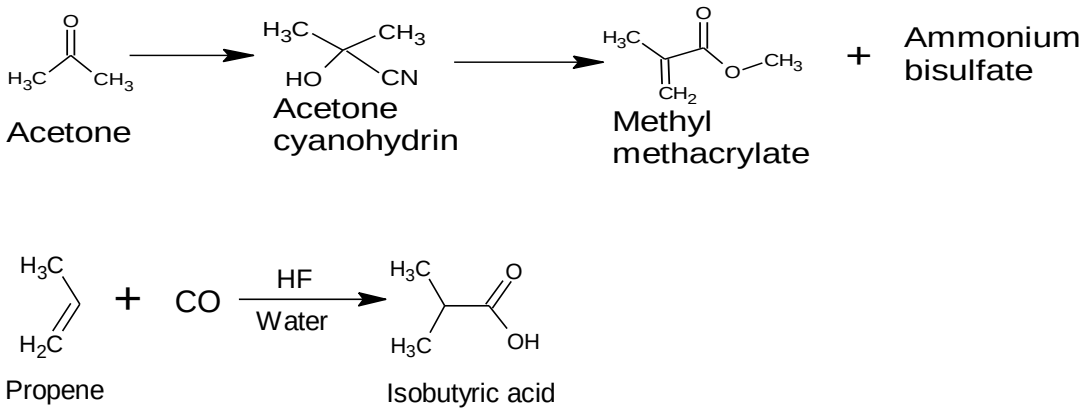
Alcohol Dehydrogenases Deletion to Increase Yield of Isobutyrate

Jun Zhou, Prof. Kechun Zhang

Introduction

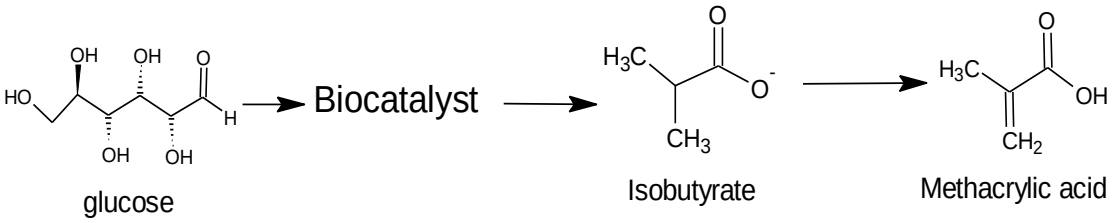
Methacrylic acid is one of the most crucial chemical feedstocks in the material industry and there is a high quantity demand of methyl methacrylate (MMA) which is the ester form of methacrylic acid. Every year million tons of methyl methacrylate is produced to synthesize polymethyl metharylate (PMMA) which is a thermoplastic material and it is used as a light or shatter-resistant alternative to glass. Therefore, to come up with a method for producing methacrylic acid and methyl methacrylate becomes a popular topic of research in biosynthesis area. There are several traditional methods being used in the industry and the most common one is reacting acetone with hydrogen cyanide to generate acetone cyanohydrins and then treat it with sulfuric acid to form methyl methacrylate. However, because of side products and reaction condition, these two traditional methods require high quality of controlling harmful chemicals and hard to increase the yield of target product. What's worse, the reactants are not renewable and emission will damage the environment.

Figure 1: Two traditional synthesis processes.



However, we have designed a new biosynthesis strategy to produce methacrylic acid. In this proposed work, glucose is converted to isobutyrate or 3-Hydroxyisobutyrate. By using oxidative dehydrogenation of isobutyrate and dehydration of hydroxyisobutyrate we could generate methacrylic acid.

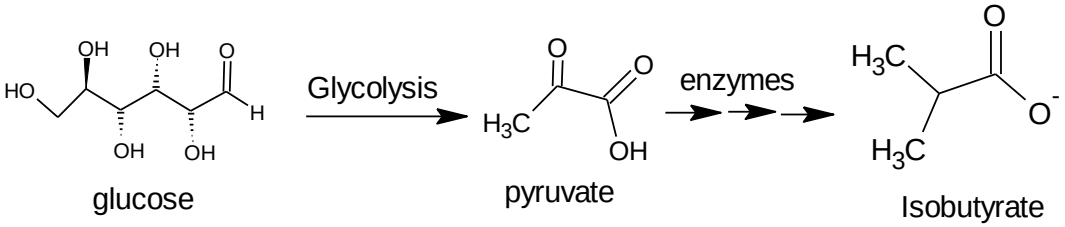
Figure 2: Biosynthesis process of methacrylic acid.



