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ORIGINAL ARTICLE

Characterization of vitamin B_{12} in *Dunaliella salina*

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Abstract Vitamin B_{12} is one of nature's complex metabolite which is industrially produced using certain bacteria. Algae could be an alternative source of vitamin B_{12} and in this study, vitamin B_{12} from a halotolerant green alga, *Dunaliella salina* V-101 was purified and characterized. The extract of *Dunaliella* was purified by passing through Amberlite XAD-2 and EASI-extract vitamin B_{12} immunoaffinity column. The total vitamin B_{12} content in purified sample fractions was 42 ± 2 μg/100 g dry weight as determined by the chemiluminescence method which was almost close to 49 ± 2 μg/100 g dry weight as estimated by microbiological method. Further quantification of total vitamin B_{12} using gold nanoparticle (AUNPs) based aptamer showed 40 ± 0.8/100 g dry weight. There was a good correlation among all the methods of quantification. Adenosylcobalamin, a form of vitamin B_{12} which is a cofactor for methylmalonyl CoA mutase was identified by HPLC. Upon quantification, *Dunaliella* was found to contain 34 ± 4 μg of adenosylcobalamin for 100 g dry biomass. Authenticity of adenosylcobalmin was confirmed by tandem mass spectrometry (MS/MS), selected ion recording (SIR) and multiple reaction monitoring (MRM) studies.

Keywords *Dunaliella salina* · Adenosylcobalamin · Chemiluminescence · Vitamin B_{12} immunoaffinity column, gold nanoparticle based aptamer

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Introduction

Vitamin B_{12} is an essential nutrient for the maintenance of myelin sheath surrounding nerve cells in humans (Herbert 1996) that promotes growth, cell development, fat and carbohydrate metabolism. It is also essential for the rapid synthesis of DNA during cell division, particularly the bone marrow tissue responsible for red blood cell formation (Stabler 1999). The biologically active forms of vitamin B_{12} are methylcobalamin and adenosylcobalamin but it is cyanocobalamin that is used in vitamin supplements, pharmaceuticals and fortification of food. Industrially vitamin B_{12} is produced through certain bacteria only. Plants are generally believed neither to synthesize nor utilize cobalamin for growth hence strict vegetarians have a greater risk of developing vitamin B_{12} deficiency. However some freshwater green algae, *Chlamydomonas reinhardtii* (Watanabe et al. 1991) and *Chlorella vulgaris* (Watanabe et al. 1997) contain vitamin B_{12}. When dried purple lavers were fed to vitamin B_{12} deficient rat, it was observed that the algal B_{12} is bioavailable to rats (Takenaka et al. 2001). While in halotolerant green algae there is little information available on the B_{12} content.

Dunaliella salina a halophilic green microalga is known for its β-carotene content. It produces large amounts of carotenoids and it is being used in dietary supplements and cosmetics. Carotenoids, glycerol, vitamin C, vitamin E are the important intracellular components in *Dunaliella* (Baz et al. 2002). It has a significant effect on decreasing the risk of lung, esophagus, pancreas, stomach, breast, skin, colon and ovary cancers (Poppel and Goldbohm 1995; Baz et al. 2002; Murthy et al. 2005; Raja et al. 2007). Prevention of tumors, extending the immunology response and neoplastic transformations are the other benefits reported (Wald et al. 1988; Challem 1997; Murthy et al. 2005; Raja et al. 2007). However *Dunaliella* has

not been studied much for the presence of vitamin B_{12} and its forms.

The aim of the present study was to evaluate the total vitamin B_{12} and the form of vitamin B_{12} in the *Dunaliella* culture.

