Mini-review

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Metabolic strategies for microbial glycerol overproduction

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Abstract

Glycerol as a platform chemical has wide applications in chemical, pharmaceutical, and food industries and is a useful feedstock for the production of bio-based chemicals. There is an increasing demand for sustainable glycerol production and microbial glycerol production is a promising alternative. The paper highlighted this new trend and summarized recent research progress on microbial glycerol production, and focused mainly on the efforts achieved in rational design of *S. cerevisiae*, as well as *Escherichia coli*, to overcome the limitations of glycerol production in these two microorganisms. More efforts are required for overcoming hurdles in yield and productivity enhancement for large scale production.

Keywords: metabolic strategies; glycerol fermentation; *saccharomyces cerevisiae*; *escherichia coli*

INTRODUCTION

Glycerol has wide applications in the chemical, pharmaceutical, and food industries for manufacturing of an array of chemicals, drugs, food and beverages, personal care items, lubricants, adhesives, emulsifiers, plastics, and other products.[1,2] As a versatile biodegradable platform molecule, glycerol has the potential to be developed into diverse products including polymers, ethers, polyesters, nylons, as well as important intermediates like propylene glycol. The demand for these high-value products hence drives progression in the technologies required for conversion of glycerol.[3] For instance, recently, Nippon Shokubai and Arkema have reported efforts to derive acrylic acid, a petrochemical with average annual revenue of $7 billion, from alternative feedstock, namely glycerol (IHS Chemical report, 2011).

Currently, glycerol is mostly obtained as a waste product from biodiesel plants via transesterification, hence the increase in new applications due to rapid development of the biodiesel market leading to higher volumes and lower prices of glycerol produced.[4] It is also recovered as a by-product in the processing of oils, for example, during saponication of fats in soap making. Industrially, the most significant process of glycerol synthesis is by propylene, in a method developed in the late 1930s.[5] However, due to the increasing costs of propylene, these routes of production have gradually become less attractive economically.[1,5,6] World production of glycerol ranges from 500 000 to 750 000 tonnes annually, with the United States being one of the dominant players in the market.[3]

Other than current chemical routes, its production by microorganisms has also been explored over the years. Many microorganisms including yeast, bacteria and algae produce glycerol. In fact, glycerol formation as a byproduct in the fermentation of alcohol was discovered by Pasteur in 1858.[5] Microbial glycerol production was attempted during the early 1940s based on Neuberg's investigation on alcoholic fermentation, but failed to gain importance industrially when compared with the synthetic chemical routes due to low yields of glycerol and difficulties encountered in purification and extraction of the target from fermentation broth.[1,6] Due to a new trend in the bioenergy industry where fats and oils are cracked down directly to hydrocarbons, it is probable that glycerol will not be produced in the biodiesel industries in future.[7] Yet, due to its multifaceted applications, the demand for glycerol remains. Furthermore, because of the inevitable decline in fossil resources and rapid advancement in biotechnology, there has been renewed interest in the production of platform chemicals, including glycerol and its derivatives, from alternative renewable sources.[8]

Recently, there have been increasing research efforts and progress on improving glycerol production in microorganisms. Since glycerol production by osmotolerant yeast has been previously reviewed,[6] this paper will focus mainly on the efforts made in the metabolic engineering of *Saccharomyces cerevisiae*, as well as *Escherichia coli*, to overcome the limitations of glycerol production in these two microorganisms.

GLYCEROL PRODUCTION PATHWAY IN YEAST

Glycerol is an important metabolite formed naturally in *S. cerevisiae*. In glycolysis, glucose is assimilated to form fructose-1,6-bisphosphate, which is then divided into two 3-carbon molecules, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate (DHAP). The production of glycerol continues from DHAP, via the reduction of DHAP by glycerol-3-phosphate dehydrogenase ($Gpd1_p$) to form glycerol-3-phosphate, a process

