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Enhancing vitamin B_{12} in lupin tempeh by *in situ* fortification

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ABSTRACT

Tempeh is a traditional, fungal fermented Indonesian product, usually made from soybeans. Tempeh is known to contain vitamin B_{12} which is essential for a healthy human diet. Therefore, tempeh is of particular interest for vegan diets since B_{12} is normally found only in animal derived products. The vitamin B_{12} in tempeh is associated with the presence of opportunistic pathogens like *Klebsiella pneumoniae*. Levels of B_{12} in tempeh do not sustain the recommended daily intake though. In addition, the use of a food-grade bacterium instead of *K. pneumoniae* is preferred. Lupin can serve as alternative substrate for soybeans due to its similar protein content, resulting in 'lupin tempeh'. In this study, *Propionibacterium freudenreichii*, a food-grade, vitamin B_{12} producing bacterium, was used in co-culture with *Rhizopus oryzae* to produce B_{12}-enriched lupin tempeh. A significant increase of vitamin B_{12} content (up to 0.97 μg/100 g) was achieved by fermenting lupin using a mixed starter of *R. oryzae* and *P. freudenreichii*. Other parameters, such as texture and volatile organic compounds, were not affected by the bacterial co-inoculation. Therefore, these results are promising for *in situ* vitamin B_{12} fortification of lupin tempeh making it a sustainable protein source for a healthy human diet.

1. Introduction

A growing population increases the demand for dietary protein. This can no longer be sustained only by animal products and therefore other protein sources from plants are used for both feed and food. Soybeans are one of the most important plant based protein sources and Europe is greatly dependent on its import (70%) (de Visser, Schreuder, & Stoddard, 2014). Native European legumes, such as white lupin (*Lupinus albus*), yellow lupin (*Lupinus luteus*), and narrow-leafed lupin (*Lupinus angustifolius*), are promising alternatives for soybeans and could overcome this dependency. Lupin has environmental benefits of proven potential for recovery of poor and contaminated soil (Lucas et al., 2015), and its nutritional quality (digestibility, protein and dietary fiber content) is good (Fudiyansyah, Petterson, Bell, & Fairbrother, 1995; Pollard, Stoddard, Popineau, Wrigley, & MacRitchie, 2002). Certain lupine varieties produce seeds with a high content of quinolizidine alkaloids which cause symptoms of poisoning in humans (Gremigni, Wong, Edwards, Harris, & Hamblin, 2001). Especially the seeds of sweet varieties of *L. angustifolius* were found to have low alkaloid levels (< 0.01% (wt/wt) (Ruiz, White, & Hove, 1977). The majority of cultivated lupin is currently used for animal feed. New opportunities for using this nutritious legume for food applications are being explored. The addition of lupin flour and kernels in for instance bakery products has already been studied (Clark & Johnson, 2002; Pollard et al., 2002) and did not affect product acceptability. For the production of legume-based food, fermentation is well suitable because of reduced off-flavour, higher digestibility, longer shelf life and enhanced nutritional value (Couto & Sanromán, 2006; Steinkraus, 1994). Tempeh is one of those examples of mould fermented foods and the production process has shown to work for lupin as well as soy (Agosin, Diaz, Aravena, & Yañez, 1989; Fudiyansyah et al., 1995).

Tempeh is a solid-state fungal fermentation of cooked seeds and seed-processing by-products (Nout, Sarkar, & Beuchat, 2007). At ambient temperature (25–30 °C), the fermentable substrates are incubated for 1–2 days to allow spore germination and subsequently growth of mycelium. During fermentation, enzymatic activity of the mould leads to a significant increase in water-soluble nutrients (Agosin et al., 1989). This enhances digestion, biosynthesis of B vitamins and transformation of (soy) isoflavones into antioxidant compounds (Nout et al., 2007), albeit that raw lupin has less anti-nutritional factors then soy (Fudiyansyah et al., 1995). Among the B vitamins, the presence of vitamin B_{12} in tempeh is remarkable since no plant-based foods contain this vitamin unless fermented or contaminated (Herbert, 1988). It is known that not the fungi, but bacteria such as *Klebsiella pneumoniae* (Okada, Hadioetomo, & Niekuni, 1983) and *Citrobacter freundii* (Keuth & Bisping, 1993; Wiesel, Rehm, & Bisping, 1997) are responsible for vitamin B_{12} production in tempeh. Both bacteria can be present in tempeh naturally or added to the tempeh starter (Nout & Kiers, 2005). The use of a Generally Recognized As Safe (GRAS) bacterium over opportunistic pathogens is preferred. Since all strains from the species

