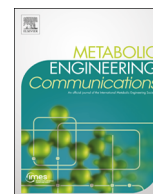




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Development of a plasmid addicted system that is independent of co-inducers, antibiotics and specific carbon source additions for bioproduct (1-butanol) synthesis in *Escherichia coli*

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ABSTRACT

Synthetic biology approaches for the synthesis of value-based products provide interesting and potentially fruitful possibilities for generating a wide variety of useful compounds and biofuels. However, industrial production is hampered by the costs associated with the need to supplement large microbial cultures with expensive but necessary co-inducer compounds and antibiotics that are required for up-regulating synthetic gene expression and maintaining plasmid-borne synthetic genes, respectively. To address these issues, a metabolism-based plasmid addiction system, which relies on lipopolysaccharide biosynthesis and maintenance of cellular redox balance for 1-butanol production; and utilizes an active constitutive promoter, was developed in *Escherichia coli*. Expression of the plasmid is absolutely required for cell viability and 1-butanol production. This system abrogates the need for expensive antibiotics and co-inducer molecules so that plasmid-borne synthetic genes may be expressed at high levels in a cost-effective manner. To illustrate these principles, high level and sustained production of 1-butanol by *E. coli* was demonstrated under different growth conditions and in semi-continuous batch cultures, in the absence of antibiotics and co-inducer molecules.

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1. Introduction

The advent and use of synthetic biology approaches has allowed the scientific community to engineer microorganisms for the production of a variety of value-based products, such as human insulin, proteases, and antibiotics (Chance and Frank, 1993; Adrio and Demain, 2014; Thykaer and Nielsen, 2003). Recently, the microbial production of biofuels, such as 1-butanol, has attracted great interest and gained momentum due to several environmental, economic and political factors. The chemical properties of 1-butanol, its high energy density and low hygroscopicity, and its compatibility with the current infrastructure, make it an attractive candidate for a transportation fuel, compared to ethanol (Peralta-Yahya and Keasling, 2010).

Clostridium acetobutylicum, a Gram-positive strict anaerobic bacterium, has historically been used for the microbial production of 1-butanol, as well as acetone and ethanol. The genes responsible for 1-butanol production in *C. acetobutylicum* were identified (Boynton et al., 1996; Fontaine et al., 2002) and heterologous expression of these

genes has been accomplished in several different microorganisms, such as *Bacillus subtilis* (Nielsen et al., 2009), *Lactobacillus brevis* (Berezina et al., 2010), *Pseudomonas putida* (Nielsen et al., 2009), *Saccharomyces cerevisiae* (Steen et al., 2008), and *Escherichia coli* (Atsumi et al., 2008; Shen et al., 2011; Bond-Watts et al., 2011; Gulevich et al., 2012) with particular interest in *E. coli* due to its proven industrial usage and associated extensive genetic and biochemical knowledge. Early constructs resulted in low yields of 1-butanol produced by wild-type organisms (Atsumi et al., 2008). To overcome this, multiple metabolic and genetic factors were altered in efforts to increase 1-butanol production. For example, with *E. coli* changes in the growth conditions or medium, the use of plasmids or promoters for expression of the 1-butanol genes, the elimination of competitive pathways, and the use of homologous non-clostridial genes have all played a significant role in raising the level of 1-butanol production to impressive levels (Shen et al., 2011).

As opposed to chromosomal expression, plasmid-based systems are often used for up-regulated heterologous expression of synthetic genes in a non-native organism; this is the case for genes that are required for 1-butanol production. The use of a plasmid-based system has many advantages, as it allows for (1) an increase in enzyme pools by gene dosage, (2) control of gene expression if

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an appropriate promoter and co-inducer are chosen, and (3) rapid construction of different combinations of genes due to facile manipulation of plasmids, as opposed to insertion of genes on the chromosome. However, if one chooses to scale-up to industrial production levels, there are two major drawbacks with this laboratory bench method. The first is the combined cost associated with supplementing cultures with a co-inducer to induce gene expression and the use of antibiotics to maintain plasmid stability. Secondly, large scale-ups invite potential ecological issues associated with the usage of large amounts of antibiotics, such as the rise in antibiotic resistant bacterial strains.

To overcome reliance on antibiotics, the metabolism-based plasmid addiction system (PAS) was devised (Voss and Steinbuchel, 2006). Essentially, the PAS relies on the strict natural selection of plasmid-containing cells, due to the expression of a plasmid-encoded gene(s) that is required for the viability of the bacterium (Kroll et al., 2010). Therefore, cells that maintain a plasmid containing the essential gene(s) and a suite of value-based product gene(s) are viable and able to produce the desired product.

