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Review

Biotechnological application of cellular functions of the methylotrophic yeast [1]

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1. Introduction: methylotrophic yeast as a basic tool in biotechnology

Basically, two conventional microbial methods have been established for the production of useful compounds. One is the fermentative method, the other the enzymatic method (Fig. 1). With the fermentative method, the metabolite itself is a useful compound, and its production is coupled with cell growth. Good examples of this method are antibiotic and amino acid fermentations. With the enzymatic method, cells are simply used as biocatalysts, and cell growth is not necessary. Not only metabolites but also artificial compounds can be used as reaction substrates. These two conventional methods are supported and inspired by basic science, i.e., microbiology, biochemistry, and genetics.

Since the late 1970s, recombinant DNA technology was introduced into the conventional microbial methods yielding new concepts in these fields. Fermentative metabolite production may be greatly enhanced by amplifying the biosynthetic genes, or even the metabolic pathway itself may be modified by introducing foreign genes to produce new products which could never have been produced by the original host. These technologies are now referred to as 'metabolic engineering'. As to the enzymatic method, a super enzyme with favored characteristics such as high thermostability or high specificity, could be obtained through enzyme engineering.

Since the discovery of the methylotrophic eucaryote, *Candida boidinii* (initially identified as *Kloeckera*) [1], the methylotrophic yeast has been studied extensively both in academic and applied fields. In the 1970s, the overall pathway for methanol-dissimilation and -assimilation was elucidated by purifying each enzyme responsible for C_1-metabolism, some of which were found to be compartmented into single-membrane-bound organelles, peroxisomes (Fig. 2). In the early 1970s, production of single cell protein (SCP) using methanol as a cheap carbon source, attracted much attention which resulted

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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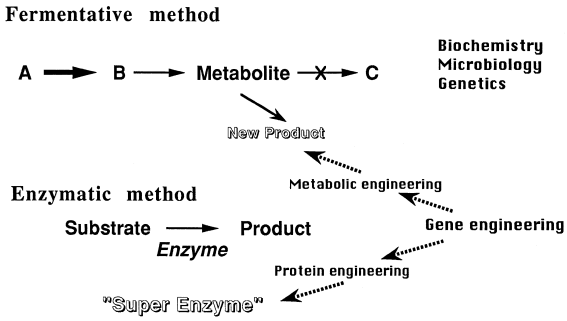


Fig. 1. Fermentative and enzymatic methods in microbial production based on biochemistry, microbiology, and genetics. Dotted arrows represent the direction of progression of 'gene engineering'. Through metabolic engineering, we could obtain a new product. Similarly, through protein engineering, we reach the goal, 'Super enzyme'. See details in the text.

in the establishment of the high-cell density cultivation method. In the late 1980s to early 1990s, recombinant DNA technology was established with several methylotrophic yeast strains, *Pichia pastoris*, *Hansenula polymorpha*, and *C. boidinii*. Heterologous gene expression with these strains has now become a basic technology in molecular biology and for the production of pharmaceutical proteins [2–4].

The methylotrophic yeast has unique C_1 metabolic functions which have been applied to the development of production of useful chemicals [5]. And in the last decade, the mechanism of peroxisome assembly has begun to be elucidated at the molecular level using these methylotrophic yeasts as model organisms [6]. These studies have medical importance in connection to human peroxisomal genetic disorders (e.g., Zellweger syndrome). Here, we summarize our recent studies with the methylotrophic yeast, *C. boidinii*, based on the concept 'application of cellular functions to the production of useful chemicals', and also try to predict developments in the field. The cellular functions discussed here include (1) metabolism, (2) gene expression, and (3) posttranslational processes, e.g., protein translocation to organelle and protein folding.

2. Exploring new metabolic functions

2.1. Enzyme production and one-step bioconversion with a unique methylotrophic enzyme

A simple utilization of metabolic function in the methylotrophic yeast is the use of unique

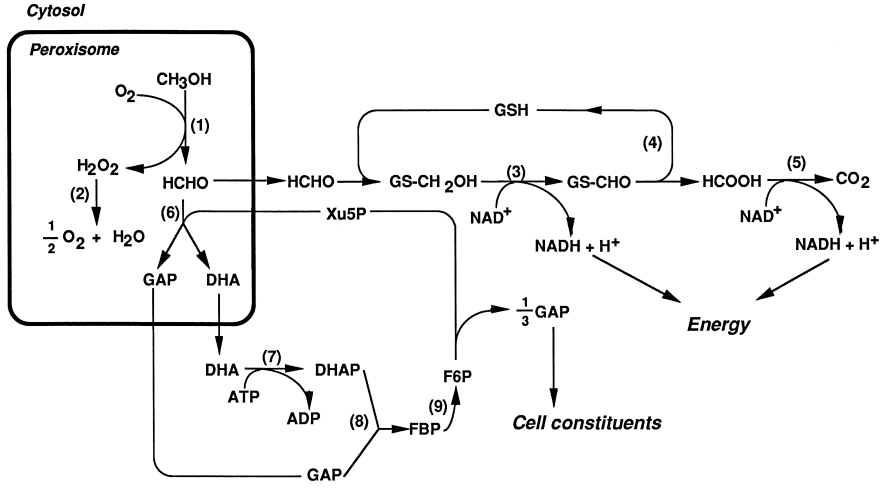


Fig. 2. C_1-Metabolic pathway of the methylotrophic yeast established in the late 1970s. Glutathione-independent formaldehyde oxidation pathway (Fig. 4) is not described in this figure. Formaldehyde oxidation pathway is now considered to contribute to formaldehyde detoxification functions. (1) Alcohol oxidase (AOD); (2) catalase (CTA); (3) glutathione-dependent formaldehyde dehydrogenase (GD-FALDH); (4) *S*-formylglutathione hydrolase; (5) formate dehydrogenase (FDH); (6) dihydroxyacetone synthase (DHAS); (7) dihydroxyacetone kinase (DHAK); (8) fructose 1,6-bisphosphate aldolase; (9) fructose 1,6-bisphosphatase.

