KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF SCIENCE
FACULTY OF BIOSCIENCES
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

CHARACTERIZATION OF MICROORGANISMS INVOLVED IN THE SECOND STAGE OF SOY SAUCE FERMENTATION (MOROMI) AND STUDIES ON THE SHELF LIFE OF SOY SAUCE.

A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MSC. FOOD SCIENCE AND TECHNOLOGY.

BY

HANNAH KYERAA ANSAH (MRS.)

DECLARATION

This is to declare that this research work presented in this thesis was carried out by Mrs. Hannah Kyeraa Ansah of the Department of Biochemistry and Biotechnology of the Faculty of Biosciences, College of Science, Kwame Nkrumah University of Science and Technology and has not been submitted to any other University for a degree. It is entirely the candidates own account of research except the references cited which have been duly acknowledged.

…….
Hannah Kyeraa Ansah (Mrs.)
(STUDENT)
DATE: ……

…….
Prof. V. P. Dzogbefia (Mrs.)
(Supervisor)
DATE: ……

…….
Dr. K. Obiri-Danso
(Supervisor)
DATE: ……

DEDICATION

This thesis is dedicated to the Ansah and Hinneh families for their prayers, love and support throughout these years.

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ABSTRACT

Microorganisms play an important role in the fermentation of soy sauce. The aim of this study was to identify and characterize the microorganisms that are involved in the second stage of soy sauce fermentation and the shelf life studied. The microorganisms were identified using biochemical and Analytical Profile Identification (API) system. The pattern of distribution of total microbial numbers, lactic acid bacteria, yeast and fungi were similar as their numbers generally decreased with time. During the fermentation, lactic acid bacteria (numbers) were the most dominant followed by yeasts (numbers) and fungi (numbers). Twenty strains of lactic acid bacteria were isolated from the soy sauce mash during moromi fermentation and these were observed mostly in the first three weeks of fermentation. *Tetragenococcus halophilus* (also known as *Pediococcus halophilus*) had the highest number, followed by *Lactococcus lactis, Lactobacillus delbrueckii, Lactobacillus plantarum* and *Lactobacillus acidophilus* respectively. Two of the lactics could not be identified. Twenty-five strains of yeasts and molds were also isolated during moromi fermentation. The predominant yeast strain was *Zygosaccharomyces rouxii* (*Z. rouxii*), which was followed by *Trichosporon mucoides, Cryptococcus humicolus, Cryptococcus laurentii, Candida ciferrii, Pichia ohmeri* and *Candida famata* respectively. No significant changes were observed in microbial load, colour, moisture content, pH, and all the sensory attributes (colour, saltiness, pungency, umami and overall acceptability) of the locally produced soy sauce over the six months storage under room temperature. These results indicate that soy sauce can locally be produced by using starter culture to shorten the moromi stage fermentation and can stay on the shelf for more than six months.

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CHAPTER ONE

INTRODUCTION

Soy sauce, a popular fermented food in China, Japan and much of the rest of Asia is an indispensable culinary component used as flavouring for an enormous range of dishes. It is acidic, salty, amino acid-rich and mildly alcoholic. The predominant amino acid present, glutamic acid, is the component that is mainly responsible for the flavour enhancing properties of the sauce (Chad, 2005).

Soy sauce is a brown liquid with a peculiarly meaty flavour that is used in Oriental cooking to flavour meat and vegetables, often as part of a marinade, and also as a table condiment (Hoversnail, 2000). It is made by fermenting boiled soybeans, roasted wheat or barley, and salt. There are several stages to the process. Generally, the mixture is inoculated with a starter culture of two *Aspergillus* molds (*A. oryzae* and *A. soyae*) and allowed to ferment. This is mixed with a strong salt solution and allowed to ferment for eight to twelve months. The reactions in this last period create a complex blend of substances contributing to the final flavour (Noda *et al.,* 1980).

Protein energy malnutrition (PEM) is a major public health problem in most developing countries. The reason is that sources of protein of high biological value, particularly those of animal origin, are expensive, and most families are not able to afford them. It has therefore become necessary to look for other alternative sources of protein, especially those from plant sources. Of these plant proteins, legumes come first as the most valuable and inexpensive source

of high-quality proteins, also serving as good sources of other nutrients. Of the legumes, soybean has the potential for alleviating PEM in the developing world (Nti and Larweh, 2003).

Native to East Asia, soybeans (*Glycine max)* have been a major source of protein for people in Asia for more than 5,000 years (Messina *et al.,* 1994). Soybeans are high in protein (more than any other legume) and fiber, low in carbohydrates and are nutrient-dense. Phytochemicals in soybeans protect the heart against oxidation, inhibit blood clot formation, function as antioxidants and also exert anti-inflammatory actions (Messina *et al.,* 1994*).* Soybeans, compared with other legumes, are higher in essential fatty acids, and are a good source of calcium, magnesium, lecithin, riboflavin, thiamin, fiber, folate (folic acid), and iron (Kataoka, 2005). Studies have shown that soybeans may help to protect against osteoporosis since when people eat soy foods in place of animal proteins, far less calcium is excreted. Protein in soybeans may also be protective against heart disease, and the isoflavones in soybeans may help to thwart development of certain cancers. The nutritional magnitude of the versatile soybean is impressive (Kataoka, 2005).

Soybeans can provide all the nutrients humans need to survive; containing up to 48% protein, 11% carbohydrates, and 18% valuable essential fatty acids. Their high quality complete protein is far more than any other plant supplying a full and a well-balanced mixture of all the essential amino acids. The bean contains more than 100% of the dietary requirements of lysine, methionine, cystine, threonine and tryptophan. Their protein content rivals that of meat, but much easier to digest and assimilate. They are a good source of lecithin, vitamins A, B, E, potassium and phosphorus (Erickson, 1995).

Soy sauce is generally not produced in Ghana even though raw soybean is cultivated in the country. Ghana is one of the countries in Africa where governments have shown considerable interest in the production and utilization of soybeans. In spite of the recognition of its nutritional benefits, soybean is still new in many Ghanaian homes. This is because traditional food uses as well as simple technologies for processing have not yet been fully established (Plahar *et al.,* 1995). Currently, research and extension efforts are being intensified for widespread production and utilization of the crop in Ghana, through the development of home-level and small-scale processing technologies for soy-based foods for rural and urban populations (Nti and Plahar, 1998). Moreover, the eating habits of Ghanaians have changed which has necessitated the use of soy sauce in Ghanaian dishes as in fried rice.

Soy sauce is classified into fermented soy sauce and chemical soy sauce (acid decomposed) according to production type. Chemical production of soy sauce means adding hydrochloric acid to hydrolyse the soybean protein to accelerate production rather than fermenting soybean over a long period of time. While brewing soy sauce with long fermentation periods takes about four months, producing it chemically takes only about a week (Chen, 2002). It is however known that chemical production of soy sauce is dangerous from the nutrition viewpoint since carcinogenic substances such as 3-monochloropropan-1, 2-diol (3-MCPD) and 1,3-dichloropropanol (1,3-DCP) may form in the production process (Chen, 2002).

Soy sauce has been produced successfully in Ghana through fermentation from a locally cultivated soybean (Anidaso) (Arthur, 2003). Therefore, characterizing the microorganisms involved in the fermentation may have several benefits. It can enable starter culture application to shorten the moromi stage fermentation. Secondly, key microbial populations can be identified or profiled and be kept at optimal levels to ensure that key biotransformation rates remain constant. Thirdly, interfering or competing microbial populations can be detected and controlled more easily. Fourthly, the presence of microbial predators can be detected and controlled. Fifthly, nutrient levels can be minimized while optimizing microbial performance (www.programs.weber.edu).

Shelf life, or the maximal period of time during which the predetermined quality attributes of food are retained, is a critical factor in the consumer acceptability and the economic feasibility of a product. The shelf life of a product is therefore critical in determining both its quality and profitability (Kroll, 1995). Considering the benefits of soy sauce, it is crucial to characterize the microorganisms involved in the fermentation during the moromi stage and establish the shelf life of the locally produced soy sauce in Ghana.

The purpose of this study therefore was to produce soy sauce from the locally cultivated soybean (Anidaso) using *Aspergillus oryzae* as the starter culture for the first stage (Koji) fermentation. The study was carried out to identify and characterize the microorganisms involved in the second stage of the fermentation in soy sauce production and to determine the shelf life and any changes, which occurred during storage of the soy sauce produced.

CHAPTER TWO

LITERATURE REVIEW

2.1.0 SOY SAUCE

2.1.1 Background

Soy sauces (shoyu) are light to dark brown liquids with a meat-like salty flavour used in cooking and as a table condiment. Traditionally made in China, Japan, Korea, Thailand, the Philippines, Indonesia and Malaysia, soy sauce is now also produced in Europe and the Americas (Nunomura, and Sasaki, 1987).

Shoyu, a popular liquid condiment with an annual production of about 1.2 million kl is consumed at about 10 liters per capita per year in Japan (Hesseltine, 1983). Shoyu is now sold worldwide. A growing amount of shoyu is being produced in the United States by fermentation. The Japanese Agricultural Standard (JAS) defines shoyu as made from heat-treated soybeans and wheat cultured with the koji molds, *Aspergillus oryzae and Aspergillus soyae.* The koji is mixed with salt water to make a mash, moromi. The moromi is fermented with yeast and lactobacilli and then aged. JAS recognizes five types, of which the Koikuchi type represents 85% of all the shoyu consumed in Japan (Hesseltine, 1983).

2.1.2 Characteristics of Good Quality Soy Sauce

A good quality soy sauce should have the following characteristics:

- 1.5-1.8% total nitrogen (one half must be free amino acids):
- Reducing sugar (mainly glucose);
- 2-2.5% ethanol;
- 1-1.5% polyalcohol (primarily glycerol);

- 1-2% organic acid (primarily lactic);
- 4.7-4.8 pH and
- 17-18% Sodium chloride, etc. (Hesseltine, 1983).

2.1.3 Koji Production

For making koji, wheat kernels are roasted at 170-180°C for a few minutes, and then coarsely crushed. Whole soybeans or defatted soybean grits are moistened and cooked with steam under pressure. Currently, defatted flakes are generally used. These two materials are mixed and inoculated with seed mold *(A. oryzae and A. sojae).* The molds are especially selected for high enzyme activity and the ability to grow rapidly in very thick substrates. The depth of the fermenting mass is 30-40 cm. Koji fermentation is typically carried out on a large perforated stainless-steel plate, 5 × 12 m for 2-3 days. Temperature and moisture levels are carefully controlled: 30°C, 40-43% moisture, with the moisture decreasing to 25-30%. This first fermentation is to produce desirable enzymes (Hesseltine, 1983).

Yokotsuka, (1981) stated that, good koji fermentation should: (a) give good flavor product; (b) produce a good amount of spores; (c) have a high percentage spores that germinate and grow rapidly; (d) have high enzymatic activity; (e) have good genetic stability; (f) give colour to the product, (g) produce no mycotoxins and (h) produce mash that is easy to press. Mutation has been used to improve strains of the koji mold. Inoculation of the wheat and soybean is at the rate of 0.1-0.2%. The incubation

temperature for maximum mycelia growth is 30-35°C, followed by a lowering of the temperature to 20-25°C for maximum enzyme production (Hesseltine, 1983).

2.1.4 Moromi fermentation

The second fermentation step is preparation of the mash, mixing salt water (18-23%) and 120-130% volume to the koji. The moromi is then transferred into deep fermentation tanks of 50-300 kl, now usually resin-coated iron tanks. The moromi is held for four to eight months, depending on the temperature with occasional mixing with compressed air to ensure uniform blending and to promote microbial growth. Although the mold in the koji is killed, the enzymes produced hydrolyze most of the protein to amino acids and low-molecular weight peptides. About 20% of the wheat starch is used by the koji molds, and the remainder is converted to simple sugars, of which more than one half is fermented to lactic acid and alcohol by the lactobacilli and the yeasts, respectively (Hesseltine, 1983).

Initially, lactic acid is produced, followed by the yeast fermentation. The major lactobacillus is *Pediococcus halophilus. Torulopsis* (now = *Candida)* is believed to give a good volatile flavor to the finished product (Hesseltine, 1983). These organisms are protected from contamination to a large degree by the high salt in the moromi and lack of aeration. In addition, *Candida etchellsii* and *C. versatilis* also may occur in the moromi and add flavor. Following fermentation, the liquid is pressed from the moromi, refined, pasteurized, and packaged. Pasteurization is necessary to inactivate the enzymes and to kill the yeast and bacteria population (Hesseltine, 1983).

