Journal of Life Sciences 11 (2017) 11-18
doi: 10.17265/1934-7391/2017.01.002

Metabolomics Study in Methylotrophic Yeast: A Minireview

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Abstract: Methylotrophic yeast has been used as a cost-effective and valuable host for expression of recombinant protein due to its unique methanol utilisation pathway. It has an AOX (alcohol oxidase) protein which has been characterised to be a strong and tightly methanol-inducible dependent promoter. Metabolomics is the systematic study and inclusive analysis of small molecules called metabolites in a biological system. Metabolomics plays an important part in connecting the phenotype and genotype gap because it magnifies the modifications in the proteome and provides a better phenotype representation of an organism. This quantitative study has provided a new perception on the metabolic burden derived from the overexpression of recombinant protein in methylotrophic yeast. In this review, we discuss the fundamental aspect of metabolomics in methylotrophic yeast followed by their latest developments.

Key words: Methylotrophic yeast, metabolomics, methanol utilisation pathway.

1. Introduction

Methylotrophic yeast has been recognised as a cost-effective and valuable host for production of a wide range of heterologous proteins. It has advantages over prokaryotic expression systems, for example, ease in handling and cultivation, cheap and simple media requirements. It is comparable to mammalian cells, and the capability to perform essential eukaryotic post-translational modification thus producing functional recombinant proteins [1].

Metabolomics is the inclusive and quantitative appraisal of endogenous metabolites and challenges to methodically distinguish and quantify metabolites from biological samples. Due to the fast turnover of intracellular metabolites, it is essential to have dependable and reproducible procedures and techniques for sampling and sample treatment. Sampling metabolites is a significant issue, particularly for planktonic cells, as the sampling process may disturb metabolic system [2]. There are a few techniques broadly used for extracting metabolites from yeast cells, for instance, freeze-thaw, sonication, hot water, boiling ethanol, permeabilization utilizing chloroform and treatment with outrageous pH [3]. However, a universal sample preparation protocol does not exist. Recently, studies were carried out to develop a better protocol to extract metabolites from yeasts cells through different kinds of methods [2, 4-6].

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2. Methylotrophic Yeast

Methylotrophs are microorganisms that have the ability to utilise all carbon sources including the 1-carbon (C_1-) compound. Methylotrophs have a number of metabolic pathways for assimilating and dissimilating C_1- compound [7]. Prokaryotic methylotrophs are able to utilise a range of C_1-compounds such as methanol, methylamine and

methane, whereas eukaryotic methylotrophs can only utilise methanol as the carbon source [8]. Eukaryotic methylotrophs only include a few yeast genera which is in the family of *Pichia, Hansenula, Turolopsis* and *Candida*. They include *Candida boidinii*, *Candida methanolovescens, Ogataea angusta* (previously known as *Hansenula polymorpha*), *Ogataea methanolica* and *Komagataella pastoris* (previously known as *Pichia pastoris*) (BioCyc). Recently, the use of methanol as carbon source to replace coal and petroleum is getting more attention because methanol is cheaper and non-food substrate (suitable as feedstock in biotechnological and chemical processes) [9].

The first methylotrophic yeast was isolated in 1969 [10]. Various ranges of media and protocols were developed to grow *K. pastoris* using methanol as sole carbon source [11]. During the 1970s, the production of SCP (single cell protein) from *K. pastoris* started to gain interest because of its high ability to reach high cell densities in continuous culture [12]. Over decades, methylotrophic yeasts are now becoming a popular and widely used host system for production of heterologous proteins. Nowadays, more than 500 proteins have been cloned and expressed using this system [13]. Among all the methylotrophic yeasts, *K. pastoris* is the most commonly used yeast.

Since methylotrophic yeasts are eukaryotes, they offer more advantages compared to prokaryotes expression system. A large range of proteins that require accurate level of post-translational development which cannot be expressed in prokaryotes, have successively been produced in the methylotrophic yeasts [1]. The methylotrophic yeasts provide high eukaryotic post-translational modification such as glycosylation, proteolytic processing, protein folding, and disulphide bond formation [13]. Eventually, they produce stable, correctly folded recombinant protein and functional recombinant protein. Besides, methylotrophic yeasts have the ability to grow on minimal medium to a very high cell density, and also are genetically easier to be manipulated and cultured than the mammalian cell [14].

