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Screening and isolation of vitamin B₁₂ producing *Pseudomonas* sp. from different natural sources

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Abstract

Vitamin B₁₂ is an essential vitamin playing a vital role in red blood cell formation, maintenance of central nervous system (CNS) and involved in metabolism of every cell in body. It is widely used as dietary supplement worldwide and used to treat anemia. The present study focuses on isolation and extraction of vitamin B₁₂ producing *Pseudomonas* sp. from rhizosphere, non-rhizosphere soil and fish gut (*Tilapia* sp.). The vitamin B₁₂ producers and non-producers were enumerated from the samples. Vitamin B₁₂ producing *Pseudomonas* sp. from fish gut was analyzed using auxonography method. Further, the vitamin B₁₂ from all the isolated bacterial samples were purified and quantified using high performance liquid chromatography technique (HPLC). Rhizosphere soil (68.4%) showed highest population of vitamin B₁₂ producers than non-rhizosphere soil (40%) and fish gut (60%). *Pseudomonas* sp. from rhizosphere soil (R6) showed higher vitamin B₁₂ production (11.54 mg/l) whereas non-rhizosphere soil and fish gut showed 5.25 mg/l and 6.68 mg/l. Control strain, *Propionibacterium freudenreichii* - 1950 had production of 8.1 mg/l which lower than the strain-R6. These wild type organisms when optimized or genetically modified can synthesis high amounts of vitamin B₁₂ which supports to overcome the vitamin B₁₂ deficiency worldwide.

1. Introduction

Cobalamin (vitamin B₁₂) was identified to be the largest B vitamin isolated by Dr. E. Lester Smith (1948) in the UK. Vitamin B₁₂ is a cry-stalline red component. It was discovered during 1926, that something present in raw liver can be used for a treatment of anaemia. Vitamin B₁₂ is also called as cobalamin due to the presence of cobalt molecule and some types varies by methyl-, cyano, adnosyl- and hydroxocobalamin (B₁₂ b). Vitamin B₁₂ is synthesized extensively by microbes, but is also present in animal tissues due to ingestion, or prevalence of the microorganisms in the gut. Moreover, grazing "meat animals" accumulates heavy metals from the environment, thus it is suggested that animal sources of B₁₂ are not as "good" a source as supposed to be (Allen, 2009; Helliwell *et al.*, 2011).

B₁₂ is a vitamin necessary for hematopoiesis and promptly growing tissues. Methylcobalamin synthesis requires cobalamin and is seen in the central nervous system (CNS) and brain where transportation of methyl groups (-CH₃) to proteins in the myelin occurs. This is the major cause why B₁₂ deficiency leads to anemia (other blood disorders) and neurological disorders like Alzheimer's. Several diseases occur due this one reason. Also, it is likely to have a deficiency of B₁₂ in the CNS even though the blood levels of B₁₂ are "normal", termed as non-anaemic deficiencies. In such serious cases,

B₁₂ is generally administered as the dietary availability of B₁₂ can be as low as 1% of the total ingested for mega B₁₂ doses (Allen, 2012; Hudson, 2010).

Mild B₁₂ deficiency is major common in oldage white men and rare in black and Asian American women. High levels of homocysteine, is most strongly correlated with low cobalamin concentration, is also most frequent in elder whites, and accompanied by renal inefficacy is more frequent in blacks and Asian Americans (Wheby, 1972). Hence, vitamin B₁₂ is very essential in the diet because they play a vital role in growth and metabolism of the living cells. In order to satisfy the growing demands, commercial production process for vitamin B₁₂ were initiated. The current annual world production of vitamin B₁₂ is estimated at about 12000 kg. Approximately, 3500 kg cyanocobalamin, 2000 kg hydroxycobalamin, 1000 kg of coenzyme B₁₂ and a small amount of methylcobalamin is supplied to the pharmaceutical industry; the remainder goes to the animal feed industry. For swine and poultry feeds, 10-15 mg vitamin B₁₂ is added per ton of feed, since animal protein can be replaced with less-expensive vegetable protein is fortified with vitamin B₁₂ (Green *et al.*, 2017; Moll and Davis, 2017).

Microorganisms most commonly exploited for the production of vitamin B₁₂ are *Propionibacterium shermanii*, *Propionibacterium freudenreichii* and *Pseudomonas denitrificans*. Merck began production of vitamin B₁₂ by *Pseudomonas denitrificans* in 1952 and have improved the efficiency of the culture more than 30 folds relative to the production of the original soil isolates by genetic manipulations and microbial screening carried out by production units and research laboratories (Belzer *et al.*, 2017; Garcia and Guerrero-Legarreta, 2005).

