Original Research Article

Evaluation of physicochemical and nutritional contents in Soybean fermented food tempeh by *Rhizopus oligosporus*

ABSTRACT:

The goal of the present study was to evaluate physicochemical, sensory characteristics and nutritional contents in soybean fermented food tempeh and verify the viability *of Rhizopus oligosporus* in these products. A fermented product "Tempeh", is made from soaked and cooked soybeans or cereal grains of mixed culture fermentation by a diverse group of microflora like bacteria, yeasts and molds. Filamentous fungi are used in the preparation of fermented foods for the improvement of taste and the nutritional value. *Rhizopus oligosporus* is considered as most preferred species in tempeh fermentation among the tempeh culture so, it is most widely used and one of the known molds in vegetal substrates fermentation. In the fermentation process for the production of desirable quality, flavor, and aroma mold synthesize enzymes that hydrolyse the components which eliminates antinutritional constituents improving the nutritional quality of the fermented product. So, Tempeh is a striking product from sensual, health and cost-effective points of view. Sensory analysis and nutritional viability evaluated the effects of P^H, temperature, relative humidity, aeration, moisture and ash contents, inoculum size and shelf life and its mineral values including protein, carbohydrate and lipid content.

Keywords: Molds, Tempeh, fermented food product, Nutritional contents, physicochemical analysis

Introduction:

In the last decades, the food industry has incorporated the consumption of residues resulting from food production. Cheese industry one of the major sector of food industry generates bulk of milk serum as the remains from cheese production. A number of studies have been carried out aiming at finding a better use for the milk whey reducing pollution caused by its disposal, causes environmental problem due to its high content of organic materials and high biological demand of oxygen necessary for lactose deterioration **(Zavareze *et al.*, 2010).** Whey having high nutritional value contains significant amounts of proteins and lactose, (Antunes *et al*., 2007). The beverages production by the use of the milk serum has been studied by several researchers such as Antunes *et al.*, (2007) and Bürger *et al.*, (2011), who investigated methods to reduce the costs of its production aiming at attaining a product with enriched characteristics and chemical composition and expanded use. Another industry trend is the production of different fermented soy food products including Tempeh usually involve the activity of molds, aspergillus, rhizopus, mucor and actinomucour several species of yeasts and lactic acid bacteria. During fermentation process is usually the action of micro-organisms on a food's carbohydrates, many nutrients in food can be converted. These nutrients can be the food's proteins, fats, vitamins, minerals, and phytonutrients. In fermented soy foods, proteins are often more digestible minerals in soy foods can become more soluble and many phytonutrients, including isoflavones like genistein and daidzein. In some cases, when fermentation changes the digestibility of protein in soy foods it develops unique health supportive properties of their own. For example, an important storage protein is Conglycinin and its fellow, glycinin, account for as much as 80% of the total proteins in soybeans. It is often broken down into smaller peptides that serve as antioxidants, boost immune function, and prevent excessive inflammatory response during the process of fermentation. These whole food-based forms of soy stand in clear peculiarity to highly processed varieties of soy like soy protein essence. At the same time, researcher's provision for the health benefits of soy foods is even robust for fermented versus non-fermented soy foods. So here, one great option is tempeh.

Tempeh is fermented soy food that came from the island of Java in Indonesia at least hundreds of years ago and is fermented with the mold *Rhizopus oligosporus*. For the fermentation of tempeh a period of several days or longer, and usually carried out at temperatures of 85-90°F/29-32°C. Tempeh is usually obtained in a cake-like form bound with dense white mycelium. To understand more about tempeh, it cannot only about fermentation of soybeans into tempeh, but about fermentation of foods in general. Pakistan is a developing country where there are roughly 35 million people live below the poverty line and there are huge food crisis with around 20% food inflation rate. Tempeh is a cheap protein source and can be used as substitute of meat. Tempeh also contain various important nutrients that are tied to an impressive array of health benefits, including decreased risk of heart disease and strokes due to low fat content, osteoporosis, cancer and digestive disorders, losing excess weight in addition to easing some of the symptoms of menopause. In view of above facts the objectives of the present study are to:

- Isolate *Rhizopus oligosporus* from local soil samples.
- Tempeh fermentation using soybean as substrate.
- Optimize process parameters*:* acidity, temperature, relative humidity, inoculums size, depth of beans, time course, aflatoxin
- To evaluate nutritional content of tempeh
- To determine moisture content and ash content
- To study the shelf life of tempeh
- Organoleptic evaluation of tempeh.

