

Improvement of High Vitamin B₁₂ Thua nao by Mixed Cultures of Soybean Oligosaccharide Utilizing Bacteria and Yeasts.

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ABSTRACT

The traditional production of “Thua nao” involves predominantly *Bacillus* spp. fermentation on soybean substrates. It is a high protein fermented with ammonia smell but without vitamin B₁₂ content. Based on soybean fermented sources and oligosaccharide (raffinose) utilizing capability, three potent microbial isolates with different characteristics to improve Thua-nao fermentation have been selected. They are a high proteolytic *Bacillus* sp. B4, a high vitamin B₁₂ producing *Klebsiella* sp.KB2 and a pleasant aroma contributing yeast, *Candida* sp. KB1. Cocultures fermentation of *Bacillus* sp. B4 and *Klebsiella* sp.KB2 could improve the quality of Thua-nao by enhancing more soluble protein and vitamin B₁₂ content in the fermented soybean up to 91.43 µg/100gdw as nine times higher than control fermentation with only *Klebsiella* sp.KB2. Furthermore, the local Thua-nao isolate, *Candida* sp.KB1 at its optimum condition added in high vitamin B₁₂ Thua-nao could decrease significantly the strong ammonia smell without interfere on the vitamin B₁₂ fermentation of the product.

INTRODUCTION

Thua nao is a Thai traditional non-salted product fermented by *Bacillus* spp. It is produced in northern Thailand and utilized as a substitute for fish paste (Sundhagul et al., 1972). To make traditional Thua nao, soybeans are washed, soaked overnight, cooked thoroughly until soft, draining and fermenting naturally without inoculation and fermenting in banana leaf lined baskets for 3 or 4 days until surface of the beans are covered with a sticky, viscous, colorless gum and are accompanied by a pungent, ammonialike odor (Steinkraus, 1996). *Bacillus* spp. strain was found to be the dominant microflora of

Thua-nao. This bacteria can not produce vitamin B₁₂ which is essential for vegetarian diets based on soybean substrates. There have been previous research about vitamin B₁₂ in another kind of fermented soybean, tempeh. It is produced through fermentation of soybean cake by *Rhizopus oligosporus*. Various kinds of bacteria (*Klebsiella pneumoniae*, *Citrobacter freundii*, *Bacillus megaterium*, *Streptomyces olivaceus* etc.) have been selected and used in mixed cultures with tempeh mold to enrich vitamin B₁₂ in tempeh product (Liem et al., 1977; Okada et al., 1985b; Supramo, 1988; Keuth and Bisping, 1993). Keuth and Bisping (1993) test 37 different tempeh isolates according to their vitamin B₁₂ producing abilities and determined *C. freundii* 259 strains. Prior to extensive applications of *C. freundii* as an additional constituent of tempeh inocula, pathogenic influences have to be excluded. The first investigations into the pathogenicity of *Citrobacter* were carried out by Keuth and Bisping (1994). By means of polymerase chain reaction analysis they could not detect any specific genes encoding typical enterotoxins and shiga-like toxins.

The aim of this work was (1) to determine the contents of protein digestibility and vitamin B₁₂ from coculture fermentation of *Bacillus* sp. B4 and *Klebsiella* sp.KB2 under various conditions, (2) to reduce the strong ammonia smell by addition another local Thua-nao yeast isolate, *Candida* sp.KB1 to the optimal cocultures obtained from (1).

MATERIALS AND METHODS

1. Microorganisms

Bacillus sp. B4 : This strain with potent protease activity was isolated from local Thua-nao (Yongsmith et al., 1999) and used throughout the experiments. The culture was kept in the stock nutrient agar medium. Bacterial inoculum preparation was carried out in nutrient broth. It was identified as *Bacillus amyloliquefaciens*.

