Effect of Plasmid Addition on the Production of 1-Butanol from CO_2 in Isolated *S.Elongatus* from the Sea Sediment of Konak - Izmir

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Abstract

For a sustainable future it is essential to close the global carbon cycle. Oxidised forms of carbon, in particular CO_2, can be used to synthesise energy-rich organic molecules. Engineered cyanobacteria have attracted attention as catalysts for the direct conversion of CO_2 into reduced fuel compounds.1-Butanol is a promising gasoline replacement compared to the more commonly used ethanol due to several advantages. Specifically, 1-butanol is less corrosive and has a higher energy density than ethanol. In this study, *Synechococcus elongatus* was isolated from the sea sediment in Konak and the effects of CO_2 isolated from the same region on the production of 1-butanol was investigated. Addition of plasmid slightly increased the CO_2 utilisation rates. The effects of environmental factors (NO_3-N, SO_4-S, NaCl, H_2, O_2) on the ratio of 1-butanol production to CO_2 removal were investigated. Under optimized conditions *S.elongatus* metabolized the CO_2 according to Monod kinetic (K_s = 1.5 mg L^{-1} and μ = 0.21 day^{-1}). Under high NO_3-N, SO_4-S, NaCl, and O_2 concentrations 1-butanol produced with un-competitive inhibition with a high K_i value of 2.3 mg L^{-1} indicating the low inhibition in *S.elongatus* with plasmid. The competitive inhibition constant (K_i) is low (0.70 mg L^{-1}) exhibiting the high competitive inhibition at high concentration for the operational conditions given above.

Keywords: 1-butanol, carbondioxide, cyanobacteria, monod, plasmid.

1. Introduction

Cyanobacteria have great potential as a platform for biofuel pro duction because of their fast growth, ability to fix carbon dioxide gas, and their genetic tractability. The Cyanobacteria allows for the use of CO_2 at higher concentrations than that of ambient air (Lan and Liao, 2011) and could potentially allow for the use of concentrated CO_2 emissions from waste industrial sources. The development of new technologies for production of alternative fuel became necessary to circumvent finite petroleum resources, associate rising costs, and environmental concerns due to rising fossil fuel CO_2 emissions (Kruse and Lindblad, 2012). Several alternatives have been proposed to develop a sustainable industrial society and reduce greenhouse emissions. The idea of biological conversion of CO_2 to fuel and chemicals is receiving increased attention. In particular, the direct conversion of CO_2 with solar energy to biofuel by photosynthetic microorganisms such as microalgae and cyanobacteria has several advantages compared to traditional biofuel production from plants (Lan and Liao, 2014). The direct conversion of solar energy into liquid fuel using photosynthetic microorganisms is an attractive alternative to fossil fuels. There are several advantages to using organisms such as microalgae and cyanobacteria: their readily available genetic tools and sequenced genomes; their higher growth rate compared to plants; and their ability to thrive in areas that cannot support agriculture. Utilization of these organisms can provide a way of resolving the potential conflict between the use of land for food or for biofuel production (Oliver *et al.*, 2011). Compared to ethanol, isobutanol is a better candidate for gasoline replacement due to its low hygroscopicity, higher energy density and high compatibility with current infrastructure. Recently it has been reported that production of 1-butanol in cyanobacteria from CO_2 was achieved by transferring a modified CoA-dependent pathway into the autotrophic organism (Kusakabe *et al.*, 2003). Since this CoA-dependent pathway was derived from strict anaerobic bacteria, it was a challenging task to express such a pathway in cyanobacteria, which produce oxygen during photosynthesis. To synthesize 1-butanol, 5 genes were integrated into the *S.elongatus* PCC7942 genome: *hbd*, *crt*, and *adhE2* (Lan and Liao, 2011). Butanol can be used directly in any gasoline engine without modification and/or substitution, because it has sufficiently similar characteristics to gasoline as a liquid fuel. For example, in 2005, a gasoline car with unmodified engine was fueled with 100% butanol and successfully ran almost 10,000 miles across the USA (Lan and Liao, 2014). This result clearly demonstrates that biobutanol is one of the most powerful alternative fuel. The genetic tractability of cyanobacteria makes them attractive targets for use in metabolic engineering for renewable technology (Oliver *et al*., 2011). *Synechococcus elongatus* sp. PCC 7942 (*S.elongatus* 7942) has a number of unique sigma factors involved in regulation in response to light and circadian rhythm (Kusakabe *et al*., 2003). In this study, 1-butanol was produced from the CO_2 trapped from the Konak region in İzmir metropolitan air (Turkey) using *S.elongatus* isolated from Konak sediment in İzmir bay. The air was trapped from Balcova and the CO_2 inside air was used to produce 1-butanol in İzmir Turkey via photosynthesis. The effects of increasing NO_3-N, SO_4-S, NaCl, H_2, O_2 concentrations on the 1-butanol levels and on the yield of 1-butanol production to CO_2 used were investigated. The effects of plasmid addition on the 1-butanol production yields were investigated while the effect of increasing NO_3-N, SO_4-S, NaCl, H_2, O_2 concentration on Monod and inhibition kinetics were evaluated.

