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(54) **ENGINEERED CYANOBACTERIA WITH ENHANCED UV TOLERANCE**

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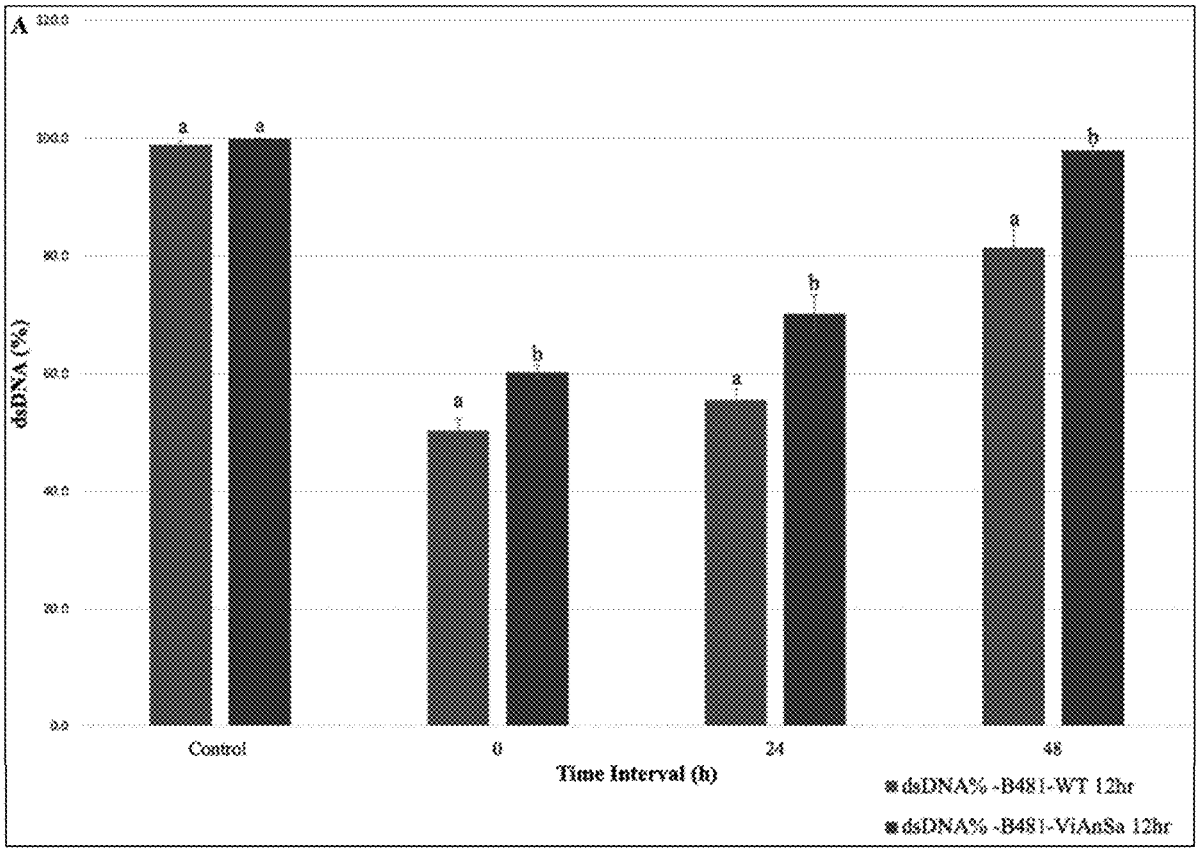
(57) **ABSTRACT**

A recombinant strain of *F. diplosiphon* was made by transforming wild type *F. diplosiphon* with a pGEM-7Zf (+) plasmid containing the photolyase gene (phrA) via electroporation. The recombinant strain was designated B481-ViAnSa and overexpressed the phrA gene to result in enhanced UV tolerance compared to wild type *F. diplosiphon.*

Specification includes a Sequence Listing.



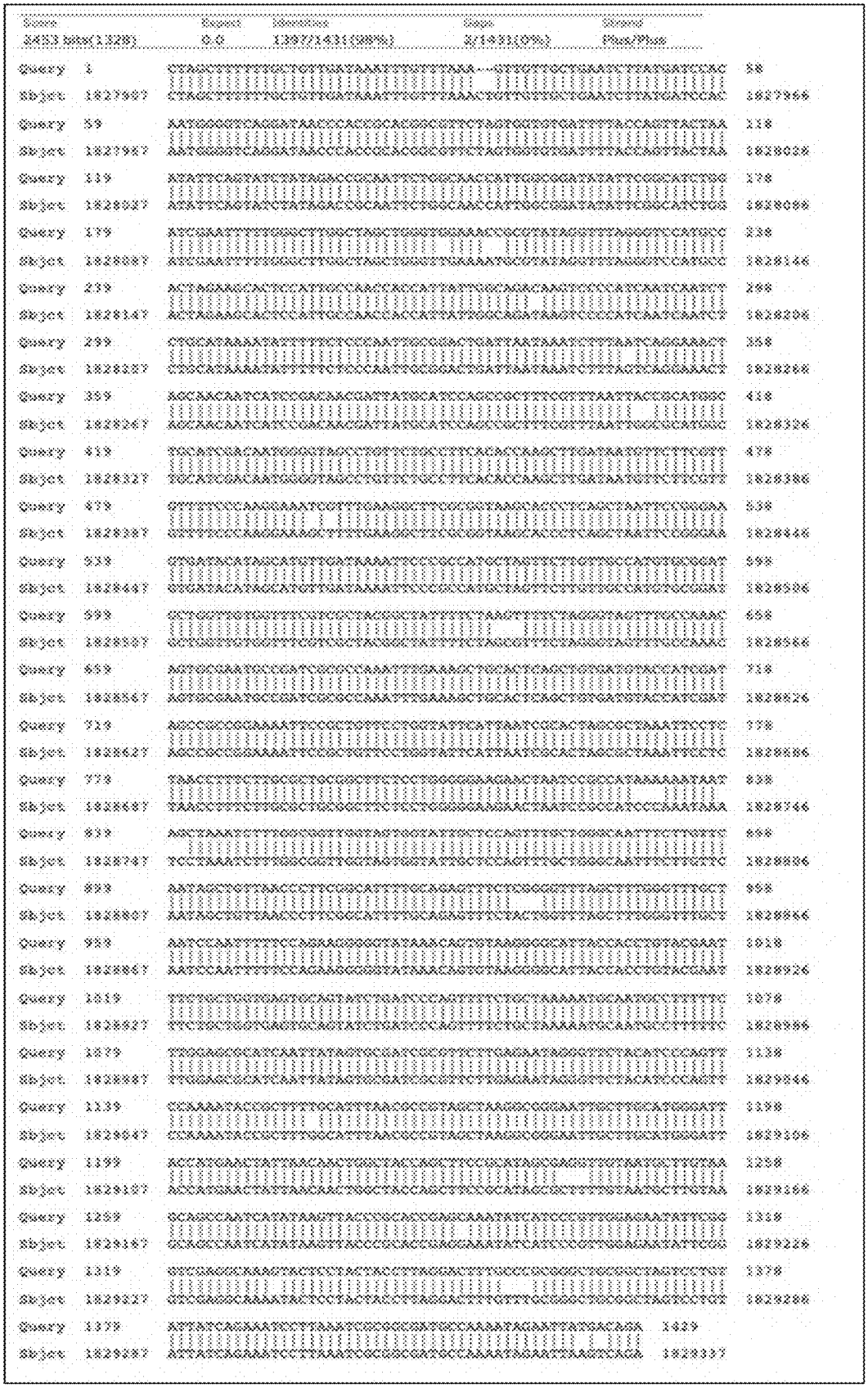


FIGURE 1

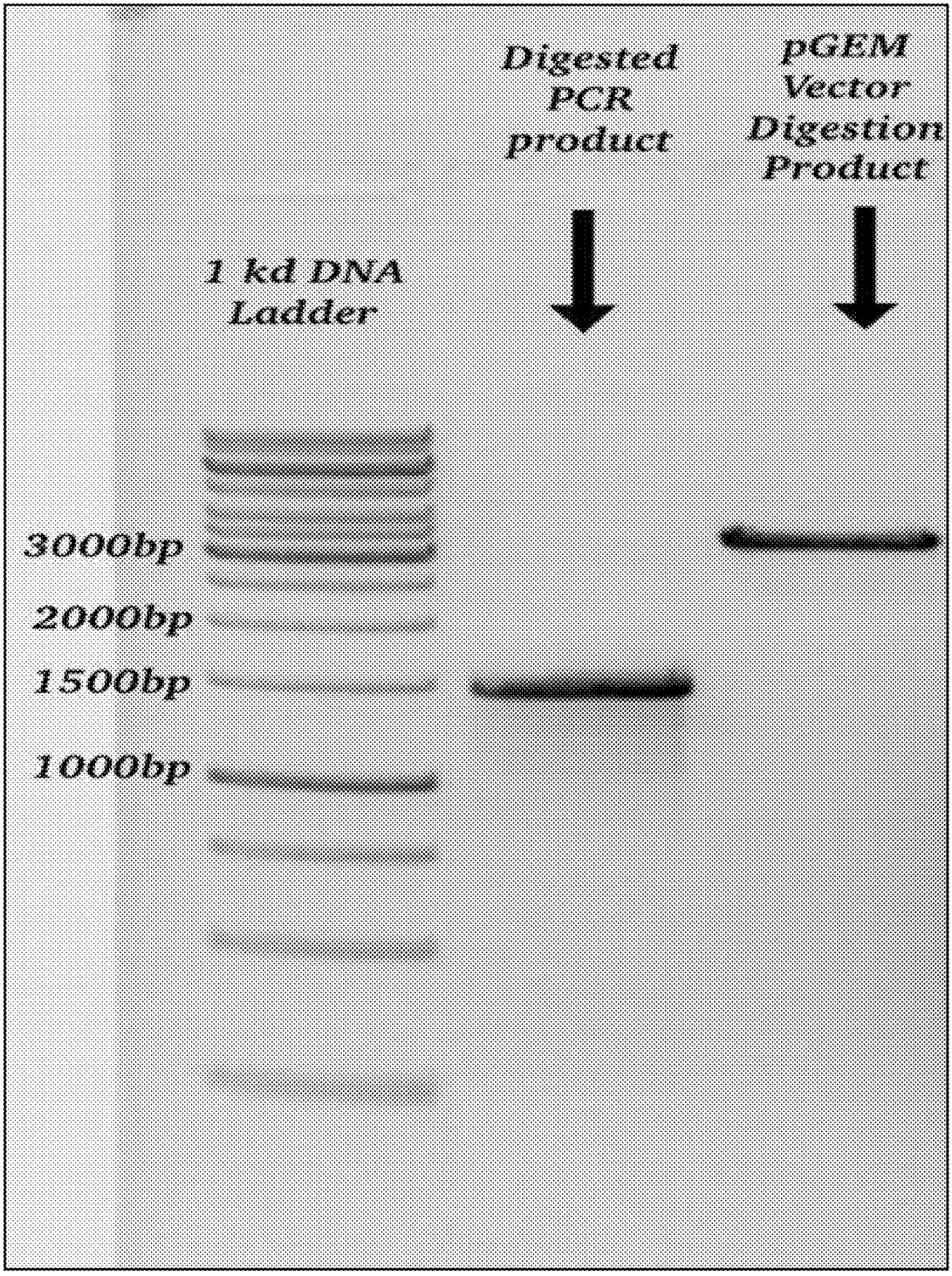

Digested PCR product
pGEM Vector Digestion Product
1 kd DNA Ladder
3000bp
2000bp
1500bp
1000bp

