Screening of Bacteria Producing Vitamin B_{12} from Tua-Nao in Thailand

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Abstract

From Tua-Nao, a non-salt fermented soybean food of Thailand, bacteria which can be utilized for the improvement of Natto bacterium by cell fusion technique were screened. Screening was carried out for vitamin B_{12}-producing bacteria which are heat-resistant and lysozyme-sensitive. Tua-Nao samples were collected at Lam Phun, Phrao, Doi Saket and Mae Hong Son in northern Thailand. Bacterial counts in Tua-Nao samples were markedly different in number and the ratio of the number of heat-resistant bacteria to the number of total bacteria was also different. Vitamin B_{12} content varied and some samples did not contain vitamin B_{12} at all. The lysozyme sensitivities and vitamin B_{12}-producing ability of bacteria separated from Tua-Nao samples after heating were examined. Among 120 isolates, about 20 were lysozyme-sensitive and one (TN02-13) produced vitamin B_{12}. However the isolate (TN02-13) was a mixture of two bacterial isolates (TN02-13a and TN02-13b) and TN02-13b was found to produce vitamin B_{12} and to be lysozyme-sensitive. Thus a vitamin B_{12}-producing bacterium was obtained from Tua-Nao. This bacterium was regenerated from a protoplast form into a bacillary form.

Additional key words : fermented soybeans, Natto, cell fusion

INTRODUCTION

It was observed[1] that Tua-Nao in Thailand contained vitamin B_{12} as in the case of Tempeh in Indonesia. Vitamin B_{12}-producing bacteria in Tempeh which were already examined[2], consisted of *Klebsiella pneumoniae, K.pneumoniae* sub.*ozaenae, K.terrigena* or *K.planticola, Enterobacter cloacae*, etc.[3] However these bacteria are Gram negative and can not be transformed into protoplasts. Therefore

they could not be used for cell fusion. Here, Tua-Nao samples were screened for the presence of vitamin B_{12}-producing bacteria that could be used for cell fusion with Natto bacteria which are unable to produce vitamin B_{12}.

MATERIALS AND METHODS

Materials: Tua-Nao samples were collected at Lam Phun, Phrao, Doi Saket and Mae Hong Son in the northern area of Thailand. They were obtained directly from the production centers during the period November 7 to 21 in 1991. The appearance and quality were highest for Mae Hong Son, lowest for Phrao and intermediate for Lam Phun and Doi Saket.

Activation and preparation of B-12 assay microorganism: Lactobacillus leichmannii ATCC 7830 was used as a standard assay microorganism. To recover the activation, the agar stab of the stock culture was transferred into a new agar medium "Nissui stock medium" (NISSUI SEIYAKU Co., Tokyo, Japan) successively 10 times every day. Then it was inoculated in 5ml of broth culture "Nissui inoculum medium" (tubes 13mmD x 100mmH with caps, 5ml x 2) and incubated at 37℃ for 16hr. Cells were harvested and washed two times with sterile "Nissui basal medium". Cells were suspended in 165ml sterile "Nissui basal medium", then 55ml of 50% glycerin was added (final 12.5% glycerin). The mixtures (2.2ml each) were put into vials and stored at -80℃.

Extraction of vitamin B-12 from Tua-Nao: To 2g of Tua-Nao, 2.5ml of 0.2M acetate buffer (pH4.5), 0.1ml of KCN solution (0.5mg/ml) and 15ml of water were added and the mixture was shaken, then heated at 100℃ for 30min. After cooling, 0.2ml of 10% meta-phosphate and water were added to reach a volume of 20ml. Centrifugation was performed to obtain the supernatant, then solutions at the dilution rates of 1/1, 1/10 and 1/100 were prepared for the bio-assay. They were kept at -20℃ until use.

