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COMPARATIVE ANALYSIS OF THE INFLUENCE OF THREE DIFFERENT GENETIC CONSTRUCTS ON KICD PROTEIN EXPRESSION

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Abstract
The relevance of this work stems from the need to develop sustainable methods for biofuel production, specifically isobutylene, using genetic engineering. The aim of this study is a comparative analysis of three distinct genetic constructs to optimize the expression of the α-ketoisocaproate dioxygenase (*KICD*) gene found in *Rattus Norvegicus* in *Escherichia coli* strain BL21(DE3). The research examines 3 vectors: the high-copy plasmid pUC19 with a lac promoter, the low-copy inducible plasmid pET-28(a)+ with a T7 promoter, and a modified pET-28(a)+ construct containing the synthetic constitutive promoter PJ23118. It is assumed that different vectors will have varying effects on the metabolic load of the cell and the expression level of the target gene as well as the produced protein function. The primary methods for evaluating efficiency are qPCR to quantify the mRNA level of the *KICD* gene, SDS-PAGE to confirm correct folding of the protein and its presence inside the cell and gas-chromatography to quantitatively assess the final function of the protein resulting in isobutylene formation. This fundamental research is aimed at creating a sustainable platform for isobutylene biosynthesis with optimal conditions for both cell viability and biofuel production.
Keywords: genetic engineering, metabolic engineering, isobutylene, constitutive promoter, protein expression, qPCR, SDS-PAGE, biofuel.

СРАВНИТЕЛЬНЫЙ АНАЛИЗ ВЛИЯНИЯ ТРЁХ РАЗЛИЧНЫХ ГЕНЕТИЧЕСКИХ КОНСТРУКЦИЙ НА ЭКСПРЕССИЮ БЕЛКОВ KICD

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Аннотация
Актуальность данной работы обусловлена необходимостью разработки устойчивых методов производства биотоплива, в частности изобутилена, с использованием методов генной инженерии. Целью данного исследования является сравнительный анализ трех различных генетических конструкций для оптимизации экспрессии гена α-кетоизокапроатдиоксигеназы (*KICD*), обнаруженного у *Rattus Norvegicus*, в штамме *Escherichia coli* BL21(DE3). В исследовании рассматриваются 3 вектора: высококопийная плазмида pUC19 с lac-промотором, низкокопийная индуцибельная плазмида pET-28(a)+ с T7-промотором и модифицированная конструкция pET-28(a)+, содержащая синтетический конститутивный промотор PJ23118. Предполагается, что различные векторы будут по-разному влиять на метаболическую нагрузку на клетку, уровень экспрессии целевого гена, а также на функцию произведенного белка. Основными методами оценки эффективности являются qPCR для количественного определения уровня мРНК гена *KICD*, SDS-PAGE для подтверждения правильного сворачивания белка и его наличия внутри клетки, и газовая хроматография для количественной оценки конечной функции белка, приводящей к образованию изобутилена. Это фундаментальное исследование направлено на создание устойчивой платформы для биосинтеза изобутилена с оптимальными условиями как для жизнеспособности клеток, так и для производства биотоплива.

Ключевые слова: генная инженерия, метаболическая инженерия, изобутилен, конститутивный промотор, экспрессия белков, qPCR, SDS-PAGE, биотопливо.

Modern biotechnology and industrial microbiology are increasingly turning to metabolic engineering techniques to create producer strains of valuable compounds. One of the pressing challenges is the search for sustainable energy sources, particularly biofuels, such as isobutylene, a valuable gaseous fuel and feedstock for the petrochemical industry which is traditionally obtained from fossil sources. Therefore, the development of biological platforms for its synthesis represents a significant scientific and applied problem.