enzymes present in this organism. In this respect, alcohol oxidase (AOD), catalyzing the first step in methanol oxidation, has a large potential in applications, including generation of H_2O_2 as a bleaching agent in detergents, oxygen scavenging in food and other products, synthesis of organic compounds, analytical detection of various alcohols, and use in enzyme mixtures for treatment of wastewater [7]. In the case of AOD, the advantage of continuous culture operation compared to batch cultivation has been well documented. In a methanol-limited chemostat with low dilution rate, this enzyme can amount to 30 to 50% of total proteins.

Metabolic production can be enhanced by separating the growth and product formation phases. We applied this scheme to the production of useful aldehydes from various alcohols. Cells having high AOD activity were used to study oxidation of a physiological substrate, methanol, together with non-physiological substrates, ethanol and allyl alcohol, for the production of corresponding aldehydes. Biological production of acetaldehyde and allyl alcohol has the following advantages compared to conventional chemical processes. (1) Chemically synthesized acetaldehyde cannot be used as food additive. When ethanol produced through alcohol fermentation is used as the reaction substrate, the acetaldehyde produced will be of a purely biological nature, and the product can be used as food additive. (2) Acrolein is industrially produced through direct oxidation of propylene. However, it is difficult to separate the product from the reaction mixture because the product becomes contaminated with large amounts of acetaldehyde and acetone as by-products. A biological process with a highly specific reaction is needed.

For an efficient product formation, both high catalyst concentration and continuous synthesis of fresh catalyst are required to compensate for the loss of catalyst due to inactivation by the reaction products. In the case of formaldehyde production, the isolation of a glucose-catabolite-repression-insensitive mutant enabled first-phase chemostat cultivation with a mixture of glucose–methanol so that a high alcohol oxidase productivity of the cells resulted in maximum productivity of aldehyde formation; 3.8 times higher than when the parent strain was grown on methanol alone [8,9].

Table 1 summarizes the results obtained with *C. boidinii* cells. The productivity and catalytic stability of cells are improved by preincubation at 37°C or by treatment with a cationic detergent (cation M2) [10]. The optimum temperature of 4°C for aldehyde production prevents the inactivation of AOD during this reaction. Ethanol oxidation was also described with *P. pastoris* cells for the production of acetaldehyde [11,12].

2.2. Multi-step bioconversion with purified methanol-assimilating enzymes

The first stage in the methanol-assimilating pathway of the methylotrophic yeast involves AOD, catalase (CAT), dihydroxyacetone synthase (DHAS), and dihydroxyacetone kinase

Table 1
Production of aldehydes from alcohols by *C. boidinii* cells

Substrate/product	Cell treatment	Concentration		Yield (%)	Reaction time (h)
		Substrate	Product		
Methanol/formaldehyde	Heat	3.0 M	1.09 M	36	10
	Cation M2	3.0 M	1.16 M	39	7
			1.38 M	46	20
Ethanol/acetaldehyde	Heat	0.43 M	0.43 M	99	1.5
		1.74 M	1.71 M	98	18
Allyl alcohol/acrolein	Heat	862 mM	536 mM	62	2

(DHAK) (Fig. 2). We applied this multi-enzyme system to produce [1, 3-^{13}C]dihydroxyacetone phosphate [13]. Besides these four enzymes, the production reaction utilizes purified preparation of another exogenous enzyme, adenylate kinase (ADK) to regenerate ATP (Fig. 3A). The product, ^{13}C-labeled dihydroxyacetone phosphate, is a convenient raw material for preparation of various kinds of ^{13}C-labeled sugars, which are useful for ^{13}C NMR diagnosis [14]. DHAS catalyzes a transketolase reaction between formaldehyde and D-xylulose 5-phosphate in vivo, and it can also utilize hydroxypyruvate as an acceptor of formaldehyde in vitro. [^{13}C]Methanol and hydroxypyruvate were used as starting materials. This reaction leads to the formation of dihydroxyacetone and CO_2. The benefit of this system is that the generated CO_2 can be spontaneously eliminated from the reaction system avoiding product inhibition of the DHAS-catalyzed reaction. Catalase was also added to support oxidation of [^{13}C]methanol to formaldehyde through its peroxidative function and to supply oxygen to AOD. After extensive optimization of each reaction step, the production system was established as described in the legend to Fig. 3. The reaction substrates (methanol, hydroxypyruvate, H_2O_2, and ATP) were fed at 40 min-intervals. Under the optimum reaction conditions, a fed-batch reaction afforded 185 mM [1,3-^{13}C] dihydroxyacetone phosphate from [^{13}C]methanol; and the molar yield of the ester relative to methanol added was 93% (Fig. 3B).

2.3. Methylformate synthase: a key enzyme in a novel formaldehyde oxidation pathway

Formaldehyde is a key intermediate which occupies the central position in the C_1-metabolism (Fig. 2). In the dissimilatory pathway of the methylotrophic yeast, formaldehyde is oxidized to formate in a glutathione (GSH)-dependent reaction catalyzed by GSH-dependent formaldehyde dehydrogenase (EC 1.2.1.1) and *S*-formylglutathione hydrolase (EC 3.1.2.12). Subsequently, formate is oxidized to CO_2 by formate dehydrogenase (EC 1.2.1.2).

