Purification, Identification, and Characterization of Methylcobalamin from *Spirulina platensis*

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The present study reports methylcobalamin in *Spirulina platensis* using high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), microbiological assay, chemiluminescence assay, liquid chromatography−mass spectrometry (LC−MS), and tandem mass spectrometry (MS/MS). Extraction of vitamin B_{12} from *S. platensis* was carried out without using cyanide. Partial purification was achieved using Amberlite XAD-2 followed by elution with 80% (v/v) methanol. Activated charcoal facilitated removal of impurities in *S. platensis* extract and in further purification of vitamin B_{12}. The purified fraction was identified to contain methylcobalamin as analyzed by HPLC and TLC. Authenticity of methylcobalamin was further confirmed by LC−MS and MS/MS. Quantitation of methylcobalamin in a test sample of *S. platensis* biomass was performed using microbiological assay and chemiluminescence assay and was found to be 38.5 ± 2 and 35.7 ± 2 μg/100 g of dry biomass, respectively.

KEYWORDS: *Spirulina platensis*; methylcobalamin; HPLC; chemiluminescence; LC−MS; MS/MS

INTRODUCTION

Vitamin B_{12} is a biologically active corrinoid, a group of cobalt-containing compounds with a macrocyclic pyrrole ring (*1*). Vitamin B_{12} acts primarily as a cofactor for enzymes, such as methionine synthase and methylmalonyl CoA mutase. It helps in maintenance of the myelin sheath, growth, cell development, and fat and carbohydrate metabolism in mammals (*2*). Vitamin B_{12} is mainly synthesized by certain bacteria that are associated with the gut flora of animals, contributing to the requirement of this vitamin. Since plants have no ability to synthesize vitamin B_{12} because of the absence of cobalamin-dependent enzymes (*3, 4*), strict vegetarians (vegans) have a greater risk of developing vitamin B_{12} deficiency (*5*) and, hence, need to depend upon vitamin B_{12}-fortified foods or vitamin B_{12}-containing dietary supplements to meet the requirement. Some seaweeds, viz., [*Porphyra yezoensis* (nori)], and microalgae (*Chlorella*) can supply adequate amounts of bioavailable vitamin B_{12} when consumed by strict vegetarians (*6−8*). *Spirulina platensis* is one of the most widely consumed cyanobacteria as a food supplement and contains substantial amounts of vitamin B_{12} and its analogue (*9*). In nature, true forms of vitamin B_{12} include hydroxocobalamin, adenosylcobalamin, and methylcobalamin. Analogues are similar in structure to true forms but differ in the nucleotide region of the corrin ring and ligands attached to cobalt (*10*). Vitamin B_{12} analogues in *S. platensis* were reported to be biologically inactive (*11, 12*) and found not to interfere in mammalian B_{12} metabolism (*13*).

The scope of the present study was to identify the true vitamin B_{12} among hydoxocobalamin, adenosylcobalamin, and methylcobalamin in *S. platensis* because it is gaining importance in health food formulations.

MATERIALS AND METHODS

Chemicals and Instruments. Cyanocobalamin, hydoxocobalamin, adenosylcobalamin, methylcobalamin, luminol, and urea−hydrogen peroxide were obtained from Sigma-Aldrich (Bangalore, India). Amberlite XAD-2 was obtained from Supelco, Sigma-Aldrich (Bangalore, India). The vitamin B_{12} assay medium was obtained from Himedia, Bangalore, India. Methanol was of high-performance liquid chromatography (HPLC) grade, and all other reagents used were of analytical grade. The thin-layer chromatography (TLC) sheet was from Merck (20 × 20 cm silica gel 60 F_{254}). A UV/vis spectrophotometer (UV-160A) and reverse-phase HPLC (SCL-10-AVP) were of Shimadzu, Kyoto, Japan. A HPLC column (300 × 4.6 mm, μm bondapak, particle size of 10 μm, C18 with 125 Å) was procured from Waters Corporation, Milford, MA. The lumino-meter was from Luminoskan TL plus, Thermolab Systems, Finland. Data acquisition was performed with decimal HyperTerminal TL plus software. The column used for electrospray ionization−mass spectrometry (ESI−MS) was Acquity UPLC HSS T3, 50 × 2.1 mm, 1.8 μm. Positive-ion tandem mass spectrometry (MS/MS) experiments were performed in product mode on a triple quadrupole TQD mass spectrometer (Waters Corporation, Milford, MA). Triple-distilled water was used for the preparation of the solvent system for HPLC analysis.

