The Islamic University-Gaza
Deanship of Graduate Studies
Faculty of Science
Biological Sciences Master Program
Microbiology

# Isolation of *Aspergillus oryzae* and New Aroma Production for Soy Sauce

by

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**Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Biological Sciences/ Microbiology**

**1429هـ - 2008م**

## Declaration

The study presented in this thesis was started and completed by the author, a post graduate student in the Faculty of biological science of the Islamic University, Gaza, Palestine, under supervision of Dr. Tarek Elbashiti & Dr. Abboud Elkichaoui.

I certify that the work presented in this thesis has not been submitted to any other University. Any help received in preparing this thesis and all sources used, have been specifically acknowledged.

Amal Fayyad

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## Abstract

*Aspergillus oryzae* is member of flavi group, used in fermentation of oriental foods for centuries. The purpose of this study is to isolate and characterize *Aspergillus oryzae* strain for using in soy sauce production with specific aroma, depending on local plants as thyme and dill.

The isolates were cultured on Potato Dextrose Agar (PDA), *Aspergillus flavus* and *parasiticus* Agar (AFPA) was used to differentiate the isolates from *Aspergillus flavus* and *Aspergillus parasiticus* depended on the reverse color. C Zapeck Yeast Extract Agar (CYA) was used in identification.

The preparation of the soy sauce was carried out by two stages. The first was Koji**,** which was prepared by mixing isolates and reference strains with steamed soybeans and crushed millet for three days. The second was the brine, which consisted of koji and salt solution. This stage was maintained for three months at 30°C by continuing stirring. The brine, then, was filtered, pasteurized and aroma plants were added. The enzymes of *A. oryzae* had the essential role in the fermentation process in addition to the lactic acid bacteria, *Z. rouxii* and *C. versitils*.

The results of analysis of soy sauce showed that the pH was 4.16, 4.65, 4.25, the concentration of ethanol was 0.11%, 0.57%,0.92% (v/v), NaCl concentration was 14.04%, 16.38%, 15.4% (w/v) and Ca concentration was 123, 127, 102 (mg/100g) for the reference, rice isolate and soybean isolate, respectively. In addition, ash contents, total solids and moisture of our product were closed to the commercial one. The color of the soy sauce was dark brown with thyme and dill aroma. It can be concluded that the quantity of water was very critical in the fungus growth. Through the brine fermentation, proteins and carbohydrates were decomposed into their fragments a process accompanied by production of many flavors. The addition of dill and thyme were gave a specific aroma to the final product. The high salt concentration was very necessary to prevent any decay in the product.

**Keywords:** *A. oryzae*, Koji , Brine, Soy sauce and Aroma.

## عزل فطر الاسبرجلس واستخدامه في إنتاج صلصة الصويا بنكهات جديدة


### الخلاصة

ينتمي فطر الاسبرجلس أوريزا الى عائلة الفلافس ويستخدم في تخمير الغذاء في الشرق الأقصى منذ عدة قرون.

الهدف الرئيس للدراسة هو عزل الفطر والتعرف على خصائصه و استخدامه في انتاج صلصة الصويا بنكهات مختلفة تعتمد على نباتات محلية مثل الزعتر والجرادة.

لقد قمنا بعزل فطر الأورايزا من خلال تنميته على أوساط غذائية هي, (PDA) لنمو الفطر و (AFPA) التي تميزه عن الفلافس السام و(CYA) التي تستخدم لتعريف الفطر. كذلك الفطر الضابط تمت زراعته على هذه الأوساط.

مرت عملية إنتاج صلصة الصويا بمرحلتين الأولى تسمى Koji والثانية Brine فالأولى تكونت من مزيج من الفطر المعزول إضافة الى حبوب من فول الصويا المبخرة و كذلك الدخن المجروش وتركت لمدة ثلاثة أيام ، أما الثانية فهي عبارة عن Koji مع محلول ملحي حيث تركت لمدة ثلاثة أشهرعلى درجة حرارة 30˚م مع التحريك المستمر للخليط. في النهاية يصفى الخليط ويبستر وتوضع به النباتات العطرية. تعتبر أنزيمات الأورايزا مصدرا أساسيا لعملية التخمر ويشاركها في ذلك بكتيريا حمض اللاكتيك وخميرة روكسي والكانديدا.

أظهرت نتائج التحليل للمنتج لكل من الفطر الضابط والمعزول من الأرز والمعزول من حبوب الصويا على النحو التالي:

درجة الحموضة 4.16، 4.65، 4.25 و تركيز الكحول, 0.11%، 0.57% , 0.92%

،تركيز الملح 14.04%، 16.38% , 15.4%و تركيز الكالسيوم 127, 123، 102 ملجم.

إضافة لذلك كانت نسب الرماد والرطوبة والمواد الصلبة موافقة لمنتجات تجارية في مناطق أخرى . المنتج النهائي ذو لون بني وبنكهة الزعتر والجرادة. ولقد استنتجت الدراسة ضرورة ضبط كمية الماء اللازمة لنمو الفطر اثناء مرحلة Koji . وكذلك تبين في اثناء تحليل البروتين والكربوهيدرات انطلاق نكهات عطرية واضافة الزعتر والجرادة أعطته نكهة مميزة وأيضا ضبط كمية الملح للمنتج ضرورية لحمايته من التحلل.



الكلمات المفتاحية: فطر الأسبرجلس أورايزا، koji ، Brine، صلصة الصويا، النكهة.

## Dedication

**To my parents**

# Acknowledgment

All the praises and thanks be to Allah for all things that subjected to my study. .

I would like to express my sincere appreciation to the following people for their contribution towards the successful completion of this thesis.

- Dr. Tarek Elbashiti & Dr. Abboud Elkichaoui for their valuable supervision and guidance throughout this study.
- Thawatchai for supplying me a control microorganism.
- Dr. Ahmed Thabet and Mohamed Mater for their assistance in samples analysis. .
- The laboratory staff of the biology and Medical Technology Department for their helpful support all the time .
- Thanks to Environmental & rural research center at the Islamic University-Gaza for helping in protein analysis.

- Thanks to the Deanship of science and research at the Islamic University-Gaza.
- Many thanks to my supporting family and friends

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## List of abbreviation

| | |
|---|---|
| AFPA | Aspergillus flavus and parasiticus Agar |
| ACP | Acid phosphatase |
| CYA | C- zapeck yeast Extract Agar |
| EPA | Environmental protection Agency |
| 4-EG | 4-ethylguaiacol |
| FPC | Flatulence-producing compounds |
| (FDP) | Fructose 1,6-diphosphate |
| GRAS | Generally Regarded As Safe |
| HEMF | 4-hydroxy-2 (or 5)-ethyl-5(or 2)-methyl-3(2H)- furanone |
| ISTA | International seed testing association |
| (KDG) | 2-keto-3-deoxy-D-gluconate |
| PMN | Pre manufacture notice |
| (PTWI) | Provisional tolerable weekly intake |
| (PDA) | Potato Dextrose Agar |
| SERMs | Selective estrogen receptor modulators |
| SPS | Shoyu polysaccharides |
| SmF | Submerged fermentation |
| SSF | Solid-state fermentation |

# Chapter 1

# Introduction

*Aspergillus oryzae* is a member of the *A. flavus* group of a spergillus species. The *A. flavus* group, which also includes *A. sojae, A. nomius and A. parasiticus* are defined by the production of spore chains in radiating heads which range in color from yellow-green to olive brown. *A. flavus* and *A parasiticus* are known to produce the potent carcinogen aflatoxin. *A. oryzae* and A. soji have been used for producing food grade amylase and fermentation of oriental foods for centuries **[1, 2]**.

Soy sauce is a dark brown salty liquid with a peculiar aroma and a meaty taste. It is the chief savory-seasoning agent in Oriental cookery, but it is becoming increasingly popular in many other regions of the world. Industrialization has altered the production process, changed the raw materials used, standardized the products and modified somewhat their characteristics **[3].**

The preparation of soy sauce by traditional methods can be shown into the following figure (Figure 1.1) **[4]**.

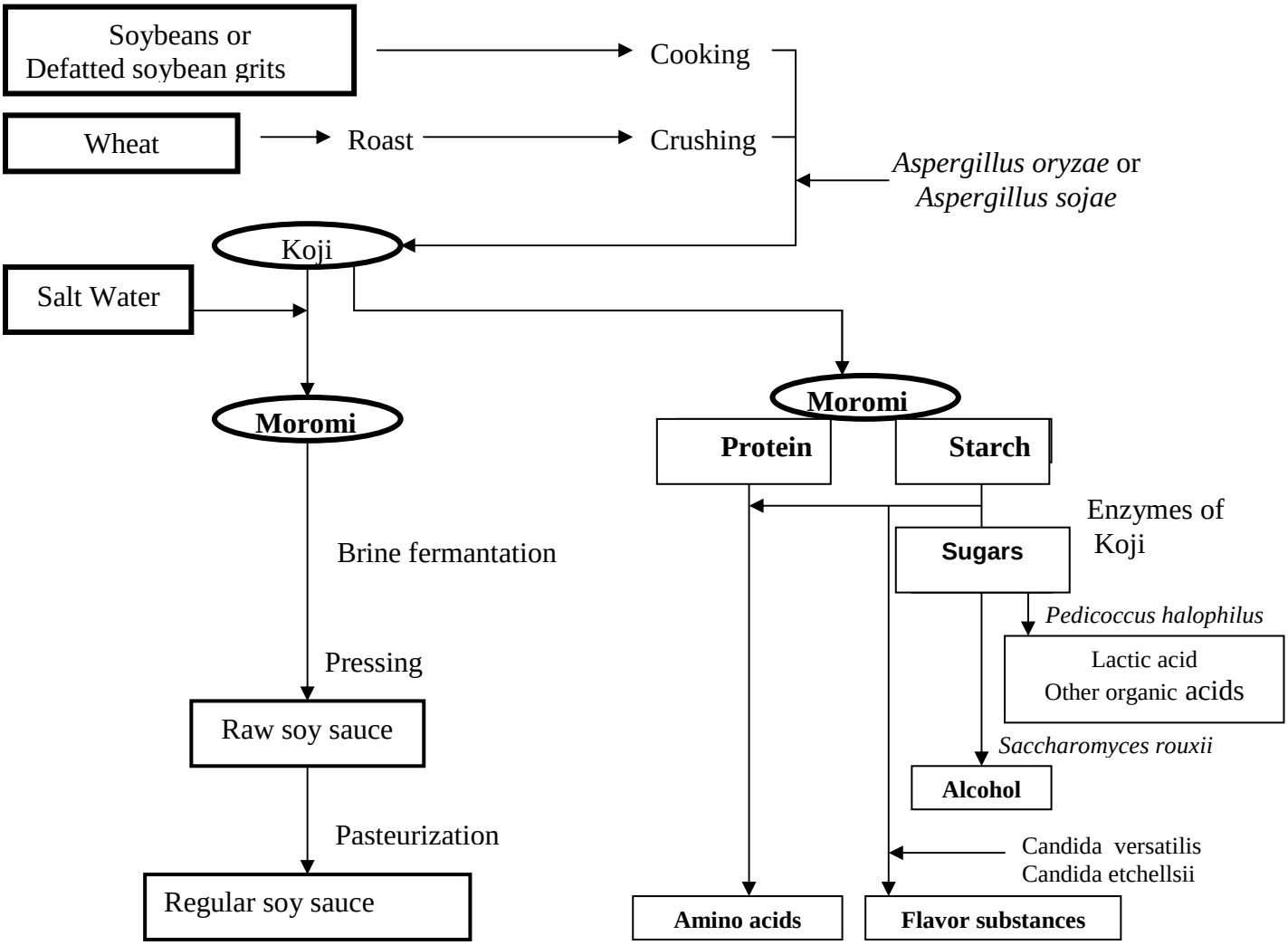



**Figure (1.1)** The manufacturing process of fermented soy sauce

This traditional process can be broken down into two sequential fermentation steps:

The first, called koji fermentation, a solid substrate aerobic fermentation of cooked soybeans or steamed soya flakes, along with wheat. Normally a strain of *A. oryzae* is used at 25-30°C. The hydrolysis of constituent starch, protein and pectin is accomplished in 2-3 days **[5]**.

The second fermentation step, called moromi fermentation, begins by combining the fermented bean wheat mixture with salt brine, creating a mash. The high salt concentration and the high water content of the mash creates a selective growth environment, which favors the proliferation of halophiles, or

The fermentation process enables the proteins and carbohydrates in soybeans to be broken down into amino acids and simple sugars respectively, making soy sauce easily assimilable. The proteinases, amylases, and other enzymes of the koji continue to act throughout the holding period. There are three stages in the curing

**1**- Lactic acid fermentation by:

**A**- Lactic acid bacteria e. g, *Lactobacillus delbrueckii*, which makes the koji acid enough to prevent spoilage and acidifies the mash,

**B**- *Bacillus subtilis* and other bacilli, which grow in the koji to improve flavor and make the soy sauce less turbid,

**C**- *Pediococcus halophilus*, which increases the acid in the mash, thereby stimulating the yeasts, contributing to essential aromas and flavors, decreasing color intensity, and reducing the activity of the mold proteinases.

**2**- Alcoholic fermentation by yeasts such as *Saccharomyces rouxii* and *Zygosaccharomyces soyae*

**3**- Completion of the fermentation and aging **[4, 8]**.

In the initial stage of the soy sauce, pH is around 6 and some oxygen is present. These circumstances support the growth of the salt tolerant aerobic *coryneform* bacteria. After oxygen is consumed, pH is still high and growth of *Tetragenococcus halophila* is favored. Due to acetate and lactate production, pH drops to 4.5. *T. halophila* cannot grow anymore then and therefore cannot compete with yeast, which can grow reasonably well at low pH. However, since in traditional production, the yeast fermentable sugars are depleted during growth of *T. halophila*, only a limited increase in yeast numbers is observed and no obvious fermentation takes place **[9]**.

Pressing-moromi to produce soy sauce after the aging of Moromi is completed; Moromi is squeezed into soy sauce. Just like in the traditional way, Moromi is wrapped in a cloth and squeezed slowly as long as three days. On the first day, soy sauce drops out of the cloth on its own weight. On

the second day, the Moromi wrapped in the cloth is squeezed with a low-pressure. On the third day, the pressure is added gradually, and that is left in the cloth is only grounds with very little liquid **[10, 11]**.

The squeezed raw soy sauce will be heated up to a certain temperature. This is not only for sterilization but also for standardizing aroma and color and increasing stability of soy sauce by stopping enzyme's activity. This process is called (heating). It is an important process of soy sauce production. After heating process, soy sauce will be checked by inspectors with not only chemical composition but also color, aroma, and taste by using their eyes, nose, and tongue **[10, 11]**.

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic, anti-estrogenic, and hypocholesterolemic effects, in mammalian species, they have also been reported to have beneficial anti-carcinogenic effects **[12]**.

The average protein contents of common millet is 14·4 % and the crude fiber content of the millets ranged from 3·2% to 4·7%. In general, the mineral contents are high compared with those of other common cereal grains. In particular, the high level of calcium (0.24%) in finger millet was noteworthy **[13]**. A high levels of Ca, Fe, K, Mg and S were found in *Thymus vulgaris,* and *Anethum graveolens* **[14]**.

The most active antibacterial plants against both gram-positive and gram-negative bacteria were *Thymus vulgaris* and *Thymus origanium*. The organic and aqueous extract from the same plants showed different activities; the organic extract showed the same or greater activity than the aqueous extract **[15]**.

The extracts from different parts of *Thymus vulgaris*, also show the presence of a large number of flavonoids and vitamin E, compounds of great interest in food industry for their antioxidant activity. Leaves and flowers of this plant are

of interest as flavorings, as well as being natural antioxidants for the food industry **[16]**.

## 1.1 Significance and Objectives

Soy sauce has been extensively produced and studied worldwide. However, to our knowledge no production unit or previous published research were conducted in the Gaza strip. It is usually imported from the outside. The present study will produce for the first time high quality and low priced soy sauce with specific aroma in the Gaza strip and its chemical ingredients were investigate. The general aim of this preliminary study, therefore, is to isolate and characterize *A. oryzae* strain and use it in soy sauce production.

The following specific objectives were achieved:

1. To isolate the *A. oryzae* from different contaminated sources.
2. To characterize of the *A. oryzae.*
3. To produce new aroma of soy sauce with *Anethum graveolens* and *Thyme vulgaris.*
4. To identify the chemical gradients of the new product.

# Chapter 2
# Literature Review

## 2.1 Fungi used in fermentation

### 2.1 1.The *Aspergillus* Group

The genus Aspergillus represents a grouping of a very large number of asexual fungi whose taxonomy is based on morphological features. The genus has been divided into groups based on attributes of the spores, conidiophores, and sclerotia. Because this separation of individual species into groups is based on morphological or physiological characteristics, it has resulted in somewhat tenuous and overlapping classification **[17]**.

In 1926 a first classification of these fungi was proposed describing 11 groups within the genus A reexamination of the genus was published by Thom and Raper identifying 14 distinct groups. Some of these groups consist of pathogenic fungi (e.g., *A. fumigatus, A. flavus, and A. parasiticus*), but most important for industrial applications are some members of the group of black aspergilli (*A. niger and A. tubingensis*) **[18]**.

The genus *Aspergillus* is a diverse group of common molds and the approximately 175 species are inhabitants of virtually all terrestrial environments, when conditions in indoor situations are favorable for fungal growth. Most species have relatively low moisture requirements and some are extremely xerophilic (dry tolerant), allowing them to colonize areas that cannot support other fungi and where only minimal or intermittent moisture is available. Their rapid growth and production of large numbers of small, dry, easily aerosolized spores makes them a significant contaminant concerning Indoor, air quality and potential human exposure-related illnesses. A few species are common opportunistic human pathogens, including *A. fumigatus*, the most common agent of aspergillosis, and *A. niger*, a common agent of otomycosis (ear infection). Aspergillosis is now considered the second most common type of

fungal infection requiring hospitalization in the United States. *Aspergillus* species are also well-known allergens (type I or atopic allergy), and *A. fumigatus* is one of the most prevalent causes of type III allergy or hypersensitivity pneumonitis, and allergic sinusitis **[19]**.

