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Research article

Vitamin B_{12} production in soybean fermentation for tempeh

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Abstract: Most studies have found that vitamin B_{12} in tempeh is produced by contaminating bacteria specifically, *Klebsiella* sp. and *Citrobacter freundii*, during fungal fermentation. This study is to evaluate the effect of starter culture on the vitamin B_{12} and isoflavone aglicone content of soybean fermentation for tempeh production. In this study, soybeans were washed, soaked in water overnight, dehulled and sterilized by boiling at 100 ℃ for 30 min. Three starter cultures (10^3 CFU g^{-1}) namely *Rhizopus oligosporus*, *Klebsiella* sp. and *Saccharomyces cerevisiae* were then inoculated as follows, soybeans + *R. oligosporus* + *Klebsiella* sp. (SRK), soybeans + *R. oligosporus* + *S. cerevisiae* (SRSc), soybeans + *R. oligosporus* + *S. cerevisiae* + *Klebsiella* sp. (SRScK), and Soy + *R. oligosporus* (SR) and soybeans + *Klebsiella sp* (SK). Inoculated soybeans were then incubated at 30 ± 2 ℃ for 40 hours (*tempeh*-style). The growth of *Klebsiella* sp., *S. cerevisiae*, *R. oligosporus* and the production of vitamin B_{12} as well as isoflavone aglicones were observed. The results showed the highest vitamin B_{12} 3.15 mg 100 g^{-1} was found in tempeh SRSc, followed by 2.88 mg 100 g^{-1} and 1.64 mg 100 g^{-1} in tempeh SR and SRScK, respectively. In addition, vitamin B_{12} in tempeh SRK was the lowest (0.81 mg 100 g^{-1}). All the starter cultures were able to hydrolyze daidzin and genistin, but the amount of daidzein and genistein was tripled and doubled, respectively when *Klebsiella sp* was inoculated to the soybean fermentation. The study suggested that *S. cerevisiae* contributes to the production of vitamin B_{12}, while *Klebsiella sp* contributes to production of daidzein and genistein in soybean fermentation for tempeh production.

Keywords: tempeh of soybean fermentation; vitamin B_{12}; isoflavones; *Klebsiella* sp.; *S. cerevisiae*; *R. oligosporus*

1. Introduction

Vitamin B_{12}, also known as cobalamin, plays an important role in the functioning of the brain and nervous system, and in the formation of red blood cells. Vitamin B_{12}, occurring naturally in food, derives from bacterial synthesis and therefore occurs only in foods of animal origin. Small amounts can be found in plant products due to the presence of bacteria or due to microbial contamination. Plant food such as blue-green algae contains of pseudo vitamin B_{12} which is in active in humans. In foods, cobalamins are bound to proteins and glycoproteins and must be released by enzymatic and acid hydrolysis in the gut. Dairy foods, milk and milk products, eggs, meat and seafoods are the main natural sources in the diet. Tempeh, solid state boiled-dehulled soybean fermentation using starter culture of *Rhizopus oligosporus*, is rich of antioxidants and contains vitamin B_{12} [1,2]. Few scientific literatures mentioned the vitamin B_{12} values of tempeh. Areekul et al. [3] found between 0.18 and 4.1 Mcg vitamin B_{12} analogues per 100 g tempeh from tempeh sold in market in Jakarta, and tempeh sold in Toronto, Canada contained of 148 ng of vitamin B_{12} per g [4]. During the soybean fermentation, enzymatic activities of *R. oligosporus* leads to a significant increase in water-soluble nutrients, enhancing the biosynthesis of B vitamins and transformation of soy-isoflavones into antioxidant compounds. Soybean itself contains low or undetectable of vitamin B_{12}, yet when soybeans are fermented to produce tempeh, a considerable amount of vitamin B_{12} was found, about 0.7 to 0.8 μg/100 g [5]. It is known that vitamin B_{12} contained in tempeh is synthesized by *Klebsiella pneumoniae* and *Citrobacter freundii* [6]. These bacteria were thought as the contaminating bacteria during processing of tempeh making. With these values of vitamin B_{12}, tempeh may be as an additional diet of vitamin B_{12} from plant-derived food sources. The RDI of vitamin B_{12} for adults is set at 2.4 μg/day in Indonesia [7], and as well in The US and Japan (RDA) [8]. Recently, industrial production of vitamin B_{12} has been carried out through microbial fermentation, mostly use *Pseudomonas denitrificans, Propionibacterium shermanii* [9]; however, this process has some drawbacks such as a long fermentation process and expensive media requirements

