PRODUCTION AND EVALUATION OF TEMPEH WITH ENHANCED NUTRIENTS

THESIS

Submitted to

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Solan

In the partial fulfillment of the requirement for the degree of

Master of Science in Biotechnology

By

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DECLARATION

This is to declare that the project report having title "Production and evaluation of tempeh with enhanced nutrients." presented by Shivangi Jaryal fulfills the requirements for the award of degree of master of science in Biotechnology from Jaypee University of Information Technology, Waknaghat, Solan.

This thesis has not been submitted in part or in its entirety to any other institute for the granting of any other degree.

Signature of supervisor:

Name of supervisor:

Designation:

Date:

ACKNOWLEDGMENT

Words cannot describe how grateful I am towards my guide, Dr Anil Kant Thakur, Associate Professor of Biotechnology and Bioinformatics department, who introduced me to the world of research and monitored me with infinite patience, and without whose invaluable guidance and unwavering cooperation, the present dissertation would not have been possible. I would like to express my recognition to our PhD scholars Diksha, Suhani Bhagta and Lalita Sharma for their invaluable support. I am grateful to the biotechnology department's lab personnel for their constant motivation and cooperation. I would like to thank my classmate Ashish Guleria for his immense support in my lab work. I am really grateful to the Civil Engineering department's lab personnel for providing me work authorization in their lab. I would also want to thank the Jaypee University of Information Technology (JUIT) officials for their participation and support.

Signature of student:

Name of student:

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1. Introduction:

Tempeh is defined as a conventional food which belongs to Indonesia. Originally this fermented food is made by the dehulling of the soya beans which are soaked earlier and then there is the process of fermentation in which there is solid state with the *Rhizopus oligosporus* species of fungus. As a result there is the formation of a structure which is cake-like in appearance which can be made for eating purposes with nutritional facts [1]. The tempeh is basically made with the soya beans but the research has also been done on different types of other sources, such as maize, lentils, barley and thus has resulted in the disparity of the nutrition. For some commercially feasible and productive reasons, there is the major production of tempeh in the Indonesian market. Most commonly the products which is tempeh like are made up of maize and soya beans. The maize and soya beans are in the ratio of 30:70. This is so because the cost of maize is less than the soya beans and this alternative is economically feasible.There is a significant portion of the population who has brought and to the consumption of meat and have become vegetarian or vegan by consuming the alternatives of meat. The process of fermentation raises the nutritional characteristics of the taken source of protein and decreases the quantity of those components which are antinutritional [12]. There are some compounds which are responsible for the health initiation like as γ-Aminobutyric acid are also produced during fermentation [31]. Along with the enhancement in the nutritional aspect there are also the sensory changes occurring after the process of fermentation.

Also the tempeh is having a large amount of minerals and probiotics along with vitamins. So it can be considered as a nutritional enhancement in the diet. In many countries which are industrialized, the demand for this fermented food tempeh is increasing day by day [24]. This is due to the increase in the interest of people for the opting vegan as well as vegetarian diet because the tempeh has almost ten essential amino acids in it [3].Tempeh is consumed as a high protein source which is vegetarian.

Due to a large amount of nutrient amount, a number of researches have been done on the production of tempeh along with this tempeh is used by the population for the issue of malnutrition in the third world countries. [1]. During the formation of the tempeh, the fermentation process is carried on by the fungal strain of the Rhizopus, in which no mycotoxin is described. But, in some studies it is shown that there are some Rhizopus strains which are actually endosymbiotic bacteria which are basically toxin forming. [35] Suitable selection is being demanded for the particular strain of the Rhizopus in the industrial production of the tempeh [9]. Sometimes the use of the *Aspergillus oryzae* as an inoculum for tempeh formation has produced mycotoxins to some extent [4].

Tempeh is a healthy and nutritious food product because of the presence of bioactive compounds like the isoflavones. It has a lot of nutritional features, like its nice flavors and a different texture as well [19]. The characteristics of the tempeh actually lies in some aspects like the type of material taken and the inoculum taken for the fermentation process. The type of inoculum used has a very important part in the tempeh making. This is because. It affects the quality of tempeh. Commonly, there is the usage of a starter culture having *Rhizopus oligosporus* [10]. There are some other microorganisms which are used in the fermentation of the soya beans, such as *R. oryzae* and *R. stolonifer*.

These three strains of Rhizopus can ferment soybeans and form tempeh. But the most commonly used is the *R. oligosporus* because this one strain remains maximum of the nutritional aspects of the soybeans and therefore enhances the digestibility for the proteins as well [25]. This strain synthesizes much more protease enzyme as compared to others and increases the production of alpha amylase enzyme. Along with all these fungal strains there was also the involvement of the yeast and bacteria for the fermentation and these also contribute for the production of the secondary metabolites. [19].

There is a type of yeast which is found in the formation of tempeh, known as *Saccharomyces cerevisiae*, and it is the microorganism which is producing the βglucan [26]. The yeast can grow along with fungus during the process of the fermentation of soybean but after the addition of the carbon sources, as a result there is the production of β-glucans in the tempeh production [28].

The study aims the production of tempeh with different sources to enhance of vitamins and minerals along with the testing that whether the nutritional aspects have increased or not. Following are the objectives of the experiment:

1. To prepare tempeh using soybean and starter culture.
2. To prepare tempeh using spinach and beetroot for estimation of iron content.
3. To prepare tempeh supplemented with kidney beans for estimation of protein content.
4. To prepare tempeh supplemented with cow milk.
5. To determine the total phenolic content in the different supplements.

2. Review of literature:

2.1 Soybean and its chemical composition:

Soybean is the most important seed legume that contributes for maximum of the protein concentration in the world for the feeding of the population and 25% for the global vegetable oil production. This is the most important crop in the aspect of the harvested area. Soybeans are often green, yellow, or dark in color and grow in units encasing tasty seeds. Protein 43.2 g percent, calcium 240 mg percent, iron 10.4 mg percent, phosphorus 690 mg percent, thiamin 0.73 mg percent, fat 19.5 g percent, riboflavin 0.39 mg percent, and niacin 3.2 mg percent are the usual nutrient concentrations of soybean [3].

2.2 Soy food and health benefits:

Soybean is really a fundamental ingredient in classic Asian cuisine that has been utilized for thousands of years. Because of the presence of protein content in high amount of the proteins, soya beans and the foods made by the soya beans are typical important nutritional alternatives for the vegetarians. Soybeans are abundant in isoflavones, which are polyphenols possessing the estrogenic effects. [2] Soybean is a superior vegetable protein source to other legumes due to its high concentration of protein and low carbohydrate level.

When contrasted to other nutritious foods, tempeh has a variety of health benefits. Several researches suggest that tempeh contains antioxidants that are important to save the body by the action of the radicals (free). Further research defines that it could also treat chronic diarrhea in children due to the presence of antimicrobial chemicals that limit the growth of *Salmonella typhi*, *E. coli*. [25]

2.3 Zearalenone metabolites in Tempeh:

The contamination with the toxins of a raw material along with the actual formation of the toxins in the process of fermentation is related to the food safety of the product. In the case of tempeh, fermentation is basically done by the using of strains which are fungal. These fungal strains are of genera named as Rhizopus where no formation of mycotoxin is visualized. But also, there are some strains of Rhizopus which forms the bacteria which is producing the endosymbiotic toxin. The particular strain selection for the industrial purposes of tempeh formation is demanding. [9]. *Aspergillus oryzae* is a fungal strain can also stop the growth of the Mycotoxins in a minor way [4]. Some raw materials are detected to have mycotoxins in some cases. There are a number of studies showing that the maize could be greatly contaminated with the estrogenic predominantly named as Fusarium mycotoxin zearalenone which is known as ZEN [17].