In addition to the morphological techniques traditionally applied, new molecular and biochemical techniques have been used in the reclassification of this group of *aspergilli*. These analyses resulted in the clear distinction of eight groups of black aspergilli (*A. niger, A. tubingensis, A. foetidus, A. carbonarius, A. japonicus, A. aculeatus, A. heteromorphus, and A. ellipticus*) . Products of several of these species have obtained a Generally Regarded As Safe status (GRAS), which allows them to be used in food and feed applications. The black aspergilli have a number of characteristics, which make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of major importance to the food and feed industry **[18]**.

Identification of the hyphomycetes is primarily based on microscopic morphology including conidial morphology, especially septation, shape, size, color and cell wall texture, the arrangement of conidia as they are borne on the conidiogenous cells, e.g., solitary, arthrocatenate, blastocatenate, basocatenate or gloiosporae, the type conidiogenous cell, e.g.,non-specialized or hypha-like, phialide, annellide or sympodial and other additional features such as the presence of sporodochia or synnemata. For identification, PDA and cornmeal agar are two of the most suitable media to use and exposure to daylight is recommended to maximize culture color characteristics. *Aspergillus* colonies are usually fast growing, white, yellow, yellow-brown, brown to black or shades of green, and they mostly consist of a dense felt of erect conidiophores. Conidiophores terminate in a vesicle covered with either a single palisade- like layer of phialides (uniseriate) or a layer of subtending cells (metulae) which bear small whorls of phialides (the so-called biseriate structure). The vesicle, phialides, metulae (if present) and conidia form the conidial head. Conidia are one-celled, smooth- or rough-walled, hyaline or pigmented and are

basocatenate, forming long dry chains which may be divergent (radiate) or aggregated in compact columns (columnar). Some species may produce Hülle cells or sclerotia **[20]**.

### 2.1.1.1 General features of *Aspergillus oryzae*

Scientific classification **[21]**.

| | |
|---|---|
| Kingdom | *Fungi* |
| Division | *Deuteromyc*ota |
| Class | *Eurotiomycetes* |
| Order | Aspergillals |
| Family | *Aspergilluceae* |
| Genus | *Aspergillus* |
| Species | *A .oryzae* |


**Figure (2.1)** Conidial head or fruiting body of *A. oryzae* –producing spores

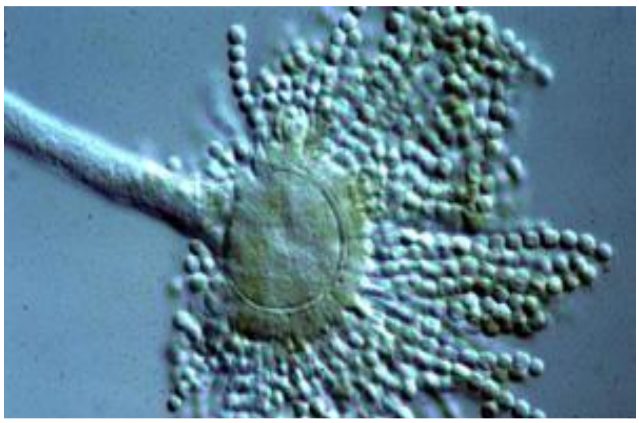


**Figure (2.2)** Conidial head or fruiting body of *A. flavus* –producing spores

*A. oryzae* (Figure 2.1) **[22]** is a member of the *A. flavus* group of *Aspergillus* species. The conidiophores are roughened and colorless. The spores themselves have conspicuous ridges and echinulations (spines). *A. oryzae/flavus* species have never been connected to a sexual or teleomorphic stage. However, the teleomorphic stages of other *Aspergillus* species have been demonstrated by the formation of cleistothecia **[23, 24]**. The *A. flavus* is shown in (Figure 2.2**) [25]**.

In nature, selection places limits on conidial size as may be critical to dispersal or survival Conidia of domesticated yellow-green *aspergilli* from strains of *A. oryzae (Ahlburg) Cohn* and *A. sojae* are used in the preparation of koji inoculum, germinate approximately 3 h sooner than conidia produced by related wild species **[26]**.

Although the details of the genetic relationship between *A. oryzae* and *A.flavus* remain unclear, the two species are so closely related that all strains of *A. oryzae* are regarded by some as natural variants of *A. flavus* modified through years of selection for fermenting of foods. *A. oryzae* is regarded as not being pathogenic for plants or animals, though there are a handful of reports of isolation of *A. oryzae* from patients. There are also several reports of products of *A. oryzae* fermentations, e.g. α amylase, that seem to be associated with allergic responses in certain occupations with high exposure to those materials. *A. oryzae* can produce a variety of mycotoxins when fermentation is extended beyond the usual time needed for production of these foods. While wild *A. flavus* isolates readily produce a flatoxins and other mycotoxins, *A. oryzae* has not been shown to be capable of aflatoxin production **[23]**.

Because *A. oryzae* has GRAS status for use in the food industry, efforts have been made to develop molecular methods to unambiguously distinguish *A. oryzae* from *A. flavus*. These methods include restriction fragment length polymorphism, amplified fragment length polymorphism, electrophoretic karyotyping, isozyme profiling and analysis of ribosomal DNA internal transcribed spacer regions. Generally, these methods have not been successful in unambiguously separating *A. oryzae* as a distinct species **[24]**.

#### 2.1.1.2 Use of *Aspergillus oryzae*

*A. oryzae* has been used for centuries in the production of many different oriental foods such as soy sauce, sake and miso. As a "koji" mold, *A. oryzae* has been used safely in the food industry for several hundred years. It is also used to produce livestock probiotic feed supplements. The koji mold enzymes

were among the first to be isolated and commercialized nearly 100 years ago. In koji preparation, *A. oryzae* also produces a low-molecular-weight iron-chelating compound, termed deferriferrichrysin, a type of siderophore **[17, 24, 27, 28, 29]**.

*A. oryzae* is currently used in the production of organic compounds such as glutamic acid, and several enzymes that are of potential use commercially, for example, amylase, protease, β-galactosidase, lipase, and cellulase. While these enzymes could be used as Toxic Substances Control Act (TSCA) products, several of them have been more often used in food processing. In 1989, Environmental Protection Agency (EPA) reviewed a pre manufacture notice (PMN) for a strain of *A. oryzae* modified for enhanced production of a lipase enzyme to be used primarily in detergent formulations for the removal of fat-containing stains. In 1994, EPA reviewed a PMN for a similar strain of *A. oryzae* modified for enhanced production of a cellulase gene for use in detergents as a color-brightening agent **[17]**.

Submerged fungal fermentations are widely used in the production of enzymes, antibiotics and organic acids, which have many applications in the food, medicine, pharmaceutical, chemical and textile industry .However, their filamentous growth characteristic creates a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scales. It is well documented that the fungal culture exhibits two major morphologies observed as pellet or mycelia, which are very much determined by several environmental and genetic factors These are; type of the strain, pH and composition of the media, inoculation ratio, type of the inoculum, agitation speed, aeration rate, feeding rate, and genetic factors of the culture **[30,31]**.

#### 2.1.1.3 Some important enzymes of *Aspergillus oryzae*

Koji contains high amounts of catalytic enzymes, including alpha-amylase (starch to simple sugar converter), proteolytic enzymes, including protease's (3 types are known, one is active at acid, one at alkaline and one at neutral

pH). Other enzymes include; sulfatases, nucleases, phosphatases, transglycosidases, peptidases, acylase, ribonucleo-depolymerase, mononucleotide phosphatase, adenyl-deaminases and purine nucleosidases.

**1 – Alpha -amylase** is composed of a group of ubiquitous endoglycosidases that hydrolyze 1,4-glucosidic linkages in polysaccharides containing three or more α- 1,4-linked D-glucose units yielding a mixture of maltose and glucose. Discovery in 1884 by Takamine in the United States of the production of the α-amylase of *A. oryzae* by solid-state fermentation on wheat bran opened up the commercial use of enzymes in cereal technology, although major break through in industrial enzymology took place first in the post-World War II period **[29]**.

In1891 Dr. Jokichi Takamine filed patent applications for "Taka koji" from *A. oryzae* This formed the basis for Dr. Takamine's fermentation process for the industrial production of a fungal amylase, the first of its kind. The method of fermentation suggested by Takamine is still used in the production of certain enzymes today. Later in 1926, Dr. James B. Sumner was able to determine that enzymes are actually proteins. By 1930, enzyme therapy was moving in two directions. One involved the study of fungal enzymes and the other focused on the study of animal enzymes **[33]**. Alpha -amylase is added to flour to compensate for the low natural content of amylases of cereal flour and so enhance carbohydrate fermentation by yeast **[34]**.

**2**- **Cellulase** refers to a group of enzymes that act together to hydrolize cellulose to glucose. Although cellulases are distributed throughout the biosphere, they are manifest in fungi and microbial sources. During the last few years it has become permissible in most countries to add fungal cellulase to white bread dough to break up roughage **[34]**.

**3**- **D-galactosidases** such as α-galactosidases, It hydrolyses variety of simple oligosaccharides and more complex polysaccharides. The efficiency of crude α extracelluar -galactosidases from *Cladosporium cladosporides*, *A. oryzae* and *A. niger* reduced the raffinose oligosaccharides content in chickpea

flours by 100%, while germination reduced the raffinose content by 69% and stachyose content by 75% . Other traditional techniques reduced the raffinose content by 13-49% and stachyose content by 10-32% **[35, 36]**.

Alpha extracellular Galactosidase from mutant strain *A. oryzaeH26-10-7* was found to have five times higher catalytic activity on the synthetic substrate o-nitrophenyl-D-galactopyranoside (ONPG) compared to the wild type enzyme. Moreover, the mutant enzyme was more thermo resistant compared to the wild type **[37]**.

Galactosidases are used to improve the gelling capacity of galactomannans, which have applications in the food industry as well in the cosmetic and pharmaceutical industries. Additionally, they reduce the concentration of raffinose and other oligosaccharides in soybean milk, cowpea meal, and sugar beet syrup **[18]**.

**4- Lactase,** a disaccharidase enzyme produced by *A. oryzae* and *A. niger*, is used extensively in the food and drug industries **[38]**.

**5- Fructose 1,6-diphosphate** (FDP) aldolase and 2-keto-3-deoxy-D-gluconate (KDG) aldolase were identified in cell-free extracts of four *A. oryzae* strains grown on D-glucose as sole source of carbon. *A. oryzae NRRL 3435* gave the highest enzymatic activity for the two enzymes and selected for further studies. Studies on the properties of the two key enzymes indicated that the optimum conditions for the activities of FDP aldolase and KDG aldolases occurred at pH 8.5, 45 °C and pH 8.0, 55 °C, respectively. Tris-acetate buffer and phosphate buffer showed the highest enzymatic activity for these two enzymes respectively. KDG aldolase was stable at 55°C for 60 minutes however FDP aldolase was found to be less stable above 45°C. Onthe other hand the two aldolases showed a high degree of stability towards frequent freezing and thawing. Dialysis of the extracts caused a decrease in the enzymatic activity of KDG aldolase, and an increase in FDP aldolase activity **[39]**.

**6- Acid phosphatase** , *A. oryzae* produce three types of acid phosphatase (ACP-I, ACP-II, and ACP-III) in a submerged culture by using only phytic acid as the phosphorous substrate. The optimum pH for the activities of the three enzymes was in the range of 4.5 to 5.5. Analysis of the substrate specificities of these enzymes revealed that ACP-I and ACP-III were acid phosphatases, and ACP-II was a phytase **[40]**. Phytase initiates the release of phosphorus from phytate (myo-inositol hexakisphosphate) hydrolysis of phytate also prevents protein–phytate complex formation, leaving more free protein available to be digested and adsorbed **[41].**

These enzymes were produced during different periods of mycelial growth, ACP-II was produced during the early phase of cultivation (around 24 h), and ACP-I was produced between 24 to 72 h., ACP-III was detected after the production of ACP-I and ACP-II had ceased. The release of phosphate from phytic acid was expected to be due to the cooperative hydrolysis of these enzymes **[40]**.

**7**- **Glutaminase** is generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauce The enzyme has received significant attention in the food industry owing to its potential as a flavor modulating agent, as it increases the glutamic acid content of the food imparting savory flavor. This unique taste called umami, elicited by meat, fish and vegetable stocks, has been confirmed as the fifth basic taste beside sweet, acid, salty and bitter **[42,43]**.

**A -The relationship between glutaminase and other compounds**

Glutamic acid is formed by two mechanisms during fermentation; firstly, hydrolytic release from the raw material due to protease and peptidase activities, and secondly, deamination of free glutamine catalyzed by glutaminase **[42,43].**

Studies demonstrated that the proteinases did not directly participate in the release of glutamic acid and that leucine amino peptidase greatly contributed

to it, two forms of glutaminases, a free form and a binding form, are known to exist in *A. oryzae* **[44]**.

Glutaminases from koji molds have been shown to be markedly inhibited by salt concentrations above 15% (w/v), which are typical conditions for soy sauce production **[43,45,46]**.

*A. oryzae* has been used to obtain high levels of heterologous proteins, such as aspartic proteinase and lipase and recently active human proteins, e.g. lactoferrin and lysozyme have been produced in recombinant strains of this organism. Despite the extensive industrial use of *A. oryzae*, little is however, known about the correlation between morphology, growth and protein production-both for this species and for other species of protein producing filamentous fungi. Current research suggests that protein secretion in filamentous fungi is intimately associated with the process of growth at the hyphal tip , Submerged culture was better than solid culture in the production of proteinases and peptidases from *A. oryzae 460*. On the contrary, solid culture was better than submerged culture in the production of α - amylase, carboxymethyl cellulase, and pectinlyase from the same fungus **[47]**.

The soy sauce mash (raoroml) made with the enzyme preparation from submerged culture was highly viscous and the soy sauce produced was characteristic in low contents of alcohol and reducing sugar, low pH value, and less aroma. Soy sauce made with the enzyme preparation from solid culture was superior on these points to that from submerged culture. Wheat bran was best as the raw material for the enzyme preparation in easy koji making, large amounting produced, and low cost. In enzyme production from a solid culture, addition of urea (0.8% to wheat bran) nearly doubled the leucine aminopeptidase for Leu-GIy-GIy. The incubation period was reduced to 30 to 40 h from 50 to 60 h using germinated spores and moisture-controlled culture with forced aeration **[47]**.

Soy sauce made with a preparation of proteases from yellow-green *Aspergilli* contains less glutamic acid than soy sauce made by the traditional shoyu koji

method. Thus, an acid treatment was developed to increase this amino acid in enzyme-made shoyu. Amide bonds of glutamine and asparagine in protein molecules were hydrolyzed at 100° C for 30 min with 1.3 N HC1 (acid treatment). Using this method, glutamic acid per total nitrogen freed from various proteins by the concerted action of proteinases and peptidases of yellow-green *Aspergillus* increased to 1.0 to 3.8 times that of control (no acid treatment). An increase of about 31% of glutamic acid per total nitrogen resulted from the acid treatment method in soy sauce made with an enzyme preparation of proteases **[47, 48]**.

**B- Glutamate role**

Glutamic acid in its free form and monosodium glutamate (MSG) are used to enhance flavor in foods prepared at home, in restaurants, and by the food processing industry. Glutamate occurs in two optical isomeric forms; the L-form is the one that possesses the ability to enhance flavor. Glutamate is a major component in all proteins, and occurs as a free acid in a variety of vegetables, meats, and sea products the range varies between 6.7 and 658 mg/100 g in fresh food to 0.05 and 6830 mg/ 100 g in processed foods Glutamate also plays an essential role in many metabolic processes. Studies have shown that the body uses glutamate as a nerve impulse transmitter to the brain, and injections of glutamate in laboratory animals have resulted in nerve cell damage **[49]**.

**8 - β-xylanase**. Xylose is considered the main sugar component involved in the amino-carbonyl reaction during shoyu fermentation, and is considered to be released synergistically from hemicellulose in soybean and wheat by β -xylanase and β -xylosidase produced by shoyu koji mold, *A. oryzae* or *A. sojae*. Some reports suggest that *A. oryzae* or *A. sojae* produces various xylanases, and that some of them have been purified and characterized. On the other hand, there are only a few reports on β -xylosidase. It is very important to investigate the enzymatic properties and expression systems of xylanase and β -xylosidase from *A. oryzae* or *A. sojae* to control their enzyme productions in shoyu koji. Only one study was demonstrated that the use of a

low xylanolytic enzyme-producing *A. oryzae* strain in shoyu koji decreased the concentration of xylose in shoyu moromi mash and the color intensity of shoyu products **[50]**.

**9 – Esterase**. Soy yeasts showed at least 10 times higher esterase activity than the other yeasts used for fermented beverages, the yeast esterase was not greatly affected by the pH or NaCl concentration. Soy koji cultured with *A. sojae* or *A. oryzae* showed very high ester-splitting activity. By gel-filtration of koji esterase, the i-amylacetate (i-AmAc) decomposing fraction was obtained. This fraction showed a decrease of activity at lower pH or higher NaCl concentration. Koji esterase decreased its activity in moromi but remained over the entire moromi period. Koji esterase exhibited a higher activity than yeast esterase in fermenting moromi. These strong esterase activities are thought to be one of the causes of the low concentration of ester flavor in soy sauce **[51]**.

### 2.1.2 *Zygosaccharomyces rouxii*

Scientific classification **[21]**.

| | |
|---|---|
| Kingdom | *Fungi* |
| Division | *Ascomycota* |
| Class | *saccharomycetes* |
| Order | *saccharomycetales* |
| Family | *saccharomycetaceae* |
| Genus | *zygosaccharomyces* |
| Species | *zygosaccharomyces rouxii* |

The predominant yeast of soy sauce fermentation is *S. rouxii. S. rouxii* begins to grow when the pH value has decreased to around 5.0. It produces 2-3% of ethyl alcohol and around 1% glycerol in addition to many of the flavor components of moromi this yeast can be grown in a 25% salt solution and in 80% glucose **[4]**.

