# MOLECULAR IDENTIFICATION OF MICROALGAE BTM 11 AND ITS LECTIN ISOLATION, CHARACTERIZATION, AND INHIBITION ACTIVITY

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## Abstract

BTM 11 is unknown species of microalgae which has active compounds that can inhibit viruses. Lectin is a carbohydrate-binding protein that is found in microalgae with antiviral and antibacterial activities. The purpose of this study was to perform identification, isolation, characterization, and assay of lectin inhibitory activity of microalgae BTM 11. The result shows that microalgae BTM 11 has homology with *Cyanobacterium* (99%) and *Geitlerinema sp* (98%). Lectin of microalgae BTM 11 has molecular weight of 17 kDa. Lectin protein activity of microalgae BTM 11 was able to inhibit the enzyme activity of RNA helicase hepatitis C by 57.90% and 27.55%. In addition, the protein was able to suppress the activity of *Staphylococcus aureus ATCC 6538*, *E. coli EPEC K.1.1.* and *Salmonella typhii ATCC 25241*. Activitiy of lectin was stable at 30 °C and unaffected by the action of the enzyme. These results indicate that lectin of microalgae BTM 11 could be an alternative to antiviral and antibacterial proteins.

**Keyword**: microalgae BTM 11, lectin, antibacterial, antiviral

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## Introduction

Development of science and knowledge in molecular biology, in particular on the characteristic assessment of genetic materials, has resulted in a tremendous progress for the study of an organism and its use for human welfare. In general, molecular techniques give advantages on identifying an organism by making the asessment more accurate and faster. Studies on the molecular identification of species and phylogeny are inseparable with DNA isolation and gene amplification. The 16S rRNA is a gene of the mitochondrial genome that is oftenly used as a standard in the study of species identification and phylogeny (Cole *et al.* 2013).

Microalgae BTM 11 was isolated from Batam Sea and it has many benefits in its chemical compounds. Regarding the potency of microalgae BTM 11, molecular identification of microalgae BTM 11 is required.

Mustopa *et al.* (2015) stated that the crude extract of microalgae BTM 11 showed a high inhibitory activity against RNAase of hepatitis C with a colorimetric *Adenosine Tri Phosphate* (ATP) testing. In addition, microalgae BTM 11 contains a flavonoid component, that is valuable for hepatitis C virus infection (HCV) treatment. The activity of purified polysaccharide isolated from microalgae BTM 11 showed inhibitory effect against HCV helicase enzyme RNAase for 87.7%, while its crude extract showed inhibitory activity of 64.65%. The declining inhibitory activity observed in the crude extract was related to the precipitated non-polysaccharides compounds including protein.

One of proteins produced by algae is a lectin. This non-immunoglobulin protein specifically binds to carbohydrate molecules (Huskens and Dominique 2012). It is commonly found in various organisms such as sponges, algae, prokaryotic, fish, plants, fungi, cereals, vegetables, and fruits. The activity of lectin is mostly influenced by the pH and temperature. Some lectins produced by algae were observed as low molecular weight protein, have monomeric function, stable to temperature changes and form aggregation. Lectin was reported to have anti-carcinogenic

and antiviral activity (Hori *et al.,* 2009). It directly prevents the fusion of the virus to the cell membrane by binding mannose-rich glycan and part of its glycoprotein (Moura *et al.* 2006).

Currently, the research development on algae-originated lectin is mainly focusing on elucidating its capability as antiviral, antifungal, and antibacterial. Some previous research have been highlighted the benefical potency of this algae for some disease treatment especially those of *Enteromorpha* (Ambrosio *et al.* 2003), *Ulva lactuva* (Wang *et al.* 2004), and *Codium barbatum* (Praseptiangga *et al.* 2012) due to their ability to bind monosaccharide. Huskens and Dominique (2012) described lectin on the algae was able to inhibit HIV replication, and actively attacked Ebola virus and hepatitis C. There is no study on lectin, particularly on its potency against the hepatitis C virus and pathogenic bacteria, had been published previously. Therefore, this study is aimed to identify microalgae BTM 11 species, isolate, characterize and test the inhibitory activity of microalgae BTM 11 protein.

