Title: Design of efficient organisms using a combination of inverse metabolic engineering and metabolic evolution

Authors: Pornkamol (Apple) Unrean, Cong Trinh and Friedrich Srienc

Department of Chemical Engineering and Materials Science/BioTechnology Institute, University of Minnesota 1479 Gortner Avenue, Saint Paul, MN 55108, USA

Abstract

In this study, we demonstrate the use of combined inverse metabolic engineering and metabolic evolution for rational strain development. We have applied these two approaches for designing the efficient *E. coli* strains for the production of primary metabolites such as ethanol as well as for the production of complex secondary metabolites like lycopene. Inverse metabolic engineering based on Elementary Mode Analysis was employed to predict a set of multiple-gene deletion required for the cell to function according to an efficient pathway. Metabolic evolution of growth-based selection was used for co-selection of an increased production of the product. We have recently constructed the design strains and characterized their performances. Experimental results have shown that the designed strains achieved a better performance than the wild type under identical conditions. The results, therefore, confirm the validity of these applied strategies for rational strain development.

Introduction

The creation of organisms that are capable of performing in the most efficient way for the production of desirable metabolites is an intriguing goal in metabolic engineering. Developing organisms to achieve a better product yield and productivity often requires the simultaneous manipulating of pathways and optimizing of regulatory effects. Previously, these optimizations were often accomplished by random mutagenesis and high-throughput screening which are relatively time- and cost-consuming process and may lead to only suboptimal phenotype. In this study, we demonstrate a rational, systematic way of strain development using a combination of metabolic engineering based on Elementary Mode Analysis and metabolic evolution. In designing *E. coli* mutants for the most efficient production of desired products, we apply Elementary Mode Analysis for systematic optimization of biosynthesis pathway of the products. In combination with Elementary Mode Analysis, we exploit evolutionary process to overcome metabolic regulation limiting the synthesis of the desired products.

A metabolic network can function according to many different pathway options. Elementary Mode Analysis is a flux balance-based stoichiometric model that has emerged as a powerful tool for rigorously dissecting a metabolic network into its basic building blocks of the metabolic structure. We apply Elementary Mode Analysis for analyzing the metabolic network of an *E. coli* strain. Evaluation of all feasible fluxes identified by Elementary Mode Analysis permits us to identify potential genes deletion required for re-routing the operating pathway of an *E. coli* cell into the efficient one for production of desired products. Later, metabolic evolution in a growth-based selection scheme was employed on the knockout strains that are designed by Elementary Mode

Analysis. The evolutionary process allows for a co-selection of a mutant *E. coli* with an improved cell growth and synthesis of the product. Here, we describe the capacity of two optimized *E. coli* strains derived from the combined approaches of metabolic engineering and metabolic evolution. One *E. coli* strain is optimized for efficient production of ethanol from glycerol and another is optimized for efficient production of lycopene from glucose. The experimental results are also verified with the prediction from Elementary Mode Analysis.

Result

Strain design using Elementary Mode Analysis

The metabolic models of ethanol producing *E. coli* and lycopene producing *E. coli* are constructed. Elementary Mode Analysis on the constructed models, then, identifies total of available Elementary Modes (or pathway options). For a rational design of *E. coli* mutants for the most efficient production of ethanol from glycerol and for the most efficient production of lycopene from glucose, we use Elementary Mode Analysis to examine the effect of gene deletion on the production of the products in the cell. Gene knockout is simulated by removing the enzymatic reaction corresponding to that gene from the metabolic network model. The phenotype of that specific knockout mutant is, then, represented by a combination of remaining Elementary Modes. We sequentially screen the simulation result and identify an optimal set of gene knockouts that could restrict the mutants to operate according to the efficient-product producing modes. The designed mutant with these multiple gene deletions for efficient production of ethanol from glycerol is designated as TCS099/pLOI297 and for efficient production of lycopene from glucose is designated as LYC018/pACEBI. All Elementary Modes remain in both strains are directed to efficient ethanol production and to efficient lycopene production respectively as well as to maintain cell growth.

Strain Construction

To validate the prediction of strain design by Elementary Mode Analysis, we have constructed the designed multiple-genes knockout mutants TCS099 and LYC018. TCS099 and LYC018 have all targeted knockout genes as identified by Elementary Mode Analysis for efficient synthesis of ethanol and for efficient synthesis of lycopene

respectively removed from their chromosomes. Gene disruption in the mutants is verified by PCR amplification using primers specific to that deleted genes and gel electrophoresis. The designed mutant TCS099 is transformed with plasmid pLOI297 and then tested for its performance on production of ethanol. Likewise, for production of lycopene, the designed strain LYC018 is transformed with plasmid pACEBI containing lycopene synthesis genes and tested for its capacity of lycopene synthesis.