Microflora of Tempeh

Overall culture of tempeh includes *Rhizopus oligosporus, R. oryzae,* R. *arrhizus, R. stolonifera*, *Mucor spp, lactic acid bacteria, Citrobacter freundii* or *Klebsiella pneumonia and* probiotic *Lactobacillus reuteri. Rhizopus oligosporus* is the dominant tempeh fungus (Sharma and Sarbhoy, 1984), although some other moulds, such as *R. oryzae* and *Mucor* spp, may also contribute to the flavour, texture or nutritive value (Wiesel *et al.*, 1997). Lactic acid bacteria may contribute to the microbial safety **(Nout *et al.*, 1987a**; Ashenafi and Busse, 1991b). Vitamin B_{12} production by bacteria, such as *Citrobacter freundii* or *Klebsiella pneumoniae* (Liem *et al.*, 1977; Okada *et al.*, 1985a; Suparmo, 2008; Keuth and Bisping, 1993; Wiesel *et al.*, 1997), has received special attention. However, these two species are both potentially pathogenic (Badger *et al.*, 1999; Struve and Krogfelt, 2004). Recently, also the probiotic *Lactobacillus reuteri* was reported to produce vitamin B_{12} Yeasts are frequently detected in tempeh, but their role is still unknown (Samson *et al.*, 1987).

Rhizopus oligosporus

After long time of tempeh manufacture and consumption, however, it was the Dutch scientist Prinsen Geerligs, who identified the tempeh most active mould for the first time in 1895. *R. oligosporus* is considered as most preferred species in tempeh fermentation among the tempeh culture (Ahmad and Sarbhoy, 1984), due to properties such as rapid growth at high temperature (30-42C°), an inability to ferment sucrose, high photolytic and lipolytic activities and production of strong antioxidants (Steinkraus *et al.*, 1983). During tempeh fermentation, the soybean is degraded by *R. oligosporus* enzymes, such as carbohydratases (e.g. polygalacturonase, endocellulase, xylanase, arabinanase and small quantities of α-D-galactosidase, β-B-galactosidase, β-D-xylosidase, α-L-arabinofuranosidase and α-D-glucosidase), lipases, proteases and phytases (Nout and Rombouts, 1990). In contrast, Rehms and Barz (1995) reported that *R. oligosporus* did not produce α-galactosidase and consequently cannot degrade flatulence-causing compounds such as stachyose and raffinose. *R. oligosporus* can inhibit the growth and aflatoxin B_1 accumulation of *Aspergillus flavus* and *A. parasiticus* (Nout, 1989). *R. oligosporus* has been reported to produce 4 to 5 anti-bacterial compounds during soybean tempeh fermentation (Anon, 1980; Wang *et al.*, 1969; Nowak & Steinkraus, 1988). The fungus also produces phenolic compounds that inhibit the growth of pathogenic bacteria such as *Helicobacter pylori* (Berghofer *et al.*, 1998; McCue *et al.*, 2003; Correia *et al.*, 2004a; Vattem *et al.*, 2004). An antibacterial protein has been purified from *R. oligosporus*, with activities against *Bacillus* spp. (especially against *Bacillus subtilis*), *Staphylococcus aureus* and *Streptococcus cremoris* (Kobayasi *et al.*, 1992).

Material and method

Organism:

Rhizopus oligosporus, NRRL-2710 was used for the preparation of tempeh. The culture was maintained on potato dextrose agar media. Potato dextrose agar media was prepared by adding 3.8 grams PDA in 100 ml distilled water followed by heating. Composition of potato dextrose agar media is given in table 1. Potato dextrose agar media was purchased from ACROS Chemical Corporation. Then culture was incubated at 30-37 C° in an oven (MEMMERT 854, West Germany) for 24 hours. After incubation, culture was stored in refrigerator at 4 C°.

Preparation of tempeh

Soya beans soaking overnight at room temperature (25°C), and then boiled for 10-15 minutes. Now the hot beans were spread in a thin layer and cooled to room temperature; subsequently, they were inoculated with a spore suspension of *R. oligosporus.* The inoculated soya beans were packed firmly in perforated plastic bags, and incubated at 30°C for 24 h, yielding fresh tempeh.

