Mitsuyoshi Ueda *Editor*

Yeast Cell Surface Engineering

Biological Mechanisms and Practical Applications

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Preface

Cell surface engineering, in contrast to the conventional intracellular expression, has many attractive features. This engineering is especially effective when yeasts are used as a host, because eukaryotic modifications that are often required for functional use can be added to the surface-displayed proteins/peptides. This engineering, leading to the generation of the so-called arming (molecular display) technology, can be employed for basic and applied research purposes. In this book, various strategies for the construction of surface-engineered yeasts and the diverse applications of this technology to industrial processes such as biofuel and chemical productions, pollutant removal, and health-related processes, including in vitro antibody preparation and oral vaccines, are outlined. In addition, this engineering is suitable for protein engineering and directed evolution through high-throughput screening that is made possible by the feature that proteins/peptides displayed on cell surface can be directly analyzed using intact cells without concentration and purification. Actually, novel proteins/peptides with improved or developed functions have been created.

Sakyo-ku, Kyoto, Japan — Mitsuyoshi Ueda

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Part I
Principle

The cell surface engineering has also been established in gram-negative bacteria, gram-positive bacteria, and phages. So far, many proteins/peptides have been successfully displayed with maintenance of their functions. In contrast to bacteria and phages, yeasts are equipped with the quality control and modification systems of eukaryotic secretory pathways. Therefore, in the case of target proteins that have a high molecular mass or require glycosylation modification, yeasts are suitable hosts for cell surface display. In addition, simultaneous display of multiple kinds of proteins/peptides on the same cell surface can be performed in yeasts by using different auxotrophic markers, leading to the enhanced potential of surface-engineered yeasts. The principles are described.

Chapter 1: Principle of Cell Surface Engineering of Yeast (Dr. Mitsuyoshi Ueda)

Chapter 1
Principle of Cell Surface Engineering of Yeast

Mitsuyoshi Ueda

Abstract The cell surface is a functional interface between the interior and exterior of cells. Surface proteins are responsible for most of the cell surface functions, serving as cell–cell adhesion molecules, specific receptors, enzymes, and transport proteins. Cells have systems for anchoring and confining surface proteins to particular domains on the cell surface. Fundamental analyses of how these molecules are targeted and localized to the cell surface have been performed. For applications, the cell surface should be exploited by making use of the known transport mechanisms of proteins to the cell surface. The use of the abovementioned systems to display heterologous proteins on the cell surface of microorganisms is expected to facilitate the segregation of produced polypeptides and the production of microbial biocatalysts, whole-cell adsorbents, and live vaccines. The use of the cell surface of living cells is also attractive for many applications in microbiology and molecular biology.

Keywords Cell surface engineering · Molecular display · GPI anchor · Arming yeast · GPI-anchor attachment signal sequence

The cell surface is a functional interface between the interior and exterior of cells. Surface proteins are responsible for most of the cell surface functions, serving as cell–cell adhesion molecules, specific receptors, enzymes, and transport proteins. Some surface proteins extend across the plasma membrane, whereas others are bound by noncovalent or covalent interactions to cell surface components. Cells have systems for anchoring and confining surface proteins to particular domains on the cell surface. Fundamental analyses of how these molecules are targeted and localized to the cell surface have been performed. For applications, the cell surface

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should be exploited by making use of the known transport mechanisms of proteins to the cell surface. The use of the abovementioned systems to display heterologous proteins on the cell surface of microorganisms is expected to facilitate the segregation of produced polypeptides and the production of microbial biocatalysts, whole-cell adsorbents, and live vaccines. The use of the cell surface of living cells is also attractive for many applications in microbiology and molecular biology (Ueda and Tanaka 2000; Kuroda and Ueda 2013; Ueda 2016).

Surface expression systems were first reported by showing that peptides could be fused to the docking proteins (p111) of a filamentous phage without affecting its ability to infect *Escherichia coli* (Scott and Smith 1990). This led to the development of phage display systems (Chiswell and McCaBerty 1992, 2018 Nobel Prize Chemistry). These systems have already facilitated the isolation of specific ligands, antigens, and antibodies from complex libraries (Hoogeoboom 1997). However, hybrids of larger polypeptides with the major coat protein of a phage were not readily incorporated into the phage particles. The bacterial surface may be more suitable for displaying higher numbers of proteins. On the surface of gram-negative bacteria, which have an outer membrane as their outermost cell surface, a number of heterologous proteins have been displayed (Little et al. 1993; Georgiou et al. 1993, 1997), and these proteins were fused to the surface-exposed termini of the outer membrane proteins (Francisco et al. 1992, 1993). Lipoproteins (Harrison et al. 1990), fimbriae (Hedegaard and Klemm 1989), and flagellar proteins (Newton et al. 1989) have also been displaying heterologous proteins on the cell surface. Gram-positive bacteria have been used for the purpose of displaying proteins on the bacterial surface, and the surface display of heterologous proteins was achieved using proteins localized on the cell wall (Gunneriusson et al. 1996; Samuelson et al. 1995; Schneewind et al. 1995). If the system for cell surface display is intended to be applied to bioindustrial processes for food, alcoholic beverages, medicines, and so on, it needs to be safe. For practical use, the most suitable microorganism is the yeast *Saccharomyces cerevisiae*, which has a "generally regarded as safe" status and can be used in the production of food and pharmaceuticals. Thus, *S. cerevisiae* is a useful organism for the development of a cell surface expression system. It is also useful as a host for genetic engineering, since it enables the folding and glycosylation of expressed eukaryotic heterologous proteins and can be subjected to many genetic manipulations. Moreover, the yeast can be cultivated to a high cell density in a cheap medium.

S. cerevisiae has a thick and rigid cell wall (Fig. 1.1), of about 200 nm, which lies outside the plasma membrane, which is in contrast to that of bacteria (Balloi 1982). The cell wall of *S. cerevisiae* is mainly composed of mannoproteins and β-linked glucans (Fleet 1991) and has a bilayered structure consisting of an internal skeletal layer of glucans, composed of β-1,3- and β-l,6-linked glucose (Manners et al. 1973a, b), and a fibrillar or brushlike outer layer, which is composed predominantly of mannoproteins (Horisberger and Vonlanthen 1977). These proteins are linked to glucans through covalent bonds. Two types of mannoproteins are present in the cell wall of *S. cerevisiae* (Cid et al. 1995; Klis 1994). Mannoproteins loosely associated with the cell wall through noncovalent bonds are extractable with sodium dodecyl sulfate (SDS). When the isolated cell wall is solubilized with hot SDS, about 60

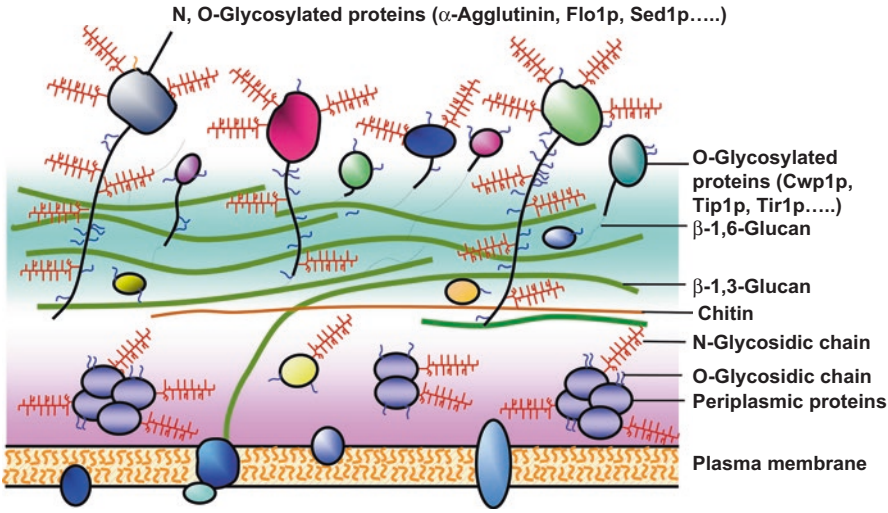

Fig. 1.1 Structure of cell surface of *S. cerevisiae*

low-molecular-weight proteins are released (Valentin et al. 1984). Other types of mannoproteins are extractable by glucanase and are released by β-1,3- or β-1,6-glucanase digestion of the glucan layer of the cell wall but not by SDS extraction (Fleet and Manners 1977). Among these glucanase-extractable mannoproteins on the cell surface of *S. cerevisiae*, the mating-type-specific agglutinins (Lipke and Kurjan 1992), which mediate the direct cell–cell adhesion between cells of the opposite mating type during mating and represent minor cell wall components, are assumed to be located on the outermost surface. Mating-type a and α cells express a-agglutinin and α-agglutinin, respectively (Terrance et al. 1987; Watzele et al. 1988). α-Agglutinin is encoded by the AGαl gene (Lipke et al. 1989) and interacts with the binding subunit of the agglutinin complex of a-type cells (Cappellaro et al. 1991). a-Agglutinin consists of a core subunit encoded by the AGA1 gene (Roy et al. 1991), which is linked through disulfide bridges to a small binding subunit encoded by the AGA2 gene (Cappellaro et al. 1991). Both α-agglutinin and the core subunit of a-agglutinin are composed of a secretion signal region, an active region, a support region rich in serine and threonine, and a putative glycosylphosphatidylinositol (GPI) anchor attachment signal, which presumably exists in heavily O-glycosylated forms (Cappellaro et al. 1991; Roy et al. 1991; Wojciechowicz et al. 1993). α-Agglutinin has a predicted length of 650 amino acids before processing.

GPI anchors have been found in many eukaryotic plasma membrane proteins ranging from the coat proteins of protozoa to mammalian cell-adhesion molecules (Cross 1990; Fredette et al. 1993; Homans et al. 1988; Dustin et al. 1987). The structure of the GPI anchor (Fig. 1.2) is highly conserved among molecules from various organisms (Ferguson and Williams 1988). The core structure of the yeast GPI anchor is similar to that found in other eukaryotes (Lipke et al. 1989; Conzelmarm et al. 1988; Leidicb et al. 1994), consisting of ethanolamine phosphate

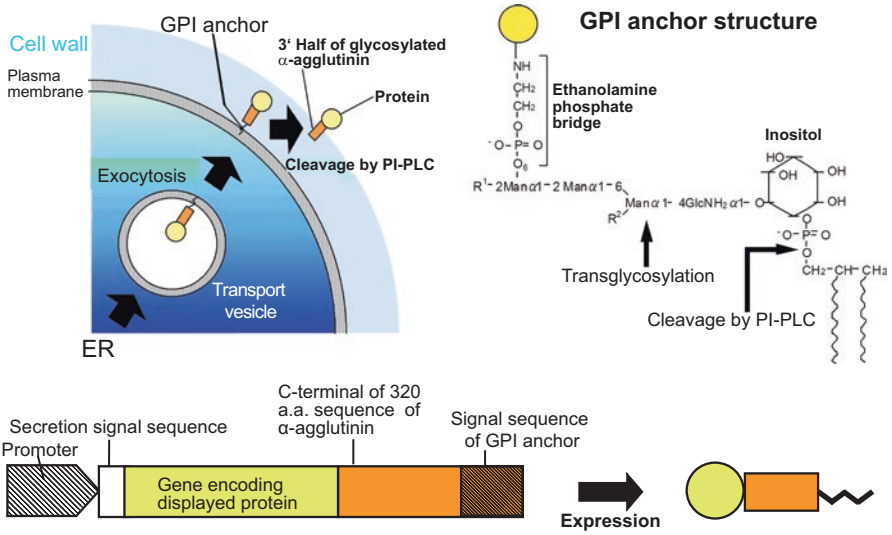

Fig. 1.2 Transportation of GPI-anchor protein, GPI anchor structure, and protein display system via GPI-anchor protein transportation

Table 1.1 GPI-anchor attachment signal

Cell surface protein	GPI anchor attachment signal
Agα1	TSTSLMISTYEG KASIFFSAELGSIIFLLLSYLLF
Aga1	TSSMVTISQYMG SGSQTRLPLGKLVFAIMAVACNVIFS
Flo1	STASLEISTYAG SANSLLAGSGLSVFIASLLLAII
Cwp1	QAPNTVYEQTEN AGAKAAVGMGAGALAVAAAYLL
Cwp2	SSTETISQQTEN GAAKAAVGMGAGALAAAMLL
Tip1	VETASNAGQRVN AGAASFGAVVAGAAALLL
Sed1	SASSHSVVINSN GANVVVPGALGLAGVAMLFL
Tir1/Srp1	ATKAVSEQTENG AAKAFVGMGAGVVAAAAMLL

(6)-mannose(αl,2)-mannose(αl,6)-mannose(αl,4)-glucosamine(αl,6)-inositol phospholipid. The lipid composition among yeast GPI anchors varies. Many cell surface proteins in yeast, for example, Agnl (Lipke et al. 1989), Agal (Roy et al. 1991), Flol (Watari et al. 1994), Sedl (Hardwick et al. 1992), Cwpl, Cwp2, Tipl, and Tirl/Srpl (Table 1.1) (Van der Vaart et al. 1995), have GPI anchors that play important roles in the surface expression of cell surface proteins and are essential for the viability of yeasts. These glycophospholipid moieties are covalently attached to the C-termini

of proteins, and their primary function is to allow for the stable association of proteins with the membrane. GPI-anchored proteins contain hydrophobic peptides at their C-termini. After the completion of protein synthesis, the precursor protein remains anchored in the endoplasmic reticulum (ER) membrane by the hydrophobic carboxyl-terminal sequence, with the rest of the protein being located in the ER lumen. In less than a minute, the hydrophobic carboxy-terminal sequence is cleaved at the ω site (an arrow in Table 1.1) and concomitantly replaced with a GPI anchor, presumably by the action of a transamidase. Because of the covalently linked lipid anchor, the protein remains membrane-bound, exposed initially on the luminal side of the ER and eventually on the cell exterior (Fig. 1.2).

The localization of both a-agglutinin and α-agglutinin to the cell surface occurs through the secretory pathway (Tohoyama and Yanagishima 1985, 1987). Protein secretion in *S. cerevisiae* comprises transfer through various membrane-enclosed compartments constituting the secretory pathway. Secreted proteins are first translocated into the lumen of the ER and then transported from the ER to the Golgi apparatus and finally from there to the plasma membrane in membrane-enclosed vesicles (Schekman and Novick 1982; Schekman 1992). Fusion of the Golgi-derived secretory vesicles with the plasma membrane releases the secreted proteins into the cell exterior. Posttranslational proteolytic modification of the precursors of secretory peptides occurs late in the secretory pathway (in the *trans* cisternae of the Golgi apparatus and secretory vesicles). The Kex2 endopeptidase is located in the *trans* cisternae of the Golgi apparatus in *S. cerevisiae* to remove the proregion of precursors, such as the α-factor pheromone (Wagner et al. 1987). It was proposed that α-agglutinin is further transported to the outside of the plasma membrane through the general secretory pathway in a GPI-anchored form, then released from the plasma membrane by a phosphatidylinositol-specific phospholipase C, and finally transferred to the outermost surface of the cell wall (Lu et al. 1994). Cell wall anchorage of α-agglutinin was accomplished by the addition of a β-1,6-glucan to the GPI anchor remnant of α-agglutinin, in a manner dependent on the prior addition of a GPI anchor to a-agglutinin (Lu et al. 1995; Kapteyn et al. 1996).

In order to target heterologous proteins to the outermost surface of the glycoprotein layer of the cell wall, information on α-agglutinin on a molecular scale was utilized (Murai et al. 1997). The anchoring signal of α-agglutinin was combined with the signal of the secreted enzymes using genetic engineering techniques. Figure 1.2 shows the general structure of the gene necessary for the display of an enzyme on the cell surface. The C-terminal half of α-agglutinin (320 amino acid residues) contains a GPI anchor attachment signal at the C-terminal end, like other cell surface proteins, and is used as an anchoring domain for heterologous proteins since these proteins are covalently linked with glucans.

Schreuder et al. (1993, 1996) reported the successful targeting of α-galactosidase from *Cyamopsis tetragonoloba* seeds, as a reporter, to the cell wall of *S. cerevisiae*. Many proteins/peptides, including those with a relatively large molecular mass and glycosylation requirements, have been successfully displayed on the cell surface of yeast using the cell wall-anchoring domain of α-agglutinin. In the α-agglutinin-based display system, target proteins/peptides are fused to the secretion signal sequence at the N-terminus and to the cell wall-anchoring domain (including the

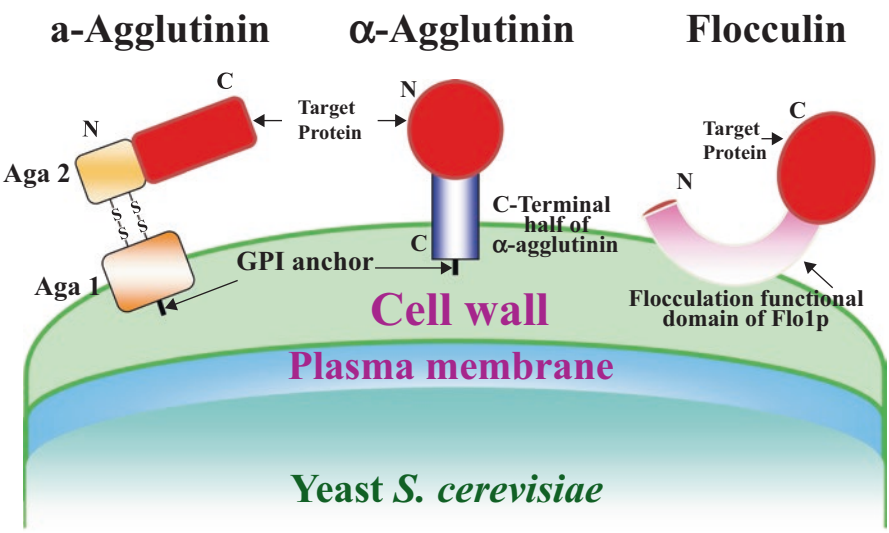

Fig. 1.3 Cell surface display system in *S. cerevisiae.* a-Agglutinin-based display, α-agglutinin-based display, and Flo1p-based display are illustrated

GPI anchor attachment signal sequence) at the C-terminus. Several strategies have been employed to improve the efficiency of cell surface display by *S. cerevisiae*. Vector engineering using a high-copy-number plasmid and an improved host strain enhances the efficiency of the α-agglutinin-based display system (Kuroda et al. 2009). On the other hand, a-agglutinin has been used in the C-terminus-free display system. a-Agglutinin consists of Aga1p and Aga2p subunits. The secreted Aga2p subunit is linked to the Aga1p subunit via two disulfide bonds, which are incorporated into the cell wall. Therefore, in the a-agglutinin-based display system, target proteins/peptides are fused with the C-terminus of Aga2p. In addition, this system has also been used in the N-terminal-free display by fusing target proteins/peptides with the N-terminus of Aga2p (Boder and Wittrup 1997; Wang et al. 2005). Additionally, screening from a cDNA library identified five genes whose overexpression improved the efficiency of the a-agglutinin-based display system (Fig. 1.3) (Wentz and Shusta 2007).

Flo1p, which is a GPI-anchored cell wall protein involved in flocculation, is available for the N-terminal-free display in a similar manner (Sato et al. 2002). Although other GPI-anchored cell wall proteins (Cwp1p, Cwp2p, Tip1p, Sed1p, YCR89w, and Tir1p) have the potential to act as cell wall-anchoring domains, they are less frequently used because of the previous successful performance of α- and a-agglutinin-based display systems (Van der Vaart et al. 1997). Flo1p is also available for the C-terminus-free display, in which truncated Flo1p without the GPI anchor attachment signal sequence is used as an adhesive region. Pir proteins (Pir1–4p) have been used as anchoring proteins in the C-terminus-free display, which permits the extraction of displayed proteins/peptides from the cell wall by alkali treatment (Abe et al. 2004).

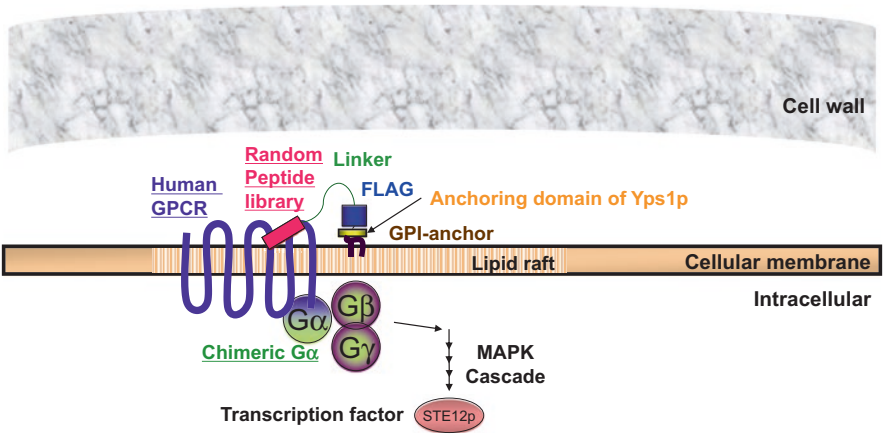

Fig. 1.4 Structure of cell membrane protein (the pheromone receptor Ste2p, which leads to activation of G proteins, the MAPK cascade, and the transcription factor Ste12p) of *S. cerevisiae* and its application. Membrane-displayed alpha-factor (peptide agonist of the yeast pheromone response pathway) activates the pheromone receptor Ste2p, which leads to activation of G proteins, the MAPK cascade, and the transcription factor Ste12p

The methylotrophic strain *Pichia pastoris* can grow on an economical carbon source and allows for high-density culture. Therefore, it is also a suitable host for use in large-scale fermentation cultures of surface-engineered cells. In *P. pastoris*, both N- and C-terminus-free display systems have been established using cell wall proteins (α-agglutinin, a-agglutinin, Flo1p, Pir1p, Sed1p, and Tip1p) from *S. cerevisiae* via the same strategy (Fig. 1.3) (Tanino et al. 2006; Wang et al. 2007, 2008).

The oleaginous yeast *Yarrowia lipolytica* is a heterothallic and dimorphic yeast and has high potential for secreting heterologous proteins, which is a preferred feature in industrial uses. Several cell wall proteins in *Y. lipolytica* have been identified. YlCWP1 and YlPIR1 from *Y. lipolytica*, which are GPI-anchored cell wall proteins, allow N-terminus- and C-terminus-free display systems, respectively (Yuzbasheva et al. 2011).

Almost all displayed proteins/peptides in yeast are localized to the cell wall, as described above. However, the display of proteins/peptides on the plasma membrane is desirable when there is a requirement for interaction with membrane proteins such as receptors. Membrane display of proteins/peptides is performed using the anchoring domain of Yps1p, a GPI-anchored plasma membrane protein. The fusion of this domain with the C-terminus of the peptide ligand allows for display on the plasma membrane and activation of either endogenous or heterologous G protein-coupled receptors (GPCRs) in *S. cerevisiae* (Fig. 1.4) (Hara et al. 2012a, b).

The *S. cerevisiae* strains are the first examples of surface-engineered yeasts in which active enzymes targeted to the cell surface endow the cells with new beneficial properties. These surface-engineered yeast strains were termed “arming yeasts” (Anonymous 1997). The displayed enzymes are also regarded as types of self-immobilized enzymes on the cell surface, with this feature being passed on to daughter cells as long as the genes are retained by the cells. This display system should further enhance the status of *S. cerevisiae* as a novel and attractive microor-

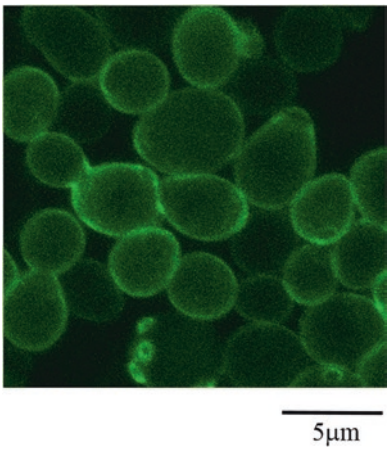

Fig. 1.5 Display of GFP on the yeast cell surface

Fig. 1.6 Illustration of arming yeast

ganism, a "charming yeast (Figs. 1.5 and 1.6)," owing to the fact that it can act as a whole-cell biocatalyst as a result of the surface expression of various enzymes. This is particularly important when the target substrates cannot be taken up by the cells, and this system will make it possible to produce renewable self-immobilized biocatalysts. In studies of the surface display of proteins, previously reported systems were used mainly to obtain possible single-chain antibodies, as in the phage display system, or to explore the use of surface-displayed enzymes as catalysts. On the

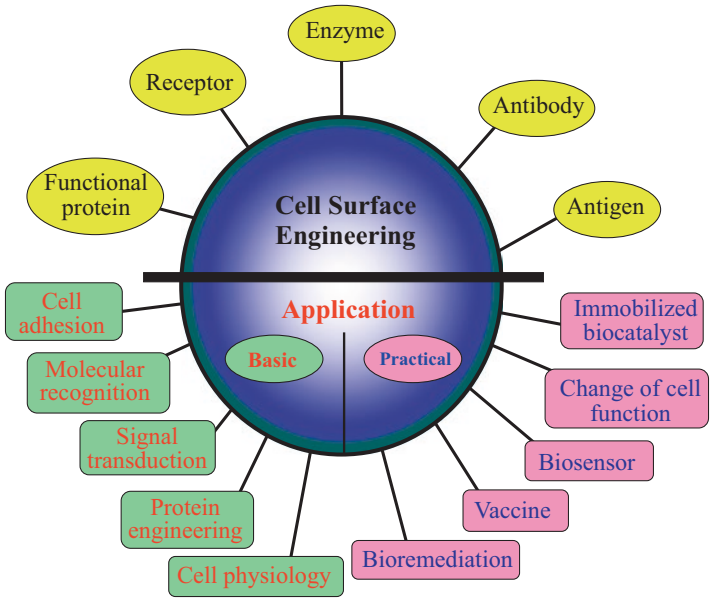

Fig. 1.7 Various applications of cell surface engineering

other hand, cellular engineering studies have focused on changing or improving intracellular metabolic capabilities by the addition or deletion of certain enzymes. Here, cell surface engineering enabled combination of the cell surface display system with the endowment of additional metabolic functions on yeast cells for the first time. Thus, the cell surface can be regarded as a new target for providing additional metabolic functions. This research heralds a new era for cellular or metabolic engineering, not only in yeast but also in all living cells. We believe that cell surface engineering will enable novel abilities to be conferred on all living cells and open up a new field in biotechnology (Fig. 1.7).

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Part II
Whole-Cell Biocatalyst

The arming technology has been applied to yeasts for the construction of whole-cell biocatalysts that can perform saccharification and fermentation. Starch and cellulose are major components in grain and cellulosic biomass, respectively. Therefore, the cell surface display of enzymes for hydrolytic degradation of these components is attempted in order to achieve consolidated bioprocessing (CBP). Furthermore, arming technology has been applied to bioadsorption of toxic metal ions and rare-metal ions, leading to bioremediation and resource recovery. To remove or degrade environmental pollutants other than heavy metal ions, arming yeasts for bioremediation and sensing of pesticides etc. have been constructed. Finally, arming technology has been also used in molecular breeding of stress-tolerant *S. cerevisiae*.

Chapter 2: Energy Production: Biomass – Starch, Cellulose, and Hemicellulose (Dr. Kouichi Kuroda (Japan, Kyoto))

Chapter 3: Energy Production: Biomass – Marine (Dr. Toshiyuki Takagi (Japan, Kashiwa))

Chapter 4: Energy Production: Biodiesel (Dr. Chiaki Ogino (Japan, Kobe) and Dr. Jerome Amoah (Japan, Kobe))

Chapter 5: Cleanup of Pollution: Heavy Metal Ions and Environmental Hormones (Dr. Kouichi Kuroda (Japan, Kyoto))

Chapter 6: Recovery of Rare Metal Ions (Dr. Kouichi Kuroda (Japan, Kyoto))

Chapter 7: Preparation of Functional Cells: Improvement of Stress Tolerance (Dr. Kouichi Kuroda (Japan, Kyoto))

Chapter 8: Bio-sensing Using Cell Surface Display: Principles and Variations of a Cell Sensor (Dr. Seiji Shibasaki (Japan, Kobe))

Chapter 9: Application of Cell Surface Engineering to Biosensing System (Dr. Shin-ichiro Suye (Japan, Fukui))

Chapter 2
Energy Production: Biomass – Starch, Cellulose, and Hemicellulose

Kouichi Kuroda

Abstract Starch, cellulose, and hemicellulose are promising renewable feedstock from terrestrial plants for biofuel production. Cell surface engineering was applied to the construction of whole-cell biocatalyst for the direct production of ethanol from these polysaccharides by displaying enzymes on yeast cell surface. The environmental polysaccharides can be efficiently degraded into monosaccharides by the synergistic effects between the displayed enzymes. The generated monosaccharides are quickly incorporated into the cells and assimilated into ethanol by intracellular metabolic activities. In this chapter, ethanol production from starch, cellulose, and hemicellulose by surface-engineered yeasts is introduced, and the advantages of cell surface display of enzymes, such as their suitability for consolidated bioprocessing (CBP), are described.

Keywords Cell surface engineering · Consolidated bioprocessing · Ethanol production · Starch · Cellulose · Hemicellulose

1 Introduction

1.1 Situation of World Energy

As symbolized by the rapid fluctuations of energy price in recent years, economic growth in developing countries triggers (i) shortage of resource and energy and (ii) global warming, leading to a threat to the world in the near future. According to the recent world energy outlook (WEO) 2017 (International Energy Agency 2017), the global energy demand will rise more slowly than that in the past but is estimated to increase by 30% in 2040 compared to that in the present. Most of the increase is accounted for by emerging countries other than OECD (Organization for Economic Co-operation and Development), especially China, India, and Middle East

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countries. In the breakout of energy resources, it is estimated that coal consumption will be about twice the current amount. Demand for crude oil is also expected to increase greatly, and, in terms of production, even if newly developed oil fields and LNG (liquefied natural gas) are added, concern about the shortage of crude oil would still exist. Although shale gas and shale oil have been known for a long time as potential natural gas and oil resources, escalation of crude oil prices stimulated and prompted the development of mining technologies for shale gas and shale oil (International Energy Agency 2017). Through further technical developments, the production cost of shale oil has declined greatly but is still quite high compared to the cost in large oil fields in the Middle East.

Another major global issue is the accumulation of carbon dioxide due to the increased use of fossil fuels; and climate change due to global warming is a matter of great concern. According to the WEO report, the amount of carbon dioxide emissions related to global energy would increase slightly till 2040, which is far from enough to avoid the serious impacts of climate change (International Energy Agency 2017). Along with the future international cooperations and efforts for reduction of emission, if the concentration of atmospheric carbon dioxide can be maintained at 450 ppm in 2030, the temperature rise will be suppressed by 2 °C. However, this goal cannot be achieved unless we make major policy changeovers and huge investments under the agreement of countries all over the world. Therefore, in addition to reducing the absolute amount of energy use, it is necessary to drastically reduce the consumption of fossil fuel and shift to alternative energy sources. To this end, the use of renewable energy as well as the development of energy-saving equipment is desirable.

1.2 Biomass Feedstock for Energy Production

Biomass energy is one of the promising renewable energies. Biomass is a generic term for organic matter synthesized from water and carbon dioxide by photochemical action of sunlight. Carbon dioxide generated by the combustion of biomass is absorbed by biomass again through photochemical action, so carbon neutrality without increase in net carbon dioxide emission can be realized. Since biomass has low energy density, it is intended not only to burn but also to improve the utilization efficiency by converting it to biogas or liquid fuel by chemical/biochemical processes. Biomass can be classified as follows: (i) biomass used in large quantities as resources (resource crops), (ii) biomass whose existence is known but not sufficiently used and whose utilization can be increased by development (unused biomass), and (iii) valuable biomass that can be used but discarded without being used (waste biomass). In the early stages of biomass energy development, attempts had been made to produce ethanol from the starch of grain biomass such as corn and sugarcane and replace some of the gasoline. However, there is a limitation to the availability of this biomass because they are food crops, and there is competition with food supply. Lignocellulosic biomass is a promising feedstock for ethanol

production by engineered yeasts for the following reasons: (i) cheap and readily available because of more abundant renewable resource than grain biomass and (ii) noncompetitive with food supply. Therefore, in the development of the second generation of biomass energy, lignocellulosic biomass such as waste wood, agricultural waste (rice straw), and herbaceous biomass has been utilized as raw materials for ethanol production.

The main polysaccharide components of lignocellulosic biomass are cellulose (40–50%), hemicellulose (25–35%), and lignin (15–20%). Among them, cellulose is most abundant on earth and is embedded in the cell wall matrix, together with hemicellulose and lignin. In cellulose, a long straight chain of D-glucose is formed by β-1,4 glucosidic linkage, converting the cellulose into the amorphous state due to strong hydrogen bond between the microfibrils. Hemicellulose (glucomannan, mannan, xylan, and xyloglucan), nonhomogeneously distributed in the plant cell wall, is bound to cellulose via hydrogen bond and to lignin via ester and glycoside bonds. Lignin is a hydrophobic and complicated organic polymer, consisting of phenylpropane monomers. The rigid structure of lignin strengthens the physical properties of cell wall but makes cellulosic biomass difficult to degrade.

1.3 Consolidated Bioprocessing (CBP) by Cell Surface Engineering

Ethanol production from cellulosic biomass includes the following four bioprocesses: (i) production of cellulases and hemicellulases, (ii) hydrolytic degradation of cellulose and hemicellulose, (iii) C6 sugar fermentation, and (iv) C5 sugar fermentation. Simultaneous saccharification and cofermentation (SSCF) is a major process for ethanol production. Meanwhile, the process of performing these four bioprocesses in a single fermenter is called "consolidated bioprocessing" (CBP) (Fig. 2.1) (Lynd et al. 2005). In SSCF, the process of enzyme production is separately required, but enzymes are produced in a single fermenter in CBP. Since CBP can make ethanol production more efficiently and downsize the fermenter, it is superior to the other ethanol production processes in terms of reduced cost of production and equipment. To actually realize CBP, it is necessary to construct single species or consortium of multiple species of microorganisms that can produce enzymes, degrade polysaccharides, and perform fermentation. The yeast *Saccharomyces cerevisiae* can perform ethanol fermentation and thus becomes an ideal host for ethanol production in CBP by endowing its saccharification ability.

The technology of cell surface engineering that enables the display of functional proteins on cell surface is suitable for strategy of constructing yeast for CBP system. Using this technology, novel whole-cell biocatalysts that display enzymes have been constructed (Kuroda and Ueda 2011, 2013, 2014). In *Saccharomyces cerevisiae*, 10^5–10^6 target proteins are displayed on the cell surface of a single cell by using α-agglutinin as an anchoring protein. When displaying enzymes, yeast cells

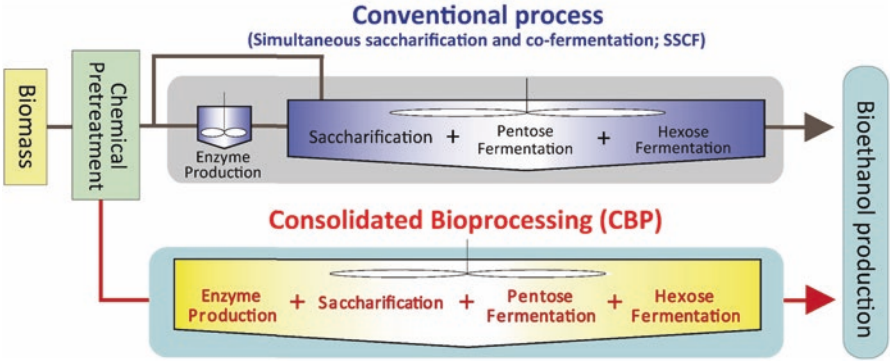

Fig. 2.1 Consolidated bioprocessing (CBP) performing enzyme production, saccharification, pentose fermentation, and hexose fermentation in a single fermenter

function not only as the host for enzyme production but also as the carrier for immobilizing enzymes. Thus, only by a simple operation of cell cultivation, enzyme production and immobilization on the cells can be simultaneously and automatically achieved, and a large amount of whole-cell biocatalyst is easily prepared at low cost. In addition, the displayed enzymes can be easily separated from the product by cell recovery and are functional during the long durations of reaction due to the improved stability of enzymes. Another advantage is that the concentration of monosaccharides in the medium is kept almost zero, because monosaccharides generated by degradation of polysaccharides on yeast cell surface are rapidly imported into the cells. This feature is advantageous in the prevention of bacterial contamination during ethanol production. Furthermore, several kinds of enzymes can be displayed simultaneously on the single yeast cell. The simultaneous display enables multistep reactions on the cell surface and prompts a synergistic reaction between the different kinds of enzymes due to the short distance between them (proximity effect) (Bae et al. 2015).

2 Ethanol Production from Starch

Starch consists of amylose and amylopectin and is one of the most convenient materials in biomass. In the strategy for conversion of starch into ethanol, starch is first degraded to glucose by *Aspergillus*, and then the generated glucose is assimilated into ethanol by *S. cerevisiae*. Since yeast cannot directly utilize starch, it is necessary to confer the ability to degrade starch into glucose, so that they can qualify for CBP. Cell surface display of a starch-degrading enzyme allows yeast to utilize starch directly as a sole carbon source. Glucoamylase from *Rhizopus oryzae* is an exo-type amylolytic enzyme that cleaves α-1,4-linked and α-1,6-linked glucose effectively from starch (Ashikari et al. 1986). A yeast strain displaying glucoamylase from *R. oryzae* was constructed by cell surface engineering technology using

α-agglutinin as an anchoring protein, which grew aerobically on starch, used as a sole carbon source, to produce ethanol (Murai et al. 1997). Additional display of α-amylase, an endo-type amylolytic enzyme from *Bacillus stearothermophilus*, on glucoamylase-displaying yeast improved cell growth on starch-containing medium, compared to the yeast displaying only glucoamylase (Murai et al. 1999). By using another anchoring protein, Flo1p, α-amylase from *Streptococcus bovis* was displayed along with display of glucoamylase from *R. oryzae* by using the anchoring domain of α-agglutinin (Shigechi et al. 2004). The constructed yeast was able to degrade raw corn starch and produce ethanol by assimilating the generated glucose under an anaerobic condition. Therefore, co-display of amylolytic enzymes on yeast cell surface enables the integration of multiple conventional processes from two different microorganisms into a single process.

3 Ethanol Production from Cellulose

Cellulose is a high-molecular-weight polysaccharide consisting of β-1,4 glycoside-linked linear chains of glucose and is a major component of lignocellulosic biomass. There are crystalline and amorphous regions in cellulose, and multiple kinds of enzymes are required for cellulose degradation. This feature makes the degradation of cellulose more difficult than that of starch. Three kinds of cellulolytic enzymes, including endoglucanases (EGs), cellobiohydrolases (CBHs), and β-glucosidases (BGLs), play an important role in cellulose degradation (Fig. 2.2). Endoglucanases act on the amorphous region of the cellulose chain and randomly

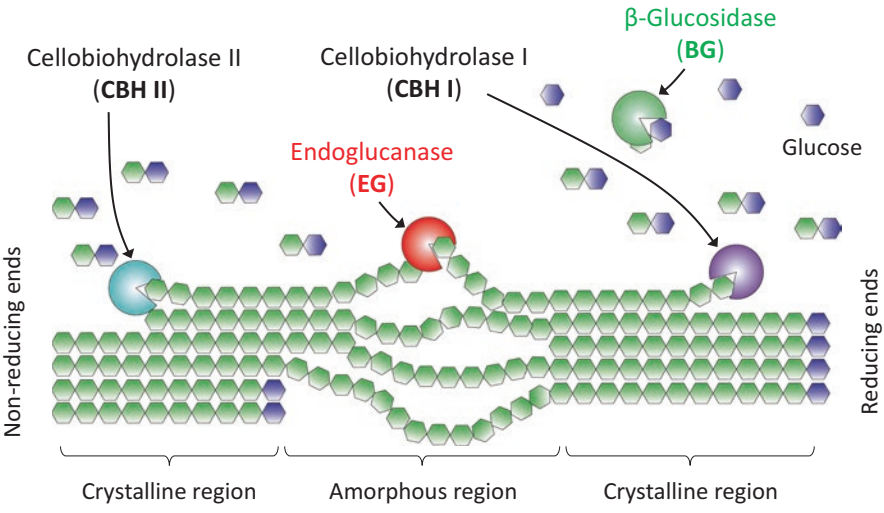

Fig. 2.2 Enzymatic hydrolysis of cellulose by the synergistic action of cellulases (cellobiohydrolases, endoglucanase, and β-glucosidase)

cleave internal bonds to create new reducing or nonreducing ends. Cellobiohydrolases cleave cellobiose and cellooligosaccharide from the ends of the exposed cellulose chain. By the endo-exo synergism of EGs and CBHs, cellulose chains are efficiently degraded to soluble cellobiose and cellooligosaccharide (Medve et al. 1994). β-Glucosidases hydrolyze the products generated by CBHs into glucose. Therefore, the co-display of these enzymes on yeast cell surface can endow yeast cells with the ability to degrade cellulose into glucose.

Carboxymethyl cellulase (CMCase) and β-glucosidase 1 (BGL1) from *Aspergillus aculeatus* were co-displayed on yeast cell surface. The degradation and assimilation of cellooligosaccharides were performed by this surface-engineered yeast in the medium containing cellooligosaccharides as the sole carbon source (Murai et al. 1998). Then, the yeast strain co-displaying BGL1 from *A. aculeatus* and endoglucanase II (EG II) from *Trichoderma reesei* was constructed. This strain showed the ability to grow in the medium containing barley β-glucan as the sole carbon source and directly fermented 45 g/L β-glucan to produce 16.5 g/L ethanol (Fujita et al. 2002). As a further attempt, EG II and cellobiohydrolase II (CBH II) from *T. reesei* and BGL1 from *A. aculeatus* were displayed on yeast cell surface (Fig. 2.3). The cellulolytic enzyme-displaying yeast achieved simultaneous saccharification and fermentation of 10 g/L phosphoric-acid-swollen cellulose (PASC) to produce 2.9 g/L ethanol (Fujita et al. 2004). Although the displayed EG II showed slight activity against PASC, the cellobiose production from PASC by EG II- and CBH II-co-displaying yeast significantly exceeded that by EG II-displaying yeast. This result indicates the importance of CBH II and the synergism between EG II and CBH II.

The display ratio of these cellulolytic enzymes is an important factor that determines the degradation efficiency of lignocellulosic biomass. However, the control of the display ratio of multiple kinds of enzymes was a difficult challenge. A cock-

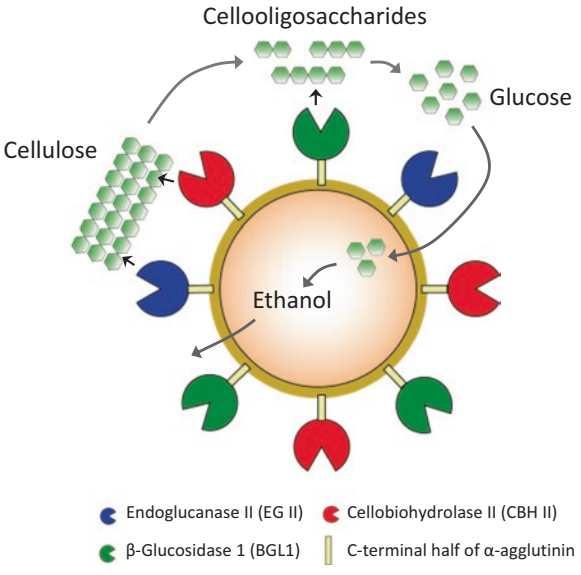

Fig. 2.3 Ethanol production from cellulose by surface-engineered yeast co-displaying cellulolytic enzymes. Endoglucanase II (EG II), cellobiohydrolase II (CBH II), and β-glucosidase 1 (BGL 1) were displayed by fusion with cell wall-anchoring domain of α-agglutinin

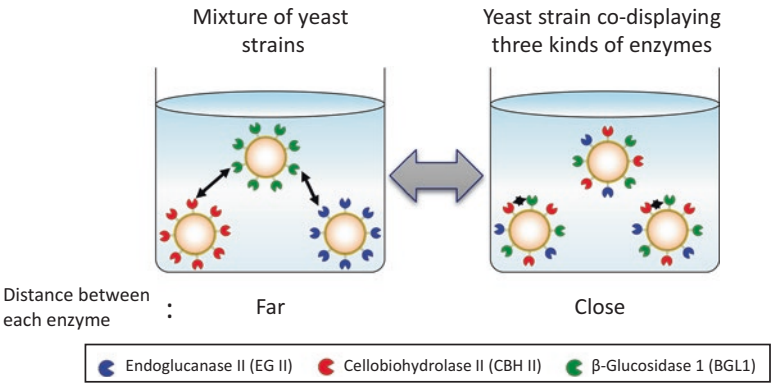

Fig. 2.4 Comparison of PASC-degrading activity between the yeast strain co-displaying cellulolytic enzymes (EG II, CBH II, and BGL1) and the mixture of EG II-displaying yeast, CBH II-displaying yeast, and BGL1-displaying yeast. Mixture of yeast strains was prepared by mixing three kinds of surface-engineered yeasts in a ratio corresponding to the display ratio of enzymes displayed on yeast co-displaying enzymes

tail δ-integration system and evolutionary engineering approach were employed to optimize the display ratio of cellulolytic enzymes by controlling the copy number of the integrated gene expression cassettes. As a result, the yeast strain with optimized display ratio of EG II, CBH II, and BGL1 showed improved degradation ability of PASC (Yamada et al. 2010). Proximity effect promotes synergistic effect of cellulolytic enzymes when they are present within a short distance from each other, which is also an important factor to be considered to improve the degradation efficiency. Actually, Bae et al. (2015) evaluated the proximity effect between the three kinds of cellulolytic enzymes (EG II and CBH II from *T. reesei* and BGL1 from *A. aculeatus*) displayed on yeast cell surface (Fig. 2.4). PASC-degrading activity of surface-engineered yeast co-displaying these enzymes was compared to that of a mixture of EG II-displaying yeast, CBH II-displaying yeast, and BGL1-displaying yeast. The yeast co-displaying the enzymes showed 1.25- or 2.22-fold higher activity than mixture of yeasts displaying single enzyme with an optical density at 600 nm (OD_{600}) of 10 or 0.1, respectively (Fig. 2.5) (Bae et al. 2015). This indicates that proximity effect between the displayed enzymes improves degradation efficiency and that this effect is more prominent at lower OD_{600}.

