BIODIESEL FUEL PRODUCTION USING FUNGUS WHOLE CELL BIOCATALYSTS

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BIODIESEL FUEL PRODUCTION USING FUNGUS WHOLE CELL BIOCATALYSTS

糸状菌全細胞触媒を用いたバイオディーゼル燃料生産

SHINJI HAMA

2006

PREFACE

This is a thesis submitted by the author to Kobe University for the degree of Doctor of Engineering. The studies collected here were carried out between 2000 and 2006 under the direction of Professor Hideki Fukuda at the Laboratory of Biochemical Engineering, Division of Molecular Science, Graduate School of Science and Technology, Kobe University.

First of all, the author expresses the sincerest gratitude to research advisor, Professor Hideki Fukuda, for his continuous guidance and invaluable suggestions, and encouragement in the course of the studies. Next, the author expresses hearty gratitude to Professor Akihiko Kondo for his enormously beneficial discussions and kind support. The author is also deeply grateful to Professor Shigeo Kato, Professor Yasukiyo Ueda, Associate Professor Hideki Yamaji, and Assistant Professor Tomohisa Katsuda for their informative advice and hearty encouragement through the work.

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CONTENTS

INTRODUCTION

Alternative fuels derived from a renewable source have recently attracted considerable attention due to the diminishing petroleum reserves and environmental consequences of exhaust gases from petroleum-fuelled engines. Plant oils are promising as a potential renewable source, however, the direct use of such oils for diesel engines leads to many problems because of their high viscosity and free fatty acid content. Consequently, considerable effort has gone into developing the synthesis methods of plant oil derivatives. Transesterification, which is the displacement of alcohol from an ester by other alcohols including methanol, propanol and buthanol, has been widely used to reduce the viscosity of plant oils, thereby enhancing the physical properties as renewable fuels. Thus, fatty acid alkyl esters (better known as biodiesel) obtained by this process can be used as an alternative fuel for diesel engines. Used oils can also be utilized for production of biodiesel, helping to reduce the cost of wastewater treatment and generally assisting in the recycling of resources.

During the last decade, environmental benefits have accelerated the worldwide production of biodiesel-fuel. In Europe and the United States, biodiesel-fuel production now exceeds 100 million liters and is expected to further increase in the near future. In most cases, biodiesel can be used in a blend with petroleum diesel, which helps the introduction of this fuel into conventional engine systems. In Japan, small quantities of biodiesel from used oils have been recently utilized in garbage trucks and municipal buses in several localities. Since Japan uses approximately 40 billion liters of light oil per year, more widespread use of the clean energy source is necessary to reduce an air pollution and depletion of resources.

This thesis forms part of a study on the intracellular lipase localization in filamentous fungi and application of intracellular lipase for biodiesel-fuel production. Lipase-catalyzed transesterification is promising for industrial biodiesel-fuel production. Moreover, direct use of lipase-producing filamentous fungi leads to a significant reduction of production cost. This chapter describes the technological backgrounds relating to the present study, focusing particularly on an enzymatic process using fungus whole-cell biocatalysts.

Lipase production by filamentous fungi

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze a variety of reactions such as hydrolysis, transesterification, and ester synthesis at the interface between substrate and water or in non-aqueous organic solvent (Sarda and Desnuelle, 1958; Zaks and Klibanov, 1984). Due to the catalytic versatility shown in Figure 1, they have received considerable attention for biotechnological applications in a wide range of processes (Jaeger and Eggert, 2000).

Hydrolysis

$$R_1\text{-}COO\text{-}R_2 + H_2O \longrightarrow R_1\text{-}COOH + R_2\text{-}OH$$

Esterification

$$R_1\text{-}COOH + R_2\text{-}OH \longrightarrow R_1\text{-}COO\text{-}R_2 + H_2O$$

Transesterification

- **Acidolysis**

$$R_1\text{-}COO\text{-}R_2 + R_3\text{-}COOH \longrightarrow R_1\text{-}COO\text{-}R_3 + R_2\text{-}COOH$$

- **Alcoholysis**

$$R_1\text{-}COO\text{-}R_2 + R_3\text{-}OH \longrightarrow R_1\text{-}COO\text{-}R_3 + R_2\text{-}OH$$

- **Interesterification**

$$R_1\text{-}COO\text{-}R_2 + R_3\text{-}COO\text{-}R_4 \longrightarrow R_1\text{-}COO\text{-}R_4 + R_3\text{-}COO\text{-}R_2$$

- **Aminolysis**

$$R_1\text{-}COO\text{-}R_2 + R_3\text{-}NH_2 \longrightarrow R_1\text{-}CONH\text{-}R_3 + R_2\text{-}OH$$

Figure 1 Types of reaction catalyzed by lipase.

Lipases have been found in many species of animals, plants and microorganisms (Antonian, 1988). In particular, lipases from filamentous fungi have a great biotechnological potential in the terms of their versatile characteristics and secretory capacity of this organism. More than 30 lipases have been so far isolated from *Rhizopus* strains and characterized (Haas and Joerger, 1995). These lipases from *Rhizopus* species generally exhibit a high 1,3-regiospecificity towards triacylglycerols, which makes them useful enzymes in lipid modification process (Table 1). Several suppliers currently provide the commercial products of non-recombinant crude lipases from *Rhizopus* strains (e.g. Amano Enzyme Inc.: Lipase F-AP15 derived from *Rhizopus oryzae*).

Table 1 Lipase production by *Rhizopus* species.

Microorganism	Application	Reference
R. arrhizus	Ester and glyceride synthesis	Bell et al., 1978
	Synthesis of monoglyceride	Chunhua et al., 2002
R. chinensis	Interesterification of oils and fats	Nakashima et al., 1988, 1989, 1990
	Esterification of short-chain fatty acids	Xu et al., 2002
R. delemar	Hydrolysis of triglycerides	Iwai et al., 1974
R. japonicus	Hydrolysis of tricaprin	Aisaka and Terada, 1981
R. oryzae	Resolution of 2-alkanols	Molinari et al., 1998
	Selective esterification of sardine fatty acids	Hiol et al., 2000
	Synthesis of biodiesel	Ban et al., 2001
	Synthesis of flavour esters	Gandolfi et al., 2001

Recently, investigation of the taxonomic diversity in the genus *Rhizopus* showed that, in spite of some minor variations, all hitherto diverse species can be combined into *Rhizopus oryzae* (Schipper, 1984). Consistent with this taxonomic re-classification, it has been demonstrated that all known sequences of lipases from *Rhizopus* species (e.g. *R. delemar*, *R. javanicus*, *R. niveus* and *R. oryzae*) have an identical amino acid sequence, and suggested that the different properties of these lipases are not due to the existence of different genes but to either different production conditions affecting the glycosylation pattern of these lipases, or to proteolytic cleavage products derived from the mature, the pro- or the pre-pro-lipase (Beer et al., 1998). It is thus significant to investigate the relationship between a degree of post-translational cleavage and properties (e.g. activity, stability and localization) in the lipase produced by this organism.

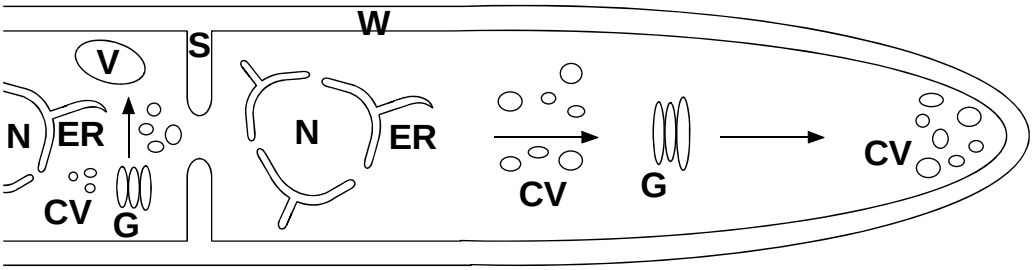
In Part I, lipase localization in filamentous fungi was investigated. For this purpose, lipase from *R. oryzae* was selected because Kaieda et al. (1999) found that it efficiently catalyzes the methanolysis of plant oils in solvent-free and water-containing system, and because direct use of lipase-producing *R. oryzae* cells was found to be effective in catalyzing the reaction (Ban et al., 2001). In Chapter 1, *R. oryzae* lipase responsible for the intracellular methanolysis activity was first identified.

Molecular-biological techniques in filamentous fungi

During the last two decades, the development of molecular-biological techniques has opened up new ways to utilize filamentous fungi for heterologous and homologous protein production. Of the fungi employed in gene manipulation, *Aspergillus awamori*, *Aspergillus niger* and *Aspergillus oryzae* have been frequently used for recombinant protein production, since these strains exhibit a high expression level (Punt et al., 2002). In the case of lipase, Huge-Jensen et al. (1989) reported that *A. oryzae*, into which the cDNA encoding *Rhizomucor miehei* triglyceride lipase precursor was introduced under the control of α-amylase gene promoter, efficiently secreted the mature lipase after the collect processing, demonstrating the utility of this organism as a host for recombinant lipase production.

Moreover, the production level of recombinant proteins in fungi has been recently improved by several strategies shown in Figure 2. Most of the strategies are generally adopted to dissolve the bottlenecks in the secretory process including transcription, translation, transport and secretion. The application of a gene fusion strategy has been especially successful as reported by Gouka et al. (1997a). When the heterologous genes were fused to highly expressed fungal genes (e.g. glucoamylase), the higher production level was obtained even in the case of mammalian protein. In general, the fungal protein used in this strategy is believed to improve the translocation into the endoplasmic reticulum, to facilitate folding and to prevent degradation of the heterologous protein. Thus, it should be taken into consideration to construct the expression system suitable for the target protein and the secretory pathway in a host strain.

Besides the strategies shown in Figure 2, the development of an improved promoter for *A. oryzae* has now been successful. Minetoki et al. (1998) found that high-level expression of *amyB*, *glaA* and *agdA* promoters depends on region III which is highly conserved sequence in these promoters, and that the introduction of multiple copies of the region III fragment into them resulted in a significant increase in promoter activity at the transcriptional level. The use of these improved promoters consequently led to success of high-level protein production in *A. oryzae* (Ichishima et al., 1999; Kanamasa et al., 2003; Honda et al., 2005).



	Nucleus	Cytoplasm	ER	Golgi	Post-Golgi
	Transcription	**Translation**	**Transport**	**Transport**	**Secretion**
Key processes	• pre-mRNA processing	• mRNA stability	• signal recognition • translocation • folding/modification • glycosylation	• protein processing • glycosylation	• post-Golgi processes • cell wall passage
Bottlenecks	• transcription control • AU-rich sequences	• mRNA destabilizing elements	• tertiary protein structure • shortage of foldases or chaperones • ER proteolytic degradation	• incorrect processing	• proteolytic degradation
Solutions	• expression of transcription factor (1) • GC-rich region (2, 3)	• gene-fusion (4, 5) • modification of UTR (6) and codon usage (7)	• gene-fusion (4, 5) • overproduction of foldases or chaperones (8) • protease-deficiency (9, 10)	• overproduction of processing enzymes (11)	• protease-deficiency (9, 10) • optimization of culture condition (12)

Figure 2 Generalized overview of the fungal secretion pathway and the putative limiting steps in protein production (Revised from Gouka et al., 1997a).

* Translation of mRNA starts in the cytoplasm, where ribosomes bind to the mRNA. As soon as the signal peptide has emerged from the ribosome, it is bound by the signal-recognition particle (SRP). Next, translation stops, the complex is targeted to the ER (with a reticular network structure in fungi) membrane via an interaction with the SRP receptor, and the translation starts again. When the newly synthesized protein enters the ER lumen, the N-terminal signal peptide is processed, and then maturation (folding, glycosylation, and processing) starts. These processes occur while the protein in the vesicles is transported from the ER to the Goldi (with dot-like structure in fungi). The protein is then transported to the cell wall, and finally secreted into the extracellular medium.

CV: cytoplasmic vesicles; ER: endoplasmic reticulum; G: Golgi(-like) compartment; N: nucleus; S: septum; V: vacuole; W: cell wall.
Corresponding references are: (1) Gomi et al., 2000; (2) Gouka et al., 1997b; (3) Romanos et al., 1992; (4) Contreras et al., 1991; (5) Nyyssonen and Keranen, 1995; (6, 7) Koda et al., 2004, 2005; (8) Valkonen et al., 2003; (9) Broekhuijsen et al., 1993; (10) Roberts et al., 1992; (11) Mizutani et al., 2004; and (12) O'Donnell et al., 2001.

However, despite much research on these improvements of protein production, studies on the secretory pathway in *A. oryzae* have not been given much attention. For a higher expression level of heterologous protein and a control of its localization, it is of great importance to investigate and understand the molecular processes governing the secretory system in this organism. The present study in Chapter 2, therefore, visualized the secretory pathway in *A. oryzae* using green fluorescent protein (GFP).

GFP, originally isolated from the marine jellyfish *Aequorea Victoria*, is a bioluminescent substance. GFP produces a strong green fluorescence under aerobic conditions and, unlike other reporter gene products such as β–glucuronidase and luciferase, can be used without destructive or invasive techniques and without addition of substrates. These features have made GFP valuable reporter to study gene expression and protein localization in living cells (Chalfie et al., 1994). Recently, GFP-chimeras have been used in filamentous fungi to provide *in vivo* visualization of specific organelles and processes including nuclear migration and protein secretion (Sulmann et al., 1997; Fernandes-Abolus et al., 1998; Gordon et al., 2000a, b). In *A. oryzae*, Kitamoto and co-workers have reported the successful *in vivo* observations of organelle dynamics (Maruyama et al., 2001, 2002; Ohneda et al., 2002; Masai et al., 2003, 2004). Among them, the gene construct of the secretory protein ribonuclease T_1 fused to GFP has made possible the *in vivo* observation of the secretory pathway in *A. oryzae* hyphae, and revealed the distribution pattern of fluorescence under stress including cold shock and addition of a protein transport inhibitor.

Although many secretory proteins alter their behaviors following the post-translational proteolysis (Ishisaka et al., 1999; Taniguchi et al., 2002), little attention has been so far paid to *in vivo* visualization of the molecular dynamics in fungi under the various types of proteolytic processing. The present study therefore investigated the secretory process of several truncated forms of *Rhizopus oryzae* lipase (ROL), which exhibits a unique localization pattern (discussed in Part I, Chapter 1). The ROL fused to GFP visualized its secretory process and revealed the role of the N-terminal region of ROL in a protein secretory pathway of filamentous fungi.

Immobilization of filamentous fungi within biomass support particles

In order to make an enzymatic process more convenient, enzymes have been immobilized into some kinds of supports after recovery from the cell or the culture broth and additional purification steps. However, enzymes recovered through such operations are generally unstable and expensive (Macrae, 1989), and consequently there has been considerable attention in the direct use of immobilized cells as whole-cell biocatalyst (Nikolova et al., 1993). The several advantages of immobilized cells are: (i) operations for enzyme extraction and/or purification are unnecessary; (ii) products can be easily recovered from immobilized cells; (iii) operational stability is generally high; and (iv) application to multiple enzyme reactions is possible. Such attractive features and potential have allowed the immobilized cell systems to be utilized in a wide variety of processes.

A technique using porous biomass support particles (BSPs: Atkinson et al., 1979) is quite promising among the cell immobilization methods so far employed, since it does not require the chemical additions and the cell growth prior to immobilization. This "natural" method can be performed using various types of particles such as polyester, polyurethane, and polyvinyl formal resin, and therefore it has been applied to a wide variety of microbial, animal and plant cell systems. In particular, since filamentous fungi employed in this study are easily and passively immobilized within BSPs because of their conformation, many investigations on fungus immobilization process have been successfully carried out (Table 2).

Table 2 Application of filamentous fungi immobilized within BSPs.

Microorganism	BSPs	Product	Reference
Aspergillu niger	PUF	Gluconic acid	Vassilev et al., 1993
Mucor ambiguus	PUF	γ-Linolenic acid	Fukuda and Morikawa, 1987
Penicillium chrysogenum	PUF	Penicillin	Kobayashi et al., 1990
Phanerochaete chrysosporium	PSF	Manganese peroxidase	Ürek et al., 2004
Trametes hirsuta	SS	Laccase	Couto et al., 2004
Trichoderma viride	SS	Cellulase	Webb et al., 1986
Rhizopus arrhizus	PUF	Lipase	Elibol and Özer, 2000
Rhizopus chinensis	PUF	Lipase	Nakashima et al., 1988, 1989
Rhizopus oryzae	PUF	Lipase	Ban et al., 2001

PUF, polyurethane foam; PSF, polystyrene foam; SS, stainless steel

Fukuda and co-workers (1988-1991, 2001), in a series of articles, reported a promising process for the industrial interesterification and methanolysis of plant oils. Unlike the conventional methods using extracellular lipase, the dried cells of *Rhizopus* species immobilized within polyurethane foam BSPs were directly utilized as a lipase catalyst. Figure 3 shows the surface and cross-sectional micrographs of the immobilized *Rhizopus oryzae* cells. As can be seen in these illustrations, the fungus formed a dense film near the surface of the BSPs, and furthermore the adhesion of cells to the support seems to have been strong. Dried *Rhizopus chinensis* cells using this method were successfully applied to continuous interesterification of fats and oils (Kyotani et al., 1991). Interestingly, it was found that the specific intracellular lipase activity of the *R. chinensis* immobilized within BSPs increased 4-7 fold compared with that obtained in the suspension cells. Cell aggregation following immobilization seems to be a common trigger for the enhancement of intracellular lipase production in *Rhizopus* species (Nakashima et al., 1990). In the present study, a further investigation was undertaken to determine the effect of immobilization on the intracellular lipase production and localization in *R. oryzae* cells (described in Part I, Chapter 1).

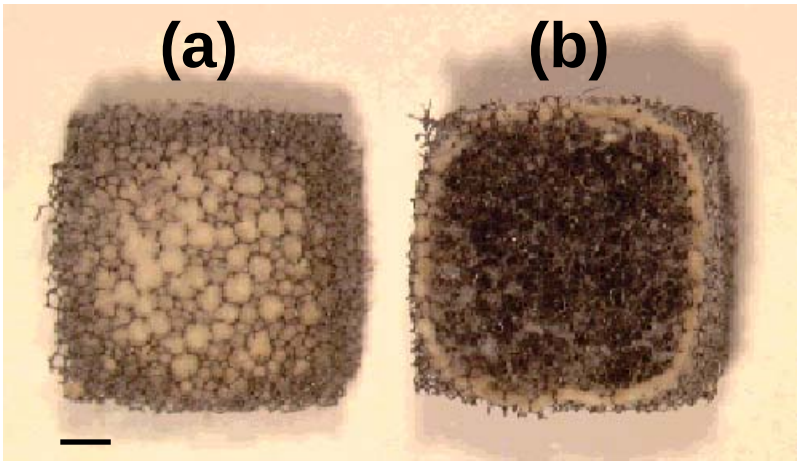


Figure 3 Surface (a) and cross-sectional (b) micrographs of BSP-immobilized *R. oryzae* cells. A 6-mm cubic particle with polyurethane foam was used as a support. Bar, 1 mm. (From Ban et al., 2001)

Biodiesel-fuel production

Part II discusses the improvement of biodiesel-fuel production using whole-cell biocatalysts. Biodiesel-fuel consists of methyl esters produced by transesterification of plant oils with methanol (i.e. methanolysis, Figure 4). The attractive features of biodiesel-fuel are: (i) it has high cetane numbers and flash points, that is, the possibility of a strong candidate to replace conventional diesel; (ii) its combustion does not increase current atmospheric levels of CO_2 since it is derived from plants, not petroleum; (iii) it is biodegradable; and (iv) it provides a substantial reduction in SOx emissions and considerable reduction in CO and suspended particulate matter. As a consequence of its advantages, there is considerable interest in exploring and developing the use of biodiesel-fuel all over the world (Ma and Hanna, 1999).

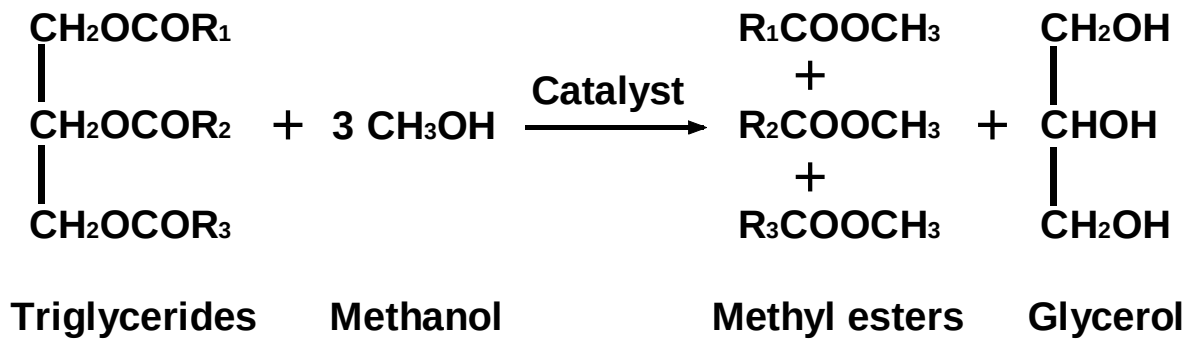


Figure 4 Reaction scheme of methanolysis.

A number of processes have been recently developed for biodiesel-fuel production. Although chemical transesterification using alkali-catalysis gives high conversion of methyl esters in short reaction time, it has several drawbacks including the difficulty in the recovery of glycerol and salt, the need for catalyst exclusion, and the energy-intensive nature of the process. In addition, Saka and Kusdiana (2001) reported that supercritical methanol treatment without any catalyst is sufficient to convert triglycerides and fatty acids to their corresponding methyl esters. However, since this method requires a high temperature of 350°C, a pressure of 45 MPa and large amount of methanol, further investigations such as continuous operation and scale-up are necessary for industrial application.

On the other hand, enzymatic transesterification using lipase has recently become more attractive for biodiesel-fuel production, since it provides a promising process to overcome the problems described above. Many researchers have so far reported the effective methanolysis reactions using extracellular lipase (Fukuda et al., 2001). In previous studies on methanolysis using *Candida antarctica* lipase, Shimada et al. (1999) found that the stepwise addition of methanol was useful to avoid lipase inactivation. Kaieda et al. (1999, 2001) investigated the methanolysis of soybean oil with non-regiospecific and 1,3-regiospecific lipases in a water-containing system without an organic solvent, and found that lipase from *Rhizopus oryzae* efficiently catalyzed the reaction in the presence of 4-30% water. Interestingly, despite of 1,3-regiospecificity of *R. oryzae* lipase, the methyl ester content reached 80-90% by stepwise addition of methanol to the reaction mixture. As to an explanation for this phenomenon, they demonstrated that acyl migration from the *sn*-2 position to the *sn*-1 or *sn*-3 position in partial glycerides spontaneously occurred, and thus a high methyl ester content above 80% was achieved. This reaction system is significantly advantageous to convert waste oils, since such oils used as a substrate inevitably contain a certain amount of water.

With the aim of reducing the cost of industrial biodiesel-fuel production, Ban et al. (2001) investigated the direct use of lipase-producing *R. oryzae* cells immobilized within BSPs as a whole-cell biocatalyst. When methanolysis was carried out with stepwise addition of methanol using BSP-immobilized cells, the methyl ester content in the reaction mixture reached 80-90%, which is almost the same production level with that achieved using extracellular lipase (Kaieda et al., 1999). To stabilize *R. oryzae* cells, they further examined the cross-linking treatment with 0.1% glutaraldehyde solution, and found that the intracellular lipase activity of the cells thus obtained was maintained during six batch cycles (Ban et al., 2002). They mentioned that a high methyl ester concentration is the major factor in the loss of lipase activity during repeated methanolysis, and that the treatment with glutaraldehyde is effective in preventing the intracellular lipase from the attack by methyl esters produced and from the leakage.