Acidity

To determine the optimum initial P^H for tempeh fermentation, the initial P^H before inoculation at PH ranging from (4, 4.5, 5, 5.5, 6). To adjust the P^H acetic acid was used.

Temperature

In context to determine the temperature suitable for fermentation process, inoculated cotyledons were incubated at 25 C°, 28 C° and 30 C°.

Effects of Aeration

To determine the aeration rate during fermentation process, the depth of soybean was varied in sterilized plastic bags from 1.0 cm to 4.5 cm.

Effects of Inoculums Size

Inoculums Size was varied from 10 µl to 500 µl per 15 grams soybeans in each sterilized plastic bags to know the best concentrations of inoculums for fermentation of soybean.

Relative Humidity

Relative humidity of inoculated cotyledons were maintained by refrigerator incubator (FOC 225I, Italy) at 20, 40 and 60.

Time Course of Fermentation

To determine the fermentation time course, tempeh was incubated for 18, 20, 22 and 24 hours,

Shelf Life

Tempeh was stored for 24 hours and was evaluated for flavor, taste, appearance, texture.

Moisture and Ash Content

Moisture content was determined by the method of (Udoidem 2016).Known weight (10.9g) of fermented sample was placed in Petri dish and dried it in oven at 100 ^{o}C for 24 hour. Final weight of sample was determined and moisture content of tempeh were expressed in percentage (%). Ash content was determined by the method outlined by AOAC (1984).Pre-weight sample was ashed by heating at 500^{o}C in a muffle furnace until residue was whitish grey. The ash content per unit weight was calculated and expressed as percentage (%).

Protein and Fat content

Protein analysis was carried out by kjeldahl method. Tempeh was fermented with sulfuric acid in the presence of mercury oxide or copper sulphate which reduced organic nitrogen the presence of catalysts which reduced organic nitrogen into ammonium sulphate, followed by distillation in the presence of sodium hydroxide, liberating ammonia gas. Then distillate was collected into boric acid solution, and the borate anions formed were titrated with standardized hydrochloric acid solution. The milliequivalents of acid required for titration are used to calculate the nitrogen content in the sample (chang, 1998). Fat analysis of tempeh was carried out by Soxhlet extraction method of prepared tempeh. Tempeh was placed inside thimble and loaded into the main chamber of the Soxhlet extractor. Then Soxhlet extractor placed onto a flask containing the ethanol. The solvent was heated to reflux and traveled to distillation arm in form of vapors, and flood into the chamber housing the thimble of solid. After the chamber filled with warm solvent and some of the desired compound dissolved in solvent in every cycle. After many cycles the desired compound concentrated in the distillation flask. After extraction the solvent is removed, typically by means of a Rotary evaporator, yielding the fat content.

Aflatoxin analysis

Aflatoxin was determining by method described by (Pons et al., 1966). Tempeh sample was prepared in labortary and was tested in Pakistan council for scientific and industrial research (PCSIR) for aflatoxin analysis.

Results

Physicochemical analysis

Effects of pH on tempeh fermentation

The low acidic pH of substrate during the production of tempeh is very important in controlling the growth of pathogen or food spoilage organisms. Acidity was varied from 4.0-6.0.The growth of mold at pH 4.0 was thick white mycelium. At the end of fermentation, the beans were bound together by mycelium forming a firm cake like products. The taste of tempeh was acceptable.

Effects of depth of beans on tempeh fermentation

The supply of oxygen is very essential for the mold growth. Effects of different depth of soybean for fermentation were evaluated in polythene bags. Thickness was varied from 1.0 cm to 4.5 cm. The mold growth was rapid while the thickness of the cake was 2.0 cm because oxygen supply was sufficient for tempeh fermentation.

Effects of temperature on tempeh fermentation

Incubation temperature has great influence on the growth rate of mold culture. The inoculated soybean was incubated at 25 C^o, 28 C^o and 30 C^o. The mold growth was rapid at 30 C^o. The fermented product was not of good quality than that at high temperature soybean tends to dry out; consequently, the mold growth was suppressed.

Effects of inoculums size on tempeh fermentation

Inoculum size is an important factor in tempeh fermentation. Excess inoculum promoted rapid and uniform tempeh fermentation and too little inoculum allowed bacteria to grow which suppress *Rhizopus oligosporus* growth. In Present study, inoculum size was varied from 10 µl to 500 µl. Optimum inoculum size was 90 µl for tempeh fermentation in polythene bags.

