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RESEARCH

Engineering an Isoprenoid Pathway in *Escherichia coli* for Production of 2-Methyl-3-buten-2-ol: A Potential Biofuel

Dinesh Gupta · Michael L. Summers · Chhandak Basu

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Abstract 2-Methyl-3-buten-2-ol (MBO) is a natural volatile 5-carbon alcohol produced by several pine species that have the potential to be used as biofuel. MBO has a high energy content making it superior to ethanol in terms of energy output, and due to its volatility and lower solubility in water, MBO is easier to recover than ethanol. Pine's MBO synthase enzyme utilizes the intermediate dimethylallyl pyrophosphate (DMAPP) produced by the methyl-erythritol-4-phosphate isoprenoid pathway for the production of MBO. In this study, we performed metabolic engineering of *Escherichia coli* to express an alternate mevalonate dependent pathway for production of DMAPP, along with a codon optimized *Pinus sabiniana* MBO synthase gene. This heterologous expressed pathway carried out the conversion of an acetyl CoA precursor to DMAPP leading to production of MBO.

Keywords Biofuel · *E. coli* · 2-Methyl-3-buten-2-ol · Metabolic engineering

Abbreviations

DIG	Digoxigenin
DMAPP	Dimethylallyl pyrophosphate
MBO	2-Methyl-3-buten-2-ol
MEP	Methyl-erythritol-4-phosphate
MVA	Mevalonate
IPTG	Isopropyl-β-D-thiogalactopyranoside
HMGS	3-Hydroxy-3-methylglutaryl-CoA synthase
IPP	Isopentenyl pyrophosphate
GC	Gas chromatography
FID	Flame ionization detector

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D. Gupta · M. L. Summers · C. Basu (✉)
Department of Biology, California State University, 18111 Nordoff Street, Northridge, Los Angeles, CA, USA
e-mail: chhandak.basu@csun.edu

Background

Both increasing demand for petroleum products and dependence on nonrenewable fossil fuel has spurred research into efficient production of renewable, eco-friendly alternative sources of biofuel. To enhance efficiency and integration into existing infrastructure, new biofuels should have physicochemical properties comparable with gasoline. Bioethanol has been used as gasoline supplement, however, its low energy density combined with high purification cost inherently limits its optimal use [1]. While attempts are being sought to increase efficiency and reduce the overall cost of ethanol production [2, 3], identification and production of other potential renewable biofuels such as short chain volatile alcohols and hydrocarbons are under investigation [4, 5]. The search for substances with enhanced chemical properties compared to ethanol is driving explorations in this area [6–9].

MBO ($C_5H_{10}O$) is a short chain volatile alcohol naturally produced by several indigenous pine trees of western North America [10–12]. The energy content of MBO is calculated to be 106,000 BTU/gallon, and is more comparable with petroleum (111,000–125,000 BTU/gallon) than ethanol (85,000 BTU/gallon).[1] MBO is also less soluble in water than ethanol, allowing capture of the volatile MBO from the headspace of a culture without requirement

[1] URL: http://www.wisys.org/uploads/media/WSTS_Guay_and_Singsaas_PP.ppt. Accessed: 2013-October-03.

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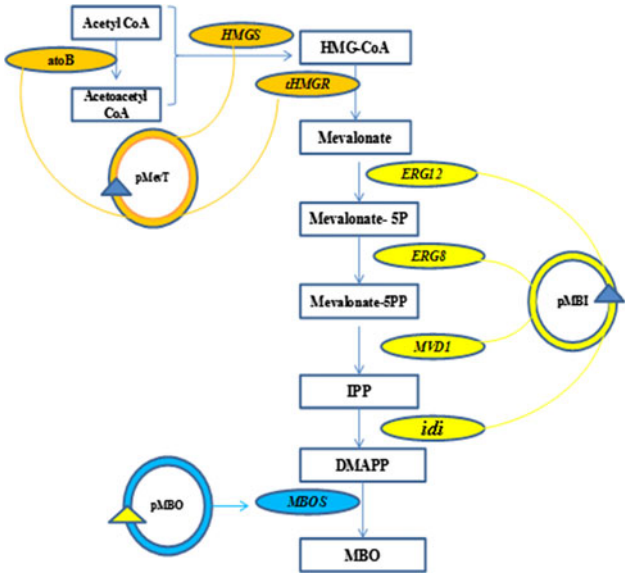