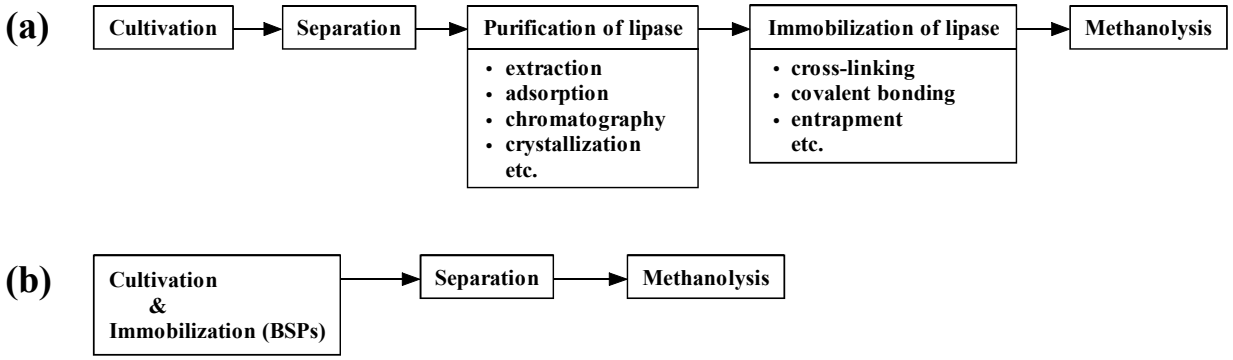


Figure 5 Comparison of lipase production processes for methanolysis with extracellular (a) and intracellular (b) lipases. (From Fukuda et al., 2001)

Figure 5 shows comparison of lipase production process for methanolysis with extracellular and intracellular lipases. Since the main hurdle to commercialize the enzymatic process is the cost of lipase production, the use of whole-cell biocatalysts seems to be significantly advantageous because of the simplicity of the process. For industrial application, however, it is necessary to further stabilize the cells without complex operations after cultivation. In the present study, a novel method utilizing the functions of cell membrane was developed to stabilize the whole-cell biocatalysts (Part II). This method requires no operations after batch cultivation, and is thus useful for practical biodisel-fuel production.

Modification of cell membrane composition

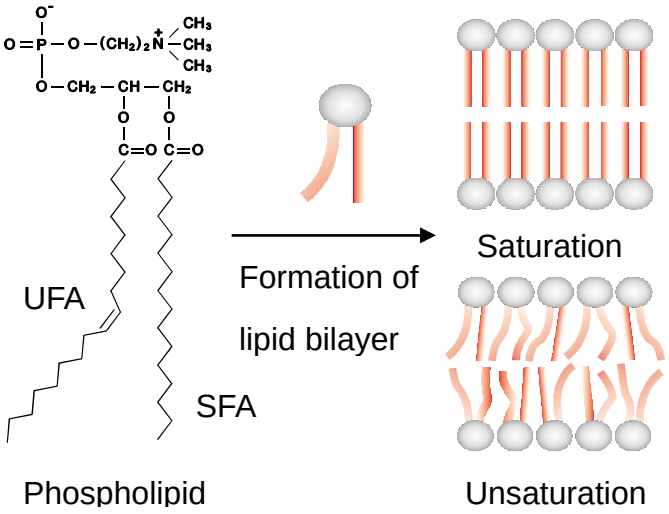
A major component of biological cell membrane is phospholipid, which consists of a head group and two acyl groups (fatty acids). In microorganisms, roles of yeast plasma membrane in the cell metabolism are well discussed, suggesting that small changes in plasma membrane composition can modify the transport and tolerance of metabolites (Keenan et al., 1982; Hazel and Williams, 1990; Valero et al., 1998).

In general, fatty acid membrane composition is highly valuable and clearly influenced by environmental factors such as temperature (Hunter and Rose, 1972), dissolved oxygen (Bardi et al., 1999), and osmotic pressure (Guillot et al., 2000). Addition of lipids into culture medium is an effective way to obtain a desired membrane composition, since these

exogenous lipids are incorporated intactly into cell membrane (Jenkins and Courtney, 2003; Stillwell and Wassall, 2003). The variation in fatty acid composition can alter the membrane properties as illustrated in Figure 6. Membrane permeability is generally increased with fatty acid unsaturation, while saturated membrane shows a high rigidity, due to the difference in a space occupation ratio (the area of fatty acid molecule in the lipid bilayer). Such alterations in membrane properties lead to a significant adaptation of cells to an environmental stress.

Figure 6

Schematic representation of membrane lipid bilayer composed of saturated (SFA) or unsaturated (UFA) fatty acids.



In studies on the ethanol tolerance of *Saccharomyces cerevisiae*, Mizoguchi et al. (1996, 1998) reported that palmitic acid-enriched cells, which include an exogenous palmitic acid in their own phospholipids, showed the lower permeability coefficient than linoleic acid-enriched cells, and that the maintenance of the cell membrane as a permeability barrier leads to a high ethanol tolerance. As for tolerance to toluene, cell membrane rigidity was again found to be a physical barrier to prevent solvent penetration into the cells (Ramos et al., 1997). It is thus supposed that alterations in fatty acid membrane composition contribute to a development of whole-cell biocatalysts possessing a high stability.

The present study in Part II investigated the effect of fatty acid membrane composition on *R. oryzae* cells for use as whole-cell biocatalysts in biodiesel-fuel production. Fatty acid composition of *R. oryzae* cells was controlled by the addition of various fatty acids, and their stability in repeated methanolysis was examined.

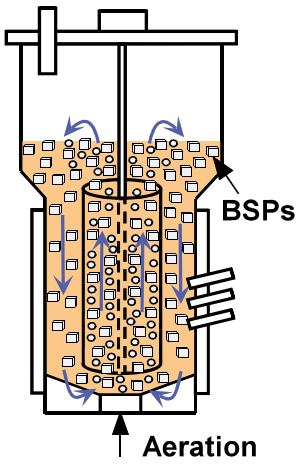
Large-scale production of biodiesel using whole-cell biocatalysts

To apply the fungus whole-cell biocatalysts in industrial biodiesel production, studies on the use of bioreactors in a large scale is necessary. Bioreactors can be generally divided into several different categories, including stirred tank reactors, fluidized bed reactors, and fixed bed reactors, according to the flow pattern. A stirred tank reactor (STR) is the most commonly used reactor and has the advantages of easy control of temperature and pH, since perfect mixing can be easily achieved. However, the use of STR in immobilized cell systems leads to the problem that immobilized cells are exposed to a rather high degree of mechanical shearing. This may severely damage the particles, resulting the inhibition of cell immobilization and the leakage of intracellular lipases. On the other hand, a fluidized bed reactor (FBR) can offer good mixing and mass transfer properties without a severe damage to the particles, since the fluidization in FBR can be carried out by liquid, gas or broth. This reactor seems to be useful for living immobilized cells where oxygen supply is necessary.

In previous studies on application of FBR for intracellular lipase production, Nakashima et al. (1989) utilized a circulating bed fermentor (CBF) in immobilized cell culture of *Rhizopus chinensis*. In the CBF, air is introduced through the lower retaining plate, and good circulation of particles is achieved at a comparatively low aeration rate. Thus, the hydrodynamic stress around particles in the CBF would be lower than that in other types of reactor. As a consequence of its advantages, this culture system could effectively produce large amounts of immobilized cells with high lipase activity.

Figure 7

Diagram of air-lift bioreactor for preparation of fungus whole-cell biocatalysts in biodiesel production.
Air is introduced from the bottom and a good circulation is obtained through the draft tube. Cells become well immobilized within BSPs as a natural consequence of their growth during cultivation.



Oda et al. (2005) investigated the scale-up cultivation of lipase-producing *Rhizopus oryzae* cells immobilized within polyurethane foam biomass support particles (BSPs). As can be seen in Figure 7, a good circulation of BSP-immobilized cells in culture medium was obtained by the use of air-lift bioreactor (20-l) with a draft tube, which has a high oxygen transfer rate (Fukuda et al., 1979). As to an advantage of this system compared with flask cultivation, they found that a higher methanolysis activity and almost same cell density within BSPs could be achieved in a short cultivation time, presumably because of a good oxygen supply by aeration. Thus, the whole-cell biocatalysts possessing a high methanolysis activity obtained in this process seems to be useful for industrial biodiesel production.

The present study in Part III investigated the scale-up methanolysis using whole-cell biocatalysts obtained by the air-lift bioreactor. A packed bed reactor (PBR), which is one of the fixed bed reactors, was used for methanolysis. A PBR has the advantages of simplicity of operation and high reaction rates. The catalysts packed in this reactor have high specific interfacial areas of solid-liquid contact. Moreover, when a recyclable system is used, the reactor can be operated at a high fluid velocity by recycling of the reaction mixture, which enhances bulk mass transfer coefficients between the substrate and the cell surface, and also achieves a good mixing of the reaction mixture. In the present study, methanolysis was carried out using a column reactor packed with dried *R. oryzae* cells immobilized within BSPs. Control of the reaction conditions enabled to obtain a high conversion of methyl esters and a higher lipase stability.

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SYNOPSIS

Part I
Lipase localization in fungus whole-cell biocatalysts

Chapter 1 Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles

The present study in Part I reports the intracellular lipase localization in filamentous fungi for use as whole-cell biocatalyst. In the first study described in Chapter 1, the lipase localization of *Rhizopus oryzae* cells was determined to identify the lipase responsible for the methanolysis activity of fungus whole-cell biocatalysts. Western blot analysis showed that *R. oryzae* cells produce two types of lipase with different molecular masses of 34 and 31 kDa; the former (ROL34) was localized in the cell wall, while the latter (ROL31) was mainly bound to the cell membrane. It was found that cell immobilization within reticulated polyurethane foam biomass support particles strongly inhibits the secretion of membrane-bound lipase into the culture medium. An investigation of the relationship between ROL34 and ROL31 suggested that ROL31 originates from the cleavage of a 28-amino-acid residue at the N-terminus of ROL34. It thus seems likely that the variation in lipase localization is due to the difference in post-translational proteolysis of the lipase precursor. The addition of olive oil to the culture medium led to the retention of increased amounts of lipase within the cell. This phenomenon was further confirmed by an immunofluorescence labeling of hyphal cells. When cells were cultivated with various substrate-related compounds such as olive oil and oleic acid, the intracellular methanolysis activity strongly correlated with the relative amounts of the membrane-bound lipase, which suggests that ROL31 localized in the membrane plays a crucial role in the methanolysis activity of *R. oryzae* cells.

Chapter 2 Visualization of secretory pathway using ROL-GFP fusion proteins expressed in *Aspergillus oryzae*

The present study in Chapter 2 investigated the expression and *in vivo* visualization of *Rhizopus oryzae* lipase (ROL) using *Aspergillus oryzae* as a host. A precursor of ProROL fused to the secretion signal was successfully expressed, processed and secreted into the culture medium to produce the lipase with an additional 28-amino-acid residue at N-terminus of the mature region (N28). Expression of the N-terminally truncated forms of ROL suggested the significant role of the N28 sequence in the secretory process. These secretory processes were visualized *in vivo* by using ROL-GFP fusion proteins. In the cells producing ROL with the N28 sequence, fluorescence, which initially formed reticular networks, was predominantly located in the dot-like organelles, hyphal septa, and the cell wall in the later period of cultivation. It was therefore suggested that ROL-GFP was successfully secreted into the culture medium through the typical secretory process in eukaryotic cells and that a basic visualization of the secretory process of ROL was established in *A. oryzae*. Interestingly, the expression of mature ROL fused to GFP induced fluorescence accumulation without its translocation into ER, indicating the crucial role of the N28 sequence in protein transport. To further investigate the role of the N28 sequence, this sequence was fused directly to GFP, which is originally a cytoplasmic protein. Regardless of whether the secretion signal is present or not, GFP without the N28 sequence was distributed evenly to the hyphal cytoplasm, whereas the presence of this sequence between the secretion signal and GFP induced the protein translocation into ER, and consequently resulted in enhanced secretion of GFP. The N28 sequence fused to GFP was efficiently cleaved by post-translational proteolysis to give an original protein in the culture medium. These findings suggest that the N28 sequence facilitates the protein translocation into ER of *A. oryzae* and can be used as a secretion enhancer even in the case of cytoplasmic protein.

Part II
Stabilization of lipase-producing *Rhizopus oryzae* cells for biodiesel-fuel production

Construction of whole-cell biocatalysts with modified membrane lipids for biodiesel-fuel production

Part II discusses the improvement of biodiesel-fuel production catalyzed by lipase-producing *Rhizopus oryzae* cells. The present study investigated the effect of cell-membrane fatty acid composition on biodiesel-fuel production in order to stabilize the lipase activity of *R. oryzae* cells as whole-cell biocatalyst. The fatty acid composition of the cell membrane was easily controllable by addition of various fatty acids to the culture medium. It seemed likely that these exogenous fatty acids were incorporated intactly into the cellular lipids during cultivation even when not originally present in the cells. Oleic or linoleic acid-enriched cells showed higher initial methanolysis activity than saturated fatty acid-enriched cells, among which palmitic acid-enriched cells exhibited significantly greater enzymatic stability than unsaturated fatty acid-enriched cells. It was assumed that fatty acids significantly affect the permeability and rigidity of the cell membrane, and that higher permeability and rigidity lead to increases in methanolysis activity and enzymatic stability, respectively.

When the optimal fatty acid ratio of 0.67, indicated by R_f [= oleic acid/ (oleic acid + palmitic acid)], was adopted for repeated methanolysis reactions, both methanolysis activity and enzymatic stability were maintained at significantly elevated levels, with methyl ester content of around 55% even in the tenth batch cycles. These findings indicate that *R. oryzae* cells possessing high methanolysis activity and stability can be obtained by controlling the fatty acid composition of the cell membrane, and used as whole-cell biocatalyst for practical biodiesel-fuel production.

Part III
Large-scale production of biodiesel using fungus whole-cell biocatalysts

Biodiesel-fuel production using a packed-bed reactor with lipase-producing *Rhizopus oryzae* cells

The present study in Part III developed a system of methanolysis using a packed-bed reactor with lipase-producing *Rhizopus oryzae* cells, that were immobilized within biomass support particles (BSPs) during batch cultivation in a 20-l air-lift bioreactor. The cuboidal BSPs with high interfacial area of cell-substrate contact were advantageous for use as whole-cell biocatalyst packed in a limited space. Emulsification of the reaction mixture containing oils and water improved the reaction rate in continuous-flow mathanolysis. When the flow rate varied in a range from 5 to 55 l/h, higher reaction rate and final methyl ester content in the first cycle were obtained with increasing flow rate. In the fifth cycle, however, the flow rate of 25 l/h showed the highest production level of methyl esters. It was assumed that a fluid-shear force at high flow rate caused the cell exfoliation from BSPs, while inefficient mixing of the reaction mixture at low flow rate allowed the BSPs to be covered with the hydrophilic layer containing a high concentration of methanol, thereby leading to a significant decrease in lipase activity. Continuous-flow methanolysis at 25 l/h achieved a high methyl ester content of over 90% in the first cycle, and maintained a high value of around 80% even after ten cycles. This production level was much higher than that in batch reaction so far reported by our group, suggesting that the packed-bed reactor with immobilized *R. oryzae* cells is greatly advantageous for repeated methanolysis in terms of protecting the cells from a physical damage and an excess amount of methanol. Consequently, the process presented here is considered to be promising for industrial biodiesel-fuel production.

Part I

Lipase localization in fungus whole-cell biocatalysts

Chapter 1 Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze a variety of reactions such as hydrolysis, transesterification, and ester synthesis. Owing to their catalytic versatility, lipases have received considerable attention with a view to biotechnological applications in a wide range of processes (Jaeger and Eggert, 2002). Filamentous fungi have a great potential to produce many types of lipase extracellularly, which are generally used as purified enzymes. However, many of the practical applications of such fungal lipases are limited by economic disadvantages caused by complex purification procedures and instability (Fukuda et al., 2001).

The direct use of lipase-producing fungal cells immobilized within porous biomass support particles (BSPs) as whole-cell biocatalysts represents an attractive process for creating new applications, particularly in the bulk production of commodity-type products such as biodiesel and polyesters, because it requires no purification or further immobilization process (Atkinson et al., 1979). In previous studies on interesterification processes for fats and oils, Nakashima et al. (1988) used acetone-dried *Rhizopus chinensis* cells immobilized within polyurethane foam BSPs as whole-cell biocatalysts and found that cell immobilization and the addition of oils to the culture medium enhance intracellular lipase production. A study by our group showed that immobilized *Rhizopus oryzae* cells efficiently catalyze the methanolysis of plant oils, the products of which can be used as biodiesel fuel (Ban et al., 2001).

In efforts to develop whole-cell biocatalysts possessing a high intracellular methanolysis activity, much attention has been paid to intracellular lipase production and the accumulation of large amounts of lipases inside the cells (Nakashima et al., 1989; Ban et al., 2001). A characterization of intracellular lipases has also been performed and suggested that filamentous fungi immobilized within BSPs produce lipase isoforms inside the cells

(Adamczak and Bednarski, 2004). However, the localization of the fungal lipases responsible for methanolysis activity within mycelial cells remains unknown. Therefore, in the present study, we investigated lipase localization in *R. oryzae* cells to identify the lipase responsible for the methanolysis activity of fungus whole-cell biocatalysts.

Materials and methods

Microorganism and media

All experiments were carried out using *Rhizopus oryzae* IFO 4697, which produces a 1,3-position-specific lipase. A lyophilized culture of IFO4697 was obtained from the Institute for Fermentation, Osaka (IFO, Osaka, Japan). It has been deposited at the NITE Biological Resource Center (NBRC, Chiba, Japan), as *Rhizopus oryzae* NBRC4697. Per liter of distilled water, the basal medium contained: polypeptone 70 g; $NaNO_3$ 1.0 g; KH_2PO_4 1.0 g; and $MgSO_4 \cdot 7H_2O$ 0.5 g. Its pH was initially adjusted to 5.6. Various oils and fatty acids were added to the medium at 30 g/l.

Sakaguchi flasks (500-ml) containing 100 ml of the basal medium were sterilized by autoclaving and aseptically inoculated with spores from a fresh agar slant using 4% potato dextrose agar. These flasks were incubated at 30°C for 24-120 h on a reciprocal shaker (150 oscillations/min; amplitude 70 mm). When the cells were immobilized, 150 BSPs were added to each flask before autoclaving. The BSPs used for cell immobilization were 6-mm cubes of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch. The cultivated cells were separated from the culture broth by filtration, washed with tap water for 1 min and with acetone for 5 min to remove adherent fatty acids, and dried at room temperature for more than 48 h before use in subsequent experiments. Biomass in suspension culture was determined by measuring dry cell weight, whereas immobilized biomass concentration within a BSP was measured as described previously (Ban et al., 2001).

Lipase activity assay

Lipase activity was determined by hydrolysis and methanolysis reactions. The composition of the reaction mixtures was as follows: for hydrolysis: olive oil 2.0 g, 0.1 M acetate buffer (pH 5.6) 9.0 ml, and 0.05 M $CaCl_2$ 1.0 ml were added to a 50-ml screw-cap bottle subject to stirring in a water bath (30°C, 250 rpm); for methanolysis: soybean oil 1.93 g, 0.1 M phosphate buffer (pH 6.8) 0.3 ml, and methanol 0.07 g were added to a 30-ml screw-cap bottle on a reciprocal shaker (30°C, 150 oscillations/min). Both reactions were initiated by the addition of either dried cells (50 mg of suspension cells or 10 BSPs) or 1 ml of culture broth. After 10 min, the hydrolysis was terminated by the addition of 40 ml of 99.5% ethanol followed by free fatty acid titration with 0.1 M NaOH. The methyl esters produced by 2.5-h methanolysis were quantified using a GC-18A gas chromatograph (Shimadzu Co., Kyoto, Japan) as reported previously (Ban et al., 2001). The hydrolysis and methanolysis activities that respectively liberate 1 μmol of fatty acids and methyl esters per min were defined as 1 unit (U).

Analysis of lipase localization

Five milligrams of dried cells was frozen at –84°C overnight, crushed using an SK-Mill (Funakoshi Co. Ltd., Tokyo, Japan), and suspended in 300 μl of 20 mM Tris-HCl buffer (pH 7.5). After centrifugation at 12,000 rpm for 5 min, the supernatant was collected as a cytoplasmic fraction. The precipitate was treated by shaking with 300 μl of 1% (w/v) Triton X-100 solution at 30°C for 20 h to release membrane-bound proteins into the supernatant. Cell-wall proteins were then prepared by incubating the precipitate at 95°C for 5 min in 300 μl of Tris-HCl buffer supplemented with 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol.

The proteins in each fraction were electrophoresed in 12.5% SDS–polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Millipore Co., Boston, MA, USA) for 1 h at 2 mA/cm^2 and room temperature. After blocking with 5% skimmed milk, the

membrane was allowed to react with primary rabbit anti-ROL IgG (Takahashi et al., 1998) and then with a secondary alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega Co., Madison, WI, USA). Lipase was then detected by staining the membrane with nitroblue tetrazolium (NBT) (Promega) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega) according to the protocol specified by the supplier. The membrane was scanned on a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA), and then the intensity of the lipase bands was calculated using Quantity One (Bio-Rad). The N-terminal sequence of the lipase blotted onto the membrane was determined by automated Edman's degradation using a protein sequencer (Model 492, Applied Biosystems, Tokyo, Japan).

Immunofluorescence detection of lipase

Immunofluorescence labeling was performed as follows. The cultivated cells were washed twice with 50 mM potassium phosphate buffer containing 150 mM sodium chloride (PBS, pH 7.4) and incubated in a blocking solution (PBS with 1% bovine serum albumin) at 4°C for 1 h to prevent nonspecific binding. The cells were then incubated with rabbit anti-ROL IgG in PBS at 4°C overnight, washed with PBS, and labeled at 4°C for 2 h using goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR, USA) at a dilution of 1:200. The cells were then washed with PBS twice and observed under a fluorescence microscope.

Results and discussion

Comparison of lipase activity between suspension and immobilized cells

Table 1 shows the cell growth and lipase activity of *R. oryzae* cells cultivated in suspension and immobilized cell cultures. Although the cell growth followed a similar profile, cell immobilization resulted in a significant difference in lipase activity. When the cells were cultivated in suspension, both the intracellular methanolysis and hydrolysis activities

decreased sharply with increasing cultivation time, while the extracellular hydrolysis activity remained relatively high throughout. In contrast, the immobilized cells showed much higher intracellular lipase activity than the suspension cells in both methanolysis and hydrolysis and maintained significantly higher values even in the later period of cultivation. Furthermore, the extracellular lipase activity of immobilized cells was only approximately half that of suspension cells.

In *Rhizopus chinensis* cells, Nakashima et al. (1990) reported that cell aggregation followed by immobilization within BSPs is a trigger for enhancement of intracellular lipase production. A morphological change in *R. oryzae* cells thus also appears to be responsible for the increase in intracellular lipase activity. Given the low extracellular lipase activity of the immobilized cells, cell immobilization does appear to be effective in inhibiting lipase secretion.

Table 1 Comparison of growth and lipase activities between suspension and immobilized cells.

Cultivation time (h)	Cell concentration[a]		Intracellular methanolysis activity[d] ($\times 10^2$U/mg)		Intracellular hydrolysis activity[e] ($\times 10^2$U/mg)		Extracellular hydrolysis activity (U/ml)	
	S[b] (g/l)	I[c] (mg/BSP)	S	I	S	I	S	I
24	1.58	1.36	2.93	7.24	16.8	87.6	18.7	8.02
48	3.06	2.76	0.998	2.88	14.6	51.0	13.3	7.92
72	4.88	2.90	0.620	3.08	10.2	32.3	14.5	7.80
96	5.76	3.20	0.285	2.01	6.96	20.3	15.8	8.30
120	5.86	3.41	0.307	1.81	4.36	19.3	11.6	5.56

Cells were cultivated by suspension and immobilized cultures.