Bio-assay of vitamin B-12: To the tubes, assay medium (2.5ml of twofold concentrated "Nissui basal medium", NISSUI SEIYAKU Co., Tokyo, Japan), standard vitamin B_{12} (0.02-0.1ng/tube) or test extracts and distilled water were added to reach a volume of 5ml. The tubes were autoclaved at 121℃ for 5min. After cooling in running water, test microorganism for the assay (referred to as assay microorganism hereafter) was inoculated using 1ml syringe mounted on a STEPPER, a stepwise distributor (NIPRO,TERUMO). The tubes were incubated at 37℃ for 23hr, then the growth rate of the microorganism was determined by optical density at 600nm. Vitamin B_{12} content was determined by comparing the growth on standard vitamin B_{12} with unknown samples.

Standard vitamin B-12 solution: 10mg of cyanocobalamin was dissolved in 100ml of 25% ethanol (100 μ g/ml) and 5ml each of the solution was added into capped tubes and stored at 4℃ after wrapping the tubes with alumifoil (to avod light). At the time of use, the solution was diluted (1/1000) two times (0.1ng/ml). For standard curves, 0, 0.2, 0.4, 0.6, 0.8 and 1.0ml were taken, corresponding to 0, 0.02, 0.04, 0.06, 0.08 and 0.1ng vitamin B_{12} per tube.

Extraction of bacteria from Tua-Nao: Bacteria were extracted with sterile 0.1M phosphate (pH7) and half of the extract was heated at 100℃ for 10min, while the other half was not heated. Bacterial counts in both heated and unheated extracts were performed. The bacterial counts in heated extracts corresponded to the *Bacillus* group and those in the unheated extracts to the total number of bacteria.

Cell preparation to extract vitamin-12: Bacterial extracts from Tua-Nao were heated at 100℃ for 20min and 1ml portions were transferred to CP (Cell production) medim (10ml) then cultivated at 37℃ for 1 day. Cells were centrifuged from the 5ml culture and stored at -80℃ to extract vitamin B_{12} later. Other portions of the culture

(1.5mlx2)were mixed with 0.5ml of 50% glycerin, and stored at -80℃ for bacterial sources.

CP (Cell production) medium: A part and B part were prepared and separately autoclaved. Ingredients of A part (700ml) consisted of 5g of glucose, 0.5g of $MgSO_4 \cdot 7H_2O$ and 68.5g of sucrose. Ingredients of B part (300ml) consisted of 2g of $(NH_4)\ SO_4$, 3.5g of K_2HPO_4, 1.5g of KH_2PO_4, 1g of Na-citrate ($2H_2O$), 1ml of biotin (5mg/50ml) and 0.1ml of $CoCl_2$ (0.1% as Co). At the time of use, A and B parts were mixed in the ratio of 7:3. Sucrose was added to achieve a high osmotic pressure so that the cell walls would become prone to lysozyme attack. $CoCl_2$ was added to obtain vitamin B_{12}.

Extraction of vitamin B-12 from bacterial cells: To the bacterial cells (cells from 5ml culture), 1ml of acetate buffer (0.2M, pH4.5), 0.04ml of KCN solution (0.5mg/ml) and 3.86ml of water were added. The suspension was heated at 100℃ for 30min. After cooling, 0.1ml of 10% mata-phosphate was added and a clear solution was obtained by centrifugation. The solution was diluted (1/1, 1/10) with water and 0.2 ml each was applied for the vitamin B_{12} test.

Isolation and cultivation of B-12 producing bacteria: Bacteria were isolated from cells which contained vitamin B_{12}. From stock cultures at -80℃ , colonies were prepared, picked up, transferred into 10ml of CP medium and cultured at 37℃ for 1 day. Culture portions (0.7ml) were used for the lysozyme test. Five ml portions were used to collect cells for the vitamin B_{12} test. Other portions (1.5mlx2) were mixed with 0.5ml of 50% glycerin and stored at -80℃ as in the case of the bacterial sources.