There is an unequal distribution of ZEN in the several parts of grain and the high concentration of this toxin was detected from the by-products by cleaning the hulls as compared to clean cereal grain [11]. There are also severe effects of these toxins observed, which are caused by the ZFN hormone like structure. This has an interaction with estrogen receptors, which ultimately causes the disorder of hormones There are some studies which have shown that by the exposure of ZFN there is the association of the premature children development[5]. The results have shown that this toxin is immensely changed into several metabolites like zearalenol, zealrone-16-glucoside, and ZEL sulfate, as shown in Figure 1.

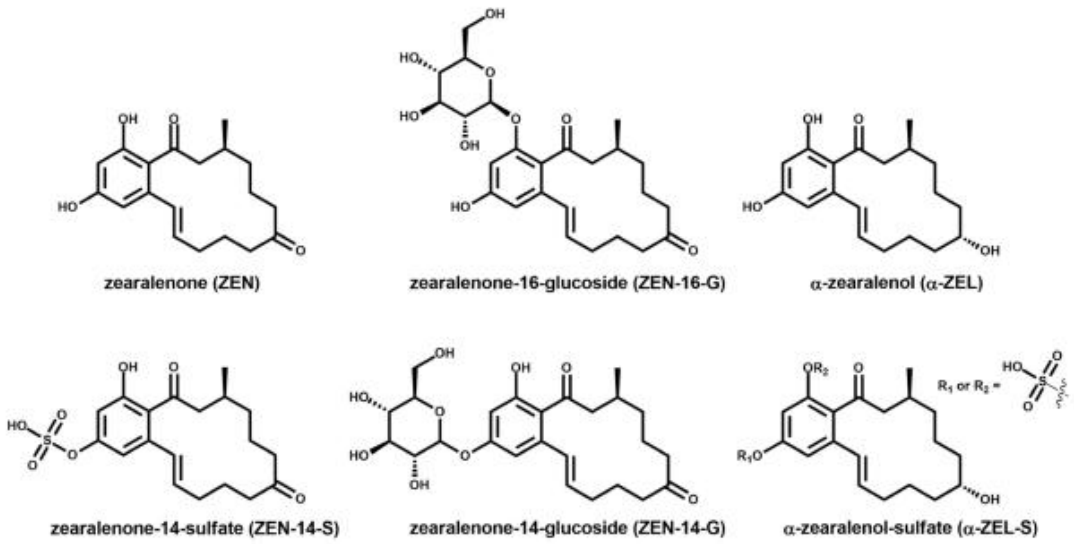


Figure 1: Structures of zearalenone and metabolites of zearalenone. [16]

2.4. Enhancement of vitamin B12 tempeh made by lupin:

Tempeh is a healthy, high in protein; affordable soy food that is globally accepted made by using fermented beans, mainly soybeans. Lots of studies has been conducted since early 1900 to till present time suggesting tempeh fermentation as a health beneficial, cheap and sustainable food processing technology to produce rich in protein by using various raw materials like soybeans, legumes and grains. Beside a comprehensive study that has been conducted further studies are needed on modification on tempeh fermentation technology and its effect on various diseases related to human health. The aim of this research is to increase the quantity of vitamin B12 in the tempeh made by the lupin by the help of biosynthesis which was bacterial by the use of bacterium P of good grade [34]. As a result this study aims to determine the standard conditions of fermentation for the vitamin B12 enhancement in the tempeh of lupin which is made by the fermentation of mixed culture. Lupin is also considered as an alternative for the proteins.

Propionibacterium freudenreichii is which is producing vitamin B12 and it is a food grade bacteria. *Rhizopus oryzae* is also used as a co-culture for the production of the vitamin B12 contained tempeh made by lupin. A particular increase in the protein was being observed but the other properties like the texture, flavor was not being changed. As a result vitamin B12 enriched lupin tempeh was produced [33].

2.5 Fermentation of tempeh by *Lactobacillus plantarum*:

Due to the increase in the intake of the foods which are containing the high fat is directly connected to the ubiquity of obesity as well as the syndromes which are metabolically inactive along with abnormal. There is a fungal strain *R. oligosporus*, belonging to the family of Mucoraceae, which is most commonly used as a starter inoculum for the tempeh formation. This fungal strain *R.Oligosporus* inhibits the growth of other microorganisms which create or form the mycotoxins, it actually grows well with the LAB which is the lactic acid bacteria. This bacteria can perform the production of β-glycosidase. This enzyme is responsible for the catalysis of the hydrolysation into aglycones of glucoside isoflavones having more bioavailability.This results in the growth of a fermented food with the inoculating of *R.oligosporus* along with the *Lactobacillus plantarum*. This is really a good and practical approach for the increasing of the tempehs bioactivity. This study is all about the betterment effects created by the *L.plantarum* in the fermented food tempeh made by soya beans on a particular diet which is containing high fat as well as abnormal metabolism of carbohydrates. This was further induced in the hyperglycemic variety of the rats and further it was evaluated [32].Then there was the incubation process done for both of the microorganisms, which were *R.oligosporus* and *lactobacillus* simultaneously with each other.

This process decreased the evaluation of the homeostatic model of the resistance from the insulin, cholesterol, lipoproteins having the low density along with the triglycerides. This actually increased the lipoprotein quantity in the high density of the rats which are HFD. Therefore as a result there was a gradual increase in the lactic acid bacteria number along with some other components like the bile juice, fatty acids having the short chain quantity in the feces of those rats which are HFD, triglycerides and cholesterol. The results revealed the regulation of the levels of lipid and the serum glucose levels as well by the help of the lactic acid bacteria by the editing of the microbiota in the internal manner. This resulted in the decrease in the synthesis of the cholesterol and the increase of the lipolysis as well. The fermented food tempeh which was produced from the two microorganisms, lactic acid bacteria along with the *R. oligosporus* can be proved as a beneficial dietary supplement for the population and especially for those people whose metabolism of carbohydrates is inactive or abnormal [29]

2.6 Production of Vitamin B12 in soybean fermentation:

Maximum studies have shown that there is the production of vitamin B12 in tempeh. This production is due to those bacteria which causes contamination during the fermentation by fungus. These bacteria are *Citrobacter freundii* and *Klebsiella*. In this article, the fermentation was done as usual. The soybeans were washed properly. Then they were soaked for the whole night, then their dehulling was done proceeding with the sterilization at 100 degree Celsius by boiling for half an hour.

The three starter cultures were taken, named as *Klebsiella*, *Saccharomyces cerevisiae* and the *Rhizopus oligosporus* as 1103 CFUg −1. Then these three inoculums were inoculated in the different samples of soya beans.Then these soybeans which were inoculated with the different cultures were given incubation of 30 degree celsius for 36 to 40 hours [20]**.**

Then the growth of the *R.oligosporus*, *Klebsiella* was observed in a fully grown tempeh along with this, the vitamin B12 production was also evaluated.After this, the isoflavone aglycones were also observed.