2.2 Benefits of Fermented foods

Fermented foods are those foods, which have been subjected to the action of microorganisms or enzymes that cause desirable biochemical changes and significant modification of the food (Campbell-Platt, 1994). Fermented foods have been with us since humans arrived on earth. They will be with us far into the future as they are the source of alcoholic foods/beverages, vinegar, pickled vegetables, sausages, cheeses, yoghurts, leavened and sour-dough breads, vegetable protein amino acid/peptide sauces and pastes with meat-like flavours, etc. (Steinkraus, 1997). Fermented foods and beverages are of great significance because they provide and preserve vast quantities of nutritious foods in a wide diversity of flavours, aromas and textures that enrich the human diet. They globally provide about 20-40% of our food supply (Campbell-Platt, 1994).

Food fermentation represents one of the oldest known uses of biotechnology and the main advantages of food fermentation can be categorized as follows (Campbell-Platt, 1994, Steinkraus, 1994; 1997):

Food fermentation develops a diversity of appealing exteriors, textures, aromas and flavours in food substrates. The increasing popularity of different types of e.g., fermented milks has as much to do with different textures that are created during the fermentation, as with significant flavour changes. The formation of diacetyl by heterofermentative lactic acid bacteria in fermentations of yoghurt and butter is not only important as a major flavour component, but it may also help inhibit less desirable microorganisms. Many fermented foods are ready-to-eat foods, such as cheese, sufu, wine, pickled vegetables,

vinegar etc. Food could get a longer shelf life through lactic acid, alcoholic, acetic, and alkaline fermentations. Alcohol has antimicrobial effects and contributes to the stability and preservation of alcoholic beverages. Food fermentations not only enrich the food substrates with protein, essential amino acids and fatty acids, but also with vitamins. Biosynthesis of B vitamins in food fermentations has been recognized to be of major nutritional significance, especially in the area where high-carbohydrate diets, particularly maize and sorghum diets can be deficient in essential B vitamins. Food fermentations also have functions in digestibility, bioavailability, and detoxification. For instance, the practice of soaking and fermenting peeled bitter cassava tubers allows the endogenous linamarase to hydrolyse the linamarin thus rendering the cassava tubers safe to eat as fermented products, such as West-African *gari* (Campbell-Platt, 1994, Steinkraus, 1994; 1997).

Fermented foods generally have a very good safety record even in the developing world where foods are often manufactured by people without formal training, and under conditions of poor hygiene.

2.2.1 Fermented soybean foods and their benefits

During the past several decades, soybeans have become an increasingly important agricultural commodity, with a steady increase in annual production. Major groups of soybean foods include traditional soybean foods, soy oil, soy protein products (e.g. used in bakery, breakfast cereals and infant formulas), new-generation soybean foods (e.g. meat alternatives like soy burgers), soy-enriched foods (e.g. soy snacks) and functional

soy ingredients/dietary supplements (e.g. phytochemicals like lecithin, isoflavones, tocopherols and sterols) (Liu, 2000).

Traditional soybean foods have been consumed in Asia for many centuries, and they remain popular and can be classified into two categories: non-fermented and fermented. Non-fermented soybean foods include soymilk, tofu, soy-sprouts and others, whereas fermented soybean foods include among others, soy sauce, sufu, miso, tempe and natto (Kiers, 2001).

The traditional fermentation process serves several functions, including the enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids, vitamins, polyamines, carbohydrates and numerous anti-oxidants and phytosterols (Haard *et al.,* 1999). The fermentation process, for example, with lactobacilli, increases the quantity, availability, digestibility and assimilation of nutrients in the body (Gorbach, 1990).

It is being increasingly recognized that while, for example, soy and soy protein isolates have potentially anti-nutritive value due to their high phytate and oxalic acid levels, cultured soy products such as miso, natto, and tempeh have enhanced nutritive bioavailability without the possible ill effects of the uncultured soy supplements (Haard *et al.,* 1999, Sarnat *et al.,* 2002). Uncultured soy with its high phytate levels has been shown in a number of studies to block absorption of vital nutrients such as calcium (Ghanem and Hussein, 1999). The use of soy as a culturing medium is also advantageous because iron is naturally available in soy (Sarnat *et al.,* 2002). Iron in an elemental form is potentially

toxic and pro-oxidative, but a lack of iron in the body potentiates development of anaemia and can thus be lethal (Ghanem and Hussein, 1999). Culturing soy has been shown to enhance the bioavailability of iron and copper, hence rendering the delivery of these nutrients in their most advantageous forms (Howard, 1999).

According to the World Health Organization (WHO, 2000), the "healthiest life expectancy" of the Japanese than any people on earth could be attributed to their extensive consumption of cultured soy products such as miso and natto, together with accompanying foods like ginger. The abandonment by consumers in developing countries of their nutritionally rich traditional fermented foods in exchange for the "sophisticated" dietary products could have detrimental effects. It was reported that continuous intake of cola beverages which have empty calories instead of indigenous fermented cereal drinks could impact negatively on many consumers when the extensive medical benefits of consuming the traditional dietary sources are lost (Haard *et al.*, 1999, Sarnat *et al.*, 2002).

In Asia, the traditional fermented soy foods are considered to have more health promoting benefits when consumed in moderate amounts than the super-processed soy products that are consumed in the West (Messina and Barnes, 1991, Messina *et al.*, 1994). It has been suggested that the fermentation process increases availability of isoflavones in soy (Golbitz, 1995, Hutchins *et al.*, 1995). For example, a study (Baggott, 1990) of the culturing method involved in the production of the Japanese traditional food miso came to the conclusion that the culturing process itself led to a "lower number of cancers per animal" and a "lower growth rate of cancer compared to controls." The researchers also indicated that it was not the presence of any specific nutrient that was

cultured along with the soybean paste but rather the cultured soy medium itself that was responsible for the health benefits associated with miso consumption. Miso, a fermented or probiotic form of soyabean, is particularly rich in the isoflavone aglycones, genistein and daidzein, which are believed to be cancer chemopreventatives (Gotoh *et al.,* 1998). The fermentation process is thought to convert the isoflavone precursors genistin and daidzin to their active anti-cancer isoflavone forms, genistein and daidzein (Takahashi *et al.,* 1995).

A Korean study concluded that soy was one of the foods that decreased the rates of certain cancers such as stomach cancer while heavy salt consumption and cooking methods increased the rates (Lee, 1995). Diets that are high in fermented soy products indicates that such diets may reduce the risk of breast, colon, lung and stomach cancers, as well as offer some protection from cardiovascular diseases, osteoporosis and menopausal symptoms (Lee, 1995, Barnes, *et al.*, 1995, Stephens 1997).

2.3 BASIC PRINCIPLES OF FERMENTATION

2.3.1The diversity of fermented foods

Numerous fermented foods are consumed around the world. Each nation has its own types of fermented food, representing the staple diet and the raw ingredients available in that particular place. Although the products are well known to the individual, they may not be associated with fermentation. Indeed, it is likely that the methods of producing many of the world's fermented foods are unknown and came about by chance. Some of

the more obvious fermented fruit and vegetable products are the alcoholic beverages - beers and wines. However, several more fermented fruit and vegetable products arise from lactic acid fermentation and are extremely important in meeting the nutritional requirements of a large proportion of the world's population (Battcock and Axam-Ali, 1998).

When microorganisms metabolize and grow they release by-products. In food fermentations the by-products play a beneficial role in preserving and changing the texture and flavour of the food substrate. For example, acetic acid is the by-product of the fermentations of some fruits. This acid not only affects the flavour of the final product, but more importantly also has a preservative effect on the food. For food fermentations, microorganisms are often classified according to these by-products. The fermentation of milk to yoghurt involves a specific group of bacteria called the lactic acid bacteria (*Lactobacillus* species). This is a general name attributed to those bacteria, which produce lactic acid as they grow. Acidic foods are less susceptible to spoilage than neutral or alkaline foods and hence the acid helps to preserve the product. Fermentations also result in a change in texture. In the case of milk, the acid causes the precipitation of milk protein to a solid curd (Battcock and Axam-Ali, 1998, and Hicks, 2002).

Nearly all food fermentations are the result of more than one microorganism, either working together or in a sequence. For example, vinegar production is a joint effort between yeast and acetic acid forming bacteria. The yeast converts sugars to alcohol, which is the substrate required by the *acetobacter* to produce acetic acid. Bacteria from different species and the various microorganisms - yeasts and molds -all have their own

preferences for growing conditions, which are set within narrow limits. There are very few pure culture fermentations. An organism that initiates fermentation will grow there until its by-products inhibit further growth and activity. During this initial growth period, other organisms develop which are ready to take over when the conditions become intolerable for the former ones (Hesseltine, 1985; Battcock and Axam-Ali, 1998).

In general, growth will be initiated by bacteria, followed by yeasts and then molds. There are definite reasons for this type of sequence. The smaller microorganisms are the ones that multiply and take up nutrients from the surrounding area most rapidly. Bacteria are the smallest of microorganisms, followed by yeasts and molds. The smaller bacteria, such as *Leuconostoc* and *Streptococcus* grow and ferment more rapidly than their close relations and are therefore often the first species to colonise a substrate (Mountney and Gould, 1988).

2.3.2 Organisms responsible for food fermentations

The most common groups of microorganisms involved in food fermentations are:

- Bacteria
- Yeasts
- Molds

2.3.3 Bacteria

Several bacterial families are present in foods, the majority of which are concerned with food spoilage. As a result, the important role of bacteria in the fermentation of foods is

often overlooked. The most important bacteria in desirable food fermentations are the *lactobacillaceae, which* have the ability to produce lactic acid from carbohydrates. Other important bacteria, especially in the fermentation of fruits and vegetables, are the acetic acid producing *acetobacter* species (Battcock and Axam-Ali, 1998).

2.3.4 Lactic Acid Bacteria

The lactic acid bacteria are a group of Gram-positive bacteria, non-respiring and non-spore forming cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, all fermented milks, cassava (to produce *gari*) and most "pickled" (fermented) vegetables. Historically, bacteria from the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* are the main species involved. Several more have been identified, but play a minor role in lactic fermentations (Lindgren and Dobrogosz, 1990; Battcock and Axam-Ali, 1998).

Lactic acid bacteria carry out their reactions - the conversion of carbohydrate to lactic acid plus carbon dioxide and other organic acids - without the need for oxygen. They are described as microaerophilic as they do not utilize oxygen. Because of this, the changes that they effect do not cause drastic changes in the composition of the food. Some of the family are homofermentative, that is they only produce lactic acid, while others are heterofermentative and produce lactic acid plus other volatile compounds and small amounts of alcohol. *Lactobacillus acidophilus, L. bulgaricus, L. plantarum, L. caret, L. pentoaceticus, L. brevis* and *L. thermophilus* are examples of lactic acid-producing

bacteria involved in food fermentations. All species of lactic acid bacteria have their own particular reactions and niches, but overall, *L. plantarum* – a homofermenter -produces high acidity in all vegetable fermentations and plays the major role. All lactic acid producers are non-motile gram-positive rods that need complex carbohydrate substrates as a source of energy. The lactic acid they produce is effective in inhibiting the growth of other bacteria that may decompose or spoil the food. The lactic acid bacteria are a diverse group of organisms with a diverse metabolic capacity. This diversity makes them very adaptable to a range of conditions and is largely responsible for their success in acid food fermentations (Cintas, *et al*., 1997; Battcock and Axam-Ali, 1998).

Despite their complexity, the whole basis of lactic acid fermentation centres on the ability of lactic acid bacteria to produce acid, which then inhibits the growth of other non-desirable organisms. (Battcock and Axam-Ali, 1998). 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. Homofermenters, convert sugars primarily to lactic acid, while heterofermenters produce about 50% lactic acid plus 25% acetic acid and ethyl alcohol and 25% carbon dioxide. These other compounds are important as they impart particular tastes and aromas to the final product. The heterofermentative lactobacilli produce mannitol and some species also produce dextran (Noda, *et al*., 1980; Battcock and Axam-Ali, 1998).

Table 2.1Major lactic acid bacteria in fermented plant products.