Lysozyme test: Portions (0.7ml) of culture were transferred into 1.2ml SM buffer (osmotic buffer), and to them 100 μl of a lysozyme solution (final con. 250 μg/ml or 12,775 units/ml)or 100 μl of water (control) was added. The solutions were incubated at 42℃ for 45 min. Decrease of the optical density at 600nm was observed by comparison with control samples.

SM buffer: Ingredients per 900ml consisted of 154g of sucrose (0.5M), 2.08g of maleic acid (0.02M), 4.44g of $MgSO_4 \cdot 7H_2O$(0.02M). The pH value was adjusted with 0.1N of NaOH solution to 6.5, then the volume was brought to 900ml. Nine ml was added to each tube, and autoclaved.

SMB buffer: This buffer which consists of SM buffer with 1% bovine serum albumin, is used to preserve the structure of the protoplasts. A 1g of aliquot of bovine serum albumin (SIGMA) was dissolved in 10ml of sterile SM buffer, then 1ml each was poured into 9ml of sterile SM buffer through a membrane filter (pore size 0.2 μ). A 0.9ml of aliquot was added to sterile vials that were kept in a refrigerator.

Protoplast formation and regeneration test: Portions (0.7ml) of stock culture with glycerin were transferred into 1.2ml of SMB buffer, and 100 μl of lysozyme solution (final conc. 250 μg/ml or 12,775 units/ml) was added, then incubated at 42℃ for 45 min. After incubation, the lysozyme-treated solutions were diluted successively by taking 0.1ml into 0.9ml of SMB buffer. A 0.1ml portion at 10^{-4} level was mixed with the PR (Protoplast Regeneration) medium, an osmotic medium. Another 0.1ml portion at 10^{-4} level was also mixed with the nutrient agar medium, a non-osmotic medium. The media were incubated for 2-4 days, and the colony number was counted. Colonies counted in the osmotic medium (PR medium) corresponds to the number of regenerated bacteria, and the colonies counted in the non-osmotic medium (nutrient agar medium) corresponds to the number of lysozyme-resistant bacteria.

PR (Protoplast regeneration) medium: A part and B part were separately prepared as follows. Ingredients of A part (700ml) consisted of 5g of glucose, 2.5g of $MgSO_4 \cdot 7H_20$, 30g of polyvinyl

pyrrolidone and 8g of agar. Ingredients of B part (300ml) consisted of 2g of $(NH_4)_2 SO_4$, 5g of casamino acids, 3.5g of KH_2PO_4, 1.5g of KH_2PO_4, 200ml of 1M sodium succinate (pH7.3), 1g of Na-citrate($2H_2O$) and 1ml of biotin (5mg/50ml). A 7ml aliquot of A part was put into screw-capped tubes, while a 3ml aliquot of B part was put into different screw-capped tubes, and autoclaved. At the time of use, A part was dissolved at 100℃, then A and B part were kept at 56℃ and mixed just before being poured into petri dishes.

Fig.1 illustrates a flow chart for the screening of bacteria producing vitamin B_{12}.

RESULTS AND DISCUSSION

a. *Development of vitamin B-12 assay*: The growth of *Lactobacillus leichmannii* ATCC 7830, a standard assay microorganism, fluctuated markedy in each experiment. To alleviate this shortcoming, the microorganism should be frozen after confirmation that the growth depends on the vitamin B_{12} content. One hundred vials containing the microorganism were prepared and stored at -80℃ in 12.5% glycerin. Fig.2 shows a typical curve of the response of the microorganism stored at -80℃. The growth response depended on the

