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Improvement of Vitamin B6 Production from *Rhizobium* sp. 6-1C1 by Random Mutation

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Abstract

Rhizobium sp. 6-1C1, vitamin B6 producing bacterium was subjected to mutagenesis by gamma irradiation, ethyl methanesulfonate (EMS) and ethidium bromide (EtBr). To screen for various forms of produced vitamin B6 and thermotolerant mutants, optimized chemical mutagen and gamma irradiation mutants were grown at 50°C. Amount of vitamin B6 was determined by agar diffusion assay. Only mutants treated by gamma irradiation were able to grow at 50°C. Based on amount of vitamin B6 production and their morphological features, 10 mutants were selected from 689 mutant isolates. For mutant stability, ten selected mutants were stable both 37 and 50°C for vitamin B6 production for a studied period (10 generations). Two isolates (08-361 and 10-94) and isolate 10-8 showed significantly ($p < 0.05$) higher amount of vitamin B6 than wild type at 37 and 50°C, respectively. Reverse phase HPLC analysis showed that the forms of vitamin B6 produced mutants were pyridoxamine 5′-phosphate (PMP) pyridoxamine (PM) and pyridoxal 5′-phosphate (PLP) in culture media. This characteristic makes these mutants good candidates for potential biotechnological and biocatalytic applications.

Keywords: vitamin B6, production, agar diffusion assay

1. Introduction

Vitamin B6, a water soluble vitamin, is found in various forms, i.e., pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorelated derivatives. Among three phosphorelated derivatives, pyridoxal 5′-phosphate (PLP) is the biological most active form and used as cofactor for many important enzymatic reactions. The deficiency of vitamin B6 has influenced human health problem. Recently, PM, a potent antioxidant, has become well respected in the therapeutic field for treatment of diabetic complications and in a

variety of conditions (including aging, altherosclerosis, and Alzheimer disease) (1).

Vitamin B6 production currently use chemical processes. Microbial production of vitamin B6 may therefore be an interesting alternative to chemical processes. Many microorganisms are capable of producing vitamin B6 (2-6), including thermophilic bacterium, *Geobacillus* sp.H6a (7).

Gram negative bacterium, *Rhizobium* sp. 6-1C1, is capable of producing extracellular vitamin B6. The major form of produced vitamin B6 was PM, with a small amount of PMP. This bacterium could grow in media at all temperature tests (25, 30, 37, 40 and 45°C) except at 45°C. The highest vitamin B6 production was shown at 37°C (6). For potential applications, e.g., reduce cost for cooling system, reduce contamination of mesophilic micro-organisms, thermotolerant and various forms of produced vitamin B6 are required in this bacterium.

In this study, we describe the screening of random mutant of *Rhizobium* sp. 6-1C1 that able to grow at 50°C by gamma radiation, ethyl methane sulfonate (EMS) or ethidium bromide (EtBr) and to identify form of producing vitamin B6 by reverse phase HPLC.

2. Materials and Methods

2.1 Materials

Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride and ethyl methane sulfonate (EMS) were obtained from Sigma. Pyridoxamine 5′-phosphate was purchased from Serva. Pyridoxal 5′-phosphate and ethydium bromide were obtained from Fluka. Pyridoxine Assay Medium (PAM) was purchased from Difco. The yeast *Saccharomyces carlbergensis* TISTR 5345 was obtained from TISTR (Thailand). All other chemicals were of analytical grade.

2.2 Micro-organism and growth condition

The wild type of *Rhizobium* sp. 6-1C1, was collected from the soil in Thailand (6). The mutant strains were derived from the *Rhizobium* sp. 6-1C1 after gamma irradiation. All stock cultures were stored in 15% glycerol at -20°C. Before each experiment, 20 µl of stock cultures was grown aerobically in 2 ml synthetic medium (SM) broth for 12 h. For vitamin B6 production, 20 µl of starters were aseptically transferred into 2 ml synthetic medium (SM) broth for 24 h in the dark. Composition of the SM was 2% glucose, 2% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.0005% $FeSO_4 \cdot 7H_2O$ (pH 5.8).

2.3 Measurement of amount of vitamin B6

The extracellular vitamin B6 was determined by agar diffusion assay using *Saccharomyces carlsbergensis* TISTR 5345 and PAM agar (6). The yeast was grown in yeast malt (YM) agar medium at 30°C for 24 h. The yeast cells were aseptically transferred from the fresh YM agar medium to sterile tube containing 2 ml of distilled water, and adjusted their turbidity at OD660 equal to 1. The cell suspensions were added to PAM agar in the ratio of 1:100 and incubated at

30°C for 6 h. Holes with 0.5 mm-diameter were punched on the PAM agar plate. Ten µl samples were loaded into each hole. After 12 h incubation at 30°C, the diameter of turbidity zones was measured and comparing with that of standard vitamin B6 (0.25-2 ng).

