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Elementary Flux Mode Analysis of *Streptomyces Fradiae* Metabolic Network for an Increased Neomycin Yield

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ABSTRACT

Neomycin is an antibiotic that can naturally be produced by *Streptomyces fradiae*. Herein, elementary flux mode analysis is used to identify potential gene targets for improvement of neomycin production yield in *S. fradiae*. To achieve the goal, a metabolic network of *S. fradiae* is firstly reconstructed by incorporation of 7 key metabolic pathways including glycolysis, tricarboxylic acid cycle, pentose phosphate, pyruvate oxidation, glutamate synthesis from oxoglutarate, glutamate synthesis from glutamine and neomycin synthesis. The main carbon and nitrogen sources for the production are glucose, glutamine and ammonia. After analyzing the reconstructed network by using METATOOL, 230 elementary modes producing neomycin are obtained. These elementary modes show 10 different theoretical yields, ranging from 0.17 to 1 mole of neomycin produced per mole of glucose uptake. It is observed that the modes providing the highest yield use the glucose to synthesize neomycin without entering the glycolysis pathway. From the analysis, it is suggested that the average yield of neomycin in *S. fradiae* could be improved by knocking out the genes gdh2, gdhA, ssgdh, GLD, gcd, PTS-GLC-EIIA and crr to decrease the total carbon flux in the glycolysis pathway.

Keywords: Elementary flux modes, Neomycin, *Streptomyces fradiae*

INTRODUCTION

One of the important antibiotic products of *Streptomyces fradiae* is neomycin that has high pharmaceutical benefits (Cammack et al, 2014). It also works actively against streptomycin-resistant bacteria. Nevertheless, neomycin supply still does not meet the demand of consumers. Previously, Vastrad, B. M. and Neelagund, S. E. (Vastrad and Neelagund, 2014) attempted to improve the production yield of neomycin in *S. fradiae* in solid state fermentation by optimizing physical parameters, i.e., inoculum size, substrate particle size, incubation temperature, initial pH, initial moisture level and incubation period, as well as chemical parameters, i.e., additional carbon and nitrogen sources. Later, they used Placket-Burman design to optimize culture medium of in *S. fradiae* in solid state fermentation (Vastrad and Neelagund, 2011). It was found that ammonium chloride, sodium nitrate, L-histidine, and ammonium nitrate were the significant nutritional factors affecting neomycin production. With the advances in sequencing and metabolic engineering, many of the biosynthetic pathways responsible for the production of pharmaceutically valuable compounds have been altered towards improvement of production yield (Schuster et al, 2000). In this work, a metabolic engineering technique namely elementary flux mode analysis (EFMA) is employed to analyze the metabolic network of neomycin synthesis of *S. fradiae*, hoping to identify potential gene targets for further pathway engineering towards an improved neomycin yield.

METHODOLOGY

The metabolic network of neomycin biosynthesis in *S. fradiae* was reconstructed using information mainly from KEGG (Kaneihisa et al, 2004) and literature (Bonarius et al, 1997). Elementary flux modes (EFMs) of the network were then computed by using the METATOOL program (Jevremovic et al, 2008) (Kamp and Schuster, 2006). Lists of stoichiometric reaction equations, the declaration of reversible and irreversible reaction and the declaration of internal and external metabolites were the input data for analysis in this program. EFMs at the stationary state obtained from the program were further analyzed for optimal and suboptimal neomycin yield, optimal and suboptimal neomycin pathways, and dispensable and indispensable enzyme sets. The enzyme sets in each EFM were the key to identify potential targets because of its suitable information to predict the effect of enzyme deficiencies or knock out mutation. The mutation of *S. fradiae* is useful for assisting the design of the new metabolic pathway to improve the type strain in the increasing of neomycin production. Neomycin yields on glucose, ammonia and

glutamine were calculated from stoichiometry of EFMs. In addition, energy gain (ATP ratio) which is the ratio of ATP released and glucose uptake was also calculated from the stoichiometry of EFMs. The pathways providing maximum neomycin yields and ATP ratio were considered. Enzyme knockout analysis (Chen et al, 2010) was performed to suggest rational design for improvement of average yield of neomycin.