In the amino-acid metabolism of this yeast, alpha-ketobutyrate is a key-intermediate; some α-ketobutyrate is needed to synthesize isoleucine, the isoleucine synthesis starts with the deamination of threonine. The deamination of threonine is catalyzed by threonine deaminase [L-threonine hydro-lyase (deaminating)] and results in the formation of α -ketobutyrate and ammonia. α-ketobutyrate is converted further toward isoleucine by acetohydroxy acid synthase [acetolactate pyruvate-lyase (carboxylase)]. This enzyme catalyzes the conversion of α -ketobutyrate and pyruvate into α -aceto-alphahydroxybutyrate and also the conversion of two molecules of pyruvate into α -acetolactate, which is converted via α -ketoisovalerate to valine and leucine **[52]**.

*Z. rouxii*, a salt tolerant yeast, is important for the flavor development in soy sauce. In soy sauce, *Z. rouxii* produces ethanol, higher alcohols, and 4-hydroxy-2 (or 5)-ethyl-5(or 2)-methyl-3(2H)- furanone (HEMF). HEMF is considered as one of the important compounds for soy sauce flavor. The metabolism of *Z. rouxii* was investigated by separately adding the amino acids threonine, cystathionine, and the branched-chain amino acids. It seemed that the addition of threonine severely inhibited the growth of *Z. rouxii*, which

resulted in the accumulation of significant amounts of glycerol and only small amounts of higher alcohols. On the other hand, the addition of the branched-chain amino acids increased the production of the higher alcohols isobutyl alcohol and active amyl plus isoamyl alcohol via the Ehrlich pathway. In addition, the added amino acids also influenced the specific activities of the enzymes catalyzing the formation or conversion of α-ketobutyrate in *Z. rouxii*. Despite this, it seemed that the α-ketobutyrate pool size in *Z. rouxii* was tightly regulated all the time, resulting in no accumulation of α-ketobutyrate in both the supernatant and cells **[52, 53, 54]**.

### 2.1.3 Candida (Torulopsis)

Scientific classification **[21]**.

| | |
|---|---|
| Kingdom | *Fungi* |
| Division | *Ascomycota* |
| Class | *Ascomycetes* |
| Order | *saccharomycetales* |
| Family | *saccharomycetaceae* |
| Genus | *candida* |
| Species | *candida versatilis* |

Species begin to grow after alcohol fermentation and produce specific flavor substances in soy sauce. Normally, two species exist as natural flora in moromi and their industrial characteristics are almost the same. These Candida species are more aerobic than *Z. rouxii*. They can grow in a 26% salt solution as can *Z. rouxii.* The pH range, temperature range, and growth factors vary with the existence of salt. Inositol is an essential factor for the growth in the medium in the presence of salt. Usually, natural flora of Candida species are used for fermentation, and the most important property of these yeasts is to produce a large amount of excellent flavor substances in a high salt concentration brine **[4]**. *C. versatilis* can convert ferulic acid (F A) to 4-ethylguaiacol (4-EG) .The bioconversion pathway from FA to 4-EG consists of two steps:

**1**- FA is first converted to 4-Vinylguaiacol (4-VG) by FA-decarboxylase.

**2**- The 4-VG thus derived is converted to 4EG by –VG reductase **[55].**

## 2.2 Bacteria in fermentation

### 2.2.1 *Tetragenococcus halophila* (*Pediococcus halophilus*)

Lactic acid bacteria (LAB) form a phylogenetically diverse group and are defined as Gram-positive, nonsporing, catalase-negative, devoid of cytochromes, of anaerobic habit but aerotolerant, fastidious, acid tolerant, and strictly fermentative bacteria that secrete lactic acid as the major end product of sugar fermentation **[56]**.

Two types of soy sauce can be distinguished: a Chinese type, made of soybeans only, and a Japanese type, made of equal amounts of wheat and soybeans. The lactic acid bacterium *T. halophila* (until recently known as *P. halophilus* grows in both types during the brine fermentation **[57]**.

The main purpose of adding the lactic acid bacteria is to reduce the pH to its final value of 4.7-4.8 from its initial value of 6.5-7.0. This prepares for growth of the yeast such as *S. rouxii*. Many different types of *P. halophilus* strains have been reported, especially as they relate to the pattern of acid formation from carbohydrates. Acid can be produced from arabinose, galactose, maltose, mannitol, alpha-methylglucoside, and dextrin (Table 2.1) **[4].** These bacteria are able to grow in 24% salt solution. Certain vitamins and amino acids are required for their growth **[4]**.

**Table (2.1):** Fermentation pattern of *pediococcus* strains isolated from moromi

| Group | Substrate and acid formation | | | | | No. of strains | % |
|---|---|---|---|---|---|---|---|
| | Arabinose | Lactose | Melibiose | Sorbitol | Mannitol | | |
| 1 | - | - | - | - | - | 221 | 13.9 |
| 2 | + | - | - | - | - | 167 | 10.5 |
| 3 | - | + | - | - | - | 12 | 0.8 |
| 4 | - | - | + | - | - | 96 | 6.1 |
| 5 | - | - | - | + | - | 169 | 10.7 |
| 6 | - | - | - | - | + | 223 | 14.1 |
| 7 | + | + | - | - | - | 16 | 1.0 |
| 8 | + | - | + | - | - | 17 | 1.1 |
| 9 | + | - | - | + | - | 21 | 1.3 |
| 10 | + | - | - | - | + | 132 | 8.3 |
| 11 | - | + | + | - | - | 6 | 0.4 |
| 12 | - | + | - | + | - | 3 | 0.2 |
| 13 | - | + | - | - | + | 26 | 1.6 |
| 14 | - | - | + | + | - | 67 | 4.2 |
| 15 | - | - | + | - | + | 60 | 3.8 |
| 16 | - | - | - | + | + | 113 | 7.1 |
| 17 | + | + | + | - | -- | 0 | 0 |
| 18 | + | + | - | + | | 6 | 0.4 |
| 19 | + | + | - | - | + | 74 | 4.7 |
| 20 | + | - | + | + | - | 31 | 2.0 |
| 21 | + | - | + | - | + | 42 | 2.6 |
| 22 | + | - | - | + | + | 16 | 1.0 |
| 23 | - | + | + | + | - | 1 | 0.1 |
| 24 | - | + | + | - | + | 0 | 0 |
| 25 | - | + | - | + | + | 2 | 0.1 |
| 26 | - | - | + | + | + | 39 | 2.5 |
| 27 | + | + | + | + | - | 0 | 0 |
| 28 | + | + | + | - | + | 1 | 0.1 |
| 29 | + | + | - | + | + | 5 | 0.3 |
| 30 | + | - | + | + | + | 15 | 0.9 |
| 31 | - | + | + | + | + | 0 | 0 |
| 32 | + | + | + | + | + | 3 | 0.2 |
| Total | | | | | | 1586 | 100 |

*Strains that produce acid only,* ( **-**) *: strains that not produce acid* (**+**) *:*

## 2.3 Plants

### 2.3.1 Plants used for aromas

#### 2.3.1.1 *Thymus vulgaris*

Scientific classification **[58]**.

| | |
|---|---|
| Kingdom | *Plantae* |
| Division | *Magnoliophyta* |
| Class | *Magnoliopsida* |
| Order | *Lamiales* |
| Family | *Lamiaceae* |
| Genus | *Thymus L.* |
| Species | vulgaris |

**Figure (2.3)** *Thymus vulgaris*

Thyme [Figure 2.3) **[59]** is a popular aromatic plant widely used as a spice in food processing, perfumes and popular medicine. The genus thymus belongs to the family *Lamiaceae* and contains approximately 350 species located mainly in Europe, the Mediterranean region and Western Asia. Thymus *vulgaris L* has been used for aromatising liqueurs, to give flavouring to cheese, soups, meat, fish, poultry and sauces, and is consumed in infusion. Eight chemotypes have been found from this species: geraniol, linalool, a-terpineol, carvacrol, thymol, trans-thujan-4-ol/terpinen-4-ol,12 1,8-cineole13 and p-cymene and thymol. It has also been shown that the contribution of some groups of compounds to the odour of the flavouring, such as aldehydes with low carbon numbers, some esters, guaiacol and its derivatives and terpenic derivatives, is much more important than that of ketones, furan and pyran derivatives, acids, phenol and its derivatives and syringol **[60]**.

The phenolic monoterpenes in thyme, thymol and carvacrol (Figure 2.4) **[61]**, are the primary compounds that contribute to the characteristic aroma of its essential oil They are also known to inhibit lipid peroxidation **[62]**.

Natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among navonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates, and tannins can act as antioxidants or have a synergistic effect when used together with phenolic antioxidants. Currently, materials, which inhibit lipid oxidation, can be obtained from plant materials, food waste, microorganisms and animal cells **[63]**.

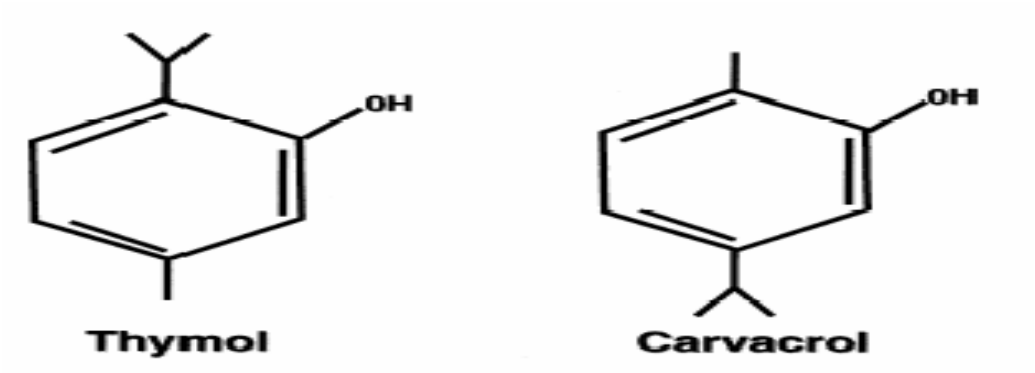



**Figure (2.4)** Structure of two plant essential oils thymol (5-ethyl-2-isopropylphenol) and carvacrol (5-isopropyl-2-methylphenol)

**2.3.1.2 *Anethum graveolens***

Scientific classification **[64]**.

| | |
|---|---|
| Kingdom | *Plantae* |
| Division | *Magonoliphyta* |
| Class | *Magnoliopsida* |
| Order | *Apiales* |
| Family | *Apiaceae* |
| Genus | *Anethum L.* |
| Species | *A. graveolens* |

**Figure (2.5)** *Anethum graveolens*

Many essential oils extracted from higher plants have shown antimicrobial activity against various pathogenic microorganisms. In the traditional system of Indian medicine, the seeds of spices are used as antiseptic, stomachic, carminative, stimulants and prevent flatulence and colic **[65]**.

*A. graveolens L*.(Figure 2.5) **[66]** is a member of the *Apiaceae* family and is more commonly known as dill. The plant is used both medicinally and as an aromatic herb and spice in cookery. The fruit of *A. graveolens* has been used for medicinal purposes to relieve digestive problems and to stimulate milk for nursing mothers. Previous phytochemical studies have identified the monoterpene carvone (Figure 2.6**) [67]** to be the main constituent (50%–60%) of the essential oil. This monoterpene has a calming effect and is used in gripe water preparations **[68, 69]**.

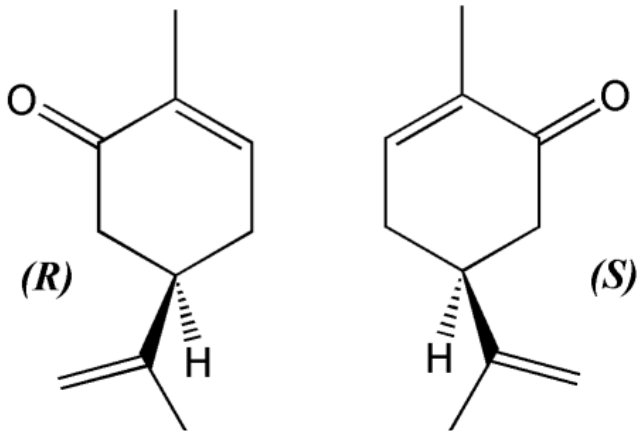



**Figure (2.6)** Carvone

## 2.3.2 Plants used in fermentation

### 2.3.2.1 Soybean

Scientific classification **[70]**.

| | |
|---|---|
| Kingdom | *Plantae* |
| Division | *Magnoliophyta* |
| Class | *Magnoliopsida* |
| Order | *Fabales* |
| Family | *Fabaceae* |
| Genus | *Glycine* |
| Species | *G. max* |

**Figure (2.7)** Soybeans

Since soybean proteins constitute a source of high quality proteins and are rich in lysine, their amino acid profile fits very well with that of cereal proteins.

In addition, soybean proteins have other advantages such as low cost and many interesting functional properties All this has provoked that soybean flour and other soybean preparations were added in the manufacturing of cereal derived products **[71]**. The soybean is a member of the tribe *Phaseoleae*, the most economically important of the legume tribes [**72]**.

The soybean is grown as a commercial crop in over 35 countries worldwide. Of the major oilseeds traded in international markets, the commercial standard for soybean moisture content is 14%. Literature reports indicate that the ranges for crude protein, fat, ash, neutral detergent fibre ( NDF), acid detergent fiber ( ADF), and carbohydrate content are 32 - 43.6%, 15.5 - 24.7%, 4.5 - 6.4%, 10.0 - 14.9%, 9.0 - 11.1%, and 31.7 – 31.8%, respectively on a dry matter (DM) basis **[72]**. Soybeans contain less than 20% (wt/wt) sugars, including a large variety of carbohydrates such as melibiose, sucrose, raffinose, stachyose, and cell wall polysaccharides **[73]**.

However, galactosyl poly- and oligo-saccharides (i.e., β-galactomannans and α-1, 6-galactosides), known as flatulence-producing compounds (FPC), still exist after soybean meal processing . Contents of α-1,6-galactosides (raffinose, 1.0%; stachyose, 4.6%and β-galactomannans 1.2% are relatively high in soybean meals, and these FPC are not digestible by pigs because they lack enzymes targeting α-1,6-galactosyl bonds and β-1,4-mannosyl bonds. Undigested FPC negatively affect energy and protein digestibility and growth due to increased osmolarity and rate of passage through the gut. Flatulence producing factor is used largely by hindgut microorganisms producing gases and causing nausea and discomfort one efficient approach to alleviate the antinutritional effects of α-1, 6-galactosides and β-alactomannans in soybean meal is applying exogenous enzymes, such as α-1,6-galactosidase, β-1,4-mannanase, and β-1,4-mannosidase **[74]**.

The essential amino acid profile of the soybean, plus cystine, which can partially replace methionine, is shown in (Table 2.2) **[72]**. Fatty acid content of the seed as reported in these literature sources is shown in (Table 2 .3) **[72],** and the nutrient composition of soybeans is shown in (Table 2.4) **[75]**.

**Table (2.2)** Amino acid composition of soybean seed

| Amino Acid | Range, % of Dry Matter |
|---|---|
| Arginine | 2.45 - 3.1 |
| Cystine | 0.45 - 0.67 |
| Histidine | 1.0 - 1.22 |
| Isoleucine | 1.76 - 1.98 |
| Leucine | 2.2 - 4.0 |
| Lysine | 2.5 - 2.66 |
| Methionine | 0.5 - 0.67 |
| Phenylalanine | 1.6 - 2.08 |
| Threonine | 1.4 - 1.89 |
| Tryptophan | 0.51 - 0.67 |
| Valine | 1.5 - 2.44 |

**Table (2.3)** Fatty acid composition of soybean seed

| Fatty Acid | Range % of Dry Matter |
|---|---|
| Palmitic, C16:0 | 1.44 - 2.31 |
| Stearic, C18:0 | 0.54 - 0.91 |
| Oleic, C18:1 | 3.15 - 8.82 |
| Linoleic, C18:2 | 6.48 - 11.6 |
| Linolenic, C18:3 | 0.72 - 2.16 |
| Arachidic, C20:0 | 0.04 - 0.7 |

**Table (2.4)** The nutrient composition of soy beans

| **Nutrient** | **100 g cooked soybeans** |
|---|---|
| Moisture (%) | 62.6 |
| Energy (kJ) | 706 |
| Protein (g) | 16.6 |
| Fat (g) | 9.0 |
| Saturated fatty acids (g) | 1.3 |
| Monounsaturated fatty acids (g) | 2.0 |
| Polyunsaturated fatty acids (g) | 5.1 |
| Carbohydrate (g) | 4.8 |
| Dietary fibre (g) | 1.6 |
| NSP total (g) | 2.9 |
| Soluble NSP (g) | 0.1 |
| Insoluble NSP (g) | 2.0 |
| Calcium (mg) | 102 |
| Iron (mg) | 5.1 |
| Magnesium (mg) | 86 |
| Phosphorous (mg) | 247 |
| Potassium (mg) | 515 |
| Sodium (mg) | 1 |
| Zinc (mg) | 1.2 |
| Copper (mg) | 0.41 |
| Vitamins: Thiamin (mg) | 0.16 |
| Ribloflavin (mg) | 0.29 |
| Niacin (mg) | 0.4 |
| A (RE) | 1 |
| E (mg α-TE) | 0.35 |
| Folic acid (µg) | 54 |

#### 2.3.2.1.1 Antinutrients in Soybeans

**A - Trypsin Inhibitors**

Raw soybeans are known to contain two separate protease inhibitors:

**1-** Proteins with a molecular weight of about 20,000 Da and a specificity directed primarily against trypsin, known as the Kunitz trypsin inhibitor.

**2-** Those that have a molecular weight between 6,000 to 12,000 Da and are capable of inhibiting chymotrypsin as well as trypsin at independent binding sites, referred to as the Bowman-Birk trypsin inhibitor **[76]**. Trypsin inhibitor activity ranging from 100 to 184 Trypsin Units Inhibited (TUI)/mg protein has been reported. The activity of these inhibitors is destroyed when the bean or meal is toasted or heated during processing **[77]**.

**B- Lectins**

The lectins in soybeans are tetrameric glycoproteins that have a specific affinity to terminal N-acetyl-D-glucosamine and D-galactose. These lectins were originally referred to as hemagglutinating factor or soyin, and it was estimated that they accounted for one-half of the growth inhibition produced by raw soybeans fed to rats. Lectins are glycoproteins with the ability to bind carbohydrate-containing molecules on the epithelial cells of the intestinal mucosa, with toxicity determined by the extent of this binding **[76,78]**.

