

SPECIES DETERMINATION OF GREEN ALGAE ISOLATED FROM JEPARA COASTAL REGION BASED ON MICROBIOLOGICAL, ECOPHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION FOR IMPROVEMENT OF CAROTENOID PRODUCTION

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ABSTRACT

A local isolate of green algae called C1 from Jepara waters is usually used as a source for carotenoid supplement for animal fisheries in the local area. This indigenous algae has been successfully purified. Although the local isolate was known as eucaryotic green algae *Dunaliella*, our previous molecular study by 18S rDNA analysis to determine the species of this organisms showed negative result. In order to improve carotenoid production especially detection of biosynthetic pathway from the organisms investigated in this study, the main purpose of this study was species determination of local isolate of green algae based on microbiological, ecophysiological and molecular characterization.

The results of this research indicated that local isolate of green algae possesses Cyanobacteria characteristic, especially *Synechocystis*. Analyses with 16S rDNA sequence from genome of green algae isolates were also in accordance with these results, showing close similarities with *Synechocystis* 16S rDNA sequence. However, it should be noted that, instead of having *Synechocystis* dominant feature, it was also found that local isolate of green algae exhibit different characteristic in having chlorophyll a, chlorophyll b and lack of phycobillins. This character was typical for aberrant Cyanophyta, *Prochlorophyta*.

Key words : a C1- isolate, *Dunaliella*, Cyanobacteria, 16S rDNA, *Prochlorophyta*

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INTRODUCTION

The recent discoveries of health related beneficial properties attributed to carotenoids have spurred great interest in their production. Carotenoids, some of which are provitamin A, have range of

diverse biological function and actions, such as species specific coloration, photo protection, and light harvesting, and they serve as precursors of many hormones (Vershinin, 1999 in Lee and Schmidt-Dannert, 2002). Carotenoids are used

commercially as food colorants, animal feed supplements and, more recently, as nutraceuticals for cosmetic and pharmaceutical purposes. The demand and market for carotenoids are anticipated to change drastically with the discovery that carotenoids exhibit significant anti-carcinogenic activity and play an important role in the prevention of chronic diseases (Lee and Schmidt-Dannert, 2002).

For many years, it was accepted that carotenoid was synthesized through the well known acetate/mevalonate pathway. However, recent studies have demonstrated photosynthetic organisms such as green algae, *Scenedesmus obliquus*, *Chlorella fusca*, *Chlamydomonas reinhardtii* use a new non-mevalonate pathway known as deoxyxylulose 5-phosphate (DXP) pathway for their carotenoid biosynthesis. The exclusive occurrence of the non-MVA pathway for the biosynthesis of plastidic isoprenoids and of sterols might represent a general feature of many green algae (Lois *et al.*, 1998; Lichtenthaler, 1999). A local isolate of an algal species from BBAP Jepara called C-1 isolate, was found potentially useful as source of carotenoids in food additives or as food supplement in fish farming. It was suspected as representing a strain of *Dunaliella*, based only on morphological identification (Sutanto-pers.comm; Kusumaningrum *et al.*, 2004; Kusumaningrum *et al.*, 2006). Thus, it was of great interest to know if this local isolate of algae would also follow the non-MVA pathway for carotenoid biosynthesis. Further research in detecting biosynthetic pathway from this "*Dunaliella*" by genetic engineering techniques, has faced several problems that might be caused by misnamed of the species. It is apparent that microbial identifications based only on morphological characterization have, until recently, failed to achieve the necessary requirements of prediction, stability and objectivity (Priest and Austin, 1993). Therefore, it is important to examine identification of species based on molecular technique using ribosomal DNA

sequence, for supporting microbiological and ecophysiological characterization.

The genes for ribosomal rRNAs are particularly suitable as targets for identifying most organisms in delivering objective result. Molecules of RNA are valuable as indicators for identifications of species because the rRNA are essential elements in protein synthesis. Therefore, the rRNA present in all living organisms. The rRNA genes contain both highly conserved sequences and variable regions. The conserved functions of these molecules have changed very little during evolution. Thus, rRNAs from even the most taxonomically distant organisms, that share virtually no DNA sequence homology, will have rRNA sequences in common, and, therefore, relatedness can be assessed (Logan, 1994). Ribosomal RNA is probably unique amongst macromolecules in this respect. Some segments of rRNA evolve more rapidly than others and sequence variation occurs between closely related organisms allowing comparisons to be made at the species level. Phylogenetic lines of descent may be inferred from rRNA sequences. The 16S rRNA has been used extensively for comparative sequencing studies in procaryotic organisms and 18S rDNA for eucaryotic organisms (Priest and Austin, 1993).

Molecular approach provide complementary data for microbial identification. Therefore, the aims of this research is to identify of species based on microbiological and ecophysiological characterization supported by molecular techniques using 16S rDNA approach.