Fig. 1 MBO production via MVA pathway involving synthetic operons. Shown in *orange* are genes present in pMevT. *atoB* acetoacetyl-CoA thiolase, *HMGS* HMG-CoA synthase, *tHMGR*: truncated HMG-CoA reductase; Shown in *yellow* are genes present in pMBI. *ERG12* mevalonate kinase, *ERG8* phosphomevalonate kinase, *MVD1* mevalonate pyrophosphate decarboxylase, *idi* IPP isomerase; Shown in *blue* is the gene present in pMBO. *MBOS* MBO synthase from *Pinus sabiniana.* In the above MVA pathway, *atoB*, and *idi* were isolated from *E. coli*, and all other genes were isolated from *Saccharomyces cerevisiae*. The T5 and *lac* promoters are indicated by *yellow* and *blue triangles*, respectively. Figure was adapted from: [15, 23] (Color figure online)

of expensive post harvesting steps. This volatile nature of MBO also increases production efficiency by preventing excess accumulation of MBO in the growth media and subsequently its inhibitory effect on microbial culture. In addition to being an ideal candidate for renewable biofuel, MBO can also be used as starting biomaterial to produce number of value added compounds. 3-Methyl-2-butenol, one of MBO isoform, can potentially be used in synthesis of vitamin A, vitamin E, flavor or fragrances, and many other pharmaceutically significant molecules [9].

The large family of organic compounds known as isoprenoids has been recognized as important source of flavor, fragrances, and medicine [13, 14]. Many pharmaceutically important compounds have been synthesized through metabolic engineering of the isoprenoid biosynthetic pathway. For example, artemisinin, a potential antimalarial drug [15] and taxol, an anticancer compound [16], are commercially produced in microbial systems. There are also recent reports on potentials of isoprenoid molecules for use as renewable biofuels [17–19]. Of relevance to this work is the observation that an intermediate in isoprenoid synthesis is the substrate for MBO synthase.

Two isoprenoid biosynthetic pathways exist in plants: chloroplasts produce the MBO precursor, dimethylallyl diphosphate (DMAPP), through methyl-erythritol-4-phosphate (MEP) pathway, whereas in the cytosol, DMAPP is produced by the mevalonate (MVA) pathway [19–21]. In the MEP pathway, pyruvate and glyceraldehyde 3-phosphate condense to produce deoxy-xylulose-5-phosphate which is converted to DMAPP following six subsequent enzymatic reactions [22]. This pathway also exists in most of prokaryotic bacteria, including *Escherichia coli*. In the MVA pathway, DMAPP is produced from acetyl-CoA precursors using several enzymatic reactions as shown in Fig. 1. In *Pinus sabiniana*, DMAPP is directly converted to MBO, catalyzed by MBO synthase [23]. Although the gene for MBO synthase is encoded by nuclear genome, a short *N*-terminal signal sequence targets this protein to chloroplast where the DMAPP precursor is present due to its production via the MEP pathway [23, 24]. Recently, MBO has also been reported as one of the metabolites in tert-Amyl alcohol (TAA) degradation. In *A. tertiaricarbonis* L108, MBO was produced during TAA degradation in small amounts (less than 5 %) [25].

