BIOCONVERSION OF TUNA PROCESSING WASTE INTO FISH SAUCE

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DECLARATION

I, Soyiri Ireneous Ngmenlanaa, wholly undertook this thesis research work in the Department of Nutrition and Food Science, University of Ghana and at the microbiology and chemistry divisions of the Food Research Institute of the CSIR under the joint supervision of Dr. K. Tano-Debrah and Dr. W .A. Amoa-Awua respectively.

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DEDICATION

Dedicated to ABRAHAM, my ancestor and mentor - the man of great FAITH.

CITATION

“Without pure cultures the study of bacteriology becomes a hopeless endeavor”

ACKNOWLEDGEMENT

I am most grateful to God for seeing me through to this point in life. I also acknowledge and express my deep appreciation to many wonderful people who have made this project possible.

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ABSTRACT

Fish sauce is a fermented fish product. It is a liquid extract of fish tissues produced by fermenting fish and/or fish processing by-products, sometimes mixed with a carbohydrate source, under a salt-saturated condition. It is typical of the oriental countries where it is consumed as a condiment. Analysis of a typical fish sauce suggests varying degree of hydrolysis of the proteins and other macromolecules of the fish, which are typical of microbial and enzymatic actions, although the high salt concentration is very restrictive to the growth of many microorganisms. The purpose of the work was to investigate the feasibility of producing fish sauce using some tuna processing wastes and the physico-chemical and microbiological changes associated with the biodegradation of tuna tissues in the production of the sauce. Tuna processing wastes obtained from a cannery were characterized physically (by determining the proportions of bones and flesh), physico-chemically and microbiologically. Sub-samples of the tissues were then formulated into appropriate media, adding glucose and sodium chloride (at pre-determined concentrations) and fermented for the production of the sauce. The yield of sauce, the physico-chemical changes of the substrate and the microfloral characteristics of the fermentation were determined. The effect and the type of the carbohydrate used on the yield and quality of the sauce were also investigated. The tuna waste comprised of 80-94% soft tissues, 4.2-18.9% bone and 1.2-1.6% of fins. The proximate composition was as follows: protein, 21.24 - 23.41 *%,* crude fat 2.17 - 2.54 %, total ash 3.76 - 9.24 % and moisture 67.09 - 76.81 %. The microbial counts were 1.0×10^{7} - 5.4×10^{9} cfu/ml aerobic mesophiles, 5.1×10^{6} - 8.51×10^{8} facultative anaerobes and 1.0×10^{3} - 9.3×10^{3} yeasts. The pH and the moisture content of the extracts produced during the fermentation fell as the fermentation period increased, from 5.64 to 4.45 and 83 % to 73%, respectively. The salinity of the extract however, increased from 10.1 to

16 %. The total protein and free amino nitrogen content of the extract ranged from 11.2 tol8.0 % and 5.0-8.3 to 6.4-36.6mg/ml respectively. The titrable acidity increased from 0.9-1.0 to 1.2-2.5 %, whilst total lipids content ranged from 2.3 and 4.0 %. The yield of extract produced (sauce) over the fermentation period ranged from 128 - 295 ml per kg of fermenting mixture. Generally, the physico-chemical characteristics of the sauce produced using glucose as the carbohydrate source were comparable to literature values. Heavy metals were also not detected beyond their permissible levels. Histamine levels ranged from 13.95 - 34.41 ppm, far below the recommended maximum permissible level of 50 ppm. SDS-gel electrophoretograms of samples of the fish extract taken at various times during fermentation showed a consistent and gradual degradation of macro protein molecules. The test for presence of pathogens was negative in all samples. The predominant bacteria isolated during the 49-day period were: *Micrococcus, Planococcus, Corynebacterium, Peptococcus, Streptococcus, Staphylococcus, Bacillus Moraxella* and *Branhamella* spp. The predominant yeasts were *Pichia capsulata, Candida vaccinii, Rhodotorula becarum, Rhodotorula pustula,* and *Sporobolomyces roseus.* Some of these, particularly the *Bacillus* and *Staphylococcus* spp. demonstrated high proteolytic activity. It maybe concluded that fish sauce could be processed from tuna processing wastes through a bioconversion process.

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CHAPTER ONE

1.0 INTRODUCTION

Human activities depend solely on materials and products from the environment. These materials are usually channelled through various processes to make products; and inevitably waste is generated in various forms. This happens at various stages in the natural resource cycle from production of goods and services through to consumption and during disposal. Therefore the maintenance of the integrity of the environment strongly depends on the waste management plan used. Food processing is one of such human activities that contribute considerably to waste in general. Of the kinds of waste generated from the food industry, solid waste among others (i.e. Liquid, Gas, and Vapour/Steam), is of critical importance, as it tends to present a high polluting potential if not handled appropriately. Appropriate waste management practices include waste re-use, recycling, composting / manure etc. The choice of waste re-use in the food industry or agro-based industry presents the opportunity to turn out new products by channelling waste as a raw material through novel processes. Bioconversion is a low cost process in which biotechnology is applied to transform organic materials into desired products.

The principle of bioconversion involves the use of living organisms to modify substances that are not normally used for growth. The term “Bioconversion” is sometimes used interchangeably with “Biotransformation”, to describe the process that involves changes, which is usually specific. For instance in the production of gluconic acid from glucose, *Aspergillus niger* is used in the fermentation. In the process, the carbonyl functional group (-CHO) is oxidized by glucose oxidase, in the presence of water and oxygen, to a carboxylic functional group (-COOH) in gluconic acid. This is an example of a transformation carried out by nitrogen-limited non-growing cells. The application of bioconversion in the pharmaceutical industry for instance

involves processes that use a biocatalyst in a continuous culture or batch culture system. The processing alternative biocatalyst in this case could include dried cells, immobilized cells, cell extracts and purified enzymes (Schlegel, 1995). They could thus be prepared in more specific ways to carry out bioconversions for desired products. The concept of bioconversion is important as it has major advantages over chemical procedures. Usually in bioconversion processes, enzymes carry out very specific reactions under mild conditions, and larger water-soluble molecules can be transformed. Unicellular bacteria, actinomycetes, yeasts and moulds have been used in such processes. The concept of bioconversion has thus found application in the handling of waste.

"Waste" may be defined as any material which technology does not yet know how to use at a given time and place. The term may thus be meaningful only in a specific context. This therefore suggests that waste is an inevitable or necessary product of any given process. All over the world daily human activities, industrial and agricultural operations result in the generation of large quantities of wastes. A lot of the waste so generated has often resulted from inefficient processes used in production systems. In Ghana, traditional processes, which are still adopted in the medium to large-scale production of certain goods and services; and are quite inefficient usually, turn out a lot of wastes, which are discarded. These wastes are largely solid and liquid. Vapour and gaseous wastes to a lesser extent are also produced, but none of these categories of wastes have a clearly defined treatment plan in the country. Among the various types of wastes, solid waste is one that cannot be conveniently ignored or overlooked in any particular situation once it is generated, Ghana generates annually, about 2.9 million metric tons of solid waste, of which Accra alone contributes about 990 tons of solid waste per day (EPA of Ghana, 1997) The annual increase in solid waste in Accra alone (which forms about 15% of the entire population of Ghana) is about 7 5%. Meanwhile the organic component of waste in general is 52%, a lot of

which can be attributed to agro based/food products. This situation calls for a retrospective look at the situation of waste generation and its management in Ghanaian food industries.

Bioconversion processes have been used directly or indirectly in waste management. The use of solid waste in sanitary landfills and composting, involve the use of microorganisms in the degradation process. The treatment of various kinds of wastes generated by man entails bioconversion processes. Protozoa, sewage fungi, algae and bacteria are known to be involved in the degradation, active decomposition, recovery, and cleaning of sewage during its treatment. Bioconversion processes have also been applied in the biodegradation of environmental pollutants. This is exemplified in the bioremediation of alkyl benzyl solfonates, chlorinated hydrocarbons and oil pollutants, particularly during spillage at high seas.

In Ghana, just like most African countries, fermented foods form the bulk of the diet of most people. The production of most of these fermented foods involve bioconversion processes. *Koobi, kako* and *ewule* (Esuman, 1992), *bonmone* (Nerquaye-Tetteh *et al,* 1978), and *dcnvadawa/ ini* (Odunfa, 1981) are fermented foods in West Africa whose processing involves proteolytic bacteria like *Bacillus subtilis.* The processing of traditional African fermented cheese from milk curd - *Wagashi,* (FAO, 1997), Palm wine (Yannick, 1996), *Pi to* (Sefa-Dedeh *et al*., 1989), *Agbelima* (Amoa-Awuah and lacobsen, 1996) and *Moree* (Halm *et al.,* 1993) involve bioconversion processes. Microorganisms, enzymes and other biochemicals are involved in the biotransformation of most raw foods into these products. This not withstanding, in Ghana, certain aspects of bioconversion in food waste management are not practiced, even though their potential is high and they could be exploited to reduce the losses in food processing thus enhancing food security. The application of modern bioconversion / biotransformation techniques for biologically active substances and food active production has become a common practice.

Modified microorganisms and their metabolites have been used in the optimization of fermentation processes. For instance, an increase in beer production quality and effectiveness is attained by the use of modified microorganisms (Variants of *Saccharomyces cereviceae).* In other applications, this process is made use of to recapture some essential nutrients generally trapped in food wastes. In such instances the nature and composition of the waste material is important in determining the kind of process to use.

Food processing wastes are composed of largely organic compounds and few other inorganic compounds. These are usually in a junk and are disposed off; meanwhile the proximate composition of most food industry waste reveals that the quantity of nutrients lost to man because of the lack of technology to maximally exploit industrial raw materials is high. Hence priority should be given to food processing wastes not only because of the benefits that can be derived from it but also to check the high losses resulting from inefficient processing systems which usually lead to high economic losses. Also agro-based industrial wastes usually have a high biological oxygen demand (BOD), which implies a high polluting potential or effect and thus must be handled appropriately. Hence the idea of recapturing essential nutrients locked up in fish processing wastes in a form that will make it available for direct human consumption borders on food security. Normally fish is wholly consumed excluding the scales, fins and bones However in a typical tuna cannery, the canning process involves the knobbing (beheading) of big fish to produce the head, viscera and by-products, which are nutritionally of high value. The canning of tuna *(Thunnus* spp.) may result in a loss of about 60-70% of the raw fish. A way to optimize the use of the total fish catches therefore may be to reuse some of the waste generated from some of these conventional processes. In Ghana, appropriate technology for agro-based waste management systems that make use of microorganisms (in bioconversion processes) is limited. The concept of zero emission is not practised. The concept of zero emission in industry

describes the model in which all industrial inputs can be converted into final products, or where waste is converted into value-added inputs in another chain of production. In the end the manufacturing line becomes a series of production cycles and recycling systems. In this way industries may be organized into "clusters" within one single corporation, or in interdependent sets of industries, such that the whole discharges no waste into the air, soil or water. Hence the fish processing industry, which generates so much waste after processing, could be the main supplier of raw material for the production of fish sauce. The residue from the fish sauce production could then be passed on to the next industry (Animal feed mills) as a value added raw material. This will ensure that the fish processing waste is maximally exploited for direct human nutrition.

1.1 RATIONALE FOR THE STUDY

Fish contributes a significant amount of animal protein to the diets of people worldwide. It is estimated that between 15 and 20 percent of all animal proteins come from aquatic animals. Fish is highly nutritious and serves as a valuable supplement in diets lacking essential vitamins and minerals. About 75 percent of the world fish catch are used for human consumption. The remainder is converted into fish meal and oil used mainly for animal feed (including farmed fish). The World's Fish Trade has been growing in both volume and value over the last few decades, but this has mainly been appreciated in developed nations. World reports have often provided misleading information about African fish imports and exports. However, FAO reports particularly in 1987 indicated the misleading reportage of African fish imports exceeding exports This report went further to cite the net outflow of fish mainly shellfish and other high value species from the continent to other parts of the world. The Nominal Catches of fish by principal producers of tunas worldwide from 1992 to 1998, and then in Ghana spanning the

period 1989 to 1998, as well as the annual fish production by source in metric tonnes from 1991 - 2000 has been reported (MOFA, 2001 and FAO, 2000). In all cases it can be obseived that the annual catch keeps increasing. Ghana's Southern border, which is washed by currents of the Gulf of Guinea, is quite rich in fishery resources, particularly tuna fish and this is of high economic value. Marine and Inland fish has been exploited in various forms depending on the species and their handlers. This has included artisanal fish processors and heavy industrial processors (fish canneries). The artisanal fish-processing sector in Ghana and Africa as a whole has received tremendous attention in the past years and it has been particularly encouraged to develop or innovate means of fish handling. Heavy fish processing industries (mostly canneries) are few in the countiy and most of them are located along the coast particularly in the metropolitan areas. One of such industry that processes only tuna fish handles about 185 tons of tuna a day. So much processing wastes (estimated at 55-70%), containing valuable nutrients which could be exploited in advanced food processing, to make them available for direct human consumption is not used as such but for different purposes. Since malnutrition in developing countries has always been an important issue to contend, 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.

Considering again the fact that Ghana is among the leading suppliers of processed tuna worldwide, it is important to really consider the maximal utilization of tuna processing by-products, since large quantities of waste are inevitably produced daily. This study therefore involved the bioconversion of tuna processing wastes into fish sauce - a way to reduce the resulting pollution of the environment. Fish sauce is however an important fermented fish product in some parts of the world. It is in the likeness of soy sauce, and it is a brown liquid seasoning with a characteristic odour. It is basically produced from a mixture offish and salt by fermentation for long periods.

The characteristic components of the waste are not known. The microbial load of the thrash fish is quite high and could be exploited in bioconversion. The tuna processing waste is however not exploited in any way prior to its use in the feed mills. The involvement of microorganisms in bioconversion processes is well acknowledged but their succession and exact role in the processing of fish sauce from tuna is yet to be established. Moreover information on the nature of tuna *(Thunnus* spp) protein/ molecular breakdown during fermentation is limited. It is therefore necessary to exploit the potential of utilizing tuna processing wastes in processing fish sauce. In this regard some valuable nutrients could be recaptured for direct human consumption. It is therefore anticipated that the study would provide fundamental information on the characteristic composition of the tuna processing waste and also explore the possibility of hydrolyzing tuna tissues to produce a sauce. The study would also provide a fundamental microbial profile of organisms involved in the fermentation process.

1.2 OBJECTIVES

1.2.1 The main objectives are:

To develop a process for the production of fish sauce using tuna canning waste; and to investigate the physico-chemical and microbiological changes associated with the biodegradation of tuna tissue into fish sauce.

1.2.2 The specific objectives are:

 To characterize tuna processing wastes from a tuna processing industry and to establish its potential as a raw material for the production of fish sauce;

To produce fish sauce from the tuna waste and investigate the physico-chemical characteristics of the fish sauce produced including its safety with respect to the presence of heavy metals and histamine;

•I* To study the microbial succession during the bioconversion of tuna processing wastes and the ability of the microorganisms to hydrolyse the fish protein and carbohydrate.

To investigate the microbiological safety of fish sauce produced from tuna processing wastes with respect to food borne pathogens.

CHAPTER TWO

2.0 LITERATURE

2.1 WASTE GENERATION AND MANAGEMENT

World wide eating habits tend to place more emphasis on precooked, packaged convenience foods, and these have led to a very rapid rise in the per capita consumption of commercially prepared foods (Sell, 1992). Along with this increased consumption comes also an increase in the waste generated. For instance the American food processing industry annually produces some 800 million lb BOD, 392 million lb. soluble solids, and 800 million tons of solid waste residuals in canning and freezing plants alone (Anon, 1973). This constitutes less than 17% of the total domestic waste generated in the United States. Meanwhile additional wastes are produced by the meat and poultry slaughtering, fish processing and dairy industries. In developing countries on the other hand, up to 85% of the waste stream is made up of food, textiles, vegetable matter and other biodegradable materials (Cunningham and Saigo, 1995). The capital city of Ghana, Accra, alone generates approximately 1000 tons/day of waste and has an annual generation rate of $3.7x10^4$ tons/year (Fobil, 2002). Hence it was reported to have an average per capita per day of 0.376 Kg waste/person/day.

Food wastes have been described to include those portions of the raw materials for which there is little demand and many of these are peelings, for example, that are not being used for human consumption or converted to pet or livestock feed (Sell, 1992). Essentially there are three methods to eliminate all pollutants and these include the ‘'recovery and reuse within the same plant”, “recovery and sale of wastes to other manufacturers" and then “bringing the waste producer and user together in one industrial complex” (Nemerow, 1995). Regardless of the treatment chosen, minimization of wastes generated is very advantageous. These wastes that are

formed can preferably be converted to useful byproducts. Many of the sludges contain large quantities of protein, the B vitamins (that is vitamin B12, thiamin, riboflavin, and niacin), pectin, and other nutrients that can be recovered for use or used directly as food supplement (Sell, 1992).

2.2 BIOCONVERSION AND FISH PRESERVATION

2.2.1 Fish preservation techniques and post-harvest losses

Fish are the most numerous of vertebrates with more unknown species than known species (Huss, 1988). The importance of fish in the diet of man coupled with the trends in fish production and utilization over generations has had a significant impact on its evolution. In the food evolution of man, he has gone through several stages of acquiring food from hunting and gathering to more organized cultivation and production systems. This has resulted in diverse fish handling and processing practices, which include curing, salting, smoking and drying which have virtually been unaltered. However modern developments have centred on understanding and controlling these processes to achieve the standardized product demanded by today's market (Horner, 1997). Such processes include Chilling, freezing and canning.

Artisanal fisheries rarely discard fish however they do lose a substantial amount of the value of their catch before it can be eaten. Particularly in tropical countries, high temperatures mean that fish can spoil while still in the boat, at landing, during storage or processing, on the way to market and while waiting to be sold. In Africa, some estimates put post-harvest losses at 20 to 25 percent and sometimes as much as 50 percent (FAO, 2000).

One method of fish preservation, which from time immemorial has been developed for food generally to have longer storage, is fermentation (Mackie *et al.,* 1971). Accordingly, fermentation is taken to mean the transformation of organic substances into simpler compounds by the action of enzymes or microorganisms. This 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 and according to Cutting (1955), factors that influenced the utilization of salted fish products cut across political, equipment, religious observations and the need to satisfy protein needs in some regions of the world. Thus new product development in the salted fish industry has been greatly transformed. The variations evolving in the method of salting of fish from one region of the world to another, has resulted in varied products. Modifications in the method of salting, which was the addition of mixtures of salt and sugar to whole fatty herring to obtain a product called "tidbits", after maturation in barrels has been reported (Aim, 1965).

A number of factors have favoured the continued use of fermentative processes to preserve or produce acceptable products from fish. This, according to Mackie, *et al.,* (1971), include the inexpensive method of preservation involving the use of whole fish, a slight demand on technology not requiring chilling, storage or complex transport and distribution facilities. It also includes the benefit of a relatively high ambient temperature given that deteriorative changes can be kept in check by the use of salt.

In regions where the advanced exploitation of fermented fish has led to the development of products like fish sauces of the "nouc-mam" type (Cambodia and Vetanam), "Nampla" (Thailand), "Shottsuru" (Japan), "Bekasang" (North Sulawesi, and the Moluccas) and fish pastes such as "Bagoong" (Philipines) etc, the difficulty in preventing enzymatic and bacterial changes during the relatively slow natural processing method used, results in the confirmation of

characteristic flavours on the products by the fermentative changes (Irianto and Irianto, 1998, Mackie, ***etal,*** 1971; Lee 1990). All of these methods are subject to local variations and customs and numerous additives such as carbohydrates in the form of starches and fruits/fruit juices of various kinds are added. Some of these may have the effect of lowering the pH or introduce proteolytic enzymes as in the case of the addition of pineapple.

2.2.2 Bioconversion in fish processing and microbiology offermented fish/fishery products

The process of fermentation has been biochemically defined as the metabolic process in which carbohydrates and related compounds are partially oxidized with the release of energy in the absence of any external electron acceptors (Atlas, 1997). Eventually the electron acceptors here are the organic compounds produced directly from the breakdown of the carbohydrates. Fermented foods have always played an important role in providing food security and making better human nutrition. Thus livelihoods and social well being of a lot of people worldwide particularly the vulnerable and the marginalized have been enhanced by their dependence on fermented foods. Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. These foods have aromas and flavour characteristics that result directly or indirectly from the fermenting microorganisms (Jay, 2000). Table 2.1 shows the class of microorganisms that can be involved in fresh and spoiled fish/marine products. Some of these have also been shown to play important roles in fermented fish/ fishery products.

The ecology of microorganisms in food fermentation in general is an important process and a necessary tool that can aid in understanding the process. The growth and activity of microorganisms involved in fermentation are dependent on both extrinsic and intrinsic factors (Chiou, 1999). For instance the growth of yeast, coupled with the subsequent production of

alcohol in food restricts the growth and /or activities of contaminating microorganisms. In the case of bacterial growth on acidic foods, microorganisms like lactic acid bacteria tend to grow with the availability of simple sugars. Food products that contain polysaccharides but not significant levels of simple sugars are normally stable to the activities of yeasts and lactic acid bacteria due to the lack of amylase in most of these organisms (Jay, 2000). Since lactic acid bacteria are unable to act on unhydrolysed starch, it is sometimes necessary therefore to supply an amylolytic agent, (Stanton and Yeoh, 1977) derived from enzymes in a different material e.g. malt to accomplish their task in the normal fermentation process. This is very necessary for the successful preservation of fish by biological fermentation, as lactic acid bacteria is generally known to ferment the sugars present to organic acids resulting in a low pH medium in which the growth of putrefactive organisms and pathogens are inhibited. In this regard the amount of added carbohydrate and salt concentration primarily control the extent of acid fermentation and the keeping quality (Lee, 1990).

Recent findings have tried to support the possible role of microbial degradation in the formation of fermented fishery products and thus clear the controversy as to the importance of enzymes and microbes particularly in salt fermented products. Even though there has been no empirical evidence as to the clear-cut roles of enzymes vis-a-vis microorganisms in the fermentation of fish; some microbes have been identified to be of major importance. *Leuconostoc mesenteroides* and *Lactobacillusplantamm,* according to Souane (1987), play a major role in such processes as acid forming bacteria for the preservation of fish as well as the generation of acceptable flavour during fermentation. Before then, von-Hofsten and Wirahadikusumah (1972), had observed that the successful preservation of fish by biological fermentation methods is dependent on the production of lactic acid. Generally in biological silage, lactic acid bacteria ferment the sugars

present to organic acids, which usually result in the lowering of the pH of the fermenting medium. Sufficiently reduced pH inhibits the growth of putrefactive organisms and pathogens.

Table 2.1 Genera of bacteria, yeast, and molds mosts often found on fresh and spoiled fish and other seafoods

Bacteria	**Gram**	**Prevalence**	**Yeasts**	**Prevalence**	**Molds**	**Prevalence**
Acinetobacter		X	*Candida*	XX	*Aspergillus*	X
Aeromonas	-	XX	*Ciyptococcus*	XX	*Aureobasidium (Pullularia)*	XX
Alcaligenes	-	X	*Debaryomyces*	X	*Penicillium*	X
Bacillus	+	X	*Hansenida*	X	*Scopulariopis*	X
Corynebaterium	+	X	*Pichia*	X		
Enterobacter		X	*Rhodotorula*	XX		
Enterococcus	+	X	*Sporobolomyces*	X		
Escherichia		X	Trichosporon	X		
Flavobacterium		X				
Lactobacillus	+	X				
Listeria.	+	X				
Microbacterium	+	X				
Moraxella	-	X				
Photobacterium	-	X				
Pseudomonas	-	XX				
Psychrobacter		X				
Shewanella		XX				
Vibrio		XX				
Weisse/la	+	X				

Note: X = Known lo occur: XX = most frequently reported.
Source:Jay, 2000.

Ijong (1996) reviewed literature concerning the utilization of lactic acid bacteria in Bakasang (a fish sauce) fermentation and it was reported that the process generally favours the growth of halophiles and lactic acid bacteria. Accordingly, these organisms contribute to the product quality and characteristics such as flavour. Several reports have also shown the use of LAB as a starter

culture for fermented fish/meat products (Dyett, 1981; Lopez, 1989; Olympia *etal,* 1989, Morzel *etal,* 1997)

2.2.3 Fish fermentation

The historical background of fish fermentation in Asia and other regions of the world has been reviewed (Lee, 1990; Mackie *et al.,* 1971; Essuman, 1992; and Irianto and Irianto, 1998). This has been classified according to the particular kind of product and region of production with respect to the technology involved (Lee, 1990; and Esssuman, 1992) in Tables 2.2-2.4 and Fig.2.1. In the low salt fermented products shown in Table 2.3, carbohydrate foods are added as a source of carbon for the process. The amount of added carbohydrate and salt concentration primarily control the extent of acid fermentation and keeping quality. Many Asian countries and some African countries produce salt cured and dried fish products as shown in Tables 2.2 and 2.4, but the role of fermentation in these products is not fully understood as in the case of a lot of fermented foods worldwide. Nevertheless, fermented foods do have an important role in stimulating appetite by providing unique aromas and flavours (Irianto and Irianto, 1998). Accordingly, the raw materials used in the processing of fermented foods in Asia include whole fish, comminuted fish and viscera from marine and fresh water fishes.

Contrary to the fact that fermented fish have been considered a product of the Orient, African fermented fishery products have also existed (Table 2.4). In Africa, salting and drying of fish for preservation is accompanied by fermentation but the period is short (a few days) and the product is not transformed into a paste or sauce (Essuman, 1992). Usually the products of fermented fish have a characteristic strong odour. This smell of fermented fish has been attributed to the effect of microorganisms usually involved in the bioconversion process. In Africa and the world at large, the availability of salt and the food habits of the people have contributed significantly in

moulding the different processing techniques employed in fish fermentation. Even though these methods vary greatly from one region to another, they can still be grouped in main techniques that, according to Essuman (1992), include fermentation with salting and drying, fermentation and drying without salting, and fermentation with salting but no drying (Figure 2.1).

