°C for 30 min, removed and cooled in the dessicator to room temperature (1 h). Each dish was weighed to the nearest milligram. Between 3 to 5 g of sample was accurately weighed into each dish. The dishes were placed on a hot plate under a fume hood. The temperature was then gradually

increased. Heating was continued until smoking ceased and samples were observed to be thoroughly charred. The dishes with the samples were then placed in the centre of the furnace and ashed overnight at 550°C, removed into the dessicator, cooled for 1 h and then weighed. Ash was determined by difference in the following manner:

If Weight (g) of sample = $W_1$

Weight (g) of ash = $W_2$

Then Ash (% by weight) = $[(W_2/W_1) \times 100]$

The ash contents of RB, WB, PS, FB and MRT were determined as above. All samples used were lyophilized; determinations were done in triplicate.

### 3.7.4. Observations - Ash Content

In table 10 are shown the ash contents of RB, WB, PS, FB and MRT.

### 3.7.5. Discussion - Ash

The ash content of RB was 5.8%. The removal of the pulp to obtain WB resulted in a reduced ash content (Table 10). The PS ash content was greater than that of WB. This may have been due to the removal of the testa since MRT had a lesser ash content than the WB. An increase in the ash content was obtained during fermentation. This may have been due to the reduction in the organic portion of the fermenting beans.

Table 10. Ash Contents.

| Sample | Ash (g/100 g Sample)* |
|---|---|
| Raw Beans (RB) | 5.8 ± 0.1 |
| Whole Beans (WB) | 4.9 ± 0.0 |
| Processed Substrate (PS) | 6.0 ± 0.1 |
| Fermented Beans (FB) | 7.0 ± 0.1 |
| Mechanically Removed Testa (MRT) | 4.4 ± 0.0 |

* Mean of triplicate determinations ± standard deviation.

## 3.8. TOTAL CARBOHYDRATES

Carbohydrates are the most abundant and widely distributed food components (Pomeranz and Melaon, 1978). They include:

(a) Monosaccharides (polyhydroxy aldehydes or ketones);

(b) Oligosaccharides formed by the linear linking of 3 to 8 monosaccharides joined by glycosidic linkages, and

(c) Polysaccharides which can either be structural (cellulose, hemicellulose, lignin) and/or nutrient materials (starch, glycogen) which form metabolic reserves in plants and animals.

In food composition tables, the carbohydrate content is usually given as 'total carbohydrate by difference' i.e. on a wet basis it is the percentage of material left after water, protein, fat and ash are subtracted from 100. It may similarly be calculated on a dry weight basis (Pomeranz and Meloan, 1978).

In tables 11a and 11b are shown the total carbohydrate contents of WB, PS, FB and MRT. The MRT had the highest total carbohydrate content at 92.0 g/100 g of the sample. This high value was expected from the low amounts of fat and protein previously reported for this material. About half of the whole bean constituents was found to be carbohydrate. The removal of the testa during the processing resulted in a decrease of the total carbohydrate content and corresponding increases in fat and protein contents. Fermentation of the beans further reduced the total carbohydrate content. The microorganisms present on the PS presumably consumed a large portion of the carbohydrates during the bioconversion.

Tables 11a & 11b. Total Carbohydrate Contents.

(11a)

| Component | g/100 g Sample* | | | |
|---|---|---|---|---|
| | Samples | | | |
| | WB | PS | FB | MRT |
| Fat | 15.4 | 22.3 | 35.1 | 0.5 |
| Protein | 28.8 | 38.5 | 39.4 | 3.1 |
| Ash | 4.9 | 6.0 | 7.0 | 4.4 |
| Carbohydrates By Difference | 50.9 | 33.2 | 18.5 | 92.0 |

(11b)

| Sample | Total Carbohydrate Contents (g/100 g Sample)* |
|---|---|
| Whole Beans (WB) | 50.9 |
| Processed Substrate (PS) | 33.2 |
| Fermented Beans (FB) | 18.5 |
| Mechanically Removed Testa (MRT) | 92.0 |

* Dry weight basis (lyophilized samples).

The reduction in the carbohydrate content of FB is an indication of the importance of carbohydrate to the process of African locust beans fermentations.



## 3.9. TOTAL AVAILABLE CARBOHYDRATES AS GLUCOSE

The monosaccharides, oligosaccharides and polysaccharides (i.e. the components of total carbohydrates as discussed above) are not equally 'available' in terms of human nutrition. The reason for this lies in the relative ease or difficulty of the breakdown of their glycosidic linkages by enzymes. For example, the polysaccharide starch with α-(1-4) glycosidic linkages is easily broken down by enzymes secreted in the mouth and intestines and is therefore considered 'available'. The same is true of monosaccharides and disaccharides such as glucose, sucrose, etc. However, the polysaccharide cellulose, the main structural component of most plant tissues, is considered 'unavailable' to humans since the digestive tract does not secrete enzymes capable of splitting the β-(1-4) glycosidic linkages. Most of it, along with other 'unavailable' carbohydrates such as hemicelluloses and lignin, passes through the body unchanged. A broad distinction of the two types has been made by Osborne and Voogt (1978) who proposed that the 'available' carbohydrate fraction in food may be considered to be composed of the sum of the monosaccharides, disaccharides, dextrins, starches and glycogens. The 'unavailable' carbohydrate may be taken as the sum of the naturally occurring cellulose, hemicelluloses, gums, pectins and other polysaccharides that may be added to foods in low levels as technological aids (e.g. agars, carageenans, locust bean gum, etc.). Clegg (1956) employed a modified anthrone method to estimate the amounts of starch and soluble sugars in cereals. This method has been designated 'total available carbohydrate method' by Osborne and Voogt (1978), even though it measures only the hydrolyzed starches and reducing sugars present in food.

### 3.9.1. Objectives - Total Available Carbohydrates

The objectives for this set of experiments were:

(1) To determine the total available carbohydrate contents of the processed substrate (PS) and the fermented beans (FB).

(2) To determine and compare the total available carbohydrate contents of the processed fermenting substrate (PFS) and radappertized beans (RADB) on a time sequenced basis.

### 3.9.2. Principle - Total Available Carbohydrates

The material of interest is digested with perchloric acid. The hydrolyzed starches (perchloric acid action) together with reducing sugars are determined colorimetrically at 630 nm. Anthrone reagent is employed to develop the color. The total available carbohydrate is expressed as glucose.

### 3.9.3. Materials and Methods - Total Available Carbohydrates As Glucose

Both lyophilized and wet samples were used. The method employed was that of Clegg (1956) as modified by Osborne and Voogt (1978). Each sample was accurately weighed (1 g of lyophilized sample and 2.5 g of the wet sample), hydrolyzed and then extracted. Glucose standards and blanks

were also prepared. Detailed accounts of the procedure are given in appendix C, Fig. C2. The optical density (O.D) of the samples, standards and blanks were read at 630 nm against reagent blanks in a Beckman model 25 spectrophotometer (Beckman, U.S.A.). A standard curve was prepared for glucose. The amount of total carbohydrate in each sample was determined as follows:

If Weight (g) of sample = W

Absorbance of dilute standard = a

Absorbance of dilute sample extract = b

Then Total available carbohydrate as % Glucose

$$= (25 \times b)/(a \times W)$$

3.9.3.1. Methods : Total Available Carbohydrate Contents (TACC): Total available carbohydrate contents of lyophilized PS, FB and of wet samples of PS and FB were determined.

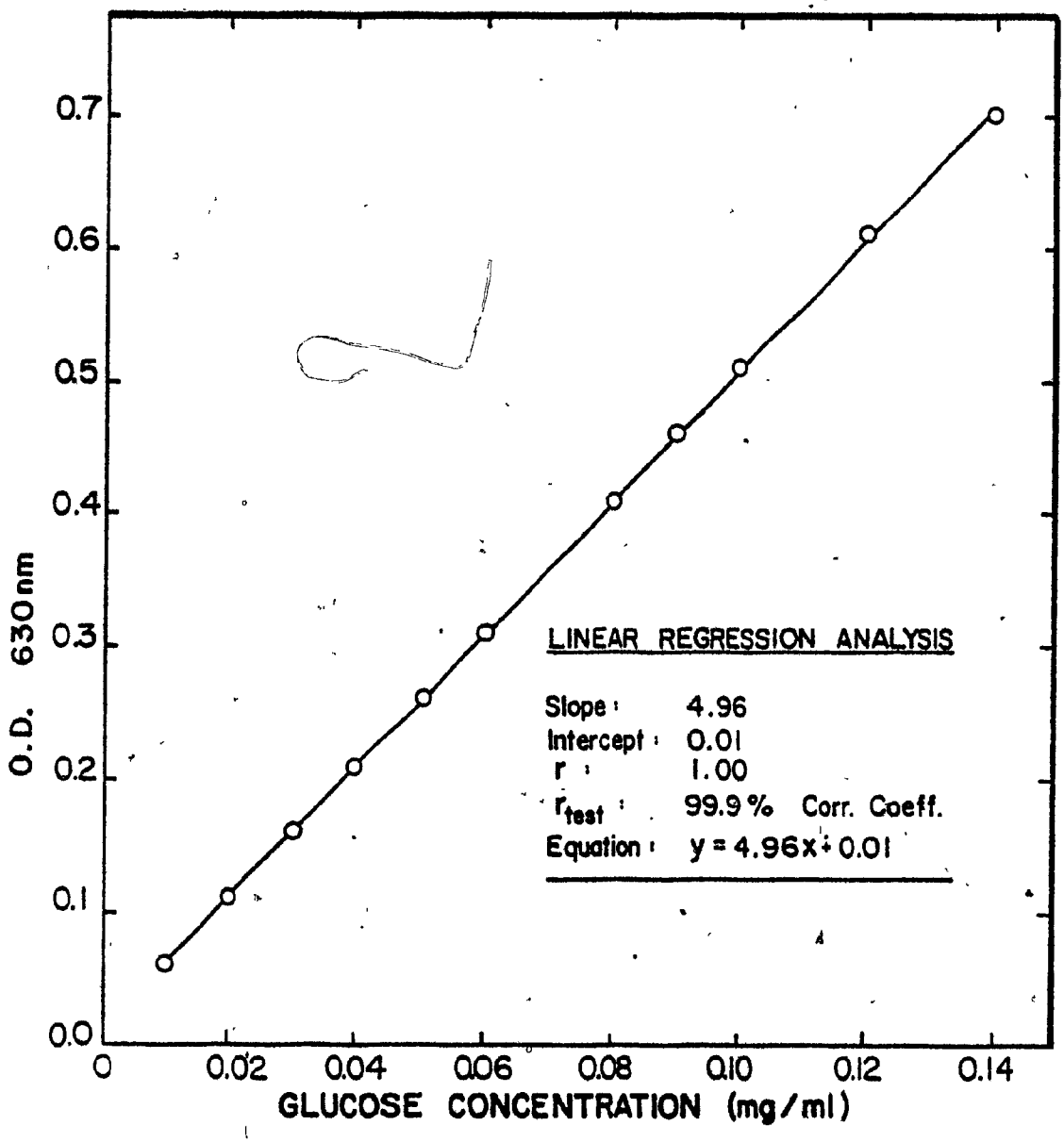
3.9.3.2. Methods : Time Sequenced Total Available Carbohydrate Contents: The total available carbohydrate contents of PFS and radappertized beans (RADB) incubated at 37°C, were determined on a time sequenced basis. Both lyophilized and wet samples were analyzed.

### 3.9.4. Observations - Standard Glucose Curve

The standard glucose curve between 0.01 and 0.14 mg glucose per ml is shown in Fig. 10. Detailed data for the curve are reported in appendix A, Table A6.

Fig. 10: Glucose Standard Curve.

Figure 10


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D. 630nm
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
GLUCOSE CONCENTRATION (mg/ml)
LINEAR REGRESSION ANALYSIS
Slope : 4.96
Intercept : 0.01
r : 1.00
$r_{test}$ : 99.9% Corr. Coeff.
Equation : y = 4.96x + 0.01

3.9.4.1. Observations: Total Available Carbohydrate Contents: The total available carbohydrate contents of LPS, LFB, wet processed substrate (WPS) and wet fermented beans (WFB) are reported in table 12.

3.9.4.2. Observations: Time Sequenced TACC: In Fig. 11 are shown the time sequenced total available carbohydrate contents of lyophilized PFS and RADB and wet PFS and RADB incubated at 37°C. Detailed data are reported in appendix A, Table A7.

### 3.9.5. Discussion - Total Available Carbohydrates

A very high correlation coefficient (1.00) was obtained for the standard glucose curve data. Osborne and Voogt (1978) have stated that the curve is a straight line between 0-0.15 mg/ml glucose. Regression analysis for the glucose standard curve (Fig. 10) gave the straight line equation of:

$$y = 4.96x + 0.01$$

$$y = O.D_{630\ nm}$$

$x$ = Glucose concentration (mg/ml)

The equation may be used to determine the amounts of TACC as glucose present in a sample.

In table 12 are shown the TACC of LPS, LFB, WPS and WFB. The lyophilized samples had higher values of TACC than those of the wet samples. In all cases the TACC were less than 3%.

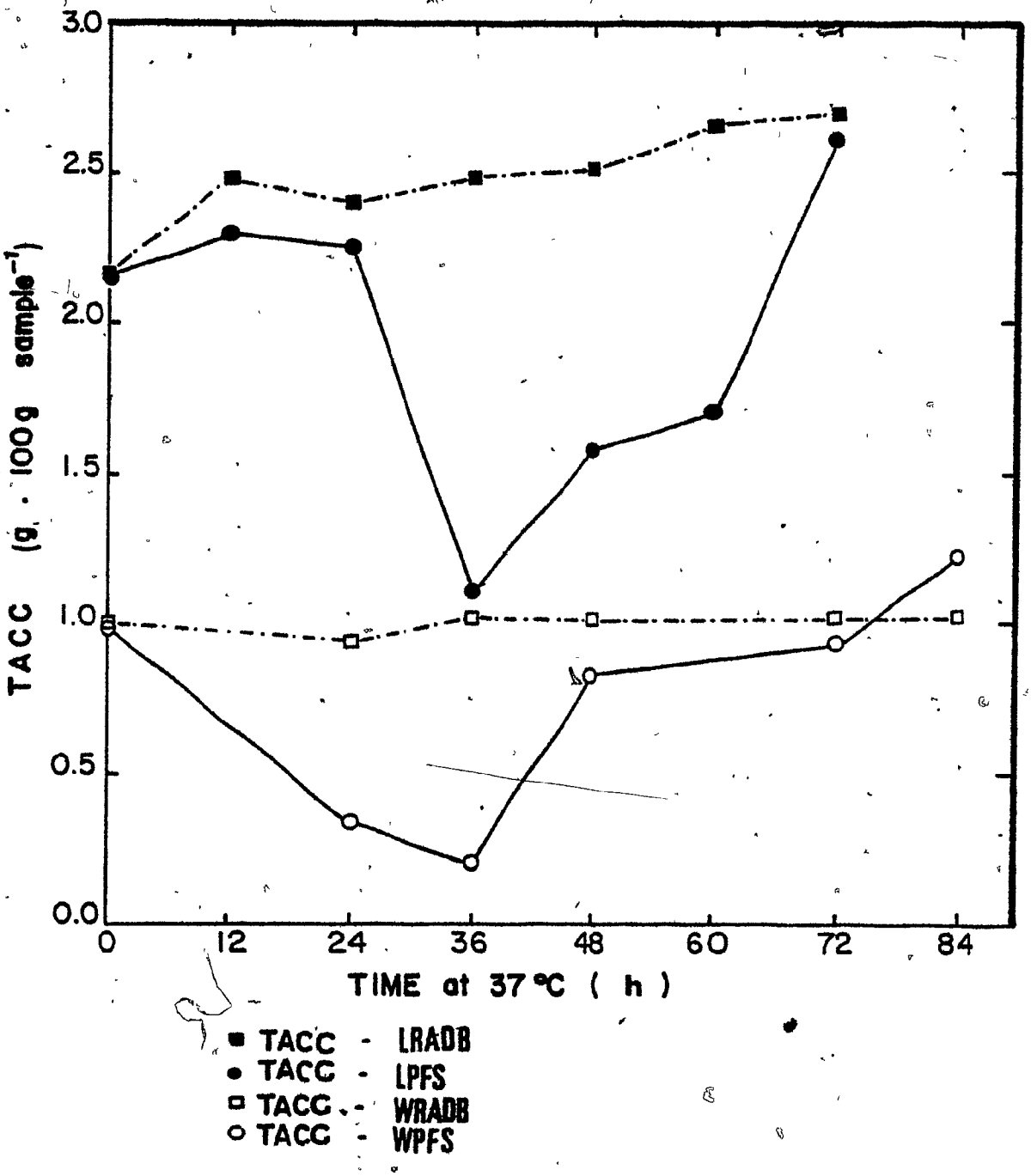
Table 12. Total Available Carbohydrate Contents.

| Sample | Total Available Carbohydrate Contents As Glucose (g/100 g Sample)* |
|---|---|
| Lyophilized Processed Substrate (LPS) | 2.16 |
| Lyophilized Fermented Beans (72 h). (LFB) | 2.61 |
| Wet Processed Substrate (WPS) | 1.06 |
| Wet Fermented Beans (72 h) (WFB) | 0.93 |

*Mean of 3 determinations.

Fig. 11: Time Sequenced Total Available Carbohydrate Contents (TACC) of Lyophilized Radappertized Beans (LRADB), Lyophilized Processed Fermenting Substrate (LPFS), Wet Radappertized Beans (WRADB) and Wet Processed Fermenting Substrate (WPFS).

Figure 11


3.0
2.5
2.0
1.5
1.0
0.5
0.0
TACC (g . 100g sample$^{-1}$)
0
12
24
36
48
60
72
84
TIME at 37 °C ( h )
■ TACC - LRADB
● TACC - LPFS
□ TACC - WRADB
○ TACC - WPFS

The time sequenced TACC are shown in Fig. 11. As expected, the lyophilized samples also had higher TACC values than the corresponding wet samples at all the times investigated. Both the radappertized lyophilized and radappertized wet beans samples had fairly constant TACC values. However, the TACC values for the lyophilized processed fermenting substrate (LPFS) and wet processed fermenting substrate (WPFS) varied markedly during the time course experiment. For the lyophilized, fermenting material the TACC did not change much between Ø to 24 h. Between 24 to 36 h there was a dramatic drop in TACC. From 36 to 72 h a steady rise was found in TACC values. The WPFS yielded a similar TACC - Time curve.

The shape of the TACC curves of the fermenting beans may be due to the activities of the microorganisms present. A small microbial population at the start of the incubation would consume TACC at a relatively slow rate; hence the constant initial TACC values. With time, an increase in microbial population resulted in a higher TACC utilization rate, causing the decrease. The subsequent rise in TACC may be due to contributions from microbial polysaccharide. A mucoid substance in the form of a grey, thin pellicle was observed to be produced on the processed beans during fermentation. This may have contributed to the TACC. Alternatively microbial populations may have produced exogenous carbohydrases which increased the TACC by making more carbohydrate 'available'.

In general, the samples analyzed in this study had low TACC values. This was probably due to the material containing very little or no starch. The absence of starch was confirmed by testing the material and a control

of soluble potato starch with iodine. Anon (1922) has reported that no true starch was found from the pulpy carbohydrate material adhering to the Parkia beans. The absence of starch in some other legume seeds has also been reported. For example, mature seeds of soybeans have been found to contain no starch (Bils and Howell, 1963 and Ogren and Rinne, 1973). Lupine seeds, of some species, were reported to have a starch content of 0.3 to 0.5% (Cerning-Beroard and Filiatre, 1976). It was therefore not unexpected that starch was not present in the samples analyzed. What was determined by the 'available carbohydrate' test was probably the free reducing sugar content in the materials analyzed. Clegg (1956) also found low levels of sugars in several cereals and cereal by-products i.e. in the range 0.8 to 3.3%.

## 3.10. GROSS ENERGY

The energy value of a food is measured in terms of kilojoules (kJ) per unit mass. It is believed that an average individual requires about 6000-8500 kJ per day from food intake. According to Osborne and Voogt (1978) food constituents provide approximate energy values of the following magnitudes:

| | Energy (kJ/g) |
|---|---|
| Available carbohydrate | 16 |
| Protein | 17 |
| Fat | 38 |

Since various foods have these constituents in varying amounts, their gross energy (G.E) contents will be different.

### 3.10.1. Objectives - Gross Energy

The objective of the experiment was to determine the gross energy of the processed fermenting substrate on a time sequenced basis.

### 3.10.2. Principle - Gross Energy

A known quantity of sample is combusted in a bomb calorimeter. The heat released during the process is determined and expressed as gross energy of the combusted material.

### 3.10.3, Materials and Methods - Gross Energy

A bomb calorimeter (Parr Instrument, Moline, Illinois, U.S.A.) was used. The detailed procedure is given in appendix C, Fig. C3. The gross energy of the processed fermenting substrate was determined after Ø, 12, 24, 36, 48, 60 and 72 h incubation at 37°C. Lyophilized samples were used.

### 3.10.4. Observations - Gross Energy Content

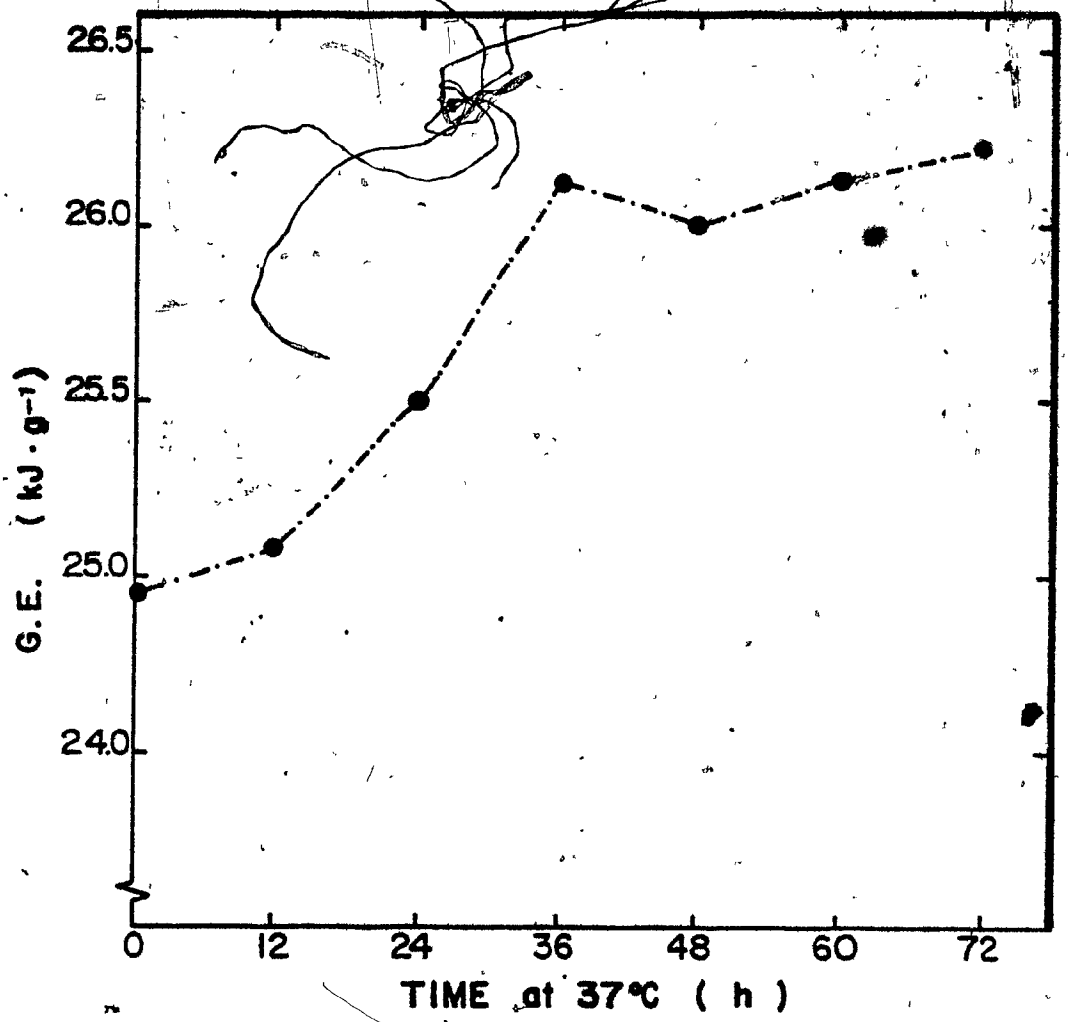
The time sequenced gross energy contents of the processed fermenting substrate (PFS) are shown in Fig. 12. Detailed data are presented in appendix A, Table A8.

### 3.10.5. Discussion - Gross Energy Content (GEC)

The GEC for the processed fermenting substrate increased progressively during incubation. The increase may have been due to the utilization of carbohydrates by the microorganisms present. Their activities resulted in a decrease in the carbohydrate content and simultaneously in increases in the fat and protein contents. Fats have a much higher GEC value than carbohydrates (Osborne and Voogt, 1978) so that their increased content led to a higher GEC of the substrate during the fermentation (Fig. 12). This result was consistent with earlier results reported in this chapter.

Fig. 12: Time Sequenced Gross Energy (G.E.) Contents (kJoules.$g^{-1}$).

Figure 12


26.5
26.0
25.5
25.0
24.0
G.E. ( kJ · g⁻¹ )
0
12
24
36
48
60
72
TIME at 37°C ( h )

## 3.11. TEXTURE

One reason among several others cited in the literature for fermenting plant materials, especially seeds, is to produce a product of enhanced organoleptic quality (flavor, aroma, color and texture). Flavor, aroma and color are usually determined by subjective evaluation. However, objective force measuring instruments are often the basis of texture evaluation. According to Szczesniak (1966), all food texture measuring devices have five essential elements:

(1) Driving mechanism.

(2) Probe element in contact with the food.

(3) Applied force.

(4) Sensing element.

(5) Read out system.

The measurement of the textural properties of food is essentially an exercise in measuring the strength of materials although, perhaps, 'weakness of materials' might be a better description since most foods are preferred when in a structurally weakened state (Bourne et al., 1966).

During Parkia filicoidea Welw. fermentations, the processed substrate was observed to become progressively softened. Partial solubilization of the beans was also observed when the substrate was left for longer than the 72 h usual period of bioconversion. It was also observed that radappertized beans (RADB) retained their original, relatively hard texture throughout. In order to quantify these observations a test known as the 'Magness Taylor Puncture Test' or simply the 'Puncture Test' (Bourne, 1965, 1976) was used. In the test, a probe is forced into the food and the force

required to do this is recorded. To carry out the test a universal texture evaluation instrument, an Instron machine model 1132 (Instron Limited, England and U.S.A.), used in food testing systems was employed. The Instron machine has been used to measure the texture of fruits, bread, cheese, raw meat, cooked meat, pork slices, frankfurters, cherries and potato chips. The machine has also been used to determine the effect of processing methods (blanching, cooking, canning, freezing, dehydration, radiation, chemical and osmotic treatments) on food texture (Matz, 1962; Kulwich et al., 1963; Bourne, 1965, 1976; Simon et al., 1965; Bourne et al., 1966 and Bourne and Mondy, 1967). Most rheologists have chosen to measure and report maximum force when the Instron machine is used due to the complexities of calculating the true stress and strain (Bourne, 1979).

### 3.11.1. Objectives - Texture

The objectives were:

(1) To determine, using an objective measurement technique, the texture of raw beans (RB), whole beans (WB), processed substrate (PS) and fermented beans (FB).

(2) To determine as in (I) above the texture of processed substrate (PS) and radappertized beans (RADB) on a time sequenced basis.

### 3.11.2. Principle - Texture

Texture was quantified by the puncture test. In this a probe is forced into the material at a constant speed; the maximum force required to do this is determined from the output on a strip chart and is taken as the texture measurement.

### 3.11.3. Materials and Methods - Texture

In the objective determination of texture the Instron Universal Testing Machine Model 1132 (Instron Corporation, U.S.A.) was used. The machine consists of two parts:

(a) The drive mechanism which drives a moving crosshead in a vertical direction by means of twin lead screws at selected speeds in the range 0.05 to 50 cm/min, and

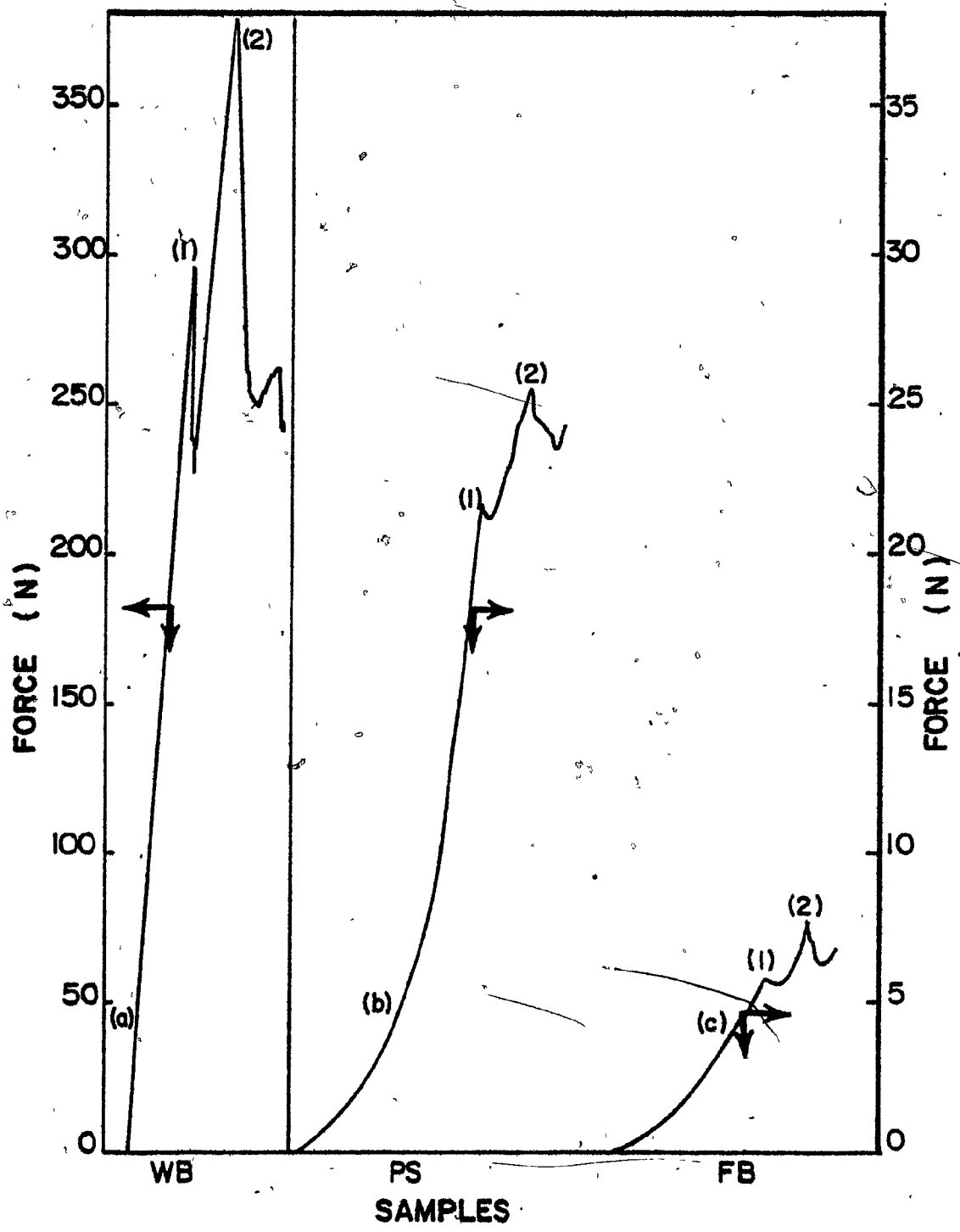
(b) The load sensing and recovery system, which consists of electric bonded-wire strain gauges whose output was fed to a strip chart recorder. The punch probe (1.6 mm diameter), attached to the moving crosshead, was driven into the surface of the sample at constant speed (10 mm/min). The force applied throughout the penetration period was recorded on the strip chart through the load sensing and recovery system. The peak force (i.e. maximum force recorded on the strip chart) and the bioyield (force at the point at which the probe just penetrated the skin or outer layer of the sample being tested) were determined (See Fig. 13).

Fig. 13: Force (Newton) Required for (a) Whole Bean, (b) Processed Substrate, and (c) Fermented Bean.

(1) Bioyield

(2) Peak Force

Figure 13


350
300
250
200
150
100
50
0
35
30
25
20
15
10
5
0
FORCE (N)
FORCE (N)
(1)
(2)
(a)
(b)
(c)
WB
PS
FB
SAMPLES

Materials and test parameters were:

| | | |
|---|---|---|
| Instrument | : | Instron Universal Machine |
| Test | : | Punch (1.6 mm diameter) |
| Sample | : | Beans (or as specified) |
| Fixture | : | Punch probe |
| Machine drive speed | : | 10 mm/min |
| Chart speed | : | 100 mm/min |
| Force range | : | 50 Newton full scale (or as specified) |

The instron used is shown in Fig. 14.

3.11.3.1. Methods: Texture: The Instron machine was used as described under section 3.11.3. Samples tested were raw beans (RB), whole beans (WB), processed substrate (PS) and fermented beans (FB). Twenty representative units of each sample were tested.

3.11.3.2. Methods: Time Sequenced Texture: The samples used were processed substrate (PS) and radappertized beans (RADB). These were incubated at 37°C. At Ø, 12, 24, 36, 48, 60 and 72 h after incubation, 20 representative units of each sample were tested as described under section 3.11.3.

3.11.4.1. Observations: Texture: In table 13 are given the results of the objective texture measurements of RB, WB, PS and FB. In Fig. 13 are shown some examples of force - deformation curves obtained from the punch test for WB, PS and FB.

3.11.4.2. Observations: Time Sequenced Texture: The time sequenced bioyield ($\bar{BY}$) and peak force ($\bar{PF}$) of the processed substrate and radappertized

Fig. 14: Instron Universal Testing Machine (Table Model) with Recorder.

Figure 14



Table 13. Texture by Instron.

| Sample | Peak Force and Bioyield (Newton)* | |
|---|---|---|
| | Peak Force ($\bar{PF}$) | Bioyield ($\bar{BY}$) |
| Raw Beans (RB) | 749.2 ± 131.1 | 571.3 ± 62.5 |
| Whole Beans (WB) | 350.0 ± 67.7 | 245.7 ± 44.1 |
| Processed Substrate (PS) | 26.0 ± 2.0 | 20.6 ± 1.0 |
| Fermented Beans (FB) | 7.4 ± 3.0 | 5.9 ± 2.0 |

* Mean of 20 determinations ± standard deviation.

beans are shown in Fig. 15. Detailed data for these are provided in appendix A, Table A9.

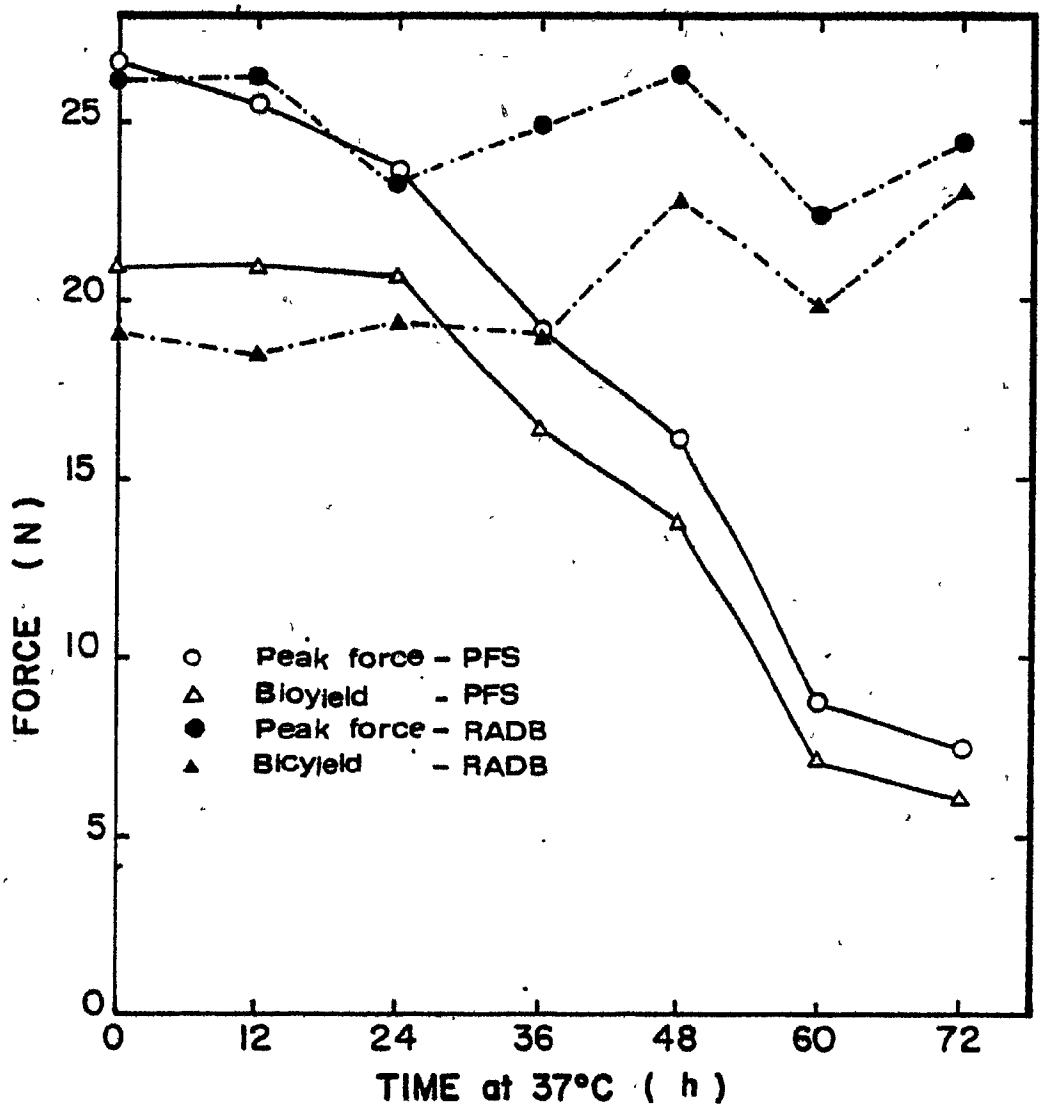
### 3.11.5. Discussion - Texture

The RB had the greatest bioyield and peak force (Table 13) indicating that this material is the hardest sample tested. The removal of the pulp caused a reduction in the bioyield and peak force. This may have been due to water absorbed by WB during pulp removal. Processing of WB to obtain PS resulted in a very large decrease in both the peak force and bioyield. Rockland and Jones (1974), Sefa-Dedeh et al. (1978) and Varriano-Marston and Omana (1979) have reported that heating non-oilseed legumes at 100°C resulted in the separation of intact cells, the separation being due to the breakdown of the middle lamellae. However, this characteristic was not found in the soybean (an oilseed legume) microstructure after boiling (Sefa-Dedeh and Stanley, 1979). The large amount of water being absorbed by the PS during the long boiling as well as the removal of the testa may have been the major reasons for its relative weakness or softness when compared to RB and WB.

Fermentation further decreased the peak force and bioyield (Table 13, Fig. 13). This may have been due to microbial activities such as the production of hydrolytic enzymes (carbohydrases and proteases) which hydrolyzed a portion of the PS and further weakened the bean tissues. Therefore, both the bioyield and peak force were much reduced in the FB when compared to the other samples.

Fig. 15: Time Sequenced Peak Force and Bioyield of Processed Fermenting Substrate (PFS) and Radappertized Beans (RADB).

Figure 15


25
20
15
10
5
0
FORCE ( N )
0
12
24
36
48
60
72
TIME at 37°C ( h )
Peak force - PFS
Bioyield - PFS
Peak force - RADB
Bicyield - RADB

The time sequenced peak force and bioyield (Fig. 15) of the processed, fermenting substrate decreased progressively with time. This may also have been due to microbial activities. However, both parameters remained fairly constant in the radappertized beans. Pomeranz and Meloan (1978) have stated that 'carbohydrates govern the rheological properties of most foods of plant origin'. It is noted that the proportion of carbohydrate decreased in the PS and was further reduced in the FB as reported earlier (Tables 11a and 11b). These reductions may have contributed to the structural weakening of both the PS and FB and therefore to the reductions in bioyield and peak force of these materials. High standard deviations were obtained with the Instron machine. This may have been due to variations within biological materials employed in this study. The results, however, agreed with observations of textural changes made subjectively. On a large production scale textural changes measured objectively, with the Instron machine, may be used to characterize Parkia fermentations.

## 3.12. DENSITY

The density of a material is defined as mass per unit volume (White, 1966):

$$\text{i.e.} \quad \rho = m/v$$

Zenz and Othmer (1960) have characterized 3 terms commonly applied to mass-volume relationships: 'True, Apparent and Bulk densities'. According to these authors the true density refers to the material making up the particle i.e. its mass per its true volume. If the material should contain minute fissures, cracks or pores, these interparticulate voids would reduce the density to an 'apparent density'. When a known mass of material is poured into a measuring cylinder to obtain its bulk volume; the mass per unit bulk volume computed is the bulk density. The true volume of a material may be determined from the difference between the bulk volume and volume of the interparticulate voids. The interparticulate voids volume may be determined by filling the voids in the bulk volume with a known volume of a liquid. It may also be found by fluid displacement as in the Archimedes principle.

In the depulping and dehulling of the raw beans (RB) and whole beans (WB) respectively, some of the beans floated on the water employed in both operations. When these seeds were examined they were found to be biodeteriorated or malformed. The implications of these are:

(i) quality of beans may be verified by their densities, and

(ii) the density parameter may be used for the grading of Parkia beans.

### 3.12.1. Objectives - Density

The objectives were:

(1) To determine the bulk and the particle (true) densities of whole beans (WB).

(2) To determine whether there is a correlation between the mass and the bulk volume of whole beans.

### 3.12.2. Principle - Density

The mass of a material and its volume (i.e. bulk volume and true volume) are determined at a known temperature and pressure. The quotient of mass per unit bulk volume gives the bulk density. The particle density of the material is the mass per true volume or actual volume.

### 3.12.3. Materials and Methods - Density

Accurately weighed, healthy, whole beans (moisture content = 8.7%) were used. The bulk volume was determined by the bulk space occupied by the beans in air, at 101.3 kPa and 20 $\pm$ 2°C, using a graduated cylinder. The particle volume of the whole beans was determined from the amount of water displaced when the whole beans were fully immersed in water in the graduated cylinder at 101.3 kPa and 20 $\pm$ 2°C. Both the bulk densities and particle densities were computed as under section 3.12.2. above.

The relationship between the mass of whole beans and its bulk volume was determined by varying the mass and measuring the corresponding bulk volume with a graduated cylinder at 101.3 kPa and 20 ± 2°C.

### 3.12.4. Observations - Density

The bulk density and particle density of WB are given in table 14. In Fig. 16 is shown the relationship between mass and bulk volume of WB. The data for these are provided in appendix A, Table A10.

### 3.12.5. Discussion - Density

The bulk density of whole beans was found to be 780 kg/m$^3$ (Table 14). The corresponding particle density was 1235 kg/m$^3$. The bulk density could be used for the quality grading of whole beans. Since the particle density of healthy whole beans is greater than that of water, it may be used to separate biodeteriorated or malformed beans from healthy ones. Seed density has been reported to correlate with oil content of flax seeds (Zimmerman, 1962). The use of particle density in this manner for Parkia seeds could not be ruled out, though this was not determined during this study.

The whole beans mass correlated very well with the mean bulk volume at 20 ± 2°C and 101.3 kPa (Fig. 16). The regression analysis gave 99.9%

correlation coefficient with a regression equation:

$$y = 0.77x + 0.28$$

where $y$ = whole beans mass (g)

$x$ = corresponding mean bulk volume (ml)

The equation can be used to determine the approximate weight of whole beans on a large scale if the bulk volume is known. The high correlation coefficient found is an indication of possibly good geometrical features of healthy whole beans. A sample with highly varied dimensions would give a poor correlation coefficient.

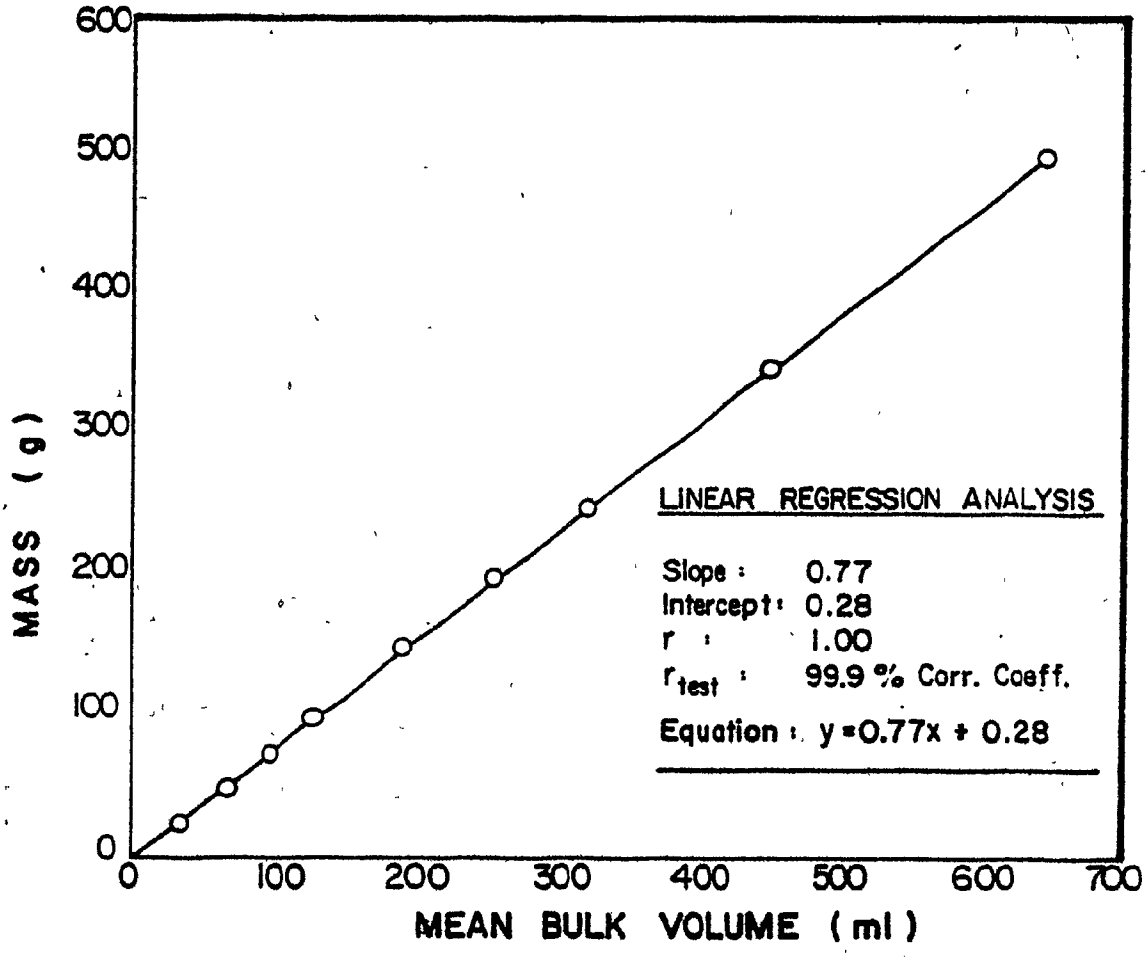
Table 14. Bulk and Particle Densities of African Locust Beans (Whole Beans) at Temperature 20 ± 2°C and Pressure 13 kPa.

| Replicate No. | Mass (kg) | Bulk Volume (ml) | Volume of Water Displaced (ml) | Density ($kg/m^3$) | | Specific* Gravity |
|---|---|---|---|---|---|---|
| | | | | Bulk | Particle | |
| 1 | 1.0 | 1260 | 820 | 794.0 | 1220.0 | 1.2 |
| 2 | 1.0 | 1280 | 820 | 781.0 | 1220.0 | 1.2 |
| 3 | 1.0 | 1300 | 820 | 769.0 | 1220.0 | 1.2 |
| 4 | 1.0 | 1300 | 800 | 769.0 | 1250.0 | 1.3 |
| 5 | 1.0 | 1280 | 800 | 781.0 | 1250.0 | 1.3 |
| 6 | 1.0 | 1280 | 820 | 781.0 | 1220.0 | 1.2 |
| 7 | 1.0 | 1260 | 820 | 794.0 | 1220.0 | 1.2 |
| 8 | 1.0 | 1280 | 800 | 781.0 | 1250.0 | 1.3 |
| 9 | 1.0 | 1300 | 800 | 769.0 | 1250.0 | 1.3 |
| 10 | 1.0 | 1280 | 800 | 781.0 | 1250.0 | 1.3 |
| $\bar{X}$ ± S.D. | - | - | - | 780 ± 9.0 | 1235.0 ± 16.0 | 1.3 ± 0.1 |

* Values computed from particle density.