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Therefore, the present study focuses on isolation and identification of vitamin B₁₂ producing *Pseudomonas* sp. from soil. Screening was performed using auxonography technique. The yield from *Pseudomonas* sp. was compared with the standard strain *Propionibacterium freudenreichii*.

2. Materials and Methods

2.1 Collection of soil sample and isolation of *Pseudomonas* sp.

2.1.1 Collection of sample from the rhizosphere and non-rhizosphere region and isolation of *Pseudomonas* sp. (Baya *et al.*, 1981)

Soil samples were collected from the rhizosphere region of tomato plants and non-rhizosphere regions by a sterile spatula collected samples were incorporated into a sterile container and transported to the laboratory. One gram of soil was serially diluted and spread plated on nutrient agar, cetrimide agar and King's B medium. The inoculated plates were incubated at 30°C for 24 h. After incubation, colonies were selected from the plates according to the pigmentation, colony morphology, Gram's reaction and oxidase test. The selected colonies were taken by the loop and streaked on nutrient agar; cetrimide agar and King's B medium plates and incubated at 30°C for 24 h. The pure cultures were maintained on nutrient agar slants and stored for further studies.

2.1.2 Isolation of *Pseudomonas* sp. from fish gut (Sugita *et al.*, 1990)

Live *Tilapia* fishes were collected from Erode fish market and dissected to take out the intestine, under aseptic condition. The intestine was cut in to small pieces and macerated in mortar and pestle with the addition of sterile distilled water. It was serially diluted and pure cultures of *Pseudomonas* sp. were obtained as per the above procedure.

2.2 Identification of *Pseudomonas* sp.

The *Pseudomonas* sp. were identified based on colony morphology, Gram's reaction, motility and pigmentation followed by a series of biochemical tests such as oxidase, catalase, acid from glucose, denitrification, urease, ammonia salt medium, oxidative-fermentation (OF) test, asculin test, *etc.*

2.3 Screening of isolates for vitamin B₁₂ production

2.3.1 Bacterial strains used

Two bacterial strains used in the study were purchased from microbial type culture collection (MTCC) center, Chandigarh, India. A mutant strain, *Brevendimonas vesicularis*-910, was used for auxonography technique and *Propionibacterium freudenreichii*-1950, was used as control bacterium for vitamin B₁₂ production.

2.3.2 Auxonography technique for the detection of vitamin B₁₂ synthesizers (Udaka, 1981)

The *Pseudomonas* sp. isolated were screened for its ability to synthesis vitamin B₁₂. For this purpose, a special technique was used. In this technique, double layered medium was used. Nutrient agar is used as first layer. On it, the isolated organisms were swabbed. For the isolated organisms were swabbed on the separate plates. After 24 h of incubation at 30°C, minimal agar medium was over layered on it. Then the mutant strain, *Brevendimonas vesicularis*, was spread on the over layered medium. It was again kept for incubation at 30°C for 24 h. Growth of *Brevendimonas vesicularis*

over the above layer indicates the isolated microorganisms are vitamin B₁₂ producers.

Propionibacterium freudenreichii is used as control strain for the synthesis of vitamin B₁₂, was also subjected to auxonography method. The bacterial strains which were identified as vitamin producers were used for study mass production and vitamin synthesis.

2.4 Production of vitamin B₁₂ by fermentation process

2.4.1 Inocula development

One loopful of stock organisms were inoculated into 5 ml of nutrient broth and kept at 30°C for 24 h. Then, it was made up to 20 ml and 150 ml consequently. Finally, 150 ml was transferred to production medium.

2.4.2 Production process (Ye *et al.*, 1996)

The production medium was prepared as per the above mentioned composition and sterilized it in an autoclave at 121°C for 15 lbs for 15 min. The fermenter vessel was also sterilized. The medium was poured into the vessel aseptically and was again sterilized. After sterilization, the vessel was connected with the control apparatus and the pH was adjusted to 7.4. It was regulated by connecting NaOH (8N) and HCl (8N). The antifoaming agent (castor oil) used was sterilized in a hot air oven.

