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Influence of *Lactobacillus casei* in Production of High Vitamin B_{12} -Tempeh

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ABSTRACT

The use of *Lactobacillus casei* during soaking of soybeans at 30°C for 15 hr did not influence the vitamin B_{12} content of the resulting tempeh produced not only by *Rhizopus oligosporus* but also by the *Rhizopus* and *Propionibacterium shermanii* 1250. Moreover, the vitamin B_{12} in tempeh produced by mixed cultures of *Lactobacillus* and *Propionibacterium* (147.4 ng/100g) was significantly lower than that of tempeh produced by the *Propionibacterium* alone (185.3 ng/100g). It was found that the *Propionibacterium* was inhibited at low pH due to the acid produced by the *Lactobacillus* and it could not compete for the common substrate. Consequently, inoculation of *Lactobacillus* was resulted in decreasing of vitamin B_{12} normally produced by the *Propionibact erium.*

INTRODUCTION

Vitamin B_{12} is essential and involved in the development of pernicious anemia in humans unable to absorb the vitamin (Wokes *et al.*, 1955). Manifestation of deficiency in Vitamin B_{12} include neurological symptoms, anemia, pancytopemia, hypothermia and severe weight loss (Davis *et al.*, 1981). Vitamin B_{12} is produced exclusively by microorganisms like *Pseudomonas*, *Clostridium* and some of the *Streptomyces* (Florent and Ninet, 1979). Moreover, vitamin B_{12} production using *Propionibacterium* have been reported by several investigators (Leviton and hargrove, 1952 ; Yongsmith and Apiraktivongse, 1983).

Tempeh, a fermented soybean product of Indonesia, is popular among vegetarians in Asia and in the western countries. It is produced through fermentation of soybean cake by *Rhizopus oligosporus*. Nutritionally, tempeh is considered as a good source of protein and containing an appreciable amount of vitamin B complex when produced from pure culture fermentation (Murata *et al.*, 1967). However, a mixed cultures of *Klebsiella pneumoniae*, *Bacillus megaterium* or *Streptomyces olivaceus* and *R. oligosporus* contained also vitamin B_{12} (Supermo and Markakis, 1987).

Lactobacilli are rods, microaerophilic, catalase negative and gram positive bacteria which ferment sugars to yield lactic acid as main product. *Lactobacillus casei* is a homofermentative lactic acid bacteria which ferments sugar chiefly to lactic acid with small amounts of acetic acid, carbon dioxide, and trace products (Frazier and Westhoff, 1988). It is not evident that *Lactobacilli* can produce vitamin B_{12}.

Vitamin B_{12} production by *Propionibacterium* and its interaction with the lactic acid bacteria was mostly investigated not only in the submerged fermentation but also in the semi-solid fermentation. Leviton and Hargrove (1952) reported that lactic acid bacteria involved in the synthesis of vitamin B_{12} by *Propionib acterium*. Moreover, Yongsmith and Kittipornpanich (1987) developed a process for increasing vitamin B_{12} levels in soybean solid waste and a semi-solid substrate, by mixed fermentation of lactic acid bacteria and propionic acid bacteria.

In this study, an attempt was made to investigate the effect of *Lactobacillus casei* in vitamin B_{12} production in tempeh using a solid state fermentation by *Propionibacterium shermanii* and *Rhizopus oligosporus*.

MATERIALS AND METHODS

Preparation of Inocula

Rhizopus oligosporus : the *Phizopus* which was isolated by Krusong (1987) from tempeh bought from a market in Bogor, Indonesia was prepared as

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powder inoculum. Rice (800g) was half cooked for 30 min in a rice pan. The cooked rice was cooled and inoculated with a spore suspension of *R. oligosporus* and spread to a loose layer approximately 1 cm thick in a tray covered with cheese cloth and then incubated at 35°C. After sporulation, the moldy mass was dried up to 45°C, pulverized and mixed with sterile wheat powder. The number of spores were checked using haemacytometer and should contain 10^8 spores for a gram in the final product.

Lactobacillus casei : the *Lactobacillus* isolated from sour milk (Yongsmith, 1977) was maintained in Glucose yeast Extract Peptone (GYP) agar composed of (g/l of deionized water) glucose 10.0, yeast extract 10.0, peptone 10.0, calcium carbonate 10.0 and agar 15.0. The pH was adjusted to 6.8 before sterilization. GYP broth without $CaCO_3$ was used as the propagation medium for *L. casei*. After incubation at 35°C for 24-48 hr, *L. casei* was used in subsequent experiments.