Methods and Materials

From previous work, a synthetic metabolic pathway in E. coli is constructed and the resultant whole-cell can convert glucose to isobutyrate. Through glycolysis glucose is metabolized to pyruvate and then via different appropriate enzymes glucose finally could produce methacrylic acid. Possible aldehyde dehydrogenases are picked up and tested by cloning plasmid and use HPLC analysis to quantify the fermentation products. The enzyme PadA has been characterized as the best enzyme to catalyze the oxidation of isobutyraldehyde.

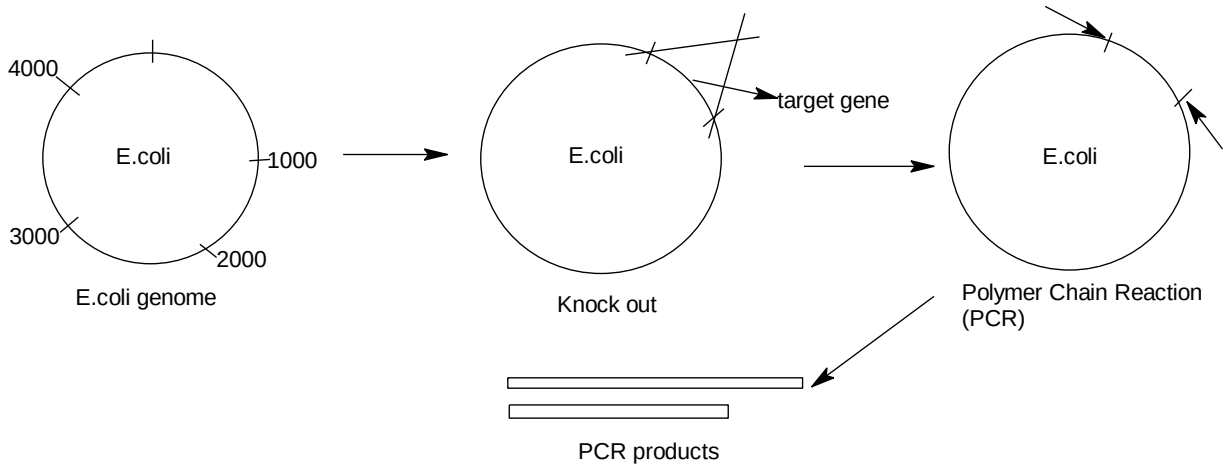
Figure 3: Synthesis metabolic pathway in E.coli.



Even though PadA does make a good yield of producing isobutyrate, there are some endogenous alcohol dehydrogenases responsible for producing alcohol which is a byproduct and the yield of isobutyrate is expected to increase. To improve the yield up

to a high level, alcohol dehydrogenases have to be deleted so that the final strain could specifically produce target products. From the E. coli genome, six alcohol dehydrogenases were found as predicted correct genes to be deleted: yqhD, adhE, adhP, eutG, yiaY and yigB. Before this proposal starts, knocking out gene yqhD has been proved as a correct deletion to increase the yield of isobutyrate. The resultant strain without yqhD can be a control and another strain with yqhD and adhE knocked out can be a starting strain to test whether adhE is a functional gene.

Figure 4: E.coli gene deletion process.