Table 2.2 Classification of high-salt fermented fish products in different countries

Country	Fish sauce	Cured fish	Fish pas
Burma	Ngan-pya-ye	-	Ngapi
Cambodia	Nuoc-man	-	Prahoc
China	Yu-lu		-
Indonesia	Ketjap-ikan	Pedah	Trassi
Japan	Shottsuru	Shiokara	-
Korea	Jeot-kuk	Jeotka	
Malaysia	Budu		Belacan
Philippines	Patis	Bagoiong	Bagoong
Sri Lanka	Blood pickle	Jaadi	-
Thailand	Nampla	-	Kapi
Vietnam	Nuoc-man	-	Manca

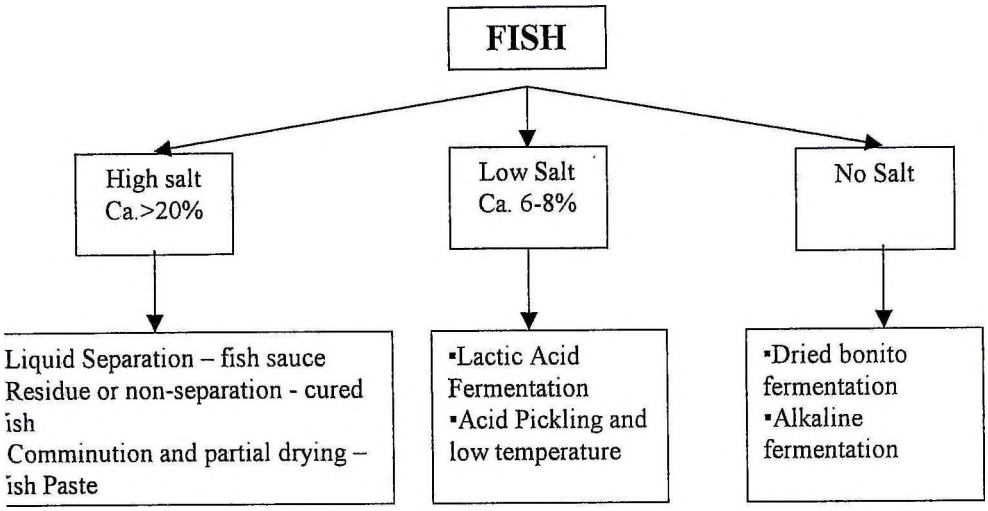
Source: Lee, 1990

Table 2.3 Classification of low-salt fermented fish products in different counties

Country	Lactic fermented	Acid pickling
Norway	Rakeorret	-
England	-	Tidbits
Germany	-	Schnell-mattjies
Japan	Narezushi	
Korea	Sikhae	-
Malaysia	Bekasam	-
Philippines	Burong-isda, Balao-balao	-
Thailand	Pla-som, Pla-ra, Pla-chom	-
Vietnam	Mum-tom	

Source: Lee, 1990

Fig. 2.1 **Classification of fermented fish products**



Source: Lee, 1990

Table 2.4 Traditional methods of fish fermentation in Africa local name of product and fermentation process

Country	Local/Common Name of Product	Fermentation Period	Drying Period	Packaging
Burundi	Ndagala	2-5 days (normally during drying, no salting).	2-5 days on ground or rack	Sacks, polythene bags
Chad	Salaga	Overnight (3-6 hours), Prior salting	3-7 days	Baskets, sacks
La Cote d'Ivoire	Gyagawere, adjonfa	6 hours to 3 days with salting	3-5 days on grass, nets, mats or raised platforms	Baskets, sacks
The Gambia	Guedji	Overnight to 2 days with salting	3-5 days on raised platforms	Sacks
Ghana	Momone, koobi, kako, ewule	Overnight to 3 days with salting	3-5 days on straw, nets, stones	Sacks, baskets
Mali	Djegue, jalan	Overnight, no salting	3-7 days on grass, mats or ground	Sacks, mates and ropes
Senegal	Guedj, tambadiang, yeet	Overnight to 2 days with salting	3-7 days	Sacks, baskets
The Sudan	Fessiekh, kejeick, terkeen, mindeshi	10-20 days with salting	No drying (fessiekh) 3-7 days (kejeick)	Cartons, cans, polythene bags
Uganda	Dagaa	3-6 hours without salting	2-5 days	Sacks, baskets

Source: Essuman, 1992

Among the products named in Table 2,4, it is only Fessiekh, the Sudanese fermented fishery product that has characteristics comparable to fish sauce. The product is processed by fermenting small fatty fish in whole, from the Nile for several days (Mackie *et al.,* 1971). The processing methods of Fessiekh could create avenues for microbial infection and risks of botulism, hence very rigorous microbiological tests are needed to be carried out on the finished products (Essuman, 1992).

The introduction of Nuoc-mam an Asian fermented fish sauce into the food delivery system in Cote d'Ivoire was justified by its nutritional value and seen as a possible solution to malnutrition in Africa (Faubeau, 1977). Reference is made to the establishment of a factory for the production of nuoc-mam, and highlights on how it fits into the food regime in Cote d'Ivoire explained (Faubeau, 1977).

2 3 **FISH / FISHERIES IN FOOD SECURITY**

2.3.1. Limitations in the exploitation offish as food in the processing industry

The constraint in the maximal exploitation and utilization of fish according to FAO (1987) covers fresh fish marketing, smoking, drying, freezing, canning and fishmeal processing. Even though each particular process has its attendant general and peculiar constraints, certain challenges seem to cut across all areas particularly in West Africa. Apart from the weak purchasing power of the population to obtain fresh fish, which is most desired, there is the lack of research, new product development, demonstration, training and quality control in the fish processing industry. There is therefore the need for a total integration of the fishery industry at all levels to maximize the capacity of related institutions for the utilization of fish. Secondly, the lack of communications, equipment and supplies in the fisheries sector and their effect on the utilization offish cannot be

overlooked. Also the lack of arrangements and activities to promote the utilization and consumption of unconventional and under-utilized species demands pertinent attention. The point in highlighting these limitations is to establish the fact that the recapture of nutrients from fish processing wastes can be given a priority to fishery development to enhance fish consumption

For instance the fishmeal industry in Ghana, (a lot of which are animal feed mills), obtain their main raw material from the wastes of canning and filleting operations. In between these two giants that is the canning and fishmeal industries, can be trapped important nutrients from the fish trash for human consumption. This is attainable through the bioconversion of fish wastes to generate a "fish sauce" and a by-product, which could even, be a better raw material for the animal feed mills. This is because fermentation (bioconversion) in general is known to be an appropriate technique for enhancing food quality by increasing the bioavailability of nutrients (Battcock and Azam-Ali, 1998).

2.3.2 ***Fish sauce production: advances and limitations***

Fish sauce production (fermentation) is common in Southeast Asian countries, since it offers a simple and low cost preservation method (Kumalaningsih, 1989). In Indonesia for instance, fish is a very important source of dietary protein - relatively cheaper than meat, and Bakasan is a traditionally fermented fish sauce produced in the region from small whole sardines *(Sardine sp.* or *Stelophours sp.)* and or the gut of big fish *(Katsuwonus pelamis*) obtained as a by-products from smoking processes (Ijong, 1996). In most of these Southeast Asian countries where fish sauces are common food condiments, even though the traditional method of their production are often presented with supporting historical background, the principle of operation is basically the same Fish sauce production offers a simple and low cost preservation method for fish and the product is mainly home based. Fermentation of fish has thus been extensively used in Southeast Asia not only in the production of fish sauce but other flavoured products.

Yean (1998), reports of the production of fish sauce from so many sources under different conditions by various researchers. Some of these include production of fish sauce from silver belly, salted at a ratio of 1:4 at 50°C; the use of *Amblygaster sirm* for fish sauce as a means of preserving seasonal gluts and also caranx, sardine and horse mackerel have all been found to be suitable for the preparation of nam-pla (a Thai fish sauce).

In the Orients, traditionally, the production of fish sauce can take up to twelve months; however, much research has been carried out to check this limitation of time. This involves hastening the fermentation process with increased temperature in the manufacture of fish sauce (patis) and thus reducing the time from 12 months to 2 months (Mabesa *et al,* 1990). In the study, a ten percent (10%) increase in protein and total yield was achieved and the quality of the product was comparable to fish sauce produced traditionally. Apart from the long period of time, which has been a challenge to the production of fish sauce, the salty taste and fishy odours have limited their consumption as a mam course. There are possibilities for the development of fish sauce with lactic acid bacteria fermentation of fish-carbohydrate mixtures in the presence of little amounts of salt and the process has been reported (Ijong, 1996, Hesseltine, 1991, Mabesa *et al.* 1990, Kumalaningsh, 1989). This results in a shorter time than that of the fish/salt products, which are primarily dependent on autolytic processes. Hence not withstanding the role of salting in inhibiting the growth of pathogenic organisms and also improving the end product texture, rapid acid fermentation by lactic acid bacteria has been shown to be essential for the effective inhibition of spoilage and pathogenic bacteria in fermented foods (Paludan-Muller, 1998). A low salt fermentation method for conserving trash fish waste under SE Asian conditions has also been reported by Stanton and Yeoh (1977). In their study, salt-generated organic acid fermentation systems using local carbohydrates assisted by malt substitutes were investigated. In this study the

amylolytic properties of the enzymes in malt were exploited in transforming unhydrolysed starch and making it available for lactic acid bacteria.

In the technological approaches to utilizing by catch in low cost products of human consumption, Yean (1998) reviewed and evaluated the significance of utilizing by-catch. It was evident that using by-catch species in a variety of conventional and novel products could avert considerable wastage. The quest for a market-driven approach to product development and the requirement to stimulate the search for novel raw materials like tuna processing wastes in obtaining a product (Fish sauce) to fill the emerging gap between supply and demand for fish is candid.

2 4 FISH SPOILAGE AND NEED FOR PRESERVATION

2.4.1. Spoilage of fish — biochemical and microbiological changes

Normally fish undergoes many processes of physical and chemical changes caused by biochemical and microbiological factors. This usually results in the ultimate decay of the fish unless the process is intervened. The post-mortem changes, which take place in fish tissue, occur in phases beginning with slime secretion on the surface followed by rigor mortis. Then autolysis as enzymatic decomposition of tissues comes to play during which period microbiological spoilage would have also set in (Bykowski and Dutkiewicz, 1996, Huss, 1988, Siebert and Schimitt, 1963).

According to Siebert and Schmitt (1963), autolysis is very complex event requiring complex measuring systems. There is therefore good reason to assume that the initial steps in deterioration of fish consist of hydrolytic reactions which are catalysed by enzymes from fish tissues themselves; thereby, the penetration of microorganisms is facilitated and nutrients are formed which would promote bacterial growth (Bramstedt 1961). 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. The decomposition involves proteins, lipids and carbohydrates and one compound's decomposition can influence others at various rates. During autolysis, great changes occur in the structure of muscle tissue, which becomes softer and very often falls into layers along the myosepts (Bykowski and Dutkiewicz, 1996). Thus the proteolysis process leads to decrease in the capacity of tissues to retain tissue juice, resulting in toughness of texture of the final product. The degradation of proteins creates ideal conditions for the growth of spoilage bacteria (Aagaard, 1972).

Bacteria are able to decompose proteins, but products of proteolysis such as amino-acids and other low-molecular nitrogenous compounds provide better nourishment. Microorganisms cause the decomposition of not only proteins but also other compounds containing nitrogen, lipids to peroxides, aldehydes, ketones, and lower aliphatic acids (Bykowski and Dutkiewicz, 1996). The penetration of bacteria into fish tissue and microbiological decomposition begins with autolysis and these processes are practically parallel. Their rate and intensity are temperature dependent (Liston, 1963).

2.4.2 The need for fish preservation

In Africa as a whole, artisanal fish processing remains the predominant and most important method of fish preservation (Essuman, 1992). Marine and Freshwater fishing is very important in Ghana supplying 66 % of all protein for human nutrition (I.I.E.D, 1992). Smoking, sun drying, salting, fermentation, grilling and frying are principally used in the processing; they may either be used alone or combined in order to achieve the desired product (Horner, 1997). The post mortem changes that occur and which affect quality, calls for processing to avert further losses,

which is usually sparked off from the point of catch, (Bykowski and Dutkiewicz, 1996). According to Turner, 1998, the spoilage of fish is brought about by two causes First of all, Autolysis, which involves the deterioration of the bodily tissues after death by enzymes, which are part of the biological makeup of the organism and then secondly by bacteria already present in the fish, which grow rapidly under favorable conditions and produce enzymes, which break down the fish tissues. Usually the quantity of bacteria in the body of a fish is small but they are often introduced through handling, cleaning, washing, etc. Hence there is the need to preserve the fish. The term "preserved fish" according to Oetterer, (2001), refers to fish prepared by enzymatic curing, or maturation, in which salt is added and this acts on the muscles, viscera, microorganisms and enzymes. The developing microorganisms which produces lactic acid, results in the lowering of pH and making the product resistant to the development of putrefying bacteria (Oetterer, 2001).

2.5 SPONTANEOUS FERMENTATION AND MICROFLORA OF FISH SAUCE PRODUCTION

2.5.1 Spontaneous fermentation

Fish sauce processing in the Oriental countries where it originated, traditionally involves a spontaneous or mixed culture fermentation process. Hence the source of raw material and composition of the fermenting medium/mixture may influence the species of microorganisms involved. Mixed culture fermentations offer a number of advantages over pure culture fermentations, which, according to Hesseltine (1991) include a higher product yield and growth rate of microorganisms. Also mixed cultures in spontaneous fermentation protect microorganisms that are in a stable association and hence there is maximal utilization of the substrate used in the fermentation process. Other advantages include microorganisms

complementing one another as they exclude from the system unwanted microbes and the utilization of cheap and impure substrates,

The disadvantages of mixed culture fermentation as indicated by Hesseltine (1991) include the formation of more variable products than in pure culture fermentations, problem of detecting and controlling contamination, controlling optimum balance of the microorganisms involved and the difficulty in the scientific study of mixed cultures. Furthermore it is difficult defining the product and microorganisms for patent and regulatory procedures.

2.5.2 Microorganisms commonly involved in traditional food fermentation

Microorganisms by virtue of their metabolic activities, contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life and safety (Holzapfel, 1997). Several authors have reported the presence of a wide range of microorganism during the fermentation of fish sauce (Dyett, 1981; Lopez, 1989, Olympia *et al.,* 1989, Ijong, 1996; Morzel *et al,* 1997). Among the microorganisms much discussed include *Bacillus* spp. lactic acid bacteria particularly Lactobacilli and yeasts. According to Campbell-Platt (1987), the bacteria involved in fish sauce fermentation are salt-tolerant (*Micrococcus*, *Staphylococcus* and *Bacillus*) and lactic acid bacteria (*Pediococcus* and *Lactobacillus).*

2.5.2.1 Yeasts and Moulds

Many Asian fermented foods like Miso (prepared from rice and soy beans with *Aspergillus oiyzae* and yeasts), Soy sauce (prepared from soy beans and wheat by a mixed-culture fermentation with *Aspergillus oiyzae,* yeasts and lactobacilli) and Tempe (prepared from soy beans with *Rhizopus oligosporus)* have become internationally popular (Ebine, 1989, Fukushima, 1989, Yoshizawa and Ishikawa, 1989). Plant material containing fermentable sugars provide

suitable substrates for yeasts species of *Saccharomyces, Candida, Torula, Hansenvla,* etc. (Holzapfel, 1997). All these species are associated with spontaneous or mixed culture fermentation systems as may be exemplified by a combined lactic and alcoholic fermentation of fish sauce. Yeasts and moulds of mixed floral composition (Candida, *Saccharomyces, Trichosporon, Kluveromyces* and *Debaryomyces* spp.) and associated with the fermentation of maize dough during the processing of a West African traditional food "kenkey" have been investigated by Jespersen *et al*, 1994. The use of amylolytic moulds and yeasts cultured in rice flour and dried, have also been proven to be beneficial in low salt fermentation and conservation of fish waste (Stanton and Yeoh, 1977).

2.5.2.2 *Bacillus* spp.

These are rod-shaped microorganisms, gram-positive, sporing, catalase producing and capable of sporulating aerobically (Sneath, 1986). This group of microorganisms are known to be technologically beneficial as they produce a wide range of commercially significant enzymes, as well as a number of cyclic linear polypeptides antibiotics (Fogarty *et al.,* 1974). These enzymes include amylases, proteases, b-glucanases and isomerases. Others are cellulases, hemicellulases, and pectinases including polygalacturonase lyase, penicillinases including P-lactamase and penicillin amidase, a-amylase, P-amylase, amyloglucosidase, nucleases cell wall lytic enzymes and acid neutral and alkaline proteases. A review of literature (Odunfa, 1981; Antai and Ibrahim, 1986; Steinkraus, 1991) reveals that *Bacillus* spp. mainly *Bacillus subtilis* are the organisms responsible for the fermentation of most traditional foods in which there is an extensive hydrolysis of protein to amino acids and peptides The resultant products obtained are mostly used as condiments to flavour cooked food and may also serve as a subsidiary source of protein. *Bacillus* spp. are identified classically using morphological and biochemical characteristics.

2.5.2.2.1 Lactic acid bacteria

Lactic acid bacteria are dominated by the genus *Lactobacillus.* Lactobacilli are regular gram-positive rods, catalase negative, oxidase negative fermentative and which grows both aerobically and anaerobically. *Lactobacillus* spp. are reported to be involved in the fermentation of most acidic foods (Souan *e et al.,* 1989; Halm *etal.,* 1993; Amoa-Awua *et al.,* 1995; Ijong, 1996; Jay, 2000). The species of the Lactobacilli can be differentiated by the fermentation of 17 different cardohydrates, using a modified MRS-broth with the added carbohydrate in mention.

Lactic acid bacteria and *Bacillus* spp., which are mostly amylolytic, may play an important role in fermentation systems with low salt content and a blend of ingredients. According to Souane *et*

al. (1989), such a condition could lead to the development of varieties of halotolerant microorganisms including some *Bacillus* spp. and lactic acid bacteria, which could compete freely, leading to the selection and dominance of bacteria. Lactic acid bacteria fermentation is known to improve flavour and quality of fermented products.

2.6 EXPLOITATION OF THE BIOCONVERSION PROCESS

2.6.1 The utilization of carbon as energy source for bacteria

The nature in which microorganisms use organic compounds as both carbon and energy sources fall under the classification chemotrophs, phototrophs, autotrophs, heterotrophs etc. The process in each energy source is mainly an oxidation of organic or inorganic compounds or high reaction.

2.6.1.1 Carbohydrate metabolism by microbes

Carbohydrates are usually polyhydroxy aldehydes and ketones or substances that hydrolyze to yield polyhydroxy aldehydes and ketones (Solomons, 1992). Microorganisms and many bacteria in particular are unable to metabolize carbohydrates (sugars) directly. Bacteria that however metabolize sugars solely by respiratory pathways produce carbon (IV) oxide (Volk *et al.,* 1991). The catabolism of sugars, which involves a series of steps or blocks of reactions usually has the first step resulting in the conversion of carbohydrate into a form that is a substrate for one of the central catabolic pathways.

According to Volk *et al,* (1991), the ability of a bacterial species to use a particular sugar is independent on the ability to synthesize the enzymes that convert that sugar into glucose or another central metabolite. Sugars from many carbohydrate sources could be made available in this form via hydrolysis (i.e. chemical and / or enzyme mediated).

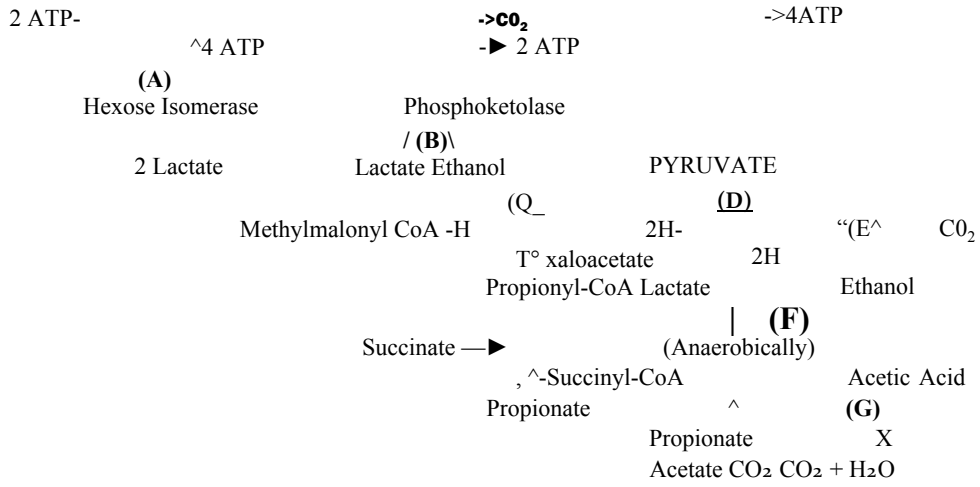
In cereal grains the process of sprouting and mashing of malt provides a hydrolytic process in which malt enzymes at about 65 °C releases or frees a lot of reducing sugars from the mash grain. Malting is essentially the same process as occurs when seeds fall to the ground or are planted, moistened by water and germinate. During germination, rootlets (sprouts) and a nascent stem (acrospire) emerge simultaneously as enzymes are produced or activated and the cellular structure and composition are modified. The resultant product obtained can be used as a substrate for fermented beverages and as food adjuncts. It is also known that malt enzymes covert added carbohydrate into fermentable sugars (Sfat and Doncheck, 1981, Sefa-Dedeh *et al.,* 1989).

The metabolism of glucose and subsequent fermentation of pyruvic acid provides the floodgate for the possibility of a series of fermentation reactions including Ethanolic Fermentation, Mixed acid Fermentation, Butylene-glycol Fermentation, Propionic-acid Fermentation, Butyric Acid, Butanol Acetone Fermentation etc, the end-products of each of these kinds of fermentation is illustrated (Fig 2.2).

2.6.1.2 Conversion of carbohydrates to lactic acid

Carbohydrates are hydrolysed by alpha and beta amylases to Maltose, which is further, acted upon by Maltase to Glucose. The generalized pathway for the production of lactic acid mainly involves Homofermentative lactics and Heterofermentative lactics. As shown in the pathway illustrated (Fig 2.2), further down, lactate could still be generated from glucose by the incorporation of 2H to pyruvate, under anaerobic conditions however this is broken down to propionate Acetate and Carbon IV oxide. The lactic acid produced is effective in inhibiting the growth of other bacteria that may decompose or spoil food (Battcock and Azam-Ali, 1998),

Fig 2.2 **Generalized pathways for the production of some fermentation products from glucose by various organisms.**



A.Homofermentative lactics, B.Heterofermentative lactics; C and D: *Propionibacterium;* E: *Saccharomyces* spp; F: *Acetobacter* spp, *GAcetobacter* "overoxidizers"

Source: Jay, 2000

Lactic Acid Bacteria (LAB) carry out their reactions - the conversion of carbohydrates to lactic acid plus carbon dioxide and other organic acids - without the need for oxygen (Battcock and Azam-Ali, 1998). The group of bacteria that are involved in the production of lactic acid have been described as non-motile gram positive rods and are known to require a complex of carbohydrate substrate for their energy requirement. The lactic acid bacteria can adapt to a range of conditions and thus have been known to be responsible for the large success in acid food fermentations. This is possible because of their diversity in the group of microorganisms as well as their diversion in metabolic capacity.

The whole basis of lactic acid fermentation according to Battcock and Azam Ali, (1998), centres on the ability of lactic acid bacteria to produce acid, which then inhibits the growth of other non-desirable organisms Species of the genera *Streptococcus* and *Leuconostoc* produce the least acid. Next are the heterofermentative species of *Lactobacillus,* which produce intermediate amounts of acid, followed by the *Pediococcus* and lastly the homofermenters of the *Lactobacillus species,* which produce the most acid (Battcock and Azam-Ali, 1998). Homofermenters, convert sugars primarily to lactic acid while heterofermenters produce about 50 % lactic acid and 25 % acetic acid plus ethyl alcohol then 25 % carbon dioxide as illustrated in the Figure 2.2.

Table 2.5 shows Homo-and Heterofermentative lactic acid bacteria and according to Jay (2000) the heterolactics are more important than the homolactics in producing flavour and aroma components such as acetylaldehyde and diacetyl from citrate.

The Homofermenters produce lactic acid through the glycolytic or Embden-Meyerhof pathway whilst the Heterofermenters use the 6 - phosphogluconate / phosphoketolase pathway. In the case of heterofermenters, appreciable amounts of ethanol, acetate and carbon IV oxide are produced.

According to Axelsson (1998), all lactic acid bacteria except *Leuconostoc,* group III *Lactobacilli, Oenococci* and *Weissellas* use the glycolytic pathway, requiring normal conditions and an excess of sugar with limited oxygen.

In the application of biotechnology to fermented foods, Beuchat (1995) has grouped the major lactic acid bacteria in fermented plant products. These fall into three groups le. Homofermenter, Facultative homofermenter, and obligate heterofermenter as shown in Table 2.6.

Table 2.5 Homo - and Heterofermentative Lactic Acid bacteria

Homofernientative Lactate			Heterofermentative Lactate		
Organisms	**Configuration**	**% G + C**	**Organisms**	**Configuration**	**% G+C**
Lactobacillus			Lactobacillus		
L. acidophilus	DL	36.7	L. brevis	DL	42.7-46.4
L. alimentarius	L(D)	36-37	L. buchneri	DL	44.8
L. bulgaricus	D(-)	50.3	L. cellobiosus	DL	53
L. casei	L (+)	46.4	L. coprophilus	DL	41.0
L. coryniformis	DL	45	L. fermentum	DL	53.4
L. curvatus	DL	43.9	L. hilgardii	DL	40.3
L. delbrueckii	D(-)	50	L. sanfrancisco	DL	38.1-39.7
L helveticus	DL	39.3	L. trichoides	DL	42.7
L jugurti	DL	36.5 - 39.0	L. fructivorans	DL	38-41
L. jensenii	D(-)	36.1	L. pontis	DL	53-56
L. lactis	D(-)	50.3	Leuconostoc		
L. leichmannii	D(-)	50.8	L. cremoris	D(-)	39-42
L. plantarum	DL	45	L. dextranicum	D(-)	38-39
L. salivarius	L (+)	34.7	L. lactis	D (-)	43-44
Pediococcus			L. mesenteroides	D(-)	39-42
P acidilactici	DL	44.0	L. gelidum	D(-)	37
P. cerevisiae	DL		L. carnosum	D(-)	39
P. pentosaceus	DL	38	L. mesenteroides		
P. damnosus			sub sp.		
P. dextrinicus			mesemtarpodes		
P. inopinatus			subsp. cremoris		
P. parvulus			subsp.		
Tetragenococcus			dextranicum		
T. halophilus	L	36.5	L. argentinum		
T. muriaticus			L. citreum		
Streptococcus			L. fallax		
S. bovis	D(-)	38-42	L. pseudomesenteroides		
S. thermophilus	D (-)	40	Carnobacterium		
Lactococcus			C. divergens		33.0-36.4
L. lactis subs			C. mobile		35.5-37.2
Lactis			C. gallinarum		34.3-36.4
biovar diacetylactis	L (+)	38.4 -38.6	C. piscicola		33.7-36.4
L lactis subsp.			Weissella		
Cremoris	L (+)	38.0-40.0	W. confusa	DL	44.5-45.0
L. lactis subsp.			W. hellenica	D(-)	37-47
hordniae		35.2	W. halotolerans	DL	45
L. garvieae		38 3-38.7	W. kandleri	DL	39
L. plantarum		36.9-38.1	W. minor	DL	44
L. raffinolactis		40.0-43	W. paramesenteroides	D (-)	38-39
Vagococcus			W. viridescens		DL 43
V. fluvialis		33.6	Oenococcus		
V. salmonmarum		36.0-36.5	O. oeni	DL	38-42

Note: DL 25% to 75% of lactic acid is ofthe L-coiifiguralion; D or L = the Isomer recorded Makes up to 90% or more of lactic acid' 0 (LV I (D) "the isomer in parentheses represents up to 15-20% of total lactic acid.