The most important property of methylotrophic yeasts as a host is the presence of a strong methanol-inducible and strongly regulated by *pAOX1* (alcohol oxidase 1 promoter) encoded from the *AOX1* (alcohol oxidase 1) gene [15] which encoded the key enzymes in methanol utilisation pathway. This strongly regulated promoter has the advantage of overexpression of proteins. *pAOX1* is strongly depressed when growing on glucose, glycerol and ethanol. *pAOX1* is fully induced only when methanol is used as the carbon source [16]. A lot of heterologous proteins were successfully cloned and expressed in *P. pastoris* , for example, thermostable L2 lipase in year 2013 [17].

3. Methanol Utilisation Pathway

All methylotrophic yeasts share a common methanol utilisation pathway which tightly regulates the expression at the level of transcription [18]. Fig. 1 shows the outline of methanol utilisation pathway in methylotrophic yeasts [19].

The initial stage in methanol utilisation pathway is the oxidation of methanol by AOX (alcohol oxidase) to formaldehyde and hydrogen peroxide, both are highly toxic compounds. The toxic hydrogen peroxide is cleaved to oxygen and water by the action of CAT (catalase). Formaldehyde is a centric intermediate of two subdivisions in methanol utilisation pathway [7]. Formaldehyde is either oxidised by two following dehydrogenase reactions (dissimilation pathway) or assimilated in the cell metabolism by condensation with Xu_5P (xylulose 5-phosphate). In the assimilation pathway, a segment of formaldehyde is condensed to Xu_5P by DAS (dihydroxyacetone synthase) to form DHA (dihydroxyacetone) and GAP (glyceraldehyde 3-phosphate), which are utilized for the synthesis of cell constituents and the recovery of Xu_5P in cytosol of cell [9].

