Metabolic Engineering of *E.coli* to Produce 1,4 Butanediol

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INTRODUCTION

There are many concerns today about non-renewable resources needing to be replaced by sustainable alternatives. Non-renewable resources, such as fossil fuels, account for a major source of plastics, fuels, and other chemicals. In 2010, 191 million barrels of petroleum and natural gas were used in the production of plastics[1]. In order to supply fuels for the nation's energy needs, 82% of America's energy comes from fossil fuels, 66.4% of all electrical energy and 95.4% of all transportation energy[2]. Reducing our reliance on the use this non-renewable resource would be greatly beneficial. This issue has led to many approaches to a search for biofuel production through renewable sources such as corn and other bioproducts.

Residue Type	Quantity (tons per year)	L-Arabinose (% mass)	Total Mass of L-arabinose (tons per year)
Corn Stover	240 million	2.70%	6.5 million
Orange Peels	12 million	14%	1.7 million
Beet Pulps	25 million	20%	5 million

Table 1: Shows the quantity of L-arabinose from various sources of waste biomass generated per year

The bioproducts used are also important to take into consideration. Lignocellulosic biomass, such as corn stover[4], orange peels[5] and beet pulp[5], can be used as a renewable feedstock. As seen in table 1, L-arabinose is abundant in this waste biomass and could be used to produce Through metabolic engineering of *E.coli*, waste products from this waste biomass can be converted into valuable chemicals. More specifically, through this project, *E.coli* was engineered to convert L-arabinose to 1,4 Butanediol (BDO) through the metabolic pathway shown below.

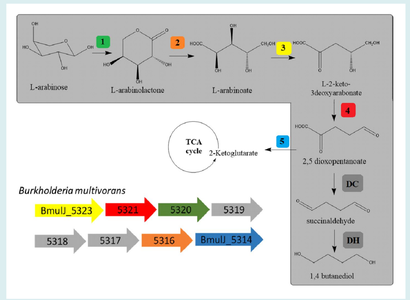

Figure 1: The pathway introduced into *E.coli* is shown above. To prove this pathway was active once implemented, three plasmids were used. Plasmids for steps 1-5 (P1) and steps 1-5 (P2) were constructed. A previously constructed plasmid (P3) containing dehydrogenase (DH) and decarboxylase (DC) genes was used as well. The color of each step corresponds to the genes of *Burkholderia multivorans*.

METHODS

Normally within *E.coli*, L-arabinose is metabolized through the pentose phosphate pathway. So, in order to build the metabolic pathway steps 1-5 seen in figure 1, genes from *Burkholderia multivorans* were cloned into a plasmid (P1). Growth assay data was then taken on the strain as it went through the TCA cycle. After this growth was confirmed, a second plasmid (P2) containing genes for steps 1-4 (in figure 1) was constructed and transformed into a cell with the last DC and DH steps (from P3), as well. Fermentation data were then taken to determine the overall BDO yield.

Plasmid Cloning: The P1 plasmid constructed was cloned from the operon of *B. multivorans*. This plasmid, containing genes BmulJ 5323, 5321-5320, 5316 and 5214, was then transformed into BW25113 and growth assay data was taken. The P2 plasmid was constructed with the same genes minus BmulJ 5214. This plasmid along with previously constructed plasmid P3 containing the genes for the decarboxylase (DC) and dehydrogenase (DH), was transformed into BW25113 and BW25113 Δicd strain.

Growth Assay: For each experiment, the strains were inoculated in 2xYT with appropriate antibiotics. Then the OD at 600 nm was measured and normalized. Once induced with IPTG the OD was again measured every few hours. This was done for BW25113, without P1, and BW25113 Δicd with and without P1.

Enzyme Assay: Each gene was individually cloned into its own plasmid, produced, purified and assayed to check its in vitro activity. The genes were each cloned into pZE-his plasmids and then transformed into BL 21 strain. The transformed cells were then grown in 2xYT medium until OD, when they were then induced with IPTG. After the protein expression was induced, the cells were lysed and the protein purified through a HisPur Ni-NTA resin column. The purified proteins were then each assayed and kinetic data was taken.

