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In vivo Quantification of Alkanes in *Escherichia coli*

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[Abstract] Microbial production of alkanes employing synthetic biology tools has gained tremendous attention owing to the high energy density and similarity of alkanes to existing petroleum fuels. One of the most commonly studied pathways includes the production of alkanes by AAR (acyl-ACP (acyl carrier protein) reductase)-ADO (aldehyde deformylating oxygenase) pathway. Here, the intermediates of fatty acid synthesis pathway are used as substrate by the AAR enzyme to make fatty aldehyde, which is then deformylated by ADO to make linear chain alkane. However, the variation in substrate availability to the first enzyme of the pathway, *i.e.*, AAR, via fatty acid synthesis pathway and low turnover of the ADO enzyme make calculation of yields and titers under *in vivo* conditions extremely difficult. *In vivo* assay employing external addition of defined substrates for ADO enzyme into the medium helps to monitor the influx of substrate hence providing a more accurate measurement of the product yields. In this protocol, we include a detailed guide for implementing the *in vivo* assay for monitoring alkane production in *E. coli*.

Keywords: ADO, Alkanes, Enzyme assay, Biochemistry, Biofuels, Gas chromatography

[Background] Research on alkane production using engineered microbes has gained significant popularity as it provides an attractive alternative to reduce dependence on fossil fuels while mitigating the climate change effects (Lee *et al.*, 2008; Knothe, 2010; Lu, 2010; Schirmer *et al.*, 2010; Tan *et al.*, 2011). Various pathways have been uncovered or artificially assembled for the production of alka(e)nes in microbes (Schirmer *et al.*, 2010; Mendez-Perez *et al.*, 2011; Rude *et al.*, 2011; Akhtar *et al.*, 2013; Howard *et al.*, 2013; Rui *et al.*, 2014). However the highest reported titres for alkane production so far have been in *E. coli* using the AAR-ADO pathway (Figure 1) (Fatma *et al.*, 2018). AAR catalyzes the reduction of fatty acyl-ACP or fatty acyl-CoA into fatty aldehydes using NADPH which is subsequently converted into alkanes by ADO (Marsh *et al.*, 2013). *E. coli* is the most widely used host for heterologous production of biofuel candidates due to a broader knowledge of its cellular metabolic network as compared to other hosts. Heterologous co-expression of cyanobacterial AAR and ADO in *E. coli* results in production and secretion of alkanes (Schirmer *et al.*, 2010). However, quantification of alkanes by the ADO enzyme and determination of the *in vivo* efficacy of the ADO enzyme cannot be determined due to the variation in the substrate availability by the first enzyme AAR of the pathway. Reports on differences in the solubility of AAR and hence its activity (Kudo *et al.*, 2016) indicate that using AAR as a source of substrate for measuring *in vivo* activity of ADO is not a suitable approach. Hence, we developed an *in*

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vivo enzyme assay, which involves addition of the substrate aldehyde exogenously to the medium that is taken up by the growing cells and gets converted to alkane by the heterologously expressed intracellular ADO enzyme, thus giving a more reliable and accurate measurement of its *in vivo* efficacy.

