MICROBIOLOGY, CHEMISTRY AND QUALITY OF FERMENTED FISH (*Pseudotolithus* sp.) LANHOUIN, AND ITS IMPROVEMENT AS A FOOD CONDIMENT

This thesis is submitted to the University of Ghana, Legon, in partial fulfilment of the requirement for the award of Ph.D degree in Food Science

By

VICTOR BIENVENU ANIHOUVI

Department of Nutrition and Food Science

University of Ghana, Legon

July, 2006

DECLARATION

This dissertation is the result of research work undertaken by Victor Bienvenu ANIHOUVI in the Department of Nutrition and Food Science, Faculty of Science, University of Ghana, Legon, under the supervision of

Professor George S. AYERNOR

Professor Joseph D. HOUNHOUIGAN

Dr Esther Sakyi-Dawson

Sign …….
Victor Bienvenu Anihouvi
(Student)

Sign ……

Prof. George Ayernor
(Principal supervisor)

Sign ……

Prof. Joseph Hounhouigan
(Co-supervisor)

Sign ……
Dr Esther Sakyi-Dawson
(Co-supervisor)

ABSTRACT

Lanhouin, a traditionally fermented fish product, widely used as condiment in the Gulf of Benin, is processed by spontaneous fermentation. The socio-economic importance of *lanhouin* and the processing methods used were investigated through a survey on *lanhouin* processors and retailers; the quality characteristics of market *lanhouin* as well as the physico-chemical and microbiological changes during the spontaneous fermentation of *lanhouin* processed in three different occasions were also investigated. The predominant microorganisms were identified and a controlled fermentation using the predominant isolates as starter cultures was carried out.

The survey showed that the production and marketing of *lanhouin* is mainly carried out by women, constituting, for most of them, the main source of income. On the processing procedure, two variants in the method were identified, but both lead apparently to the same final product. Though many types of fish can be used for *lanhouin* processing, processors preferred cassava fish (*Pseudotolithus* sp.) followed by lasser African threadfin (*Galeoides decadactylus*) and king fish (*Scomberomorus tritor*).

Results from the optimization studies indicated that the optimal fermentation conditions were ripening time of 8 hrs, salt concentration of 27.50-30.00 % and fermentation time of 4-5 days. These conditions gave the best quality characteristics of *lanhouin* in terms of total viable count, sodium chloride and histamine contents.

In the course of processing fish into *lanhouin*, the pH increased from 6.83 to 7.30 and then decreased to 6.98 after 8 days of fermentation. Total volatile nitrogen, free fatty acid and sodium chloride contents of samples increased from 70.50 to 260.15 mg N/ 100 g, 3.60 to 8.15% and 1.50 to 12.05 % after 8 days of fermentation respectively, whilst the moisture content decreased from 73.00 to 46.88 %. Histamine contents (9.05-14.1 mg/100 g) in these samples were lower than the recommended level of 20 mg / 100 g, whilst the majority (75 %) of the market samples contained histamine contents (21.4-39.7 mg/100 g) higher than the maximum allowable level.

In general, after a slight increase during the first day of fermentation, the microbial population of the fermenting fish decreased with fermentation time and reached levels of 10^4 cfu / g after 8 days of fermentation. *Bacillus* spp. and *Staphylococcus* spp. were the predominant genera of

the *lanhouin* microbiota and accounted for 48.70 and 27.30% of the total of isolates respectively. *Bacillus subtilus*, *Bacillus licheniformis*, *Staphylococcus lentus* and *Staphylococcus xylosus* were the dominant species of each genus.

The ability of these predominant organisms to ferment fish for controlled fermentation of *lanhouin* was investigated and the results showed that *lanhouin* fermentation could be carried out using a single starter culture of *Bacillus subtilis* and a mixed starter culture of *Bacillus subtilis* and *Bacillus licheniformis* as well as a mixed starter culture of *Bacillus subtilis* and *Staphylococcus xylosus.*

There was a marked difference in the aroma profiles of the spontaneously fermented fish and the fish which was fermented using a starter culture. A total of ninety four aroma compounds were detected in the fish spontaneously fermented for 2, 4, 6 and 8 days. Of these 82 were positively identified with a quality index higher than 70 % and comprised 15 aliphatic hydrocarbons, 6 aromatic hydrocarbons, 9 esters, 9 ketones, 7 acids, 5 alcohols, 9 amines, 2 amides, 7 aldehydes, 2 pyrrole, 6 thiazoles, 2 furan and 3 phenols. The predominant aroma compounds detected over the fermentation period were nitrogen-containing compounds and lipid-derived components. A total of forty one aroma compounds were detected in the inoculated samples of *lanhouin*. Pyrrole, thiazoles, furan and phenols were not identified in the inoculated samples of *lanhouin* in contrast to the spontaneous fermentation.

DEDICATION

To my wife and children for the extreme sacrifices they endured during the gestation period of this work, to my sisters and brothers and to my late father and mother.

ACKNOWLEDGEMENTS

I express my profound gratitude to Prof. G.S. Ayernor, Department of Nutrition and Food Science, University of Ghana, Prof. J.D. Hounhouigan, Department of Nutrition and Food Science, University of Abomey-Calavi-Benin and Dr Esther Sakyi-Dawson, Department of Nutrition and Food Science, University of Ghana, for their particular interest in the work and for their advice and guidance towards the realisation of the goals of this study.

Acknowledgements are extended to Prof. M.C. Nago, Dean, Faculty of Agriculture (FSA), University of Abomey-Calavi, Republic of Benin, for his interest in the research work and for granting permission to carry out this programme. I am grateful to Prof. S. K. A. Danso, Director of Ecological Laboratory, Department of Geography and Human Resource, University of Ghana, for his assistance and provision of laboratory facilities. The valuable advice given by Dr F. K. Saalia, Department of Nutrition and Food Science, University of Ghana and Dr W.K.A. Amoa-Awua, Food Research Institute, are gratefully acknowledged.

I thank Messers Sarquah Mercer and Mustapha Elegba, all members of Ecological laboratory, Department of Geography and Human Resource, University of Ghana, and Messers Stephen Kwame Nyamekye and Richard Kumah, Department of Nutrition and Food Science, for their technical assistance. My gratitude also goes to Mr Ireneous Soyiri who spent his time reading some parts of this thesis. I would also like to thank my Beninese colleagues: Dr Moutaïrou Egounlety, Dr Joseph Dossou, Dr Romain Dossa, Dr Noël Akissoé, Dr Paulin Azokpota, Mr Sathurnin Houndji, Mrs Générose Dalodé, Mr Polycarpe Kayodé, Mr Issa Amadou, Mrs Thérèse Gnonlonfoun, Mr Christophe Soudo and Benoît Anago for their interest in my work.
I also thank the group of *lanhouin* processors with whom I worked, in particular Mrs Miassi Florentine who spent her time processing the laboratory samples of *lanhouin*.
Financial support for this research work was received from Benin Government through the "training of trainers Programme" of the University of Abomey-Calavi. I am very grateful to the Government of the Republic of Benin for funding this work. My sincere gratitude also goes to DANIDA/ENRECA Project, for providing financial support for API kits.

The patience and the encouragement of my wife, Mrs ANIHOUVI SIVOAKOU Agathe and my children, Eudes, Candice and Joanita is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

1.0 INTRODUCTION

1.1 The fishing sector in Benin

Fisheries in the Republic of Benin, comprise of the artisanal and industrial sub-sectors, and contribute to about 4 % of the Gross Domestic product (GDP) (Anon., 2005a). In 2004, total fish landings were estimated at about 41, 877 tonnes. Of this, the industrial landings were estimated at about 10 tonnes, which were mainly exported as frozen products (Anon., 2005a). Exports to the neighbouring countries are not recorded but represent significant amounts. In Benin the fisheries sector employs 600,000 Beninese and accounts for about 60 % of all animal proteins consumed. The artisanal fisheries sub-sector provides about 94% of the domestic fish supply and accounts for 31% of national intake of animal protein (Anon., 2005b). In Benin, artisanal fishery catches are sold fresh and mainly processed according to the storage conditions and taste of consumers. As a matter of fact, Benin is a tropical country and there are limited cold storage facilities at the landing sites and in the markets. Consequently, a large part of the artisanal catch that cannot be marketed immediately is dried, fermented or smoked for preservation.

Fish consumption in Benin was estimated at 10.8 kg per capita in 1995 (Horemans, 1998). This level of consumption is less than the world average of 13.2 kg in 1992 (Horemans, 1998; Lem, 2005). In 2002, world average per capita consumption of fish was estimated to be about 16.2 kg, 21 percent higher than in 1992 (FAO, 2004; Lem, 2005). This growth is largely attributable to China, whose estimated share of world fish production increased from 16 % in 1992 to 33 % in 2002. If China is excluded, fish supply per capita would be 13.2 kg, almost the same as in 1992 (FAO, 2004). The world fish production was estimated to be about 133 million metric tonnes in 2003 with 6 % percent from African continent (Lem, 2005).

1.2 Utilization of fish resources in developing countries

Fish and fish products play a very significant role in the diet of the world's population (FAO, 2000; Gram, 2003). It is estimated that between 15-20 percent of all animal proteins come from aquatic animals and about 75 % of world fish production goes into human consumption, either fresh or in some processed condition (FAO, 2004). For low-income populations in developing countries, fish is often the only source of protein to which they have access.

Fish represents a valuable source of micronutrients, minerals, essential fatty acids and proteins in the diet of many countries. It is estimated that fish contributes up to 180

kilocalories per capita per day, but reaches such high levels only in a few countries where there is a lack of alternative protein foods, and where a preference for fish has been developed and maintained, for example in some small island developing states. More commonly, fish provide about 20 to 30 kilocalories per capita per day (FAO, 2000).

Although people have developed the taste for traditional fish products, fresh fish is generally more valuable and preferred by consumers, and usually brings better returns to the fishermen (Sefa-Dedeh *et al.*, 1995). Notwithstanding, access to fresh fish can be a problem in rural areas due to the lack of refrigeration and shortage of ice (Nautilius, 1997). Therefore, most consumers in these areas get access mainly to cured fish. In contrast, people in the urban areas tend to consume more fresh fish and less cured fish; the cured fish is generally used only in small quantities as flavouring. In 2002 the proportion of cured fish was higher in Africa (16%) and Asia (11%) compared with other continents (FAO, 2004). Thus, the improvement of the quality of fish through technological advances is important to reduce post harvest losses so as to utilize the countrys' resources to their full potential. In Africa, post harvest losses are estimated at 20 to 30 percent, and sometimes as much as 50 percent (Horemans, 1998). Since malnutrition in developing countries has always been an important problem, it is quite imperative that much of the fish produced be retained to supplement the general protein intake and alleviate some of the challenges of malnutrition. In addition, it will be of more importance if value is added to the harvested fish to make it more available.

1.3 Handling, processing and storage of fresh fish in developing countries

A key factor limiting fish utilization is its extreme perishability due especially to bacterial and autolytic spoilage which usually occurs at the same time, after the death, during processing and sometimes during storage, and both contribute to normal spoilage processes. As reported by Adams *et al.*, (1987), fish flesh offers to microorganisms conditions of good nutrient availability coupled with a moderate pH and high water activity. In tropical regions, these conditions coupled with a high ambient temperature and unsanitary conditions cause fish spoilage within 12 hours (FAO, 1981; Adam et *al.*, 1987). The action of bacterial and autolytic spoilage can result in the formation and accumulation of undesirable and poisonous substances (Ababouch, 1990; Halasz et *al.***,** 1994; Eerola et *al.*, 1996; Silla-Santos, 1996).

Various authors have reported that fresh fish is not well handled and stored in developing countries. The handling and processing techniques need to be improved upon to enhance the

quality of cured products, increase the availability and nutritional values of the fish to consumers as well as profitability for the producers (Sefa-Dedeh *et al.*, 1995; Njai, 2000). The current methods of fish handling and processing are generally inadequate and result in major fish losses. Often, catches are landed ashore without ice, under climatic conditions often involving temperatures of up to 32°C and relative humidity of 90 per cent. Under these conditions the fish deteriorate very rapidly, especially since they are rarely provided with any protection against sun. Onshore fish handling in the artisanal sector is often poor; fish are usually scooped out of the boats with all sorts of containers, such as buckets. This method of unloading the catch by hand takes considerable time during which the temperature of the fish also increases considerably. For those reasons, spoilage rates are extremely high in tropical regions compared to those in temperate climates and according to observations, species such as sole, can become completely spoilt 10 to 12 hours after catching, and catfish in 5 to 7 hours (Watanabe, 1982; FAO, 1989). In addition, the environment in which fishes are processed is generally unhygienic paving the way for microbial contamination and production of food toxicants such as histamine (Sefa-Dedeh, 1995; Ababouch, 1990). This shows that there are basic educational problems, such as lack of awareness of the importance of hygiene and the importance of using ice.

1.4 Traditional methods of fish preservation

In tropical regions traditional processes such as drying, salting, smoking and fermentation are used for fresh fish preservation. The preservation involves one or a combination of these methods in order to achieve the desired products. Sun drying of fish is one of the simplest and cheapest methods of curing fish. The quality of the product depends on climatic conditions since the product is at the mercy of the weather. Some big fishes are always salted before sun drying. Among the salted fishes, some are allowed to ferment for a period before drying (FAO, 1971). Salted, fermented and sun dried fish is generally known as “fermented fish” (Beddows, 1985). It is mainly used for flavouring stews and soups although some people especially in Ghana, use it as the main source of protein in their diet, grilled and eaten with *kenkey* (Beddows, 1985; Abbey *et al.*, 1994).

Traditional fermented fish products are popular throughout the world and particularly in South East Asia (Lee, 1990; Gram, 2003) and the West African countries of Senegal, Ghana, Togo and Bénin (Essuman, 1992). Campbell–Platt (1987) defines fermented foods as foods,

which have been subjected to the action of microorganisms so that desirable biochemical changes cause significant modification of the food. In tropical regions the ambient conditions (high temperature, high humidity) provide ideal conditions for fermentation. Left alone, most foodstuffs will ferment naturally, some with desirable end results and others with less desirable and even poisonous end-products (Rolle, 1997). However with knowledge of the fermentation process, conditions for fermentation can be modified to encourage the growth of beneficial microorganisms.

The use of fermentation as a low-cost method of fish preservation is commonly practised all over the world. It is a highly appropriate technique for use in developing countries and in remote areas where access to sophisticated equipment is limited. The traditional fermentation process is a relatively efficient, low energy preservation process, which increases the shelf life and decreases the need for refrigeration or other forms of food preservation technology. The process demands little energy nor sophisticated equipment and requieres no electric power. Fermentation reduces cooking times, which in turn reduces the energy consumption required for preparation of foods (Odunfa, 1985a). In general, fermentation improves the keeping quality and tends to inhibit microbial growth including that of pathogens. For example plant-based fermented foods may be protected against spoilage and pathogenic microorganisms by a combination of factors generated by lactic acid bacteria including production of organic acids, hydrogen peroxide, antibiotic-like substances and lowering of oxidation-reduction potential (Cooke *et al*., 1987; Nout *et al*., 1989; Hounhouigan, 1994). In some cases the microorganisms are destroyed especially if salt is added or the moisture content lowered (Mensah *et al*., 1991).

Most of the traditional fermented products plants are rural and informal and the fermentation processes are handed down from generation to generation. Activities are carried out by mostly illiterate women as the major executors (Sefa-Dedeh, 1993). The methods of processing were developed in homes and improvements were based on the observations of practitioners. There is little interest in knowing the role of microorganisms and the physical and chemical changes that occur in the products. What is recognized are changes in colour, odour and taste that result from modifications of the process or variation in ingredients.

Disadvantages of the indigenous fermentations technologies are listed to include high labour input, low efficiency, the time consuming nature of the process, lack of hygienic practice and

quality assurance (Lartey, 1975). Home-made products have sometimes been found to be the source of infection: for example, home-made sausages in parts of Europe have been found to transmit trichinellosis (Stratton *et al*., 1991); traditional fermented fish products in parts of the world have been found to transmit botulism and histamine poisoning (FAO, 1975; Essuman, 1992; Silla-Santos, 1996).

Different types of traditional fermented fish products have been identified by various workers (FAO, 1971; Lee, 1990, Essuman, 1992). Their nature depends largely on the extent of fermentation, which has been allowed to take place. Fermented fish products can be categorized as:

- Fish which retains its original texture
- Pastes
- Liquids/sauce

In the case of the first category, Essuman (1992) has identified three techniques in West African countries:

- Fermentation with salting and drying
- Fermentation and drying without salting
- Fermentation with salting and without drying

Traditional fermentation of food in West African countries usually involves the use of mixed cultures in solid substrate fermentation because the traditional fermentations are mostly carried out spontaneously and natural microbial populations usually occur as mixed cultures (Hounhouigan, 1994; Amoa-Awua, 1996). Solid substrate fermentation refers to any fermentation that occurs on a solid or semi-solid substrate (Aidoo *et al*., 1982). It has been used in the production and preservation of a variety of indigenous fermented foods. For example maize is fermented in Bénin and Ghana by lactic acid bacteria and yeasts (Halm *et al*., 1993; Jespersen *et al*., 1994; Hounhouigan, 1994).

1.5 Importance of fermented fish products in Benin

Fermented fish plays an important role in the diets of most people in Asia, Africa and other parts of the World (Beddows, 1985; Yean, 1998). They are mostly used as preferred condiments. In certain under-privileged areas, fermented fish is mostly used as a low-cost protein substitute. Salted and fermented fish called *lanhouin*, is used extensively in the diets of Beninese. Areas of production in Bénin include mainly Atlantic and Mono Districts in the south of the country. In these areas the production of *lanhouin* is a major activity in the

coastal landing centers after the production of smoked fish. It is the main activity of the women of the two ethnics groups called *Xla* and *Mina* inhabitants of these areas. About 3000 metric tonnes of *lanhouin* are produced each year (Anihouvi *et al.*, 2005). In Bénin, *lanhouin* serves different markets: domestic, urban and rural markets, and sub-regional markets that contribute to an expanding market. Exports to neighboring countries (Togo and Ghana) are not recorded but represent significant amounts.

1.6 Statement of the problem

Traditionally in Benin, many commodities (maize, sorghum, millet and fish) are processed by the cottage industries into fermented foods, which constitute the most important part of the staple foods, beverage, weaning foods and condiments consumed in the country. Recent studies carried out on cereal-based products showed the diversity of the micro-organisms involved in the fermentations (Hounhouigan, 1994). Many other traditional fermented foods also need investigation. Included is *lanhouin*, a salted and fermented fish highly consumed in Benin as condiment. *Lanhouin* is produced by spontaneous and largely uncontrolled fermentation under very unhygienic conditions which may lead to microbial contamination and production of food toxicants such as histamine in the product. Recent work carried out by Abbey *et al.*, (1994) on *momone*, a *lanhouin*-like product, established that this fermented fish contain very high levels of histamine (105–172 mg /100g). These levels exceed the hazardous limit of 20 mg / 100g stipulated by the Australian National Food Authority, the European Economic Community and the Food and Drug Administration (USA). Despite a lack of official information in Bénin on food poisoning linked to *lanhouin* consumption, there is a potential for more than just sporadic amine poisoning. There is therefore a need to investigate *lanhouin* in order to identify the micro-flora responsible for the fermentation and the biochemical changes, brought about in the product. Possibilities for improvement of the processing or the product, which appear feasible, will be formulated and promoted. Essuman (1992) has identified a number of problems encountered during the traditional processing of fermented fish. These include lack of proper hygienic practices, rudimentary packaging and contamination of product and non-identification of micro-organisms involved in the fermentation. The need for future work using pure cultures of isolated organisms to shorten the period of fermentation was suggested by Yankah (1988).

1.7 Aim/overall objective

This work was carried out to define the product and process characteristics of *lanhouin* and to develop the traditional product into a modern food condiment which can be adopted by the formal food industries and produced as a commercial flavouring agent.

1.7.1 Specific objectives

The specific objectives of this work are:

i. To investigate the traditional method of *lanhouin* processing and its socio-economic importance in Bénin.

ii. To characterise the chemical and microbiological quality of traditional *lanhouin* sold in Benin.

iii. To evaluate the biochemical and microbiological changes which occur during the processing and spontaneous fermentation of *lanhouin*.

iv. To isolate and identify the predominant microorganisms involved in the spontaneous fermentation of *lanhouin*.

v. To improve the quality of *lanhouin* through controlled fermentation using a starter culture.

vi. Make recommendations for upgraded production of *lanhouin* as a food condiment.

2.0 LITERATURE REVIEW

Fermentation is one of the oldest and most economical techniques of producing and preserving foods in the world, particularly in developing countries (Odunfa, 1985a; Hall, 1997). It is a dynamic process during which several reactions proceed simultaneously depending on many conditions including microflora, substrate and environmental factors. Fermented products are in general, culturally accepted and in many cases improvement in nutritional quality and digestibility of food, highly appreciated tastes, flavour and/or textures are obtained (Olympia, 1992; Rolle, 1997). The contribution of fermented foods to the human diet is important in developing countries where many fermented products, especially cereal-based, root or tuber-based and fish-based foods are an integral part of the daily food intake. The fermentation process has been exploited in fish catches and historically, has been associated with salt treatment. The use of salt for the preservation of fish has continued to this day (Gram, 2003).

2.1 Composition of the edible part of fish

The edible part of fish mainly consists of muscle tissue. The chemical composition of fish muscle tissue varies between and within species, and depends on environment, season, age and sex (Ito and Watanabe, 1968; Huss, 1988; Love, 1997) and could also be favorably compared to mammal muscle tissue. The principal components are protein, lipid, carbohydrate, ash and water. Examples of the variation between the constituents in fish are shown in Table 2.1. The composition of beef muscle has been included for comparison.

As can be seen from Table 2.1, a substantial variation is observed for the constituents of fish muscle. The variation in the chemical composition of fish is closely related to feed intake, migratory behaviour, seasonal variation and sexual changes in connection with spawning (Watanabe, 1971; Poulter, 1982).

Table 2.1 Main components (%) of fish and beef muscle

Component	Content in fish muscle (%)			Content in meat muscle (%)
	min	normal	max	
Protein	6	16-21	28	20
Lipid	0.1	0.2-25	67	3
Carbohydrate		< 0.5		1
Ash	0.4	1.2–1.5	1.5	1
Water	28	66-81	96	75

Source: Gram (2003)

These factors are observed in wild, free-living fishes in the open sea and inland waters. Fish raised in aquaculture may also show variation in chemical composition, but in this case, several factors are controlled, thus the chemical composition may be predicted. To a certain extent, the fish farmer is able to control the composition of the fish by selecting the farming conditions. According to Wheeler *et al*. (2003) factors such as feed composition, environment, fish size, and genetic traits all have an impact on the composition and quality of the aquacultured fish. However, the factor having the most pronounced impact on the chemical composition is considered to be the feed composition (Watanabe, 1971). The lipid fraction is the component showing the greatest variation. Although the protein fraction is rather constant in most species, variations have been observed such as protein reduction occuring in salmon during long spawning migrations and in Baltic cod during the spawning season (Wheeler *et al*., 2003).The variations in lipid, protein and water contents in various fish species are shown in Table 2.2.

Table 2.2 Chemical composition of the fillets of various fish species

Species	Scientific name	Water %	lipid %	protein %	energy kJ/ 100 g
Blue whiting[a]	*Micromesistius poutassou*	79-80	1.9-3.0	13.8-15.9	314-388
Cod[a]	*Gadus morhua*	78-83	0.1-0.9	15.0-19.0	295-332
Herring[a]	*Clupea harengus*	60-80	0.4-22.0	6.0-19.0	
Plaice[a]	*Pleuronectes platessa*	81	1.1-3.6	15.7-17.8	332-452
Salmon[a]	*Salmo salar*	67-77	0.3- 14.0	21.5	
Trout[a]	*Salmo trutta*	70-79	1.2-10.8	18.8-19.1	
Tuna[a]	*Thunnus spp.*	71	4.1	25.2	581
Carp[b]	*Cyprinus carpio*	81.6	2.1	16.0	
Sabalo[c]	*Prochilodus platensis*	67.0	4.3	23.4	
Pacu[c]	*Colossoma macropomum*	67.1	18.0	14.1	
Tambaqui[c]	*Colossoma brachypomum*	69.3	15.6	15.8	
Corvina[c]	*Plagioscion squamosissimus*	67.9	5.9	21.7	

Sources: (a;b) Murray and Burt (1969); (c) Huss (1988)

2.1.1 Lipids content of fish muscle tissue

Fish muscle tissue is defined as lean, moderately fatty or fatty. Lean fish muscle tissue contains less than 1% fat, moderately fatty fish meat between 1 – 5% while the fat content of fatty fishes is usually greater than 5% (Love, 1997, Wheeler *et al*., 2003). The lipid content of fillets from lean fish is low and stable whereas the lipid content of fillets from fatty species varies considerably. However, the variation in the percentage of fat is reflected in the percentage of water, since fat and water normally constitute around 80 % of the fillet. This means that these two components show fluctuation in opposite directions. When fishes are accumulating fats in their muscle tissue due to heavy feeding, the moisture content of the tissues is gradually lowered. *Vice-versa* the tissues again absorb moisture when the fatty

substances of the tissue are gradually broken down during metabolism (Ito and Watanabe, 1968; Huss, 1988; Love, 1997; Wheeler *et al*., 2003).

The fat content of fish depends on species, season, maturity, feeding status, age and the type of food (Watanabe, 1971; Huss, 1988). For example, Watanabe (1971) examined fresh water fish from Zambia and found a variation from 0.1 to 5 % in fat content of four species including both pelagic and demersal ones. The majority of fat in fish is composed of triglycerides; beside the triglycerides there are also fatty substance of different characters, the phospholipids and the sterols (Huss, 1988; Love, 1997; Wheeler *et al*., 2003). Fish lipids are mainly composed of fatty acids of a highly unsaturated nature; for example in cod liver oil, 32 % of the fatty acids are highly unsaturated (Eskin *et al*., 1971; Ramanathan and Das, 1992). Whether a fish is lean or fatty the actual fat content has consequences for the technological characteristics postmortem (Ramanathan and Das, 1992; Morrissey, 1997; Wheeler *et al*., 2003). The changes taking place in fresh lean fish may be predicted from knowledge of biochemical reactions in the protein fraction, whereas in fatty species changes in the lipid fractions have to be included. The implication may be that the storage time is reduced due to lipid oxidation, or special precautions have to be taken to avoid this (Ramanathan and Das, 1992; Love, 1997; Wheeler *et al*., 2003). The unsaturated character of the fish fats makes them very vulnerable to the action of oxygen; fats are easily oxidized to hydroperoxide and these are broken down further to a great number of chemical compounds, some of which are aldehydes that smell badly (Huss, 1975; Huss, 1988; Ramanathan and Das, 1992). Decomposition of fats into fatty acids and glycerol is the first symptom of spoilage of fatty substances and the percentage of free fatty acids (FFA) of the fat is an indication of the freshness of fatty fish (Pearson, 1976; Huss, 1988; Tungkawachara *et al*., 2003). Similar to amino acids, some fatty acids are essential elements in human nutrition; nowadays it is known that an important essential nutrient is the fatty acid linolic, acid which occurs, in a minor concentration in fish fats (Huss, 1988; Huss, 1995).

2.1.2 Protein content

Based on the dry matter content of fish tissue, the proteins are the most important constituents, both qualitatively and quantitatively (Huss, 1988; Love, 1997). In the qualitative sense, fish proteins contain all the essential amino acids like milk, eggs and mammalian meat proteins, and have a very high biological value (Huss, 1988; Love, 1997) (Table 2.3).

Table 2.3 Essential amino-acids (percentage) in various proteins

Amino-acids	Fish	Milk	Beef	Eggs
Lysine	8.8	8.1	9.3	6.8
Tryptophan	1.0	1.6	1.1	1.9
Histidine	2.0	2.6	3.8	2.2
Phenylalanine	3.9	5.3	4.5	5.4
Leucine	8.4	10.2	8.2	8.4
Isoleucine	6.0	7.2	5.2	7.1
Threonine	4.6	4.4	4.2	5.5
Methionine-cystine	4.0	4.3	2.9	3.3
Valine	6.0	7.6	5.0	8.1

Source: Huss (1988)

The proteins of fish muscle tissue can be subdivided based on their solubility in solvents such as brine and water (Huss, 1988; Love, 1997; Yongsawatdigul *et al.*, 2000).

- A water-soluble fraction adds up around 20 % of the proteins. This group is called "myogene" and consists mainly of globular proteins having enzymatic activity. After the death of the organism these proteins are responsible for the uncontrolled progress of reactions that pave the way for the intruding bacteria (Herbert *et al.*, 1976; Poulter, 1982; Huss, 1988; Yongsawatdigul *et al.*, 2000).
- The most important group of the fish proteins consists of the muscle-fiber- proteins. This group is of fibrillar nature, which contributes around 75 % of the proteins (compared with 40 % in mammals) and is soluble in salt solution of around 5%. The most important components are actin and myosin, which, during contraction of the muscle fibres, form a complex named actomyosin (Eskin *et al.*, 1971; Huss, 1988; Love, 1997).
- Finally there is an insoluble protein fraction amounting to about 5 % (compared with 17 % in mammals). These proteins ("collagens") form the connective tissue surrounding the muscle fibres and are also present in the skin.

The varying amounts and types of collagen in different fishes may have an influence on the textural properties of fish muscle (Connell, 1970; Love, 1997). Treatment with high salt concentrations or heat of fish proteins may lead to denaturation, after which the native protein structure would have irreversibly changed (Connell, 1970; Huss, 1988; Hsu *et al.*, 1993; Love, 1997; Choi *et al.*, 2000). When the proteins are denatured under controlled conditions, their properties may be utilized for technological purposes. A good example is the production

of surimi-based products, in which the gel forming ability of the myofibrillar proteins is used (Jaczynski *et al*., 2002).

2.1.3 Nitrogen-containing extractives

Other compounds of fish muscle tissue are the nitrogen-containing extractives of non-protein nature (NPN). The nitrogen-containing extractives can be defined as the water-soluble, low molecular weight nitrogen-containing compounds of non-protein nature (Eskin *et al*., 1971). This non-protein nitrogen fraction includes volatile bases such as ammonia and trimethylamine oxide (TMAO), creatine, free amino acids, nucleotides and in the case of cartilaginous fish, urea (Huss, 1988; Love 1997;). Trimethylamine oxide constitutes an important part of the non-protein nature fraction in marine species which is found in all marine fish species in quantities from 1 to 7 % of the muscle tissue (dry weight), but is virtually absent from freshwater species and from terrestrial organisms (Hebard *et al*., 1982; Huss, 1988; Hall, 1997). However a study of Nile perch and tilapia from Lake Victoria revealed TMAO levels of 150-200 mg /100 g in fresh fish (Poulter, 1982).

The amount of TMAO in the muscle tissue depends on the species, season, and fishing ground. In marine fish this nitrogen containing substance is present in amounts varying between 100 mg N/kg in flat fishes and 700 mg N/kg in round fishes, while the highest amount up to 3000 mg N/ kg is found in elasmobranches such as rays, sharks and cod (Tokunaga, 1970; Hebard *et al*., 1982). TMAO is supposed to play a role as regulator of the osmotic pressure in the living fish (Love, 1980).

The NPN-fraction also contains a fair amount of free amino acids. These constitute 630mg/100 g light muscle in mackerel (*Scomber scombrus*), 350-420 mg/ 100g in herring (*Clupea harengus*) and 310-370 mg/100 g in capelin (*Mallotus villosus*) (Huss, 1988). Their relative importance varies with species; taurine, glycine and histidine seem to dominate in most fish. However, histidine has attracted much attention because it can be decarboxylated microbiologically to histamine (Ababouch *et al*., 1990; Collette, 2001; Kim *et al*., 2001). Fish species such as tuna and mackerel have a high content of histidine (Ababouch *et al*., 1990; Kim *et al*., 2002b).

2.1.4 Vitamins and mineral contents

Fish muscle is a relatively poor source of water-soluble vitamins of B-group: 0.05 mg / 100 g for thiamine, 0.3mg / 100g for riboflavin, 4 mg / 100 g for nicotinic acid and 50 µg / 100g for folic acid (Huss, 1988). In general, all fatty fish are rich in vitamin A, D and E but vitamin C

is absent in fish muscle. For mineral elements, fish flesh is regarded as a valuable source of calcium, phosphorus and magnesium in particular, but also of iron, copper and selenium. Some of the vitamins and mineral contents of fish muscle tissue are listed in Tables 2.4 and 2.5.

Table 2.4 Vitamin contents of fish muscle

Fish	A (IU/g)	D (IU/g)	B_1 (thiamine,μ /g)	B_2 (riboflavin,μ /g)	B_6 (μ /g)	Niacin (μ /g)
Cod fillet	0–50	0	0.7	0.8	1.7	20
Herring fillet	20-400	300-1000	0.4	3.0	4.5	40
Cod-liver oil	200-10 000	20-300	-	3.4	-	15

Source: Huss (1988)

Table 2.5 Mineral contents of fish flesh

Element	Average value (mg/100 g)	Range (mg/100 g)
Sodium	72	30-134
Potassium	278	19-502
Calcium	79	19-881
Magnesium	38	4.5 -452
Phosphorus	190	68- 550

Source: Huss (1988)

2.2 Post mortem changes occurring in fresh fish

The first changes occuring in fish after death are those of sensory changes including appearance, texture, odour and taste (Trucco, 1982; Huss, 1988; Lowe *et al*., 1993; Nakayama *et al*., 1994).

2.2.1 Rigor mortis

Rigor mortis is the first post-mortem process that has a major influence on the appearance and structure of fish muscle and this may affect meat quality (Trucco, 1982; Lowe *et al.*, 1993; Nakayama *et al*., 1994). Onset of rigor occurs when the adenosine- triphosphate (ATP) content of the muscle drops below a critical level (Trucco, 1982). Since the detachment of the

myosin and actin filaments are ATP dependent, the post mortem time for the onset of rigor is affected by the ATP-content in the muscle at the time of death, which is affected by handling stress (Korhonen, *et al*., 1990; Lowe *et al*., 1993; Mochizuki and Sato, 1994). When the ATP content drops, as is the case after death, further movement of filaments becomes impossible. The muscles become hard and stiff, the whole body becomes inflexible and the fish is in "rigor mortis", normally occurring shortly after death, but sometimes after a few hours (Connell, 1970; Korhonen *et al*., 1990; Lowe *et al*., 1993; Mochizuki and Sato, 1994). The rate of the onset and resolution of rigor varies from species to species and is affected by temperature, handling, size and physical condition of the fish (Korhonen *et al*., 1990; Lowe *et al*., 1993; Mochizuki and Sato, 1994) (Table 2.6). Cooling and handling are responsible for prolonging duration of rigor mortis. Thus, fish kept cool undergo rigor mortis at a later stage after death than those kept at higher temperatures, but observations, especially on tropical fish show the opposite effect of temperature with regard to the onset of rigor (Trucco, 1982). Explanation for this observation has been suggested by Nakayama *et al*. (1994) who have shown that the onset of rigor mortis in carp (*Cyprinus carpio*) depends on the difference in sea temperature and storage temperature. When the difference is large the time from death to onset of rigor is short and vice versa. The importance of rigor mortis in fish is recognized by the fishing industry, since in addition to retarding microbial spoilage, it makes the fish stiff, which is generally recognized by the consumer as a sign of good quality (Korhonen, *et al*., 1990; Mochizuki and Sato, 1994).

The dissolution of rigor mortis makes the muscle relax again and it becomes limp, but no longer as elastic as before rigor (Connell, 1970; Korhonen *et al*., 1990).The resolution of rigor is a process still not completely understood but always results in the subsequent relaxation of the muscle tissue and is thought to be related to the activation of one or more of the naturally-occurring muscle enzymes (Connell, 1970; Korhonen *et al*., 1990). The softening of the muscle during resolution of rigor is coincidental with the autolytic changes. Among the changes, one of the first to be recognized was the degradation of ATP to form adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) (Korhonen *et al*., 1990; Love, 1997).

The degradation of ATP proceeds in the same manner with most fish but the speed of each individual reaction greatly varies from one species to another and often progresses coincidentally with the perceived level of spoilage as determined by trained analysts. Saito *et al*. (1959) were the first to observe this pattern and to develop a formula for fish freshness

based on these autolytic changes, the so-called K-value. This value expresses the relationship between inosine and hypoxanthine and the total amount of ATP related compounds:

$$K\,(\%) = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]} \times 100$$

Where [ATP], [ADP], [AMP], [IMP], [Ino] and [Hx] represent the relative concentrations of these compounds in fish muscle measured at various times during chilled storage.

The K or "freshness" index gives a relative freshness rating based primarily on the autolytic changes which take place during post mortem storage of the muscle. The higher the K value, the lower the freshness level. Unfortunately, for some fish species (Atlantic cod) a maximum K value is reached well in advance of the shelf life as determined by trained judges, and K is therefore not considered reliable as a freshness index for all marine fish. Since autolysis always follows the same course in fish, hypoxanthine has also been used as a freshness criterion in some instances, but according to Ehira (1976) this may be misleading if different species are compared. For example some fish such as horse mackerel (*Trachurus japonicus*) accumulate inosine (Ino) while others, such as many flat fish; accumulate hypoxanthine (H_X). It is now widely accepted that the loss of flavour in fish flesh is attributed to the degradation of inosine monophosphate (IMP). None of the nucleotide catabolites are considered to be related to the perceived changes in texture during the autolytic process except ATP whose loss is associated with rigor mortis.

Table 2.6 Onset and duration of rigor in various fish species

Species	Conditions	Tempera-tures °C	Time from death to onset of rigor (hours)	Time from death to end of rigor (hours)
Cod (*Gadus morhua*)	Stressed	0	2-8	20-65
Cod (*Gadus morhua*)	Stressed	10-12	1	20-30
Cod (*Gadus morhua*)	Stressed	30	0.5	1-2
Cod (*Gadus morhua*)	Unstressed	0	14-15	72-96
Blue Tilapia (*Areochromis aureus*)	Stressed	0	1	
Blue Tilapia (*Areochromis aureus*)	Unstressed	0	6	
Japanese flounder (*Paralichthys olivaceus*)	Stressed	0	3	>72
Japanese flounder (*Paralichthys olivaceus*)	Stressed	5	12	>72
Japanese flounder (*Paralichthys olivaceus*)	Stressed	10	6	72
Japanese flounder (*Paralichthys olivaceus*)	Stressed	15	6	48

Sources: Korhonen *et al.*, (1990); Nakayama *et al.*, (1994).

2.2.2 Changes in pH

The pH of muscle is considered as one of the more important factors affecting quality and is associated with changes in colour, carcass grading characteristics, tenderness, microbial spoilage and water holding capacity of the muscle (Love, 1980; Love 1997; Benjakul *et al*. 1997). The changes in pH during the onset of rigor mortis do not occur as a result of bacterial action but rather of enzymatic action in the muscles; post mortem glycolysis results in the accumulation of lactic acid, which in turn lowers the pH of the muscle (Huss, 1988; Benjakul *et al*. 1997). In general, fish muscle contains a relatively low level of glycogen compared to mammals and hence less lactic acid is generated after death (Eskin *et al*., 1971; Huss, 1995).

Consequently, the ultimate pHs of fish are usually higher than those observed for post mortem mammalian muscle: beef muscle for example, often drops to pH levels of 5.1 in rigor mortis whereas in cod this decrease is usually from pH 7.0 to pH 6.3 -6.9; in large mackerel and in tuna values of 5.8- 6.0 and 5.4 -5.6 have been recorded respectively (Huss, 1988). No change in pH value is observed for other species of fish such as capelin (*Mallotus villosus*).

Post–mortem lowering of pH affects the physical properties of the muscle, principally a decrease in water–binding capacity of the protein has been observed (Benjakul *et al*. 1997; Hall, 1997).

2.2.3 Changes in eating quality

The characteristic sensory changes in fish post mortem vary considerably depending on fish species and storage method (Huss, 1995; Hall, 1997; FAO, 2001). In the quality assessment of fresh fish, the organoleptic evaluation is most important. The newly caught fish has a bright shining appearance; the gills are bright red or red-brown without any covering slime and the texture is generally soft; the fish is very fresh with a species specific taste and odour (Connell, 1975; Huss, 1988**;** Tungkawachara *et al.*, 2003).

The changes in the eating quality of chilled fish during storage can be assessed by daily organoleptic examination of cooked flesh. A characteristic pattern of the deterioration of fish stored in ice can be differentiated into the following four phases (Shewan, 1977; Huss, 1988; Peters *et al*., 1995; Benjakul *et al*. 1997) (Table 2.7).

- Phase 1: During the first six days there is no marked spoilage. The fish is very fresh with a species specific taste and odour
- Phase 2: In the following four days there is a loss of characteristic odour and some slight odours may develop but the fish is still found acceptable.
- Phase 3: After ten days, there are signs of early spoilage with some off-flavours. Spoilage odours are described as sour, stale, sickly sweet and fruity. The texture becomes either soft and watery or tough and dry. In the case of fatty fish, rancidity can be detected.
- Phase 4: After a fortnight the fish can be characterized as spoiled and putrid; profuse and very offensive spoilage odours are perceived and described as sulphidy, cabbage-like, faecal and ammoniacal

On the other hand, in aseptically prepared fish flesh stored at 0°C, no such spoilage odours are perceived after the same storage period (Shewan, 1977; Peters *et al.*, 1995).

Table 2.7 Organoleptic changes during storage of white fish at 0° and 4°C

Stage I	0 – 6 days at 0°C; 0- 3 days at 4°C
	No marked spoilage
	Firm flesh
Stage II	7 – 10 days at 0°C; 3 – 5 days at 4°C
	Some strengthening of odour, musty, mousey
	Soft flesh
Stage III	11 –14 days at 0°C; 6 – 7 days at 4°C
	Some sourness, slight sweet, malty to fruity odour
	Stale appearance
	Soft flesh
Stage IV	15 days or more at 0°C; > 7 days at 4°C
	H_2S and other sulphidy odours, stale, cabbage-like
	faecal and strong ammoniacal odours
	Soft slimy flesh

Sources: Shewan (1977); Huss (1988)

2.2.4 Autolytic spoilage of fish

Autolysis can be defined as "self-digestion". The process of autolysis as described by Bykowski and Dutkiewicz (1996) starts on the death of the fish leading to decomposition of basic compounds of tissues which takes place under the influence of enzymes from fish tissues themselves. The decomposition involves proteins, lipids and carbohydrates and decomposition of one compound can influence others at various rates.

It has been reported by various authors that there are at least two types of fish spoilage: bacterial and enzymatic (Huss, 1975; Love, 1980; Gram *et al*., 2002a; Gram *et al*., 2002b). Uchyama *et al*., (1974) showed that for cod and yellowtail tuna, enzymatic changes related to fish freshness preceded and were unrelated to changes in the microbiological quality. In some species (squid, herring), the enzymatic changes precede and therefore predominate the spoilage of chilled fish.

During autolysis, great changes occur in the structure of muscle tissue, which becomes softer and very often falls into layers along the myosepts (Bykowski *et al*., 1996; Hall, 1997). Many

proteases have been isolated from fish muscle and the effects of proteolytic breakdown are often related to extensive softening of the tissue (Lin *et al*., 1996; An, 1998). The low molecular weight peptides and free amino-acids produced by the autolysis of proteins not only lower the commercial acceptability of fish, but autolysis also has been shown to accelerate the growth of spoilage bacteria by providing a superior growth environment for such organisms (Love, 1997; FAO, 2001; Gram *et al*., 2002a). The induction of bacterial spoilage in capelin by autolysis also resulted in the decarboxylation of amino-acids, producing biogenic amines and lowering the nutritive value of the fish significantly. The autolytic changes affecting the edibility of fresh and frozen fish are summarized in Table 2.8.

Table 2.8 Summary of autolytic changes in chilled fish

Enzymes	Substrates	Changes encountered	Prevention/inhibition
Glycolytic enzymes	Glycogen	Production of lactic acid; pH of tissue drops; Loss of water-holding capacity in muscle; High temperature rigor may result in gaping	Fish should be allowed to pass through rigor at temperatures as close to 0°C as possible Pre-rigor stress must be avoided
Autolytic enzymes, involved in nucleotide breakdown	ATP ADP AMP IMP	Loss of fresh fish flavour, gradual production of bitternes with Hx	Same as above Rough handling or crushing accelerates breakdown
Cathepsins	Proteins, peptides	Softening of tissue making processing difficult or impossible	Rough handling during storage and discharge
Chymotrypsin, trypsin, carboxy-peptidases	Proteins, peptides	Autolysis of visceral cavity in pelagics	Problem increased with freezing/thawing or long- term chill storage
Collagenases	Connective tissue	Gaping" of fillets softening	Connective tissue degradation related to time and temperature of chilled storage
TMAO demethylase	TMAO	Formaldehyde-induced toughening of frozen gadoid fish	Store fish at temperature ≤ -30°C Physical abuse and freezing/thawing accelerate formaldehyde-induced toughening

Sources: Love (1980); Huss (1988); Huss (1995); Hall (1997)

2.2.5 Bacteriological changes in fish body

2.2.5.1 Bacterial flora of newly caught fish

Although the flesh and the blood of newly caught healthy fish are sterile, the skin, gills and the intestines may carry considerable bacterial loads (Shewan, 1977; Huss, 1988). In cooler regions, psychrophiles are more abundant and mesophiles represent only 5 percent of the total flora, while in tropical waters the latter make up about 55 percent (Shewan, 1977). Quantitatively, the total number of organisms varies enormously: 10^2 to 10^7 cfu /cm^2 on the skin surface; both gills and gut contain between 10^3 and 10^9cfu/g (Gillespie *et al.*, 1975; Shewan, 1977; Liston, 1982).

Quantitatively and more qualitatively, the bacterial flora on newly caught fish depends on the environment where it is caught than on fish species (Shewan, 1977; Hall, 1997 Gram and Huss, 2000). Thus, very low numbers of microorganisms (10 –100/cm^2 skin) were found in fish caught from clean cold waters while much higher counts are found in fish caught from polluted areas or warm tropical waters (Shewan, 1977; Huss, 1988; Hall, 1997).

Newly caught fish from temperate waters were dominated by a large number of psychrophilic, aerobic or facultative anaerobic gram-negative bacteria belonging to the genera *Pseudomonas, Alteromonas, Moraxella, Acinobacter, Flavobacterium* and *Vibrio* (Shewan, 1977). In addition, Gram-positive organisms such as *Bacillus, Micrococcus, Clostridium* and *Lactobacillus* could also be found in varying proportions in microbial flora of fish in temperate water (Shewan, 1977; Gram and Huss, 2000) (Table 2.9). On the contrary, in fish from tropical and subtropical areas, mesophilic gram-positive genera such as *Bacillus* and *Micrococcus* seem to predominate (Shewan, 1977) but according to Lima dos Santos (1978), gram-positive bacteria do not normally prevail in microbial flora of tropical fish. This conclusion from Lima dos Santos (1978) was later challenged by several studies, which found that the microflora on tropical fish species was very similar to the flora on temperate species (Gram, 1989; Gram *et al.*, 2002a).

