

**Development of a high-performance heterologous protein expression
system in the methylotrophic yeast *Ogataea minuta* and its applications
in biopharmaceutical protein production**

Masashi Tsuda

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Contents	
General Introduction	1
 Chapter I	
Development of a high-performance heterologous protein expression system in the methylotrophic yeast <i>Ogataea minuta</i>	
 Section I-1	4
Selection of the methylotrophic yeast <i>Ogataea minuta</i> as a high-producing host for heterologous protein expression	
 Section I-2	24
Development of a protein production system using <i>Ogataea minuta</i> alcohol oxidase-deficient strain under reduced-methanol-consumption conditions	
 Section I-3	42
Optimization of heterologous protein production process using genetically engineered <i>Ogataea minuta</i> toward industrial-scale manufacturing	
 Chapter II	
Applications of developed <i>Ogataea minuta</i>-based heterologous protein expression system in biopharmaceutical protein production	
 Section II-1	65
Production of Kex2 proteinase in a genetically modified <i>Ogataea minuta</i> system	
 Section II-2	72
Production and characterization of Norovirus VLPs vaccine candidates in a genetically modified <i>Ogataea minuta</i> system	
 Conclusion	90
 References	92
 Acknowledgements	102
 Publications	103

General Introduction

The global demand for recombinant proteins, including biopharmaceuticals and industrial enzymes, has increased significantly in recent years. More than 200 biopharmaceuticals have been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), with annual sales exceeding US\$ 343 billion in 2021 (1). Additionally, the industrial enzyme market is valued at around US\$ 6 billion, while the entire recombinant protein market is valued at over US\$ 125 billion in 2021 (2). To satisfy the growing demands for recombinant proteins, various heterologous protein production systems have been established using representative host organisms including mammalian cells such as Chinese hamster ovary (CHO) cells, insect cells, yeast, and *Escherichia coli*. Among these systems, methylotrophic yeast has been proposed as a particularly promising host for the production of diverse heterologous proteins (3).

Since the isolation of methylotrophic yeasts by Ogata et al. in 1969, various species – including *Pichia*, *Komagataella*, *Candida*, and *Ogataea* – have been developed as industrial expression platforms (4-8). Methylotrophic yeast systems are preferable for commercial production from the perspective of biological safety, given that they have been allocated generally recognized as safe (GRAS) status by the FDA. These systems also have several other advantages: the ability to secrete proteins into the culture broth; a eukaryotic protein-quality-control system; a post-translational modification system; and rapid growth (9). Notably, these systems utilize methanol-inducible promoters such as alcohol oxidase 1 (*AOX1*) promoter, which is strongly induced by methanol and also repressed by glucose and glycerol, making it particularly effective for controlled recombinant protein expression (10, 11). The methanol utilization (MUT) pathway, which has been well refined and integrated to assimilate and dissimilate methanol, is widely conserved in methylotrophic yeast species. In the MUT pathway, alcohol oxidase 1 (AOX1) plays an important role as the first-step enzyme in the oxidation of methanol to formaldehyde (12).

Although methanol-inducible promoters are powerful tools for protein production systems, methanol and related metabolites including formaldehyde and formic acid are known to be toxic, causing acidosis and other clinical symptoms (13). Accordingly, it would be necessary to construct a safety-compliant facility capable of handling large volumes of methanol under explosion-proof and fire-resistant conditions to ensure worker safety, which would entail significant costs. Therefore, reducing the methanol consumption rate is considered a promising strategy for improving methanol-inducible methylotrophic yeast systems, including that of *Ogataea minuta*.

Previous studies have shown that protease-deficient strains of *O. minuta* can be used for glyco-engineered monoclonal antibody (mAb) production (14). However, comparative evaluation of available strains had not been systematically performed. I evaluated three *O. minuta* strains—NBRC 0975, NBRC 10402, and NBRC 10746—for their suitability as heterologous expression hosts. Further I isolated a high-performance host strain from NBRC 10746 suitable for industrial applications in recombinant protein manufacturing (Chapter I Section I-1).

To establish a heterologous protein production system with lower methanol consumption, I disrupted the *AOXI* gene, which encodes alcohol oxidase: a key enzyme in the methanol assimilation pathway and generated a mutant strain which is not able to utilize methanol (Δ aox strain). The expression activity of the *AOXI* promoter was analyzed under induced conditions with lower methanol consumption in the Δ aox strain. The protein production capacity between the wild-type and Δ aox strain was compared to demonstrate that the Δ aox strain exhibited a comparable protein production level to that of the wild-type strain (Chapter I Section I-2).

To improve the productivity of the heterologous protein production system using the Δ aox strain under lower methanol consumption, culture conditions were optimized. The optimized cultivation process was subsequently applied to the manufacturing of a therapeutic protein candidate. Industrial-scale cultivation in a 4,500-L bioreactor was performed and successfully yielded approximately 2 kg of the target therapeutic protein candidate (Chapter I Section I-3).

The optimized production system was applied to the manufacturing of the industrially relevant serine protease Kex2, which is widely utilized for the removal of affinity tags and the maturation of precursor proteins. Production of Kex2 protease using this system resulted in an approximately 1.6-fold increase in enzyme yield compared to that achieved with the conventional expression system (Chapter II Section II-1).

Norovirus is a major causative agent of acute gastroenteritis, and the development of effective treatments and vaccines is urgently needed, due to the risk of severe symptoms in infants and the elderly. Among potential strategies, the use of virus-like particles (VLPs) as a vaccine candidate is considered particularly promising. The optimized production system was also applied to the manufacturing of the VP1 protein, which constitutes norovirus VLPs. The purified VP1 protein was analyzed by a transmission electron microscopy (TEM), and the characteristic VLP morphology was clearly observed. Furthermore, binding assays were conducted using *in vitro* ELISA to assess the interaction of the VLPs with anti-VLP antibodies and target proteins. The results confirmed that the produced VLPs possess their antigen-binding capacity. In addition, an *in vivo* assay was performed in mice to evaluate the vaccine potential of the produced VLPs. The results demonstrated that the

produced VLPs successfully elicited an antibody response, indicating their potential as a norovirus vaccine candidate (Chapter II Section II-2).

Chapter I

Development of a high-performance heterologous protein expression system in the methylotrophic yeast *Ogataea minuta*

Section I-1

Selection of the methylotrophic yeast *Ogataea minuta* as a high-producing host for heterologous protein expression

Introduction

Three strains were historically deposited as *O. minuta* var. *minuta*, which have been deposited at the National Institute of Technology and Evaluation (NITE) as the Biological Resource Center, NITE (NBRC) collection (<https://www.nite.go.jp/en/index.html>). One of these strains is NBRC 0975, derived from NRRL Y-441 in the Agricultural Research Service (ARS) Culture Collection at the Northern Regional Research Laboratory (NRRL), which was isolated from fermented mushrooms (15). Another of these strains is NBRC 10402, derived from ATCC 26176 in the American Type Culture Collection (ATCC), which was isolated from a rotten azalea flower by Oki et al. (16). The third strain, NBRC 10746, is derived from DSM 70718 in the German Collection of Microorganisms and Cell Cultures (DSMZ), which was isolated from forest soil by Henninger and Windisch (17).

Kuroda et al. reported that protease-deficient strains of *O. minuta* NBRC 10746, a methylotrophic yeast, were effective for monoclonal antibody (mAb) production (18). They also reported that the suppression of yeast-specific *O*-mannosylation caused increased mAb production and enhanced antigen-binding activity (14). Furthermore, Suzuki et al. have reported that mAb production was 16-fold higher in engineered *O. minuta* NBRC 10746 with reinforced chaperones than in the parental strain (19).

I am interested in their ability to produce heterologous proteins and also their characteristics such as the D1/D2 domain of large subunit (LSU) rRNA, electrophoretic karyotype, and *AOX1* loci as analyzed by pulsed-field gel electrophoresis (PFGE) with Southern blotting. In this chapter, I report the abilities

of the above three NBRC strains as hosts for heterologous protein expression and the establishment of a host strain, that is particularly effective for heterologous protein expression, isolated from NBRC 10746.

Materials and Methods

Yeast strains and media

The *O. minuta* strains were obtained from the NBRC collection (Tokyo, Japan). Synthetic dextrose agar (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% glucose, 0.002% L-tryptophan) was used for regeneration of the spheroplasts after transformation. Yeast extract peptone dextrose (YPD) agar (1% Difco™ yeast extract, 2% Bacto™ peptone, 2% glucose, 0.004% adenine) was used for the general cultivation, and 2xYP-P6-GG medium (2% Difco™ yeast extract, 4% Bacto™ peptone, 0.5% glucose, 2% glycerol, 0.1 M potassium phosphate buffer, pH 6.0) was used for the expression of CLuc (20, 21). Representative clones were stored as suspensions in 17% glycerol at –80 °C. 5-Fluoroorotic acid (5-FOA) agar (1% Difco™ yeast extract, 2% Bacto™ peptone, 2% glucose, 0.004% adenine, 2% Bacto™ agar, 0.1% 5-FOA) was used for the selection of *URA3* pop-out clones.