Homofermenter	**Facultative homofermenter**	**Obligate heterofermenter**
Enterococcus faecium	*Lactobacillus bavaricus*	*Lactobacillus brevis*
Enterococcus faecalis	*Lactobacillus casei*	*Lactobacillus buchneri*
Lactobacillus acidophilus	*Lactobacillus coryniformis*	*Lactobacillus cellobiosus*
Lactobacillus lactis	*Lactobacillus curvatus*	*Lactobacillus confusus*
Lactobacillus delbrueckii	*Lactobacillus plantarum*	*Lactobacillus coprophilus*
Lactobacillusleichmannii	*Lactobacillus sake*	*Lactobacillus fermentatum*
Lactobacillus salivarius		*Lactobacillus sanfrancisco*
Streptococcus bovis		*Leuconostoc dextranicum*
Streptococcus thermophilus		*Leuconostoc mesenteroides*
Pediococcus acidilactici		*Leuconostoc paramesenteroides*
Pedicoccus damnosus		
Pediococcus pentocacus		

Source: *Beuchat (1995).*

2.3.5 Yeasts

Yeast is a unicellular fungus, which reproduces asexually by budding or division, especially the genus *Saccharomyces* which is important in food fermentations (Walker, 1988). Yeasts and yeast-like fungi are widely distributed in nature. Like bacteria and moulds, they can have beneficial and non-beneficial effects in foods. Most yeasts are larger than most bacteria. The most well known examples of yeast fermentation are in the production of alcoholic drinks and the leavening of bread. For their participation in these two processes, yeasts are of major importance in the food industry (Battcock and Axam-Ali, 1998; Aidoo, *et al* 2006).

Some yeasts are chromogenic and produce a variety of pigments, including green, yellow and black. Others are capable of synthesizing essential B group vitamins (Mai, *et al*., 2002). Although there is a large diversity of yeasts and yeast-like fungi, (about 500 species), only a few are commonly associated with the production of fermented foods. They are all either ascomycetous yeasts or members of the genus *Candida*. Varieties of the *Saccharomyces cervisiae* genus are the most common yeasts in fermented foods and beverages based on fruit and vegetables. All strains of this genus ferment glucose and many ferment other plant-derived carbohydrates such as sucrose, maltose and raffinose. In the tropics, *Saccharomyces pombe* is the dominant yeast in the production of traditional fermented beverages, especially those derived from maize and millet (Adams and Moss, 1995).

2.3.6 Molds

Molds are also important organisms in the food industry, both as spoilers and preservers of foods. Certain molds produce undesirable toxins and contribute to the spoilage of foods. These molds are frequently found in foods and can tolerate high concentrations of salt and sugar. However, others impart characteristic flavours to foods and others produce enzymes, such as amylase for bread making. Molds from the genus *Penicillium* are associated with the ripening and flavour of cheeses. Molds are aerobic and therefore require oxygen for growth. They also have the greatest array of enzymes, and can colonise and grow on most types of food (Battcock and Axam-Ali, 1998, and Aidoo *et al*., 2006).

2.5 Functions of Lactic Acid Bacteria and Yeasts in Soy Sauce Fermentation

There are two specific fermentation stages involved in soy sauce production namely aerobic koji fermentation, which involves the use of *A. oryzae* or *Aspergillus sojae*, and an anaerobic moromi or salt mash, which undergoes lactic acid bacteria and yeast (*Zygosaccharomyces rouxii)* fermentations (Aidoo, *et al.,* 2006). The two main groups of enzymes produced by *A. oryzae* during koji fermentation are amylases, and proteinases. Lipase activity has also been reported. These major enzymes hydrolyze carbohydrates and proteins to sugars and amino acids and low molecular weight peptides, respectively. These soluble products are essential for yeast and bacterial activities during moromi fermentation (Aidoo, *et al.,* 1994; Chou & Rwan, 1995). During the fermentation,

Tetragenococcus halophila initially proliferates and produces lactic acid, which lowers the pH to 5.5 or less. Acid-tolerant dominant yeasts, notably *Z. rouxii*, grow and produce about 3% (w/v) alcohol and several compounds which add characteristic aroma to soy sauce (Aidoo, *et al.*, 2006).

Zygosaccharomyces rouxii is the dominant moromi yeast, which produces alcohol and several compounds that add characteristic aromas to soy sauce. Also other yeasts such as *Candida versatilis* and *Candida etchellsii* also produce phenolic compounds, i.e. 4-ethylguaiacol and 4-ethylphenol, which contribute to soy sauce aroma (Aidoo, *et al.*, 2006). Nearly 300 types of flavour compounds have been identified in Japanese soy sauce (Nunomura & Sasaki, 1992). *Zygosaccharomyces rouxii* produces flavour compounds including alcohols, glycerol, esters, 4-hydroxy-5-methyl-3 (3 H)-furanone (HMF), 4-hydroxy-2 (or 5)-ethyl-5 (or 2) -methyl-3 (2 H)-furanone (HEMF). Of the furanones, HEMF produced by *Z. rouxii* and *Candida spp*. gives Japanese-type soy sauce its characteristic flavour (Aidoo, *et al.*, 2006). This compound is also reported to have antitumour and antioxidative properties (Nagahara *et al.*, 1992; Koga *et al.*, 1998). Higher alcohols such as isobutyl alcohol, isoamyl alcohol and 2-phenyl ethanol, produced by *C. versatilis*, are also important flavour constituents of soy sauce (Aidoo, *et al.*, 2006).

2.6 Production of Soy Sauce by Acid Hydrolysis

Soy sauce is a major seasoning component of the East and South East Asian diet and is becoming increasingly popular worldwide. Two main methods are used in the production of soy sauce (Nunomura and Sasaki 1987, and Luth, 1995). In the traditional manner of

preparation, a mixture of whole or defatted soybeans and whole wheat (which is sometimes roasted) is hydrolysed by enzyme fermentation after being cooked in steam. The mass is then fermented for up to 18 months, principally with *Aspergillus oryzae* and *Aspergillus sojae,* before separation of the sauce by pressing. Chemically hydrolysed sauce is prepared by refluxing the soy meal and wheat with hydrochloric acid for 12-16 hours. The hydrosylate is neutralized with sodium hydroxide (Crews *et al.,* 2003). Chlorinated propanols are produced during the manufacture of the savoury food ingredient acid hydrolysed vegetable protein (acid-HVP). The reaction leads to the formation of a number of chlorinated propanols including monochloropropanols, dichloropropanols and monochloropropanediol (VelõÂ sÏ ek *et al*. 1978). The most important chloropropanols in terms of both levels present and toxicity are 1,3-dichloro-propan-2-ol (1,3-DCP) and 3-monochloro-propane-1, 2-diol (3-MCPD). The FAO/WHO Joint Expert Committee on Food Additives concluded in (2001) that because of its carcinogenicity, 1,3-DCP was an undesirable contaminant in food and that levels should be reduced to as low as technologically possible (Olsen, 1993). 1,3-DCP has been assessed by the UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC 2001) and the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM, 2001). The committees declared that `it is prudent to assume that 1,3-DCP is a genotoxic carcinogen and that exposures to 1,3-DCP should be reduced to as low a level as technologically feasible' and it is prudent to assume that 1,3-DCP may possess genotoxic activity *in vivo*' (Crews *et al.,* 2002).

In 1978, VelõÂ sÏ ek *et al*. reported 0.94mg /kg of 1,3-DCP in an acid-hydrolysed soup seasoning. Surveys of chloropropanols in acid-HVP carried out in 1990 and 1992 by the UK Joint Food Safety and Standards Group found 1,3-DCP in only one sample out of 74, subject to a limit of detection of 0.02 mg/kg (JFSSG 1999). Industry action has reduced the level of contamination by chloropropanols of acid-HVP prepared in Europe. On the other hand Meierhans *et al.* in 1998 stated that high levels of 3-MCPD have been reported in some samples of soy sauce. The 3-MCPD contamination was as the result of the addition to the sauces of acid-HVP prepared by older techniques or by the use of acid hydrolysis of some or all of the vegetable protein used to prepare the sauce. Crews *et al.*, (2002) were of the view that regardless of cause, it seemed likely that products with very high levels of 3-MCPD might also contain other chloropropanols, particularly 1,3-DCP.

The chemical hydrolysis process for soy sauce is rapid and therefore more cost effective than enzyme fermentation, but the product lacks many of the essential flavours of fermented product (Crews *et al.*, 2003).

2.7 Methods of Microbial Characterization

Microorganisms have been classified and identified on the basis of a variety of characteristics including morphological, growth, tolerance, metabolic, biochemical, and genetic. Recently there has been a tendency to determine definitive classification and taxonomic assignment by nucleic acid hybridization, 16S rRNA sequence analysis, and other molecular genetic techniques **(**Bascomb and Manafi, 1998). After classification has been established, characteristics are selected for the identification of unknown isolates.

Commercial kits based on such a process are available for the identification of clinically important bacteria (Bascomb and Manafi, 1998).

Most growth-dependent tests require at least an overnight incubation; others, based on the ability to utilize a single carbon or nitrogen source, may require as long as 7 days. The molecular genetic techniques are still time-consuming and less amenable to routine application. Moreover, most of the techniques available now are for the specific detection of a limited number of taxa. Alternatively, determinations of the enzymatic activities of isolates with a variety of synthetic substrates (Bascomb 1980, D'Amato, 1980) can be used for identification and give similar results to those obtained by other characterization methods. The identification of bacteria based on enzyme patterns offers simple and rapid results **(**Bascomb and Manafi, 1998).

The first commercial kit with tests for specific enzymes contained Patho-Tec paper strips (Narayan *et al***.,** 1967), a method that evolved to the Micro-ID system (Barry and Badal**,** 1979) for the identification of clinically important gram-negative rods, mainly members of the Enterobacteriaceae. The Micro-ID kit included tests for –galactosidase, cytochrome oxidase, lysine and onithine decarboxylases, tryptophanase and urease. Other kits in the form of cards, micro titer trays, or multi chamber strips are now available for the identification of certain taxonomic classes of bacteria. The largest number is available for the identification of clinically important aerobic and facultatively anaerobic gram-negative bacteria (e.g., API 20E, MicroScan conventional overnight and MicroScan Rapid GN Identification Systems, and Vitek GNI Card). Fewer kits are available for the identification of gram-positive cocci, staphylococci, streptococci, anaerobic cocci, and

yeasts. API 20E, MicroScan conventional overnight, and Vitek GNI Card include growth-dependent tests and a few enzyme tests; in general, they do not use the ability of enzyme tests to provide results rapidly. A specific enzyme test for –galactosidase based on utilization of the synthetic substrate o-nitrophenol-D-galactopyranoside (ONPG) or substrates with other synthetic moieties is included in most kits for identification of the *Enterobacteriaceae*. Some kits are completely manual, whereas others offer automation with instruments for all or some of the following tasks: inoculation, incubation, determination of test results, and identification. Dade MicroScan Rapid Identification Systems for gram-negative rods and gram-positive cocci are the fastest systems, providing identification in 2 to 2.5 h by measuring enzymatic activities fluorometrically with a high correct identification rate (Achondo *et al*., 1995).

The commercial identification systems also provide databases of expected results, and an unknown isolate is assigned to one of the taxa in the database either by using a code book or by using an automated system and computer-based identification. Most of these kits have been designed for clinically important groups of bacteria, as reflected in the taxa, tests, and expected results included in the databases. Some may be applicable to isolates taken from different environments (Langlois *et al*.**,** 1983, Watts and Yancey 1994). These kits can also be used for characterization of microorganism groups other than those specified in the database (Biehle *et al*., 1996).

In (1998), Bascomb and Manafi reported that commercial identification kits have been optimized for the sets of taxa included in their databases. The kits require regulatory approval and are expected to provide a high level of accurate identification in comparison

with an acceptable reference method. Conversely, characterization kits are designed to provide an easy and reproducible method for testing a variety of unknown isolates. They are very useful in taxonomic studies, and the results used for the construction of identification kits. These kits are not accompanied by a database, and some may have methods for computer analysis of the data including classification and identification of unknown isolates.

Studies of the clarification of the taxonomic position of individual taxa have used laboratory-prepared tests (Kämpfer, 1992, Kämpfer *et al*., 1991) as well as commercial characterization and identification kits. Only the latter contain information on expected results for specified taxa in each of the kit tests.

The API ZYM system (API System; bioMérieux, Paris, France) is a semi quantitative micro method designed for the detection of 19 enzymatic activities (Humble *et al*., 1977, Waitkins *et al*., 1980). The use of API enzyme research kits detecting 20 glycosidases, 10 esterases, 57 arylamidases, alkaline and acid phosphatases, and phosphoamidase has been reported (Milliere et *al.*, 1989**,** Manafi and Rotter, 1992**,** Manafi *et al.*, 1993).

In addition, kits based on growth-dependent tests to determine the utilization of amino acids, organic acids, and carbohydrates are available. The API 50CH (Kerr *et al***.,** 1990), 50AA, and 50OA (Kersters, *et al.,* 1984) tests are strip based; the results are indicated by changes in the colour of the pH indicators. The Biolog system (Biolog Inc., Hayward, Calif.) is micro titer tray based and is dependent on the detection of substrate-specific dehydrogenases with tetrazolium salt as an indicator (Miller and Rhoden**.** 1991). The MAST ID system (Mast Laboratories Ltd., Bootle, England) provides a means of

determining the metabolic activities of a number of isolates by agar plate and multipoint inoculator techniques (Geary and Stevens**.** 1989, Geary *et al***.,** 1989). Combinations of these systems permit the detection of over 340 biochemical reactions. A number of studies report the use of commercially available characterization kits alone or in combination with test batteries prepared in their own laboratories (Bridge and Sneath**,** 1983).