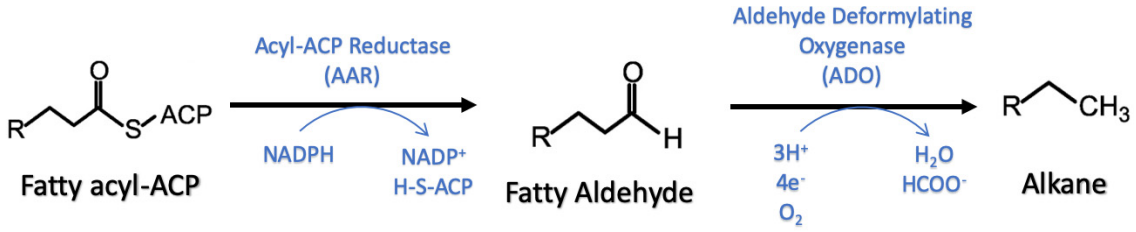

Figure 1. Schematic representation of AAR-ADO pathway for alkane production

Materials and Reagents

1. 2 ml micro-centrifuge tubes (Tarson, catalog number: 500020)
2. Culture tubes (55 ml, Borosil, catalog number: 9820U08)
3. GC vials (Agilent, catalog number: 5190-2280)
4. Parafilm (Sigma, catalog number: P7793)
5. 0.2 µm filter (mdi Membrane Technologies, catalog number: SYNN0301MNXX104)
6. *E. coli* DH5α strain overexpressing ADO enzyme cloned in pQE30 vector (strain source: Invitrogen, vector source: Qiagen)
7. Hexadecanal (TCI America, catalog number: H1296)
8. Octadecene (TCI America, catalog number: S0348)
9. Sodium phosphate dibasic (Na_2HPO_4) (Merck, catalog number: 255793)
10. Potassium dihydrogen phosphate (KH_2PO_4) (Sigma, catalog number: P5655)
11. Sodium chloride (NaCl) (Sigma, catalog number: S9888)
12. Ammonium chloride (NH_4Cl) (Sigma, catalog number: 213330)
13. Magnesium sulphate heptahydrate ($MgSO_4·7H_2O$) (Sigma, catalog number: 230391)
14. Calcium chloride ($CaCl_2$) (Sigma, catalog number: C4901)
15. Ferric chloride hexahydrate ($FeCl_3·6H_2O$) (Sigma, catalog number: 236489)
16. Zinc chloride ($ZnCl_2$) (Sigma, catalog number: 746355)
17. Sodium molybdate dihydrate ($Na_2MoO_4·2H_2O$) (Sigma, catalog number: 331058)
18. Copper sulphate ($CuSO_4$) (Sigma, catalog number: C1297)
19. Boric acid (H_3BO_3) (Sigma, catalog number: B7901)
20. Thiamine hydrochloride (Sigma, catalog number: T4625)
21. Glucose (Sigma, catalog number: G8270)
22. Bis Tris (Amresco, catalog number: 0715-250G)
23. Triton X-100 (Amresco, catalog number: M143-1L)
24. Ethyl acetate (Merck, catalog number: 1096232500)

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25. IPTG (Sigma, catalog number: I6758)
26. LB broth (HiMedia, catalog number: M1245)
27. Ampicillin (Sigma, catalog number: A9393)
28. M9 salts (see Recipes)
29. M9 modified medium (see Recipes)

Equipment

1. Table top centrifuge (Eppendorf, models: 5418R and 5810R)
2. Incubator shaker (Kuhner, model: ISF-1)
3. GC FID (Agilent, model: 7890 A System, equipped with HP-5 column with catalog no. 19091J-413)
4. Spectrophotometer (GE Healthcare, model: Ultrospec™ 2100)
5. Vortex (Sigma, catalog number: Z258423-1EA)
6. Autoclave (Natsteel, model: 24 SR)

Procedure

A. *In vivo* culture

1. Inoculate *E. coli* strains harbouring the plasmids carrying ADO gene (Figure 2) from plates streaked and incubated overnight at 37 °C (plates originally streaked from glycerol stocks) in 5 ml LB broth medium with 5 µl of appropriate antibiotics (ampicillin–100 µg/ml used in this study) in 55 ml culture tubes (1 ml of Ampicillin stock of 100 mg/ml was prepared by adding 100 mg of Ampicillin powder in 1 ml of water followed by filter sterilization).

