THE MICROBIAL ACTIVITIES INVOLVED IN THE ALKALINE FERMENTATION OF SOYBEANS INTO DAWADAWA

BY

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DECLARATION

I declare that, this work was carried out by myself at the Food Research Institute (FRI) and the Department of Nutrition and Food Science, University of Ghana, Legon under the Supervision of Dr. W. K. Amoa-Awua (FRI) and Dr. (Mrs.) Esther Sakyi-Dawson (Legon) and that it has not been presented in part or whole to any University for the award of a degree.

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DEDICATION

Dedicated to my husband Alfred Kofi Darkwa,
my children Maame Koomah and Paa Kweku Darkwa
and my parents Mr. and Mrs. Opai Tetteh.

ABSTRACT

This study was initiated to identify the dominant microbial species and investigate their activities during the fermentation of soybeans to dawadawa.

The dominant microorganisms present in both the boiled and roasted soydawadawa, which were spontaneously fermented for 72 h, were isolated, characterized and identified using an API kit. The proteinase and α-amylase activities of the microorganisms were determined. Quality indices such as pH, moisture, protein and fat contents of the soydawadawa were measured. The breakdown of proteins during fermentation of soydawadawa was studied using Gel electrophoresis. The trend in the level of sugars during fermentation of soydawadawa was carried out using the Lane and Eynon's method. Aromatic compounds (GC-MS method) produced by the different types of soydawadawa were also determined.

Results showed that *Bacillus* species were the dominant micro-organisms found and which increased from 10^3 to 10^{11} cfu/g and 10^3 to 10^9 for the boiled and roasted soydawadawa from the start to the end of fermentation respectively. Lactic acid bacteria (10^4 to 10^6 cfu/g) were also found. *Bacillus subtilis* accounted for 48% of the representative isolates taken from various stages of fermentation. Other *Bacillus* species accounted for 8% to 16% of the isolates. All the identified *Bacillus* species demonstrated proteolytic activity in the order *Bacillus subtilis* >*Bacillus formis* > *Bacillus cereus* > *Bacillus pumilus*. At $\alpha = 0.05$, proteolytic activity was significantly affected by fermentation time and production method. However, there was no interaction between these two factors.

α-amylase activity was positively affected by fermentation time, increasing with increase in fermentation time.

Hydrolysis of proteins during fermentation was confirmed in soydawadawa by an increase in the fraction of lower molecular weight proteins and more distinct bands. The level of sugars increased in the first 24 hours but decreased subsequently as fermentation progressed.

Aroma compounds produced were more in the roasted products than the boiled. Both products were dominated by 3-Hexanol and 9, 12-Octadecanoic acid. However, Tetradecanoic acid and 1,2-Benzene dicarboxylic acid were found only in the roasted product.

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1.0 INTRODUCTION

In developing countries, the diet of most people is based on processed cereal grains such as sorghum, rice and maize and on root crops such as cassava and fruits or vegetables such as plantain. These foods by virtue of the fact that they are consumed in large quantities provide some amount of protein, yet the quantities are low. Food legumes due to their high protein content generally constitute the natural protein supplement to staple diets (Singh and Rachie, 1985). They are also perfect alternatives for costly meat and fish proteins and help to supplement cereal grains which lack lysine. They are healthy and satisfying, blend well with a full range of flavours and as the traditional foundation for many ethnic dishes, can give consumers an exotic taste experience (Ihenkoronye and Ngoddy, 1992). Grain legumes are therefore very important in the diets of the people in most developing countries. Apart from their high protein content, legumes contain good amounts of B-Vitamins and minerals at adequate levels (Kordylas, 1991).

In recent times, there has been a widespread effort to combat the seemingly persistent problem of malnutrition among the ever-increasing population particularly in the developing countries. In view of this, priority has been placed on the need for an effective increase in the exploitation of the many avenues for a good source of new and unconventional low-cost and high quality protein foods.

Dawadawa is the Hausa name for a strong smelling thick dark coloured paste of fermented African locust bean seeds (*Parkia biglobosa*). Although "Dawadawa" is

used as a food condiment, it contributes to the protein and calorific content of food. Steinkraus and Van Veen (1971) stated that traditionally fermented protein rich foods offer excellent possibilities for improving the diets of people around the world. Dawadawa is a good source of lysine needed to complement cereal foods with inadequate lysine content (Annegers, 1974). It has no cyanogenic glucosides and is nutritionally important (Schery, 1972).

Dawadawa is prepared by boiling the seeds of African locust bean to soften the testa. It is then dehulled, reboiled to soften, drained, fermented and dried. In Ghana, dawadawa has always been processed traditionally from the African locust bean seeds by women in the Northern Regions of Ghana. Low- income families use dawadawa as a low-cost meat substitute and they generously add it to soups or stews and sorghum or millet-based dumplings and porridge. Dawadawa is also popular in several West and Central African countries such as Nigeria where it is also called "iru" and in Burkina Faso where it is known as "soumbala". As the population of Africa increases there will be more demand for animal protein sources as well as other protein sources such as dawadawa or similar vegetable protein products.

The process of making dawadawa requires little capital but is constrained by the following:

1. The African locust beans whose seeds are used to make dawadawa grow in the wild.
2. The seeds are produced seasonally.
3. Long hours are used in hunting for seeds
4. There is the risk in climbing trees for seeds

5. Processing of the seeds into dawadawa is time consuming and laborious.

Soybean *(Glycine max)* like the locust bean is a grain legume, cultivated in many areas of the world. It has for several centuries contributed significantly to the nutritional requirements of the people in many East and South East Asian countries forming a major part of the traditional diet (Singh *et. al.* 1987). It is fermented into products such as natto, miso and tempeh and are processed to give soymilk and soy sauce which is a principal flavouring and sauce in Eastern Asia (Carrao *et. al.* 1994).

Soybean is gradually becoming very popular in Ghana as an important source of protein and oil. It contains 40% high quality protein (dry matter basis) with a good balance of the essential amino acids closely approximating standards established by FAO (Weigartner, 1987). Its oil, 20-32% (dry matter basis), is quite desirable because it contains a large proportion of unsaturated fatty acids (FAO, 1989).In recent years the use of soybeans for producing dawadawa has been introduced into Nigeria and is also being promoted in Ghana by Women in Agriculture and Development (WIAD) and Food Research Institute (FRI). The reasons for promoting soybeans for the production of dawadawa are that soybeans are:

1. rich in proteins, fat, minerals and vitamins
2. easy to grow
3. obtained all year round.
4. processed into dawadawa which has been found to be acceptable to consumers.

Even though a lot of scientific investigations have been carried out to evaluate microbiological and biochemical changes which occur during the fermentation of

African locust bean seeds into dawadawa very little work has been carried out to explain the fermentation of soybeans into dawadawa. It is therefore important that the microorganisms responsible for the fermentation of soybeans into dawadawa are evaluated and the extent of protein modification during soydawadawa production investigated. Such information will help to upgrade the current traditional process used for soydawadawa production and facilitate its promotion and adoption in dawadawa consuming parts of the country.

1.1 OBJECTIVES

This study was initiated to:

1. Identify the dominant microbial species involved in the fermentation of soybean into dawadawa and characterize some of their technological properties.
2. Investigate the modification of protein during soydawadawa production and define the amino-acid profile of the product.
3. Study the effect of different processing methods on the composition and quality of soydawadawa.

2.0 LITERATURE REVIEW

2.1 FERMENTATION

Fermentation is a process which relies upon the enzymatic reactions of micro-organisms normally present or added as starter culture to a food to cause change of properties, evolution of heat and effervescence. Although fermentation has long been recognized as one of the means of preserving foods, the phenomenon has also been employed to develop desirable characters such as flavour and texture. In the Far East however, fermentation processes used in most food preparations have been found to cause reduction or elimination of its antinutrients. A traditional food such as tempeh is a well known example (Steinkraus *et al.* 1960, 1965, 1983; Shallenberger *et. al.* 1967).

Fermentation improves digestibility and increases the concentration of anti-oxidants and vitamins. The micro-organisms involved in fermentation are not only catabolic, breaking down several complex vitamins and other growth factors (Potter, 1968). The industrial production of such materials as riboflavin, vitamin B_2 and the precursor of vitamin C are largely by special fermentation processes. Fermentation also causes the liberation of nutrients locked into plant structures and cells by indigestible materials. Certain bacteria, yeasts and moulds break down indigestible protective coatings and cell walls both chemically and physically. These micro-organisms during fermentation release certain enzymes which split cellulose, hemicellulose and related polymers which are not digestible by man into simpler sugars and sugar derivatives (Potter, 1968).

Fermentation could either be in the liquid state or solid state. Solid substrate fermentation is defined as the growth of micro-organisms on solid materials without the presence of free liquid. This covers the fermentation of plant derived solids to produce foods for human consumption. In the case of the liquid substrate fermentation, there is the presence of free liquid (Paredes-Lopez and Harry, 1988).

2.2. *PARKIA BIGLOBOSA* (AFRICAN LOCUST BEAN)

The taxonomy of African locust bean trees has been in a state of flux until recently. Because of this, various reports in the literature have referred to this same tree as *Parkia filicoidea, Parkia. bicolor* and *Parkia. biglobosa.* The related species *Parkia. filicoidea* is indigenous to the forests of East and Central Africa. The *Parkia. bicolor* is found in the forest regions of West Africa. The fruit provides a constant source of valuable protein in the dry season. The Locust bean tree is also used for medicinal purposes and as a source of mouth wash to relieve toothaches. The bean husks (seed coats) are used with indigo dye to improve the lustre of fabrics, while the tree bark yields a red tannin for dying leather. The dry yellow powdering pulp is rich in sweet carbohydrate and can be mixed with cereal, meat or soup. It can also be made into candy or be pressed into cakes for preservation (Abbiw, 1990).

Dawadawa is the name given to fermented African Locust bean seeds (*Parkia* species) and is an important condiment in the Northern Regions of West Africa. Dawadawa as known often by the Hausa name has other names such as *iru* in Yorubaland of Nigeria, *Ogiri-igala* in Iboland, *Kpalugu* among the Kusasis and Dagombas of northern Ghana, *Koligo* and *Tsogo* in Ghana; *Kinda* in Sierra Leone and *netetou* or *soumbala* in Gambia and other French speaking countries. (FAO, 1989; Campbell-Platt, 1980; Ihekoronye and Ngoddy, 1985).

The steps involved in preparation of dawadawa from African locust bean seeds are shown in Figure 2.1. The process is believed to involve a number of proteolytic changes in the substrate due to bacterial action. The fermentation which is by chance inoculation is by various sub-species of the *Bacillus subtilis* group (Odunfa, 1981; FAO, 1989). The African locust bean seeds are boiled in water to soften the seed coat or testa and then dehulled by pounding in a mortar or by rubbing them between the palms. The cotyledons are washed and boiled further for 1-2 h. These are spread in trays or baskets and wrapped in many layers of jute sacks or leaves. The seeds ferment for about 36 h or longer. The fermented seeds have a greyish sticky mucilage covering and a strong ammoniacal smell. The colour also changes from light brown to dark brown.

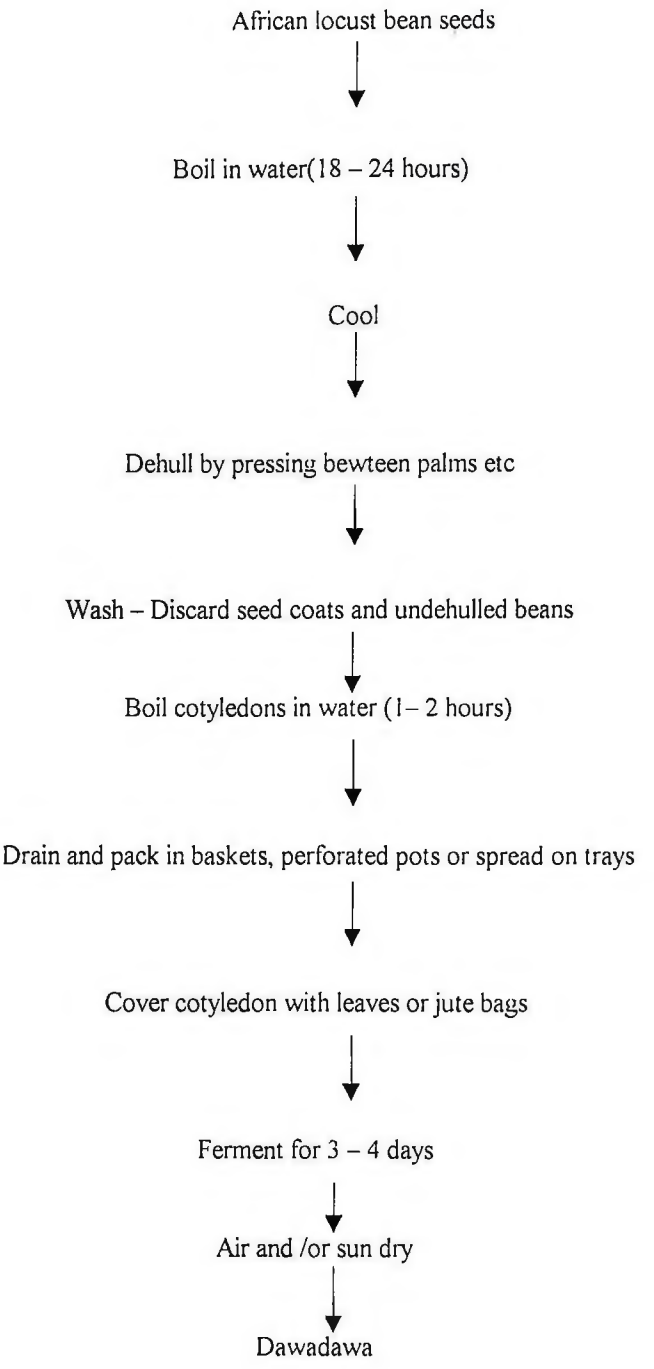


Fig. 2.1 **Flow diagram of the processing of African locust bean into dawadawa.**

Different ethnic groups process dawadawa from different raw material as shown in Table 2-1

Table 2-1 TYPES OF DAWADAWA

Ethnic group	Raw material	Shape	Bean
Dagarti	Locust Bean Soybean	Ball	Paste
Dagomba	Locust Bean mixture or Locust Bean	Cylindrical Ball and Flat	Bean Paste
Frafra	Locust Bean	Ball	Bean Paste
Kussasi	Locust Bean Soybean mixture or Locust bean groundnut mixture	Cylindrical Ball and Flat	Bean Paste
Mossi	Locust Bean	Ball	Bean

Source: Campbell – Platt (1980)

2.2.1 Microbiological And Physico-Chemical Changes During The Fermentation of Dawadawa

Dawadawa fermentation is a solid-substrate fermentation where the growth of micro-organisms occur without the presence of free liquid. The physiological and microbiological changes that occur during fermentation of African locust bean seeds into dawadawa are presented in Table 2-2.

Table 2-2 Physical And Microbiological Changes During Fermentation Of *Parkia* Seeds

Fermentation time (h)	Moisture Content (%)	Temp. (°C)	pH	Plate Count	
				Aerobic	Anaerobic
0	43.0	25	7.0	0	$5.0x10^4$
12	48.0	30	7.2	0	$2.4x10^6$
24	54.0	42	7.5	$1.8x10^4$	$1.2x10^6$
36	56.0	45	8.1	$2.5x10^6$	$3.0x10^5$

(Odunfa 1983)

Several physico-chemical changes occur during fermentation of African locust bean seeds The temperature of the fermenting seeds increase from about 30 °C to a maximum of about 50 °C in 24h. The pH increases to about 8.1 during the first 30h of fermentation, due in part to the production of ammonia. The most significant biochemical change that occurs during dawadawa fermentation is protein hydrolysis. This is due to the high proteinase activity which results in rapid amino acid production (Odunfa, 1983).

Odunfa (1983) studied the extracellular enzymatic activities of the micro-organisms in fermenting African locust beans. The enzymes α -galactosidase, β- galactosidase, sucrase, proteinase, amylase and lipase were detected in fermenting dawadawa. Glycosidases hydrolyze African locust bean oligosaccharides (stachyose, raffinose and sucrose) and other complex sugars during fermentation. The high temperature which develops during the fermentation hastens enzymatic activities and biconversion. The optimum activity of the glycosidases occur at 12h and 24h of fermentation respectively. Invertase activity is highest at 36 h. whilst that of amylase is fairly low and evident only in the first 24h of fermentation

(Odunfa, 1983). Presumably all the starch is hydrolyzed during this period and is not detected in fermented dawadawa (Watson, 1971).

The sugars produced during fermentation through amylolysis provide easily utilizable substrates for the micro-organisms. Although oil constitutes 31 to 40% of the locust bean seeds, lipase activity is low in fermenting African locust beans and there is fluctuation in activity throughout the fermentation. Campbell-Platt (1980), suggested that lipolytic activity occurred in the later stages of African locust bean fermentation. This observation was based on the increase in the number of lipolytic micro-organisms. Another significant change during the fermentation of African locust bean seeds is a decrease in percentage of free fatty acids i.e. from 0.6% in the cooked unfermented African locust bean seeds to 0.1% in dawadawa (Odunfa, and Adesomoju, 1983). The decrease in free fatty acids is desirable since large amounts of free fatty acids in foods can result in objectionable taste and cause rancidity. However they sometimes produce characteristic flavours in some foods.

2.2.2 Micro-Organisms associated with Dawadawa fermentation

Only bacteria have been reported to be associated with the dawadawa fermentation Ikenebomeh (1982), reported a few fungi in the dawadawa fermentation as contaminants. Campbell-Platt (1980), reported that *Penicillium*, fermenting oval budding yeasts, and the film yeast *Candida* were incidental contaminants. They constituted 3% of the total microbial isolates. The majority of the bacteria found in dawadawa are aerobic while approximately 10% are anaerobic after 36h.of fermentation (Table 2-2).

