Journal homepage www.jzs.univsul.edu.iq
Journal of Zankoy Sulaimani
Part-A- (Pure and Applied Sciences)

Construction of Local Isolates of Cyanobacteria for Ethanol Production

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Article info

Original: 13.08.2015
Revised:18.01.2016
Accepted:30.01.2016
Published online:
20.06.2016

Key Words:
cyanobacteria,
Synechococcus,
Ethanol, Sulaimani.

Abstract

Cyanobacteria can use solar energy and convert carbon dioxide into biofuel molecules in one single biological system. In this research, *Synechococcus* sp. was isolated from Saray Subhan Agha fresh water, a pure culture of *Synechococcus* was obtained by several subculturing on BG11 media. For the production of ethanol by *Synechococcus* sp. *pyruvate decarboxylase (PDC)* and *alcohol dehydrogenseII (ADH II)*, genes from *Zymomonas mobilis* ATCC (29191), were amplified by PCR and cloned into the pSyn_1/D-TOPO® Vector. The *Synechococcus* and *Synechococcus elongates* transformed with constructed vector (pSyn_1/D-TOPO®) that harboring the two ethanol fermenting genes. The transformation was performed using a double homologous recombination system to integrate the *PDC* and *ADHII* genes into the local isolates of *Synechococcus* sp. and *Synechococcus elongatus* chromosome under the control cyanobacterial weak constitutive nickel inducible promoter. The recombinant *Synechococcus* cells grow in different concentrations of $NiSO_4$ (1, 2.5, 5, 7.5, 10) µM in BG11 media, under different temperature (15, 30, 45) ˚C and different light intensity (10, 50, 150) µE. The enzymatic ethanol assay kit was used to determine ethanol concentration produced by both recombinant *Synechococcus* sp. and recombinant *Synechococcus elongatus*. Highest ethanol concentration obtained by those cultures containing five µl $NiSO_{4,}$ which incubated under continues light of 50µE at 30˚C (Optimum um condition for ethanol production by recombinant *Synechococcus* cells). The amount of ethanol produced by local isolates of *Synechococcus* sp. was 0.00103 g/l, whereas for *Synechococcus elongatus* was 0.0138 g/l. The amount of ethanol produced by those *Synechococcus* cultures containing different concentrations of $NiSO_4$ were incubated under continuous light of (10 and 150) µE and temperature of (15 and 45) ˚C was less than those cultures were incubated under light of 50 µE and temperature of 30˚C.

Introduction

Energy shows a significant role in our life. Fossil fuels meet most energy demand. Petroleum yields are the main transportation fuel, and coal is mostly used for producing electricity whereas natural gas is increasingly used for heating [1]. Due to the increasing global energy demand and limited fossil fuel reserves, one of the most intimidating challenges facing science in the 21st century is to deliver solutions for addressing global energy needs in the future in a sustainable manner. There is also an increasing awareness that the utilization of fossil raw materials and fuels increase the net discharge of carbon dioxide (CO_2) in the atmosphere and contribute to the "greenhouse effect". Thus, besides the energy problem, the other important challenge is to predict how Earth's ecosystems will respond to global climate change. CO_2 is measured to be the most prevalent greenhouse gas, and the buildup of CO_2 causes global climate change [2]. The growth of renewable energy sources has established significant interest in recent years owing to the depletion of fossil fuels, the ever-increasing demand for energy, and concerns over climate change. A promising source of renewables is the reprocessing of CO_2 into practical fuels and fine chemicals by photosynthetic organisms using solar energy. There are, however, growing concerns over the used methods for making biodiesel from crops and biomass. The problems comprise high production costs and a reduction of land available for rising edible crops. These subjects highlight the need for a new generation of biofuel technology [3]. Biofuels are expected to play a fundamental role in the development of a sustainable, economic, and environmentally safe source of energy. Microbes offer great potential for applications in technology-based biofuel production. As a candidate for biofuel-producing microbial systems, cyanobacteria are attractive because they incorporate the favorable characteristics of prokaryotic and plants [4]. Cyanobacteria possess certain properties that have entitled them to be one of the most promising feedstocks for bioenergy generation. They own greater photosynthetic levels and growth rates compared to other algae and higher plants. Also, Cyanobacteria grow easily with basic nutritional requirements. Another benefit is that cyanobacteria, being prokaryotes, can much more freely be genetically engineered to improve the production of biofuels as opposite to eukaryotic algae [5]. *Synechococcus* is a unicellular freshwater photoautotrophic cyanobacterium with rod-shaped cells [6]. They are gram-negative cells [7]. In last years, workers have attained partial achievement in expressing foreign genes in this cyanobacterium, as well as other transformable strains. For example, using the human carbonic anhydrase gene *ca*II to study CO_2-concentrating mechanisms, and also an investigation of *E. coli* and human superoxide dismutase genes to examine oxidative stress. *E. coli pet* genes used to increase salt stress resistance. *Bacillus thuringiensis* larvicidal genes used to progress bioinsecticidal hosts All the previous genes been expressed in *Synechococcus* sp. at appropriately high levels to generate distinct phenotypes [8]. Pyruvate decarboxylase is central to home ethanol fermentation and catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde with the issue of CO2. Acetaldehyde produced by this reaction reduced to ethanol by alcohol dehydrogenase. These two enzymes (PDC and ADH) are sufficient to convert intracellular pools of pyruvate and NADH to ethanol [9]. Many bacteria can yield some ethanol, the obligately fermentative bacterium *Zymomonas mobilisis* one of few prokaryotes that generate ethanol as the predominant fermentative product. In this bacterium, PDC and ADH are very abundant [8]. Deng and Coleman in 1999 [8] and Wang *et al*., in 2012 [10], described the photosynthetic creation of ethanol using genetically engineered cyanobacterium *Synechococcus* 7942. They expressed artificial operon of *PDC-adh* (genes originally from *Zymomonas mobilis*) under a P_{lac} and P_{rbc} promoters via a shuttle vector pCB4. The *pdc-adh* expression cassette incorporated into the chromosome of *Synechocystis* 6803 at the *psbA2* locus, driven by the light inducible strong P_{psbA2} promoter. Expression of *pdc/adh* resulted in ethanol assembly by the engineered *Synechocystis* under high light (~1000 $\mu E/m^2/s$) conditions [11].