Shake Flask Fermentation: The strains were incubated in minimal medium overnight. The grown up culture was then transferred into a conical shake flask with 2xYT and induced with IPTG. At 250 rpm, the flasks were then incubated at 30°C for 48 hours in anaerobic conditions. The solution was then measured by HPLC. The two strains used were BW25113 with P1 and P3 and BW25113 with P2 and P3. The initial feeds for each were 40g/L L-arabinose and 20g/L of both Glucose and L-arabinose.

RESULTS

Growth Assay: Within BW25113, L-arabinose is metabolized through the TCA cycle. However, the growth of BW25113 Δicd was hindered because this gene within the TCA cycle is essential for producing 2-ketoglutarate. Once the new P1 plasmid was introduced, cell growth recovered slightly. This shows that the proposed pathway is active.

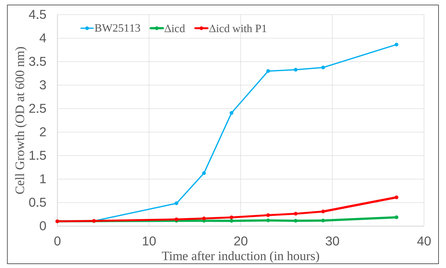

Figure 2: Growth assay data comparing BW25113, BW25113 Δicd, and BW25113 Δicd with P1. From these results it was concluded that the introduced genes allow for the proposed metabolism to occur.

Enzyme Assay: To further confirm the enzyme activity of the introduced metabolic pathway, each enzyme was purified and characterized. All kinetic data is shown below in table 2. As seen, BmulJ 5320 had the highest K_{cat} while 5323 and 5321 were relatively low. 5321 was the rate-limiting step because it had the highest K_m (9.7 mM). This enzyme limiting production of 2,5-dioxopentanoate explains why cell growth did not fully recover in the growth assay.

Enzyme	Substrate	Organism	Km (mM)	Kcat (sec^{-1})	Km/Kcat ($mM^{-1}sec^{-1}$)
BmulJ 5320	L-arabinose	B. multivorans	3.14±0.12	101.4±5.2	32.3
BmulJ 5323	L-arabinoate	B. multivorans	2.05±0.05	0.17±0.01	0.083
BmulJ 5321	2-keto-3-deoxyarabonate	B. multivorans	9.69±0.2	0.2±0.02	0.023

Table 2: Kinetic data of the enzymes BmulJ 5320, 5323, and 5321. As BmulJ 5316 is a ring-opening step, it was assumed to be spontaneous. And 5314 is insignificant to the production of BDO.

Shake Flask Fermentation: To see which conditions were best for BDO production, various feeds and plasmids were tested. As seen in figure 3, the strains fed only L-arabinose were not able to produce as much BDO as when glucose and L-arabinose were fed. When comparing P1 and P2 fed both glucose and L-arabinose, P2 was able to produce higher yields of BDO.

RESULTS

It also consumed more glucose, as L-arabinose was not able to be consumed by the TCA cycle. By the end, there was also around 6 g/l L-arabinose unconsumed but still produced 5.6 g/L BDO. This level of BDO production is around 61% of the theoretical maximum.

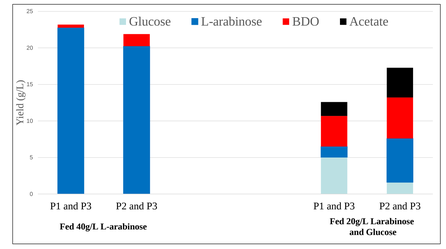

Figure 3: Best results from each strain and feed complied. All plasmids transformed into BW25113

CONCLUSIONS

To summarize, the gene cluster taken from B. multivorans was transformed into *E.coli* and capable of producing BDO. These enzymes were active both in vivo and in vitro, through fermentation and enzyme assay data. The best strain with this pathway was capable of quite high yields of 5.6 g/L BDO which is 61% of the theoretical maximum.

Some improvements could be made to this process, such as optimizing the feed or improving the enzymes. From optimizing the feed of glucose and L-arabinose, the cost of scaling this production up could be minimized.

As for improving enzymes, protein engineering of enzymes or looking to other organisms for similar genes could work. This project could be replicated on these other organisms to determine which is best for BDO production.

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