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Estabilização plasmidial através da complementação do gene *icd*^{NAD} e bioprodução de 1,3-propanadiol em *Escherichia coli* recombinante

Dissertação apresentada ao Programa de Pós-graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Mestre em Microbiologia.

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CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB nº **904/2017** referente ao projeto intitulado: ***"Estudo da produção de 1,3-propanodiol em biorreator e a sua relação com a disponibilidade de oxigênio dissolvido no meio"*** sob a responsabilidade de ***Jung Hun Park*** e orientação do(a) Prof.(a) Dr.(a) ***José Gregório Cabrera Gomez***, do Departamento de Microbiologia, foi analisado pela **CEUA** - Comissão de Ética no Uso de Animais e pelo **CEPSH** – Comitê de Ética em Pesquisa com Seres Humanos, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da Lei nº 11.794, de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº 466 de 2012.

São Paulo, 21 de agosto de 2017.

Profa. Dra. **Luciane Valéria Sita**
Coordenadora CEUA ICB/USP

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Decl. CEPSH.03.2019

DECLARAÇÃO

Em adendo ao Certificado de Isenção **CEP nº 904/2017**, aprovado em 21/08/2017, e por solicitação do Prof. Dr. **José Gregório Cabrera Gomez**, do departamento de Microbiologia, informo que o título do projeto do aluno **Jung Hun Park** foi alterado para ***"Estabilização plasmidial através da complementação do gene icd^{NAD} e bioprodução de 1,3-propanodiol em Escherichia coli recombinante"***, devido às adequações que o projeto sofreu durante o seu desenvolvimento.

São Paulo, 09 de dezembro de 2019.

Profa. Dra. **Camila Squarzoni Dale**
Coordenadora da CEPSH-ICB/USP

Profa. Dra. **Luciane Valéria Sita**
Coordenadora CEUA-ICB/USP

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RESUMO

Park JH. Estabilização plasmidial através da complementação do gene *icd*^{NAD} e bioprodução de 1,3-propanodiol em *Escherichia coli* recombinante. [Dissertação (Mestrado em Microbiologia)] – Instituto de Ciências Biomédicas, Universidade de São Paulo; 2020.

O 1,3-propanodiol (1,3-PDO) é um componente primário para a produção de biopolímeros de alto valor agregado. Este composto pode ser produzido por algumas bactérias em microaerobiose, utilizando glicerol como fonte de carbono. Em um projeto anterior do Laboratório de Bioprodutos, uma cepa de *Escherichia coli* MG1655 foi transformada com um plasmídeo de produção (pBBR1MCS2 :: *dha*) contendo genes de *K. pneumoniae* (operon *dha*) necessários para a conversão de glicerol em 1,3-PDO. No entanto, este plasmídeo é mantido através do uso da canamicina ao meio de cultura, resultando em custos adicionais e questões de biossegurança que podem afetar a produção em larga escala. Para superar este problema, foi proposta uma estratégia de complementação do gene *icd* para estabilizar o plasmídeo de produção, sem a necessidade de antibióticos. O gene codifica uma Isocitrato desidrogenase NAD-dependente e é essencial para o crescimento da bactéria em meio mínimo.

Este projeto teve como objetivo construir uma cepa de *E. coli* com o gene *icd* deletado em seu cromossomo, mas complementada com o mesmo gene no plasmídeo de produção.

A cepa complementada com *icd*$^{NAD}_{(pJ23100)}$ foi testada no ensaio de frasco agitado e a produção de 1,3-PDO sem antibiótico mostrou resultados promissores, produzindo a mesma quantidade de produto que a cepa de controle cultivada com antibiótico. No ensaio de biorreator, a cepa apresentou um fenótipo incomum, no qual a cultura morria ao adicionar anti-espumante e o oxigênio dissolvido (DO) no meio cair repentinamente. Apesar deste problema, uma solução foi encontrada diminuindo gradualmente a DO para atingir a microaerobiose. O rendimento de 1,3-PDO pela cepa complementada por *icd*$^{NAD}_{(pJ23100)}$, sem antibiótico, foi comparável à cepa controle sem a complementação genética usando antibiótico. Apesar disso, foi detectada uma considerável perda de plasmídeo na população de bactérias utilizando a estratégia de complementação gênica. Portanto, uma combinação com um outro sistema de estabilização poderia aumentar ainda mais a produção de 1,3-PDO. Acredita-se que a morte da cultura de células no biorreator esteja relacionada ao aumento da produção de NADH, promovida pela super-expressão do gene *icd*^{NAD}.