Materials and Methods

Sample collection

Five water samples collected from Saray Subhan Agha spring *Synechococcus* sp. were isolated using solidified BG 11 medium.

DNA extraction

DNA extracted from *Zymomonas mobilis* ATCC 29191 by using Genomic DNA Mini Kit (Blood/Cultured Cell from Geneaid , Taiwan).

Amplification of PDC and ADH II genes

The primers (CyberGen AB, Sweden) (Table 1) for amplification of *PDC* and *ADHII* designed from the *zymomonas mobilis* ATCC 29191 complete genome (gene bank accession number NC_018145.1).The amplification was carried out by PCR thermocycler (Techne, USA) and GoTaq® Green Master Mix kit

(Promega, USA) according to manufacturer's instructions in a final volume 100µl reactions. The PCR thermocycler program started with 2 minutes denaturation at 94°C, and after 30 cycles the program was ended with 5 minutes elongation followed by a 4°C unlimited hold. PCR products imagined under UV light after resolution by gel electrophoresis. DNA fragments from primers, polymerases, unincorporated nucleotides, and salts were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan).

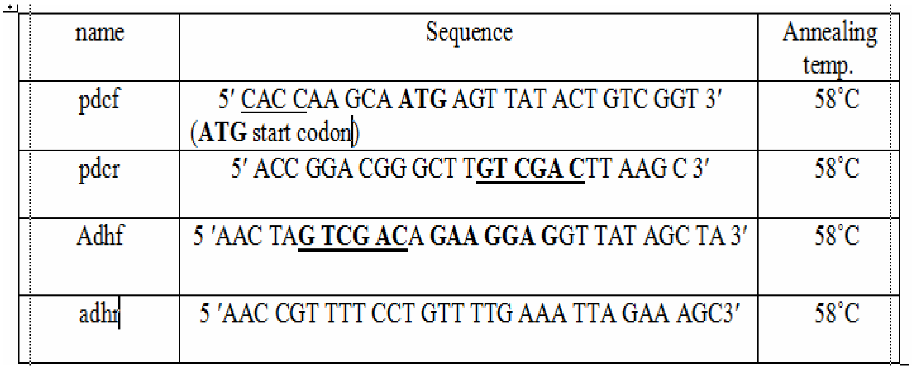
Cloning of PDC and ADH II genes

PDC and *ADHII* products were blunt-ended using a Quick Blunting Kit (NEB, USA). The Quick Blunting Kit was used to convert DNA with incompatible 5´or 3´ overhangs to 5´phosphorylated, blunt-ended DNA for efficient blunt-end ligation into DNA cloning vectors. TOPO ligation did cloning of PDC to pSyn_1/D-TOPO®Vector (pSyn_1/D-TOPO®pbh1 construction). The ligation was carried out according to manufacturer's instructions. Blunt ended pdc ligated into the vector in a salty medium (200mM NaCl, 10mM $MgCl_2$). TOPO® Cloning reaction performed in sterile 1.5 microcentrifuge tube. The ligation mixture was used for transformation.

Transformation

The Competent cells prepared using the calcium chloride ($CaC1_2$) method, and then Transformation was done according to Sambrook *et al.*, 1989 [12]. The competent cells were transformed with ligation mixture according to Sambrook *et al.*, 1989[12]. The plasmid prep was done according to manufacturer's instructions of High-Speed Plasmid Mini Kit (Geneaid, Taiwan).