Moisture and Ash content

Tempeh fermentation is considered as exothermic reaction because of the release of moisture during and after fermentation moisture content of tempeh was determined by oven drying tempeh sample at 100^oC for 24 hours. The moisture content of tempeh sample was 62.38%. Ash content was determined by pre-weight tempeh sample was ashed by heating at 500^oC in a muffle furnace until residue was whitish grey. The ash content per unit weight was 4.12 %.

Nutritional contents:

Protein and Fat content

Rhizopus oligosporus produce a variety of enzyme like proteases which cause significant increase in protein content of tempeh. Kjeldahl method was used for determination of protein content in tempeh sample. Result showed that protein content in tempeh sample was 37.38 %. During tempeh fermentation, *Rhizopus oligosporus* produce lipases enzymes which break down lipids in tempeh and *Rhizopus oligosporus* consumes these small fatty acids molecules for their energy requirement. Fat content was determined by soxhlet extraction method and calculated value of fat content in tempeh samples was 17.31%.

Aflatoxin content

Tempeh sample was prepared in laboratory and was tested in Pakistan council for scientific and industrial research (PCSIR) for aflatoxin analysis. Results showed that tempeh was free of aflatoxin because mould *Rhizopus oligosporus* does not produce aflatoxin itself as well as inhibits the growth of those species which produce aflatoxin (Ko, 1974).

DETERMINATION OF SOME OF PARAMETERS AFTER TEMPEH FERMENTATION

Assays	Values
Humidity	20-60 R.h.
P^H	4.0-6.0
Temperature	25-30 C°
Moisture content	62.38%
Ash content	4.12%
Proteins	37.38 %
Lipids	17.31%
Aflatoxin	Absent

Discussion:

The present study describes propagation of *Rhizopus oligosporus* on dehulled soybeans as substrates. Soybean Tempeh was prepared by *Rhizopus oligosporus* NRRL-2710.

Acidity

In normal condition, pH of tempeh varies from 4-6. The initial pH increased from 4.5 to 6.0 after 26 h at 28 C° or 18 h at 38 C°. Tempeh fermented for 48 h at 28 C° or 20 h at 38 C° resulted in the pH leveling off around 7.5 to 8.0.R. oligosporus can grow as well at pH 3 as at pH 4 or 5. However, there was a significant difference between pH 5 and pH 6. Thus, this mold could be used in a protected fermentation. This would be desirable in circumstances where sterilization is not possible due to lack of equipment or cost (Omosaiye et al 1978) The most favorable pH range for the growth of most fungi is from pH 4 to 7 (Litchfield, 1968).The preferable pH of beans is of a range of 4.0 to 5.0. At this pH range, the growth of contaminating bacteria would be inhibited, but not that of the tempeh mold. The tempeh mold will be inhibited when the pH drops below 3.5.

Temperature

Temperature is another important factor in tempeh manufacture, according to the (Frankland et al., 1982) that the speed of fermentation is determined by the incubation temperature .Incubation temperatures above 40 C° and below 25 C° will not produce good tempeh temperature of 37-38 C° will produce tempeh within 22hr; a temperature of 28-30 C° will take up to 48 h to produce tempeh. When fermentation temperature is as low as 25 C°, an acceptable tempeh could be produced. However, the fermentation required as long as 5 days to complete. In contrast, fermentation at 37 C° required only 1 day. Thus, it can be concluded that a temperature slightly above room temperature is the best for *tempeh* fermentation (LIU, 1997).

Relative humidity

Relative humidity is defined as the ratio of the partial pressure of water vapors in an air parcel of air to the saturated vapor pressure of water vapor at a prescribed temperature. Winarno and Reddy (1986) reported a pilot plant process requiring 18 h incubation at 35-38 C° and 75-78% relative humidity (r.h.). Optimum Relative humidity was reported as 60 and 65, 75% and 90% (Steinkraus 1985a). At relative humidity >75% undesirable fungal sporulation**.** Martinelli and Hesseltine (1964) elevated the relative humidity by placing a tray of water in the bottom of an incubator set at 31 C°. A similar procedure using black common beans was conducted at 37 C° with a relative humidity of 70% (Paredes-Lopez *et aI*., 1987). .Relative humidity was maintained at 75% by wetting a Whatman No. 1 filter paper disc with a saturated solution of sodium chloride (Rockland, 1960).