[a] Dry cell weight was measured.

[b] Cells cultivated in suspension culture.

[c] Cells cultivated in immobilized culture.

[d] Intracellular methanolysis activity divided by dry cell weight.

[e] Intracellular hydrolysis activity divided by dry cell weight.

Lipase localization in suspension and immobilized cells

To determine lipase localization in *R. oryzae* cells, Western blot analysis was performed. As can be seen in Figure 1, *R. oryzae* cells produced mainly two types of lipase with different molecular masses of 34 and 31 kDa. Inside the cells, the 34-kDa lipase (ROL34) was bound to the cell wall and the 31-kDa lipase (ROL31) to the cell wall or membrane. Lipase in the cytoplasmic fraction was difficult to detect because of its small amount (data not shown). It should be noted that in the suspension cells the amount of membrane-bound lipase decreased sharply with cultivation time (Figure 1A), while in the immobilized cells large amounts of lipase remained even in the later period of cultivation (Figure 1B). It was also found that cell immobilization strongly inhibits the secretion of ROL31 into the culture medium.

Many researchers have reported that filamentous fungi tend to allow their lipases to localize mainly in the cell wall (Toskueva et al., 1988; Hoshino et al., 1991; Abbadi et al., 1995). However, Davranov et al. (1983) found that the fungus *Oospora lactis* produces two different lipases, one of which (43 kDa) is localized in the periplasmic space and can be released into the medium, while the other (40 kDa) is tightly bound to the membrane and can be solubilized by detergent treatment. Although the properties of the lipases have been thoroughly discussed, the mechanism of such a variation in lipase localization remains unknown. Under the conditions employed in this study, *R. oryzae* cells showed a lipase localization similar to that of *O. lactis*, although their profiles changed with growth and cell morphology. It was speculated that the variation in lipase localization with molecular mass is due to the difference in the amino acid sequence of whole molecules or to an additional peptide segment (about 3 kDa) in ROL31. The reason is discussed elsewhere in this report.

In fungal fermentation, it has been widely reported that cell morphology greatly affects the productivity of antibiotics, organic acids, and enzymes (Papagianni, 2004) and it has been demonstrated that suspension cells are favorable for enzyme secretion (Mackenzie et al., 1994; Papagianni and Moo-Yong, 2002). Although the relationship between cell morphology and enzyme secretion depends on fungal strain and enzyme type, cell

immobilization that induces pellet formation was found to strongly inhibit the secretion of *R. oryzae* lipase, particularly from the membrane.

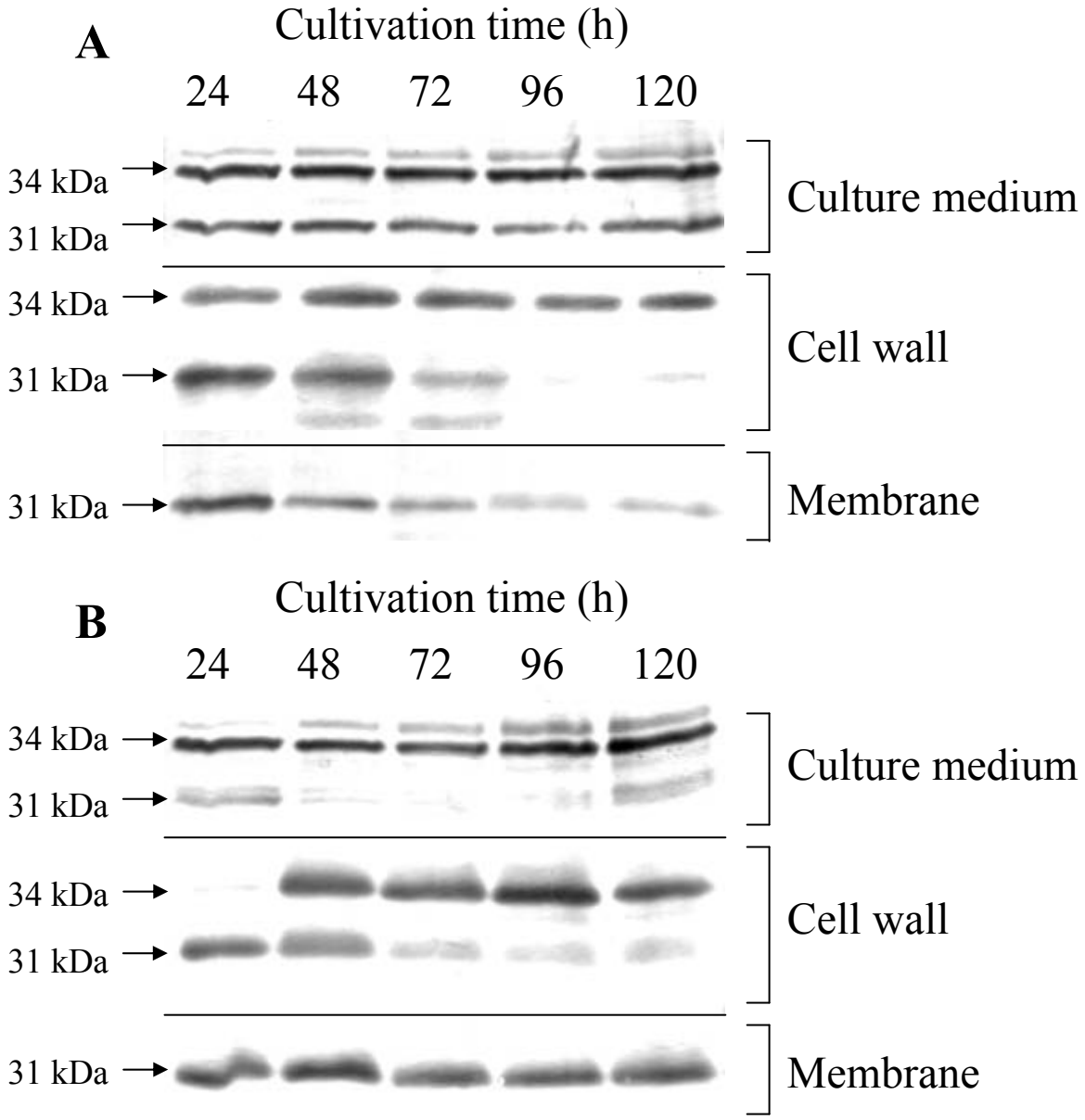

Figure 1 Western blot analysis of *Rhizopus oryzae* lipase extracted from culture medium, cell wall, and membrane. *R. oryzae* cells were cultivated in suspension (A) and immobilized (B) cell culture. The basal medium without oils and fatty acids was used for cultivation. Fifteen μl of the lipase solution in each cellular fraction was subjected to SDS-PAGE electrophoresis.

Relationship between ROL34 and ROL31

When ROL34 was incubated at 4°C, we found that it gradually shifted to ROL31 over a few days (Figure 2). The small amounts of serine proteases present in the lipase solution are likely to be responsible for this phenomenon, as the shift was strongly inhibited by adding phenylmethylsulfonyl fluoride (PMSF). This result indicates that ROL31 originates from a limited proteolysis of ROL34 by specific serine proteases.

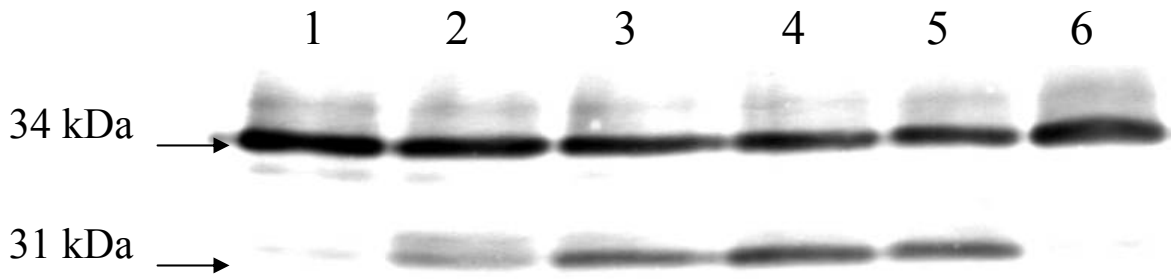

Figure 2 Shift of ROL34 to ROL31. *R. oryzae* lipase (F-AP15, Amano Enzyme Inc.) was dissolved in distilled water at a concentration of 0.33 mg/ml, and then 15 μl of the lipase solution was subjected to SDS-PAGE electrophoresis and detected by Western blotting. Lanes 1-5: *R. oryzae* lipase solution kept at 4°C for 0, 1, 3, 6, and 12 days, respectively; Lane 6: *R. oryzae* lipase solution kept at 4°C for 12 days with phenylmethylsulfonyl fluoride (PMSF).

The N-terminal amino acid sequences of ROL34 and ROL31 were “D-D-N-L-V” and “S-D-G-G-K”, respectively, which clearly indicates the processing site of the lipase precursor. The *R. oryzae* lipase (ROL) precursor consists of a signal sequence (26 amino acids), a prosequence (97 amino acids), and a mature region (269 amino acids), as deduced from the nucleotide sequence (Beer et al., 1996). Of these regions, the prosequence contains a Lys–Arg sequence at amino acids –30 to –29 from its C-terminus, which is a Kexin-like protease recognition site (Ueda et al., 2002). Figure 3 illustrates a schematic representation showing the precursor and processing site of ROL. The N-terminal sequence of ROL34 is identical to the sequence of the precursor between residues 97 and 101 (“D-D-N-L-V”), and that of ROL31 to the N-terminal 5-amino-acid sequence of the mature region (“S-D-G-G-K”). It was thus concluded that the processing at a C-terminal site of the Lys–Arg sequence produces ROL34, while ROL31 is the mature lipase produced by the cleavage of the N-terminal 28-amino-acid residue of ROL34.

Note that the N-terminal 28-amino-acid residue of ROL34 is critical for the determination of the lipase localization. Generally, in eukaryotic cells, the topology of proteins in the membrane is determined at the translocation across the endoplasmic reticulum (ER) membrane after the cleavage of a signal peptide (Schülein, 2004). Once secretory proteins are transported into the ER, they locate in the cell wall and are finally secreted extracellularly. On the other hand, proteins integrated into the ER membrane finally accumulate in the cell membrane as they do in the ER membrane. Thus, the fact that ROL34 is localized hardly in the membrane and mostly in the cell wall led us to hypothesize that the N-terminal 28-amino-acid residue of ROL34 plays an important role in the translocation of ROL across the ER membrane. In studies of COS1 cells, Buscà et al. (1998) reported that the carboxyl terminal region of human lipoprotein lipase is necessary for its exit from ER. The N-terminal region of ROL34 may have a similar role. Further studies of the role of this region in the secretory pathway are under investigation.

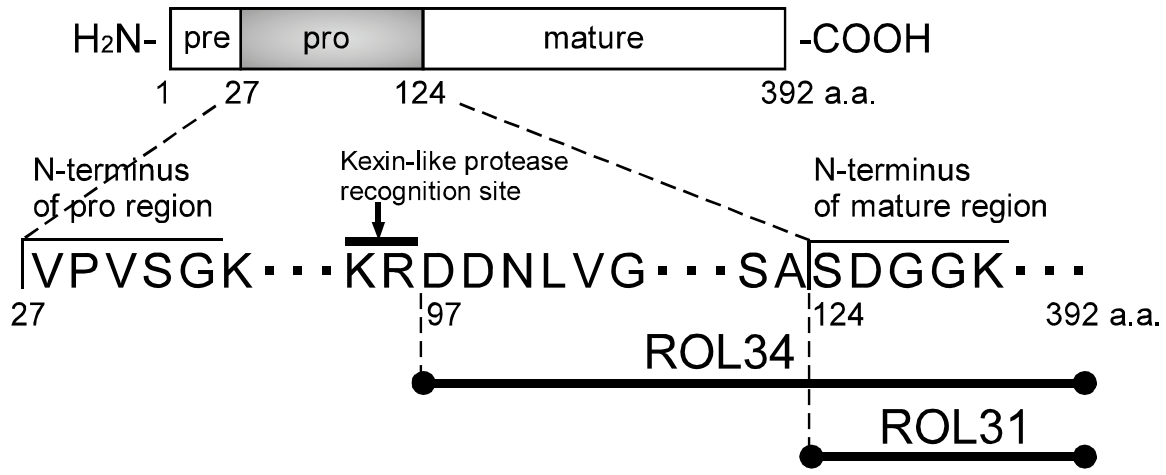

Figure 3 Schematic representation showing precursor and processing site of ROL. After the cleavage of the pre region, the two patterns of processing occur in the pro region. First, the processing at a C-terminal site of the Lys–Arg sequence produces ROL34. Second, the complete cleavage of the pro region gives a mature lipase with a molecular mass of 31 kDa (ROL31). In the secretory pathway, ROL34 is localized in the cell wall and easily secreted into the culture medium, while ROL31 is tightly bound to cell membrane.

Effect of olive oil on lipase activity and localization

It is well known that substrate-related compounds of lipase such as triglycerides and fatty acids act as inducers for lipase production in filamentous fungi (Long et al., 1996). In a previous study utilizing *R. oryzae* cells as whole-cell biocatalysts, Ban et al. (2001) tested various substrate-related compounds such as olive oil, oleic acid, oleyl alcohol, methyl caprate and Tween 80, and found that the addition of olive oil or oleic acid to the culture medium enhances the intracellular methanolysis activity. The effects of olive oil on lipase production and localization in suspension cells were therefore investigated.

As can be seen in Table 2, the presence of olive oil enhanced intracellular lipase production. It is noteworthy however that the extracellular hydrolysis activity was much higher in the absence of olive oil. Since the *R. oryzae* cells used in the study were able to produce lipase constitutively regardless of whether substrate-related compounds were present or not, it seems likely that these compounds are effective in retaining lipase within the cells. Western blot analysis of cells cultivated with olive oil showed that lipase secretion was strongly inhibited and that large amounts of lipase were localized in the cell wall and membrane (Figure 4), suggesting that the lipase-retaining effect caused the increase in intracellular lipase activity.

Table 2 Effect of addition of olive oil on growth and lipase activities.

Cell	Cell concentration[c] (g/l)	Intracellular lipase activity[d] ($\times 10^2$U/mg)		Extracellular hydrolysis activity (U/ml)
		Methanolysis	Hydrolysis	
Control[a]	5.76	0.285	6.96	15.8
Olive oil[b]	11.2	6.20	25.6	1.07

Cells were cultivated by suspension culture for 4 d with and without olive oil.
To investigate the effect of olive oil, the basal medium containing 30 g/l olive oil was used as culture medium.
[a] Cells cultivated in the basal medium without olive oil.
[b] Cells cultivated in the basal medium containing 30 g/l olive oil.
[c] Dry cell weight.
[d] Intracellular methanolysis or hydrolysis activity divided by dry cell weight.

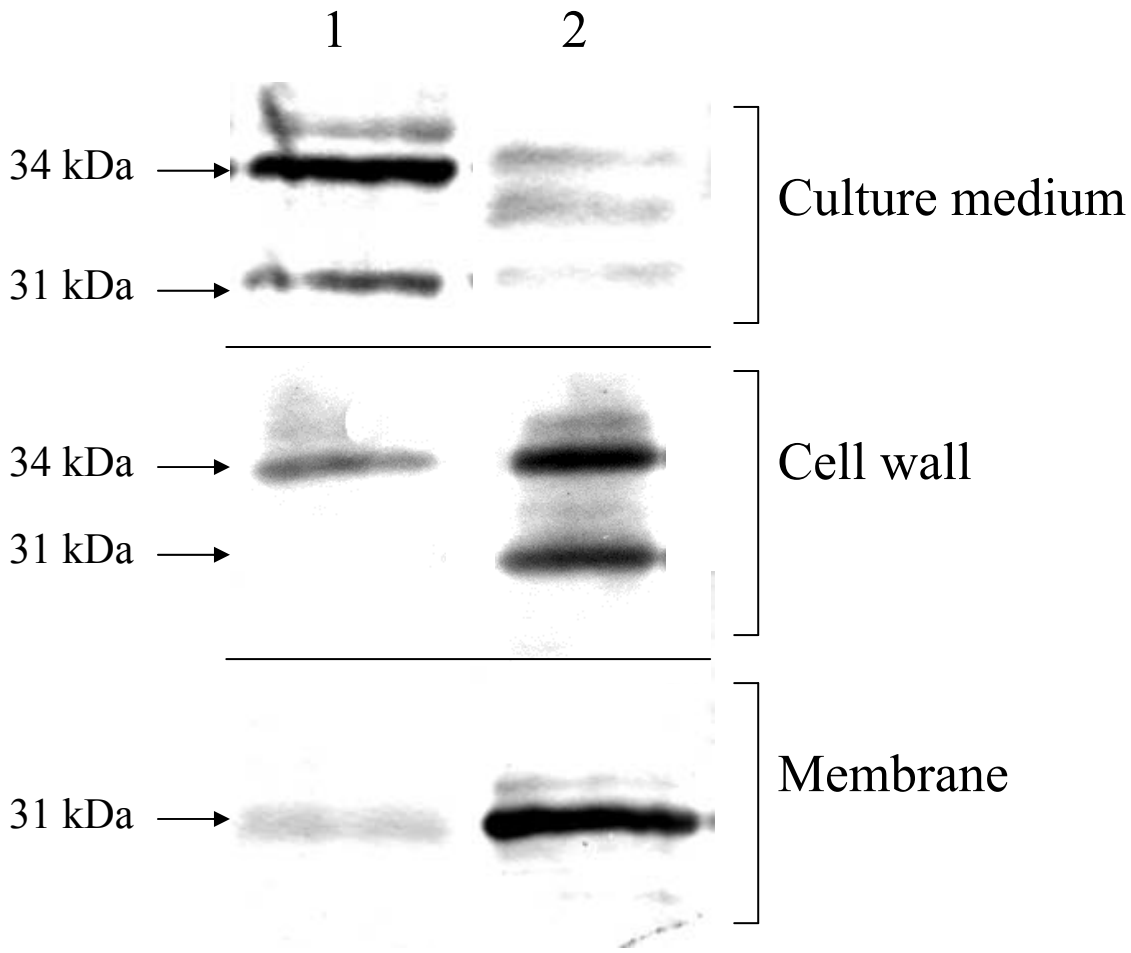

Figure 4 Effect of addition of olive oil on lipase localization. *R. oryzae* cells were cultivated in suspension for 4 days with and without 30 g/l olive oil and the lipases in each fraction were detected by Western blot analysis. Fifteen μl of the lipase solution in each cellular fraction was subjected to SDS-PAGE electrophoresis. Lane 1: cells cultivated without olive oil; lane 2: cells cultivated with 30 g/l olive oil.

Immunofluorescence detection of lipase on cells

To further investigate the effect of olive oil on lipase localization, an immunofluoresce labeling of cells was performed. The green fluorescence of immunostained lipase was clearly observed in the hyphal cell wall of cells cultivated with olive oil (Figure 5). It was thus concluded that substrate-related compounds such as olive oil are effective in retaining lipase within *R. oryzae* cells.

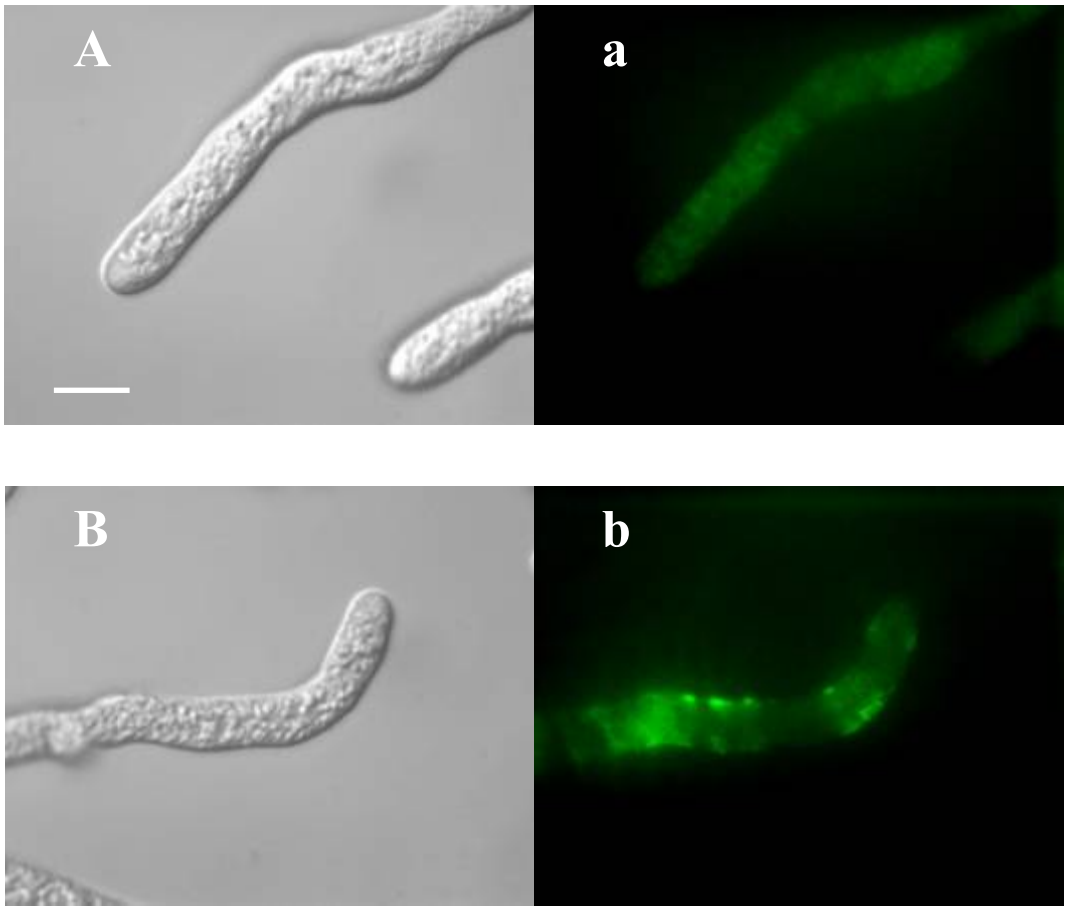

Figure 5 Microscopy of immunofluorescence-labeled *R. oryzae* cells. Differential interference contrast micrographs (panels A and B) and fluorescence micrographs (panels a and b) are shown. Panels A and a: *R. oryzae* cells cultivated without olive oil; panels B and b: *R. oryzae* cells cultivated with olive oil. Bar: 10 μm.

In a previous paper (Hama et al., 2004), we demonstrated that certain lipids can be incorporated intactly into the cell membrane of *R. oryzae* and suggested that the resulting modification of membrane composition alters membrane properties such as permeability and rigidity. Such a difference in membrane function may affect the lipase secretion rate and lipase retention ability of *R. oryzae* cells. Another possible explanation is the modification of lipase by lipids. In studies of *Aspergillus kawachii*, Iwashita (2002) reported that an extracellular soluble polysaccharide, derived from cell-wall components and bound to the β-glucosidase, greatly affects enzyme localization. Since lipids are the main substrates for lipase, it is possible that modification by lipids changes the characteristics of lipase to result in a different localization. Further studies from the view of the lipid–lipase interaction are currently under consideration.