Fig. 16: Mass of Whole Beans (g) Versus Mean Bulk Volume (ml) of Whole Beans at 20°C and 101.3 kPa.

Figure 16


600
500
400
300
200
100
0
MASS (g)
0
100
200
300
400
500
600
700
MEAN BULK VOLUME (ml)
LINEAR REGRESSION ANALYSIS
Slope : 0.77
Intercept : 0.28
r : 1.00
$r_{test}$ : 99.9 % Corr. Coeff.
Equation : y = 0.77x + 0.28

## 3.13. MASS BALANCE

The mass balance or material balance is based upon the law of conservation of mass. The law of mass conservation states that whatever amount of material enters a system must also leave that system. In the writing of a mass balance two fundamental steps are involved: (i) Selection of the system, and (ii) the selection of the basis (Charm, 1978). The selection of the system involves the arbitrary location of a boundary while the basis is usually expressed in terms of weight of some material or component entering or leaving the system. The general equations employed in a mass balance according to Charm (1978) are:

Mass entering system = Mass leaving system

and

Mass of component entering the system = Mass of component leaving the system.

Fermentation involves materials entering and leaving a system. During the process, it is usual to have a gradual loss in mass from the system. The reduction in mass, in fermentation, is frequently due to carbon dioxide, water and other gaseous compounds being liberated. This loss may range between 5 to 10% (Whitaker, 1978).

### 3.13.1. Objectives - Mass Balance

The objectives of the mass or material balance experiment were:

(1) To determine the amount of bioconverted product that could be obtained from a known weight of the raw beans (RB).

(2) To determine the weight loss from the processed fermenting substrate (PFS) and radappertized beans (RADB) incubated at 37°C on a time sequenced basis.

### 3.13.2. Principle - Mass Balance

Weights or mass of beans are determined from the starting raw beans through the processing stages to the completed fermented product. Mass balances are then calculated from the determined weights. Initial, intermediate and final weights are determined on a time sequenced basis for PFS and RADB.

### 3.13.3. Materials and Methods - Mass Balance

The materials used included RB, WB, PS, PFS, FB and RADB. An electronic Mettler PL 1200 balance (Mettler Instrument, Switzerland) was employed for weighing. The methods employed to obtain the WB, PS and RADB were as previously described. The process of fermentation from raw beans (RB) to the fermented beans (FB) is presented in flowchart form in Fig. 17 below. The weights of the solid materials lost during the processing in the form of pulp, testa, biodeteriorated sample and non-processed beans were determined after these had been air dried to constant weight.

3.13.3.1. Methods: Process Mass Balance: One kilogram of raw beans (RB) was treated as in Fig. 17. All solids entering and leaving the system were weighed.

3.13.3.2. Methods: Weight Losses: The weight losses of PFS and RADB were determined on a time sequenced basis. The sample was weighed before, during and after incubation at 37°C for a specified period. Weight losses were determined from weight differences.

3.13.4.1. Observations: Process Mass Balance: The mass balance data are presented in flowchart form in Fig. 18. This figure constitutes the process mass balance on a wet basis.

3.13.4.2. Observations: Weight Losses: In Fig. 19 are shown the weight loss curves for processed fermenting substrate (PFS) and radappertized beans (RADB). The detailed data for these are reported in appendix A, Table A11.

### 3.13.5. Discussion - Mass Balance

In Fig. 17 the flowchart of the African locust bean fermentation was shown. The quantities of materials involved, on a wet weight basis, were presented in Fig. 18. The major losses of material during the process were due to pulp removal, biodeteriorated sample, dissolved solids, non-processed beans and testa removal. On a wet weight basis (Fig. 18)

Fig. 17: Flowchart of African Locust Bean Fermentation.

BIOD: Biodeteriorated Sample

DS : Dissolved Solids

NPB : Non-processed Beans

Figure 17

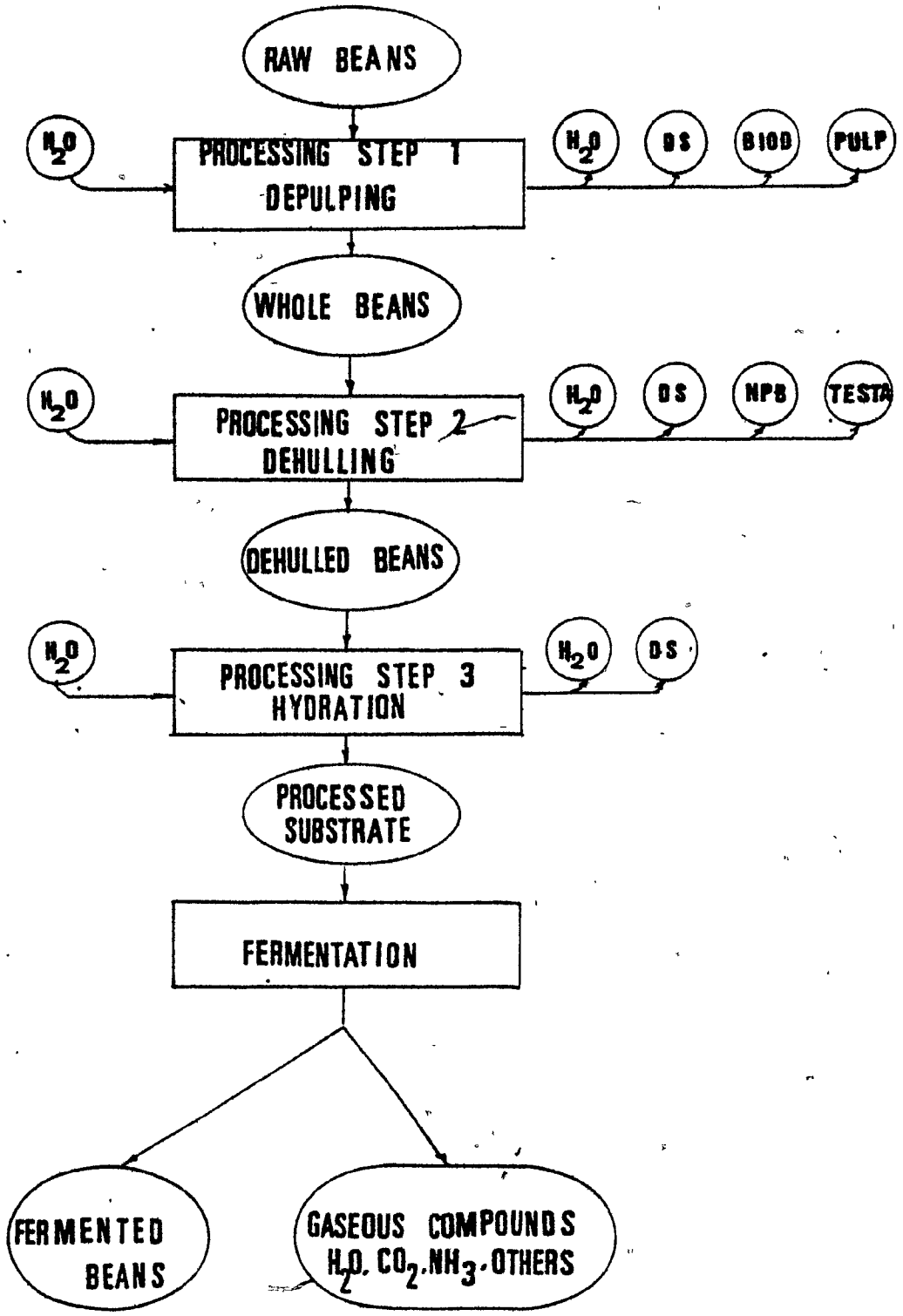

RAW BEANS
$H_2O$
PROCESSING STEP 1
DEPULPING
$H_2O$
DS
BIOD
PULP
WHOLE BEANS
$H_2O$
PROCESSING STEP 2
DEHULLING
$H_2O$
DS
NPB
TESTA
DEHULLED BEANS
$H_2O$
PROCESSING STEP 3
HYDRATION
$H_2O$
DS
PROCESSED SUBSTRATE
FERMENTATION
FERMENTED BEANS
GASEOUS COMPOUNDS
$H_2O$. $CO_2$. $NH_3$. OTHERS

Fig. 18: African Locust Bean Fermentation Mass Balance (Wet Weight Basis).

BIOD: Biodeteriorated Sample

DS : Dissolved Solids in Water Effluent

* : Not Determined Quantitatively

( ): Values in grams. Average of 9 determinations.

Figure 18

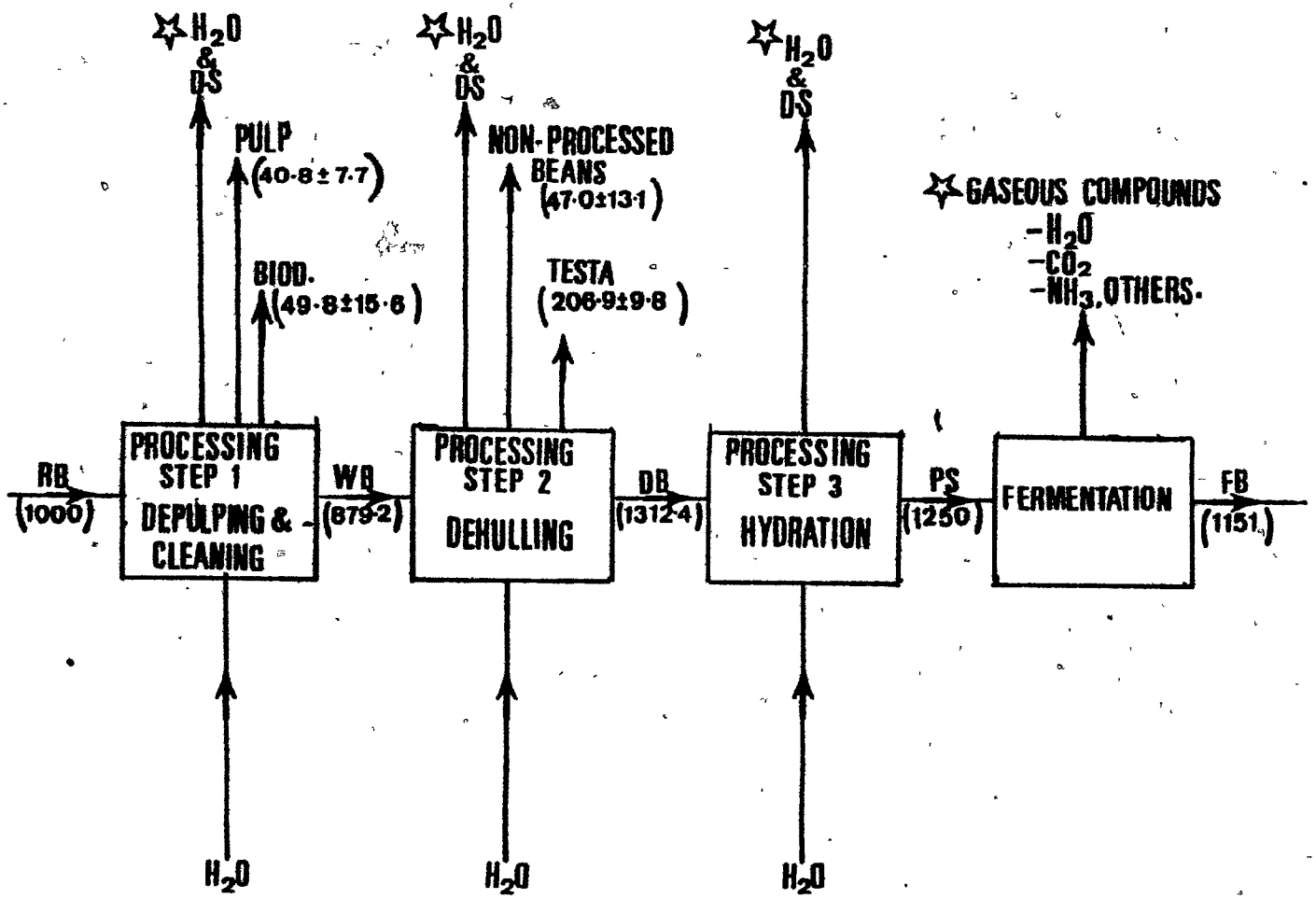
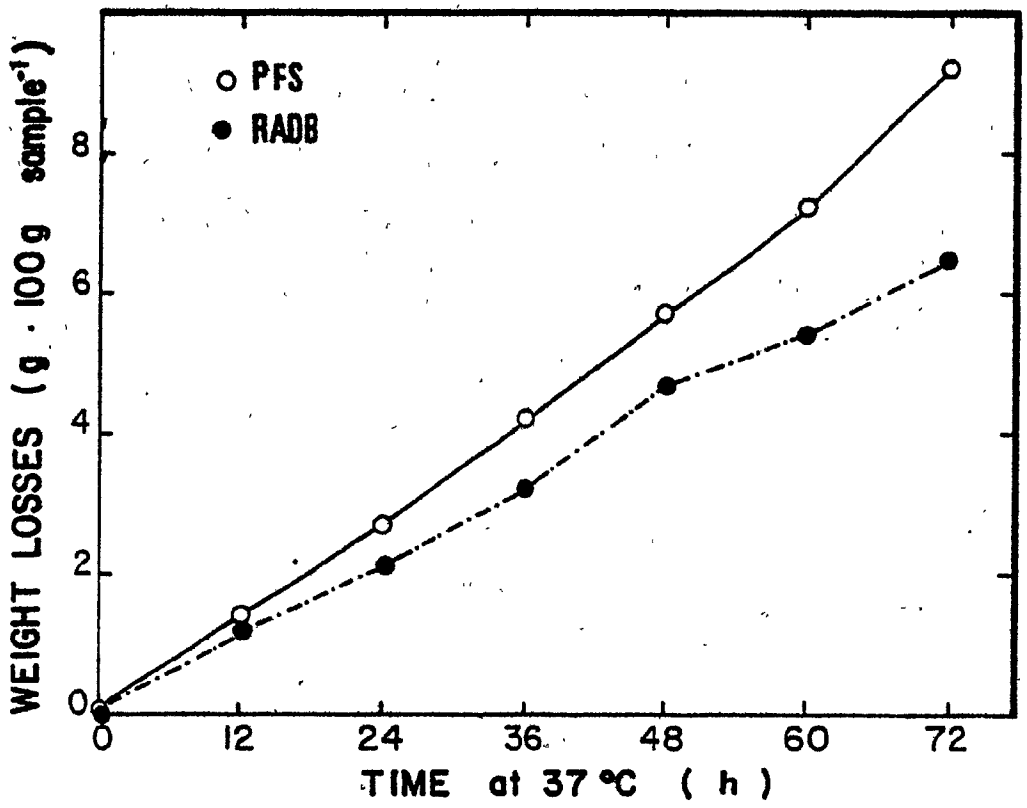

$H_2O$ & DS
PULP (40·8 ± 7·7)
BIOD. (49·8±15·6)
$H_2O$ & DS
NON-PROCESSED BEANS (47·0±13·1)
TESTA (206·9±9·8)
$H_2O$ & DS
GASEOUS COMPOUNDS
$-H_2O$
$-CO_2$
$-NH_3$, OTHERS.
RB (1000)
PROCESSING STEP 1 DEPULPING & CLEANING
WB (879·2)
PROCESSING STEP 2 DEHULLING
DB (1312·4)
PROCESSING STEP 3 HYDRATION
PS (1250)
FERMENTATION
FB (1151)
$H_2O$
$H_2O$
$H_2O$

Fig. 19: Time Sequenced Weight Losses at 37°C of Processed Fermenting Substrate (PFS) and Radappertized Beans (RADB).

Each graphic point is a mean value of 7 determinations.

Figure 19


PFS
RADB
WEIGHT LOSSES ( g · 100 g sample⁻¹ )
0
2
4
6
8
0
12
24
36
48
60
72
TIME at 37 °C ( h )

about 1.2 kg of FB could be obtained from 1 kg of RB. In table 15 are presented the wet weights and calculated solids during processing and fermentation. On a dry weight basis, about 0.43 kg of FB solids could be obtained from 1 kg of RB solids (Table 15). The loss in solids was due to processing and fermentation. The losses in solids from stage to stage are also presented in table 15.

The mass balances, on a dry weight basis, for fat, protein, ash and carbohydrate are presented in tables 16, 17, 18 and 19. The energy balance during fermentation is shown in table 20. The losses and gains of energy and of the various constituents during fermentation, are presented in table 21. Tables 16 through 21 were constructed from the results of weighings in this experiment in conjunction with earlier reported results of analyses in this chapter. As shown in table 21, during fermentation losses occurred in the dry mass, protein and carbohydrate while ash remained constant. These type of losses are usual for fermentation. They may be due to the activities of microorganisms. A gain in fat content was observed during fermentation. The source of the fat gained was not entirely clear.

It may have been due to overestimation of the FB fat content. This may happen if solids other than fat in the FB have been extracted with the actual fat. It is precisely because of this possibility of extracting other materials during the Soxhlet fat extraction, that the fat has been designated 'crude fat'. Another possibility is that it may be due to microbial synthesis of glycolipids which may be extracted with the fat. A calculated energy loss of 9% was obtained during fermentation. This

Table 15. African Locust Beans Mass Balance During Processing and Fermentation (Wet and Dry Weight Basis).

| Sample | Wet Weight (g) | % $H_2O$ (From Figure 6) | Calculated Solids (g) | % Loss of Solids† |
|---|---|---|---|---|
| Raw Beans (RB) | 1000.0 | 6.40 | 936.0 | |
| | | | | 14 |
| Whole Beans (WB) | 879.2 | 8.70 | 802.7 | |
| | | | | 30 |
| Dehulled Beans (DB) | 1312.4 | 57.00* | 564.33 | |
| | | | | 18 |
| Processed Substrate (PS) | 1250.3 | 63.00 | 462.61 | |
| | | | | 14 |
| Fermented Beans (FB) | 1151.4 | 65.30 | 399.54 | |

* Not previously reported.
Obtained as in Section 3.3.3.

† From stage to stage.

Table 16. Fat Mass Balance.

| Sample | Dry Weight (g) | % Fat (From Table 7) | Fat (g) | % Loss* |
|---|---|---|---|---|
| Raw Beans (RB) | 936.0 | 14.81 | 138.6 | |
| | | | | 11 |
| Whole Beans (WB) | 802.7 | 15.37 | 123.4 | |
| Dehulled Beans (DB) | 564.3 | - | - | 16 |
| Processed Substrate (PS) | 462.6 | 22.29 | 103.1 | |
| | | | | -36 (37.1 g) |
| Fermented Beans (FB) | 399.5 | 35.10 | 140.2 | |

* From stage to stage.

Table 17. Protein Mass Balance.

| Sample | Dry Weight (g) | % Protein (From Table 8) | Protein (g) | % Loss† |
|---|---|---|---|---|
| Raw Beans (RB) | 936.0 | 28.0* | 262 | |
| | | | | 12 |
| Whole Beans (WB) | 802.7 | 28.8 | 231 | |
| | | | | 23 |
| Dehulled Beans (DB) | 564.3 | - | - | |
| | | | | 12 (21 g) |
| Processed Substrate (PS) | 462.6 | 38.5 | 178 | |
| Fermented Beans (FB) | 399.5 | 39.4 | 157 | |

* Not previously reported.
Obtained as in Section 3.5.3.1.

† From stage to stage.

Table 18. Ash Mass Balance.

| Sample | Dry Weight (g) | % Ash (From Table 10) | Ash (g) | % Loss* |
|---|---|---|---|---|
| Raw Beans (RB) | 936.0 | 5.8 | 54.3 | |
| | | | | 28 |
| Whole Beans (WB) | 802.7 | 4.9 | 39.3 | |
| Dehulled Beans (DB) | 564.3 | - | - | 29 |
| Processed Substrate (PS) | 462.6 | 6.0 | 27.8 | |
| | | | | -4 |
| Fermented Beans (FB) | 399.5 | 7.0 | 28.0 | |

* From stage to stage.

Table 19. Carbohydrate Mass Balance.

| Sample | Dry Weight (g) | % Carbohydrate (From Table 11b) | Carbohydrate (g) | % Loss* |
|---|---|---|---|---|
| Raw Beans (RB) | 936.0 | - | - | |
| Whole Beans (WB) | 802.7 | 50.9 | 408.6 | |
| Dehulled Beans (DB) | 564.3 | - | - | 62 |
| Processed Substrate (PS) | 462.6 | 33.2 | 153.6 | |
| Fermented Beans (FB) | 399.5 | 18.5 | 73.91 | 52 (79.7 g) |

* From stage to stage.

Table 20. Energy Balance*.

| Sample | Dry Weight (g) | Energy Content kJ/g From Fig. 12 | Gross Energy kJ | % Loss* |
|---|---|---|---|---|
| Processed Substrate (PS) | 462.6 | 25.0 | $1.16 \times 10^4$ | |
| | | | | 9.0 |
| Fermented Beans (FB) | 399.5 | 26.2 | $1.05 \times 10^4$ | |

* During fermentation.

Table 21. Loss and Gain during Fermentation for Beans derived from the Original 1000 g Raw Beans.

| Constituent Loss or Gain | Quantity |
|---|---|
| Dry mass loss | 63 g |
| Protein loss | 21 g |
| Carbohydrate loss | 80 g |
| Ash loss | Ø g |
| Fat Gain | 37 g |
| Energy loss | 9 % |

may be due to microbial utilization and loss of gaseous products liberated during the process. The overall mass and component balance for the African locust bean fermentation is presented in Fig. 20. The values reported in Fig. 20 may serve as a guide for the future scale up of the process.

During the bioconversion of the processed substrate (PS) to the fermented beans (FB) weight losses were recorded for the processed fermenting substrate (PFS). The loss increased with fermentation time at 37°C (Fig. 19). Losses, though smaller, were also recorded for the RADB sample. This is an indication that the loss suffered was not entirely due to microbial activities. At 72 h a loss of 6.5% was recorded for RADB, which may have been due to moisture exchange with the environment. The actual loss in weight which may be due to microorganisms was about 3% i.e. 3 g/100 g of PS (Fig. 19).

Fig. 20: Mass and Components Balance of the African Locust Bean Fermentation (All Quantities in g).

A: Ash

C: Carbohydrate

F: Fat

M: Moisture

P: Protein

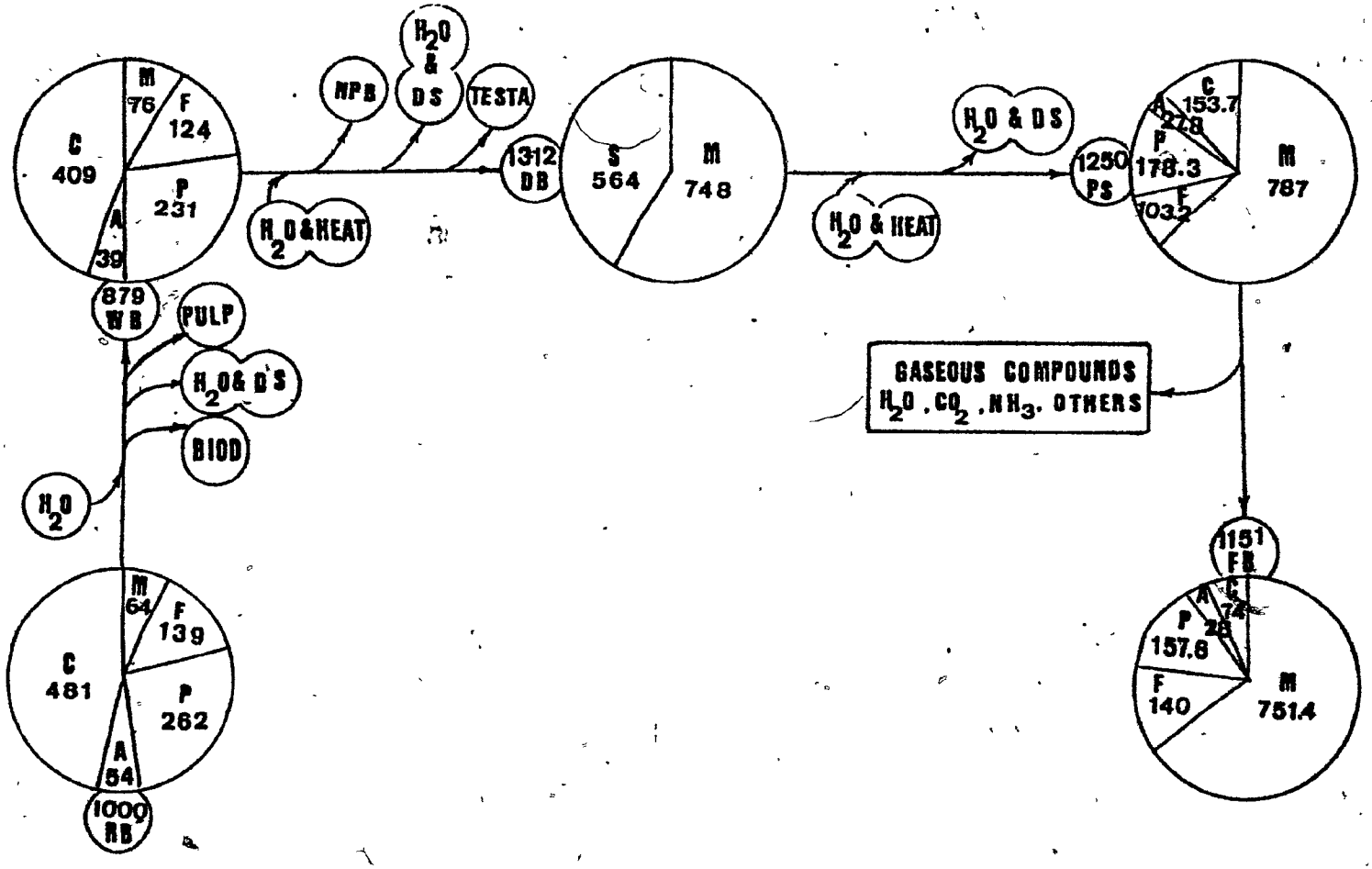
NPB: Non-processed Beans

S: Solids

DS: Dissolved Solids

BIOD: Biodeteriorated Samples

Figure 20


M
76
F
124
C
409
P
231
A
39
879
WB
PULP
$H_2O$& D S
BIOD
$H_2O$
M
64
F
139
C
481
P
262
A
54
1000
RB
$H_2O$&HEAT
NPB
$H_2O$
&
DS
TESTA
1312
DB
S
564
M
748
$H_2O$ & HEAT
$H_2O$ & DS
1250
PS
C
153.7
A
27.8
P
178.3
F
103.2
M
787
GASEOUS COMPOUNDS
$H_2O$ . $CO_2$ . $NH_3$. OTHERS
1151
FB
C
74
A
28
P
157.6
F
140
M
751.4

# CHAPTER 4

# MICROBIOLOGICAL ASPECTS OF THE FERMENTATION

# 4. MICROBIOLOGICAL ASPECTS OF THE FERMENTATION

In the process of bioconversion, which fermentation is, microorganisms play a central role. Their growth, and conditions affecting it, is central to the success of the operation. This chapter contains the results of experimental investigations on the microbiological aspects of the solid substrate fermentation of the African locust bean (Parkia filicoidea Welw.). The raw beans (RB), processed substrate (PS), fermented beans (FB), radappertized beans (RADB) and sterilized beans (SB) were all studied. A hypothesis is proposed for the process of bioconversion. The direct involvement of microorganisms in the fermentation was experimentally studied. The effects of temperature, sodium chloride, oxygen and water activity on the fermentation were also studied. The pH and titratable acidity were determined as indices of fermentation. Each experiment is reported as a complete unit made up of: Objectives, Principle, Methods, Observations and Discussion. This approach is followed throughout this chapter except where no experimental detail is needed.

## 4.1. GENERAL MATERIALS AND METHODS

This section and its subsections deal with materials and methods common to several of the other sections below. Reference shall be made to this section and its subsections as needed.

### 4.1.1. Source of Substrate for Microbiological Studies

The source of the substrate was as described under section 3.1. The sample of raw beans (RB) to be used for the microbiological counts was aseptically obtained in order not to introduce extraneous microflora into the RB. About 1 kg representative sample of the RB was aseptically transferred with a sterilized spoon from the lot into sterilized, wide mouthed, mason jars upon receipt of the RB. The mason jars containing the RB were covered with sterilized covers. A representative amount of this sample was used for microbiological counts. The remaining RB inside the jar was placed in a polyethylene bag which had been rinsed with 70% alcohol and shaken dry. The polyethylene bag with the sample was kept at 20 $\pm$ 2°C. Samples were obtained from this source when needed for the microbiological analysis of the RB.

### 4.1.2. Preparation of Processed Substrate and Fermented, Radappertized and Sterilized Beans

The preparation of processed substrate and its bioconversion to fermented beans were carried out as described under section 3.2. Strict personnel

working area cleanliness was maintained during processing. The bench was disinfected with lysol and containers used to hold the beans were thoroughly cleaned and rinsed with boiling water before use. Prior to dispensing, the beans were covered with sterilized aluminium foil. Sterilized dispensing spoons were used to transfer the beans into the fermentation units. The radappertized beans (RADB) were obtained as described under section 3.2. In this study two radiation units at two different locations, both supplying γ-ray source with cobalt-60, were used. The unit locations were: (1) Macdonald College (Pilot Plant), Ste. Anne de Bellevue, P.Q., Canada, and (2) Institut Armand-Frappier, Laval Des Rapides, P.Q., Canada. The two units are of identical make, i.e. Gamma Cell 220, Model GC 220 Type B (AECL). The second unit was used because of the higher radiation level in it at the time of this study. The sample to be irradiated was centrally located in the radiation unit draw tube. This ensured 100% irradiation of the sample (Idziak, 1980 - Personal communication). In appendix D, Fig. D1 is indicated the radiation strength at varying positions within the tube in the Macdonald unit. The duration for the required dosage was calculated from a table provided by Dr. Idziak of the Department of Microbiology, Macdonald College, P.Q., Canada (Appendix D, Table D1) for the Macdonald College Pilot Plant unit. A standard curve was provided at Armand-Frappier (Appendix D, Fig. D2). At the time of the experiment it was 368.85 x $10^3$ rads $h^{-1}$ ( 3.7 kGy $h^{-1}$). The irradiated beans were employed as control or as otherwise described in the text. The samples were irradiated at 20.0 $\pm$ 2.0°C. The sterilized beans (SB) were obtained by autoclaving the processed substrate (PS) in petri-plates (100 mm x 15 mm) fermentation units at 121°C for 15 min.

### 4.1.3. Preparation of Sample Homogenate

The sample homogenate was prepared in order to release the microorganisms from the solid substrate into the fluid medium. This allowed for their isolation and enumeration. For this process a bottom driven single speed Waring commercial blender with metal blending jars each of 1 litre capacity having autoclavable covers was used. Weighings were carried out with a tared balance of capacity 2,500 g and sensitivity 0.1 g. All materials such as forceps, spoons and spatulas used in contact with the samples were wrapped with aluminium foil and autoclaved before use at 121°C for 15 min. These were dried at 100°C overnight and allowed to cool to room temperature before they were used. Pipettes of capacities 0.1 ml, 1 ml and 10 ml were sterilized in canisters dried at 100°C, cooled and then used. The diluent used for the sample homogenate was 0.1% peptone dispensed in dilution bottles with screw caps or rubber bungs and autoclaved at 121°C for 15 min. Bottles containing 180 ml diluent were used to prepare $10^{-1}$ dilution; the 90 ml and 99 ml blanks, also in bottles and sterilized, were used for progressive dilutions.

In the preparation of the homogenate an autoclaved blender jar was tared and 20.0 $\pm$ 0.1 g of representative sample was aseptically weighed into it. Peptone diluent, 180 ml, was added. The sample was blended for a total of 90 seconds in six steps, each lasting 15 seconds. A pause of 5 seconds was observed between steps. This procedure was used to cut down on possible heat development which might affect microbial recovery and counts and also to reduce chances of foaming. The $10^{-1}$ dilution was obtained in the above manner. One ml of the $10^{-1}$ dilution was

measured aseptically into a 99 ml dilution blank or 10 ml into a 90 ml blank. The inoculated dilution blank was then shaken vigorously 25 times in a 30 cm arc (Thatcher and Clark, 1975). The process was repeated to progressively prepare dilutions of $10^{-2}$, $10^{-3}$, $10^{-4}$, etc. or as required. The homogenate dilutions were used to isolate and count the viable microorganisms in the sample of interest.

### 4.1.4. Enumeration of Microorganisms in Samples

Depending on the experiment, as shall be stated in the test, one or the other of three methods were used to determine the viable counts of microorganisms present in the homogenate. These were: (1) Pour Plate or Standard Plate Count Method (SPC), (2) Surface Plate or Surface Spread Method (SPM), and (3) Drop Plate Method (DPM). In all cases plates were incubated at selected temperatures. The incubator temperature was monitored during the experiment with a mercury in glass thermometer installed in a beaker of water. A colony counter and tally register were used to count the colonies on the agar medium.

For the SPC procedure 1 ml aliquots from suitable dilutions ($10^{-1}$ - $10^{-7}$) were aseptically added to duplicate sets of sterile petri dishes. About 10-15 ml melted and tempered (45°C) agar was then added. The inoculum and agar were aseptically mixed and allowed to solidify at room temperature (20 $\pm$ 2°C). A sterility check was provided as control with only medium in the petri dish. The inoculated plates and sterility check plates were incubated at 37 $\pm$ 2°C or as specified in the text

for 24 to 72 h. Plates having 30-300 colonies were counted and colony forming units per gram sample (CFU/g) was computed from the counts.

In the SPM procedure pre-poured, solidified and dry surfaced media plates were employed. The prepared media were each inoculated with 0.1 ml aliquots of selected dilutions. The inoculum was spread on the agar surface by a glass spreader sterilized by alternative flaming and cooling in 70% alcohol. A separate spreader was used for each plate. Duplicate plates were prepared for each dilution. A sterility check was provided with two uninoculated plates. All plates were incubated at 37 $\pm$ 2°C or as otherwise stated in the text for 24-72 h. Plates containing 30-300 colonies were counted and the CFU/g was computed for each sample tested.

With the DPM procedure a 25 µl Eppendorf Pipette (Eppendorf, Germany) and sterilized fitting pipette tips were used. The outside dish bottom of poured plates were each divided into 6 segments with a felt pen. Each segment was marked with the dilution of the corresponding inoculum to be used for that segment and then aseptically inoculated with 25 µl of the sample. Plates were allowed to dry at room temperature and then incubated at 37 $\pm$ 2°C or as otherwise specified for 24-72 h. All segments with 20 or less colonies were selected for counts. The viable number of microorganisms present per gram (CFU/g) was then calculated.

## 4.2. PRELIMINARY MICROBIOLOGY

The general viable counts on a food sample are usually determined by counting colonies that develop after plating and incubation. The number of microorganisms counted is generally affected by a variety of nutritional and physical requirements of the diverse population usually present on foods. For foods which have been adequately studied, standard microbiological methods exist and these can be used by investigators. However, for new foods, it is important that preliminary microbiological tests be carried out on such aspects as the diluent to be used, temperature of incubation, etc. After the preliminary tests, suitable values for these factors are selected on the basis of conditions which give the highest viable counts.

### 4.2.1. Objectives - Preliminary Microbiology

The objectives of this experiment were to test various diluents and isolation methods and to select a temperature for the fermentation and determine the best enclosure system for the solid substrate fermentation (SSF).

### 4.2.2. Principle - Preliminary Microbiology

Comparisons are made among different diluents, methods of isolation, several incubation temperatures and different enclosure systems on the

basis of viable counts obtained on defined media.

### 4.2.3. Materials and Methods - Preliminary Microbiology

The microbiological procedures employed for viable counts were as described under sections 4.1.3. and 4.1.4. above. For the comparisons of diluents, isolation methods and incubation temperatures, the sample used consisted of 72 h fermented beans. The diluents tested were: distilled tap water, physiological saline (0.85% NaCl - prepared by the addition of 1 litre distilled water to 8.5 g sodium chloride) and 0.1% peptone (1.0 g dissolved in 1 litre distilled water). All diluents were dispensed into labelled dilution bottles with either screw caps or rubber bung corks and then sterilized at 15 psi for 15 min. Homogenate of the 72 h fermented beans and subsequent dilutions were prepared using each of the selected diluents in turn. Inoculation of Plate Count Agar (PCA, Difco) was carried out using both pour plate and surface spread methods as described above. Sufficient plates were prepared from each selected dilution so that different temperatures could be tested for incubation. The enclosure systems were tested in the following manner: Processed substrate (PS) was incubated in -

(a) Sterile petri dish without filter papers,

(b) with PS sandwiched between sterilized filter papers in sterile petri dish, and

(c) as in (b) after addition of 2% (W/W) sodium chloride to the PS.

All the fermentation units (a), (b) and (c) were incubated at 37 ± 2°C for 72 h. Homogenate and dilutions were prepared from each unit type and counts obtained on defined media. The media used were de Man Rogosa Sharp (MRS), Plate count agar (PCA) and Wort agar (WA). Media constituents are given in appendix E.

### 4.2.4. Observations - Preliminary Microbiology

In tables 22, 23 and 24 are presented the viable counts obtained by the use of different diluents, different isolation methods and temperatures of incubation of plates and varied fermentation system units respectively.

### 4.2.5. Discussion - Preliminary Microbiology

As judged by the viable counts obtained (Table 22) any of the three diluents studied could be used with success. However, 0.1% peptone was selected since slightly higher counts were obtained with this diluent. Harrigan and McCance (1976) have stated that for a food sample, it is important to test a range of diluents. They recommended the choice of the diluent which gave the highest recovery for subsequent use. It has been reported that some diluents may be toxic to microorganisms, especially if the time of contact between the homogenate and diluent is prolonged (Winslow and Brooke, 1927; Butterfield, 1932; Gunter, 1954 and King and Hurst, 1963). As a general purpose diluent 0.1% peptone water

Table 22. Viable Counts of Fermented Bean Homogenate Prepared with Different Diluents.

| Diluent | Viable Counts* | |
|---|---|---|
| | $CFU \cdot g^{-1}$ | $\log_{10} CFU \cdot g^{-1}$ |
| Tap Distilled Water | $1.0 \times 10^9$ | 9.0 |
| Peptone Water (0.1%) | $1.7 \times 10^9$ | 9.2 |
| Physiological Saline (0.85%) | $1.3 \times 10^9$ | 9.1 |

* Counts on Plate Count Agar (PCA).
Plates were incubated at 37 ± 2°C.
Average of 3 experiments; 2 plates per experiment.

NOTE: Sterility check plates as Control (2 plates)
- No viable count.

Table 23. Enumeration of Viable Microorganisms of Fermented Beans at Different Temperatures on PCA Using Different Isolation Methods.

| Isolation Method | CFU·g$^{-1}$ (x 10$^{-7}$)* | | | |
|---|---|---|---|---|
| | 25°C | 30°C | 37°C | 45°C |
| Drop Plate | 1.1 | 31 | 128 | 86 |
| Pour Plate | 3.0 | 52 | 150 | 140 |
| Spread Plate | 3.6 | 49 | 130 | 120 |

* Average counts of six plates; 3 experimental runs; 2 plates per experiment.

Diluent: 0.1% peptone water.

Table 24. Viable Counts of Beans Fermented Using Different Fermentation System Units.

| Unit Type | CFU·g$^{-1}$* MRS | PCA | WA |
|---|---|---|---|
| A | $1.5 \times 10^9$ | $2.0 \times 10^9$ | $1.0 \times 10^8$ |
| B | $8.9 \times 10^9$ | $9.1 \times 10^9$ | $4.0 \times 10^8$ |
| C | $6.0 \times 10^9$ | $6.7 \times 10^9$ | $3.0 \times 10^8$ |

* Average counts for six plates; 2 experiments; triplicate plates per experiment.

A = Unit without filter paper.

B = Unit with filter paper.

C = As in B + 2% NaCl (W/W).

(pH 6.8 - 7.0) appears however to have an adequate protective effect (Straka and Stokes, 1957). Since the homogenate and dilutions were used immediately (0-15 min) after preparation the question of toxicity due to extended contact between food sample and diluent may not be crucial.

The viable counts were highest for the plates incubated at 37°C (Table 23) for all the three isolation methods (drop plate, pour plate and spread plate). The 37°C was therefore selected and used except where stated differently in the text.

There was little difference between the pour plate and spread plate viable counts. The count for the drop plate was somewhat lower than for the other methods. In this case the choice of method was based on the type of experiment and this will be stated in the appropriate sections.

The type B unit (Table 24) was selected for subsequent experiments except where otherwise stated. This unit gave the highest counts. This may be due to a higher availability of moisture on the solid substrate. The type A unit may allow more rapid moisture exchange which could cause a reduction in counts.

## 4.3. MICROBIOLOGY OF RAW BEANS, PROCESSED SUBSTRATE AND FERMENTED BEANS (SAMPLES)

Whereas the African locust bean fermentation has been carried out in West Africa, possibly for centuries, no previous report on the microbiological aspects of the fermentation has to the author's knowledge appeared in the literature. This information gap prompted the interest in this study. The first report on the isolation of the microorganisms involved in the solid substrate fermentation of African locust beans was made very recently by Ikenebomeh, Kok and Blackwood (1981). It has been noted that there are many indigenous food fermentations all over the world of which scientific understanding (microbiological and otherwise) is still lacking (Hesseltine, 1965 and Steinkraus, 1981). Dawadawa, with respect to microbiological aspects, is one of these. Therefore, the study was undertaken.

### 4.3.1. Objectives - Microbiology of Samples

The objective of this experiment was to determine the total viable counts of raw beans (RB), processed substrate (PS) and fermented beans (FB).

### 4.3.2. Principle - Microbiology of Samples

The samples are each homogenized and serial dilutions are prepared from them. Viable counts are obtained after plating out on suitable media and incubation.

### 4.3.3. Materials and Methods - Microbiology of Samples

The samples employed were raw beans (RB), processed substrate (PS) and fermented beans (FB). Homogenates and subsequent dilutions of each of these were prepared and viable counts carried out as under sections 4.1.3. and 4.1.4. The pour plate method was used in the isolation process. The media used were MRS, PCA, potato dextrose agar (PDA), tomato juice agar (TJA) and WA. Detailed constituents of these media and their preparation are provided in appendix E. Incubation was carried out at selected temperatures and counts made after 18-72 h. The colony forming units per gram (CFU/g) of each sample was computed.

### 4.3.4. Observations - Microbiology of Samples

The microbiological counts of the raw beans (RB), processed substrate (PS) and fermented beans (FB) samples are shown, in order as listed, in Figs. 21, 22 and 23.

### 4.3.5. Discussion - Microbiology of Samples

The viable counts of the RB were shown in Fig. 21. An average count for all the media employed at 37°C was about $1 \times 10^5$ CFU/g for this material. There was a reduction in microbial population when the beans were processed (Fig. 22). The PS counts at 37°C gave a 5 log reduction in both MRS and

Fig. 21: Viable Counts: $\log_{10} CFU \cdot g^{-1}$ of Raw Beans on Different Media at Varied Temperatures of Incubation.

Figure 21

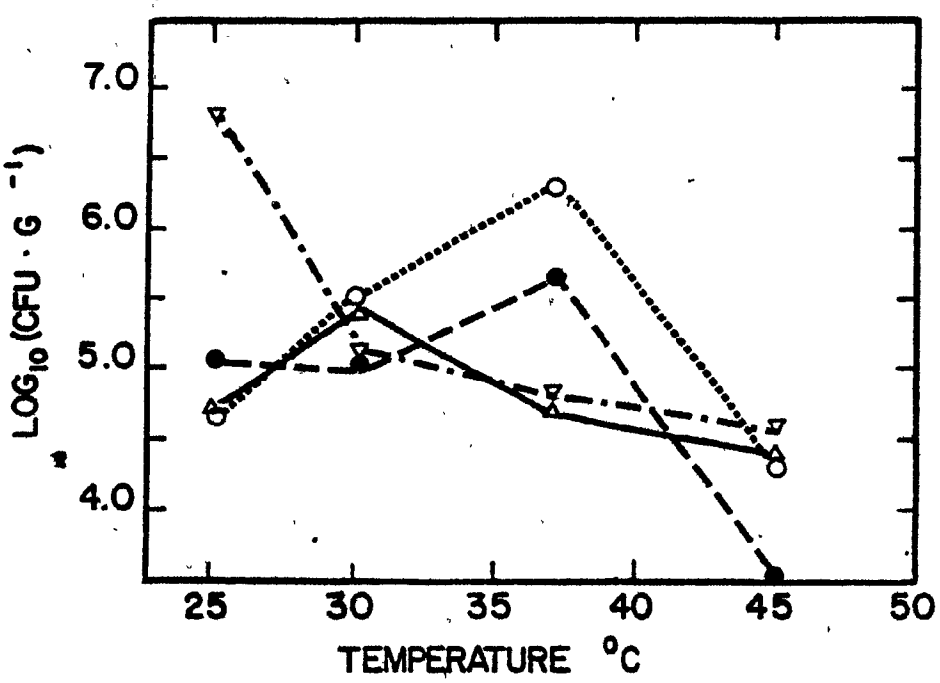



PLATE COUNT AGAR
LACTOBACILLI MRS AGAR
POTATO DEXTROSE AGAR
TOMATO JUICE AGAR

Fig. 22: Viable Counts: $\log_{10} CFU \cdot g^{-1}$ of Processed Substrate.

MRS: de Man Rogosa Sharpe medium

PCA: Plate Count agar

PDA: Potato Dextrose agar

TJA: Tomato Juice agar

WA : Wort agar

Figure 22

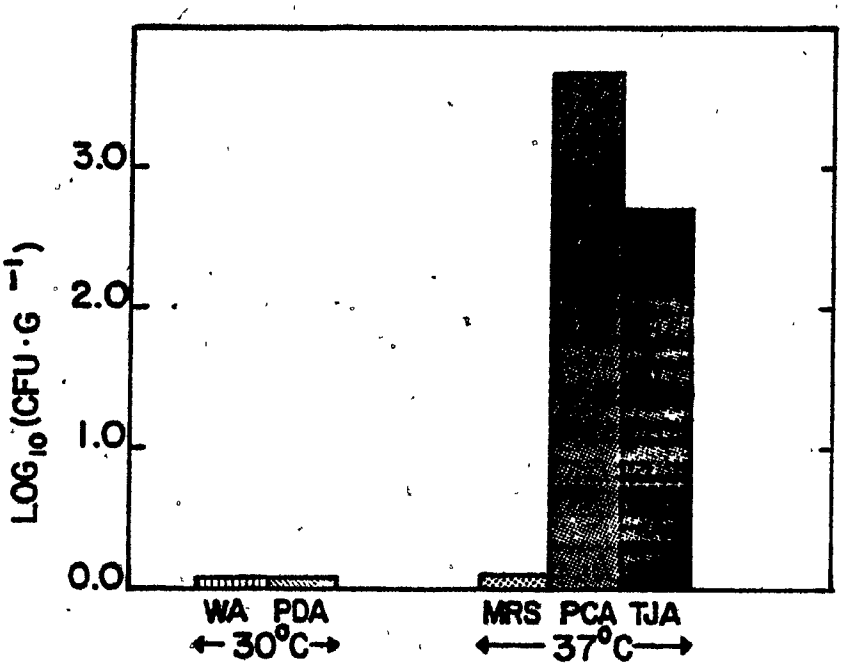

3.0
2.0
1.0
0.0
$LOG_{10}$ (CFU · G $^{-1}$)
WA PDA
← 30°C →
MRS PCA TJA
← 37°C →

Fig. 23: Viable Counts: $\log_{10}$CFU·g$^{-1}$ of Fermented Beans.