FIGURE 2A

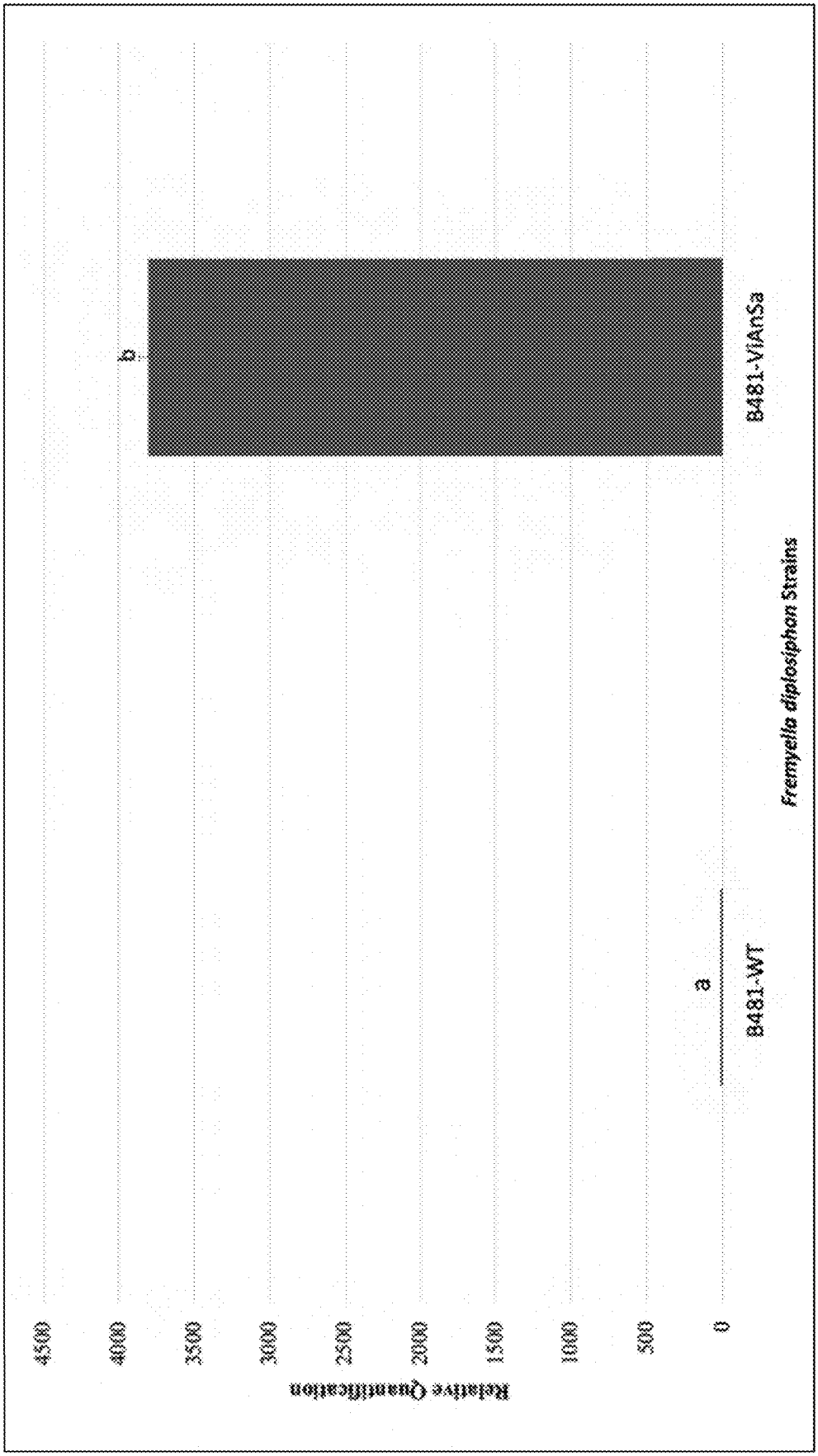

Relative Quantification
4500
4000
3500
3000
2500
2000
1500
1000
500
0
a
b
B481-WT
B481-ViAnSa
Fremyella diplosiphon Strains

FIGURE 2B

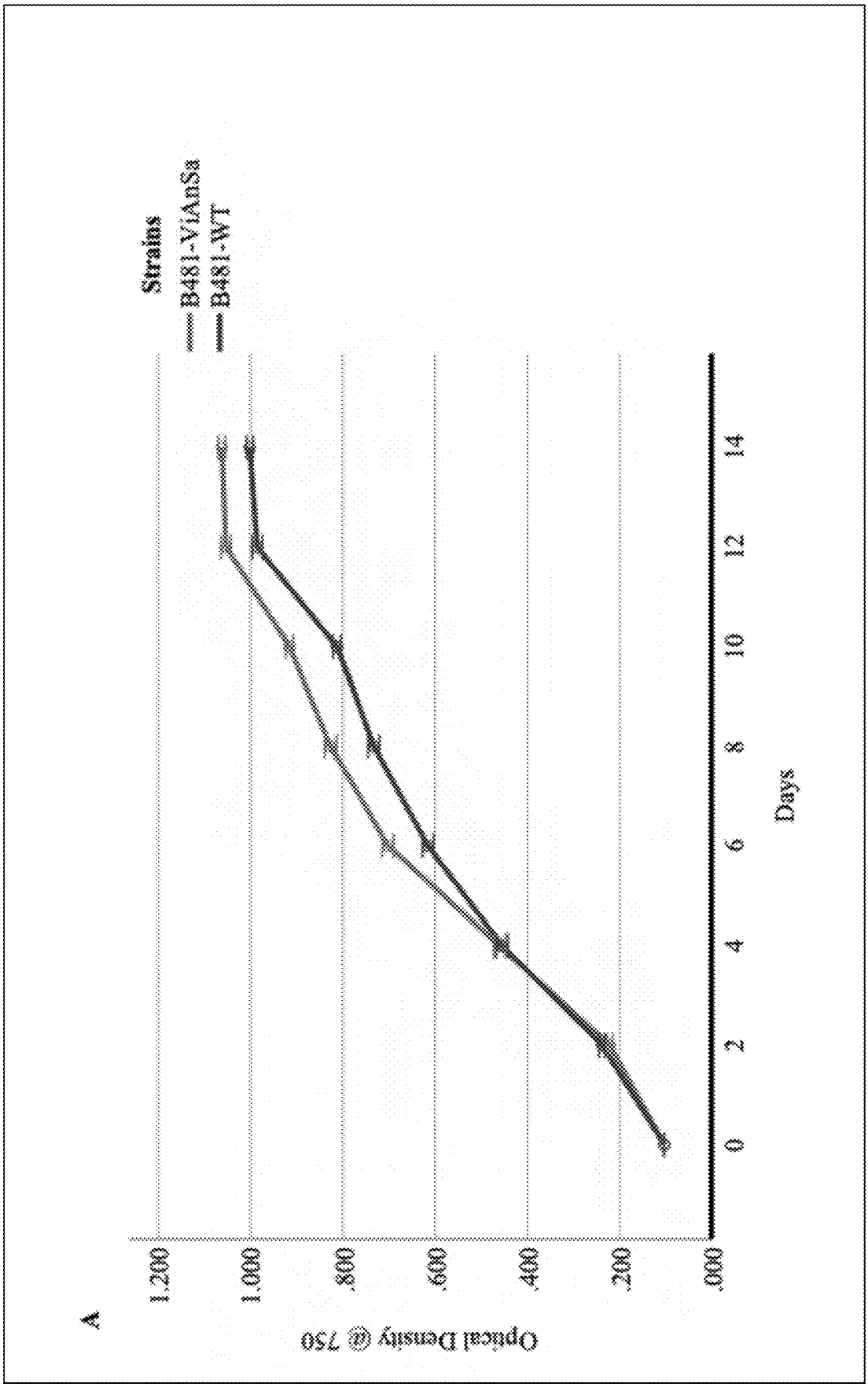

A
Optical Density @ 750
1.200
1.000
.800
.600
.400
.200
.000
0
2
4
6
8
10
12
14
Days
Strains
B481-VIAnSa
B481-WT