2.7.1 *Streptococcaceae* and Related Organisms

Cells arranged in pairs or tetrads, with no catalase activity

(i) *Pediococcus.* The genus *Pediococcus,* contains five species associated with lactic acid fermentations of vegetables, grain mashes, and cheese (*P. acidilactici*, *P. damnosus*, *P. dextrinicus, P. parvulus,* and *P. pentosaceus*). Some strains are used as starter cultures in silage and various fermented sausages (Colman and Efstratiou**,** 1987). One species (*P. damnosus*) is an important spoilage agent of beer (Poutrel and Ryniewicz**.** 1984); two species (*P. acidilactici* and *P. pentosaceus*) have been isolated from human specimens. The phylogenetic interrelationships within the genus have been discussed by Collins *et al.* (1990). Differentiation within the genus is based on tests used in the clinical laboratory, such as formation of acid from carbohydrates and the ability to grow in 6.5% NaCl, and in food microbiology, in particular the ability to grow at low pH, in different beers, and in the presence of hops. Schemes for separating the taxon from others and for differentiation within the genus have been provided (Facklam and Elliott. 1995, Facklam *et al*., 1989).

(ii) *Tetragenococcus.* The genus *Tetragenococcus* contains one species, *Tetragenococcus halophilus* (Collins *et al*., 1990); this species was previously included in the genus *Pediococcus* but is susceptible to vancomycin. The genus represents a distinct line of descent quite separate from aerococci and pediococci (Collins *et al*., 1990). It is important in the fermentation of soy moromi to produce soy sauce **(**Stiles and Holzapfel, 1997**).**

2.8 Shelf life studies

The modern food industry has developed and expanded because of its ability to deliver a wide variety of high quality food products to consumers on a nation wide and worldwide basis. This feat has been accomplished by building stability into the productions through processing, packaging and additives that enable foods to remain fresh and wholesome throughout the distribution process. Consumer demands for convenience have fueled new innovations in the food product development, packaging and chemical industries, and widespread desire for products to use in the microwave oven has added further impetus to this effort (Sewald and De Vries, 2003).

2.8.1 Processed Food Deterioration

The principal mechanisms involved in the deterioration of processed foods are

1. Microbiological spoilage sometimes accompanied by pathogen development.
2. Chemical and enzymatic activity causing lipid breakdown, color, flavor, and texture changes

3. Moisture and/or other vapor migration producing changes in texture, water activity and flavor. Formulation and processing variables which affect these mechanisms and which can be used to control deterioration include: (1) moisture and water activity; (2) pH; (3) heat treatments; (4) emulsifier systems; (5) preservatives and additives; and (6) packaging (Sewald and De Vries, 2003).

2.8.2The importance of Moisture and Water Activity

Water has been called the universal solvent because it is a requirement for growth and metabolism of microbes, and the support of many chemical reactions, which occur in food products. Water occurs in food in both the free and bound states. Free water is just that, it is free for chemical reactions, to support microbiological growth, and to act as a transporting medium for compounds. In the bound state, water is not available to participate in these reactions as it is tied up by water-soluble compounds such as sugar, salt, gums, etc., (osmotic binding) and by the surface effects of the substrate (matrix binding). These water-binding effects reduce the vapor pressure of the water over the food substrate (Sewald and De Vries, 2003).

2.8.3 Microbiology and the Shelf life of Food Products

Shelf life of food products is most familiar to consumers through the open dating. The shelf life of food products is affected, for the most part, by their microbiological status. These products pose the highest food safety risk and have the shortest shelf life because they are the most susceptible to microbiological deterioration and the possibility of the

growth of pathogenic organisms. Bacteria need certain conditions for growth namely available moisture, the proper pH, the right temperature, nutrients and time. By controlling these conditions one can prevent the growth of these organisms and extend the shelf life. The growth of yeasts, mold, spoilage and pathogenic bacteria, etc., can be monitored by microbiological methods. Other noticeable reactions such as gas production, syneresis (phase separation), and changes in colour or viscosity can give further indications as to what might have to be changed in the formulation or packaging to increase the shelf life. Microbiological shelf life determinations can be accomplished in real time and are therefore quite accurate (Sewald and De Vries, 2003).

2.8.4 Chemical Deterioration

Chemically based deterioration of packaged food products can be classified into three different mechanisms: oxidation of lipids, enzymatic degradation and non- enzymatic browning. All three can occur simultaneously in food systems and are accelerated to some extend or another by increasing storage temperatures (Sewald and De Vries, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Source of raw materials

The soybeans (*Glycine max*), Anidaso variety was obtained from the Legumes and Grains Development Board of the Ministry of Food and Agriculture, Kumasi and the wheat (*Triticum aestivum*) from Irani Brothers, Accra.

Aspergillus oryzae, (ATCC 46249), th*e* starter culture was obtained from the America Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, U.S.A.

3.1.1 Sample preparation

The soybeans were spread on aluminum tray and all foreign particles removed by handpicking.

3.2 Preparation of starter culture

The starter culture, *Aspergillus oryzae* (ATCC 46249) which was stored on agar slants in the freezer was activated by sub-culturing on malt extract agar. The media was prepared according to manufacturer's instructions, poured into test tubes, cotton wool plugged and autoclaved at 121^{o}C for 15 minutes. The test tubes were slanted until the media became solid on cooling. The culture (*Aspergillus oryzae*) was sub cultured onto the freshly prepared slants and then incubated for 72 hours at 26^{o}C. A green/brightly yellow coloured growth of *Aspergillus oryzae* was observed after the incubation.

3.3 Preparation of "KOJI"

One kilogram each of the soybeans was weighed into three separate plastic containers. The beans were washed thoroughly under running water and steeped in standing water at room temperature for 15 hours. The water was changed once during this period. The water was drained off the beans, poured into a stainless steel canister and pressure-cooked (Griffin, England) at 121^{o}C for one hour. The cooked beans were cooled and blended into a mash using a blender (WARING Commercial Heavy Duty Blender U.S.A).

Wheat, 0.85kg each was roasted in an electric cooker at 180^{o}C for 20 minutes. It was cooled and crushed into approximately 5 pieces per grain using the mill (WARING Commercial Heavy Duty Blender U.S.A).

3.3.1 Mixing and inoculation

One kilogram each of the mashed soybeans was mixed with 0.85kg of the crushed roasted wheat in a ratio of 54% to 46%. Each of the semi- solid mixture was spread on aluminum trays and labeled. Two of the trays containing the mixture were inoculated with a culture of *Aspergillus oryzae* and the third tray served as the control. Each tray was inoculated by mixing three inoculation loops of the culture in 100ml of sterilized distilled water to knead the mixture of soybean and wheat thoroughly. They were incubated at room temperature for five days, but were turned on day 3 to allow maximum growth of the mycelia. The mixture at this stage is called "koji".

Plate A1: Inoculated Koji **Plate A2: Uninoculated Koji**

+

Plate B: Moromi Fermentation

Plate C: Koji Mixed With 18% Salt **Plate D: Bottled Soy Sauce**

3.4 Brine preparation

Nine hundred grams of sodium chloride (NaCl) was weighed into a container containing five thousand milliliters (5000ml) of water to make 18% brine.

3.4.1 Brine fermentation (Moromi)

Three plastic containers containing 5000ml 18% brine each was mixed with the "Koji." The mixture (moromi) was then allowed to ferment for three months.

3.5 Harvesting

The moromi was poured into a white polyester cloth after the fermentation and then pressed to drain off the liquid portion into sterilized 10litres plastic containers and the solid soybean portion was discarded. The liquid portion (soy sauce) was stored to age.

3.5.1 Clarification

One gram of alum was added to a litre of the soy sauce and allowed to settle overnight to help clarify the product and reduce turbidity.

3.5.2 Addition of Caramel

Three hundred milliliters (300mls) of industrial caramel was mixed to 900mls of soy sauce to deepen the colour of the soy sauce.

3.5.3 Pasteurization

The soy sauce was bottled and pasteurized at $80^{o}C$ for 15 minutes using a water bath (Clifton, Nickel Electro Ltd, England).

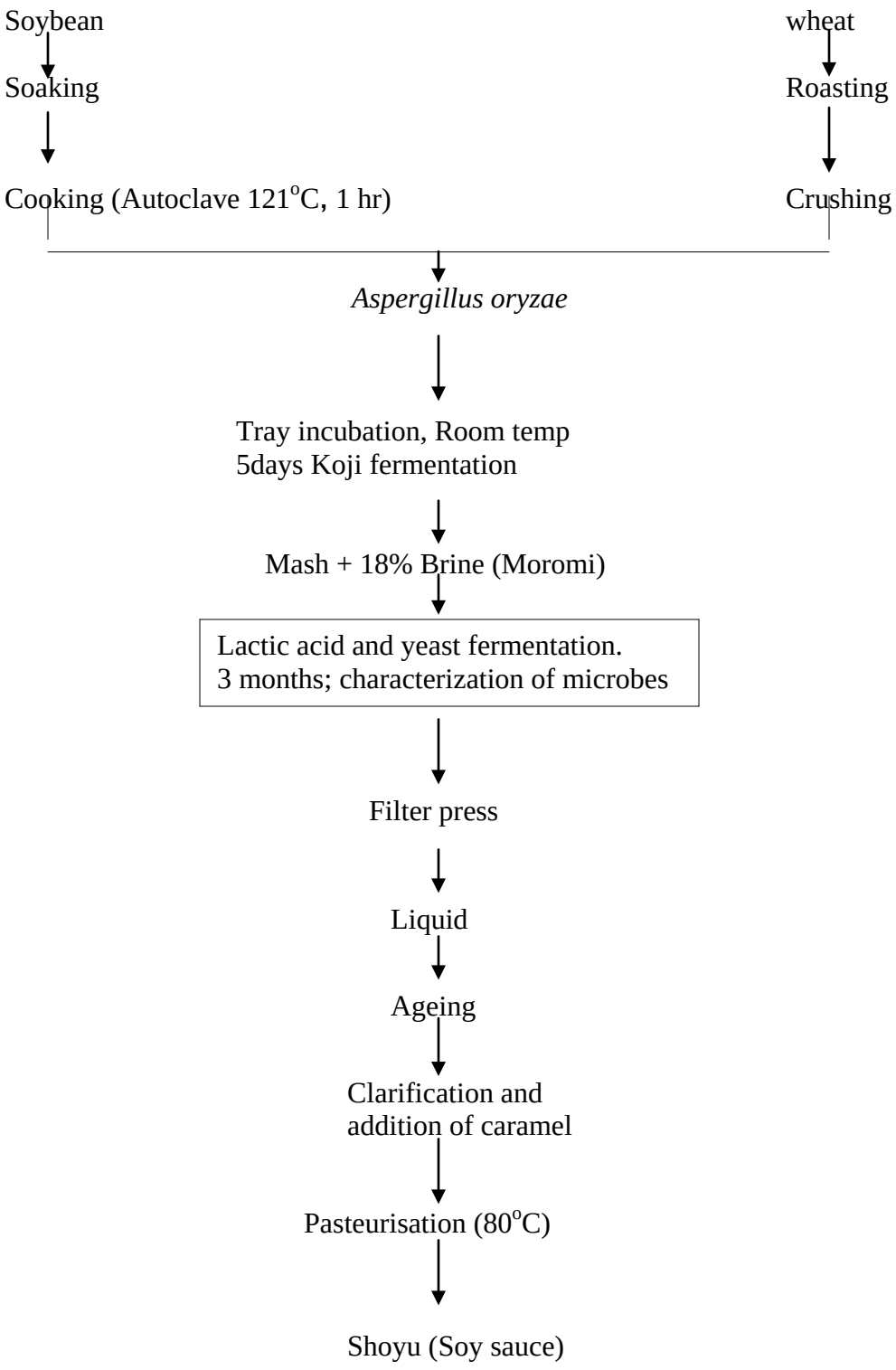

Fig. 3.1: Flow diagram for the production of soy sauce

3.6 Sample Collection

Weekly five-milliliters of brine fermentation were collected in sterilized Bijou bottles for 12 weeks during the moromi stage of fermentation to assay for microbial numbers.