Inoculum size

The inoculation levels of *R. oligosporus* strongly influenced tempeh fermentation**.** The excess inoculum promoted rapid and uniform fermentation. Wang *et aI*. (1975) concluded that if too little inoculum was used, bacteria would be allowed to grow. Like With inoculation at approximately 10^2 spores/g moist, the fungus grew more slowly and a tempeh cake with dense mycelial growth was not obtained until after 28 to 32 h (Nout and Kiers, 2005). When *R. oligosporus* was inoculated at approximately 10^4 spores/g moist substrate, a tempeh cake with dense mycelial growth was obtained after 20 h (Nout and Kiers, 2005) For optimal fermentation, Wang et al. (1975) recommended that 1 x 10^6 spores per 100 g of cooked soybeans be used. On the other hand, fermentation failures and excessive heat production were reported to be caused by insufficient packing density with pockets of air and heavy inoculation. However, the growth was uneven (Nout and Kiers, 2005), probably due to oxygen limitation in the center.

Aeration

Aeration is one of important factor for production of tempeh which affects the quality of tempeh. Most of the researchers prepared tempeh within range of 2-5 cm of bean's thickness. Frankland et al., (1982) performed experiment at laboratory scale in which he placed soybeans in a plastic bag and flatten the contents out to a cake about 2.0 cm thick and reported that the area of the cake is not important, but the thickness should always be about 2.0 cm to have food quality of tempeh. Same as hackler *et al.,* (1963) inoculated beans were spread on stainless steel pans (25.4 X 35.6 X 6.4 cm) to a depth of approximately 2.54 cm and covered with metal covers and incubated at 37 C°. A freeze-dried starter culture of R. oligosporus NRRL 2710 was added at 1% (w/w) of the wet substrate and mixed thoroughly for 3 min. The inoculated substrates were packed into sterile plastic Petri plates(diameter 87 mm, depth 12.5 mm(1.25 cm), each plate containing approximately 42 g (Davey, 1991).The inoculated substrate is transferred to a confined space and a slight pressure is applied from outside. Traditionally, this is achieved by wrapping small quantities in plant leaves or by covering 4-6 cm thick beds with banana leaves or polythene sheets, which may be weighted down with clay bricks (Nout and Rombouts, 1990). American vegetarians' consume tempeh burgers of about 1.5-cm thickness Beans and starter are mixed homogeneously into 3–5 cm thick beds (Bates *et al*., 1977).

Moisture and Shelf life

Tempeh fermentation is an example of solid substrate fermentation that involves the growth of microorganisms on solid organic materials in the absence or near absence of free water. In general, high relative humidity and good absorbency of the substrate are absolutely needed for proper Tempeh. The production of polysaccharidases as well as their specific activities during tempeh fermentation was found to depend on water activity of the soybean substrate (liu, 1997). The nutritional implications of the tempeh fermentation and reported that fresh tempeh contains 60% moisture. Fresh tempeh cakes must be consumed within 1 or 2 days or the mold proteolytic enzymes will cause ammonia to form, which results in an undesirable taste. Storage stability of tempeh can be extended by drying, frying, dehydration, freezing, and other preservation methods. Wang and Hesseltine (1979) reported that shelf life could be prolonged by freezing, drying, or canning. Steinkraus et al. (l965b) cut the fermented tempeh into 2.5-cm squares and placed the squares into a hot-air dryer in order to lower the moisture level to 2 to 4%.) concluded that tempeh remained stable without refrigeration for 24 to 48 h after harvesting. Therefore, freshly made tempeh can be stored for several days at room temperature without adversely affecting the nutritional or organoleptic properties. Steinkraus et al. (1965b) reported that dehydrated tempeh could be stored in plastic storage bags for several months at room temperature without noticeable changes in color or flavor, changes in reducing substances, soluble solids, and soluble nitrogen content of tempeh occurred. Iljas *et al* (1970) studied the storage stability of tempeh using canning and found that a shelf life of 10 weeks could be attained without significant alterations in the acceptability of tempeh resulting.Having an effect or making an impression on sense organs; usually used in connection with subjective testing of foods and drug products known as organoleptic evaluation. Organoleptic evaluation includes testing of tempeh by aroma, taste, appearance, and texture and mycelia growth. Organoleptically, tempeh scored best at the end of the first phase of fermentation (30 h at 32 C°), kept its good quality during the second phase (one additional day at 32 C°), and deteriorated rapidly during the third phase (Sudarmadji and Markakis, 1978). Signs of deterioration appeared as loss of pleasant taste, smell and texture.