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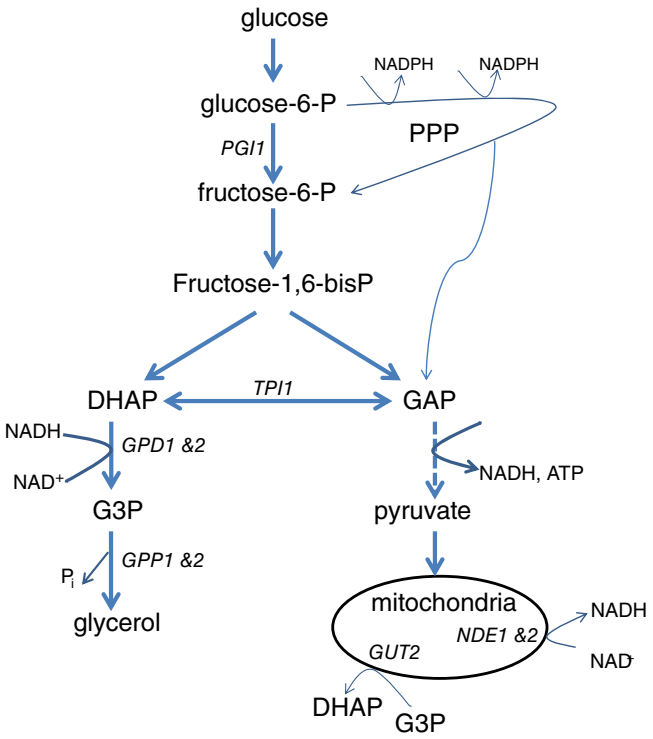

Figure 1. A simplified glycerol biosynthetic pathway in *S. cerevisiae*. DHAP, dihydroxyacetonphosphate; GAP, glycerolaldehyde-3-phosphate; PPP, pentose phosphate pathway; *TPI1*, triose phosphate isomerase gene; GPD1&2, glycerol-3-phophate dehydrogenase 1&2; GPP1&2, glycerol-3-phosphatase gene 1 and 2.

requiring NADH as co-factor. This is followed by the dephosphorylation of glycerol-3-phosphate by glycerol-3-phosphatase ($Gpp2_p$) to produce glycerol, as depicted in Fig. 1.

Under anaerobic conditions, glycerol biosynthetic pathway plays a vital role in maintaining the redox balance in *S. cerevisiae*. Excess reducing equivalents formed during cell synthesis reactions in anaerobic conditions are oxidized through glycerol production. Glycerol thus serves as a sink for the excess NADH, and is produced in more significant amounts in anaerobic rather than aerobic conditions.[6,9,10]

Glycerol also functions as a major osmolyte in *S. cerevisiae*. In response to environmental osmotic changes, cells adjust their intracellular concentration of osmolytes so as to maintain cell turgor, cellular activity and growth.[11] In *S. cerevisiae*, the high osmolarity glycerol (HOG) pathway is activated when external osmolarity is increased, allowing the accumulation of glycerol so as to prevent loss of water, and to reduce the difference in transmembrane osmotic pressures.[12] As for efflux of glycerol from the *S. cerevisiae* cells during osmoregulation, the channel protein Fps1p is a major porin involved in glycerol transport across the membrane, and is linked with the activation of the HOG pathway.[13] *FPS1* encodes for a channel protein responsible for secretion of glycerol out of the yeast cell. Deletion of this gene reduced glycerol production and increased ethanol levels. However the opposite effect was not seen when over expressing FPS1. By deleting the control region to the N-terminal of the Fps1p protein it produced a constitutively open channel that resulted in higher levels of glycerol production and secretion together with lowered ethanol yields.[38]

There are two isoenzymes, $Gpd1_p$ and $Gpd2_p$ in *S. cerevisiae*, and it has been found that these have separate roles under different conditions. $Gpd2_p$ contributes to a larger extent to glycerol formation in anaerobic conditions, as reported in a study[14] by Nissen *et al.*, in which mutants lacking *GPD2* gene had significantly lower yields of glycerol and specific growth rates. In contrast, mutants with *GPD1* deleted did not have obvious growth deficiencies or lower glycerol yields. The importance of glycerol in maintaining redox balance in yeast under anaerobic conditions was elucidated by the lack of growth and glycerol formation of double deletion *gpd1*Δ*gpd2*Δ mutants, which only revived with the provision of an external electron acceptor.[10,14] While *GPD1* was less important in anaerobic conditions, it was found to be of significance in the osmoregulation of *S. cerevisiae*. The *gpd1*Δ mutants were discovered to be unable to accumulate high levels of glycerol to maintain pressure under osmotic stress, whereas wild type cells under the same conditions displayed increased $Gpd1_p$ activity.[6,15,16]