In the case of control strain, *Propionibacterium freudenreichii* - 1950, the media composition were polypeptone: 12.5 g, casaminoacid: 11.0 g, yeast extract: 2.5 g, MgCl₂·6H₂O: 0.4 g, K₃PO₄: 1.76 g, CoCl₂·6H₂O: 0.018 g, FeSO₄·7H₂O: 0.01 g, calcium pantothenate: 0.004 g, biotin: 0.0003 g, glucose: 40 g, distilled water: 1 liter. After the injection of inoculum through the rubber bush on the cover of the vessel using a sterile syringe and needle, a continuous aerobic fermentation method was followed for seven days and stirrer speed was adjusted at 500 rpm, in case of *Pseudomonas* sp. For the *Propionibacterium* sp., an intermittent aerobic and anaerobic method of fermentation was used. In it ten hours of anaerobic fermentation followed by fourteen hours of aerobic fermentation was done. The stirrer speed was adjusted to 500 rpm. The fermentation was allowed to continue for 7 days.

2.5 Assay of vitamin B₁₂ by high performance liquid chromatography (HPLC) (Lambert *et al.*, 1992)

The assay was performed using a computer and software program based HPLC (Varian-Prostar), the star chromatography workstation version. Its body consists of a solvent delivery module as mobile phase container, a chromatography column for the separation of compounds, a sample injection point and a UV-visible detector. The HPLC analysis was done at Tamilnadu Agricultural University, Coimbatore.

Column conditions were given as below:

Column	:	C ₁₈
Mobile phase	:	Acetonitrile
Flow rate	:	0.5 ml/min
Detection at	:	254 nm
Sample rate	:	10 Hz
Run time	:	20 to 30 min
Run mode	:	Analysis
Measurement	:	Peak area

Standard

1mg of vitamin B₁₂ (Himedia) was diluted with 10 ml distilled water. From this, 20 ml was injected into the HPLC.

Calculation

Concentration of standard

The concentration of standard in 20 ml is

$$\frac{1 \text{ mg}}{10 \text{ ml}} = \frac{1000 \text{ } \mu\text{g}}{10000 \text{ } \mu\text{l}} = \frac{0.1 \text{ } \mu\text{g}}{1 \text{ } \mu\text{l}}$$

So, in 20 ml = 0.1 μg \times 20 = 2 mg = 0.002 mg

Therefore, the injected sample contain 0.002 mg / 0.02 ml

Retention time of standard = 4.702 min

The area of standard = 1507106 counts

Therefore, one count of standard = $\frac{0.002}{1507106} = 1.33 \times 10^{-9}$

2.6 Quantitative analysis of vitamin B₁₂ produced by control and *Pseudomonas* strain

For 1 g of sample,

One count of standard \times sample area count \times

$$\frac{\text{Amount of solvent used for the dilution of sample}}{\text{Amount of sample taken}}$$

$$= 1.33 \times 10^{-9} \times \text{sample area count}$$

$$\times \frac{\text{Amount of solvent used for the dilution of sample}}{0.02}$$

3. Results

3.1 Screening of vitamin B₁₂ producing microorganisms from rhizosphere and non-rhizosphere soil

Thirty-eight strains of *Pseudomonas* sp. were isolated from rhizosphere soil of tomato plant, twenty-six found to be vitamin B₁₂ producers. It was found that 68.4% of the *Pseudomonas* sp. isolated from rhizosphere were able to produce vitamin B₁₂ (Figure 1). From the non-rhizosphere soil sample, 20 *Pseudomonas* sp. strains were isolated. Only 8 (40%) of them were found to be the vitamin B₁₂ producers, whereas other strains were found to be no producers. This may be due to the need of high level of specific nutrients for vitamin B₁₂ producing *Pseudomonas* sp., which may be lacking in non-rhizosphere soil, unlike rhizosphere soil.

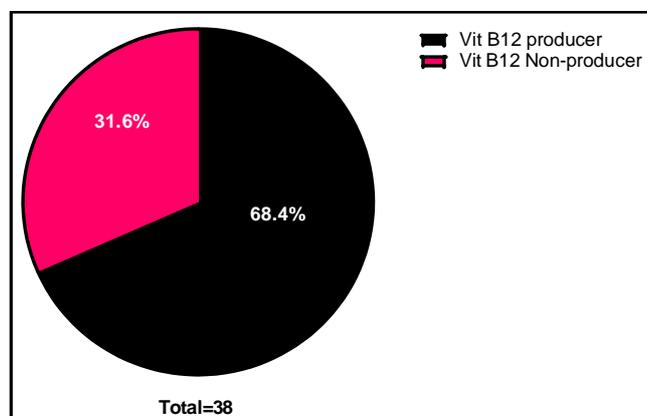


Figure 1: Screening of vitamin-B₁₂ from rhizosphere soil.