MRS: de Man Rogosa Sharpe medium

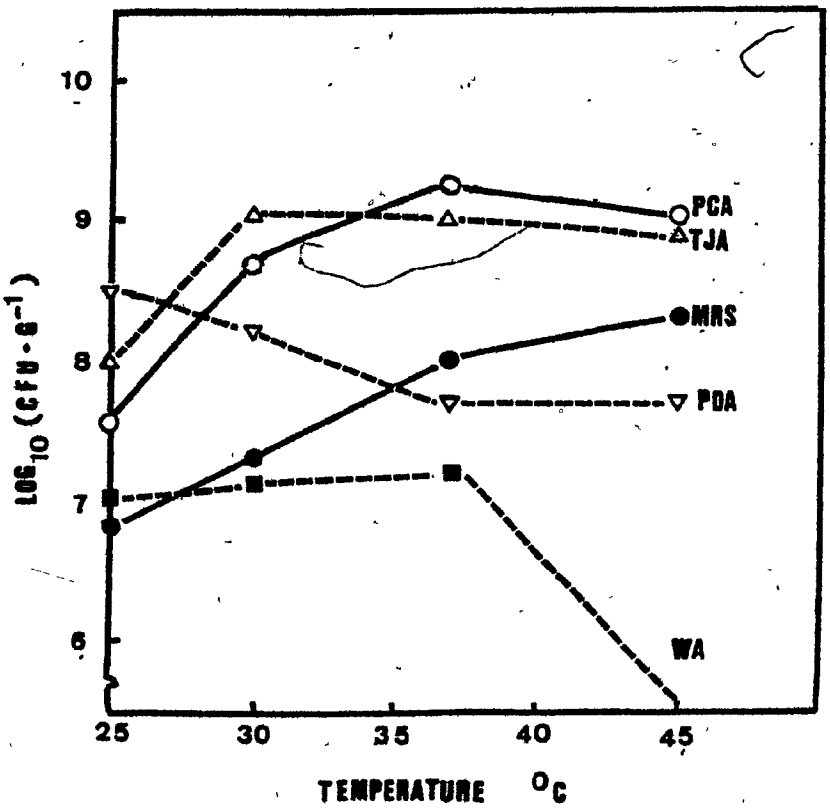
PCA: Plate Count agar

PDA: Potato Dextrose agar

TJA: Tomato Juice agar

WA : Wort agar

Figure 23


10
9
8
7
6
$LOG_{10}$ (CFU · g$^{-1}$)
PCA
TJA
MRS
PDA
WA
25
30
35
40
45
TEMPERATURE °C

PDA, a 4 log reduction for WA and 3 and 2 log reductions for PCA and TJA, respectively, as compared to values with the same media for the RB. The reductions may be due to the processing of the beans which involved heating on a hot plate for 8 h and further boiling after the testa had been removed. Processing of raw materials prior to fermentation has been reported to be an effective way of reducing microbial population prior to fermentation. The fermented beans gave high counts at 37°C for all the media employed i.e. above $1 \times 10^7$ CFU/g (Fig. 23). Since no additional nutrient was provided with the solid substrate, it is likely that the solid substrate per se supported microbial growth. Viewed against the reduced number of microorganisms recovered with the PS ($1 \times 10^4$) CFU/g and higher population of up to $1 \times 10^9$ CFU/g in the FB, it was concluded that strong interaction exists between the substrate (PS) and its microflora during incubation at 37°C.

## 4.4. MICROBIOLOGY OF PROCESSED FERMENTING SUBSTRATE (PFS), RADAPPERTIZED BEANS (RADB), AND STERILIZED BEANS (SB)

The microbiological study of RB, PS and FB (section 4.3.5.) above has shown that a large microbial population developed on the FB. The possible interaction between the PS and the microorganisms was inferred. The question then arose whether microorganisms present on or within the FB were responsible for bioconversion or not. Similarly, it could be asked whether it was possible to have bioconversion of the beans in the absence of these microorganisms. If the answer to the latter question was positive, then the role of microorganisms in dawadawa production could be deemed redundant. These lines of thoughts led to this investigation. There are some literature reports in which fermentation of locust beans has been assumed (no microbiological data). In this exercise scientific evidence of the role of microorganism was sought.

### 4.4.1. Objectives - Microbiology of PFS, RADB and SB

The objective was to carry out the microbiological examination of PFS, RADB and SB on a time sequenced basis at 37 $\pm$ 2$^{o}$C.

### 4.4.2. Principle - Microbiology of PFS, RADB and SB

Samples are incubated at the required temperature. At pre-determined time intervals homogenate and dilutions are prepared for each sample. Selected media are inoculated and counts are obtained.

4.4.3. Materials and Methods - Microbiology of PFS, RADB and SB

The samples employed were processed substrate which, when incubated at 37 ± 2°C was called processed fermenting substrate (PFS); others were RADB and SB. The methods of preparation for these were as described under section 4.1.2.

4.4.3.1. Methods: Microbiology of PFS and SB: Viable counts for the PFS and SB were obtained from their homogenate and dilutions made in 0.1% peptone water. The enumeration for each sample was carried out at Ø, 12, 24, 36, 48, 60 and 72 h after incubation at 37 ± 2°C. The pour plate method was used and the media employed were MRS, PCA, TJA and WA.

4.4.3.2. Methods: Microbiology of PFS and RADB: Viable counts were obtained for PFS and RADB as under section 4.3.3.1. Prior to the counts the amount of radiation dosage required to obtain radappertized beans was determined as detailed below. A Gamma Cell 220 Type B (AECL) with a cobalt-60 radiation source (Armand-Frappier unit, Laval) was used to irradiate the beans. From the standard curve provided at Institut Armand Frappier, Laval (Appendix D, Fig. D2 ) the time required for a pre-determined total amount of irradiation dosage on the sample was calculated. The dosages employed are provided in table 25. The samples were irradiated, then incubated at 37 ± 2°C for 72 h and counts obtained from them. The amount of radiation dosage required to achieve radappertization of the PS was determined and subsequently used to obtain RADB (1.1 Mrad ≡ 11 kGy).

Table 25. Total Exposure Time and Calculated Irradiation Dosage.

| Time (min) | Calculated Dosage* | |
|---|---|---|
| | Mrad | kGy |
| 16 | 0.1 | 1.0 |
| 33 | 0.2 | 2.0 |
| 49 | 0.3 | 3.0 |
| 65 | 0.4 | 4.0 |
| 82 | 0.5 | 5.0 |
| 98 | 0.6 | 6.0 |
| 114 | 0.7 | 7.0 |
| 130 | 0.8 | 8.0 |
| 147 | 0.9 | 9.0 |
| 163 | 1.0 | 10.0 |

* Radiation Source: Cobalt-60 (AECL).

4.4.4.1. Observations: Microbiology of PFS and SB: The microbiological counts on PFS and SB are shown in Fig. 24.

4.4.4.2. Observations: Microbiology of PFS and RADB: The effect of γ-irradiation dose levels on the microbial population are shown in Figs. 25 and 26. The results of viable counts of PFS and RADB are shown in Fig. 27. The overall result for PFS, RADB and SB are presented in Fig. 28.

4.4.5. Discussion - Microbiology of PFS, RADB and SB

In Fig. 24 are shown the curves obtained for PFS and SB samples. High viable counts were obtained with PFS sample on all the isolation media employed. However there was no microbial recovery with the SB sample on any of the media used. The PFS was observed, after 72 h incubation, to have been bioconverted to the FB. The SB, in contrast, was not and was found to retain the characteristic beany flavor of the PS. The significance of this is that autoclaved samples will not undergo spontaneous fermentation and that bioconverted beans had microorganisms associated with them. The ultimate agents of bioconversion are enzymes. Apart from the destruction of microbial life to obtain SB, autoclaving will also destroy any indigenous beans' enzymes present. Pederson (1979) has stated that although microbial activities and their enzymes are responsible for the major fermentation processes, yet, the activities of enzymes indigenous to the food cannot be entirely divorced from the process. To eliminate the uncertainty about the source of enzymes (microorganisms or indigenous

Fig. 24: Viable Counts: $\log_{10}CFU \cdot g^{-1}$ of Processed Fermenting Substrate (PFS) and Sterilized Beans (SB).

Note: No viable counts with SB. All curves are at zero. They are presented in this manner for clarity.

Figure 24

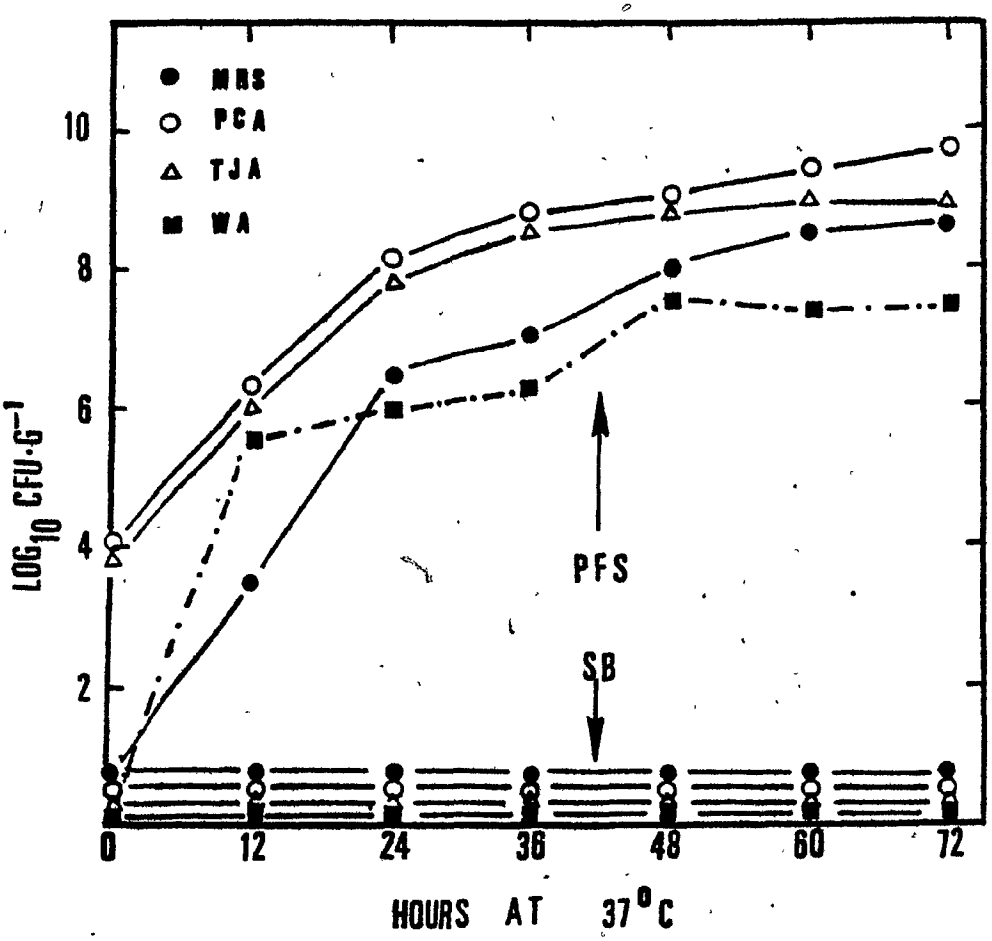

MRS
PCA
TJA
WA
10
8
6
4
2
0
12
24
36
48
60
72
$LOG_{10}$ CFU·G$^{-1}$
PFS
SB
HOURS AT 37°C

Fig. 25: Viable Counts: $\log_{10}$CFU·g$^{-1}$ at Various Dosage of γ-radiation after 72 h Incubation at 37°C on MRS and PCA.

MRS: de Man Rogosa Sharpe medium

PCA: Plate Count agar



Figure 25

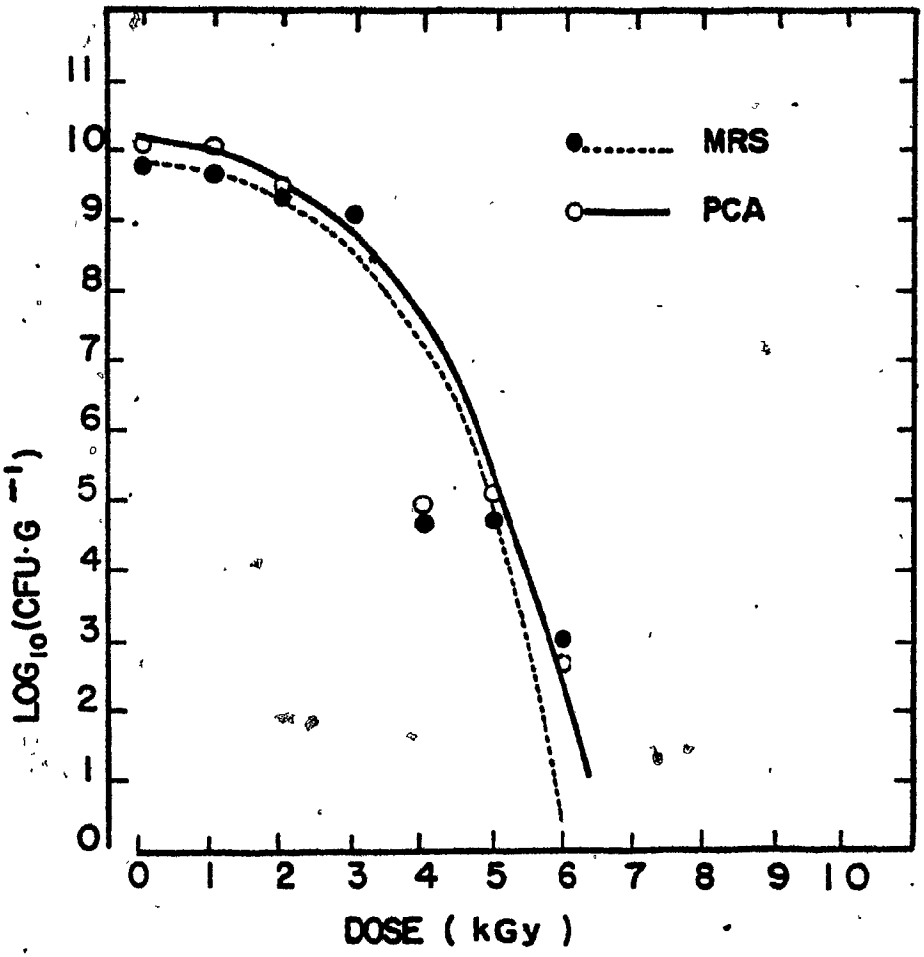

MRS
PCA
$LOG_{10}(CFU \cdot G^{-1})$
DOSE ( kGy )
0 1 2 3 4 5 6 7 8 9 10
0 1 2 3 4 5 6 7 8 9 10 11

Fig. 26: Viable Counts: $\log_{10}$CFU·g$^{-1}$
at Various Dosage of γ-radiation
after 72 h Incubation at 37°C
on TJA and WA.

TJA: Tomato Juice agar

WA : Wort agar

Figure 26

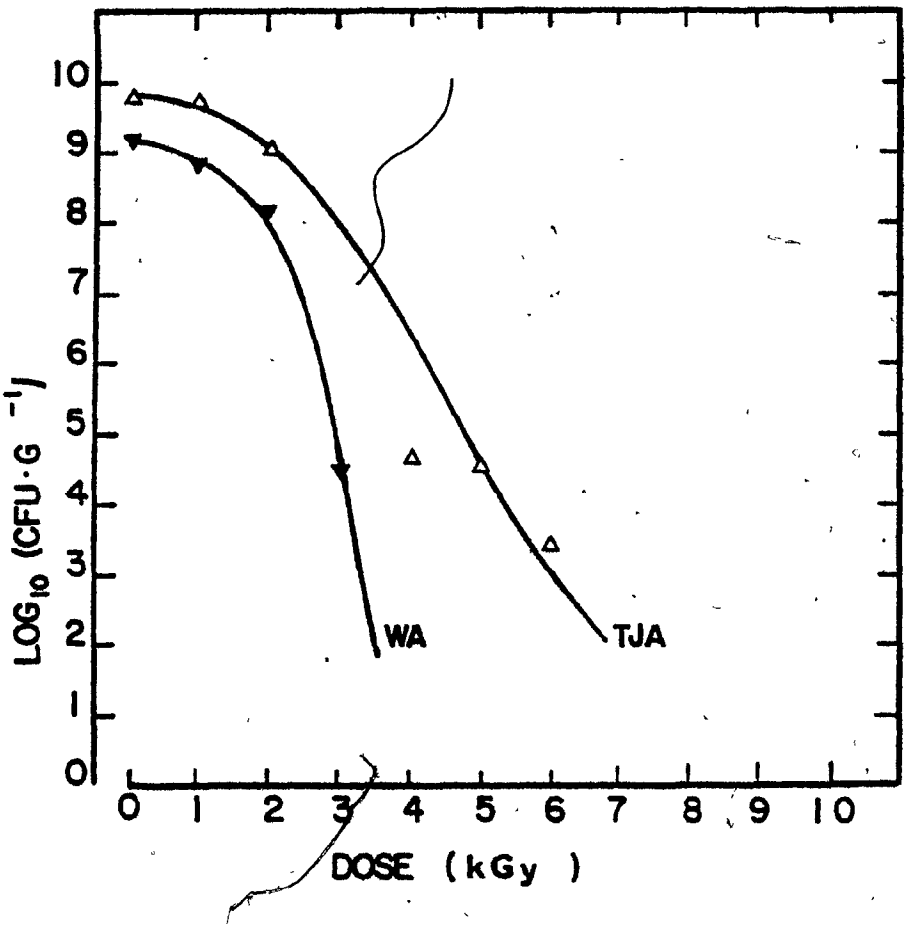

LOG10 (CFU·G -1)
0
1
2
3
4
5
6
7
8
9
10
DOSE (kGy)
WA
TJA

Fig. 27: Time Sequenced Viable Counts: $\log_{10}$CFU·g$^{-1}$ of Radappertized Beans (RADB) and Processed Fermenting Substrate (PFS).

Note: No viable counts from RADB. All curves are at zero. They are presented in this manner for clarity.

Figure 27

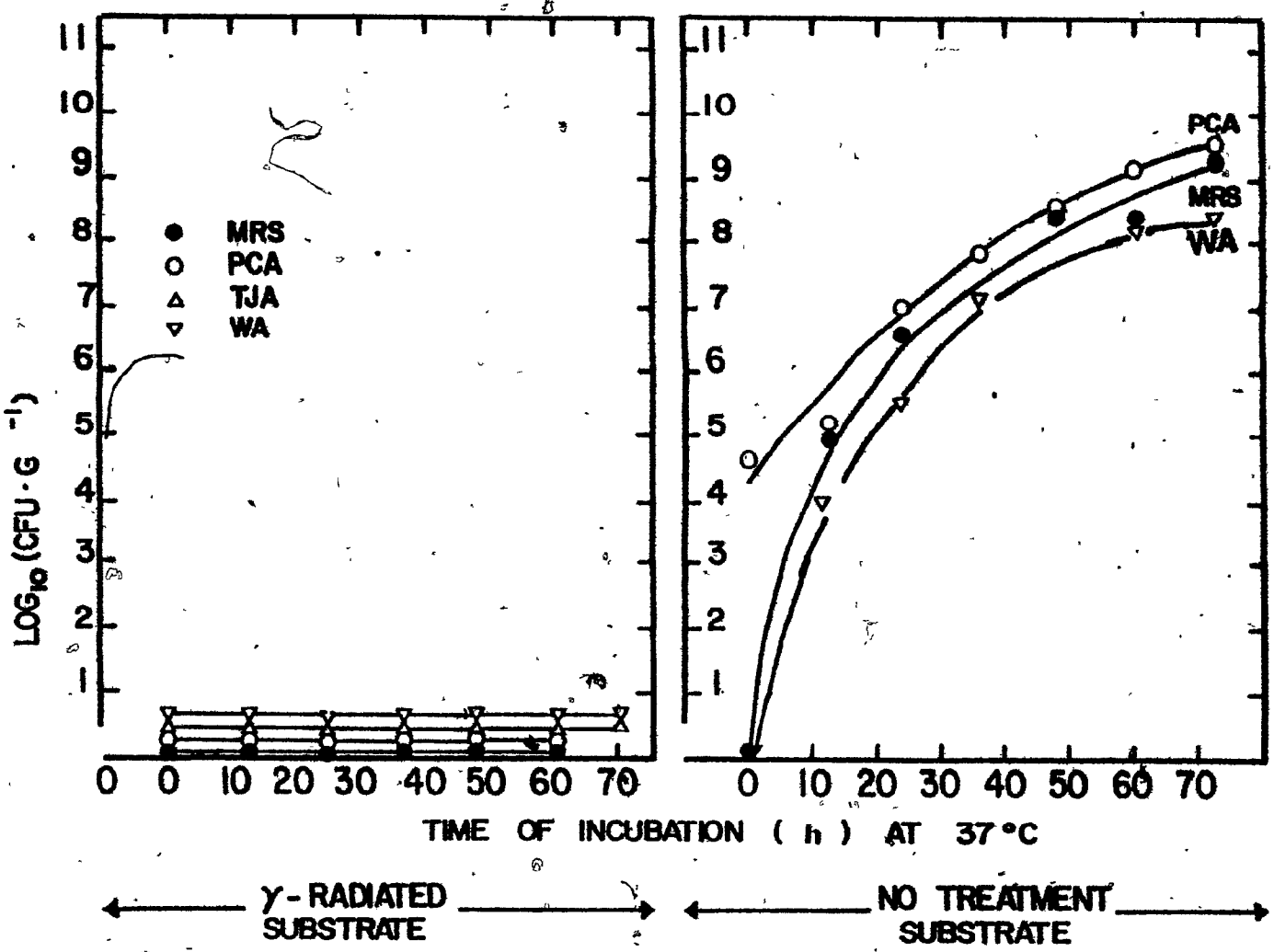
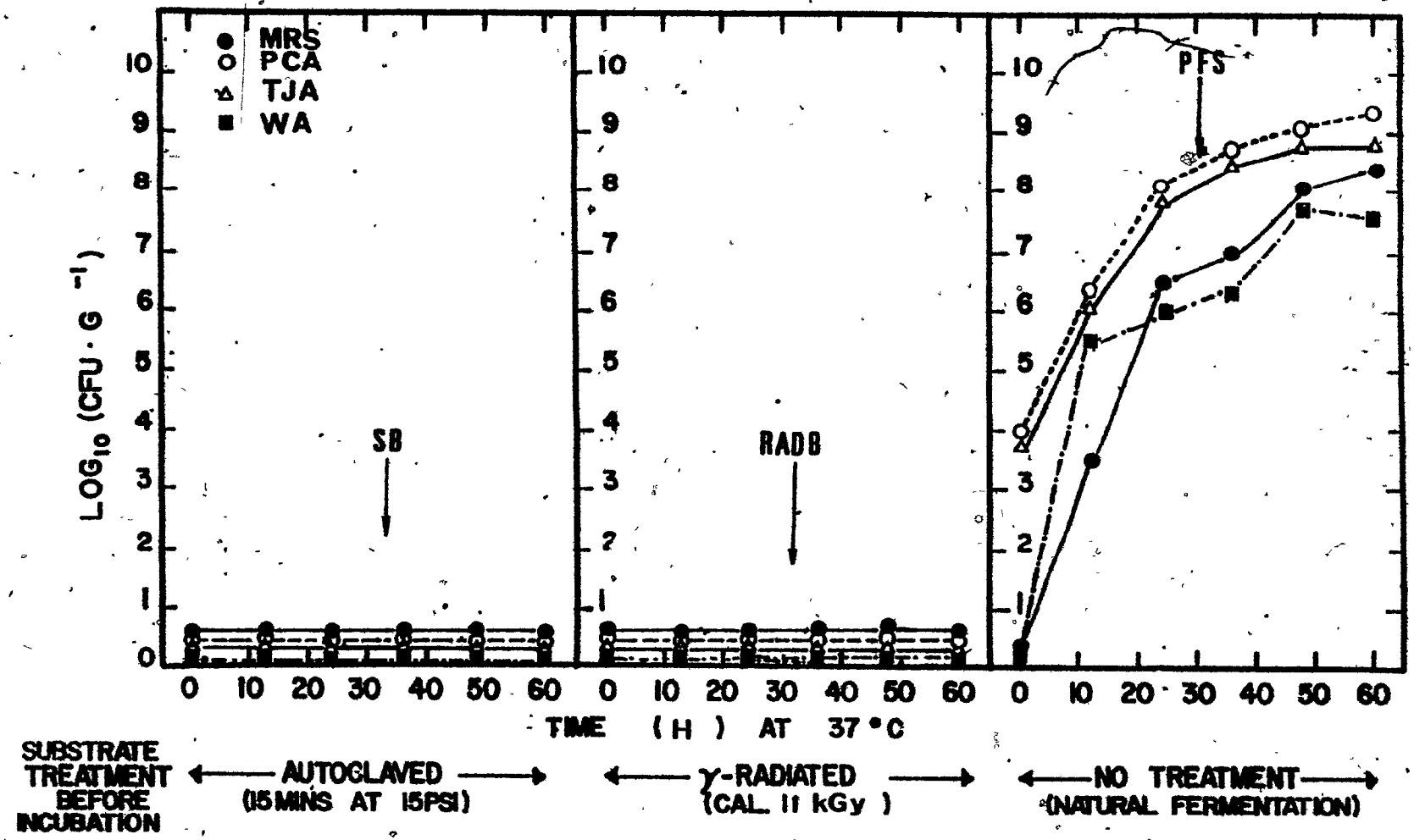

MRS
PCA
TJA
WA
$LOG_{10}$ (CFU · G $^{-1}$)
0 0 10 20 30 40 50 60 70
TIME OF INCUBATION ( h ) AT 37 °C
γ - RADIATED SUBSTRATE
NO TREATMENT SUBSTRATE

Fig. 28: Viable Counts: $\log_{10}$CFU·g$^{-1}$ of Processed Substrate, Autoclaved (SB), Radappertized (RADB) and Undergoing Natural Fermentation (PFS).

Note: No viable counts from SB and RADB. All curves are at zero. They are presented in this manner for clarity.

Figure 28


MRS
PCA
TJA
WA
SB
RADB
PFS
LOG10 (CFU · G -1)
0 10 20 30 40 50 60
TIME (H) AT 37 °C
SUBSTRATE TREATMENT BEFORE INCUBATION
AUTOCLAVED (15 MINS AT 15PSI)
γ-RADIATED (CAL. 11 kGy)
NO TREATMENT (NATURAL FERMENTATION)

beans' enzymes), a method had to be found to eliminate the microbial population while leaving the beans' enzymes unaffected. Depending on the amount employed, γ-irradiation or ethylene oxide may be used to achieve sterilization of the beans without destroying the indigenous enzymes. It has been stated that γ-irradiation is more effective than ethylene oxide in the reduction of the microbial population on spices (Vajdi and Pereira, 1973). However, the choice of γ-irradiation in this exercise was based on the relative convenience of securing its use rather than on the comparison of its effectiveness with ethylene oxide. The term 'radappertized' as employed in this study was in accordance with the guidelines of "The Commission on tentative Classification of Food Irradiation processes with Microbiological objectives" (Goresline et al., 1964). According to the commission the term signifies the application to foods of ionizing radiation sufficient to reduce the number and activity of viable microorganisms to such an extent that very few if any are detectable in the treated food by any recognizable method.

In Figs. 25 and 26 are presented the microbial counts obtained after treating the processed substrate (PS) with varying ionizing radiation dosages. A dose of 8 kiloGray (kGy) was found to be enough to treat the PS to give a RADB sample in which the presence of viable microorganisms was not detected on any of the media used. A much higher dose (11 kGy) was then selected in order to include a margin of safety for the process. Viable counts for PFS and RADB (11 kGy) were presented in Fig. 27. Microbial viable counts were obtained from the PFS on the isolation media used and none from RADB on similar media. Spontaneous fermentation occurred in the PFS samples but not with the RADB samples. The dose of

11 kGy used to obtain RADB was most likely not sufficient to inactivate any indigenous bean enzymes, if these were indeed still present after the processing steps to obtain the PS. A dose of up to 1000 kGy is needed to inactivate all the indigenous enzymes in foods (Grünewald, 1961 and Jay, 1978). The inference therefore, from Fig. 27, was that microorganisms are very important to the fermentation of African locust beans and that their enzymes are the agents of bioconversion.

In Fig. 28 are shown the three treatment (PFS, RADB and SB) curves. Both RADB and SB had no microbial counts on any of the media used and they were not bioconverted. The PFS had viable counts of up to $10^9$ CFU/g and was bioconverted. The results of experiments reported in this section agree with those reported in chapter 3 on texture (section 3.11.).

## 4.5. pH AND TITRATABLE ACIDITY

Microorganisms, as reported above, are important to the process of bioconversion of the processed substrate (PS) of African locust beans to dawadawa. For these organisms to propagate the PS must provide an appropriate biophysical environment and biochemical nutrients. Biophysical environmental parameters include pH, temperature, oxygen activity, water activity and others. The pH plays an important role in the determination of the types of microorganisms which can grow adequately on or within a given food. Most molds and yeasts will grow at low pH while bacteria, in general, favor a pH near neutrality. Some bacteria, especially the acid forming ones, have preference for a moderate pH. Some other bacteria, especially the actively proteolytic bacteria, will grow in an environment with a high (alkaline) pH (Frazier and Westhoff, 1978). Variations in the pH of a particular food or substrate can be brought about by the activities of the microorganisms present in or on it. However, different foods have different abilities to allow or resist changes in their pH values when they are acted upon by microorganisms. This variability is due primarily to the differences in buffer capacity of the foods. Pederson (1979) has stated that the presence of buffering substances such as certain salts and proteins allows the continuation of fermentation without a large change in the pH of some foods such as milks, meats and seeds. In contrast, the leafy vegetables have low buffering capacity and therefore their fermentation by lactic acid bacteria will result in an appreciable decrease in pH. The change of the pH and titratable acidity during the process of African locust beans bioconversion is studied in this experiment.

### 4.5.1. Objectives - pH and Titratable Acidity

The objective of this experiment was to determine the pH and the titratable acidity of processed fermenting substrate (PFS), radappertized beans (RADB) and sterilized beans (SB), incubated at 37 $\pm$ 2°C, on a time sequenced basis.

### 4.5.2. Principle - pH and Titratable Acidity

A $10^{-1}$ homogenate of the sample is prepared with water. The pH of the homogenate is determined with a single electrode pH meter. For the titratable acidity, a $10^{-1}$ homogenate of the sample is prepared with carbon dioxide free water and filtered. The filtrate is titrated against standardized NaOH.

### 4.5.3. Materials and Methods - pH and Titratable Acidity

The samples used were PFS, RADB and SB. For the pH measurements a $10^{-1}$ homogenate of each sample was made with sterilized distilled water cooled to room temperature (20 $\pm$ 2°C). The pH meter used was a single electrode pH Controller type 45 (Chemtrix Inc., Hillsboro, Ore., U.S.A.). The meter was switched on for about 30 min and its readings adjusted with standard buffers pH 4.01 and pH 6.86 before it was used for the determinations. Measurements were carried out on $10^{-1}$ homogenate

slurry of each sample in triplicate. The slurry was stirred continuously with a magnetic stirrer during the process. Samples used were incubated at 37 ± 2°C and determinations were made on a time sequenced basis.

The titratable acidity was determined for all the samples after incubation for Ø, 12, 24, 36, 48, 60 and 72 h at 37 ± 2°C. Tap distilled water was boiled to eliminate dissolved carbon dioxide and then cooled to 40°C. The decarbonated water was used to prepare a $10^{-1}$ homogenate of each sample i.e. 10 g of sample in 90.0 ml of decarbonated water. The slurried solution was stirred vigorously with a heavy glass rod and then passed through a funnel lined with Whatman No. 1 filter paper to remove the solid particles. A 10 ml aliquot of the filtrate, representing 1 g of the sample, was titrated with standardized 0.1 N NaOH (standardized by titrating against potassium hydrogen phthalate solution). The indicator employed was phenolphthalein and 0.5 ml (10 drops) of this was added to the filtrate before titration. The end point was indicated by a color change from colorless or slightly colored filtrate to purplish red or pink. To eliminate error due to possible colored samples the end point of the titration which occurred at pH 8.6 was also determined for all titrations with a pH meter. The color change was stable for at least 2 h at 20 ± 2°C. As a blank, the indicator, 0.5 ml phenolphthalein, was added to 10 ml of the decarbonated water used in the preparation of the homogenate slurry. This mixture was then titrated and the titre subtracted from the sample titre. Three titrations per sample were carried out and the mean value determined. The total titratable acidity was calculated using the relationship given by the AOAC (AOAC, 1970, 16.201).

1 ml 0.1 N NaOH titre ≡ 0.0090 g lactic acid

≡ 9.0 mg lactic acid

Values were expressed as either mg per g of sample or as percent titratable acidity.

### 4.5.4. Observations - pH and Titratable Acidity

The pH and titratable acidity of processed fermenting substrate (PFS), radappertized beans (RADB) and sterilized beans (SB) are presented in Figs. 29 and 30.

### 4.5.5. Discussion - pH and Titratable Acidity

The pH and titratable acidity of PFS, RADB and SB were present in Figs. 29 and 30. The pH of the PFS increased progressively with the incubation time from acid to alkaline. The corresponding time course determinations for RADB and SB gave pH values which remained constant and acidic throughout. The increased pH observed in PFS was due to microbial activities. The pH of RADB and SB remained constant since the viable microorganisms had been eliminated in these samples. The rise in pH with progressive fermentation is not a usual feature of many fermentations, especially those mediated by lactic acid bacteria. However, as found from this experiment, bioconversion of the African locust beans was always accompanied by a rise in the pH of the fermenting

Fig. 29: Time Sequenced pH and Titratable Acidity of PFS and RADB.

PFS : Processed Fermenting Substrate

RADB: Radappertized Beans

T.A.: Titratable Acidity

Figure 29

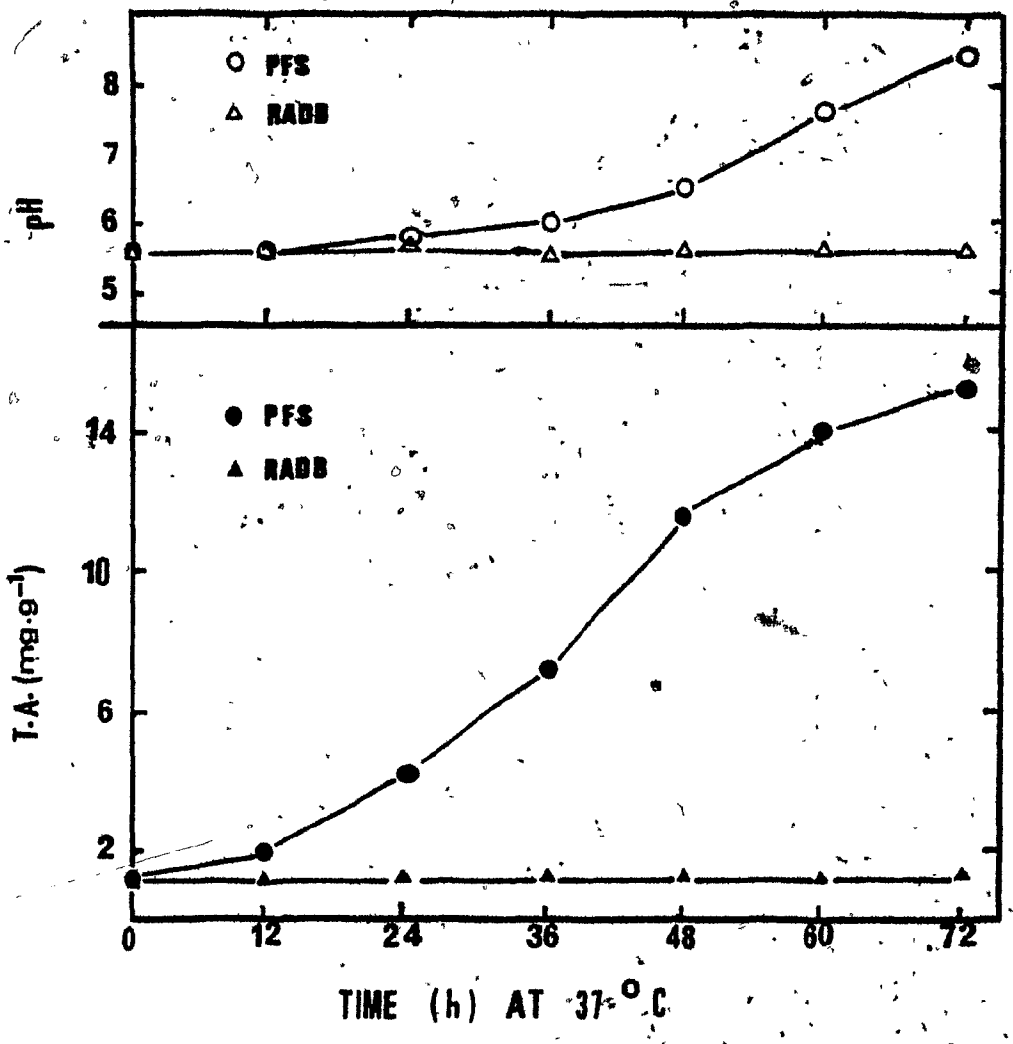

PFS
RADB
pH
8
7
6
5
PFS
RADB
T·A (mg·g⁻¹)
14
10
6
2
0
12
24
36
48
60
72
TIME (h) AT 37 °C

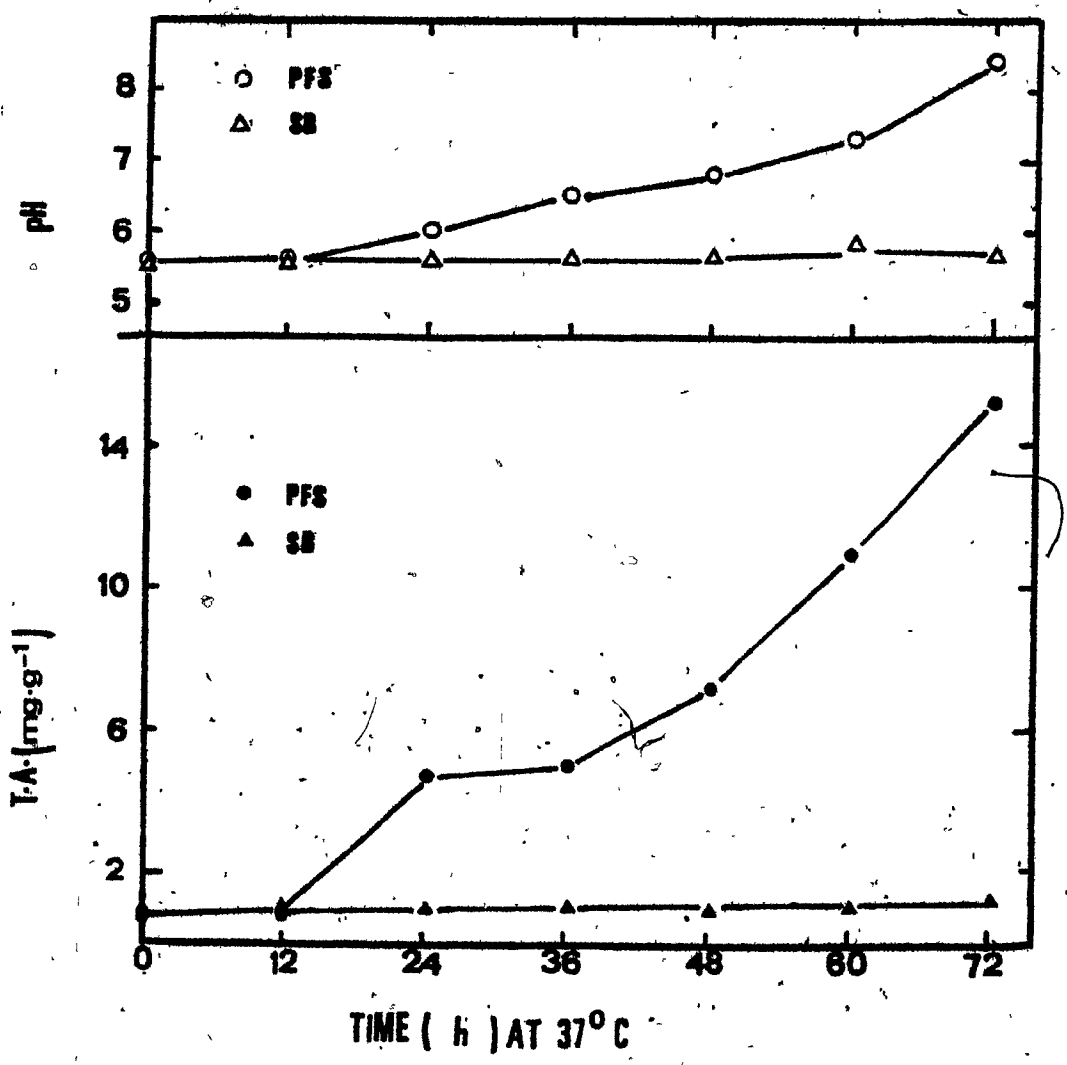
Fig. 30: Time Sequenced pH and Titratable Acidity of PFS and SB.

PFS : Processed Fermenting Substrate

SB : Sterilized Beans

T.A.: Titratable Acidity

Figure 30


PFS
SB
pH
8
7
6
5
PFS
SB
T.A. (mg.g⁻¹)
14
10
6
2
0
12
24
36
48
60
72
TIME ( h ) AT 37° C

substrate (PFS). There was also a progressive increase in the titratable acidity of the PFS with time. This was not the case with RADB and SB samples in which titratable acidity remained fairly constant throughout the period of the experiment (Figs. 29 and 30). The increased titratable acidity was due to the activities of microorganisms. The simultaneous increases in both pH and acidity of PFS are unusual. In a situation of this nature neutralization would be expected. However, increased titratable acidity as well as increased pH characterized the African locust bean fermentation. Since the pH is simple to measure it would be a very useful parameter in process control.

In the determination of the pH a $10^{-1}$ homogenate was used. This procedure yielded similar values for subsequent analyses of the same sample. When the solid substrate was tested on a preliminary basis by inserting the electrode into the solid mass the pH measured varied markedly from 6 to 9.5 on the same fermenting substrate at the same time. This probably resulted from the inhomogeneity of the distribution of microorganisms responsible for the fermentation on the processed substrate. The $10^{-1}$ homogenate eliminated this problem. The determination of the final pH of the reactants during the titratable acidity experiment eliminated possible error due to colored filtrates. It should be noted that the pH and titratable acidity values reported are not necessarily always the same. They should be taken as trend values i.e. as the trend lines or curves for the pH and titratable acidity. These values may change as a result of initial microbial population on the PS, biophysical conditions and biochemical nutrients. However, whenever there is a spontaneous fermentation of PS, the pH and titratable acidity curves will be of the type shown in Figs. 29 and 30 above.

## 4.6. EFFECT OF TEMPERATURE

Temperature very strongly influences the growth of microorganisms. At high temperatures microbes are destroyed while at freezing temperatures their activity is decreased. Therefore, growth of microorganisms is promoted mainly within these two extremes, usually -5 to 80°C (Stanier et al., 1970). Microorganisms vary in their reaction to the temperature of the microhabitat; consequently it is possible to group these organisms into three classes as psychrophilic, mesophilic and thermophilic. All these types are capable of bringing about biochemical changes in food substances depending on the type of food and the temperature at which the food is kept. Each type of microorganism has its optimum, minimum and maximum temperatures of growth. The most rapid growth usually occurs at the optimum temperature. Microorganisms are involved in the process of Parkia bioconversion; the temperature which will promote their rapid growth in this substrate will consequently promote rapid bioconversion. This 'best' temperature was sought in this experiment.

### 4.6.1. Objectives - Effect of Temperature

The objective in this experiment was to determine the optimum temperature at which the bioconversion of the processed substrate (PS) to fermented beans (FB) will occur.

### 4.6.2. Principle - Effect of Temperature

PS is incubated at selected temperatures for 72 h. The viable counts are obtained from homogenate. The temperature which promotes the highest viable counts is determined. Time sequenced viable counts are also determined for some of the temperatures. The pH and titratable acidity are determined as indices of fermentation.

### 4.6.3. Materials and Methods - Effect of Temperature

The samples used were processed substrate (PS) already prepared for the fermentation process in their units. Incubators were used, set at 4°C, 15°C, 25°C, 30°C, 37°C, 45°C and 55°C. Each temperature chosen had a maximum fluctuation range of ± 2°C. The isolation media used for the viable counts were MRS, PCA, PDA (acidified with sterile tartaric acid), TJA and WA.

For the determination of viable counts, prepared PS in fermentation units were incubated at selected temperatures for 72 h. Some PS incubated at temperatures 30°C to 45°C could actually be described as processed fermenting substrate (PFS). Homogenates and dilutions of these were prepared. The viable counts at each chosen temperature were determined on the isolation media using the drop plate method. For the time sequenced experiments, incubation temperatures of 25°C, 30°C, 37°C and 45°C were used. These were selected based on the counts obtained from the original set of temperatures chosen. For all the temperatures investigated the pH

and titratable acidity were also determined as indices or indicators of bioconversion. The methods employed for pH and titratable acidity were as described under section 4.5.3.

### 4.6.4. Observations - Effect of Temperature

The effect of temperature on viable counts of PS incubated for 72 h at different temperatures is shown in Fig. 31. The detailed data are presented in appendix F, Table F1. The corresponding pH and titratable acidity (TA) values are shown in Fig. 32. The data are shown in appendix F, Table F2. In Fig. 33 A, B, C and D are presented the viable counts on a time sequenced basis. The raw data obtained are given in appendix F, Table F3. The corresponding pH and TA are shown in Fig. 34. The data for these are given in appendix F, Table F4.

### 4.6.5. Discussion - Effect of Temperature

The processed substrate (PS) which was incubated for 72 h at 4°C gave no viable counts on any of the media employed for isolation and enumeration (Fig. 31). At 15°C counts were obtained on PCA and TJA but none were found on MRS, PDA and WA. There was no count on acidified PDA from all the PS samples incubated at different temperatures. The MRS, PCA, TJA and WA gave viable counts from the PS incubated at 25°C, 30°C, 37°C and 45°C. The PS incubated at 55°C dried out before the

Fig. 31: Viable Counts: $\log_{10}$CFU·g$^{-1}$ of PS Incubated at Different Temperatures for 72 h.