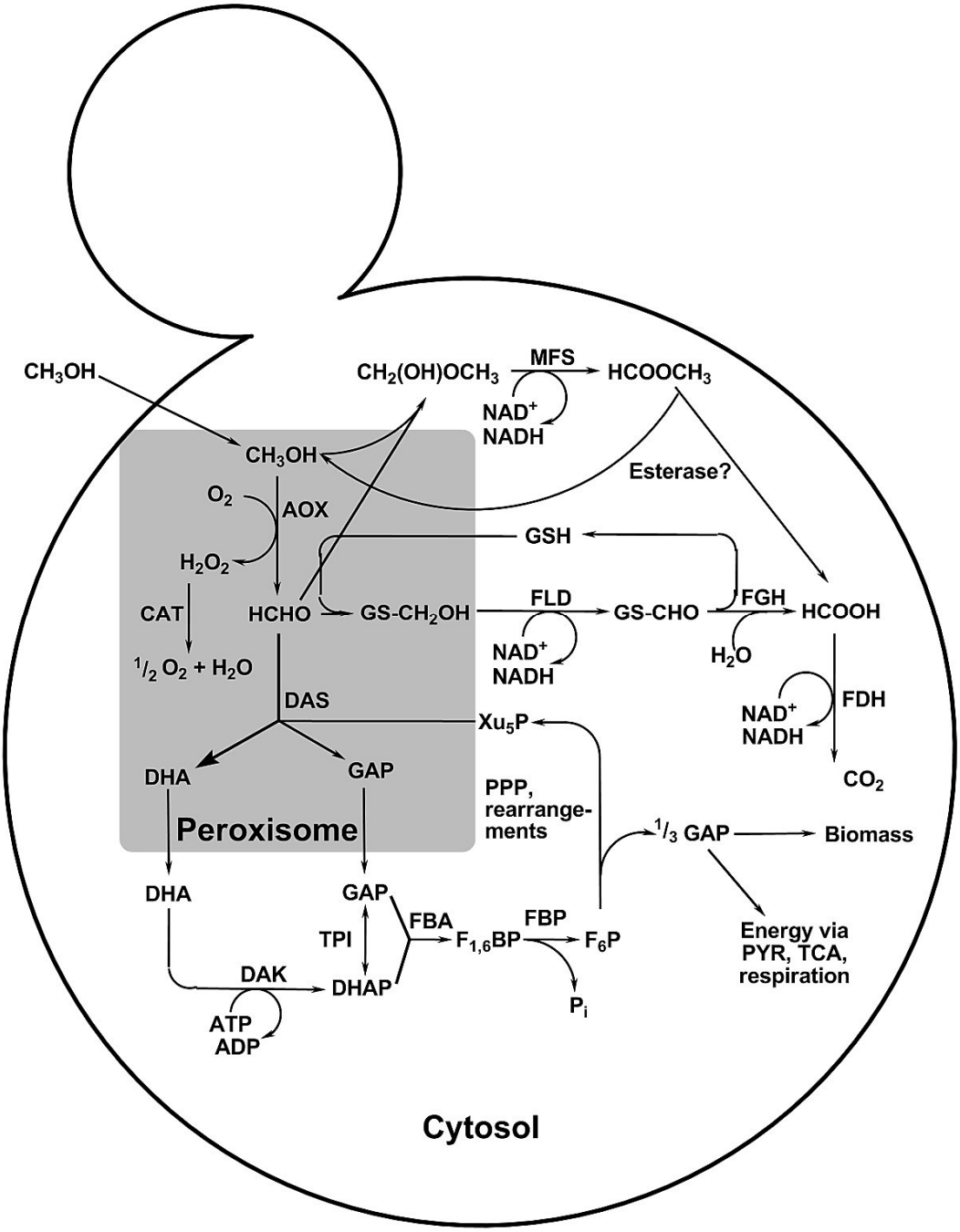

Fig. 1 Outline of methanol utilisation pathway in methylotrophic yeasts. The metabolites and the respective enzymes present in methanol metabolism system are shown.

Enzymes: AOX: alcohol oxidase (EC 1.1.2.13); DAS: dihydroxyacetone synthase (EC 2.2.1.3); CAT: catalase (EC 1.11.1.6); FLD: formaldehyde dehydrogenase (EC 1.2.1.1); FGH: S-formylglutathione hydrolase (EC 3.1.2.12); FDH: formate dehydrogenase (EC 1.2.1.2); MFS: methylformate synthase; DAK: dihydroxyacetone kinase (EC 2.7.1.29); TPI: triosephosphate isomerase (EC 5.3.1.1); FBA: fructose-1,6-bisphosphate aldolase (EC 4.1.21.13); FBP: fructose-1,6-bisphosphate (EC 3.1.3.11); Metabolites: DHA: dihydroxyacetone; GAP: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; $F_{1,6}$BP: fructose 1,6-bisphosphate; F_6P: fructose 6-phosphate; Xu_5P: xylulose 5-phosphate; GSH: reduced form of glutathione; P_i: phosphate; PYR: pyruvate; PPP: pentose phosphate pathway; TCA: tricarboxylic acid cycle [19].

Another bit of formaldehyde is further oxidised to CO_2 by the cytosolic dissimilation pathway. In the dissimilation pathway, formaldehyde instinctively reacts with GSH (glutathione) to S-hydroxymethylglutathione followed by two consecutive reactions which are catalysed by NAD^+-dependent FLD (formaldehyde dehydrogenase) and an NAD^+-dependent FDH (formate dehydrogenase) to carbon dioxide. The whole process is located in the cytosol. The coenzymes, NADH generated from the two subsequent dehydrogenase reactions are being utilized as part in energy production for growth on methanol [20]. The dissimilation pathway enzymes not only assume parts in energy production, it also play a part in the detoxification of formaldehyde and formate, respectively [18]. Three enzymes in dissimilation pathway FLD, FDH and S-FGH (formylglutathione hydrolase) take part in the detoxification of formaldehyde and regenerates GSH [21]. Nakagawa et al. [22] carried out the knockout studies on FLD and FDH strains of *C. boidinii* and *P. methanolica*. They reported that the FLD knockout phenotype is more severe than FDH knockout, which was explained by the higher toxicity of formaldehyde compared to formate [22].

Generally, the expression of genes in methanol utilisation pathway is repressed by glucose and ethanol and tightly induced by methanol. When methylotrophic yeasts are grown in methanol, the key enzymes (AOX, DAS and FDH) can reach up 30% (AOX) or 20% (DAS, FDH) of the total soluble protein in induced cell culture [11].