FIGURE 3A

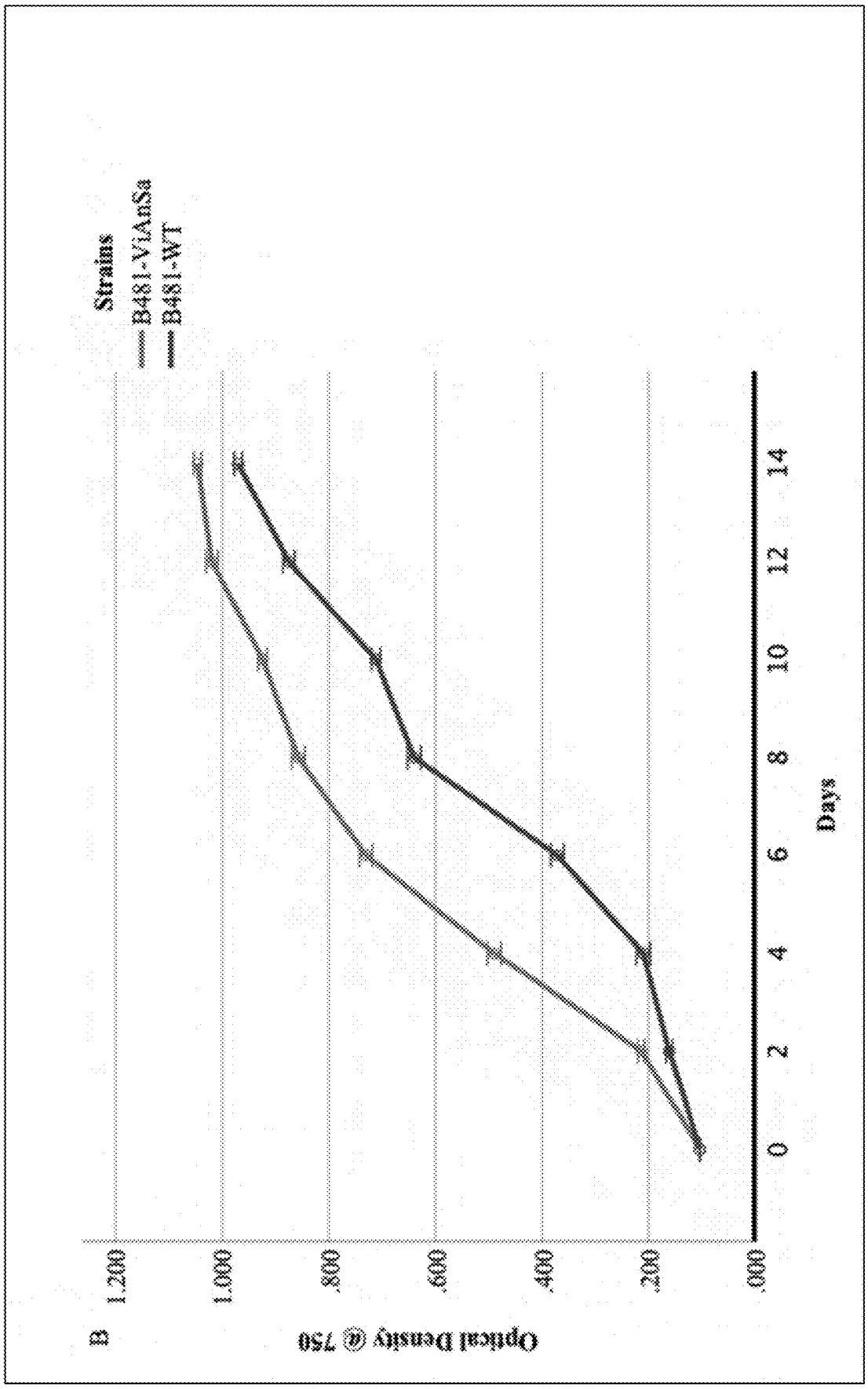

B
Optical Density @ 750
1.200
1.000
.800
.600
.400
.200
.000
0
2
4
6
8
10
12
14
Days
Strains
B481-ViAnSa
B481-WT

FIGURE 3B

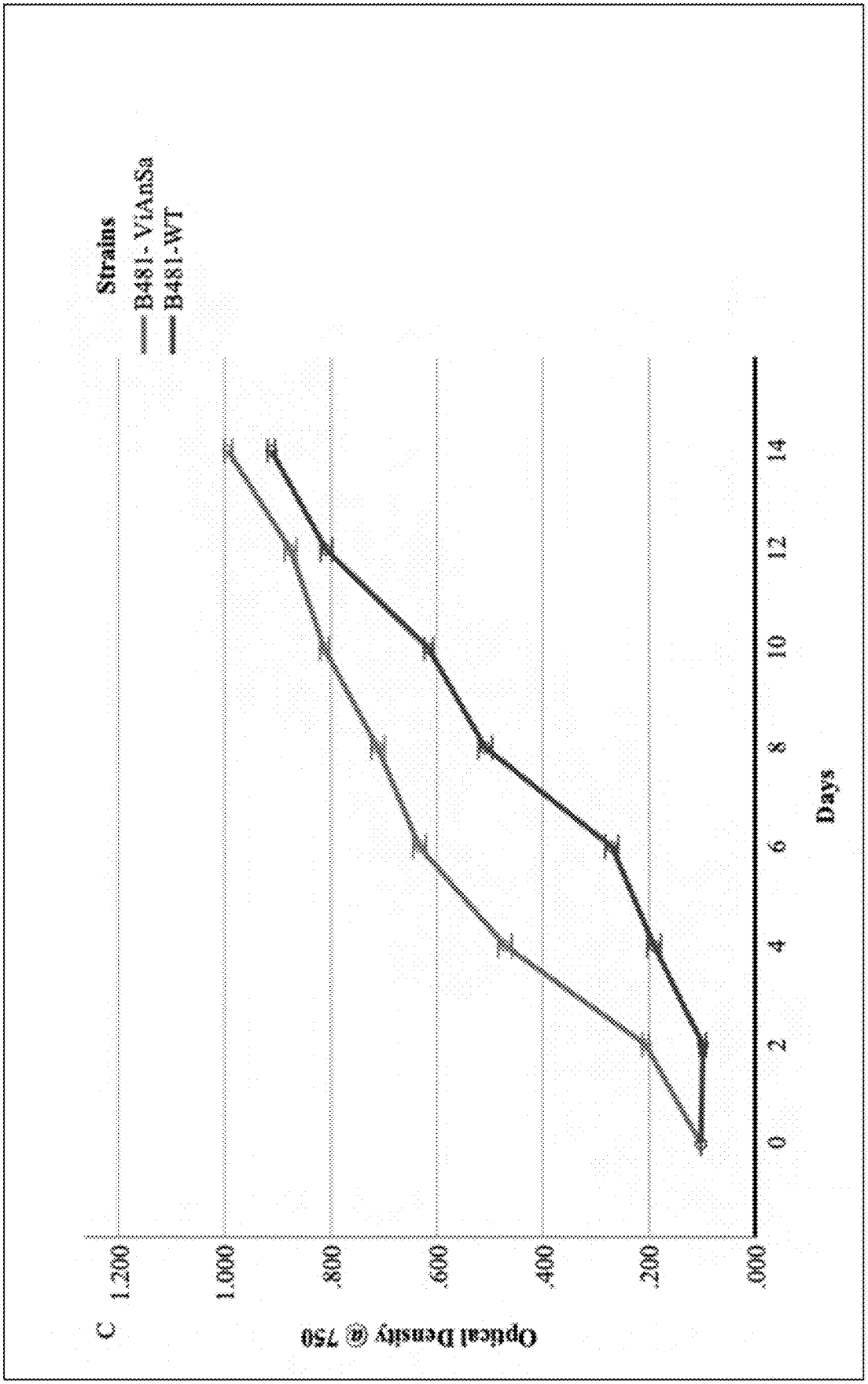

C
Optical Density @ 750
1.200
1.000
.800
.600
.400
.200
.000
0
2
4
6
8
10
12
14
Days
Strains
B481-VIAnSa
B481-WT

FIGURE 3C

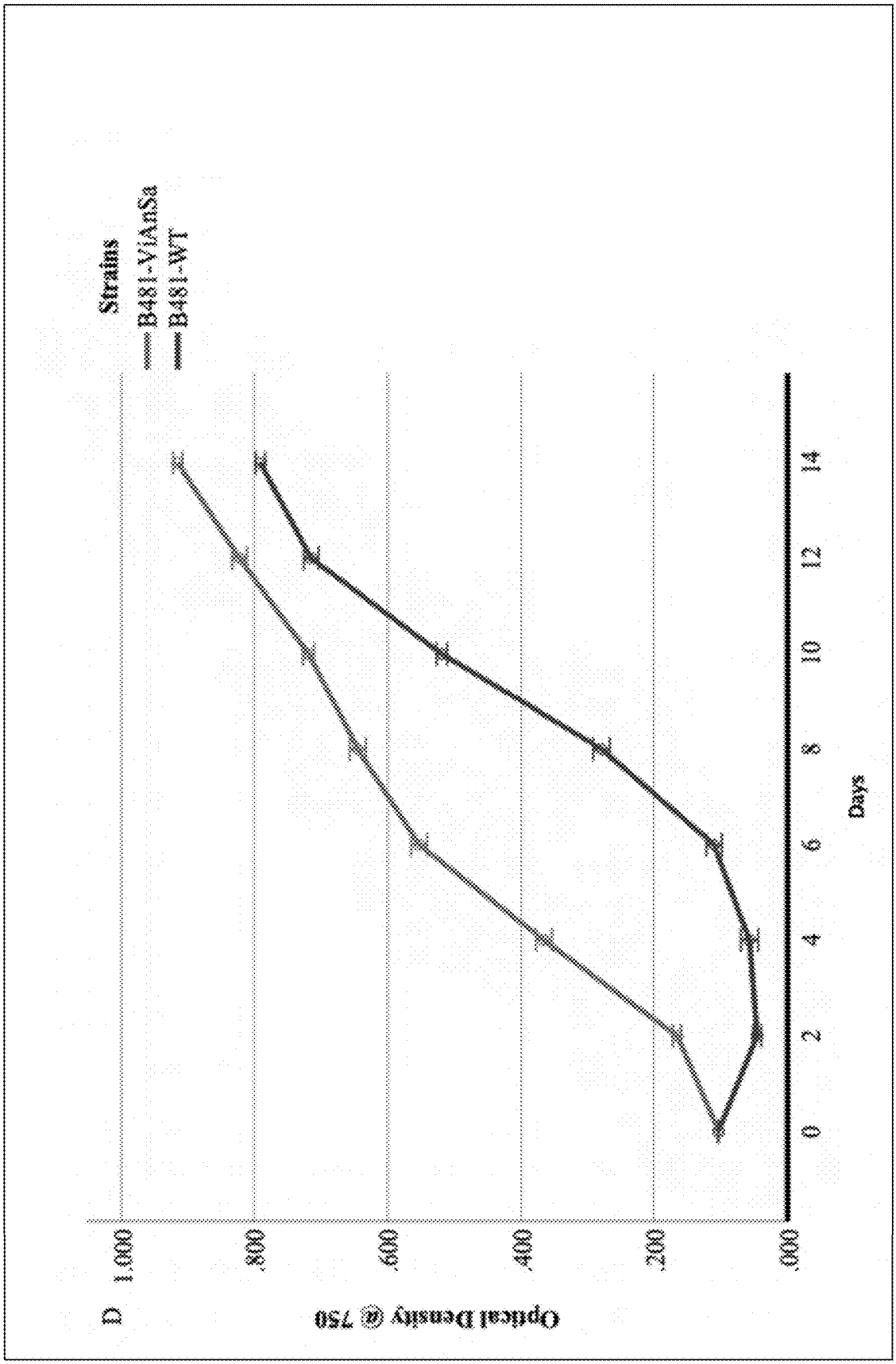

Strains
B481-VlAnSa
B481-WT
Optical Density @ 750
1.000
.800
.600
.400
.200
.000
0
2
4
6
8
10
12
14
Days