2. Materials and Methods

2.1. Analytical procedure

Cultures were grown in an orbital shaker at 120 rpm under a continuous fluorescent white light illumination of 34 W L^{-1}. The *S.elongatus* in 100 mL sediment samples were grown in 250 mL erlenmeyer flasks. Closed bottles contained 20 mL of liquid culture inoculated to a starting an MLVSS concentration of 50 mg L^{-1} was monitored at after 30 h cultivation at 20 °C at a UV power of 5 W m^{-2}. *Synechocystis elongatus* plasmid PCC7942 was obtained from American Type Culture Collection (ATCC) and was transformed to *S.elongatus* by grown in BG11 medium. The morphology of *S.elongatus* species was observed using a fluorescence microscope (Olympus, Japan). 1-butanol and CO_2 concentrations were measured in an Agilent GC-MS. 3-hidroxybutyryl-CoA dehydrogenase (Hbd), Trans-2-enoyl-CoA reductase (Ter) and Aldehyde/alcohol dehydrogenase (AdhE2) enzyme activities were measured in a spectrophotometer. The air was trapped using air bags **(Figure 1)**. The atmospheric gas was passed from an absorbant ped with a flow rate of 0.083 m^3 min^{-1} containing *Azotobacter vinelandi* culture grown on a specific medium. In this step the N_2 gas present in the air samples was eliminated since this bacteria used N_2 gas for growth during non simbiotic N_2 fixation. The *Azotobacter vinelandi* medium consisted from 08% K_2HPO_4; 0.02% KH_2PO_4, 0.02% $MgSO_4$ 0.01% $CaSO_4$; 0.0015% $FeSO_4/7H_2O$; 0.00025% g $NaMoO_3$ and from 0.5% sucrose. Then the remaining gas was passed from an absorbant ped containing *Nitrosomonas* sp. bacteria at a flow rate of 0.083 m^3 min^{-1}. This bacteria used the O_2 in the air. As *Nitrosomonas* medium Medium A, Medium B, Medium A-Z and A-2 Trace element medium were used. A medium consisting of $(NH_4)_2SO_4$ 0.1 g; K_2HPO_4 0.1 g; NaCl 0.2 g; $MgSO_4.7H_2O$ 0.05 g; $FeCl_3$ trace; $CaCO_3$ 1.0 g and tap water 100 ml. Medium B was made up as follows: NaCl 0.3 g; $MgSO_4.7H_20$ 0.14 g; $FeSO_4.7H_20$ 0.03 g and $(NH_4)_2SO_4$ 0.66 g. They are dissolved in 90 ml distilled water. The ‘A-2’ trace element mixture contains $LiSO_4$ 0.01 g; $CuSO_4$ 0.02 g; $ZnSO_4$ 0.02 g; H_3B0_4 0.22 g; $Al_2(SO_4)$ 0.02 g; $SnCl_2$ 0.01 g; $MnCl_2$ 0.14 g; $NiCl_2$ 0.02 g; $CoSO_4$ 0.02 g; $TiCl_4$ (15% solution) 0.13 ml; Kl 0.01 g; KBr 0.01 g and distilled water, 360 ml. As a result the air was purified from N_2 and O_2 and ontained %99.9 CO_2 with the exception of some NOx gases.