MRS: de Man Rogosa Sharpe medium

PCA: Plate Count agar

PDA: Potato Dextrose agar

TJA: Tomato Juice agar

WA : Wort agar

Figure 31

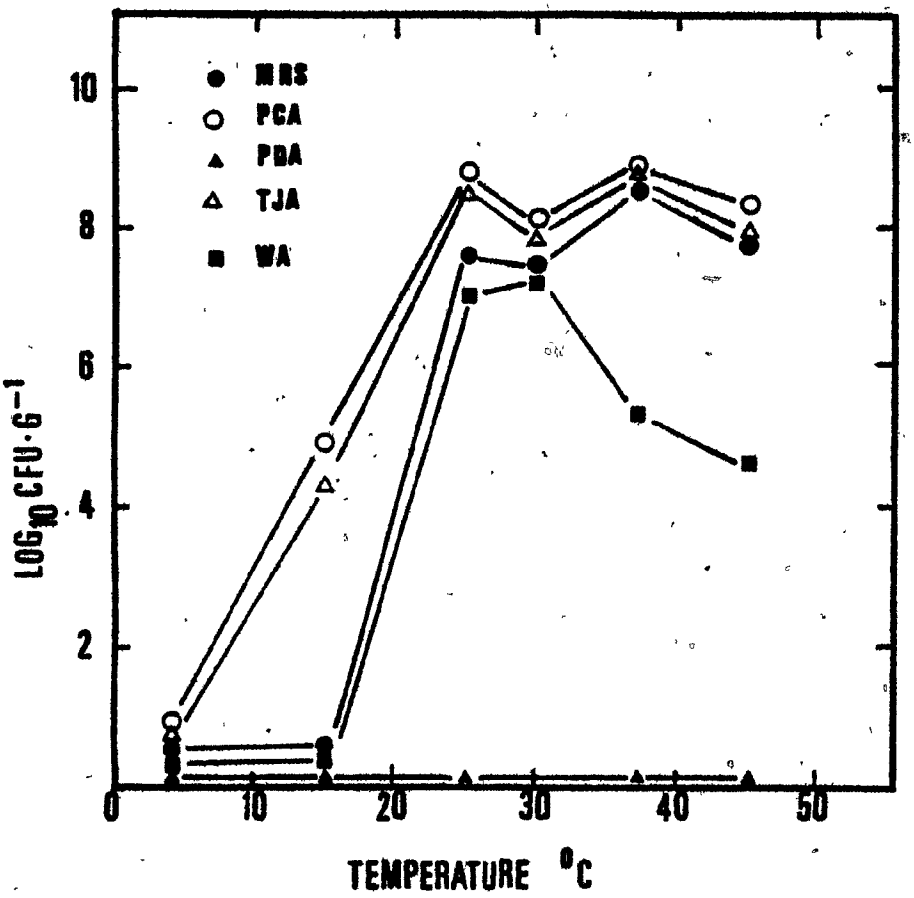
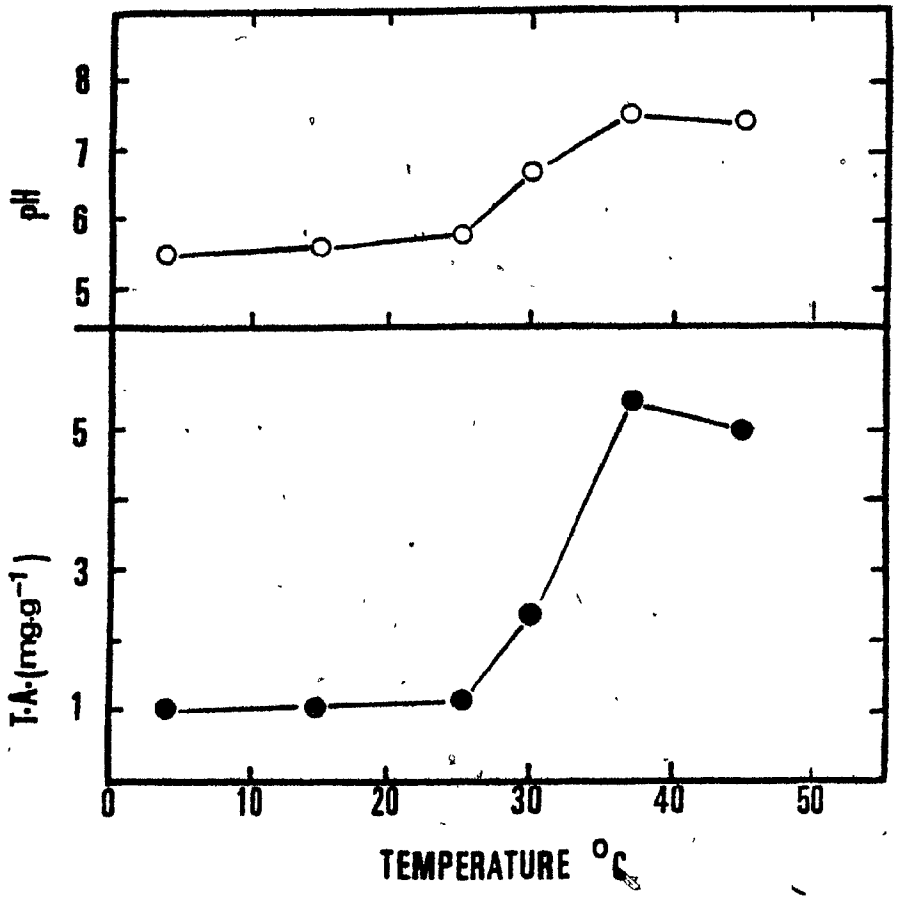

MRS
PCA
PDA
TJA
WA
10
8
6
4
2
0
10
20
30
40
50
$LOG_{10}$ CFU·G$^{-1}$
TEMPERATURE °C

Fig. 32: pH and Titratable Acidity (T.A.) of PS after Incubation for 72 h at Different Temperatures.

Figure 32


pH
8
7
6
5
T·A·(mg·g⁻¹)
5
3
1
0
10
20
30
40
50
TEMPERATURE °C

Figs. 33A & 33B: Time Sequenced Viable Counts ($\log_{10}$CFU·$g^{-1}$) of Processed Substrate Incubated at (A) 25°C and (B) 30°C on MRS, PCA, Acidified PDA, TJA and WA.

MRS: de Man Rogosa Sharpe medium

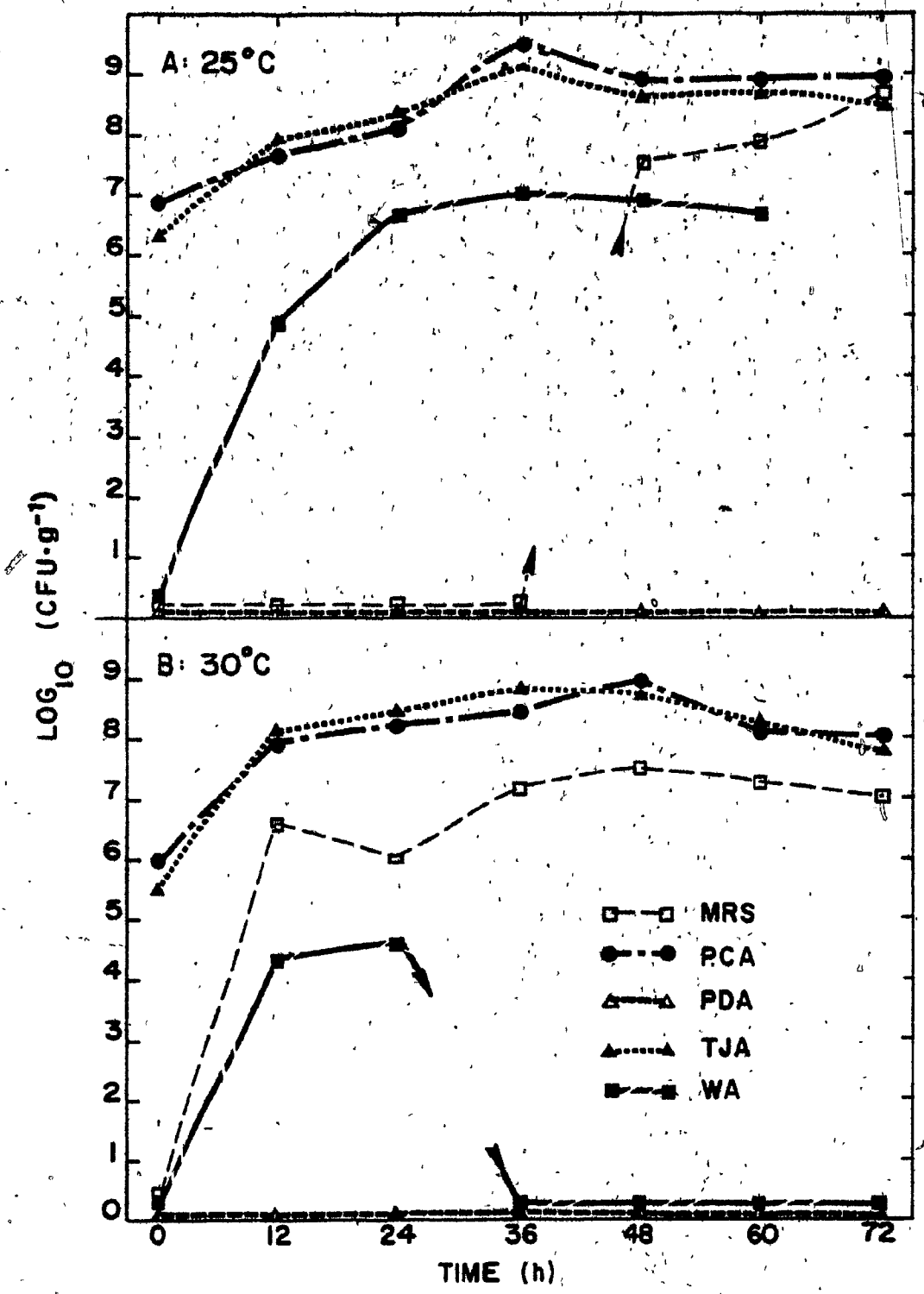
PCA: Plate Count agar

PDA: Potato Dextrose agar

TJA: Tomato Juice agar

WA : Wort agar

Figures 33A and 33B


A: 25°C
B: 30°C
LOG10 (CFU·g-1)
TIME (h)
0
12
24
36
48
60
72
MRS
PCA
PDA
TJA
WA

Figs. 33C & 33D: Time Sequenced Viable Counts ($\log_{10}$CFU·g$^{-1}$) of Processed Substrate Incubated at (C) 37°C and (D) 45°C on MRS, PCA, Acidified PDA, TJA and WA.

MRS: de Man Rogosa Sharpe medium

PCA: Plate Count agar

PDA: Potato Dextrose agar

TJA: Tomato Juice agar

WA : Wort agar

Figures 33C and 33D

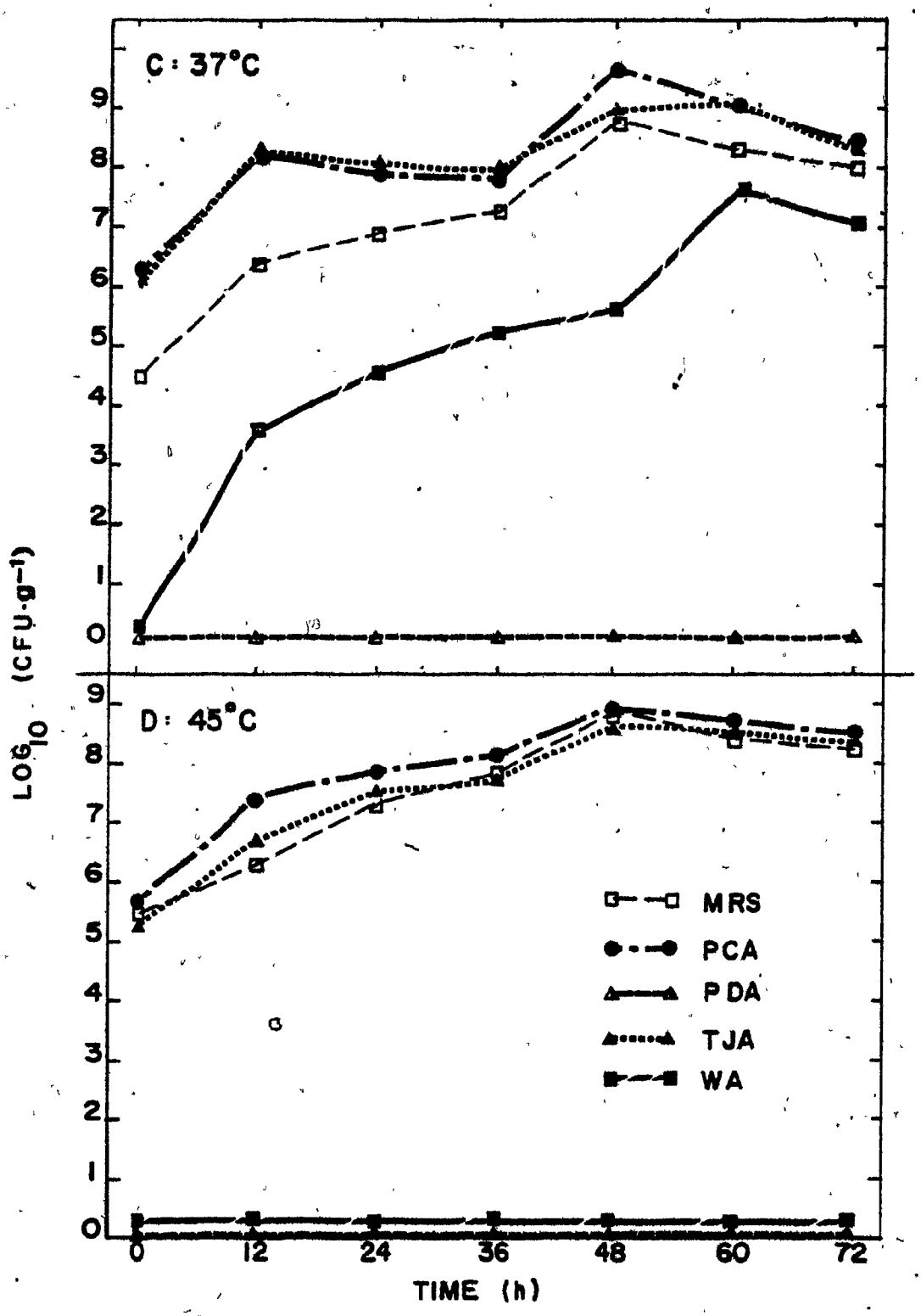
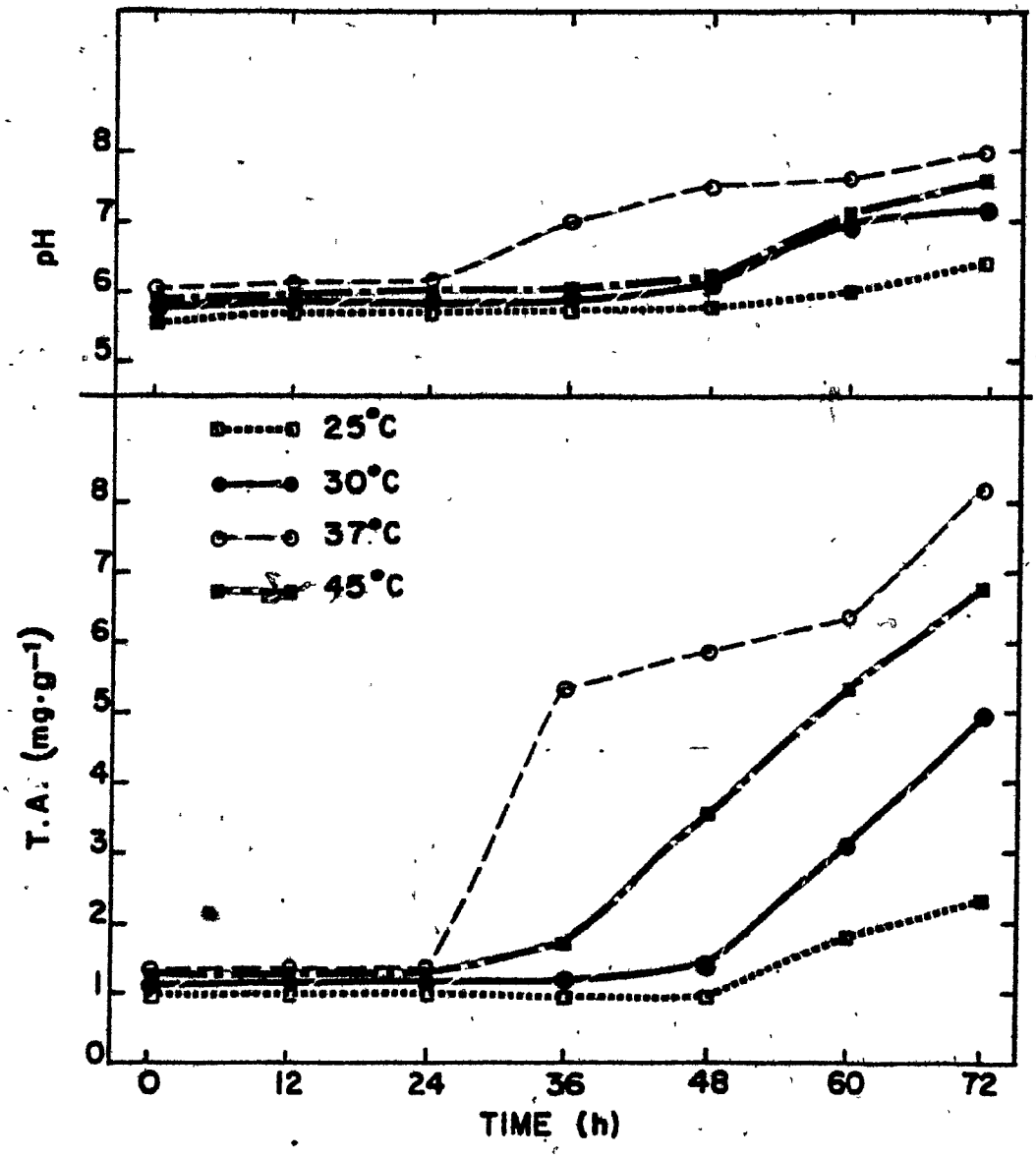

C : 37°C
D : 45°C
LOG10 (CFU·g-1)
TIME (h)
0
12
24
36
48
60
72
MRS
PCA
PDA
TJA
WA

Fig. 34: Time Sequenced pH and Titratable Acidity (T.A.) of PS Incubated at 25, 30, 37 and 45°C.

Figure 34


pH
8
7
6
5
25°C
30°C
37°C
45°C
T.A. (mg·g⁻¹)
8
7
6
5
4
3
2
1
0
0
12
24
36
48
60
72
TIME (h)

72 h could elapse. It was therefore not possible to carry out counts for the PS at this temperature. The relatively high recovery of microorganisms on PS samples incubated between 25 and 45°C on the different media employed is an indication of the range of temperature within which the fermentation could be carried out.

The pH and TA values for PS incubated for 72 h at different temperatures are shown in Fig. 32. At 4°C, 15°C and 25°C the values remained relatively constant. There were steady increases in these values between 25 to 45°C. The maximum pH and TA values were found at 37°C followed by 45°C values and then 30°C before 25°C. From these values, 37°C would appear to be the temperature of choice for the fermentation. The viable counts obtained for the time sequenced experiments at 25°C, 30°C, 37°C and 45°C were shown in Fig. 33 A, B, C and D. There was no recovery on acidified PDA even when the inoculated medium was incubated beyond the 72 h to 120 h. Acidified PDA was used for the possible detection and isolation of molds. It appeared from this experiment that molds are not important to the process of bioconversion of the African locust beans. Reduced counts were obtained on WA and at 45°C no counts were obtained on this media. WA is a media recommended for the cultivation of yeasts (Difco, 1974). Relatively high counts were obtained on the remaining media used i.e. MRS, PCA and TJA. Both MRS and TJA are recommended for the cultivation of Lactobacilli and the lactic acid bacteria (Difco, 1974 and Harrigan and McCance, 1976). The plate count agar (PCA) or Tryptone Glucose Yeast Extract Agar is the Standard Methods Agar of the American Public Health Association (APHA, 1960) and it is a non-selective medium for the general viable

counts of bacteria in foods (Harrigan and McCance, 1976). From the recovery on these media it seemed that more than one type of microorganism may be present on the fermenting PS.

When time sequenced pH and TA were determined (Fig. 34), the lowest values were recorded at 25°C. There were slight increases in the values at 30°C and more increases at 45°C. The maximum values were recorded for both parameters at 37°C. The increases started after 24 h of incubation at 37 and 45°C. Extended time lags of 48 and 36 h were obtained for 25 and 30°C respectively before increases could be detected in the PS samples at these temperatures (Fig. 34). The implication of the extended lag is that it would take a relatively longer period to obtain bioconversion at 25 and 30°C when compared to the time it would take at 37 and 45°C. An important deduction from the pH and titratable acidity values is that even though large numbers of microorganisms may be found on the PS, this alone does not guarantee that fermentation will occur. The temperature condition must be satisfied. This in turn will allow rapid bioconversion. On the basis of data obtained from this experiment 37°C appeared to be a satisfactory temperature for Parkia fermentations and it is as such recommended.

## 4.7. THE EFFECT OF SALT (NaCl)

The addition of salt to fermentation substrates has been reported in the literature to play a useful role in some food fermentations such as sauerkraut, cheese and various Oriental foods. Pederson and Albury (1954) and Pederson (1979) have stated that salt, up to 2.25%, added to shredded cabbage gave sauerkraut with good organoleptic qualities. However, when the percentage of added salt was very high, poor organoleptics (color, flavor and texture) resulted in the kraut. An appropriate salt level may select microorganisms useful to the fermentation, decrease contamination and improve product consistency. In this experiment the observations of the effect of salt on Parkia fermentations are reported.

### 4.7.1. Objectives - Effect of Salt

The objective of this experiment was to study the effect of salt addition (0-5%) on the bioconversion of the processed substrate.

### 4.7.2. Principle - Effect of Salt

Various quantities of salt are added to processed substrate (PS). Microbial counts, organoleptic scores and pH developed are determined after incubation for the required length of time at an appropriate temperature.

### 4.7.3. Materials and Methods - Effect of Salt

Sodium chloride was aseptically added to the processed substrate (PS) and thoroughly mixed with it. The mixture was then packed into fermentation units. The amount of salt added was varied between 0-5% (W/W) as indicated in the flowchart below (Fig. 35). The fermentation units were incubated at 37 $\pm$ 2$^{o}$C for 72 h after which their contents were analyzed. Microbial counts were obtained on selected media of MRS, PCA, TJA and WA using the SPM procedure (section 4.1.4.). Organoleptic score was based on whether the PS had fermented or not. The pH was determined as described in section 4.4.3.

### 4.7.4. Observations - Effect of Salt

In Fig. 36 are shown the curves obtained for the microbial counts and the pH developed at different salt percentages. The organoleptic scores as well as the developed pH are presented in table 26.

### 4.7.5. Discussion - Effect of Salt

The viable microbial counts obtained when different amounts of salt were added to the PS were shown in Fig. 36. Between 1 to 2 log reduction in viable counts, depending on the media, were found when 5% salt was added to the PS before fermentation. An almost similar trend was found when

Fig. 35: Flowchart for the Determination of the Effect of Salt on the Process of Bioconversion.

Figure 35

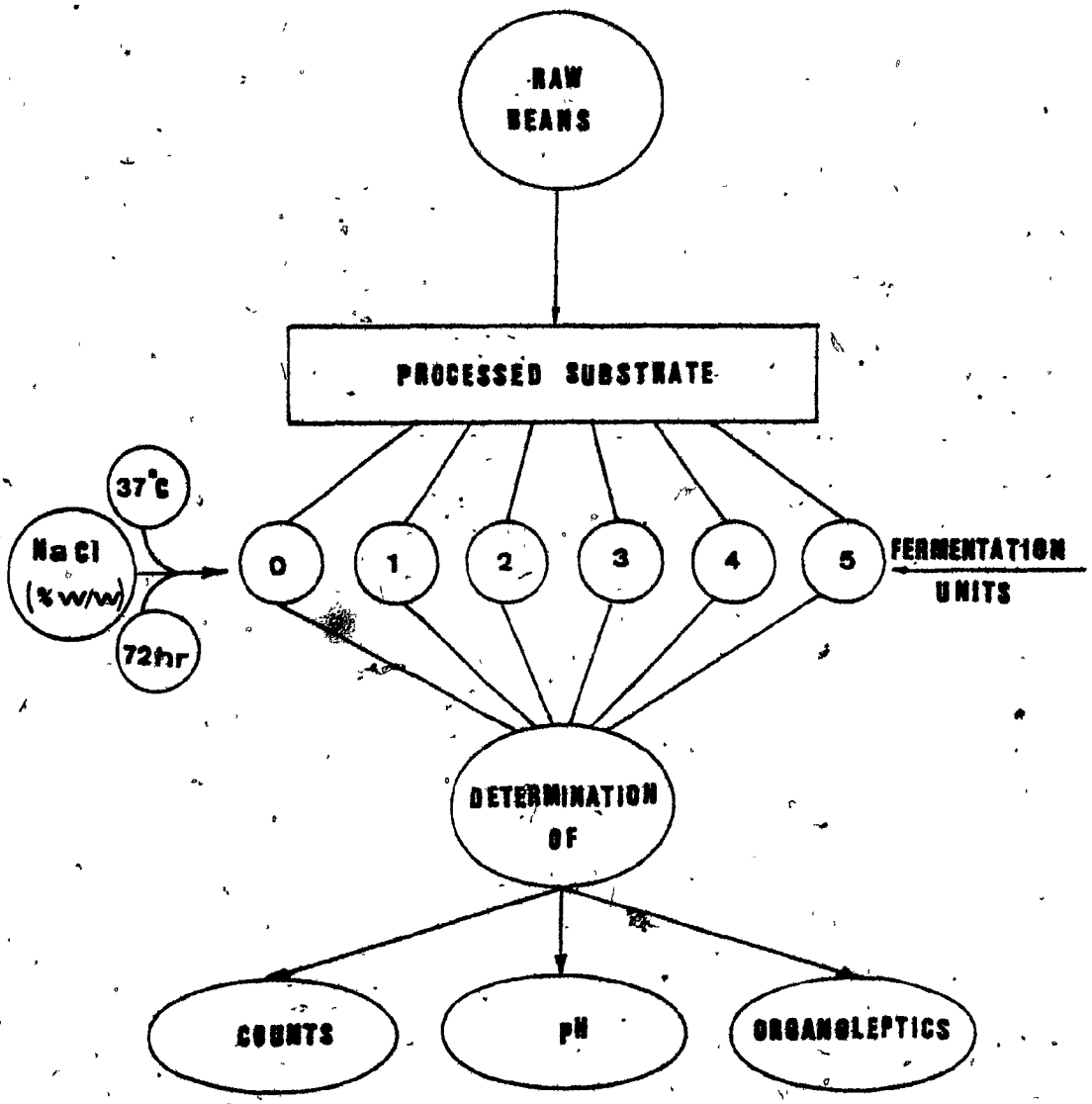

RAW BEANS
PROCESSED SUBSTRATE
37°C
NaCl (%w/w)
72hr
0
1
2
3
4
5
FERMENTATION UNITS
DETERMINATION OF
COUNTS
pH
ORGANOLEPTICS

Fig. 36: Effect of Salt (NaCl) on Viable Counts ($\log_{10}$CFU·$g^{-1}$) and pH Developed on Processed Substrate (PS) after Incubation at 37°C for 72 h.

Figure 36

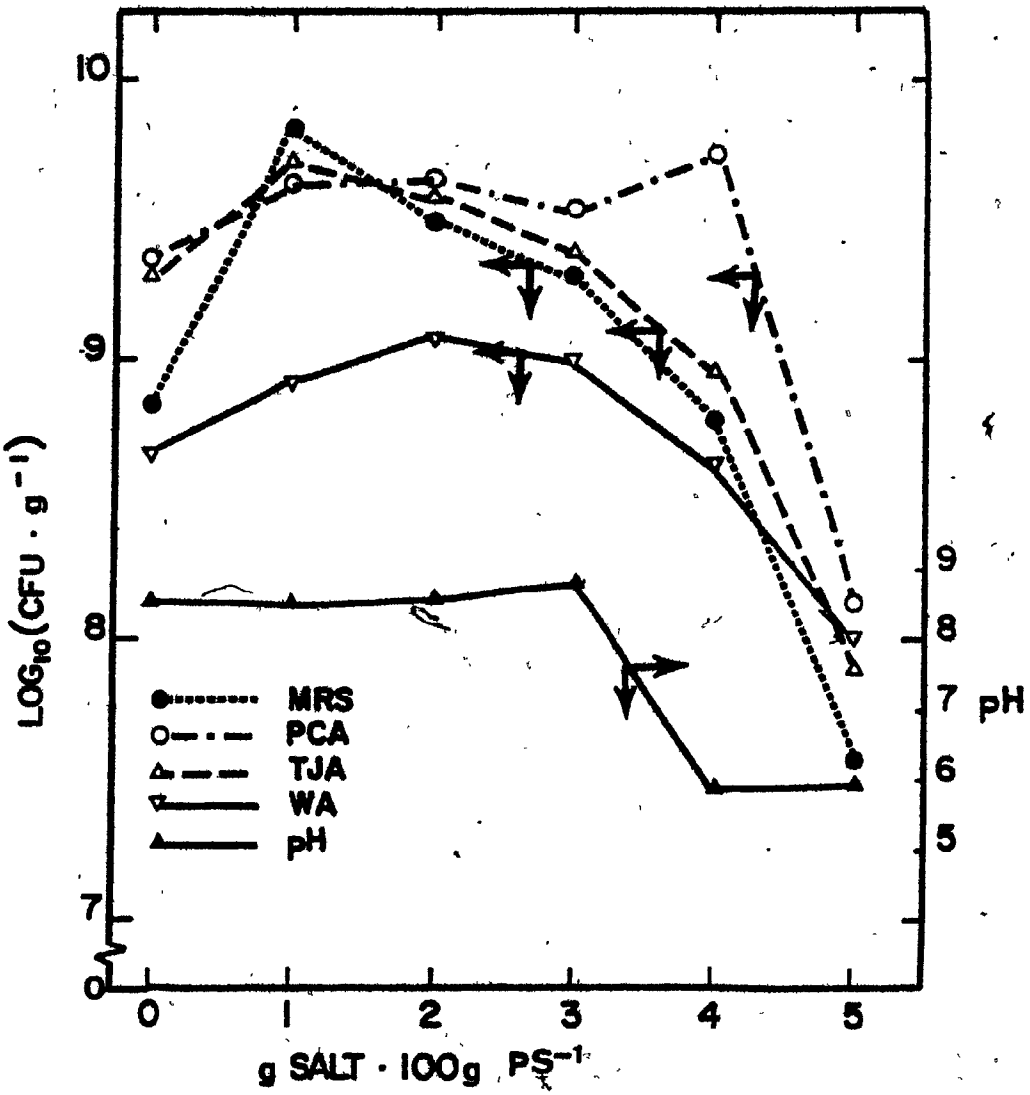

10
9
8
7
0
1
2
3
4
5
6
pH
MRS
PCA
TJA
WA
pH
$LOG_{10}$(CFU · $g^{-1}$)
g SALT · 100g $PS^{-1}$

Table 26. The Effect of Salt on the Organoleptics and pH Developed.

| % Salt Added | Organoleptic Score | pH* |
|---|---|---|
| 0 | + + + | 8.60 ± 0.45 |
| 1 | + + + + | 8.50 ± 0.15 |
| 2 | + + + | 8.50 ± 0.30 |
| 3 | + + + | 8.85 ± 0.35 |
| 4 | + | 5.90 ± 0.10 |
| 5 | + | 5.90 ± 0.10 |

* Mean of 3 determinations ± standard deviation.

Score: + = Poor, no fermentation.
+ + = Fair, marginal.
+ + + = Good, fermentation.
+ + + + = Very good, fermentation.

4% was used. The exception at 4% was PCA medium which gave high viable counts. Relatively higher counts ($\geq 1 \times 10^9$ CFU/g) were obtained at 0-3% salt levels from the PS after incubation. Although the counts on WA were less than $1 \times 10^9$ CFU/g at 0-3% they were higher than counts for 4 and 5% salt on this medium. The pH developed between 0-3% of added salt was alkaline. The pH dropped to acidic level when 4 and 5% salt were added to the PS and then incubated (Fig. 36). These observations related very well to the organoleptic scores developed on the incubated PS (Table 26). Although there was microbial growth when 4 or 5% NaCl was added to PS the fermentation product was not obtained. There was fermentation in the range 0-3% NaCl addition and the pH was high. The 1% level of added salt was found to endow the PS with slightly better organoleptic scores compared to Ø, 2 and 3%. Slightly higher counts were recorded for the PS treated with 1% salt level. The higher counts may explain the reason for better organoleptic scores at 1% of added salt. The failure of the PS to ferment when 4 or 5% salt was added may be due to reduced moisture. The higher percentage of salt may have created a stressed microenvironment for the bioconverting microorganisms. In this type of situation the organisms may reduce their biochemical activities and as a result the PS would not be bioconverted. Therefore the pH remained acidic (Fig. 34). Either Ø or 1% of salt is recommended for the fermentation. Although 2% and 3% will also result in fermentation, the added costs involved, with no significant gain in product quality, make Ø% or 1% of added salt better choices. The experiment worked well with NaCl crystals i.e. when this was added as solid. The addition of brine water is not recommended as this tended to give very diverse and organoleptically inferior products.

## 4.8. EFFECT OF WATER ACTIVITY (Aw)

The term water activity (Aw) was introduced by Scott in 1953 (Scott, 1953) to describe the amount of unbound water i.e. available water to microorganisms in growth media and foods It is determined by calculating the ratio of the vapor pressure of an aqueous solution to that of pure water at a given temperature.

$$\text{i.e. } Aw = P/Po = \frac{ERH}{100\%}$$

where:

Aw = Water activity

P = Vapor pressure of water in food (kPa)

Po = Vapor pressure of pure water at the same temperature (kPa)

ERH = Equilibrium relative humidity (%)

The Aw affects chemical, enzymatic and microbial changes and the overall stability of food products (Rockland and Nishi, 1980). When Aw is reduced, microbial growth is suppressed, although reduction hardly results in outright cell death but usually rather in a lowered rate of growth. Smaller population and reduction in one or more secreted metabolites are often observable features (Troller, 1980). Enzyme stability and reactivity may also be affected (Schwimmer, 1980). Several authors (Mead, 1969; Christian and Stewart, 1973; Roberts and Ingram, 1973; Acott and Labuza, 1975 and Boylan et al., 1976) have reported on the effect of Aw on the growth of microorganisms in foods and the suppression of secondary metabolite generation (i.e. toxins) at low Aw values.

Since Aw affects the microbial population, their enzymes and metabolites, it can be employed to prevent food spoilage due to microorganisms and to encourage desirable changes such as in the fermentation of foods. Therefore, a knowledge and proper manipulation of the Aw with respect to process and product development may enhance organoleptic product acceptability. The enumerated advantages of a knowledge of the operating Aw in a particular fermentation led to this experiment.

### 4.8.1. Objectives - Effect of Aw

The objectives of this experiment were to determine the viable microbial counts of processed substrate (PS) incubated in various Aw chambers at 37°C for 72 h and to determine the corresponding organoleptic scores and pH developed at the different selected Aw values.

### 4.8.2. Principle - Effect of Aw

Processed substrate (PS) is prepared in fermentation units (section 3.2.). The units are transferred into pre-equilibrated Aw chambers at 37°C for 72 h. The viable counts, organolopetic scores and pH values are then determined.

### 4.8.3. Materials and Methods - Effect of Aw

Chambers in which the water activity was controlled in the range of 0.11 to 0.96 were prepared by using various salts as previously described by Rockland (1960). The desired Aw environments were prepared with various salts dissolved in water contained in dessicators with the aid of a magnetic stirrer at 37°C. The process was continued by adding more solute until equilibrium was reached i.e. when no more solute would dissolve in the water. The closed chambers were placed in an incubator at 37°C for 72 h before the introduction of labelled fermentation units into them. The units were placed on top of wire gauze shelves which separated the liquid and saturated gaseous phases in the chambers. The salts used to obtain each desired Aw are listed in table 27 below. The surface plate method (section 4.1.4.) was used to determine viable counts after 72 h of incubation. The media used were MRS, PCA, TJA and WA. The corresponding organoleptic and pH developed at each Aw level investigated were also determined. The organoleptic scores were based on whether fermentation had taken place or not. Control units were also provided. These units were incubated in air at 37°C in the same incubator. The viable counts, organoleptics and pH developed were also determined for the control units at the end of the 72 h incubation periods.

### 4.8.4. Observations - Effect of Aw

The effect of water activity on viable counts is shown in Fig. 37. The corresponding organoleptic scores are presented in table 28 along with the observed pH values.

Table 27. Salts Employed to Prepare Various Aw Environments at 37°C.

| Required Aw | Saturated Salt Solution Used at 37°C | |
|---|---|---|
| 0.11 | Lithium chloride | LiCl |
| 0.23 | Potassium acetate | $K(CH_2COO)$ |
| 0.32 | Magnesium chloride | $MgCl_2$ |
| 0.41 | Potassium carbonate | $K_2CO_3$ |
| 0.57 | Sodium bromide | $NaBr.2H_2O$ |
| 0.65 | Sodium nitrate | $NaNO_3$ |
| 0.75 | Sodium chloride | NaCl |
| 0.84 | Potassium chloride | KCl |
| 0.96 | Ammonium sulfate | $(NH_4)_2SO_4$ |

Fig. 37: Effect of Water Activity (Aw) on Viable Counts ($log_{10}CFU \cdot g^{-1}$) and pH Developed on PS Incubated at $37^{o}C$ in Different Aw Chambers for 72 h.

Figure 37

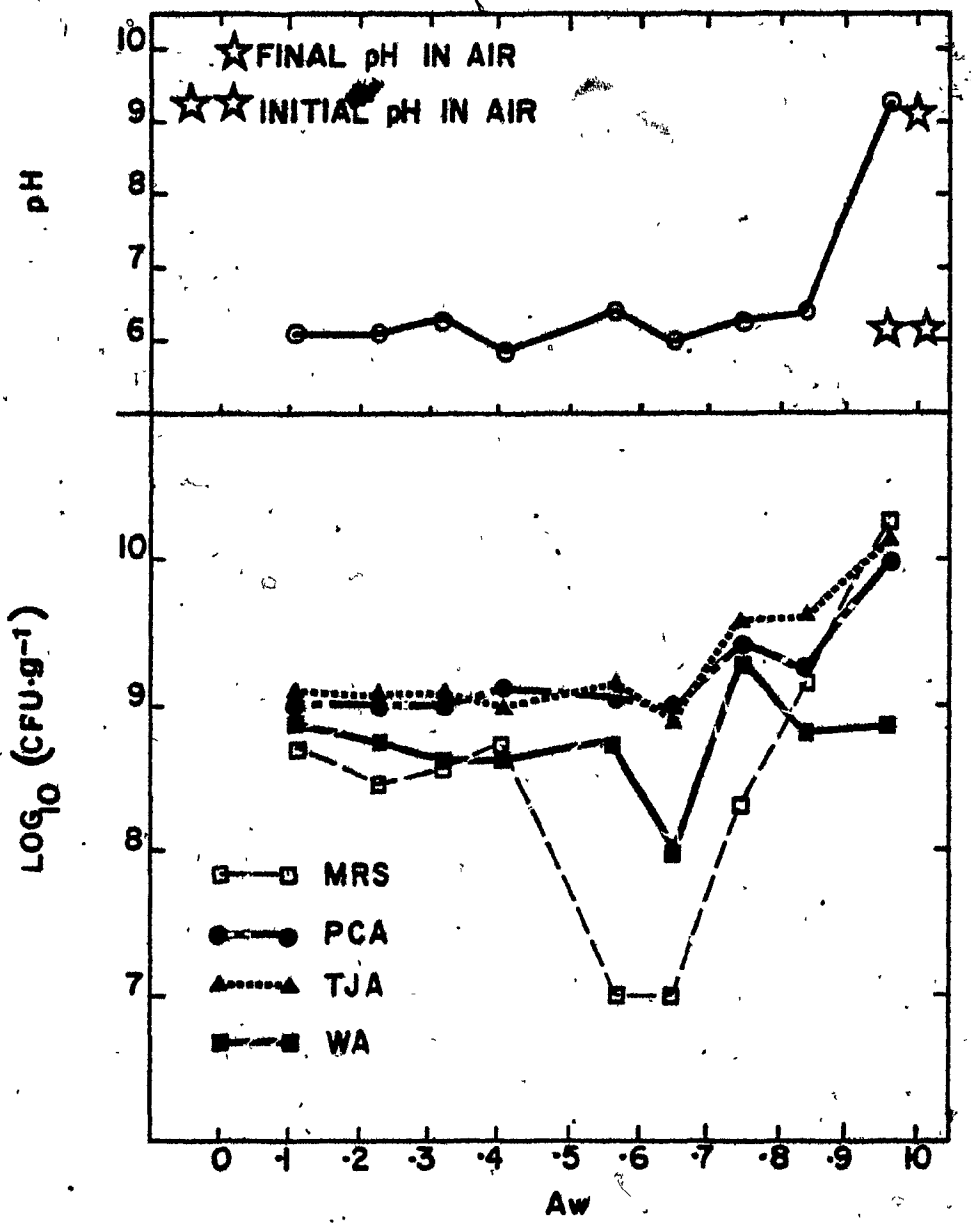

FINAL pH IN AIR
INITIAL pH IN AIR
pH
10
9
8
7
6
LOG10 (CFU.g-1)
10
9
8
7
MRS
PCA
TJA
WA
0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 10
Aw

Table 28. Organoleptics and pH Developed by PS at Various Aw at 37°C for 72 hours.

| Aw | Organoleptic Score | pH* |
|---|---|---|
| 0.11 | + | 6.10 ± 0.10 |
| 0.23 | + | 6.10 ± 0.10 |
| 0.32 | + | 6.30 ± 0.20 |
| 0.41 | + | 5.80 ± 0.30 |
| 0.57 | + | 6.40 ± 0.30 |
| 0.65 | + | 6.00 ± 0.20 |
| 0.75 | + | 6.30 ± 0.20 |
| 0.84 | + | 6.40 ± 0.20 |
| 0.96 | + + + + | 9.30 ± 0.40 |
| Control† | + + + | 9.10 ± 0.50 |
| Initial¶ | + | 6.10 ± 0.10 |

* Mean of 3 determinations ± standard deviation.

† PS incubated in air.

¶ Initial organoleptic and pH of PS.

Score: + = No fermentation;
+ + + = Fermentation;
+ + + + = Fermentation, very good product.

### 4.8.5. Discussion - Effect of Aw

The viable counts and developed pH at varied Aw were presented in Fig. 37. The counts were relatively high in all cases, however, between 0.5 to 0.7 Aw, reduced counts were obtained i.e. a decrease in viable counts on all the media employed. The reason for the decrease in counts is not completely clear. There was, however, a literature report on the progressive decrease in the survival rate of Staphylococcus aureus in dried foods when the Aw was raised in the range 0.22 to 0.53 (Christian and Stewart, 1973). Between 0.11 to 0.84 Aw the pH values remained relatively constant and acidic. The organoleptic scores of the PS also remained unchanged i.e. no fermentation (Table 28). When the Aw was raised to 0.96 increased viable counts were obtained on MRS, PCA and TJA. The pH increased from an initial value of 6.1 (Table 28) to 9.3. The increased and alkaline pH indicated that fermentation had occurred at this water activity level. The organoleptic scores developed (presence or absence of fermented product) were given in table 28. At Aw 0.11 - 0.84 fermentation product was not obtained. When the PS was placed in the 0.96 Aw chamber, very good fermented product was obtained. A control incubated in air also yielded the product. The PS usually has a pH in the range 5.60 to 6.20. If all environmental factors are satisfied, the pH may increase from 1 to 3 units in the fermented product.

The use of saturated salt solutions to generate the required Aw has been noted to have the advantage of maintaining constant humidity over a long period as long as the amount of salt present is above saturation level (Karel, 1975). However, when the Aw values are lowered as a result of

added solutes a hostile microenvironment is created. This affects the physiological activities of the microorganisms present in such a microenvironment. These organisms, in order to survive, must develop mechanisms to combat the hostile environment. This requires input of energy possibly diverted from other cellular activities. As a result of reduced energy, activities such as enzyme secretion for bioconversion may be curtailed. This may explain the lack of fermentation under reduced Aw condition. It has been suggested that during the osmotic stress created by high solute concentration in the environment it is possible that the microorganisms accumulate, intracellularly, a physiologically mild but osmotically active compound to counter the osmotic imbalance across their membrane. These osmotically active compounds were collectively called 'compatible solutes' (Gould and Measures, 1977). The compatible solutes include potassium ions in halophilic bacteria, polyols in yeasts, proline in osmotolerant bacteria, γ-amino butyric acid in moderately tolerant bacteria and glutamic acid in the least osmotolerant bacteria. In chapter 3 reference was made to high glutamic acid present in the amino acid analysis report. This acid may perform an osmoregulatory function in the microorganisms associated with the Parkia fermentations.

It is important to note that the Aw values reported were those operating within the enclosed equilibrated chambers. The author cannot say whether the same Aw as that of the enclosed chamber was operating on or within the processed substrate in the fermentation units. What was known was that under reduced Aw bioconversion would not occur. For fermentation to occur in the PS, there must be sufficient moisture i.e. available water. The Aw 0.96 was found to give a very good product.

## 4.9. EFFECT OF OXYGEN ($O_2$)

All forms of life, including microorganisms, require oxygen either in the free state as molecular oxygen ($O_2$) or in a combined state (CHO) as in a carbohydrate to carry out their metabolic activities. Microorganisms are grouped in accordance to their relative ability to utilize oxygen as aerobes, anaerobes, facultative anaerobes and microaerophiles. The aerobic strains are capable of using molecular oxygen ($O_2$) whereas the anaerobic ones can only utilize oxygen in the combined form (CHO). Others, such as facultative anaerobes will grow in the presence or absence of molecular oxygen while the microaerophilic bacteria generally survive better under reduced oxygen tension. Consequently, the amount of oxygen present in a particular food or in its environment determines to a large extent the type of microorganisms that will colonize it and as such the type of biochemical activities that may be expected to occur. Under atmospheric conditions foods can be, though very slowly, completely oxidized. For example, sugars and fats are oxidized to carbon dioxide and water while ammonia, carbon dioxide and water are the products formed from amino acids. Under reduced oxygen levels microbial activities will give rise to alcohols, lactic, acetic and formic acids from sugars. Proteins are degraded to amino acids, ammonia, hydrogen sulfide, sulfites, carbon dioxide and water. Glycerol and fatty acids, usually are the end products of microaerophilic utilization of fats. When the situation is completely anaerobic the degradation of proteins will give rise to butyl alcohol, mercaptans and hydrogen sulfide, all of which are foul smelling compounds. In this case the process is referred to as putrefaction

(Weiser et al., 1971). Since microorganisms are involved in the process of Parkia bioconversion, they must exhibit some form of oxygen dependence on the processed substrate. The results of experimental studies of such relationships are presented below.

### 4.9.1. Objectives - Effect of $O_2$

The objective of this experiment was to determine the relationship of molecular oxygen to the process of Parkia bioconversion.

### 4.9.2. Principle - Effect of $O_2$

Processed substrate is incubated under aerobic and anaerobic conditions. Homogenates are prepared from each set condition. The viable counts, developed pH and titratable acidity are then determined from the prepared homogenates.

### 4.9.3. Materials and Methods - Effect of $O_2$

The sample used was PS in fermentation units. GasPak Anaerobic jars with GasPak Anaerobic Systems (BBL) were employed. Detailed instructions for the use of these are well documented on each GasPak envelope and the enclosed literature with each box of GasPak Anaerobic Systems (BBL, 1977). The prepared fermentation units containing the processed substrate were

separated into two groups. One group was incubated under anaerobic conditions in GasPak Anaerobic Jars (BBL). Anaerobic conditions were created by the addition of 10 ml of water to the content of each GasPak pre-positioned in the GasPak Anaerobic Jar. The jar was immediately closed and sealed. The GasPak anaerobic indicator was used to monitor the effectiveness of the developed anaerobic condition in each GasPak Anaerobic Jar. Checks were also made for visible condensation in the jar within 30 min of closing the jar. Only jars with visible condensation were acceptable (BBL, 1977). All jars were incubated at 37 $\pm$ 2°C. The second group was incubated in air, to serve as controls, also at 37 $\pm$ 2°C. Time sequenced viable counts were carried out, only for the units incubated anaerobically, on MRS, PCA, TJA and WA using the SPC method (section 4.1.4.). Time sequenced pH and titratable acidity developed were also determined as described in section 4.4.3. In these latter determinations both the anaerobic and the aerobic control units were analyzed.

### 4.9.4. Observations - Effect of $O_2$

In Fig. 38 are shown the time sequenced viable counts obtained from PS samples incubated anaerobically at 37°C. The corresponding pH and titratable acidity (T.A.) developed in the PS are shown in Fig. 39. The pH and TA of the control samples are also shown in Fig. 39.

Fig. 38: Time Sequenced Viable Counts ($log_{10}CFU \cdot g^{-1}$) of PS Incubated Anaerobically at $37^{o}C$.