Odunfa (1981), first reported that the predominant fermentation micro-organism was a *Bacillus*, possibly *Bacillus subtilis* and other species. Later Odunfa and Adesomoju (1983), confirmed the presence of *Bacillus pumilis, B. licheniformis* and *B. subtilis* in the fermentation. Similar findings have been reported elsewhere (Campbell-Platt 1980; Ikenebomeh, 1982). In some Nigerian samples, *Pediococcus* and two strains of *Staphylococcus saprophyticus* were detected (Odunfa, 1981). Adewuyi (1983), confirmed the presence of *Bacillus subtilis* strains in dawadawa fermentation. He reported that *Bacillus subtilis* is responsible for production of acceptable dawadawa. All isolates of the *Bacillus subtilis* from dawadawa were found to be proteolytic, with only a few strains being amylotytic (Adewuyi, 1983). Campbell-Platt (1980), found that *Bacillus* species composed 95% of both proteolytic and amylolytic isolates, and 76% of lipolytic isolates. They were thermotolerant, grew at 50°C with an optimum temperature at 35°C to 40°C. They were facultative anaerobes and grew over a wide range of pH. Diawara *et. al.* (1998) found the dominating *Bacillus* species in dawadawa produced in different parts of Burkina Faso to be *Bacillus subtilis*.

2.2.3 Nutritional Composition And Quality

Campbell-Platt (1980) reported that on a moisture free basis, dawadawa contains 38.5% protein, 31.2% fat, and 23,6% carbohydrate, compared to unfermented African locust bean seeds, which have 30% protein, 15% fat and 49% carbohydrate. Fetuga *et. al.* (1973) observed a small decrease in sulfur-containing amino acids and a greater decrease in aspartic and glutamic acids as a result of fermentation of locust beans. Like many dry beans, locust beans are low in the sulfur-containing amino acids, cysteine and methionine (Fetuga et. al., 1973). Eka (1980) found dawadawa to be low in the essential amino acids,

leucine, isoleucine, phenylalanine, and tryptophan. However, the essential amino acids in the main meal in which dawadawa is used as a condiment help to complement the low levels in dawadawa.

Unsaturated fatty acids account for about 60 to 80% of total lipids in dawadawa (Busson, 1965; Girgit and Turner 1972). Linoleic acid is the major fatty acid in dawadawa and others found in appreciable amounts include palmitic, stearic and oleic acids (Odunfa, 1983; Girgit and Turner, 1972). Much of the available reducing sugars and other carbohydrates are utilized by micro-organisms during fermentation. The raffinose family of oligosaccharides and sucrose decreased significantly during fermentation (Odunfa, 1983).

Fully fermented dawadawa contains very little reducing sugars. Watson, (1971) found less than 0.1% glucose, 0.3% fructose and no starch or sucrose in fermented dawadawa. Dawadawa contains appreciable amounts of folate (Keshinro, 1983; Hug *et. al.* 1983). Out of 24 foodstuffs analysed, dawadawa was the second highest in folate content after sweet potatoes (Hug *et. al.* 1983). Folate content in dawadawa ranges from 0.89 to 0.95 μg/g on a dry weight basis. Significant amounts of folate can be derived from dawadawa to satisfy the adult RDA requirement for folate.

Dawadawa contains most of the important minerals adequate to meet the RDA requirements with the exception of calcium which is deficient in the diet of many West Africans (Oke, 1972). The digestibility of fermented beans averages 97.6% with a range of 93.2 to 99.4% (Umoh and Oke, 1974). The digestibility of protein measured as protein efficiency ratio (PER), net protein utilisation (NPU), and biological value (BV) of

fermented African locust beans were found to be higher than that of the raw seeds (Fetuga *et. al.* 1973; Umoh and Oke, 1974).

2.2.4 Toxicological Aspects

The levels of toxic substances such as oxalic acid, phytic acid and hydrocyanic acid are high in unfermented locust beans. However, some of these toxic substances are reduced during cooking and fermentation of dawadawa (Table 2-3). Although hydrocyanic acid is present in African locust beans, its level is not harmful to the body (Oke, 1969). Soluble oxalate is present in low levels in dawadawa and is toxic to animals. There is an observed decrease in the phytic acid and oxalic acid content of dawadawa during fermentation which makes more mineral elements available (Eka, 1980). Phytohaemagglutinins in various species of *Parkia* are normally destroyed by cooking and are not found in fermented dawadawa. Studies by Alozie *et al.* (1980) showed that aflatoxins are not present in dawadawa and is attributable to the alkaline pH which is unfavourable to mould growth.

Table 2-3 Vitamin and Toxicant Content of Fermented and Unfermented Dawadawa
(Eka, 1980; Campbell Platt, 1962)

Component	Fermented Dawadawa	Unfermented Dawadawa
Thiamin (mg/100g)	1.35	0.65
Riboflavin (mg/100g)	1.30	0.45
Niacin (mg/100g)	5.30	7.50
Oxalate (g/100g)	0.12	0.21
Phytic acid P (g/110g)	7.50	15.00

2.3 USES OF SOYBEAN

While the use of soybeans for food has a long history in china, the far East and Southeast Asia, it is still at an incipient stage in other regions of the world. In this connection, it is a pleasing tendency that with peoples increasing awareness of health and their changing dietary habits, they are becoming more interested in soybean products. Considering the value of soybeans as a protein source and ease of handling as a material, a campaign for more soybean consumption as human food will play a very important role in solving the world's food issues (Horii, 1997). Horii (1997), found that soybeans had the effect of controlling the accumulation of fat in the liver and the body. He also found that linoleic acid which accounts for about 50% of soybeans fatty acids together with linolenic acid have the function of reducing the amount of cholesterol that settles on the wall of vessels. In addition, the phospholipids, mainly lecithin which is high in soybeans, prevent the

settlement of cholesterol on vessel walls. Because soybeans contain these substances, they are expected to help control the occurrence of hypertension.

2.3.1 Fermented Soybeans

Lee *et. al.* (1983) investigated the natural fermentation of soybean for 7days at ambient temperature. They found out that, the content of riboflavin increased from 98 to 309.4 µg/100g dry matter, relative nutritive value from 78.66 to 94.59% and available lysine from 6.56 to 7.38 mg/gN respectively. Also during fermentation, the activities of protease and lipase increased with progress of proteolysis during fermentation.

Fermentation of soybean into a dawadawa type of product using whole cotyledons in four days was undertaken by Barimalaa *et al.* (1994). Isolates from fermenting cotyledons showed *Bacillus subtilis* and *Bacillus lichenformis* as the major fermenting micro-organisms. The fermentation increased moisture, protein and fat contents of cotyledons. Total available carbohydrate reduced in 48h to less than 50% of value at the start of fermentation but then increased to 92% on the fourth day. The increase was attributed to secretion of slime by fermenting micro-oganisms. Fermentation also reduced the trypsin inhibitor activity of cotyledons.

Several articles in the literature make reference to the clinical significance of *Bacillus* species in the food industry. However *Bacillus* species mainly *Bacillus subtilis* have been identified as the main organisms responsible for the alkaline fermentation of legumes such as the African locust bean seeds, melon seeds, oil bean, sesame seeds, castor oil bean and flutted oil bean into tradtional products in West Africa (Odunfa and Oyewole 1986; Antai and Ibrahim 1986; Steinkraus 1991). The fermentation of soybeans into natto, thua-nao and Kinema in Asia are also reported to be carried out by *Bacillus subtilis* (Suzukin and Ohta 1979; Campbell Platt, 1987; Steinkraus 1991).

Nikkuni (1997) reported a conspicuous and charming characteristic of fermented foods from soybean. He reported that tempeh, miso and other fermented soybean foods have some antioxidant substances which are non-existent in raw soybeans. Among the important types of fermented foods are tempeh, soy sauce or "shoyu", miso, "sufu" or chinese cheese and natto. Tempeh, a popular food in Indonesia and Malaysia, is a fermented soybean product made by the action of *Rhizopus oligosporus* on cooked and dehulled soybeans. The dehulled and boiled soybeans are innoculated with some tempeh from a previous fermentation, and finally wrapped in banana leaves and allowed to ferment until the mycelium of the tempeh mould has grown over and through the soybeans to make a solid mass.

Soy Sauce represents one of the largest uses of soybeans in the Orient and is used extensively as a condiment. It is a dark brown liquid, with a pleasant aroma used primarily as a flavoring agent. Its high salt content of about 18% makes it an adjunct for many bland foods with which it is used. "Tamari" is a soy sauce made in China, in which the proportion of soybeans is higher than regular soy sauce. "Chau yan" is a Chinese name and "toyo" the Phillipine name for this condiment. In Indonesia soy sauce called "Ketjap" is made from black soybeans. Fermentation is dependent on three micro-organisms a mould *Aspergillus oryza*, a yeast *Zygosaccharomyces soja* and a bacterium *Lactobacillus delbruekii*.

Miso is a fermented food product prepared in Japan, China, Taiwan, Phillipines, Indonesia and other countries in the Orient. It is essentially a fermented blend of rice, soybeans and sometimes barley or malt. It has the consistency and colour of peanut butter. It is used as a flavoring substance for foods and as a spread for bread. A two-stage fermentation is used, the first, an aerobic fermentation by strains of *Aspergillus oryzae* and the second, an anaerobic fermentation carried out by *Saccharomyces rouxii*. A mixture of strains of *Aspergillus oryzae* are innoculated into steamed rice and fermented for 48 h at 30°C. Before spore fermentation occurs, mycelial growth is arrested and this is known as the Koji. Simultaneously soybeans are washed, soaked, steamed and cooked. These are blended with salt in the proportion of 4 parts moulded rice or Koji, 10.4 parts soybean, 2 parts salt and 1 part old miso and water. This blend is fermented aerobically for 7 days at 28 °C and then two months longer at 35 °C. It is then placed in a vat where the second fermentation,

essentially anaerobic, continues for two months after which it is allowed to age for two weeks at room temperature (Pederson, 1971). Several types of miso are made "Kome miso" made with soybeans and milled rice, "Mugi miso" made with soybeans and barley, and "Mame miso" made with soybeans alone,

Sufu is prepared by mould fermentation of cakes of finely ground precipitate soybeans. Soybean milk is cooked to eliminate the bean-like flavour and then pressed, cut into cubes called "tofu", sprayed with acid-saline solution, innoculated with a mould culture, usually *Mucor* spp. and incubated at 12-20 °C for 3-7 days. The mouldy cubes known as phetzes are placed in a solution of 12% NaCl and 10% ethanol and then aged up to 2 months. "Fuyo", "Fu-ju" and "Tsofu" are other names applied to Sufu.

Natto is known as a highly nutritious food containing protein, fat and various vitamins (Ueda, 1989). During natto fermentation, soybean protein which has been denatured by cooking process, is hydrolyzed by proteases produced by *Bacillus subtilis* into peptides and amino acids (Ueda, 1989). The percent liberation of amino acids i.e. the ratio of free amino acid content to the total amino acid content is 8.0% for natto and 1.1% for soybeans (Nikkuni *et. al.* 1995). Natto is known as a highly nutritious food containing protein, fat and various vitamins (Ueda, 1989). Recently, natto is also attracting much attention as a food having several physiological functions including the action of lowering blood pressure (Sumi, 1990).

2.4 READY – TO –USE API KIT

In the present work, comprehensive carbohydrate utilization profiles of isolates were determined in a ready-to-use API kits in addition to classical biochemical tests used to characterize and identify microbial isolates. The biochemical profile of *Bacillus* species were determined in API 50 CH kit (BioMérieux SA) using API 50 CHB media (BioMérieux SA). This allowed the utilization of 49 carbohydrates by each tested strain to be studied. In the API kit, the carbohydrates substrates are present in a dehydrated form and are rehydrated with the inoculated ready to use medium. Fermented carbohydrates produced a decrease in pH which was detected by a colour change of the incorporated indicator. The result constituted a biochemical profile of the strain and the species could be identified by comparison to a reference table or a database.

API tests have been found to give more reproducible results than the classical tests in the identification of *Bacillus* species. (Logan and Berkerley 1981). The carbohydrate substrates present in API 50 CH include glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β methyl-xyloside, galactose, D-glucose,D-fructose, D-mannose,L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α methyl –D-mannoside, α methyl –D-glucoside, N acetyl glucosamine, amygdaline and arbutin. The rest are esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, inuine,melezitose, D-raffinose, amidon, glycogen, xylitol, β gentiobiose, D-turanose, D-lyxose, d-tagatose, D-fucose, L-arabitol, glucanate, 2 ceto-gucanate and 5 ceto-gucanate

2.5 ELECTROPHORESIS

Electrophoresis is concerned with the migration of charged components within a fluid medium under the influence of an electric field. The charged components may be molecular, for example proteins, or cells or even charged particles. Similarly, the fluid medium may be liquid or gaseous, but in biological systems it is only electrophoresis in the former that is of interest (Gordon and Macrae, 1992).

The basis of electrophoretic separation is that molecules with different charges or sizes will migrate at different velocities. If there is an interaction between the charged molecules and the support material, then the situation may be complicated further. Support material is used to reduce other effects such as convection, which would disrupt the migration of the components as discrete bands (Gordon and Macrae, 1992).

Electrophoresis of the total cellular proteins in polyacylamide gels provide a partial separation in which individual bands mostly represent several proteins.

2.5.1 SDS Polyacrylamide-Gel Electrophoresis.

The original electrophoretic systems used to determine protein fingerprints involved polycrylamide rod gels used in non-denaturing conditions, but more recently sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has found greater application (Priest and Austin, 1993). SDS-PAGE is a low cost reproducible and rapid method for quantifying, comparing and characterizing proteins. It separates proteins based primarily on their molecular weight. SDS binds along the length of the polypeptides chain and the length of the reduced SDS-protein complex is proportional to its molecular weight. The mobility of charged molecules in gels is a function of both their size and charge, so it is

quite possible for molecules with the same electrophoretic mobility (under specified conditions) to be quite different in terms of relative molecular mass. Proteins may associate in buffer solutions and hence migrate during electrophoresis as aggregates thereby not reflecting their true charge or relative molecular mass. To overcome these problems, a method which includes an anionic detergent, such as sodium dodecyl sulphate (SDS), into the buffer system is used. SDS binds to hydrophobic sites within the protein, hence reducing the possibility of hydrophobic bonding between protein molecules. It also imparts a large negative charge to the protein units, its contribution largely swamping any effect of charged groups within the protein. Under these conditions, it has been found that the electrophoretic mobility of a protein is inversely related to the log of its relative molecular mass (Gordon and Macrae, 1992).

2.6 GAS CHROMATOTOGRAPHY-MASS SPECTROMETRY (G.C-MS)

Food products comprise trace levels of numerous flavour components dispersed in a highly complex and often nonhomogenous matrix. To enhance the sensitivity of analytical methods preconcentration is important. A number of techniques are commonly employed for extracting and concentrating flavour components from products (Jennings, 1980: Bemelmans, 1981: Teranishi and Kint, 1993). They include solvent extraction, headspace sampling and distillation. Each technique has its own advantages and disadvantages and often compliment one another. To choose an optimal method one must consider the analytical objectives, type of products and the type of flavour constituents likely to be present. The first of these tasks is generally carried out using GC coupled with mass spectrometry.

Gas Chromatography is ideally suited to the analysis of volatile components in the environmental or in the headspace above biological materials. Analysis of volatiles is important in various areas including determination of flavour and odour components of foods (Williams, 1971).

Gas Chromatography achieves separation of mixtures by partition of components between a mobile gas phase and a stationary phase. The stationary phase could be an involatile liquid coated onto an inert solid support (Gas Liquid Chromatograpy) or comprises particles of a solid absorbent (Gas Solid Chromatography). The time taken for a molecule to pass through a GLC column is known as the retention time, tr, and is dependent on the partition coefficient k..

$$K = \frac{\text{mass of vapour dissolved in unit column length of stationary phase}}{\text{mass of vapour dissolved in unit column length of mobile phase.}}$$

The separation of two components in a mixture is dependent on the difference in their retention times (Dtr) and the mean peak width (Wb) of the bands. GLC has become a major analytical technique which gives excellent separation of components from many complex mixtures (Gordon and Macrae, 1992).

Mass spectrometry is a powerful technique for the identification of pure compounds. It can also be used for confirmation of the purity of a sample and for quantitative analysis of mixtures. The mass spectrum consist of a series of peaks of varying intensity plotted against the mass-to-charge ratio (m/y).Mass spectrometry combines high specificity with great sensitivity, since amount of less than a picogram of some compounds can be detected.

GC-MS represents a very powerful combination of separation and structural identification techniques. It is required for many non-routine studies involving the analysis of complex mixtures. The advantages of a mass spectrometer are that it provides a sensitive specific and universal method of detection. Full structural identification of unknown components is often possible from the mass spectrum.

GC-MS rely on the separation of mixtures into individual components which can readily be identified by mass spectrometry. Packed column GC-MS is best performed by enrichment of the eluent with a molecular separator. This device selectively removes carrier-gas molecules from the gas flow entering the mass spectrometer (Gordon and Macrae, 1992).

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Soybean (*Glycine max*) was purchased from Makola Market,Accra-Ghana.

Microbiological media

Plate count agar (PCA) Difco 0479-17-3, Detroit U.S.A

Man, de Rogosa and Sharpe agar (MRS) Merck 1,10660 Darmstadt, Germany.

Malt agar (MA) OxoidCM59, England

Yeast extract Difco, 0127 17 - 9, U.S.A

Bacteriological peptone - Oxoid 1375, England.

Agar - Sigma 91H01625, U.S.A

Nutrient agar - Difco Detroit, U.S.A

Nutrient broth - Difco 36201JA

3.2 METHODS

3.2.1 Field Survey

A brief field study was carried out in Nima, Mateheko and Madina all suburbs of Accra to select two major sources of traditional dawadawa production. The field study involved the use of questionnaires and informal interviews of dawadawa sellers. Information gathered included the following: types of dawadawa available, raw materials for its preparation and its availability, methods of production, ingredients used, packaging, marketing and consumers' perception of good quality dawadawa (Appendix 1).

Two experienced dawadawa producers were selected to produce the two different types of dawadawa used as control for laboratory studies.

All other experimental work were carried out at the Food Research Institute, Accra, Ghana and at the Department of Nutrition and Food Science, University of Ghana, Legon.

3.2.2 **Production of Soydawadawa**

Two different types of soydawadawa were prepared as shown in the flow diagram (Figure 3-1). One type was made from boiled soybeans and the other from roasted soybeans as shown in Figure 3.1

Soybean seeds were cleaned, boiled for 30 min cooled and dehulled by pressing between palms or by pounding in a mortar. The dehulled beans were washed to get rid of the seed coat and undehulled beans. It was further boiled for 1h, drained and packed into baskets lined with banana leaves. This was covered with more leaves and left to ferment for 72h to obtain boiled soydawadawa. For the roasted soydawadawa, the soybean bean seeds, after cleaning were roasted for 30 min, cooled and dehulled and further treated as in the boiled one.