We found that a considerable amount of methylformate accumulated in the culture media of methylotrophic yeasts. When *P. methanolica* was grown on a medium containing 2% (v/v) methanol, methylformate accumulated until the cells reached mid-exponential phase, at which point the maximum methylformate level (ca. 0.5 mM) was observed. Further studies revealed that methylformate formation was found to be catalyzed by a new type of alcohol dehydrogenase, which was named methylformate synthase (MFS). MFS was induced on methanol-medium, and methylformate accumulation and MFS ac-

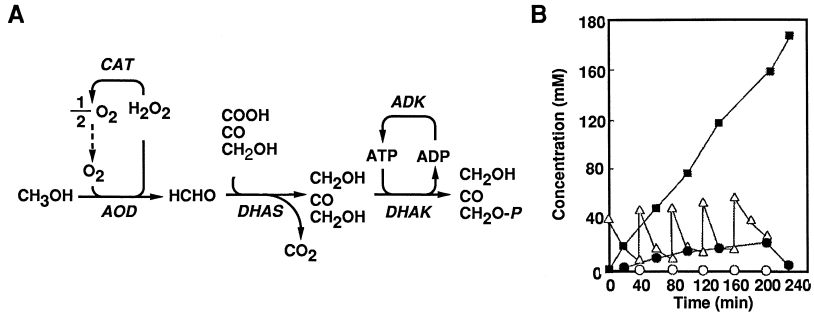


Fig. 3. Enzymatic preparation of [1,3-^{13}C]dihydroxyacetone phosphate (DHAP) from [^{13}C]methanol and hydroxypyruvate using the methanol-assimilating system of the methylotrophic yeast. (A) Reaction scheme. (B) Fed-batch reaction. The starting reaction mixture contained potassium phosphate buffer (pH 7.0, 100 mM), $MgCl_2$ (5 mM), thiamin pyrophosphate (0.5 mM), ATP (40 mM), [^{13}C]methanol (40 mM), hydroxypyruvate (80 mM), H_2O_2 (50 mM), DHAS (2 U/ml), DHAK (4 U/ml), adenylate kinase (ADK) (10 U/ml), and CAT (200 U/ml). Methanol (40 mM), hydroxypyruvate (80 mM), H_2O_2 (50 mM), and ATP (40 mM) were fed at 40-min intervals. Closed square, DHAP. Open triangle, methanol. Closed circle, dihydroxyacetone. Open circle, formaldehyde.

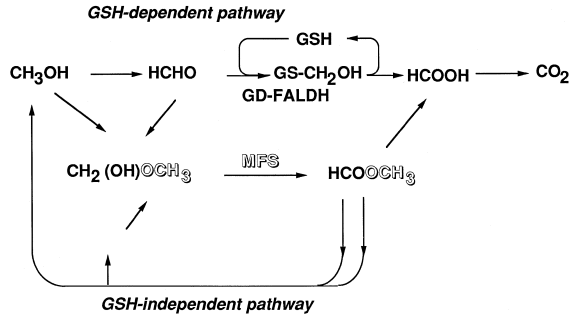


Fig. 4. Glutathione-dependent and glutathione-independent pathway of formaldehyde oxidation in the methylotrophic yeast. MFS, methylformate synthase.

tivity were enhanced by the addition of formaldehyde in the growing medium [15]. MFS was purified to an apparent homogeneity from cell-free extract of *P. methanolica* and *C. boidinii* [16]. MFS was composed of four identical subunits of 42 kDa, and included two zinc atoms per subunit. The reaction of MFS required formaldehyde, NAD^+, and a high concentration (0.5 M) of methanol in the assay mixture. From these results, we proposed a novel GSH-independent formaldehyde oxidation pathway (Fig. 4).

From a biotechnological aspect, MFS could provide a new approach to the production of water soluble esters in aqueous reaction mixture. Methylformate accumulated up to the concentration of 138 mM under aqueous conditions when cells having high MFS activity were used in the reaction [17]. This type of reaction, i.e., dehydrogenation of hemiacetal, provides a novel strategy to produce water-soluble esters in vitro.

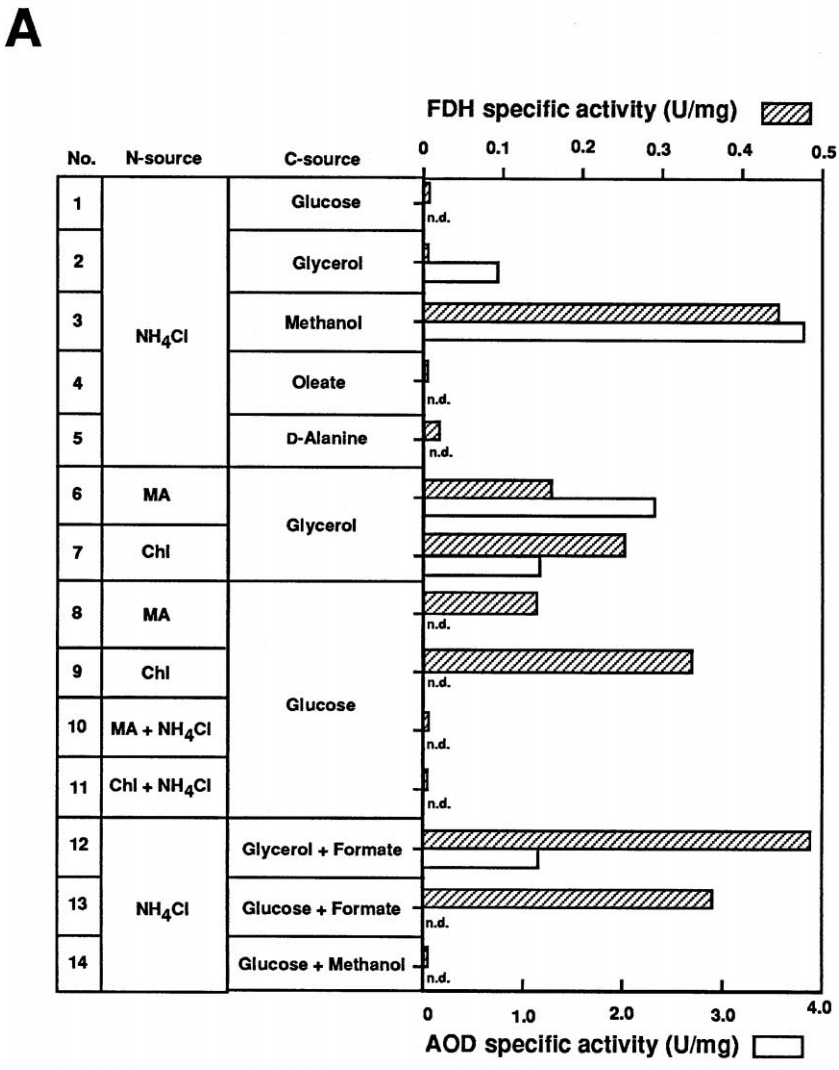
2.4. Formate dehydrogenase: its regulation and physiological role in the growth on C_1-compounds