Every starter culture was able to hydrolyse all the starter cultures where components named as the genistein and the daidzein as well.After this it was observed that the quantity of the daidzein and the ganistin as well was almost doubled or tripled, particularly when the bacterial species *Klebsiella* was used as a starter culture in the soyabean for the process of fermentation. This study revealed that the *Saccharomyces cerevisiae* helps in the vitamin B12 production whereas the bacterial species *Klebsiella* helps in the genistein and daidzein production in the process of the fermentation of the soya beans for the production of tempeh. [20].

2.7 Microorganisms role in the production of tempeh:

There are some complex communities of microbes which help in the colonization of the soybean tempeh and its development as well as growth initiated when the raw materials are being soaked [25].There are several stages in the tempeh fermentation in which these microbes are involved significantly. Basically microbes perform the acidification of the soybeans during the stage of soaking. There is a chance of contamination in a cross manner. This contamination can occur during the processing of the tempeh, like the handling of tempeh during its soaking or packing or may be drying.

There may be a case of contamination during the inoculation of the starter culture or may be the inoculum composition may be affected. There are several microorganisms which are used for formation of the tempeh as bacteria as well as molds. Basically the mycelium of the molds is very important inoculum for the tempeh production and they are considered as a tremendous source of fermentation as they do production in a very efficient manner when the cotyledons of the soya beans are brought along with the mycelia of the molds. This fermented food product is actually produced in a very small scale in the industry of home where the fermentation is controlled very poorly.

The process of the fermentation is unmanagedly performed under these conditions which are aseptic by the help of inoculum which contains the fungus and this fungus is added at the starting of the process of fermentation. There are a number of organisms which perform the fermentation of the soybeans and form the tempeh. [16]. Due to the use of various organisms there is the change in the quality as well as the flavor of the tempeh in the country Indonesia. The most important fungal strains for the production of the tempeh are the *R. oligosporus*, *R. oryzae* and *R. stolonifer* with *R. arrhizus* as well. The fungal strain *Rhizopus* is required for the various processes in the fermentation which are the increasing of the nutrients in the soybeans like fats, carbohydrates and the proteins which are soluble as well [18]. But every one of the species the tempeh or performs the fermentation of a different quality as well as different flavor. If the tempeh produced with the help of the *Rhizopus oligosporus* strain of the fungus then the concentration of the amino acids which are free is the highest, as compared with the tempeh produced with the help of the starter culture of the *Rhizopus oryzae* and the *Rhizopus stolonifer* as well [22].

This species *Rhizopus* produces that enzyme which is detected to degrade the substrates of the soya beans which are its carbohydrates namely the arabinose, polygalacturonase, endocellulase and the xylase as well but not the proteases and lipases are able to be degraded by this fungal species [25]. According to the studies, the tempeh fermentation with the help of the single culture which is *Rhizopus oligosporus*, the content of raffinose is made less by 60% and the content of the stachyose by 10%. [26].

2.8 Molds and Bacteria viability in Tempeh made with carbon dioxide which is supercritical:

There is the presence of carbon dioxide which is supercritical or the formation of the food product. This actually has a result on the inactivation of microbes. Its quality is greatly affected from the fungal growth of due to the zero involvement of heat due to the importance of the production of the texture which is compact and stiff in nature, along white in color along with the properties which are functional along with the consumer acceptance.[5] This research deals with the observation of the molds, life of the molds in this fermented food. After the processing with the carbon dioxide as well as the detection of the production conditions which are best for the maintenance of the growth of mold and the reduction of the microorganisms in the fermented food tempeh. Results of this study revealed that there were particular reactions or interactions among the incubation and the pressure. This resulted in the revealing of the reactions with the reduction in the number of bacteria and the molds increasing [26]. This increased the period of incubation for the viability of bacteria as well as mold. Reducing the number of bacteria as well as increased with the incubation period which is long. Detecting the molds was not possible after twenty minutes with the carbon dioxide being critical or supercritical.

The bacteria were particularly reduced to some colony forming units per gram. Secondly the best technique for the production of the foods was the use of carbon dioxide which was supercritical for ten minutes. The molds were grown at a good rate by the treatment of the carbon dioxide while they were stored at thirty degree Celsius. Along with this there was a production of the mycelia which was white in color. This also increased the value of color as well as the acceptability of tempeh. When the mold was inactivated, this reaction was actually reversible. This caused it to grow again while storing in the particular criteria [20].

The composition of the matrix of the tempeh could give protection from the severe effects of the carbon dioxide which was supercritical.It was observed that more resistance was seen in the bacteria which were gram positive ones as compared to those bacteria which were gram negative ones.

Concludingly, the carbon dioxide which was super critical could perform as the technique of pasteurization which was cold type for the tempeh. This method could be taken as a new technique for tempeh preservation [20].

2.9 Analysis of Nutritional Contents Tempeh:

To make Tempeh, soybeans are fermented using a fungus called Rhizopus oligosporus or Tempeh starter. To increase the health benefits of tempeh various studies have been conducted. One study suggests use of *S. cerevisiae* as an inoculum along with *R. oligosporus* during the fermentation process. Results indicated that antimicrobial activity against *E. coli* and a very high amount of β-glucan content was observed in mixed inoculum culture rather than single inoculum. This β-glucan has many health benefits such as anticancer, anti-diabetic and antihypercholesterolemic which minimize the problem of other diseases of body. [5]

3. Material and methods:

Materials:

Magnesium nitrate, Hydrochloric acid, Hydroxylamine hydrochloride solution, alpha, alpha-dipyridyl solution, Ferric nitrate, orthophenanthroline, Follins reagent, Bovine serum albumin, Bradford reagent, Lactophenol cotton blue.

Methods:

3.1. To prepare standard TEMPEH using soybean and starter culture

Procedure:

1. Raw, dry soybeans are hydrated by soaking in cold water overnight or for a shorter length of time if warm water is used, according to [16]. The quantity of the soybeans and the starter culture is shown in Table 1.

2. To reduce the pH of the solution and soybeans to 5 or below, lactic acid or acetic acid is added to the water. This process is done to prevent the growth of unwanted bacteria.

3. The acidic atmosphere has little effect on the Rhizopus mold. Mold growth rate was shown to be steady when the pH was at or above 3.5, but was somewhat slower when the beans were more acidic.

4. After hydration, the skins on each bean are removed to allow the mold to grow more easily. This stage is completed with the beans rubbing, manually with the help of hands. After that, the beans are in their peeled stage and they are boiled for 90 min in the water at 100°C.

5. Then the beans are supposed to cool at 37-38 °C. This is the accurate temperature for the inoculating of starter culture. After this there is the drying of soya beans then the *Rhizopus* starting culture is added to the beans.

6. The culture, or inoculums, must be present in sufficient quantities. Incubation is to be given at 28°C [16]. The flow chart for the protocol of tempeh production is shown in Figure 2.