4 Ethanol Production from Hemicellulose

Hemicellulose is also a major component of lignocellulosic biomass and mainly consists of β-1,4-xylan. β-1,4-Xylan is a complex polysaccharide and is composed of a backbone of β-1,4-linked xylopyranoside. Xylan is degraded into xylose through the hydrolysis of xylan to xylooligosaccharides by endo-β-xylanase and the

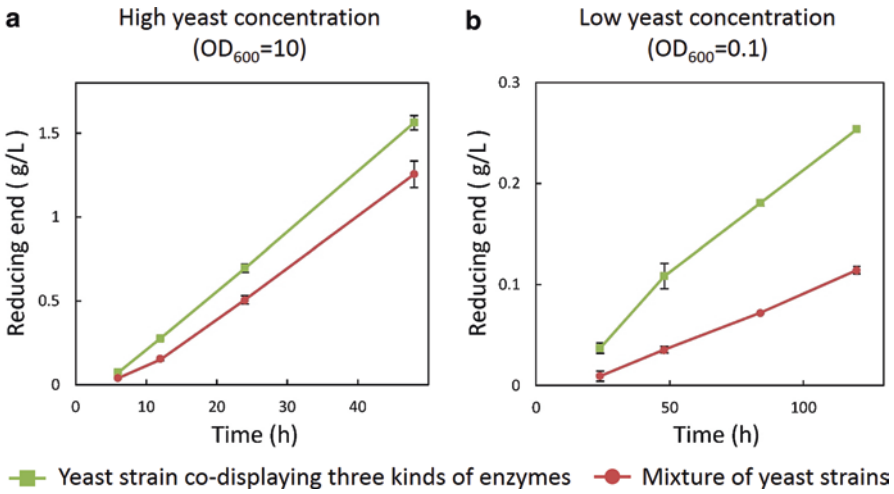

Fig. 2.5 Proximity effect between cellulolytic enzymes co-displayed on yeast cell surface. PASC-degrading reactions were performed by using high (**a**) or low (**b**) concentrations of yeast cells. The reducing ends generated by yeast cells were quantified using the 2,4-dinitrosalicylic acid (DNS) assay

subsequent hydrolysis of xylooligosaccharides to xylose by β-xylosidase (Kulkarni et al. 1999). In addition, the ability to metabolize xylose is required for ethanol production. As the first step toward ethanol production from xylan, these enzymes were displayed on yeast cell surface. Xylanase II from *T. reesei* (XYN II) and β-xylosidase from *Aspergillus oryzae* (XylA) co-displayed on yeast cell surface showed the activity, leading to the successful conversion of xylan to xylose (Katahira et al. 2004). The second step is to convert the produced xylose to xylulose that can be assimilated to ethanol by native metabolic pathway of yeast. There are two available pathways for achieving this step. One is the pathway including conversion of xylose to xylitol by xylose reductase (XR) and conversion of xylitol to xylulose by xylitol dehydrogenase (XDH). However, in this pathway, the difference in coenzyme specificity between XR and XDH causes intracellular redox imbalance and the accumulation of by-products including xylitol and glycerol (Van Vleet and Jeffries 2009; Matsushika et al. 2009). Even so, XR and XDH from *Pichia stipites* and xylulokinase (XK) from *S. cerevisiae* were overproduced in the yeast cells co-displaying XYN II and XylA, resulting in direct production of 7.1 g/L ethanol from birchwood xylan under anaerobic conditions (Katahira et al. 2004). Therefore, the simultaneous saccharification and fermentation of xylan were achieved by a combination of cell surface engineering and metabolic engineering in yeast.

In the other pathway, xylose is directly converted to xylulose by xylose isomerase (XI). The advantage of this pathway is that it does not need coenzymes for the conversion reaction; thus the intracellular redox imbalance can be avoided. However, there are only a few reports showing the successful intracellular production of active XIs (Kuyper et al. 2003; Walfridsson et al. 1996; Brat et al. 2009), and it was a chal-

lenge to realize this pathway in yeast. Ota et al. (2013) attempted to convert xylose to xylulose outside yeast cells by displaying XI from *Clostridium cellulovorans* because environmental xylulose in the medium can be easily imported into the cells through different uptake mechanisms, whereas xylose uptake is inefficient. The constructed yeast displaying XI grew in the medium containing xylose as the sole carbon source and directly produced 0.5 g/L ethanol from xylose under anaerobic conditions (Ota et al. 2013). Metal cations such as Mg^{2+}, Mn^{2+}, or Co^{2+} were reported to improve the catalytic activity and stability of XIs (Callens et al. 1988). Therefore, further study optimized a specified metal cation in the surface-engineered yeast displaying XI from *C. cellulovorans*. Co^{2+} enhanced the activity of the XI displayed on yeast cell surface. Addition of 3 mM Co^{2+} in the xylose-containing medium improved ethanol yields and xylose consumption rates by 6.0- and 2.7-fold, respectively (Sasaki et al. 2017). Furthermore, xylan saccharification and fermentation were achieved by yeast co-culture system consisting of two kinds of yeast strains: (i) XI-displaying yeast and (ii) yeast co-displaying endo-1,4-β-xylanase from *Saccharophagus degradans* (Xyn11B) and β-xylosidase from *Aspergillus niger* (XlnD) (Fig. 2.6). Under the optimized inoculation ratio of both the yeast strains (1:1), 6.0 g/L ethanol was produced from 10% (w/v) xylan (Sasaki et al. 2017).

Both a hexose and a pentose generated from cellulose and hemicellulose are present in lignocellulosic biomass that is used as an actual raw material. Therefore,

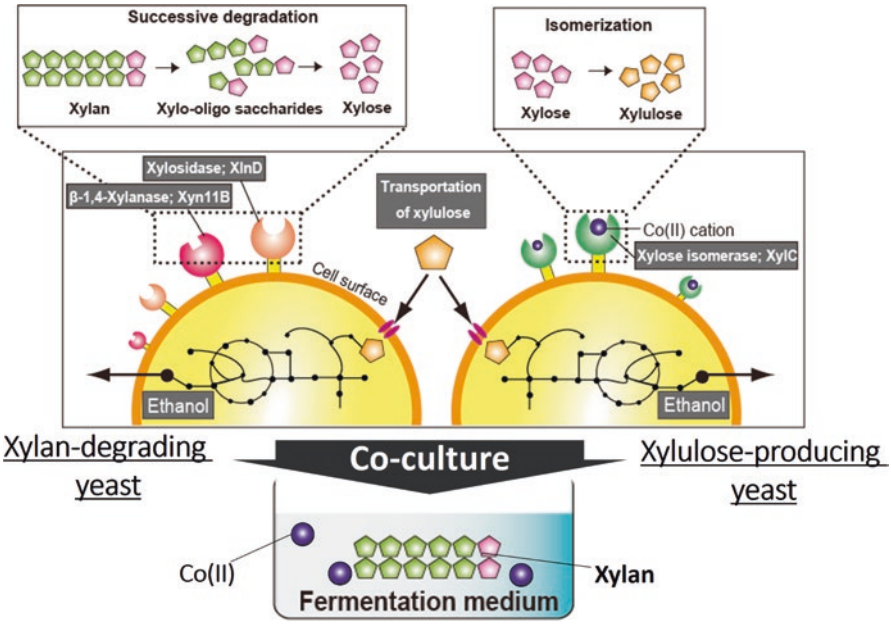

Fig. 2.6 The yeast co-culture system for xylan saccharification and ethanol fermentation. Xylan is degraded into D-xylose by Xyn11B- and XlnD-co-displaying yeast. The D-xylose is converted into D-xylulose by XylC-displaying yeast. Both the yeast strains assimilate the D-xylulose into ethanol

simultaneous assimilation of both types of sugars is required for efficient ethanol production from an actual lignocellulosic biomass. However, a hexose such as glucose is preferentially taken up and assimilated by yeast cells, whereas the uptake and assimilation of a pentose such as xylose start after the depletion of the hexose. In addition, the uptake of xylose is slower than that of glucose, due to the lack of xylose transporter in yeast, making it difficult for the simultaneous assimilation of both the sugars. Some attempts to solve this problem were made by using cell surface engineering. Cell surface display of BGL1 and intracellular production of XR, XDH, and XK were integrated in the yeast cells. The constructed yeast strain degraded xylose and cellooligosaccharides in mixed sugars to produce ethanol by preventing the inhibition of xylose consumption by glucose (Katahira et al. 2006). This could be due to the immediate uptake of the generated glucose from cellooligosaccharides, thereby keeping the glucose concentration low. In the mixed sugars containing 3% (w/v) glucose and 2% (w/v) xylose, xylose uptake by XI-displaying yeast was shown to be faster than that by the yeast producing XI intracellularly (Sasaki et al. 2017), suggesting the advantage of converting xylose to xylulose on cell surface.

5 Summary

Energy production from biomass including starch, cellulose, and hemicellulose is one of the promising approaches for sustainable society. Cell surface engineering technology was applied to the construction of novel whole-cell biocatalysts for degrading polysaccharides into monosaccharides for easier uptake and the subsequent assimilation to ethanol. Using the surface-engineered yeasts displaying various enzymes, multistep reactions can be catalyzed in one type of cell. Therefore, the cell surface display of enzymes is an effective strategy for the construction of microorganisms suitable for CBP. In addition, proximity effect between the displayed enzymes promotes synergistic effect of enzymes to improve reaction efficiency. The easy import of the monosaccharides produced on the cell surface allows sugar concentrations to be almost zero in the medium, thus preventing contamination. Therefore, CBP will be increasingly prevalent by cell surface engineering owing to these advantages in the surface-engineered yeasts, together with metabolic engineering and synthetic biology.

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Chapter 3
Energy Production: Biomass – Marine

Toshiyuki Takagi

Abstract The creation of third-generation biofuels from marine macroalgae is being considered as one of the best alternatives for bioresources because these fuels have no adverse impacts on food supplies. Marine macroalgae aquaculture is highly productive without the necessity for arable land, freshwater resources, pesticides, and fertilizer. Commercial-scale aquaculture of marine macroalgae is used in many countries to produce both food products and their biochemical constituents. Furthermore, the macroalgae contain little or no lignin; therefore, sugars from these biomasses can be released through simple biorefinery processes. Thus, marine macroalgae are expected to be a promising alternative to traditional biomass feedstocks, such as corn and sugarcane, for biofuel production. Several breakthroughs in the fermentative process have demonstrated the potential for marine macroalgae as an energy source. This chapter presents the fermentation biotechnologies for these marine biomasses using yeast cell surface engineering.

Keywords Bioethanol · Marine macroalgae · Yeast cell surface engineering · Laminarin · Alginate · Mannitol

1 Introduction

1.1 Potential of Marine Macroalgae as an Energy Source

The utilization of bioethanol is attracting attention as a clean and sustainable energy source. Compared with petroleum products, bioethanol is less toxic and discharges fewer air pollutants (John et al. 2011); therefore, the development of biotechnologies to enable efficient bioethanol production is essential for sustainable energy in the future. Bioethanol is typically produced from sucrose- and starch-containing feedstocks such as corn, sugarcane, wheat, and sugar beets (Ogbonna et al. 2001;

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Quintero et al. 2008; Wang et al. 2002); however, these biomasses are also edible crops, which result in competition between their use for energy production and their value as foodstuff.

On the other hand, marine macroalgae (i.e., seaweed) are a promising alternative feedstock as biomass for bioethanol production because they contain little or no lignin. The low concentrations or absence of lignin facilitates the release of the necessary carbohydrates using simple biorefinery processes (Wargacki et al. 2012). Thus, using marine macroalgae is an effective method by which low-cost bioethanol can be refined. Marine macroalgae are classified into the following three classes based on their photosynthetic pigments and color schemes: green algae, red algae, and brown algae (Demirbas 2010). They are abundant in the shallow waters of the ocean up to a depth of 118 m, where 0.1% photosynthetic light is available (Vijay et al. 2017). In addition, their growth rate far exceeds that of terrestrial plants, mainly because water for growth is not a limiting factor in an ocean environment (Ross et al. 2008). Brown algae are characteristically larger and have higher productivity than green or red algae. Two genera of brown algae—*Undaria* and *Laminaria*—account for ~50% of the world's aquacultural production of marine macroalgae (Kim 2011). The largest of the brown algae, *Macrocystis* spp., commonly known as giant kelp, grow up to 45 m long and dominate kelp forests along the west coasts of North and South America and at scattered locations in the South Pacific Ocean, including South Africa, southern Australia, New Zealand, and several sub-Antarctic islands (Steneck et al. 2002). The development of the biotechnologies necessary for fermentation of their carbohydrate components is essential for the effective utilization of these species. This chapter focuses on the biotechnologies for fermentation of the carbohydrates in brown algae using yeast cell surface engineering.

1.2 World Production of Marine Macroalgae

In many countries, marine macroalgae are commercially produced for both food and their useful biochemicals. Based on 2006 data (Roesijadi et al. 2010), the annual wild harvest of marine macroalgae was estimated to be approximately 1.1 million wet metric tons (WMT), while the annual aquaculture-based world production of marine macroalgae was estimated to be approximately 15.1 million WMT (Table 3.1). The top ten countries that harvested wild marine macroalgae were those in Asia, South America, Central America, and Europe plus Iceland, Russia, and Australia; however, aquaculture-based production of marine macroalgae is concentrated in Asian countries, mainly in China, which accounted for 72% of all marine macroalgae farming in 2006 (Table 3.1) (Roesijadi et al. 2010). The primary genera for aquaculture-based production are *Saccharina* (*Laminaria*), *Undaria*, *Pyropia* (*Porphyra*), *Eucheuma*/*Kappaphycus*, and *Gracilaria* (Table 3.2) (Pereira and Yarish 2008). Unlike conventional means by which edible crops are produced,

Table 3.1 Top ten producers of wild stock harvests and cultured marine macroalgae plus monetary value (USD) of cultured stock in 2006 (Roesijadi et al. 2010)

Harvests of wild stock			Aquaculture				
Source	Production (metric ton)	% of total	Source	Production (metric ton)	% of total	Value US$1000s	$/metric ton
World total	1,143,273	100.00	World total	15,075,612	100.00	7,187,125	476.74
China	323,810	28.32	China	10,867,410	72.09	5,240,819	482.25
Chile	305,748	26.74	Philippines	1,468,905	9.74	173,963	118.43
Norway	145,429	12.72	Indonesia	910,636	6.04	127,489	140.00
Japan	113,665	9.94	Republic of Korea	765,595	5.08	269,657	352.22
Russian Federation	65,554	5.73	Japan	490,062	3.25	1,051,361	2,145.36
Ireland	29,500	2.58	Korea DPRp	444,300	2.95	244,365	550.00
Mexico	27,000	2.36	Chile	33,586	0.22	52,394	1,560.00
Iceland	20,964	1.83	Malaysia	30,000	0.20	4,500	150.00
France	19,160	1.68	Vietnam	30,000	0.20	15,000	500.00
Australia	15,504	1.36	Cambodia	16,000	0.11	4,000	250.00

Table 3.2 Aquaculture production (metric tons) and monetary value (USD) of the primary genera of marine macroalgae (Pereira and Yarish 2008)

Genus	Production (metric ton)	Value US$1000s	Three main producers
Laminaria	4,075,415	2,505,474.9	China (98.3%), Japan (1.2%), and South Korea (0.5%)
Undaria	2,519,905	1,015,040.5	China (87.1%), South Korea (10.4%), and Japan (2.5%)
Porphyra	1,397,660	1,338,994.7	China (58%), Japan (25.6%), and South Korea (16.4%)
Eucheuma and *Kappaphycus*	1,309,344	133,325.2	Philippines (92%), China (7.5%), and Tanzania (0.5%)
Gracilaria	948,282	385,793.7	China (93.7%), Vietnam (3.2%), and Chile (2.1%)
Total	10,250,606	5,378,629	

aquaculture-based production of marine macroalgae does not require arable land and is conducted using saltwater, thereby avoiding competition for freshwater resources that are necessary for field-crop production. Given these advantages, marine macroalgae are being considered as the third-generation biomass for producing biofuels and biochemicals, circumventing any adverse impacts on food supplies (Goh and Lee 2010; Enquist-Newman et al. 2014).

2 Carbohydrates in Brown Algae

The carbohydrates found in brown algae comprise alginate, laminarin, mannitol, cellulose, and hemicellulose. Lignocellulose biomass, such as that from softwoods, corn cobs, and wheat straw, is mainly composed of cellulose, hemicellulose, and lignin (Bajpai 2016), while brown algae contain a high content of laminarin, alginate, and mannitol; therefore, in this chapter, we have summarized the molecular breeding using yeast cell surface engineering for the utilization of laminarin, alginate, and mannitol.

Laminarin in brown algae is a β-1,3 storage glucan (a glucose polysaccharide) with β-1,6 cross-linked branches (Kadam et al. 2015). The molecular weight of laminarin in *Eisenia bicyclis* is approximately 5 kDa and is within the range of 20–25 glucose moieties (Fig. 3.1a) (Nelson and Lewis 1974; Alderkamp et al. 2007). The high degree of β-1,6 branching makes this laminarin more soluble. As a long-term storage compound in brown algae, the laminarin content can be as high as 35% of the dry weight of the algae, depending on the species, harvesting season, habitat, and method of extraction (Kadam et al. 2015).

Alginate is a complex copolymer composed of α-L-guluronic acid and its C5-epimer β-D-mannuronic acid, linked by 1,4-glycosidic bonds between the pyranose rings of the two monosaccharide components (Fig. 3.1b) (Draget et al. 2005). Alginate is one of the major components of brown algae and comprises 22–30% and 25–44% of the dry weight of *Ascophyllum nodosum* and *L. digitata*, respectively (Qin 2008). In the opportunistic pathogen *Pseudomonas aeruginosa*, alginate is known for its important role in the formation of thick, highly structured biofilms (Hentzer et al. 2001). The biological function and physiological properties of alginate

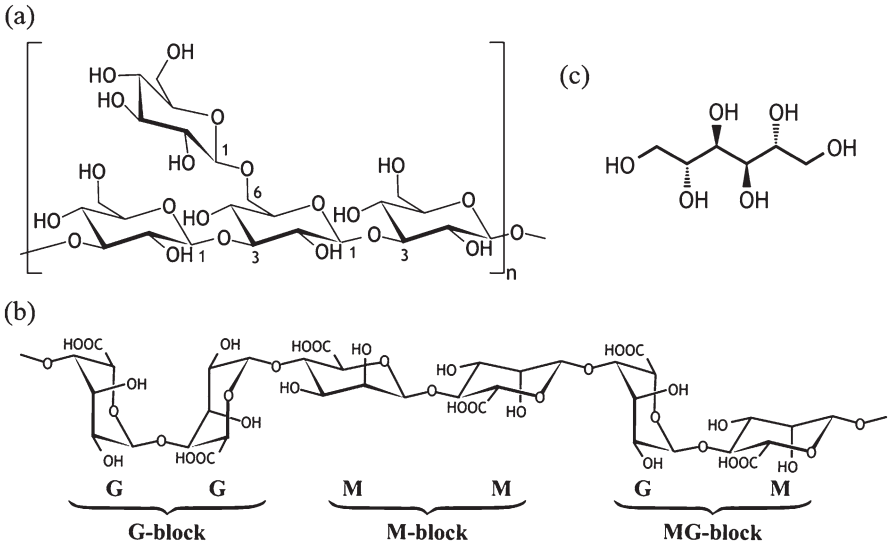

Fig. 3.1 Chemical structures of laminarin (**a**), alginate (**b**), and mannitol (**c**)

are thought to contribute to the strength of the cell walls in brown algae in addition to impairing their flexibility (Smidsrød and Draget 1996). Alginate consists of three sequences of blocks: homopolymeric blocks of mannuronic acid (M-block) and of guluronic acid (G-block) and blocks with a random sequence (MG block) (Davis et al. 2003) (Fig. 3.1b). The difference in the molecular conformation between the M-block and G-block is believed to be chiefly responsible for the variable affinity of alginates to heavy metals (Davis et al. 2003).

Mannitol is another carbon storage compound found in brown algae and is likely to act as an osmoprotectant or local osmolyte (Dittami et al. 2009) (Fig. 3.1c). Mannitol can represent up to 20–30% of the dry weight of the algae, depending on the species, harvesting season, and habitat (Reed et al. 1985). This polyol is one of the primary photosynthetic products and is important for their increasing salt and osmotic tolerance (Conde et al. 2011). The biosynthesis of mannitol from fructose-6-phosphate is catalyzed by the enzymatic reaction of mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase (Groisillier et al. 2013).

3 Bioethanol Production from Laminarin Using Yeast Cell Surface Engineering

Laminarinases, such as β-1,3-glucanase and β-1,6-glucanase, hydrolyze laminarin into glucose. Bioethanol production from laminarin has been attempted with several microorganisms including bacterium, yeast, and fungus (Adams et al. 2009, 2011; Horn et al. 2000a, b; Lee and Lee 2011; Motone et al. 2016; Mountfort and Rhodes 1991). Using focused proteomic analysis, Motone et al. (2016) have found a new laminarinase, Gly5M, in the marine bacterium *Saccharophagus degradans*, and have successfully constructed Gly5M-displaying *Saccharomyces cerevisiae* using yeast cell surface engineering technology. To identify laminarinase, a comparative analysis was conducted on the proteome expressed in *S. degradans* when cultured in the presence of the laminarin or glucose as a sole carbon source. Based on the proteome analysis, 92 laminarin specifically produced proteins, including six carbohydrates, were successfully identified in the samples from three biological replicates of each carbon source. Among the carbohydrases, Gly5M was selected for displaying on the yeast cell surface by comparing its catalytic machinery and molecular weight. Gly5M-displaying *S. cerevisiae* showed laminarin-degrading activity and generated mainly gentiobiose, which is a disaccharide of glucose with a β-1,6-bond. For further degradation of gentiobiose into glucose, *Aspergillus aculeatus* β-glucosidase (BG)-displaying *S. cerevisiae* was also constructed. The coculture of Gly5M- and BG-displaying yeasts produced bioethanol directly from laminarin. The adjustment of the initial cell density of Gly5M- and BG-displaying yeasts was the key factor for efficient bioethanol production from laminarin, and the best mix of Gly5M- and BG-displaying yeasts (35 and 15 in terms of optical density $[OD]_{600}$) successfully produced 5.2 g/L bioethanol from 20 g/L laminarin (a conversion efficiency of 46%).

4 Bioethanol Production from Alginate and Mannitol

4.1 Bacterial Strategies for Alginate Assimilation and Its Application for Bioethanol Production

A large number of alginate-assimilating bacteria have been isolated from seawater, soil, or wastewater (Wong et al. 2000), and the alginate-utilization mechanisms of several bacteria have been characterized (Hayashi et al. 2014; Kabisch et al. 2014; Takagi et al. 2016a). The powerful biomass-degrading marine bacterium *S. degradans* expresses genes that encode for multienzyme complexes that can degrade brown algae carbohydrates, including alginate, laminarin, and cellulose (Weiner et al. 2008). The alginate-assimilation process has been investigated by using comparative proteomic analysis of *S. degradans* cultured with glucose, pectin, or alginate as a sole carbon source (Takagi et al. 2016a) (Fig. 3.2). *S. degradans* produces endo-type and exo-type lyases for cleaving the glycosidic linkages of alginate and producing unsaturated oligosaccharides and a monosaccharide. The products of alginate degradation are incorporated into periplasmic space by TonB-dependent receptors and are further transported into the cytoplasm with a major facilitator superfamily transporter. The unsaturated monosaccharide is spontaneously and/or enzymatically converted into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) (Takase et al. 2010). The enzymatic conversion of the unsaturated monosaccharide into DEH is catalyzed by KdgF, which is annotated as a cupin-like protein (Hobbs et al. 2016). DEH is converted to 2-keto-3-deoxy-D-gluconic acid (KDG) by DEH reductase (DehR) in the cytoplasm. KDG can then be metabolized into pyruvate and glyceraldehyde-3-phosphate (GAP) by KDG kinase (KdgK) and 2-keto-3-deoxy-6-phosphogluconate aldolase (KdgpA) and fed into central metabolic pathways (Takase et al. 2010). A Gram-negative alginate-assimilating bacterium, *Sphingomonas* sp. strain A1, has been also investigated and has provided more details of the alginate-assimilating processes and utilization of alginate for bioethanol production (Takase et al. 2010; Takeda et al. 2011). The alginate incorporation process of *Sphingomonas* sp. strain A1 differs from that of *S. degradans*. When grown on alginate, *Sphingomonas* sp. strain A1 cells form a mouth-like pit on the cell surface for reorganizing and directly transporting alginate into the periplasm. After alginate is degraded into DEH by various endo- and exo-type alginate lyases in the cytoplasm, DEH is metabolized to pyruvate and GAP through the same metabolic pathway described above. Takeda et al. (2011) successfully constructed metabolically engineered *Sphingomonas* sp. strain A1 for direct bioethanol production from alginate. The pathway of ethanol production derived from the ethanologenic bacterium *Zymomonas mobilis* was introduced into *Sphingomonas* sp. strain A1 and deleted the gene that encodes lactate dehydrogenase for suppressing byproduct. The constructed recombinant strain A1 produced 13 g/L bioethanol in three days directly from 60 g/L alginate.

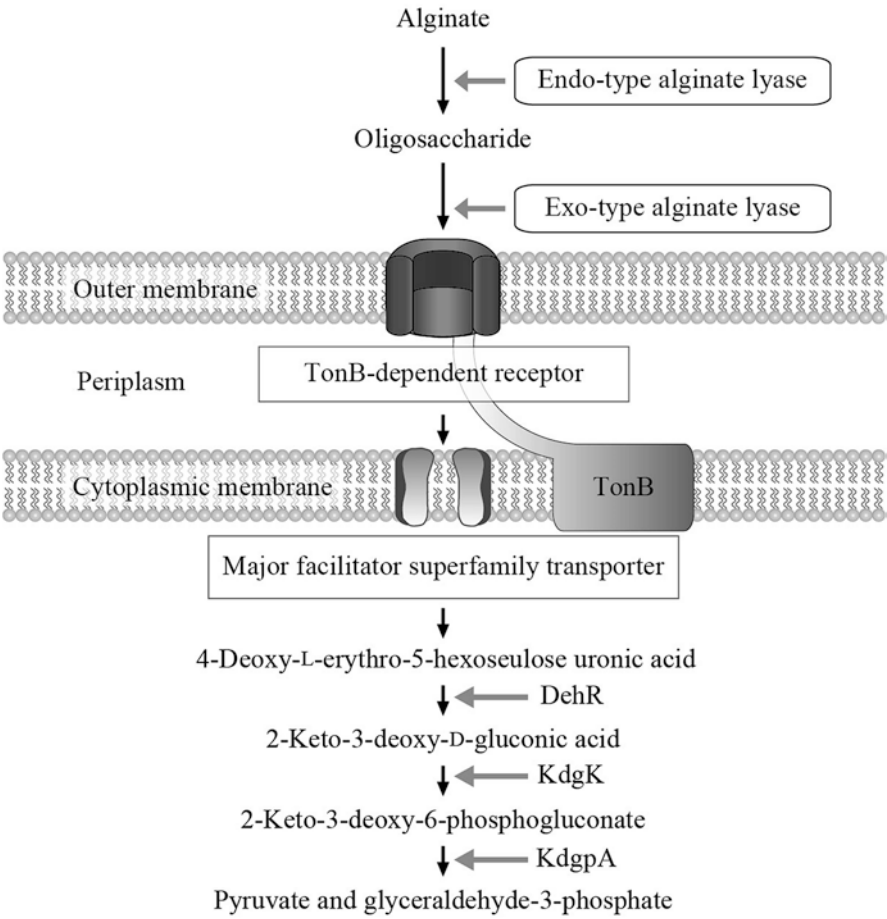

Fig. 3.2 Alginate assimilation mechanism of *Saccharophagus degradans.* Alginate is degraded into monosaccharide by endo- and exo-type alginate lyases. The monosaccharide are converted into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) during transportation into the cytoplasm by TonB-dependent receptor and the major facilitator superfamily (MFS) transporter. DEH is converted to 2-keto-3-deoxy-D-gluconic acid (KDG) by DEH reductase (DehR). KDG can then be metabolized into pyruvate and glyceraldehyde-3-phosphate by KDG kinase (KdgK) and 2-keto-3-deoxy-6-phosphogluconate aldolase (KdgpA) and fed into the central metabolic pathways

4.2 Alginate Degradation Using Yeast Cell Surface Engineering

Among the bioconversion processes used to produce bioethanol, alginate degradation into DEH using the alginate lyases displayed on the yeast cell surface is the essential step in the direct fermentation of carbohydrates to produce bioethanol. Liu et al. (2009) have reported pioneering research on the display of alginate lyase on

the yeast cell surface. The alginate lyase from *Vibrio* sp. QY101 was displayed on the cell surface of yeast *Yarrowia lipolytica* by fusion with the cell wall-anchoring domain of YlCWP1. The alginate lyase-displaying *Y. lipolytica* can degrade polymannuronic acid, polyguluronic acid, and sodium alginate into oligosaccharides; however, the production of the oligosaccharides is insufficient to produce bioethanol from alginate. Although *Y. lipolytica* is an attractive host for lipid production, *S. cerevisiae* is the most commonly used yeast for industrial bioethanol fermentation because of its high ethanol productivity, high stress tolerance, and well-established genetic engineering techniques (Argueso et al. 2009; Cavka and Jönsson 2014; Hughes and Qureshi 2010; Ledesma-Amaro and Nicaud 2016). Takagi et al. (2016b) have successfully displayed various alginate lyases derived from *S. degradans* on the yeast cell surface by fusion with the cell wall-anchoring domain of α-agglutinin. Among the lyases, Alg7A-, Alg7D-, and Alg18J-displaying *S. cerevisiae* showed endo-type alginate lyase activity. In contrast, Alg7K-displaying *S. cerevisiae* showed exo-type alginate lyase activity. Alg7A-, Alg7D-, Alg18J-, and Alg7K-displaying *S. cerevisiae* could degrade both polymannuronic and polyguluronic acid. Furthermore, *S. cerevisiae* that codisplayed endo- and exo-type alginate lyases were constructed for enhanced efficiency of alginate degradation. The *S. cerevisiae* that codisplayed Alg7A and Alg7K had maximum alginate-degrading activity, producing 1.98 g/L reducing sugars in a 60-min reaction (a conversion efficiency of 36.8%).

4.3 Bioethanol Production from Alginate Using Engineered S. cerevisiae

S. cerevisiae lacks not only the ability to degrade alginate but also the ability to assimilate DEH; therefore, the introduction of the DEH transporter and components of the DEH metabolic pathway (DehR, KdgK, and KdgpA) into *S. cerevisiae* is necessary for this species to assimilate DEH. Enquist-Newman et al. (2014) have succeeded in introducing the DEH metabolic pathway into *S. cerevisiae.* First, to identify a DEH transporter from the alginate-assimilating fungi *Asteromyces cruciatus*, *S. cerevisiae* strain BAL2193 to be subjected to transporter expression was constructed by genome integration of the genes for DEH assimilation (*dehR* from *Sphingomonas* sp. strain A1, *kdgK* from *S. degradans*, and *kdgpA* from *V. splendidus*). Ac_DHT1 was identified as the DEH transporter based on comparative RNA-sequencing analysis of *A. cruciatus* cultured in the presence of alginate or glucose as a primary carbon source and a complementary approach using a constitutive expression cDNA library isolated from *A. cruciatus.* BAL 2193 harboring pDHT1-1 showed improved growth in the screening medium containing DEH as a sole carbon source compared to BAL 2193 harboring an empty vector. Because DEH metabolism into ethanol generates a redox imbalance, the ability to metabolize mannitol was introduced into *S. cerevisiae*. The conversion of mannitol into D-fructose by

mannitol-2-dehydrogenase produces excess reducing equivalents. After adapting to the improved growth on the medium containing both DEH and mannitol, the resulting BAL3215 strain produced 36.2 g/L bioethanol from 98 g/L sugar mixture (DEH and mannitol at a ratio of 1:2).

To directly produce bioethanol from alginate, Takagi et al. (2017) have further bred *S. cerevisiae* using a combination of metabolic and yeast cell surface engineering (Fig. 3.3). The alginate-assimilating *S. cerevisiae* recombinant strain Alg1 was created by genome integration of the genes for codisplaying endo-type (Alg7A) and exo-type (Alg7K) alginate lyases and assimilating DEH. The strain Alg1 showed

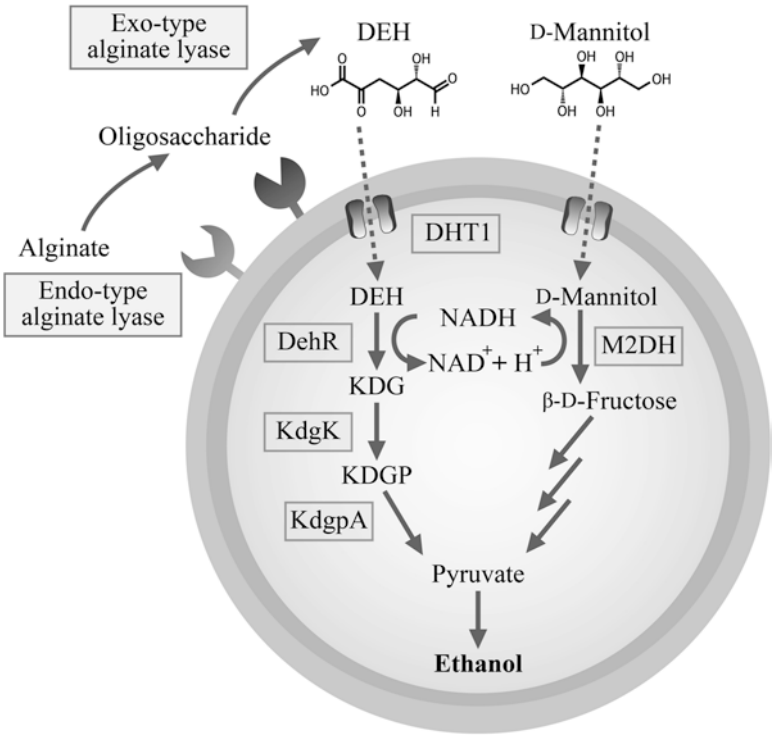

Fig. 3.3 Direct bioethanol production using alginate and mannitol from yeast constructed by a combination of metabolic and yeast cell surface engineering. Alginate is first degraded into oligosaccharides by endo-type alginate lyase displayed on the yeast cell surface. These oligosaccharides are sequentially broken down into a monosaccharide by the displayed exo-type alginate lyase. The monosaccharide is spontaneously converted into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH). DEH is transported into the cytoplasm by a DEH transporter (DHT1). DEH is converted to 2-keto-3-deoxy-D-gluconic acid (KDG) by DEH reductase (DehR). KDG can then be metabolized into pyruvate and glyceraldehyde-3-phosphate by KDG kinase (KdgK) and 2-keto-3-deoxy-6-phosphogluconate aldolase (KdgpA) and fed into the central metabolic pathways. In the mannitol assimilation process, mannitol is transported into the cytoplasmic space by a mannitol transporter. Mannitol is converted into D-fructose by mannitol-2-dehydrogenase (M2DH). The cometabolism of alginate and mannitol enables the regulation of the redox balance in *Saccharomyces cerevisiae*

alginate-degrading activity and better growth in the yeast extract-peptone (YP) medium containing 20 g/L sodium alginate than the negative control strain lacking *dehR*. Because wild-type *S. cerevisiae* was known to acquire the ability to assimilate mannitol during extended culture in a medium containing mannitol as the sole carbon source (Chujo et al. 2015), strain Alg1 was cultured in this medium to regulate the redox balance by the cometabolism of alginate and mannitol. The resulting strain AM1 could assimilate both alginate and mannitol; hence, the optimal conditions for the displayed alginate lyases were unsuitable for bioethanol fermentation by *S. cerevisiae*, and an adjustment of the pH and temperature was a critical factor for efficient bioethanol production from alginate and mannitol. Under optimal conditions (pH 6.0, 37 °C), strain AM1 produced 8.8 g/L bioethanol from 60 g/L sugar mixture (alginate and mannitol at a ratio of 1:2). Although efficient direct bioethanol production from raw brown algae is still challenging, Sasaki et al. (2018) have conducted an unprecedented trial using a coculture system of alginate- and mannitol-assimilating strain AM1 and cellulase-displaying *S. cerevisiae* strain CDY (Bae et al. 2015). Strain CDY displays three types of cellulases on the cell surface—endoglucanase, cellobiohydrolase, and β-glucosidase. After pulverizing the algae, removing the phlorotannins, and performing acid hydrolysis, the resulting brown algae acid hydrolysate was used as a carbon source in YP medium. The AM1/CDY coculture system produced 2.1 g/L bioethanol from 50 g/L brown algae *Ecklonia kurome.*

5 Summary

In this chapter, biotechnologies used in the fermentation of carbohydrates from brown algae using yeast cell surface engineering were introduced. Remarkable progress has been made in the fermentation of brown algae carbohydrates, especially laminarin, alginate, and mannitol, and their conversion into bioethanol. To realize efficient bioethanol production from all carbohydrates in brown algae without excessively loading yeast, the control of the extracellular environment in the coculture system is important. Thus, future studies for optimizing fermentation conditions and yeast genetic modifications, including cell surface and metabolic engineering, will be necessary to produce industrial-scale bioethanol from brown algae.

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Chapter 4
Energy Production: Biodiesel

Chiaki Ogino and Jerome Amoah

Abstract Biodiesel as a renewable fuel can be used in current diesel engines without further modifications. The conventional chemical methods for synthesizing biodiesel can be replaced by more environmentally friendly and low energy requiring enzymatic methods. To reduce the cost of enzymatic methods, whole-cell lipases with high hydrolytic activities which are prepared by the simultaneous cultivation and immobilization of intracellular lipase displaying microbes have been developed. The elimination of multiple steps of enzyme separation, purification, and immobilization of extracellular lipases is promising for improved process intensification. Aside from using refined plant and animal oils as feedstock, whole-cell lipases efficiently produce high yields of biodiesel from unconventional feedstock such as nonedible plant oils and microbial oils, and this is expected to further reduce the cost and improve the sustainability of biodiesel production. This chapter discusses the advances in biodiesel synthesis via whole-cell biocatalysis. Various protein engineering technologies and reaction engineering strategies crucial for the development of whole-cell biocatalysis in biodiesel production have been evaluated. Current challenges facing the industrialization of this technology have been discussed. Addressing these challenges with novel strategies is expected to overcome the barriers in the industrialization of whole-cell biocatalysis of biodiesel and unlock the prospects this technology holds.

Keywords Biodiesel · Whole cell · Lipase · Methyl esters · Oil · Transesterification

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1 Introduction

Fossils have been the major source of fuel over the past decades due to its readily availability. However, with a global increasing concern on the environmental degradation caused by the exploration and use of these fossils and increasing energy demand have sparked the need for more sustainable forms of energy (Demirbas and Balat 2006). Recent advances in biotechnology are paving way for the production of various greener and renewable forms of energy from biomass, and biodiesel has become a plausible substitute for petroleum diesel. As of 2013, biodiesel accounted for a 78% share of the total biofuel consumption in Europe's transportation industry, and its consumption in the United States of America had increased more than 100-folds over the previous decade (Koçar and Civaş 2013, EIA). Biodiesel consists of a mixture of fatty acid methyl esters (FAME) with varying chain length, and it can be readily used in compression-ignition engine without any modification. It is known to produce lower methane emissions and a 41% reduction in the life cycle of greenhouse gases (Hill et al. 2006).

Biodiesel has conventionally been produced from plant and animal oils with chemical methods including the use of NaOH with acyl acceptors such as methanol and ethanol. The use of this conventional method however faces challenges as high amount of energy and complicated post-processing are required. Furthermore, to avoid the competition of feedstock with food sources, low-grade, nonedible oils are being used. Unfortunately, the low purity of these oils presents technical challenges in the use of this conventional method, as 0.5% (w/w) free fatty acid (FFA) in oils rather produces soap and not FAME (Lotero et al. 2005). To alleviate these challenges, enzymatic-mediated processes have been developed by various researchers. The enzymes which are used for these processes are hydrolases which act on ester bonds in carboxylic esters and are categorized as EC 3.1.1.3 lipases. They require only mild reaction temperatures, and most of these enzymes can convert both triglycerides and free fatty acids into fatty acid methyl esters. In addition to the aforementioned advantages, enzymes are highly selective and side reactions are avoided leading to easier separation and product recovery (Ranganathan et al. 2008; Du et al. 2008; Christopher et al. 2014).

Despite these advantages, lipases are generally expensive and contribute significantly to the total cost of biodiesel production. The successful repetitive use of lipases has been suggested to reduce the cost of lipase, and this can be achieved through immobilization of the lipase. The conventional lipase immobilization method is in itself a cost-intensive procedure. This generally requires cultivation of the enzyme-producing microbes, separation, purification, and the immobilization of the extracellular lipase. The concept of whole-cell immobilization thus seeks to address this challenge. This technique only requires a simultaneous cultivation and immobilization of the cell producing the lipase. This drastically reduces the cost of immobilization and further offers an efficient reuse of the lipase. Other challenges faced by lipase-catalyzed biodiesel production are the low reaction rates and loss of activity of the lipase particularly due to the presence of short-chain alcohols in the reaction medium.

This chapter provides a cutting-edge discussion on the historical development of strategies, current challenges, and future opportunities of using whole-cell biocatalysts in the production of biodiesel. A comprehensive analysis on using whole-cell

biocatalysts on vast range of feedstocks geared toward reducing the total cost of biodiesel production through the use of less expensive materials is also presented. Ultimately, the industrial use of whole-cell biocatalysts in biodiesel production with insights into the integrated process chain is further discussed.

2 Design of Whole-Cell Biocatalysts for Biodiesel Production

Whole-cell biocatalysts have a unique advantage in biodiesel production due to the ease with which they can easily be separated from reaction mixture. This advantage is achieved because of the localization of the lipase on cells of their host. In conventional methods, the lipase is overproduced intracellularly, and the cells are used as biocatalysts for the synthesis of methyl esters (Fig. 4.1a). Matsumoto et al. (2001) cloned a gene encoding lipase from *Rhizopus oryzae* IFO4697 (ROL) and constructed an intracellular overproduction system of a recombinant ROL with a prosequence (rProROL) in *Saccharomyces cerevisiae* MT8-1. These systems often require treatment prior methyl ester synthesis due to the poor mass transfer of substrate to the active sites of the lipase. Improvement of the accessibility of substrate to the enzyme's active site can be achieved through air-drying, freeze-thawing, incubation in isopropyl alcohol, or cetyltrimethylammonium bromide solution (Liu et al. 2000; Gowda et al. 1991). These techniques are geared toward the improvement of the activity of the whole-cell biocatalyst via permeabilizing the host cell to enhance better flow of both substrate and products.

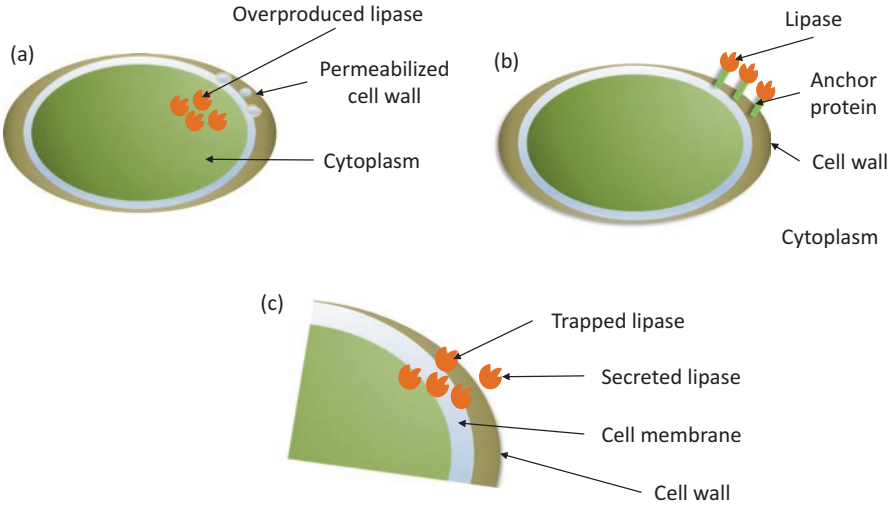

Fig. 4.1 Schematic representation of whole-cell lipase expression systems constructed by (**a**) intracellular overproduction of lipase enhanced by cell permeation, (**b**) using cell wall anchor protein to display lipase on the cell surface, and (**c**) trapping secreted lipase within the cell wall and cell membrane of the host cell

Contrary to this conventional method, latter approaches target the display of the lipase on the surface of the host cell (Fig. 4.1b). The cell-wall anchor protein of the 3′ region of FLO1 gene, FS, which encodes flocculation functional domain of Flo1p is fused with the N terminus of rProROL from *R. oryzae* to achieve a cell-surface display of FSProROL on *S. cerevisiae* MT8-1/. This system showed a hydrolytic activity of 61.3 IU/g (dry cell weight) and produced 78.3% methyl ester in 72 h as compared to 71% in 165 h by the conventional intracellular overproduction system (Matsumoto et al. 2002). The authors further observed that the initial reaction rate of the FSProROL was comparable to free ROL and thus the cell-surface displayed FSProROL may have the same level of accessibility to substrate as the free ROL.