MATERIALS AND METHODS

Culture of algae

Laboratory cultures were grown in 100 ml flasks. The microcosms consisted of 100 mL cultures in 4 erlenmeyer. All cultures were grown in a temperature of 30°C. For induction of β -carotene synthesis, cells were grown in a sulfate-depleted media (MgCl₂

instead of $MgSO_4$), under intense illumination conditions 600 lux and with 2 – 4 ppm O_2 passing to the liquid (Rabbani *et al.*, 1998)

Preparation of Media

The medium Bold Basal Medium (BBM) used and modified from Stein (1998) in Bidwell, J.P. and Spotte S., 1983) consisted of KH_2PO_4 17,5 g/l; $CaCl_2 \cdot 2H_2O$ 2,5 g/l; $MgSO_4 \cdot 7H_2O$ 7,5 g/l; $NaNO_3$ 25 g/l; K_2HPO_4 7,5 g/l; $NaCl$ 2,5 g/l; Na_2EDTA 10 g/l; KOH 6,2 g/l; $FeSO_4 \cdot 7H_2O$ 4,98 g/l; H_2SO_4 1 ml/l; “Trace Metal” solution 1 ml/l (H_3BO_3 2,86 g/l; $MnCl_2 \cdot 4H_2O$ 1,81 g/l; $ZnSO_4 \cdot 7H_2O$ 0,222 g/l; $NaMoO_4 \cdot 5H_2O$ 0,39 g/l; $CuSO_4 \cdot 5H_2O$ 0,079 g/l; $Co(NO_3)_2 \cdot 6H_2O$ 0,0494 g/l); H_3BO_3 11,5 g/l. The ingredients were dissolved in 1 L of distilled water. The pH was adjusted to 6.8 with HCl or NaOH. Sterilization was done by autoclaving at 15 lb/in² (103 kPa and 120°C). The medium was using by adding 0.1 ml solution to each 10 mL of seawater.

Microbiological and Ecophysiological Characterization

Morphological and microbiological characterization was done according to Holt *et al.*, (1994) and Logan (1994). Cultural characterization consist of colony shape, margin, elevation, surface appearance, opacity, texture, pigmentation and appearance of growth in broth. Morphological characters include cell shape, curvature, size and arrangements. Pleomorphisms, formation of daughter cell, cell division and reproduction, presence and arrangement of flagella, gliding motility, presence or lack of cell walls, presence or lack of nucleus walls, presence or lack of cell sheath and staining reactions such as Gram.

Ecophysiological characterization was conducted according to Borowitzka dan Borowitzka (1988) and Ben-Amotz (1993)

including the maximum and minimum temperatures permitting sustained growth, reproducibility, temperature tolerance, atmospheric requirements such as aeration and illumination, also growth at different NaCl concentrations (0 – 30 %). Ecophysiological experiment was measured by cell count and cell density absorbancies at $OD_{600\text{ nm}}$. Illumination was observed at 660 \square Einstein. $m^{-2} \cdot sec^{-1}$ or 600 lux (Rabbani *et al.*, 1998). Measurement of pigments concentration was done by extracting the specimen with methanol or acetone to check if residual color (blue to red) caused by the non-organic soluble phycobillins remains in the cell (Goodwin and Britton, 1988; Holt *et al.*, 1994). Chlorophyll concentration were analyzed by extracting cell pellet with methanol until the pellet color is dissappeared. Concentration of chlorophyll was measured by $OD_{663\text{ nm}}$ and $OD_{645\text{ nm}}$, then calculated with formulas (Harborne, 1984; Goodwin and Britton, 1988) :

$$\begin{aligned} \text{Total chlorophyll} &= 17.3 A_{645} + 7.18 A_{663} \text{ mg/ml} \\ \text{chlorophyll a} &= 12.21 A_{663} - 2.81 A_{645} \text{ mg/ml} \\ \text{chlorophyll b} &= 20.13 A_{645} - 5.03 A_{663} \text{ mg/ml} \end{aligned}$$

DNA Extraction

Preparation of a green algae DNA isolate was carried out by modification of CTAB methods (Ausubel *et al.*, 1995). Culture of algae about 15 ml were centrifugated 13,000 rpm for 3 minutes. Pellet was pulverized on cold mortar and pestled to a fine powder. One ml of warm CTAB extraction buffer [(2% (w/v) CTAB, 100 mM Tris-HCl pH 8; 20 mM EDTA pH 8; and 1.4 M NaCl, 1% (w/v) pre warmed on 65°C] was added to the pulverized algae and mixed to wet thoroughly. 25 μ l Lisozyme enzyme with concentration of 25 mg/ml was added to suspension, homogenized and incubated in waterbath on 37°C for 1 hour with occasional mixing. 750 μ l of SDS 10% was added,

incubated again in waterbath on 37°C for 1 hour. The extraction was incubated in waterbath on 65°C for 1 hour with occasional mixing. The homogenate was extracted, with an equal volume of chloroform, and mixed well by inversion. The homogenate was centrifugated 5 min at 13.000 rpm in microcentrifuge. The top (aqueous) phase was recovered and the supernatant was removed. The nucleic acid was precipitated by adding 0.6 vol isopropanol and 1/10 vol Sodium asetat 3 M. The suspension was incubated over night on -20°C. The suspension was centrifugated 5 min at 13.000 rpm. The pellet was washed with 100 μ l etanol 70 %, air dried and resuspended in 50 μ l TE buffer (10 mM Tris pH 8; 1 mM EDTA pH 8). DNA was purified with RNaseA and incubated in waterbath on 37°C for one hour. DNA was kept on -20°C or used directly for PCR.

Amplification of 16S rDNA

The 16S rDNA fragment was amplified by PCR using spesific primers in 16S-23S ribosomal genes spacer region. Sequence of forward primer was 5'-AGAGTTTGATCMTGGCTCAG-3', reverse primer was 5'-TACGGYTACCTTGTTACGACTT-3' corresponding to base pairs 1541 respectively (Garcia-Martinez *et al.*,1999). PCR was carried out in mixture containing 50 ng of genomic DNA, 2,0 mM of MgCl₂, a 0,2 mM concentration of each deoxynucleoside triphosphate, 2,5 pmol of each primer, and 1,8 U of Taq Polymerase and ddH₂O until volume 25 μ l. PCR conditions were performed with *hot start* for 2 minutes at 94°C, denaturation for 1 minute at 94°C, *annealing* for 1 minute at 50°C, polimerization for 1 minute at 72°C, extra extention at 72°C for 2 minutes, with 30 cycles of PCR reactions. In this PCR, a single DNA fragmen of 1,5 kb was amplified.

