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Exploration of vitamin B12-producing bacteria from Indonesia Eutrophic Lake: A new strategy to improve microalgae biomass production

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Abstract. *Prabaningtyas S, Ardyati T, Suharjono, Retnaningdyah C. 2021. Exploration of vitamin B12-producing bacteria from Indonesia Eutrophic Lake: A new strategy to improve microalgae biomass production. Biodiversitas 22: 4538-4544.* Biofuel producing autotrophs and heterotrophs organisms are mostly found in freshwaters such as lakes. Preliminary research showed that Ranu Grati has a high diversity of microalgae and bacteria. Microalgae are known as one source of biofuels due to the high content of lipid. The growth of microalgae is strongly influenced by vitamin B12, which is synthesized by bacteria. The role of vitamin B12 in the metabolism of algae is mainly as a co-enzyme for vitamin B12-dependent methionine synthase. The purpose of this research was to observe the bacterial diversity based on NGS analysis, to explore vitamin B12 produced by bacteria from Ranu Grati lake, and also to identify the highest potency of vitamin B12-producing bacteria based on 16S rRNA gene sequencing. The study was carried out by taking a water sample at a depth of 50 cm at five stations. Isolation of total bacterial DNA was carried out using FastDNA Spin Kit for Soil. The metagenomic method of Next Generation Sequencing (NGS) was used as an initial study of bacterial diversity. Vitamin B12-producing bacteria was isolated using PYBG agar (phytone peptone, trypticase peptone, lab-lemco powder, bacto yeast extract, glucose, agar). Isolated bacteria were screened for production of vitamin B12. The potential isolates were identified base on 16S rRNA gene sequence similarity. The results of the metagenomic study showed that the genus of potential bacteria producing vitamin B12 included *Bacillus*, *Pseudomonas*, *Propionibacterium*, *Enterobacter*, *Escherichia*, *Acinetobacter*, and *Flavobacterium*. The results of screening with PYBG media obtained 30 isolates of vitamin B12 producing bacteria. Ten efficient vitamin B12-producing isolates were identified as *Lysinibacillus fusiformis* (isolate G2V1), *Bacillus cereus* group (isolate G2V25, G2V24, G2V13, G2V9, G2V8), *Alcaligenes faecalis* (isolate G2V22), *Delftia acidovorans* (isolate G2V14A) and the genus *Delftia* (isolate G2V19). The isolate G2V1 (*Lysinibacillus fusiformis*) was the highest producer of vitamin B12 which was able to produce 33,783 ug/mL of vitamin B12 at 4 days incubation time.

Keywords: Bacteria, lake, Ranu Grati, vitamin B12

INTRODUCTION

The bacterial community associated with microalgae is an important factor in producting microalgae biomass and lipid production (Wang et al. 2014). One of the efforts made in the bioenergy production process its co-culture, which adds bacteria to the algae culture. Research on co-culture that has been carried out includes: The research is a co-culture between *Chlorella* sp. with *Azospirillum* sp. showed a faster growth of microalgae (de-Bashan et al. 2008). Co-culture was also carried out between *Chlorella vulgaris* and *Bacillus licheniformis* which proved this bacterium as a promoter of accelerating the growth of *Chlorella vulgaris* (Liang et al. 2013). Research by Higgins and Vandergheynst (2014) shows that co-culture between *Chlorella minutissima* and *Escherichia coli* can increase the biomass and lipid content of *Chlorella minutissima* (Higgins and VanderGheynst 2014).

Ranu Grati is a large lake in East Java, with various activities around the lake, which can cause the community structure to change. The preliminary study result show that these lakes include eutrophic lakes with a secchi disk depth (brightness) of 75-110 cm, medium microalgae diversity index, moderate dominance, and abundance of high microalgae (Prabaningtyas et al. 2018). In lake water, bacteria and microalgae interact with each other (Kazamia et al. 2014). Favorable interactions include bacteria providing inorganic carbon for microalgae. Bacteria also supply several minerals, hormones, and vitamins to microalgae (Ramanan et al. 2016). Several studies were conducted to isolate lake bacteria, for example research on bacteria that have the potential to produce indole-3-acetic acid (IAA) (Sulistya et al. 2020) and amylase enzymes that can be co-cultured with microalgae (Nisa et al. 2020).