Data were analyzed using SPSS 17.0 version for Windows. Differences with $p < 0.05$ were considered to be statistically significant using the one-way ANOVA.

2.4 Strain improvement by gamma irradiation

Rhizobium sp. 6-1C1 was inoculated into 2 ml SM medium until turbidity (OD600) reached 0.15 (approximately 10^7 CFUs/ml). Then the bacterial suspensions were irradiated at 0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 4 kGy, respectively. The irradiation experiment was done at the Office of Atomic for Peace (Bangkok, Thailand). Cobalt 60 was the gamma–ray source used in this experiment. An amount of 0.1 ml of the irradiated samples was incubated at 37°C for total bacteria counts. The remaining irradiated samples were incubated at 50°C for 24 h before spreading onto SM agar at 50°C until seeing colonies.

2.5 Strain improvement by mutagenesis

Rhizobium sp. 6-1C1 was inoculated into 1 ml SM medium until turbidity (OD600) reached 0.15 (approximately 10^7 CFUs/ml). Then the bacterial suspensions were mixed with 0.1, 0.2 and 0.5 % (v/v) EMS and 5, 10 and 20 µg/ml EtBr, respectively. These suspensions with mutagens were incubated in the dark at room temperature for 2 h, before plated onto SM agar and incubated at 50°C until seeing colonies. All plates were incubated at 50°C for at least 5 days.

2.6 Stability studies of mutants

Mutants obtained by the above methods were studied for their stability for vitamin B6 production for 10 generations. Mutants, after every cultivation were collected by 15 % (v/v) glycerol stock and used for inoculating next fermentation.

2.7 Form of producing vitamin B6

Vitamin B_6 compounds were investigated by the reversed-phase isocratic HPLC method described by Argoudelis (8). The supernatant of medium was injected to a 4.6 mm X 25 cm hydrosphere C18 column (YMC, Japan) with pre-column buffer (0.15 M sodium dihydrogen phosphate, pH 2.5). Column effluent was monitored with a Shimadzu spectrofluorometer detector RF-10A at excitation 290 nm and emission 389 nm with a flow rate 0.1 ml/min. The post-column buffer (1 g/L sodium bisulfite) was used for the derivatization of PLP.

3. Results and Discussion

3.1 Selection of bacterial strains for mutagenesis

The 689 mutant isolates were obtained from the wild type *Rhizobium* sp. 6-1C1 through 0.8 and 1.0 kGy Gamma irradiation. The survival rate of 0.2-0.6 KGy was higher than 0.8 and 1.0 kGy but no mutant could grow at 50°C. In addition, no mutant was observed at 37°C for irradiation at 2-4 kGy. For EMS and EtBr mutagenesis, no mutant could grow at 50°C.

We had two reasons for using temperature at 50°C for screening mutants. Firstly, temperature higher than 50°C could rapidly catalyze the spontaneous Maillard reaction in the SM broth. This phenomenon had effect on the decrease in amount of produced vitamin B6. The action of vitamin B6, especially PM could inhibit the Maillard reaction by interfering with Post-Amadori oxidative reactions (9-10). Production of vitamin B6 from thermophilic bacterium, *Geobacillus* sp.H6a was also supported this occurrence (7). Secondly, high temperature could use as marker for screening mutants. Temperature change also has affected to enhance the mutant phenotype. Furthermore, if mutants were selected at temperature as that of wild type growth, it was difficult to screening population of mutants from that of wild type.

Among 689 mutant isolates, ten isolates were selected because they showed higher vitamin B6 production after the third time of screening. These mutants had some phenotype which differed from wild type. Wild type's colonies were circular, convex with an entire margin and mucilaginous after 24 h on SM plate. Most mutant's colonies were circular, convex with an undulate margin and faster mucilaginous than wild type on SM plate. The quantities of vitamin B6 were measured by agar diffusion assay in cultivated media at 37 and 50°C for 24 h as shown in Figure 1. All mutants could produce vitamin B6 at 37 and 50°C. Isolate 08-403, 10-4, 10-8, 10-94 and 10-102 showed high vitamin B6 production at 50°C.