3.3 CHEMICAL ANALYSES

A) pH

Fermenting dawadawa samples weighing 10g were homogenized in a blender with 90ml of distilled water and the pH determined with a pH meter (PHM series Lab. pH meter; PHM 92, Radiometer, Copenhagen, Denmark).

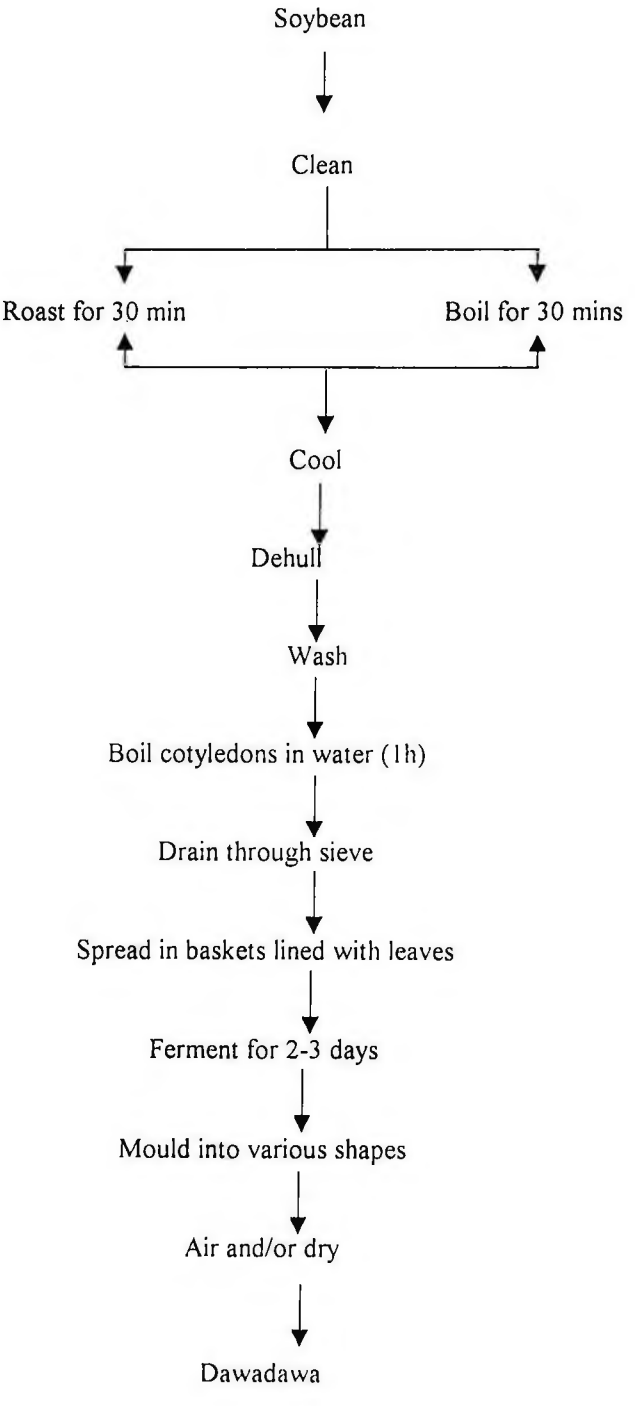


Figure 3.1: Flow diagram for preparation of dawadawa from soybean

B) TITRATABLE ACIDITY OF DAWADAWA

Titratable acidity was determined by the titration of 80ml of filtrate obtained from 10g of dawdawa samples macerated in a blender in 250ml distilled water, against 0.1N NaOH with 1% phenolphthalein. 1ml of 0.1N NaOH was taken as equivalent to 9.008 x 10^{-3}g lactic acid.

C) PROXIMATE ANALYSES

i) Moisture

Moisture content was calculated by drying the well-mixed sample at 130.°C to constant weight (AOAC 1990). For all samples, 2g were weighed into moisture dishes and dried in an air oven set at 130 °C to constant weight. Dishes were cooled in a desiccator and reweighed. The percentage moisture was calculated as:

$$\% \text{ moisture} = \frac{\text{Loss in weight}}{\text{initial weight of sample}} \times 100$$

This was calculated on Dry Matter Basis

ii) Protein Content

The protein content of samples was determined by multiplying total nitrogen, estimated by micro-kjeldahl method, by 5.7 (AOAC 1990). For all samples, 2g were weighed on filter paper and digested with concentrated sulphuric acid in a kjeldahl flask. It was then distilled into 2% boric acid and back titrated with 0.1M HCL with methyl red as indicator. Protein content was calculated as:

% Protein = % Nitrogen x 6.25

This was calculated on Dry Matter Basis.

iii) Fat Content

Fat content was determined by ether extraction using the Soxhlet-petroleum ether extract method (AOAC 1990). For all samples, 2g were weighed into a soxhlet paper thimble, covered with cotton wool and placed in the extraction unit of the soxhlet apparatus. A weighed clean oven dried soxhlet flask was attached to the lower end of the extraction unit which was filled with petroleum ether and heated for some hours. The percentage of fat was calculated as:

$$\% \text{ Ether extract} = \frac{\text{Increase in Flask weight}}{\text{weight of sample}} \times 100$$

This was calculated on Dry Matter Basis

iv) Ash

Ash content was measured by heating the sample at 600°C until the difference between the successive weighings was (less than or equal) < 1mg (AOAC 1990). For all samples about 2g of the dried sample was transferred into a weighed crucible (Silica dishes) and heated for about 2 h to red hot (600°C) in a furnace. The fine ash was weighed after cooling in a desicattor to give the ash content.

$$\% \text{ Ash} = \frac{\text{Weight of Ash}}{\text{Dry sample weight}} \times 100$$

This was calculated on Dry Matter Basis

v) Carbohydrate

Carbohydrate content was calculated by difference:

% Carbohydrate = 100 - (% Moisture + % Ash + % Fat + % Protein

This was calculated on Dry Matter Basis

3.4 MICROBIOLOGICAL ANALYSES

3.4.1 Sampling of Fermenting Soydawadawa

The two types of dawadawa were sampled in duplicate on 4 separate occasions precisely at 0h, 24h, 48h and 72h of fermentation. Samples of 10g of soydawadawa at 0, 24h 48h and 72h of fermentation were aseptically collected into stomacher bags (Seward Medical, London, England) for analysis. Soydawadawa samples were taken from within the fermenting mass after the surface layers had been removed aseptically using a sterile spatula.

For all samples, 10g were added to 90ml sterile diluent containing 0.1% peptone, 0.85% NaCl, with pH adjusted to 7.0 and homogenized in a stomacher (Lab Blender, Model 4001, Seward Medical) for 120s at high speed. From appropriate ten-fold dilutions, enumeration of aerobic mesophiles was carried out on Plate Count Agar (PCA, Difco) incubated at 37°C for 3 days. Lactic acid bacteria were enumerated on de Man, Rogosa and Sharpe Agar (MRS Merck) incubated anaerobically in an anaerobic jar with anaerocult A (Merck) at 37°C for 4 days. Mould and Yeast counts were enumerated on Malt Agar (MA, Oxoid) containing 100mg chloramphenicol

(Chloramphenicol selective supplement Oxoid) and 50mg chlotetracycline (sigma C-4881, St. Louis, USA) per litre and incubated at 30°C for 7 days.

3.4.2 ISOLATION OF DOMINATING MICROORGANISMS

The highest dilution plate or suitable plate with a total of about 30 colonies in each quadrant was used for the isolation. Distinct individual colonies were picked with a sterile innoculating loop and subcultured in the corresponding broth medium and streaked onto the agar substrate repeatedly until pure cultures were obtained.

3.4.3 INITIAL CHARACTERIZATION OF ISOLATES

***Bacillus* species**

Isolates from PCA were subcultured in nutrient broth/agar and *Bacillus* species were characterised using morphological examination and biochemical test comprising of colony and cell morphology, Gram reaction and catalase production. The proportion of *Bacillus* species in the isolates was used to calculate the total numbers of the species present in the sample.

Gram reaction was carried out according to a modification of the method of Lillie (1928) by Parry *et al.* (1983). For catalase production a loopful of culture was mixed into a drop of 3% hydrogen peroxide on microscope slide and observed for the production of gas bubbles to indicate production of catalase. Oxidase test was carried out using strips of Oxidase paper. (Lillie (1928) by Parry et. al. 1983)

Lactic acid bacteria

Isolates from MRS were subcultured in MRS medium and examined by Gram reaction, catalase production, oxidase test, aerobic and anaerobic growth, colony and cell morphology.

Yeasts and moulds

Isolates from MA were subcultured in yeast-malt-peptone-glucose broth and agar (MYPG) containing 3g, yeast extract (Difco) bacteriological peptone (Oxoid) 10g, glucose (Merck) with or without 15g, agar (sigma) and examined by colony and cell morphology.

3.4.4 MAINTENANCE OF ISOLATES

Plate Count Agar (PCA) isolates were maintained on PCA slants stored at 4°C. Colonies isolated from de Man, Rogosa and Sharpe (MRS) Agar were preserved by subculturing in MRS broth and vortexing 1ml of culture broth with 1ml of 50% glycerol in a Cryotube and frozen immediately at 40°C. Malt Agar (MA) isolates were maintained on MYPG agar slants stored at 4°C.

3.4.5 IDENTIFICATION OF *BACILLUS* SPECIES

Bacillus species were recognised from initial tests of PCA isolates as Gram positive, catalase positive rods with phase bright spores. The species of *Bacillus* isolates were identified according to Claus and Berkerley (1986) and Parry *et. al.* (1983). Tests performed included both morphological examination and biochemical tests. In the morphological examination, the diameter and length of cell were measured and the

shape and position of spores noted. The biochemical tests carried out were anaerobic growth, acid production from D-glucose, hydrolysis of casein and starch, growth at pH 5.7, in 6.5% (w/v) NaCl and 10% (W/V) NaCl, at 37°C and 65°C. The identity of selected *Bacillus* isolates were confirmed by testing cultures for the fermentation of 49 carbohydrates in API 50 CHB galleries (Bio Merieux SA).

3.4.6 Biochemical tests for characterization of isolates

(i) Protein Hydrolysis (Casein utilisation)

For decomposition of casein, plates of skim milk agar were prepared from 100g of skim milk powder and 20g of agar (Sigma 91H01625, USA) dissolved in 1000ml of distilled water. The plates of skim milk agar were each inoculated with a single streak of the culture incubated at 37°C and examined for clear zones around growth at 3 to 7 days to indicate decomposition of casein and proteolytic activity.

(ii) Starch Hydrolysis

For hydrolysis of starch, plates of starch agar were prepared from 10g of starch and 23g of nutrient agar (Difco) dissolved in 1000ml distilled water were each inoculated in duplicate with the test organism and incubated at 37°C for 3 to 5 days. Hydrolysis of starch was determined at 3 and 5 days by flooding plates with iodine solution. Unhydrolysed starch changed colour to blue-black within 15 to 30 min. Hydrolysis of starch was indicated by clear zones around colonies.

(iii) Growth in NaCl

To test for growth in 6.5% sodium chloride (Merck KG, 1.06404, Darmstadt, Germany) tubes containing 3ml of nutrient broth (Difco 36201JA), with 6.5% (W/V)

sodium chloride were inoculated with the test organism and incubated at 37°C and observed for growth after 7 and 14 days. This was repeated for growth in 10% NaCl.

(iv) Growth at pH 5.7

To test for growth at pH 5.7, Sabouraud dextrose agar slants containing 10g of peptone (Oxoid), 40g of dextrose and 15g of agar (sigma) dissolved in 1000ml distilled water with pH 5.6 and sabourand and dextrose broth containing 10g of peptone (Oxoid) and 20g dextrose dissolved in 1000ml distilled water with pH 5.7, were inoculated with isolates which had previously been grown in nutrient broth (Difco). The tubes were observed for growth for up to 14 days after incubation at 30°C.

(v) Acid production

To test for acid production from D (+) Glucose, 3ml slants containing 150 µl of 10% filter sterile solution of D (+) Glucose (Merck 8342, Darmstadt) and a basal medium containing 1g of diammonium hydrogen phosphate, 1g of potassium chloride, 0.2g of magnesium sulphate (Analytical), 0.2g of yeast extract, (Difco, 0127-17-9, USA) and 15g of agar (Sigma, 91H01625, USA), 0.006g of bromcresol purple dissolved in 1000ml distilled water with pH 7.0, were inoculated with the test organism and incubated at 30°C. Acid production was indicated by a change in colour of the medium from purple to yellow.

(vi) Identification using API 50CHB kit

The species of *Bacillus* isolates were identified by assaying selected cultures in API 50 CHB galleries (Bio Merieux SA). Isolates were grown in 10ml nutrient broth at 37°C for 48h, plated out on Nutrient Agar and incubated at 37°C for 24h. Bacteria were

harvested from the plates with a sterile swab. The cells were resuspended in 3ml sterile distilled water and its turbidity adjusted to 3 Mcfarland by comparing with a Mcfarland turbidity standard. 150 µl of the bacterial suspension was transferred into one tube of 50CHB medium (Bio Mereux SA) and used to innoculate the copules of the API 50 strips containing the dehydrated substrate using a sterile pipette. The strips were placed in the boxes containing 10ml of distilled water which has been distributed into the honey comb to maintain moist conditions and incubated at 37°C. The strips were read at 24h and 48h and the strain identified by referring to a reference table.

3.4.7 IDENTIFICATION OF LACTIC ACID BACTERIA

(i) Initial identification

Gram-positive catalase-negative MRS isolates were examined by gas production from glucose in MRS broth with durham-tube, gas production from MRS broth in which glucose was replaced with gluconate as sole carbon source, growth at 15°C and 45°C and Hugh and Leifson test (Hugh and Leifson 1953).

(ii) Gas Production

Production of gas from glucose was tested for by inoculating MRS broth containing a durham tube with the test organism and incubating at 30°C for at least 72h. Gas production was indicated by an air bubble trapped inside the inverted durham tube.

(iii) Growth at different temperatures

For growth at 15°C and 45°C, two tubes containing MRS broth were inoculated with the test organism and incubated at 15°C and 45°C up to 14 days and examined for significant growth.

(iv) Hugh & Leifson's test

Two tubes each containing 1 litre distilled water, 2g of peptone (Oxoid L37 England) 5g of NaOH, 0.3g of K_2HPO_4 (Sigma p5504) and 15ml of 0.2% bromothymol blue (sigma B 7271) were used. 1% glucose (Merck 8342) was added after passing through a sterile filter and the pH of the solution in the tubes adjusted to pH 7.1. The two tubes were innoculated with the test organism and one of the tubes topped with paraffin oil to obtain anaerobic conditions. After incubation at 30°C for 2 to 7 days, fermentative reactions were indicated by yellow colouration in the aerobic and anaerobic tubes due to formation of acid. Oxidative reactions were indicated by yellow coloration in the top section of the aerobic tube whilst anaerobic tubes maintained their original colour of blue.

Gram-positive, catalase-positive cocci were tentatively identified by mode of glucose breakdown, tetrad formation, growth at 15°C and 45°C, growth at pH 4.2 and 9.3 on 10% (w/v) and 15% (w/v) NaCl agar. Cultures identified as *Staphylococcus* occurred singly or in pairs, fermented glucose with the production of CO_2, were able to grow at 15°C and 45°C and also mostly in up to 10% NaCl (w/v) but not 15% NaCl (w/v).

v) Identification of *Lactobacillus* species

Regular Gram-positive rods and very short rods/coccoids which were catalase negative, oxidase negative, fermentative and grew both aerobically and anaerobically were considered to belong to the genus *Lactobacillus*. The *Lactobacillus* species were classified into obligately homofermentative, facultatively heterofermentative and obligately heterofermentative by their ability to produce ($C0_2$) from glucose and gluconate.

3.5 DETERMINATION OF SUGARS

The Lane and Eynon's method was used. The sugar solution was neutralized and clarified. The sugar solution was placed in a 50-ml burette. A preliminary titration was first performed by pipetting 10 or 25 ml of mixed Fehling's solution into a 300 ml conical flask. 15 ml of the sugar solution from the burette was added to the mixed Fehling's solution and boiled on an asbestos-covered gauze. Quantities of the sugar solution were added further (1 ml at a time) at 10-15 seconds intervals until the blue colour was nearly discharged. 3-5 drops of aqueous methylene blue solution (1%) were added and titration continued until the indicator was completely decolourised.

An accurate titration was performed by repeating the titration. Almost all the sugar solution required to effect reduction of the copper was added before heating. It was then boiled gently for two minutes and 3-5 drops of the methylene blue indicator were added. The titration was completed within a total boiling time of 3 minutes. The blue colour was fully discharged with the liquid showing an orange-red colour at the end-point.

The proportions of the various sugars equivalent to 10 or 25 ml of Fehling's solution were read from a given table.

Sucrose was determined in a solution which had been inverted and contained no other sugar. The inversion was done on a portion containing about 0.5g sucrose in a 100 ml volumetric flask with acid and neutralised. This solution was titrated against Fehling's solution and the amount of invert sugar produced was obtained by reference to the tables.

% Invert sugar x 0.95 = % sucrose

3.6 CHARACTERISATION OF THE PROTEINS OF SOYDAWADAWA USING GEL ELECTROPHORESIS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Excel Gel XL SDS 12-14, Pharmacia Biotech AB 71-7137 00) was used to characterize and compare the proteins in the soydawadawa.

3.6.1 Sample Preparation

From a sample of soydawadawa 0.05g was weighed and added to 500µl of Tris buffer, pH 7, containing 1.576g Tris/HCl (sigma T-7149), 0.372g EDTA (Sigma E-5134) per 100ml Milli-Q water. 500mg of glass beads was added to facilitate disruption of the cells and the sample was then placed on ice for 1min and then vortexed for 1min repeating 9 times. The sample was centrifuged at 5,000xg for 10min and the supernatant diluted with 90µl of Tris buffer, pH 8, containing 1.576g Tris/HCL (SigmaT-7149), 0.372g EDTA (Sigma E-5134) per 1000ml of milli-Q water. 100µl of sample buffer containing 10.0ml Tris buffer, 1.0ml Bromophenol blue solution, 20.0ml Sodium dodecyl sulfate (SDS) solution, 5.0ml Glycerol solution, 1.0ml Dithiothreitol (DTT) solution was added to 100ul of diluted sample and boiled for 5min. The Bromophenol blue (Merck 8122), contained xg in 1.0ml milli-Q water, the SDS solution 2.00g of SDS in x ml of milli-Q water and the Glycerol solution 0.25g of Glycerol (Merck 4092) in 5.0ml milli-Q water. After boiling, sample was certrifuged at 5000xg for 5min.