Vitamin B12-producing bacteria in Ranu Grati has not been explored. These potential bacteria can accelerate the growth of microalgae, because they provide nutrients to microalgae. It has been known that many species of unicellular algae require vitamin B12, which is supplied mainly by bacteria in the aquatic ecosystem (Jones et al. 2010; Kazamia et al. 2014; Ramanan et al. 2016). Vitamin B12 is a co-enzyme of the methionine synthase enzyme which is an enzyme that plays a role in the formation of methionine. Methionine is one of the amino acids which is

the start codon in the formation of proteins (Bromke and Hesse 2015).

This study aims to isolate and identify vitamin B12-producing bacteria from Ranu Grati. Vitamin B12 is needed by microalgae for its growth. So if this vitamin B12-producing bacterial isolate is co-cultured with microalgae, it is expected to obtain high biomass and lipid content from microalgae.

MATERIALS AND METHODS

Study site and sampling station

One of the lakes in Grati Sub-district, Pasuruan District, East Java, Indonesia with an area of ± 198 Ha, irrigates 1080 ha of rice fields, is a place of tourism, fishing area and floating net cage. Ranu Grati is a eutrophic lake with the deep was about 7-150 m. Furthermore, five sampling stations in the Ranu Grati have been selected. The five sites were distributed over the lake at the deeps 50 cm. Bacterial samples were collected from water at 5 stations and each station 3 repetitions. The location of the sampling station is as follows: Station 1: S 07°43' 45.9", E 113°00'46.2"; station 2: S 07°43'45.9", E 113º 00' 46.2"; station 3: S 07°44' 04.7", E 113°00' 31.9"; station 4: S 07°43' 39.3", E 113°00' 11.6"; station 5: S 07°43' 38.9", E 113°00' 32.2" (Figure 1).

Analysis of physico-chemical parameters

The physic-chemical parameters of water collected from lake were analyzed using standard methods. The concentration of the total nitrogen was analyzed using Total Kjeldahl method. The content of dissolved inorganic phosphorus (DIP) was determined using the phosphorus molybdenum blue colorimetric method (Paytan and McLaughlin 2007). Water transparency was determined in situ using the Disc method (Lee et al. 2015). Salinity, concentrations of DO (dissolved oxygen), temperature, and pH were measured using Refractometer Atago S-28E, DO meter Lutron 5510, pH meter Lutron 5509, respectively (Gao and Song 2005).

Samples collection

Genomic DNA was extracted from water samples using the NGS method, at least 3000 ml of lake water is needed and was taken for each station (Ávila et al. 2017). Both water samples were taken using the sterile "water sample bootle"–and then transferred to a sterile bottle. Water samples from all stations were transported in an icebox and stored at 4°C with ice gell (Grant et al. 2014). Water samples 3000 mL from 5 stations were immediately transported to the laboratory, pre-filtered using 20-µm paper filters, and filtered through 0.22-µm pore-size filter membranes for metagenomic analysis. The filters were stored at –20°C until further processing (Ávila et al. 2017). The bacterial communities attached to the filter membrane were inserted into the microtube for analysis of bacterial diversity using the metagenomic DNA method.

Metagenomic DNA extraction

Isolation of total bacterial DNA was carried out using FastDNA Spin Kit for Soil. Cells are lysed enzymatically with Protease than centrifuge with a standard microcentrifuge. Added protease and lysis buffer to the spin column with the sample. Lysate buffering conditions adsorb DNA to the spin column membrane. Several washing steps ensure that proteins and other contaminants were not retained in the membrane and purify DNA. DNA was eluted in elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) (Walden et al. 2017). The DNA obtained was then amplified using universal primers, namely 27f and 1492r (Yu et al. 2013). The diversity and composition of bacterial communities in water samples was investigated by NGS. The NGS was carried out with Illumina Novaseq6000 in Novogen.