Sourcc: Jay, 2000.

Table 2.6 Major lactic acid bacteria in fermented plant products

Homofermenter	Facultative homofermenter	Obligate heterofermenter
Enterococcusfaecium	*Lactobacillus bavaricus*	*Lactobacillus brevis*
Enterococcus faecalis	*Lactobacillus casei*	*Lactobacillus buchneri*
Lactobacillus acidophilus	*Lactobacillus coryniformis*	*Lactobacillus cellobiosus*
Lactobacillus lactis	*Lactobacillus curvatus*	*Lactobacillus confuses*
Lactobacillus delbrueckii	*Lactobacillus plantarum*	*Lactobacillus coprophillus*
Lactobacillusleichmamnii	*Lactobacillus sake*	*Lactobacillus fermentum*
Lactobacillus salivarius		*Lactobacillus sanfrancisco*
Streptococcus bovis		*Leuconostoc dextranicum*
Streptococcus thermophilus		*Leuconostoe mesenteroides*
Pediococcus acidilactici		*Leuconostoc paramesenteroides*
Pedicoccus darrmosus		
Pediococcus pentocacus		

Source: Beuchat, 1995

The basis of this division is that for the homofermenter, there is the presence or absence of the key enzymes of homo- and heterofermentative sugar metabolism. The presence or absence of fructose-1,6-diphosphate aldolase and phosphoketolase account for facultative homofermenter and obligate heterofermenters respectively.

2.7 PROTEOLYSIS DURING FISH FERMENTATION

2.7.1 Fish tissue breakdown

As mentioned earlier, the breakdown of fish tissue entails bacterial and enzymatic activities. Uchyama and Ehira (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. In others, autolysis contributes to varying degrees to the overall quality loss in addition to microbially-mediated processes.

In the production of fish sauce, the fish would undergo enzymic proteolysis a process where enzymes in the gut of the fish would react with the salt, producing an acrid and potent-smelling brine (Mabesa, *etal.,* 1990, Mackie, *et al.,* 1971) which is collected and then bottled.

2.7.2 Electrophoretic protein fingerprints

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a low cost reproducible and rapid method for quantifying, comparing and characterizing proteins. Electrophoresis of proteins in polyacrylamide gels provide a partial separation in which individual bands mostly represent several proteins In recent times SDS-PAGE has found greater application in the determination of protein fingerprints (Priest and Austin, 1993). The polypeptide chain binds to the SDS and therefore the length of the reduced SDS-polypeptide complex is directly proportional to its molecular weight. Hence the separation of proteins is based primarily on their molecular weight.

A gradient gel is one which has an acrylamide concentration varying uniformly from, typically 8 % at the top of the gel to 18 % acrylamide at the bottom of the gel. According to Wilson and Walker (1995), there are two advantages for running gradient gels (as SDS gels with a stacking gel): First, a much greater range of protein M_r values can be separated than on a fixed-percentage gel. The second advantage of gradient gels is that proteins with very similar M_r values may be resolved, although they cannot otherwise be resolved in fixed percentage gels

2.8 IMPORTANT QUALITY INDICES IN FISH PRODUCTS

2.8.1 Histamine in fish

Histamine is a causative agent of scombroid poisoning and is formed as a result of time /temperature abuse of certain species of fish. According to Leurs *et al.,* (2001), it is an aminergic neurotransmitter, playing an important role in the regulation of several (patho) physiological processes and in mammals; histamine is synthesized in a restricted population of neurones located in the brain. The consequent effect of this is that several brain functions like sleep/ wakefulness, hormonal secretion, cardiovascular control, thermoregulation, food intake, and memory formation are impaired. These might translate to symptoms in man like headache, fast heartbeat, skin sensations stomach pain, anxiety and agitation, and respiratory problems. As a result of its medical importance the U.S. FDA has established guidelines, which regulates the histamine level in fishery products to a maximum of 5mg/g of fish. Eventhough the monitoring of histamine levels at each handling and processing step is a critical step in tuna industry, histamine levels at the receiving points continues to be the main critical point assuring safety of tuna products (Collette, 2001). Other world bodies like the FAO of the United Nations and the World Health Organization have equally established HACCP programmes to improve the safety of the products. According to Collette (2001), The rapid chilling of fish immediately after death to the internal temperature below 10 °C or less within 6 to 9 hours of death depending on the size of the fish has been the most commonly used strategy of preventing the formation of scombrotoxin, and the practice has proven to be effective as the incidences of histamine is substantially reduced since the adaptation of HACCP.

In the use of Molecular techniques to detect histamine- and biogenic amine-forming bacteria, Velazquez, J.B., 2001, found the detection of *Morganella morganii* and *Stenotrophomonas*

maltophilia -involved in histamine and cadaverine formation in albacore- useful for the direct detection of these biogenic amine-producing bacteria in other marine species and in other foodstuffs potentially able to cause histamine poisoning. On the other hand WEI and An, 2001, also determined the Microbial contribution of histamine and biogenic amines formation in seafoods and it was mainly observed that *M. morganii,* unlike other commonly present bacterial species in fish, highly correlates with the increase in histamine content in muscles of a variety of fish. In addition to histamine formation, *Morgariella morganii* was able to convert other naturally present free amino acids to toxigenic biogenic amines, which can potentiate the toxicity of generated histamine further.

2.5.2 Heavy metals in foodstuffs (marine products)

Heavy metals occur as natural constituents of milk and meat (for example, zinc, copper and selenium) or as contaminants from environmental or industrial sources (for example, lead, tin, cadmium, mercury and arsenic). While Maximum Residue Limits (MRLs) have not been established, generally, for heavy metals in food products (O'Keeffe *et al,* 2001), there are specifications or Provisional Tolerable Weekly Intake (PTWI's) for heavy metals. It has been indicated that the concentration of some heavy metals, lead, mercury, and cadmium have increased greatly in the aquatic environment (Kinsella, 1987). The cause of this increase has been the increased discharge of these and other heavy metals in the form of wastes and spillages into water bodies. This suggests that aquatic organisms stand a very high risk of heavy metal poisoning.

Mercury is found in concentrations ranging from 0.005-0.05 mg/kg in foodstuffs. The main contributor is methyl mercury in fish, which contains up to 2-4 mg/kg. The average level of

mercury in fish is 0-0.08 mg/kg (National Food Agency of Denmark, 1995) In fish, the major amount of mercury is methyl mercury (Beliles, 1994; Berlin, 1986). Sims *et al.* (1977), have reported on the excessive intake of mercury and lead by humans and how these can damage the central and peripheral nervous systems and impair kidney function. Mercury toxicity can affect the brain and central nervous system and arsenic can also affect the liver and kidney (Dunn, 2002). Lasky *et al.* 2002, have reported on the higher than previously recognized concentrations of arsenic in chicken combined with the increasing levels of chicken consumption. Their report also suggests there may be a need to review assumptions regarding overall ingested arsenic intake.

Cadmium is found in most foodstuffs in the range of 0.005-0.1 mg/kg (Fnberg et al., 1986). Epidemiological investigations have also implicated cadmium as a carcinogen (Koller, 1980), and the same heavy metal has been reported to induce toxic free radical species producing lipid peroxidation and causing liver damage (Gil *et al.,* 1989).

It is also generally known that selenium toxicity may lead to loss of hair and nails, lesions of the skin nervous system and teeth (CEIM, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

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3.1 SAMPLES

3.1.1 Sample acquisition

Tuna processing wastes: Raw and pre-cooked tuna processing wastes were obtained from the Pioneer Food Cannery, Tema, Ghana. These were packaged aseptically in sterile polythene bags transported to the laboratory in an ice chest.

Maize: A high quality seed grade maize was purchased from the Ghana Seed Growers Association.

Fermented cassava doush (Aebelima) and fermented com dough (Moree): 72 hour-fermented samples were obtained from commercial producers. They were packed separately in sterile polyethylene bags and transported to the laboratory in ice packs.

Fruits: Samples of fresh pineapple, mango, banana, and plantain were purchased from a grocery shop at Madina, Accra.

Sugar and Sodium Chloride: Sucrose, glucose and sodium chloride that were all laboratory grade chemicals (AnalaR), were used.

3.1.2 Sample Preparation

The raw and precooked tuna processing wastes were separated into bones, fins, gills and muscles. The muscle portions of the raw and precooked samples were mixed (1:1) using a Hobart bowl

chopper (Model No. 84142PED, Spec. No. 8163, The Hobart Mfg. Co. Ltd., Ontario, Canada) for 3 min. The blend was packaged in polyethylene bags and kept in the freezer until used.

Mai tins and Mashing, of Maize: The maize grains were sorted, cleaned and steeped in water for 24 hours. They were spread on a moistened piece of cloth for 72 hours to germinate. During this period they were moistened daily by sprinkling water on the grams. After germination they were dried in a solar drier and then milled into flour. The mashing was carried out by mixing with water(ratio of 1:2) and heating in a fermenter at 50 °C for 2 hours to enable the enzymes present break down the starches into simple sugars.

Fruits: The fruits were peeled and separately blended using a laboratory warring blender (HGB550 Model 36BL29, Waring products division, USA). Samples of the blends were taken for the determination of brix and the rest frozen for future use.

3.1.3 Raw material characterization

3.1.3.1 Physical characterization of fish waste

The raw tuna processing wastes were hand-sorted into their bone, fins & scales and soft tissues fraction and weighed.

3.1.3.2 Proximate analyses.

Protein, crude fat, moisture, and total ash of the samples were determined using standard AO AC (1990) methods described.

3.1.3.3 Microbiological characterization

General-purpose media (Plate Count Agar [PCA] and Tryptone Soy Agar [TSA]) were used to determine the bacterial populations in the fish processing wastes. MRS was used to determine the population of lactic acid bacteria and the coliforms were determined using TSA and violet red bile agar (VRBA).

3.2.1 Production of Fish Sauce

The process flow chart for the production of fish sauce is shown in figure 3.2. Fish sauce was produced using deboned fish waste, 10 % salt and different sources of carbohydrate to facilitate the growth of microorganism.

The blended deboned fish waste was mixed with a carbohydrate source (10 % w/w) and NaCl (10 % w/w), packed into fermenting jars (1000 g per jar) and then placed in an incubator at 37.0 °C for fermentation. Fermentation was allowed to proceed over seven weeks. Control experiments were set up in which: I. - No carbohydrate source; II - No salt added to the fish waste and HI. - Sample containing a carbohydrate source (10 % w/w) and NaCl (10 % w/w) was autoclaved at 121°C for 15 minutes prior to incubation to inactivate inherent microorganisms and enzymes.

3.2.1 Production offish sauce using glucose, sucrose and some locally fermented foods as source of carbon

This formed part of the preliminary studies carried out to investigate the possibility of fermenting the tuna processing waste into a sauce-like product. Equal portions of raw and precooked tuna processing waste together with 10% Sodium Chloride and a different source of carbohydrate for each set up were blended together and incubated at 37 °C for 40 days. These set ups contained

the following: 10 % glucose, 5 % sucrose, 10 % maize malt (flour), 20 % maize malt (flour), 10 % fermented cassava dough, 10 % fermented maize dough and a control (without any sugar),

3.2.2 *Production offish sauce using some selected foods containing high amount of simple sugars as source of carbon*

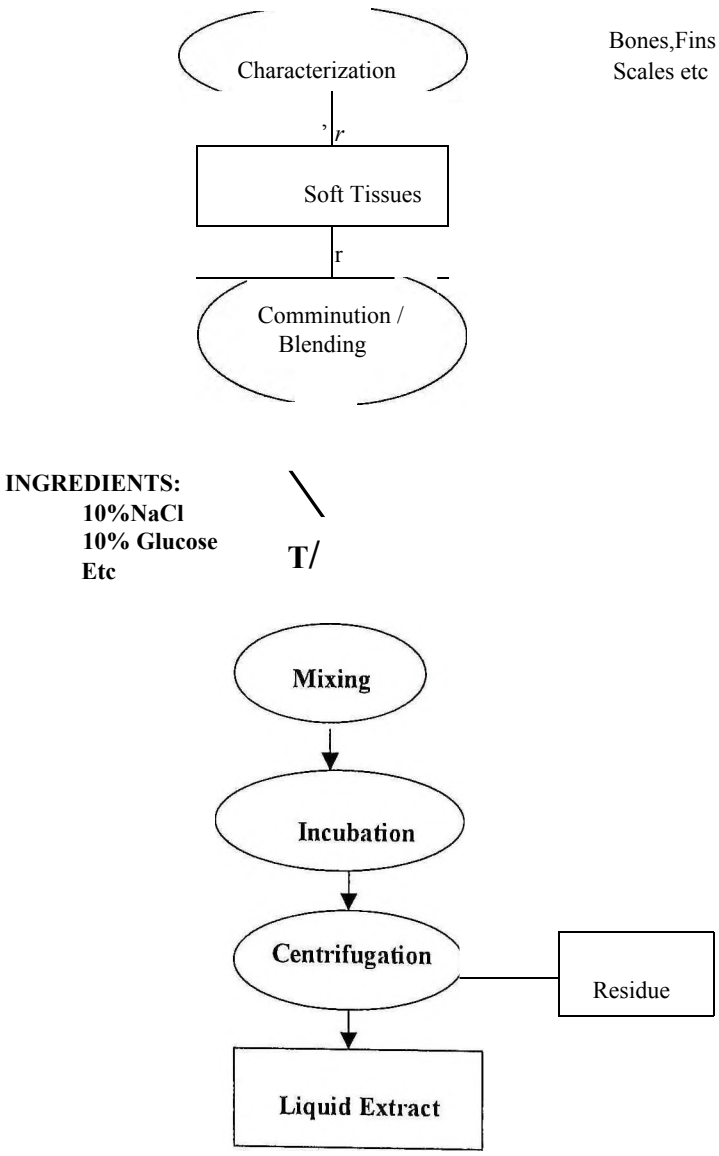
As described in section 3.2.1 above equal portions of uncooked and pre-cooked tuna waste were blended with 10 % of selected food sample each weighed at 80 % moisture. The six experimental set-ups consisted of the following: pineapple, mango, banana, plantain, malt mash and glucose. They were incubated as usual.

Table 3.1: Formulations of ingredients for the production of fish sauce

Sample (Food Material Used)	**Weight of blended tuna processing waste**	**Weight NaCl**	**Weight of** CHO*
SUC (5% w/w Sucrose)	85.0	10	5.0
SAL (No added sugar)	90.0	10	0
MAL (Malt Mash Extract)	84.9	10	5.1
PIN (Pineapple Pulp)	86	10	4.0
MAN (Mango Pulp)	84.6	10	5.4
BAN (Banana Pulp)	83.8	10	6.3
PL A (Plantain Pulp)	83.4	10	6.6
AGB (Agbelima)	82.6	10	7.4
MOR (Moree)	82.6	10	7.4
MALH (Malt Flour)	70.0	10	20
MALF (Malt Flour)	80.0	10	10
CGLUG (Control to check Glucose)	90	10	0
CGLUS (Control to check Salt)	90	0	10
GLU (10% w/w Glucose)	80	10	10

*Kind of carbohydrate used - taken at 80% moisture.

Figure 3.1 FLOW DIAGRAM FOR THE LABORATORY PROCESSING OF FISH SAUCE.



3.3 PHYSICO-CHEMICAL ANALYSES

The physico-chemical changes associated with the fermentation of the fish sauce were monitored The following characteristics were determined: moisture, sodium chloride, pH, total acid, total nitrogen protein, total lipid, free ammo nitrogen, free fatty acids, thiobarbituric acid number, heavy metals and histamine. The analyses were done on extracts collected at various stages of the fermentation. Samples of the extracts were filtered through Whatman No. 4 filter paper (Whatman Co. USA) and the filtrate used for the determination.

3.3.1 Moisture content

The moisture content was determined by drying one gram of well-mixed sample at 105 °C in an air oven (7B 1660 C CAT No. OVH 200 01 OH, Gallenkamp) to a constant weight (AOAC, 1990 method 44-15).

3.3.2 Sodium Chloride (NaCl) content

The NaCl Content was estimated by titration with silver nitrate. (AOAC, 1975 method 18.030-18.031).

3.3.3 pH

This was determined by a pH meter (Radiometer LAB pH meter, PHM 923.1 3412 NO 17 Analytical A/S, Denmark.) as described in AOAC, 1990 method 44-15.

3.3.4 Titrable acidity

The Total Acid as percent, w/w of lactic acid was determined according to the method of Nout *et al.* (1989). In this procedure, pH determination preceded the titration of the sample with 0.1N NaOH (AnalaR grade) to an end point at pH 8.5. The solution was stirred continuously for 10 min with a magnetic stirrer and pH adjusted again for replicate determination with 0. IN HC1.

The total acid was calculated as follows:

Total Acid (%, w/w) = volume (ml) 0. IN NaOH x 10.3 x90

3.3.5 Total lipid

The Bligh and Dyer (1959) method for the determination of total lipid was used. In the process ten (10) grams of the sample was weighted accurately into a 200ml-homogenizing flask. After the addition of 10ml of water, 20ml of chloroform and 40ml of methanol the mixture was homogenized for lmin. A further 20ml methanol was added and the mixture was homogenized for 1mm. A further 20ml of chloroform was added into the flask and the mixture homogenized for 30 seconds. A final 30-second homogenisation was done after addition of 20ml of water. The homogenate was transferred to glass centrifuge tubes and centrifuged at 2000xg for 20min. After centrifugation, the aqueous layer was removed by suction. The lipid in 20ml of chloroform was then determined after evaporation in a dried, weighted flask, initially on a steam bath and finally in an oven at 105°C for 30min. The weight of the fat was multiplied by 2 to give the weight of total lipid in the sample.

3.3.6 Free fatty acids (FFA).

This was done as described in the AOAC (1990) method 26.060

3.3.7 Total nitrogen protein

Total Nitrogen was determined using AOAC (1990) method 47-021.

3.3.8 Free amino nitrogen (FAN)

Free Amino Nitrogen (FAN) was determined with the colorimetric Ninhydnn method (AOAC, 1975 method 24.041 / 24.043) using a spectrophotometer (Spectronic 21D, Milton Roy, Belgium).

3.3.9 Gel electroph oresis

SDS-PAGE was earned out to investigate the breakdown of proteins during bioconversion. Fish extract was taken at predetermined times throughout the fermentation period and prepared for use. Exactly 10 pi of tuna extract was diluted with 90 pi of Tns Buffer (g/L, Milli-Q Water; 1 576 g, Tris/HCl [Sigma 7149]; 0.372 g, EDTA [Sigma E-513]; pH 8.0). A 100 pi of the sample buffer containing per litre 10 ml Tris buffer, 1 ml Bromophenol blue solution (g/ml, Milli-Q Water; 0.020 g, Bromophenol blue [Merck 8122]); 20 ml SDS solution (g/20 ml, Mlli-Q Water, 2.00 g, SDS [Pharmacia Biotech 7-13 13-0]), 5 ml, Glycerol solution [g/5 ml, Milli-Q Water; 25 g, Glycerol [Merck 4092]); 1 ml, Dithiothreitol (g/ml, Milli-Q Water; 0.154g, DTT [Sigma D-9779]) solution and 100 ml Milli-Q Water was added and then boiled for 15 min. This was then centrifuged at 5000 g for 2 min. The prepared sample was then loaded at 10 pi per well on a SDS pre-cast gel plate (ExcelGel™ SDS, gradient 8-18, Pharmacia Biotech). The running conditions were 600 V; 50 mA; 30 W and 80 min.

3.4.1 Thiobarbituric acid number

The method of Ramanathan and Das (1992) was used. Ten (10) ml of each sample was homogenized in 25 ml of deionized water using a stomacher (Lab Blender, Model SE I IPP, Struers Seward Medical, Medical, UK) at 3,000 rpm for 1 min; 25 ml of 10% trichloroacetic acid (T-4885 Lot 50H0541, SIGMA Chemical Co., USA) were then added to the homogenate and homogenized using a magnetic stirrer at 600 rpm for 1 min. It was then filtered through Whatman No. 2 filter paper, (Whatman Co. Ltd., USA) One millilitre of 0.06 M TBA (Prod 3040844, Lot 1894837 702, BDH, Poole, England) was added to 4ml aliquots of the filtrate, which were then heated in boiling water bath for 10 min. The tubes were cooled and the absorbances read at 532 nm using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). For the blank, 0.06 M Thiobarbituric acid was added to the filtrate samples after cooling.

3.4.2 Determination of heavy metals

The heavy metals were determined by Atomic Absorption Spectrum method using a Perkin Elmer (Model 3110, PERKIN ELMER - AAS, USA) equipment. The following metals were determined: Cadmium, Selenium, Mercury and Arsenic. The condition and assay settings of the instrument are shown in Table 3.2. Standard curve for each metal was prepared using standard solutions obtained from the Ghana Standards Board.

Table 3.2 Assay settings of the AAS Instrument

SETTINGS	CADMIUM	SELENIUM	MERCURY	ARSENIC
Wavelength (nm)	228.8	204.0	253.7	193.7
Slit width (nm)	0.7	0.7	0.7	0.7
Lamp current (mA)	240	280	210	
Sensitivity (ppm)	0.028	2.9	4.2	1.0
Sensitivity check	1.5	150.0	200.0	45 0
Lamp type	EDL	EDL	EDL	EDL
Replicate Reading	5	5	5	5
Linear ranee (ppm)	2.0	_	300	_

The values read on the AAS were in ppm (parts per million). The instrument was operated with the following llow rates: Adjusted regulator pressures to air= 50 psi, acetylene = 10-15 psi, Flow rates adjusted to "FUEL" = 2 and "OXIDANT" = 4.

3.4.3 Determination of histamine in fish sauce

Colorimetric method was used (CSIR-FRI Laboratory Manual, 2001) and this involved the reaction of histamine with a diazo aromatic compound to form a colour complex, which enabled histamine to be estimated by column chromatographic technique. A 4.0 ml sample of fish sauce (4.0 ml) was blended with 100 ml of 2.5 % Tnchloroacetic acid solution and filtered. The filtrate was then neutralized to a pH 7.0 with IN Potassium hydroxide and 0.75 ml applied to a column of Amberlite (1 g of resin in 10 ml of 0.2 N acetate buffer - pH 4.63). The column was then washed with 100 ml buffer to remove all interfering substances after which Histamine was eluted with exactly 25 ml of 0.2 N hydrochloric acid. A blank was performed using a similar volume of 2.5 % TCA.

A 2.0 ml of chilled diazo reagent (0.894 g p-bromoaniline in 9 ml concentrated HC1 and diluted with distilled water to 100 ml plus 5 % Sodium nitrite) was added to the mixture and allowed to stand at 0 °C for 10 min prior to the measurement of absorbance. 1 ml of the eluent HC1 eluate was added to 15 ml of 5 % Sodium carbonate in a stoppered test tube previously chilled in ice water bath and the absorbance determined at 495 nm using distilled water as reference in a Double beam Uvikon Spectrophotometer (UVIKON 940, Kontron instruments, Tegimenta AG CH-6343, Switzerland)

A standard curve was prepared using 0-80 p.g histamine/ml 0.2N HC1 and the concentration of histamine calculated as:

Concentration in ppm = K x (F/E) x Cone, (ig/ml.

Where the definition for K is given as:

K = (25ml HC1 / 75ml IN KOH) x 4ml of sample taken = 1.3.

F = Volume after neutralization.

E = Volume of extract after filtration.

3.5 MICROBIOLOGICAL ANALYSIS

Samples were taken on predetermined days (0, 3, 5, 7, 14, 21, 28, 35, 42 and 49), for the isolation and identification of the dominant microflora.

3.5.1 Sampling Procedure and incubation

The samples of extracts were taken from three different depths (i.e. surface, middle and the bottom) in the fermenting jar These samples were serially diluted with peptone water (0 1% peptone, 0.85% NaCl pH: 7.2 ± 0.2) and plated for aerobic counts, yeast counts and facultative anaerobe count.

The mould and yeasts were enumerated on Malt Agar (MA, DIFCO 211320 [0113-17]), containing 100 ppm of chloramphenicol (Chloramphenicol Selective Supplement Oxoid). This was incubated at 30 °C for a period 3 to 5 days. The facultative anaerobes (lactic acid bacteria) were enumerated in DeMan Rogosa and Sharpe (MRS Agar, DIFCO 28821).

Incubation for the MRS Agar plates was done anaerobically in an anaerobic jar with Anaerocult®. (A 1.13829. Microbiologie, MERCK Kga A, Darmstadt, Germany) at 30.0°C for five days. The total aerobic count was done on PCA and TSA incubated at 35 °C for 72 hours and 30 °C for 72 h respectively.

For the halotrophic organisms, the media TSA and MA as described above was engineered to contain 10 % NaCl additionally.

3.5.2 Isolation of dominant microflora

Colonies totalling 15-20 in number per plate (mostly covering an area greater than 15 % of the total plate) were isolated into the corresponding broth medium and streaked continually on agar plates until pure cultures were obtained.

3.5.2.1 Maintenance of isolates

The PCA and TSA isolates were maintained on PCA and TSA slants respectively and stored at 4 °C. The MRS isolates were sub-cultured in MRS broth and stored in a 50 % glycerol in a ratio of 1:1 at -20 °C. The yeast isolates were stored on malt agar slants at 4 °C, The method of enumeration for the halotrophic microorganisms was the same as for those carried out for the aerobic mesophiles and yeasts, the only difference was with the step up concentration of NaCl in agar plates and broths.

3.5.3 Characterization and identification of isolates

3.5.3.1 Aerobic mesophiles and lactic acid bacteria.

Isolate from TSA and PCA were examined to determine their colony and cell morphology. Gram reaction, catalase tests, casein hydrolysis, starch hydrolysis and oxidation/ fermentation test were also done for identification to the genera (Benson, 1990,). Other secondary tests carried out to aid in the identification of these bactenal isolates are described below. The various bacteria isolated were identified to the genus level according to the classifications described by Cowan and Steel, 1974; Sneath, 1986, Kandler and Weiss, 1986 and Clause and Berkeley, 1986.

3.5.3.1.1 Gram reaction

Gram reaction was carried out using a modified method of Party *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.

3.5.3.1.2 Catalase test

This method was adopted from the method described by Cowan and Steel, 1975. A loop full of the culture was examined for catalase production by mixing it into a drop of 3 % hydrogen peroxide on a microscopic slide. The slide was then observed for the production of gas bubbles to indicate the production of catalase.