Consumption of soy based foods may have health benefits due to their functional ingredients, especially isoflavones. In soybeans, isoflavones are in the form of isoflavone glycosides. The two most widely studied of isoflavones from soybeans are daidzin and genistin, which are not easily absorbed by the intestinal absorptive cells because of their large hydrophilic structures [10,11]. However, hydrolytic enzyme-producing microorganisms can hydrolyze isoflavone glucosides by the enzyme β-glucosidase to form of aglicones, daidzein and genistein which have antioxidant properties. Hydrolysis of isoflavone glycoside, and variation of individual isoflavone aglycones are influenced by microbial types [12–14] reported that bacteria are more effective in converting daidzin and genistin into their aglycone forms than molds. Aspergillus and Streptomyces isolated from soybean fermented foods are known as best producer of ortho-dihydroxyisoflavone (ODI), hydroxylated isoflavones which is produced during fermentation of soybeans. This study conducted to analyze the vitamin B_{12} and the isoflavone aglycone contents produced during soybean fermentation using co-inoculated of *R. oligosporus, S. cerevisiae*, and *Klebsiella sp* as starter cultures.

2. Materials and methods

2.1. Starter cultures and inoculum preparation

Starter cultures used in this study: *Rhizopus oligoporus was* isolated from tempeh, *Saccharomyces cerevisiae* isolated from commercial *Fermipan* dried yeast product, and *Klebsiella sp* non-pathogenic bacteria were isolated from the tempeh [15]. Except for *Klebsiella sp* which was cultured on nutrient agar and its stock culture was maintained at −20 ℃ in 20% glycerol, all the starter cultures were freshly prepared. For inoculums preparation, the bacteria and yeast were grown in nutrient broth at 37 and 32 ℃ respectively for 24 h. The cells were harvested and resuspended in sterile distilled water and properly adjusted to obtain a concentration of 10^5 and 10^5 CFU mL^{-1} respectively. For mold preparation, the mycelium was streaked on potatoes dextrose agar and grown at 27 ℃ for 120 h. The spores were harvested, resuspended in sterile distilled water and properly adjusted to obtain a concentration of 10^5 cell mL^{-1}.

The data obtained was analysed by the use of Excel Microsoft program 2016.

2.2. Tempe making

Soybeans in this research were purchased in the *Primkopti* Bandar Lampung. Tempeh was produced in a microbiology laboratory as follows; 300 g of soybeans were soaked in clean water overnight at room temperature, and then dehulled manually. Dehulled soybeans were boiled in clean water with a ratio of 1:3 (soybeans: water) for 30 minutes, drained, air dried at room temperature, and then 100g aseptically inoculated with the cultures at defined number of cells which was 10^5, 10^5, and 10^5 CFU $100g^{-1}$ for *R.oligosporus, S. cerevisiae*, and *Klebsiella sp*, respectively. Inoculated soybeans were packed in perforated plastic packaging and incubated at 32 ± 2 ℃ for 40 hours. Five types of fermented soybeans with the addition of different inoculated cultures were produced in this study, namely (1) soybeans + *R. oligosporus* + *Klebsiella sp* (SRK), soybeans + *R. oligosporus* + *S. cerevisiae* (SRSc), soybeans + *R. oligosporus* + *S. cerevisiae* + *Klebsiella sp*. (SRScK), and Soybean + *R. oligosporus* (SR) and soybeans + *Klebsiella sp*. (SK). Separate soybeans were also fermented as a control without inoculation. The experiments were made in three replication.