There are a few examples of the use of metabolism-based plasmid addicted systems for value-based product formation. The first example used *Ralstonia eutropha* strain H16. Plasmid expression of the essential 2-keto-3-deoxy-6-phosphogluconate (KDPG)-aldolase gene coupled with a cyanophycin synthetase gene, resulted in cyanophycin production with either fructose or gluconate as carbon source (Voss and Steinbuchel, 2006). A second example was cyanophycin production in *E. coli* (Kroll et al., 2011). Like the first example, plasmid addicted value-based product formation was medium- and carbon-source dependent. Another example was the plasmid-based expression of a synthetic 1-butanol operon in an *E. coli* mutant strain that restored anaerobic growth; as a consequence, 1-butanol was produced (Shen et al., 2011). However, in this instance, expression of the plasmid-based operon was dependent on co-inducer, isopropyl β -D-1-thiogalactopyranoside (IPTG), addition. Recently, the production of ethanol was accomplished in *E. coli* and, similarly to other examples, plasmid addiction was carbon source-dependent (Wong et al., 2014).

To date, all recently employed PAS systems negate the requirement of antibiotics for plasmid stability. However, each of these PAS systems relies on specific constraints; for example, the use of a specific carbon source or medium-specific growth condition, as well as the need for co-inducer supplementation for up-regulated gene expression. There is one exception that does not rely on any constraints; however, in this example the product titer was less than that of the control strain (Kroll et al., 2009). The aforementioned constraints limit the flexibility for industrial scale production. Addressing these issues, we now report the development of a plasmid addicted 1-butanol production system in *E. coli* that

negates the need for expensive co-inducers and antibiotics, and is not limited by medium, carbon source, or growth condition. Without the constraints usually associated with metabolism-based plasmid addicted value-based product synthesis, this system produced significant yields of 1-butanol using a test strain of *E. coli* during semi-continuous batch culture.

2. Materials and methods

2.1. Reagents

The chemicals used were acquired from Sigma-Aldrich (St. Louis, MO) or Fisher Scientifics (Pittsburgh, PA). Oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO). Phusion High-Fidelity DNA polymerase and restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Invitrogen T4 ligase was obtained from Life Technologies (Grand Island, NY).

2.2. Bacterial strains

E. coli strain JM109 (Yanisch-Perron et al., 1985) was used for propagation of plasmids and strain BW25113 (Datsenko and Wanner, 2000) was used for plasmid-addicted 1-butanol production.

2.3. Plasmid and strain construction

Plasmids and strains used in this study are listed in Table 1. To construct plasmids p62, p72, p91, p91BuOH(1), and p91BuOH(1)*lptB*, the multiple cloning site from pUC19, particularly the nucleotide sequence that spans the *SacI* and *HindIII* restriction sites, was subcloned into plasmid 3716 (a derivative of pLO11 (Schwarze et al., 2010) with a pBAD promoter and the *araC* gene (kindly provided by Dr. Oliver Lenz, Berlin, Germany)), replacing the existing nucleotide sequence between the *SacI* and *HindIII* restriction sites. As a result, the number of available restriction sites for cloning purposes was expanded, resulting in the construction of plasmid p62. A *NcoI* restriction site was introduced within the *araC* gene of p62 by site directed mutagenesis, thus resulting in plasmid p72. This allows one to replace the existing pBAD promoter with a promoter of choice flanked by *NcoI* and *SacI* restriction sites. The promoter sequence of the chromosomal *cbbL* gene from *R. eutropha* strain H16 was amplified from genomic DNA and cloned into pUC19. Site-directed mutagenesis was then performed on the *cbbL* promoter region. Specifically, the nucleotide sequence of the – 35 element within the *cbbL* promoter was changed to the consensus *E. coli* nucleotide sequence (TTGACA).

Table 1
Plasmids and strains used in this study.

| Plasmid or strain | Relevant characteristics | Reference or source |
|------------------------|---|------------------------------|
| <i>Plasmids</i> | | |
| pUC19 | General cloning vector | New England Biolabs |
| #3716 | Derivative of pLO11, contains the pBAD promoter and <i>araC</i> gene | Oliver Lenz, Berlin, Germany |
| p62 | #3716 Derivative with pUC19 multiple cloning site | This work |
| p72 | p62 Derivative with <i>NcoI</i> restriction site in <i>araC</i> gene | This work |
| p91 | p72 Derivative with modified <i>R. eutropha cbbL</i> promoter | This work |
| p91BuOH(1) | Synthetic butanol operon (<i>hbd</i> , <i>crt</i> , <i>ter</i> , <i>atoB</i> , <i>adhE2</i>) | This work |
| p91BuOH(1) <i>lptB</i> | Synthetic butanol operon with essential <i>lptB</i> gene | This work |
| <i>Strains</i> | | |
| BW25113 | Wild-type strain | ATCC, Manassas, Virginia |
| RKE01 | BW25113 Δ <i>lptB</i> p91BuOH(1) <i>lptB</i> | This work |
| RKE03 | BW25113 Δ <i>lptB</i> Δ <i>adhE</i> p91BuOH(1) <i>lptB</i> | This work |
| RKE05 | BW25113 Δ <i>lptB</i> Δ <i>adhE</i> Δ <i>pta</i> p91BuOH(1) <i>lptB</i> | This work |
| RKE07 | BW25113 Δ <i>lptB</i> Δ <i>adhE</i> Δ <i>pta</i> Δ <i>ldhA</i> p91BuOH(1) <i>lptB</i> | This work |
| RKE09 | BW25113 Δ <i>lptB</i> Δ <i>adhE</i> Δ <i>pta</i> Δ <i>ldhA</i> Δ <i>frdABCD</i> p91BuOH(1) <i>lptB</i> | This work |

