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Review

Advances in engineering methylotrophic yeast for biosynthesis of valuable chemicals from methanol

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ABSTRACT

Methylotrophic yeasts and bacteria, which can use methanol as carbon and energy source, have been wildly used as microbial cell factories for biomanufacturing. Due to their robustness in industrial harsh conditions, methylotrophic yeasts such as *Pichia pastoris* have been explored as a cell factory for production of proteins and high-value chemicals. Methanol utilization pathway (MUT) is highly regulated for efficient methanol utilization, and the downstream pathways need extensively constructed and optimized toward target metabolite biosynthesis. Here, we present an overview of methanol metabolism and regulation in methylotrophic yeasts, among which we focus on the regulation of key genes involved in methanol metabolism. Besides, the recent progresses in construction and optimization of downstream biosynthetic pathways for production of high value chemicals, such as polyketides, fatty acids and isoprenoids, are further summarized. Finally, we discuss the current challenges and feasible strategies toward constructing efficient methylotrophic cell factories may promote wide applications in the future.

1. Introduction

Methylotrophic yeasts and bacteria have been successfully exploited as cell factory hosts for production of heterologous proteins, particularly for biopharmaceuticals and industrial enzymes [1,2]. In general, both yeasts and bacteria are ideal chassis hosts for construction of cell factories owing to their rapid growth and readily accessible to genetic manipulations tools. However, many valuable proteins or enzymes are derived from eukaryotes like fungi, plants or animals, which make them challenging to express functional proteins in prokaryotes due to the differences in cellular environments between eukaryotes and prokaryotes [3]. Therefore, methylotrophic yeasts display advantages over methylotrophic bacteria for the production of eukaryotes derived proteins and value chemicals such as phytoterpenoids [4]. So far, four categories of methylotrophic yeasts have been characterized, including *Pichia pastoris*, *Candida albicans*, *Hansenula polymorpha*, and *Pichia methanolica*, among which *P. pastoris* is the most wildly used species for the production of heterologous proteins [2–4].

P. pastoris and *H. polymorpha* can use methanol as a single carbon and energy source, which provides a great opportunity to establish a methanol biotransformation process to relieve the food security stresses in current sugar based bio-refineries. The methanol is a plenty feedstock that can be obtained from coal or hydrogenization from CO_2. There are mainly three methanol catabolism pathways in nature: The xylulose monophosphate (XuMP) cycle, the ribulosemonophosphate (RuMP) cycle or serine cycle. The XuMP cycle is present solely in yeasts [5]. Although the metabolic engineering system is currently not well established, *P. pastoris* has been considered as a wide-spread recombinant protein expression host in both academia and industries [3]. *H. polymorpha* is also a promising host for recombinant protein production because of its unique methanol-assimilating property [6]. Furthermore, thermo-tolerant *H. polymorpha* can grow at high temperatures up to 37–43 °C. Therefore, *H. polymorpha* can be protected from contamination during large scale fermentation and surpasses the cooling costs [6].

Engineering heterologous multi-step pathway to synthesize value chemicals by using *P. pastoris* or *H. polymorpha* is a promising sustainable route. Actually, genetic engineering *P. pastoris* for the production of various chemicals, especially terpenoids, polyketide, and some other high-valued chemical compounds, is developing rapidly in recent years (Fig. 1) [3]. In previous studies, the methylotrophic yeast usually use glucose as the raw material for

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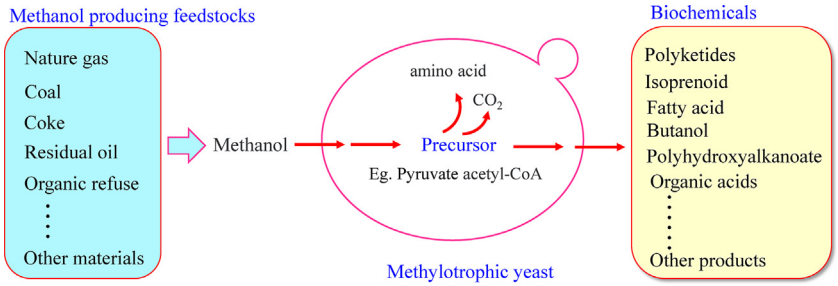

Fig. 1. Engineering methylotrophic yeast of producing various metabolites. Methanol, derived from diverse sources, could be transformed into precursor, such as pyruvate and acetyl-CoA, for the production of valuable chemicals through genetic engineering of methylotrophic yeast.

initial growth, and then use methanol as the inducer for protein expression [7]. However, it needs more time for protein production when using glucose firstly and methanol secondly. The use of one-stage fermentation method could shorten the fermentation time. For example, one-stage fermentation of methanol with high biomass of xylanase production was achieved in *P. pastoris* [8]. The attempts of using methanol as the sole carbon and energy source open a door for increasing the application of this methylotrophic yeast for the production of proteins and metabolites of interest with less time and lower costs.

