Accepted Manuscript

Production of Acetol from Glycerol Using Engineered Escherichia coli

Hongliang Zhu, Xianyang Yi, Yi Liu, Hongbo Hu, Thomas K. Wood, Xuehong Zhang

PII: S0960-8524(13)01493-4
DOI: http://dx.doi.org/10.1016/j.biortech.2013.09.062
Reference: BITE 12427

To appear in: Bioresource Technology

Received Date: 30 July 2013
Revised Date: 12 September 2013
Accepted Date: 16 September 2013


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Production of Acetol from Glycerol Using Engineered *Escherichia coli*

Hongliang Zhu¹, Xianyang Yi¹, Yi Liu¹, Hongbo Hu¹*, Thomas K. Wood², Xuehong Zhang¹

¹Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China, 200240

²Department of Chemical Engineering, Pennsylvania State University, University Park, PA 16802-4400, USA

**Keywords:** acetol; glycerol; methylglyoxal
Abstract

*Escherichia coli* Lin43 is a strain which has some mutations in glycerol kinase (GlpK) and the repressor for the glycerol 3-phosphate regulon (GlpR). When exposed to glycerol, it quickly accumulates lethal levels of methylglyoxal, which is a precursor of acetol; acetol is important for the manufacture of polyols, acrolein, dyes, and skin tanning agents. This work reports the engineering of *E. coli* Lin 43 for the conversion of glycerol into acetol. First, the glyoxalase system was interrupted by deleting the *gloA* gene, which increased the acetol yield by 32%. In addition, the aldehyde reductase YqhD was overexpressed which led to an increase of acetol production by 11.4 fold. Acetol production was optimized by varying the cell density, glycerol concentration, supplemental carbon source, pH and temperature. Under the optimal conditions (OD$_{600}$ = 20, 20 g/L glycerol, 2 g/L succinate, pH7.0, and 28°C), we obtained 5.4 g/L acetol in 21 h.
1. Introduction

Due to the increase in environmental concerns and diminishing supplies of fossil fuels, biodiesel, a good alternative and renewable fuel, has attracted much attention because it could reduce net greenhouse effects (Almeida et al., 2012). As a result, biodiesel production increased rapidly in the past few years. For example, the global biodiesel production in 2010 was about 5 billion gallons, which is 4.7 fold more than that in 2005 (Almeida et al., 2012). However, biodiesel production based on triglycerides will produce about 10% (w/w) glycerol as the main byproduct. The increasing production of biodiesel results in an increased production of glycerol. It was estimated that the global biodiesel market would reach 37 billion gallons by 2016, which means about 4 billion gallons of crude glycerol will be generated. (Yang et al., 2012). The excess of glycerol produced in the biodiesel industry is leading to a decrease in glycerol prices and glycerol is now considered a waste rather than a co-product (Yazdani & Gonzalez, 2007). Therefore, it is imperative to discover potential value-added applications of glycerol and provide an ideal platform for chemical and pharmaceutical industries. A great number of studies have used diverse chemical and biological approaches to convert glycerol to various value-added products (Barbelli et al., 2012; Clomburg & Gonzalez, 2013; Posada et al., 2012; Wang & Yang, 2013).

Acetol, a C3 keto alcohol, is an important intermediate used to produce polyols and acrolein (Mohamad et al., 2011). It is also widely used as a reduced dye in the textile industry and as a skin tanning agent in the cosmetic industry (Soucaille et al.,
Acetol can be synthesized from glycerol via dehydration (Chiu et al., 2008; Yamaguchi et al., 2010) or from propylene glycol via dehydrogenation (Sato et al., 2008). However, the high cost of acetol by chemical processes reduces its industrial application and markets (Soucaille et al., 2008a).

Bioconversion of glycerol is considered to be a more sustainable option, since it offers an environmentally friendly approach without the use of high temperatures/pressures and the addition of toxic organic solvents. However, there are only a limited number of publications for bioconversion of glycerol to acetol. It was reported that three strains of *Clostridium thermosaccharolyticum* could produce acetol from *D*-xylose and *D*-glucose, and the highest yield reached was 1.47 g/L (Cameron & Cooney, 1986). Cameron *et al.* used *S. cerevisiae* expressing the *mgsA* gene of *E. coli* to produce acetol. However, the best titers reported are below 0.1 g/L (Cameron et al., 1999). Moreover, Soucaille *et al.* claimed that 1.40 g/L and 1.63 g/L of acetol could be obtained respectively by two different genetically engineered *E. coli* strains from glucose (Soucaille et al., 2008a; Soucaille et al., 2008b).