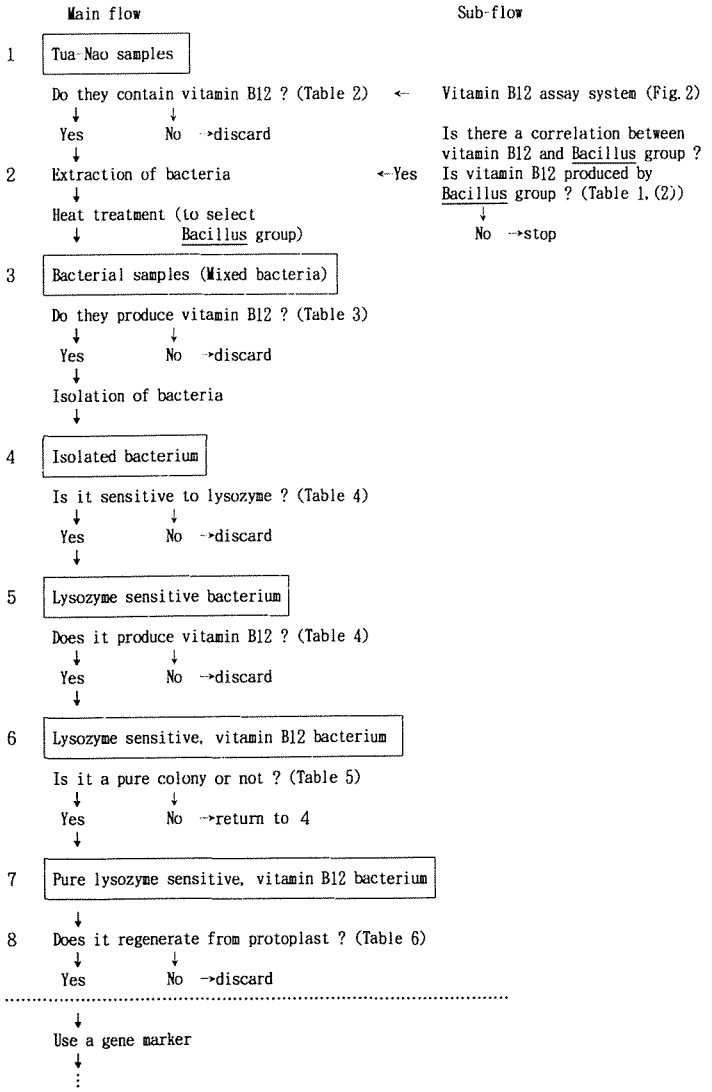

Fig 1. Flow chart for screening of bacteria producing vitamin B_{12}.

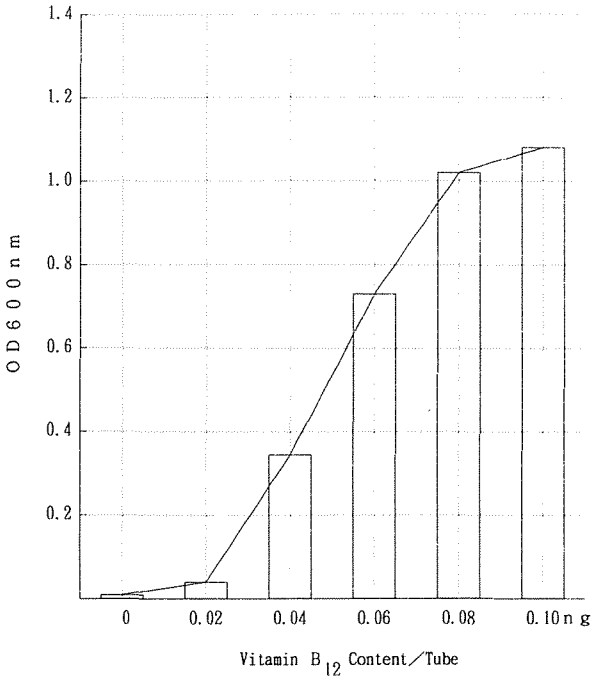

Fig 2. Vitamin B_{12} Response Curve
Test microorganism for assay: Frozen *Lactobacillus leichmannii* ATCC 7830 was used at -80℃ in 12.5% glycerin.

vitamin B_{12} content within limited concentrations (0.02-0.08ng/tube). The response did not change in the current study.

b. *Bacterial count in Tua-Nao and growth on Tua-Nao samples from various origins*: The results of bacterial counts are shown in Table 1. Bacterial counts were examined in heated and unheated extracts. Counts in heated extracts ranged from 10^7 to 10^8/g fresh Tua-Nao, while counts in unheated extracts ranged from 10^7 to 10^9/g fresh Tua-Nao. The ratio (heated /unheated extracts) varied from 0.4% to 100%. High ratio values were recorded in dried Tua-Nao and some wet samples.