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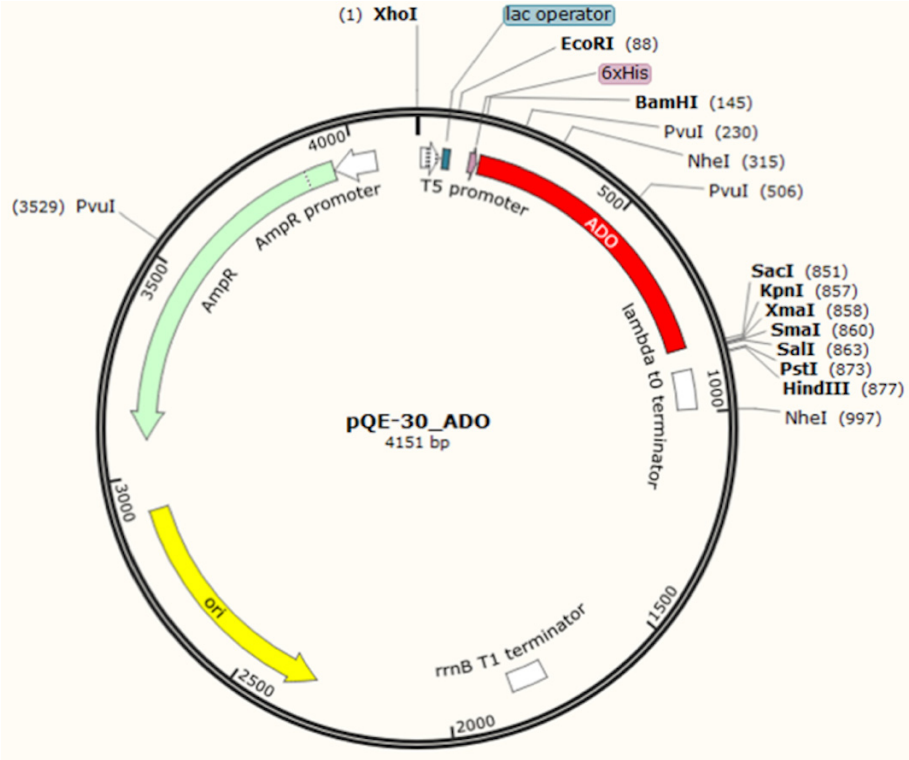

Figure 2. Schematic representation of the plasmid involved in alkane pathway construction. Plasmid vector map of pQE_ADO, carrying ado gene in pQE30 plasmid at the restriction sites *Bam*HI/*Sac*I.

2. Incubate the samples at 37 °C overnight in an incubator shaker set at 180 rpm.
3. Use 50 µl (OD_{600} ranging from 3-4) of the primary inoculum grown overnight to inoculate 3 ml secondary culture having M9 modified medium (Recipe 1) supplemented with 2% glucose, 1 mg/L thiamine and 100 µg/ml ampicillin.
4. Induce the culture with 0.01 mM IPTG (3 µl of 10 mM IPTG to 3 ml culture) at the time of secondary inoculation in M9 medium as the plasmid pQE30 carrying ADO gene is inducible by IPTG.
5. Add 100 mg/L hexadecanal (dissolved in absolute ethanol) exogenously to the culture medium at the time of secondary culture inoculation and seal the culture tubes with parafilm to prevent any loss of substrate or product due to evaporation (15 µl of 20 mg/ml stock added to 3 ml culture).
6. Incubate the culture tubes at 30 °C (or higher to test for thermostable enzymes) in an incubator shaker set to 120 rpm for 48 h.

B. Hydrocarbon extraction

1. Measure the optical density at 600 nm of the samples to estimate the growth before proceeding with extraction of alkanes.

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2. Take equal volumes (0.75 ml) of sample and ethyl acetate (containing 10 mg/L Octadecene added as internal standard–from a 100 ml stock of ethyl acetate containing 1.265 µl of 99% 1-Octadecene) in a 2 ml microcentrifuge tube.
3. Vortex the samples for 20 min and centrifuge at 15,700 *x g* for 3 min at room temperature.
4. Collect the upper fraction and aliquot it into a fresh GC (gas chromatography) vial.
5. Analyze the samples using gas chromatography (GC).

C. Hydrocarbon analysis

1. Set the hydrocarbon analysis method on the gas chromatography (GC) system equipped with HP-5 column of 30 m length, 0.32 mm internal diameter and 0.25 mm film thickness. The FID detector is used for analysis. The hydrocarbon analysis method is to be set as follows. The oven temperature programme is set as: initial 100 °C held for 3 min, increase temperature to 250 °C at the rate of 10 °C min^{-1} and held at 250 °C for additional 10 min. The total run-time of the programme will be 28 min. The inlet and detector temperature are to be maintained at 150 °C and 280 °C, respectively.
2. Run pentadecane, hexadecanal, 1-octadecene, and hexadecanol standards of known quantity (Figure 3) prior to sample analysis and use the area under curve to calculate the concentration of metabolites in the test samples.
3. Run the test samples in GC machine (Figure 4) and quantify the pentadecane content with respect to its standard.