Correlation between methanolysis activity and lipase localization

Various substrate-related compounds influence lipase production in microorganisms (Dalmau et al., 2000). Therefore, it seems likely that studying lipase localization in immobilized *R. oryzae* cells in the presence of these compounds would reveal the correlation of such localization with the methanolysis activity of the whole-cell biocatalyst. As can be seen in Figure 6, lipase secretion into the culture medium was strongly inhibited by the addition of olive oil or oleic acid containing unsaturated fatty acyl chains, whereas in the case of saturated fatty acids such as palmitic and stearic acid, large amounts of lipase were secreted. The solubility of these compounds in the culture medium may have been the cause of their difference in the lipase-retaining effect. In the cell wall, large amounts of ROL34 were localized and there was no significant difference in between the compounds. In the membrane, however, cells cultivated with olive oil or oleic acid retained larger amounts of ROL31. As can be seen in Figure 7, there was a significant correlation between intracellular methanolysis activity and the amount of ROL31 localized in the membrane. Also of interest is the fact that cells cultivated without substrate-related compounds, which retain large amounts of ROL34 in the cell wall, showed the lowest intracellular lipase activity (see lane 1 in Figure 6 and letter

"a" in Figure 7). These findings suggest that ROL31 localized in the membrane plays a crucial role in the methanolysis activity of *R. oryzae* cells.

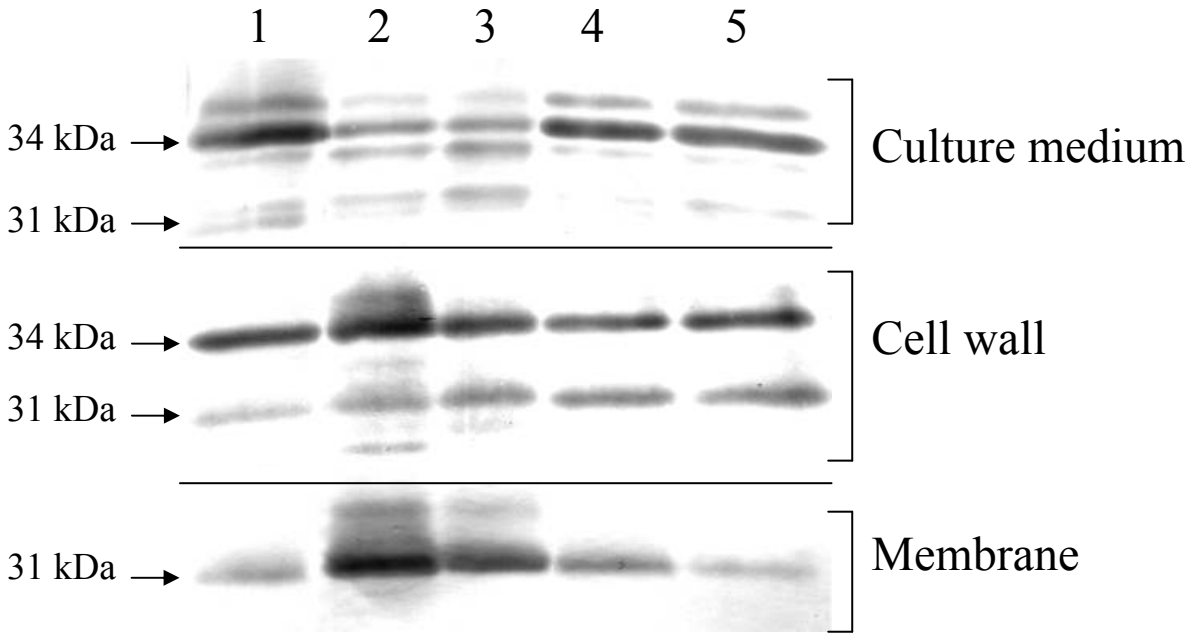

Figure 6 Effects of various substrate-related compounds on lipase localization. *R. oryzae* cells were cultivated by immobilized cell culture (150 BSPs and 100 ml of the basal medium) for 4 days with various substrate-related compounds. Lane 1: cells cultivated without oils or fatty acids; lanes 2-5: cells cultivated with olive oil, oleic acid, palmitic acid, and stearic acid, respectively, at a concentration of 30 g/l. Fifteen μl of the lipase solution in each cellular fraction was subjected to SDS-PAGE electrophoresis.

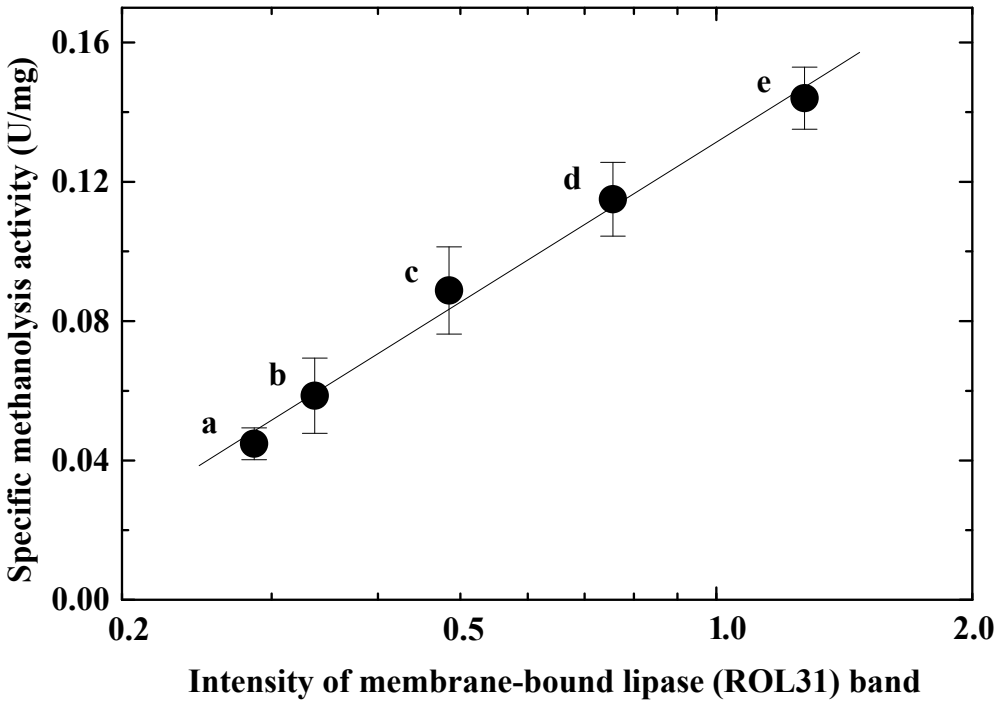

Figure 7 Correlation between specific methanolysis activity and amount of membrane-bound lipase (ROL31). Letters in plots show immobilized cells cultivated for 4 days without oils or fatty acids (a), and with stearic acid (b), palmitic acid (c), oleic acid (d), and olive oil (e), respectively, at 30 g/l. The horizontal axis shows the sum of the intensities of the pixels inside the bands corresponding to each membrane-bound lipase detected by Western blot analysis under the same conditions as those in Figure 6. The specific methanolysis activity of the cells shows a linear relationship with the intensity of the membrane-bound lipase (ROL31) band in a semilogarithmic plot.

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Chapter 2 Visualization of secretory pathway using ROL-GFP fusion proteins expressed in *Aspergillus oryzae*

Introduction

Aspergillus oryzae, which has a long history of the commercial exploitation for fermented food products including sake, soy sauce, and soybean paste, has attracted considerable attention as a host for heterologous protein production due to its high secretory capacity and the status as a GRAS (Generally Regarded As Safe) organism (Christensen et al., 1988; Barbesgaard et al., 1992). Industrial importance of *A. oryzae* has recently accelerated the establishment of its genomics (Machida, 2002) and therefore future demand for this organism is expected to further increase in a wide variety of fields such as fermentation technology and cell biology. However, heterologous protein production in the filamentous fungi has sometimes resulted in low-level production, which presumably depends on bottlenecks in the post-transcriptional pathway rather than on low gene expression (Gouka et al., 1997a). Although a number of strategies that attempted to overcome the bottlenecks have been so far developed (Contreras et al., 1991; Gouka et al., 1997b; Koda et al., 2004), limited knowledge on the secretory pathway in this organism leads to a difficulty in further improving the production levels of heterologous protein. It is thus necessary to gain a better understanding of the molecular basis of the secretion process in *A. oryzae*.

One of the methods to obtain such a detailed knowledge is *in vivo* visualization of the secretion process dynamics. Recently, green fluorescent protein (GFP) has been used in filamentous fungi to provide *in vivo* visualization of specific organelles (Ohneda et al., 2002; Suelmann et al., 1997) and secretion processes (Gordon et al., 2000a; Masai et al., 2003). The studies using these techniques have so far revealed the distribution patterns of the GFP fusions in hyphae under stress which was artificially caused by the addition of a protein transport inhibitor or by cold shock (Gordon et al., 2000b; Khalaj et al., 2001; Masai et al., 2004). However, in spite of the fact that many secretory proteins alter their behaviors following the

post-translational proteolysis, little attention has been paid to *in vivo* visualization of the molecular dynamics in *A. oryzae* under the various types of proteolytic processing.

For the *in vivo* visualization of secretory protein, we focused on the GFP fused to a lipase derived from fungus *Rhizopus oryzae* (ROL), which consists of pre, pro and mature regions in a precursor protein with a unique pattern of intracellular localization. In a previous study using *R. oryzae* cells, we found that ROL with molecular mass value of 34 kDa (ROL34) was localized in the cell wall and easily secreted into culture medium, while ROL31, which originates from the cleavage of the additional 28-amino-acid residue at the N-terminus of ROL34, was mainly bound to the cell membrane (Hama et al., 2006). These variations in intracellular localization may be a key to gain a better understanding of the molecular processes governing the secretory system in *A. oryzae*. The present study therefore investigated the expression of ROL and *in vivo* visualization of ROL-GFP fusion proteins. Here we report an important role of the N-terminal region of ROL in the secretory pathway and suggest its application for the heterologous protein production in *A. oryzae*.

Materials and methods

Strains, media, and growth conditions

The *Escherichia coli* strain used for a genetic manipulation was Novablue (Novagen Inc., Madison, WI, USA) and was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, w/v) containing 0.1 mg/ml of ampicillin. The host strain *A. oryzae* niaD300 is a *niaD* mutant strain derived from the wild-type strain RIB40 (Minetoki et al., 1996) and routinely maintained on a Czapek-Dox (CD) medium [2% glucose, 0.2% $NaNO_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% KCl (w/v), 0.8 M NaCl, 0.001% (v/v) trace elements solution (2% $CuSO_4 \cdot 5H_2O$, 1% $FeSO_4 \cdot 7H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot 7H_2O$, 0.1% $AlCl_3$, w/v), adjusted to pH 5.5] containing 1.5% (w/v) agar. Fungal transformants were selected on a 1.5% (w/v) agar-containing CD-NO_3 medium, in which

$NaNO_3$ replaced $NaNO_2$, and their spores were suspended in 0.01% (w/v) Tween 80. The solution containing spores were used to aseptically inoculate Sakaguchi flasks (500 ml) containing 100 ml of complete medium (2% maltose·H_2O, 5% polypepton, 1% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, w/v, adjusted to pH 6.8). These flasks were incubated at 30°C on a reciprocal shaker (150 oscillations/min; amplitude 50 mm).

Construction of expression plasmids

Constructed plasmids and oligonucleotide primers used in this study are summarized in Figure 1 and Table 1, respectively. The gene encoding the secretion signal derived from triacylglycerol lipase (*tglA*; Toida et al., 2000) was amplified from *A. oryzae* niaD300 chromosomal DNA using two primers tglA-ss-fw-Hind III and tglA-ss-rv-Spe I. The amplified fragment was inserted into the plasmid pNGA142 (Minetoki et al., 1998). The gene encoding ProROL was amplified from *R. oryzae* IFO4697 chromosomal DNA using two primers ProROL-fw-Spe I and ROL-rv-Sph I, then inserted into the plasmid pNGA142 constructed above. The resulting plasmid was named pNGA142ssProROL. These PCR experiments were carried out using *pfu* turbo polymerase (Stratagene, La Jolla, CA, USA). The deletion mutants at the N-terminus of the prosequence of ProROL were amplified by PCR using pNGA142ssProROL as a template and inserted into pNGA142 containing the secretion signal. The gene encoding mature ROL with the additional N-terminal 28-amino-acid residue (N28ROL) was amplified using two primers N28ROL-fw-Spe I and ROL-rv-Sph I, while the gene encoding mature ROL alone (mROL) was prepared using primers mROL-fw-Spe I and ROL-rv-Sph I. The resulting plasmids were named pNGA142ssN28ROL and pNGA142ssmROL, respectively.

The gene encoding green fluorescent protein (GFP) was amplified from pEGFP (Clontech Laboratories, Palo Alto, CA, USA) using two primers GFP-fw-Sph I and GFP-rv-Xba I, then inserted into pNAN8142 (Minetoki et al., 1998). The resulting plasmid was named pNAN8142GFP. For the expression of genes encoding the ROL-GFP fusion protein, the *ROL* genes (ProROL, N28ROL, and mROL) without stop codon were amplified

from plasmids containing the corresponding *ROL* gene as a template. Primers used in this PCR were tglA-ss-Sal I and ROL-rv-Sph I-2. The resulting fragments were then inserted into pNAN8142GFP. These plasmids were named pNAN8142ssProROL::GFP, -ssN28ROL::GFP, and -ssmROL::GFP, respectively. *GFP* gene was amplified from pNAN8142GFP using primers GFP-fw-Spe I and GFP-rv-Sph I, then inserted into the plasmid pNGA142 with the secretion signal. The fragment containing the secretion signal and *GFP* was digested with *Sal* I and *Sph* I, then ligated into pNAN8142. The resulting plasmid was named pNAN8142ssGFP. The DNA fragment containing the secretion signal and the N-terminal 28-amino-acid residue of N28ROL (N28) was amplified from the plasmid pNGA142ssN28ROL using two primers tglA-ss-fw-Sal I and N28-rv-Sph I, then inserted into pNAN8142GFP. The *Sph* I site between N28 and GFP was converted to "AAGCGT" sequence, which encodes Lys-Arg in amino acid, by site-directed mutagenesis using four primers N28-KR-GFP-fw, N28-KR-GFP-rv, tglA-ss-fw-Sal I, and GFP-rv-Xba I. The resulting plasmid was named pNAN8142ssN28GFP. These PCR experiments using plasmid as a template were carried out by KOD Plus polymerase (Toyobo Co. Ltd., Osaka, Japan). Successful constructions of desired plasmids were confirmed by nucleotide sequencing (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems, Tokyo, Japan).

Transformation procedure

Transformation of *E. coli* and *A. oryzae* was carried out according to the methods described by Hanahan (1983) and Gomi et al. (1987), respectively. *A. oryzae* protoplasts were prepared from mycelia grown at 30°C for 48 h using Yatalase (Takara Bio Inc., Shiga, Japan). The constructed plasmids were digested with *Bam* HI prior to transformation.

Lipase activity assay

Lipase activity was determined by hydrolysis reaction. The composition of the reaction mixture was as follows: 2.0 g of olive oil, 9.0 ml of 0.1M acetate buffer (pH 5.6), and 1.0 ml of 0.05M $CaCl_2$ were added to a 50-ml screw-cap bottle subject to stirring in a water

bath (30°C, 250 rpm). Addition of 1.0 ml of culture broth initiated the reaction. After 10 min, hydrolysis was terminated by addition of 40 ml of 99.5% ethanol followed by free fatty acid titration with 0.1M NaOH. The hydrolysis activity which liberates 1 μmol of fatty acids per min was defined as 1 unit (U).

Western blot analysis

A volume of 15 μl of culture broth was electrophoresed in 12.5% SDS–polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Millipore Co., Boston, MA, USA) for 1 h at 2.0 mA/cm^2 and room temperature. After blocking with 5% skimmed milk, the membrane was allowed to react with primary rabbit anti-ROL IgG (Takahashi et al., 1998) or rabbit anti-GFP IgG (Funakoshi Co. Ltd., Tokyo, Japan), and then with secondary alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega Co., Madison, WI, USA). Lipase or GFP was detected by staining the membrane with nitroblue tetrazolium (NBT; Promega) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Promega) according to the protocol specified by the supplier.

Fluorescence assay

A. oryzae mycelia were suspended in 50 mM potassium phosphate buffer containing 150 mM sodium chloride (pH 7.4), placed onto glass slides, covered with a cover slip, and observed under a fluorescence microscope (BZ-8000, KEYENCE Co., Osaka, Japan). A 480 nm/30 nm excitation filter and a 510 nm barrier filter were used for the observation of GFP and GFP fusions. Images (1,360×1,024 pixels) were collected, exported as JPEG files, and processed using Adobe Photoshop.

Fluorescence intensity of culture medium was measured with Wallac 1420 multilabel counter (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). A volume of 50 μl of the culture medium was loaded into a 96 well plate. The excitation wavelength was set at 485 nm and the emission of fluorescence was detected at 535 nm.

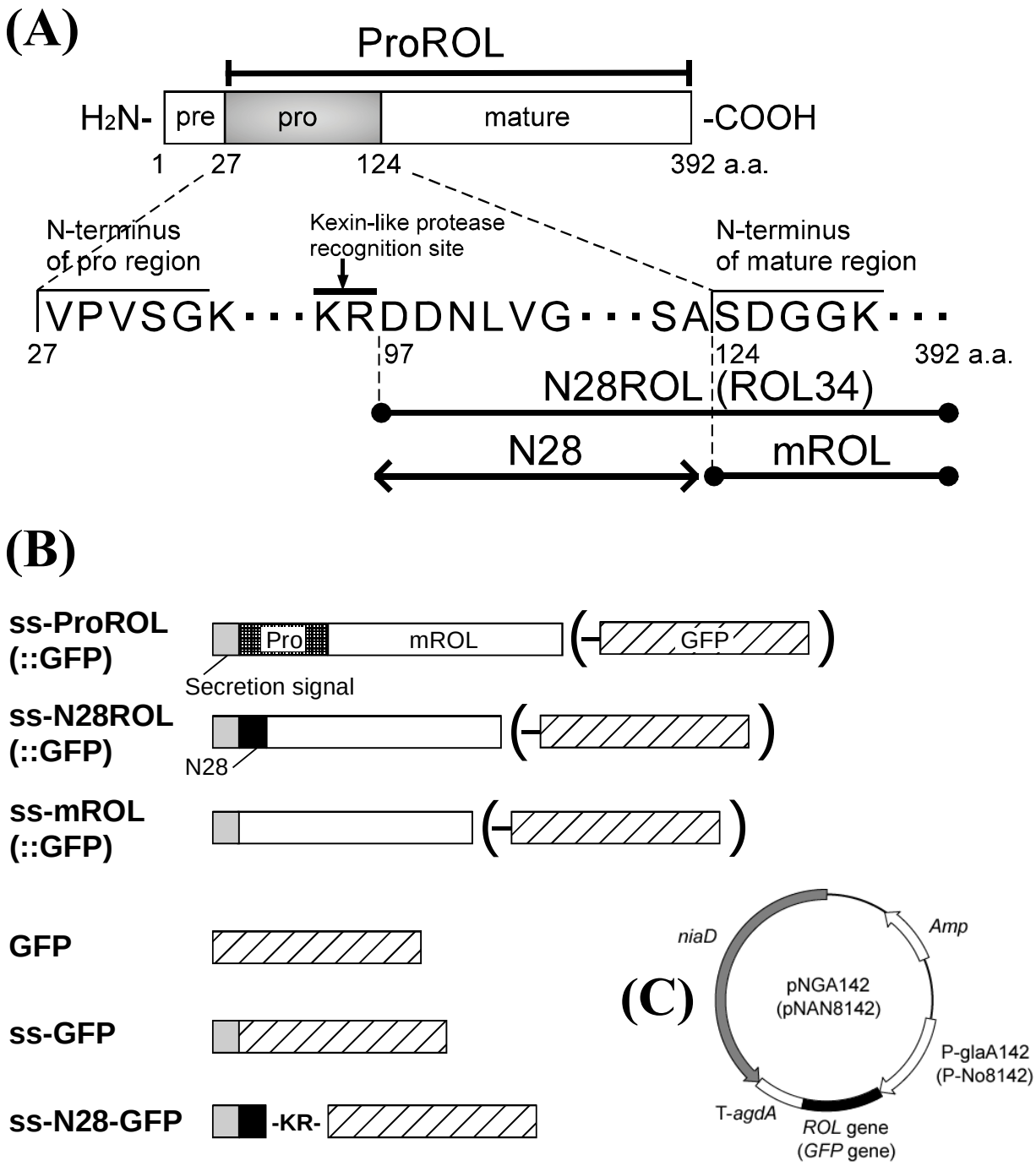

Figure 1 Schematic representation of ROL precursor (A) and gene constructs (B) cloned in the fungal expression vector pNGA142 or pNAN8142 (C). Plasmid constructs were expressed under the control of P-glaA142 or P-No8142 that are the improved promoter regions of the *glaA* (Hata et al., 1992) and No. 8AN (Ozeki et al., 1996) genes, respectively. The *niaD* gene was used as a selectable marker.

Table 1 Primers used in this study.

Name	Sequence (5' to 3')
tglA-ss-fw-Hind III	TAC*AAGCTT* ATGCATCTTGCTATCAAGTCTCTCTTTGTC
tglA-ss-fw-Sal I	ATCG*GTCGAC* ATGCATCTTGCTATCAAGTCTCTCTTT
tglA-ss-rv-Spe I	AG*ACTAGT* TCTCTCAACCAGAGCATTGCTG
ProROL-fw-Spe I	CA*ACTAGT* GTTCCTGTTTCTGGTAAATCTGGATC
ROL-rv-Sph I	GTAA*GCATGC* TTACAAACAGCTTCCTTCGTTGATATC
ROL-rv-Sph I-2	TTTA*GCATGC* CAAACAGCTTCCTTCGTTGATATC
N28ROL-fw-Spe I	CT*ACTAGT* GATGACAACTTGGTTGGTGGCATGAC
mROL-fw-Spe I	AG*ACTAGT* TCTGATGGTGGTAAGGTTGTTGCTGC
N28-rv-Sph I	CGCC*GCATGC* GGCGCTGTTGGTAGAGCTAG
GFP-fw-Spe I	TA*ACTAGT* ATGGTGAGCAAGGGCGAGGAGC
GFP-fw-Sph I	TACC*GCATGC* ATGGTGAGCAAGGGCGAGGAGC
GFP-rv-Sph I	GTAA*GCATGC* TTACTTGTACAGCTCGTCCATG
GFP-rv-Xba I	AA*TCTAGA* TTACTTGTACAGCTCGTCCATG
N28-KR-GFP-fw	CTACCAACAGCGCCAAGCGTATGGTGAGCAAGGG
N28-KR-GFP-rv	CCCTTGCTCACCATACGCTTGGCGCTGTTGGTAG

Sequences in italic indicate the recognition sites of restriction enzyme.

Results

Expression of *Rhizopus oryzae* lipase in *Aspergillus oryzae*

A previous study using *R. oryzae* cells suggested the significant role of the N-terminal region of ROL in intracellular localization (Hama et al., 2006). We thus investigated the expression of N-terminally truncated forms of lipase from *R. oryzae* IFO4697 under the control of P-glaA142 in *A. oryzae* cells. Figure 2A shows the time course of lipase production in the cultures of *A. oryzae* transformants. Lipase activity of the cells integrated with pNGA142ssProROL or -ssN28ROL increased with cultivation time to reach 9.5 and 7.9 U/ml after 96 h, respectively. On the other hand, the cells integrated with pNGA142ssmROL showed no lipase activity throughout the cultivation. As can be seen in Figure 2B, Western blot analysis of these culture broths revealed that the formers (ProROL and N28ROL) produced the lipase with molecular mass value of 34 kDa corresponding to ROL34 in the

previous report (Hama et al., 2006), while almost no lipase band corresponding to ROL was detected when the mature region of ROL (mROL) fused to the secretion signal was expressed in *A. oryzae* cells. These results indicate that ROL was successfully expressed, processed and secreted into the culture medium except for the case of complete deletion of the prosequence, and suggest that the N-terminal 28-amino-acid residue of ROL34 plays a crucial role in the secretion of ROL to culture medium.