FIGURE 3D

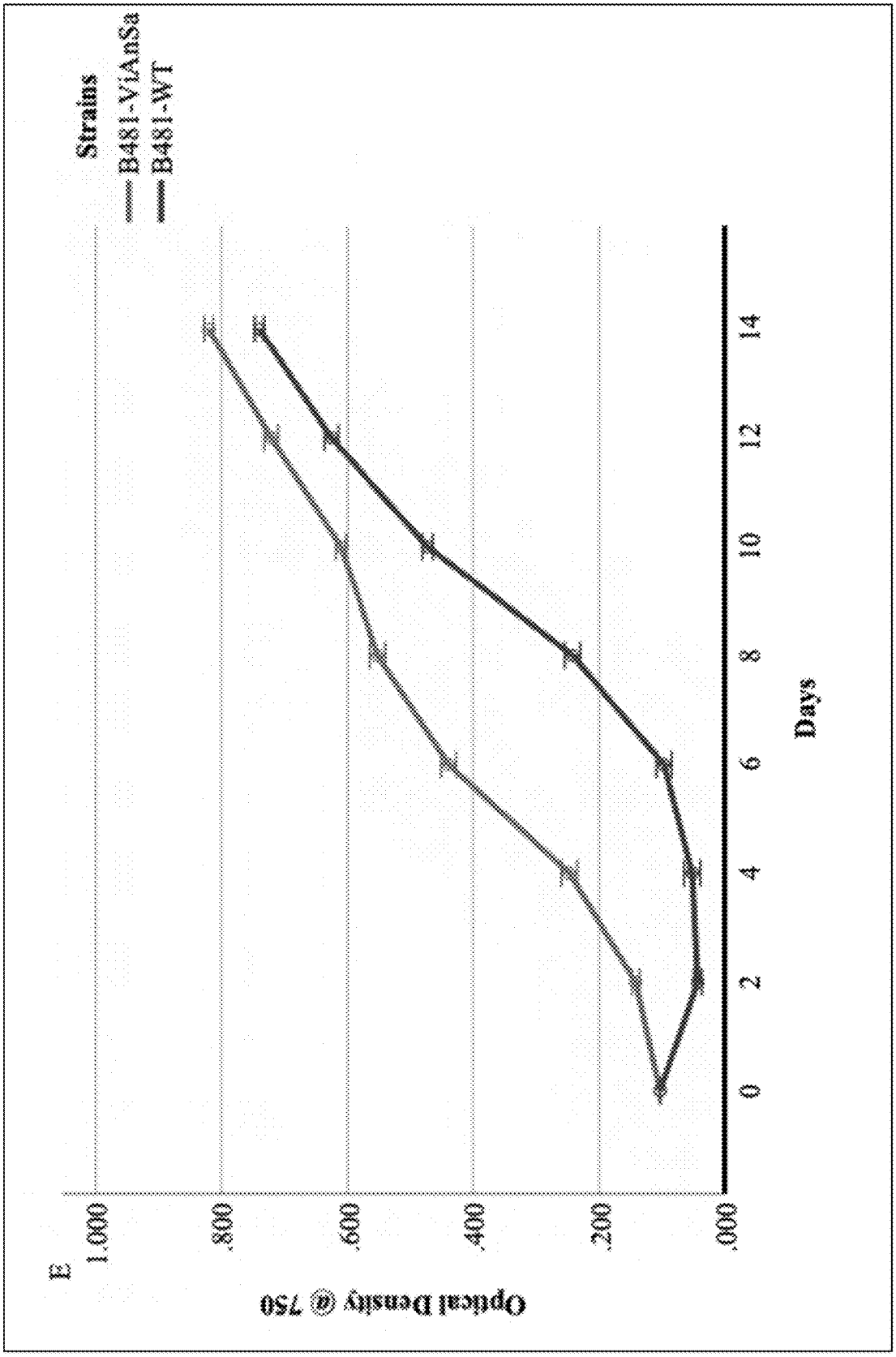

Strains
B481-ViAnSa
B481-WT
Optical Density @ 750
E
1.000
.800
.600
.400
.200
.000
0
2
4
6
8
10
12
14
Days

FIGURE 3E

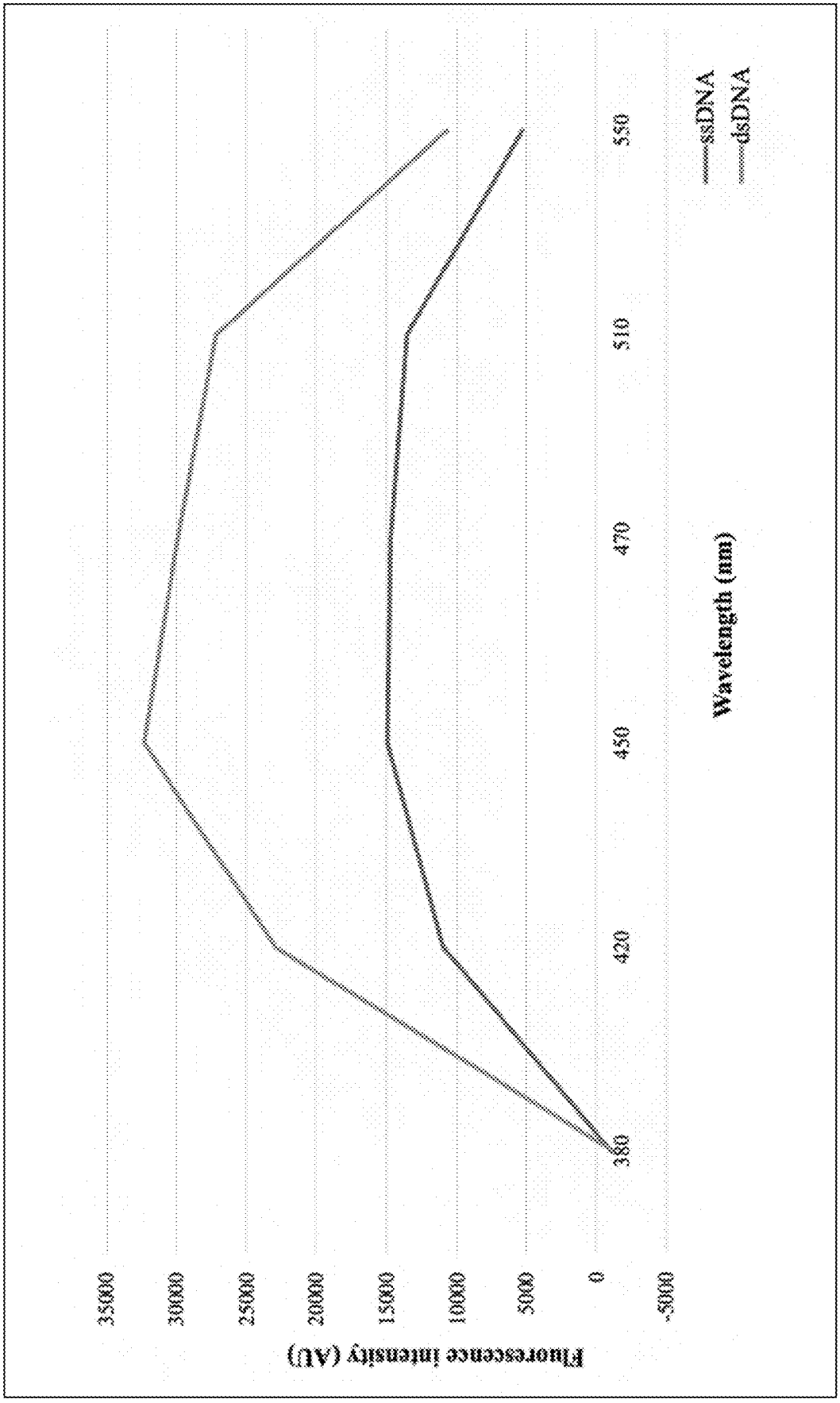

Fluorescence intensity (AU)
35000
30000
25000
20000
15000
10000
5000
0
-5000
380
420
450
470
510
550
Wavelength (nm)
ssDNA
dsDNA

FIGURE 4

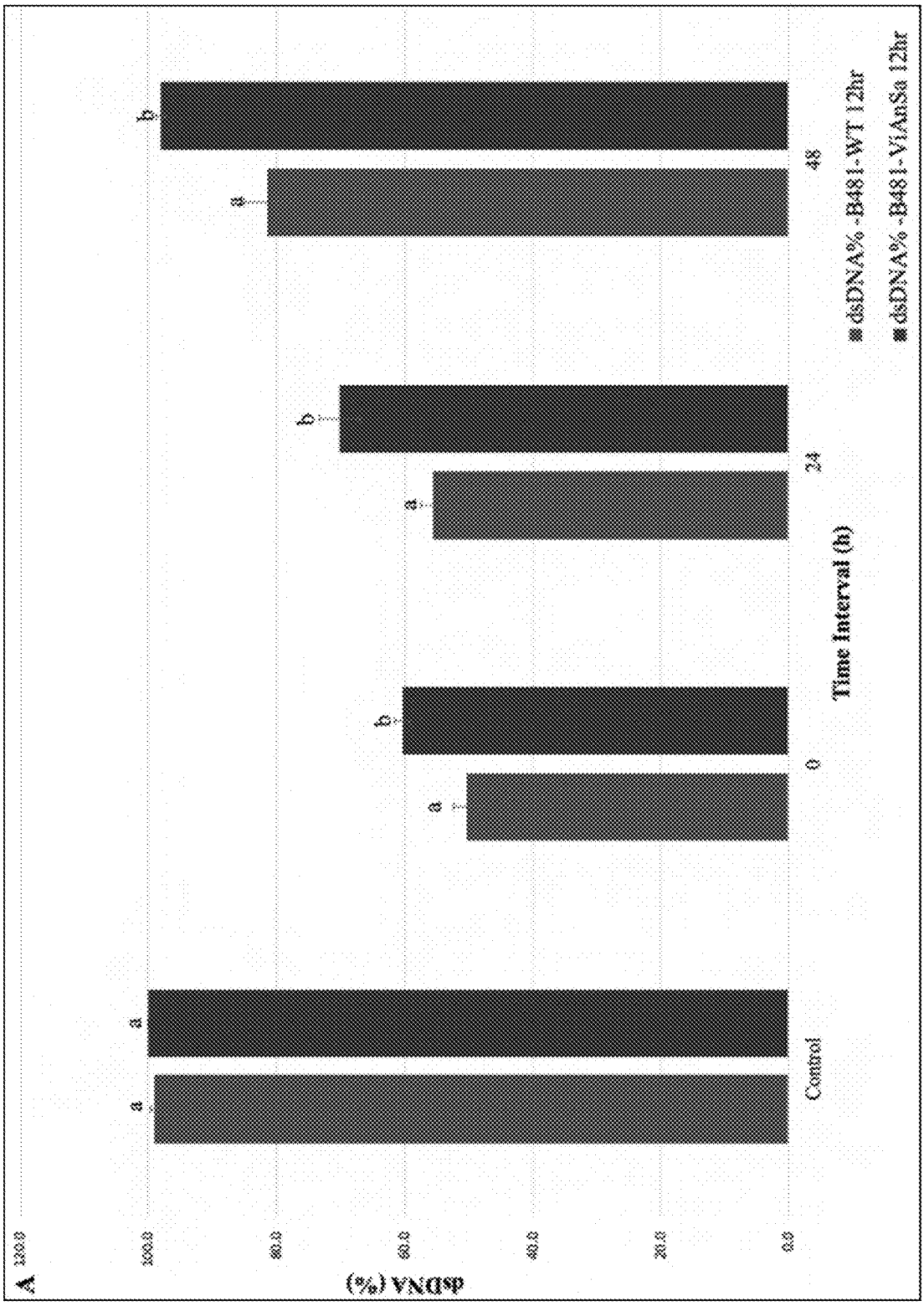

A
dsDNA (%)
100.0
80.0
60.0
40.0
20.0
0.0
Control
0
24
48
Time Interval (h)
dsDNA% -B481-WT 12hr
dsDNA% -B481-ViAnSa 12hr
a
b