Similar to Gpd_p, Gpp_p is encoded by two homologous isogenes *GPP1* and *GPP2*. Expression of *GPP1* was found to be induced in anaerobic conditions, and *GPP2*, in conditions of external osmotic stress. *GPP1* may also substitute well for *GPP2* as single mutants were found to be unaffected in high osmolarity, but deletion of *GPP1* resulted in arrested anaerobic growth.[17,18] These two homologous genes have been found to be essential, but not rate-limiting in the production of glycerol. The *gpp1*Δ*gpp2*Δ mutant did not produce glycerol, and was also sensitive to osmotic shock and anaerobicity.[17]

METABOLIC ENGINEERING STRATEGIES FOR YEAST

Strategies for improving glycerol yield are on the basis of increasing flux to the glycerol production pathway and mainly focused on overexpression of the key enzymes involved in glycerol production to direct carbon flux to glycerol, as well as increasing the availability of DHAP and cytosolic NADH to drive glycerol production.[6,19] The strategies employed can be grouped broadly into the below methods:

Deletion of triosephosphate isomerase

Tpi1p is responsible for the isomerization of glyceraldehyde-3-phosphate and DHAP. By deleting *TPI1* gene, the carbon flux is restricted to an equimolar distribution of glucose to glyceraldehyde-3-phosphate and DHAP. With inactivated Tpi1p, the maximum theoretical yield of 1.0 mol glycerol mol^{-1} glucose (0.51 g g^{-1}) is created.[19–21] However, accumulation of DHAP leads to inactivation of myo-inositol-3-phosphate synthase, leading to inositol auxotrophy of tpi1Δ mutants, which are unable to grow in the absence of inositol.[22] Thus, the toxic effect of DHAP accumulation becomes a driving force for glycerol formation. In *tpi1*Δ mutants, there is no net gain of ATP in glycolysis via substrate level phosphorylation. It was observed such mutants were unable to grow on glucose as a sole carbon source, and were only able to grow by respiratory metabolism. Growth of *tpi1*Δ mutants was also found to be inhibited by glucose concentrations above 0.1%.[1,20] With only *tpi1* deleted from *S. cerevisiae* cells grown on ethanol and glucose, Compagno *et al.* achieved a glycerol yield of 85% that of the theoretical yield.[20]

For DHAP to be completely converted to glycerol in a *tpi1*Δ mutant, NADH availability in the cytosol for the reduction of DHAP to glycerol-3-phosphate is an important factor. Competing reactions for cytosolic NADH will result in DHAP accumulation, and

inhibition of cell growth.[19,23] To increase the availability of cytosolic NADH for driving glycerol production, Overkamp *et al.*[19] investigated the effect of removing $Nde1_p$ and $Nde2_p$, the mitochondrial external NADH dehydrogenases, and $Gut2_p$, a mitochondrial glycerol-3-phosphate dehydrogenase, from the *tpi1*Δ mutant, so as to prevent oxidation of cytosolic NADH by the mitochondrial respiratory mechanism. The external mitochondrial NADH dehydrogenases serve to oxidize NADH to NAD^+ in the cytosol, while the Gut2p converts glycerol-3-phosphate back to DHAP. Removal of these three genes allows the available cytosolic NADH to be used for DHAP reduction to glycerol, and prevent the dissimilation of glycerol. In shake flask cultures, Overkamp *et al.* achieved higher specific growth rate on glucose using the *tpi1*Δ*nde1*Δ*nde2*Δ*gut2*Δ strain compared with the single deletion *tpi1*Δ strain, but the growth rate was still lower than the wild-type. The quadruple mutant strain was adapted to grow at higher glucose concentration and a yield of 0.99 mol mol^{-1} glucose was achieved in aerated batch culture.[19] In order to overcome the inositol auxotrophy in *tpi1*Δ mutants, a recent study by Cordier *et al.*[21] overexpressed *GPD1*, glycerol-3-phosphate dehydrogenase, which reduced the levels of DHAP, and prevented the inhibition of myo-inositol synthase. *ALD3* encoding NAD^+-dependent aldehyde dehydrogenase was also overexpressed, and *ADH1* gene encoding the major alcohol dehydrogenase was further deleted. These strategies prevented the consumption of NADH by $Adh1_p$ in the conversion of acetaldehyde to ethanol, and increased cytosolic NADH by overexpression of *ALD3* acting on acetate formation. With NADH levels increased for favourable glycerol production, they achieved a yield of 0.46 g g^{-1} glucose, at a production rate of 3.1 mmol (g biomass $h)^{-1}$.