Klebsiella sp.KB2 : The highest vitamin B₁₂ producer was selected amongst 8 *Klebsiella* spp. isolates from various fermented soybean products (Apirak, 2001). Bacterial stock cultures were maintained on Tryptic-soy agar slants (17 g peptone from casein, 3 g peptone from soymeal, 2.5 g D(+)Glucose, 5 g Sodium chloride, 2.5 g Dipotassium hydrogen phosphate and 15 g l⁻¹ agar)

Candida sp.KB1: This strain was isolated from local Thua-nao with oligosaccharides (raffinose, stachyose) utilizing and pleasant odor contributing capacities. It was kept in the stock yeast extract malt extract

agar medium slant (5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g raffinose and 15 g l⁻¹ agar)

2. Preparation of soybean

Dry dehulled soybeans seed were washed with clean water and soaked overnight. After decanting the water, approximate 125 g (wet weight) of soaked beans were put into each of several 500 ml flask and autoclaved at 121°C for 50 minutes.

3. Thua-nao produced using monoculture fermentation

Soybean (125g wet weight) were inoculated with bacteria suspension of *Bacillus* sp. B4 or *Klebsiella* sp.KB2 (inoculum size 5% of 10⁶ cell ml⁻¹ of cell suspension). The inoculated bean were incubated at room temperature (30°C).

4. Preparation of Thua-nao by mixed cultured fermentation

Soybean (125g wet weight) were inoculated with bacteria suspension of *Bacillus* sp. B4 and *Klebsiella* sp.KB2 (each inoculum size 5% of 10⁶ cell ml⁻¹ of cell suspension). The inoculated bean were incubated at room temperature.

5. Variation of fermentation parameters.

For analysis the influence of incubation temperature, fermented soybean was carried out at 25, room temperature, 30, 37 and 45 °C. The influence of different numbers of bacterial cells (*Klebsiella* sp.KB2) on vitamin B₁₂ formation was investigated by inoculating soybean with suspensions of 10⁶ to 10⁹ cell ml⁻¹. Cobalt (II) sulfate heptahydrate and 5,6-dimethylbenzimidazole at a concentration range of 0 to 0.4 mg of bacterial suspension ml⁻¹ was added to the bacterial suspension, with which the beans were incubated before solid substrate fermentation with the *Bacillus* sp. B4.

6. Effect of *Candida* sp.KB1 on the fermentation.

Soybean (125g wet weight) were inoculated with bacteria suspension of *Bacillus* sp. B4 (inoculum size 5% of 10⁶ cell ml⁻¹ of cell suspension), *Klebsiella* sp.KB2 (under optimum parameter obtained from experiment 5.) and *Candida* sp.KB1 (inoculum size 5% of 10⁶ cell ml⁻¹ of cell suspension). The inoculated beans were incubated at optimum condition

7. Analysis

7.1 Microbial examination

The fermenting soybeans was examined after incubation for 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 72 h. The growth of bacteria in fermentd soybean was examined by spread out on plate count agar, while yeast examined by spread out on yeast extract malt extract agar.

7.2 Determination of vitamin B₁₂.

One gram pulverized sample was homogenized with 9.0 ml extraction solution (0.1 M acetate buffer, pH 5.5 containing 0.1% KCN) and autoclaved for 15 min. The samples were cooled and centrifuged at 7,000 rpm for 10 min. Then the clear solution was diluted to desired concentration. Vitamin B₁₂ in Thua-nao was determined by the microbiological assays method with *Lactobacillus leichmanii* (ATCC 7830) as the test organism. The assay procedure followed the official methods of analysis of the AOAC(1975).

7.3 Determination of soluble protein

The assay for soluble protein was based on Lowry et al., 1951.

7.4 Determination of proteolytic activity

The assay for proteolytic activity was based on Keay and Wildi (1970). Beans (2g wet weight) were blended with 10 ml of 0.05 M phosphate buffer, pH 7.0 and filtrated with Whatman No.4 filter paper. 1 ml of the crude extract was added to 1 ml of 1.5% soluble casein and incubated at 40°C for 10 minutes. The reaction was terminated by adding 2 ml of 0.4M trichloroacetic acid soluble (TCA). The mixture was filtered through Whatman No.1 filter paper. The filtrate mixture 0.5 ml was added 2.5 ml of 0.4M NaCO₃ solution, 1N folin-ciocalteu reagent 0.5 ml and incubated at 40°C for 10 minutes. The optical density of the mixture was measured at 660 nm. The Blank contained the same mixture but with the phosphate buffer 0.05 M simultaneously with the enzyme extract. One unit of proteolytic activity was defined as the amount which produced 1 µg of tyrosine per minute under the assay conditions