Glycerol can be transformed to acetol via the methylglyoxal (MG) bypass pathway, in which DHAP is converted to MG and subsequently metabolized to acetol and 1,2-propanediol (Cooper, 1984). MG is very toxic and arrests growth of *E. coli* at millimolar concentrations. Thus, its production is tightly controlled in the cells. In order to improve the metabolic flux through this pathway, it is essential to overexpress MG synthase and the genes converting glycerol to DHAP and at a low phosphate condition (Altaras & Cameron, 1999; Clomburg & Gonzalez, 2011).
E. coli strain Lin43, which lacks fructose-1, 6-bisphosphate inhibition of glycerol utilization, synthesizes excessive MG during unregulated glycerol metabolism (Lin, 1976). As a result, cells are killed because of the accumulation of cytotoxic MG (Freedberg et al., 1971). In microorganisms, MG can be converted to acetol by aldo-keto reductases (Cameron & Cooney, 1986; Ko et al., 2005). E. coli Lin43 strain can accumulate MG when the cells are using glycerol as a carbon resource. Therefore, it is feasible to produce acetol from glycerol by strain Lin43 if MG can be rapidly converted to acetol.

The aims of this study were to obtain a genetically engineered Lin43 strain which can efficiently produce acetol from glycerol. By using resting cells, we obtained 5.38 g/L of acetol in 21 hours. To our knowledge, this is highest report of production of acetol from glycerol by E. coli and the highest yield.
2. Material and methods

2.1 Strains and plasmids

The strains, plasmids and primers used in this study are listed in the Table 1. For deleting and overexpressing genes, we used the Keio collection and the ASKA library, respectively. Kanamycin (50 μg/mL) was used for pre-culturing the isogenic knock-outs. Chloramphenicol (30 μg/mL) was used for the strains harboring pCA24N and its derivatives. The gloA gene in Lin43 was disrupted by P1 phage transduction (Silhavy et al.).

2.2 Culture medium and growth conditions

Cells were cultivated aerobically in medium A containing 34 mM NaH$_2$PO$_4$, 64 mM K$_2$HPO$_4$, 20 mM (NH$_4$)$_2$SO$_4$, 0.3 mM MgSO$_4$, 0.001 mM FeSO$_4$, 0.001 mM ZnCl$_2$, 0.01 mM CaCl$_2$, 15 mM sodium succinate, and 0.04% (g/g) casein hydrolysate (Tanaka et al., 1967). Liquid cultures were routinely incubated in 250-mL-baffled Erlenmeyer flasks at 37 °C and 130 rpm.

2.3 Production of acetol by the resting cells

To obtain the active biomass of the resting cells, the cells were grown in medium A in the presence of the appropriate antibiotics at 37 °C and 180 rpm to an OD600 of 0.3 and induced with 1 mM of IPTG. After 3 h, the cells were harvested by centrifugation (5000 g, 4 °C, and 8 min), washed with phosphate buffer (100 mM, pH 7.0), and resuspended in the phosphate buffer containing glycerol at 20 g/L.

Biotransformations were performed at 37 °C and 180 rpm aerobically.

2.4 Analytical methods
Concentrations of acetol, glycerol, and MG were measured by high performance liquid chromatography (HPLC; Agilent, model 1260) using a Rezex ROA-Organic Acid H\(^+\) column (300 mm × 7.8 mm; Phenomenex, Torrence, CA, USA) and a differential refractive index (RI) detector (Altaras & Cameron, 1999). Prior to analysis, each sample was centrifuged at 5000g for 8 min and the supernatant was filtered through 0.45 μm membranes. The operation conditions were: mobile phase, 5 mM H\(_2\)SO\(_4\); column temperature, 60 °C; flow rate, 0.5 mL/min. The retention time of MG, glycerol and acetol are 16.7 min, 18.2 min and 22.7 min, respectively.

3. Results and discussions

3.1 The Deletion of gloA gene increased acetol yield

MG, the precursor of acetol, is a toxic metabolite known for its inhibition of cell growth and cell death (Booth et al., 2003; MacLean et al., 2002). There are several pathways for the degradation of MG in E. coli. The glyoxalase system, which consists of glyoxalase I (encoded by gloA) and II (encoded by gloB), is the dominant route to transform MG (MacLean et al., 2002). In order to improve the yield of acetol, the glyoxalase system in Lin43 strain was disrupted by deleting the gloA gene. As shown in Fig.1, the gloA-deficient strain reached the yield of acetol as 0.044 g/g glycerol, which was 32% more than that of the parent strain. These results indicate that the disruption of the glyoxalase system improves the carbon flux flow to acetol and improved its yield. Therefore, the Lin43 ΔgloA strain was used for the further studies.

