

Preliminary studies on tempeh flour produced from three different *Rhizopus* species

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Abstract. Tempeh which is culturally an Indonesian food was produced by fermenting soybeans with different *Rhizopus* species - *Rhizopus oryzae*, *Rhizopus stolonifer* and *Rhizopus oligosporus*. The tempeh was subsequently dried and milled. The tempeh flours were subjected to proximate analysis, physicochemical (total titratable acidity, pH and water absorption capacity) and microbiological tests. Selected minerals such as calcium, potassium, iron and zinc were analyzed. Vitamin contents were evaluated. Sensory evaluation was conducted in order to determine the acceptability of the samples. The statistical analysis was carried out using the Analysis of Variance (ANOVA) at 95% confidence limit and Duncan test. The pH and total titratable acidity ranged from 6.8 to 7.0 and 0.032 to 0.077 g/100 g, respectively. No significant difference existed among the water absorption capacities of the samples. The average percent crude protein was 44.27, 44.62 and 44.85% for the three samples. The fat content ranged from 16.45 to 17.12% while the % crude fibre content ranged from 0.38 to 0.42. There was no statistically significant difference in the Vitamin B2, Vitamin C and Vitamin D content of the samples while a significant difference existed in the Vitamin B1 and Niacin content among the samples. The result of the mineral content analysis revealed that the potassium, iron, zinc and calcium contents ranged from 0.14 to 0.17, 0.011 to 0.014, 0.0046 to 0.0050 and 0.19 and 0.21%, respectively. Yeast and Coliform was not detected in the samples. The total bacterial count ranged from 0.5×10^5 to 1.6×10^5 cfu/g, the lactic acid bacteria ranged from 18.0×10^5 to 22.4×10^5 cfu/g while the mould count ranged from 0.8×10^5 to 1.4×10^5 cfu/g. There was no significant difference in the aroma and texture of the flour samples. The tempeh flour produced using *R. oligosporus* and the one produced using *R. oryzae* were compared in terms of colour and overall acceptability.

Keywords: Tempeh, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Rhizopus oligosporus*.

INTRODUCTION

Soybean seeds are very rich in protein, oil and also minerals particularly calcium and iron but low in sugar contents. Soybeans also have high lysine content. Consequently, they afford very nutritious foods for all. Soybean is known for its ability to be processed into different other products to meet with the human diet in most developing countries. One economical way of preventing under nourishment is to consume protein in the form of soybeans (Kumar et al., 2007).

Utilization of soybean is one of the more promising means of alleviating the shortage of good quality protein in developing countries (Aworh et al., 1987), because it provides high quality protein, cholesterol-free fat and good amount of omega-3 fatty acids. (Messina et al. (1994a, b) recommended the incorporation of soy foods into the diet for the prevention or treatment of heart ailments and cancer. A number of soy-fortified products like snacks, extruded products, bread, soy-fortified *atta*,

paneer, *dahi* and noodles have been developed (Yadav et al., 2007).

Fermentation causes biochemical and nutritional changes in seeds. Fermentation improves the digestibility of many foods, increases nutritional values, and provides important living enzymes and beneficial microorganisms to our diet (Bost, 2006). There is a breakdown of certain constituents, reduction of anti-nutritional factors in grains and the synthesis of B-vitamins (Egounlety and Aworh, 2000; Egounlety, 2002). The soy carbohydrates in tempeh become more digestible as a result of the fermentation process. In particular, the oligosaccharides associated with gas and indigestion are greatly reduced by the *Rhizopus* culture. The fermentation process also reduces the phytic acid in soybeans (Amanda, 2011). Processing methods, such as, sprouting and fermentation has been reported to improve the nutritional and functional properties of plant seeds (Jirapa et al., 2001; Yagoub and Abdalla, 2007). For instance, sprouting or germination has been reported to improve digestibility, bioavailability of vitamins, minerals, amino acids, proteins and phytochemicals, and decrease anti-nutrients and starch of some cereals and legumes (Kyler and McCready, 1975; Lopez et al., 1983; Camacho, 1992; Asiedu et al., 1993; Egli, 2001; Helland et al., 2002; Egli et al., 2004) and thereby improve protein and iron absorption.

