Molecular breeding of yeast *Saccharomyces cerevisiae* for effective ammonia production from food processing wastes

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Contents

Abbreviations

IS: Internal standard

ESI: Electrospray ionization

MRM: Multiple reaction monitoring

PCR: Polymerase chain reaction

SDC: Synthetic dextrose

PCR: Polymerase chain reaction

FAD: Flavin adenine dinucleotide

MTBSTFA: *N-tert*-**Butyldimethylsilyl-*N*-methyltrifluoroacetamide**

DAFC: Direct ammonia fuel cell

NCR: Nitrogen catabolite repression

GPI: Glycosylphosphatidylinositol

PLP: Pyridoxal 5'-phosphate

Introduction

There has been a growing interest in environmental initiatives among researchers since the declaration of the Sustainable Development Goals (SDGs) (Capua and Giovannini 2019). SDGs include the declaration about environmental problems such as global warming and clean energy because these problems cause climate change and ocean acidification due to the consumption of a vast amount of fossil fuels. For example, while the Haber–Bosch method is essential for fertilizer production, this process consumes a large number of fossil fuels, so there is a need to introduce alternative methods (Schrock 2006). The Harbor–Bosch process has been used for over 100 years and has produced more than 100 million tons of ammonia per year. The two major nitrogen fixation processes, Haber–Bosch process and fossil fuel combustion generated about 1.1×10^{13} ammonia. Currently, these chemical processes produced approximately 55% of ammonia; however, this process requires about 2%–3% of the total energy generated globally in fossil fuels to cleave the triple bond of nitrogen molecules at high pressures (150–350 atm) and temperatures (350°C–550°C). With this method, fossil fuels are used in large quantities, which is one of the factors that contribute to the environmental burden (Canfield et al. 2010; Erisman et al. 2008; Schrock 2006; Smil 1991).

This concern about increase in the fossil fuel consumption has caused the society to adapt a sustainable approach using an alternative energy source like biofuels (Lopes 2015; Wernick and Liao 2013). While bioethanol is a typical biofuel, we have focused on ammonia production as a next-generation biofuel (Miura and Tezuka 2014). Ethanol is carbon-based and produces carbon dioxide after combustion. On the other hand, ammonia emits only water and nitrogen when completely degraded by catalysis, which means no greenhouse gases are released into the environment and is deemed a more environmentally friendly fuel (Wang et al. 2013). In addition, ammonia could be produced from food processing wastes like soybean residue (okara, in Japanese). When soybean residue is landfilled, it produces nitrogen dioxide due to the action of microorganisms in the environment, which puts a burden on the environment. Therefore, the production of ammonia from food processing wastes could be effective for the low-cost refining of biofuels.

Ammonia usage for various industries

The value of ammonia has been increasing in recent years (Nuutinen and Timonen 2008). Ammonia is primarily used as a fertilizer, but also in chemical synthesis and fiber synthesis (Muller and Beller 1998; Wakida et al. 2004). Recently, primary amines have been successfully synthesized from ammonia (Park et al. 2020). Amines are critical intermediates in the chemical industry for producing dyes, pesticides, and pharmaceuticals. Primary and secondary amines are particularly important because of their access to biologically active substances and, more generally, fine chemicals (Muller and Beller 1998). Therefore, ammonia is an increasingly valuable compound.

Recently, a novel application has emerged using ammonia as a hydrogen carrier in a society where hydrogen is formed as the primary fuel (Fig. 1) (Lin et al. 2020; Miura and Tezuka 2014; Valera-Medina et al. 2018). Unlike carbon in our current society, hydrogen does not produce carbon dioxide when it is burned, making it a next-generation fuel. Ammonia is expected to act as a carrier of hydrogen for various reasons. Ammonia 1 mol includes 1.5 mol of hydrogen, and has a high volumetric hydrogen fraction ($121 \, \mathrm{kg \, m^{-3}}$) and weight fraction (17.8 wt%) suitable for storage (Lan et al. 2012; Wang et al. 2013). In addition, ammonia can easily be transported as liquid below $-33.4°C$ at atmospheric pressure or 8.5 atm at room temperature. Ammonia is also safe to transport because the flammable range is 16%–25% in air. Moreover, the infrastructure for the transportation of ammonia has already been established for agriculture and industry. Along with the chemical properties, ammonia fuel cells have been used to produce a 1 kW of energy from ammonia, which is equivalent to hydrogen, opening up the possibility of using ammonia itself as energy (Kishimoto et al. 2020). These discoveries have led to the possible development of ammonia-fueled vehicles because ammonia can be efficiently converted into energy by a direct ammonia fuel cell (DAFC) (Fig. 2) (Zhao et al. 2019).
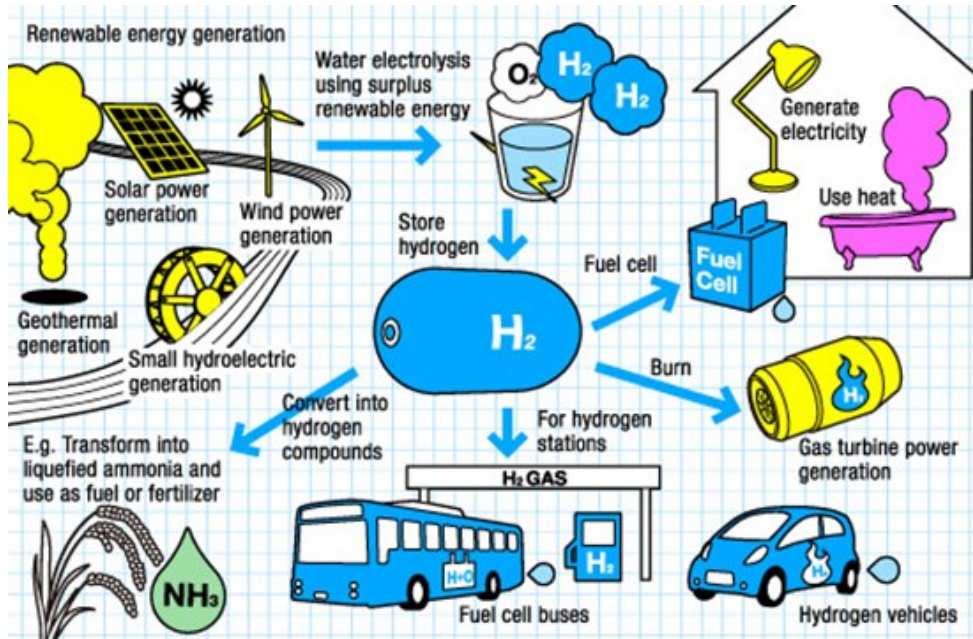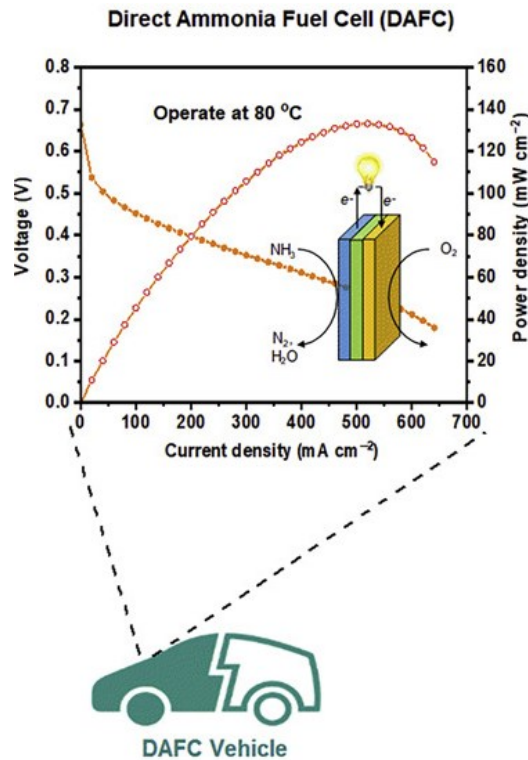
Fig. 1 Hydrogen society

(http://hori.way-nifty.com/synthesist/images/2011/12/30/renewableh2society.jpg)

Fig. 2 Carbon-neutral transportation system driven by ammonia fuel cell

(Modified from Zhao et al. 2019)

Ammonia production by biological methods

Ammonia is produced from nitrogen molecules in the air mainly by nitrogen-fixing bacteria; however, this natural process is not enough to cover the need of the world's demand and thus relies exclusively on the Haber–Bosch process (Das 2019). Approximately 50% of the ammonia used by the Haber–Bosch process produces nitrogen fertilizer for food production. In other words, about half of the world's population is thought to be fed by the Haber–Bosch process (Fig. 3).
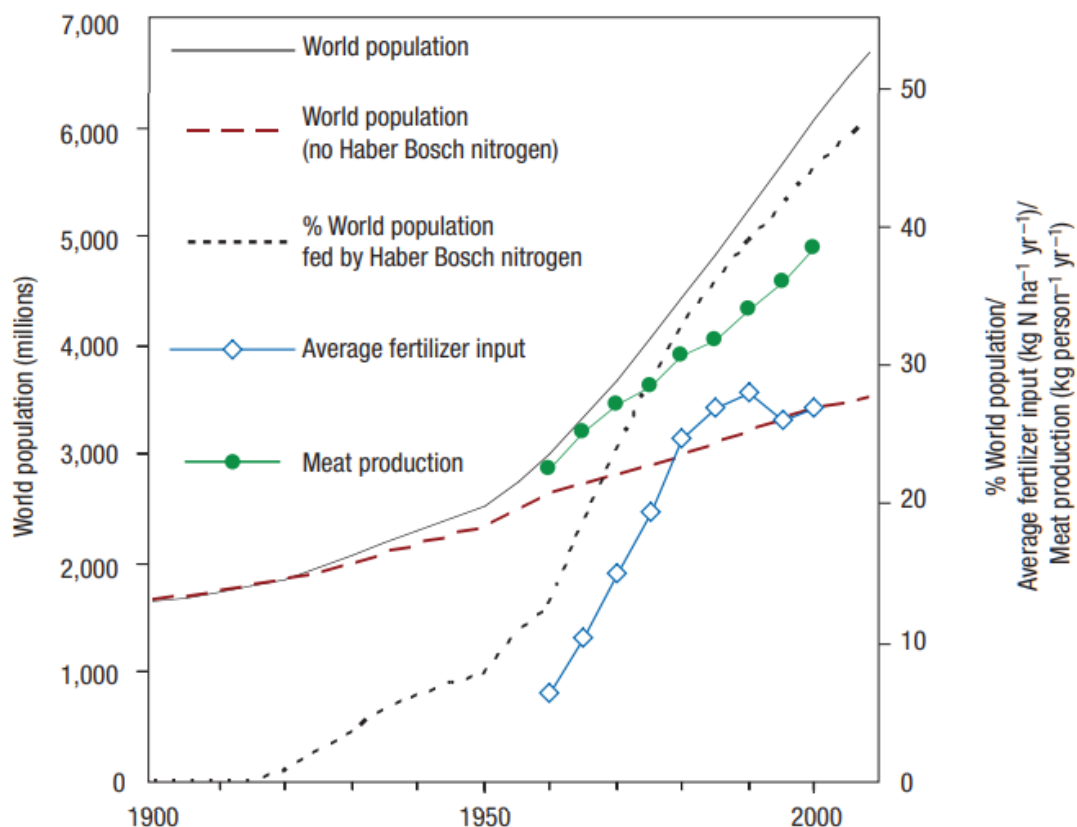
Fig. 3 Trends in human population and nitrogen use throughout the twentieth century (Erisman et al. 2008)

Since the Haber–Bosch process has a large environmental burden, methods are being explored using nitrogen-fixing bacteria at mild conditions. Rhizobia is a type of Diazotroph, a collective of specific gram-negative bacterial groups of *Alphaproteobacteria* and *Betaproteobacteria*, forms nodules in the roots of the host, and sometimes in the stem, and coexists with legumes as host plants to fix nitrogen. The representative diazotroph *Azotobacter vinelandii* is the most widely studied (Lindstrom

and Mousavi 2019; Noar and Bruno-Barcena 2018; Philippot et al. 2013). This diazotroph has been used in many studies to independently synthesize ammonia from nitrogen molecules using a series of enzyme-protein complexes (e.g., nitrogenase).