Table 1: Primer sequences of PDC and ADHII genes



name	Sequence	Annealing temp.
pdcf	5′ CAC CAA GCA **ATG** AGT TAT ACT GTC GGT 3′ (**ATG** start codon)	58°C
pdcr	5′ ACC GGA CGG GCT T**GT CGA C**TT AAG C 3′	58°C
Adhf	5 ′AAC TA**G TCG AC**A **GAA GGA G**GT TAT AGC TA 3′	58°C
adhr	5 ′AAC CGT TTT CCT GTT TTG AAA TTA GAA AGC3′	58°C

1-The underlined nucleotides represented the sequence that pair with the overhang of the vector.
2-The bold and underlined nucleotides represent the restriction site
3-The bold nucleotides represent the Ribosome Binding Site (RBS).

The confirmation for the presence of cloned fragment did by PCR amplification and Restriction digestion of recombinant plasmid (pSyn_1/D-TOPO®pbh1) with *Sal* I restriction enzyme. For Cloning of *ADHII* on pSyn_1/D-TOPO®pbh1 Vector, the Recombinant ppbh1containing pdc amplicon was cut with *sal1* restriction enzymes to form sticky end. *A sal1* recognition site that located directly downstream of *PDC* stop codon selected as a method of choice for ppbh1 linearization. Linearized vector with sticky ends was blunt ended with Quick Blunting Kit. Gel electrophoresis analyzed the result. Ligation of Purified *ADHII* blunt ended into Linearized pSyn_1/D-TOPO®pbh1 Vector (pSyn_1/D-TOPO®pbh2 construction) was carried out using T4 DNA ligase (Promega, USA). Colony PCR did the confirmation for the presence of cloned fragment. Single Bacteria colony directly used as a template for PCR without isolating pure DNA by Colony Polymerase chain reaction (colony PCR). The confirmation of cloning was also done by Restriction Digestion of Recombinant Plasmid (pSyn_1/D-TOPO®pbh2) with *Eco*RI restriction enzyme. The digested product analyzed by 1% agarose gel electrophoresis.

Synechococcus Transformation

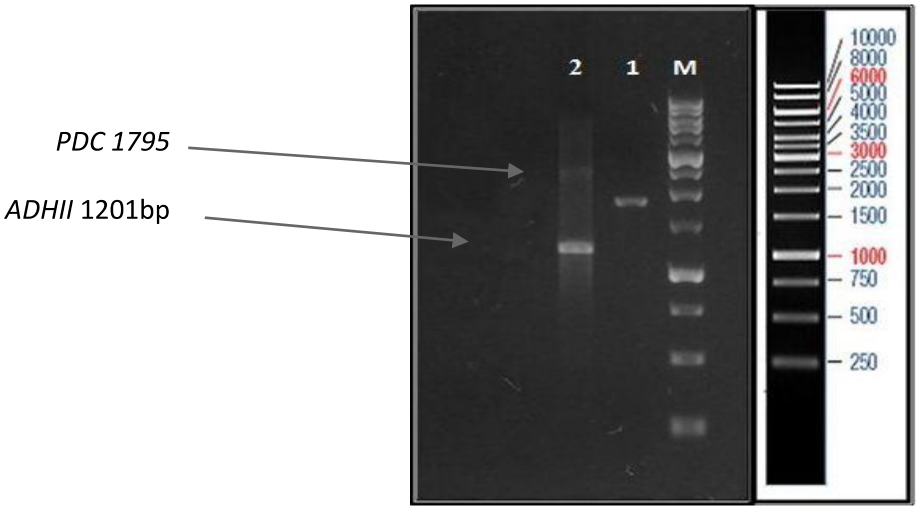
Synechococcus sp. and *Synechococcus elongatus* transformed with Recombinant Plasmid (pSyn_1/D-TOPO®pbh2) by growing them photo autotrophically in broth BG11 medium at 30°C under the continuous light intensity of 50µE. After three days, the optical density of the two cultures was measured at 750 nm. The OD_{750} of local isolates of *Synechococcus* sp. was reached to 1.4, but for *Synechococcus elongatus* the OD_{750} reached to 1.2. *Centrifugation harvested Synechococcus cultures*; Supernatant was removed the by pipetting. The cells resuspended in 1 ml of Gibco® BG-11 medium, One microliter of supercoiled recombinant plasmid DNA (pSyn_1/D-TOPO®ppbh2 containing *PDC* and *ADHII* gene) added to the resuspended cells incubated at 30˚C for 8 hrs. transformation mixture spreaded on separate BG-11 Agar plates containing 10 µg/ml spectinomycin. The plates incubated at 30˚C under continuous illumination of 50 µE for 5-7 days in incubator. Transformants screened for the integration of the two genes by direct colony PCR.

Ethanol Concentration Assay

BG11 broth medium was prepared, Nickel sulfate solution with 5 µM added into conical flasks containing BG11 medium, then each flask inoculated with local isolates of *Synechococcus* sp. and one conical flask without nickel sulfate was inoculated with *Synechococcus* sp. The conical flasks incubated for 48hrs. at 30 ˚C under continuous lighting of 30 µE, the other remaining conical flasks were used for *Synechococcus elongatus* after the addition of nickel sulfate.The conical flasks incubated for 48hrs. at 30 ˚C under continuous illumination of 50 µE. Determination of ethanol concentration of liquid culture was done by using enzymatic ethanol detection kit (Boehringer Mannheim / R-Biopharm, Germany).

