MINISTRY OF RESEARCH, TECHNOLOGY AND HIGHER EDUCATION
UNIVERSITAS BRAWIJAYA
FACULTY OF AGRICULTURAL TECHNOLOGY
Jl. Veteran, Malang 65145 East Java Indonesia,
Phone. +62 341 580106 Fax. +62 341 568917 e-mail : ftp_ub@ub.ac.id

Dear Dr. Maria Erna Kustyawati,

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Who produces vitamin B_{12} in Tempeh

[*1]Maria Erna Kustyawati and [1]Peni PujiAstuti

Type the author addresses here

1*Corresponding author, E-mail: mariaernakustyawati@gmail.com

Abstract. Since 20 years ago people have known that vitamin B_{12} in tempeh is produced by activity of bacteria especially *Klepsiella sp* and *Citrobacter freundii* during fungal fermentation. In this study *Klebsiella sp* and *Saccharomyces cerevisiae* were used as inoculums along with *Rhizopus oligosporus* in soybean fermentation for the making of tempeh (T). Each inoculum was inoculated separately along with *R.oligosporus* on to dehulled cooked soybeans as follows: soybeans + *R.oligosporus* + *Klebsiella sp* (TRK), soybeans + *R.oligosporus* + *S.cerevisiae* (TRS), soybeans + *R.oligosporus* + *S.cerevisiae* + *Klebsiella sp*. (TRSK), and Soy + *R.oligosporus* (TR) and soybeans + Klebsiella sp. (TK). Inoculated soybeans were then incubated at 30°C for 36 hours. Observations were made on the growth of *Klebsiella sp, S.cerevisiae and R. Oligosporus,* and vitamin B_{12} in tempeh. The results showed that the highest vitamin B_{12} was 3.15 mg/100g in TSR, followed by 2.88 mg/100g and 1.64 mg/100g were in TSKR and TR respectively. Meanwhile, vitamin B12 in TKR, 0.81mg/100g, was lower than in TK which was 0.96mg/100g. Nevertheless, the total number of bacteria in the tempeh was the highest (10^8 CFU / g) compared with the total number of *S. cerevisiae* (104CFU / g) or the total number of *R.oligosporus* (10^3CFU / g) in the tempeh. This suggests that the role of bacteria in producing vitamin B_{12} was less than that of *S. cerevisiae or R.oligosporus*. The conclusion was that *S. cerevisiae* has a significant contribution to the production of vitamin B_{12} in tempeh.

1. Introduction

It has been several decades ago, people known that vitamin B_{12} contained in tempe is synthesized by *Klebsiella pneumoniae and Citrobacter freundii* which are a contaminating bacteria during process of tempeh (Keuth and Bisping, 1994; Watanabe, 2013). Co-inoculation of non-patogenic *Klebsiella pneumoniae* with *Rhizopus oligosporus*. *Rhizopus* is the main role of fermentation in the manufacture of tempeh but bacteria and yeast grow together and have an important contribution to the nutritional and functional properties of tempeh. Tempe is solid state fungal fermentation of cooked-dehulled soybeans by *Rhizopus oligposporus* activities at incubation temperature of 27-30^{o}C for 36-40 h. During 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 (Nout et al., 2007). Tempe is an extraordinary food because it contains vitamin B_{12} which is the only vitamin absent from plant-derived food sources unless contaminated or processed with B_{12}-synthesising microorganisms (Watanabe et al., 2014). Soybean itself contains low or undetectable of vitamin B_{12}, yet when soybeans are fermented to produce tempeh, the amount of vitamin B_{12} increases to 0.7 to 0.8 ug/100g. With these values of vitamin B_{12}, tempeh is a promising source of B12 for vitamin-derived foods such milk (0.3-0.4 ug/100g, meat (3ug/100g), and eggs (0.9-

1.4 ug/100g) (Watanabe, 2007). Industrial production of Vitamin B_{12} occurs through microbial fermentation, mostly use *Pseudomonas denitrificans, Propionibacterium shermanii*; however, this process has some drawbacks such as long fermentation cycle and expensive media requirements.