Lectin levels can vary from 37 to 323 Hemagglutinating Activity Units (HU)/ mg protein). Lectins are rapidly degraded upon heating. In one study, lectin levels dropped approximately 100- fold when the raw soybean was processed into defatted, toasted soybean meal **[77]**.

**C- Phytoestrogens**

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic, anti-estrogenic, and

hypocholesterolemic effects, in mammalian species. These compounds have been implicated in adversely affecting reproduction in animals fed diets containing large amounts of soybean meal. However, it is not universally accepted that the isoflavones are antinutrients as they have also been reported to have beneficial anti-carcinogenic effects **[77]**.

**D- Stachyose and Raffinose**

Soybean oligosaccharides are a group of soluble low molecular weight oligosaccharides in soybean seeds, which include sucrose, stachyose and raffinose. Soybean oligosaccharides are defined as non-digestible oligosaccharides (NDOS) or non-digestible sugars (NDS) except sucrose, since human gastrointestinal tract does not possess α- galactosidase enzyme essential for hydrolysis of the α-1, 6 galactosyl linkages.Therefore oligosaccharides are supposedly involved in flatulence. The presence of these oligosaccharides impedes the full utilization of the soybean products **[79]**.

Many researches have been carried out to reduce the oligosaccharides content in legume seeds or in soybean products by processing techniques such as soaking, cooking, irradiation, germination, fermentation and enzyme treatment. However, many clinical researches have suggested that oligosaccharides, with approximately 30 to 50% caloric value of sucrose, may contribute to the growth of beneficial bacteria in the intestines, prevention of cancer, lowering the levels of blood cholesterol and reducing the risk of coronary heart disease, modulating the immune response, and stimulation of minerals absorption **[79]**.

These compounds are present in defatted toasted soybean meal, as well as in raw soybeans. The raffinose content of soybean seeds ranges from 0.1-0.9 g per 100g on fresh weight basis, while stachyose content is from 1.4-4.1 g per 100 g **[77]**.

**E- Phytic Acid**

Phytic acid (myo-Inositol 1,2,3,4,5,6-hexakis [dihydrogen phosphate]) is present in soybeans. This compound chelates divalent mineral nutrients

including calcium, magnesium, iron, and zinc, rendering them unavailable to monogastric animals consuming the beans. In fact, phytic acid chelation of zinc present in corn-soybean meal diets used for growing swine requires supplements of zinc to avoid a parakeratosis condition. It is becoming common for feed formulators to add a phytic acid degrading enzyme, phytase, to swine and poultry diets to release phytin-bound phosphorus, so that the amount of this mineral added to the diet can be decreased, potentially reducing excess phosphorus in the environment. Phytic acid naturally occurs in soybeans and most soybean products and can make up to 1 - 1.5 g per 100 g of the dry weight **[77]**.

phytic acid occurring in grains acts as an antioxidant by the formation of chelates with prooxidant transition metals. Although phytic acid is generally regarded as an antinutrient due to its mineral binding activity, it is known to reduce the risk for colon and breast cancer in animals **[80]**.

**2.3.2.2 Millet**

Scientific classification **[21]**

| | |
|---|---|
| Kingdom | *Plantae* |
| Division | *Magnoliophyt* |
| Class | *Liliopsida* |
| Order | *cyperales* |
| Family | *Poaceae* |
| Genus | *Panicum* |
| Species | *P miliaceum* **[** |

**Figure (2.8)** *Panicum miliaceum*

Recent studies have shown that cereal grains contain constituents that have demonstrated health benefits for humans, such as antioxidants and anti-disease factors. For instance, phytic acid was found to play a major role in the treatment of cancer, hypercholesterolemia, hypercalcuria and kidney stones **[82]**.

Other studies have also demonstrated that diets high in carbohydrate, rich in dietary fiber, and largely of cereal origin, allowed withdrawal of oral hypoglycaemic agents or a reduction of insulin dose in diabetic subjects **[82, 83]**.

Millet (Figure 2.8**) [84]** would be an alternative protein source for people sensitive to the common protein source. There was no report about allergic reaction to millet. However, little information on characteristics of millet is available. Highly purified millet protein without changing the characteristic of millet flour would contribute to better understanding of physiological effects of millet protein.The protein concentration of millet varies from species to species in the range of 9.3-12.7%, but is generally high compared to those of rice (6.8-7.4%) and wheat (10.5%) **[85]**.

Five millet types are common: common millet (Panicum miliaceum), Foxtail millet (Setaria italica), Finger millet (Eleusine coracana), Pearl millet (Pennisetum typhoideum) and Barnyard millet (Eichinochloa frumentacea). Generally, millets are consumed widely as subsistence or emergency foods. The worldwide food shortage in recent years has aroused renewed interest in millet as evidenced by the release of improved millet cultivars in South Asia. However, little published information is available on the nutritional composition of these new cultivars **[13]**.

Finger millet (Eleusine coracana) is one of the important minor cereals and constitutes a staple food for a large segment of the population in the Indian subcontinent as well as of many in the African countries, The seed coat of the millet is an edible component of the kernel and is a rich source of phytochemicals, such as dietary fibre and polyphenols, and is also a very good source of minerals, especially calcium **[86, 87]**.

Seeds were analyzed for fatty acid, amino acid and mineral contents. They contained 12 mg/g total fatty acid, 42% of which was oleic acid (C18:1n-9), 21% palmitic acid (C16:0), 25% linoleic acid (C18:2n-6) and 4% α-linolenic

acid (C18:3n-3). The α-linoleic acid/-linolenic acid ratio (6.5:1) was within the World Health Organization (WHO) recommendation (5:1 to 10:1) **[88]**.

Pearl millet is particularly rich in zinc and iron and has a high level of fat when compared to other cereals. However, due to the presence of hard seed coat and high fiber content, it has poor consumer appeal. In addition, the presence of anti-nutritional compounds such as phenols and tannins reduces its preference and for this reason, it is not ideal in the preparation of weaning foods. It has been suggested that various processing methods could reduce the anti-nutritional factors and improve the nutritional quality of pearl millet . Soaking is an essential processing method, which is simple and saves fuel as it facilitates cooking. Sprouting facilitates reduction in anti-nutritional compounds and increases enzymatic activities in minor millets **[89, 90, 91]**.

The poor starch and protein digestibility of cereals is caused by phytic acid and polyphenols that bind to enzymes in the digestive tract and thus inhibit utilization of proteins and carbohydrates. This adverse effect can be overcome by fermentation, germination or extrusion. Fermentation and germination improve digestibility by partial hydrolysis of storage proteins and carbohydrates by endogenous and microbial enzymes, whereas extrusion and other forms of cooking improve digestibility of starch by solubilization of the granules, gelatinization and formation of maltodextrins **[92]**.

## 2.4 Soy sauce

### 2.4.1 The wonderful world of traditional soy sauce

(Jiang is  a soy sauce precursor, was first produced in China as far back as 500 B.C. In the classic mode of seemingly all oriental stories that find their way to the occident, a Zen priest brought epicurean enlightenment from China to Japan some thousand years later. Thereafter, soy sauce evolved as adistinctly Japanese culinary institution. It was not until the 1800s that soy sauce began to find its way to the United States, along with oriental laborers. By 1972, demand for traditionally prepared soy sauce was robust enough to

lead the Kikkoman Corporation (purveyors of fine soy sauce since the1600s) to open their first American production facility, in Walworth Wisconsin) **[93]**.

Fermented soy sauce is prevalently used in Asian countries and is rapidly propagating into North American and European markets because of its unique appetizing flavor. The authentic soy sauce production takes 6-8 months and is composed of several steps. Though many types of fermented soy sauce are marketed, (deep-colored" soy sauce is the predominant one among various types of soy sauce produced from wheat and soybeans. **[94, 95].**

Further, deep-colored fermented soy sauce can be classified two types according to the soybeans used as starting materials. Originally, in Japan, soy sauce had been produced from wheat and whole soybeans since the 16th century. However, soy sauce production using wheat and defatted soybeans was developed just after the World War II to efficiently utilize defatted soybean resources **[94, 95]**.

Asian countries such Japan, china, Malaysia, Philippines, Thailand, Indonesia and Korea have many kinds of indigenous fermented foods made from soybeans. Each country has different methods for production of soy products dependent on preferred taste and flavour. They include soy sauce (fermented wheat and soybeans); sufu (fermented soybean protein crud); tempeh (fermented whole soybeans), Tofu (coagulating soy milk and pressing the resulting curds) and others. Various aspects of soybean production have been researched, such as technological, biochemical, microbiological, toxicological and mechanical engineering viewpoints **[96]**.

In Indonesia two kinds of soy sauces can be distinguished; kecap asin and kecap manis. The first has a salty taste and is mainly consumed on Sumatra; the second has a sweet taste due to a large amount of added sugar and is preferred by the people of Java. Kecap manis production is generally done as shown in (Figure2.9) **[94]** and the Japan sauce is done as shown in (Figure 2.10), **[97]**.

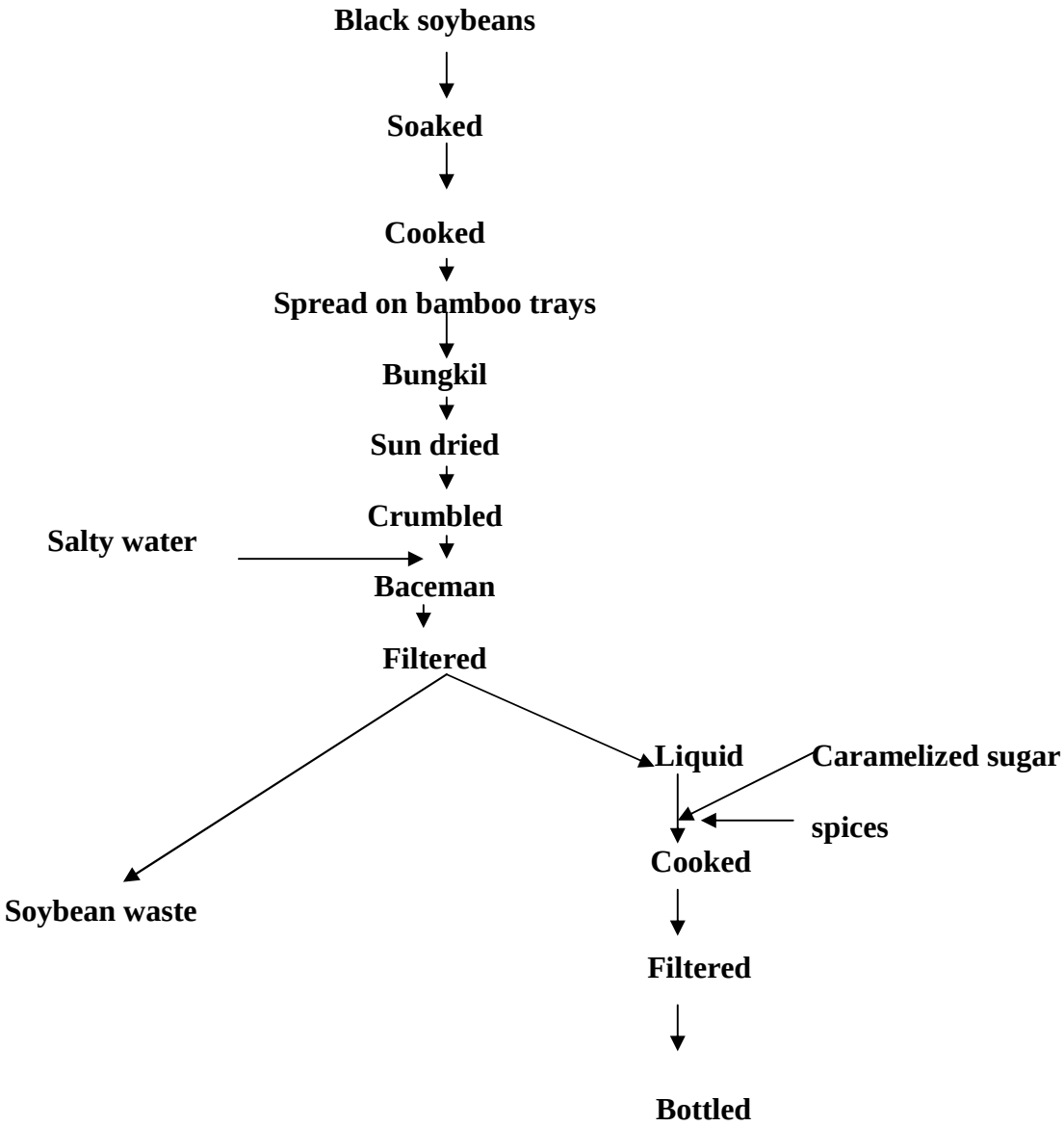



**Figure : 2.9 .** Scheme of Indonesian kecup(soy sauce)production

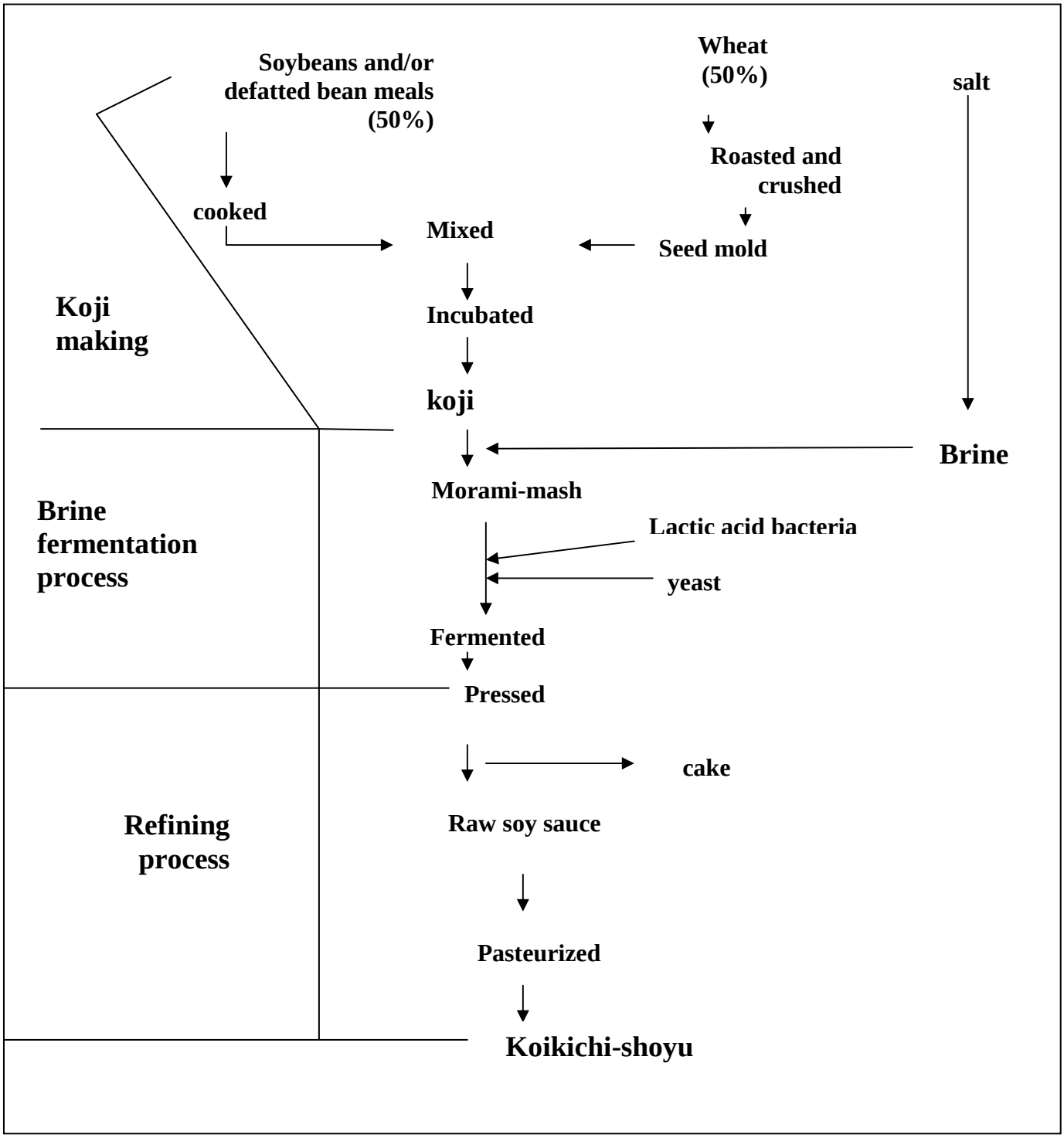



**Figure (2.10)** scheme of koikuchi soy sauce production

### 2.4.2 Fermentation

Campbell-Platt has defined fermented foods as those foods, which have been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification to the food. However, to the microbiologist, the term"fermentation" describes a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor **[98]**.

Fungal fermentations are typically performed in liquid culture medium submerged fermentation; (SmF) or on a solid substrate, such as cereals or beans solid-state fermentation; (SSF). SmF is usually the production method of choice in the West, whereas SSF is much more used in Asia. SSF can be superior with respect to productivity or yield of certain products and, in addition, fungi may produce a different spectrum of products in SSF. The reasons for this are not fully understood, but it is likely to be a consequence of the different physiology of the fungi on a solid substrate compared to submerged cultivation. Fungal physiology in SSF is not well characterized, but an understanding of physiology is required to explore the possibilities for controlling or directing product formation **[99]**.

In the course of time, it was discovered that microorganisms could modify certain compounds by simple, chemically well-defined reactions, which were further catalyzed by enzymes. Nowadays, these processes are called "biotransformation" The essential difference between fermentation and biotransformation is that there are several catalytic steps between the substrate and the product in a fermentation while there is only one or two in a biotransformation. The distinction is also in the fact that the chemical structures of the substrate and the product resemble one another in a biotransformation, but not necessarily in fermentation **[100]**.