Tempeh is a compact, sliceable cake of mold-fermented soybean cotyledons. Tempeh is an indigenous fermented food that originated from the Javanese people in Indonesia, where it is most popular (Shurtleff and Aoyagi, 1997; Aderibigbe and Kolade, 2003). In Indonesia, the traditional and original material for producing tempeh is soybean (Shurtleff and Aoyagi, 2001). However, other kinds of raw materials including legumes, cereals and press cakes of coconut and peanut have been used (Science daily, 2008). There are many recipes that can be derived from tempeh. They include burgers, snacks and meat substitutes. Tempeh is a health promoting food. It is rich in nutrients and active substances. It is the best source of vitamin B12 in vegetarian diets (Liem et al., 1977; Truesdell et al., 1987). Tempeh is cholesterol free and it helps lower total cholesterol level in the blood, thus decreasing the risk of cardiovascular diseases. Tempeh is readily digestible compared to other leguminous foods and is tolerated by patients suffering from dysentery and nutritional oedema. Tempeh contains high quality protein, thus can be used to supplement protein-energy deficient diets (Aderibigbe et al., 2010).

Vegetable proteins have made up a higher proportion of the human diet in recent years. Soybeans are the most important source of vegetable protein ingredients. The health claim allowed on food products containing soy protein by the U.S. government has created a demand for functional soy proteins in the form of flours, protein concentrates, and protein isolates (Wrick, 2003).

Tempeh was traditionally prepared by fermenting soybeans with *R. oligosporus* but this study entailed the use of two other *Rhizopus* species - *Rhizopus stolonifer* and *Rhizopus oryzae* to produce tempeh. These species can easily be cultured locally without necessarily having to get the Indonesian tempeh or order for the *R. oligosporus* powder. Physical, chemical, microbiological and sensory tests were carried out to determine if these samples compared with the conventional one. Tempeh from literature is a nutritious food with health benefits that could be harnessed for the growth and development of the vulnerable groups. This could be done by the incorporation of the flour into basic food products which are low in protein and vitamin. The incorporation of the tempeh flour should help combat malnutrition.

MATERIALS AND METHODS

Source of materials

Soybean (*Glycine max*), used for the study was purchased from a local market in Ogbomoso, Nigeria. The starter culture inoculums (*R. oligosporus*) were obtained from tempeh powder from the Indonesian Embassy, Victoria Island, Lagos. *Rhizopus stolonifer* was cultured from ripe pawpaw while *Rhizopus oryzae* was cultured from rice both purchased from a local market in Ogbomoso.

Preparation of tempeh Sub culture

Tempeh subculture was prepared by adding 50 ml of distilled water to 50 g of rice, ripe pawpaw in a clean beaker for *R. oligosporus*, *R. oryzae* and *R. stolonifer*, respectively. The beaker was covered with a piece of muslin cloth and tied with a twine. The beaker was sterilized with the content in an autoclave to destroy other organisms present in the substrates. The substrates were inoculated with the organisms. The beakers were then placed in the incubator after inoculation at 35°C for 72 h.

Preparation of tempeh

Soybeans were soaked overnight (10 to 12 h) and subsequently dehulled by rubbing between the palm of the hands. The hulls were removed by flotation in water. The dehulled beans were boiled for 20 to 30 min, drained and left for surface drying in a mesh. The beans were inoculated with 1 ml of fungal spore suspension (*R. oligosporus*, *R. oryzae* and *R. stolonifer*) separately. The inoculated beans were packed firmly in aluminium foil and incubated for 40 to 48 h. The resultant tempeh was sliced and dried in Gallenkamp oven at 70°C. The dried tempeh was milled using the Perten laboratory mill 3100.

The flour was stored in polythene bags for analysis.

Sensory evaluation

The samples were evaluated for colour, texture, aroma and overall acceptability by a semi-trained panel of 20 judges using a 7-point hedonic scale (7 = liked extremely to 1 = disliked extremely) (Larmond, 1982).

Analytical methods

Total titratable acidity and pH

Titratable acidity, expressed as lactic acid, was measured by titration with 0.1 N NaOH to the phenolphthalein endpoint. The pH was monitored by using a glass electrode digital pH meter (Jenway 3505 pH meter).

Total soluble solids

Total soluble solids were determined at 20°C by the Joslyn (1970) method, using an Abbe refractometer (Bellingham and Stanley LTD, London).