The amount of nitrogen fixed annually is 2.4×10^{12} mol of N by cultivation-induced nitrogen fixation from fodder legumes (Field et al. 1998; Gruber and Galloway 2008). Therefore, the biological process generated ammonia around 45% of the total nitrogen produced on earth (Canfield et al. 2010). The production of ammonia by microorganisms at ambient temperature and pressure has been attempted in recent years since this approach does not put a burden on the environment (Das 2019). Nitrogen-fixing microorganisms have also been studied, but they have been only partly successful under aerobic conditions, as oxygen easily and irreversibly deactivates the nitrogen-fixing enzymes (Noar and Bruno-Barcena 2018). Many engineered microorganisms can produce ammonia from amino acids and other substances found in food and other products, rather than nitrogen in the air (Choi et al. 2014). However, nitrogenases are very difficult to handle because they are susceptible to oxygen and are easily and irreversibly deactivated. Therefore, there have been few successes with expression of the subunit in heterologous hosts like yeast (Lopez-Torrejon et al. 2016).

Potential of soybean residue as food processing wastes for biofuel production

More than 3.9 million tons of soybean residues (okara) are produced each year as a byproduct of the production of soy milk and tofu (Fig. 4), with over 40% of this residue being landfilled or incinerated due to expiring (Liu et al. 2016; Vong et al. 2016). Most of the landfilled soybean residue is converted to the greenhouse gas nitrogen dioxide by microorganisms in the soil (Canfield et al. 2010; Galloway et al. 2008; Montzka et al. 2011). Therefore, ammonia production from soybean residue would divert nitrogen and reduce an environmental burden. Although soybean residue contains a high amount of protein (~31%), there are few studies on microorganisms that produce ammonia from soybean residue for practical applications, mainly in amino acid media (Choi et al. 2014; Huo et al. 2011; Kumar et al. 2016; Vong et al. 2016). The lack of ammonia production is probably due to the residue containing substances that inhibit the intracellular conversion of amino acids to ammonia (Huo et al. 2011).

Therefore, a method to produce ammonia from okara in a way that is independent of intracellular metabolism should be developed.

Fig. 4 Okara, soybean residue (Photo from https://allabout.co.jp/gm/gc/298549/)

Nitrogen catabolism by microorganisms and strategies for ammonia production

Besides the production of ammonia from air using microorganisms, ammonia can be produced from biomass, such as food processing wastes. Unlike nitrogen in the air, biomass contains amino acids that are immobilized nitrogen. Amino acids can be converted to ammonia by breaking them down using the catabolizing enzymes of many microorganisms. Most of these enzymes are easy to produce, unlike the nitrogen-fixing nitrogenase that is inactivated by oxygen. Therefore, several attempts have been made to produce ammonia from media containing amino acids (Choi et al. 2014; Huo et al. 2011; Mikami et al. 2017). For example, knocking out the *codY* gene, a global regulator of branched-chain amino acids, and overexpressing the *leuDH* gene in *Bacillus* promotes the deamination and production of ammonia from amino acid media in 46.6% of the theoretical yields (Choi et al. 2014). In addition, the glutamine assimilation gene *glnA* was knocked out and the decarboxylase gene *kivD* was overexpressed in *Escherichia coli* to produce ammonia from the amino acid medium at 47.8% yield (Fig. 5) (Mikami et al. 2017). These systems have a potential tradeoff between growth and production, since the ammonia production occurs in the cell. In a study using engineered *B. subtilis* for ammonia biosynthesis the production plateaued on day 6 and did not increase on day 7 (Choi et al. 2014). A state of equilibrium may have reached between ammonia production and maintenance of cell growth, implying a problem with using metabolic engineering techniques to produce compounds necessary for growth.
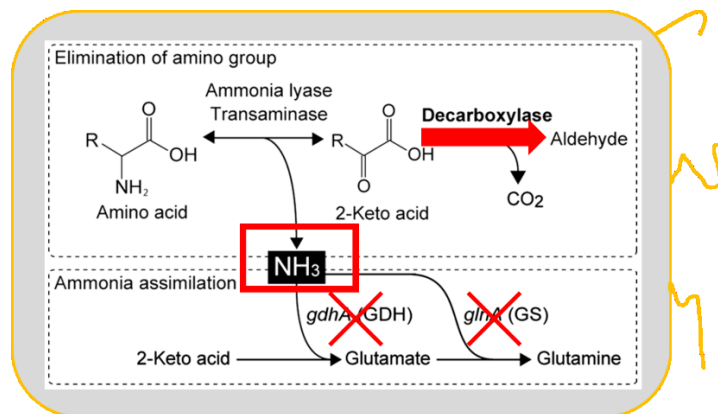
Fig. 5 Knockout of ammonia assimilation genes of *E. coli* to produce ammonia from amino acid media (Modified from Mikami 2018): (GDH-glutamine dehydrogenase, GS-glutamine synthase)

Nitrogen catabolism of yeast

Yeast (*Saccharomyces cerevisiae*) is a fungi, used in the production of numerous substances and has various advantages because yeast remains stable under various environmental conditions, such as low pH and has a low risk of contamination (Kuroda and Ueda 2016; Liu et al. 2019). Studies show that knocking out amino acid assimilation genes *GLN1* and *GDH1/3* of *S. cerevisiae*, causes a non-increase in production (Magasanik 2003; Mikami 2018). This might be because ammonia assimilation in yeast is extremely complexed and partially elucidated (Zhang et al. 2018). This a little ammonia production from knocking out nitrogen assimilation-associated genes to avoid nitrogen assimilation results also occurs in the bacteria *E. coli* (Magasanik 2003). Therefore, I think that metabolic engineering is not effective on ammonia production from food processing wastes and ammonia production outside the cells to avoid assimilation in the cells is effective.
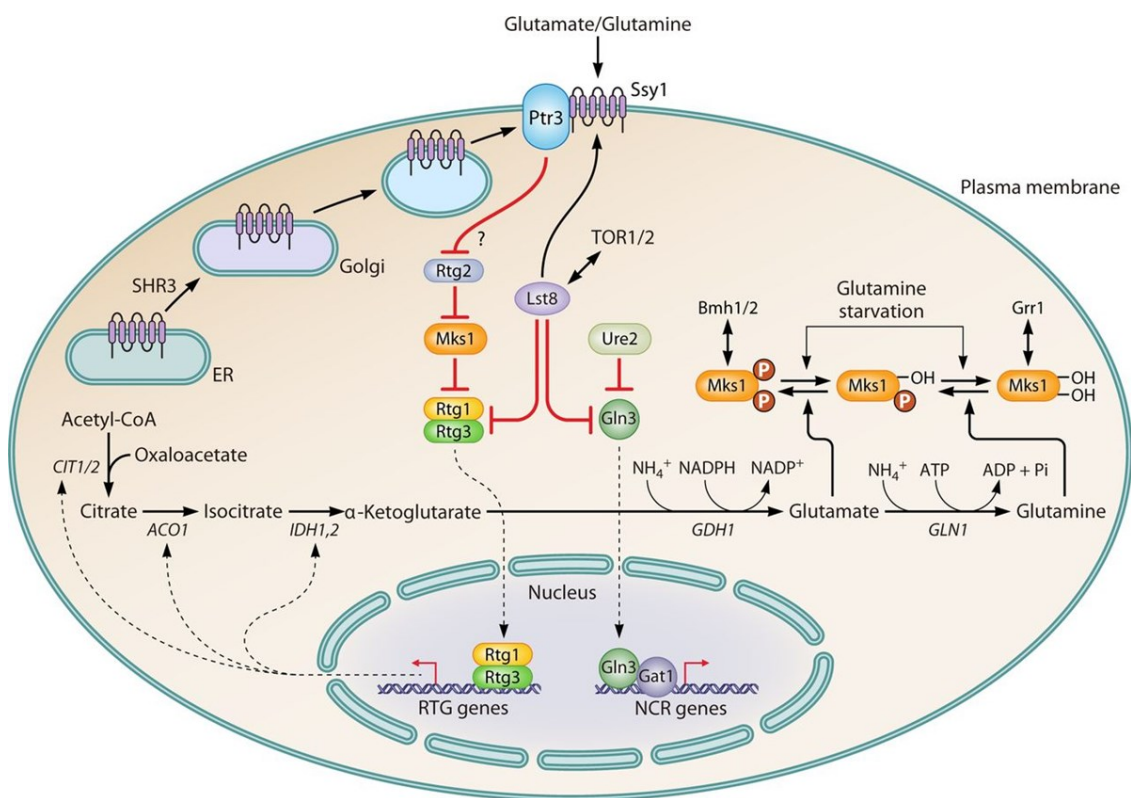
Fig. 6 Ammonia assimilation-related genes of *S. cerevisiae* (Zhang et al 2018)

Surface engineering technology of yeast cells for nitrogen catabolism

The efflux of ammonia from cells was examined by displaying amino acid catabolic enzymes using a cell surface engineering system to avoid ammonia assimilation and toxicity in the cell. Each N-terminal secretion signal was fused with an α-agglutinin cell wall anchor protein containing a C-terminal glycosylphosphatidylinositol (GPI)-anchored attachment signal sequence to display the target protein using cell surface engineering (Fig. 7)(Kuroda and Ueda 2011; Kuroda and Ueda 2013; Ueda 2016). Cell surface engineering allows yeast to display approximately 10^5–10^6 target proteins on the surface of each cell, and then these yeasts can be directly used as a biocatalyst. In addition, the labeled enzymes are anchored to the cell wall, by which the proteins are stabilized, and reusability of the yeast is increased to reduce costs (Inokuma et al. 2014; Miura et al. 2015; Motone et al. 2016; Takagi et al. 2016).

Therefore, this engineering approach was examined to produce ammonia to display amino acid catabolic enzymes on the surface of yeast cells.
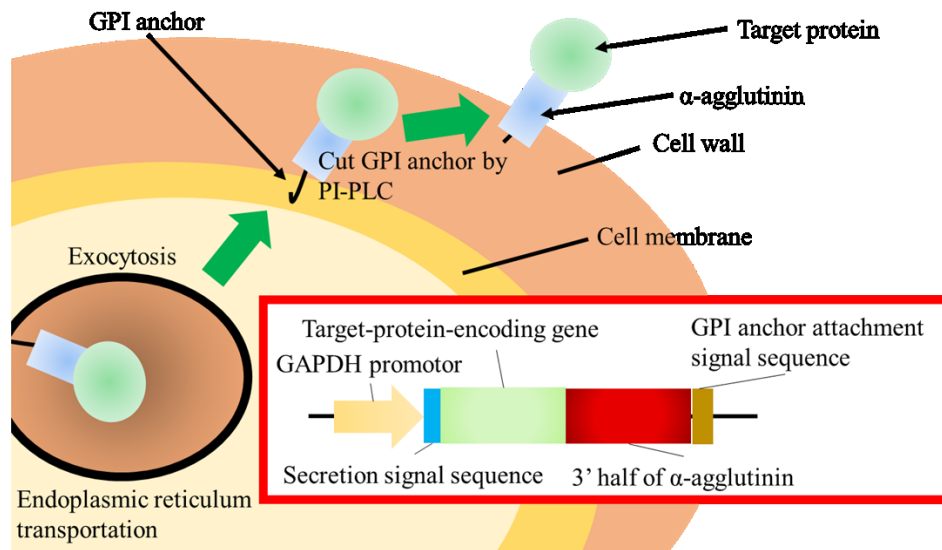
Fig. 7 Concept of cell surface engineering system for displaying enzymes on the cell surface and molecular gene design of cell surface display system (Modified from Miura et al. 2015)

Amino acid catabolite enzyme

Amino acid catabolic enzymes that produce ammonia from amino acids include deaminases and transaminases (Brown et al. 2008; Lu et al. 2013; Pollegioni et al. 2013). Glutamine ammonia-lyase is particularly suitable for displaying on the surface of yeast cells because this enzyme does not require any cofactor for catalysis.

Other enzymes can also catabolize amino acids like L-amino acid oxidase. The enzyme can convert other amino acids besides glutamine into ammonia for more effective ammonia production from soybean residue. Although several amino acid oxidases are known, not many have been successfully established in heterologous expression systems. For example, well-known oxidases in snake venom, L-amino acid oxidases, are active against hydrophobic amino acids such as leucine, methionine, and aromatic amino acids such as histidine and phenylalanine, but not against hydrophilic amino acids such as glutamine and aspartic acid (Table 1). Another L-amino acid oxidase from sea horse is active against arginine and lysine but shows little activity against other amino acids (Table 1).