This promoter was shown to have high activity in *E. coli* (Jeffke et al., 1999). The modified promoter was then amplified using primers flanked by NcoI and SacI restriction sites, and the amplicon was cloned into p72, resulting in plasmid p91.

The *hbd* and *crt* genes were amplified from *C. acetobutylicum* genomic DNA and cloned into plasmid pBBR1MCS-3. Specifically, the *hbd* gene was cloned into the KpnI and XhoI restriction sites, and the *crt* gene was cloned into the XhoI and SmaI restriction sites, resulting in plasmid pBBR1MCS-3HC. The *ter* gene was amplified from *Treponema denticola* genomic DNA and cloned into the SmaI and SpeI restriction sites of pBBR1MCS-3HC, resulting in plasmid pBBR1MCS-3HCT. Then the entire *hbd*, *crt*, and *ter* nucleotide region from pBBR1MCS-3HCT was amplified and cloned into the SacI and XbaI multiple cloning site of p91, resulting in plasmid p91HCT. Next, the *atoB* gene from *E. coli* was amplified from genomic DNA and cloned into the XbaI multiple cloning site of p91HCT, resulting in plasmid p91HCT*atoB*. The *adhE2* gene was amplified from *C. acetobutylicum* genomic DNA and cloned into the XbaI and HindIII multiple cloning sites of p91HCT*atoB*, resulting in plasmid p91HCT*atoBadhE2*. This plasmid was designated as p91BuOH(1). Finally, the *lptB* gene was amplified from *E. coli* genomic DNA and cloned between the *hbd* and *crt* genes of p91BuOH(1), using an available XhoI restriction site. The resulting plasmid was designated as p91BuOH(1)*lptB*.

To construct the plasmid addicted 1-butanol producing strains, plasmid p91BuOH(1)*lptB* was first used to construct strain RKE01, an *lptB* chromosomal gene deletion strain carrying a previously described *lptB* allele (Sherman et al., 2014). The *adhE* gene was inactivated in *E. coli* strain RKE01, resulting in strain RKE03. Next, the *pta* gene was inactivated in strain RKE03, resulting in strain RKE05. Strain RKE07 was constructed by inactivation of the *ldhA* gene in strain RKE05. Finally, the *frdABCD* genes were inactivated in strain RKE07, resulting in strain RKE09.

All amplicons and constructed plasmids were sequenced to ensure that no mutations occurred. In terms of a ribosome-binding site (RBS) for translation of each gene, the RBS within the *cbbl* promoter was used for the *hbd* gene and the remaining genes used an optimized RBS (Sichwart et al., 2011). All chromosomal deletion alleles were transferred using P1vir transduction by selecting for the kanamycin-resistance cassette that was used to replace the deleted gene of interest (Silhavy et al., 1984) and confirmed by genomic polymerase chain reaction sequence analysis. After successful transduction of each deletion allele, the kanamycin cassette was excised as previously described (Cherepanov and Wackernagel, 1995).

2.4. 1-Butanol production medium

Production of 1-butanol was performed in Terrific Broth (TB) (12 g tryptone, 24 g yeast extract, 2.31 g KH₂PO₄, 12.54 g K₂HPO₄, per liter of water) supplemented with glycerol or glucose.

2.5. Culture conditions for 1-butanol production

Single colonies were seed cultured in 10 ml TB with 12.5 µg/ml tetracycline at 30 °C with 200 rpm shaking in a rotary incubator shaker overnight and inoculated at 5% of the total culture volume into vessels for testing different 1-butanol production conditions. Four different growth conditions were examined: aerobic; anaerobic; semi-aerobic; and high cell density. For the aerobic 1-butanol production condition, 150 ml of TB was used in 250 ml shake flasks. Flasks were incubated at 30 °C with 200 rpm shaking in a rotary incubator shaker. For anaerobic 1-butanol production, 50 ml of TB in 160 ml serum bottles were purged with N₂ for 5 min, closed with a thick butyl rubber stopper and sealed with an aluminum crimp. Cultures were incubated at 30 °C without shaking. For semi-aerobic 1-butanol production, 5 ml of TB in a 12 ml capped test tubes were incubated