Figure 1. The locations for water sampling in Ranu Grati lake, Grati Sub-district, Pasuruan District, East Java, Indonesia

Identification of vitamin B12-producing bacteria

Ten potential and efficient vitamin B12-producing bacterial strains were isolated using the QIAmp DNA Mini Kit (Qiagen, Germany). Top Taq Master Mix Kit Reagent was used to amplify 6S rRNA gene with PCR machine, primers namely 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT CTT ACG ACT T-3'). The amplicons of 16S rRNA gene were sequenced at 1st Base Laboratories, Malaysia. Phylogenetic trees and genetic distances with the Maximum Likelihood were calculated with The MegaX software (https://www.megasoftware.net/), including the 1000-replication bootstrap test (Wake 2016).

Isolation and screening of vitamin B12-producing bacteria

Isolation of Vitamin B12 Producing Bacteria was carried out by spread the sample water on selective PYBG agar media (trypticase peptone 1%, phytone peptone 0.5%, lab-lemco powder 0.24%, bacto yeast extract 0.2%, glucose 0.2%, agar 1%, pH 7.5) (Sugita et al. 1994). The vitamin B12 concentration of the culture medium was assayed using the *Salmonella typhimurium*. The isolate of vitamin B12-producing bacteria was grown in PYBG Broth. Cells were harvested in the exponential growth phase. The lysate solution was obtained by centrifugation and used for supporting the growth of a reporter strain *Salmonella typhimurium* (Raux et al. 1996) for the bioassay. The isolate cell lysate, for which the production of B12 was to be quantified, was added to *S. typhimurium* culture broth to stimulate its growth. The extent of the growth of *S. typhimurium* reflects the amount of vitamin B12 in the cell lysate. Semiquantitative estimation of vitamin B12 in a sample by reference to a standard curve (Grant et al. 2014; Nguyen-Vo et al. 2018; Raux et al. 1996).

RESULT AND DISCUSSION

Physicochemical parameters of lake water

The results showed that the waters of Ranu Grati found contaminated with high organic matter (BOD levels 8.30-8.72 mg/L) with trophic status classified as mesotrophic to hypertrophic, Total phosphate (TP) 0.05-0.08 mg/L and total Kjeldahl N (TKN) 0.23-0.32 mg/L). DO levels were at a range value 7.47-9.87 mg/L, while pH value 8.73-9.07. Some environmental factors were not significantly different in all stations, such as light intensity, temperature, and conductivity, while the brightness, pH and DO appear to differ markedly at several stations (Prabaningtyas et al. 2019).

Community structure and abundance of bacteria in the lake water

Bacterial communities are highly important for the cycling of organic and inorganic matter in freshwater environments; however, little is known about the diversity of bacteria in a tropical system. This research used 16S rRNA gene NGS to investigate the bacterial communities of Ranu Grati lake, East Java Province, Indonesia, with eutrophic status. Lakes are suitable habitats for bacterial and microalgae communities. Eutrophic lake conditions are always accompanied by an abundance of bacteria (Kazamia et al. 2014). Based on 16S rRNA gene metagenomic analysis showed that the abundance of bacteria in Ranu Grati was high. The total number of OTUs (Operational Taxonomy Units) is 399 (Figure 2).

To explore the communities' OTUs distribution patterns, we determined alpha diversity measures for all samples (the species estimators ACE and Chao1, and the Shannon and inverse Simpson diversity indexes) (Ávila et al. 2017).

