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Improvement of High Vitamin B_{12} Thua nao by Mixed Cultures of Soybean Oligosaccharide and the Use of 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 food with ammonia smell but without vitamin B_{12} 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 amyloliquefaciens*, a high vitamin B_{12} 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 B_{12} contents in the fermented soybean up to 91.43 µg/100gdw as nine times higher than control fermentation inoculated with *Klebsiella* sp. KB2 alone. Furthermore, the local Thua-nao isolate, *Candida* sp. KB1 could significantly decrease the strong ammonia smell without interference on the vitamin B_{12} content of the product.

Key words: Thua nao production, mixed cultures, vitamin B_{12} enrichment

INTRODUCTION

Thua nao is a Thai traditional non-salted product fermented by *Bacillus* spp. It is produced in the northern Thailand and utilized as a substituent for fish paste (Sundhagul *et al*., 1972). To make traditional Thua nao, soybeans are washed, soaked overnight, cooked thoroughly until soft, drained and fermented naturally without inoculation and leave on 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, ammonia-like odor (Steinkraus, 1996). *Bacillus* spp. strains were found to be the dominant microflora of Thua - nao. They can not produce vitamin B_{12} which is essential for vegetarian diets based on soybean substrates. There have been previously research about vitamin B_{12} in another kind of fermented soybean so called tempeh. It is produced through fermentation of soybean cake by *Rhizopus oligosporus*. Various kinds of bacteria eg. *Klebsiella pneumoniae, Citrobacter freundii, Bacillus megaterium, Streptomyces olivacens,* etc., have been selected and used as mixed cultures with

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the tempeh mold to enrich vitamin B_{12} in the tempeh product (Liem *et al*., 1977; Okada *et al*., 1985; Supramo, 1988; Keuth and Bisping, 1993). Keuth and Bisping (1993) tested 37 different tempeh isolates according to their vitamin B_{12} producing abilities and determined as *C. freundii* 259 strains. Prior to extensive applications of *C. freundii* as an additional constituent of tempeh inocula, pathogenous 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 aims of this work were (1) to determine the contents of protein digestibility and vitamin B_{12} from coculture fermentation of *Bacillus* sp. B4 and *Klebsiella* sp.KB2 under various condition, and (2) to reduce the strong ammonia smell by addition of another local Thua-nao yeast isolate, *Candida* sp.KB1 to the optimal cocultures obtained from the first experiment.

MATERIALS AND METHODS

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 B_{12} producer was selected among 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 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 agar).

Preparation of soybean

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

Preparation of Thua-nao using monoculture fermentation

Soybean (125g wet weight) was inoculated with bacteria suspension of *Bacillus* sp. B4 or *Klebsiella* sp. KB2 (inoculum size 5% of 10^6 cell/ml of cell suspension). The inoculated bean was incubated at room temperature (30°C).

Preparation of Thua-nao by coculture fermentation

Soybean (125g wet weight) was inoculated with the bacterial suspensions of *Bacillus* sp. B4 and *Klebsiella* sp. KB2 and incubated at room temperature. The inoculum size of each strain was 5% of 10^6 cell/ml of cell suspension.

Variation of fermentation parameters

For analysis of the influence of incubation temperatures, fermentation of soybean was carried out at 25, room temperature, 30, 37 and 45°C. The influence of different concentrations of bacterial cells (*Klebsiella* sp. KB2) on vitamin B_{12} formation was investigated by inoculating soybean with suspensions of 10^6 to 10^9 cell/ml. Cobalt (II) sulfate heptahydrate and 5,6-dimethylbenzimidazole at a concentration range of 0 to 0.4 mg ml^{-1} of which bacterial suspension were added to the bacterial suspension, with which the beans were

incubated before solid substrate fermentation with the *Bacillus* sp. B4.