RESULTS

1. Thua-nao fermentation of monoculture system.

Thua-nao was fermented with inoculum size 5% (v/w) pure culture of *Bacillus* sp. B4 or *Klebsiella* sp.KB2. Fig.1 shows that the vitamin B₁₂ content of Thua-nao fermented with pure culture of *Klebsiella* sp.KB2 was 10.15 µg/100gdw higher than that fermented with only *Bacillus* sp. B4. Eventhough monoculture of *Bacillus* sp. B4 was unable to produce vitamin B₁₂ in the product, the *Bacillus* sp. B4 reached a population of 5.8×10^{10} cell/gdw within 36 h incubation earlier than *Klebsiella* sp.KB2 which reached its maximum growth at 2.1×10^{10} cell/gdw after 48 h incubation. Thua-nao was fermented with *Bacillus* sp. B4 showed high proteolytic activity 826.40 units/gdw and good characteristics in the release of soluble protein when compared with fermented soybean by *Klebsiella* sp.KB2.

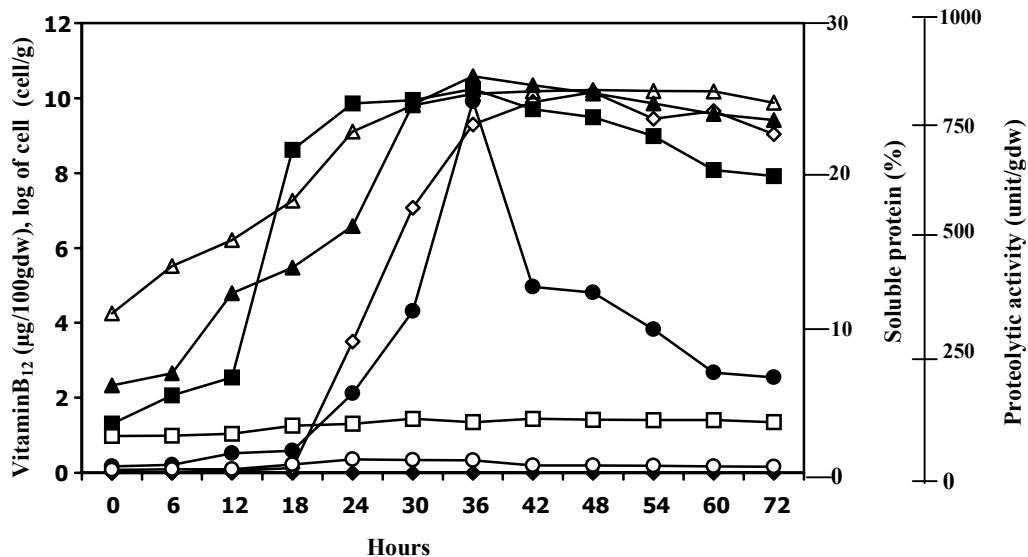


Fig.1 The amount of vitaminB₁₂, soluble protein, proteolytic activity and cell number of thua-nao produced using monoculture fermentation of *Bacillus* sp. B4 and *Klebsiella* sp.KB2.

- ◆ , vitaminB₁₂ of thua-nao produced by *Bacillus* sp. B4
- ◇ , vitaminB₁₂ of thua-nao produced by *Klebsiella* sp.KB2
- ▲ , cell number of *Bacillus* sp. B4
- △ , cell number of *Klebsiella* sp.KB2
- , soluble protein of thua-nao produced by *Bacillus* sp. B4
- , soluble protein of thua-nao produced by *K. pneumoniae*
- , proteolytic activity of thua-nao produced by *Bacillus* sp. B4
- , proteolytic activity of thua-nao produced by *K. pneumoniae*

2. Thua-nao fermentation with cocultures of *Bacillus* sp. B4 and *Klebsiella* sp.KB2.

The cocultures fermentations of soybeans differed remarkably to monoculture fermentation. Growth of the *Bacillus* sp. B4 and *Klebsiella* sp.KB2 by cocultures was lower than monoculture fermentation. Vitamin B₁₂ content of Thua-nao fermented with a mixed culture of *Bacillus* sp. B4 and *Klebsiella* sp.KB2 was 15.45 µg/100gdw higher than that fermented with *Klebsiella* sp. KB2

only (10.15 $\mu\text{g}/100\text{gdw}$). Moreover, the proteolytic activity and soluble protein accumulation of cocultures were improved than that of individual *Bacillus* sp.B4 as shown in Fig.2.