In polluted waters from tropical regions, among the mesophiles bacteria from human and animal origin may be present, including indicator-organisms and pathogens (Shewan, 1977; Huss, 1988). In clean temperate waters, these organisms from human and animal origin disappear rapidly, but it has been shown that *Escherichia coli* and *Salmonella* can survive for very long periods in tropical waters and once introduced may almost become indigenous to the environment (Shewan, 1977; Huss 1988).

As far as freshwater fish are concerned, the bacterial loads on the skin, gills and gut contents were lower than on marine fish (Huss, 1988). A high proportion of gram-positive bacteria such as *Micrococcus*, *Bacillus* and *Streptococcus* on freshwater fish was reported by Liston (1982), while Shewan (1977) pointed out the presence of the genus *Aeromonas* in all fresh water fish and its absence in marine fish.

Table 2.9 Bacterial flora on fish caught in clean, unpolluted waters

Gram-negative	**Gram-positive**
Pseudomonas	*Bacillus*
Moraxella	*Clostridium*
Acinetobacter	*Micrococcus*
Shewanella putrefaciens	*Lactobacillus*
Flavobacterium	Coryneforms
Cytophaga	
Vibrio Photobacterium	
Aeromonas	

Sources: Shewan, 1977; Huss, 1988

2.2.5.2 Changes in bacterial flora of fish during storage and spoilage

During the catching and handling of fish, bacteria load on the outside (skin and gills) will increase with bacteria from the nets, fish boxes, hands and clothes of fishermen and sometime ice as well (Njai, 2000). While the fish is alive, bacteria growth and invasion into the flesh are prevented by the body's natural defense system (Huss, 1988). When the fish dies, the defence mechanism breaks down and bacteria can start to multiply and invade the flesh by moving between the muscle fibers (Connell, 1975; Shewan, 1977; Gram *et al*., 2002a). Fishes spoil at very different rates, and differences in surface properties of fish have been proposed to explain this (Shewan, 1977; Huss, 1988; Gram *et al*., 2002a). When fish is stored in ice, the bacteria will grow with a doubling time of approximately 1 day and will, after 2-3 weeks, reach numbers of 10^8-10^9 cfu/g flesh or cm^2 skin (Gillespie and Macrae, 1975; Shewan, 1977; Gram, 1989). During ambient storage, a slightly lower level of 10^7-10^8 cfu/g is reached in 24 hours (Shewan, 1966; Shewan, 1977; Gram, 1989). Qualitatively, this increase of micro-flora is accompanied by the emergence of some species. For example, under aerobic iced storage, the flora is composed of *Pseudomonas* spp. and *Alteromonas putrefaciens* as predominant

genera irrespective of the initial flora of the fish and this is certainly due to the shorter generation times of these microorganisms at chilled temperatures (Gillespie and Macrae, 1975; Shewan, 1977). During anaerobic storage, bacterial counts of fish never reach the same level as seen in iced fish (Dalgaard, 1993; Huss, 1995). In addition, the qualitative aspect may differ; the anaerobic conditions favouring bacteria groups such as *Aeromonas*, *Achromobacter* and *Micrococcus* spp. and consequently the *Pseudomonas* never predominate (Lima dos Santos, 1978; Huss, 1988; Gram, 1989).

It is important to make a clear distinction between the terms spoilage flora and spoilage bacteria. The first describes the bacteria present on the fish when it spoils whereas the latter are the specific groups that produce the off-odours and off-flavours associated with spoilage (Shewan, 1977; Dalgaard, 1993). A large part of the bacteria present on the spoiled fish have played no role whatever in the spoilage. It is not an easy task to determine from the bacteria isolated from the spoiled fish those causing spoilage, and it requires extensive sensory, microbiological and chemical studies (Dalgaard, 1993). Table 2.10 gives an overview of changes in total counts and the specific spoilage bacteria of fresh fish products stored in ice and at ambient temperatures.

2.2.6 Biochemical changes induced by bacteria during storage and spoilage of fresh fish

The bacteria spoilage has the most conspicuous influence upon the decrease of keeping quality of fresh fish, although the influence of the denaturation of proteins and rancidity of lipids are not negligible (Shewan, 1977; Huss, 1988; Gram, 2003). The most important changes involved during fish decomposition include:

- Autolysis: primary changes which lead to the formation of amino-acids from protein or to certain types of intermediate products such as peptides and;
- Secondary changes including those which lead to the formation of products such as trimethylamine, volatile sulphur compounds, aldehydes, ketones, esters, hypoxanthine as well as other low molecular weight compounds (Shewan, 1977; Huss, 1988; Essuman, 1992; Hall, 1997; Benjakul *et al*., 1997; Gram, 2003).

According to Bykowski and Dutkiewicz (1996), autolysis is a very complex event requiring complex measuring systems. The penetration of bacteria into fish tissue and microbiological decomposition begins with autolysis and these processes are practically parallel (Huss, 1988; Hall, 1997; Jaczynski and Park, 2002). The substrates for the production of volatile compounds are the carbohydrates (lactose, ribose), nucleotides (inosine mono-phosphate,

inosine) and other non protein nitrogen molecules (Shewan, 1977; Huss, 1988; Benjakul *et al*., 1997; Tungkawachara *et al*., 2003).

The amino acids are particularly important substrates for the formation of hydrogen sulphides and ammonia (Shewan, 1977; Huss, 1988). Substrate and off-odour compounds produced by bacteria during spoilage of fish are shown in Table 2.11.

Table 2.10 Dominating microflora and specific spoilage bacteria of fresh white fish (cod)

Storage temperature	Packaging atmosphere	Dominating microflora	Specific spoilage organisms (SSO)
0°C	Aerobic	Gram-negative psychrotrophic, non-fermentative rods *(Pseudomonas* spp.*, S. putrefaciens, Moraxella, Acinetobacter)*	*S. putrefaciens* *Pseudomonas* [3]
0°C	Vacuum	Gram-negative rods; psychrotrophic or with psychrophilic character (*S. putrefaciens, Photobacterium)*	*S. putrefaciens P. phosphoreum*
0°C	MAP[1]	Gram-negative fermentative rods with psychrophilic character (*Photobacterium*) Gram-negative non-fermentative psychrotrophic rods (*Pseudomonas, S. putrefaciens*) Gram-positive rods (LAB[2])	*P. phosphoreum*
5°C	Aerobic	Gram-negative psychrotrophic rods *(Vibrionaceae, S. putrefaciens)*	*Aeromonas* spp. *S. putrefaciens*
5°C	Vacuum	Gram-negative psychrotrophic rods *(Vibrionaceae, S. putrefaciens)*	*Aeromonas* spp. *S. putrefaciens*
5°C	MAP	Gram-negative psychrotrophic rods *(Vibrionaceae)*	*Aeromonas* spp.
20-30°C	Aerobic	Gram-negative mesophilic fermentative rods *(Vibrionaceae, Enterobacteriaceae)*	Motile *Aeromonas* spp. *(A. hydrophila)*

[1] MAP - Modified Atmosphere Packaging (CO_2 containing); [2] LAB: Lactic Acid Bacteria
[3]Fish caught in tropical waters or freshwaters tend to have a spoilage dominated by *Pseudomonas* spp.

Sources: Huss (1995); Dalgaard (1993)

Table 2.11 Substrate and off-odour compounds produced by bacteria during spoilage of fish

Substrate	Compounds produced by bacterial action
Inosine	hypoxanthine
Carbohydrates and lactate	acetic acid, CO_2 and H_2O
Methionine	hydrogen sulphide,
Cysteine	dimethyl sulphide
	methyl mercaptan
Glycine, leucine, serine	esters of acetic, propionic, butyric
	and hexanoic acid
Trimethylamine oxide	trimethylamine
Urea, amino-acid	ammonia

Sources: Shewan (1977); Huss (1988)

Hydrogen sulphide (H_2S), dimethyl sulphide ($[CH_3]_2S$) and methyl mercaptan (CH_3SH) are produced from methionine and cysteine respectively and are responsible for the sulphidy and cabbage-like odours (Herbert *et al*., 1976; Tungkawachara *et al*., 2003). *Alteromonas putrefaciens* and some *Vibrionaceae* were identified as hydrogen sulphide and trimethylamine producers (Herbert *et al.*, 1976; Huss, 1995; Gram and Huss, 2000). On the contrary, neither *Pseudomonas* nor *Photobacterium phosphoreum* produce significant amounts of Hydrogen sulphide (Herbert *et al.*, 1976). The esters of lower fatty acids such as acetic, butyric, propionic and hexanoic acid perceived as fruity odours are produced from amino acids such as glycine, serine and leucine (Miller *et al*., 1973; Shewan, 1977; Benjakul *et al*., 1997). A number of volatile aldehydes, ketones and esters are produced by *Pseudomonas* spp. (Miller *et al.*, 1973; Benjakul *et al*., 1997). Fruity, rotten and sulphidryl odours and flavours are typical of the *Pseudomonas* spoilage of iced fish and in particular the fruity off-odours are produced by *Pseudomonas fragi* (Shewan, 1974; Huss, 1988). A pyrazine derivative from 2 methoxy-3-isopropyl pyrazine is responsible for the stale potato-like odours (Miller *et al*., 1973). Ammonia is produced by oxidation of amino acids and amines (Shewan, 1974; Huss, 1988).

In a case of marine fish trimethylamine (TMA) is also produced from trimethylamine oxide (TMAO) by the facultative anaerobes that use TMAO as a hydrogen acceptor. The marine environment bacteria such as *Alteromonas, Photobactetium, Vibrio, Alteromonas putrefaciens* and also *Aeromonas* and the *Enterobacteriaceae* are mainly associated to TMAO reduction

(Eskin *et al*., 1971; Huss, 1995). The most important spoilers of fish stored aerobically or packed in ice or at ambient temperatures and their typical spoilage compounds produced are indicated in Table 2.12.

Table 2.12 Typical spoilage compounds during spoilage of fresh fish stored aerobically or packed in ice or at ambient temperatures

Specific spoilage organisms	Typical spoilage compound
Alteromonas putrefaciens	TMA, H_2O, CH_3SH, $(CH_3)_2S$, Hx
Photobacterium phosphoreum	TMA, Hx
Pseudomonas spp.	Ketones, aldehydes, esters, non-H_2S sulphides
Vibrionaceae	TMA, H_2S
Anaerobic spoilers	NH_3, acetic, butyric and propionic acids

Sources: Shewan (1977); Huss (1988)

2.2.6.1 Reduction of trimethylamine oxide

Fish spoils rapidly because the fish tissue becomes less acid after death than tissue of warm blooded animals and fish contains trimethylamine oxide (TMAO) which stimulates anaerobic growth of spoilage bacteria (Raa, 1981; Huss, 1995, FAO, 2001). TMAO can be used by spoilage bacteria as an electron acceptor when oxygen is depleted (Raa, 1981; Benjakul *et al*., 1997). The enzymatic reduction of TMAO under anaerobic conditions proceeds as follows:

$$CH_3\,CHOHCOOH + (CH_3)_3\,NO \longrightarrow CH_3\,COCOOH + (CH_3)_3\,N + H_2\,O$$

The pyruvic acid, however, is oxidized according to

$$CH_3\,COCOOH + (CH_3)_3\,NO \longrightarrow CH_3\,COOH + (CH_3)_3\,N + CO_2$$

The overall reaction can be written as follows:

$$CH_3\,CHOHCOOH + 2(CH_3)_3\,NO \longrightarrow CH_3\,COOH + 2(CH_3)_3\,N + CO2 + H_2O$$

The TMAO reduction is mainly associated with the genera of bacteria typical of the marine environment *(Alteromonas, Photobactetium, Vibrio* and *S. putrefaciens),* but is also carried out by *Aeromonas* and intestinal bacteria of the *Enterobacteriaceae* (Tungkawachara *et al*., 2003). The reduced component trimethylamine (TMA) is present in relatively large amounts

in marine fish. The level of TMA found in fresh fish rejected by sensory panels varies between fish species, but is typically around 10-15 mg TMA-N/100 g in aerobically stored fish and at a level of 30 mg TMA-N/100 g in packed fish (Huss, 1988; Dalgaard, 1993).

The trimethylamine (TMA) constitutes the most important compound of the total volatile bases (TVB), also called total volatile nitrogen (TVN) in cod and other gadoid fishes, until spoilage (Eskin *et al*., 1971; Connel, 1975; Shewan, 1977). However, in spoiled fish where the TMAO supplies are depleted and TMA has reached its maximum level, TVB levels still increase due to formation of ammonia (NH_3) and other volatile amines. The development of TMA in many fish species is associated with a production of dimethylamine (DMA) and hypoxanthine. Hypoxanthine can be formed by bacteria action, but it can also be formed by the autolytic decomposition of nucleotides and the rate of autolysis is less than the bacterial formation (Huss, 1975; Dalgaard, 1993). Both Huss (1988) and Dalgaard (1993) observed a linear correlation between the two contents (TMA and hypoxanthine) during iced storage of packed cod. Numbers of bacteria including *Pseudomonas* spp., *Alteromonas putrefaciens* (Shewan, 1977; Gram *et al*., 2002a) and *Photobacterium phosphoreum* (Shewan, 1977) were identified as hypoxanthine producer from inosine or inosine mono-phosphate.

2.2.6. 2 Formation of biogenic amines in foods and fish products

The principal amines of interest in fish muscle may conveniently be divided into two groups: the low molecular-weight (TMA, DMA) commonly used as indicators of eating quality in lean fresh and frozen fish, and the biogenic amines, which are formed from microbial decarboxylation of amino acids and are implicated in scombroid poisoning (Eitenmiller et al., 1982; Ababouch, 1990; Eitenmiller, 2001; Kim et al.,. 2002a). Biogenic amines such as histamine, cadaverine, putrescine, tryptamine, tyramine, spermine and spermidine are organic compounds, which are derived from the corresponding amino acids when the carboxylic acid group on the amino acid is removed by enzymic reactions (Frank, 1985; Ababouch et al., 1988; Ababouch, 1991; Collette, 2001). If foods are mishandled during storage and processing, proteins within the foods may break down to free amino acids, which may also be naturally present within the food (Ababouch et al., 1988; Eerola et al., 1996; Collette, 2001). When the foods are contaminated with bacteria containing decarboxylase enzymes, these free amino acids undergo decarboxylation to produce biogenic amines (Ahmed, 1991; Silva et al., 1998; Kim et al., 2001; Kim et al., 2002b). For instance, histidine is decarboxylated to produce histamine, lysine is decarboxylated to produce cadaverine and putrescine can be

produced from two free amino acids, glutamine and arginine (Eitenmiller et al., 1982; Brink et al., 1990; Halasz et al., 1994) (Table 2.13).

Table 2.13 Some important biogenic amines and their amino-acids precursors

Biogenic amines	Precursors
Phenylethylamine	Phenylalanine
Tyramine	Tyrosine
Histamine	Histidine
Tryptamine	Trytophane
Cadaverine	Lysine
Putrescine	Arginine

Sources: Love (1980); Brink *et al.*, (1990)

Biogenic amines may be present in various foods, including cheese, meat, dairy products, vegetables fruits, fermented foods and fish and fish products (Stratton *et al.*, 1991; Eerola *et al*., 1996; Kerr *et al*., 2002). Species of many genera such as *Bacillus, Pediococcus, Lactobacillus Citrobacter, Clostridium, Escherichia, Klebsiella, Photobacterium, Proteus, Pseudomonas, Salmonella, Shygella, Streptococus* and *Morganella morganii* are capable of decarboxylating one or more amino acids (Ferencik, 1970; Rice *et al*., 1976; Taylor *et al*., 1978; Taylor *et al*., 1979; Eitenmiller *et al.*, 1982; Russell and Maretic, 1986; Taylor, 1986; Ababouch *et al*., 1988; Kim *et al*., 1999; Kim *et al*., 2002b). These bacteria are capable of producing hazardous amounts of histamine in a very short period of time when fish are held at elevated temperatures; as a result, contaminated fish may not appear spoiled but still be hazardous to consume (Deng-Fwu *et al*., 1995; Kerr *et al.,* 2002).

Most amines are heat stable and some decarboxylases remain active even after pasteurisation. This implies that the amount of amine once formed will not be reduced during processing and might even increase during storage. The amount of biogenic amines formed is influenced by factors such as microbial growth, availability of free amino acids, the presence of decarboxylase enzymes and elevated temperature conditions (Halasz *et al*., 1994; Kim *et al*., 1999). The enzyme involved in the production of histamine, histidine decarboxylase, requires temperatures greater than 15°C with an optimum temperature of 30°C for production of histamine (Chen *et al*., 1988; Silva *et al.*, 1998; Kim *et al*., 1999). Thus, in tropical areas

where fish are often caught in temperatures exceeding 20°C if these fish are not refrigerated immediately, conditions are favourable for biogenic amines production (Eitenmiller *et al*., 1982; Chen *et al*., 1988; Ahmed, 1991**;** Collette, 2001).

Many biogenic amines have been reported in scientific literature, however histamine, putrescine and cadaverine have often been documented in clinical studies with histamine being linked to food poisoning and putrescine and cadaverine potentiating the toxicity of histamine (Eitenmiller *et al*., 1982; Veciana-Nogues *et al*., 1990: Ababouch *et al*., 1988; Ahmed, 1991; Kerr *et al*., 2002). Reviews in scientific literature indicate that scombroid species including mackerel, tuna, bonito, albacore, bluefish, butterfly fish and kingfish contained high levels of histamine within their flesh and are frequently involved in histamine poisoning (Taylor, 1986; Taylor and Bush, 1988; Ababouch *et al.*, 1988; Ahmed, 1991; Kerr *et al*., 2002). Histamine contents of some food substances are show in Table 2.14.

Histamine is formed in foods by enzymic decarboxylation of free histidine and various studies have established that decarboxylation results largely from the action of bacteria, which possess the enzyme, histidine decarboxylase (Arnold and Brown, 1978; Ababouch *et al*., 1988; Colette, 2001) (Figure 2.1). However, some early studies indicated that a minor part of histamine might result from the breakdown of fish flesh by autolysis (Tomiyasu *et al*., 1957; Kimata, 1961).

Table 2.14 Histamine content of some fermented fish products

Foods substances	Histamine (mg/100g)
Canned fish	0.2 - 10.0
Dried fish	0.2 – 30.1
Salted fish	2.2 – 9.8
Salted dried fish	0.1– 30.0
Smoked fish	1.7 – 34.5
Fish sauces and paste	18 - 32
Other fermented fish	12 - 83

Sources: *Stratton et al.*, (1991); den Brinker *et al*., (1995)

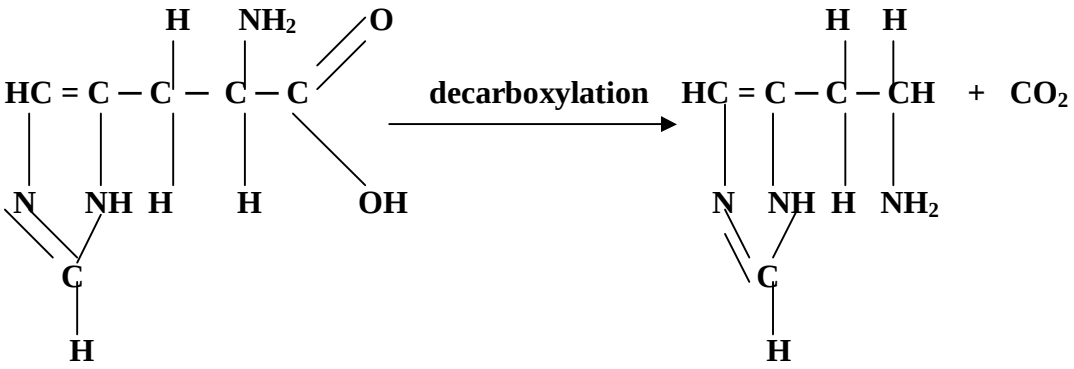


Figure 2.1 The formation of histamine from histidine

The level of biogenic amines in fresh fish is very low and their presence is related to spoilage (Veciana-Nogues *et al*., 1990). Levels of histamine above 10 mg/100g indicate that fish have been mishandled during storage or processing (Veciana-Nogues *et al*., 1990; den Brinker *et al*., 1995). For this reason, histamine, putrescine, tyramine and in general all biogenic amines, have been proposed by various authors as indicators of the quality and/or indexes of microbial spoilage of food, and in particular of fish and fish products (Hui and Taylor, 1983; Veciana-Nogues *et al*., 1990; Collette, 2001). Enteric bacteria appear to be the most important histamine-producing bacteria in fish. *Proteus morganii, Klebsiella pneumoniae* and *Enterobacter aerogenes* belong to the category of prolific histamine producers while *Hafnia alvei, Citrobacter freundii* and *Escherichia coli* are slow producers of histamine (Ferencik, 1970; Taylor, 1979; Ababouch *et al*., 1988; Kim *et al*, 2002a; Kim *et al*., 2002b).

The presence of biogenic amines in fermented products has been investigated by various authors (Rice and Koehler, 1976; Voigt and Eitenmiller, 1977; Bauer *et al*., 1994; Maijala *et al*., 1995; Eerola *et al*., 1996). During fermentation, complex bacterial activities occur and therefore high levels of biogenic amines could be expected to be found in these products. According to Brink *et al*., (1990) all products in which lactic acid fermentation take place (except yorghurt) might contain considerable amounts of cadaverine, histamine, putrescine and tyramine. High levels of biogenic amines have been reported in fermented sausages from retail markets (Rice *et al*., 1976; Vidal-carou *et al*., 1990; Bauer *et al*., 1994). In contrast, in yeast fermented alcoholic beverages, levels of biogenic amines were found in very low concentrations (0.23 to 11.4 mg/ml) in 13 varieties of alcoholic beverages produced in Taiwan (Yen and Chandra, 1988). Kerr *et al*., (2002) observed that amine levels in canned tuna

implicated in an outbreak of scombroid poisoning in humans ranged from 1.30, 1.16, 2.37, 12.80 and 116 mg/100 g fish for putrescine, spermine, spermidine, cadaverine and histamine respectively. Investigations on fish sauce, fish paste and dried fish imported from Asian countries confirmed high levels of biogenic amines (Wootten *et al*. 1989). Dried fish products contained putrescine and cadaverine at levels up to approximately 220 mg/ 100g and 330 mg/100g respectively and 80.3mg/ 100g of histamine were reported by Wootten *et al.* (1989). Work carried out by Abbey *et al*., (1994) on certain fermented mackerel species showed that they contain very high levels of histamine (105–172 mg / 100g). These levels exceeded the hazardous limit of 20 mg / 100g stipulated by the Food and Drug Administration-USA (FDA, 1982) and the European Economic Community (CEE, 1990). The Australian National Food Authority (ANFA) has established recently, a hazard action level for histamine in fish and fish products of 20 mg /100 g (ANFA, 2002).

Ingestion of food containing small amounts of histamine has little effect on humans, but in larger amounts histamine can be toxic (Taylor, 1986; Ababouch, 1990; Eitenmiller, 2001). Histamine poisoning is a chemical intoxication resulting from the ingestion of high amounts of histamine. The incubation period of histamine poisoning is short; poisoning effects can occur within several minutes to a few hours following ingestion of a meal containing high levels of histamine (Taylor, 1986; Ababouch, 1990; Eitenmiller, 2001). The duration of illness is usually short and, in most cases, symptoms such as flushing, oral burning or a blistering sensation and perspiration pass within a few hours. Less frequent symptoms include vomiting, diarrhoea, stomach pain, headaches, swelling of the tongue, facial swelling and dizziness (Taylor and Bush, 1988; den Brinker *et al*., 1995). However, histamine has an important role in human metabolism such as the release of stomach acid. The intestinal tract of humans contains the enzymes diamine oxidase (DAO) and histamine-N-methyl transferase (HMT), which convert histamine to harmless degradation products (den Brinker *et al*., 1995). If a high level of histamine is present in the diet, the capacity of DAO and HMT to detoxify histamine will be limited, resulting in toxic effects as the histamine enters the bloodstream (Taylor, 1986). The biogenic amine levels present a food poisoning hazard especially when coupled with additional risk factors such as the ingestion of amine oxidase inhibiting drugs, alcohol, other food amines and gastrointestinal disease (Maijala, 1994). Putrescine and cadaverine have been associated with potentiating the toxicity of histamine in humans but these compounds may also have independent toxicity effects on humans, although further

research work is required in this area (den Brinker *et al.*, 1995). Other biogenic amines tyramine and 2-phenylethylamine have been linked with food induced migraine headaches and should also be included in the Food Standards Code (Izquierdo-Pulido *et al*. 1994).

2.2.6.3 Effect of hygiene, handling and processing on the production of biogenic amines in fish

Handling and storage conditions, processing methods and environments could affect the production of biogenic amines in foods (Kranner *et al.*, 1991; Maijala, 1994). High levels of biogenic amines can be prevented if proper hygienic care during processing is practiced, contamination and microbial activity often due to mishandling and temperature abuse during storage is controlled and low initial contamination of the food is taken care of (Eitenmiller *et al*., 1982; Kranner *et al.*, 1991; Maijala *et al*., 1995a, Silva *et al*., 1998; Kerr *et al*., 2002); Joosten and Stadhouders (1987) established that biogenic amines were not produced in Gouda and Maasdam cheese made from pasteurized milk with sufficient hygienic care. Silva *et al*., (1998) observed that the histamine content of delayed iced tuna was significantly higher than those immediately iced on board; after 8 days of storage, histamine contents were 4.14 mg/100 g and 8.10 mg /100g for immediately iced and delayed iced tuna respectively. For fish stored at temperatures of 0°C or below, negligible histamine production occurred (Smith *et al.*, 1980; Benjakul *et al*., 1997; Collette, 2001).

In addition, the quality and storage life of many fish decreases if they have not been gutted. This evidence seems to show a relationship between gutting and histamine formation. Thus, Hardy *et al*., (1976) and Fernandez-Salguero *et al*., (1979) observed that the histamine content in the ungutted mackerel was ten (10) times more than in the gutted fish after storage at ambient temperature for 140hrs.

It could be possible to use antimicrobial agents together with ice to inhibit microbial growth and histamine production (Maijala *et al*., 1995b; Eerola *et al*., 1996). In some marinated or pickled products, acetic acid has been used to lower the pH and encourage formation of lactic acid bacteria, which are safe microbes (Maijala *et al*., 1995b; Eerola *et al*., 1996). Other important parameters suggested for preventing the accumulation of amines is the control of natural fermentation by addition of amine-negative starter cultures (Taylor and Speckhard, 1984; Holzapfel, 1997). However, Rice and Koehler (1976) reported that the use of *Pediococcus cerevisiae* and *Lactobacillus plantarum* caused no difference in tyramine

contents. Bauer *et al*., (1994) also reported that the addition of starter culture did not affect formation of biogenic amines.

In contrast, work carried out by Maijala *et al*., (1995b) showed that amine–negative *Pediococcus pentosaceus* added as starter culture decreased the levels of histamine, tyramine and cadaverine formed during ripening of dry sausages. These results therefore support the use of starter culture to decrease formation of biogenic amines.

Taylor and Speckhard (1984) observed that potasium sorbate at 0.50 percent effectively inhibited growth and histamine production by *Proteus morganii* and *Klebsiella pneumoniae*. In addition, sodium salts of hexameta phosphate and polyphosphate at 2 percent slowed down the histamine production rate (Taylor and Speckhard, 1984). According to Toyama (1982) addition of glucose or ribose reduces the histamine content of fish meal and this is due to the maillard reaction between the amino group of histamine or histidine and reducing sugars. Wood ash also possesses the capability to reduce moisture content and increase pH to levels unfavourable for most bacteria and thereby preventing the formation of biogenic amines (Clucas, 1982). It was shown by Watts and Brown (1982) that a carbon dioxide (CO_2) modified atmosphere could reduce the formation of biogenic amines in pacific mackerel *(Scomber japonicus).*

2.2.7 Rancidity of lipid fraction

The most important changes taking place in the lipid fraction are oxidative processes of a purely chemical nature, but enzymatic (microbial or tissue enzymes) degradation may also play a role (Huss, 1988; Benjakul *et al*., 1997). The relative significance of these reactions mainly depends on storage temperature and fish species. Fatty fish are, of course, particularly susceptible to lipid degradation which can create severe quality problems such as rancid flavours and odours as well as discoloration (Huss, 1988; Pikul *et al*., 1990; Benjakul *et al*., 1997). Two types of rancidity can be found: lipid oxidation, which is a reaction involving unsaturated lipids and oxygen, and lipid autolysis, which is an enzymatic hydrolysis with free fatty acids (FFA) and glycerol as major products.

2.2.7.1 Lipid oxidation

Lipids in fish are very labile due to the high number of unsaturated fatty acids. In fact, large amounts of polyunsaturated fatty acids found in fish lipids make them highly susceptible to

oxidation by an autocatalytic mechanism; this is also known as autoxidation (Demeyer *et al.*, 1974; Huss, 1988; Pikul *et al.*, 1990).

Lipid oxidation may be a significant problem and it results in the formation of off-odours and flavour and the reduction or destruction of essential fatty acids (Eskin *et al.*, 1971; Huss 1988). Lipid oxidation is initiated as described below by abstraction of a hydrogen atom from the central carbon of the pentadiene structure found in most fatty acid acyl chains containing more than one double bond:

$$-CH = CH-CH_2-CH = CH- \longrightarrow -CH = CH-CH-CH = CH- + H$$

Contrary to the native molecule, the lipid radical (L) reacts very quickly with atmospheric oxygen making a peroxy-radical (LOO), which again may extract a hydrogen from another acyl chain resulting in a lipid hydroperoxide (LOOH) and a new radical L (Love, 1980; Huss, 1988; Love, 1997; Tungkawachara *et al.*, 2003). This propagation continues until one of the radicals is removed by reaction with another radical or with an antioxidant whose resulting radical is much less reactive. The hydroperoxides are broken down and catalyzed by heavy metal ions, to secondary auto-oxidation products of shorter carbon chain-lengths (Eskin *et al.*, 1971; Huss, 1988; Love, 1997; Tungkawachara *et al.*, 2003). These secondary products are mostly aldehydes, ketones, alcohols, small carboxylic acids and alkanes. Several of the aldehydes can be determined as "thiobarbituric acid-reactive substances".

The oxidation may be initiated and accelerated by heat, light and several organic and inorganic substances such as Cu and Fe (often found in salt), which are very active catalysts, thus having a strong pro-oxidative effect (Huss, 1988; Ramanathan and Das, 1992; Hornor, 1997). Fatty acid hydroperoxides may also be formed enzymatically, catalyzed by lipoxygenase which is present in variable amounts in different fish tissues (Ramanathan and Das, 1992). This enzyme is unstable and is probably important for lipid oxidation only in fresh fish. Cooking or freezing/thawing rather effectively destroys the enzyme activity. Living cells possess several protection mechanisms directed against lipid oxidation products. An enzyme, glutathione peroxidase, exists which reduces hydroperoxides in the cellular membranes to the corresponding hydroxy-compounds (Huss, 1988; Morrissey, 1997). This reaction demands supply of reduced glutathione and will therefore cease post mortem when the cell is depleted of that substance. The membranes also contain the phenolic compound α-tocopherol (Vitamin E), which is considered the most important natural antioxidant (Eskin *et al.*, 1971; Huss, 1988; Morrissey, 1997). Other compounds, for example carotenoids, ascorbic acid as well as citric acid may also function as antioxidants (Eskin *et al.*, 1971; Huss, 1988).

The acids prevent oxidation mostly by inactivating the metal ions (Huss, 1988; Ramanathan *et al*., 1992). The Wood smoke contains phenols, which may penetrate the fish surface during smoking and thereby provide some protection against lipid oxidation (Huss, 1988).

2.2.7.2 Lipid autolysis

This type of lipid degradation involves both lipolytic activity (lipid hydrolysis) and in the case of fatty fish decomposition, a lipoxydative activity (Huss, 1988; Benjakul *et al*. 1997; Gram, 2003). During storage the lipid hydrolysis may be caused by microbial or endogenous lipases leading to the formation of a considerable amount of free fatty acids (FFA); the phenomenon is more profound in ungutted than in gutted fish probably because of the involvement of digestive enzymes (Huss, 1988; Benjakul *et al*., 1997**;** Gram, 2003). Triglyceride in the fat is cleaved by triglyceride lipase originating from the digestive tract or excreted by certain microorganisms.

In lean fish, for example Atlantic cod, production of free fatty acids also occurs, even at low temperatures. The enzymes responsible are believed to be cellular phospholipases, in particular phospholipase A_2, although a correlation between the activity of these enzymes and the rate of appearance of FFA has not yet been firmly established (Huss, 1988; Morrissey, 1997**)**. Fatty acids bound to phospholipids at glycerol-carbon atom 2 are largely of the polyunsaturated type, and hydrolysis therefore often leads to increased oxidation as well (Love, 1980; Huss, 1988). Furthermore, fatty acids themselves may cause a "soapy" off-flavour.

2.3 Traditional methods of fish fermentation

Fermented fish is any fishery product, which has undergone degradative changes through the microbiological activity either in the presence or absence of salt (FAO, 1993). For a large number of the world's population, fermented fish products contribute significantly to the diet by supplementing the protein intake (Beddows, 1985). The amino acids composition of fish is such that it complements those obtained from cereals, ensuring that a good utilization of dietary nitrogen is attained (Beddows, 1985; FAO, 1989). Fermented foods are agricultural products, which have been converted by enzymatic activities of microorganisms into desirable food products, whose properties are considered more attractive than those of the original raw materials. In addition to its external attractive properties, its nutritional value and keeping qualities are in many cases better than that of the raw materials (Beddows, 1985). If the

manufacturing procedures are properly followed foods are usually safe for consumption. All these beneficial properties of the final products increase the economic value of the commodity (Steinkraus, 1985).

The fermentation of fish is a technological process traditionally available in West Africa for improving the shelf life of fish and improving its nutritional quality as well. Most fermented fish are currently produced traditionally by spontaneous and largely uncontrolled fermentation (Nerquaye-Tetteh *et al*., 1978; Beddows, 1985; Yankah, 1988; Essuman, 1992; Abbey *et al*., 1994). The traditional fermentation of food in Benin usually involves the use of mixed cultures in solid or semi solid substrate fermentation (Hounhouingan, 1994). Aidoo *et al*., (1982) defined solid substrate or solid state fermentation as any fermentation process in which the substrate is not a free liquid and may take place on a solid or semi-solid substrate or in a nutritionally inert solid support which provides some advantage to the micro-organisms with respect to access to nutrients. Salted tilapia (*koobi*) and *momone* are listed as some indigenous Ghanaian fish products, which are produced by traditional solid-substrate fermentation (Essuman, 1992; Abbey *et al*., 1994; Kingley-Ekow, 1999). *Lanhouin*, a salted and fermented fish is also an indigenous Beninese fish product, which is produced by traditional solid–substrate and mixed culture fermentation. The advantages of solid substrate fermentation include superior productivity, simpler techniques, reduced energy requirements, low waste water output and improved product recovery (Aidoo *et al*., 1982). Disadvantages include limitations on types of substrates used; substrates being heterogeneous during fermentation, and complexity of nutrients available requiring a complex of enzymes for their degradation (Aidoo *et al*., 1982; Bol *et al*., 1991).

According to Amoa-Awua (1996), mixed culture fermentations (the use of culture from one or more microbial groups) are prevalent because the traditional fermentations are mostly carried out spontaneously and a natural microbial population usually occurs as mixed cultures. The advantages of the spontaneous mixed culture solid substrate fermentation are that they rely on various micro-organisms to produce different enzymes to break down the substrate and require minimal handling skills of the traditional processors (Aidoo *et al*., 1982; Amoa-Awua, 1996). Other advantages of mixed culture fermentation over single pure culture fermentations are that product yield may be higher, growth rate of microorganisms may be higher, protection against contamination may be greater and remarkable stable associations of microorganisms may occur permitting better utilization of substrates (Hesseltine, 1991; Amoa-Awua, 1996). The disadvantages of this type of fermentation are that variable products

may be produced and there may be difficulty in controlling the optimum balance among the microorganisms involved (Hesseltine, 1991; Amoa-Awua, 1996). Other disadvantages are that very little control can be exercised over the fermentation process and the product is often of variable quality with inherent quality risks (Hesseltine, 1991; Amoa-Awua, 1996).

Various processing methods in fish fermentation are used in different localities and the particular method used depends largely on availability of salt and the food habits of the local people (Beddows, 1985; Olympia, 1992; Essuman, 1992; Gram, 2003). Three main techniques have clearly emerged as methods commonly practised in many African countries: these techniques are:

- Fermentation with salting and drying,
- Fermentation and drying without salting and
- Fermentation with salting without drying (Essuman, 1992).

Some of the major fermented fish produced in Ghana are: *momone, koobi* and *Kado* (Nerquaye-Tettteh *et al.*, 1978; Yankah, 1988; Essuman, 1992). According to Nerquaye-Tettteh *et al.*, (1978) during processing of *momone*, iced or frozen fish is never used. This is because the final product obtained from the use of iced fish does not have the desirable soft textural characteristics of fermented fish.

A momone-like product called *lanhouin* is mostly produced in Benin. *Lanhouin* is widely used for its flavour enhancing properties. Various species of fish such as cassava fish, kingfish, Senegal jack, milk shark, Atlantic horse mackerel, Congo dentex, lasser African threadfin and catfish, etc. can be used for *lanhouin* processing (Anihouvi *et al.*, 2005). A few major fermented fish consumed in African countries are shown in Table 2.15.

In Asia, and in particular those areas with an extensive coastline and high ambient temperatures such as Thailand, Kampuchea, Malaysia, Philippines and Indonesia, the use of fermentation as a preservation method for fish has been practised since the earliest of times (Beddows, 1985; Gram, 2003). According to Lee, (1990) fermented fish products consumed in Asia are largely divided into three groups: high-salt fermented fish products, low-salt fermented products and non-salt fermented fish products.

Table 2.15 Some of traditional fermented fishes produced in African countries

Country	Local name	Fermentation time	Drying time	Packaging
Burundi	Ndagala	2 to 5 days without salting	2-5 days on the grown or elevated racks	Sacks, polythene bags
Ivory Cost	Gyagawere, adjonfa	6 hours to 3 days after salting	3 to 5 days	Baskets, sacks
Gambia	Guedj	Overnight to 2 days after salting	3 to 5 days on the elevated racks	Sacks
Ghana	Momone, koobi, kado, ewule	Overnight to 3 days after salting	3 to 5 days	Sacks, baskets
Mali	Djegue, jalan	Overnight without salting	3 to 7 days	Sacks, mates and ropes
Uganda	Dagaa	3 to 6 hours without salting	2 to 5 days	Sacks, baskets
Senegal	Guedj, tambadiang, yeet	Overnight to 2 days after salting	3 to 7 days	Sacks, baskets
Sudan	Fessiekh, kejeick, terkeen, mindeshi	10 to 20 days after salting	No drying for (fessiekh) 3 to 7 days for (kejeick)	Cartons, cans, polythene bags
Chad	Salanga	3 to 6 days without salting	3 to 7 days	

Source: Essuman (1992)

- High-salt fermented fish products contain about 20 % salt and are represented by fish sauce (*patis* in Philippines, *nuoc-man* in Cambodia and *nampla* in Thailand) and fish paste (*kapi* in Philippines, *bagoong* in Thailand).

- Non-salt fermented products are represented by solid-state bonito and some alkaline fermentation of flat or fat fish (Figure 2.2).

- Low-salt fermented fish products contain between 6 and 8 % salt and are subdivided into lactic fermented products with added carbohydrate and acid pickling associated with low temperature.

Fish-salt-carbohydrate mixtures (lactic fermented product) consist of *pla-ra* in Thailand and *burong-isda* in the Philippines. In this category, a lactic fermentation occurs; a rapid decrease of pH (<4.5) within the first 48 hours and rapid proliferation of lactic acid bacteria with concomitant decreases in spoilage micro-organisms count contribute to the extended shelf life (Adams *et al.*, 1987). This category of products with their lower salt contents offer the advantage of being consumed as a main course, rather than in the condiment role of the high salt-fish sauces and pastes (Adams *et al.*, 1987). Fish sauce is often used to give rice flavour and aroma and up to 50 ml may be consumed over two meals while fish pastes are widely used as a condiment (Beddows, 1985). Some of major fermented fish products of East-Asia countries are represented by Table 2.16.

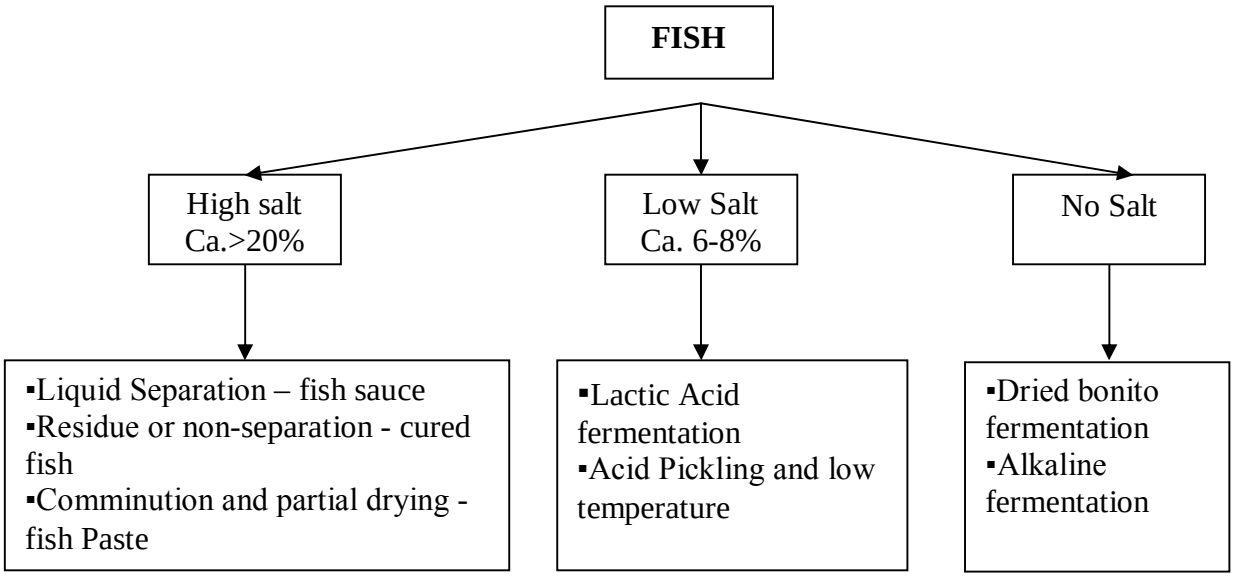


Figure 2.2 Classification of fermented fish products of East-Asia countries

Sources: Lee (1990); Soyiri (2002); Gram (2003)

Table 2.16 Some traditional fermented fish of East-Asia countries

Countries	Local names	Fermentation time
Philippines	Patis (sauce)	3 months to one year
	Bagoong (paste)	3 months to one year
	Nam-pla (sauce)	5 months to one year
Thailand	Kapi (paste)	15 days to one year
	Pla-ra (lactic fermentation)	3 months to one year
Vietnam	Nuoc-mam (sauce)	3 months to one year
	Shottsuru (sauce)	6 months
Japon	Ika-Shoyu (sauce)	6 months
	Shiokara (paste)	Weeks to one year
	Budu (sauce)	40 days
Malaysia	Belacan (paste)	1 month

Sources: FAO (1971); Ko (1982); Olympia (1992)

2.4 Processing of fish for lanhouin production

Various species of fish can be used for *lanhouin* processing. The fermentation periods vary from three (3) to eight (8) days and depend on the species of fish and marketing conditions. At the end of fermentation, the fish are sun dried for 2 to 4 days. For processing, the fish is scaled, gutted and sometimes cut into pieces and then left overnight at ambient temperature in fly free enclosure. The next day, the seemingly spoiled fish is washed. Dry salt is rubbed into gills, the belly cavity and on the surface. After this first salting, the fish is arranged in a basket, a can or a hole, covered with old cement paper bag and old clothes and allowed to ferment for three (3) days at room temperature before being removed, washed lightly and dried. Sometimes on the third day after the fish has been removed and washed lightly, a

second salting may be done and fermentation allowed to progress until the 8th day before sun drying.

2.5 Importance of salting

The initial treatment most commonly used is the salting of fish either by immersion in brine or by mixing with dry salt (Waterman, 1976; Beddows, 1985; Hall, 1997; Gram, 2003). This results in removing some of the water to the fish muscle, thus tending to firm the flesh, inhibit the action of bacteria, prevent some enzymatic activities while enhancing others and affects the stability of the proteins (Ko, 1982; FAO, 1989; Love, 1997). Salt also imports a flavour to the product (Beddow, 1985; Love, 1997). Salting requires minimal equipment, but the method of use is important.

Traditional methods involve rubbing salt into the flesh of the fish or making alternate layers of salt and fish. There are often problems with these methods because the concentration of salt in the flesh is not sufficient to preserve the fish, as it has not been uniformly applied (Beddows, 1985; FAO, 1989**;** Love, 1997). A better technique is brining which involves immersing the fish into a pre-prepared solution of salt (Beddows, 1985; FAO, 1989; Love, 1997). The advantage is that the salt concentration can be controlled easily, and salt penetration is more uniform.

In Bénin, where solar salt is available and inexpensive, fermented fish is heavily salted. Solar salt, however, is noted for its poor microbiological quality (Sefa-Dedeh *et al*., 1976; FAO, 1981; Essuman, 1992; Love, 1997). In fact, certain types of bacteria, which can live in strong brine or in solid salt, have been found practically in every case where salt obtained from seawater was used. These halophilic bacteria, which can grow at salt concentrations of 13 % and above, confer a reddish colour on salted foods (Beddows, 1985; Essuman, 1992; Sefa-Dedeh, 1993).

Very pure sodium chloride would be the most desirable for curing purposes. However, the end product of fish salted with the pure sodium chloride is not desirable as compared to fish salted with salt containing small amounts of calcium and magnesium (Beddow, 1985; Horner, 1997). The salt to be used for fish curing in the tropics should have approximately 0.50 % calcium and magnesium impurities, although in the case of greater levels of such impurities in the salt, Ca^{2+} and Mg^{2+} ions bind with protein and form a barrier to the passage of Na^{2+} ions to the thicker part of the fish flesh (Horner, 1997). The chemical composition of the two types of salt (solar salt and artisanal salt) usually used for fish curing in Benin are summarized in

Table 2.17. Owing to the improvement of Benin solar salt processing, its sodium chloride content can be favourably compared to that of imported solar salt from France (Table 2.17).

