Chapter 7
Preparation of Functional Cells: Improvement of Stress Tolerance

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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

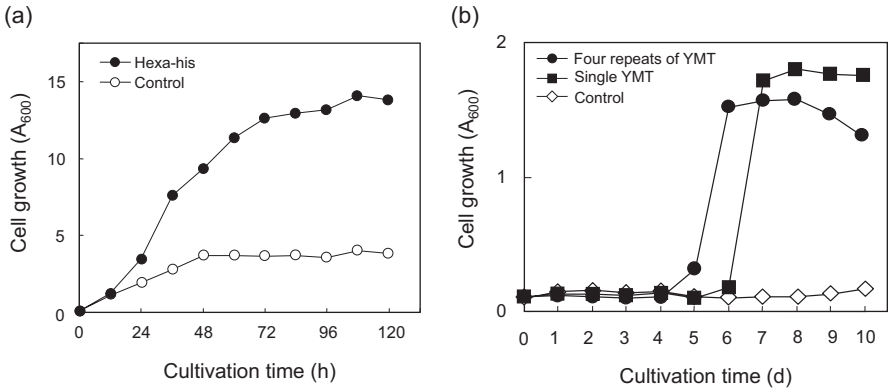

Fig. 7.1 Cell growth of surface-engineered yeasts in the medium containing heavy metal ions at a toxic concentration. (**a**) The hexa-His-displaying yeast in medium containing 2 mM Cu^{2+} and (**b**) yeast displaying tandem repeats of metallothionein in medium containing 70 μM Cd^{2+}

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-

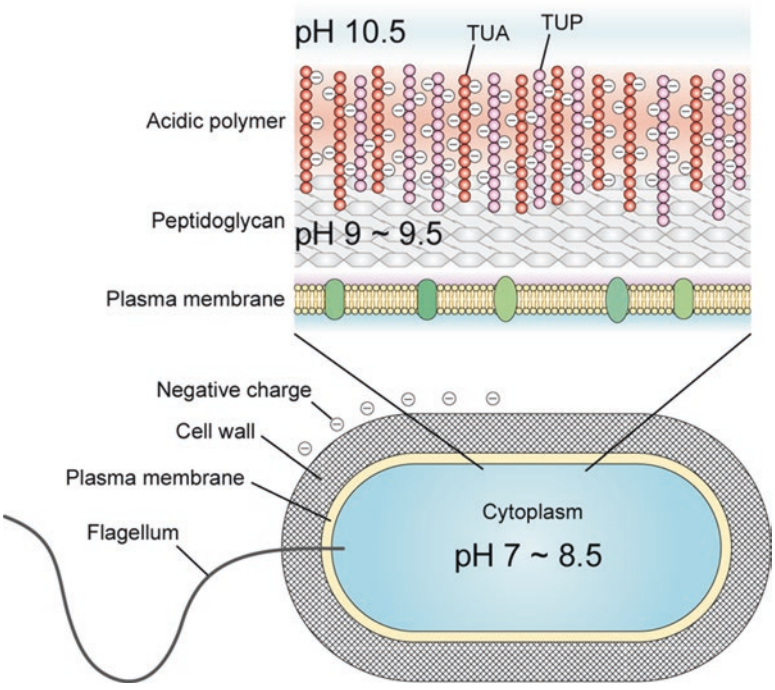

Fig. 7.2 Cell surface property of *Bacillus lentus* C-125. *TUA* teichuronic acid, *TUP* teichuronopeptide

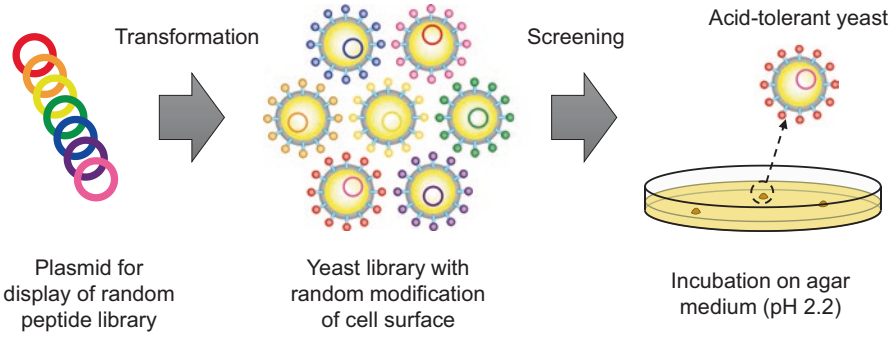

Fig. 7.3 Strategy for construction of acid-tolerant yeasts by random modification of cell surface and subsequent screening

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

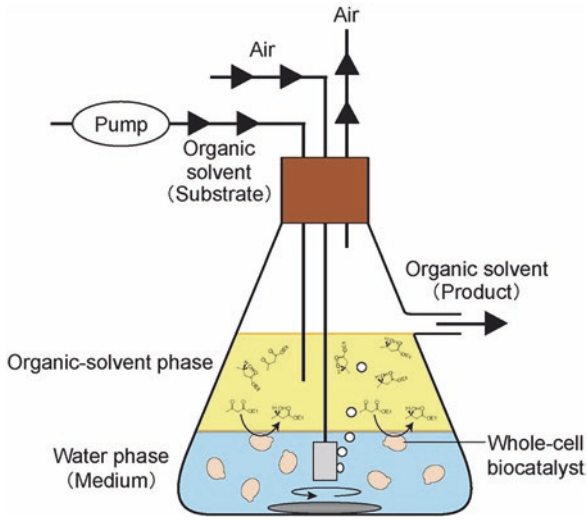

Fig. 7.4 Continuous bioproduction by whole-cell biocatalyst in aqueous/organic solvent biphase system

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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