### 2.4.3 Importance of soy sauce

#### 2.4.3.1 Soy sauce as antioxidants

Phytoestrogens comprise a class of several different chemicals produced by a variety of plants .Of these, the soy isoflavones (particularly genistein) are of greatest interest because of the widespread human consumption of soy, due largely in Western countries to extensive advertising by the soy industry for potential human health benefits **[101]**.

Soybean and soybean products, containing various amounts of phenolic compounds, have been shown to possess antioxidative ability. Concentrations of phenolic compound were reported to increase in soybean. For example, the antioxidative activity of fermented soybean products such as miso, tempeh and natto, inoculated with *A. oryzae*, *Rhizopus oligosporum* and *Bacillus natto*, respectively, was significantly higher than in non-fermented steamed soybean **[102].**

It was suggested that the liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genestein by the catalytic action of β -glucosidase during fermention resulted in the increased antioxidative activity of miso and tempeh, while it was also reported that a significant increase in the formation of a water-soluble antioxidative fraction, not the free aglycone, lead to the enhanced antioxidative activity of natto. Furthermore, the use of diazein and geistein during the fermentation of Japanese soybean has yielded o-hydroxyisoflavones, a potent antioxidant **[102].**

#### 2.4.3.2 Soy sauce and some diseases

Epidemiological studies and nutritional surveys have led researchers to identify dietary components that may have significant health benefits. Researchers have identified both antioxidants and phytoestrogens as potentially beneficial bioactive food components for example, soy-rich diets (the so-called a sian diet) correlate with a low incidence of **cardiovascular** disease, **osteoporosis**, and **estrogen-related cancers** such as **breast and**

**endometrial cancer.** Soy- supplemted diets relive symptoms of menopause such as hot flashes. Because of these finding, soy-based diets have become more popular in the United States. Soybeans contain several phytoestrogens (plant estrogens), some of which become biologically active only after microorganisms in the gut metabolize them. Phytoestrogens can block the activity of normal estrogen by binding to estrogen receptors and may elevate the level of serum proteins that bind this sex hormone. Through this dual action, they may lower the risk of certain cancers correlated with elevated plasma estrogen levels **[75, 98,103,104,105]**.

A case–control study among Asian–American women detected a 30% decreased risk of pre- and postmenopausal women breast cancer for women who reported eating tofu more than once a week as compared with women who ate tofu less than once a month. In a study from Singapore, consumption of 55 g or more soy products per day was protective in premenopausal but not in postmenopausal women. Studies from China and Japan did not detect a protective effect of soy intake. However, two recent studies reported a lower breast cancer risk with increasing isoflavonoid excretion in urine **[106]**.

**Fibrinolytic enzymes** are agents that dissolve fibrin clots. Recently many food derived fibrinolytic enzymes have been found in various traditional Asian foods. Fibrinolytic enzymes can be found in a variety of foods, such as Japanese Natto, Tofuyo, Korean Chungkook-Jang soy sauce, and edible honey mushroom. Enzymes have been purified from these foods, and their physiochemical properties have been characterized. Fermented shrimp paste, a popular Asian seasoning, was shown to have strong fibrinolytic activity. These novel fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing **heart disease**, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular disease **[107]**.

The protective effects of soy may also be exerted in other steps involved mechanistically in cancer development, such as phase I and II metabolism of carcinogens. Induced levels of the phase II enzymes, glutathione S-transferase (GST) and quinone reductase (QR) were proposed as suitable biomarkers for identifying compounds likely to inhibit carcinogenesis. There is evidence for a relationship between induction of phase II enzyme activity by dietary nonnutritive compounds and anticarcinogenic effects in the dimethyl benza anthracene (DMBA) induced animal tumor model There is also initial evidence that soy or its isoflavones can induce antioxidant and phase II enzymes **[103]**.

Recent studies have shown that cells have two types estrogen receptors; α and β Human estrogens have more affinity to α receptors, whereas, isoflavones have high affinity to β-receptors. β receptors exist in brain, bone, bladder and vascular epithelium, being important in the function of non-steroid estrogens **[108]**.

Isoflavones have a chemical structure resembling that of estrodiol- 17β, the most potent mammalian estrogen. The major isoflavones, namely, genistein and daidzein, have several features in common with estradiol-17β, including an aromatic A ring with hydroxyl group in the same plane at a distance similar to that in estradiol (Figure 2.11) **[97]**.

Indeed, isoflavones appear to have both estrogenic and antiestrogenic effects, like selective estrogen receptor modulators (SERMs), depending on the target tissue. Therefore, rather than classifying soy isoflavones as "estrogens", they should be judged more correctly as natural SERMs. Reproductive cells, especially those of the breast and uterus, are rich in estrogen receptor α (ERα), whereas other cells (such as those in the bone) have greater amounts of estrogen receptor β (ERβ) than ERα . This differential distribution of the two types of estrogen receptors, and the greater affinity of the isoflavones for ER β in relation to ER α suggests that the isoflavones have different effects in different tissue. Despite the beneficial effects of isoflavones on postmenopausal health are still controversial, there are some researches, including epidemiological studies, suggest that

isoflavones may help to alleviate postmenopausal symptoms and protect against chronic diseases such as hormone-dependent cancer (e.g. breast and endometrial cancer), cardiovascular diseases and osteoporosis. Thus, in terms of both health promotion and chronic disease prevention, the potential public health impact of daily soy consumption could be important, especially in postmenopausal women. Although many commercial soy capsules containing isoflavone extract are now available in many Western countries, soybeans and soy food (such as tofu, soy flour, soy milk, etc.), which provide the main sources of isoflavones, are consumed in significant amounts in Asian countries because they are inexpensive and high in quality protein. The purpose of this trial was to compare the pharmacokinetics of plasma isoflavones, daidzein and genistein, in postmenopausal Thai women after a single dose of orally administered commercial soy extract capsules and soy beverage **[104,109,110]**.

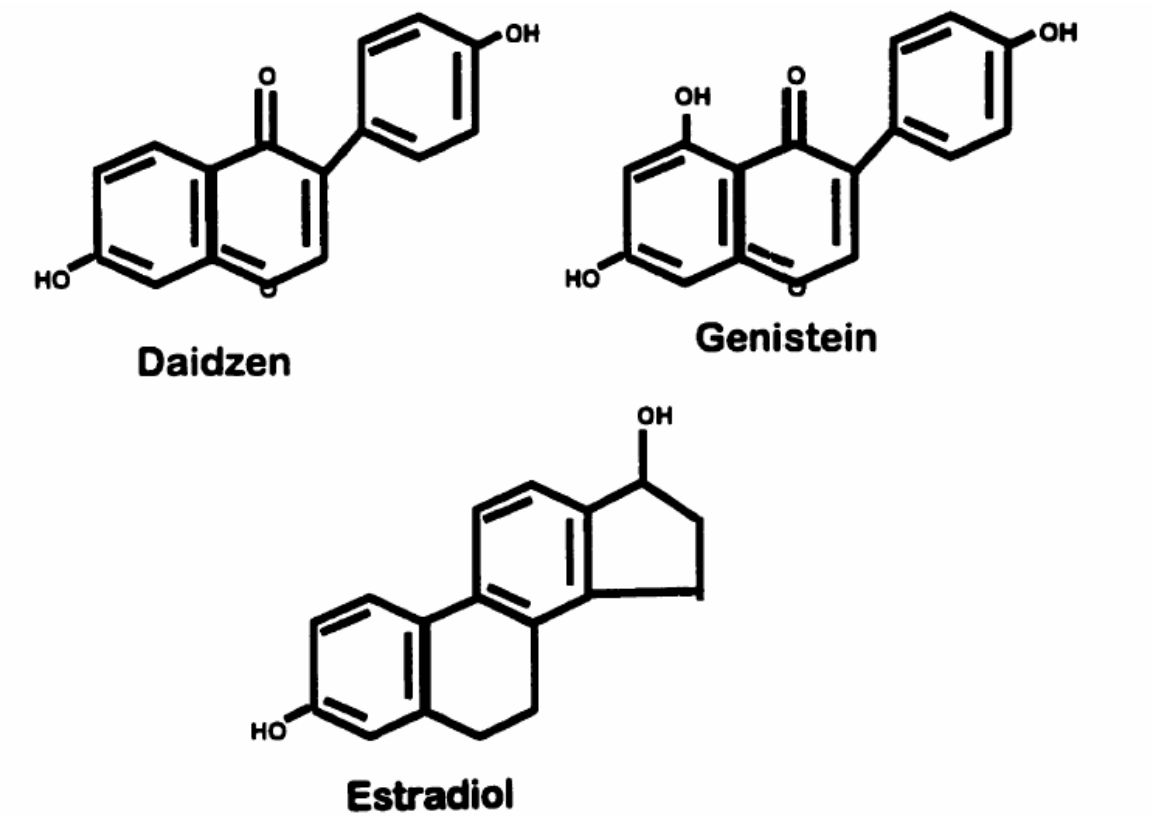



**Figure (2.11)** Principal isoflavones aglycones of soy are genistein and daidzen. The female hormone estradiol is also shown

#### 2.4.3.3 Soy sauce facilitate digestion

Soy sauce promotes digestion, because the consumption of a cup of clear soup containing soy sauce enhances gastric juice secretion in humans. Soy sauce possesses antimicrobial activity against bacteria such as *Staphylococcus aureus, Shigella flexneri, Vibrio cholera, Salmonella enteritidis, nonpathogenic Escherichia coli* and pathogenic *E. coli O157:H7*. Soy sauce also contains an antihypertensive component. An angiotensin I-converting enzyme inhibitor having antihypertensive effects was found in soy sauce. The active compound was identified as nicotianamine, which comes from soybeans **[111]**.

#### 2.4.3.4 Soy sauce as anti allergic activity

Soy sauce is a traditional fermented seasoning of East Asian countries and is available throughout the world. In Japanese soy sauce (shoyu), soybeans and wheat are the two main raw materials, used in almost the same quantity. Proteins of the raw materials are completely degraded into peptides and amino acids by microbial proteolytic enzymes after fermentation, and no allergens of the raw materials are present in soy sauce. In contrast, polysaccharides originating from the cell wall of soybeans are resistant to enzymatic hydrolyse. These polysaccharides are present in soy sauce even after fermentation and termed shoyu polysaccharides (SPS). Soy sauce generally contains about 1% (w/v) SPS and SPS exhibit potent antiallergic activities in vitro and in vivo. Furthermore, an oral supplementation of SPS is an effective intervention for patients with allergic rhinitis in two double-blind placebo-controlled clinical studies. In conclusion, soy sauce would be a potentially promising seasoning for the treatment of allergic diseases through food because of its hypoallergenicity and antiallergic activity **[112]**.

### 2.4.4 Soy sauce production

#### 2.4.4.1 Koji

The manufacture of soy sauce generally involves two stages of fermentation, the koji stage and the moromi stage. Koji is a Japanese word describing the preparation of mould growth on cooked cereals and/or soybeans. It serves as

an enzyme source for the conversion of natural plant constituents to simpler compounds. Koji for Chinese soy sauce is made from a mixture of wheat flour and steamed soy beans with strains of *A.* or *A. soyae* as the koji starter **[113]**. On the other hand, roasted wheat and steamed defatted soybean meal are often used by the Japanese when they prepare koji. Soy sauce koji, with its dark-green appearance, pleasant aroma and sweet taste with a slightly bitter note, contains high protease and amylase activity and is generally regarded as of superior quality. During the koji stage of fermentation, proteins are broken down to peptides and amino acids by proteolytic enzymes, especially by neutral and alkaline proteases. Polysaccharides are hydrolysed to oligosaccharides, disaccharides and monosaccharides, mainly by α-amylase secreted by the mould, although some invertase activity has also been detected. Lipids are also acted upon by the lipase present in the koji **[113]**.

#### 2.4.4.2 Brine

The dried beans are mixed with salty water (12-26% sodium chloride), resulting in the baceman. This is placed outside under the sun and kept for 2 to 120 d, after which the mash is filtered and the liquid cooked together with sugar and spices **[94]**. In soy sauce production, enzymic hydrolysis, lactic acid fermentation and ethanol fermentation of the mash take place in a batch process over a period of 6 months.' The lactic acid fermentation is important in controlling the pH of the mash and preparing appropriate conditions for the yeast fermentation to produce ethanol. The lactic acid fermentation process also plays an important role in the quality control of the mash and has an influence on soy sauce flavor and taste **[6]**.

Three overlapping phases could be distinguished in brine fermentation:

**1-** Amino acid production (judged from formol nitrogen production)

**2**- Fermentation by lactic acid bacteria (judged from acetate and lactate production)

**3**- Yeast fermentation (judged from ethanol and glycerol production) **[114]**.

### 2.4.4.3 Pasteurization and blending

A heater is used to pasteurize the filtrates of the aged mash at 80 °C in order to stop the greater part of microbial and enzymatic reactions and to bring about the sedimentation of incompletely degraded protein compounds by coagulation. It helps to enhance their flavour and colour and none of the nutrients is lost. Sometimes, some ingredients, for example, sodium glutamate, sugar, maltose or spices, may be added to make the special flavor of soy sauce when soy sauce is blended. Chemical preservatives are very often added to soy sauce to prevent spoilage by microorganisms. The chemical preservatives most widely used in China are sodium benzoate at a concentration of 0.01% **[115]**.

The addition of preservatives to food has been carried out for centuries. Some methods of preservation are based on the use of natural substances, such as essential oils. However, the most commonly encountered food preservatives are weak acids, such as sorbic, benzoic, propionic and acetic acids and sulphur dioxide (sulphite), some of which occur naturally in foods (fruits and vegetables). Weak-acid preservatives are widely used in sugar containing, low-pH foods such as fruit juices, beverages, wine, dressings and sauces, in which spoilage is most often caused by yeasts, moulds and lactic acid bacteria **[116]**.

### 2.4.4.4 Thickening

In sauces thickened with potato starch xanthan gum blends, penetration force, adhesiveness, and stringiness increased, and the same trends could be observed in sauces thickened with blends of oat starch with oat hydrolysates. In sauces containing either the blends of oat starch with xanthan gum or three component blend of oat starch with oat hydrolysate or xanthan gum these textural parameters decreased on storage, although in the latter blends changes in stringiness were negligible **[117]**.

### 2.4.4.5 Bottling

Packaging is the tool that protects and contains goods so that the environmental impact on the food in the package is minimized. Effective packaging is vital to the health and welfare of the consumer. The materials and methods used to package food have changed more in the past 10 or 15 years than over the preceding 150 years. However, there is scanty research information on the effects of packaging in aluminum on food items. The data on aluminum concentration in food items are scarce, although aluminum containers are widely used to cook, freeze, or wrap foods the quality, safety, and nutritional content of packaged foods has not been thoroughly researched for certain newly packaged products. The desire for higher quality and safer food with a longer shelf life has led to increased interest in the interaction between foods and food packaging **[118]**.

The migration of food packaging elements to the contacted food has received some attention over recent years. Food packaging interactions can be defined as interplay between food, packaging, and the environment, which produces an effect on the food and/or package reported that aluminum was earlier considered to be a non-risk element and its toxicological evaluation ratio was only recently presented in the report of the Joint WHO/FAO Expert Committee on Food Additives. Now that a provisional tolerable weekly intake (PTWI) of 7 mg/ kg body weight has been established for aluminum, it is even more important to pursue and collect data through studies on this topic **[118]**.

### 2.4.5 Characteristics of soy sauce

### 2.4.5.1 Color

Soy sauce color is a pivotal factor in evaluating its commercial value, Superfluous coloration vitiates its quality remarkably and diminishes product value. Therefore, intensive efforts have been made to preclude coloration in manufacturing processes for usukuchi type soy sauce; also, further development of such technology is considered an absolute necessity.

Coloration of soy sauce is caused by melanoidin, which is formed by non-enzymatic reaction with sugar and amino acid in what is known as amino-carbonyl reaction. Sugar contributes to this aminocarbonyl reaction; pentoses, such as xylose and arabinose, have higher reactivity than hexoses such as glucose and galactose **[119]**.

Browning reactions occurring in food systems may be broadly classified into non-enzymatic and enzymatic reactions. Non-enzymatic browning results from oxidation, caramelisation, or the Maillard reaction. Enzymatic browning is due to oxidation of phenolic compounds by the action of oxido- reductase enzymes, e.g. polyphenol oxidase or ortho-diphenol oxidase, tyrosinase, laccase or paradiphenol oxidase, as well as peroxidase . Both non-enzymatic and enzymatic browning can cause destructive changes in the appearance and organoleptic attributes of food products, leading to short shelf life and lower market value. Browning is important when food is processed and preserved.**[120,121].**

Mash fermentation (moromi fermentation) and pasteurization (cooking) of raw soybean paste or raw soy sauce, prior to bottling; affect the browning in these products. About 50–60% of browning in soy sauce is developed during mash fermentation, and the remaining occurs during pasteurization. Both developments of browning are considered to stem from the Maillard reaction. Polyphenol oxidase-like enzymes, such as tyrosinase and laccase, are prevalent among fungi, and soybean is rich in phenolic compounds **[120,121]**.

### 2.4.5 2 Aroma

4- Hydroxy-3[2H]-furanones are exceptional aroma compounds due to their attractive flavor and low odor thresholds. They are biosynthesized by plants, microorganisms, and insects but are also formed during the thermal treatment of food in the so-called Maillard reaction. The furanones are formed during fermentation, indicating an enzymatically catalyzed bioformation performed by *Z. rouxii.* Few attempts were undertaken to identify the precursors of 4-hydroxy-3[2H]-furanones in soy sauce. D-Xylulose-5-phosphate, D-ribulose-

5-phosphate, and D-sedoheptulose- 7-phosphate were determined as potential precursors of homofuraneol and norfuraneol **[122]**.

4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, Furaneol), a potent aroma compound found in many fruits such as strawberry, mango, pineapple, and raspberry, is extensively used as food flavoring due to its low odor threshold and flavor enhancing properties. Aside from fruits, HDMF has been isolated from yeasts, bacteria, and insects, and it is formed chemically during the so-called Maillard reaction, the carbons in HDMF originate exclusively from exogenously supplied D-fructose 1,6- diphosphate **[122]**.

