Noda Institute for Scientific Research GRANT
2014 Young Investigator Research Grant
Outline of Research Result

Elevation of lipid productivity by enhancing the pentose phosphate pathway in *Aspergillus oryzae*

Koichi TAMANO

National Institute of Advanced Industrial Science and Technology

Research aims

Recently, the focus has shifted to biofuels generated from plant biomass owing to the increasing global demand for fossil fuels and carbon neutral characteristics of plant biomass-derived biofuels leading to a lesser effect on global environment. The filamentous fungus *Aspergillus oryzae* can convert biomacromolecules from plant biomass to many varieties of small molecules by both catabolism and assimilation. Thus, *A. oryzae* has been used to develop high production systems for lipids, such as free fatty acids (FFAs) and acylglycerol, as source materials of biofuels and chemical products.[1), 2)] In this study, genes encoding enzymes of the pentose phosphate pathway (PPP), which generates cytosolic NADPH required for lipid biosynthesis, were subjected to overexpression. Consequently, an increase in the FFA productivity was expected.

Methods

Overexpression mutants for genes encoding enzymes composing the PPP were constructed from the *A. oryzae faaA* disruptant with a defective *pyrG* (ΔfaaA_pyrG-). Native promoters were replaced with the *tef1* promoter (P*tef1*), which has constitutively high transcriptional activity. For promoter replacement, DNA fragments harboring, in order, a 1-kb fragment of the 5′-UTR of the replaced gene, the *pyrG* marker cassette, a 1028-bp P*tef1* fragment, and a 1-kb fragment from a start codon of the replaced gene were prepared by fusion PCR using KOD-PLUS DNA polymerase (Toyobo). Transformants were subjected to single-spore isolation, followed by clone check using PCR. The constructed overexpression mutants were cultured in 50 mL Czapek-Dox liquid medium (modified to contain 10% glucose) at 30°C at 200 rpm for 120 hours. The cultured hyphae were used to quantify intracellular FFAs. Hyphae were washed twice by 100 mL Milli-Q water and lyophilized overnight. Using approximately 50 mg lyophilized hyphae, FFAs were extracted in chloroform after disruption with a beads cell disrupter MS-100 (TOMY). Extracted FFAs were precipitated by evaporation. The precipitate was dissolved in 6% Triton X-100/6% ethanol, and 5 μL solution was used for FFA quantification by the Free Fatty Acids, Half Micro Test kit (Roche).

Results

The FFA productivity of *A. oryzae* has been increased by modifying its metabolism by metabolic engineering techniques. Previously, the acyl-CoA synthetase gene *faaA* disruptant (ΔfaaA) had been identified to most increase its FFA productivity (9.2-fold increase compared to the parental RIB40 strain).[2)] To further increase FFA productivity, the PPP was selected as a modification target because it generates cytosolic NADPH required for lipid biosynthesis. Because whole transcriptome data of *A. oryzae* metabolism imply that insufficient synthesis of cytosolic NADPH limits FFA production, enhancement of the PPP was considered effective in further increasing FFA productivity. Therefore, individual genes encoding enzymes composing the PPP were overproduced for this enhancement.

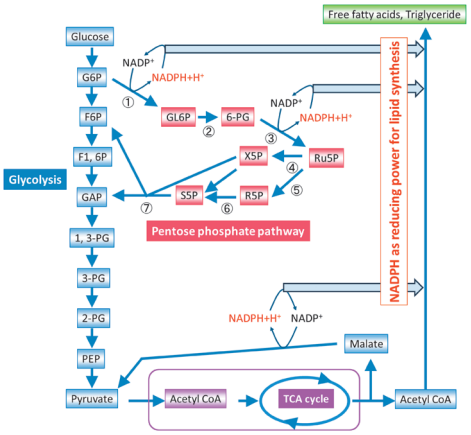
Genes encoding enzymes functioning in the PPP were selected based on a previous study on a genome scale metabolic model of *A. oryzae*[3)] and on homology search results for identified PPP enzyme-encoding genes of *Saccharomyces cerevisiae* by BLASTP. A list of selected genes encoding enzymes composing the PPP is described in Fig. 1.

Overexpression mutants of the selected genes were constructed from ΔfaaA_pyrG- using *pyrG* as a selection marker. FFA productivities of the constructed mutants were measured after liquid culture. Consequently, overexpression of AO090023000345, which was predicted to encode transketolase, caused a 1.4-fold increase in FFA productivity (Fig. 2A). Total dry cell weight of hyphae increased by 1.2 fold in the AO090023000345-overexpressing *faaA* disruptant (ΔfaaA_tktOE) than in ΔfaaA. Therefore, by multiplying these increase rates, the total FFA production yield per unit volume of culture increased by 1.7 fold due to overexpression (Fig. 2B).