at 30 °C with 200 rpm shaking. In addition, 50 ml of TB in 160 ml serum bottles were closed with a thick butyl rubber stopper and sealed with an aluminum crimp. These 50 ml semi-aerobic cultures were incubated at 30 °C with 200 rpm shaking in a rotary incubator shaker overnight and then moved to an incubator without shaking. For high cell density 1-butanol production conditions, 150 ml TB in 250 ml shake flasks were used. Flasks were incubated at 30 °C with 200 rpm shaking in a rotary incubator shaker overnight. The next morning, the cells were collected by centrifugation at 10,000g for 10 min at 4 °C. The cell pellet was then resuspended in 25 ml TB. The cell suspension was transferred to 160 ml serum bottles (purged with N₂ for 5 min for high density anaerobic growth, closed with a thick butyl rubber stopper, sealed with an aluminum crimp, and then autoclaved). Cultures were incubated at 30 °C without shaking.

To determine the relative stability and duration of 1-butanol production of semi-continuous aerobic batch cultures, 250 ml shake flasks containing 150 ml TB supplemented with 2% glycerol was initially used. Batch cultures were incubated at 30 °C with 200 rpm shaking in a rotary incubator shaker. This first batch culture was inoculated with 7.5 ml of an overnight 30 ml seed culture and cultured for 72 h. Then 135 ml of culture from the first batch culture was removed and replaced with 135 ml of fresh medium, resulting in the second batch culture. Likewise, the second batch culture was used to create the third batch culture. 1-Butanol was measured at the end of each batch cycle (72 h) since this time frame resulted in maximal butanol production under the conditions described. Thus, the stability of the described PAS system was monitored over 210 h.

Unless otherwise noted, the culture pH was adjusted to around 7 using 5 N NaOH on a daily basis for all cultures. Samples were taken daily to monitor cell growth (OD₆₀₀), substrate (glycerol or glucose) consumption, and production of 1-butanol, butyric acid, ethanol, and acetic acid during the fermentation.

2.6. Detection of 1-butanol, glycerol, and glucose

The quantification of 1-butanol was performed independently in two different laboratories, each using a GC-2014 (Shimadzu, Columbia, MD) gas chromatograph (GC) equipped with a flame ionization detector and a Stabilwax DA (Restek, Bellefonte, PA) column (30 m, 0.32 mm ID, 0.25 µm film thickness). For the first method, the GC oven temperature was initially held at 50 °C, raised with a gradient of 10 °C/min until a temperature of 125 °C was reached, then raised with a gradient of 20 °C/min until a temperature of 220 °C was reached; this was held for 10 min. Helium was used as the carrier gas. 1 µl of spent medium was injected in a split ratio of 1:5. For the second method, the GC was operated at an injection temperature of 200 °C with 1 µl of sample injected with an auto injector (AOC-20i, Shimadzu). The column temperature was initially held at 80 °C for 3 min, then increased at a constant rate of 30 °C per min to 150 °C, and held at 150 °C for 3.7 min. Both methods yielded identical measurements of 1-butanol levels in the culture media. The second method was also used to quantify ethanol, acetate, and butyrate. Fermentation broth samples were analyzed for their glycerol and glucose consumption using a high performance liquid chromatograph (HPLC) equipped with an organic acid analysis column (Bio-Rad HPX-87H) and a refractive index detector (Shimadzu RID-10A) at 45 °C, with 0.007 M H₂SO₄ as the eluent at 0.6 ml/min.

3. Results

3.1. Construction of metabolism-based plasmid addicted 1-butanol production strains

A metabolism-based plasmid addiction system relies on plasmid-based expression of an essential gene in a strain where

the chromosomal essential gene is inactivated. There are two types of metabolism-based plasmid addition systems used: a catabolism- and an anabolism-based system (Kroll et al., 2010). The catabolism-based system relies on an essential gene that is involved in the metabolism of an essential carbon or energy source, while the anabolism-based system relies on an essential gene for a required anabolic pathway. In this study, we used both metabolism-based plasmid addition systems for the production of 1-butanol. The essential anabolic system that we targeted was lipopolysaccharide (LPS) biogenesis, specifically the essential *lptB* gene. LPS is a cell surface glycolipid that is required for viability of *E. coli*. LptB is also essential for survival because it functions as the ATPase of the ABC transporter that powers LPS transport to the cell surface (Sherman et al., 2014; Sperandeo et al., 2008). We targeted LPS biogenesis for plasmid addition, as LPS synthesis is essential regardless of carbon source or growth condition (aerobic or anaerobic). The essential catabolic system that we targeted was fermentation, specifically essential genes that encode alcohol dehydrogenase, lactate dehydrogenase, and fumarate reductase enzymes. It was previously demonstrated that inactivation of these genes prevents anaerobic growth of *E. coli* and that 1-butanol production rescues growth by restoring redox balance (Shen et al., 2011). Therefore, our system establishes a stringent basis for maintaining plasmid stability by providing two essential plasmid-based metabolic systems under anaerobic growth conditions, the preferred growth condition for industrial production (due to cost savings associated with not having to maintain a rigorous aerobic environment).