RESULTS AND DISCUSSION

Reconstruction of metabolic pathways in *S. fradiae*

The reconstructed network of neomycin synthesis is shown in Figure 1. This was a simplified network of biochemical reactions starting from glucose to neomycin. This metabolic network consisted of 7 key metabolic pathways including glycolysis, tricarboxylic acid cycle, pentose phosphate, pyruvate oxidation, glutamate synthesis from oxoglutarate, glutamate synthesis from glutamine and neomycin synthesis. In total, there were 83 metabolites and 78 reactions.

Pathways analysis based on glucose uptake

By using METATOOL to analyze this network, 416 EFMs were obtained, 230 of which were the modes leading to neomycin. Each EFM was an overall reaction possessing a set of enzymes. 10 different theoretical neomycin yields on glucose by mole were observed, i.e., 1, 0.62, 0.5, 0.44, 0.41, 0.36, 0.33, 0.25, 0.2 and 0.167. There were 48 EFMs providing the maximum theoretical yield of 1 mole of neomycin per 1 mole of glucose. These EFMs indicated a direct conversion of glucose to neomycin without going through the glycolysis pathway. This is sensible because, through the glycolysis pathway, glucose is converted to ribulose 5-phosphate (DRIBUP) and carbon dioxide that is later released to atmosphere. The second highest yield was 0.62. The major difference between the modes in this yield and the modes in yield 1 is that the glucose is uptake into the cell and then transformed to be Beta-D-Glucose 6-phosphate (BDGLC6P) by reaction 78. After that, BDGLC6P is further transformed to DRIBU5P with releasing of carbon dioxide leading to the loss of carbon. Moreover, glucose is further transformed to pyruvate. Before entering the TCA cycle, pyruvate is reduced to acetyl coA (ACTCOA) by reaction 59. After the TCA cycle, oxaloacetate (OXACT) is converted back to pyruvate by reaction 48. In these 2 reactions, there is some carbon loss in the form of carbon dioxide which is also the main reason of the lower yield compared to the mode in yield 1. For the modes that has yield below 0.62

which are 0.5, 0.44, 0.41, 0.36, 0.33, 0.25, 0.2 and 0.167, there is no significant difference that can specify the main reason of carbon lost compared to modes in yield 0.62.

Pathways analysis based on uptake ammonia

From 230 EFMs that can produce neomycin, only 120 EFMs consumed ammonia. The maximum theoretical yield was 0.5 moles of neomycin per 1 mole of ammonia. To reach the maximum theoretical yield of 0.5, ammonia must be fed to reaction R7 as shown in Figure 3 and used in neomycin synthesis directly but there was some ammonia lost at reaction R79. For the other lower yields, it was observed that reaction R7 was not occurred so ammonia has not been used to produce neomycin but there is another nitrogen source which is glutamine.

Pathways analysis based on uptake glutamine

EFM could suggest how to improve the wild type strain for a desired product on glutamine as a new nitrogen source. There were 8 yields of 230 EFMs on glutamine uptake which are 0.125, 0.24, 0.25, 0286, 0.291, 0.3, 0.36 and 0.5. To reach the maximum theoretical yield of 0.5, glutamine must be fed to reaction R1 for the neomycin synthesis. The EFMs that contains reaction R79 cannot reach the highest yield because there is some nitrogen lost in reaction R79 as can be seen from Figure 4.

Pathways analysis based on ATP

This part presents energy contained in cells in form of these internal free energy sources; ATP, ADP and AMP, and the reducing equivalent-in form of NADPH and NADH. The energy cost could be used as another criteria to determine the best EFMs in terms of energy usage. The way to calculate internal energy was the summation of ATP and all reducing equivalents; NADPH and NADH, which are changed in form of ATP. In this work, energy gain for neomycin synthesis was calculated in term of ATP ratio. The relation of ATP ratio to neomycin yield is shown in Figure 5. Figure 5 indicates that the higher the neomycin yield, the more ATP can be generated. The four modes that can give the highest of both neomycin yield and ATP ratio are observed as shown in Table 1. From Table 1, first, for the neomycin yield equal to 1 which is the maximum theoretical yield, there are 2 different amounts of released ATP; 123 and 105 mole ATPs (Mode 397 and 391 respectively). Although these 2 EFMs give the same neomycin yield, each EFM produces a different amount of ATPs because of having different synthesis pathways. Mode 397 gives higher ATP than mode 391 which means that mode 397 is an effective pathway. Also, for the

neomycin yield equal to 0.62 that requires 24.462 and 22.154 ATPs yield (Mode 238 and 277 respectively), mode 238 can produce more ATP for 0.62 neomycin yield.