## Materials and Methods

### Materials

The used materials were microalgae BTM 11 isolate and primers 8F (5'-AGA GTTTGA TCA TGGCTC AG-3'; position 8 to 27bp), and 15R (5'-AAGGAG GTG ATC CAA CA-3').

### Methods

**Culture of Microalgae BTM 11 (Mustopa *et al.* 2015)**

Isolate of microalgae BTM 11 was cultured for 7-10 days in the media SWBT (10 L). The culture was processed at room temperature conditions with aeration , then harvested with centrifugation for 10 min at 8500 g. Pellets were taken and dried at 40 ° C for 24-48 h. Dry biomass was stored at -20 °C.

**Molecular Identification of Microalgae BTM 11**

The sample (30 mg) was crushed with liquid nitrogen in a 2 ml tube, 1 ml of buffer Cetylmethyltrimethyl ammonium bromide (CTAB) was added, followed by incubation at 65 °C for 1 h. Samples were added to 1 ml chisam, mixed for 1-5 min until homogeneous, and then centrifuged at 12000 rpm for 15 min. The upper phase was transferred into 1.5 ml tube, sodium acetate (3M) 2/3 and 1/10 volume of isopropanol were added and incubated at -20 °C for 2 h. The tubes were centrifuged at 12.000 rpm for 10 min. The pellet was washed with cold 70% ethanol and centrifuged at 12.000 rpm for 5 min. The pellets were dried overnight and re-suspended in ddH2O and RNAase. The DNA was identified using 16S-rRNA primers. The process was pre-denaturation PCR at 94 °C for 3 min. The PCR condition consisted of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min. The PCR reactions were performed by 30 cycles and final extension at 72 °C for 5 min. PCR results were visualized by electrophoresis using 1% agarose gel. The obtained data were analyzed by bioinformatics covering nucleotide sequence analysis and alignment analysis using some software such MEGA5, ClustalW, Bioedit, and software tools from the NCBI data from Genbank.

**Isolation and Purification of Lectin of Microalgae BTM 11 (Praseptiangga *et al.* 2012 and Sampaio 1998, modified)**

Dried biomass (4 g) was crushed using liquid nitrogen. Sample was stirred at 4 °C with 40 ml of Tris Buffer Saline (TBS). Sonication was operated at an amplitude: 45%, cycle: 0.5 time: 20 sec for three times. Samples were centrifuged at 13.500 g for 30 min, and the supernatant was precipitated using ammonium sulfate at concentration of 75%. The result was centrifuged at 13.500 g for 30 min and eluted using TBS. The pellet was purified by gel filtration chromatography Sephadex G-50 which was with TBS buffer eluent. The extract (2 ml) was added slowly into the column by dropping on the top surface of the gel. Each column fraction was filled by ± 2 ml to the fraction 25.

**Analysis of molecular weight using *Sodium Dodesil Sulfat Poliakrilamide* (SDS PAGE) (Laemmli 1970)**

Polyacrylamide gel was made by mixing separation and stacking buffer, sucrose, polyacrylamide, distilled water, TEMED and ammonium persulfate. Samples were mixed with loading dye and denatured at 95 °C for 10 min. Once electrophoresis device was

prepared, the samples and the molecular weight markers (for comparison) were inserted into the electrophoresis wells. The gel was then stained with brilliant blue commasie.

**Hemagglutination Assay (Praseptiangga 2012)**

Hemagglutination activity assay used 2% human erythrocytes (RBC) that were washed with 0.85% NaCl. Serial dilution of 0.85% NaCl (25µl) on a plate with addition of RBC (25µl) were homogenized gently for 30 min and incubated at room temperature for 1 h. Positive test result was indicated by RBC spreading in the well, while the negative result was observed when RBC was on the one point in the bottom of the well.