Results and Discussion

Pyruvate is a common intermediary of the primary metabolism of many organisms. Two steps, catalyzed by a pyruvate decarboxylase and an alcohol dehydrogenase, are required to convert pyruvate into ethanol [13]. The ethanologenic bacterium, *Zymomonas mobilis* , is capable of a second type of anaerobic fermentation commonly mentioned to as an alcoholic fermentation in which pyruvate is break down to acetaldehyde and CO_2 by pyruvate decarboxylase (PDC). Acetaldehyde is then reduced to ethanol by ADH regenerating NAD^+ Alcoholic fermentation results in the metabolism of 1 molecule of glucose to two molecules of ethanol and two molecules of CO_2 [14]. The genes which encode both of these enzymes in *Z. mobilis* have been amplified by polymerase chain reaction using the primer pair’s pdcf, pdc r, adhf, and adhr to obtain sufficient DNA for cloning (Figure 1).



(Figure 1): Amplification of *PDC* and *ADHII*

Lane M: 1Kb DNA ladder

Lane 1: 1759bp PCR amplified a product of *PDC* gene from *Z.mobilis* genome.

Lane 2: 1201bp PCR amplified a product of *ADHII* gene from *Z.mobilis* genome.

Cloning of PDC and ADHII genes

PDC clone was Constructed by insertion of amplified PDC gene into pSyn_1/D-TOPO® vector. The 1759 bp of *PDC* gene cloned into pSyn_1/D-TOPO® vector. The vector carries the gene for spectinomycin resistance for selection on *E.coli* and *Synechococcus* sp. The vector also carries nickel promoter for regulated expression of the recombinant gene of interest. After that, chemically prepared competent *E.coli*/DH5α cells were transformed with TOPO® cloning reaction mixture. For measuring the efficiency of the transformation, host cells should also be transformed with supercoiled pSyn_2/control vector. After overnight incubation at 37˚C, distinct colonies were observed figure (2). Plasmid minipreps were prepared from all colonies and subjected to PCR amplification with the primer pairs pdc f and pdcr. The product was a single fragment of the same size as that given by amplification of *PDC* from *Zymomonas mobilis* genome, strongly suggesting the plasmids contained the desired insert (figure 3). Single digestion of prepared plasmids with *SalI* performed and produced 5554bp (figure 4) the confirmed recombinant vector with *PDC* was named pSyn_1/D-TOPO®pbh1 (figure 5).

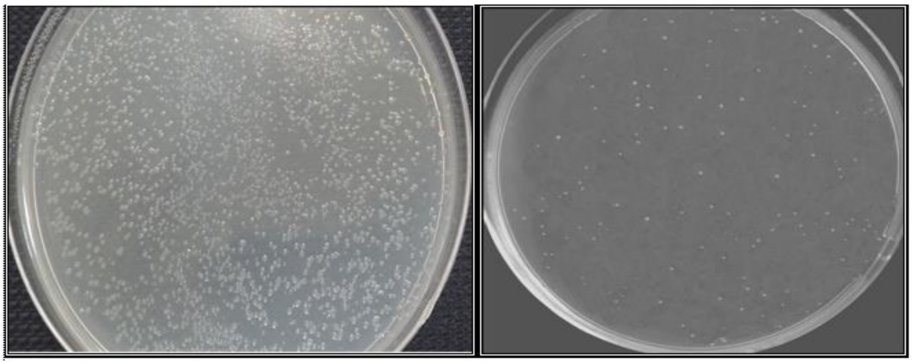


Figure (2): Transformation of *E.coli* / DH5α with pSyn_1/D-TOPO®pbh1 construct.

A: Control plate *E.coli* / DH5α cells transformed with control vector pSyn_1/D-TOPO® containing the Spectinomycin resistance marker.

B: *E.coli* / DH5α cells transformed with construct pSyn_1/D-TOPO®pbh1 vector containing the Spectinomycin resistance marker

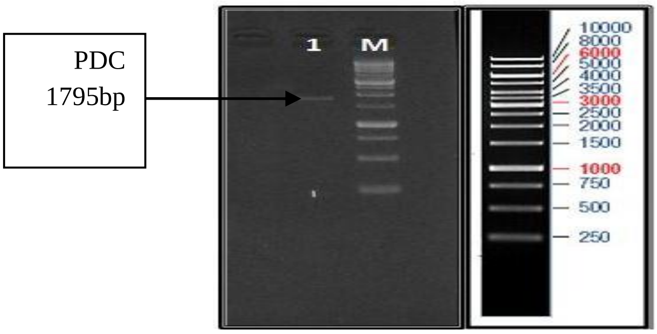


Figure (3): PCR amplification is confirming the ligation of *PDC* using pSyn_1/D-TOPO®pbh1 as a template. Lane M: 1Kb DNA ladder; Lane 1: PCR amplification of PDC (1759bp) from pSyn_1/D-TOPO®pbh1 construct.