Hebeloma cylindrosporum has mycorrhizal for absorbing ammonia into their cells from amino acids in the soil (Table 1)(Chalot et al. 2006; Nuutinen et al. 2012; Nuutinen

and Timonen 2008). Therefore, the conversion rate from 20 proteinogenic amino acids into ammonia by L-amino acid oxidase from *H. cylindrosporum* (HcLAAO)-displaying yeast is expected to be higher than previous studies (Bloess et al. 2019). Furthermore, since HcLAAO cannot produce ammonia from some amino acids, such as proline, serine, threonine, cysteine, and aspartic acid, further yeast breeding is required to efficiently produce ammonia from all proteinogenic amino acids.

HcLAAO should be displayed on the yeast strain that knocks out the transcriptional regulator of the amino acid assimilation gene, *gln3*.

Instead of Harbor–Bosch methods, the production of ammonia with (engineered) microbial cells would be promising and eco-friendly approach to solve the global problem of ammonia demand and develop a sustainable society.

Table 1 L-amino acid oxidase successfully heterologously expressed (Modified from Bloess et al. 2019; Bregge-Silva et al. 2012; Chen et al. 2012; Kasai et al. 2010; Kommoju et al. 2007; Yang et al. 2005)

Origins	Organism	Specificity	Cloned host
Snake	*Calloselasma rhodostoma*	Leu, Met, His, Phe	*P. pastoris*
	Daboia russelii		*E. coli*
	Lachesis muta		
Sea hare	*Aplysia californica*	Arg, Lys	*P. pastoris*
Fish	*Platichthys stellatus*	Arg, Lys	
Fungi	*Hebeloma cylindrosporum*	Ala, Ile, Leu, Met, Phe, Trp, Val, Asn, Gln, Tyr, Arg, His, Lys, Glu	*E. coli*

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Chapter I

Ammonia production from soybean residues by yeast cell surface-displayed glutaminase

Amino acid is major nitrogen source of food processing wastes like soybean residue. It contains many glutamine residues in food as a substrate for ammonia production (Eppendorfer and Bille 1996; Ninomiya 1998). In order to utilize food processing wastes effectively, glutaminase (YbaS) from *Escherichia coli* was selected among glutamine ammonia lyases, which is used by *E. coli* to protect itself by neutralizing host gastric acid; it is known to be highly efficient in converting glutamine to ammonia (Lu et al. 2013).

In this chapter, I constructed YbaS-displaying yeast to produce ammonia from amino acids outside the cells. The degradation of glutamine and successive ammonia production was quantified by LC-MS/MS and enzyme assay. Furthermore, YbaS-displaying yeast produced ammonia from glutamine in soybean residue treated with proteases and cellulases.

Materials and methods

Strains and media

E. coli strain DH5α [F−, ϕ80d*lacZ*ΔM15, Δ (*lacZYA-arg*F) U169, *end*A1, *hsd*R17 (r^{-k}, m^{+k}), *sup*E44, *thi*-1, λ−, *rec*A1, *gyr*A96, *rel*A1, *deoR*] was employed for recombinant-DNA- manipulation-host and the recombinant cells were grown in Luria–Bertani medium [1% (w/v) tryptone (Becton, Dickinson and Company, MI, USA), 0.5% (w/v) yeast extract (BD), and 1% (w/v) sodium chloride] containing 100 μg/mL ampicillin (Meiji Seika Pharma, Tokyo, Japan).

Saccharomyces cerevisiae strain BY4741/*sed1*Δ (*MAT*a, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0, *YDR077w::KanMX4*), obtained from EUROSCARF (Frankfurt, Germany), was used for display of YbaS on the yeast cell surface because it is known that *sed1*Δ resulted in an increase of the quantity of protein on the yeast cell surface (Kuroda et al. 2009). Yeast host cells were grown in synthetic complete without uracil (SC-Ura) medium

[0.15% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (BD), 0.5% (w/v) ammonium sulfate (Wako Pure Chemical Industries, Osaka, Japan), 0.19% (w/v) yeast synthetic drop-out medium supplements (Sigma-Aldrich, MO, USA), 2% (w/v) glucose] and transformed into the competent cells. To produce ammonia by YbaS-displaying yeast, the transformants were cultured in SC-Ura buffered at pH 5.5 with 200 mM 2-morpholinoethanesulfonic acid (MES) (Nacalai Tesque, Kyoto, Japan).

Pretreatment of soybean residues (okara)

Soybean residues (Nihon Beans, Tokyo, Japan), called okara, were obtained commercially at the supermarket in Kyoto city. These residues [14% (w/w)] were diluted in 50 mM MES (pH 5.5) and sterilized by autoclave. All the enzymes described below were purchased from Amano Enzyme (Nagoya, Japan). The protease mixture solution contained 2 mg/mL ProteAX, Peptidase R, PROTIN SD-AV10, Protease M "Amano" SD, PROTIN SD-NY10, THERMOASE R PC10F, and Protease A "Amano" SD in 50-mM MES (pH 5.5). The cellulase mixture solution contained 2 mg/mL Hemicellulase "Amano" 90, Cellulase A "Amano" 3, Mannanase BGM "Amano" 10, Cellulase T "Amano" 4, and Pectinase G "Amano" in 50-mM MES (pH 5.5). These mixtures were filtrated through a 0.45 μm PVDF filter (Merck Millipore, MA, USA) and added into sterilized soybean residues. The mixture of soybean residues containing these enzymes was shaken at 250 rpm at 55 °C for 72 h (Bio-Shaker BR-300LF, Japan). The mixture was incubated at 80 °C for 30 min to denature the enzymes. Then, the mixture was purified by filtering with ADVANTEC131 (3 μm particle retention capacity; Toyo Roshi Kaisha, Tokyo, Japan) after the filtration with ADVANTEC2 (5 μm particle retention capacity; Toyo Roshi Kaisha). After that, the flow-through was filtrated through a 0.45 μm PVDF filter (Merck Millipore) for sterilization and used for the substrate of ammonia production.

Plasmid construction for displaying glutaminase on the cell surface

The genomic DNA of *E. coli* K-12 (Baba et al. 2006) strain was grown in Luria-Bertani medium at 37°C for 12 h and extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Primers YbaS-F (5'-GTTTCTGCCAGATCTATGTTAGATGCAAAC-3'), YbaS-R (5'-

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AGATCCACCCTCGAGTCAGCCCTTAAACAC-3'), and KOD FX Neo (Toyobo, Osaka, Japan) were used to amplify the glutaminase gene (YbaS) from *E. coli* genomic DNA by polymerase chain reaction (PCR). The PCR-amplified YbaS was digested with BglII and XhoI, followed by insertion into the vector pULD1 (Kuroda et al. 2009). The constructed plasmid was named pULD1-YbaS. pULD1-s (Kuroda et al. 2009) is a pULD1 analog containing a strep-tag instead of a FLAG-tag, and it was employed as a negative control of immunofluorescence labeling and ammonia production.

Yeast transformation

The constructed plasmid pULD1-YbaS was introduced into *S. cerevisiae* BY4741/sed1Δ by using the lithium acetate method (Ito et al. 1983) using the Frozen-EZ Yeast Transformation Kit (Zymo Research, CA, USA). The transformant was grown on SC-Ura plate medium at 30 °C for 2 days.

Immunofluorescence labeling

To confirm the display of YbaS on the yeast cell surface, immunofluorescent labeling of the FLAG tag was performed as follows. Detection of the fluorescence of Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific, MA, USA) on the cell surface indicates that the enzyme-FLAG tag fusion is displayed on the cell surface because the FLAG tag is inserted between the enzyme and the cell-wall-anchoring domain. The yeast cells were incubated in phosphate-buffered saline (PBS; pH 7.4) containing 1% bovine serum albumin for 30 min before immunostaining for blocking. A mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) was used at a dilution rate of 1:300 and a mixture of cells and antibody was incubated for 1.5 h at room temperature with rotator. After washing with PBS (pH 7.4), Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) as the secondary antibody was used at a dilution rate of 1:300 at room temperature with rotator for 1.5 h. After washing with PBS (pH 7.4), the cells were observed using an inverted microscope IX71 (Olympus, Tokyo, Japan) through a U-MNIBA2 mirror unit with a BP470-490 excitation filter, a DM505 dichroic mirror, and a BA510-550 emission filter (Olympus). Live images were obtained by using the Aqua Cosmos 2.0 software (Hamamatsu Photonics, Shizuoka,

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Japan) to control a digital charge-coupled device camera (Hamamatsu Photonics).

Ammonia production by glutaminase-displaying yeast

The yeast cell cultures were centrifuged at 3000×g for 5 min at 4 °C after pre-cultivation in SC-Ura buffered at pH 5.5 for 36 h at 30 °C. The harvested cells were washed with sterilized distilled water to remove materials derived from medium. The cells were suspended in sterilized distilled water and cell suspension was inoculated into 200-mM MES buffer (pH 5.5) containing 37.3 g/L glutamine (Maximum concentration to resolve into water at 37°C) or pretreated soybean solution containing 85.2 mg/L glutamine to an OD600 of 1. The reaction mixtures in 1.5 mL microtubes were incubated with shaking at 1000 rpm (Bio-Shaker M·B-024, Japan) at 37 °C and sampled at each time point. The reaction mixtures were centrifuged at 15,000 rpm for 1 min at 25°C and the supernatants were collected and incubated for 20 min at 80°C to denature residual enzymes. The ammonia concentration in the heated supernatant of the reaction mixture was measured using F-kit Ammonia (J.K. International, Tokyo, Japan). The measurement was based on the 340 nm absorbance of NADH, which is converted to NAD+ by the enzyme reaction with 2-oxoglutarate and ammonia. All the ammonia yields presented in this manuscript were obtained by eliminating the background ammonia concentration as shown below. In the case of ammonia yields produced from glutamine solution, the minimum ammonia concentrations produced by each yeast cell harboring pULD1-YbaS (0 h) or pULD1-s (0 h) were subtracted from those by yeast cells harboring pULD1-YbaS (0, 6, 12, 24, 36, 48 h) or pULD1-s (0, 6, 12, 24, 36, 48 h), respectively. In another case of ammonia yields produced from pretreated soybean residues, the minimum ammonia concentrations produced by yeast cells harboring pULD1-YbaS (0 min) or pULD1-s (20 min) were subtracted from those by yeast cells harboring pULD1-YbaS (0, 5, 10, 20, 40, 60 min) or pULD1-s (0, 5, 10, 20, 40, 60 min), respectively. The conversion efficiencies from glutamine to ammonia was calculated as follows: (ammonia concentration at each time point/glutamine concentration at 0 min).

LC–MS/MS quantification of amino acids

To perform quantification of amino acids using LC–MS/MS accurately, I added L-

glutamine-13C5,15N2, and labeled amino acid standards [50 μg/mL algal hydrolysate amino acid mixture (U-13C, 97%–99%; U-15N, 97%–99%; Cambridge Isotope Laboratories CNLM-452-0.5)] as an internal standard (IS) into pretreated soybean residues and the reaction mixture after reacting with the cell surface engineered yeasts. The residual glutamine concentration in glutamine solution was quantified as shown below. LC experiments were conducted using HPLC (Nexera System; Shimadzu, Kyoto, Japan). Chromatographic separation was performed by using a Scherzo SS-C18 column (100 mm × 3 mm, 3 μm; Imtakt, Kyoto, Japan) with gradient elution with a mobile phase composed of eluents A (0.1% formate in water, v/v) and B (100-mM ammonium sulfate and 40% acetonitrile in water, v/v). The mobile phase was programmed as follows: isocratic elution of 5% B for the first 3 min, followed by the linear gradient elution of 5%−100% B from 3 to 8 min. After the solvent composition of 100% B had been held from 8 to 11 min and then changed to 5% B for the next 2 min, it was returned to its starting condition and proceeded for re-equilibration. The supernatants of pretreated soybean residues were incubated with YbaS-displaying yeast or yeast harboring pULD1-s (1 μL), and these were injected onto the column and the flow rate was 0.3 mL/min for the first 3 min and 0.6 mL/min for the next 10 min.