**Determination of Protein Content (Pierce Biotechnology 2013)**

Protein content of sample obtained from the highest inhibitory activity, which was measured quantitatively using Bovine Serum Albumin (BSA). Sample and working reaction were incubated at 37 °C for 30 min, and the reaction product was read at a wavelength of 540 nm. Distilled water was used for blank instead of fraction sample

**Protein Characterization to Temperature and Enzyme (Todorov *et al.* 2010)**

Variation on temperature treatments (30, 50, 70, 90°C) were used to determine the optimum temperature of protein. The characterization of the enzyme was incubated for 2 h after the addition of trypsin, proteinase-K, and catalase (concentration added for each enzyme was 1mg / ml). The enzyme activity of the lectin was stopped by heating at 100 °C for 5 min, then tested its inhibitory activity.

**Expression and Purification of RNA Helicase HCV (Utama *et al.* 2000)**

E. coli BL21 (DE3) pLysS (2 ml) that carry the expression vector pET-21b / HCV NS3 helicase was inoculated into 400 ml of liquid LB medium containing 400 ug / ml ampicillin, and then cultured overnight in the shaking incubator (150 rpm) at 37 °C. When it reached OD600 ± 0.3, isopropyl β-D-thiogalaktopiranosidase (IPTG) of 0.3 M was added. The culture was incubated to obtain OD600 at ± 1. The culture was centrifuged at 4 °C with a speed of 4000 rpm for 10 min. Pellets were stored at -20 °C, before it was resuspended using freeze and thaw method (which is repeated 3 times) in 20 ml of buffer B (10 mM Tris-HCl pH 8.5; 100 mM NaCl, 0.25% Tween 20), then sonicated (amplitude 40; 0.5 cycles; 3x15 second time; a time interval of 1 minute). The cell suspension was centrifuged at 10.000 rpm at 4 °C for 20 min to obtain a supernatant. RNA helicase enzyme in the supernatant was purified using affinity chromatography method (resin) for 3 h at 4 °C, then centrifuged at 3500 rpm for 7 min. Resin pellet was washed 2 times using buffer B. The resin was eluted to release the enzyme with 150 mL of elution buffer solution (400 mM imidazole in buffer B), using the rotator 4 °C for overnight. Samples were centrifuged at 4 °C at 3.000 rpm for 1 min. The supernatant was the RNA enzyme.

**Activity of Colorimetric ATPase RNA Helicase (Utama *et al.* 2000)**

Inhibiton of RNA helicase with colorimetric ATPase assay. Extract of sample (5µL) was added to reaction mixture (50µL) containing 10mM MOPS buffer (pH 6.5), 2mM ATP, 1mM MgCl2, appropriate RNA helicase, and incubated at room temperature for 40min. The reaction was stopped with dye solution 100 µL (water : 0.081% malachite green : 5.7% ammonium molybdate in 6N HCl : 2.3% polyvinyl alcohol = 2 : 2 : 1 : 1). After the addition of 25µL of 30% sodium citrate, the inhibition activity was measured at OD620/405.

**Antibacterial Properties of Lectin (Lay 1994)**

*Staphylococcus aureus* ATCC 6538, *E. coli EPEC* K.1.1 and *Salmonella typhii* ATCC 25 241 were cultured in agar medium at 37 °C and 150 rpm for overnight. The bacteria were diluted in 0.85% NaCl added to the agar plate. The protein sample was dripped onto the wells of 50 ml (± 6 mm diameter), incubated at 37 °C for ± 24 h. Diameter of inhibition zone was observed.

## Results

**Cultivation of Microalgae BTM 11**

Microalgae BTM 11 was cultured on modified medium that contain mineral such as $NaNO_3$, $Na_2HPO_4$, $KH_2PO_4$, ferric amonium citrate, $Na_2EDTA$, citrite acid, $CaCl_2$,$2H_2O$, and

sterilized sea water. The pH of culture condition was maintained at 7 to 8 by aeration treatment, keeping $CO_2$ flow to exchange with environment properly. Cultivation was done on 10 L of cultured algae producing dried mass approximately as much as 7,24 gram. The algae is ready to be harvested when it produced very dense pigment at day of 10 to 14 (Mustopa *et al*., 2015).