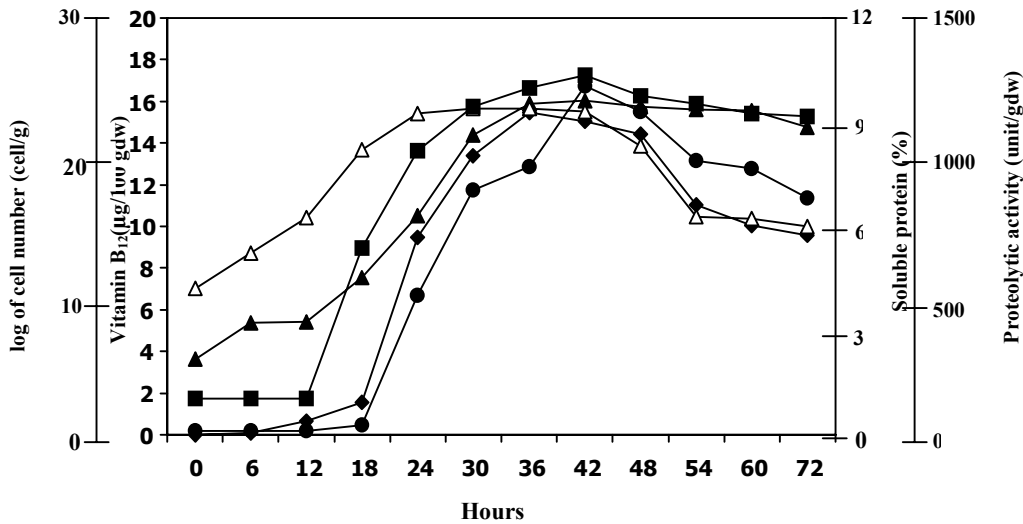


Fig.2 The amount of vitaminB₁₂, soluble protein, proteolytic activity and cell number of thua-nao produced using cocultures of *Bacillus* sp. B4 and *Klebsiella* sp.KB2.

◆ vitaminB₁₂, ▲ cell number of *Bacillus* sp. B4,
 △ cell number of *Klebsiella* sp.KB2, ■ soluble protein,
 ● proteolytic activity

3. Variation of fermentation parameters

The influence of fermentation parameters such as incubation temperature, initial numbers of initial cells (*Klebsiella* sp.KB2), inoculum volume (% of initial inoculum), Cobalt(II)-sulfate-heptahydrate, and 5,6-dimethylbenzimidazole production was carried out. Results showed that inoculum size, 8% of 10^8 cells/ml of *Klebsiella* sp.KB2 with the addition of vitamin B₁₂ precursor such as $\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$ and 5,6-dimethylbenzimidazole (DB1) at 0.3 and 0.2 mg/ml respectively of bacterial suspension affected vitamin B₁₂ content as high as 91.43 $\mu\text{g}/100 \text{gdw}$. (Fig. 3)