Figure 1. Air trapping devise and empty nylon bags

Figure 2. Air purification devise

3. Results and Discussion

3.1. Enumeration of *S.elongatus isolated from Konak sediment and S.elongatus with* PCC 7942 plasmid

S.elongatus cynobacteria numbers isolated from konak sediment were as high as *S.elongatus* containing PCC 7942 plasmid using the CO_2 trapped from the Konak-Izmir district air (**Table 1**).

3.2. 1-butanol$_{produced}$/CO$_{2utilized}$ yields for S.elongatus isolated from Konak sediment and S.elongatus with plasmid in Izmir metropolitane

The maximum 1-butanol concentration produced from the polluted air containing 0.17 mg L^{-1} O_2 is 99 mg L^{-1} with an 1-butanol$_{produced}$/$CO_{2utilized}$ yield of 88% at 21°C temperature, using 34W L^{-1} light intensity, at pH = 6.70 at 100 mV redox potential in the *S.elongatus* isolated from the Konak bay sediment. The O_2 and the CO_2 trapped from the Balcova region air **(Table 2**). The maximum 1-butanol concentration produced from the *S.elongatus* containing plasmid with an air containing 0.17 mg L^{-1} O_2 is 90.0 mg L^{-1} with an 1-butanol$_{produced}$/$CO_{2utilized}$ yield of 89% at 21°C temperature, at 34W L^{-1} light intensity, at pH = 6.60 at 100 mV redox potential. It was found that no significant 1-butanol yields was obtained in both *S.elongatus*.

Table 1. Enumeration of *S.elongatus* isolated from Konak sediment and *S.elongastus* containing PCC 7942 plasmid under laboratory conditions

	Cyanobacteria Sizes		Number of Cyanobacteria (colony mL^{-1})
	lengths (μm)	widths (μm)	
Konak sediment and air	3 - 15	0.4 – 6	11800 *S.elongatus*
Konak sediment and air	2 - 14	0.4 – 5	11300 *S.elongatus*
Konak sediment and air	2.2 - 12	0.4 –5.24	10600 *S.elongatus*
Konak sediment and air	1.9 - 11	0.3 – 398	11400 *S.elongatus* with plasmid
Konak sediment and air	1.9 – 14	0.4 – 3.7	11600 *S.elongatus* with plasmid
Konak sediment and air	2.4 – 15	0.3 – 3.5	11500 *S.elongatus* with plasmid

3.3. Enzyme activities

Hbd and Ter enzyme activities are high at both S.elongatus species. The CO2 from the Konak air in Izmir was trapped and purified from the N2 and O2. No significant differences in both S.elongatus types was obtained (Table 3). It was found that the Hbd enzyme activities were high then that Ter enzyme in both S.elongatus. The CO2 trapped from the air was used as carbon source and it was converted to the 1- butanol via photosynthesis process. During metabolic pathway the production of Hbd and Ter enzymes catalyse the formation of 1-butanol from CO2.

3.4. Effects of different NO3-N, SO4-S, NaCl, H_2 and O_2 concentrations on 1-butanol productions

The 1-butanol productions were slightly higher in *S.elongatus* containing plasmid than that in *S.elongatus* isolated from Konak sediment in Izmir. The results showed that NaCl, NO_3-N and SO_4-S affect negatively the 1-butanol production at high concentrations (4 and 5 mg L^{-1}) in *S.elongatus* without plasmid (**Table 4**). Salt contained both osmotic pressure and ionic stress. NaCl cause fouling in the membrane water channels and the cells were disrupted with osmotic pressure and the electron transporting system inactivated causing decrease in photosynthesis of CO_2 to 1-butanol. High NO_3-N and SO_4-S concentrations decrease the photoshynthesis and decrease the 1-butanol production from CO_2. The *S.elongatus* containing plasmid is more resistant to high NaCl, NO_3-N and SO_4-S concentrations. The maximum specific growth rate (μmax) of *S.elongatus* with plasmid is high compared to *S.elongatus* isolated one. The *S.elongatus* with plasmid did not exhibited death at high NaCl, NO_3-N and SO_4-S concentrations. The inhibition level is low in *S.elongatus* with plasmid indicating a high inhibition constant (K_i = 2.3 mg L^{-1}) at high NO_3-N, SO_4-S, and NaCl (3, 4 and 5 mg/L) concentrations. The K_i is low in *S.elongatus* isolated from Konak sediment with high competitive inhibition