Data analysis

Representative data

For detailed data analysis, please refer to the article of Shakeel *et al.* (2018). The representative GC chromatograms for all the standards are shown in Figure 3. The area under curve of known quantities of these standards are used to quantify metabolites in the test samples. The representative GC chromatograms for the typical *in vivo* samples containing alkane are shown in Figure 4. The area under curve of each metabolites is used to calculate their concentrations by comparing it with the area under curve of corresponding standards of known quantities.

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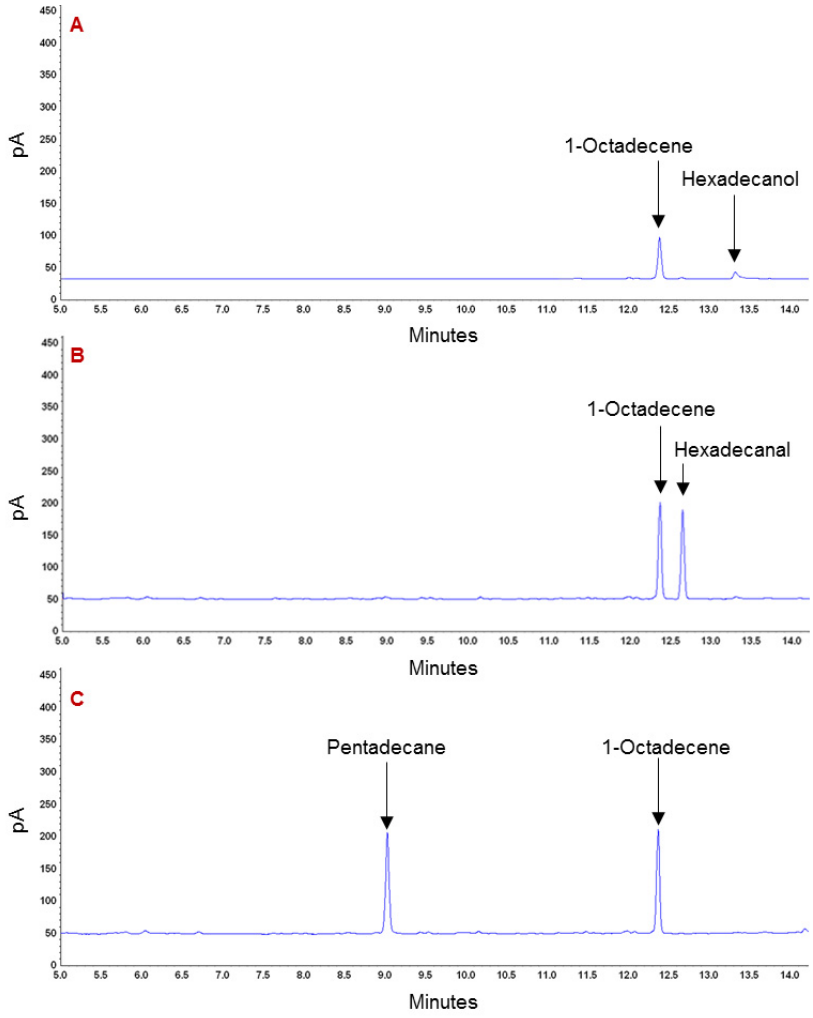

Figure 3. GC chromatograms of the standard metabolites. A. Chromatogram of Hexadecanol and 1-Octadecene. B. Chromatogram of Hexadecanal and 1-Octadecene. C. Chromatogram of Pentadecane and 1-Octadecene. The 1-Octadecene was used as an internal standard for checking extraction efficiency, while other metabolites are either substrate (Hexadecanal) or products (Hexadecanol and Pentadecane) observed after the hydrocarbon extraction.

