Research Article

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Axenic purification and cultivation of an Arctic cyanobacterium, *Nodularia spumigena* KNUA005, with cold tolerance potential for sustainable production of algae-based biofuel

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A psychrotolerant cyanobacterium, *Nodularia spumigena* KNUA005, was isolated from a cyanobacterial bloom sample collected near Dasan Station in Ny-Ålesund, Svalbard Islands during the Arctic summer season. To generate an axenic culture, the isolate was subjected to three purification steps: centrifugation, antibiotic treatment and streaking. The broad antibacterial spectrum of imipenem killed a wide range of heterotrophic bacteria, while the cyanobacterium was capable of enduring both antibiotics, the remaining contaminants that survived after treatment with imipenem were eliminated by the application of an aminoglycoside antibiotic, kanamycin. Physical separation by centrifugation and streaking techniques also aided axenic culture production. According to the cold-tolerance test, this mat-forming cyanobacterium was able to proliferate at low temperatures ranging between 15 and 20°C which indicates the presence of cold-tolerance related genes in *N. spumigena* KNUA005. This suggests the possibility of incorporating cold-resistance genes into indigenous cyanobacterial strains for the consistent production of algae-based biofuel during the low-temperature seasons. Therefore, it is needed to determine the cold-tolerance mechanisms in the Arctic cyanobacterium in the next research stage.

Key Words: algae-based biofuel; Arctic cyanobacterium; axenic culture; cold-tolerance

INTRODUCTION

Over 90% of commercial algae biomass production is currently with large-scale open-pond systems. Such systems usually suffer from low production rates during the cold seasons (Sheehan et al. 1998, Benemann 2008). Hence, cyanobacteria isolated from the polar regions offer interesting potential for the production of biomass and biofuel due to their psychotrophic characteristics as Tang et al. (1997) previously reported that many high-latitude cyanobacterial strains remain active at low temperatures.

However, obtaining axenic cultures is essential for genetic, physiological and taxonomic research. Although numerous methods to produce axenic cultures of cyanobacteria have been suggested (Rippka 1988, Ferris and Hirsch 1991, Bolch and Blackburn 1996, Choi et al. 2008), it is still very difficult to eliminate all contaminating bacteria from environmental samples. Since cyanobacteria are a very diverse group, exhibiting enormous variations in growth, morphology and metabolic capabilities, any particular approach cannot guarantee success of purification.

The objectives of the current study were to axenically

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obtain cyanobacteria from an Arctic freshwater bloom sample by using a combination of antibiotics and physical separation techniques and to test the isolate's cold-tolerance capability. Our eventual goal is to discover the mechanisms of cold tolerance in Arctic cyanobacteria, identify and isolate genes with high activity. These genes will then be incorporated into indigenous cyanobacteria for consistent biofuels production during the low-temperature seasons.

MATERIALS AND METHODS

Sample collection and cyanobacterial isolation

Arctic cyanobacterial bloom samples were taken from the temporal water runoff region, located approximately 10 km east of Dasan Station in Ny- Ålesund, Svalbard Islands in August 2009 (Table 1, Fig. 1). Samples were then taken to the laboratory and 1 mL of bloom samples were inoculated into 100 mL BG-11_0 medium (Rippka et al. 1979) with cycloheximide (Sigma, St. Louis, MO, USA) at a concentration of 250 μg mL^{-1}. The flasks were incubated on an orbital shaker (Vision Scientific Co. Ltd., Bucheon, Korea) at 160 rpm and 15°C until cyanobacterial growth was apparent.

Axenic culture production

Well-grown cyanobacterial cultures (1.5 mL) were centrifuged at × 3,000 g for 15 min. Resulting pellets were streaked onto BG-11 agar supplemented with imipenem (100 μg mL^{-1}) (Choongwae Pharma Corporation, Seoul, Korea) and cycloheximide (20 μg mL^{-1}) and incubated in the dark for 24 hours to eliminate bacterial contamination. Plates were then incubated in a light : dark cycle (16 : 8 hours) at 15°C and filamentous growth was observed daily. When visible to the naked eye, emerging cyanobacterial filaments were aseptically transferred to fresh BG-11 plates to separate cyanobacteria from contaminating bacteria. Cyanobacterial filaments were then streaked onto R2A and LB agar plates (Becton, Dickinson and Company, Sparks, MD, USA) and incubated in the dark to check the axenic status of the culture for 7 to 14 days.