Electrophoresis was run at x°C for 15min and temperature set to 15°C. About 2-4ml of kerosene was smeared onto the cooling plate of the electrophoresis equipment. The

gel (Excel Gel XL SDS 12-14, Pharmacia Biotech AB 71-7137-00) was positioned on the cooling plate with the cut corner on the gel corresponding to the anodic (+) side of the cooling plate .Buffer strips were positioned on the gel. The sample applicator strip was positioned about 5mm from the cathodic (-) buffer strip and left there during the electrophoresis. Gels were stained by silver staining. They were soaked for 30 min in fixing solution containing per 250ml distilled water, 100ml ethanol and 25ml glacial acetic acid. Gels were then placed for 30 min in sensitizing solution containing per 250ml distilled water, 75ml ethanol, 1.25ml of 25% (w/v) glutardialdehyde, 0.5g sodium thiosulphate and 17g sodium acetate and washed 3 times for 5 min each in distilled water. Gels were then stained for 20 min in a silver solution containing per 250ml distilled water, 25ml of 2.5% (w/v) silver nitrate solution and 0.1ml of 37% (w/v) formaldehyde and washed twice for 1 min each in distilled water. The stained gels were developed for 2 to 5 min in a developing solution containing per 250ml distilled water, 6.25g sodium carbonate and 0.05ml of 37% (w/v) formaldehyde. The developing reaction was stopped after 2 to 5 min by placing the gels in a stop solution containing per 250ml distilled water, 3.65g EDTA-$Na_2.2H_2O$ for 10 min and washed 3 times for 5 min each in distilled water. Gels were preserved by soaking for 20 min in preserving solution containing per 250ml distilled water, 75ml of 87% (w/w) glycerol and covered with cellophane preserving sheets.

3.7 DETERMINATION OF AROMA COMPOUNDS

A GC -MS Hewlett Packard 6890 GC, 5973 MS, Avondale Pennsylvania was used to separate the soydawadawa extract into individual components which were readily identified by the mass spectrometer.

3.7.1 Sample Preparation And Extraction Procedures

Soydawadawa sample weighing 2g was mixed with 10ml distilled water and 10ml n-hexane in a 100ml separating funnel. The mixture was shaken vigorously for about a minute and allowed to separate into aqueous and solvent layers. The solvent layer was filtered through whatman No. 1 filter paper filled with some anhydrous sodium sulphate into a 2ml screw head vial which was then tightly capped. This extract was injected into the GC-MS.

3.7.2 Gas Chromatography And Mass Spectrometry.

Aroma compounds present in samples were detected by injecting into a Hewlett Packard 6890 gas chromatography (Hewlett-Packard, Avondale, Pennsylvania) equipped with a fused silica capillary column, (Hp-SMS column) connected to a Hewlett-Packard 5973 mass spectrometer (Hewlett-Packard, Avondale, Pennsylvania).

The column pressure in the Gas Chromatograph column flow was approximately 10ml/min and split temperature for the run was 45°C, Ramp and Total Run time, 22min. Analytical grade reagents (NaCl) were used.

3.8 ENZYME ASSAYS

Organisms were streaked on casein and starch agar. Those which were more efficient in degrading the casein were selected and their proteinase activities were assayed by using the method described by Young and Wood (1977). Similarly, organisms which

were more efficient in degrading starch on agar plates were selected and their α-amylase activities were assayed by using the method described by Bernfeld (1955).

3.8.1 Preliminary Screening of cultures for Proteinase and α-amylase activity

Cultures were streaked on surface dried plates of casein agar and starch agar (Gordon *et. al.* 1973), and incubated at 37°C. After 3 d plates were flooded with iodine solution and the diameters of the clearing zones were used as an assessment of proteinase (casein agar) and α-amylase (starch agar) activities.

3.8.2 Preparation of Extraction Buffer for Proteinase activity

The extracting buffer was 0.1M sodium hydrogen phosphate, pH 6.5. The assay method used was that of Young and Wood (1977). It has been found useful for analysing proteinases in the presence of reducing sugars normally found in food substances.

Soydawadawa weighing 5g was added to 50ml of 0.1M sodium hydrogen phosphate and ground in a mortar to prepare an extract. The suspension was then washed with 5ml petroleum ether to extract the oil and centifuged at 5000 rpm. The supernatant was stored in a deep-freezer at -20°C. Assays and analyses were carried out on duplicate fermentations and for each sample three determinations were made at each time interval.

3.8.3 Determination of Proteinase Activity

5ml of the extract was added to 10ml of 2% solution of light soluble casein (BDH) and incubated at 35°C for 30 min. The reaction was terminated by adding 10ml of 10%

trichloroacetic acid (TCA) solution. The mixture was filtered through whatman No. 1 filter paper. The optical density of the filtrate was obtained by reading the absorbance at 275nm with an SP6 250 spectrophotometer. The blank contained the same mixture but with the TCA added simultaneously with the enzyme extract.

Enzyme activity was expressed in terms of an arbitrary unit called an XS unit, and is defined as: 'An enzyme extract which under the stated experimental conditions produced a filtrate with an optical density of 0.500 when measured in a 10mm path length cell, had a strength of 36 XS units per gram' (Young and Wood, 1977 a).

3.8.4 Preparation of Extraction Buffer for α-Amylase Activity

The extracting buffer was 1M potassium hydrogen phosphate, pH 6.5. The assay procedure described by Bernfeld (1955) was used.

The extract was prepared as in the proteinase activity with 1M potassium hydrogen phosphate, pH 6.5 as the extracting buffer.

3.8.5 Determination of α-Amylase Activity

2 ml of the extract was mixed with 1 ml of 1% starch solution and incubated for 1 h at 40°C. The reaction was stopped by adding 3ml dinitrosalicylic acid reagent (DNS). The mixture was heated in a boiling water bath for 5 min, cooled in cold water, and then diluted with 18 ml water. The optical density of the resultant solution was obtained by reading the absorbance at 550 nm, using an SP6 250 spectrophotometer. The blank was similarly treated except that the DNS was added before adding the starch solution. The amount of the reducing sugars formed was calculated from a standard curve prepared with known concentrations of maltose (Bernfeld, 1955).

3.9 DETERMINATION OF AMINO ACIDS IN SOY DAWADAWA

This was done using the Pico-Tag Method, and an Amino Acid Analyzer. Basically an internal standard (norleucine) was added to the protein/sample and hydrolysed with 6M hydrochloric acid. The hydrolysed samples were evacuated to dryness, neutralised with Triethlamine (TEA) and sodium acetate. The samples were derivatised with phenylisothiocyanate (PITC). The derivatives (PTC - AA) were analysed with reverse phase HPLC and UV detection. List and grade of chemicals used for the amino acid analysis is shown in Appendix 2.

3.9.1 Working Solutions

The solutions used in the analysis were each prepared as follows:

6M hydrochloric acid

1000 ml 37% hydrochloric acid was added to water in 2000 ml volumetric flask. Mixture when cold was diluted to mark with water.

Internal standard (6.25m M Nor).

0.82g norleucine was weighed, transferred to 1000ml volumetric flask with 6M hydrochloric acid and diluted to mark with hydrochloric acid. It was stored refrigerated.

2.5m M norleucine

0.328g norleucine was weighed, transferred to 1000ml volumetric flask and dissolved in 17 ml 6M hydrochloric acid. It was diluted to mark with water and aliquots stored at - 20°C.

0.1 M Dithiothreitol (DTT)

0.1542g DTT was weighed in 10 ml graduated centrifuge tubes, dissolved in water and diluted to mark. (This was used the same day).

5m M hydroxyproline and taurine

0.655g hydroxyproline and 0.626g taurine, were weighed, transferred to 1000 ml volumetric flask with 17 ml 6M hydrochloric acid and diluted to mark with water. Aliquots were stored at - 20°C.

Standard 1.1.2, 5m M

1ml 2.5mM amino acid standard H and 1 ml 2.5mM norleucine were pipetted and vortexed thoroughly. This was stored at - 20°C.

Standard 2.1 mM incl. hydroxyproline and taurine

2ml standard 1.1.25 mM and 0.5ml 5mM hydroxyproline and taurine were pipetted, into 4ml sample vials and vortexed thoroughly. This was stored at - 20°C.

0.2 M Sodium acetate

1.7206g water free sodium acetate was weighed, transferred to 100ml volumetric flask and diluted to mark with water. This was stored refrigerated.

Redry Solution

Methanol, 0.2M sodium acetate and Triethylamine (TEA) were mixed in relation 2:2:1 in 4 ml sample vials and stored in freezer at (-20°C).

Derivatisation Solution

Methanol, water, Triethylamine (TEA) and Phenylisothiocyanate (PITC) were mixed in relation 7:1:1:1 and stored at room temperature for 1 h). Opened ampoules of PITC were divided into 5 x 200µl in 4ml chromacol tubes, blanketed with nitrogen (30s) and stored at - 20°C up to 3 weeks.

Stock EDTA SOLUTION

0.100g EDTA was weighed, transferred to 100ml volumetric flask with water and diluted to mark and stored refrigerated.

Eluent A (Acetate buffer), Sample Diluent

38.0g sodium acetate was weighed in 2000ml beaker and 200 ml water, 1.0 ml TEA and 500 µL stock EDTA Solution were added and mixed thoroughly on a magnetic stirrer. PH was adjusted to 6.1 using Concentrated acetic acid. 1880 ml of this mixture was measured in a measuring cylinder and filtered through triton free filter to 2000 ml reservoir bottle. 120 ml acetonitrile was measured and added. This was kept in refrigerated storage.

Eluent B (60% acetonitrile)

600ml acetontirile and 400ml water were measured separately in a measuring cylinder and mixed in 2000ml reservoir bottle. 250µl Stock EDTA solution was added, degased for 20 sec and stored refrigerated.

3.9.2 Procedure

Sample corresponding to 30 mg protein was weighed into flat bottom flasks. 60 ml 6M hydrochloric acid, 5 ml 6.25mM norleucine and 300 µL 0.1MD TT were added and mixed. Flasks were placed in a steel container and placed in a pre heated heater (110°C). Hydrolysis was done for 22 hours at 110°C.

Samples were cooled in cold water, transferred to 100 ml volumetric flask, diluted to mark with water, and mixed thoroughly. This was filtered through 0.45 µm Millipore filter, with 5 ml syringe, into 4 ml chromacol tubes and stored at -20°C.

Vacuum work station was connected and started to achieve derivatisation. 2 x 20 µl standard was pipetted together with 20 µl of every sample in 6 x 50 mm tubes. Tubes were placed in reaction vials and these were further placed in the vacuum station. They were evacuated to dryness for 45 min. 30 µl redry solution was added, vortexed thoroughly and evacuated to dryness for 45 min. 20 µl derivatisation solution was added and vortexed thoroughly. Samples were left under atmospheric pressure for exactly 10 min and evacuated for 15 min. 40 µl methanol was added, vortexed thoroughly and evacuated to dryness for 2 h.

The dried samples were dissolved in 200 µl sample diluent and vortexed thoroughly. The supernatant was transferred into 1 ml syringe. The supernatant was then filtered through Whatman No. 1 filter paper into 4 ml sample vials which were inserted onto the HPLC system.

For analysis, samples were run on HPLC system controlled by Millenium 2010. Sample set was started with an equilibration time of 10 min and continued with condition column, run time being equal to gradient time. 10 µl of standard and sample was injected into the column. Eluents were degased and stabilized by continuous helium supply. Eluents were pre-heated and column heater with temperature control was used to keep temperature on column constant. Gradient eluting was run, detecting samples by uv absorbance at 254 nm. Peak areas were measured for both the standard and samples and corrected by internal standard.

4.0 RESULTS AND DISCUSSION

4.1 FIELD SURVEY

A brief field study confirmed that different types of raw materials are fermented into dawadawa and that the preferred raw material for preparing dawadawa varies from one ethnic group to the other. 60% of the dawadawa found on the markets were made from African locust beans, with 25% from Soybean, 10% from a mixture of African locust beans and soybean mixture and 5% from a mixture of African locust beans and groundnuts (*Arachis hypogaea*).

The processing method used by all the ethnic groups is the same and the dried products are stored in traditional earthernware pots, baskets or boxes. They are used almost daily in cooking. About 8g to 12g at a time is crumbled into a pot of soup or stew as a seasoning. This is enough for a meal for about 4 people. Several workers including Ogbadu and Okagbue (1988) have reported that both African locust beans and soybeans are used as raw material by traditional dawadawa processors.

As to how much dawadawa is often taken per person per day, the consumers gave amounts that ranged between 2g to 4g respectively per person per day.

According to consumers interviewed, dawadawa could be roasted in whole and used like a piece of fish in eating kenkey, gari or any other food. Simmons (1976) found that the average daily per capita intake of "dawadawa" among some Hausas of Northern Nigeria constitutes 1.4% of the daily calories intake and 5% of the total protein intake. Lawson (1965) found the average per capita per day consumption of dawadawa in Togo and Ghana to be 4 and 2g respectively. On the other hand, Dema (1965) found that the Yorubas of South Western Nigeria consume 10g per day per

person, whilst the overall consumption estimated for parts of Nigeria by Nicol (1959) range from 1 to 17g per person per day. Ogunbunmi and Bassir (1980) reported that dawadawa contributes appreciable amounts of protein to the diet of Nigerians.

The results of the survey showed that the preparation of the dawadawa depends on the type of raw material used. To make soybean dawadawa, the soybeans are first roasted for about 30 minutes to a brown colour and pounded to remove the seed coat or testa. The dehulled soybeans are cooked in water for 1 h, drained using a sieve and spread in a basket lined with leaves after cooling. The basket is covered with more leaves and placed in a warm place for 2 to 3 d for fermentation to take place. The fermented beans are moulded into various shapes, sun dried and stored as dawadawa in pots, baskets, etc. (Figure 3.1)

In the case of the African locust bean, the beans are boiled for 24h to soften the seed coat or testa. The boiled seeds are put in a mortar and pounded slightly with a pestle or pressed by foot to remove the softened testa. The seeds are rubbed between the palms or against the walls of a basket. The cotyledons are then washed thoroughly and the testa removed. The washed bean cotyledons are boiled again for 1 to 2 h in a metallic pot and the hot bean cotyledons drained through sieves or baskets. They are spread in a basket lined with leaves after cooling and covered with more leaves. Millet flour or wood ash is sprinkled on the bean cotyledons before fermenting for 72 h. After fermentation, they are moulded into various shapes, sun dried for a day or two and stored as dawadawa in pots, baskets etc. (Figure 2-1). In the case where groundnuts are added to either the African locust bean or soybean to prepare the dawadawa, the groundnuts are given the same treatment as the soybean.

The main differences found between the method of production of the various types of dawadawa were mainly in time and energy used. The method of production of soybean dawadawa saves time, conserves energy and thus lead to increased production. In terms of product acceptance, consumers rated dawadawa processed from the African locust beans as the best product. This was followed by dawadawa processed from soybeans and lastly dawadawa processed from a mixture of African locust beans and soybean.

On the other hand, dawadawa prepared from a mixture of African locust beans and groundnuts is not very well patronised. According to consumers interviewed, a good quality dawadawa has a strong ammonia-like smell and a dark brown colour. After fermentation, the seeds are either moulded in seed form or pounded into a paste before moulding.

Figures 1 and 2 indicate boiled and roasted soydawadawa.

FIG 4-1 BOILED SOYDAWADAWA

FIG 4-1 BOILED SOYDAWADAWA

FIG 4-2 ROASTED SOYDAWADAWA

4.2 CHEMICAL COMPOSITION OF SOYDAWADAWA

The proximate composition of soydawadawa is shown in Table 4-1. The percentage moisture of the soydawadawa was generally higher than the soybeans. However, as expected, the roasted product had a slightly lower moisture content than the boiled product because the boiled product absorbed water during boiling. Work by Barimalaa *et al.* (1994) showed an increase in moisture, protein and fat contents of cotyledons during fermentation of soybeans into dawadawa. However, this study though confirming an increase in protein content showed a decrease in the fat content of the soydawadawa during fermentation. Obizoba and Atu (1993) in their work on the chemical evaluation of some food condiments of Nigeria also confirmed an increase in the protein content of African locust bean dawadawa during fermentation. They observed that the 4-day fermentation period caused the highest increases in protein of the dawadawa and stated that fermentation for 4-days offers a greater advantage than other periods for production of nutritious and cheap food condiments in Nigeria.

The increase in protein content during fermentation of dawadawa was explained by Lee *et. al.* (1983) in their work on the nutritional evaluation of naturally fermented soybean and the enzymatic activity changes during the preparation. They found that, the activities of protease and lipase increased during fermentation with progress of proteolysis and a release of more free amino acids. Heat has been known to cause an improvement in some proteins particularly in Legumes. The enhancement of the nutritive value of soybean protein by moderate heat is generally attributed to the destruction of the heat-labile antitryptic factor naturally present in the raw bean. Dry heating is less effective than steam in enhancing nutritional value and the presence of

water partially prevents the adverse effect of excessive heating (Harris and Von Loesecke, 1960). This may explain why the protein content of the boiled soydawadawa was higher than that of the roasted soydawadawa. The chemical compostion of dried soybean seeds was given by Carrao *et. al.* (1994) as water - 5.0 - 9.4%, protein - 29.6 - 50.3%, Fat - 13.5 - 24.2% and Ash - 3.3 - 6.4%. The chemical composition of the dried soybean seeds found in this study falls within the ranges given by Carrao *et. al.* (1994) for yellow seeded cultivars of soybeans used in this study.

Table 4-1 Proximate chemical composition of Soydawadawa

% Dry Matter Basis	% Soybean	% Soydawadawa (BOILED)	% Soydawadawa (ROASTED)
Moisture	8.8	25.0	24.0
Ash	5.2	3.5	5.0
Protein	42.8	45.5	43.2
Fat	36.7	26.7	21.5

* Average values for 2 sets of samples

4.3 CHANGES IN PH AND TITRATABLE ACIDITY DURING FERMENTATION

The pH of soybeans changed from acid thorough neutral to alkaline during fermentation (Table 4-2). A progressive increase in the titratable acidity was recorded from the beginning to the end of the fermentation period. Changes in the pH and titrable acidity during fermentation are shown in Table 4.2. The pH of boiled soybeans increased from about 6.6 to 8.3 and roasted soybeans from about 6.4 to 8.2 during 72 h. Titratable acidity of boiled soybeans increased from about 0.1 to 0.4 and roasted

soybeans from about 0.1 to 0.4 during 72 h. The simultaneous increase in pH and acidity is unusual since one would expect the pH to drop as acids are produced.