**Identification of Microalgae BTM 11**

DNA Isolation of microalgae BTM was conducted to prepare DNA template for PCR 16S-rRNA gene amplification. Amplification of DNA fragment of the 16S-rRNA gene was carried out by annealing at optimum temperature, 45 °C for 3 min. The PCR product with size of 1500 bp (Figure 1) was purified and sequenced to obtain its genetics information. Then, the sequence of the 16S-rRNA gene microalgae BTM 11 was uploaded on BlastN (BasicLocal Alignment Search Tool- nucleotide) at the NCBI web site (National Center for Biotechnology Information) to ascertain the PCR amplified product was correct and identify the species. Microalgae BTM 11 exhibited homology with Cyanobacterium (99%) and *Geitlerinema sp* (98%) as shown in Table 1. Furthermore, phylogeny tree result (Figure 2) confirms that microalgae BTM 11 is forming a distict clade with *Cyanobacterium* KR998335.1 and *Geitlerinema sp* FJ042947.1 with 100% homology.

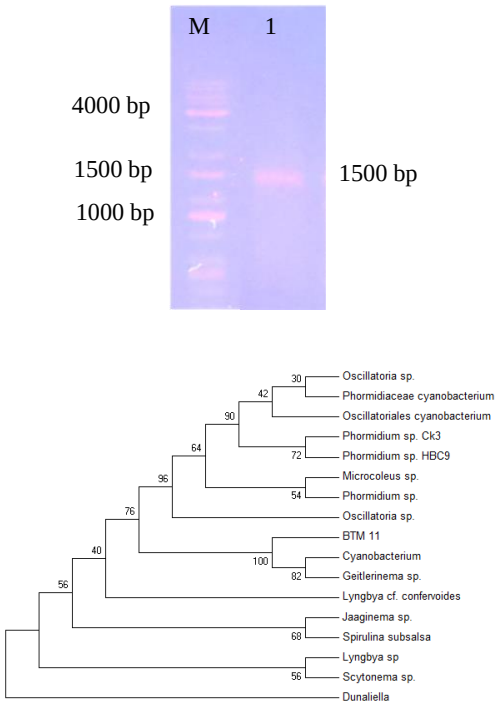


Figure 1. Amplification of 16S BTM 11 (above) and phylogenetic tree (below)

Table 1. Species identification

| Results | Homology | Access code |
|---|---|---|
| Geitlerinema sp. | 98% | FJ042947.1 |
| Cyanobacterium | 99% | KR998335.1 |