3.6.1 Isolation Procedures

Serial dilutions 10^{-1} to 10^{-20} of the brine fermentation were prepared in 9ml volumes of sterile distilled peptone water. Using a fresh pipette tip, 1ml aliquots of each dilution was transferred into sterile petri dishes having a label corresponding to the dilution. To each dilution in the sterile petri dish, was added 15ml of molten De Mann Rogossa and Sharpe Medium (MRS, BIOTEC Laboratories Ltd. United Kingdom) for lactic acid bacteria, Plate Count Agar (PCA, BIOTEC Laboratories Ltd. United Kingdom) for aerobic plate count, Cassava Dextrose Agar (CDA, 250g of cassava, 9g of agar and 5g of glucose to make 500mls) for fungi and Malt Extract Agar (MEA, Oxoid Ltd, Hampshire, England) for yeast to cover the base of the petri dishes. The petri dishes were swirled clockwise and anticlockwise to ensure uniform mixing and allowed to set. PCA plates were incubated at 37℃ whilst MEA and CDA plates were incubated at 30^{o}C all for 24 to 48 hours. MRS plates were also incubated anaerobically at 37^{o}C 48 to 72 hours.

After 24 hours of incubation, colonies on plates showing growth were counted using the Colony Counter (STUART Scientific Colony Counter, UK). Representative colonies on the corresponding media were kept for further identification.

3.6.2 Maintenance of Pure Culture

The cultures were purified by continual streaking on agar plates. Pure isolates were kept on slants in Bijou bottles in the freezer.

3.7 Identification of isolates

3.7.1 Gram staining

Gram staining was done for all the bacteria isolates. This was done to identify the shape and arrangement of the bacteria cells. A purple colour indicates a Gram positive bacteria cell whilst a pink or orange colour indicates a Gram negative.

3.7.2 Catalase Test

This was done to determine the presence of the enzyme catalase, which is capable of hydrolyzing hydrogen peroxide (H_2O_2) into water and oxygen with a visible effervescence. The isolates were put on slides and 3% hydrogen peroxide solution added. Visible effervescence indicates a catalase positive organism.

3.7.3 Spore Staining Test

This was done for some isolates which showed signs of sporing using Schaefer and Fulton's method. The isolates were put on slides and 5% malachite green solution added, and then steamed for about 2 minutes. They were washed under running water and counterstained with 50% aqueous safranin for 15 seconds. Rinsed with water, drained off and viewed under oil. A bacterial body staining red with the spores staining green indicated a sporing bacteria.

Representative colonies of the MRS plates were mostly Gram positive, catalase negative rods and non-spore forming which suggested they belong to the Lactic acid bacteria group.

3.8 Phenotypic Identification

API 20C was used for yeast and fungi identification and characterization and API 50CH was used for lactic acid bacteria identification and characterization. The species of *Lactobacilli* were identified by assaying cultures in API 50 CHL galleries (Bio Merieux, SA, France) using API 50 CHL medium (Bio Merieux). MEA isolates were assayed in API 20 C using C medium (Bio Merieux).

3.8.1 API 50 CH for Lactic Acid Bacteria

3.8.1.1 Preparation of the strip

An incubation box was prepared by distributing about 10ml of sterile distilled water into the honey-combed wells of the tray to create a humid atmosphere. The strain reference was put on the elongated flap of the tray. The three strips (0-19, 20-39 and 40-49 were removed from their packaging and separated into five smaller strips (0-9, 10-19, 20-29, 30-39 and 40-49) and placed in the incubation tray.

3.8.1.2 Preparation of the inoculum

The bacteria culture was picked with the help of a sterile swab and mixed with 2ml sterile distilled water in a bijou bottle to make a heavy suspension. 0.5ml of this suspension was transferred to 5ml sterile distilled water in a bijou bottle to make turbidity

equivalent to 2 on the McFarland scale. Using a fresh pipette, 1ml of the 2 McFarland suspension was transferred to an ampoule of API 50 CHL medium and homogenized.

3.8.1.3 Inoculation of the strip

The tubes were filled with the inoculated API 50 CHL medium and overlaid with sterile mineral oil. The strips were incubated at 37^{o}C for 48 hours.

3.8.1.4 Reading the strip

All the tests were read after both 24 and 48 hours of incubation. A positive test corresponds to acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the Esculin test (tube no. 25), a change from purple to black was observed. Identification was done using the Analytical Profile Index.

3.8.2 API 20 C Aux for Yeasts and Molds

3.8.2.1 Preparation of the strip

An incubation box was prepared by distributing about 5ml of sterile distilled water into the honey-combed wells of the tray to create a humid atmosphere. The strain reference was put on the elongated flap of the tray. The strip was removed from its package and placed in the incubation tray.

3.8.2.2 Preparation of the inoculum

A portion of the yeast colony was picked with a sterile swab and mixed with 2ml 0.85% NaCl medium, by making successive touches to make a suspension with turbidity

equivalent to 2 McFarland. Using a pipette, 100ul of the previous suspension was transferred into an ampoule of C Medium. It was gently homogenized with the pipette avoiding the formation of bubbles.

3.8.2.3 Inoculation of the strip

The cupules were filled with the suspension obtained in the ampoule of C Medium. The formation of bubbles was avoided by placing the tip of the pipette against the side of the cupule. Care was taken not overfill or under fill the cupules. The tray was covered with the lid and incubated at 30^{o}C for 48-72 hours.

3.8.2.4 Reading the strip

The strips were read both at 48 and 72 hours of incubation with the 0 cupule serving as control. A cupule more turbid than the control indicates a positive reaction, which were recorded on the results sheets. Identification was done using the Analytical Profile Index.

Plate E: An example of a Lactic acid bacteria isolate on API 50 CH test strip

Plate F: An example of a Yeast isolate on API 20 C test strip

3.9 Shelf Life Studies

Samples of the soy sauce were stored at room temperature. Monthly, samples of the soy sauce were collected and the total microbial numbers, changes in colour, moisture content, pH and sensory qualities determined for six months.

3.9.1Colour Measurement

The colour of the soy sauce was determined with the Minolta Chroma meter using L* a* b* colour system with white tile as standard (CR 200, Minolta Camera Co. Ltd, Japan).

3.9.2 Measurement of pH

The pH was monitored using a digital pH meter with an electrode (Mettler Delta 350, UK).

3.9.3 Plate Count

Serial dilutions 10^{-1} to 10^{-10} of the pasteurized soy sauce were prepared in 9ml volumes of sterile distilled water. Using a fresh pipette tip, 1ml aliquots of each dilution was transferred into sterile petri dishes having a label corresponding to the dilution. To each dilution in the sterile petri dish, was added 15ml of molten Plate Count Agar (PCA) media to cover the base of the petri dishes. The petri dishes were swirled clockwise and anticlockwise to ensure uniform mixing and allowed to set. All plates were incubated at 37^{o}C for 24 hours.

3.9.4 Sensory Test

Monthly, sensory tests were carried out to evaluate the preference and aesthetic qualities of the soy sauce by a team of panelists for six months as part of the shelf life studies.

Panelists were served with 3 coded samples of fried rice prepared with samples of the soy sauce as food condiment. They were asked to observe, taste and smell each one and indicate their perception for each by using the values 9 to 1 (9 point hedonic scale) to access the intensity of saltiness, pungency, colour, umami and overall acceptability.

3.10 Statistical Analysis

Completely Randomised Design (CRD) was used in this experiment and the data was subjected to the analysis of variance using the Genstat (1995) statistical package.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Microbial population of the fermenting extract

4.1.1 Aerobic Plate Count

Initial mean aerobic mesophiles in the fermenting moromi of the treated sample was high ($4.18x10^{19}$cfu/100ml) but dropped sharply to $1.03x10^{10}$ cfu/100ml after seven days of fermentation (Fig. 4.1). This may be due to the composition of the fermenting moromi, which was high in salt (18% brine) and thus unsuitable for the survival of most microorganisms that were not tolerant of high salt concentrations. Hicks, (2002) stated that high salt concentrations around 18%, effectively limits growth to a few desirable osmophilic microorganisms.

There was however a gradual rise in aerobic plate counts numbers from the 14^{th} day until it almost stabilized from the 56^{th} day onwards (Fig. 4.1). However, weekly variations in numbers were statistically significant ($P< 0.05$) but the treated sample compared to the control was not significant ($P> 0.05$). This might be due to changes in the fermenting medium. Aerobic plate count numbers in the control fermentation were generally higher than the treated sample (Fig 4.1). This might be due to the spontaneous fermentation which occurred in the control sample as compared to the treated sample.

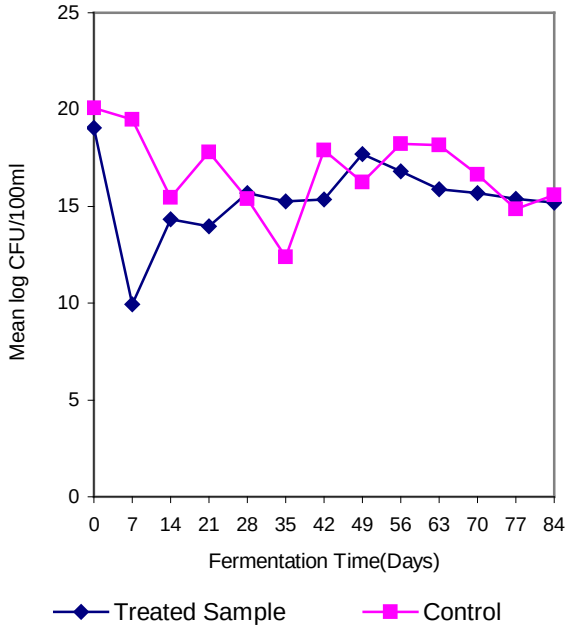

Fig 4.1: Changes in aerobic plate numbers during moromi fermentation

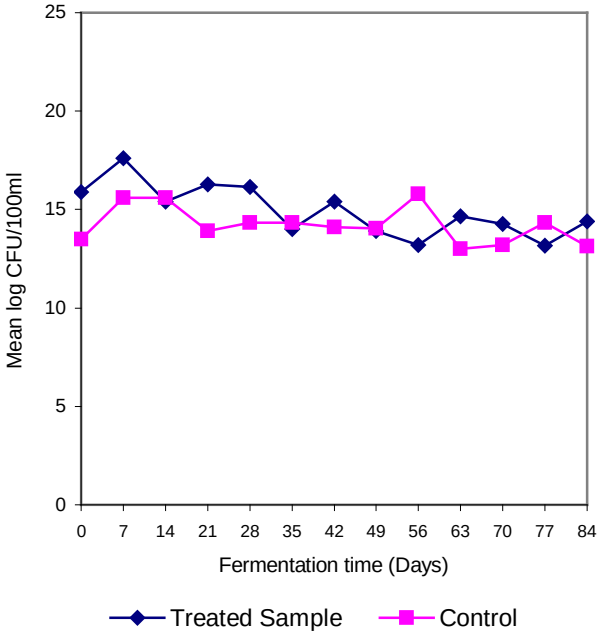

Fig 4.2: Changes in Lactic Acid Bacteria numbers during moromi fermentation

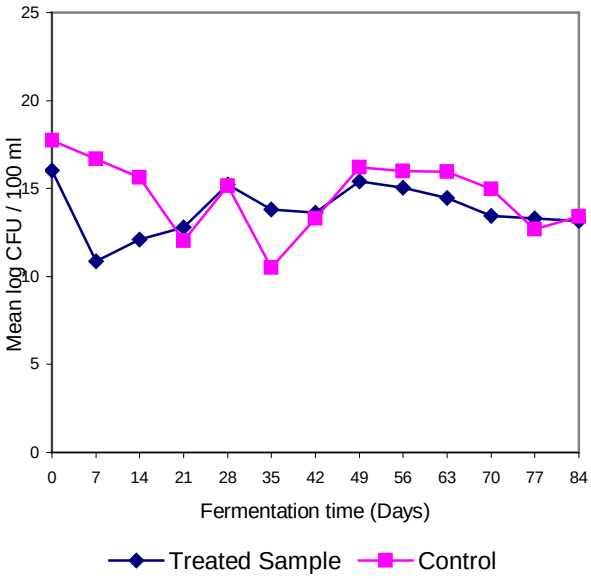

Fig 4.3: Changes in fungi numbers during moromi fermentation

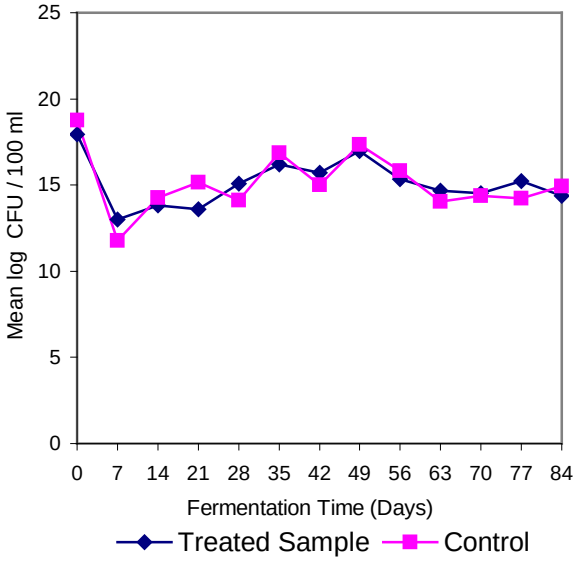

Fig4.4: Changes in yeast numbers during moromi fermentation

4.1.2 Lactic acid bacteria

Initial mean Lactic acid bacteria numbers at the beginning of the bioconversion process was $1.51x10^{16}$ cfu/100ml for the treated sample, but rose to $6.88x10^{17}$ cfu/100ml after seven days of fermentation (Fig.4.2). This supports the fact that about 20% of converted simple sugars is used by lactobacilli as a substrate to produce lactic acid (Hesseltine, 1983).