The bacterial growth in the Mae Hong Son sample of Tua-Nao was optimum and the ratio (heated/unheated extracts) was high (100, 100%). The growth on the Phrao sample was the worst, and the ratio (heated/unheated extracts) was low (2.0, 2.2, 8.6%) except for the dried samples. The high ratio corresponds to a high ratio of *Bacillus*

Table 1. Bacterial count in Tua-Nao.

Tua-Nao			Bacterial count/g fresh		Ratio(%)
No.	Origin	Procedure	Heated[1]	Unheated	Heated/Unheated
TN01	Lam Phun	One day[2]	3.4×10^7	8.4×10^9	0.4
TN02	Lam Phun 1	Two day[3]	3.3×10^8	5.1×10^8	64.7
TN03	Lam Phun 2	Two day[3]	1.9×10^8	1.1×10^8	>100
TN04	Lam Phun	Mashed	8.7×10^8	5.4×10^9	16.1
TN05	Phrao	Two days[3]	1.9×10^7	2.2×10^8	8.6
TN06	Phrao	Mashed	4.6×10^7	2.1×10^9	2.2
TN07	Phrao	Balls	8.8×10^7	4.3×10^9	2.0
TN08	Phrao 1	Dried	5.8×10^7	3.9×10^7	>100
TN09	Phrao 2	Dried	9.6×10^8	1.2×10^9	80.0
TN10	Doi Saket	Two days[3]	4.5×10^7	3.0×10^8	15.0
TN11	Doi Saket	Mashed	2.7×10^8	5.5×10^8	49.1
TN12	Mae Hong Son	Two days[3]	2.0×10^8	1.8×10^8	>100
TN13	Mae Hong Son	One days[2]	2.4×10^8	8.5×10^7	>100
TN14	Mae Hong Son	Dried	6.3×10^8	6.2×10^8	>100

1) Bacteria were heated at 100℃ for 10 min.
2) One day =After one day of fermentation
3) Two days =After two days of fermentation

Table 2. Vitamin B_{12} Content in Tua-Nao.

Tua-Nao			Bio-assay value	Vitamin B_{12}
No.	(origin	Procedure)	(OD at 600nm)	(μ g/100g fresh)
TN01	(Lam Phun	One day)	0.182	2
TN02	(Lam Phun 1	Two days)	0.101	1
TN03	(Lam Phun 2	Two days)	0.007	0
TN04	(Lam Phun	Mashed)	0.107	1
TN05	(Phrao	Two days)	0.011	0
TN06	(Phrao	Mashed)	0.455	3
TN07	(Pharao	Balls)	0.561	5
TN08	(Pharao 1	Dried)	0.376	3
TN09	(Pharao 2	Dried)	0.600	6
TN10	(Doi Saket	Two days)	0.000	0
TN11	(Doi Saket	Mashed)	0.109	1
TN12	(Mae Hong Son	Two day)	0.017	0
TN13	(Mae Hong Son	One day)	0.060	0
TN14	(Mae Hong Son	Dried)	0.349	3

Sample preparation: Tua-Nao 2g→B_{12} extract→Fill up to 20ml→Dilution 1/10→Assay volume 0.1ml

group and the low ratio to a low ratio of *Bacillus* group.

c. *Vitamin B-12 content in Tua-Nao samples*: The results are shown in Table 2. The values were expressed as μ g per 100 gram of fresh samples. Phrao samples, in which the ratios (heated/unheated extracts) were low, showed relatively higher values than others. Mae Hong Son samples, in which the ratios (heated/unheated extracts) were high, showed a very low value, suggesting that vitamin B_{12} may not be produced by the *Bacillus* group.