This review summarizes the respective advantages of methylotrophic yeasts for heterologous gene expression. As for the production of high-valued chemical compounds and the construction of microbial cell factories, methylotrophic yeasts are considered to be more preferred hosts. Using *P. pastoris* as an example, the available information on MUT pathway in *P. pastoris*, regulation of key enzymes in the MUT pathway, production of target proteins and metabolites under methanol induction, and prospects of using methanol as the sole carbon and energy source for synthetic biology were also summarized.

2. Methylotrophic yeasts for heterologous gene expression

2.1. The differences between methylotrophic yeasts and bacteria

Yeasts can use xylulose 5-phosphate (Xu5P) for the transformation of formaldehyde to DHAP and GAP, and further form Xu5P through xylulose monophosphate (XuMP) pathway. In methylotrophic bacteria, however, there are two pathways that can catalyze formaldehyde [9]. The first pathway includes the fixation of formaldehyde into hexulose 6-phosphate (H6P) via ribulose 5-phosphate (Ru5P). H6P is then transformed into fructose 6-phosphate (F6P), which is then used to regenerate ribulose 5-phosphate by using fructose 1,6-bisphosphate or 2-keto-3-deoxy-6-phosphogluconate as the intermediate. The second pathway is the serine cycle that can metabolize formaldehyde to produce glycine and reproduces serine. In addition to the pathways that use formaldehyde as the starting reactant, the chemolithoautotrophic bacteria may use the ribulose bisphosphate pathway (RuBP) for carbon assimilation at the level of CO_2 [10].

Both methylotrophic fungi and bacteria can achieve high growth density in fermentation culture. Compared with methylotrophic bacteria, the methylotrophic fungi contain a variety of organelles, in which the heterogenous enzymes and biochemical metabolites can be produced and modified. Besides, organelles in methylotrophic fungi like peroxisome can improve the stability and increase the content of heterogenous enzymes and also beneficial for biochemical metabolites [11,12].

2.2. The advantages of methylotrophic yeasts as the expression host

As the representative of methylotrophic yeasts, *P. pastoris* are tightly regulated by methanol. *P. pastoris* possesses the advantages in ease of use and relatively rapid expression time with post-translational modification system and lipid composition (Table 1). Until now, *P. pastoris* has been considered to be the most well established system for the production of heterologous proteins, which makes it an ideal host for microbial cell factories in the biotechnological industry [13]. *H. polymorpha* also has some advantages in thermotolerance and capacity to grow at higher rates on simple, defined media compared with other methylotrophic yeasts. The thermo-tolerance of *H. polymorpha* makes it a suitable host for the production of mammalian (including human) proteins with the low risk of contaminations in large scale fermentations (Table 1). As for the *C. boidinii*, which can be strictly induced by methanol, will be in good controllability in the fermentation process. Consequently, the methylotrophic yeasts have their specific advantages, which make it extremely important to select an appropriate strain according to the characteristics of heterogenous proteins and the fermentation conditions (Table 1).

3. Regulation of methanol metabolism pathway in methylotrophic yeasts

3.1. Methanol metabolism pathway

There are two steps for methanol metabolism in methylotrophic yeast: the first step is methanol oxidation toward formaldehyde and then formaldehyde could be assimilated to central metabolism by dihydroxyacetone synthase (DAS, EC 2.2.1.3) [14]. In addition to the assimilation pathway, formaldehyde can also be dissimilated via formate to carbon dioxide with the formation of two molecules of NADH for growth on methanol [15], which is catalyzed by the NAD-dependent formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) [16].

The first step of methanol oxidation in *P. pastoris*is is catalyzed by alcohol oxidases (AOX), which were encoded by two genes, *AOX1* and *AOX2* [17]. The major alcohol oxidase AOX1 can comprise

Table 1
The comparison of methyltrophy-type bacteria and methylotrophic fungi as chassis organisms.

Chassis organisms	Operation flexibility	Transformation stability	Growth density	Growth rate	Thermostability	Protein modification
Bacillus methanolicus	+++	++	+++	+++	++	–
Pichia pastoris	++	+++	+++	++	–	+++
Hansenula Polymoupha	++	+++	+++	++	++	+++

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