In order for plasmid addicted 1-butanol production to occur without constraints, we have developed a system whereby the cell must maintain a plasmid that expresses a synthetic 1-butanol operon as well as an essential gene. With such a construct, there is no requirement of antibiotics to maintain plasmid stability (Fig. 1). Moreover, to eliminate the need for adding expensive exogenous co-inducer compounds to drive gene expression, the modified *R. eutropha cbbL* promoter (Section 2) was chosen to constitutively express at high levels the synthetic 1-butanol operon (plasmid p91BuOH(1)). To alleviate the need for antibiotic, the essential *lptB* gene was cloned within the synthetic 1-butanol operon (plasmid

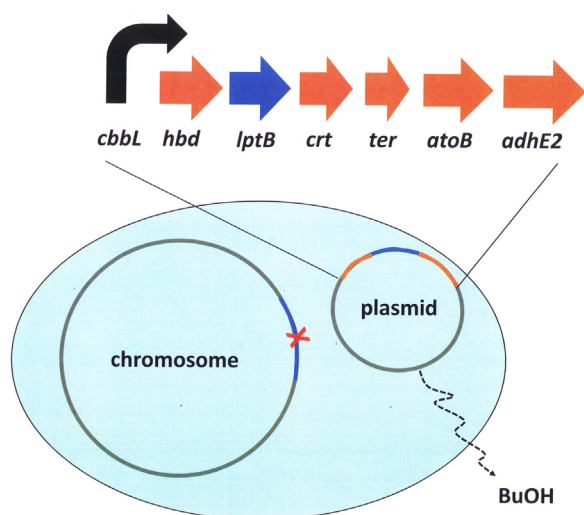


Fig. 1. The *lptB* gene plasmid addicted 1-butanol production system. The chromosomal *lptB* gene (blue) was inactivated and placed within the synthetic plasmid-based 1-butanol operon (orange) to construct plasmid p91BuOH(1)*lptB*. The constitutive *cbbL* gene promoter was used to drive expression of the essential synthetic operon. In this system, the requirement of co-inducer to drive gene expression was negated and the use of antibiotic was not required for plasmid stability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

p91BuOH(1)*lptB*) (Fig. 1). Clearly, expression of the synthetic 1-butanol operon and essential *lptB* gene all rely on the constitutive promoter, thus negating the need for co-inducer; plasmid stability was maintained by virtue of expression of the essential *lptB* gene, as opposed to any requirement for the presence of antibiotic.

With the rationale provided in Fig. 1, plasmids p91BuOH(1) and p91BuOH(1)*lptB* were separately transformed into *E. coli* strain BW25113; deletion of the chromosomal *lptB* gene was performed via P1vir phage transduction. As expected, the strain that contained plasmid p91BuOH(1) was not viable due to the absence of either a chromosomal or plasmid-based essential *lptB* gene. However, plasmid p91BuOH(1)*lptB* rescued growth in the chromosomal *lptB* gene deletion mutant strain due to expression of the plasmid-based essential *lptB* gene, thus resulting in the construction of strain RKE01.

3.2. Plasmid addicted 1-butanol production

The selection of genes for the synthetic 1-butanol operon used in this study was based on prior knowledge. It had been previously demonstrated that clostridial and non-clostridial gene products are capable of the multistep conversion of acetyl-CoA to 1-butanol. Therefore, we acquired specific 1-butanol genes that catalyze this conversion to construct a synthetic 1-butanol operon. Strain RKE01, which is addicted to plasmid p91BuOH(1)*lptB*, contains all the required genes for 1-butanol production (Fig. 1).