Chemicals and general recombinant DNA techniques

E. coli JM109, restriction enzymes, and DNA modifying enzymes were purchased from Takara Bio Inc. (Kusatsu, Japan). The media, growth conditions, and general recombinant DNA techniques used in this study were in accordance with the work of Sambrook et al. (22). Zeocin™ was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All oligonucleotides in this study were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan). Polymerase chain reaction (PCR) amplification was performed using the GeneAmp® PCR System 9700 (Thermo Fisher Scientific). Automated DNA sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (Thermo Fisher Scientific). Transformation of *O. minuta* was performed as previously described (19).

PFGE and Southern blotting

PFGE was conducted in accordance with the procedure described by Schwartz et al., with slight modifications (23, 24). A CHEF Genomic DNA Plug Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with lyticase was used to prepare the plugs for PFGE. The chromosomal DNA in the plugs was separated in 1% Pulsed Field Certified Agarose™ gel (Bio-Rad). PFGE was run for 24 h under the following conditions: 6 V/cm, 90 to 120 s switching time, and 120°, with the CHEF Mapper® system (Bio-Rad). After electrophoresis, the gel was stained with ethidium bromide and sequentially treated with 100 mL of 0.25 M HCl (2 times, 10 min), 100 mL of 0.5 M NaOH/1.5 M NaCl (2 times, 15 min), and 100 mL of 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl (2 times, 15 min) (25). The separated chromosomal DNA was then transferred to the nylon membrane and positively charged (Roche

Applied Science, Mannheim, Germany) by 0.4 M NaOH overnight. The membrane was rinsed three times in 2× SSC [0.03 M sodium citrate, 0.3 M NaCl (pH 7.0)] and heated for 20 min in an oven at 120 °C. The probe to detect the *AOXI* gene was prepared in accordance with the standard protocol of the PCR DIG Probe Synthesis Kit™ (Roche) provided by the supplier. The part of the *AOXI* coding region of NBRC 10746 genomic DNA that was PCR-amplified by primers 1 and 2 was labeled with DIG-dUTP. All primers used are listed in Table 1-1. The heated membrane was hybridized with the labeled probe and treated with the DIG Wash and Block Buffer Set™ (Roche). The resulting membrane was detected by Anti-Digoxigenin-AP, Fab fragments™ (Roche). Detection was performed using CDP-Star, Ready-to-Use™ (Roche).

Internal transcribed spacer (ITS) sequencing

The D1/D2 domains of the LSU rRNA sequences of the three *Ogataea* strains were amplified and sequenced as described by Kurtzman and Robnett (26, 27). The DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) accession numbers for the LSU rRNA sequences of NBRC 0975, NBRC 10402, and NBRC 10746 are LC580394, LC580395, and LC580396, respectively. The D1/D2 domains of the LSU rRNA sequences of the strains belonging to the genus *Ogataea* and related species were obtained from the DDBJ database (<http://www.ddbj.nig.ac.jp/>), as per the work of Kurtzman and Robnett (27). All sequences were aligned, and a phylogenetic tree was created by neighbor joining (28) with the two-parameter distance correction of Kimura (29) using MEGA ver. 5.0 (30). Bootstrap support (31) for the tree was determined from 1,000 replications.

Cloning of deduced *AOXI* regions in the *Ogataea* strains and construction of expression plasmids

The primer pairs were designed from an alignment of deduced *AOXI* regions, which were analyzed using Genetyx®-SV/RC ver. 11 (Genetyx, Tokyo, Japan). The primer pairs were obtained from DDBJ/GenBank/EMBL. Primers 3 and 4 were used to amplify the deduced *AOXI* coding region in NBRC 0975. The amplified 0.5-kb DNA fragment was sequenced using primers 3 and 4. The DNA fragment containing the deduced *AOXI* gene and its promoter region was cloned from NBRC 0975 by inverse PCR. Genomic DNA from NBRC 0975 was digested by HindIII and purified using the QIAquick® Nucleotide Removal Kit (Qiagen, Hilden, Germany). Purified DNA was ligated using the Mighty Mix DNA Ligation Kit (Takara Bio), precipitated by ethanol, and resuspended in Tris-EDTA (TE) buffer as a PCR template. PCR was conducted using PrimeSTAR® Max DNA Polymerase (Takara Bio), in accordance with the standard protocol provided by the supplier. The fragment amplified using

primers 5 and 6 as designed based on the above partially sequenced *AOXI* region was cloned using the Zero Blunt[®] TOPO[®] PCR Cloning Kit (Thermo Fisher Scientific) and sequenced to design primers 7 and 8 for the second amplification. The resulting fragment was amplified and sequenced using primers 7 and 8. The same process was repeated with EcoRI and SacI until the DNA fragment containing the deduced *AOXI* coding region and its promoter region reached the neighboring open reading frame (ORF). The deduced *AOXI* promoter region (1-kb) was cloned using primers 5 and 6, as designed based on the above sequenced region in NBRC 0975. Conversely, the deduced *AOXI* genes were amplified and sequenced using primers 9 and 10 from NBRC 10402 and NBRC 10746 genomic DNA. In a similar manner, the deduced *AOXI* promoter regions were amplified and sequenced using primers 11 and 12 from NBRC 10402 and NBRC 10746 genomic DNA. Next, HindIII and XbaI restriction enzyme sites used for vector construction were attached to each promoter region at the 5' and 3' ends by PCR. Each deduced 1-kb *AOXI* promoter region was amplified using primers 13 and 14 for NBRC 0975, primers 12 and 15 for NBRC 10402, and primers 16 and 17 for NBRC 10746. The two deduced *AOXI* sequences cloned from NBRC 0975 and NBRC 10402 have been deposited in DDBJ/GenBank/EMBL under accession numbers LC582401 and LC582402, respectively. The plasmid pOMEA-Z1-10746 was constructed as follows: The *AOXI* promoter region (submitted to DDBJ/GenBank/EMBL under accession number AB242209), with HindIII and XbaI restriction enzyme sites attached at the 5' and 3' ends, was amplified from NBRC 10746 genomic DNA using primers 16 and 18. Then, the *AOXI* terminator region, with BamHI and KpnI restriction enzyme sites attached at the 5' and 3' ends, was amplified from NBRC 10746 genomic DNA using primers 19 and 20. Next, the fused *AOXI* promoter-terminator fragment containing the XbaI and BamHI restriction enzyme cloning sites was amplified from a mixture of the above amplified fragments by overlapping PCR with primers 16 and 20. Finally, the *AOXI* promoter-terminator cassette, double-digested with HindIII and KpnI restriction enzymes, was inserted into HindIII-KpnI-cleaved pOMGZ (19) to generate pOMEA-Z1-10746. The plasmid pOMEA-Z1-0975, in which the deduced *AOXI* promoter region was derived from NBRC 0975, was constructed as follows: The deduced *AOXI* promoter region was amplified by PCR from NBRC 0975 genomic DNA with primers 13 and 14, which were designed based on the sequence of the region cloned from NBRC 0975. Then, the resulting fragment harboring HindIII and XbaI restriction enzyme sites at the 5' and 3' ends was inserted into pOMEA-Z1-10746 digested with HindIII and XbaI to generate pOMEA-Z1-0975. The plasmid pOMEA-Z1-10402, in which the deduced *AOXI* promoter was derived from NBRC 10402, was constructed as follows: The deduced *AOXI* promoter region was amplified by PCR from NBRC 10402 genomic DNA using primers 12 and 15, which were designed based on the sequence of the cloned region from NBRC 10402.

Then, the resulting fragment harboring HindIII and XbaI restriction enzyme sites at the 5' and 3' ends was inserted into pOMEA-Z1-10746 digested with HindIII and XbaI to generate pOMEA-Z1-10402. Moreover, the plasmid pOMEU1 was constructed as follows: The *AOX1* promoter-terminator cassette from pOMEA-Z1-10746 was digested by HindIII and KpnI and introduced into pOMEGPU1 digested with HindIII and KpnI to generate pOMEU1 (14).

***URA3* disruption in NBRC 10746**

The *O. minuta* *URA3* promoter region (submitted to DDBJ/GenBank/EMBL under accession number AB242207) was amplified from NBRC 10746 genomic DNA using primers 21 and 22. The *URA3* terminator region was amplified from NBRC 10746 genomic DNA using primers 23 and 24. The fused *URA3* promoter-terminator fragment was amplified by overlapping PCR from a mixture of the above amplified fragments using primers 22 and 23. The *URA3* promoter-terminator cassette was introduced to generate *URA3*-disrupted strains. The transformed cells were regenerated on 5-FOA plate medium at 30 °C for approximately 3 days (32). The transformants were transferred on the same selection medium to confirm resistance. *URA3* disruption was subsequently confirmed by PCR using primers 22 and 23.

