CHAPTER II

REVIEW OF LITERATURES

Fermented soybeans foods have been an intricate part of the orient diet for millennia. Soybeans have been an important source of protein in Asia for centuries. To improve their flavor, and perhaps also their nutritional value, soybeans are often converted into various food products by fermentation with molds or bacteria.

Tempeh

Tempeh can be made from substrates other than soybeans. Other varieties of tempeh include five major types : legume tempeh, cereal grain tempeh, soyblends and cereal grain tempeh, presscake tempeh, and nonleguminous seed tempeh. Soybeans are the most common substrates used for the preparation of tempeh, followed by wheat and soy wheat blend (Hachmeister and Fung, 1993). One of the most popular fermented soybean products is tempeh which is widely used for food in the orient, especially in Indonesia (Zamora and Veum, 1979). However, in the last two decades it has attracted attention of the western world, particularly north America, because of its unique flavor, sliceable meat-like texture, and nutritional attributes.

The traditional fermentation is very simple. It is a household art in Indonesia and therefore varies from place to place. It serves as a major source of protein and vitamins for the people (Lim, 1991). The essential steps of tempeh fermentation are the same. Some recent studies (Mak,1986) in which five species of *Rhizopus* (*R. arrhizus, R. microsporus, R. oligosporus, R. oryzae, and R. stolonifer*) were used to prepare tempeh, showed that only two of them *R. oligosporus* and *R. oryzae* could give good tempeh. The other three could also

result in tempeh, but not of comparable good quality. This confirms the opinion that the best molds for tempeh preparation is *Rhizopus oligosporus* (Steinkraus, 1983). *R. oligosporus* is the principle species used in Indonesia for tempeh fermentation. One strain, *R. oligosporus* NRRL 2710, is the recommended strain for producing tempeh commercially (Wang and Hesseltine, 1981). The soybeans are washed and soaked in water overnight, during which time the beans double their dry weight and undergo bacterial acid fermentation reducing the pH to 5.0 or lower. Lowering the initial pH allows the mold to grow (Steinkraus, 1983) while bacterial growth is suppressed (Nout et al., 1985; Tanaka et al., 1985; Nout and Rombouts, 1990). An alternate process is to boil the beans in water and then allows the beans to soak overnight. The general purpose of boiling is to facilitate hull removal. The hulls are then removed manually and the loosened hulls are floated away with water. It is essential that all hulls be loosened from the cotyledons as the mold cannot grow on intact beans. The dehullled beans are then boiled with excess water for varying lengths of time (usually about 30 min), cooled, surface dried, and inoculated with tempeh from previous fermentation or with mold grown on dried leaves. Hesseltine et al. (1963) used a spore suspension of *Rhizopus oligosporus* grown on potato dextrose agar slants for 5 to 7 days. Martinelli and Hesseltine (1964) suggested that excess inoculum promoted rapid and uniform fermentation. Wang et al. (1975) concluded that if too little inoculum was used, bacteria would be allowed to grow. For optimal fermentation, Wang et al. (1975) recommended that $1x10^6$ spores per 100 g of cooked soybeans be used. Traditionally, the inoculated cotyledons are then wrapped in small packets using wilted banana leaves and allowed to ferment at room temperature for a day. Shallow aluminium foil or metal trays with perforate or woven mesh bottoms and covers or perforated plastic film covers also have been successfully used in commercial production of soybean tempeh. During fermentation, white mycelia of the mold invade and cover the substrate mass to bind the soybean together (Beuchat,1984). The mold fermentation results in a matrix of dense, cottony, mycelia in which cooked soybeans are embedded, forming a compact greyish-white cake. Tempeh possesses a pleasant aroma which can be described as nutty, cheesy, and mushroomy (Mital and Garg, 1990).

Nutritional quality of tempeh

Tempeh is easily digested and is even tolerated by patients suffering from dysentery and nutritional edema (Ko and Hesseltine, 1979). Recovery from diarrheal disease was reported to be faster (Mahmud, Herman, and Karyadi, 1985) with tempeh-based formula and resulted in better weight gain (Karyadi, 1985). Winarno and Reddy (1986) reviewed the nutritional implications of tempeh fermentation and found that fresh tempeh contains about 60% moisture. Similar findings were reported by Shurtleff and Aoyagi (1979), with fresh tempeh containing 60.4% moisture. On a dry matter basis, tempeh contains approximately 55 % crude protein, 14% fat, 28% carbohydrates, 3% fiber, and 3% ash (Winarno and Reddy, 1986).