Interestingly, in the liquid culture, ΔfaaA_tktOE did not form hyphal clusters. The ΔfaaA hyphae formed clusters, while the ΔfaaA_tktOE hyphae appeared to be dispersed in the culture medium (Fig. 3).

On the other hand, overexpression of the genes encoding other enzymes in the PPP did not cause an increase in FFA productivity. In addition, except for AO090023000345, two genes were predicted to encode transketolase in *A. oryzae*. However, overexpression of these genes also did not increase the FFA productivity.

Together with the PPP, the malic enzyme reaction, which



Reaction number	Gene ID	Annotation
①	AO090005001427	Glucose-6-phosphate 1-dehydrogenase
②	AO090005000789	6-phosphogluconolactonase
③	AO090003000121	6-phosphogluconate dehydrogenase
③	AO090001000547	6-phosphogluconate dehydrogenase
④	AO090023000209	Ribose 5-phosphate isomerase
⑤	AO090012000303	D-ribulose-5-phosphate 3-epimerase
⑥	AO090012000526	Transketolase
⑥	AO090023000345	Transketolase
⑥	AO090001000322	Transketolase
⑦	AO090020000520	Transaldolase

Fig. 1. Illustration of cytosolic NADPH generating metabolism (left), and a list of genes of *A. oryzae* that encode enzymes predicted to function in the pentose phosphate pathway (right).

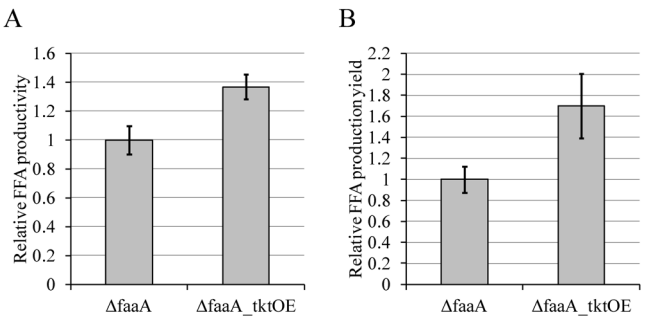


Fig. 2. FFA productivity (A) and the production yield (B) of ΔfaaA_tktOE relative to those of ΔfaaA.

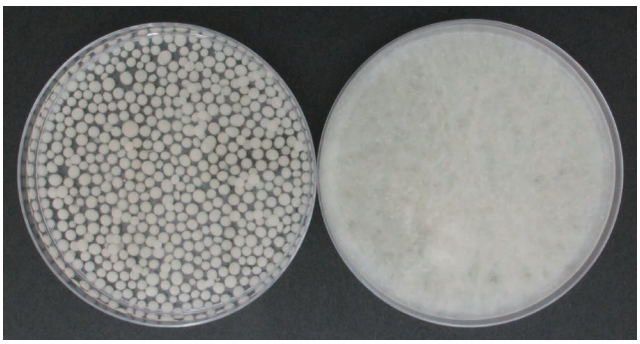


Fig. 3. A photograph of the liquid cultured ΔfaaA (left) and ΔfaaA_tktOE (right) in petri dish.

results in pyruvate synthesis from malate derived from the TCA cycle, generates cytosolic NADPH. Overexpression mutants for genes (AO090011000876, AO090038000621) predicted to encode malic enzyme were constructed from ΔfaaA_pyrG-. However, the FFA productivity did not increase in these mutants.

Conclusion

By overexpression of the predicted transketolase gene AO090023000345 of the PPP in the *A. oryzae faaA* disruptant, FFA productivity was increased by 1.4 fold. In addition, the hyphal biomass increased by 1.2 fold. Consequently, overexpression of this gene resulted in a 1.7-fold increase in FFA production yield per liter of liquid culture. However, overexpression of genes encoding enzymes directly involved in NADPH synthesis, such as glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme, did not contribute to an increase in FFA productivity.

References

1) Tamano, K. *et al*. (2013) Increased production of fatty acids and triglycerides in *Aspergillus oryzae* by enhancing expressions of fatty acid synthesis-related genes. *Appl. Microbiol. Biotechnol*. **97**: 269–281.
2) Tamano, K. *et al*. (2015) Increased production of free fatty acids in *Aspergillus oryzae* by disruption of a predicted acyl-CoA synthetase gene. *Appl. Microbiol. Biotechnol*. **99**: 3103–3113.
3) Vongsangnak, W., Olsen, P., Hansen, K., Krogsgaard, S., and Nielsen, J. (2008) Improved annotation through genome-scale metabolic modeling of *Aspergillus oryzae*. *BMC Genomics* **23**: 245.