FIGURE 5A

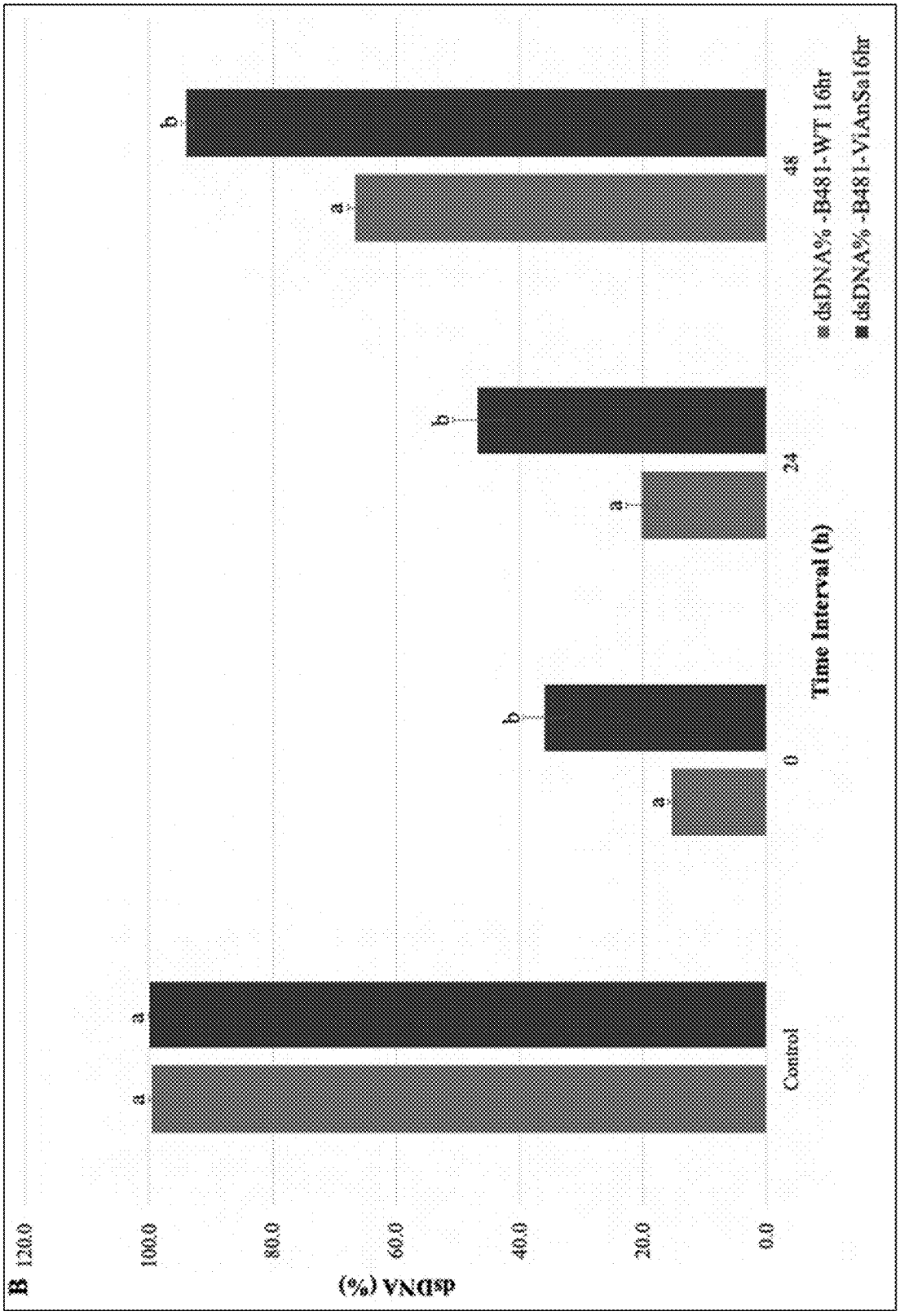

B
dsDNA (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Control
0
24
48
Time Interval (h)
dsDNA% -B481-WT 16hr
dsDNA% -B481-ViAnSa16hr
a
b

FIGURE 5B

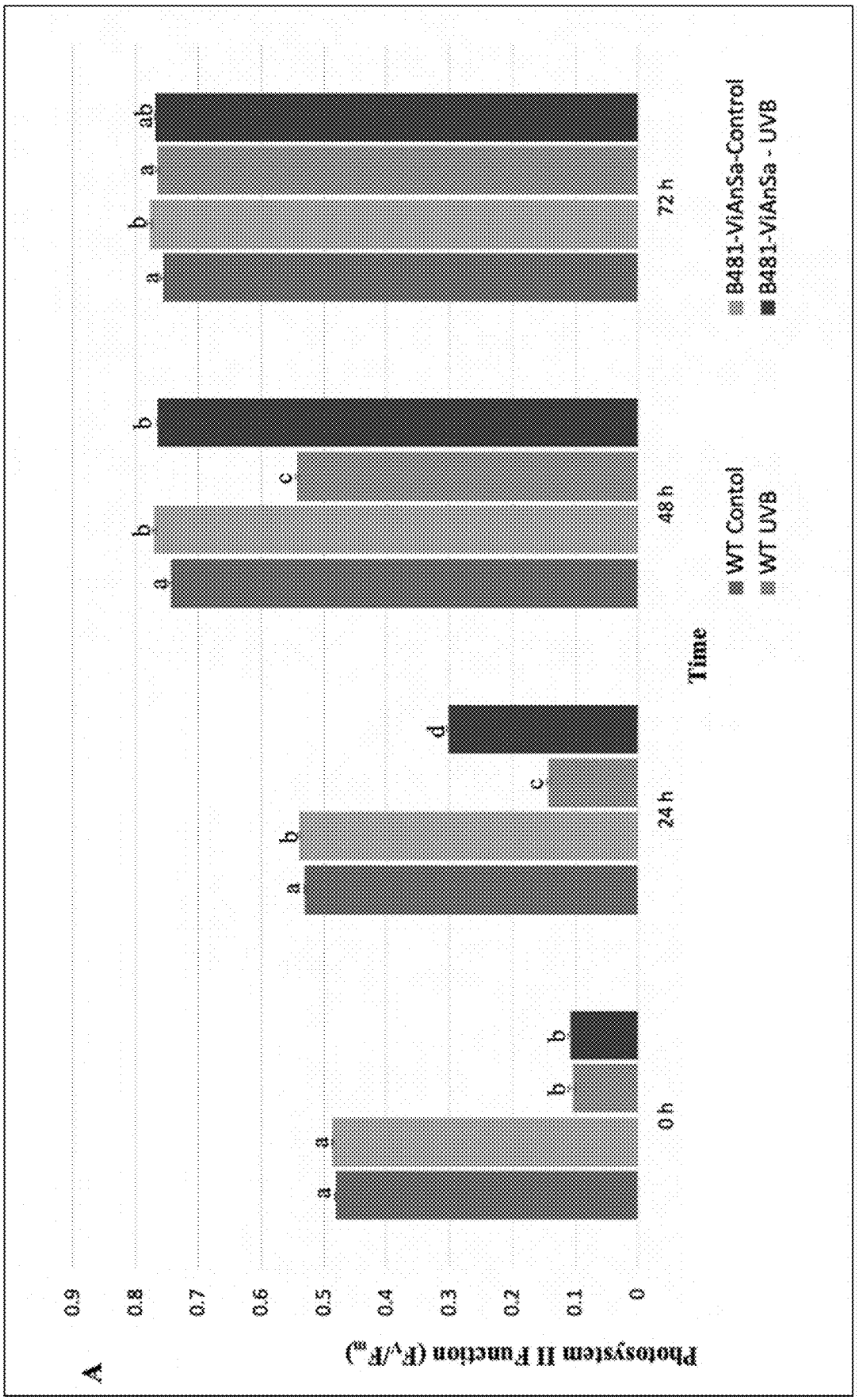

A
Photosystem II Function (F_v/F_m)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a a
b b
a b c d
a b c b
a b a ab
0 h
24 h
48 h
72 h
Time
WT Contol
WT UVB
B481-ViAnSa-Control
B481-ViAnSa - UVB

FIGURE 6A

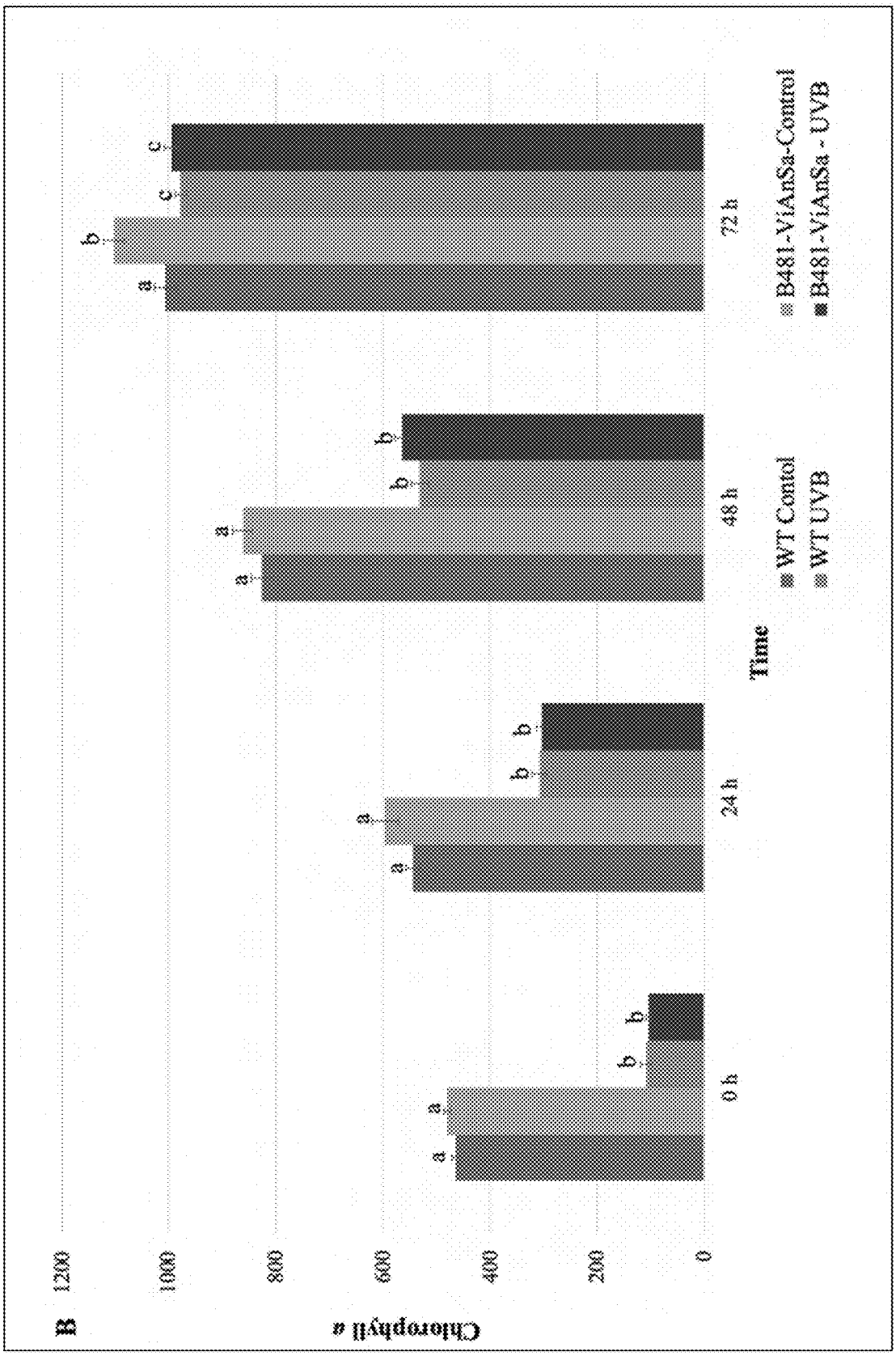

B
Chlorophyll a
1200
1000
800
600
400
200
0
0 h
24 h
48 h
72 h
Time
WT Control
WT UVB
B481-ViAnSa-Control
B481-ViAnSa - UVB

FIGURE 6B

ENGINEERED CYANOBACTERIA WITH ENHANCED UV TOLERANCE

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to bioengineered cyanobacteria with enhanced properties.