Materials and methods

Chemicals and instruments

Cyanocobalamin (CN-Cbl), Adenosylcobalamin (Adl-Cbl), methylcobalamin (Me-Cbl), hydroxocobalamin (OH-Cbl), luminol, urea hydrogen peroxide diethylpyrocarbonate (DEPC), silver nitrate, gold (III) chloride and trisodium citrate were procured from Sigma Aldrich chemicals Co. (St. Louis, MO, USA). Amberlite XAD-2 was obtained from Supelco (Sigma Aldrich Co. Bangalore, India). Vitamin B_{12} assay medium was obtained from Himedia, Bangalore, India. Vitamin B_{12} RNA aptamer sequence (5′ GGA ACC GGU GCG CAU AAC CAC CUC AGU GCG AGC AA 3′) was adapted from Lorsch and Szostak (1994) report. All pyrimidines were 2′ fluoro modified and obtained from Trilink Biotechnologies (San Diego CA, USA). Stock solutions were prepared in DEPC treated water. Methanol was of HPLC grade. HPLC (SCL-10-AVP) was from Shimadzu Kyoto, Japan. Reverse-phase HPLC Column (4.6 × 300 mm, μ bondpack, particle size 10 μm) was from Waters Corp. (Milford MA, USA). Luminometer from Luminoskan TL plus, thermolab systems (Helsinkin, Finland). Data acquisition was performed with decimal HyperTerminal TL plus software. EASI-EXTRACT vitamin B_{12} immunoaffinity column was procured from R-Biopharma, Scotland. Column used for ESI-MS was Acquity UPLC HSS T3 2.1 × 50 mm,1.8 μm. Positive ion MS/MS experiments was performed in product mode on a triple quadrupole Xevo TQD mass spectrometer (Waters Corp., Milford USA). Triple Distilled water was used for the preparation of solvent system for HPLC analysis. All other reagents used were of the highest purity commercially available.

Organism and culture

D. salina strain V-101, was obtained from Centre for Advanced Studies in Botany, Madras University, Chennai. Liquid culture of *D. salina* was maintained in modified AS100 medium (MAS100) (Vonshak 1986). The stock culture was maintained under 16 h light intensity of 1.5 ± 0.2 Klux at 25 ± 1 °C. The outdoor culture was grown in a circular pond at ambient temperature ranging from 24 to 28 °C. The culture growth was monitored by measuring optical density at 560 nm. The biomass was harvested by centrifugation and washed twice with distilled water, lyophilized and stored at −80 °C until further use.

Extraction

The lyophilized *Dunaliella* cells of 100 g were added to 0.1 mol/L acetate buffer with pH 4.8. Total vitamin B_{12} was extracted from the cell suspension by boiling with 20 mg of KCN. The solution containing algal cells was boiled for 30 min at 98 °C and centrifuged at 10,000 *g*. The extraction was repeated twice and the supernatant was pooled and used for the assay. The extraction was carried out in dark. The combined supernatant fractions were loaded on to a column of Amberlite XAD-2 resin which had been washed with methanol and then equilibrated with distilled water. The sample was eluted with 80 % (*v*/*v*) methanol solution in the dark. The eluate containing a corrinoid compound was evaporated to dryness using rotavapor (Buchi B 490) and dissolved in distilled water.

To evaluate the forms of B_{12} the biomass was suspended in triple distilled water and autoclaved at 121 °C for 10 min. The homogenate was centrifuged at 10,000 *g* for 10 min. The cooled supernatant was used for B_{12} analysis. The whole extraction process was carried out in dark. Partial purification was done by passing through Amberlite XAD-2 resin column.

Purification using immunoaffinity column

The partially purified sample obtained was passed through the EASI-EXTRACT vitamin B_{12} immunoaffinity column which contained monoclonal antibody with high affinity and specificity to vitamin B_{12}. The column was washed with 20 ml of 2 % Tween 20 in water followed by 10 ml water. Elution of the vitamin was carried out with 100 % HPLC grade methanol. The eluted sample was evaporated to dryness under reduced pressure and analyzed using HPLC.

High performance liquid chromatography

The concentrated sample was analyzed on a reverse phase μBondapack HPLC column (4.6 × 300 mm, particle size 10 μm). Elution of the vitamin B_{12} compound was carried out with a linear gradient of methanol [from 0 to 90 % of a 50 % (*v*/*v*) methanol solution containing 0.1 % (*v*/*v*) acetic acid] for 40 min by measuring the absorbance at 361 nm and 546 nm. The flow rate was 1 ml/min (Watanabe et al. 2000). The retention times of authentic standards and sample were recorded.