Organism and Culture. *S. platensis* strain (SP6) isolated and maintained at Central Food Technological Research Institute (CFTRI) was used for the experimental purpose. The algal cells were aseptically cultured in modified Zarrouk's media (*14*) at 25 °C on a rotary shaker (40 rpm). The media contained 16.0 g of $NaHCO_3$, 0.5 g of K_2HPO_4, 2.5 g of $NaNO_3$, 1.0 g of K_2SO_4, 1.0 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.08 g of Na_2EDTA, 1 mL of A5

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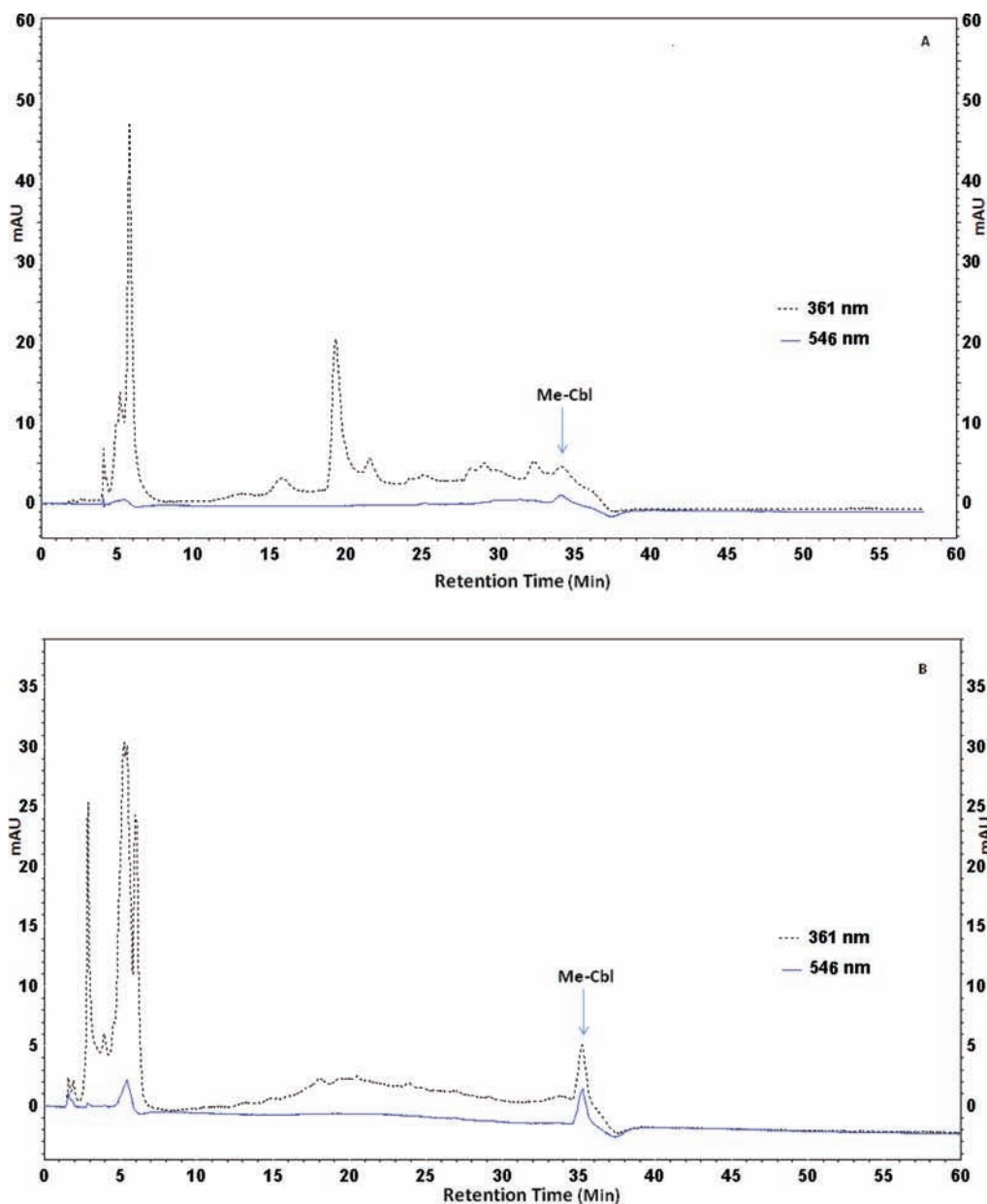
Figure 1. HPLC chromatogram of the (A) sample after passing through Amberlite XAD-2 and (B) sample after passing through activated charcoal.