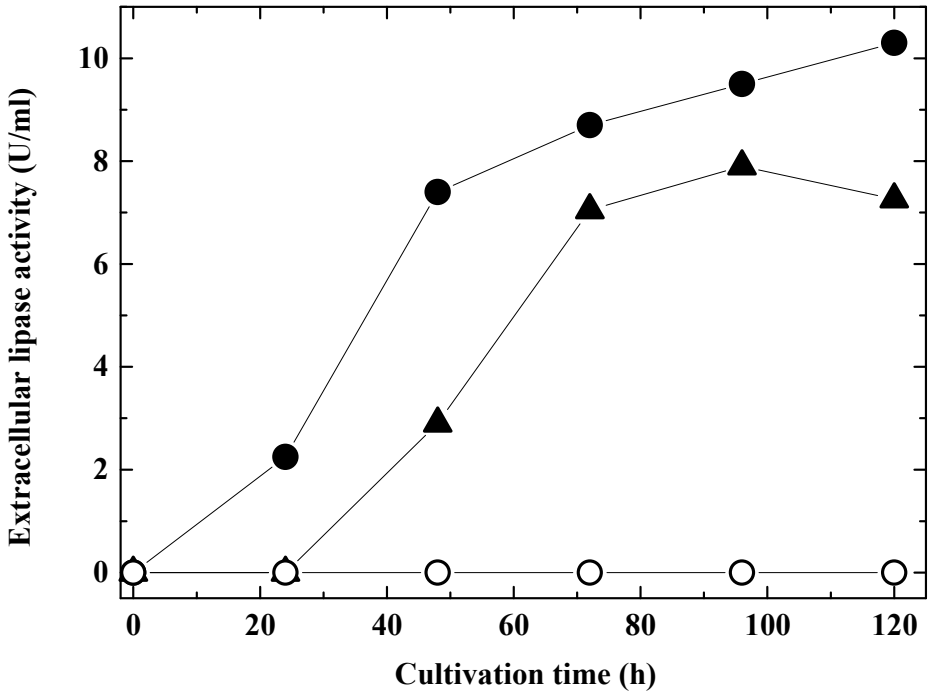

Figure 2A Time course of extracellular lipase activity of *A. oryzae*. The cells were integrated with the plasmids pNGA142ssProROL (circles), -ssN28ROL (triangles) and -ssmROL (open circles), respectively.

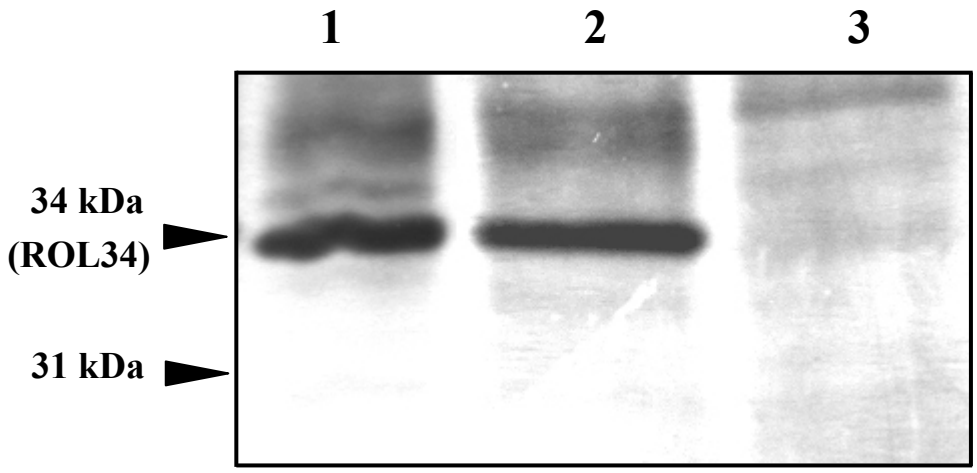

Figure 2B Western blot analysis of extracellular ROL expressed by *A. oryzae*. Lane 1-3: cells integrated with the plasmids pNGA142ssProROL, -ssN28ROL and -ssmROL, respectively. *A. oryzae* transformants were cultivated in complete medium for 96 h. The primary antibody used was a rabbit anti-ROL IgG.

Visualization of ROL in *A. oryzae* hyphae by expression of ROL-GFP fusion protein

To investigate the secretion process of ROL, *in vivo* visualization of ROL was carried out by the use of ROL-GFP fusion protein. The fusion protein with the expected molecular mass value of 61 kDa (34 kDa of ROL and 27 kDa of GFP) was detected in the culture medium, and the secretion profiles were nearly consistent with the data in Figure 2 (data not shown). Figure 3 shows the observations of the fusion proteins in *A. oryzae* hyphae under the fluorescence microscope. Among these three transformants, similar fluorescence patterns were observed between the cells integrated with pNAN8142ssProROL::GFP and -ssN28ROL::GFP. The brighter fluorescence with a reticular network, which probably shows endoplasmic reticulum (ER), was clearly visible in the early period of cultivation (48 h). In 96 h-cultures, a large proportion of the fluorescence was located on the cell walls, while the fluorescence with dot-like structures remained in intracellular regions. In addition, high fluorescence was located at the hyphal septa throughout the cultivation. These fluorescence patterns of the secretion pathway in *Aspergillus* species were similar to that in the studies on glucoamylase-GFP (Gordon et al., 2000) and ribonuclease T_1-GFP (Masai et al., 2003) fusion proteins. The results described above thus indicate that a basic visualization of the secretory process of ROL was established in *A. oryzae*.

In contrast, the expression of mature ROL directly fused to the secretion signal induced significant differences both in the fluorescence patterns and in the fungal morphology (Figure 4). In the initial period of cultivation (24 h), the fluorescence showed many vesicular structures distributed to a wide range of the intracellular regions, while these small vesicles accumulated in the vicinity on the edge of one compartment after 48 h. In the hyphal septa, however, no fluorescence was observed even in the later period of cultivation (96 h). Moreover, the cells grew in highly branched form, which was similar to that of the hyphae treated with a protein transport inhibitor (Khalaj et al., 2001). Thus, expression of the mature ROL seems to be toxic for *A. oryzae* cells. Given little fluorescence with a reticular network showing ER structure, the N-terminal 28-amino-acid residue of ROL appears to play an important role in protein transport into ER of *A. oryzae*.

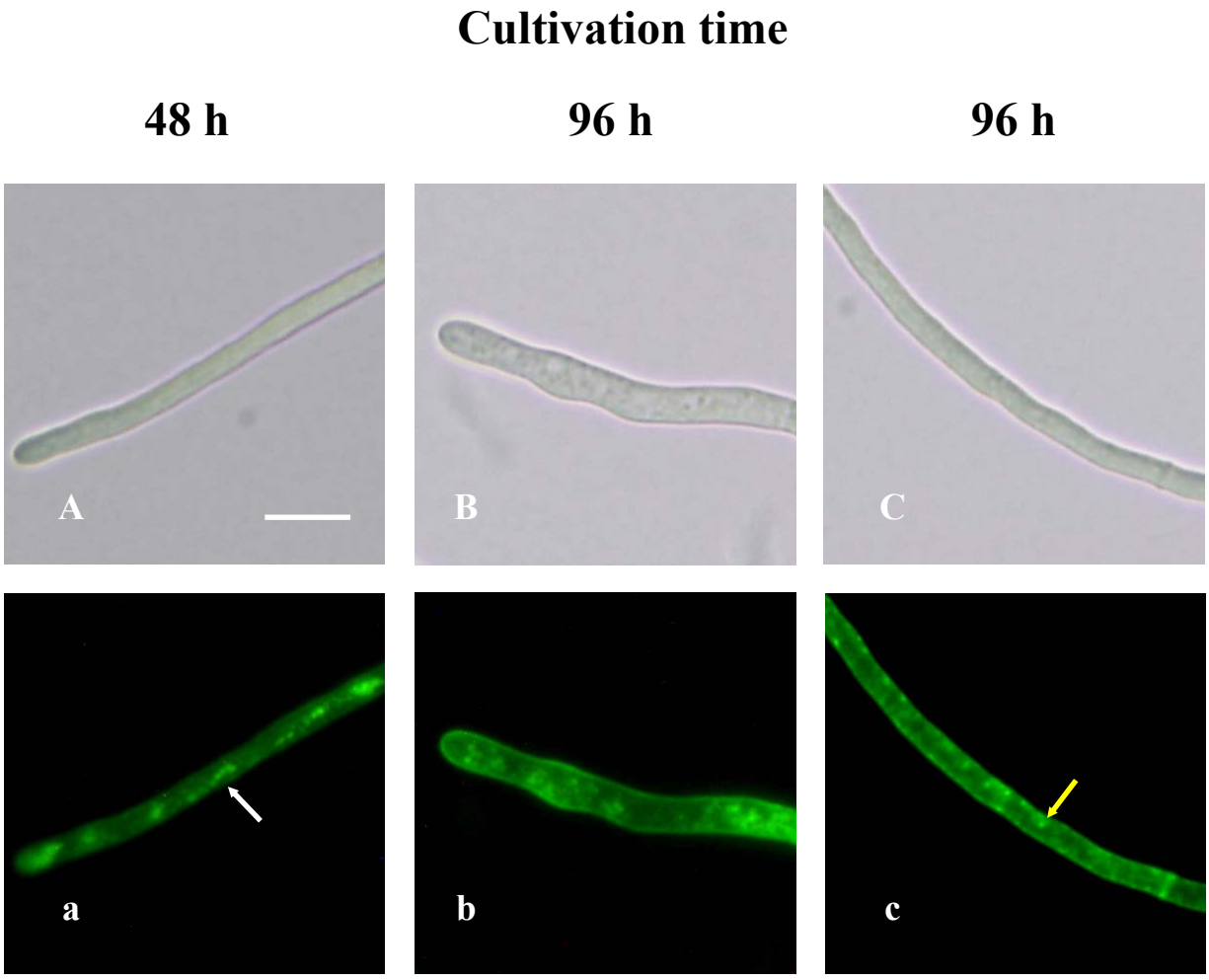

Figure 3 Fluorescence microscopy of *A. oryzae* hyphae integrated with the plasmid pNAN8142ssProROL::GFP. Differential interference contrast micrographs (panels A, B, and C) and fluorescence micrographs (panels a, b, and c) are shown. The white and yellow arrows indicate the ER structure and the dot-like structures, respectively. Bar, 10 μm. Note the bright fluorescence with reticular networks (panel a), in the cell wall (panel b), with the dot-like structures and in the hyphal septa (panel c).

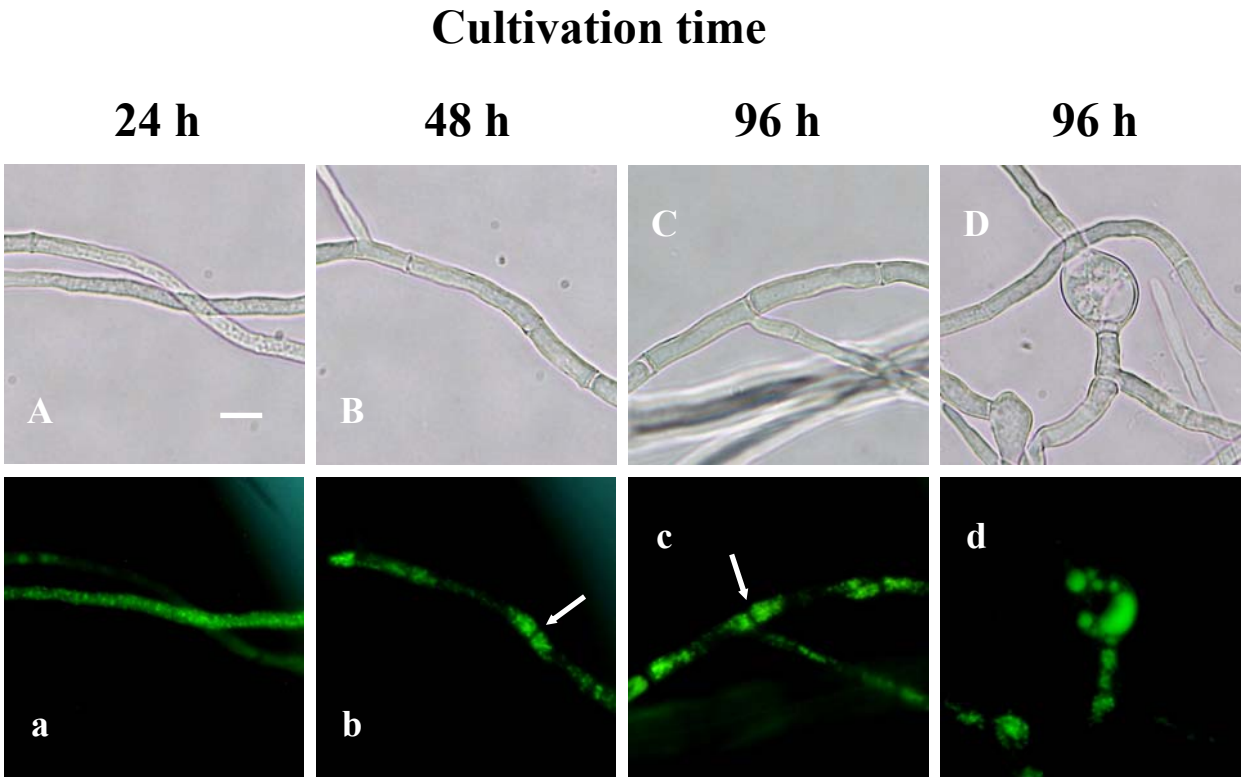

Figure 4 Observation of fungal morphology and fluorescence of *A. oryzae* hyphae expressing the mature ROL fused to GFP. *A. oryzae* integrated with the plasmid pNAN8142ssmROL::GFP was cultivated in complete medium for 24-96 h. Differential interference contrast micrographs (panels A, B, C, and D) and fluorescence micrographs (panels a, b, c, and d) are shown. The white arrow indicates the position of hyphal septa. Bar, 10 μm. Note the fluorescence accumulation around the septa (panels b and c) and the large vacuoles inside the balloon-like structures (panels D and d).

Effect of N-terminal region from ROL on transport of GFP in *A. oryzae*

To further investigate the role of the N-terminal region from ROL in protein secretory pathway, this region was fused directly to GFP, which is originally a cytoplasmic protein (Haseloff, 1999). Figure 5 shows the observations of the cells expressing GFP under the fluorescence microscope. In the case of the cells with GFP alone (Figure 5A), the fluorescence was distributed evenly in the hyphal cytoplasm throughout the cultivation, while the cells producing GFP with the secretion signal (Figure 5B) showed the fluorescence pattern similar to that of the former, though bright fluorescence was visible in the hyphal septa, which is often reported in the studies on the secretory proteins (Gordon et al., 2000a; Masai et al., 2003). GFP accumulated in the cytoplasm continued to emit clear fluorescence even in the later period of cultivation (96 h), indicating that GFP expressed in *A. oryzae* is very stable and not toxic for this organism. In contrast, when the N-terminal 28-amino-acid residue from ROL34 was fused to GFP (Figure 5C), the 48 h-cultured hyphae showed the fluorescence with a reticular network, which probably originates from the localization in ER. A large proportion of such fluorescence was disappeared in the later period of cultivation (96 h) except for hyphal septa. Secretion profiles of GFP were then investigated by measurement of fluorescence intensity and by Western blotting. Figure 6 shows the relative fluorescence intensity of GFP secreted into the culture medium. The cells expressing GFP with the secretion signal showed the slightly higher fluorescence intensity than those with GFP alone. On the other hand, when the N-terminal region from ROL was inserted between the secretion signal and GFP, the fluorescence intensity increased significantly with cultivation time to give approximately 3-fold higher value after 6 day than its absence. Furthermore, as can be seen in Figure 7, the cells secreted the GFP extracellularly with molecular mass value of 27 kDa corresponding to GFP alone, indicating that the N-terminal region from ROL was efficiently cleaved at the C-terminal end of Lys-Arg site by post-translational proteolysis. These findings suggest that the N-terminal region from ROL facilitates the protein translocation into ER and thus effective secretion can be obtained even in the case of cytoplasmic protein.

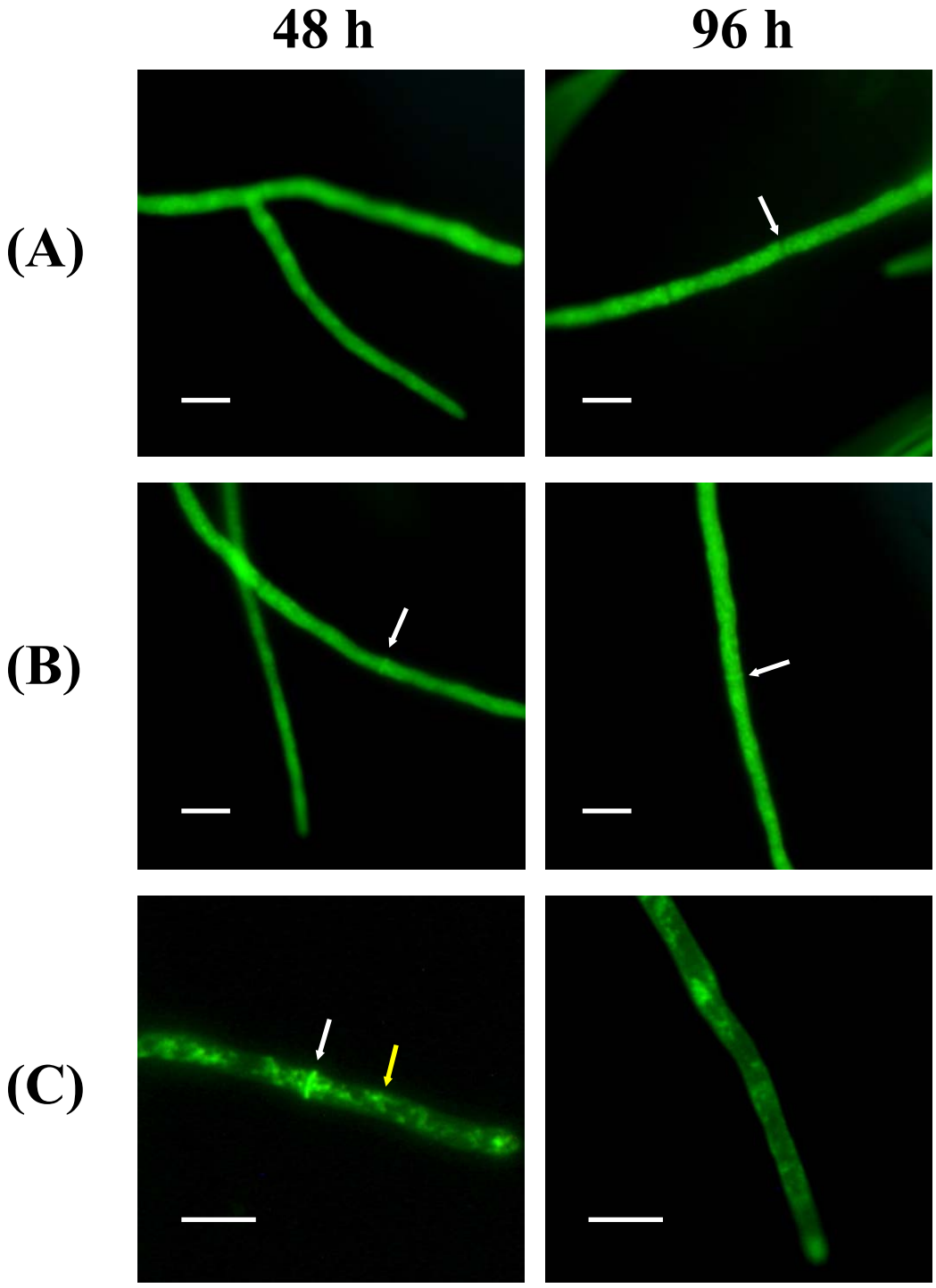

Figure 5 Distribution of GFP expressed in *A. oryzae* hyphae. The plasmid pNAN8142GFP (A), -ssGFP (B), or -ssN28GFP (C) was introduced into *A. oryzae*. Cells were cultivated in complete medium for 48 or 96 h. The white and yellow arrows indicate the position of hyphal septa and the ER structure, respectively. Bar, 10 μm.

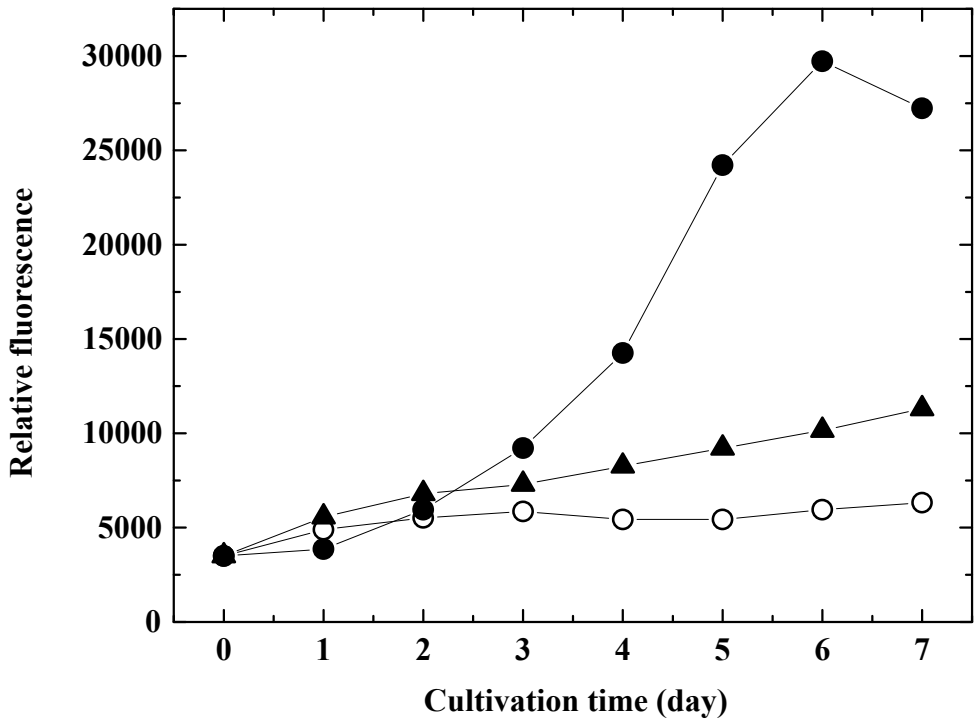

Figure 6 Relative fluorescence of GFP secreted into culture medium. *A. oryzae* cells were integrated with the plasmids pNAN8142GFP (open circles), -ssGFP (triangles), and -ssN28GFP (circles), respectively. Complete medium was used for cultivation.