A previous work on the aggregation of *Rhizopus* species revealed that the immobilization of the mycelium in polyurethane biomass support particles (BSPs) altered the morphology of the cells from a pulp-like structure to a pellet-like structure, and this caused an enhancement in the intracellular activities of the lipase of the *Rhizopus* species (Nakashima et al. 1990). In a further investigation of the use of *Rhizopus oryzae* immobilized in BSPs, Ban et al. (2001) successfully produced 90% biodiesel from soybean oil when the culture medium of cells was spiked with 30 g/L olive oil. The extracellular activities reported in this study were rather lower when the mentioned techniques were applied. It was hypothesized that these techniques seem to maintain lipase within the cells. To elucidate this, Western blot analysis was performed on ROL immobilized into BSPs in the presence of olive oil, and the results were further confirmed by an immunofluorescence labeling of hyphal cells (Hama et al. 2006). This analysis revealed that the immobilization of the mycelium on BSPs and the addition of olive oil enhance the localization of lipase on the cell surface. Immobilization of the cells results as a natural course of growth within the spores of the BSPs. Two lipases with molecular masses of 31 kDa and 34 kDa were observed in the culture medium for the cells cultivated in suspension. A quick reduction in the 31 kDa lipase was observed for the culture medium containing BSPs with a relatively stable amount of the lipase on the cell membrane of the mycelium. Generally, secretory lipases from the eukaryotic *R. oryzae* are transported into the endoplasmic reticulum (ER) membrane and into the cell wall which are eventually secreted extracellularly. However, the lipase with 31 kDa molecular mass was integrated into the ER membrane and accumulates in the cell membrane. This form of localization of lipases may depend on the host strain and the type of lipase, but the results of various researchers indicate the localization of lipases on the cell surface of the host (Hoshino et al. 1991; El Abbadi et al. 1995; Davranov et al. 1983; Toskueva 1988). These studies show another phenomenon of cell surface display of lipases in host cells (Fig. 4.1c) which are currently under intense research as whole-cell biocatalysts in biodiesel production.

A number of microorganisms have been used as whole-cell biocatalyst for the production to biodiesel. These microorganisms generally possess lipases on their cell body, and these lipases are responsible for the hydrolysis of ester bonds in carboxylic esters. The extent to which these lipases hydrolyze the ester bonds is expressed as activity and varies from lipase to lipase. In an effort to discover whole-cell biocatalysts possessing “super lipases” with high activity and effectiveness for producing

biodiesel, researchers are exploring various techniques which include screening of wild types, immobilization techniques, application of protein engineering, and engineering of biodiesel process parameters.

In the late 1980s, works by Nakashima et al. (1988, 1989) led to the discovery of high lipase activity of whole-cell *Rhizopus chinensis* through cell immobilization. It was revealed that the use of BSPs further improves the intracellular activity of the whole-cell biocatalyst compared to freely suspended cells. Among a number of support particles tested, a polyurethane material of 635 μm pore size and 0.97 porosity was found to be the best. Cells immobilized within this material showed high intracellular lipase activity of 3750 U/mg which is almost threefold higher than the freely suspended cells and low extracellular lipase activity which is about 1.5-fold lower than the freely suspended cells. The other polymeric materials tested in this work included nylon, polyester, cellulose, polyvinyl alcohol, and stainless steel. Polyurethane BSPs were thus further investigated for the immobilization of six other *Rhizopus* species. Immobilization of the cells into the matrix of the polyurethane material indeed improved the intracellular activities of all six species tested. A later work using *Rhizopus oryzae* showed that the cells form a dense sheath near the surface of the polyurethane cuboid (Ban et al. 2001). Thus, this phenomenon seems to increase the arrest of lipases on the surface of the cells that are trapped in the spores at the near surface of polyurethane BSPs by inducing the formation of pellet-like structures in this region as a result of natural course of growth.

With this phenomenon of cell membrane-bound lipases and advances in genetic engineering which allows the integration of lipase-encoding genes into host microorganisms, novel whole-cell biocatalytic systems have been developed for biodiesel synthesis (Tamalampudi et al. 2007; Hama et al. 2008; Adachi et al. 2011). These works reported on high intracellular activities in whole-cell *Aspergillus oryzae* expressing *Candida antarctica* lipase B (CalB), *Fusarium heterosporum* lipase, and a co-expression of *F. heterosporum* lipase and mono- and diglyceride lipase (Table 4.1). In spite of the fact that lipases are highly substrate-specific, feedstock for biodiesel is very diverse. The ability to express lipases of different specificities in whole-cell biocatalysts thus broadens the avenue for selecting and matching the appropriate lipase to feedstocks.

3 Application of Whole-Cell Biocatalysts in Biodiesel Production

The concept of whole-cell biocatalysis is gaining grounds in biodiesel industry as robust varieties of whole-cell lipases have been developed. Its attractiveness in this industry stems from the cost reduction achieved through one-step lipase production and immobilization. Following previous works of producing whole-cell biocatalysts with high lipase activities, the first use of whole-cell biocatalyst for biodiesel synthesis was developed using refined soybean and methanol as feedstock (Ban et al. 2001).

Table 4.1 Hydrolytic activities of whole-cell lipases constructed by different methods

Lipase	Intracellular lipase activity[a]	Extracellular lipase activity[a]	Reference
R. oryzae	2.67 U × 10^3/mg	283 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus japonicus	2.96 U × 10^3/mg	21 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus niveus	6.25 U × 10^3/mg	76 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus oligosporus	2.80 U × 10^3/mg	71 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus delemar	9.48 U × 10^3/mg	183 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus javanicus	8.33 U × 10^3/mg	158 U × 10^3/mL	Nakashima et al. (1990)
Rhizopus chinensis	3.75 U × 10^3/mg	198 U × 10^3/mL	
F. heterosporum-mono-, di-glyceride lipase B	18.5 U/g[b]	–	Adachi et al. (2011)
CalB	14.1 U/g[b]	–	Adachi et al. (2013)
F. heterosporum[c]	16.3 U/g[b]	–	Takaya et al. (2011)
F. heterosporum	54 IU/g[d]	504 IU/L[d]	Hama et al. (2008)
Rhizopus miehei	130.5 U[e]	–	Adamczak and Bednarski (2004)
Yarrowia lipolytica	90.3 U[e]	–	Adamczak and Bednarski (2004)
R. oryzae	474.5 IU/L[d]	–	Matsumoto et al. (2001)

Note: The activities presented in Table 4.1 where determined using different methods as such it would be erroneous to compare the activities presented in this table. However, these values show that the methods used for constructing the whole-cell lipases resulted in significant intracellular lipase activities

[a]Lipase activity determined using olive oil as substrate

[b]Lipase activity determined using p-nitrophenyl butyrate (pNPB)

[c]Double copies of the lipase

[d]Lipase activity determined by 2,3-dimercaptopropan-1-ol tributyl ester prior crushing of whole cell

[e]Lipase activity determined using olive oil substrate stabilized with Arabic gum

3.1 Mechanism for Whole-Cell Lipase Biocatalysis in Biodiesel Synthesis

Whole-cell biocatalysis of biodiesel from oil occurs via the transesterification of oils and an acyl acceptor. Methanol is widely used as the acyl acceptor as it results in a reaction rate and produce biodiesel with desirable properties. Biocatalysis of transesterification occurs at the active site of whole-cell lipases under suitable reaction conditions. Although the conformation of the active site varies slightly from lipase to lipase, it is made up of a catalytic triad consisting of Ser, His, and Asp

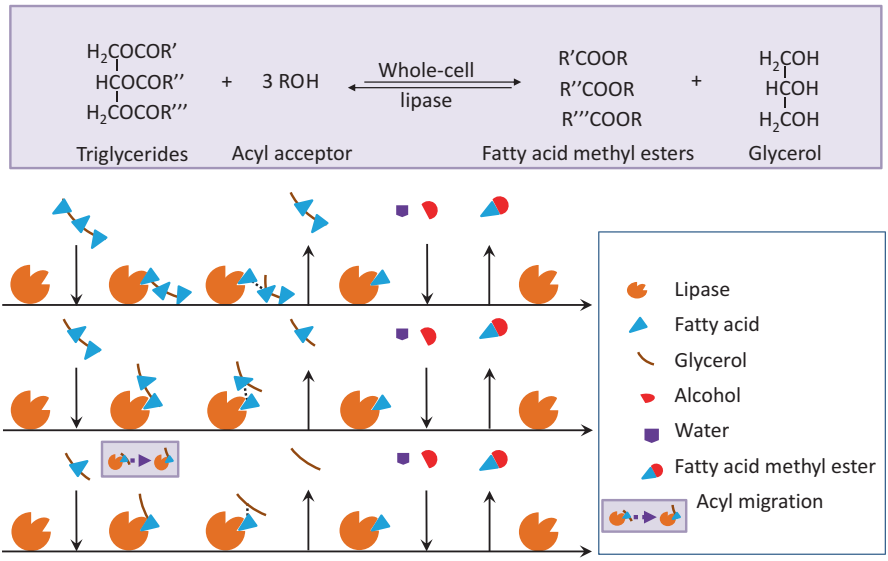

Fig. 4.2 Reactions involved in whole-cell lipase catalyzed synthesis of fatty acid methyl esters (biodiesel) and an overview of the mechanism (Ping Pong Bi Bi)

amino acid residues (Brady et al. 1990; Winkler et al. 1990). The hydroxyl group of serine functions as an electron-rich center and attacks the carbonyl carbon of triglyceride to form a lipase-substrate intermediate. A diglyceride is formed by the transfer of protons to the alkyl oxygen of the substrate via histidine of the active site resulting in a lipase-acyl complex. In the presence of alcohol, this complex reacts with oxygen atoms to subsequently form the fatty acid alkyl ester. The released diglyceride is further taken up as a substrate to produce a second fatty acid alkyl ester and a monoglyceride. Eventually, a third fatty acid alkyl ester is formed from the monoglyceride with glycerol as a by-product (Al-Zuhair et al. 2007). This series of reaction is generally referred to as the Ping-Pong Bi Bi mechanism (Fig. 4.2). The carboxyl moieties of the aspartic acid in the catalytic triad stabilize the serine and the histidine. The catalytic triad itself is buried under a short surface helix referred to as the lid, and it is stabilized by hydrophobic and electrostatic interactions. The displacement of this lid by aqueous-nonaqueous interface is suggested to be the basis for lipase activation.

3.2 Feedstock and Reaction Parameters for Whole-Cell Lipase-Mediated Biodiesel Production

As efforts are being made to produce robust and high efficient lipases, the cost of feedstock and social concerns such as competing resources for food and energy have also instigated the search of appropriate biodiesel feedstock. This presents

another challenge on the use of lipases for biodiesel production. The high substrate specificity and the sensitivity of lipases to reaction conditions could easily be hindered by components of nonconventional feedstocks. In the following sections, the application of whole-cell biocatalysts for the efficient biodiesel production from various feedstocks is presented.

3.3 Conventional Feedstock

The first reported work of whole-cell biocatalysis employed the use of an sn-1,3-positional specificity lipase, *R. oryzae* lipase immobilized within the pores of polyurethane BSPs (Ban et al. 2001). This whole-cell biocatalyst which was cultivated under the support of olive oil successfully attained a 90% methyl ester yield from refined soybean oil. The reported biodiesel yield was comparable to that obtained with extracellular lipase (Kaieda et al. 1999). Refined soybean and other plant oils are referred to as conventional feedstocks for biodiesel production and are mainly made up of triglycerides with saturated and unsaturated fatty acids ranging in chain length from C16 to C18. Stoichiometrically, 66% methyl ester yield is expected from the sn-1,3-regiospecific lipase. However, a further evaluation of this whole-cell biocatalyst revealed that *R. oryzae* catalyst promotes acyl migration from sn-2 of monoglycerides to 1,3-positions, and this is facilitated by the water content in the reaction mixture (Oda et al. 2005).

As proteins, lipases are usually denatured by short-chain alcohols such as methanol and ethanol, which on the other hand are the commonly used acyl acceptors for the transesterification of oils. Despite the availability and affordability, these short-chain alcohols denature the enzymes by disrupting the conformation of the proteins resulting in an alteration of the active sites of the lipases and lead to deactivation. To overcome this challenge, a number of microorganisms from oil containing soil were screened, and the whole-cell biocatalyst of the yeast strain, *Rhodotorula mucilaginosa* P11189, was found to have a high methanol tolerance, producing 83.3% fatty acid methyl esters from palm oil with six molar equivalents of methanol (Srimhan et al. 2011). The stoichiometric molar equivalent of methanol required for the transesterification of triglycerides 3:1. However, since the transesterification of oils is a reversible reaction, an excess amount of methanol is usually required. Most enzymatic biodiesel reactions exhibit signs of deactivation with 1.5 molar equivalent making lipase inactivation by short-chain alcohol one of the bottlenecks of using whole-cell biocatalysts for biodiesel production.

In addition to using methanol-tolerant lipases, other techniques such as stepwise methanol addition, solvent addition, and glycerol removal have been discovered to reduce the risk of lipase inactivation by short-chain alcohols. Shimada and his colleagues successfully produced 98.4% of soybean oil to fatty acid methyl ester using Nov 435, an extracellular *C. antarctica* lipase B immobilized on acrylic resin and known to be one of the effective immobilized lipases (Shimada et al. 1999). The procedure involves a strategic addition of aliquoted methanol to the reaction mix-

ture at several points along the time course of the transesterification, and this method has been successfully adapted for whole-cell biocatalysis. A three-step methanol addition at 24 h interval was used to achieve 87% fatty acid methyl ester from palm oil using a whole-cell *Aspergillus* sp. lipase (Xiao et al. 2010).

The addition of suitable solvents dissolves methanol and reduces the deactivation effect on whole-cell biocatalysts. This strategy has been adopted as a method for reducing the deactivation effect methanol on whole-cell biocatalysts as it avoids the sophisticated stepwise addition of methanol. The choice of solvent should however factor in the tolerance of the lipase to avoid irreversible inactivation by the solvent. Although the ionic liquids [BMIM][BF4] and [EMIM][BF4] were good solvents for conversion of soybean oil to fatty acid methyl ester using whole-cell *R. oryzae* and 4:1 molar equivalent of methanol, [EMIM][TfO] resulted in poor yield (Arai et al. 2010). It was suggested that the ionic liquid as a solvent served as a reservoir for methanol to suppress lipase deactivation. In an effort to identify suitable solvents for whole-cell biocatalysis for biodiesel, organic solvents with different polarity-hydrophobicity (log *P*) were investigated (Huang et al. 2012). It was revealed that solvents with a higher log *P*, that is, more nonpolar, resulted in higher methyl ester yield from soybean oils using *Rhizomucor miehei* lipase-displaying *Pichia pastoris* whole cells. Conventional feedstocks which are often referred to as first-generation feedstock are mainly made of triglycerides. These triglycerides are generally immiscible with acyl acceptors such as methanol and thus the biocatalysis in biodiesel synthesis is thought of to be an interfacial reaction usually involving an aqueous and nonaqueous interface. This aqueous-nonaqueous interface is said to be responsible for the activation of most lipases as such the disruption of the sheath of water around whole-cell biocatalysts by hydrophilic solvents tends to contribute to the deactivation of the enzymatic process. Solvent-tolerant strains of *R. miehei* lipase (RML) have been developed by combining multiple alignments with site-directed mutagenesis. The variants of RML which were immobilized on the cell surface of *P. pastoris* using the *N*-terminal 874 amino acids of Flo1p were found to have 1.1- to 5-fold of the activity of native lipases in the presence of heptane (Han et al. 2011). Despite these novel techniques of using solvents, the extra cost and the potential toxic nature of organic solvents limit the use of this technique.

3.4 Unconventional Feedstock

Conventional feedstocks including soybean oil, sunflower oil, and other plants oils are sources of food and thus their use for fuel production raises concerns on food scarcity. Furthermore, these feedstocks are expensive and contribute significantly to the cost of biodiesel production, making up about 60–80% of the total cost. Reducing the cost of biodiesel production through the use of whole-cell biocatalysts goes hand in hand with choosing cheaper and more sustainable oil feedstock. A new class of feedstock referred to as second-generation feedstock has been paired with whole-cell biocatalysts for biodiesel production. These feedstocks include waste cooking

oils, yellow grease, and other nonedible oils such as *Jatropha* oils. The fatty acid profile of the nonedible oils from *Calophyllum inophyllum* provides compatible properties for biodiesel. Its general high content in free fatty acid makes the conventional alkaline biodiesel conversion impossible. Under optimized conditions, 92% of biodiesel can be achieved with whole-cell *R. oryzae* at 35 °C and 15% v/v water (Arumugan and Ponnusami 2014). The presence of toxic components such as phorbol esters renders *Jatropha* oil nonedible but attractive as a feedstock for biodiesel due to its high availability and low cost. With *Jatropha* as feedstock, immobilized whole-cell *R. oryzae* produced 80.2% FAME yield as against 75.1% by Nov 435 (Tamalampudi et al. 2008). These crude nonedible oils often contain high amounts of free fatty acids. Most EC 3.1.1.3 lipases are effective in hydrolyzing free fatty acids and are usually capable of converting free fatty acids into FAME in the process of esterification. In fact, the reaction rate of esterification of most lipases is higher than the transesterification of triglycerides. As a by-product, water is liberated during esterification of free fatty acids leading to an increase in water content in the reaction medium. The poor water tolerance of Nov 435 is suggested as the cause of low FAME yield due to enzyme deactivation by water. To elucidate the methanolysis of unrefined oils containing high amounts of free fatty acids, a partial oil hydrolysate mainly consisting of free fatty acids and 24% triglyceride was used as a substrate for biodiesel synthesis. Approximately 75.3% FAME was produced by Nov 435, whereas 93% FAME was produced by whole-cell *A. oryzae* expressing *F. heterosporum* lipase (Amoah et al. 2016a). Most of the whole-cell biocatalyst developed for biodiesel synthesis are known to have high tolerance for water. The water liberated as the by-product is suggested to enhance the transesterification activity of the whole-cell lipase through the facilitation of acyl migration of *sn*-2 positioned fatty acid to *s*n-1,3-position. The application of whole-cell biocatalyst for this class of feedstock is cost-effective considering the simplicity of the reaction process.

A more interesting class of feedstock is oleaginous organisms especially microalgae. Although there is no accepted definition for this class of feedstock, they are sometimes included as a second-generation feedstock or referred to as a third-generation feedstock or more generally as advanced feedstock. These oils are viewed as the most sustainable feedstock for the future due to the significant high yields. The microorganisms that produce the oil can be cultivated on non-arable land and grow rapidly, and their cultivation can be merged with the treatment of various waste streams (Chisti 2007; Singh et al. 2011; Ho et al. 2010). These advanced feedstocks vary from conventional feedstocks in quality and composition, as such special techniques are required for conversion by whole-cell biocatalysts. Recent works thus focus on the effective conversion of unconventional oils to biodiesel using whole-cell biocatalysts. Whole-cell *Aspergillus* sp. immobilized on BSP was found to be superior to whole-cell *Candida* sp. using *Scenedesmus obliquus* oils as a feedstock. A Box-Behnken response surface methodology (RSM) approach for reaction optimization reveals that water content between 1% and 5% does not alter the FAME yield catalyzed by the *Aspergillus* sp. FAME yield was however influenced by a temperature range of 35–55 °C, and it was found that the conversion of oil to FAME depends on the collective influence of the reaction parameters (Guldhe et al. 2016).

Parameter interactions such as temperature and number whole-cell loadings interaction and water content and lipase loading interaction show significant influence on this reaction system. A three-step methanol addition approach was employed to the optimized reaction parameter, and 90.8% FAME was achieved (Guldhe et al. 2016). Biodiesel fuel properties depend largely on the quality and composition of the feedstock; however, conversion techniques and post-production steps can influence major fuel properties such as the methyl ester content which the European standards (EN14214) have set a minimum value of 96.5%. Ninety-seven percent FAME was successfully produced from the microalgal strain, *Chlamydomonas* sp. JSC4, using *F. hetersporum* lipase-expressing *A. oryzae* whole-cell biocatalyst. Although this oil feedstock contained high amounts of phospholipids which usually interfere in enzymatic biodiesel process, the high water tolerance of the whole-cell biocatalyst was swiftly used to achieve high yield of biodiesel with a onetime addition of methanol (Amoah et al. 2017). The reuse stability of this whole-cell biocatalyst was found to be more than threefold higher than a lipase cocktail containing the commercially available *Thermomyces lanuginosus* lipase liquid preparation and *Candida cylindracea* lipase after four batches of biodiesel production. The quality of oils has detrimental effect on the whole-cell catalysis by decreasing the reaction velocity and resulting in low FAME yields. Microbial oils vary in quality depending on the strain, cultivation conditions, extraction, and other handling techniques. Oils from *Nannochloropsis gaditana* extracted with a nonpolar solvent hexane result in a lower polar lipid content of 37.4% compared to 49.0% by a more polar solvent, ethanol. Methanolysis of these oils with different polar lipid contents utilizing immobilized whole-cell *R. oryzae* results in a rapid reaction rate for lipids containing lower polar content to achieve a FAME content of 83% where as 58% FAME was achieved from the oils containing higher polar lipid content (Lopez et al. 2016). Moreover, the reuse stability of the whole cells was greatly inhibited by the presence of polar lipids. Polar lipids in the form of phospholipids in itself do not deactivate lipases; however, the coexistence of phospholipids and methanol promotes the inhibitory effect of methanol. It was discovered that, in the use of *F. hetersporum* lipase-expressing *A. oryzae* whole-cell biocatalyst for the conversion of oils containing phospholipids, reverse-micelles formation is facilitated by the presence of phospholipids, and these reverse micelles disrupt the aqueous-nonaqueous interface required for lipase activation (Amoah et al. 2016b; Li et al. 2014). The result of this is a low reaction rate and subsequent deactivation of the lipase by extended residence time of methanol in the reaction mixture. The inhibitory effect of short-chain alcohols on whole-cell biocatalysts can be eliminated by substituting these alcohols with other acyl acceptors. This was demonstrated by using methyl acetate as the acyl acceptor in the conversion of *Chlorella salina* oil using the immobilized yeast strain, *Rhodotorula mucilaginosa* as a whole-cell biocatalyst. To achieve a high conversion rate in the reversible transesterification process, an extra amount of alcohol is added to shift the equilibrium to the product side. An average of 4:1 methanol to oil ratio has generally led to effective FAME production. However, an oil to methyl acetate molar ratio of 1:4 only resulted in about 22% biodiesel, but a further increase to 1:12 molar ratio improved the yield to about 56% (Surendhiran et al. 2014). No signifi-

Table 4.2 Whole-cell lipase-mediated biodiesel production from unconventional feedstocks

Whole-cell lipase	Feedstock	Yield (%)	Remarks	Reference
R. oryzae	*C. inophyllum* oil	92	A flow rate of 25 L/h is favorable for BDF production in PBR	Arumugan and Ponnusami (2014)
R. oryzae	*Jatropha* oil	80.2	Whole-cell *R. oryzae* was showed higher water tolerance than Nov 435	Tamalampudi et al. (2008)
F. heterosporum	*Chlamydomonas* sp. JSC4 lipid	97	Low agitation with high water content	Amoah et al. (2017)
R. oryzae	*N. gaditana*	83	Lipids containing higher polar lipids results in lower reaction rate	Lopez et al. (2016)
R. mucilaginosa	*Chlorella salina*	85	The alcohol acyl acceptor was replaced with methyl acetate	Surendhiran et al. (2014)
CalB + RML	Waste frying oil	91.2	A combination of CalB and RML separately displayed on *P. pastoria* in *t*-butanol and isooctane solvent medium	Jin et al. (2013)
CalB + RML	Gutter oil (waste oil dredged from gutters)	90.1	A combination of CalB and RML separately displayed on *P. pastoria* in *t*-butanol and isooctane solvent medium	Jin et al. (2013)

cant deactivation of the whole-cell biocatalyst can be observed with this acyl acceptor. However, a further increase might result in a dilution of the reaction system and slow down the rate of reaction. Under optimized conditions, 85% FAME yield is obtained from this microalgal strain using the *R. mucilaginosa* whole-cell biocatalyst. A summary of research findings employing the use of whole-cell lipases and unconventional feedstocks has been summarized in Table 4.2.

These results presented by researchers reveals that whole-cell biocatalysis for biodiesel production is affected by the feedstock, water content, type and strategy of acyl acceptor addition, agitation, and the presence or absence of organic solvents. Other parameters include temperature and reaction time. These factors are interrelated and needless to say are dependent on the strain of lipase and type of lipase-producing whole cells. All these parameters are factored in the design of bioreactors for biodiesel production.

4 Bioreactor Design for Whole-Cell Biocatalysis of Biodiesel

For the industrialization of whole-cell biocatalyst for biodiesel production, a number of reactor configurations have been designed. The heterogeneous nature of whole-cell biocatalysts and the reaction medium make the use of stirred tank reactor

(STR) and packed-bed reactor (PBR) more attractive. Stirred bed reactor involves the agitation of the entire reaction mixture through the driving force of a propeller. The propeller facilitates the fine distribution of water bubbles through the reaction mixture, enhancing the creation of aqueous-nonaqueous interface required for lipase activation. STR can either be used for batch processes or continuous processes. Various liquid-solid separation methods can be applied to the reaction mixture at the end of the reaction to separate the whole cells from the reaction mixture to subsequent batch operations. Depending on the feedstock, appropriate washing technique may be required to remove impurities such as glycerol, remaining alcohols, and other polar remnants of oils. The unloading of products and reloading of feedstock lead to a low throughput in batch STR. In continuous mode of operating STR, the reactor is equipped with an in-line liquid-solid separator, usually a membrane. The continuous operation mode offers a better throughput, but reaction control can be complicated especially in situations where feedstock quality varies widely (Tan et al. 2010). Despite the effective reaction interface provided in STR, the shear stress resulting from the driving force of the propeller causes abrasion of the whole cells leading to leaching and physical damage of the whole cells (Adachi et al. 2013). The result of this is the drastic decrease in FAME in the subsequent reuse of the whole cells.

Packed-bed reactor is attractive for the long-term use of whole-cell lipases as physical damage to whole cell is at a minimal. In PBR, the whole-cell biocatalysts are stationed in a column, and the reaction mixture is allowed to flow through the tube at a regulated flow rate. The FAME yield is influenced by the flow rate of the reaction mixture through the stationary whole-cell lipase with a higher flow rate resulting in a higher FAME yield. A change in flow rate from 5 to 55 L/h results in a change in FAME yield from 87% to 91% using *R. oryzae* whole-cell biocatalyst (Hama et al. 2007). The main agitation force in PBR is usually the flow of the reaction mixture through the whole cells. Although this promotes the longevity of the whole cells as less physical damage is caused, this reactor is limited by inefficient formation of aqueous-nonaqueous interfacial area. To improve the efficiency of this reactor type, an external agitation system such as ultrasound is introduced to the reaction mixture prior injection into the packed bed. This promotes the emulsification of the reaction mixture and enhances the distribution of aqueous-nonaqueous interface across the packed bed for efficient reaction. To further improve the longevity of the whole cells, the upward flow of reaction mixture is preferred as this prevents the compression of the whole cells and subsequent pressure drops (Fjerbaek et al. 2009). PBR has been successfully used for the conversion of various plants oils in whole-cell-mediated biodiesel production with modifications such as treatment of the whole cells with glutaraldehyde (Xiao et al. 2011). Although the design of bioreactors for whole-cell biocatalysis is still in its infancy, an in-depth analysis of bioreactors for enzymatic biodiesel has been reviewed (Poppe et al. 2015; Christopher et al. 2014). Lessons from these can find novel applications in the design of appropriate bioreactors for whole-cell biocatalysis of biodiesel.

5 Prospects and Challenges of Whole-Cell Biocatalyst-Mediated Biodiesel Production

A life cycle analysis on biodiesel production suggests that in comparison to chemical methods, enzymatic biodiesel production reduces terrestrial ecotoxicity by 40% and fresh water aquatic and marine aquatic toxicity by 12% and 10%, respectively (Harding et al. 2008). The omission of the neutralization step and the lower energy requirements have been attributed to these reductions. The fewer processing steps and lower energy requirements in producing whole-cell biocatalyst stand to further improve these reductions achieved by enzymatic methods.

There is currently no in-depth report on the techno-economic analysis of biodiesel production using whole-cell lipases, but a close analysis of the current reports on free and immobilized lipase provides a gist of the economic direction of whole-cell-mediated biodiesel production. An estimated cost of the commercially available lipase Lipozyme IM (extracellular *T. lanuginosus* lipase immobilized on non-compressible silica gel) per kilogram of biodiesel produced is $0.14 compared to $0.006 for alkali catalyst (Fjerbaek et al. 2009). This price has been slashed down to $0.03/kg by a Chinese company which uses immobilized lipase of *Candida* sp. 99–125 as a biocatalyst and waste cooking oil as feedstock (Christopher et al. 2014). In other studies, the cost of biodiesel production using immobilized lipase was reported as $2414.63/tonne, whereas $7821.37/tonne was reported for free lipase (Jegannathan et al. 2011). The reuse potential exhibited by immobilized lipase was the major factor for the lower production cost. The elimination of separation and purification steps in whole-cell lipase preparation thus shows potential further reduction of the lipase cost for biodiesel production through process intensification (Fig. 4.3). Recent trends also focus on the use of cheap medium for the cultivation of whole-cell lipases, and this is expected to further reduce the cost of lipase.

Despite these potentials, biodiesel production via whole-cell biocatalysis is yet to see a boom in its industrialization. Laboratory scale experiments have shown steady progress in the development of robust strains and novel process engineering to optimize the yield in whole-cell lipase-mediated biodiesel production, yet significant amounts of unconverted intermediates such as mono- and diglycerides and free fatty acids remaining in the reaction medium. This does not only lead to process inefficiency but also a low-quality biodiesel fuel. Low lipase activities and specificities and limited mass transfer have been suggested to be contributors to this problem. This can be observed in the slow reaction rates requiring long reaction time to achieve the desired FAME content. Further, the use of live microbes, most of which are genetically modified organisms (GMO), handling of these whole cells raises concerns on the safety of this process on an industrial level.

Novel protein engineering strategies can lead to the development of "super whole cells" with multiple lipase expressions and high activities as well as better methanol tolerance capable of effectively enhancing reaction rate (Fig. 4.4). Efforts in process engineering such as mixing of lipases with different activities and in-line product extraction to enhance conversion of intermediates may contribute to a better process

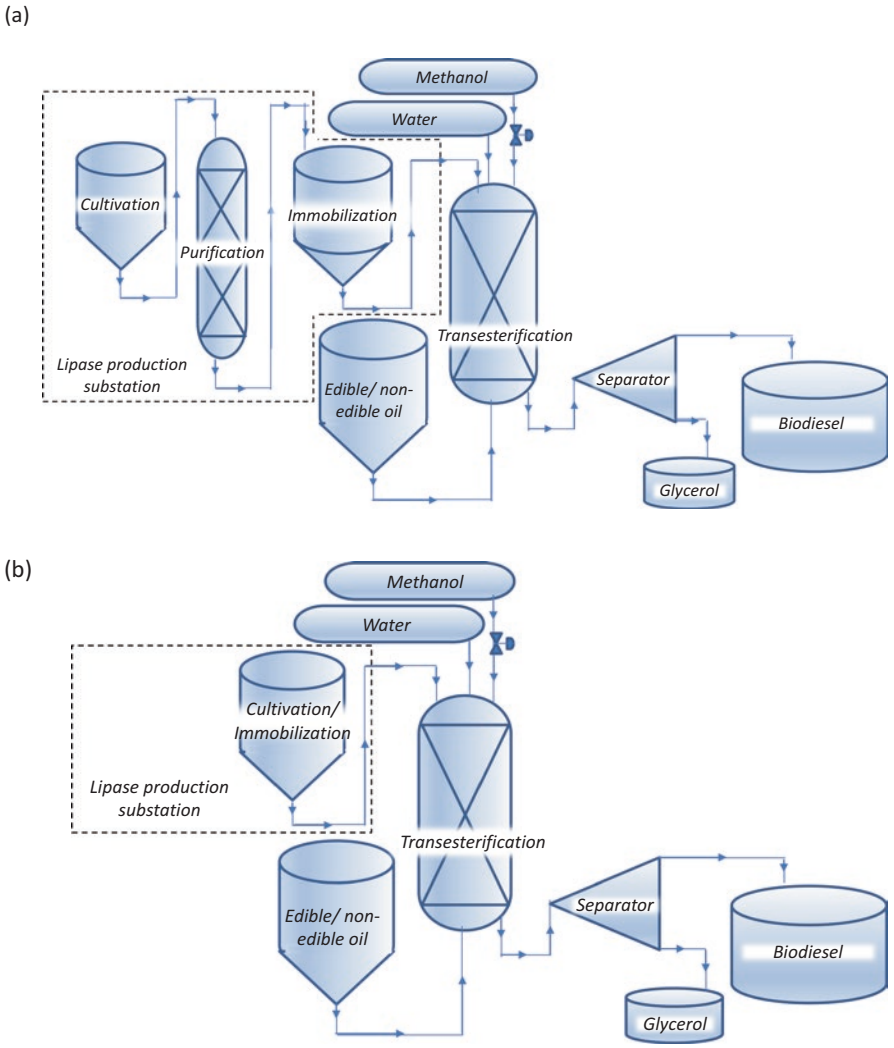

Fig. 4.3 Process layout for enzymatic biodiesel production using (**a**) conventional immobilized lipase and (**b**) whole-cell lipase. The lipase production substation for conventional immobilized lipase requires multiple process steps of cultivation, purification, and immobilization, whereas a simultaneous cultivation and immobilization are used for whole-cell lipase preparation

efficiency. Besides the technological developments, a robust supply chain can be considered for the proper mobilization of logistic can reduce the risk of environmental impact (Hama et al. 2018). This can facilitate proper aseptic handling of whole-cell lipases and provide essential strategies for the containment of these live microbes if they are to be used on a large scale.

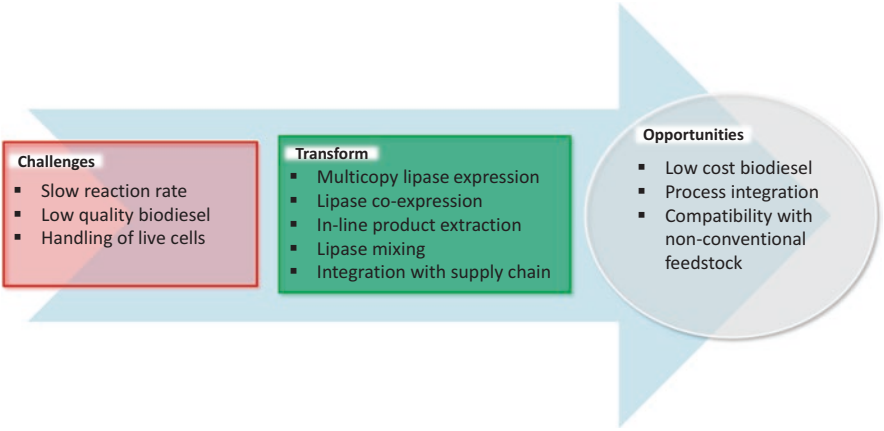

Fig. 4.4 Challenges and opportunities of whole-cell lipase in biodiesel industry

6 Conclusions

Enzymatic methods show high potential to replace chemical methods for biodiesel production due to the lower temperature requirements, environmental friendliness, and the possibility to utilize a broader range of feedstock. The advances in lipase technology with the inception of whole-cell lipases alleviate concerns about the cost of lipases through its cheap production cost by the elimination of costly lipase separation and purification steps, couple with high reuse stability. Whole-cell lipases have been to be versatile in its application with both conventional and unconventional feedstock which could not be achieved with traditional chemical methods. This, however, depends on the whole-cell lipase and the reaction strategy which through advanced research continues to make huge progress. The success of this technology is expected to further make biodiesel production cheaper through the use of cheap oil feedstock such as nonedible plant oils and microbial oils. Nonetheless, technical challenges such as mass transfer barriers and low-quality biodiesel properties should be addressed through novel protein engineering and process engineering strategies to ensure the industrialization of this technology. Containment measures for handling live genetically modified microbes on large scale should be considered for the safe application of this robust technology.

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Chapter 5
Cleanup of Pollution: Heavy Metal Ions and Environmental Hormones

Kouichi Kuroda

Abstract Adsorption of environmental pollutants, such as toxic heavy metal ions and endocrine-disrupting compounds (EDCs), by microorganisms contributes to the cleaning of the environment and industrial wastewater. In the conventional methods, metal ions were accumulated in microbial cells isolated from the nature, and intracellular accumulation was further improved by overexpressing metal-binding proteins. Recently, cell surface adsorption by displaying metal- or EDC-binding proteins/peptides on the cell surface is a novel and effective strategy for the construction of bioadsorbents. Adsorption on the cell surface is rapid and reversible, leading to easy recovery of the adsorbed metal ions without cell breakage. Therefore, bioadsorbents used for the adsorption can be reused or regenerated for further use. These features are useful not only for the effective environmental cleanup but also for the recovery of adsorbed metal ions.

Keywords Cell surface engineering · Environmental cleanup · Bioadsorption · Heavy metal · Endocrine-disrupting compound

1 Introduction

In developed or developing countries, faster economic growth expanded industrial-scale production and promoted urbanization. During this industrialization, economy was always regarded as the top priority, and neither the nation nor the citizens paid any attention to the environment. This resulted in environmental pollution that developed into a social problem impairing the health of people. In particular, water pollution is one of the most important types of environmental pollution that needs to be sincerely addressed because water is an invaluable resource for every creature. The water of seas and rivers is equipped with self-cleaning characteristic which purifies water by removing pollutants during natural circulation. In other words,

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planktons that inhabit the seas and rivers clean the water by eating the pollutants. However, if water pollution increases above a certain threshold level, its self-cleaning action is abolished. Besides, some chemical contaminants, such as heavy metal ions, cannot be cleaned by self-cleaning action. They accumulate in the seas and rivers and contaminate these precious water resources. As a result of environmental pollution, the wastewater standards are getting increasingly strict, and the importance of environmental cleanup and saving water resources is increasing.

Metals are important for living organisms and affect our daily life in various ways. In terms of in vivo function, metals can be roughly classified into the trace metals that are indispensable for living organisms such as humans and nonessential toxic metals. It is necessary to actively ingest essential trace metals from the foods, and their insufficient uptake causes deficiency disorders posing a problem for different life activities. Besides, the nonessential toxic metals are released into the environment by industrial activities and are concentrated in vivo along the food chain, resulting in serious health risks. However, it should be noted that even an essential metal can exhibit toxicity when present in excess amounts and even a toxic metal does not show toxicity at low concentrations. For example, copper and zinc act as cofactors for enzymes that catalyze various in vivo reactions. Thus, while they are indispensable, their accumulation at high in vivo concentrations causes inhibition of enzyme activity by indiscriminate binding with the enzymes. Thus, living organisms have mechanisms for maintaining adequate levels of intracellular metal ions regardless of their concentration in the environment (metal ion homeostasis). Some proteins respond to the excess of metal ions by suppressing their uptake and efflux or by sequestering the excessive metal ions in a nontoxic form. When the metal ions are deficient in vivo, cells actively import them from the environment and utilize the intracellularly stored metal ions. These biological mechanisms for metal ion homeostasis found in the living organisms can be efficiently employed for the cleanup of metal-contaminated wastewater.

Endocrine-disrupting compounds (EDCs) are also an important class of environmental pollutants that need to be removed from the hydrosphere. EDCs have been reportedly found in surface waters, wastewater, sediments, groundwater, and drinking water. EDCs interfere with the endocrine systems of various organisms such as fishes, mollusks, and mammals at very low concentrations, leading to sexual aberrations (Mills and Chichester 2005). In case the EDCs are not removed from polluted water bodies, EDCs released into the hydrosphere persist and can continue to harm. Various chemical compounds that induce estrogen-like responses, such as pesticides, pharmaceuticals, and industrial chemicals, have been identified as sources of EDCs (Giesy et al. 2002). EDCs are classified into different classes, namely, (i) natural estrogens such as estrone (E1), 17β-estradiol (E2), and estriol (E3); (ii) natural androgens such as testosterone (T), dihydrotestosterone (DHT), and androsterone (A); (iii) artificial synthetic estrogens or androgens, such as ethynylestradiol (EE2), norgestrel (N), and trenbolone (Tr); (iv) phytoestrogens such as isoflavonoids and coumestrol; and (v) industrial compounds such as bisphenol A and nonylphenol (Liu et al. 2009). Biological techniques for adsorption of EDCs can thus be utilized for the cleanup of these environmental pollutants.

2 Strategies for Removal of Environmental Pollutants

For environmental protection, it is necessary to prevent the release of pollutants such as metal ions and endocrine disrupters into the environment and remove or decontaminate those that have already been released into the ecosystem. However, it is difficult to remove these pollutants from the environment because they cannot be chemically degraded. In order to remove the metal ions present in wastewater, it is necessary to adsorb these contaminating metal ions on some adsorbent as the first step and to efficiently release the adsorbed metal ions by a change in the physicochemical parameters as the second step. There are two main methods for the removal of pollutants from wastewater: (i) physicochemical techniques and (ii) bioremediation that involves the use of microorganisms as a bioadsorbent. The conventional physicochemical methods include various precipitation and ion-exchange techniques but are often ineffective and costly when applied for low concentrations of pollutants (Kapoor and Viraraghavan 1995; Marques et al. 1991). Recently, the use of biological methods is gaining attention as a promising method for the removal of pollutants. Among these bioremediation techniques, much effort has been made in the development of bioadsorbents (Gadd and White 1993; Eccles 1999; Lovley and Coates 1997). Metal adsorption by microorganisms is advantageous in terms of cost, the ability to adsorb even low concentration of metal ions, and the potential to adsorb target metal ions specifically.

Adsorption of metal ions by microorganisms involves two processes: (i) metabolism-independent bioadsorption on the cell surface by the cell wall polysaccharides, associated proteins, and functional groups (cell surface adsorption) and (ii) transport into the cell by the transporters involved in uptake of essential metal ions and the subsequent sequestration (intracellular accumulation). These processes are thus promising targets for improving metal ion adsorption ability of microorganisms.

3 Intracellular Accumulation of Metal Ions

Intracellular accumulation has been a topic of major focus in the metal ion adsorption by microorganisms. After the import of metal ions into the cells, cysteine-rich metal-binding proteins are produced and sequester metal ions into biologically inactive forms to maintain the balanced concentration of metal ions in the cells. Metallothioneins (MTs), phytochelatins (PCs), and glutathione (GSH) are the typical metal-binding proteins that play an important role in cellular metal homeostasis. These innate mechanisms of cellular metal homeostasis can be utilized for the intracellular accumulation of metal ions. Yeast *Saccharomyces cerevisiae*, one of the appropriate microorganisms for bioadsorption, has the ability to accumulate various metal ions (Vasudevan et al. 2002; Volesky and May-Phillips 1995). Researchers have tried to improve the uptake and intracellular accumulation of metal ions by

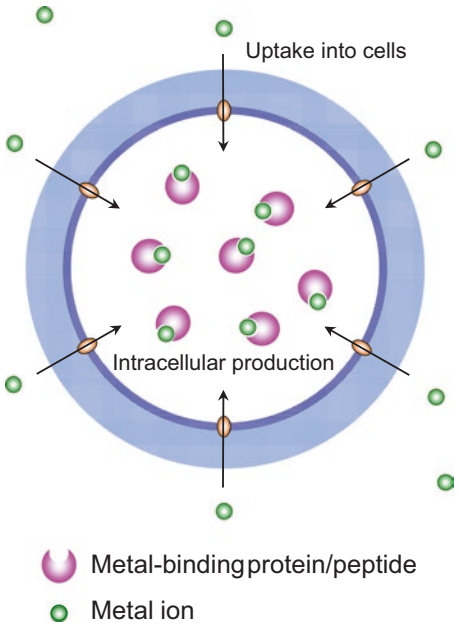


Fig. 5.1 Intracellular accumulation by overexpressing metal-binding protein/peptide in the cytoplasm

overexpressing metal-binding proteins, such as cysteine-rich metallothioneins inside the microorganisms (Fig. 5.1) (Berka et al. 1988; Hou et al. 1988; Pazirandeh et al. 1995; Sayers et al. 1993). However, this strategy depends on the cellular metabolic activities, and the microorganisms that are used for intracellular accumulation must be capable of tolerating the toxicity of accumulated metal ions. Furthermore, recovery of the accumulated metal ions is cumbersome and difficult due to the requirement of cell breakage and purification. This limits the use of bioadsorbents based on intracellular accumulation for removal of environmental pollutants.

4 Cell Surface Adsorption of Metal Ions and Endocrine-Disrupting Compounds

It is desirable not only to remove the metal ions from the environment but also to recover and utilize them efficiently. Adsorption of metal ions on the cell surface is a suitable alternative. In comparison to the intracellular accumulation, the cell surface adsorption offers several advantages. Adsorption proceeds rapidly on the cell surface of living as well as dead cells. A large surface area to the volume ratio of the small sized bioadsorbents provides a large area of contact for interacting with the environmental metal ions. Metal adsorption capacity of bioadsorbents is functional as long as the cell surface is intact. Furthermore, the metal ions adsorbed on the cell surface can be easily recovered without any need of cell breakage, thereby allowing

repeated use of these bioadsorbents. Therefore, cell surface engineering is a promising approach for the construction of novel bioadsorbents (Kuroda and Ueda 2010, 2011b; Li and Tao 2015). In the cell surface engineering, various functional proteins/peptides are displayed on the cell surface in fusion with the cell wall-anchoring domain of cell wall proteins. Yeast *Saccharomyces cerevisiae* is one of the most suitable microorganisms for bioadsorption and is amenable to cell surface engineering. Among the cell surface proteins, α-agglutinin is a mannoprotein that plays an important role in the sexual adhesion of mating-type **a** and α cells (Lipke and Kurjan 1992). Using the cell-wall-anchoring domain of α-agglutinin, many functional proteins/peptides have been successfully displayed on the cell surface of *S. cerevisiae* (Kuroda and Ueda 2011a, 2013, 2014).