(2.8 g of H_3BO_3, 1.8 g of $MnCl_2 \cdot 4H_2O$, 0.2 g of $ZnSO_4 \cdot 7H_2O$, 0.074 g of $CuSO_4 \cdot 5H_2O$, and 0.015 g of MoO_3), and 1 mL of B6 [0.02 g of NH_4NO_3, 0.09 g of $K_2Cr_3(SO_4)_4 \cdot 24H_2O$, 0.05 g of $NiSO_4 \cdot 7H_2O$, 0.02 g of $Na_2SO_4 \cdot 2H_2O$, 0.04 g of $Ti(SO_4)_3$, and 0.04 g of $CO(NO_3)_2 \cdot 6H_2O$]. The suspension cultures were scaled up serially from a 150 mL Erlenmeyer flask, 5 L carboy, an inoculum pond of 500 L, and then to 5000 L in race way ponds (*15*). The biomass was harvested by gravity filtration, washed twice with distilled water, lyophilized, and stored at −80 °C until use for effective stability of vitamin B_{12} (*16*).

Extraction of Vitamin B_{12}. A total of 1 kg of lyophilized biomass of *S. platensis* was suspended in triple-distilled water and autoclaved at 121 °C for 10 min. The homogenate was centrifuged at 10000*g* for 10 min. The cooled supernatant was adjusted to a pH of 6.0 and was analyzed for vitamin B_{12} (*17*). For purification, the sample was loaded onto Amberlite XAD-2, prepared as a methanolic suspension of the resin packed to a bed height of 15−16 cm. The column was equilibrated with water (*18*). The sample was eluted with 80% (v/v) methanol and concentrated using Rotavapor (Buchi). The concentrate was further purified over activated charcoal and analyzed for vitamin B_{12} by HPLC.

Vitamin B_{12} Analysis. *HPLC.* The sample was injected in to a reverse-phase HPLC column pre-equilibrated with solvent. Vitamin B_{12} was eluted with a linear gradient of methanol [from 0 to 90% of a 50% (v/v)

aqueous methanol solution containing 0.1% (v/v) acetic acid] for 40 min, with a flow rate of 1 mL/min (*19*). The retention times of authentic standards of hydoxocobalamin, cyanocobalamin, adenosylcobalamin, and methylcobalamin were recorded.

Assay of Vitamin B_{12} Using Escherichia coli. The *E. coli* (ATCC 11105) strain was grown in maintenance medium at 37 °C and mixed with vitamin B_{12} assay agar and pour-plated (*20*). Wells of 5 mm in diameter were bored in the solid agar media. Standard vitamin B_{12} and purified sample (50 μL) were inoculated into the wells. Triple-distilled water was used as the control. The plates were incubated at 37 °C for 24 h, and the zone of growth was recorded.

Assay of Vitamin B_{12} Using Lactobacillus delbrueckii. Vitamin B_{12} was assayed by the microbiological method using *L. delbrueckii* MTCC 911. The standard vitamin B_{12} (range of 0.01−0.2 μg/mL) was prepared in distilled water for analysis. HPLC eluant was assayed for vitamin B_{12} activity. The turbidity (% T) of *L. delbruekii* test culture was measured at 600 nm using a Shimadzu spectrophotometer (UV-160A) (*21*).

Chemiluminescence-Based Assay. Studies on the chemiluminescence reactions were carried out using a luminometer. The reactions were carried out in a polystyrene cuvette. Optimized concentrations of luminol and vitamin B_{12} were added to each cuvette followed by the addition of urea−H_2O_2. This results in the production of signals that was measured in

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terms of chemiluminescence units (*22*). The signals were plotted at 10 s intervals for a period of 10 min. An increase in the chemiluminescence unit was observed commensurate with vitamin B_{12} levels.