Microbiological assay of vitamin B_{12}

Lactobacillus delbrueckii MTCC 911 strain obtained from Microbial Type Culture Collection, CSIR-IMTECH, Chandigarh, India was used for microbiological assay. The standard vitamin B_{12} was prepared in the range of 0.01–0.2 μg/ml for the analysis. Purified fractions from HPLC were

collected, pooled, concentrated and used for the assay. The turbidity (%T) of *Lactobacillus delbrueckii* test culture was measured at 600 nm (Watanabe et al. 1998).

Chemiluminescence assay of vitamin B_{12}

Purified fractions from HPLC were concentrated and subjected to chemiluminescence (CL) assay. Chemiluminescence reactions were carried out using a luminometer and in a polystyrene cuvette. The CL signals were plotted at 10-s intervals for a period of 10 min. The sample was prepared according to Kumar et al. (2009). An optimized concentration of luminol and vitamin B_{12} was added to the cuvette followed by urea– H_2O_2. This results in CL signals. The generation of signals measured in terms of chemiluminescence units (CLU) was caused due to the reaction between luminol and urea hydrogen peroxide with vitamin B_{12} (Kumar et al. 2009). An increase in CLU can be observed in the presence of vitamin B_{12}.

Gold nanoparticle based RNA aptamer detection

For the detection of vitamin B_{12}, RNA aptamer-based colorimetric sensor using gold nanoparticles were employed as described in the earlier report (Kumudha et al. 2015). AUNPs were prepared and the concentration and the size of AUNPs were determined using an ultraviolet-visible (UV-vis) spectrophotometer. The mixture containing aptamer and optimized concentration of AUNPs was shaken gently for 10 min at room temperature and incubated for 10 min along with the purified sample. The change in the color and spectra of the sample after addition of NaCl were measured using UV-vis spectrophotometer in a wavelength range from 400 nm to 700 nm.

UPLC-ESI-MS

UPLC-ESI-MS was carried out in a UPLC/Xevo TQD Tandem quadrupole with nominal mass measurement in positive mode. The cone and desolvation gas were set to 28 and 1000 L/H, respectively. Sample source conditions were as follows: capillary voltage, 3.00 kV; sample cone voltage, 10 V; extraction cone voltage, 2 V; source temperature, 135 °C; and desolvation temperature, 450 °C; cone gas flow (L/H) 10; collision gas flow (ml/min) 0.21; LM 1 resolution 15.00; HM 1 resolution 15.00; Ion energy 1, 0.50; MS Mode entrance, 50.00; MS Mode collision energy, 1.00; MS Mode exit 50.00; MSMS Mode entrance, 1.00; MSMS mode collision energy, 1.00; MSMS Mode exit, 0.5; LM 2, resolution, 15.00; HM 2, resolution, 15.00; Ion energy 2, 1.0; Gain, 1.00 and multiplier −522.98. 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. Column used was Acquity UPLC HSS T3 2.1 × 50 mm, 1.8 μm. 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. Column temperature was set at 35 °C (Kumudha et al. 2010).

MS/MS experiments

Positive ion MS/MS experiments were performed in product mode on a triple quadrupole TQD mass spectrometer (Waters corporation Milford USA). The instrument was operated with the following instrumental conditions: Calibration dynamic 1, capillary (kV) 3.00; cone (V) 10.00; extractor (V) 2.00; RF (V) 0.10; source temperature (°C) 135; desolvation temperature (°C) 450; cone gas flow (L/H) 10; desolvation gas flow (L/H) 1100; collision gas flow (ml/min) 0.21; LM 1, resolution 15.00; HM 1, resolution 15.00; Ion energy 1, 0.50; MS mode entrance 50.00; MS mode collision energy, 1.00; MS mode exit, 50.00; MSMS mode entrance,1.00; MSMS mode collision energy 1.00; MSMS mode exit 0.50; LM 2, resolution 15.00; HM 2, resolution 15.00; Ion energy 2, 1.00; Gain, 1.00; multiplier −522.98.

Results and discussion

Vitamin B_{12} in *Dunaliella salina*

In the present study, *Dunaliella* cells were grown in outdoor circular cement ponds and the fresh biomass was lyophilized and used for the estimation of total B_{12}. In order to quantify the total vitamin B_{12}, cyanide was added in the extraction protocol so as to convert all the natural forms such as OH-Cbl, Adl-Cbl and Me-Cbl to cyanocobalamin.

