Abstract

Zymomonas mobilis is an ethanol-producing bacterium known for its high tolerance and yield. It utilises sugars via a highly active Entner–Doudoroff pathway, resulting in a rapid glucose consumption rate. *Z. mobilis* has an incomplete tricarboxylic acid cycle (TCA) and a deficient respiratory chain, which makes it dependent on glucose-to-ethanol fermentation for energy production and redox balance. A significant portion of the carbon flux (95-97%) is directed towards ethanol production, which leads to reduced biomass formation. These characteristics render *Z. mobilis* an excellent host for producing non-native compounds at high yields, provided the flux through the native ethanol pathway can be effectively eliminated.

Z. mobilis was engineered to produce acetoin and D-(-)-2,3-butanediol (2,3-BDO) by heterologously expressing the key pathway genes, acetolactate synthase (*alsS*)/acetohydroxyacid synthase (*ahas1*), acetolactate decarboxylase (*alsD*), and R, R-2,3-butanediol dehydrogenase (*bdhA*), through integration at two neutral gene loci. The engineered strain produced acetoin and 2,3-BDO at low yields, as most carbon flux (77-88%) was directed towards ethanol production. To improve yields, we targeted the ethanol pathway by deleting the *adh1* and *adh2* genes based on previous work in our lab while concurrently integrating the acetoin/2,3-BDO pathway genes (*ahas1, alsD*, and *bdhA*). The Zm_ΔA1ΔA2_HS1D strain showed negligible ethanol production (~0.8 g/l). However, the Zm_ΔA1ΔA2_HS1D strain did not consume more than 10-12 g/l of glucose, and there was only a slight increase in acetoin yield compared to the wild-type strain. The decreased glucose consumption was due to the combined effect of the low activity of the heterologous genes and the strain's inability to regenerate $NAD(P)^+$ without ethanol production. The Zm_ΔA1ΔA2_HS1D strain was further improved by expressing a strong *alsS* gene from *Bacillus subtilis* to reroute more pyruvate flux towards 2,3-BDO and acetoin formation. To regenerate cofactor equivalents, the *nox* gene encoding NADH oxidase from *Lactococcus lactis* was also expressed. The Zm_ΔA1ΔA2ΔM_HS1DXS strain produced acetoin with a yield of 0.27 g/g of glucose, corresponding to 54.5% of the maximum theoretical yield (MTY). However, only 50-65% of the carbon flux was diverted towards acetoin, 2,3-BDO, and glycerol, indicating the accumulation of other metabolites.

we deleted the *pdc* gene in the engineered acetoin/2,3-BDO production strain Zm_ΔA1ΔA2_HS1DX to improve yields. The Zm_ΔA1ΔA2ΔP_HS1DXS strain (with the *pdc* gene deletion) did not produce ethanol under any growth conditions. Under aerobic condition,

the strain achieved an acetoin yield of 0.38 g/g of glucose, corresponding to 76% of the MTY. The Zm_ΔA1ΔA2ΔP_HS1DXS strain was cultivated in a reactor under different dissolved oxygen (DO) levels to optimize the growth conditions for acetoin/2,3-BDO production. Under 30-35% DO, the strain produced 39.09 g/l of acetoin and 7.86 g/l of 2,3-BDO from 128 g/l of glucose. At a DO level below 10%, the strain produced 11.22 g/l of 2,3-BDO from 30 g/l of glucose, achieving a 2,3-BDO yield close to 75% of the MTY. This study demonstrated the ability of a single recombinant strain to produce both acetoin and 2,3-BDO at high yields, with the production profile being dependent on the aeration conditions. The strain exhibited robust growth in bioreactors and demonstrated the ability to consume high glucose concentrations. The Zm_ΔA1ΔA2ΔP_HS1DXS strain was further engineered to increase acetoin yield by deleting the *bdhA* gene. The Zm_ΔA1ΔA2ΔP_KS1DXS strain (with *bdhA* deletion) produced acetoin with a yield of 0.44 g/g (88% of the MTY). The strain consumed up to 80 g/l of glucose in a shake flask.

Z. mobilis was further engineered to produce valine or valine pathway intermediates by redirecting carbon flux from ethanol towards valine. The valine pathway genes were integrated at the *adh1* and *adh2* gene loci. The strong *B. subtilis* ALS was selected to catalyse the conversion of pyruvate to acetolactate, the first step towards valine production. For the subsequent two steps, native ketol-acid reductoisomerase (*ilvC*) and dihydroxy-acid dehydratase (*ilvD*) genes were overexpressed by placing them under the control of a strong *Ppdc* promoter. *B. subtilis* NADH-specific L-leucine dehydrogenase *(*BCD) was selected to catalyse the last reductive step. The Zm_ΔA1ΔA2_S.Zm.CD.D strain did not produce valine, likely due to the inability of the native ILVC and ILVD enzymes to pull flux downstream towards valine synthesis. To address this limitation, the Zm_ΔA1ΔA2_S.Zm.CD.D strain was engineered to express the *E. coli ilvC* gene, resulting in the generation of a new strain, Zm_ΔA1ΔA2 ΔAT_S.Zm.CD.D.EcC. However, valine production was not detected.

In this study, *Z. mobilis* was engineered to completely eliminate ethanol production by introducing an alternative pathway for redox regeneration. This work demonstrated that the *pdc* gene is not essential in *Z. mobilis* and can be deleted if an alternative route for redox equivalents regeneration is provided (NOX in this study). *Z. mobilis* was engineered to produce acetoin and 2,3-BDO at higher yields by completely redirecting carbon flux from ethanol towards target products.