Hesseltine (1965), suggested that the simultaneous increase in pH and acidity during fermentation of legumes may be due to the high buffering capacity of the legume beans and the proteolytic activities of *Bacillus subtilis* leading to ammonia release. This phenomenon is characteristic of most vegetable protein fermentations. This observation was also made by Odunfa (1985), when he found that proteolysis was the major biochemical change in fermenting African locust bean during *iru* fermentation. Wagenknecht *et. al.*, (1961), made similar observations about tempeh fermentation. The pH of the fermenting soybeans increased despite the large amount of acid that was liberated. They suggested that liberated ammonia or other basic end products of protein decomposition was the cause of this.

The final product had comparatively lower pH and titratable acidity values. This could be due to the fact that during the subsequent drying of the product after 72 h of fermentation to obtain the final product, there was loss of water leading to reduced water activity as well as reduced microbial activity which as a result reduced the release of ammonia as well as the pH of the fermenting medium.

Table 4-2: Changes in pH and Titratable acidity (expressed as lactic acid) during the fermentation of soybeans into dawadawa.

FERMENTATION TIME	BOILED SOYDAWADAWA		ROASTED SOYDAWADAWA	
	PH	Acidity	pH	Acidity
0 h	6.59	0.07	6.41	0.12
24 h	6.87	0.39	6.57	0.42
48 h	8.00	0.35	7.90	0.51
72 h	8.25	0.42	8.15	0.42
* Final Product	6.38	0.02	6.11	0.02

[a] Average values for 2 sets of samples

* Soydawadawa dried after 72h of fermentation.

4.4 MICROBIAL POPULATION OF FERMENTING SOYDAWADAWA

The viable counts obtained on the soydawadawa are shown in Table 4-3. The counts obtained for the MRS Agar plates were considerably lower than those on the PCA Agar plates. The low level of micro-organisms at the start of fermentation is likely to have resulted from the method of processing which involved heating of the beans. It is possible that this served as a selective mechanism for the fermenting micro-organisms

At 37°C, the processed beans (i.e. at 0 h) count on PCA was about 10^{3} cfu/g for both the boiled and roasted soydawadawa whereas that of fermented beans (72 h) was about 10^{11} cfu/g and 10^{9} cfu/g.

4.4.1 **Aerobic Mesophilic count**

In all fermenting soydawadawa samples, the population of aerobic mesophiles enumerated on PCA consisted of a variety of Gram-positive catalase positive rods bearing phase bright spores as well as Gram-negative bacteria and Gram-positive catalase-negative rods at the start of fermentation (Table 4-3). The population of aerobic mesophiles of all fermented products after 48 h fermentation were dominated by the Gram positive catalase positive rods. In most fermenting samples, the Gram-positive catalase negative rods and coccobacilli found at the start of fermentation were greatly reduced in the flora of the final fermented product. The Gram-negative bacteria present at the start of the fermentation were not found in the flora of the final fermented product.

The Gram-positive, catalase-positive rods bearing phase bright spores which grew on PCA and were subcultured in nutrient broth or agar, were considered to be *Bacillus* species. The Gram-positive catalase-negative rods were similar in colony and cell morphology to colonies which were later found to dominate growth on de Man Rogosa Sharpe (MRS) Agar plates.

The *Bacillus* species were often present at moderately high levels, about 10^9 cfu/g. Some Gram-negative bacteria were found in all two types of samples but were usually present at levels of less than 10^3 cfu/g.

4.4.2 **Growth On de Man Rogosa Sharpe (MRS) Medium**

The initial population of soydawadawa enumerated anaerobically on MRS agar consisted of Gram-positive catalase-negative rods and coccobacilli with rods as the

dominant type. Representative colonies of the Gram-positive catalase-negative rods and coccobacilli were found to be oxidase-negative non-sporing and strictly fermentative. They were therefore classified as lactic acid bacteria. The Gram-positive catalase-negative rods and coccobacilli found on PCA plates were also determined to be lactic acid bacteria. The population of the fermenting soydawadawa samples on MRS sometimes contained several relatively large colonies which were determined to be yeasts by morphological examination. The population of lactic acid bacteria increased from a level of 10^{3} to 10^{6} cfu/g and 10^{4} to 10^{6} cfu/g for boiled soydawadawa and roasted soydawadawa respectively during fermentation (Table 4-3).

Table 4-3: Microbial population of Bacteria and Yeast of fermenting Soydawadawa (cfu/g).

FERMENTATION TIME	BOILED SOYDAWADAWA Count (cfu/g)	ROASTED SOYDAWADAWA Count (cfu/g)
Start of Fermentation		
Aerobic mesophiles ([a])	2.5×10^3	9.0×10^3
Lactic acid bacteria ([b])	6.1×10^3	6.3×10^4
Yeasts ([c])	1.5×10^3	2.0×10
After 24 hours		
Aerobic mesophiles	4.6×10^9	1.2×10^9
Lactic acid bacteria	2.5×10^5	5.0×10^5
Yeasts	2.7×10^5	1.0×10
After 48 hours		
Aerobic mesophiles	1.2×10^9	9.0×10^9
Lactic acid bacteria	1.9×10^6	7.5×10^6
Yeasts	3.1×10^2	4.6×10^3
After 72 hours		
Aerobic mesophiles	1.5×10^{11}	1.8×10^9
Lactic acid bacteria	1.4×10^6	5.9×10^5
Yeasts	No growth	No growth
Final Product		
Aerobic mesophiles	6.2×10^8	5.1×10^8
Lactic acid bacteria	5.4×10^3	2.2×10^4
Yeasts	No growth	No growth

a. Counts on plate count Agar including both Gram-positive and Gram-negative bacteria.

b. Enumerated on MRS Agar reflecting Gram-positive, catalase-negative rods and coccobacilli.

c. Determined on Malt Agar.

Table 4-4: Morphological and biochemical characteristics of *Bacillus* isolates

TEST	ISOLATE								
	1	2	3	4	5	6	7	8	9
Cell Diameter > 1.0	-	+		+	-	-			
Spore Shape	C	O/E	E	E	E	E	O	C	C
Spore position	T	T/C	C	T/C	C	T/C	C	C	C
Gram stain	+	+	+	+	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+	+	+
Oxidase reaction	+	+	+	+	+	+	+	+	+
Anaerobic growth	-	V	+		-	-	V	+	+
Acid from D-Glucose	+	+	+	+	+	+	V	+	+
Acid from L-arabinose	+	-	+	+	+		+	+	+
Acid from D-Mannitol	+	-	+	+	+	+	+	+	+
Gas from Glucose					-				
Casein hydrolysis	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+		+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+	+
Growth at 65°C	-		-		-	-			
Growth at 30°C	+	+	+	+	+	+	+	+	+

Species of *Bacillus* identified

1. *B. subtilis*
2. *B. cereus*
3. *B. licheniformis*
4. *B. subtilis*
5. *B. subtilis*
6. *B. firmus*
7. *B. subtilis*
8. *B. subtilis*

   B. subtilis

C - Central / Circular

E- Elongated / Ellipsoidal

O - Oval

T- Terminal

4.4.3 Yeast and Moulds

The viable counts obtained for the malt agar plates were very low (Table 4-3). By morphological examination, the large colonies on the malt agar plates were determined to be yeast. However, after 72 h of fermentation, there was no growth. Further investigations were not carried out to identify the exact species of yeast cells. Ikenebomeh (1982) and Campbell-Platt (1980) have reported the incidence of a few fungi in the dawadawa fermentation as contaminants. Thus the incidence of a few yeast cells in the soydawadawa in this study could be as a result of contamination. The bacteria outgrew the fungi and possibly created an environment that was not conducive for the growth of fungi.

4.5 PATTERN OF MICROBIAL GROWTH

Microbial counts increased from the start of fermentation to the end of fermentation after 72 h (Table 4-2). However, there was reduction in microbial counts for the final product. This reduction in microbial counts for the final product could be due to reduced water activity in the final product after drying which possibly in combination with other factors, resulted in depressed microbial growth and biochemical activities in general. Although microbial counts generally increased with time of fermentation, the rate of increase of aerobic mesophiles was higher than that of the lactic acid bacteria due to increased pH in the sample. High pH normally promotes growth of aerobic mesophiles like *Bacillus* species but is not very conducive for growth of lactic acid bacteria.

Work by Ikenebomeh (1989) on the influence of salt and temperature on the natural fermentation of African locust beans showed that there was no microbial growth on

acidified PDA known to select for yeasts and moulds. This indicates that moulds and yeasts are not involved in the fermentation of the African locust bean seeds. However, in the present work, microbial growth on Malt Agar indicated the presence of yeasts, though they may not play much role in the fermentation process. With increased pH during fermentation, the yeast disappeared in the latter days of fermentation.

4.6 OCCURRENCE OF DIFFERENT *BACILLUS* SPECIES

The most frequently isolated cultures formed colonies with irregular margins and rough ridged or ringed surfaces often producing exudate. They had small cells with circular centrally placed spores, and were identified as strains of *Bacillus subtilis*.

The morphological and biochemical tests showed that most of the *Bacillus* isolates from the fermenting soydawadawa produced acid from D-glucose, L-arabinose, D-xylose and D-mannitol. They also hydrolysed casein and starch, reduced nitrate, grew at pH 5.7 and 6.8 and in 6.5% NaCl, (Table 4.4). These *Bacillus subtilis* strains generally utilized ribose, α-methyl-D-glucoside, amygdalin, esculin, salicin, L-arabinose, cellobiose, maltose, saccharose, trehalose, inuline, D-raffinose, amidon, glycogen, glycerol, D-xylose, D-glucose, D-fructose, D-mannose, inositiol, mannitol, sorbitol, arbutin, D-turanose, melibiose and gentiobiose, in API 50 CH galleries (Table 4-5).

Table 4-5: Percentage of identified species from boiled and roasted soydawadawa which fermented various carbohydrates

Carbohydrate	*B. subtilis*	*B. cereus*	*B. licheniformis*	*B. pumilus*	*B. firmus*
Glycerol	100	0	100	100	100
Erythritol	0	0	0	0	0
D-arabinose	0	0	0	0	0
L- arabinose	100	100	100	100	0
Ribose	100	0	100	100	0
D-xylose	83	0	100	100	0
L-xylose	0	0	0	0	0
Adonitol	0	0	0	0	0
β-methyl-xyloside	0	0	0	0	0
Galactose	0	0	0	100	0
D-glucose	100	100	100	100	100
D-fructose	100	100	100	100	100
D-mannose	100	0	100	100	0
L-sorbose	0	0	0	0	0
Rhamnose	0	0	0	0	0
Dulcitol	0	0	100	0	0
Inositol	100	0	100	100	0
Mannitol	100	0	100	100	100
Sorbitol	100	0	0	100	0
α-methyl-D-mannoside	0	0	100	100	0
α-methyl-D-glucoside	100	0	100	100	0
N acetyl glucosamine	33	100	100	100	100
Amygdaline	83	0	100	100	0
Arbutin	100	0	100	100	0
Esculin	100	100	100	100	100
Salicin	100	0	100	100	0
Cellobiose	100	0	100	100	0
Maltose	100	100	100	100	100
Lactose	17	0	0	0	100
Melibiose	100	0	0	0	0
Saccharose	100	100	0	0	0
Trehalose	100	100	100	0	100
Inulin	100	0	0	0	100
Melezitose	0	0	0	0	0
D-raffinose	100	0	0	100	0
Amdion	100	100	100	100	0
Glycogen	100	100	100	0	100
Xylitol	0	0	0	0	0
β-gentiobiose	17	0	100	0	0
D-turanose	83	0	0	100	0
D-lyxose	0	0	0	0	0
D-tagatose	0	0	0	0	0
L-fucose	0	0	0	0	0
D-arabitol	0	0	0	0	0
L-arabitol	0	0	0	0	0
Gluconate	100	0	0	0	0
2 Ceto-gluconate	0	0	0	0	0
5-Ceto-gluconate	0	0	0	0	0

Within the *Bacillus subtilis* isolates, variations could be observed in the colony morphologies pointing to the possible presence of different strains of the species. Other *Bacillus* species identified were *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus cereus* and *Bacillus firmus*. Isolates identified as *Bacillus licheniformis* had opaque rough star shape colonies which were very strongly attached to the surface of nutrient agar and difficult to scrape off. They could grow anaerobically. Isolates identified as *Bacillus cereus* generally had oval colonies with pear shaped ends with a medium to coarse matt appearance. Isolates identified as *Bacillus pumilus* had small creamy coloured colonies with moist raised surfaces. Variations were observed in the proportions of the different *Bacillus* species in the two sets of samples examined but *Bacillus subtilis* accounted for over half of the *Bacillus* population in the two samples. At all stages of the fermentation, the dominant species was *Bacillus subtilis* (Table 4-6).

Table 4-6: Distribution (percentage) of different *Bacillus* species in Soydawadawa. (Final Product).

% BACILLUS SPP	% OF ORGANISM	
	BOILED SOYDAWADAWA	ROASTED SOYDAWADAWA
B. subtilis	50	48
B. pumilus	20	20
B. licheniformis	6	7
B. cereus	16	16
B. firmus	8	9

* A total of 224 isolates from two samples of soydawadawa taken at a 24 hour interval for 3 days.

The characteristics reported for the dominant bacterial isolates are similar to those of *Bacillus subtilis* described by Gordon (1973). Odunfa (1981) reported the presence of *Bacillus subtilis* in fermenting African locust beans, while *Bacillus* species amongst others were also reported to be involved in the fermentation of dawadawa (Ikenebomeh *et al.*; 1981; Antai and Ibrahim, 1986).

4.7 THE ROLE OF *BACILLUS* SPECIES IN SOYDAWADAWA FERMENTATION

Fairly severe heat pre-treatments are given to the soybeans during the preparation of the Boiled and Roasted Soydawadawa. This pretreatment could be described as a spore activation process favouring selection for *Bacillus* species by virtue of their heat resistant spores. The findings that *Bacillus* species play a dominant role in the fermentation of a traditional staple product is unexpected due to the reported clinical importance of the species (Parry *et. al.* 1983).

Since the major constituents of soybeans are proteins, fats and carbohydrates, the organisms responsible for fermenting them must be capable of utilizing these three constituents. Most of the organisms isolated from the fermented beans are known to possess such characteristics. *Bacillus* species have a proteolytic ability and are also able to break down oils (Frazier 1967; Forgarty and Griffin 1973). The results of this work demonstrating the central role of *Bacillus subtilis* in the fermentation of soybeans into soydawadawa is in agreement with the findings of other investigators who have studied the fermentation of soybeans in other countries.

Tamang (1993) and Sakar *et. al.* (1994) in their work on Kinema, an indigenous non-salted fermented soybean food of the Himalayan regions showed the presence of *Bacillus subtilis*, *Enterococcus faecium*, *Candida parapsilosis* and *Geotrichum candidum*. Of the organisms isolated from Kinema, *B. subtilis* was the only one found to play a role in the fermentation (Sakar and Tamang, 1994).

Ogbadu and Okagbue (1988) in their work on the bacterial fermentation of African locust bean for dawadawa production also found *Bacillus subtilis* and *Bacillus pumilus* to be the dominant organisms in the fermenting locust beans with *Bacillus subtilis* dominating the fermentation process.

The findings of the present work are similar to those reported for African locust bean dawadawa. Campbell-Platt (1980) as reported by Odunfa (1985) found about 31% of microorganisms isolated from numerous dawadawa samples collected from different countries to be *Bacillus subtilis*. In some samples Campbell-Platt (1980) found *Bacillus subtilis* to constitute 61-69% of all isolates. *Bacillus subtilis* has also been confirmed as the predominant species in dawadawa fermentation in Nigeria by Odunfa (1981), Ikenebomeh (1989), Adewuyi (1983) and Odunfa and Oyewole (1986).

4.8 CHARACTERIZATION OF THE LACTIC ACID BACTERIA POPULATION

Over half of all Gram-positive catalase-negative rods and coccobacilli enumerated on MRS from fermenting soydawadawa samples and previously determined to be lactic acid bacteria were found to be facultatively heterofermentative lactobacilli. This was done by examination of their ability to produce CO_2 from glucose and gluconate (Table 4-7). There were also some homofermentative *Lactobacilli*. The *Lactobacillus*

species were non-oxidative, metabolized glucose fermentatively in Hugh and Leifson medium, grew at pH 4.7 and 9.6 but not in 6.5% NaCl and 18% NaCl. The species of the *lactobacilli* were not identified.

Table 4-7 Biochemical characteristics of Lactic acid bacteria isolated from Boiled and Roasted Soydawadawa

TEST	1	2	3	4	5	6
Tetrad formation	-	-	-	-		
Gram stain	+	+	+	+	+	+
Oxidase test	-	-		-		
Catalase test	+	-		-		
Anaerobic growth	+	+	+	+	+	+
CO_2 from glucose	-	+		+	+	
Growth at 45^0 C				+		V
Growth in 6.5% NaCl	+	+	+	-	+	+
Growth at pH 4.4	+	+	+	V	+	+
Fermentative (H & L)	+	+	+	V	+	+
Oxidative (H & L)				V		

* absent

\+ present

V Variable

4.9 TOTAL REDUCING SUGARS

The sugar levels showed a remarkable fluctuation with the length of fermentation. The reducing sugar level increased during the first 24h but subsequently decreased (Figure 4-3) This trend in sugar levels during fermentation has also been described by Odunfa (1985) in his studies on the biochemical changes in fermenting African locust bean *(Parkia biglobosa)* during 'iru' fermentation. He explained that, the initial rise in the reducing sugar level may not be due to the amylase activity. They might be produced from the hydrolysis of oligosaccharides, present in the unfermented bean (Odunfa, 1983). These sugars are easily untilisable by the *Bacillus* species involved in the fermentation. Although carbohydrates constitute 11-15% of the unfermented beans no starch has been reported in the fermented beans (Watson, 1971). Eka (1980) also recorded a decrease in the carbohydrate level of unfermented locust beans and fermented beans. However a similar work by Odunfa (1982) during "ogiri", a fermented melon (*citrullus vulgaris*) product showed an initial decrease in the sugar level. He explained that the initial decrease in the sugar level may be due to the initial population of bacteria which preferentially utilise the soluble sugars in the melon (Odusote, 1977) found that melon contains 2.5% soluble sugar and 11% starch by weight. After the exhaustion of the sugars, the initial population is succeeded by amylolytic bacteria which hydrolyse the starch, thereby increasing the sugar level in the fermenting melon. Odusote (1980) found that the latter stage of "ogiri" fermentation was charcterised by α- amylase producing *Bacillus* species.