For a long time, the formaldehyde oxidation pathway was believed to be involved in energy generation when cells were grown on methanol as a single carbon source (Fig. 2). Recently, there has been some discrepancy regarding the role of formate dehydrogenase (FDH) in the methylotrophic growth of yeast cells. Bystrykh et al. [18] reported that an FDH-deficient mutant of *H. polymorpha* could not grow on methanol. On the other hand, Sibirny et al. [19] reported that neither GSH-dependent formaldehyde dehydrogenase nor FDH was essential to the energy supply for the methylotrophic growth of *H. polymorpha*. However, the data of both groups were very limited, especially those on FDH-deficient strains. FDH-mutant strains from both groups were selected after random mutagenesis, and their only experimental criterion was the deficiency in FDH activity.

FDH from *C. boidinii* is the most commonly used enzyme for regenerating NADH from NAD^+ in many bioreactor reactions, and it is commercially available. Also, since FDH activity is induced with methanol medium and FDH accumulates up to 20% of soluble proteins in the cell extract, high-level expression using the FDH promoter can be expected. However, there had been no detailed study on the regulation of FDH at the molecular level.

To reveal the physiological roles of FDH in methylotrophic yeasts, we cloned the *FDH1* gene coding for FDH from *C. boidinii* genome and derived a strain with *FDH1* deleted (*fdh1Δ* mutant strain) by means of the *C. boidinii* gene disruption technique to determine the knock out effect on growth on various carbon and nitrogen sources. Also, we investigated its regulation by growth carbon and nitrogen sources at the molecular level.

In a batch culture experiment, although the *fdh1Δ* strain was still able to grow on methanol, its growth was greatly inhibited and a toxic level of formate was detected in the medium. In a methanol-limited chemostat culture at a low dilution rate (0.03 to 0.05 h^{-1}), formate was not detected in the culture medium and the *fdh1Δ* strain showed only one-fourth the growth yield of the wild type strain. Expression of *FDH1* was analyzed by both enzyme activity and at the mRNA level (Fig. 5). It was induced by choline or methylamine (used as a nitrogen source), as well as by methanol (used as a carbon source). Induction of *FDH1* was not repressed in the


A
FDH specific activity (U/mg)
No.
N-source
C-source
0
0.1
0.2
0.3
0.4
0.5
1
NH4Cl
Glucose
n.d.
2
Glycerol
3
Methanol
4
Oleate
n.d.
5
D-Alanine
n.d.
6
MA
Glycerol
7
Chl
8
MA
Glucose
n.d.
9
Chl
n.d.
10
MA + NH4Cl
n.d.
11
Chl + NH4Cl
n.d.
12
NH4Cl
Glycerol + Formate
13
Glucose + Formate
n.d.
14
Glucose + Methanol
n.d.
0
1.0
2.0
3.0
4.0
AOD specific activity (U/mg)
Chl : Choline
MA: Methylamine
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
FDH1
ACT1

presence of glucose when cells were grown on methylamine, choline, or formate, and expression of *FDH1* was regulated at the mRNA level (Fig. 5B).

Growth on methylamine or choline as a nitrogen source in a batch culture was compared between the wild type and the *fdh1Δ* mutant. Although the growth of the *fdh1Δ* mutant was impaired and the level of formate was higher in the *fdh1Δ* mutant than in the wild type strain, the growth defect caused by *FDH1* gene disruption was small and less severe than that caused by growth on methanol. As judged from these results, the main physiological role of FDH with all of the *FDH1*-inducing growth substrates seems to be detoxification of formate. Also, during growth on methanol, FDH seems to contribute significantly to the energy yield. Our results also suggested that *FDH1* expression was regulated mainly not by methanol but by formate, since *FDH1*-expression was observed with glucose/formate medium. To investigate the molecular basis for these control mechanisms, further studies to identify upstream activating sequences (UAS) in the *FDH1* promoter are now in progress.