Variations in counts of lactic acid bacteria between the treated sample and control were not significant ($P> 0.05$). Similarly, weekly changes in lactic acid bacteria numbers were not significant ($P> 0.05$). However, lactic acid bacteria numbers for the treated sample were generally higher than that of the control. This could be due to the use of *Aspergillus oryzae* as the starter culture, which gave high proteolytic, amylolytic enzyme levels, thus releasing more substrate for growth in the treated sample as compared to the control (Sugiyama, 1984).

4.1.3 Yeasts

Yeasts numbers in both treated and control samples were initially high, $3.89x10^{18}$ cfu/100ml and $7.30x10^{18}$ cfu/100ml, respectively (Fig.4.3) but dropped drastically after seven days of fermentation to $1.0x10^{14}$ in the treated sample and $6.00x10^{11}$ in the control. This may be due to the composition of the fermenting medium, which was unsuitable for the survival of some of the yeast cells since the moromi was made up of 18% salt concentration. The high salt concentration effectively limits the growth to a few desirable microorganisms (Hicks, 2002). The yeast numbers generally decreased with time with the control generally higher than the treated sample.

Variations in counts of yeasts between the treated sample and control samples were not significant (P> 0.05). However, weekly changes in yeast numbers were significant (P< 0.05). This might be due to changes in the fermenting medium.

4.1.4 Fungi

Initial mean fungi numbers were high for the treated sample, $2.63x10^{16}$ cfu/100ml but dropped within seven days of fermentation (Fig.4.4). The fungi numbers for the control were generally higher than the treated sample, which may be due to uncontrolled fermentation in the control as compared to the treated sample. Variations in counts of fungi between the treated sample and the control were not significant (P> 0.05). Similarly, weekly changes in fungi numbers were not significant (P> 0.05).

The pattern of distribution on PCA, CDA, MRS and MEA were similar as their numbers generally decreased with time. Comparingly, during the fermenting period, lactic acid bacteria numbers were the most dominant microorganisms followed by the yeasts and fungi numbers. This is similar to that reported by Soyiri *et al.* (2003). The pattern of lactic acid bacteria growth in this study agrees with the findings of Soyiri *et al.* (2003) who showed that growth on de-Mann Rogossa and Sharp (MRS) medium had slightly lower counts compared to total microbial numbers on Plate Count Agar (PCA) in their work on fish sauce from tuna waste in Ghana.

4.2 Characterization of some microorganisms

4.2.1 Lactic acid bacteria

Twenty strains of gram-positive, catalase-negative regular rods, very short rods and coccoids isolated anaerobically on MRS agar were examined by assaying cultures in API 50 CHL galleries.

The most frequently occurring isolate *Tetragenococcus halophilus* fermented L-arabinose, ribose, galactose, glucose, fructose, mannose, *N*-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, celiobiose, maltose, trehalose, melezitose, genitiobiose, and gluconate. The second group of isolates identified as *Lactococcus lactis* fermented ribose, galactose, glucose, fructose, mannose, *N*-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, celiobiose, maltose, trehalose, and gentiobiose. The next was *Lactobacillus delbrueckii*, which fermented glucose, fructose, mannose, *N*-acetyl glucosamine, maltose, and saccharose. The fourth group which were identified as *Lactobacillus plantarum* also fermented L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, α-methyl-mannoside, *N*-acetyl glucosamine, amygdaline, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, melezitose, raffinose, gentiobiose, D turanose and gluconate. One isolate was identified as *Lactobacillus acidophilus* and fermented galactose, glucose, fructose, mannose, *N*-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, celiobiose, maltose, lactose, saccharose, trehalose and gentiobiose.

Table 4.1: Percentage of lactic acid bacteria isolates identified

Species	%
Tetragenococcus halophilus	45
Lactococcus lactis	15
Lactobacillus delbrueckii	15
Lactobacillus plantarum	10
Lactobacillus acidophilus	5
No identification	10

Calculated as ((number of isolated species /total number of isolated species)* 100)

The microorganisms that were observed in the first three weeks of fermentation were mostly lactic acid bacteria. *Tetragenococcus halophilus* (also known as *Pediococcus halophilus*) had the highest percentage (45%), followed by *Lactococcus lactis* (15%), *Lactobacillus delbrueckii* (15%), *Lactobacillus plantarum* (10%) and *Lactobacillus acidophilus* (5%) respectively. Two of the lactics could not be identified (Table 4.1). Tanasupawat *et al.* (2002) reported that the major *Lactobacillus spp.* in moromi fermentation is *Pediococcus halophilus.* Aidoo *et al.* (2006) also noted that *Tetragenococcus halophilus* (*T. halophilus*) initially proliferates and produces lactic acid, which lowers the pH to 5.5 or less. This was also observed in this study (pH 5.5 to 5.2) during the first three weeks of fermentation. Lactic acid produced by the lactic acid bacteria is effective in inhibiting the growth of other bacteria that may decompose or spoil food. The lactic acid bacteria are a diverse group of organisms with a diverse metabolic capacity. This diversity makes them very adaptable to a range of conditions and is largely responsible for their success in acid food fermentations (Battcock and

Axam-Ali, 1998). In addition to lactic acid, they produce organic acids such as acetic acid, which help to control the dominant yeast *Zygosaccharomyces rouxii* in moromi (O'toole, 1997). Roling *et al.* (1996) indicated that the organic acids formed during the growth of *T. halophila* have a preserving effect on the final product. *T. halophila* has been shown to reduce the pH of the moromi, which then sustained the growth of soy yeast and prevent the proliferation of undesirable microorganisms (Roling *et al.,* 1994; Roling and van Verseveld, 1996). Choi *et al.* (2002) reported that *Lactobacillus delbrueckii* completely hydrolyzed soybean isoflavone glucosides into isoflavone aglycones. *Lactobacillus acidophilus* and *Lactobacillus plantarum,* which were also identified in this work, are believed to produce antibiotics and are therefore important in soy sauce fermentation (Tanasupawat *et al.,* 2002). The lactic acid bacteria isolated from the produced soy sauce in this study is similar to that isolated from soy sauce in Thailand by Tanasupawat *et al.* (2002).

4.2.2 Yeasts

Twenty-five strains of yeasts were isolated from the soy sauce during moromi fermentation for characterization. The predominant microorganisms observed after the fourth week of fermentation were *Zygosaccharomyces rouxii* (*Z. rouxii*), followed by *Trichosporon mucoides* (Table 4.2).

The most frequently occurring yeast *Zygosaccharomyces rouxii* (36%) fermented glucose, glycerol, galactose, sorbitol, α-methyl-D-glucoside, maltose, saccharose,

terhalose and raffinose. This was followed by *Trichosporon mucoides* (24%)**,** which fermented glucose, glycerol 2-keto-D-gluconate, D-xylose, xylitol, galactose, inositol, α-methyl-D- glucoside, *N*-acetyl-D-glucosamine, celiobiose, lactose, maltose, trehalose and melezitose. *Cryptococcus humicolus* (12%) also fermented glucose, glycerol, 2-keto-D-gluconate, D-xylose, xylitol, galactose, inositol, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, celiobiose, lactose, maltose, trehalose, melezitose and raffinose. The next was *Cryptococcus laurentii* (8%) and they fermented glucose, 2-keto-D-gluconate, D-xylose, xylitol, galactose, inositol, α-methyl-D- Glucoside, *N*-acetyl-D-glucosamine, celiobiose, lactose, maltose, trehalose, melezitose and raffinose. *Candida ciferrii* (8%) fermented glucose, glycerol, 2-keto-D-gluconate, arabinose, D-xylose, adonitol, xylitol, galactose, inositol, sorbitol, *N*-acetyl-D-glucosamine, celiobiose, maltose, saccharose, trehalose, and raffinose. *Candida famata* (4%) fermented glucose, glycerol, 2-keto-D-gluconate, arabinose, D-xylose, adonitol, xylitol, galactose, sorbitol, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, celiobiose, lactose, maltose, saccharose, trehalose, melezitose and raffinose. *Pichia ohmeri* (8%) also fermented glucose, glycerol, 2-keto-D-gluconate, arabinose, D-xylose, adonitol, xylitol, galactose, inositol, sorbitol, α-methyl-D- Glucoside, *N*-acetyl-D-glucosamine, celiobiose, maltose, saccharose, trehalose, and raffinose.

Table 4.2: Percentage of yeast and mold isolates identified

Species	%
Z. rouxii	36
Trichosporon mucoides	24
Cryptococcus humicolus	12
Cryptococcus laurentii	8
Candida ciferrii	8
Candida famata	4
Pichia ohmeri	8

Calculated as ((number of isolated species /total number of isolated species)* 100)

According to Roling *et al.,* (1996) and Aidoo *et al.,* (2006), *Z. rouxii* is the dominant moromi yeast, which produces alcohol and several compounds that add characteristic aromas to soy sauce. The presence of *Z. rouxii* therefore, was very important since it is responsible for the characteristic flavour of soy sauce. *Z. rouxii* is known to ferment glucose or other sugars into alcohol under high salt concentration during soy sauce fermentation and *Candida spp.* were described as concerned with the production of aroma in soy sauce (Roling *et al.,* 1994; Noda *et al.,* 1980).

Z. rouxii produces flavour compounds including alcohols, glycerol, esters, 4-hydroxy-5-methyl-3 (3 H)-furanone (HMF) and 4-hydroxy-2 (or 5)-ethyl-5 (or 2) -methyl-3 (2 H)-furanone (HEMF). Of the furanones, HEMF produced by *Z. rouxii* and *Candida spp*. gives Japanese-type soy sauce its characteristic flavour (Aidoo *et al.,* 2006). Hayashida *et al.* (1997) showed that inoculation of *Z. rouxii* into a soy sauce mash free from lactic acid bacteria resulted in the formation of the flavour-active compound HEMF and that this soy sauce had the highest concentration of HEMF (around 50-250 mg/L), the major flavour note in soy sauce. This compound is also reported to have anti-tumour and anti-oxidative

properties (Nagahara *et al.*, 1992; Koga *et al.*, 1998). HEMF has also been identified as a sweet-tasting compound with a caramel-like odour, which significantly contributes to the flavour of some fermented foods such as soy sauce (Blank and Fay, 1996). Pariza, (1994) reported that inclusion of the compound HEMF in the diet of mice at 4 mg/kg body weight significantly reduced carcinogen-induced forestomach neoplasia. Several other workers: Lee (1995), Barnes *et al*. (1995) and Stephens (1997) noted that fermented soy products might reduce the risk of breast, colon, lung and stomach cancers, which is as the result of HEMF produced by *Z rouxii*. *Candida spp.* also produces phenolic compounds, i.e. 4-ethylguaiacol and 4-ethylphenol, which contribute to soy sauce aroma (Aidoo *et al.*, 2006; Hesseltine, 1983). The presence of *Pichia spp, Triscosporon spp*, and *Cryptococcus spp* in this work has also been reported by Noda *et al.* (1980) as not so important in the production of soy sauce, but are occasionally observed during brine fermentation.

According to Mountney and Gould (1988), fermentation is generally initiated by bacteria, followed by yeasts and then molds. There are definite reasons for this type of sequence. The smaller microorganisms are the ones that multiply and take up nutrients from the surrounding area most rapidly. Bacteria are the smallest of the microorganisms, followed by yeasts and molds. The smaller bacteria, such as *Leuconostoc* and *Streptococcus* grow and ferment more rapidly than their close relations and are therefore often the first species to colonise a substrate.