The shanon index is more than 6, indicating species diversity and high community stability. Some lakes have a Shanon Wiener index between 1-6, for example, Lake Baikal in Siberia 3.65-6.03, then Lake Heviz in Hungary 2,1-2,90 (Kurilkina et al. 2016; Krett et al. 2017). Simpson index 0.967, indicating that the dominance is high (this index value is between 0-1). The Chao1 index is an index that estimates the minimum amount of OTU (richness) in a population. Chao 1 shows 399 OTUs and this value is the same as the ACE (Abundance-Based Coverage Estimator). Based on taxonomic groups obtained from the sample are as follows (Figure 3.). *Acinetobacter* 19.34% - 33.04%, *Methylotenera* 18.827%- 27.04%, *Methylophilus* 1.168% - 2.00%, *Ottowia* 3.566% - 6.09% and *Synechococcus* 1.322% - 2.26%.

The bacteria that are being explored are vitamin B12 producing bacteria. The results of bacterial NGS can be determined by the level of their genus. Of the 100 genera with the highest abundance is shown in Figure 4. From this figure it can also be seen that the genus of vitamin B12 producing bacteria. These results indicate several bacterial genera of vitamin B12 producing bacteria found from water samples in Ranu Grati, namely, *Bacillus*, *Pseudomonas*, *Propionibacterium*, *Enterobacter*, *Escherichia*, *Acinetobacter and Flavobacterium*. *Acinetobacter* is a genus with the highest relative abundance compared to other genera (Figure 4).

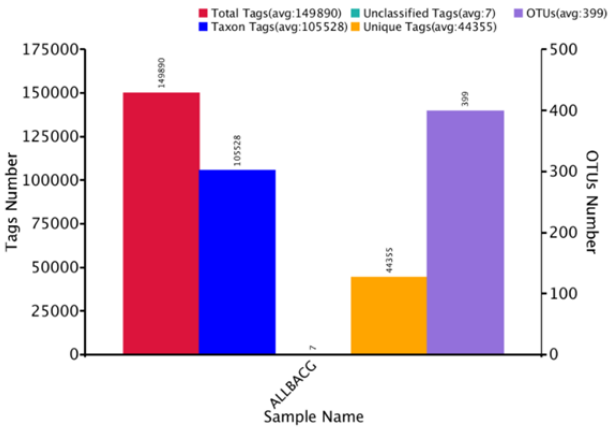

Figure 2. The abundance of bacteria in Ranu Grati Lake, East Java. Sample name is ALLBACG

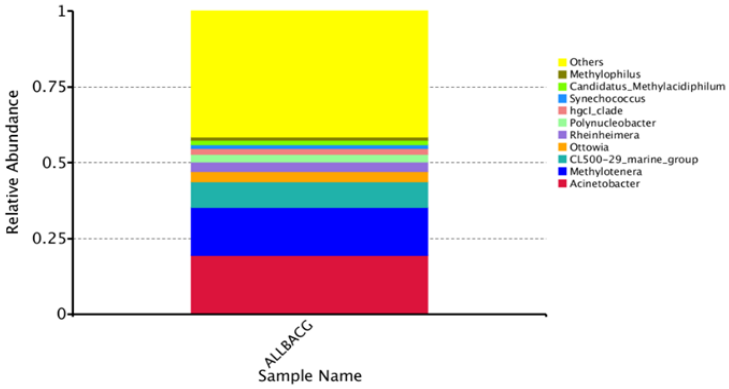

Figure 3. The Relative Abundance of The Top 10 Genera of Bacteria from Ranu Grati. Sample name is ALLBACG