The measurement of pH was carried out with a pH meter model 501 (Crison, Barcelona, Spain). All samples were homogenized prior to pH measurements. Measurements were made three times for testing the accuracy then the mean was taken.

2.3. Microbial enumerations

Each of fermented soybeans was analyzed for its total number of bacteria, yeast and molds at the starting and the end of fermentation. A total of 15 g tempeh was taken, mixed with 135 mL of 0.1% peptone water, homogenized with a stomacher for 30 seconds, and a series of dilutions from 10^{-1} to 10^{-8} was made in duplicate. Then one mililiter is taken from certain dilutions for plating microorganisms done by spread plate method on EMB agar (BBL Microbiology Systems. Cockeysville, Md.), MEA (Difco), and PDA (Oxoid) for *Klebsiella sp*., *S. cerevisiae*, and *R. oligosporus*, respectively. Incubation of the plates was at 37, 27, 32 ℃ for *Klebsiella sp*., *S. cerevisiae*, and *R. oligosporus* respectively, for 24 to 48 hours.

2.4. Vitamin B12 analysis

The method of vitamin B_{12} analysis was performed according to the procedure run by Lawrence [16] with small modification on the application of UAE. Sample preparation method applies water ultrasonic assisted extraction (UAE), in which ultrasonic water extraction was used for extraction. A total of 0.5 grams of ground tempeh was weighed and place into 100 mL Erlenmeyer containing of 20 mL of milli-Q water. Sample was extracted by using ultrasonic equipped with heater for 30 minutes. Then, sample volume was set for 25 mL by adding milli-Q-water, and centrifuged at 3000 rpm for 10 min. Supernatant was pipetted using syringe equipped with filter holder so that 2 mL of supernatant was obtained, and then filtered using paper filter of 13 mm in diameter and 0.2 μm pore size. The filtrate was then placed in the vial bottle. The sample was ready to be injected into the HPLC (Shimadzu, CBM-20A controller, LC 20AD solvent delivering unit, CTO 10A column oven, SPD M20-A photo diode array detector). HPLC running condition was using Agilent C-18 5 um (125 × 4.6 mm) column, column temperature of 35 ℃, mobile phase (water: acetonitrile: buffer phosphate 10 nM = 80:10:10), isocratic mobile phase method with flow rate 1 mL min^{-1}, injection volume of 20 μL, wavelength detector 360 nm, and running time of 20 min.

Standard solution was prepared as following, weigh 5 mg of standard cyanocobalamin, dissolve it in 100 mL of milli-Q-water so that a concentration of 50 $\mu g.mL^{-1}$ is obtained. Then the standard was diluted into a working solution with a concentration of 0.5 $\mu g.mL^{-1}$, 1 $\mu g.mL^{-1}$, 2 $\mu g.mL^{-1}$, and 4 $\mu g.mL^{-1}$.

2.5. Daidzein and genistein analysis

For quantification of daidzein and genistein, fermented soybean samples were freeze-dried and stored at −20 ℃ until used. The extraction of isoflavones from fermented soybeans and unfermented soybeans, and their quantification by HPLC were assayed followed the procedures worked by El-Shazy et al. [17] with small modification and was assayed at the Centre Technology and Innovation Lab. Univ. of Lampung. HPLC-MWD instrument (Agilent 1200 series), Zorbax 5μm (4.6 mm × 150 mm) column, and UV detector (λmax = 254 nm). The mobile phase consisted of 100% methanol and 10 mmol L^{-1} of ammonium acetate buffer (60:40) containing 1 mL of trifluoro-acetic acid per liter of solvent mixture. This was set at a flow rate of 0.6 mL min^{-1}. Standards of daidzein and genistein were obtained from Sigma. 1gram of the freeze-dried sample was added to 10mL of 80% (v v^{-1}) aqueous methanol and extracted under agitation for 24 hours at room temperature. The homogenates were centrifugated at 15000rpm for 30min and the methanol obtained were used to determine daidzein and genistein concentrations. Injection volumes of isoflavone standards and of the samples were set at 100 μL throughout the run time of 30min. Single standards were also prepared for peak identification. Calculation of the isoflavone concentration was dry basis (μg g^{-1} fermented soybean).