3.2 Overexpression of the genes for the conversion of MG to acetol
MG can be converted to acetol by the action of aldehyde reductase which is encoded by \textit{yqhD} gene (Clomburg & Gonzalez, 2011; Ko et al., 2005), and YqhC is a transcriptional activator of \textit{yqhD} (Lee et al., 2010). In addition, MG can be converted to acetol by aldo-keto reductases (AKRs) from \textit{E. coli} and the mammalian sources (Ko et al., 2005). Some AKRs, such as YafB and AKR from human liver (Cao et al., 1998), have large values of \(k_{\text{cat}}/K_m\) on MG. Therefore, overexpression of the \textit{yqhD} gene, aldo-keto reductases from \textit{E. coli} (encoded by \textit{yafB}) and human liver (\textit{akr}), and the \textit{yqhC} gene were investigated. As shown in Fig.2, the concentration of acetol produced by the overexpressed strains increased compared to the control. The yield of acetol by \textit{yqhD} overexpression reached 0.58 g/L, which was 11.4-fold higher than that of the control. Overexpression of \textit{yafB}, \textit{akr}, and \textit{yqhC} also increased the yield by 6.4, 5.2 and 1.8 fold, respectively.

The \textit{E. coli} aldehyde reductase YqhD has drawn the attention of researchers when it was used to produce 1, 3-PD using recombinant \textit{E. coli} (Jarboe, 2011). YqhD has reductase activity for a broad range of short-chain aldehydes (Jarboe, 2011) and has successfully been used for the production of 1, 2-propanediol at high yield and titer (Clomburg & Gonzalez, 2011). Our results show that YqhD is more suitable than several kinds of aldo-keto reductase for acetol production. Thus, the strain Lin43 Δ\textit{gloA} pCA24N-\textit{yqhD} was selected for further study.

\textit{3.3 Production of acetol by the resting cells}

In order to produce MG from glycerol, strain Lin43 was grown in medium A for full expression of the \textit{glp} operon and then the cells were exposed to glycerol.
(Freedberg et al., 1971). Moreover, it has reported that high density of cells can increase the resistance to MG (Fraval & McBrien, 1980). Furthermore, there is only one step from MG to acetol. Thus, it is feasible to transform glycerol to acetol using resting cells of Lin43 ΔgloA pCA24N-yqhD. First, the possibility of production of the acetol by resting cells was investigated. It was found that the resting cells in phosphate buffer with 20 g/L glycerol could produce 0.51 g/L acetol at OD$_{600}$ = 0.75 (equal to 0.31 g cell dry weight per liter), which is about the same level as the exponentially growing cells (0.58 g/L, Fig.2). The results indicate that the resting cells do not lose activity for converting glycerol to acetol in phosphate buffer.

3.4 Effect of supplemental carbon sources on acetol production

It was reported that the specific activity of glycerol kinase in strain Lin43 increased by 6.3 fold on succinate compared to glucose (Zwaig et al., 1970). Therefore, the effect of supplemental carbon sources on acetol production was investigated. Several kinds of carbon sources (citrate, glucose, pyruvate, succinate and sucrose) were added to the conversion medium to 67 mmol C/L, respectively. As shown in Fig.3, addition of succinate and glucose increased acetol production by 71% and 21% respectively compared with the control, while addition of citrate and pyruvate showed slight effects on acetol production but sucrose decreased acetol production by 10%. Moreover, the yield of acetol increased by 30% when succinate was added. Furthermore, the effect of succinate concentration on acetol production was studied. As shown in Fig.4, the highest acetol concentration was obtained with 2 g/L succinate.
3.5 Effect of temperature on MG concentration

Temperature is an important factor for biotransformations. First, we conducted the biotransformation process at 37°C. As shown in Fig. 5, the concentration of acetol reached a maximum at 13 h and then remained unchanged. In addition, the glycerol consumption stopped and the concentration of MG reached 5.4 mM. Cells lose viability when the MG concentration in the growth medium reaches 0.6 mM (Booth et al., 2003). Therefore, these results indicate that overproduction of MG results in cell death. It was further confirmed that the cells were lost the ability to transform glycerol to acetol when they were collected and reused (data not shown). In order to alleviate the damage of MG, we conducted the transformation experiments at lower temperature, 28 °C. The results show that the resting cells produce acetol slower than those at 37 °C at the first 10 h, but the concentration of acetol increases with the incubation time. As a result, the acetol production at 28 °C is 41% higher than at 37 °C after 72 hours fermentation. Moreover, the MG concentration almost remains constant at a low level at 28 °C. However, a lower productivity is obtained because it needs longer times to reach a high acetol production. Other approaches, such as NADPH regeneration and keeping a low glycerol concentration by fed batch cultivation, may be effective for the decrease of MG concentration.