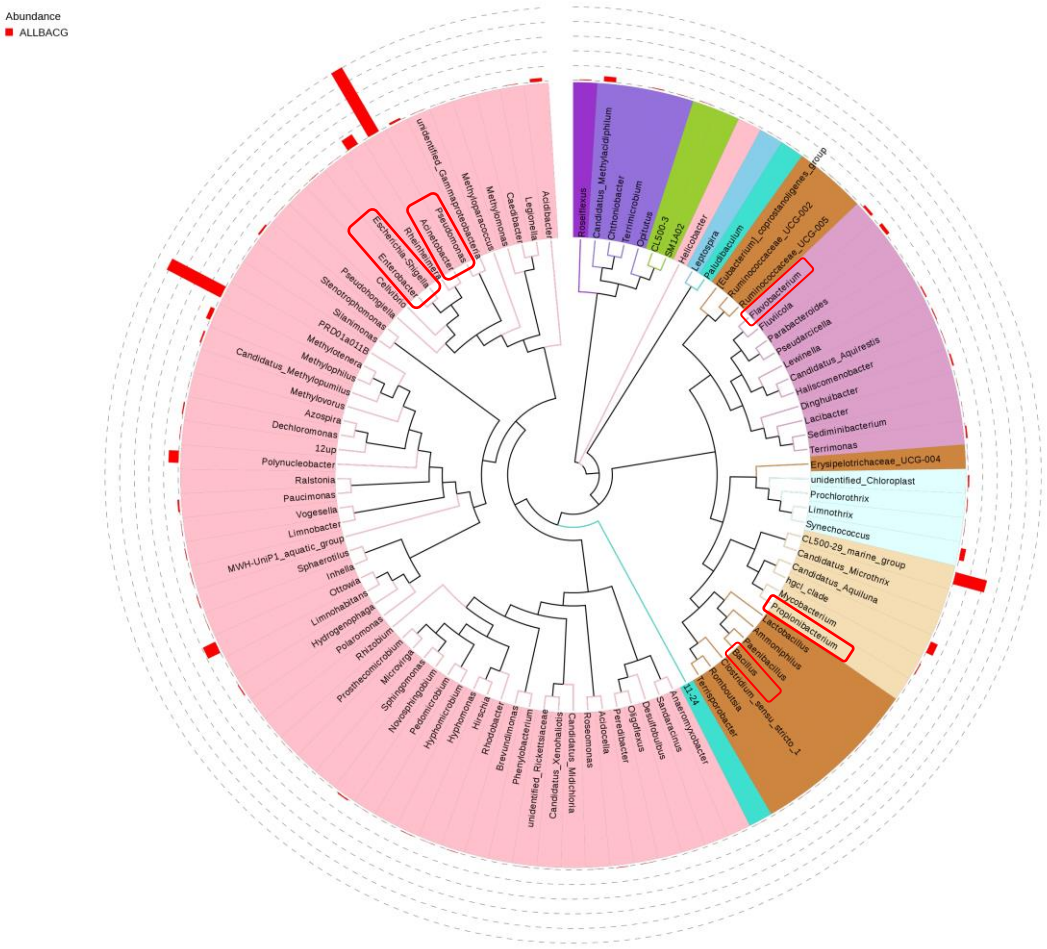

Figure 4. The abundance of bacterial genera found in Ranu Grati. The red circle in the image shows the genus of vitamin B12 producing bacteria

Vitamin B12-producing bacteria from water of Ranu Grati lake

The results of isolation with PYBGF selective media obtained 30 bacterial isolates. The 10 highest vitamin B12 producing bacteria were identified based on 16S rRNA gene sequence similarity. The results showed that G2V1 and G2V2 isolates were identified as *Lysinibacillus fusiformis*, G2V25, G2V24, G2V13, G2V9, G2V8 as *Bacillus cereus* species, G2V22 isolate as *Alcaligenes faecalis*, G2V14A isolate as *Delftia acidovorans* and G2V19 belong to the genus of *Delftia*. The phylogenetic tree of the 10 isolates is shown in Figure 5.