***Propionibacterium shermanii* 1250** : the *Propionibacterium* was maintained in complete medium agar stab. The complete medium used for cultivation was composed of (g/l of deionized water) glucose 10.0, yeast extract 5.0, acid hydrolysed of casein 1.0, pancreatic digested casein 1.5, biotin 0.0003, calcium pantothenate 0.004, NaH_2PO_4 1.6, K_3PO_4 1.6, $MgCl_2 . 6H_2O$ 0.4, $FeSO_4 . 7H_2O$ 0.01 and $CoSo_4 . 7H_2O$ 0.012. The pH was adjusted to 6.8 before sterilization. After incubation at 35°C for 24-48 hr, *P. shermanii* 1250 was used in subsequent experiments.

Tempeh Perparation

Soybeans, Phraputthabath vareity, were washed thoroughly under running water and soaked in distilled water acidified with 1% of 80% lactic acid for 2 hr at 70°C, dehulled by hand, and then dried at 80°C until the surface appeared dry. Fifty grams of beans were placed in each petri dish and autoclaved for 15 min at 121°C (Krusong *et al.*, 1991).

The cooled, sterile soybean cotyledons were inoculated with the appropriate culture(s). Inoculated beans were incubated at 35°C for 18-24 hr.

The tempeh was blanched by steaming for 30 min and dried at 80°C overnight, pulverized and kept in the refrigerator. About 1.0 g of each dried, pulverized tempeh was extracted for its vitamin B_{12} content.

Addition of *Lactobacillus casei* during soaking of soybeans

Mixed fermentation with *L. casei* during soaking of soybeans was conducted. Soybeans were subjected to three treatments : *treatment 1* (control) where the beans were unfermented and unsoaked ; *treatment 2* the beans were soaked in distilled water acidified with 1% of 80% lactic acid for 2 hr at 70°C ; and *treatment 3* the beans were soaked in distilled water inoculated with 10% *L. casei* for 15 hr at 30°C. This is a modified method based on the study of the addition of *L. brevis* during hydration of beans (Steinkraus *et al.*, 1965).

Beans in treatments 2 and 3 were dehulled by hand. Fifty grams were placed in each petri dish, autoclaved for 15 min at 121°C, cooled and inoculated with 50 mg *R. oligosporus* powdered inoculum for tempeh making and 1 ml *P. shermanii* 1250 and 250 mg *R. oligosporus* powdered inoculum for vitamin B_{12} production in tempeh.

Vitamin B_{12} Production in Tempeh by Mixed Culture of *Lactobacillus casei* and *Propionibacterium shermanii*

After pretreatment of soybeans as mentioned in tempeh preparation, the cooled and sterile bean cotyledons placed in petri dishes were inoculated with the four treatments as followed : *treatment 1* (control) 250 mg *R. oligosporus* powdered inoculum ; *treatment 2* 1 ml *L. casei* in GYP broth and 250 mg *R. oligosporus* powdered inoculum ; *treatment 3* 0.5 ml *L. casei*, 0.5 ml *P. shermanii* 1250 inoculum and 250 mg *R. oligosporus* powdered inoculum ; *treatment 4* 1 ml *P. shermanii* 1250 and 250 mg *R. oligosporus* powdered inoculum.

Vitamin B_{12} Determination

As recommended by Liem *et al.*, (1977), Ro *et al.* (1979) and Truesdell *et al.* (1987), a cyanide-buffer solution was used for extraction of vitamin B_{12}. Samples were extracted in 0.1M acetate buffer (pH 5.5) containing 0.1% KCN.

Vitamin B_{12} in tempeh was determined by microbiological assay with *Lactobacillus leichmanii* (ATCC 7830) as the test organism. The assay procedure followed the official method of analysis of AOAC (1975).

RESULTS AND DISSCUSSION

Soybeans contain generally less than 100 ng of vitamin B_{12} per 100 g (Liem *et al.*, 1977). Steinkraus *et al.* (1960) reported that the amount of vitamin B_{12} in soybeans was found to be 15 ng per 100 g. However, soybeans of the Phraputthabath vareity used in this study contained 30-36 ng/100g of vitamin B_{12}. Moreover, increase in vitamin B_{12} in soybeans was achieved by fermentation (Steinkraus *et al.*, 1960).

Lactobacillus casei is homofermentative *Lactobacillus* which ferments sugar chiefly to lactic acid with small amounts of acetic acid, carbon dioxide and trace products (Frazier and Westhoff, 1988). It is not evident that *Lactobacilli* can produce vitamin B_{12}. Leviton and Hargrove (1952) reported that in the presence of *Propionibacterium*, they participate in the synthesis of vitamin B_{12} by mixed culture fermentation.

Table 1 Yield of vitamin B_{12} in tempeh made by different methods of soybean soaking.