Strain RKE01 was grown in small-scale cultures using 5 ml TB medium in 12 ml screw capped test tubes supplemented with 2% glycerol under semi-aerobic conditions and then examined for the production of 1-butanol, in the absence of co-inducer and antibiotic. Strain RKE01 produced 56 mg/L of 1-butanol (Fig. 2). However, previous studies had indicated that increased 1-butanol production occurred after inactivation of the synthesis of fermentative products such as ethanol, acetate, lactate, and succinate (Shen et al., 2011). Therefore, the orderly and additive deletion of genes encoding enzymes of these fermentative pathways was performed using RKE01 as parent strain. This resulted in the construction of strains RKE03($\Delta adhE$), RKE05($\Delta adhE \Delta pta$), RKE07($\Delta adhE \Delta pta \Delta ldhA$), and RKE09($\Delta adhE \Delta pta \Delta ldhA \Delta frdABCD$). Inactivation of the alcohol dehydrogenase gene (*adhE*) in strain RKE03 increased 1-butanol production by 3.8-fold (Fig. 2). In strain RKE05, the additional inactivation of the phosphotransacetylase gene (*pta*) resulted in an additional 4.3-fold increase in 1-butanol production (Fig. 2). Next, a further 1.2-fold increase in 1-butanol production was obtained when the lactate dehydrogenase gene (*ldhA*) was additionally inactivated in strain RKE07 (Fig. 2).

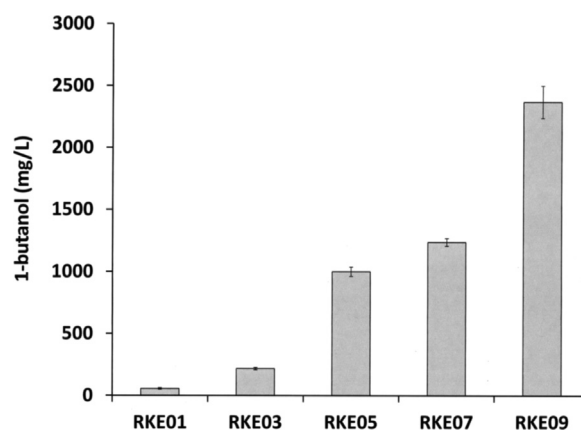


Fig. 2. Small-scale semi-aerobic 1-butanol production in plasmid addicted strains. Strains RKE01, RKE03 ($\Delta adhE$), RKE05 ($\Delta adhE \Delta pta$), RKE07 ($\Delta adhE \Delta pta \Delta ldhA$), and RKE09 ($\Delta adhE \Delta pta \Delta ldhA \Delta frdABCD$) were cultured and 1-butanol levels monitored as described in Section 2.

Lastly, when the fumarate reductase genes (*frdABCD*) were also inactivated, in strain RKE09, a level of 2.3 g/L of 1-butanol was produced (Fig. 2). In summary, the step-wise inactivation of the indicated genes resulted in a 40-fold increase in butanol production in strain RKE09 as compared to strain RKE01. According to previous findings (Shen et al., 2011; Bond-Watts et al., 2011), this level of butanol produced may be enhanced even further to 15–30 g/L by an additional modification, however for the purposes of this investigation we were primarily interested in demonstrating stable 1-butanol production in the absence of co-inducers and antibiotics.

Strain RKE09 was further examined for 1-butanol production in larger-scale cultures in TB medium under different growth conditions. Aerobic, anaerobic, semi-aerobic, and high cell density growth conditions were examined with the supplement of 2% glycerol. 1-Butanol production increased to 4.7 g/L (yield: 0.23 g butanol/g of glycerol used, productivity: 0.047 g 1-butanol synthesized/L/h) under aerobic growth conditions but decreased somewhat under anaerobic, semi-aerobic, and high cell density growth conditions (Table 2 and Fig. 3). The maximum level of 1-butanol production (4.7 g/L) compared favorably to the previously mentioned initial small-scale semi-aerobic production studies (2.3 g/L). Aerobic growth was further examined by increasing glycerol levels from 2% to 5%, however, no significant increase in 1-butanol production occurred. Anaerobic, semi-aerobic, and high cell density 1-butanol production was also reexamined, by using 2% glucose as opposed to 2% glycerol. The production of 1-butanol increased with 2% glucose supplementation, as compared to 2% glycerol (Table 2). Similarly, when glucose was increased to 5%, 1-butanol production was further improved under these conditions (Table 2).

3.3. Antibiotic requirement for 1-butanol production

In larger-scale experiments using varying growth parameters, the requirement for tetracycline addition was examined to determine whether maximal 1-butanol production was affected by its presence or absence; e.g. to ensure that plasmid-borne genes were maintained in strain RKE09. While it was demonstrated that strain RKE09 produced 1-butanol under small-scale test tube growth conditions without antibiotic addition, it was important to verify that production levels were maintained in larger cultures. Therefore, experiments were repeated and tested under aerobic, anaerobic, semi-aerobic, and high cell density growth conditions with larger scale cultures, using media supplemented with 5% glucose. Cultures were grown in the presence or absence of antibiotic (tetracycline). The production of 1-butanol was monitored in these cultures and it was clear that 1-butanol levels in strain

RKE09 were similar whether the antibiotic was present or absent (Table 3).