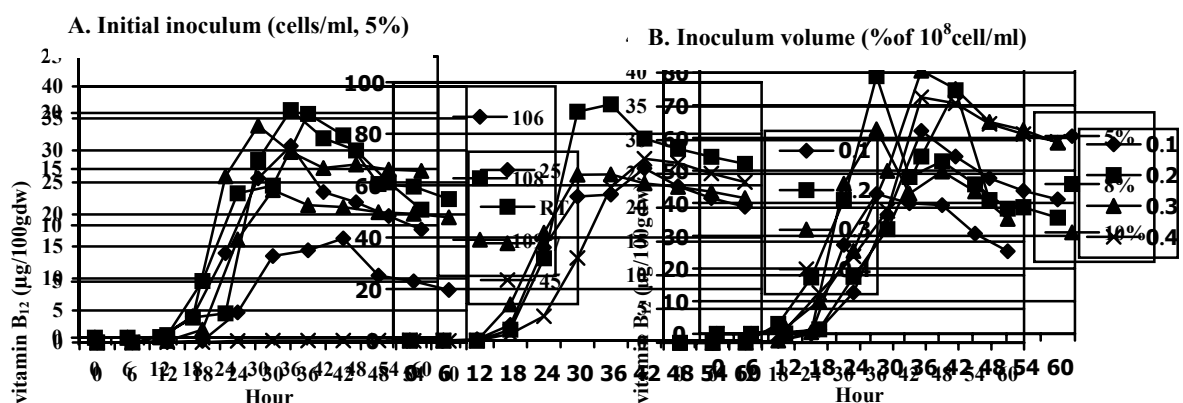


Fig. 3 Factors affecting vitamin B₁₂ fermentation of coculture fermented when inoculated with 5% of 10⁶ cells/ml of *Bacillus* sp. B4.
 A,B : initial inoculum and inoculum volume of *Klebsiella* sp.KB2. ,
 C : Temperature and D,E : chemical addition

In order to reduce the strong smell of ammonia in the high vitaminB₁₂ Thua-nao fermentation, an innovation system of mixed cultures of *Bacillus amyloliquefaciens* B4, *Klebsiella* sp.KB2 and *Candida* sp.KB1 was subsequently used. Fig.4 show after 36 hours incubation at room temperature, the developed Thua-nao fermentation could reduce ammonia odor, while not affect on vitaminB₁₂ or proteolytic enzyme yields.

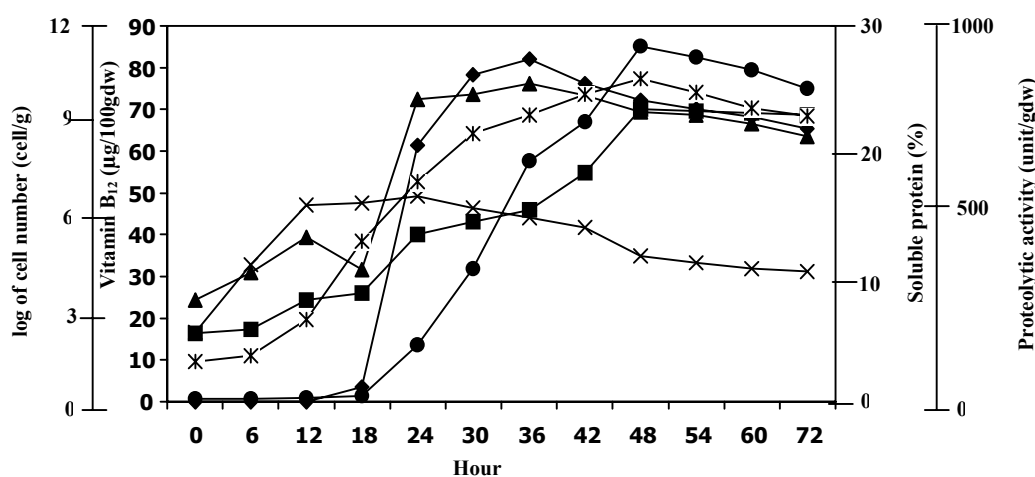


Fig.4 Fermentation time course of vitaminB₁₂, soluble protein, proteolytic activity and cell number of thua-nao produced using mixed cultures of *Bacillus* sp. B4, *Klebsiella* sp.KB2 and *Candida* sp KB1.
 ◆, vitaminB₁₂, ■ cell number of *Bacillus* sp. B4,
 ▲ cell number of *Klebsiella* sp.KB2, X cell number of *Candida* sp.KB1, * soluble protein, ● proteolytic activity

Fermentation time course of vitamin B₁₂ production between monoculture, coculture of *Bacillus* sp. B4 and *Klebsiella* sp. KB2 and mixed cultures of the three different good characteristic were thus compared in Fig. 5.