3.6 Screening other basic parameters for acetol production

The toxicity of MG depends on many parameters, such as the characteristics of the growth medium and the growth phase of the cells (Ferguson et al., 1998b). In order to enhance the acetol production and alleviate the damage of MG, the effects of
phosphate buffer pH, glycerol concentration, cell mass, and the cells phase on acetol production were investigated. The optimal glycerol concentration was found to be 20 g/L, as shown in Fig. 6A. Acetol production increased slightly as the glycerol concentration increased from 10 g/L to 20 g/L and decreased at 40 g/L. As shown in Fig. 6B and C, acetol production was highest at pH 7.0 and cell OD$_{600}$ = 20 (equal to 8.2 g cell dry weight per liter). In addition, cells from exponential and stationary phase were used for acetol production. The results showed that acetol produced by exponential cells is only 30% as those of stationary cells (Fig.6D). Similar results were obtained when *E. coli* cells were exposed to the toxic electrophile N-ethylmaleimide (Ferguson et al., 1998a). Under the optimized conditions, we obtained 5.4 g/L acetol in 21 h at 28°C when concentrated cell mass (OD$_{600}$=20) of strain Lin43 ΔgloA pCA24N-yqhD was used. To our knowledge, it is the highest reported yield to date (Table 2).

4. Conclusions

Acetol is an important organic intermediate or starting material for the chemical industry. By deleting *gloA* and overexpression of *yqhD* in strain *E. coli* Lin43, here we obtained a recombinant strain that increased the acetol production and yield. Moreover, we increased the acetol production by using a resting cell system. After optimization of process conditions, this strain produced 5.4 g/L acetol. As strain Lin43 accumulates excess MG, methods to reduce the MG concentration are the key point for further improvements in acetol production by strain Lin43.
Acknowledgements

This work was supported by 973 Programs of China (2012CB721005).

References


(−)–1, 2–Propanediol and Acetol by Clostridium thermosaccharolyticum. Nat.


Low-pressure packed-bed gas phase conversion of glycerol to acetol. AIChE J.,
54, 2456-2463.


for the production of 1,2-propanediol from glycerol. Biotechnol. Bioeng., 108,
867-879.

Microbiol., 38, 49-68.

RpoS and Dps in survival of exposure of both exponential- and stationary-phase
Escherichia coli cells to the electrophile N-ethylmaleimide. J. Bacteriol., 180,
1030-1036.


93, 642-648.


Figure Captions

Fig. 1. Production of acetol in strain Lin43 ΔgloA.

Left: Lin43 strain; right: Lin43ΔgloA strain. Acetol was produced 24 hours after adding glycerol into the growth medium when OD600 reached 0.75. Transformation was conducted aerobically at 37 °C.

Fig. 2. Effect of overexpression of the gene of akr, yafB, yqhC, and yqhD on acetol production in strain Lin43.

Glycerol was added to the medium to 20g/L when OD600 reached 0.75, then the samples were analyzed after 24 hours aerobic incubation at 37 °C.

Fig. 3. Effect of supplemental carbon sources on the production of acetol by resting cells of Lin43ΔgloA/pCA24N-yqhD.

The samples were analyzed at 24 hours after transferring the resting cells into the conversion medium containing 2 g/L succinate (equivalent to approximately 67 mmolC/L) or other carbon sources (equivalent to approximately 67 mmolC/L). The biotransformation was conducted aerobically at 37 °C.

Fig. 4. Effect of succinate concentration on the production of acetol by resting cells of Lin43ΔgloA/pCA24N-yqhD.

The samples were analyzed at 24 hours after transferring the resting cells into the conversion medium with different concentrations of succinate. The biotransformation
was conducted aerobically at 37 °C.

Fig.5. Time course of the fermentation by the resting cells of *E. coli* Lin43\(\Delta\)gloA/pCA24N-yqhD at 28 °C and 37°C.

The products were detected after transferring the resting cells into the conversion medium containing 2 g/L succinate. The transformation was conducted aerobically with OD\(_{600}\) at 3.0 at 37 °C and 28 °C, respectively.