Methods of soaking	pH (of soaking water)		Yield of vitamin B_{12}* (ng/100g)
	Initial	Final	
None (dry beans)		-	36 a
Distilled water acidified with 1% of 80% lactic acid for 2 hr at 70°C	4.5	5.5	36 a
Distilled water inoculated with 10% *L. casei* inoculum for 15 hr at 30°C	4.4	4.2	35 a

Means of three replications ; means in the same column followed by the same letters are not significantly different by 5% DMRT

L. casei possibly influences the vitamin B_{12} content of the product because it provides lactate which then serves as substrate for the *Propionibacterium*, to produce the vitamin. The main reasons for using *L. casei* in tempeh fermentation is to decrease the pH of tempeh so that fermentation time is prolonged before ammonia is liberated.

Initial soaking of the beans in distilled water either with or without *L. casei* did not influence the vitamin B_{12} content of the mold fermented product (Table 1). The pH changes in soaking water were then investigated. In acidified soaking water (1% of 80% lactic acid for 2 hr at 70°C), the pH increased from 4.5 to 5.5 because of the removal of substances such as nitrogen compounds in the beans by the combination effect of acid and heat. On the other hand, there was a decrease in pH of soaking water inoculated with 10% *L. casei* inoculum for 15 hr at 30°C because of acid production during the fermentation of sugars by *L. casei*. This result was found to be the same in the preliminary test of inoculating the soaking water with the bacteria *L. plantarum* or *L.brevis* after a 15 hr prefermentation at 32°C followed by a drop in pH to 5.5. It was also reported by Steinkraus *et al.* (1960, 1956).

Influence of the Addition of *Lactobacillus casei* during Soaking of Soybeans on Vitamin B_{12} Production in tempeh using *Propionibacterium shermanii* 1250

During soaking, acid fermentation by bacteria usually takes place as exemplified by falling of pH to 4.5 mentioned in the previous study. However, the pH can be lowered further by the addition of lactic acid. The soaked soybeans were dehulled in running tap water which caused the increase in pH to 6.3. This pH was suitable for growth of *P. shermanii* 1250. After sterilization, the beans were inoculated with *P. shermanii* 1250 and *R. oligosporus*.

Vitamin B_{12} produced in resulting tempeh was shown in Table 2. It was found that vitamin B_{12} produced by *P. shermanii* 1250 in tempeh pretreated with *L. casei* during soaking of beans was not significantly different from the tempeh which was artificially acidified with lactic acid. The amount of lactic acid remaining in soybean cotyledons before tempeh fermentation in both cases of soaking treatments were not different because of the dilution of acids during dehulling in running tap water. Moreover, *P. shermanii* 1250 metabolized lactate for production of vitamin B_{12} in tempeh.

Influence of *Lactobacillus casei* and *Propionibacterium shermanii* 1250 on Yield of Vitamin B_{12} Production in Tempeh

Propionibacterium shermanii 1250 significantly increased the yield of vitamin B_{12} in tempeh (185.3 ng/100g) as shown in Table 3. Although it is nothworthy that *P. shermanii* preferentially metabolizes lactate which is, in general, an end product of fermentation for

Table 2 Vitamin B_{12} production by *Propionibacterium shermanii* 1250 in tempeh as affected by different methods of soybean soaking.

Methods of soaking	Yield of vitamin B_{12}* (ng/100g)
None (dry beans)	36.0 b
Distilled water acidified with 1% of 80% lactic acid for 2 hr at 70°C	182.5 a
Distilled water inoculated with 10% *L. casei* inoculum for 15 hr at 30°C	181.4 a

Means of three replications ; means in the same column followed by the same letters are not significantly different by 5% DMRT

many bacteria (Lee *et al.*, 1974), production of vitamin B_{12} in tempeh produced by mixed culture of *R. oligosporus* and *L. casei* was not significantly different from tempeh produced without bacteria inoculation. It could support the fact that lactic acid fermentation did not influence the vitamin B_{12} content of tempeh as first mentioned by Liem *et al.* (1977). Mixed culture fermentation of *L. casei* and *P. shermanii* 1250 together with *R. oligosporus* produced 147.4 ng of vitamin B_{12} per 100 g. However, its vitamin content is significantly lower compared to that produced in tempeh inoculated with *P. shermanii* 1250 and *R. oligosporus*. This was due to the inhibition of growth of *P. shermanii* 1250 caused by the low pH brought about when *L. casei* grew and competed to use the degraded sugars (Lee *et al.*, 1976). In fact, *Lactobacillus* and *Propionibacterium* normally exhibit features typical of commensal interaction even though a number of complicating factors, such as a large maintenance requirement of *Lactobacillus* and the growth inhibition of *Propionibacterium* at low pH, are present (Lee *et al.*, 1976). Moreover, Lee *et al.* (1974) reported that, in nature, *P. shermanii* usually coexist with lactic acid bacteria. However, due to their low growth rate, they can not survive in competition for the common substrate with lactic acid bacteria.