Effect of *Candida* sp. KB1 on the fermentation

Soybean (125g wet weight) was inoculated with bacterial cell suspension of *Bacillus* sp. B4 (inoculum size 5% of 10^6 cell/ml of cell suspension), *Klebsiella* sp. KB2 (under optimum parameter obtained from experiment 5.) and *Candida* sp. KB1 (inoculum size 5% of 10^6 cell/ml of cell suspension). The inoculated beans were incubated at the optimum conditions.

Analysis

1. Microbial examination

Growth of the bacterial and yeast in fermented soybean was determined by spread plate technique on plate count agar and yeast extract malt extract agar, repectively after incubation for 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 72 h.

2. Determination of vitamin B_{12}

One gram of pulverized sample was homogenized with 9.0 ml 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. Clear supernatant solution was diluted to a desired concentration.Vitamin B_{12} in Thua-nao was determined by the microbiological assay method with *Lactobacillus leichmanii* (ATCC 7830) as the test organism and followed the official analysis methods of the ATCC (1972).

3. Determination of soluble protein

Assay for soluble protein was based on Lowry *et al*. (1951).

4. Determination of proteolytic activity

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 filtered through Whatman No.4 filter paper. One ml of the crude extract was added to 1 ml of 1.5% soluble casein and incubated at 40°C for 10 min. The reaction was stopped by adding 2 ml of 0.4M trichloroacetic acid soluble (TCA). The mixture was filtered through Whatman No.1 filter paper. The filtrate mixture of 0.5 ml was added to the solution of 2.5 ml of 0.4M Na_2CO_3 and 0.5 ml of 1N folin-ciocaltau reagent then incubated at 40°C for 10 min. Optical density of the mixture was measured at 660 nm. Blank contained the same mixture supplemented with the 0.05 M phosphate buffer without the enzyme extract. One unit of proteolytic activity was defined as the amount of enzyme which produce 1 µg of tyrosine per min under the assay conditions.

RESULTS

1. Thua-nao fermentation of monoculture system

Thua-nao was fermented with 5% (v/w) pure inoculum of *Bacillus* sp. B4 or *Klebsiella* sp.KB2. Figure1 showed that the vitamin B_{12} content of Thua-nao fermented with pure culture of *Klebsiella* sp.KB2 was 10.15 µg/100gdw at 36 h and no vitamin B_{12} was detected from *Bacillus* sp. B4 fermentation. Even though monoculture of *Bacillus* sp. B4 was unable to produce vitamin B_{12} 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 fermented with *Bacillus* sp. B4 showed high proteolytic activity 826.40 units/gdw and good characteristics in the release of soluble protein when compared withsoybean fermented by *Klebsiella* sp. KB2.

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

The coculture fermentations of soybean differed remarkably to those monoculture fermentations. Growth of the *Bacillus* sp. B4 and *Klebsiella* sp. KB2 by coculture was lower than that in the monoculture fermentation. Vitamin B_{12} content of Thua-nao fermented with coculture of