However, investigations are still continuing to identify vitamin B_{12}-producing bacteria in the *Bacillus* group in Tua-Nao.

d. *Vitamin B-12 test in mixed bacterial cells from Tua-Nao*: Mixed bacterial cells were prepared from cultures using heated bacterial extracts of Tua-Nao which were found to contain vitamin B_{12}. Vitamin B_{12} was extracted from the mixed bacterial cells then the content was examined. Results are shown in Table 3. Five samples, namely TN01, 02, 06, 07 and 09 were selected as sources of vitamin B_{12}-producing bacteria.

e. *Lysozyme test in bacteria isolated from Tua-Nao*: Bacteria were isolated from the mixed bacterial cells (TN01, 02, 06, 07, 09) which showed a vitamin B_{12} activity. Isolates (total 120) were numbered from 1 to 24 for every sample (TN01, 02, 06, 07 and 09). Then the lysozyme sensitivity was examined. Approximately 20 lysozyme-sensitive bacteria were identified among 120 isolates (Table 4).

f. *Vitamin B-12 test in lysozyme-sensitive bacteria from Tua-Nao*: In twenty lysozyme-sensitive bacteria the vitamin B_{12} activity was examined. One isolate (TN02-13, namely No.13 from TN02) was able to produce vitamin B_{12} (1ng/5ml culture) among the 20 lysozyme-sensitive bacteria (Table 4).

Table 3. Vitamin B_{12} test in mixed bacterial cells extracted from Tua-Nao.

No.	Sample[1)]	(Origin of Tua-Nao)	Vitamin B_{12} (ng/5ml culture)
TN01	Mixed bacterial cells	(Lam Phun One day)	1
TN02	Mixed bacterial cells	(Lam Phun 1 Two day)	1
TN04	Mixed bacterial cells	(Lam Phun Mashed)	0
TN06	Mixed bacterial cells	(Phrao Mashed)	1
TN07	Mixed bacterial cells	(Phrao Balls)	5
TN08	Mixed bacterial cells	(Phrao 1 Dried)	0
TN09	Mixed bacterial cells	(Phrao 2 Dried)	1
TN11	Mixed bacterial cells	(Doi Soket Mashed)	0.5
TN14	Mixed bacterial cells	(Mae Hong Son Dried)	0

1)Bacterial cells extracted from Tua-Nao →Heat treament (100℃, 20min)→Culture→Culture 5ml → Mixed bacterial cells →B_{12} extract →Fill up 5ml →Dilution 1/1, 1/10→Bio-assay 0.2ml

Table 4. Lysozyme Test and B_{12} in Isolated Bacteria from Tua-Nao Samples.

Isolate No.	OD600nm -Lysozyme	OD600nm +Lysozyme	Rate of decrease	Vitamin B_{12} (ng/5ml culture)
TN02-02	0.373	0.221	0.41	0
TN02-13	0.448	0.310	0.31	1
TN02-18	0.373	0.190	0.49	0
TN02-20	0.310	0.175	0.44	0
TN02-23	0.205	0.190	0.07	0
TN02-24	0.147	0.062	0.58	0
TN06-03	0.147	0.121	0.18	0
TN06-13	0.397	0.373	0.06	0
TN07-02	0.373	0.330	0.12	0
TN07-04	0.397	0.351	0.12	0
TN07-05	0.373	0.351	0.06	0
TN07-14	0.448	0.397	0.11	0
TN07-15	0.205	0.190	0.07	0
TN07-18	0.254	0.238	0.06	0
TN07-20	0.190	0.175	0.08	0
TN07-22	0.290	0.272	0.06	0
TN09-03	0.238	0.221	0.07	0
TN09-05	0.373	0.330	0.12	0
TN09-10	0.397	0.330	0.17	0
TN09-20	0.397	0.373	0.06	0