CLuc expression

Codon-optimized DNA sequences encoding CLuc (luciferase secreted from the ostracod *C. noctiluca*) (20, 21) fused with the α -mating factor secretion signal (α F signal) sequence were synthesized with XbaI and BamHI at the 5' and 3' ends, respectively, by GeneArt™ (Regensburg, Germany). The synthesized fragments were introduced into pOMEA-Z1-0975, pOMEA-Z1-10402, and pOMEA-Z1-10746 at the XbaI and BamHI sites, resulting in pOMEA-Z1-0975/CLuc, pOMEA-Z1-10402/CLuc, and pOMEA-Z1-10746/CLuc, respectively. In the same way, DNA encoding CLuc fused with the α F signal sequence was introduced into pOMEU1 at the XbaI and BamHI sites, resulting in pOMEU1/CLuc. CLuc production was carried out in accordance with the method of Suzuki et al. (19), with slight modifications. For the selection of CLuc-producing cells, transformants were cultivated in the individual wells of a 96-well deep-well plate at 28 °C for 4 days with reciprocal shaking at 310 rpm. To evaluate the productivity at a larger fermentation scale, selected clones were cultivated in a test tube at 28 °C for 4 days with reciprocal shaking at 250 rpm. After removal of the cells by centrifugation (2,200 × g), CLuc in culture broth was quantified using a CLuc reporter assay kit (Atto, Tokyo, Japan).

***URA3* excision from the transformants containing pOMEU1/CLuc by homologous recombination**

O. minuta transformants containing the functional *URA3* gene were cultivated in YPD medium in a test tube at 30 °C for approximately 2 days with reciprocal shaking at 310 rpm. Then, 0.1 mL of cultured broth was transferred onto 5-FOA plate medium and incubated at 30 °C for 4 days. The colonies growing on the 5-FOA plate medium were transferred onto the same plate to confirm 5-FOA resistance. *ura3* null mutants were confirmed by PCR amplification with primers 22 and 23.

Table 1-1 Primers used in this study.

Primer #	Nucleotide Sequence (5' to 3')
1	CTTATATCCACCCAACCATGAGAAACAA
2	GAAACCAGAAATAACAGAGTAGTGCAT
3	GTACTGGCTCAAGTGGATCAACAGG
4	GAAACCGGAAATGACAGAGTAATGCA
5	TGACAACCTTCTCGCACTTGGAGGACGTGAG
6	TCAGACCAACCGAGGCGGAGCTTGCTACTG
7	AGCGGGTTGTCATTTTGTGGTGC
8	CTTGTATCTGTATCGATGCAATGCCAAG
9	TCTAGAATGGCTATTCCTGACGA
10	GGATCCTTAGAATCTAGCCAGACCCT
11	TCATGGAAGATTTCCTGC
12	TCTAGATTTTTTCGTAAGTCGTA
13	AAGCTTTCTCTAATGGCAATCTG
14	TCTAGATTCAATTTGGTCTTAA
15	AAGCTTTCATGGAAGATTTCCTGC
16	ATCAAGCTTTCATGGAAGATTTCCTGC
17	ATCTCTAGATTTTTTCGTAAGTCGTA
18	GAACCCGGGAACAGAATCTAGATTTTTTCGTAAGTCGTAAG
19	CTGTTCCCGGGTTCCTGGATCCGAGACGGTGCCCGACTC
20	GCGGTACCGTTAGTGGTACGGGCAG
21	AGTCACGTGACCCGAAAAGTCGCCTGTTTATCTG
22	CCAAGGAGGAAGAAATT
23	GGTACCAGTACTGGAAA
24	CAGATAAACAGGCGACTTTTCGGGTCACGTGACT

Results

Comparison of the three strains as hosts for heterologous protein expression

To evaluate the three strains as hosts for heterologous protein expression, CLuc was used as a reporter protein. Yeast CLuc reporter assay is a promising tool for high-throughput analysis using secretory luciferase. First, pOMEA-Z1-10746/CLuc was introduced into NBRC 0975, NBRC 10402, and NBRC 10746. Thirty-two clones were picked up after transformation and cultivated in a 96-well deep-well plate under the conditions described in the Materials and Methods section. The secreted CLuc was measured based on its activity in culture broth. No CLuc secretion was observed in NBRC 0975 and NBRC 10402. In contrast, almost all transformants from NBRC 10746 secreted CLuc into culture broth (Fig. 1-1A). To examine the possibility that the *AOX1* promoter of NBRC 10746 did not work in NBRC 0975 and NBRC 10402, I then expressed CLuc in NBRC 0975, NBRC 10402, and NBRC 10746 under the control of their own *AOX1* promoters (Fig. 1-1B). As a result, all three of the strains secreted CLuc. This suggested that the transcription in each strain was controlled by a different regulatory system under an independent *AOX1* promoter. Transformants from NBRC 10746 secreted larger amounts of CLuc than those from NBRC 0975 and NBRC 10402. Meanwhile, transformants from NBRC 10402 showed higher biomass than those from NBRC 0975 and NBRC 10746.

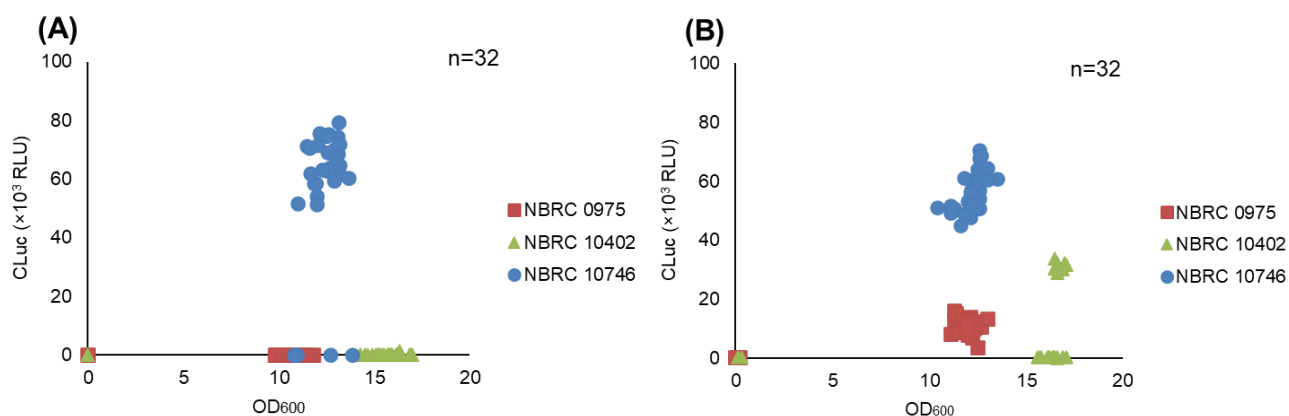


Fig. 1-1 Correlation between the CLuc expression level regulated by the *AOX1* promoter and biomass of the 3 strains. Transformed cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days in 96-well deep-well plate. Culture supernatants were subjected to CLuc assay. (A) Comparison using the *AOX1* promoter derived from NBRC 10746. (B) Comparison using the *AOX1* promoter derived from each 3 strains.

Single-colony isolation of NBRC 10746 for heterologous protein expression

Since NBRC 10746 was selected from the tested strains as a high-producing strain, as shown in Fig. 1, a further single-colony isolation was continued to establish stable host cells with high potential for heterologous protein expression. The six clones of the *ura3* disruptants, which were isolated under the conditions described in the Materials and Methods section, were electro-transformed by pOMEU1/CLuc for CLuc expression. Each of the 32 clones from the six above clones was picked up from individual growing colonies and cultivated in a 96-well deep-well plate. The activity of secreted CLuc in culture broth was measured (Fig. 1-2).