Table 2.17 Chemical composition of solar and artisanal salt of the Republic of Benin and imported salt (Guérande-France)

Physico-chemical components	Solar salt	Artisanal salt	Guérande salt (France)
Humidity (%)	6.31	10.38	5.90
Impurity (%)	0.11	0.27	0.31
Sodium chloride (%)	87.00	82.10	90.70
Potassium (%)	0.17	0.30	0.20
Calcium (%)	0.55	0.51	0.13
Magnesium (%)	0.54	1.00	0.67
Iron (mg/kg)	20.00	19.00	80.00

Source: PIRRATES (1990)

2.6 Effects of fermentation on the nutritional quality of fish

Fermentation is used for the production of flavoured fish products, which are added to a variety of diets and contribute greatly to the general nutrition of large populations, particularly in Tropical Africa and South-East Asia (Ko, 1982; Beddows, 1985; Essuman, 1992; Gram, 2003). Volatile bases are produced during the fermentation of fish and these contribute to the characteristic flavour and odour of fermented fish products (Nerquaye-Tetteh *et al*. 1978; Ko, 1982; Olympia, 1992). According to Nerquaye-Tetteh *et al.* (1978) and Abbey *et al*., (1994) the values of volatile bases (TVN) produced during *momone* processing were high and compared well with those reported by Amano, (1972), Mackie *et al.*, (1974) and Gram, (2003) for various fermented fish products from South-East Asia. Amano, (1972) and Mackie *et al*., (1974) reported nitrogen loss of about 30 to 35 % for some South-East Asia fermented fish products. Yankah, (1988) has also reported nitrogen loss of 25 % on *momone* fermented over two days. Since fermented fish is usually used in small quantities as a condiment and not as a main protein source, the nutritional loss due to processing might not be very important (Nerquaye-Tetteh *et al*., 1978; Ko, 1982; Beddows, 1985).

2.7 The predominant microflora and their role in the fermentation of fish

Microorganisms by virtue of their metabolic activities, contribute to the development of characteristic properties such as aroma, visual appearance, texture, taste, shelf life and safety (Beddows, 1985; Huss and Valdimarson, 1990; Gram, 2003). Several authors have reported the presence of a wide range of microorganisms during fermentation of fish products (Beddows, 1985; Adams *et al*., 1985; Adams *et al*., 1987; Olympia, 1992; Huss and Valdimarson, 1990; Oronsaye, 1991; Gram, 2003; Achinewhu *et al*., 2004). Salted and fermented fish products flora originate from the natural microbial population of fish, from salt and micro-organisms introduced during the process from fermentation tanks, equipments, the environment and workers (Ko, 1982; Watanabe, 1982; Beddows, 1985; FAO, 1989; Essuman, 1992).

The high salt content of salted and fermented fish products leaves only salt tolerant bacteria to survive (Ko, 1982; Beddows, 1985; FAO, 1989; Huss and Valdimarson, 1990). Adams *et al*., (1985) recognised two different types of fish fermentation: fish–salt-carbohydrate mixture represented by an acid fermentation in which lactic acid bacteria are responsible for acidifying the product and fish–salt formulation represented by alkaline fermentation in which *Bacillus* spp. and micrococci are considered to be responsible for the fermentation. Work by Knochel and Huss, (1984) showed that the microflora of salted herrings were mainly composed of 70 % halophilic Gram negative aerobes, 20% microcoques halotelerant aerobes and 3 % yeasts. According to Campbell-Platt (1987) and Gram (2003), bacteria involved in fish sauce fermentation are salt-tolerant bacteria (*Micrococcus*, *Staphylococcus* and *Bacillus*) and lactic acid bacteria (*Pediococcu*s and *Lactobacillus*). Ko (1982) reported that a study carried out on four fermented fish sauces including *nampla* from Thailand, *patis* from the Philippines and *kaomi* and *ounago* from Japan, showed that the microflora in these products were halotolerant rather than halophilic and *Bacillus* species predominated in three of the products. In addition, this study revealed that *Bacillus pumilus* is present in the early stage of the *patis* fermentation and *Bacillus licheniformis* during all stages of the *nampla* fermentation. This suggested that spore-forming bacilli may play an active role early in the fermentation process. The occurrence of *Micrococcus colpogenes* and *Micrococcus varians* in one month old *patis* also indicated the possible involvement of non-spore-forming organisms in the early stages of some fish sauce fermentation. With the exception of *Candida clausenii* in the *patis*, yeasts were not found in the other fish sauces (Ko, 1982).

Studies done by Olympia (1992) on the production of *burong bangus*, a fermented rice-fish mixture revealed *Streptococcus* spp., *Pediococcus* species, *Leuconostoc* and *Lactobacillus* to be organisms responsible for the fermentation. Work by Soyiri (2002) on fish sauce produced from fermented tuna waste showed that the predominant genera in the 49-day product consisted of *Bacillus* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium*. *Streptococcus*, *Corynebacterium* and *Staphylococcus* were predominant during the early stage of the fermentation and *Bacillus* took over during the latter part. Lopetcharat *et al*., (2001) observed *Staphylococcus* spp., *Bacillus* spp. and *Micrococcus* spp. as the predominant microorganisms during the processing of fish sauce made from pacific whiting.

Work carried out by Nerquaye-Tetteh *et al*., (1978) on *momone* samples collected from open markets showed that the dominant group of organisms isolated from the fish samples were Gram positive *Micrococcus* (72 %), spore-forming *Bacillus* (4.4 %) and *Staphylococcus* (3.2 %). Yankah (1988) and Abbey *et al.*, (1994) also observed the Gram-positive halophilic aerobic micrococci and bacilli to be the predominant microorganisms in this product. Recent reports by Oronsaye (1991) and Achinewhu (2004) identified *Bacillus pumilis*, *Bacillus licheniformis* and *Staphylococcus epidermidis* as the representative strains which, are involved in the solid state fermentation of fish.

The aroma, flavour and texture in fermented fish products have been claimed to be derived from the activity of various halophilic bacteria (Ko, 1982; Beddows, 1985; Gram, 2003). According to Ko (1992) *Leuconostoc mesenteroides*, *Pediococcus cerevisiae* and *Lactobacillus plantarum* play the major acid-producing role in *burong dalag*, a fish paste made in the Philippines. Gram (2003) reported *Bacillus* spp., *Micrococcus* spp. and halobacterium to be the main organisms which are involved in fish sauce production and are responsible for the production of volatile compounds. It was also reported that halophilic *Bacillus* species and *Staphylococcus* species are responsible for the production of volatile acids in a type of fish sauce produced in Thailand (Ko, 1982).

The increase in fermentation time has been observed to enhance the flavour, aroma and texture of the product. This development coincides with increased numbers of *Bacillus* species (Nerquaye-Tetteh *et al*., 1978; Beddows, 1985). Volatile acids and low molecular weight volatile compounds such as methyl, ketone and carbonyl compounds have been found to contribute significantly to the flavour and aroma of fermented fish products (Ko, 1982; Olympia, 1992). Organic acids in particular have been shown to be associated with the aroma of fish sauce (Gram, 2003).

2.8 Microorganisms commonly involved in solid substrate fermentation of fish

Solid substrate fermentation of fish is the fermentation in which fish retains its original texture. Microorganisms much discussed in this type of fish fermentation include *Bacillus* spp. and Micrococcaceae.

2.8.1 *Bacillus* species

Sneath (1986) defined *Bacillus* spp. as rod-shaped microorganisms, sporing, Gram-positive, catalase producing and capable of sporulating aerobically. According to Fogarty *et al.*, (1974) this group of organisms are known to be technologically important as they produce a range of industrially significant enzymes as well as a number of cyclic or linear polypeptide antibiotics. Enzymes of commercial importance produced by *Bacillus* spp. include amylases, proteases, β-glucanases and isomerases. Others enzymes produced by the genus are cellulases, hemicellulases, pectinases including polygalacturonase lyase, penicillinases including β-lactamase and penicillin amidase, α-amylase, β-amylase, amyloglucosidase, nucleases, cell wall lytic enzymes and acid, neutral and alkaline proteases. Many authors have observed that *Bacillus* spp. mainly *Bacillus subtilis* are micro-organisms responsible for the fermentation of most traditional foods in which there is an extensive hydrolysis of protein to amino acids and peptides (Odunfa, 1985a; Streinkraus, 1991; N'Dir *et al.*, 1997; Omafuvbe *et al.*, 2000). The resultant products of this fermentation are mostly used as condiments to flavour cooked food but also serve as low cost sources of protein (Nerquaye-Tetteh *et al.*, 1978; Beddows, 1985; Odunfa, 1985b; Yankah, 1988; Streinkraus, 1991; Abbey *et al.*, 1994; N'Dir *et al.*, 1997; Omafuvbe *et al.*, 2000). Classical methods including both morphological and biochemical characterization as well as the API test can be used for *Bacillus* identification.

2.8.2 Micrococcaceae

They are spherical in cell shape, non-sporing, Gram positive and catalase positive organisms. Micrococcaceae included both staphylococci and micrococci. By the late 1950s distinctions were being made between those cocci, that fermented glucose anaerobically (the *Staphylococcus*) and those that oxidized the sugar or did not attack it (the *Micrococcus*). Microorganisms have been used successfully in many fields including food technology where they have been used as starter cultures in milk processing, meat processing and vegetable processing. According to Hammes *et al.*, (1985) and Hammes (1986), the starter culture preparations available on the German market for use in meat processing at the time were

mono-culture and mixed cultures containing groups of organisms such as Micrococcaceae with the species *Micrococcus varians, Micrococcus cryophilus, Staphylococcus xylosus, Staphylococcus simulans* and *Staphylococcus carnosus*. These strains of staphylococci and micrococci have the enzyme nitrate reductase and are partly responsible for reddening and for stabilization of the cured red colour (Bacus, 1984; Lucke, 1985). Both lactic acid formers and nitrate reductase together with the type and quality of the initial material, influence flavour and texture of the end product (Bacus, 1984; Lucke, 1985; Adams, 1986).

2.9 Packaging and storage of salted and fermented fish products

One of the most important problems listed by various authors about traditional fermented products in general and fermented fish products in particular is their rudimentary packaging (Lartey, 1975; Essuman, 1990). Fish packaging can result in the products damage and add to losses (Sefa-Dedeh, 1995). As a matter of fact, packaging forms an important part of food processing because it facilitates handling during storage and distribution within the marketing chain. Thus, packaging material must possess certain characteristics, such as adequate strength to protect the packaged product from damage, it must be readily available and easy to use, and should be clean to prevent contamination by undesirable substances and prevent recontamination by insects and microorganisms. Traditional packaging materials usually used for fermented fish products include, according to the type of products: jute bags, cane baskets, leaves, polythene bags, glass bottles, and oil cans (Essuman, 1990; Essuman, 1992). The major advantage of these products is their low cost, but the type of packaging is necessarily restricted. Glass bottles and even plastic bags are used for better quality products.

In West African countries where fermented fish products retain their original texture, packaging material such as jute bags, baskets and polythene bags are used to get the fish to distant markets (Essuman, 1990; Essuman, 1992).

Under climatic conditions often involving temperatures of up to 32°C and a relative humidity of 90 per cent, storage of salted and fermented dried fish leads to substantial losses due to bacterial and mould attacks, insect infestation and mites (Sefa-Dedeh, 1995). In addition, insect pests are potential carriers of pathogenic bacteria (Waterman, 1976).

Another potential storage problem of salted and fermented dried fish is the continuous bacterial and enzymatic activities within the fermented product. Work carried out by Abbey *et al*., (1994) revealed that stored products continued to undergo further fermentation resulting in an unstable finished product. This process could eventually cause unwanted textural

changes of the fermented fish and may be regarded as deteriorative (Essuman, 1992; Abbey *et al*., 1994). During storage, the halophilic bacteria can confer a pink or reddish colour on salted and fermented fish products (Essuman, 1992). Reddish colour occurs in two stages:

- A rose pink colour appears on the salted fish; this can be easily washed off and the fish suffers little damage at this stage.
- The fish becomes brown and discoloured. The fish easily falls, particularly when held up by the tail. Unpleasant odours are produced because of indole and hydrogen sulphide formation (Beddows, 1985; Essuman, 1992). Another type of spoilage known as "dun" can also occur. "Dun" is caused by moulds that form brown spots on the flesh side of salted fish (Essuman, 1992).

Different treatments in order to control insect infestation, microbial growth as well as enzymatic activity during fermentation and storage have been suggested by various workers (Proctor, 1976; Asastyasih and Madden, 1986; Borquez and Gonzalez, 1994; Abbey *et al*., 1994). Asastyasih and Madden (1986) observed that acetic acid, garlic and white pepper had a repellent effect on certain species of insect. Garlic acid was used to control microbial growth during storage of momone (Abbey *et al*., 1994). Repellent and microbial effects of some insecticides such as pyrethrins and piperonyl butoxide have been reported by various authors (Proctor, 1976; Borquez and Gonzalez, 1994). FAO/WHO in 1986 gave the approval for the use of low toxic compounds such as an organophosphate insecticide, pirimiphos-methyl. A number of experiments carried out by Rattagool *et al*., (1988) in Thailand and Esser (1988) in Indonesia proved that pirimiphos-methyl was effective in controlling blowfly infestation.

3.0 METHODOLOGY

3.1 Materials

3.1.1 Fresh fish and salt

Fresh cassava fish (*Pseudotolithus* sp.) was purchased at the Cotonou seaport (Bénin). The fish was placed in an ice-chest filled with ice and transported to a local processing site. The fish was selected on the basis of its popularity (Anihouvi *et al*., 2005). Solar salt used for fish curing was obtained from the local market.

3.1.2 Market samples of *lanhouin*

Twenty five traditonally processed samples of *lanhouin* made from cassava fish (*Pseudotolithus* sp.) and twenty five others from king fish (*Scomberomorus tritor*) were purchased from processors and retailers in the processing sites and the markets. Samples were collected into sterile plastic bags, stocked in an ice-chest filled with ice, and transported to the laboratory for analyses.

3.2 Methods

3.2.1 Field investigation work

Field investigation was conducted on processors of *lanhouin*. A preliminary survey was conducted in order to identify production sites and pre-test the questionnaire (appendix 1). The survey was in the form of interviews administered through a questionnaire as well as observations of the processors at work. Forty one (41) persons including 21 processors and 20 retailers were interviewed. The questionnaire focused on the socio-economic status of the processors, the raw fish used for *lanhouin* production, preparation of the raw fish, the materials used, the fermentation process and its duration, sensory characteristics and storage of *lanhouin*.

3.2.2 Processing of cassava fish into *lanhouin*

Fresh fish was processed (with the help of one experienced *lanhouin* processor) for laboratory studies using the procedure described by processors during the field study (Anihouvi *et al*., 2005). The fish samples were scaled, gutted through a slit at the anal opening, washed clean, and then left overnight at room temperature (28-30°C) in a fly-free enclosure (Fig.3.1). The next day, the seemingly spoiled fish were washed and dry salt rubbed onto the flesh, the gills

and the degutted cavity (Plates 3.1 and 3.2). The fish were then wrapped with cloth, arranged in a basket and allowed to ferment at room temperature for 3 to 8 days after which they were sun dried for 2 to 4 days under mosquito netting material to prevent the contact of flies with the product (plate 3.3).

Plate 3.1 Scaling and gutting of fresh cassava fish

Plate 3.2 Salting of ripened cassava fish

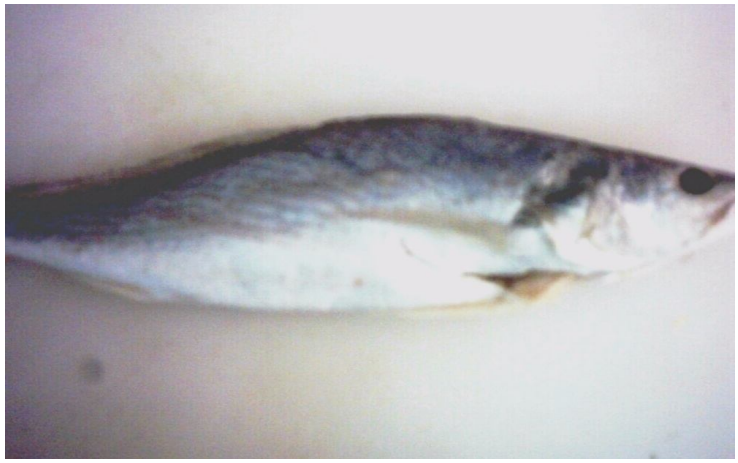


Plate 3.3 Fermented fish *lanhouin*

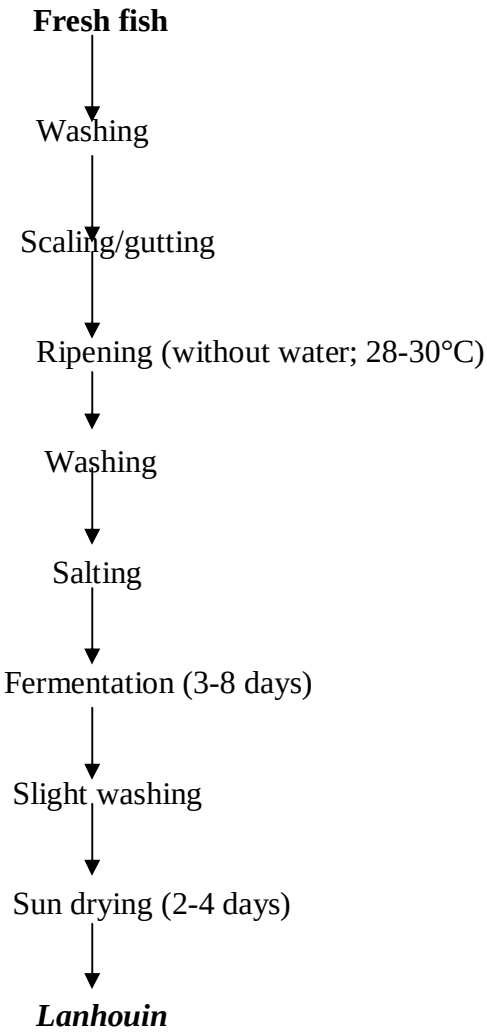


Fig. 3.1 Flow diagram of laboratory *lanhouin* processing

3.2.3 Optimization of the spontaneous fermentation conditions for *lanhouin* production

Response surface methodology (Myers, 1971) was used for the optimization of process characteristics.

3.2.3.1 Experimental design for response surface methodology

A Central Composite Rotatable Design (CCRD) of the experiment was set up using the Statgraphics plus 3.0 software with experimental variable number K = 3, for independent variables including ripening time (X_1), salt concentration (X_2) and fermentation time (X_3). Range of ripening time, salt concentration and fermentation time were obtained based on information from the local *lanhouin* processors. The dependent variables studied included the following: total viable count, sodium chloride and histamine contents of the salted and fermented fish. Twenty sample combinations were generated by the software in experimental design using the design matrix and variable combinations in experimental runs as shown in Tables 3.1 and 3.2 below. The experiments conducted on the various combinations were then tabulated accordingly and analysed using stepwise regression analysis.

Table 3.1 Process variables and their limits used in the Central Composite Rotatable Design for k = 3

Independent variables	Code	Variable levels (y)				
		- 1.682	-1	0	+1	+1.682
Ripening time (hours)	X_1	0	3.04	7.5	12	15
Salt concentration (%)	X_2	20	23	27.5	32	35
Fermentation time (days)	X_3	3	4.0	5.5	7	8

Table 3.2 Design matrix and variable combinations in experimental runs

Serial N°	Level codes			Levels (y)		
	RT (X_1)	SC (X_2)	FT (X_3)	Ripening time (h)	Salt concentration (%)	Fermentation time (days)
1	-1	-1	-1	3.04	23.00	4.01
2	-1	1	1	3.04	32.00	7.00
3	1	-1	1	12.00	23.00	7.00
4	1	1	-1	12.00	32.00	4.01
5	0	0	0	7.50	27.50	5.50
6	0	0	0	7.50	27.50	5.50
7	-1	-1	1	3.04	23.00	7.00
8	-1	1	-1	3.04	32.00	4.01
9	1	-1	-1	12.00	23.00	4.01
10	1	1	1	12.00	32.00	7.00
11	0	0	0	7.50	27.50	5.50
12	0	0	0	7.50	27.50	5.50
13	1.682	0	0	15.00	27.50	5.50
14	-1.682	0	0	0.00	27.50	5.50
15	0	1.682	0	7.50	35.00	5.50
16	0	-1.682	0	7.50	20.00	5.50
17	0	0	1.682	7.50	27.50	8.00
18	0	0	-1.682	7.50	27.50	3.00
19	0	0	0	7.50	27.50	5.50
20	0	0	0	7.50	27.50	5.50

RT: ripening time SC- salt concentration F T- fermentation time

3.2.3.2 Samples treatment during optimization process

Equal amounts of fish were fermented with sodium chloride concentrations of 20%, 23%, 27.5%, 32% and 35% by weight of fresh fish after 0, 3.04, 7.5, 12 and 15 hours of ripening (Tables 3.1and 3.2). The fishes were treated following the procedure described in 3.2.2 and allowed to ferment at room temperature (28-30°C) for 3, 4, 5.5, 7 and 8 days (Tables 3.1and 3.2). The average weight of the individual fresh fish piece was 250.00 ± 6.50g.

3.2.3.3 The optimization process

Stepwise multiple regression analyses was conducted on the data from the Central Composite Rotatable Design to relate ripening time, salt concentration and fermentation time to total viable count, sodium chloride (NaCl) and histamine contents of the samples. The response surface models were generated and presented as 3-dimensional plots in the function of 2 factors (fermentation time and salt concentrations) whilst the ripening time was kept constant. Adequacy of the model equation for predicting optimum response values was checked by conducting the lack of fit test. Diagnostic analyses were also made to check for the violation of assumptions. Three optimal processing conditions of the fermentation procedures of the fish were determined from the mathematical models. In order to get these optimal values, the first partial derivatives of the regression equations were done according to X_1, X_2 and X_3 and sorted.

3.2.3.4 Statistical analysis

All the statistical analysis and graphical presentations were done using Statgraphics (Graphics Software Systems, STCC, Inc., Rockville, USA). The significant probability was set at $p < 0.05$.

3.2.4 The processing of cassava fish into *lanhouin* using the optimal fermentation conditions

The fish was processed as described in 3.2.2 using the optimum operating conditions evolved from the optimization study. A ripening time of 8 hours and salt concentration of 30 % by fresh

fish weight were mainly used. For the monitoring of fermentation, samples were taken at predetermined periods (0, 1, 2, 3, 4, 6, 8 and 10 days) for microbiological and chemical analyses.

3.2.5 Sensory evaluation of laboratory samples of *lanhouin*

Coded laboratory and market samples of *lanhouin* were presented to a panel of 24 untrained judges. The ballot sheet is given in Appendix 4. The multiple paired comparisons test was used. The test was designed to determine which, of the two samples of *lanhouin* was liked more in terms of aroma, texture, colour, taste and overall acceptability. The samples evaluated had been fermented for 4 and 8 days, respectively.

3.2.6 Production of modified *lanhouin* using a starter culture

Strains of predominant microorganisms originally isolated during the spontaneous fermentation study of *lanhouin* were used as starter cultures after characterization. The three predominant organisms isolated were utilised as single and mixed starter cultures to inoculate the fish flesh samples. The experimental design is given in Table 3.3.

Table 3.3 Starter cultures tested during the inoculated fermentation of *lanhouin*

Microorganisms	Starter cultures
A	A
	B
	C
B	A × B
	A × C
C	B × C
	A × B × C

Seven (7) combinations of starter cultures were used for fermentation according to the experimental design previously described.
Three (3) single inoculation experiments and four (4) mixed inoculation experiments

- A non-inoculated sample was used as Control

3.2.6.1 Inoculum source and preparation

Colonies of organisms previously isolated during the spontaneous fermentation were grown overnight at 30°C in Nutrient Broth (oxoid CM0001) and cells were harvested by centrifugation at 3000 rpm for 10 min. The pellet was then washed once in 10 ml sterile peptone physiological salt solution (1 g peptone, 8.5 g NaCl in 1000 ml distilled water, pH 7.2) and diluted in sterile saline solution to give a concentration of 10^3-10^4 c.f.u / ml, checked as viable count in Nutrient Agar.

3.2.6.2 Preparation of fresh fish

Fresh fish (*Pseudotolithus* sp.) was purchased at the seaport and transported in an ice chest to the laboratory. The fish was washed, scaled, gutted, beheaded and washed twice before filleting. The fish flesh was then separated from the skin and bones and then ground using a blender (Waring Commercial Blender 35B64, USA).

3.2.6.3 Formulation and fermentation of fish mixture

Salt and glassware were sterilized at 121°C for 15 mins. The ground fish flesh was collected into a baker and pasteurized at 80°C for 30 mins using an electric steam sterilizer, followed by rapid cooling to about 30°C. During pasteurization, the vane of the sterilizer was left open to avoid an increase of pressure. The fish mixture was prepared as follows: 150g of sodium chloride was added to 1000g pasteurized ground fish flesh. The ingredients were mixed by hand under aseptic conditions and approximately 200g packed into 500 ml sterilized conical flask. 2 ml suspension of vegetative cells containing approximately 10^4 cells/ml was inoculated into the 200g pasteurised fish flesh; the mixture was then homogenised using a sterilized spatula and the conical flask covered with cotton wool (Plate 3.4).

For the mixed fermentation, the suspensions of different culture starters were mixed equally and 2 ml of the final mixture inoculated into 200 g of pasteurised fish mixture. The inoculated fish mixtures were incubated at 35°C for 48 hours. Duplicate samples were taken at 0, 12, 24 and 48 h of fermentation for microbiological and chemical analyses. 6 g of potato starch was added to the

fermented samples which were then pasteurized for 30 minutes at 80°C (as indicated above) to stop fermentation. The potato starch was added to bind the fish flesh.

Plate 3.4 Inoculated fermentation of ground fish flesh with *Bacillus* species

3.3 Microbiological analyses

3.3.1 Enumeration of microorganisms from *lanhouin* samples

Ten (10) grams of each sample (cut from the head, middle and tail region of the fish) was weighed into a sterile stomacher bag with 90 ml of sterile diluents containing 0.1% peptone, 0.8 % Nacl with pH adjusted to 7.2 and macerated for 2 mins in a stomacher blender (Lab Blender, Model 400). 1 ml of the homogenate was serially diluted and used for enumeration of microorganisms.

3.3.1.1 Total aerobic mesophilic bacteria

The total aerobic mesophilic bacteria were investigated according to the Nordic Committee on Food Analysis n° 86, 2nd edition, 1986. 1 ml of appropriate dilutions were plated out in duplicate on Plate Count Agar (PCA, Oxoid CM 463). The plates were incubated at 30°C for 72 h. Only plates showing between 30 and 300 colonies were counted.

3.3.1.2 Halophilic bacteria

Halophilic bacteria were enumerated on Plate Count Agar containing 15 % sodium chloride (NaCl).

3.3.1.3 Micrococcaceae

Micrococci were enumerated according to Rodríguez *et al*., (1994) using spread plate. 0.1 ml of appropriate dilutions was placed in duplicate on Mannitol Salt Agar (MSA, Oxoid CM 85) plates, which were then incubated for 48 h at 30°C.

3.3.1.4 *Bacillus* species

Bacillus species were enumerated according to Stevenson *et al*. (1992) using Dextrose Tryptone Agar (DTA, Oxoid CM 75). Plates were incubated for 48 h at 35°C.
Bacillus spores were enumerated on DTA after 10 ml of the 1:10 dilution was heated at 80°C for 10 min.

3.3.1.5 Yeasts and moulds

Yeasts and moulds were enumerated on Malt Extract Agar (MEA, Oxoid CM 59) containing 2 ml of sterilised lactic acid, 10 % per 100 ml of medium, according to the method described by Mislivec *et al*., (1992). The plates were incubated at 25°C for 3-5 days.

3.3.1.6 Enterobacteriaceae

Enterobacteriaceae were enumerated using Nordic Committee on Food Analysis (NCFA) No. 44, 4th ed., 1995 method. In the procedure, 1 ml of appropriate dilution was transferred into a sterile petri dish after which 5 ml of Tryptone Soy Agar (TSA, Oxoid CM 131) was added and pre-incubated at 20 – 25^{o}C for 2 h. Following this, the plate was over laid with 10 – 15 ml of molted Violet Red Bile Agar (VRBA, Oxoid CM 107). After solidification, the dishes were incubated for 24h at 30°C and 44°C for coliform and faecal coli respectively.

3.3.1.7 *Salmonella*

Salmonella were detected on Xylose-Lysine-Desoxycholate Agar (Oxoid CM 469) after pre-enrichment in Buffered Peptone Water (Oxoid CM 509) and selective enrichment in Rappaport-Vassiliadis Soy peptone Broth (RV, Oxoid 669) according to the Nordic Committee on Food Analysis, n° 71, 4th ed., 1991[UDC 579.842.14] method. For the pre-enrichment, 25 g of fish samples were treated with Buffered Peptone Water in a ratio of 1: 9. The mixture was shaken and incubated at 37^{o}C for 18-20 h. 0.1 ml of pre-enrichment mixture was transferred to 10 ml Rappaport-Vassiliadis Soy Peptone Broth (Oxoid CM 669), which had been pre-warmed to the incubation temperature. Incubation was done in a water bath at 42^{o}C for 23 ± 3 h. A loopful of the material from the enrichment broth was then inoculated onto Xylose Lysine Desoxycholate Agar plates. The plates were then incubated at 37^{o}C for 24 h. Typical *Salmonella* colonies have a black centre with a slightly reddish transparent zone due to a change in the colour of the indicator.

3.3.1.8 *Clostridium* species

Clostridium spp. were detected according to the method N° 95, 3rd ed., 1997 [UDC 576.851.57] of the Nordic Committee on Food Analysis. In the procedure, 1ml of suitable dilutions were

inoculated into tubes containing 10 ml of Iron Sulfite Agar (Oxoid CM 79). After solidification the tubes were incubated under anaerobic conditions at 37°C for 48 h.

3.3.2 Characterization and identification of predominant microorganisms isolated during the spontaneous fermentation of *lanhouin*

3.3.2.1 Isolation, purification and maintenance of microorganisms

The colonies on media were randomly picked from selected plates (having 30-300 colonies per plate) to obtain representative strains at different fermentation times. The isolates were purified by successive subculturing in Nutrient Broth (NB, Oxoid CM 0001) and on Nutrient Agar (NA, Oxoid CM 3), observed under microscope before maintaining on Nutrient Agar slant and stored at 4ºC for identification.

3.3.2.2 Initial characterization of isolates

Isolates on DTA were subcultured on Nutrient Agar and *Bacillus* species recognized after initial tests comprising colony and cell morphology, Gram reaction and catalase test. The remaining isolates were identified to the genus level according to the methods described by Cowan and Steel (1974); Collins and Lyne (1984) and Benson, (1990). The tests performed included, oxidase production, aerobic and anaerobic growth, starch and casein hydrolysis, gelatin liquefaction, acid production from glucose and oxidation/fermentation (Hugh & Leifson's test) in addition to those carried out for *Bacillus* spp..

3.3.2.2.1 Gram reaction

Gram reaction was carried out using a modified method of Parry *et al* (1983). Isolates were smeared on slides and covered with crystal solution for 20 s. Crystal violet solution was washed off with distilled water and excess water drained. The smear was then covered with gram iodine solution and left to stand for 60 s and then poured off. The smear was then flooded with 95 % ethyl alcohol for 15 s. The alcohol was rinsed off and the smear covered with safranin for 20 s. This was then gently washed for a few seconds and blotted with bibulous paper. The slide was then examined under oil immersion.

3.3.2.2.2 Catalase activity

For catalase activity, a loopful of the culture was mixed into a drop of 3 % hydrogen peroxide on a microscope slide. The slide was then observed for the production of gas bubbles to indicate the production of catalase (Cowan and Steel 1974).

3.3.2.2.3 Oxidase test

The test was carried out according to the method of Kovacs (1956) described by Cowan and Steel (1974). A piece of Whatman No.2 filter paper was placed in a petri dish and 2 to 3 drops of 1% tetramethyl-*p*-phenylenediamine dihydrochloride (BDH 2596) were added. A loopful of the test organism from a pure colony was smeared on a small area of the impregnated filter paper. A positive reaction was shown by the development of dark purple colour within 10-15s.

3.3.2.2.4 Starch hydrolysis test

Starch hydrolysis test was carried out according to the method described by Harrigan (1998). Starch Agar containing 10 g starch (Merck 1259), 23 g Nutrient Agar (Oxoid CM 3) and 1000 ml distilled water was prepared, poured into sterile petri dishes, inoculated in duplicate with the test organisms and incubated at 30°C for 5 days. Hydrolysis of starch was determined at 3 and 5 days by flooding the plates with 95% ethanol. The clear zones around colonies indicated hydrolysis of starch. Unhydrolysed starch became white and opaque within 15 to 30 minutes.

3.3.2.2.5 Casein hydrolysis

Casein hydrolysis was determined according to Harrigan (1998). Skim Milk Agar plates containing 20 g agar N°1 (Oxoid L 11), 100 g skim milk (Difco 0032-17-3) and 1000 ml distilled water were inoculated with single streaks, incubated at 30°C and examined for clear zones around growth at 2, 7, and 14 days indicating decomposition of casein and proteolytic activity.

3.3.2.2.6 Acid production from glucose

For acid production from glucose, 3 ml slants containing 150 microlitres of 10 % filter sterile solution of D (+) Glucose (Merck 8337) and a basal medium containing per litre distilled water: 1

g, diammonium hydrogen phosphate (Merck 11570), 1 g, potassium chloride (Merck 117601), 0.2 g, magnesium sulphate (Merck 115573), 0.2 g, yeast extract (Oxoid L 21) and 15 g, agar (Oxoid L 11), 0.006, bromocresol purple (Merck 111376), pH 7.0 were inoculated with the test microoganism and incubated at 30^{o}C. Acid production was indicated by a change in the colour of the medium from purple to yellow (Cowan and Steel, 1974; Benson, 1990).

3.3.2.2.7 Hugh and Leifson's test

For oxidation / fermentation test, a basal medium containing per litre distilled water: 2 g peptone (Oxoid L 34), 5 g NaOH, 0.3 g K_2HPO_4 (Merck) and 15 ml of 2 % bromocresol blue (Merck 3026) was prepared. The pH was adjusted to 7.1 and the medium sterilised at 115^{o}C for 20 mins. The basal medium was melted before use, cooled down to 45^{o}C and 1% glucose (Merck 8337) sterile filtered into the medium (4 ml sterile 50% glucose solution per 200m1 of the basal media) and dispensed aseptically in sterile tubes. Two tubes were inoculated with each organism and one of the tubes topped with paraffin oil to obtain anaerobic conditions. After incubation for 2 to 7 days at 30°C, fermentation reactions were indicated by yellow colouration in both aerobic and anaerobic tubes due to formation of acid. Oxidation reactions were indicated by yellow colouration in the top section of the aerobic tubes whilst anaerobic tubes maintained their original colour (Cowan and Steel, 1974; Benson, 1990).

3.3.2.3 Identification of *Bacillus* species

The isolates recognised as *Bacillus* were identified to species level according to Parry *et al*., (1983) and Claus and Berkeley (1986). Both morphological examinations and biochemical tests were performed. In the morphological examinations, the shape and positions of spores were noted. The biochemical tests carried out included: Voges-Proskauer reaction, pH in Voges-Proskauer medium, acid production from D-glucose, D-xylose, D-mannitol and L-arabinose, hydrolysis of starch and casein, liquefaction of gelatin, nitrate reduction, growth at pH 5.7 and 6.8, tolerance to 7 % NaCl and growth at 50°C.

Starch hydrolysis and casein decomposition were investigated using the methods described in 3.3.2.2.4 and 3.3.2.2.5 respectively. The results of identification were confirmed by tests conducted in API 50 CH strips (BioMérieux).

3.3.2.3.1 Acetyl methyl carbinol production

Aliquots of 5 ml of Voges-Proskauer broth containing per litre distilled water: 5 g, tryptone (Oxoid L 42) and 15 g, yeast extract (Oxoid L 21) , pH 6.5, were inoculated in triplicates with the test organism for acetyl methyl carbinol production after 3, 5 and 7 days incubation at 30°C. The resultant culture was mixed with 3 ml of 40 % (w/v) sodium hydroxide and 1 mg of creatine (Merck 5205) and acetyl methyl carbinol production was indicated by the development of a red colour within 30 to 60 min. The pH of the 7-day-old culture was measured before it was tested for carbinol production (Cowan and Steel, 1974; Benson, 1990).

3.3.2.3.2 Hydrolysis of gelatine

For liquefaction of gelatine, the test cultures were inoculated on Gelatine Agar containing: 4 g gelatine (Oxoid L 8), 50 ml distilled water and 1000 ml Nutrient Agar (Oxoid CM 3). The plates were incubated at 37°C for 3 days after which they were flooded with saturated ammonium sulphate solution (Merck 1211). Hydrolysis of gelatine was indicated by a halo around colonies of organisms producing gelatinase (Cowan and Steel, 1974; Benson, 1990).

3.3.2.3.3 Nitrate reduction

Test tubes with Durham's tubes containing 10 ml of nitrate broth [5 g neutralized bacteriological peptone (Oxoid L 37), 1 g potassium nitrate, 3 g beef extract (Merck) and 1000 ml distilled water] were inoculated with the test organisms and incubated at 30°C for up to 14 days. After 3 and 7 days, cultures were tested for the presence of nitrite by the addition of 1 ml of 0.8% sulphanilic acid (Fluka 96090) solution in 5 N acetic acid followed by 1ml of 0.5% α-naphtylamine (Analar 10164) solution in 5 N acetic acid to observe for the development of a red colour within 2 mins and the accumulation of gas in the Durham's tubes. Red colour showed the presence of nitrite and indicated that nitrate had been reduced. Cultures negative at 7 days were tested again after 14 days. About 5 mg of zinc dust (Merck 1110025) were added to tubes still appearing negative to reduce any residual nitrate to nitrite. True negative cultures now turned red indicating the presence of nitrate and consequently the absence of reduction (Cowan and Steel, 1974; Benson, 1990).

3.3.2.3.4 Acid production from carbohydrates

The acid production test from carbohydrates was carried out according to the method described in 3.3.2.2.6 using 150 micro litres of 10% filter sterile solutions of L(+) Arabinose (Merck1492), D(+) Xylose (Merck 8689) and D(-) mannitol (Merck 5987) separately.

3.3.2.3.5 Growth in 7% sodium chloride

For growth in 7% sodium chloride, tubes containing 3 ml of Nutrient Broth (Oxoid CM 67), with 7% (w/v) sodium chloride were inoculated with the isolates and incubated at 30°C and observed for growth after 7 and 14 days (Cowan and Steel, 1974; Benson, 1990).

3.3.2.3.6 Growth at pH 5.7 and 6.8

Growth at different pH values was studied by adjusting the pH of Sabouraud Dextrose Agar slants [15 g agar (Oxoid L 11), 10 g peptone (Oxoid L 34), 40 g dextrose (Merck 11 4353), 1000 ml distilled water, pH 5.7] and Sabouraud dextrose broth [20 g dextrose (Merck 11 4353), 10 g peptone (Oxoid L 34), 1000 ml distilled water, pH 6.8] to different levels using 1 N hydrochloric acid or 10 % (w/v) sodium hydroxide. The tubes were inoculated with test organisms, which had previously been grown in Nutrient Broth (Oxoid CM 67), incubated at 30°C and observed for growth for up to 14 days (Cowan and Steel's, 1974; Benson, 1990).

3.3.2.3.7 Growth at different temperatures

For growth at 50°C the test organisms were inoculated on Nutrient Agar (Oxoid CM 3) and incubated at 50°C and examined for growth.

3.3.2.3.8 API 50 CH and API 50 CHB medium test

For the API test, isolates were grown on Nutrient Agar at 35°C for 16 –18 hrs and the cell were heavy suspended in 1 ml of sterile saline water containing per litre distilled water 9 g NaCl. A certain number of drops of this suspension (noted S) were transferred into NaCl 0.85% medium and its turbidity adjusted to 2 McFarland. Then, a quantity of the bacterial suspension corresponding to 2 S was transferred into one tube of API 50 CHB medium containing 10 ml of medium. The API 50 CH strips were placed in the incubation trays into which 10 ml of distilled

water had been distributed into the honeycomb to maintain moist conditions and the tubes of the strips containing the dehydrated substrate filled with the inoculated API 50 CHB medium using a sterile pipette. The strips were incubated at 30°C and read after 24 and 48 h of incubation and the strain identified by referring to the API reference table.

3.3.2.4 Identification of *Micrococcus* species

The isolates recognised as *Micrococcus* were identified to species level according to Schleifer *et al*. (1981) and Kocur (1986). Tests performed included: starch and gelatin hydrolysis, nitrate reduction, Voges-Proskauer test, phosphatase, growth on Nutrient Agar with 7.5 and 10 % NaCl, and aerobic acid production from D-glucose, D-mannose, galactose and lactose. Aerobic acid production from galactose, D-glucose, D-mannose and lactose was determined as described in 3.3.2.2. Acetoin production, nitrate reduction and growth on Nutrient Agar with 7.5 and 10 % NaCl were investigated according to the methods described in 3.3.2.3.

3.3.2.4.1 Phosphatase

Phenolphthalein Diphosphate Phosphate Agar containing, 1000 ml nutrient agar (Oxoid CM 3), 10 ml phenolphthalein diphosphate 1% solution (Merck) was inoculated with the test cultures and the plates incubated at 30°C for 3 to 5 days. 0.1 ml of ammonia solution (Analar 100115Q) was placed in the lid of the Petri dish and the medium was inverted above it. Free phenolphthalein liberated by phosphatase reacted with the ammonia and phosphatase positive colonies became bright pink (Cowan and Steel, 1974; Benson, 1990).

3.3.2.5 Identification of *Staphylococcus* species

The isolates tentatively recognised as *Staphylococcus* were identified to species level according to Kloos and Schleifer (1986). The main tests carried out included: phosphatase activity, nitrate reduction, acetoin production test, growth on Nutrient Agar with 10 and 15 % NaCl, coagulase test, and aerobic acid production from D-glucose, D-xylose, D-mannitol, D-galactose, maltose and sucrose.

Aerobic acid production from D-glucose, D-xylose, D-mannitol, α- lactose, D-galactose, maltose and sucrose was carried out as described in section 3.3.2.2. Nitrate reduction, acetoin production test**,** growth on nutrient agar with 10 and 15 % NaCl and growth on nutrient agar at 45°C were

investigated as described in 3.3.2.3 and 3.3.2.4. The results were confirmed using API Staph strips (REF 20500, BioMérieux).

3.3.2.5.1 Coagulase test

For coagulase test the slide method was used. The cultures of test organisms were emulsified in a drop of sterile distilled water on a slide. A straight wire was dipped into rabbit plasma (Ref 55181) and stirred with the bacterial suspension and observed for visible clumping within 10-20s (Collins and Lyne, 1984).

3.3.2.5.2 API Staph test

For the API Staph test, the isolates were grown on P agar (peptone, 10 g; yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; agar, 15 g; distilled water, 1litre) for 18 -24 h at 36°C ± 2°C and a homogeneous bacterial suspension with a turbidity equivalent to 0.5 McFarland was prepared using API Staph medium. The API strips were then inoculated by filling only the microtubes with the inoculated API Staph medium. To ensure anaerobiosis in the ADH (arginine dihydrogenase) and URE (urease) tests, the cupules were filled with mineral oil. The inoculated strips were incubated at 36°C ± 2°C for 18- 24 h.

3.3.2.6 Enzymatic analysis

3.3.2.6.1 Proteolytic activity of predominant organisms

For proteolytic activity of predominant organisms, plates of Skim Milk Agar containing per litre distilled water: 100 g, Skim Milk (Difco 0032-17-3) and 20 g, Agar (Oxoid L11), were inoculated with single streak of inoculum, incubated at 30°C for 3 days and examined for clear zone around the colony to indicate proteolytic activity. The width of the clear zone around the colony was measured each day with a ruler (Cowan and Steel, 1974; Benson, 1990).

3.3.2.6.2 Lipolytic activity of predominant organisms

Lipolytic activity was examined on tubes of tributyrin agar containing per litre distilled water: 5 g, peptone (Oxoid L 37), 3 g, yeast extract (Oxoid L21), 10 g, tributyrin (Sigma T-8626) and 12 g, agar (Oxoid L11). The tubes containing the medium were inoculated with the test organisms

and incubated at 30°C for 3 days. A clear zone developed in the tube indicated degradation of tributyrin. The extent of lipolytic activity was determined each day by measuring the depth of the clear zone (Cowan and Steel, 1974; Benson, 1990).

3.4. Chemical analysis of lanhouin samples

The physico-chemical and biochemical changes associated with the fermentation of the fish were monitored. The following characteristics were determined: pH, titratable acidity, water activity, moisture, total volatile nitrogen, free fatty acids, crude protein, sodium chloride, thiobarbituric acid number, histamine and flavours components. The analyses were carried out on the samples at various stages of fermentation.

3.4.1 pH determination

Fish flesh samples weighing 20 g were blended and 80 ml of distilled water was added. The mixture was homogenized using a stomacher (Lab Blender, Model 400) and the pH determined with a pH-meter (Hanna Instrument HI 9318).

3.4.2 Titratable acidity

Titratable acidity as percent, w/w of lactic acid was determined according to the method of AOAC (1995). In this procedure, 5ml of the suspension used for pH determination was mixed with 75 ml distilled water. Two drops of phenolphthalein were then added and the mixture titrated against 0.1N sodium hydroxide.

3.4.3 Water activity

Water activity was measured with a thermo-hygrometer recorder C056696. This method consists of sealing the fish in a container, which is held at a constant temperature until the water vapour in the air reaches equilibrium with the moisture in the product; a probe determines the humidity above the product. The meter measured both the temperature and equilibrium relative humidity. The water activity was determined from the relationship (Troller *et al*., 1992):

$$Aw = \frac{ERH}{100}$$

Where Aw = water activity and

ERH = equilibrium relative humidity percent

3.4.4 Moisture content

Moisture was determined using AOAC, (1995) method 950.46. About 5 g of blended fish muscle were weighed into a metal dish containing about 20 g of treated sand. The sample was mixed with the sand using the glass rod to achieve as homogeneous a mixture as possible. The dish, its contents including the rod were dried to constant weigh at 103 ± 2°C.

3.4.5 Total volatile nitrogen

Total volatile nitrogen (TVN) was determined according to Pearson (1976). Ten (10) grams of raw fish or fermented fish samples were macerated with 100 ml tap water. The macerated sample was transferred to a macro-Kjeldahl distilling flask. 2 g magnesium oxide and 200 ml tap water were added. A 500 ml receiving flask containing 25 ml of 2 % (w/v) boric acid solution and 2-3 drops of protein indicator (methyl red: bromocresol green 1: 5) was connected to the distillation apparatus. The distillate collected in boric acid solution was back titrated with 0.1N sulphuric acid. The TVN expressed as mg N per 100 g flesh was obtained by multiplying the titration (less blank) by 14.