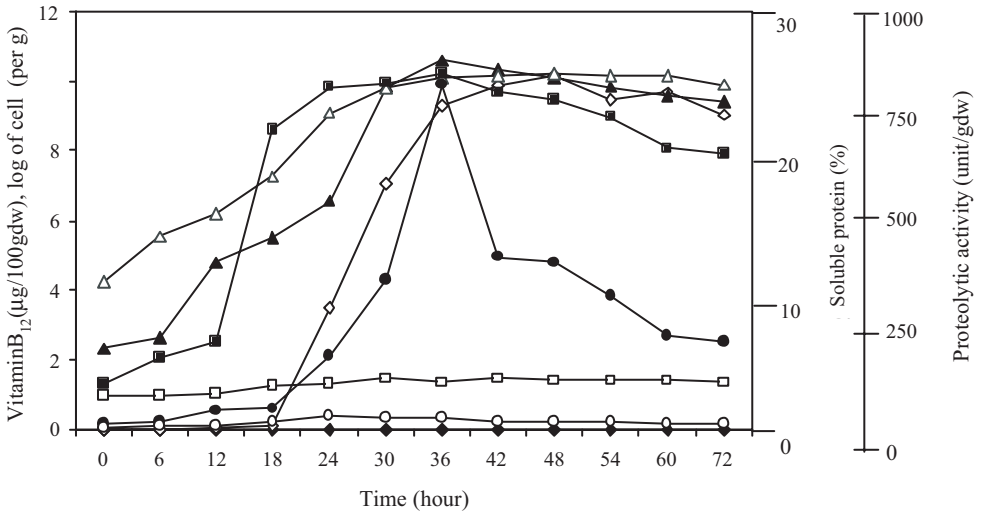


Figure1 Time courses of Thua-nao fermentation using monoculture of *Bacillus* sp. B4 or *Klebsiella* sp. KB2.

◆ , vitaminB_{12} of thua-nao produced by *Bacillus* sp. B4
◇ , vitaminB_{12} 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 *Klebsiella* sp. KB2
● , proteolytic activity of thua-nao produced by *Bacillus* sp. B4
○ , proteolytic activity of thua-nao produced by *Klebsiella* sp. KB2

Bacillus sp. B4 and *Klebsiella* sp. KB2 was 15.45 µg/100gdw which was 50% higher than that fermented with only *Klebsiella* sp. KB2 only (10.15 µg/100gdw).

Moreover, the proteolytic activity and soluble protein accumulation in the coculture were also improved than that of individual *Bacillus* sp. B4 (Figure 2).

3. Variation of fermentation factors

The influence of fermentation factors such as incubation temperature, initial numbers of bacterial cells (*Klebsiella* sp. KB2), inoculum size (% of initial inoculum), Cobalt(II)-sulfate-heptahydrate and 5,6-dimethylbenzimidazole (DBI) affecting the vitamin B_{12} production was determined. Results showed that inoculum size, 8% of 10^8 cells/ml of *Klebsiella* sp. KB2 together with the addition of vitamin B_{12} precursors, $CoSO_4.7H_2O$ and 5,6-dimethylbenzimidazole (DBI) at 0.3 and 0.2 mg/ml of bacterial suspension respectively, affected vitamin B_{12} content to as high as 91.43 µg/100 gdw (Figure 3).

4. Effect of *Candida* sp. KB1 on high vitamin B_{12} Thua-nao fermentation

In order to reduce the strong smell of ammonia in the high vitamin B_{12} Thua-nao fermentation, an innovative system of mixed cultures of *Bacillus amyloliquefaciens* B4, *Klebsiella* sp. KB2 and *Candida* sp. KB1 was subsequently used. Figure 4 showed that after 36

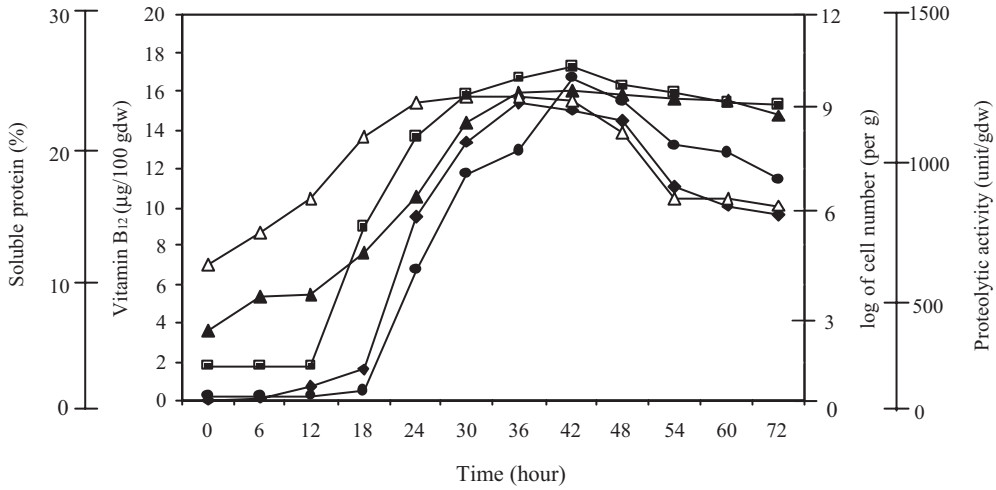


Figure 2 Time course of Thua nao fermentation using cocultures of *Bacillus* sp. B4 and *Klebsiella* sp. KB2.
◆ vitaminB_{12}, ▲ cell number of *Bacillus* sp. B4, △ cell number of *Klebsiella* sp. ■ KB2, soluble protein, ● proteolytic activity