3.2 Screening of vitamin B₁₂ producing microorganism from gut of fish

A total of 10 *Pseudomonas* sp. strains were isolated from the gut of fish, *Tilapia* sp. Out of this ten, when screened for vitamin B₁₂ production by auxonography, six were found to be the vitamin B₁₂ producers (Figure 2). It is found that 60% of the fish gut *Pseudomonas* sp. was able to produce vitamin B₁₂. The organisms isolated from the intestine of fish caught from the river Cauvery, Erode show high load of organism and high frequency of vitamin production among *Pseudomonas* sp. than the non-rhizosphere soil. Comparatively the *Pseudomonas* sp. of rhizosphere soil. (68.4%) were producing vitamin B₁₂ more frequently than fish gut (60%) or non-rhizosphere (40%) soil *Pseudomonas* sp.

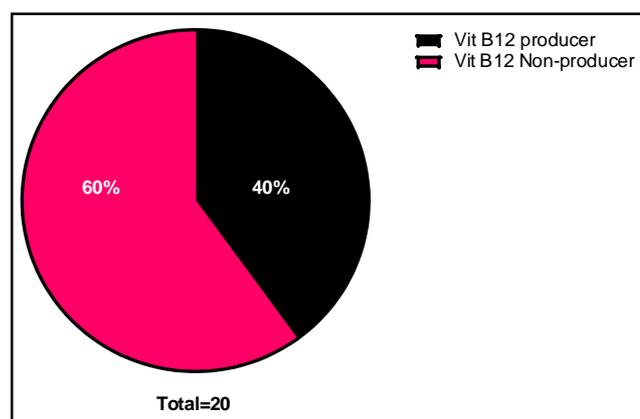


Figure 2: Screening of vitamin-B₁₂ from non-rhizosphere soil.

3.3 Assay of vitamin B₁₂ producers by HPLC

High performance liquid chromatography (HPLC) is one of the most sensitive methods for the determination of the vitamin B₁₂. HPLC allows fraction of elutes to be collected. Using HPLC very low concentrations (100-1000 pg/ml) in biological materials have to be determined (Lambert *et al.*, 1992). So, we used this method combined with UV-visible spectrometric analysis, where sensitivity will be more. Using this method, for the quantification of corrinoids, the "true cobalamin (cbl)" forms can be distinguished from cbl analogues, using intrinsic factors and heptacorrin as binders of the radio isotope dilution assay (RIDA) (Dgatali *et al.*, 1990).

There must be a change in yield happens according to the slight variations of fermentation parameters, medium used, alterations in percentage of the components and change in the organism. Control strain (*Propionibacterium freudenreichii*) produced 8.1mg/l of vitamin B₁₂. From rhizosphere soil, Strain R6 showed highest production of 11.54 mg/l and the lowest was observed on R18 (4.3 mg/l). Vitamin B₁₂ production from non-rhizosphere soil ranged from 1.17 mg/l to 5.25 mg/l. Strain NR3 showed highest production. Strain F2 from *Tilapia* sp. showed highest production of 6.68 mg/l (Figures 3 and 4). Figures 5 and 6 show HPLC analysis of vitamin B₁₂ synthesized by *Propionibacterium freudenreichii* and R6 isolate.

The ability of each of the organisms to produce vitamin B₁₂ differs in great extends. The wild type shows the above mentioned result (Table 4) of vitamin B₁₂ synthesis even without adding precursor in the media. With simulated fermentation condition or with genetically improved strains of this organism may increase the yield enormously.