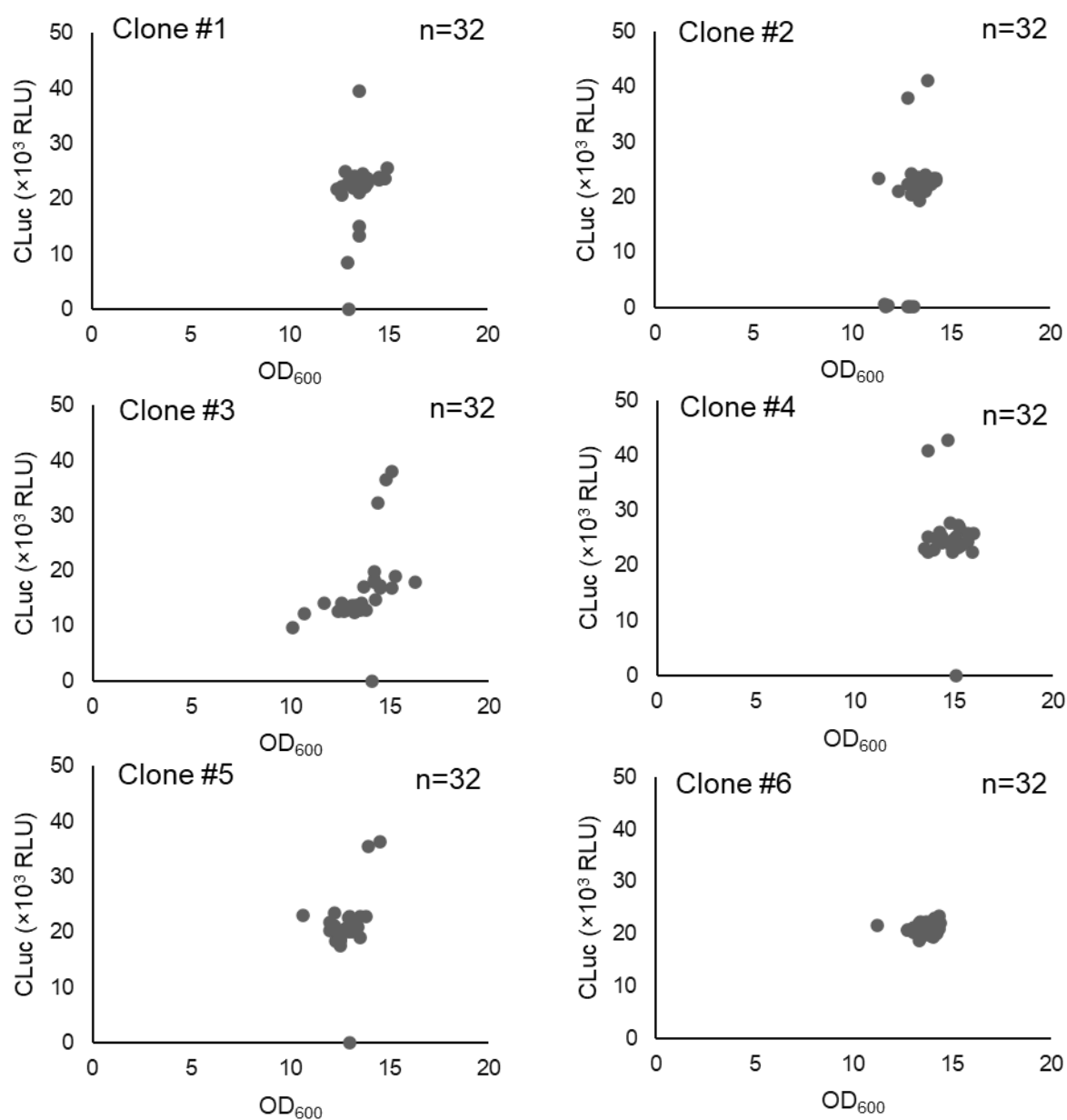


Fig. 1-2 Correlation between the CLuc expression level and biomass of the 6 clones that were selected as *ura3* disruptants derived from NBRC 10746. Transformed cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days in 96-well deep-well plate. Culture supernatants were subjected to CLuc assay.

The top five clones of the transformants derived from each of the six *ura3* disruptants were recultivated in a test tube, and I selected the greatest producer among the tested clones (Fig. 1-3A). The *URA3* gene of the resulting clone was removed (popped-out) by selection for general 5-FOA resistance. As a result, the *URA3* popped-out clone #5-30, named tat06213, was selected as a preferable host for exogenous protein expression. To confirm the protein expression performance of tat06213, the CLuc expression plasmid pOMEU1/CLuc was repeatedly introduced into the *ura3* disruptant #5, a parent of tat06213. Each of the 32 transformants from clones #5 and tat06213 was picked up and cultured in a 96-well deep-well plate. The activity of secreted CLuc in culture broth was measured. The 32 transformants obtained from tat06213 showed 2.1-fold higher CLuc activity than those from clone #5 (Fig. 1-3B). This strongly suggested that tat06213 established by single-colony isolation had higher potential for heterologous protein expression than NBRC 10746.

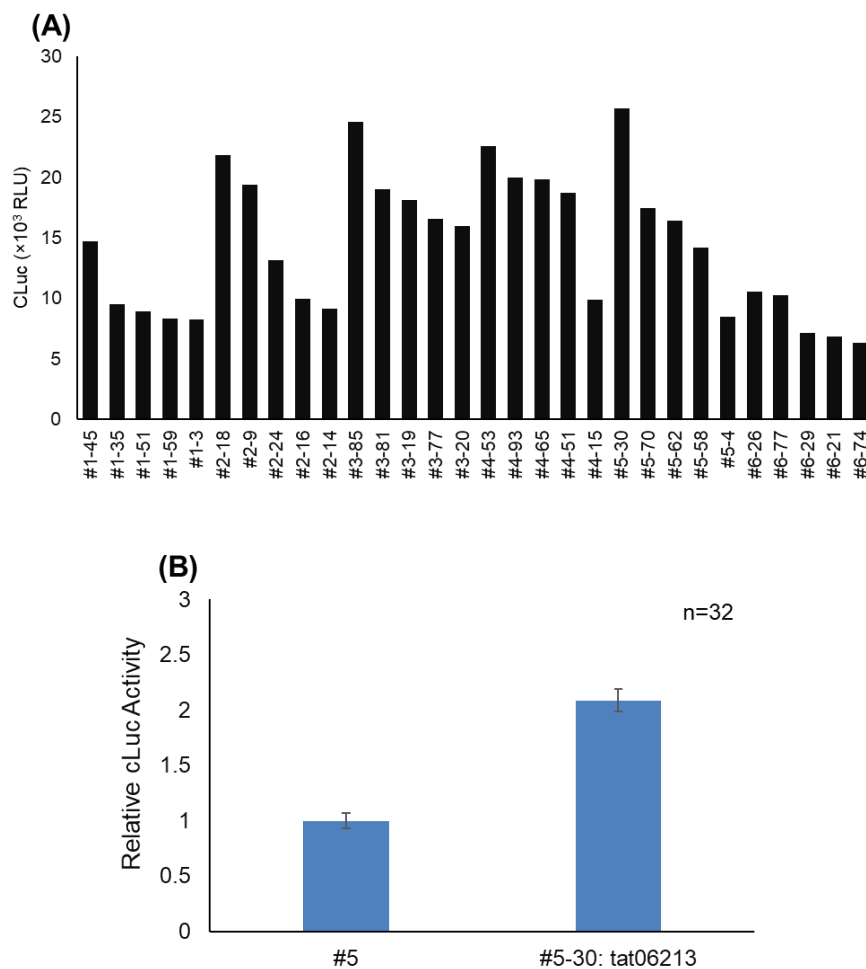


Fig. 1-3 (A) Quantified CLuc expression levels of the top 5 clones selected from each of the 6 *ura3* disruptants. (B) Comparison of the CLuc expression levels between #5 and #5-30 (tat06213). Transformed cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days in tube. Culture supernatants were subjected to CLuc assay.

Taxonomic characterization of the *O. minuta* strains

As the three strains that were historically deposited as *O. minuta* var. *minuta* in the NBRC collection showed quite different levels of heterologous protein production, I compared these three strains by taxonomic analysis. The sequence of the LSU D1/D2 domain of rRNA from each of the three strains was analyzed. The sequence of NBRC 10402 showed 99.7% identity to that of NBRC 10746, whereas the sequence of NBRC 0975 showed 96.7% identity to that of NBRC 10402 or NBRC 10746 (Table 1-2). Based on the phylogenetic tree drawn by aligning the nucleotide sequences of the LSU D1/D2 domain in various species belonging to the *Ogataea* clade, NBRC 10402 and NBRC 10746 were categorized into a different cluster from NBRC 0975, a type strain of *O. minuta* var. *minuta* (Fig. 1-4).

Table 1-2 Identity of the large subunit D1/D2 domain in each of the 3 strains.

	NBRC 0975	NBRC 10402	NBRC 10746
NBRC 0975	—	96.7	96.7
NBRC 10402		—	99.7
NBRC 10746			—
(% Identity)			