HEMF in miso and soy sauce is considered to formed by the combination of compound containing 5 carbons produced through the amino –carbonyl reaction under mild conditions of fermentation, and the chemical compounds of 2 carbons provided by the yeast **[123]**. While *Z. rouxii* produces ethanol and aroma components, *C. versatilis* produce specifically phenolic compounds such as (4-EG) and 4- ethylphenol which add characteristic aromas to soy sauce. It is considered that the optimum 4-EG content in conventional soy sauce is 1-3 ppm . The content of 4-EG was maximal at the pH values of 3.5-4.0 and decreased gradually above 4.0. 4-EG content was maximal at 30-33 °C **[124]**.

Application of aroma extract dilution analysis (AEDA) to the volatiles isolated from a commercial Japanese soy sauce revealed 30 odor-active compounds in the flavor dilution (FD) factor range of 8-4096, among which 2-phenylethanol showed the highest FD factor of 4096, followed by 3-(methylsulfanyl)propanal (methional), The tautomers 4-hydroxy-5-ethyl-2-methyl- and 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (4-HEMF), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF), and 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone), all showing FD factors of 1024 **[125]**.

### 2.4.5.3. Taste

Taste can be measured with a multichannel taste sensor, which responds to different taste qualities by a unique pattern of output signals. The sensor has lipid membranes as transducers and computer as a data analyzer. Electric potentials of eight kinds of lipid membrane in the sensor change depending on a variety of taste substances. The multichannel sensor easily distinguishes among qualities of five basic tastes sour, bitter, sweet, salty and umami. In addition, some kinds of beverages such coffee, mineral water, milk and aqueous drink. It has the sensitivity, durability and reproducibility superior to those of humans **[126]**.

### 2.4.5.4 Salt

Salt is a major ingredient of sauces and related products such as Worcestershire sauce, tomato ketchup, soy sauce, mayonnaise and salad cream, mustards, mint sauce, and horseradish sauce. Typical levels are 2.3% in tomato ketchup, 1.5%, and 2% in mayonnaise and salad cream, respectively, but levels in soy sauce may be above 10% In addition to its contribution to the overall savory flavor of the sauce, salt has a significant preservative effect in these products **[127]**.

# CHAPTER 3

# Materials and Methods

## 3.1 Collection of samples

Salt, Millet (*Panicum miliaceum*), dill seeds (*Anethum graveolens*), thyme (*Thymus vulgaris)* and wheat (*Triticum aesvum*) were obtained locally, rice (*Oryza sativa L*) and soy beans (*Glycine max*) were purchased from Egypt.

## 3.2 Microorganisms

A reference strain of *A. oryzae* was obtained from Thailand (faculty of science, mahidol university).

## 3.3 Media

- Aspergillus differentiation agar (base) was obtained from (sigma; India)
- Potato dextrose agar was obtained from (himedia; India)
- Chloramphenicol supplement was purchased from (liofilchem; Italy)

## 3.4 Chemicals

$NaNO_3$, KCl, $MgSO_4–7H_2O$, $ZnSO_4–7H_2O$, $CuSO_4–7H_2O$, $K_2HPO_4$, Agar, Yeast extract, Sucrose were obtained from labs of the faculty of science, IUG.

## 3.5 Isolation of *A. oryzae*

The international seed testing association techniques especially (ISTA) (Agar plate method) was used to detect the *A. oryzae* from (Rice, soybeans and wheat seeds) **[128]**. The sample of seeds was divided into three groups each group was represented by fifteen Petri plates, (15 for soybeans, 15 for wheat and 15 for rice) ten seeds were inoculated into each plate. The first group was untreated. the second group was treated with HOCl 7 % for 5 minutes and thoroughly washed in sterilized distilled water before plating on PDA. The third group was treated with HOCl 3.5% for 5 minutes and then washed in sterilized distilled water before plating **[128]**. All plates were incubated at

30°C and examined after 4 days. Samples of spores assumed to be *A. oryzae were* plated on PDA and incubated at 30°C for 5 days until sporulation. Five pure cultures of isolates for each sample were obtained by sub-culturing on PDA slants for further identification. Spores mold obtained from Thailand were inoculated on PDA then transferred to PDA slants to be used as a reference strain.

### 3.5.1 Differentiation on AFPA selective medium

The isolates and reference strain were inoculated in triplicate on AFPA selective medium, incubated at 30°C for 48-72 h and observed for reverse color. All the plates were incubated at 30°C, and observed every two days for one week for observing the changes in the reverse color.

### 3.5.2 The morphological characteristics

The isolates and reference strain were plated on CYA (Czapek conc. 1ml, $K_2HPO_4$ 1g, yeast extract 5g, sucrose 30g, agar 15g and distilled water 1L) at 25°C for 7 days. The morphological characteristics of isolates and reference were compared **[1]**.

### 3.5.3 Slide preparation.

1. A drop of 70% alcohol was placed on a microscope slide.
2. The filaments of *A. oryzae* were immersed in the drop of alcohol.
3. One or at most two drops of the lacto-phenol were added before the alcohol dries out.
4. The cover slip was held between forefinger and thumb, touched one edge of the drop of lactophenol with the cover slip edge, and lowered gently, avoiding air bubbles.

The microscopic characteristics of isolates and the reference were examined according to **[129]**.

## 3.6 Soy sauce

### 3.6.1 The materials

Three hundred and thirty grams of soybeans, 300g millet, 61.41g sea salt, thyme, dill seeds, glass containers, glass bottles, trays, incubator, water and a blender.

### 3.6.2 Microorganism

A reference strain and isolates *A. oryzae* from (rice and soybeans). This organism was selected because it is abundant in protease, and amylase production, lack in toxins forming and yielded a good flavor and aroma in soy sauce.

### 3.6.3 Preparation of starter

Five grams of crushed millet spores of *A. oryzae* and 2 ml of distilled water were placed in Petri dish and incubated at 30°C for 4 days.

This was done using three strains (a reference, soybean isolate and rice isolate).

### 3.6.4 Koji production

#### 3.6.4.1 The Materials

Four-tenths gram of starter for each strain (a reference, soybean isolate and rice isolate) crushed millet and soybeans approximately 1:1. Incubator, autoclave, plastic sieving, trays, deep containers and spoons.

#### 3.6.4.2 Method of preparation of koji

1. Steaming soybeans, crushed millet
2. Starter
3. Mixing of starter and crushed seeds
4. Incubation for 3 days

## 3.7 Preparation of spore suspension.

Starter (for each sample) was used to prepare the spore suspension of *A. oryzae.* The spores were contained in bottles keeping at 4°C for 12 h and were used later, when using, it was adjusted with sterile distilled water. The spore count was determined by using the haemocytometer and recording the number of spore per milliliter.

## 3.8 Treatment of soybeans

Three hundred and thirty grams of soybeans was soaked in water for 15 h, the amount of water used for soaking was triple the amount of soybeans. During soaking, the water was changed every four hours; the weight was increased from 330 g to 753 g. Then the soybeans were crushed in a blender.

### 3.8.1 The cooking of soybeans

Different cooking conditions for soybeans are considered such as pressure cooking, steaming and normal cooking **[96]**. Pressure steaming is chosen in this experiment. The duration of steaming time will help to determine the texture and the color of sauce. Steaming at high pressure for 30 min lead to optimal nutritional value.

### 3.8.2 Draining

After steaming, the crushed soybeans were placed on a cloth to drain in sun light for 3.1/2 h, the weight was reached 528g.

## 3.9 The roasting of millet

Three hundred grams of millet was roasted in a tray at weak gas flame for 15 min. During roasting, regular stirring was used to avoid any burning, and then they were crushed by a blender and kept away from contamination. Five hundred and twenty eight grams of crushed soybeans and 300 g of crushed millet were mixed then divided into three equal parts.

## 3.10 Mixing

Four-tenths gram of starter (for three strains) are added for each part to the crushed seeds on trays, wet by water, and mixed. This mixture was placed in plastic sieving, which put on deep container, covered by plastic tray, and incubated for 72 h at 30°C. The mixture was stirred twice daily to expel $CO_2$ and to increased the $O_2$ concentration.

## 3.11 Brine fermentation

### 3.11.1 Materials

- Koji 276 g for each part.
- Salt 61.41 g for each part.
- Water 375 ml for each part.
- Glass containers 3 containers
- Thyme, dill seeds.

### 3.11.2 Methodology

A glass container was used to process a mixture of koji (276 g) with salt (61.41g) and 375 ml $H_2O$. Then the container was placed in incubator for fermented ageing at 30°C for 3 months. During the ageing, two times stirring was used to accelerate the brine fermentation.

### 3.11.3 Pressing

The moromi is wrapped in finely woven cotton cloth, then pressed to extract the raw soy sauce.

### 3.11.4 Pasteurization and bottling

The raw soy sauce was pasteurized at 80°C for 30 min. At the end of pasteurization, the spices were added, left at room temperature, then filtered and bottled.

## 3.12 Chemical analyses of the soy sauce

### 3.12.1 pH measurement

The pH was measured using (a pH meter 211). Glass calomel electrode was dipped into soy sauce at room temperature.

### 3.12.2 Ash analysis

Crucibles were labeled on the bottom with a lead pencil and weighed. About 4 -5 g of sample was weighed out into weighed crucibles. The crucibles were dried in an oven at 100°C, then heat gently over a Bunsen burner until the sample charred. Transfer to muffle furnace at about 550°C and left until light grey ash result. The ash content was calculated by:

% of ash = weight of ash / Weight of sample ×100 **[130]**.

### 3.12.3 Moisture and total solids analysis

Dried crucibles were labeled and weighed. About 4-5 g of sample was weighed out and placed on a steam bath until the excess water has evaporated, dried in an oven at 105°C for 5 h, and cooled. After that, the crucibles were weighed again to measure the moisture loss.

% moisture = loss in weigh of sample / weight of sample ×100

% total solids = 100% - % moisture [**130]**.

### 3.12.4 Nitrogen and Protein analysis

Protein content in soy sauce was determined by the Kjeldahl method. Three samples of 0.1 g were digested in sulphuric acid in the presence of potassium sulphate and mercuric oxide as a catalyst. There after, each sample was placed in distillation unit, (1002 Kjeltec System). The acid solution was made alkaline by a sodium hydroxide solution. The ammonia was distilled into boric acid and the acid was simultaneously titrated with 0.1M HCL. The nitrogen content was calculated by:

Nitrogen%= 100 (A×B×.014) / C

Crude protein% = nitrogen in sample × 6.25

Where A= hydrochloric acid used in titration, B = normality of standard acid, C = weight of sample **[131]**.

### 3.12.5 Salt content analysis (Mohr titration)

Five 5 ml of the soy sauce was diluted to 250 ml, 25 ml of the diluted brine was transfered into a 250 ml conical flask, 1 ml of potassium chromate indicator was added and titrated with 0.1 M silver nitrate solution until reddish brown color appeard. The salt content was calculated by:

% (m / v) salt in brine = 58.5 ×0.1 ×T/ 5

Where T = mean titer of 0.1 M silver nitrate in ml [**130]**.

### 3.12.6 Ethanol analysis

Alcohol was analyzed by distillation method. Ten ml of a sample and 125 ml of distilled water were prepared in distillation flask, about 95ml of distillate was obtained into a 100 ml volumetric flask, the flask was filled to the mark with distilled water and mixed, in conical flask 10ml $K_2Cr_2O_7$ 0.2 N, 5 ml $H_2SO_4$ conc. and 10 ml distillate were prepare and boiled for 10 min., 500 ml was transferred to the flask, 300 ml of distilled water was used and 1 g of kI then the all shaked , titrated with sodium thiosulfate 0.1 N

Ethanol /g = 1.15 ($V_1n_1$-vn) R / C

$V_1$ = volume of chromate for titration, $n_1$ = chromate normality, v =sodium thiosulphate for titration, n = thiosulphat normality, R = dilution factor, C = sample volume **[132]**.

### 3.13.7 Calicum analysis

Ca was analyzed by using Atomic absorption spectrophotometer (A. Analyst 100 perkin–Elmer), standard solutions of different concentrations are prepared for Ca ( 5-10-15-20-30-40-50 ppm ). The values are calculated from the standard curve which was drawn according to the values resulted from the spectrophotometer of each concentration.

# Chapter 4

# Results

## 4.1 Isolation of *A. oryzae*.

The agar plate method was used to isolate *A. oryzae.* After 4 days of incubation, *A. oryzae* was observed growing on three types of seeds with varying degrees (Table 4.1). The determination of fungus type was depended on its specific yellow green color.

**Table (4.1)** The growth of *A. oryzae* on contaminated seeds in different concentration of HOCl

| Seed / state | untreated | Treated by HOCl 7% | Treated by HOCl 3.5% |
|---|---|---|---|
| Soybeans | 5+ | 5+ | 5+ |
| Rice | 4/5+ | -- | 1/5+ |
| Wheat | 2/5+ | -- | 2/5+ |

*Symbols: -, no visible growth; 1/5+, visible growth on only one Petri; 2/5+, visible growth on two Petri ; 4/5+, visible growth on four Petri; 5+, visible growth on all five Petri*

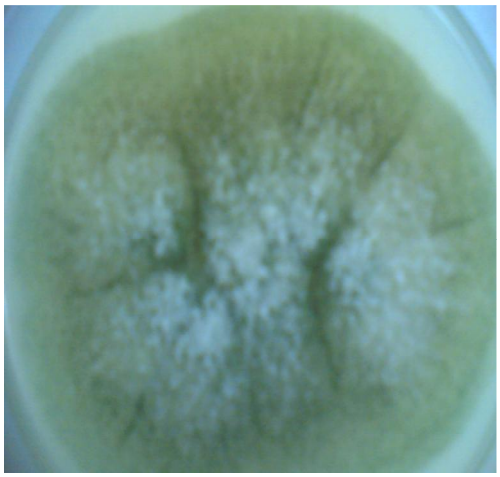
Three pure cultures assumed to be *A. oryzae* were obtained from contaminated seeds. All isolates showed yellow green colony color on PDA. Moreover, All the isolated colonies of *A. oryzae* and reference strain were produced orange-yellow reverse color within 48 h of incubation at 30°C on AFPA. These isolates did not show any change in the reverse color during incubation for further one week at 30°C as the reference strain. The isolates were also inoculated on CYA to be compared with the reference strain to carry out the macroscopic characteristics of the isolates and the reference.

### 4.1.1 The Macroscopic characteristics of isolates and the reference

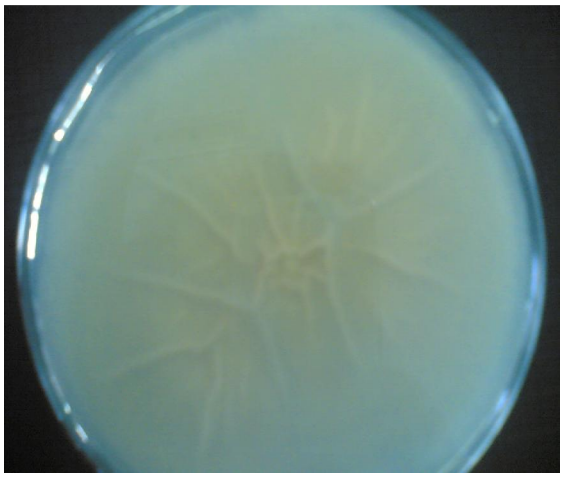
The macroscopic characteristics of isolates and reference of *A. oryzae* are summarized in (Table 4.2)

**Table (4.2)** Macroscopic characteristics of isolates compared with the reference strain of *A. oryzae* observed after 7 days of incubation at 25°C on CYA medium.

| characteristics | Soybean isolate | Rice isolate | Wheat isolate | reference |
|---|---|---|---|---|
| Colony diameter | 55 mm | 75 mm | 60 mm | 56 mm |
| Colony color | White centre green yellow periphery | White centre green yellow periphery | White centre green yellow periphery | White centre green yellow periphery |
| Colony reverse | Pale yellow | Pale yellow | Pale yellow | Pale yellow |
| Colony texture | wet | Wet | wet | wet |
| Conidial color | Yellow green | Yellow green | Yellow green | Yellow green |
| Nature of pore | powdery | powdery | powdery | powdery |



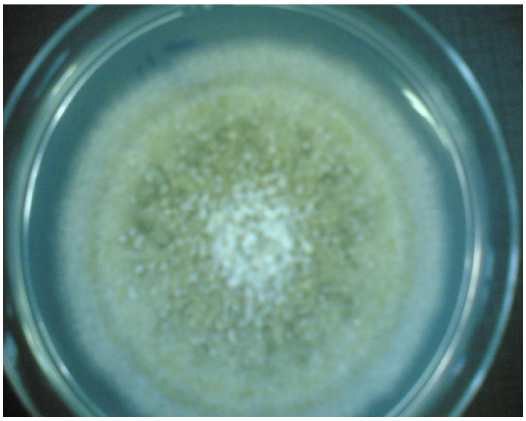
**A**



**B**

**Figure (4.1)** A colony surface of *A. oryzae* isolated from rice grown on CYA (A)
A colony back of *A. oryzae* isolated from rice grown on CYA (B)

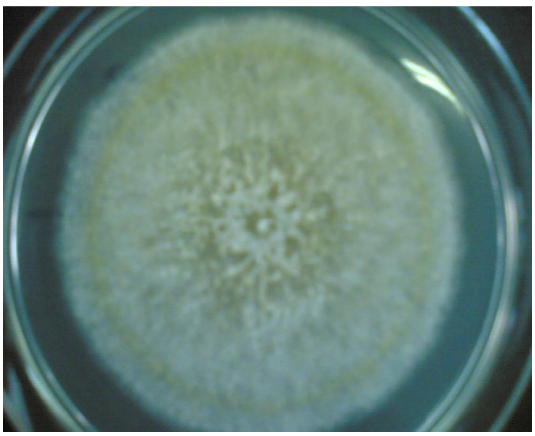
According to the figures (4.1- 4.4) and table (4.2), the color of all colonies of *A. oryzae* varied gradually from each other depending on growth density of the fungus.