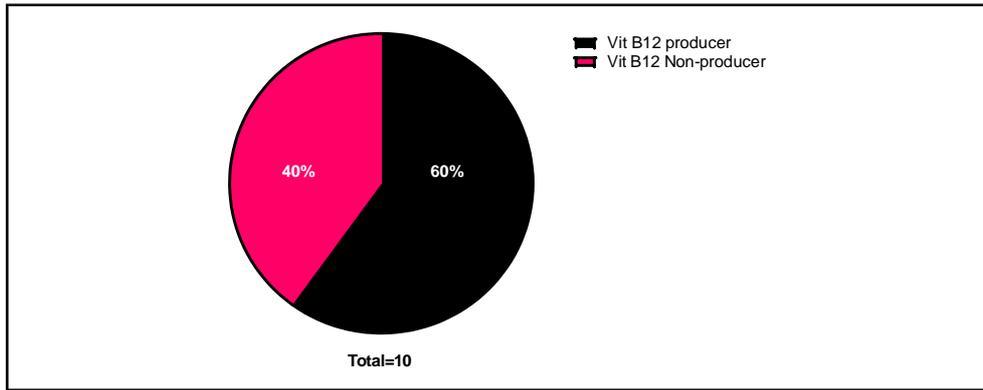


Figure 3: Screening of vitamin-B₁₂ from fish gut (*Tilapia*)

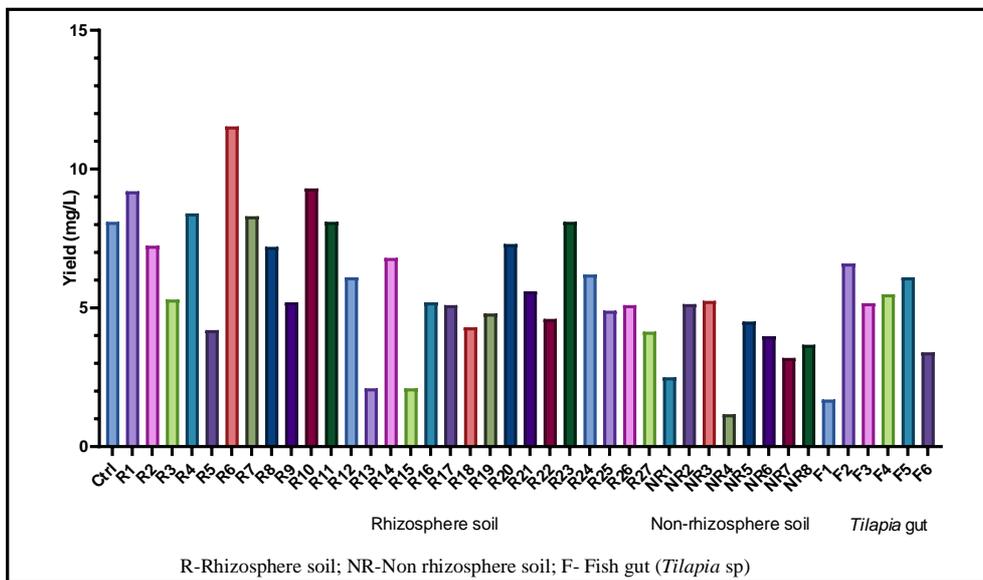


Figure 4: Quantification of vitamin B₁₂ using HPLC.

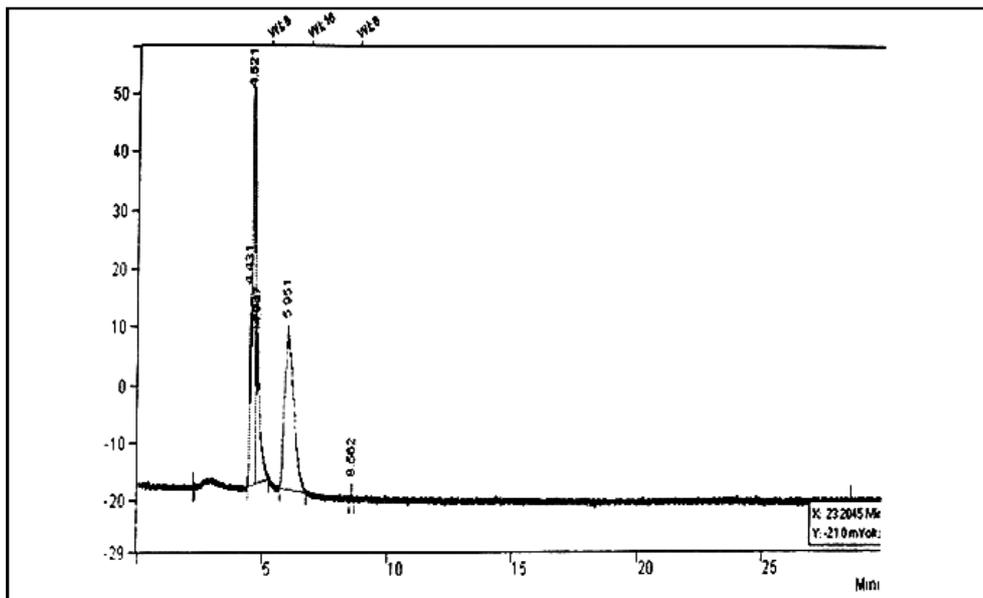


Figure 5: HPLC analysis of vitamin B₁₂ produced by *Propionibacterium freudenreichii*.