Description of the Background

[0002] The negative impact of fossil fuels on the environment and human health has sparked enormous interest in the development of biofuels as a renewable energy source. While cyanobacteria are an ideal third-generation feedstock for a variety of fuels including biodiesel, ethanol, and biogas, these photosynthetic organisms face an immense threat due to the global climatic changes. In recent years, a decrease in stratospheric ozone layer due to excessive release of air pollutants such as chlorofluorocarbons, organobromides, and reactive nitrogen species has resulted in increased solar UV-B (280-320 nm) reaching the Earth's surface. Several physiological and biochemical processes such as motility, photo-orientation, and CO_2 uptake in cyanobacteria are impaired by UV radiation. In addition, it is known to adversely impact biomolecules in these organisms, with nucleic acids being the primary targets.

[0003] Studies by Rastogi et al. and Castenholz & Garcia-Pichel have reported that cyanobacterial genomic function and fidelity are adversely affected by UV-B, as the DNA molecules directly absorb UV-B radiation inducing DNA strand breaks. A variety of mutagenic and cytotoxic DNA lesions including cyclobutane-pyrimidine dimers (CPDs), 6-4 photoproducts (6-4PPs), and their Dewar valence isomers are induced, disrupting genomic integrity. In addition, cyanobacterial UV-B induced-DNA degradation due to thymine dimerization has been confirmed in *Anabaena, Nostoc,* and *Scytonema* sp. Additionally, UV-B-induced DNA lesions (CPDs and 6-4PPs) can also cause primary and secondary breaks, since they are associated with transcription and replication blockages, leading to the collapse of replication forks in CPD-containing DNA.

[0004] To combat the negative effects of radiation stress, cyanobacteria employ a variety of direct and indirect defense strategies that enable tolerance to fluctuating UV levels. The first line of defense employed by most cyanobacterial species is the avoidance by migration through self-shading or mat formation. Cyanobacteria such as *Anabaena* sp. *Nostoc-commune* and *Scytonema* sp. have the capacity to produce UV-absorbing compounds mycosporine-like amino acids and scytonemin as a response to UV radiation. Although cyanobacteria use these defense mechanisms to combat UV stress, these repair systems can be rapidly overwhelmed by sustained UV radiation. However, some species employ photoreactivation, a process in which photolyase is activated by the blue wavelength of solar light, to reverse and modify nitrogenous bases to their normal state followed by thymine dimer formation caused by UV radiation.

[0005] *Fremyella diplosiphon* is a well-studied cyanobacterial species that has great potential as a third-generation biofuel agent due to its fatty acid methyl esters. In addition to growth in varying light intensities by modifications of its light-harvesting complexes, the organism is extremely amenable to genetic transformation. Efforts to enhance value-added traits such as halotolerance and cellular lipid content in this species have enabled unique environmental applications. A report by Vass et al. has indicated that phr A gene plays a role in DNA repair mechanism of *Synechocystis* sp. PCC 6803, and mutant cells lacking the gene were highly susceptible to UV-B damage.

SUMMARY OF THE INVENTION

[0006] The development of a UV-B tolerant strain would be invaluable to maximize its potential for biofuel production in scale-up systems. An object of the present invention was to overexpress the photoreactivation (phr A) gene in *F. diplosiphon* B481-WT to enhance UV-B tolerance. Successful transformation was confirmed using RT-qPCR and fluorometric analysis of DNA unwinding assays, and photosynthetic efficiency evaluated as a measure of photosystem II functionality, which is known to be adversely impacted by UV-B. Additionally, fatty acid methyl ester (FAME) profile of the transformant compared favorably to the wildtype, confirming its usefulness as a biofuel.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The foregoing summary, as well as the following detailed description of the preferred invention, will be better understood when read in conjunction with the appended figures. In the figures:

[0008] FIG. **1** shows the basic local alignment search tool analysis of *F. diplosiphon* B481-WT photolyase gene (SEQ. ID.NO. 3) on the National Center for Biotechnology Information showing 97.82% similarity to the photolyase gene in Nostoc sp (SEQ.ID.NO. 4).

[0009] FIG. **2**A shows an electrophoresis gel of polymerase chain reaction of amplification product of double digested photoreactivation gene from extracted vector in lane 2 and pGEM-7Zf vector in lane 3.

[0010] FIG. **2**B is a chart showing quantification of photolyase transcript levels in *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa). Error bars indicate ΔCt values at a 95% confidence interval across four replicates.

[0011] FIG. **3**A is a chart showing growth of *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains irradiated under UVB (3.0 W m^{-2}) for 0 min and grown in BG11/HEPES media. Different letters above final time point indicate significance among treatment means ($P<0.05$).

[0012] FIG. **3**B is a chart showing growth of *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains irradiated under UVB (3.0 W m^{-2}) for 20 min and grown in BG11/HEPES media. Different letters above final time point indicate significance among treatment means ($P<0.05$).

[0013] FIG. **3**C is a chart showing growth of *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains irradiated under UVB (3.0 W m^{-2}) for 40 min and grown in BG11/HEPES media. Different letters above final time point indicate significance among treatment means ($P<0.05$).

[0014] FIG. **3**D is a chart showing growth of *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains irradiated under UVB (3.0 W m^{-2}) for 80 min

and grown in BG11/HEPES media. Different letters above final time point indicate significance among treatment means ($P<0.05$).

[0015] FIG. **3**E is a chart showing growth of *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains irradiated under UVB (3.0 W m^{-2}) for 160 min and grown in BG11/HEPES media. Different letters above final time point indicate significance among treatment means ($P<0.05$).

[0016] FIG. **4** is a chart showing fluorescence excitation of *F. diplosiphon* DNA-bound Hoechst 33258. Emission data (emission peak 450 nm) were obtained using the maximum wavelength of the excitation peak at 343 nm. The dsDNA was not subjected to alkaline unwinding while ssDNA was subjected to complete alkaline unwinding.

[0017] FIG. **5**A is a chart showing percentages of double stranded DNA in *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains after exposure to UVB radiation (3.0 W m^{-2} (~8.0 μmol $m^{-2}s^{-1}$)) for 12 h. Different letters above standard error bars indicate significance between percentages ($p<0.05$).

[0018] FIG. **5**B is a chart showing percentages of double stranded DNA in *F. diplosiphon* wild type (B481-WT) and transformant (B481-ViAnSa) strains after exposure to UVB radiation (3.0 W m^{-2} (~8.0 μmol $m^{-2}s^{-1}$)) for 16 h. Different letters above standard error bars indicate significance between percentages ($p<0.05$).

[0019] FIG. **6**A is a chart showing evaluation of photosystem II activity in *F. diplosiphon* B481-WT and B481-ViAnSa strains after 12 h UVB radiation. Different letters above error bars indicate significance differences ($p<0.05$).

[0020] FIG. **6**B is a chart showing evaluation of chlorophyll a content in *F. diplosiphon* B481-WT and B481-ViAnSa strains after 12 h UVB radiation. Different letters above error bars indicate significance differences ($p<0.05$).

DETAILED DESCRIPTION OF THE INVENTION

[0021] To enhance UV stress tolerance in this species, we overexpressed the photoreactivation gene (phrA) that encodes for photolyase DNA repair enzyme in the wild type *F. diplosiphon* (B481-WT) by genetic transformation. Our efforts resulted in a transformant (B481-ViAnSa) with a 3,808-fold increase in the phrA mRNA transcript level, with enhanced growth under UVB stress. Additionally, DNA strand breaks in the transformant were significantly lower after 12 and 16 h of UV radiation, with significantly higher dsDNA recovery in B481-ViAnSa (98.1%) compared to B481-WT (81.5%) at 48 h post irradiation. Photosystem II recovery time in the transformant was significantly reduced (48 h) compared to the wildtype (72 h). Evaluation of high-value fatty acid methyl esters (FAMEs) revealed methyl palmitate, the methyl ester of hexadecenoic acid (C16:0), to be the most dominant component, accounting for 53.43% of the identified FAMEs in the transformant. The present invention thus presents an increased UV tolerant recombinant *Fremyella diplosiphon* and a method for enhancing UV tolerance in cyanobacteria, thus paving the way to large-scale open or closed pond cultivation for commercial biofuel production.

MATERIALS AND METHODS

Strains and Culture Conditions

[0022] *F. diplosiphon* strain (B481-WT) obtained from the UTEX algal repository (Austin, USA) was grown in liquid BG-11 medium containing 20 mM HEPES (hereafter termed as BG-11/HEPES) to an exponential growth phase (optical density at 750 nm of ~0.6). Cultures were grown under continuous shaking at 170 rpm and 28° C. in an Innova 44R incubator shaker (Eppendorf, Germany). The photosynthetic light in the shaker had peak wavelengths at 437 nm and 600-650 nm with an intensity adjusted to 30 μmol $m^{-2}s^{-1}$ using the model LI-190SA quantum sensor (Li-Cor, USA).

RNA Isolation and Complementary DNA Synthesis

[0023] Total RNA was extracted from *F. diplosiphon* grown to an exponential phase (7 days) using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol with modifications. The concentration and purity of the extracted RNA was tested on an agarose gel and A260/280 absorbance ratio measured using a Nanodrop 2000 (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using the high-capacity RNA to cDNA kit (Life Technologies, USA). A 20 μ1 reaction mixture containing 1000 ng of RNA, 2×reverse transcription buffer (RT), and 10×RT random primers was incubated at 37° C. for 60 min followed by 95° C. for 5 min. Synthesized cDNA was aliquoted and stored at −20° C.