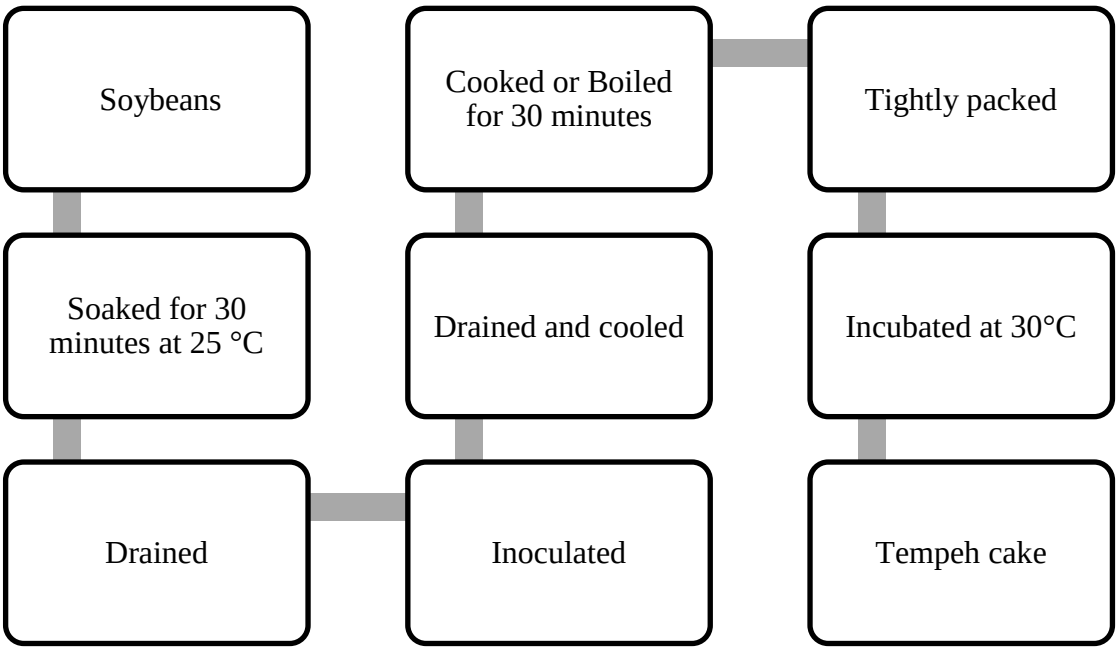


Figure 2: Flow chart for the protocol of tempeh production.

Table 1: Composition of ingredients for the production of tempeh (Standard).

S. No.	**Constituent**	**Quantity**
1	Soya Beans	500g
2	Starter Culture	1g

3.2. To perform the staining of tempeh by lactophenol cotton blue.

3.2.1: Procedure:

1. A drop of 70% ethanol onto a microscope slide was added.

2. The culture was inoculated in the alcohol drop.

3. Before the alcohol dried off, one or two drops of lactophenol cotton blue stain was added.

4. The coverslip was held between the forefinger and thumb, and carefully lowered one side of the drop of mountant with the coverslip edge, avoiding air bubbles. [6]

3.3. To prepare tempeh using spinach and beetroot for the estimation of iron content.

3.3.1: Procedure for beetroot supplemented tempeh:

1. Step 1, 2 and 3 are repeated the same as procedure 3.1. The quantity of the soybeans, beetroot and the starter culture is shown in Table 2.

2. After the dehulling, the beans are boiled with the beetroot extract for 30 minutes and then cooled down.

3. Dry the beans. Then add starting culture on the beans. The inoculated beans are subjected to incubation.

Table 2: Composition of ingredients for the production of beetroot supplemented tempeh.

S. No.	Constituent	Quantity
1	Soya Beans	500g
2	Starter Culture	1g
3	Beetroot	250 g

3.3.2: Procedure for spinach supplemented tempeh:

1. Step 1, 2 and 3 are repeated the same as procedure 3.1. The quantity of the soybeans and the starter culture along with the incubation conditions is shown in Table 3.

2. After the dehulling, the beans are boiled with the spinach extract for 30 minutes and then cooled down.

3. After the beans have cooled and dried. Then the starting culture is added over beans. These inoculated beans are subjected to incubation at 28°C.

Table 3: Composition of ingredients for the production of spinach supplemented tempeh.

S. No.	Constituent	Quantity
1	Soya Beans	500g
2	Starter Culture	1g
3	Spinach	250g

3.3.3: Estimation of iron content:

Solutions preparation:

(a) Solution of magnesium nitrate (50 percent w/v)

50 g Mg $(NO_3)^2.6H_2O$ dissolved in water, diluted to 100 mL in water

b) Hydrochloric acid in concentrated form

(10 percent w/v)

c) Hydroxylamine hydrochloride solution

Dilution of 10 g H_2 N OH. HCl in 100 ml water was done.

(d) Acetate buffer solution

8.3g Sodium acetate in anhydrous form was dissolved in water, and then 12 ml glacial acetic acid was added, and the solution was diluted to 100 ml.

e) (0.1 percent w/v) alpha, alpha-dipyridyl solution

100 ml of water with 0.1 g alpha, alpha-dipyridyl was diluted and its dilution was performed up to 100 ml.

f) (0.01 mg Fe/ml) iron standard solution

(i) 0.3512 g Fe $(NH_4)^2$ $(SO_4)^2.6H_2O$ was dissolved in water and then diluted to 100 mL with 2 drops of conc. HCl.

(ii) Dilution 5 mL of solution (f) in 250 mL with water.

(g) (0.1 percent w/v) orthophenanthroline solution

At 80 °C, 0.1 g of o-Phenanthroline in 80 mL of water was dissolved, then chilled, and then diluted to 100 mL with water. This solution was kept in a cool, dark location.

Sample preparation:

1. 2.5 grams amount of properly homogenized sample of tempeh was taken in a crucible.
2. The sample was dried in a water bath as it contained extra water. The sample was heated on a crucible in a muffle furnace at 450°C for 6 h until all volatile materials have escaped and the smoke has stopped.
3. The dish was placed in muffle furnace at temperature 450°C.
4. The process was continued to 6 h at 450°C until the ash was nearly carbon-free
5. 5 mL of concentrated HCl was poured into the dish, After this the upper portion of the dish was rinsed. Put the dish in waterbath and allow to evaporate.
6. Accurately 2 mL of concentrated HCl addition was done. Heat the dish in a water bath for 5 min.
7. Rinse the sample with water and pour in a flask, cooled.

Iron Determination:

1. Pipette 10 mL solution in a flask and add 1mL solution of hydroxylamine hydrochloride.

2. After 5 minutes, 5mL of buffer solution was added. Then 2mL of dipyridyl solution was added.

3. The solution's absorbance at 510 nm was determined.

4. Using the absorbance data, calculation of the Fe concentration of an aliquot of ash solution [22]

3.4: To prepare tempeh supplemented with kidney beans for estimation of protein content.

3.4.1: Procedure for fermentation:

1. Step 1, 2 and 3 are repeated the same as procedure 3.1. The quantity of the soybeans along with the kidney beans and the starter culture is shown in Table 3.

2. After the dehulling, the beans are boiled with the water for 30 minutes and then cooled down.

3. After the beans have cooled and dried. Then starting culture is added. The inoculated beans are subjected to incubation at 28°C for 48 hours.

Table 4: Composition of ingredients for the production of kidney beans supplemented tempeh.

S. No.	Constituent	Quantity
1	Soya Beans	500g
2	Starter Culture	1g
3	Kidney Beans	250g

3.4.2: Procedure for quantification of protein content:

Protein Extraction:

1. 100 mg of sample was diluted in 8 mL distilled water. Then homogenization was done with an electric blender. After this incubation was done for 24 hours at 60°C.

2. The samples were centrifuged for 15 minutes at 4000 g at 4 C. The pellet was re-dissolved in 8 mL of the solvent and incubated for 24 hours at 23 or 60 °C.