Increasing availability of cytosolic NADH while keeping Tpi1p activity

The above strategies with *TPI1* deleted limited the theoretical maximum yield to 1.0 mol mol^{-1} glucose, and led to the constraints of requiring all cytosolic NADH produced to be consumed by DHAP. Geertman, *et al.*[23] believed that higher glycerol yields could be achieved by keeping the *TPI1* while increasing availability of NADH and ATP instead. The levels of glycerol and ethanol production correspond to the levels of NADH in yeast cells. Decreasing NADH in the cytosol causes a reduction in glycerol production, while decreasing NADH within the mitochondria was reported to decrease ethanol production within *S. cerevisiae*.[24] With the restriction on carbon flux removed, ATP can be generated by the respiratory dissimilation of pyruvate formed from glyceraldehyde-3-phosphate.[23] In order to remove competing reactions for cytosolic NADH, Geertman *et al.* deleted the pyruvate decarboxylase genes *PDC1*, *5* and *6*, the mitochondrial external NADH dehydrogenase genes *NDE1*, *2*, as well as *GUT2*, the gene encoding the glycerol-3-phosphate shuttle. The deletion of the other three genes *NDE1*, *2* and *GUT2* serve the same purpose mentioned earlier in preventing the mitochondrial respiratory mechanism from oxidizing cytosolic NADH.[19] This *pdc1*Δ*pdc5*Δ*pdc6*Δ*nde1*Δ*nde2*Δ*gut2*Δ mutant produced glycerol at a yield of 0.90 mol mol^{-1} glucose. In addition to these deletions, Geertman studied if the theoretical maximum yield of 1.0 mol mol^{-1} glucose could be exceeded in aerobic cultures by overexpressing the genes *FDH1*, encoding formate dehydrogenase, and *GPD2*, glycerol-3-phosphate dehydrogenase. Formate is a commonly used electron donor, and the conversion of formate to carbon dioxide by FDH1 reduces NAD^+ to NADH, regenerating the cofactor required in cells.[25] In this case, the action of formate dehydrogenase on formate would provide an additional source of cytosolic NADH. It was found that the overexpression of *FDH1* and *GPD2* in the *pdc1*Δ*pdc5*Δ*pdc6*Δ*nde1*Δ*nde2*Δ*gut2*Δ strain resulted in efficient formate oxidation and glycerol production, and with formate co-feeding, a highest reported yield of 1.08 mol mol^{-1} glucose was achieved.[23,25]

Nevoigt[26] proposed another method in his review, which was simulated to produce 1.66 mol glycerol mol^{-1} glucose, but was restricted by the efficiency of the *gpsA* gene. In this method, *PGI1* was deleted. Its deletion in theory routes glycolysis through the pentose phosphate pathway. The carbon flux is directed to the pentose phosphate pathway and produces, in theory, 2 mol NADPH per mol glucose. However *pgi1*Δ mutants have been shown to be unable to utilize glucose or fructose as the sole carbon sources[27] and the introduction of an NADPH-dependent DHAP reductase here served to remediate this problem. By swapping the genes *GPD1* and *GPD2* with a *Bacillus subtilis* NADPH dependent DHAP reductase (encoded by *gpsA*), Nevoigt[26] successfully expressed the *gpsA* gene into the *S. cerevisiae pgi1*Δ mutant, but compared with the *pgi1* mutant, the increase in glycerol was only 35% higher due to the low specific activity of *gpsA* in yeast.