Identification and cloning of the photoreactivation gene

[0024] To identify homologs of the phr A gene in B481-WT, the forward (5'AAGCTT-TATGTGGCACACGACTGTACC3') (SEQ ID NO. 1) and reverse (5'GGATCCGGTTATTTGACCAATTGATAAC3') (SEQ ID NO. 2) primers with EcoR1 and BamHI restriction sites were designed. Complementary DNA synthesized as described above was used as template for phr A gene amplification. PCR conditions were set in a C1000 Touch thermocycler (Bio-Rad, USA) as follows: 95° C. for 2 min; 40 cycles at 95° C. for 30 s and an annealing temperature of 51.4° C. for 30 s, followed by a final elongation step at 72° C. for 45 s. Amplified products were electrophoresed on a 1.5% agarose gel, bands excised at the expected size ranges, and DNA extracted using the gel recovery mini pre-kit (Zymo Research, USA).

[0025] The amplified gene products and pGEM-7Zf vector containing T7 promoter were double digested with EcoR1 and BamHI restriction enzymes (Promega, USA), and purified using Zymo DNA clean and concentrator kit. Inserts were ligated into the vector at the digested restriction sites with T4 DNA ligase (New England BioLabs, USA) and pGEM-7Zf-phr A expression plasmid constructed to overexpress the photolyase gene. The ligated plasmids were transformed into *E. coli* FB5α competent cells via heat shock at 42° C. for 20 s followed by incubation on ice for 5 min. The transformed cells were plated on Luria Bertoni (LB) agar plates containing 80 mg L^{-1} ampicillin and incubated for 16 h at 37° C. Twenty resistant single colonies were randomly selected, transferred to liquid LB medium containing 80 mg L^{-1} ampicillin and grown at 37° C. for 16

h. Plasmids were extracted using the Zyppy plasmid miniprep kit (Zymo Research, USA) and the insert confirmed by PCR and Sanger sequencing.

Electroporation-Mediated Transformation of the phr A Gene in *F. diplosiphon*

[0026] Expression plasmid containing the phr A gene was transformed into *F. diplosiphon* B481-WT according to parameters described by Tabatabai et al. Competent cells (40 μL) were mixed with ligated purified plasmid DNA and electroporated using a GenePulser Xcell with CE module (Bio-Rad, USA) at 200 Ω resistance, 1.0 kV, and 25 μF capacitance. After incubation on ice for 20 min, the transformant was grown in BG11/HEPES liquid medium for 16 h and plated on LB agar containing 80 mg L^{-1} ampicillin. To verify insertion of the phr A gene, PCR was performed using gene specific primers as mentioned above in para. [0021] and products visualized on a 1.5% agarose gel.

Quantitation of Gene Overexpression in the Transformant by Reverse Transcription-Quantitative PCR (RT-qPCR)

[0027] Total RNA from the wildtype and the transformant were extracted, cDNA was synthesized as mentioned above in para. [0019], and RT-qPCR performed to quantify gene overexpression. Gene-specific primers for the phr A gene were designed and real-time amplifications performed using SYBR green master mix (Applied Biosystems, USA) in a Thermal Cycler CFX96 Real-Time machine (Bio-Rad, USA). Amplifications were performed under the following conditions: 95° C. for 20 s; and 40 cycles at 50.9° C. for 30 s. Four replicates were maintained for each treatment type and the experiment repeated. Relative quantification (RQ) data of the transformant was analyzed using the ΔCt method with CFX Manager 3.1 (Bio-Rad, USA) with the B481-WT with pGEM-7Zf vector as control. The 16S rRNA was used as the internal control and fold-change values calculated.

Detection of DNA Breakages using Fluorometric Analysis of DNA Unwinding Assay

[0028] Fluorometric analysis of DNA unwinding (FADU) assay was performed to determine DNA breakages as described previously by Rastogi et al. *F. diplosiphon* wildtype and transformant strains were grown to logarithmic phase under culture conditions described above at para. [0017]. Cultures were diluted to an OD_{750nm} of 0.3 and 30 mL culture exposed to 12 and 16 h of UV-B radiation in an open petri dish. Three samples were tested in this assay: UVt-sample (exposed to UV), ds-sample (double stranded sample; not UV treated or subjected to alkaline condition), and ss-sample (subjected to alkaline condition to fully unwind the DNA).

[0029] After centrifuging 1.0 mL of each sample at 3000×g for 10 min, the pellets were washed with 1 mL of TE buffer (Tris-HCl 10 mM, EDTA 1 mM), followed by 20 μL 0.5 M EDTA and 164 μL TE buffer. The cell suspension was centrifuged at 3000×g for 10 min, and 16 μL of lysozyme (50 mg/mL) added to the pellet and incubated at 37° C. for 90 min to lyse the cell walls. To the suspension, 15 μL of 10% SDS, 10 μL of 4M NaCl, 15 μL of proteinase K (6 mg/mL), and 60 μL of TE buffer was added and incubated at 60° C. for 30 min for complete cell lysis. Finally, 300 μL of 0.1 M NaOH was added to each sample and subjected to different unwinding protocols as described below.

[0030] ss-Sample. The cell extract was sonicated for 2 min, incubated at 20° C. for 30 min, neutralized by adding 300 μL of 0.1 M HCl, and sonicated for 15 s to fully unwind dsDNA and the lowest level of background fluorescence estimated.

[0031] ds-Sample. To estimate total fluorescence, cell extract was neutralized by adding of 300 μL of 0.1 M HCl, incubated at 20° C. for 30 min, and sonicated for 15 s to prevent the unwinding of dsDNA.

[0032] UVt-Sample. Cell extract was incubated at 20° C. for 30 min under alkaline condition, neutralized by adding 300 μL of 0.1 M HCl, and sonicated for 15 s to diminish the single as well as double-stranded DNA regions. This sample set was used to estimate the UV-induced DNA breaks.

[0033] After processing each sample as mentioned above, 20 μL of 20 mM Hoechst 33258 (bisbenzimide) DNA probe in 0.6 M phosphate buffer (pH 7.6) was added and centrifuged at 10,000×g for 5 min. Fluorescence intensity of 200 μL it supernatant was measured using a microplate reader (Agilent BioTek Synergy H1 Hybrid, USA) at 343 nm with emission between 380 and 550 nm. The percentage fraction (% F) of dsDNA was calculated using the formula, F=(UVt−ss)/(ds−ss)×100 where ss, ds, and UVt corresponded to fluorescence intensities of ss, ds, and UVt samples respectively.

Physiological Evaluation of the Transformant Exposed to UV-B

Evaluation of Growth and Stability

[0034] The wildtype and transformant strains were grown in liquid BG11/HEPES media to logarithmic phase under culture conditions described above in para [0017]. Cultures were adjusted to an OD_{750} of 0.1 with BG11/HEPES media and irradiated under UV-B (3.0 W m^{-2}) for 0 to 160 min in a UV crosslinker (Fisher Scientific, USA). Three biological replicates were maintained, and cells not irradiated with UV-B served as control. Growth of the strains at OD_{750} nm was measured for a period of 14 days. Stability of the transformant was tested on BG11/HEPES plates containing 80 mg L^{-1} ampicillin for a ten day-period under culture conditions described above and exposed to UV-B for 30 min per day. Stability and presence of the gene was confirmed by RT-qPCR after 18 generations of subculture.

Evaluation of Photosynthetic Pigment Levels

[0035] Photosynthetic efficacy of the wildtype and transformant was quantified as a measure of PSII activity and chlorophyll a content, which provides an estimate of the well-being of photosynthetic cells. To allow maximal irradiation and avoid cell shadowing, cultures grown to an OD_{750nm} of 0.3 were placed in open petri dishes and irradiated in a UV-B crosslinker (Fisher Scientific, USA) for 60 min. Cultures were grown under conditions mentioned in para. [0017] for three days to allow cell recovery and PSII functionality measured after 24, 48, and 72 h using a MINI-PAM (Walz, Effeltrich, Germany) to measure minimal and maximal fluorescence yield (Fo and Fm). Based on these parameters PSII quantum yield (Fv/Fo) was calculated using the equation Fv/Fm=(Fm−Fo)/Fm. In addition, chlorophyll a (chlα) was measured at the excitation of 420 nm

and emission of 680 nm using a microplate reader (Agilent BioTek Synergy H1 Hybrid, USA) and photosynthetic efficacy compared.

Characterization of Lipids in the Wildtype and Transformant *F. diplosiphon* Grown under Simulated UV-B Conditions

[0036] Lipid profile of the wildtype and transformant exposed to simulated UV-B conditions (Omaykey UV-B lamps) were compared using GC-MS. Cultures adjusted to 0.1 at OD_{750} were grown in 5×7×6 containers and exposed to UV-B for 4 h each day for 15 days to simulate the sun's UV-B radiation effects. Three replicated treatments were maintained and OD_{750} measured every three days for a period of 12 days and growth rate calculated. Simultaneous lipid extraction and transesterification was performed as described previously by Tabatabai et al. and fatty acid composition analyzed at the Mass Spectrometry Facility at Johns Hopkins University (Baltimore, MD) using the Shimadzu GC17A/QP5050A GC-MS systems (Shimadzu Instruments, USA). Identification of FAMEs was accomplished by comparing each GC/MS mass spectrum to the Lipid Web archived FAME spectra.

Statistical Analysis

[0037] Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's honest significant differences post-hoc test at 95% confidence intervals ($p<0.05$). The single factor, fixed-effect ANOVA model, $Yij=\mu+\alpha Gi+\varepsilon ij$, was used where Y is the variable being measured in strain I and biological replicate j. The μ represents mean growth with adjustments from the effects of strain (αG), and εij is the experimental error from strain I and biological replicate j.

RESULTS AND DISCUSSION

Identification, Cloning, and Expression of Photolyase phr A Gene in *F. diplosiphon*

[0038] *F. diplosiphon* UV-B tolerance was enhanced by taking advantage of the gene expression system of a plasmid vector containing the photolyase gene. Gel electrophoresis of the double digested vector construct revealed bands at the expected sizes of ~1,500 bp and ~3,000 bp for phr A gene and pGEM-7Zf plasmid respectively (FIG. **2**A). The high similarity of 97.82% to the phr A gene from B481-WT (SEQ.ID.NO. 3) indicated homology to the photolyase gene in Nostoc sp (SEQ.ID.NO. 4). (FIG. **1**). Quantification of the phr A gene transcript levels in the transformant revealed a 3,808-fold increase ($p<0.05$) compared to the wildtype strain (FIG. **2**B). The phr A-overexpressing *F. diplosiphon* strain was designated as B481-ViAnSa and the sequences deposited at NCBI Genbank with the accession number MW357071.

[0039] Given that cyanobacteria use solar energy for essential energy-dependent processes, harmful UV-B radiation affects several physiological and biochemical processes such as photosynthesis, growth, survival, cell differentiation, genome integrity and total lipid profiles. Therefore, we evaluated the efficacy of the transformant under UV-B conditions at an intensity of 3.0 W m^{-2} at the surface of the cell culture. Our results revealed significantly high UV-B tolerance in the transformant radiated for 20 to 160 min. While a significant reduction ($p<0.05$) in growth of B481-WT was observed even at 20 min of UV-B exposure, the transformant (B481-ViAnSa) showed no significant reduction of growth at exposure time of 40-160 min (Table 1).