3.4. Long-term production of 1-butanol using the PAS system and the constitutive promoter

An experiment was devised whereby strain RKE09 was cultured until it maximally produced 1-butanol in the absence of co-inducers and antibiotics. After 72 h, cells from this initial culture were used to inoculate fresh media in a second culture, which was allowed to grow for 72 h as before. Cells from this second culture were then used to inoculate a third culture and maintained for another 72 h. This successive 216 h experiment indicated that there was no instability or substantial decrease in 1-butanol production over this time (Fig. 4). Thus, the plasmid was stably maintained over this time without the addition of antibiotics, and the promoter was also actively maintained, resulting in stable gene expression over the time of this experiment.

4. Discussion

The ability to use microorganisms as biological factories for the production of value-based products is becoming commonplace due to (1) increased metabolic knowledge of selected host microorganisms, thus allowing for the manipulation of native biochemical pathways and (2) the use of synthetic biology principles whereby key genes encoding for desired foreign biochemical pathways may be used for synthesis of desired value-based products. In order to

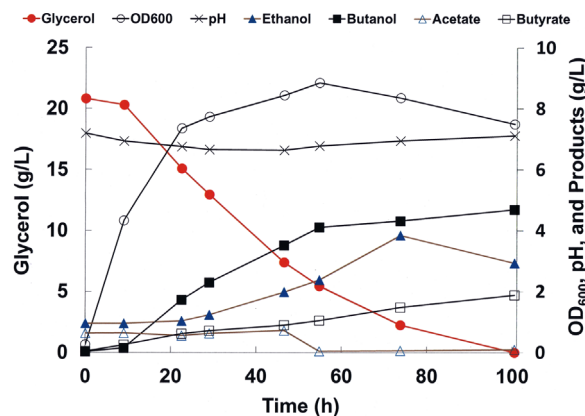


Fig. 3. Fermentation kinetics of RKE09 under aerobic growth conditions with pH controlled at 7.0 with 5 N NaOH. Aerobic growth consisted of a culture volume of 150 ml in a 250 ml flask. Gas chromatography was used to measure the levels of 1-butanol, ethanol, acetate, and butyrate. High performance liquid chromatography was used to measure glycerol levels.

Table 2

Large-scale 1-butanol production in strain RKE09 under different growth conditions with media supplemented with either glycerol or glucose.

| | 1-Butanol (titer, yield, and productivity) | | | | | | | | |
|--------------------------------|--|-------------|----------------------|-----------------|-----------------|----------------------|-------------|-------------|----------------------|
| | 2% Glycerol | | | 2% Glucose | | | 5% Glucose | | |
| | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) |
| Aerobic ^a | 4.7 | 0.23 | 0.047 | ND ^b | ND ^b | ND ^b | 3.3 | 0.084 | 0.036 |
| Anaerobic ^c | 1.1 | 1.02 | 0.0035 | 2.6 | 0.14 | 0.028 | 4.1 | 0.15 | 0.044 |
| Semi-aerobic ^d | 1.4 | 0.30 | 0.015 | 2.2 | 0.28 | 0.028 | 3.6 | 0.26 | 0.038 |
| High cell density ^e | 1.6 | 0.042 | 0.006 | 2.1 | 0.38 | 0.0088 | 3.4 | 0.20 | 0.029 |

^a 150 ml Culture in a 250 ml non-sealed flask.

^b Not determined.

^c 50 ml Culture in a N₂ purged 160 ml sealed serum bottle.

^d 50 ml Culture in a 160 ml sealed serum bottle.

^e 25 ml Culture (resuspended from 150 ml culture) in a N₂ purged 160 ml sealed serum bottle.

Table 3
1-Butanol production in strain RKE09 in the presence or absence of antibiotic.

| Antibiotic ^a | 1-Butanol (titer, yield, and productivity) | | | | | | | | | | | |
|-------------------------|--|-------------|----------------------|------------------------|-------------|----------------------|---------------------------|-------------|----------------------|--------------------------------|-------------|----------------------|
| | Aerobic ^b | | | Anaerobic ^c | | | Semi-aerobic ^d | | | High cell density ^e | | |
| | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) | Titer (g/L) | Yield (g/g) | Productivity (g/L/h) |
| + | 3.3 | 0.084 | 0.036 | 4.1 | 0.15 | 0.044 | 3.6 | 0.26 | 0.038 | 3.4 | 0.20 | 0.029 |
| – | 3.1 | 0.084 | 0.033 | 4.4 | 0.14 | 0.039 | 4.4 | 0.26 | 0.020 | 4.3 | 0.23 | 0.045 |

^a Tetracycline at 12.5 µg/ml was used where indicated.

^b 150 ml Culture in a 250 ml non-sealed flask.

^c 50 ml Culture in a N₂ purged 160 ml sealed serum bottle.

^d 50 ml Culture in a 160 ml sealed serum bottle.