Using steering chemicals in media

The addition of chemicals in the culture media to manipulate or steer the direction of metabolic pathways is a well-established subject. Presence of bisulphate during both fermentation and aerobic conditions leads to the production of bisulfate–acetaldehyde complexes,[28] inhibiting further catabolic breakdown of acetaldehyde to acetic acid and ethanol, via *ALD* and *ADH1*. The absence of $Adh1_p$ activity presents a NADH block, requiring the cell to redirect carbon metabolism towards glycerol production, in order to reduce the buildup of excess NADH, through conversion of DHAP to glycerol-3-phosphate. Alternatively, increasing and maintaining the pH of the media above pH 7, using sodium bicarbonate produced an increase in glycerol, through the breakdown of acetaldehyde into acetic acid and ethanol directly, circumventing the activities of Ald_p and $Adh1_p$. Both these avenues could in theory completely redirect the reduction of NADH away from ADH1 if sulphites and alkaline are present at 1 mol mol^{-1} glucose levels. However, it was noted that the use of steering chemicals only achieved 60% of the theoretical yield,[29] furthermore, the large amounts of sulphite and alkaline salts required both inhibits growth[28] and impedes downstream extraction of glycerol.

Adaptive evolution

Random mutagenesis was first used by Wright *et al.*[29] to isolate yeast strains overproducing glycerol. Subsequent developments termed evolution engineering involves the use of an organism's innate ability through its genetic plasticity to adapt to, thrive under different, often taxing conditions and pass down these traits down future generations. This strategy was used by Overkamp *et al.*[19] to produce a *tpi1*Δ*nde1*Δ *nde2*Δ*gut2*Δ adapted to high glucose concentrations for glycerol production. Pyruvate decarboxylases ($Pdc1_p$, $Pdc2_p$, $Pdc5_p$ and $Pdc6_p$) catalyses the reduction of pyruvate to acetaldehyde, and since deletion of the above isoforms usually results in a glucose intolerant strain, adaptive evolution strategies are used to initially produce pyruvate decarboxylase mutants tolerant in glucose.[23]

Another adaptive selection method[30] produced glycerol overproducing strains of *Candida glycerinogenes* in high phosphate media. One of these, with a glycerol production rate of 129 g L^{-1}

in 84 h, was noted to have higher rates of glycerol-3-phosphotase activity when cultured in high phosphate media. Knowledge of any changes the sequences in the coding or upstream promoter regions of this gene or its activators will give new insights on phosphate metabolism and utilization.

In addition, *E. coli* were evolved to overproduce glycerol by deleting *tpiA*, *mgsA*, and *edd* genes and insertion of *S. cerevisiae* genes *GPD1* and *GPP2*.[31] Among the results was a new fusion protein of $Gpd1_p$ and $Gpp2_p$ with higher and more specific enzyme activities. Given the more rapid growth rate and greater genome plasticity of the prokaryote, *E. coli* presents itself as a better candidate for use in evolution engineering methods.

Using a solely adaptive evolution strategy under sulfite selection, Kutyna *et al.*[32] isolated moderately higher glycerol producing strains of *S. cerevisiae*. By back crossing them to the parental strain, they were able to demonstrate that tolerance to sulfite and glycerol production are separate traits, caused by different genetic perturbations and are not co-inherited together. High glycerol production strains sensitive to high sulfite concentrations were isolated. Increased glycerol production was also found to be a recessive trait, suggesting that loss of function mutations are causative of this trait. As shown in the above examples, evolutionary strategies can be utilized to overcome genetic bottlenecks, in order for cells to produce viable quantities of desired products and produce new insights on cellular mechanisms.