Sequencing and Phylogenetic Analysis

The 16S rRNA fragment was sequenced on BPPT Jakarta. Sequencing process involves several steps. First step was purification of amplification product of PCR by Qiagen Purification Kit. The next step was *cycle seq* process. The reaction composition consists of DNA template, primers, buffer, ddH₂O and *big dye* (DNA polymerase enzyme, ddNTP, and dNTP). The last step was sequencing using ABI Prism 310 *sequencer*.

Sequence of 16S rDNA of a green algae isolate was used to search its homology, process of comparing a new sequence with all other known sequences in the database. Then attempting to infer the function of the new sequence by assessing the matches and their biological annotations as described in the database. Sequence analysis was analyzing by similarity (homology). Sequence data was submitted to GenBank website at www.ncbi.nlm.nih.gov and European Bioinformatics Services website at www.ebi.ac.uk. Setting up database search was using BLASTN Program.

Database searches and phylogenetic analyses also performed for the *DXS* gene of several species. Homologous protein sequences were retrieved from public and proprietary genomic sequence databases. Preliminary sequence data were also obtained from *GenBank*. The nucleotides were aligned using the program of CLUSTALW version 1.7 with the BLOSUM62 similarity matrix and gap opening and extension penalties of 10.0 and 0.05, respectively. Phylogenetic trees were constructed by maximum-parsimony (MP) and neighbor-joining (NJ) methods for each set of alignments.

RESULTS AND DISCUSSION

Microbiological and Morphological characterization

According to microscopic view as illustrated in **Fig 1.** and **Fig 2,** morphological

characteristics of C1-isolate of green algae are unicellular, solitary, spherical or elongate in shape, widely oval before division and after division hemispherical; the cell length was 5 - 6.5 μm . Cells of C1-isolate of green algae are non motile cells and do not have flagella. They have two cell wall, the color of the cell is bright green and turn to greenish yellow on the sixth day of growth. Forming green colonies on solid media look form aggregates of the cell. Cells are always surrounded by narrow, fine, pinkish colour envelopes. Cellular reproduction is by division into two

morphologically equal, hemispherical daughter cells (binary fission), which reach the original globular shape before next division, cells divide in one planes in successive generations in broth media, the envelopes around cells will split together with dividing cells. Daughter cells separate after division and grow into the original size and shape before next binary fission. Daughter cells held together by mucilaginous sheath. Cell of C1-isolate of green algae was stain gram negative, this characteristic is closely related to a typical feature of Cyanobacteria.

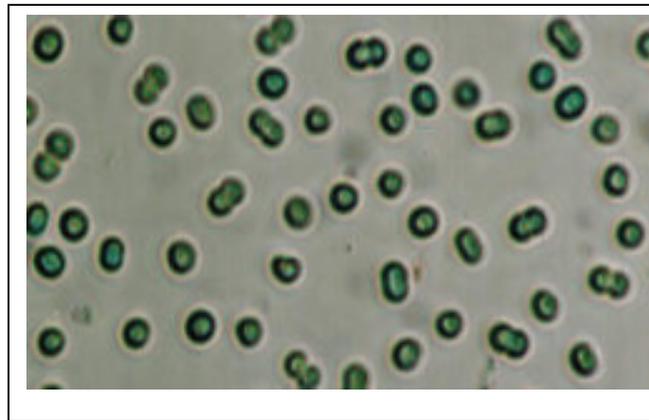


Fig1. Microscopic View of a C1-isolate of Green Algae 48 hours

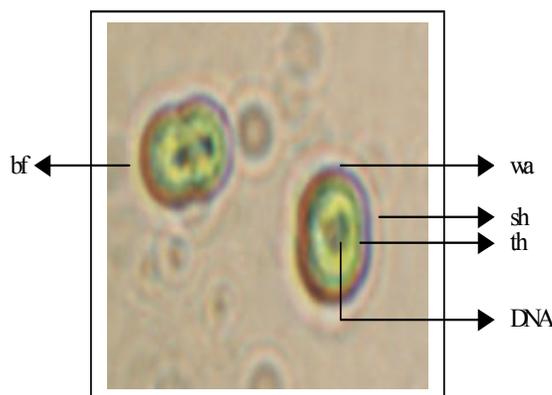


Fig 2. Microscopic View of a C1-isolate of Green Algae 120 hours

Table 1. Characteristic of a C1-Green Algae Isolate

Characteristic	C1-Isolate	Cyanobacteria (<i>Synechocystis</i>)
1. Cellular organization	procaryotic	procaryotic
2. Growth temperature	-20°C – 30 °C	26 – 39 °C
3. salinity	0 – 30%	-
4. source of energy and carbon	photoheterotroph	Photoheterotroph, photoautotroph
5. habitat	Sea Waters	Tropical, fresh water
6. unicellular	+	+
7. coccoid or spherical	+	+
8. binary fission in 2 successive planes	+	+
9. Extracellular sheath	+	+
10. Chlorophyll a	+	+
11. Chlorophyll b	+	-
12. Phycobillin	-	+
13. filament	-	-
14. thylakoid	+	+
15. cell diameter	2 – 7 µm	5 – 6 µm
16. motility/movement	slow gliding	slow gliding
17. Cell	solitary	solitary
18. Colonies	Forming colonies	Never forming colonies
19. Cell color	Bright green	pale blue-green, olive- green, bright- green or pinkish chromatoplasma
20. Color of sheath	pink	Yellow pigment or red- blue pigment
21. Gram stain	Gram negative	Gram negative
22. Cell division	Binary fission	Binary fission
23. Reproduction	solitary cells	solitary cells