The potency of vitamin B12 producing bacteria

Based on potential assay of 10 bacterial isolates that produce vitamin B 12 show that G2V1 isolate has highest

to produce vitamin B12 (33,783 ug / mL) (Figure 6). The potential assay of 10 bacterial isolates that produce vitamin B12 shows that the G2V1 isolate has the highest to produce vitamin B12 (33,783 ug/mL) (Figure 6). Isolate G2V1 produces the highest vitamin B12 on 4 days of incubation. This isolate is the efficient producer of vitamin B12 with the highest yield. The results also showed that on 4 days of incubation, the amount of vitamin B12 produced was higher than the other isolates. The presence of the bacteria (G2V1 / *L. fusiformis*) investigated in different habitats such as the drinking chlorinated water, in the presence of stress factors (arid climate, high or low temperatures, depleted soil, and the occurrence of disinfectants) indicates their ability to easily adapt to new living conditions expanding their habitat (Roi et al. 2014). *Lysinibacillus fusiformis* which prior to 2007 was known as *Bacillus fusiformis*, are gram-positive bacteria with rod-shaped cells. They form ellipsoidal or spherical spores arranged at the center or terminally in convex sporangia; their temperature optimum is 37°C (range of 16–45°C), and optimal pH is 7–8 (range of 5.5–9.5). These bacteria are mobile (Roi et al. 2014; Zhao et al. 2015).

Vitamins may be considered the ideal signaling currency among microbes: because they provide important cofactors, they are needed and recognized everywhere (Grant et al. 2014). Heterotrophic bacteria living in water environment transform matter and energy cycling in it. This is so, among other things, because of their abilities, including the ability to synthesize vitamins of group B, contained in many enzymes participating in the processes of transformation and mineralization of various organic compounds. The presence of vitamins in water environment is one of the factors conditioning the growth and development of many groups of organisms, such as phytoplankton, zooplankton, macrophytes and bacteria themselves (Donderski and Sokół 1990).

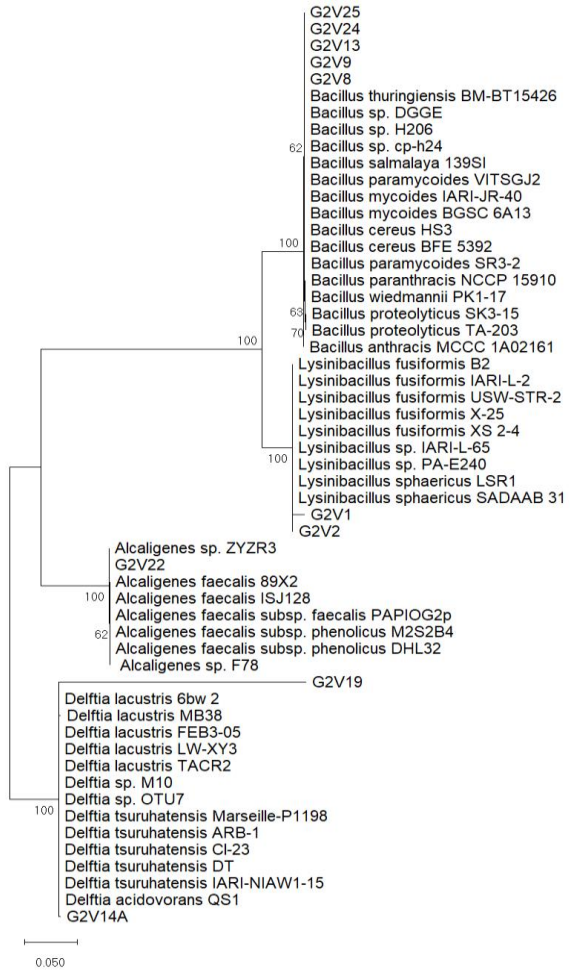

Figure 5. Phylogenetic Tree of The 10 Highest Vitamin B12-producing Bacteria Isolated from Ranu Grati lake and reference strains based on 16S rDNA sequence similarity according Maximum Likelihood Algorithm with 1000 bootstrap