3. Results and discussion

The number of *R.oligosporus, S. cerevisiae, and Klebsiella sp* inoculated into soybeans for tempeh production were 10^3, 10^5 and 10^3 cells g^{-1}, respectively. Table 1 shows that the addition of *S. cerevisiae* and *Klebsiella* sp. either together or separately did not affect the growth of *R. oligosporus*.

R oligosporus in tempeh is not a contaminant microorganism and instead it has to be deliberately added into the soybeans. It was possible that R. oligosporus played a role in supporting the growth of other microorganism in the soybeans fermentation, because during fermentation *R. oligosporus* produces several enzymes including lipase, protease, and phytase which hydrolyzes carbohydrate, lipid, and protein from the soybeans and produces fatty acids and amino acids which are then utilized by bacteria and yeast [18]. In addition, they also function as growth control of microorganisms in tempeh, preventing the tempeh consumer from diarrhea and flatulence. *R.oligosporus* produces antibiotics which actively kill certain bacteria in tempeh i.e. *Staphylococcus aureus, Bacillus subtilis, and Clostridium sp* which is known to produce gas in the intestines [19]. *S. cerevisiae* was able to grow together either with *R. oligosporus* or *Klebsiella sp*. during fermentation of tempeh making, it is possible to obtain carbon and nitrogen sources from soybeans as well as fatty acid and amino acids produced by *R. oligosporus*. Meanwhile, the wide pH tolerant and that Klebsiella is a bacterial contaminant in tempeh which may be the reason for the high number of Klebsiella found in tempeh. The pH of tempeh in this experiment was found between 6.60 to 7.40 (Tabel 2).

Table 1. The total number of microorganism in soy fermentation inoculated with various cultures.

Type of isolates in soy fermentation	Microbial number (CFU g^{-1}) at 0 h of fermentation			40 h of fermentation		
	Ro	Sc.	K	Ro	Sc.	K
SR	2.6×10^3	ND	ND	3.4×10^6	ND	2.0×10^8
SRSc	2.8×10^3	3.6×10^3	ND	4.5×10^6	1.2×10^6	1.1×10^8
SRScK	4.4×10^3	6.3×10^3	1.0×10^3	5.6×10^5	3.7×10^5	2.5×10^8
SRK	5.0×10^3	ND	2.7×10^3	3.0×10^5	ND	1.9×10^8
SK	ND	ND	2.1×10^3	ND	ND	2.7×10^8

Note: The data in the table were average value of three replications. ND not detected. SR is soy + *R. oligosporus,* SRSc is soy + *R. oligosporus* + *S. cerevisiae,* SRScK is soy + *R. oligopsorus* + *S. cerevisiae* + *Klebsiella sp,* SRK is soy + *R.oligosporus* + *Klebsiella sp,* SK is soy + *Klebsiella sp.* Ro *is R. oligosporus,* Sc *is S. cerevisiae,* K *is Klebiella sp.*

Biosynthesis of vitamin B_{12} is limited to some bacteria and archaea, and its production depends on type of microorganisms involved during fermentation. Soybeans only used as control of the tempeh production in this experiment contained very little vitamin B_{12} that it would not be of nutritional significance. This finding (0.36 mg$100g^{-1}$) was close with data reported by Liem et al. [4] and Areekul et al. [3], in which the amount was found to be 0.39 ng/g and 0.15 ng g^{-1}, respectively. *R. oligosporus* was an essential microorganism in the production of tempeh, but when *Klebsiella sp* was used as culture to soybean fermentation, tempeh did not produce (Figure 1). When *R. oligosporus* was as an inoculum in soybean fermentation it yielded vitamin B_{12} of about 2.88 mg $100g^{-1}$, *R. oligosporus* co-inoculated with *Klebsiella sp* yielded lower vitamin B_{12} (0.81 mg $100g^{-1}$). The first data (2.88 Mcg 100 g^{-1}) was close with the data reported by Liem et al. [4] which was 66.0 ng g^{-1}. The presence of *S. cerevisiae* during tempeh fermentation was beneficial from the nutritional point of view. When *S. cerevisiae* co-inoculated with *R. oligosporus*, a high vitamin B_{12} was produced (3.15 Mcg100 g^{-1}) in tempeh. *S. cerevisiae* is rich in cobalt and a vitamin B_{12} producer [20,21], which may contribute to an increase in vitamin B_{12} of the tempeh. In addition, *S. cerevisiae* co-inoculated with *R. oligosporus* in soybean fermentation produced tempeh that contain β-glucan [22] and a new aroma of yeasty which is preferred by some tempeh consumers [23]. Yeasty aroma of tempeh produced by