Reproduction by solitary cells. Comparison of cell morphology between the C1-isolate of green algae and literature from Cyano-database on **Fig. 2.** showed that their cells were resembles to member of Cyanobacteria, *Synechocystis*, (confirmed also by 16S rDNA alignment). The comparison characteristic between these two organisms are presented in **Table 1.**

Ecophysiological characterization of C1-isolate of green algae

Ecophysiological characterization of the C1-isolate of green algae was carried out by

growth and factor influencing growth including temperature, salinity and light. The C1-isolate exhibit ranges of tolerance for ecophysiological factors that determine their limits of growth. When the culture was tested in survival on the extreme temperature minus 20°C and without light, it was still able to live. Although low temperatures restrict the rates of growth and enzymatic activities of organisms, they did not kill these organisms. Therefore, when the culture is thawed, they can grow. Supporting the previous results, it can be explained that prokaryotic organization of green algae isolates cells similar to cyanobacteria which allows them to grow under extreme conditions, where they

may thrive in the absence of competition from other algae.

Cyanobacteria could grow over a wide range of temperatures. If the C1-isolate was a member of cyanobacteria, it is possible that it might follow an alternative system of cyanobacteria photosynthesis in keeping its growth under anaerobic conditions (anoxygenic photosynthesis). In the absent of oxygen, some non-heterocystous cyanobacteria show nitrogenase activity in ordinary vegetative cells. They may fix nitrogen at night or in oxygen-depleted region. (Cohen *et al.*, 1975 in Sze, 1993).

The growth of the C1-isolate as illustrated in **Fig. 3**. was seven days under illumination 600 – 1000 lux on the room temperature. High light intensity is generally detrimental to many algae. At a very intense illumination, the algal cells generally bleach. According to Orset and Young (2000), illumination will affect growth and had a marked effect both on growth of alga (that will be suppressed by low and high

illumination) and on the accumulation of β -carotene. Research on *D. bardawil* showed that under stress condition such as high light intensity, the cells turn orange due to massive β -carotene formation under high light intensities (Rabbani *et al.*, 1998). The β -carotene globules in *Dunaliella* will protect the cell against the high intensity irradiation to which it is exposed in the natural habit by absorbing energy in the blue region of the spectrum. Strains unable to accumulate β -carotene die when exposed to high irradiation, while the β -carotene-rich *Dunaliella* strains flourish (Shaish *et al.*, 1991 in Ben-Amotz, 1993).

As shown in **Fig 2**. the cell of C1-isolate also turns their colour into yellow-brown starting on day fourth under the treatment of high light intensities when the growth decreased gradually. Analysis of total pigment production on C1-isolate also exhibit an increase pigment production as illustrated in **Fig. 4**.