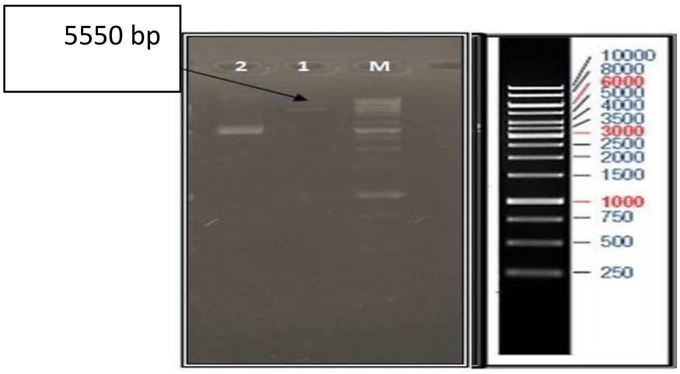


Figure (4): Restriction digestion of pSyn_1/D-TOPO®pbh1 with *SalI*
Lane M: 1Kb DNA ladder; Lane 1: Recombinant plasmid (pSyn_1/D-TOPO®pbh1) digested with *SalI* resulting in a 5554bp linear fragment.; Lane2: Recombinant plasmid (pSyn_1/D-TOPO®pbh1).

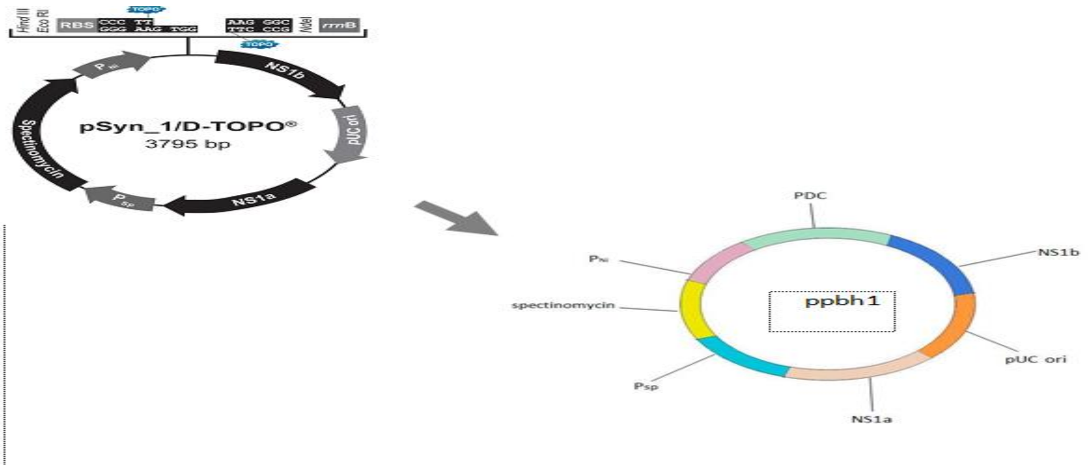


Figure (5): Genetic Map of pSyn_1/D-TOPO®ppbh1 and pSyn_1/D-TOPO®

pSyn_1/D-TOPO®pbh2 Constructed by forming an operon of *PDC* and *ADHII* under the control of nickel promoter. *ADHII* gene must also cloned into the downstream of *PDC* gene in pSyn_1/D-TOPO®pbh1 vector. The *pet* operon that consisted of pet genes (*PDC* and *ADH*II) from *Z.mobilis* was also constructed and expressed in *Lactobacillus casei* under the control of Gram-positive promoter regions [15]. For inserting the *ADHII* gene to the downstream of *PDC* gene in pSyn_1/D-TOPO®pbh1 vector, the vector was linearized with *SalI* restriction enzyme. Blunt ended *ADHII* (1201bp) fragment inserted the pSyn_1/D-TOPO®pbh1 plasmid. Ligation involves creating a phosphodiester bond between the 3'-hydroxyl group of one DNA fragment and the 5'-phosphate group of another and is equivalent to repairing nicks in a duplex strand. The enzyme most frequently used to ligate fragments is bacteriophage T4 ligase [12]. The temperature, time necessary for successful ligation and the optimal temperature is a cooperation flanked by that of ends (the *Tm* of ends are generally 12-16˚C) and the optimal temperature (25˚C) for activity of T4 DNA ligase [16]). After that, ligation mixture is transformed to prepare chemically competent *E.coli*/DH5α cell. To measure the efficiency of the transformation, the host cell should also transformed with supercoiled pSyn_2/control vector. After 18 hrs. incubation at 37˚C, colonies were observed (Figure 6). Screening methods are required to confirm the presence and/or orientation of the intended insert. Colony PCR was performed using the primer pairs adhf and adhr (figure 7). The product was a single fragment of 1201 bp after analyzing results of gel electrophoresis. Plasmid minipreps are also prepared and subjected to restriction digestion by *EcoRI* restriction enzyme. The products were 2525bp and 4212bp (figure 8), which is strongly suggesting the plasmids contained the desired insert. The confirmed recombinant vector with *PDC* and *ADHII* was named pSyn_1/D-TOPO®ppbh2 (figure 9).