Saccharomyces cerevisiae is unicellular yeast and one of the most explored organisms in terms of industrial applications. It is used for bread making and various fermented food products and beverages partly due to its contribution to flavour as well as vitamin B_{12} (Fakruddin et al., 2017). In addition to traditional alcoholic and fermented products, *Saccharomyces cerevisiae* has been used for diverse industrial purposes such as (i) lactose fermentation to ethanol, to produce lactose-free milk for people with lactose intolerance; (ii) the production of various alditols, such as glycerol or D-glucitol; (iii) protein production from alkanes and pulp-paper waste; (iv) providing enzymes, such as β-fructofuranosidase (invertase), α- and β-galactosidase and lipase; (v) production of compounds for research purposes, such as, novel carbon-carbon bonds and methyldiols of aldehydes and (vi) as biocontrol agents because they have antifungal activity, and (vii) cell biomass production (yeast food and feed), ingredient production, additives and as a processing aid for food processing, such as antioxidants, aroma, color, taste and vitamins, probiotic yeast, and yeast biocatalysts. *Saccharomyces cerevisiae* co-inoculated with *Rhizopus oligosporus* in fermentation of tempeh making, enhanced the aroma of tempeh by masking the beany aroma containing in tempeh (Kustyawati et al., 2017). Modification technique of tapioca by using *S.cerevisiae* also beneficial in term of increasing of protein in modifeied tapioca (Kustyawati et al., 2015). This study was aimed to investigate the growth of *Saccharomyces cerevisiae, and Klebsiella sp* co-inoculated individually or together with *R.oligosporus* in fermentation of tempe making, and to evaluate the production of vitamin B_{12} in tempeh.

2. Materials and methods

2.1. Tempe making

Soybeans in this research were purchased in the Primkopti BandarLampung, and Klebsiella sp was non-patogenic bacteria isolated from the tempeh (Ayu et al., 2014). Tempe making was produced in the Microbiology laboratory according to the procedure done by Kustyawati et al., (2009) with modifying in inoculation stages as follows, 300 g of soybeans were soaked in clean water overnight at room temperature, then manually removed the skin. Next, soybeans were boiled in clean water with a ratio of 1: 3 (soy: water) for 30 minutes, drained, dried at room temperature, and ready to be inoculated with certain cultures. Inoculation was carried out as follows: 100 g of cooked dehulled soybeans were inoculated with 1ml of defined number of spore suspension of *R. oligosporus* and 1ml of suspension cell of certain bacteria and yeast. Inoculated Soybeans were packed in perforated plastic packaging and incubated at 32oC for 40 hours. Five types of tempeh with the addition of different inoculated cultures produced on this study, namely (1) soybeans + *R.oligosporus* + *Klebsiella sp* (TRK), soybeans + R.oligosporus + S.cerevisiae (TRS), soybeans + *R.oligosporus* + *S.cerevisiae* + *Klebsiella sp*. (TRSK), and Soy + *R.oligosporus* (TR) and soybeans + *Klebsiella sp.* (TK). Soybeans without inoculation as a control negative (Soy). Tempe making was made in duplicate.

2.2. Microbial analysis

Each of tempeh made was analyzed for its total number of bacteria, yeast and molds at the starting and the end of fermentation by growing culture on appropriate media. A total of 15 g tempeh was taken, mixed with 135 ml of 0.1% peptone water, homogenized with a stomacher for 5 minutes, and a series of dilutions from 10-1 to 10-8 was made in duplicate. Then one ml is taken from certain dilutions. Planting microorganisms was done by surface plate count method on the suitable solid media (BGBL agar, MEA, and PDA were for *Klebsiella sp, S.cerevisiae, and R.oligosporus* respectively), and incubated at suitable temperature and time. The data obtained were analyzed descriptively and displayed in graphical form.

2.3. Vitamin B_{12} analysis

The analysis of vitamin B_{12} was done following the procedure run by in house method of LCIT Laboratory, University of Lampung. A total of 0.5 grams of tempe powder was weighed and put into 100 mL Erlenmeyer containing of 20 mL millique water. Each sample was verified using ultrasonic equipped with heater for 30 minutes. Sample volume was set for 25 mL, then centrifuged. Approximately of 2 mL supernatan was pipetted using syiringe equipped with a filter holder and filtered using paper filter with 13 mm in diameter and 0.2 mm pore. The filtrate was then placed in the vial bottle. The sample is 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 5um 125x4.6 mm column, column temperature of 35oC, mobile phase (water: acetonitrile: bufferphosphate 10nM = 80:10:10), isocratic mobile phase method, with flow rate 1 mL/min, injection volume of 20uL, wavelength detector 360 nm, and run time of 20 min.

3. Result and discussion

Table 1. Effect of variety culture inoculation of tempeh making on the growth of microorganism and vitamin B_{12} production.