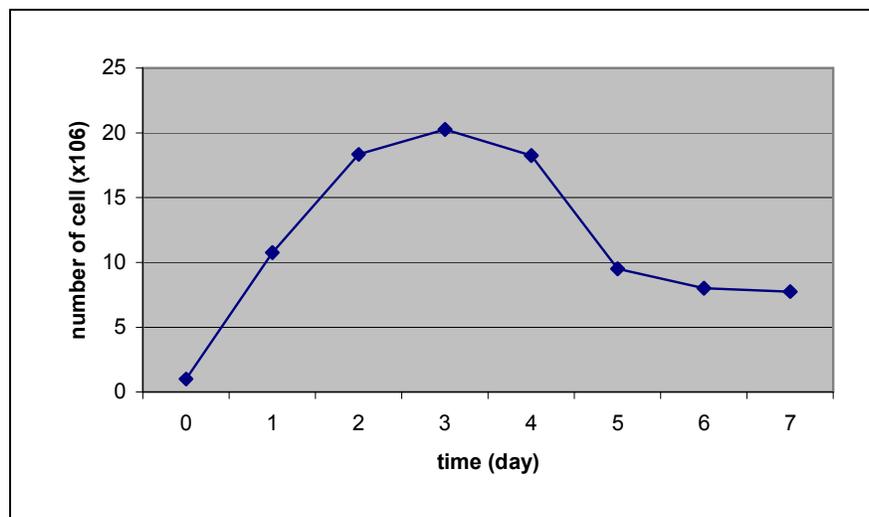


Fig 3. Growth curve of C1-isolate on Walne media

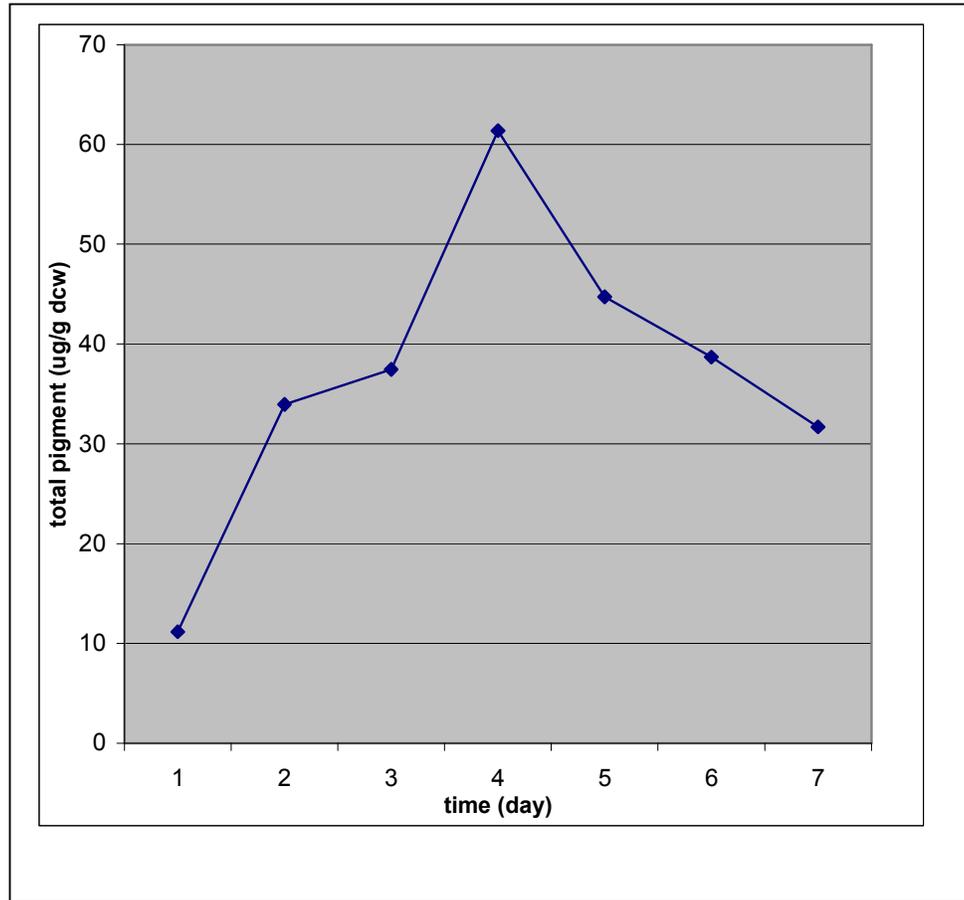


Fig 4. Production of total pigment of C1- isolate

The result of salinity treatment for the local isolate of green algae with a variety concentration of NaCl from 0% until 30% presented in **Fig 5.**, shows that the optimal concentration of NaCl for growth was 5% and 10%. Many marine bacteria typically grow best at salt concentration of about 3%. The outer membranes of marine bacteria require at least 1,5% NaCl to maintain their integrity (Atlas, 1995). In all concentration of NaCl, there is no significant difference on the growth and colour of the culture after several days, as illustrated in **Fig 5.** This

result clearly shows agreement with previous experiment which exhibits similarities of local isolate of green algae with cyanobacteria in toleration ability on high salt concentration, as may occur in tide pools and lakes when evaporation concentrates salts (Sze, 1993). Some studies also display that green algae *Dunaliella* showing a remarkable adaptation to a variety of salt concentration from as low as 0,2% to salt saturation of about 35% (Borowitzka & Borowitzka, 1988; Ben-Amotz, 1993).

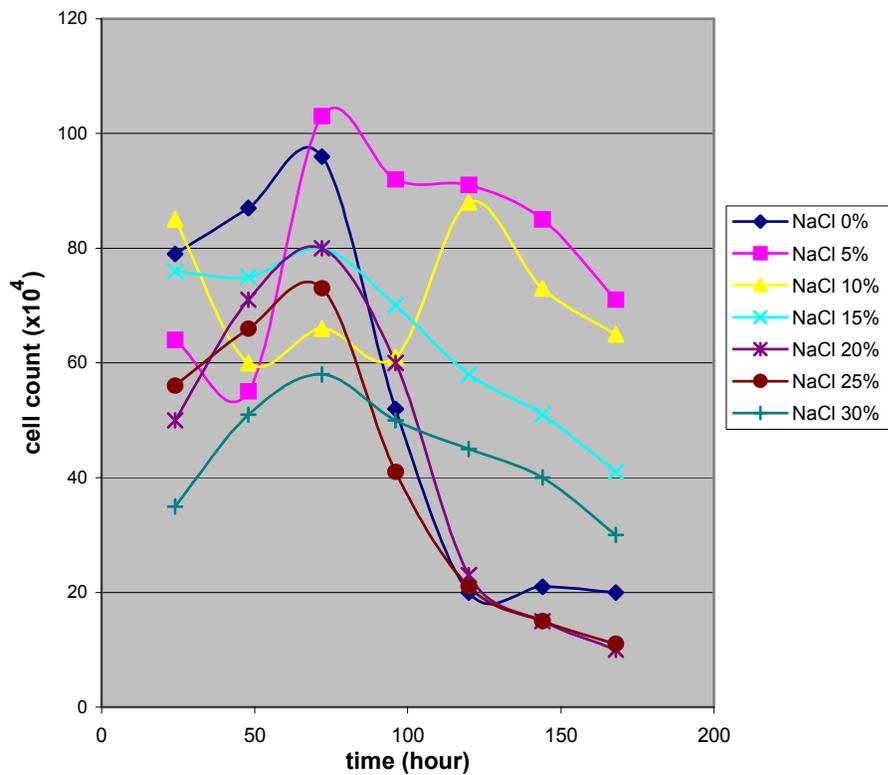


Fig. 5. Effect of salinity on growth of C1- isolate

Some green algae will change their cell colors after several days under salinity treatment as shown by *D. salina*. On salinity 0,5-2,0 M *D. salina* appeared yellow-green after less than one week of growth (Wong *et al.*, 2000). It has been observed that *Dunaliella* osmoregulates by varying the intracellular concentration of the photosynthetic glycerol in response to the extracellular osmotic pressure. On growth in media containing different salt concentration, the intracellular glycerol concentration is directly proportional to the extracellular salt concentration and

maintains the cell water volume and the required cellular osmotic pressure.