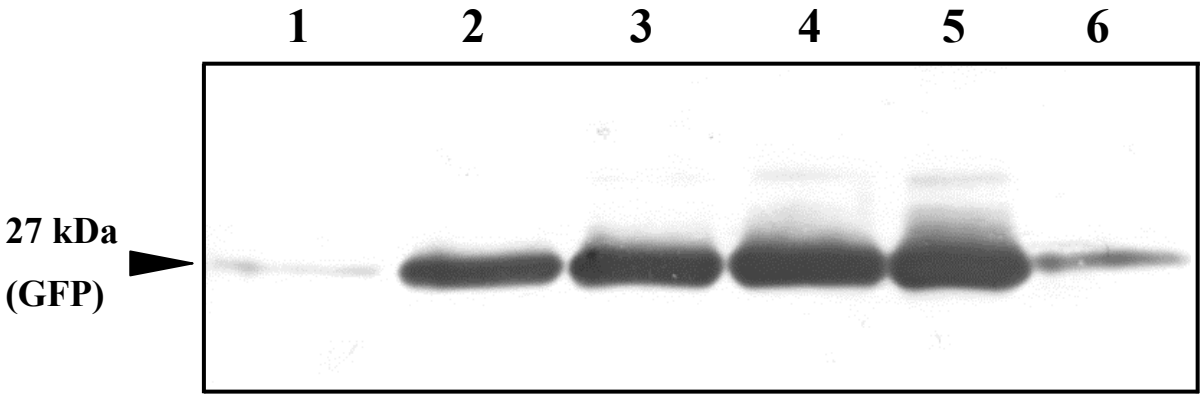

Figure 7 Western blot analysis of GFP secreted into culture medium by *A. oryzae* cells. Lane 1-5: cells integrated with pNAN8142ssN28GFP and cultivated in complete medium for 1, 2, 3, 4 and 5 days, respectively; Lane 6: cells integrated with pNAN8142GFP and cultivated in complete medium for 4 days. The primary antibody used was a rabbit anti-GFP IgG.

Discussion

In the present study, we investigated the expression and *in vivo* visualization of *R. oryzae* lipase (ROL) using *A. oryzae* as a host. The fact that the ROL fused to its prosequence is successfully expressed, processed, and secreted to culture medium (Figure 2) indicates that the expression system employed in this study would be adequate to examine the secretory pathway of *A. oryzae*. Since ROL presents great opportunities in a variety of industrial application such as biodiesel production (Kaieda et al., 1999) and resolution of chiral compounds (Matsumoto et al., 2004), it has been so far expressed in several microorganisms including *Escherichia coli* (Beer et al., 1996), *Saccharomyces cerevisiae* (Takahashi et al., 1998), and *Pichia pastoris* (Minning et al., 1998). Although *Rhizomucor miehei* lipase, which is closely related with ROL (>55% homology), has been already expressed in *A. oryzae* (Huge-Jensen et al., 1989), the present study is the first attempt in the expression of recombinant ROL using *A. oryzae* as a host.

A precursor of the ProROL was secreted in the form of ROL34 with the additional 28-amino-acid residue (N28) to the mature ROL, which is presumably because of KexB-type processing (Mizutani et al., 2004; Hama et al., 2006). Expression of the N-terminally truncated forms of ROL suggested a crucial role of the N28 sequence in the secretory process of ROL (Figure 2). In previous studies on the expression of ROL, it was found that even small amount of the mature active lipase is toxic on translocation in the periplasmic space in *E. coli*, and that the prosequence of ROL modulates the enzyme activity of the mature lipase so as not to damage the host (Beer et al., 1996). Similar results on such functions of the prosequence were obtained in yeast (Takahashi et al., 1998, 2001). It thus seems likely that *A. oryzae* cells make some responses to the toxicity of the mature ROL and that the N28 sequence has an effect of reducing the enzyme toxicity and/or a facilitatory effect of transporting the lipase in this organism. These variations in the lipase expression would contribute to reveal the secretory process in *A. oryzae*.

In vivo visualization of the secretory process in *A. oryzae* by the use of ROL-GFP

fusion proteins allowed us to gain a better understanding of its molecular dynamics. In the cells producing ROL34 fused to the N-terminus of GFP, the fluorescence, which initially formed reticular networks, was predominantly located in the dot-like organelles, septa, and the cell wall in the later period of cultivation (Figure 3). The finding that the structure of ER in *Aspergillus* species forms a reticular network has been so far reported with *A. nidulans* and *A. niger* transformed with an ER-tagged GFP construct (Fernandez-Abalose et al., 1998; Gordon et al., 2000a; Derkx and Madrid, 2001). In addition, since punctate nodes can be seen when Golgi bodies in *Pisolithus tinctorius* hyphae are labeled with a fluorescent probe (Cole et al., 2000), the dot-like organelles observed in Figure 3 are considered to be Golgi bodies of *A. oryzae*. The results described above thus suggest that ROL-GFP was successfully expressed, targeted to the ER, and transported to the Golgi apparatus according to the typical secretory process in eukaryotic cells. It should be also noted that brighter fluorescence was visible in the hyphal tips (panel "a" in Figure 3), supporting the hypothesis that protein secretion takes place at the tips of growing hyphae (Wosten et al., 1991). Moreover, accumulation of the fusion protein at septa was observed (panel "c" in Figure 3), which has been reported in the cases of other secretory protein (Gordon et al., 2000b; Khalaj et al., 2001; Masai et al., 2004). These results therefore indicate that a basic visualization of the secretory process of ROL was successfully established in *A. oryzae*.

Compared to the results described above, the expression of mature ROL fused to GFP induced significant differences in the fluorescence pattern. The fusions accumulated in the vicinity on the edge of hyphal compartment (Figure 4). Furthermore, the cells grew in highly branched form and occasionally showed balloon-like structures, where fluorescence accumulation was visible with large vacuoles (panel "d" in Figure 4). These fungal phenotypes are frequently associated with stress such as addition of protein transport inhibitors (Gordon et al., 2000b; Khalaj et al., 2001; Masai et al., 2004) and disruption of enzymes responsible for the cell integrity (Horiuchi et al., 1999; Fillinger et al., 2001). It thus seems likely that quality-control system of *A. oryzae* cells recognized the toxicity of mature ROL, inhibited its secretory process, and accumulated it for degradation. Given little

fluorescence with ER structures throughout the cultivation, it is speculated that *A. oryzae* cells accumulated the mature ROL without its translocation into the ER lumen, and that the N28 sequence plays an important role in protein transport into the ER of *A. oryzae*.

In order to clarify our hypothesis, the N28 sequence was fused directly to GFP, which is originally a cytoplasmic protein. Regardless of whether the secretion signal is present or not, GFP without the N28 sequence was distributed evenly to the hyphal cytoplasm (Figure 5A and B). Since GFP is certainly a cytoplasmic protein and seemed to be non-toxic for *A. oryzae*, the characteristics of the protein such as folding rate and its own sequence might cause the difficulty in translocation into ER. On the other hand, the presence of the N28 sequence between the secretion signal and GFP induced the protein translocation into the ER (Figure 5C), and consequently resulted in enhanced secretion of GFP (Figure 6). In spite of the fact that GFP does not have significant toxicity for this organism, we were able to observe the facilitatory effect of the N28 sequence on protein secretion similar to that in the ROL. Although we cannot unequivocally exclude the possibility that the N28 sequence reduced the toxicity of protein, it was demonstrated that this sequence facilitates the protein translocation into ER of *A. oryzae* even in the case of cytoplasmic protein.

Insertion of a KexB-cleavage site into the junction between the N28 and GFP resulted in efficient processing from a fusion protein to mature GFP, indicating that an original protein can be finally obtained by post-translational proteolysis. Since the KexB protein is localized in Goldi membranes (Mizutani et al., 2004), it can be assumed that the N28 sequence is cleaved in a Goldi compartment after facilitating the protein translocation into ER. Consequently, these results suggest that the N28 sequence can be used as a secretion enhancer for the heterologous protein production in *A. oryzae*.

A number of studies using different organisms have shown that amino- or carboxy-terminal domains play a crucial role in protein transport (Buscà et al., 1998; Ishisaka et al., 1999; Tani et al., 2004). As a result of their function, some promote a protein secretion and some retain a protein in organelles. To explain these phenomena, many mechanisms have been proposed, including regulation of enzyme activity, facilitation of collect folding, and

interaction with membrane. Our previous study using *R. oryzae* cells (Hama et al., 2006) showed that a precursor of ROL undergoes two types of post-translational processing: mature ROL accumulates in the cell membrane, while ROL with the N28 sequence (ROL34) is localized in the cell wall and easily secreted extracellularly. Since the topology of protein in the membrane is determined during ER insertion (Schülein, 2004), the N28 sequence seems to facilitate the protein translocation across ER membrane and consequently control the protein localization in combination with a secretion signal. In studies of ROL-lipid interaction *in vitro*, Sayari et al. (2005) found that the N28 sequence at N-terminus of mature ROL increases the penetration power of ROL into phospholipid layers. This sequence therefore may contribute to the protein penetration into ER membrane. Our results show that the facilitatory effect of the N28 sequence is not specific for mature ROL, but applicable to other non-secretory proteins such as GFP. In conclusion, the findings presented here suggest that the role of the N28 sequence as a secretion enhancer is useful for the heterologous protein production in *A. oryzae*.

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Part II

Stabilization of lipase-producing *Rhizopus oryzae* cells for biodiesel-fuel production

Construction of whole-cell biocatalysts with modified membrane lipids for biodiesel-fuel production

Introduction

Biodiesel (fatty acid methyl ester), which is derived from triglycerides by transesterification with methanol (i.e. methanolysis), has attracted considerable attention during the past decade as a renewable, biodegradable, and nontoxic fuel. Utilization of lipase as a catalyst for biodiesel-fuel production has great potential compared with chemical methods using alkaline catalyst, since no complex operations are needed either to recover the glycerol or to eliminate the catalyst and salt.

Methanolysis can be carried out using extracellular (Mittelbach, 1990; Nelson et al., 1996; Kaieda et al., 1999; Shimada et al., 1999; Samukawa et al., 2000; Watanabe et al., 2000) or intracellular (Ban et al., 2001, 2002; Matsumoto et al., 2002) lipases, but the former require purification by procedures that may be too complex for practical use and the lipases recovered are generally unstable and expensive. Direct use of intracellular lipase as whole-cell biocatalyst therefore represents an attractive process for practical biodiesel-fuel production, since it requires no purification or immobilization process when prepared using a technique based on porous biomass-support particles (BSPs) (Atkinson et al., 1979).

In earlier methanolysis studies utilizing *Rhizopus oryzae* cells immobilized within BSPs as whole-cell biocatalyst, Ban et al. (2001, 2002) reported that stepwise addition of methanol in the presence of 4–20% water resulted in a peak methyl ester (ME) content in the reaction mixture of 80–90% without organic solvent pretreatment; and that the lipase activity of *R. oryzae* cells treated with 0.1% glutaraldehyde (GA) solution was maintained without significant decrease during six batch cycles, with ME content in each cycle reaching 70–83% within 72 h. From an economic point of view, however, preparation of BSP-immobilized cells without GA-treatment is preferable.

To stabilize the lipase activity of *R. oryzae* cells without GA-treatment, the present

study investigated the effect on methanolysis of *R. oryzae* membrane fatty acid composition, and used modification of the latter to construct a whole-cell biocatalyst with significantly improved stability.

Materials and methods

Microorganism and media

All experiments were carried out using *R. oryzae* IFO 4697. The basal medium contained per liter of distilled water: polypepton 70 g; $NaNO_3$ 1.0 g; KH_2PO_4 1.0 g; and $MgSO_4 \cdot 7H_2O$ 0.5 g. Various fatty acids (FAs) were added to the basal medium at a concentration of 30 g/l. When two FAs were mixed, they were added in such a manner that the total concentration of FAs was 30 g/l. The pH of the medium was initially adjusted to 5.6 and then allowed to follow its natural course.

Preparation of BSP-immobilized cells

Sakaguchi flasks (500-ml) containing 100 ml of the basal medium with BSPs, sterilized by autoclaving, were inoculated by aseptically transferring spores from a fresh agar slant using potato dextrose agar, and incubated at 30°C for 90 h on a reciprocal shaker (150 oscillations/min; amplitude 70 mm). When linoleic acid was added to the basal medium, the acid was separately sterilized by microfiltration using a 0.22 μm pore size filter paper. The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation. Immobilization was effected by placing 150 particles inside a flask together with the pre-sterilized medium. The BSPs used for immobilization were 6-mm cubes of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of over 97% and a pore size of 50 pores per linear inch. BSP-immobilized cells were separated from the culture broth by filtration, washed with tap water for 1 min and with acetone for 5 min, and dried at room temperature for approximately 48 h before use as

methanolysis or hydrolysis catalyst.

The cell concentration within the BSP was determined as follows. Ten particles were taken and washed with acetone several times to remove adherent fatty acids, and then they were dried at 80°C for 24 h. The particles plus dried cells were weighed and treated with an aqueous solution of sodium hypochlorite (approximately 10% v/v) to remove biomass. The cleaned particles were rinsed, dried, and reweighed. The biomass was estimated from the difference between the weights.

Enzyme reaction by BSP-immobilized cells

The methanolysis and hydrolysis reactions were carried out at 30°C in a 30-ml or 50-ml screw-cap bottle with incubation on a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The composition of the reaction mixtures in the batch operations were as follows: for methanolysis: soybean oil 1.93 g, 0.1 M phosphate buffer (pH 6.8) 0.3 ml, and methanol 0.07 g, were added with 10 BSPs to a 30-ml screw-cap bottle; for hydrolysis reaction: same composition as for methanolysis but without methanol. Both mixtures were incubated for 2.5 h.

In repeated methanolysis, the reaction mixture [soybean oil 9.65 g, 0.1 M phosphate buffer (pH 6.8) 1.5 ml, and methanol 0.35 g] was dispensed with 50 BSPs into a 50-ml screw-cap bottle and incubated for 72 h. The molar equivalent of methanol to 9.65 g soybean oil was 0.35 g. To fully convert the oil to its corresponding methyl esters, 0.35 g of methanol was added twice: when the methyl ester content in the reaction mixture reached approximately 30% and 60%. For repeated use, the BSP-immobilized cells were dried at room temperature after washing with tap water and methanolysis was carried out several times with a fresh reaction mixture.

The methyl ester (ME) and free fatty acid (FFA) contents in the reaction mixture were quantified using a GC-18A gas chromatograph (GC) (Shimadzu Co., Kyoto, Japan) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA, USA). Samples (150 μl) were taken from the reaction mixture at specified times and centrifuged to

obtain the upper layer. For GC analysis, 100 μl of the upper layer and 20 μl tricaprylin were precisely measured into a 10-ml bottle, to which a specified amount of anhydrous sodium sulfate as a dehydrating agent and 3.0 ml hexane were added. Tricaprylin served as the internal standard for GC analysis. A 1.0-μl aliquot of the treated sample was injected into a GC-18A gas chromatograph connected to a DB-5 capillary column (0.25 mm×10 m) to determine the ME and FFA contents in the reaction mixture. The ME and FFA contents were, respectively, determined as the ratio of MEs and FFAs converted to the reaction mixture without water and glycerol. The column temperature was held at 150°C for 0.5 min, raised to 300°C at 10°C/min, and maintained at this temperature for 3 min. The temperatures of the injector and detector were set at 245 and 320°C, respectively.

The methanolysis and hydrolysis activities that respectively liberate 1 μmol of methyl esters and fatty acids per min were defined as 1 unit (U). The initial reaction rate of ME production was defined as the ME production rate during 2.5-h reaction from initiation.

Determination of fatty acid composition of membranes

After washing with acetone to remove adherent fatty acids, the BSP-immobilized cells were scraped up with a spatula and approximately 50 mg of dry cell was harvested for lipid extraction. This was sonicated with 3 ml of acetone for 15 min and the resulting extract was evaporated to dryness at 40°C in a vacuum desiccator. Phospholipids and non-phosphorus lipids were separated using Sep-Pack Silica Cartridges (Waters Co., Milford, MA, USA) as described by Juaneda et al. (1985). After adsorption of the sample, non-phosphorus lipids were eluted with 30 ml of chloroform and phospholipids collected with 20 ml of methanol.

The formation of fatty acid methyl esters (FAMEs) was carried out by the method of Pugh et al. (2000). The fraction containing phospholipids was suspended in a 15-ml Teflon-lined screw-cap tube with 3 ml methanol/toluene (4:1, v/v). By slowly adding 200 μl of acetyl chloride, acid-catalyzed transesterification was carried out in a water bath at 50°C with moderate stirring for 1 h.

After addition of 5 ml of 6% (w/v) Na_2CO_3 to neutralize the reaction mixture, it was

vortex-mixed and the contents transferred to a 15-ml centrifuge tube, where the solution was centrifuged at 8000 × *g* for 5 min and a portion of the upper toluene layer collected for FAME analysis.

The analysis was performed using a GC-18A gas chromatograph (Shimadzu Co., Kyoto, Japan) connected to a DB-5 capillary column (0.25 mm × 10 m; J & W Scientific, Folsom, CA, USA). The column temperature was maintained at 150°C for 0.5 min, raised to 225°C at 5°C/min, and maintained at this temperature for 3 min. The temperatures of the injector and detector were set at 245 and 320°C, respectively. FAME peaks were identified by comparison of their retention times with those of a standard solution.

Results and discussion

Effect of various fatty acids on lipase activity and cell growth

To investigate the effect of fatty acid species on lipase activity and cell growth, cells were cultivated with BSPs in the presence of various fatty acids. Table 1 shows ME content after 2.5-h methanolysis, specific methanolysis and hydrolysis activities, and cell concentration at 15% water content in the reaction mixture.

For all fatty acids apart from decanoic ($C_{10:0}$) acid, both ME content and specific methanolysis activity were much greater in the presence of the acid than without it. Two of the unsaturated fatty acids (UFAs), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acid, paticularly enhanced ME content and specific methanolysis activity. Specific hydrolysis activity showed an almost identical pattern of enhancement to methanolysis activity except in the case of stearic acid ($C_{18:0}$). With decanoic acid, however, no cell growth was observed, probably because it acted as a strong inhibitor. Such an inhibitory effect of short-chain fatty acids on mycelial growth was also observed in *Aspergillus oryzae* (Fukushima et al., 1991) and *Ganoderma lucidum* (Yang et al., 2000).

Table 1 Effect of various fatty acids on lipase activity and cell growth.

Fatty acid added[a]	Methyl ester content (wt%)[b]	Specific methanolysis activity (U/mg)[c]	Specific hydrolysis activity (U/mg)[d]	Cell concentration (mg/BSP)
Decanoic acid ($C_{10:0}$)	n.d.[e]	n.d.	n.d.	n.d.
Lauric acid ($C_{12:0}$)	3.54	0.057	0.023	2.68
Myristic acid ($C_{14:0}$)	7.32	0.079	0.068	3.98
Palmitic acid ($C_{16:0}$)	7.92	0.101	0.111	3.39
Stearic acid ($C_{18:0}$)	5.26	0.069	0.055	3.29
Oleic acid ($C_{18:1}$)	14.9	0.129	0.134	5.01
Linoleic acid ($C_{18:2}$)	14.6	0.110	0.102	5.77
Control[f]	3.86	0.049	0.063	3.40

[a] Fatty acid added to medium at 30 g/l.
[b] Methyl ester content of reaction mixture after 2.5-h.
[c] Methanolysis activity divided by dry cell weight.
[d] Hydrolysis activity divided by dry cell weight.
[e] Not detected.
[f] No fatty acid added to medium.

In a previous paper (Ban et al., 2001), we reported preliminarily that, in tests of various substrate-related compounds such as oleic acid, olive oil, oleyl alcohol, methyl caprate, and Tween 80, the presence of oleic acid and olive oil was found to enhance methanolysis activity. In the case of *Rhizopus chinensis* (Nakashima et al., 1988) and *Candida rugosa* (Dalmau et al., 2000), lipidic substrates and fatty acids were again found to have a significant inducing effect on intracellular lipase production. Moreover, the present study in Chapter 1 demonstrated that the addition of oleic acid led to the accumulation of a large amount of membrane-bound lipase (see Chapter 1, Figure 6). The addition of UFAs such as oleic acid and linoleic acid to culture medium appears thus to be an important method for enhancement of methanolytic lipase activity.

Effect of various fatty acids on fatty acid composition of cell membrane

Table 2 shows the fatty acid cell membrane composition of *R. oryzae* cells following addition of various fatty acids to the medium. Although neither lauric acid ($C_{12:0}$) nor myristic acid ($C_{14:0}$) was found in the control without addition of fatty acid, when added to the medium, both accounted for a significantly higher proportion than other fatty acids. Ishikawa et al. (1979) also reported that fatty acids added to the medium were incorporated intactly into *Saccharomyces cerevisiae* cellular lipids such as triglyceride and phosphatidylcholine even when not originally present in the cell. It was thus concluded that both lauric and myristic acid were incorporated intactly by *R. oryzae* cells during cultivation.

Following addition of palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$), the most abundant fatty acid component in each case was the one added. This pattern of exogenous fatty acid behavior reflected that seen in *S. cerevisiae* (Ishikawa et al., 1979) and *Mycoplasma laidlawii* (Tourtellotte et al., 1970) cells and suggests that the fatty acid composition of *R. oryzae* cells could be controlled easily by addition of various fatty acids.

Table 2 Fatty acid membrane composition of *R. oryzae* cells grown in various fatty acids.

Fatty acid added	Fatty acid composition of membrane (%)										
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	UFA[a]	SFA[b]	UFA/SFA[c]
Lauric acid ($C_{12:0}$)	41.6	2.99	12.3	0	4.28	18.3	17.7	2.83	38.8	61.2	0.635
Myristic acid ($C_{14:0}$)	0	53.7	9.72	0	4.24	17.0	15.4	0	32.4	67.7	0.479
Palmitic acid ($C_{16:0}$)	0	0	75.6	0	3.07	10.9	10.4	0	21.3	78.7	0.271
Stearic acid ($C_{18:0}$)	0	0	9.87	0	65.3	11.9	13.0	0	24.9	75.2	0.331
Oleic acid ($C_{18:1}$)	0	3.35	9.71	5.33	3.69	65.8	12.1	0	83.2	16.8	4.97
Linoleic acid ($C_{18:2}$)	0	0	7.42	0	3.65	30.3	58.7	0	89.0	11.1	8.04
Control[d]	0	0	26.6	1.48	18.7	20.7	23.8	8.74	54.7	45.3	1.21

[a] Percentage of unsaturated fatty acids.

[b] Percentage of saturated fatty acids.

[c] Ratio of unsaturated to saturated fatty acids.

[d] No fatty acid added to medium.

Repeated methanolysis by UFA- and SFA-enriched cells

To investigate the effect of UFA- and SFA-enriched cells on lipase activity, repeated methanolysis reactions were carried out. Figures 1a and 1b show the time course of ME content in methanolysis by UFA- and SFA-enriched cells, respectively. In Figure 1a, a high ME content of over 70% was achieved using both oleic acid- (UFA/SFA = 4.97) and linoleic acid-enriched (UFA/SFA = 8.04) cells in the first batch cycle. However, both ME content and the initial reaction rate of ME production decreased sharply with each cycle to give an ME content of less than 30% at the end of the fourth batch cycle. With the control (UFA/SFA = 1.21), meanwhile, ME content was only 47% in the first batch cycle, and methanolysis activity decreased in the same way as with oleic acid- and linoleic acid-enriched cells, with the result that almost all catalytic activity was lost by the fourth batch cycle. In contrast, in the case of palmitic acid-enriched (UFA/SFA = 0.271) cells, ME content in the first batch cycle was nearly 65%, and almost no significant decrease was observed in either ME content or initial reaction rate of ME production during four batch cycles (Figure 1b). With stearic acid- (UFA/SFA = 0.331) and myristic acid-enriched (UFA/SFA = 0.479) cells, however, the rate of ME production decreased significantly from the initial level.