Several researchers stated that the improvement of the tempeh protein efficiency ratio (PER) during fermentation can be attributed to better availability of amino acids from the tempeh and greater digestibility of the tempeh protein (Iljas et al., 1970; Smith et al., 1964). Hackler et al. (1964) found that the nutritional value of tempeh decreased as the time of fermentation increased. However, these researchers discovered that the PER value remained constant throughout the course of fermentation times of 0, 12, 24, 36, 48, 60, and 70 hours. In 1979, Zamora and Veum reported that fermentation of cooked soybeans inoculated with *R. oligosporus* improved the digestibility and net protein utilization (NPU) in rats fed diets containing tempeh.

Chemical and biochemical changes in tempeh

Rhizopus spp. produce a variety of enzymes, including carbohydrases, lipases, proteases, and other enzymes. Steinkraus (1983) stated that levels of pectinase increase with mycelial growth of *R. oligosporus*. In contrast, Whitaker (1978) mentioned that *R. oligosporus* produces little pectinase activity. Hesseltine (1965) reported that this organism produced very little pectinase and amylase. Polygalacturonase is a major enzyme of *R. oligosporus, R. stolonifer* (Manachini, Fortina,and Parini, 1987), and *Rhizopus arrhizus* (Sachde, Al-Bakir, and Sarhan,

1987). Other carbohydrases of *R. oligosporus* in tempeh include endocellulase, xylanase, arabinase, α-D-galactosidase, and others. Although carbohydrase activity of *R. oligosporus* is minimal during fermentation, the mold does produce substantial amounts of lipase (Beuchat and Worthington, 1974) and protease (Wang and Hesseltine, 1965). Lipase activity in tempeh fermented with *R. oligosporus* was maximized at 24 hours of fermentation and was fully inactivated by heating at 60°C for 10 min according to Souser and Miller (1977). Nahas (1988) studied the production of extracellular lipase by *R. oligosporus* and found that maximum lipase activity occurred at 25°C after 3 days at pH 6.5. In addition, several strains of *Rhizopus* were able to degrade aflatoxin B_1 to a nonfluorescent compound aided by monooxygenase systems (Bol and Smith, 1989; Nout, 1989).

Changes in protein and amino acids

Although Murata, Ikehata, and Miyamoto (1967) found no large differences in protein content between soybean tempeh and unfermented soybeans, these researchers observed an increase from 1 to 85 times in free amino acids during fermentation. Kao and Robinson (1978) reported that lysine and methionine in horsebeans and tryptophan in soybeans increased considerably during tempeh fermentation. Stillings and Hackler (1965) observed an increase in free amino acid content and in ammonia as fermentation time increased. Most tempeh is not fermented over 30 hours since the organoleptic properties are adversely affected when the tempeh passes from the transitional into the deterioration phase (Sudarmadji and Markarkis, 1978). Therefore, the essential amino acid index of tempeh fermented for 24 hours is slightly higher than that of raw soybeans or the unfermented control (Stillings and Hackler, 1965).

The effect of tempeh fermentation on total nitrogen content is negligible, but increases of free amino acids take place (Nout and Rombouts, 1990). Although the amino acid pattern as quantified by the essential amino acid index is hardly effect during a 24-hour fermentation period (Stillings and Hackler, 1965), longer fermentation times result in losses of certain amino acids, such as

lysine, arginine, and threonine. Winarno and Reddy (1986) reported that tempeh fermented over 60 hours reduced in lysine content of 25%. Stillings and Hackler (1965) found that a 72-hour fermentation period reduced the lysine and arginine content in raw soybeans by 8.9 and 13.5%, respectively.

One of the important functions of the mold in such food fermentation is the synthesis of enzyme which decompose complex compounds such as starch and protein into smaller molecules. Such enzymatic activity may also decrease or eliminate antinutritional components such as phytic acid and, consequently, improve the food value of fermented product (Mital and Garg, 1990). As stated earlier, the effect of tempeh fermentation on total nitrogen content is negligible; however, the processing steps, including dehulling, soaking, and cooking, play a larger role in the loss of nitrogen and the loss of solids.