3. Molecular characterization of C1-isolate

The result of 18S rDNA analysis as a typical method to identify molecular eucaryotic species revealed negative result. Electroferogram of a C1-isolate 16S rDNA amplification was shown one clear band as illustrated by **Fig. 6**.

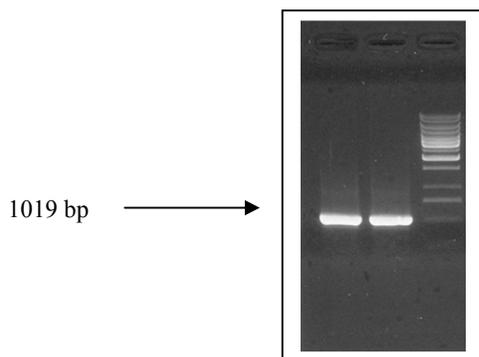


Fig 6. Electroferogram 16S rRNA of a green algae isolate

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>[gi|24817732|dbj|AB058249.1] Cyanobacterium sp. MBIC10216 gene for 16S rRNA,
partial sequence
Length=1252

Score = 613 bits (309), Expect = 4e-173
Identities = 315/317 (99%), Gaps = 0/317 (0%), Strand=Plus/Minus

Query 536  ACTTCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGGATTACCCGAGTATGC 595
          |||
Sbjct 1252  ACTTCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGGATTACCCGAGTATGC 1193

Query 596  TGACCTGCGATTACTAGCGATTCTCTCATGCAGGCGAGTTTCAGCCTGCAATCTGAA 655
          |||
Sbjct 1192  TGACCTGCGATTACTAGCGATTCTCTCATGCAGGCGAGTTTCAGCCTGCAATCTGAA 1133

Query 656  CTGTGGCTGGGTTTGATGAGATTCGCTCCACCTCGCGGTTTCGCACCCCTTTGTCCCAAC 715
          |||
Sbjct 1132  CTGTGGCTGGGTTTGATGAGATTCGCTCCACCTCGCGGCTTCGCTCCCCTTTGTCCCAAC 1073

Query 716  CATTGTAGTACGTGTGTAGCCAAGACGTAAGGGGCATGCTGACTTGACGTACATCCCCAC 775
          |||
Sbjct 1072  CATTGTAGTACGTGTGTAGCCAAGACGTAAGGGGCATGCTGACTTGACGTACATCCCCAC 1013

Query 776  CTTCTCCGAGTTCTCCCGGCGGTCTCCCTAGAGTCCCAACTTAATGCTGGCAACTAA 835
          |||
Sbjct 1012  CTTCTCCGAGTTCTCCCGGCGGTCTCCCTAGAGTCCCAACTTAATGCTGGCAACTAA 953

Query 836  GGACGAGGGTTGCGCTC 852
          |||
Sbjct 952  GGACGAGGGTTGCGCTC 936
    
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Fig 7. The multiple alignment analysis result of 16S rDNA sequence of green algae isolate with *Cyanobacterium* sp. MBIC1021 (Query = 16S rDNA sequence of C1-isolate, Sbjct = 16S rDNA sequence of *Cyanobacterium* sp. MBIC1021)

Surprisingly, a control conducted by amplification and analysis of 16S rDNA fragments revealed close homology with 16S rDNA sequence from some member of Cyanobacteria especially with *Cyanobacterium* sp. MBIC1021 in 99% similarity (**Fig. 7**) and 95% similarity with *Synechocystis* PCC6308 (**Fig.8**)

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>|gi|14625357|dbj|AB039001.1| Synechocystis PCC6308 gene for 16S rRNA,
partial sequence
Length=1435

Score = 682 bits (344), Expect = 0.0
Identities = 403/420 (95%), Gaps = 2/420 (0%) , Strand=Plus/Minus

Query 434 CCTTCCGGTACGGCTACCTTGTACGACTTCACCCAGTCACTAGTCCCACCTTCGGGCGC 493
      |||
Sbjct 1435 CCTTCCGGTACG-CTACCTTGTACGACTTCACCCAGTCACTAGTCCCACCTTCGGCAT 1377

Query 494 CTCCCTCCATTA-CGGTTGAGATAACGACTTCGGGCGTGACCAACTTCCATGGTGTGACG 552
      |||
Sbjct 1376 CCCTCTCCGTAAACGGTTGAGGTAACGACTTCGGGCGTGACCAACTTCCATGGTGTGACG 1317

Query 553 GGCGGTGTGTACAAGACCCGGGAACGGATTACCCGAGTATGCTGACCTGCGATTACTAG 612
      |||
Sbjct 1316 GGCGGTGTGTACAAGACCCGGGAACGGATTACCCGAGTATGCTGACCTGCGATTACTAG 1257

Query 613 CGATTCTCCTTCATGCAGGCGAGTTTCAGCCTGCAATCTGAACTGTGGCTGGGTTTGAT 672
      |||
Sbjct 1256 CGATTCTCCTTCATGCAGGCGAGTTTCAGCCTGCAATCTGAACTGGGCTGGGTTTGAC 1197

Query 673 GAGATTCGCTCCACCTCGCGGTTTCGCACCCCTTTGTCCCAACCATTGTAGTACGTGTGT 732
      |||
Sbjct 1196 AGGATTCGCTCCACTTCGCAGTTTCGCCTCCCTTTGTCCCAACCATTGTAGTACGTGTGT 1137

Query 733 AGCCCAAGACGTAAGGGGCGATGCTGACTTGACGTATCCCCACCTTCTCCGAGTCTCC 792
      |||
Sbjct 1136 AGCCCAAGACGTAAGGGGCGATGCTGACTTGACGTATCCCCACCTTCTCCGAGTCTCC 1077