These findings indicate that oleic acid- and linoleic acid-enriched cells have higher initial methanolysis activity than SFA-enriched cells, among which palmitic acid-enriched cells exhibit significantly greater enzymatic stability than UFA-enriched cells. Mizoguchi and Hara (1997) report that, for cells grown in medium, palmitic acid-enriched cells have a lower membrane permeability coefficient than linoleic acid-enriched cells, as well as a greater ethanol tolerance and higher cell-membrane integrity. Moreover, cell viability in the presence of ethanol is lower in linoleic acid-enriched cells than in palmitic acid-enriched cells, suggesting that cell viability correlates with membrane permeability (Mizoguchi and Hara, 1996). These results suggest that the higher membrane permeability of oleic- and linoleic acid-enriched cells may produce an increase in initial methanolysis activity, and that the higher ethanol tolerance of palmitic acid-enriched cells may contribute to higher enzymatic stability in methanolysis.

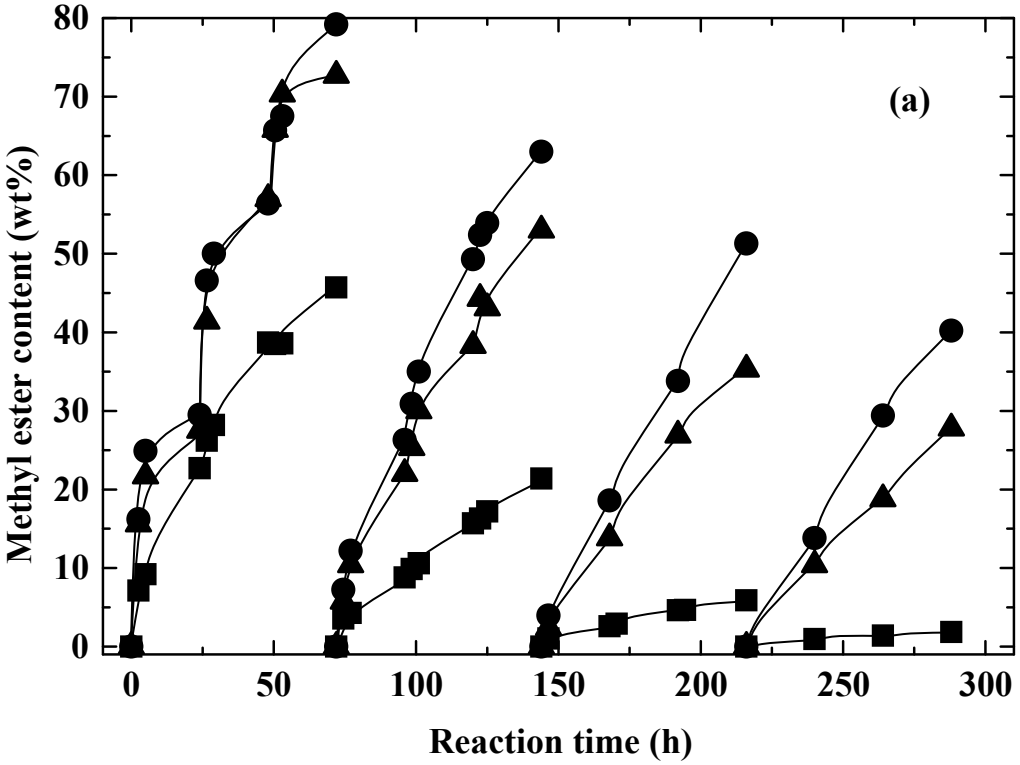

Figure 1a Time course of methyl ester content during repeated methanolysis reaction by UFA-enriched cells. Symbols: (●) oleic acid-enriched cells; (▲) linoleic acid-enriched cells; and (■) control (no fatty acid added to medium).

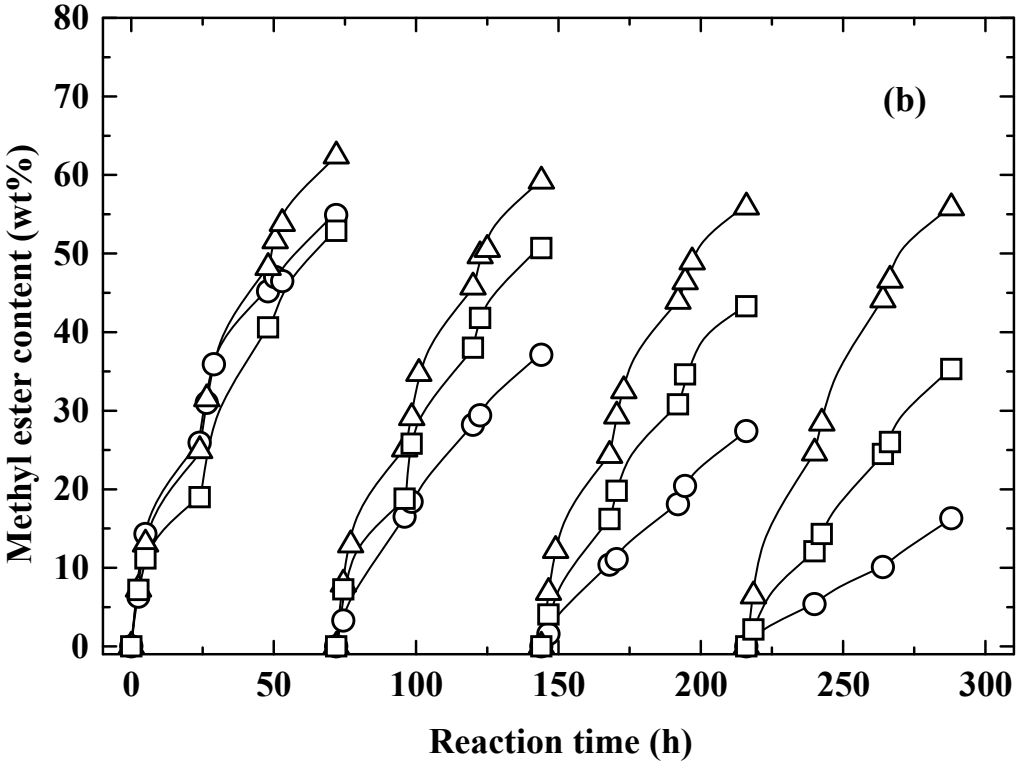

Figure 1b Time course of methyl ester content during repeated methanolysis reaction by SFA-enriched cells. Symbols: (○) myristic; (△) palmitic; and (□) stearic acid-enriched cells.

Comparing to the palmitic acid-enriched cells, myristic acid- and stearic acid-enriched cells showed the lower stability in methanolysis, suggesting the importance of lipase localization inside the cells. As mentioned in Part I, a large proportion of intracellular lipase produced by stearic acid-enriched cells was localized in the cell wall. Moreover, in spite of the low UFA/SFA value in the control (UFA/SFA = 1.21), the methanolysis activity decreased sharply with each cycle, suggesting that the lowest amount of membrane-bound lipase (see Chapter 1, Figure 6 and 7) led to the significant decrease in methanolysis activity. Thus, it seems likely that a control of fatty acid composition is effective in stabilizing the membrane-bound lipase of *R. oryzae* cells.

Methanol tolerance of UFA- and SFA-enriched cells

In the previous study, we (Ban et al., 2002) reported that a high methyl ester concentration is one of the major factors in the loss of intracellular lipase activity during repeated methanolysis reaction. The present study, however, showed no significant difference in the lipase inactivation between oleic acid- and palmitic acid-enriched cells treated with methyl esters (data not shown). Since methanol is another possible factor in the lipase inactivation (Kaieda et al., 2001), the effect of methanol treatment on the lipase activity was investigated. As can be seen in Figure 2, palmitic acid-enriched cells maintained the higher lipase activity than oleic acid-enriched cells, suggesting that a high methanol tolerance of the former led to the significant stabilization in methanolysis.

Several studies have shown that fatty acids affect the properties of the plasma membrane. Many of them have reported that membrane permeability is significantly affected by fatty acid composition in experiments using lipid vesicles (Langner and Hui, 2000; Locher and Leuschner, 2000) and living cells (Mizoguchi and Hara, 1997). As for tolerance to ethanol (Mizoguchi and Hara, 1997) and toluene (Ramos et al., 1997), cell membrane rigidity appears to prevent their penetration. However, the effect of fatty acids on the plasma membrane are still uncertain in terms of the mechanism of action and their location within the cell. Further studies from these standpoints are currently under consideration.

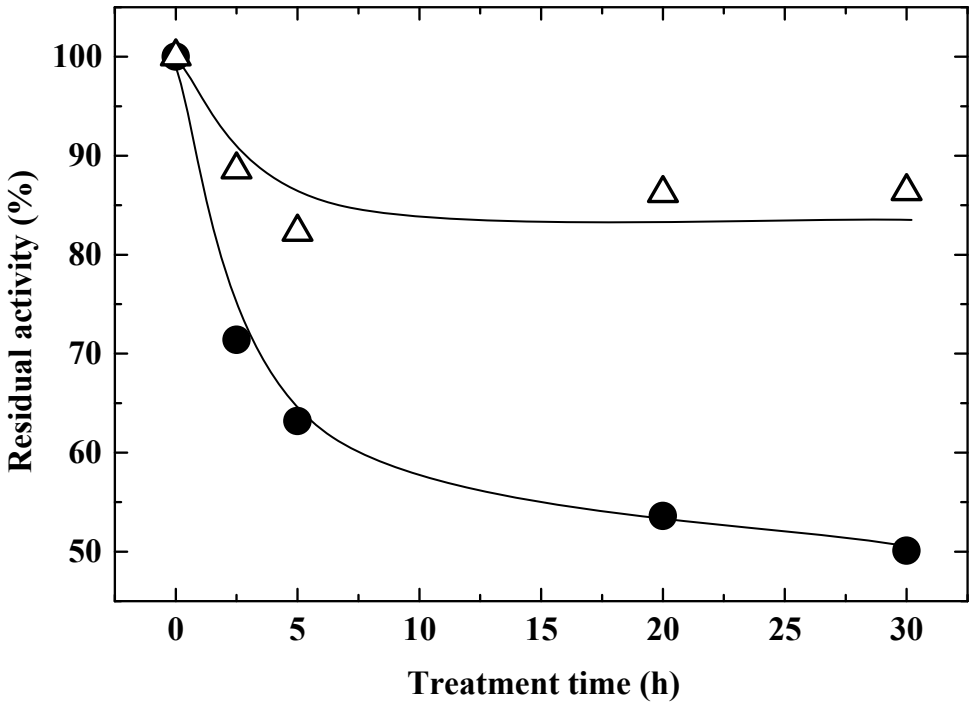

Figure 2 Residual activity of UFA- and SFA-enriched cells treated with methanol. Symbols: (●) oleic acid; and (△) palmitic acid-enriched cells. Ten BSPs with dried cells were incubated in mixtures containing 28.95 g of methyl oleate and 1.05 g of methanol on a reciprocal shaker (30°C, 150 oscillation/min). The horizontal axis shows the incubation time. The vertical axis shows ME production rate of the incubated cells during 2.5-h reaction as a percentage of that before the incubation. Reaction conditions are described in Materials and methods.

Effect of mixture composition of oleic and palmitic acid on methanolysis activity and enzymatic stability

The addition of UFAs such as oleic and linoleic acid is effective in increasing an initial metanolysis activity due to their lipase-inducing effect and higher cell-membrane permeability, whereas the addition of SFA, especially palmitic acid, increases the cell-membrane rigidity, resulting the significant stabilization in metanolysis. *R. oryzae* cells possessing both higher methanolysis activity and stability may thus be obtained by addition of fatty acid to the culture medium in the form of a mixture of oleic or linoleic acid with palmitic acid. To investigate the optimal composition of the mixture of oleic and palmitic acid added to the culture medium, two cycles of repeated batch operation were carried out using mixtures with varying ratios of oleic to palmitic acid. Figure 3 shows ME content after 2.5-h in the first batch cycle and the initial reaction rate of ME production in the second batch cycle as a percentage of that in the fast. This figure reflected a degree of enzymatic stability, and the higher it was the greater the enzymatic activity that could be expected.

The fatty acid ratio was defined as:

$$R_f = \frac{\text{Oleic acid (g)}}{\text{Oleic acid (g)} + \text{Palmitic acid (g)}}$$

where the denominator shows the constant “3 (g)”. When the fatty acid ratio indicated by R_f was increased, there was no significant acceleration of the decrease in initial reaction rate up to 0.67, beyond which however it decreased rapidly.

ME content showed its maximum value at R_f of 0.67, and decreased at R_f values greater or lesser than this critical level. It is thus concluded that there exists an optimal R_f value of 0.67 which yields both higher methanolysis activity and higher stability.

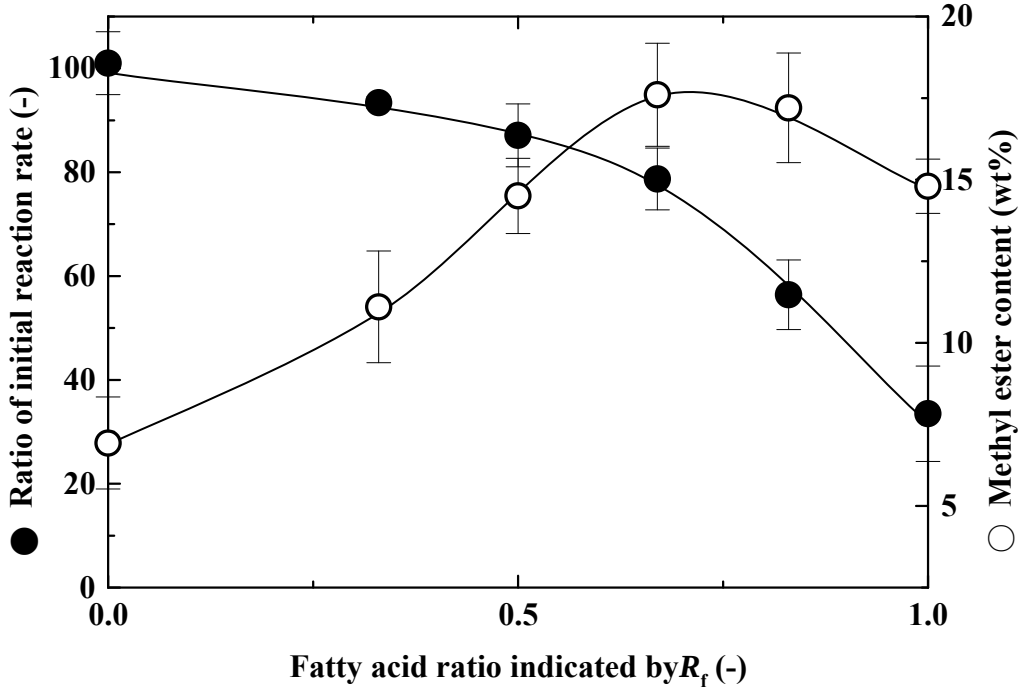

Figure 3 Effect of fatty acid ratio (R_f) on development of initial reaction rate and methyl ester content. The left-axis reflecting an enzymatic stability shows ME content after 2.5-h in the second batch cycle as a percentage of that in the first, while the right-axis reflecting an initial methanolysis activity shows ME content after 2.5-h in the first batch cycle. A mixture of oleic and palmitic acid was added to culture medium at a total concentration of 30 g/l.

Repeated methanolysis by whole-cell biocatalysts with various R_f ratios

Figure 4 shows the time course of ME content in repeated methanolysis using *R. oryzae* cells obtained by various R_f ratios. The figure shows that, at R_f value of 1.0, both ME content and the initial rate of ME production decreased sharply with each cycle to give an ME content of only 5% at the end of ten batch cycles. In contrast, at R_f value of 0.83 and 0.67, the production rates decreased gradually with each cycle, and, in the latter case in particular, ME content was maintained at a high value of around 55% even after ten batch cycles. These results are consistent with the data in Figure 3 and indicate that the optimal value of R_f in terms of both ME content and ME production rate is around 0.67.

The findings outlined above indicate that BSP-immobilized cells are significantly stabilized by cultivation at optimal levels of R_f, and could be used as whole-cell biocatalyst for practical biodiesel-fuel production.

Concluding remarks

In the present study, to enhance both the lipase activity and the stability of *R. oryzae* cells immobilized within BSPs, the effect of their membrane fatty acid composition on biodiesel-fuel production was investigated. Lipase activity was increased by the use of unsaturated fatty acid-enriched cells, while the addition of palmitic acid enhanced the stability in repeated batch methanolysis. Based on these findings, we developed cells possessing both higher activity and higher stability by using an optimal admixture of oleic and palmitic acid to produce cells with optimal membrane fatty acid composition.

The stabilization method developed in this study needs no complex operation after cultivation. Hence, whole-cell biocatalysts with modified membrane lipids appear to have a great potential for industrial biodiesel-fuel production. Further studies on a large-scale production of biodiesel are necessary.

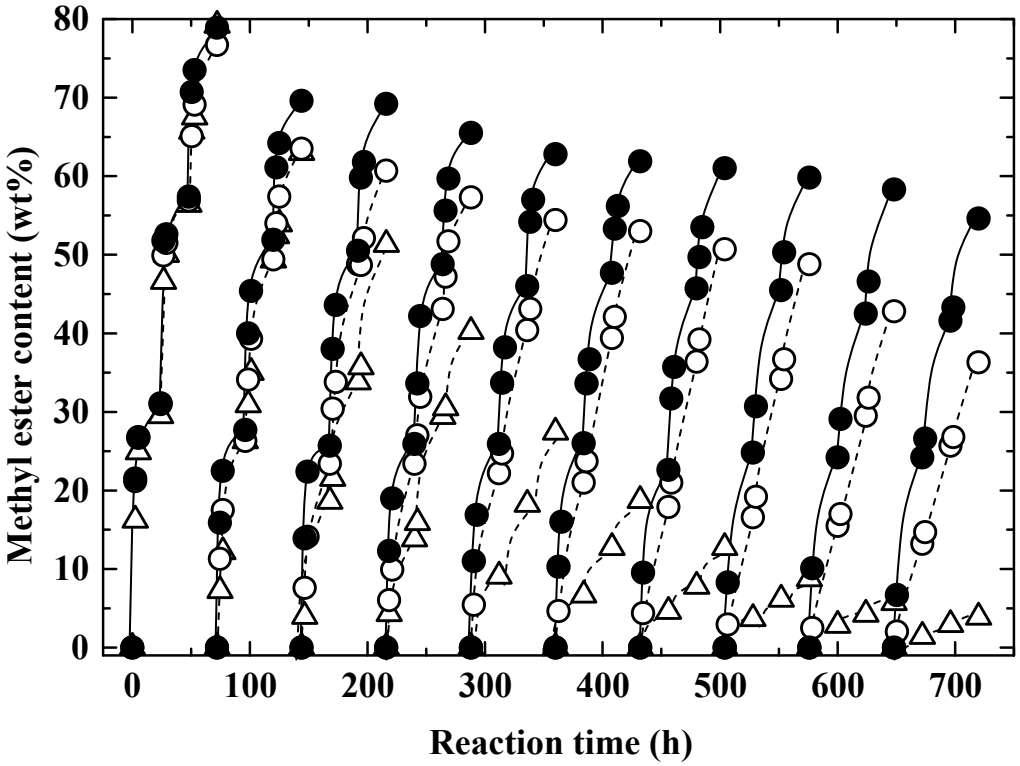

Figure 4 Time course of methyl ester content during ten repeated batch cycles using cells cultivated at various R_f values. Symbols: (●) R_f =0.67; (○) R_f =0.83; and (△) R_f =1.0.

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Part III

Large-scale production of biodiesel using fungus whole-cell biocatalysts

Biodiesel-fuel production using a packed-bed reactor with lipase-producing *Rhizopus oryzae* cells

Introduction

Biodiesel (fatty acid methyl esters), which is produced by methanolysis of triglyceride, has recently attracted considerable attention as a renewable, biodegradable, and nontoxic fuel. During the past decade, environmental benefits of using this fuel have accelerated developing the processes of biodiesel production all over the world (Ma and Hanna, 1999). Of the several processes so far employed, chemical technology using alkaline catalyst has been the most widely applied since it gives a high conversion of methyl esters in a short reaction time. However, this process has several drawbacks including the difficulty in recycling glycerol, the need to eliminate the catalyst and salt, and the energy-intensive nature, leading to a limitation of the availability of biodiesel (Zhang et al., 2003).

To overcome these drawbacks, many attempts have been made to develop an enzymatic process using extracellular or intracellular lipase as catalyst (Shimada et al., 1999; Kaieda et al., 2001; Matsumoto et al., 2001, 2002). The latter process, in which lipase-producing microorganisms are directly utilized as whole-cell biocatalysts, is much simpler since it requires no purification or further immobilization step when cells are prepared using a technique based on porous biomass support particles (BSPs; Atkinson et al., 1979). In a previous study, we (Ban et al., 2001, 2002) found that dried *Rhizopus oryzae* cells immobilized within BSPs efficiently catalyze the methanolysis in the presence of 4-20% water, and that the lipase activity of *R. oryzae* cells treated with 0.1% glutaraldehyde solution was maintained during 6 batch cycles, with the methyl ester content in each cycle reaching 70-80% within 72 h. Thus, the direct use of lipase-producing *R. oryzae* cells is an effective way to reduce the cost of lipase production which is the main hurdle to commercialization of the enzymatic process.

For industrial biodiesel production, the scale-up cultivation of immobilized *R.*

oryzae cells was performed successfully with a 20 l air-lift bioreactor (Oda et al., 2005). Repeated methanolysis using the dried cells maintained a high methyl ester content (65-80%) during 20 batch cycles. However, no study on methanolysis using a bioreactor containing the whole-cell biocatalysts has been so far reported to assess their availability for practical biodiesel production in a large scale.

The present study therefore developed a system of methanolysis using a packed-bed reactor (PBR) containing the dried *R. oryzae* cells immobilized within BSPs. Reaction conditions were examined to increase the reaction rate and the lipase stability.

Materials and methods

Microorganism, culture media and biomass support particles (BSPs)

All experiments were carried out using the filamentous fungus *Rhizopus oryzae* IFO4697, which has a 1,3-positional specificity lipase. The fungus was routinely maintained on an agar slant made from 4% potato dextrose agar and 2% agar powder. Per liter of distilled water, the basal medium contained: polypepton 70 g; $NaNO_3$ 1.0 g; KH_2PO_4 1.0 g; and $MgSO_4 \cdot 7H_2O$ 0.5 g. Glucose or olive oil as sole carbon source was added to the medium at a concentration of 10 and 30 g/l, respectively. The pH of the medium was initially adjusted to 5.6 and then allowed to follow its natural course. The BSPs were 6×6×3 mm cuboids and 6 mm cubes of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch.

Experimental apparatus

For immobilized cell culture, *R. oryzae* cells were grown in a 20 l air-lift bioreactor (Kansai Chemical Engineering Co. Ltd., Hyogo, Japan). Dimensions of this reactor were described in a previous paper (Oda et al., 2005). The bioreactor can be thermally sterilized by steam injection and circulate a culture medium by aeration.

The packed-bed reactor (PBR) used for methanolysis consisted of a glass column (25 mm i.d. × 400 mm length), a tygon tube and a peristaltic pump. Recycling the reaction mixture continuously operated the bioreactor packed with dried BSP-immobilized cells. The schematic diagram of this system was shown in Figure 1.

Immobilized cell culture by air-lift bioreactor

Sakaguchi flasks (500-ml) containing the basal medium with glucose were sterilized by autoclaving at 121°C for 20 min, aseptically inoculated with spores from a fresh agar slant, and then incubated on a reciprocal shaker at 30°C for 24 h (150 oscillations/min; amplitude 70 mm). The resultant culture medium (5% inoculation size) was transferred into the 20 l air-lift bioreactor containing 10 l of the basal medium with 30 g/l of olive oil and 24,000 BSPs (6×6×3 mm cuboids). The bioreactor was then aerated at 2.5 vvm at 30°C. The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during air-lift cultivation.