Nutritional content

Soybean source of protein

Protein is one of most essential nutrients among other nutritive elements. Tens and thousands of children in developing countries die every day due to disease caused by protein deficiencies**.** Soybean is considered by many agencies, including the US food and Drug administration, to be a source of complete protein. A complete protein is one that contains significant amounts of all the essential amino acids that must be provided to the human body because of the body's inability to synthesize them. For this reason, soy is a good source of protein, amongst many others, for many vegetarian and vegans or for people who cannot afford meat (USDA, 2004). All around the world, soybean are known due to their rich protein content but increasingly, soyfoods are being recognized as having potential roles in the prevention and treatment of chronic diseases, most notably cancer and heart disease. There are also potential roles for soyfoods with respect to osteoporosis and kidney disease. Soybean is thought of Asian origin (kowal and kassan, 1978).Soybean was taken to the united states in 1804, but there was little commercial production until the 20th century .since then, soybean has been processed into comparatively simple food products; processing includes water extraction (soy milk)with coagulation calcium salt(tofu), roasting (kinako) and fermentation (miso, natto, tempeh, and soy sauce).Of all legumes, soybean crop proteins have reached the highest degree of refinement and extent of development; and are added to a wide variety of processed foods (Wolf and Cowan, 1975). soybean is rich with following nutrients: protein 39 %;(crude protein 44%); lipids 17-20%: carbohydrate 18%: digestible fiber 40% and minerals 5%. Soybeans have played very important role in Asian culture, both as a food and as a medicine. In comparison to most other legumes, soybeans are much higher in protein (~35% of energy), which may be particularly important for developing countries. However; it is not only the amount of protein in soybeans that is notable, but also the amino acid pattern of soy protein. Soy protein is very efficiently produced; approximately 25, 10 and 5 times more protein is produced by soybeans per acre as compared with beef, milk and wheat production, respectively.Because of the proteins semi digestive state, it makes a good protein source for people with gastro-intestinal upsets (i.e. POW's, AIDS, third world countries) (Varzakas, 1986). From a nutritional perspective, soy protein may hold many advantages over animal proteins above and beyond the fact that soybeans are low in saturated fat and, of course, cholesterol-free. Of utmost importance is the hypocholesterolemic effect of soy protein (Bakhit *et al*., 1994), so with help of this property of soybean, soy protein represents a safe, viable and practical non pharmacologic approach to lowering cholesterol. The hypocholesterolemic effects of soy protein may be of particular benefit to patients with chronic renal in sufficiency, because elevated levels of cholesterol can exacerbate disease progression**.** The oxidation of low-density lipoprotein (LDL) cholesterol may play a critical role in this regard; consequently, the suppression of LDL-cholesterol oxidation by soyprotein may be still another benefit of soy protein not only to kidney disease patients, but also to the general public. For this reason, the kidney disease patients would benefit as much by substituting soy protein for animal protein as by restricting overall protein intake. Soy protein may also help to promote bone health. Factors affecting urinary calcium excretion play critical roles in determining calcium balance and bone mineral density. The hypercalciuric effect of protein has been proposed as one factor contributing to the high rates of osteoporosis in Western countries (Abelow *et al.*, 1992), where protein intake greatly exceeds requirements. However, in comparison with animal proteins, soy protein causes much less calcium to be excreted in the urine. Parenthetically, the isoflavones in soybeans may also directly inhibit bone absorption (Brandi 1992). Research on the potential health benefits of soyfoods is particularly intriguing with respect to cancer prevention and treatment. Epidemiologic data suggest the consumption of as little as one serving of soyfoods (i.e., one cup soymilk, 5 cup tofu) per day lowers risk for a wide range of cancers (Messina *et al.,* 1994).

Conclusions

In conclusion, this study demonstrated that with the growing demand for soy foods, tempeh is now becoming more and more available throughout the country. Plain soy tempeh that has been made from soy and Rhizopus mold with and without the addition of soy-grain combinations flavored with soy sauce. Tempeh that says "pre-cooked" and "ready to eat" foods contains a good source of protein, phosphorus, vitamin B_{12}, and magnesium which are also more delicious, healthier, digestible and absorbable form due to the process of fermentation.