hours of incubation at room temperature, the developed Thua-nao fermentation could reduce ammonia odor, without affecting the vitaminB_{12} production or proteolytic enzyme activity.

Fermentation time courses of vitamin B_{12} production between *Bacillus* sp. B4 monoculture, coculture of *Bacillus* sp. B4 and *Klebsiella* sp. KB2 and mixed cultures of the three different good characteristic strains of *Bacillus* sp. B4, *Klebsiella* sp. KB2 and *Candida* sp. KB1 were thus compared in Figure 5.

DISCUSSION

The results of our investigation demonstrated that *Klebsiella* sp. KB2 was responsible for vitamin B_{12} formation in Thua-nao. In fermentation with monoculture of *Bacillus* sp. B4, there was an increase in proteolytic enzyme activity (826.40 units/gdw) and soluble protein (25.59%) but not on vitamin B_{12} production. In contrast, monoculture of *Klebsiella* sp. KB2 was essential for vitamin B_{12} production in fermented beans (10.15 µg/100gdw). Coculture of *Klebsiella* sp. KB2 to Thua-nao fermented by *Bacillus* sp. B4 improved the vitamin B_{12} production (15.45 µg/100gdw), proteolytic activity (1,255.08 unit/gdw) and soluble protein (25.87%). During Thua-nao fermentation by this coculture, *Bacillus* sp. B4 exhibited an increase of proteolytic enzyme as well as soluble protein while *Klebsiella* sp. KB2 was essential for vitamin B_{12} production.

In our investigation, conditions for the vitamin B_{12} production by this model of the mixed cultures was thus optimized. Best conditions were that 8% of 10^8 cell/ml inoculum of *Klebsiella* sp. KB2 with the addition of vitamin B_{12} precursors, 0.3 mg/ml $CoSO_4.7H_2O$ and 0.2 mg/ml 5,6-dimethylbenimidazole (DBI) of bacterial suspension result in 91.43 µg/100gdw vitamin B_{12} to the Thua-nao. Vitamin B_{12} production was correlated with inoculum size, optimum content of cobalt and 5,6-dimethylbenzimidazole. The vitamin B_{12} content of Thua-nao fermented by *Bacillus* sp. B4 and *Klebsiella* sp. KB2 under the optimum condition was nine times higher than the

control which was fermented with only *Klebsiella* sp. KB2. However, 91.43 µg vitamin B_{12}/100gdw was produced by *Klebsiella* sp. KB2 after 36 h which was equivalent to about 0.32 µg vitamin B_{12}/wet weight of bean and therefore sufficient for daily requirement of 1 µg (Herbert *et al*., 1984) if we consume 3-4 g of Thua-nao wet weight.