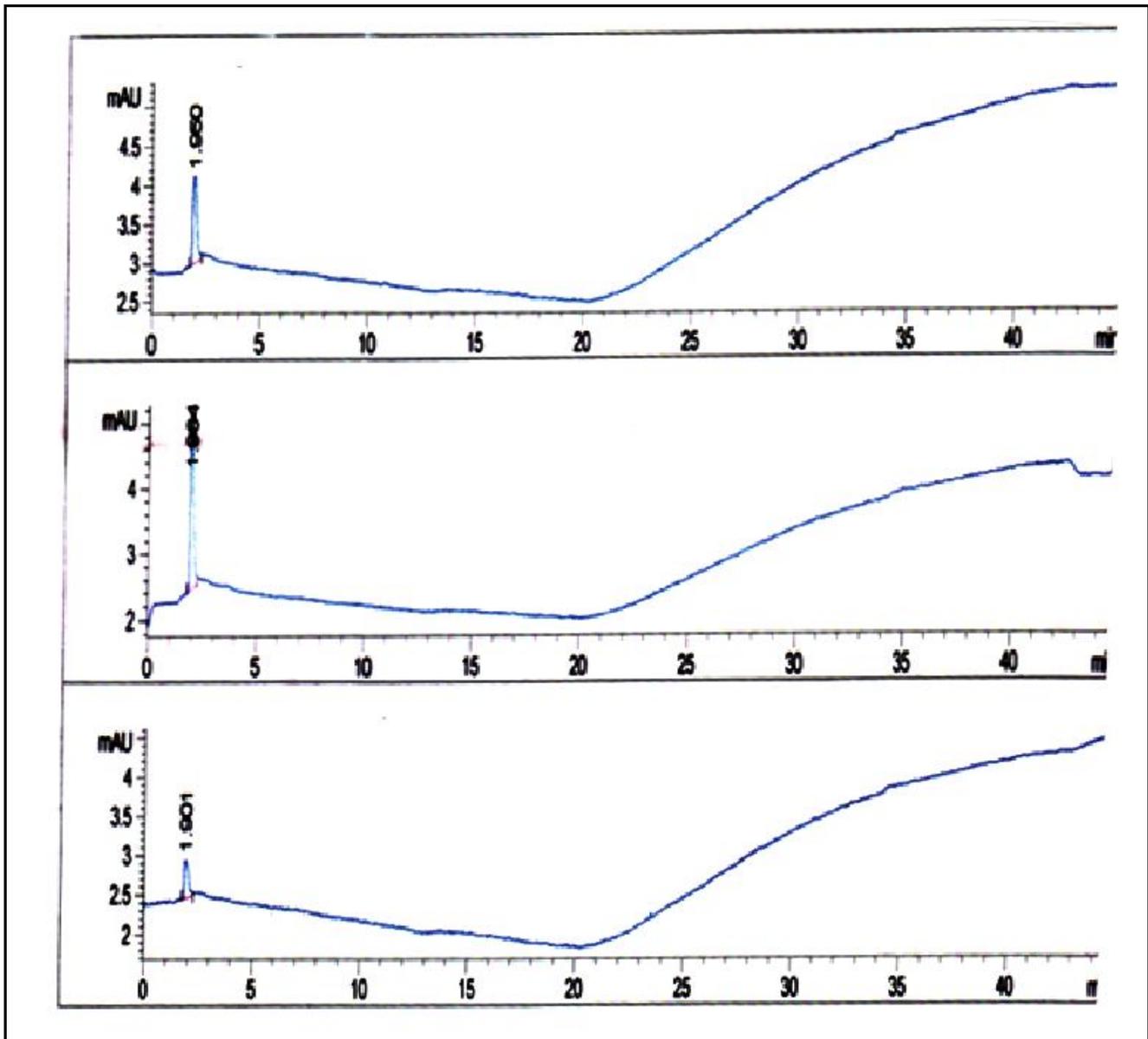


Figure 6: HPLC analysis of vitamin B₁₂ synthesized by R6 isolate.