Type of the tempeh	Mold number (CFU/g)	Number of yeast (CFU/g)	Number of bacteria (CFU/g)	Vit B12 (mg/100g)
Kedelai + *R. oligosporus*	3.35×10^3 ±1.32	5.9×10^2	2.04×10^9 ±2.03	2.88± 0.01
Kedelai + *R. oligosporus* + *S. cerevisiae*	4.5×10^3 ±1.16	1.2×10^7 ±1.66	1.09×10^9 ±2.35	3.15 ±0.01
Kedelai + *R. oligosporus* + *S. cerevisiae* + *Klebsiella Sp*.	5.6×10^3 ±2.01	3.7×10^7 ±2.11	2.54×10^{10} ±1.85	1.64± 0.0
Kedelai + *R. oligosporus* + *Klebsiella Sp.*	3.0×10^2 ±1.44	0	1.86×10^{10} ±2.41	0.81± 0.0
Kedelai + *Klebsiella Sp.*	0	0	2.69×10^{12} ±2.56	0.96± 0.01

Table 1 showed that adding *S.cerevisiae* or *Klebsiella sp* together or separately did not affect the growth of *R.oligosporus*. *R.oligosporus* in tempeh is not contaminant microorganism instead it has to be deliberately added into the soybeans. *R.oligosporus* produces several enzymes during fermentation 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. Thus *R.oligosporus* plays a role in suppoting the growth of other microorganism in fermentation of tempeh making. The low number of bacteria in this experiment can be caused by antibiotic produced by *R.oligosporus* (Kobayasi et al., 1992). *S.cerevisiae* can live together with *R.oligosporus* or *Klebsiella sp* during fermentation of tempeh making. They use carbon and nitrogen source from soybean as well as fatty acid and amino acid produced by *R.oligosporus*. On the other hand, *Klebsiella sp* grew very high during fermentation because they are used to be a contaminant microorganism. Therefore it can be said that *R.oligosporus* serves as bacterial growth control while supporting microbial growth.

Biosynthesis of vitamin B_{12} is limited to some bacteria and therefore the production depends on microbial fermentation. Fang et al., 2017, that microbial de novo biosynthesis of vitamin B_{12} occurs through two alternative routes, they are aerobic and anaerobic pathways, in bacteria and archaea,

respectively. Production of vitamin B_{12} during fermentation of tempeh making may be influenced by the inoculated cultures. Tempeh making with adding *R.oligosporus*, and *S.cerevisiae* contained highest vit B_{12}, but tempe making added with R. oligosporus and Klebsiella contained the lowest vitamin B_{12}. Even though the fermentation of soybeans with adding *Klebsiella sp* did not produce tempeh, it produced vitamin B_{12}. *R.oligosporus* could be better vitamin B_{12} producing than *Klebsiella sp* in this experiment. Since soybean itself contains low or undetectable of vitamin B_{12}, it indicated that *Klebsiella* sp was responsible on producing vitamin B_{12} in fermented soybean. Nevertheless, when *Klebsiella sp* was co inoculated with *R.oligosporus*, the amount of vitamin B_{12} in tempeh was low. This can be explain that either *R.oligosporus* or *S.cerevisiae* can produce antibiotic which may inhibit the growth of bacteria including Klebsiella sp. On the other hand, *S.cerevisiae* can produce vitamin B_{12} and is rich of chromium (Klis et al., 2006; Fakruddin et al., 2017), and therefore addition of this can increase the vitamin B_{12} in tempeh. The production of vitamin B_{12} in tempeh inoculated with adding *S.cerevisiae* in this experiment (3.19 mg/100g) was quiet lower than that of done by Kustyawati et al (2009) which was 3,95 mg/100g. Recently *Propionibacterium freundenreichii* the food grade producer of vitamin B_{12} has been used to enrich vitamin B_{12} in tempe making (Watanabe F et al., 2014) and in lupin tempeh (Fudiyansyah et al., 1995; Chamlagain, 2016; Wolkers-Rooijackers et al., 2018). Even though exture, taste and overall acceptance of lupin tempeh were no different from soy tempeh, this discovery may not be accessed by tempeh consumers in Indonesian who are familiar with the taste of tempeh. *P. freundenreichii* was normally used in ripening of cheese making with its contribution to fatty compounds, important flavor in cheese (Mukdsi et al.,2014).

4. Conclusion

Tempe was a model in this experiment. The co-inoculation of *Klebsiella sp* and *S.cerevisiae* with *R.oligosporus* was to reveal most of the responsibility for vitamin B_{12} production in tempeh making. *S.cerevisiae* was most contributor of vitamin B_{12} in tempeh and support the growth of *R.oligosporus* Co-inoculation of *Klebsiella sp* with either *R.oligosporus or S.cerevisiae* inhibited vitamin B_{12} production in tempeh, although it did not affect growth.

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