3.5.3.1.3 Oxidase test

The method was adopted from the method described by Benson (1990), and modified slightly as descnbed here. A peace of Whatman No.2 filter paper was placed in a petri dish and several drops of oxidase test reagent was added. A loop full of the culture organism from a pure colony was smeared on a small area of the filter paper. After 10-15 s the observed result was recorded on a descriptive chart.

3.5.3.1.4 Casein hydrolysis

The determination of Casein hydrolysis was done following the method described by Harigan (1998). In the process, skim milk agar plates containing g/1 distilled water; 100, skim milk powder and 20, agar were inoculated with single streaks and incubated at 30 °C and examined for clear zones around growth at 2,7, and 14 d indicating deposition of casein and proteolytic activity.

3.5.3.1.5 Starch hydrolysis

Harigan, 1990, describes this method. Starch agar was prepared to contain g/1 distilled water, 10, starch and 23, nutrient agar and poured into sterile petri dishes for use. The plates were inoculated with the test microorganism and incubated at 30 °C for 5 d. The starch hydrolysis was determined at 3 and 5 d by flooding the plates with 95 % ethanol. The clear zones around colonies indicated hydrolysis of starch Un-hydrolyzed starch became white and opaque within 15 to 30 min

3.5.3.1.6 Acid Production from carbohydrate

For acid production from carbohydrate, 3ml slants containing 150 microlitres of 10 % filter sterile solution of either D (+) Glucose, L (+) Arabinose, D (+) Xylos or D (-) manitol and a basal medium containing g/1 distilled water; 1, diammonium hydrogen phosphate, 1, potassium chloride, 0.2, magnesium sulphate, 0.2, yeast extract and 15, agar, 0.006, bromocresol purple, pH 7.0 were inoculated with the test microoganism and incubated at 30 °C. The acid production was indicated by a change in the colour of the medium from purple to yellow.

3.5.3.1.7 Hugh & Leifson test CH/L)

The basal medium was prepared with Peptone, 2 g; NaCI, 5 g, K_2HPO_4, 0.3 g Agar, 3 g, Distilled water, 100 ml; Bromothymol blue (0.2 % solution) 15ml. The pH was kept at 7.1 and sterilised at 115 °C for 20 mm.

The basal medium was melted before use, cooled down to 45°C and 1% glucose was sterile filtered into the medium (4 ml sterile 50% glucose solution per 200ml of the basal media) and dispense aseptically in sterile tubes.

Two tubes of the H & L media were inoculated with the test microorganism (i.e. bacteria in mention). Paraffin oil was added to one of the tubes to obtain anaerobic conditions. After incubation at 30 °C (for the period 2, 7 and/or 14 days) a fermentative reaction was shown by the formation of acid (both tubes would turn yellowby the indicator bromothymol blue) in the aerobic tube and in the anaerobic tube. An Oxidative reaction was shown by the formation of acid in the top of the aerobic tube (no formation of acid in the anaerobic tube).

Non glucose-fermenting strains do not show the formation of acid in any of the tubes, whilst an alkali change would be indicated by blue colour due to the assimilation of peptone.

These species of isolates were identified by determining their pattern of carbohydrate fermentation, together with other characteristics described above and comparing these to known carbohydrate fermentation profile (Claus and Berkeley 1986).

3.5.3.2 Yeasts

Isolates from MA were sub-cultured and examined for their colonial morphology, cell morphology, texture, colour and the nature of growth of colonies. Their fermentation and assimilation of carbon compounds in sugars were also tested to permit identification to species level (Kreger Van Rij, 1984; Kurtzman and Fell, 1998).

3.5.3.2.1 Assimilation and fermentation of carbohydrates

Assimilation of CHO was determined, using the methods described by Kreger Van Rij (1984) and Kurtzman and Fell, (1998). A basal medium (prepared to contain 4.5 g yeast extract, 7.5 g peptone,

1.0 ml Bromothymol blue solution and 1.0 L distilled water) was adjusted to pH 6.4. Tubes containing 4 ml of this solution and an inverted Durham tube were autoclaved at 121°C for 15 mins Two millilitres of 6 % sterile filtered sugars (glucose, galactose, maltose, sucrose, lactose, melibiose or 12 % raffinose) was added to each tube. The tubes were inoculated with the yeast isolates and then incubated at 30 °C for 14 d. The assimilation of the carbohydrate and production of acid was indicated by a change in color from red to yellow. For the control experiment in each case, one of the tubes was left un-inoculated. The presence of gas bubbles trapped in the Durham tube was used as an indication of carbohydrate fermentation.

3.5.4 Test for Food Borne Pathogens

The methods used to test for the presence of food bome pathogens were adopted from methods described by the Nordic Committee on Food Analysis (NMKL), and modified slightly to suit as described below.

3.5.4.1 Detection of *Staphylococcus* spp.

The method was adapted from the NMKL No. 66, 3.ed. 1999. In the test, a sample of the fish sauce, about 10 ml was well mixed before making suitable dilutions in a diluent (Saline peptone containing, g/L Distilled water; 1.0 g, Peptone; 8.5 g, NaCl; pH 7.2 ± 0.2 and autoclaved at 121 °C for 15 min.). An amount of 0.1 ml of each dilution of the sample was transferred to the surface of Baird-Parker agar plates (g/L Distilled water; 63 g, Baird-Parker Medium (Oxoid CM275), 50 ml, SR54; pH 6.8 ± 0.2). The inoculum was evenly spread with a sterile bent rod and allowed to dry for 15 min. at room temperature. The plates were inverted and incubated for 24 ± 3 h and reincubated to a total of

48 ± 4 h at 37.0 ± 1.0 °C. Typical colonies *of Staphylococcus* spp (black or grey, shining and convex), diameter 1.0 - 1.5 mm after 24 h incubation and 1.5 - 2.5 mm after 48 h incubation and with each colony surrounded by a clear zone were isolated and tested for coagulase positive as a confirmatory test. After incubation for at least 24 h an opalescent nng immediately in contact with the colony may appear in this clear zone. Atypical colonies lack the clear and the opaque zone or these zones are barely visible. Atypical colonies may present a narrow white edge,

3.5.4.2 Detectionof *Bacillus cereus*

Bacillus cereus was tested for using the NMKL No. 67 4th ed. 1997, As a pretreatment, 10 ml of fish sauce was incubated in a water bath for 30 min, at 30.0 ± 1.0 °C prior to inoculation. Serial dilutions were made and 0.1 ml of each dilution of the sample plated out on the surface of a Bacillus Cereus Agar Base plate containing g/L, Distilled water; 20.5 g, Bacillus Cereus Agar Base (Oxoid CM617); 2 ml, SR99 and 25 ml, SR47 at pH 7.2 ± 0.2. The plates were incubated in an inverted position at 30.0 ± 1.0 °C for 24 ± 3 h. Colonies numbering 10 - 100, large irregular and greyish-white, surrounded by a well-defined hemolytic zone which Microscopically have ellipsoidal or cylindrical pores, that are centrally or terminally positioned in the sporangium are recognized as typical of *Bacillus cereus.*

3.5.4.3 Detection of *Clostridiumperfringens*

The NMKL No. 95 3rd ed. 1997 was used in the test for *Clostridium perfringens.* A sample of fish sauce (10 ml) was well mixed and prepared into suitable dilutions in adiluent (Salinepeptone containing, g/L Distilled water; 1.0 g, Peptone; 8.5 g,NaCl; pH 7.0 ± 0.2andautoclaved at 121"C

for 15 min.). About 5 ml of Tryptose Sulphite Cycloserine (TSC) agar containing g/L, distilled water; 46 g, TSC (Oxoid CM587); pH 7.6 ± 0.2, was poured into a sterile petri dish and evenly distributed. After the agar had solidified, 1 ml of each dilution of the sample was pipetted on the agar surface in triplicates, then about 15 ml of TSC agar previously held at 45 ± 1 °C was poured and mixed thoroughly before agar solidified. The petri dishes were incubated anaerobically in an inverted position using Anaerocult® (A. 1.13829. Microbiologie, Merck KgaA, Darmstadt, Germany) at 37.0 + 1.0 °C for 24 ± 3 h. Dishes containing 10 - 100 black colonies were read and further identified by spreading 3 - 1 0 black colonies on Blood - Free Pyruvate Clostridium Perfringens (BCP) agar (Hood *et al,* 1990). Alternatively Perfringens Agar Base containing g/500ml, distilled water; 23g, BCP (Oxoid CM587); 25 ml, SR47; 1 vial, SR93; pH 7.6 ± 0.2 was used to double check the outcome. The plates were incubated anaerobically for 24 + 3 h at 37.0 ± 1.0 °C. The pure culture was then stab-inoculated into the lactose medium (g/L, distilled water; 8 0 g, Brain Heart Infusion, 2.5 g, Na_2HPo_4, 5.0 g, Galactose; 3.0 g, Agar; pH 7.3 ± 0.2 autoclaved at 121 °C for 15 min.), incubated anaerobically for 24 ± 3 h at 37.0 ± 1.0 °C.

The medium was examined for gas production (Durham tubes) and for change of colour from red to yellow as would be indicated by the fermentation of lactose with gas production.

3.S.4.4 Detection of *Salmonella* spp.

According to the NMKLNo. 71, 5th ed., 1999 for the determination of *Salmonella* spp., samples of fish sauce were each treated to a pre-enrichment medium containing g/L, distilled water; 20g, Buffered Peptone Water (Oxoid CM509); pH 7.2 ± 0.2 in a ratio of 1: 9 parts of Buffered

peptone water. This was shaken and incubated at 37.0 ± 1.0 °C for 18 ± 2 h. The pre-enrichment broth was then mixed prior to removal and 0.1 ml transferred to 10 ml Rappaport-Vassiliadis Soy peptone (RV) broth containing g/L, distilled water; 30 g, RV (Oxoid CM669); pH 5.2 + 0.2, which had been pre-warmed to the incubation temperature. Incubation was done in a water bath at 42.0 ± 0.2 °C for 23 ± 3 h. A loop of the material from the enrichment broth was inoculated on a Xylose Lysine Decarboxylase (XLD) agar plate (g/L, distilled water; 53.5 g, XLD [LAB M 048395]; 4.5 g, Agar [LAB M Q 21767/184]; pH 7.4 ± 0.2. The plates were then incubated in an inverted position at 37.0 ± 1.0 °C for 24 ± 3h.

Typical *Salmonella* colonies were to be detected as having slightly transparent zone of reddish colour due to indicator change in the medium and a black centre.

3.5.4.5 Detection of Coliform Bacteria

In the test for the detection of Coliform Bacteria, NMKL No. 44, 4th ed., 1995 was adapted. In the procedure, a sample of fish sauce was mixed thoroughly and serially diluted up to a 10‘9 dilution. One ml was transferred into a sterile petri dish after which 5 ml of Tryptone Soy Agar (TSA) containing g/L, distilled water; 40g, TSA (Oxoid CM131); 2.5 g; Agar (LAB M Q 21767/184); pH 7.3 ± 0.2, previously tempera.ted to 45.0 ± 1.0 °C was added mixed with inoculum and pre-incubated at 20 - 25 °C for 2 h.. Following this 10 - 15 ml molten Violet Red Bile Agar (g/L, distilled water; 38.5 g, VRBA [Oxoid CM107]; 5.5 g, Agar [LAB M Q21767/184]; pH 7.4 ± 0.2) previously cooled to 45.0 ± 1.0 °C was overlayed to a depth ratio between the top and the bottom layers being at least 2:1. The dishes were inverted and incubated at 37.0 + 1.0 °C for 24 ± 3 h.

Coliform bacteria were counted as all dark red colonies, which have a diameter of at least 0.5 mm and surrounded by a red precipitation zone. A confirmation was done with selected suspect colonies. Five colonies of each type were picked into Brilliant Green Bile Lactose Broth (BGLB) containing g/L, distilled water; 52 g, BGLB (LAB M 049084); pH 7.2 ± 0.2 and incubated at 37.0 ± 1.0 °C for 24 ± 3 h.. The production of gas in the durham tube indicates a positive reaction The population density from the colony count was calculated with respect to the degree of dilution.

CHAPTER FOUR

4.0 RESULTS, DISCUSSION AND CONCLUSION.

This study involved the examination of some properties of tuna processing waste, a preliminary study followed by the production of fish sauce using glucose and sucrose as substrates for fermentation The physico-chemical and microbiological characteristics of the fish sauce produced from tuna processing waste were determined. Following this various substrates from selected food materials were exploited for the same purpose.

4.1 CHARACTERIZATION OF TUNA PROCESSING WASTES.

4.1.1 Physical characterisation of fish wastes.

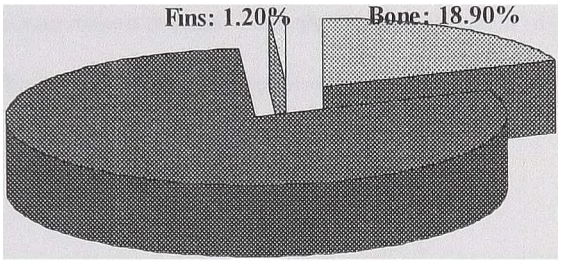
The proportions of the major components of the wastes are presented in Fig. 4.1 and 4.2. The major components were fish muscles. These were slightly less in the pre-cooked samples than in the raw sample. It was generally observed that 80-94% of the waste comprised soft fermentable tissues, 4.2-18 9% was mainly bone and the fins comprised 1.2-1.6%. Analysis of variance of the proportion of soft tissues suggested that the composition of the pre-cooked and raw samples were similar ($p>0.05$). A comparison of the proportion of the bone and fins showed significant difference between the raw and pre-cooked samples ($p>0.05$).

4.1.2 Proximate composition of tuna processing wastes

The proximate composition of the wastes is presented in Table 4.1 and it shows a high content of protein and relatively low fat content in both samples.

Fig. 4.1

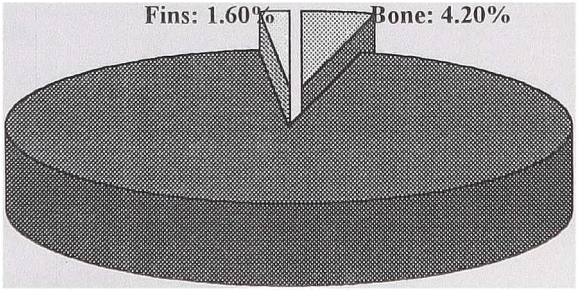
% PRE-COOKED TUNA PROCESSSING WASTE



Soft Tissues: 80.00%

Fig. 4.2

% RAW TUNA PROCESSING WASTE



Soft Tissues: 94.20%

The ash content was relatively high for the pre-cooked sample and it reflects the rather high bone proportion of the latter. Generally however, the compositions of the raw and pre-cooked samples were similar. The proximate composition of the samples suggests the suitability of the waste samples as raw material for a bioconversion process. They also suggest that a lot of proteinaceous matenal is discarded as waste. These could be hydrolyzed during fermentation to yield a liquid (fish sauce) which would be for direct human nourishment.

Table 4.1 Proximate composition of tuna processing wastes

.... **ANALYSES** (%j.....	**PRE-COOKED SAMPLE**	**RAW SAMPLE**
Moisture	67.09 ±1.57	76.81 ±0.95
Total Ash |	9.24 ±1.12	3.76 ±1.76 1
i Crude Fat	2.54 ±0.12	2.17 ±0.04
i Crude Protein	21.24 ±0.80	23.41 • o.ol |
i Carbohydrate J	<1.0	<1.0

4.2 POTENTIAL FOR THE UTILIZATION OF TUNA PROCESSING WASTES IN THE PRODUCTION OF FISH SAUCE.

The tuna processing wastes was characterised and a greater portion consisted of soft fermentable tissues. This is of relevance in the production of fish sauce since fermentation leads to the hydrolysis of the tissue proteins into smaller molecular weight proteins, peptides and amino acids. These

components make up the fish sauce. Tuna processing wastes contained 21 - 23 % protein This is quite significant and can be exploited to supplement the protein intake of man.

4.3 PRODUCTION OF FISH SAUCE FROM TUNA WASTE

4.3.1 Preliminary investigation

Preliminary studies were carried out to investigate the possibility of fermenting the tuna processing waste into a sauce-like product. Equal portions of raw and precooked tuna processing waste together with 10% Sodium Chloride and a different source of carbohydrate that is glucose, sucrose, maize malt, fermented cassava dough, fermented maize dough and a control (without any sugar) were blended together and incubated at 37 °C for 40 d.. The blend was observed for evidence of liquefaction of the tuna tissues. The percentage yield of extract was determined after the 40 days (Table 4.2). The control sample got rotten before the 20^{lh} day apparently because of the lack of control over spoilage microorganisms. Samples prepared using sucrose and glucose gave considerable yield in extract whilst the samples containing 10% malted maize flour produced very little sauce. However samples containing 20% malted maize flour and the fermented traditional products (cassava dough and maize dough) did not yield any sauce. This could be due to the fact that there was reduced water activity in the sample containing the higher level of malted maize flour. In the fermented sample it is likely that there were very little utilizable sugars left to support microbial growth since fermentable sugars originally present would have been used up during the previous fermentation of the maize and cassava dough. The table below summarizes the results of this aspect of the study.

Table 4.2 Yield of fish from tuna wastes containing different carbohydrate sources

Sample	% Yield of extract after 40 days.	Observations
10% Glucose	35	Gradual liquefaction was observed with no visible fungal growths
5% Sucrose	33	Gradual liquefaction was observed with no visible fungal growths
10% Maize Malt	16	Delayed liquefaction was observed
20% Maize Malt	0	There was no visible mould growth on the surface or within probably because of the very low moisture content of the fermenting medium.
10% Fermented cassava dough and 10% Fermented maize dough	<1	Mould growth was observed in the samples between the 3rd and 7lb day of incubation after which they disappeared these moulds which had white colonies and some very pale grayish-green ones as well, were identified as *Penicillium species* according to Sampson *el al,* 1995
0% Carbohydrate (Control)		The sample went bad with signs of deterioration - very pungent odour.

Based on the results of the preliminary studies on the production of fish sauce, sucrose and glucose were selected as the most suitable carbohydrates for the production of fish sauce from tuna wastes

Plate 1. Fish sauce production using four different carbohydrate sources - fermented cassava dough (AGB), Sucrose (SUC), fermented maize dough (MOR), and Glucose (GLU).

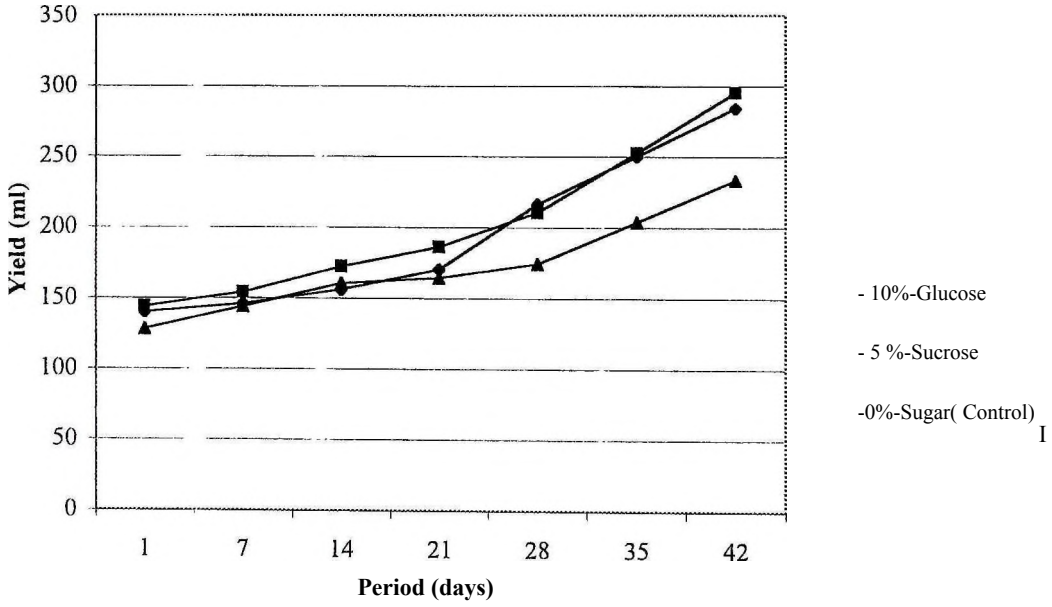
Plate 1. Fish sauce production using four different carbohydrate sources - fermented cassava dough (AGB), Sucrose (SUC), fermented maize dough (MOR), and Glucose (GLU).

4.3.2 Production offish sauce using glucose and sucrose as carbohydrate sources.

4.3.2.1 Changes in yield of fish sauce (Rate of Hydrolysis).

There was gradual but consistent liquefaction of the fish tissue dunng the 42 days of bioconversion (Fig. 4.3). There appears to be a slight increase in the rate of hydrolysis after 21 days of bioconversion. The yield of the extract from the samples prepared using glucose and sucrose appeared comparable, but all together significantly different ($p>0.05$) from the control (containing no added sugar).

Fig. 4.3 Volume of extract obtained from tuna wastes during bioconversion



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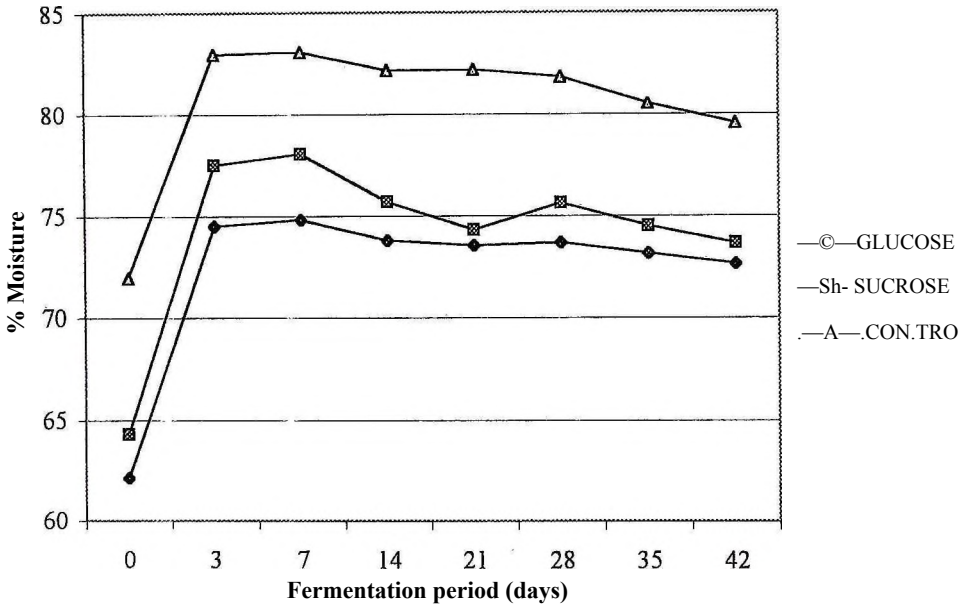
4.3.2.2 Physico-chemical changes during bioconversion of tuna processing waste into fish sauce

In the fermentation process, the sauces produced from two sets of fermentation and a control (involving the use of sucrose, and glucose as added sugar) had pH's ranging from 4 45 to 8.00 pH units over the fermentation period. The moisture content varied from 72.6-74.5 % to about 79.6-83 0 % over 49 d fermentation. The variations of salinity of the extract over the period of fermentation ranged from 10.1 % to between 13.4 & 20.0 %. The total protein of the extract at the beginning of fermentation was 11.2 % and this increased over the period of fermentation to about 16.7-18.0 %. Free Amino Nitrogen ranged between 5.0-8.3 % to 6.4-36.6 mg/ml. The acidity, which was measured with reference to lactic acid over the period of fermentation, ranged from 0.9-1 0 to 1 .2-2.5 %. Total lipids in the sauces ranged between 2.3 and 4.0 % over the period. The volume of extract produced over the fermentation period for the three samples ranged between 128-144 ml and 233-295 ml.

4.3.2.2.1 Moisture content

Figure 4.4 illustrates the variation in moisture content of the tuna extract during bioconversion. At the start of biconversion, the moisture content of the fermenting mixture was between 62 % and 65 % for the sample, which either glucose or sucrose had been added and 72 % in the control samples. There was an increase of over 10 % in moisture content in all samples during the first 3 days of bioconversion. However after this, a slight decrease in the moisture content of all samples were observed till the end of bioconversion.

fig. 4.4 Moisture content of extracts from fermenting tuna wastes containing different sugars.



At 95 % Confidence Interval there was no statistically significant difference within the three samples being compared (p= 0.3778) and equally no significant difference in moisture content of the extract dunng the period of bioconversion within samples (p= 0.4468). The change in the solute component of the extract obtained with time is an indication of tissue breakdown and this was rather slow thus accounting for the insignificant change in the moisture content of the extract during fermentation

4 3.2.2.2 Changes in pH

The pH of all three samples remained fairly constant around pH 5.5 during the first 21 days of fermentation (Fig. 4.5). After 21 days however there was a significant pH drop in the samples containing sugars; but there was an increase in the non-sugar sample. By the end of the 42 days, the sugar samples had final pHs of about 4.4 whilst the control sample was 8.0. The latter sample had gone extremely bad and had an odour of deteriorated fish. The increase in pH of the control sample was probably due to putrefaction leading to the formation of basic nitrogenous compounds. The drop in pH in the sample containing sugars could be attributed to the production of acids during bioconversion. According to Frazier and Westhoff (1988), the trend in pH reduction may be explained as due to the involvement of lactic acid bacteria that are able to ferment sugars (glucose in particular) to produce lactic acid, which then results in the lowenng of the pH.

The differences in pH within the various samples could be attributed to the availability of fermentable sugars coupled with the population of lactic acid bacteria at the time. Lee *et al.,* 1986, had also indicated such an inverse relationship between pH and lactic acid, and also pH and acid forming bacteria/yeast in a lactic fermented fish product called Sikhae, Autoclaved samples were included to confirm the involvement of these microorganisms or enzymes in bioconversion process

and none of these samples showed any change in pH during the incubation period. This confirmed the involvement of microorganisms and enzymes in the bioconversion process since autoclaving destroyed the microflora and enzymes present.

4.3.2.2.3 Changes in Titrable Acidity

Tljere was a gradual rise in acidity of the fish extract in samples to which glucose or sucrose was added as carbohydrate source. This indicated acidic fermentation in these samples. In the control sample no such increase in acidity was observed. Surprisingly the level of acidity in this sample remained fairly constant after 21 days though the measurement of pH in this sample showed a rise from pH 5. 6 to pH 8.0 between the 21^{s1} and 42nd day.