alcohol, ester, and aromatic group such as styrene, caryophyllene, phenol, and maltol during fermentation.

The recommended dietary allowance of vitamin B_{12} is 2.4 μg/day for adults. When the concentration of vitamin B_{12} in tempeh was 3.15 Mcg$100g^{-1}$, it becomes visible for the consumer to get his or her daily requirement of vitamin B_{12} by consumption of approximately 76.2 g of tempeh. The bioavailability of vitamin B_{12} in tempeh in this study was not investigated. Mo et al. [24] found the bioavailability of tempeh was 0.016–0.072 Mcg $100g^{-1}$ which is very low. This could be because they made tempeh from soybean fermentation with *Rhizopus microsporus var. microsporus* LU573 and did not use the vitamin B_{12} producing bacteria. However, Bor et al. [25] mentioned that a daily vitamin B_{12} intake of 6 Mcg is sufficient to maintain a steady-state concentration of plasma vitamin B_{12}. Bioavailability of dietary vitamin B_{12}, daily body loss of vitamin B_{12}, and human healthiness of normal gastrointestinal function are important to consider for consumption of tempeh as a source of daily vitamin B_{12} intake. When compared to cooked lean meat which contains 33 μg $100g^{-1}$ of vitamin B_{12} and the bioavailability of vitamin B_{12} which is 0.9 μg from 100 g of mutton ground patties, tempeh can be a potential source of vitamin B_{12} from non-meat origin. Provided that the potential use of bacteria producing vitamin B_{12} or yeast in soy fermentation is attempted to increase the quantity of vitamin B_{12} to make tempeh a reliable source of vegetable vitamin B_{12}.

Recently *Propionibacterium freundenreichii*, the food grade producer of vitamin B_{12}, has been used in co-culture with *R. oryzae* to enrich vitamin B_{12} in lupin tempeh [26]. An increase of vitamin B_{12} content (up to 0.97 μg100 g^{-1}) was achieved in lupin tempeh and its texture, taste and overall acceptance were not affected by the bacterial co-inoculation [27]. Nevertheless, lupin tempeh have distinct flavour and aroma that may be unacceptable to consumers who are familiar with the taste of soy tempeh. *Propionibacterium freundenreichii* was usually used in the preparation of cheese making with its contribution to fat compounds, an important flavour in cheese [28]. Regardless of whether tempeh can be fermented with substantial amount of vitamin B_{12} producing bacteria, its overall digestibility, rich in protein content and antioxidant. This can make it potential meat alternative and functional food.

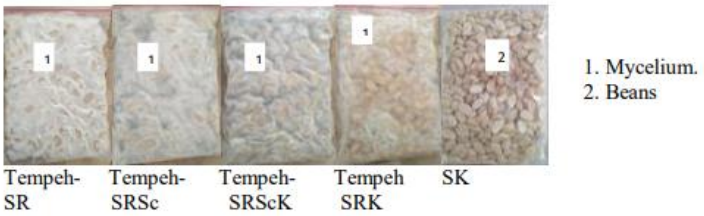

Figure 1. The profile of tempeh produced by the use of different cultures. SR is soy + *R. oligosporus*), SRSc is soy + *R. oligosporus* + *S. cerevisiae*, SRScK is soy + *R. oligosporus* + *S. cerevisiae* + *Klebsiella sp*, SRK is soy + *R.oligosporus* + *Klebsiella sp*, SK is soy + *Klebsiella sp*. All of the tempeh except SK showed the white mycelium binds the beans into a compact, cake-like texture.