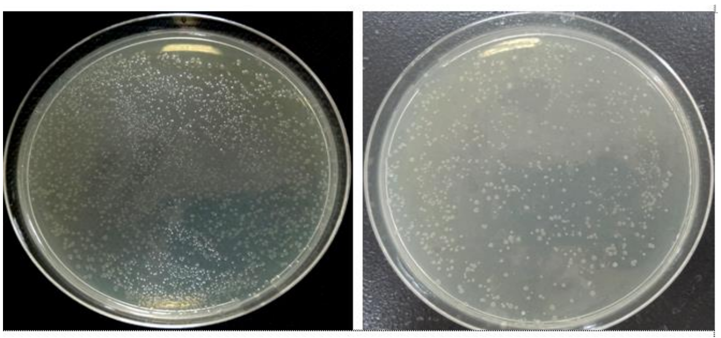


Figure (6): Transformation of *E.coli* /DH5α with pSyn_1/D-TOPO® ppbh2 construct

A: Control plate *E.coli* /DH5α cells transformed with control vector pSyn_1/D-TOPO® containing the Spectinomycin resistance marker.

B: *E.coli*/ DH5α cells transformed with construct pSyn_1/D-TOPO®pbh2 vector containing the Spectinomycin resistance marker.

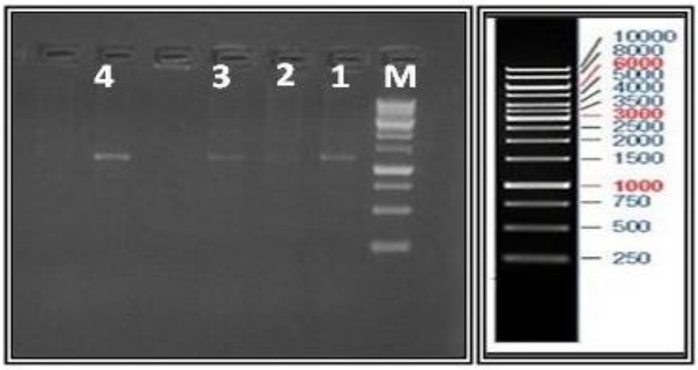


Figure (7): Colony PCR amplification of 1201bp *ADH* gene using *E.coli*/ DH5α cells as a template. (Note Lanes 1-4 are identical)

Lane M: 1Kb DNA ladder.

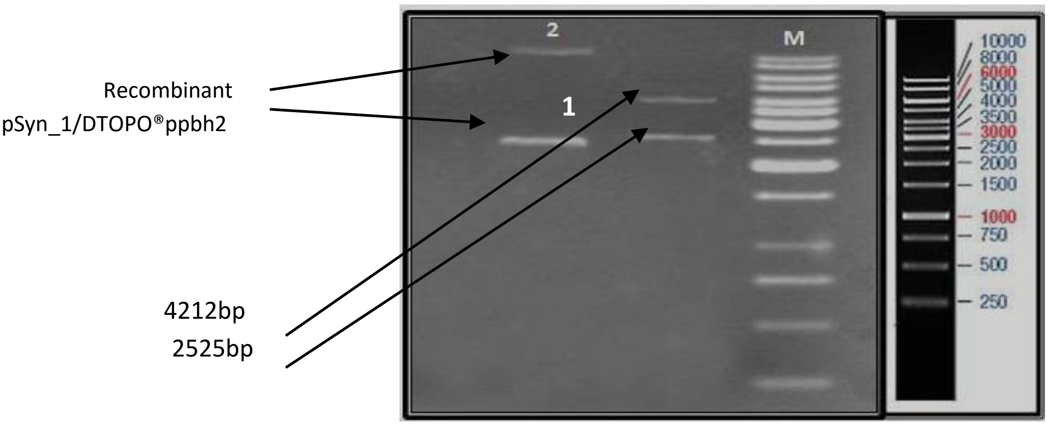


Figure (8): Restriction digestion of pSyn_1/D-TOPO® ppbh2

Lane M: 1Kb DNA ladder; Lane1: Digestion of recombinant ppbh2 with *EcoR1* restriction enzyme resulting in 2525bp and 4212bp fragments; Lane 2: recombinant pSyn_1/D-TOPO®ppbh2 vector.