4.1 Adsorption of Heavy Metal Ions on the Yeast Cell Surface

To endow the yeast cells with an ability to adsorb heavy metal ions on the cell surface, metal-binding proteins/peptides were displayed by α-agglutinin-based cell surface display system (Fig. 5.2). In this display system, the intended metal-binding proteins/peptides to be displayed are expressed as a fusion protein with a signal sequence for secretion and the cell-wall-anchoring domain of α-agglutinin fused to the N- and C-terminus of metal-binding proteins/peptide, respectively. The gene sequences encoding these components are fused in frame with the sequence

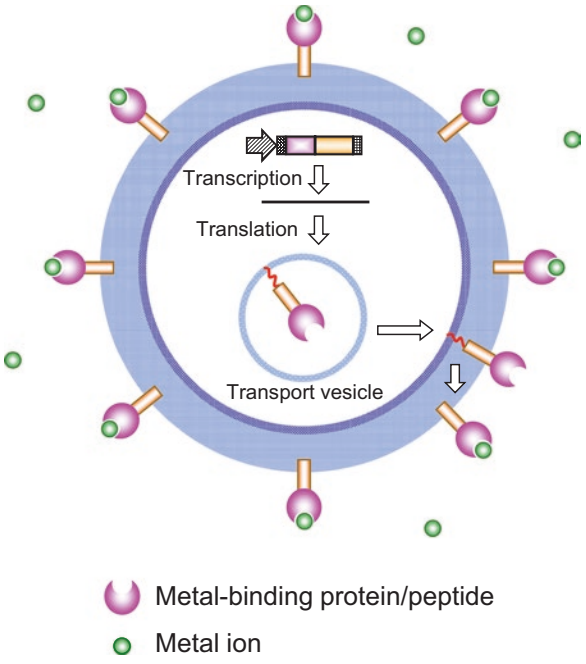


Fig. 5.2 Cell surface adsorption of metal ions by displaying metal-binding protein/peptide on the cell surface

encoding the metal-binding protein/peptide and are expressed from a constitutive promoter such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (Fig. 5.3). A metal-binding hexa-histidine peptide ($[His]_6$; hexa-His) was displayed on the yeast cell surface (Kuroda et al. 2001). Hexa-His is a chelator of divalent heavy metal ions and is widely used as an affinity tag in protein purification (Hochuli et al. 1987). Similarly, a metal-binding protein, yeast metallothionein (YMT) was displayed on the yeast cell surface (Kuroda and Ueda 2003). YMT is involved in maintaining the homeostasis of divalent heavy metal ions in the cells and protects the cells from heavy metal-derived toxicity by binding and sequestering the excess of heavy metal ions due to a large number of cysteine residues. The constructed yeast cells displaying hexa-His or YMT were tested for bioadsorption by incubating them in the solution containing 100 μM of Cu^{2+}, Cd^{2+}, or Ni^{2+}. The heavy metal ions adsorbed on the cell surface were desorbed and recovered by treating the yeast cells with ethylenediaminetetraacetic acid (EDTA). The hexa-His- or YMT-displaying yeast cells showed an increased adsorption and recovery of Cu^{2+} and Ni^{2+} or Cd^{2+}, respectively (Fig. 5.4) (Kuroda et al. 2001; Kuroda and Ueda 2003). Additionally, hexa-His- or YMT-displaying yeast could grow in the medium containing Cu^{2+} or Cd^{2+} at the concentrations at which wild-type yeast could not grow (Kuroda et al. 2001; Kuroda and Ueda 2003). Therefore, the increased adsorption of heavy metal ions on the yeast cell surface by cell surface display of metal-binding proteins/peptides is an effective mechanism for improving cellular tolerance to the heavy metal ions.

Several improvements have been made to further enhance the utility of the yeasts displaying metal-binding proteins/peptides described above. The process of separating the surface-engineered yeasts after adsorption of heavy metal ions for the cleanup of a contaminated hydrosphere was improved. Another function was added to allow the yeast cells to be recovered quickly and easily from the hydrosphere by allowing the yeast cells to undergo self-aggregation upon detecting heavy metal ions inside the cells after adsorption, leading to easy separation of cells (Fig. 5.5) (Kuroda et al. 2002). To this end, Gts1p, which causes yeast cells to show strong aggregation when overexpressed, and the *CUP1* promoter which allows induction of transcription in the presence of Cu^{2+} incorporated into the cells from the environment were utilized. The fusion gene consisting of *CUP1* promoter and an open reading frame of *GTS1* was constructed and introduced into the hexa-His-displaying

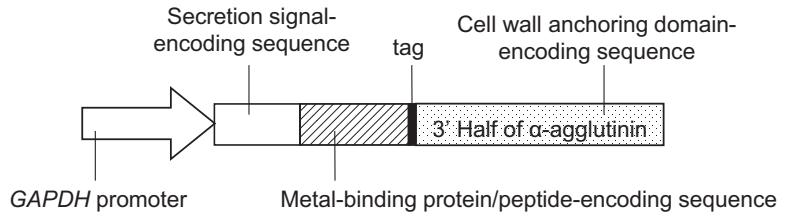


Fig. 5.3 The fusion gene construct used for the cell surface display of metal-binding protein/peptide

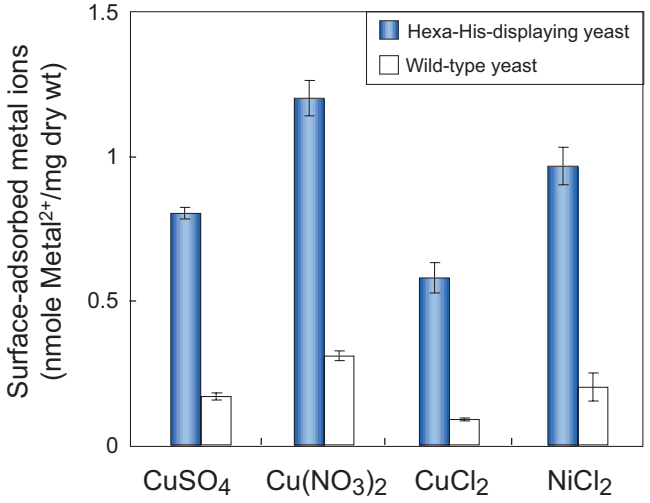


Fig. 5.4 Adsorption of heavy metal ions on the cell surface by hexa-His-displaying yeast

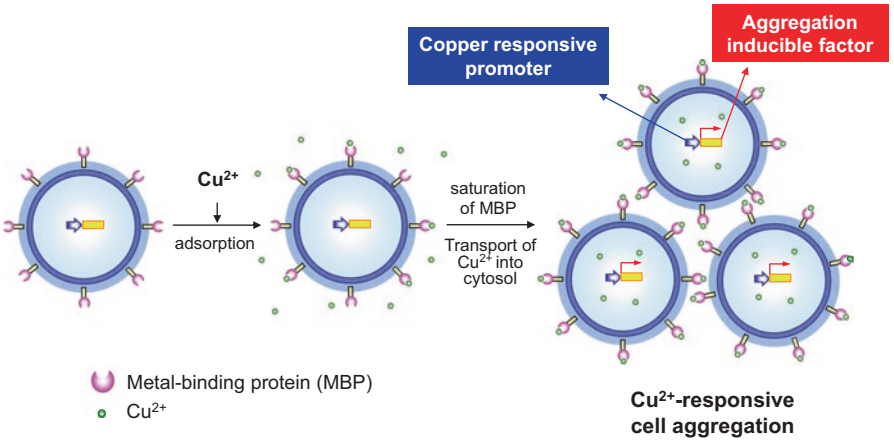
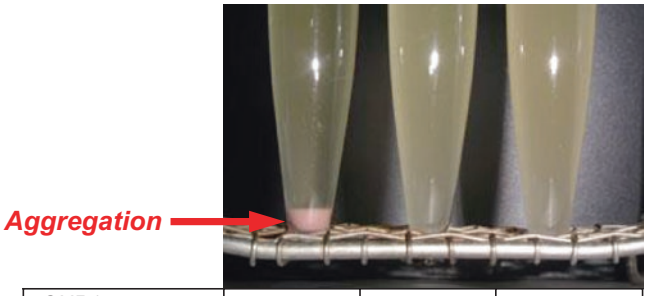


Fig. 5.5 Surface-engineered yeast with Cu^{2+} adsorption ability and Cu^{2+}-responsive cell aggregation

yeast cells. The resulting yeast cells were able to aggregate and settle out in response to Cu^{2+} in the medium (Fig. 5.6). Even a few micromoles of Cu^{2+} in the medium were detected, and cell aggregation occurred within several hours. The constructed yeast is intelligent because it starts cell aggregation after adsorption of Cu^{2+} and its utility as a bioadsorbent can be improved further.

The second target is the property of metal-binding proteins/peptides to be displayed on the cell surface. To further enhance the ability to adsorb heavy metal ions, attempts were made to display a fusion of multiple YMTs in tandem to increase the metal adsorption sites (Kuroda and Ueda 2006). The adsorption and recovery of



CUP1 promoter -*GTS1* ORF	+	+	−
Cu^{2+}	+	−	+

Fig. 5.6 Cell aggregation in response to Cu^{2+} in the medium: Test tubes were allowed to stand static for 5 min after 24 h incubation in the medium containing 100 μM Cu^{2+}

Cd^{2+} on the cell surface of the yeasts displaying YMT tandem repeats were increasingly enhanced with the increasing number of tandem repeats. Therefore, the potential of surface-engineered yeasts as a bioadsorbent is dependent on the metal-binding ability of the displayed proteins/peptides.

4.2 Adsorption of Endocrine-Disrupting Compounds on the Yeast Cell Surface

Cell surface engineering has been used to construct novel yeast strains with an ability to adsorb endocrine-disrupting compounds (EDCs). Specifically, the ligand-binding domain of the rat estrogen receptor (ERLBD) was displayed on the yeast cell surface (Yasui et al. 2002). ERLBD-displaying yeast showed strong binding activity to the fluorescent 17β-estradiol, which is an analogue of the natural ligand of the estrogen receptor, and the binding activity was comparable to that of the native estrogen receptor. The constructed yeast strain allows the measurement of the binding affinity of target hormone-like compounds to the steroid hormone receptors and is useful for the removal of EDCs from the environment.

5 Conclusions and Perspectives

Cell surface engineering is a powerful technology for allowing cell surface adsorption of environmental pollutants, since it is metabolism-independent, rapid, and reversible. Therefore, the adsorbed metal ions can be easily desorbed without cell breakage, allowing reuse/regeneration of the surface-engineered yeasts. Owing to

these features, cell surface adsorption by surface-engineered yeasts is a promising strategy for the removal as well as recovery of toxic metal ions from the polluted environments. By engineering the proteins/peptides displayed on the yeast cell surface, yeast cells with increased ability to adsorb various environmental pollutants can be constructed. Therefore, the cell surface adsorption is a biotechnological strategy that holds great potential for utilization in environmental cleanup.

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Chapter 6
Recovery of Rare Metal Ions

Kouichi Kuroda

Abstract Metals are essential materials that play an important role in several processes of life. Some metals are necessary as trace nutrients but can be toxic at excessively high levels. Other metals are nonessential and toxic even at trace amounts. Along with the development of the high-tech industry in recent years, the use of metals, such as rare metals having various useful characteristics, has been promoted. Rare metals are also called industrial vitamins and are indispensable for various high-tech products. Therefore, their importance has been increasing in recent years. Cell surface adsorption of metal ions can be used not only for removing toxic metal ions but also for recovering useful and rare metal ions. By displaying the proteins that can bind to rare metal ions on the cell surface, it is possible to construct surface-engineered yeasts that can adsorb rare metal ions such as molybdate or uranyl ions. It is important to possess the ability to selectively recover target metal ions from complex ion mixtures, which can be achieved using protein engineering. For example, the introduction of an amino acid mutation into the molybdate-binding protein (ModE) displayed on the yeast cell surface has allowed for the selective adsorption of tungstate ions. Creation of novel metal-binding proteins/peptides with the ability to selectively sequester target rare metal ions is crucial for further development of the technology required to improve our capability of recovering rare metal ions from the aquatic environment using genetically engineered microorganisms.

Keywords Cell surface engineering · Bioadsorption · Rare metal · Resource recovery · Resource recycling

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1 Introduction

Metals are often regarded as a cause of environmental pollution. However, with the possibility of recycling, used metals become a useful and renewable resource. The ability to capture and reuse metals would also benefit the availability of metals. Especially, the demand for rare metals has increased in recent years because rare metals are indispensable for high-tech products, such as automobiles, digital consumer electronics, and information-related devices. Therefore, rare metals are called vitamins of the modern industry. However, compared with other metals, rare metals are not only found in small amounts but also are eccentrically located and highly dependent on producing countries and have a remarkably low substitution ability. The international prices of rare metals are rising and tend to fluctuate owing to the vulnerable supply structure. Although rare metals are globally scarce, large amounts of rare metals are accumulated in high-tech products, and they can be regarded as resources if they can be isolated from their source. Therefore, technologies that efficiently recover rare metals from nature, discarded high-tech products, and industrial wastewater are required to effectively utilize our resources.

2 Strategies for Recovering Metal Ions from the Hydrosphere

To recover metal ions from the hydrosphere, conventional physicochemical techniques, such as precipitation, ion exchange, electrochemical methods, solvent extraction, and membrane filtration, have been used. However, they are costly, ineffective, and not environmentally friendly. Furthermore, it is difficult to recover target metal ions selectively using these conventional methods. Bioadsorption, in which biological functions of microorganisms are used for metal adsorption, is expected to be a promising approach to recover rare metal ions. There are two types of metal adsorption by microorganisms. One is intracellular accumulation by metal-binding proteins/peptides produced in the cytoplasm, and the other is adsorption on the cell surface via polysaccharides, cell surface proteins, and functional groups. Intracellular accumulation is cumbersome owing to the necessity of cell disruption in order to recover the metals. In addition, these bioadsorbents cannot be reused, leading to rising costs. On the other hand, cell surface adsorption is rapid and reversible, allowing the easy recovery of the adsorbed metal ions by mild chemical treatment without cell disruption (Fig. 6.1). Therefore, the reuse of these bioadsorbents is possible. Furthermore, cell surface adsorption is functional in living and dead cells, as long as cell surface structure is maintained. Therefore, the cell surface adsorption is suitable for recovery of metal ions from the hydrosphere.

Cell surface engineering, in which various functional proteins/peptides are displayed on the cell surface by fusing them with the cell wall-anchoring domain of cell wall proteins, has provided a means for cell surface adsorption in the recovery

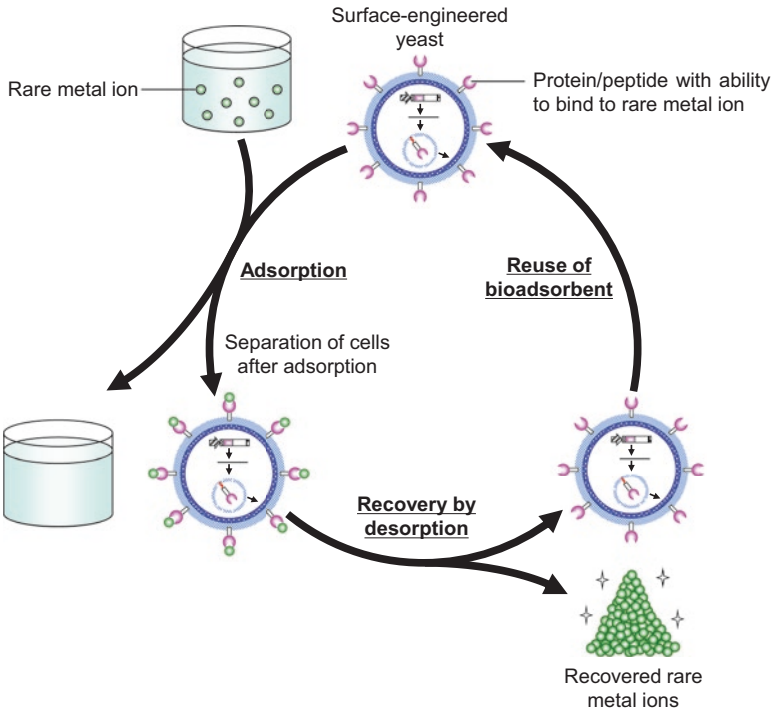

Fig. 6.1 Adsorption and recovery system of rare metal ions using surface-engineered yeast

of metal ions (Kuroda and Ueda 2010, 2011). In cell surface engineering of the yeast *Saccharomyces cerevisiae*, α-agglutinin, a mannoprotein involved in sexual adhesion of mating-type **a** and α cells, is utilized as a cell wall-anchoring domain (Kuroda and Ueda 2013, 2014; Ueda and Tanaka 2000). The ability to adsorb target metal ions can be conferred to cells by displaying metal-binding proteins/peptides on the cell surface. Metal adsorption using cell surface engineering can be customized to target any metal ions by selecting or newly creating metal-binding proteins/peptides according to the target metal ion.

3 Adsorption and Recovery of Molybdate Ions on the Yeast Cell Surface

The cell surface adsorption of metal ions can be applied to the recovery of rare metal ions with high utility in the industry and the development of beneficial bioadsorbents in terms of resource recovery. To this end, biomolecules capable of binding rare metal ions are necessary. Essential trace metals are cofactors of cellular metalloproteins and are indispensable for various enzymatic reactions. The

metal-recognizing and metal-binding capacity of such proteins can be utilized for constructing a bioadsorbent. Molybdenum, a rare metal whose international price has increased in the recent years, has features such as hardness and heat resistance and is highly useful in special steel, electronic parts, catalysts, and lubricants. As the first attempt to recover rare metals, a bioadsorbent that adsorbs and recovers molybdenum was constructed by cell surface engineering. There are molybdenum-binding proteins that utilize molybdenum as a cofactor in various organisms (Hille 2002). In *Escherichia coli*, ModE acts as a transcription factor that regulates the expression of a transporter required for the uptake of environmental molybdenum (Self et al. 2001). It consists of a DNA-binding domain, a linker domain, and a molybdate-binding domain and induces the transcription of downstream genes. Therefore, the full length or the C-terminal molybdate-binding domain of ModE was displayed as a molybdate-binding molecule for the construction of molybdate-adsorbing yeast *Saccharomyces cerevisiae* (Nishitani et al. 2010). Both of the engineered yeast strains showed increased adsorption of molybdate ions when compared to control yeast (Fig. 6.2). About 50% of the adsorbed molybdate ions could be recovered by the treatment of yeast cells with papain, a well-characterized cysteine protease (Nishitani et al. 2010). Therefore, cell surface adsorption of metal ions is effective not only for toxic heavy metal ions but also for rare metal ions.

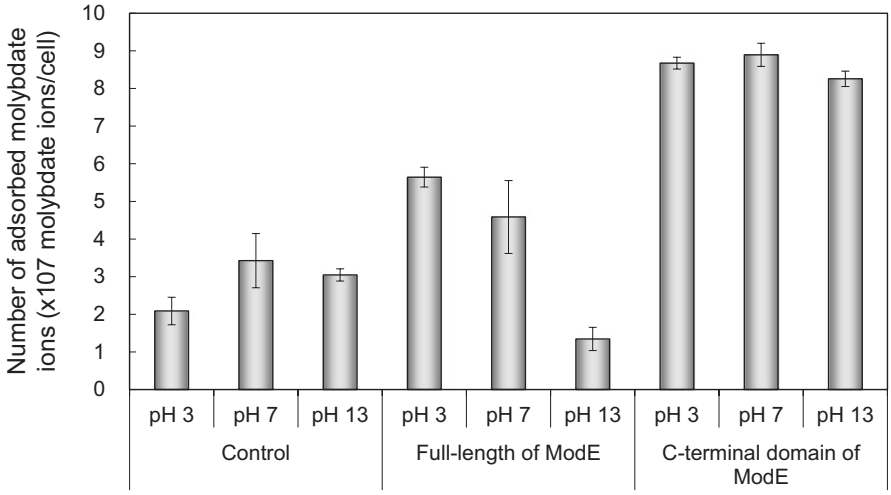

Fig. 6.2 Adsorption of molybdate ions by yeast cells displaying the full-length or C-terminal domain of ModE. The surface-engineered yeasts and its control strain (display of strep-tag) were incubated in a 10 mL solution containing 100 μM molybdate ions at an OD_{600} of 20 for 2 h at 3 °C. The amount of residual molybdate ions in the supernatant was quantified by inductively coupled plasma mass spectrometry (ICP-MS)

4 Adsorption of Uranyl Ions on Yeast Cell Surface

Uranium is another high-demand rare metal. It is used for fuels that generate nuclear power. Uranium is mainly supplied from mining. If the demand of uranium increases in future, securing new supply sources and producing technologies will be necessary. In addition to mines, uranium exists in a small amount in seawater and other hydrosphere components, and its reserves far exceed the minable uranium. For the adsorption of such low concentrations of metal ions, bioadsorption using biological functions is effective. Therefore, molecular breeding of yeast with the ability to adsorb uranyl ions on the cell surface was performed by displaying a uranyl ion-binding protein on the yeast cell surface. As a uranyl ion-binding protein, a NikR mutant protein from *E. coli* was displayed on the cell surface. The NikR protein consists of 2 domains, 47 amino acids at the N-terminus and 86 amino acids at the C-terminus. The N-terminal domain shows homology with a transcription factor and has DNA-binding ability, while the C-terminal domain shows high-affinity nickel ion-binding ability (Schreiter et al. 2003). Although NikR is originally a protein with nickel ion-binding ability, a NikR mutant introduced with three amino acid mutations (Val72Ser, His76Asp, Cys95Asp) in the C-terminal domain can bind uranyl ions (Wegner et al. 2009). Therefore, the C-terminal domain of the NikR mutant (NikR-Ct) was displayed on the yeast cell surface (Kuroda et al. 2014). Because NikR-Ct binds uranyl ions as a dimer, two NikR-Ct were tandemly fused using a flexible peptide linker consisting of amino acids with small side chains (Gly and Ser) and displayed on the cell surface (Fig. 6.3). The lengths of the linker between the two NikR-Ct and the linker between NikR-Ct and α-agglutinin were variously set, and the optimum combination of linker lengths was determined. The lengths of both linkers influenced the adsorption ability. The constructed yeast displaying NikR-Ct tandem repeats with optimized linker lengths showed enhanced adsorption of uranyl ions compared to control yeast (Kuroda et al. 2014).

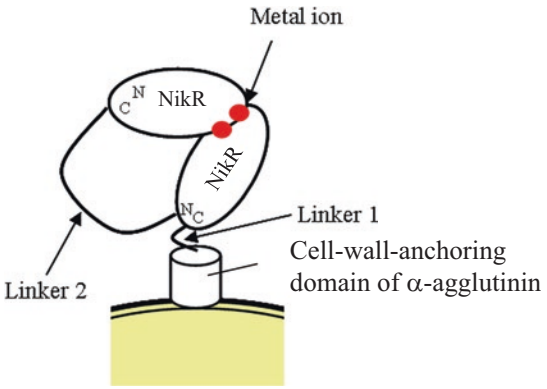

Fig. 6.3 Cell surface displays of NikR-Ct tandem repeat on yeast cell surface. Two NikR-Ct were tandemly fused via a flexible linker 2 and fused with cell wall-anchoring domain of α-agglutinin via a flexible linker 1

5 Recovery of Platinum(0) by the Reduction of Platinum Ions

Platinum is a rare metal used in catalytic converters and fuel cells. Its crustal abundance is extremely low, and the Republic of South Africa accounts for about 70% of platinum production. Therefore, for a stable and continuous supply of platinum, technologies to recover platinum ions from the hydrosphere are required. Cell surface adsorption has been developed by displaying metal-binding proteins/peptides on the cell surface for the selective recovery of rare metal ions. In this system, rare metals are recovered as ions in a concentrated aqueous solution. In a further attempt, a yeast strain capable of reducing platinum ions and recovering them in solid form from the aqueous solution was constructed. In nature, sulfate-reducing bacteria (SRB) reduce metal ions such as chromate and uranium (Michel et al. 2001; Payne et al. 2002). The genus *Desulfovibrio*, one of the SRB, was reported to reduce $PtCl_4^{2-}$ in aqueous solutions to elemental platinum [Pt(0)] by hydrogenases on the cell membrane (Riddin et al. 2009). However, the growth rate of SRB is slower than that of yeast, and SRBs are more difficult to handle because they are anaerobic prokaryotes. Therefore, a hydrogenase from *Desulfovibrio vulgaris* or *D. desulfuricans* was displayed on cell membrane of the yeast *S. cerevisiae*. The display system on the cell membrane has been developed by using a part of Yps1p form *S. cerevisiae* as an anchoring domain to the cell membrane (Hara et al. 2012). The anchoring domain of Yps1p from *S. cerevisiae* was fused with the hydrogenases to allow for their display outside the cell. Under anaerobic conditions without any electron donors, hydrogenase-displaying yeasts reduced platinum ions and produced black nanoparticles in $PtCl_4^{2-}$ solution (Fig. 6.4). The recovery of platinum in a solid state

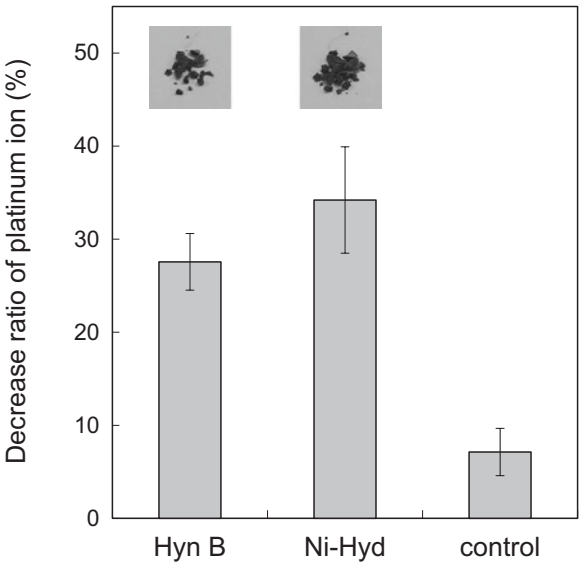

Fig. 6.4 Platinum reduction by hydrogenase-displaying yeasts. For reduction reaction, the surface-engineered yeasts were anaerobically incubated in phosphate-buffered saline (pH 7.4) containing 100 μM platinum ions at 37 °C for 72 h with shaking. After incubation, cells and black precipitates (pictures in the graph) were separated from the reaction solution by centrifugation. The residual platinum ions in the supernatant were quantified by ICP-MS

from an aqueous solution is practically useful because platinum nanoparticles can be easily handled, collected, and recycled.

6 Selective Adsorption by Modification of Metal-Binding Proteins

Cell surface engineering has allowed for yeast cells to adsorb heavy metal ions and rare metal ions. However, there is a high possibility that multiple metal ions coexist in complex solutions such as industrial wastewater. In these situations, if adsorption selectivity can be imparted to the surface-engineered yeast cells, they become more attractive as a resource-recovering yeast. In the conventional intracellular accumulation method, it is difficult to selectively incorporate the desired metal ions, but by displaying a protein with selective binding ability on the cell surface, there is a possibility that adsorption selectivity can be imparted. To this end, a protein with selective highly binding ability is necessary. Therefore, the creation of ModE mutants with selective binding ability was attempted by modification of ModE from *E. coli* normally used for molybdenum adsorption. Wild-type ModE can bind to molybdate as well as tungstate ions, which are homologous elements of molybdate ions. Hydrogen bonds via six amino acid residues are used for binding to these two metal ions of ModE. A single amino acid mutation was introduced into the amino acid residues of ModE, and the resulting protein was displayed on the yeast cell surface (Kuroda et al. 2012). Site-directed mutation at the fusion gene for cell surface display of ModE resulted in substitutions with amino acids with the OH side chain contributing to hydrogen bonding and different side chain lengths (Ser126Thr, Thr163Ser, Thr163Tyr) and substitution of a basic amino acid for an acidic amino acid (Arg128Glu). In the yeast displaying the ModE mutants (Ser126Thr, Arg128Glu, Thr163Ser), the ability to adsorb both metal ions was lost. However, in the case of Thr163Tyr mutation, only the ability to adsorb molybdate ions was lost while retaining the ability to adsorb tungstate ions (Fig. 6.5) (Kuroda et al. 2012). Using such amino acid mutations, it was possible to create a novel protein that selectively binds to tungstate ions. Therefore, the molecular breeding of yeast showing selective adsorption of tungstate ions can be performed by cell surface display of the created ModE mutant.

An alternative high-throughput strategy for identifying novel proteins/peptides with selective binding to target rare metal ions is to screen a protein/peptide library with random amino acid sequences. Flexibility in amino acid sequences based on the combination of 20 amino acids is an advantage in the creation of a useful protein/peptide for selective adsorption of rare metal ions. For this strategy, cell surface engineering is a very useful molecular tool that allows direct analysis of the displayed proteins/peptides on intact cells without the purification or concentration processes that are necessary for conventional intracellular and extracellular expressions (Ueda 2004; Georgiou et al. 1997; Wittrup 2001). In cell surface engineering,

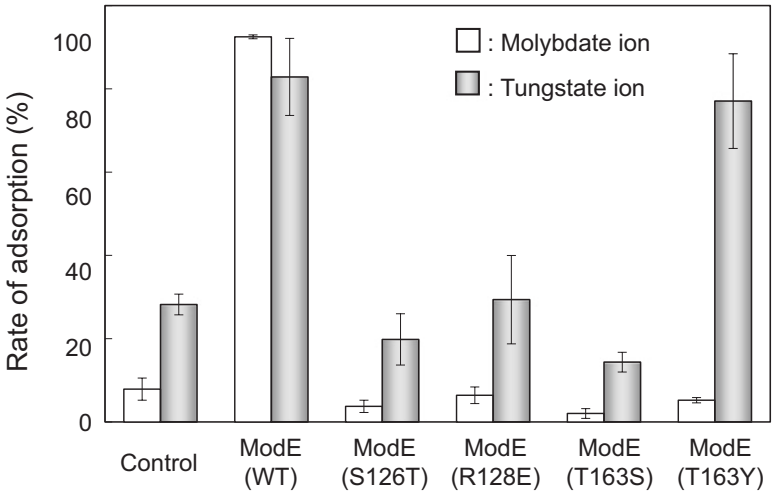

Fig. 6.5 Adsorption of molybdate and tungstate ions by surface-engineered yeasts displaying ModE mutants. The surface-engineered yeasts and its control strain (display of strep-tag) were incubated in 10 mL solution containing 100 μM molybdate ions and 100 μM tungstate ions with an OD_{600} of 20 for 2 h at 30 °C. The amount of residual molybdate and tungstate ions in the supernatant was quantified by ICP-MS

surface-engineered yeast cells can be treated as microparticles covered with the displayed proteins/peptides, allowing for high-throughput screening of displayed libraries to select proteins/peptides showing the desired function (Fig. 6.6). Furthermore, introduction of random amino-acid mutation is performed by random mutagenesis of the expression plasmid, and the amino-acid sequence of isolated proteins/peptides can be determined rapidly by analyzing the DNA sequence on the plasmid. Therefore, creation of a novel metal-binding protein/peptide and construction of surface-engineered bioadsorbents can be performed at the same time by using cell surface engineering. Actually, novel peptides with an ability to bind to gold, nickel, cadmium, and zinc ions were created from a random peptide library by cell surface engineering (Brown 1997; Kjaergaard et al. 2001; Mejare et al. 1998). In yeast cell surface engineering, proteins with large molecular mass can be successfully displayed; this serves as an advantage when compared to the bacterial display system. Proteins such as metal sensors and transcription factors that respond to metal ions can recognize specific metal ions based on their strictly structured binding pocket. Therefore, the screening of protein libraries with regions of random amino-acid sequences would be more promising for the selective binding of target metal ions.

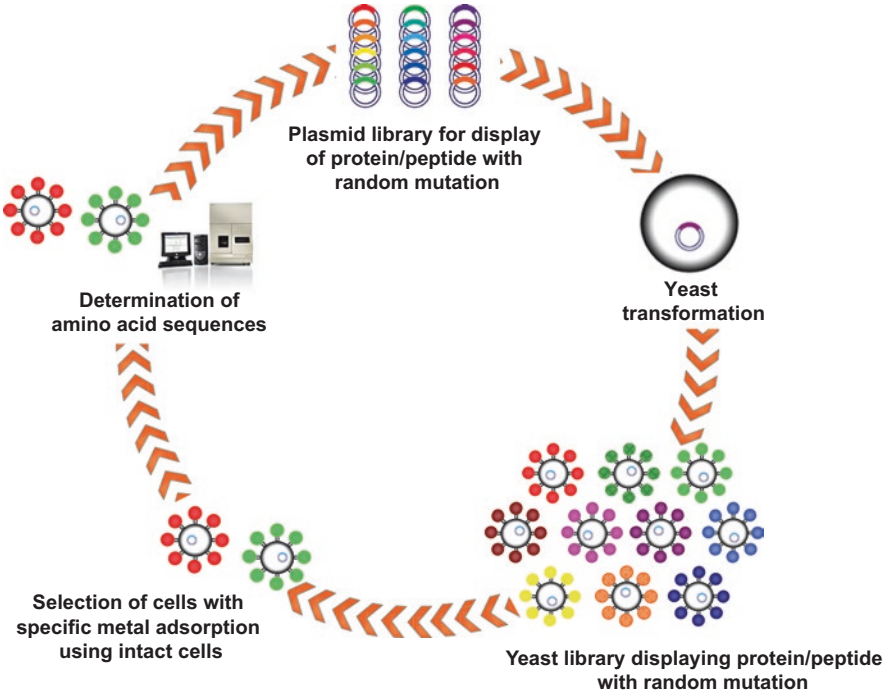

Fig. 6.6 High-throughput creation of a novel metal-binding protein/peptide by cell surface engineering

7 Conclusions and Perspectives

A novel strategy for metal ion adsorption and recovery has been established by cell surface engineering where proteins/peptides with metal ion recognition and binding capacity were displayed on the cell surface as an adsorption site. The cell surface adsorption of metal ions can be utilized to overcome the difficulties in conventional methods. They offer advantages of efficient recovery of metal ions adsorbed by bioadsorbents, reuse of bioadsorbents, and high selectivity. One of the major features of cell surface engineering is that this strategy can deal with diverse metal ions by creating metal-binding molecules according to the target metal ions. As introduced in this chapter, it is possible to custom-make yeast bioadsorbents with an ability to adsorb and recover toxic heavy metal ions as well as rare metal ions. By endowing bioadsorbents with selective adsorption ability, the choice of target aqueous solutions to recover metal ions is expanded, contributing to the development of resource recovery using bioadsorbents.

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Chapter 7
Preparation of Functional Cells: Improvement of Stress Tolerance

Kouichi Kuroda

Abstract In bioproduction using microorganisms such as yeast *Saccharomyces cerevisiae*, cells are under various stresses such as acid, base, heavy metal, organic solvent, and temperature stress. To overcome decreases in bioproduction efficiency because of the reduced biological activity of stressed cells, the stress tolerance of the cells must be enhanced. The cell surface is the first site at which a cell interacts with the external environment and plays an important role in the stress response, adaptation to stress, and protection from physical stress. Therefore, modification of cell surface properties is an attractive approach for enhancing stress tolerance. Cell surface engineering to display proteins/peptides on the cell surface can be used for surface modification. The display of a metal-binding protein/peptide successfully improved tolerance to toxic heavy metal ions via cell surface adsorption of metal ions. Furthermore, random modification of the cell surface by displaying a random protein/peptide library and subsequent screening successfully enhanced tolerance to acid and organic solvent. Therefore, various stress-tolerant yeasts can be constructed by modifying the cell surface according to the type of stress.

Keywords Cell surface engineering · Stress tolerance · Toxic heavy metal · Acid · Organic solvent

1 Introduction

The burden on the global environment has increased in recent years, and thus eco-friendly production of useful substances has attracted attention. Particularly, studies have focused on producing substances with biocatalysts utilizing the biological functions of microorganisms, a process known as bioproduction. The advantages of bioproduction include its multi-step reactions, reduction of by-products and isomers, mild reaction conditions, and energy-saving potential compared to

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conventional chemical processes. However, improvement of bioproduction efficiency is necessary for practical application. Therefore, metabolic designs that introduce metabolic pathways responsible for substance production and improve efficiency are being actively examined along with the development of omics analysis to comprehensively analyze diverse biological phenomena. Additionally, stress tolerance of cells used for bioproduction is an important factor in improving production efficiency. The environment in which bioproduction is carried out often differs from the ordinary culturing conditions suitable for cells, and cells undergo various stresses depending on the bioproduction reaction, such as exposure to acid, base, heavy metal, organic solvent, and temperature stress. A final or intermediate product that is hydrophobic or cytotoxic can cause cell stress. This may affect the metabolic pathway responsible for bioproduction, reducing bioproduction efficiency. Therefore, to maintain metabolic activity and promote bioproduction even in a stressful environment, it is necessary to enhance cellular stress tolerance.

The cell surface is composed of a cell wall and cell membrane with various functions such as morphological maintenance of cells by the robust cell wall, identification and transmission of various factors from the external environment, and tolerance to cell-threatening external causes. Pathogenic bacteria initially contact the host defense system via the cell surface. The properties and functions of the cell surface are very important in adhesion and fixation to the host cell. Therefore, the cell surface is a promising modification target for improving cellular stress tolerance. Furthermore, because the cell surface can be easily modified by cell surface engineering (Kuroda and Ueda 2013, 2014; Ueda and Tanaka 2000), the use of this effective strategy is increasing. In this chapter, the molecular breeding of stress-tolerant yeast *Saccharomyces cerevisiae* by modifying the cell surface is described.

2 Heavy Metal Tolerance

In bioproduction using biomass as a raw material, heavy metal ions that are leaked into the system place a burden on the cells as a stress factor. Living organisms take up various metal ions from the growing environment, and metal ions become concentrated in vivo via the food chain. Some metal ions function as cofactors for various enzymes and support the activities in cells by catalyzing intracellular reactions. However, some toxic heavy metals ions exert toxic effects by replacing the cofactor in the enzyme. Interactions between microorganisms and metal ions include adsorption on the cell surface and uptake/accumulation of metal ions in cells. Reducing the amount of heavy metal ions incorporated into cells could be an effective strategy for improving the tolerance to heavy metal ions.

Through cell surface engineering, proteins/peptides such as hexa-His and metallothionein which bind to heavy metal ions were displayed on the yeast cell surface to adsorb and remove heavy metal ions from the hydrosphere (Kuroda et al. 2001; Kuroda and Ueda 2003). Hexa-His is often used as a tag for protein purification based on affinity with a divalent heavy metal ion such as nickel. Metallothionein

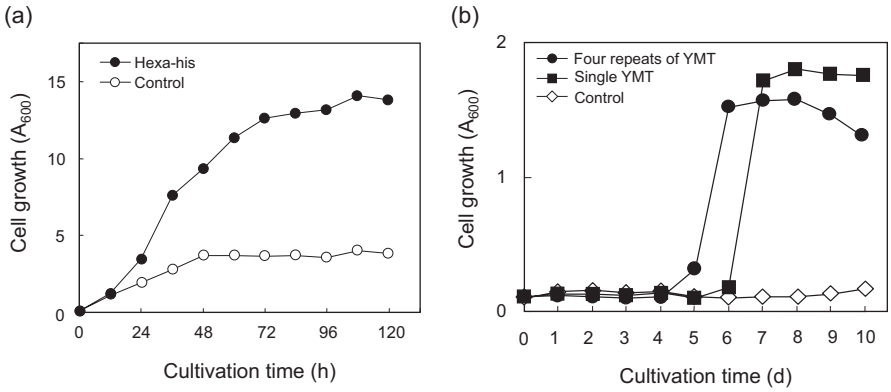

Fig. 7.1 Cell growth of surface-engineered yeasts in the medium containing heavy metal ions at a toxic concentration. (**a**) The hexa-His-displaying yeast in medium containing 2 mM Cu^{2+} and (**b**) yeast displaying tandem repeats of metallothionein in medium containing 70 μM Cd^{2+}

plays an important role in maintaining the homeostasis of the intracellular heavy metal ion concentration by sequestering excessive heavy metal ions into a nontoxic form in cells. These surface-engineered yeasts showed an increased adsorption ability of heavy metal ions such as copper, nickel, and cadmium ions on the cell surface, as described in Chap. 5. Furthermore, hexa-His-displaying yeast or metallothionein-displaying yeast grew in medium containing 2 mM Cu^{2+} or 80 μM Cd^{2+}, in which wild-type yeast cannot grow, respectively (Fig. 7.1). Multiple repeats of metallothionein were displayed on the cell surface by fusing metallothioneins in tandem to increase the number of binding sites (Kuroda and Ueda 2006). Increasing the number of displayed metallothioneins further enhanced both Cd^{2+} adsorption ability and Cd^{2+} tolerance according to the repeating number. These results suggest that adsorption on the cell surface is a cellular tolerance mechanism to protect against heavy metal ions. Therefore, the modification of the cell surface by cell surface engineering is effective not only for imparting bioadsorption ability for metal ion removal but also as a molecular breeding method for yeast showing tolerance to toxic heavy metal ions.

3 Acid Tolerance

Pretreatment of raw materials under acidic conditions and acid catalysts are often used in a variety of chemical conversion processes. For example, during the pretreatment of biomass feedstock for biofuel production, organic acids such as acetic acid are released. Because the generated acids flow into cells and decrease the intracellular pH, the cells must discharge excessive protons through plasma membrane H^+-ATPase by ATP hydrolysis (Eraso and Gancedo 1987; Holyoak et al. 1996). In addition to reducing enzymatic activity under intracellular acidic conditions, energy

consumption increases to maintain intracellular pH. As a result, the growth rate of a whole-cell biocatalyst decreases, which decreases bioproduction efficiency. To overcome this limitation, the acidic conditions are neutralized, but this process is not cost-effective. Therefore, yeast cells functioning as biocatalysts and able to grow even under acidic conditions to maintain cellular metabolic activity would be advantageous.

The cell surface was reported to play an important role in overcoming acid or alkaline stress in the environment. The alkaliphile *Bacillus lentus* C-125 strain, an extremophile, can grow under alkaline conditions (pH: 6.8–10.8) (Aono 1995). The cell wall of this strain consists of peptidoglycan, teichuronic acid (TUA), and teichuronopeptide (TUP). TUA and TUP are highly negatively charged acidic polymers and are thought to prevent hydroxide ion from flowing into cells (Fig. 7.2) (Aono 1987; Tsujii 2002). *Candida albicans* induces structural modification of cell wall mannans in response to acidic conditions (pH 2.0) to cope with acid stress (Kobayashi et al. 1994). Therefore, the cell surface is a promising target for modification to improve acid tolerance, and various features can be obtained by displaying proteins/peptides on the cell surface. The properties of the yeast cell surface were randomly modified to improve acid tolerance by displaying a peptide library con-

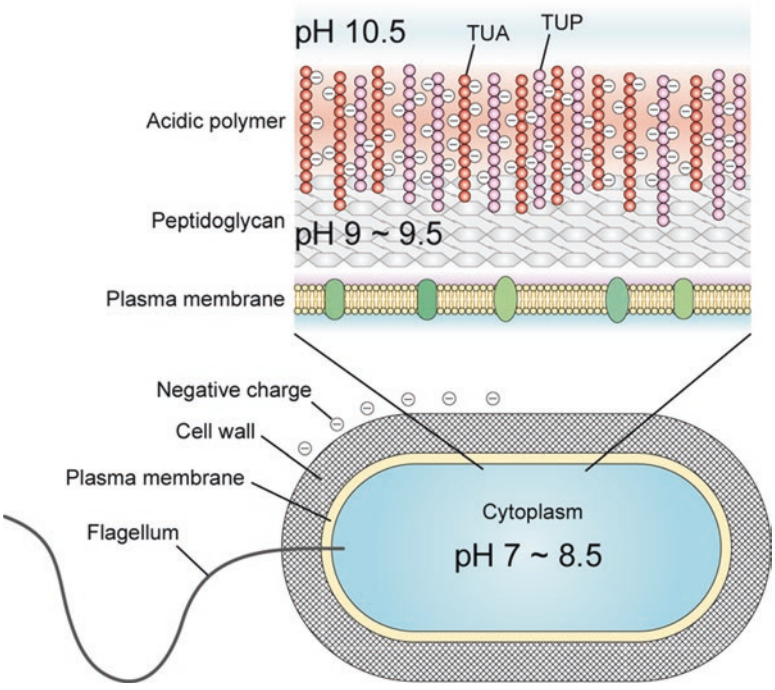

Fig. 7.2 Cell surface property of *Bacillus lentus* C-125. *TUA* teichuronic acid, *TUP* teichuronopeptide

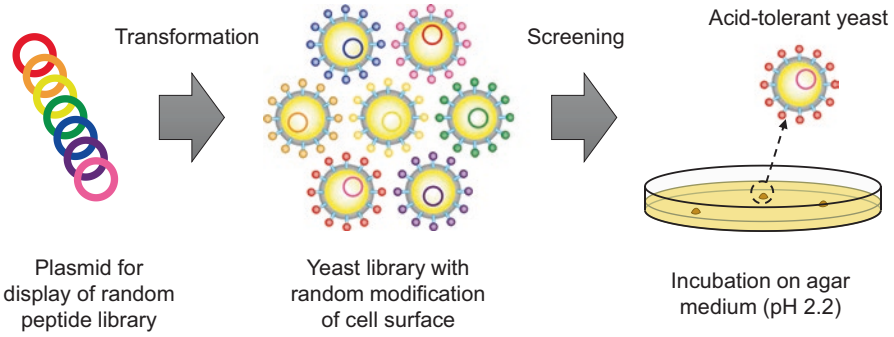

Fig. 7.3 Strategy for construction of acid-tolerant yeasts by random modification of cell surface and subsequent screening

sisting of 25 amino acid residues with random sequences (Matsui et al. 2009). The yeast library displaying peptides with random amino acid sequences was inoculated onto agar medium (pH 2.2) to select acid-tolerant yeasts (Fig. 7.3). Although the wild-type strain cannot survive at pH 2.2, an acid-tolerant yeast that grew at pH 2.2 was successfully isolated. The peptide displayed on the acid-tolerant yeast was a novel peptide (Scr35) containing a relatively large number of hydrophobic and basic amino acids. The identified Scr35 peptide showed high homology with a part of the hypothetical membrane-spanning protein PTO1510 from *Picrophilus torridus*, which can grow even at approximately pH 0 and 65 °C. The higher theoretical isoelectric point of the displayed Scr35 peptide (pI 9.98) appeared to have a buffering effect against acidic conditions on the cell surface. Furthermore, Scr35-displaying yeast showed better growth under low-glucose conditions and high-glucose uptake activity.