TLC Separation and Analysis. A concentrate of HPLC eluant was spotted on a silica gel TLC sheet and developed with 2-propanol/NH$_4$OH (28%)/water (7:1:2, v/v/v) at room temperature in the dark (*23*), and the R_f was recorded.

HPLC−ESI−MS Analysis. This was carried out in a tandem quadrupole with exact mass measurement in positive mode. The cone and desolvation gas were set to 28 and 1000 L/h, respectively. Sample source

Figure 2. TLC of the (A) standard Me-Cbl and (B) HPLC fraction of Me-Cbl from the *S. platensis* sample.

conditions were as follows: capillary voltage, 3.00 kV; sample cone voltage, 28 V; extraction cone voltage, 3 V; source temperature, 120 °C; and desolvation temperature, 400 °C; cone gas flow, 25 L/h; collision gas flow, 0.10 mL/min; LM 1 resolution, 15.00; HM 1 resolution, 15.00; ion energy 1, 0.50; MS mode entrance, 50.00; MS mode collision energy, 2.00; and MS mode exit, 50.00. Samples were introduced into the mass spectrometer through a direct-flow injection UPLC system for solvent delivery at the flow rate of 0.6 mL/min. A linear gradient of 10 mM ammonium formate and 0.1% formic acid in water (A) and 10 mM ammonium formate and 0.1% formic acid in methanol (B) was used. The column temperature was set at 35 °C. MS of the sample and standard was recorded.

MS/MS Experiments. Positive-ion MS/MS experiments were performed in product mode on a triple quadrupole TQD mass spectrometer (Waters Corporation, Milford, MA). The instrument was operated with the following instrumental conditions: source temperature, 120 °C; desolvation temperature, 400 °C; capillary voltage, 3.00 kV; cone voltage, 28 V; extraction cone, 3 V; drying and cone gas, nitrogen; entrance, 1.00; collision energy, 20.00; exit, 0.50; LM 2 resolution, 15.00; HM 2 resolution, 15.00; ion energy 2, 1.00; gain, 1.00; multiplier, 511.00. MS/MS, selected ion recording (SIR), and product ion spectra of the sample and standard were recorded.

RESULTS AND DISCUSSION

Extraction of Vitamin B_{12}. True forms of vitamin B_{12} are hydoxocobalamin, adenosylcobalamin, and methylcobalamin, which are unstable during extraction upon exposure to light. To stabilize these true forms, many researchers have used cyanide to form a stable molecule of cyanocobalamin and, therefore, also in the extraction of vitamin B_{12} in *S. platensis* (*13*, *16*). Use of cyanide in the extraction procedure will not help in identifying true forms of vitamin B_{12}, because cyanide converts all natural forms to cyanocobalamin. In the present study, we used an extraction method without cyanide to identify true forms of vitamin B_{12}. The culture of *S. platensis* was grown in media devoid of Co salts to avoid the formation of inactive corrinoid compound in algae (*24*).