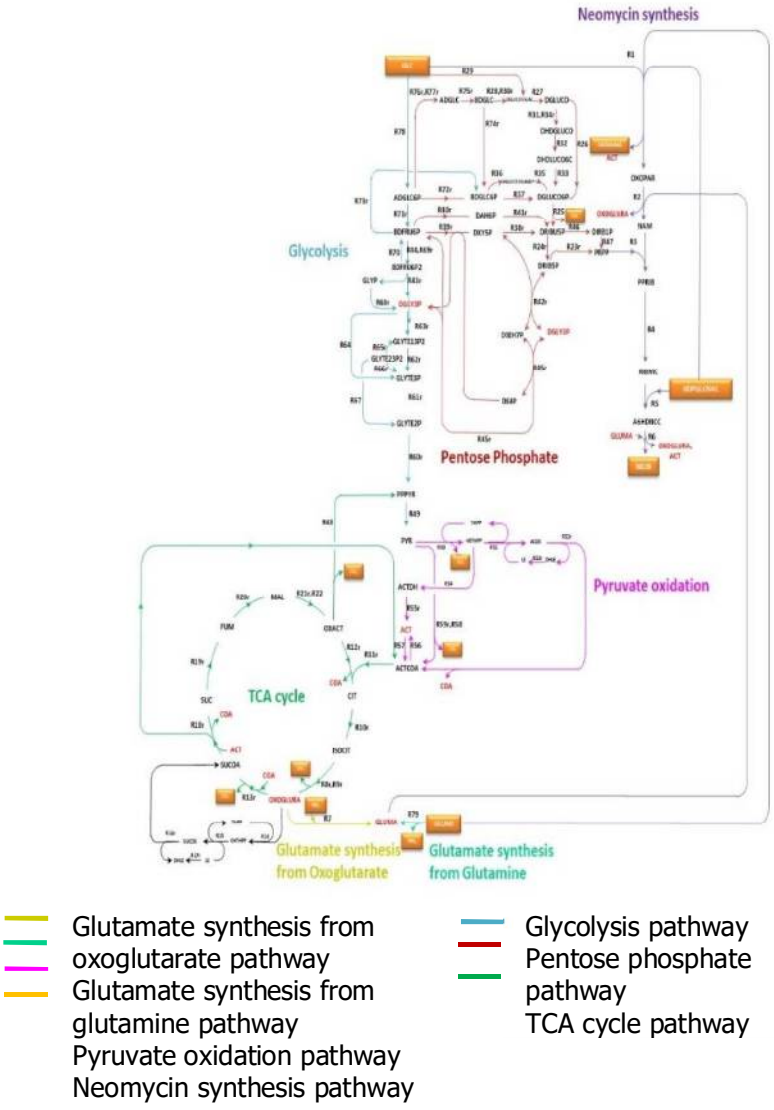

Figure 1. The reconstructed network of neomycin synthesis. Internal metabolites are represented by black capital letters, while the repetitive internal metabolites are written in red letters. External metabolites are shown in orange blocks. All reactions are shown as R (irreversible) or r (reversible) followed with reaction ID.