The mobile phase was programmed as follows to measure glutamine from pretreated soybean residues: isocratic elution of 5% B for the first 5 min, followed by linear gradient elusions of 5%−10% B from 5 to 10 min and 10%–100% B from 10 to 20 min. The solvent composition of 100% B had been held from 20 to 23 min and then changed to 5% B for the next 2 min, and it was returned to its starting condition and held for re-equilibration. The heated supernatants of pretreated soybean residues incubated with YbaS-displaying yeast or yeast harboring pULD1-s (1 μL) was injected onto the column. The flow rate was 0.3 mL/min for the first 6 min and 0.6 mL/min for the successive 23 min and the column temperature was set at 40°C throughout the analysis. Glutamine concentration was quantified using the multiple reaction monitoring (MRM) mode of triple quadrupole mass spectrometry (LCMS-8060; Shimadzu) equipped with an electrospray ionization source (ESI). The MS conditions were as follows: electrospray voltage of 4.0 kV, capillary temperature at 300 °C and N2 sheath gas of 10 L/min. Each parameter of the MRM mode was as shown below: precursor ion (m/z) of 147.1, product

ion (m/z) of 56.2, Q1 pre-bias of −2 V and collision energy of −32 V. The MS conditions of amino acids obtained from soybean residues are shown in Additional file 1 in Table S1. IS of amino acids; L-glutamine-13C5,15N2, and labeled amino acid standards [50 μg/mL algal hydrolysate amino acid mixture (U-13C, 97%–99%; U-15N, 97%–99%; Cambridge Isotope Laboratories CNLM-452-0.5)], was spiked in the samples for quantification of amino acids obtained from soybean residues.

Results

Construction of cell surface engineered yeast displaying glutaminase

To produce ammonia from glutamine outside the cells, I displayed glutaminase (YbaS), one of the amino-acid-catabolizing enzymes from *E. coli* (Lu et al. 2013), on the yeast cell surface. The glutaminase gene (YbaS) was inserted into pULD1, a cassette vector for the efficient display of proteins, to display YbaS on the yeast cell surface (Fig. 1a). The constructed pULD1-YbaS was introduced into yeast competent cells. Immunofluorescence labeling was performed with mouse monoclonal anti-FLAG M2 antibody.to confirm the display of YbaS on the yeast cell surface. The green fluorescence by Alexa Fluor 488 was observed at the yeast cell surface indicate that the YbaS-FLAG tag fusion had been successfully displayed there. In contrast, fluorescence was not observed in yeast transformed with negative control plasmid, pULD1-s, for displaying only a strep-tag (Fig. 1b).
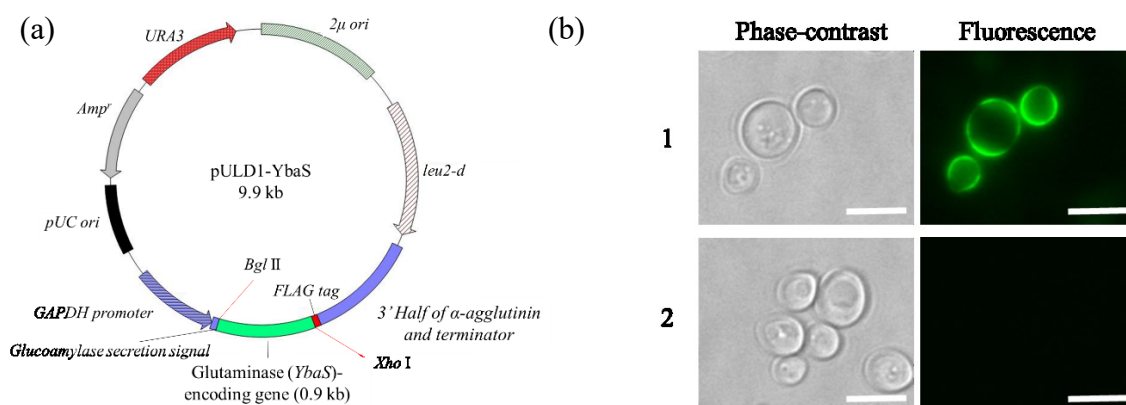
Fig. 1 Yeast cell surface display (a) The constructed plasmid for display of glutaminase from *E. coli* (YbaS) on the cell surface and (b) Fluorescence observation of cells using immunofluorescence labeling. An anti-FLAG antibody and Alexa Fluor 488 anti-mouse IgG antibody were used for labeling of the FLAG-tag fused to displayed proteins. Phase-contrast micrographs was shown at left column and fluorescence micrograph (right column). The first row indicates pULD1-YbaS; the second row indicates pULD1-s. The scale bars indicate 5 μm.

Ammonia production from glutamine by glutaminase displayed on the yeast cell surface

In order to monitor the ammonia production by YbaS-displaying yeast on the cell surface, we measured the ammonia concentration in each sample by enzyme assay methods. We monitored the glutamine converted by YbaS-displaying yeast with LC–MS/MS (Fig. 2). YbaS-displaying yeast successfully produced 3.34 g/L ammonia at 48 h, whereas the control yeast harboring pULD1-s produced almost no ammonia (Fig. 3). The results suggest that YbaS-displaying yeast can produce ammonia from glutamine with high efficiency (83.2%) for 48 h.
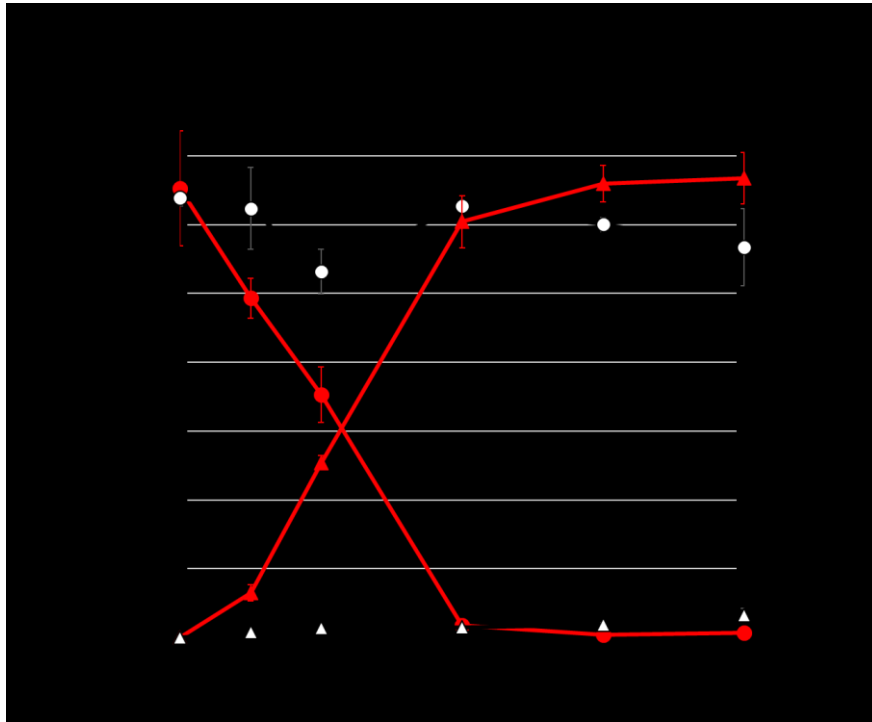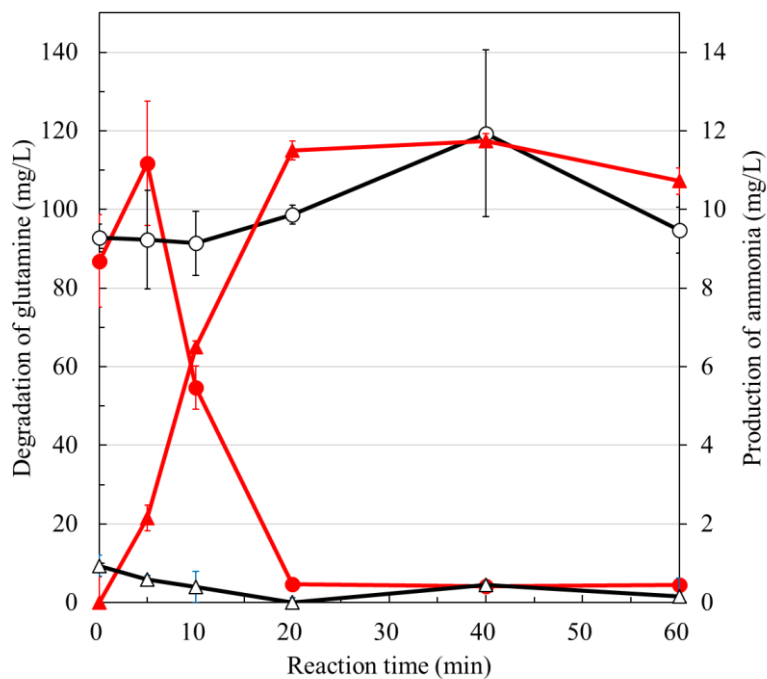
Fig. 2 Ammonia production from glutamine using YbaS-displaying yeast

The ammonia produced by YbaS-displaying yeast (pULD1-YbaS, red triangle) and control yeast (pULD1-s, open black triangle), and the residual glutamine by YbaS-displaying yeast (pULD1-YbaS, red circle) and control yeast (pULD1-s, open black circle) were measured. Values are given as mean ± SE with biological triplicates.

Ammonia production from pretreated soybean residues by glutaminase-displaying yeast

Since I succeeded in producing ammonia from glutamine solution by YbaS-displaying yeast, I employed this yeast for ammonia production from glutamine included in soybean residues pretreated with proteases and cellulases. These proteases thought to degrade proteins contained in soybean residues and produce amino acids as products, and cellulases degrade cellulose associated with proteins and might increase the efficiency of protein degradation. YbaS-displaying yeast produced 11.7 mg/L ammonia from glutamine included in pretreated soybean residues at 40 min, whereas negative control, yeast harboring pULD1-s, did not produce ammonia (Fig. 3). This indicates that YbaS-displaying yeast was able to produce ammonia from glutamine included in the pretreated soybean residues as food processing wastes.

Fig. 3 Ammonia production from pretreated soybean residues using YbaS-displaying yeast

The ammonia produced by YbaS-displaying yeast (pULD1-YbaS, red triangle) and control yeast (pULD1-s, open black triangle), and the residual glutamine by YbaS-displaying yeast (pULD1-YbaS, red circle) and control yeast (pULD1-s, open black circle) were measured. Values are given as mean ± SE with biological triplicates.

Discussion

To establish a biological technique for efficient ammonia production, it seemed to be important to avoid ammonia assimilation that occurs inside yeast cells. In this study, I displayed glutaminase, YbaS, on the yeast cell surface to produce ammonia from glutamine contained in pretreated soybean residues outside the cells. YbaS-displaying yeast produced 3.34 g/L ammonia, which was 1.4 times and 7.3 times higher than the ammonia concentration in the previous studies by metabolically engineered *B. subtilis* and *E. coli* (Fig. 2) (Choi et al. 2014) (Mikami et al. 2017). Therefore, I succeeded in producing high-concentration ammonia using yeast for the first time. In addition, the results indicate that the ammonia production by YbaS-displaying yeast was not inhibited any other factors like the toxicity of ammonia because it is known that a high concentration of ammonia above 0.1% (w/v) in the solution causes poor growth of yeast due to its toxicity, rather than an increase in pH (Santos et al. 2012). However, in this system, even though cells are damaged by the toxicity of ammonia, displayed enzymes could function and produce ammonia in our system, because whether the cells are dead or alive does not affect the function of the enzyme displayed on the cell surface. Therefore, I have shown that extracellular ammonia production by cell surface engineering is effective to avoid ammonia assimilation and toxicity to yeast.

All the previous studies on the production of ammonia by bacteria focused on intracellular production by metabolic engineering. For example, the yield of ammonia production by metabolically engineered *B. subtilis* was limited to 46.6% after incubation for 7 days, because it is thought that ammonia is assimilated into the cell components (Choi et al. 2014). In contrast, we extracellularly produced ammonia from glutamine solution with high conversion efficiency of 83.2% (Fig. 2). However, 83.2% conversion efficiency can be further increased in the future by some other methods. In our experiment, the yeast in the reaction solution used may have been nitrogen-starved and may have taken up ammonia and glutamine into the cells. Since the transporter works mainly for uptake in the nitrogen-starved-situation, the disruption of glutamine transporter (Schreve et al. 1998) or ammonia transporter (Marini et al. 1997) may contribute to further improvement of the production efficiency.