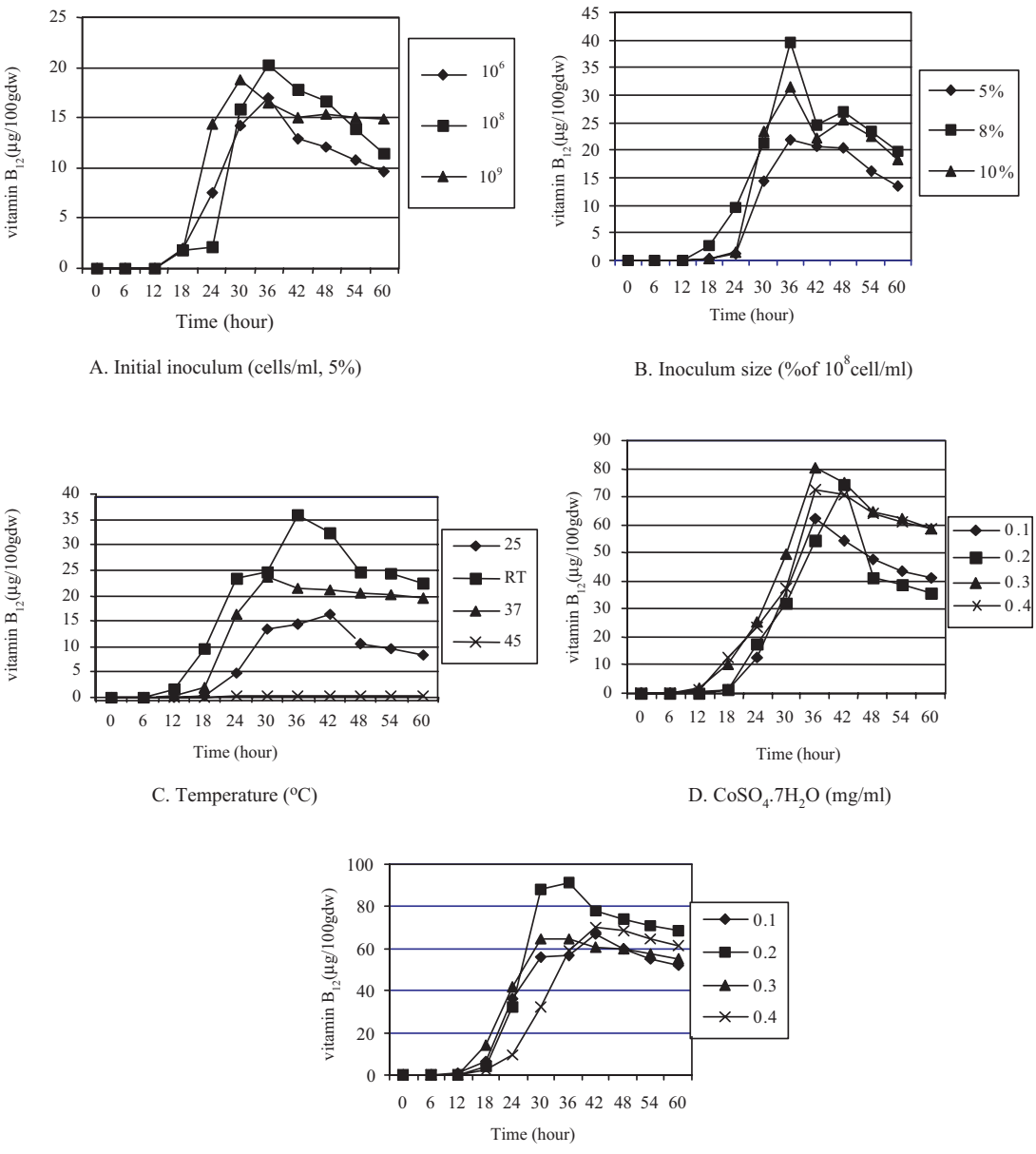


Figure 3 Factors affecting vitamin B_{12} fermentation by coculture fermentation with 5% of 10^6 cells/ml of *Bacillus* sp. B4 as basal starter.
A, B : initial inoculum and inoculum size of *Klebsiella* sp.KB2., C : Temperature, D : $CoSO_4.7H_2O$ and E : Dimethylbenzimidazolyl

Thua-nao as sold in market places often has an unpleasant smell and, even a stronger and persistent smell of ammonia. This smell could be noticed when soybeans fermented with either monoculture of *Bacillus* sp. B4 or the mixed cultures of *Bacillus* sp. B4 and *Klebsiella* sp. KB2. Interestingly, such smell was remarkably reduced when the fermenting mass was inoculated with *Candida* sp. KB1 which as traditional isolated from Thua-nao and *Candida* sp. KB1 also showed oligosaccharides utilizing capacity. The presence of *Candida* sp. KB1 had thus an detectable effect on the reduction of strong ammonia smell of the Thua-nao product but no interferent effects on the