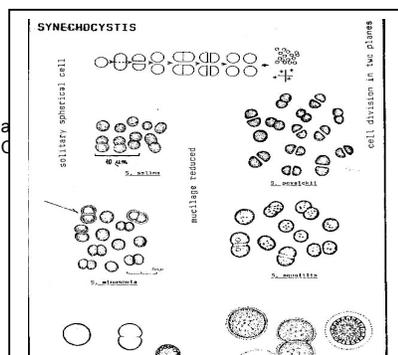
Query 793 CCGGCGGTCTCCCTAGAGTCCCAACTTAATGCTGGCAACTAAGGACGAGGGTTGCGCTC 852
      |||
Sbjct 1076 CCGGCGGTCTCCCTAGAGTCCCAACTTAATGCTGGCAACTAAGGACGAGGGTTGCGCTC 1017
    
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Fig 8. The multiple alignment analysis result of 16S rDNA sequence of green algae isolate with *Synechocystis* PCC6803 (Query = 16S rDNA sequence of C1-isolate, Sbjct = 16S rDNA sequence of *Synechocystis* PCC6803)

However, ecophysiological, morphological and microbiological analysis supports the molecular analysis in showing more similarities with feature of *Synechocystis* instead of *Cyanobacterium*. *Synechocystis* as shown in Fig 9. has twenty four described species and three unclear taxa. Its taxonomic position with higher hierarchy was *Cyanophyceae*, *Chroococcales*, *Merismopediaceae*, *Merismopedioideae*. The common characteristic of this species is solitary cells, never forming colonies. The cell length *S. salina* was 10 µm, while *S. minuscula* 5 µm. Mainly it has thin, fine, colourless mucilaginous envelopes with homogeneous content or with several prominent granules,

sometimes with visible chromatoplasma, pale blue-green, olive-green, bright-green or pinkish; different but stable pigment ratios in related species (and populations); cell division by binary fission. Several species are planktonic in fresh water reservoirs or in salinic or sea waters, others grow in metaphyton of pools, of thermal and mineral springs, of salinic (brackish) swamps or in moors (Lewin 1983). According to Holt *et al.* (1994) *Synechocystis* is unicellular, coccoid, or spherical, with binary fission central but in 2 or 3 successive planes at right angles to each other. Agregates sometimes occur with amorphous slime holding together. Cells are 2-7 µm in diameter, most strain in the 2-4 µm range; thylakoid is present.

Species Determination of Green Alga
 Characterization for Improvement of C



microbiological, Ecophysiological and Molecular

Fig 9. *Synechocystis* (Cyano-Base)

Despite the similarities between the local isolate of green algae and *Synechocystis*, there is a clear several different factors as we can see from **Table 1**. which has to be considered among the two species. Blue green algae has phycobilins as their principal accessory pigments. Extraction of the Cyanobacteria pellet with methanol or etanol will cause blue to red residual color which corresponds to its pigment phycobilins. It has been proved in our experiment that the local isolat of green algae displays a green to yellow color of the residu. The result of this analysis shows that cell of green algae isolate only has chlorophylls a and b but lack phycobiliproteins. It shows that these

characteristics are considered not corresponding to typical characteristic of Cyanobacteria especially *Synechocystis*. However, Lewin (1983) reported the discovery of a unicellular prokaryotic alga which was not a cyanobacterium. The alga, later named Prochloron, resembles the coccoid cyanobacterium *Synechocystis*, which its cells have chlorophylls a and b and lack phycobiliproteins. Prochloron resembles cyanobacteria in its cell structure and biochemistry (Lewin and Cheng, 1989 in Sze, 1993), but its photosynthetic pigments are similar to green algae. **Table 2** shows different characteristics among these three species.

Table 2. Comparison of Cyanophyta, Prochlorophyta and Green Algae (Sze, 1993)

	Cyanophyta	Prochlorophyta	Chlorophyta
Cellular organization	prokaryotic	prokariotyc	eukaryotic
Chlorophylls	a	a,b	a,b
Phycobiliproteins	present	absent	absent
Thylakoids	solitary	paired	stacked (2-6)

Lewin (2002) mentioned that Prochlorophyta was assigned to a new algal sub-class in showing clearly photosynthetic prokaryotes like cyanophytes, but it contains no blue or red bilin pigment. However, since their possible phylogenetic relationships to ancestral green-plant chloroplasts have not

received support from molecular biology, it now seems expedient to consider them as aberrant cyanophytes.

CONCLUSION

Characterization of C1-green algae isolate from Jepara Sea Waters based on ecophysiological, microbiological and molecular analysis clearly shows a prokaryotic characteristic. Based on the experiment results, it can be assumed that the species of green algae isolate was the member of Cyanobacteria, and it was not *Dunaliella*. Instead of showing similarities with *Synechocystis*, green algae isolate also provides some characters which resemble to Prochlorophyta. In conclusion, C1-isolate of green algae from Jepara Seawaters possess some characters similar to both of two species, Prochlorophyta and *Synechocystis*.