References:

1. Abelow, B. J., Holford, T. R., & Insogna, K. L. (1992). Cross-cultural association between dietary animal protein and hip fracture: a hypothesis.*Calcified tissue international*, *50*(1), 14-18.
2. Ahmad, N., & Sarbhoy, A. K. (1995). New powdery mildews from India.*Mycological Research*, *99*(3), 374-376.
3. Añón, M. C., & Calvelo, A. (1980). Freezing rate effects on the drip loss of frozen beef. *Meat science*, *4*(1), 1-14.
4. Ashenafi, M. (1991). Growth of Listeria monocytogenes in fermenting tempeh made of various beans and its inhibition by Lactobacillus plantarum. *Food Microbiology*, *8*(4), 303-310.
5. Bakhit, R. M., Klein, B. P., Essex-Sorlie, D., Ham, J. O., Erdman Jr, J. W., & Potter, S. M. (1994). Intake of 25 g of soybean protein with or without soybean fiber alters plasma lipids in men with elevated cholesterol concentrations. *The Journal of nutrition*, *124*(2), 213-222.
6. Brandi, M. L. (1992). Flavonoids: biochemical effects and therapeutic applications. *Bone and Mineral*, *19*, S3-S14.
7. Brockerhoff, H., Hoyle, R. J., Hwang, P. C., & Litchfield, C. (1968). Positional distribution of fatty acids in depot triglycerides of aquatic animals. *Lipids*, *3*(1), 24-29.

8. Chang, I. S., & Lee, C. H. (1998). Membrane filtration characteristics in membrane-coupled activated sludge system—the effect of physiological states of activated sludge on membrane fouling. *Desalination*, *120*(3), 221-233.
9. Correia, M., Silva, M. R., Matos, I., Magalhães, R., Lopes, J. C., Ferro, J. M., & Silva, M. C. (2004). Prospective community-based study of stroke in Northern Portugal Incidence and case fatality in rural and urban populations. *Stroke*, *35*(9), 2048-2053.
10. Frankland, J. C., Hedger, J. N., & Swift, M. J. (1982). *Decomposer basidiomycetes: their biology and ecology*. Cambridge University Press.
11. Horbar, J. D., Badger, G. J., Carpenter, J. H., Fanaroff, A. A., Kilpatrick, S., LaCorte, M., ... & Soll, R. F. (2002). Trends in mortality and morbidity for very low birth weight infants, 1991–1999. *Pediatrics*, *110*(1), 143-151.
12. Keuth, S., & Bisping, B. (1993). Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation.*Journal of Applied Bacteriology*, *75*(5), 427-434.
13. Kobayashi, M., Nagasawa, T., & Yamada, H. (1992). Enzymatic synthesis of acrylamide: a success story not yet over. *Trends in biotechnology*, *10*, 402-408.
14. Kontessis, P., Jones, S., Dodds, R., Trevisan, R., Nosadini, R., Fioretto, P., & Viberti, G. (1990). Renal, metabolic and hormonal responses to ingestion of animal and vegetable proteins. *Kidney international*, *38*(1), 136-144.
15. Kowal, J. M., & Kassam, A. H. (1978). *Agricultural ecology of savanna. A study of West Africa*. Oxford University Press.
16. Liem, I. T., Steinkraus, K. H., & Cronk, T. C. (1977). Production of vitamin B-12 in tempeh, a fermented soybean food. *Applied and environmental Microbiology*, *34*(6), 773-776.
17. Liu, K. (1997). Chemistry and nutritional value of soybean components. In*Soybeans* (pp. 25-113). Springer US.
18. Messina, M. J., Persky, V., Setchell, K. D., & Barnes, S. (1994). Soy intake and cancer risk: a review of the in vitro and in vivo data. *Nutrition and cancer*, *21*(2), 113-131.
19. Nout, M. J. R., & Kiers, J. L. (2005). Tempe fermentation, innovation and functionality: update into the third millenium. *Journal of Applied Microbiology*, *98*(4), 789-805.