Changes in carbohydrates

Processing of soybeans into tempeh brought about favorable changes, including reduction in levels of starch and flatulence-causing oligosaccharides such as raffinose and stachyose (van der Riet et al., 1987). Slight increase in the concentrations of the unspecified monosaccharides and raffinose during fermentation are due to the attack by α-galactosidase on stachyose and by other chemical interactions between the other sugars. Hence, monosaccharides and raffinose, which are end products of enzymatic degradation, increase with fermentation, whereas sucrose and starch concentrations are reduced by fermentation (van der Riet et al., 1987). van der Riet et al. (1987) found that processing of soybeans resulted in a much greater loss of soluble carbohydrate material than did fermentation. Cooking as well as the soaking step are necessary to reduce certain carbohydrates.

Calloway, Hickey, and Murphy (1971) found that tempeh was virtually nonflatulent when fed to humans. One reason for this important phenomenon is the fact that stachyose, raffinose, and other flatulence causing carbohydrates are reduced. van der Riet et al. (1987) found that stachyose and raffinose contents

decreased with fermentation. Sucrose concentrations also were lower in fermented tempeh than in soybean. Reduction of the oligosaccharide content during the fermentation step is due primarily to the hydrolysis of the sugars by the mold *R. oligosporus* (Steinkraus, 1983).

Changes in lipids

R. oligosporus has strong lipase activity (Wang and Hesseltine, 1966) and therefore breaks soybean lipids down into free fatty acids during fermentation (Sudarmadji and Markarkis, 1977). Of the free fatty acids that were liberated, only linolenic acid was used by *R. oligosporus*, based on the findings of Hesseltine (1965). Glycerides in raw soybeans are broken down into free fatty acids during the first 30 hours of fermentation (Sudarmadji and Markarkis, 1978). Wagenknecht et al. (1961) observed changes in lipids during tempeh fermentation and identified the free fatty acids that were liberated as palmitic, stearic, oleic, linoleic, and linolenic acids, with linoleic acid predominating. Murata et al. (1967) found an increase in oleic acid content, but a decrease in linoleic acid content after fermentation of soybeans. Total free fatty acid composition rose from 39 mg/100 g of unfermented soybeans to 10,678 mg/100 g of tempeh during a 90-hour fermentation (Sudarmadji and Markarkis, 1978). Stearic, oleic, linolenic, linoleic, and palmitic acids increased 559, 557, 250, 232, and 138 times, respectively, in the fermented tempeh.

Changes in vitamins

van der Riet et al. (1987) found that fermentation soybeans reduced thiamin concentrations to undetectable levels; however, riboflavin and nicotinic acid concentration increased significantly over the 72-hour fermentation period as a result of synthesis by *R. oligosporus* NRRL 2549. Despite the fact that the thiamin concentration slightly decreased with fermentation, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid concentrations increased during the conversion of soybeans into tempeh (Murata et al., 1967). These B vitamins increased further when tempeh fermentation was carried on longer than 48 hour.

Steinkraus (1983) stated that vitamins B_{12} was produced by *Klebsiella pneumoniae*, which was a desirable and possibly essential micro-organism in the natural fermentation process of tempeh. Liem, Steinkraus, and Cronk (1977) suggested that soybean tempeh contained micro-organisms capable of producing vitamin B_{12}. Nicholas (1952) found that some vitamin B_{12} was produced by *Aspergillus niger* ; however, no reports had shown *Rhizopus* species to be vitamin B_{12}-producing organisms. In a study conducted by Liem et al. (1977), pure tempeh molds obtained from different sources did not produce vitamin B_{12}. However, a bacterium that accompanies the mold during fermentation was found to produce vitamin B_{12} in commercial tempeh purchased in Canada.

Microbiological safety and quality

In fact, *R. oligosporus* inhibited the growth, sporulation, and aflatoxin production of *Aspergillus flavus* (Ko, 1974). In 1969, Wang, Ruttle, and Hesseltine discovered an antibacterial compound from a soybean product fermented by *R. oligosporus*, which produced a compound that inhibited the growth of lactic acid bacteria. The antibiotic produced by *R. oligosporus* behaved as some antibiotics in that bacterial growth was stimulated when in low concentrations (Wang et al., 1969).