Furthermore, I showed that YbaS-displaying yeast can be applied to ammonia production from food processing wastes, soybean residues. In this study, pretreated soybean residues were used as a representative type of food processing wastes because soybean residues are produced during the process of making tofu. Almost all the glutamine from pretreated soybean residues were converted into ammonia by YbaS-displaying yeast at 40 min (Fig. 3). These results indicate that the YbaS-displaying yeast can efficiently produce ammonia from food processing wastes and that ammonia production by YbaS on the yeast cell surface was not prevented by any other materials such as metal ions and sugars in pretreated soybean residues.

For producing ammonia from soybean residues, protein and cellulose in soybean residues had to be degraded by proteases and cellulases, and I quantified the concentrations of amino acids. In a previous study, soybean residues were treated with a strong acid, but the pretreatment places a burden on the environment because of the need for post treatment to neutralize the acid (Kumar et al. 2016). In addition, glutamine was surely decomposed to glutamic acid by acid treatment in the previous study. In contrast, I used proteases and cellulases for degrading soybean residues under mild conditions (pH 5.5). I could accurately quantify the concentrations of 16 proteinogenic amino acids, with the exceptions of cysteine, tryptophan, asparagine, and alanine from pretreated soybean residues. To compare the production efficiency of amino acids from proteins in the previous study, it was thought to be sufficient to quantify only essential amino acids other than tryptophan (Kumar et al. 2016). I show that the amount of glutamine produced from soybean residues was 84.8 mg/L. As a result, total essential amino acid content, excluding tryptophan produced from soybean residues in this study, was estimated about 4.97 g/100 g (Fig. 4). This was because all the proteases have specificity and it is difficult to completely convert all proteins to free amino acids, even if many different types of protease are used. To improve the conversion rate, it might to be efficient to optimize the amount and types of proteases and cellulases, or the reaction time and reaction temperature for degrading soybean residues. In the future, it is expected that all protein from soybean residues will be converted for ammonia production by the co-display of proteases, contributing to the low-cost utilization of food processing wastes.
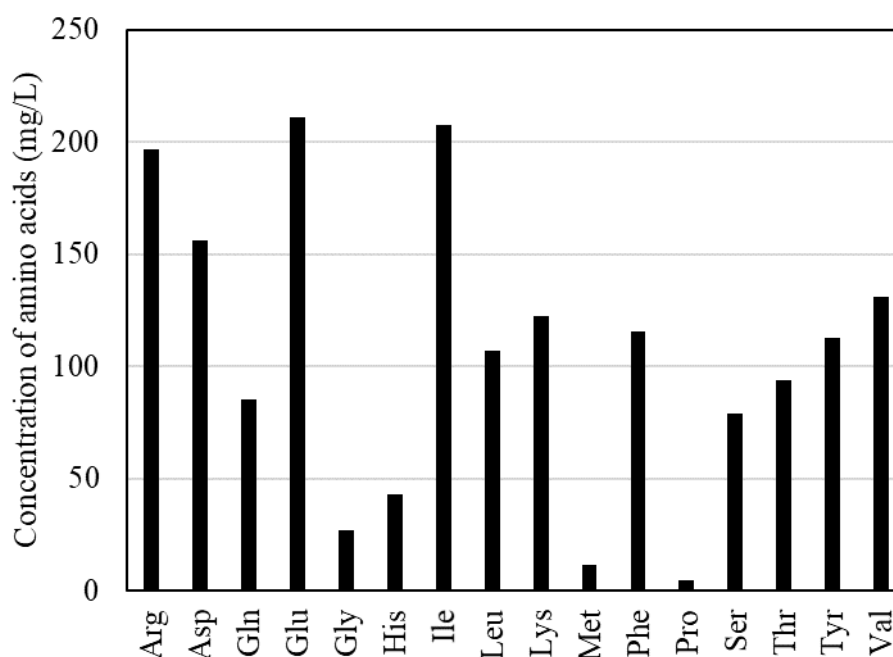
Fig. 4 Amino acids contained in pretreated soybean residues

The amino acids contained in pretreated soybean residue were measured using LC-MS/MS. Values are given as biological singlicate.

In chapter I, I showed the YbaS-displaying yeast produced high concentrations of ammonia from glutamine solution and from pretreated soybean residues at high efficiency outside the cells. This is the first report describing an approach suitable to meet the SDGs of extracellular ammonia production from food processing wastes by surface-engineered yeast that could avoid ammonia assimilation into the cells. Therefore, the yeast cell-surface-display system could contribute to the efficient production of essential materials for cell growth or poisonous products at high concentration in yeast such as ammonia.

Summary

Ammonia is an essential substance for agriculture and the chemical industry. The intracellular production of ammonia in yeast (*Saccharomyces cerevisiae*) by metabolic engineering is difficult because yeast strongly assimilates ammonia, and the knockout of genes enabling this assimilation is lethal. Therefore, we attempted to produce ammonia outside the yeast cells by displaying a glutaminase (YbaS) from *Escherichia coli* on the yeast cell surface. YbaS-displaying yeast successfully produced 3.34 g/L ammonia from 32.6 g/L glutamine (83.2% conversion rate), providing it at a higher yield than in previous studies. Next, using YbaS-displaying yeast, we also succeeded in producing ammonia from glutamine in soybean residues (okara) produced as food processing wastes from tofu production. Therefore, ammonia production outside cells by displaying ammonia-lyase on the cell surface is a promising strategy for producing ammonia from food processing wastes as a novel energy resource, thereby preventing food loss.

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Chapter II

Improved ammonia production from soybean residues by cell surface-displayed L-amino acid oxidase on yeast

There are amino-acid-catabolizing enzymes, which produces ammonia from amino acids, such as amino acid deaminase, amino acid transaminase, and ammonia-lyase (Brown et al. 2008; Hartman 1968; Lu et al. 2013; Molla et al. 2017; Pollegioni et al. 2013). In chapter I, I succeeded to produce ammonia from glutamine in pretreated soybean residue by cell surface engineered yeast displaying glutaminase on the cell surface. However, I thought that 20 proteinogenic amino acids should be converted, not just glutamine, into ammonia for more effective ammonia production from soybean residue. *Hebeloma cylindrosporum* among various microorganisms has mycorrhizal for uptaking ammonia into their cells and the ammonia is produced from amino acids in soil. To produce ammonia, the fungi has L-amino acid oxidase (HcLAAO) with fairly broad specificity (Bloess et al. 2019; Chalot et al. 2006; Kuroda et al. 2009; Nuutinen and Timonen 2008). Therefore, the conversion rate from 20 proteinogenic amino acids into ammonia by HcLAAO-displaying yeast is expected to be higher than previous chapter (Chapter I) (Bloess et al. 2019; Nuutinen et al. 2012).

In this chapter, I attempted to produce ammonia from amino acids included in proteinogenic amino acid-containing solution and the food processing waste soybean residues by displaying HcLAAO on the yeast cell surface.

Materials and methods

Construction of plasmids for yeast cell surface display of L-amino acid oxidase and yeast transformation

The codon-optimized sequence of the HcLAAO was synthesized (Integrated DNA Technologies, Coralville, IA, USA) and inserted into pULD1 (Kuroda et al. 2009). The constructed plasmid was named to pULD1-HcLAAO. pULD1-s (Kuroda et al. 2009) is a pULD1 derivative as described in chapter I. *S. cerevisiae* strain BY4741/sed*1*Δ (*MAT*a,

*his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0, *YDR077w::KanMX4*), obtained from EUROSCARF (Frankfurt, Germany), was used for the cell surface display of HcLAAO and the negative control. The constructed plasmid was introduced into *S. cerevisiae* BY4741/Δ*sed1* as described in chapter I.

Ammonia production from amino acids by ʟ-amino acid oxidase-displaying yeast

Yeast cells were pre-incubated in SDC medium for 72 h at 30°C. The yeast cell cultures were centrifuged at 6,000 × *g* for 5 min at 4°C and washed with PBS (pH 7.4). After the washing, the cells treated with sodium phosphate buffer (pH 3.0) for 0, 10 or 60 min and washed with PBS (pH 7.4) again. The cells were suspended in sterilized distilled water and these were inoculated into 50-mM HEPES buffer (pH 5.5) containing amino acids or pretreated soybean residues and OD600 values were adjusted to equivalent to 20. All the reaction mixtures were shaken at 250 rpm (Bio-Shaker M·B-024, Japan) at 30°C and sampled at each time point. Ammonia concentration in the supernatant was measured by using F-kit Ammonia (J.K. International, Tokyo, Japan). The efficiency of ammonia production from amino acids were obtained by eliminating the background ammonia yields as shown below. In the case of yields of ammonia produced from amino acids, the ammonia yields produced by each yeast cell harboring pULD1-HcLAAO were subtracted from those by yeast cells harboring pULD1 respectively. The conversion efficiency from glutamine to ammonia was calculated as follows: (ammonia yields at each time point/ amino acids concentration added in the mixture).

Effect of preculture-time, temperature, acid treatment time, FAD addition and cell density on ammonia production

To evaluate the effect of preculture time on ammonia production, I preincubated HcLAAO-displaying yeast cells for 24 to 120 h used in the ammonia production. To determine the optimal temperature for ammonia production, I incubated reaction samples at 25°C or 30°C. To determine the effect of acid treatment, I measured the ammonia concentration using conditions in the ammonia production by ʟ-amino acid oxidase-displaying yeast after 0 to 60 min incubation of cells with 50-mM of phosphate buffer (pH 3.0). To determine the effect of flavin adenine dinucleotide (FAD) addition, I added

FAD into reaction solutions at the final concentration of 10 μM. To determine optimal cell density, we added at the HcLAAO-displaying yeast cells (optical density at 600 nm was equivalent to 10, 20, or 30) into reaction mixtures.

Measurement of hydrogen peroxide (H$_2$O$_2$) production

I assayed L-amino acid oxidase activities by measuring the production of hydrogen peroxide (H$_2$O$_2$) as follows: a reaction mixture contained 1-mM of each amino acid, 3-mM of 4-amino antipyrine, 6.7 units of peroxidase (Tokyo Chemical Industry, Tokyo, Japan), 50-mM of HEPES (pH 7.0), and the HcLAAO-displaying yeast at the OD600 values were adjusted to equivalent to 20. The production of H$_2$O$_2$ was measured at room temperature for 5 min by absorbance at 562 nm by the VMax® microplate reader (Molecular Devices, Tokyo, Japan).

GC-MS/MS quantification

Amino acids derivatizations were prepared as follows. The pretreated soybean residues of samples treated with enzymes as described above were reconstituted were dried. Esterification was performed by adding 25 μL of acetonitrile and *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), and the samples were heated for 30 min at 60 °C. The solutions were transferred into autosampler glass vials equipped with micro inserts, and the samples into vials were sealed and subjected to the GC–MS analysis. All analyses were performed by using a SHIMADZU GC-MS-QP2010ultra and an AS1310 autosampler from Shimazu (Kyoto, Japan). The column used in this chapter was a fused-silica capillary column InertCap 5MS/NP (30 m length, 0.25 mm I.D., and 0.25-μm film thickness) from GL-sciences (Tokyo, Japan) with a bonded phase (5% diphenyl and 95% dimethylpolysilarylene) and used with carrier gas helium. The oven program was set at 150 °C and kept for 2 min, and then increased at 8 °C /min to 270 ℃, and finally held at that temperature for 8 min. The injector temperature was 250 °C and the injection was the split mode (splitter 1:20) at 250 °C with an injection volume of 1 μL. Mass spectra (99 eV, electron impact mode) scan range was m/z 45-800 amu with a mass scan time of 0.3 s and the interface temperature 270 °C. The flow rate was 1.5 mL/min, column head pressure 73 kPa and total flow rate: 20.0 mL/min.

The total analytical run time was 25 min.