Several attempts have been made in the past to engineer native bacterial MEP pathways to increase DMAPP precursor pools, however, limited success was reported due to a "tight regulatory mechanism" [15, 19, 26]. To overcome this limitation, the MVA pathway in *E. coli* was engineered so that the "upper half" of the mevalonate pathway genes were expressed from plasmid pMevT, and "lower half" genes were expressed from plasmid pMBI [15], which together constitute complete set of genes to efficiently convert acetyl CoA to DMAPP (Fig. 1). Following the addition of a codon optimized *P. sabiniana* MBO synthase gene [23] to this genetic background, we demonstrate the feasibility of MBO production in *E. coli*.

Materials and Methods

Bacterial Strains and Plasmids

Escherichia coli DH5α and SoluBL21 (AMS Biotechnology) strains were used as expression hosts in Luria–Bertani (LB) or Terrific Broth growth media. The pMBO plasmid [23] harboring *P. sabiniana* MBO synthase gene (GenBank No. JF719039) optimized for expression in *E. coli*, was generously provided by Dr. Thomas D. Sharkey, Michigan State University. For heterologous MVA pathway expression in *E. coli,* two plasmids (pMevT and pMBI) encoding *S. cerevisiae* mevalonate pathway genes were kindly provided by Dr. Jay D. Keasling, University of California, Berkeley. All other important properties of bacterial strains and plasmids used in this study are listed in Table 1.

Table 1 Bacterial strains, plasmids, and primers used in this study

	Relevant properties	Source
Strains		
E. coli DH5α	Expression host	Lab stock
E. coli SoluBL21	Expression host (DE3) with IPTG inducible T7 polymerase (Cm^S)	Lab stock
E. coli BG101	*E. coli* DH5α harboring pMevT and pMBI	This work
E. coli BG102	*E. coli* DH5α harboring pMBO, pMevT and pMBI	This work
E. coli BG103	*E. coli* SoluBL21 harboring pMevT and pMBI	This work
E. coli BG104	*E. coli* SoluBL21 harboring pMBO, pMevT and pMBI	This work
Plasmids		
pMevT	Ori p15A, Cm^R, P_{lac}, AtoB-HMGS-tHMGR	[15]
pMBI	Ori pBBR1, Tet^R, P_{lac}, MK-PMK-PMD-*idi*	[15]
pMBO	Ori pMB1, Kan^R, P_{T5}, *P. sabiniana* MBO synthase (MBOS)	[23]
Primers		
HMGS-f	5′-GAATTAAGGAGGACAGCTAAATGAAACTCTCAACTAAACTTTG	[15]
HMGS-r	5′-AGTGTAATCCTCCTTATTTTTTAACATCGTAAG	[15]
idi-f	5′-ATCCCGGGAGGAGGATTACTATATGCAAACGGAACACGTC	[15]
idi-r	5′-ATCCCGGGTTATTTAAGCTGGGTAAATG	[15]
MBOS-f	5′-GCTTGTAGCGCGATTCAAACGGAA	This work
MBOS-r	5′-AGACGCCTTTCATGTACTCTGGCA	This work

Introduction and Analysis of Heterologous Pathways in *E. coli*

Chemically competent *E. coli* cells were sequentially transformed with the pMevT and pMBI plasmids using standard chemical transformation [27], resulting in *E. coli* strains BG101 (DH5α) and BG103 (SoluBL21). Sequential plasmid transformations were performed; selected transformants were made chemically competent before subsequent transformations. To test growth defects caused by expression of the MVA pathway genes as mentioned previously [15], the overnight culture of transformed *E. coli* BG101 was subcultured in LB media supplemented with antibiotics at 37 °C shaking at 200 rpm. After 2 h of culture initiation, the culture was induced with 0.5 mM IPTG or water (for minus IPTG controls) and incubated. To study the growth characteristics, optical density of 1 ml cultures was measured at 600 nm before and after 3, 6, 9, and 24 h of IPTG induction using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). After confirming growth defects in strains containing the introduced mevalonate pathway genes, pMBO was similarly transformed into BG101 and BG103, and selected using triple antibiotic selection to produce strains BG102 and BG104, respectively.