3. Strong and regulatable gene expression of the methylotrophic yeast

3.1. Molecular breeding system: gene disruption and intracellular protein production as the basis for bioconversion

The yeast gene expression system is considered advantageous in the following respects: (1) yeast cells are easy to handle the production system can be scaled up to an industrial level; (2) eucaryotic intracellular structures, which are important for secretion, modification and folding of the proteins exist in yeast cells as well as in higher organisms. *C. boidinii* shows potential as a producer of useful metabolites such as citric acid, ATP, and aldehydes [5]. Gene expression coupled with molecular breeding system may be considered a basic technology for metabolic engineering.

A transformation system for *C. boidinii* was established by deriving a uracil auxotroph strain (*ura3*), and constructing a chromosomal integrative vector and an autonomous replicating vector [20,21] (Fig. 6A–B). Any specific gene in *C. boidinii* can be efficiently deleted by one-step gene disruption, if it is not lethal (Fig. 6C) [22]. The alcohol oxidase gene (*AOD1*), which is efficiently expressed during growth on methanol but repressed on glucose or ethanol, was cloned from the *C. boidinii* genome [23]. Heterologous gene expression in *C. boidinii* under the *AOD1* promoter was investigated using the *Saccharomyces cerevisiae* adenylate kinase (*S. cerevisiae ADK1*)-expression vector pTRex. The single integrant strain was grown on various carbon sources, and the ADK activity was followed to characterize the transcriptional control of the *C. boidinii AOD1* promoter [24]. The pattern of ADK regulation in the transformant was essentially the same as the *C. boidinii AOD1* regulation, i.e., (a) cells grown on methanol or methanol plus glycerol medium showed highly induced levels of ADK activity, (b) their activities were repressed to low levels when they were grown in the presence of glucose or ethanol in the medium, (c) glycerol-grown cells represent a middle-level expression (Fig. 7). The produced ADK protein accumulated up to 28% of the total soluble proteins on glycerol plus methanol medium, and all of the produced ADK was in a soluble, active, and

Fig. 5. Regulation of *FDH1*-expression in *C. boidinii*. (A) FDH and AOD activities in extracts of cells grown on the indicated carbon and nitrogen sources. Chl., choline; MA, methylamine; n.d., not detected. (B) Northern analysis. Twenty micrograms of total RNA was loaded on each lane and probed with *FDH1* or *C. boidini ACT1* (actin) DNA. The *C. boidinii ACT1* DNA was used as the control of constitutive expression. The lane numbers correspond to the combinations of carbon and nitrogen sources listed in (A).

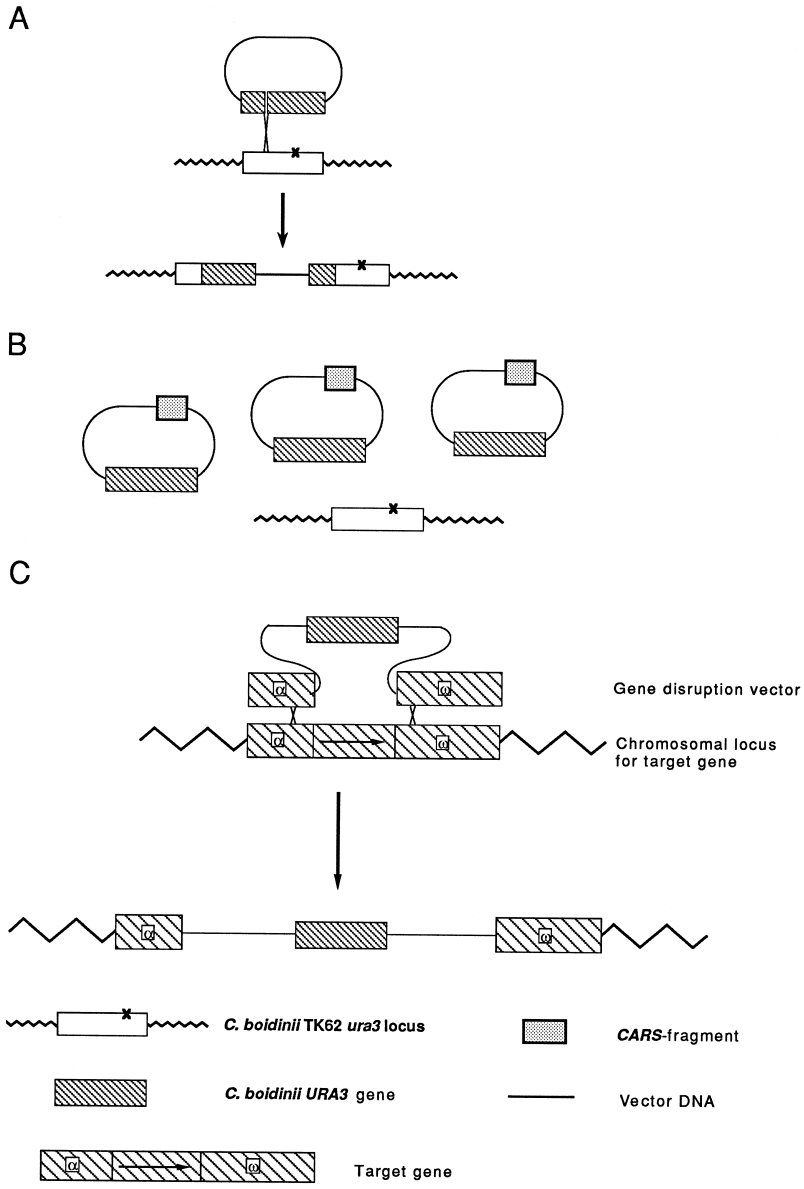


Fig. 6. Molecular breeding system in *C. boidinii*. (A) Integrative transformation. (B) Transformation via ARS (autonomous replicating sequence)-based vector. (C) Gene disruption.

modified form as was expressed in *S. cerevisiae* (Fig. 7).