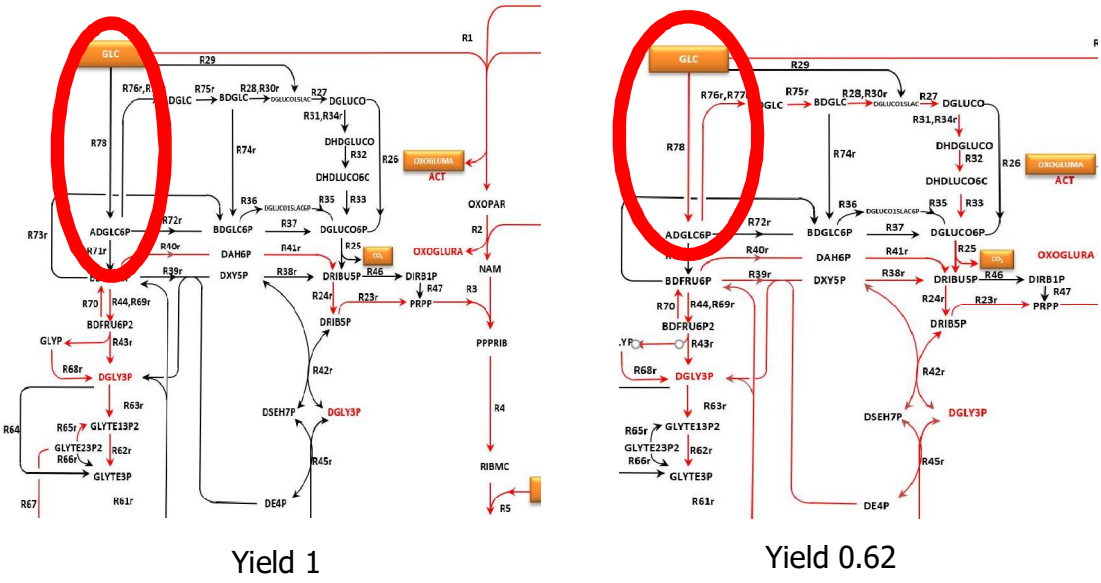

Figure 2. The different active pathway between yield 1 and yield 0.62 based on glucose uptake