3. During both incubations, the samples were shaken constantly. After that, the samples were centrifuged for 15 minutes at 4000 rpm at 4°C. Supernatants were mixed, amino acid analysis was performed on the final extracts. The flowchart of this procedure is shown in Figure 3.

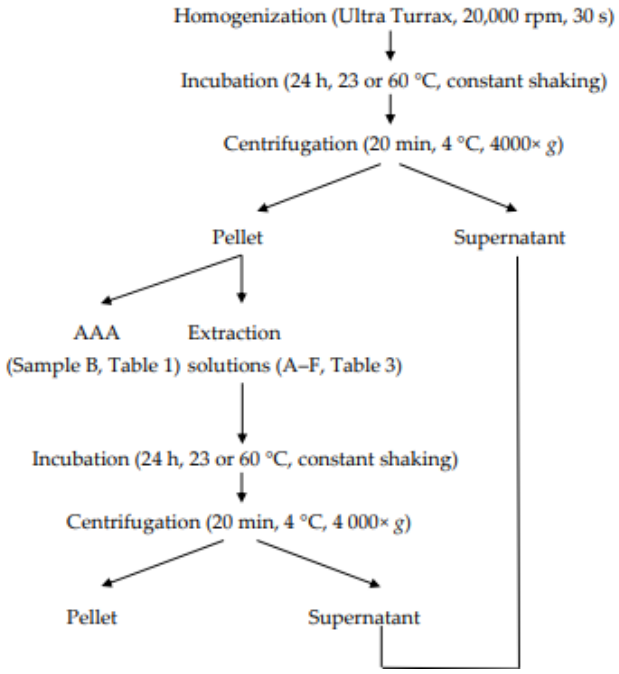


Figure 3: Process of extraction of proteins [15].

Enzymatic Pre-Treatment:

1. With few adjustments, the enzyme pretreatment was carried out according to [Harnedy and Gerald 2016].

2. 1 g of tempeh was dissolved in 28 mL of 0.05 M sodium acetate buffer. Then the homogenization was done for 30 seconds, and incubated at 40°C in a shaker for 30 min.

3. The sample was then added to a 2 mL sodium acetate buffer then incubation was done at 40°C for 18 h.

4. Samples were then centrifuged for 15 minutes at 4000 g at 4°C.Discard the supernatant and pipette out the pellet.

5. After this, pellets were dissolved in 8 mL 0.1 M NaOH, incubated for 24 hours at 60 °C in an incubator shaker. Then centrifugation was done at 4000 rpm for 15 min at 4 °C.

Bradford Test:

1. 0.1 mg/ml stock solution of BSA, the standard was made.

2. In deionized water, the unknown samples were diluted at 5-50 g/ml. The value should be within the absorbance reader's linear range.

3. Dilution of the Bradford reagent in 2.5 times in deionized water was there.

4. Duplicate wells were filled with 0 BSA stock solutions for creating the 0-5 g BSA calibration curve.

5. In triplicates, add the sample to distinct wells.

6. Each well was filled with diluted Bradford reagent.

7. At least 5 minutes for color development were allowed, but no more than 60 minutes.

8. Deionized water was used as the blank.

9. The absorbance was carried at 590 nanometers.

3.5. 4. To prepare tempeh supplemented with cow milk.

1. Step 1, 2 and 3 are repeated the same as procedure 3. Incubation temperature was 28°C for 36 hours and the quantity of the soybeans along with the kidney beans and the starter culture is shown in Table 5.

2. After the dehulling, the beans are boiled with the cow milk for 30 minutes and then cooled down.

3. Dry the beans. Then add starting culture on the beans. The inoculated beans are subjected to incubation.

Table 5: Composition of ingredients for the production of cow milk supplemented tempeh.

S. No.	Constituent	Quantity
1	Soya Beans	500g
2	Starter Culture	1g
3	Milk	1L

3.6: To determine the total phenolic content of tempeh in the different supplements.

3.6.1 Calibration Curve Preparation of Standard Gallic Acid:

1. The total phenolic contents (TPC) of the fruits, seeds, and bark extracts were determined using the Folin–Ciocalteu colorimetric method reported by [7] with some modifications.

2. Gallic acid standard solution was made by dissolving 10 mg in 10 mL methanol (1 mg/mL).

3. From the standard solution, several concentrations of gallic acid solutions in methanol (25, 50, 75, and 100 g/mL) were produced.

4. To make a final volume of 10 mL, 5 mL of 10% Folin–Ciocalteu reagent (FCR) and 4 mL of 7% Na_2CO_3 were added to each concentration.

5. The blue-colored mixture was thoroughly mixed before being incubated at 40°C for 30 minutes in a water bath. The absorbance was measured at 760 nm against a blank.

6. The FCR reagent does the oxidation phenols , producing a dark blue color that may be detected with a UV spectrophotometer.

7. All of the tests were done in triplicate, and the calibration curve was plotted using the average absorbance values obtained at various doses of gallic acid.

3.6.2 Determination of Total Phenolic Components.

1. Folin Ciocalteu technique was used for the detection of Total phenolic compounds in the different alternatives of the tempeh prepared.
2. 10 g tempeh was cut into small pieces. 20 mL hot water addition was there and then the homogenization of mixture was done.
3. The sample was then heated and filtered through a filter cloth.
4. An aliquot of 80mL was combined with 200mL of 0.25 Folins reagent.
5. Add 1 mL of Na_2CO_3 solution in and let the sample sit for 2 hours before detecting the absorbance was detected at 725 nm.
6. Samples were examined three times, with the average of the results calculated.

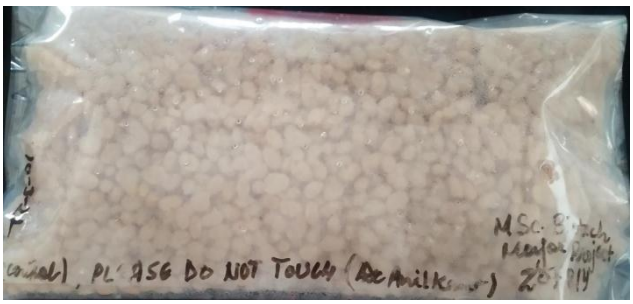
4. Results and Discussion:

4.1: To prepare standard Tempeh using soybean and starter culture

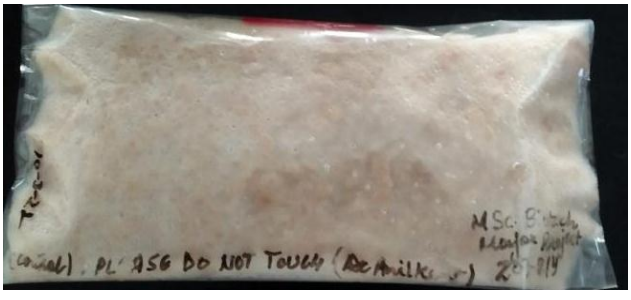
The standard tempeh started showing growth of the fungi and the soya beans started to combine with each other by the fungus on Day 2. On day 3 there was the formation of a compact structure which was cake-like. After that, the tempeh was cooked with the Indian flavors.

A.

Day 1



Day 2



Day 3

B.

Cake like formation

Cooked tempeh

Figure 4: Progression of fungal growth and formation of tempeh after different intervals of incubation [Standard tempeh].