Table 1. Description of sampling point

Sampling point	Temperature (°C)	Salinity (‰)	pH
Arctic glacial area (78° 55' N, 11° 57' E)	9.8	8.8	8.7

Fig. 1. Cyanobacterial bloom in temporal water runoff from melting glacier ice in Ny-Ålesund, Svalbard Islands.

Contaminating bacteria that survived the imipenem treatment were identified by 16S rRNA gene sequencing (Lane 1991). Non-axenic (bacterially contaminated) cyanobacterial culture was further incubated on BG-11 agar with kanamycin (100 μg mL^{-1}) (Duchefa Biochemie, Haarlem, The Netherlands) for another 24 hours in the dark and the rest of the purification steps were repeated until a pure culture of the cyanobacterium was obtained (Fig. 2).

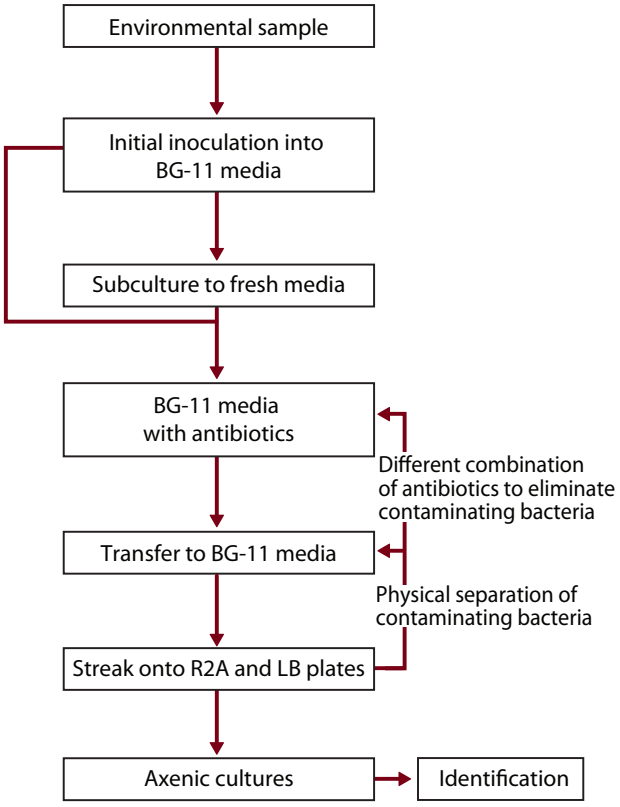

Fig. 2. Schematic of obtaining axenic cultures from environmental samples.

Morphological identification

The strain was grown in BG-11$_0$ medium (without nitrogen) for 10 days. Live cells were harvested and suspended in sterile dH_2O and inspected at × 400 magnification on a Zeiss Axioskop 2 light microscope (Carl Zeiss, Korea Co. Ltd., Seoul, Korea) equipped with differential interference contrast optics.

Molecular identification

PCR conditions and the primer sets CYA106F, CYA781R(a), and CYA781R(b) were used for 16S rRNA sequence analysis as described by Nubel et al. (1997). The phycocyanin encoding operon intergenic spacer (PC-IGS) region was amplified using the primer pair, PCβF and PCαR specific for cyanobacteria (Neilan et al. 1995). Region ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) *rbc*LX was amplified with primers CW and DW described previously (Rudi et al. 1998). Synthesis of the primers used and DNA sequencing were performed at the Macrogen facility (Macrogen, Seoul, Korea).

Cold-tolerance test

A seed culture of *Nodularia spumigena* KNUA005 was inoculated into both BG-11$_0$ and BG-11(+) media in triplicate and incubated in a light : dark cycle (16 : 8 hours) on an orbital shaker at 160 rpm and 10, 15, 20 and 25°C for 21 days. Cyanobacterial density was determined by measuring the optical density of a culture at 750 nm on an Optimizer 2120UV spectrophotometer (Mecasys Co. Ltd., Daejeon, Korea). Then, growth curves were drawn to reveal the effect of temperature on *N. spumigena* KNUA005.