Dot Blot Analysis

To confirm the compatibility and stability of three plasmids to coexist in an *E. coli* expression host, dot blot analysis was performed on strain BG102. Plasmid DNA from various strains of *E. coli* was extracted with GeneJET Plasmid Miniprep kit (Fermentas Life Science, USA). An overnight culture of BG102 from a freezer stock was used to inoculate 5 ml of LB broth with antibiotics and 0.5 mM IPTG in 4 tubes for plasmid extraction for dot blot analysis. Plasmids were denatured by boiling, applied to a positively charged nylon membrane, and UV cross-linked.

Probes were PCR amplified from purified plasmids, HMGS from pMevT, *idi* from pMBI, and MBOS from pMBO (Table 1), using Dream Taq Master Mix (Fermentas, USA). All oligonucleotide primers were purchased from Integrated DNA Technology, USA. PCR condition included an initial denaturation at 95 °C for 5 min, 30 PCR cycles with denaturation at 95 °C for 30 s, annealing for 30 s (HMGS: 53.5 °C, *idi*: 57 °C, MBOS: 55.2 °C), and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplified products were gel purified using NucleoSpin Gel and PCR clean-up kit (Macherey–Nagel, Germany), and digoxigenin (DIG) labeled using DIG High Prime Labeling Kit (Roche Applied Science, USA) following manufacturer's instructions and protocols described previously [28]. The pBIN-mGFP5-ER plasmid [29] was used as negative control in each experiment. The hybridization was performed at 42 °C as reported before [28]. The hybridized blot was washed as recommended by the manufacturer, and hybridization detected by anti-DIG antibody conjugated with alkaline phosphatase, using NBT/BCIP (nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl-phosphate in 67 % DMSO) for color detection.

MBO Production and Quantification by Gas Chromatography (GC)

To assay the amount of MBO produced by *E. coli* strains BG102 and BG104, overnight cultures inoculated from freezer stocks were used to inoculate 3 ml of LB broth containing antibiotics and 0.5 mM IPTG in sealable tubes. The culture tubes were then capped with rubber stoppers, crimp sealed, and incubated for 48 h at 37 °C shaking at 200 rpm. After incubation, gas from the headspace was sampled and analyzed by GC–FID using a Hewlett-Packard, HP6890 Gas Chromatograph equipped with a HP-5 column (crosslink 5 % PhMe siloxane 30 m × 0.25 µM film thickness) at 90 °C with helium as the carrier gas at a flow rate of 1.1 ml/min using a 1:20 split ratio. MBO was identified by comparing retention time with a MBO standard (Sigma-Aldrich, USA). The amount of MBO produced by bacterial cultures was calculated by comparing the peak area of the chromatograph against MBO standard peak area prepared in identical sealed vials containing an equal volume of water. The production of MBO was further optimized using Terrific Broth and oxygenation using overnight cultures of *E. coli* BG104 grown in the same media as used in the experiment. Oxygen enrichment in culture tubes was performed by blowing pure oxygen into the headspace of culture tubes and quickly capping the tubes in front of blowing oxygen. All sets of experimental tests (cultures in LB, LB with O_2, Terrific Broth, and Terrific Broth with O_2) were carried out in triplicates, and MBO production was analyzed by using GC–FID as described above.

MBO Toxicity Assay

To test MBO toxicity to the cell growth, *E. coli* strain BG104 was grown at 37 °C for 2 h in LB medium supplemented with antibiotics in a 125 ml flask. This stock was used to aliquot 3 ml of bacterial culture to individual sterile vials with 20 mM glucose. Various amounts of liquid MBO (Sigma-Aldrich, USA) ranging from 0.5 to 30 µl were added, and the vials were immediately caped and crimp sealed to minimize the escape of volatile MBO. Culture density was measured spectrophotometrically at 600 nm after 10 h of incubation.