The growth was observed after 24 hours. Some spores of the *R. oligosporus* were visualized and the separate soya beans started to form a whole structure. Then on the day third, after 36 hours the inoculated beans were visualized. There was a formation of a full cake-like compact structure and that structure was tempeh as shown in figure 4[A]. The proper compact growth of tempeh was visualized after 36 hours which is similar to the prior studies [19]. The observed structure was actually hard, but can be cut into the thin or thick slices. The cooking time for tempeh was 10 minutes, which gave it a good fragrance along with the good shape and texture as well as shown in figure 4[B]. The raw texture of tempeh was different as it was hard but when it was cooked, its texture became soft.

4.2: To perform the staining of tempeh by lactophenol cotton blue.

When the inoculated fungus was stained with lactophenol cotton blue stain. Then visualization was done. Then it was visualized that the fungal spores were having a large diameter along with this there was the detection of the irregularity in the shape and size of the spores. The spores were highly differentiated on the basis of their volume.

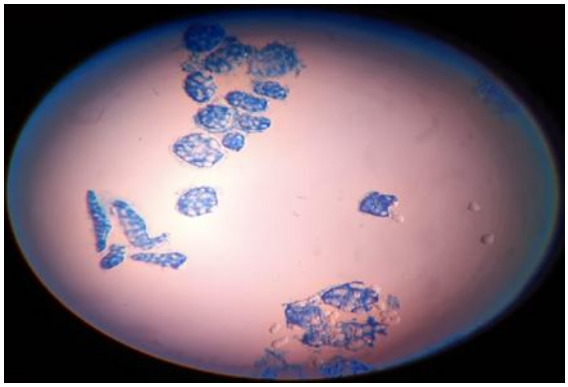


Figure 5: Microscopic view of *Rhizopus oligosporus* stained with Lactophenol cotton blue.

According to the prior studies, the spores of the *R.oligosporus* are very irregular in shape, they have very large and unequal diameter. Along with this, there is a huge difference in the size of the spores [30]. This is quite similar to our study, in which there is the inequality in the sizes of spores. Along with this the shape and the diameter of the spores is also irregular and unequal as shown in Figure 5.

4.3 To prepare tempeh using spinach and beetroot for the estimation of iron content.

4.3.1: Progression of fungal growth of beetroot supplemented tempeh:

Day 1

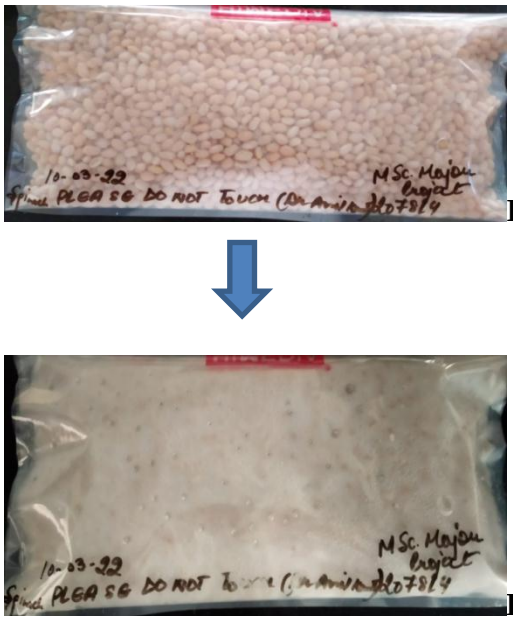
Day 3

Figure 6: Progression of fungal growth of tempeh supplemented with beetroot extracts.

After 24 hours, no fungal growth was observed in the beans, the beans did not show any compactness. On day 3, after 36 hours there was mild growth of the fungus observed and the beans started forming a cake-like structure in some texture.

Then after 48 hours, there was the formation of a proper and a compact cake like structure. The structure was soft as compared to the standard tempeh and here it took 24 hours more than standard tempeh. This beetroot extract treated tempeh was totally different on the basis of color and texture form the standard tempeh. This tempeh was having slightly pinkish orange in color and had a little softness as shown in figure 6.

4.3.2: Progression of fungal growth of spinach supplemented tempeh:



Day 1

Day 3

Figure 7: Fungal growth of tempeh supplemented with spinach.

It was observed that after 24 hours, no fungal growth was seen in the beans, the beans did not show any compactness. After 36 hours there was very mild growth of the fungus observed and the beans started forming a cake-like structure with little texture. Then after 48 hours, day 4 there was a mild growth of fungus observed. Then on day 5 there was a formation of a proper and a compact cake like structure.

The structure was a little hard as compared to the standard tempeh and here it took 36 hours more than standard tempeh. This spinach extract treated tempeh was totally different on the basis of color and texture form the standard tempeh. This tempeh was having a slight dark color and had a little hardness as shown in figure 7.

4.3.3: Quantification of Iron content:

Total iron concentration of the tempeh was calculated from the regression equation of calibration curve (y= 3.7777x + 0.067 and R2 = 0.9982) and expressed as mg Ferric nitrate equivalents per gram of sample in dry weight (mg/g). The total iron content of all the three treatments varied between 0.480mg/g to 0.541mg/g as shown in Table 7. Total iron content was detected in the control or the standard tempeh and the tempeh made with the treatment of spinach and beetroot. Total iron content was observed highest for the tempeh which was treated with the beetroot, followed by the tempeh which was treated with the spinach as compared with the standard tempeh. The absorbance for different concentrations of Ferric Nitrate was detected, as shown in Table 6 and the standard graph was plotted against the concentration and absorbance for ferric nitrate, in figure 8.

Table 6: Absorbance of Ferric Nitrate (Standard).

S.No	**Concentration**(mg/ml)	**Absorbance** (λmax= 510 nm)
1	0.0	0.172
2	0.2	0.152
3	0.4	0.205
4	0.6	0.297
5	0.8	0.361
6	0.12	0.514
7	0.16	0.681

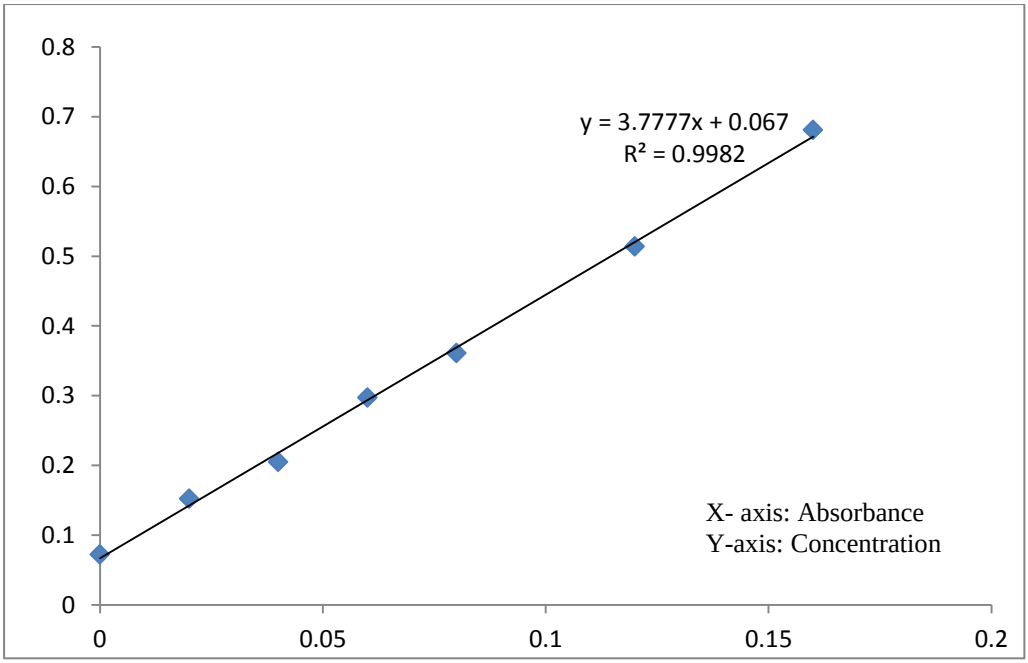


Figure 8: Standard curve for Ferric nitrate (TIC).