Fig 4-3

Total reducing sugars determination

Total sugars of:

BSD	(0h)=	80
BSD	(24h)=	130
BSD	(48h)=	65
BSD	(72h)=	15
BSD	(final product)=	5
RSD	(0h)=	78
RSD	(24h)=	125
RSD	(48h)=	68
RSD	(72h)=	12
RSD	(final product)=	5

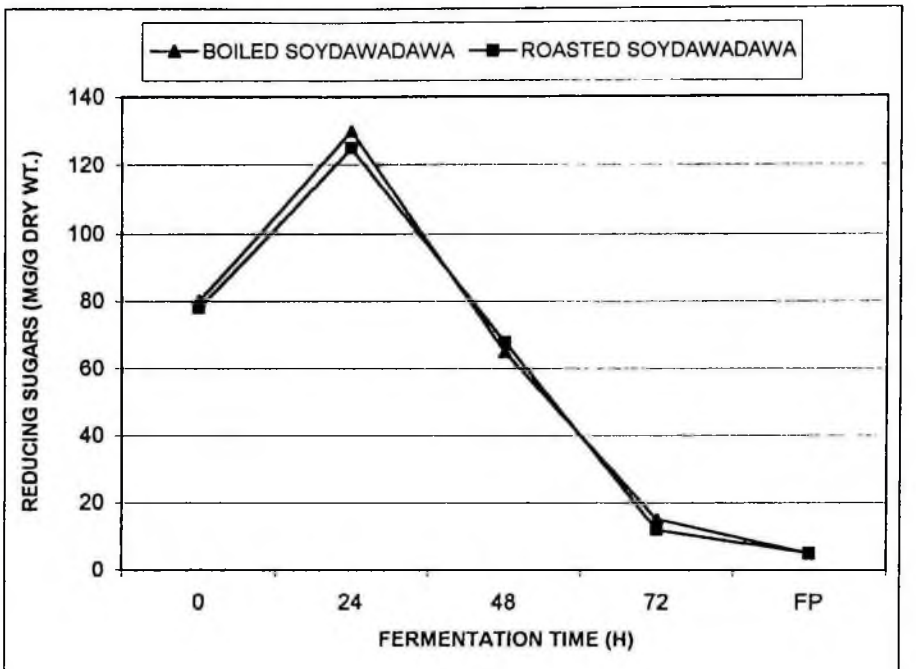


FIG. 4-3 TOTAL REDUCING SUGARS

4.10 ENZYMATIC ACTIVITY IN SOYDAWADAWA DURING FERMENTATION

All isolates of *Bacillus* species, the dominating microorganisms showed a similar pattern of enzymatic activity with respect to α-amylase and proteinase activity. All *Bacillus* isolates showed high proteinase activity and nearly all except *Bacillus pumilus* showed high amylase activity.

Fig. 4.4

Diameter of clear zone on skim milk agar indicating extent of proteinase activity of micro-organism.

Microorganism

BS 1 = *Bacillus subtilis* 1

BS 2 = *Bacillus subtilis* 2

BS 3 = *Bacillus subtilis* 3

BS 4 = *Bacillus subtilis* 4

BS 5 = *Bacillus subtilis* 5

BS 6 = *Bacillus subtilis* 6

BC = *Bacillus cereus*

BP = *Bacillus pumilus*

BL = *Bacillus licheniformis*

BF = *Bacillus firmus*

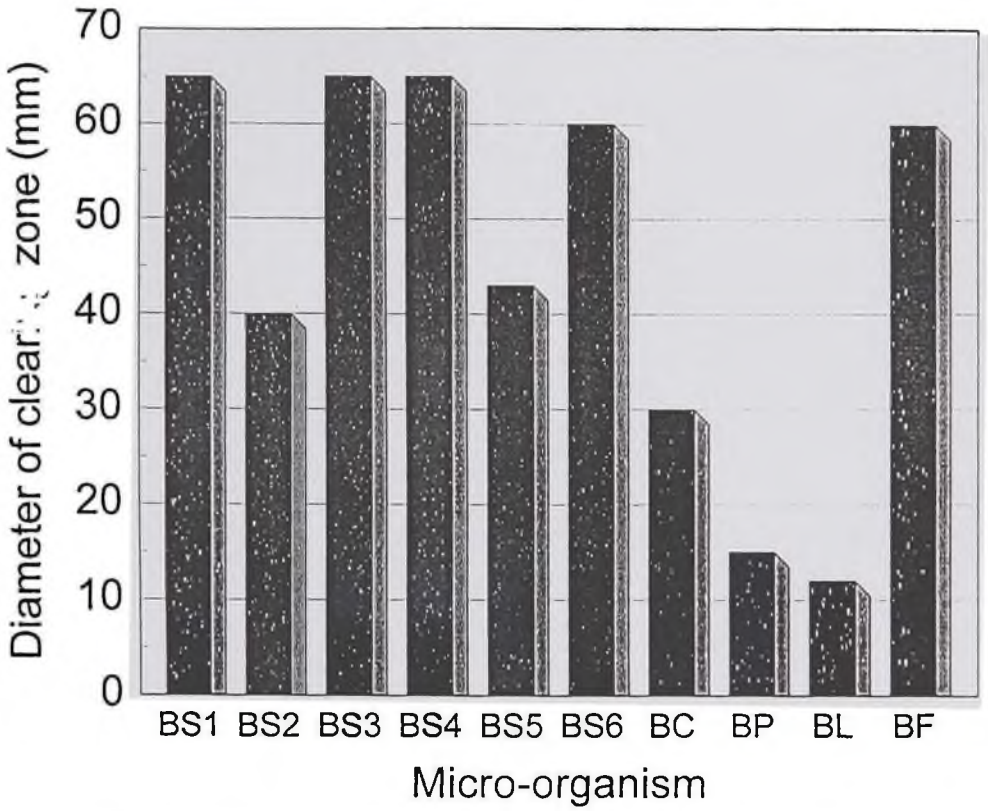


Fig.4.4Diameter of clear zone on skim milk agar indicating extent of proteinase activity of micro-organisms

Fig. 4.5

Proteolytic activity in soydawadawa during fermentation.

BSD – Boiled soydawadawa

RSD – Roasted soydawadawa

Proteinase activity of :

BSD	(0h)=	0.18
BSD	(24h)=	1.00
BSD	(48h)=	3.00
BSD	(72h)=	4.10
BSD	(final product)=	3.50
RSD	(0h)=	0.30
RSD	(24h)=	1.10
RSD	(48h)=	2.50
RSD	(72h)=	3.30
RSD	(final product)=	2.50

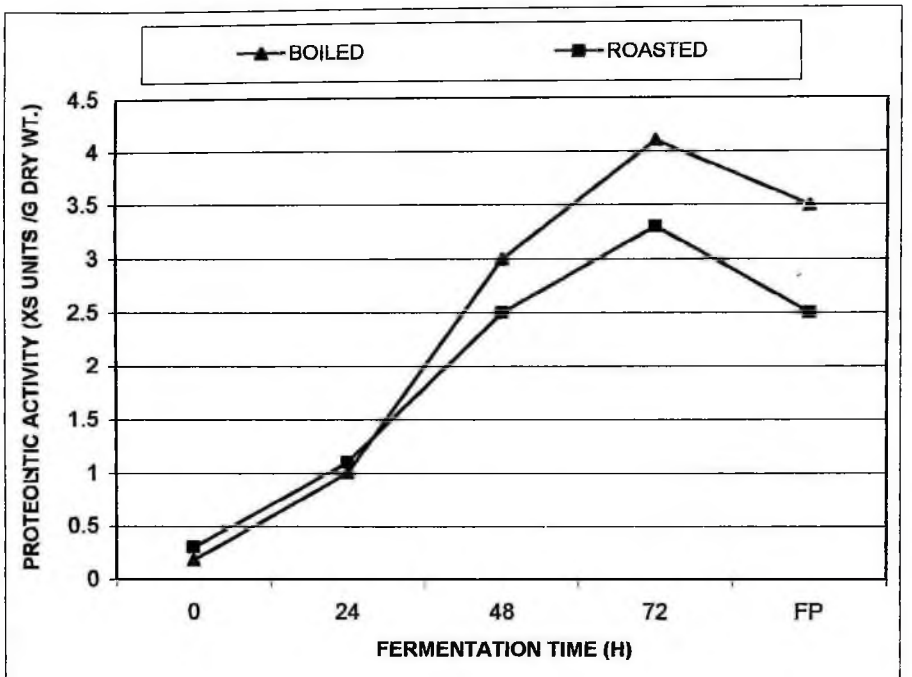


FIG. 4-3 PROTEOLYTIC ACTIVITY IN SOYDAWADAWA DURING FERMENTATION.

4.10.1 **Proteinase Activity**

The proteinase activity of the *Bacillus* isolates was determined by their ability to break down skim milk.

All the *Bacillus* isolates had proteinase activity which was exhibited by their ability to disintegrate skim milk when directly placed onto sterile skim milk agar plates.

All the strains of *Bacillus subtilis* exhibited a higher level of proteinase activity than the other *Bacillus* species. *Bacillus licheniformis* isolates examined showed very low proteinase activity and were in fact the least proteolytic with a clearing zone of about 12 mm in diameter (Figure 4.4). The results indicated that strains within the species group differed in the quantity of extracellular proteinase secreted. Also, the proteinase activity increased with increase in time of fermentation.

The most significant biochemical change that occurs during dawadawa fermentation is protein hydrolysis (Odunfa, 1985). High proteinase activity gives rise to rapid amino acid production.

The proteinase activity within the fermenting soydawadawa was also determined by measuring the level of enzyme activity at specific times during the period of fermentation. The proteinase activity in the boiled and roasted soydawadawa medium is shown in Fig. 4-5.

At the start of fermentation, there was detectable proteinase activity despite the fact that there were mostly spores present. The proteinase activity in the *Bacillus* isolates decreased in the order of BSI = BS3 = BS4>BS6 = BF>BS5>BS2>BC>BP>BL as shown in Fig. 4.4. In the few hours of fermentation, there was a rapid increase in proteinase activity with the activity reaching a maximum level between 60 and 72h. This was almost followed by an abrupt proteinase activity decline at the end of the fermentation period when the product was dried.

The above observation falls within expectation. Normally bacteria have a peculiar pattern of growth. At the log phase or initial stage where no multiplication takes place little enzyme activity is observed. This phase is followed by the logarithmic phase where bacteria numbers approximately double with each interval time to come to a stationary and decline phase where no multiplication occurs and numbers decrease. In industrial production of many important secondary metabolites, product formation generally occurs in the idiophase, i.e. after the initial period of rapid growth as reported for penicillin. Yokotsuka (1972) and Yamamoto *et al.* (1972) also concluded that around 60 h is an optimal cultivation time for enzyme production.

The results obtained from Fig. 4-5 were subjected to statistical analysis using two-way Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT). The levels of significance chosen was at α = p(0.01) (Appendix 3). Both fermentation time and method of production significantly affected proteinase activity (Table 4-9). Proteinase activity increased with fermentation time. The proteinase activity of the final product was lower and this could be attributed to the reduced microbial load of the final product. The boiled soydawdawa showed higher proteinase activity than the roasted soydawadawa as shown in Table 4-9. However the interaction between the

type of soydawadawa and time of fermentation did not have any significant effect on proteinase activity. Table 4-9. Proteinase activity was affected by only fermentation time and type of soydawadawa.

Table 4-8 Mean effect of fermentation time and type of Soydawadawa on the Proteinase activity of *Bacillus Species*

Fermentation Time/h	Proteinase activities in (units)	
	Boiled Soydawadawa	Roasted Soydawadawa
0	0.20	0.30
24	1.00	1.10
48	3.00	2.45
72	4.10	3.60
Final product	3.50	2.45
Proteinase Activity Mean *	2.36	1.98

α = 0.05

4.10.2 α- Amylase Activity

Bacillus pumilus did not show any α-amylase activity irrespective of prolonged incubation. However, the other *Bacillus* isolates showed high α-amylase activity. The production of amylolytic enzymes by representatives of the genus *Bacillus* is common but not general. Species such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus circulans*, *Bacillus maecerans*, *Bacillus coagulans*, *Bacillus stearothermophilus* and *Bacillus brevis* produce α-amylase. (Zemek *et. al.*, 1980).

The level of activity in the *Bacillus* isolates decreased in the order of BS2 = BS5>BS3>BS4 = BL>BS6>BC>BF>BP (Fig. 4.6). However the interaction between the type of soydawadawa and time of fermentation did not have any significant effect on α-amylase activity. Table 4-10. α-amylase activity was affected by only fermentation time and type of soydawadawa.

Boiled soybeans processed into soydawadawa did not show any detectable α-amylase production at the start of fermentation as shown by the roasted soydawadawa in Fig. 4.7. The trend in production of the α-amylase was similar to the production of proteinase except that after 48 h both the boiled and roasted soydawadawa showed similar levels and almost overlapped. Only fermentation time significantly affected α-amylase activity (Table 4-10).

Figure 4.6

Diameter of clear zone on starch agar indicating extent of α-amylase activity of micro – organisms

Microorganism

BS 1 = *Bacillus subtilis* 1

BS 2 = *Bacillus subtilis* 2

BS 3 = *Bacillus subtilis* 3

BS 4 = *Bacillus subtilis* 4

BS 5 = *Bacillus subtilis* 5

BS 6 = *Bacillus subtilis* 6

BC = *Bacillus cereus*

BP = *Bacillus pumilus*

BL = *Bacillus licheniformis*

BF = *Bacillus firmus*

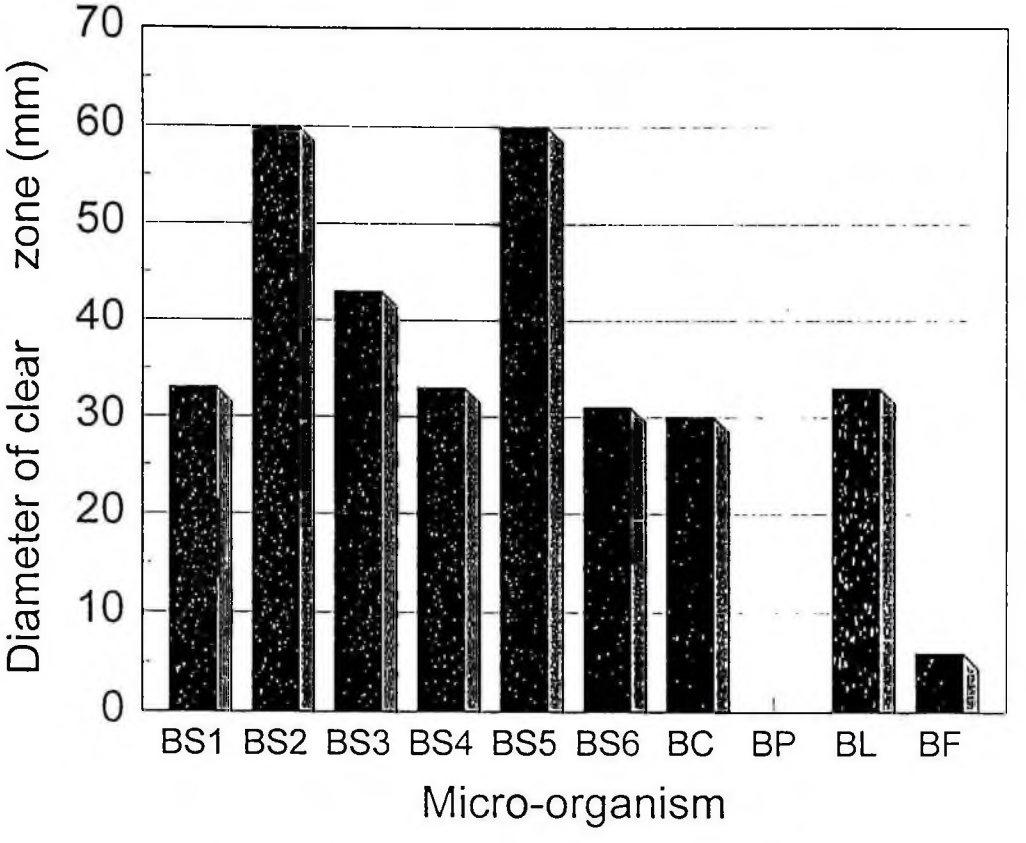


Fig. 4-6Diameter of clear zone on starch agar indicating extent of α -amylase activity of micro-organisms

Fig 4.7

α - Amylase activity in soydawadawa during fermentation.