Results and Discussion

Effect of MVA Pathway Induction on Growth

The growth curve (Fig. 2) showed that the exponential growth rate of *E. coli* strain BG101 is similar with or without IPTG induction of the MVA pathway genes. However, IPTG induction causes the culture to enter into stationary phase sooner and reach a lower cell density than the uninduced control. Expression of the complete set of MVA genes cloned under control of the *lac* promoter (in pMevT and pMBI plasmids) can lead to accumulation of the end-product DMAPP to toxic concentrations [15]. Thus, the lower cell yield after IPTG induction, as compared to control culture, is most likely due to production of excess precursor metabolites.

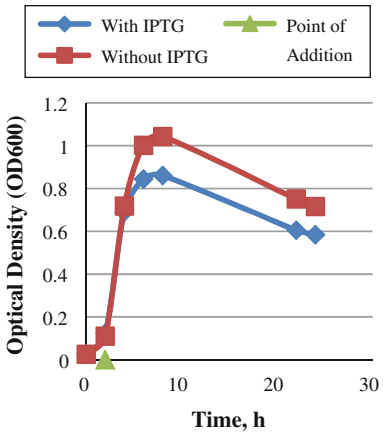

Fig. 2 Comparative study of growth characteristics of *E. coli* BG101, harboring pMevT, and pMBI plasmids, with or without IPTG addition. Induction of complete mevalonate pathway genes cloned under P_{lac} promoter accumulate inhibitory levels of precursor metabolites such as DMAPP, a final product of mevalonate pathway, causing observed growth retardation of *E. coli* culture induced with IPTG upon entry into stationary phase

Confirmation of Heterologous MVA Pathway and MBO Synthase Genes in Transformed *E. coli*

Three genes from the respective plasmids (*HMGS* from pMevT, *idi* from pMBI, and *MBOS* from pMBO) were used as specific probes after labeling with DIG. The probes did not show nonspecific hybridization to a blotted control plasmid, and hybridized to total plasmids isolated from the triple plasmid-bearing strain BG102. This indicates that the three plasmids were compatible in the host strain as shown in duplicate samples (Fig. 3) as predicted by their three different origins of replication (Table 1). The sampling of plasmids from strain BG102 was done in same condition as used in MBO production, indicating that pMevT, pMBI, and pMBO were stable in the host cell during experimental conditions. After establishing the presence of all three plasmids, the strain was subjected to further tests for MBO production.

MBO Production Assay by Gas Chromatography

The negative controls, culture media with no cells, as well as samples containing cultures of recombinant *E. coli*