Partial Purification Using Amberlite XAD-2 and Charcoal. Water extract obtained from dry *S. platensis* biomass was
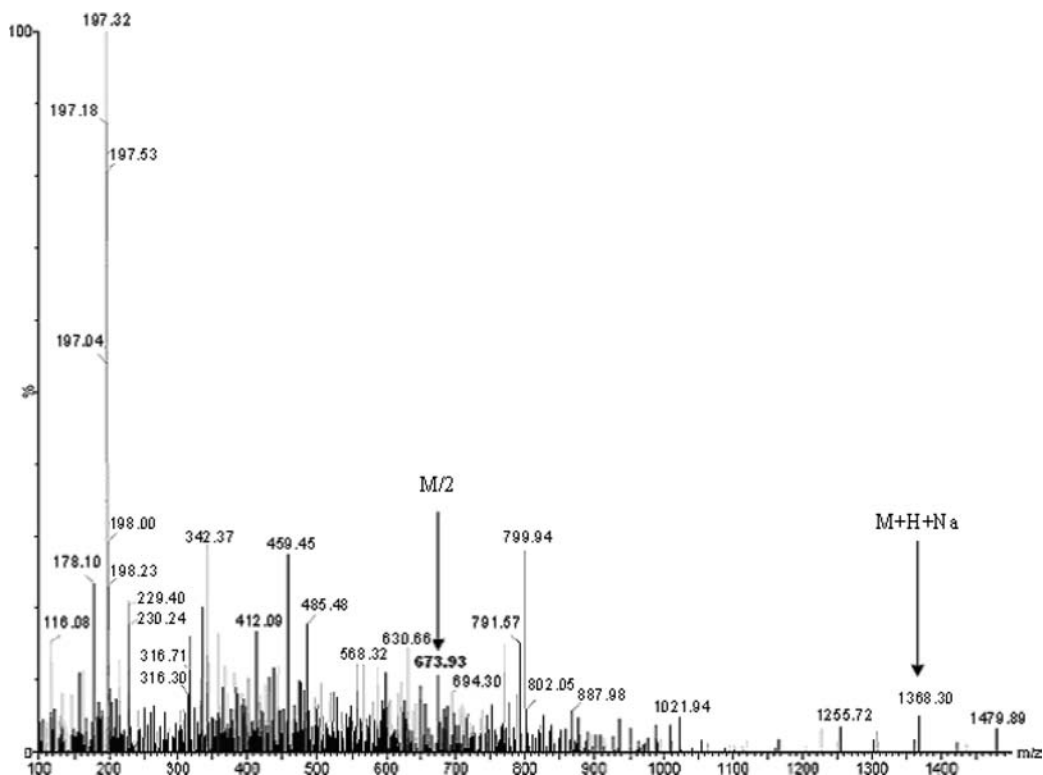

Figure 3. Mass fragmentation pattern of the sample. Methylcobalamin *m/z* 1344; M/2, 673; M + H + Na, 1368.
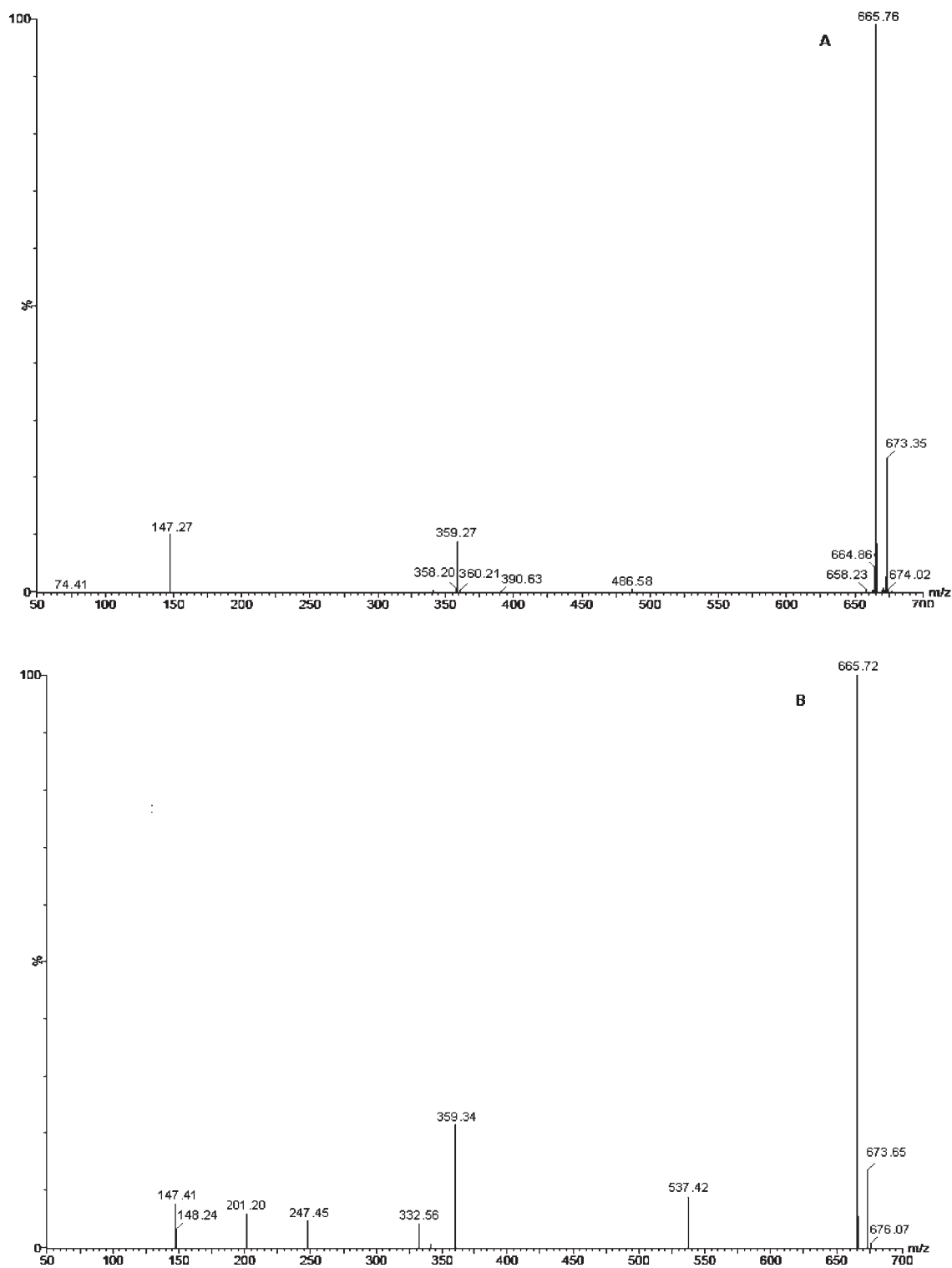
Figure 4. MS/MS of the (A) standard methylcobalamin and (B) sample.