4 Organic Solvent Tolerance

In bioproduction involving hydrophobic substances or products, organic solvents are required for solubilization. An aqueous/organic solvent biphasic system is often utilized in these cases. A whole-cell biocatalyst of microorganisms is present in the aqueous layer and interacts with hydrophobic substrates in the organic solvent layer at the interface of the two phases (Fig. 7.4). If the final product is hydrophobic, potential product inhibition can be reduced by separating biocatalysts from the substrate and product at the interface. However, the problem of this biphasic system is that cells are affected by the toxic organic solvents. Organic solvents are incorporated into cell membrane lipids to denature membrane-bound proteins and disrupt essential membrane functions, leading to cytotoxic activity (Sikkema et al. 1995). Therefore, to prevent a decrease in the bioproduction efficiency caused by

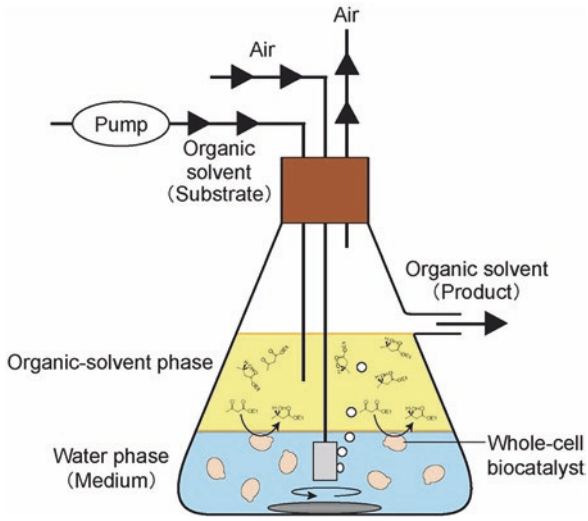

Fig. 7.4 Continuous bioproduction by whole-cell biocatalyst in aqueous/organic solvent biphase system

cytotoxicity of organic solvents in the biphasic system, the organic solvent tolerance of microorganisms such as yeasts should be improved for efficient bioproduction.

Random modification of the yeast cell surface by cell surface engineering was also employed to improve organic solvent tolerance. A random protein library constructed from random DNA fragments generated from *S. cerevisiae* cDNA was displayed on the cell surface. The resulting yeast library was inoculated onto agar medium overlaid with *n*-nonane, and the yeast cells grew even in the presence of *n*-nonane were isolated (Zou et al. 2001, 2002). A surface-engineered yeast with improved organic solvent tolerance was successfully isolated. The protein displayed on the cell surface of the isolated yeast was the structurally uncharacterized domain of the YGR193C gene product and is highly hydrophilic according to hydropathy plot analysis. Therefore, the cell surface of the isolated yeast likely became more hydrophilic upon displaying the protein, which improved organic solvent tolerance. The organic solvent-tolerant *S. cerevisiae* KK-211 strain was isolated by long-term serial culture in the presence of isooctane (Kanda et al. 1998). Wild-type yeast tends to adhere to isooctane droplets, whereas the KK-211 strain showed minimal adherence and dispersed in the aqueous phase (Miura et al. 2000). Even in this case, cell surface properties were modified, leading to improved organic solvent tolerance.

5 Conclusions and Perspectives

The cell surface is an important cellular component forming the outermost layer of yeast cells and is the first site of interaction with the surrounding environment to transmit environmental information to cells. Additionally, the cell surface plays an important role in protecting against physical stresses, stabilizing internal osmotic

conditions, maintaining cell shape, and enabling protein scaffolding. To adapt to environmental conditions, the cell surface is dynamically remodeled in response to extracellular stress factors despite the rigidity of the cell wall. Cell surface engineering which enables the display of any proteins/peptides on the cell surface is a powerful tool for modifying cell surface properties. By imparting new properties to the yeast cell surface, cellular tolerance to heavy metal ions, acids, and organic solvents was successfully improved. Particularly, random modification of the cell surface by displaying a random protein/peptide library and subsequent high-throughput screening was shown to be a promising strategy for improving stress tolerance, even when the tolerance mechanism is unclear. A strategy using cell surface engineering can be applied for constructing yeasts with improved tolerance to other stresses in addition to those described in this chapter. Furthermore, modification of a master regulator such as a transcription factor responsible for comprehensive gene expressions is also an effective strategy for enhancing stress tolerance (Kuroda and Ueda 2017; Satomura et al. 2014). Therefore, the integration of both strategies would further enhance stress tolerance.

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Chapter 8
Bio-sensing Using Cell Surface Display: Principles and Variations of a Cell Sensor

Seiji Shibasaki

Abstract The cell surface is the interface between intracellular and extracellular compartments. Intracellular and extracellular information is exchanged via transport of molecules across the cell surface. Receptors, binding proteins, transporters, and channels play important roles in the exchange of information between intracellular and extracellular compartments. In the cytosol, some information is eventually carried to the promoter regions of genes in the nucleus. The sensing ability of these cells can be applied for the development of biosensors. Because *Saccharomyces cerevisiae* has played a role in both conventional fermentation and as a host cell for heterologous protein production, using yeast as potential biosensors would enhance their utility in microbial technology. Cell surface engineering has been adapted to facilitate further real-time, noninvasive studies on biosensors. Here, we describe that representative yeast cell surface engineering for the generation of cell sensors will provide powerful analytical solutions in biochemistry and biotechnology.

Keywords Bio-sensing · Monitoring · Cellular information · Fluorescent protein

1 Introduction

1.1 General Principles and Backgrounds of Biosensors

Bio-sensing or biomonitoring is an indispensable technology for biological and medical studies. Target analytes need to be purified from samples before quantification to ensure precise and accurate analysis. Bio-sensing of metabolites or proteins needs real-time output because the quantity of the target molecules changes rapidly owing to enzyme or self-degradation. In addition, invasive processes involving tissue destruction and digestion are unfavorable, since they inhibit physiological

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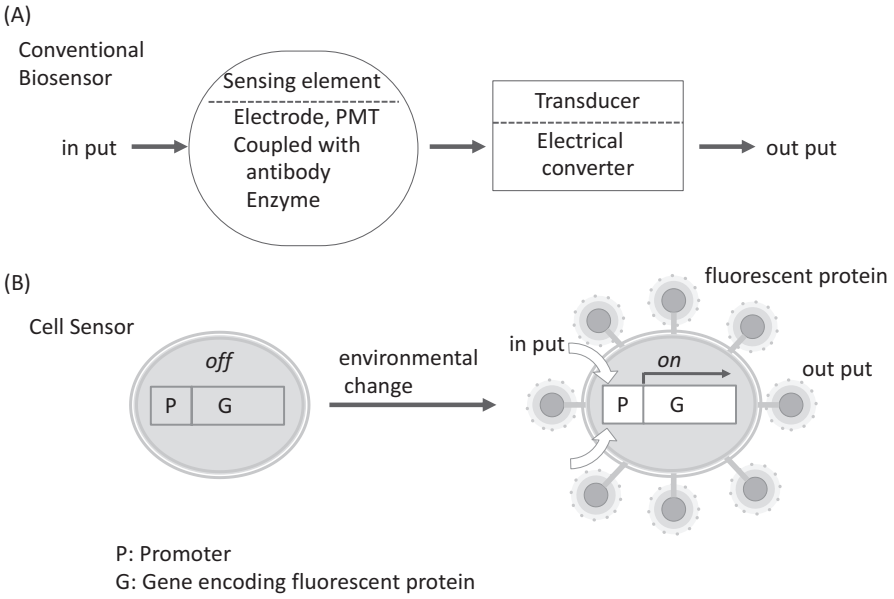

Fig. 8.1 General concepts of biosensors and cell sensors

activities. Real-time, noninvasive bio-sensing would yield improvements in not only electronic devices but also bio-sensing modules.

In general, a biosensor consists of a sensing element and a signal-transducing element (Fig. 8.1a). The sensing element is usually an electric component such as an electrode, which, in many cases, contains bioactive molecules such as enzymes and antibodies. In conventional biosensors, information received via molecules should be transformed into signals by signal-transducing elements to generate the output. To generate a novel type of biosensor, yeast cell surface engineering has been applied to endow yeast cells with the ability to transform received signals using a promoter, i.e., an analyte-responsive DNA sequence (Fig. 8.1b) (Shibasaki et al. 2009; Shibasaki and Ueda 2010, 2014a).

1.2 Combination of Promoter and Fluorescence Reporter for Arming Yeasts

In terms of possibilities for use in bio-sensing, the yeast cells have advantages over bacterial cells: microscopic observations and cell sorting analysis are easier for yeast cells because they are approximately ten times larger than bacterial cells. If yeasts are genetically modified to generate a biosensor, they can express both prokaryotic and eukaryotic genes. Additionally, yeast has long been used in

fermentation and bio-production (Shibasaki et al. 2008a, b) and is therefore convenient for both laboratory-scale and large-scale cultivation. Considering these factors, cell surface engineering has been applied to endow yeast cell an ability of bio-sensing.

Construction of biosensors via cell surface engineering is based on fairly simple principles, as described in Chap. 1. The promoter region of any gene that can respond to environmental changes can be used as a sensing element, and a fluorescent protein-encoding gene is used as a signal-transducing element to emit fluorescence and thereby present the output. Genetic construction of these elements is depicted in Fig. 8.3. The fluorescent protein-encoding gene is inserted between secretory signal sequence- and α-agglutinin-encoding genes. This fusion gene is then regulated by the appropriate promoter region that responds to any changes in the concentrations of molecules of interest. In *S. cerevisiae*, because several types of auxotrophic markers can be used simultaneously in the same cell, different information can be sensed by using suitable promoters and transduced by appropriate reporters. Several types of fluorescent proteins are available to enable the presentation of different signals by the cell.

1.3 Fluorescent Reporter

As reporter molecules, green fluorescent protein (GFP) and its variants are well investigated as signal-transducing elements of cell sensors, which will be introduced later in this chapter. GFPs are more useful than previous biological reporter molecules, i.e., bioluminescent molecules such as luciferase, because they do not require cofactors other than molecular oxygen to emit fluorescence. In previous studies using arming yeast for bio-sensing, the feasibility of jellyfish (*Aequorea victoria*) GFP display on yeast cell surface was examined; fluorescent intensity-enhanced variants of GFP are now available to use instead of GFP. Amino acid substitutions were introduced into these variants to generate stable fluorescence and emit at wavelengths other than that of green (Fig. 8.2). Enhanced GFP (EGFP) and similar variants have promoted many studies in biochemistry and biotechnology (Day and Davidson 2009; Sawano and Miyawaki 2000). Different emission wavelengths are useful to simultaneously generate outputs for multiple signals. Recently, different types of fluorescent proteins have emerged in biological studies from species other than *A. victoria*. For example, the fluorescence spectra of *Discosoma sp*. red fluorescent protein (DsRed) and its variants do not overlap that of *A. victoria* GFP variants (Bevis and Glick 2002). A DsRed variant, mCherry, has an emission maximum at 610 nm, far from that of EGFP at 509 mm (Fig. 8.3b) (Shaner et al. 2004). These reporter proteins can be applied for not only α-agglutinin-based cell surface display systems for bio-sensing (Sect. 2) but also other types of bio-sensing strategies (Sects. 3 and 4).

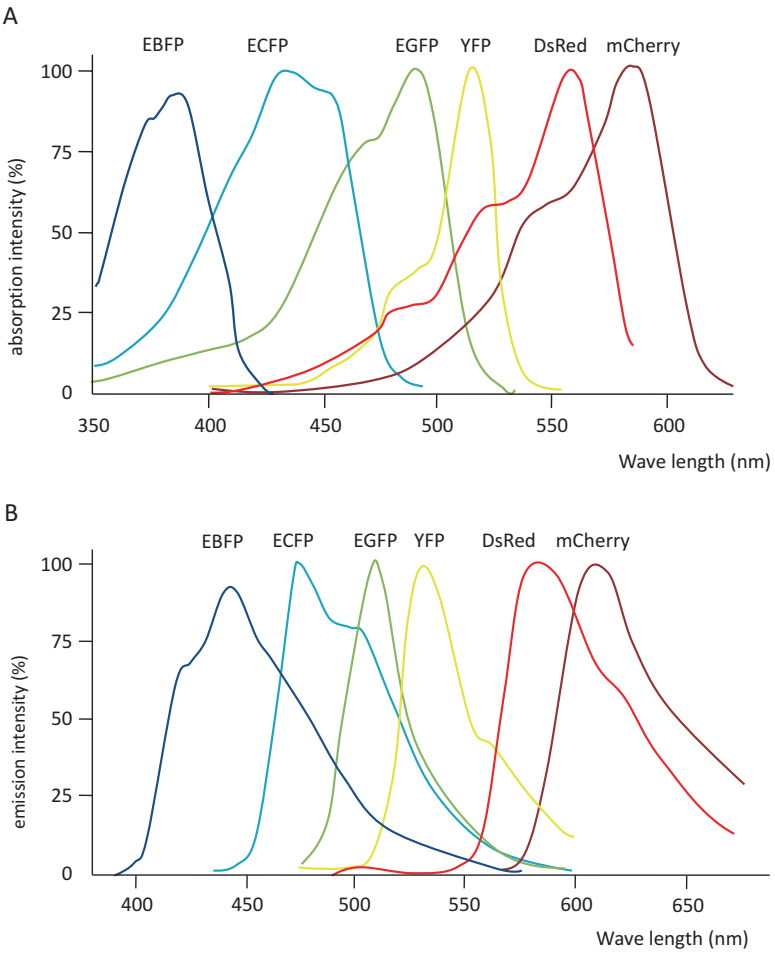

Fig. 8.2 Fluorescence spectra of proteins that could be used in cell sensors. (**a**) Excitation spectra; (**b**) emission spectra. The profiles of the spectra of proteins were combined based on the methods described in these studies: EGFP and ECFP (Sawano and Miyawaki 2000), EBFP (Day and Davidson 2009), EYFP (Martin et al. 2008), DsRed (Bevis and Glick 2002), mCherry (Shaner et al. 2004)

2 Application of α-Agglutinin-Based Cell Surface Engineering for Biosensors

2.1 Glucose Bio-sensing

Glucose is an essential nutrient in the blood. Medical devices can be used to detect and analyze blood glucose concentrations. Regulation of glucose levels is an important factor in yeast fermentation technology, as glucose is an important nutrient in

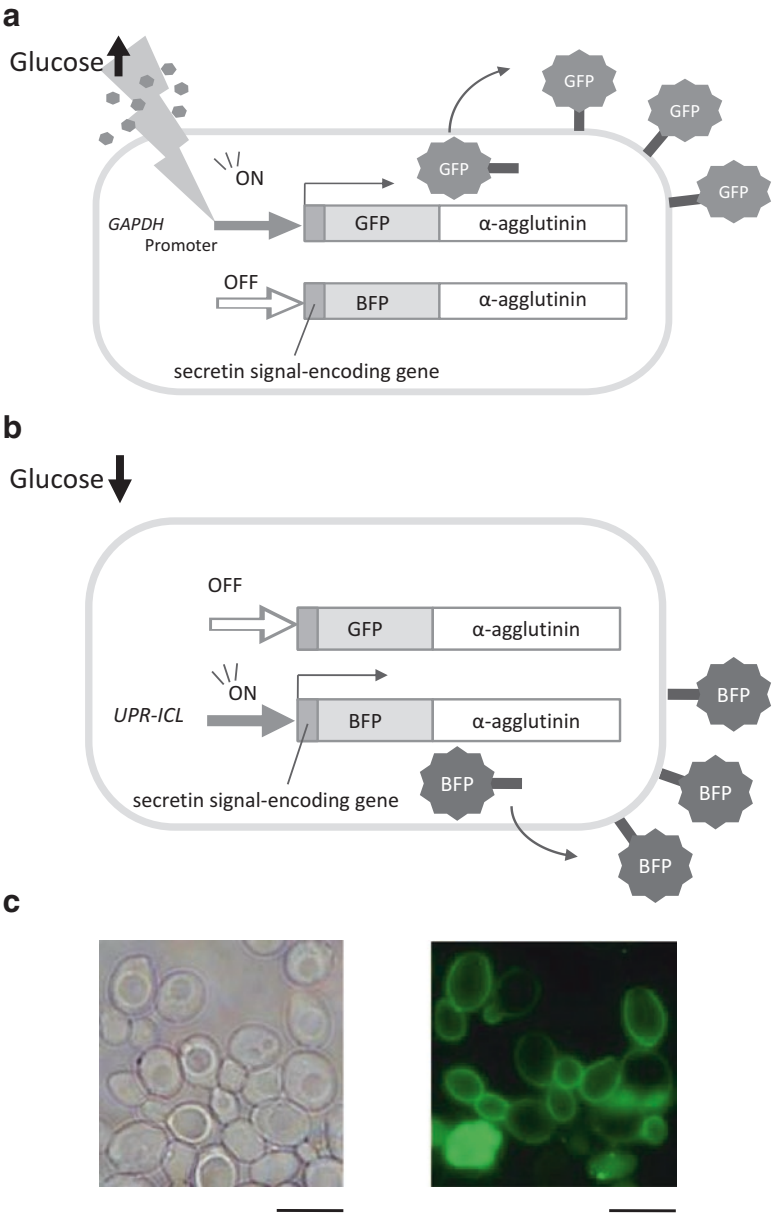

Fig. 8.3 Fluorescent switching by using two different promoters. (**a**) GFP display is induced by growing the cells in media containing glucose. (**b**) BFP display is induced by growing the cells in glucose-depleted media. (**c**) GFP-displaying yeast; light field micrograph (left) and fluorescence micrograph (right), bar = 5 μm

culture media for yeast and other microorganisms. Glucose concentration in media can be measured using electrodes (Xu et al. 2004; Koschinsky and Heinemann 2001); however, intracellular glucose is not easily measured using conventional devices.

The sensing element of the cell sensors is introduced via genetic engineering. To develop a glucose-sensing cell sensor, two different types of inducible promoter regions were first used, which respond to glucose concentration. One is the *GAPDH* promoter derived from the gene encoding glyceraldehyde-3-phosphate dehydrogenase. It is often used to express any gene in yeast cells in glucose-rich conditions. Another type of promoter is the *UPR-ICL* sequence, which responds to decreasing concentrations of glucose. The *UPR-ICL* is the upstream region of the gene encoding isocitrate lyase in *Candida tropicalis* and has been used to activate gene expression under glucose depletion.

The first model of a fluorescent reporter on the cell surface was investigated using a combination of *GAPDH* and GFP. Simultaneously, a combination of *UPR-ICL* and blue fluorescent protein (BFP) was also investigated for sensing glucose depletion. Genetic constructs for those proteins and α-agglutinin as an anchor on the cell surface have been integrated into the chromosome of *S. cerevisiae* MT8-1 strain (Ye et al. 2000). Fluorescence and immunofluorescence microscopic analyses have illustrated intracellular and extracellular expression of GFP and BFP in response to glucose concentration in living cells (Shibasaki et al. 2001a, b). Fluorescence spectroscopy has also revealed switching from green to blue fluorescence from the cell surface in real time during culture in response to changes in glucose concentration (Fig. 8.3).

2.2 Ion Bio-sensing

Similar to glucose sensing, ammonium ion and phosphate ion sensing have also been explored as models for ion sensing. First, a promoter cassette plasmid was constructed to integrate various sequences that can respond to analytes of interest (Fig. 8.4). Plasmids pIDC1 (cyan fluorescent) and pIDY1 (yellow fluorescent) can be used to fuse analyte-responsive promoters to fluorescent reporters and the α-agglutinin fusion protein-encoding gene in host *S. cerevisiae* genome (Shibasaki et al. 2001c).

To demonstrate ammonium ion sensing using cell surface engineering, the upstream region of *MEP2*, which encodes an ammonium ion transporter, was introduced into pIDY1 to display EYFP on the yeast cell surface under low-NH_4^+ conditions. *MEP2* is induced by nitrogen scarcity although it is repressed under the presence of NH_4^+. To demonstrate phosphate ion sensing, the *PHO5* promoter was selected and introduced into pIDC1 to express ECFP during phosphate depletion. *PHO5* is induced by low inorganic phosphate ion conditions. Both genetic constructs were introduced into the genome of the *S. cerevisiae* MT8-1 strain to create ion-sensing cells.

Each ion-sensing cell was analyzed via flow cytometry for fluorescence, and the increasing rate of relative fluorescence was calculated in units rfu/h. For ammonium

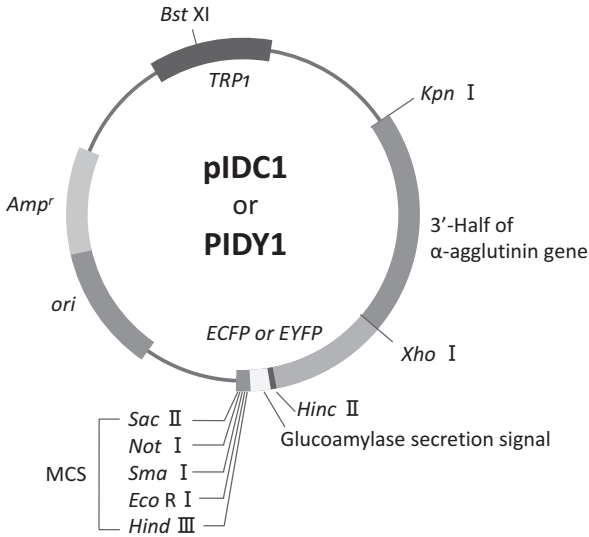

Fig. 8.4 Promoter cassette-type plasmids for ECFP and EYFP are available to emit different colors from the same cell surface by induction of the applied promoter

ion sensing, this engineered cell could display EYFP in response to decreases in extra- and intracellular NH_4^+. However, for phosphate ion sensing, the fusion gene ECFP-α-agglutinin was induced by the *PHO5* promoter in low-phosphate ion conditions. The relationship between the concentrations of the ions was successfully evaluated on the basis of the rate of change in fluorescence.

2.3 Monitoring of Protein Expression Levels

Considering the importance of *S. cerevisiae* for fermentation and foreign protein production (Contador et al. 2011; Leber et al. 2015), the cell itself can be used for the output of information on protein production levels via cell surface engineering. As a model of investigations for the monitoring of protein production levels, the *GAL1* promoter was selected to produce protein and β-galactosidase or human interferon ω, as shown in Figs. 8.5a and b (Shibasaki et al. 2003). *GAL1* expression levels can be regulated by galactose, and the *GAL1* promoter has therefore been used for the production of several proteins using *S. cerevisiae*. As for protein expression, the fusion gene encoding EGFP-α-agglutinin protein was placed under the control of the *GAL1* promoter in another plasmid with a different auxotrophic maker (Fig. 8.5c). Both protein production and fluorescence from EGFP expressed on the cell surface were monitored, and the correlation between fluorescence and production level was confirmed up to 24 h in each culture. Additionally, the intracellular and extracellular galactose concentrations were reflected fluorescent signal intensity from the cell surface during the culture. Thus, the intracellular production levels

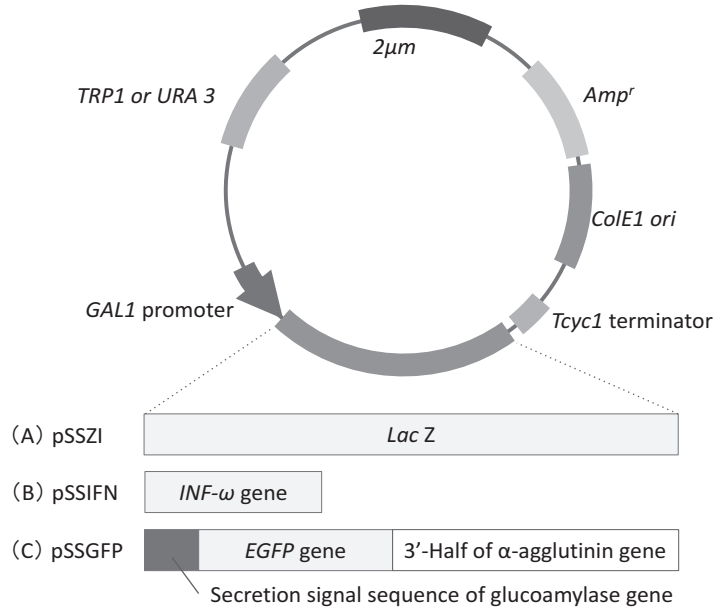

Fig. 8.5 Plasmid constructions of *GAL1*-controlled protein production and EGFP display. Each plasmid can induce the production of (**a**) β-galactosidase, (**b**) interferon omega, (**c**) EGFP-α-agglutinin

of a protein and the concentrations of carbon sources were efficiently evaluated by means of cell surface-displayed reporters in a noninvasive manner.

2.4 Single-Cell Analysis

GFP-displaying yeast cell has been used for not only glucose sensing but also quantitative analysis of yeast cell surface proteins. Fluorescent proteins on the cell surface were quantified via imaging analyses to reveal the number of molecules that could be displayed by cell surface engineering, using α-agglutinin as a scaffold (Shibasaki et al. 2001a). Serial images of a single cell displaying EGFP were obtained using confocal laser scanning microscopy. After obtaining serial sections, signals of intracellular auto-fluorescence and cytoplasmic EGFP were eliminated so that fluorescence intensity from only the cell surface could be determined using the TRI image processing system. The result revealed that 10^4 EGFP molecules were displayed on the surface of a cell harboring the fusion gene EGFP-α-agglutinin in the genome; however, 10^5 EGFP molecules were expressed on the surface of a cell harboring a multi-copy type plasmid encoding the same gene.

3 Sensing by a Membrane-Anchoring System at the Cytosolic Face

Increasing proteomic studies have advanced our understanding of the dynamic profile of cellular proteins under various conditions. Although comprehensive analysis of proteins using proteomic big data is becoming an indispensable technique in biology (Shibasaki et al. 2014a, b, 2016), tools for the investigation of molecular interactions between each protein still play an important role in revealing the molecular networks in a cell. The yeast two-hybrid system and protein fragment complementation assay have been used to investigate intracellular protein-protein interactions for a long time (Shibasaki et al. 2008b). These systems do not require any purification steps to analyze the interaction of two proteins of interest. This advantage is based on the premise that proteins are expressed with a different domain of a protein, whose activity will be recovered if the two domains interact closely. However, if the proteins of interest do not interact with nuclear pores or are not concentrated adequately to interact in the cytosol, protein–protein interaction may not be detected correctly.

Considering these situations, a novel tool for the detection of an intracellular protein–protein interaction in the cytosol without localizing in the nucleus has been developed using cell membrane proteins (Shibasaki et al. 2006). This method can accumulate target proteins at the plasma membrane and it is regarded as different type of cell surface display. In this study, fluorescence resonance energy transfer (FRET) was used to detect interactions between two proteins of interest (Martin et al. 2008). The Z-domain of Protein A and Fc fragment of immunoglobulin G are known to interact with each other and, hence, were selected and used as an interaction model (Shibasaki et al. 2007). The Z-domain-ECFP fusion protein was anchored on the cytosolic face of the plasma membrane by the minimal membrane-target domain (MTD) of Snc2p (Fig. 8.6). This MTD consists of 31 amino acid residues at the C-terminal of Snc2p. However, the fusion protein EYFP-Fc fragment was produced in the cytosol of cells anchoring the Z-domain-ECFP. An

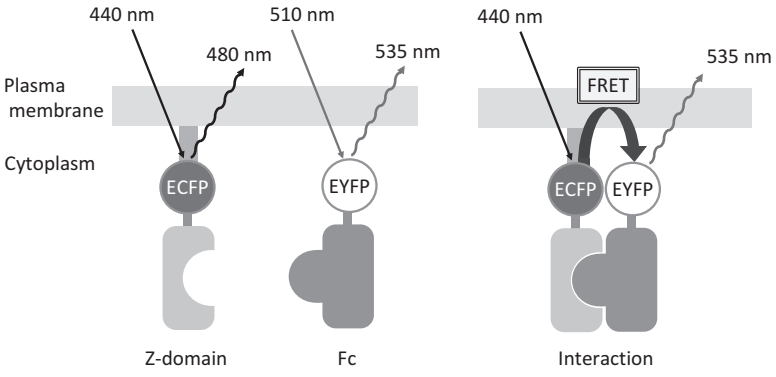

Fig. 8.6 The sensing strategy for protein-protein interactions by membrane-based display

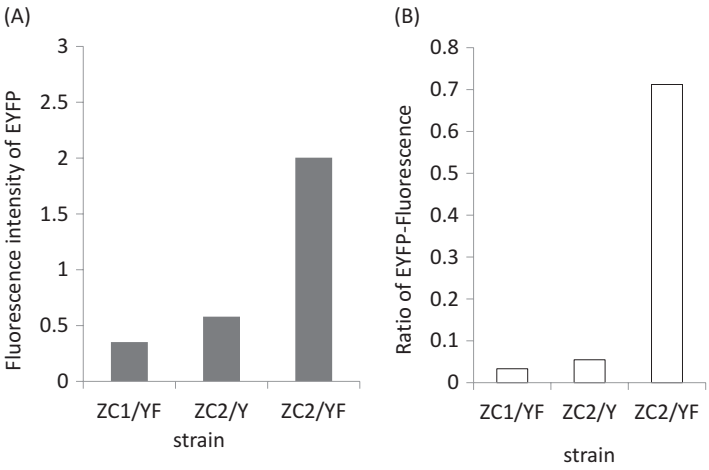

Fig. 8.7 FRET signal was sensed in the partner protein-anchored plasma membrane. (**a**) Fluorescence of EYFP at the excitation wavelength of ECFP. (**b**) Ratio of the fluorescence of the plasma membrane/cytoplasm. Among the Z-domain-ECFP-anchored yeast cells (ZC2), EYFP fluorescence was confirmed for EYFP-Fc fusion protein-producing cells (ZC2/YF). Strain ZC1 is not anchored to the Z-domain-ECFP, and EYFP fluorescence was confirmed to be limited in these cells despite the production of EYFP-Fc (ZC1/YF). The ZC2/Y strain produced EYFP without the Fc-fragment

inverted microscope equipped with an AquaCosmos/FRET system (Hamamatsu Photonics, Hamamatsu, Japan) and a CCD camera (Hamamatsu, Japan) was used to acquire FRET images. On processing ECFP and EYFP images using AquaCosmos software program, the FRET ratio was calculated (Fig. 8.7). These results suggest that protein–protein interactions in the cytoplasm could be effectively detected via FRET coupled with molecular display using MTD. Moreover, this technology is expected to complement conventional systems such as the two-hybrid system because this system can help researchers study protein–protein interactions in real time.

4 Sensing Ligands by Displaying a Fusion-Type Receptor

Cell surface receptors initiate cellular functions in response to the information presented by its target ligands. Therefore, combinations of receptors and fluorescent proteins have been examined for use as biosensors. The ligand-binding ability of these receptors was first demonstrated in a mammalian receptor—rat estrogen receptor—on the yeast cell surface (Yasui et al. 2002). The ligand-binding region of the rat estrogen receptor was displayed on yeast cells using α-agglutinin-based cell surface engineering. The binding ability of the receptor was successfully evaluated

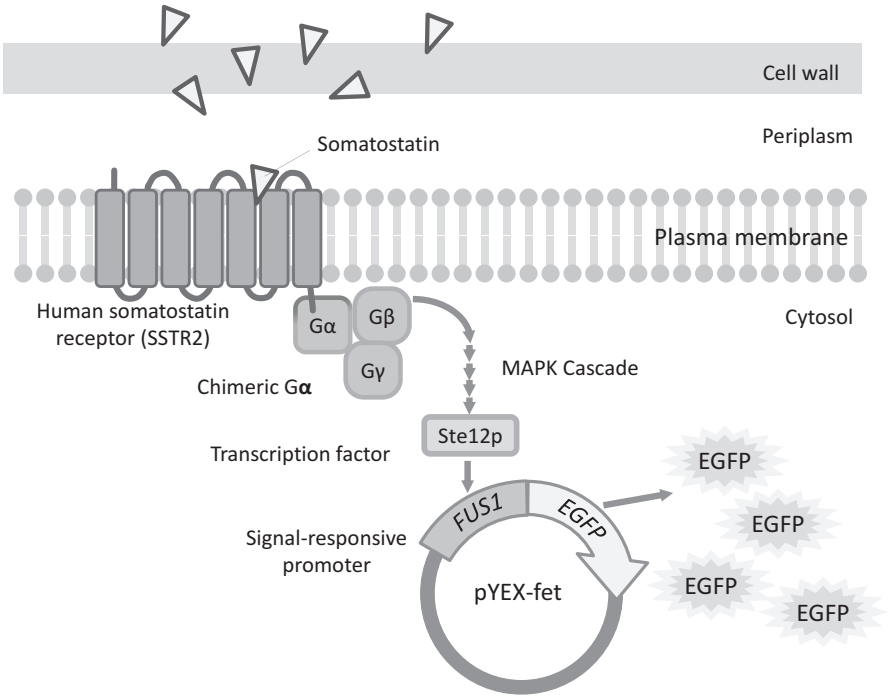

Fig. 8.8 A ligand sensing system using a combination of receptors and fluorescent proteins

using fluorescent 17β-estradiol as the ligand, and the results of the study suggested that the mammalian receptor can be displayed on cell surfaces and bind to ligands while maintaining its functional conformation.

After a few years, G protein-coupled receptors (GPCRs) and EGFP were used to develop an arming yeast with ligand-sensing ability. Among the many human GPCRs, somatostatin receptor (SSTR2) was displayed on the yeast cell surface in one of its active forms. In addition, a chimeric Gα subunit was constructed by introducing the C-terminal five amino acids of mammalian Gi to yeast Gpa1p for activation of the transcription factor ste12p via the MAPK cascade when SSTR2 received a functional ligand (Fig. 8.8). Moreover, pYEX-fet, which serves as the *FUS1* promoter in EGFP, is introduced into cells harboring SSTR2 and chimeric Gα. When these cells were exposed to exogenously added somatostatin, EGFP fluorescence was detected in a dose-dependent manner. Thus, yeast cells displaying a GPCR were shown to relay the information obtained from binding to ligands through a signal transduction cascade. These receptors could be used for sensing not only a particular known ligand but also for contributing to the development of methods for screening novel ligands or agonists (Hara et al. 2012).

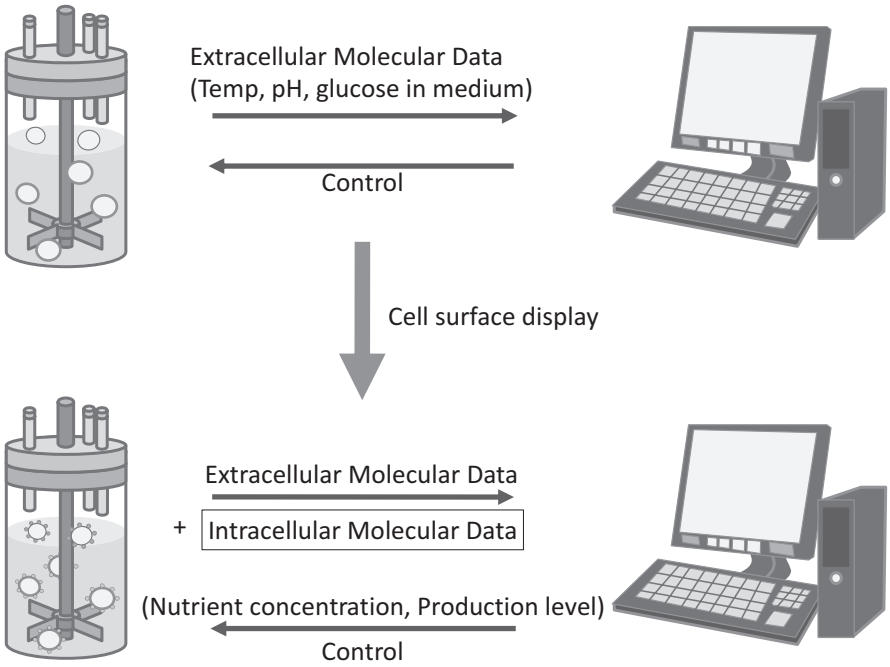

Fig. 8.9 A sensor created by cell surface engineering enables the control of multiple parameters

5 Summary

In this chapter, several variations and possibilities of bio-sensing by cell surface engineering were introduced. A yeast cell surface display of the fluorescent reporter would yield alternatives to conventional sensing in biotechnology. In particular, in the α-agglutinin-based display system, cell sensors could both receive and transform intracellular and extracellular information (Fig. 8.9). The presented yeast model of the combination of a suitable promoter and fluorescent protein is expected to be developed in other species as well, and these models can then be applied in cell surface engineering.

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Chapter 9
Application of Cell Surface Engineering to Biosensing System

Shin-ichiro Suye

Abstract Organophosphorus compounds (OPs) such as pesticides, fungicides, and herbicides are highly toxic but are nevertheless extensively used worldwide. To detect OPs, we constructed a yeast strain that co-displays organophosphorus hydrolase (OPH) and enhanced green fluorescent protein (EGFP) on the cell surface using a Flo1p anchor system. The strain with highest activity showed 20-fold greater OPH activity than *Escherichia coli* using the ice nucleation protein anchor system. OPs degradation releases protons and causes a change in pH. This pH change results in structural deformation of EGFP, which triggers quenching of its fluorescence, thereby making this cell useful for visual detection of OPs (20 mM paraoxon) in a microchamber chip. The authors also report the real-time detection of OPs using a sol–gel silica planar waveguide doped with EGFP and OPH on a yeast-cell surface display. The waveguide was pumped at 488 nm, and it emitted green fluorescence at the far field. The green fluorescent light at 550 nm changed by 50% from the original power 1 min after application of OPs.

Keywords Organophosphorus compounds · Optical biosensing · Optical waveguide

1 Introduction

Cell surface engineering has various advantages of biocatalyst for biosensing. Here, we will refer to the detection system of organophosphorus compounds (OPs) as an example. Organophosphorus compounds (OPs) such as pesticides, fungicides, and herbicides are highly toxic but are nevertheless used worldwide. The continued use of

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OPs increases the possibility that delayed cholinergic toxicity and neurotoxicity are induced (Tuovinen et al. 1994). Developed countries inspect imported agricultural food products for toxic OPs, and the growing public concern about their safety in modern agriculture has stimulated the development of methods for OPs detection.

Current analytical techniques, such as chromatography, immunoassay, and the use of enzyme biosensors based on inhibition of cholinesterase activity, are highly sensitive but have disadvantages such as high cost or lengthy procedures (Cortada et al. 2009; Lee et al. 2003). Organophosphorus hydrolase (OPH) effectively hydrolyzes OP esters, including pesticides, such as parathion (Tuovinen et al. 1994).

Microbial biosensors for OPs have been developed using recombinant *Escherichia coli* intracellularly expressing the OPH gene, but the response was slow because of the mass-transport limitation of substrates and products across the cell membrane (Ranina et al. 1996). Therefore, *E. coli* was engineered to express OPH on the cell surface, and the INP anchor was used to improve OPH display on the surface of *E. coli* (Shimazu et al. 2001).

2 Organophosphorus Hydrolase and EGFP Co-displaying Yeast

2.1 OPH Activity Cell Surface-Engineered Yeast Strain Using Flo1p Anchor System

We constructed a yeast strain that displayed OPH from *Flavobacterium* species, the cell surface-engineered yeast-displayed OPH using glycosylphosphatidylinositol (GPI) anchor system (Takayama et al. 2006). Details of this system are shown in Fig. 9.1a. Yeasts displaying OPH exhibited greater activity than *E. coli* displaying OPH using the ice nucleation protein (INP) anchor system (Shimazu et al. 2001). However, it is possible that OPH activity might be suppressed in yeast strains when using GPI anchor system. Because the OPH active site is located near the C-terminus, the 30-half of a-agglutinin of the GPI anchor might be sterically hindered (Matsumoto et al. 2002). Therefore, we adopted an optimal anchor system for the location of the OPH active site. Yeasts displaying OPH using Flo1p anchor system were constructed. Flo1p is a lectin-like cell-wall protein in *Saccharomyces cerevisiae*. Details of Flo1p are shown in Fig. 9.1b. Using Flo1p anchor system, a recombinant lipase having its active site near the C-terminus was displayed on the yeast, and it showed higher activity than that obtained using GPI anchor system. Using Flo1p anchor system, we attempted to construct OPH-displaying yeasts that exhibited greater activity compared with that obtained using GPI anchor system.

Fig. 9.1 (continued) GPI anchor attachment signal sequence are genetically fused to the C-terminal region of the target protein. (**b**) Flo1p fusion protein. The N-terminal regions of target proteins such as OPH are fused to the Flo1p flocculation functional domain. (**c**) Design of Flo1p (FL/FS) fusion gene for display of protein on the yeast cell surface

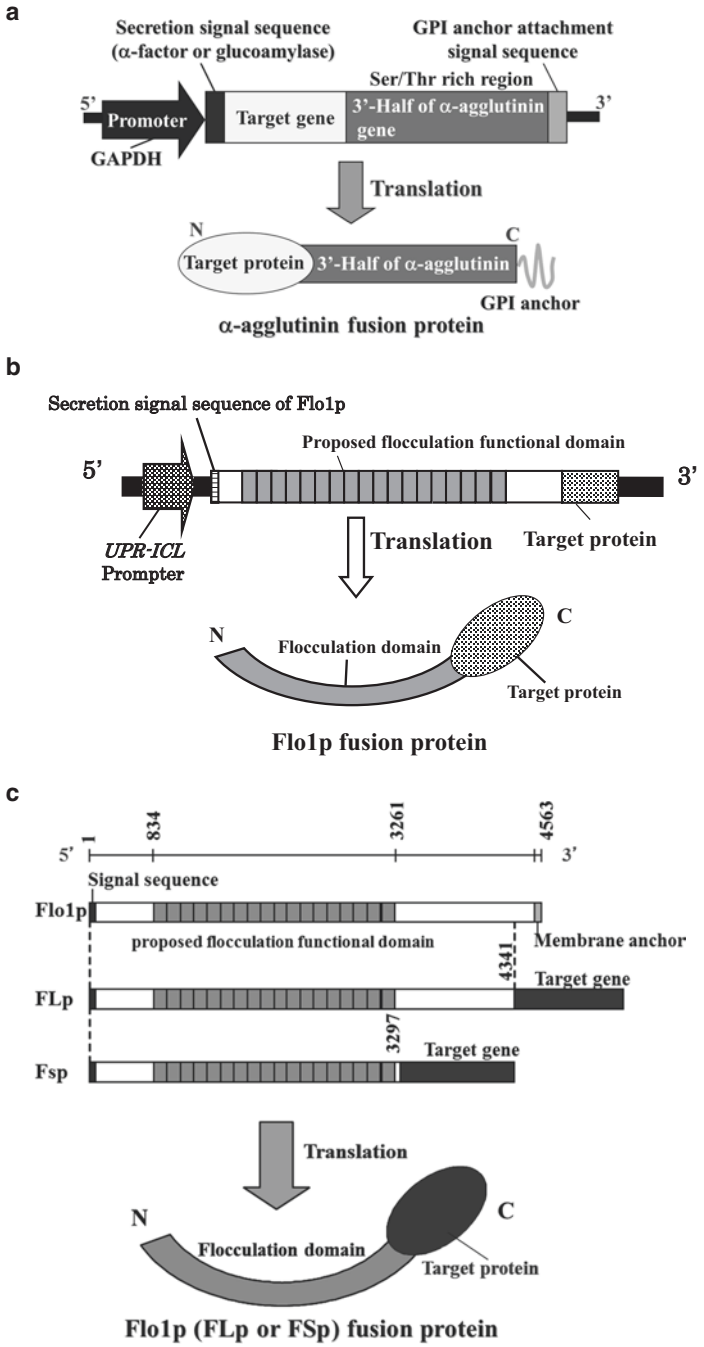

Fig. 9.1 The α-agglutinin fusion gene and Flo1p fusion gene for display of protein on the yeast cell surface. (**a**) α-Agglutinin fusion protein. The secretion signal sequence of α-agglutinin and the

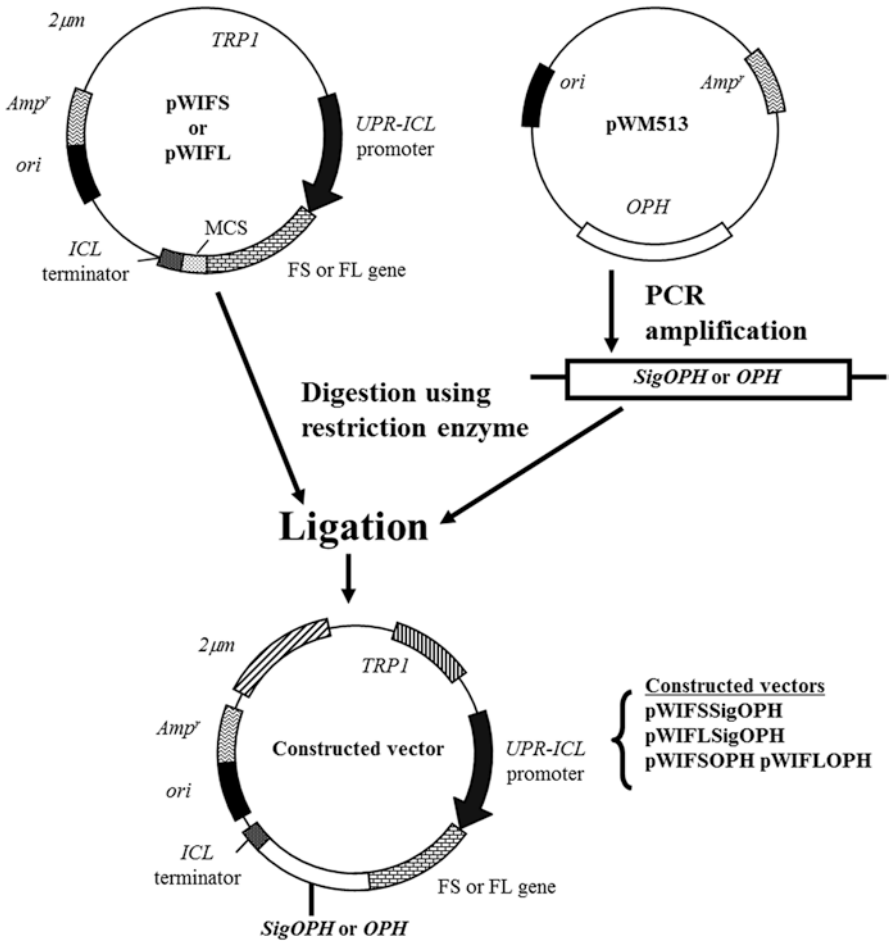

Fig. 9.2 Scheme for the construction of plasmids for display of OPH on the yeast cell surface using Flo1p anchor system. The gene-encoding OPH is amplified by PCR with pWM513, as the template. pWIFS and pWIFL are the plasmid vectors for expressing the OPH on the yeast cell surface. The amplified fragment is digested and introduced in each plasmid vector. The resulting plasmids are named pWIFSSigOPH, pWIFLSig-OPH, pWIFSOPH, and pWIFLOPH

2.2 Construction of Plasmids for Cell Surface Display

Plasmids for OPH display using Flo1p anchor system were constructed as follows. The gene-encoding OPH was amplified using PCR with pWM513 (Takayama et al. 2006) as the template (Fig. 9.2). pWIFS and pWIFL were the plasmid vectors used for expressing OPH on the yeast cell surface (Fig. 9.2). The FL gene comprised almost all the sequence of Flo1p, and the FS gene consisted of part of Flo1p without 1044 bp of 30 downstream of the FL gene (Fig. 9.1c) (Matsumoto et al. 2002). The

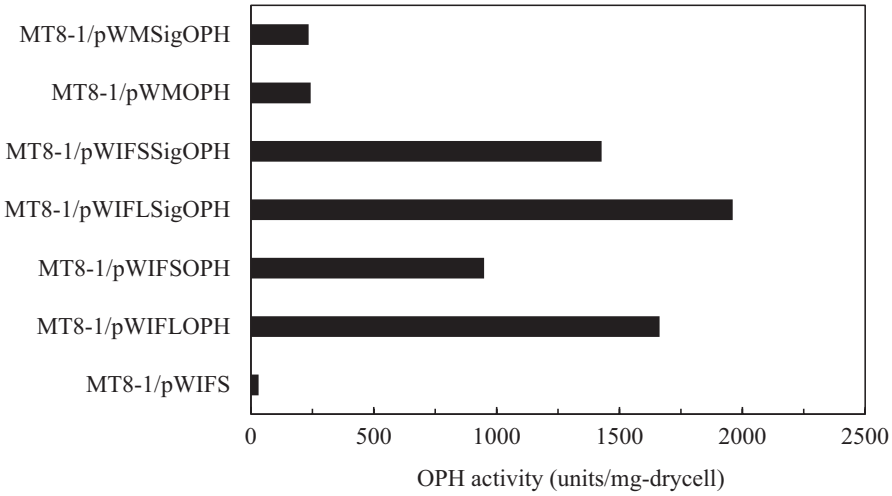

Fig. 9.3 Comparison of OPH activity of yeast cells transformed with various plasmids. The y axis shows the OPH activity, which expressed as formation of 1 nmol of PNP per min per mg dry cells (units/mg dry cell). The x axis shows the strains. Strains MT8-1/pWIFSSigOPH, MT8-1/pWIFL-SigOPH, MT8-1/pWIFSOPH, and MT8-1/pWIFLOPH are constructed using Flo1p anchor system (Fukuda et al. 2010). MT8-1/pWM-SigOPH and MT8-1/pWMOPH that were constructed (Takayama et al. 2006) are positive controls using GPI anchor system. MT8-1/pWIFS is a negative control using Flo1p anchor system, which was not inserted OPH gene to the vector. Data are averages based on three independent measurements

amplified fragment was digested and introduced in each plasmid vector (Fig. 9.2). The resulting plasmids were named pWIFSSigOPH, pWIFLSigOPH, pWIFSOPH, and pWIFLOPH (Fig. 9.2).