Palavras-chave: 1,3-propanodiol. *icd*. NADH. Estabilização plasmidial. *Escherichia coli*

ABSTRACT

Park JH. Plasmid stabilization by icdNAD gene complementation and bioproduction of 1,3-propanediol in recombinant Escherichia coli. [Dissertation (Master thesis in Microbiology)] – Instituto de Ciências Biomédicas, Universidade de São Paulo; 2020.

1,3-propanediol (1,3-PDO) is a primary component for the production of high added value biopolymers. This compound can be produced by some bacteria in microaerobiosis, using glycerol as a carbon source. In the previous project in our laboratory, an *Escherichia coli* MG1655 strain was transformed with a production plasmid (pBBR1MCS2::*dha*) containing *K. pneumoniae* genes (operon *dha*) necessary for the conversion of glycerol to 1,3-PDO. However, the plasmid is maintained by adding kanamycin to the medium, resulting in an additional cost and biosafety issues for large-scale production.

To overcome this problem, it was proposed a strategy of *icd* gene complementation to stabilize the production plasmid without the need for antibiotic. The gene codes for a NAD-dependent Isocitrate dehydrogenase and is essential for growth in minimal medium.

This project aimed to construct an *E. coli* strain lacking *icd* in its chromosome, complemented with the same gene in the production plasmid.

The *icd*$^{NAD}_{(pJ23100)}$ complemented strain was tested in shake flask assay and the production of 1,3-PDO without antibiotic showed promising results, yielding the same amount of product as the control strain cultivated with antibiotic. In the bioreactor assay, the strain showed an unusual phenotype, in which the culture died when anti-foam was added and Dissolved Oxygen (DO) in the medium suddenly dropped. Despites of this problem, a solution was found by gradually decreasing the DO in order to achieve microaerobiosis. The yield of 1,3-PDO by *icd*$^{NAD}_{(pJ23100)}$ complemented strain, without antibiotic, was comparable to the strain without gene complementation using antibiotic. Despites of that, considerable plasmid loss was detected in gene complementation strategy, therefore a combination with another stabilization system could enhance the 1,3-PDO production even further.

Cell culture death in bioreactor was suggested to be linked to the overproduction of NADH, promoted by *icd*$^{NAD}_{(pJ23100)}$ overexpression.

Keywords: 1,3-propanediol. *icd*. NADH. Plasmid stabilization. *Escherichia coli*

ABSTRAKT

1,3-Propandiol (1,3-PDO) ist eine Hauptkomponente für die Herstellung von Biopolymeren mit hoher Wertschöpfung. Diese Verbindung kann von einigen Bakterien unter Verwendung von Glycerin als Kohlenstoffquelle hergestellt werden. In dem vorherigen Projekt in unserem Labor wurde ein *Escherichia coli* MG1655-Stamm mit einem Produktionsplasmid (pBBR1 :: *dha*) transformiert, das K. pneumoniae-Gene (Operon *dha*) enthielt, die für die Umwandlung von Glycerin zu 1,3-PDO erforderlich sind. Das Plasmid wird jedoch durch Zugabe von Kanamycin zu dem Medium aufrechterhalten, was zu zusätzlichen Kosten- und Biosicherheitsproblemen bei der Produktion in großem Maßstab führt.

Um dieses Problem zu überwinden, wurde eine Strategie der icd-Genkomplementierung zur Stabilisierung des Produktionsplasmids ohne die Notwendigkeit eines Antibiotikums vorgeschlagen. Das Gen kodiert für eine NAD-abhängige Isocitrat-Dehydrogenase und ist für das Wachstum in Minimalmedium essentiell.

Dieses Projekt zielte darauf ab, einen *E. coli*-Stamm zu konstruieren, dem *icd* im Chromosom fehlt und der mit demselben Gen im Produktionsplasmid pBBR1MCS2 :: *dha* komplementiert ist.