During 72 hr of fermentation time as shown in Figure 1., It was found that vitamin B_{12} content in tempeh inoculated with *L. casei* and *R. oligosporus* did not change. On the other hand, vitamin B_{12} content produced in tempeh inoculated with mixed culture of *L. casei* and *P. shermanii* 1250, together with *R. oligosporus* increased in 24 hr, then, decreased until 72 hr of fermentation. This was because the pH was

Table 3 **Influence of *Lactobacillus casei* and *Propionibacterium shermanii* 1250 on yield of vitamin B_{12} in tempeh.**

Culture inoculum	Yield of vitamin B_{12}* (ng/100g)
250 mg *R. oligosporus* powdered inoculum	36.0 c
1 ml *L. casei* inoculum, 250 mg *R. oligosporus* powdered inoculum	36.0 c
0.5 ml *L. casei* inoculum, 0.5 ml *P. shermanii* 1250 inoculum, 250 mg *R. oligosporus* powdered inoculum	147.4 b
1 ml *P. shermanii* 1250 inoculum, 250 mg *R. oligosporus* powdered inoculum	185.3 a

* Means of three replications ; Means in the same column followed by the same letters are not significantly different by 5% DMRT

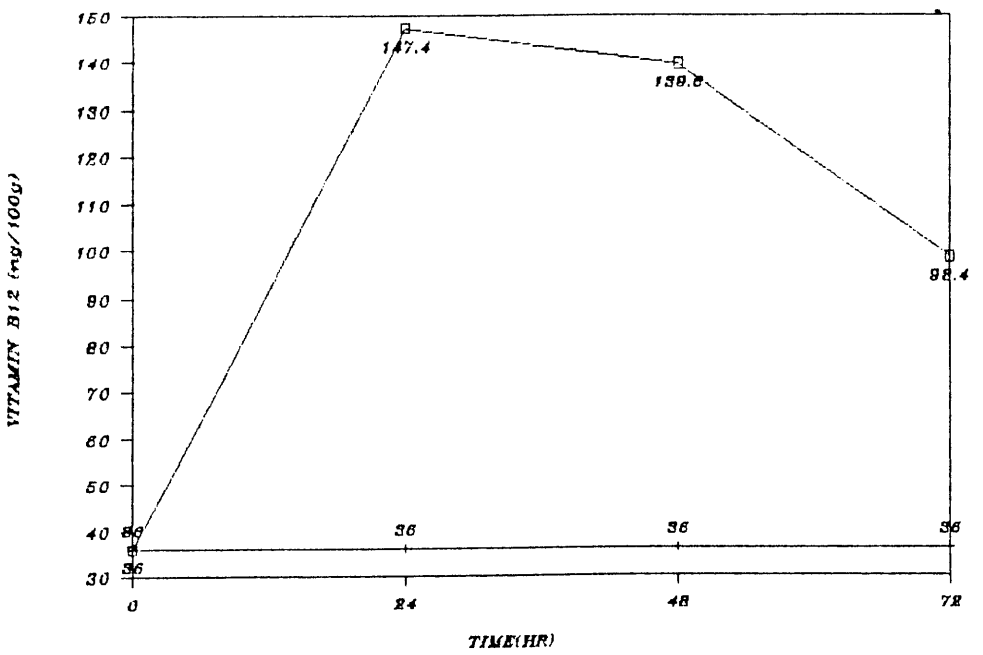

Figure 1 **Content of vitamin B12 in tempeh inoculated with : (1) *Lactobacillus casei* and *Rhizopus oligosporus* powdered inoculum (+) ; (2) *L.Casei, Propionibacterium shermanii* 1250 and *R.oligosporus* powdered inoculum (□).**

lowered by the acid produced by *L. casei* which inhibited the growth of *P. shermanii* 1250. Therefore, without buffering the fermentation mashes, *L. casei* could not be used for co-fermentation with *P. shermanii* 1250 because it decreased the production of vitamin B_{12} by *P. shermanii* 1250 in this solid state fermentation of tempeh.

However, *L. casei* which decreased the pH of tempeh allowed a longer fermentation time before ammonia was liberated. Since *R. oilgosporus* has high proteolytic activities ; deamination following hydrolysis released ammonia, causing pH to rise. Above pH 7.0, substantial amount free ammonia is released to kill the mold. This is the reason for encouraging the influence of *L. casei* in tempeh fermentation.

CONCLUSION

The preliminary results showed that there is high possibility to increase the vitamin B_{12} content in tempeh by adding *Propionibacterium shermanii* 1250, isolated from Swiss cheese. The optimization process for the production of high vitamin B_{12} -tempeh by mixed fermentation of the *Propionibacterium* and the mold, *Rhizopus oligosporus*, will be reported later.

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