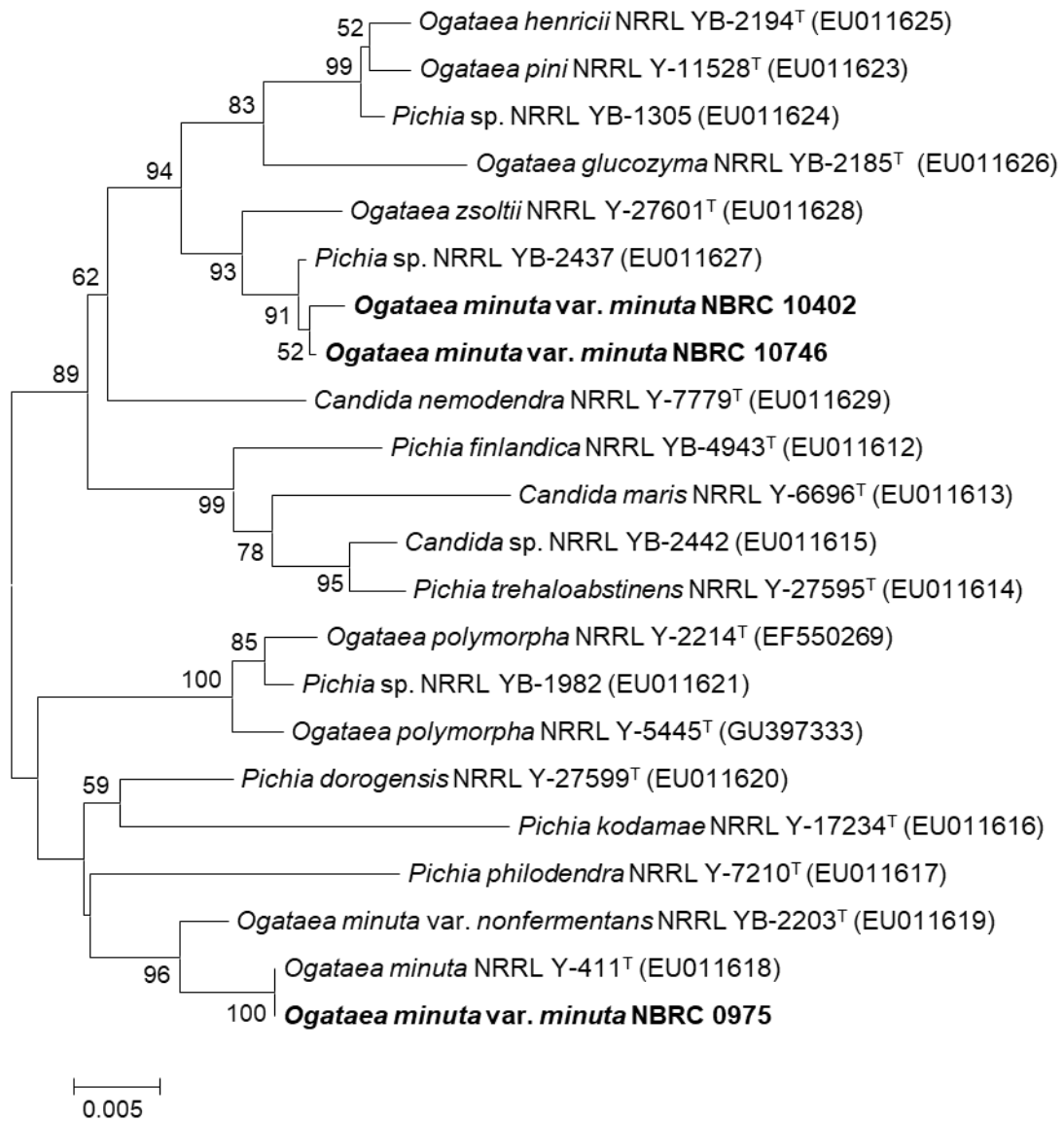


Fig. 1-4 Phylogenetic tree created by neighbor joining based on the sequence of the D1/D2 domain of large subunit rRNA.

Comparison of sequences of *AOXI* promoter and coding regions among the *Ogataea* strains

Since it was revealed that NBRC 10402 and NBRC 10746 belonged to a different cluster from NBRC 0975, as shown in Fig. 4, I compared the sequences of the deduced *AOXI* coding and promoter regions that were cloned from each strain, as described in the Materials and Methods section.

The nucleotide sequence of the deduced *AOXI* coding region of NBRC 0975 showed 80.2% and 78.4% identity to those of NBRC 10402 and NBRC 10746, respectively. Meanwhile, the nucleotide sequence of the deduced *AOXI* coding region of NBRC 10402 showed 89.6% identity to that of NBRC 10746. This indicated that the nucleotide sequences of the deduced *AOXI* coding regions differed among the three strains (Table 1-3, upper). The amino acid sequence of the deduced *AOXI* coding region of NBRC 0975 showed 83.5% identity and 94.6% similarity to that of NBRC 10402 and 85.3% identity and 96.0% similarity to that of NBRC 10746. Meanwhile, the amino acid sequence of the deduced *AOXI* coding region of NBRC 10402 showed 92.5% identity and 97.8% similarity to that of NBRC 10746 (Table 1-3, lower).

Table 1-3 Comparison of the nucleotide (upper) and deduced amino acid (lower) sequences of the *AOXI*-encoding gene cloned from each of the 3 strains.

	NBRC 0975	NBRC 10402	NBRC 10746
NBRC 0975	—	80.2	78.4
NBRC 10402		—	89.6
NBRC 10746			—
			(% Identity)
	NBRC 0975	NBRC 10402	NBRC 10746
NBRC 0975	—	83.5/94.6	85.3/96.0
NBRC 10402		—	92.5/97.8
NBRC 10746			—
			(% Identity/% Similarity)

Furthermore, the sequences of the deduced *AOX1* promoter regions were compared (Table 1-4, upper). Since the TATA box (TATAAA) plays an important role in transcription (33), the domains between the putative TATA boxes and translation start sites were also compared (Table 1-4, lower). The deduced promoter sequence of NBRC 0975 showed 53.5% and 53.1% identity to those of NBRC 10402 and NBRC 10746, respectively. The sequence between the putative TATA box and deduced *AOX1* translation start site of NBRC 0975 showed 42.1% identity to that of NBRC 10402 and 52.5% identity to that of NBRC 10746. Meanwhile, the deduced promoter sequence of NBRC 10402 showed 79.1% identity to that of NBRC 10746. The sequence between the putative TATA box and deduced *AOX1* translation start site of NBRC 10746 showed 85.5% identity (Table 1-4).

Table 1-4 Identities of the sequences of the deduced *AOX1* promoter regions (upper) and the domains between the putative TATA boxes and translation start sites (lower).

	NBRC 0975	NBRC 10402	NBRC 10746
NBRC 0975	—	53.5	53.1
NBRC 10402		—	79.1
NBRC 10746			—
			(% Identity)
	NBRC 0975	NBRC 10402	NBRC 10746
NBRC 0975	—	42.1	52.5
NBRC 10402		—	85.5
NBRC 10746			—
			(% Identity)

The length of the sequence after the putative TATA box was 76 bp in NBRC 0975 and approximately 120 bp in both NBRC 10402 and NBRC 10746. Notably, the sequences after the putative TATA box were clearly different among NBRC 0975, NBRC 10402, and NBRC 10746 (Fig. 1-5). Additionally, I performed homology searches of deduced *AOXI* promoter regions for putative binding sites of a transcription factor to reveal whether there are any differences in such regions among the three strains. The three strains of interest have several putative Trm2p binding sites, consisting of a core 5'-CYCC-3' motif (34), and NBRC 10746 has the highest number of similar binding sites among the three strains (Table 1-5).

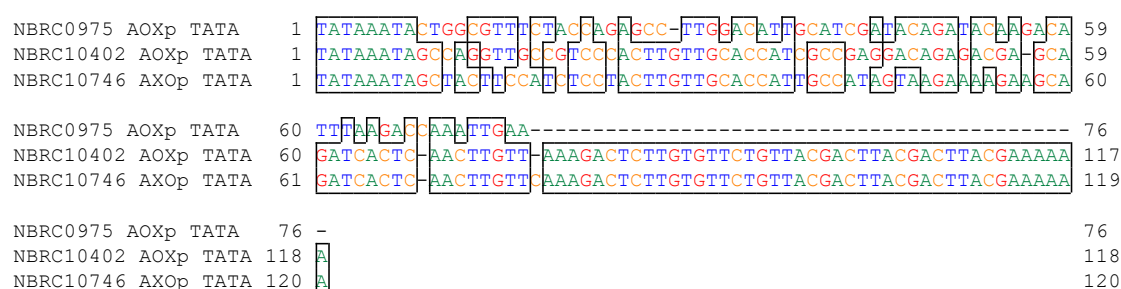


Fig. 1-5 Multiple nucleotide sequence alignment between the putative TATA box and translation start site in each of the 3 strains.

Table 1-5 The number of deduced binding sites of Trm2p in the deduced *AOXI* promoter regions.

NBRC 0975	NBRC 10402	NBRC 10746
7	9	11

Electrophoretic karyotype comparison by PFGE

For further investigation of the genomic features of the three strains, chromosome structure was analyzed by PFGE (35), which is a useful tool for investigating larger DNA fragments. PFGE was performed to compare the size of each chromosome, followed by Southern blotting with the *AOXI* gene from NBRC 10746 as a probe. After ethidium bromide staining, the gel showed clearly visible differences in the size and number of chromosomes among the three strains. NBRC 0975 showed at least eight chromosome bands with estimated sizes of approximately 2.3, 1.7, 1.4, 1.3 (multiple bands), 1.2, and 1.1 Mbp. NBRC 10402 showed five chromosome bands with estimated sizes of approximately 2.3, 1.9, 1.5, 1.3, and 1.2 Mbp. NBRC 10746 showed at least eight chromosome bands with estimated sizes of approximately 2.3, 2.1, 1.8, 1.6, 1.4, 1.3, 1.2, and 1.1 Mbp. The *AOXI* locus was detected on the chromosome of NBRC 10746 of approximately 1.6 Mbp in size in Southern blotting with its own *AOXI* gene probe. Meanwhile, the deduced *AOXI* loci of NBRC 0975 and NBRC 10402 were detected by the *AOXI* gene probe of NBRC 10746 on a different chromosome with different signal intensities (Fig. 1-6).