By using P1 transduction, all the resultant strains with corresponding genes knocked out can grow up as colonies on the surface of kanamycin plate. After this, the polymer chain reaction method can amplify a piece of DNA across several orders of magnitude and generate millions of copies of a particular DNA sequence in the resultant strains. Under UV light, the particular lengths of the PCR products indicate if the corresponding genes have been knocked out. Then the final five new E.coli strains will be tested for their fermentation performance. There are three different aldehydes used in the fermentation, 2-methylbutanal, 3-methylbutanal and isobutylaldehyde. During fermentation, aldehydes will be converted to either acid or alcohol. All five new resultant strains will be tested by HPLC analysis to see the

quantities of acid and alcohol generated from fermentation. Since different cells in fermentation will grow in different rate, the starting optical density of all cells should be the same. Optical density (OD) measures the concentration of cells in the solution. There are in total 5 resultant strains and each strain will grow in three different aldehydes so we prepare 15 flasks. For each flask, concentration of aldehyde is 2g/L (2μL/1mL solution) and mix with 200μL cells. Lastly, we add various volumes of fermentation medium to dilute until the OD measurements are the same. When measuring OD, we dilute target cell solution 20 times because OD values could not excess 1.000. After fermentation, it is necessary to measure OD again before run the HPLC analysis because we have to figure out what rate the cells grow at. On account of the fact that the peak of isobutanol for the fermentation with 2-methylbutanal will appear latest (at around 48 min) we set the HPLC running time of one single trial as 60mins for safety. And the peak area of peaks representing isobutanol is the parameter to show the production level of isobutanol because the more isobutanol is generated, the large the peak area will be. Therefore compared with the control strain △yqhD, if any of the 5 new strains lead to a decreasing production level of the alcohol isobutanol, the corresponding genes are correct to be deleted. As expectation, with all genes responsible for producing byproducts in the E. coli strain deleted, the final strain will improve the yield of isobutyrate biosynthesis.

Results and Analysis

Table 1: The Length of the PCR product for corresponding gene.

Strain with Corresponding Gene Knocked Out	Length (Successfully Knocked Out) in kb	Length (Not Knocked Out) in kb	Actual Length From Operation

dkgA	1.7	1.2	1.7
adhP	1.7	1.4	1.7
eutG	1.7	1.6	1.7
yiaY	1.8	1.6	1.8

From table 1, the actual length measurement of the PCR products indicates that all four alcohol dehydrogenases have been successfully knocked out. In addition, all fermentation performance for these four new E.coli resultant strains has been finished. In order to make the results more convincible and consistent we have done two trials of fermentation and in each trial we test products by different aldehydes for comparison. With the help of Optical Density of fermentation product, we could see whether knocking out the particular alcohol dehydrogenase actually decreases the production level of alcohol.

Table 2: 1st Fermentation Product and HPLC analysis Result by using aldehyde 2-methylbutanal.

Strain with Corresponding Gene Knocked Out	Optical Density of Fermentation Product	Peak Area for isobutanol
yqhD (1)	0.310	8666.14
yqhD and adhE (2)	0.565	13105.9
yqhD, adhE and dkgA (3)	0.242	29888.8
yqhD, adhE and yiaY (4)	0.608	16059.2
yqhD, adhE and adhP (5)	0.585	10163.1

Table 3: 1st Fermentation Product and HPLC analysis Result by using aldehyde 3-methylbutanal.

Strain with Corresponding Gene Knocked Out	Optical Density of Fermentation Product	Peak Area for isobutanol
yqhD (1)	0.107	20484.9
yqhD and adhE (2)	0.420	19029.68
yqhD, adhE and dkgA (3)	0.361	23230.7
yqhD, adhE and yiaY (4)	0.572	20050.2
yqhD, adhE and adhP (5)	0.581	20132.6

Table 4: 1st Fermentation Product and HPLC analysis Result by using aldehyde isobutylaldehyde.

Strain with Corresponding Gene Knocked Out	Optical Density of Fermentation Product	Peak Area for isobutanol
yqhD (1)	0.530	9013.82
yqhD and adhE (2)	0.417	4899.37
yqhD, adhE and dkgA (3)	0.236	58132.2
yqhD, adhE and yiaY (4)	0.610	3347.92
yqhD, adhE and adhP (5)	0.580	8973.32

Table 5: 2nd Fermentation Product and HPLC analysis Result by using 2-methylbutanal.

Strain with Corresponding Gene Knocked Out	Optical Density of Fermentation Product	Peak Area for isobutanol

yqhD (1)	0.225	16467.9
yqhD and adhE (2)	0.257	3269.81
yqhD, adhE and dkgA (3)	0.335	14274.5
yqhD, adhE and yiaY (4)	0.404	16755.2
yqhD, adhE and adhP (5)	0.500	31448.7

Table 6: 2nd Fermentation Product and HPLC analysis Result by using isobutylaldehyde.