| | (b) | (c) | (d) | (e) | (f) | (g) | (h) | (i) | (j) | (k) | (l) | (m) | (n) | (o) | (p) | (q) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cyanobacterium (a) | | | | | | | | | | | | | | | | |
| Geitlerinema sp (b) | 0.000 | | | | | | | | | | | | | | | |
| Jaaginema sp (c) | 0.065 | 0.065 | | | | | | | | | | | | | | |
| Lyngbya cf (d) | 0.062 | 0.062 | 0.082 | | | | | | | | | | | | | |
| Lyngbya sp. (e) | 0.066 | 0.066 | 0.072 | 0.065 | | | | | | | | | | | | |
| Microcoleus sp. (f) | 0.040 | 0.040 | 0.069 | 0.060 | 0.077 | | | | | | | | | | | |
| Oscillatoria sp.(g) | 0.041 | 0.041 | 0.067 | 0.061 | 0.078 | 0.005 | | | | | | | | | | |
| Oscillatoria_sp.(h) | 0.040 | 0.040 | 0.069 | 0.057 | 0.077 | 0.005 | 0.006 | | | | | | | | | |
| Oscillatoriales (i) | 0.041 | 0.041 | 0.067 | 0.061 | 0.078 | 0.005 | 0.000 | 0.006 | | | | | | | | |
| Phormidiaceae (j) | 0.041 | 0.041 | 0.067 | 0.061 | 0.078 | 0.005 | 0.000 | 0.006 | 0.000 | | | | | | | |
| Phormidium sp.(k) | 0.043 | 0.043 | 0.069 | 0.063 | 0.080 | 0.006 | 0.001 | 0.008 | 0.001 | 0.001 | | | | | | |
| Phormidium sp. (l) | 0.044 | 0.044 | 0.071 | 0.064 | 0.082 | 0.004 | 0.006 | 0.009 | 0.006 | 0.006 | 0.008 | | | | | |
| Phormidium sp. (m) | 0.043 | 0.043 | 0.069 | 0.063 | 0.080 | 0.006 | 0.001 | 0.008 | 0.001 | 0.001 | 0.000 | 0.008 | | | | |
| BTM 11(n) | 0.006 | 0.006 | 0.072 | 0.066 | 0.074 | 0.046 | 0.047 | 0.044 | 0.047 | 0.047 | 0.048 | 0.050 | 0.048 | | | |
| Dunaliella (o) | 5.564 | 5.564 | 5.724 | 5.399 | 6.050 | 5.316 | 5.346 | 5.310 | 5.346 | 5.346 | 5.343 | 5.310 | 5.343 | 5.561 | | |
| Scytonema sp. (p) | 0.127 | 0.127 | 0.125 | 0.113 | 0.100 | 0.113 | 0.119 | 0.116 | 0.119 | 0.119 | 0.121 | 0.111 | 0.121 | 0.131 | 5.974 | |
| Spirulina (q) | 0.089 | 0.089 | 0.080 | 0.094 | 0.109 | 0.097 | 0.098 | 0.100 | 0.098 | 0.098 | 0.100 | 0.102 | 0.100 | 0.095 | 5.599 | 0.147 |

Figure 2 Genetic matrix of amplification 16S BTM 11

**Isolation, Purification, Protein Characterization of Protein Microalgae BTM 11**

Ammonium sulfate with 75% saturation was used for protein precipitation. In this study, precipitation of lectin from the supernatant of microalgae microalgae BTM 11 is based on the solubility. The resulting pellet was crude protein of lectin that was eluted with TBS buffer. Color of crude protein extract is solid green. The pellet obtained from precipitation of microalgae BTM 11 was then purified by gel filtration chromatography using sephadex G-50 with eluen TBS bufer with 10mM EDTA. Figure 3 indicates that fraction 6 and 7 were the highest and the most observable bands. The gel filtration was done in 25 fractions with a volume of 2 ml in each fraction.

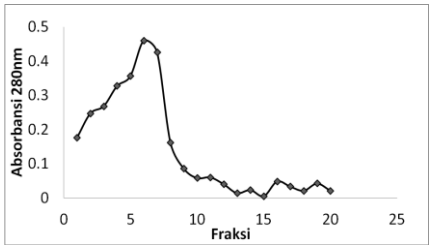


Figure 3. Sephadex gel purification of G-50 protein microalgae BTM 11

Table 2. Total concentration of protein

| Purification steps | Vol (mL) | Protein (mg/mL) | Total of Protein (mg) |
|---|---|---|---|
| Metabolite extraction | 30 | 9,6 | 288.0 |
| Crude Extract | 7 | 7.51 | 52.57 |
| Purification | 0,3 | 3.04 | 22.8 |

Table 2 describes the total concentration of protein in the crude extract of 52.57 mg, whereas the purified fractions contained 22.8 mg. The results show that concentrating protein tends to lower the protein content.

Molecular weight of protein was analyzed using polyacrylamide gel electrophoresis in SDS-PAGE. Figure 4 shows a thick band of lectin protein (17 kDa). Impure part appeared on the gel was detected in lane 3, indicating a non target protein. Elution buffer 0,05 M Tris HCl pH 8, 0,15 M NaCl, and 0,01 M EDTA was not perfectly purified.