Using high temperature selection, 10 mutants with highly produced vitamin B6 were chosen from induction with gamma radiation. It is well known that radiation from gamma ray has effects on the genetic material of the cell, possibly leading to cell death and permanent changes within daughter cells. Our results agree with report

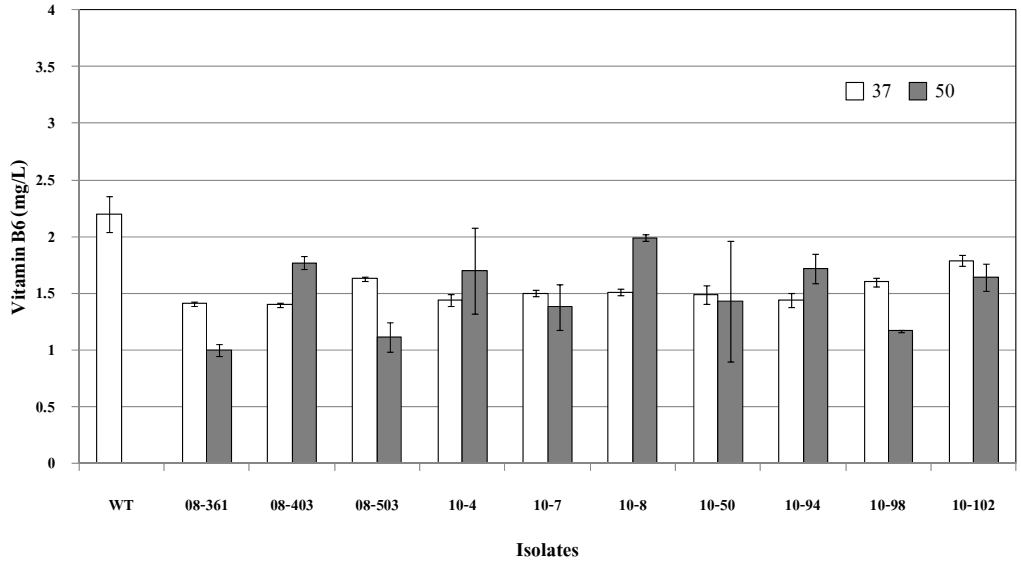

Figure 1. Amount of vitamin B6 production of wild type and mutant isolates after incubation at 37 and 50°C.

of Min et al. (11). It indicated that DNA damage due to gamma-ray radiation also increased with increasing dose rates. Meanwhile no mutant was found from induction of EMS and EtBr. This might be the cell lethality and more severe genetic damage caused by higher mutagen concentrations.

3.2 Mutant stability for vitamin B6 production

The result presented in Figure 2 indicated that ten mutants were stable both 37 and 50°C for vitamin B6 production for a studied period (10 generations). Two isolates (08-361 and 10-94) showed significantly higher amount of vitamin B6 than in the WT at 37°C. Furthermore, isolate 10-8 showed significantly higher vitamin B6 production than wild type at 50°C. The relationship between amount of vitamin B6 and cell growth showed that was primary metabolite kinetics, indicating the increase in vitamin B6 coincided with the increase in optical density at 600 nm. This occurrence was found in *Rhizobium* sp. 6-1C1 and *Geobacillus* sp.H6a (6-7). So, it suggested that the difference of vitamin B6 production might be caused by the difference of cell growth.

The stability of mutant was determined by several subcultures. All mutants could well adapt themselves after the mutagenesis, as indicated by an increase of the amount of vitamin B6 (Figure 2). Several subcultures of mutants have been helped to increase their phenotypic stability. Furthermore, morphology of mutants has still unchanged from first generation. From these data, it is possible to be irreversible mutation. As we known, microorganisms could adapt to change in the environment. By applying this knowledge, several times of subculture in the same environment would induce the consistency of mutation.