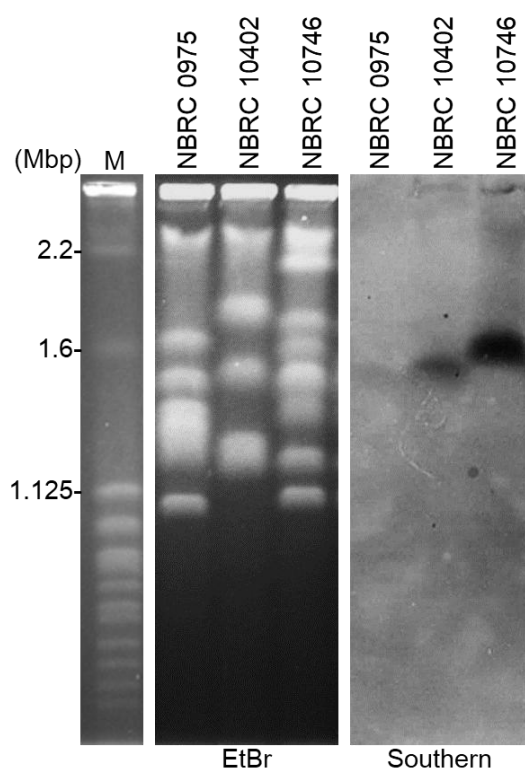


Fig. 1-6 Electrophoretic karyotype analyzed by pulsed-field gel electrophoresis and Southern blot analysis of each strain: NBRC 0975, NBRC 10402, and NBRC 10746.

Discussion

I have developed a heterologous protein expression system using genetically engineered NBRC 10746, the outcomes of which were previously reported (14, 19). I have continued to expand the *Ogataea* expression system from three *Ogataea* strains found in the NBRC collection. NBRC 0975 is derived from NRRL Y-441 in the Agricultural Research Service (ARS) Culture Collection at the Northern Regional Research Laboratory (NRRL), which was isolated from fermented mushrooms by Henrici (15). NBRC 10402 is derived from ATCC 26176 in the American Type Culture Collection (ATCC), which was isolated from a rotten azalea flower by Oki et al. (16). NBRC 10746 is derived from DSM 70718 in the German Collection of Microorganisms and Cell Cultures (DSMZ), which was isolated from forest soil by Henninger and Windisch (17). These strains were historically deposited as *O. minuta* var. *minuta* in the NBRC collection. Here, upon finding the three *Ogataea* strains (NBRC 0975, NBRC 10402, and NBRC 10746), the question arose of which strain is the best host for heterologous protein expression. I performed an evaluation to select the best strain for heterologous protein expression using a CLuc reporter assay and revealed that NBRC 10746 showed the highest CLuc activity in culture broth (Fig. 1-1). The biomass of NBRC 10402, which was determined using optical density, was approximately 120% higher than that of NBRC 10746. This indicates that the production ability per cell in NBRC 10746 would be around 250% higher than that in NBRC 10402. In terms of developing commercial fermentation processes, higher cell density may cause some difficulties: increased oxygen requirements, difficulty recovering supernatant from the culture broth, and so on. I selected NBRC 10746 as a parental strain for further development of an optimal host strain because of the high productivity per cell and adequate cell mass.

Generally, vials distributed from culture collections are inhomogeneous. Therefore, monoclonal selection is an effective procedure to isolate specimens with uniform characteristics from inhomogeneous groups. It is anticipated that cells particularly suited to heterologous protein expression might be isolated by screening for specific purposes. As shown in Fig. 1-3B, tat06213 showed higher CLuc activity than the parental clone #5, meaning that tat06213 was successfully isolated from NBRC 10746 under monoclonal evaluation with CLuc secretion, as expected. As the above data revealed the difference in ability to produce heterologous proteins among the three *Ogataea* strains, I compared their genotypic features. NBRC 10746 was first reported as *Pichia lindnerii* in 1975 (17), and was later reclassified as the same species as NBRC 0975 based on DNA homology (36). However, when NBRC 0975 was reclassified with *O. minuta* var. *minuta* based on a partial rRNA sequence (37, 38), NBRC 10746 was also reclassified with the same species as NBRC 0975 as *O. minuta* var. *minuta*. However,

based on sequence analysis of the longer LSU D1/D2 domain of each strain, a phylogenetic tree was created by neighbor joining. The results showed that NBRC 10402 and NBRC 10746 were grouped into different clusters far from NBRC 0975, a type strain of *O. minuta* var. *minuta* (Fig. 1-4), indicating that NBRC 0975 is quite different from NBRC 10402 and NBRC 10746. The electrophoretic karyotype and *AOX1* loci analyzed by PFGE and Southern blotting showed different patterns and signal intensities among the three strains (Fig. 1-6). The difference of signal intensities of *AOX1* loci detected by the probe prepared from the NBRC 10746 gene could have resulted from the difference in nucleotide sequences of the *AOX1*-encoding gene among the three strains. These findings revealed genomic and electrophoretic karyotype differences among the three strains that were deposited as *O. minuta* var. *minuta*, not only between NBRC 0975 and NBRC 10402 or NBRC 10746 but also between NBRC 10402 and NBRC 10746. Peter et al. performed taxonomic analysis of CBS 10795, which is registered as a synonym of NBRC 10402 in the Japan Collection of Microorganisms, and proposed it as a new strain "*Ogataea saltuana*" (39). The data related to NBRC 10402 agree with the proposal for renaming by Peter et al., while it may also be necessary to rename NBRC 10746 upon further taxonomic analysis.

For heterologous expression, promoters of methanol-inducible genes are often adopted by methylotrophic yeasts because of the powerful promoter activity that is strictly regulated in response to carbon sources (12). Among the methanol-inducible promoters, the *AOX1* and homologous promoter is widely used (5). The regulatory mechanism of the *AOX1* promoter has been well studied in *Komagataella phaffii*. Several regulatory transcription factors are reported to be responsible for control of the *AOX1* promoter (40). There are also differences in genomic sequences of the deduced *AOX1* coding and promoter regions among the three strains. *AOX1* loci analyzed by PFGE and Southern blotting were shown to be present on chromosomes of different sizes (Fig. 1-6). The low sequence identity of the deduced *AOX1* coding regions among the three strains is consistent with the results of Southern blot analysis as detected with the probe that was prepared from NBRC 10746 genomic DNA. The difference in protein productivity as identified among the three strains is possibly due to different *AOX1* promoter activities derived from different promoter sequences. According to Yoko-o's model, *MPP1* and *TRM2* play important roles in regulating the *AOX1* promoter activity and the expression of the gene of interest (41). Mpp1p is a putative Zn(II)₂Cys₆-type transcription factor that preferentially binds to 5'-CGG-3' triplets in the promoter sequence (42). I searched for this triplet in *AOX1* promoter sequences of the three strains and revealed such an abundance of binding candidates that it is difficult to speculate on deduced binding sites. Therefore, I searched for sequences homologous to the Mit1-W2A, Mit-W2B, and Mit1-W3A domains of the *K.pAOX1* promoter sequence

(34). Contrary to my expectations, however, the three strains were revealed not to have homologous sequences in *AOXI* promoters. Trm2p is a putative C2H2-type transcription factor that preferentially binds to the 5'-CYCC-3' motif in the promoter sequence (34). I searched for sequences homologous to this motif and revealed that the three strains have several deduced binding sites, with NBRC 10746 having the most (Table 1-5). The finding that deduced binding sites of Trm2p as a positive promoter regulator were most frequently observed may support NBRC 10746 having the highest productivity. Meanwhile, Yoko-o et al. proposed that the activity of the *AOXI* promoter is mainly regulated by MPP1. For further discussion of the difference in productivity among the three strains, it is necessary to identify the binding site of Mpp1p in the *AOXI* promoter sequences. If the Mpp1p binding site is identified, it would be a good clue to reveal more details about the regulatory mechanism of the *AOXI* promoter and speculate on the cause of the difference in productivity among the strains in this study. Furthermore, it appears that clarification of the sequence of binding sites would also be useful to optimize the *AOXI* promoter and make it more sophisticated as a high-producing promoter, as several studies focusing on deletion and/or duplication of a transcription factor binding site and an upstream activation sequence have been reported (43, 44). Additionally, the ability to perform heterologous protein expression is determined by not only transcription but also translation, secretion, and extracellular protease activity. It might thus be a good strategy to investigate all of these factors for host cell engineering to enhance recombinant protein production.

In this study, I clarified several differences among three *Ogataea* strains and selected the best strain from among those of the *Ogataea* genus deposited in the NBRC collection. The clone #5-30 named tat06213 established from NBRC 10746 should be a promising host for heterologous protein expression.

Section I-2

Development of a protein production system using *Ogataea minuta* alcohol oxidase-deficient strain under reduced-methanol-consumption conditions

Introduction

Various heterologous protein production systems have been developed. Among these production systems, methylotrophic yeast might be a particularly convenient host for heterologous proteins due to several advantages: the ability to secrete proteins into the culture broth; a eukaryotic protein quality control system; a post-translational modification system; and rapid growth (3, 9). One of the key advantages of methylotrophic yeast production systems is the use of methanol both as a sole carbon and energy source, along with the availability of strong methanol-inducible promoters (11). The methanol utilization (MUT) pathway, which has been well refined and integrated to assimilate and dissimilate methanol, is widely conserved in methylotrophic yeast species. In the MUT pathway, alcohol oxidase 1 (AOX1) plays an important role as the first-step enzyme in the oxidation of methanol to formaldehyde (12). In *Pichia pastoris* (syn. *Komagataella phaffii*), since its first adoption for recombinant protein production in 1987, the *AOX1* promoter has been an attractive option due to its unique feature of being highly suppressed by glucose and glycerol and strongly induced by methanol (10, 11).