MRS: de Man Rogosa Sharpe medium

PCA: Plate Count agar

TJA: Tomato Juice agar

WA : Wort agar

Figure 38

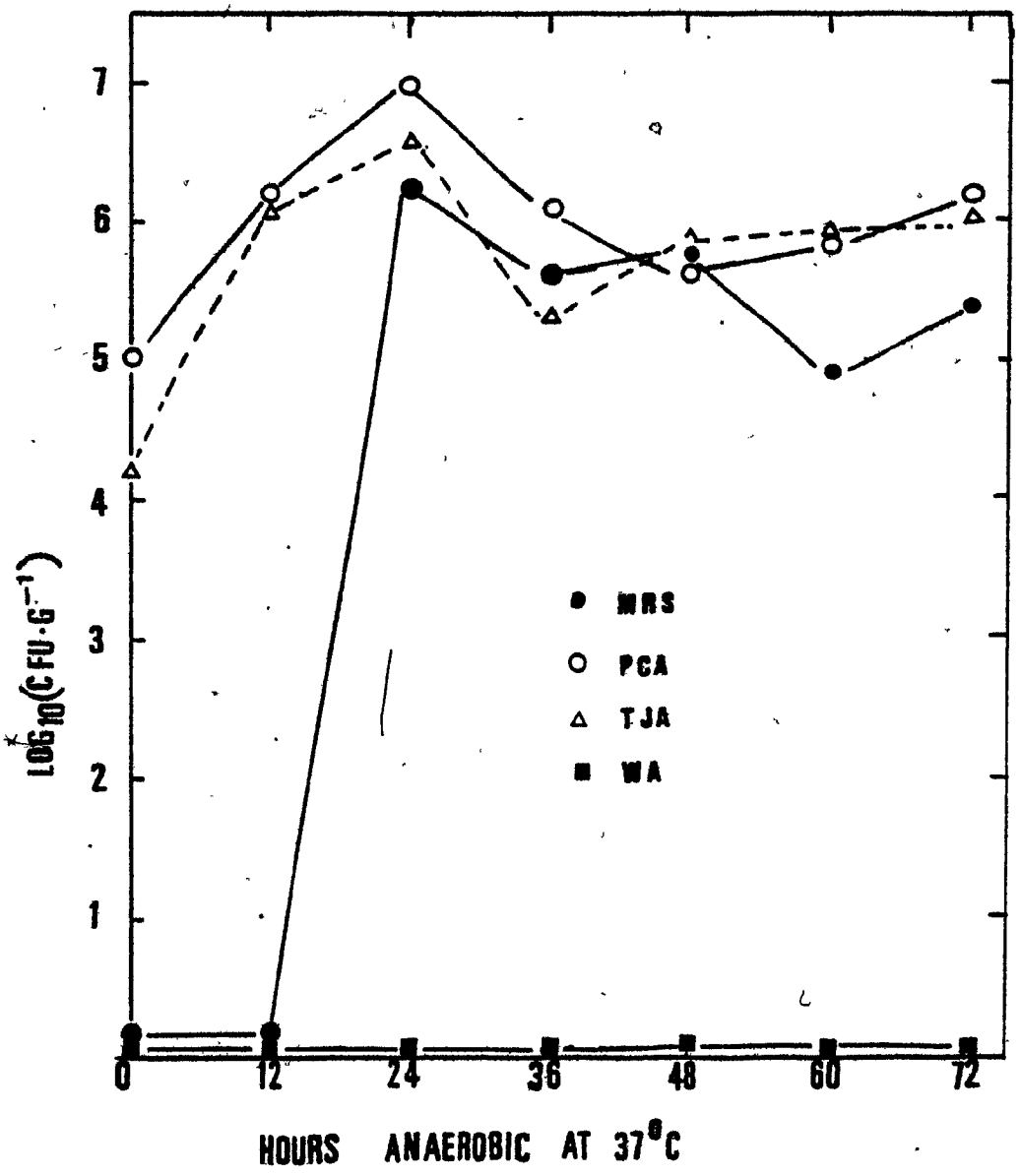
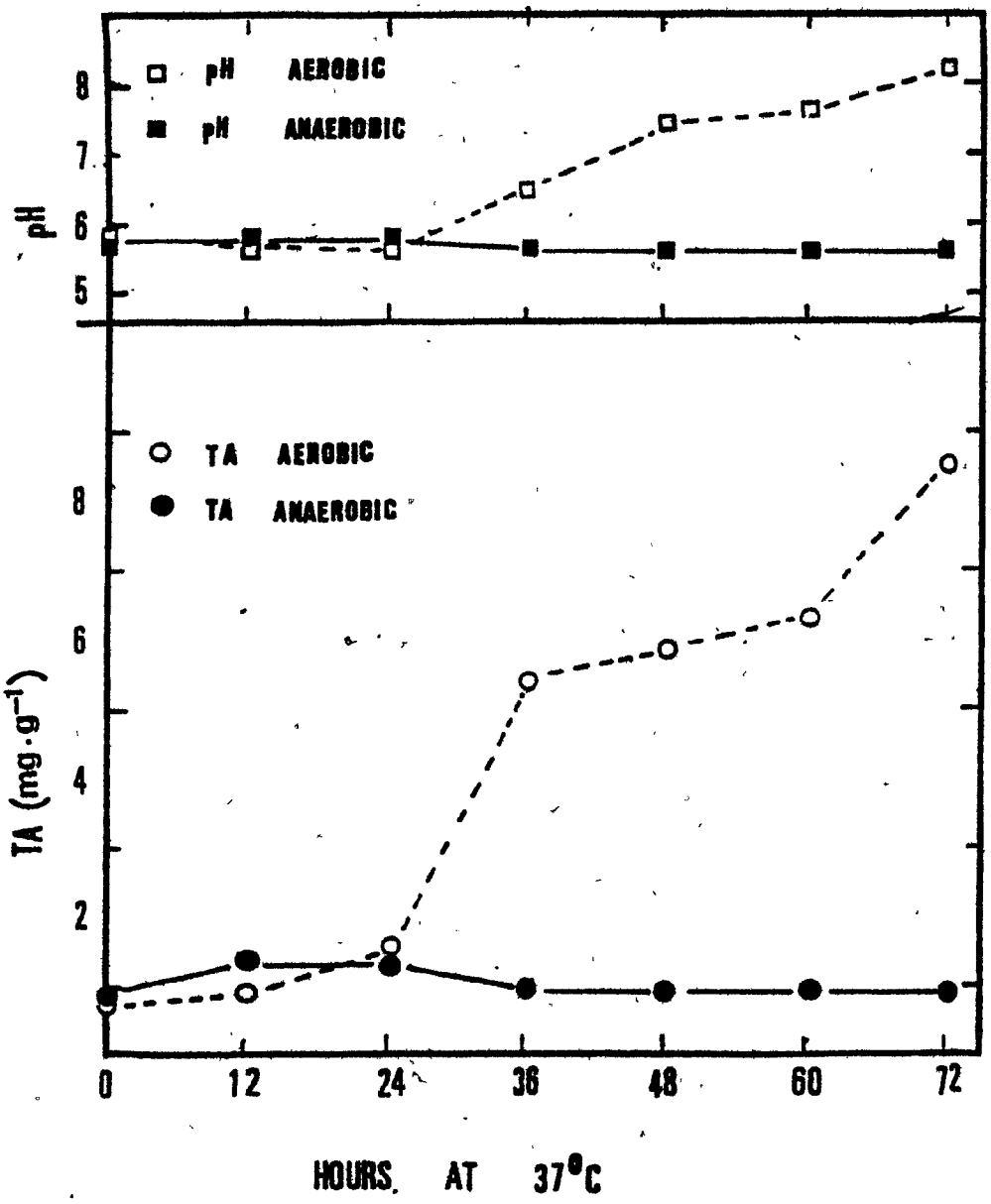

7
6
5
4
3
2
1
0
12
24
36
48
60
72
MRS
PCA
TJA
WA
HOURS ANAEROBIC AT 37°C

Fig. 39: Time Sequenced pH and TA Developed on PS Incubated Anaerobically and Aerobically at 37°C.

TA: Titratable Acidity

Figure 39


pH AEROBIC
pH ANAEROBIC
pH
8
7
6
5
TA AEROBIC
TA ANAEROBIC
TA (mg·g⁻¹)
8
6
4
2
0
12
24
36
48
60
72
HOURS AT 37°C

### 4.9.5. Discussion - Effect of $O_2$

The general viable counts under anaerobic condition (Fig. 38) were poor ($1 \times 10^7$ CFU/g maximum) when compared to similar counts made under aerobic conditions (about $1 \times 10^9$ CFU/g; table 22). The low anaerobic counts may have been due to some of the species' inability to tolerate the anaerobic condition. The developed pH and titratable acidity (T.A.) of the PS remained low and fairly constant throughout the period of anaerobic incubation (Fig. 39). The samples incubated aerobically developed high pH and T.A. (Fig. 39) and were fermented within 72 h. Under strictly anaerobic conditions the correct type of bioconversion will not occur i.e. the PS will not form dawadawa. There has been one literature report in which African locust bean, Parkia filicoidea, was said to ferment in an anaerobic situation (Stanton and Wallbridge, 1969). Our experience, from this experiment, showed that this was not so.

## 4.10. HYPOTHESES OF BIOCONVERSION

A hypothesis can be defined in one of several ways as:

(1) A tentative assumption made in order to draw and test its logical or empirical consequences, or

(2) an interpretation of a practical situation or condition taken as the ground for action, or

(3) a formulation of a natural principle based on inference from observed data.

Prior to industrial scale up, it is usual for fermentation processes to be studied and investigated on a laboratory scale in small units. To avoid loss of time and resources the fermentation scientist always designs experiments with a view of the ultimate goal in mind. An experiment tests a hypothesis and the hypothesis has to be precisely written with its assumptions and implications thoroughly examined and understood. According to Davis and Blevins (1979) an experiment should be designed in such a manner that 'it unequivocally demonstrates that the hypothesis is either true or false'. However, they observed that such an experiment is rare and that in practice it is usually a matter of to what relative degree the data support or contradict the hypothesis.

For a long time the bioconversion of the hard seeds of Parkia to the soft texturized dawadawa has evoked the author's interest. The fact that containers and carried-over inoculum could perpetuate the process satisfies part of that inquiry. However, when Parkia beans were imported and plastic containers and petri dishes which had never been used before for dawadawa

production were employed, the fermented product was obtained. In this case there were no carried-over inoculum or containers loaded with the right type of microorganisms. The conviction then was strong that the beans themselves must carry the right type of microorganisms. The question then follows that if this were true how could the microorganisms survive the harsh processing conditions and then develop to a level to bring about bioconversion in 72 h? Searching for the answers to these questions resulted in the postulation of the hypothesis of bioconversion as outlined below and in the flowchart shown in Fig. 40.

HYPOTHESES:

(1) The spontaneous fermentation of the solid substrate of P. filicoidea Welw. and related species is due principally to sporulating microorganisms.

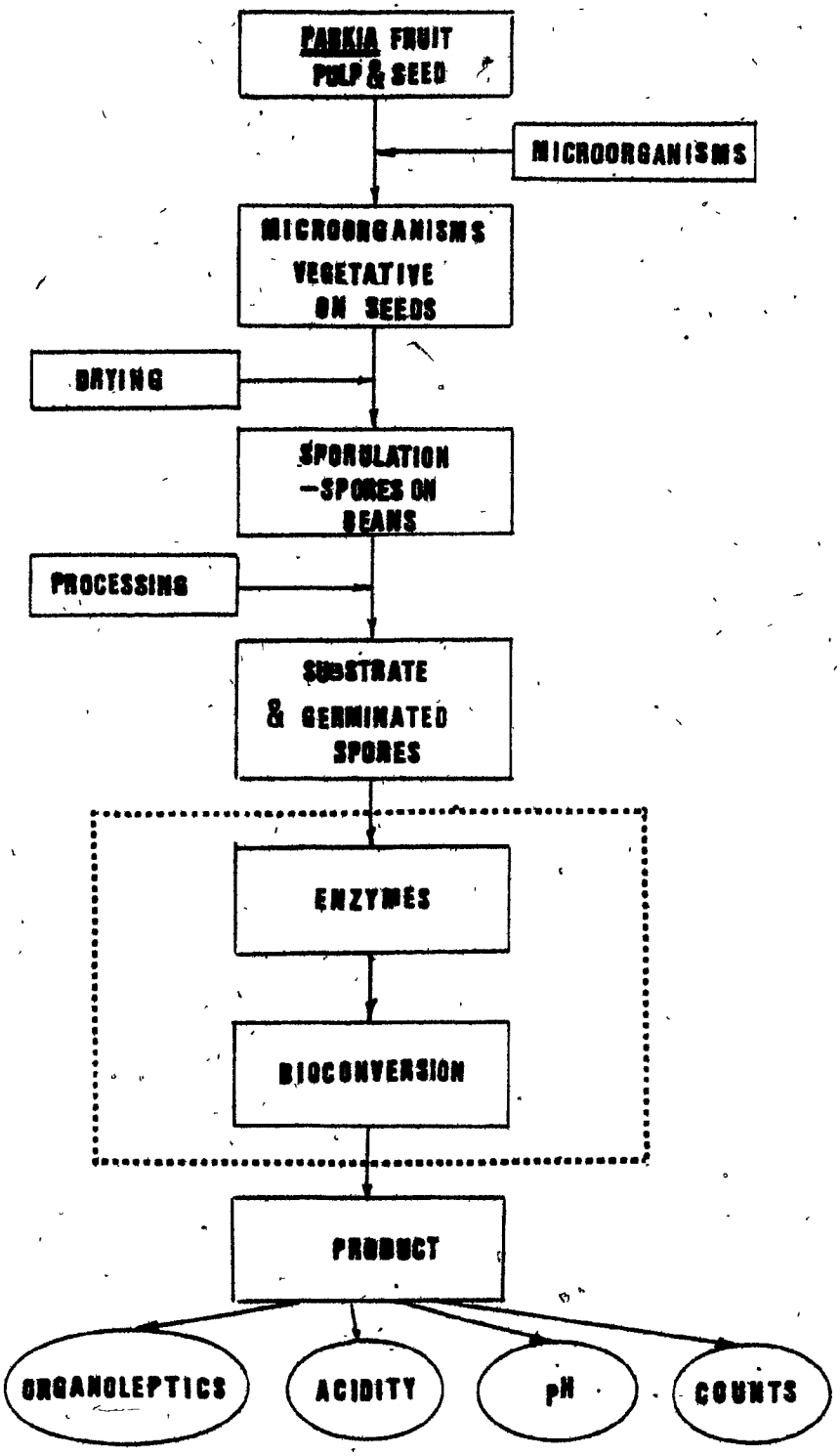
(2) The spores produced were resistant and able to survive 8 h or more of boiling over a hot plate. The heating and subsequent processing provided the necessary heat shock to allow vegetative germination of spores.

(3) The vegetative cells, now on the processed substrate, derived energy from the substrate, produced biocatalysts in the form of extracellular enzymes which carried out the process of bioconversion. The biocatalysts produced are so effective that the substrate is softened and texturized. Solubilization and deamination may even occur if the reaction is allowed to continue for longer than the usual 72 h.

As a corollary it is assumed that the initial vegetative cycle of the organism developed and derived energy from the adhering pulpy material,

Fig. 40: Hypothesis of Bioconversion of *Parkia* Beans to Product (Dawadawa) Flowchart Form.

Figure 40


PARKIA FRUIT PULP & SEED
MICROORGANISMS
MICROORGANISMS VEGETATIVE ON SEEDS
DRYING
SPORULATION —SPORES ON BEANS
PROCESSING
SUBSTRATE & GERMINATED SPORES
ENZYMES
BIOCONVERSION
PRODUCT
ORGANOLEPTICS
ACIDITY
pH
COUNTS

mostly sweet carbohydrate, on the seeds. During sun drying (usual method employed to preserve the Parkia seeds in Nigeria) continued loss of moisture from the pulp created microenvironment problems for the microorganisms. The organisms therefore sporulated to perpetuate their existence.

It is further assumed that the survival of the microorganisms through the harsh processing, and from generation to generation in the hands of those who know little or no microbiology is an indication that a strong survival mechanism is present. Sporulation seems to fit this mechanism. A probable alternative to sporulation is thermostability. The microorganisms in this case are thermostable i.e. resistant to heat and temperatures of 100°C. This may be due to their genetic make up and enzymes present in them.

If the proposed hypothesis of sporulation were to be true then it could be verified in the laboratory. The subsequent sections were designed partly towards this end.

## 4.11. MODIFIED KOCH'S POSTULATES

A hypothesis was postulated for the bioconversion of the processed substrate (PS) to dawadawa (section 4.10.). In earlier sections strong interaction was reported to occur between the microorganisms and the PS during the process of bioconversion. However, diverse kinds of microorganisms are associated with the process i.e. counts on MRS and TJA are indicative of the presence of lactic acid bacteria while PCA counts indicate a mixed population. The major interest however was to find out which type or types of microorganisms are responsible for the bioconversion. This led to the use of modified Koch's postulates. Koch's postulates deal with disease causing microorganisms and their relationship with their hosts. The postulates are four in number. These are listed below:

(1) Association - The microorganisms responsible for a particular disease are always found in association with that particular disease.

(2) Isolation in pure form - The organisms can be isolated from the diseased tissue in pure form.

(3) Re-infection of healthy tissue - When pure isolate is employed to inoculate a healthy tissue of the host, the tissue will develop similar symptoms like that of the original diseased tissue from which the isolate was obtained.

(4) Re-isolation - The organisms can be re-isolated from the newly infected tissue.

These postulates were employed with the Parkia fermentation with two modifications:

(i) The ability of the organism to cause disease was modified to its ability to cause fermentation.

(ii) The healthy tissue was taken as the radappertized beans (RADB) in which microorganisms had been eliminated.

### 4.11.1. Objectives - Modified Koch's Postulates

The objectives of this experiment were:

(i) To obtain pure isolates.

(ii) To obtain photomicrographs of the pure isolates.

(iii) To test these isolates' ability to cause bioconversion of radappertized beans (RADB).

### 4.11.2. Principle - Modified Koch's Postulates

Pure isolates are obtained by streaking isolated colonies on solid media. Slides are prepared from the pure cultures and their photomicrographs taken. Individual pure isolates are employed to inoculate radappertized beans (RADB). The pH developed with time on RADB is followed and it is taken as a measure of a particular isolate's ability to bioconvert the substrate to dawadawa.

4.11.3. Materials and Methods - Modified Koch's Postulates

The samples used included raw beans (RB), processed substrate (PS), processed fermenting substrate (PFS), fermented beans (FB) and radappertized beans (RADB). The media employed to obtain pure isolates included MRS, PCA, PDA, TJA and WA. Phase contrast microscope (Carl Zeiss) was used to obtain photomicrographs of isolates. A counting chamber (Petroff-Hausser Bacteria Counter, Phila., U.S.A.) was used to determine the number of bacteria in broth media.

4.11.3.1. Methods: Pure Isolates: Pure isolates were obtained from RB, PS, PFS and FB throughout the investigation period. Colonies were obtained as described in sections 4.1.3. and 4.1.4. above. Colonies which were sufficiently differentiated macroscopically by their colonial morphology and separated from other colonies were isolated by streaking onto similar, solidified surface dried agar media. The bacterial isolates obtained from the streaked plates were transferred into broth media corresponding to those of the agar media from which the isolates were obtained. From the broth media they were transferred into microslants. All incubations between the transfers were done at 37 $\pm$ 2$^{o}$C. The microslants were stored at 4$^{o}$C. Acidified PDA medium was used to isolate the molds. These were isolated into test tube slants of PDA, developed at 30$^{o}$C and then stored at 4$^{o}$C.

4.11.3.2. Methods: Photomicrographs: A phase contrast microscope (Carl Zeiss) was used. Agar, 1.5 g, was dissolved completely in 89.2 ml of

water by constant stirring with a magnetic stirrer on a hot plate in a fume hood. Formaldehyde (37%), 10.8 ml, was added to the dissolved agar and then stirred to mix completely. The formaldehyde agar was kept in a water bath at 45-50°C and was used in preparing the slides. A clean slide was dipped into the agar for 5 seconds, withdrawn and drained of excess agar. One side of the slide was wiped clean and the thin agar layer side was allowed to set before a drop of the broth culture was added. A coverslip was placed on top of the culture and gently pressed to expel excess water and to make the cells stationary. Phase contrast photographs of the stationary cells were taken. For the molds, slant cultures were employed. Both light and phase contrast photographs of these were taken.

4.11.3.3. Methods: Testing Isolates: Bacterial isolates selected for the test were grown in their respective broth culture media (i.e. MRS broth, m Plate count broth, tomato juice broth) to a population of $1 \times 10^6$ cells per ml, (counting chamber). To prepare an inoculum a 10 ml portion of the growth medium was aseptically harvested and spun down at 10,000 g for 10 min in a refrigerated (4°C) centrifuge. The cells were washed twice, aseptically, by resuspending the pellet of cells with 10 ml sterilized distilled water and spinning down at 10,000 g. The cells were again suspended in 10 ml of sterilized distilled water by vortex. The final suspension was used as the inoculum. Radappertized beans (RADB) were inoculated with 4% (V/W) of the inoculum. Controls were provided by processed substrate (PS) and by RADB samples which had not been inoculated. The inoculated RADB and the controls were incubated at $37 \pm 2$°C. The pH developed in each sample was followed on a time sequenced

basis and was used as a measure of bioconversion. Molds were similarly tested. In this case the inoculum of each isolate tested was obtained from slant cultures. Spores were aseptically obtained by shaking the slant culture on the PDA with sterilized distilled water and sterilized glass beads. The outline of the process is given in flowchart form in Fig. 41.

4.11.4.1. Observations: Pure Isolates: The pure isolates obtained from RB, PS, PFS and FB are presented in table 29.

4.11.4.2. Observations: Photomicrographs: Phase contrast photomicrographs of pure isolates from RB, PS, PFS and FB are shown in Figs. 42 to 52.

4.11.4.3. Observations: Testing Isolates: In Fig. 53 are shown the pH curves obtained with one of the pure isolates and also for the controls employed.

### 4.11.5. Discussion - Modified Koch's Postulates

About 1000 pure isolates were obtained from RB, PS, PFS and FB during the course of this study. The majority of these were Gram positive, sausage shaped spore forming rods. The organisms isolated were all coded using the media on which they were cultured (Table 29). The Bacillus spp. was regularly found associated with the fermentation and was isolated on almost all the media used including unacidified PDA. It will not develop on acidified PDA medium. Its spores were cylindrical and sub-terminally

Fig. 41: Basic Flowchart of Isolation of Pure Cultures and Testing Individual Isolate for Ability to Bioconvert the RADB. (Uninoculated RADB and PS were used as controls).

Figure 41

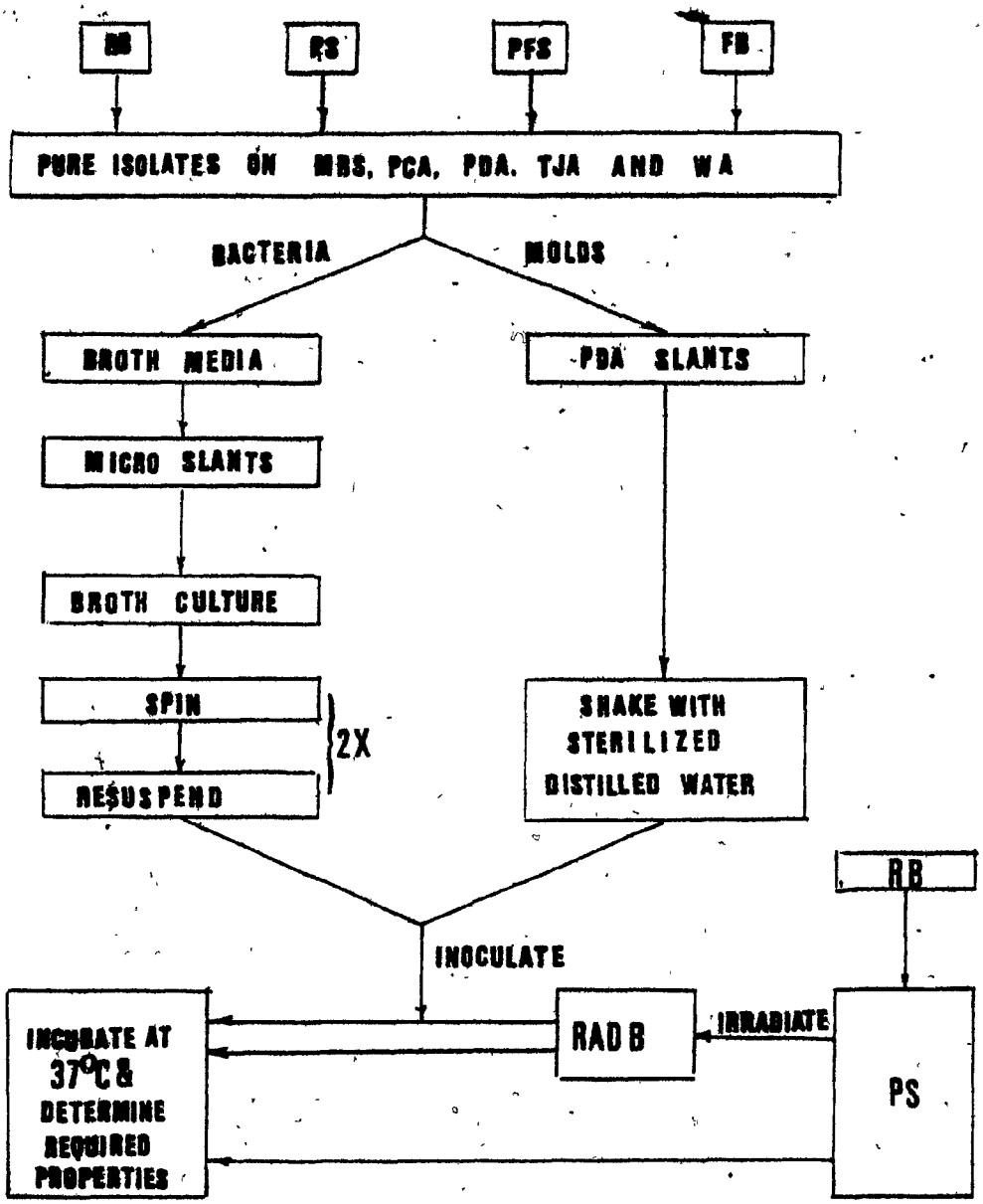

MS
RS
PFS
FB
PURE ISOLATES ON MRS, PCA, PDA, TJA AND WA
BACTERIA
MOLDS
BROTH MEDIA
PDA SLANTS
MICRO SLANTS
BROTH CULTURE
SPIN
RESUSPEND
2X
SHAKE WITH STERILIZED DISTILLED WATER
RB
INOCULATE
INCUBATE AT 37°C & DETERMINE REQUIRED PROPERTIES
RAD B
IRRADIATE
PS

Table 29. Pure Isolates.

| Pure Isolate* Code | Microscopic Description of Cells |
|---|---|
| MRS - 1 (13) | Bacillus - sporulating sausage shaped cells |
| MRS - 2 ( 5) | Elongated asporogenous rods |
| PCA - 1 (21) | Bacillus - sporulating sausage shaped cells |
| PCA - 2 ( 1) | Coccobacilli |
| TJA - 1 (11) | Bacillus - sporulating sausage shaped cells |
| TJA - 2 ( 3) | Coccobacilli |
| TJA - 3 ( 1) | Slender rods |
| WA - 1 ( 9) | Sausage shaped rods |
| WA - 2 ( 1) | Large bead-like cells |
| PDA - 1 ( 2) | Phycomycetes - Rhizopus spp. |
| PDA - 2 ( 1) | Ascomycetes - Aspergillus spp. |
| PDA - 3 ( 1) | Ascomycetes - Penicillium spp. |

* Isolates obtained from RB, PS, PFS, and FB.

Parenthesis: Probable number of strains of the same organisms isolated; based on macroscopical features of colonies.

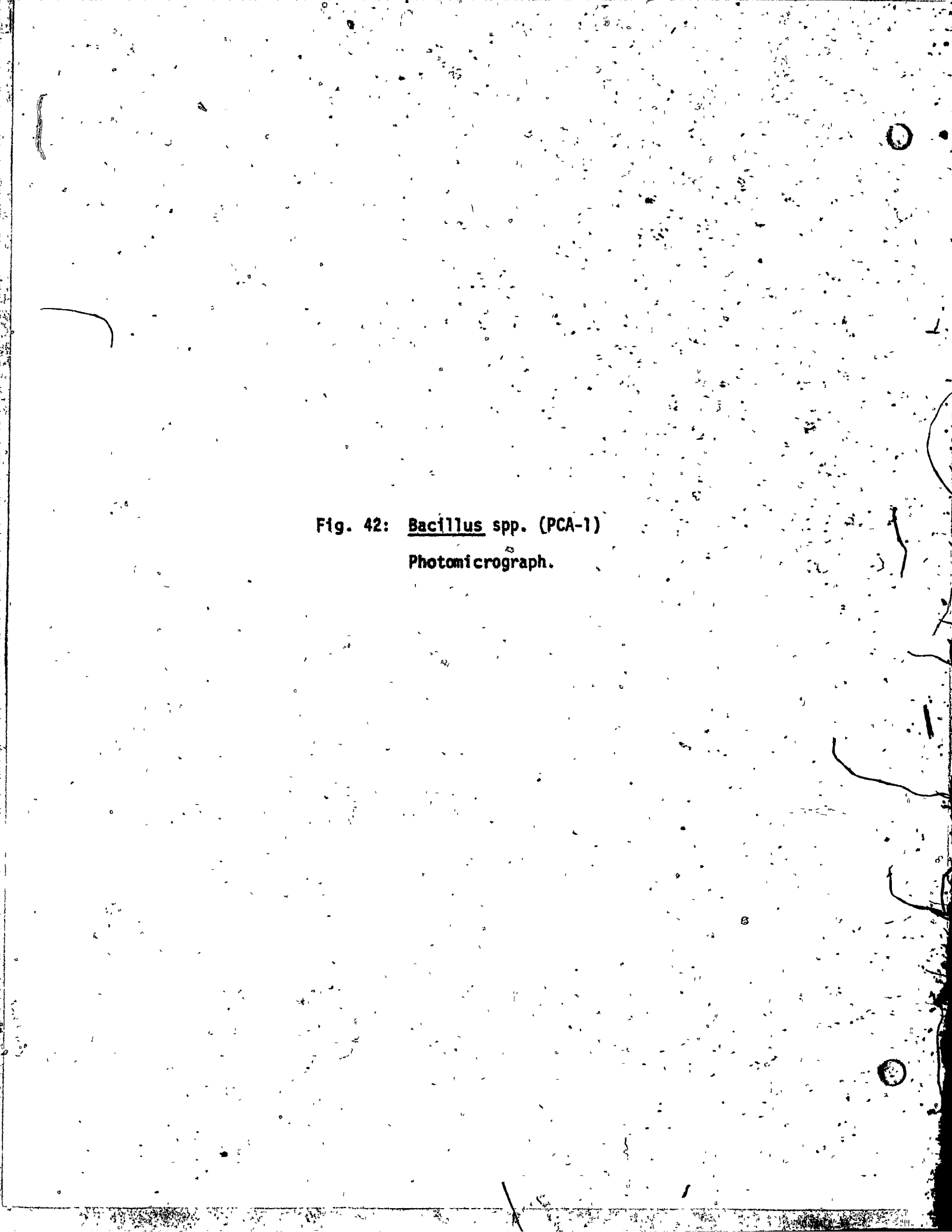


Fig. 42: Bacillus spp. (PCA-1)
Photomicrograph.

Figure 42



Fig. 43: Bacillus spp. (PCA-NS)
Photomicrograph.

Figure 43



Fig. 44: Bacillus spp. (Young Cells; PCA-1) Photomicrograph.

Figure 44



Fig. 45: Asporogenous Rods (MRS-2) Photomicrograph.

Figure 45


1.0 μm

Fig. 46: Coccobacilli (TJA-2)
Photomicrograph.

Figure 46

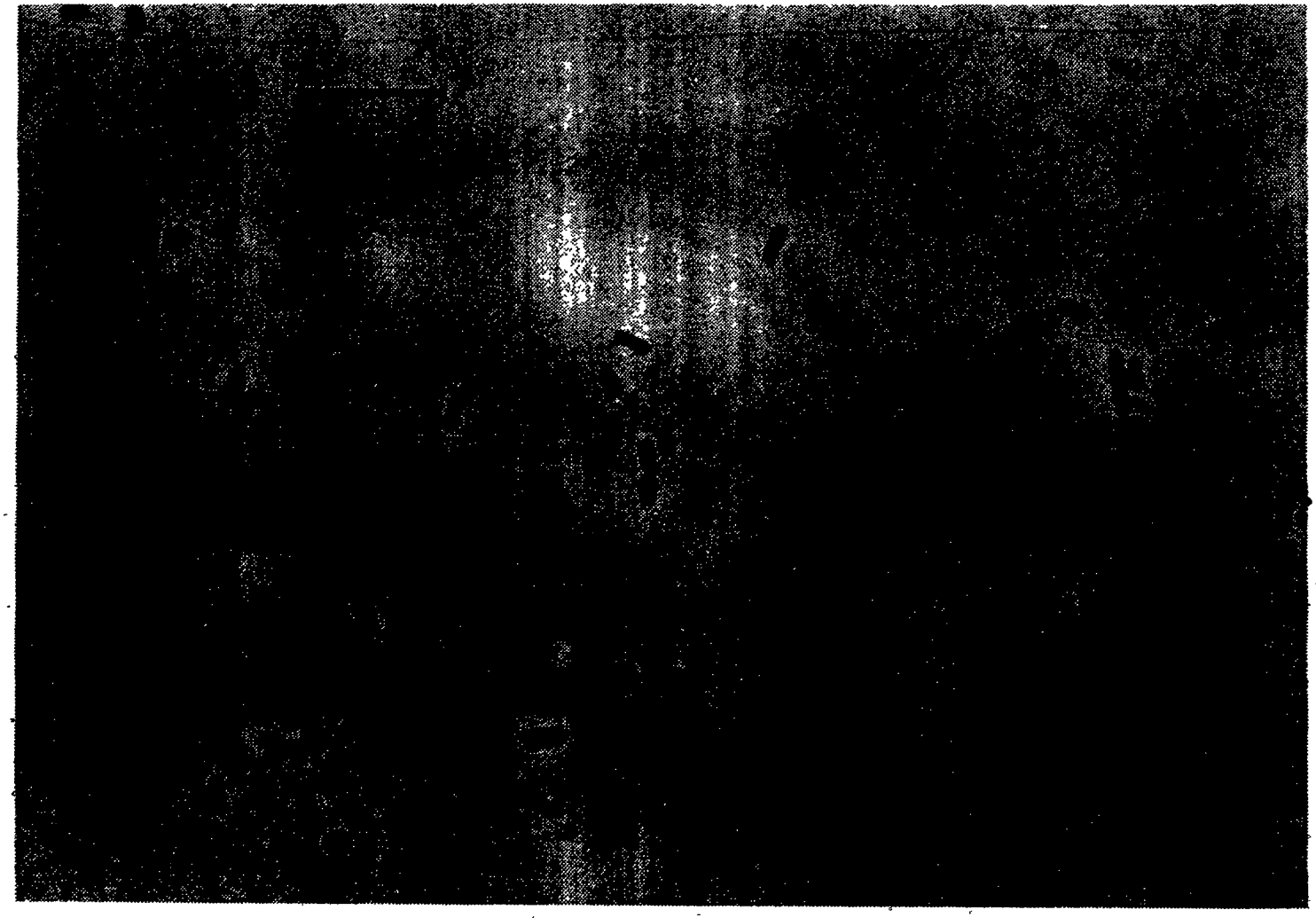
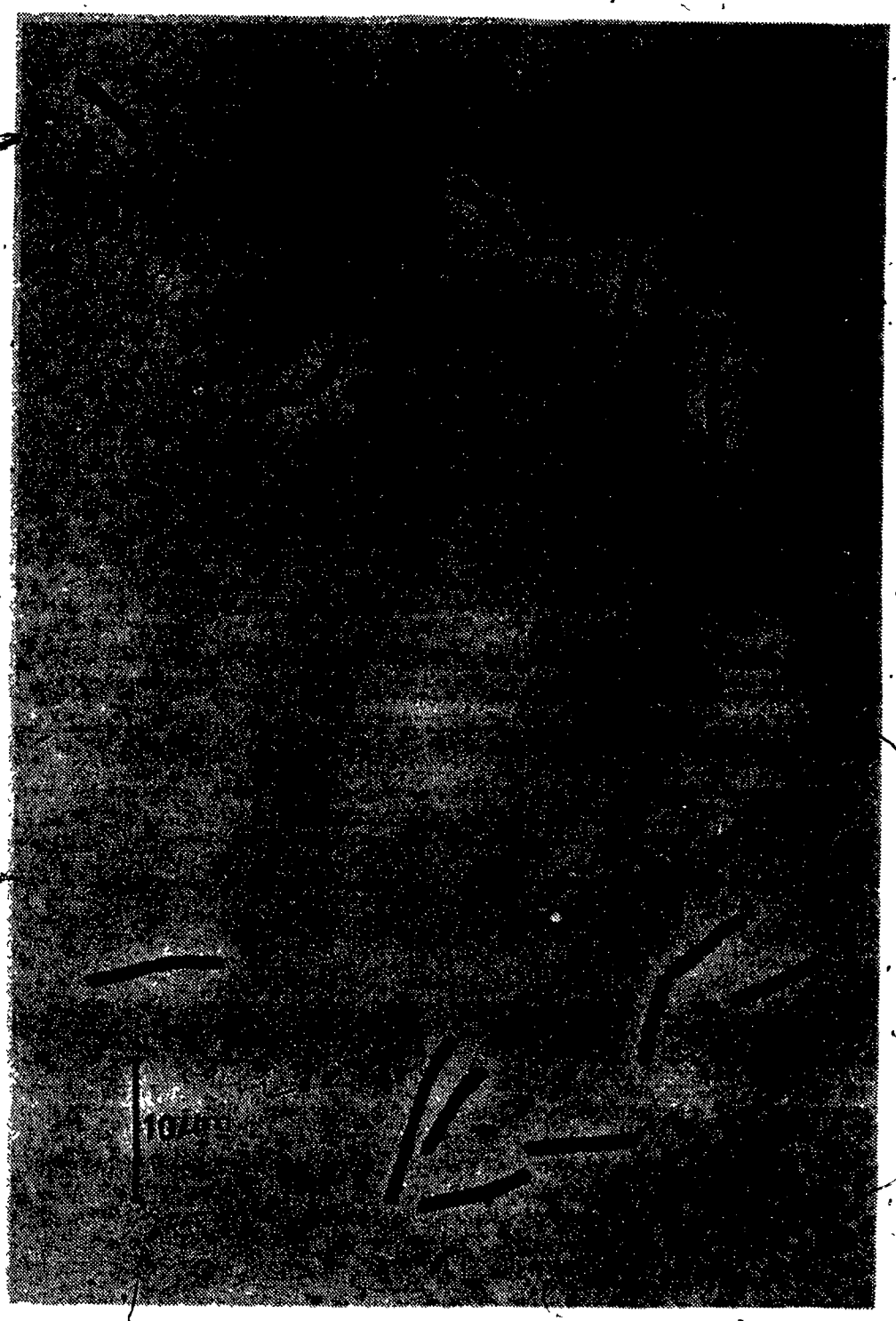


Fig. 47: Slender Rods (TJA-3)

Photomicrograph.

Figure 47



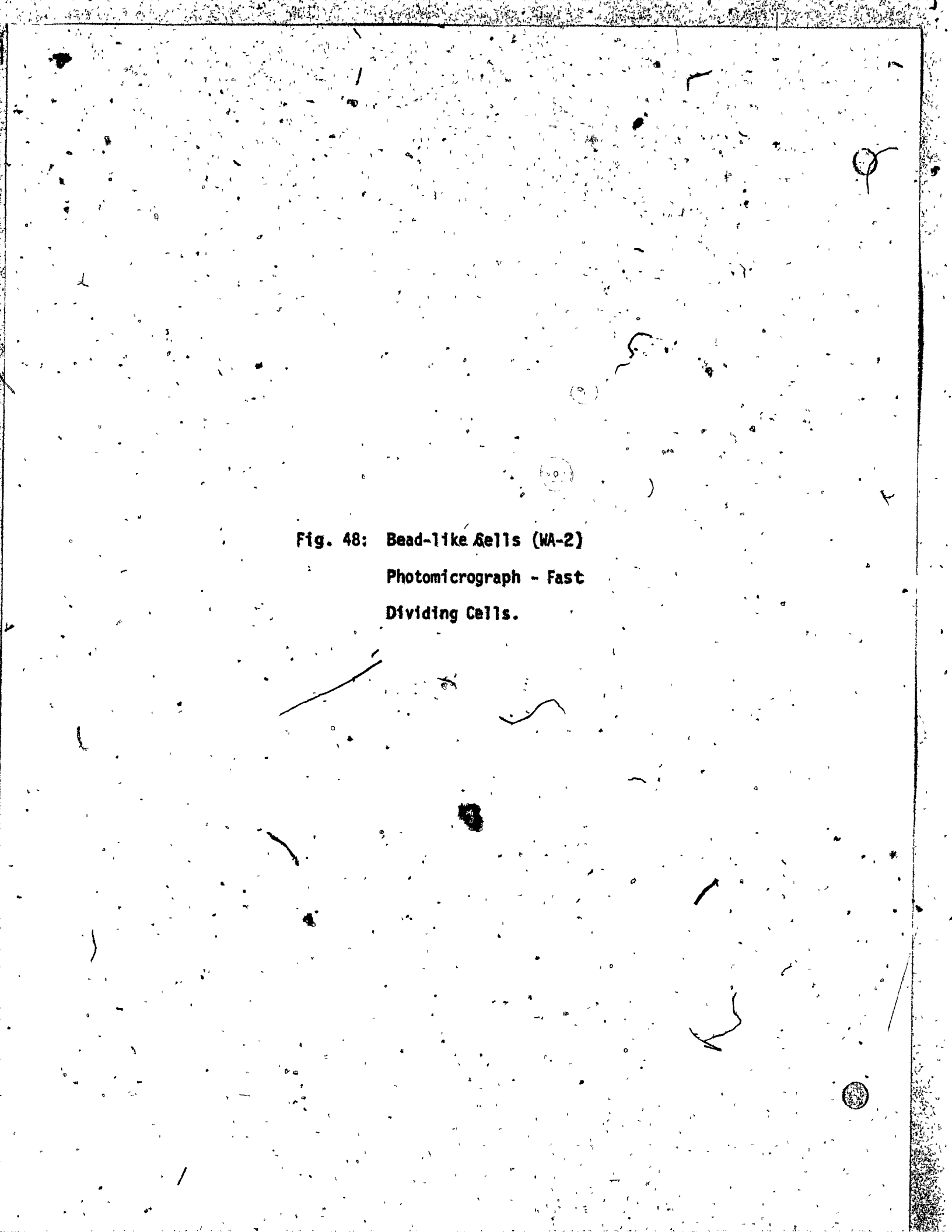
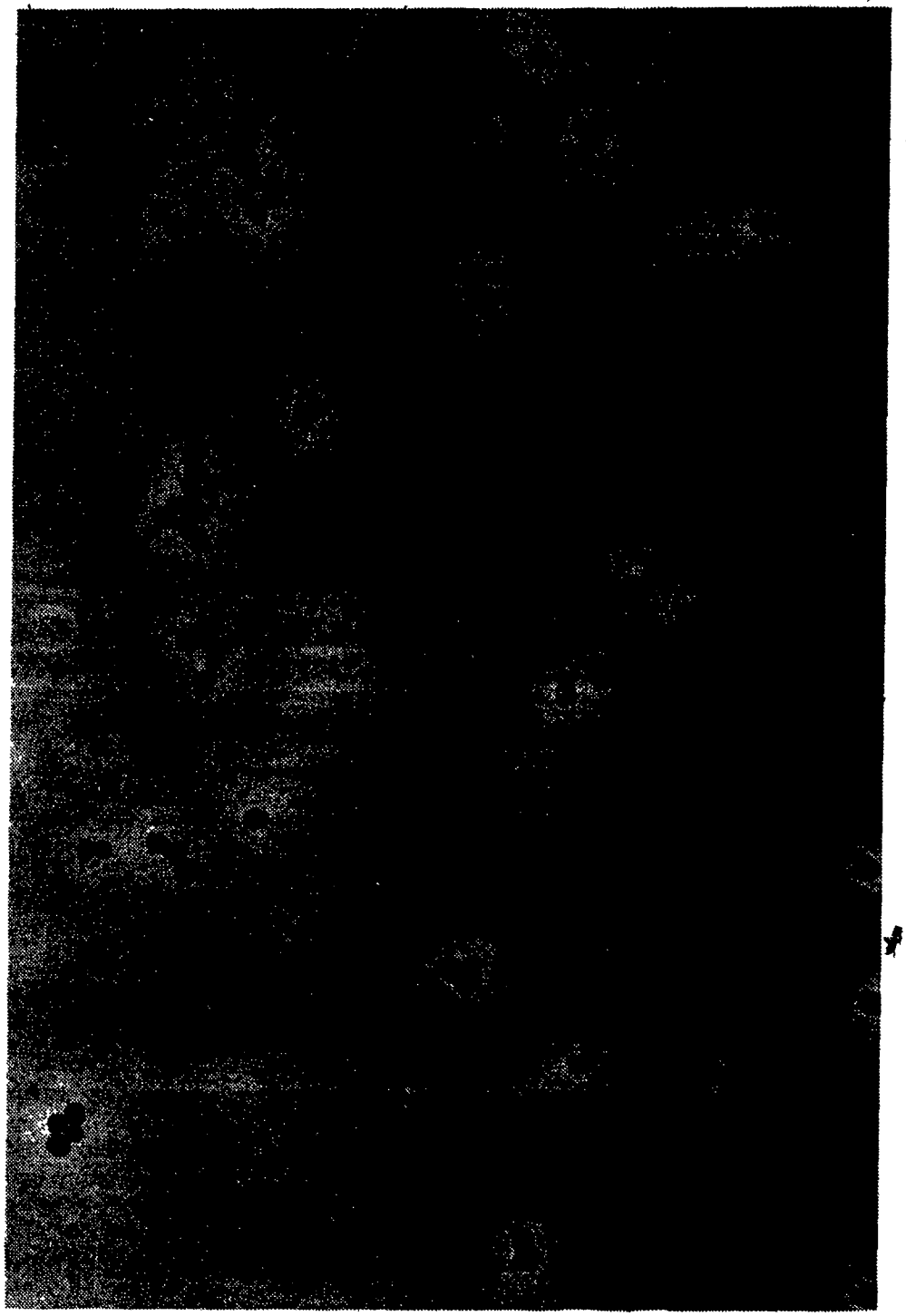


Fig. 48: Bead-like Cells (WA-2) Photomicrograph - Fast Dividing Cells.

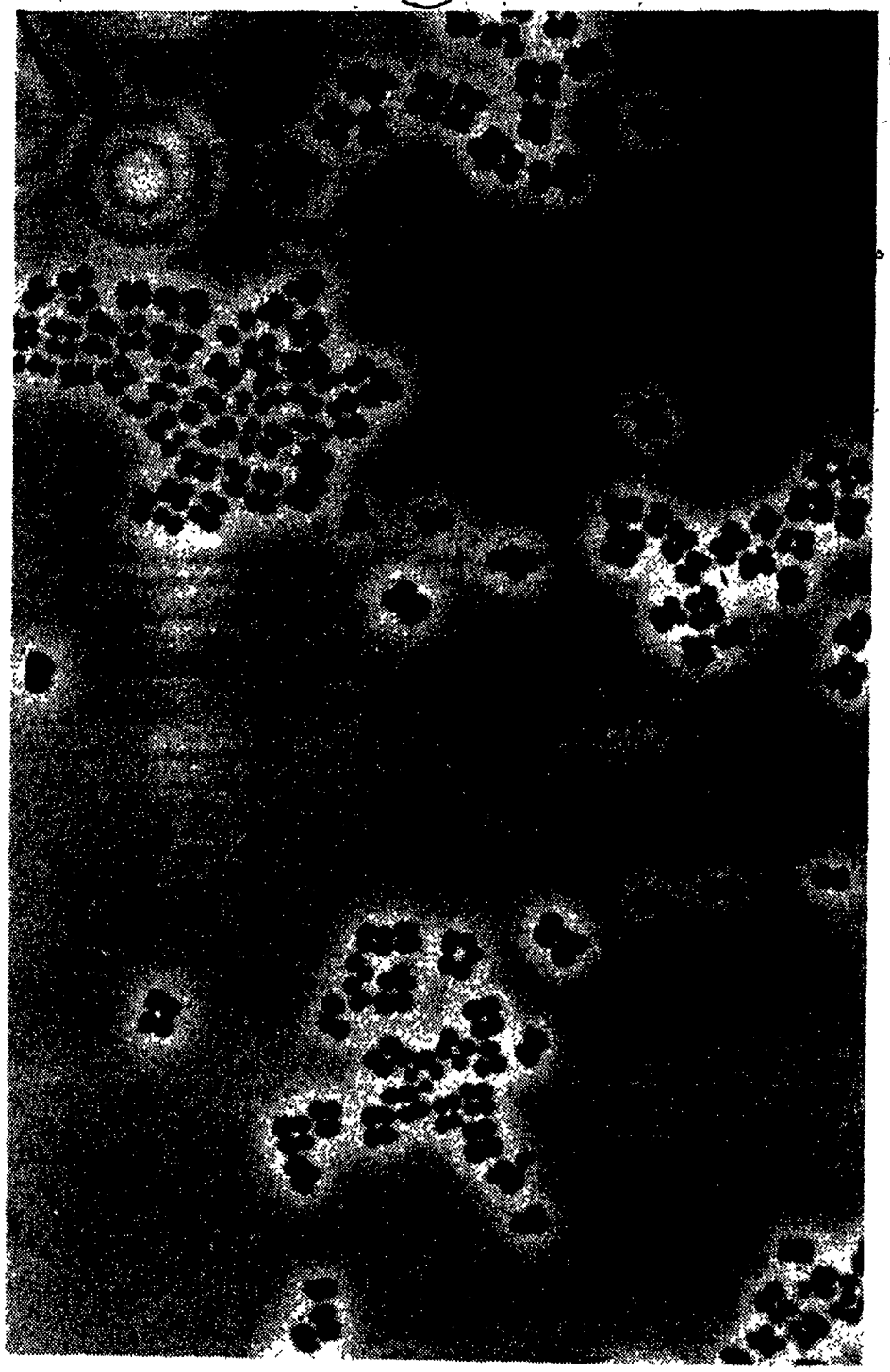
Figure 48



Ftg. 49: Bead-like Cells (WA-2) Photomicrograph - More Cells.