Fig. 4.5 **pH of tuna extract during fermentation into fish sauce**

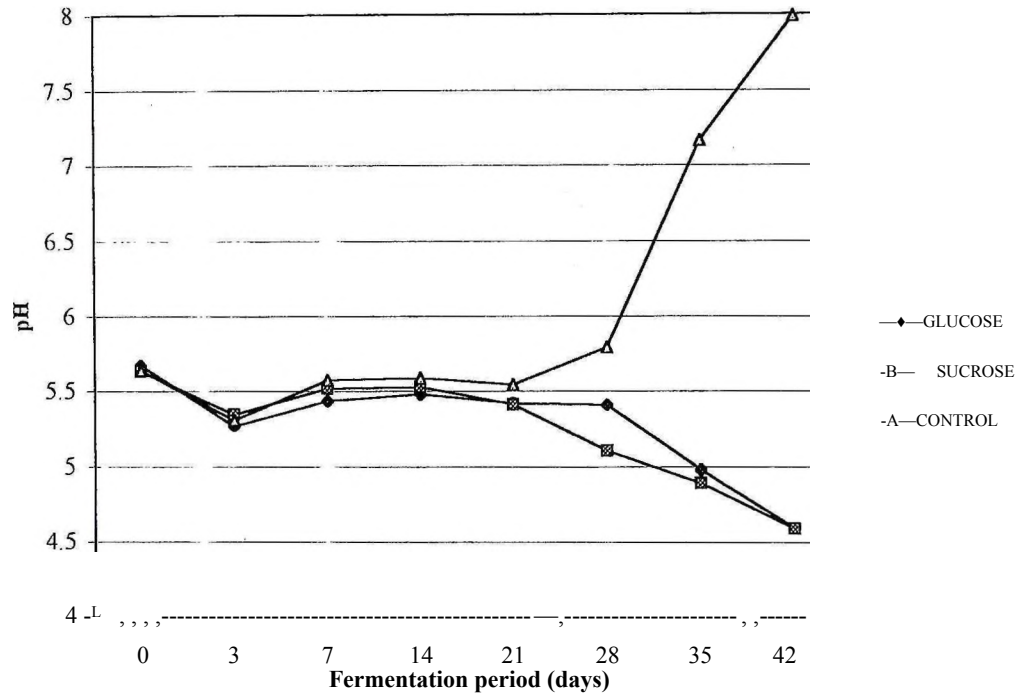
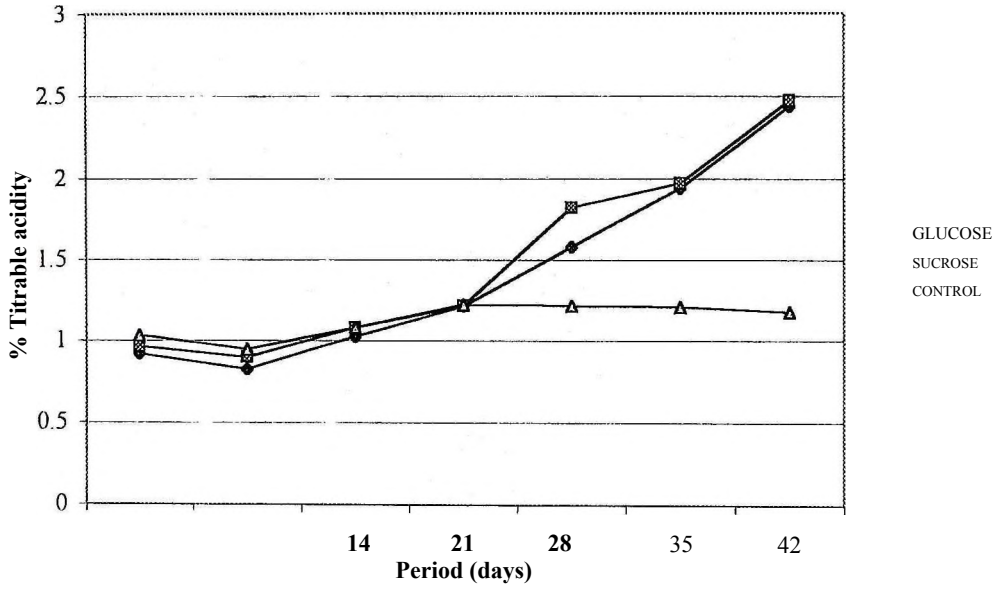


Fig.4.6 Percentage Titrable Acidity (w/w lactic acid) of tuna extract during fermentation



4.3.2.2.4 Sodium Chloride content (NaCl)

The salinity of the extract was found to increase in all samples during bioconversion (Fig 4 7) The difference in salt content during the penod of fermentation was not significant for the control and sucrose samples Multiple range analysis showed no significant difference in salt concentrations in all samples up to the seventh day. Subsequently, there was a gradual increase in salt concentration in all the samples up to the last day, with the control sample having the highest concentration The absence of fluctuations in salinity is a reflection of the inherent homogeneity of the fermentation systems set up in the laboratory. This was also reported by Thongthai and Siriwongpairat, (1978) The highest concentration of sodium chloride in the control sample could be due to the absence of the other solutes in the medium i.e. sucrose or glucose.

4.3.2.2 5 Protein and Free Amino Nitrogen (FAN)

The samples to which carbohydrate sources were added showed increases in protein content and free amino nitrogen of the fish extracts dunng bioconversion (Fig. 4.8 and 4.9). This could be explained by the combined effect of autolysis and microbial degradation of the fish muscle, which is usually associated with fish sauce production According to Ijong (1996) increases in total free ammo nitrogen and total soluble nitrogen occur concurrently during the production of fish sauce Statistical analysis of the variance show that the total Protein nitrogen generally increased with fermentation time, and the control sample differed significantly from the samples containing sucrose and glucose (p= 0.0619). The glucose sample showed significant difference in free amino nitrogen after the fourth week (p^ 0 05),

Fig. 4.7 **Sodium Chloride contents of extracts from fermenting tuna wastes with time**

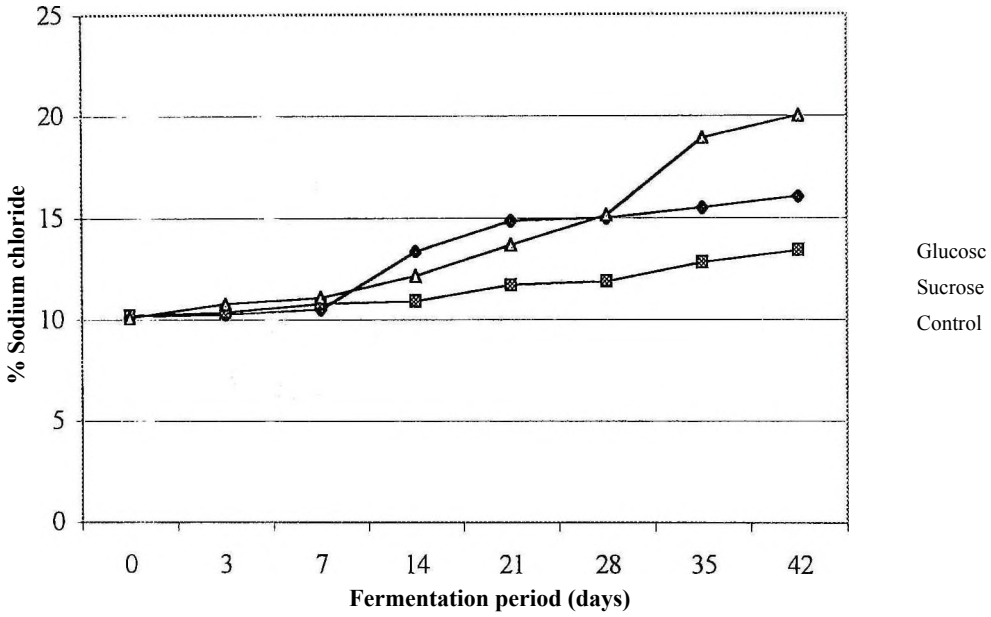


Fig.4.8 Total protein nitrogen (%) of extract from fermenting tuna waste using different sugars

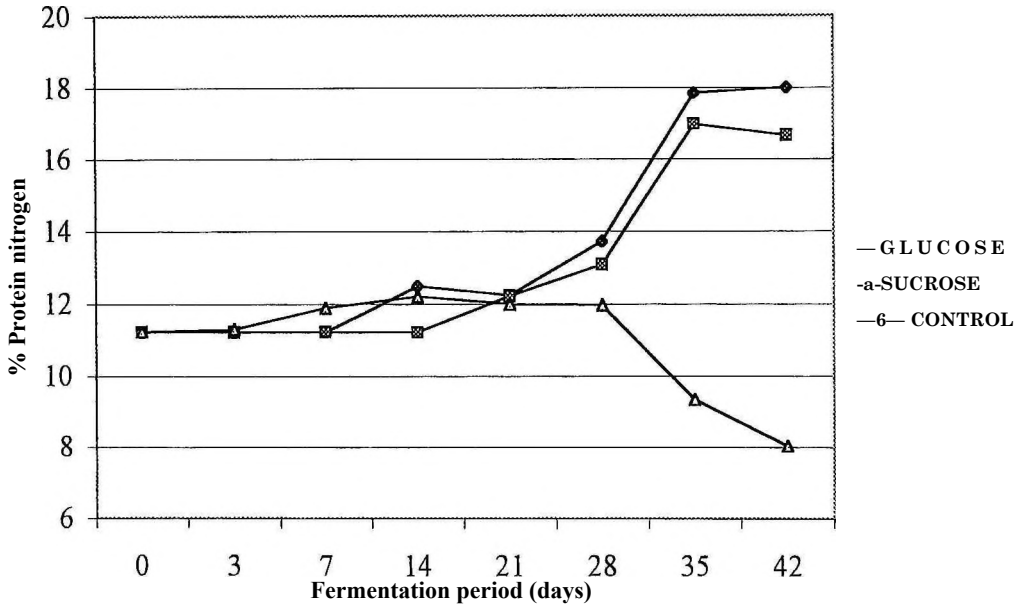
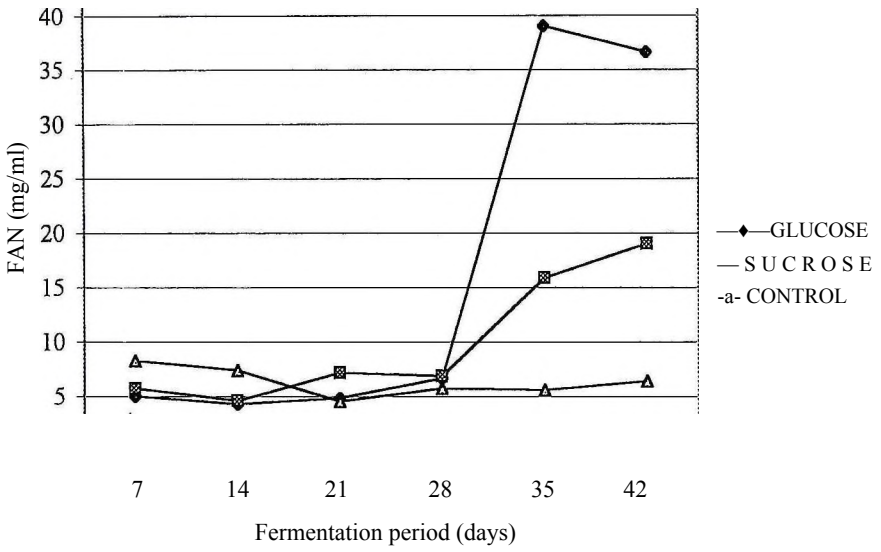


Fig. 4.9 Free Amino Nitrogen (mg/ml) of extract from the fermenting tuna waste containing different sugars.

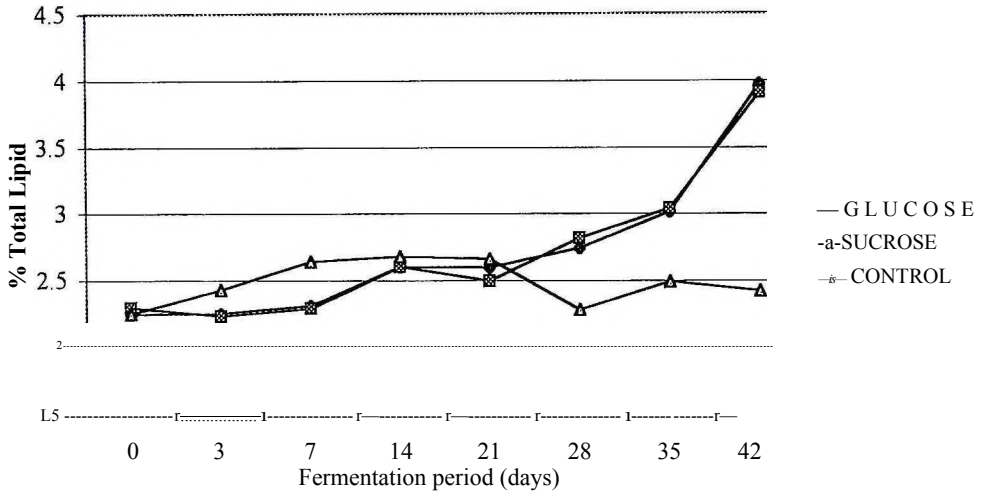


4.3.2.2,6 Changes in Lipid

The changes in total lipids of the fish extract during the fermentation period are shown in Figure 4.10. There was a gradual increase in % total lipid up to about the 5"' week after which the glucose and sucrose samples showed a remarkable difference. The lipids in the fish are very labile due to the high number of unsaturated fatty acids (Huss, 1988). Some hydrolysis takes place and this usually leads to the formation of free fatty acids.

Fig. 4.10 Lipid content of tuna extract during the fermentation

tuna waste with different sugars.



4.3.3 Use of alternate sources of simple sugars (glucose and sucrose) for the bioconversion of tuna wastes

The results of the present work has so far showed that incorporation of glucose or sucrose into tuna processing wastes as a source of carbon for microbial growth during fermentation has proved successful in the production of fish sauce. For a more practical formulation for the production of fish sauce from tuna wastes, locally available food / materials containing high levels of glucose or sucrose was used as carbon source in the formulation. The food materials used as sources of simple sugars for the bioconversion process were banana, plantain, mango, pineapple and malted maize. For comparative purposes, glucose was included in the studies.

Table 4.3 Sucrose content of food samples used as sources of simple sugars for tuna waste bioconversion.

Food Sample	**% Mass Sucrose**
Maize Malt	19 50±4.50
Pineapple	7 40.-.2.75
Banana	17.00.1150
Plantain	1 18.05 - 3.20
Mango	12.50±1.90

Table 4.3 shows the sucrose content of various food samples used as replacement for glucose and sucrose. The % sucrose in the various preparations was quite high except for the pineapple and mango pulp samples The values were generally not significantly different ($p>0.05$), but Multiple

Range Analysis showed some differences, (appendix 3A). These sugars are known to support microbial growth by providing the needed carbon in bioconversion processes. Other entities which are present in some of the food materials and also believed might contribute to the process are enzymes. For instance an enzyme (bromeline) in pineapple is known to be proteolytic. Also the malted maize mash, considering the changes that take place during its preparation could contain lots of similar enzymes.

4.3.3.1 Yield of fishsauce

Figure 4.11 shows the comparative yield of the sauce (extract) produced using the various food materials as sources of simple sugars for bioconversion. There were significant differences in yield of extract produced from the use of vanous food materials as sources of carbon for microbial growth. The highest yield of fish sauce was obtained from pineapple followed by glucose, plantain, banana, malted maize and mango respectively.

The yield observed from the use of each food sample in each case did not directly correlate to their fermentable sugar content indicated. Pineapple, which had the least brix, rather gave the highest yield. The most probable explanation is that the inherent exogenous proteolytic enzymes in both the sugars compound and the wastes must have significantly influenced the protein tissue hydrolysis of the tuna tissues more than the microbial endogenous enzymes. Howard and Dougan, (1974), suggested the addition of proteolytic enzymes of vegetable origin to facilitate the process of fish sauce manufacture.

Fig. 4.11 Yield of tuna extract after 50 day fermentation of samples with different carbohydrate source

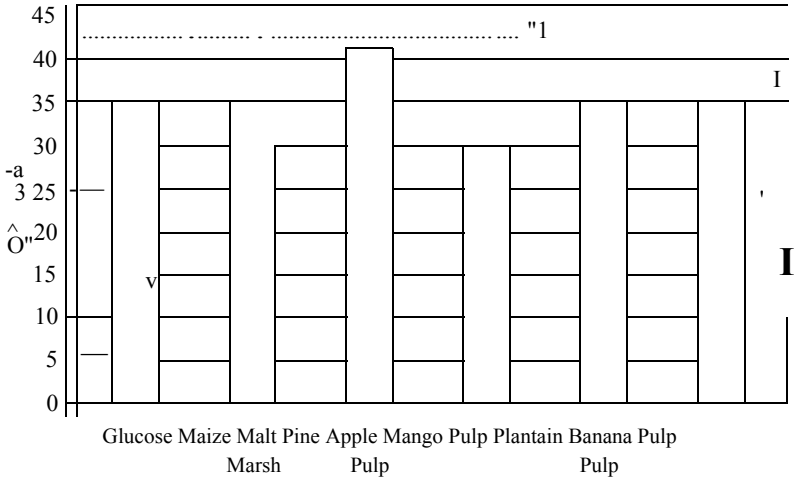


Plate 2 **Fish sauce produced from 6 different sugar sources (Glucose, Malt, Pineapple, Mango, Plantain, Banana).**

Plate 2 **Fish sauce produced from 6 different sugar sources (Glucose, Malt, Pineapple, Mango, Plantain,Banana).**

4.3.3.2 pH of tuna extract.

The changes in pH associated with the fermentation of tuna processing wastes using various carbon substrates are shown in Figure 4.12, Generally the pH appeared to decrease with time. The Glucose substrate appeared to have attained the lowest pH at the end of the fermentation period.

4.3.3.3 Free fatty acids (FFA)

Table 4.4 presents the free fatty acid content which is an indication of the deterioration in oil-hydrolysis of triglyceride to produce un-esterified fatty acids. Among the six samples studied the relative proportions of free fatty acids in the sauce produced had no significant difference between some samples: banana and mango; plantain and glucose samples. The rest however had a significantly different free fatty acid level (Refer to MRA appendix A17).

Fig 4.12 **pH of tuna extract during fermentation of samples.**

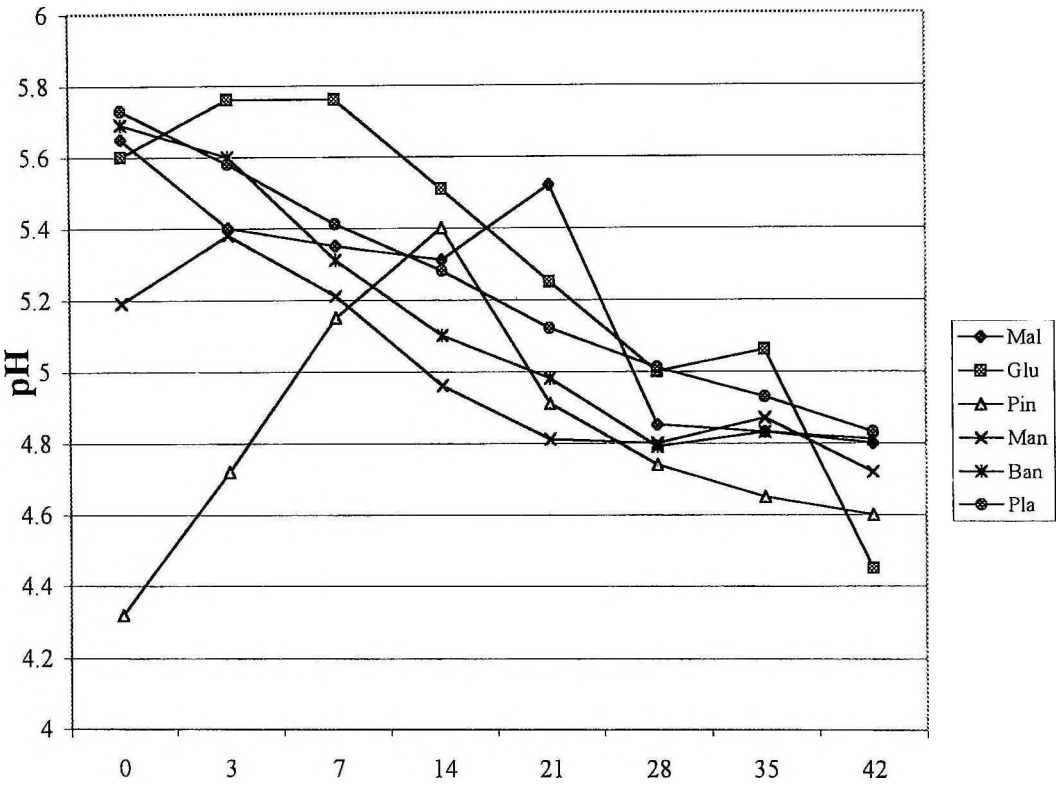


Table 4.4 Free fatty acid (FFA) levels in fish sauces produced

Sample	% Free Fatty Acids
ij GLTJ (Glucose substrate)	38.07 ± 0.11
|- MAL (Malt substrate)	! 37.22 ± 0.34
P PIN (Pineapple substrate)	41.7410.18
!; MAN (Mango substrate)	34.40 ±0.16
PL A (Plantain substrate)	38.97 ±0.35
}! BAN (Banana substrate)	35.53 ±0.17

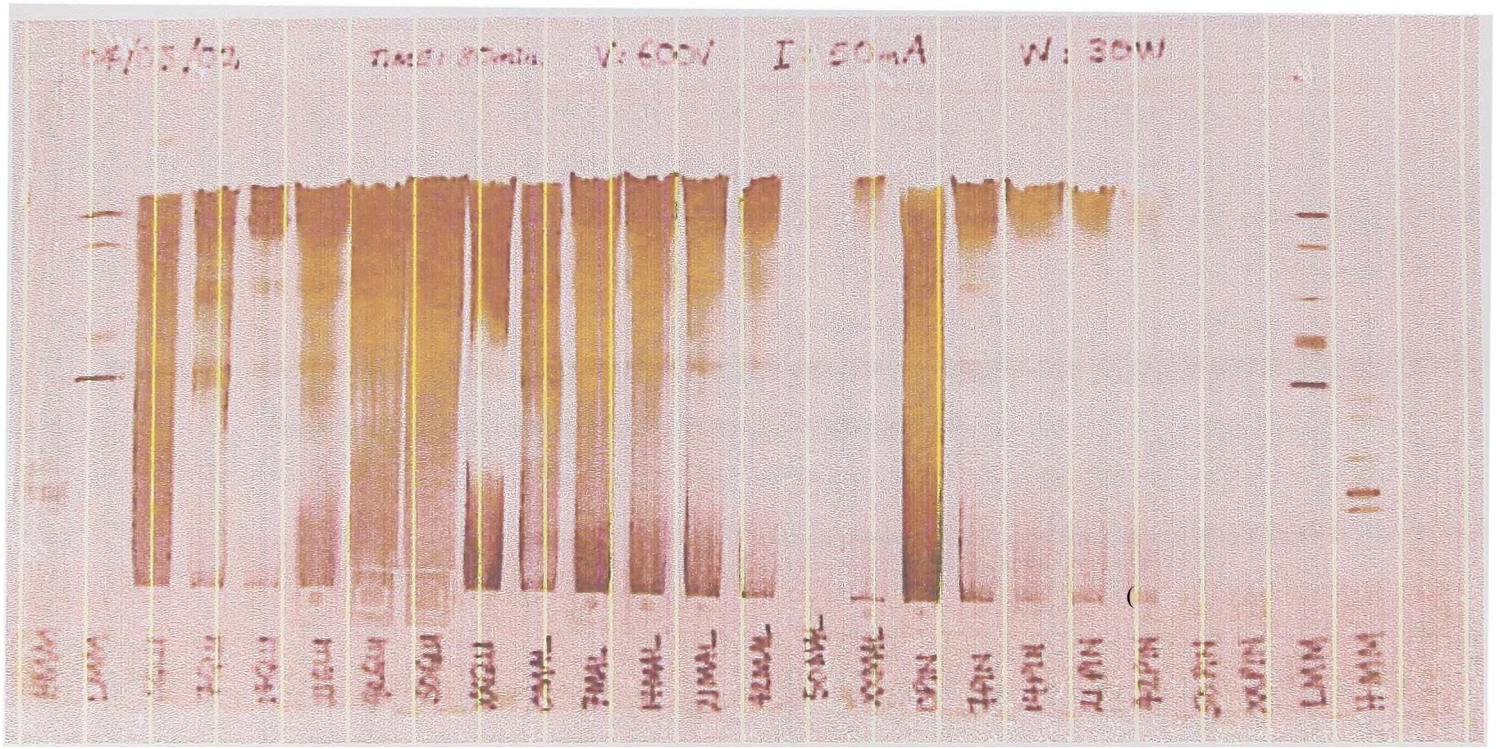
4.4.3.4 SDS-gel protein fingerprints of fish sauce run on 8-18 % gradient gel.

The protein profiles of the fermenting tuna extract are depicted in the electrophoretograms presented as Plate 3 and 4. A critical examination of the bands on the gel plates revealed that there was a consistent reduction in the size of the bands with time during the bioconversion process. As this occurred the initial bands noticed at the lower part of the gel plate on the early days of bioconversion disappeared with time. These however reappeared at higher levels of the gel gradient where they were trapped, forming different bands according to their molecular sizes. This observation supports the fact that the tuna tissues were degraded during the process, resulting in the solublization of proteins and hence the increased protein content of the sauce. The pattern of the electrophoretograms and thus the protein degradation process (Plates 3 and 4) reflects the type of sugar used in the fermentation. The rate of degradation was different with each source, being fastest with the pineapple sample.

Plate 3 SDS - whole protein fingerprints of fish sauce produced using Glucose, Malt and Pineapple as additional substrate run on 8 — 18 % gradient gel.

Lanes 1 and 25 represent high molecular weight protein marker (standard). Lane 2 and 24 represent low molecular weight protein marker (standard). Lanes 3 to 9, Fish extract taken from Glucose sample on days 0, 7, 14, 21, 42, 50 and beyond day 50. Lanes 10 to 16, Fish extract taken from Malt sample on days 0, 7, 14, 21, 42, 50 and beyond day 50. Lanes 17 to 23, Fish extract taken from Pineapple sample on days 0, 7, 14, 21, 42, 50 and beyond da

Plate 3 SDS - whole protein fingerprints of fish sauce produced using Glucose, Malt and Pineapple as additional substrate run on 8 -18 % gradient gel.