Purification of *Dunaliella* extracts on Amberlite XAD −2 ensures efficient binding of the vitamin B_{12} from the complex matrix (Fenton and Rosenberg 1978). Further purification on EASI-EXTRACT vitamin B_{12} immunoaffinity column enables concentration of the analyte and removal of interfering compounds allowing accurate quantification of vitamin B_{12} (Marley et al. 2009). Immunoaffinity columns have also been reported to be highly efficient for the purifiction of vitamin B_{12} (Heudi et al. 2006). The eluant was analysed by HPLC and the profile observed was similar to that of the standard cyanocobalamin. The purity of the peak was confirmed with spiked standard cyanocobalamin. The fraction was collected and subjected to further confirmation and quantification by chemiluminescence and microbiological assay. It is reported that Co^{2+} enhances the photon production during luminol and H_2O_2 reaction (Kumar et al. 2009). In a chemiluminescence reaction, photons are produced and are directly proportional to

the vitamin B_{12} concentration. The sample was quantified by chemiluminescence assay and it was found to contain 42 ± 2 μg of cyanocobalamin for 100 g dry biomass of *Dunaliella*.

Microbiological assay of vitamin B_{12} is one of the most frequently applied methods for routine analysis of vitamin B_{12} (Kralova et al. 1982). The intensity of turbidity caused by cell growth is proportional to vitamin B_{12} concentration. The quantification of cyanocobalamin in the eluted sample from HPLC was carried out by microbiological assay using *Lactobacillus delbrueckii* MTCC 911. The sample was quantified and was found to contain 49 ± 2 μg of cyanocobalamin for 100 g dry biomass of *Dunaliella*.

Total cyanocobalamin in the sample was quantified using gold nanoparticle based RNA aptamer. Aptamer using AUNPs have been considered as an on-site detection method with high specificity and sensitivity (Cho et al. 2008). These aptamers are short DNA or RNA sequences which have high affinity towards its analyte. The AUNPs were treated with vitamin B_{12} aptamer along with the sample. The addition of salt to the solution creates electrostatic repulsion between negatively charged AUNPs and results in aggregation of AUNPs leading to a red-to-purple colour shift in the presence of vitamin B_{12}. UV-vis studies provided quantitative results and based on the spectral data, the vitamin B_{12} in the sample was estimated at 40 ± 0.8/100 g dry weight.

Identification of form of vitamin B_{12}

To evaluate the forms of vitamin B_{12} found in *Dunaliella*, the extraction was carried out in aqueous solution without cyanide. Lyophilized sample was used for extraction since it exhibited higher stability for biologically active vitamin B_{12} (Takenaka et al. 2001). Forms of vitamin B_{12} in lyophilized *Dunaliella* cells were determined after extracting the lyophilized algal cells in aqueous condition and purifying vitamin B_{12} by passing the extract through Amberlite XAD-2 column and immunoaffinity column. HPLC analysis of eluant for vitamin B_{12} was carried out using a suitable solvent system which separated all forms of vitamin B_{12}. Retention time (RT) of standard OH-Cbl, Adl-Cbl and Me-Cbl were found to be 20.1, 29.2 and 35.2 mins respectively. Purified samples were injected into HPLC to identify forms of vitamin B_{12} based on RT of standards. It was observed that the retention time of the sample was identical to that of standard Adl-Cbl. The form of vitamin B_{12} was detected at 546 nm along with 361 nm wavelengths. A peak at 29.2 min having absorbance at 546 nm similar to Adl-Cbl was observed (Fig. 1a and b). Further, the purity of the peak was confirmed with spiked standard Adl-Cbl. The Adl-Cbl fraction was collected and subjected to further confirmation and quantification by chemiluminescence and microbiological