3.4.6 Free fatty acids

Free fatty acids (FFA) were determined by the method of Pearson (1976). One gram of the crude fat extract from the fish sample was mixed with a neutral solvent (mixture of 25 ml diethyl ether with 25 ml alcohol and 1 ml of 1 % phenolphthalein, neutralised with 0.1M sodium hydroxide) and titrated with aqueous 0.1 M NaOH.

3.4.7 Crude protein

Crude protein was estimated using AOAC (1995) method 981.10. 1.5–2.5 g of blended fish flesh sample was transferred to a Kjeldahl flask and 0.5 g $CuSO_4 \cdot 5\ H_2O$, 15 g K_2SO_4 and 25 ml sulphuric acid and some glass beads were added. The mixture was digested at 420°C for 90 min. 50 ml distilled water was added to the mixture. The digestion tube containing the diluted digest was attached to the distillation unit and 75 ml of 33 % sodium hydroxide added. The distillate collected into 25ml boric acid solution with indicator was back titrated with 0.2 N HCL. Blanks were prepared and treated similarly.

3.4.8 Salt as sodium chloride

The salt level was estimated by titration against silver nitrate (AOAC, 1995; method 937.09). Known volumes of silver nitrate ($AgNO_3$) and nitric acid (HNO_3) were added to 10 g blended fish meat, and the mixture boiled for 15 min. After cooling, 50 ml of distilled water and 5 ml of ferric indicator were added to the mixture followed by titration with 0.1N ammonium thiocyanate (KSCN).

3.4.9 Histamine

Histamine content was determined using AOAC, (1995) method 977.13. The histamine was extracted from the finely minced fish flesh using 40 ml methanol. The extract was then passed through an anion exchange resin (Bio-Rad AG 1-X8, 50–100 mesh) to remove interfering substances. Histamine in the eluent was reacted with O-phthalicdicarboxaldehyde (OPT) to generate a fluorescent compound. The corresponding fluorescence was then read on a calibrated fluorometer -Turner model 450.

3.4.10 Thiobarbituric acid number

Thiobarbituric acid value (TBA) was measured according to Pearson (1976). Ten (10) g of sample was macerated in 50 ml water using a stomacher blender (Lab Blender Model 400) for 2 mins and washed into a distillation flask with 47.5 ml water. 2.5 ml of 4 M hydrochloric acid were added followed by antifoaming preparation and a few glass beads, and the mixture heated so that 50 ml distillate could be collected in 10 min. Five (5) ml of TBA reagent (0.2883g/100 ml of glacial acetic acid) was added to 5 ml aliquots of the distillate, which were then heated in

boiling water for 35 min. The tubes were cooled and the absorbance read at 538 nm using a spectrophotometer (Shimadzu UV- 240). A blank was similarly prepared using 5 ml water and 5 ml TBA reagent.

3.4.11 Determination of aroma compounds of *lanhouin* samples by GC-MS

Aroma compounds associated with the laboratory samples of *lanhouin* obtained by spontaneous and inoculated fermentations were investigated using the simultaneous steam distillation and extraction (SDE) method (Likens-Nickerson, 1966) and gas chromatography (GC) /mass spectrometry (MS) analysis.

3.4.11.1 Sample preparation and extraction procedures

A fermented fish sample weighing 10 g was mixed with 200 g distilled water in a 1 litre Erlenmeyer flask to obtain a 5 % slurry (w/v) and one (1) milliliter internal standard solution (50 ppm, methyl octanoate in H_20) added to the fermented fish slurry. Six (6) milliliters of a mixture of pentane and diethyl ether (1:1) were placed in a 9 ml pear-shaped solvent flask. Both flasks were connected to the distillation apparatus and extraction of volatiles carried out for 30 mins, after the sample started boiling. The aqueous-solvent mixture was then placed in a freezer to freeze out the aqueous solution and the solvent extract phase was poured off, dried over about 2g of anhydrous sodium sulfate (Na_2SO_4) and concentrated to about 100 mg by gently blowing nitrogen gas over the surface. The concentrated extract was then analyzed for aroma compounds using GC-MS.

3.4.11.2 Gas chromatography and Mass spectrometry

Detection of aroma compounds present in extracts of the fermenting fish samples were performed on a Hewlett-Packard HP 6890 Gas Chromatograph (Hewlett-Packard, Avondable, Pennsylvania) equipped with a Hewlett-Packard DB-WAX column (30 m × 0.25 µm i.d., × 0.25 mm film thickness) and connected to a Hewlett-Packard 5973 Mass Spectrometer (Hewlett-Packard, Avondable, Pennsylvania). 100 microlitre extracts were injected (split ratio 1:20) using the temperature program: 10 min at 40°C, increased to 240°C at 6°C /min, and held constant at 240°C for 30 min. Identification of aroma compounds was done in the Total Ion mode, scanning a mass to charge ratio (m/z) of range between 25 and 550. Further identification was obtained by

probability-based matching with mass spectra in the G 1033A NIST PBM Library (Hewlett-Packard) containing 75,000 reference spectra as well as by matching with the mass spectra and retention indices of the standard reference compounds used.

3.4.12 Statistical analysis

Statistical package for Social Science (SPSS version 10) was used. Data analyses involved one-way analysis of variance (ANOVA). Mean differences were determined using Ducan's Multiple Range Test. Significant difference was established.

4.0 RESULTS AND DISCUSSION

This study involved field investigations conducted on *lanhouin* processors and retailers, as well as the examination of the quality characteristics of *lanhouin* on sale in urban and rural areas; preliminary work followed by the study of natural fermentation of cassava fish (*Pseudotolithus* sp.) including mainly, the optimization of the fermentation conditions, isolation and identification of the predominant organisms involved in the fermentation. Inoculated fermentation for modified *lanhouin* production was also investigated. The physico-chemical and microbiological changes of the fish during processing were determined.

4.1 Field investigation

4.1.1 Social profile of *lanhouin* processors

The survey of *lanhouin* producers showed that the production and retailing of *lanhouin* is carried out mainly by women. This is the main economic activity of the women of the *Xla* and *Mina* ethnic groups of the coastal region of Benin. Most of the processors interviewed (95%) have family relations with the fishermen. All the forty-one (41) processors interviewed were females aged between 18 and 50 years. In general, they have a low educational level. Most of them (85%) had no formal education; a very small number (5%) had secondary education and the remaining (10%) had only primary education. The production of *lanhouin* constitutes for seventy five percent (75%) of the processors main source of income. Ninety five percent (95%) of the processors interviewed work independently whilst five percent (5%) work in groups. Three categories of processors were identified: the large scale processors who produce more than 200 kg of *lanhouin* per month, the medium scale processors who produce between 25 and 50 kg per month and the small scale processors who produce less than 25 kg per month.

4.1.2 Commercialization of *lanhouin*

In Benin, *lanhouin* serves different markets: namely urban and rural markets as well as the sub-regional markets. Medium and small-scale processors mainly sell their products in urban and rural markets in Benin. Another important category of stakeholders in the *lanhouin* sector are the retailers who distribute the product throughout the different markets of the country as found through the survey (Figure 4.1). Exports to the neighboring countries of Togo and Ghana are mainly done by women traders from Togo and Ghana (65%) and by the large scale processors

(35%) from Benin. Exports to these neighboring countries are not recorded but represent significant amounts. According to the large scale processors who are engaged in the transborder trade of *lanhouin*, more than 50 % of their production is exported to Togo and Ghana. Each year, about 3000 metric tonnes of *lanhouin* is produced. In 2001 this represented about 10 % of the marine catch and 2.5 % of the total catch (Gbaguidi, 2001).

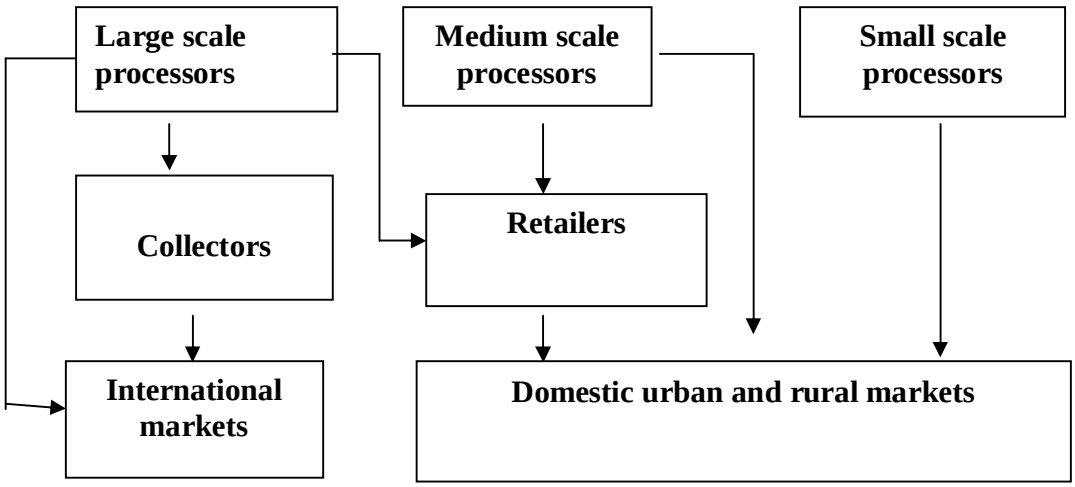


Figure 4.1 Flow diagram of the marketing of *lanhouin*

4.1.3 Production and storage of *lanhouin*

During the survey, all the twenty one (21) *lanhouin* processors interviewed said that normally, they do not ice the fish after purchase because the product that results from the use of iced fish does not have the desirable soft textural characteristics of the fermented fish. The processing sites were located close to the beach; processing activities were carried out late in the evening or early in the morning and this was to avoid high temperature during the first stage of processing and to prevent contact of fish with flies. None of the processors interviewed claimed they had any training in the handling and the processing of fish. They said that their knowledge in the fish handling and processing had been acquired through observing fellow processors as well as many years of experience in *lanhouin* processing.

For *lanhouin* processing the usual raw materials used are fresh fish, which had not been subjected to any cooling preservation and the addition of coarse salt. The survey showed that many different types of fish are used to produce *lanhouin* and those mentioned by the processors are showed in Table 4.1.

Ninety seven percent (97%) of the processors claimed that they used cassava fish (*Pseudotolithus* sp.) primarily for *lanhouin* processing; ninety one percent (91%) of the processors claimed usage of lesser African threadfin (*Galeoides decadactylus*) as their second choice for *lanhouin* processing whilst seventy seven percent (77%) of the processors claimed they used king fish (*Scomberomorus tritor*) as their third choice for *lanhouin* processing.

Very slight variations were observed in the procedures used for *lanhouin* processing. In general, fresh fish are left overnight at room temperature, after gutting or without gutting for spoilage to set in. When the fish are not gutted they are immersed in sea water while the gutted fish are not immersed in sea water. However, fish, which has become stale as a result of poor handling are ready raw material for processing and are not left overnight. According to the processors, this first step, which involves leaving the fish overnight, is very important for the production of good, soft textured and well flavoured *lanhouin*. Ninety two percent (92%) of processors interviewed gutted their fish before leaving them overnight whilst eight percent (8%) did not.

A second variation in the processing is the period during which, gutted or ungutted fish are left at room temperature. This varied between 10 and 15 h. The fish are then washed, drained and salted with coarse salt to fish weight ratio of 20 to 35 %. After salting, the fish are arranged in a basket, in a polyethylene bowl or in a hole, covered and allowed to ferment for 3 to 8 days.

Table 4.1 Types of fish commonly used in Benin for *lanhouin* processing

Local name	Common name	Scientific name
Agbanmandoui/ Zadou	Kingfish/Spanish mackerel	*Scomberomorus tritor*
Ekan /Djoké	Cassava croaker	*Pseudotolithus* sp.
Figni	Senegal jack	*Caranx senegallus*
Finvi	-	*Larimus peli*
Gbohloué	Milk shark	*Rhizoprionodon acutus*
Glanmatan / Kobi	Longfin pompano	*Trachinotus goreensis*
Guinfio / Guinlénou	Royal threadfin	*Pentanemus quinquarius*
Hawui	Bigeye grunt	*Brachydeuterus auritus*
Kokoui	Bastard grunt	*Pomadasys incisus*
Kokovi	-	*Corvina nigrita*
Kpankpan	Crevalle jack	*Caranx hippos*
Lizi	Guachanche barracuda	*Sphyraena guachancho*
Signivi	Atlantic horse mackerel	*Trachurus trachurus*
Sika-Sika	Congo dentex	*Dentex congoensis*
Tchikoué	Lesser african threadfin	*Galeoides decadactylus*
Zozrovi	Atlantic bumper	*Chloroscombrus chrysurus*

After three days of fermentation, the fish is removed from the tank, washed lightly and dried for 2 to 4 days. If fermentation exceeds 3 days, the fish is salted again with a ratio of 15 to 25 % of the salt used for the first salting and then arranged again in the same tank for 5 days.

Seventy seven percent (77%) of processors surved used a basket for fermentation whilst twenty one percent (21%) used polyethylene can and two percent (2%) used a hole dug in the ground. On the mode of storage of *lanhouin*, the majority of processors (94%) stated that they store their product in baskets while the remaining (6%) stored the *lanhouin* in a can or hole.

Utensils and other simple pieces of equipment such as knives used by *lanhouin* processors are showed in Table 4.2.; the flow diagrams of the artisanal processing of *lanhouin* as revealed by field study are shown in Figure 4.2.

Table 4.2 Utensils, etc used for *lanhouin* processing

Processing steps	Utensils, etc
Washing	Bowl
Gutting	Knife
Salting	Bowl
Fermentation	Basket, can, hole, claypot
Washing off salt	Bowl
Storage and packaging for sale	Basket, cement paper bag

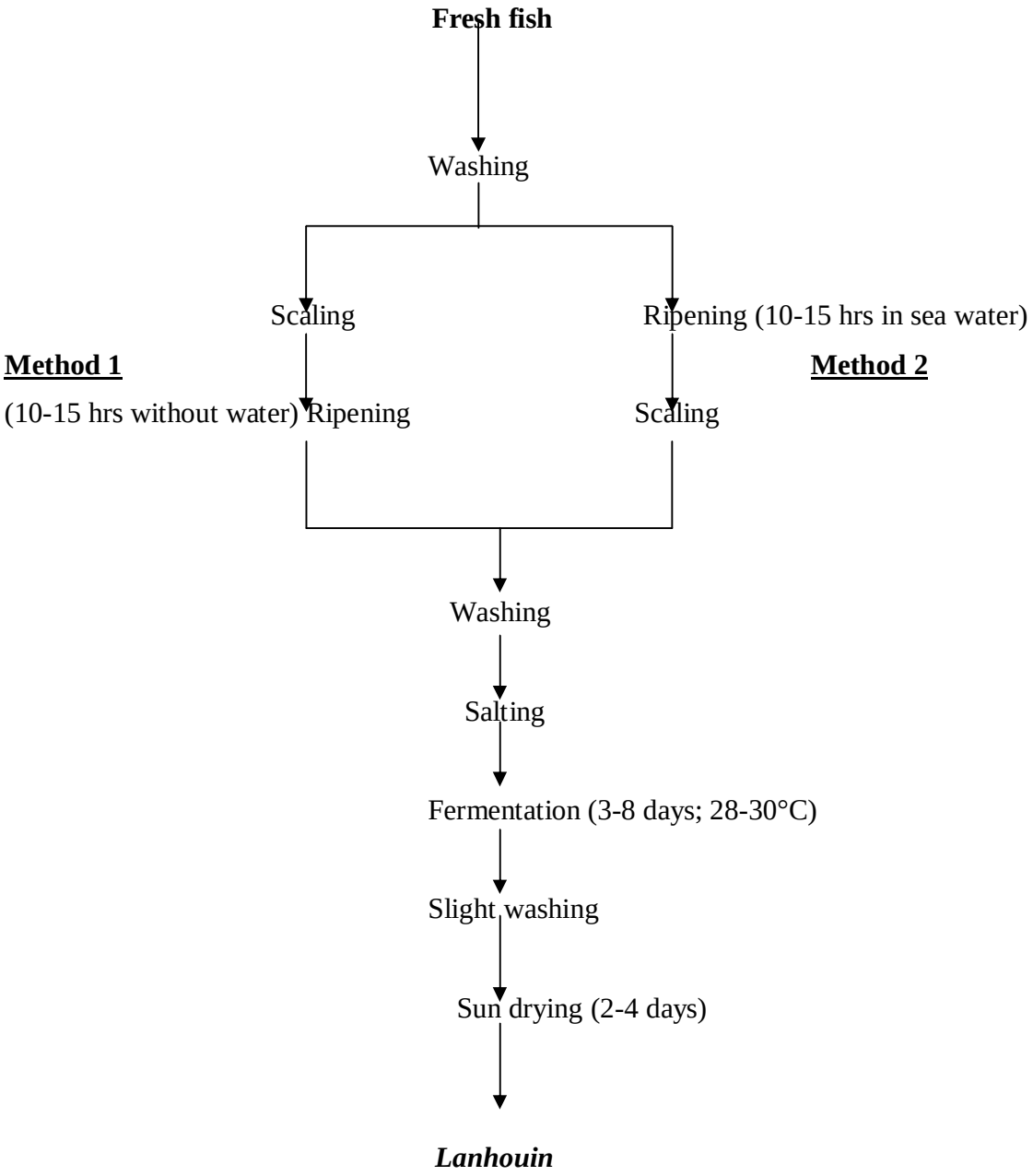


Figure 4.2 Flow diagrams of the traditional processing of *lanhouin*. In both procedures, the fish is sometimes salted a second time if fermentation exceeds 3 days.

4.1.4 Major problems encountered in the *lanhouin* sector

The major problems observed with the *lanhouin* processors are the general unhygienic conditions of the processing environment as well as the equipment used for processing. Other improper handling practices noticed include the use of dirty water for washing the fish and improper packaging of the product. Washing the fish with dirty water can cause contamination of the fish. Using dirty water contaminated with faecal matter could harbour coliforms such *Esherichia coli*, which is a prolific histamine producer. The ratio of salt used changed from one production site to another and from one processor to the other, and is not usually quantified. As salt is the only preservative agent used, low salt fermentation could possibly permit the growth of some pathogenic organisms in the product. In addition the salt is not stored under good conditions and most of the time the salt is reused in subsequent fermentations. This practice could be a potential source of contamination by halophilic bacteria. Flies also were a big problem to the processors leading to the illegal use of household insecticides to prevent flies setting on the product. The use of insecticides for this purpose poses health hazard to the consumers.

Processors usually packaged the *lanhouin* in baskets covered with old sacks, old clothes or cement paper bags during fermentation, storage and when transporting the product to the market. The unhygienic nature of these materials could be potential sources of microbial or other types of contamination.

4.2 Quality characteristics of lanhouin on sale in the urban and rural markets of Benin

Lanhouin samples made from cassava fish (*Pseudotolithus* sp.) and king fish (*Scomberomorus tritor*) were collected from processors and retailers in the markets and the processing sites for characterization. The results of chemical and microbiological analyses performed on the samples are presented below.

4.2.1 Physico-chemical characteristics of market samples of *lanhouin*

The physico-chemical composition of market samples of *lanhouin* are showed in Table 4.3. The moisture content of samples varied between 45.3 and 61.6 % with an average of 50.1 and 56.6 % for cassava fish and king fish respectively. King fish *lanhouin* recorded higher moisture contents and significant differences ($p < 0.05$) were observed within and between the fish species. The

difference in moisture content could be attributed to variable drying times and level and types of salt used for the curing. However, the moisture content seems to be a rough indicator of the susceptibility of the product to undergo microbial spoilage. A major factor, which determines the microbial, chemical and enzymatic stability of foods, is the water activity (A_w) (Owens and Mendoza, 1985; Troller *et al*., 1992).

Table 4.3 Chemical characteristics of market samples of *lanhouin* from two different species of fish

	Cassava fish (lean) n = 25	King fish (fatty) n = 25
Moisture (%)	50.1 ± 4.9a	56.6 ± 5.0b
Water activity	0.71 ± 0.06a	0.77 ± 0.10 b
pH	7.3 ± 0.6[1] a	7.6 ± 0.3b
Free fatty acids (% oleic acid)[2]	12.5 ± 1.5a	31.9 ± 4.7b
Thiobarbituric acid value (mg malonaldehyde / kg)[2]	5.7 ± 0.4a	8.3 ± 1.1b
Total volatile nitrogen (mg N/100g)[2]	294.5 ± 29.8a	374.5 ± 15.3b
Crude protein (% nitrogen × 6.25)- (g/100 g)[2]	26.5 ± 3.1a	24.6 ± 3.7a
Salt (%)[2]	7.3 ± 1.6a	5.2 ± 1.0b
Histamine (mg/100g)[2]	21.4 ± 4.0a	33.1 ± 6.6b

n: number of samples analysed, each sample in duplicate;

[1]Means ± Standard Deviations (SD); [2]Wet weight basis

a,b:Means with different letters in a row are significantly different ($p < 0.05$)

The water activity values of the samples ranged from 0.65 to 0.77 for cassava fish and 0.67 to 0.87 for king fish. The highest values of water activity (A_w) were recorded on the samples obtained from king fish and significant difference ($p < 0.05$) was observed between the measurements. These values of water activity were relatively low and not favourable for enzymatic activity and proliferation of microorganisms during storage including food poisoning

bacteria. The major histamine producing bacteria are active within A_w range of 0.91 to 1.0 (Troller *et al*., 1992).

pH values of the majority of *lanhouin* samples (78.5 %) were above 7 (6.7 – 7.9) (Table 4.3). There was a statistically significant difference ($p < 0.05$) between these values. These pH values should be considered as normal for this kind of product, since *lanhouin* is prepared from seemingly deteriorated fish. Similar higher values of pH, above 7, were reported by Nerquaye-Tetteh *et al*., (1978), Yankah (1988) and Abbey *et al*., (1994) for *momone,* a *lanhouin*-like fermented fish in Ghana. No literature on the recommended pH range of *lanhouin* is available. Similar fermented fish known as *Pedah siam* is processed in Thailand. For this product fresh fish is fermented unlike the partially deteriorated fish used for *lanhouin*. The standard pH requirement for *Pedah siam* is 6.0 - 6.4 with a pH of 6.5 or higher considered as indicative of poor quality (FAO, 1971).

Free fatty acids (FFA) contents were very high for both species of fish and ranged from 27.2 to 36.6 % and 11 and 14 % oleic acid for king fish and cassava fish respectively. The thiobarbituric acid values (TBA) varied between 5.3 and 6.1 mg malonaldehyde /kg for cassava fish whereas that obtained from king fish ranged between 7.2 and 9.4 mg. Significant difference ($p < 0.05$) was observed in the values within and between the species. High levels of free fatty acids and thiobarbituric acid number is an indication of a product that had undergone both microbial and chemical spoilage with its attendant rancidity (Pearson, 1976; Huss, 1988; Horner, 1997). According to Pearson (1976) most fat acidity begins to be noticeable to the palate when the free fatty acids values calculated as oleic acid is about 0.5 –1.5 %.

As expected, the total volatile nitrogen (TVN) levels were high for all samples with the average content of 294.5 ± 29.8 mg N/100 g for cassava fish and 374.5 ± 15.3 for king fish (Table 4.3). Similar TVN values were recorded on *momone,* a *lanhouin* like-product by Nerquaye-Tetteh *et al*., (1978) and Abbey *et al*., (1994). The level of total volatile nitrogen (TVN) in fish is commonly used as a spoilage indicator (Pearson, 1976; Silva *et al*., 1998; Kerr *et al*., 2002). TVN measurements indicate the extent of the breakdown of proteins due to bacterial and enzymatic action, leading to amines production and thus a low nutritional value of the product (FAO, 1971; Pearson, 1976; Kerr *et al*., 2002). Pearson (1976) suggests that for white–fleshed fish, TVN levels below 200 mg N/kg indicate that the fish is fresh, whereas the fish would be rejected for

human consumption when the TVN level exceeds approximately 500 mg N/kg (Silva *et al*., 1998).

The Protein levels recorded for the *lanhouin* samples ranged from 23.4 to 29.6 % for cassava fish while that of king fish varied between 20.9 and 28.3 %. A significant difference ($p < 0.05$) was observed within species but no significant difference was noted ($p > 0.05$) between species. These protein contents of the samples agreed with those reported by other workers (Nerquaye-Tetteh *et al*., 1978; Abbey *et al*., 1994).Yankah (1988) observed protein content of 57% mainly for fish fermented for two days. The variations observed in protein levels could probably be due to the variation in fish species, the level and type of salt used and the period of fermentation, which determined the degree of proteolysis during processing.

Levels of salt in all *lanhouin* samples appeared low (5.2-7.3%) when compared to the values of 10 and 15% recorded for *momone* by Nerquaye-Tetteh *et al*., (1978). However salt concentrations of 4- 6% were also reported by Abbey *et al*., (1994) for *momone*. This range of salt concentration (5.2-7.3%) is too low to inhibit microbial histidine decarboxylase activity (Gunaratme *et al*., 1995). In laboratory cultures, histamine production by bacteria may occur at 8 % salt (Ababouch, 1990) and up to 12 % salt (Gunaratme *et al*., 1995).

The histamine content of cassava fish samples ranged between 17.4 and 25.4 mg / 100g whereas that obtained for king fish varied from 26.5 to 39.7 mg/ 100g. These levels exceeded the maximum allowable level of 20 mg / 100g stipulated by the Food and Drug Administration (USA), the European Economic Community (EEC) and the Australian National Food Authority (ANFA) (FDA, 1982; CEE, 1990; ANFA, 2001). Seventy five (75%) of *lanhouin* samples obtained from cassava fish showed histamine contents higher than the allowable level of 20 mg/100g whilst all the samples (100 %) from king fish showed histamine levels higher than the recommended level. The high level of histamine in the samples could be an indication of mishandling during storage and processing, and could also be the result of the low salt concentration of samples (Ababouch, 1990; Ahmed, 1991; Silva *et al*., 1998).

4.2.2 Microbiological status of market samples of *lanhouin*

The microbiological status of market samples of *lanhoin* are summarized in Table 4.4. Total aerobic mesophilic counts of the majority (83.5%) of the samples were high and varied from 5.4 to 6.6 Log (cfu/g) for cassava fish and 6.2 to 7.8 Log (cfu/g) for king fish. The predominant microorganisms were Micrococcaceae followed by *Bacillus* spp.. Similar microfloras have been reported by various workers for *momone* and other traditional fermented fish products (Nerquaye-Teteh *et al.*, 1978; Yankah, 1988; Abbey *et al*. 1994). Coliforms and faecal coliform counts (Log cfu/g) were lower than 1 for most of the samples; however in 6 % of the samples, the coliforms counts (Log cfu/g) which ranged between 1.47 and 1.84 were observed. Mould counts (Log cfu/g) were also lower than 1 for all samples while *Clostridium* spp. counts varied between 1.68 and 1.80. *Staphylococcus aureus* was absent in the majority (82.3%) of the samples; however *Staphylococcus* counts lower than 10 cfu/g were observed in the remaining of the samples. No *Salmonella* and no yeasts were detected in any of the samples. The presence of coliform bacteria, *Staphylococcus aureus* and *Clostridium* spp., even though in few numbers in some of the samples, is still significant and show that there is need to improve the handling and processing procedures for *lanhouin.* The absence of the pathogen *Samonella* in all the samples is worth noting as this may have been the result of the salt concentration (5.2-7.3%) of the samples. It is expected that controlled material handling and fermentation will lead to lower levels of histamine and better microbial status in the product.

Table 4.4 Microbiological status of market samples of *lanhouin* (Log cfu/g) from two different species of fish

	Cassava fish n = 20	Kingfish n = 20
Total aerobic mesophilic count	6.0 ± 0.6[1]a	7.0 ± 0.8b
Halophilic count	5.0 ± 0.5a	5.8 ± 0.4a
Micrococci	5.0 ± 0.6a	5.4 ± 0.4a
Bacilli	3.7 ± 0.5a	4.1 ± 0.3a
Coliforms	< 1	< 1
Faecal coli	< 1	< 1
Moulds	< 1	< 1
Clostridium spp.	1.6 ± 0.2a	1.8 ± 0.2a
Salmonella [c]	absent	absent
Staphylococcus aureus	absent	absent

n: number of samples analysed, each sample in duplicate

[1] Means ± Standard Deviations (SD); [c] Search in 25 g sample

a,b:Means with different letters in a row are significantly different ($p < 0.05$)

4.3 Study of the spontaneous fermentation of cassava fish into lanhouin

The most important difference in the use of fermented fish between the people of the tropics and the rest of the world is related to the physical form in which they are used and the procedures employed in preparing them. The physical form and the quality of the fermented fish require very careful control of salt concentration as well as the duration of the procedure. During processing of fish into lanhouin, the fermentation period ranges between 3 and 8 days, the salt concentrations from 20 to 35 % and the ripening time from 0 to 15 hours (Anihouvi et al., 2005). The physical form and quality of the end product is highly dependent on the ability to control these factors. Thus it is important to determine processing conditions that would yield the best quality, mainly low histamine content of the product. Traditionally processed lanhouin was found to contain high levels of histamine (Anihouvi et al., 2006). In this regard, response surface methodology (RSM)

could be used to determine the most appropriate and adequate processing conditions. This part of the work was aimed at using RSM to study the optimal processing conditions for the fermentation of fish to yield low histamine content. This was followed by three different fermentation trials using the optimal processing conditions evolved from the optimization study. Microbiological and chemical changes occurring during the fermentation were investigated and the predominant organisms were identified.

4.3.1 Optimization of the spontaneous fermentation process conditions

Responses of three dependent variables (total viable count, final sodium chloride and histamine contents of the samples) to fermentation conditions for the improvement of *lanhouin* quality were followed. Estimated regression coefficients for each dependent variable obtained from these responses by stepwise multiple linear regressions are given in Table 4.5. The analysis of variance for the model for the three response variables (Appendix 2) indicated that the model was statistically acceptable at 5%, possessing no lack-of- fit (F). Therefore, the model could be used to predict the three response variables with satisfactory R^2 value higher than 80 %.

Table 4.5 Reduced model coefficients[a] estimated by stepwise multiple linear regression for Optimization of the fermentation conditions

Factors	Coefficients		
	Total viable count	NaCl	Histamine
Constant	6.65563	- 22.2161	88.5229
Linear effect			
X_1	0.1659*	0.5045*	-
X_2	-	1.6391**	-5.7265***
X_3	-	-	0.5878**
Quadratic			
X_1^2	- 0.0093*	- 0.0213	0.0197***
X_2^2	- 0.0014**	- 0.0355	0.0954***
X_3^2	- 0.0415***	-0.1782***	-
Interactions			
X1X3	-	-	-
X2X3	-	0.1172*	-
R^2	80.7	81.0	90.3
F	19.1	19.0	9.6

[a] Model on which X1 = ripening time; X2 = salt concentration; X3 = fermentation time
- : Variables not included in the model
*** Significant at $p < 0.001$
** Significant at $p < 0.01$
* Significant at $p < 0.05$

4.3.1.1 Effect of process variables on the total viable count of fermenting fish samples

The best explanatory model equation obtained for total viable count (TVC) during the fermentation of cassava fish for *lanhouin* production was:

$Z = 6.65563 + 0.165911X_1 - 0.00932644X_1^2 - 0.00149717X_2^2 - 0.0415606X_3^2$ with an R^2 of 80.7%, adjusted R^2 = 75.6 % and MSE = 0.14.

There was a strong and significant influence of the quadratic effects of fermentation time, salt concentration and ripening time on the total viable count of the fermented fish samples. However, the quadratic effect of fermentation time and salt concentration was highly significant ($p< 0.001$) compared to that of ripening time ($p<0.05$). The model could explain 80.7% of the variations in total viable count, meaning only 19.3 % of the variations were due to other factors not included in the model. The model was considered adequate with satisfactory R^2 value (>.80 %) and non significant F (19.1 %) (Table 4.5).

The response surface curves (Fig.4.3 a-c) generated showed a linear relationship of fermentation time. This suggested that the total viable count of the samples decreased as the fermentation progressed while the relationship of salt concentration with the response was curvi-linear. In addition, the response surface plots (Fig.4.3 a-c) showed that increasing the ripening time resulted in an increase in the initial bacterial load of the samples.

Fig.4.3 Total viable count of the fermenting fish samples at (a) 0 h, (b) 8 h and (c) 15 h of ripening time

(a)- Effect of fermentation and salt concentration on TVC at 0 h of ripening

(b)- Effect of fermentation and salt concentration on TVC at 8 h of ripening

(c)- Effect of fermentation and salt concentration on TVC at 15 h of ripening

Regression equation:

$Z = 6.65563 + 0.165911X_1 - 0.00932644X_1^2 - 0.00149717X_2^2 - 0.0415606X_3^2$

Where X_1= ripening time

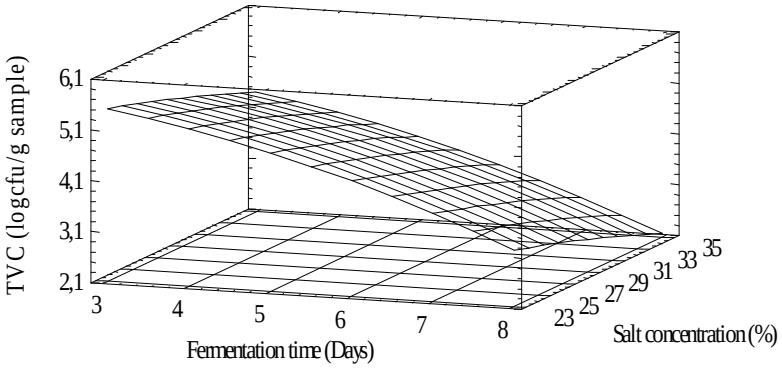
X_2 = salt concentration

X_3 = fermentation time

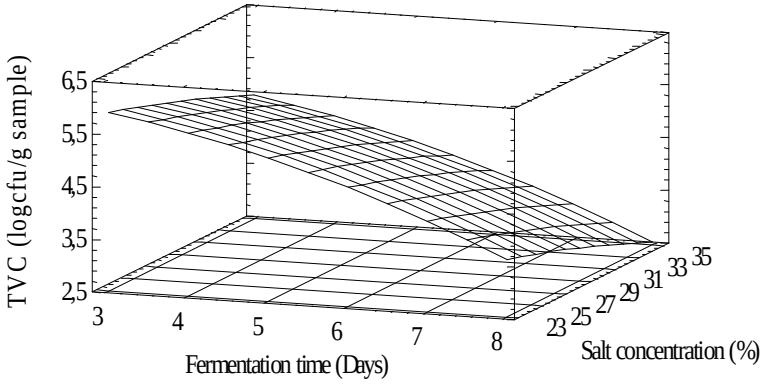
Z = total viable count of the fermenting fish samples, and

R^2 = 80.7%

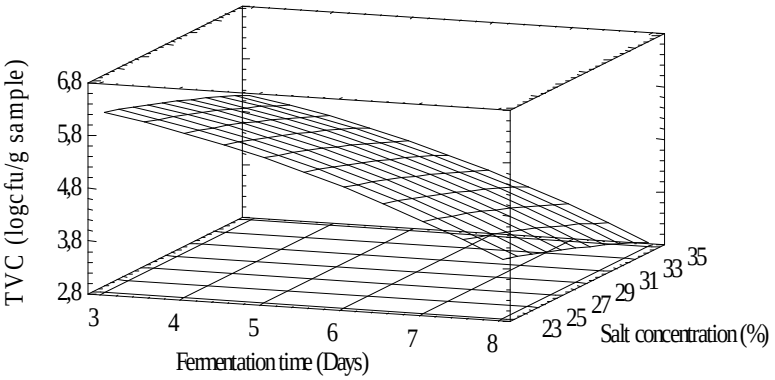
(a


TVC (logcfu/g sample)
6,1
5,1
4,1
3,1
2,1
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(b)


TVC (logcfu/g sample)
6,5
5,5
4,5
3,5
2,5
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(c)


TVC (logcfu/g sample)
6,8
5,8
4,8
3,8
2,8
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

4.3.1.2 Effect of process variables on the final sodium chloride content of fermenting fish samples

The reduced model obtained for final sodium chloride (NaCl) content when cassava fish was processed for *lanhouin* production was:

$Z = -22.2161 + 0.50458X_1 + 1.63911X_2 - 0.0213859X_1^2 - 0.0355826\ X_2^2 - 0.178205X_3^2 + 0.117226X_2X_3$ with an $R^2 = 81.0$ %, adjusted $R^2 = 72.2$ and MSE = 1.8.

For NaCl content, the regression coefficients showed that both ripening time (X1) and salt concentration (X2) had significant linear effects (($p \leq 0.05$). The quadratic effect of fermentation time (X3) was highly significant ($p \leq 0.001$) (Table 4.5). Significant interaction ($p \leq 0.01$) was also observed between the salt concentration (X2) and fermentation time (X3). The largest value of estimated regression coefficient for X2 (1.63) (Table 4.5) indicated that sodium chloride (X2) was the most important linear variable influencing NaCl content of the samples. The positive value confirmed that NaCl content of the samples increased with increasing X2. Ripening time (X1) was the second most important linear variable with a regression coefficient of 0.50 (Table 4.5). The model could explain about 81% of the variations in NaCl content of samples, indicating that about 19 % of the variation was due to other factors not included in the model. The model was considered adequate with satisfactory R^2 value (> 80 %) and non-significant F (19.0 %) (Table 4.5). The analyses of the response plots (Fig. 4.4 a–c) showed that the longer the ripening time and the fermentation time, the higher the NaCl content of the samples. A similar observation was noted with all the salt concentrations used to treat the fermenting fish samples.

Fig.4.4 Final NaCl content of the fermenting fish samples at (a) 0 h, (b) 8 h and (c) 15 h of ripening time

(a)- Effect of fermentation and salt concentration on final NaCl content at 0 h of ripening
(b)- Effect of fermentation and salt concentration on final NaCl content at 8 h of ripening
(c)-Effect of fermentation and salt concentration on final NaCl content at 15 h of ripening

Regression equation:

$Z = -22.2161 + 0.50458X_1 + 1.63911X_2 - 0.0213859X_1^2 - 0.0355826\ X_2^2 - 0.178205X_3^2 + 0.117226X_2X_3$

Where X_1= ripening time

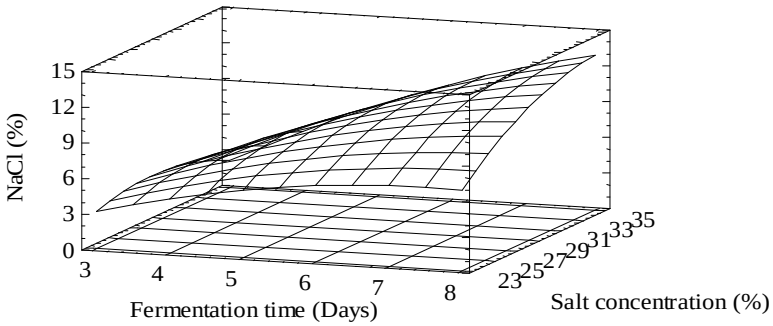
X_2 = salt concentration

X_3 = fermentation time

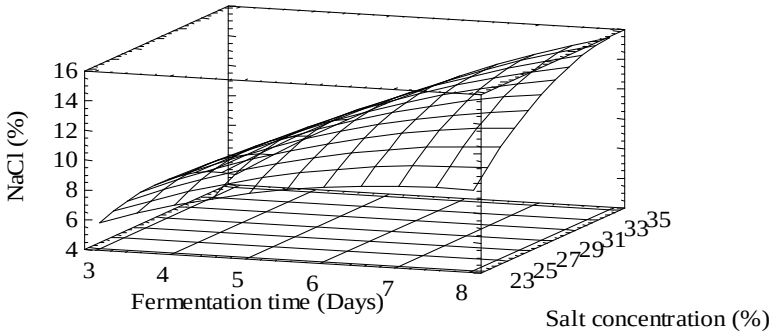
Z = NaCl content of the fermenting fish samples, and

R^2 = 81.0 %.

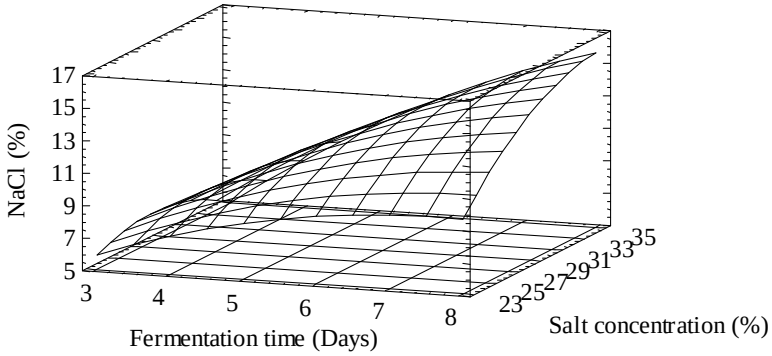
(a)


NaCl (%)
15
12
9
6
3
0
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(b)


NaCl (%)
16
14
12
10
8
6
4
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(c)


NaCl (%)
17
15
13
11
9
7
5
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

4.3.1.3 Effect of process variables on the histamine content of fermenting fish samples

The regression model obtained for histamine content when the cassava fish was used for the fermentation was:

$Z = 88.5229 – 5.72654X_2 + 0.587899\ X_3 + 0.0197967X_1^2 + 0.0954959X_2^2$

with an R^2 of 90.3 % , adjusted R^2 of 87.7 % and MSE = 1.06.

From the regression coefficients (Table 4.5), there was a strong and significant ($p \leq 0.001$) influence of linear effects of salt concentration (X2) and fermentation time (X3). The strong and significant quadratic effects ($p \leq 0.001$) of ripening time (X1) and salt concentration (X2) on the histamine content of the fermented fish samples was also observed. Salt concentration (X2) was the most important linear factor affecting histamine content of the samples and had the highest regression coefficient (-5.72), followed by fermentation time (X3). The model could explain 90.3% of the variations in histamine content, meaning only 9.7 % of the variations were due to other factors not included in the model. The model was considered adequate with satisfactory R^2 value (> 80 %) and non significant F (9.6 %). The response plots (Fig.4.5 a–c) showed that an increase in ripening time resulted in an increase in histamine content. The plots also described a curvi-linear relationship between salt concentration and the response. This implies that the histamine content of the samples reduced on defined range of salt concentration.

In summary, from this study it appeared that the Central Composite Rotatable Design (CCRD) and Response Surface Methodology (RSM) can be used effectively to estimate the effect of ripening time, salt concentration and fermentation time, and their interactions on the optimal processing conditions of fish fermentation for *lanhouin* production. The results showed that the ripening time, salt concentration and fermentation time influenced significantly ($p \leq 0.05$) most of the quality index of the fermented fish. Based on the results from study parameters, mainly histamine levels in the samples, ripening time of 8 h, salt concentration of 25-30 % and fermentation time of 4–5 days could be recommended as the optimum fermentation conditions required to achieve the optimum quality of the fermented fish. The regions that satisfy these conditions were obtained by superimposing Fig. 4.3 a-c, Fig. 4.4 a-c and Fig.4.5a-c over each other to obtain the shaded areas. These conditions give the best quality characteristics of *lanhouin*

in terms of TVC level (10^4-10^5 cfu/g), final NaCl content, between 9-10 %, and histamine content of less 15 mg / 100g sample. A salt concentration above 7% is desirable for creating an unfavourable environment for microbial activity and hence increases the shelf life of the product (Horner, 1997; Gram, 2003). Decrease in histamine content of fish products have been reported as a result of lower enzymatic and microbial deterioration activities in the product and sodium chloride salt used as antimicrobial agent could be used to achieve this objective (Horner, 1997).

Fig.4.5 Histamine content of the fermenting fish samples at (a) 0 h, (b) 8 h and (c) 15 h of ripening time

(a)- Effect of fermentation and salt concentration on histamine content at 0 h of ripening
(b)- Effect of fermentation and salt concentration on histamine content at 8 h of ripening
(c)-Effect of fermentation and salt concentration on histamine content at 15 h of ripening

Regression equation:

$Z = 88.5229 – 5.72654X_2 + 0.587899\ X_3 + 0.0197967X_1^2 + 0.0954959X_2^2$

Where X_1= ripening time

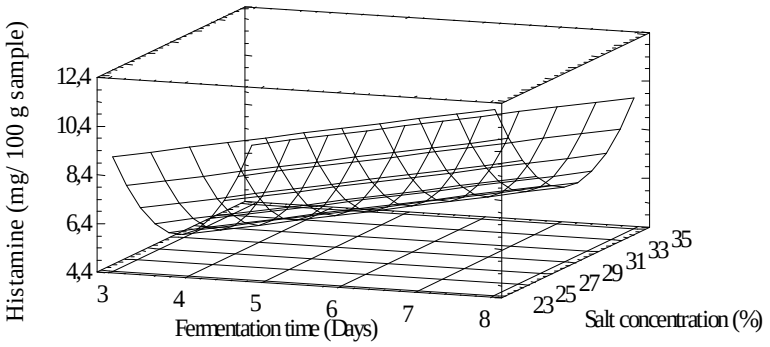
X_2 = salt concentration

X_3 = fermentation time

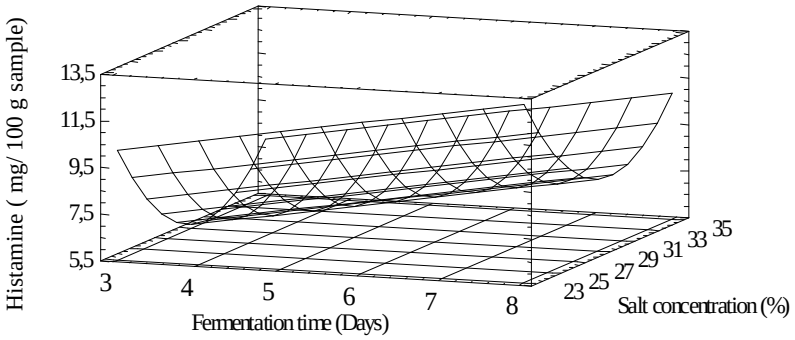
Z = NaCl content of the fermenting fish samples, and

R^2 = 90.3 %.