3.2. Energy supply in the bioconversion system

Previous studies of ATP production with *C. boidinii* cells suggested that the phosphorylation of AMP to ATP catalyzed by ADK was the rate-limiting step [25]. As methanol-induced transformants had ca. 10,000-fold enhanced levels of ADK activity, ATP production with these transformants was investigated to seek whether the enhancement in ADK activity would increase ATP productivity in *C. boidinii* cells [26]. The wild type strain was low in ADP and ATP productivities. In contrast, cells of transformant strain phosphorylated AMP to ADP efficiently, reflecting the high activity of ADK

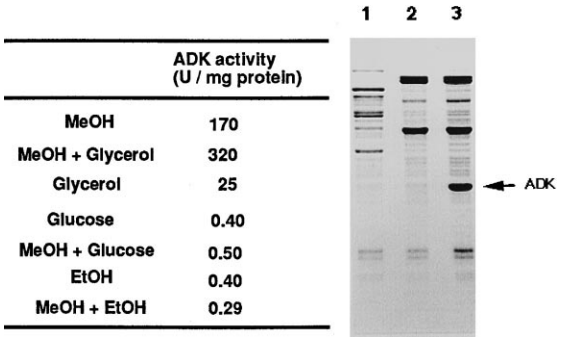


Fig. 7. Expression of *S. cerevisiae ADK1* under the *AOD1* promoter in *C. boidinii*. Regulation of ADK by carbon sources and SDS-PAGE analysis of intracellular soluble proteins (15 μg). Lane 1, glucose-grown transformant; lane 2, methanol-grown wild type; lane 3, methanol-grown transformant.

in the cells. Consequently, the transformant showed very high ATP productivity. ATP production was performed with successive feeding of adenosine under controlled pH. As shown in Fig. 8, after 42 h, the ATP concentration in the reaction mixture was 230 mM (117 g/l), and the conversion efficiency was 88% [26].

As many bioconversion reactions require ATP as a substrate or energy source, simultaneous expression of ADK and other heterologous enzymes should enable us to establish a bioconversion system with *C. boidinii* that does not require continuous addition of ATP. Such a

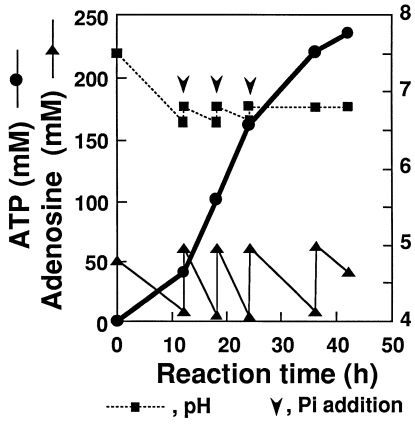


Fig. 8. ATP production from adenosine with cells of *C. boidinii* transformant. The reaction was performed at a 20-ml scale. Arrows show the addition of potassium phosphate for pH adjustment.

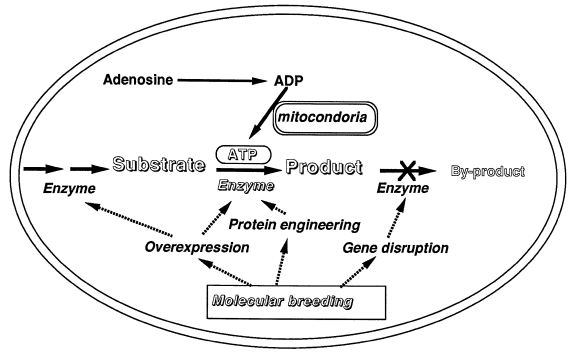


Fig. 9. A novel bioconversion system using *C. boidinii* transformant cells. Intracellular overproduction of a enzyme results in the amplification of a specific reaction, and by disrupting the gene of enzyme that forms the by-products, unwanted reactions can be eliminated. ATP production system will make possible an energy-coupled bioconversion with *C. boidinii* cells.

system would provide a novel strategy for further metabolic engineering in *C. boidinii* (Fig. 9).

4. Protein production in organelles: molecular mechanism of peroxisomal protein transport and protein folding

4.1. The peroxisome is the best place to produce heterologous 'oxidase'

Intracellular production of an enzyme protein in a large amount is the basis to preparing highly catalytic cells to produce useful chemicals. In the methylotrophic yeast under highly methanol-induced conditions, much of the intracellular volume (up to 80%) is occupied by single membrane organelles, peroxisomes. The peroxisome is a class of ubiquitous eucaryotic organelle where various kinds of oxidative metabolisms are executed. H_2O_2 generated by the oxidase reaction is decomposed by catalase which is also present in this organelle. From a biotechnological point of view, peroxisomes of the methylotrophic yeast are attractive organelles in which 'to pack' the produced heterologous proteins where they are protected from degradation by cytosolic proteases or when proteins of a toxic nature are present in the cytosol.

Most peroxisome matrix proteins are destined for peroxisomes by a 3-amino acid sequence, -SKL and its derivatives (Peroxisome Targeting Signal 1: PTS1), located at the extreme carboxyl end [6]. As such, an enzyme protein can be easily targeted to peroxisomes just by adding three amino acids as long as the addition does not affect its activity.

Various kinds of oxidases are commonly used as clinical diagnostic enzymes since H_2O_2 generated by the reaction can be easily detected by a color-developing reaction using peroxidase. We have been investigating fungal fructosyl amine oxidases (FAOD) which can be used for the determination of glycated proteins. Since glycation of blood proteins is not affected by transient increases in blood glucose, the levels of the glycated proteins are good indices for monitoring diabetes mellitus patients during therapy.