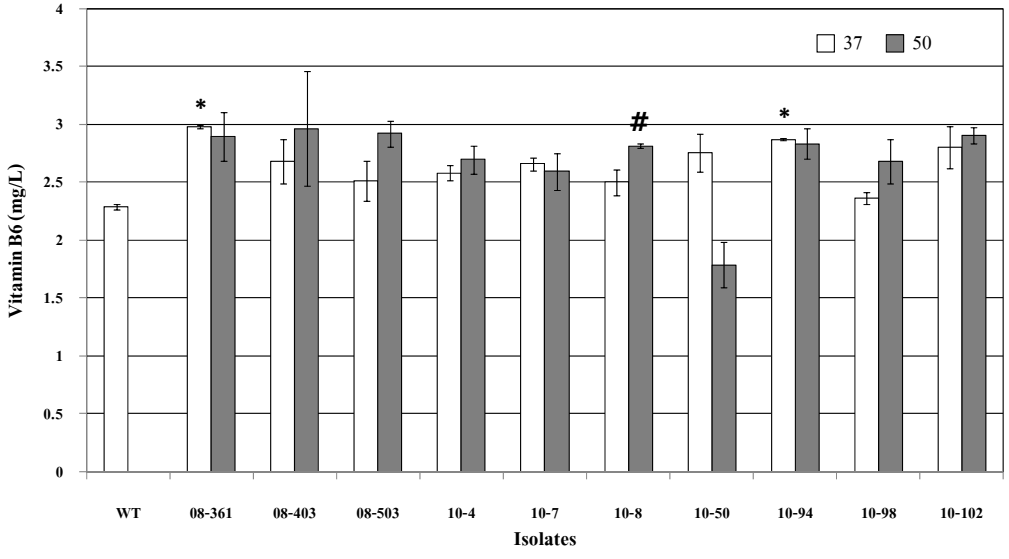

Figure 2. Stability of vitamin B6 production of mutant isolates at 37 and 50°C. (*) and (#) indicated the amount of vitamin B6 production which are significantly different from that of WT at $p<0.05$ at 37 and 50°C, respectively.

At 50°C, the isolate 10-8 showed significantly higher vitamin B6 production than wild type. From our review literature, there was only one report about random mutation in vitamin B6 producing bacteria. A vitamin-B6-producing mutant, BA 1, was selected by treatment of *Bacillus subtilis* with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, that excrete 2-5 mg of vitamin B6/L. Form of producing vitamin B6 in this mutant is not known (12). Although amount of vitamin B6 production from isolate 10-8 (2.8 ±0.1 mg/L) was lower than that from previous report. But it showed the good

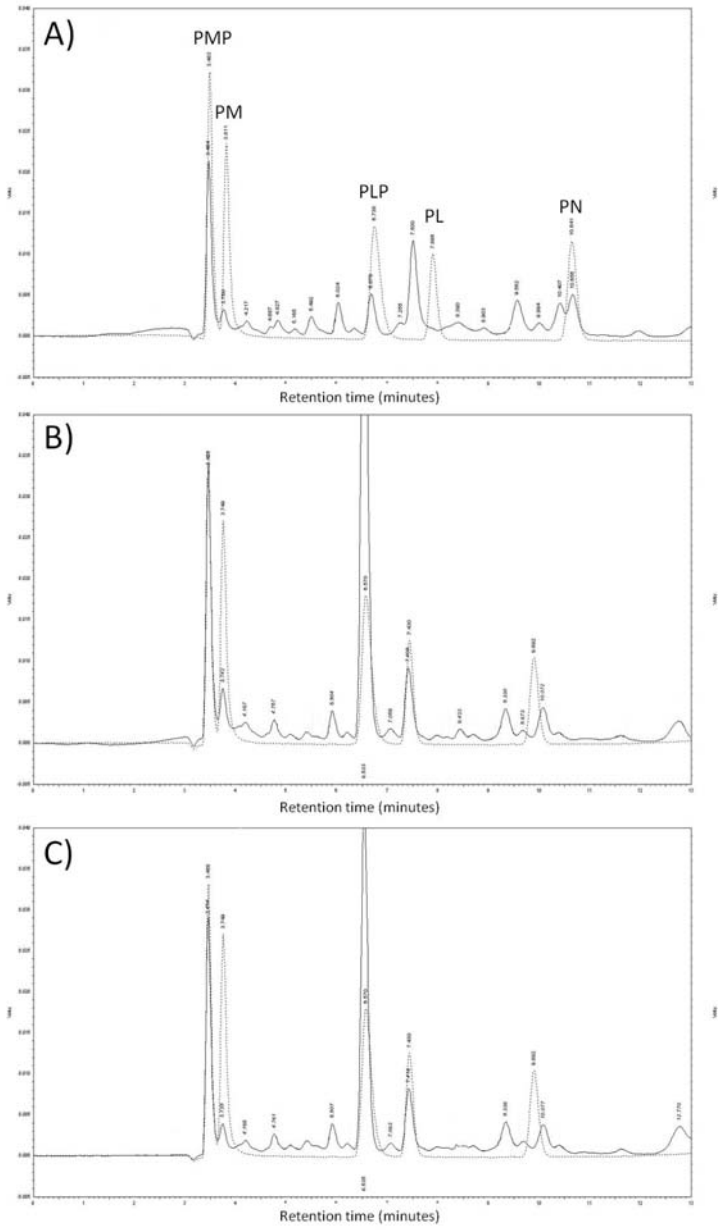

Figure 3. HPLC Chromatogram of vitamin B6 of the mutants (full line) comparing with the standard solution of vitamin B6 (dashed line). A) The supernatant of culture broth of isolate 10-8 after 24 h growth at 50°C, B) isolate 10-94 after 24 h growth at 37°C, C) isolate 08-361 after 24 h growth at 37°C.

characteristic, the growth at broad range of temperature (37 – 50°C) which could help in reducing stress from temperature fluctuation in fermentation. Furthermore, there is not report about vitamin B6 producing bacteria by gamma radiation.