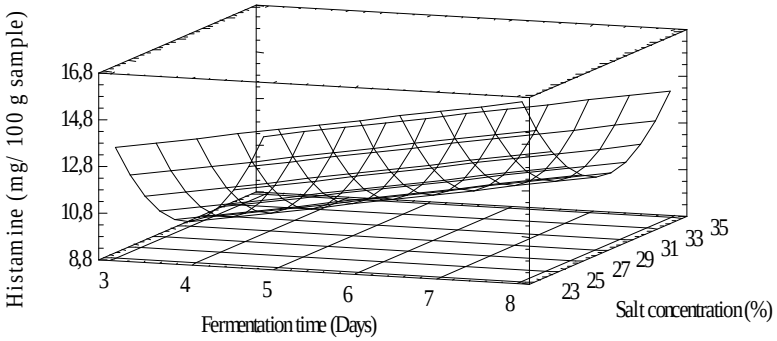
(a)


Histamine (mg/ 100 g sample)
12,4
10,4
8,4
6,4
4,4
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(b)


Histamine (mg/ 100 g sample)
13,5
11,5
9,5
7,5
5,5
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

(c)


Histamine (mg/ 100 g sample)
16,8
14,8
12,8
10,8
8,8
3
4
5
6
7
8
Fermentation time (Days)
23
25
27
29
31
33
35
Salt concentration (%)

4.3.2 Microbiological changes during the spontaneous fermentation of *lanhouin*

Cassava fish was fermented using the optimum conditions of fermentation generated from the modelling studies i e. ripening time of 8 h and salt concentration of 30%. The microbiological changes in the fermenting fish samples were investigated and the predominant organims identified

4.3.2.1 Microbiological status of raw fish and salt

The microbiological status of fresh cassava fish, ripened fish and salt used to treat the fish is presented in Table 4.6. The total viable count (TVC) determination was used as a general-purpose test to cover a wide range of microorganisms that may be present in the food (Huss, 1988; Gram, 2003). The TVC of fresh fish and ripened fish ranged from 6.4×10^4 to 4.0×10^5 and 2.5×10^5 to 1×10^6 respectively and were both within acceptable limits for tropical fish (Huss, 1988). As expected, the microbial population in the ripened fish was higher than the microbial level recorded in the fresh fish. It was observed that the total viable count in the ripened fish increased by 4 fold the microbial load of raw fresh fish (Table 4.6). The results also showed that the halophylic bacteria counts on the fresh and ripening fish varied between $1.6 \times 10^3 - 4 \times 10^3$ and $2 \times 10^3 - 8.1 \times 10^4$ respectively, while Enterobacteriaceae population of the fresh fish increased from 1.6×10^2 to 5.1×10^2 at the end of the ripening time (Table 4.6). Halophylic bacteria count in the salt ranged from 7.8×10^2 to 1.3×10^3 (Table 4.6).

Table 4.6 Microbiological status of raw fish and solar salt used to treat the fish

Samples	Bacterial population (cfu/g)		
	TVC	Halophylic count	Enterobacteriaceae
Fresh cassava fish (n = 6)	*$(2.3 \pm 1.7) \times 10^5$	$(2.8 \pm 1.2) \times 10^2$	$(1.6 \pm 0.2) \times 10^2$
Ripened fish (n = 6)	$(6.2 \pm 3.7) \times 10^5$	$(4.1 \pm 3.9)\ 10^4$	$(5.1 \pm 0.5) \times 10^2$
Salt (n = 3)	-	$(1.0 \pm 0.2) \times 10^3$	ND

n- Number of samples; TVC- Total viable count; ND- Not determined;
*Means ± Standard Deviations (SD)

4.3.2.2 Microbial population of the fermenting fish samples of *lanhouin*

The growth of various bacteria species during the natural fermentation of cassava fish is shown in Figure 4.6. After an increase during the first day of fermentation, the population of aerobic mesophiles enumerated on Plate Count Agar (PCA) showed a decrease up to 6 days of fermentation and then a gradual decrease occurred up to the latter stages of fermentation, whilst the control samples (unsalted fish) showed increases in aerobic mesophilic count during the fermentation periods. The increase in the population of aerobic mesophiles during the first day of fermentation could be due to the fact that bacterial and autolytic spoilage had already started, since the fish was treated with salt in a partially deteriorated state. Another probable source of increase in the population of aerobic mesophiles could be from the addition of salt (FAO, 1989; Essuman, 1992; Hornor, 1997). The decrease in the population of aerobic mesophiles after 1 day of fermentation could be attributed to the inhibition of the natural flora by the high salt concentration (Fig.4.6). This inhibition could be due to the bactericidal and bacteriostatic property of salt, and the dehydration or osmotic action of salt, resulting in a lower water activity of the fermenting fish samples, thus making it impossible for certain microorganisms to survive in the environment (Proctor, 1976; Horner, 1997). The results showed that the population of aerobic mesophiles in the samples decreased from 1.8×10^6 to 3.0×10^5 and 8.9×10^4 cfu/g after 4 and 8 days respectively (Fig.4.6). Halophilic bacteria count (HBC) decreased to 5.6×10^4 cfu/g at the eighth day of fermentation after reaching a peak of 7.2×10^5 cfu/g during the first two days of fermentation (Fig.4.6).

This gradual decrease observed suggested that the less halophilic organisms were eliminated, giving way to the more halophilic organisms already present in the fish and the added salt (Baross and Lenovich, 1992; Gram, 2003). In a similar manner, counts of micrococci enumerated on Mannitol Salt Agar and *Bacillus* spp. enumerated on Dextrose Tryptone Agar showed a slight increase and reached a peak on the first and the second day of fermentation respectively. Both *Bacillus* spp. and the micrococci were present at moderately high levels, about 10^4 and 10^5 cfu /g, respectively (Fig.4.6). These halophilic organisms showed low tolerance to high salt concentrations because their numbers decreased with fermentation time suggesting they were halotolerant rather than halophilic. A similar decreasing trend of aerobic mesophiles and HBC

during high salt fermentation of fish has been reported by Yankah (1988), Subasinghe *et al*., (1990); Abbey *et al*., (1994) and Achinewhu *et al*., (2002).

Bacillus spp. were also present in the samples as spores and their numbers increased from an initial number of 3.0×10^1 to 5.9×10^2 cfu /g after 8 days of fermentation. The presence of spores in dried fish products has been reported by Dube *et al.*, (2004). In the case of *lanhouin,* the spores could also be picked up from the basket and the cloth used to wrap the fish during the fermentation. A similar observation was made by Amoa-Awua and Jakobsen (1995) during the processing of cassava into "agbelima". During the fermentation, the *Bacillus* spore count increased because of the adverse conditions of high salt concentration. Thus, though the *Bacillus* count decreased, more of the remaining cells sporulated to withstand the severe conditions.

The population of Enterobacteriaceae was low at the start of fermentation (3.2×10^2 cfu/g) and decreased further to counts of less than 10 cfu/g after two days of fermentation. No yeasts were detected at any stage of fermentation.

At the early stages of fermentation, PCA plates had representative colonies consisting of both Gram-positive and Gram-negative bacteria. Representative colonies on the MSA plates were mostly Gram-positive, catalase-positive, oxidase-positive or negative cocci. Colonies on the DTA plates were Gram-positive, catalase-positive spore forming rods suggesting they were largely *Bacillus* spp.

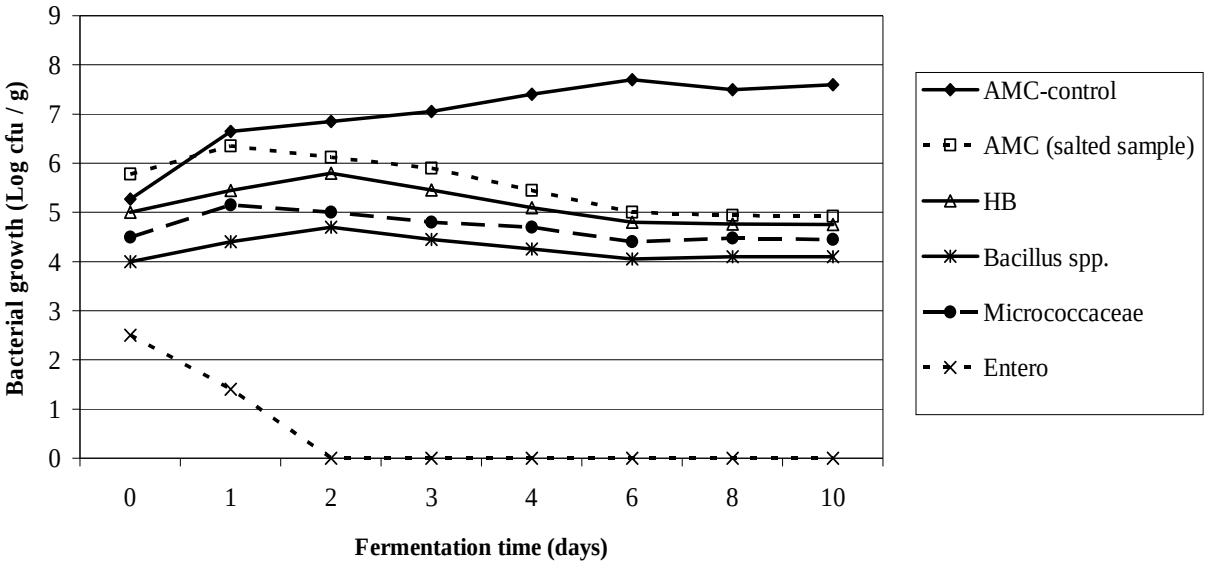


Fig. 4.6 Growth of bacterial species during the spontaneous fermentation of cassava fish treated with 30 % salt concentration

AMC-aerobic mesophilic count; HB- halophilic bacteria; Entero- Enterobacteriaceae; Control- fish fermented without salt

4.3.2.3 Microorganisms isolated during the spontaneous fermentation of *lanhouin*

The isolates obtained at different stages of fermentation consisted of a variety of Gram-positive, catalase-positive rods and cocci as well as Gram-positive, catalase-negative cocci and Gram-negative bacteria. These Gram-positive and Gram-negative bacteria accounted for 92.7 % and 7.3 % respectively.

a. Characteristics of Gram-positive isolates

Gram-positive isolates were identified to the genus level based on their colony and cell morphology and biochemical tests performed, and included *Bacillus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. (Table 4.7).

b. Characteristics of Gram-negative isolates

Gram-negative isolates were classified into three groups based on their colony and cell morphology as well as their biochemical characteristics. The results showed that the Gram-negative bacteria isolated belonged to *Pseudomonas* spp., *Achromobacter* spp. and *Alcaligenes* spp. (Table 4.8).

Table 4.7 Characteristics of Gram-positive microorganisms isolated during the spontaneous fermentation of cassava fish into *lanhouin*

Tests	Bacterial species				
	Bacillus spp.	*Corynebacte-rium* spp.	*Staphylococcus* spp.	*Micrococcus* spp.	*Streptococcus* spp.
Shape	R	R	R	S	S
Mobility	V	-	-	-	+
Spore	+	-	-	-	-
Catalase	+	+	+	+	-
Oxidase	V	-	-	V	-
Growth anaerobic	V	+	+	-	+
Growth in air	+	+	+	+	+
Casein hydrolysis	+	-	-	-	-
Starch hydrolysis	+	-	-	-	+
Acid from glucose	V	+	+	V	+
Oxidation/fermentation	F/O/-	F	F	O/-	F

R: rod-shaped, S: sphere, coccus; O: oxidation; F: fermentation; V: variable reactions in different species of the genus; +: positive reaction; -: negative reaction.

Table 4.8 Characteristics of Gram-negative microorganisms isolated during the spontaneous fermentation of cassava fish into *lanhouin*

Tests	*Pseudomonas* spp.	*Achromobacter* spp.	*Alcaligenes* spp.
Shape	R	R	R
Mobility	+	-	+
Catalase	+	+	+
Oxidase	+	+	+
Growth anaerobic	+	-	-
Growth in air	+	+	+
Acid from glucose	+	+	-
Gelatin liquefaction	-	+	-
Oxydation/fermentation	O/-	O	-

R: rod-shaped; O: oxidation; +: positive reaction; -: negative reaction.

c. Identification of *Bacillus* species

The isolates identified as *Bacillus* species were rod-shaped with spores but on few instances without spores. Some were motile whilst others were non-motile. They were all aerobic with a few facultative anaerobes. All the cultures classified under this group were catalase-positive. However for oxidase, glucose (acid) test and Hugh and Leifson's test, the reactions were different probably due to the difference in strains of the *Bacillus* species. Most of the *Bacillus* species isolated and characterized hydrolysed starch, gelatin and casein. They reduced nitrate, produced acid from L-arabinose, D-xylose, D-mannitol and D-glucose; produced acetoin from Voges-Proskauer medium but failed to grow at 50°C (Table 4.9). They grew in 7 % sodium chloride (NaCl) and at pH 5.7 and 6.8 (Table 4.9). In addition, most of isolated cultures of *Bacillus* species had colonies with round or irregular margins and ridged surface. These colonies had small cells with circular and centrally placed spores (Table 4.9). These isolates utilized glycerol, L-arabinose, ribose, D-xylose, glucose, fructose, mannose, inositol, mannitol, sorbitol, α-methyl-D-glucoside, amygdaline, arbutine, esculine, salicine, celiobiose, maltose, melibiose, saccharose,

trehalose, inuline, raffinose, amidon, glycogene, gentiobiose and D-turanose in API 50 CH strips (Table 4.10). They were identified as *Bacillus subtilis*. Variations were observed in the colony morphology among *Bacillus subtilis* isolates suggesting the presence of different strains of the species.

Other *Bacillus* species identified were *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium* and *Bacillus mycoides* (Table 4.9). Isolates identified as *Bacillus cereus* generally had oval colonies whilst isolates identified as *Bacillus licheniformis* had opaque rough star shaped colonies which were strongly attached to the surface of the medium (Plates 4.1 – 4.5) and could grow under anaerobic conditions and at 50°C. *Bacillus cereus* produced acid only from D-glucose whilst *Bacillus licheniformis* was able to produce acid from D-glucose, D-xylose, D-mannitol and L-arabinose. Isolates identified as *Bacillus megaterium* showed yellow moderately dull and slightly rugose colonies whereas *Bacillus mycoides* formed distinctive rhizoid colonies but this ability to form rhizoid colonies was lost with time and renewed cultures gave oval colonies with a lengthy end on nutrient agar. Unlike the other *Bacillus* isolates, *Bacillus mycoides* and *Bacillus megaterium* failed to produce acid from D-xylose, D-mannitol and L-arabinose. The fermentation of sugars in API 50 CH galleries by different *Bacillus* species is presented in Table 4.10.

Table 4.9 Morphological and biochemical characteristics of *Bacillus* spp. isolated during the spontaneous fermentation of cassava fish

Tests	Bacterial isolates					
	B. subtilis	*B. licheniformis*	*B. subtilis*	*B. megaterium*	*B. mycoides*	*B. cereus*
Spores round	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Anaerobic growth	-	+	-	-	+	+
Voges-Proskauer Test	+	+	+	-	+	+
pH in V-P medium < 6	-	+	-	-	+	+
pH in V-P medium > 7	-	-	-	-	-	-
Acid from D-glucose	+	+	+	+	+	+
Acid from L-Arabinose	+	+	+	-	-	-
Acid from D-Xylose	+	+	+	-	-	-
Acid from D-Mannitol	+	+	+	-	-	-
Hydrolysis of casein	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	+	+
Growth at pH 6.8	+	+	+	+	+	+
Growth at pH 5.7	+	+	+	+	+	+
Growth in 7% NaCl	+	+	+	+	-	+
Growth at 50°C	-	+	-	-	-	-

+: positive reaction; -: negative reaction

Table 4.10 Utilization of active ingredients in API 50 CH by *Bacillus* species during the spontaneous fermentation of cassava fish

Carbohydrates	*B. subtilis*	*B. licheniformis*	*B. megaterium*	*B. mycoides*	*B. cereus*
Glycerol	100	100	2	15	70
Erythritol	0	0	0	0	0
D-arabinose	0	0	0	0	0
L-arabinose	100	100	2	0	0
Ribose	91	100	10	100	93
D-xylose	80	100	10	0	1
L-xylose	1	0	0	0	0
Adonitol	1	0	0	0	0
β methyl-D xyloside	1	0	0	0	0
Galactose	25	100	2	31	1
Glucose	100	95	70	100	100
Fructose	100	100	54	100	100
Mannose	100	100	0	1	1
Sorbose	0	6	0	0	0
Rhamnose	0	34	0	0	0
Dulcitol	0	0	0	0	0
Inositol	63	100	0	1	0
Mannitol	91	90	24	0	0
Sorbitol	100	100	0	0	0
α-methyl-D mannoside	0	0	0	0	0
α-methyl-D glucoside	86	100	0	0	0
N acetyl glucosamine	10	59	40	100	100
Amygdalin	67	100	0	14	31
Arbutin	95	90	0	62	85
Esculin	100	100	2	56	93
Salicin	100	100	0	62	70
Cellobiose	97	100	0	43	62
Maltose	100	100	60	100	100
Lactose	0	37	24	31	0
Melibiose	50	100	2	0	1
Saccharose/sucrose	100	100	65	50	56
Trehalose	100	100	36	100	100
Inulin	81	60	0	0	0
Melezitose	0	0	10	0	0
Raffinose	100	51	30	0	0
Starch	100	100	45	100	0
Glycogen	80	100	60	100	0
Xylitol	1	0	0	0	0
Gentiobiose	80	64	0	0	1
D turanose	100	95	2	1	14
D lyxose	0	0	0	0	0
D tagatose	1	70	0	0	0
D fucose	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	0	35	0	14	62
2 keto gluconate	0	0	0	0	0
5 keto gluconate	0	0	0	0	0

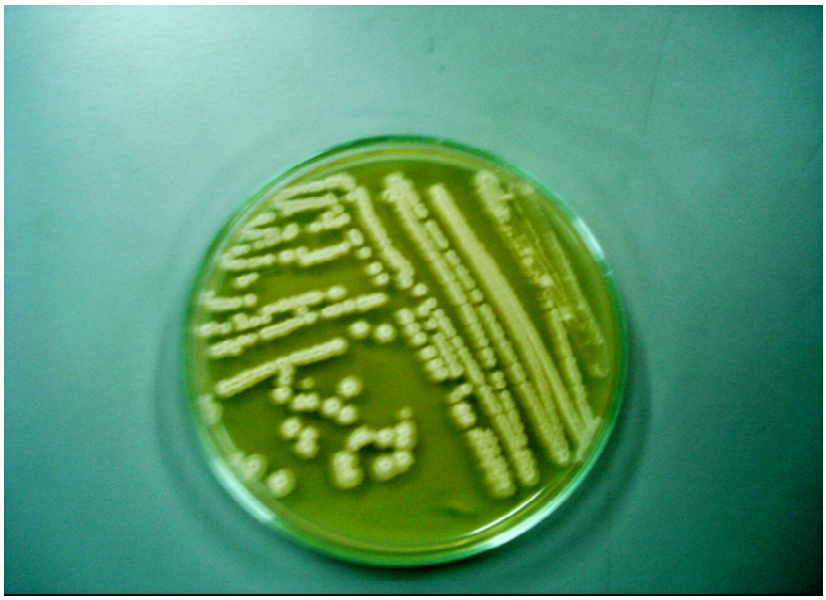


Plate 4.1 Pure culture of *Bacillus licheniformis* growing on nutrient agar plate

Plate 4.2 Pure culture of *Bacillus subtilis* growing on nutrient agar plate

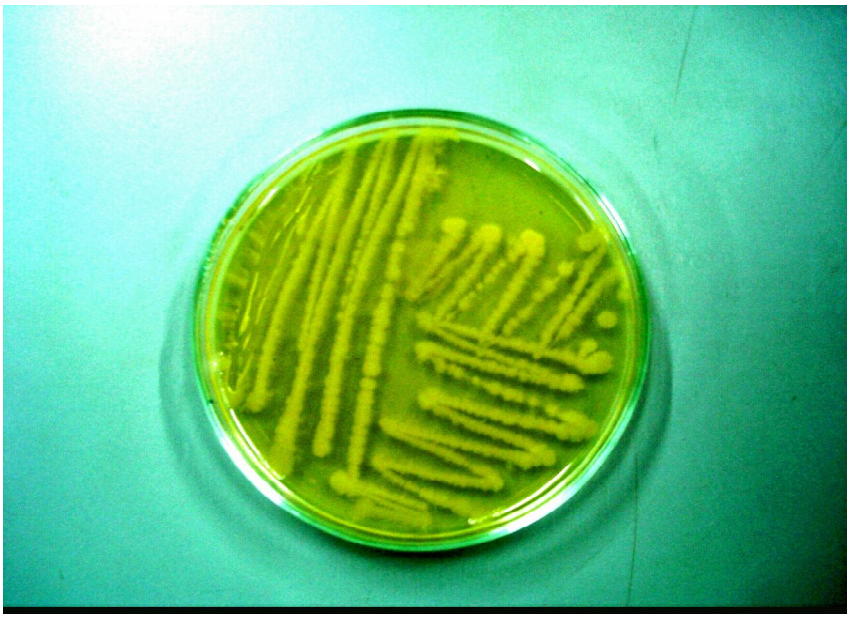


Plate 4.3 Pure culture of *Bacillus megaterium* growing on nutrient agar plate

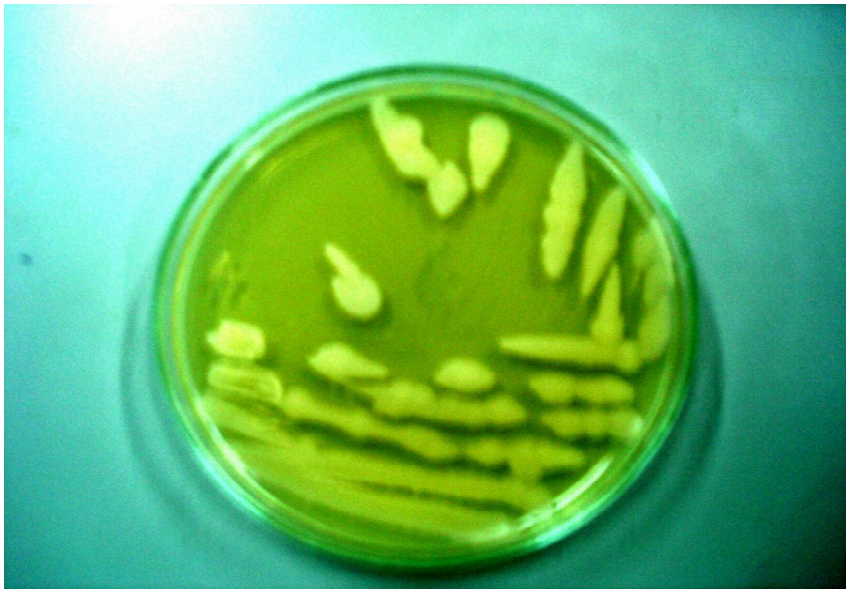


Plate 4.4 Pure culture of *Bacillus mycoides* growing on nutrient agar plate

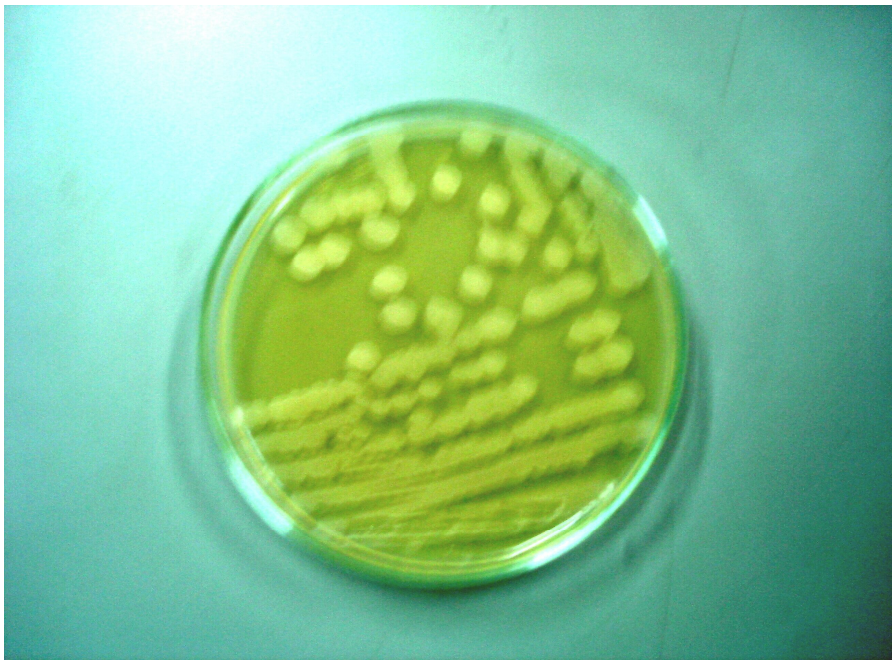


Plate 4.5 Pure culture of *Bacillus cereus* growing on nutrient agar plate

d. Identification of *Staphylococcus* species

Isolates identified as *Staphylococcus* spp. were spherical in shape occurring singly, in pairs as well as in a few tetrads. They were facultative anaerobes and most tested catalase positive. For the oxidative and fermentative test (Hugh & Leifson's test), the cultures produced acid in fermentation both under aerobic and anaerobic conditions. The majority of the isolates were oxidase positive, grew in 10 % NaCl, reduced nitrate, produced acid aerobically from D-mannitol, maltose and sucrose but were unable to produce acid from D-xylose and D-galactose, or acetyl methyl carbinol from Voges-Proskauer medium and could not grow in 15 % NaCl and at 45°C (Table 4.11). These isolates were identified as *Staphylococcus lentus*. Other *Staphylococcus* species identified was *Staphylococcus xylosus*.

Staphylococcus lentus had small white or cream colonies, 1 to 5 mm in diameter, whilst *Staphylococcus xylosus* had big orange-yellow colonies, 4 to 10 mm in diameter (Plates 4.6 and 4.7). *Staphylococcus xylosus* strains were oxidase negative; they were able to produce acid from D-mannitol, D-xylose, Maltose, Sucrose and Lactose but failed to produce acid from D-galactose and grew at 45°C (Table 4.11). *Staphycococcus lentus* strains were generally unable to utilize xylitol, β-naphtyl phosphate, L-arginine and urea whilst *Staphylococcus xylosus* strains failed to utilize D-melibiose, D-raffinose, methyl-αD-glucopyranoside and L-arginine in API Staph galleries (Table 4.12). No coagulase-positive strain was found.

Table 4.11 Morphological and biochemical characteristics of *Staplylococcus* spp. isolated during the spontaneous fermentation of cassava fish

Characteristics of isolates	*Staphylococcus xylosus*	*Staphylococcus lentus*	*Staphylococcus lentus*
Colony diameter on P agar >5 mm	+	-	-
Oxidase	-	+	+
Aerobic growth	+	+	+
Anaerobic growth	+	W*	w
Aerobic acid from:			
D-mannitol	+	+	+
D-xylose	+	-	-
D-galactose	-	-	-
Maltose	+	+	+
Sucrose	+	+	+
Lactose	+	+	-
Nitrate reduction	+	+	+
Phosphatase	-	-	-
Coagulase	-	-	-
Voges Proskauer	-	-	-
Growth at 45°C	-	-	-
Growth on nutrient agar with			
10 % NaCl	+	+	+
15 % NaCl	-	w	w

* w= weak; +: positive reaction; -: negative reaction

Table 4.12 Utilization of active ingredients in API Staph by *Staphylococcus* spp. isolated during the spontaneous fermentation of cassava fish

Active ingredients	*Staphylococcus lentus*	*Staphylococcus xylosus*
	Percentage of strains giving positive reactions	
D-glucose	100	100
D-fructose	92	100
D-mannose	100	92
D-maltose	100	81
D-lactose	90	90
D-trehalose	100	95
D-mannitol	100	90
Xylitol	7	40
D-melibiose	100	9
Potassium nitrate	92	82
β-naphthyl phosphate	25	75
Sodium pyruvate	60	70
D-raffinose	100	10
D-xylose	100	82
D-saccharose (sucrose)	100	87
Methyl αD-glucopyranoside	28	10
N-acetyl-glucosamine	100	80
L-arginine	0	5
Urea	0	90

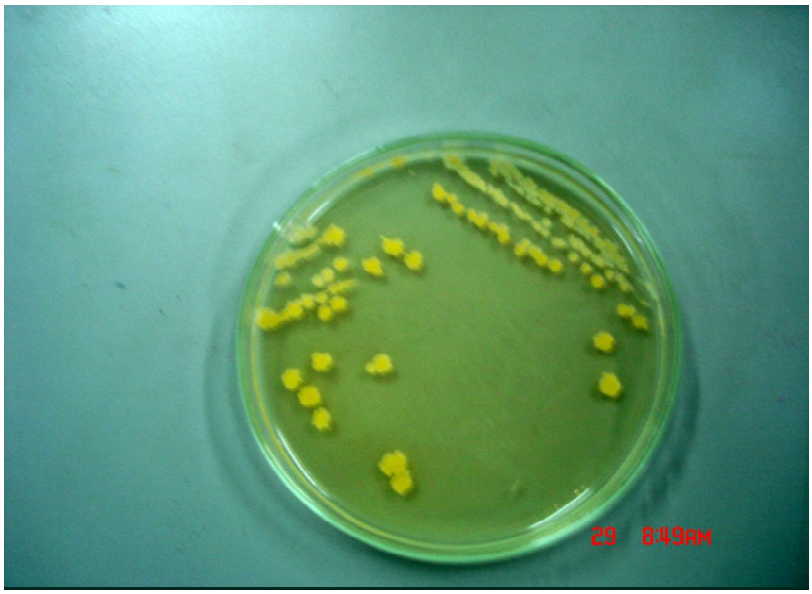


Plate 4.6 Pure culture of *Staphylococcus xylosus* growing on nutrient agar plate

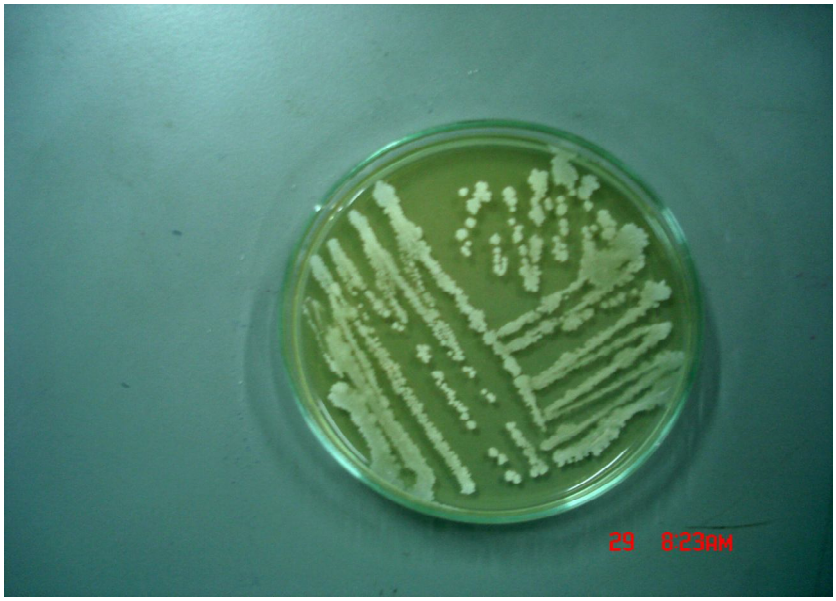


Plate 4.7 Pure culture of *Staphylococcus lentus* growing on nutrient agar plate

e. Identification of *Micrococcus* species

Micrococcus species were observed as spherical cells occurring mostly in pairs, tetrads and irregular clusters; they were non-motile and did not have any spores. All the isolates had smooth and convex yellow colonies (Plate 4.8). None of the isolates were able to produce acid aerobically from glucose, galactose, lactose and mannose, produce acetoin from Voges Proskauer medium, reduce nitrate to nitrite, hydrolyse starch or grow in 10% NaCl. The isolates were able to grow at 37°C and in 7.5 % NaCl, and hydrolyse gelatin (Table 4.13). This group of isolates was identified as strains of *Micrococcus luteus*. These *Micrococcus* strains were unable to utilize any of the active ingredients in API Staph galleries (Table 4.14).

Plate 4.8 Pure culture of *Micrococcus luteus* growing on nutrient agar plate

Table 4.13 Morphological and biochemical characteristics of *Micrococcus* spp. isolated during the spontaneous fermentation of cassava fish

Characteristics of isolates	*Micrococcus luteus*
Pigment on P agar	Y
Mobility	-
Oxidase	+
Aerobic acid from:	
Glucose	-
Mannose	-
Lactose	-
Galactose	-
Phosphatase	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Voges Proskauer	-
Nitrate reduction	-
Growth at 37°C	+
Growth on nutrient agar with	
7.5 % NaCl	+
10 % NaCl	-

Y: yellow; +: positive reaction; -: negative reaction

Table 4.14 Utilization of active ingredients in API Staph by *Micrococcus* spp. isolated during the spontaneous fermentation of cassava fish

Active ingredients	*Micrococcus luteus*
	Percentage of strains giving positive reactions
D-glucose	2
D-fructose	4
D-mannose	0
D-maltose	1
D-lactose	0
D-trehalose	1
D-mannitol	0
Xylitol	0
D-melibiose	0
Potassium nitrate	8
β-naphthyl phosphate	15
Sodium pyruvate	1
D-raffinose	0
D-xylose	0
D-saccharose (sucrose)	1
Methyl αD-glucopyranoside	0
N-acetyl-glucosamine	1
L-arginine	11
Urea	11

4.3.2.4 Predominant microflora during the spontaneous fermentation of cassava fish into *lanhouin*

The numbers and nature of the microorganisms isolated from the fermenting fish are a reflection of the immediate environment of the samples. There were both halophilic and non-halophilic organisms. The predominant bacteria isolated were largely halophylic gram-positive types:

Bacillus spp. (48.7%), *Staphylococcus* spp. (27.3%) and *Micrococcus* spp. (9.4%), and to a lesser extent *Streptococcus* (4.7%) and *Corynebacterium* spp. (2.6%) (Table4.15). Among bacteria *Micrococcus, Staphylococcus* and *Bacillus* spp. are bacteria expected to be found in fish from warm waters (Huss, 1988). These organisms could also have come from the salt used to treat the fish. Salt from various parts of Africa are known to be of poor microbiological quality (Sefa-Dedeh *et al*., 1976; Essuman, 1992). Microorganisms belonging to the genera *Bacillus, Micrococcus* and *Staphylococcus* have been isolated from various types of solar salt (Sefa Dedeh *et al*., 1976). The salt used to treat the fish had a halophylic bacteria load ranging between 7.8×10^2 and 1.3×10^3 cfu /g (Table 4.6). Lactic acid bacteria (*Streptococcus* and *Corynebacterium*) were found in very low numbers and this could be due to the high salt concentration. Only salt-tolerant microorganisms are able to survive in products containing high concentrations of salt. Salt concentration usp to 7 % result in inhibition of lactic acid bacteria **(**Horner, 1997; Gram, 2003).

The few Gram-negative aerobic microorganisms isolated included *Alcaligenes, Achromobacter* and *Pseudomonas* spp., psychrophilic rods mostly involved in fish mishandling (Huss, 1988; Horner, 1997; Love, 1997; Gram, 2003). These organisms are widely distributed in soil, fresh and sea water and are economically important in food spoilage, especially of fish and meat (Huss, 1988; Horner, 1997).

The results showed that *Bacillus* spp. were the dominant microorganisms followed by *Staphylococcus* spp. and *Micrococcus* spp. (Table 4.15). This is in agreement with the findings of Nerquaye-Teteh *et al*., (1978) who reported the predominant microorganisms isolated from *momone* samples collected from the open market to be Gram-positive *Bacillus* spp., micrococci and staphylococci. The high numbers of *Bacillus* spp. could be due to the fact that these spore forming bacteria were able to adapt to the more unfavorable conditions than the non-spore formers.

Among the micropopulation of *Bacillus* species, *Bacillus subtilis* accounted for 43.8%. The remaining members of the genus represented 34.2%, 15%, 4.1% and 2.7% for *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mycoides* and *Bacillus cereus* respectively (Table 4.16). Thus, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megaterium* were the

predominant species of the genus. However more limited growth was exhibited by *Bacillus megaterium*; this species represented only 15% of *Bacillus* species and 7.4% of the total isolates (Table 4.16). Representative species of *Staphylococcus* were found to be *Staphylococcus lentus* and *Staphylococcus xylosus*. They accounted for 14% and 9.3% of the total number of colonies isolated; non-identified staphylococci represented 4% of the total number of colonies isolated (Table 4.16). The only species of *Micrococcus* identified was *Micrococcus luteus*.

Work carried out by Yankah (1988) revealed the presence of four (4) *Bacillus* spp. in a momone, a *lanhouin*–like product fermented for two (2) days. Report by Oronsaye (1991) indicated *Bacillus pumilis* and *Bacillus licheniformis* as bacteria responsible for fermentation of fish at ambient temperatures. *Micrococcus luteus* and *Bacillus megaterium* were isolated during the indigenous fermentation of fish sausage in Australia (Aryanta *et al*., 1991). Recent work by Achinewhu *et al*., (2004) identified *Bacillus licheniformis* and *Staphylococcus epidermidis* as the predominant organisms which are involved in the spontaneous fermentation of *Sardinella.*

The results of the present work varied from those of Oronsaye (1991), Aryanta *et al*., (1991) and Achinewhu *et al*., (2004) who did not report the presence of *Bacillus subtilis*, *Staphylococcus lentus* and *Staphylococcus xylosus* in solid substrate fermentation of fish.

Bacillus species as well as *Staphylococcus* species isolated showed moderate proteolytic and lipolytic activities. *Micrococcus luteus* showed weak proteolytic activity and negative lipolytic activity. However, greater proteolytic activity was exhibited by *Bacillus subtilis* and *Bacillus megaterium* (Table 4.17). The proteolytic and lipolytic activities exhibited by these organisms could contribute to aroma development in *lanhouin*. These organisms are reported to produce metabolites that contribute to flavour and odour development in various fermented fish products (Adams, 1986; Saisithi, 1987; Gram, 2003). In addition, the proteolytic activity of these microorganisms could be responsible for major textural changes during the fermentation of fish (Lin *et al*., 1996; Gram, 2003).

The results of the present work showed that the halophylic organisms involved in the fermentation of fish showed low tolerance to high salt concentrations because their numbers decreased with fermentation time; this suggests that these microorganisms were halotolerant rather than halophilic. Similar observations have been reported by various workers (Ko, 1982; Subasinghe *et al*., 1990).

On the safety aspect, *Bacillus cereus* as well as *Bacillus megaterium* may be associated with food poisoning but are widely distributed in nature and can be isolated from a wide variety of foods in which they may be present as part of the normal biota (Collins and Lyne, 1984). Not all strains of *Bacillus cereus* produce toxins and the presence of *Bacillus cereus* has been reported in several African and Asian fermented foods including *agbelima*, soybean *dawadawa* and cocoa (Amoa-Awua and Jakobsen, 1995; Dakwa *et al*., 2005; Kuni, 2005). There are several references in the literature to the use of *Bacillus* species in the food industry. *Bacillus subtilis* is cited in the list of microorganisms used as starter cultures for cured meat products (Liepe, 1983). *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 into traditional products in West Africa (Odunfa, 1985b; Steinkraus, 1991). The fermentation of soy beans into *afitin*, *iru*, *sonru* and *dawadawa* in West Africa regions are also reported to be carried out by *Bacillus subtilis* (Omafuvbe *et al.*, 2000; Azokpota *et al.*, 2005).

Both *Staphylococcus lentus* and *Staphylococcus xylosus* have been rarely associated with human or animal infections (Kloos and Schleifer, 1986) and *Staphylococcus xylosus* is even used as starter culture in dry sausage ripening (Metz, 1993). The absence of *Staphylococcus aureus*, a known enterotoxin producer, means that the fermented fish *lanhouin* might be safe for consumption.

Table 4.15 Microorganisms isolated during the spontaneous fermentation of *lanhouin*

Representative groups	Genera	% total colonies isolated
Gram-positive (92.7 %)	*Bacillus* spp.	48.7
	Staphylococcus spp.	27.3
	Micrococcus spp.	9.4
	Streptococcus spp.	4.7
	Corynebacterium spp.	2.6
Gram-negative (7.3 %)	*Pseudomonas* spp.	-
	Alcaligenes spp.	-
	Achromobacter spp.	-

Table 4.16 Composition of predominant organisms isolated during the spontaneous fermentation of cassava fish into *lanhouin*

Genera	Species	% colonies in the genus	% total colonies isolated
Bacillus	*subtilis*	43.8	21.3
	licheniformis	34.2	16.7
	megaterium	15.0	7.4
	mycoides	4.1	2.0
	cereus	2.7	1.3
Staphylococcus	*lentus*	51.2	14.0
	xylosus	34.1	9.3
	Staphylococcus spp	14.6	4.0
Micrococcus	*luteus*	100	9.4

Table 4.17 Proteolytic and lipolytic activities of predominant organisms isolated during the spontaneous fermentation of cassava fish

Isolates	Proteolytic activity[a]	Lipolytic activity[b]
Bacillus subtilis [1]	++	+
Bacillus subtilis [2]	++	+
Bacillus licheniformis	+	+
Bacillus megaterium	++	+
Staphylococcus lentus [1]	+	+
Staphylococcus lentus [2]	+	+
Staphylococcus xylosus	+	+
Micrococcus luteus	+	-

+ = 1-5 mm ; ++ = 6-11 mm

[a] diameter of clear zones on skim milk agar after 3 days of incubation at 30°C

[b] diameter of clear zones on tributyrin agar after 3 days of incubation at 30°C

[1] Strain isolated at the commencement of the fermentation
[2] Strain isolated after 3 days of fermentation

4.3.2.5 Microbial succession during the spontaneous fermentation of *lanhouin*

The results showed that mixed populations of microorganisms are involved in the fermentation of cassava fish with the predominant organisms being bacterial species. The occurrence of the predominant organisms and their frequency distribution are shown in Table 4.18 and Fig. 4.7. *Bacillus, Micrococcus, Staphylococcus, Streptococcus,* and *Corynebacterium* initiated the fermentation process, however, *Bacillus, Staphylococcus* and *Micrococcus* species were the predominant organisms identified at the start of the fermentation. *Bacillus* and *Staphylococcus* persisted up to the latter stages of fermentation whilst *Streptococcus* and *Corynebacterium*, and *Micrococcus* were not detected after two (2) and four (4) days of fermentation respectively. *Pseudomonas* and *Achromobacter* initially present in the fermenting fish samples were not detected two (2) days after salting. In fact, these microorganisms associated with fish spoilage are halophobic and will not grow in salt concentrations exceeding 5% (Baross, 1992). Other workers have explained this pattern of growth to be due to differences in the sensitivity of each bacteria to salt concentration (Baross, 1992; Horner, 1997; Gram, 2003).

Among the micropopulation of *Bacillus* species, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. mycoides* and *B. cereus* were detected on the first day of the fermentation but only *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus megaterium* persisted up to the latter stages of fermentation. *Bacillus cereus* and *Bacillus mycoides* died off after one (1) and two (2) days of fermentation respectively. Despite the presence of *Bacillus megaterium* after eight (8) days of fermentation, their numbers started to decrease two (2) days after salting.

The only strain of *Micrococcus luteus* identified was not detected after four (4) days of fermentation whereas both *Staphylococcus lentus* and *Staphylococcus xylosus* were present at all stages of fermentation. However, more limited growth was exhibited by *Staphylococcus xylosus*.

Table 4.18 Occurrence of predominant genera during the natural fermentation of cassava fish for *lanhouin* production

Fermentation time (days)	Predominant genera							
	B. subtilis	*B. licheniformis*	*B. megaterium*	*B. mycoides*	*B. cereus*	*S. lentus*	*S. xylosus*	*M. luteus*
0	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+
2	+	+	+	+	-	+	+	+
3	+	+	+	-	-	+	+	+
4	+	+	+	-	-	+	+	-
6	+	+	+	-	-	+	+	-
8	+	+	+	-	-	+	+	-

B. – *Bacillus*; S.- *Staphylococcus***;** M. **–** *Micrococcus*
+: present; -: absent

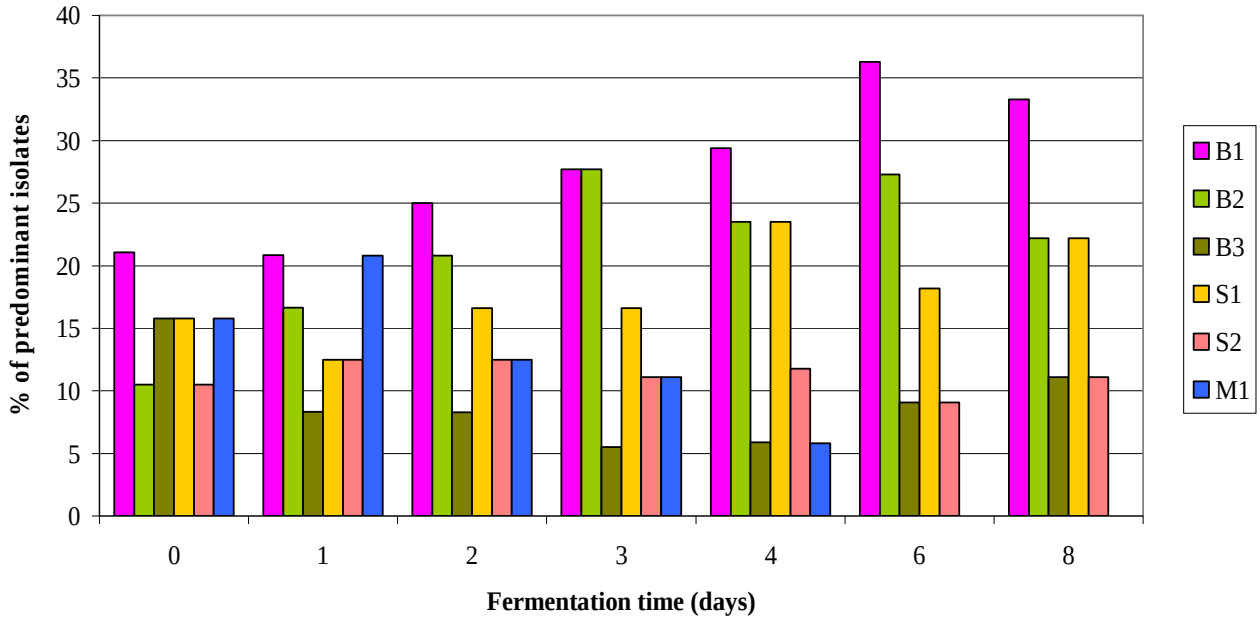


Fig. 4.7 Frequency distribution of predominant organisms isolated from the fermenting fish samples

B1- Bacillus subtilis; *B2- Bacillus licheniformis*; *B3-Bacillus megaterium*; *S1-Staphylococcus lentus*; *S2-Staphylococcus xylosus*; *M1- Micrococcus luteus*

4.3.3 Physico-chemical changes during the spontaneous fermentation of *lanhouin*

The physico-chemical changes during the spontaneous fermentation of cassava fish were investigated through monitoring of pH, titratable acidity, moisture, sodium chloride, protein, total volatile nitrogen, histamine, free fatty acids and thiobarbituric acid number.