2.3 Effect of the Anchor System on OPH Activity

As shown in Fig. 9.3, MT8-1/pWIFLOPH using Flo1p anchor system showed six-fold greater OPH activity compared with MT8-1/pWMSigOPH or MT8-1/pWMOPH using GPI anchor system, indicating the effective display of OPH with an active site located near the C-terminus. We investigated the effect of Flo1p length on OPH activity. The optimal whole cell activity of MT8-1/pWIFSOPH was only 960 units/mg dry cell wt, whereas MT8-1/pWIFLOPH had 1600 units/mg dry cell (Fig. 9.3). Matsumoto et al. (2002) demonstrated that the yeast cells displaying FL-anchor protein had higher flocculation ability than that of the FS-anchor protein and that the flocculation ability of the FL-anchor protein was sufficient to absorb on the cell surface. Therefore, a large amount of the FL-OPH fusion protein would be displayed stably on the cell surface compared with the FS-OPH fusion protein, and MT8-1/pWIFLOPH would exhibit high activity. This result shows that the

C-terminal region of the FL-anchor protein (consisting of 3297–4341 bp of DNA sequence) was a significant domain for flocculation of the Flo1p-OPH fusion protein on the cell surface.

2.4 Effect of the OPH Secretion Signal on OPH Activity

Next, the effect of the OPH secretion signal on OPH activity was investigated. MT8-1/pWIFSOPH and MT8-1/pWIFLOPH contained only the secretion signal sequence of Flo1p in the 5′-upstream region of Flo1p-OPH sequence in each transformed plasmid, whereas MT8-1/pWIFSSigOPH and MT8-1/pWIFLSigOPH contained two secretion signal sequences for OPH in the 50-upstream region of OPH and a secretion signal sequence for Flo1p in the 50-upstream region of Flo1p in each transformed plasmid. MT8-1/pWIFSSigOPH showed high activity (1440 units/mg dry cell) compared with MT8-1/pWIFSOPH (960 units/mg dry cell) (Fig. 9.3). While the flocculation ability of the FS-anchor protein was insufficient, in contrast to the FL-anchor protein (Matsumoto et al. 2002), the function of the OPH secretion signal might contribute to the efficient expression of OPH and would complement its activity. Furthermore, MT8-1/pWIFLSig-OPH showed highest activity (1920 units/mg dry cell). A synergy between the FL/FS anchor and the secretion signal of OPH would be occurred toward activity of OPH on the yeast cell surface. Displaying OPH on the yeast cell surface using Flo1p anchor system resulted in higher OPH activity than GPI anchor system. In particular, MT8-1/pWIFLSigOPH showed eightfold higher OPH activity than MT8-1/pWMSigOPH or MT8-1/pWMOPH. We constructed a biocatalyst for Ops detoxification and detection based on the yeast-displayed OPH using Flo1p anchor system. The OPH activity of this strain was about 20-fold more efficient than that obtained with *E. coli* using the INP anchor system. The strains we used may not only be useful for the detoxification of OP nerve agents but also for the rapid detection of OPs. For example, the fluorescence intensity of GFP depends on pH; GFP displayed on *E. coli* showed 65% attenuation in fluorescence for a pH change from 8 to 6 (Shi and Su 2001).

3 OPs Detection on a Cell Chip with Yeast Coexpressing Hydrolase and EGFP

Organophosphorus hydrolase (OPH) effectively hydrolyzes a range of organophosphorus esters, including pesticides, such as parathion, coumaphos, and acephate, and chemical warfare agents, such as soman, sarin, VX, and tabun (Dumas et al. 1989). The catalytic hydrolysis of these compounds releases protons and changes the pH (Mulchandani et al. 1999). The measurement of released protons and their correlation with OP concentration forms the basis of potentiometric measurement,

and OPH immobilized on the surface of the potentiometric electrodes have been developed (Mulchandani et al. 1999). To construct a simple biosensor for direct and rapid detection of OPs, we adopted enhanced green fluorescent protein (EGFP) for the detection of the protons released from the hydrolysis of OPs. EGFP has higher fluorescence intensity than wild-type GFP, and this fluorescence intensity changes in proportion to the pH value ranging from 4.0 to 8.0 (Kneen et al. 1998). This pH change causes structural deformation of EGFP and triggers quenching of its fluorescence.

Shibasaki et al. (2001) demonstrated that the yeast cells displaying EGFP show the higher fluorescence intensity for 24 h, and they suggested that EGFP denatured by acid (pH 2) would be renatured by shifting the pH to 7.4.Thus, EGFP is suitable for detection of protons released from hydrolyzed OPs. Previously, we constructed the yeast cell displaying OPH from *Flavobacterium* species by cell surface engineering (Takayama et al. 2006). Cell surface engineering allows display of proteins on the cell surface of the yeast *Saccharomyces cerevisiae* using Flo1p anchor system. The use of whole yeast cells as an alternative biological catalyst without the high cost of enzyme purification has been explored (Takayama et al. 2006). Flo1p is a lectin-like cell-wall protein in *S. cerevisiae* (Matsumoto et al. 2002), and the N terminus of the target protein OPH is fused with the Flo1p flocculation functional domain. In this study, we have successfully created yeast cells co-displaying EGFP and OPH on the cell surface using Flo1p anchor system. Furthermore, we adopted a microchamber chip for the detection of EGFP quenching (Fukuda et al. 2007). The chip was placed in the single yeast cell into each microchamber, and its behavior was observed during culture, and a target cell was picked up under the microscopic observation. Catalytic reactions of a single yeast cell displaying lipase or β-glucosidase has been previously observed in a microchamber (Fukuda et al. 2007). We used this unique cell chip protocol together with the single yeast cell co-displaying EGFP and OPH to detect OPs degradation.

The fluorescence intensity changes of EGFP on the yeast cell surface were investigated in a pH range of 4.0–8.0. The fluorescence intensity of the co-displaying strains decreased with decrease in pH values (data not shown). Therefore, for the investigation of fluorescence quenching of EGFP against pH change, a buffer of pH 8.0 was adopted as the starting pH value. EGFP fluorescence quenching due to OPs degradation of the co-displaying single cell was observed by fluorescence microscopy using a cell chip as follows: the co-displaying strains were cultured in SD-W medium for 72 h at 30 °C. Cells were harvested and washed with 2 mM HEPES-NaOH buffer containing 50 μM $CoCl_2$ (pH 8.0). Washed cells were resuspended with 2 mM HEPES-NaOH buffer (final concentration $OD_{600\ nm} = 0.05$). Cells were placed in a cell chip (Muranaka Medical Instruments, Osaka, Japan; Fig. 9.4). After 10 min, a cover glass was placed on the chip gently. Ten microliters of 20 mM of paraoxon was introduced into the microchamber from the cover glass side. On the cell chip, quenching of EGFP fluorescence on the cell surface of a single cell was observed using fluorescence microscopy. In the absence of paraoxon, quenching of EGFP fluorescence did not occur on the cell surface of the single cell co-displaying OPH and EGFP (data not shown). On the other hand, in the presence of 20 mM

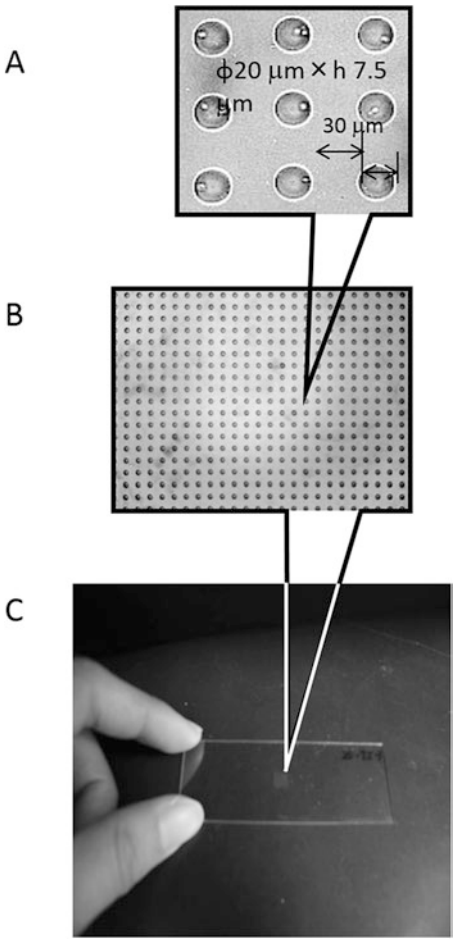

Fig. 9.4 Yeast cell chio. (**a**) Cell chip; (**b**) chamber surface; (**c**) enlarged view of the chip. Chamber diameter, 20 μm; height; 7.5 μm; 100 × 100 = 10,000 MicroChambers

paraoxon, the gradual fluorescence quenching of EGFP on the single cell surface was observed within 40 s (Fig. 9.5). These results suggested that the degradation of paraoxon on the cell surface was accompanied by a pH change of the cell surface surroundings by production of protons. As a result, structural deformation of EGFP was caused, triggering the fluorescence quenching of EGFP (Fig. 9.6). Furthermore, 0.5 mM paraoxon caused fluorescence quenching of EGFP on the single cell surface in a microchamber (data not shown). Fluorescence measurement of cell suspensions is not suitable for fluorometric measurement because the high-density cell mass in the suspension causes turbidity and inhibits fluorescence intensity measurement. On the other hand, the fluorescence quenching on the single yeast cell could be detected in a microchamber of the cell chip. Therefore, we suggest that both OPH and EGFP co-displaying yeast in combination with a cell chip have sufficient sensitivity for the visual detection of OPs. In addition, the co-displaying yeast strain can also produce large amounts of OPH and EGFP without any tedious purification steps. In this

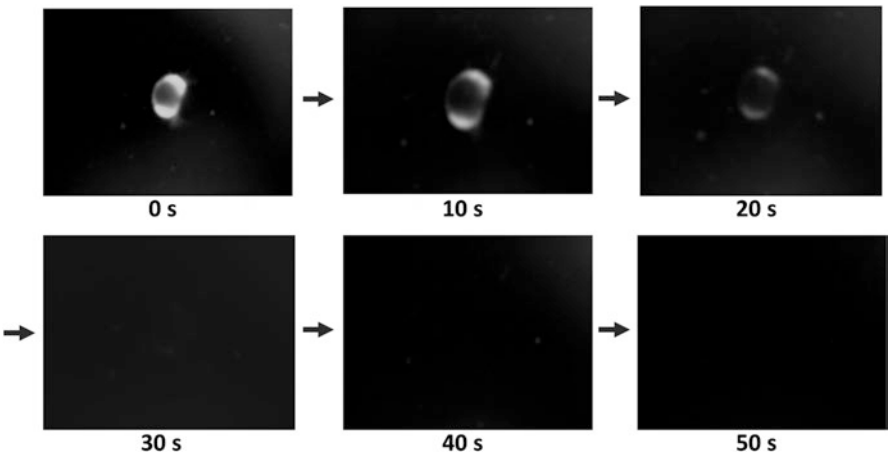

Fig. 9.5 Fluorescence quenching of EGFP on a single yeast cell using a cell chip. Paraoxon degradation-induced quenching of EGFP fluorescence of a single cell co-displaying OPH and EGFP in a MicroChamber. Ten microliters of 20 mM of paraoxon was introduced

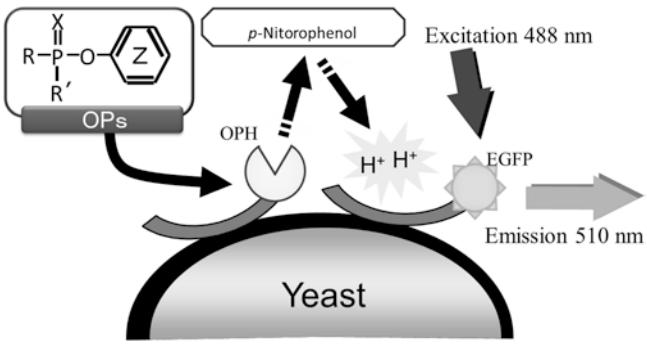

Fig. 9.6 Schematic of fluorescence quenching of EGFP based on OP degradation. The reaction of OP degradation releases protons and changes local pH, which causes structural deformation of EGFP and triggers the quenching of fluorescence

study, the yeast strain we constructed, combined with a cell chip, demonstrated the potential of a new biosensor chip.

4 Detection of OPs Based on a Sol-Gel Silica Planar Waveguide Doped with an EGFP and an OPH

The electrical and optical biosensors have been designed to function independently for detecting biomaterials. Electrical and optical connections between the biosensors must be employed for monitoring potentially hazardous biomaterial. The

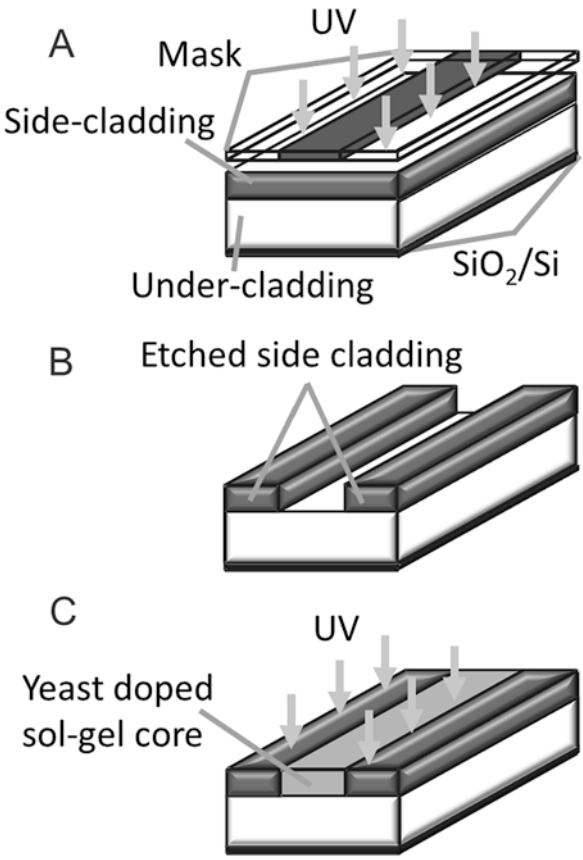

Fig. 9.7 Fabrication process for the EGFP- and OPH-doped waveguide device for detecting the OP compound. (**a**) The side cladding layer coated on the under cladding/SiO_2/Si layer is radiated using UV light though the photolithographic mask for patterning of side cladding. (**b**) The EGFP-doped waveguide device is developed in isopropanol to remove the nonirradiated part. (**c**) The EGFP- and OPH-doped sol-gel core solution is spin coated between the sidewalls of the side cladding. UV is radiated to partially promote the silica network in the sol-gel core. After UV radiation, the EGFP-doped sol-gel waveguide device is cleaved to couple light from the SM fiber

sol-gel silica waveguide sensor is easily butt-coupled with an optical fiber using UV curable resin. The silica waveguide (2 × 5 μm) is packaged with a standard method for waveguide devices such as optical modulators (Enami et al. 2007a).

When the OPH and EGFP displayed on the yeast cell surface were doped in the sol-gel silica waveguide core and pumped at 488 nm, the waveguide detected the OP compound by the change in green fluorescence power. This study demonstrates the detection of the OP compound by the change in the green fluorescence power in a sol-gel silica waveguide doped with the EGFP and OPH on a yeast cell surface display.

The EGFP and OPH immobilized on a yeast cell surface were doped in a sol-gel core (4 μm wide, 4 μm high, 5 mm long). A SM waveguide biophotonic device was fabricated, as shown in Fig. 9.7 (Enami et al. 2007b). The process of the sol-gel silica waveguide fabrication was described in a previous report (Enami et al. 2007a). Sol-gel solutions with molar ratios of 85% methacryloxypropyltrimethoxysilane (MAPTMS) to 15% index modifier [zirconium (IV)-*n*-propoxide (ZrPO)] and 88% MAPTMS to 12% ZrPO were prepared for the sol-gel core and cladding, respec-

tively. An 8 μm thick sol-gel under cladding was spin coated over a SiO_2 (6 μm thick)/Si substrate and baked at 150 °C for 1 h, as shown in Fig. 9.7a. The 4 μm thick sol-gel side cladding layer was coated on top of the under cladding and wet etched in isopropanol after UV light in a mask aligner was radiated over the side cladding layer through a photolithographic mask for defining the waveguide side cladding structure. The nonirradiated part of sol-gel side cladding layer was removed from isopropanol and baked at 80 °C for 24 h Fig. 9.7b).

Glycerol (0.3–0.7% v/v) was added to the sol-gel core solution for improving the penetration of the OP solution (Ma et al. 2008) into the sol-gel silica core. We optimized the amount of glycerol in the sol-gel silica core after observing that the OP solution penetrated into the fabricated sol-gel silica waveguide within 10 min. The EGFP and OPH immobilized on the yeast cell in the buffer solution were mixed with the sol-gel core solution at the 2.56 fM doping level EGFP. The sol-gel core solution was coated on the sol-gel cladding etched on the substrate and filled in the trench between the sol-gel side cladding, as shown in Fig. 9.7c). The reversed ridge core was radiated with UV light in the mask aligner for 10–15 min without postbaking. This process accelerated the hydrolysis of the sol-gel silica core and partially cross-linked the sol-gel silica network in the core without deactivation of the living EGFP and yeast cells, which are sensitive to high temperatures. It also maintained an optimized porous size in the silica. The OP solution can penetrate the optimized sol-gel silica core within 10 min. The immobilized EGFP and OPH on the yeast cell surface also had an optimized distance in the nanosized volume to react with the OP compound and change the pH, which resulted in the change in the green fluorescence power. The fabricated waveguide was cleaved along the Si crystal axis to have the straight end facet with a coupling loss of 1–2 dB with SM fiber (mode field diameter of 4.2 μm) that can be reduced further with optimization of the refractive index difference between the core and cladding. The refractive index was controlled (e.g., within 0.0001) by varying the doping level of ZrPO. The waveguide propagating loss was 1 dB per 5 mm at 515 nm.

A laser at 488 nm was propagated from a diode through the SM fiber and butt-coupled into the EGFP- and OPH-doped waveguide. The green fluorescent light emitted from the waveguide was collected and focused by a 20 X microscope objective lens on a spectrometer with a 1.02 nm wavelength resolution through a dichroic filter (T ~10^{-5} at 422–530 nm) for removing the 488 nm pumping light. The fluorescent light received by the spectrometer was digitally processed and transferred to a computer for analyzing the fluorescence spectrum, which monitors the change in the fluorescence power at 550 nm (Fig. 9.8). As an OP compound, 20 mM paraoxon was mixed with 10% methanol in deionized (DI) water (10% methanol/DI water). While monitoring the fluorescence spectrum in the waveguide, 4 μl of paraoxon solution was dropped on the waveguide core. When the paraoxon penetrated the sol-gel core, the fluorescence power reduced at each wavelength. The change in the fluorescence power at 550 nm is shown as an example in Fig. 9.9. After 20 mM paraoxon was applied to the waveguide, the fluorescence power was reduced by 50% in 1 min and to 20% of the original fluorescence power in 6 min on account of the photobleach effect of the EGFP. To confirm if the reduction was caused by the

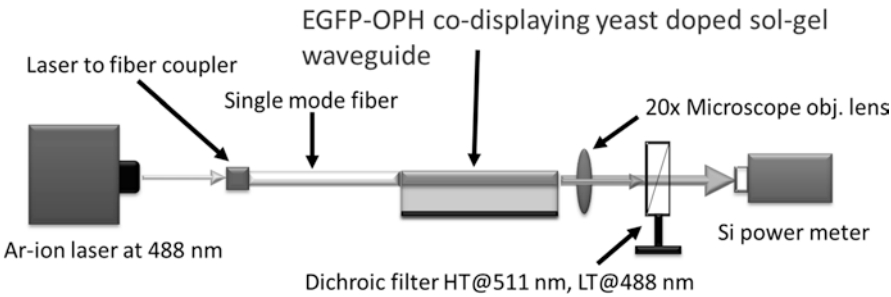

Fig. 9.8 Schematic of the experimental setup. EGFP and OPH displaying on the yeast cell surface are directly doped in the sol-gel silica core. The pump laser is coupled from the SM fiber into the waveguide device. Fluorescent light from the device is collected using a 20 X microscope objective lens and focused on the spectrometer though the dichroic beam splitter to filter the pump light. The fluorescence spectrum is analyzed on a computer. Paraoxon, an OP compound, is mixed with 10% methanol/DI water and applied to the waveguide

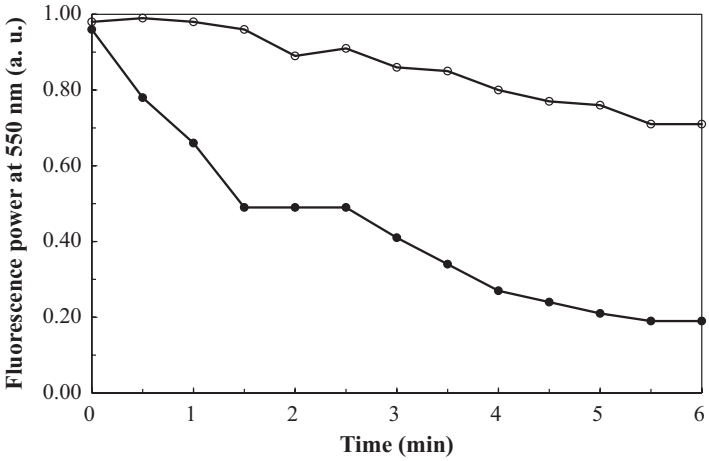

Fig. 9.9 Change in the fluorescence power after application of 20 mM paraoxon mixed with 10% methanol/DI water. Solid triangles represent output power after application of 10% methanol/DI water. Solid circles represent output power after application of 20 mM OP mixed with 10% methanol/DI water

OP compound, 4 μl of 10% methanol/DI water was applied to another waveguide on the same substrate. The fluorescence power was reproduced and linearly reduced to approximately 70% of the original value in 6 min. From these results, we concluded that the change in the fluorescence power at each wavelength was caused solely by 20 mM OP compound in 10% methanol/DI water. When the mixing amount of glycerol is less than 0.1% volume in the sol-gel silica core solution, the reduction in the fluorescence power was similar to the results obtained from the 10% methanol/DI water applications. On the other hand, when the mixing amount of glycerol is more than 1% volume in the sol-gel silica core solution, the thin-film quality of the

spin-coated core on the cladding degraded and developed visible spots on the film, which did not work as a low-loss waveguide.

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Part III
Medical Applications

Improvement of the affinity of antibodies for target biomolecules is an important and essential challenge in diagnostic and therapeutic development. Single-chain variable fragments (scFv) are those in which the light and heavy chains are linked by a flexible linker. Artificial IgGs are freely and speedily prepared with arming technology. Construction of an oral vaccine is also attempted by displaying antigen on the cell surface of *S. cerevisiae*.

Chapter 10: Engineering Antibodies and Alternative Binders for Therapeutic Uses (Dr. Wataru Aoki (Japan, Kyoto))

Chapter 11: Oral Vaccine Development Using Cell Surface Display Technology (Dr. Seiji Shibasaki (Japan, Kobe))

Chapter 10
Engineering Antibodies and Alternative Binders for Therapeutic Uses

Wataru Aoki

Abstract Yeast surface display is one of the most successful in vitro protein engineering platforms. In vitro selection enables antibodies screening with specific functions in the primary screening process, which is difficult when using the classical hybridoma systems. Moreover, eukaryotic quality control systems assist in displaying the complex proteins, and the applicability of flow cytometry facilitates the quantitative evaluation of protein libraries. Based on these characteristics, yeast surface display has broad applications from antibody screening and maturation to the high-throughput evaluation of de novo designer proteins. In this chapter, we review yeast surface display technologies, highlighting their roles in protein engineering and in therapeutic uses.

Keywords Yeast surface display · Antibody · Alternative binder · Directed evolution

1 Introduction

Monoclonal antibodies (mAbs) are the most successful proteins used for biological therapy and for diagnostic reagents. The major characteristics of mAbs are their binding accuracy (high specificity and affinity) for the intended targets together with their less toxicity to patients compared to conventional small molecule therapeutics. Many antibody therapeutics have been approved worldwide, demonstrating the potential of this class of therapeutics.

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For antibody discovery, two approaches are predominant: animal immunization and surface display technologies. Animal immunization is an effective method because we can screen antibodies from available 10^{13} naïve clonal repertoire (Greiff et al. 2017), but there are several problems to be solved. When antigens are well conserved between species or toxic to the animals, generation of antibodies could be difficult. In addition, animal immunization is time-consuming, and antibody screening is costly and requires substantial effort. To overcome these problems, in vitro selection, display technologies, such as phage display or cell surface display, are widely used (Schofield et al. 2014; Boder et al. 2000). In vitro selection enables cheap and high-throughput selection of antibodies against desired antigens, even if they are conserved in various species or toxic to animals. It is widely believed that antibodies generated from naïve antibody libraries exhibit weaker affinity compared to those from animal immunization (Schofield et al. 2014), but improved library design enables generation of high-affinity antibodies as a primary selection, and a plethora of mutagenesis strategies enable effective affinity maturation to produce antibodies with equilibrium dissociation constants (K_d) at the fM level (Boder et al. 2000). Another advantage of in vitro selection is the availability of various selection forces during the selection process. This assists in obtaining various functional antibodies with conformational specificity, pH sensitivity, high thermal stability, etc. Besides conventional antibody formats, alternative smaller binding molecules, such as single-domain antibodies (Hamers-Casterman et al. 1993) and antibody mimetics (Yu et al. 2017), have been developed, using yeast surface display. These small binders are becoming important tools for super-resolution microscopy and highly sensitive diagnostics.

This paper reviews display technologies with special emphasis on yeast surface display, highlighting its role in protein engineering for therapeutics uses. In addition, the paper focuses on noncanonical antibody formats, antibody mimetics, and novel therapeutic applications.

2 Yeast Surface Display Technology

Yeast surface display is a powerful platform for protein engineering (Boder and Wittrup 1997), allowing intended target proteins to be fused with cell surface proteins and tethered to the cell wall. Cell surface anchoring of the target proteins establishes genotype-phenotype linkage at the single cell level, enabling rapid construction of mutant libraries. Protein characteristics (affinity, stability, enzymatic kinetics, etc.) can be easily evaluated without purification. Furthermore, yeast surface display is compatible with fluorescence-activated cell sorting (FACS) that assists in evaluating the functions of displayed proteins in a rapid and quantitative manner. The budding yeast *Saccharomyces cerevisiae* is the most commonly used species, and some studies reported the use of the methylotrophic yeast *Pichia pastoris* (Jacobs et al. 2008; Tanino et al. 2006; Powers et al. 2001) that shows high protein production.

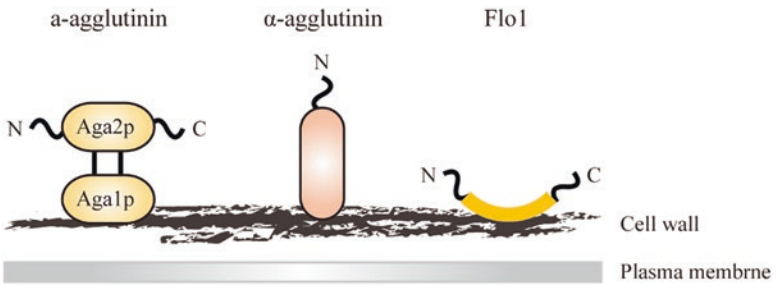

Fig. 10.1 Host cell wall proteins used in yeast surface display and their schematic structures

Heterologous target proteins are fused with various host cell wall proteins, which include a-agglutinin, α-agglutinin, Flo1p, Cwp1p, Cwp2p, Sed1p, Tip1p, YCR89w, Spi1, and Pir1–4 (Mei et al. 2017; Andreu and Del Olmo 2017) (Fig. 10.1). Among these, a-agglutinin and Flo1p are representative proteins that can be used for fusion of heterologous proteins at both N-terminal and C-terminal ends. Another approach is to use an adapter-directed display system with modular features capable of displaying heterologous proteins without direct fusion to host cell wall proteins (Wang et al. 2010). In this system, heterologous proteins are displayed with the aid of the small interactive coiled-coil adapters GR1 and GR2. These anchoring proteins are different from tertiary structures with possible fusion orientations. For some heterologous proteins, protein orientation is important for their activities. For example, an alternative antibody mimetic (fibronectin domain) exhibited 40–90% of reduced enrichment efficiency when switched from C-terminal to N-terminal display (Stern et al. 2016). Since there is no universal anchoring protein, choosing the most appropriate protein for any given target protein is essential. For example, the human antibody formats are fused to the C-terminus of the Aga2p subunit of a-agglutinin. Nanobodies, a camelid single domain antibody format, are successfully displayed at both N-terminal and C-terminal ends with a variety of anchoring proteins (Aga2p (Kruse et al. 2013), Sag1 [α-agglutinin]) (Ryckaert et al. 2010) and for a synthetic tether (McMahon et al. 2018).

Eukaryotic surface display possesses several advantages against other display systems, these include bacterial surface and phage display. Eukaryotic cells have sophisticated quality control machinery for protein folding and can display various antibody formats. Phage and bacterial display systems sometimes encounter difficulties due to misfolding. Thus, co-expression with molecular chaperones is critical in increasing the solubility and binding activity of single-chain variable fragments (scFv) (de Marco and De Marco 2004; Sonoda et al. 2011; Wang et al. 2013). Because of this quality control machinery, yeast surface display can produce antibodies that are too complex to display in phage and bacterial display systems. Bowley et al. compared phage display and yeast surface display, using the same HIV-1 immune scFv cDNA library and showed that yeast display sampled the antibody repertoire more fully than phage display (Bowley et al. 2007). Co-expression of protein disulfide isomerase in yeast can increase the efficiency of antibody dis-

play (Wentz and Shusta 2007). Another advantage of yeast surface display compared to phage display is its compatibility with FACS, which enables a quantitative discrimination of binding kinetics, and use of expression tags can normalize binding affinity with expression level. However, the library size that can be screened by state-of-the-art FACS is limited to 10^8–10^9. To increase throughput and the possibility of identifying rare clones, non-specific cells must be removed using magnetic-activated cell sorting to reduce library size from 10^9 to 10^7 unique clones (Siegel et al. 2004; Chao et al. 2006; Ackerman et al. 2009).

Although yeast surface display possesses various advantages, it also has some drawbacks; one is the avidity effect. Each yeast cell could display around 10^5 molecules on its surface (Shibasaki 2001), and screening could result in isolation of antibodies with relatively weak affinity. The impact of avidity is emphasized when the selection is against oligomeric antigens (Sheehan and Marasco 2015). To solve the problem, Stern et al. developed a titratable avidity reduction method using dithiothreitol to release displayed proteins in a controllable manner (Stern et al. 2017). This method reduced a number of displayed molecules to 3000–6000 per yeast cell, aiding in isolating binders with higher affinities. Another disadvantage is that the library size in yeast surface display is more reduced than the phage display. Yeast surface display can routinely generate 10^7–10^9 unique clones (Chao et al. 2006) while phage display generates 10^{11} unique clones (Bazan et al. 2012). However, recent optimization of a yeast transformation process produced a library size of 10^{10}, comparable to that of phage display (Benatuil et al. 2010).

Some studies have taken the advantage of phage display and yeast surface display. For example, primary screening is carried out with aid of the larger scale of phage display, while secondary screening takes advantage of the flexibility and quantification of FACS-based yeast selection (Bidlingmaier et al. 2015; Ferrara et al. 2012). Patel et al. developed another selection strategy in which the same antibody libraries are displayed on both phage and yeast surfaces via a cross-species display and successfully obtained more unique clones (Patel et al. 2011).

3 Antibodies and Antibody Mimetics Used in Yeast Surface Display

This section briefly describes types of antibodies and antibody mimetics used in yeast surface display. Yeast surface display have been successfully used to display various binder molecules such as scFv fragments (Boder and Wittrup 1997; Chao et al. 2006), Fab fragments (van den Beucken et al. 2003; Walker et al. 2009; Rosowski et al. 2018), full-length IgG antibodies (Rakestraw et al. 2011), and T cell receptors (Smith et al. 2015) (Fig. 10.2). Even when using a eukaryotic yeast surface display system, production and display of full-length IgG antibodies are difficult due to their structural complexity (Horwitz et al. 1988). Rakestraw et al. carried out a directed evolution of a secretory leader and found that a mutant α-mating factor 1 leader peptide showed a 180-fold improvement in the secretion of

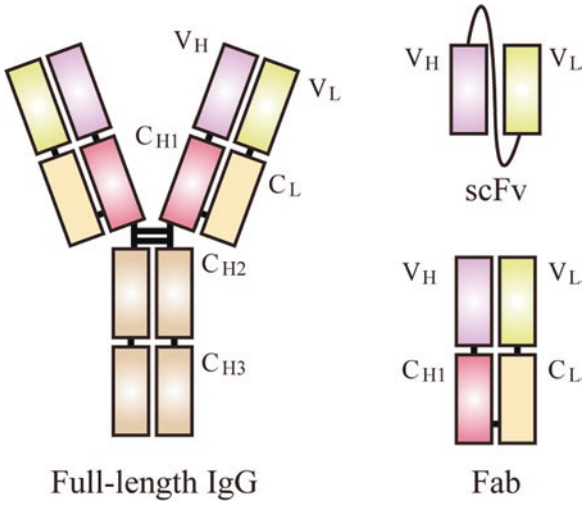

Fig. 10.2 Representative antibody formats displayed on yeast cell surface

functional full-length human IgG (Rakestraw et al. 2009). Cell surface display of functional full-length IgG has been achieved, using several approaches. Rakestraw et al. developed the secretion and capture technology, SECANT system, which relied on in vivo biotinylation of the target protein followed by its capture on the avidinated yeast cell surface (Rakestraw et al. 2011). Rhiel et al. developed another approach named REAL-Select (Reversible Expression of Antibody Libraries for Selection) without requiring intracellular modification of IgG (Rhiel et al. 2014). In this approach, a streptavidin-ZZ domain fusion protein is immobilized on biotinylated cell surfaces, and full-length antibodies are captured by binding to the ZZ domain. Besides full-length antibodies, a Fc fragment with antigen-binding (Fcab) has been proposed as an alternative construct (Wozniak-Knopp et al. 2010, 2017; Leung et al. 2015). Fcab is an antibody format in which the C_H3 domain is randomized to produce target protein-binding ability while retaining many of the key properties of the wild-type Fc fragment. With this process, it is easy to replace the Fc portion of a monoclonal antibody with a Fcab fragment, enabling an easy construction of bispecific antibodies.

Alternative binding scaffolds, which have been thoroughly reviewed (Konning and Kolmar 2018), have emerged as a novel class of binding biomolecules. These proteins include single-domain antibodies (e.g., V_HHs in camelids (Desmyter et al. 1996; Revets et al. 2005) and vNARs in sharks (Stanfield et al. 2004; Grzeschik et al. 2018)), fibronectin type III domain (Fn3) (Koide et al. 1998; Chen et al. 2013), designed ankyrin repeat proteins (DARPins) (Pluckthun 2015), anticalins (Gebauer and Skerra 2012), GFP-based binders (GFAbs) (Pavoor et al. 2009), etc. These alternative binders are small compared to the conventional antibody formats and exhibit greater stability, solubility, and improved tissue penetration (Weidle et al. 2013). In addition, alternative binding scaffolds are easy to produce in *Escherichia*

coli, thereby reducing the production costs. Furthermore, researchers have developed much smaller alternative binders that include fynomers (63 a.a.) (Grabulovski et al. 2007), affitin (65 a.a.) (Mouratou et al. 2007), Sso7d (63 a.a.) (Gera et al. 2011), affibodies (58 a.a.) (Lofblom et al. 2010), and knottin (20–50 a.a.) (Moore et al. 2012). Some papers further improved the characteristics of these small binders. For example, Sso7d is small, having high thermal stability, and lacks cysteins and glycosylation sites; however, Sso7d is highly positively charged leading to specificity constraints for target epitopes and leading to non-specific binding to mammalian cell membranes. Traxlmayr et al. developed charge-neutralized Sso7d and reported that charge neutralization facilitated an evolutionary adaptation of Sso7d to differentially charged epitopes (Traxlmayr et al. 2016). In general, charge neutralization is not straightforward because it can affect protein solubility, stability, affinity, specificity, and productivity. Case et al. compared three approaches to introduce charged-to-neutral mutations to affibodies (Case and Hackel 2016) and found that a synthetic consensus design, based on the amino acid distribution in functional mutants, was the most robust. It is known that some alternative binders are prone to lose stability upon introduction of mutations, and researchers have tried to develop novel small binders with high-affinity and retention of stability. Kruziki et al. systematically screened protein structures from PDB against several criteria (e.g., stability retention upon mutation, lack of disulfides, and large available binding surfaces) and found that the 45-residue T7 phage gene 2 protein (Gp2) could produce a more superior binding scaffold (Kruziki et al. 2015). De novo ligand discovery yielded target-specific Gp2 as strong as 200 ± 100 pM affinity, having T_ms from 65 to 80 °C, allowing in retaining the activity after a thermal denaturation.

Biodistribution mechanisms, such as extravasation and tissue penetration, are limited in conventional antibody formats because of their large size (full-length antibody 150 kDa, Fab 50 kDa, and scFv 27 kDa). Due to a superior biodistribution of small alternative binders, they have been developed for the applications of targeted molecular therapy, diagnostics, and imaging. For example, their small size is ideal for permeability across vasculature, which facilitates uptake by tumors (Schmidt and Wittrup 2009; Yuan et al. 1995). Furthermore, the in vivo clearance rate of alternative binders is rapid, and it is an appropriate tool for imaging because of its high signal to noise ratios (Stern et al. 2013; Miao et al. 2010). Some papers further improved the biodistribution of alternative binders. For example, Hackel et al. showed off-target uptake of Fn3 is directly tunable through hydrophilic charge mutations (Hackel et al. 2012). Small binders are also appropriate for use in single-molecule super-resolution imaging because of their small molecular weights. It has been shown that, by targeting GFP with camelid V_HH, nanometer spatial resolution is easily achievable from any GFP-tagged proteins with minimal linkage error (Ries et al. 2012).

Yeast surface display can be used to engineer antibodies with special features. For example, it has been used to engineer catalytic antibodies that recognize transition states to accelerate enzymatic catalysis (Okochi et al. 2007; Lin et al. 2003) and protease-inhibitor antibodies by grafting peptide inhibitor motifs (Aoki et al. 2011; Nam et al. 2017).

4 Design of Screening Strategies

4.1 Preparation of Antigens

Membrane proteins that permit communication between cells are important research tools and useful for the therapeutic purpose. However, it is difficult to develop antibodies against membrane proteins because their isolation is still a bottleneck. This section focuses on trials to screen antibodies against membrane proteins.

Generally, it is necessary to purify the target proteins to generate specific antibodies. When a target membrane protein has ectodomain, a domain that extends into the extracellular space, screening against isolated ectodomains that have been produced as soluble recombinant is possible, using the usual yeast surface display protocols (Chan et al. 2017). Without an ectodomain, a purification of membrane proteins becomes difficult. Membrane proteins are typically not abundant, and heterologous expression often leads to a bottleneck because of its unpredictable compatibility in a host. A highly hydrophobic and/or amphipathic nature of membrane proteins leads to challenges to purify a homogeneous and stable sample (Pandey et al. 2016). To solve these problems, many variables need to be optimized, which include construct design, expression system, culture conditions during expression, detergent choice, purification methods, and additives in the purification process (Moraes et al. 2014).

In the field of antibody screening against membrane proteins and its application, one of the most prominent studies is the structural analysis of the active state of the β_2-adrenergic receptor (β_2AR) carried out by Kobilka's group (Rasmussen et al. 2011; Steyaert and Kobilka 2011). β_2AR is one of the GPCRs and mediates physiological responses via interaction with epinephrine (Manglik et al. 2017). GPCRs have intrinsic conformational heterogeneity, which is a major hurdle in obtaining their crystal structures. In addition, it is difficult for natural agonists to stabilize GPCR structure due to their weak affinity, hence obtaining crystal structures of active GPCR conformations is quite challenging. Kobilka's group screened anti-β_2AR nanobodies, using phage display, and found that a nanobody stabilized the agonist-bound, active-state structure of β_2AR (Rasmussen et al. 2011; Pardon et al. 2014). Nanobody has a unique three-dimensional structure with a long protruding CDR3 loop with the ability to bind cryptic epitopes, largely inaccessible using conventional antibodies (De Genst et al. 2006) (Fig. 10.3). Using the anti-β_2AR nanobody, they succeeded in determining the active-state crystal structure of β_2AR bound to the strong agonist BI-167107. Furthermore, Kobilka's group improved the affinity of the anti-β_2AR nanobody with the aid of yeast surface display and succeeded in determining the crystal structure of the active state of β_2AR bound to a weak natural agonist. Despite the importance of this study, crystal structure analysis of membrane proteins is still difficult because of their intrinsically unstable nature. Thermostabilization of receptor proteins by a small number of point mutations has become a promising approach to overcome this problem (Jazayeri et al. 2016; Rucktooa et al. 2018).