Table 2. 1-butanol$_{produced}$/CO$_{2utilized}$ yields

Samples	1-butanol (mg L^{-1})	1-butanol$_{produced}$/ CO$_{2consumed}$	Cyanobacteria type
Konak sediment and air	98	0.89	*S.elongatus* with PCC7942
Konak sediment and air	98	0.87	*S.elongatus* with PCC7942
Konak sediment and air	98	0.90	*S.elongatus* with PCC7942
Konak sediment and air	97	083	*S.elongatus* isolated
Konak sediment and air	96	0.81	*S.elongatus* isolated
Konak sediment and air	97	0.82	*S.elongatus* isolated

Table 3. Enzyme activity measurements in both *S.elongatus* species

Samples	Hbd (μg mL^{-1})	Ter (μg mL^{-1})	Cyanobacteria type
Konak sediment and air	0.099	0.0032	*S.elongatus* PCC7942
Konak sediment and air	0.098	0.0030	*S.elongatus* PCC7942
Konak sediment and air	0.096	0.0028	*S.elongatus* PCC7942
Konak sediment and air	0.089	0.0031	*S.elongatus* isolated
Konak sediment and air	0.086	0.0024	*S.elongatus* isolated
Konak sediment and air	0.0089	0.0026	*S.elongatus* isolated

3.5. Cost analysis

The total cost for 1-butanol production from 1 m^3 CO_2 using *S.elongatus* isolated from the Balcova sea is only 1.30 Euro while the total cost is 1.60 Euro for *S.elongatus* PCC 7942 (**Table 5**). Since *A.vinelandi* and *Nitrosomonas sp.* were isolated from the soils the cost used only for media were taken into consideration in the calculation of total cost.

4. Conclusions

In this study it was found that 1-butanol as an alternative of gasoline can be effectively produced from the CO_2 present in air of Konak located in Izmir Turkey using *S.elongatus* isolated from Izmir Bay sediment *and* with *S.elongatus* with PCC 7942 plasmid..

Table 4. Effect of some environmental conditions on 1-butanol productions and Monod kinetic

Samples	1-butanol production (mg L^{-1})	NO_3-N (mg L^{-1})	SO_4-S (mg L^{-1})	K_i (mg L^{-1})	NaCl (mg L^{-1})	H_2 (mg L^{-1})	O_2 (mg L^{-1})	μ_{max} (h^{-1})	X_{max} (mg L^{-1})
Konak	87	1	1	-	1	1	1	0.03	100
Konak	87	2	2	-	2	2	2	0.04	100
Konak	70	3	3	2.1	3	3	3	0.02	50
Konak	60	4	4	1.2	4	4	4	0.02	50
Konak	56	5	5	0.7	5	5	5	0.01	30
With plasmid	89	1	1	-	1	1	1	0.04	100
With plasmid	89	2	2	-	2	2	2	0.04	100
With plasmid	89	3	3	-	3	3	3	0.04	100
With plasmid	88	4	4	-	4	4	4	0.04	100
With plasmid	80	5	5	2.3	5	5	5	0.03	80

Table 5. Cost analysis for 1-butanol production in both *S.elongatus* strains

	1-butanol production
Isolation cost for *S.elongatus*	0.20 Euro
Purchasing of *S.elongatus* PCC 7942	0.30 Euro
BG – 11 media	1 liter: 0.20 Euro
Electricity	60 W light power: 0.50 Euro Illumination for 35 days
CO_2 purification Media used for growths of *A.vinelandi* and *Nitrosomonas sp.*	0.40 Euro
1 m^3 1-butanol cost in *S.elongatus* isolated from Konak sediment	Total cost: 1.30 Euro / m^3
1 $m^3$1-butanol cost in *S.elongatus* with PCC 7942 plasmid	Total cost: 1.60 Euro / m^3

Althoughout the *S.elongatus* containing plasmid exhibited slightly higher 1-butanol productions is more resistant to high NO_3-N, SO_4-S and NaCl concentrations than *S.elongatus* without plasmid. At high inorganic compound concentrations the *S.elongatus* growth exhibited a high competitive inhibition with a low K_i value of 0.7 mg/l at isolated genous. The cost of the 1-butanol production is cheaper than the 1 m^3 of commercial gasoline (3.9 Euro) and the CO_2 emissions in Izmir metropolitan can be reduced in this way

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