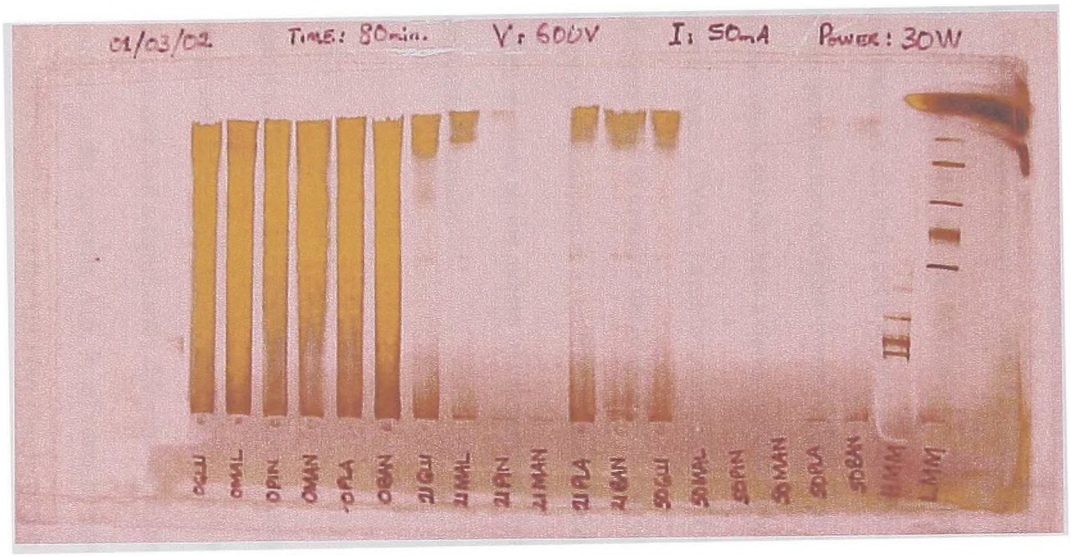


Lanes 1 and 25 represent high molecular weight protein marker (standard). Lane 2 and 24 represent low molecular weight protein marker (standard). Lanes 3 to 9, Fish extract taken from Glucose sample on days 0, 7, 14, 21, 42, 50 and beyond day 50. Lanes 10 to 16, Fish extract taken from Malt sample on days 0, 7,14, 21, 42, 50 and beyond day 50. Lanes 17 to 23, Fish extract taken from Pineapple sample on days 0, 7, 14, 21, 42, 50 and beyond da

Plate 4 **SDS— whole protein fingerprints of fish sauce produced using Glucose, Malt, Pineapple, Mango, Plantain and Banana as additional substrates run on 8 — 18 % gradient gel.**

Lanes 1 to 6 represents the six samples taken on day zero. Lanes 7 to 12 represents the six samples taken on day 21, and Lanes 13 to 18 represents the six samples taken on day 50. Lanes 19 and 20 represent high and low molecular weight protein markers respectively

Banana as additional substrates run on 8 -18 % gradient gel.



Lanes 1 to 6 represents the six samples taken on day zero. Lanes 7 to *12* represents the six samples taken on day 21, and Lanes 13 to 18 represents the six samples taken on day 50. Lanes 19 and 20 represent high and low molecular weight protein markers respectively

4.3.3.5 Quality and safety of fish sauce produce from tuna wastes using glucose, malt, pineapple, mango, plantain and banana.

4.3.3.5.1 Thiobarbituric acid number (TBA)

The TBA numbers shown in Table 4.5 indicate the differences in lipid oxidation in the sauces produced from the various substrates. TBA value is a measure of the extent of oxidative deterioration in oily fish, but it can fall again at latter stages of fish spoilage (FAO / SIFAR, 2001). All the samples had different levels of TBA (i.e. at the 95% Cl) except for mango and banana samples, which did not show a significant difference (appendix A15). Dimethylamine (DMA), Total volatile bases (TVB), Trimethylamine (TMA), Peroxide value (PV) and hypoxanthine are all indicators used to measure the level of deterioration of fish / fish products. All these indices were however not measured as a result of the lack of facilities. It is therefore not possible in this study to attempt to relate all the indices of deterioration; even though such comparison would have given a better comprehension

4.3.3.5.2 Histamine

Table 4.6 shows the histamine levels of the various samples. The difference between the banana and mango samples was not significant ($p<0.05$). This was the same for plantain and glucose samples. The rest each had a significantly different histamine levels as demonstrated by the *Multiple Range Analysis* in the appendix A16. In all cases however histamine levels were below the maximum acceptable level set by the US FDA (5mg/g of fish) to regulate fishery products; as a result of its medical importance (Collette, 2001). The measurement of the amount of histamine in fish is used as a guide to curtail the potential of fish for causing scombroid poisoning. The differences observed can

be explained in two ways. In the first instance, numerous bacteria (e.g. *Morganella morganii),* containing histidine decarboxylase, which is responsible for the conversion of free histidine to histamine, have been the main contributor to the histamine formation in fish (Wei and An, 2001). Since microbial growth and activity depends largely on their growth medium, the different samples in this study could support histamine formation differently. Secondly, according to Eitenmiller (2001), Microbial histidine decarboxylase responsible for histamine formation in foods has been quite completely characterized and these are either pyruvoyl- or pyridoxal-P-dependent enzymes. Since each of the fermenting waste had a unique composition that could contain various enzymes in varying degrees, they could result in the formation of histamine differently.

4.3.3.5.3 Heavy metals in fish sauce

According to the Table 4.7, mercury was not detected in any of the samples tested. However, Joint FAOAVHO Expert Committee on Food Additives (JECFA) has in 1978/1988 established a Provisional Tolerable Weekly Intake (PTWI) at 0.005 mg/kg body weight for mercury (Baum, 2001). Mercury is highly toxic to man and is known to easily penetrate cell walls, getting absorbed in fatty tissues, into nerve and brain cells where it causes irreparable damage. In the other heavy metals measured, there was a significant difference among all the samples; however a *multiple range analysis* showed some interaction between the malt and banana samples. Cadmium, which was observed in all the samples in a range of 0.21 to 0.32 ppb, was far below the maximum permitted levels for even for drinking water (0.005 ppm). Cadmium is a cumulative poison and highly toxic to the human organism but shows no signs of being an essential trace element in biological processes (Sciortino and Ravikumar, 1999). JECFA (1993) has established a PTWI at 0.007 mg/kg cadmium of body weight.

Table 4.5 Thiobarbiric acid number (TBA) of fish sauces produced

Sample	Thiobarbituric Acid Number
j ' GLU	3.3110.32 .
MAL	1.33 ± 0.12 |
PIN	1 10.±0.04 **j**
MAN	| 1.19 ±0.11
PLA	I 0 84 +0 19
BAN	j 1.16 ± 0.20 *i*

Table 4.6 Histamine levels in fish sauces produced

Sample	Histamine Level in pmm
GLU	13.95 + 0.14 |
,..... MAL ..... |	21.18 + 0.98 i
PIN' i	23.25 ±0.62
MAN |	34.4116.18 ||
j| PLA	28.44 ±1.97 |
!j BAN **j**	20.76 1 2.05

Table 4.7 Heavy metals in fish sauces produced

..... Sample	Heavy Metal Measured in ppb			
	Cadmium (Cd) j	Selenium (Se) j	Arsenic (As)	Mercury (Hg) i
j.....GLU".... ']	0.21 ±0.00	5.4 ±0.2	4.16 ±0.23	0
! MAL	0.32 l 0 00 ;	5.9 ±0.4	15.39 ±0.57	o
I.....PEN.....	0.32 ±0.00	6.9 ±0.5	12..02±1.54 i	0
P MAN j	0.30 + 0.05	| 6.5 ±1.2	| 20.70 ±1.85	0
| PLA	0.32 ±0.00	| 5.5 ±0.5	| 16.82 ±0.91 :	o
j BAN	0.31 ±0.00	| 5.4 ±0.3	! 9.15 ±0.67	! 0 !

Table 4.8 Quality characteristics of fish sauce produced from tuna processing wastes supplemented with glucose.

Quality Index of sample	Characteristics of fish sauce	
	Lab. Sample	**Reference figures (Ijong 1996)**
Colour	Dark Brown	Brown, yellow-brown, light brown
Moisture (%)	72.61 ± 1.15	66.30 ± 1.82 -78.07 ±6.13
Salt (%)	16.03 ±0.05	8.14 ± 0.56 - 18.70 ±0.21
Total Protein N (%)	18.01 ±0.10	12.31 ±0.90- 17.75 ±1.90
Total FAN mg/ml	36.59 ±0.10	31.21 ±0.49-98.90 ± 1.60
Total lipid (%)	3.28 ±0.19	0.10 + 0.01 - 3.00 ± 0.10
Free Fatty Acid	38.07+ 1.78	
pH	4.43 ±0.12	5.95 ±0.10-6.73 ±0.06
Acidity as (w/w) Lactic acid %	2.43 ±0.01	1.21 ±0.06-2.03 ±0.36
TBA Number	3.31± 0.01	
Histamine (ppm)	13.95 ±0.05	-

Table 4.8 summarises some key quality characteristics of the fish sauce produced from tuna processing waste using glucose as a supplement in the fermentation process. These characteristics compares with Indonesian traditional fish sauces reported by Ijong, (1996), even though total protein as well as total lipid appear to be higher than the traditional product. These slight differences could be attributed mainly to the raw material used.

4.4 MICROBIOLOGICAL STUDIES

4.4.1 Microbial population of tuna wastes.

Growth on Plate Count Agar (PCA), and Tryptone Soy Agar (TSA), referred to as aerobic mesophiles, showed the presence of the different types of microorganisms in the fish processing waste (Table 4.9). The pre-cooked and raw samples were similar with respect to the quality of microbes. As expected the microbial population in the raw uncooked waste was higher than with the pre-cooked samples.

Growth on de-Man Rogosa and Sharp (MRS) plates referred to as Gram positive and facultative anaerobes had slightly lower colonial counts compared to the Aerobic mesophiles counts. The Aerobic mesophiles and facultative anaerobes were however more predominant than other microorganisms (Yeasts and Coliforms).

It was observed that the pre-cooking of the tuna reduced the population of aerobic mesophiles and the Gram positive and facultative anaerobes by about 100 fold in the waste (Table 4.9). Generally the aerobic mesophiles were the predominant microflora ($6.6xl0^9$ cfu/g in the uncooked tuna waste, and about $2.6x10^7$ cfu/g in the cooked tuna waste).

The population of yeasts (i.e. growth on MA) and coliforms (growth on VRBA) were fairly low, between $l.OxlO^3$ - $9.3x10^3$ and $1.6xl0^3$ - $1.4xl0^3$cfu/g respectively.

Table 4.9 Microbial population in colony forming units/g of tuna processing wastes

Microorganism	Precooked Sample	I; Raw Sample (uncooked)
ı Aerobic Mesophiles[a]	(l.OxKff	1 5.4x10° 1
I Aerobic Mesophiles[B]	\.....26xi'o[7].....	]j.....6.6xio[5].....1
; Counts on MRS Agar	51x1q6·····	| 8.5x10[s] ||
i Yeasts[c]	| l.OxlO-.....	| 9 3x10[s] ||
| *Coliform	! i.6xio'	if 1.4x1 O' ||

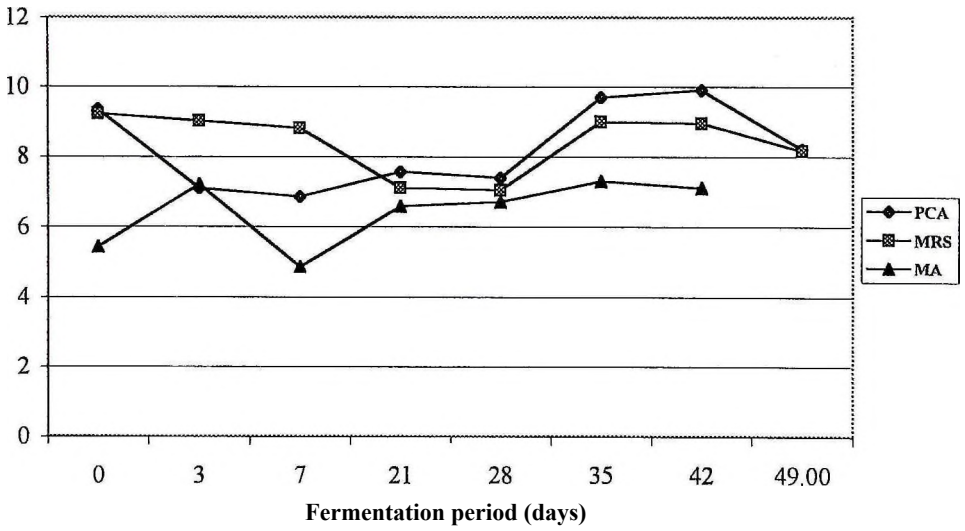
[a] Counts on Plate Count Agar.
[b] Counts on Tryptic Soy Agar.
'Counts on Malt Agar supplemented with chloramphenicol (lOOppm).
^Enumerated on VRBA.
[m] Mean value

The total microflora in the wastes as received was quite high particularly for the raw tuna processing waste (Table 4.9). This is important, as it will provide a good source for the selection of important species, which are relevant to the production of fish sauce through spontaneous fermentation. Even though lower counts were observed for yeasts, it is not very significant as yeasts and moulds have been reported to play little role in the traditional fish sauce fermentation (Thongthai and Siriwongpairat, 1989).

4.4.2 Microbial population offish sauce fermentation using Malt as a supplement.

The Figure 4.13 represents the population curves of Aerobic mesophiles, Facultative anaerobes which were gram positive, catalase negative rods and cocci and Yeasts in the fermentation system containing malted maize flour as added sugar. The population of microorganisms fluctuated between 4 and 10 Log cfu/ml.

Fig. 4.13 Microbial population in log cfu/ml of fish extract from tuna waste containing 10% w/w Malt maize flour.



PCA: Aerobic mesophiles counts on Plate Count Agar.
MRS: Facultative anaerobes counts on de-Man Rogosa and Sharp Agar.
MA: Yeasts counts on Malt Agar supplemented with chloramphenicol (lOOppm).

The initial population of Aerobic mesophiles, Facultative anaerobes and Yeasts were 9.34 ± 0.21 log cfu/g, 9.22 ± 0.48 log cfu/g and 5.43 ± 0.42 log cfu/g respectively. The fluctuation of the yeasts and bacterial populations over the period of fermentation could be the result of the introduction of the

complex carbohydrate source provided by the malted maize flour. The Facultative anaerobes (largely Lactic acid bacteria) survived more in this medium than in the medium containing glucose as sole carbohydrate (when compared to Fig. 4.14). This is because, all lactic acid producers need complex carbohydrate substrates as a source of energy (Batcoock and Azam-Ali, 1998).

The survival of the yeast up to the fifth week is probably due to the malt-containing medium. Malt, which is known to include plant, desired carbohydrates like sucrose, maltose, glucose, fructose and raffinose and which are also known to be utilized by yeast associated with fermented foods (Kurtzman and Fell, 1998) could contribute significantly in this regard. However yeasts and moulds play little role in traditional fish sauce fermentation (Thongthai and Sinwongpairat, 1989).

4.4.3 Microbial populations offish sauce fermentation using glucose as a supplement.

At the beginning of the bioconversion process, counts for this sample were quite high at levels

1. $lxl0^{s}$ - $2.0xl0^{8}$ cfu/ml for both the aerobic mesophiles and the facultative anaerobes. The pattern of distribution was similar as their numbers generally decreased with time. Representative colonies of the MRS plates were mostly Gram-positive, catalase-negative rods, coccobacilli and cocci. They were also found to be oxidase negative and non-sporing, suggesting they were largely lactic acid bacteria. The PCA and TSA plates had representative colonies consisting of both gram-positive and gram-negative bacteria. The Yeasts and Coliforms were fewer in numbers at the start, 7.4x1 O' and $1.4x10^{3}$ cfu/ml, but the Yeasts survived longer up to the 21^{s1} day (Table 4.10). The counts of Aerobic mesophiles and Yeasts that require salt for survival as shown as Halo-TSA and Halo-MA were not observed at all after the 5^{th} day of bioconversion.

4 4 3 1 Identification of dominant microflora in fish extract produced using glucose as additional substrate

4.4.3.1.1 Identification of gram-negative bacteria

Out of the 162 bacterial strains isolated, 7 were gram negative. All strains were however grouped according to their colony and cell morphology into two (B-I and B-II). Table 4.11 summarises the charactenstic features used to identify the gram-negative bacterial isolates. The group B-I were observed as rods whilst group B-II were observed as cocci. The rods were very short and plump with most of them approaching coccus shape. They both occurred in pairs and short chains. They were all growing aerobically, with non-pigmented colonies, which largely tested oxidase positive and catalase-positive. They did not produce any acid with carbohydrates in the Hugh & Leifson test.

Table 4.10 Total microflora counts in cfu/ml of fish fermenting extract

Days	TSA[a]	PCA[b]	MRS[0]	MA[d]	VRBA[e]	Halo-TSA[f]	Halo-MA[8]	Control Exp.[h]
0	(2.0x10[s])[m]	1.8x10[s]	1.1x10[s]	7.4x10[3]	1.4xl0[3]	5.2xl0[4]	5.2xl0[4]	0
3	6.4x10[s]	3.2xl0[6]	6.8x10[s]	3.9xl0[3]	l.OxlO[3]	3.3xl0[3]	2.0xl0[3]	0
5	1.9x10[s]	4.3x10[s]	4.7xl0[5]	2.4x10[3]	6.8xl0‘	2.1xl0[3]	8.2xl0[2]	0
7	3.6xl0[5]	2.7xl0[4]	1.3xl0[4]	1.8xl0[3]	3.1x10*	0	0	0
14	1.5xl0[7]	7.5x10[s]	1.3x10[s]	1.5xl0[3]	0	0	0	0
21	1.6x10‘‘	5.6xl0[4]	6.7xl0[3]	1.9xl0[2]	0	0	0	0
28	1.4xl0[3]	2.8xl0[3]	5.8xl0[4]	0	0	0	0	0
35	3.1xl0[2]	3.5xl0[2]	1.2xl0[6]	0	0	0	0	0
42	4.8xl0[2]	1.7xl0[2]	0	0	0	0	0	0
49	2.0x10[2]	7. lxlO[1]	0	0	0	0	0	0

[3] Aerobic mesophile counts on Tryptic Soy Agar.
[b] Aerobic mesophile counts on Plate Count Agar.
[c] Facultative anaerobe counts on MRS Agar (Gram-positive, catalase-negative rods, coccobacilli and cocci.
[d] Yeasts counts on Malt Agar supplemented with chloramphenicol (lOOppin).
[e] Enumerated on VRBA.
Enumerated on TSA supplemented with 10% NaCl for halotrophic microorganisms
[s]Enumerated on MA supplemented with 10% NaCl for halotrophic microorganisms
[h] Control sample was autoclaved at 12loC for 15 min prior incubation.
[m] Mean value

Table 4.11 Characteristics of typical forms of Gram-negative bacteria isolated.

CHARACTERISTIC	Isolates of Gram-negative bacteria	
	B-I |	B-II
Shape	Sphere; Coccus j	Rod-shaped jj
Motility	{	
Growth in air	+ j	+ ||
i Growth anaerobically		
j Catalase	j+ j	+ p
j Oxidase	I + <	+ ||
| Glucose acid	i - [	
I Carbohydrates: I fermentation/oxidation [F/O/-]	-F/-0	-F/-0 ||
;j"Species suspected	j ***Moraxella*** |	***Branhamella***

Legends: F=Fermentation; 0=0xidation; +=85-10% Strains are positive; - =0-15% Strains are positive.

The above tests suggested that the gram-negative microorganisms isolated B-I belonged to *Moraxellci* spp. whilst group B-II were *Branhamella* spp. *Moraxella.* and *Brcinhamella* spp. are known to be parasitic on the mucous membranes of man and other warm-blooded animals (Bovre, 1984). *Moraxella* spp. is known to appear an obligate parasite of the eye, the upper respiratory tract, and the genital tract (Volk *et al.,* 1991). *Branhamella* spp. are also known to be part of the normal pharyngeal flora and one spp in particular (*B. catarrhalis)* is recognised as an important respiratory pathogen causing acute bronchitis, pneumonia, and otitis media (Volk *el al,* 1991). However according to Huss, (1988) the bulk of the aerobic flora consists mainly of gram-negative psychrophilic asporogenous rods such as *Pseudomonas* and *Achromobaeler* or *Moraxella, Vibrio*

and Flavobacterium. Furthermore, the presence of human pathogens on the fish waste as received is related to heavy human pollution of the environment.

These gram negative bacteria; from their nature, must have been contaminants resulting from the handling of the tuna processing waste. Even though they are of public health importance, they did not survive the fermentation period for the first week. This was largely due to the composition of the fermenting medium (lowpH and [NaCl]>10%), which was unsuitable for their survival.

4.4.3.1.2 Identification of Gram positive bacteria

Of the 162 strains of aerobic mesophiles isolated from TSA plates, 155 were gram-positive. They were put under 7 different groupings based on their colony and cell morphology. Their characteristics and importance are described below

Group B+I

These were observed as spherical cells occurring mostly in pairs, tetrads and irregular clusters. They were non-motile and did not have any spores. They also produced acid to some extent (i.e. for most samples). The distinguishing characteristics of this group of organisms observed are summarized in Table 4.12.

Group B+II

In this group of isolates it was observed that the cells were mostly spherical, occurring singly, in pairs or in clusters of three and/ or tetrads. Most of them were motile with flagella. They were aerobic and tested catalase positive. The colonial morphology was quite distinct from other groups.

The colonies were yellow-orange in colour on Tryptone soy agar as well as on nutrient agar. They did not however hydrolyse starch.

Group B+III

These were rod-shaped with curved or tapering ends with no endospores. They were non-motile and observed as facultative anaerobes and catalase positive. They produced acid from glucose in Hugh and Leifson's test. Table 4.12 shows the summarised distinguishing characteristics of B+III group of isolates.

Group B+IV

Isolates of group B+IV were non-sporing cocci and survived better under anaerobic conditions during incubation. Their surface colonies on tryptone soy agar were very tiny (<0.5mm) circular with entire margins, convex, shiny and smooth. Their catalase reaction and cellular arrangements were not very distinctive. However, cells were observed to occur in pairs, tetrads, irregular masses or chains. (Details on characteristic features are shown on Table 4.12)

Group B+V

This group of isolates were cocci, were non-motile, aerobic as well as facultative anaerobic. They tested negative to catalase as well as oxidase. The cells were mostly spherical and occurred in pairs or chains. They fermented glucose with the production of acid which was observed in colour change in the Hugh and Leifson's test. Unlike other cultures, colonies of B+V did not show any increase after prolonged incubation.

Group B+VI

The group of cultures were spherical in cell shape occurring singly, in pairs as well as a few tetrads, which divided characteristically in more than one plane to form irregular clusters. They were facultative anaerobes and most tested catalase positive. In the oxidative and fermentative test (Hugh & Leifson's test) the cultures produced acid in fermentation both in aerobic and anaerobic conditions. Typical colonies observed were smooth, raised, entire and translucent. Single colony size ranged between 6 and 8 mm in diameter on Tryptone Soy Agar. The colonies became slimy after storage for a few days; succeeding the initial incubation period.

Group B+VII

The B+VII isolates 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 grouped under B+VII were catalase positive. However for oxidase, glucose (acid) test and Hugh and Leifson's test, the reactions were different, probably depicting differences in strains of *Bacillus* spp.

Table 4.12 shows summary results of some of the characteristic features used in identifying the Gram-positive bacterial groups. It was therefore inferred from the various characteristic features observed that the gram-positive bacteria isolated could be grouped under the following species: *Micrococcus* (B+I), *Planococcus* (B+II), *Corynebacterium* (B+HI), *Peplococcus* (B+IV), *Streptococcus* (B+V), *Staphylococcus* (B+VI) *and Bacillus* spp (B+VII).

Table 4.12 Characteristics of typical forms of gram-positive bacteria isolated.

ISOLATED GROUP OF MICROORGANISMS	Shape	Motility	Spores	Casein Hvd	Starch Hvd	Gmowth anaerobic	Growth in air	Catalase	Oxidase	Glucose	Hugh & Leifson's	BACTEIRA IDENTIFIED
B+I	S		"		-	-	+	+		D	*01-*	*Micrococcus*
B+n	S	+	-	+	-	-	-	+	-	+	F/-	*Planococcus*
B+m	R	"	-	-	-	+	+	+	-	-/+	-/F	*Corynebacterium*
B+rv	S	-	"	-		+	-V	+V	-	+V	-/	*Peptococcus*
B+V	S	-/+	-	"	+	+	+	-		+	F	*Streptococcus*
B+VI	S	-	-	-	-	+	+	+	-	+	F	*Staphylococcus*
B+vn	R	D	+	+	+	D	+	+	d	D	F/O/-	*Bacillus*

Legends:R=Rod-shaped; S=Sphere, Coccus; F= Fermentation; Oxidation; +=85-10% Strains are positive; -=0-15% Strains are positive.

4.4.3.1.3 Characteristics and Identification of isolated Yeasts

The yeasts isolates taken from the MA plates could be grouped into five based on their colony and cell morphology described below.

Group Y I

The cells of these species were spheroidal to ellipsoidal in singles, in pairs and occasionally in small clusters. Their colonies grew in butyrous form and were creamy-white in colour, glistening and mucoid in appearance. The cultures produced a slight pleasant odor. There were one or two hat-shaped ascospores per ascus and budding could be described as lateral.

Group Y II

The colonies observed were smooth and entire. The color was pale dull yellowish brown. Their cells were predominantly globose with some slightly ovoid in shape. Those that formed short chains were more than the single cells.

Group Y III

Cream coloured colonies were observed and these were smooth, convex colonies with an entire to slightly undulating margins. Microscopy revealed ovoid and elongated cells.

Group YIV

These species had white, mucoid, glossy, smooth and convex colonies with entire margin. The cells however were ovoid, long ovoid to cylindrical and pear-shaped.

Group Y V

Colonial morphology showed some pseudohyphae in some instances. Colonies were mostly brownish-orange, dull or shiny with butyrous, smooth, reticulate or warty flat colonies. The margins were eroded while some were also entire. The cells were ellipsoidal to cylindroidal and single Budding observed was polar with short denticles.

Representative colonies from these groups were each tested for the assimilation of sugars as a means of inferring the species to which they belong. The result of this is summarized in Table 4.13.

On the bases of all the observations made and in addition to their ability or inability to utilize these sugars (Table 4.13), the following Yeasts were identified: *Pichia capsulata; Candida vaccinii; Rhodolorula becarum; Rhodotorula pustula* and *Sporobolomyces roseus.*

Table 4.13 Fermentation and Assimilatioh of carbon from various sugars by yeast

isolated during the fermentation of fish sauce.