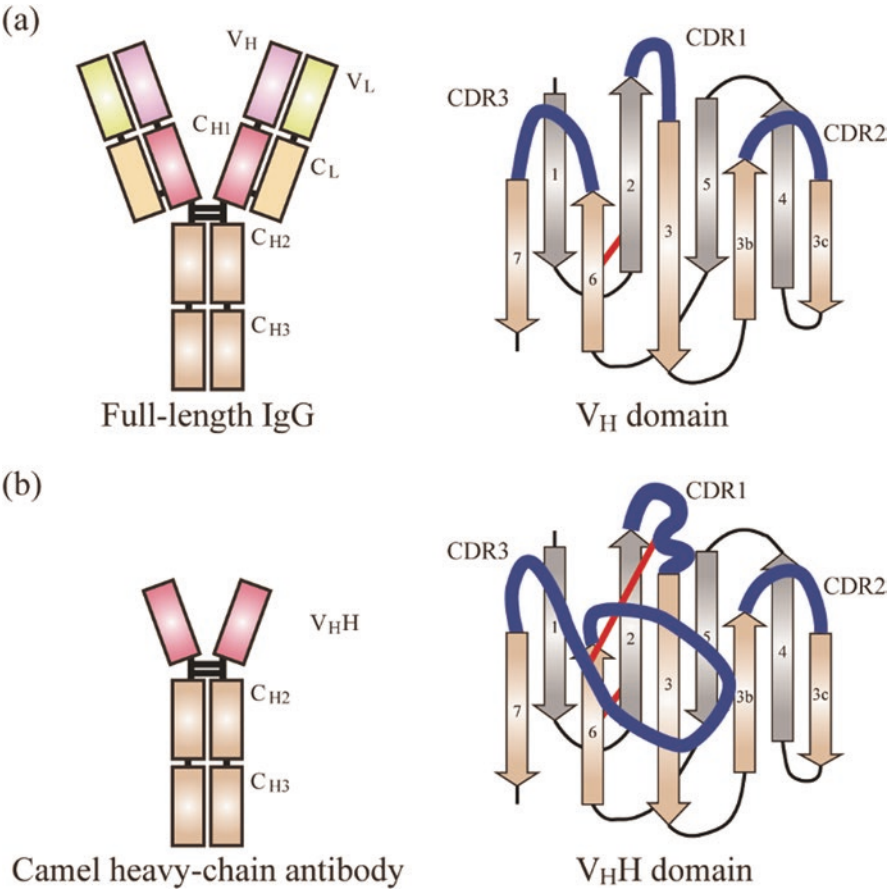

Fig. 10.3 Schematic representations of CDRs. (**a**) Full-length IgG and V_H domain. (**b**) Camel heavy-chain antibody and V_HH domain (nanobody)

In this wise, purification of membrane proteins is a challenging and time-consuming process. To bypass this problem, researchers have developed more direct approaches to engineer antibodies to membrane proteins in which whole cells or detergent-solubilized whole-cell lysates are used as antigens (Tillotson et al. 2013a, 2015a). Wang et al. showed that anti-fluorescein scFv can be enriched by successive rounds of yeast biopanning against a fluorescein-labeled mammalian cell monolayer (Wang and Shusta 2005) (Fig. 10.4a). They also applied the biopanning method to rat brain endothelial cells. Using a naïve human scFv library displayed by *S. cerevisiae*, they are able to identify 34 unique lead antibodies (Wang et al. 2007). They found that some of these antibodies were internalized by the cells. This biopanning approach was extended to obtain cancer-specific antibodies. For example, Zorniak et al. applied yeast display biopanning against glioblastoma stem-like cells and obtained a collection of antibodies that were differentially selective for these cells (Zorniak et al. 2017). One of these antibodies was selective for five distinct

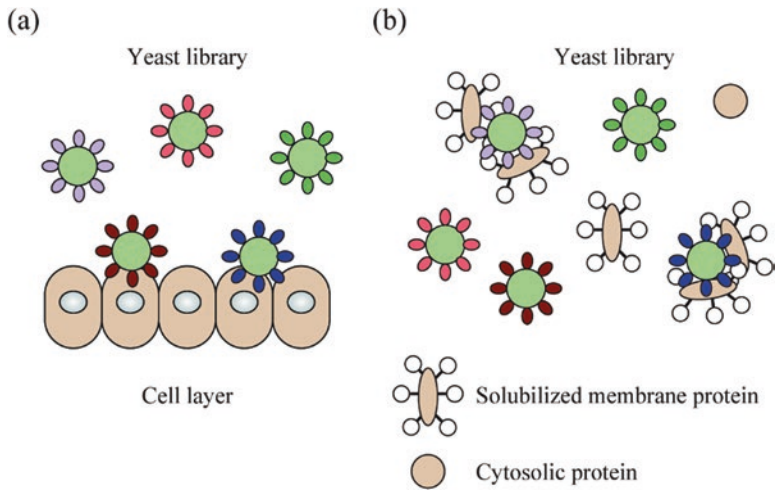

Fig. 10.4 Direct approaches to engineer antibodies to membrane proteins. (**a**) Yeast biopanning against mammalian cells. (**b**) Solubilized whole cell lysate as an antigen source

patient-derived glioblastomas and succeeded in visualizing orthotopic glioblastoma xenografts in vivo after conjugation with a near-infrared dye. Biopanning has also been applied to alternative binders (fibronectin domain, Gp2, and affibodies), and the elements that have dictated ligand enrichment and yield have been quantitatively identified (Stern et al. 2016). As a result, it was found that extended flexible linkers between ligand and yeast cell improved enrichment ratios in the biopanning process. Richman et al. also reported another approach called a density centrifugation, which facilitated a rapid selection of proteins with high affinities for mammalian cell surfaces (Richman et al. 2006).

Although biopanning is an easy method to identify antibodies against membrane proteins, it is not compatible with a high-throughput and quantitative FACS screening. Thus, Shusta's group proposed a method in which solubilized whole cell lysate was used as an antigen source (Fig. 10.4b). Using this approach, they find that scFv displayed on yeast cell surfaces retains its activity on a variety of detergent solutions and that antibody screening and immunoprecipitation are possible via the antigen-antibody interaction in solubilized whole cell lysates (Cho et al. 2009; Cho and Shusta 2010). Furthermore, affinity maturation of anti-transferrin receptor scFv in whole cell lysate successfully improved its equilibrium binding affinity by three- to sevenfold (Tillotson et al. 2013b).

4.2 Construction of Antibody Libraries

The quality and diversity of antibody libraries are determined by the immunoglobulin gene source. There are mainly two sources of establishing the library repertoire: the mRNA of B cells from immunized donors (immune library) and healthy donors

(naïve library). The immunized library is used for screening the antibodies against certain antigens, and the naïve library against all types of antigens. In addition, some reports have used synthetic or semisynthetic libraries (McMahon et al. 2018; Hanes et al. 2000; Moutel et al. 2016) diversified by design. These methodologies have different properties in terms of size, quality, framework, and CDR composition (Ponsel et al. 2011).

To construct antibody libraries from immune B cells, the heavy- and light-chain genes are amplified from mRNAs and associated depending on the antibody format to be displayed. There are two types of fragment association: randomly paired and natively paired. Many methods are used for constructing randomly paired libraries. Feldhaus et al. developed a method to generate a combinatorial Fab library, using yeast mating (Weaver-Feldhaus et al. 2004). In this method, a set of yeast strains and a two-vector system for cell surface display of heavy chain and light chain are constructed. Through mating of the haploid yeast libraries, a large-scale heterodimeric Fab library can be displayed on yeast cell surfaces. Yeast mating is a useful method. Optimizing the process, it can be applied to large-scale (10^9) Fab library construction (Baek and Kim 2014) and affinity maturation via light-chain shuffling (Lou et al. 2010).

Screening antibodies using random pairing have led to the development of dozens of experimental or approved drugs (Bradbury et al. 2011; Ecker et al. 2015); however, the non-cognate pairing of V_H and V_L sequences frequently produces poor biophysical properties and lower selectivity compared to authentic human immunoglobulins (Ponsel et al. 2011; Jayaram et al. 2012). To address this problem, novel approaches have been developed to display natively paired Fab or scFv libraries on yeast from the immunized animals, and these have succeeded in developing broadly neutralizing anti-HIV-1 antibodies and anti-PD-1 antibodies efficiently (Adler et al. 2017; Wang et al. 2018). These in vitro methods of displaying natively paired antibodies have advantages in comprehensiveness and in turnaround time compared to the hybridoma-based discovery of natively paired antibodies. Adler et al. compared antibody discovery with natively paired to randomly paired scFv libraries, which were developed from the same mice immunized with IL-21R (Adler et al. 2018). They found that natively paired antibodies had advantages in sensitivity and specificity for antibody screening over those from the randomly paired library that exhibited higher false-negative and false-positive rates. High-affinity antibodies can be obtained from randomly paired antibody libraries from immunized animals, but, in some cases, it can be as a result of the isolation of natively paired antibodies (Wang et al. 2016).

It is difficult to obtain high-affinity antibodies by in vitro cell surface selection from the naïve library because of lack of somatic hypermutation. Thus, many studies have optimized affinity maturation processes after isolation of lead antibodies from a naïve library. Many in vitro affinity maturation papers use error-prone PCR in combination with gene shuffling and targeted mutagenesis. For example, Wittrup's group mimicked the full spectrum of mutagenesis strategies of B cells (random point mutagenesis, insertions, deletions, receptor revision, and chain shuffling), using yeast homologous recombination (Swers et al. 2011). This method enabled a

reduction of the probability of trapping in local optima and improved affinity by 10,000-fold in three rounds of screening. Simplified affinity maturation processes have also been developed. Tiller et al. developed a method in which they identified sites in V_HH CDRs permissive to mutagenesis while maintaining antigen binding by computational and experimental alanine scanning. They also mutagenized the positions to encode the most frequently occurring residues at each CDR position based on the natural antibody diversity. Although the process is easy and assists in the improvement of affinity by fivefold, an effective affinity maturation requires a more comprehensive mutational strategy (Tiller et al. 2017a). Most of the previous studies on affinity maturation target the binding sites on CDRs for mutation, but other residues can affect affinity. For example, Sharma et al. reported that a conservative substitution of a non-CDR residue at the interface of TCR variable domains increased affinity by 60-fold (Sharma and Kranz 2018). The result indicated that subtle changes at significant distances from the binding site could strongly affect ligand binding. Another approach to improving the affinity maturation process is to use computational design. Lippow et al. used an iterative computational design procedure that focused on electrostatic binding contributions and single mutants to obtaining mutations that improved the affinities of antibody drugs (Lippow et al. 2007). They also improved the affinity of the anti-epidermal growth factor receptor drug cetuximab from tenfold to 52 pM, combining multiple designed mutations.

Robust affinity maturation processes have also been optimized for alternative binders. Wittrup's group investigated the robustness of a selection process to obtain stable, high-affinity Fn3. By combining error-prone mutagenesis, loop length diversification, and shuffling of mutagenized loops, they succeeded in isolating an anti-lysozyme Fn3 by affinity maturation with an affinity of 1.1 pM (Hackel et al. 2008). Based on the analysis of an intermediate population, they found that all of recursive mutagenesis, loop length diversity, and loop shuffling processes are critical components in obtaining high-affinity and stable Fn3.

During a library diversification, a balance between variation to improve functionality and the mutant destabilization is important. Wittrup's group compared two diversification strategies for Fn3: partial wild-type conservation strategy and tailored amino acid composition strategy (Hackel et al. 2010). The design elements were compared with seven target proteins by direct competition in a single tube. They found that the structurally biased, tailored diversity library is the more efficient approach in obtaining a generation of superior binders.

In evolved antibody sequences, site-wise constraints are observed in a nonspatial gradient from diverse to conserved. Hence, the possible existence of such constraints in non-antibody scaffolds has also been evaluated. For example, a hydrophilic fibronectin domain was evolved against six targets, and the result of the sequences was investigated to ascertain whether they exhibited a nonspatial, site-wise gradient of amino acid diversity (Woldring et al. 2015). The results demonstrated a range of diversities and site-wise amino acid preferences, which showed that implementation of site-wise constrained diversity enabled a selection of high-affinity binders without affinity maturation (Woldring et al. 2015, 2017).

4.3 Designing Selection Forces

Using display technologies in a screening process, selection forces can be flexibly designed. For example, display technologies can select antibodies that can distinguish orthologue or homologue proteins from the primary screening. Such selection can be assessed post-isolation in the classical hybridoma approach. In this section, we review different examples of design selection process for obtaining antibodies with specific characteristics.

Broadening antibody specificity without losing affinity is important to developing antibody therapeutics against viral infections. Botulism is a disease caused by botulinum neurotoxin (BoNT), which has multiple subtypes. For example, BoNT subtypes A1 and A2 differ by 10% at the amino acid level (Smith et al. 2005). Marks' group tried to develop a dual-selection strategy to increase cross-reactivity of scFv against these BoNT subytpes (Garcia-Rodriguez et al. 2007). In the dual-selection strategy, the primary screening round is carried out against the A1 subtype and next four rounds against the A2 subtype at decreasing concentrations. They further carried out affinity maturation with a similar strategy. Starting from an affinity for the A1 subtype at 136 pM and for the A2 subtype at 109 nM, they succeeded in improving affinity to 115 pM and 87 pM, respectively. Broadening antibody specificity might require sequential selection against variant antigens, as Marks' group did, but simultaneous selection against multiple antigens might not be appropriate. Chakraborty et al. developed an *in silico* model of affinity maturation and investigated the appropriate selection forces for obtaining cross-reactive antibodies (Wang et al. 2015). They found that the simultaneous presence of variant antigens led to conflicting selection forces, which could frustrate maturation. Additionally, they suggested that sequential immunization could be important for inducing cross-reactive antibodies.

Targeting specific epitopes is critical to developing effective antibodies for revealing molecular interactions. Especially, binders that recognize conformational epitopes are useful in several respects, as partially described in the previous section (e.g., work by Kobilka's and Shusta's groups). In a classic example, Siegel's group obtained conformation-specific scFvs against calmodulin (CaM) (Weaver-Feldhaus et al. 2005). They carried out affinity maturation against two different CaM conformations, apo-CaM and Ca^{2+}-CaM, and succeeded in screening conformation-specific scFvs. In streamlining the discovery of epitope-specific binders, an effective approach helps in designing positive and negative selections, using two alternative target molecules, a wild-type and a mutant with an amino acid substitution at the desired epitope (Mann and Park 2015). These positive and negative screening methods are effective to bias the evolution of specificity. Park's groups succeeded in screening Fn3 that specifically inhibited the activity of Erk-2 in mammalian cells (Mann et al. 2013). Puri et al. also developed a similar competitive panning of a yeast display library and obtained antibodies against dengue virus envelope protein domain III successfully (Puri et al. 2013). Another approach to obtaining conformation-specific antibodies is by using single-molecule real-time gene

sequencing as exemplified by Colby's group (Doolan and Colby 2015). In this approach, a mutant prion protein (PrP) library was displayed by yeast, and multiple anti-PrP antibodies were applied to the library. The authors sorted mutants with diminishing antibody binding by FACS, and the responsible mutations were identified by next-generation sequencing. By statistically analyzing the results, they determined the critical epitope for each antibody and detected secondary-structure-dependent and tertiary-structure-dependent contacts. Kruse's group developed a platform to obtaining conformation-specific nanobodies with the aid of yeast surface display (McMahon et al. 2018). They constructed a fully synthetic yeast display nanobody library based on an alignment of structurally characterized nanobodies from PDB. To obtain conformation-specific nanobodies, they carried out primary screening against agonist-bound β_2AR and depleted variants that bound an inactive β_2AR conformation, using a counterselection strategy. They further applied this strategy and succeeded in obtaining active-state-specific nanobody clones against the A_{2A} adenosine receptor.

Protein stability, especially thermal stability, is an important factor that affects the utility of antibodies. Thermal stability correlates with chemical stability, protease resistance, and protein productivity. For example, scFv with an improved thermal stability exhibited less sensitivity to serum protease (Willuda et al. 1999). Hence, improving thermal stability is one of the major goals in the protein engineering field (Traxlmayr and Shusta 2017; Traxlmayr and Obinger 2012). Several approaches have been developed in improving thermal stability. The first is to obtain antibodies that are secreted efficiently. An efficient secretion correlates with thermal stability because incorrectly folded proteins are degraded by ER-associated degradation machinery (Traxlmayr and Obinger 2012). Another approach is to directly obtain thermally stable antibodies. For example, Obinger's group designed a strategy to obtain thermally stable IgG1-Fc scaffolds (Traxlmayr et al. 2012, 2013). In their approach, they subjected yeast cells of IgG1-Fc to heat stress and isolated well-folded mutants by FACS. After the isolation, plasmid DNAs were amplified from the yeast cells, and their sequences were determined. After several selection rounds, they succeeded in obtaining proteins with increased thermal stability.

pH dependence is another important characteristic for functional antibodies (Igawa et al. 2014), and it is attracting attention with respect to its enhanced clinical potential and to its chromatographic applications. Histidine scanning can be useful for obtaining pH-dependent antibodies (Schroter et al. 2018). Hock's group constructed the combinatorial histidine scanning libraries and simultaneously screened antibodies with both high affinity at pH 7.4 and pH sensitivity (Schroter et al. 2015). As a result, they succeeded in isolating antibodies whose dissociation rate constants at pH 6.0 increased from 230- to 780-fold compared to pH 7.4. Tillotson et al. used a histidine saturation mutagenesis strategy to screen for anti-transferrin receptor scFvs with markedly increased dissociation at pH 5.5 (Tillotson et al. 2015b). One such scFv resulted an increased internalized fraction 2.6-fold greater than that of a non-pH-sensitive scFv. Histidine scanning can be applied to alternative binders, and several researchers have succeeded in conferring pH sensitivity to Fn3s (Heinzelman et al. 2015) and IgNARs (Konning et al. 2016).

Haptens are known as difficult targets for obtaining antibodies because of their low molecular weights, low immunogenicities, and their cross-reactivity with protein carriers. Hence, designing appropriate selection forces for binders of haptens could be useful. Blake's group proposed a competitive FACS method that uses a hapten conjugate and soluble hapten (Sun et al. 2016). Using this competitive method, they successfully identified antibodies against methylated versus unmethylated phenanthrenes, which could be used as petroleum contamination markers.

4.4 Epitope Mapping Using Yeast Surface Display

Yeast surface display can be used for epitope mapping which is an important process in antibody development. Surface display of antigens eliminates the necessity to purify antigens and enables a rapid identification of antibody epitopes. In a classic example, each domain of an antigen is displayed on a yeast cell surface, and rough epitope mapping can be achieved by identification of yeast cells with neutralizing activity (Levy et al. 2007). Another classical approach is to display randomized antigen on yeast cell surfaces, and this approach can identify residues that are critical for binding (Spangler et al. 2010; Mata-Fink et al. 2013; Makiya et al. 2012). Development of next-generation sequencers (NGS) enables more precise and efficient epitope mapping of a panel of antibodies (Van Blarcom et al. 2018). Using NGS, Rajpal's group tried epitope mapping of anti-alpha toxin antibodies (Van Blarcom et al. 2015) (Fig. 10.5). They displayed a rationally designed antigen library on yeast cell surfaces. This library was designed with point mutations that uniformly covered the protein surface without affecting the structural integrity. As a next step, they incubated the mutant antigen library with alpha-toxin-specific antibodies. Yeast-producing alpha toxin variants with decreased antibody binding were isolated by FACS. Finally, they conducted high-resolution epitope mapping using deep sequencing of the mutant alpha toxin sequences of the isolated yeast cells. Deep mutational scanning has also been applied to high-throughput residue-level epitope mapping of anti-hemagglutinin nanobodies, revealing that cross-neutralizing nanobodies can bound to a conserved pocket in the hemagglutinin stem region

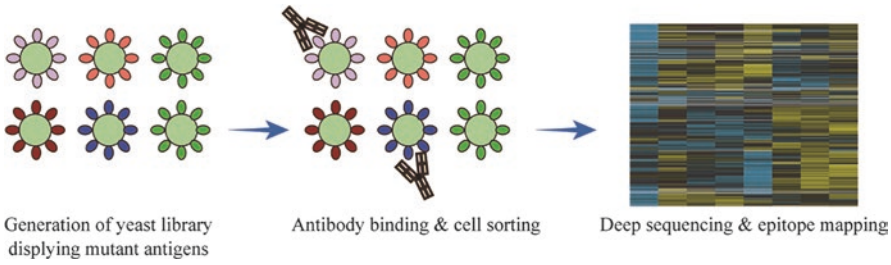

Fig. 10.5 Precise epitope mapping using yeast surface display and next-generation sequencers

(Gaiotto and Hufton 2016). Varadarajan's group improved the epitope mapping process and developed a method to identify ligand binding sites and conformational epitopes with a single amino acid resolution by combining cysteine scanning and deep sequencing (Najar et al. 2017). They found that construction of a cysteine scanning library and labeling of cysteine residues consistently abrogated binding to the target protein, whereas, in alanine scanning, many residues in physical contact are insensitive to alanine substitution.

Cell surface display of antigens is useful for not only epitope mapping but vaccine development (Tamaru et al. 2006). Yeast surface display can produce a large number of antigens without purification, and β-glucan can act as an adjuvant (Rodriguez et al. 2009). Antigen-displaying yeast cells having these characteristics can be used as safe and cheap vaccines. For example, Shibasaki et al. displayed enolase 1 of the pathogenic fungus *Candida albicans* on the *S. cerevisiae* cell surface and found that oral delivery of the *S. cerevisiae* cells to mice improved the survival rate of animals subsequently challenged with *C. albicans* (Shibasaki et al. 2013). Other than *C. albicans*, there are several reports aimed at developing yeast-based vaccines against reovirus (Luo et al. 2015) and influenza (Lei et al. 2016).

5 Novel Applications of Yeast Surface Display to Protein Engineering for Therapeutic Uses

Based on our previous discussion, almost studies on yeast surface display have tried to isolate and optimize promising binders, using known protein structures. However, recent breakthroughs by David Baker assist in the de novo design of novel protein structures with atomic-level accuracy and provide opportunities for screening an unbiased protein shape and sequence space larger than the set of natural proteins (Rajagopalan et al. 2014; Bjelic et al. 2014).

To reveal the sequence determinants of folding, Baker's groups investigated rules to design proteins folded into unique structures stabilized by a number of weak interactions (Rocklin et al. 2017). Combining computational protein design, next-generation gene synthesis, yeast surface display, high-throughput protease susceptibility assays, and deep sequencing, they carried out massively parallel design, synthesis, and testing for the global analysis of protein folding. More specifically, 15,000 de novo designed mini proteins, 10,000-point mutants, 30,000 negative controls, and 1000 natural proteins were synthesized, and their protease susceptibility was quantified using yeast surface display. As a result, they identified more than 2500 stable designed proteins in four basic folds, and the results enabled them to systematically examine how protein sequences determined folding and stability in an uncharted protein space. By further iterative design and experimental cycles, the success rate of de novo protein design increased from 6% to 47%, and they succeeded in producing stable proteins unlike natural ones. The protein shape and sequence space designed by Baker's groups are vastly larger than those of natural

proteins and are unbiased by selection for novel functions. Hence these novel protein structures could exhibit superior characteristics to natural ones. The designed mini-proteins exhibited higher stability compared to known sized monomeric proteins in the PDB database.

Baker's group further tried to design therapeutic mini-protein binders, using a similar approach (Chevalier et al. 2017). They designed 22,660 mini-proteins of 37–43 residues, targeting influenza hemagglutinin and botulinum neurotoxin B, and 6286 control sequences. They displayed these designed proteins on yeast cell surfaces and identified 2618 high-affinity binders by deep sequencing. These binders exhibited good biophysical characteristics. Unlike antibodies, they retained their binding abilities after exposure to high temperature. They demonstrated their clinical applicability and found that three sequential doses of the identified mini-proteins via intranasal or intravenous administration every 2 weeks produced little or no antibody response. Furthermore, intranasal delivery of mini-protein binders against influenza 24 h prior to viral challenge led to 100% prophylactic efficacy. Small binders have advantages of stability and amenability to chemical synthesis compared to antibodies and selectivity and designability compared to small molecules. With these advantages, small binders might bridge the gap between small molecule and antibody therapeutics.

6 Conclusions and Outlook

Yeast surface display is an effective methodology for engineering proteins with desired characteristics, and it has led to successful therapeutic applications. However, there are still several problems to be solved in the field of antibody screening and engineering.

In display technologies, immobilization of target proteins could affect protein functions. For example, the orientation of displayed proteins is important as shown in the case of an anti-CD3ε scFv, which showed threefold higher affinity with free N-terminus (Wang et al. 2005). In another example, affinity maturated anti-CaM scFv (K_d = 1 nM) lost binding activity after conversion to a soluble form (Weaver-Feldhaus et al. 2005). scFvs possess lower stability than full-length mAbs, and they require further stabilization (Grewal et al. 2016). To solve the immobilization problem, some researchers have proposed switchable display and secretion systems to achieve a rapid and easy evaluation of soluble antibody fragments (Van Deventer et al. 2015; Shaheen et al. 2013). This system enables high-throughput evaluation of soluble antibodies without plasmid construction. Recently, Shembekar et al. used droplet microfluidics to evaluate the binding abilities of secreted antibodies while maintaining genotype-phenotype linkage (Shembekar et al. 2018). Though throughput and a signal/noise ratio of the system are not high, high-throughput direct evaluation of soluble antibodies will be a powerful platform for antibody engineering.

Superior drug-like properties other than high affinity are necessary for successful antibody therapeutics (Ponsel et al. 2011; Jain et al. 2017). However, drug-like

properties are often evaluated in late stages of the development process. It will be of high value (by minimizing the risks inherent in drug development) if it is possible to evaluate developability such as specificity (Kelly et al. 2017a, b, 2018; Xu et al. 2013; Tiller et al. 2017b), aggregability, productivity, solubility, in vivo clearance rate (Kelly et al. 2015), and immunogenicity, during the high-throughput selection process.

Another problem to be solved is the high-throughput production of a proteome-wide antibody set against the proteomes of various organisms. The Human Protein Atlas project was launched to construct a complete proteome map in all major tissues in the human body (Uhlen et al. 2005, 2010, 2015). This project requires highly specific antibodies to all protein-coding human genes and costs a lot. Some researchers have tried to develop a high-throughput antibody screening system to construct proteome-wide antibodies using animal immunization, phage display, and yeast display (Colwill 2011; Mersmann et al. 2010; Hust et al. 2011; Dubel et al. 2010; Schofield et al. 2007; Ferrara et al. 2015; Zhao et al. 2014; Bowley et al. 2009). However, these systems still require improvement to generate a diverse antibody set with high fidelity. Low cost but reliable methodologies for generating proteome-wide antibody sets will lead to protein atlas projects in various non-model organisms.

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Chapter 11
Oral Vaccine Development Using Cell Surface Display Technology

Seiji Shibasaki

Abstract Oral vaccines are an attractive medical application of cell surface engineering technology. After the discovery of the effectiveness of vaccines, several fundamental methods to produce antigenic proteins have been developed. Recently, display and evaluation methods for production of oral vaccines using cell surface engineering have been established. *Saccharomyces cerevisiae* and *Lactobacillus casei* have been used to display antigens and studied as delivery tools for antigens. These microorganisms function not only as vehicles for antigens but also as adjuvant materials. In this chapter, we describe examples of oral vaccines developed to combat candidiasis using these two host cells.

Keywords Oral vaccine · Candidiasis · Enolase · *Saccharomyces cerevisiae* · *Lactobacillus casei*

1 Introduction

1.1 Vaccination to Treat Infectious Diseases

Infectious diseases are the leading cause of death in humans worldwide. The two fundamental approaches to improving public health during the past century have been sanitation and vaccination, which have dramatically reduced deaths from infectious diseases (Janeway et al. 2005). Four representative types of vaccines are shown in Fig. 11.1. Inactivated whole pathogens generally retain a high proportion of antigens found in the live pathogen. However, frequent booster administration and adjuvants are needed to confer and maintain protection. In general, live-attenuated pathogens are regarded as more effective than inactivated whole pathogens because they can induce high titers of pathogen-neutralizing antibodies. Unfortunately, attenuated vaccines are associated with the risk of pathogen

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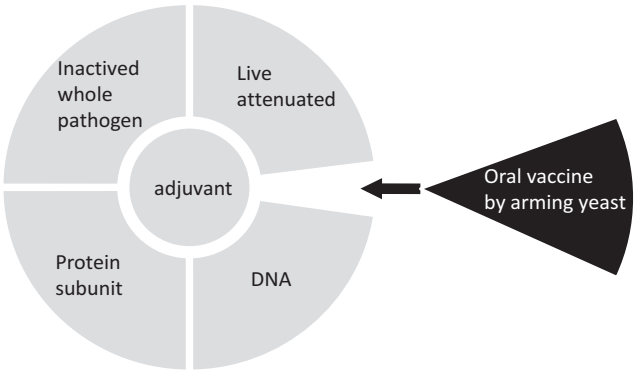

Fig. 11.1 General category of conventional vaccines (gray). Oral vaccination by arming yeast (black) is novel method (The figure is modified from Chambers et al. 2016)

reversion to the virulent form, resulting in disease (Chambers et al. 2016). Protein subunits or recombinant proteins are regarded as safe vaccine antigens (Pua et al. 2017), although the processes required for purification of antigenic proteins are complicated. Moreover, the time required to produce sufficient amounts of protein for clinical use is often long. Therefore, novel tools are needed for large-scale production of vaccines against a wide variety of infectious diseases, and different biotechnological approaches should be examined to eliminate complicated steps in vaccine production.

1.2 Novel Approaches for Production of Antigenic Protein

Cell surface engineering has recently provided convenient tools for protein production, called surface display technology or molecular display technology (Shibasaki and Ueda 2014, 2010). Using this technology, foreign proteins can be produced on the surface of host cells. If whole cells (such as genetically modified yeast cells) displaying foreign protein on the cell surface could be applicable for vaccine, purification steps can be omitted, thereby speeding up the vaccine production process (Shibasaki et al. 2003, 2007). Therefore, surface display technology may be an appropriate novel tool for the development of oral vaccines (Fig. 11.2). Although virus, bacterial cells, and mammalian cells are available as hosts for cell surface display of recombinant proteins (Shibasaki et al. 2009), safety should be considered for administration in humans. Easy handling is also important to develop novel vaccines for various infectious diseases. The yeast *Saccharomyces cerevisiae* and the bacterium *Lactobacillus casei* have been extensively investigated for production of antigenic protein and as potential oral vaccines (Shibasaki and Ueda 2016).

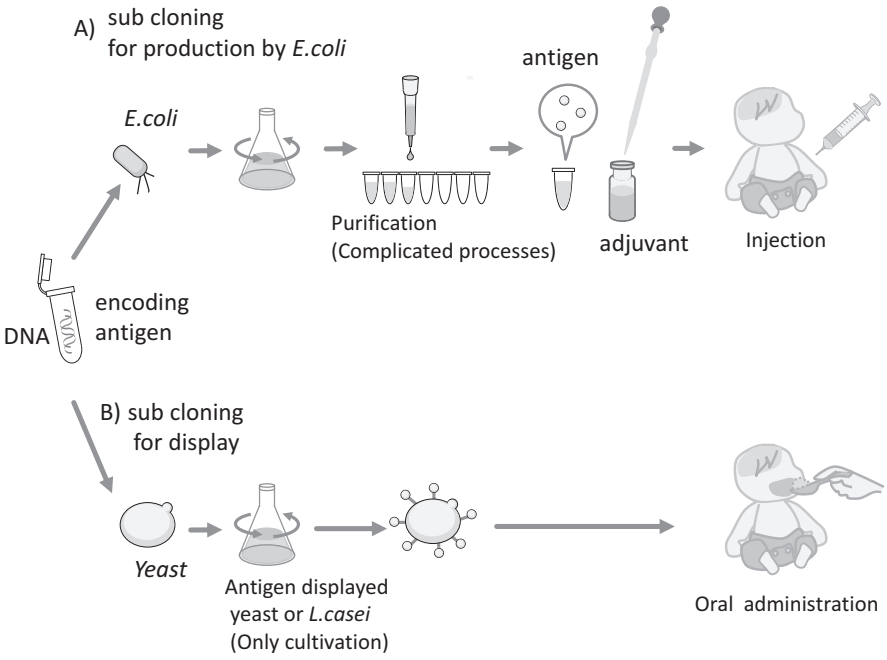

Fig. 11.2 Conventional recombinant vaccine (upper) and oral vaccine produced by cell surface display on antigen (lower)

1.3 Candidiasis: A Model Infectious Disease

Combatting infectious diseases with vaccines is a major research area in medicine. As a model disease that can be targeted by oral vaccine using cell surface engineering, candidiasis is often studied as a type of fungal infections caused by *Candida* species (Paramythiotou et al. 2014). Systemic or superficial candidiasis occurs when host immunity is compromised by chemotherapies for cancer, the administration of immunosuppressants, or the presence of acquired immunodeficiency syndrome. Typically, chemotherapy for candidiasis should be started immediately after diagnoses (Glöckner and Cornely 2013). However, clinical treatments for candidiasis remain unsatisfactory because of delayed diagnosis and a lack of reliable tools to detect *Candida* species in patients (Noble and Johnson 2007). Therefore, efficient prevention by vaccination in addition to therapies is urgently needed to manage candidiasis.

Prevention of candidiasis using vaccines is currently a major focus of several laboratories (Scriven et al. 2017; Shibasaki et al. 2016). To date, several potential antigens have been reported for vaccine development (Green et al. 2013; Clancy et al. 2008). For creation of an oral vaccine by cell surface engineering, *C. albicans* Eno1p, which has well-characterized immunological responses (Li et al. 2011), has been extensively studied. In this chapter, applications of cell surface engineering for

development of oral vaccines against *Candida* infections using *S. cerevisiae* and *Lb. casei* are discussed.

2 Yeast Cell Surface Display of the Antigen

2.1 Architecture of the Display System Using S. cerevisiae

S. cerevisiae has a cell wall consisting of polysaccharides and proteins (Shibasaki et al. 2008; Klis et al. 2002). Polysaccharide networks, such as β-1,3-glucan, β-1,6-glucan, and chitin, provide chemical and physical strength to the cell wall. β-Glucans are recognized as macromolecules that stimulate the immune system (Stier et al. 2014). Therefore, display of an antigenic protein on the yeast cell surface permits the cell to function as an adjuvant when used as a vaccine. In addition, *S. cerevisiae* has a "generally recognized as safe" (GRAS) status, suggesting that it may be suitable for oral administration if it is developed as a vaccine.

In early studies of cell surface antigen display, an antigen from red sea bream iridovirus (Nakajima et al. 1999) was displayed on the surface of yeast cells to develop an oral vaccine for use by fisheries (Tamaru et al. 2006). Notably, dried yeast cells are currently used for feeding of fish, suggesting that this method may be suitable as an injectable vaccine (Nakajima et al. 1999).

2.2 Display of Eno1p on the Surface of S. cerevisiae

As described above, *C. albicans* Eno1p was displayed on the surface of *S. cerevisiae* cells (Shibasaki et al. 2016) via introduction of the pULD1-eno1 plasmid into yeast strain BY4741. Confirmation of successful transformation with this plasmid was performed using auxotrophic selection and colony direct polymerase chain reaction (PCR). In addition, display of Eno1p on the cell surface of BY4741/eno1 was also observed by immunofluorescence microscopy using Alexa Fluor 488 (Fig. 11.3). Furthermore, the fluorescence intensity of yeast cells stained with Alexa Fluor 488 was quantified using a multiwell plate reader (Shibasaki et al. 2001). As a result, the authors showed that the amount of displayed Eno1p increased for the first 24 h of the growth cycle.

Cells used for oral administration of Eno1p-displaying yeast to mice were grown for 24 h, which was the length of culture when cells showed the largest number of Eno1p-fusion proteins on the surface. Mice were vaccinated by oral administration of the cells four times over the course of a 7-week period before challenge with a lethal dose of *C. albicans*. The average titer of antibodies against Eno1p generated after oral administration Eno1p-displaying cells was 5.2×10^3, although the value varied substantially between animals (1×10^2–5.2×10^4; Fig. 11.4). The yeast

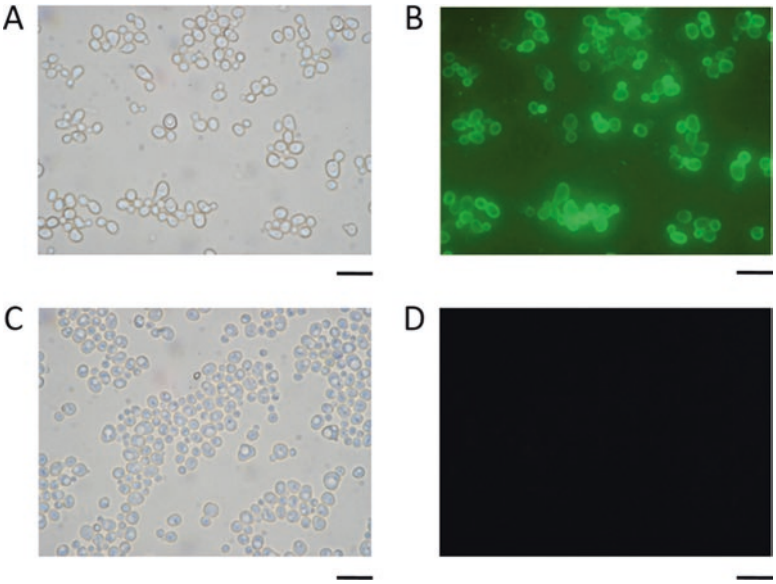

Fig. 11.3 Microscopic observation of *S. cerevisiae* by bright-field microscopy (**a**, **c**) and fluorescence microscopy (**b**, **d**). Eno1p-expressing cells (**a**, **b**). Control cells, strain BY4741 (**c**, **d**). Bar = 10 μm

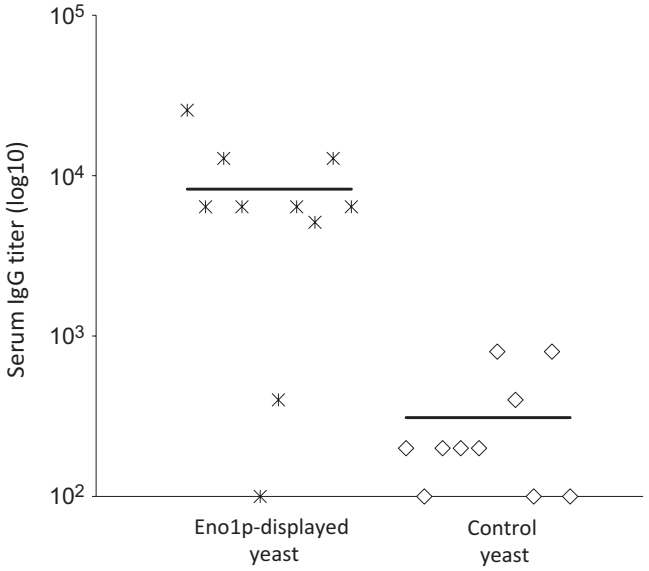

Fig. 11.4 Antibody responses following oral administration of yeast cells. Serum derived from mice administered yeast displaying Eno1p (cross) and mice administered control yeast (diamond)

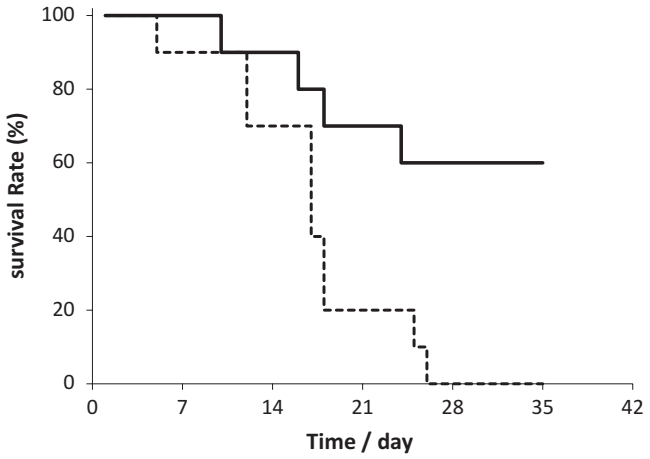

Fig. 11.5 Survival rates in mice administered *S. cerevisiae* expressing Eno1p (solid line) and in mice administered control *S. cerevisiae* (dotted line)

showed sufficient immunological stimuli in almost all mice. Investigation of survival rates after challenge with *C. albicans* for 35 days indicated that 60% of mice receiving oral administration of Eno1p-displaying cells survived longer than mice receiving oral administration of control cells (Fig. 11.5). This survival rate was better than that associated with the more conventional modes of immunogen administration, i.e., subcutaneous injection and intranasal administration (Shibasaki et al. 2013).

3 *Lactobacillus* Cell Surface Display of the Antigen

3.1 Architecture of the Display System Using Lb. casei

Lb. casei has been previously used in oral vaccine development and is also a GRAS organism (Schiraldi et al. 2006). For example, antigen display of human papillomavirus (HPV) 16 E7 on the surface of *Lb. casei* cells has been investigated using M6 protein originating from *Streptococcus pyogenes*. *Lb. casei* cells displaying E7 antigen made it possible to induce immune responses in mice against HPV (Bermúdez-Humarán et al. 2004). Additionally, cell surface engineering has also been successfully used to produce the HPV16 E7 protein on the surface of *Lb. casei* using poly-gamma-glutamic acid synthetase complex A from *Bacillus subtilis* as an anchoring protein (Poo et al. 2006).

3.2 *Display of Eno1p on the Surface of* Lb. casei

The plasmid pPG-Eno1 was introduced into *Lb. casei* 525. Subsequently, transformed cells were confirmed by performing auxotrophic selection and colony-direct PCR (Shibasaki et al. 2014). Oral administration with *Lb. casei* 525 cells (control) or Eno1p-expressing *Lb. casei* cells was performed four times during a 7-week period in mice. The average anti-Eno1p antibody titer generated after oral administration of Eno1p-expressing *Lb. casei* cells was 7.5×10^2 and that of control *Lb. casei* 525 was 1.3×10^2 (Fig. 11.6). The antibody titer in mice administered Eno1p-expressing *Lb. casei* (5.2×10^3) was around one-seventh that of mice administered Eno1p-expressing yeast (Fig. 11.4). These average antibody titers generated by *Lb. casei* and yeast indicated that yeast induced a better immunological response against Eno1p. Compared with mice treated with control *Lb. casei*, mice administered *Lb. casei* cells displaying Eno1p had increased survival rates after challenge with a lethal dose of *C. albicans.* Oral administration of *Lb. casei* cells displaying Eno1p on the surface protected 10% of the mice against candidiasis (Fig. 11.7).

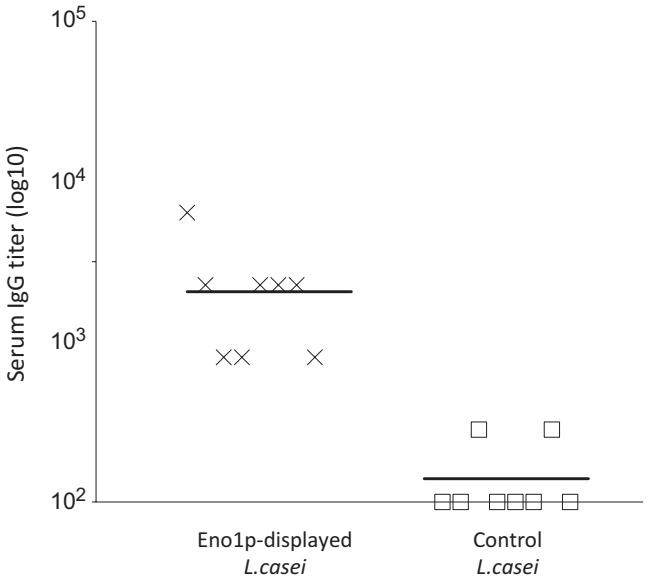

Fig. 11.6 Antibody responses in mice following oral administration of *Lb. casei* cells. Serum derived from mice administered *Lb. casei* cells expressing Eno1p (crosses) and control *Lb. casei* cells (squares)

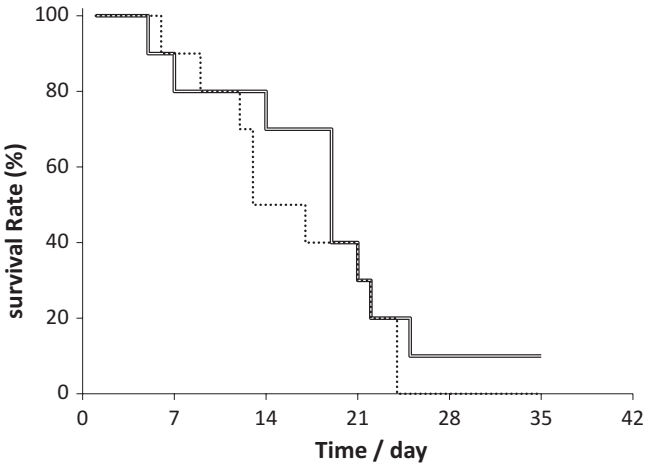

Fig. 11.7 Survival rates in mice administered *Lb. casei* expressing Eno1p (double line) and in mice administered control *Lb. casei* (dotted line)

4 Summary

The studies described in this chapter suggested that suitable antigens could be displayed on the surfaces of orally delivered yeast cells constructed by cell surface engineering. These technologies may provide convenient, effective oral vaccines against various infectious diseases. Moreover, this yeast-derived oral vaccine can be prepared rapidly because, unlike proteins produced in *Escherichia coli*, it does not require a complicated purification step (Fig. 11.1). The adjuvant characteristics of *Lb. casei* have been previously reported (Ogawa et al. 2006), and the cell wall component beta-glucan has been identified as a potent adjuvant in *S. cerevisiae* (Berner et al. 2008). These advantages also can be applied to emerging pandemics if the DNA sequences of target antigens can be screened using high-throughput technologies, such as proteomics (Shibasaki and Ueda 2017; Shibasaki et al. 2016).

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Part IV
Protein Engineering

The advantages of arming technology, including direct analysis of target proteins/peptides using intact cells without concentration and purification, are suitable for high-throughput screening of protein/peptide libraries containing random or comprehensive mutations. Therefore, protein engineering has been performed by display of randomly and/or comprehensively mutated proteins/peptides and subsequent high-throughput screening. For example, luciferase is also an interesting target for protein engineering, as it plays an important role in the pyrosequencing system of next-generation DNA sequencers. A mutant luciferase library is constructed by arming technology, and interesting mutant luciferases with improved specific activity and dATP discrimination are obtained through only three step-wise screenings. Further, the technological innovation of the system for single cell analysis and isolation would lead to more efficient protein engineering and directed evolution by arming technology.

Chapter 12: Combinatorial Engineering (Dr. Mitsuyoshi Ueda (Japan, Kyoto))
Chapter 13: Enzyme Evolution (Dr. Natsuko Miura (Japan, Sakai))

Chapter 12
Combinatorial Engineering

Mitsuyoshi Ueda

Abstract In the case of the yeast display system, the correspondence between the genotype (introducing the gene) and the phenotype (expressing the gene) can be clarified by determining the DNA sequence encoding the displayed proteins by simply providing primers on either side of the introduced gene. Furthermore, it is not necessary to purify the mutated proteins individually. Protein particles (clusters) or whole-cell biocatalysts with individually mutated proteins can thus be prepared easily after cultivation. These innovative methods are expected to lead to breakthroughs in protein engineering instead of the conventional protein engineering.