Der mit $icd^{NAD}_{(pJ23100)}$ komplementierte Stamm wurde im Schüttelkolben-Assay getestet und die Herstellung von 1,3-PDO ohne Antibiotikum zeigte vielversprechende Ergebnisse und ergab die gleiche Produktmenge wie der mit Antibiotikum kultivierte Kontrollstamm. Im Bioreaktor-Assay zeigte der Stamm einen ungewöhnlichen Phänotyp, bei dem die Kultur starb, wenn Antischaum zugesetzt wurde und der gelöste Sauerstoff (DO) in dem Medium plötzlich abfiel. Trotz dieses Problems wurde eine Lösung gefunden, indem der DO allmählich verringert wurde, um eine Mikroaerobiose zu erreichen. Die Ausbeute an mit 1,3-PDO durch $icd^{NAD}_{(pJ23100)}$ komplementiertem Stamm ohne Antibiotikum war vergleichbar mit dem Stamm ohne Genkomplementierung unter Verwendung von Antibiotikum. Trotzdem wurde ein beträchtlicher Plasmidverlust in der Genkomplementierungsstrategie festgestellt, weshalb eine Kombination mit einem anderen Stabilisierungssystem die 1,3-PDO-Produktion noch weiter steigern könnte.

Es wurde vermutet, dass der Zellkulturtod im Bioreaktor mit der durch Überexpression von $icd^{NAD}_{(pJ23100)}$ hervorgerufenen Überproduktion von NADH zusammenhängt.

1 INTRODUCTION

1,3-propanediol (1,3-PDO) is an organic compound that can be used to produce a diverse range of materials, such as adhesive, paints, solvent, cosmetics and lubricant, but its main usage has been directed to the production of polymers, namely Polytrimethylene terephthalate (PTT) (1–3). This polyester is formed by a transesterefication reaction between 1,3-PDO and terephthalic acid. PTT has an improved elasticity, higher durability and stain resistance, making it attractive for manufacturing of noble grade material such as synthetic carpet, textile fibers, and thermoplastics for engineering purpose (4,5).

1,3-PDO can be produced by chemical synthesis using petroleum-based source. However, with the increasing demand for this polymer and the rising awareness for sustainable production, the industrial biotechnology has emerged as an alternative method, using microorganisms to produce 1,3-PDO (3,6).

Due to the constant evolution of increasingly efficient biological processes and the use of glycerol as a carbon source, the production of 1,3-PDO by biotechnological approach has an economic potential in the market. Gylcerol is a renewable source, produced as a secondary product from the biodiesel industry. Over the years, with the consolidation of biodiesel fuel in the market, the supply of crude glycerol has also increased, resulting in a dramatic drop of its price (7). Lastly, the surplus and lower added value of glycerol have attracted researches encouraging the use of this carbon source as a raw material in the bioindustry, including 1,3-PDO production (8).

1.1 From glycerol to 1,3-propanediol

The metabolic branch of 1,3-PDO is better described in bacteria, such as *Klebsiella pneumoniae*, *Citrobacter freundii* and *Clostridium butyricum*, which contains the necessary genes clustered in an operon (9,10). In the case of *K. pneumoniae* for example, the *dha* operon is composed of six characterized genes. *dhaB*, *dhaC* and *dhaE* encode for subunits of Glycerol dehydratase (DhaBCE), an enzyme responsible for converting glycerol to 3-hydroxypropionaldehyde (3-HPA). This process inactivates DhaBCE, and another enzyme (DhaGF) plus vitamin B_{12} is needed for its recovery to the active form. In the next metabolic step, 1,3-propanediol oxidoreductase (DhaT)

plus NADH (Nicotinamide Adenine Dinucleotide [reduced]) converts 3-HPA to 1,3-propanediol (Figure 1) (11–13).

The production of 1,3-PDO is done by a reducing pathway, while the oxidative pathway of glycerol consumption is related to the production of energy, metabolites and reducing equivalents (NADH and NADPH), necessary for biosynthesis and for the production of 1,3-PDO itself. The equilibrium between the both paths is, therefore, crucial for an efficient production.