Table 2. Effect of isolated cultures in producing vitamin B_{12}, daidzein, and genistein during tempeh fermentation.

Isolated culture	Initial pH	Final pH	Water content (%)	Vit B_{12} (Mcg100 g^{-1})	Daidzein (Mcg g^{-1})	Genistein (Mcg g^{-1})
SR	6.2	6.40	65.1 ±0.19	2.88 ±0.50	276.17 ±0.20	492.96 ±1.0
SRSc	6.2	6.10	65.23 ±0.80	3.15 ±0.70	224.37 ±0.20	465.12 ±0.90
SRScK	6.2	6.60	65.14 ±0.10	1.64 ±0.9	753.96 ±0.15	1072.87 ±0.90
SRK	6.2	6.60	65.24 ±0.20	0.81 ±2.00	781.76 ±0.15	1071.85 ±1.0
SK	6.2	7.40	65.91 ±0.50	0.39 ±1.9	782.17 ±0.30	1072.51 ±0.70
Soybean only	6.2	6.20	65.0 ±0.50	0.36 ±0.42	0.69 ±0.20	0.25 ±0.60

Note: The data in the table were mean of three replications with standard deviation. SR is soy + *R. oligosporus*, SRSc is soy + *R. oligosporus* + *S. cerevisiae*, SRScK is soy + *R. oligopsorus* + *S. cerevisiae* + *Klebsiella sp*, SRK is soy + *R.oligosporus* + *Klebsiella sp*, SK is soy + *Klebsiella sp*.

In all, the tempeh that was analyzed showed that genistein was larger amount than daidzein (Table 2). Most of the inoculated cultures, *R.oligosporus, S. cerevisiae and Klebsiella sp* may effectively hydrolyze daidzin and genistin during the soy fermentation. In tempeh fermentation co-inoculated with *Klebsiella sp*, it was noticed that the amount of daidzein was three-fold, and genistein was doubled compared to that of without *Klebsiella sp*. Differences in the biotransformation of both daidzin and genistin were found when the addition of *Klebsiella sp* to the soy fermentation produced high amount of both daidzein and genistein. In addition, inoculation of the mold *R. oligosporus* into the soy fermentation produced low amount of either daidzein or genistein. Even though the activity of β-glucosidase was not analyzed in this study, the production of daidzein and genistein indicated that there was biotransformation of isoflavones catalysed by the β-glycosidase. Our results are not in agreement with the work done by El-Shazly et al. [17] where the amount of daidzein was larger than genistein in fermented soy flour with *Bacillus licheniformis*. The type of soybean fermentation applied to measure isoflavones, and the selective action of the β-glucosidase, might explain the different findings in this study. Solid state fermentation as a process in which microorganisms are grown in solid material without the presence of free liquid was applied in this study while submerge fermentation was applied in another study. According to Fang et al. [9], hydrolysis of soy isoflavones by microbial β-glucosidase depend on substrate specificity. Beside hydrolyzed from daidzin and genistin, daidzein and genistein may also be derived from formononetin and biochanin-A [29]. In addition, the bacterial-synthesized β-glucosidase can be bound to the cell walls or secreted into the periplasmic space [30]. Borges et al. [31] found that soaking, cooking and fermentation times in the tempeh making influenced the production of isoflavone, and the highest yield of genistein and daidzein was fermentation with *R. oligosporus* for18 hours, but the beans and the culture used were no cause for concern.

4. Conclusions

R. oligosporus is the main isolated culture in tempeh production. Inoculation of *Klebsiella* to cooked dehulled soybean fermentation did not produce tempeh. *S. cerevisiae* is the most potential contributor of vitamin B_{12} in tempeh. *Klebsiella sp*. co-inoculation with either *R. oligosporus or S.*

cerevisiae inhibit the production of vitamin B_{12} in tempeh, although it does not affect their growth. *Klebsiella sp* contributes to the production of daidzein and genistein in tempeh.

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Conflict of interest

All authors declare no conflict of interest.

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