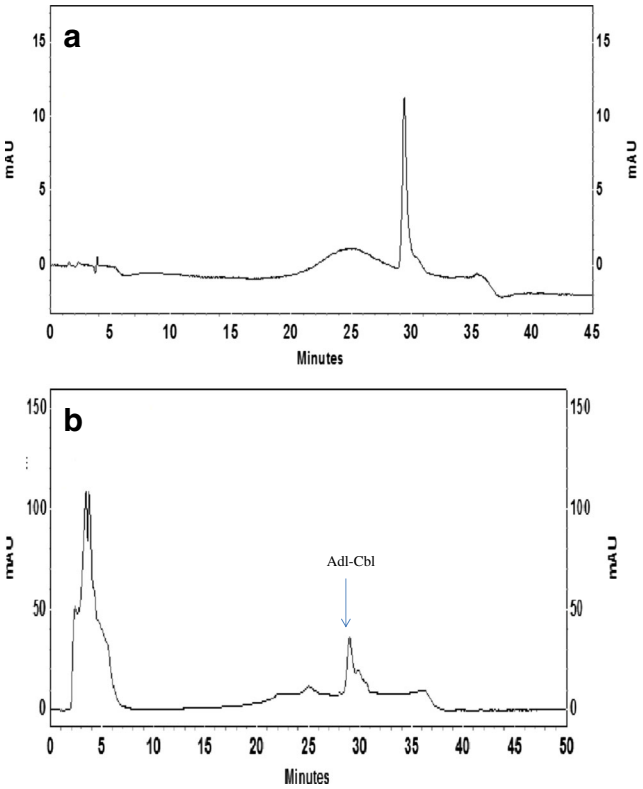

Fig. 1 **a** HPLC chromatogram of the standard Adenosylcobalamin. **b** HPLC chromatogram of the *Dunaliella* extract

assay. In chemiluminesence method 27 ± 4 μg of adenosylcobalamin was observed for 100 g dry biomass of *Dunaliella* while microbiological assay showed 34 ± 4 μg of adenosylcobalamin for 100 g dry biomass of *Dunaliella*. Similar correlation between microbiological and chemiluminesence assay was observed for methylcobalamin in *Spirulina* sample (Kumudha et al. 2010).

LC-MS and MS/MS of the sample

The LC-MS analysis of the standard and the purified sample was carried out. The MS TIC (total ion chromatogram) observed for Adl-Cbl standard showed the ionization in ESI positive mode. The UV data and the corresponding full scan (MS) data of standard were compared with the purified samples to confirm the presence of Adl-Cbl in the ESI positive mode. Though the mass of Adl-Cbl is 1579.58 m/z, the spectra showed that it is doubly charged and thus mass 790 m/Z was observed instead of 1581 m/Z. (Fig. 2a). The MS/MS of the standard Adl-Cbl showed the doubly charged ion (Fig. 2b). The purified sample of *Dunaliella* also showed the doubly charged ion of Adl-Cbl (Fig. 3a). Since the intensity of the sample was low, MS/MS, selected ion recording (SIR) and multiple reaction monitoring (MRM) were performed for the mass to confirm the presence of Adl-Cbl (Fig. 3b). Adenosylcobalamin is one of the two active forms of vitamin