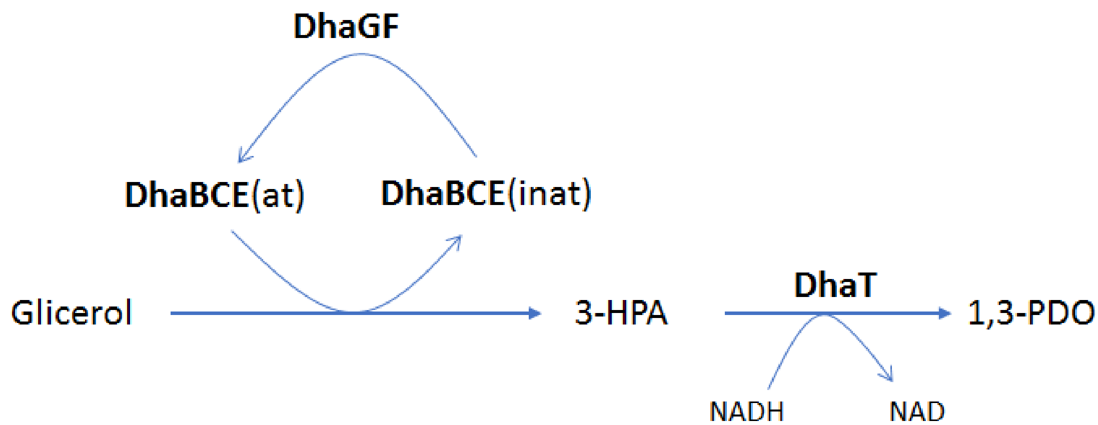

Figure 1: 1,3-propanediol generation from glycerol. Glycerol dehydratase (DhaBCE) depends on DhaGF and coenzyme B12 for regeneration to its active form. 3-hydroxypropanoaldehyde (3-HPA) is reduced to 1,3-propanediol (1,3-PDO) by NADH-dependent enzyme 1,3-propanediol oxidoreductase (DhaT) (11).

The production of 1,3-PDO is also described to be improved under microaerobiosis (14). In this scenario, key regulators of the central metabolism, such as Arc system and FNR, are activated to modulate the transition of the metabolism to a microaerobic condition. As the oxygen concentration is low, less NADH is used in the respiratory chain, resulting in a surplus of NADH that can be directed to 1,3-PDO production (15). However, during this condition, NADH can also be used for the formation of byproducts that might be important for NAD regeneration and maintenance of a proper redox balance in the cell. ArcA and FNR can also positively regulate genes such as *poxB* and *pfl*, that decreases the level of NAD reduction by preventing the carbon flow through NADH generating pathways.

Aiming to prevent the loss of NADH to undesireble byproducts, deletion of genes related to the formation of organic acids is recurrent. In the same way, overexpression of NADH generating enzymes or even *arcA* deletion are also reported to increase the amount of 1,3-PDO (16–20).

1.2 Redox ratio (NADH/NAD) and the central metabolism regulation.

The proper control of the cell metabolism is very important to cell adaptation against environmental changes. ArcAB and FNR systems are the major regulators of *E. coli* metabolic shift between aerobic, microaerobic and anaerobic condition (21). It modulates the expression of many genes from the central metabolism, coordinating the metabolic flux in order to respond rapidly to changes in oxygen availability (22).

The Arc (aerobic respiration control) system is composed of two elements: ArcA, which upon phosphorilation is resposible for regulating the expression of various genes; and ArcB, a membrane redox-sensing protein (23). In microaerobiosis, ArcB is autophosphorilated and transfers its phosphate group to ArcA (ArcA-P) (24,25). Active ArcA-P represses the expression of TCA cycle and glyoxylate shunt related genes. On the other hand, the operon from pyruvate formate lyase catabolic brach (*pfl*) is up-regulated (26). In the respiratory chain, the cytochrome bo operon (*cyoABCDE*) is repressed, while cytochrome bd (*cydAB*) is activated (27–29). This modulation on cytochrome expression in very important, because cytochrome bd has higher affinity to oxygen compared to cytochrome bo, making it more suitable during microaerobiosis, since the oxygen concentration is very low in this scenario (30). *cydAB* activation is also suggested to provide an optimal intracelular environment, by rapidly reducing the available oxygen to H_2O. Oxygen-free cytoplasmic environment is essential for the proper functioning of Pyruvate formate lyase (PFL), because this enzyme, when active, irreversibly reacts to oxygen and loose its function (31). Therefore, once cytochrome bd expression is activated, the flux of pyruvate can now be directed to PFL (32).

FNR is also responsible for regulating essential genes for metabolic shift. The regulator is activated in microaerobiosis, but it is only fully active during anaerobiosis (32). FNR also takes place in repressing TCA cycle genes and activating fermentation related enzymes, but represses both *cyoABCDE* and *cydAB* expression (33).

The shift from aerobic environment to microaerobiosis needs a coordination between ArcA and FNR activation, which ultimately will block TCA cycle and activate Acetil-CoA generation via PFL. The success of this transition also depends on other genes, such as *cydAB* for maintaining a suitable micro-environment and the proper functioning of these enzymes.