RESULTS

Axenic production of the culture

After imipenem treatment and physical separation by streaking onto BG-11 agar plates, two strains of contaminating bacteria still survived and co-existed with the cyanobacterial culture. These bacteria, CB1 and CB2, were identified as *Rhizobium* sp. and *Brevundimonas* sp., respectively (Table 2). However, kanamycin treatment successfully removed the contaminants from the cyanobacterium. The purity of the culture was verified when no bacterial growth was observed after incubating for 14 days after streaking the culture onto R2A and LB agar plates.

Identification of the axenic Arctic cyanobacterium

Molecular characterization showed that the Arctic cyanobacterium was *N. spumigena* and all results inferred from 16S rRNA, PC-IGS and *rbc*LX sequence analyses were in agreement (Table 3). The isolate's morphological features also suggested that the isolate was *N. spumigena* (Fig. 3). The trichomes were straight or slightly sinuous, the vegetative cells were discoid-shaped and the heterocysts were both at intercalary and terminal.

Cold-tolerance of *Nodularia spumigena* KNUA005

As shown in Fig. 4A, *N. spumigena* KNUA005's optimal growth temperature in BG-11$_0$ was 20°C, but it was also able to grow well at 15°C. However, this heterocystous organism showed a tendency of growing slower in BG-11(+) medium than in nitrogen-free BG-11$_0$ medium (Fig. 4B). There was little or no cyanobacterial growth in either BG-11$_0$ or BG-11(+) media at 10°C, but the cyanobacterium

Fig. 3. Light microscope images of *Nodularia spumigena* KNUA005. Scale bars represent 20 μm.

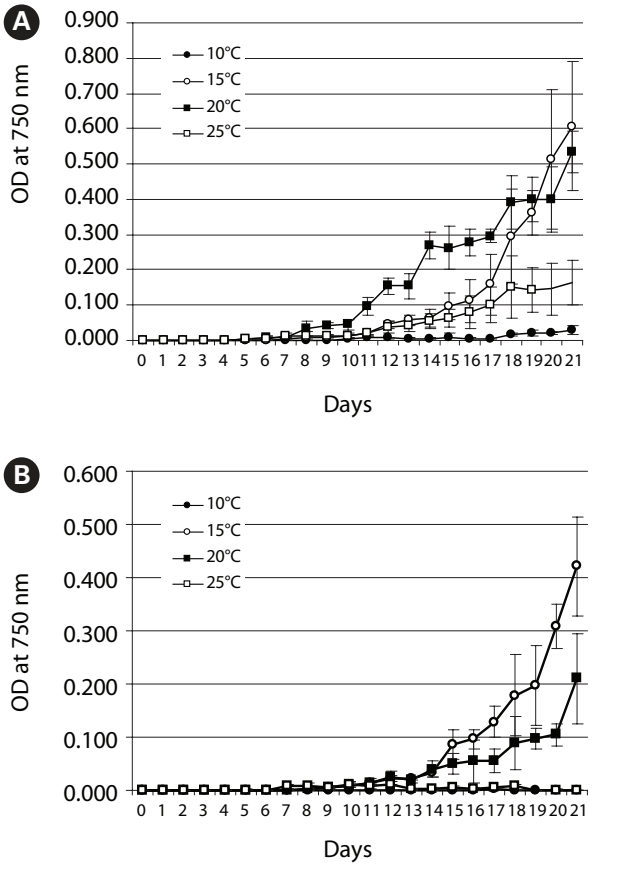

Fig. 4. Growth curves of *Nodularia spumigena* KNUA005 grown in BG-11$_0$ (A) and BG-11(+) (B) media. OD, Optical density.

Table 2. Identification of contaminating bacteria isolated from the cyanobacterial culture

Bacterial isolate	Accession number	Length (bp)	Closest match (GenBank accession number)	Overlap (%)	Sequence similarity (%)	Taxonomic affinity
CB 1	HM241944	1,407	*Rhizobium* sp. 525W (AB262326)[a]	100	98	*Rhizobium* sp.
CB 2	HM241945	1,383	*Brevundimonas variabilis* ATCC 15255^T (AJ227783)	100	99	*Brevundimonas variabilis*

[a] The closest cultured match.