α - Amylase activity of *Bacillus* species

BSD	(0h)=	0.00
BSD	(24h)=	0.20
BSD	(48h)=	0.30
BSD	(72h)=	0.80
BSD	(final product)=	0.65
RSD	(0h)=	0.10
RSD	(24h)=	0.25
RSD	(48h)=	0.32
RSD	(72h)=	0.82
RSD	(final product)=	0.68

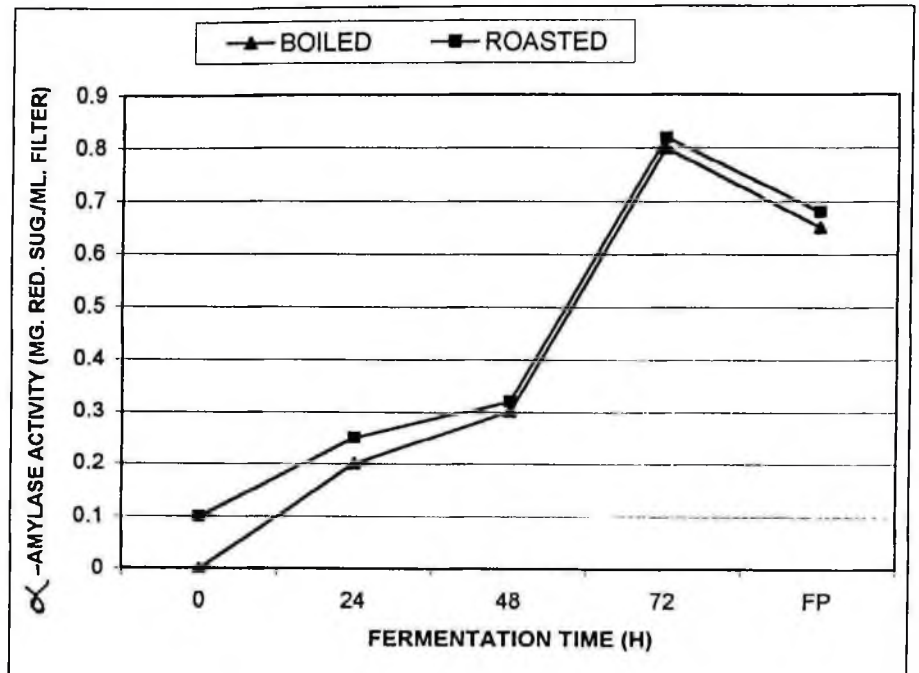


FIG. 4-7 α-AMYLASE ACTIVITY IN SOYDAWADAWA DURING FERMENTATION

Table 4-9 Mean effect of fermentation time and type of Soydawadawa on α-Amylase activity of *Bacillus Species*

Fermentation Time/h	α-Amylase Activity in (units)	
	Boiled Soydawdawa	Roasted Soydawadawa
0	0.00	0.10
24	0.20	0.25
48	0.30	0.32
72	0.80	0.82
Final product	0.65	0.68
α-Amylase Activity Mean	0.39	0.43

α = 0.05

4.11 AMINO ACID PROFILE OF SOYDAWADAWA

The amino acid profile of soybeans did not change much with fermentation (Table 4-11). The fermented soydawadawa were low in tryptophan and tyrosine. Blackburn (1968) found that tryptophan and tyrosine are extensively destroyed on acid hydrolysis in the presence of carbohydrates. He suggested that appreciable amounts of carbohydrates associated with the protein produce acid degradation products of the sugars, such as hydroxymethyl furfural and that these may interact with amino acids to cause loss.

Since soybean contains quite a high amount of carbohydrates, about 40g/100g, it is expected that the loss of amino acids during acid hydrolysis will be high. However,

with fermentation of the soybeans into soydawadawa, most of the carbohydrates and available reducing sugars are utilized by microorganisms, reducing the loss of amino acids. There were generally slight increases in the concentration of the amino acids. This could be due to amino acids from the microorganisms as they increased during fermentation. With the use of acid hydrolysis in this present work for the determination of the protein profile, the trends in the above mentioned amino acids was well expected.

Methionine content was quite low in both unfermented soybeans and fermented soydawadawa. This is because methionine has been identified as the limiting amino acid in soybeans meals (Almquist and Grau, 1944). Eka, (1980) reported that dawadawa produced from African locust bean is low in the essential amino acids, leucine, isoleucine, phenylalanine and tryptophan. However, this work showed that dawadawa produced from soybean was low in only tryptophan with the other essential amino acids mentioned above comparatively higher. The deficiency of some of the essential amino acids in dawadawa produced from African locust bean affects its value as a source of high quality protein. Thus with soydawadawa improving upon this quality attribute, it could be regarded as a good source of high quality protein in the meal. Moreover, the amino acids of the main meal will not be solely relied upon to complement the low levels of essential amino acids in traditional dawadawa.

The amino acid content of the Roasted Soydawadawa was slightly lower than that of the Boiled Soydawadawa. Normally, dry heating is less effective than steam in enhancing the nutritional value of soybean protein, and the presence of water partially prevents the adverse effect of excessive heating (Harris and Von Loesecke, 1960).

This phenomenon may account for the observation that the Roasted Soydawadawa had a slightly lower amino acid content. There was a decrease in aspartic and glutamic acids up to 48 h of fermentation. Fetuga *et. al.* (1973) also observed similar results in the fermentation of African locust bean dawadawa.

Table 4-10: Amino Acid Profile of fermented and unfermented Soybean Dawadawa (mg/aa)

AMINO ACID	FERMENTED BOILED SOYDAWADAWA					FERMENTED ROASTED SOYDAWADA					UNFERMENTED SOYBEAN
	0h	24h	48h	72h	F.P	0h	24h	48h	72h	F.P*	
Isoleucine	228	227	232	280	276	225	223	230	272	269	230
Leucine	426	422	434	490	487	422	421	432	466	464	430
Lysine	338	336	342	400	398	332	331	341	392	390	340
Methionine	76	72	78	80	78	72	67	62	77	76	80
Cysteine	67	65	64	66	65	64	63	67	96	94	70
Phenylalanine	272	267	274	310	310	263	257	270	303	303	270
Tyrosine	207	205	213	200	198	203	200	192	196	193	210
Threonine	232	228	248	240	240	210	198	242	230	230	240
Tryptophan	89	86	89	80	78	85	83	79	77	75	90
Valine	302	300	312	300	300	280	276	335	289	288	310
Arginine	262	261	274	350	335	250	247	273	335	333	270
Histidine	147	144	153	160	157	132	120	154	158	157	150
Alanine	278	269	264	270	270	268	262	282	268	268	280
Aspartic	335	322	325	330	328	325	312	310	321	318	750
Glutamic	257	243	258	270	265	263	242	234	272	267	670
Glycine	233	229	246	260	256	238	235	242	252	250	240
Proline	229	218	338	340	335	229	226	234	339	336	240
Serine	324	318	316	320	320	298	296	312	318	316	330

* F.P. - final product

4.12 CHARACTERISATION OF PROTEINS IN SOYDAWADAWA

Hydrolysis of protein in soydawadawa during fermentation was investigated by electrophoretically determining the protein profiles of samples. The protein profiles are shown in Figure 4-8 and the profiles could be used to distinguish between the different fragments or types of protein-Lanes 3-11 showed bands which were less clear than those of lanes 13-25. The experimental procedure in which soybeans were defatted before fermenting resulted in more pronounced breakdown of the proteins than the treatment in which soybeans were fermented before defattening.

The bands observed could have possibly resulted from the breakdown of soy proteins into soy amino acids by proteinase enzymes. This occurs during fermentation of soydawadawa since the key mechanism which occurs is the production of proteinase by the dominant *Bacillus* species.

As stated earlier, the number of dominant microorganisms present in the fermenting soydawadawa increased with the progress of fermentation. Their cumulative enzymatic activities also increased simultaneously, especially their proteolytic activity. Thus more enzymes or proteinases were produced to breakdown the protein in the soydawadawa, making available more amino acids. This supports the results obtained by the electrophoresis.

The protein profiles of both types of soydawadawa during fermentation showed an increase in the fraction of lower molecular weight proteins confirming the hydrolysis of proteins during fermentation The protein bands (fig 4-7) became more distinct with the progress of fermentation. This could be attributed to specificity in attack of proteases on the proteins, resulting in the accumulation of protein fragments of certain lengths.

Fig. 4-8

Protein profile of soydawadawa at different stages of fermentation

1= High molecular weight protein (standard).

2= Low molecular weight protein (standard)

3= Raw soybean

4= Boiled but not fermented

5, 6, 7= Boiled and fermented for 24h, 48h, 72h.

8= Roasted but not fermented

9, 10, 11= Roasted and fermented for 24h, 48h, 72h.

12= Roasted but not fermented

13= Raw soybean defatted and fermented

14, 15, 16= Roasted and

17= Boiled but not fermented

18,19, 20=

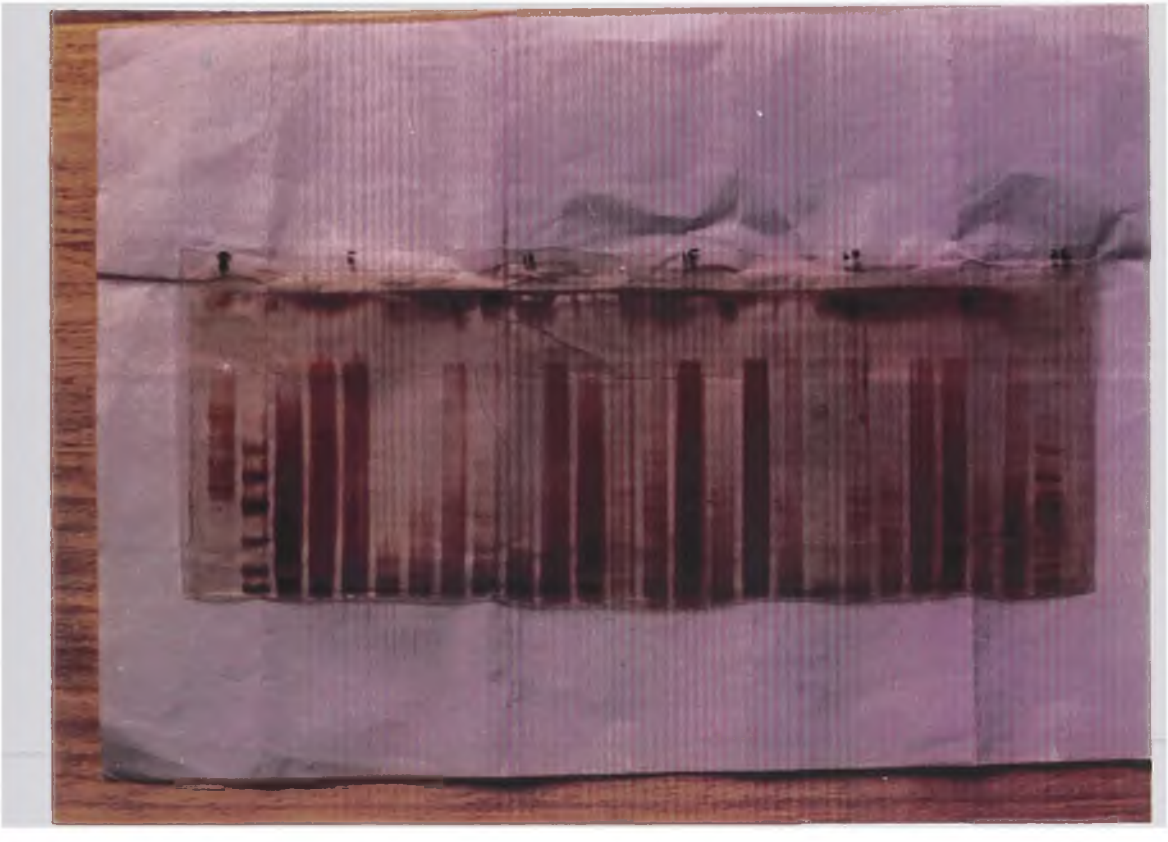
21= Boiled but not fermented

22= Boiled, defatted, boiled

23; 24,25= Boiled and fermented for 24h, 48h, 72h

Lanes 4 – 12 Beans were fermented before defattening

Lanes 13 – 26 Beans were defatted before fermenting.



4.13 AROMA PROFILE OF SOYDAWADAWA

Table (4-11) shows that a lot of difference were observed in the numbers and types of aroma compounds present in the two different types of soydawadawa suggesting that the aroma of soydawadawa is greatly affected by the method of production i.e. heat pretreatment. More aroma compounds were detected in the roasted soydawadawa and the compounds were also present at quite high levels. A few compounds however were detected in both the roasted and boiled soydawadawa. Acetophenone and pyrazines were found to be components related to protein degradation during maillard browning in roasted foods. This may explain the presence of pyrazines in the roasted soydawadawa. Octadecanoic and hexanol were detected in both samples of soydawadawa throughout the fermentation. This could have resulted from the hexane which was used as the solvent to extract the aroma. Tetradecanoic acid was detected in only the roasted sample throughout the period of fermentation. On the whole, aroma compounds detected were basically, alcohols, ketones, aromatic and aliphatic organic acids and phenols.

Table 4-12 also shows that some aroma compounds were produced as a result of the fermentation. In the boiled soydawadawa, phenyl ethyl alcohol and 2,4-bis (1, 1 dimethyl ethyl phenol) which were not detectable at the start of fermentation were detected in the fermented product. Similarly, in the roasted soydawadawa 1, 2-benzenedicarboxylic acid, 2-methoxy-phenol and 2, 5-dimethyl pyrazine were produced during fermentation because even though they were present in the fermented product they were not detected at the start of fermentation.

The flavour impressions of many foodstuffs implies a complex mixture of aroma substances and that many traditional foodstuffs and beverages are flavoured in situ by the action of microorganisms e.g. the use of proteases produced by bacteria in the meat industry (Schermers *et al.* 1976). Many vegetable proteins such as soybeans possess characteristic beany flavours and odour which limit their sale and use because the flavour and/or odour is unappealing to large numbers of potential consumers. Non pathogenic bacteria have however been found to proliferate such foods and remove the flavour and/or odour from it (Hanson, 1974). Wang *et al.* (1968) found that bacteria or moulds during the fermentation of soybeans into various foodstuffs help to improve the flavour and perhaps also add to their nutritional value.

Honig *et. al.* (1976) found roasting to be necessary in order to produce soy protein products with flavour scores approaching the blandness of wheat flour. Although flavour scores were not done on the soydawadawa, one could perhaps deduce from the aroma compounds produced that the roasted product was more flavourful than the boiled one. Hesseltine, (1965) also found fermentation to improve or modify flavour, taste and texture of foods. Vandore, (1967) found dry method of cooking such as grilling and roasting to produce meats of superior flavour.

Table 4-11: Major aroma compounds detected by GC-MS during the fermentation of soydawadawa.

AROMA COMPONENT	BOILED SOYDAWADAWA			ROASTED SOYDAWADAWA		
	0h	24h	48h	0h	24h	48h
3 - Hexanol	+	+	+	+	+	+
9, 12 - Octadecanoic acid	+	+	+	+	+	+
Phenyl ethyl alcohol	-	+	+	-		
2, 4-bis (1,1-dimethyl ethyl phenol)			+	+		+
Tetradecanoic acid	-			+	+	+
1, 2-Benzenedicarboxylic acid	-			-	+	+
2 - methoxy - phenol	-		-			+
2, 5 - dimethyl pyrazine	-	-				+

5.0 CONCLUSIONS

The heat pretreatment given to soybeans before processing may favour the selection of heat resistant *Bacillus* spores and hence makes them the predominant micro-organisms responsible for the fermentation of soybeans into dawadawa.

The *Bacillus* species identified in this work are mainly, *Bacillus subtilis*, *B. licheniformis*, *B. pumilus*, *B. firmus* and *B. cereus*. This confirms the work of previous workers who had also noted the dominance of *Bacillus* species over other micro-organisms during dawadawa fermentation.

Hydrolysis of proteins during fermentation has been confirmed by an increase in the fraction of lower molecular weight proteins in the protein profiles of both types of soydawadawa. The attack of proteases on the proteins also increased with the progress of fermentation. This is evidenced by more distinct protein bands in the electrophoresis gels as fermentation progressed. This phenomenon is due to the accumulation of protein fragments of certain lengths.

The *Bacillus* species that were identified showed proteolytic activity and are important for the proteolysis associated with soydawadawa production. Some products of this proteolysis are responsible for the characteristic strong smell of soydawadawa. The pH of the fermenting beans confirms the alkaline nature of the process. The compounds responsible for the alkalinity could be mainly volatile nitrogenous compounds because the dried final products do not have the high pH found in the fermenting mass. This alkalinity enhances the growth of the *Bacillus* species.

The form of heat used for the pretreatment had a significant effect on the aroma compounds and hence the aroma of the resulting end product. This work identified the

main organic acids and volatile aroma compounds developed during the fermentation process. The major aroma compounds identified included 9,12-octadecanoic acid, phenyl ethyl alcohol, 2, 4-*bis* (1, 1-dimethyl ethyl phenol), Tetradecanoic acid, 1, 2 – Benzene dicarboxylic acid, 2 – methoxy – phynol and 2, 5 – dimethyl pyrazine

6.0 RECOMMENDATIONS

The identification of *Bacillus* species as the dominant micro – organisms responsible for soydawadawa production and their enzymatic activities could serve as a first step in the process of developing a starter culture. This starter culture could be used for producing soydawadawa with consistent high quality.

It is also recommended that technological properties such as proteinase and the α-amylase activities of the starter culture should be typed. Optimization of the fermentation parametres should be done to reduce fermentation time for the production of soydawadawa.

REFERENCES

Abbiw D. 1990. Useful plants of Ghana: Intermediate Technology Publications Ltd; London, PP 48-254.

Adewuyi. E.Y. 1983. Studies on the Optimization of process conditions for locust bean (*Parkia biglobosa*) fermentation, MSc dissertation. Department of Botany, University of Ibadan.

Almquist, H. J. and Grau, C. R. 1944. Amino Acids in Soybean Varieties. Poultry Science. 23, 34.

Alozie, T. C., Rotimi, C. N. and Oyibo, B. B. 1980. Production of aflatoxin by *Aspergillus flavus* (UBMI) in some Nigerian indigenous beverages and foodstuffs. Mycopathol. Mycol. Appl. 70, 125.

Annegers, J. F. 1974. Protein quality of West African foods. Ecol. Food Nutr. 3, 125-130.

Antai, S. P. and Ibrahim, M. H. 1986. Microorganisms associated with African locust bean (*Parkia filicoidea* welw.) fermentation for dawadawa production. J. Appl. Bacteriol. 61, 145-148.

AOAC (1990) Official Methods of Analysis, 15th edn. Virginia. Association of Official Analytical Chemists.

Arcega, B.1969. The Manufacture of Sauce. NIST Tech. Bulletin. No.9.

Barimalaa, I.S., Achinewhu, S.C., Yibatama, I. & Amadi, E.N. 1994. Studies on the solid substrate fermentation of soybeans (*Glycine max* var Merril). J. Appl. Bacteriol. 80.

Bemelmans, J. M. H. 1981. Isolation and concentration of volatiles from foods. Chpt.2 in "Isolation, separation and Identification of volatile compounds in Aroma research", ed. H. Maarse and R. Belz, pp. 4-59. Akademie-Verlag, Berlin.

Bernfeld, P. 1955. Amylases and Proteinases. In Methods in enzymology, vol 1. (Colowicz S. P. and Kaplan N. O. eds). Pp. 149-158. New York: Academic press.

Blackburn, S. 1968. Amino Acid Determination, Methods and Techniques. Marcel Dekker, Inc; New York Pp. 125.

Busson, F. 1965. Plantes Alimentaires de l'ouest African, Direction de la Cooperation Culturelle et Technique. Paris, p. 272.

Campbell- Platt, G. 1987. Fermented foods of the world, A Dictionary and Guide. London; Butterworths.