Figure 49





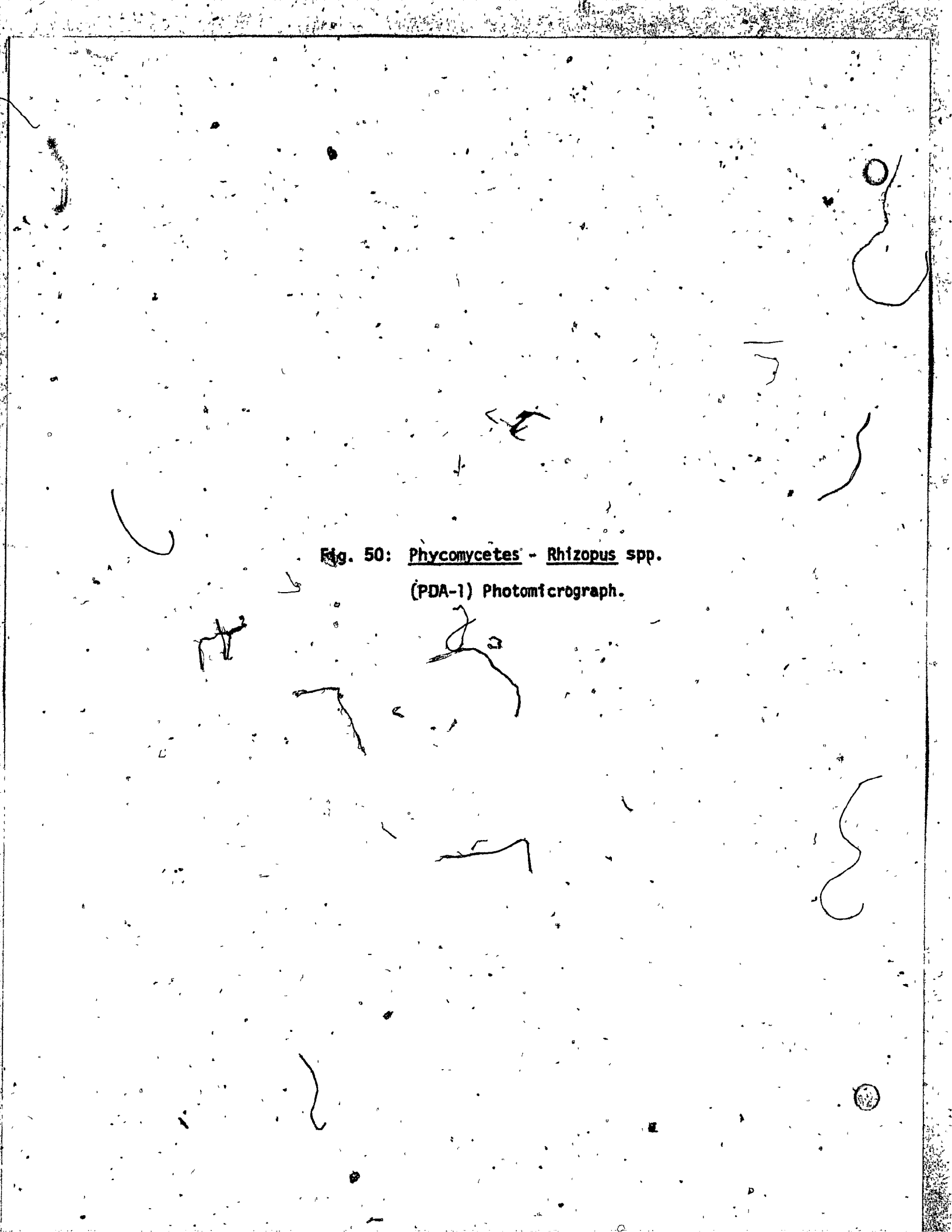


Fig. 50: Phycomycetes - Rhizopus spp. (PDA-1) Photomicrograph.

Figure 50



Fig. 51: Ascomycetes - Aspergillus spp. (PDA-2) Photomicrograph.

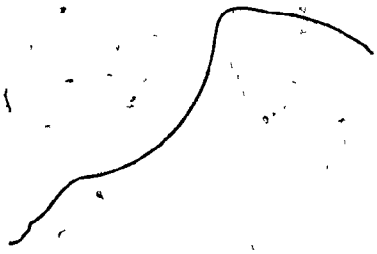


Figure 51



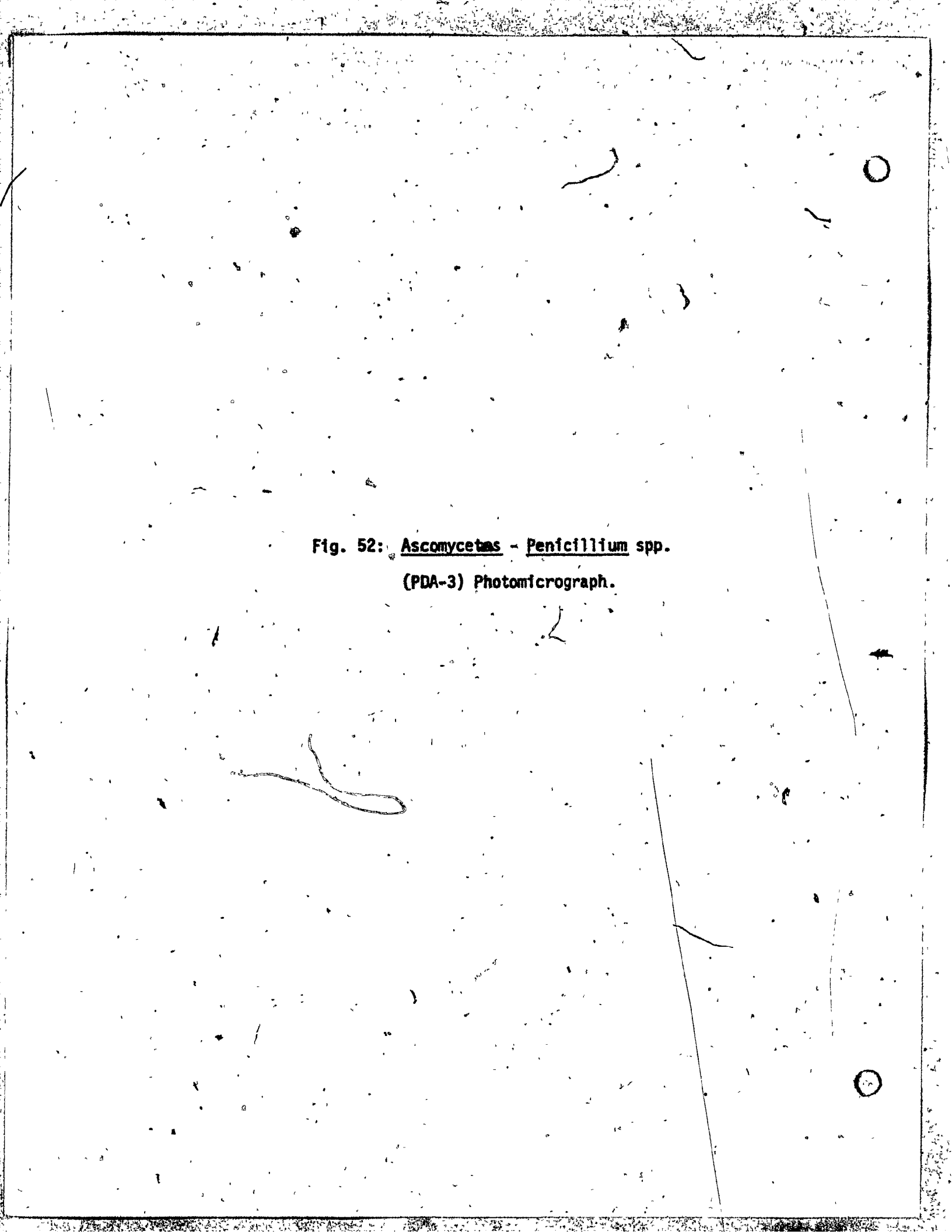


Fig. 52: Ascomycetes - Penicillium spp. (PDA-3) Photomicrograph.

Figure 52

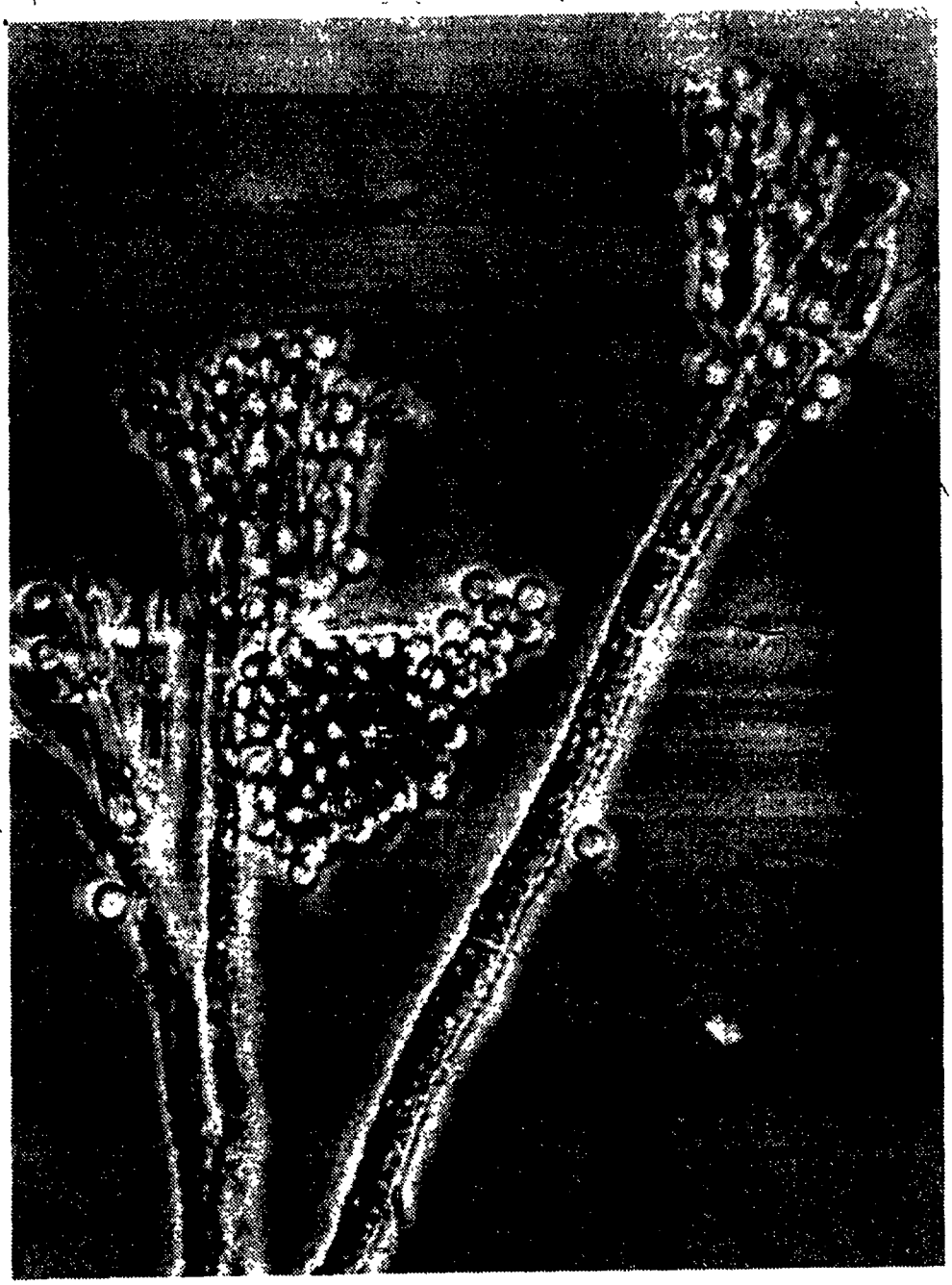
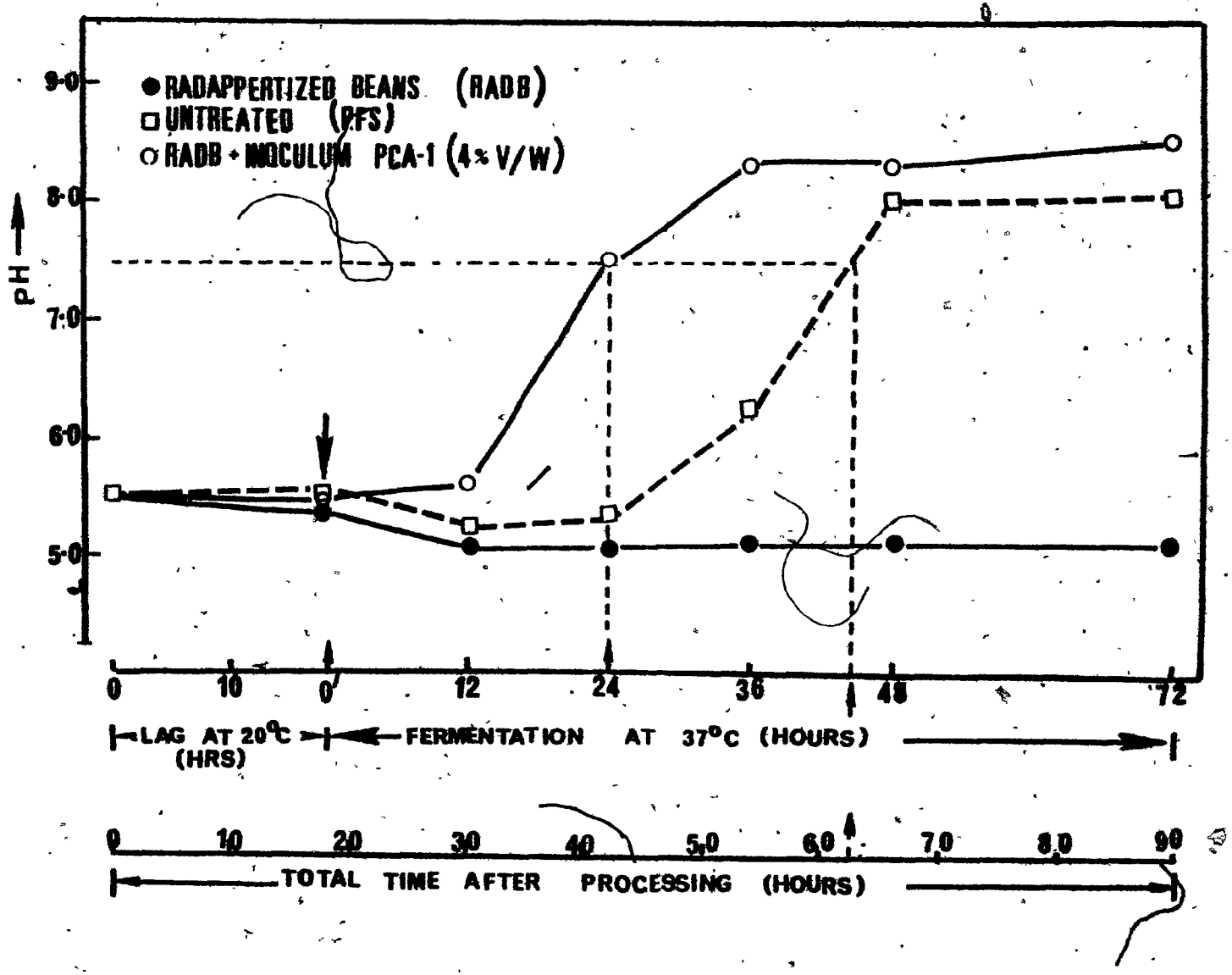


Fig. 53: Time Sequenced pH Developed on RADB Inoculated with Pure Culture PCA-1 and Controls of Untreated PS and Uninoculated RADB.

Figure 53


● RADAPPERTIZED BEANS (RADB)
□ UNTREATED (PFS)
○ RADB + INOCULUM PCA-1 (4% V/W)
pH
9·0
8·0
7·0
6·0
5·0
0
10
0
12
24
36
48
72
LAG AT 20°C (HRS)
FERMENTATION AT 37°C (HOURS)
0
10
20
30
40
5·0
6·0
7·0
8·0
90
TOTAL TIME AFTER PROCESSING (HOURS)

located with thick exosporium (Figs. 42 and 43). Other isolates included Coccobacilli, asporogenous rods and slender rods. These latter ones were probably lactic acid bacteria. Bead-like cells which divide very rapidly were isolated on WA. This isolate was from RB and it may be a yeast. All the isolates on acidified PDA were from RB. These were all molds (Table 29).

During the course of this study about 500 phase contrast photomicrographs of pure isolates were obtained. Figures 42 to 52 were some representatives of these. In Fig. 42 were shown the cylindrical spores as well as in Fig. 43. The organisms shown in Fig. 43 were isolated from a fermented bean sample obtained from Nigeria during the course of this study. It was coded PCA-NS. It was presented here to show similarity to the PCA-1 isolate and because it was used in the next section. The young cells of PCA-1 were shown in Fig. 44. The linked chain-like sausage cells type arrangement was usual with young cells. The chain was usually disrupted as soon as one of the cells in the middle started to sporulate. Figures 45, 46 and 47 were obtained on MRS and TJA. These were presumed to be lactic acid bacteria. Bead-like cells were obtained from WA (Figs. 48 and 49). They divided very rapidly and were presumed to be yeast cells. The molds were primarily from the RB. They included Phycomycetes - Rhizopus spp. (Fig. 50), Aspergillus spp. (Fig. 51) and Penicillium spp. (Fig. 52). Molds were not isolated from processed fermenting substrate except on rare occasions as contaminants.

The pure culture inoculation of RADB gave positive results with the inoculum of Bacillus spp. (MRS-1, PCA-1, TJA-1). Other isolates listed

(Table 29) had no effect on RADB. They were not presented in Fig. 53 so as to allow for clarity in the figure. The PCA-1 inoculum converted RADB to the fermented product within 24 h as indicated by the alkaline pH. The uninoculated control (PS) took 62 h to achieve the same level of pH. The radappertized control (RADB) had an intial fall in pH and then maintained a constant acidic pH (Fig. 53). The acidic pH was an indication that RADB on its own was not bioconverted. The Bacillus spp., from the data obtained from this experiment, is the principal organism responsible for the solid substrate fermentation of African locust beans. The direct inoculation of the substrate with the PCA-1 isolate will reduce by 48 h the time required for the fermentation.

To conclude the experiment, the PCA-1 was reisolated from the fermented products in line with Koch's postulate. The results obtained from this section justified the proposed hypothesis for Parkia fermentation in section 4.10. above.

## 4.12. PURE CULTURE INOCULATIONS

The use of pure culture inoculation in fermentation originated from the so-called "back-slopping" technique. In this method a portion of a recently fermented food is employed as an inoculum source for the succeeding batch of substrate. The process, though still practiced today in many parts of the world (Daly et al., 1973 and Baccus and Brown, 1981) is susceptible to contamination by hazardous microorganisms. Therefore the current and modern trend is to inoculate the substrate with pure inoculum. This gives safe, consistent, unique and superior product. In addition better shelf life and shorter fermentation schedules are obtained (Baccus and Brown, 1981). The microorganisms which are presently exploited to promote nutritional and organoleptic properties of foods were recently reviewed (Smith and Palumbo, 1981). In this experiment the effect of the addition of pure cultures of microorganisms to processed African locust beans was studied.

### 4.12.1. Objectives - Pure Culture Inoculations

The objective of this experiment was to determine the effect of inoculating radappertized beans (RADB) with known microorganisms and with some of the pure isolates obtained from processed fermenting substrate (PFS) on a time sequenced basis.

### 4.12.2. Principle - Pure Culture Inoculations

RADB is inoculated with known microorganisms. The pH developed on RADB on a time sequenced basis is determined. The absence or presence of fermented product is also determined.

### 4.12.3. Materials and Methods - Pure Culture Inoculations

The RADB was obtained as described under section 3.2. The microorganisms used in the study were as listed in table 30 below. The method used to obtain and test the inoculum from each of the microorganisms used was as detailed in section 4.11.3.3. Each microorganism was grown in its own broth medium as listed in table 30 and harvested as detailed in section 4.11.3.3. The pH developed on the substrate (RADB) by each of the microorganisms used was determined on a time sequenced basis. Organoleptic score was based on whether the RADB was fermented by the added inoculum or not.

### 4.12.4. Observations - Pure Culture Inoculations

In fig. 54 are shown the time sequenced pH developed by the different pure cultures of microorganisms employed to inoculate RADB. The organoleptics developed by each organism on RADB is presented in table 31.

Table 30. Microorganisms Employed for Pure Culture Inoculations.

| Microorganisms | MAC # | ATCC # | NRRL # | Culture Medium |
|---|---|---|---|---|
| *Streptococcus lactis* | 383 | 11454 | - | MRS Broth |
| *Coccobacilli* - (PCA-2) | LAB | - | - | MRS Broth |
| *Leuconostoc mesenteroides* | 765 | 9135 | - | MRS Broth |
| *Bacillus* spp. (PCA-1) | LAB | - | - | mPC Broth |
| *Bacillus* spp. (PCA-NS) | LAB | - | - | mPC Broth |
| *Bacillus subtilis* | 194 | 6633 | B543 | BHIB |

MAC: Macdonald College.

ATCC: American Type Culture Collection.

NRRL: Northern Regional Research Laboratories.

LAB: Isolates from PFS.

BHIB: Brain Heart Infusion Broth.

Fig. 54: Time Sequenced pH Developed on RADB by Pure Culture Inoculation with Different Microorganisms.

Figure 54

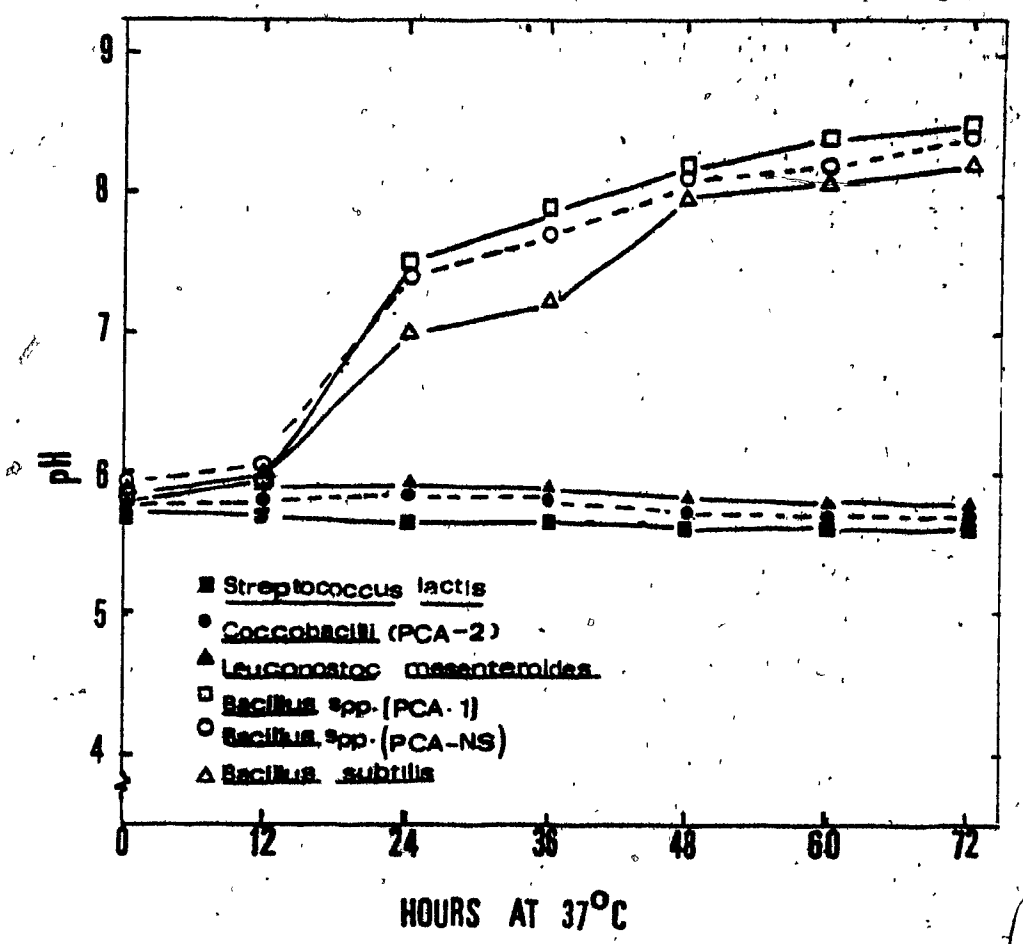

9
8
7
6
5
4
pH
0
12
24
36
48
60
72
HOURS AT 37°C
Streptococcus lactis
Coccobacilli (PCA-2)
Leuconostoc mesenteroides
Bacillus spp. (PCA-1)
Bacillus spp. (PCA-NS)
Bacillus subtilis

Table 31. Organoleptic Scores after Pure Culture Inoculations of RADB at 37°C.

| Microorganisms | Organoleptics | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time in Hours at 37°C | | | | | | |
| | ø | 12 | 24 | 36 | 48 | 60 | 72 |
| Streptococcus lactis | - | - | - | - | - | - | - |
| Coccobacilli (PCA-2) | - | - | - | - | - | - | - |
| Leuconostoc mesenteroides | - | - | - | - | - | - | - |
| Bacillus spp. (PCA-1) | - | - | + | + | + | + | ++ |
| Bacillus spp. (PCA-NS) | - | - | -/+ | + | + | + | ++ |
| Bacillus subtilis | - | - | -/+ | + | + | + | ++ |

Score: - No fermentation.
-/+ Start of fermentation.
+ Fermentation.
++ Fermentation and partial solubilization.

## 4.12.5. Discussion - Pure Culture Inoculations

The pH developed on RADB by the different types of microorganisms used as sources of inoculum were shown in Fig. 54. Streptococcus lactis, Coccobacilli (PCA-2) and Leuconostoc mesenteroides did not cause increased pH on RADB. The pH values obtained for these three types of microorganisms were fairly constant and acidic. A slight lowering of the pH value was noted in the curve obtained with Streptococcus lactis. The acidic pH values are indications that these three types of microorganisms will not, on their own, bioconvert the processed substrate. However, when pure cultures of Bacillus spp. (PCA-1), Bacillus spp. (PCA-NS) and Bacillus subtilis were employed with RADB there were dramatic increases in the pH values of RADB (Fig. 54). These latter groups of microorganisms raised the pH of RADB to 7.0 or higher within 24 h of inoculation. These results indicate that the Bacillus spp. earlier isolated and Bacillus subtilis would each on its own bioconvert processed substrate.

The time sequenced organoleptics developed by the pure inoculum of the microorganisms tested were presented in table 31. Those isolates which did not cause increase in the RADB pH value were also found to lack the ability to bioconvert since their substrate (RADB) remained fresh at the end of the 72 h incubation. All the Bacillus spp. tested (table 31) caused the RADB to bioconvert within 24 h and at 72 h solubilization had already started. The result showed that the isolated Bacillus spp. are the principal microorganisms responsible for the bioconversion of processed substrate of African locust beans. The activity of Bacillus subtilis on

RADB showed similarity to those of the other Bacillus spp. discussed. This suggests a possible link between Bacillus spp. (PCA-1), Bacillus spp. (PCA-NS) and Bacillus subtilis.

Bacillus subtilis (Bacillus natto) has been reported to be the principal organism responsible for the fermentation of soybeans to natto. Pending detailed classification of the Bacillus spp. isolated from the African locust bean fermentation the name Bacillus dawadawa is tentatively suggested for the organism.

# CHAPTER 5

# BIOCHEMICAL AND ENZYMOLOGICAL ASPECTS OF THE FERMENTATION

## 5. BIOCHEMICAL AND ENZYMOLOGICAL ASPECTS OF THE FERMENTATION

The importance of microorganisms to the fermentation process is their ability to secrete enzymes with which they carry out various biochemical activities ascribed to them. These biochemical activities result in the bioconversion of the substrate to the product. Many microbial enzymes have been described and more than a thousand patented although only about fifty of these are presently of industrial importance (Aunstrup et al., 1979). The proteases and amylases produced by the genus Bacillus, with an annual production of 800 tons or 52% of the total microbial enzymes produced industrially, form the largest portion. The reasons for the predominance of this genus in the production of enzymes are several. First, they comprise a group of chemoorganotrophs that can be easily maintained and cultivated and yet are markedly heterogeneous in character. Psychrophiles, mesophiles, thermophiles and alkalophilic, neutrophilic and acidophilic species exist within the genus. In addition, all the listed 48 species have been known to secrete a variety of soluble extracellular enzymes. Some produce amylases that can liquefy starch under pressure at 110°C (Priest, 1977) and proteases that are stable and active at pH 12.0 (Aunstrup et al., 1972). Fogarty et al. (1974a, b) and Kelly and Fogarty (1976) have compiled lists of Bacillus species which produce amylases and proteases. They reviewed the different types of these enzymes which are produced extracellularly with some of their important characteristics. In this chapter the enzymes involved in Parkia bioconversion are discussed. The results of experiments carried out with extracts from fermented beans (FB) and radappertized beans

(RADB) are presented. As in the earlier chapters, experiments are presented in modules of Objectives, Principle, Methods, Observations and Discussion.

## 5.1. GENERAL MATERIALS AND METHODS

In this section and its subsections are presented materials and methods common to some of the other sections below. They are described here and shall be referred to as the need arises in the text.

### 5.1.1. Source of Substrate for Biochemical and Enzymological Studies

The source of substrate was as described under section 3.1.

### 5.1.2. Preparation of Processed Substrate and Fermented, and Radappertized Beans

The preparation of processed substrate and its bioconversion to fermented beans were carried out as previously described (section 3.2.). The radappertized beans (RADB) were also obtained as in section 3.2.

### 5.1.3. Preparation of Sample Extract

A $10^{-1}$ homogenate of a required sample was made with 90 ml sterilized distilled water as diluent and 10 g of the sample in a sterilized blender. The homogenate was passed through a funnel lined with Whatman filter paper No. 1. The filtrate was spun down in a refrigerated centrifuge (4°C) at

10,000 g for 10 min. The supernate was filtered through a sterilized millipore unit with sterilized filter (0.45 µm) into a previously sterilized receptacle. The filter sterilized material was designated as the 'extract'. When not used immediately, the extract was kept at 4°C until required. When comparisons were to be made, the samples to be used were obtained at the same time and investigated similarly.

## 5.2. SOME BIOCHEMICAL ACTIVITIES OF THE ISOLATES

The presence or absence of certain enzymes is employed to characterize some genera and species of bacteria. The ability of a microorganism to carry out some standard conversions is based on these biocatalysts. Therefore, in the characterization of an organism, these enzymes are searched for, in addition to morphological characterization. In this section some characteristics and biochemical activities of the bacterial isolates obtained in section 4.11. and those obtained under anaerobic conditions (section 4.9.) are reported upon.

### 5.2.1. Objectives - Some Biochemical Tests

The objectives of this experiment were to carry out some biochemical tests with the isolates obtained during the course of the experiments described in chapter 4 and to tentatively characterize these isolates.

### 5.2.2. Principle - Some Biochemical Tests

Some standard basic tests as outlined in standard microbiology texts are carried out for each isolate. Standard schemes are used to interpret results obtained from the tests.

## 5.2.3. Materials and Methods - Some Biochemical Tests

The bacterial isolates employed were as listed in table 29. The predominant isolates obtained during the anaerobic incubation of processed substrate (PS) at 37°C were also tested. The media used for biochemical tests included starch agar, milk agar and tributyrin agar. The methods of preparation of these media are given in appendix E. Tests such as Gram reaction and catalase activity were also carried out with the isolates. All these tests have been well documented in standard texts of basic microbiology, food and dairy microbiology, identification methods, etc. (Harrigan and McCance, 1976; Mitruka, 1976 and Speck, 1976).

In the determination of carbohydrase activity, the isolate to be tested was streaked onto starch agar and incubation was carried out at 37 ± 2°C for between 24 to 72 h. The plate was then flooded with Gram's iodine. The lack of blue color around a colony was taken as a positive reaction whereas a blue color was read as a negative reaction. The lack of blue color around a colony indicated that the microorganisms had the ability to break down starch.

In testing for lipase activity, tributyrin agar plates were used. The isolates were each streaked onto the surface. Those isolates which showed clearing around the developed colonies were taken as having a positive reaction while those which lacked clearing were taken to exhibit a negative lipase activity.

The ability to hydrolyze proteins was also tested by streaking milk agar plates with the isolates. Clearing of the opaque medium around colonies indicated the presence of protease activity. To eliminate the problem of

false positives, the milk agar plates were flooded with dilute HCl. Only clearings which persisted after flooding were recorded as positive. All inoculated plates were incubated at 37 ± 2°C for 24 to 72 h before the reactions were scored.

In the characterization of the predominant microorganisms which developed on PS incubated anaerobically, colony type, Gram reaction, catalase reaction, presence or absence of spores, microscopic cell shape and arrangements were all determined.

### 5.2.4. Observations - Some Biochemical Tests

The results of tests carried out on the isolates obtained during aerobic and anaerobic incubation of PS at 37°C are presented in tables 32 and 33.

### 5.2.5. Discussion - Some Biochemical Tests

In table 32 were presented some of the biochemical activities and colonial characteristics of the isolates obtained during the process of Parkia fermentation. Two major groups predominated. These were Bacillus spp. and the lactic acid bacteria. As discussed earlier (sections 4.11. and 4.12.), the Bacillus spp. were the principal microorganisms responsible for bioconversion. However, this should not entirely rule out the role of lactic acid bacteria in the process of bioconversion. They may also play a role in the development of the 'nutty flavor' of the fermented beans.

Table 32. Isolates from PS Incubated Aerobically at 37°C.

| Isolate | Characteristics | | | | | Tests | | | | | Tentative Identification Of Genus* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Colony and Diameter at 37°C | Cell Type | Cell Arrangement | Mucoid Secretion | Spore | Gram | Catalase | Carbohydrase | Lipase | Protease | |
| MRS-1 | Large 5 - 10 mm | Rods | Sausage, single, pairs, chains | + | + | + | + | + | - | + | Bacillus spp. |
| MRS-2 | Pinpoint 0.5 - 1 mm | Rods | Elongated, singly, varied | - | - | + | V | W | - | - | Lactic acid bacteria |
| PCA-1 | Large 7 - 10 mm | Rods | Sausage, singly, pairs, chain | + | + | + | + | + | - | + | Bacillus spp. |
| PCA-2 | Pinpoint 0.5 - 1 mm | Coccobacilli | Singly, pairs | - | - | + | + | - | - | - | Lactic acid bacteria |
| TJA-1 | Large 7 - 10 mm | Rods | Sausage, singly, pairs, chains | + | + | + | + | + | - | + | Bacillus spp. |
| TJA-2 | Pinpoint 0.5 - 1 mm | Coccobacilli | Singly, pairs | - | - | + | + | W | - | - | Lactic acid bacteria |
| TJA-3 | Medium 1 - 4 mm | Rods | Slender, singly, pairs | - | - | + | V | W | - | - | Lactic acid bacteria |
| WA-1 | Large 3 - 7 mm | Rods | Sausage, long chains | - | V | + | + | + | - | + | Bacillus spp. |
| WA-2 | Small 1 - 2 mm | Cocci Bead-like | Singly, pairs, varied | - | - | V | + | - | - | - | - |

* Based on identification schemes of: Gordon (1973); Harrigan and McCance (1976) and Mitruka (1976).

- : Negative reaction.
+ : Positive reaction.
V : Variable reaction.
W : Weak reaction.

Table 33. Isolates from PS Incubated Anaerobically at 37°C.

| Isolation Media | Characteristics | | | | | | Tentative Identification* |
|---|---|---|---|---|---|---|---|
| | Colony | Gram | Catalase | Spore | Aerobic Growth | Cell Arrangement | |
| MRS | Convex Pinpoint (0.5 - 1 mm) White | + | V | - | + | Coccoid in varying arrangements pairs, tetrad short chains, etc. | Lactic Acid Bacteria |
| PCA | Convex Pinpoint (0.5 - 1 mm) White | + | V | - | + | | |
| TJA | Convex Pinpoint (0.5 - 1 mm) White | + | V | - | + | | |

* Based on identification schemes of: Gordon (1973); Harrigan and McCance (1976) and Mitruka (1976).

- : Character not present or negative reaction.

+ : Positive reaction.

V : Variable reaction.

Under anaerobic incubation conditions almost all of the isolated organisms were found to be lactic acid bacteria. Since these organisms could not bioconvert the PS (section 4.9.) it was concluded that their role to the process could only be considered as secondary. The anaerobic organisms were found to be Gram +ve, asporogenous, catalase variable and coccoid in varying arrangements (Table 33). The tentative identification of genus as reported in tables 32 and 33 were based on standard published schemes as indicated in the footnotes to the tables.

## 5.3. HYDROLYTIC ENZYMES AND AMMONIA

The major solid constituents of the processed substrate (PS) are carbohydrates, proteins and lipids. The fermentation takes place on the solid substrate. Therefore, the microorganisms involved in bioconversion must obtain their food from this material. To do this, they must have the ability to hydrolyze some portions of the major constituents of the PS. This means that extracellular enzymes must be released to accomplish the breakdown of the PS macronutrients. Extracellular enzymes released by microorganisms include carbohydrases (act on carbohydrates), lipases (act on lipids and fats) and proteases (act on proteins). If these enzymes are released onto the substrate exterior by the bioconverting microorganisms, it should be possible to detect them from liquid extract of the solid substrate undergoing fermentation. Hydrolysis of protein frequently gives rise to ammonia. This should also be detectable. The detection of the extracellular enzymes and the product ammonia ($NH_3$) are the basis of the experiment reported in this section.

### 5.3.1. Objectives - Hydrolytic Enzymes and Ammonia

The objective of this experiment was to test qualitatively the prepared extracts of processed substrate (PS), processed fermenting substrate (PFS) and radappertized beans (RADB) for the presence of carbohydrases, lipases, proteases and ammonia on a time sequenced basis.

### 5.3.2. Principle - Hydrolytic Enzymes and Ammonia

The sample extract is obtained with sterilized distilled water. The hydrolytic enzymes are qualitatively tested for on starch agar, milk agar and tributyrin agar. Ammonia is tested for by the use of Nessler's reagent.

### 5.3.3. Materials and Methods - Hydrolytic Enzymes and Ammonia

The samples used were processed substrate (PS), processed fermenting substrate (PFS) and radappertized beans (RADB) incubated at 37 $\pm$ 2°C. The PS and RADB were obtained as described in section 3.2. At Ø, 12, 24, 36, 48, 60 and 72 h after incubation extracts were obtained from the samples as previously described (section 5.1.3.). In testing for the hydrolytic enzymes starch agar, milk agar and tributyrin agar were used. The exterior of the base of each plate was marked with felt pen into sectors. Each sector was labelled to correspond to the extract to be used for the sector (Fig. 55 below) and then inoculated with 0.05 ml of the prepared extract. After inoculation, the plates were incubated at 37 $\pm$ 2°C and results interpreted for each type of extract as described in section 5.2.3.

The production of ammonia was determined by testing the extract with Nessler's reagent (7 g KI + 10 g HgI + 10 g KOH made up to 100 ml with distilled water). A filter paper was soaked with the reagent and a drop of the extract was added to the reagent on the filter paper. The

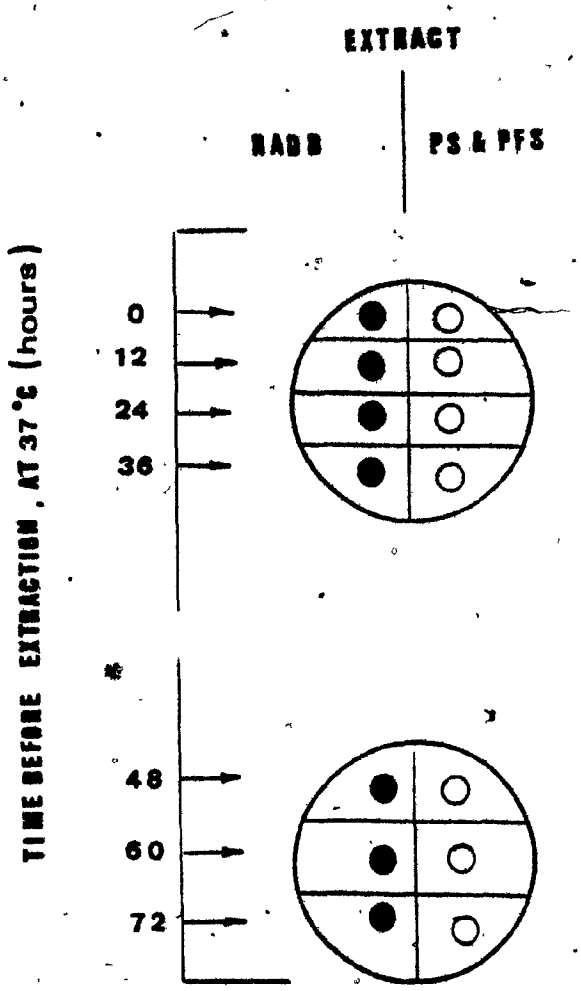
Fig. 55: Extracellular Hydrolytic Enzymes Detection Set Up on a Time Sequenced Basis.

PFS : Processed Fermenting Substrate

PS : Processed Substrate

RADB: Radappertized Beans

Figure 55


EXTRACT
RADB
PS & PFS
TIME BEFORE EXTRACTION, AT 37°C (hours)
0
12
24
36
48
60
72

development of an orange to brown or dark brown color after the addition of the extract indicated the presence of ammonia. Control tests were also carried out by testing the water used for the extract preparation with the reagent. Pale yellow or no color reaction indicated a negative result (Harrigan and McCance, 1976 and Mitruka, 1976).

### 5.3.4. Observations - Hydrolytic Enzymes and Ammonia

In table 34 are presented the time sequenced results obtained for the qualitative determination of hydrolytic enzymes (carbohydrases, lipases and proteases) and ammonia from the prepared sample extracts.

### 5.3.5. Discussion - Hydrolytic Enzymes and Ammonia

In table 34 were given the results of the qualitative hydrolytic enzymes and ammonia determinations. The RADB sample gave negative results for all the extracts. The PS undergoing natural fermentation yielded both carbohydrase and protease activities. These enzymes were secreted onto the substrate by the microorganisms associated with the processed fermenting substrate (PFS). These extracellular enzymes will react with the substrate to bring about bioconversion and to enable the microorganisms to obtain the required nutrients for various physiological and metabolic activities. No lipase activity was detected in any of the sample extracts tested (Table 34).

Table 34. Time Sequenced Qualitative Determination of Hydrolytic Enzymes and Ammonia from Prepared Sample Extracts.

| Extract | | Reaction on | | | Nessler's Reagent ($NH_3$) |
|---|---|---|---|---|---|
| | | Starch | Tributyrin | Casein | |
| RADB | Ø | - | - | - | - |
| | 12 | - | - | - | - |
| | 24 | - | - | - | - |
| | 36 | - | - | - | - |
| | 48 | - | - | - | - |
| | 60 | - | - | - | - |
| | 72 | - | - | - | - |
| PS | Ø | - | - | - | - |
| PFS | 12 | W | - | W | - |
| | 24 | + | - | + | - |
| | 36 | + | - | + | - |
| | 48 | + | - | + | - |
| | 60 | + | - | + | W/+ |
| | 72 | + | - | + | + |

RADB - Radappertized beans.

PS - Processed substrate.

PFS - Processed fermenting substrate.

Numerals - Indicate hours at 37 ± 2°C before extract was prepared.

\- : Negative reaction.

\+ : Positive reaction.

W : Weak reaction.

Ammonia was not detected in any of the RADB extracts. It was detected only after 60 h of incubation of the PS at 37 $\pm$ 2°C. The presence of ammonia can be organoleptically (smell) detected after 72 h incubation at 37 $\pm$ 2°C. This most probably is due to its release in gaseous form from the solid substrate. The release of ammonia may be an indication of active proteolysis.

## 5.4. PROTEOLYTIC AND NON-PROTEOLYTIC MICROORGANISMS

Hydrolysis of casein, an opaque skim milk agar, is often used to determine proteolysis by microorganisms on agar plates (Lee, 1976). A clear zone is formed on the medium by proteolytic bacteria as a result of the conversion of insoluble casein into soluble nitrogenous compounds (peptides and amino acids). However, Frazier and Rupp (1928) have observed that clear zones could be produced on milk agar by bacteria that produce acid from fermentable carbohydrates in the medium. This false clearing or interference would affect the results obtained. Interference by false positives could be eliminated if the plates are flooded with a protein precipitant such as: mercuric chloride or 1% hydrochloric acid or 1% tannic acid solution or mercuric chloride in 20% hydrochloric acid (Harrigan and McCance, 1976 and Mitruka, 1976). Casein is said not to be hydrolyzed if the cleared area disappeared after flooding with the precipitant. The need to flood milk agar plate is a serious disadvantage (Lee, 1976). Therefore the use of another medium has been proposed (Martley et al., 1970). This medium consists of Standard Method Agar (SMA) to which sodium caseinate, trisodium citrate and calcium chloride have been added. After the addition, the medium is then called Standard Methods Caseinate Agar (SMCA). The SMCA is said to have greater sensitivity in the detection of the early stages of casein breakdown. When casein is hydrolyzed, a precipitation zone is formed around the proteolytic microorganism. The precipitation was reported to be due to the formation of insoluble paracaseins in the transparent medium. Both methods were used in this experiment.

### 5.4.1. Objectives - Proteolytic and Non-proteolytic Microorganisms

The objectives were to obtain a permanent record of both the proteolytic and non-proteolytic colonies of microorganisms associated with Parkia fermentation and to tentatively characterize the two groups of microorganisms.

### 5.4.2. Principle - Proteolytic and Non-proteolytic Microorganisms

The two colonies, developed on the casein agar (milk agar) and standard method caseinate agar (SMCA) plates, are photographed. Pure isolates are obtained and characterized.

### 5.4.3. Materials and Methods - Proteolytic and Non-proteolytic Microorganisms

The media employed were milk agar (casein agar) and SMCA. The methods for the preparations of these media are given in appendix E. Both media were inoculated with pure cultures of proteolytic and non-proteolytic microorganisms obtained during Parkia fermentation. The microorganisms employed were PCA-1 (proteolytic) and PCA-2 (non-proteolytic) (see table 32). The media were heavily inoculated with pure colonies of these two microorganism. The colonies developed on the agar media were photographed. The two groups were again characterized as to their

Gram's reaction, catalase reaction, presence or absence of spores and general microscopic morphology as a check.

### 5.4.4. Observations - Proteolytic and Non-proteolytic Microorganisms

The proteolytic and non-proteolytic colonies of microorganisms associated with Parkia fermentations are shown in Figs. 56, 57 and 58. Their characteristics are presented in table 35.

### 5.4.5. Discussion - Proteolytic and Non-proteolytic Microorganisms

Two major groups of microorganisms were present during the fermentation of processed substrate to dawadawa. The two types were - Proteolytic (P) and non-Proteolytic (NP) microorganisms. Their colonies were shown in Figs. 56, 57 and 58. The clearing produced on the milk agar by the proteolytic (P) microorganisms persisted after the plates were flooded with protein precipitant (mercuric chloride in 20% hydrochloric acid). The non-proteolytic (NP) types had no clearing around them. These latter colonies, however, could not be easily distinguished from the white background of the milk agar. They were well shown in Fig. 57 on SMCA. The precipitate around the proteolytic colonies was that of insoluble paracasein. The non-proteolytic microorganism had no precipitate

Fig. 56: Proteolytic (P) and Non-proteolytic (NP) Microorganisms of Parkia Fermentation on Milk Agar (Casein Agar).