Although methanol-inducible promoters are powerful tools for protein production systems, methanol and related metabolites including formaldehyde and formic acid are known to be toxic, causing acidosis and other clinical symptoms (13). Caution is also needed when handling and storing methanol, given its flammability and explosiveness. Furthermore, an excessive concentration of methanol during fermentation may cause the substitution of methionine residues due to the misincorporation of *O*-methyl-L-homoserine (45). As such in some cases, it might be necessary to control the product variant ratio in order to ensure the quality of the biopharmaceutical product. To overcome issues related to promoter induction using methanol, several approaches such as de-repression (via the removal of other carbon sources that repress promoter activity), de-repressed promoter engineering, and transcription factor engineering have been attempted in *K. phaffii*. The *AOX1* promoter is not only strongly induced by methanol but also repressed by some carbon sources such as glucose and glycerol. To reduce the required amount of methanol, a protein production system under conditions in which promoter activity

is de-repressed by adjusting the feeding rate of the repressive carbon sources was developed in *K. phaffii* (46-50). Several studies have also reported that the methylotrophic yeast *O. minuta* is an attractive host for producing heterologous proteins, including monoclonal antibodies (19, 51). In *O. minuta* production systems under methanol induction conditions, methanol consumption has not been controlled to ensure that enough methanol is supplied to the culture. In the present study, I investigated the conditions for the methanol-based induction of the *AOX1* promoter in *O. minuta* and generated an alcohol oxidase-deficient (Δ aox) strain via homologous recombination with a *URA3* pop-in/pop-out system to decrease the required methanol supply. The culture conditions were evaluated using the *O. minuta* Δ aox strain under mixed feed conditions to achieve equivalent or higher production of heterologous proteins under low-methanol conditions.

Materials and Methods

Yeast strains, plasmids, and media

O. minuta tat06213 (52) was used as a host strain for protein production. All *O. minuta* strains used in this study are listed in Table 2-1. The plasmid pOU1/PDER-PKA was introduced to generate strains that co-overexpress chaperones for protein production, namely, PDI1, ERO1, and KAR2, to improve the productivity of the protein of interest (19). Codon-optimized DNA sequences encoding human serum albumin (HSA, GenBank accession number: AGI02589.1) fused with the α -mating factor secretion signal (α F signal) sequence were synthesized with XbaI and BamHI at the 5' and 3' ends, respectively, by GeneArt™ (Regensburg, Germany). The synthesized fragments were introduced into pOMEA-Z1-10746 (52) at the XbaI and BamHI sites, resulting in pOMEA-Z1-10746/HSA. Plasmids pOU1/PDER-PKA and pOMEA-Z1-10746/HSA were introduced into tat06213 to generate tat19913. Plasmids pOU1/PDER-PKA and pOMEA-Z1-10746/HSA were introduced into *O. minuta* tat10113 to generate tat20313. Plasmid pOMEU1/CLuc (52) was introduced into tat06213 to generate tat07313. Plasmid pOMEU1/CLuc was introduced into tat10113 to generate tat09513. The electroporated cells were plated on synthetic dextrose (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% glucose, 0.002% L-tryptophan) with 2% agar. Yeast extract peptone dextrose (YPD) (1% Difco™ yeast extract, 2% Bacto™ peptone, 2% glucose, 0.004% adenine) was used for the general cultivation, and YPD with 2% agar was used for cell growth on plate media. 2xYP-P6-GG medium (2% Difco™ yeast extract, 4% Bacto™ peptone, 0.5% glucose, 4% glycerol, 0.1 M potassium phosphate buffer, pH 6.0) was used to produce the HSA protein. Representative clones were stored as suspensions in 17% glycerol at –80 °C.

Chemicals and general recombinant DNA techniques

E. coli JM109, restriction enzymes, and DNA-modifying enzymes were purchased from Takara Bio Inc. (Kusatsu, Japan). The media, growth conditions, and general recombinant DNA techniques used in this study were in accordance with the work of Sambrook and Russell (22). Zeocin™ was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Hygromycin was purchased from WAKO Pure Chemicals, Co., Ltd. (Osaka, Japan). All oligonucleotides in this study were synthesized by Fasmac Co., Ltd. (Atsugi, Japan). Polymerase chain reaction (PCR) amplification was performed using the GeneAmp® PCR System 9700 (Thermo Fisher Scientific). Automated DNA sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (Thermo Fisher Scientific). Transformation of *O. minuta* was performed as previously described (19).

Disruption of *AOX1* gene in *O. minuta*

The plasmid pOMEU1 (52) was introduced into tat06213 to generate intermediate strains harboring the *URA3* marker gene and duplicate promoter-terminator cassettes of the *AOX1* gene. The transformed cells were regenerated on synthetic dextrose plate medium at 30 °C for approximately 3 days. The transformants were transferred on the same selection medium to confirm that transformation had been performed successfully. The selected strain harboring the *URA3* marker gene and duplicate promoter-terminator cassettes was cultivated in YPD medium in a test tube at 30 °C for approximately 2 days with reciprocal shaking at 300 rpm. Then, 0.1 mL of the culture broth was transferred onto 5-fluoroorotic acid (5-FOA) plate medium and incubated at 30 °C for 4 days. The colonies growing on the 5-FOA plate medium were transferred onto another plate with the same medium to confirm 5-FOA resistance. *aox1*-null mutants (Δ aox) were confirmed by PCR amplification with the following primers: AOX ORF fw, 5'-ATG GCT ATT CCT GAC GAA TT-3'; AOX ORF rv, 5'-TTA GAA TCT AGC AGA CCC TTC-3'. The clone confirmed as the Δ aox strain was named tat10113.

HSA expression and preparation for RNA isolation

HSA production was carried out in accordance with the method of (19), with slight modifications as follows. For the selection of HSA-producing cells, transformants were cultivated in the individual wells of a 96-well deep-well plate at 28 °C for 4 days with reciprocal shaking at 300 rpm. To evaluate the productivity at a larger fermentation scale, selected clones were cultivated in test tubes containing YPD medium at 28 °C with reciprocal shaking at 300 rpm. After overnight incubation, test tubes containing 5 mL of 2xYP-P6-GG medium were inoculated to an initial optical density (OD) at 600 nm of 0.1. After overnight incubation, each carbon source was added, such as 1% (v/v) methanol, 1% (w/v) glycerol, and a mixture of carbon sources including 0.5% (v/v) methanol and 0.5% (w/v) glycerol. After the cultivation period, the cells were removed by centrifugation ($2,200 \times g$) and HSA secreted into the culture broth was subjected to SDS-PAGE analysis. Centrifuged cells were subjected to RNA isolation. For this, the cells were first processed with Yeast Processing Reagent (for total RNA preparation) (Takara), after which NucleoSpin RNA (Macherey–Nagel, Düren, Germany) was used for total RNA isolation.

CLuc expression

CLuc production was carried out in accordance with the method of Tsuda et al. (52) with slight modifications. For the selection of CLuc-producing cells, transformants were cultivated in the

individual wells of a 96-well deep-well plate at 28 °C for 4 days with reciprocal shaking at 300 rpm. After removal of the cells by centrifugation ($2,200 \times g$), CLuc in the culture broth was quantified using a CLuc reporter assay kit (Atto, Tokyo, Japan).

SDS-PAGE

The culture broth containing secreted HSA was heated at 70 °C for 10 min in NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Heat-treated samples were analyzed with the NuPAGE® System (Invitrogen) using 10% Bis-Tris gels (12 wells, 7 cm wide, 1 mm thick) and NuPAGE® MES SDS Running Buffer (Invitrogen) for 30 min at a constant voltage of 200 V, followed by Coomassie Brilliant Blue Staining using EzStain Aqua (Atto).

Bioreactor cultivation

In a preculture, frozen stocks of *O. minuta* strains were thawed and streaked onto YPD plates. The plates were incubated at 30 °C for 2 days. Colonies grown on the YPD plates were cultured in test tubes containing 5 mL of YPD at 30 °C with reciprocal shaking at 250 rpm for 24 h. Ten milliliters of collected cultured broth was transferred to baffled flasks containing 40 mL of 2xYP-P6-GG medium and cultured at 30 °C with rotary shaking at 250 rpm for 16 h. Fed-batch culture was then carried out in a 3-L bioreactor (Biott, Tokyo, Japan) containing 1.5 L of 3xYP-P6-GG medium (3% Difco™ yeast extract, 6% Bacto™ peptone, 0.5% glucose, 2% glycerol, 0.1 M potassium phosphate buffer, pH 6.0). The inoculation volume was 5% (v/v) of the starting volume. The stirrer speed was varied to keep the dissolved oxygen concentration at 25% at a constant aeration rate. Eight hours after the inoculation, glycerol solution was continuously supplied for 12 h at 0.33 g/h. For the methanol-utilizing strains, methanol was continuously supplied at 4.5 g/h after bolus feeding of methanol at 1.0%. For the Δ aox strain, glycerol was continuously supplied at 3.3 g/h after bolus feeding of methanol at 1.0%. The concentrations of carbon sources in the culture media were measured using a flow injection analyzer (BF-9; Oji Scientific Instruments, Amagasaki, Japan). In this system, the carbon sources were converted to hydrogen peroxide by the enzymes immobilized on columns, and the amount of generated hydrogen peroxide was measured by the electrochemical method.