3.3 Form of producing vitamin B6

The supernatant of mutant cultured medium was identified form of vitamin B6 by isocratic HPLC. The chromatogram (Figure 3) showed pyridoxamine 5′-phosphate (PMP) pyridoxamine (PM) and pyridoxal 5′-phosphate (PLP), after comparison with the standard solution of vitamin B6. Interestingly, forms of produced vitamin B6 of mutants have differed from that of wild type which produced only PMP and PM.

Form of produced vitamin B6 from isolate 10-8, 10-94 and 08-361 were PM, PMP and PLP which differ from that of wild type (Figure 3). PLP is a cofactor for many important enzymatic reactions such as glutamate decarboxylase in γ-aminobutyric acid (GABA) production (13). This characteristic makes these mutants good candidates for potential biotechnological and biocatalytic applications.

4. Conclusion

In this work, mutants of *Rhizobium* sp. 6-1C1 induced by gamma radiation showed that are able to resist high temperature (50°C) and produced various forms of vitamin B6 compared to wild type.

5. Acknowledgement

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6. References

(1) Ericson KL, Maloney VM, Mahuren D, Coburn SP, Degenhardt TP. *N*-Methylpyridoxamine: novel canine vitamin B_6 urine metabolite. Bioorg Med Chem Lett. 2008; 18: 1845-8.

(2) Ishida M, Shimura K. Vitamin B_6 production with a cell-suspension of *Achromobacter cycloclastes*. Agr Biol Chem. 1970; 34: 327–34.

(3) Nishio N, Sakai K, Fujii K, Kamikubo T. Utilization of *n*-paraffins and vitamin B_6 production by *Pechia gulliermondii* wickerham. Agr Biol Chem. 1973; 37: 553–9.

(4) Tani Y, Nakamatsu T, Izumi Y, Ogata K. Extracellular formation of vitamin B_6 by marine and terrestrial micro-organisms and its control. Arg Biol Chem. 1972; 36: 189–97.

(5) Tazoe M, Ichikawa K, Hoshino T. Production of vitamin B_6 in *Rhizobium*. Biosci Biotechnol Biochem. 1999; 63: 1378–82.

(6) Trongpanich Y, Phimwapi S, Niamsanit S, Wangsomnuk PP, Boonmee M, Siri S. Isolation and characterization of bacteria capable of producing pyridoxamine (PM) and pyridoxamine 5′–phosphate (PMP), vitamin B_6 compounds. J Gen Appl Microbiol. 2007; 53: 295–9.

(7) Anutrakunchai C, Niamsanit S, Wangsomnuk PP, Trongpanich Y.

Isolation and characterization of vitamin B6-producing thermophilic bacterium, *Geobacillus* sp. H6a. J Gen Appl Microbiol. 2010; 56: 273-9.

(8) Argoudelis CJ. Simple high-performance liquid chromatographic method for the determination of all seven vitamin B_6 –related compounds. J Chromatogr A. 1997; 790: 83-91.

(9) Voziyan PA, Hudson BG. Pyridoxamine as a multifunctional pharmaceutical: targeting pathogenic glycation and oxidative damage. Cell Mol Life Sci. 2005; 62: 1671–81.

(10) Arribas-Lorenzo G, Morales FJ. Effect of pyridoxamine on acrylamide formation in a glucose/asparagines model system. J Agric Food Chem. 2009; 57: 901–9.

(11) Min J, Lee CW, Gu MB. Gamma-radiation dose-rate effects on DNA damage and toxicity in bacterial cells. Radiat Environ Biophys. 2003; 42: 189-92.

(12) Pflug W, Lingens F. Vitamin B6 biosynthesis in *Bacillus subtilis*. Hoppe Seylers Z Physiol Chem. 1978; 359 (5): 559-70.

(13) Komatsuzaki N, Shima J, Kawamoto S, Momose H, Kimura T. Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. Food Microbiol. 2005; 22: 497-504.