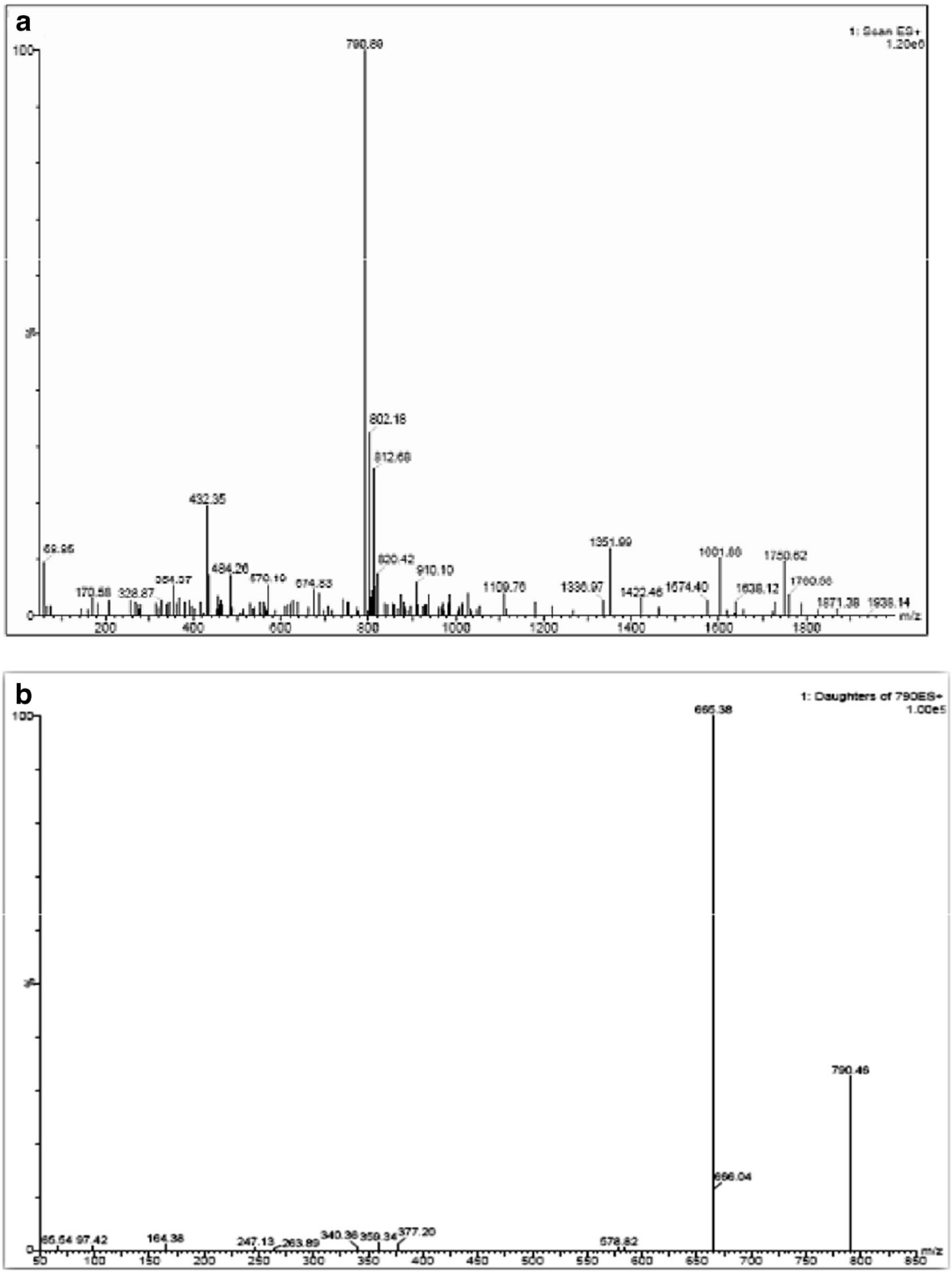

Fig. 2 **a** Mass spectrum of standard adenosylcobalamin. **b** MS/MS of standard adenosylcobalamin

B_{12}. It plays an essential role in the production of blood and maintenance of normal cerebral and nervous functioning in the human body. Also known as cobamamide or dibencozide, adenosylcobalamin occurs naturally in animal derived food types such as fish, meat, eggs and milk.

Not many algae have been studied extensively for the vitamin B_{12} content, its forms and bioavailability. *Porphyra yezoensis*, an ebible puple laver is one among few algae to be characterized and reported to contain five types of biologically active B_{12} compound. *Spirulina*, a cyanobacteria is also