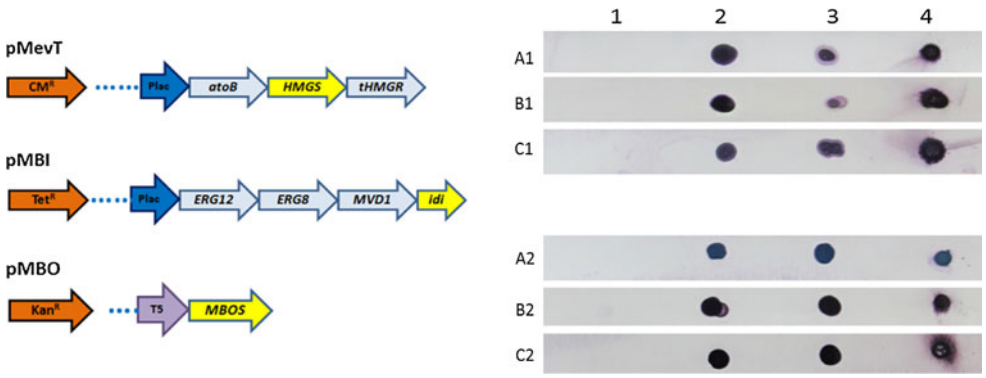

Fig. 3 Confirmation of recombinant *E. coli* BG102 by *dot blot* analysis. Three *highlighted* (*yellow*) genes HMGS (from pMevT), *idi* (from pMBI), and MBOS (from pMBO) were PCR amplified, DIG labeled and used as labeled probe to confirm the presence of plasmids pMevT, pMBI, and pMBO, respectively. *Spot 1* pBIN-mGFP5-ER (negative control); *spot 2* respective plasmid itself (positive control); *spot 3* total plasmid isolate from *E. coli* BG102; *spot 4* probe itself. *Blots A1* and *A2* (replicates) pMevT confirmation; *Blots B1* and *B2* (replicates): pMBI confirmation; *Blots C1* and *C2* (replicates): pMBO confirmation (Color figure online)

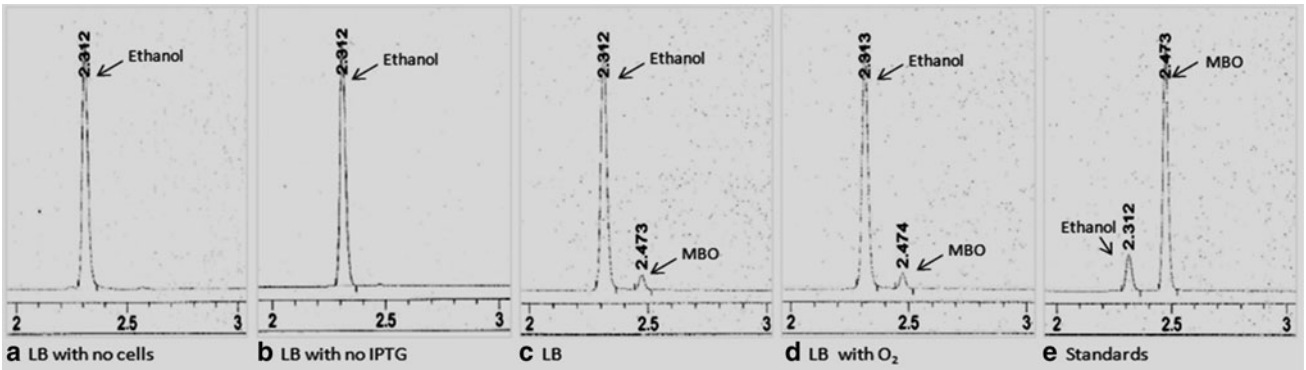

Fig. 4 MBO production in *E. coli* BG104. Gas chromatography profiles of control cultures, **a** (media with all antibiotics, IPTG, but no cells) and **b** (culture not induced with IPTG) showed only single peak of ethanol, while same cultures (in LB and LB + O_2) induced with 0.5 mM IPTG (**c** and **d,** respectively) showed two characteristic peaks of ethanol and MBO. MBO and ethanol peaks were confirmed by comparison with a pure MBO and ethanol standards (**e**). Times of elution (retention) from *column* are indicated at the *bottom* of each figure in minutes. Also, retention time difference between ethanol and MBO was found constant in each case (0.161 min)

strains BG102 and BG104 without IPTG induction showed only the presence of ethanol (ethanol was used to dissolve the chloramphenicol and tetracycline antibiotics) in its headspace air as indicated by the peak at 2.31 min retention time (Fig. 4a, b). BG102 and BG104 cultures showed a new peak at 2.47 min specific to IPTG induction (Fig. 4c, d). These peaks were determined to be MBO by comparing its retention time on gas chromatograph against its standard (Fig. 4e). Based upon comparison to standard under identical conditions, MBO production by *E. coli* strain BG102 was calculated in the range of 20 µl/l, whereas under same culture conditions, 31.5 µl/l MBO was produced by *E. coli* SoluBL21 strain (BG104). The increased production in the SoluBL21 (BG104) background may be due to the lack of an OmpT protease, allowing higher amounts of a limiting enzyme to be present in the cells.