GLYCEROL PRODUCTION IN *ESCHERICHIA COLI*

Although glycerol is not a natural metabolite of *E. coli*, due to the versatility of *E. coli,* there has been interest in the engineering of *E. coli* to produce glycerol. A recent patent by Bulthius *et al.*[33] from Dupont describes a method for production of glycerol from recombinant *E. coli* by expressing a gene with glycerol-3-phosphate dehydrogenase activity, and another with glycerol-3-phosphate phosphatase activity. Performing the cultures in shake flasks, Bulthius *et al.* managed to achieve 3.8 g L^{-1} in 24 h in *E. coli* transformed with both *GPP2* and *DAR1* (glycerol-3-phosphate dehydrogenase) genes. Chotani *et al.*[36] reported a near-theoretical yield and over 200 g L^{-1} glycerol produced by an engineered *E. coli* strain in a fed-batch fermentation culture. Alternative pathways to glycerol production include the dephosphorylation of dihydroxyacetone phosphate (DHAP) to dihydroxyacetone (DHA), followed by reduction to glycerol, and the isomerization of DHAP to glyceraldehyde-3-phosphate (GAP) by triose phosphate, followed by dephosphorylation of GAP to glyceraldehyde, and reduction by glycerol dehydrogenase to glycerol.[34,35]

In 2007, Salles *et al.*[31] evolved an *S. cerevisiae* pathway in *E. coli* to produce glycerol. Three major deletions were performed in *E. coli*, namely the deletions of *tpiA*, *mgsA* and *edd*. TpiA is similar to $Tpi1_p$ in *S. cerevisiae*, and functions to interconvert DHAP and GAP. Likewise to the *S. cerevisiae tpi1*Δ mutant, accumulation of DHAP is detrimental to the *E. coli* mutant. In order to prevent the conversion of DHAP to methylglyoxal, the *mgs* gene was thus removed. To prevent the evolution of the double mutant to produce GAP and pyruvate via the Entner-Doudoroff pathway, the edd gene was further removed. Upon creation of this Δ*tpiA*Δ*mgsA*Δ*edd* mutant, the genes *GPD1* and *GPP2* encoding the glycerol production pathway from yeast were cloned into the strain. This recombinant *E. coli* strain was then evolved for higher glycerol yield, resulting in the production of a fusion protein with G3P dehydrogenase and G3P phosphatase activities, leading to a yield of 1 mol mol^{-1} glucose, high titer of 130 g L^{-1}, and productivity of 16 g L^{-1} day^{-1} in glucose-limited fed-batch cultures.[31]

In considering the unfavorable economics of intensive aerobic processes at large scale, a recent study[38] in our group was attempted to develop a micro-aerobic/anaerobic fermentation process for glycerol production in *E. coli* Δ*adhE* Δ*ldhA* Δ*frdC* Δ*tpiA* Δ*mgsA* mutant carrying yeast glycerol biosynthetic pathway. The mutant was capable of growing anaerobically and produced glycerol with up to 90% of theoretical yield. This study[37] also demonstrated that the endogenous acetate pathway for ATP generation was indispensable for growth of the penta mutant under anaerobic conditions. However, further efforts are required to improve the glycerol titer and productivity in this mutant for an industrially viable fermentation process.

LOOKING FORWARD

Glycerol is emerging as a useful feedstock for the production of bio-based chemicals and development of a sustainable bioprocess is essential for the production of glycerol. Such a process should operate at higher yield and productivity and should also be capable of utilizing diversified feedstocks. Two hurdles, yield and productivity enhancement for large-scale production and efficient utilization of C5 sugars for better utilization of lignocellulosic feedstocks, however, remain to be overcome for economical microbial production of glycerol. To increase the yield of aerobic glycerol production above 1 mol mol^{-1} glucose, it is essential to maintain an intact *TPI1* gene, to knock out competing NADH utilization pathways and to introduce additional NADH producing pathways. In addition, *in silico* metabolic network analysis to identify gene targets for improving the yield of glycerol should also be performed.

As another consideration, economics of aerobic fermentation processes are affected at large scale due to energy intensive aeration process. The major bottleneck for micro-aerobic/anaerobic production is the ability to generate additional ATP. Hence, successful implementation of the strategies for additional ATP generation under such condition will enable us to create a platform strain for the production of glycerol and other bio-based chemicals on a larger scale. Further the utilization of diploid strains will be very handy to improve productivity.

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