Group	Fermentation							Assimilation							
	6% Glucose	6% Galactose	6% Sucrose	6% Maltose	6% Lactose	6% Trehalose	12% Raffmose	6% Glucose	6% Galactose	6% Sucrose	6% Maltose	6% Lactose	6% Melibiose	CO2 (g)	YEASTS SPECIES IDENTIFIED
Y I	+	-	-	-	-	+	-	+	-	-	+	V	"	-	*Pichia capsulata*
YH	+	-	+	-	-	-	S	+	+/1	+	-	-	-	+	*Candida vaccinii*
YEI								+	-	+	+	-	-	+	*Rhodotorula becarum*
Yrv								+	V						*Rhodotorula pustula*
Y V								+	V	V	+	-	-	+	*Sporobolomyces roseus*

Plate 5 **A pure culture of *Pichia eapsulata* growing on a yeast extract agar plate**

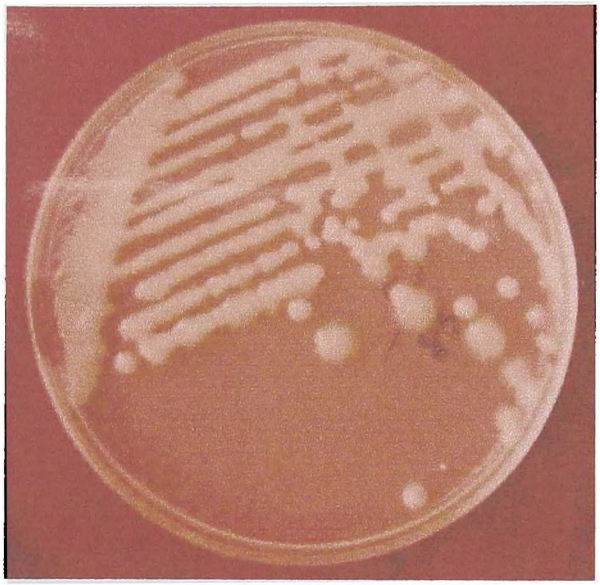


Plate 6 Cultures of *Candida vaccinii* growing on a yeast extract agar plate

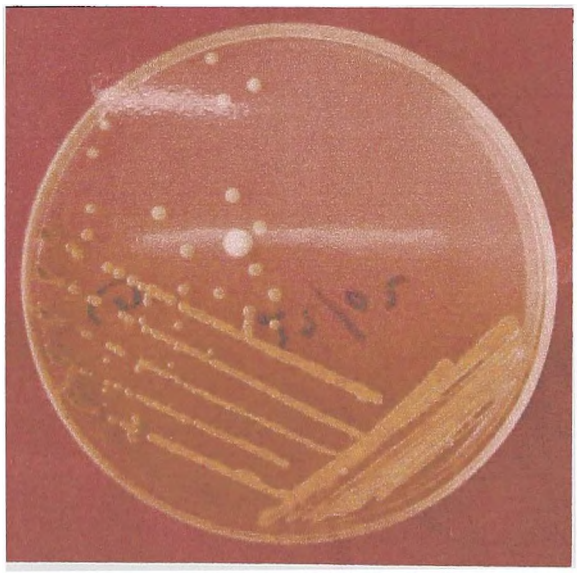


Plate 7 **Pure culture of *Rhodotorula becarum* growing on a yeast extract agar plate**

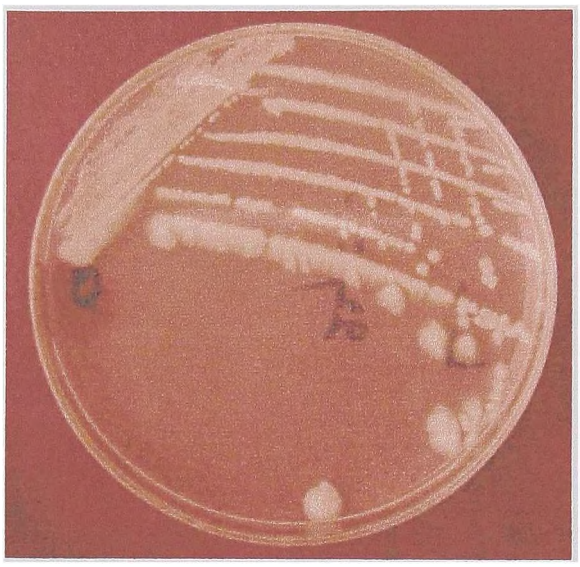
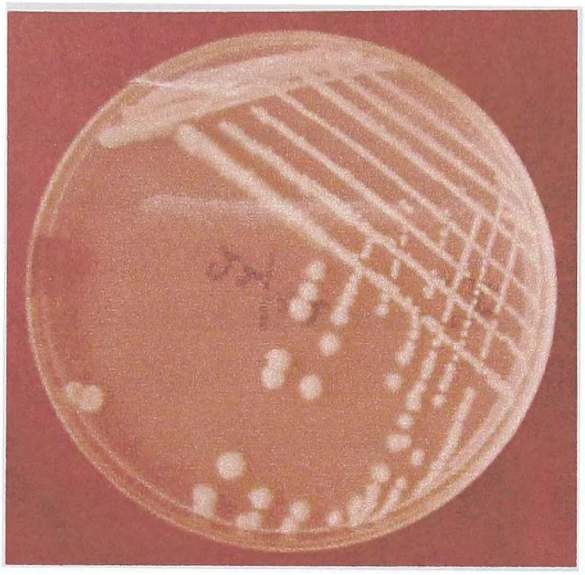


Plate 8 **Pure culture of *Rhodotorula pustula* growing on a yeast extract agar plate**

Plate 9 **Pure culture of *Sporobolomyces roseus* growing on a yeast extract agar plate**



4.4.4 Dynamics of microbial population in glucose supplemented fermentation.

The microbial load of the tuna processing waste was very high (with the raw waste having a higher count than the pre-cooked waste); for Bacteria, Yeasts and facultative anaerobes.

Table 4.14 Microorganisms isolated during the bioconversion of tuna wastes

Aerobic Mesophiles		**Yeasts**
Gram-Positive Bacteria	Gram-Negative Bacteria	
Micrococcus spp. *Planococcus* spp *Coiynebacterium* spp. *Peptococcus* spp *Streptococcus* spp *Staphylococcus* spp. *Bacillus* spp.	*Moraxella* spp. *Branhamella* spp	*Pichia capsulata,* *Candida vaccinii,* *Rhodotorula becarum,* *Rhodotorula pus tula,* *Sporobolomyces roseus.*

During the fermentation period the total counts for Bacteria reduced from log 8.25 cfu/ml to about log 1.85cfu/ml, whilst that of the yeasts fell from log 3.87 cfu/ml to log 0 cfu/ml (i.e. by the 28^{lh} day).

4.4.4.1 Dominant micro flora involved in the fermentation of tuna waste into fish sauce

The numbers and nature of the microorganisms isolated are a reflection of the immediate environment of the sample. This obviously includes the degree of handling / level of pollution. The predominant bacteria isolated during the fermentation period were largely gram positive: *Bacillus,*

Staphylococcus, *Streptococcus*, *Corynebacterium* spp. and to a lesser extent; *Peptococcus, Micrococcus* and *Planococcus* spp. The few gram negative aerobic flora consisted *of Moraxella* and *Branhamella* spp. *Moraxela* spp., as a psychrofilic gram negative rod involved in fish mishandling has earlier been reported by Huss, (1975). He has also indicated that among other bacteria, *Micrococcus* and *Bacillus* spp. are expected particularly for fish coming from warm waters. Lactic acid bactena appear to dominate the bacteria isolated. It has been shown to improve the production and quality of Indonesian fish sauce produced from *Sardinella sp.* (Ijong and Ohta, 1995).

4.4.4.2 Microbial succession in glucose supplemented fermentation

The successions of the microorganisms (log cfu/ml) isolated during the 49-day bioconversion process from the feimented medium containing glucose as added sugar are shown in Fig. 4.14 following. A mixed population was observed, predominated by bactenal species. There were halophilic and non-halophilic microorganisms. As depicted in the figure, the total counts of the species observed generally decreased with fermentation period. Yeasts were not detected from the third week of fermentation and coliforms disappeared after 7 days. No growth was observed in the control sample.

The initial number of yeasts was found to be 3.87 ± 0.72 log cfu/ml (or per gram) on Malt Extract Agar, while Coliforms gave 3.16 ± 0.37 log cfu/g on violet red Bile Agar. The bacteria gave 8.30 ± 0.29 and 8.25 ± 0,66 log cfu/g for Tryptone Soy Agar and Plate Count Agar respectively - which was quite high comparatively. Lactic Acid bacteria had an initial count of 8.04 ± 0.36 log cfu/g. Generally, survival of microorganisms appeared to decrease with time. The exception was the primary rise of bacteria where there was an increase in log cfu/ml in the second week. The cause of

this primary rise in total population at around the 14^{lh} day (as indicated by both PCA & TSA media) is explained by their succession discussed below. The fluctuation in numbers of lactic acid bacteria is characteristic of it in mixed culture fermentation involving a medium containing a complex source of carbohydrate. Yeasts did not survive beyond three weeks while coliforms were totally absent after a week.

4.4.4.2.1 Bacteria

In the study of the bacterial succession using TSA as a growth medium, 162 strains were isolated under aerobic conditions. In the 49-day study period, the genera of bactena isolated include *Streptococci*, *Micrococci, Planococci, Bacillus, Moraxella, Lactobacilli* and then some untallied strains. Out of these, *Branhamella, Moraxella* and 4 other untallied strains were gram negative, the rest were all gram positive bacteria. The predominant genera were *Bacillus sp.. Staphylococci*, *Streptococci* and then *Corynebacterium.* The less predominant ones are indicated in the Table 4.15 below. Among the dominant bacterial species: *Streptococcus* spp., *Corynebacterium* spp. *and Staphylococcus* spp. were predominant during the early stages of the fermentation. *Bacillus* spp. however took over during the latter part. Also since the sample contained at least 10 % sodium chloride, the bacteria isolated must have been salt tolerant.

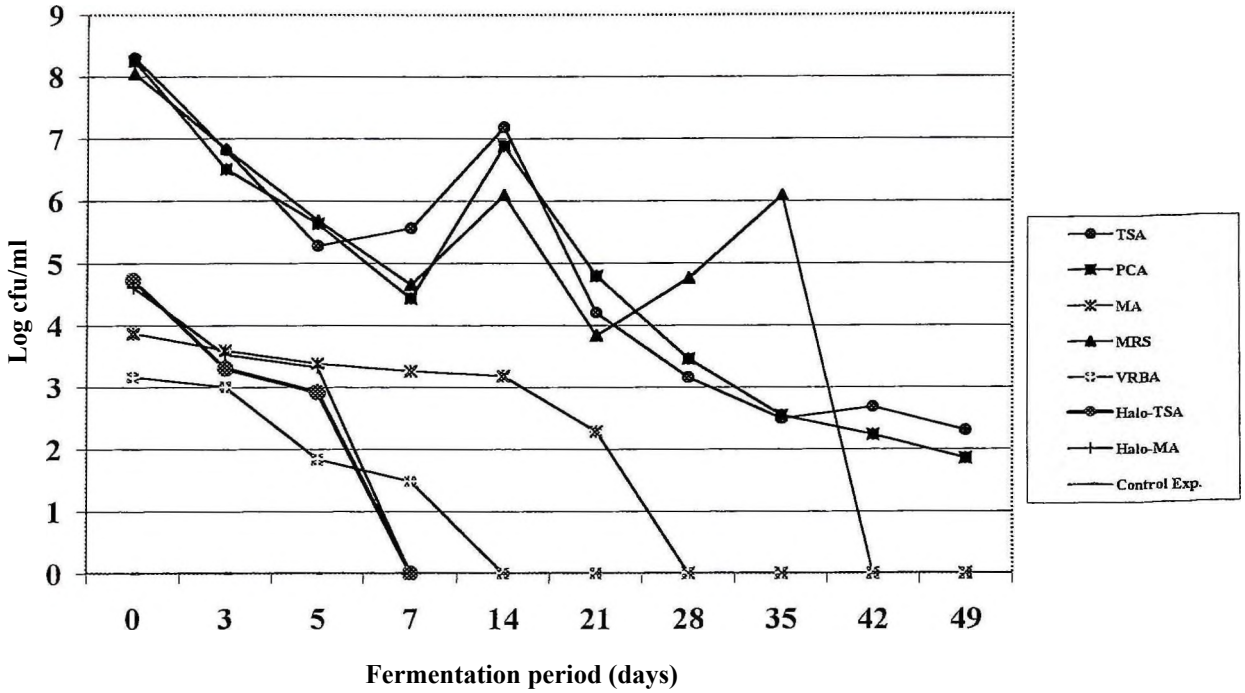


Fig. 4.14 Total microflora counts (Log cfu/ml) of the fermenting extract.

Table 4.15 Composition (% total colonies counted) of bacterial spp. in the sauce-like extracts collected during the fermentation

% Total	Bacterium.	% Composition of species on the nth day of fermentation									
		0	3	5	7	14	21	28	35	42	49
19	*Streptococcus* spp.	70	50	18	31		-	-	-	-	-
13	*Corynebacterium* spp.	12	-	6	44	"	-	67	6	-	-
1.2	*Branhamella* spp	12	-	-	-	-	-	-	-	-	-
0.6	*Peptococcus* spp.	-	5	-	-	-	-	-	-	-	-
28	*Staphylococcus* spp.	-	25	59	25	67	69	13	22	"	-
1.2	*Micrococcus* spp.	-	-	6	-	-	-	-	-	7	*
1.2	*Planococcus* spp.	-	-	-	-	13	-	-	-	-	-
30	*Bacillus* spp.	-	-	-	-	-	31	20	72	93	94
4	*Moraxella* spp.	6	15	12	-	-	-	-	-	-	~
2	Un-tallied	-	5	-	-	20	-	-	■	~	6

Presented in Table 4.16 is an illustration of the yeasts isolated during the fermentation process *Candida* spp. and *Pichia* spp. were apparently predominant, however the absence of *Candida vaccinii* on the 3rd and 7th days probably indicates a period of dormancy. This same reason could equally apply to *Rhodotorula becarum,* which after first appearance did not survive two weeks. *Candida vaccinii* was the only surviving yeasts on the 21st day of bioconversion (2.28 Log cfu/ml ~ 10^2 cfu/ml).

The other less dominant yeasts was *Sporobolomyces roseus* and *Rhodotorula* spp. These yeasts must have been responsible for the fruity, musty taint observed during the first month of fermentation. This assertion is in reference to Lee *et al.,* (1989) who also reported of a similar observation for unripe fermenting fish in barrels.

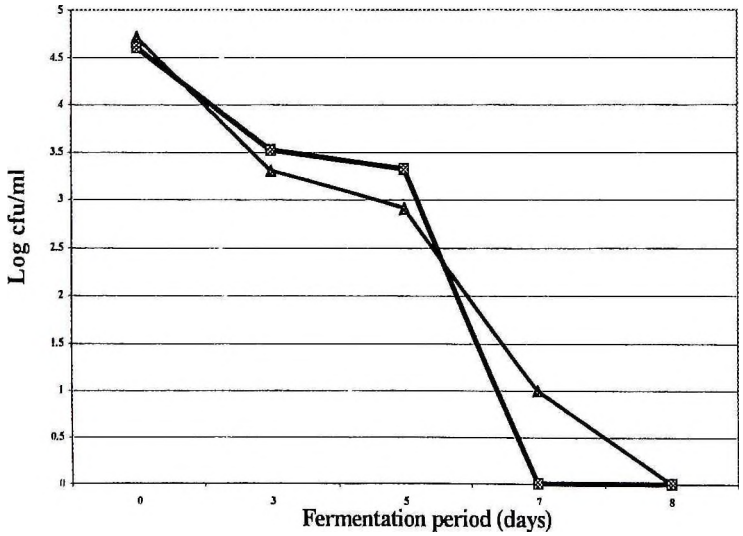
Table 4.16 Composition of yeast species in the sauce-like extract collected during fermentation

Yeasts	% Composition of species on the n"[1] day						
	0	3	5	7	14	**21**	**28**
Candida vaccinii	36.8	-	13.3	-	40	100	-
Sporobolomyces roseus	5.3	-	-	-	-	-	-
Pichia capsulata	42.1	100	83.7	93.3	60	-	-
Rhodotorula pustida	15.8	-	-	-	-	-	-
Rhodotorula becarum	-	-	-	6.7		-	-

4.4.4.2.2 Halotrophic microorganisms

The illustration on Fig. 4.15 represents the pattern of growth for a group of halotrophic microorganisms isolated from engineered microbial media. None of the organisms was isolated after the 8lh day. These decreasing trends could probably be due to the fact that majority of halotrophic organisms thrive best under anaerobic conditions. These halotrophic organisms, a lot of which are known to belong to the *Halobacterium species,* and have also been shown to dominate other bacteria in the first three weeks of traditional fish sauce fermentation, did not survive long because the fermenting medium was not kept anaerobic. A further examination of these cultures revealed that they form part of the major bacterial population whose counts fell to minimum numbers in the liquid extract eight (8) days after fermentation.

Fig. 4.15 Halotrophic microorganisms in the glucose supplemented fermentation.



This is indicated by the bacterial counts for PCA & TSA in Fig. 4.14. Again, a comparison of the results illustrated in Fig. 4.14 and Fig. 4.15 suggests that the presence of these strictly halotrophic bacteria seem to inhibit the growth in numbers of other bacteria. This is so because there was a secondary rise in log cfu/ml of bacteria on the 14th day; just after the strictly halotrophic bacteria were totally out of the system (the fermenting medium).

4.4.5 Microbial degradation of starch and protein

In Table 4.17 the starch and protein hydrolyzing ability of bacterial isolates showed that *Bacillus* spp. and *Staphylococcus* spp. demonstrated appreciable protein hydrolyzing ability. Some of the other species also showed starch-utilizing abilities. The observations suggest that the *Bacillus* spp. and *Staphylococcus* spp. would play significant roles in the bioconversion of tuna waste into fish sauce.

Table 4,17 Protein and starch hydrolyzing ability of bacterial isolates on fermenting substrate.

Bacterium	Proportion (%)of isolates of each bacterial specie capable of hydrolysis	
	Casein Hydrolysis	**Starch Hydrolysis**
Streptococcus spp.	0	100
Corynebacterium spp.	0	95
Branhcimella spp.	0	*
Peptococcus spp.	0	0
Staphylococcus spp.	33	7
Micrococcus spp.	0	50
Planococcus spp.	0	0
Bacillus spp.	40	98
Moraxella spp.	0	0

***No growth was detected.**

4.4.6. The role of dominant bacteria and yeasts associated with the fermentation of fish sauce

Micrococcus spp. primarily inhabits the skin of mammals and has secondary habitat in meat and dairy products, soil and water. They are close to *Staphylococcus* spp., which are also known to occur in natural populations associated with skin mucous membranes of warm-blooded animals. Some of these species are mainly respiratory or fermentative, and could make use of a variety of carbon and energy sources aerobically to produce acid (Kloos and Schleifer, 1986). In these organisms the main product of glucose fermentation is lactic acid, and in the presence of air the main product is acetic acid and carbon IV oxide. Both products play important roles in streamlining the bioconversion process. *Planococcus* spp. is known to be distributed in seawater, marine clams, shrimps and prawns. *Planococcus* spp., according to Kocur (1986), shows specific relationship to the genus *Bacillus* spp. The occurrence of Bacillus spp. is not necessarily related to the natural habitat because of passive distribution and persistence of spores. *Bacillus* spp. is known to occur in most foods and dominates in processes like fermentation (Amoa-Awuah *et al.,* 1995; Odunfa, 1981). The genus is particularly recognized for its role in lactic acid fermentation where it reduces pyruvate to lactate (Prescott *et al.,* 1993). *Corynebacterium* spp. is also known to be involved in fermentation of sugars and has been identified in some African fermented foods. *Bacillus, Lactococcus, Micrococcus* and *Staphylococcus* spp. have also been confirmed as indigenous micro flora in fish, fish sauce. As fermentation progresses, their populations and number of spp. decline with the changing brine environment (Doyle *et al.,* 1997).

Pichia capsulata was among the yeasts isolated. Studies on isolates of this yeast obtained mainly from coniferous trees, soil or humus, lichen and cattle dung have shown that the yeast produces

copious amounts of polysaccharides by synthesising mannans and phosphomannnans derived from their environment (Kurtzman and Fell, 1998). Their ability to meet this nutritional requirement could probably account for their relatively long survival compared to the other yeasts. *Rhodotorula becarum* and *Rhodotorula pusiula* are also known to have been isolated from dolphin (mammal) skin and Black arrants. The species was distinguished as occasionally having a primitive pseudomycelium, producing carotenoid pigments and lacking the ability to ferment (Kurtzman and Fell, 1998). This could explain the short survival of most of the yeast species during the fermentation penod. *Sporobolomyces roseus* is known to have been isolated among others mycotic lesion of mucous membranes. The species according to Burg (1974) belongs to the so-called "sugar" fungi, which use honey-dew for growth. *Candida* spp. appears to be the only yeast presently known to impact positively on the fermentation process. Various species of yeasts (*Candida* inclusive) can be grown on waste materials, thus recycling these substances into useful sources of food (Atlas, 1997). Yeasts on the whole however play little role or no role in traditional fish sauce fermentation (Thongthai and Siriwongpairat, 1990), and the short life span of yeasts in the fermentation of fish sauce has also been demonstrated by Lee (1990).

4.5 CONCLUSIONS AND RECOMMENDATION

4.5.1 CONCLUSIONS

Both precooked and raw tuna wastes obtained from fish processing factory had 80 - 94 % soft tissues and were considered to be suitable for the production of fish sauce. The tuna waste had 67 - 77 % moisture, 4 - 9 % total ash, 2 % fat, 21-23 % protein and less than 1 % carbohydrate. To be able to produce fish sauce it was necessary to add a carbon source in the form of simple sugars, which could support growth of some of the resident microflora responsible for hydrolyzing the fish tissue. Carbohydrate sources, which were used successfully to produce fish, sauce were Glucose, Sucrose, Malt, Pineapple, Mango, Banana, Plantain, the most successful being Glucose and Sucrose

During the bioconversion process moisture and pH dropped from 83 to 73 % and 5.6 to 4.5 respectively whilst titrable acidity increased from 0.96 to 2.5 %. The total protein and free amino nitrogen of the sauce respectively increased from 11 to 18 % and from 5.02 to 36.6 mg / ml. Similarly total lipid and volume of sauce produced per kilogram increased tremendously from 2 to 40 % and 128 to 295 ml respectively.

The resident microflora in the tuna waste was found to be responsible for the hydrolysis of fish tissue during the bioconversion process. Genera of microorganisms, which were isolated in high numbers and increased during the fermentation, were *Bacillus, Staphylococcus, Streptococcus* and *Corynebacterium.* Of these *Bacillus* and *Staphylococcus* were found to possess proteolytic activity and considered to be the dominant microflora, which were responsible for the production of the fish

sauce through hydrolysis of the fish protein. The solubilization or hydrolysis of the fish during bioconversion was confirmed by SDS - PAGE profile of the fish protein, which showed increases in the smaller molecular weight protein in the extract. The fish sauce produced was found to be safe since it contained no pathogens and low levels of heavy metals and histamine, which according to the US FDA standards are far below the maximum permissible levels indicated earlier in the literature and discussion.

4.5.2 RECOMMENDATION

The following recommendations are made for future research.

The study could not cover sensory analysis and hence it is recommended that further studies should attempt to do a sensory analysis of the fish sauce using a standard product (e.g. Soy Sauce or Maggi Sauce) as control.

Also, the identification of the isolated microorganisms using molecular techniques (genotyping) would be relevant in the possible development of a starter culture for the process of fish sauce production using tuna wastes.

It would also be relevant if future studies particularly identify histamine forming microorganisms as well as the biogenesis of histamine during the fermentation of tuna fish into fish sauce.

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APPENDICES

APPENDIX A

Table A1

Source of] Sum of	d.f.	Mean square	F-ratio	Sig. level

variation	Squares				
Main Effects	.6203076	4	.1550769	1.007	.4145
SIN a. replicates	.0013335	1	.0013335	.009	.9273
SIN a samples	.5716605	2	.2858303	1.856	.1686
SIN a. days	.0473135	1	.0473135	.307	.5882
Residual	6.6221804	43	.1540042		
Total (Corr.)	7.2424880	47			

0 missing values have been excluded.

Table A2 Analysis of Variance for SIN b waste propotion

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	26313.880	5	5262.776	90.894	.0000
SIN b. replicates	3.245	2	1.622	.028	.9724
SIN b samples	.005	1	.005	.000	.9928
SIN b.days	26310.630	2	13155.315	227.208	.0000
Residual	694.79960	12	57.899967		
Total (Corr.)	27008.679	17			

0 missing values have been excluded

Table A2b Multiple range analysis for SIN b. Propotion by SIN b co

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	6	1.400000	*
1	6	11.550000	*
2	6	87.100000	*

Table A3 Analysis of Variance for SIN c brix

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	331.27000	6	55.211667	8.058	1.0000
SIN c replicates	39.80233	2	19.901167	2.905	1.0000
SIN c.samples	291.46767	4	72.866917	10.635	1.0000
SIN c days	.000000	0	1.000000	.146	1.0000
Residual	54.814333	8	6.8517917		
Total (Corr.)	386.08433	14			

0 missing values have been excluded.

Table A3b Multiple range analysis for SIN c. brix by SIN c .sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	3	7.400000	*
5	3	12.500000	**

3	3	16.916667	**
4	3	18.050000	**
1	3	19.500000	*

Table A3c Multiple range analysis for SIN c . brix by SIN c . da

Method Level	95 Percent Count	Confidence Average	Intervals Homogenous Groups
0	15	14.873333	*

Table A3d Multiple range analysis for SIN c . brix by SIN c.re

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	5	12.870000	*
1	5	14.890000	*
3	5	16.860000	*

Table A4 Analysis of Variance for SIN d yield

Source of vanation	Sum of Squares	Af.	Mean square	F-ratio	Sig. level
Main Effects	93273.643	9	10363.738	77.991	.0000
SIN d. replicates	7.714	1	7.714	.058	.8137
SIN d, samples	6569.440	2	3284.720	24.719	.0000
SIN d. days	86696.488	6	14449.415	108.738	.0000
Residual	4252.2619	32	132.88318		
Total (Corr.)	97525.905	41			

0 missing values have been excluded.

Table A4b Multiple range analysis for SIN d .yield by SIN d .sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	14	172.75000	*
1	14	195.50000	*
2	14	201.89286	*

Table A4c Multiple range analysis for SIN d. yield by SIN d. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	6	137.33333	*
2	6	148.33333	**

3	6	161.91667	**
4	6	174.25000	*
5	6	200.83333	*
6	6	236.00000	*
7	6	271.66667	*

Table AS Analysis of Variance for SIN e. moisture

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Mam Effects	1 1656E0011	10	1.1656E0010	1.000	.4613
SIN e. replicates	1 1656E0010	1	1.1656E0010	1.000	.3344
SIN e. samples	2.3306E0010	2	1.1653E0010	1.000	.3777
SIN e. days	8.15 96E0010	7	1.1657E0010	1.000	.4468
Residual	4.3127E0011	37	1.1656E0010		
Total (Corr.)	5.4783E0011	47			

0 missing values have been excluded.