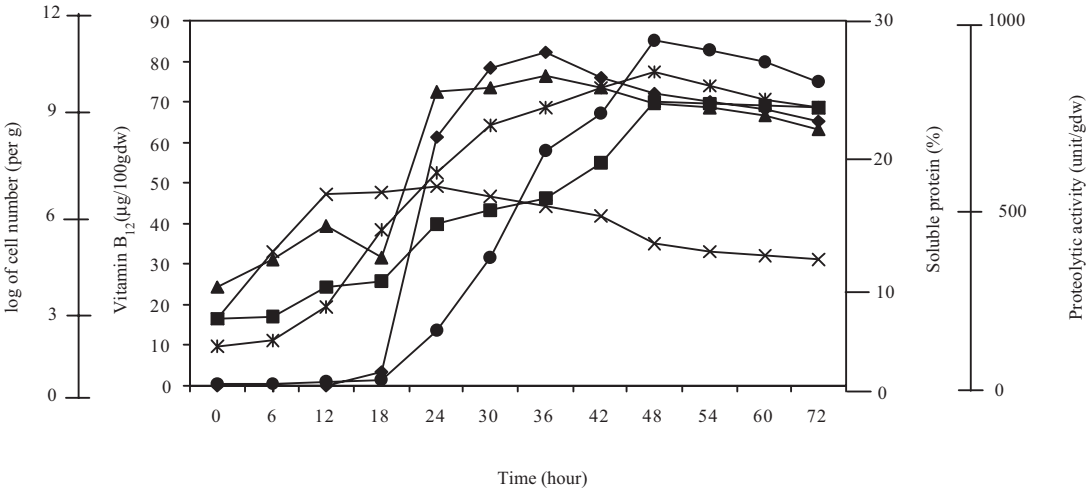


Figure 4 Time course of Thua-nao fermentation using mixed cultures of *Bacillus* sp. B4 ,*Klebsiella* sp. KB2 and *Candida* sp.KB1.
◆, vitaminB_{12}, ■ cell number of *Bacillus* sp. B4, ▲ cell number of *Klebsiella* sp. KB2, **X** cell number of *Candida* sp. KB1, * soluble protein, ● proteolytic activity

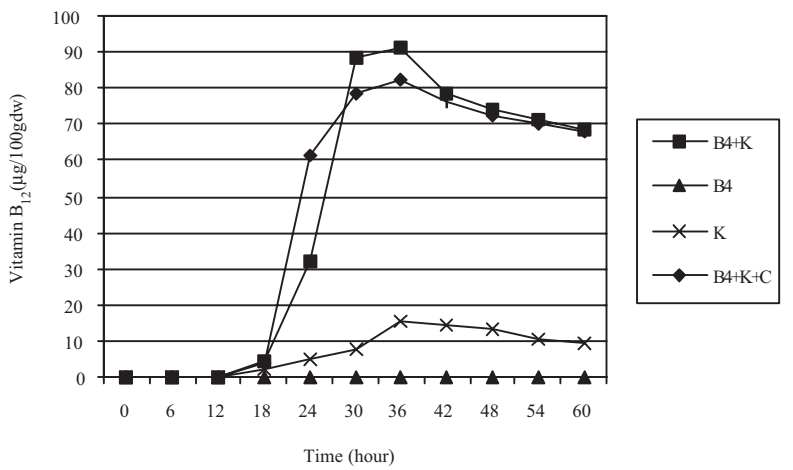


Figure 5 Fermentation time course of vitamin B_{12} production by monoculture, coculture and mixed culture of *Bacillus* sp. B4 (B4), *Klebsiella* sp. KB2 (K) and *Candida* sp. KB1 (C).

growth of *Bacillus* sp. B4, *Klebsiella* sp. KB2, proteolytic activity and vitamin B_{12} content in the product.

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