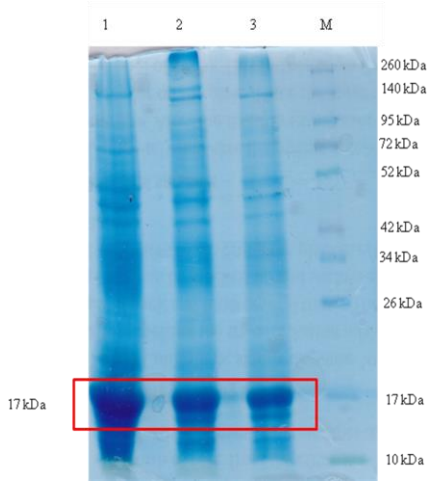


Figure 4. SDS-Page Microalgae Protein BTM 11 (1: metabolite, 2:precipitated protein 3: gel filtration purification)

**Haemagglutination Assay**

Agglutinin activity is minimal number of the samples that can lead to blood agglutination, and observed macroscopically. Haemagglutination assay result is presented in Table 3. Lectin of microalgae BTM 11 had highest hemagglutination activity on O blood type with a value 64 titer test (without dilution). Sampel (2x and 3x dilution) affected haemagglutination activity. Total concentration of the lectin affected haemagglutination activity.

Table 3. Titer of Haemagglutination assay

| Sample | Haemagglutination Activity | Note |
|---|---|---|
| Crude extract | 64 | + |
| Crude extract (2x dilution) | 16 | + |
| Crude extract (3x dilution) | 8 | + |

**Activity of Calorimetric ATPase RNA Helicase**

The result show that sample (40x dilution) was able to suppress activity of Hepatitis C RNA helicase by 57.90%, while the other (80x dilution) was 27.55%. Lectin of microalgae BTM 11 was able to act as an antivirus (Figure 5).

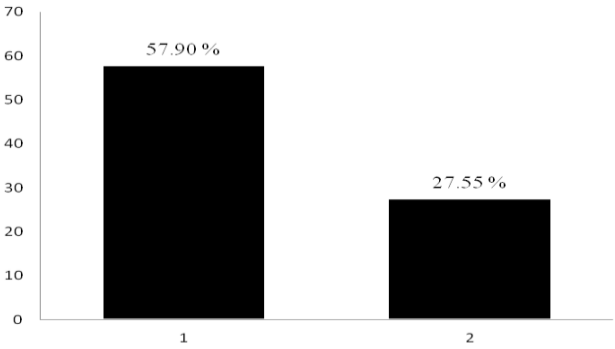


Figure 5 Inhibition of RNA Helicase (1: protein dilution 40x; 2: protein dilution 80x)

**Antibacterial Activity of Microalgae Protein**

Pathogenic bacteria such as *Staphylococcus aureus* ATCC 6538, *E. coli* EPEC K.1.1. and *Salmonella typhii* ATCC 25241 were used to test antibacterial effect of protein from crude extract. Inhibition zone was formed due to the addition of microalgae protein of microalgae BTM 11. Lectin protein showed inhibitory activity on all three bacteria (Table 4 and 5). The lectin is capable to inhibit the action of bacteria by damaging the cell membrane.

Tabel 4. Microalgae protein BTM 11 antibacteria activity

| Bacteria | Inhibition zone (mm) | | |
|---|---|---|---|
| | K+ (CMP) | K- (TBS) | Protein |
| *S.typhii* | 23 | - | 7,5 |
| *S.aureus* | 20,5 | - | 7.5 |
| *EPEC K.11* | 22,5 | - | 6,75 |

Table 5. Inhibition zone of bacteria

| **Control -** | **Control +** | ***S. typhii*** | ***Staphilococcus .aureus*** | ***EPEC K.11*** |
|---|---|---|---|---|
|  |  |  |  |  |

**Characterization of Lectin**

Lectin which was treated at 30 °C showed inhibition zone of 7.5 mm (Table 6), which indicates resilience of lectin activity. Degradation of protein binding ability of lectin occurred at 60 °C. Table 7 shows that the activity of lectin due to the addition of trypsin, proteinase-K, and catalase was smaller compared with the positive control. This evidence suggests that the activity of the lectin is unaffected by enzyme.