Table 7: Concentration of total iron content (TIC) in different supplements of Tempeh.

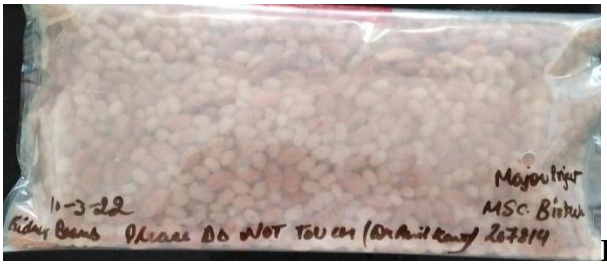
S.No.	Sample	TIC(mg/g)
1.	Control	0.480
2.	Beetroot	0.541
3.	Spinach	0.496

It was observed that the total iron content in the standard tempeh was 0.480mg/g of tempeh, the tempeh which was treated with the beetroot contained 0.541mg/g and the tempeh which was supplemented with spinach contained 0.496mg/g as shown in Table 7. So the treatment of the beetroot and the spinach in the tempeh will increase the tempeh iron content.

In the previous studies the total iron content in the raw tempeh was 0.67 mg/g of tempeh [28].So it could be concluded that the tempeh supplemented with spinach and beetroot contains an equivalent content of iron as compared to the standard tempeh prepared in the prior studies.

4.4: To prepare tempeh supplemented with kidney beans and estimation of protein content

4.4.1 Progression of fungal growth of tempeh supplemented with kidney beans:



Day 1

Day 3

Figure 9: Progression of fungal growth of tempeh supplemented with kidney beans.

The growth was observed after 24 hours. After 24 hours, no fungal growth was observed in the beans, the beans did not show any compactness. On day 3, after 36 hours there was very mild growth of the fungus observed and the beans started forming a cake-like structure with proper texture as shown in figure 9. Then on day 5 there was a formation of a proper and a compact cake like structure.

But as in the prior studies the growth of the tempeh made with the red kidney beans has shown the proper growth along with the compact cake like structure in 48 hours. The growth of tempeh in our studies is 24 hours late, as compared to the prior studies done. [13]

4.4.2 Quantification of Proteins:

Total protein content of the tempeh was calculated from the regression equation of calibration curve ($y=0.9182x + 0.7288$ and $R^2= 0.9893$) and expressed as mg BSA bovine serum albumin equivalents per gram of sample in dry weight (mg/L) as shown in Figure 9. The absorbance of BSA standard for the concentration is shown in Table 8.The total protein content of all the two treatments varied between 0.56mg/g to 0.90mg/g as shown in Table 9. Total protein values were detected in the control or the standard tempeh and the tempeh made in the half proportion of the kidney beans. Total protein value was observed higher for the kidney bean and soy tempeh as compared with the standard tempeh.

Table 8: Absorbance of BSA standard.

S.No.	**Concentration (mg/ml)**	**Absorbance (λmax= 590nm)**
1	0	0.02
2	0.2	0.974
3	0.4	1.278
4	0.6	1.472
5	0.8	1.643
6	1	1.869

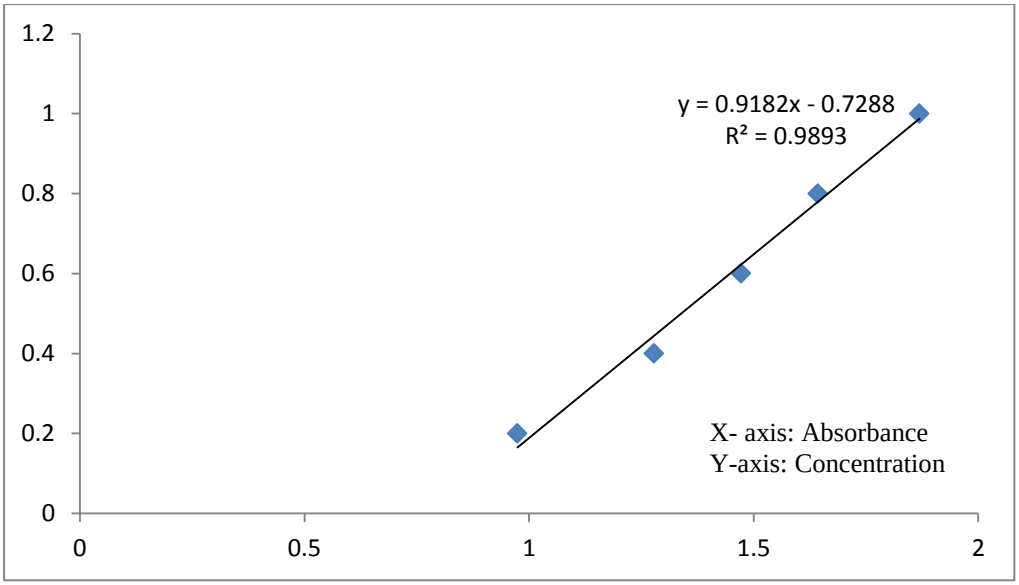


Figure 10: Standard curve for BSA for total protein estimation.

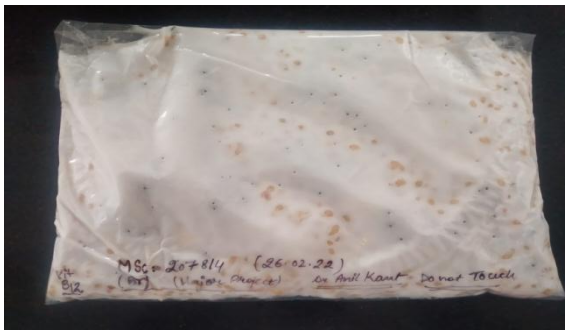
Table 9: Total protein content in different samples of tempeh.

S.No.	**Sample**	**Total Protein Content** (mg/g)
1.	Control	0.56
2.	Kidney Beans	0.90

It was observed that the protein content was 0.56 mg/g in the standard tempeh and the addition of the kidney beans in the proportion of the soya beans increases the protein content as 0.90 mg/g as shown in Table 9. In the previous research, the protein content determination of tempeh gembus meatballs revealed a relationship between the inclusion of tempeh gembus in meatballs and protein content. Protein quantity in all different treatments varied considerably from controls and between treated groups. The protein content of tempeh gembus meatballs in the control group was 0.12 mg/g [27]. Both the protein values (of prior studies and our studies) are comparatively different.

4.5. To prepare tempeh supplemented with cow milk.

Day 1



Day 3

Figure 11: Progression of fungal growth in tempeh supplemented with cow milk.