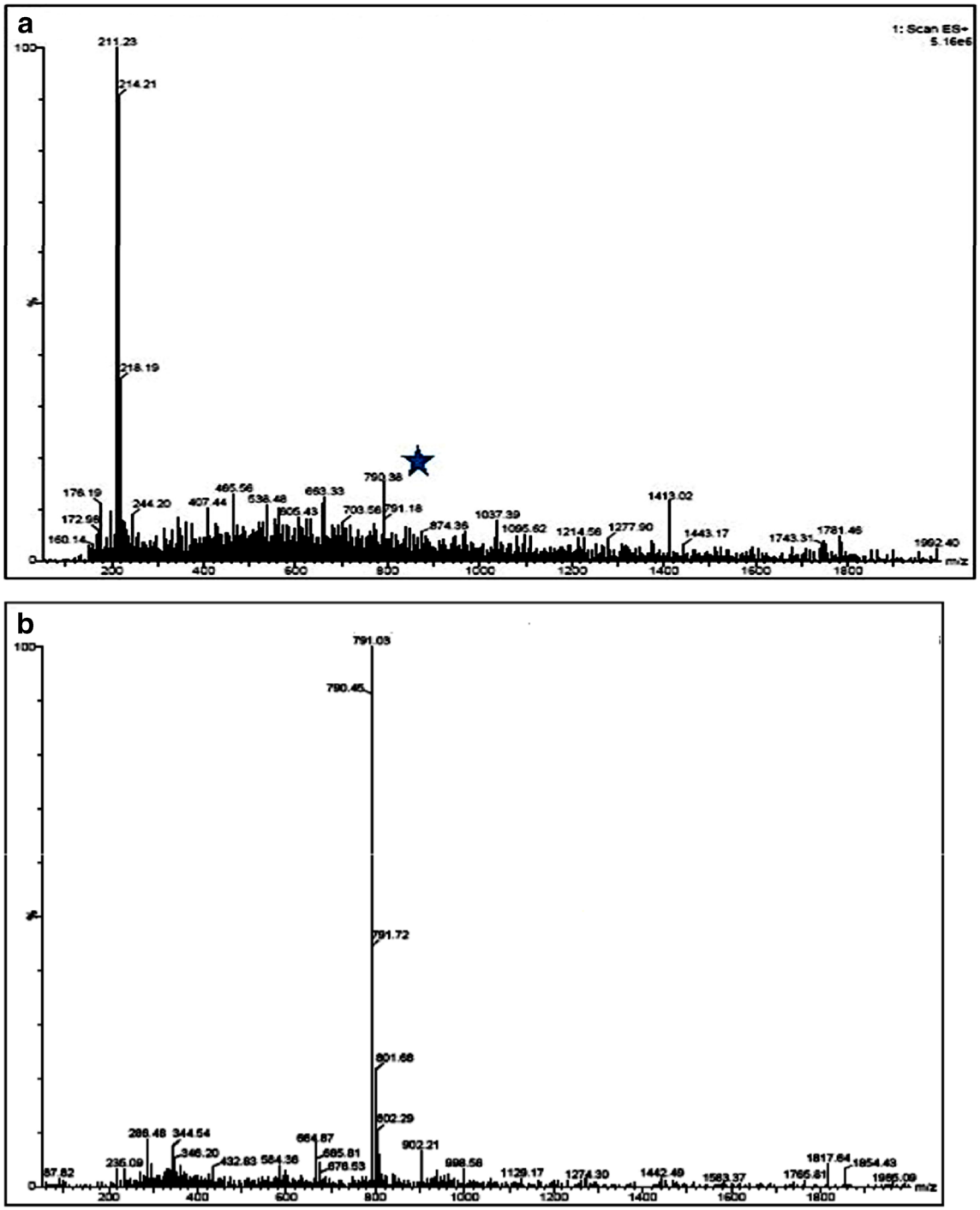

Fig. 3 **a** Mass spectrum of *Dunaliella* extract. **b** MS/MS of *Dunaliella* extract

reported to contain methylcobalamin, a form of vitamin B_{12} (Kumudha et al. 2010). Marine algae are known to be extremely rich in vitamin B_{12} (Croft et al. 2005), but many of them have not been characterized for vitamin B_{12}. In the present study, the marine alga, *Dunaliella* contains a total of 49 ± 2 μg of vitamin B_{12} and 34 ± 4 μg of Adl-cbl per 100 g dry biomass of *Dunaliella*. Adl-cbl being one of the biologically active forms of vitamin B_{12} it is of great relevance to human health.

Conclusion

The recognition of algae as a source of vitamin B_{12} will necessitate a change in our understanding of algal communities and is likely to have profound implication for the exploitation of algae, both as a food source and for biotechnological application. The present findings highlight the presence of adenosylcobalamin in *Dunaliella salina* which was quantified

and characterized using microbiological, chemiluminescence assay along with MS/MS and gold nanoparticle based aptamer studies. Vitamin B_{12} is known to be deficient in the vegetarian diet, and algae could be an alternative source of vitamin B_{12}. The present results suggest that *Dunaliella* can be used as a source of vitamin B_{12} apart from its other nutrients.

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