Strain with Corresponding Gene Knocked Out	Optical Density of Fermentation Product	Peak Area for isobutanol
yqhD (1)	0.479	11469.5
yqhD and adhE (2)	0.498	10100.6
yqhD, adhE and dkgA (3)	0.387	21449.1
yqhD, adhE and yiaY (4)	0.460	23843.6
yqhD, adhE and adhP (5)	0.251	41138.8

- The resultant strains are labeled in order from (1) to (5).

YqhD is proved as a correct alcohol dehydrogenase to be deleted for decreasing production level of isobutanol. From table 2, the control strain △yqhD (1) has OD 0.310 and the corresponding peak area is 8666.14 while strain with one more alcohol dehydrogenase adhE (2) knocked out has OD 0.565 and it has the corresponding peak area 13105.9 for isobutanol. The strain (2) has double concentration but the peak area is only 4439.76 which is a relative small amount difference. Similarly, from table 3 the control strain (1) with OD 0.107 and isobutanol peak area is 20484.9. However, the strain (2) has OD 0.420 which is much larger than the control and it only has a

alcohol peak with area 19029.68 which is even smaller than the control. We can get the same observations for the rest of the tables. This result suggests that adhE is one of the correct alcohol dehydrogenases responsible for generating isobutanol and required to be deleted.

For the rest of the resultant strains, we use strain (2) as a control since strains (3), (4) and (5) are built up from the strain (2). That means the last three strains has only one more dehydrogenase knocked out compared with strain (2). For the strain (5), in table 2, it has OD value 0.585 which is more concentrated than the strain (2) but it has just a small isobutanol peak with area 10163.1 which is much less than what strain (2) did. From table 3, strain (5) has OD value 0.581 which is relative large amount difference but the difference of alcohol peak area is even less than 1000. These results show that dehydrogenase adhP is a correct gene required to be deleted.

Strain (4) has yqhD, adhE and yiaY alcohol dehydrogenases knocked out. From table 4, we could easily tell that with yiaY knocked out, although the resultant strain has a large OD value 0.610, it only has the corresponding peak for isobutanol with area 3347.92 while the control strain (2) has a smaller OD value 0.417 but generates more isobutanol because it has peak area of 4899.37. However, from the other four tables, strain (4) has a larger OD values than what strain (2) does and meanwhile strain (4) has a higher production level of isobutanol. Because of the inconsistency, the observations cannot prove yiaY as the dehydrogenase responsible for producing alcohol but it is most likely to be a correct one for deletion. Finally, we could consider the strain with yqhD, adhE and dkgA knocked out as a failure strain to increase the yield of isobutyrate. Whenever the strain (2) has a large OD value or not it always produces great amount of isobutanol. This result shows that there is a functional interference occurring within the living cells. And the interference may be caused by the combination of those three particular dehydrogenases knockout. That means there

are some other possible strains with some other dehydrogenases including dkgA that can produce less alcohol.

Conclusion

We have shown that all four possible alcohol dehydrogenases adhE, adhP, eutG and yiaY have been successfully knocked out. By varying fermentation aldehydes, a variety of different fermentation products have been prepared and analyzed by HPLC. As a result, we have identified two particular dehydrogenases adhE and adhP as appropriate targets necessary to be deleted. With these two deletions, the production level of corresponding alcohol isobutanol successfully decreased. Most importantly, we have found out from the dkgA deletion case that more deletion does not mean a better strain due to the disruption of internal physiological state. This provides a better sense for continuing research on other predicted alcohol dehydrogenases deletions and meanwhile it suggests that further exploration on this research is necessary. The two dehydrogenases yiaY and dkgA have a great impact on decreasing isobutanol production so by using appropriate deletion a number of new resultant strains will effectively improve the yield of isobutyrate.