g. *Re-isolation, Vitamin B-12 test*: Re-isolation was performed for theTN02-13 isolate and it was found that it consists of a mixture of TN02-13a and TN02-13b isolates. TN02-13a did not display any vitamin B_{12}-producing ability unlike TN02-13b. The value was 30ng/5ml culture (Table 5), and it was even higher than the original value (1ng/5ml culture). The increase in the value may be ascribed to the fact that most of the original TN02-13 isolates consisted of TN02-13a which does not produce vitamin B_{12}. TN02-13b, which produces vitamin B_{12} was sensitive to lysozyme also (Table 5). Thus the screening of vitamin B_{12}-producing bacteria (-rium) from Tua-Nao could be achieved.

h. *Re-generation test*: TN02-13b which was found to produce vitamin B_{12} could also be regenerated from the protoplast form to the

Table 5. Vitamin B_{12} Test and Lysozyme Test after Re-isolation.

Isolate	Bio-assay[1)] value (OD at 600nm)	Vitamin B_{12} (ng/5ml culture)	OD600nm -Lysozyme	OD600nm +Lysozyme	Rate of decrease
TN03-13a	0.040	0	-	-	-
TN03-13b	0.797	30	0.285	0.198	0.31

1)Culture 2.5ml Cent. →Cells →B-12 extract→Fill up 5ml →Dilution 1/10 →Bio-assay 0.2ml

bacillary form (Table 6).

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Table 6. Regeneration test.

Isolate	Number of colonies/0.1ml	
	Regenerated[1)]	Lysozyme-resistant[2)]
TN03-13b	137	1

1) Osmotic medium was used.
Osmotic medium : PR medium (0.2M sodium succinate)
2) Non-osmotic medium was used.
Non-osmotic medium:Nutrient agar medium
Cell suspension after lysozyme treatment was diluted with SMB buffer at 10^{-4}, then 0.1ml was analyzed.

REFERENCES

1) Okada, N., Hadioetomo, R. S., Nikkuni, S., Katoh, K. and Ohta, T. (1983). Vitamin B-12 Content of Fermented Foods in the Tropics. *Rep. Natl. Food Res. Inst.* **43** : 126-129. (in English).
2) Okada, N., Hadioetomo, R. S., Nikkuni, S. and Itoh, H. (1985). Isolation of Bacteria Producing V.B-12 from Fermented Soybean Tempeh of Indonesia. *Rep. Natl. Food Res. Inst.* **46** : 15-20. (in English).
3) Okada, N., Hariantono, J., Hadioetomo, R. S., Nikkuni, S. and Itoh, H. (1985). Survey of Vitamin B-12 producing Bacteria Isolated from Indonesian Tempeh. *Rep. Natl. Food Res. Inst.* **47**: 49-56 (in English).

タイのトゥアナオよりビタミンB_{12}生産菌の分離

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摘　要

タイの無塩大豆発酵食品より，細胞融合による納豆菌の改良に利用可能なバクテリアを探索した。検索は，リゾチーム感受性かつ耐熱性のビタミンB_{12}生産菌を求めて行った。トゥアナオ試料は北タイのラムプーン，プラオ，ドイサケット，メーホーンソンより採取した。トゥアナトのバクテリア数は試料によりかなり異なった。耐熱性／非耐熱性の比も大きく異なった。またトゥアナオはビタミンB_{12}含量が異なるのみでなく，含まないものもあった。ビタミンB_{12}を含むトゥアナオ試料より，熱処理後バクテリアを分離し，リゾチーム感受性，ビタミンB_{12}生産能を調べた。分離株120株のうち，リゾチーム感受性を示す約20株を得，そのうちビタミンB_{12}生産能を有する１株（TN02-13）を見いだした。本菌は混合菌（TN02-13a，TN02-13b）であったが，再分離後TN02-13bにリゾチーム感受性かつビタミンB_{12}生産能があることが分かった。また本菌はプロトプラストから桿菌へ再生した。

キーワード：大豆発酵食品，納豆，細胞融合