Water-holding capacity (WHC) and oil-holding capacity (OHC)

Twenty-five millilitres of distilled water or commercial olive oil were added to 1 g of dry sample, stirred and incubated at 40, 60 or 80°C for 1 h. Tubes were centrifuged at 3000 × g for 20 min, the supernatant was decanted, and the tubes were allowed to drain for 10 min at a 45° angle. The residue was weighed and WHC and OHC calculated as g water or oil per g dry sample, respectively (Rodriguez-Ambriz et al., 2008).

Viscosity

The viscosities of the samples were determined as described by Fagbemi (1999). Flour was dispersed in water at 8% (w/v) concentration using a magnetic stirrer (1000 rpm) and heated from 30 to 95°C in a waterbath and kept at this temperature for 20 min. The slurry obtained was stirred constantly and cooled at room temperature. The viscosity was measured using a Brookfield Viscometer, model DV-E (Brookfield engineering laboratories, Inc, Middleboro, MA, USA) using spindle 3, at 50 rpm.

Proximate analysis

The protein content of the samples was determined by the micro kjedahl method as described by AOAC (2000).

The protein content, fat content, ash content, moisture content and crude fiber were determined by the AOAC methods (2000).

Mineral and vitamin content analysis

For the mineral analysis, finely ground dried samples were digested in a mixture containing perchloric acid and concentrated nitric and hydrochloric acids. From the digest, potassium, iron, zinc and calcium were determined using a Perkin Elmer Atomic Absorption Spectrophotometer model 703 with an acetylene/air flame (IITA, 1982).

Vitamin B1 (Thiamin), B2 (Riboflavin) and Niacin (B3) were analyzed using 5 g of the ground samples. Vitamin B1 and B2 were measured using the method described by Okwu and Ndu (2006) while the method of the Association of Vitamin Chemist described by Okwu (2004) was used to determine the niacin content of the samples. The absorbance of Vitamin B1, B2 and niacin extracts of the samples was measured with spectrophotometer (Jenway model) at wavelengths of 360, 510 and 470 nm, respectively. Vitamin C was measured using 1 g of ground sample using the new spectrophotometric method, Leuco Malachite Green (LMG) as described by Nielsen (2003). The absorbance of vitamin C extract at the wavelength of 620 nm was measured with spectrophotometer. The concentration of ascorbic acid was established from calibration graph of absorbance against ascorbic acid concentrations between 0.8 and 8.0 µg. Vitamin D was determined by the method reported by Perales et al. (2005). Each experiment was carried out in three replicates.

Microbiological analysis

The total viable count was determined using plate count agar while the mould and yeast count were determined using violet red bile agar.

Statistical analysis

Samples were analyzed in triplicate and the figures were then averaged. The data collected from the studies were subjected to analysis of variance ANOVA. The means were compared using Duncan's multiple range test with a probability $P \leq 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Results of the proximate analysis of the tempeh samples obtained by fermentation of soybeans with *R. oligosporus*, *R. oryzae* and *R. stolonifer* are shown in Table 1. Various studies have shown that fermentation

Table 1. Proximate composition of the tempeh flour samples.

Sample code	Crude protein (%)	Crude fibre (%)	Fat content (%)	Ash content (%)	Moisture content (%)	Carbohydrate content (%)
Tempeh (<i>Rhizopus oligosporus</i>)	44.62 ± 0.01 ^a	0.38 ± 0 ^a	16.45 ± 0.01 ^c	5.60 ± 0 ^a	3.00 ± 0 ^a	33.52 ± 0.005 ^c
Tempeh (<i>Rhizopus oryzae</i>)	44.85 ± 0 ^a	0.40 ± 0 ^a	17.12 ± 0 ^a	5.72 ± 0.05 ^c	2.50 ± 0 ^a	32.57 ± 0.01 ^b
Tempeh (<i>Rhizopus stolonifer</i>)	44.27 ± 0 ^a	0.42 ± 0.07 ^b	16.68 ± 0.05 ^b	5.61 ± 0.05 ^b	2.50 ± 0 ^a	33.55 ± 0 ^a

Uncommon superscripts along columns indicate statistically significant difference (P < 0.05).

Table 2. Vitamin and mineral content.