20. Nout, M. J. R., & Rombouts, F. M. (1990). Recent developments in tempe research. *Journal of Applied Bacteriology*, *69*(5), 609-633.
21. Nout, M. J. R., & Rombouts, F. M. (1990). Recent developments in tempe research. *Journal of Applied Bacteriology*, *69*(5), 609-633.
22. Nout, M. J. R., De Dreu, M. A., Zuurbier, A. M., & Bonants-van Laarhoven, T. M. G. (1987). Ecology of controlled soyabean acidification for tempe manufacture. *Food Microbiology*, *4*(2), 165-172.
23. Nowak, J., & Steinkraus, K. H. (1988). Effect of tempeh fermentation of peas on their potential flatulance productivity as measured by gas production and growth of Clostridium perfringens. *Nutrition reports international*, *38*(6), 1163-1171.
24. Okada, Y. (1985). Surface deformation due to shear and tensile faults in a half-space. *Bulletin of the seismological society of America*, *75*(4), 1135-1154.
25. Omosaiye, O., Cheryan, M., & Matthews, M. E. (1978). Removal of oligosaccharides from soybean water extracts by ultrafiltration. *Journal of Food Science*, *43*(2), 354-360.
26. Pons, W. A., Robertson, J. A., & Goldblatt, L. A. (1966). Objective fluorometric measurement of alfatoxins on TLC plates. *Journal of the American Oil Chemists' Society*, *43*(12), 665-669.
27. Rehms, H., & Barz, W. (1995). Degradation of stachyose, raffinose, melibiose and sucrose by different tempe-producing Rhizopus fungi.*Applied microbiology and biotechnology*, *44*(1-2), 47-52.
28. Rockland, L. B. (1960). Saturated salt solutions for static control of relative humidity between 5° and 40° C. *Analytical Chemistry*, *32*(10), 1375-1376.
29. Samson, S. D., & Alexander, E. C. (1987). Calibration of the interlaboratory 40 Ar□ 39 Ar dating standard, MMhb-1. *Chemical Geology: Isotope Geoscience Section*, *66*(1), 27-34.
30. Sharma, K., McCue, P., & Dunn, S. R. (2003). Diabetic kidney disease in the db/dbmouse. *American Journal of Physiology-Renal Physiology*,*284*(6), F1138-F1144.
31. Steinkraus, K. H. (1983). Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. *Antonie van Leeuwenhoek*,*49*(3), 337-348.
32. Steinkraus, K. H. (1983). Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. *Antonie van Leeuwenhoek*,*49*(3), 337-348.

33. Struve, C., & Krogfelt, K. A. (2004). Pathogenic potential of environmental Klebsiella pneumoniae isolates. *Environmental microbiology*, *6*(6), 584-590.
34. Suparmo, P. M. (2008). Uji ketergunaan situs web jaringan perpustakaan asosiasi perguruan tinggi Katolik di Indonesia (APTIK) bagi mahasiswa yang sedang menulis skripsi pada tahun akademik 2006/2007 di Universitas Sanata Dharma Yogyakarta. *Info Persadha*, *6*(1).
35. Turecki, G., Grof, P., Cavazzoni, P., Duffy, A., Grof, E., Ahrens, B., ... & Vojtechovsky, M. (1998). Evidence for a role of phospholipase C-y1 in the pathogenesis of bipolar disorder. *Molecular psychiatry*, *3*(6).
36. Udoidem, I. U. (2016). *Characteristics of Traditional Snacks Produced From Citrullus vulgaris S, Glycine max L, Arachis hypogea L and Sclerotium tuberygii* (Doctoral dissertation).
37. Varzakas, T., Escudero, I., & Economou, I. G. (1999). Estimation of endoglucanase and lysozyme effective diffusion coefficients in polysulphone membranes. *Journal of biotechnology*, *72*(1), 77-83.
38. Vatten, L. J., & Skjærven, R. (2004). Is pre-eclampsia more than one disease?. *BJOG: an International Journal of Obstetrics & Gynaecology*,*111*(4), 298-302.
39. WANG, H. L., Swain, E. W., Hesseltine, C. W., & Heath, H. D. (1979). Hydration of whole soybeans affects solids losses and cooking quality.*Journal of Food Science*, *44*(5), 1510-1513.
40. Wang, J. C. (1969). Variation of the average rotation angle of the DNA helix and the superhelical turns of covalently closed cyclic λ DNA. *Journal of molecular biology*, *43*(1), 25-39.
41. Wiesel, P., Mazzolai, L., Nussberger, J., & Pedrazzini, T. (1997). Two-kidney, one clip and one-kidney, one clip hypertension in mice.*Hypertension*, *29*(4), 1025-1030.