4.3.3.1 Proximate composition of raw fish

The physico-chemical characteristics of fresh cassava fish (*Pseudotolithus* sp.) and fresh ripened (left overnight) cassava fish used for the production of *lanhouin* is presented in Table 4.19. The fresh cassava fish samples had low levels of fat and high levels of protein. The samples also had low contents of total volatile nitrogen (TVN) and histamine which was an indication of being fresh and having good microbiological status since histamine level can be used as microbiological index of fresh fish (Ababouch, 1990; Collette, 2001; Kim *et al*., 2002b). The histamine levels in the fresh cassava fish were very low and did not pose any toxicological problem to man. This is in agreement with the findings of Fernandez and Mackie (1979) that the histamine content of fresh fish is very low and its appearance is indicative of spoilage. In contrast, the TVN content of the fresh ripened fish was found to be high (70.20 mg N/100g) and this could be attributed to enzymatic and microbial activities in the fish flesh as reported by Ababouch (1990), Collette (2001) and Gram (2003). For fresh fish, TVN content that exceeds approximately 50 mg N/100 g would be rejected for human consumption (Pearson, 1976; Silva *et al*., 1998). It was observed that the fresh fish lost about 6.5 % of its moisture content during the ripening time probably due to autolysis and proteolysis actions. Autolysis and proteolysis lead to decreases in the capacity of fish tissues to retain water (Bykowski and Dutkiewicz, 1996; Horner, 1997; Gram, 2003).

Table 4.19 Physico-chemical characteristics of fresh and ripened cassava fish

Parameters	Fresh cassava fish (n = 6)	Fresh ripened cassava fish (n = 6)
pH	6.28 ± 0.12[1]	6.83 ± 0.20
Moisture (%)	78.60 ± 1.50	73.50 ± 1.54
Crude fat (%)[2]	0.85 ± 0.04	ND
Crude protein (% nitrogen × 6.25)- (g/100 g)[2]	19.20 ± 1.00	19.87 ± 1.20
Total ash (%)[2]	1.30 ± 0.06	ND
Total volatile nitrogen (mg N/100g)[2]	16.00 ± 0.80	73.20 ± 3.50
Histamine(mg/100g)[2]	1.10 ± 0.11	7.60 ± 0.45

n: number of samples analysed, each sample in duplicate

[1]Means ± Standard Deviations (SD)

[2]Wet weight basis

ND- non determined

4.3.3.2 Effect of fermentation on pH and titratable acidity of the fermenting fish samples

Figure 4.8 illustrates the variation in pH and titratable acidity of the fish during the indigenous fermentation for *lanhouin* production. The pH of the fermenting fish samples showed a rise during the first two days of fermentation and reached a pH value of 7.30 ± 0.10, possibly due to the formation of nitrogenous basic compounds, and then a gradual decrease occurred while the pH value of the control samples increased gradually throughout fermentation (Fig. 4.8). The titratable acidity increased slightly during the first 72 h and then decreased gradually. It

was observed that the rise in pH coincided with the period of increase of *Bacillus* spp. and micrococci counts suggesting high microbial proteolytic activity. The very low gradual production of acid could be due to the presence of very little fermentable carbohydrates in the fish or to the presence of few numbers of lactic acid bacteria in the fermenting fish during the early stages of fermentation. This increase in acidity may also be due to the high buffering capacity of the fish flesh (Dakwa *et al.*, 2005). The decrease in pH observed after the first two days could suggest the ability of the fermenting organisms to produce acid in the presence of high concentrations of salt (Achinewhu *et al.*, 2004). This decrease in pH could also be due to the decrease in *Bacillus* spp. and micrococci counts, and consequently, the decrease in proteolytic activity and the formation of nitrogenous basic compounds in the fermenting fish samples. Similar decreases in pH during high salt fermentation of fish have been reported by Subasinghe *et al*. (1990), Abbey *et al*. (1994), Achinewhu *et al*. (2002) and Achinewhu *et al*. (2004). However the pH values recorded during the present study were higher than those reported by other workers notably Subasinghe *et al*. (1990) and Achinewhu *et al*. (2004). This could be due to the fact that fresh fish is used for the fermentation reported by the other workers unlike the partially deteriorated fish used for *lanhouin* production. The pH values of laboratory samples were however lower than the values recorded in the samples collected from the open market (Anihouvi *et al*., 2006) and this could be seen as the result of good handling of fresh fish and the use of adequate salt concentration to treat the fish.

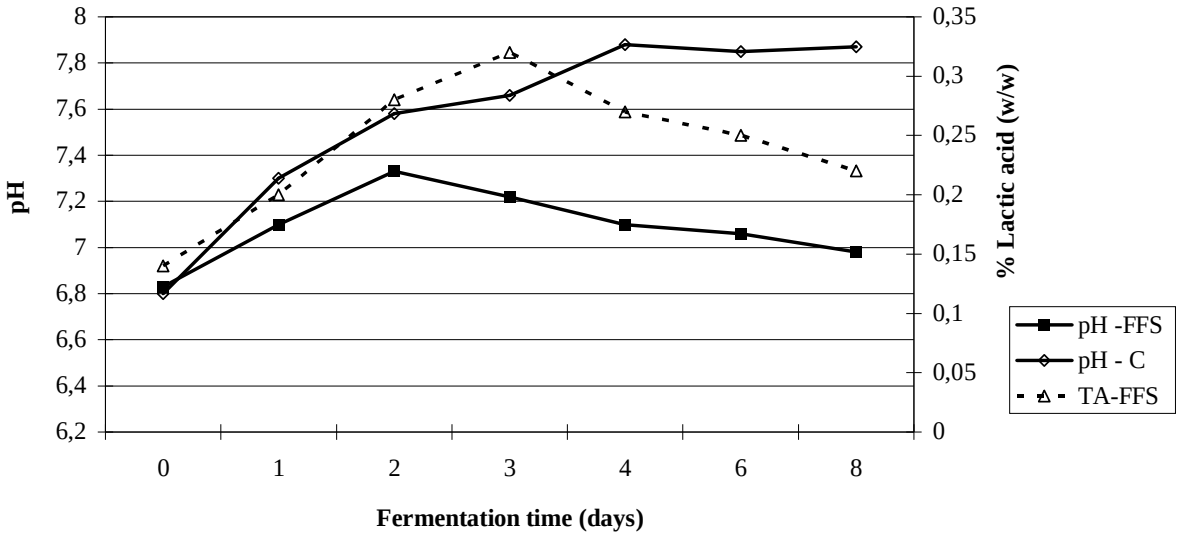


Fig. 4.8 Changes in pH and titratable acidity during the spontaneous fermentation of cassava fish

FFS: fish fermented with salt; C: control (non-salted fish); TA: titratable acidity of fish fermented with salt

4.3.3.3 Effect of fermentation on moisture, sodium chloride and protein contents of the fermenting fish samples

Figure 4.9 shows the variation in moisture, sodium chloride (NaCl) and protein during the spontaneous fermentation of cassava fish. Moisture content of the fermenting fish samples varied from 73 % to mean values of 51.68 and 46.88 % after 4 and 8 days of fermentation, respectively. At the same time, a gradual increase in sodium chloride (NaCl) content of the samples was recorded from 6.11 % to mean values of 18.8 and 25.7 % (dry weight basis) after 4 and 8 days of fermentation respectively. The decrease in moisture content of the samples was due to the penetration of sodium chloride into the fish flesh by dialysis; this displaced water from the fish tissue to the outside due to the osmotic pressure between the fish muscle and the brine (Hornor, 1997). A similar relationship between the moisture and NaCl contents during solid substrate fermentation of fish has been reported by other workers (Kingsley-Ekow, 1999; Itou *et al*., 2000). During the salting period, fish were strongly dehydrated and a decrease in body weight by up to 35.78 % due to salt penetration was observed in the samples. Decreased moisture and high salt content of food products have been reported to lower the enzymatic deterioration as well as microbial and chemical activities in the product. This is said to increase the shelf life of the product (Lopez, 1987; Horner, 1997; Kingsley-Ekow, 1999).

Total protein content of the samples decreased from the initial concentration of 75.6 % (dry weight basis) to mean values of 60.1 and 54.8 % (dry weight basis) after 4 and 8 days of fermentation, respectively (Fig. 4.9). These levels of protein in laboratory samples were much higher than those found in the market samples of *lanhouin* (Anihouvi *et al*., 2006), and other values reported by Nerquaye-Teteh *et al*., (1978) and Abbey *et al*., (1994). The decrease in protein content of the samples revealed a loss of 27.5 % of the initial protein content after 8 days of fermentation; this could mean that proteins are broken down by enzymatic and microbial activities. When the proteins are broken down into peptides and amino acids, they are lost in the exudates leading to lower protein content in the fish samples. It was observed that the decrease in protein was more drastic during the first three days of fermentation and this was followed by gradual decrease as sodium chloride content of the samples increased (Fig.4.9).

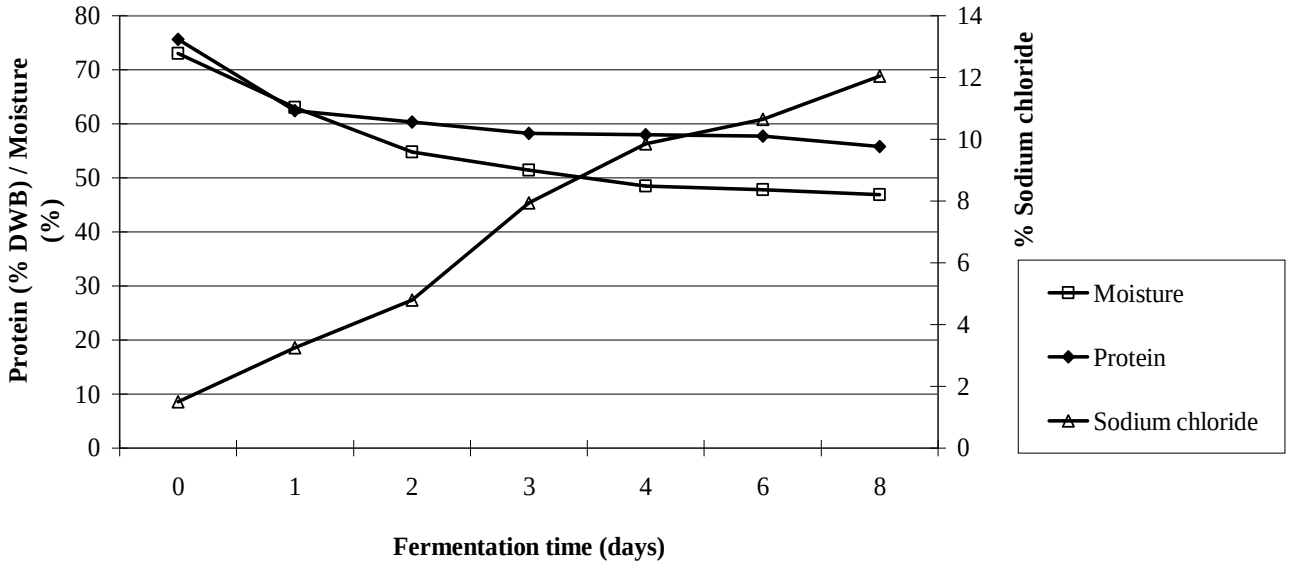


Fig. 4.9 Effect of fermentation on moisture, protein and sodium chloride contents of fermented fish samples

DWB - dry weight basis

4.3.3.4 Effect of fermentation on total volatile nitrogen and histamine contents of the fermenting fish samples

Figure 4.10 shows the changes in total volatile nitrogen (TVN) and histamine contents of the fermenting fish samples. TVN content of the fermenting fish samples increased from the initial value of 70.5 mg N/100 g to mean values of 229.75 and 260.15 mg N / 100 g after 4 and 8 days of fermentation, respectively. The increase in total volatile nitrogen (TVN) resulted from the formation of nitrogenous basic compounds due to the degradation of protein (Person, 1976; Huss, 1988; Gram, 2003). The TVN values obtained from the test samples were lower than those recorded in market samples of *lanhouin* (Anihouvi *et al*., 2006) and appeared to be consistent with other TVN values reported in various fermented fish by other authors (Young *et al*., 2000; Kuda *et al*., 2001; Gram, 2003). The histamine contents of the salted samples were low and varied between 8.30 and 13.55 mg / 100 g sample whilst the unsalted samples showed the highest level of histamine. This tends to indicate the inhibiting role of salt to histamine production. Under high salt conditions, only halophilic bacteria are favoured and since most histamine-producing bacteria are not halophylic, histamine production would be impaired. At salt concentrations of 9.75 % obtained after 4 days of fermentation in *lanhouin* test samples, most of the histamine decarboxylating bacteria may have been rendered incapable of histamine production. This confirms work carried out by other workers which suggest that salt may play an important role in the inhibition of histamine production (Wootton *et al*., 1989; Kingley-Ekow, 1999). The low histamine content of the test samples could be seen as a result of good handling and shortening of ripening time. Total volatile nitrogen and histamine were analysed in each of the samples to establish if there was a link between the two parameters. If this relationship did exist, the TVN determination, which is quite rapid, could be used as a simple test to assess possible histamine content. The results showed that there was no relationship between histamine and TVN contents as seen from Fig.4.10 in which the TVN content of the samples increased continuously with fermentation time whilst histamine increased slightly for a period, decreased and appeared to increase again.

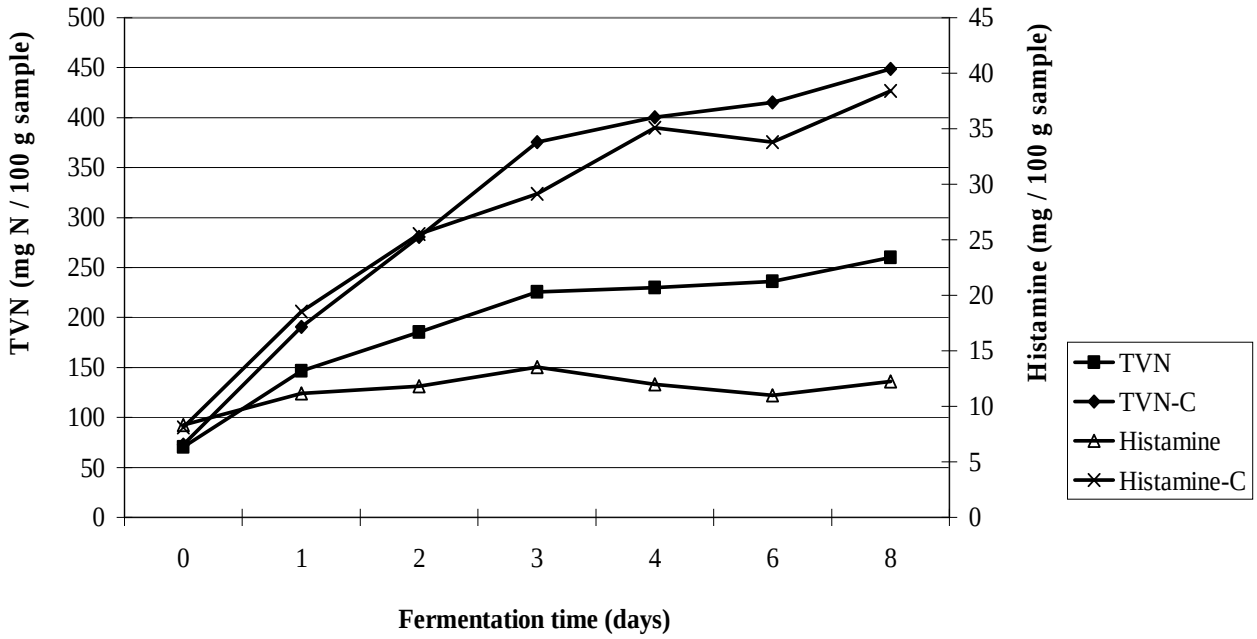


Fig. 4.10 Effect of fermentation on total volatile nitrogen and histamine

TVN-C: TVN-control; Histamine-C: histamine control

4.3.3.5 Effect of fermentation on free fatty acid and thiobarbituric acid number contents of the fermenting fish samples

The effect of fermentation on free fatty acid (FFA) and thiobarbituric acid value (TBA) of *lanhouin* test samples is shown in Figure 4.11. Both FFA and TBA values are a measure of the extent of oxidative deterioration in oily fish, but they can fall further at latter stages of fish spoilage (FAO / SIFAR, 2001). FFA and TBA increased with fermentation time and reached a peak of 8.35 % of oleic acid and 1.98 mg malonaldehyde / kg after four (4) and six (6) days respectively. These levels of FFA and TBA recorded on the test samples were greatly lower than those determined on retail *lanhouin* samples and other *lanhouin*-like products (Abbey *et al*., 1994; Anihouvi *et al*., 2006). This could be the result of good handling, short ripening time and the quality of the salt as well as the salt concentration used to treat the samples. High levels of FFA and TBA are characteristics of products that have undergone both microbial and biochemical spoilage (Hornor, 1997; Tungkawachara *et al*., 2003). As a matter of fact during ripening and fermentation a considerable amount of free fatty acids could appear because triglyceride in the fat is cleaved by triglyceride lipase originating from the fish flesh or that is excreted mainly by certain microorganisms (Pearson, 1976; Huss, 1988; Benjakul *et al*., 1997). Short ripening time and an adequate salt concentration resulted in low bacterial load and consequently to low degradation of fatty acids due to microbial activity. In a similar manner several substances such as Cu and Fe often found in salt are very active catalysts, thus having strong pro-oxidative effects, can increase the risk of fat autoxidation yielding to the formation of secondary products such as aldehydes whose several kinds can be determined as "thiobarbituric acid-reactive substances" (TBARS) (Ramanathan and Das, 1992; Benjakul *et al*., 1997; Horner, 1997). The salt used to treat the fish contained relatively low levels of iron, about 2 mg Fe / 100 g (PIRRATES, 1990).

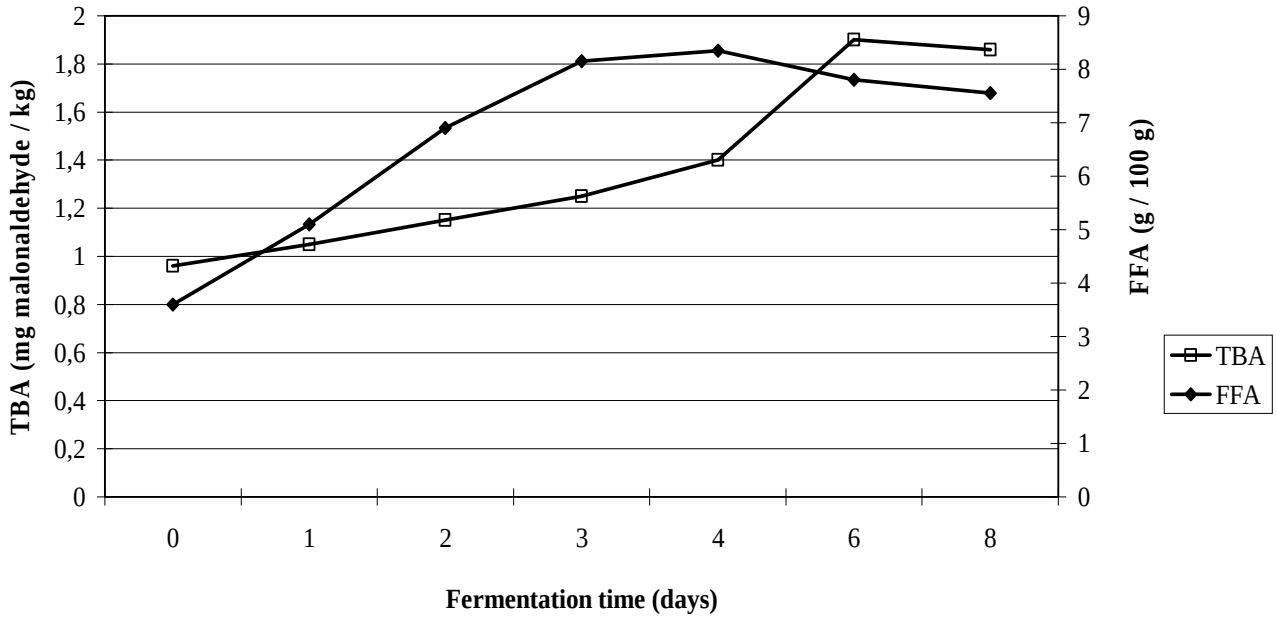


Fig. 4.11 Effect of fermentation on free fatty acid and thiobarbituric acid value

4.3.3.6 Aroma compounds of laboratory samples of *lanhouin* obtained by the spontaneous fermentation

The aroma compounds isolated from laboratory *lanhouin* samples were identified based on comparisons of chromatographic retention time between the internal standard and samples of aroma constituents, and the mass spectra GI033A NIST PBM library (Fig.4.12- 4.16 and Table 4.20). A total of 94 aroma compounds were detected from *lanhouin* samples obtained by spontaneous fermentation of cassava fish over a period of eight (8) days. Of these 82 compounds were positively identified with a quality index higher than 70% and comprised mainly 15 aliphatic hydrocarbons, 6 aromatic hydrocarbons, 9 esters, 9 ketones, 7 acids, 5 alcohols, 9 amines, 2 amides, 7 aldehydes, 2 pyrroles, 6 thiazoles, 2 furan and 3 phenols. Hydrocarbons and other lipid-derived compounds such as aldehydes, alcohols and esters comprised the majority of volatile compounds from the fermented fish samples. Few numbers of aroma compounds were detected in fresh cassava fish and consisted mainly of aldehydes and alcohols which were present in relatively high concentrations (Table 4.21). According to Serrini *et al*. (1994) and Prost *et al*. (1998) fresh fish flavour is due to volatile aldehydes and alcohols, which are mainly derived from the oxidative deterioration of polyunsaturated fatty acids (PUFA). The major groups of compounds found in the *lanhouin* samples obtained by the spontaneous fermentation have been reported by other workers on other fermented fish and these are attributed to the action of various microorganisms including *Bacillus* spp., *Staphylococcus* spp., *Micrococcus* spp. and some gram-negative species (Ko, 1982; Gram, 2003).

The results showed that a sequential build up of aroma compounds takes place during the spontaneous fermentation. This was probably due to the breakdown of various components of fish, mainly proteins and lipids into other aroma compounds as the fermentation progressed as a result of enzymatic and microbial activities. However some of the compounds showed sharp increases or decreases with fermentation time.

Aliphatic hydrocarbons could be derived from lipid degradation and may not play a significant role in the fermented fish flavour, since they are known to possess a relatively weak aroma (Chi-Tang *et al*., 1981; Chung *et al*., 2002).

Ketones may be produced by oxidation/degradation of PUFA or amino acid degradation (Cha *et al*., 1998; Spurvey *et al*., 1998). Among the six lower acids detected, hexadecanoic acid occurred in the highest amount (Table 4.21). Aldehydes mainly derived from oxidative degradation of PUFA are responsible for oxidized aromas of foods and are important in many

food products (Baek *et al*., 1997; Prost *et al*., 1998). According to Cadwallader *et al*., (1994) (Z)-4-heptenal is responsible for undesirable fishy and rancid odours in cooked alligator meat. Microbial species such as *Pseudomonas* are associated with the production of a number of volatile aldehydes (Benjakul *et al*., 1997). 3-fluoro-4-hydroxy-Benzaldehyde was the most abundant aldehyde (2.1ppm) followed by 5-Fluoro-2-nitrobenzaldehyde (1.6 ppm) in 4 day samples whilst (Z)-4-heptenal (34.2ppm) followed by 5-Ethylcyclopent-1-ene-Carboxaldehyde (25.9 ppm) was predominant in 8 day samples (Table 4.21). Alcohols may have been produced by lipid oxidation (chemical or enzymatic) (Kawai, 1996; Cha *et al*., 1998; Chung *et al*., 2002), but they generally do not contribute to the overall flavour because of their high threshold values unless they are unsaturated (Le Guen *et al.*, 2000). Among esters, 2-methyl-propyl heptanoate and Ethyl 2,4-decadienoate were detected only in samples fermented for 8 days. The presence of 2-methyl-propyl heptanoate and ethyl 2,4-decadienoate in other fermented fish products has been reported by Cha *et al*., (1998). However, dibutyl phthalate was the most important ester found in the fish and it was present at all the stages of fermentation except in 8 day fermented fish (Table 4.21). Only two furan compounds were detected in all the *lanhouin* samples: 3-Methyl-2-(2-methyl-2-butenyl)-furan (1.3 ppm) and Carboxyfuran (37.7 ppm) (Table 4.21). Furan was reported for the first time as seafood volatile in turbot (*Scophtalamus maximus*) by Prost *et al*., (1998). According to Spurvey *et al*., (1998), its positive or negative contribution to food aroma is debatable.

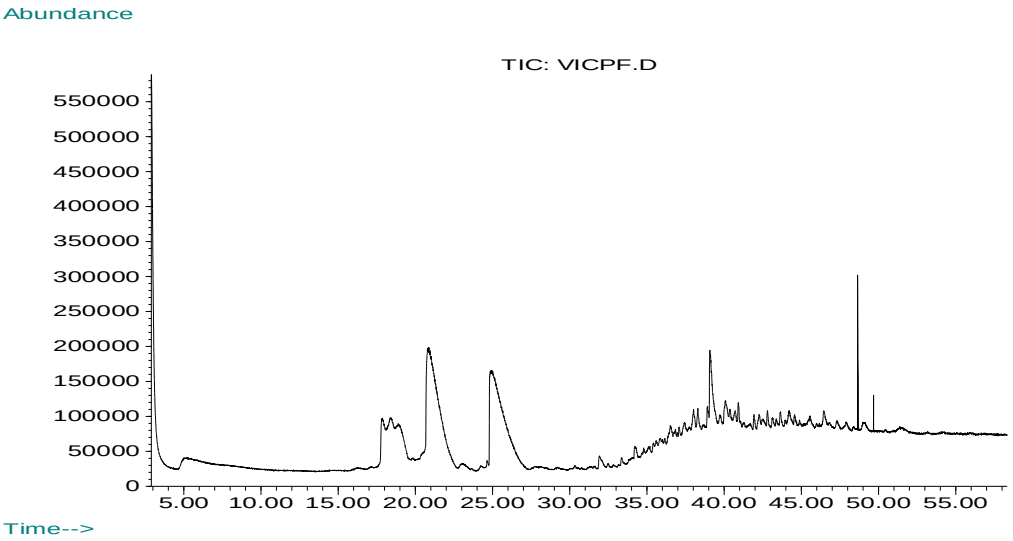


Fig.4.12 Gas chromatogram of aroma compounds from fresh cassava fish

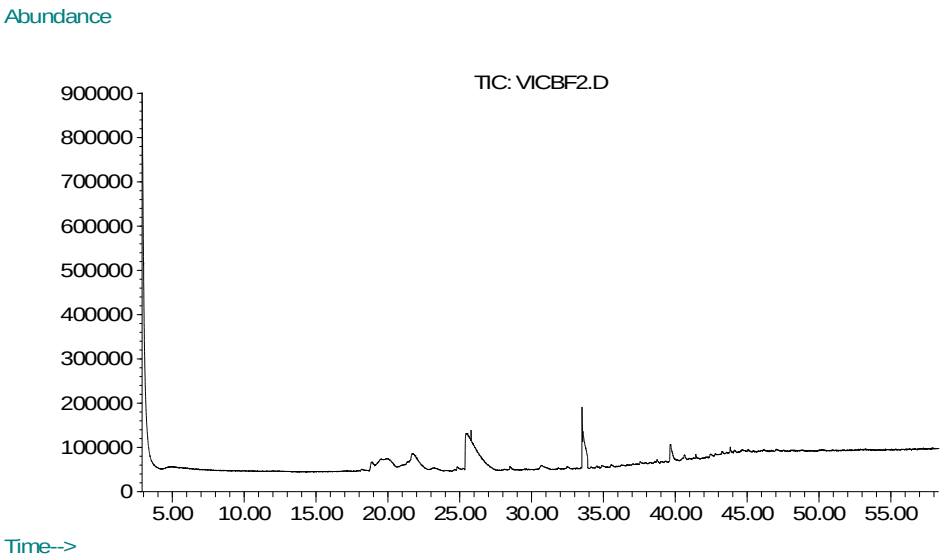


Fig. 4.13 Gas chromatogram of aroma compounds from cassava fish spontaneously fermented for 2 days

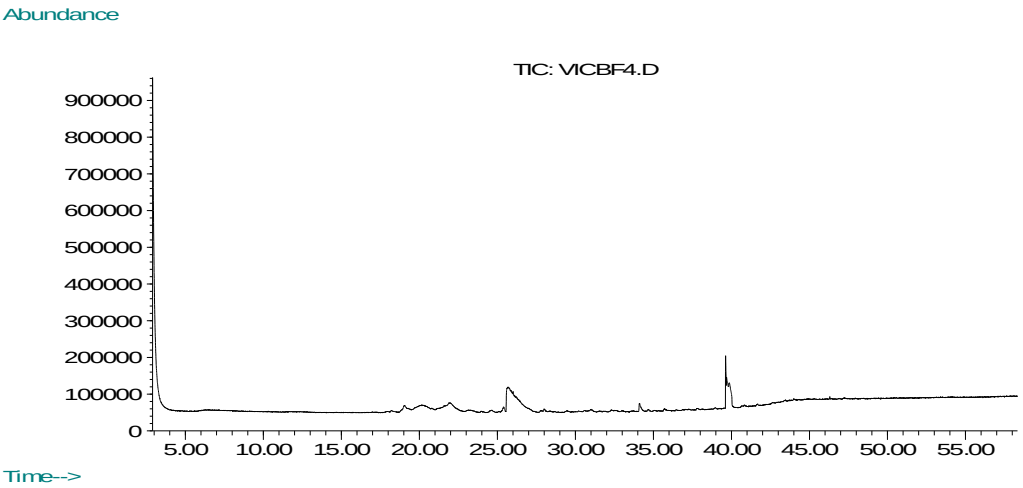


Fig. 4.14 Gas chromatogram of aroma compounds from cassava fish spontaneously fermented for 4 days

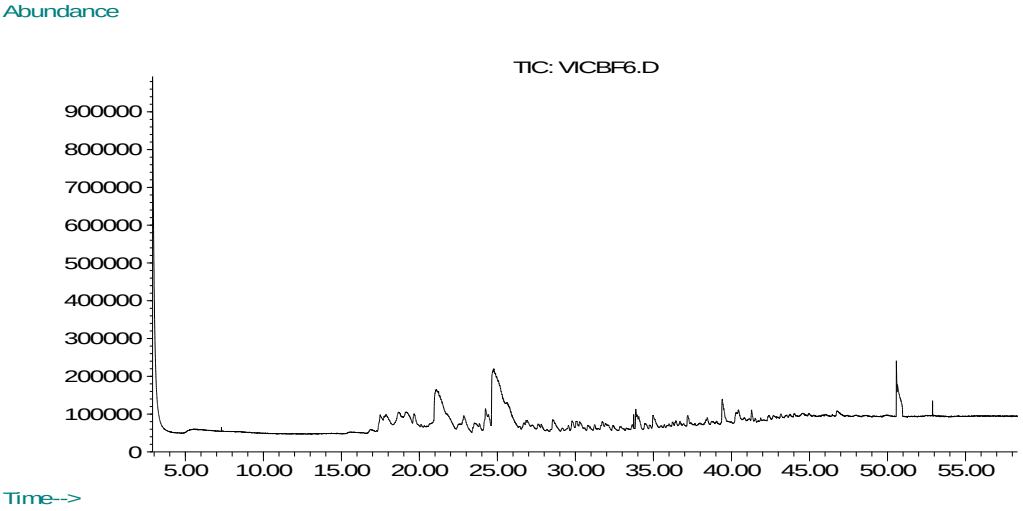


Fig. 4.15 Gas chromatogram of aroma compounds from cassava fish spontaneously fermented for 6 days

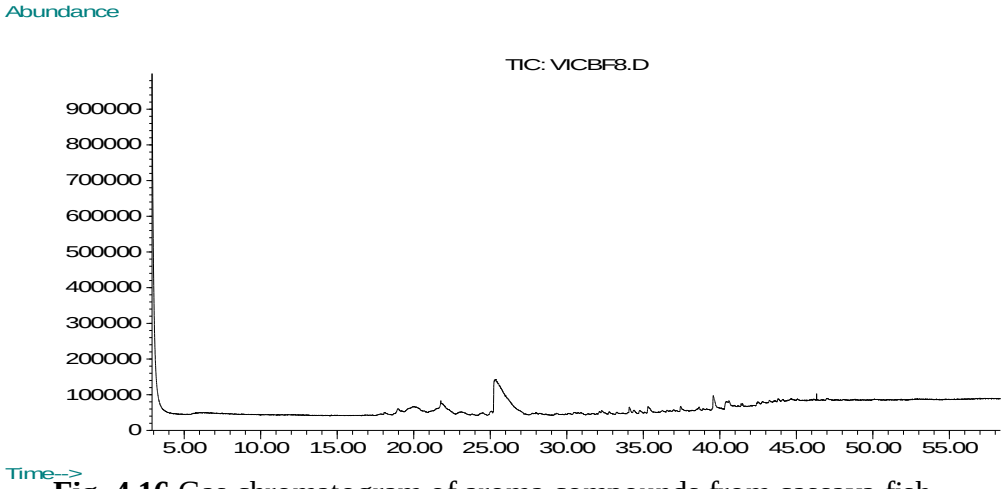


Fig. 4.16 Gas chromatogram of aroma compounds from cassava fish spontaneously fermented for 8 days

Table 4.20 Volatile aroma compounds identified during the spontaneous fermentation of cassava fish

Aroma compounds	Identification	
	R T (min)	Q I
Aliphatic hydrocarbons (15)		
2,2-dimethyl-3-Octyne	32.615	**
1,2-dimethyl-3-pentyl-4-propyl-Cyclohexane	12.732	*
3,4-bis (1-methylethenyl)-1,1-dimethyl-Cyclohexane	14.017	*
3-ethyl-2-methyl-, (Z)- 1,3-Hexadiene	15.680	**
1-pentyl-Cyclohexene	36.604	**
2-chloro-1,1,3,3,3-pentafluoro-1-Propene	49.861	**
1R-Ethoxy-3-cis-methoxy-5-cis-methylcyclohexane	32.773	**
3,4-Diethyl-3-hexene	18.331	**
Cyclododecyne	9.570	**
1-Thienylcyclohexene		
1,1,3,4-tetramethyl-Germacyclopent-3-ene	48.266	**
Cyclododecane	34.224	***
1,2-Cyclononadiene	8.864	**
1,4-dimethyl-cis-Cyclohexane	28.443	**
Decahydro-1,2-dimethyl-Naphthalene	36.282	**
Aromatic hydrocarbons (6)		
[2-(methylsulfinyl)ethenyl]-Benzene	36.137	***
3,5-dichloro-1-methoxy-Benzene	43.600	**
1-methoxy-2-methyl- Benzene	6.891	**
1-bromo-4-(ethoxymethyl)- Benzene	47.878	**
1,3-dimethoxy-Benzene	49.379	**
Chloro-Benzene	17.815	***
Esters (9)		
Ethyl palmitate	36.449	***
3-Pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester	4.241	**
1,3-Cyclobutanedicarboxylic acid, 1-chloro-, dimethyl ester	7.913	**
Dibutyl phthalate	39.659	***
Cycloheptane pentanoic acid, 2-oxo-, methyl ester	45.677	***
Benzyl acetate	29.537	*
Benzenepropanethioic acid, .beta.-oxo-, O-ethyl ester	49.832	**
2-methyl-propyl heptanoate	4.934	**
Ethyl 2,4-decadienoate	54.921	**
Ketones (9)		
4-(methylamino)-3-octen-2-one	7.212	*
3-phenyl-5(4H)-Isoxazolone	40.600	**
3,4-dihydroxy-3-Cyclobutene-1,2-dione	54.510	**
1-[(3-hydroxy-6-methyl-2-pyridinyl)thio]- 2-Propanone	47.255	**
4-ethoxyoctahydro-8a-methyl-1(2H)-Naphthalenone	32.001	**
3-methyl-2(1H)-Pyridinethione	36.916	**
Octahydro-trans-2(1H)-Naphthalenone	38.076	**
4-methoxy- Cyclohexanone	47.100	**
2-amino- 4(1H)-Pyrimidinone	57.398	**

Table 4.20 (continued)

Aroma compounds	Identification	
	RT (min)	QI
Acids (7)		
5-Heptylpyrimidine-2-carboxylic acid	25.254	**
2,4,5-trimethyl Benzoic acid	28.256	**
1,2-Dithiolane-3-pentanoic acid	54.540	**
Hexadecanoic acid	40.300	***
8-Nonynoic acid	40.247	***
3,5-diamino-Benzoic acid	14.665	**
[(2-methylpropyl)thio]-Acetic acid	51.530	**
Alcohols (5)		
5-methyl-2- Cyclohexanol	21.080	**
1,4-Eicosanediol	45.325	**
1,3-Cyclohexanediol	29.309	**
3-methoxybenzyl alcohol	32.651	**
Decahydro-1,4-Naphthalenediol	31.725	*
Amines (9)		
Bicyclo[2.2.2]octan-1-amine	28.244	**
2-Hydroxy-4-hydroxyaminopirimidine	9.227	*
2-Benzothiazolamine	20.165	**
1-Chloro-2-diethylaminoethane	19.551	**
1-(cyanoacetyl)-Piperidine	35.966	**
6-methyl-2-Benzothiazolamine	31.486	**
1-(1-Cyanocyclohexyl) morpholine	19.335	**
2,3-diethyl-5-methyl- Pyrazine	10.664	**
4-[1-(1-methylethyl)-1-propenyl]- Morpholine	48.271	**
Amides (2)		
2-Cyclobutene-1-carboxamide		
N-phenyl-Ethanethioamide	21.080	**
	50.479	**
Aldehydes (7)		
3.5-dimethoxybenzaldehyde	24.201	**
5-Fluoro-2-nitrobenzaldehyde	16.076	**
3-fluoro-4-hydroxy-Benzaldehyde	24.945	**
Hexadecanal	34.984	**
Propylhydrazone-Isobutyraldehyde	39.840	**
5-Ethylcyclopent-1-ene Carboxaldehyde	11.818	**
(Z)-4-heptenal	11.160	*
Pyrroles (2)		
Indole (1H-Benzo [b] pyrrole)	34.134	***
2,5-Dimethyl-1-propylpyrrole	32.597	**

Table 4.20 (continued)

Aroma compounds	Identification	
	RT (min)	QI
Thiazoles (6)		
2,4-diphenyl-Oxazole	49.748	****
1-Butyl-1H-1,2,4-triazole	24.728	****
5-Ethyl-2-isopropyl-4-methyloxazole	8.404	**
5-hydroxymethyl-4-trifluoromethyl-Imidazole	30.314	**
2-(N,N',N'-Trimethylhydrazino)-1,3-Benzothiazole	18.253	**
3,4-dimethyl- Isothiazole	36.145	**
Furan (2)		
3-Methyl-2-(2-methyl-2-butenyl)-furan	16.273	**
Carboxyfuran	43.333	**
Phenol (3)		
2,6-dimethoxy-Phenol	48.560	**
2-methoxy-4-(1-propenyl)-Phenol	16.029	**
3-methoxy-2,4,5-trimethyl-Phenol	58.022	**

RT: retention time; QI: quality index

Quality index= degree of agreement between mass spectrum of sample and mass spectrum in database on a scale from 0 to 100;

*** Quality index > 90

** Quality index between 80 and 90

* Quality index between 70 and 80

Table 4.21 Relative concentration of predominant aroma compounds identified per fermentation time during the spontaneous fermentation of cassava fish

Aroma compounds in ppm*	Fresh fish	Fermentation time (days)			
		2	4	6	8
Aliphatic hydrocarbons (8)					
Cyclododecane	39.4	nd	nd	nd	nd
3,4-bis (1-methylethenyl)-1,1-dimethyl-Cyclohexane	10.3	24.9	nd	25.2	nd
3-ethyl-2-methyl-, (Z)- 1,3-Hexadiene	7.9	22.0	nd	nd	nd
2-chloro-1,1,3,3,3-pentafluoro-1-Propene	nd	24.4	nd	nd	nd
1R-Ethoxy-3-cis-methoxy-5-cis-methylcyclohexane	nd	nd	18.9	19.1	21.1
1,2-Cyclononadiene	nd	nd	15.6	23.7	27.5
1,4-dimethyl-cis-Cyclohexane	nd	nd	10.2	25.9	25.2
Decahydro-1,2-dimethyl-Naphthalene	nd	nd	12.5	nd	31.4
Aromatic hydrocarbons (6)					
[2-(methylsulfinyl)ethenyl]-Benzene	nd	28.2	24.5	0.7	17.5
3,5-dichloro-1-methoxy-Benzene	nd	48.9	2.9	nd	nd
2-methoxy-4-(1-propenyl)-Phenol	nd	nd	nd	nd	nd
1-bromo-4-(ethoxymethyl)- Benzene	nd	nd	19.1	20.7	22.3
3-methoxy-2,4,5-trimethyl-Phenol	nd	nd	21.4	21.2	23.2
Chloro-Benzene	100.4	nd	nd	nd	nd
Esters (6)					
Ethyl palmitate	nd	nd	nd	30.7	nd
Dibutyl phthalate	263.2	120.7	113.4	81.9	nd
Cycloheptane pentanoic acid, 2-oxo-, methyl ester	nd	22.0	19.7	16.8	14.7
Benzyl acetate	nd	nd	16.5	15.1	15.4
2-methyl-propyl heptanoate	1.3	nd	nd	nd	nd
Ethyl 2,4-decadienoate	2.1	nd	nd	nd	nd
Acids (4)					
[(2-methylpropyl)thio]-Acetic acid	0.8	12.3	17.3	nd	nd
2,4,5-trimethyl Benzoic acid	2.5	23.7	nd	nd	nd
1,2-Dithiolane-3-pentanoic acid	nd	nd	47.4	48.1	48.7
Hexadecanoic acid	nd	nd	38.2	52.9	60.4
Aldehydes (6)					
5-Fluoro-2-nitrobenzaldehyde	nd	nd	1.6	nd	nd
3.5-dimethoxybenzaldehyde	10.0	15.3	nd	17.1	nd
3-fluoro-4-hydroxy-Benzaldehyde	nd	nd	2.1	nd	nd
Hexadecanal	21.6	28.9	35.1	70.8	72.3
(Z)-4-heptenal	10.8	nd	nd	nd	34.2
5-Ethylcyclopent-1-ene Carboxaldehyde	13.3	nd	nd	nd	25.9
Pyrroles (1)					
Indole	nd	39.0	77.7	165.8	182.7
Alcohols (3)					
5-methyl-2-Cyclohexanol	15.7	29.7	nd	nd	nd
1,4-Eicosanediol	12.3	5.5	24.7	nd	nd
Decahydro-1,4-Naphthalenediol	nd	nd	nd	nd	13.1
3-methoxybenzyl alcohol	26.4	1.3.	nd	nd	nd
1,3-Cyclohexanediol	16.2	1.5	nd	nd	nd

nd: compounds not detected

***** Values are means of determinations from two fermentation trials

4.3.4 Quality characteristics of laboratory samples of *lanhouin*

The microbiological status and data on the physico-chemical composition of laboratory *lanhouin* samples prepared from cassava fish are presented in Tables 4.22 and 4.23. On the microbiological aspect, the total viable count for all the samples ranged between 2.6×10^4 and 1.8×10^5 and were within acceptable limits. *Staphylococcus aureus* and *Clostridium* spp. were not detected in any of the samples in contrast with commercial *lanhouin* samples (Anihouvi *et al*., 2006). On the physico-chemical aspects, the water activity (A_w) of the samples was low as were the free fatty acid (FFA) and the thiobarbituric acid number (TBA). The decrease in A_w contributes to eradicating microrganisms that could not survive in less humid environments (Troller *et al*., 1992). It is also known that the stability of salted and dried food products depends on their water activity, a measure of the available water, which is able to react chemically or to support the growth of microorganisms such as bacteria and moulds (Beddow, 1985; Troller *et al*., 1992). The A_w levels observed in the samples range between 0.59-0.70 and are relatively low to support microbial proliferation and enzymatic activity during storage (Table 4.22). However there is a need to investigate the packaging of the product which could increase the keeping quality of *lanhouin* during storage. In warm and humid regions such as Benin, fermented and dried foods can take up water during storage and this could lead to spoilage. However, vacuum sealed packaging storage was not investigated as a result of the lack of facilities. The histamine levels in all the samples were lower than the recommended level of 20 mg/100g stipulated by the Food and Drug Administration (USA), the European Economic Community (EEC) and the Australian National Food Authority (FDA, 1982; EEC, 1990; ANFA, 2001). Despite the effect of the fermentation, the *lanhouin* samples contained relatively high contents of protein and relatively low contents of total volatile nitrogen. The absence of food borne pathogens such as *Staphylococcus aureus* and *Clostridium* spp., and the low content of histamine, indicate that the laboratory samples of *lanhouin* were safe for consumption.

Table 4.22 Microbiological status (cfu/g) of laboratory samples of *lanhouin* obtained by the spontaneous fermentation

Parameters	Laboratory *lanhouin*	
	4 days fermentation (n = 10)	8 days fermentation (n = 10)
Total viable count	$(1.4 \pm 0.4 \times 10^5$	$(3.3 \pm 0.7) \times 10^4$
Staphylococcus aureus	Absent	Absent
Enterobacteria	Absent	Absent
Clostridium spp.	Absent	Absent

[1] Means ± standard deviations; n- Number of samples analysed

Table 4.23 Physico-chemical composition of laboratory samples of *lanhouin* obtained by the spontaneous fermentation

Parameters	Laboratory *lanhouin*	
	4 days fermentation (n = 10)	8 days fermentation (n = 10)
Water activity	0.67 ± 0.03	0.61 ± 0.02
Moisture (%)	48.15 ± 1.50	43.85 ± 1.20
Protein (% nitrogen × 6.25)- (g/100 g)[2]	29.40 ± 2.00	32.74 ± 1.80
Fat (%)[2]	0.66 ± 0.03	0.78 ± 0.02
Total volatile nitrogen (mg N / 100 g)[2]	225.10 ± 14.75	260.15 ± 9.15
Histamine (mg / 100 g)[2]	10.15 ± 1.10	12.45 ± 1.65
Free fatty acids (% oleic acid)[2]	8.10 ± 0.15	10.75 ± 0.18
TBA (mg malonaldehyde / kg)[2]	1.35 ± 0.08	4.05 ± 1.02

[1] Means ± standard deviations; [2] Wet weight basis; n- Number of samples analysed

4.3.5 Sensory evaluation of laboratory samples of *lanhouin*

Twenty four panellists used a multiple paired comparison test to comparatively evaluate their preference for the colour, aroma, texture and taste of 4 different samples of *lanhouin*. There were 2 *lanhouin* samples prepared using the optimized process and 2 market *lanhouin* samples.