4. Discussion

Vitamin B₁₂ is an essential vitamin plays a vital role in red blood cell formation, maintenance of central nervous system (CNS) and involved in metabolism of every cell in body. It is widely used as dietary supplement worldwide and used to treat anemia (Hunt *et al.*, 2014). The present study focuses on isolation and extraction of vitamin B₁₂ producing *Pseudomonas* sp. from rhizosphere, non-rhizosphere soil and fish gut (*Tilapia* sp.).

Rhizosphere soil (68.4%) showed highest population of vitamin B₁₂ producers than non-rhizosphere soil (40%) and fish gut (60%). This is due to the easy availability of nutrients on rhizosphere soil. *Pseudomonas* sp. from fish gut maintains a mutualistic relationship with the fish and thus, the chance of finding these types of organisms in fish gut and the productivity is also high. But, in the non-

rhizosphere soil, there is very less chance to make associations and the organisms must acquire nutrients from a more limited environment. This may prevent most of the organisms from extra production of compounds (Keshavarz and Moghadam, 2017; Watanabe and Bito, 2018).

In an earlier work, Baya *et al.* (1981) described that 62.5% of phosphate solubilizing bacteria from rhizosphere soil was synthesizing vitamin B₁₂ due to which, there was high incidence of vitamin accumulation in the root region. These vitamins produced by phosphate solubilizers contribute significantly to the plant growth. Cook and Lochhead (1959) report further confirms this phenomenon. Vitamin at root zone may be synthesized by plants, rhizosphere bacteria or both. It is true that green plants generally produce evident quantities of vitamins to meet their own needs. Yet, the root may not be able to produce all the vitamins they need

and there is evidence that the exogenous B vitamins can be absorbed by roots, producing favorable effect on respiration, protein synthesis and nutrient transfer (Rempe, 1973). These results well correlate with the present study.

The amount of vitamin B₁₂ which is present in animal tissue is too low for use in commercial production. Activated sludge from sewage treatment contains 4-10 mg B₁₂/kg, but isolation from this source is expensive due to the problem of separating the various forms B₁₂ analogs. Considering the production of vitamin B₁₂ by chemical process, it is almost impractical as it needs a series of a variety of steps of reaction, as high as 70. Vitamin B₁₂ was first obtained commercially as a byproduct of *Streptomyces* fermentation for the production of the antibiotics streptomycin, chloramphenicol, or neomycin, with a yield of about 1mg/liter. As the demand for vitamin B₁₂ increased, fermentation processes were developed with higher yielding strains. Commercial production is currently carried out easily by fermentation (Fang *et al.*, 2017).

Ye *et al.* (1996) have reported 6.64 mg/l of vitamin B₁₂ being produced by *Propionibacterium freudenreichii*. In our results, 8.12 mg/l of vitamin B₁₂ was synthesized by control strain *Propionibacterium freudenreichii*. Ye *et al.* (1996) followed a periodic operation with variation of oxygen supply and acquired high level vitamin B₁₂ of 9.54 mg/l. There must be a variation in the results when altering the fermentation parameters. The above mentioned authors describe the cycle of oxygen supply dependent on the cell growth rate calculated using online measurements of optical density of the fermentation broth. Another study conducted by Marwaha *et al.* (1983) followed 84 h of first stage anaerobic fermentation and another 84 h of aerobic fermentation with whey permeate medium and *Propionibacterium shermanii* showed higher production of vitamin B₁₂.

Pseudomonas sp. from rhizosphere soil (R6) showed higher vitamin B₁₂ production (11.54 mg/l) whereas non-rhizosphere soil and fish gut showed 5.25 mg/l and 6.68 mg/l. These wild type organisms when mutated or optimized can synthesis high amounts of vitamin B₁₂.

5. Conclusion

Samples from rhizosphere, non-rhizosphere and fish gut were screened for *Pseudomonas* sp. production. Rhizosphere soil showed highest production than the other two and the control bacteria (*Propionibacterium freudenreichii*). Further, the isolated organism can be improved for the production by a series of mutation and screening in the laboratories. Yield can also be enhanced by inhibiting the secondary metabolites which obstruct with the synthesis and the growth of organism or by adding analogues to the compounds which inhibit the desirable characters of an organism.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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