TABLE 1

Pairwise comparison of mean growth differences between the wildtype (B481-WT) and (B481-ViAnSa) *Fremyella diplosiphon* strains.

			Pairwise Comparisons Mean Difference				
						95% Confidence Interval for Difference	
Variable	(i) Strains	(j) Strains	Mean Difference	Std. Error	Significance	Lower Bound	Upper Bound
Control	B481-phrA	B481-WT	0.05	0.002	<.001	0.046	0.054
	B481-WT	B481-phrA	−.050*	0.002	<.001	−0.054	−0.046
20 min	B481-phrA	B481-WT	0.168	0.002	<.001	0.164	0.172
	B481-WT	B481-phrA	−0.168	0.002	<.001	−0.172	−0.164
40 min	B481-phrA	B481-WT	0.162	0.002	<.001	0.159	0.166
	B481-WT	B481-phrA	−0.162	0.002	<.001	−0.166	−0.159
80 min	B481-phrA	B481-WT	.209*	0.002	<.001	0.205	0.213
	B481-WT	B481-phrA	−.209*	0.002	<.001	−0.213	−0.205
160 min	B481-phrA	B481-WT	.157*	0.002	<.001	0.154	0.161
	B481-WT	B481-phrA	−.157*	0.002	<.001	−0.161	−0.154

Based on estimated marginal means

*The mean difference is significant at the .05 level.

[0040] Interestingly, we also observed a significantly rapid growth recovery of the B481-ViAnSa strain compared to B481-WT (FIG. **3**). Furthermore, irradiation of B481-ViAnSa for 20 min significantly ($p<0.05$) increased growth rate over a 14-day period compared to the non-irradiated transformant, indicating exceptional growth performance under UV stress.

Comparison of DNA Strand Breakages in the Transformant and Wildtype *Fremyella diplosiphon* Strains

[0041] FADU assay, an accurate and powerful method for quantitative analysis of DNA damage, was used to measure DNA strand breaks in the transformant engineered with the

phr A (B481-ViAnSa). Quantification of dsDNA damage detected by fluorescence analysis of the fluorochrome bound DNA revealed maximal fluorescence at 450 nm while it was lower in ssDNA (FIG. **4**). The difference between upper and lower fluorescence limit of ds-and ss-samples provided a more reliable analysis of DNA strand breaks in UVt samples since the amount of DNA damage in treated cells is expressed by the difference in fluorescence intensities. Using this assay, we detected significantly higher ($p<0.05$) DNA damage in both B481-WT and B481-ViAnSa strains exposed to UV-B at 12 and 16 h (FIG. **5**) compared to the untreated control (sample-ds). While our results indicated a significant reduction ($p<0.05$) in the dsDNA of both strains exposed to UV-B, the transformant exhibited significantly less dsDNA breakages compared to wildtype. In addition, significantly high ($p<0.05$) dsDNA of 60.3, 70.2 and 98.1% were observed in B481-ViAnSa compared to B481-WT (50.4, 55.6, and 81.5%) at 0, 24, 48 h post-UV-B irradiation. Interestingly, we noted significantly higher ($p<0.05$) dsDNA recovery in B481-ViAnSa (98.1%) compared to B481-WT (81.5%) after 48 h (FIG. **5**B).

[0042] These results show that higher photolyase activity results in more efficient DNA repair in the transformant. In addition, this strain exhibited a significantly higher ($p<0.05$) percentage of dsDNA at 16 h of UV-B radiation. Comparison of gene transcription in the transformant and DNA damage showed an inverse correlation. While the phr A gene overexpression in the transformant was significantly high ($p<0.05$) compared to the wildtype, DNA damage as indicated by FADU assay was low. These results indicate that the overexpression of the photolyase gene reduced thymine dimers caused by UV-B.

Evaluation of Photosystem II Activity and Chlorophyll a Content in the Transformant, B481-ViAnSa

[0043] The ratio of variable and maximum fluorescence (Fv/Fm) of the dark-adapted chlorophyll α fluorescence parameter was used to measure photochemical efficiency of photosystem II reaction centers. Comparison of photosystem II activity and chlorophyll a content between the wildtype and transformant strains did not reveal significant differences (FIG. **6**). However, we noticed a significant difference ($p<0.05$) in the photosystem II (PSII) recovery rate of the UV-treated transformant compared to the wildtype. While the transformant PSII recovered in 48 h following UV-B radiation at an intensity of 3.0 W m^{-2} for 1 h, the wildtype strain took 72 h, indicating enhanced photolyase gene activity in the transformant contributing to UV stress tolerance.

Fatty Acid Methyl Ester Composition in UV-B Irradiated and Non-Irradiated *Fremyella diplosiphon* Strains, B481-ViAnSa and B481-WT

[0044] *F. diplosiphon* possesses valuable biodiesel qualities, which can maximize biofuel production. Hence, we compared the high-value saturated and unsaturated FAMEs in the transformant to the wildtype strain. Methyl palmitate, the methyl ester of hexadecenoic acid (C16:0), was found to be the most dominant FAME component, accounting for 53.43% and 51.69% in B481-ViAnSa and B481-WT, respectively. Methyl octadecenoate (C18:0), the second abundant FAME, accounted for 30.12% and 33.02% in B481-ViAnSa and B481-WT respectively. This was followed by methyl octadecenoate (C18:1), which accounted for 22% in B481-ViAnSa and 23.02% in B481-WT. Additionally, we detected methyl tetradecanoate (C14:1), methyl hexadecanoate (C16:1), and methyl octadecadienoate (C18:2) in both strains (Table 2).

TABLE 2

Quantitative composition of fatty acid methyl esters in transesterified lipids of non-irradiated and UVB radiated *F. diplosiphon* wild type (WT) and transformant (B481-ViAnSa) strains.

	Nonirradiated		UVB Irradiated	
FAMEs Type	B481-ViAnSa	B481-WT	B481-ViAnSa	B481-WT
Methyl palmitate (C16:0)	53.43%	51.69%	42.47%	41.74%
Methyl octadecanoate (C18:0)	30.12%	33.02%	23.33%	24.18%
Methyl octadecanoate (C18:1)	22%	23.02%	15.99%	17.05%
methyl tetradecanoate (C14:1)	5.19%	5.07%	1.18%	2.07%
Methyl hexadecanoate (C16:1)	4.76%	4.59%	0.87%	0.81%
Methyl octadecadienoate (C18:2)	3.01%	3.13%	0.91%	0.78%

[0045] Interestingly, UV-B radiation significantly reduced ($p<0.05$) the percentage of all FAME components, including methyl palmitate, which was reduced by 20.51% and 19.25% in B481-ViAnSa and B481-WT respectively (Table 2). Due to the exposure of cultures to simulated UV-B radiation for 4 continuous hours, a reduction of FAMEs is expected. However, we observed significantly higher ($p<0.05$) amounts of saturated FAMEs in both strains irradiated with UV-B when compared to the untreated control.

[0046] In summary, our results indicate that overexpression of the phr A gene enhanced *F. diplosiphon* UV stress tolerance, with enhanced PS II reversal rate and no negative impact on lipids. Considering future projections of increased UV-B radiation reaching the earth's surface due to environmental pollution and depletion of the ozone layer, this invention paves the way for cultivating *F. diplosiphon* in large-scale outdoor systems.

[0047] It will be appreciated by those skilled in the art that changes could be made to the preferred embodiments described above without departing from the inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and scope of the present invention as outlined in the present disclosure and defined according to the broadest reasonable reading of the claims that follow, read in light of the present specification.

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```

1. A recombinant cyanobacterium comprising at least one polynucleotide encoding a phr A gene.

2. The recombinant cyanobacterium according to claim **1**, wherein the recombinant cyanobacterium is *Fremyella diplosiphon.*

3. The recombinant cyanobacterium according to claim **1**, wherein the recombinant cyanobacterium has a higher UV tolerance as compared to wild-type cyanobacterium.

4. The recombinant cyanobacterium according to claim **1**, wherein the at least one polynucleotide is present in one or more plasmids.

5. The recombinant cyanobacterium according to claim **1**, wherein the recombinant cyanobacterium is more resistant to UV-induced DNA damage as compared to wild-type cyanobacterium.

6. The recombinant cyanobacterium according to claim **1**, wherein the recombinant cyanobacterium shows a higher dsDNA recovery following DNA damage as compared to wild-type cyanobacterium.

7. A method for producing biofuel comprising: growing a recombinant cyanobacterium comprising at least one polynucleotide encoding a phr A gene under conditions suitable for production of a lipid for biofuel production; and isolating the lipid.

8. The method according to claim **7**, wherein the recombinant cyanobacterium is *Fremyella diplosiphon.*

9. The method according to claim **7**, wherein the recombinant cyanobacterium has a higher UV tolerance as compared to wild-type cyanobacterium.

10. The method according to claim **7**, wherein the recombinant cyanobacterium is more resistant to UV-induced DNA damage as compared to wild-type cyanobacterium.

11. The method according to claim **7**, wherein the recombinant cyanobacterium shows a higher dsDNA recovery following DNA damage as compared to wild-type cyanobacterium.

12. The method according to claim **7**, wherein at least one polynucleotide encoding a phr A gene is incorporated in one or more plasmids and integrated into genome of the cyanobacterium.

13. A method for producing a recombinant *Fremyella diplosiphon* having increased tolerance to UV exposure compared to wild type *Fremyella diplosiphon* comprising: introducing at least one polynucleotide encoding a phr A gene into *Fremyella diplosiphon* wherein the recombinant *Fremyella diplosiphon* has a higher UV tolerance as compared to wild-type *Fremyella diplosiphon.*

14. (canceled)

15. The method for producing a recombinant *Fremyella diplosiphon* according to claim **13**, further comprising a step of incorporating at least one polynucleotide encoding a phr A gene in one or more plasmids and transforming one or more plasmids into a genome of the *Fremyella. diplosiphon.*

16. (canceled)

17. (canceled)

18. A method for producing a recombinant *Fremyella diplosiphon* that is more resistant to UV-induced DNA damage as compared to wild type *Fremyella diplosiphon* comprising: introducing at least one polynucleotide encoding a phr A gene into *Fremyella diplosiphon.*

19. The method for producing a recombinant *Fremyella diplosiphon* according to claim **18**, further comprising a step of incorporating at least one polynucleotide encoding a phr A gene in one or more plasmids and transforming one or more plasmids into a genome of the *Fremyella. diplosiphon*

20. A plasmid comprising at least one nucleic acid encoding a phr A gene.

21. A recombinant strain of *Fremyella diplosiphon* comprising the characteristics of that deposited in MW357071, or a mutant thereof.

22. A composition comprising the recombinant strain of claim **1**.

23. laimsA composition comprising the recombinant strain of claim **21**.

* * * * *