The soya beans were boiled with cow milk for 30 minutes and then dehulled. The boiled and dehulled soybeans were inoculated with the starter culture of fungus, the soya bean was packed in a polyethene bag with the pinned holes over it. Then the packed beans were subjected to the incubation of 30 degree Celsius. After this, the growth was observed after 24 hours. Then on the day third, after 36 hours the inoculated beans were visualized as shown in Figure 11. There was a formation of a full cake-like compact structure and that structure was tempeh. This tempeh took the same amount of time to ferment as the time taken by standard tempeh. This cow milk extract treated tempeh was the same in structure and texture, when compared with the standard tempeh.

Table 10: Comparative day wise growth of standard tempeh and tempeh supplemented with different ingredients.

Day	Control	Spinach	Beetroot	Cow milk	Kidney Beans
1	**Nill**	**Nill**	**Nill**	**Nill**	**Nill**
2	Mild growth	No growth	No growth	Mild growth	No growth
3	Extreme growth	Very mild growth	Mild growth	Extreme growth	Very mild Growth
4	Cakelike formation	Mild growth	Cake like formation	Cake like formation	Mild Growth
5	Refrigerated	Cake like formation	Refrigerated	Refrigerated	Extreme Growth
6	Refrigerated At -80 °C	Refrigerated At -80 °C	Refrigerated At -80 °C	Refrigerated At -80 °C	Refrigerated At -80 °C

The full growth of tempeh was observed in the standard sample along with the tempeh treated with cow milk was visualized in 3 days. But the tempeh was treated with the beetroot, spinach and the kidney beans detected in 5 days. The observed structure of the standard tempeh was actually hard, but can be cut into the thin or thick slices. But the alternative treatments made the new fermented products with different texture and the shape as shown in Table 10.

4.6 To determine the total phenolic content of tempeh in different supplements.

Total phenolic content of the tempeh was calculated from the regression equation of calibration curve (y=0.0033x + 0.0775 and R2= 0.9864) and expressed as mg Gallic acid equivalents (GAE) per gram of sample in dry weight (µg/g) as shown in Figure 12. The total phenolic content of all the three treatments varied between 0.98µg/g to 1.62µg/g as shown in Table 12. TPC values were in the cow milk, kidney beans, beetroot and spinach. TPC value was observed highest for the cow milk treated tempeh, and the lowest was for the spinach treated tempeh.

Table 11: Absorbance of Standard (Gallic Acid)

S.No.	**Concentration** (mg/ml)	**Absorbance(** λmax= 760nm)
1	0	0.00
2	25	0.153
3	50	0.256
4	75	0.309
5	100	0.407

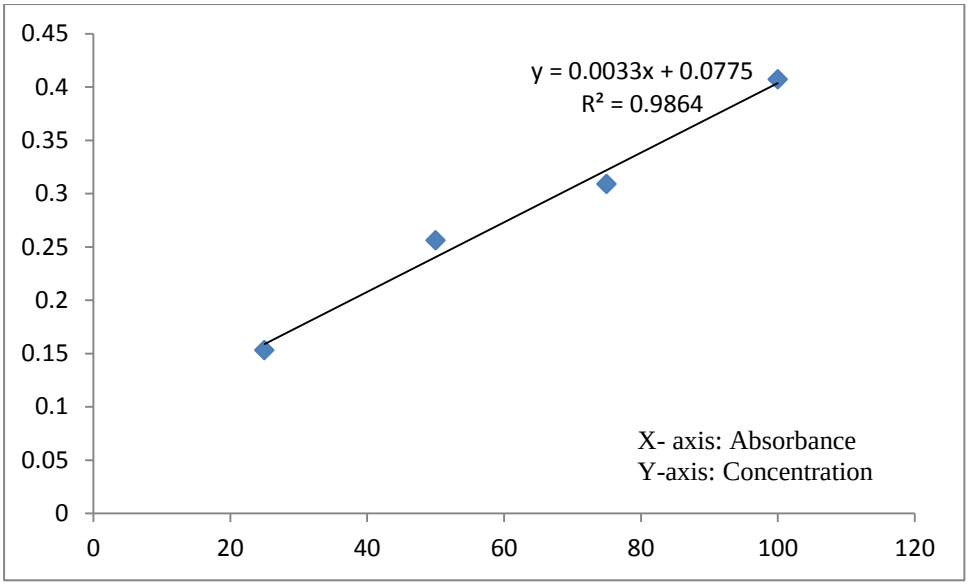


Figure 12: Standard curve of Gallic acid (TPC).

Table 12: TPC in different samples of Tempeh.

S.No.	**Tempeh treated with supplements**	**TPC**(µg/g)
1.	Control	1.09
2.	Kidney beans	1.29
3.	Beetroot	1.15
4.	Cow Milk	1.62
5.	Spinach	0.98

Total phenolic contents in different types of tempeh made by the extracts of beetroot, kidney beans, cow milk, spinach and kidney beans were determined by the Folin–Ciocalteu (F–C) method using gallic acid as the standard. The absorbance values obtained at different concentrations of gallic acid were used for the construction of the calibration curve. Therefore the Total phenolic content (TPC) was detected maximum in the tempeh which was treated with the cow milk as 1.62 µg/g of tempeh extract followed by the tempeh which was made in the proportion with kidney beans and then the beetroot treated tempeh. Lowest content of the TPC was observed in the spinach treated tempeh and the standard tempeh as 0.98 µg/g.

The total amount of the phenolic contents in several alternatives of tempeh made by the spinach, beetroot, kidney beans and cow milk extracts were detected by Folin Ciocalteu method by the use of gallic acid standard curve. Values of the absorbance detected in the several concentrations of the gallic acid were then used for formation of the calibration curve. The maximum TPC value was observed for the cow milk followed by the kidney beans, and the lowest was for the spinach extract fermented tempeh. But in the previous study, the tempeh extract had a lower total phenolic content of 6.58 μg/g of tempeh extract. [8]. These results are much higher as compared to our studies.

5. Summary:

Tempeh is an Indonesian food made using fermented beans (mostly soybeans). To make Tempeh, soybeans are fermented using a fungus called Rhizopus oligosporus or Tempeh starter. This fungus helps bind the soybeans together and gives it a cake-like structure. The three strains of *Rhizopus* can ferment soybeans and form tempeh. But the most commonly used is the *R. oligosporus* because this one strain remains maximum of the nutritional aspects of the soya beans and therefore enhances the digestibility for the proteins as well. The study aims the production of tempeh with the help of several plant and animal sources to enhance vitamins and minerals along with the testing that whether the nutritional aspects have increased or not.

The tempeh is fermented with the extracts of beetroot, spinach, cow milk and the kidney beans for the increase of iron and protein content. Then this was followed with the testing of phenol, iron and protein content in the different samples of tempeh. The results were as; the iron content of beetroot was higher than the standard tempeh prepared, the protein content was increased in the same amount which was made in the half of the proportion of the soya beans. The maximum TPC value was observed for the cow milk supplemented tempeh i.e. 1.09µg/g and the minimum was for the spinach supplemented tempeh i.e. 0.98µg/g. The maximum total iron concentration value was observed for beetroot supplemented tempeh i.e. 0.541mg/g and minimum was for spinach i.e. 0.496mg/g. The protein content of kidney beans i.e. 0.904g/g.

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