concentrated and passed through Amberlite XAD-2. The eluant was analyzed by HPLC, and the profile is shown in **Figure 1A**. The isolation of cobalamins on Amberlite XAD-2 columns was found to be suitable because it efficiently binds the vitamin B_{12} from the complex matrix (*18*). The fractions collected from XAD-2 were further purified using charcoal. The eluate from the XAD column was either dissolved in water or in 80% methanol for further separation of vitamin B_{12}. Activated charcoal efficiently bound vitamin B_{12} from the aqueous extract but not from that of 80% methanol. The activated charcoal-purified sample when

analyzed by HPLC showed more prominent peak matching with standard methylcobalamin (**Figure 1B**). This is an important observation not explored previously for the purification of vitamin B_{12}.

HPLC Analysis of Vitamin B_{12}. HPLC analysis for vitamin B_{12} was carried out using a suitable solvent system that separated all forms of vitamin B_{12}. The retention times (RTs) of hydoxocobalamin, adenosylcobalamin, and methylcobalamin were found to be 20.1, 29.2, and 35.2 min, respectively. Purified *S. platensis* extracts were injected into HPLC to identify true forms of vitamin

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B_{12} compared to the RTs of standards. We found that the RT at 35.2 min was identical to that of methylcobalamin. The true form of vitamin B_{12} was detected at 546 nm, along with 361 nm wavelengths. It is reported that vitamin B_{12} absorption at 361 nm is ~3 times more than that at 546 nm (25). Therefore, analysis was performed at both of the wavelengths. After each step of purification, there was a gradual decrease in the impurities, as evident from the spectra (**Figure 1B**). It is evident from **Figure 1A** that the *S. platensis* extract after passing through XAD-2 has many peaks, whereas **Figure 1B** showed only few peaks, suggesting minimum impurities in the sample after passing through activated charcoal. A peak at 35.2 min having absorbance at 546 and 361 nm, similar to methylcobalamin, was observed. Further, the purity of the peak was confirmed with spiked standard methylcobalamin. The peak at 5−6 min having an absorbance maximum at 361 nm was also observed (**Figure 1A**). It is interesting to know that this peak did not match any of the standard vitamin B_{12} forms analyzed and may be due to the conjugated nature of the compound.

TLC Analysis of Vitamin B_{12}. The HPLC fraction (peak eluting at 35.2 min) was analyzed by silica gel TLC. The R_f values of the purified *S. platensis* sample was compared to the standard methylcobalamin and found to be similar to the values of authentic methylcobalamin (**Figure 2**).