After cultivation, the BSP-immobilized cells were separated from the culture broth by filtration, washed with tap water, and dried at room temperature for around 24 h. To stabilize the lipase activity, the died cells were treated with 0.1% (v/v) glutaraldehyde solution at 25°C for 1 h, washed with tap water, dried at room temperature for more than 24 h, and then used as whole-cell biocatalyst for methanolysis.

Methanolysis reaction

Methanolysis reaction was carried out both in a screw-cap bottle and in the PBR. In batch reaction, the reaction mixture consisted of 3.86 g of soybean oil, 0.14 g of methanol, 0.2 ml of distilled water, and 20-40 particles of dried BSP-immobilized cells. A 30-ml screw-cap bottle containing this mixture was incubated on a reciprocal shaker (30°C, 150 oscillations/min).

In continuous-flow reaction, the PBR contained 96.5 g of soybean oil, 3.5 g of methanol (one molar equivalent to 96.5 g of soybean oil), 5 ml of distilled water, and 1,000

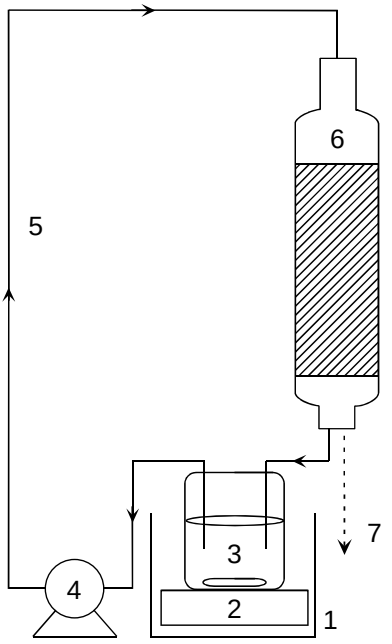
particles of dried BSP-immobilized cells. The reaction mixture was emulsified by ultrasonication for 5 min prior to reaction. Continuous-flow methanolysis was performed at room temperature in the PBR, where a pump circulated the reaction mixture at various flow rates. To fully convert the oil to its corresponding methyl esters, at least three molar equivalents of methanol are necessary. As an excess amount of methanol leads to a significant inactivation of lipase, stepwise addition of methanol was conducted to maintain the methanol content at the desired level. After one reaction cycle, a flesh reaction mixture replaced the old one to start the next cycle. To compare the reaction efficiency with batch operation, methanolysis was carried out in a 150-ml screw-cap bottle containing the same volume of the reaction mixture (room temperature, 150 oscillations/min).

Analytical methods

The methanolysis products were analyzed as described in a previous paper (Ban et al., 2001) with slight modifications. The methyl ester (ME) content in the reaction mixture was determined using a GC-18A gas chromatograph (Shimadzu Co., Kyoto, Japan) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA, USA). The temperatures of the injector and detector were set at 245 and 320°C, respectively. The column temperature was initially maintained at 150°C for 0.5 min, raised to 300°C at 10°C /min, and finally maintained at this temperature for 10 min.

Reaction mixtures were observed under a microscope (BZ-8000, KEYENCE Co., Osaka, Japan). Images (1,360×1,024 pixels) were collected and exported as JPEG files.

Immobilized cell concentration within a BSP was measured as described previously (Ban et al., 2001).



1. Ultrasonic device
2. Magnetic-stirring machine
3. Reservoir of substrates
4. Peristaltic pump
5. Tygon tube
6. Glass column packed with immobilized cells
7. Products

Ultrasonication was employed prior to reaction.

Figure 1 Schematic diagram of packed-bed reactor.

Results and discussion

Selection of particle size to increase the interfacial area of cell-substrate contact

As reported in a previous paper (Ban et al., 2001), *R. oryzae* cells form a dense film near the surface of BSPs and rarely exist inside the particles due to oxygen starvation. Since we could expect that an interfacial area of cell-substrate contact affects the reaction efficiency in a PBR, the effect of particle size on methanolysis was preliminary investigated. Figure 2 shows the relationship between the reaction rate and the total volume of BSPs (6×6×3 mm cuboids and 6 mm cubes) used in methanolysis. In both cases, the reaction rate showed a linear relationship to the total volume of BSPs. However, the slope of the linear plots in the cuboidal BSPs was higher than that in the cubic BSPs, indicating that the former BSPs with high interfacial area of cell-substrate contact is significantly advantageous for use as catalyst packed in a limited space. Subsequent experiments were therefore carried out using the bioreactor packed with the cuboidal BSPs.

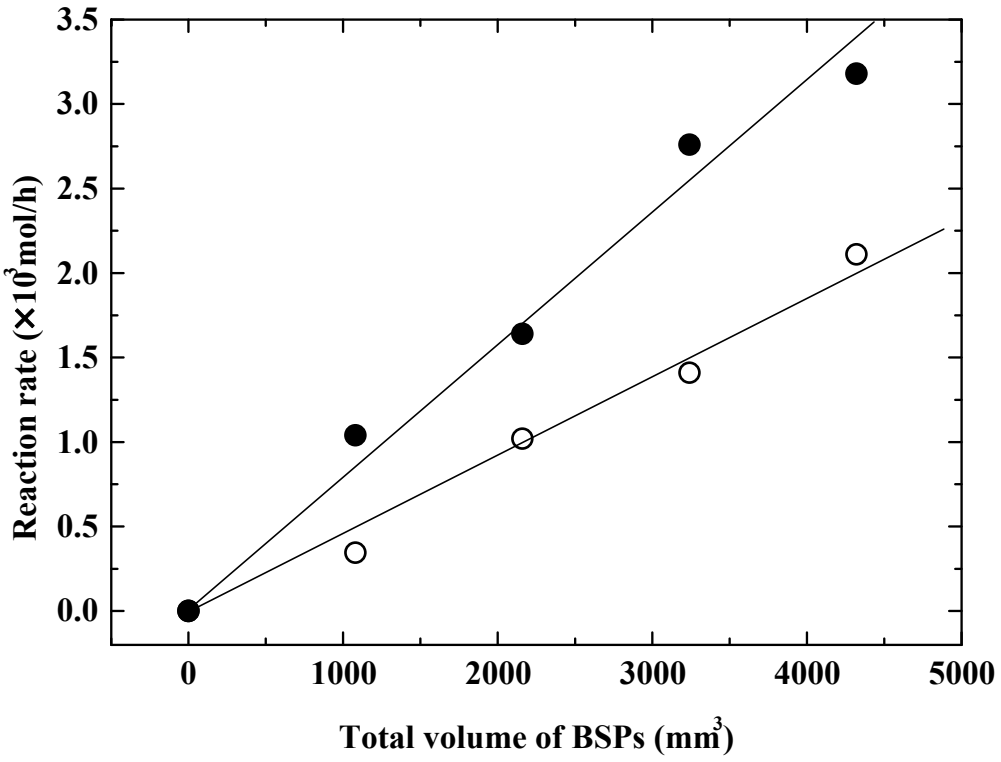


Figure 2 Relationship between reaction rate and total volume of BSPs. Symbols: (●) cuboidal and (○) cubic BSPs. Methanolysis was carried out in a screw-cap bottle as described in Materials and methods. Reaction rate of methanolysis was defined as the ME production rate during 1-h reaction from initiation.

Emulsification of reaction mixture

Figure 3A shows the microscopic photographs of the reaction mixture with and without ultrasonication. Without ultrasonication, water droplets with various sizes (larger than 100 μm) were observed, while after ultrasonication, small water droplets with less than 5 μm were distributed evenly to the reaction mixture, indicating that the reaction mixture containing oils and water was well emulsified. Figure 3B shows the time course of ME content in continuous-flow methanolysis using the PBR containing these reaction mixtures. When the reaction mixture was emulsified prior to the methanolysis, a higher reaction rate was obtained to reach an ME content of 75.5% at 29 h. This production rate was much higher than that obtained in our previous study (Ban et al., 2002; Hama et al., 2004; Oda et al., 2005). Because the lipase catalysis occurs in the interfacial layer between the hydrophobic and hydrophilic phase (Rostrup-Nielsen et al., 1990), much larger surface area of the water/oil interface seemed to lead the increased accessibility of lipase to substrates. It was thus suggested that emulsification of the reaction mixture is effective in continuous-flow methanolysis. Subsequent experiments were carried out using the emulsified substrates.

A previous study by our group (Kaieda et al., 1999) found that *R. oryzae* lipase efficiently catalyzes the methanolysis in a water-containing system without an organic solvent. Although this reaction system is advantageous especially for methanolysis of used oils, there may be a difficulty in dispersing water in a large scale. However, the PBR without emulsification unexpectedly showed a high production level with the ME content of 63% at 29 h, suggesting that the PBR with recycling-flow system can originally offer a good mixing of the reaction mixture. There is recently a growing interest in developing the microemulsion systems containing a surfactant (Orlich and Schomäcker, 2001; Shin et al., 2002). Thus, combinations with these techniques would further improve the reaction efficiency.

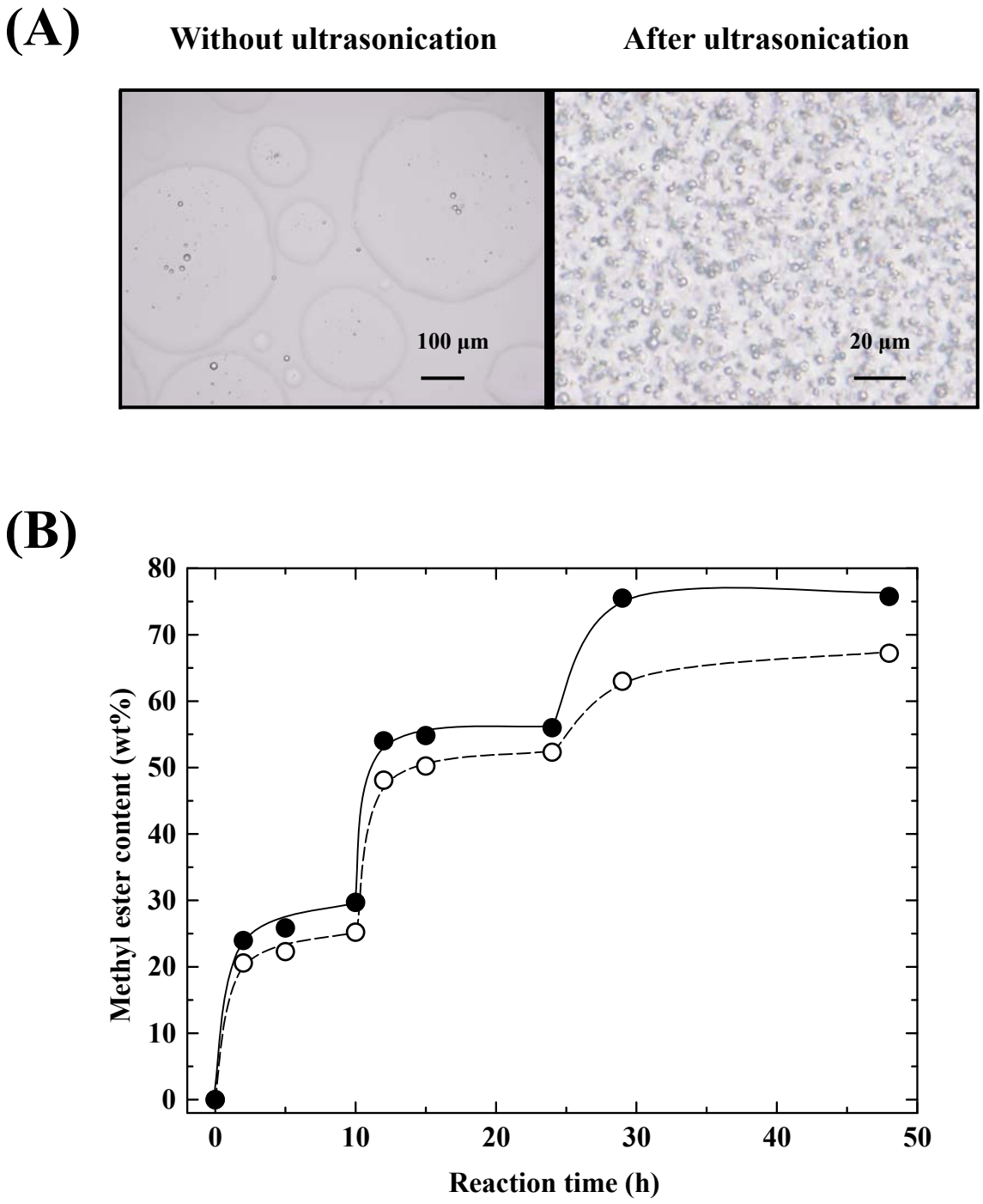


Figure 3 Effect of ultrasonic treatment on methanolysis. Microscopic photographs of reaction mixture (A) and time course of methyl ester content in reaction mixture (B). Symbols: (●) with and (○) without ultrasonication. Methanol was added stepwisely at 0, 10, 24 h.

Effect of flow rate on methanolysis in PBR

The lipase activity and stability were investigated at the several flow rates ranging from 5 to 55 l/h. To increase a final ME content, four molar equivalents of methanol were added to soybean oil stepwisely at 0, 10, 24 and 48 h. As can be seen in Table 1, higher reaction rate and final ME content in the first cycle were obtained with increasing flow rate. In the fifth cycle, however, methanolysis at the flow rate of 25 l/h showed the highest production level, whereas the significant decrease in a final ME content was observed at 5 and 55 l/h. In addition, measurement of immobilized cell weight after five cycles revealed that increasing flow rate accelerated a degree of cell exfoliation from BSPs.

These results led to the hypothesis that there are two factors responsible for lipase stability during continuous-flow methanolysis: physical damage to immobilized cells and effect of methanol. Several studies have shown that a high degree of shear severely damages an immobilized lipase, which is located at the exterior surface of particles (Arcos et al., 2000). As mentioned earlier, the immobilized fungal cells are located at the surface of BSPs. Hence, a fluid-shear force at high flow rate seemed to severely damage and exfoliate the cells, thereby leading to the loss of lipase activity.

Although we could expect that the flow at 5 l/h scarcely damaged the cells, lipase inactivation was observed significantly with repeating reaction. This phenomenon may be explained by the effect of methanol on lipase activity. Visual observations at low flow rate indicated that BSPs adsorbed a large proportion of hydrophilic layer containing water and glycerol as by-product. In studies of continuous-flow methanolysis using immobilized *Candida antarctica* lipase, Watanabe et al. (2000) suggested that unreacted methanol migrates from reaction mixture to glycerol layer covering the particles, with the result that the lipase is inactivated by high concentration of methanol in the glycerol layer. It was thus assumed that inefficient mixing of reaction mixture caused the covering of BSPs with hydrophilic layer, thereby leading to a significant inactivation of lipase by high concentration of methanol in the hydrophilic layer. Consequently, we concluded that the flow rate of 25 l/h is the optimal value to obtain a high lipase activity and stability during continuous-flow methanolysis.

Table 1 Effect of flow rate on repeated methanolysis reaction in PBR.

Flow rate (l/h)	R_i[a] ($\times 10^2$mol/h)	Conversion[b] (wt%)		Cell weight[c] (mg/BSP)
		First cycle	Fifth cycle	
5	3.20	86.9	66.9	1.77
25	3.89	88.7	81.4	1.68
55	4.64	90.9	63.3	1.28

[a] Initial reaction rate defined as ME production rate during 2-h methanolysis.
[b] Methyl ester content of reaction mixture after 50-h.
[c] Dry cell weight immobilized within a BSP was measured after 5 cycles. Before reaction, dry cell weight was 2.11 mg/BSP.

Repeated methanolysis in batch and continuous-flow system

Figure 4 shows the time course of ME content during 10 cycles of methanolysis in the PBR and in a screw-cap bottle. Both cases reached a high ME content of over 90 % in the first cycle. When methanolysis was carried out in batch system, the reaction rate of ME production decreased sharply with each cycle to give an ME content of only 10% at the end of ten batch cycles. In contrast, ME production rates in the PBR decreased gradually with each cycle, and a high ME content of around 80% was maintained even in the tenth cycle.

In a previous study utilizing BSP-immobilized *R. oryzae* cells, we (Oda et al., 2005) found that an ME content reaching 65-80% was obtained during repeated methanolysis, in which three molar equivalents of methanol were added stepwisely at 0, 24, and 48 h. To achieve a higher level of ME production, the present study attempted to add four molar equivalents of methanol stepwisely at 0, 10, 24, and 48 h in each cycle. However, the lipase activity was significantly decreased during repeated methanolysis using a screw-cap bottle subject to vigorous shaking, which is probably explained by both a cell exfoliation (0.93 mg/BSP after 10 cycles) and a high concentration of methanol. In the latter case, it is assumed that inefficient mixing of reaction mixture led to an excessive accumulation of methanol in the hydrophilic layer covering the BSPs, although a motion of BSPs during shaking enables a comparatively high production rate in the first cycle. Consequently, these results suggest that the packed-bed reactor is significantly advantageous for repeated methanolysis in terms of protecting cells from a physical damage and an excess amount of methanol.

The findings outlined above indicate that a packed-bed reactor with lipase-producing *R. oryzae* cells can be used for continuous-flow methanolysis to achieve a high production level of methyl esters. Since the cost of lipase production is the main hurdle to commercialization of the enzymatic process, the direct use of intracellular lipase as whole-cell biocatalyst is desired from the viewpoint of industrial application. Thus, the process presented here is considered to be promising for industrial biodiesel-fuel production.

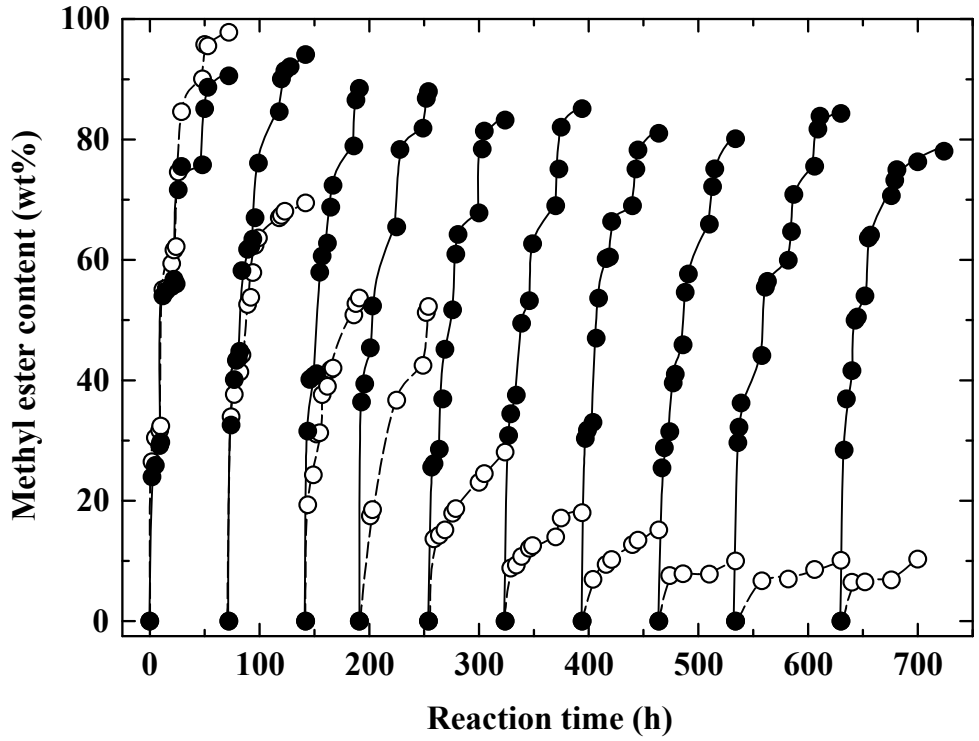


Figure 4 Time course of methyl ester content during ten repeated cycles in batch and continuous-flow methanolysis. Symbols: (●) continuous-flow and (○) batch reaction.

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GENERAL CONCLUSION

The studies collected here were carried out to determine the lipase localization in filamentous fungi and to apply the fungus whole-cell biocatalysts for biodiesel-fuel production.

Rhizopus oryzae cells produced two types of lipase with different molecular masses of 34 and 31 kDa; the former (ROL34) was localized in the cell wall, while the latter (ROL31) was mainly bound to the cell membrane. An investigation of the relationship between ROL34 and ROL31 suggested that ROL31 originates from the cleavage of an additional 28-amino-acid residue at the N-terminus of ROL34. It seems therefore likely that the variation in lipase localization is due to the difference in post-translational proteolysis of the lipase precursor. Cell immobilization within biomass support particles (BSPs) was found to strongly inhibit the secretion of membrane-bound lipase into the culture medium, while the addition of olive oil to the culture medium led to the retention of increased amounts of lipase within the cell. Thus, these two methods appear to be effective in developing cells possessing a high intracellular lipase activity. The intracellular methanolysis activity strongly correlated with the relative amounts of the membrane-bound lipase, which suggests that ROL31 localized in the membrane plays a crucial role in the methanolysis activity of *R. oryzae* cells.

To clarify the role of the 28-amino-acid residue (N28) at the N-terminus of ROL34, *R. oryzae* lipase (ROL) was expressed using *Aspergillus oryzae* as a host. Expression of the N-terminally truncated forms of ROL suggested a crucial role of the N28 sequence in the secretory process. *In vivo* visualization of these secretory processes suggested that ROL with the N28 sequence was successfully secreted into the culture medium through the typical secretory process in eukaryotic cells, and revealed that the expression of mature ROL induced protein accumulation without its translocation into endoplasmic reticulum (ER). These results indicate a significant role of the N28 sequence in protein transport. The presence of the N28 sequence between the secretion signal and GFP induced the protein translocation into ER, leading to the enhanced secretion of GFP into the culture medium. Therefore, these findings

suggest that the N28 sequence facilitates the protein translocation into ER even in the case of cytoplasmic protein and can be used as a secretion enhancer for the heterologous protein production in *A. oryzae*.

In order to stabilize the lipase activity of *R. oryzae* cells as whole-cell biocatalyst, the effect of cell-membrane fatty acid composition on biodiesel-fuel production was investigated. The fatty acid composition of the cell membrane was easily controllable by addition of various fatty acids to the culture medium. Oleic or linoleic acid-enriched cells showed higher initial methanolysis activity than saturated fatty acid-enriched cells, among which palmitic acid-enriched cells exhibited significantly greater enzymatic stability than unsaturated fatty acid-enriched cells. It was assumed that fatty acids significantly affect the permeability and rigidity of the cell membrane, and that higher permeability and rigidity lead to increases in methanolysis activity and enzymatic stability, respectively. Based on these findings, we developed cells possessing both higher activity and higher stability by using an optimal admixture of oleic and palmitic acid to produce cells with optimal membrane fatty acid composition.

A system of methanolysis using a packed-bed reactor with whole-cell biocatalysts was developed. Lipase-producing *R. oryzae* cells were immobilized within cuboidal BSPs during batch cultivation in a 20-l air-lift bioreactor. An investigation of flow rate suggested that a fluid-shear force at high flow rate caused the cell exfoliation from BSPs, while inefficient mixing of the reaction mixture at low flow rate allowed the BSPs to be covered with hydrophilic layer containing a high concentration of methanol, thereby leading to a significant decrease in lipase activity. Continuous-flow methanolysis at 25 l/h achieved a high methyl ester content of over 90% in the first cycle, and maintained a high value of around 80% even after ten cycles. Comparison with batch reaction suggested that the packed-bed reactor is greatly advantageous for repeated methanolysis in terms of protecting cells from a physical damage and an excess amount of methanol. Consequently, the process presented here is considered to be promising for industrial biodiesel-fuel production.

PUBLICATION LIST

Part I

Hama, S., Tamalampudi, S., Fukumizu, T., Miura, K., Yamaji, H., Kondo, A., and Fukuda, H. (2006). Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. Journal of Bioscience and Bioengineering, **101(4)**, 328-333.

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