NADH/NAD ratio is related to *E. coli*'s central metabolism regulation, as ArcBA and FNR regulated pathways seem to be indirectly activated due to higher redox ratio in the cell (23,29,34–36).

There is also a correlation between NADH/NAD balance and whether the metabolism is directed towards respiration or fermentation. For example, a NADH/NAD ratio about 0.75 is found during anaerobiosis, and 0.02 in fully aerobic environment (32). The ratio is lower in aerobiosis because most of NADH produced is also consumed in the respiratory chain, highlighting the role of respiratory chain in maintaining the redox state lower. It is suggested that these redox ratios are ideal for the proper functioning of the metabolism in different conditions of oxygen availability.

1.3 1,3-PDO production by a recombinant *Escherichia coli*.

The use of *Klebsiella pneumoniae* is not of interest to the industry since it is pathogenic and produce secondary compounds that hampers the purification of the product of interest (37). On top of that, *K. pneumoniae* has not been extensively studied, making it more difficult to enhance the productivity by studying its metabolism and genetic modifications (8). To solve these problems several research groups, have cloned the *dha* operon and expressed in a recombinant *E. coli*. Since then, 1,3-PDO production was extensively studied and progress has been made by optimization and genetic modifications in this strain (38–40). A previous project in our laboratory also sought to construct a recombinant *E. coli* harboring the necessary genes for 1,3-PDO production. Firstly, the *dha* operon from *K. pneumoniae* was cloned into pBBR1MCS2 plasmid, resulting in pBBR1::*dha* (plasmid map in Appendix A). This production plasmid was then used to transform *Escherichia coli* MG1655, which was confirmed to be able to produce of 1,3-propanediol (19).

Further modifications in this recombinant strain were also made. In order to increase NADH availability and enhance the production, the wild type *icd* gene (icd^{NADP}), which codes for a NADP-dependent Isocitrate dehydrogenase, was substituted to a NAD-dependent icd (icd^{NAD}). The production of 1,3-PDO after this modification has increased significantly, confirming the importance of NADH availability. Furthermore, the impact of oxygen supply on 1,3-PDO production in this strain was also studied. The results showed that lower supply of oxygen was beneficial for the production, as described in the literature (19).

The last modification in this strain was the deletion of the *yqhD* gene. It codes for a NADPH dependent-alcohol dehydrogenase that shares a similar function as DhaT, converting 3-HPA to 1,3-PDO (41,42). Metabolic models indicated that YqhD activity is harmful for the synthesis of the product, since it drains NADPH for the synthesis of 1,3-PDO in detriment of cell biomass biosynthesis and even other activities in which NADPH is needed, such as to deal with oxidative stress. Therefore, the deletion of *yqhD* resulted in a higher synthesis of 1,3-PDO from glycerol. (20).
Until this moment the strain *E. coli* MG1655^{NAD} Δ*yqhD* harboring pBBR1::*dha* had the best production of 1,3-PDO in our laboratory.

1.4 Antibiotic-free plasmid stabilization.

E. coli is the most used bacterial chassis in biotechnology. In 2009, almost 30% of protein based recombinant pharmaceuticals were obtained by *E. coli* (43). The production of these compounds is done by heterologous expressions from the genes of interest, and the simplest way of inserting this desirable DNA fragment in the cell is through plasmids. However, keeping these plasmids stable in the bacteria through generations needs a strong selective pressure favoring the cells with plasmid, such as antibiotics in the medium associated to a resistance gene in the plasmid.

Although the use of antibiotics is sufficient to maintain the vector, it leads to an increased production cost, requires purification from the compound of interest, and treatment of the product and the biological waste carrying the gene of resistance. Bioproducts with lower added value that can be alternatively produced by chemical industry are more sensitive to this increase of cost.

Moreover, the emergence of resistant bacteria against antibiotics in the last decades have concerned health agencies and global organizations (FDA, EMEA and WHO) that are recommending increasing limitations in the use of antibiotics and genetic elements carrying antibiotic resistance. In the near future, it is likely that these limitations will become even more restrictive to the industries (44).