4.3 Changes in pH and Temperature during fermentation

In the fermentation process, the treated sample had pH values ranging from 4.12 to 5.46 whilst the control ranged from 4.24 to 5.91 (Table 4.3). The pH of the treated sample remained fairly constant for the first 14 days of fermentation after which there was a significant drop in pH ($P< 0.05$). The control also remained fairly constant for the first 21 days of fermentation after which there was a significant drop in pH ($P< 0.05$). Roling and Verseveld (1996) reported a drop in pH during soy sauce fermentation and attributed it to the production of acids during the bioconversion process, which was due to the involvement of lactic acid bacteria that were able to ferment sugars to produce lactic acid. The differences in pH between the treated sample and the control could be attributed to the availability of fermentable sugars coupled with the population of lactic acid bacteria at the time, as well as the presence of enzymes inherent in the raw material used or produced by microbes in the treated sample as compared to the control.

The temperature was fairly constant during the fermentation period with the control generally higher than the treated sample. The temperature of the treated sample ranged from 29.00 to 33.96 whilst the control ranged from 29.40 to 34.00 (Table 4.3). Variations between the treated sample and the control were not significant ($P> 0.05$) however weekly variations were significant ($P< 0.05$). Bajracharya *et al.* (1992) reported fermentation temperature for moromi between 30-55^{o}C and therefore the fermentation temperatures for both the treated sample and the control were within acceptable limits.

Table 4.3 Changes in pH and temperature during moromi fermentation of soy sauce

Fermentation Time	Treated Sample		Control	
(Days)	pH	T (℃)	pH	T (℃)
0	5.46	29.00	5.91	29.40
7	5.43	30.40	5.87	30.91
14	5.35	30.70	5.82	31.10
21	5.21	30.71	5.42	31.22
28	4.72	31.40	5.32	31.50
35	4.26	31.53	5.42	32.00
42	4.24	31.89	5.40	32.21
49	4.37	32.20	4.80	32.43
56	4.62	32.23	4.85	32.88
63	4.35	32.76	4.67	33.11
70	4.51	32.90	4.61	33.25
77	4.33	33.12	4.51	33.44
84	4.12	33.96	4.24	34.00

4.4 Shelf life studies

Shelf life, or the maximal period of time during which the predetermined quality attributes of food are retained, is a critical factor in the consumer acceptability and the economic feasibility of a product (Kroll, 1995).

The modern food industry has developed and expanded because of its ability to deliver a wide variety of high quality food products to consumers on a nation wide and worldwide basis. This feat has been accomplished by building stability into the productions through processing, packaging and additives that enable foods to remain fresh and wholesome throughout the distribution process. Consumer demands for convenience have fueled new innovations in the food product development, packaging and chemical industries, and

widespread desire for products to use in the microwave oven has added further impetus to this effort (Sewald and De Vries, 2003). The shelf life of a product is therefore critical in determining both its quality and profitability (Kroll, 1995).

4.4.1 Plate Count of Soy Sauce

Plate count of the pasteurized samples ranged from 6.00 x 10^4 to 11.00 x 10^4 cfu/ml (Table 4.4). The difference in number between the treated samples (A^1 and B^1) and the control (C^1) were not significantly different (P> 0.05). This might be due to pasteurization, which arrested the microorganisms from further fermentation. Hicks (2002) showed that the pasteurization heat inactivates organisms or enzymes of spoilage significantly in foods. Changes in microbial numbers over the six months period were not significant and this also contributed to the stability of the products on the shelf. The non-significant change in microbial numbers is also due to the right pH and moisture content, which did not support the growth of microorganisms (Sewald and De Vries, 2003). According to Labuza (2006), a food product is spoilt when the number of microbes is around 10 x 10^7 cfu/ml. Therefore, the total microbial numbers in this study were within acceptable limits for the six months period. The non-significant difference in microbial numbers was also due to the high salt concentration in the soy sauce. High salt concentration makes water unavailable for microbial growth and cells of microbes become plasmolysed (Hicks, 2002).

Kim *et al.* (2003) indicated that for seasoned *tofu*, shelf life estimates based on a microbial criterion of 10^6 cfu/g represented a more conservative estimate than those based on sensory quality and concluded that microbial quality was generally to be a common and sensitive criterion for shelf life determination. Therefore this shelf life studies using microbial count, as an index was very important since action by microorganisms is a common means of food spoilage. Singh and Anderson, (2004) reported that the growth of most microorganisms can be prevented or slowed down by adjusting initial microbial numbers and temperature of storage, reducing water activity, lowering pH, the use of preservatives and use of proper packaging.

Table 4.4 Plate count (10^4 CFU) of soy sauce samples stored over a six-month period

Month	Sample		
	A^1	B^1	C^1
1	6.00a	7.00a	9.00a
2	7.00a	6.00a	10.00a
3	7.00a	8.00a	10.00a
4	8.00a	9.00a	11.00a
5	6.00a	6.00a	10.00a
6	7.00a	7.00a	10.00a

A^1 =Treated sample 1, B^1 = Treated sample 2 and C^1 = Control
Samples (A^1, B^1 and C^1) with the same superscript are not significantly different at ($p > 0.05$)
Figures with the same letter are not significantly different ($p > 0.05$)

4.4.2 Colour

The product colour readings from the chroma meter ranged from 41.15 to 42.24 (Table 4.5). The colour of the treated samples (A^1 and B^1) after the fermentation period was dark/reddish brown whilst the control (C^2) was light brown in colour. The samples were

further darkened with the addition of caramel. Treated sample one (A^1) was measured to be the darkest and the control (C^2) the least dark. There were significant differences between the treated samples and the control. The differences could be due to the use of starter culture in A^1 and B^1 but none in C^2. The development of the dark/reddish brown colour is by Maillard reaction; some of the amino acids and sugars produced by proteolytic and amylolytic enzymes released by the starter culture undergo this reaction to give the colour (Kikkoman, 2004). According to Yokotsuka, (1981), good koji fermentation gives colour to the product and this was achieved by the use of the starter culture. The intense dark colour could be attributed to the intensive Mailard reaction as a result of good fermentation processes. Colour changes over the six-month period were not significantly different which means the products were stable on the shelf. Sewald and De Vries (2003) indicated that chemical reaction such as Mailard browning reactions and enzymatic activity causing colour changes is a principal mechanism involved in the deterioration of processed food. However this did not occur in the produced soy sauce because of its acidity. Singh and Anderson (2004) showed that Mailard browning reaction is very dependent on pH and seldom occurs at low pH. They further stated that, browning reactions such as caramelization can occur with carbohydrates but require higher temperatures than products would typically be subjected to during distribution and storage. Enzymatic activity did not also occur in the produced soy sauce because of the pasteurization, which inactivated the enzymes of spoilage significance (Hicks 2002).

Table 4.5: Color (L* values) of soy sauce stored over a six-month period

Month	Sample A[1]	B[1]	C[2]
1	41.19a	41.20a	42.24a
2	41.30a	41.30a	42.30a
3	41.16a	41.16a	42.31a
4	41.15a	41.16a	42.28a
5	41.15a	41.16a	42.33a
6	41.30a	41.30a	42.31a

A^1 =Treated sample 1, B^1 = Treated sample 2 and C^2 = Control
L lightness /darkness: 100/0
Samples (**A^1**and **B^1**) with the same superscript are not significantly different ($p > 0.05$)
Color changes over the six months period are not significantly different ($p > 0.05$).

4.4.3 pH

The pH of the samples ranged from 4.73 to 4.88 which agrees with the suggested pH range of 4.7 to 4.8 by Hesseltine (1983). The treated sample (B^1) had the highest pH whilst the control (C^2) had the least (Table 4.7). The pH of the treated samples (A1 and B1) were significantly different from the control (C^2). This may be due to different fermentation processes that took place in the treated samples and the control. The pH variations in the soy sauce samples over the six months storage period were not significant. The stable moisture content and microbial numbers might have contributed to the stability of the pH since these factors may have caused changes in biochemical reactions. Hicks (2002) noted that fermentation is a form of preservation used to slow down spoilage factors through the production of alcohol and acids and this is normally combined with pasteurization. Battcock and Axam-Ali (1998) also stated that most food spoilage organisms cannot survive in either alcoholic or acidic environments. Therefore,

the production of these end products prevented the soy sauce from spoilage and extended the shelf life.

Table 4.6: pH of soy sauce samples stored over a six-month period

Month	Sample		
	A^1	B^1	C^2
1	4.87a	4.87a	4.75a
2	4.84a	4.80a	4.73a
3	4.82a	4.88a	4.70a
4	4.80a	4.81a	4.65a
5	4.86a	4.80a	4.73a
6	4.73a	4.86a	4.70a

A^1 =Treated sample 1, B^1 = Treated sample 2 and C^2 = Control
The pH values of samples **(**$A^1$and B^1**)** with the same superscript are not significantly different ($p > 0.05$)
The pH of samples throughout the six-month period was not significant ($p > 0.05$).

4.4.4 Sensory analysis of soy sauce.

Sensory analysis is a scientific discipline used to evoke, measure, analyze, and interpret reactions to the characteristics of food and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing (Meilgaard *et al*, 1991).

In the food industry and in academic research, sensory evaluation is used for product development, grading, quality control and assurance, marketing, measuring and predicting shelf life (Meilgaard *et al.*, 1991). It is an invaluable tool used to ensure that products meet consumer specifications and demands, thus there will be a low degree of market failure. Consumer acceptance is the ultimate determinant of a product's success in the marketplace.

The sensory characteristics used in the shelf life studies were colour, pungency, saltiness, umami and overall acceptability. This is because soy sauce is defined as a brown liquid with a salty and a pungent but pleasant meaty flavour (umami) that is used as a savoury condiment in Oriental cuisine (Hoversnail, 2000).

4.4.4.1 Colour

The panelists ranged color from 6.67 to 7.89 (Fig. 4.5). The treated samples (1 and 2) were rated as being darker than the control (3). The colours of the treated samples (1 and 2) were significantly ($p < 0.05$) darker than the control (3). The development of colour is due to browning reaction and this might have taken place well in the treated samples (1 and 2) compared to the control (3) as a result of different rates of fermentation processes. There were no significant differences in colour for all the samples over the six months storage period. This might be due to the stable microbiological condition and also the presence of salt acting as a preservative during the storage period.

4.4.4.2 Pungency

One good attribute of soy sauce is pungency and this comes from the organic acids, lactic acid and succinic acid, which are produced during fermentation and maturation of the moromi (Kikkoman, 2004). The treated samples (1 and 2) were rated as being stronger (7.83) (Fig. 4.5). The treated samples which had their pH as 4.8 were rated by panelists as more pungent than the control. Pungency over the six months storage period however did not change significantly ($P > 0.05$) which may be due to stable storage conditions.

4.4.4.3 Saltiness

The salty sensations as evaluated by the panelists were between the range of 5.95 and 6.18 (Fig. 4.5). Even though there were slight differences in the salt content of the various samples, they were not significant ($p > 0.05$). This could be due to the same treatment (18% salt), which was given to the various samples. The salt content over the six months storage period also did not change significantly ($P > 0.05$). Salt enhances the taste of the soy sauce as well as acting as a preservative.

4.4.4.4 Umami

This is translated as “delicious”, “savoury” or “meaty” flavour, which is elicited by amino acids mainly glutamic acid (Yamaguchi, 1998; Dzogbefia *et al.,* 2007). The umami taste ranged between 6.44 and 7.52 (Fig. 4.5). The umami taste was high in the treated samples compared to the control, which was also significant ($p < 0.05$). This could also be attributed to the use of the starter culture, which enhanced the synthesis of more amino acids including glutamic acid giving the treated samples its meaty flavour. The burnt, smoky/roasty (umami) is a unique flavour of soy sauce. Kumakura (2004) noted that the flavour of soy sauce gives a pleasant odour to the food in which they are used as condiments. The umami flavour is as a result of chemical reactions involving ethanol, amino acids and sugars during and after fermentation (Yamaguchi, 1998). The umami taste over the six months storage period for all the samples did not significantly change ($P> 0.05$).