Table A5b Multiple range analysis for SIN e moisure by SIN e. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	16	74.210	*
3	16	81.160	*
1	16	46821.476	*

Table A5c Multiple range analysis for SIN e moisture by SIN e. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	6	67.79	*
8	6	75.28	*
7	6	76.05	*
5	6	76.68	*
6	6	77.04	*
4	6	77.22	*
2	6	78.33	*
3	6	124743.17	*

Table A6 Analysis of Variance for SIN f NaCl

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	296.48316	10	29.648316	18.131	.0000
SIN f. replicates	.00000	1	.000001	.000	.9996

SIN f. samples	51.06622	2	25.5331 11	15.614	.0000
SIN f. days	245.41694	7	35.059562	21.440	.0000
Residual	60.504200	37	1.6352487		
Total (Corr.)	356.98736	47			

Omissing values have been excluded.

Table A6b Multiple range analysis for SIN f .NaCl by SIN f. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	1*6	11.508919	*
1	16	13.200819	*
3	16	13.979850	*

Table A6c Multiple range analysis for SIN f.NaCl by SIN f. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	6	10.151833	*
2	6	10.456783	*
3	6	10.788617	*
4	6	12.136367	**
5	6	13.148750	*
6	6	14.003367	**
7	6	15.742367	**
8	6	16.474150	*

Table A7 Analysis of Variance for SIN g protein

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	140.40020	10	14.040020	3.406	.0031
SIN g.replicates	.08242	1	082419	.020	.8898
SIN g.samples	61.67091	2	30.835454	7.480	.0019
SIN g.days	78.64687	7	11.235267	2.726	.0219
Residual	152.52253	37	4.1222306		
Total (Corr.)	292.9227	47			

0 missing values have been excluded.

Table A7b Multiple range analysis for SIN g protein by SIN g .sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	16	10.874125	*
2	16	12.976125	*
1	16	13.496063	*

Table A7c Multiple range analysis for SIN g .protein by SIN g .da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	6	11.228500	*
2	6	11.250667	*
3	6	11.451000	**
4	6	11.821667	**
5	6	11.967833	**
6	6	12.917167	**
7	6	14.232667	**
8	6	14.720667	*

Table A8 Analysis of Variance for SIN h FAN

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	2269.3689	8	283.67111	4.974	.0007
SIN h. replicates	.0000	1	.00000	.000	.9999
SIN h. samples	584.7413	2	292.37067	5.126	.0130
Soyiria.days	1684.6275	5	336.92551	5.907	.0008
Residual	1539.9673	27	57.035827		
Total (Corr.)	3809.3362	35			

0 missing values have been excluded.

Table A8b Multiple range analysis for SIN h FAN by SIN h. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	12	6.314400	*
2	12	9.870017	**
1	12	16.067850	*

Table A8c Multiple range analysis for SIN h. FAN by SIN h. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	6	5.416917	*
3	6	5.510133	*
1	6	6.343567	*
4	6	6.417850	*
5	6	20.149300	*
6	6	20.666767	*

Table A9 Analysis of Variance for SIN i. Titrable acidity

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	7.7877237	10	.7787724	10.755	.0000
SIN i. Replicates	.0000097	1	.0000097	.000	.9909
SIN i. Samples	1.1337412	2	.5668706	7.828	.0015
SIN i. Days	6.6539728	7	.9505675	13.127	.0000
Residual	2.6792666	37	.0724126		
Total (Corr.)	10.466990	47			

0 missing values have been excluded.

Table A9b Multiple range analysis for SIN i. Titrable acidity by SIN i. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
2	16	1.1238750	*
1	16	1.3523688	**
3	16	1.4972187	*

Table A9c Multiple range analysis for SIN i .Titrable acidity by SIN i. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	6	.8886833	*
2	6	.9708167	*
4	6	1.0597000	*
1	6	1.1834833	**
5	6	1.2153833	**
6	6	1.5392167	**
7	6	1.7075667	**
8	6	2.0310500	*

Table A10 Analysis of Variance for SIN j Log-microbe

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	199.58799	7	28.512570	64.246	.0000
SIN j. replicates	1.32643	2	.663213	1.494	.2463
SIN j. samples	20.33633	1	20.336333	45.823	.0000
SIN j. days	177.92523	4	44.481308	100.228	.0000
Residual	9.7636733	22	.4438033		
Total (Corr)	209.35167	29			

0 missing values have been excluded.

Table AlOb Multiple range analysis for SIN j. Log-microbe by SIN j, sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	16	5.4700000	*
2	16	7.1166667	*

Table AlOc Multiple range analysis for SIN j. Log-microbe by SIN j. me

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
5	6	3.1700000	*
4	6	3.4900000	♦
3	6	7.8200000	*
1	6	8.3716667	*
2	6	8.6150000	*

Table All Analysis of Variance for SIN k. Log-microbe

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	111.01138	10	11.101138	4.973	.0001
SIN k. replicates	13311	1	.133106	.060	.8110
SIN k. samples	62.62774	2	31.313871	14.029	.0000
SIN k. days	39.2221 1	7	5.603158	2.510	.0323
Residual	82.586506	37	2.2320677		
Total (Corr.)	193.59788	47			

0 missmg values have been excluded.

Table Allb Multiple range analysis for SIN k. Log-microbe by SIN k. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
3	17	5.7923529	*
1	16	8.2593750	*
2	15	8.4313333	*

Table Allc Multiple range analysis for SIN k. Log-microbe by SIN k. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
8	6	5.4533333	*
3	6	6.8216667	**
5	6	7.0450000	**
4	6	7.0916667	**
2	6	7.7700000	**
1	6	7.9816667	*
7	6	8.6600000	*
6	6	8.6916667	*

Table A12 Analysis of Variance for SIN 1. Log-microbe

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	199.56445	8	22.173828	4.934	.0011
SIN 1. replicates	00011	1	.000113	.000	.9961
SIN 1. samples	81.28125	1	81.281250	18.086	.0003
SIN 1. days	118.28309	7	16.897584	3.760	.0079
Residual	98.868838	22	4.4940381		
Total (Corr.)	298.43329	31			

0 missing values have been excluded.

Table A12b Multiple range analysis for SIN 1. Log-microbe by SIN 1. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	16	5.2293750	*
2	16	8.4168750	*

Table A12c Multiple range analysis for SIN I. Log-microbe by SIN 1. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
8	4	4.0600000	*
7	4	4.4825000	**
5	4	5.6900000	***
6	4	6.5575000	***
4	4	6.6225000	***
3	4	8.8450000	**
2	4	9.0700000	*
1	4	9.2575000	*

Table A13 Analysis of Variance for SIN m. Log-microbe

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	100.9645	9	11.218828	2.449	.0418
SIN m. replicates	.00053	1	.000528	.00	.9916
SIN m. samples	42.57338	1	42.573378	9.295	.0058
SIN m. days	58.39555	7	8.342221	1.821	.1334
Residual	100.76312	22	4.5801418		
Total (COIT.)	201.73257	31			

0 missing values have been excluded.

Table A13b Multiple range analysis for SIN m. Log-microbe by SIN m. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
1	16	5.9525000	*
2	16	8.2593750	*

Table A13c Multiple range analysis for SIN m. Log-microbe by SIN m. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
8	4	4.3900000	*
7	4	6.4075000	**
5	4	6.4900000	s|es|e
6	4	7.2200000	**
4	4	7.2575000	**
3	4	7,7900000	**
2	4	7.9025000	**
1	4	9.3900000	*

Table A14 Analysis of Variance for SIN n.yield

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	178.26315	6	29.710525	1000.000	1.0000
SIN n. replicates	.04941	1	.049408	3.615	1.0000
SIN n. samples	178.21374	5	35.642748	100.000	1.0000
SIN n. days	.00000	0	1.000000	73.162	1.0000
Residual	0683417	5	.0136683		
Total (Corr.)	178.33149	11			

0 missing values have been excluded.

Table A14b Multiple range analysis for SIN n. yield by SIN n. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
4	2	28.990000	*
2	2	30.960000	*
6	2	31.705000	*
5	2	34.590000	*
1	2	35.755000	*
3	2	40.805000	*

Table A14c Multiple range analysis for SIN n. yield by SIN n. ti

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
49	12	33.800833	*

Table A15 Analysis of Variance for SIN o NaCl

Source of variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
Main Effects	8.3023343	6	1.3837224	100.000	1.0000
SIN o. replicates	.0000403	1	.0000403	.340	1.0000
SIN o .samples	8.3022940	5	1.6604588	1000.000	1.0000
SIN o .days	.0000000	0	1.0000000	1000.000	1.0000
Residual	5.93667E0-004	5	1.18733E-004		
Total (Corr.)	8.3029280	11			

0 missing values have been excluded.

Table A15b Multiple range analysis for SIN o, TBA by SIN o. sa

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
5	2	.8255000	*
3	2	1.1015000	*
6	2	1.157500	*
4	2	1.1870000	*
2	2	1.3295000	*
1	2	3.3150000	*

Table A15c Multiple range analysis for SIN o. TBA by SIN o. da

Method level	95 Percent Count	Confidence Average	Intervals Homogeneous Groups
49	12	1.4860000	*

Table A16 Anal[1] ysis of Variance for SIN p. histamine

Source of variation	sum of squares	d. f	JVIean square	F-ratio	Sig. Level
MAIN EFFECTS	450.61213	6	75.102021	252.212	1.0000
SIN p. replicate	1.58573	1	1.585732	5.325	1.0000
SIN p. sample	449.02639	5	89.805279	301.589	1.0000
SIN p. day	.00000	0	1.000000	3.358	1.0000
RESIDUAL	1.4888670	5	.2977734		
TOTAL (CORR).	452.10099	11			

0 missing values have been excluded.

Table A 16b Multi ale range analysis for SIN p. histamine by SIN p. sa

Method: Level	95 percent count	Confidence Average	Internals Homogeneous Groups
1	2	13.895000	*
6	2	20.379050	*
2	2	21.073150	*
3	2	23.176500	*
5	2	27.919900	*
4	2	33.360300	*

Table A16c Multiple range analysis for SIN p. histamine by SIN p. da

Method: Level	95 Percent Count	Confidence Average	Internals Homogeneous Groups
49	12	23.300650	*

Source of variation	sum of squares	d. f.	Mean square	F-ratio	Sig. Level
MAIN EFFECTS	75.188450	6	12.531408	117.121	1.0000
SIN q. replicate	1.026675	1	1.026675	9.596	1.0000
SIN q. sample	74.161775	5	14.832355	138.627	1.0000
SIN q. day	.000000	0	1.000000	9.346	1.0000
RESIDUAL	.5349750	5	.1069950		
TOTAL (CORR)	75.723425	11			

0 missing values have been excluded.

Table A17b Multiple range analysis for SIN q. FFA . FFA by SIN q. sa

Method: Level	95 percent count	Confidence Average	Internals Homogeneous Groups
4	2	34.500000	*
6	2	35.620000	*
2	2	37.280000	*
5	2	38.520000	*
1	2	38.590000	*
3	2	42.275000	*

Table A17c Multiple range analysis for SIN q. FFA by SIN q. da

Method: Level	95 Percent Count	Confidence Average	Internals Homogeneous Groups
49	12	37.797500	*

Table A18 Analysis of Variance for SIN r Log-microbe

Source of variation	sum of squares	d.f.	Mean square	F-ratio	Sig. Level
MAIN EFFECTS	879.69010	17	51.746476	42.991	.0000
SIN r. Replicate	.00827	1	.008266	.007	.9350
SIN r. Sample	538.08895	7	76.869851	63.864	.0000
SIN r. day	341.59288	9	37.954664	31.533	.0000
RESIDUAL	170.91876	142	1.2036532		
TOTAL (corr).	1050.6089	159			

0 missing values have been excluded

Table A18b Multiple range analysis for SIN r. Log-micrbe by SIN r. sa

Method: Level	95 percent Count	Confidence Average	Internals Homogeneous Groups
8	20	.0000000	*
5	20	.9575000	**
7	20	1.1100000	**
6	20	1.1690000	**
3	20	1,9555000	*
4	20	4.6010000	*
2	20	4.6555000	*
1	20	4.7990000	*

Table A18c **Multiple range analysis for** SIN **r. Log-microbe by** SIN **r. da**

Method: Level	95 percent count	Confidence Average	Internals Homogeneous Groups
10	16	.5137500	*
9	16	.1656250	
8	16	1.3925000	**
7	16	1.4231250	**
6	16	1.8818750	**
4	16	2.4293750	**
5	16	2.9206250	**
3	16	3.5168750	**
2	16	4.2200000	**
1	16	5.1456250	3je

Table A19 Anal[1] psis of Variance for SIN **s pH**

Source of variation	sum of squares	d. f.	Mean square	F-ratio	Sig. Level
MAIN EFFECTS	7.4790211	10	.749021	1.747	.1064
SIN **s.** replicate	.0009161	1	0009160	.002	.9638
SIN s. sample	6.59433848	2	3.291924	7.702	.0016
SIN s. day	.8837202	7	.1262457	.295	.9516
RESIDUAL	15.838956	37	.44280799		
TOTAL (corr).	23.317977	47			

0 missing values have been excluded

Table A19b Multiple range analysis for SIN s .pH by histamine by SIN s. sa

Method: Level	95 Percent Count	Confidence Average	Internals Homogeneous Groups
2	16	5.254030375	*
1	16	5.2855938	*
3	16	6,0556125	*

APPENDIX B

Table Bl. MICROBIOLOGICAL MEDIA USED

Abbreviation	Name of Medium	Batch #	Manufacturer	Preparation	Supplements		pH
					Additive	Quantity	
SPS	Saline Peptone Solution		MERCK	Ig Peptone; 8.5g Salt/LH_20			7.2 ± 0.2
PCA	Plate Count Agar	247940	DIFCO	23.5g/LH_20	Bacto Agar (BA)	2.5g	7.0 ±0.2
MA	Malt Agar	211220	DIFCO	33.6g/LH_20	BA; Chloramphenicol	2.5g/100 ppm	4.7 ±0.2
MRS	Lactobacilli MRS Agar	288210	DIFCO	70g *fL* H_20	BA	2.5g	6.5 ±0.2
TSA	Tryptone Soy Agar	CM131	OXOID	40g *fL* H_20	BA	2.5	7.3 ± 0.2
VRBA	Violet Red Bile Agar	CM 107	OXOID	38.5g *fL* H_20	BA	55g	7.4 ±0.2
VRBG	Violet Red Bile Glucose Agar	CM485	OXOID	38.5g *fL* H_20	BA	55g	7.4 ±0.2
BGA	Brilliant Green Agar	048397	LAB M	52g *fL* H_20	BA	55g	6.9 ±0.2
XLD	Xylose Lysine Decarboxylase	048395	LAB M	53 .5g *IL* H_20	BA	4.5g	7.4 ±0.2
BCAB	Bacillus Cereus Agar Base	CM617	OXOID	20.5g/475ml H_2O	SR99; SR47	2ml; 25ml	7.2 ±0.2
BPM	Baird-Parker Medium	CM275	OXOID	63 g /L H_20	SR54	50ml	6.8 ±0.2
PAB	Perfringens Agar Base	CM587	OXOID	23 g/500ml H_20	SR47; SR93	25ml; lvial	7.6 ±0.2

NOTE: Table continued on the next page with same headings a above

Abbreviation	Name of Medium	Batch #	Manufacturer	Preparation	Additive	Quantity	pH
RV	Rappaport-Vassiliadis	CM669	OXOID	30g/LH_20			5.2 ± 0.2
BPW	Buffered Peptone Water	CM509	OXOID	20g/L H_20			7.2 ± 0.2
ASPW	Alkaline Saline Peptone Water				NaCl; Peptone	30g; 20g	
EC-B	Escherichia coli Broth	CM853	OXOID	37g/LH_20			6.9 ± 0.2
TCBS	Thiosulphate Citrate Bile Sucrose Agar	36446JA	DIFCO	89g /L H_20			8.6 ± 0.2
MEB	Malt Extract Broth	211320	DIFCO	15g/LH_20			4.7 ± 0.2
MRSB	:deMan Rogosa Sharp Broth	CM359	OXOID	52g /L H_20			6.2 + 0.2
NB	Nutrient Broth	123820JB	DIFCO	8g /L H_20			6.8 ± 0.2
NA	Nutrient Agar	G3DKUEXG	DIFCO	23 g /L H_20	BA	2-5g	6.8 ± 0.2
	Bacto Agar						
	Agar	Q21767/184	LAB M				
	Bacteriological Peptone	218560/L37	OXOID	@1% of solution			6.3
SB	Slanetz & Bartley	CM377	OXOID	42g /L H_20	BA	7.5g	7.2 ± 0.2
SM	Skim Milk (Dehydrated)	100624JA	DIFCO	10%in Medium (lOOg /L H_20)	NA; **BA**	23 g; 1.75g	6.6 ± 0.2

Table B2. GROWTH PROMOTION TESTS

Medium	Control Medium	Test Medium
Batch Number		
Date Media was Prepared		
Date Media was Used.		
Test Microorganism used.		
Distribution Factors (eg 1CT^{6}, 10$^{-/}$, 10'^{8})		
Count on Test Medium	XXXX.XXXXXXXXXXXXXX.X	
Count on Control Medium		XXXXXXXXXXXXXXXXXXXX
Growth Promotion Ratio (PR)		
Microscopy		
Remarks		
Initials		
Acceptance Criteria of PR PR: 0.7 < PR < 1 . 3		

Table B3. MORPHOLOGICAL AND BIOCHEMICAL PROPERTIES OF ISOLATES FROM MEDIA

EXAMINATION	COLONIAL ISOLATES FROM PLATE																														
	G	r-i o	<*! O	0	Ю ©	VO O	r- ©	00 ©	o\ ©	©				π-	I/)	VO		00		©		N	Tt	W r<\	VO	c*	oo	G*\ rs	©	
Shape																															
Width																															
Pigment																															
Configuration																															
Margins																															
Elevations																															
Acid Fast																															
Spores																															
Motility																															
Cocci																															
Rods																															
Growth in Air																															
Anaerobic Growth																															
Catalase Test																															
Oxidase Test																															
Glucose (Acid)																															
O / F Test																															
Casein Hydrolysis																															
Starch Hydrolysis																															
Gram Reaction																															

Table B4. EXPERIMENTAL RESULT FORM FOR GEL ELECTROPHORESIS

Date----------

Batch----------

Experiment No.-

Operator----------

ExcelGel™ SDS, gradient 8-18

Sample No	Sample Description	Cone. (mg/ml)	Volume CMD	Notes
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

Running conditions			
Time	Voltage	Current	Power
Start			

Cooling temperature °C

Pharmacia Biotech

APPENDIX C

Cl CONVENTIONAL SDS GEL ELECTROPHORESIS

1- Introduction

The method is used for conventional gel electrophoresis for separation of larger protem.

Gloves were worn during, the entire procedure (see instruction 4.1 for further details). Solution marked with "WASTE" was collected and senc for separate destruction.

2. Solutions:

The solution were prepared accordingly using manufactures protocol as follows:

2.1 Tris buffer (Tris/HCI 10 mM, EDTA 1.0 mM, pH = 8.0)

Tris/HCl (Sigma -7149).....1.576g
EDTA (Sigma E-5134)..... 0.372g
Milli-Q water, add..... 1000ml

Tris / HC1 and EDTA was dissolved in 800 ml milli-Q water, pH was adjusted to 8.0 and milli-Q water was then added to 1000 ml.

2.2 Bromophenol blue solution (2% w/v)

Bromophenol blue (Merck 8122).....0.020 g
Milli-Q water, add.....1 0ml

2.3 SDS (sodium dodecyl sulphate) solution (10 % w/v)

SDS (Pharmacia Biotech 7-13 13-0)..... 2.00g
Milli-Q water, add.....20 ml

2.4 Glycerol solution (5 % w/v)

Glycerol (Merck 4092).....0.25 g
Milli-Q water, add.....5.0nil

2.5 DTT (Dithiothreitol) solution (10 mM)

DTI (Sigma D-9779).....0.154 g
Milli-Q water, add.....10 ml

2.6 Sample buffer

Tris buffer.....10.0ml
Bromophenol blue solution.....1.0 ml
SDS solution.....20.0 ml
Glycerol solution.....5.0 ml
DTT solution..... 1.0ml
Milli-Q water, add..... 100ml

The sample buffer can be stored at -80°C.

3. Procedure:

The samples were be kept oil ice during the entire procedure.

Marker preparation

3.1.1 Low Molecular Weight standard, LMW pharmacia Biotech AB, 1**7-0464-0**1**)**

Dissolve one ampoule of the marker with 1.00 ml of sterile milli-Q water (the marker solution can be kept at -80^C for 1 year)

Add per 10 ~ of marker solution 90 ~ sample buffer

Boil the marker for 5 mm.

Centrifuge the marker at 5,000 x g for 2 min (the supernatant should be loaded on the gel).

3.1.2 High Molecular Weight standard, BMW (pharmacia Biotech AB, 17-0615-01)

Dissolve one ampoule of the marker with 1.00 ml of sterile mili-Q water (the marker solution can be kept at - 80°C for 1 year)

Add per 20 pi of marker solution 80 pi sample buffer

Centrifuge the marker at 5,000 x g for 2 mm (the supernatant should be loaded on the gel).

3.1.3 Broad range SDS Page standard (BioRad 161-0318)

Dilute 10 pi with 90 pi Tris buffer (the marker solution can be stored at - 80°C)

Dilute 5 pi of the marker solution with 5 pi sample buffer.

3.2 Sample preparation

The protein content in the samples was calculated.

The samples were diluted with Tris buffer, pH = 8,0 till the required protein concentration.

For silver staining x 10 0.2 pg protein / p

For coomassie staining: 1 -10 pg protein / pi.

Add per 10 pi sample 90 [j.1 sample buffer.

Boil the protein samples for 15 mm.

Centrifuge the samples 5,000 x g for 2 min using a Microlitre Centrifuge Model Z233M, Hermle Labortechnik GmbH, Germany (the supernatant should be loaded on the gel).

Gel electrophoresis

Loading of the sample (10(il) unto the gradient gel (Excel Gel ™ SDS, gradient 8 - 1 8 Pharmacia Biotech, 80 - 1255 - 09) via the template. The Power Supply for running conditions were: Voltage, 600V, Current, 50mA; power, 302 and Time, 120 minutes [see Appendix] for recordings on the (Experimental Result Form).

The electrophoresis equipment was turned on and set to cool at 15°C and then about 2-4 ml of Kerosene (Fluka 60710) was added onto the cooling plate of the electrophoresis equipment.

Positioning the gel (ExcelGel XL SDS 8-18 Pharmacia Biotech on the cooling plate and making sure that the cut comer on the gel correspond to the anodic side of the cooling plate and. Preventing air bubbles to be trapped beneath the gel was done carefully

The sample applicator strip was positioned on the gel and loaded for each sample maker 10 pi).

4- Safety regulations observed

4.1 Disposable nitrile gloves were worn during the entire staining procedure.

4.2 Solution marked with "WASTE" was not discarded in the dram but be collected and senc for separate destruction.

4.3 The gels contain polyacrylamide and were not touched without appropriate gloves. Besides the gels were collected for separate destruction.

The method is a modified version of the method described by the supplier in ExcelGel XL SDS 12-14 - Instructions Edt. AE, Pharmacia Biotech."

C2 SILVER STAINING PROTOCOL FOR PROTEINS

SILVER STAINING OF SDS GELS

1. Introduction

The method is used for silver staining of SDS gels from both conventional and 2 dimensional gel electrophoresis. Freshly made solutions (not older than 24 h) gave the best result. The solutions described below correspond to staining of one gel (24.5 x 18 cm). When nothing is indicated the reagent is supplied with the silver staining kit (71-1150-01, Pharmacia Biotech AB, Uppsala, Sweden). The component marked * were immediately added before use.

Gloves were worn during the entire procedure Solution marked with "WASTE" was be collected and send for separate destruction.

2. Solutions

2.1 Fixation solution

Acetic acid (100 %, Merck 0063)..... 25.0ml
Ethanol (40 % v/v) Absolute Ethanol..... 100ml
MilliQ..... 125ml

For staining ofone gel 2 x 250 ml fixation solution is required.

2.2 Sensitizing solution-

Ethanol absolute..... 75ml
Sodium thiosulphate (5% w/v)*.....0.10ml

Glutardialdehyde (25% w/v)*..... 1.25 ml
Milli-Q water, add.....250.0ml
Sodium acetate $3H_20$..... 170g

2.3 Silver solution

Silver nitrate solution (2.5 % w/v)..... 25.0 ml
Formaldehyde (37% w/v)*.....0.10ml
Milli-Q water, add.....250.0ml

2.4 Developing solution

Sodium carbonate (1 packet)..... 6.25g
Formaldehyde (37% w/v)*.....0.05ml
Milli-Q water, add.....250.0 ml
Stir vigorously to dissolve the sodium carbonate.

2.5 Stopping solution

EDTA-$Na_2 2H_20$ (1 packet)..... 3.65g
Milli-Q water, add..... 250.0 ml

2.6 Preservation solution:

Glycerol (Merck 4092)..... 25.0 ml
Milli-Q water, add..... 250.0 ml

3. Procedure:

3.1 Fixation

Add to the gel 250 ml fixation solution.
Agitate the gel gently on a horizontal shaker for 15 min.
Discard the fixation solution.
Add 250 ml new fixation solution.
Agitate the gel gently on a horizontal shaker for 15
Discard the fixation solution.

3.2 Sensitizing

Add to the gel 250 ml sensitizilig solution
Agitate the gel gently on a horizontal shaker for 30 min.
Discard the sensitizing solution

3.3 Washing

Wash the gel 4 min by adding 250 ml milli-Q water.
Discard the milli Q-water
Repeat the washing procedure four times in total.

3.4 Silver reaction

Add to the gel 250 ml silver reaction solution.
Agitate the gel on a horizontal shaker for 20 min.

Discard the silver reaction solution

3-5 Washing

Wash the gel 1 min by adding 250 ml milli-Q water
Discard the milli-Q water
Repeat the washing procedure twice

3.6 Developing

Add to the gel 250 ml developing solution
Agitate the gel on the horizontal shaker for 2 5 min. until the right it colouris achieved.
Discard the developing solution

3.6 Stopping

Add to the gel 250 in stopping solution
Agitate the gel on the horizontal shaker for 10 ml.
Discard the stopping solution.

3.7 Washing

Add to the gel *250* ml of milli-Q water
Agitate the gel on the horizontal shaker for 4 min.
Repeat the washing procedure four times in total

3.8 Preserving

Add to the gel 250 ml preserving solution.
Agitate the gel on the horizontal shake- for 20 min.
Discard the preserving solution.

3.9 Drying

Place the gel on a glass plate.
Wet the gel with distilled water.
Wrap the gel gently in plastic film
Avoid air bubbles between the gel and the plastic film.