After screening of FAOD-producing microorganisms, we showed that FAOD from several fungi could be used for the determination of glycated human serum albumin (HSA) [27,28]. Fig. 10 shows one such example using FAOD from *Aspergillus terreus* GP1. Another interesting enzyme is FAOD from *Penicillium janthinelum*, which shows high affinity to fructosyl valine, a model compound for glycated hemoglobin. We cloned cDNAs coding for FAOD from cDNA libraries of *A. terreus* GP1 and *P. janthinelum* [28]. The C-terminal tripeptides-SKL and -AKL of FAODs from *A. terreus* and *P. janthinelum*, respectively, represented typical PTS1. Peroxisomal localization of FAOD in these fungi was confirmed also by immunoelectron microscopy [29]. From these observations, these fungal FAODs were found to be peroxisomal enzymes. Although the enzyme could be produced in *Escherichia coli* in an active form, the yield was not sufficient to satisfy production [28]. When cDNAs were ligated to a high expression vector and transformed to *E. coli*, the recovered vector DNA always carried the coding sequences of FAODs in the opposite direction of transcription sug-

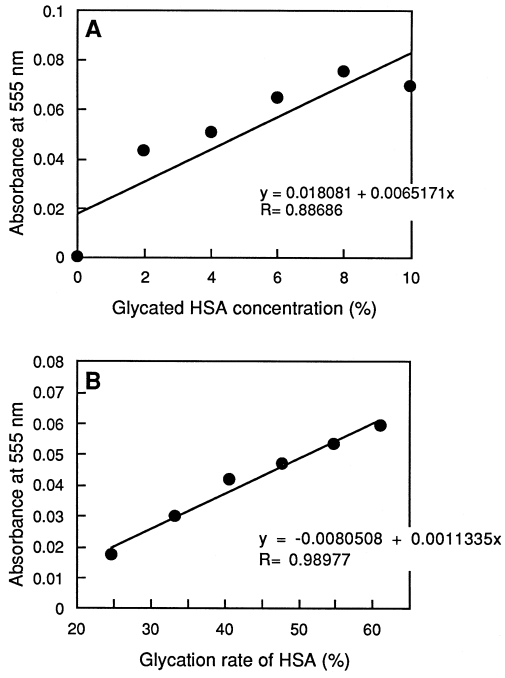


Fig. 10. Enzymatic measurement of glycated human serum albumin (HSA) with *A. terreus* FAOD. (A) Standard solutions of glycated human serum albumin at various concentrations were used for the protease-coupled FAOD assay. (B) Solutions prepared by mixing two glycated HSA solutions exhibiting different glycation rates in various ratios were used. The net glycation rates of the standard solutions were determined with the HPLC method, using automatic glyco-albumin analyzer, GAA-2000 (Kyoto Daiichi Kagaku).

gesting that a high level expression of these cDNAs was toxic to *E. coli*. We have tested *C. boidinii* as the host for production of *P. janthinelum* FAOD. FAOD produced in *C. boidinii* was translocated to peroxisomes judged by sucrose-density fractionation experiment. By using (1) the synthetic gene optimized for *C. boidinii* codon usage and (2) the *aod1*-disrupted strain, we have succeeded in production of FAOD in a high amount corresponding to 15 to 20% of soluble proteins [29]. We assume that the disruption of *AOD1*-coding gene increased (1) supply of both FAD to FAOD from alcohol oxidase; and (2) space in peroxisomes to accommodate more FAOD. In this way we were able to avoid toxicity of FAOD production since high amounts of homologous oxidases are originally located in peroxisomes of this yeast.

4.2. Posttranslational process involved in the expression of peroxisomal enzyme activity

The molecular bases for peroxisomal protein transport began to become clear with the use of yeast genetics. To date, at least 17 *PEX* (peroxine) genes had been identified with yeast cells. Methylotrophic yeasts, *P. pastoris*, *H. polymorpha*, and *C. boidinii*, are currently used as model systems in these peroxisomal studies because of their ability to proliferate massive peroxisomes [6].

During investigation of the function of a peroxisome membrane protein of *C. boidinii*, we have found that a putative peroxisomal transporter, Pmp47, was responsible for transport and folding of a specific matrix enzyme, i.e., DHAS [30,31]. Biochemical and immunoelectron microscopic observations showed that DHAS protein aggregated in the cytosol as an inclusion body while AOD was active and was imported into peroxisomes. Pmp47 was also depleted from the two peroxisome assembly mutants of *C. boidinii* [31]. In these *pex-pmp47Δ* strains as well as in *pex* strains, DHAS was in an active form in the cytosol (or nucleus). Since both AOD and DHAS have PTS1 motif sequences at their carboxyl terminal, our results were the first to show that depletion of Pmp47 could dissect the peroxisomal import pathway of these proteins.

Fig. 11 summarizes the diversity of peroxisomal protein import pathways within the PTS1-pathway and our working hypothesis. Both DHAS and AOD have a PTS1 motif sequence, NHL and ARY, respectively, which could direct non-peroxisomal protein to peroxisomes [29]. However, only DHAS seems to demand a solute transported by Pmp47 for showing its activity. The solute may be necessary for the folding of DHAS in peroxisomes (the peroxisomal chaperone model) or for the translocation of DHAS (the translocation machinery model). According to the peroxisomal chaperone model, in *pmp47Δ* cells, although both DHAS and the solute are present in the cytoplasm, DHAS could not fold into an active form because of the lack of peroxisomal factor. In peroxisome-deficient strains, all of these molecules are within the cytosol, and so DHAS could fold into an active form. According to the translocation model, DHAS could not fold into an active form in *pmp47Δ* because of the inhibition of folding by the membrane component. Peroxisome-deficient strains had enzyme activity since DHAS folding was not inhibited by the membrane component.