Note: 1 unit = 10 mm

Figure 56

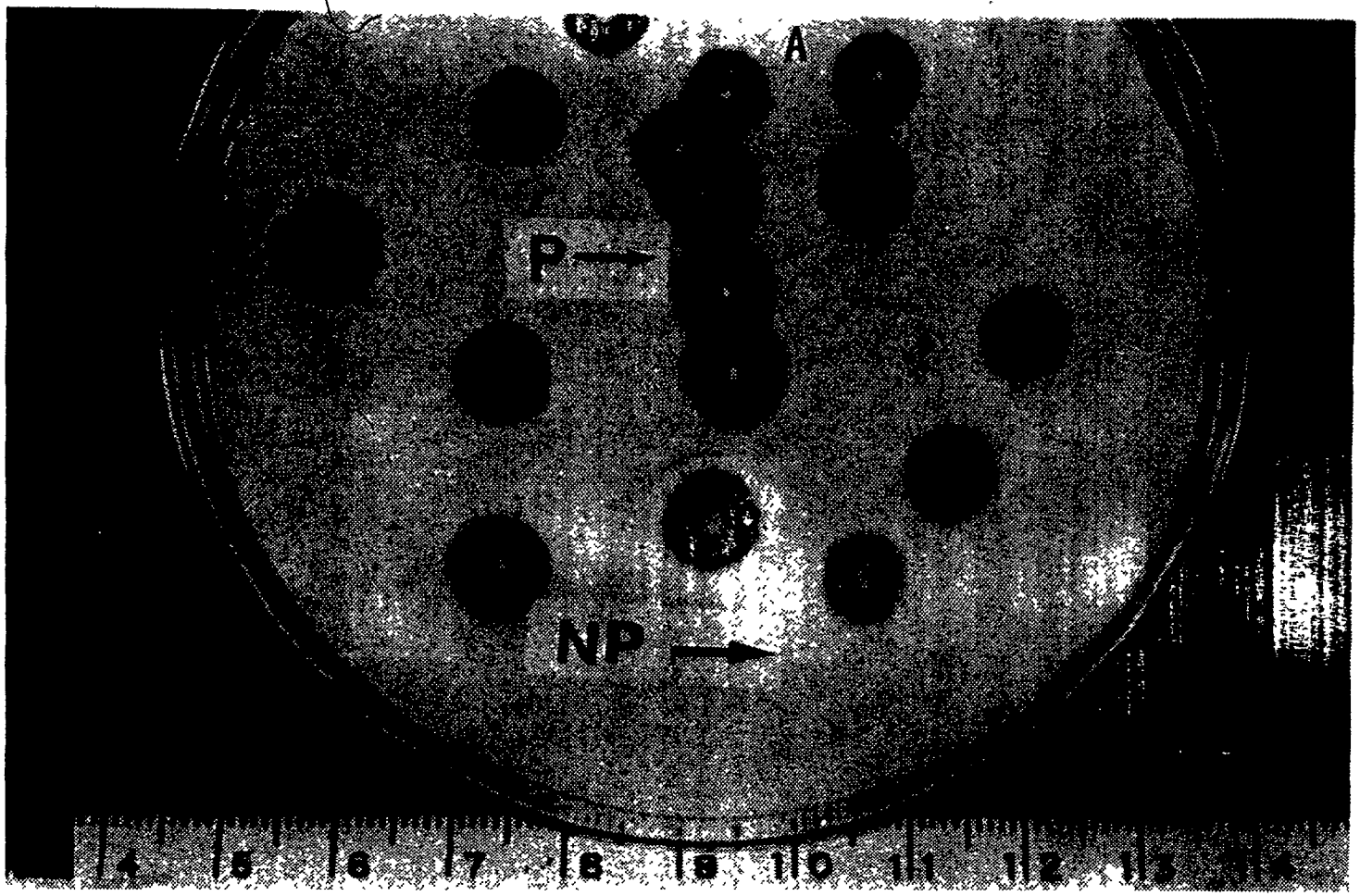

A
P
NP

Fig. 57: Proteolytic (P) and Non-proteolytic (NP) Microorganisms of Parkia Fermentation on Standard Method Caseinate Agar (SMCA).

Note: 1 unit = 10 mm

Figure 57

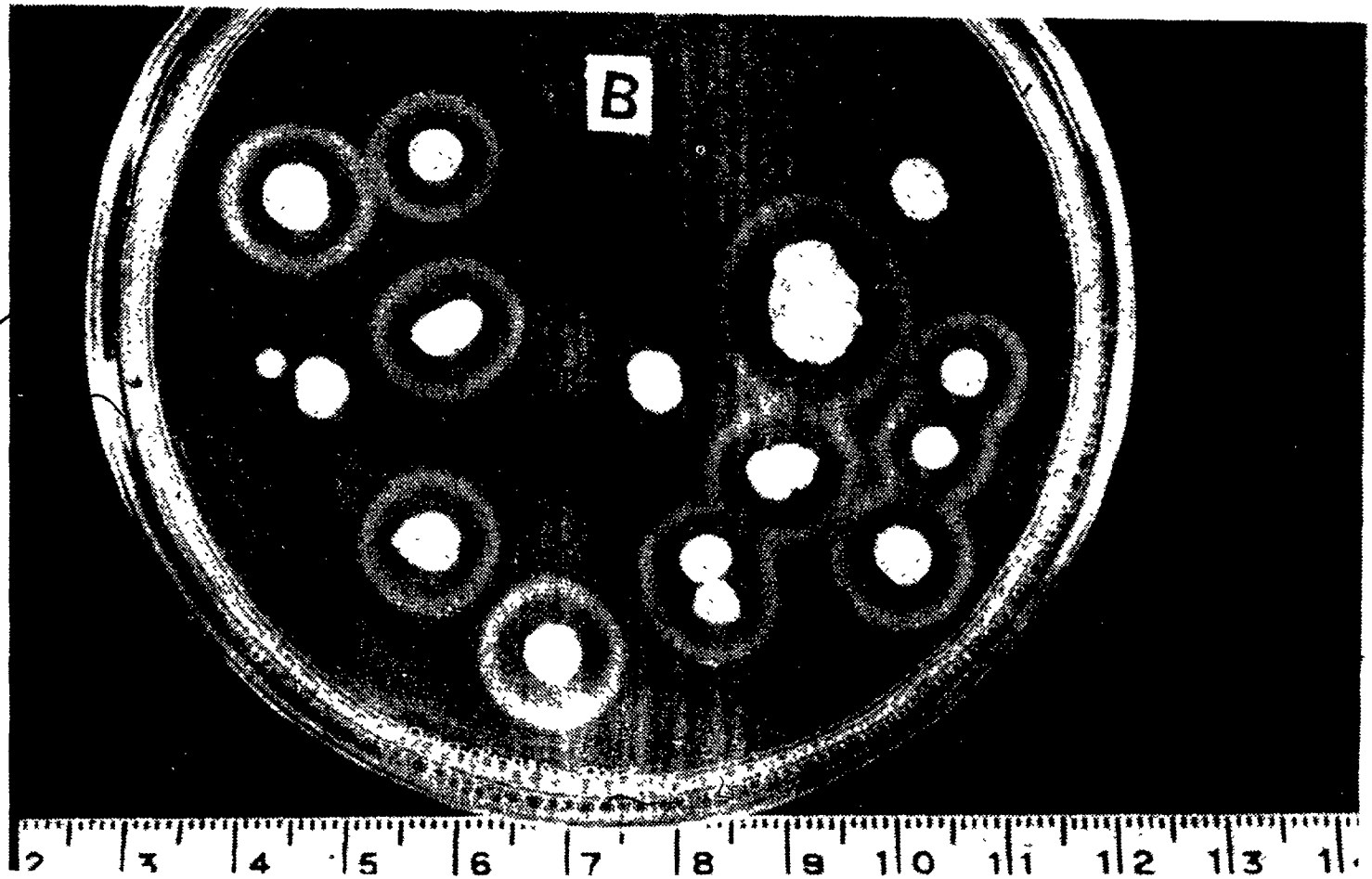

B
3
4
5
6
7
8
9
10
11
12
13

Fig. 58: Proteolytic (P) and Non-proteolytic (NP) Microorganisms on:

A - Milk Agar (Casein Agar)

B - SMCA.

Figure 58

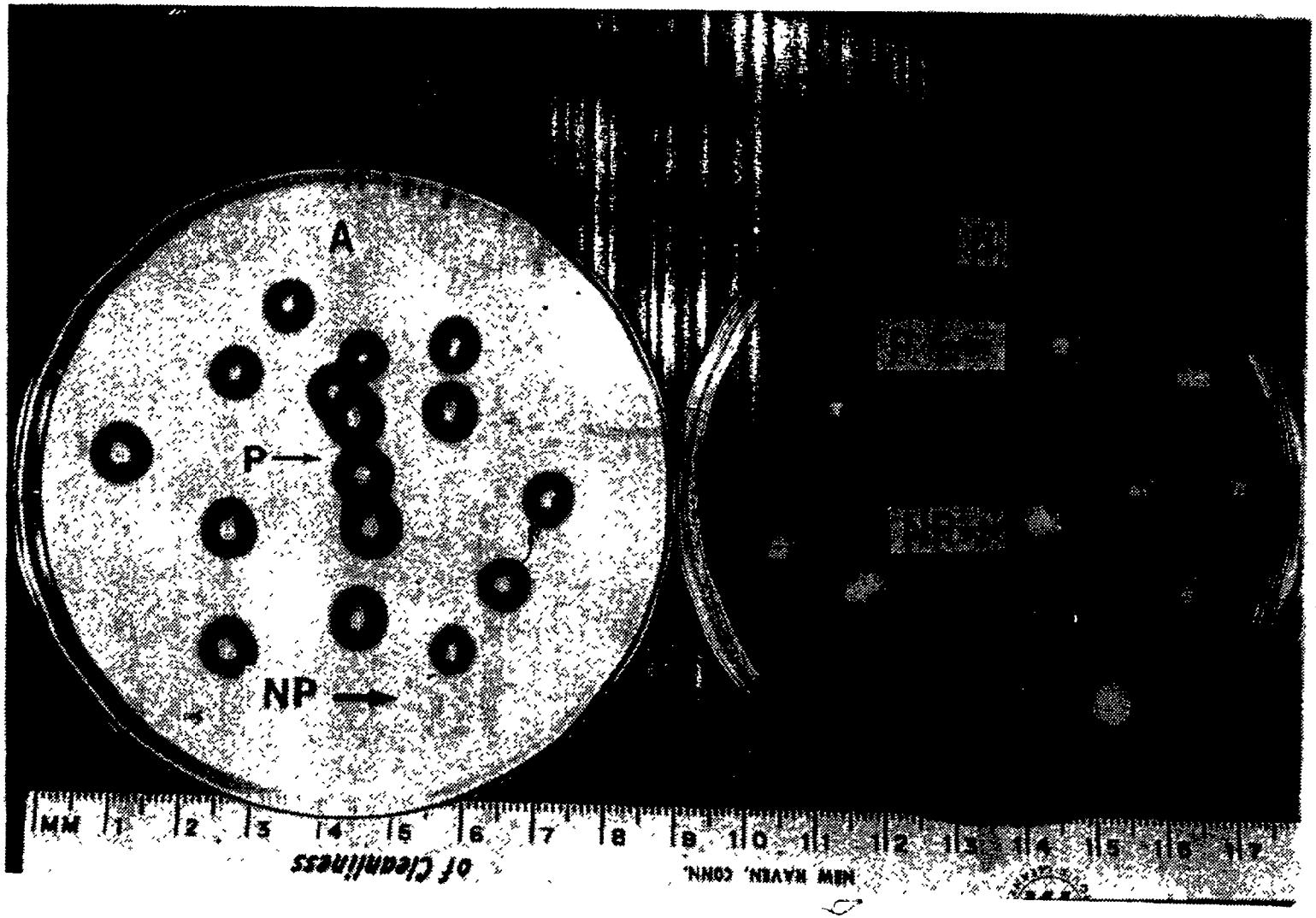

A
P
NP
MM 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
of Cleanliness
NEW HAVEN, CONN.

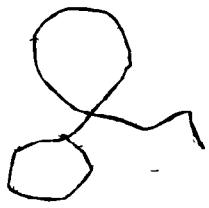
Table 35. Some Characteristics of the Proteolytic and Non-proteolytic Microorganisms Associated with Parkia Fermentation.

| Characterization | Isolates | |
|---|---|---|
| | Proteolytic (P) | Non-proteolytic (NP) |
| Gram | + | + |
| Spore | + | - |
| Catalase | + | V |
| Vegetative Cell Arrangement | Sausage shaped, singly and in chains | Cocci, Coccobacilli, singly, pairs, small chains, etc. |
| Tentative identification of genus | Bacillus spp. | Lactic acid bacteria |

- : Negative reaction.
+ : Positive reaction.
V : Variable reaction.

around their colonies. The two types of colonies (P and NP) on the two media used were shown together in Fig. 58.

They were characterized (Table 35). The proteolytic ones were Gram positive, catalase positive, spore forming sausage shaped cells. They were tentatively characterized as Bacillus spp. The second type i.e. the non-proteolytic ones were Gram positive, catalase variable and spore negative cocci and Coccobacilli. These were tentatively called lactic acid bacteria. The ability or inability of a microorganism to hydrolyze a complex food macromolecule such as protein is an extremely useful property in identification and classification of bacteria (Crabtree and Hinsdill, 1974 and Lee, 1976).



## 5.5. PROTEOLYTIC COUNTS

Determination of the concentration of proteolytic bacteria and/or their proportion in terms of total microbial counts could be useful for some foods (Levin, 1968). For example, quality such as the potential refrigerated shelf life could be determined from the proteolytic counts of the microorganisms present on or within the food. Proteolytic microorganisms were shown to be associated with Parkia fermentation (sections 5.2. and 5.3.) and to possibly be the major microorganisms responsible for bioconversion (section 5.4.). In this experiment their numbers in a typical Parkia fermentation system were examined.

### 5.5.1. Objectives - Proteolytic Counts

The objectives of this experiment were to determine the proteolytic counts and compare their numbers to the total microbial counts on a time sequenced basis and to determine the corresponding pH and titratable acidity developed.

### 5.5.2. Principle - Proteolytic Counts

Homogenates are prepared from time sequenced samples. Milk agar and Standard Method Caseinate Agar (SMCA) are inoculated. Total counts and proteolytic counts are determined. The corresponding pH and titratable acidity (T.A.) are determined for each sample.

### 5.5.3. Materials and Methods - Proteolytic Counts

The media used were milk agar and SMCA. Processed substrate (PS) was incubated at 37 $\pm$ 2°C. After Ø, 12, 24, 36, 48, 60 and 72 h of incubation, homogenates and serial dilutions were prepared (section 4.1.3.) from PS and subsequently from processed fermenting substrate (PFS). The differential media (Milk agar and SMCA) were inoculated using the SPM procedure (section 4.1.4.). Plates were incubated at 37 $\pm$ 2°C for 24 to 72 h. The proteolytic counts were obtained from both the milk agar and SMCA. The total counts were obtained from the SMCA by counting all the developed colonies (proteolytic and non-proteolytic). The fraction of the total number of microorganism which was proteolytic was then computed. The developed pH and titratable acidity (T.A.) were determined as in section 4.5.3.

### 5.5.4. Observations - Proteolytic Counts

In Fig. 59 are shown the time sequenced proteolytic counts on milk agar and SMCA. The total counts obtained on SMCA are also shown. The proteolytic fraction on a time sequenced basis is given in table 36. The corresponding time sequenced pH and titratable acidity developed are shown in Fig. 60.

Fig. 59: Time Sequenced Proteolytic Counts ($\log_{10}$CFU·g$^{-1}$) of PS Incubated at 37°C on Milk Agar and SMCA and Total Counts ($\log_{10}$CFU·g$^{-1}$) on SMCA.

Figure 59

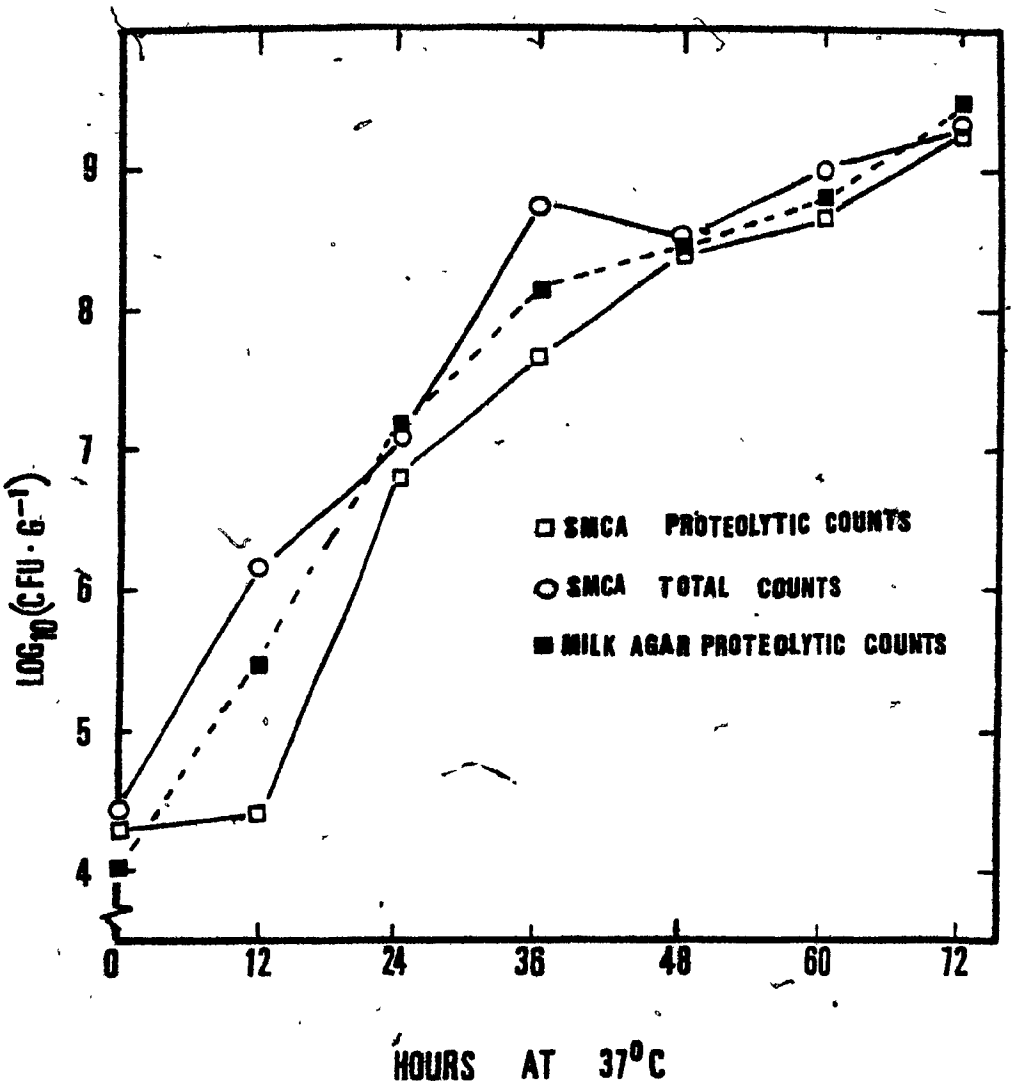

9
8
7
6
5
4
0
12
24
36
48
60
72
LOG10(CFU · G⁻¹)
SMCA PROTEOLYTIC COUNTS
SMCA TOTAL COUNTS
MILK AGAR PROTEOLYTIC COUNTS
HOURS AT 37°C

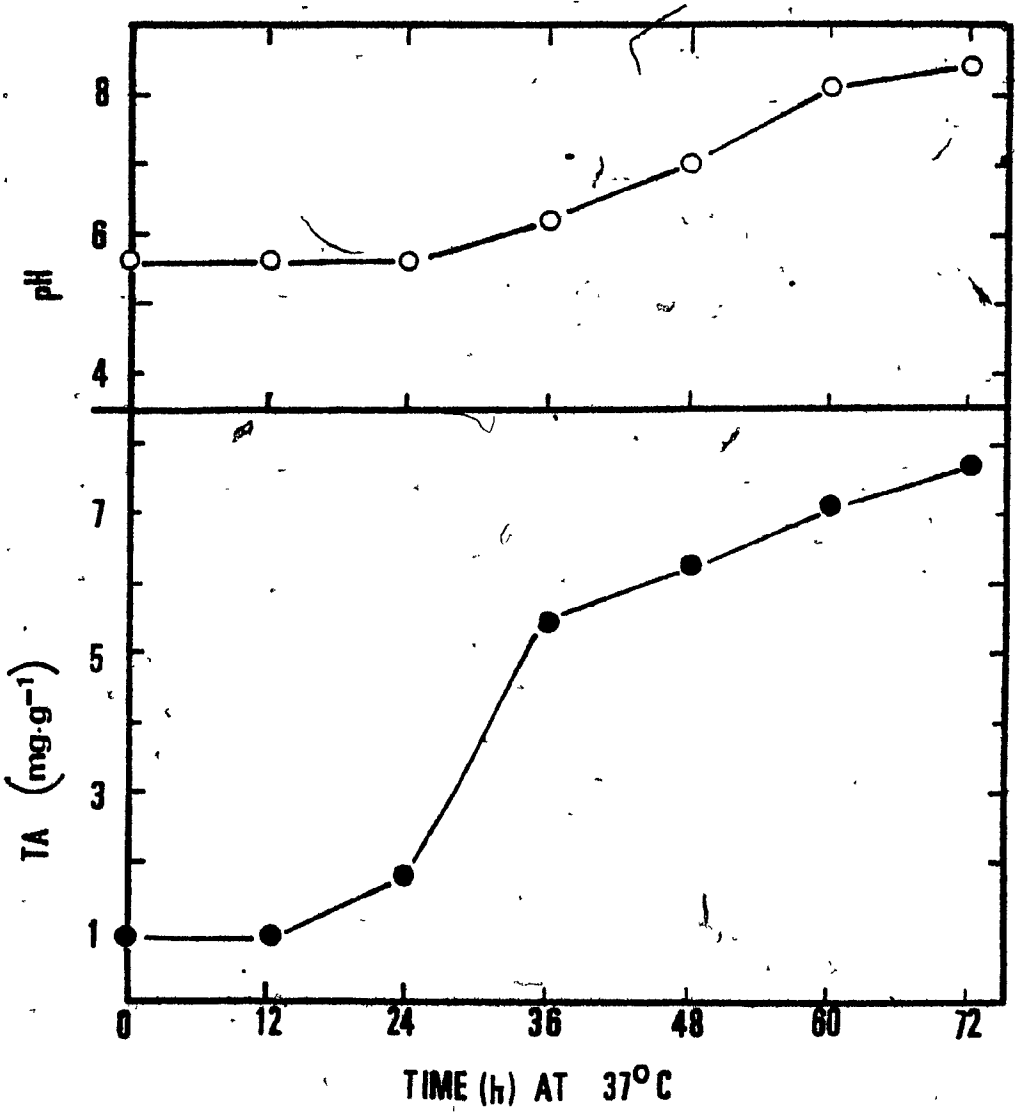
Table 36. Time Sequenced Proteolytic Counts (PC) of Processed Fermenting Substrate and the Proportion of PC to Total Counts on SMCA.

| Time at 37°C (h) | $\log_{10}$ CFU·g$^{-1}$ | | | % Proteolytic* | |
|---|---|---|---|---|---|
| | Total SMCA | Proteolytic | | | |
| | | Milk Agar | SMCA | Milk Agar | SMCA |
| 0 | 4.40 | 4.0 | 4.30 | 91 | 98 |
| 12 | 6.16 | 5.47 | 4.40 | 88 | 71 |
| 24 | 7.12 | 7.21 | 6.82 | 101 | 96 |
| 36 | 8.76 | 8.16 | 7.67 | 93 | 88 |
| 48 | 8.45 | 8.45 | 8.43 | 100 | 100 |
| 60 | 9.00 | 8.78 | 8.65 | 98 | 96 |
| 72 | 9.28 | 9.48 | 9.26 | 102 | 100 |

* Percent of total counts recorded on SMCA. Mean value of three sets of experiments.

Fig: 60: Time Sequenced pH and Titratable Acidity (T.A.) Developed by Processed Substrate and Processed Fermenting Substrate at 37°C during Proteolytic Count Experiment.

Figure 60


pH
8
6
4
TA (mg·g⁻¹)
7
5
3
1
0
12
24
36
48
60
72
TIME (h) AT 37°C

### 5.5.5. Discussion - Proteolytic Counts

High proteolytic counts were obtained from both the milk agar and SMCA (Fig. 59). The counts on milk agar were slightly higher than similar counts on SMCA. The total counts on SMCA were higher than the proteolytic counts on the same medium. This is an indication of the presence of other types of microorganisms collectively called 'non-proteolytic'.

The percentage of proteolytic microorganisms to the total counts were given in table 36. All the proteolytic microorganisms were Gram positive spore forming rods (Bacillus spp.). The genus Bacillus is known for the capability of its members to secrete large amounts of extracellular protease enzymes. Their large number on the processed fermenting substrate indicate their usefulness to the process of Parkia fermentation through their extracellular proteases. This finding also supports the earlier hypothesis that spore-forming microorganisms are responsible for the bioconversion.

The developed pH and T.A. were shown in Fig. 60. The increases in the developed pH and T.A., of the PFS with time, were indications of the bioconversion of the substrate.

## 5.6. AMYLASE ACTIVITY: QUANTITATIVE

Amylases are enzymes involved in the hydrolysis of polysaccharides, e.g. starch. They are produced by a variety of microorganisms and most especially by members of the genus Bacillus. Fogarty et al. (1974) have compiled a list of members of the genus Bacillus responsible for the secretion of different amylolytic enzymes such as amylases, pectinases, cellulases and hemicellulases. The qualitative activity of carbohydrase (amylase) during Parkia bioconversion was reported upon in section 5.3. In this section the quantitative activity of amylase produced by the microorganisms associated with Parkia bioconversion is examined.

### 5.6.1. Objectives - Amylase Activity: Quantitative

The objective of this experiment was to quantitatively determine the amylase activity present in processed substrate (PS), fermented beans (FB) and radappertized beans (RADB).

### 5.6.2. Principle - Amylase Activity: Quantitative

The addition of amylase to soluble starch results in the hydrolysis of the starch to glucose units. The reaction is stopped by the addition of 3,5 dinitrosalicylic acid (DNS) to the reaction vessel. The liberated glucose units are measured spectrophotometrically at O.D $_{540}$ nm. The

quantity of glucose liberated is read off a standard glucose curve and it is a measure of amylase activity.

5.6.3. Materials and Methods - Amylase Activity: Quantitative

The reagent used was 3,5 dinitrosalicylic acid (DNS) obtained from Matheson Coleman and Bell, Cincinatti, U.S.A. One gram of the DNS was dissolved in 20 ml of 2 M NaOH and 50 ml of water. Rochelle salt (sodium potassium tartarate, from Sigma, MO, U.S.A.), 30 g, was added and the volume made up to 100 ml with distilled water. The DNS solution was stored protected from light. The test substrate employed was soluble potato starch obtained from Sigma, U.S.A. The substrate was prepared by dissolving 1 g of the starch in 100 ml of 0.02 M phosphate buffer ($Na_2HPO_4$), pH 6.9, containing 0.0067 M NaCl. Samples analyzed were extracts of PS, FB and RADB prepared as outlined in subsection 5.1.3.

Amylase activity was determined by the modified Bernfeld (1955) method. In the determination, 1 ml of diluted enzyme source (0.5 ml extract + 0.5 ml water) was incubated with 1 ml of the test substrate at 37°C. The incubation was carried out for 30 min on a shaker (New Brunswick Scientific) operated at 200 rpm, installed inside a Uni-Therm Puffer Hubbard 2-70C selector refrigerator (Michigan, U.S.A.). The reaction was stopped by the addition of 2 ml DNS solution. The reaction vessel (test tube) containing the mixture was heated for 5 min in boiling water, 8 ml water was added, the tube vortexed and then cooled. The optical density was read at 540 nm using a Gilford spectrophotometer 240

(Gilford Instrument). A similar procedure was carried out for (buffer + extract) and (water + substrate) to obtain a blank reading. The actual O.D for each sample was determined from:

Actual O.D sample = O.D (Sample - Blank)

A standard curve was obtained with D-glucose obtained from BDH Chemicals, Montreal. The curve was used to convert the O.D values obtained from the samples to mg or µg of glucose released by a particular extract. Prior to the choice of the duration of incubation, for the quantitative amylase activity detection procedure, a preliminary test was carried out with a specific amount of the extract (0.5 ml from FB) for a time course determination of glucose released at 37°C. The amylase activity was expressed in mg or µg of glucose released per minute per ml of extract.

### 5.6.4. Observations - Amylase Activity: Quantitative

In Fig. 61 is shown the relationship between O.D $_{540}$ nm and the duration of incubation of prepared extract and test substrate at 37°C. The standard glucose curves are shown in Fig. 62. The quantitative amylase activities present in the various sample extracts are presented in table 37.

### 5.6.5. Discussion - Amylase Activity: Quantitative

There was a linear relationship between the O.D $_{540}$ nm and the time of test incubation at 37°C in the range 5 to 40 min (Fig. 61). The linear

Fig. 61: $O.D_{540}$ nm of FB Extract Incubated at 37°C with Substrate at Different Times of Incubation.

Figure 61

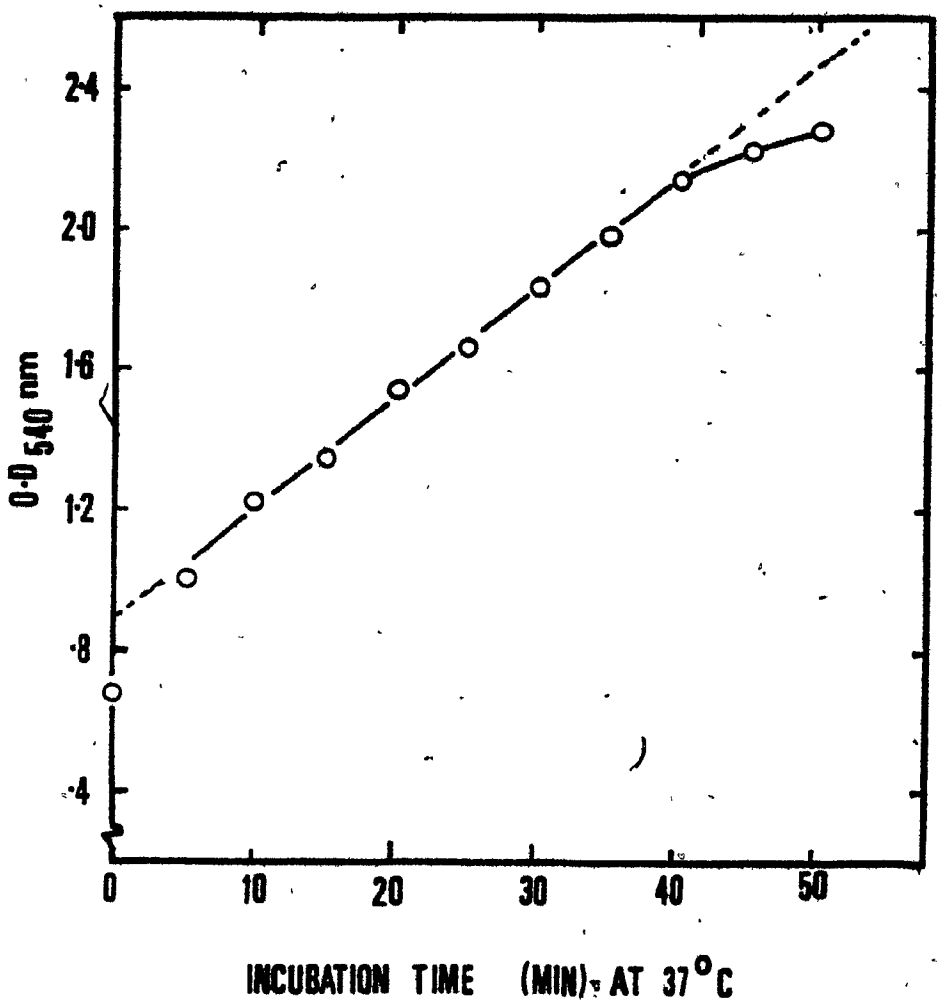

2·4
2·0
1·6
1·2
·8
·4
O·D 540 nm
0
10
20
30
40
50
INCUBATION TIME (MIN) AT 37°C

Fig. 62: Standard Glucose Curves

at O.D $_{540}$ nm.

Figure 62

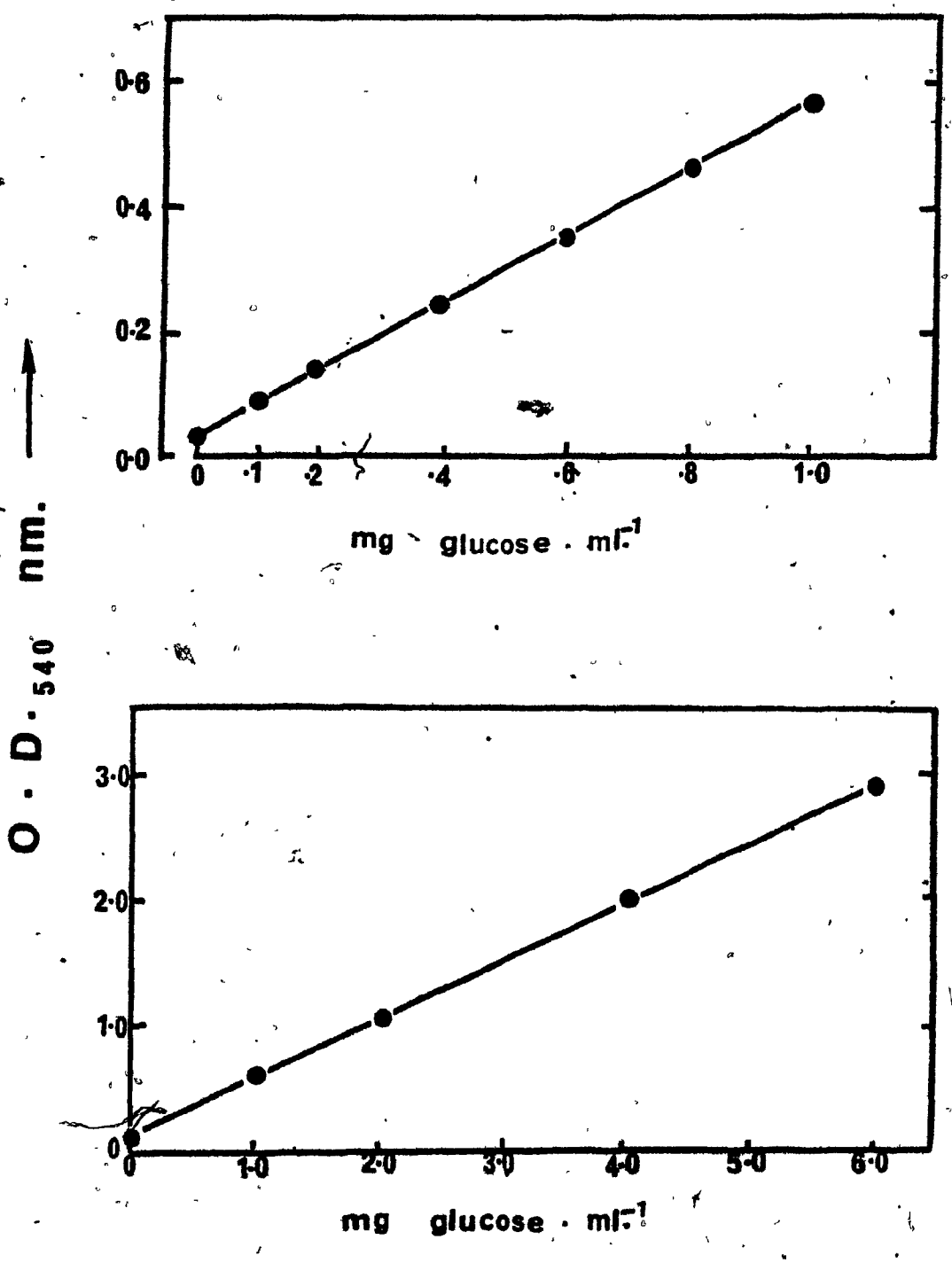

O · D · 540 nm.
0·6
0·4
0·2
0·0
0
·1
·2
·4
·6
·8
1·0
mg glucose · ml.⁻¹
3·0
2·0
1·0
0
1·0
2·0
3·0
4·0
5·0
6·0
mg glucose · ml.⁻¹

Table 37. Amylase Activity of Sample Extracts.

| Extract from | Activity (µg glucose released/min/ml extract) |
|---|---|
| PS | 4.12 |
| FB | 71.7 |
| RADB | 4.0 |

portion of the graph was employed in the selection of the duration of incubation. For this experiment it was 30 min. The glucose released increased with time of incubation as shown by increased O.D $_{540}$ nm.

The standard glucose curves were shown in Fig. 62. Two standard curves were prepared to allow for the determinations of high and low quantities of glucose released as indicated on the axes.

The quantity of glucose released by the FB extract was higher than in the other two sample extracts (Table 37). The higher value was due to the extracellular amylase secreted by the bioconverting microorganisms. The presence of extracellular amylase in the FB extract resulted in higher hydrolytic activity on the starch substrate. The PS usually had a very small microbial population (Fig. 22) which may explain the low amylase activity value from this extract. The RADB had had its population eliminated and consequently extracellular amylase enzymes from microorganisms were not present thereon. The small amylase activities obtained with PS and RADB may have either been due to the reducing sugars present in these materials or to the indigenous bean enzymes. The amylase activity of the FB was about 18 times that of PS and RADB (Table 37).

## 5.7. PROTEASE ACTIVITY: QUANTITATIVE

Extracellular protease production by microorganisms is well known and has long been used as a method of classifying, differentiating and identifying them (Breed et al., 1957; Fogarty et al., 1974b and Kelly and Fogarty, 1976). Keay (1971) has reported that microbial proteases may be divided into three groups on the basis of their pH optima for activity. These are acid, neutral and alkaline proteases. Members of the genus Bacillus produce either neutral or alkaline proteases. Some strains of Bacillus subtilis have been reported to produce both types of proteases (Fogarty et al., 1974b) at the same time while other species of Bacillus may produce these proteases either alone or simultaneously as extracellular enzymes. Some yeasts produce these enzymes intracellularly (Doi et al., 1967; Hayashi et al., 1969 and Kelly and Fogarty, 1976). There are literature reports of Bacillus spp. that grow obligatorily or preferentially at alkaline pH values and produce extracellular alkaline proteases, amylases and pectinases (Horikoshi, 1971a, b, 1972; Boyer and Ingle, 1972 and Kelly and Fogarty, 1976). Proteases, especially the alkaline proteases, are extensively employed in detergent formation, the food industry, leather tanning and in medicine. In an earlier section (5.4.) protease activity was qualitatively investigated (Figs. 56, 57 and 58) and the microorganisms responsible for its production were characterized (Table 35). In this experiment are examined, quantitatively, the activity of the extracellular proteases involved in Parkia fermentation.

### 5.7.1. Objectives - Protease Activity: Quantitative

In this experiment, the objective was to quantitatively determine the protease activity of processed substrate (PS), fermented beans (FB) and radappertized beans (RADB) after RADB had been incubated for 72 h at 37°C.

### 5.7.2. Principle - Protease Activity: Quantitative

The digestion of an azoprotein with proteolytic enzymes results in the formation of colored components soluble in trichloroacetic acid (TCA). The intensity of the color in the TCA filtrate of the hydrolyzed substrate is a function of the proteolytic activity of the enzyme solution. The extract (section 5.1.3.) is used to digest azoalbumin. The O.D of the colored filtrate is measured at 440 nm. Protein in the extract is determined by the Lowry (1951) method. The enzyme activity is expressed as $\Delta O.D_{440}$/min/ml extract.

### 5.7.3. Materials and Methods - Protease Activity: Quantitative

The test substrate used was azoalbumin obtained from Sigma Chemical Co., MO, U.S.A. It was made up in phosphate buffer pH 7.4 to obtain a 1.25% (W/V) solution. Trichloroacetic acid (TCA; $CCl_3.COOH$) used was a product of BDH Chemicals, Montreal, Canada. Distilled water was used with TCA to make a 5% (W/V) solution. The protease enzyme sources were extracts of PS, FB and RADB prepared as outlined in section 5.1.3.

The proteolytic activity was determined by the modified colorimetric method of Charney and Tomarelli (1947). In the determination, 1 ml of diluted extract (1:1 extract with distilled water) was added to 1 ml of test substrate solution in a test tube at 37°C. Test incubation at 37°C was carried out for 30 min on a shaker (New Brunswick Scientific) operated at 200 rpm installed inside a Uni-Therm Puffer Hubbard 2-70C selector refrigerator. After the test incubation, 8.0 ml of 5% TCA solution was added to the contents of the tube. The TCA precipitated the large peptides as insolubles. The soluble portion was separated out by either passing the mixture through Whatman No. 1 filter paper or alternatively the mixture was centrifuged at 9,500 g for 20 min at 4°C. The supernatant was collected and to 5 ml of this 5 ml of 0.5 M NaOH was added and the contents mixed. The O.D of the mixture was determined with a Gilford Spectrophotometer 240 (Gilford Instrument, Ohio, U.S.A.). The color developed was stable for at least 2 h. The above procedure was repeated for the blank (water + buffer).

Then $\Delta O.D_{440} = O.D_{440\ Sample} - O.D_{440\ Blank}$

The enzyme activity was expressed as

$\Delta O.D_{440}$/min/ml extract.

The determination of the amount of protein in the extract was carried out using the Lowry (1951) method. The standard protein used was lysozyme. A standard curve of this was obtained at $O.D_{750}$ nm (Gilford Spectrophotometer 240). The $O.D_{750}$ nm of the appropriately diluted extracts, tested for protein by Lowry method, were obtained and referred to the standard curve to obtain mg protein/ml of each extract.

### 5.7.4. Observations - Protease Activity: Quantitative

The quantitative protease activities of PS, FB and RADB are presented in table 38. In table 39 are given the mg protein per ml of individual sample extract. The standard curve for lysozyme is shown in Fig. 63.

### 5.7.5. Discussion - Protease Activity: Quantitative

The highest protease activity was recorded for the FB extract. The values for PS and RADB were both low compared to that of FB (Table 38). There was no bioconversion of RADB and the PS was yet to ferment. The sources of increased protease activity in FB were proteolytic microorganisms. As earlier reported (section 5.4.) the proteolytic microorganisms of Parkia fermentation are Bacillus spp. These microorganisms were present in large numbers only on FB. They were eliminated, by the irradiation treatment, from RADB while PS had only a very small population of microorganisms (Fig. 22).

The extract of FB (Table 39) had a much higher protein content when compared to values obtained for PS and RADB extracts. The higher protein content of FB extract might have been due to the microorganisms associated with this material. The microorganisms secreted soluble extracellular enzymes which were themselves proteins and they could be detected by the Lowry method employed. Therefore, they may have contributed to the protein content in the extract from the FB. Hydrolysis of some proteins in the substrate (PS) during fermentation by protease enzymes

Table 38. Protease Activity Quantitative.

| Extract from | Mean* $O.D_{440\ nm}$ | $\Delta O.D_{440\ nm}$ | $\Delta O.D_{440} \times 10^4$/min/ml |
|---|---|---|---|
| Blank | 0.0075 | - | - |
| PS (1:1)† | 0.018 | 0.0105 | - |
| FB (1:1) | 0.0905 | 0.083 | - |
| RADB (1:1) | 0.01675 | 0.00925 | - |
| PS¶ | - | 0.021 | 7.0 |
| FB¶ | - | 0.166 | 55.0 |
| RADB¶ | - | 0.0185 | 6.2 |

* Mean value of 4 determinations.

† Sample extract diluted (1:1) with distilled water.

¶ Calculated.

Table 39. Extract Soluble Protein.

| Extract from | Mean* O.D 750 nm | Mean-Blank | mg protein# | mg protein/ml |
|---|---|---|---|---|
| Blank | 0.041 | - | - | - |
| PS (1:5)† | 0.250 | 0.209 | 0.06 | - |
| FB (1:50) | 0.1949 | 0.1539 | 0.04 | - |
| RADB (1:5) | 0.238 | 0.197 | 0.05 | - |
| PS¶ | - | - | - | 0.3 |
| FB¶ | - | - | - | 2.0 |
| RADB¶ | - | - | - | 0.25 |

* Mean value of 4 determinations.

# Values obtained from standard curve.

† Parenthesis: extract diluted with distilled water.

¶ Calculated.

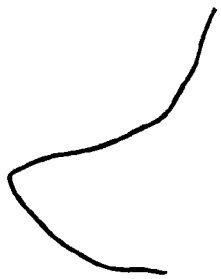
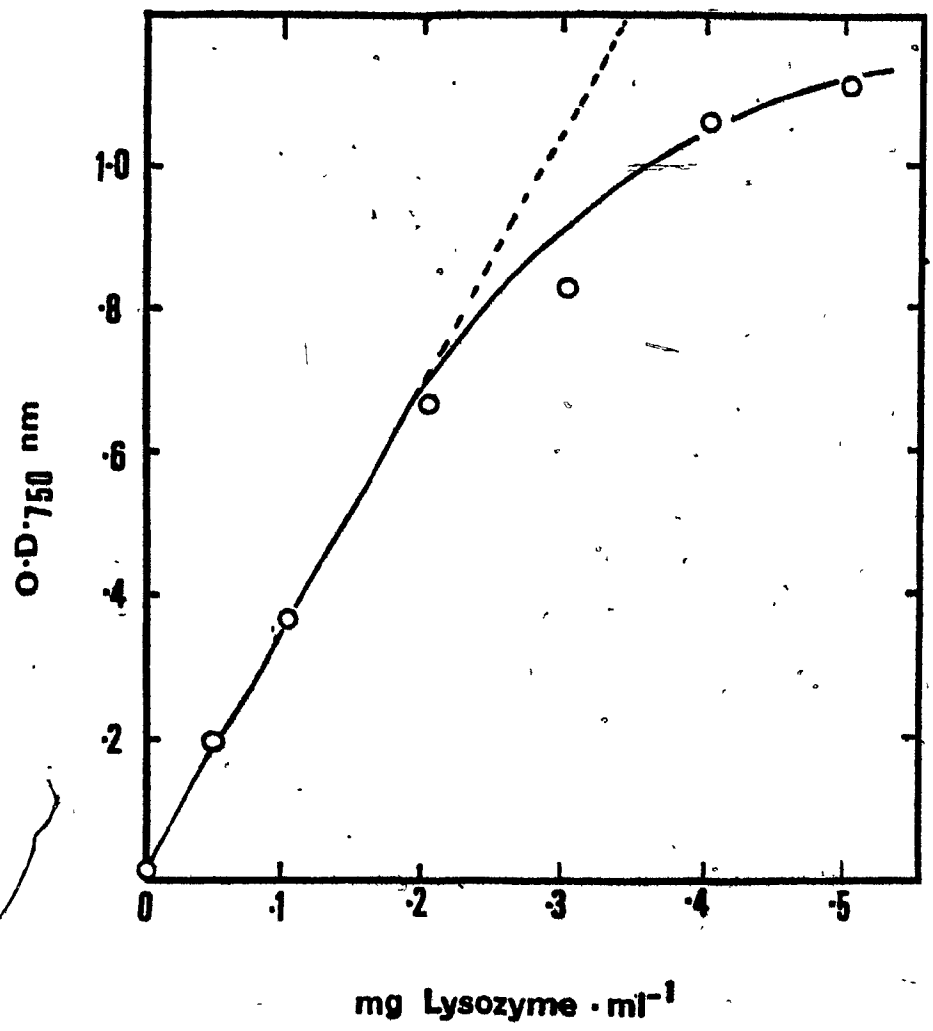


Fig. 63: $O.D_{750}$ nm against mg lysozyme per ml. Lowry Protein Standard Curve.*

*Each point is a mean value of 4 determinations.

Figure 63


1·0
·8
·6
·4
·2
0
·1
·2
·3
·4
·5
O·D·750 nm
mg Lysozyme · ml⁻¹

may also give rise to low molecular weight proteins which may be soluble. If these were present in the extract they would also contribute to the higher protein content of FB extract. The values reported in table 39 may be used together with those of tables 37 and 38 to compute the specific activity values if needed. The standard curve for lysozyme (Fig. 63) was employed to determine the amount of protein present in each extract.