After constructing a recombinant plasmid that carry both *PDC* and *ADHII* genes pSyn_/D-TOPO®ppbh2 vector, both *Synechococcus* sp. and *Synechococcus elongates* were transformed with pSyn_/D-TOPO®ppbh2. Liquid cultures of both *Synechococcus* sp. and *Synechococcus elongatus* were grown to an OD_{340} of 1-2. The cells centrifuged and resuspended in fresh BG11 media, and then the samples were transferred to eppindorf tubes containing pSyn_1/D-TOPO®ppbh2 vector and control vector. The tubes were wrapped in aluminum foil to exclude light and incubated at 30˚C for 8 hrs before being incubated for 2 hrs without aluminum foil, then the samples were plated on fresh BG11 plates containing spectinomycin, after incubation for 7 days, many colonies were produced from cells treated with pSyn_1/D-TOPO® ppbh2 and control vector. To confirm the integration of the two genes, colony PCR was performed using primer pairs pdcf, pdcr, adhf and adhr. The results revealed the successful integration of the two genes into *Synechococcus* chromosome via homologous recombination. Daniel and Mcfadden (1986) [17] described that the period of incubation of cells with DNA might affect the transformation. Lightfoot and Wootton (1988) [18] reported that the increase of transformation efficiency of the strain *Synechosystis* sp. 6301 was detected when the cells were incubated with DNA in darkness. The same mechanism of transformation was also performed by (Golden and Sherman, 1984) [19], they observed the increasing in transformation frequency by incubating cells with DNA in darkness with the strain *Anacystis nidulance* 7942, they reported that the transformation frequency of *Anacystis nidulance* increased with the length of time in which recipient cells were incubated with

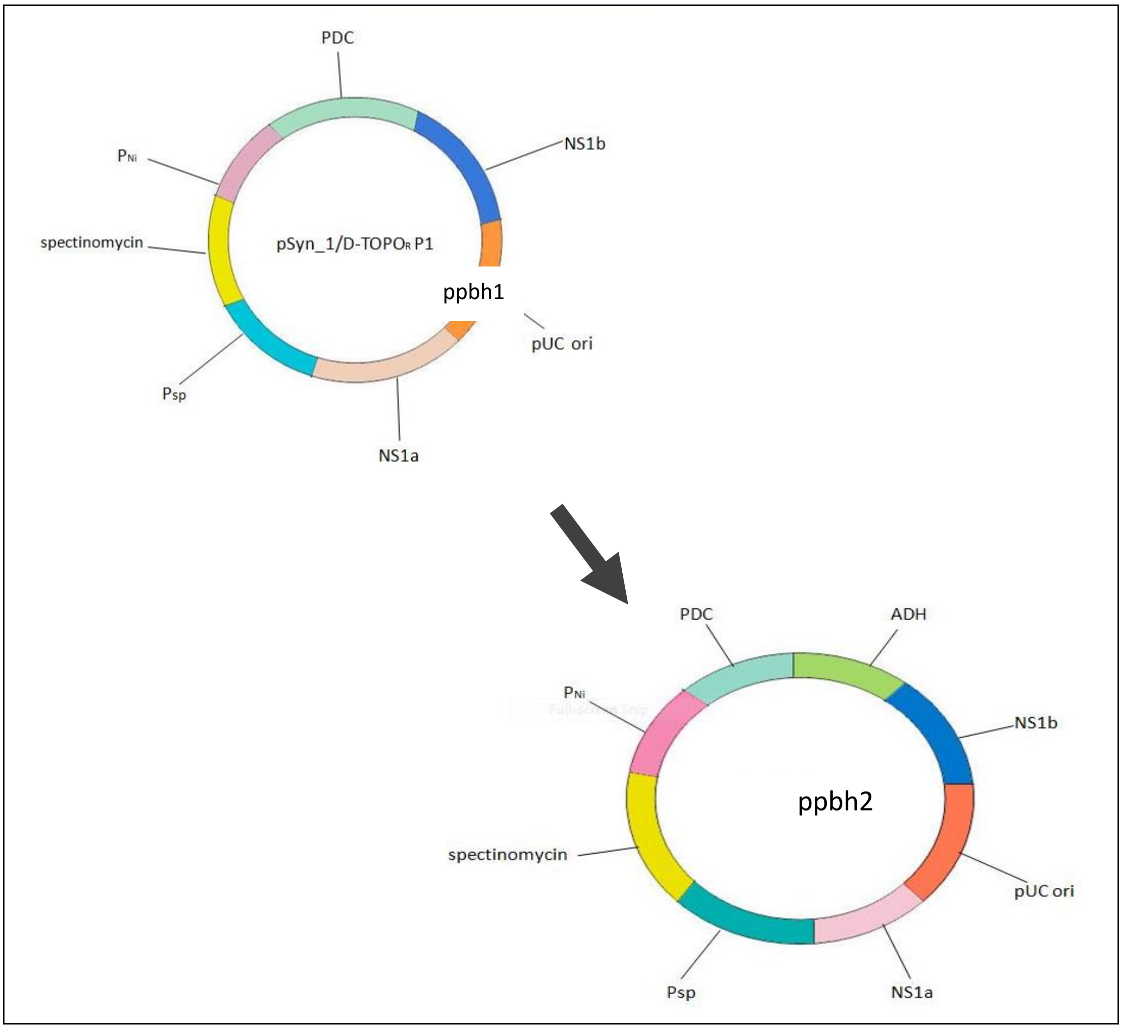


Figure (9): Genetic Map of Recombinant Plasmid pSyn_1/D-TOPO®pbh1 and pSyn_/D-TOPO®ppbh2 vector.