The results of multiple paired comparison test are shown in Table 4.24. The Friedman's test was used to statistically determine the significance of the differences noted. For each pair, market samples versus market samples, market samples versus laboratory samples and laboratory samples versus laboratory samples, there were statistically significant differences between samples. The laboratory *lanhouin* fermented for 4 days and 8 days were the most preferred followed by market *lanhouin* fermented for 4 and 8 days respectively for aroma and taste. For the colour, a significant difference ($p < 0.05$) was also noted within and between the samples with laboratory *lanhouin* fermented for 4 days being most preferred, followed by market *lanhouin* fermented for 4 days, laboratory *lanhouin* fermented for 8 days and market *lanhouin* fermented for 8 days. In contrast, for the texture, the laboratory *lanhouin* fermented for 8 days was preferred to laboratory *lanhouin* fermented for 4 days; the differences in preference for texture observed were not statistically significant ($p > 0.05$). Overall most (85%) of the panellists preferred the laboratory *lanhouin* fermented for 4 days because of its aroma and taste. Summary of scores for individual product and attributes as well as Friedman's least significant difference (LSD) test are given in Appendix 5.

Concerning the aroma, conditions which favour lipid oxidation in fish during fermentation may directly or indirectly contribute to the development of the odour of fermented fish samples. Salting and fermentation act to reduce the moisture and increase the salt content of the fermenting fish samples with fermentation time, thus creating suitable conditions for the oxidation of the fish lipid with the formation of off-odours and flavours reduction (Love, 1980; Horner, 1997). In addition, the degradation of protein with the formation of nitrogenous basic compounds and some undesirable compounds such as indole increased with the fermentation time and could have a significant impact on the aroma of the fermented fish samples. All this could explain the reason why the panellists preferred the aroma of the samples fermented for 4 days. The desirable light colour (or whiteness) of the laboratory samples fermented for 4 days could be attributed to the high concentration of salt on the skin

of the fish during the fermentation; the occurrence of Maillard reaction involving free amino groups and carbonyls and the formation of brown pigments as fermentation progressed could also contribute to the development of the colour and this may influence the degree of lightness of the colour of the samples as the fermentation progressed (Smith and Hole, 1991). The involvement of proteins in biochemical reactions in the fish during the fermentation would be also reflected in the texture of the fermenting fish samples. Consequently, the fish fermented for 8 days (which were more soft) were preferred than those fermented for 4 days (which were less soft).

Table 4.24 Rank sum totals for different samples of *lanhouin* evaluated

	Fermented fish samples			
Attribute	LLF4	LLF8	MLF4	MLF8
Aroma	126*	110	103	93
Taste	127	116	114	87
Colour	127	101	108	96
Texture	112	116	111	105

LLF4: laboratory lanhouin fermented for 4 days; LLF8: laboratory lanhouin fermented for 8 days; MLF4: market lanhouin fermented for 4 days; MLF8: market lanhouin fermented for 8 days.
* Higher number indicates higher degree of preference

4.4 Production of modified lanhouin using starter culture: inoculated fermentation of cassava fish flesh

Strains of the two predominant organisms (*Bacillus subtilis* and *Bacillus licheniformis*) and strains of *Staphylococcus lentus* and *Staphylococcus xylosus* initially isolated during the spontaneous fermentation of *lanhouin* were used as starter cultures for the controlled fermentation of *lanhouin*. The changes occurring during this controlled/inoculated fermentation as well as the role of the two predominant organisms were investigated. The ability of *Bacillus* species to produce histamine was also investigated. Certain species of *Bacillus* can produce histidine decarboxylase enzyme yielding to histamine production (Taylor, 1986; Ababouch *et al*., 1990).

The minced flesh used was pasteurised at 80°C for 30 mins followed by rapid cooling to about 30°C. During heating the natural microflora on the fish was destroyed and this

facilitated the growth of the microorganisms used as starter culture. In addition, the enzymes present in the fish flesh were inactivated, thus preventing any external influences except those of the inocula introduced into the fish for the fermentation purpose.

4.4.1 Growth of microorganisms during the inoculated fermentation of cassava fish flesh with *Bacillus* species as starter culture

The growth of microorganisms during the controlled fermentation of fish is shown in Table 4.25. The trend of total viable counts in the fermenting fish samples inoculated with single and mixed starter cultures of *Bacillus* species were similar. For all fermentations, a gradual increase in total viable counts was observed with final counts of 4.8×10^6 cfu / g, 1.6×10^6 and 3.7×10^6 cfu / g after 48 h of fermentation for *Bacillus subtilis, Bacillus licheniformis* and a mixture of *B. subtilis* and *B. licheniformis* respectively, while no growth was observed in the control sample. However *Bacillus subtilis* exhibited more limited growth in mixed culture with *B. licheniformis* compared to *B. subtilis* used as single inoculum. The ability of test microorganisms to grow varied significantly ($p < 0.05$) between *Bacillus* species used as single or mixed starter cultures.

Table 4.25 Changes in total viable count during the inoculated fermentation of cassava fish flesh with *Bacillus* species as starter cultures

Fermentation time (h)	Log cfu/ g sample			
	BS	BL	BS+BL	Control
0	4.33 ± 0.10^{a}	4.34 ± 0.08^{a}	4.36 ± 0.06^{a}	0
12	4.88 ± 0.09^{b}	4.67 ± 0.11^{ab}	4.50 ± 0.10^{abc}	0
24	6.60 ± 0.12^{c}	6.14 ± 0.06^{ac}	6.18 ± 0.11^{abd}	0
48	6.8 ± 0.13^{d}	6.20 ± 0.10^{ad}	6.56 ± 0.14^{bcd}	0

BS-*Bacillus subtilis*; BL- *Bacillus licheniformis*; control: non-inoculated sample

[1] Means ± standard deviations of three different fermentations;

a, b, c, d: Means with different letters in a row are significantly different ($p < 0.05$)

4.4.2 Changes in pH during the inoculated fermentation of cassava fish flesh with *Bacillus* species as starter cultures

For all the *Bacillus* species used as single and mixed inocula, the pH values of the fish mix samples increased from their initial values of about 6.5 as the fermentation progressed and reached final values of 7.10, 6.93 and 6.98 after 48h of fermentation for *Bacillus subtilis, Bacillus licheniformis* and the mixed inoculum of *B. subtilis* and *B. licheniformis* respectively. However *Bacillus subtilis* used as single inoculum gave the most alkaline product (Fig.4.17). A statistically significant difference ($p < 0.05$) was noted on the pH values between *Bacillus* species used as single or mixed starter cultures. The pH of the non-inoculated sample (control) remained the same throughout fermentation since there was no microbial activity (Fig.4.17). The strain of *Bacillus subtilis* used had a greater ability to increase the pH than the strain of *Bacillus licheniformis* and it could be concluded that the *B. subtilis* strain had a higher proteolytic activity than the *B. licheniformis* strain. The pH values recorded with single and mixed starter cultures of *Bacillus subtilis* were similar to those recorded for the spontaneous fermentation of *lanhouin*.

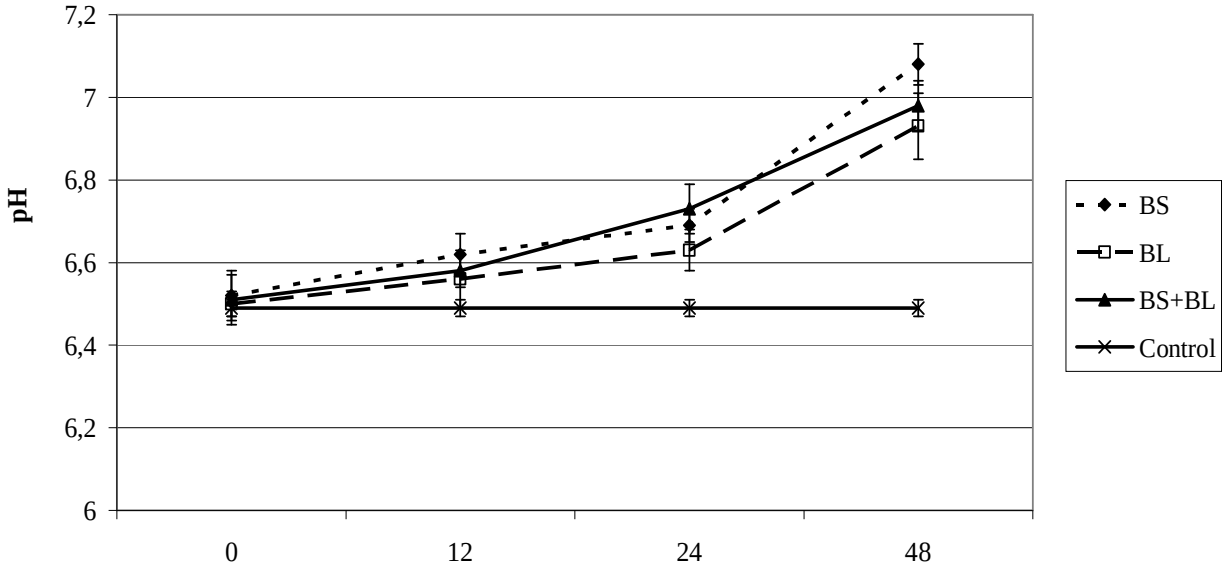


Fig.4.17 Changes in pH during the inoculated fermentation of modified *lanhoiun* with *Bacillus* species

BS- *Bacillus subtilis*; BL-*Bacillus licheniformis*; Control- sterile and non-inoculated fish mixture

4.4.3 Changes in proteolytic activity during the inoculated fermentation of cassava fish flesh with *Bacillus* species as starter cultures

The proteolytic activity of the microorganisms used as inocula was monitored through the total volatile nitrogen (TVN) level as the result of breakdown of proteins by the extracellular protease produced by the test organisms. The breakdown of proteins leads to the formation of nitrogenous basic compounds indicated by the ammoniacal smell of the inoculated fish samples. As shown in Fig. 4.18, the single and mixed cultures fermentations with the Bacillus species exhibited different proteolytic activities. Bacillus subtilis used as single inoculum exhibited the highest level of proteolytic activity (Fig.4.18). In addition, it was observed that the level of proteolytic activity developed by Bacillus licheniformis as a mono starter culture was lower compared to Bacillus licheniformis used as mixed culture with Bacillus subtilis. From this observation, it can be inferred that the increase in the proteolytic activity of the mixed culture was due to the presence of Bacillus subtilis. The dominance of Bacillus subtilis over the other Bacillus species during the spontaneous fermentation of lanhouin may be due to its ability to produce higher levels of proteolytic enzymes. The gradual increase in the TVN content of the inoculated fish samples during fermentation was due to increasing proteolytic activity whilst no such observation was made in the control sample since there was no microbial activity (Fig.4.18). The levels of TVN content were significantly different ($p< 0.05$) between the *Bacillus* species used as single or mixed starter cultures.

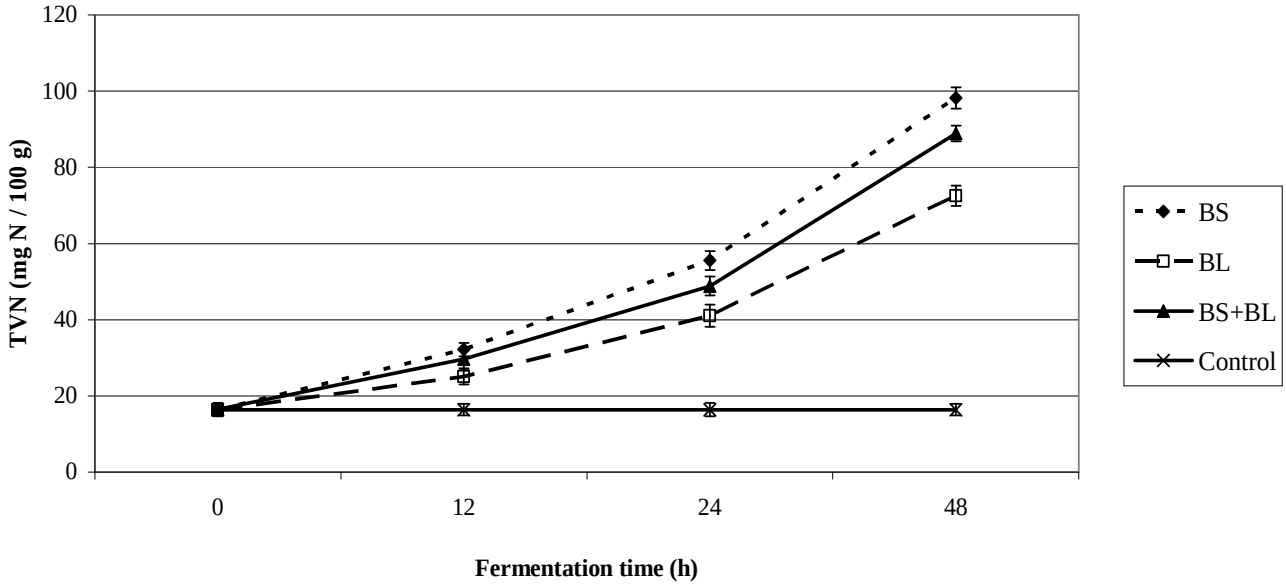


Fig. 4.18 Change in TVN during the inoculated fermentation of modified *lanhouin* with *Bacillus* species

BS-*Bacillus subtilis* ; BL-*Bacillus licheniformis* ; Control-sterile and non-inoculated samples

4.4.4 Performance of other starter cultures tested during the inoculated fermentation of cassava fish flesh

Performances of other starter cultures tested are summarised in Table 4.26. The use of either *Staphylococcus lentus* or *Staphylococcus xylosus* did not result in much increase in pH of the fish mixture, though *Staphylococcus xylosus* showed slightly higher increases. The pH values obtained after 48 h of fermentation were 6.55 and 6.67 for *Staphylococcus lentus* and *Staphylococcus xylosus* respectively. There was a statistically significant difference ($p < 0.05$) between these pH values. The results show that in the fermentations involving *Staphylococcus xylosus* as a member of a two or three-membered starter culture, the pH values obtained were higher when compared with fermentations involving *Staphylococcus lentus* as single culture; however significant differences were not observed between these pH values. Similary the TVN contents of fish mix inoculated with *Staphylococcus xylosus* as a member of starter cultures were noted to be higher than TVN levels obtained with *Staphylococcus lentus* used as a single culture (Table 4.26). The presence of *Staphylococcus lentus* in the fermenting fish mix had no significant ($p<0.05$) effect on the TVN levels whilst the presence of *Staphylococcus xylosus* significantly influenced ($p<0.05$) the TVN levels. From this observation, it appears that despite their relatively low number, *Staphylococcus xylosus* may play some role during the spontaneous fermentation of *lanhouin*.

From this investigation, it appeared that controlled fermentation of *lanhouin* can be performed by using a single starter culture of *Bacillus subtilis* as well as mixed starter cultures containing *Bacillus subtilis* and *Bacillus licheniformis*, *Bacillus subtilis* and *Staphylococcus xylosus*, and *Bacillus licheniformis* and *Staphylococcus xylosus*. However, further experiments need to be performed for a better understanding of the inoculated fermentation. This could provide comprehensive knowledge on the processing which will facilitate the development of a modern food condiment along the lines of the "bouillon cube" (Plate 4.9). For this purpose, the role of different predominant microorganisms in the development of the taste and aroma of *lanhouin* during fermentation needs to be investigated. A preliminary investigation on the aroma compounds produced by the two predominant *Bacillus* species was performed during the present study.

Table 4.26 pH and TVN of cassava fish flesh samples inoculated with other starter cultures and fermented for 48 hrs

Starter cultures	pH	TVN (mg N / g)
Control	6.49 ± 0.03^{a}	16.40 ± 0.02^{d}
S L	6.55 ± 0.12^{a}	29.65 ± 1.50^{ab}
SX	6.67 ± 0.11^{b}	36.50 ± 1.45^{ac}
SL+BS	6.99 ± 0.10^{c}	70.35 ± 1.45^{ad}
SL+BL	6.94 ± 0.07^{c}	58.92 ± 1.36^{d}
SL + BS + BL	7.00 ± 0.06^{c}	84.89 ± 0.90^{abc}
SX + BS	7.03 ± 0.06^{c}	85.86 ± 1.41^{abc}
SX + BL	6.96 ± 0.08^{c}	78.16 ± 2.09^{ad}
SX + BS + BL	7.04 ± 0.07^{c}	96.56 ± 0.83^{abd}

[1] Means ± standard deviations of three different fermentation experiments

Control- sterile and non inoculated fish mix

a, b: Means with different letters in a row are significantly different ($p < 0.05$)

SL- *Staphylococcus lentus;* SX- *Staphylococcus xylosus;* BS- *Bacillus subtilus*

BL- *Bacillus licheniformis*

A sensory evaluation test was carried out to determine the acceptability of three modified *lanhouin* samples obtained by the single and mixed fermentations with *Bacillus subtilis* and *Bacillus licheniformis*. The results showed that the samples had acceptable sensory properties of aroma, taste, colour and overall acceptability. However, the samples from the mixed fermentation with *Bacillus subtilis* and *Bacillus licheniformis* were the most preferred by the panellists (65%) in terms of aroma and taste, with proportions of 40% and 25% for aroma and taste respectively. The colour of two samples was acceptable and not significantly different

($p>0.05$). The single fermentation samples inoculated with *Bacillus subtilis* were preferred to those inoculated with *Bacillus licheniformis* in term of aroma but a significant difference was not found for the taste and the colour. From this sensory test, it appears that the mixed fermentation samples were more acceptable as compared to samples from single fermentation.

Plate 4.9 ″Bouillon cube″, expected end-product to be appropriately packaged for the modern market (modified *lanhouin* cube)

4.4.5 Aroma compounds of modified *lanhouin* obtained by the inoculated fermentation of cassava fish flesh with *Bacillus* species as starter culture

A GC-MS system was used to detect aroma compounds in extracts of the inoculated fermenting fish. A total of 41 aroma compounds were detected in the samples of modified *lanhouin* obtained by the inoculated fermentation with single cultures of *Bacillus subtilis* and *Bacillus licheniformis* and mixed cultures of *Bacillus subtilis* and *Bacillus licheniformis.* The aroma profiles are represented by the Figures 4.19-4.21. Table 4.27 lists the compounds identified which consisted of 5 aliphatic hydrocarbons, 4 aromatic hydrocarbons, 5 esters, 6 ketones, 5 acids, 4 alcohols, 8 amines, 3 aldehydes and 1 amide. In contrast to the spontaneous fermentation, aroma compounds such as furan, phenol, thiazoles and pyrroles were not detected in the inoculated samples of modified *lanhouin*. However in both types of fermentation, carbonyls and lipid-derived compounds were predominant.

The results showed that the majority of acids and ketones were detected in samples inoculated with *Bacillus subtilis* whereas amides, aromatic hydrocarbons and alcohols were identified in samples inoculated with *Bacillus licheniformis* (Table 4.27). In contrast, the majority of aldehydes and amines were detected in samples inoculated with a mixed culture of *Bacillus subtilis* and *Bacillus licheniformis*. In addition, a number of aroma compounds found in the samples spontaneously fermented were not detected in the inoculated samples and *vice- versa* (Tables 4.20 and 4.27). The chromatograms of aroma compounds from samples inoculated with *Bacillus subtilis*, *Bacillus licheniformis* and a mixed culture of *B. subtilis* and *B. licheniformis* are showed in Fig.4.19- 4.21. The profiles of chromatograms from samples of *lanhouin* spontaneously fermented for 2 days (Fig.4.13) and samples inoculated with *B. licheniformis* and a mixed culture of *B. subtilus* and *B. licheniformis*, and fermented for 2 days (Fig.4.20 and 4.21) appeared to be similar indicating the important role of *B. licheniformis* in the build-up of aroma compounds in the products.

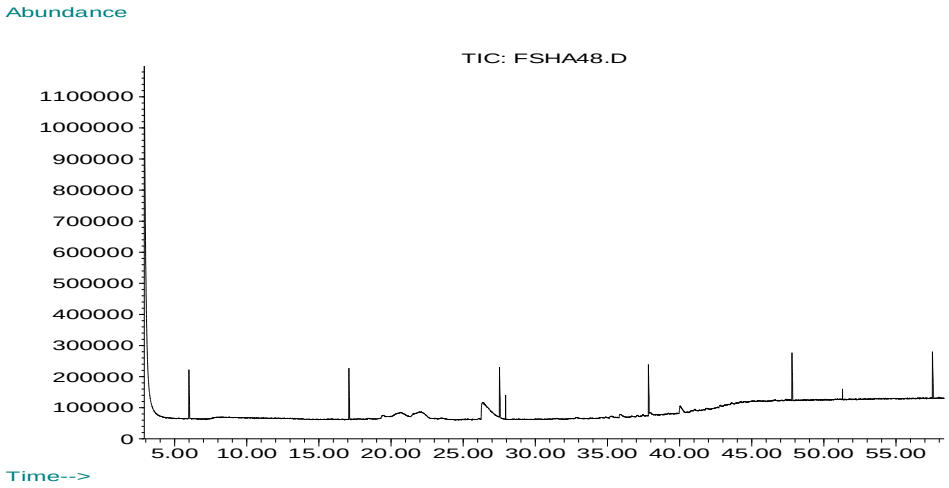


Fig. 4.19 Gas chromatogram of aroma compounds from cassava fish inoculated with starter culture of *Bacillus subtilis* and fermented for 2 days

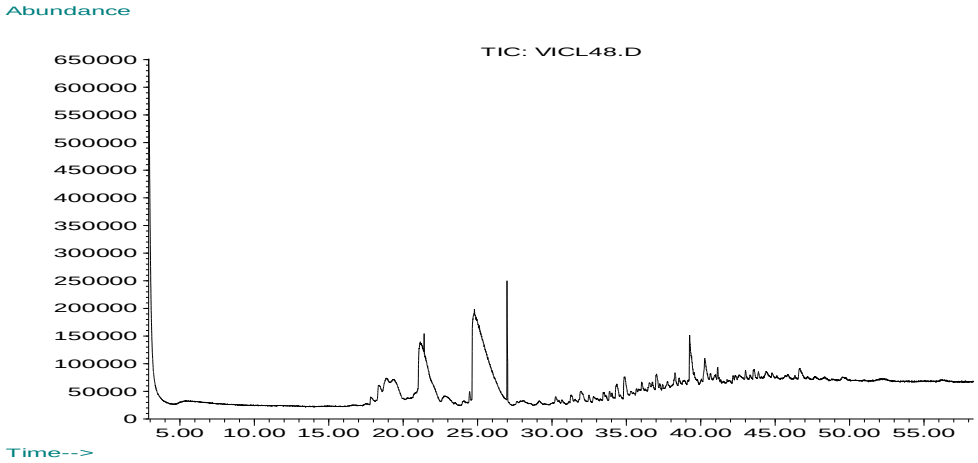


Fig. 4.20 Gas chromatogram of aroma compounds from cassava fish inoculated with starter culture of *Bacillus licheniformis* and fermented for 2 days

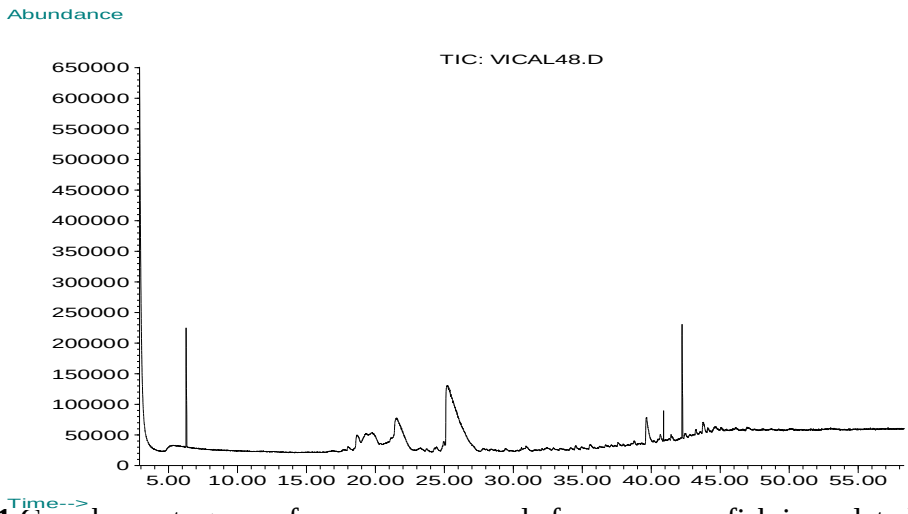


Fig. 4.21 Gas chromatogram of aroma compounds from cassava fish inoculated with a mixed starter culture of *B. subtilis* and *B. licheniformis*, and fermented for 2 days

Table 4.27 Volatile aroma compounds identified during the inoculated fermentation of cassava fish flesh with *Bacillus* species

Aroma compounds	Identification	
	R T (min)	Q I
Aliphatic hydrocarbons (5)		
Decahydro-2,3-dimethyl-Naphthalene[2]	42.013	**
(+)-valecene[2]	54.182	**
Cyclododecane[2]	34.301	***
1-methyl-4-methylene-Cyclohexane[3]	13.060	**
3,4-Diethyl-3-hexene[1]	18.331	**
Aromatic hydrocarbons (4)		
1,3-Dimethyl Benzene[2]	49.379	**
Chloro-Benzene[3]	23.055	**
1-chloro-4-ethenyl-Benzene[2]	18.719	**
1-methoxy-2-methyl- Benzene[3]	50.905	**
Esters (5)		
1 H-Pyrrole-2-Carboxylic acid, 5-ethyl-4-methyl-ethyl ester[1]	8.55	**
2-Methyl-3-Hexyne[3]	13.541	**
Dibutyl Phthalate	39.955	***
1,3-Octadiene[3]	49.755	**
Cycloheptane pentanoic acid, 2-oxo-, methyl ester[2]	45.677	***
Ketones (6)		
1,2–Cyclohexadione[1]	6.245	**
1,3-difluoro-2-propanone[1]	47.525	**
3,4,4-trimethyl-2,5-cyclohexadien-1-one[3]	41.227	**
Bicyclo[4.1.0] heptan-2-one[1]	44.522	**
6-Methyltetrahydro-1,3-oxazine-2-thione[2]	49.97	**
3-methyl-2(1H)-Pyridinethione[1]	36.916	**
Acids (5)		
1H-Imidazole-2-carboxylic acid[3]	38.076	*
5-Heptylpyrimidine-2-carboxylic acid[1]	25.254	**
Hexadecanoic acid[1]	40.300	**
[(2-methylpropyl)thio]-Acetic acid[2]	47.851	**
1,2-Dithiolane-3-pentanoic acid[1]	54.540	**
Alcohols (4)		
2-methyl-2-Propen-1-ol[3]	39.158	*
1,4-Eicosanediol[1]	45.325	**
1,3-Cyclohexanediol [3]	29.309	**
1-Tetradecanol [2]	31.950	***

Table 4.27 (continued)

Aroma compounds	Identification	
	RT (min)	QI
Amines (8)		
6- Hydroxydopamine[2]	30.416	**
2-Ethyl-3-methoxypyrazine[3]	40.368	**
5,7-dichloro-Thiazolo[5,4-d]pyrimidine[3]	42.599	**
2-Ethoxy-3-ethylpyrazine[3]	42.784	**
N-ethyl-N-(1-methylethyl)-2-Propanamine[3]	50.498	**
4-propyl-Piperidine[2]	52.534	***
2-Benzothiazolamine[1]	20.165	**
1-(cyanoacetyl)-Piperidine[1]	35.966	**
Aldehydes (3)		
4-dimethyl amino-benzaldehyde[3]	57.027	****
2-methyl 2 Pentenal[3]	38.818	*****
Hexadecanal[2]	34.851	**
Amides (1)		
2-Cyclobutene-1-carboxamide[3]	38.650	**

RT: retention time; QI: quality index

Quality index= degree of agreement between mass spectrum of sample and mass spectrum in database on a scale from 0 to 100.

[1] Samples inoculated with culture of *Bacillus subtilis*

[2] Samples inoculated with culture of *Bacillus licheniformis*

[3] Samples inoculated with mixed culture of *Bacillus subtilis* and *B. licheniformis*

*** Quality index > 90

** Quality index between 80 and 90

* Quality index between 70 and 80

4.4.6 Histamine content of modified *lanhouin* obtained by the inoculated fermentation of cassava fish flesh with *Bacillus* and *Staphylococcus* species.

Histamine contents of all fish mixes inoculated with *Bacillus subtilis* and *B. licheniformis,* and *Staphylococcus lentus* and *S. xylosus* were very low and less than 1 mg / 100 g sample (Table 4.28). In addition, the levels of histamine in the inoculated samples were similar to those recorded on the control samples (pasteurized and non-inoculated fish flesh) suggesting that histamine is not formed during the fermentation period. These results are an indication that both *Bacillus* spp. and *Staphylococcus* spp. used appear not to be histamine producers. The low amount of histamine found in the samples was probably formed before the fresh fish was treated (pasteurized and inoculated). This tends to show that histamine can survive the thermal processing and may be present in food after heat treatment.

Table 4.28 Histamine content in the modified *lanhouin* samples

Fermentation Time (h)	Histamine content (mg / 100 g sample)				
	FBS	FBL	FSL	FSX	Control
0	0.93 ± 0.01	1.01 ± 0.03	0.89 ± 0.01	0.86 ± 0.02	1.02 ± 0.02
24	1.00 ± 0.02	0.94 ± 0.01	0.91± 0.02	0.84 ± 0.01	0.93 ± 0.01
48	0.96 ± 0.01	0.93 ± 0.01	0.98 ± 0.01	0.90 ± 0.02	0.96 ± 0.02

FBS- fish mixture inoculated with *Bacillus subtilis*

FBL- fish mixture inoculated with *Bacillus licheniformis*

FSL- fish mixture inoculated with *Staphylococcus lentus*

FSX- fish mixture inoculated with *Staphylococcus xylosus*

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, the socio-economic aspects of fish processing to *lanhouin* were investigated. Fresh cassava fish (*Pseudotolithus* sp.) was spontaneously fermented for 3 to 8 days using the optimal fermentation conditions evolved from the optimization of spontaneous fermentation process characteristics. The fresh fish was also fermented for 48 h using the predominant microorganisms isolated and identified during the spontaneous fermentation. Microbiological and physico-chemical changes were monitored during the two types of fermentation. In addition, the laboratory *lanhouin* samples were evaluated for their organoleptic qualities. The main results obtained were as follows:

1. The survey of traditional processing of fish and retailing of fish products including *lanhouin* revealed that this sector was dominated by women and it was a major source of income for most of them. The study of the traditional process showed that there were problems in the handling of the fish and also hygiene practices were not taken into consideration during processing, leading to products of inconsistent quality. Improving the traditional processing techniques and hygienic quality of the product will ensure the promotion of *lanhouin* sector of the traditional fish processing industry, which will go a long way in enhancing the income of the women. Although sixteen different types of fish were cited by the processors as mostly used for *lanhouin* production, but cassava fish (*Pseudotolithus* sp.) use was predominant.

2. Histamine contents in the majority of market samples were higher than the maximum allowable level of 20 mg / 100g fish; whilst histamine contents in all laboratory samples were lower than the recommended level. This means that safe products can be produced if the process is controlled. In contrast to the majority of market *lanhouin*, the laboratory *lanhouin* samples had a better microbial status and this could be seen as a result of good handling and better processing conditions mainly, the repening time and level of salt concentration used to treat the fish.

3. Intense microbiological and biochemical activities take place during the spontaneous fermentation of *lanhouin*. Though a variety of microorganisms are involved in the

fermentation with *Bacillus* species and *Staphylococcus* species being the predominant ones. The biochemical changes resulted in a decrease of protein and moisture contents of the fermenting fish, and an increase in salt, free fatty acids, thiobarbituric acid number and total volatile nitrogen contents as fermentation time increased. The histamine content did not show this kind of linear relationship with fermentation time.
A sequential build-up of aroma compounds was observed during fermentation and the predominant ones detected over the fermentation period were nitrogen-containing compounds and lipid-derived components.

4. The processing of fish into *lanhouin* is carried out through the recommended process flow diagram showed in Fig.4.2 (method 1, with a ripening time of 8hrs and salt concentration of 25-30%). Among the different steps of the flow diagram, the status of fresh fish, the gutting, the ripening time and the salting constitute the main points where a careful control may contribute to reduce significantly the production of histamine. Consequently, those different steps constitute critical control points in *lanhouin* production.

The status of fresh fish constitutes a very important critical control point since it is a step where the fish may be contaminated through improper handling. In this respect good handling practices such a prompt icing of the fresh fish, cleaning of processing environments, good personal hygiene of *lanhouin* processors, use of potable water, cleaning of equipments and containers during all the processing constitute a general critical control point to improve the microbiological status of fresh fish and the end product.

There is a relationship between gutting and histamine formation. Gutting may help to reduce proteolytic enzymes which may speed up the autolytic process of fish and bacteria growth and consequently may increase the production of histamine. In this respect, histamine levels in the fresh fish can be controlled by gutting promptly after purchasing the fish.

The use of salt with poor microbiological quality may result in the introduction of certain types of bacteria into the fish. The control of this critical control point can be achieved by using salt with good microbiological quality.

In order to reduce lipid oxidation during processing and storage, it is important to use lean fish for *lanhouin* production.

5. The inoculated fermentation of fish with *Bacillus subtilis* as a single starter culture and as a mixed starter culture with *Bacillus licheniformis* as well as *Staphylococcus lentus* and *Staphylococcus xylosus* was found to be effective.

5.2 Recommendations for further study

A potential problem of fermented fish is the continued bacterial and enzymatic activities within the fish during storage, resulting in an unstable product. There is a need to find out how to stop the fermentation and inhibit further microbial growth and enzymatic activity in order to increase the shelf life of the product. We suggest irradiation where it is permitted.

Further studies need to be performed for a better understanding of the controlled fermentation with regard to changes in aroma profile and flavour quality, and histamine formation.

REFERENCES

Ababouch, L., Afilah, M.E., 1988. Histamine forming bacteria isolated from spoiling sardines. *Food microbiology*, 2: 385-389.

Ababouch, L., 1990. Histamine in fishery products: a review. Proceedings of the FAO expert consultation on fish technology in Africa. Abidjan, Côte d'Ivoire, 25–28 April 1988. FAO-Fisheries Report, 1990, n° 4000, Supplement, pp 44–50.

Ababouch, L., Afilah, M. E., 1991. Identification of histamine producing bacteria isolated from sardine (*Sardina pilchardus*) stored in ice and at ambient temperature (25°C). *Food Microbiology*, 8(2):127-36.

Abbey, L. D., Hodari-Okae, M. and Osei-Yaw A., 1994. Studies on traditional processing and quality of fermented fish (momone). Ghana/Netherlands Artisanal Fish Processing and Applied Research Project Report. Food Research Institute, Accra, Ghana, 48p.

Achinewhu, S.C., Oboh, C.A., 2002. Chemical, microbiological and sensory properties of fermented fish products from *Sardinella* sp. in Nigeria. *Journal of Aquatic Food Product Technology*, 11 (2): 53-59.

Achinewwhu, S.C., Amadi, E.N., Barimalan, I.S., Eke, J., 2004. Microbiology of naturally fermented fish (*Sardinella* sp.). *Journal of Aquatic Food Product Technology*, 13 (1): 47-53.

Adams, M.R., Cooke, R.D., Twiddy, D.R., 1987. Fermentation parameters involved in the production of lactic acid preserved fish-glucose substrates. *International Journal of Food Science and Technology*, 22:105-114.

Adams, M.R., Cooke, R.D., Rattagool, P., 1985. Fermented fish products of S. E. Asia. Tropical Science, 25: 61-73.

Adams, M.R, 1986. Fermented flesh foods (sausages, fish products). In Progress in industrial microbiology. Londres & New-York, Elsevier, 23:159-98.

Ahmed, F., 1991. Scombroid (histamine) fish poisoning. Committee on evaluation of the safety of fishery products, National Academy Press, Washington, DC. Seafood Safety, pp 93-96.

Aidoo, K.E., Henry, R. and Wood, B.J., 1982. Solid substrate fermentations. *Adv. Appl. Microbiology*, 28: 201-237.

Amano, K., 1972. The influence of fermentation on the nutritive value of foods, with special reference to fermented fish products of South-East Asia. In Fish in Nutrition. E. Heen and R. Kreuzer (Eds.). Londres, Fishing News (Books) Ltd., pp 180-200.

Amoa-Awua, W.K.A., 1996. The dominant microflora and their role in the fermentation of "agblima" cassava dough. PhD thesis, University of Ghana, p91.

Amoa-Awua, W.K.A, Jakobsen, M., 1995. The role of *Bacillus* species in the fermentation of cassava. *J. of Applied Bacteriol*., 79: 250-256.

An, H., 1998. Proteolytic activities in salmon muscle and its inactivation for quality assurance. From "Proceedings of the Fourth International Symposium on the Efficient Application and Preservation of Marine Biological Resources". Kangnung, Korea.

ANFA, 2001. Australian Food Standards Code; Part D Fish and Fish products. Standards D1 and D2 version 18.

Anihouvi, V.B., Hounhouigan, J.D., Ayernor, S.G., 2005. La production et la commercialisation du *lanhouin*, un condiment à base de poisson fermenté du Golfe du Bénin. *Cahiers Agricultures*, 14 (3) : 323-330.

Anihouvi, V.B., Ayernor, S.G., Hounhouigan, J.D., Sakyi-Dawson, E., 2006. Quality characteristics of *lanhouin*, a traditionally processed fermented fish in the Republic of Benin, *AJFAND*, issue10, 6(1):1-15.

Anonymous, 2005a. Produit intérieur brut du Bénin: composantes et emplois. Rapport annuel INSAE/DSEE/SCN, p40.

Anonymous, 2005b. La pêche au Bénin : atouts, contraintes et perspectives ; MDR/DE/DP Cotonou, juillet 2005, 6p.

AOAC, 1995. Official Methods of Analysis, 16th revised edition, vol. 2. Association of Official Analytical Chemists.

Arnold, S.H. and Brown W.D., 1978. Histamine toxicity from fish products. Adv. Food Res. 24:113-54.

Aryanta, R.W., Fleet, G.H., Buckle, K.A., 1991. The occurrence and growth of microorganisms during the fermentation of fish sausage. *International Journal of Food Microbiology*, 13: 143-156.

Asastyasih, M. and Madden J., 1986**.** Effect of plant products and extracts on the acceptability of fish to flies. Unpublished report of research. Institute of Fish Technology, Jakarta, Indonesia.

Azokpota, P., Hounhouigan J.D**.**, Nago, M.C. and Jakobsen M. 2006. Esterase and protease activities of *Bacillus spp* from *afitin*, *iru* and *sonru*, three African locust bean (*Parkia biglobosa*) condiments from Benin. *African Journal of Biotechnology*, 5 (3): 265-272.
Bacus, J., 1984. Utilisation of microorganisms in meat processing Letchworth, Herts., UK, Research Studies Press Ltd and New-York: J. Wiley & Sons Inc.

Baek, H.H.; Cadwallader, K.R., 1997. Aroma volatiles in cooked alligator meat. *Journal of Food Science*, 62 (2): 321-325.

Baross, J.A., Lenovich, L.M., 1992. Halophilic and osmophilic microorganisms. In: Vangerzant, C., Splittstoesser, D. F., (Eds), Compendium of methods for the microbiological examination of foods. American Public Health Association, third edition, pp99 – 238.

Bauer Von, F., Seuss, I., Paulsen, P. and Vali, S., 1994. The formation of biogenic amines in meat and meat products. ICMST 1994. S-V. 25.

Beddows, C.G., 1985. Fermented fish and fish products, in "`Microbiology of fermented foods", Vol.2, edited by B.J.Wood, Elsevier Applied Science Publishers, London, pp1-39.

Benson, H.J., 1990. Microbiological Applications, *A Laboratory Manual in General Microbiology*, fifth Edition, WCB W.M. C. Brown Publishers, United states. Pg. 40 and 134.

Benjakul, S., Seymour, T.A., Morrissey, M.T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. *J. Food Sci.* 62(4):729-733.

Bol, J. and de Vos, M.W., 1991. Fermented foods- an overview. In Biotechnological Innovations in Food Processing, ed. Green, J., Oxford: Butterworth- Heinemann, pp 45-76.

Boore K., 1986. Gram-negative bacteria. In Bergey's Manual of Systematic Bacteriology, vol. 2, Sneath, P. H. A., Baltimore: Williams and Wilkins.
Borquez, R. and Gonzalez, C., 1994. Influence of acetic acid preservation of Chilean mackerel (*Trachurus murphy*) on fish meal production. *J. Sci. Food Agric.* 66 (2): 187-192.

Brink, B.T., Danunk, C., Joosten, H.M.J., 1990. Occurrence and formation of biologically active amines in foods. *Int. J. of Food Microbiology*, 11:73-84.

Bykowski, P. and Dutkiewicz, D., 1996. Freshwater fish processing and equipment in small plants, FAO Fisheries circular. No. 905. Rome, FAO. http:/ /www. Fao.Org/ docrep/ w 0495e/ w0495E00- 02. htm

Cadwallader, K.R., Back H.H., Chung H.Y. and Moody M.W., 1994. Contribution of lipid derived components in the flavour of alligator meat. In lipids if Food Flavors, C.T.Ho and T.G. Hartman (Ed.). ACS Symposium series 558, American Chemical Society, Washington, DC.

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

Castell, C.H., Neal, W.E., Dale, J., 1973. Comparison of changes in trimethylamine, dimethylamine and extractable protein in iced and frozen gadoid fillets. J. Fish. Res. Board Can., 30 (8): 1246-8.

CEE, 1990. Proposition de règlement (CEE) du Conseil arrêtant les règles sanitaires régissant la production et la mise sur le marché des produits de la pêche. *Journal officiel des communautés européennes* C84, (33) : 58-70.

Cha, YJ, Cadwallader, K.R, 1998. Aroma-active compounds in skipjack tuna sauce. *Journal of Agricultural and Food Chemistry,* 46 (3) 1123-1128.

Chen, C.M., Marshall, M.R., Koburger, J.A., Otwell, W.S. and Wei Cheng, 1988. Determination of minimal temperatures for histamine production by five bacteria. In: proceedings of the Twelfth-Annual-Conference of the Tropical and Subtropical Fisheries,

Technological Society of the America, Florida-Univ., Gainesville-USA, Sea-GrantColl. Program, pp365-376.

Choi, Y.J., Cho**,** M.S., Park, J.W., 2000. Effect of hydration time and salt addition on gelation properties of major protein additives. *J. Food Sci.* 65(8):1338-1342.

Chung, H.Y., Yung, I.K.S.Y., Ma, W.C.J., Kim, J.S., 2002: Analysis of volatile components in frozen and dried scallops (*Patinopecten yessoensis*) by gas chromatography/mass spectrometry. *Food Res. Int.* 35: 43–53.

Claus, D., Berkeley, R.C.W., 1986. The genus *Bacillus*. In: Sneath P. H. (Ed.), Bergey's Manual of Systematic Bacteriology, Baltimore: Williams and Wilkins, 2:1105-1139.

Clucas, I.J, 1982. Fish handling, preservation and processing in the tropics. II.Report,-Tropical-Products-Institute; G145, p144.

Collart, A., 1986. Planification du développement des pêches artisanales en Afrique de l'Ouest : production et traitement du poisson, ses aspects matériels, techniques et socio-économiques. Cotonou, rapport projet DIPA, p67.

Collette, R.L., 2001. Industry practice of HACCP to prevent histamine in Tuna and other seafoods. Food Regulatory Affairs, National Fisheries Institute, Arlington, VA 22209. http://ift.confex.com/ift/2001/techprogram//paper_9642.ht

Collins, C.H., Lyne, P.M., 1984. Microbiological methods. Fifth edition, Butterworths, London, p450.

Connell, J.J., 1970. Properties of fish protein. In proteins as human food; edited by R. A. Lawrie, Butterworths, London.

Connell, J.J., 1975. Control of fish quality. Farnham, Surrey, UK. Fishing News (Books) Ltd.

Cooke, R.D., Twiddy, D.R. and Reilly, P.J.A., 1987. Lactic acid fermentation as a low-cost means of food preservation in tropical countries. *FEMS Microbiol., Rev*. 46, 369-379.

Cowan, S.T., Steel, K.J., 1974. Manual for the identification of medical bacteria, 2 nd ed., London: Cambridge University Press, pp45 –122.

Dakwa, S., Sakyi-Dawson, E., Diako, C., Takyiwa Annan, N., Amoa-Awua, W.K., 2005. Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy-dawadaw). *International Journal of Food Microbiology*, 104: 69-82.

Dalgaard, P. 1993. Fresh and lightly preserved seafood. In: Man,C.M.D. and A.A. Jones (eds) *Shelf-Life Evaluation of Foods.* Aspen Publishers Inc., London, UK., pp110-139.

Demeyer, D., Hoozec, J. and Mesdom H., 1974. Specificity of lipolysis during sausage fermentation and ripening. *J. Food Sci*., 39: 293-296.

Den Brinker, C., Kerr, M. and Rayner, C., 1995. Investigation of biogenic amines in fish and fish products. Food Safety Unit. State Chemistry Laboratory, Werribee, July 1995, pp 5-14.

Deng, F.H., Sheng, H., Chyuan, Y. and Chang, C.C. 1995. Biogenic amines in the flesh of Sailfish (Istiophorus platypterus) responsible for Scombroid poisoning. *Journal of Food Science*, 60 (5): 926- 928.

Dube, S., Dlamini, N.R, Muzungu, W., 2004. Levels and types of aerobic sporeforming bacteria associated with dried Matemba. *African Journal of Food, Agriculture, Nutrition and Development*, 4 (2).

Eerola, S., Maijala, R., Rig Sagués, A., Salminen, M. and Hirvi, T., 1996. Biogenic amines in dry sausages as affected by starter culture and contaminant amine-positive *Lactobacillus*. *J. Food Sci.* 61 (6): 1243-1246.

Ehira, S., 1976. A biochemical study on the freshness of fish. *Bull. Tokai Reg. Fish. Res. Lab.*, 88: 1-132.

Eitenmiller, R.R., Orr, J.H., Wallis, W.W., 1982. Histamine formation in fish: Microbiological and biochemical conditions. In Chemistry and Biochemistry of Marine food products, Publisher- AVI, Connecticut, edited by R. Martin pp 39-50.