R. oligosporus also has other desirable properties that ensure safety and quality of tempeh. Because *Rhizopus* spp. grow fast and quickly deplete the substrate of fermentable carbon-compounds, they outcompete slower growing fungi such as *Aspergillus* spp. (Nout, 1989). *R. oligosporus* also was reported to exhibit antimicrobial activity against some *Bacillus, Clostridium*, and *Staphylococcus* spp. (Ko and Hesseltine, 1979; Winarno and Reddy, 1986), and substances inhibiting *A. flavus* growth (Nout, 1989). According to Nout and Rombouts (1990), oxygen levels in tempeh were much lower than in the surrounding air because of fungal metabolism. Although this did not affect the growth of added *Staphylococcus aureus*, it reduced its enterotoxin A production by 92% (Nout, Notermans, and Rombouts, 1988).

Tooa-nao

Tooa-nao, a fermented soybean food, is traditionally consumed by the north and northeast Thai that closely resembles natto. Natto is a Japanese fermented soybean food prepared from whole soybeans using *Bacillus subtilis* (Djien and Hesseltine, 1979). A similar product, kinema, is known in Nepal, Sikkim, and Darjeeling districts of India. Kinema is a Nepali name which has very often been erroneously spelt as "kenima" (Batra and Millner, 1976; Ramakrishnan, 1979; Campbell-Platt, 1987). In kinema, *Bacillus subtilis* is the dominant micro-organism (Djien and Hesseltine, 1979; Sarkar et al., 1996).

Tooa-nao is served with cooked rice as a main dish or snack in the Northern. To make traditional tooa-nao, whole soybeans are washed and soaked overnight. Boil at low heat for 3 to 4 hours, then drain. Place the beans on a layer of banana leaves on a bamboo tray and also covered with banana leaves. Leave to ferment for 3 to 4 days. The cooked beans can also be wrapped in banana leaves and left in a sunny place for 2 to 3 days. The fermented soybeans are grind into a paste (Bulan Phithakpol, Warunee Varanyanond, and Suparat Reungmaneepaitoon, 1995). The fermentation process of tooa-nao may be improved further by fermenting sterilised beans with *Bacillus subtilis* which leads to more desirable fermentation within a much shorter period, compared to the traditional fermentation process (Sarkar and Tamang, 1995). After fermentation, cooked beans bind together and cover with viscous, sticky substance produced by the bacteria, ammonia odor, and musty flavor (Wang,1984; Reddy et al.,1986).

Nutritional quality of tooa-nao

Although tooa-nao is a popular traditional fermented soybean food in the northern Thailand, nutritive value information is lacking in this area. However, some researches have been conducted on the nutritive value of similar products such as natto and kinema. Hesseltine and Wang (1972) showed that there were no change in fat and fiber contents of soybean during a 24-hour period of natto

fermentation, but carbohydrate almost totally disappeares. A great increase in water-soluble and ammonium nitrogen was noted during fermentation as well as during storage. The amino acid composition remained the same. Boiling markly decreased the thiamin level of soybeans, but fermentation by *Bacillus natto* enhanced the thiamin content of natto approximately to the same level of soybeans. Riboflavin in natto greatly exceeded that in soybeans. Vitamin B_{12} in natto was found to be higher than in soybeans.

Kim et al. (1995) found that total sugar content of natto supplemented with red pepper oleoresin decreased during the 24-hour fermentation period. Amino-type nitrogen content increased gradually during the 24-hour fermentation period. Free amino acid content increased in natto. Akimoto, Matsumoto, and Imai (1993) suggested that protease activity increased continuously during 30-hour period of natto fermentation. Protein solubilization and hydrolysis were higher in natto incubated at 35 or 40° C for 18 to 20 hours. The odor of natto fermented by pure culture of *Bacillus subtilis* originated from the hydrolysis of soybean protein to peptides and amino acids (Ohta, 1986). Taira and Suzuki (1983) conducted an experiment to determine lipid content and fatty acid composition of natto during processing. The results showed that the lipid content of raw soybean were slightly less than normally fermented product, or over-fermented product. A number of interesting changes occurred during kinema fermentation. During the first 16 hour of traditional kinema fermentation, the pH of the fermenting beans decreased. Cooked soybeans contain sucrose, raffinose and stachyose (Steinkraus, 1983), and *B. subtilis* was capable of producing acid from sucrose, raffinose and their hydrolytic products, glucose, and fructose. Therefore it seems that sugars, not proteins or fats, were initially used as substances for metabolism and growth of the organisms (Sarkar and Tamang, 1995). However, after this fall, the pH started rising significantly at every 8 hours interval. This was due to proteolytic activities of the micro-organisms and consequent production of ammonia (Sarkar et al., 1993). Hydrolysis of protein to produce amines and ammonia through peptides and amino acids is responsible for this final change in pH (Odunfa, 1985; Campbell-Platt, 1987). Lipases produced during kinema fermentation break down