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Propionibacterium freudenreichii have the GRAS status and produce vitamin B_{12} under microaerophilic conditions (Martens, Barg, Warren, & Jahn, 2002) they are a good candidate for applications in food fermentation.

The estimated value of vitamin B_{12} in Indonesian soybean tempeh ranged from 0.7 μg/100 g in fresh tempeh to 8 μg/100 g during storage (Okada et al., 1983). With these values tempeh is a promising alternative source of B_{12} for animal-derived foods such as milk (0.3–0.4 μg/100 g), eggs (0.9–1.4 μg/100 g), lean red meat (3 μg/100 g) and fish (2 μg/100 g) (Watanabe, 2007). In the Netherlands, a vitamin B_{12} intake of 2.8 μg/day is recommended (Health Council of the Netherlands, 2003). Dietary deficiency of vitamin B_{12} can cause diseases of the nervous system and disturbances in cell (red blood cells) division, which may pose a risk for elderly and especially strict vegans of which breastfed infants are most at risk (Truswell, 2007). Vitamin B_{12} deficiency is becoming more and more a worldwide problem (Jeleń, Majcher, Ginja, & Kuligowski, 2013).

The aim of this research was to boost the amount of vitamin B_{12} in lupin tempeh by bacterial biosynthesis using the food grade bacterium *P. freudenreichii*. Therefore, the objectives of this study were to determine optimum fermentation parameters for enhancing vitamin B_{12} content in lupin tempeh and to evaluate the quality of lupin tempeh produced by mixed culture fermentation.

2. Materials and methods

2.1. Lupin beans

The main substrate used in this project was chopped sweet narrow-leafed lupin beans (*Lupinus angustifolius*) which has a particle size of 1–2.8 mm. The chopped lupin beans, sold as lupin bites, were purchased from Lupinfood.eu (Utrecht, the Netherlands).

2.2. Fungal spore suspension

The strain used in this project was *Rhizopus oryzae* CBS 285.55. This strain was obtained from the public culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands and was originally isolated from soy tempeh in the Netherlands. The strain, kept in 20% (v/v) glycerol stock at −80 °C, was cultured onto Malt Extract Agar (MEA, Oxoid) and incubated at 30 °C for 48 h. The mould was subcultured onto MEA slants and incubated at 30 °C for 7–10 days to obtain a fully sporulated sample. Spores were harvested by adding sterile saline solution (0.9% (wt/vol) NaCl) to the culture slants and scraping the spores off using a sterile loop. After collecting the spores in the saline solution, the suspension was ready for use.

2.3. Bacterial suspension

The vitamin B_{12} producing *Klebsiella pneumoniae* IIEMP-3 (Yulandi, Sugiokto, Febrilina, & Suwanto, 2016), originating from soy tempeh and obtained from Bogor Agricultural University (Indonesia), was used as positive control. The food grade vitamin B_{12} producing bacterium, *Propionibacterium freudenreichii* subsp. *freudenreichii* (DSM 20271, isolated from Swiss cheese), was stored as −80 °C glycerol stock. For preparing bacterial suspensions, the bacteria were cultured from the frozen stock on Violet Red Bile Glucose (VRBG) agar (Oxoid) for *K. pneumoniae* and Sodium Lactate (SL) agar (Burgess, Smid, Rutten, & Van Sinderen, 2006) for *P. freudenreichii*. Plates were incubated for 24 h at 37 °C, aerobically or 5 days at 30 °C anaerobically respectively. After incubation, one single colony of each species was transferred aseptically into 10 ml nutrient broth for *K. pneumoniae* or 10 ml of SL broth for *P. freudenreichii*. An aliquot of 1 ml of grown culture in broth medium was centrifuged at 17.000 × g for 5 min and the supernatant was discarded. The bacterial pellet was then re-suspended in 1 ml phosphate buffered saline (pH 7.2).