Fig. 6. Screening other parameters of the biotransformation. (A) Effect of glycerol on acetol production. (B) Effect of pH on production. (C) Effect of the cell concentration of on acetol production. (D) Effect of the cell growth phage on acetol production.
Glycerol consumption (g/L) vs. Acetol yield (g/g%) for strains Lin43 and Lin43 ΔgloA. The graph shows increased acetol yield with the deletion of the gloA gene.
Fig. 2: Bar graph showing Acetol concentration and yield for different strains:
- Lin43ΔgloA/pCA24N
- Lin43ΔgloA/pCA24N-yqhD
- Lin43ΔgloA/pCA24N-yafB
- Lin43ΔgloA/pCA24N-yqhC
- Lin43ΔgloA/pCA24N-akr

Y-axis: Acetol concentration (g/L) and Acetol yield (g/g)
X-axis: Strains

Legend:
- White bars: Acetol concentration
- Gray bars: Acetol yield
A. Relative acetol concentration (%) vs. Glycerol (g/L)

B. Relative acetol concentration (%) vs. pH

C. Relative acetol concentration (%) vs. OD$_{600}$

D. Relative acetol concentration (%) vs. Cell growth phase (Exponential vs. Stationary)
Table 1. Strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strains, plasmids primers</th>
<th>Description/Genotype/Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lin43</td>
<td>Hfr(PO2A) fhuA22 ΔphoA8 fadL701(T2R) relA1 glpR2(glp') pitA10 spoT1 glpK22(bR) rmpB-2 mcrB1 creC510 lacF5 rrb714 ΔlacZΔM15 hsdR514 ΔvarA ΔBAD ΔrhaBAD Δ3578 Δdeletion for gloA gene in BW25113 Lin43, ΔgloA::FRT-Km-FRT; deletion mutant for gloA gene in Lin43 Lin43 ΔgloA / pCA24N Lin43 ΔgloA, Cm; lacF5, pCA24N Lin43 ΔgloA / pCA24N-mgsA Lin43 ΔgloA, Cm; lacF5, pCA24N P₇₅₁₆::mgsA+ Lin43 ΔgloA / pCA24N-yqhD Lin43 ΔgloA, Cm; lacF5, pCA24N P₇₅₁₆::yqhD+</td>
<td>CGSC (Freedberg et al., 1971) (Baba et al., 2006) This study This study This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCA24N</td>
<td>Cm; lacF5, pCA24N</td>
<td>(Kitagawa et al., 2005)</td>
</tr>
<tr>
<td>pCA24N-mgsA</td>
<td>Cm; lacF5, pCA24N P₇₅₁₆::mgsA+</td>
<td>(Kitagawa et al., 2005)</td>
</tr>
<tr>
<td>pCA24N-yqhD</td>
<td>Cm; lacF5, pCA24N P₇₅₁₆::yqhD+</td>
<td>(Kitagawa et al., 2005)</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-gloA</td>
<td>F:5'-GCCGCCGCTATACTAAAACA-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R:5'-TGAGCGTTATCGGACATCTG-3'</td>
<td></td>
</tr>
</tbody>
</table>

*Cm* and *Km* are chloramphenicol and kanamycin resistance, respectively.
Table 2. The production of acetol by different researchers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Production concentration (g/L)</th>
<th>Fermentation time (h)</th>
<th>Yield (g/g carbon source)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium thermosaccharolyticum</em></td>
<td>Glucose</td>
<td>1.47</td>
<td>95</td>
<td>0.04</td>
<td>(Cameron &amp; Cooney, 1986)</td>
</tr>
<tr>
<td><em>Saccharomy cerevisiae</em></td>
<td>Glucose</td>
<td>&lt;0.1</td>
<td>72</td>
<td>&lt;0.005</td>
<td>(Cameron et al., 1999)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Glucose</td>
<td>1.40</td>
<td>72</td>
<td>0.09</td>
<td>(Soucaille et al., 2008a)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Glucose</td>
<td>1.63</td>
<td>-</td>
<td>0.17</td>
<td>(Soucaille et al., 2008b)</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td>Glucose</td>
<td>3.3</td>
<td>84</td>
<td>0.055</td>
<td>(Niimi et al., 2011)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Glycerol</td>
<td>5.38</td>
<td>21</td>
<td>0.37</td>
<td>This study</td>
</tr>
</tbody>
</table>
Highlights

> A recombinant *E. coli* strain Lin43 was constructed to convert glycerol into acetol.

> Deletion of the gene of *gloA* increased the acetol yield by 32%.

> Overexpression of the gene of *yqhD* increased the acetol production by 11.4 fold.

> Under the optimal conditions, the engineered strain produced 5.4 g/L acetol in 21h.