A

B

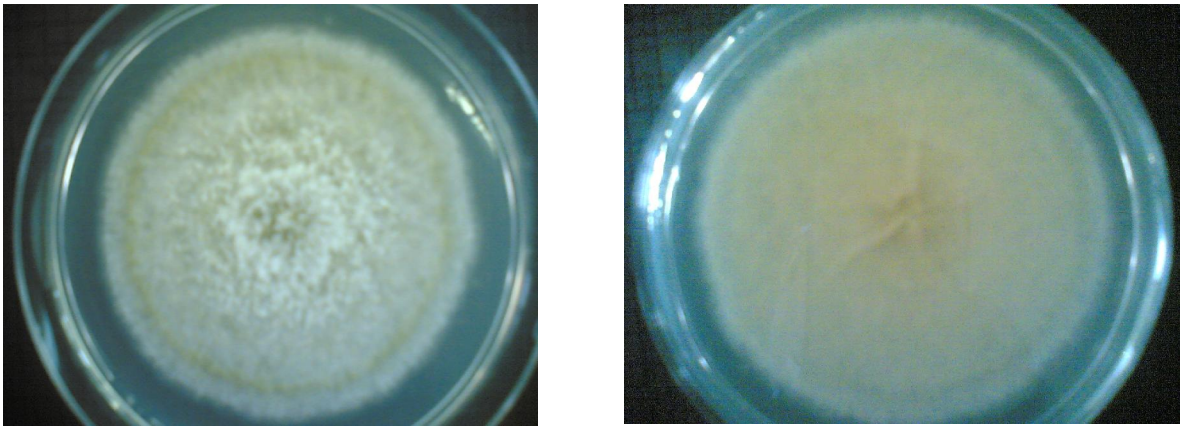
**Figure (4.2)** A colony surface of *A. oryzae* isolated from wheat grown on CYA (A)
A colony back of *A. oryzae* isolated from wheat grown on CYA (B)



A

B

**Figure (4.3)** A colony surface of *A. oryzae* as a reference grown on CYA (A)
A colony back of *A. oryzae* as a reference grown on CYA (B)



A B

**Figure (4.4)** A colony surface of *A. oryzae* isolated from soybean grown on CYA (A) A colony back of *A. oryzae* isolated from soybean grown on CYA (B)

### 4.1.2 The microscopic characteristics of the isolates and the reference of *A. oryzae*

Microscopic characteristics of the isolates compared with the reference strain *A. oryzae* were grown for 7 days at 25°C on CYA. All the isolates and the reference showed flask a vesicle with yellow green color. Phialides of the isolates and the reference were uniseriate with yellow green color. All conidia of the isolates and the reference were round. Conidiophores for the isolates and the reference were long-smooth and colorless. According to this results (Figure 4.5), the isolates and the reference are identical. Thus, the isolates are pure *A. oryzae*.

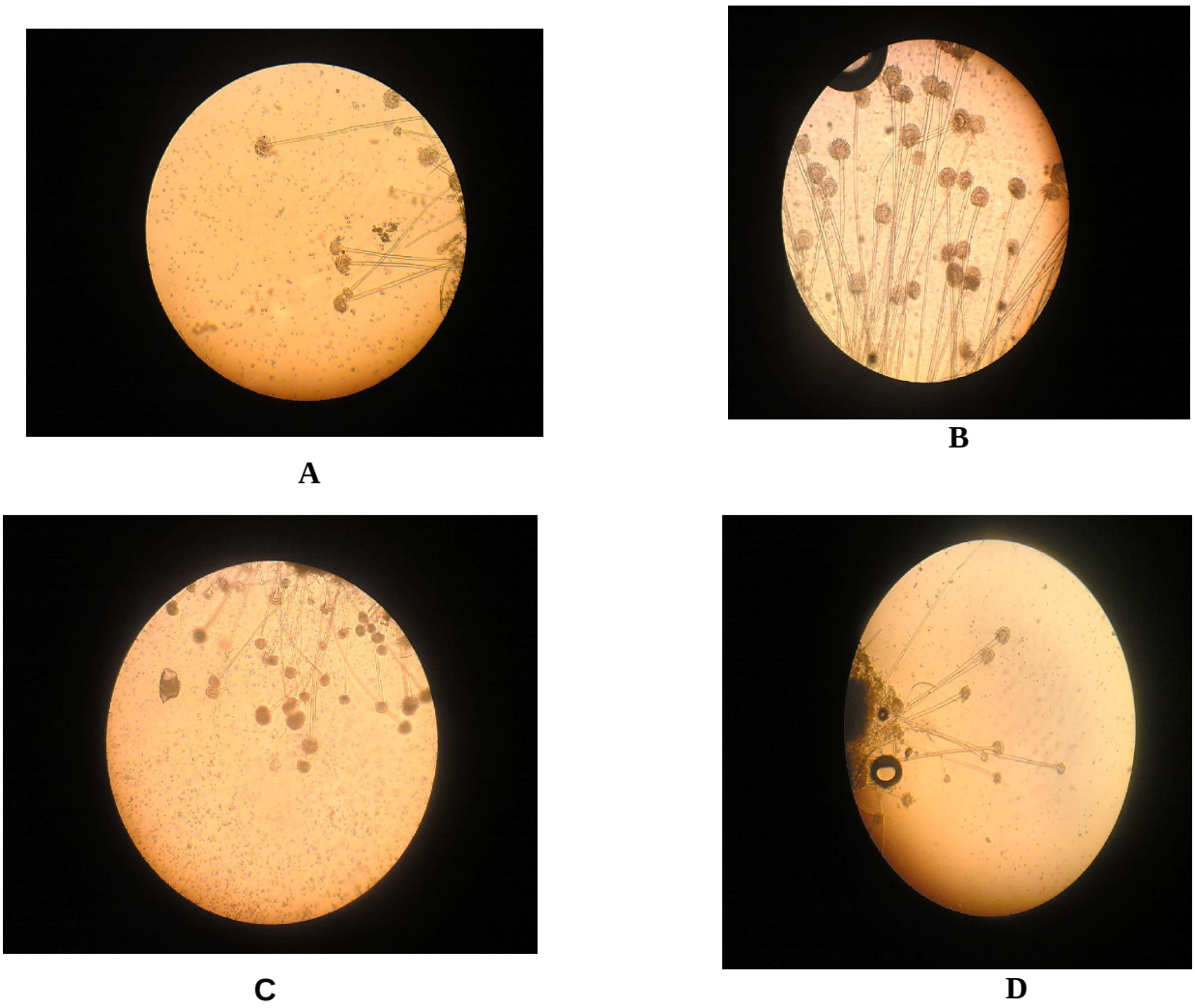



**Figure (4.5)** Fruiting heads of
A- a reference strain B - wheat isolate C - rice isolate D - Soybean isolate

## 4.2 Soy sauce production

### 4.2.1 Weight of soybeans increasing after soaking

The weight of 330 g of soybeans increased to 753 g after soaking, the maximum absorption was 128%. The weight of 753 g decreased to 528 g after steaming and drying. The steamed soybeans contained high concentration of water that was not suitable for growing the fungus. After drying, the beans were slightly wet when mixed with millet to give a good spore formation and growth of *A. oryzae*.

**Figure (4.6)** Soybeans, from which soy sauce was derived

### 4.2.2 The starter formation

The starter made in Petri dishes for the three strains (the reference, the rice isolate, the soybean isolate) (Figures 4.7-4.10). The initial growth of the fungus was indicated by vapor formation and condensing underside of the cover of Petri. Moreover, the crushed millet was covered by yellow greenish color of mycelial growth. The aerobic condition leads the mould to increase its biomass; this growing was combined by aromatic odor.

**Figure (4.7)** Millet, from which starter is derived

**Figure (4.8)** Petri Starter of rice isolate 4 days of incubation

**Figure (4.9)** Petri starter of soybean isolate after 4 days of incubation

**Figure (4.10)** Petri starter of the Reference after 4 days of incubation

### 4.2.3 Suspension of koji spores

The counting number of spore per milliliter by the haemocytometer for each strain was as follow:

*1.2x10*$^{7}$ spores /ml for a reference *A. oryzae*

1.33x10$^{7}$ spores/ml for soybean isolate *A. oryzae*

2x10$^{7}$ spores /ml for rice isolate *A. oryzae*

The number of spores of *A. oryzae* for rice isolate was more than the reference and soybean isolate.

### 4 2.4 Koji preparation

Koji was incubated for 72 h at 30°C. After 24 h of incubation, the heat of koji was raised gradually to 35°C and water started to drain. This drain was continued for 60 h then stopped at the last 12 h. At initial 36 h the *A. oryzae* was started to grow, continued in growing rapidly. The color of fungus throughout the incubation was started by white then yellow, lastly the yellow greenish was dominant .This changes were combined by releasing very clear volatile aroma. After the incubation period, the koji was dried at room temperature at 30-32°C for 23 h.

A

B The reference

C Rice isolate

D Soybean isolate

**Figure (4.11)** Immediately after Koji mold is added to soybeans and millet (A) and Koji three days after Koji mold is added, which is completely covered with Koji mold (B,C,D).

## 4.2.5 Soy sauce fermentation analysis

### 4.2.5.1 Chemical analysis of three kinds of soy sauce prepared by traditional fermentation

Chemical analysis was performed on three kinds of soy sauce that were manufactured by one technique. These included: soy sauce using starter of the reference, soy sauce using starter of rice isolate and soy sauce using starter of soybeans isolate. The results are shown in Table 4.4.

**Table (4.3)** Chemical analyses of three kinds of soy sauce products

| Types of chemical analysis | Reference soy sauce | Rice isolate soy sauce | Soy bean isolate soy sauce |
|---|---|---|---|
| pH | 4.16 | 4.65 | 4.25 |
| Ash % | 10 | 12.5 | 12.5 |
| Protein % | 8.1 | 9.8 | 7.2 |
| Nitrogen % | 1.3 | 1.5 | 1.1 |
| Moisture% | 76.6 | 43.3 | 78.3 |
| Total solids% | 23.4 | 56.7 | 21.7 |
| Ethanol % | 0.11 | 0.57 | 0.92 |
| NaCl % | 14.04 | 16.38 | 15.21 |
| Ca mg/100 g | 123 | 127 | 102 |

#### 4.2.5.2 Salt content

The value of NaCl range from 14.04 % to 16.38%, the reference NaCl was 14.04%, the rice isolate NaCl was 16.38 % and the soy isolate NaCl was 15.21%.

#### 4.2.5.3 Color of the soy sauce

Color development of brine is begun by pale yellowish, and then gradually the brown color is increased at the end of fermentation to the dark brown.

#### 4.2.5.4 Flavors of the soy sauce

The aroma of brine is begun by spices odor, then changed to pickling odor, after forty days of fermentation many odors is detected as alcohols, acetone, esters (the mango odor is the most obvious one), the strongest odor is detected in the brine which is produced by rice isolate.

#### 4.2.5.5 Aroma of the soy sauce

Each sample of sauce from a reference, rice isolate and soy isolate was divided into two divisions. One is embedded with dill and gives dill odor and the other with thyme and gives thyme odor.

#### 4.2.5.6 Ethanol

Alcohol of soy isolate sauce is the highest one

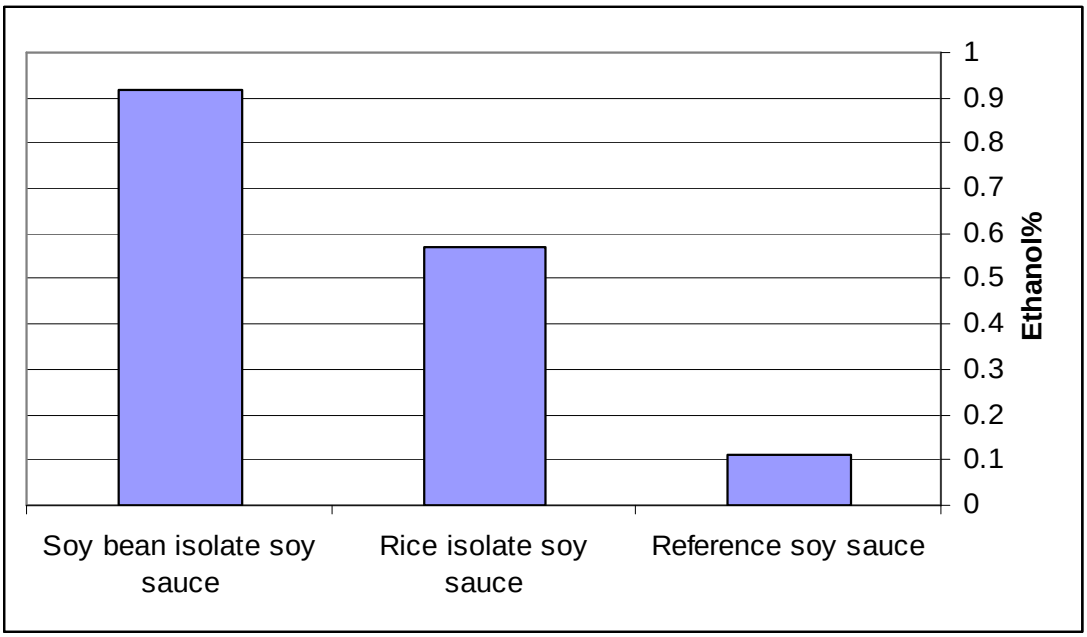



**Figure (4.12)** Ethanol percentages in three soy sauces**.**

**4.2.5.7 Total solids and moisture in soy sauces.**

The total solids (Figure 4.13) in rice isolate soy sauce were higher than reference and soy isolate soy sauces, but the moisture in soy isolate was the highest.

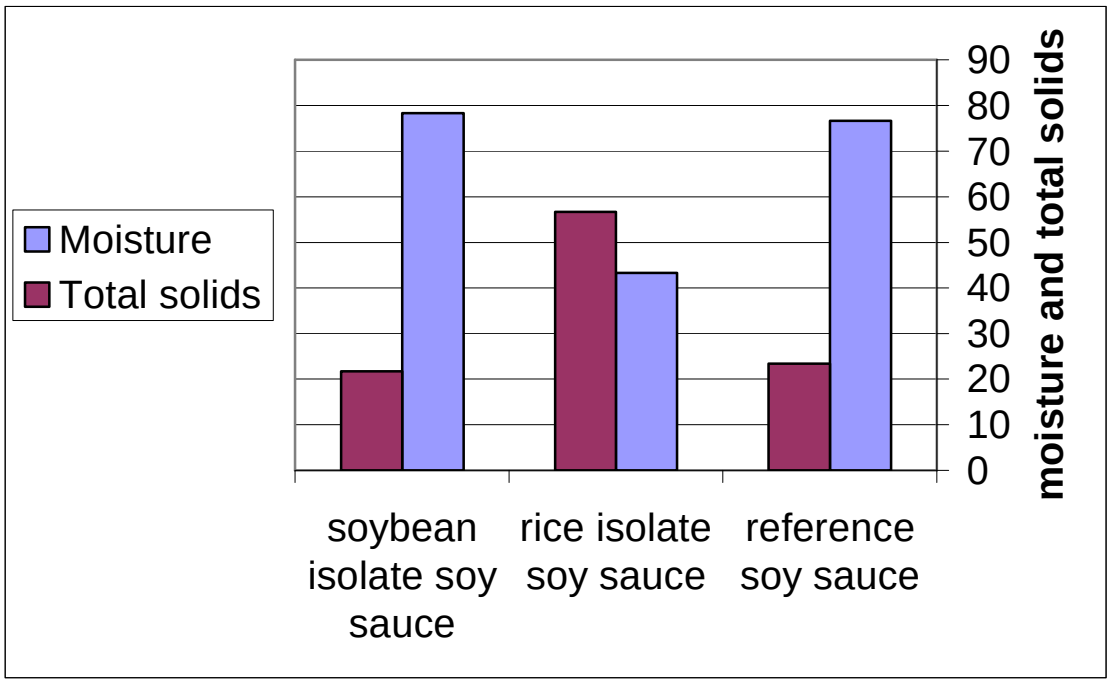



**Figure (4.13)** Total solids and moisture in soy sauces

**4.2.5.8 The nitrogen and crude protein**

The nitrogen and protein of rice isolate soy sauce is the highest quantity.

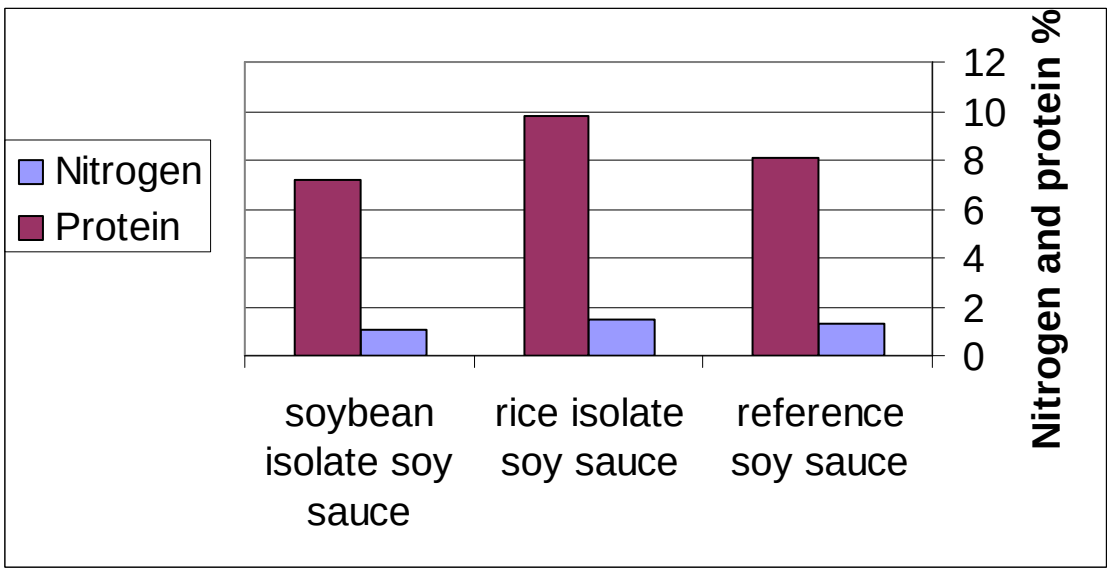



**Figure (4.14)** Nitrogen and protein in three soy sauces

#### 4.2.5.9 Calcium

The rice isolate soy sauce is the highest value of calcium.