^e 25 ml Culture (resuspended from 150 ml culture) in a N₂ purged 160 ml sealed serum bottle.

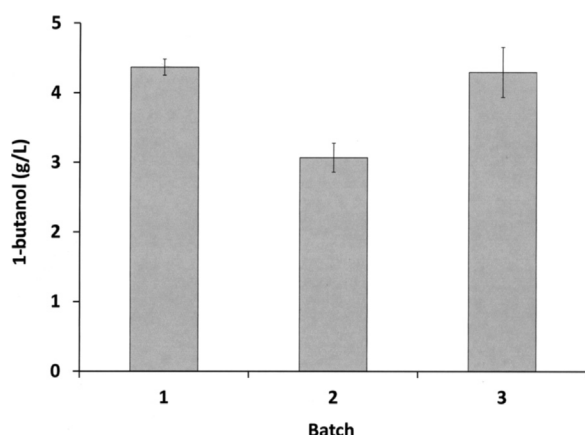


Fig. 4. 1-Butanol production by strain RKE09 grown under aerobic semi-continuous batch culture conditions. Batch cultures were grown for 72 h and 1-butanol production was measured at the end of each batch culture cycle. The preceding batch culture served as the inoculum for the ensuing batch culture.

engineer microbes to serve as metabolic factories for industrial scale production of value-based compounds the selected system must be easy to manipulate and the costs associated with growing the organism for maximal product formation must be low. In this study we have developed a metabolism-based plasmid addiction system in *E. coli* for the production of a model bioproduct, 1-butanol, which retains the benefits of a plasmid-based system but without the cost associated with co-inducers to up-regulate gene expression or antibiotics to maintain plasmid stability. Moreover, the strategy we have developed abrogates environmental issues associated with large-scale usage of antibiotics. The plasmid addiction strategy developed here involves the expression of the essential plasmid-borne *lptB* gene, which is co-expressed with genes of a synthetic 1-butanol pathway, thus ensuring that microbial growth and cell viability involves synthesis of the desired product, 1-butanol in this case, without the need for antibiotics to maintain plasmid stability. In addition, when anaerobic growth was employed, the production of 1-butanol served as a second essential system for cell viability, thus increasing the stringency for plasmid addiction. It is important to note that our system employs a promoter that allows for constitutive expression of desired genes, such that expensive cofactors such as IPTG are unnecessary. As a result, under small-scale semi-aerobic conditions, the initial plasmid-addicted strain produced 56 mg/L of 1-butanol without the use of co-inducer and antibiotics. Further development of this strain, by inactivation of competing fermentative pathways, resulted in the production of 2.3 g/L under small-scale conditions, again without the requirement of co-inducers or antibiotics. Further extensive studies were performed under larger-scale growth conditions, with supplementation of growth media with

glycerol or glucose under aerobic, anaerobic, semi-aerobic, and high cell density conditions. Using strain RKE09, a maximum titer of 4.7 g/L was produced under aerobic growth with 2% glycerol. Under anaerobic conditions, the 1-butanol titer was highest with glucose supplementation as opposed to glycerol addition, with a titer of 4.1 g/L of 1-butanol (yield: 0.15 g butanol/g of glucose utilized, and the productivity was at 0.044 g butanol produced/L/h). In addition, larger-scale cultures did not require the presence of antibiotics to maintain plasmid stability, with high levels of 1-butanol obtained in the absence of antibiotics. These studies with larger scale cultures confirmed initial results with the small culture studies and verified the stability of our PAS 1-butanol producing strain, regardless of culture size or growth mode. Finally, strain RKE09 was able to stably produce high levels of 1-butanol over a time period of over 200 h during semi-continuous growth with no hint of any instability.

5. Conclusion

Competitive yields of 1-butanol were produced without the requirement of antibiotic or co-inducer supplementation over a time period of 9 days. Furthermore, the PAS system described here is versatile, being independent of carbon source or growth mode, as compared to other systems. Finally, a PAS-based 1-butanol production strain should lend itself well to industrial-scale, low-cost production, particularly with additional strain modifications. For example, it was previously demonstrated that 1-butanol production in *E. coli* could reach levels up to 30 g/L by over-expression of the formate dehydrogenase gene and continuous removal of 1-butanol by gas stripping from a pH controlled batch fermentor (Shen et al., 2011). In addition, over-expression of the pyruvate dehydrogenase complex provided increased levels of NADH and markedly improved 1-butanol production in *E. coli* (Bond-Watts et al., 2011). Based on our initial experiments, we might realistically expect that a similarly modified strain RKE09 might continuously produce high levels of 1-butanol, however without the need for co-inducers or antibiotics. The major conclusion from this study, however, is that plasmid-addicted strains containing constitutive promoters will be highly useful for synthetic biology approaches for low-cost industrial synthesis of bioproducts and biofuels.

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