Table 3. Results from BLAST searches using 16S *rRNA*, PC-IGS, *rbcLX* sequences of *Nodularia spumigena* KNUA005

Marker gene	Accession number	Length (bp)	Closest match (GenBank accession number)	Overlap (%)	Sequence similarity(%)	Taxonomic affinity
16S rRNA	HM241943	616	*Nodularia spumigena* BY1 (AF268004)[a,b]	100	99	*Nodularia spumigena*
PC-IGS	HM241946	640	*Nodularia spumigena* PCC7804 (AF101452)[c]	91	97	*Nodularia spumigena*
*rbc*LX	HM241947	866	*Nodularia spumigena* UTEX B2092 (AJ783696)[d]	99	97	*Nodularia spumigena*

[a] The closest cultured match.
[b] Isolated from Arkona Sea, Baltic Sea.
[c] Isolated from thermal spring water, Dax, France.
[d] Isolated from Osoyoos, BC, Canada.

remained alive and subsequently grew well when placed under favourable conditions (data not shown).

DISCUSSION

Many research groups have successfully isolated cyanobacterial cultures from a variety of environmental samples. Nevertheless, cultures maintained in laboratories are mostly unialgal, not axenic cultures, which are not suitable for understanding genetic, biochemical or physiological properties of a particular taxon. In this study, we have developed a solid medium-based isolation method for effective axenic culture production of filamentous cyanobacteria.

Imipenem is a β-lactam antibiotic derived from *Streptomyces cattleya* with a broad spectrum against aerobic and anaerobic Gram positive bacteria as well as Gram negative ones through inhibiting peptidoglycan biosynthesis. Incubation in the dark for 24 hrs may have killed the majority of contaminating bacteria while the cyanobacterium remained unaffected. However, two contaminants, *Rhizobium* sp. CB1 and *Brevundimonas* sp. CB2, belonging to Gram negative bacteria survived the imipenem treatment. They were not physically separated by streaking technique either, but were finally eliminated by the application of kanamycin, which prevents mRNA translation by interacting with the 30S ribosomal subunit.

This method uses a combination of two antibiotics with different mechanisms of action in an attempt to prevent any contaminating bacterial growth. This approach may provide an effective way of axenic culture production for filamentous cyanobacteria from heavily contaminated environmental samples.

N. spumigena is known as one of the major bloom formers in late summer throughout the world (Horne and Galat 1985, Carmichael et al. 1988, Codd et al. 1994). It was also reported that many polar cyanobacteria generally show optimal growth at 15°C or above (Tang et al. 1997, Chevalier et al. 2000). According to the temperature tolerance test, *N. spumigena* KNUA005's maximal growth was attained in the range of 15 to 20°C which is consistent with the previous reports. However, the isolate exhibited a tolerance to low temperatures ranging from 10 to 15°C which suggests that there are cold-tolerance genes present in this Arctic cyanobacterium. Hence, screening and identification of these useful genes using differentially expressed gene tag profiling and the Solexa® (Illumina, Inc., San Diego, CA, USA) massive parallel sequencing approach are needed. Cold-tolerance genes will then be incorporated into native cyanobacteria for consistent production of algae-based biofuels in the next research stage.

Our research group has already obtained four potential candidates for biofuel production that were isolated from cyanobacterial bloom samples in Lake Daecheong, Korea in late summer 2009 (unpublished data). It is hoped that new genetic strains may have potential for sustainable biofuel production under unfavourable weather conditions such as those experienced in the autumn and winter seasons.

However, it should be noted that *N. spumigena* produces Nodularin, a potent hepatotoxin which may pose a health risk for humans and animals (Runnegar et al. 1988, Sivonen et al. 1989). *N. spumigena* is also responsible for a large part of nitrogen input into water bodies (Huber 1986) due to their nitrogen-fixing ability. Thereby, care should be taken when working with this isolate to prevent release into environment.

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