Results

Construction of yeast displaying L-amino acid oxidase on the cell surface

To produce ammonia from amino acids outside cells, I constructed yeast cells displaying L-amino acid oxidase derived from *H. cylindrosporum* (HcLAAO). To display HcLAAO on the yeast cell surface, the codon-optimized L-amino acid oxidase gene lacking the 18 amino acids of the putative signal sequence (GenBank accession number MH751433) (*HcLAAO*) (Bloess et al. 2019) was inserted into the linear pULD1 cut by restriction enzymes (Fig. 1). pULD1-HcLAAO was introduced into yeast, and I observed a successful display of HcLAAO on the yeast cell surface (Fig. 2).
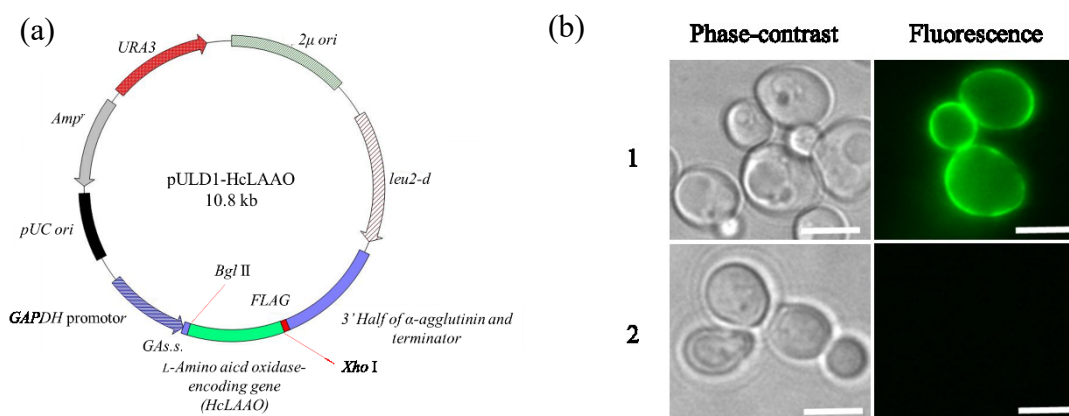
Fig. 1 Yeast cell surface display (a) The constructed plasmid for display of L-amino acid oxidase from *Hebeloma cylindrosporum* (HcLAAO) on the yeast cell surfaces and (b) Fluorescence observation of cells using immunofluorescence labeling. An anti-FLAG antibody and Alexa Fluor 488 anti-mouse IgG antibody were used for labeling of the FLAG-tag fused to displayed proteins. Phase-contrast micrographs was shown at left column and fluorescence micrograph (right column). The first row indicates pULD1-HcLAAO; the second row indicates pULD1-s. The scale bars indicate 5 μm.

Ammonia production from L-leucine

To verify the ammonia production by the HcLAAO-displaying yeast, I incubated the cells cultivated for 24 to 120 h with L-leucine. The HcLAAO-displaying yeast successfully produced 5.92-mM ammonia when the cultivation time was 72 h, whereas control yeast harboring pULD1-s produced almost no ammonia (Fig. 3a). The result suggested that the HcLAAO-displaying yeast can produce ammonia, and the maximum yield was achieved when using the yeast cells cultured for 72 h.

Since it is known that some oxidases are activated by exposure to acidic pH (Bloess et al. 2019; Kanade et al. 2006; Kenten 1957), we evaluated the effect of acid treatment on the ammonia production by the HcLAAO-displaying yeast. Although there seemed to be a tendency for acid treatment to increase the activity, it was not statistically significant under the present experimental conditions (Fig. 3b).

To examine the optimum temperature, the HcLAAO-displaying yeast was incubated at different temperatures because the L-amino acids oxidase from *Rhizoctonia solani* is known to be more active at lower temperatures (Hahn et al. 2017). However, we found that there was no difference in the ammonia concentration after incubation between 25 °C and 30 °C (Fig. 3c).
We examined the effect of FAD addition because L-amino acid oxidase generally requires FAD for their activity (Pollegioni et al. 2013). As shown in Figure 3d, the addition of FAD did not show an effect on ammonia production.
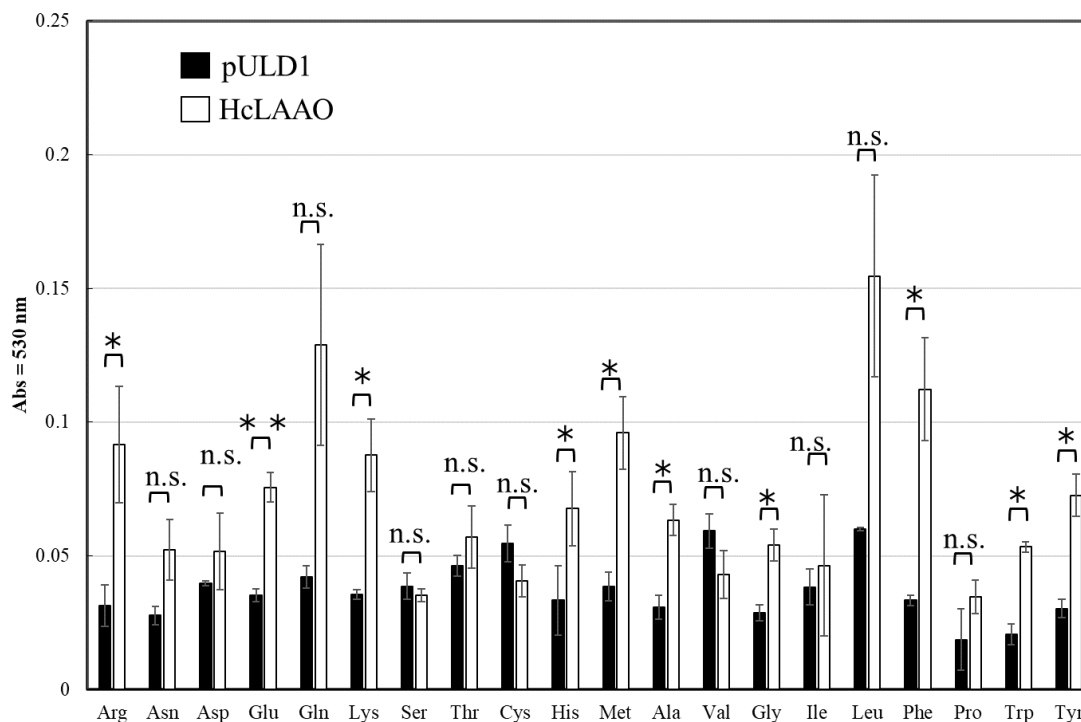
H_2O_2 production from each proteinogenic amino acid

To evaluate the specificity of the substrate, I tested H_2O_2 production from each proteinogenic amino acid at a concentration of 1-mM by the HcLAAO-displaying yeast (Fig. 4). The result showed that HcLAAO-displaying yeast tend to produce ammonia from amino acids more than 10 amino acids, ten of which (Arg, Glu, Lys, His, Met, Ala, Gly, Phe, Trp and Tyr) were statistically significant. This result was consistent with the previous studies which showed the broad substrate specificity of HcLAAO (Bloess et al. 2019; Faust et al. 2007).
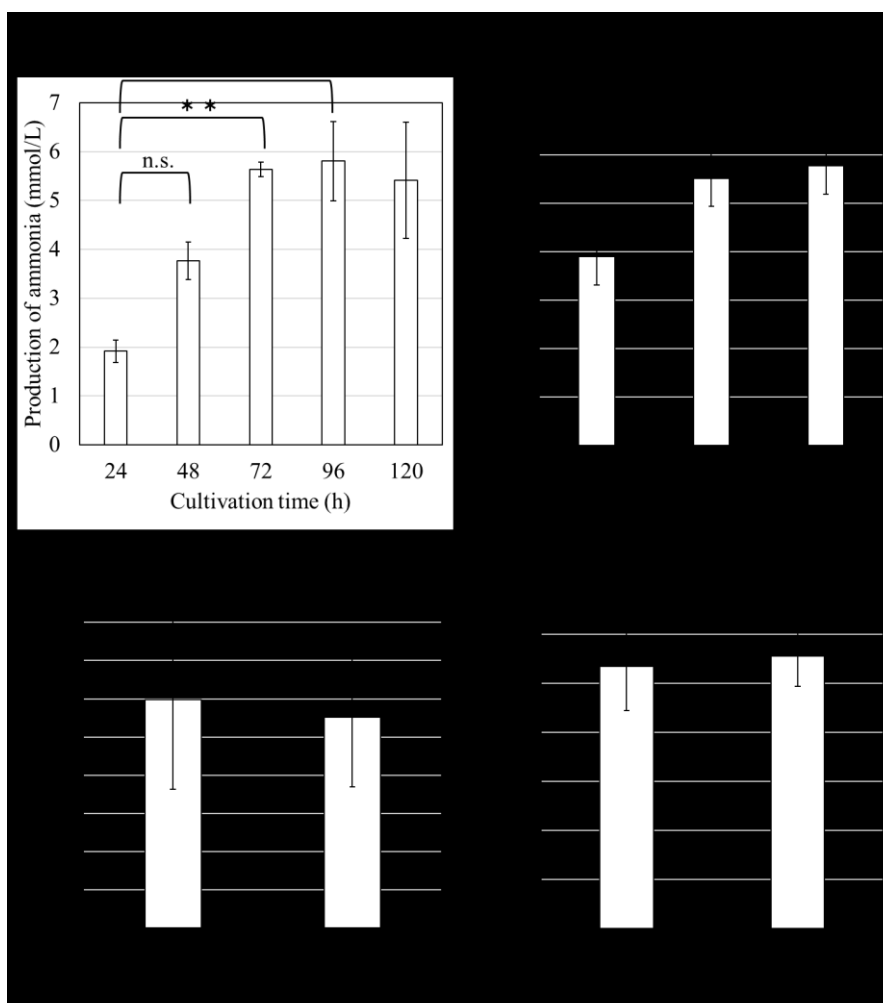
Fig. 3 Ammonia production from 50-mM of L-leucine by the HcLAAO-displaying yeast with various conditions

The amount of ammonia produced by the HcLAAO-displaying yeast (HcLAAO, white bars) with (a) different cultivation time, (b) different acid treatment time, (c) different reaction temperatures and (d) FAD addition. Values are given as mean ± SE (n=3). *$P <$ 0.05, **$P <$ 0.01, n.s.; not significant, a was tested with Dunnett's test comparison between the 24h and all other groups, b was tested with Dunnett's test comparison between the 0 min and all other groups, and c and d were tested with two-tailed paired t-test.

Fig. 4 Hydrogen peroxide (H₂O₂) production from each proteinogenic amino acid by L-amino acid oxidase displaying yeast

The amount of H_2O_2 produced by HcLAAO-displaying yeast (HcLAAO, white bars) and the control yeast (pULD1, black bars). Values are given as mean ± SE (n=3). The production of H_2O_2 was measured after incubation for 24 h at 30°C by absorbance at 530 nm.

Effect of initial cell density on ammonia production by L-amino acid oxidase-displaying yeast

Since HcLAAO-displaying yeast could produce ammonia from each of various amino acids, I attempted to produce ammonia from an amino acid mixture. The ammonia production was performed in the total initial OD600 values adjusted to equivalent to 10 to 30. Fig. 5 showed that the highest amount of ammonia was produced at the OD600 of equivalent to 30 and the conversion efficiency from 20 types of proteinogenic amino acids into ammonia was 63.0%.
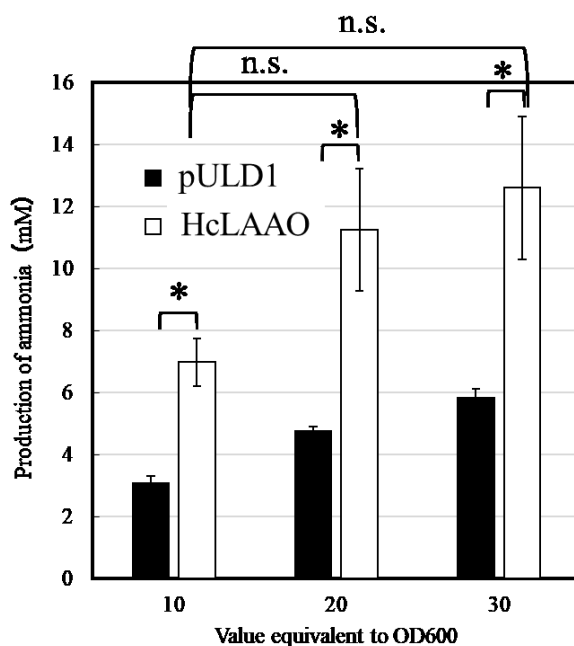
Fig. 5 Ammonia production from the proteinogenic amino acid mixture by the HcLAAO-displaying yeast.

The amounts of ammonia produced by the HcLAAO-displaying yeast (HcLAAO, white bars) and the control yeast (pULD1, black bars) were measured. Values are given as mean $\pm$ SE (n=3). n.s.; not significant, Dunnett's test comparison between the $OD_{600}=10$ and all other groups.