Chemiluminescence Analysis of Vitamin B_{12}. The HPLC fraction was collected and analyzed for cobalt-enhanced chemiluminescence. It is reported that Co^{2+} enhances the photon production during the luminol and H_2O_2 reaction (22). During the chemiluminescence reaction, photons are produced that are directly proportional to the vitamin B_{12} concentration. The sample was quantified and found to contain 35.7 ± 2 μg of methylcobalamin for 100 g of dry biomass of *S. platensis*. Chemiluminescence can detect Co of both true and pseudo forms. This assay method has a limitation for distinguishing true and pseudo forms of cobalamin. However, a significant correlation was observed between microbiological assay and chemiluminescence assay upon purification of methylcobalamin from *S. platensis*, which merits using the latter method for analysis of vitamin B_{12}.

Microbiological Assay of Vitamin B_{12}. To confirm the presence of vitamin B_{12} in *S. platensis*, the eluted sample from HPLC was subjected to microbiological assay using *E. coli*. A zone of *E. coli* growth was observed surrounding the wells containing standard vitamin B_{12}, as well as for the purified *S. platensis* fraction, confirming the presence of vitamin B_{12}. There was no growth surrounding the wells of the control containing triple-distilled water.

The quantitation of methylcobalamin in the eluted sample from HPLC was carried out by microbiological assay using *L. delbruekii*. The sample was found to contain 38.5 ± 2 μg of methylcobalamin for 100 g of dry biomass of *S. platensis* as per the standard plot of methylcobalamin. As mentioned earlier, the quantitations of methylcobalamin by microbiological assay and chemiluminescence assay were significantly similar, indicating the presence of the true form of vitamin B_{12}, which was also reported by Watanabe et al. (21) in different food samples. However, these authors have observed a significant difference in the values of microbiological assay and chemiluminescence assay in *S. platensis* extract, which may be possibly due to the extraction method employed (wherein KCN was used for extraction) and the specificity of the chemiluminescence method. It is evident from our study that the true form of vitamin B_{12} shows a good correlation between microbiological and chemiluminescence methods.

LC−MS and MS/MS of the Sample. The LC−MS analysis of the standard and the charcoal-purified sample was carried out, and the peak matching with standard methylcobalamin was

Table 1. Product Ion Scan for Standard Methylcobalamin and Sample

number	sample name	retention time (min)	positive mode	
			collision energy (eV)	product ion mass
1	standard	3.943	20	673.65 > 665.76
2	sample	3.943	20	673.65 > 665.72

ionized. The mass of the ionized peak confirms the presence of methylcobalamin in the *S. platensis* sample. The presence of methylcobalamin was further confirmed by SIR and product ion spectra. The mass of methylcobalamin is m/z 1344.38. The spectrum shows that it is doubly charged, and hence, a mass of 673.93 was observed. Because the intensity of mass 673.93 (**Figure 3**) observed in the sample was less, SIR was performed for the mass to confirm the presence of methylcobalamin in the sample. MS/MS of methylcobalamin and the sample was compared; the daughter ion of both were found to be similar (panels **A** and **B** of **Figure 4**). The product ion scan performed for both the standard methylcobalamin and sample was found to be similar, as presented in **Table 1**.

Biologically active vitamin B_{12} compounds, such as hydoxocobalamin, sulphitocobalamin, adenosylcobalamin, and methylcobalamin, were reported in *Porphyra yezoensis*, commonly known as purple laver (16). Yamada et al. (26) have also reported that methylcobalamin is predominantly found in a purple laver. Apart from these algae, methylcobalamin was found in methanol-using bacteria (27). In the present study, we have confirmed the presence of methylcobalamin in *S. platensis*. The mass of methylcobalamin in *S. platensis* determined by MS and MS/MS further substantiated the presence methylcobalamin. In our study, we found methylcobalamin to be 38.5 ± 2 and 35.7 ± 2 μg/100 g of dry biomass of *S. platensis* by microbiological assay and chemiluminescence assay, respectively. Because the vegetarian diet does not contain vitamin B_{12}, *S. platensis*, along with other nutrients, helps in meeting the recommended daily allowance of vitamin B_{12} of the vegetarian diet and also meeting the requirement of needy individuals of varied food habits or health status.

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