glycerides into easily assimilated fatty acid (Mital and Garg, 1990). Sarkar et al. (1994) reported that a marked increase in the free fatty acid of kinema compared to raw soybeans was due to lipolytic activities of the micro-organisms during kinema production. Wang et al. (1975) and Sarkar et al. (1994) suggested that the increase of crude lipids was probably due to three possible reasons. First, the active assimilation of carbohydrates and limited consumption of lipid, resulting in an accumulation of crude lipid at the end of fermentation. Next, the synthesis and accumulation of extra lipid during fermentation. Finally, dissociation of lipoprotein complexs in soya bean during fermentation, resulting in the release of ether-extractable free fatty acids.

Protein quality

It is well known that proteins differ in their nutritive values due to the variability of amino acid composition, digestibility, and availability of the digested proteins. Therefore, it is possible to rate proteins on the basis of their constituent amino acids, digestibility of proteins, availability of the digested product to the organism, and concentrations of their essential amino acids.

One useful rating system is based on the biological values (BV) of proteins. Biological values are useful for comparing proteins from a variety of sources and for judging the nutritional adequacy of a combination of proteins such as might be found in a normal diet. Several other methods have been developed for evaluating protein quality. Two of the more widely used methods are the protein efficiency ratio, the gain in body weight per gram of protein ingested, and net protein utilization, the relationship between the ingested nitrogen (protein) and gain in carcass nitrogen.

Protein score, based on the amino acid composition of a protein mixture or individual protein, permits one to predict the "performance" of dietary mixture. This method is based on the concept that the limiting value of the protein source is the concentration that fails to meet all of the essential amino acid requirements (Sarwar, 1990).

In vivo assays are generally the methods of last resource for evaluating protein quality because they are both time- and material-consuming. Furthermore, the results are open to a wide range of interpretations, yielding valid information only in the hands of an expert. For these reasons *in vitro* chemical, microbiological, or enzymic methods are preferred and when used with discrimination, serve as an indispensable complement to *in vivo* assays.

The nutritional values of dietary proteins depend primary upon the concentration and distribution pattern of their constituent amino acids. Amino acid composition data thus generally indicate the protein nutritive value of various protein sources (Bodwell, Satterlee, and Hackler, 1980). Even though the amino acid profile is important in evaluating the nutritive value of a protein, the digestibility of that protein is the primary determinant of the availability of its amino acids. The digestibility of a food protein may be obtained by using rats bioassays but this is an expensive and time consuming procedure as mentioned before (Hsu et al.,1977).

Several *in vitro* methods for the measurement of protein digestibility have been developed. Akeson and Stahmann (1964) found that a pepsin-pancreatin enzyme system gave a reasonably accurate approximation of protein digestibility. Mega, Lorenz, and Onayemi (1973) pointed out that initial rates of hydrolysis by trypsin on some commonly used protein sources were good indicators of their digestibility. Rhinehart (1975) examined several enzyme systems which included trypsin, pepsin-trypsin, trypsin-chymotrypsin, and trypsin-chymotrypsin-peptidase combinations. The results were encouraging, with correlation coefficients of 0.79, 0.72, 0.80, and 0.74 for the trypsin, pepsin-trypsin, trypsin-chymotrypsin, and trypsin-chymotrypsin-peptidase systems, respectively. Satterlee and coworkers (Hsu et al., 1977; Satterlee, Kendrick, and Miller, 1977) used *in vitro* enzymic digestion procedures to predict apparent protein digestibility as determined in rats. For most protein sources studied, there was general agreement (r=0.90) between predicted and experimentally determined apparent digestibility. This finding is consistent with the investigation of Bodwell et al. (1980) in plant protein. They found that the correlation between values from the

three-enzyme and four-enzyme methods and apparent rat digestibility were 0.89 and 0.95, respectively.

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