Ammonia production from pretreated soybean residues

Since I succeeded in producing ammonia from the amino acid mixture by HcLAAO-displaying yeast, I employed this yeast to produce ammonia from pretreated soybean residues. Fig. 6 shows that HcLAAO-displaying yeast produced 5.18-mM ammonia from amino acids included in pretreated soybean residues at 120 min, whereas negative control, yeast harboring pULD1-s and glutaminase from *E. coli* (YbaS)-displaying yeast, produced 1.57-mM and 1.37-mM, respectively. The result indicates that HcLAAO-displaying yeast was able to produce ammonia from amino acids included in the pretreated soybean residues as food processing wastes.
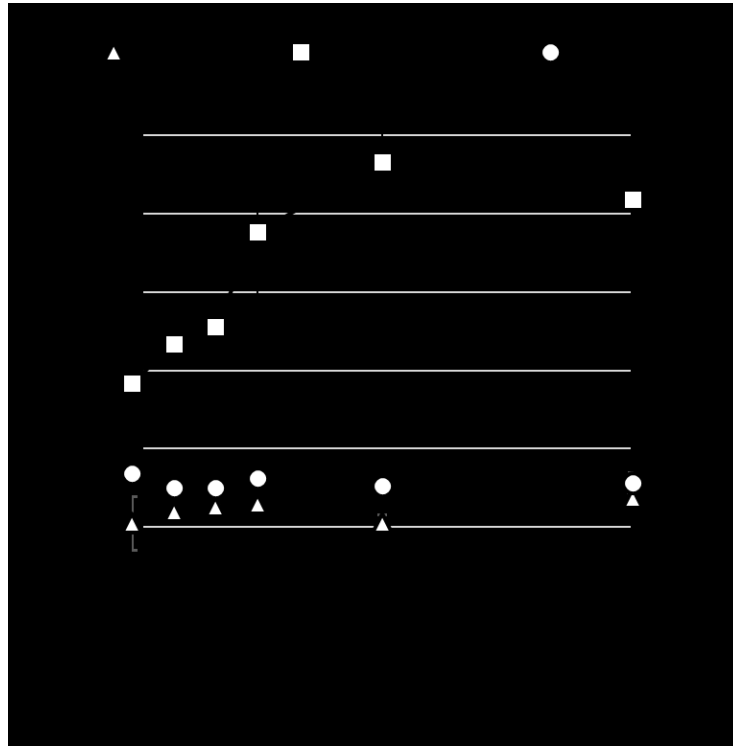
Fig. 6 Ammonia production from pretreated soybean residues by HcLAAO-displaying yeast

The amount of ammonia produced by HcLAAO-displaying yeast (HcLAAO, square), yeast constructed in the previous study as a control (YbaS, triangle), and control yeast (pULD1, circle), were measured. Values are given as mean ± SE (n=3). n.s.; not significant, **$P < 0.01$, two-tailed paired t-test.

Discussion

In this study, I constructed yeast displaying the L-amino acid oxidase with broad substrate specificity, HcLAAO, to produce ammonia from amino acids and pretreated soybean residues. Most of the previous studies on the production of ammonia by bacteria only focused on intracellular production by metabolic engineering (Choi et al. 2014; Huo et al. 2011; Mikami et al. 2017; Vong et al. 2016). For example, the yield of ammonia production by metabolically engineered *B. subtilis* was limited to 46.6% because ammonia seems to be assimilated into the cell components (Choi et al. 2014). In contrast, HcLAAO-displayed yeast produced 12.6-mM of ammonia at high conversion rate (63.0% of conversion rate) (Fig. 5). The result indicates that the conversion rate was more than 15% higher than previous studies using metabolically manipulated microorganisms (Fig. 6) (Choi et al. 2014; Mikami et al. 2017; Chapter I). To further increase the conversion efficiency, the specificity of the displayed enzyme was significant, has further broadened specificity or displaying with other enzymes for production of ammonia from the short side-chain amino acids such as serine, asparagine, and so on might be effective. Therefore, as one of candidates, an enzyme capable of producing ammonia from short-chain amino acids, L-aspartate oxidase etc. may also be effective (Job et al. 2002; Nasu et al. 1982; Nishiya and Imanaka 1998).

A 1.87-fold increase in ammonia production was observed at 72 hours of incubation time (Fig. 3a). This might be due to the increased displayed-enzyme at the increased incubation time because the amount of target protein displayed by pULD1 have already known to increase in proportion to cultivation time (Kuroda et al. 2009). In the present study, there was no difference in ammonia production in HcLAAO-displaying yeast with or without FAD added (Fig. 3d). This is probably because several FAD-containing enzymes are known to bind to intracellular FAD even after releasing from the intracellular environment because the enzymes could bind to FAD with multiple non-covalent bonds (Faust et al. 2007; Hahn et al. 2017; Yang et al. 2005).
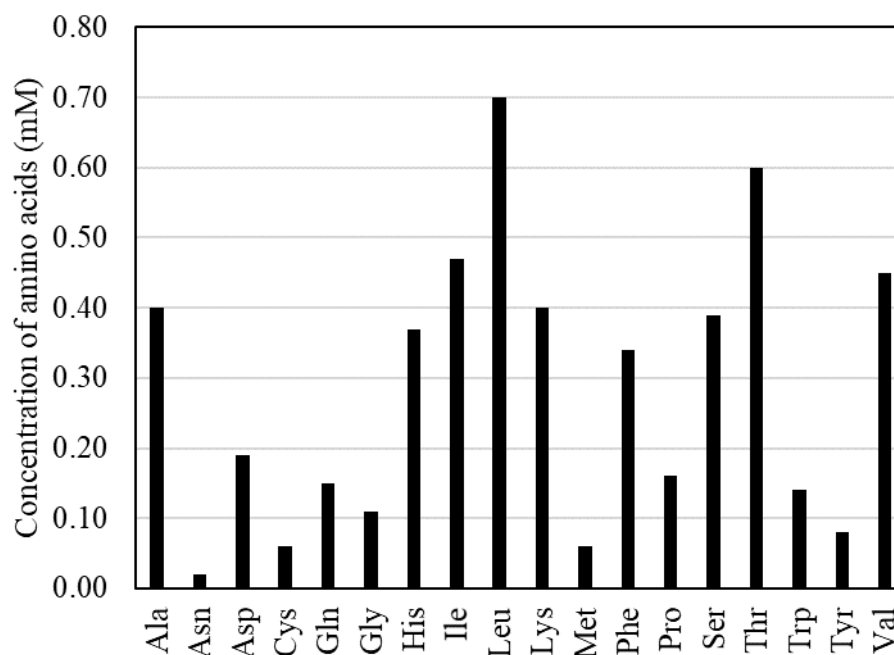
Fig. 7 Amino acids contained in pretreated soybean residues

The amino acids contained in pretreated soybean residue were measured using GC-MS. Values are given as biological singlicate.

HcLAAO-displaying yeast converted amino acids in pretreated soybean residues into ammonia and the conversion efficiency was estimated to be about 88.1% (Fig. 6 and Fig. 7). However, this estimation did not include the data of the concentration of arginine, which is converted to ammonia by HcLAAO-displaying yeast, so there is room for improvement in accuracy. Since arginine is difficult to derivatize by GC-MS due to the inhibition of multiple silylation of the nitrogen atoms, it is possible to improve the quantification further in the future by using another method such as LC-MS/MS or HPLC to quantify only arginine (Chaves Das Neves and Vasconcelos 1987; Dasneves and Vasconcelos 1987).

Summary

Ammonia plays important roles in the agriculture and chemical industry. The extracellular production of ammonia by yeast (*Saccharomyces cerevisiae*) using cell

surface engineering can be an efficient approach because the yeast can avoid growth deficiencies caused by knockout of genes for ammonia assimilation. In this study, we tried to produce ammonia outside the yeast cells by displaying an L-amino acid oxidase with wide substrate specificity derived from *Hebeloma cylindrosporum* (HcLAAO) on the yeast cell surface. HcLAAO-displaying yeast successfully produced ammonia from a mixture of 20 proteinogenic amino acids on the order of 12.6-mM (63.0% conversion rate), achieving a higher yield than that in previous studies. Next, using HcLAAO-displaying yeast, we also succeeded in producing ammonia from a food processing wastes, soybean residues (okara), derived from tofu production with the highest conversion efficiency (88.1%). Our study demonstrated that ammonia production outside cells by displaying L-amino acid oxidase is a promising strategy.

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Chapter III

Improvement of ammonia production by co-culture of amino acid catabolic enzyme-displaying yeast

In living organisms, ammonia is usually produced from amino acids and is used as a source of nitrogen for the biosynthesis of amino acids and nucleic acids (ter Schure et al. 2000). Certain kinds of mushrooms use amino acid oxidases with very broad substrate specificity to produce ammonia from amino acids (Bloess et al. 2019). In chapter I, we showed that extracellular ammonia production from amino acids is effective. Furthermore, in chapter II, it was shown that this oxidase could be presented on the surface of yeast cells to produce ammonia from mixed amino acid solutions with high conversion efficiency. However, to achieve even higher conversion efficiency, it is necessary to synthesise ammonia from amino acids unavailable to HcLAAO. Therefore, it is necessary to utilise amino acid catabolite-enzymes, which could produce ammonia from these unavailable amino acids unavailable to HcLAAO (Favrot et al. 2018; Job et al. 2002; Sarquis et al. 2004; Settembre et al. 2003; Zhao and Liu 208). Amino acid deaminase derived from CHA1 is known to produce ammonia from hydrophilic amino acids, such as L-serine/threonine (Bornaes et al. 1992). Therefore, an attempt to display this enzyme on the yeast cell surface and the co-cultivation of CHA1- and HcLAAO-displaying yeasts was performed; this technique could be used to produce ammonia from amino acids more efficiently.

Materials and methods

Strains and media

Saccharomyces cerevisiae strain BY4741 (*MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0* and *YDR077w::KanMX4*) was used for displaying HcLAAO on the yeast cell surface described in chapter II. Synthetic dextrose (SDC) medium [0.67% (w/v) yeast nitrogen base without amino acids (Sigma-Aldrich, MO, USA), 2% (w/v) glucose, 2% (w/v) casamino acids, 0.002% (w/v) L-histidine, 0.003% (w/v) L-leucine and 0.003%

(w/v) L-methionine] was used for selectively transforming and growing of the yeast host cells.

Construction of plasmids for yeast cell surface for displaying L-amino acid oxidase and L-amino acid deaminase yeast transformation

The constructed plasmids were introduced into *S. cerevisiae* BY4741 by the lithium acetate method with the frozen-EZ yeast transformation kit (Zymo Research, CA, USA). SDC medium [0.67% (w/v) yeast nitrogen base without amino acids (Sigma-Aldrich, MO, USA), 2% (w/v) glucose, 2% (w/v) casamino acids, 0.002% (w/v) L-histidine, 0.003% (w/v) L-leucine and 0.003% (w/v) L-methionine] was used for selectively transforming and growing of the yeast host cells.

Ammonia production from L-serine or L-threonine by L-amino acid deaminase-displaying yeast

Yeast cells were pre-incubated in SDC medium for 24 h at 30°C. The yeast cell cultures were centrifuged at 3,000 × *g* for 5 min at 4°C and washed with phosphate-buffered saline (PBS) (pH 7.4). After washing, the cells harbouring pULD1-CHA1 were suspended in sterilised distilled water, and these were inoculated into 50-mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.0) containing amino acids (50-mM L-serine of L-threonine), and OD_{600} values were adjusted to an equivalent of 10. All reaction mixtures were shaken at 250 rpm (Bio-Shaker M·B-024, Japan) at 30°C and sampled at each time point. Ammonia concentration in the supernatant was measured using the F-kit ammonia (J.K. International, Tokyo, Japan).

Ammonia production from amino acids by L-amino acid deaminase-displaying yeast and L-amino acid oxidase-displaying yeast

Yeast cells were pre-incubated in SDC medium for 24 h at 30°C. The yeast cell cultures were centrifuged at 3,000 × *g* for 5 min at 4°C and washed with PBS (pH 7.4). After washing, the cells harbouring pULD1-HcLAAO were treated with sodium phosphate buffer (pH 3.0) for 10 min and washed with PBS (pH 7.4) again. The cells harbouring pULD1-HcLAAO or pULD1-CHA1 were suspended in sterilised distilled

water, and these were inoculated into 50-mM HEPES buffer (pH 7.0) containing amino acids (1 mM of L-arginine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-lysine, L-serine, L-threonine, L-cysteine, L-histidine, L-methionine, L-alanine, L-valine, glycine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-tryptophan and L-tyrosine) or pre-treated soybean residues, and OD_{600} values were adjusted to an equivalent of 10. All the reaction mixtures were shaken at 250 rpm (Bio-Shaker M·B-024, Japan) at 30°C and sampled at each time point. Ammonia concentration in the supernatant was measured using F-kit ammonia (J.K. International, Tokyo, Japan).