Further optimization for MBO production was carried out using higher MBO producing SoluBL21 strain BG104. There was no difference in the amount of MBO produced

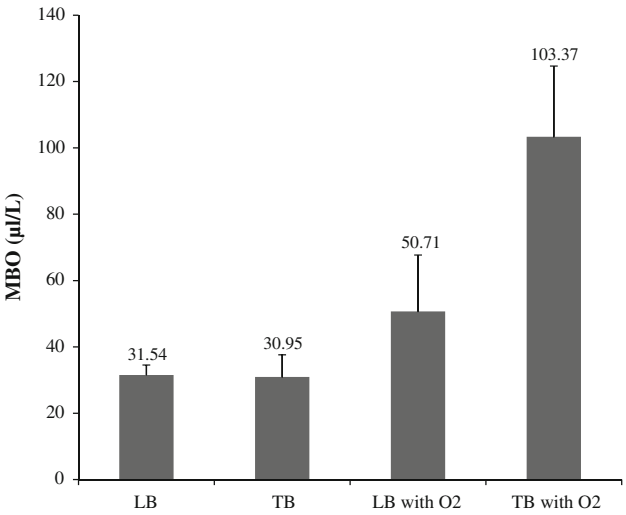

Fig. 5 Optimization of MBO production in BG104 strain using LB broth, Terrific Broth, LB broth with O_2, and Terrific Broth with O_2. The highest MBO production was 103 µl/l (0.985 mM) and was observed in Terrific Broth with O_2

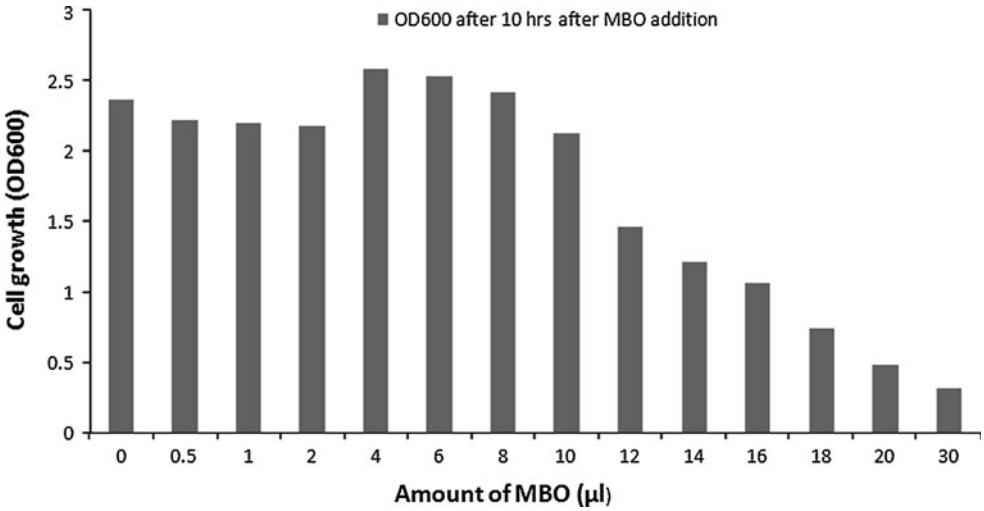

Fig. 6 Study of MBO toxicity on the growth of *E. coli* BG104. MBO-induced cytotoxicity was observed with the addition of 12 µl (38.3 mM) of MBO

when cells were incubated in LB or Terrific Broth media without oxygen. Interestingly, strain BG104 incubated without oxygen in Terrific Broth media exhibited a new peak in addition to those of ethanol and MBO that appeared at 2.39 min retention time. The identity of this compound is unknown, but did not migrate through the column identical to known isoprene controls (data not shown). In case of LB media with excess oxygen, production was increased to 50.7 µl/l (1.6 times more compared to LB without oxygen), and in case of Terrific Broth media with oxygen, production increased to 103.3 µl/l (3 times more compare to Terrific Broth without oxygen) (Fig. 5). This result showed that *E. coli* produce MBO more efficiently in aerobic conditions with a rich carbon source.