Campbell-Platt, G. 1980. African locust bean (Parkia species) and its West Africa fermented food product, dawadawa. Ecology of Food and Nutrition. 1:123 – 132.

Carrao, M. C., Panizzi, J. M., Gontijo Mandarino, G. 1994. Soybean for Human consumption, Nutritional quality, processing and utilization. FAO, Rome pp. 241 – 253.

Claus, D. and Berkerly, R. C. W (1986). The genus *Bacillus*. In Bergey's Manual of Systematic Bateriology, Vol. 2. Ed. Sneath, P.H.A, Mair, N. S, Sharpe, M. E. and Holts, J. G. pp. 1105-1139, Baltimore: Williams and Wilkins.

Dema I. S. 1965. Nutrition in Relation to Agricultural Production. Food and Agriculture Organization. Rome. 129.

Diawara, B. Sywadogo, L. Amoa-Awua, W. K. A. and Jakobsen, M. 1998. Quality system for the production of soumbala. The HACCP System. Taastrup: Waitro.

Eka, O. U. 1980. Effect of fermentation on the nutrient status of locust beans. Food Chemistry. 5 : 303-308.

FAO Food and Nutrition Paper 47/4 (1989). Utilization of tropical foods: Tropical beans FAO, Rome.

Fetuga B. L., Babatunde, G. M. and Oyenuga, V. A. 1973. Protein quality of some Nigerian Foodstuffs. I. Chemical assay of nutrients and amino acid composition. J. Sci. Food Agric. 24, 1505.

Forgarthy, W. M. & Griffin, P. J. 1973. Production and Purification of metalloproteases of *Bacillus polymyxa*. Applied Microbiology 26, 191-195.

Frazier, W. C. (1967) Food Microbiology. 2nd ed. New York: Mc Graw – Hill, p. 450.

Girgit, P and Turner, T. D. 1972. Lesser known Nigerian edible oils and fats. III. Fatty acid composition as determined by gas-liquid Chromatography, J. Sci. Food Agric. 23-25.

Gonzalez, O. N. and Uyenco, F R. 1975. Microbiology of Fermented Foods. Paper presented at the technical seminar. 20th July – 2nd August. Manila: ASEAN Sub-committee on Protein

Gordon, M. H. and Macrae, R. 1992. Instrumental Analysis in the Biological Sciences. Blackie and Sons Ltd. Edmunds, Suffolk, p. 41-43.

Gordon, R. E. Haynes, W. C. and Pang, C. H. N. (1973) The genus *Bacillus*, Handbook Nº 427. Washington, United States Department of Agriculture.

Gordon, R. E (1973) The genus *Bacillus*. In: Laskin A. I. and . Lechevalier H. A. (Eds.), CRC Handbook of Microbiology. Vol. 1, Organismic Microbiology. CRC Press, Cleveland. OH. Pp. 71-81.

Hanson, L. P 1974. Vegetable Protein Processing.. Pg. 25-26. Noyes data Corporation, New Jersey

Harris, R. S. and Von Loesecke, H. 1960. Nutritional Evaluation of food Processing. Pp. 239. John Wiley.

Hesseltine, C. W. 1965. A millennium of fungi, food and fermentation. Mycologia 57: 149-197.

Hesseltine, C. W., Smith, N., Bradle, B. and Djien, L. 1963. Investigations of Tempeh and Indonesian Food. Washington D. C: American Institute of Biological Sciences.

Honig, D. H., Warner, K. and Rackis, J. J. 1976. Soybean Protein flavour. J. Food Sci. 41, 642-646.

Horii, M. 1997. Soybean and Fermented Food culture in the world. J. Farming Japan Vol. 31-4, pp. 10-20.

Hug, R. S., Abalaka, J. A. and Stafford, W. L. 1983. Folate Content of various Nigerian foods. J. Sci. Food Agric, 34, 404, 1983.

Hugh, R. and Leifson, E. 1953. The taxonomic significance of fermentation versus oxidative metabolism of Carbohydrates by various gram negative bacteria. J. Bacteriol. 66, 24-26.

Ihekoranye, A. I and Ngoddy, P. O. 1992. Integrated Food Science and Technology for the Tropics; The Macmillan Press Ltd., London; pp. 92.

Ikenebomeh, M. J. (1989). The influence of salt and temperature on the natural fermentation of African Locust bean.

Ikenebomeh, M. J. 1982. The solid substrate Fermentation of African Locust Beans (*Parkia filicoidea*) Ph. D. thesis, Mc Gill University (McDonald Campus) Montreal.

Ikenebomeh, M. J., Kok, R. and Blackwood, A. C. 1981. Solid substrate fermentation of the African locust bean (*Parkia filicoidea* welw.). Abstract. SIM News. 31-46.

Jennings, W. G. 1980. Sample preparation. Chpt. 12 in "Gas chromatography with glass capillary columns," 2nd edition., pp. 183 – 200. Academic Press, New York.

Keshinro, O. O. 1983. The Free and Total folate activity in some commonly available foodstuffs, J. Food Chem. p. 11, 87.

Lawson, R. M. 1965. The Consumption approach to measuring Agricultural production in foodstuffs. Food Res. Inst. Stud. J. Agric. Econ. Trade Dev. (Standford). p. 5, 205.

Lee, S. Y. Park, K. W. and Min, Y. K. (1983) Nutritional evaluation of naturally fermented soybean and the enzymatic activity changes during the preparation. Korean Journal-of-food-science-and-Technology. 15 (2) p. 101-107.

Lilie, R. D. (1928) The Gram Stain. 1. A quick method for staining Gram positive organisms in the tissue. Archives of Pathology 5, 828.

Logan, N. A. and Berkerley, R. C. W. (1984) Identification of *Bacillus* strains using API system. J. Gen. Microbiol. 130 – 1871 – 1882.

Logan, N. A. and Berkerley, R. C. W. (1981) Classification and identification of the genus *Bacillus* using API tests. In The Aerobic Endospore forming Bacteria: Classification and Identification. Pp. 105-140. London: Academic Press.

Kordylas, M. 1991. Processing and Preservation of Tropical and Sub-Tropical Foods. English Language Book Society, Hong Kong. pp. 52-58.

Nicol, B. M. 1959. The protein requirements of Nigerian peasant farmers. Br. J. Nutr. 13, p7.

Nikkuni, S. 1997. Nutritive value and processing of soybeans. Food Science & Technology. Int.; 90-93.

Nikkuni, S., Karki, J.B. Vilkhu, K. S., Suzuki, T., Shindoh, K., Suzuki, C. and Okada, N. 1995. Food Science Technology, Int. 1, 107-111.

Nnam, N. M. 1995. Evaluation of nutritional quality of fermented cowpea (*vigna unguiculata*) flours. Ecology-of-food-and-Nutrition (USA). V 33 (4) p. 273-279, Tables, 32, ref.

Obizoba, I. C. and Atu-L.N. 1993. Production and chemical evaluation of some food condiments of Nigeria. Plant-Foods-for-Human Nutrition. 44:3, 249-254; 2 ref.

Odunfa, S. A. 1985. Biochemical changes in fermenting African locust bean (*Parkia biglobosa*) during iru fermentation. J. of Food Technology. 20, 295-303.

Odunfa, S. A. 1983. Carbohydrate changes in fermented locust bean (*Parkia filicoidea*) during iru preparation. Plant foods for Human Nutrition 32: 3- 10.

Odunfa, S. A. 1981. Micro-organisms associated with fermentation of African locust bean (*Parkia filicoidea*) during *iru* preparation. J. Plant Foods 3: 245-250.

Odunfa, S. A. and Adesomoju, A. A. 1983. Effects of fermentation on the free fatty acids of African locust bean during *iru* preparation. J. Plant foods.

Odunfa, S. A. and Oyewole, B. O. 1986. Identification of *Bacillus* Species from *iru*, a fermented African locust bean product. J. Basic Microbiol. 26, 101-108.

Odusote, K. O. 1980. Microbiology of melon fermentation for "ogiri" production. Report in Department of Botany, University of Ibadan, Nigeria. Pp. 28.

Ogbadu, L. J. and Okagbue, R. N. 1988. Fermentation of African locust bean (*Parkia biglobosa*) seeds: involvement of different species of *Bacillus*. Food Microbiol. 5, 195-199.

Ogunbunmi, E. M. and Bassir, O. 1980. Protein and amino acid contents of some Nigerian food Condiments. Nutr. Rep. Int. 22, 497.

Oke, O. L. 1969. Oxalic acids in plants used in nutrition. World Rev. Nutr. Diet. 11. 170. Oke, O. L. 1972. A nutrition policy of Nigeria, World Rev. Nutr. Diet. 14, 1.

Oxoid, 1979. The Oxiod Manual, 4th edn. Oxiod Ltd. Hampshire, U. K., 310 pp.

Paredes-Lopez, O and Harry, G. I. 1988. Food biotechnology review: traditional solid-state fermentation of plant raw materials. Critical-reviews-in food-science-and-nutrition. (USA). V. 27 (3) p-159-187.

Parry, J. M. Turnball, P. C. B. and Gibson J. R. 1983. A colour Atlas of Bacillus Species. Ipswich: Wolfe Medical Pub. Ltd.

Pederson, C. 1971. Microbiology of Fermented Foods. Westport: AVI.

Potter N. N. 1968. Food Science: The Avi Publishing Company, Inc. West Port, Connecticut.

Priest, F and Austin, B. 1993. Modern Bacteria Taxonomy, second edition. London: Chapman Hill.

Rose, A. H. 1982. Fermented Foods. Economic Microbiology Volume 7. Great Britain Alden Press, Oxford P. 30, 45-80.

Sakar, P. K., Tamang, J. P., Cook, P. E. and Owens, J. D. 1994. Kinema – a traditional soybean fermented food: proximate composition and microflora. Food Microbiol. 11, 47-55.

Sakar, P. K. and Tamang, J. P. 1994. The influence of process variables and inoculum composition on the sensory quality of kinema. Food microbiology 111, 317 - 325

Schermers, F. H., Duffus, J. H. and Macleod, A. M. 1976. The Role of Microorganisms in Flavour Formation. J. Inst. Brewing, 82, 170.

Schery, R. W. 1972. Other food seeds and forages. In: Plants for Man. 2nd edition. (edn) Prentice Hall, Englewood Cliffs, NJ. p. 461.

Shallenberger, R. S., Hand D. B. and Steinkraus, K. H. 1967. Changes in sucrose, raffinose and stachyose during tempeh fermentation. In report of 8th Dry Bean Research Conference, ARS, USDA, ARS -74-41, Bellaire Michigan. p. 68-71.

Simmons, E. B. 1976. Calorie and protein intake in three villages of Zaria Province. Samaru Miscellaneous Paper. No. 55. Pp. 14. Zaria, Nigeria: Institute of Agriculture Research.

Singh, S. R. Rachie, K. O. and Danhiell, K. E. 1987. Soybeans for the Tropics-Research, Production and Utilization. John Wiley and Sons Ltd., Great Britain.

Singh, S. R. and Rachie, K. O. 1985. Cowpea Research Production and Utilization John Wiley and Sons Ltd., Great Britain. Pp. 353-9.

Smith, A. K. 1971. Oriental Methods of using Soybeans as food. Peoria US Dept. of Agriculture.

Srivastav, P P., Das, H. and Sitaram-Prasad. 1990. Effect of roasting process variables on in-vitro protein digestibility (PD) of bengal gram, maize and soybean. J. of Food Chemistry, 35:1, 31-37.

Steinkraus, K. H. 1991. African alkaline fermented foods and their relation to similar foods in other parts of the world. In proceedings of a Regional workshop on Traditional African Foods – Quality and Nutrition. International Foundation for Science ed. Wesby, A. and Reilly. Pp. 87-92.

Steinkraus, K. H., Cullem, R. E., Pederson, C. S., Nellis, L. F and Gavitt, B. K. 1983. Indonesian tempeh and related "Fermentations": Handbook of indigenous Fermented Foods. Microbiology series vol. 9 Marcell Dekker, Inc. New York, N. Y. 10016.

Steinkraus, K. H. and Van Veen, A. G. 1971. Biochemical, nutritional and Organoleptic changes occuring during production of traditional fermented foods. UNESCO Symposium Proc. 3rd Int. Conf. Gran. 444-450. University of Bombay, India.

Steinkraus, K. H., Van Busen, J. P., Heckler, L. R. and Hand, D. B. 1965. A Pilot Plant Process for dehydration of tempeh. Food Technol 19:63.

Steinkraus, K. H., Hand, D. B., Van Buren, J. P and Hackler, I. R. 1961. Pilot Plant Studies on tempeh. In Proceedings of Conference on soybean products for protein in Human Foods, USDA. Pp. 75-84.

Steinkraus, K. H., Yap, B. H., Van Buren, J. P., Providenti, M. I. and Hand, D. B. 1960. Studies on tempeh – An Indonesian fermented soybean food. Res. 25:777-778.

Sumi, H. 1990. Biochemical changes during natto fermentation. J. Brew. Sci. Japan, 85, 518-524.

Suzukir, T. and Ohta, T. 1979. Studies on the biological properties of commercial natto starters. Report of the National Food Research Institute, 36, 266-272.

Tamang, J. P. 1993. Studies on the microflora of some traditional fermented foods of Darjeelling hills and Sikkim. PhD. Thesis. Darjeeling. North Bengal University, India.

Teranishi, R. and Kint, S. 1993. Sample preparation. Chpt. 5 in Acree and Teranishi (5th ed), pp. 137 – 167.

Ueda, S. 1989. *Bacillus subtilis*. Molecular Biology and Industrial Application. Maruo and Yoshikawa H. (3rd ed). Elsevier and Kodansha Ltd. Japan pp. 143-161.

Umoh, I. B. and Oke, O. L. 1974. Nutritive value of some lesser known oil seeds in rats. Nutr. Rep. Int. 9, 453.

Vandore, J. F. 1967. Fermentation of flavour compounds in foodstuffs. Msc. Thesis, Department of Agriculture. University of Edinburg, UK.

Wagenknecht, A. C., Mattick, I. R., Lewin, I. M., Hand and Steinkraus, K. H. 1961.Changes in soybean lipids during tempeh fermentation. J. Food Science. 26, 373-376.

Wang, H.L., Ruttle, D. I. and Hesseltine, C. W. 1968. Protein Quality of Wheat and soybeans after *Rhizopus oligosporus* fermentation. Journal of Nutrition. 96:109-114.

Watson, J. D. 1971. Investigation on the nutritive value of some Ghanaian Foodstuffs, Ghana J. Agricultural Science. 4, 95.

Weigartner, J. (1987) Essential amino acids of soybean protein. Journal of Food and Agriculture Organisation. 26. No. 1.

Williams, A. A., Lewis, K. J. and May, H. V. 1983.
J. Science Food Agriculture 34, 311.

Williams, D. H., Senior Reporter. Mass Spectrometry. Volume 1, The Chemical Society, London, 1971.

Yakotsuka, T. 1972. Industrial Production of Enzymes; Proceedings of the IVth I. F S, Fermentation Technology Today. p. 659.

Yamamoto, Y., Yangida, F. and Suminoe, K. 1972. Patterns of Bacterial Growth; Yippon Shokuhin Kogyo Gakkai-Shi, 19, 29. (Chem. Abstr. 1974. 81, 150, 264).

Young , F M. and Wood, B. J. B. 1977 Biochemical changes in experimental soy sauce *Kofi*. Journal of Food Technology. 12: 163 - 175

Zemek, J., Augustin, J., Borriss, R., Kuniak, L., Svabova, M. and Pacova, Z. 1981. Polysaccharides-hydrolysing enzymes in the genus *Bacillus*. Folia microbiologica 26, 403.

APPENDIX 1

UNIVERSITY OF GHANA

DEPARTMENT OF NUTRITION AND FOOD SCIENCE

QUESTIONNAIRE ON QUALITY CHARACTERISTICS OF DAWADAWA

1. Name: .....
2. Location ..... 3. Ethnicity .....
4. Familiarity with product (Years) .....
5. Source of product (Tick) (a) Own production (b) Purchased
6. If own production indicated what raw material is used (Tick)
   (a) Soybean (b) Locust bean (c) Other .....
7. Briefly describe the processing steps for this product.

8. Do you use this product? (a) Yes (b) No

9. If yes, how often do you use it? .....
10. How do you use the product? .....
11. For each usage, how much of the product do you use? .....
12. Describe what a good quality product should be like.

13. How and where do you keep the product?

14. How long does a product remain wholesome in storage?

Comments:

APPENDIX 2

Chemicals/Reagents used:

Hydrochloric acid, 37% (HCL) Merck, (art no. 1, 00317)

Norleutine, DL, sigma, (art no N-6752)

Dithiothreitol (DTT), sigma, (art no N-6752)

Dry ice

Isopropanol

Vacuum grease.

Hydroxyproline, L, Sigma, (art no. H-6002)

Taurine, Sigma, art no. T-0625

Amino Acid Standard H, Pierce, art no. 20088

Sodium acetate, water free ($Nac_2H_30_2$) Merck, art no 6268

Methanol (CH_30H) Fisons, art no. M/4056/17

Triethlamine, TEA, Aldrich, art no. 23, 962-3

Phenylisothiocyanate (PITC), Pierce 26922

EDTA, calcium disodium salt ($C_{10}H_{12}N_2O_8$ $CaNa_2$) Sigma, art no. ED2 SC.

Sodium acetate tri hydrate ($NaC_2H_3O_2$* $3H_2O$) Merck, art no. 6267

Acetic acid (CH_3COOH) RdH, art. no. 33209

Acetonitate (CH_3CN), Fisons art. no. A/0626/17

Double distilled water

APPENDIX 3 PROTEINASE ACTIVITY

Analysis of variance Table

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Replication	1	0.101	0.101	1.2108	0.299
Factor A	4	34.877	8.719	104.7176	0.0000
(Time)					
Factor B	1	0.722	0.722	8.6712	0.016
(soy)					
AB	4	0.953	0.238	2.8614	0.087
Error	9	0.749	0.083		
Total	19	370402			

APPENDIX 4 Alpha Amylase Activity

Analysis of variance Table

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Replication	1	0.002	0.002	0.2526	
Factor A (Time)	4	1.593	0.398	60.0735	0.0000
Factor B (soy)	1	0.010	0.010	1.4800	0.2547
AB	4	0.005	0.001	0.1768	
Error	9	0.060	0.007		
Total	19	1.669			