2.4. Lupin tempeh production

Approximately 800 g of lupin bits was weighed and equally divided into two plastic jars containing each 1200 ml of tap water for soaking. The pH of the soaking water was adjusted to 4.1 using lactic acid (1 M) and bits were soaked for 15 h at 30 °C. After soaking, the lupin bits were drained, rinsed with running tap water and then cooked for 20 min in water (ratio beans to water 1:3). After the cooking stage, lupin bits were drained and spread out on a metal mesh (cooling tray) with approximately 1 cm layer thickness and then dried in the open air for 2 h. After drying, circa 450 g of lupin bits were weighed into a stomacher bag.

The tempeh was prepared for each 450 g portion of lupin bits. An aliquot of 4.5 ml of *R. oryzae* spore suspension (10^6 viable spores per ml) was transferred into a sterile 15 ml falcon tube. The mixed culture was made by adding 0.9 ml of bacterial suspension into the tubes containing the fungal spore suspension. The inoculum suspension was mixed and added to the stomacher bag containing 450 g lupin bits. The lupin bits and tempeh inoculum inside stomacher bag were mixed by shaking vigorously. A clean tempeh container, a perforated lunch box (14 cm × 9 cm x 3.5 cm), was surface sterilized by 70% ethanol. Lupin bits, mixed with the inoculum, were poured from the stomacher bag into the tempeh container. The influence of different initial inoculum sizes of *P. freudenreichii* was investigated by co-inoculating lupin bits with a bacterial suspension for a final inoculum size of 10^3, 10^5 or 10^7 CFU/g of lupin bits. Two incubation temperature regimes were compared: (i) 25 °C, 44 h and (ii) the combination of 30 °C for 24 h followed by 25 °C for 20 h, referred to as 25 °C and 30 °C respectively.

2.5. Microbiological vitamin B_{12} assay

The amount of vitamin B_{12} present in the lupin tempeh was determined according to the protocol of Okada (Okada et al., 1983). The assay is based on the fact that *Lactobacillus leichmannii* ATCC 7830 cannot grow in the absence of vitamin B_{12}.

In short, two extracts from lupin tempeh were prepared (A and B). Extract A contains the active form of cyanocobalamin and B analogues of vitamin B_{12}. Both extracts were added separately to vitamin B_{12} assay medium (BD Difco™ 245710) and inoculated with 10 μL of a *L. leichmannii* suspension ($OD_{600} \approx 0.25$). After incubation at 37 °C for 48 h the OD was measured at 600 nm. A calibration curve made with pure vitamin B_{12} was prepared simultaneously. The vitamin B_{12} values of extracts A and B were calculated using the standard curve equation (see Fig. S1 in Supplement). The difference in read-out value between extract A and B represents the cyanocobalamin concentration. For determination of the final content of vitamin B_{12} in the original samples the dilution factor (20×) during extract preparation was taken into account.

2.6. Microbiological quality

Lupin tempeh samples were microbiologically examined by mixing 10 g of lupin tempeh with 90 ml sterile PPS (Peptone Physiological Salt, 1 g/L bacteriological peptone (Oxoid) and 8.5 g/L NaCl) in a sterile filter stomacher bag and homogenized (Seward, 400 circulator) at normal speed for 1 min. Total viable count was performed by spread plating appropriate dilutions onto Plate Count Agar (PCA, Oxoid). For the enumeration of lactic acid bacteria, an aliquot of 1 ml was pour-plated in duplicate in de Man, Rogosa and Sharp agar (MRS broth, Merck and 15 g/L Bacteriological agar (BA, Oxoid)) supplemented with 2 g/L Delvocid (50% Natamycin, DSM) to prevent fungal growth on the plates. In order to enumerate the viable count of vitamin B_{12} producing bacteria in the final product, an aliquot of 1 ml of tempeh containing *K. pneumoniae* IIMP-3 was pour-plated in VRBG agar. The enumeration of *P. freudenreichii* in the final product was examined by spread-plating the diluted sample onto SL agar supplemented with 2 g/L Delvocid (50% Natamycin, DSM).