Under laboratory conditions, the MBO synthase enzyme has been shown to exhibit a dual activity, catalyzing DMAPP to both MBO as well as isoprene [23]. Under these in vitro conditions, MBO production is favored over isoprene at a ratio of about 90:1 [23]. In contrast, no in vivo isoprene production from its natural pine tree host has been observed [23]. Similar to its natural host, we did not observe any detectable amount of isoprene production from *E. coli* strains BG102 and BG104. The result confirmed feasibility of developing *E. coli* host for production of volatile MBO through expression of codon optimized *P. sabiniana* MBO synthase gene and supplying host cells with DMAPP precursor through engineering of heterologous MVA genes.

MBO Toxicity Assay on Cell Growth

We used a closed system to study the toxicity of the product *i.e.,* MBO on *E. coli* BG104 (Fig. 6), because MBO is volatile and would escape from the system if not contained. To assure the *E. coli* growth, 20 mM of glucose was added to LB broth, since in a closed system *E. coli* continues to grow by fermentation. The result showed that up to 10 µl of MBO in 3 ml of culture (i.e., 31.6 mM MBO) is not inhibitory to normal growth. According to this result, about 3.3 ml of MBO per liter of cell culture can be produced without any cell growth inhibition, with an estimated cost of $2.32 for this growth medium (Supplementary Data 1). Furthermore, if a continuous system in which the product is removed from headspace is used, accumulated MBO would not inhibit production if kept below inhibitory concentrations in the culture. A two-phase gaseous/aqueous bioreactor [30] could also be used to harvest MBO, which may reduce the cost of production of MBO.

Conclusion

Engineered microbial platforms are convenient and cost-effective approaches for large scale production of recombinant proteins and their metabolic products. Several pine trees produce MBO naturally, but one of the major limitations in MBO production from its natural host is its volatile nature. The volatile nature of MBO, however, makes its recovery easier when produced from bacterial cultures. Many other biofuel compounds need to be phase separated from host cells and growth culture medium through expensive and complicated procedures. Here, we report the production of 2-Methyl-3-buten-2-ol, a naturally occurring volatile alcohol through engineering of microbial metabolic pathways.

In this work, two *E. coli* host strains, DH5α and SoluBL21, were equipped with a complete set of MVA pathway genes to convert acetyl CoA to the DMAPP precursor utilized by MBO synthase. The functionality of the introduced MVA pathway was inferred to produce excess DMAPP upon IPTG induction due to the lower growth rate during growth curve analysis and

by subsequent production experiments requiring IPTG induction for MBO expression. DMAPP was then converted to MBO, catalyzed by a codon optimized *P. sabiniana* gene encoding MBO synthase. MBO production by the SoluBL21 strain was found to be better than in a DH5α strain, perhaps attributable to the absence of a protease in this strain allowing increased enzyme activity. With this metabolic engineering strategy and experimentally determined incubation conditions, MBO production in *E. coli* BG104 was optimized to produce up to 103 μl/l (0.985 mM). This level of MBO production was far below that found to inhibit cell growth. We propose that future strain development leading to MBO production near the inhibitory concentration could be mitigated by harvesting MBO using a condenser.

Higher energy output and cost effectiveness are two major requirements for an ideal biofuel. With higher energy content and less solubility in water than ethanol, MBO is a promising candidate for future use. The report of MBO production in this paper is novel, but not cost effective yet. However, MBO production on an industrial scale must be significantly increased by media and strain optimization, and by improved bioreactor design. Since the genes in pMevT and pMBI are eukaryotic in nature, codon optimization suitable for expression in the production strain could be undertaken to further boost MBO production.

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