Metabolic evolution

According to the strain design, TCS099 and LYC018 always coproduce biomass and the products, ethanol and lycopene respectively, in a growth-associated manner. This obligated couple between cell growth and the products provide a basis for strain improvement through metabolic evolution. That is, by selecting for improved growth would also co-select for improved production of the products. TCS099 mutant designed for efficient production of ethanol and LYC018 mutant designed for efficient production of lycopene are, therefore, evolved in a serial cultivation under a growth-based selection scheme where cell growth rate of the mutants in each batch culture was monitored during the serial dilution. The results reveal the continuous increase in cell growth of both mutants over the course of evolution. After several generations, the evolved strain of TCS099 and the evolved strain of LYC018 are isolated and renamed TCS099Rd50 and LYC018E1 correspondingly.

Strain characterization

Based on Elementary Mode Analysis, TCS099/pLOI297 was rationally designed for efficient ethanol production from glycerol and LYC018/pLOI297 was rationally designed for efficient lycopene production from glucose. The performance of the

constructed strains based on the design of Elementary Mode Analysis is tested experimentally in comparison with the wild-type strain as a control in aerobic batch fermentation. The mutant LYC018/pACEBI outperforms the wild type MG1655/pACEBI for the conversion of glucose into lycopene. Similarly, TCS099/pLOI297 also outperforms the wild-type MG1655pLOI297 for the production of ethanol from glycerol. The production of ethanol and the production of lycopene are further improved in the evolved strains derived from growth-based selection evolution, TCS099Rd50/pLOI297 and LYC018E1/pACEBI comparing to their parental strains. In vivo phenotypes of all strains are also compared with predicted phenotype by Elementary Modes. The observed phenotype of all strains is in agreement with the predicted phenotypic space of Elementary Mode Analysis. These consistencies, therefore, confirm the accuracy of the Elementary Mode Model.

Discussion

In this study, we have shown an alternative way for rationally engineering efficient *E. coli* strain using the combined approach of inverse metabolic engineering and metabolic evolution. Inverse metabolic engineering via Elementary Mode Analysis has been applied for optimizing two *E. coli* strains, one for production of ethanol from glycerol and another for production of lycopene from glucose. Elementary Mode Analysis can be used to dissect a metabolic network into unique, non-decomposable pathways. A set of these pathways, therefore, presents all possible physiological states of cells at steady-state conditions. Elementary Mode Analysis is utilized to computationally search for the combination of expressed gene products that supports only a minimum number of possible pathways. A set of target gene knockouts is extracted through the sequential search of Elementary Mode Analysis aiming at minimizing the cell's pathway options while retaining the most efficiency of the desired products. The derived minimal strains can, therefore, only function on the basis of the most efficient production of the desired products. Both mutants with multiple gene deletions predicted by the model, TCS099/pLOI297 and LYC018/pACEBI, experimentally showed the improved performance on the production of the desired products, ethanol and lycopene respectively. This also confirms the validity of the Elementary Mode Analysis as a powerful tool for a rational strain design

Besides pathway optimization guided by Elementary Mode Analysis, another feasible alternative called metabolic evolution can be undertook to yield promising targets for further enhancement of ethanol or lycopene synthesis. In particular, the tightly couple between the products synthesis and the cell growth allows for a selection of an

improved ethanol-producing mutant or an improved lycopene-producing mutant through a growth-based selection scheme. The improvement of cell growth observed during a serial dilution evolution suggests an occurrence of a new strain from the parent as a result of spontaneous mutation or mutations. The evolved strains, both TCS099Rd50/pLOI297 and LYC018E1/pACEBI that are obtained from growth based selection evolution are found to co-select for increased production of ethanol product and lycopene product respectively. Both evolved strains also show distinct kinetic of product accumulation which suggests that the different modes of action and different combinatorial genes mutation between the evolved strains and their parents. In vivo phenotype of evolved strains in comparison with Elementary Mode modeling result also reveals the shift toward a predicted optimal metabolic state over the course of evolution.

In conclusion, this study underscores the application of inverse metabolic engineering based on Elementary Mode Analysis in combination with metabolic evolution for developing of two efficient *E. coli* strains, one for the production of ethanol and another for the production of lycopene. The improved performance observed in the evolved strains suggested that *E. coli* can be metabolically engineered and evolved to increase fluxes to the production of the products. These applied strategies allow us to capture apparent limitations in obtaining the most efficient performance of the designed strain caused by both metabolic pathways and regulatory mechanisms. These combined approaches could aid in efficient strain design for production of many other useful products as well.