Keywords Combinatorial bioengineering · Protein engineering · Yeast display · Innovative method · Yeast cell chip

1 Innovation in Protein Engineering

Previous research into protein engineering has concentrated on (i) isolation of genes encoding target proteins, (ii) construction of systems for overexpressing the target gene, (iii) purification of the expressed protein, (iv) crystallization of the protein and analysis of its structure by X-ray diffraction, (v) modeling by computer, (vi) analysis with random mutagenesis and site-directed mutagenesis, and (vii) improvement of protein activity and functions for practical applications.

Systems in which libraries of peptides are displayed on a phage or another biological resource have proved to be useful in the assaying and analysis of large numbers of mutated peptides for protein/enzyme improvement. Phage display is currently the most popular method for this because of its high transformation efficiency ($<10^{11}$), but it does not permit posttranslational glycosylation or proteolytic modification. Moreover, the size of the molecules that can be displayed on the phage

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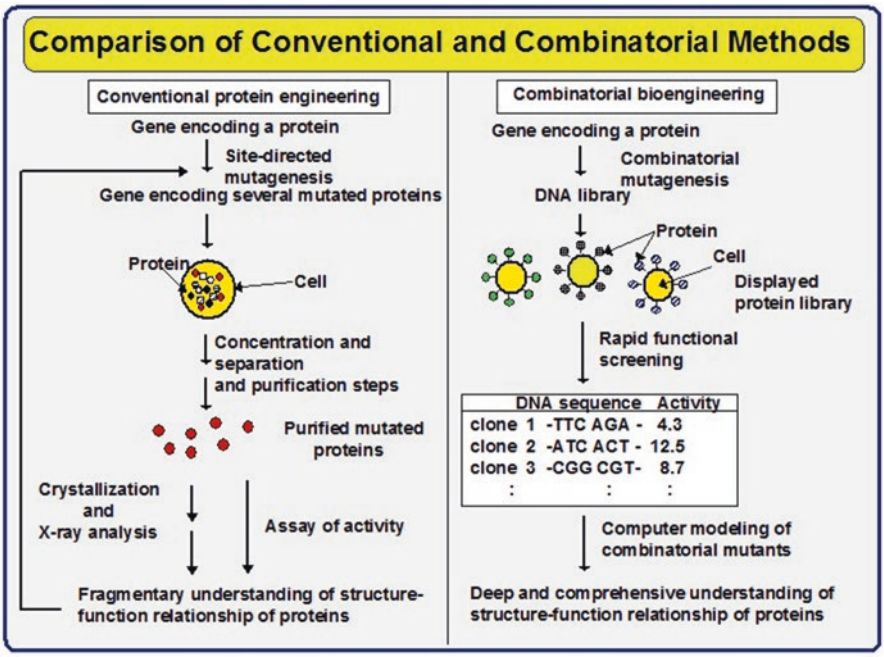

Fig. 12.1 Comparison of the conventional method and combinatorial method using yeast cell surface display (combinatorial bioengineering) for protein engineering

surface is limited, and clones can be screened only when they have improved affinity for substrates or ligands.

To further advance our understanding of protein functions, further methodological innovations have become necessary. As the yeast cell surface engineering system allows for the expression of many functional proteins as well as peptides, which is necessary for posttranslational modification, it would seem to be uniquely useful among the various display systems. Previous and conventional methods of protein engineering have been modified based on the molecular display system described in "combinatorial bioengineering" (Ueda 2004a, 2011). This modification has led to the improvement of protein engineering research strategies from the mutagenesis of individual points to that of multiple (contiguous and/or noncontiguous) and combinatorial points in the combination of structural information (Fig. 12.1). This method begins with the construction of a protein library with contiguous or noncontiguous combinatorial mutations of target domains and regions on individual cells. Next, it becomes possible to perform direct screening of target clones with a high-throughput system with a yeast cell chip and cell manipulator. In the case of the yeast display system, the correspondence between the genotype (introducing the gene) and the phenotype (expressing the gene) can be clarified by determining the DNA sequence encoding the displayed proteins by simply providing primers on either side of the introduced gene. Furthermore, it is not necessary to purify the

mutated proteins individually. Protein particles (clusters) or whole-cell biocatalysts with individually mutated proteins can thus be prepared easily after cultivation. These innovative methods are expected to lead to breakthroughs in protein engineering instead of the conventional protein engineering (Ueda 2004b).

We have successfully improved or designed lipases (Shiraga et al. 2002, 2005a, b), glucosidases (Fukuda et al. 2007a), proteases (Kato et al. 2005), peptidases (Kadonosono et al. 2007a, b, 2008), and functional proteins (Shiraga et al. 2004; Lin et al. 2004; Ito et al. 2004; Okochi et al. 2007) in combination with the single-cell screening method that we developed using micro- and nanotechnologies (Fukuda et al. 2005, 2006a, b, 2007b). One example is the improvement of lipases by the creation of a combinatorial library of the domain that determines substrate specificity (Shiraga et al. 2002, 2005a, b).

Rhizopus oryzae (ROL) lipase, which has been used to produce diesel fuel from vegetable oil, requires modification to attain a mature form and has substrate specificity with respect to long-chain substrates. Many reports about the structure of microbial lipases have been published; almost all of them, including ROL, have a lid domain in the active site, the movement of which occurs at a substrate concentration above the critical micellar level and is necessary for activation. The lid domain, which directly contacts the substrate, is an interesting domain having a major influence on the substrate specificity of the lipase. In the display of ROL, the first success was spacer-mediated display of ROL on the yeast cell surface. To investigate the relationship between the amino acid sequence of the lid domain of ROL and its substrate specificity, the six amino acids (Phe88-Arg89-Ser90-Ala91-Ile92-Thr93) constituting the lid domain were combinatorially changed, and the mutated ROLs were displayed on the yeast cell surface by cell surface engineering (Fig. 12.2). Clones exhibiting halos around plate colonies containing tributyrin or soybean oil were screened. Several clones showed clear halos on tributyrin-containing plates.

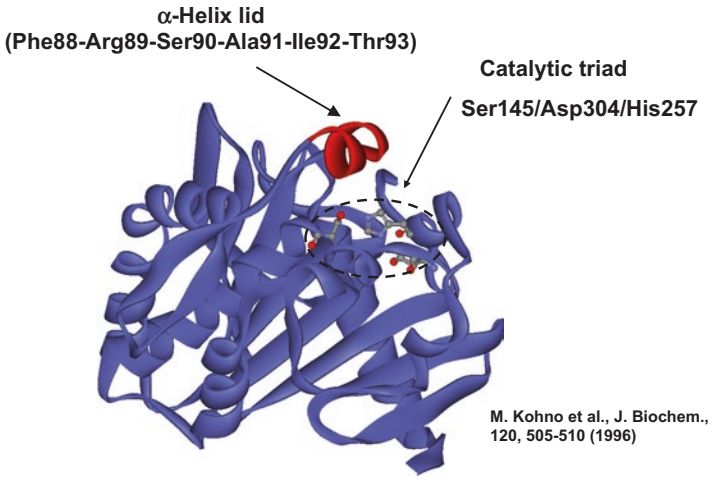

Fig. 12.2 Structure of ROL

Assays using fluorescent substrates indicated that cells displaying mutated enzymes had lower activity than those displaying the wild-type enzymes, but there were several cells that exhibited unique substrate specificity. In the mutated ROL library examined, the three mutants that formed clear halos had a conserved sequence of [basic amino acid]–[polar amino acid]–[nonpolar amino acid]. This indicated that the sequential alignment is important in allowing the lid to activate ROLs. These results indicate that not only the position of the basic amino acid but also the sequence of [basic amino acid]–[polar amino acid]–[nonpolar amino acid] is essential for the determination of substrate specificity by the lid domain.

Alteration of the chain-length specificity of ROLs was produced by combinatorial mutagenesis of the lid domain. The yeast display method is an effective technique for obtaining interesting clones from functional protein libraries by the introduction of high-throughput screening. Many reports on the study of microbial lipases by site-directed mutagenesis have been published, which requires information on conformation and electrostatic properties. However, not all studies using site-directed mutagenesis have achieved the desired results. Because of its applicability to the assay of mutants, the yeast display method allows for the combinatorial mutagenesis of multiple amino acids. Combinatorial mutagenesis of the six amino acid residues of the lid domain of ROLs readily supplied several mutant enzymes with altered chain-length specificity but a conserved amino acid sequence.

ROL-displaying *S. cerevisiae*, constructed by the Flo1p-based display system, could synthesize methylesters from triglyceride and methanol (Matsumoto et al. 2002). In addition, yeast displaying ROL could be used for the optical resolution of (*R*,*S*)-1-benzyloxy-3-chloro-2-propyl monosuccinate (Nakamura et al. 2006).

Candida antarctica lipase B (CALB) is one of the most widely used lipases. Lipases are among the enzymes that are most applicable to display on the surface of yeast cells because they are used in a wide range of industries, and the display of lipase is an attractive approach for creating a whole-cell biocatalyst. Mutated CALB displayed on yeast using the α-agglutinin-based display system has higher thermal stability (Kato et al. 2007). Furthermore, CALB-displaying yeasts constructed using the α-agglutinin or Flo1p display systems have been used for several ester syntheses with a reduced level of by-products (Inaba et al. 2009; Tanino et al. 2007, 2009). In *P. pastoris*, CALB was displayed on the cell surface using the *S. cerevisiae* α-agglutinin-based display system. Compared with CALB-displaying *S. cerevisiae*, the engineered *P. pastoris* showed higher activity, and the ability to synthesize ethyl hexanoate was enhanced (Han et al. 2009). CALB was also displayed on *P. pastoris* via the Sed1p-based display system and exhibited improved thermal stability (Su et al. 2010). Moreover, ROL, *Y. lipolytica* lipases, and *Pseudomonas fluorescens* lipases were displayed on *P. pastoris* using the Flo1p-based display system (Kuroda and Ueda 2013; Jiang et al. 2007, 2008). Furthermore, *Y. lipolytica* lipase was displayed on *Y. lipolytica* by using the Flo1p-based display system (Yuzbasheva et al. 2011).

Neurolysin is a metalloendopeptidase that cleaves the bioactive peptide neurotensin. A mutant library of neurolysin constructed by semirational mutagenesis was displayed, on which screening was performed using two fluorescence-quenching

peptides: the matrix metalloproteinases-2/9- (MMPs-2/9) and MMP-3-specific substrates (Kadonosono et al. 2008). The results revealed an attractive mutant of neurolysin that exhibited altered substrate specificity. In addition, the protease inhibitory activity of matrix metalloproteinase-2/9 was improved by cell surface engineering and an automatic single-cell pickup system.

Luciferase is also an interesting target for protein engineering, as it plays an important role in the pyrosequencing system of next-generation DNA sequencers. A mutant luciferase library was constructed by cell surface engineering, and interesting mutant luciferases with improved specific activity and dATP discrimination were obtained through only three stepwise screenings (Fushimi et al. 2013; Miura et al. 2015). Further technological innovation of the system for single-cell analysis including the development of single-cell chips (Fukuda et al. 2007b) and isolation would lead to more efficient protein engineering and directed evolution by cell surface engineering.

Isoflavone aglycones, which are bioactive and easily adsorbed by human cells, are hydrolysates of isoflavone glycosides by β-glucosidase (BGL1). Three kinds of β-glucosidases were independently displayed on *S. cerevisiae* using the α-agglutinin-based display system. Among these, BGL1-displaying yeast could convert isoflavone glycosides into isoflavone aglycones most efficiently (Kaya et al. 2008). Carnosine and chitosan oligosaccharides also show bioactivities such as antioxidant, antiglycation, cytoplasmic buffering, antitumor, and anticancer properties. The synthesis of carnosine from β-alanine and histidine was achieved through a reverse reaction catalyzed by human carnosinase (CN1)-displaying *S. cerevisiae* in organic solvents and ionic liquids (Inaba et al. 2010). Cell surface display of chitosanase from *Paenibacillus fukuinensis* enabled the production of chitosan oligosaccharides from chitosan (Fukuda et al. 2007c).

GPCRs are seven transmembrane domain proteins with the ability to mediate rapid responses to extracellular signals. They play an important role in many aspects of cellular physiology. The design and identification of peptide ligands for GPCRs are an attractive research area contributing to diagnostics (Fig. 12.3). In the study of ligand–GPCR interactions and the identification of ligands to orphan GPCRs, the display of peptide ligands specifically on the plasma membrane is important because ligands displayed only on the cell wall would be unable to access GPCRs on the plasma membrane. Hara et al. (2012a) developed a system for displaying peptide ligands on the yeast plasma membrane for the activation of GPCR signaling. First, this system was applied to the activation of Ste2 signaling in *S. cerevisiae*, where activation was detected using a Ste2-responsive *FUS1* promoter driving EGFP reporter expression. Alpha-factor displayed on the plasma membrane was shown to functionally activate the pheromone response pathway. This system has been applied to the activation of human GPCR signaling as well as the yeast pheromone response pathway using chimeric Gα protein. Somatostatin is a naturally occurring gastrointestinal hormone that regulates various endocrine and exocrine processes. After the construction of yeast producing human somatostatin receptor subtype-2 (SSTR2) and chimeric Gα protein, somatostatin was displayed on the plasma membrane. This somatostatin could activate human SSTR2 in *S. cerevisiae* (Hara et al.

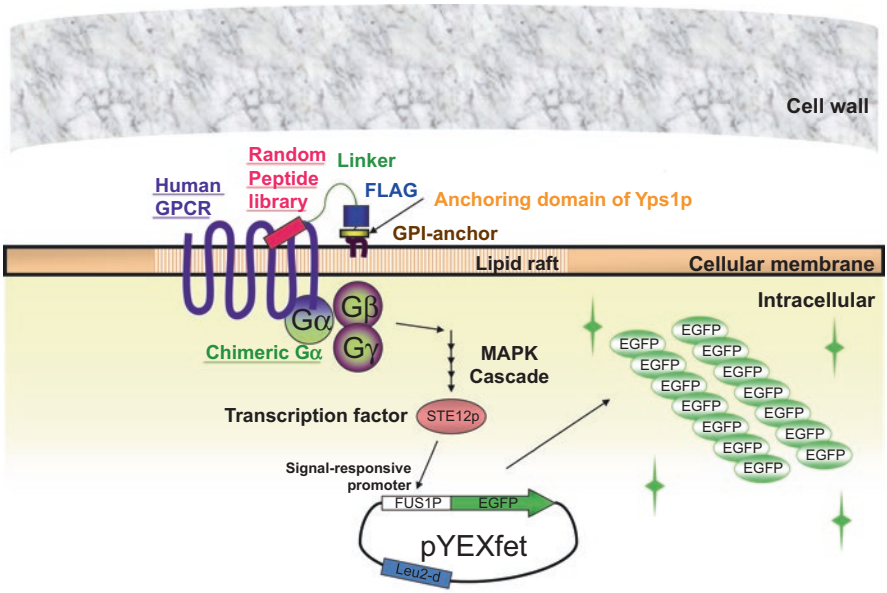

Fig. 12.3 Functional assay system by display of GPCR

2012b). This technological platform, namely, "PepDisplay," is useful for the identification of novel peptide ligands for heterologously produced GPCRs by the membrane display of peptides with random and/or comprehensive sequences and screening based on the activation of reporter genes.

2 Creation of Novel and Functional Proteins

The creation of novel and functional proteins requires the construction of a combinatorial protein library that can be displayed on the surface of *S. cerevisiae* cells using a multi-copy cassette vector. In the absence of quantitative and computational data on the structure–function relationships of proteins, rational approaches to mutagenesis have limited potential for success in rapidly altering protein molecular properties to meet predefined criteria. An alternative strategy, namely, the construction and selection of randomly mutated combinatorial libraries, has yielded numerous successes. In vitro selection from molecular libraries has rapidly come of age as a protein engineering tool. In a library, the massive number of variants that can be simultaneously surveyed using either chemical or biological approaches has a key consequence for protein engineering. Specifically, it makes it practical to mutate multiple residues of a protein simultaneously, enabling complex, nonadditive combinatorial effects to be obtained. Thus, changing the properties of proteins may not rely mostly on a detailed molecular understanding of their functions, and biological libraries can offer a powerful tool for improving protein function (Fig. 12.4) (Ueda 2004b).

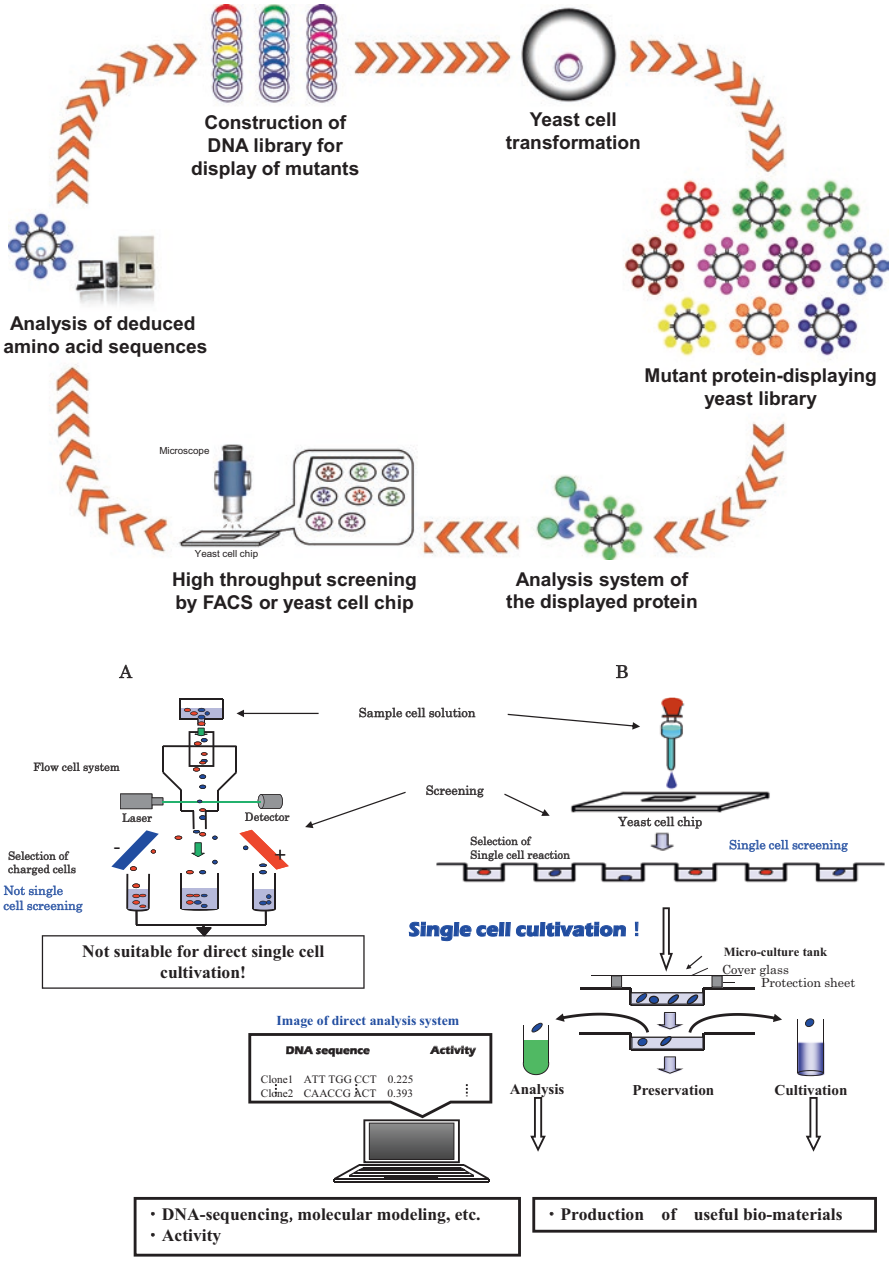

Fig. 12.4 Screening system using cell surface engineering and illustration of high-throughput design including cell chip

For the creation of novel and functional proteins, the attractive property of solvent (organic solvents, acid, etc.) tolerance was selected (Zou et al. 2002; Matsui et al. 2009). As a factor that is anticipated to facilitate a wide range of applications in bioprocesses, solvent tolerance is a cell surface-related function for which no genetic information in eukaryotic organisms has been available. In such cases, a random combinatorial protein library that can be displayed on the yeast cell surface is helpful, as renovating the cell surface may create strains with new phenotypes. A random combinatorial protein library that could be displayed on the yeast cell surface using cell surface engineering technology was therefore constructed. As a combination of both the library method and the yeast cell surface display technique, a combinatorial protein library that could be displayed on the yeast cell surface was constructed. From this library, the randomly selected colonies were isolated, and the inserted random fragments were sequenced and compared with the Genome Database. Using the method of DNA random priming or DNA synthesis, a vast pool of DNA fragments with different lengths and various structures was generated for the combinatorial library. Proteins displayed on the yeast cell surface made a major contribution to yeast breeding. When these fused random proteins are displayed on the yeast cell surface, their intrinsic localization, composition, and conformation may be somewhat altered, which increases the variability of the library and extends the range of selectable phenotypes. From this combinatorial protein library, a solvent-tolerant yeast strain, for example, was obtained by screening (Kuroda and Ueda 2017). Many solvents are highly toxic to living organisms because they accumulate in and disrupt cell membranes. There are few reports on eukaryotic organisms that can adapt to and survive these antimicrobial agents.

Metal ions play various roles in life. Some trace metal elements are indispensable for living organisms, but are toxic at high concentrations. Moreover, heavy metal ions that contaminate the environment are not chemically degraded and cause serious toxicity at high concentrations in cells. In biorefineries that produce biofuels and chemicals, biomass is used as a renewable resource. Herbaceous plants, such as rice, contain cadmium ions, and cellulosic biomass of building waste, which does not compete with food, contains chromium, copper, and arsenic as toxic heavy metal ions. Therefore, heavy metal ion tolerance can be imparted by the modification of cell surface properties to prevent the uptake of heavy metal ions. That is, metal-binding proteins/peptides can be displayed on the cell surface, thereby reducing the incorporation of metal ions into cells via metal adsorption on the cell surface.

As a chelator of divalent heavy metal ions, histidine oligopeptide (hexa-His) (Hochuli et al. 1987) has been displayed on the surface of *S. cerevisiae* cells. The cell surface display of hexa-His allows for cell growth in medium containing copper ions at toxic levels, in which the wild-type strain cannot grow (Kuroda et al. 2001). In addition, the cell surface adsorption of heavy metal ions, such as copper and nickel ions, is elevated by the display of hexa-His. Therefore, enhanced tolerance to toxic heavy metal ions could be achieved by the cell surface adsorption of metal ions and the consequent reduction of metal incorporation into the cells. As another chelator, yeast metallothionein has also been displayed on the yeast cell surface.

Metallothionein is a cysteine-rich protein with a low molecular mass; it plays an important role in metal homeostasis by sequestering heavy metal ions (Butt and Ecker 1987; Karin 1985). Metallothionein-displaying yeast shows elevated adsorption of cadmium ions, leading to cellular tolerance to cadmium ions (Kuroda and Ueda 2003). Furthermore, the metal-binding capacity of displayed metallothionein is increased by tandem fusion. An increased number of tandem repeats lead to stronger tolerance to cadmium ions as well as higher ability to adsorb on the cell surface (Kuroda and Ueda 2006). These results indicate that there is a correlation between the adsorption capacity and the strength of cellular tolerance. Therefore, the modification of the cell surface by displaying metal-binding proteins/peptides is an effective strategy for the molecular breeding of cells with enhanced tolerance to heavy metal ions.

Acid catalysts are commonly used in various chemical conversion processes, and the neutralization of the acidic conditions is indispensable for subsequent microbial reactions. When an acid flows into cells in acidic conditions, the intracellular pH is lowered, and excessive protons are excreted outside the cells. Proton excretion mainly occurs via plasma membrane H^+-ATPase by ATP hydrolysis (Eraso and Gancedo 1987; Holyoak et al. 1996; van Maris et al. 2004), which concomitantly results in the reduction of intracellular ATP levels. Increased energy consumption for pH homeostasis as well as reduced enzymatic activity at a low intracellular pH causes reductions in cell growth and bio-production efficiency. Therefore, the improvement of acid tolerance is desirable not only to lower the cost of neutralization, but also to prevent bacterial contamination.

In nature, some microorganisms, called extremophiles, can survive in extreme environments. The alkaliphile *Bacillus lentus* C-125 is an extremophile that can grow in the pH range 6.8–10.8 (Aono 1995), and its cell wall properties have been studied extensively. The cell wall of *B. lentus* C-125 consists mainly of peptidoglycans, teichuronic acid (TUA), and teichuronopeptide (TUP) (Aono 1987). TUP is a copolymer of polyglutamic acid and polyglucuronic acid. TUA is composed of galacturonic acid, glucuronic acid, and *N*-acetylfucosamine. These highly, negatively charged acidic polymers in the cell wall were suggested to prevent the influx of hydroxide ions into the cell (Tsujii 2002). Therefore, the modification of cell wall properties appears to be an effective strategy for acid tolerance.

To obtain acid-tolerant yeast, random modifications of cell surface properties have been introduced by cell surface engineering. In particular, a random peptide library consisting of 25 amino acid residues was displayed on the surface of *S. cerevisiae* cells. The constructed yeast library displaying random peptides was inoculated and screened on agar medium at pH 2.2, at which the wild-type strain could not survive (Matsui et al. 2009). Acid-tolerant yeast was successfully isolated, and the displayed peptide was identified as a novel peptide consisting of relatively hydrophobic and basic amino acids. Interestingly, this peptide had high homology with part of the hypothetical membrane protein PTO1510 from *Picrophilus torridus*, which can grow at approximately pH 0 and 65 °C (Futterer et al. 2004). Considering that the theoretical isoelectric point of the displayed peptide is 9.98, there is a possibility that this peptide can exert a buffering effect on the cell surface

under acidic conditions. In addition, random modifications of the cell surface have been introduced by displaying a random protein library derived from *S. cerevisiae* cDNA. By the screening of random protein library-displaying yeast on agar medium overlaid with *n*-nonane, an *n*-nonane-tolerant yeast was successfully isolated (Zou et al. 2001, 2002). The protein displayed on the isolated yeast was the structurally uncharacterized domain of the YGR193C open reading frame with relatively high hydrophilicity. Therefore, the cell surface is a promising target to enhance acid tolerance as well as heavy metal ion tolerance.

In conclusion, as the yeast display system allows for active enzymes with various sizes and forms to be displayed, it is expected that its use in combination with crystallization analysis and computerized modeling will facilitate combinatorial analysis of the structure–function relationship of proteins and the construction of a practical protein engineering system. Furthermore, the possibility of creating completely novel and functional proteins from random DNA alignments has been demonstrated. In conjunction with molecular display systems and high-throughput systems for combinatorial and rapid analysis of the functions of proteins derived from many genes and artificially synthesized DNA, methods of proteome analysis and protein library construction have also been developed. The combination of these systems is expected to facilitate easy and simultaneous analysis of DNA data and protein function and to greatly support the combination of genomics with proteomics.

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Chapter 13
Enzyme Evolution

Natsuko Miura

Abstract In this chapter, we discuss how to obtain enzymes with desired functions by using yeast cell surface display methods. Although useful enzymes can be found in natural environments and in databases, catalytic activities are often weak or lack specificity for desired functions (Newton MS, Arcus VL, Gerth ML, Patrick WM, Curr Opin Struct Biol 48:110–116, 2018). Numerous studies show the utility of yeast cell surface display in the investigations of enzyme evolution (Gai SA, Wittrup KD, Curr Opin Struct Biol 17:467–473, 2007; Ueda M, Biosci Biotechnol Biochem 80:1243–1253, 2016; Angelini A, Chen TF, de Picciotto S et al, Methods Mol Biol 1319:3–36, 2015). This chapter is an overview of strategies for developing enzymes with desirable functions.

Keywords Random mutation · Protein library · DNA library · Screening

1 Introduction

The major challenges of enzyme evolution studies using yeast cell surface display (YSD) technologies include the development of screening methods for suitable enzyme reactions and the choice of methods for introducing mutations (Miura et al. 2015). Screening methods need to be chosen with consideration to library sizes because screens are generally performed for more times than the number of library entries. Even when using low-throughput screening, libraries are required to include one to several thousands of entries. For example, when 3 amino acid residues are substituted with 20 amino acids, the library size = 20^3 = 8000. Recently developed ultrahigh-throughput screening methods with DNA barcoding enable the screening of more than one million cells at a time. These methods potentially allow the screening of protein libraries with completely random amino acid sequences, albeit with some fundamental limitations.

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After displaying enzymes on yeast cell surfaces, it is relatively easy to assess the binding abilities of library proteins, such as antibodies and related proteins or peptides. Thus, various antibodies, antibody alternatives, and antibody enzymes have been investigated using YSD (Angelini et al. 2015; Boder and Wittrup 1997, 2000; Cherf and Cochran 2015; Garcia-Rodriguez et al. 2007) as reviewed by Aoki (see Chap. 10). In this chapter, we discuss the methods for developing and isolating novel enzymes with specific catalytic activities other than binding. To this end, we initially provide an overview of methods for constructing protein libraries by introducing mutations. We then describe the methods and instruments for screening protein libraries and discuss future perspectives.

2 Strategies for Enzyme Evolution

2.1 Library Construction

The strategies for constructing protein libraries are partially dependent on the objectives of enzyme evolution. These include (1) improving enzymatic functions and specificities, (2) improving protein stability, and (3) generating enzymes with catalytic functions that are not found in nature (Fig. 13.1). To improve function and specificity, structure–function relationships (Thomas et al. 2003) are initially investigated and are used to inform subsequent molecular manipulations (Baier et al. 2016; Desideri et al. 1992). For this purpose, the overviews of previously reported sequence–function relationships are important, and the maps of relationships between amino acid positions and the functional effects of mutations (Fowler and Fields 2014) offer a very useful approach. Depending on the library size permitted, protein libraries can be systematically constructed using the DNA barcode technique (Mazurkiewicz et al. 2006; Sun et al. 2016), and other methods for the targeted introduction of mutations include domain swapping and proregion engineering. The second and third objectives require more careful selections of strategies, including in silico screening and the use of promiscuous enzymes (Renata et al. 2015) as starting materials.

Given that the structure–function relationships of target enzymes are often not known (Brown and Babbitt 2014), nontargeted mutagenesis approaches are often employed (Fig. 13.2). These often involve the introduction of random mutations via DNA replication errors (Shiraga et al. 2005) or DNA shuffling (Packer and Liu 2015), which have enabled the evolution of useful enzymes. In other studies, neutral drift libraries were prepared by the repeated selection of active groups of enzymes and the subsequent reintroduction of mutations that efficiently lead to novel enzyme functions (Gupta and Tawfik 2008; Kaltenbach and Tokuriki 2014). In a recent in silico study, protein libraries were prepared and optimized (Tinberg et al. 2013), and YSD were used to produce final protein products, thus validating this novel in silico strategy for enzyme evolution. Similar to other fields, the combined in silico and

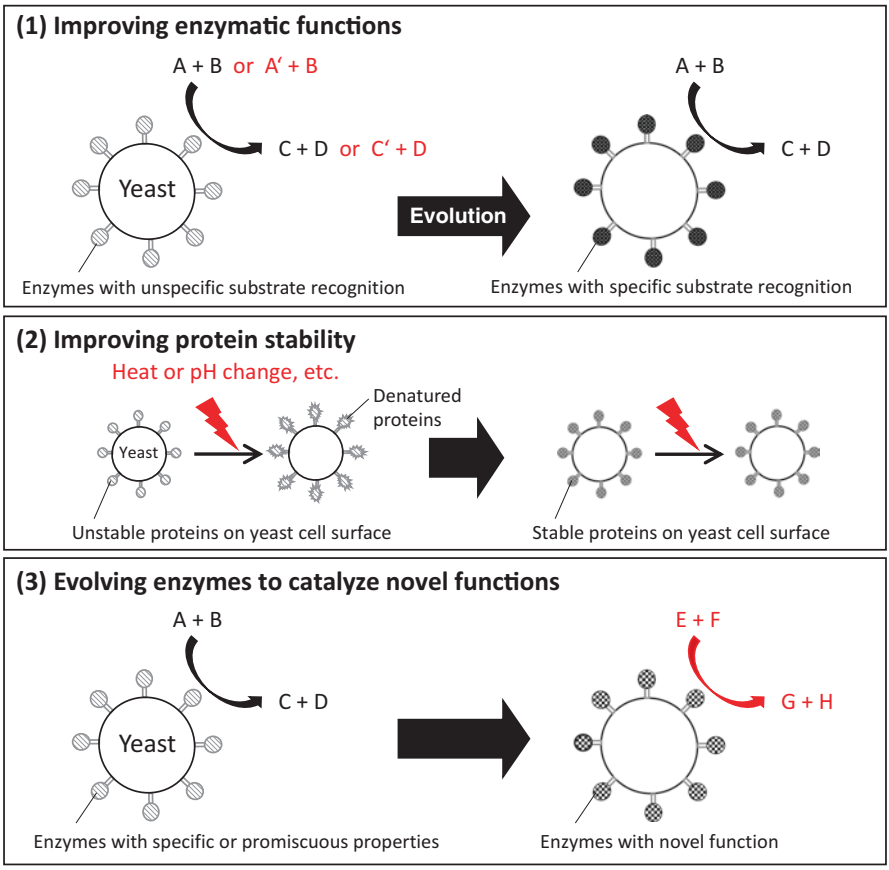

Fig. 13.1 Objectives of enzyme evolution

in vitro analyses of mutant protein sequences may soon accelerate the development of enzymes with novel functions.

2.1.1 Targeted Introduction of Mutations

When preparing smaller libraries, amino acid residues for mutation need to be selected carefully. Introducing mutations to specific amino acid residues can improve enzymatic functions and substrate specificity (Fushimi et al. 2013; Fig. 13.3a). Moreover, after acquiring the amino acid sequences and 3D structures of certain numbers of relevant enzymes, swapping similar domains of relevant enzymes can improve enzyme functions (Fischbach et al. 2007; Zhang et al. 2013; Fig. 13.3b). For enzymes with proregion sequences that are involved in protein maturation, the engineering of these proregions can also improve protein maturation

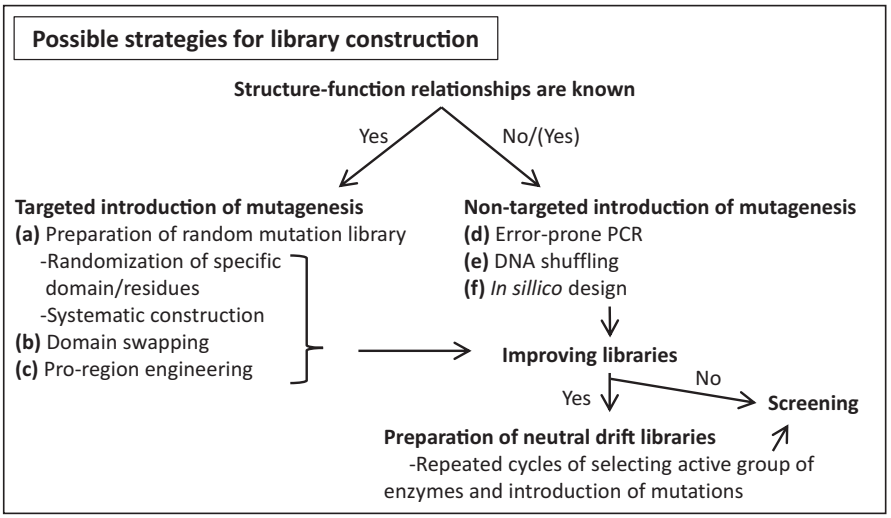

Fig. 13.2 Overview of strategies for library preparation

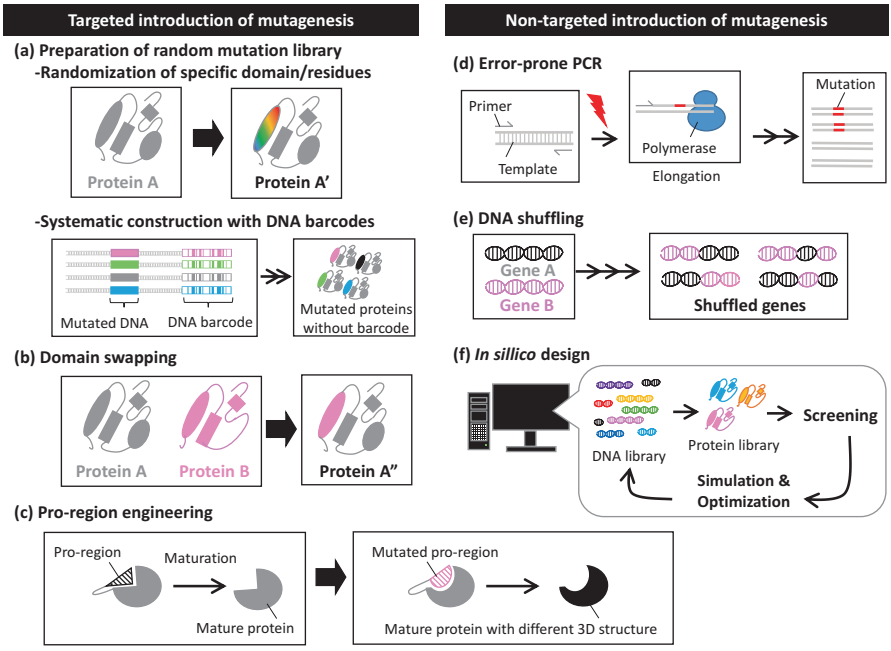

Fig. 13.3 Overview of methods for library preparation

and increase enzymatic activities (Burns et al. 2016; Nagayama et al. 2012; Fig. 13.3c). Considering that proregions are generally cleaved off during maturation, the amino acid sequences of "new" mature enzymes are similar to those of original enzymes, but their 3D structures differ from the original enzymes.

Recently, the DNA barcode technique has become a popular method for systematically preparing protein libraries (Mazurkiewicz et al. 2006; Sun et al. 2016; Fig. 13.3a). In this method, specifically tagged DNA sequences are fused with mutant DNA sequences; unlike for proteins with introduced mutations, DNA barcodes are not necessarily translated to peptide sequences. Subsequent YSD enables the screening of proteins that are fixed on yeast cells, thus resulting in the expression of the introduced DNA sequences, and the amino acid sequences of selected proteins from libraries contain specific DNA barcodes that correspond with the mutation. This technique is not very powerful when only one or two amino acids are randomized in the sequence. However, with library sizes of more than one million and broad distributions of amino acid residues in proteins, DNA barcoding is a powerful method for the convenient determinations of mutations that contribute positively to enzyme evolution. Although next-generation sequencing is required for this method, it is becoming easy to prepare DNA barcodes because free sources for generating DNA barcodes are becoming increasingly available (Buschmann 2017).

2.1.2 Nontargeted Mutagenesis

Major methods for introducing nontargeted mutations include in vivo, in vitro (Fig. 13.3d, e), and in silico techniques (Fig. 13.3f). In in vivo experiments, mutations are usually induced using chemical or physical DNA damage and repair mechanisms or by using error-prone polymerase chain reactions (PCRs; Fig. 13.3d) (Packer and Liu 2015). Given that living cells are required to induce DNA repair, mutations are also introduced into their genomes, and these can interrupt reproduction.

Error-prone PCR is an easy and effective in vitro technique for introducing random mutations (Packer and Liu 2015) and exploits the low fidelity of some DNA polymerases to enhance replication error rates (Cadwell and Joyce 1992; Leung et al. 1989). Although this method is used widely, the library sizes are necessarily small and random, thus potentially precluding the coverage of a broad range of sequences, particularly those with longer templates. Other nontargeted mutations can be performed in vitro by using DNA shuffling (Fig. 13.3e), which is performed constitutively or randomly (Packer and Liu 2015). In these experiments, the lengths of nucleotide parts with protruding or blunt ends are merged by ligation or homologous recombination. PCR can also be used to fuse the ends of nucleotide parts when nucleotide filaments have overlaps. When using DNA shuffling to construct libraries, the frameshifts and insertions of stop codons can occur. Hence, it is important to screen for and remove "junk" sequences that are not proteogenic.

The third method uses computational screening to minimize the sizes of protein libraries to be constructed (Fig. 13.3f). The design and screening of sequence sets that produce proteins with specific lengths are both performed in silico, as reported previously using phage display (Watters and Baker 2004). Furthermore, inferred protein folding and stability characteristics can be screened in silico, thus compensating for low-throughput screening (Watters and Baker 2004; Tinberg et al. 2013).

2.2 *Screening Protein Libraries on Yeast Cell Surfaces*

Several methods have been proposed to screen enzyme libraries with and without YSD (Leemhuis et al. 2009; Packer and Liu 2015). One of the easiest ways to screen protein sequences is based on the live/dead statuses of host cells, as determined by the displayed proteins. For example, when searching for mutant enzymes that degrade toxic substrates, screening can only be performed by plating library-protein bearing yeast on toxic substrates (van Rossum et al. 2013). In these screens, plating densities and culture conditions must be carefully determined to decrease the numbers of satellite colonies and increase the numbers of positive colonies carrying useful mutations. When screening substrate-degrading enzymes such as lipase, the formation of a halo can also be considered a useful marker (Shiraga et al. 2005, 2002). Given that the sizes of mutant libraries that can be screened using these assays are limited (van Rossum et al. 2013; Shiraga et al. 2002), higher-throughput screening methods are utilized for libraries with large numbers of mutants.

Higher-throughput screening is often performed using fluorescence- or luminescence-based measurements on enzymatic activities (Bershtein and Tawfik 2008; Fig. 13.4). Given that fluorescence detectors are more common than luminescence detectors, fluorescence-based determinations offer the fastest approach for the simultaneous screens of all proteins in large libraries. Alternatively, screening experiments with 96- or 384-well microtiter plates (Fig. 13.4a) are more time-consuming, but several useful enzymes have been identified in this manner (Bershtein and Tawfik 2008). Screens of proteins displayed on yeast cell surfaces

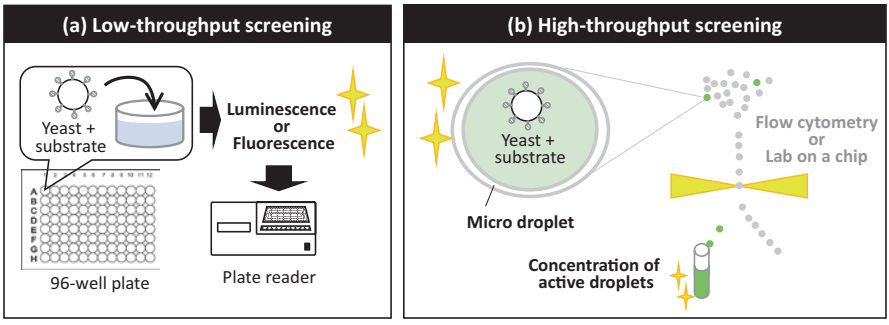

Fig. 13.4 Schematic illustrations of low- and high-throughput methods for library screening

using fluorescence-activated cell sorting (FACS) (Mei et al. 2017; Zhang et al. 2015; VanAntwerp and Wittrup 2000; Rosenfeld et al. 2015) or fluorescence-activated droplet sorting (FADS) (Nolan et al. 1988; Baret et al. 2009; Agresti et al. 2010; Fig. 13.4b) have accelerated the screening of yeast cell surface protein libraries. Furthermore, by using fluorescence emissions from enzyme reactions, conformation-specific synthetic antibodies (Paduch et al. 2013) facilitated the isolation of enzymes with desirable 3D structures, and these enzymes were subsequently validated.

3 Future Perspectives

Future developments in the field of enzyme evolution will be focused on designing mutation libraries and higher-throughput assays. For known enzymes, it may be helpful to prepare protein mutation databases with maps for amino acid positions and their functional relationships (Fowler and Fields 2014). Such studies may be applicable to artificial intelligence (AI) technologies, including deep learning, which enables AI technologies to suggest possible protein libraries with domain shuffling or amino acid point mutations. For example, in a recent study by Dehghanpoor et al. (2018), the effects of amino acid mutations were examined using deep neural networks. Increasing the numbers of predictable mutations and target protein families from these types of studies may enable the wider scientific application of these technologies.

The investigations of promiscuous enzymes (Renata et al. 2015) may accelerate various applications, including the development of novel enzymatic functions that are not found in nature, and the flexibility of enzyme evolution (Pabis et al. 2018) has been demonstrated. Directed evolution (Konning and Kolmar 2018; Traxlmayr et al. 2013, 2014; Gupta and Tawfik 2008; White and Zegelbone 2013; Shusta et al. 2000; Burns et al. 2014; Gupta and Farinas 2010; Pershad et al. 2012) may also be applied more widely as related tools and software are developed, and these tools and software will accelerate protein evolution in a broad range of scientific fields. Optimization plateaus (Goldsmith et al. 2017) and the biases of directed evolution (Cochran et al. 2006) during the preparation of mutant libraries will also need to be resolved in future studies.

The use of in silico designs and screens may become major approaches, in addition to conventional in vitro and in vivo methods. Accordingly, computationally designed enzyme libraries of proteins with enhanced enzyme activity and stability (Rosenfeld et al. 2015; Banerjee et al. 2017; Arkadash et al. 2017; Bednar et al. 2015; Butz et al. 2014; Wijma et al. 2014) are increasingly being considered. The decreased costs and increased accessibility of these methods, as well as international services for DNA synthesis, will benefit these approaches.

During high-throughput screening using FACS or FADS, it is important to develop fluorescence probes, including antibodies (Shembekar et al. 2018) or substrates (Beneyton et al. 2017), that can be used to detect the desired enzyme functions of evolved proteins. These probes have been extensively developed for

screening single cells with specific functions and could be further developed for application to the higher-throughput screening of protein libraries on yeast cell surfaces.

Although fluorescence-based screens are not appropriate for large numbers of enzymes, recent advances in automated robotic screens with the DNA barcode technique (Ko et al. 2013; Yachie et al. 2016) will enable the fluorescence-free high-throughput screening of protein libraries on yeast cell surfaces.

Enzyme evolution by yeast cell surface engineering remains under development but continues to advance via the combination of several recently developed techniques. The further recruitment of methods used in other areas of science and the sharing of established knowledge in databases for enzyme evolution will guide the free development of enzymes with desired functions.

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