Eitenmiller, R.R., 2001. Chemical aspects of histamine formation in foods. Histamine and toxigenic amines in foods. IFT Annual meeting, New Orleans, Louisiana. http://ift.confex.com/ift/2001/techprogram/session_754.htm

Esser, J.R., 1988. Prevention of insect infestation and losses of salted dried fish in Indonesia by treatment with an insecticide approved for use on fish. R. Sunness (ed.) proceedings of a symposium held in conjunction with the seventh session of the Indo-Pacific Fishery Commission Working Party on fish technology and marketing. FAO Regional Office, Bangkok, Thailand, 19 –22 April, 1988.

Essuman, K.M., 1990. Artisanal fish containers in Ghana. West Q-B; RAFR publication. FI/90/1, p32.

Essuman, K.M., 1992. Le poisson fermenté en Afrique : traitement, commercialisation et consommation. FAO Doc. Tech. Pêches, 329: p80.

Eskin, N.A.M., Henderson, H.M., Townsend, R.J., 1971. Biochemistry of foods. Academic Press, New York - San Francisco- London, p227.

FAO, 1971. Poisson fermenté et produits dérivés. Préparé par I. M Mackie, R. Hardy et G. Hobbs. FAO Fish. Rep. (Fr), 100: p62.

FAO, 1981. The prevention of losses of cured fish. FAO Fish. Tech. Pap, 219: p87.

FAO, 1989. Fish technology in Africa. A successful TCDC experience. RAFR /FI/88/1. Rome, pp2-23.

FAO, 1993. Fermented fish in Africa. A study on processing, marketing and consumption. Fish. Tech. Paper, n° 329.

FAO, 1987. Technical publication on measures for improving the utilisation and marketing of fish in West Africa. FAO: JEFAD/AMS/87/43, pp23 -25.

FAO, 1997. Fishery statistics, captures production, N° 147, vol. 84.

FAO, 2000. Fishery statistics- Capture production, vol. 86/1 1998, pp99-100.

FAO / SIFAR, 2001. Non-Sensory Assessment of Fish Quality. (FAO in partnership with Support unit for International Fisheries and Aquatic Research, SIFAR, 2001). Torry Advisory Note No. 92. http://www.fao.org/wairdocs/tan/x5990e/X5990e00.htm

FAO, 2004. Mainstreaming fisheries into national development and poverty reduction strategies: current situation and opportunities, by A. Thorpe. FAO Fisheries Circular No. 997, Rome

Fernandez-Salguero J. and Mackie I.M., 1979. Histamine metabolism in mackerel (Scomber scombrus). Studies on histamine decarboxylase activity and histamine formation during storage of flesh and liver under sterile and non-sterile conditions, *J. Food Technology*, 14: 131-139.

Ferencik, M., 1970. Formation of histamine during bacterial decarboxylation of histidine in the flesh of some marine fishes. *J. Hyg. Epid. Microbiol. Immunol.*, 14 :52-60.

Fogarty, W.M., Griffin, P.J. and Joyce, A.M., 1974. Enzymes of Bacillus species – Part 1. Process Biochemistry, 9: 11-24.

Food and Drug Administration, 1982. Defect action levels for histamine in tuna; availability of guide. Fed. Reg. 470: 40-87.

Frank, H.A., 1985. Histamine forming bacteria in tuna and other marine fish. In: Histamine in marine products: production by bacteria, measurement and prediction of formation. B.S. Pan and D. James (eds). FAO Fish. Rep., 252: 2-3

Gbaguidi A., 2001. Annuaire statistique des pêches maritimes. Rapport annuel, Ministère du Développement Rural, Direction de l'Elevage /Direction des Pêches (MDR/DE/DP, Cotonou, 80p.

Gillespie, N.C. and Macrae I.C., 1975. The bacterial flora of some Queensland fish and its ability to cause spoilage. *J Appl. Bacteriol.*, 39: 91-100.

Gil, K.D., Pal, R. and Sandhil, R.N., 1989. Effect of chronic cadmium exposure on lipid composition and peroxidation in liver and kidney in rats. *Med. Sci. res.* 17: 927-923.

Gram, L., 1989. Isolation, identification and characterization of bacteria isolated from tropical fish. PhD thesis, the technological laboratory of the Danish Ministry for Fisheries and the Royal Veterinary and Agriculture University of Denmark.

Gram, L. and Huss, H.H. 2000. Fresh and processed fish and shellfish. In: Lund, B.M., T.C. Baird-Parker and G.W. Gould (eds.) *The Microbiological Safety and Quality of Foods.* Aspen Publishers Inc., Gaitherburg, Maryland, USA. pp 472-506.

Gram, L. and Dalgaard P., 2002a. Fish spoilage bacteria-problems and solutions. In: Current opinion in Biotechnology, 13 (3): 262-266.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B. and Givskov, M., 2002b. Food spoilage-interactions between food spoilage bacteria. *International Journal of Food Microbiology* 78: 79-97.

Gram, L., 2003. Fermented fish products microbiology and technology. [Feb.13-2003]; http://www.dfu.min.dk/micro; http://www.dfu.min.dk/micro/lg.htm.

Gunaratme, S. and Samarajeewa, U., 1995. Effect of processing on histamine in fish and dried fish. Proceedings of the third Asia Pacific Food Analysis Network, Manila, Philippines.

Halasz, A., Barath, A., Simon-Sarkadi, L., Holzapfel, W., 1994. Biogenic amines and their production by micro-organisms in food - Review. Trends in Food Sci. and Tech., 5: 42-49.

Hall, G.M., 1997. *Fish Processing Technology*, 2nd Edition, Blackie Academic Professional. London pp1, 22, 24, 26, 160,170.

Hammes, W.P., Rolz, I., Bantleon, A., 1985. Mikrobiologische Untersuchung der auf dem deutschen Markt vorhandenen Starterkultupraparate fur die Rohwurstreifung. Fleischwirtsch, 65: 729-734.

Hammes, W.P, 1986. Starterkulturen in der Fleichwirt-schaft.*Chem. Mikrobiol. Technol*. Lebensm, 9: 131- 143.

Hanson, S.W., Knowles, M.J. and Al-Kasade A.S., 1985. Histamine in southeast Asia cured fish and changes in histamine levels during salting and drying. FAO fesheries Report, 317 suppl., 3: 386-392.

Hardy, R. and Smith, J.G.M., 1976. The storage of Mackerel (*Scomber scombrus*). Development of histamine and rancidity. *J. Sci. Food Agric.*, 27:595.

Harrigan, W.F., 1998. *Laboratory methods in Food Microbiology*. 3rd Edition. Academic Press. San Diego. pp 424 – 462.

Hebard, C.E., Flick, G.J. and Martins, R.E., 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. In chemistry and biochemistry of marine food products, ed. R.E. Martin et al. Westport, Connecticut, AVI Publishing Co, pp 149-304.

Herbert, R.A. and Shewan, J.M., 1975. Precursors of the volatile sulphides in spoiling North Sea Cod (*Gadus morhua*). *J. Sci. Food Agric.*, 26: 1195-1202.

Herbert, R.A., Hendrie, M.S., Gibson, D.M. and Shewan, J.M., 1971. Bacteria active in the spoilage of certain sea foods. *J. Appl Bacteriol*., 34 (1): 41-50.

Herbert, R.A. and Shewan, J.M., 1976. Roles played by bacterial and autolytic enzymes in the production of volatile sulphides in spoiling North Sea cod (*Gadus morhua*). *J. Sci. Food Agric.*, 27:89-94.

Hesseltine, C.W., 1991. Mixed culture fermentations: an introduction to oriental food fermentations. In mixed cultures in biotechnology. Ed. Zeikus, J G. and Johnson E. A., , New-York: Mc Graw-Hill, pp1-16.

Horner, W.F.A., 1997. Preservation of fish by curing (drying, salting and smoking). In Fish processing Technology, 2nd edition. Edited by G. M. Hall, Blackie Academic & Professional (Chapman & Hall) London, pp32–72.

Hounhouigan, D.J., 1994. Fermentation of maize (Zea mays L.) meal for mawè production in Bénin. Physical, chemical and microbiological aspects. PhD thesis, p99.

Horemans, B., 1998. The state of artisanal fisheries in West Africa in 1997. Programme for the Integrated Development of Artisanal Fisheries in West Africa, Cotonou- Bénin, IDAF / WP/122, p44.

Holzapfel, W., 1997. Use of starter cultures in fermentation on a household scale. *Food control*. Vol 8. No. 5/6 pp241–258.

Hui, J. and Taylor, S., 1983. High pressure liquide chromatographic determination of putrefactive amines in food. *J AOAC,* 6: 853-857.

Hsu, C.K., Kolbe, E., Morrissey, M.T. and Chung, Y.C. 1993. Protein denaturation of frozen Pacific whiting (*Merluccius produc* tus) fillets. *J. Food Sci.* 58:1055-1075.

Huss, H.H. 1988. Fresh fish: quality and quality changes. A training manual prepared for the FAO/DANIDA Training Programme on Fish Technology and Quality Control. FAO Fisheries series, 29: p132.

Huss, H.H. 1975. Principal changes during storage of wet fish. FAO/DANIDA Training Programme on Fish Technology and Quality Control. FAO Fisheries series, n° 33501, E1, p16.

Huss, H.H., Valdimarson, G., 1990. Microbiology of salted fish. FAO Food Tech. News, 10 1: p190.

Huss, H.H. (eds) 1995. *Quality and Quality Changes in Fresh Fish.* FAO Fisheries technical paper No. 348., FAO, Rome, Italy.

Ito, Y. and Watanabe, K., 1968. Variations in chemical composition in the fillet of corvina and "pescada-foguete". *Contrib. Inst. Oceanogr. Univ. Sâo Paulo* (*Ser. Techhnl.*), 5: 1-6.

Itou, K., Akahane,Y., 2000. Changes in proximate composition and extractive components of rice-bran-fermented mackerel heshiko during processing. In Nippon-Suisan-Gakkaishi, 66 (6), 1051-1058.

Izquierdo-Pulido, M., Marine-Font, A., Vidal-Carou, M., 1994. Biogenic amine formation during malting and brewing. *J. Food Sci*. 59: 104-1107.

Jespersen, L., Halm M., Kpodo K. and Jakobsen, M., 1994. The significance of yeasts and moulds occurring in maize dough fermentation for kenkey production. *Int. J. Food Microbiol*., 24: 239-248.

Kawai, T., 1996: Fish flavour. *Cri. Rev. Food Sci. Nutr.* 36: 257–298.

Kerr, M., Lawicki, P., Aguirre, S., Rayner, C., 2002. Effect of storage conditions on histamine formation in fresh and canned Tuna. Food Safety Unit, Department of Human Service. State Chemistry Laboratory, Werribee, pp5-20.

Kim, S.H. and Price, R.J., 1999. Histamine formation and bacterial spoilage of albacore harvested off the U. S. Northwest coast. *Journal of Food Science*, 64, (2): 340- 343.

Kim, S.H., Field, K.A., Morrissey, M.T., Price, R.J., Wei, C.I. and An, H. 2001. Source and identification of histamine-producing bacteria from fresh and temperature-abused albacore. *J Food Prot*. 64(7):1035-1044.

Kim, S.H., Price, R.J., Morrissey, M.T., Field, K.G., Wei, C.I. and An, H. 2002a. Histamine production by *Morganella morganii* in mackerel, albacore, mahi-mahi and salmon at various storage temperatures. *J. Food Sci*. 67(4): 1522-1528.

Kim, S.H., Price, R.J., Morrissey, M.T., Field, K.G., Wei, C.I. and An, H. 2002b. Occurrence of histamine-forming bacteria in albacore and histamine accumulation in muscle at ambient temperature. *J. Food Sci.* 67(4): 1515-1521.

Kimata, M., 1961. The histamine problem. In fish as food, Academic Press, New York and London, 1: p329.

Kingley-Ekow, G.S., 1999. A study of the effects of handling, processing and storage on the histamine content in salted, fermented Tilapia. M. Phill thesis, p133.

Kloos, W.E., Schleifer, K.H., 1986. The Genus *Staphylococcus* In: Sneath, P.H.A. (Ed.), *Bergey's Manual of Systematic Bacteriology*, Baltimore: Williams and Wilkins, 2:1013-1035.

Knochel, S. and Huss, H.H., 1984. Ripening and spoilage of sugar-salted herring with and without nitrate. 1. Microbiological and related chemical changes. *J. Food Technol*., 19:203-213.

Kocur, M., 1986. The Genus *Micrococcus*. In: Sneath, P.H.A (Ed.), *Bergey's Manual of Systematic Bacteriology*, Baltimore: Williams and Wilkins, 2: 1004-1008.

Ko, S.D., 1982. Indigenous fermented foods. In *Econ. Microbiol.* , 7: 15-38.

Korhonen, R.W., Lanier, T.C. and Giesbrecht, F., 1990. An evaluation of simple methods for following rigor development in fish. *J. Food Sci*., 55: 346-348.

Kranner, P., Bauer, F. and Hellwig, E., 1991. Investigations of the formation of histamine in foods, and food poisoning caused by histamine. Die Nahrung. 15: 109- 113.

Kuda, T., Miyamoto, H., Sakajiri, M., Ando, K., Yano, T., 2001. Microflora of fish nukazuke made in Ishikawa, Japan. In Nippon-Suisan-Gakkaishi; 67 (2) : 296-301.

Kuni, W.O., 2005. Microbial succession in cocoa bean fermentation. M.Phil. thesis, Department of Nutrition and Food Science, University of Ghana, p124.

Lartey, B.L., 1975. Some technological transformation and research needs in Ghana's indigenous food industries. UNIDO Joint Consultation on Promotion of Industrial Research and Services in Africa. Lagos 15 –22 September 1975. UNIDO.

Lee, C.H, 1990, Fish Fermentation Technology- A review. In Post harvest technology, preservation and quality of fish in Southeast Asia. Reilly, P.J.A., Parry, R.W.H. and Barile, L.E. (Eds.), IFS pp 1–13.

Lem, A. 2005. Aquaculture-world trends, opportunities for developing countries, technical and financial constraints. UNCTAD/FAO, Geneva, October 25: p48.

Le Guen, S., Prost, C., Demaimay, D., 2000. Characterization of odorant compounds of mussels (*Mytilus edulis*) according to their origin using gas chromatography–olfactometry and gas chromatography/mass spectrometry. *J. Chromat. A.* 896: 361–371.

Liepe, H.U., 1983. Starter culture in meat production. In *Biotechnology*, vol.5; ed. Rehm, H.J. and Reed G.; Basel: Verlag chemie, pp 400-424.

Lima Dos Santos, C.A.M., 1978. Bacterial spoilage of iced Amazonian freshwater catfish. UK, Loughborough Univ., Technology, (M.Sc. thesis).

Lin, T.M. and Park, J.W. 1996. Protein solubility in Pacific whiting affected by proteolysis during storage. *J. Food Sci.* 61(3): 536-539.

Liston, J., 1982. Recent advances in the chemistry of ices fish spoilage. In: Chemistry and Biochemistry of Marine Food Products. R.E. Martin, G.J. Flick and D.R. Ward (eds.).

Lopetcharat, K**.,** Choi, Y.J., Park, J.W., and Daeschel, M.D., 2001. Fish sauce products and manufacturing - a review. Food Reviews International 17(1):65-88.

Lopez, A., 1987. A complete course in canning Book. II and III, 12th ed., Baltimore USA. The canning Trade Inc.

Love, R.M., 1997. Biochemical dynamics and the quality of fresh and frozen fish. In Fish processing technology, 2nd edition. Edited by G. M. Hall, Blackie Academic & Professional (Chapman & Hall) London, pp1–26.

Love, R.M., 1980. The chemical biology of fishes. Vol. 2, Academic Press, London.

Lowe, T.E., Ryder J.M., Carragher, J.F. and Wells, R.M.G., 1993**.** Flesh quality in snapper (*Pagrus auratus*), affected by capture stress. *J. Food Sci.*, 58: 770-773.

Lucke, F.K., 1985. Fermented sausages. In: Microbiology of fermented foods, vol. 2 (edited by B.J. Wood), London, Elvisier Applied Science Publishers Ltd, pp 41-63.

Mackie, J.M., Thomson B.W., 1974. Decomposition of trimethylamine oxide during iced and frozen storage of whole and comminuted tissue of fish. Proc. Int. Congr. *Food Sci. Technol*., 4 (1): 243-50.

Maijala, R. and Eerola, S., 1993. Contaminant lactic acid bacteria of dry sausages produce histamine and tyramine. Meat Sci., 35: 387-395.

Maijala, R., 1994. Formation of biogenic amines in dry sausages with special reference to raw materials, lactic acid bacteria, pH decrease, temperature and time. Academic dissertation, Helsinki, Finland.

Maijala, R., Nurmi, E. and Fisher, A., 1995a. Influence of processing temperature on the formation of biogenic amines in dry sausages. *Meat Sci.* 39: 9-22.

Maijala, R., Eerola, S., Lievonen, S., Hill, P. and Hirvi, T., 1995b. Formation of biogenic amines during ripening of dry sausages as affected by starter culture and thawing time of raw materials. *J. Food Sci*. 60(6): 1187-1190.

Mensah, P.P.A., Tomkins, A.M., Drasar, B.S. and Harrison, J.T., 1991. Antimicrobial effect of fermented Ghanaian maize dough. *J. Appl. Bacteriol.*, 70: 203-210.

Metz, M., 1993. Starter cultures, their industrial manufacture for the meat industry. In Fleischwirtsch., English part, 73 (12): 1394-1396.

Miller, A., Scanlan, R.A., Lee, J.S., Libbey, L.M., 1973. Identification of the volatile compounds produced in sterile fish muscle by Pseudomonas fragi. *Appl. Microbiol*., 25: 952-955.

Mislivec, P.B., Beuchat, L.R. and Cousin, M.A., 1992. Yeasts and Molds. In: Vanderzant C and DF Spliistoesser (Eds). Compendium of methods for the microbiological examination of foods, 3rd ed., American Public Health Association, 16: 239-249.

Mochizuki, S. and Sato, A., 1994. Effects of various killing and procedures and storage temperatures on post-mortem changes in the muscle of horse mackerel. Nipon Suisan Gakkaishi 60 (1):125-130.

Morrissey, M.T., 1997. Low fat and reduced fat fish products. In *Healthy Production and Processing of Meat, Poultry and Fish Products* . A.M. Pearson and T.R. Dutson (Eds). Elsevier Applied Science, New York.

Murray, C.K. and Hobbs, G., 1982. Scombrotoxin and scombrotoxin-like poisoning from canned fish. *J. Hyg. Camb*. 88: 215-220.

Murray, J. and Burt, J.R., 1969. The composition of fish. *Torry Advis. Note, Aberdeen*, 38p.
Myers, R.H., 1971. Response Surface Methodoly. Allyan and Bacon: Boston.

Nakayama, T., Toyoda T., Ooi, A., 1994. Physical property of carp muscle during rigor tension generation. *Fish. Sci*. 60 (6): 717-721.

Nautilius, C., 1997**.** Marketing of Fishery products. FAO Globefish publication. [Nov.172000]<http://www.globefish.org/publications/specialseries/vols/vol.01.htm. >

N'Dir, B., Gningue, R.D., Keita, N.G., Souane, M., Laurent L., Cornelius C., Thonart, P., 1997. Caractéristiques microbiologiques et organoleptiques du nététu du commerce. Cahiers Agricultures, 6: 299-304.

Nerquaye-Tetteh, G., Eyeson K.K., Tete Marmon, 1978**.** Studies on "momone" a Ghanaian fermented fish product. Ghana. *J. Agric. Sci*., 11: 21-26.

Nickerson, G.B, Likens, S.T., 1966. Gas chromatographic evidence from the occurence of hop oil in Beer. *J. Chromatogr*., 21: 1-3.

Njai, S.E., 2000. Fishery post harvest practices in The Gambia. Fisheries Department, The Gambia. Unpublished, pp 1-20.

Nordic Committee on Food Analysis, 1986. Aerobic micro-organisms. Enumeration at 30°C in meat and meat products, 2nd ed., 86: 1-5.

Nordic Committee on Food Analysis, 1995. Coliform bacteria detection in foods, 4th ed., 44: 1-5.

Nordic Committee on Food Analysis, 1997. *Clostridium* determination in foods, 3rd ed., 95:1-4.

Nordic Committee on Food Analysis, 1992. *Staphylococcus aureus* determination in foods, 2nd ed., 66: 1-6.

Nordic Committee on Food Analysis, 1999. *Salmonella* detection in foods, 5th ed., 71: 1-9.

Nout, M.J., Rombouts, F.M. and Havelaar, A., 1989. Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic microorganisms. *Int. J. Food Microbiol*., 8: 351-361.

Odunfa, S.A., 1985a. African Fermented Foods. In: Word B.J. B. (Ed) Microbiology of Fermented Foods. London & New–York: Elsevier Applied Science Publishers, 2:155 – 191.

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

Olympia, M.S.D., 1992. Fermented fish products in the Phillippines. In: Application of Biotechnology to Traditional Fermented Foods. National Academic Press, Washington D. C., pp 131 –139.

Omafuvbe, B.O., Shonukan O.O. and Abiose, S.H., 2000. Microbiological and biochemical changes in the traditional fermentation of soybean for "soy-daddawa" Nigerian food condiment. *Food Microbiology*, 17: 469-474.

Oronsaye, J.A.O., 1991. An approach to fish processing and preservation. African Bio-Sciences Network (ABN), p125.

Owen, J.D. and Mendoza, L.S., 1985. Enzymatically hydrolysed and bacterially fermented fishery products. *J. Food. Technol*., 20: 273-293.

Parry, J.M., Turnbull, P.C.B. and Gibson, J.R. 1983. *A Colour Atlas of Bacillus Species*. Ipswich: Wolfe Medical.

Pearson, D., 1976. The chemical Analysis of Foods, 7th edition, Churchill Livingstone, Edinburgh London and New York, pp387-497.

Peters, G., Sylvia, G. and Morrissey, M.T. 1995. Determination of quality parameters for Pacific whiting. In *The Biology and Management of Hake* . Tony Pitcher and Jurgen Alheit (Eds). Chapman and Hall, London.

Pikul, J. and Fred, A. K., 1990. Lipid oxidation of chicken muscles and skin after roasting and refrigerated storage of main broiler parts. *J. Food Sci*., 55 (1): 30-37.

Poulter, R.G., 1982. Comparison of the biochemistry and bacteriology of tropical and temperate water fish during preservation and processing. Paper presented at the Symposium on Harvest and Post-Harvest Technology of Fish, Cochin, India, November, 1982, London, Trop. Dev. and Res. Inst..

Proctor, D.L., 1976. The control of insect infestation of fish during processing and storage in the tropics. In: Proceedings of the conference on the handling, processing and marketing of tropical fish, held in London, 5 th-9th july, pp307-311.

Prost, C.; Sérot, T.; Demaimay, M., 1998: Identification of the most potent odorants in wild and farmed cooked turbot (*Scophthalmus maximus*). *J. Agric. Food Chem*. 46: 3214–3219.

Raa, J., 1981. Biochemistry of microbial fish spoilage and preservation by lactic acid bacteria and added acid. In: Global ipacts of Applied Microbiology, S.O. Emejuaiwa, O. Ogunbi and S.O. Sann.(Eds), Academic Press.

Ramanathan, L. and Das, N.P. 1992. Studies on the control of lipid oxidation in ground fish by some polyphenolic natural products. *J.Agric. Food Chem.,* 40:17 – 21.

Rattagool, P., Metathip, P. and Esser, J.R., 1988. Evaluation of insecticides to protect salted-dried marine fish from insect infestation during processing and storage. In Thailand. R. Sunness (ed.), Proceeding of a symposium held in conjunction with the seventh session of the Indo-Pacific Fishery Commission Working Party on fish technology and marketing. Food and Agriculture Organisation Regional Office, Bangkok, Thailand, 19-22 April 1988.

Rice, S.L. and Koehler, P.E., 1976. Tyrosine and histidine decarboxylation activities of *Pediococcus cerevisiae* and *Lactobacillus* species and the production of tyramine in fermented sausages. *J. Milk Food Technol*. 39:166-169.

Rodríguez, M., Núñez F., Córdoba J.J., Sanabria C., Bermúdez E. and Asensio M.A., 1994. Characterization of *Staphylococcus* spp. and *Micrococcus* spp. isolated from Iberian ham throughout the ripening process. *Int. J. Food. Microbiol*. 24:329-335.

Rolle, R.S., 1997. Technical opportunities and challenges to upgrading food bioprocessing in developing countries. Review; Food and Agriculture organisation.

Russell, P.E. and Maretic, Z., 1986. Scombroid poisoning: Mini-revew with case histories. Toxicon , 24: 967- 973.

Saisithi, P., 1987. Traditional fermented fish products with reference to Thai products. *Asian Food J.*, 3: 3-10.

Sefa-Dedeh, S. and Youngs, A.J., 1976. The bacteriological quality of Ghanaian solar salt with reference to its use in fish preservation. *Ghana J. Sci.*, 16 (2): 7-13.

Sefa-Dedeh, S., 1993. Traditional food technology. In: Encyclopaedia of Foods. Food technology and Nutrition, ed. Macrae, R.; Robinson R. and Sadler M., New-York: Academic Press, pp 4600-4606.

Sefa-Dedeh, S., 1995. Traditional fish processes: Technology, quality and evaluation. In Workshop on Seeking Improvement in Fish Technology in West Africa. IDAF Technical Report. N^{o}: 66.

Sefa-Dedeh, S., Cornelius B., Sakyi-Dawson E. and Afoakwa E.O., 2003. Application of response surface methodology to study the quality chracteristics of cowpea-fortified nixtamalized maize. *Journal of Innovative Food Science and Emerging Technologies*, 4:109-119.

Serrini, G., Luzzana, U., Valfrè, F., 1994. Compounds and biochemical pathways involved in development and alteration of fish smell and flavour. *Ind. Alim.* 33: 538–544.

Shewan, J.M., 1966. Bacteriological and biochemical changes occurring during microbial spoilage of fish. Supplement to *Medlemsblad for Den Norske Veterinaerforening* (11): 13–20.

Shewan, J.M., 1974. The microbiology of fish and fish products. A progress Report. *J. appl. Bact.* 34 (2): 229-315.

Shewan, J.M., 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacteria action. In: Handling, processing and marketing of tropical fish, London, Tropical Products Inst., pp 51–66.

Silla-Santos, M.H, 1996. Biogenic amines: their importance in foods. *International Journal of Food Microbiology*, 29 (2/3): 213-231.

Silva, C.C.G., Da Ponte, D.J.B. and Dapkevicius, M.L.N., 1998. Storage temperature effect on histamine formation in big Eye Tuna and Skipjack. *Journal of Food Science*, 63 (4): 644 – 647.

Sims, G.G., Cambell, J.R., Zemlyakm, F. and Graham, J.M., 1977. Organochlorine residues in fish and fishery products from the Northwest Atlantic. Bull. Environ. Contam. & Toxic. 18: 697-705.

Smith, G. and Hole, M., 1991. Browing of salted sun dried fish. *J. Sc. Food and Agric*., 55 (2): 291-301.

Smith, J.G. M., Hardy, R. and Young, K.W., 1980. A seasonal study of the storage characteristics of mackerel stored at chill and ambient temperatures. In : Advances in fish Science and Technology, (Ed.) J. J. Connell. Fishing News Book Ltd., Farnham, U. K.

Sneath, P.H.A., 1986. Endospore-forming Gram-Positive Rodsa and Cocci. In: *Bergey's manual of systematic bacteriology*. Sneath, P.H.A. (ed), Baltimore: Williams and Wilkins, vol. 2, pp1105–1139.

Soyiri, I.N., 2002. Bioconversion of Tuna processing waste into fish sauce. M.Phil. thesis, Department of Nutrition and Food Science, University of Ghana, p189.

Steinkraus, K.H, 1985. Indigenous fermented food technologies for small-scale industries. *Food and Nutrition Bulletin*, 7(2): 21-27.

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

Stevenson, K.E. and Segner, W.P., 1992. Mesophilic aerobic sporeformers. In: Vanderzant C and DF Spliistoesser (Eds). Compendium of methods for the microbiological examination of foods, 3rd ed., American Public Health Association, 18: 265-274.

Spurvey, S., Pan, B. S., Shahidi, F., 1998. Flavour of shellfish. In: *Flavor of meat, meat products and seafoods*, 2nd ed. F. Shahidi (Ed.). Blackie Academic and Professional, London, pp159–196.

Stratton, J.E; Hutkins, R.W; Taylor, S., 1991. Biogenic amines in cheese and other fermented foods: a review; *Journal-of-Food-Protection*; 54 (6): 460-470.

Stroud, G.D., 1968. Rigor in fish- The effect on quality. Torry Advisory Note, Torry Research Station, Aberdeen, 36: p11.

Subasinghe, S., Mohideen, M.S., Vidanapathirana, S., 1990. Microbiological and biochemical changes in *Amblygaster sirm* during high salt fermentation. Post-harvest Technology, Preservation and Quality of Fish in Southeast Asia, pp 29-33.

Taylor, S.L, Guthertz, L.S., Leatherwood, M., Tillman, F. and Lieber, E.R., 1978. Histamine production by food-borne bacterial species. *J. Food Safety*, 1: 173–187.

Taylor, S.L. and Speckhard M.W., 1984. Inhibition of bacterial histamine product by sorbate and other antimicrobial agents. *J. Food Prot.*, 47: 508-511.

Taylor, S.L, Guthertz, L.S., Leatherwood, M. and Lieber, E.R., 1979. Histamine production by *Klebsiella pneumoniae* and an incident of scombroid fish poisoning. *Appl. Environ. Microbiol.*, 37: 274-278.

Taylor, S.L., 1986. Histamine food poisoning: toxicity and clinical aspects-a review CRC. Critical reviews in toxicology, 17: 91-128.

Taylor, S.L. and Bush, R., 1988. Allergy by ingestion of seafood in Handbook of Natural Toxins Vol.3. Marine Toxins and Venoms, edited by Anthony T., pp149-183.

Taylor, S.L., 1989. Histamine poisoning (scombroid fish poisoning): an allergy- like intoxication. Clin. Toxicol. 27: 225-240.

Tokunaga, T., 1970. Trimethylamine oxide and its decomposition in the bloody muscle of fish.1. TMAO, TMA and DMA contents in ordinary and bloody muscle. *Bull. Jap. Sci. Fish.*, 36 (5): 502-509.

Tomiyasu, Y. and Zenitani, B., 1957. Spoilage of fish and its preservation by chemical agents. Advances in Food Research 7: p41.

Toyama, R., 1982. The effect of drying and smoking on the nutritional properties of fish. In: Fish smoking and drying, Burt J.R. (ed.), pp 42-45.

Troller, J.A. and Scott, V.N., 1992. Measurement of water activity and acidity. In: Vanderzant C and DF Spliistoesser (Eds). Compendium of methods for the microbiological examination of foods, 3rd ed., American Public Health Association, 8: 135-151.

Trucco, R.E., 1982. Study on the evolution of rigor mortis in batches of fish. Lebensm.-Wiss. Technol., 15: 9-77.

Tungkawachara, S., Park, J.W. and Choi, Y.J. 2003. Biochemical properties and consumer acceptance of Pacific whiting fish sauce. *J. Food Sci.* 68(3): 855-860

Veciana-Nogues, M.T., Vidal-Carou, M.C. and Marie-Font, A., 1990. Histamine and tyramine during storage and spoilage of Anchovie (*Engraulis encrasicholus*): relationships with other fish sploilage indicators. *Journal of Food Science*, 55: 1192-1195.

Vidal-Carou, M.C., Veciana-Norgues, M.T. and Marine-Font, A., 1990. Histamine and tyramine during storage and spoilage of Anchovie, Engraulis encrasicholus: Relationships with other fish spoilage indicators. *J. of Food Sci.*, 55: 1192-1195.

Voigt, M.N. and Eitenmiller, P.E., 1977. Production of tyrosine and histamine decarboxylase by dairy related bacteria. *J. Food Prot.* 40: 241 – 245.

Watanabe, K.O., 1971. Physical characteristics and chemical composition of fresh bream, mud sucker, tiger fish and barb from Lake Kariba. *Fisheries Research Bull.*, 5: 153-173.

Watanabe, K.O., 1982. Fish handling and processing in tropical Africa. In: Compte rendu de la consultation d'experts FAO sur la technologie du poisson en Afrique, Casablanca, Maroc. Rapports de la FAO sur la pêche, (268) supl. pp1-5.

Waterman, J. J., 1976. The production of dried fish. FAO, Fish. Tech. Pap., 16: p52.

Williams, J.C., Field R. A., Miller, G.J. and Welke, R.A., 1983. Evaluation of TBA methods for determination of lipid oxidation in red meat from four species. *J. Food Sci.,* 48:1776-1778.

Wheeler, S. and Morrissey, M.T., 2003. Quantification and distribution of lipid, moisture and fatty acids in West Coast albacore tuna (*Thunnus alalunga*). J. *Aquatic Fd. Prod. Technol*., 12(2): 3-16.

Wood, C.D., Cole R.C., 1989. Small insulated fish containers. FAO Fisheries circular. N° 824, Rome, FAO, p80.

Wootton, M., Silalahi J., Wills R., 1989. Amine levels in some Asian seafood products. *J. Sc. Food Agric*., 49: 503-506.

Yankah, V.V. 1988. Studies on *momone*: a Ghanaian fermented fish product. B. Sciences thesis. Department of Nutrition and Food Science, University of Ghana–Legon.

Yean, Y. S., 1998.Technological Approaches to utilizing by catch in low- cost products for human consumption Fish utilization in Asia and the pacific. Proceedings of the A P F I C Symposium, Beijing, China, 24-26 Sept. 1998. (RAP Publication 1998/24) pp 13-28.

Yen, G.C., Chandra, T., 1988. Biogenic amines in alcoholic beverages produced in Taiwan, J. Sc. Food. Agric., 44 (3): 2-80.

Yongsawatdigul, J., Park, J.W., Virulhakul, P. and Viratchakul, S., 2000. Proteolytic degradation of tropical tilapia surimi. *J. Food Sci.* 65(1):129-133.

Young, J.C; Yeong, S.I.; Hee, Y.P.; Yeung, J.C., 2000. Quality characteristics of Southeast Asian salt-fermented fish sauces. Journal-of-the-Korean-Fisheries-Society; 33 (2): 98-102.

APPENDICES

Appendix 1

Survey questionnaire on *lanhouin* production and utilization in the Republic of Benin

1. Background information

1.1 Name

1.2 Age

1.3 Sex

1.4 City/area

1.5 Educational level

No formal education

Primary school

Secondary school

University

2. Where do you buy the fish?

3. Do you ice the fish after purchased from fishermen? Yes/no, why?

4. How do you transport the fish to the processing site?

5. Have you had any training in the handling, processing and storage of fish?

6. Do you produce or retail lanhouin?

7. Is there any particular reason why you produce lanhouin?

8. What quantity of lanhouin do you produce at a time?

9. What types of fish do you use for lanhouin production and why?

10. How do you process the lanhouin?

11. Do you have any idea as to the amount of salt used?

12 How long do you ferment the fish?

13. What container do you use to ferment the fish?

14. Do you package your fermented fish?

15. If yes, what packaging material do you use?

16. How do you store the lanhouin?

17. How long do you keep the lanhouin in store?

18. How do you prevent insects from attacking the product during processing and storage?

19. Where do you sell your product?

20. How do you transport the product to the market?
21. What characteristics are associated with the product?
22. Do you have any problem with lanhouin production?
23. Do you think there is the need for any improvement in lanhouin quality?

Appendix 2

Anova Summary Tables for Response Surface Methodology

Model fitting results for Total Viable Count

Multiple Regression Analysis

Dependent variable: TVC

Parameter	Estimate	Standard Error	T Statistic	P-Value
CONSTANT	6,65563	0,468953	14,1925	0,0000
X1	0,165911	0,077805	2,1324	0,0499
X1*X1	-0,00932644	0,00494922	-1,88443	0,0790
X2*X2	-0,00149717	0,00041406	-3,61582	0,0025
X3*X3	-0,0415606	0,00616489	-6,7415	0,0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	8,97446	4	2,24362	15,73	0,0000
Residual	2,13899	15	0,1426		
Total (Corr.)	11,1135	19			

R-squared = 80,7531 percent
R-squared (adjusted for d.f.) = 75,6206 percent
MSE = 0.1426

Further ANOVA for Variables in the Order Fitted

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
X1	0,180854	1	0,180854	1,27	0,2778
X1*X1	0,454496	1	0,454496	3,19	0,0944
X2*X2	1,85827	1	1,85827	13,03	0,0026
X3*X3	6,48084	1	6,48084	45,45	0,0000
Model	8,97446	4			

TVC = 6.65563 + 0.165911X1 – 0.00932644X1*X1 – 0.00149717*X2*X2 - 0.0415606*X3*X3

Appendix 2 (continued)

Model fitting results for sodium chloride (NaCl)

Multiple Regression Analysis

Dependent variable: NaCl

Parameter	Estimate	Standard Error	T Statistic	P-Value
CONSTANT	-22.2161	13.8628	-1.60257	0.1330
X1	0.50458	0.281139	1.79477	0.0960
X2	1.63911	1.00548	1.63018	0.1270
X1^2	-0.0213859	0.0178924	-1.19525	0.2533
X2^2	-0.0355826	0.0178998	-1.98788	0.0683
X3^2	-0.178205	0.120301	-1.48133	0.1623
X2*X3	0.117226	0.0482118	2.43149	0.0302

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	101.905	6	16.9841	9.23	0.0005
Residual	23.9277	13	1.84059		
Total (Corr.)	125.832	19			

R-squared = 80.9845 percent

R-squared (adjusted for d.f.) = 72.2081 percent

MSE = 1.8405

Further ANOVA for Variables in the Order Fitted

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
X1	9.14512	1	9.14512	4.97	0.0441
X2	28.9374	1	28.9374	15.72	0.0016
X1^2	1.58801	1	1.58801	0.86	0.3699
X2^2	6.53266	1	6.53266	3.55	0.0821
X3^2	44.8198	1	44.8198	24.35	0.0003
X2*X3	10.8818	1	10.8818	5.91	0.0302
Model	101.905	6			

NaCl = -22.2161 + 0.50458X1 + 1.63911X2 - 0.0213859X1^2 - 0.0355826X2^2 - 0.178205X3^2 + 0.117226X2X3

Appendix 2 (continued)

Model fitting results for histamine

Multiple Regression Analysis

Dependent variable: Histamine

Parameter	Estimate	Standard Error	T Statistic	P-Value
CONSTANT	88.5229	10.245	8.64056	0.0000
X2	-5.72654	0.746281	-7.67344	0.0000
X3	0.587899	0.186942	3.14482	0.0067
X1^2	0.0197967	0.00396762	4.98956	0.0002
X2^2	0.0954959	0.0135129	7.06704	0.0000

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	148.655	4	37.1637	35.00	0.0000
Residual	15.9256	15	1.06171		
Total (Corr.)	164.581	19			

R-squared = 90.3235 percent
R-squared (adjusted for d.f.) = 87.7431 percent
MSE = 1.0617

Further ANOVA for Variables in the Order Fitted

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
X2	60.4762	1	60.4762	56.96	0.0000
X3	10.5351	1	10.5351	9.92	0.0066
X1^2	24.6187	1	24.6187	23.19	0.0002
X2^2	53.025	1	53.025	49.94	0.0000
Total (Corr.)	148.655	4			

Histamine = 88.5229 - 5.72654X2 + 0.587899X3 + 0.0197967X1^2 + 0.0954959X2^2

Appendix 3

Anova Summary Tables for inoculated fermentation data

pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,146	7	,164	11,727	,000
Within Groups	,558	40	,014		
Total	1,704	47			

	starter culture	N	Subset for alpha = .05		
			1	2	3
Duncan(a,b)	SL	6	6.5583		
	SX	8		6.7738	
	SL+BL	6			6.9217
	SL+BS	4			6.9325
	SX+BL	6			6.9600
	SL+BS+BL	6			7.0000
	SX+BS	6			7.0350
	SX+BS+BL	6			7.0417
	Sig.		1,000	1,000	,136

a Uses Harmonic Mean Sample Size = 5,818.
b Alpha = ,05.

Total Volatile Nitrogen (TVN)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22566,051	7	3223,722	78,285	,000
Within Groups	1647,163	40	41,179		
Total	24213,214	47			

	starter culture	N	Subset for alpha = .05					
			1	2	3	4	5	6
Duncan(a,b)	SL	6	29.6550					
	SX	8		44.5813				
	SL+BL	6			59.6383			
	SL+BS	4				70.7925		
	SX+BL	6				76.1650		
	SL+BS+BL	6					84.8917	
	SX+BS	6					85.8600	
	SX+BS+BL	6						96.5650
	Sig.		1,000	1,000	1,000	,161	,798	1,000

a Uses Harmonic Mean Sample Size = 5,818.
b Alpha = ,05.

Total Viable Count (T VC)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	27,875(a)	8	3,484	324,637	,000
Intercept	875,521	1	875,521	81570,264	,000
FERMTIM	27,038	2	13,519	1259,556	,000
STARCULT	,621	2	,310	28,922	,000
FERMTIM * STARCULT	,216	4	,054	5,034	,007
Error	,193	18	,011		
Total	903,589	27			
Corrected Total	28,069	26			

	Microorganism (Starter cultures)	N	Subset 1	Subset 2	Subset 3
Student-Newman-Keuls(a,b)	BL (Bacillus Licheniformis)	9	5,5000		
	BS+ BL	9		5,7133	
	BS (Bacillus Subtilis)	9			5,8700
	Sig.		1,000	1,000	1,000
Duncan(a,b)	BL (Bacillus Licheniformis)	9	5,5000		
	BS+ BL	9		5,7133	
	BS (Bacillus Subtilis)	9			5,8700
	Sig.		1,000	1,000	1,000

a Uses Harmonic Mean Sample Size = 9,000.
b Alpha = ,05.

Appendix 4

Ballot sheet for sensory evaluation of market and laboratory *lanhouin*

Date:

Judge name:

Six samples pairs of *lanhouin* are presented to you. Note each sample code, compare each sample pair and indicate which one you prefer in terms of aroma, texture, taste and colour. Indicate by placing an X under the characteristic evaluated.

Left samples					Right samples			
Code	Aroma	Texture	Taste	Colour	Aroma	Texture	Taste	Colour
	…	….	….	….	…	..…	…	….
	....	….	…	….	...	….	…	….
	….	….	…	…	…	….	….	…
	…..	….	….	….	….	….	…	….
	…..	….	…	….	…	……	…..	…
	…	…	…	….	…	….	….	…

Comments on the overall acceptability of the products

……..……

……. ……

…….

Appendix 5

Results of multiple paired comparisons test for *lanhouin* evaluation

Aroma (a)

		Better				
	Samples code	MLF4	MLF8	LLF4	LLF8	
Less better	MLF4	-	6	19	16	**41**
	MLF8	18	-	16	17	**51**
	LLF4	5	8	-	5	**18**
	LLF8	8	7	19	-	**34**
		31	**21**	**54**	**38**	
	RST	**103**	**93**	**126**	**110**	

RST: Rank sum totals

Friedman's T = 24.08

Critical value of T at $\alpha = 0.05 = 7.28$

Least significant difference among samples for aroma

Samples	Aroma difference				
		MLF4	MLF8	LLF4	LLF8
	RST	103	93	126	110
MLF4	103	-	10	**23***	7
MLF8	93		-	**33***	17
LLF4	126			-	16
LLF8	110				-

* indicates that the two samples are significantly different at 5% level.

Critical value of Friedman's test for difference between RST, W=23, 573523

LLF4: laboratory lanhouin fermented for 4 days

LLF8: laboratory lanhouin fermented for 8 days

MLF4: market lanhouin fermented for 4 days

MLF8: market lanhouin fermented for 8 days

Appendix 5 (continued)

Taste (b)

		Better				
	Samples code	MLF4	MLF8	LLF4	LLF8	
Less better	MLF4	-	6	18	14	**38**
	MLF8	18	-	22	17	**57**
	LLF4	6	2	-	9	**17**
	LLF8	14	7	15	-	**36**
		38	**15**	**55**	**40**	
	RST	**114**	**87**	**127**	**116**	

Friedman's T = 145.58

Critical value of T at $\alpha = 0.05 = 7.28$

Least significant difference among samples for taste

Samples	Taste difference				
		MLF4	MLF8	LLF4	LLF8
	RST	114	87	127	116
MLF4	114	-	**27***	13	2
MLF8	87		-	**40***	29
LLF4	127			-	11
LLF8	116				-

* indicates that the two samples are significantly different at 5% level

Critical value of Friedman's test for difference between RST, W=23, 573523

LLF4: laboratory lanhouin fermented for 4 days

LLF8: laboratory lanhouin fermented for 8 days

MLF4: market lanhouin fermented for 4 days

MLF8: market lanhouin fermented for 8 days

Appendix 5 (continued)

Colour (c)

		Better				
	Samples code	MLF4	MLF8	LLF4	LLF8	
Less better	MLF4	-	8	19	9	**36**
	MLF8	16	-	15	17	**48**
	LLF4	5	9	-	3	**17**
	LLF8	15	7	21	-	**43**
		36	**24**	**55**	**29**	
	RST	**108**	**96**	**127**	**101**	

T = 23.08

Critical value of T at $\alpha = 0.05 = 7.28$

Least significant difference among samples for colour

Samples	Colour difference				
		MLF4	MLF8	LLF4	LLF8
	RST	108	96	127	101
MLF4	108	-	12	19	7
MLF8	96		-	**31***	5
LLF4	127			-	**26***
LLF8	101				-

* indicates that the two samples are significantly different at 5% level

Critical value of Friedman's test for difference between RST, W=23, 573523

LLF4: laboratory lanhouin fermented for 4 days

LLF8: laboratory lanhouin fermented for 8 days

MLF4: market lanhouin fermented for 4 days

MLF8: market lanhouin fermented for 8 days

Appendix 5 (continued)

Texture (d)

		Better				
	Samples code	MLF4	MLF8	LLF4	LLF8	
Less better	MLF4	-	11	16	14	**41**
	MLF8	13	-	12	14	**39**
	LLF4	8	12	-	12	**32**
	LLF8	14	10	12	-	**36**
		35	**33**	**40**	**40**	
	RST	**111**	**105**	**112**	**116**	

Friedman's T = 112.08

Critical value of T at $\alpha = 0.05 = 7.28$

Least significant difference among samples for texture

Samples	Texture difference				
		MLF4	MLF8	LLF4	LLF8
	RST	111	105	112	116
MLF4	111	-	6	1	5
MLF8	105		-	7	11
LLF4	112			-	4
LLF8	116				-

Critical value of Friedman's test for difference between RST, W=23, 573523

LLF4: laboratory lanhouin fermented for 4 days

LLF8: laboratory lanhouin fermented for 8 days

MLF4: market lanhouin fermented for 4 days

MLF8: market lanhouin fermented for 8 days

Appendix 6
PUBLICATIONS

Publication 1

Publication 2

Publication 3