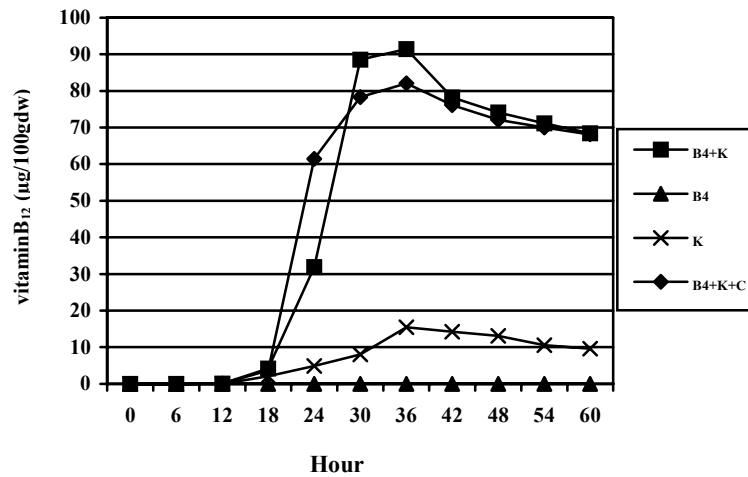


Fig. 5 Fermentation time course of vitamin B₁₂ production by monoculture, cocultures and mixed cultures of *Bacillus* sp. B4 (B4), *Klebsiella* sp.KB2 (K) and *Candida* sp.KB1 (C).

Discussion

The results of our investigation demonstrate that both *Bacillus* sp.B4 and *Klebsiella* sp.KB2 are responsible for vitamin B₁₂ formation in Thua-nao. In fermentation with monoculture of *Bacillus* sp.B4, there was an increase in proteolytic enzyme (826.40 units/gdw) and soluble protein but none of vitamin B₁₂ content. In contrast to this, monoculture of *Klebsiella* sp.KB2 was essential for vitamin B₁₂ production in fermented beans (10.15 µg/100gdw). Coculture of *Klebsiella* sp.KB2 to Thua-nao fermented by *Bacillus* sp.B4 improves the vitamin B₁₂ (15.45 µg/100gdw), proteolytic activity (1,255.08 unit/gdw) and soluble protein (25.87 mg/gdw). During Thua-nao fermentation by this cocultures, *Bacillus* sp.B4 exhibited an increase of proteolytic enzyme as well as soluble protein while *Klebsiella* sp.KB2 was essential for vitamin B₁₂ production.

In our investigation, conditions for the vitamin B₁₂ production by this model of mixed cultures was thus optimized. Best conditions were that 8% of 10⁸ cell/ml of *Klebsiella* sp.KB2 with the addition of vitamin B₁₂ precursors, 0.3 mg/ml CoSO₄.7H₂O and 0.2 mg/ml 5,6-dimethylbenimidazole (DBI) of bacterial suspension resulted vitamin B₁₂

91.43 $\mu\text{g}/100\text{gdw}$ of Thua-nao. Vitamin B₁₂ production is correlated with inoculum sizes, optimum content of cobalt and 5,6-dimethyl benzimidazole. The vitamin B₁₂ content of Thua-nao that fermented with *Bacillus* sp. B4 and *Klebsiella* sp.KB2 under optimum condition was nine times as high as control fermentation with *Klebsiella* sp.KB2 only. However, 91.43 $\mu\text{g}/100$ gdw was produced by *Klebsiella* sp.KB2 after 36 h which is equivalent to about 0.32 μg per gram wet weigh and sufficient for the daily requirement of 1 μg (Herbert, 1984) if are eats 3-4 g of Thua-nao per wet weight.

Thua-nao as sold in market places often has an unpleasant smell and, when heated give a strong and persistent smell of ammonia. This smell could not be noticed when soybeans fermented with either monoculture of *Bacillus* sp. B4 or a mixed cultures of *Bacillus* sp. B4 and *Klebsiella* sp.KB2. Interestingly, an identical objectionable smell emerged whenever the fermenting mass was inoculated with *Candida* sp.KB1(isolated from Thua-nao and oligosaccharides utilizing capacity). The presence of *Candida* sp.KB1 had detectable effect on the reduction of strong ammonia smell of Thua-nao product but no interference effects on the growth of *Bacillus* sp. B4, *Klebsiella* sp.KB2, proteolytic activity and content of vitamin B₁₂ of the fermentation.

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