Results

Construction of cell-surface engineered yeast displaying L-amino acid deaminase

To produce ammonia from amino acids outside the cells, CHA1 or HcLAAO (described in chapter II) were displayed on the yeast cell surface. The constructed pULD1-CHA1, pULD1-HcLAAO and control yeasts (pULD1-s) were introduced into the competent yeast cells. Immunofluorescence labelling was performed using mouse monoclonal anti-FLAG M2 antibody to confirm the display of HcLAAO on the yeast cell surface. Green fluorescence by Alexa Fluor 488, which was observed on the yeast cell surface, indicates that the HcLAAO-FLAG tag fusion was successfully displayed. In contrast, fluorescence was not observed in yeast transformed with the negative control plasmid, pULD1-s, for displaying only a strep-tag instead of FLAG-tag (Fig. 1).
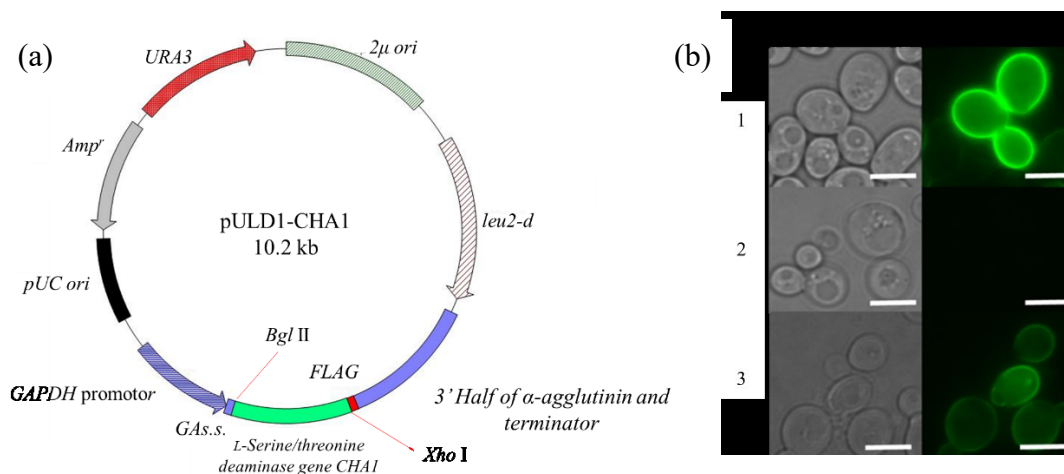
Fig. 1 Yeast cell surface display (a) The constructed plasmid for display of L-serine, threonine deaminase from *Saccharomyces cerevisiae* (CHA1) or L-amino acid oxidase from *Hebeloma cylindrosporum* (HcLAAO) on the yeast cell surfaces and (b) Fluorescence observation of cells using immunofluorescence labelling. An anti-FLAG antibody and Alexa Fluor 488 anti-mouse IgG antibody were used for labelling the FLAG-tag fused to displayed proteins. Phase-contrast micrographs are shown at the left column and fluorescence micrograph (right column). The first row indicates pULD1-CHA1; the second row indicates pULD1-s and the third row indicates pULD1-HcLAAO. The scale bars indicate 5 μm.

Ammonia production from L-serine or L-threonine solution by L-amino acid deaminase-displaying yeast

To evaluate the ammonia production by CHA1-displaying yeast to produce ammonia from L-serine of L-threonine solution, an attempt was made to monitor the ammonia production from L-serine of L-threonine solution after incubation for 24 h. Fig. 2 shows that CHA1-displaying yeast produced ammonia approximately 5.86 mM from L-serine in 24 h with a significant difference, whereas CHA1-displaying yeast did not show a significant difference in ammonia production from L-threonine.
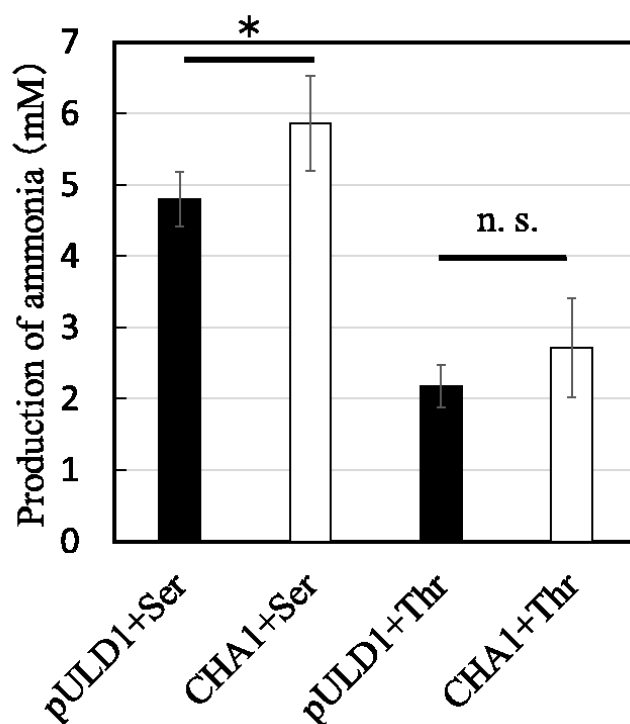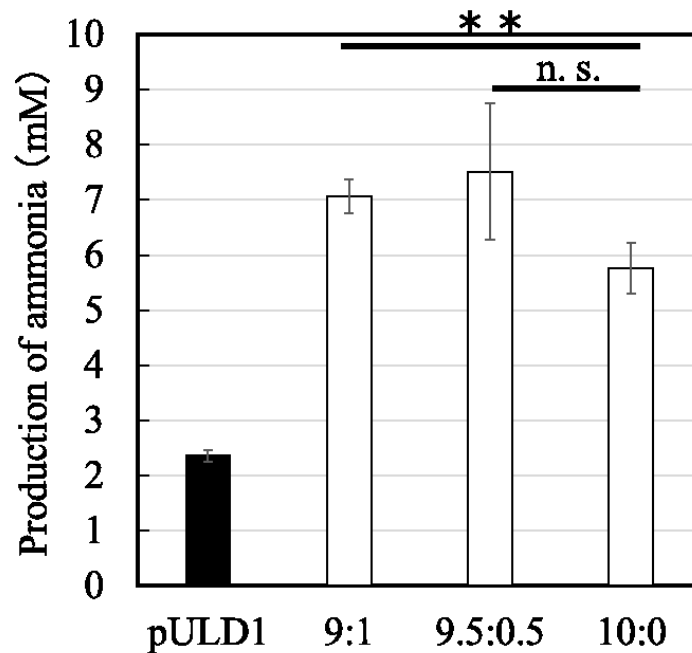
Fig. 2 Ammonia production from L-serine or L-threonine solution by CHA1-displaying yeast

The amount of ammonia produced by CHA1-displaying yeast (CHA1, white bars) and control yeast (pULD1, black bars), were measured. Values are given as mean ± SE (n = 3). n.s.; not significant, $*P < 0.05$, two-tailed paired t-test.

Ammonia production from amino acid mixture solution by the combination of L-amino acid oxidase-displaying yeast and L-amino acid deaminase-displaying yeast

To evaluate ammonia production by the combination of CHA1- and HcLAAO-displaying yeasts to produce ammonia from amino acids mixture solution, an attempt was made to monitor the ammonia production from an amino acid mixture solution after incubation for 24 h. Fig. 3 shows that 7.07 mM of ammonia was produced for 24 h when HcLAAO- and CHA1-displaying yeasts were used at a ratio of 9:1 with statistical significance.

Fig. 3 Ammonia production from amino acid mixture solution by HcLAAO-displaying yeast and CHA1-displaying yeast

The amount of ammonia produced when HcLAAO-displaying yeast, CHA1-displaying yeast (white bars) and control yeast (pULD1, black bar), were measured. Values are given as mean $\pm$ SE (n = 3). n.s.; not significant, $**P < 0.01$, two-tailed paired t-test.

Discussion

Here, yeast displaying CHA1 was constructed to produce ammonia from amino acids more effectively. The result of chapter II showed that HcLAAO-displaying yeast can produce ammonia than bacteria and YbaS-displaying yeast. However, HcLAAO-displaying yeast did not produce ammonia from some amino acids, such as serine, threonine, cysteine and asparagine (Bloess et al, 2019). In this chapter, CHA1-displaying yeast produced ammonia from L-serine with statistical significance but not from L-threonine. This might be because CHA1 requires a pyridoxal 5′-phosphate (PLP)-dependent enzyme, which was not added to the reaction mixture (Zhao and Liu 2008). This chapter showed that ammonia production from amino acids involves HcLAAO- and CHA1-displaying yeasts, there was an improvement in ammonia production by co-culture

of HcLAAO- and CHA1-displaying yeasts from almost all amino acids solution (Fig. 3). To produce more ammonia from amino acids solution, newer enzymes were explored, which produced more ammonia from amino acids, or displayed dimer or tetramer on the yeast cell surface because the oligomer enzymes on the yeast cell surface are known that that these do not work (Furukawa et al. 2006). However, most of the amino acids in food processing wastes, such as soybean residue, exist as proteins entangled by cellulose and require pre-treatment with commercially available enzymes or acid treatment (Li et al, 2013). To reduce the cost by consistent ammonia production from food waste, it would also be useful to efficiently degrade the protein and cellulose using yeast and other enzymes that present proteases instead of commercial enzymes (Bae et al. 2015; Kataoka et al. 2014).

Summary

The co-culture of HcLAAO- and CHA1-displaying yeasts was used to improve the amino acid degradation efficiency. The CHA1-displaying yeast produced ammonia from serine, whereas HcLAAO-displaying yeast did not. Therefore, co-culture of CHA1- and HcLAAO-displaying yeast seemed to be useful for effective ammonia production. Finally, it was revealed that more ammonia was produced when HcLAAO- and CHA1-displaying yeasts were used at a ratio of 9:1 than when these used at a ratio of 10:0.

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Conclusions

The successful industrial scale of ammonia production by the Haber–Bosch method has led to an increase in crop yields, resulting in an increase in population over the last century. This study has been carried out to produce ammonia from amino acid media by bacteria *E. coli* and *S. cerevisiae* toward the development of methods for effective ammonia production from biomass like food processing wastes.

In chapter I, I assess the yeast *S. cerevisiae* to test whether extracellular ammonia production is effective in improving yields. I designed yeast displaying glutaminase YbaS from *E. coli* on the cell surface to produce ammonia from glutamine. YbaS-displaying yeast produced a high yield of ammonia (3.34 g/L) from glutamine with a high conversion efficiency (83.2%). Finally, we showed that YbaS-displaying yeast directly produced ammonia from the food processing wastes, soybean residues. Ammonia production outside cells via cell surface engineering could produce ammonia from food processing wastes effectively.

In chapter II, I tested whether ammonia could be produced from amino acids in proteinogenic amino acid-containing solution and okara residues using yeast with L-amino acid oxidase from *Hebeloma cylindrosporum* (HcLAAO) on the cell surface. Ammonia production from 20 proteinogenic amino acid mixture solution HcLAAO-displaying yeast resulted in a high conversion efficiency (63.0% of the theoretical yield). Finally, we showed that HcLAAO-displaying yeast efficiently produced ammonia from amino acids in soybean residues as food processing wastes and the conversion efficiency from amino acids into ammonia was higher than the previous studies (88.1%).

In chapter III, to improve the ammonia production from amino acids, I displayed L-serine/threonine deaminase derived from *Saccharomyces cerevisiae* on the yeast cell surface to produce ammonia assimilation genes. The co-culture of the resulting strain and HcLAAO-displaying yeast were cultured in amino acid mixture solution and produced higher amount of ammonia from various amino acids than if only HcLAAO-displaying yeast had been used.

In conclusion, I have successfully produced ammonia from a mixture of various amino acids and pretreated soybean residue directly with an engineered yeast strain. This

system could contribute to solving the global problem of ammonia shortages and develop a sustainable society.

Acknowledgements

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Chapter II

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In preparation

Other publications

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