Table 6. Characterization of Microalgae Protein Temperature

| Bacteria | Control (+) | Control (-) | Inhibition Zone (mm)±SD | | | |
|---|---|---|---|---|---|---|
| | | | $30^0$C | $50^0$C | $70^0$C | $90^0$C |
| *S. typhii* | 7.0±0.0 | - | 7.50±0.0 | | - | - |

Table 7. Characterization of Microalgae Protein Enzyme

| Bacteria | Contr. (+) | Contr. (-) | Inhibition Zone (mm)±SD | | |
|---|---|---|---|---|---|
| | | | Proteinase-K | Trypsin | Katalase |
| *S. typhii* | 7,50± 0.0 | | 7,00±0.0 | 7,50±0. 58 | 7.45±0.58 |

**Discussion**

In the current study, identification and analysis of microalgae BTM 11 with 16S-rRNA gene were successfully conducted. Recently, Mustopa *et.al* (2015) had observed that the microalgae BTM 11 filaments were characterized as green colour. Microalgae BTM 11 was harvested at lag phase of growth. Microalgae BTM 11 lives in extreme environment condition, such as immoderate temperature, light, and salinity. Furthermore, because of their similarity with terrestial plants, microalgae can be easily cultured in the laboratory. Cultivation of microalgae BTM 11 can provide a consistent source of bioactive compound in appropiate culture conditions.

Microalgae BTM 11 was identified as a member of genus *Geitlerinema* sp. Hameed (2008) collected *Geitlerinema* sp. The eutrophic conditions may encourage algal blooms of *Geitlerinema* sp. UK-G-106 near Astola Island, Pasni, Balochistan, Pakistan. It was some 40 km away from East-south-East of the fishing port of Pasni. These waters were rich in variety of corals. Their study was the first kind of its nature from marine waters, which indicated that Pakistani *cyanobacterium Geitlerinema* sp. UK-G-106 contains a new class of potent bioactive oligopeptides. *Cyanobacterium Geitlerinema* sp is a promising source of new bioactive natural compounds that should be tested for further

investigations, whether it can be used as a therapeutic drug in cancer treatment. Then variation *Geitlerinema* within the 16S rDNA gene caused a distinct geographic grouping of isolates. Published report states that *Geitlerinema* sp. of Pakistan was found similar to *Geitlerinema* sp of California and coast Mexico.

The lectin protein from microalgae BTM 11 purified by filtration gel sephadex G-50 requires optimized purification to obtain one clear protein band.

During Hemaglutination assay, a number of sample shows a high specifity with erythrocytes of blood type O and it was observed macroscopically. Protein of microalgae BTM 11 positively considered as lectin. During the hemagglutination assay, lectin will agglutinate erythrocytes. Erythrocyte blood type O contains polysaccharide compounds. Each blood has a specific arrangement. Fucose is attributed to the blood type O (D'adamo 2006).

The bioactivity of lectin as antiviral in calorimetric ATPase assay shows inhibition to RNA helicase enzyme. The envelope proteins of Hepatitis C (HCV), E1, E2, are critical for viral infectivity. Both E1 and E2 are heavily glicosylated with conserved N-linked glicosylation sites. The proper glycosylation of this sites is important both for protein folding and for viral interactions with host receptors. Lectin targeting the glycan on HCV envelope E1 and E2. Lectin have potent anti HCV activity (Takebe *et al.* 2013). The lectin was able to inhibit the action of bacteria by damaging the cell membrane. According to Singh *et al.* (2012), all organisms have a polysaccharide component with a covalent bond. Therefore, lectin possibly tends to bind to polysaccharides of cell surface and damage the cell membrane.

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