Sample code	Vit B ₁ (mg/100 g)	Vit B ₂ (mg/100 g)	Vit C (mg/100 g)	Vit D (mg/100 g)	Niacin (mg/100 g)	Potassium (%)	Iron (%)	Zinc (%)	Calcium (%)
Tempeh (<i>Rhizopus oligosporus</i>)	0.12 ± 0 ^a	0.04 ± 0 ^a	1.06 ± 0 ^a	0.28 ± 0 ^a	0.26 ± 0.005 ^a	0.14 ± 0 ^a	0.011 ± 0.001 ^b	0.0046 ± 0 ^a	0.19 ± 0.005 ^b
Tempeh (<i>Rhizopus oryzae</i>)	0.19 ± 0.005 ^b	0.08 ± 0 ^a	1.77 ± 0 ^a	0.37 ± 0 ^a	0.36 ± 0.05 ^b	0.17 ± 0 ^b	0.013 ± 0 ^a	0.0050 ± 0 ^a	0.20 ± 0 ^a
Tempeh (<i>Rhizopus stolonifer</i>)	0.16 ± 0.005 ^c	0.06 ± 0 ^a	1.59 ± 0 ^a	0.42 ± 0 ^a	0.28 ± 0 ^a	0.15 ± 0 ^a	0.014 ± 0 ^a	0.0050 ± 0 ^a	0.21 ± 0 ^a

Uncommon superscripts along columns indicate statistically significant difference (P < 0.05).

can increase soluble protein (Chavan et al., 1988), and provide better essential amino acids composition as a result of *de novo* production of important amino acids (Au and Fields, 1981). There was no significant difference between the crude protein content of the samples, although the sample fermented with *R. oryzae* had slightly higher protein content than the other two samples. Crude fibre in tempeh from *R. stolonifer* was highest (0.42%) and lowest in sample from *R. oligosporus* (0.38%). Sample from *R. oryzae* had the highest fat content (17.12%) while the sample fermented with *R. oligosporus* had the lowest (16.45%). The sample fermented with *R. oryzae* had the highest ash content (5.02%) while the sample from *R. oligosporus* had the lowest (5.00%). The sample fermented with *R. oligosporus* had the highest moisture content while the other two samples had the same. The carbohydrate content in the sample fermented with *R. stolonifer* was highest and lowest in the sample fermented with *R. oryzae*. According to

Kazanas and Fields (1981), fermentation can help enrich the nutritive content of essential nutrients through microbial synthesis and improvement in protein and carbohydrate digestibility (Taur et al., 1984). However, all the samples had very low moisture contents. This should help ensure that the samples have a long shelf life and are easy to reconstitute.

Fermentation can also improve mineral availability and increase vitamin B content (Manning, 1970; Mungula et al., 2003). Various studies have shown that fermentation can increase the concentrations of vitamins, minerals and protein (Taylor and Dewar, 2000). Table 2 shows the vitamin and mineral contents of the three tempeh samples. The table indicated that there was no significant difference in the vitamin B₂, vitamin C, vitamin D, potassium and zinc content of the samples at 0.05 confidence limit. There was however significant difference in vitamin B₁, niacin, iron and calcium components of the tempeh flour samples. The niacin and

potassium content of the *R. oryzae* fermented tempeh flour were higher and significantly different from that of the other two samples. It also had higher levels of vitamin B₁ and B₂. The zinc content of *R. oryzae* and *R. stolonifer* fermented tempeh were the same. *R. oryzae* and *R. stolonifer* fermented tempeh flour had higher and significantly different iron content from *R. oligosporus* fermented tempeh flour sample. The vitamin D content of the samples ranged from 0.28 to 0.43 mg/100 g. Tempeh is known to have a health promoting effect on bone building. This can be supported by the relatively high calcium content in the tempeh samples produced in this study. There was no significant difference in the physico-chemical properties of the samples as shown in Table 3 at 0.05 confidence limit. Fermentation decreases the pH of the fermented samples (Carnovale et al., 1988). The pH of the samples showed that they were low acid samples (tempeh fermented with *R. oligosporus* and *R. stolonifer*) while sample fermented with *R. oryzae*

Table 3 Physico-chemical properties of the tempeh flours.