4. Metabolomics

Metabolomics is the systematic study and inclusive analysis of small-molecules called metabolites in an organic system. Metabolomics can also be characterized as a study in biochemistry, which plays an important role in the comprehension of the control of metabolic pathways and metabolic network in a biological system. Metabolomics comprehensive analysis includes the identification and quantification of all intracellular and extracellular metabolites [23]. Metabolomics plays an important part in connecting the phenotype and genotype gap since it magnifies the modifications in the proteome and gives an enhanced representation of the phenotype of a living being [24].

Metabolome is a complete set of small-molecule metabolites with molecular weight less than 1 kDa in a biological organism which includes hormones, signal molecules, metabolic intermediate, secondary metabolites and products of metabolism [25]. The metabolome may also contain carbohydrates, lipids, amino acids, alcohols and natural compounds [26].

For mammals, the samples used in metabolomics studies are body fluids. For example, blood and urine are the most common samples used to determine biomarker for disease in human. Other samples that can be taken from mammals are breast milk, umbilical cord blood, faecal extract and various tissues and cells. All of the samples are unique in terms of the type of information that they provide.

The metabolome in eukaryote is particularly larger than prokaryote, relatively in light of the fact that the human body contains ten times more bacterial cells than human cells, and hence contains numerous “bacterial” metabolites and also human metabolites [27]. In yeast metabolome database, the expected number of metabolites existing in the yeast metabolome is 2,027. The real number of metabolites will largely increase as the expected number is incomplete as it does exclude quite a lot of lipids, drugs and food nutrients.

5. Application of Metabolomics

In early 1900s, metabolomics studies were conducted to identify the health of an individual. For example, in China, traditional doctors used ants to evaluate the urine of patients to detect diabetes [28]. In 1971, Horning et al. [29], introduced a term named “metabolic profiling” which means identification and quantification of a range of compounds, after they

found out that compounds present in human urine can be detected by using GC-MS (gas chromatography-mass spectrometry) [30]. Metabolic profiling provides an instantaneous snapshot of the physiology of particular tissue or cell. In 1974, Hoult et al. managed to detect metabolites by using NMR (nuclear magnetic resonance) spectrometry [31]. Over 20 years, NMR has been used to study human inborn errors of metabolism [32].

Recently, metabolomics is not only a tool to diagnose disease but it becomes a significant tool to predict treatments and drug development. Metabolomics studies can be applied in three general areas: biomedical, clinical and environmental [23]. In terms of biomedical application, cancer and tumour are popular subjects in finding the information about the metabolism of the disease [30]. The clinical application of metabolomics builds the understanding of wellbeing in humans and the critical effect has been exhibited by a high number of publications. The environmental application studies the relationship between living organisms and their environment [33].

Throughout these years, plenty of metabolic pathways and metabolites recognized by other biochemical experiments are being revealed. However metabolomics is still considered a “developing field” [34]. Further studies have to be conducted to find out the unidentified metabolites and metabolic pathways.

6. Metabolomics Approaches

There are two main approaches in metabolomics: discovery metabolomics or non-targeted metabolomics and targeted metabolomics. Discovery metabolomics includes three major steps: metabolite profiling, identification and interpretation. Metabolic profiling by using statistically noteworthy variations in abundance within an extensive experimental and control samples, aims to find out and identify the unknown metabolites. After profiling, assurance of the chemical structure of these metabolites is the fundamental issue in the identification step. The last step, interpretation of functions by relating the metabolites revealed the biological processes or conditions. On the other hand, targeted metabolomics focused on validation and quantification of known metabolites in a sample.

7. Analytical Techniques in Metabolomics

Throughout the years, several analytical techniques have been applied in metabolomics, including GC-MS, LC-MS (liquid chromatography-mass spectrometry) or HPLC (high performance liquid chromatography), CE-MS (capillary electrophoresis-mass spectrometry) and NMR spectroscopy.