Reference

1. Causey, T.B., Zhou, S., Shanmugam, K.T. & Ingram, L.O. Engineering the metabolism of Escherichia coli W3110 for the conversion of sugar to redox-neutral and oxidized products: homoacetate production. *Proc. Natl. Acad. Sci. USA* **100**, 825-832 (2003).
2. Atsumi, S. et al. Engineering the isobutanol biosynthetic pathway in Escherichia coli by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Appl. Microbiol. Biotechnol.* **85**, 651-657 (2010).
3. Baba, T. et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 10.1038 (2006).
4. Datsenko, K.A. & Wanner, B.L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**, 6640-6645 (2000).
5. Esterbauer, H., Schaur, R.J. & Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11**, 81-128 (1991).
6. Gruez, A. et al. Crystal structure and kinetics identify Escherichia coli YdcW gene product as a medium-chain aldehyde dehydrogenase. *J. Mol. Biol.* **343**, 29-41 (2004).
7. Firth, A.E. & Patrick, W.M. Statistics of protein library construction. *Bioinformatics* **21**, 3314-3315 (2005).
8. Warnecke, T. & Gill, R.T. Organic acid toxicity, tolerance, and production in Escherichia coli biorefining applications. *Microb. Cell Fact.* **4**, 25 (2005).
9. Lee, I.Y. et al. Production of D-β-hydroxyisobutyric acid from isobutyric acid by Candida rugosa. *J. Ferment. Bioeng.* **81**, 79-82 (1996).
10. Miller, E.N. et al. Silencing of NADPH-dependent oxidoreductases (yqhD and dkgA) in furfural-resistant ethanologenic Escherichia coli. *Appl. Environ. Microbiol.* **75**, 4315-4323 (2009).
11. Atsumi, S. et al. Evolution, genomic analysis, and reconstruction of isobutanol tolerance in Escherichia coli. *Mol. Syst. Biol.* **6**, 449 (2010).
12. Jacob, N.M. et al. Reaching the depth of the Chinese hamster ovary cell transcriptome. *Biotechnol. Bioeng.* **105**, 1002-1009 (2010).
13. Goodhue, C.T. & Schaeffer, J.R. Preparation of L(+) β-hydroxyisobutyric acid by bacterial oxidation of isobutyric acid. *Biotechnol. Bioeng.* **13**, 203-214 (1971).
14. Kim, C.H., Hong, W.K., Lee, I.Y., Choi, E.S. & Rhee, S.K. Enhanced production of D-β-hydroxyisobutyric acid through strain improvement. *J. Biotech.* **69**, 75-79 (1999).

15. Amutha, R. & Gunasekaran, P. Production of ethanol from liquefied cassava starch using co-immobilized cells of Zymomonas mobilis and Saccharomyces diastaticus. *J. Biosci. Bioeng.* **92**, 560-564 (2001).
16. Aberhart, D.J. A stereochemical study on the metabolism of isobutyrate in Pseudomonas putida. *Bioorg. Chem.* **6**, 191-201 (1977).
17. Brock, M., Maerker, C., Schütz, A., Völker, U. & Buckel, W. Oxidation of propionate to pyruvate in Escherichia coli. *Eur. J. Biochem.* **269**, 6184-6194 (2002).
18. Hashimoto, Y. et al. Nitrile pathway involving acyl-CoA synthetase. *J. Biol. Chem.* **280**, 8660-8667 (2005).
19. Zhang, Y.X. et al. Genes encoding acyl-CoA dehydrogenase (AcdH) homologues from Streptomyces coelicolor and Streptomyces avermitilis provide insights into the metabolism of small branched-chain fatty acids and macrolide antibiotic production. *Microbiology* **145**, 2323-2334. (1999).
20. Lee, S.H., Park, S.J., Lee, S.Y. & Hong, S.H. Biosynthesis of enantiopure (S)-3-hydroxybutyric acid in metabolically engineered Escherichia coli. *Appl. Microbiol. Biotechnol.* **79**, 633-641 (2008).
21. Steele, M.I., Lorenz, D., Hatter, K., Park, A. & Sokatch, J.R. Characterization of the mmsAB operon of Pseudomonas aeruginosa PAO encoding methylmalonate-semialdehyde dehydrogenase and 3-hydroxyisobutyrate dehydrogenase. *J. Biol. Chem.* **267**, 13585-13592 (1992).