Sample code	pH	^a TTA	^b WHC (g water/g dry sample)	^c OHC	Total Soluble Solid (^o Brix)	Viscosity (mPa.S)
Tempeh (<i>Rhizopus oligosporus</i>)	6.80 ± 0 ^a	0.036 ± 0 ^a	2.77 ± 0 ^a	0.98 ± 0.02 ^a	10.4 ± 0.05 ^b	75.77 ± 0.76 ^c
Tempeh (<i>Rhizopus oryzae</i>)	7.00 ± 0.02 ^a	0.032 ± 0 ^a	2.64 ± 0 ^a	0.96 ± 0.13 ^a	8.8 ± 0.01 ^a	71.56 ± 1.23 ^b
Tempeh (<i>Rhizopus stolonifer</i>)	6.80 ± 0 ^a	0.077 ± 0 ^a	2.61 ± 0 ^a	0.98 ± 0.03 ^a	8.2 ± 0.05 ^a	69.23 ± 0.34 ^a

Uncommon superscripts along columns indicate statistically significant difference (P < 0.05). ^aTotal titratable acidity; ^bWater holding capacity; ^cOil holding capacity.

Table 4. Microbiological characteristics of the tempeh flours.

Sample code	Total bacteria count [Cfug ⁻¹ (×10 ⁵)]	Coliform count [Cfug ⁻¹ (×10 ⁵)]	Lactic acid bacteria [Cfug ⁻¹ (×10 ⁵)]	Mould count [Cfug ⁻¹ (×10 ⁵)]	Yeast count [Cfug ⁻¹ (×10 ⁵)]
Tempeh (<i>Rhizopus oligosporus</i>)	1.6	^a ND	22.4	1.0	^a ND
Tempeh (<i>Rhizopus oryzae</i>)	0.8	^a ND	18.0	1.4	^a ND
Tempeh (<i>Rhizopus stolonifer</i>)	0.5	^a ND	18.7	0.8	^a ND

^aNot Detected

Table 5. Sensory analysis.

Quality attributes	Tempeh (<i>Rhizopus oligosporus</i>)	Tempeh (<i>Rhizopus oryzae</i>)	Tempeh (<i>Rhizopus stolonifer</i>)
Colour	6.2 ^a	4.9 ^a	3.6 ^b
Aroma	4.5 ^a	4.8 ^a	4.5 ^a
Texture	5.3 ^a	5.3 ^a	4.6 ^a
Overall acceptability	5.7 ^a	5.1 ^a	4.3 ^b

had a neutral pH.

The microbiological characteristics of the tempeh samples are shown in Table 4. Yeast and coliform bacteria were not detected. Coliforms are bacteria that are associated with faecal contamination and always in company of pathogens in foods and water. Thus, the water source used for production can be ascertained as safe. Presence of Lactic acid bacteria shows its action in the course of fermentation. Lactic acid bacteria are known to produce B vitamins showing its advantageous presence in the product. The table also shows the presence of the mould which

is involved in tempeh fermentation. Fermentation can improve microbiological safety and keeping quality (El Tinay et al., 1979)

Fermentation causes changes in food quality indices including texture, flavor, appearance, nutrition and safety (Rooney and Waniska, 2004). Results of the sensory analysis as shown in Table 5 indicate that the three samples generated from this research were significantly different at 95% confidence limit in terms of colour. The sample fermented with *R. stolonifer* had the least acceptability in terms of colour because of the dark spores formed during fermentation. There

was no significant difference at 95% confidence limit in terms of aroma and sample fermented with *R. oryzae* was most preferred. It had a crayfish-like characteristic aroma. There was no significant difference in the texture of the samples. There was no significant difference at 95% confidence limit in terms of overall acceptability between the three tempeh samples produced.

CONCLUSION

Fermentation of soybeans with *R. oligosporus*, *R.*

oryzae and *R. stolonifer* produced tempeh flours of relatively high nutritional quality and relatively satisfactory sensory characteristics. Traditionally, *R. oligosporus* has been the only species used to ferment soybeans to produce tempeh but it has been discovered from this study that the use of the other two species of *Rhizopus* - *R. oryzae* and *R. stolonifer*, utilized in this study to produce tempeh was equally satisfactory in terms of the quality characteristics evaluated. They compared favourably with tempeh obtained from *R. oligosporus* - the conventional mould used for tempeh production in Indonesia. However, more extensive toxicology studies have to be carried out on the tempeh produced from *R. oryzae* and *R. stolonifer* to ascertain the safety for human consumption.

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