Metabolomics studies utilised MS (mass spectrometry) because of its high reproducible quantitative analysis, wide dynamic range, and the ability to analyse very complex biofluids. Separation (GC, LC or CE) before MS analysis is often performed to detect as many metabolites as possible because of the complex nature of samples. GC (gas chromatography) requires volatile sample while LC (liquid chromatography) is highly versatile, though, standard reversed phase separation does not adequately address the hydrophilic component of the metabolome. CE (capillary electrophoresis) is very suitable for hydrophilic metabolites. CE has a higher hypothetical separation efficiency than HPLC and is reasonable for use with a more extensive range of metabolite classes than GC [35]. Among all those three, GC-MS was the first technique to be developed in 1971.

NMR spectroscopy is the only detection technique which does not depend on partition of the analysts. The major advantage of NMR is that the sample can be recouped for further analysis. Most NMR analysis utilized ^{1}H NMR for detection of metabolites, which means any compounds containing protons can be detected by NMR. The application of ^{1}H NMR includes characterising, diagnosing and understanding the metabolic states, and is progressively connected in drug characterisation, toxicology, biomedical research

and in human nutritional research [36].

8. Metabolomics Studies in *Komagataella pastoris*

A lot of metabolomics studies have been carried out in *Saccharomyces cerevisiae* over decades, however, metabolomics studies in *K. pastoris* are getting more popular these years. A few robust and dependable speedy sampling instruments have been created and numerous quenching and separation protocols have been proposed and practised in the previous years [23]. There are a few strategies broadly used for extracting metabolites from yeast cells such as sonication, boiling ethanol, hot water, freeze-thaw, permeabilisation by chloroform and treatment with outrageous pH [3]. However, a general sample preparation protocol does not exist. The applicable sampling methods for yeasts' metabolome analysis are depending on the organism to be explored [37]. Recently, studies were carried out to develop a better protocol to extract metabolites from yeasts cells through different types of methods [2, 4-6].

The first quenching procedure was developed for *S. cerevisiae* [4]. It is an effective technique to concentrate cells and to decrease the changes of metabolite concentration during cell sampling and to remain most part unaltered throughout the years. For yeasts, immersing cells into cold 60% methanol appeared to effectively stop cellular metabolism.

Several sampling methods were compared to minimise the metabolite loss. Tredwell et al. [2] performed intracellular metabolite extraction using 4 different concentrations of methanol quenching solutions and compared each of them with the boiling ethanol extraction method as refer to Gonzalez et al. [38]. The extracted metabolites were investigated by using both GCMS and ^{1}HNMR techniques. Metabolites were assigned using Fiehn Library [5]. It was concluded that direct sampling into 60% (v/v) of cold methanol (temperature: less than -50 °C) as quenching solution and consequent extraction using boiling ethanol was the best approach.

Carnicer et al. [6] mentioned about the importance of accuracy, reliability and reproducibility of measurement of intracellular metabolite levels for metabolic studies of microbial cell factories. They worked on the combination of methanol quenching and fast cell separation using filtration to extract intercellular metabolites. They optimised the extracting methods especially for *K. pastoris*, thereby provided a corroborated procedure for metabolomics analysis of *K. pastoris*. Metabolite quantification was examined with LC-ESI-MS/MS and GC-MS based isotope dilution MS introduced by Canelas et al. [39].

Russmayer et al. [40] compared the function of centrifugation and filtration as cell separation protocol for *K. pastoris* in terms of metabolite loss. Yeast cells were sampled and quenched into cold methanol (60% methanol at -27 °C) from a chemostat cultivation using the parameters already optimized by Carnicer et al. [6] in order to measure the amount of metabolite loss for both cell separation methods. Consequently, metabolites were analysed by LC-MS/MS [41]. Russmayer et al. [40] concluded that the cell separation methods influenced directly metabolite loss during sample preparation. They proposed to use filtration for cell separation and minimizing the contact time of cells to quenching solution would be the best way to avoid extensive metabolite loss in order to determinate concentration of intracellular metabolites accurately.

9. Conclusions

In short, quantitative metabolomics is gradually becoming a key tool to characterise the metabolites in complex living systems, which provide instant fingerprint of the physiology of the cells and valuable information about metabolic system. However, in order to have reliable and valid data, it is essential to have dependable and reproducible procedures and techniques for sampling and treatment of samples.

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