donor DNA, they also described that concentration of plasmid may also affect the transformation efficiency. Chauvat *et al*. (1983) [20] did not observe an enhancement of transformation by dark incubation of *Anacystis nidulance* cells, but they found that *A.nidulance* were highly transformable only during the transition between the first and second phase of logarithmic growth. Golden and Sherman (1984) [19] hypothesized that a membrane-bound component involved in photosynthetic ion exchange and required for DNA uptake appropriated during enlightenment. Shestakov *et al*. (1982) [21] raised a similar mechanism to explain the light dependence of transformation. It seems equally that the depressed metabolism of darkened cells might favor uptake, preservation, or constant incorporation of DNA, or expression of the foreign resistance gene. With continuing the reduction of known fossil fuel funds, much research effort is being focused on the finding and utilization of renewable energy sources [8]. Cyanobacteria are becoming progressively attractive cell factory for producing renewable biofuels and chemicals due to their high photosynthetic efficacy, limited nutrient requirements, rapid growth rates, capacity to transport inorganic carbon, and their relatively simple genetic background for genetic manipulation [10]. The attempt of the present study is to engineer a pathway for ethanol synthesis in the cyanobacterium *Synechococcus* sp. by expressing the *Z.mobilis* genes encoding PDC and ADHII under the control cyanobacterial weak constitutive Ni^{2+} inducible nrsRS promoter (nickel promoter). The NrsS-NrsR two-component system regulates transcription of the *nrsBACD* operon, which involved in the resistance to an excess of Ni^{2+} ions in *Synechocystis* [22]. *PDC* and *ADHII* genes integrated into the *Synechococcus* genome by double homologous recombination using vector with the so-called neutral site (NS). In the present study, we generated an operon for ethanol production in *Synechococcus* sp. in which the *PDC* gene coding sequence was placed 5' with respect to the *ADHII* gene. No promoter sequence and transcription terminator was present between the stop codon of the *PDC* gene and the initiation codon of *ADHII* gene. Concentration of ethanol production by recombinant *Synechococcus* cells that transformed with control vector and recombinant *Synechococcu*s cells that harboring both *PDC* and *ADHII* genes were detected by Boehringer Mannheim/R-biopharm® enzymatic ethanol detection kit. No ethanol detected in the culture media inoculated with *Synechococcus* cells containing control vector. This is because they do not harbor ethanol cassette for ethanol production. The amount of ethanol produced by recombinant *Synechococcus* cells were incubated at 30 ˚C under light of 50 µE and in concentration of 5µM of $NiSO_4$; by local isolates of *Synechococcus* sp. which is (0.0103 g/l) and for *Synechococcus elongatus* is (0.0138 g/l) as shown in (Table 2).

Table 2: Concentration of ethanol production by *Synechococcus* sp. in 50µE at 30˚C in different concentrations of iSO4.

Species name	Ethanol concentration (g/l) in different $NiSO_4$ concentrations (µM)					
	B[a]	1µM	2.5µM	5µM	7.5µM	10µM
Synechococcus sp.	0.0023	0.0062	0.008	0.0103	0.0074	ND[b]
Synechococcus elongates	0.0027	0.0077	0.0101	0.0138	0.0086	ND
Synechococcus sp. (C[c])	ND	ND	ND	ND	ND	ND
Synechococcus elongatus (C)	ND	ND	ND	ND	ND	ND

The amount of ethanol produced by local isolates is less than those produced by *Synechococcus elongatus*, the variance between the levels of expression may be due to the efficiency of ribosome binding site, codons used to encode the foreign protein because some codons preferred over others in different organisms, protein size, and stability, which are important factors that affect foreign gene expression in cyanobacteria [23]. Deng and Coleman (1999) [8] expressed *pyruvate decarboxylase* and *alcohol dehydrogenase* in *Synechococcus* sp. strain PCC 7942 under the control of the promoter of cyanobacterial *rbcLS* operon encoding the ribulose-1,5-bisphosphate carboxylase/oxygenase. Deng and Coleman have used plasmids pCB4-LRpa, pCB4-LR(TF) as the repeating plasmid vector for expression of the pdc/adh cassette, and plasmid selection by ampicillin. In the present study, the non-replicating vector was used for direct integration of antibiotic resistance cassette (spectinomycin), *PDC* and *ADHII* genes into the genome at the neutral site one under the control of the nickel promoter. Thus, the strains do not need to be maintained on antibiotics after isolation. The joint results from both studies, thus, suggest that there would be much benefit in screening multiple strains of cyanobacteria using various promoter systems to determine the best combination for ethanol production.

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