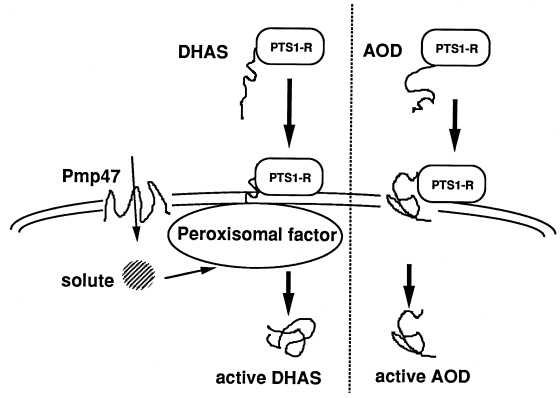


Fig. 11. A working model showing translocation of DHAS and AOD to peroxisomes in relation to the function of peroxisomal transporter Pmp47.

Understanding of the mechanism for translocation and folding of peroxisomal proteins will give us a novel strategy for the overproduction of foreign proteins within peroxisomes, and eventually, the system may be applied to bioconversion reactions in a more sophisticated and economical manner.

5. Secretory production of useful enzymes

Various proteins, including enzymes, could be produced in the growth media via secretion. To investigate secretory enzyme production using the *C. boidinii* gene expression system, *Rhizopus oryzae* glucoamylase was studied. The cDNA of glucoamylase was placed under the *C. boidinii AOD1* promoter. A transformant integrated with a single-copy expression cassette to the chromosome produced glucoamylase into

the medium in large amounts when the cells were grown on methanol or methanol plus glycerol as (a) carbon source(s). The transformant *C. boidinii* cells were grown up to ca. 95 g dry cell weight/liter medium, and the concentration of glucoamylase in the medium reached 3.4 g/l.

6. Conclusion

The methylotrophic yeast, *C. boidinii*, was initially isolated during screening for methyl acetate esterase. This project started from purely applied backgrounds, and at the time, nobody could predict such a great expansion of the use of methylotrophic yeast.

Through extensive biochemical, genetical, and cellular studies of methylotrophic yeasts, various biological functions have already been utilized and many more are sure to follow. Primarily, its unique C_1-metabolism provides a novel reaction not found in other organisms. Here, as an example, an alcohol oxidase-catalyzed reaction and a multi-step C_1-assimilating system were developed to produce ^{13}C-labeled DHAP. Although C_1-metabolism in the methylotrophic yeast had been studied extensively, current studies on methyl formate formation led to a surprising finding of yet another new pathway of formaldehyde oxidation. New findings like these will lead to the production of new metabolites. Second, the strong and inducible gene expression system of the methylotrophic yeast can be directly applied to the production of useful proteins. Third, when the molecular breeding system is coupled with gene expression, the system can be considered a novel bioconversion system. In other words, intracellular overproduction of enzymes means amplification of the reaction involved in bioconversion, and by-product formations can be eliminated by disruption of the specific gene. Fourth, the ATP-production system shown here may enable energy-coupled bioconversion with *C. boidinii* cells. Fifth, using methylotrophic yeast cells, we will be able to 'optimize' the localization of foreign proteins or 'to pack' foreign proteins into peroxisomes.

When we consider the events in gene expression following the 'central dogma' and 'post-central dogma' from the field of biotechnology, many further studies are needed to produce large amounts of proteins at an economically reasonable cost for each product or to establish a gene expression system universal for any heterologous protein (Fig. 12). In the course of these studies, the methylotrophic yeast could be 'the bridge' between applied and basic biological sciences.

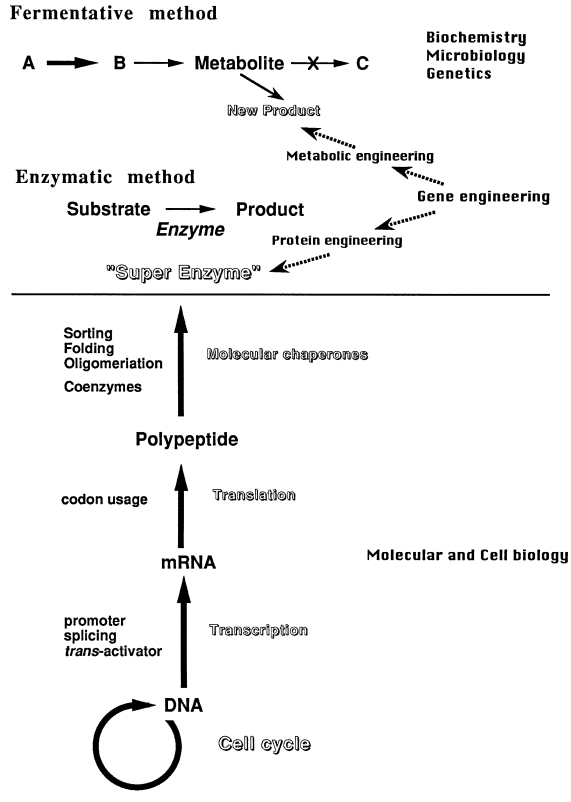


Fig. 12. Future prospects for microbial production based on molecular and cell biology. Revealing antibiotics biosynthesis led us to new findings in metabolic pathways and of enzymes, and enabled us to increase the antibiotics productivity greatly. Similarly, in the future, by following the concept of the central dogma and cell biology, revealing biosynthetic process of useful proteins may lead us to find new molecules, which may hold the key to enlighten the mechanism of protein expression.

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