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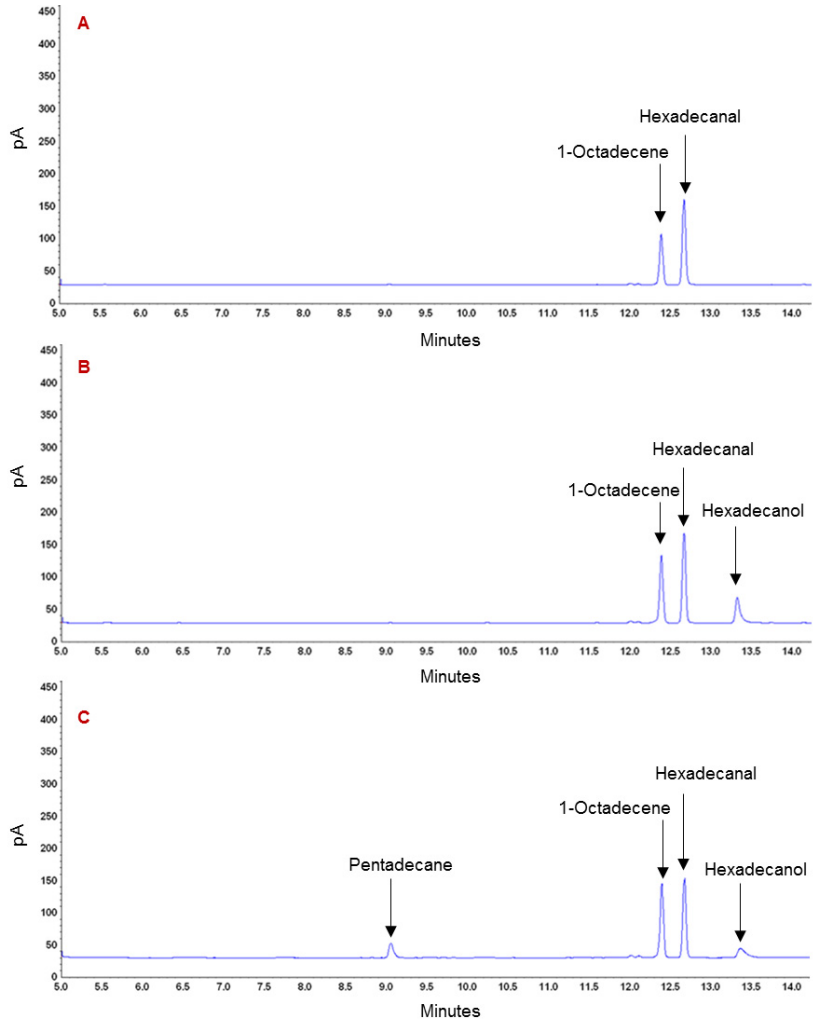

Figure 4. Functionality of ADO protein in recombinant *E. coli* after addition of exogenous precursor *i.e.* hexadecanal. A. GC chromatogram of control sample containing hexadecanal and internal standard. B. GC chromatogram of *E. coli* expressing empty pQE30 plasmid, showing peak of hexadecanal and hexadecanol. C. GC chromatogram of *E. coli* expressing pQE_ADO, showing formation of end metabolites *i.e.,* pentadecane and hexadecanol.

Recipes

1. M9 modified medium

   Components of M9 modified medium are prepared as follows:

   a. M9 salts

      For 100 ml M9 salts (10x)

      11.32 g Na_2HPO_4

      3 g KH_2PO_4

      1 g NaCl

      2 g NH_4Cl

      Volume made up to 100 ml by adding MQ water and filter sterilized using 0.2 µm filter

b. Trace element recipe

1x Trace element composition:

0.25 g/L $MgSO_4 \cdot 7H_2O$

11 mg/L $CaCl_2$

18.71 mg/L $FeCl_3 \cdot 6H_2O$

1.311 mg/L $ZnCl_2 \cdot 4H_2O$

2 mg/L $Na_2MoO_4 \cdot 2H_2O$

1.9 mg/L $CuSO_4$

0.5 mg/L H_3BO_3 (Boric acid)

1 mg/L Thiamine

All the above components are prepared individually as 100x stock in MQ water and filter sterilized using 0.2 µm filter

c. 1x Additive composition

200 mM Bis Tris (5x stock prepared)

0.1% Triton X-100 (10x stock prepared)

2% Glucose (10x stock prepared)

All the above components are prepared individually as in MQ water and filter sterilized using 0.2 µm filter

The 1x constituents from (a), (b) and (c) are mixed sequentially and volume is made up to 100 ml with MQ water autoclaved at 121 °C for 20 min.

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Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

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