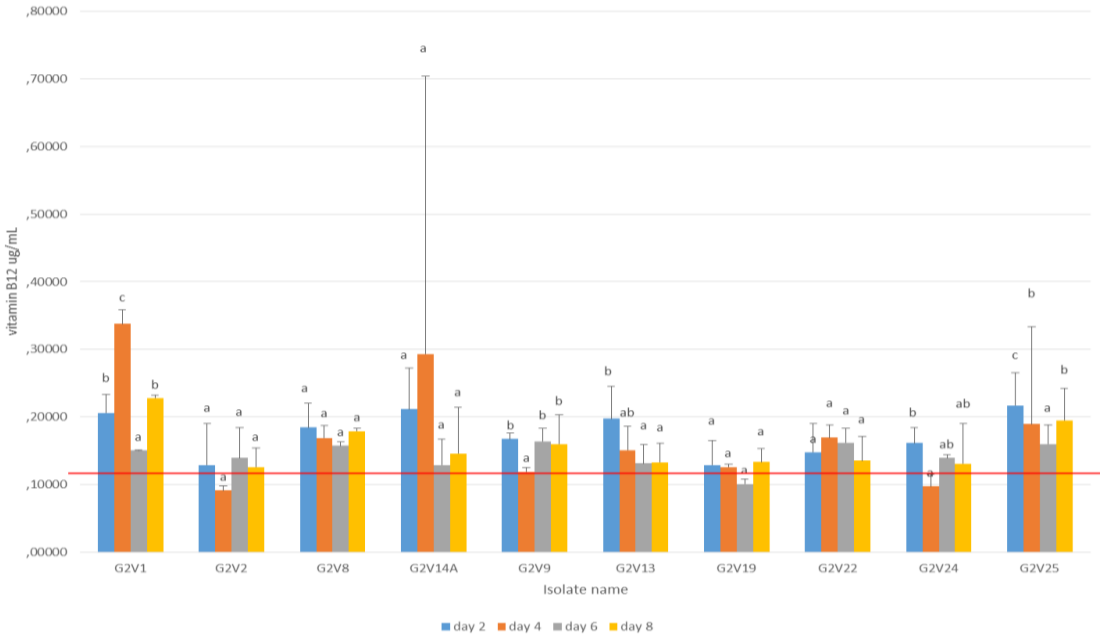

Figure 6. The Potency of Bacterial Isolates to Produce Vitamin B12. The same letter above each histogram is not significant different among treatments (p>0.05)

Vitamin B12 (cobalamin) is a water-soluble vitamin that is required by many eukaryotic organisms, but is synthesized by prokaryotes only (Martens et al. 2002). Many prokaryotic species cannot biosynthesize adenosylcobalamin, but can make it from cobalamin. These organisms are capable of transporting cobalamins into cells and converting them into the required co-enzyme forms. Even organisms such as *Salmonella typhimurium* that can make cobalamin also assimilate it from external sources if available. Absorption into cells is facilitated by the ABC transporter which absorbs cobalamin through the cell membrane (Woodson et al. 2005). Different strains show different abilities to synthesize vitamins of group B. The majority of bacteria referred to particular genera or groups were able to synthesize 2-4 vitamins simultaneously. Bacteria of the genus *Bacillus* isolated from the water of the littoral zone and those of the genus *Alcaligenes* isolated from the water of the pelagic zone were able to produce only one B-vitamin (Donderski and Sokół 1990). In conclusion, The results of the metagenomic study showed that the genus that has high potency as vitamin B12-producing bacteria included *Bacillus*, *Pseudomonas*, *Propionibacterium*, *Enterobacter*, *Escherichia*, *Acinetobacter* and *Flavobacterium*. The bacteria isolate of G2V1 can produce the highest vitamin B12 with an average of 33,783 ug/mL, based on 16S rRNA gene sequence similarity, it identified as *Lysinibacillus fusiformis*. A majority of eukaryotic phytoplankton species require an exogenous source of vitamin B12 for growth and recent field studies in some coastal and polar regions indicate that the addition of vitamin B12 alone, or with another limiting nutrient can influence the accumulation of phytoplankton biomass (Koch et al. 2011).

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