Therefore, in order to continue delivering the transition to a more sustainable way of production, it is necessary to find alternatives to reduce the cost of production and to explore alternative ways to stabilize the plasmid.
Fortunately, there are several different strategies to achieve plasmid stabilization without antibiotics. Examples are:

- Operator-Repressor Titration: an essential chromosomal gene is modified by putting its expression under the control of a *lac* operator/promoter. The expression of this gene is blocked, unless IPTG is added. However, in the presence of a multicopy plasmid also containing the *lac* operator, LacI repressor is titrated by the operators in the plasmid, allowing the expression of the essential gene and promoting cell growth (45).
- ParABS system: it allows plasmids to segregate equally to each of its daughter cells, increasing the percentage of the population harbouring the vector. The system consists in two genes *parA* and *parB*, and a sequence called *parS*. In short, ParB specifically recognizes and binds to *parS* site, then ParA interacts to this partition complex to actively segregate the plasmids in a not fully understood ATP-dependent manner (46) (47).
- Toxin-Antitoxin (TA): this method relies on two genes coding for a stable toxin and a less stable antitoxin. The differential decay is essential for the post-segregational killing, whereby the toxin will still be active even after cell division, and daughter cells lacking the plasmid will perish for not being able to replenish the antitoxin (48).
- Gene complementation: an essential gene is deleted from the bacterial chromosome and the same gene is inserted into the desired plasmid. Because the microorganism cannot grow without this gene, daughter cells that end up losing the plasmid will not be able to grow anymore, and thus plasmid borne bacteria are selected. This approach is very commonly used in yeasts (49), and have been successfully used in bacteria as well (50).

1.5 This project

The use of antibiotics in industrial biotechnology has major concerns regarding to the cost and public health safety, which may be impeditive for the production of some bioproducts. In order to address this issue, this project aims to maintain the production plasmid pBBR1::*dha* in *E. coli* without the use of antibiotics, and test the impact of this approach on 1,3-PDO production.

Any of the previously cited strategies for plasmid stabilization could be used for this purpose. However, based on previous findings that increasing NADH results in a higher production of 1,3-PDO, we wondered whether the heterologous overexpression

of *icd*^{NAD} would lead to an even higher production. For this additional purpose, the best strategy is the complementation of *icd* gene, because it is essential for the biosynthesis and *icd*^{NAD} dependent complementation can be used to deliver more NADH to the metabolism.

E. coli ∆*icd* cannot produce α-ketoglutarate, preventing its growth in minimal medium; although supply of glutamate could supplement this limitation (51). In other words, by deleting chromosomal *icd*, only pBBR1::*dha*::*icd* carrying cells would be capable of growing in minimal medium.

As one of the TCA cycle genes, *icd* is down-regulated by ArcA during microaerobiosis. In order to generate more NADH, we also proposed to increase its expression even under low oxygen condition, by simply replacing its native promoter with a constitutive promoter (pJ23100) (52). If correct, this approach should result in an antibiotic free recombinant *E. coli* with an increased capability of production, compared to the previous strain.

Because of ICD's major role in the central metabolism, we did not know whether its overexpression could negatively impact the cell growth. To avoid problems, gradual changes regarding *icd* gene were performed, by first comparing the complementation with icd^{NAD} and icd^{NADP}, and then the impact of using a constitutive promoter.

6 CONCLUSIONS

Plasmid stabilization by *icd*^{NADP} complementation turned out to be inefficient for 1,3-PDO production. The overproduction of NADPH and consequently growth inhibition is believed to the main cause for the rapid plasmid loss. On the other hand, *icd*$^{NAD}_{(pJ23100)}$ complementation showed promising results by enhancing plasmid stability without the use of antibiotic. As a result, the strain carrying this complementation system did not require antibiotic and produced the same amount of 1,3-PDO as the strain dependent of antibiotic. However, when antibiotic was added, J2018 produced 63% more (in total grams) of 1,3-PDO than H2017; and in the absence of antibiotic, J2018 produced 60% more than H2017.

icd$^{NAD}_{(pJ23100)}$ complementation does not fully stabilize the production plasmid, as evidenced by isocitrate accumulation and stability assay. However, it opens the possibility that combining this strategy with another stabilization system, might increase 1,3-PDO production even further.

It seems that *icd*^{NAD} overexpression did result in a higher amount of NADH for 1,3-PDO production. Meanwhile, the excess of NADH also affected the central metabolism regulation, causing cell cycle arrest and death upon rapid environmental change from aerobic to microaerobic condition. The actual reason for this phenotype is still unknown, and further studies comprising gene expression and metabolic flux experiments in this strain are needed. The understanding of the cause of this phenotype could add more information about the metabolism in *E. coli*.

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