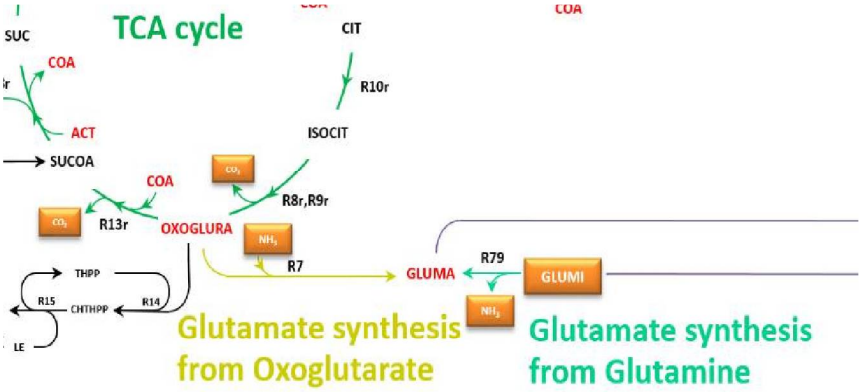

Figure 3. The active pathway of yield 0.5 based on ammonia uptake

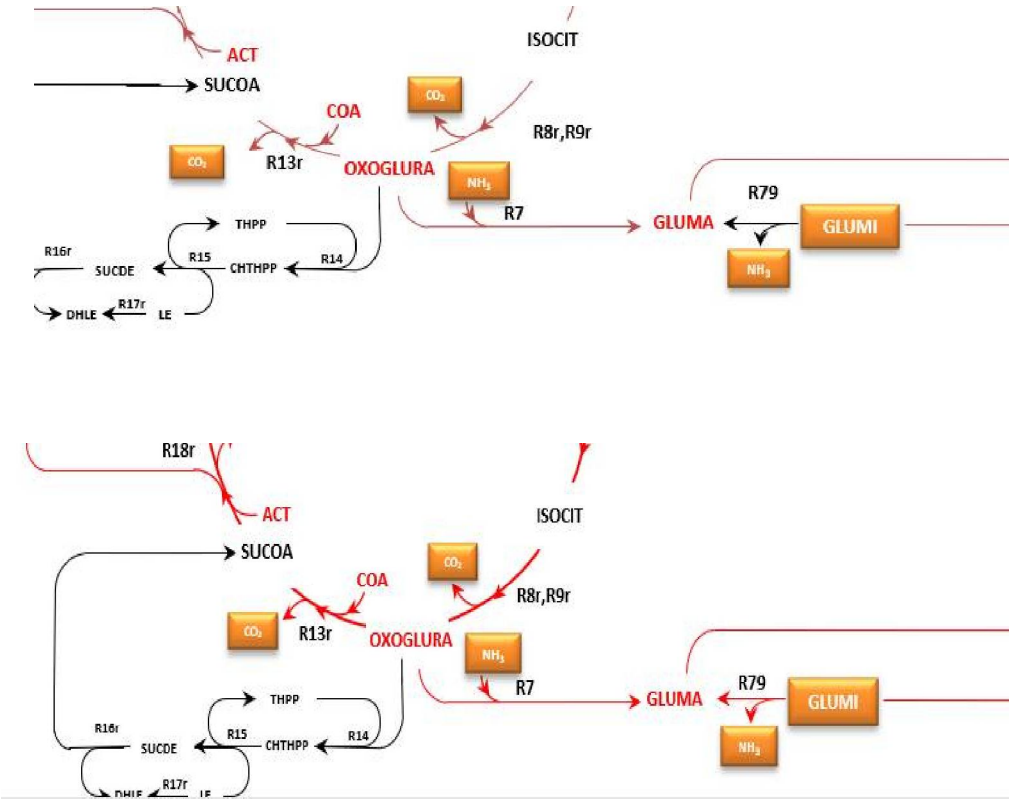

Figure 4. The different active pathway between yield 0.5 and yield 0.36 based on glutamine uptake

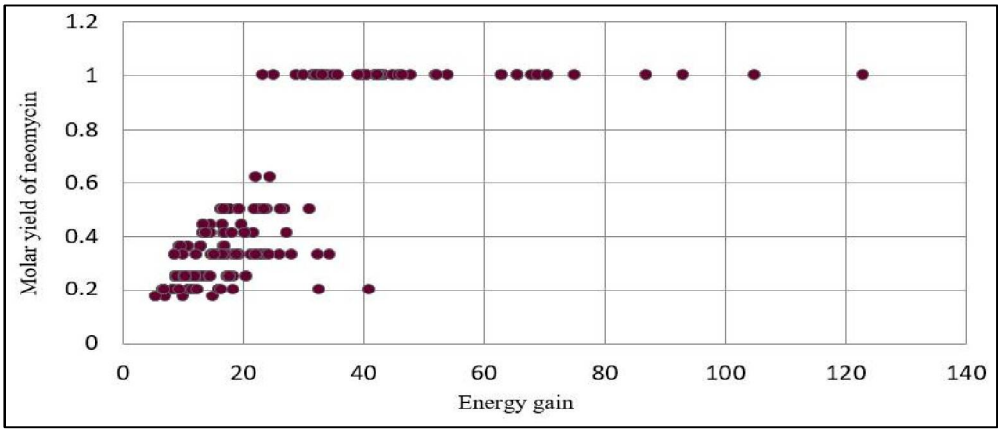

Figure 5. The relation between the neomycin yield and energy gain (or ATP ratio)

Table 1. The two highest neomycin yields and its ATP yields.

No. of reaction	Neomycin yields (mole neomycin/mole glucose)	ATPs (mole ATP/ mole glucose)
397	1	123
391	1	105
238	0.62	24.462
277	0.62	22.154

CONCLUSION

In this work, the simplified neomycin synthesis pathways of *S. fradiae* comprising 83 metabolites and 78 biochemical reactions were analyzed via METATOOL. There are 230 elementary flux modes producing neomycin and they can be classified in 10 different yields -1, 8/13, 1/2, 4/9, 7/17, 12/33, 1/3, 1/4, 1/5, and 1/6 moles of neomycin per moles of glucose. The highest yield of 1 occurring only when glucose is directly used in the neomycin synthesis pathway without entering the glycolysis pathway. Knocking out the genes gdh2, gdhA, ssgdh, GLD, gcd, PTS-GLC-EIIA and crr to prevent glucose entering glycolysis pathway could lead to more neomycin yield. In addition, to reach the maximum theoretical yield of neomycin, both ammonium and glutamine must be fed to the cell and used for neomycin synthesis directly. Also, the higher neomycin yield can be attained when the higher ATP is generated.

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