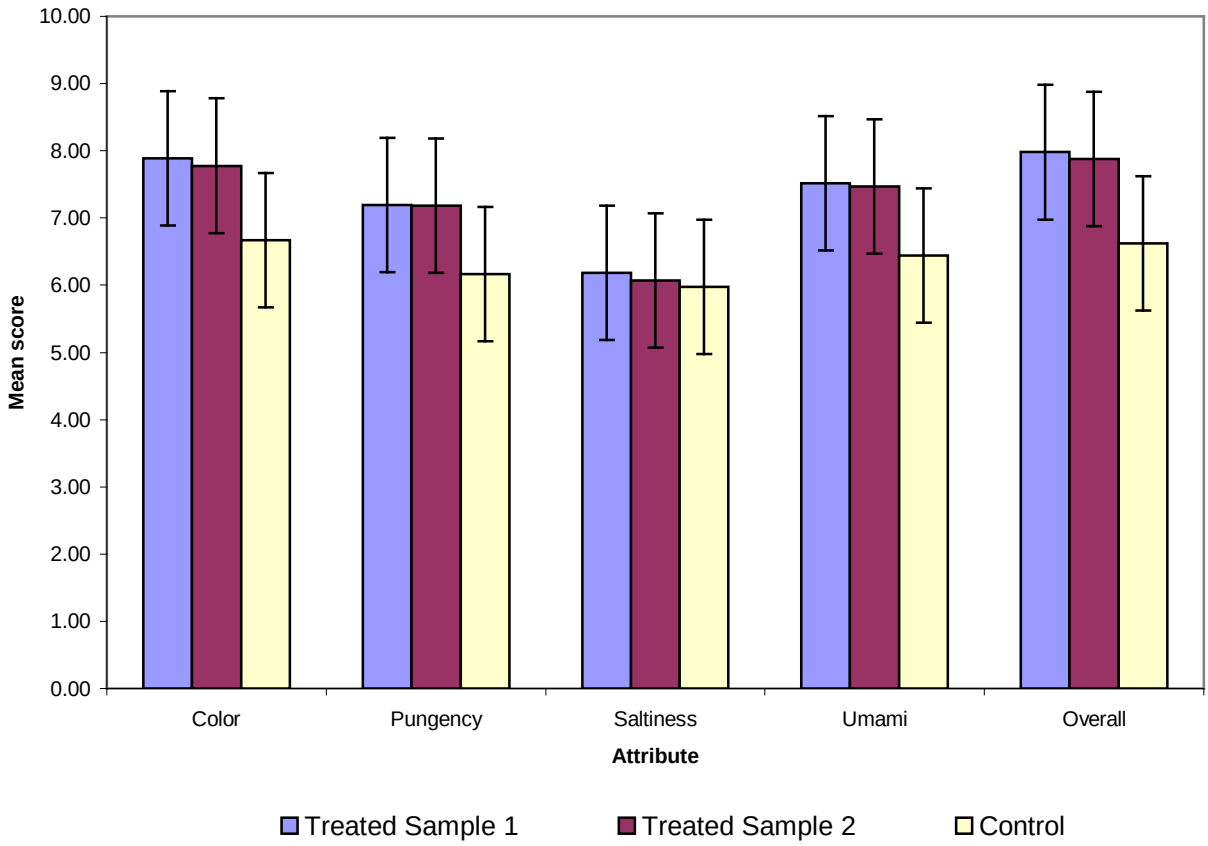

Fig. 4.5: Sensory characteristics of soy sauce samples

4.4.4.5 Overall acceptability

Treated sample 1 was the most preferred followed by sample 2 and 3 (Fig.4.5). They were rated between 6.62 (like) and 7.98 (like slightly) on a 9-point hedonic scale. The treated samples were significantly different from the control ($p < 0.05$), which could be due to the controlled fermentation that might have taken place in the treated sample as compared to the control. Yokotsuka, (1981) stated that, good koji fermentation should: (a) give good flavor product; (b) produce a good amount of spores; (c) have a high percentage spores that germinate and grow rapidly; (d) have high enzymatic activity; (e) have good genetic stability; (f) give colour to the product; (g) produce no mycotoxins and (h) produce mash that is easy to press. The treated sample 1 that had controlled

fermentation was chosen since consumer acceptability is based on good colour and flavour and this was achieved by the use of a starter culture.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Soy sauce was produced from a locally cultivated soybean (Anidaso) using *Aspergillus oryzae* as the starter culture for the first stage (Koji) of fermentation.

Five different LAB were isolated and identified namely *T. halophilus, L. lactics, L. delbrueckii, L. plantarum and L. acidophilus.* Seven different yeasts were isolated and identified namely *Z. rouxii, T. mucoides, C. humicolus, C. laurentii, C. ciferrii, C. famata and P. ohmeri.* Undoubtedly, inoculation of the soy mash with these strains of microorganisms appropriately will hasten the moromi fermentation and shorten the time.

The shelf life of the locally produced soy did not have significant changes in the microbial load, colour, moisture content, pH, and all the sensory attributes (colour, saltiness, pungency, umami and overall acceptability) over the six months storage under room temperature.

5.2 Recommendation

Further work should be carried out to compare the technological properties of the isolates in order to select the best and combination of strains for starter culture in moromi fermentation.

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Appendix 1

ANALYSIS OF VARIANCE OF PRODUCT

Microbial population of the fermenting extract
PCA

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	9.149E+38	9.149E+38	3.84	0.057
Time	12	2.397E+40	1.997E+39	8.38	<.001
Residual	38	9.055E+39	2.383E+38		
Total	51	3.394E+40			

Tables of means

Grand mean 8.E+18

Sample	1.00	2.00
	4.E+18	1.E+19

Time	0.00	7.00	14.00	21.00	28.00	35.00	42.00
	8.E+19	2.E+19	3.E+15	3.E+17	3.E+16	5.E+16	8.E+17

	49.00	56.00	63.00	70.00	77.00	84.00
	9.E+17	2.E+18	7.E+17	1.E+17	7.E+15	2.E+16

Least significant differences of means

Table	Sample	Time
rep.	26	4
d.f.	38	38
l.s.d.	8.67E+18	2.21E+19

CDA

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	6.962E+34	6.962E+34	1.05	0.314
Time	12	7.015E+35	5.846E+34	0.88	0.575
Residual	31	2.059E+36	6.642E+34		

Total	44	2.829E+36

Tables of means

Grand mean 5.E+16

Sample	1.00	2.00
	1.E+16	9.E+16

Time	0.00	7.00	14.00	21.00	28.00	35.00	42.00
	1.E+16	2.E+15	5.E+17	4.E+16	2.E+16	1.E+14	1.E+16

	49.00	56.00	63.00	70.00	77.00	84.00
	5.E+16	1.E+16	2.E+16	1.E+16	1.E+16	1.E+16

Least significant differences of means

Table	Sample	Time
rep.	26	4
d.f.	31	31
l.s.d.	1.46E+17	3.72E+17

MRS

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	2.149E+34	2.149E+34	1.21	0.293
Time	12	2.166E+35	1.805E+34	1.02	0.488
Residual	12	2.128E+35	1.773E+34		
Total	25	4.509E+35			

Tables of means

Variate: MRS

Grand mean 3.E+16

Sample	1.00	2.00
	6.E+16	1.E+15

Time	0.00	7.00	14.00	21.00	28.00	35.00	42.00
	8.E+15	3.E+17	4.E+15	1.E+16	9.E+15	5.E+14	3.E+15

Time	49.00	56.00	63.00	70.00	77.00	84.00
	1.E+14	3.E+15	1.E+15	1.E+14	1.E+14	1.E+14

Least significant differences of means

Table	Sample	Time
rep.	13	2
d.f.	12	12
l.s.d.	1.14E+17	2.90E+17

Variate: sample_(MEA)2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	12	1.192E+38	9.935E+36	4.04	0.002
Sample	2	5.415E+36	2.707E+36	1.10	0.348
Residual	24	5.896E+37	2.457E+36		
Total	38	1.836E+38			

Tables of means

Variate: sample_2

Grand mean 5.E+17

Time	0.00	7.00	14.00	21.00	28.00	35.00	42.00
	7.E+18	8.E+13	3.E+16	2.E+14	4.E+16	3.E+16	4.E+15

Time	49.00	56.00	63.00	70.00	77.00	84.00
	3.E+17	4.E+15	2.E+16	2.E+15	2.E+16	4.E+15

SAMPLE	1	2	3
	6.E+17	7.E+16	1.E+18

Least significant differences of means

Table	Time	Sample
rep.	3	13
d.f.	24	24
l.s.d.	2.641E+18	1.269E+18

Appendix 2

pH during fermentation period

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	2.6235	2.6235	9.80	0.003
Residual	50	13.3804	0.2676		
Total	51	16.0039			

Tables of means

Grand mean 4.915

Sample	1.00	Control
	4.690	5.139

Least significant differences of means

Table	Sample
rep.	26
d.f.	50
l.s.d.	0.2882

Appendix 3

Temperature during fermentation

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	1	1.674	1.674	1.03	0.315
Residual	50	81.287	1.626		
Total	51	82.961			

Tables of means

Grand mean 31.94

Sample	1.00	2.00
	31.76	32.12

Least significant differences of means

Table	Sample
rep.	26
d.f.	50
l.s.d.	0.710

Appendix 4

SHELF LIFE STUDIES

MICROBIAL LOAD

Analysis of variance

Variate: M_load

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	8.889E+07	4.444E+07	0.11	0.892
Month	5	8.222E+08	1.644E+08	0.42	0.828
Residual	28	1.088E+10	3.885E+08		
Total	35	1.179E+10			

Tables of means

Variate: M_load

Grand mean 69444.

Sample	1.00	2.00	3.00
	68333.0	71667.0	68333.0

Month	1.00	2.00	3.00	4.00	5.00	6.00
	68333.0	66667.0	75000.0	76667.0	63333.0	66667.0

Least significant differences of means

Table	Sample	Month
rep.	12	6
d.f.	28	28
l.s.d.	16482.8	23310.2

L COLOUR

Variate: Color

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	4.701678	2.350839	1233.68	<.001
Month	5	0.035978	0.007196	3.78	0.035
Residual	10	0.019056	0.001906		
Total	17	4.756711			

Tables of means

Variate: Color

Grand mean 41.572

Sample	1.00	2.00	3.00
	41.210	41.212	42.295

Month	1.00	2.00	3.00	4.00	5.00	6.00
	41.543	41.633	41.543	41.530	41.547	41.637

Least significant differences of means

Table	Sample	Month
rep.	6	3
d.f.	10	10
l.s.d.	0.0562	0.0794

pH (after pasteurization)

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	0.111901	0.055950	30.22	<.001
Month	5	0.022359	0.004472	2.41	0.061
Residual	28	0.051848	0.001852		
Total	35	0.186108			

Tables of means

Grand mean 4.7889

Sample	1.00	2.00	3.00
	4.8188	4.8371	4.7107

Month	1.00	2.00	3.00	4.00	5.00	6.00
	4.8300	4.7920	4.7993	4.7552	4.7952	4.7617

Least significant differences of means

Table	Sample	Month
rep.	12	6
d.f.	28	28
l.s.d.	0.03599	0.05089

Sensory

Color

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	162.8926	81.4463	124.02	<.001
Month	5	3.1870	0.6374	0.97	0.435
Residual	532	349.3630	0.6567		
Total	539	515.4426			

Tables of means

Grand mean 7.446

Sample	1.00	2.00	3.00
	7.889	7.778	6.672

Month	1.00	2.00	3.00	4.00	5.00	6.00
	7.322	7.522	7.400	7.511	7.522	7.400

Least significant differences of means

Table	Sample	Month
rep.	180	90
d.f.	532	532
l.s.d.	0.1678	0.2373

Pungency

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	125.4037	62.7019	67.86	<.001
Month	5	6.5926	1.3185	1.43	0.213
Residual	532	491.5519	0.9240		
Total	539	623.5481			

Tables of means

Grand mean 6.848

Sample	1.00	2.00	3.00
	7.194	7.183	6.167

Month	1.00	2.00	3.00	4.00	5.00	6.00
	6.967	6.744	6.689	6.978	6.911	6.800

Least significant differences of means

Table	Sample	Month
rep.	180	90
d.f.	532	532
l.s.d.	0.1990	0.2815

Salty

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	3.8111	1.9056	2.63	0.073
Month	5	1.8000	0.3600	0.50	0.778
Residual	532	385.1222	0.7239		
Total	539	390.7333			

Tables of means

Grand mean 6.078

Sample	1.00	2.00	3.00
	6.183	6.072	5.978

Month	1.00	2.00	3.00	4.00	5.00	6.00
	5.989	6.156	6.056	6.100	6.133	6.033

Least significant differences of means

Table	Sample	Month
rep.	180	90
d.f.	532	532
l.s.d.	0.1762	0.2492

Umami

ANOVA Table

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	2	132.4778	66.2389	76.31	<.001
Month	5	6.4444	1.2889	1.48	0.193

```
Residual                  532   461.8111     0.8681
Total                     539   600.7333
```

Tables of means

```
Grand mean 7.144

Sample      1.00      2.00      3.00
            7.517     7.472     6.444

Month      1.00      2.00      3.00      4.00      5.00      6.00
           7.000     7.222     7.100     7.022     7.278     7.244
```

Least significant differences of means

```
Table                Sample       Month
rep.                    180          90
d.f.                    532         532
l.s.d.               0.1929      0.2728
```

Means scores sensory

Sample	Color	Pungency	Saltiness	Umami	Overall
1	7.89	7.19	6.18	7.52	7.98
2	7.78	7.18	6.07	7.47	7.88
3	6.67	6.17	5.98	6.44	6.62