It is not unusual to have increased protein content in extracts from fermented products mediated by proteolytic enzymes. Increases over the initial values of soluble nitrogen compounds and free amino acids have for example been reported during tempeh fermentations (van Veen and Schaefer, 1950; Steinkraus et al., 1960, 1965; Stillings and Hackler, 1965; Murata et al., 1967 and van Buren et al., 1972). The higher activity of proteolytic enzyme and the release of ammonia (section 5.3.) during fermentation, as a result of the presence of microorganisms, are indications that the Parkia bioconversion occurs at least partly by proteolysis.

## 5.8. CULTURE PRESERVATION

The maintenance of pure stock cultures of microorganisms for educational, research, bioassay, industrial and other purposes is extremely important. It is in many laboratories a standard microbiological practice. The microorganism selected for maintenance is obtained in pure form and treated in such a way that it is kept alive for as long as possible and as close as possible to the original isolate. In industries where microorganisms are employed with processes such as fermentation they are usually preserved in a special way so as to discourage unwanted mutations. Several methods of preservation are available. These have been described in the literature (Haynes et al., 1955; Hwang, 1960, 1966; Gherna, 1981 and Hill, 1981).

Reports in earlier sections have shown that the Bacillus spp., isolated from the processed fermenting substrate, was the most important microorganism involved in the process of Parkia bioconversion. The work described in this section was directed towards the preservation of this microorganism.

Lyophilization or freeze drying was employed. Cells were grown in brain heart infusion broth (BHIB) for 12 h at 37°C to a population density of $1 \times 10^9$ cells/ml (counting chamber). They were grown in 200 ml lots of media in 1 litre flasks. Aeration was provided by shaking at 200 rpm. The cells were harvested by centrifugation in sterilized 250 ml bottles at 4°C and 10,000 g for 20 min. The supernatant was poured off and the pellet of cells was resuspended in 5 ml of sterilized 12.5% (W/V) skim

milk containing 0.5% (W/V) sucrose. The bacterial suspension, 0.5 ml at a time, was dispensed into sterilized freeze drying ampoules (Bellco Glass, New Jersey, U.S.A.). The ampoules were plugged with sterilized cotton plugs. The contents of the ampoules were frozen at -80°C (2 h) and then lyophilized at -50°C under maximum vacuum in a freeze dryer for 36 h. The ampoules containing the lyophilized cells were sealed under vacuum using a torch flame. Vacuum leaks were checked with a vacuum leak detector (Fisher Scientific Co.). Only those ampoules which glow during the check were retained, labelled and stored refrigerated in a dessicator.

The lyophilized cells should serve as a ready source of inoculum for Parkia fermentation.

# CHAPTER 6

# CONCLUSIONS

# 6. CONCLUSIONS

Based on the results of the physico-chemical studies, it was concluded that changes do occur in the substrate during processing and fermentation of African locust beans. From the process mass balance (section 3.13), losses were found to have occurred in both carbohydrate and protein content while an increase was recorded in the fat content. A loss in energy content of 9% was also found to have occurred during fermentation.

The investigations of the microbiological aspects proved that the presence of microorganisms was obligatory to the process of Parkia fermentation. These microorganisms were responsible for the physico-chemical changes observed during fermentation as confirmed by the use of RADB controls.

On a time sequenced basis, during fermentation, changes were found to occur also in sequence on the substrate. During the first 18 h of incubation no physical changes could be observed in most cases. From 18 to 36 h the PS started to change in color from light yellow to greyish light yellow. At 48 h the changes were pronounced and a thin mucoid pellicle, possibly a microbial polysaccharide, started to develop on the solid substrate. Between 48 and 72 h the microbial activities increased with partial hydrolysis and softening of the PFS. When the process was continued beyond 72 h partial solubilization of the solid substrate resulted and ammonia generation could be perceived and detected with Nessler's reagent. The objective textural determinations by Instron machine, during fermentation, gave results in support of the subjective observable physical changes.

The main microorganism responsible for the physico-chemical changes in the PS, during fermentation, was a Gram positive, sausage shaped, cylindrical spore forming, catalase positive Bacillus spp. For identification purposes it was tentatively called Bacillus dawadawa until such a time that full taxonomic characterization can be carried out. The organism was labelled PCA-1. It was also for possible taxonomic characterization that lyophilized culture of the organism was prepared. Microorganisms isolated from dawadawa obtained from Nigeria had characteristics similar to those of the PCA-1 isolate. It was called PCA-NS. In a pure culture inoculation of RADB, PCA-1, PCA-NS and Bacillus subtilis ATCC 6633 all bioconverted the PS to the product in 24 h. The pure culture inoculation of the PS reduced by 48 h the usual fermentation period for dawadawa production.

Aside from the Bacillus spp. there were also lactic acid bacteria present. These may play a role in flavor development. They would not, on their own, bioconvert the processed substrate. Other microorganisms found on FB were molds which were infrequently isolated. These produced wild mycelial growth on the substrate. They were not involved in the process of African locust bean fermentation.

One major problem with dawadawa is its instability. The product usually turned black within a few days of its production. However when dawadawa was lyophilized and its fat extracted, a superior product, very stable at room temperature was obtained. This product has been kept for over 24 months at room temperature and it is still stable. It is presumed that both moisture and fat contributed to the instability of the product.

During the process of bioconversion there were simultaneous increases in both the developed pH and titratable acidity of the processed fermenting substrate. While these types of results were unusual, they were however not unique. In the study of burong dalag, a fermented product made from fish and moist rice, simultaneous increases in both pH and titratable acidity values have also been reported (Orillo and Pederson, 1968 and Pederson, 1979). Wagenknecht et al. (1961) also found that during tempeh fermentation, the pH of soybeans increased despite the relatively large amount of acid being liberated. They suggested that liberated ammonia or other basic end products of protein decomposition were the cause of this. Throughout the duration of this investigation, from a large number of the small scale fermentations studied, the normal trends were for both pH and titratable acidity to go up. The processed substrate usually has an initial pH range 5.5 - 6.2. This became neutral and then alkaline with progressive fermentation. The acidity of the processed substrate was usually in the range 0.05 - 0.09%. This may increase to 0.7% or more in the fermented product. The final pH and titratable acidity developed after 72 h of fermentation were determined by the number of the right type of microorganisms present in the fermentation unit and the prevailing conditions of incubation such as temperature, oxygen activity, water activity, and others. There was no bioconversion under anaerobic condition.

The ultimate agents of bioconversion are enzymes. The enzymes involved in the Parkia fermentation were found to be carbohydrases and proteases. Of these two, proteases are believed to be the major cause of the bioconversion. Lipases were not detected extracellularly during active

fermentation. It has been reported by Whitaker (1978) that some of the changes observed when soybeans are fermented to tempeh might be due to the proteolysis of the substrate. For example, it has been suggested that the beany flavor of soybean may be related to the presence of this flavor compound in a bound form to soybean proteins. Hydrolysis by proteolysis during fermentation results in the loss of this organoleptic characteristic (Whitaker, 1978). The characteristic beany flavor was also perceived in the African locust bean processed substrate (PS). After fermentation the beany flavor was replaced with a nutty flavor and in some products ammonia may be perceived. These are indications of proteolysis. Whitaker (1978) has proposed a scheme involving the fermentation of high protein foods such as soybeans. It is likely that one of the pathways proposed by Whitaker (1978), especially one of those in which ammonia is being liberated, may be operative in the Parkia fermentation.

# CHAPTER 7

# RECOMMENDATIONS FOR FUTURE STUDIES

# 7. RECOMMENDATIONS FOR FUTURE STUDIES

As usual with most research undertakings, the solution of a particular problem brings into focus several others. This study has not been an exception.

The PS preparation is by far the most complex aspect of the Parkia fermentation. The operation is both tiring and time consuming. There is a need for improvement in this area to realize process scale up. It is suggested that hydrostatic separation or separation by centrifugal force be investigated. Dry dehulling and winnowing may also prove useful.

The processing time is excessively long. To reduce this the author has attempted to use an autoclave. The whole beans were sufficiently softened after 15 min at 121°C to allow the testa to be removed. However, autoclaved whole beans had had their load of bioconverting microorganisms destroyed. In addition, the resulting PS was not sufficiently softened. It is suggested that further boiling followed by pure culture inoculation be investigated.

The mass balance indicated a possible increase in fat content. It was earlier suggested that this might have been due to the extraction of materials other than fat. It was also suggested that microbial biosynthesis may be responsible for this (section 3.13.5). Further investigation of this aspect is suggested.

The Bacillus spp. (PCA-1) isolated will require a detailed characterization and taxonomic study. As pointed out by Gordon (1973) "a goodly number" of named strains of the genus Bacillus is required for this exercise. The actual role of the lactic acid bacteria though secondary, also requires further investigation.

Although some studies have been carried out on the nutritional significance of Parkia beans, no publications are available on the time sequenced investigation of this aspect of the fermentation. This is therefore suggested.

Some answers have been provided in this study to the questions of 'whys and hows' of the solid substrate fermentation of African locust beans. A lot more questions are yet to be answered. This will be done in stages by scientists, engineers and technologists. The basis has been laid with this first time sequenced physico-chemical, microbiological, biochemical and enzymological study of the process.

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# 9. APPENDICES

# APPENDIX A

Table A1. Minimum VOM Time:

| Time (h) | % Weight Loss* | | |
|---|---|---|---|
| | Whole Bean (WB) | Mechanically Dehulled Beans (MDB) | Mechanically Removed Testa (MRT) |
| ø | 0.00 | 0.00 | 0.00 |
| 6 | 9.10 | 8.30 | 13.80 |
| 12 | 9.70 | 8.50 | 14.20 |
| 24 | 10.30 | 8.70 | 14.60 |
| 36 | 10.30 | 8.80 | 14.40 |
| 48 | 10.30 | 8.90 | 14.20 |
| 60 | 10.60 | 9.10 | 14.40 |
| 72 | 10.90 | 9.60 | 14.60 |

* Each recorded value in the table is a mean of triplicate determinations; % ≡ % by weight (g/100 g sample).

Table A2. The Moisture Contents and Total Solids of RB, WB, PS, FB, MDB, and MRT as Determined by Vacuum Oven Method at 95°C and 13 kPa.

| Sample | % Moisture (g $H_2O$/100 g Sample) | % Total Solids (g solid/100 g Sample) |
|---|---|---|
| Raw Beans (RB) | 6.40 ± 0.03 | 93.60 ± 0.03 |
| Whole Beans (WB) | 8.70 ± 0.05 | 91.30 ± 0.05 |
| Processed Substrate (PS) | 63.00 ± 0.40 | 37.00 ± 0.40 |
| Fermented Beans (FB) | 65.30 ± 0.30 | 34.70 ± 0.30 |
| Mechanically Dehulled Beans (MDB) | 7.20 ± 0.10 | 92.80 ± 0.10 |
| Mechanically Removed Testa (MRT) | 13.90 ± 0.20 | 86.10 ± 0.20 |

Table A3a. Time Sequenced Moisture Content Determinations for Processed Fermenting Substrate (PFS) and Control (Radappertized Beans).

| Time at 37°C Before Analysis (h) | Moisture (g $H_2O$/100 g Substrate)* | |
|---|---|---|
| | Sample Treatment | |
| | None† | Radappertized¶ |
| ø | 67.44 ± 0.34 | 68.52 ± 0.06 |
| 12 | 67.12 ± 0.93 | 66.15 ± 1.04 |
| 24 | 67.94 ± 0.31 | 67.72 ± 0.20 |
| 36 | 69.31 ± 0.27 | 68.85 ± 0.17 |
| 48 | 69.33 ± 0.58 | 67.81 ± 0.16 |
| 60 | 67.57 ± 0.52 | 67.32 ± 0.11 |
| 72 | 65.97 ± 0.52 | 65.14 ± 0.10 |
| 84 | 65.80 ± 0.18 | 64.51 ± 0.04 |

* Each result is a mean of six determinations ± standard deviation.

† Sample undergoing natural fermentation.

¶ Control: Radappertized Beans (11 kGy ≡ 1.1 M rad).

Table A3b. Time Sequenced Total Solids Determinations for Processed Fermenting Substrate and Control (Radappertized Beans).

| Time at 37°C Before Analysis (h) | Total Solids (g Solid/100 g Substrate)* | |
|---|---|---|
| | Substrate Treatment | |
| | None† | Radappertized¶ |
| ø | 32.56 ± 0.34 | 31.48 ± 0.06 |
| 12 | 32.88 ± 0.98 | 33.85 ± 1.04 |
| 24 | 32.06 ± 0.31 | 32.28 ± 0.20 |
| 36 | 30.69 ± 0.27 | 31.15 ± 0.17 |
| 48 | 30.67 ± 0.58 | 32.19 ± 0.16 |
| 60 | 32.43 ± 0.52 | 32.68 ± 0.11 |
| 72 | 33.99 ± 0.52 | 34.86 ± 0.10 |
| 84 | 34.20 ± 0.18 | 35.49 ± 0.04 |

* Each result is a mean of six determinations ± standard deviation.

† Sample undergoing natural fermentation.

¶ Control: Radappertized Beans (11 kGy ≡ 1.1 M rad).

Table A4. Time Sequenced Fat Contents.

| Time at 37°C Before Analysis (h) | Fat (g/100 g Sample)* | |
|---|---|---|
| | Processed Substrate Treatment | |
| | None† | Radappertized¶ |
| ø | 22.49 ± 0.03 | 22.41 ± 0.51 |
| 12 | 21.56 ± 0.33 | 22.83 ± 0.32 |
| 24 | 27.94 ± 0.47 | 24.43 ± 0.16 |
| 36 | 32.45 ± 0.26 | 23.80 ± 2.19 |
| 48 | 35.45 ± 0.31 | 25.96 ± 0.03 |
| 60 | 33.79 ± 0.14 | 26.46 ± 0.12 |
| 72 | 36.90 ± 0.36 | 21.18 ± 0.01 |

* Each result is a mean of 3 determinations ± standard deviation.

† Sample undergoing natural fermentation.

¶ Control: Radappertized Beans (11 kGy ≡ 1.1 M rad).

Table A5. Time Sequenced Protein Contents.

| Time at 37°C Before Analysis (h) | Protein (g/100 g Sample)* | |
|---|---|---|
| | Processed Substrate Treatment | |
| | None† | Radappertized¶ |
| ø | 38.6 ± 0.0 | 38.6 ± 0.0 |
| 12 | 38.6 ± 0.1 | 38.6 ± 0.0 |
| 24 | 40.8 ± 0.3 | 39.0 ± 0.0 |
| 36 | 44.9 ± 0.0 | 39.1 ± 0.1 |
| 48 | 41.8 ± 0.3 | 39.6 ± 0.0 |
| 60 | 41.0 ± 0.0 | 39.5 ± 0.0 |
| 72 | 40.2 ± 0.5 | 39.5 ± 0.2 |

* Mean of duplicate determinations ± standard deviation.

† Sample undergoing natural fermentation.

¶ Control: Radappertized Beans (11 kGy ≡ 1.1 M rad).

Table A6. Glucose Standard Curve Data.

| Glucose Concentration ($mg - ml^{-1}$) | Mean $O.D._{630\ nm}$ * |
|---|---|
| 0.01 | 0.06 |
| 0.02 | 0.11 |
| 0.03 | 0.16 |
| 0.04 | 0.21 |
| 0.05 | 0.26 |
| 0.06 | 0.31 |
| 0.08 | 0.41 |
| 0.09 | 0.46 |
| 0.10 | 0.51 |
| 0.12 | 0.61 |
| 0.14 | 0.70 |

* Mean of 3 determinations.

Table A7. Time Sequenced Total Available Carbohydrate Contents.

| Time at 37°c (h) | Total Available Carbohydrates Contents* (g/100 g Sample) | | | |
|---|---|---|---|---|
| | Lyophilized | | Wet | |
| | PS | RADB | PS | RADB |
| ø | 2.16 | 2.16 | 1.06 | 1.02 |
| 12 | 2.30 | 2.48 | - | - |
| 24 | 2.25 | 2.40 | 0.35 | 0.94 |
| 36 | 1.10 | 2.48 | 0.20 | 1.06 |
| 48 | 1.58 | 2.52 | 0.83 | 1.16 |
| 60 | 1.71 | 2.66 | - | - |
| 72 | 2.61 | 2.70 | 0.93 | 1.14 |
| 84 | - | - | 1.22 | 1.18 |
| 96 | - | - | 1.30 | 1.18 |

* Mean of 3 determinations.
Determined as Glucose.

PS: Processed Substrate

RADB: Radappertized Beans

Table A8. Gross Energy Contents.

| Time at 37°C (h) | Gross Energy Contents* (kJ/g) |
|---|---|
| ø | 25.0 |
| 12 | 25.1 |
| 24 | 25.5 |
| 36 | 26.1 |
| 48 | 26.0 |
| 60 | 26.1 |
| 72 | 26.2 |

* Mean of duplicate determinations.

Table A9. Time Sequenced Texture.

| Time at 37°C (h) | Peak Force ($\bar{PF}$) and Bioyield ($\bar{BY}$) N* | | | |
|---|---|---|---|---|
| | Sample | | | |
| | Processed Substrate | | Radappertized Beans | |
| | $\bar{PF}$ | $\bar{BY}$ | $\bar{PF}$ | $\bar{BY}$ |
| Ø | 26.7 ± 1.6 | 20.9 ± 1.4 | 26.2 ± 3.0 | 19.0 ± 1.3 |
| 12 | 25.5 ± 3.5 | 21.0 ± 2.3 | 26.3 ± 0.4 | 18.5 ± 0.0 |
| 24 | 23.7 ± 3.0 | 20.6 ± 0.7 | 23.4 ± 0.4 | 19.4 ± 0.2 |
| 36 | 19.2 ± 3.5 | 16.4 ± 3.6 | 25.0 ± 3.3 | 19.0 ± 2.4 |
| 48 | 16.1 ± 3.0 | 13.8 ± 2.8 | 26.5 ± 1.6 | 22.8 ± 2.6 |
| 60 | 8.7 ± 3.3 | 7.1 ± 3.0 | 22.4 ± 8.8 | 19.8 ± 1.1 |
| 72 | 7.4 ± 3.4 | 6.0 ± 2.0 | 24.4 ± 5.0 | 23.0 ± 1.0 |

* Mean of 20 determinations ± standard deviation.
N - symbol for Newton; Newton: Unit of force.

Table A10. Weight and Apparent Volume of Whole Beans.

| Mass (g) | Mean* Bulk Volume (ml) |
|---|---|
| 0 | - |
| 25 | 32.5 ± 1.3 |
| 50 | 65.3 ± 1.5 |
| 75 | 97.7 ± 1.8 |
| 100 | 127.0 ± 1.0 |
| 150 | 192.3 ± 0.6 |
| 200 | 254.0 ± 2.0 |
| 250 | 322.7 ± 4.0 |
| 350 | 450.0 ± 1.0 |
| 500 | 646.7 ± 5.8 |
| 1000 | 1280.0 ± 20.0 |

* Mean of triplicate determinations ± standard deviation.

Table All. Losses (g/100 g Sample) at 37°C.

| Time at 37°C (h) | Sample | |
|---|---|---|
| | Processed Substrate (PS)* | Radappertized Beans* |
| ø | ø | ø |
| 12 | 1.4 ± 0.3 | 1.2 ± 0.1 |
| 24 | 2.7 ± 1.0 | 2.1 ± 0.2 |
| 36 | 4.2 ± 1.3 | 3.2 ± 0.2 |
| 48 | 5.7 ± 1.1 | 4.7 ± 0.5 |
| 60 | 7.2 ± 1.1 | 5.4 ± 0.5 |
| 72 | 9.2 ± 0.7 | 6.5 ± 0.3 |

* Mean of 7 determinations ± standard deviation.

# APPENDIX B

```
DATA PARKIA;

INPUT T R MOIST TSOLIDS FAT PRO;

CARDS;
- - - - - - - -
- - - - - - - -
- - - - - - - -

PROC PRINT;

TITLE FERMENTATION DATA;

PRO GLM;

CLASSES T R;

MODEL MOIST TSOLIDS FAT PRO = T R;

MEANS T R/DUNCAN;

TITLE ANOVA FOR FERMENTATION DATA;

*/

//
```

Fig. B1. The Statistical Computer Programme GLM Used to Analyze the Fermentation Data.

# APPENDIX C

Fig. C1. Materials and Reagents - Protein.

(i) 0.1 N HCl: Prepared by adding 10.7 ml concentrated HCl to 1.2 litres of distilled water followed by the addition of 2.5 ml methyl red as indicator. The HCl was standardized against standard NaOH.

(ii) 8% (W/V) sodium thiosulfate: 160 g sodium thiosulfate was dissolved in 2 litres of distilled water.

(iii) 50% NaOH: 1,500 g NaOH flakes was dissolved in distilled water and made up to 3 litres.

(iv) 0.1 N NaOH: 6.5 ml of the 50% NaOH was diluted to 1.2 litres with carbon dioxide free distilled water and standardized against potassium hydrogen phthalate.

(v) Potassium sulfate - mercuric oxide (catalyst): In polyethylene packages (Kel-Pak powder No. 5) obtained from Canlab.

(vi) Concentrated $H_2SO_4$ (sp. gr. 1.84): Nitrogen free, obtained from Fischer Scientific Co.

(vii) Boric acid solution: prepared by dissolving 40 g of boric acid ($H_3BO_3$) in water and diluted to 1000 ml with water.

(viii) Copper sulfate pentahydrate, potassium sulfate anhydrous, zinc chips, boiling regulators.

(ix) Mixed indicator solution: 1 g methyl red and 0.5 g methylene blue was dissolved in 500 ml ethanol (96% V/V), stored in the refrigerator in brown bottle. The indicator color change occurs at pH 5.4 (Osborne and Voogt, 1978).

(x) Kjeldahl flasks: 100 and 800 ml capacities.

(xi) Distillation apparatus, heating device, magnetic stirrer.

Fig. C2. Materials and Methods - Total Available Carbohydrates as Glucose.

Both lyophilized and wet samples were used. Each sample was accurately weighed (1 g of lyophilized sample and 2.5 g of the wet sample), and transferred quantitatively into a 100 ml graduated cylinder. Ten ml of water was added and then stirred with a long rod to disperse the sample. To this, 13 ml of 52% perchloric acid, prepared by adding 270 ml of perchloric acid (Baker Chemical Co.) to 100 ml of water and cooled before use, was added and the contents of the cylinder was stirred frequently with the glass rod for 30 min. The rod was washed down with water and the contents of the cylinder was made up to 100 ml. The diluted sample was mixed and filtered through Whatman No. 542 filter paper into a 250 ml volumetric flask. Washings with 10 ml portions of water were also transferred through the filter paper into the volumetric flask and then made up to mark with water. After mixing, 10 ml of the extract was transferred into a 100 ml flask and made up to volume with water. One ml of the sample extract was used for the determination of the total available carbohydrate. For standards, 100 mg of anhydrous glucose (Aldrich Chemical Co., U.S.A.) was dissolved in 100 ml distilled water to obtain a concentrated standard solution. Ten ml of the concentrated standard was diluted to 100 ml with water to obtain the working standard (1 ml ≡ 0.1 mg glucose). One ml of the sample extract, water and working glucose standard were each pipetted into stoppered test tubes in duplicate. Five ml of 0.1% anthrone reagent (Anthrone from Baker Chemical Co.) in sulfuric acid (prepared by addition of

760 ml sulfuric acid, specific gravity 1.84, to 330 ml of water and cooled before use) was added to the contents of each test tube. The tube was then stoppered, its contents mixed and placed in boiling water for exactly 12 min. The tubes were retrieved and cooled quickly to room temperature. The optical densities of the samples, standards and blanks were read at 630 nm against reagent blanks in a Beckman model spectrophotometer (Beckman, U.S.A.). A standard curve was prepared for glucose. The amount of total available carbohydrate in each sample was determined as outlined in the text.

Fig. C3. Materials and Methods - Gross Energy.

The materials employed included:

- Lyophilized ground samples
- Parr Instrument for pelleting
- Tared combustion capsule
- Oxygen gas cylinder with regulator valve
- Parr fuse wire
- Distilled and tap water at about 2°C below the ambient temperature
- Adiabatic calorimeter (Parr Instrument, Moline, Illinois, U.S.A.) linked to a calorimetric thermometer module (Parr 1661) with output temperature recorded on a chart (speed: 1 cm/min).

About 1 g of sample, accurately weighed, was pelleted and introduced into the bomb containing 1 ml of water. Fuse wire was looped over the sample and oxygen was admitted into the bomb to a pressure of 300 kPa. The bomb was placed in the adiabatic calorimeter. Water (2000 ml) was added to the calorimeter and stirred continuously while the pellet was ignited. The rise in temperature ($\Delta T$) was obtained from the chart output and the G.E. determined using the relationship.

$$\text{G.E.} = \frac{\Delta T \times \text{Bomb factor} - (\text{Fuse} + \text{Acid}) \text{ Heat (kJ)}}{\text{Sample weight (g)}}$$

The bomb factor was 9.88 kJ/°C.

Fuse Heat (kJ/cm) was obtained by measuring the remnant of fuse wire. Each combusted centimeter of wire contributed 4.2 joules of heat and had to be subtracted from the total heat.

Acid Heat was obtained by washing the condensate in the bomb into a beaker with glass distilled water and then titrated against $Na_2CO_3$ solution (prepared by dissolving 3.84 g $Na_2CO_3$ in 1 l of water). One ml of indicator (0.1% methyl orange) was used; the end point was yellow. One ml of titre is equivalent to 4.2 joules of heat. This heat was also subtracted from the total heat.

The G.E. was obtained in kilojoules per gram.

# APPENDIX D

Table D1. Radiation Strength (Calculated) During the Period of Investigation in the Macdonald Unit - GC 220 AECL (Source Cobalt 60)*.

| Month | Year** | | | |
|---|---|---|---|---|
| | 1978 | 1979 | 1980 | 1981 |
| January | .1637 | .1434 | .1256 | .1101 |
| February | .1619 | .1418 | .1242 | .1089 |
| March | .1601 | .1402 | .1228 | .1077 |
| April | .1583 | .1387 | .1215 | .1065 |
| May | .1566 | .1372 | .1202 | .1053 |
| June | .1549 | .1357 | .1189 | .1041 |
| July | .1532 | .1342 | .1176 | .1030 |
| August | .1515 | .1327 | .1163 | .1018 |
| September | .1498 | .1312 | .1150 | .1007 |
| October | .1482 | .1298 | .1137 | .0996 |
| November | .1466 | .1284 | .1125 | .0985 |
| December | .1450 | .1270 | .1113 | .0974 |

* Cobalt 60 $T_{\frac{1}{2}}$ = 5.27 years.
Decay factor (1 month) = 0.01096

** Each value recorded in the table multiplied by $10^6$ will give value in rads.

$10^2$ rads = 1 kGy

$10^3$ rads = 1 Mrad.

Fig. D1: Radiation Strength at Varying Positions within the Macdonald Unit.

Figure D1

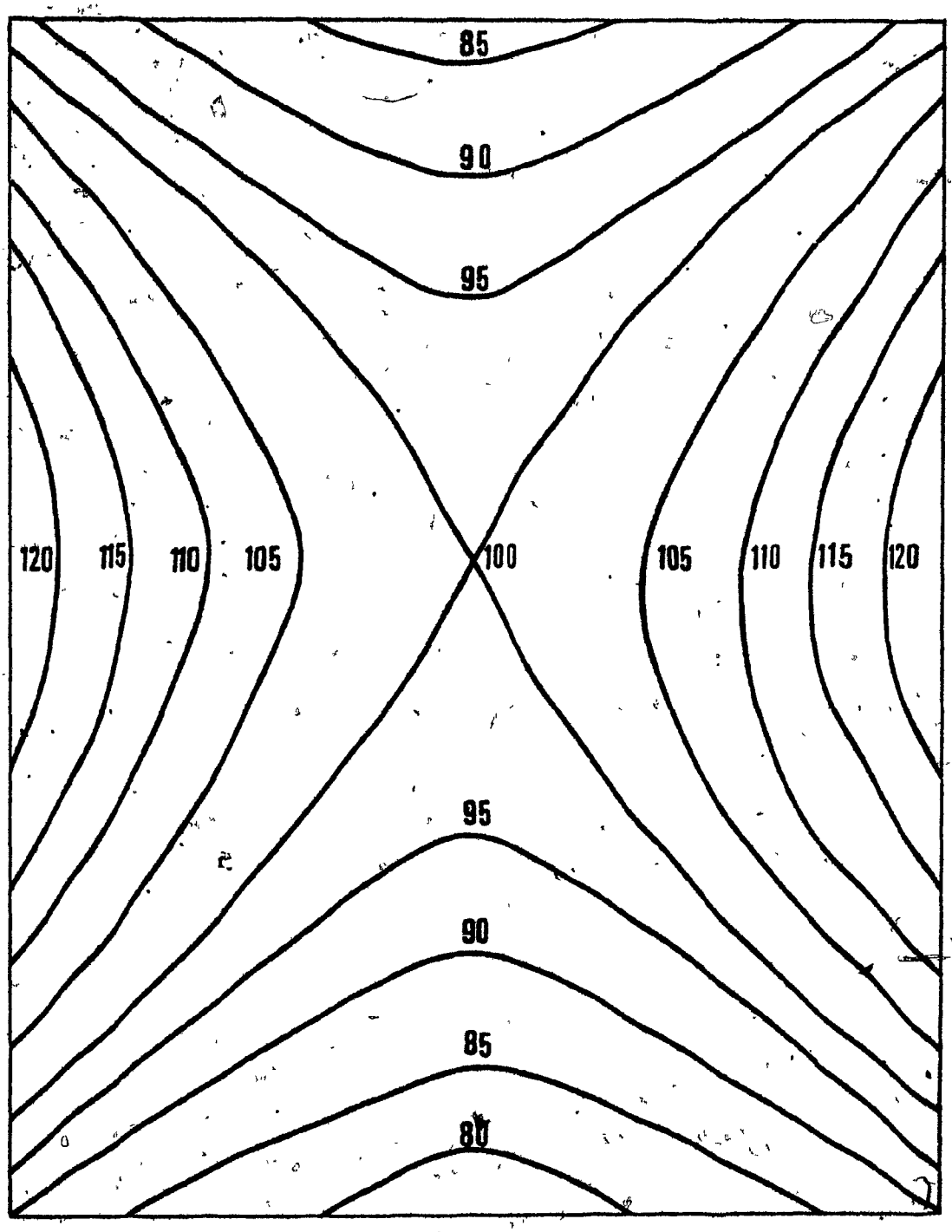
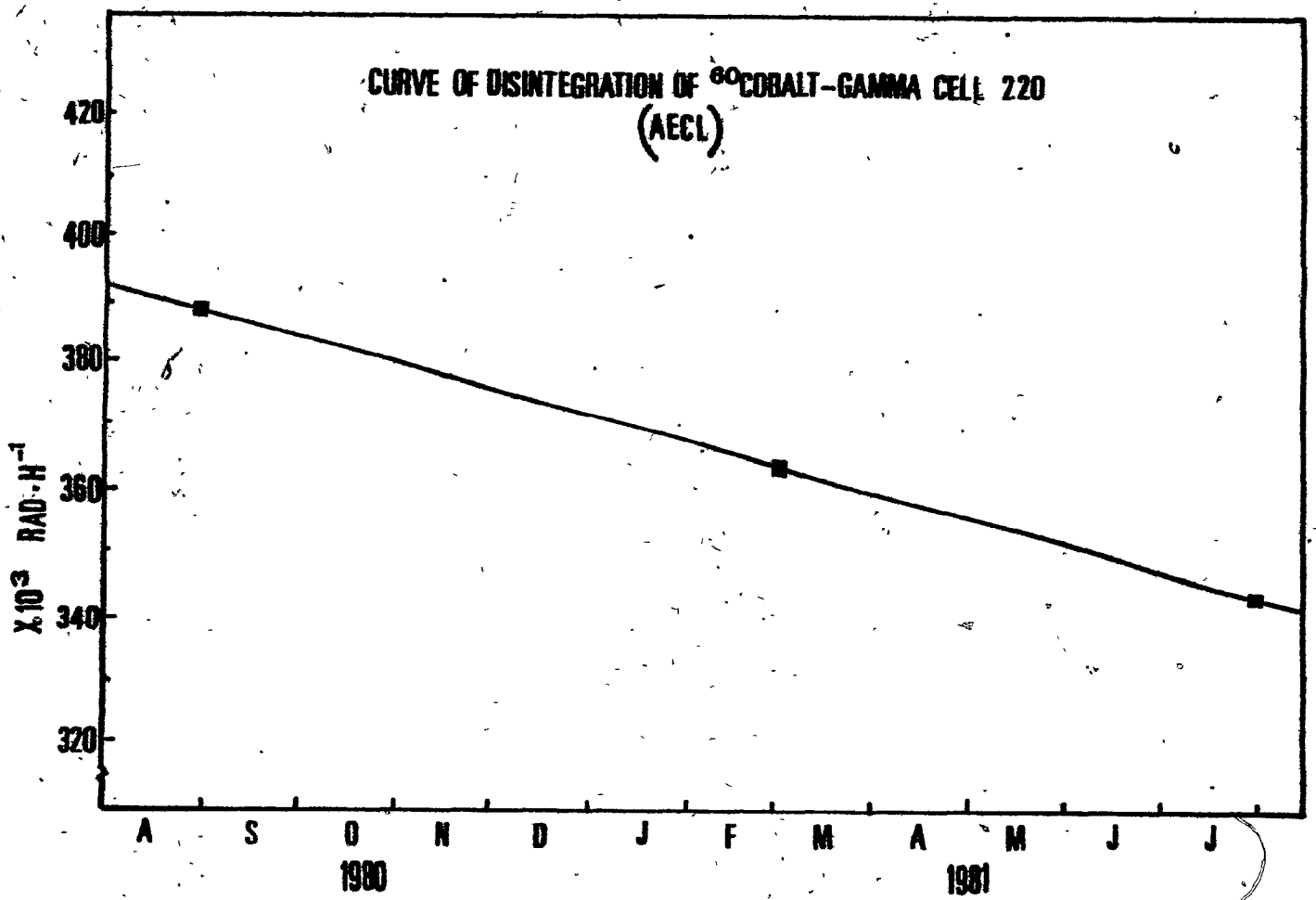

85
90
95
120
115
110
105
100
105
110
115
120
95
90
85
80

Fig. D2: Standard Disintegration Curve — Armand Frappier Unit, Laval, P.Q.

Figure D2


CURVE OF DISINTEGRATION OF $^{60}$COBALT-GAMMA CELL 220
(AECL)
420
400
380
360
340
320
X.10$^{3}$ RAD·H$^{-1}$
A
S
O
N
D
J
F
M
A
M
J
J
1980
1981

# APPENDIX E

Table E1. Brain Heart Infusion Broth (BHIB).

| Constituent | Amount (g) |
|---|---|
| Calf Brains, Infusion from | 220.0 |
| Beef Heart, Infusion from | 250.0 |
| Proteose Peptone, Difco | 10.0 |
| Bacto-Dextrose | 2.0 |
| Sodium Chloride | 5.0 |
| Disodium Phosphate | 2.5 |

Preparation: A 37 g of the dehydrated powder is added to 1000 ml of distilled water, dissolved and dispensed as required. The medium is sterilized in the autoclave at 121°C for 15 min. The final reaction pH of medium is 7.4.

Table E2. Milk Agar (Casein Agar) pH 7.2.

| Constituent | Amount |
|---|---|
| Peptone | 5 g |
| Milk powder | 100 g |
| Agar | 15 g |
| Distilled water | 1000 ml |

Preparation: The ingredients are dissolved in distilled water by boiling and stirring. The medium is sterilized at 121°C for 15 min and dispensed into sterile petri plates.

Milk agar is used for the qualitative determination of the presence of proteases.

Table E3. MRS (de Man Rogosa Sharpe) Broth.

| Constituent | Amount |
|---|---|
| Peptone (Oxoid L34) | 10.0 g |
| Lab-Lemco Powder (Oxoid L29) | 8.0 g |
| Yeast extract (Oxoid L21) | 4.0 g |
| Dextrose | 20.0 g |
| Tween 80 | (1 ml) |
| Di-potassium hydrogen phosphate ($K_2HPO_4$) | 2.0 g |
| Sodium acetate $.3H_2O$ | 5.0 g |
| Tri-ammonium citrate | 2.0 g |
| Magnesium sulfate $.7H_2O$ | 0.2 g |
| Manganese sulfate $.4H_2O$ | 0.05 g |

Preparation: A 52 g of the dehydrated powder is added to 1000 ml of distilled water, brought to boil, dispensed as required and autoclaved at 121°C for 15 min. The final pH is about 6.2.

NOTE: MRS Agar

MRS agar is prepared by the addition of 2% agar-agar to the broth constituents above.

MRS broth and MRS agar are non-selective media for the profuse growth of Lactobacilli.

Table E4. Plate Count Agar (PCA).

| Constituent | Amount |
|---|---|
| Bacto - Tryptone | 5 g |
| Bacto - Yeast Extract | 2.5 g |
| Bacto - Dextrose (Glucose) | 1 g |
| Bacto - Agar | 15 g |
| Distilled Water | 1000 ml |

Preparation: 23.5 g of dehydrated PCA powder is suspended in 1000 ml cold distilled water and dissolved completely by heating to boil. The medium is sterilized for 15 min at 121°C. The final pH at 25°C is 7.0.

Table E5. m Plate Count Broth (mPCB).

| Constituent | Amount |
|---|---|
| Bacto - Yeast Extract | 5 g |
| Bacto - Tryptone | 10 g |
| Bacto - Dextrose | 2 g |
| Distilled Water | 1000 ml |

Preparation: 17.0 g of dehydrated mPCB powder is suspended in 1000 ml cold distilled water and dissolved completely by heating to boil. The medium is sterilized for 15 min at 121°C. The final pH at 25°C is 7.0.

Table E6. Potato Dextrose Agar (PDA).

| Constituent | Amount |
|---|---|
| Potatoes, Infusion from | 200 g |
| Bacto - Dextrose | 20 g |
| Bacto - Agar | 15 g |
| Distilled Water | 1000 ml |

Preparation: 39 g of dehydrated PDA powder is suspended in 1000 ml cold distilled water and dissolved completely by heating. The medium is sterilized by autoclaving for 15 min at 121°C. Tartaric acid (10% solution) millipore filter sterilized was added to adjust the pH to 3.5. Plates were poured.

PDA (acidified) is recommended for the determination of yeasts and molds (Difco, 1974).

Table E7. Standard Methods Caseinate Agar (SMCA) pH 6.6.

| Constituent | Amount (g) |
|---|---|
| Pancreatic digest of casein (Bacto Tryptone) | 5.0 |
| Yeast extract (Difco) | 2.5 |
| Glucose | 1.0 |
| Agar (Difco) | 15.0 |
| Sodium caseinate | 10.0 |
| Trisodium citrate (Hydrated) $Na_3C_6H_5O_7.2H_2O$ | 4.41 |
| $CaCl_2.2H_2O$ | 2.94 |

Media Preparation: The SMCA was prepared using Martley et al. (1970) method. One litre of 0.015 M sodium citrate solution was prepared by dissolving 4.41 g of hydrated trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) in 1000 ml tap distilled water. The 0.015 M sodium citrate was divided into two halves of 500 ml each. 5.0 g of pancreatic digest of casein, 2.5 g of yeast extract, 1.0 g of glucose and 15.0 g of agar were rehydrated in one part of 500 ml sodium citrate to obtain Standard Method Agar (SMA). The balance (500 ml) of sodium citrate was employed to disperse 10.0 g of sodium caseinate. The dispersed sodium caseinate was added to the rehydrated and molten SMA and the mixture was autoclaved

at 121°C at 15 psi for 15 min. 1.0 M $CaCl_2.2H_2O$ (2.94 g/l) was prepared to replace 1.0 M $CaCl_2.6H_2O$ (4.38 g/l). The prepared 1.0 M solution of $CaCl_2.2H_2O$ was filter sterilized before it was added to the autoclaved media. The SMCA and $CaCl_2.2H_2O$ were thoroughly mixed and dispensed into petri plates. Plates were held at 37°C for 48 h, cooled at 20°C before use or storage at 4°C until required.

Table E8. Starch Agar Medium (pH 7.0).

| Constituent | Amount |
|---|---|
| Peptone | 5 g |
| Beef extract | 3 g |
| Soluble starch | 2 g |
| Agar | 15 g |
| Distilled water | 1000 ml |

Preparation: The ingredients are dissolved in distilled water by boiling and stirring. The medium is sterilized at 121°C for 15 min and dispensed into sterile plates.

Starch agar is used for the qualitative determination of the presence of carbohydrase.

Table E9. Tomato Juice Agar (TJA).

| Constituent | Amount |
|---|---|
| Tomato Juice (400 ml) | 20 g |
| Bacto - Peptone | 10 g |
| Bacto - Peptonized Milk | 10 g |
| Bacto - Agar | 11 g |
| Distilled Water | 1000 ml |

Preparation: 51 g of dehydrated TJA powder is suspended in 1000 ml cold distilled water and dissolved completely by heating to boil. The medium is sterilized for 15 min at 121°C. The final medium pH is 6.1.

TJA is recommended for the direct plate count and cultivation of Lactobacilli (Difco, 1974).

Table E10. Tributyrin Agar.

| Constituent | Amount |
|---|---|
| Nutrient agar | 23 g |
| Distilled water | 1000 ml |
| Tributyrin | 10 ml |

Preparation: Nutrient agar containing 23.0 g in 1000 ml distilled water is prepared and tributyrin agar is added. The two constituents are blended together in a Waring blender to emulsify the tributyrin agar. Usually preparation is by portions i.e. 200 ml nutrient agar + 2 ml tributyrin, blended and dispensed into bottles.

Sterilization: Achieved by Tyndallization. Bottles containing the media are steamed for 30 min and the media stored till the second day when the media is steamed for another 30 min followed by storage. The steam treatment is again carried out for 30 min on the 3rd consecutive day and the media poured into sterile petri plates.

Tributyrin agar is used for the qualitative determination of the presence of lipases.

Table E11. Wort Agar (WA).

| Constituent | Amount (g) |
|---|---|
| Bacto - Malt Extract | 15 |
| Bacto - Peptone | 0.78 |
| Maltose, Technical | 12.75 |
| Dextrin, Difco | 2.75 |
| Glycerol | 2.35 |
| Dipotassium Phosphate | 1 |
| Ammonium Chloride | 1 |
| Bacto - Agar | 15 |

Preparation: 50 g of dehydrated WA powder is suspended in 1000 ml cold distilled water and dissolved completely by heating to boil. The medium is sterilized for 15 min at 121°C. The medium pH is 4.8.

WA is recommended for the cultivation of yeasts (Difco, 1974).

**APPENDIX F**

Table F1. Viable Counts of PS Incubated at Different Temperatures.

| Media | $\log_{10}$ CFU·g$^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | Temperature °C | | | | | | |
| | 4 | 15 | 25 | 30 | 37 | 45 | 55* |
| MRS | ø | ø | 7.60 | 7.48 | 8.53 | 7.64 | N.D. |
| PCA | ø | 4.90 | 8.78 | 8.08 | 8.85 | 8.30 | N.D. |
| PDA | ø | ø | ø | ø | ø | ø | N.D. |
| TJA | ø | 4.30 | 8.60 | 7.85 | 8.70 | 7.83 | N.D. |
| WA | ø | ø | 7.00 | 7.30 | 5.30 | 4.60 | N.D. |

* Not determined.

Table F2. pH and Titratable Acidity (T.A.) of PS after Incubation for 72 h at Different Temperatures.

| Parameter Measured | Temperature (°C) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4 | 15 | 25 | 30 | 37 | 45 | 55# |
| pH* | 5.5 | 5.6 | 5.8 | 6.7 | 7.5 | 7.4 | N.D. |
| T.A.* (mg/g) | 1.0 | 1.0 | 1.0 | 2.3 | 5.4 | 5.0 | N.D. |

* Mean of 3 determinations.

\# Not determined.

Table F3. Viable Counts of PS Incubated at 25, 30, 47 and 45°C on a Time Sequenced Basis.

| Temp. °C | Media | $\log_{10}$ CFU·g$^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Time (h) | | | | | | |
| | | ø | 12 | 24 | 36 | 48 | 60 | 72 |
| 25 | MRS | ø | ø | ø | ø | 7.50 | 7.90 | 8.70 |
| | PCA | 6.90 | 7.65 | 8.10 | 9.51 | 8.90 | 8.90 | 8.90 |
| | PDA | ø | ø | ø | ø | ø | ø | ø |
| | TJA | 6.30 | 7.95 | 8.29 | 9.06 | 8.60 | 8.85 | 8.64 |
| | WA | ø | 4.85 | 6.72 | 7.04 | 6.94 | 6.70 | - |
| 30 | MRS | ø | 6.62 | 6.00 | 7.24 | 7.51 | 7.20 | 7.00 |
| | PCA | 5.90 | 7.99 | 8.21 | 8.43 | 8.96 | 8.08 | 8.04 |
| | PDA | ø | ø | ø | ø | ø | ø | ø |
| | TJA | 5.48 | 8.08 | 8.32 | 8.82 | 8.82 | 8.15 | 7.93 |
| | WA | ø | 4.30 | 4.60 | ø | ø | ø | ø |
| 37 | MRS | 4.48 | 6.43 | 6.90 | 7.26 | 8.83 | 8.32 | 8.04 |
| | PCA | 6.20 | 8.20 | 7.92 | 7.82 | 9.62 | 9.04 | 8.40 |
| | PDA | ø | ø | ø | ø | ø | ø | ø |
| | TJA | 6.11 | 8.29 | 8.04 | 7.87 | 8.95 | 9.04 | 8.34 |
| | WA | ø | 3.60 | 4.60 | 5.26 | 5.60 | 7.58 | 7.08 |
| 45 | MRS | 5.48 | 6.26 | 7.38 | 7.83 | 8.89 | 8.40 | 8.26 |
| | PCA | 5.54 | 7.40 | 7.85 | 8.15 | 8.85 | 8.73 | 8.53 |
| | PDA | ø | ø | ø | ø | ø | ø | ø |
| | TJA | 5.40 | 6.73 | 7.52 | 7.83 | 8.73 | 8.52 | 8.48 |
| | WA | ø | ø | ø | ø | ø | ø | ø |

Table F4. Time Sequenced pH and Titratable Acidity (T.A.) of PS Incubated at 25, 30, 37 and 45°C.

| Time (h) | pH* | | | |
|---|---|---|---|---|
| | Temperature °C | | | |
| | 25 | 30 | 37 | 45 |
| ø | 5.6 | 5.6 | 5.6 | 5.6 |
| 12 | 5.7 | 5.7 | 5.6 | 5.6 |
| 24 | 5.7 | 5.7 | 5.7 | 5.7 |
| 36 | 5.7 | 5.8 | 7.0 | 5.7 |
| 48 | 5.8 | 6.2 | 7.5 | 6.0 |
| 60 | 6.0 | 7.0 | 7.6 | 6.8 |
| 72 | 6.4 | 7.2 | 8.0 | 7.0 |

| Time (h) | Titratable Acidity* ($mg\text{-}g^{-1}$) | | | |
|---|---|---|---|---|
| | Temperature °C | | | |
| | 25 | 30 | 37 | 45 |
| ø | 1.0 | 1.0 | 1.0 | 1.0 |
| 12 | 1.0 | 1.0 | 1.0 | 1.0 |
| 24 | 1.0 | 1.0 | 1.4 | 1.2 |
| 36 | 1.0 | 1.0 | 5.4 | 1.8 |
| 48 | 1.0 | 1.4 | 5.9 | 3.6 |
| 60 | 1.8 | 3.2 | 6.3 | 5.4 |
| 72 | 2.3 | 5.0 | 8.2 | 6.8 |

* Mean of 3 determinations.

## 10. DEDICATION

To my kind parents
Margaret and James Ikenebomeh
who got me started;
To my loving wife Juliana Edo Ikenebomeh and
children Omegie, Ogie and Afe
who keep me going;
To my good aunt, Theresa Ayemere and
uncle Julius Enekhaze who give me constant support;
To my able teachers
who guided me all along;
To my country Nigeria for a better tomorrow.
Above all to God who made it possible for me
to be a part of this great academic circle -
McGill University, Montreal, Canada.

MARCEL J. IKENEBOMEH

Montreal, Canada

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