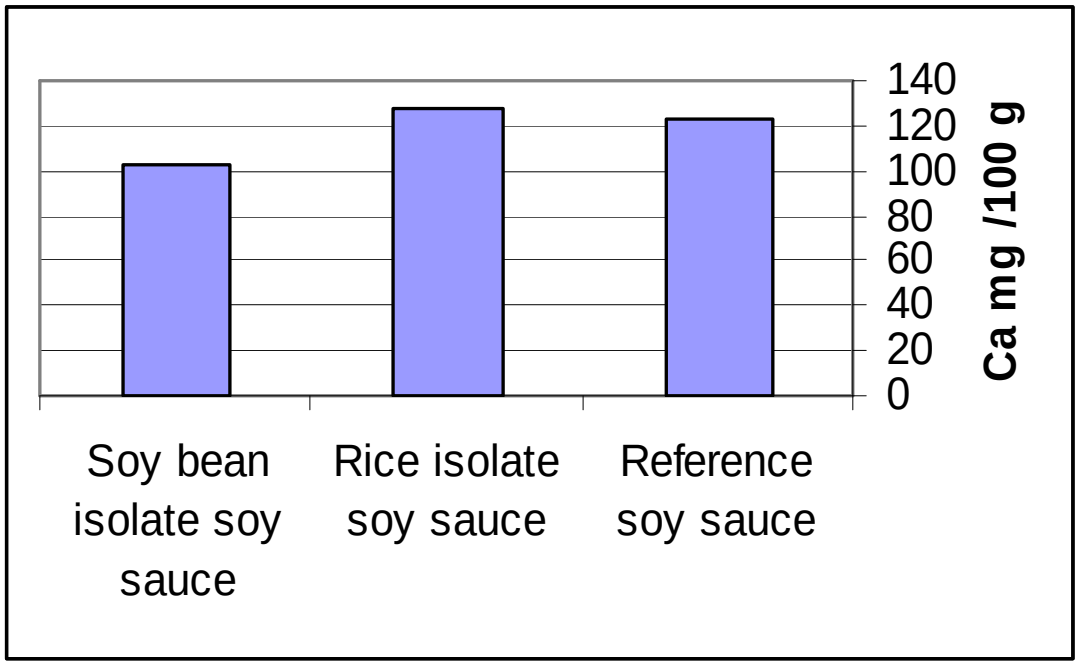



**Figure (4.15)** The calcium in three soy sauces.

# Chapter 5

# Discussion

## 5.1 Isolation and Identification of *A. oryzae*

As the main objective of this study was the isolation of *A .oryzae* from rice, soybean and wheat, and investigation of their characteristics for the production of soy sauce aroma. ISTA especially agar plate method was used for the isolation method. Observations recorded on the seventh day showed that all concentration of HOCl affected the colony growth (Table 4.1). All Petri dishes appear with yellow green color.

Antibacterial media containing compounds were used to inhibit or reduce spreading growth of molds, such as dichloran rose bengal chloramphenicol (DRBC) agar or dichloran 18% glycerol (DG18) agar were recommended for enumerating fungi in foods. AFPA designed specifically for detection of potentially aflatoxigenic species **[29]**. The isolates and the reference showed bright orange yellow reverse color for three weeks, which indicated that the strains were not toxic, this is agree with Sooriyamoorthy S.S **[1],** who reported that the isolates produced bright orange –yellow reverse color with 48 h of incubation at 30 °C.

Identification of *Aspergillus* species requires growth on media developed for this purpose, including C zapek agar, a defined medium based on mineral salts, or a derivative such as CYA, and malt extract agar. Growth on CYA 20% sucrose agar (CY20S) can be a useful aid in identifying species of *Aspergillus*. The color of the conidia can be a very useful starting point in identification, as well as phialides, or metulae plus phialides, and shape and size of vesicle, etc., *A. oryzae* (Ahlburg) Cohn is closely related to *A. flavus*, and produces colonies of similar or slightly smaller size on the standard media. However, colonies of *A. flavus* remain green as they age, whereas those of *A. oryzae* are more floccose and turn olive brown as they age **[29]**. The isolates were compared with the reference strain with respect to macroscopic colonial and morphological characteristics colonies of *A .oryzae on* CYA spreaded rapidly at 25 °C, and were yellow pale

(Figure 4.1- 4.4). The colony diameter ranges from 55-75 mm, and the conidia were yellow green color. This was agreed with Munoz G. M. D **[133]** who reported that colonies on CYA, 50-60 mm of diameter. Mycelium and color in mass was yellow greenish. Conidial heads columns had a radiating arrangement. The reverse color was cream-yellowish. All macroscopic characteristics of our isolates of spherical vesicles, phialides, and small smooth-walled conidia had characteristics of a reference *A. oryzae* as shown in (Table 4.2) and microscopic colonial as shown in (Figure 4.5).

## 5.2 The pH

Our results showed that the pH values of soy sauce of a reference, rice isolate and soybeans isolate were 4.16, 4.65 and 4.25 respectively. The raise in pH of rice isolate may be related to the mold growth on the edges and cover of the container. The acid protease might increase proteolytic hydrolysis in brine fermentation, resulting in good soy sauce. The high temperature of fermentation might decrease pH values and inactivate alkaline protease resulting in poor digestibility, causing poor flavor. At the low pH, the sugar content was low, alcohol content was moderate high. The low pH also had a good preservative effect on the soy products, as several undesirable organisms could not survive in the pH range of 3.0-3.9 **[96]**.

## 5.3 The ethanol

Ethanol was synthesized by *Z. rouxii* from the sugars, which were ample and in wide variety present during the brine fermentation. However, the kinds of sugars that were fermented to ethanol by *Z. rouxii* were limited because of high salt content of the brine solution (Table 2.1). In the brine solution, *Z. rouxii* could ferment glucose which it can not ferment maltose which is fermented in salt free medium by the same microorganism. In contrast, *C. versatilis* could ferment maltose in the brine solution. Without sugar fermentation, the yeasts would not be able to survive during the brine fermentation because of low availability of oxygen, which was caused by the low aeration rate and poor solubility of oxygen in the brine solution. Some

traces of oxygen were, however, required for this fermentation. In addition, this fermentation was only possible when the pH was lower than 5.0 **[11]**.

According to Syed Rasheeduddine **[134],** a fermented soy sauce contains usually about 1-2 % (v/v) ethanol. But, naturally fermented soy sauces in united Kingdom have 2% alcohol, and there are two kinds of soy sauce in USA and Canada, one is Kikkoman brewed soy sauce having 1.7% or more alcohol, and other is Lachoy soy sauce made by acidified hydrolysed soy protein. Our results showed that the ethanol content was 0.11, 0.75 and 0.92, in a reference; rice isolate and soy isolate sauces respectively. The quantity of ethanol was les than the Japanese and South East Asian sauces **[96]**. The low value of alcohol might be related to the long pasteurization period (30 min.) instead of (10 min.) for pasteurization. On the other hand, the millet has less carbohydrate than wheat, which used in other sauces.

## 5.4 The flavor

Our results indicated that the odor was started by spices then gradually changed to aromatic odor. The change in the odor might be explained by the change in pH of the brine, which initially was 6.0, and after 40 days the pH of the brine by using the reference strain reached 4.22, by using rice isolate was 4.84 and by using soybeans isolate was 4.37.
This drop in pH encouraged the different yeasts to grow and gave this aroma, the more flavor and aroma of rice isolate brine might be explained by an increase of proteolytic enzymes activities that released glutamic acid. That acid enhanced the flavor and aroma in the brine.

According to Bui **[96],** the flavor of soy sauce comprised of about 37 hydrocarbons, 30 alcohols, 41esters, 15 aldehydes, 5 acetals, 17 ketones, 24 acids, 16 phenols, 16 furans, 4 lactones, 4 furanones, 5 pyrones, 25 pyrazines, 7 pyridines, 11 sulfur compounds, 3 thiazoles, 3 terpenes and 8 miscellaneous compounds. This results also agreed with Lee, et al, **[135]** who reported, alcohols and esters were dominant in the volatiles of fermented soy sauce, whereas heterocyclic compounds, including pyrazines , furans, and

acids were relatively abundant in the acid-hydrolyzed soy sauce. The odor-active compounds were also evaluated. The major odor-active compounds of fermented soy sauce were acetic acid, furfuryl alcohol, 2-methoxyphenol, benzeneethanol, benzoic acid, butyric acid, 2-ethyl-4-hydroxy-5-methyl-3(2H) furanone, and 2-methylbutanal, whereas acetic acid, 2-methoxyphenol, formic acid, benzoic acid, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, butyric acid, 2,6-dimethoxyphenol, 3-hydroxy-2-methyl-4H-pyran-4-one, 2-acetyl-5-methylfuran, 2,5-dimethyl-3-ethylpyrazine, and 2-methylbutanal constituted the major odor-active compounds of acid-hydrolyzed soy sauce .

## 5.5 **The color**

The three samples were appeared by brown color, but the soy sauce color of rice isolate was darker than the others. This dark color was related to the more growth of mycelium at brine stage. Bui [96] reported that the color of miso depended on the mycelia and salt concentration related to its flavor, the darker the color, the stronger the flavor**.**

## **5.6 The salt**

The concentration of NaCl of the study product was sufficient to stop bacterial growth. These results were also confirmed by the results of Bui **[96]**. He found that sodium chloride helps to destroy staphylococci in soy sauce. However, Soy sauce with salt solution containing 10-17 % sodium chloride, pH 4.7 destroyed 90 % of the staphylococci cells in 10% NaCl solution at 980-1440 min, and of 17 % at 460-530 min.

## 5.7 KOJI

Normally raw materials are pretreated before fermentation. In the soy sauce fermentation, whole soybeans were steamed to make the soy protein more easily hydrolyzed by the proteases of *A. oryzae.* Too much moisture was introduced and crushed millet must be added to decrease the moisture of content to a level that prevents bacterial growth. The result of koji was in agreement with Bull, et al **[113**]. The growth and sporulation of *A. oryzae* was joined to the production of volatiles. The volatiles present at the mycelial phase were different from the volatiles of sporulated koji. Aeration was necessary for fungi to grow. If $O_2$ was absent, the fungi will die. This is agreed with Shankar, et al **[36]**, they found decrease in enzyme production might be due to increased bed height of the substrate in the tray that affected the aeration.

## 5.8 Brine fermentation

During the fermentation of soy sauce, proteins in the raw materials were hydrolyzed into small molecular weight peptides, amino acids and ammonia by the proteases produced by *A. oryzae*. Nitrogen constituents are the important parameter used for grading the quality of soy sauce product. According to the Chinese National Standard, Grade A soy sauce should contain total nitrogen and amino nitrogen of more than 1.4 and 0.56%, respectively **[136**].

The brine solution was kept around 30°C and semi-anaerobic conditions were applied by stirring. In the brine solution the *Aspergillus* enzymes continued to hydrolyze the soybeans and wheat and as a result, a surplus of different kinds of sugars and amino acids arised . These sugars and amino acids were consumed by salt tolerant lactic-acid bacteria (*T. halophila*) and yeasts (*Z. rouxi*i and *C. versatilis*) during the so-called brine fermentation **[11]**.

In general, several kinds of microorganisms are involved in the fermentation of various foods. When different kinds of microorganisms exist in foods, a phenomenon such as competition or antagonism was observed among these

microorganisms. Most investigation on microbial interactions focuses on the lactic acid bacteria **[137]**.

Chemical analysis of soy-fermented products indicated several peptides can be released if they manufactured under the proper conditions. These compounds are produced partly by the hydrolytic action of microbial enzymes. Fermentation detoxifies endogenous harmful chemicals while creating a variety of flavors and aromas through hydrolysis of larger molecules. This process also extends the life of the product since the final state is acidic (or alkaline) and probably contains salt. Preservation of Soy Sauce by refrigeration, canning, or drying is thus not required in developing countries **[138]**.

Breakdown of carbohydrates, lipids and proteins to sugars, fatty acids and peptides, respectively, enhances the nutrient content and reduces the water activity in soy-fermented foods. Other components such as flatulence causing oligosaccharides (stachyose, raffinose) are eliminated whilst antinutritional factors are reduced. During the fermentation, vitamins and anticarcinogenic agents were microbially produced. Bioactive peptides, antioxidants and hypocholesterolemic compounds were also generated during the process. **[138]**.

The microorganisms broke down carbohydrates, proteins and lipids in the brine by releasing enzymes into the medium.

The main proteolytic enzymes in koji (*A. sojae*) are:

**1**. Proteinase (Alkaline, Semi-alkaline, Neutral I, Neutral II, Acid I, Acid II, Acid III)

**2**. Acid carboxy peptidase (I, II, III, IV)

**3**. Leucine amino peptidase (I, II, III, IV, V, VI, VII) (Figure 5.1) **[4]**.

Peptides and amino acids can be further converted into smaller volatile molecules, which improve the flavor of brine.

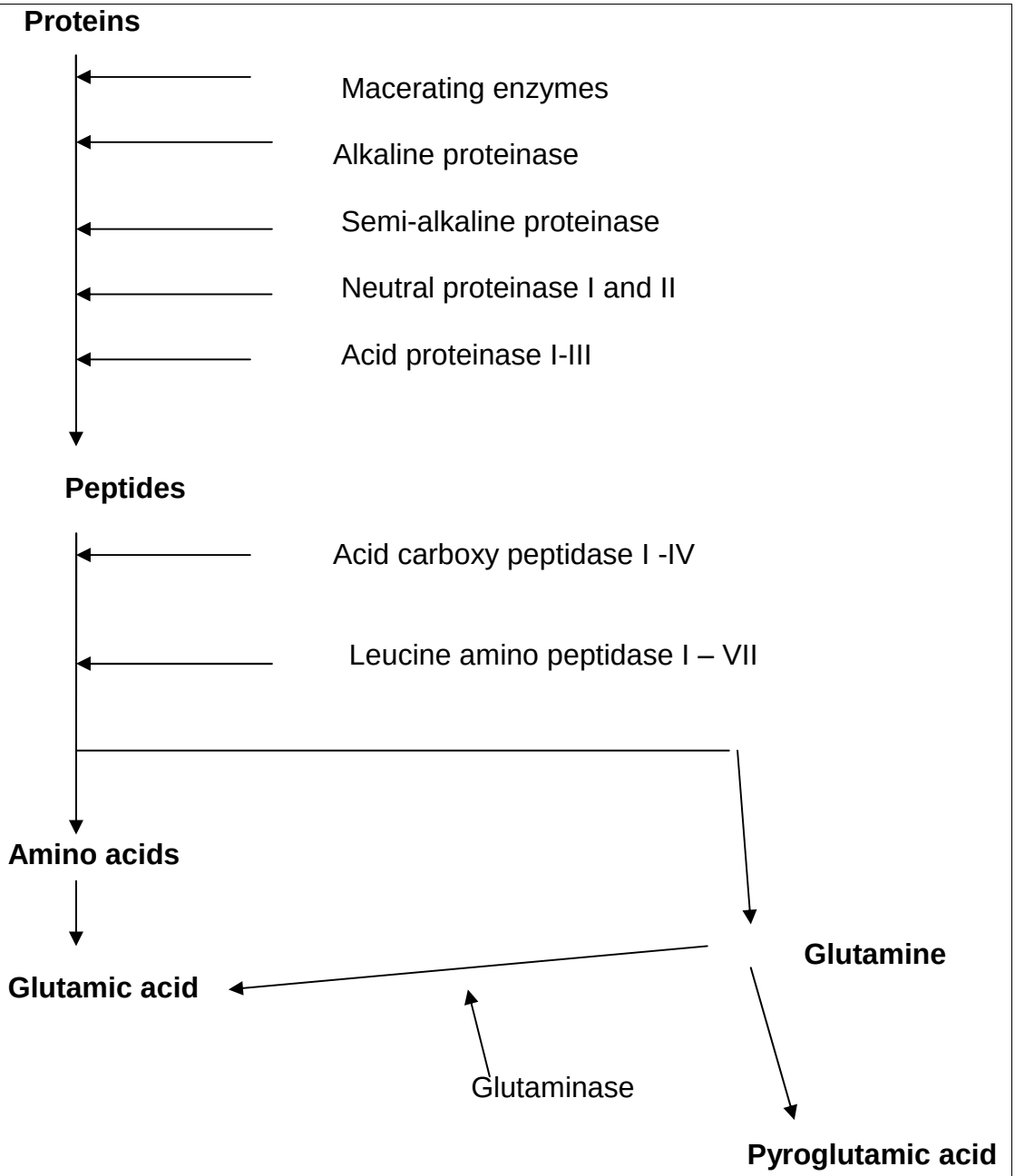



**Figure (5.1)** The role of each enzyme in koji during protein hydrolysis

# Chapter 6

## 6.1 Conclusions

The study isolated *A. oryzae* from seeds and used it in soy sauce product with new aroma. From the result of this work, the following conclusions were drawn:

1. The quantity of water in koji preparation played critical reason on *A. oryzae* growth.

2. Tane koji, (starter) prepared by growing *A. oryzae* on cooked seeds, provides different enzymes that were used in the production of soy sauce.

3. Specific bacteria and yeasts were controlling the brine pH. There enzymes produced a number of amino acids and peptides.

4. The amount of salt must be adjusted to certain concentration (17% -26%).

5. The pasteurization process was very necessary to stop further microorganism growth.

6. New aromas were obtained by specific spices as dill and thyme.

## 6.2 Recommendations

In The Gaza strip, the soy sauce has not been produced before. This preliminary study can be considered the first one in Palestine. The produced soy sauce was very salty, so it might be suitable for people on a low sodium diet. It also contained very few amount of alcohols. Therefore we should look for production of low sodium and nil alcohol soy sauce at future. The study also recommends

1. Studying specialized types of preservative-free varieties soy sauces.

2. Isolating bacterial strains of lactic acid and selecting the best for fermentation.

3. Isolating *A .oryzae* strains and selecting the best one in enzymes production.

4. Optimization factors affecting *A. oryzae* growth and soy sauce fermentation.

5. Establishing a centre for keeping bacterial and fungal strains used in the soy sauce production.

6. Establishing a large scale production of soy sauce.

## References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