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REFERENCES

- Ausubel, F., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl. 1995. *Short Protocols in Molecular Biology. A Compendium of Methods from Current Protocols in Molecular Biology*. 3rd Ed. Wiley & Sons. 2-10p.
- Atlas, R.M. 1995. *Principles of Microbiology*. Mosby. Toronto. 350 – 355p.
- Ben-Amotz, A. 1993. Production of β -carotene and Vitamins by The Halotolerant Alga *Dunaliella*. *Marine Biotechnology* Vol 1. Pharmaceutical and Bioactive Natural Products. Ed. Attaway, D.H. & Zaborsky, O.R. Enum. 411-416p.
- Bidwell, J.P. and Spotte S. 1983. *Artificial Sea Water Formulas and Methods*. Jones & Bartlett. 324-325p.
- Borowitzka, M.A. and Borowitzka, L.J. 1988. Limits to Growth and Carotenogenesis in Laboratory and Large-Scale Outdoor Cultures of *Dunaliella salina*. In *Algal Biotechnology*. Ed. Mollion, T.S et al., Elsevier. 171-180p.
- Garcia-Martinez, J., S.V Acinas, A.I. Anton, F. Rodriguez-Valera. 1999. Use of the 16S-23S Ribosomal Gene Spacer Region in Studies of Prokaryotic Diversity. *J. Microbiol Methods*. 36. 55-64p
- Goodwin, T.W. 1974. Carotenoids and Billiproteins. In *Algal Physiology and Biochemistry. Botanical Monographs*. Ed. Stewart, Blackwell. 176-180p.
- Goodwin, T.W. 1974 G. Britton. 1988. Distribution and Analysis of Carotenoids. *Plant Pigments*. Ed. T.W. Goodwin. 75 – 80p.
- Harborne, J.B. 1984. *Metode Fitokimia*. Ed. II. ITB. Bandung. 259 – 261p.
- Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, S.T. Williams. 1994. *Bergey's Manual of Determinative Bacteriology*. 9nd Ed. William & Wilkins. 377-390p.
- Johnson, E.A. and WA Schroeder. 1996. *Microbial Carotenoids: Advances in Biochemical Engineering /Biotechnology* Ed. by A. Fiechter. 141-145
- Kusumaningrum, H.P. J. Soedarsono., T. Yuwono, E. Kusdiyantini. 2004. The

- Effect of Various Salinity Level to the Growth and Characterization of *Dunaliella* sp Isolated from Jepara Waters, in Laboratory Scale. *ILMU KELAUTAN*. 9(3) : 136 –140
- Kusumaningrum, H.P. J. Soedarsono., 2006. Species Determination of Green Algae Isolated from Jepara, Indonesian Coastal Region based on Molecular Characterization and Its Correlation with *DXS* Gene Detection for Carotenoid Production. *ILMU KELAUTAN*. (in press)
- Kusumaningrum, H.P. J. Soedarsono, 2006. Molecular Determination of *Dunaliella* sp. to detecting 1-Deoxy-D-Xylulose-5-phosphate Synthase (*DXS*) Gene in Improvement of Carotenoid Production. Abstract. *Seminar Nasional Perhimpunan Mikrobiologi Indonesia*. Solo. 26-27 Agustus 2006. V 22
- Kusumaningrum, H.P. J. Soedarsono, E. Kusdiyantini. 2006. Molecular Characterization A Green Algae Isolate by 16S rRNA in Improvement of Carotenoid Production. Abstract. *Seminar Nasional SPMIPA*. Semarang. 9 September 2006. BIO 5
- Lee, P.C. and C. Schmidt-Dannert. 2002. Metabolic engineering towards biotechnical production of carotenoids in microorganisms. *Appl Microbiol Biotechnol* 60 : 1 –11
- Lewin, RA. 1983. The problems of Prochloron. *Ann Microbiol* (Paris) ; 134B (1):37-41.
- Lewin, RA. 1984. Prochloron--a status report. *Phycologia*. 23(2):203-8.
- Lewin, RA. 2002 Prochlorophyta - a matter of class distinctions. *Photosynth Res*. 73(1-3):59-61.
- Lichtenthaler. 1999. The 1-Deoxy-D-Xylulose 5-Fosphate Pathway of Isoprenoid Biosynthesis in Plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1(50) : 47-65.
- Logan, N.A. 1994. *Bacterial Systematics*, Blackwell Scientific Publications.
- Lois L.M., Campos N., S.R. Putra, K. Danielsen, M. Rohmer, A. Boronat. 1998. Kloning and Characterization of a Gene from *Eschericia coli* Encoding a Transketolase-like Enzymes That Catalyzes the Synthesis of 1-Deoxy-D-Xylulose 5-Fosphate, a Common Precursor for Isoprenoid, Thiamin and Pyridoxol Biosynthesis. *Proc. Natl.Acad.Sci.* 95 : 2105 – 2110.
- Orset, S.C. and A.J. Young. 2000. Exposure to Low Irradiances Favors the Synthesis of 9-cis β,β -Carotene in *Dunaliella salina* (Teod.). *J. Plant. Physiol.* 122 : 609 – 617
- Partensky F. Hess WR. Voulot D. 1999. Prochlorococcus, a marine photosynthetic prokaryote of global significance. *Microbiol Mol. Bio. Rev.* 63(1) : 106-27.
- Priest, F. and B. Austin. 1993. *Modern Bacterial Taxonomy*. Chapman & Hall. 73 – 80p.
- Rabbani, S., P. Beyer, J.v. Lintig, P. Hugueney, and H. Kleinig. 1998. Induced β -Carotene Synthesis Driven by Triacylglycerol Deposition in the Unicellular Alga *Dunaliella bardawil*. *Plant Physiol.* 116 (4) : 1239 – 1248
- Sandmann, G. 2001. Genetic Manipulation of Carotenoid Biosynthesis : Strategies, problems and achievements. *TRENDS in Plant Science*. 6 (1): 14– 17
- Sze, P. 1993. *A Biology of the Algae*. Second Ed. Wm.C.Brown Publ. 1 – 81p
- Wong, V., X. Liu and R. Bidigare. 2000. Dependence of Carotenoid Production on Salinity in *Dunaliella salina*. *MarBEC Summer Undergraduate Research Fellowship*. 1 –14p.