A promising approach is the modification of the metabolic pathways of bacteria, specifically *Escherichia coli*, through the introduction of heterologous genes. Majority of the research for bacterial and fungal isobutylene production is focused mainly on biosynthesis of isobutanol via fermentative pathways and its further chemical modifications resulting in isobutylene. This method, however popular and well-studied, has large limitations including toxicity of isobutanol to microorganisms thus limiting the scale of production and the need for further additional steps of substrate modifications to acquire the target product [2]. More recent research, however, has shown that isobutylene can be produced in a one-step reaction using α-ketoisocaproate dioxygenase (*KICD)* gene, originally found in the organism of the *Rattus Norvegicus*. The product of this gene catalyzes a reaction of dioxygenation of the α-ketoisocaproate, a direct precursor of L-leucine, leading to the formation of an unstable compound, β-Hydroxy β-methylbutyrate (HMB), which spontaneously decomposes to produce the desired isobutylene into the gaseous space of the bioreactor [3]. For such heterologous gene to be properly expressed in a bacterial host, codon optimization was performed, matching codon usage of the donor organism.

An analysis of the current state of research shows that optimizing the expression of heterologous genes remains a central challenge in metabolic engineering. High-copy plasmids such as pUC19, contain a fairly weak and not-well regulated (leaky) *lac* promoter which is compensated by a large number of plasmid copies per cell, however constant replication of the plasmid often lead to the depletion of cellular metabolic resources, making them unsuitable for long-term productive protein expression. An alternative is a class of low-copy plasmids, such as pET-28(a)+. This plasmid carries a very strong T7 promoter, recognized by a T7 RNA Polymerase, and is widely used for tightly-regulated and robust expression of the genes. Main disadvantages of the pET28(a)+ plasmid include its' fitness to a limited amount of *E. coli* strains as it requires a T7 RNA Polymerase expression system inside the host organism, as well as low translational efficiency and solubility of the formed proteins as experimental data shows that high levels of mRNA do not equal to correctly folded and properly functioning proteins, on the contrast, excessive mRNA overwhelms ribosomes and depletes cell's resources causing majority of the peptide chains fold incorrectly and be consumed by inclusion bodies for degradation [5]. In addition, both pUC19 and pET28(a)+ require induction of expression with IPTG which is proven to have inhibitory effect on the cell viability [1]. In response to the limitations of those systems, the field of constitutive expression using synthetic promoters is actively developing in bioengineering allowing to synthetically modify pET28(a)+ expression vector by replacing its' inducible elements (T7 promoter and lac operator) with a constitutive promoter: J23118, a synthetic promoter from the Anderson family which has shown medium strength and metabolic burden on the host cell [4]. Such approach eliminates the need for addition of toxic inducers and is hypothesized to provide a stable level of expression of the target *KICD* gene with lower metabolic burden and higher percentage of properly translated and functional proteins.

To compare the levels of transcription, translation and protein function, three variants of plasmids carrying the *KICD* gene were constructed. The vectors were selected based on an analysis of their replication characteristics and promoter elements:

1) pUC19 — a high-copy plasmid with a lac promoter;

2) pET-28(a)+ — a low-copy plasmid with a T7 promoter;

3) pET-28(a)+ PJ23118 — a modified low-copy plasmid where the native T7 promoter and its lac operator are replaced with the medium-strong synthetic constitutive promoter J23118.

All three constructs were assembled and transformed into competent cells of the producer strain *E. coli* BL21(DE3). Vector assembly involved standard steps: amplification of the insert (KICD gene), restriction, ligation and transformation. Selection of positive clones was performed via colony PCR screening.

Assessment of constructed plasmids will be carried out on both transcriptional and translational levels using qPCR assay and SDS-PAGE to measure levels of gene and protein expression, respectively. qPCR method will allow for the quantitative assessment of *KICD* gene mRNA accumulation, serving as direct evidence of promoter efficiency and plasmid stability within the cell. To determine translational efficiency, SDS-PAGE will be performed to detect fully translated KICD protein present inside the cell after incubation with or without induction. The data obtained from qPCR, SDS-PAGE and gas-chromatography will not only identify the best vector for *KICD* expression and function but also contribute to understanding the interaction of plasmid elements with the host cell's metabolism. As this project is fundamental in nature and aims to create a viable platform for biofuel production, confirming efficiency at the transcriptional, translational and functional levels is a necessary step.

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