RT-qPCR

cDNA was synthesized from 400 ng of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). To quantify gene expression, quantitative real-time PCR analysis was performed on the Bio-Rad QX100 ddPCR platform (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using cDNA

corresponding to 40 ng of the total RNA and ddPCR EvaGreen Supermix (Bio-Rad). The PCR was performed as follows: 5 min at 95 °C; 40 cycles of 95 °C for 30 s and 60 °C for 60 s; 5 min at 4 °C; and finally 5 min at 90 °C. Gene-specific oligonucleotides were as follows: ACT1(F) (5'-ACC ATG TTC CCA GGT ATT GC-3') and ACT1(R) (5'-TTG GAA AGT GGA CAG GGA AG-3') for *ACT1* cDNA; RT-PCR OmAOX Left (5'-GTA TCA ACG TCC TTG CAA CAA CA-3') and RT-PCR OmAOX Right (5'-AGG TCT TCA AGA TCG TCG TTG AA-3') for *AOX1* cDNAs; and RT-PCR HSA Left (5'-GTT CGG TGA CAA GTT GTG TAC TG-3') and RT-PCR HSA Right (5'-TGT CGT GGA AAG CAG TAC ACA TA-3') for *HSA*-encoding cDNA. The copy numbers of the *AOX1* and *HSA* genes were measured by droplet digital PCR and normalized by the *ACT1* gene.

Results

Comparison of *AOX1* promoter activity under de-repression and methanol induction conditions

In a preliminary analysis of in-house microarray data, the *AOX1* promoter activity in *O. minuta* was induced by depleting carbon sources that repressed such activity (de-repression; data not shown). To evaluate *AOX1* promoter activity under de-repression and methanol induction conditions for heterologous protein production, recombinant HSA was used as a model protein in *O. minuta*. An HSA-producing strain was generated and cultivated as described in the Materials and Methods section. In detail, tat19913 was cultured in a test tube containing 2xYP-P6-GG medium. After overnight incubation, bolus feeding of glycerol solution was supplied at 1.0% (w/v) as de-repression conditions and bolus feeding of methanol was supplied at 1.0% (v/v) as methanol induction conditions. The HSA secreted into the culture broth after cultivation was analyzed by SDS-PAGE under reducing conditions (Fig. 2-1A). The results showed that the culture conditions under methanol induction resulted in the secretion of a larger amount of HSA than that under de-repression conditions. In contrast to the rapid HSA production under the methanol induction conditions, the secreted HSA protein band remained faint for several hours after bolus feeding of glycerol in the de-repression conditions.

To investigate the causes of this repressed production of HSA under de-repression conditions, further analysis of cultured broth and cells was conducted as follows: The concentration of glycerol, the repressive carbon source, in culture broth was already insufficient at 3 h and was completely depleted at 6 h after bolus feeding of glycerol (Fig. 2-2A). On the other hand, the concentration of methanol in culture broth decreased gradually and a little methanol remained unconsumed 12 h after bolus feeding of methanol (Fig. 2-2B). The optical density (OD) at 600 nm (OD_{600}) of cultured samples was measured over the course of fermentation (Fig. 2-2C).

I also quantified the transcript levels of the genes encoding *AOX1*, HSA, and actin (*ACT1*, GenBank accession number: LC835676) by RT-qPCR using mRNA extracted at each sampling point (Fig. 2-1B). Under de-repression conditions, the transcription of *AOX1* and *HSA* was completely repressed at 3 h after bolus feeding of glycerol. After glycerol was completely depleted at 6 h after bolus feeding, the transcript levels of *AOX1* and *HSA* started increasing. In the methanol induction conditions, the transcript levels of *AOX1* and *HSA* decreased gradually, which mirrored the methanol concentration. These results indicate that, under de-repression conditions, the *AOX1* promoter achieved lower protein production than under methanol induction conditions, due to repression of promoter activity by carbon sources and the absence of methanol as a strong promoter inducer. Notably, the expected protein was produced at a certain level under de-repression conditions without the addition of methanol, although

at lower levels compared to methanol induction conditions.

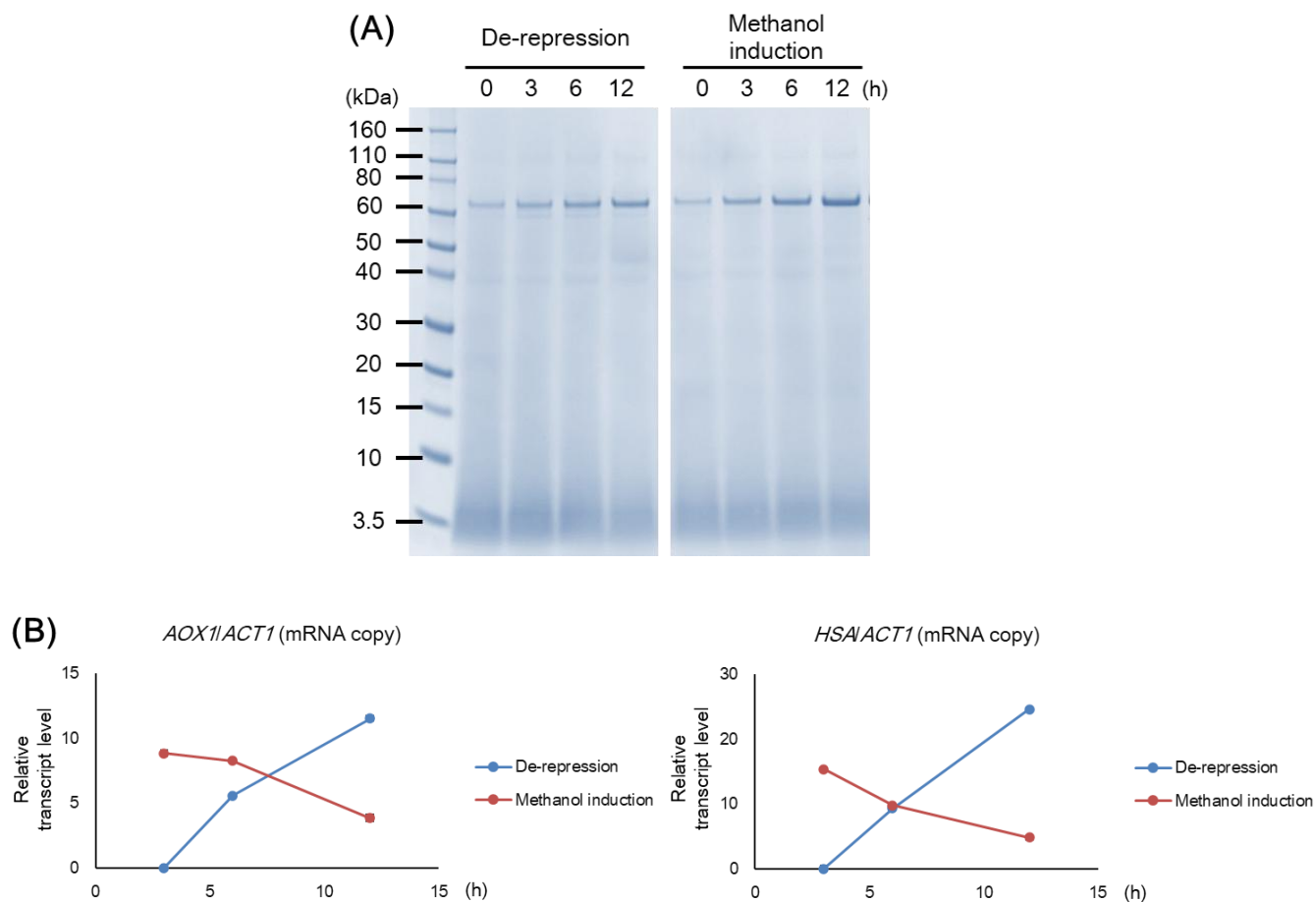


Fig. 2-1 Comparison of *AOX1* promoter activity under de-repression and methanol induction conditions. (A) CBB staining/SDS-PAGE analysis under reducing conditions of secreted recombinant HSA. (B) Comparison of the ratio of mRNA copy number by quantitative RT-PCR. The copy number of the genes encoding *AOX1* and *HSA* was normalized by dividing by that of the gene encoding actin (*ACT1*) (blue: de-repression conditions, orange: methanol induction conditions). Values are shown as mean $\pm$ S.D. of three independent experiments. The x-axis indicates the time points after carbon source bolus feeding.

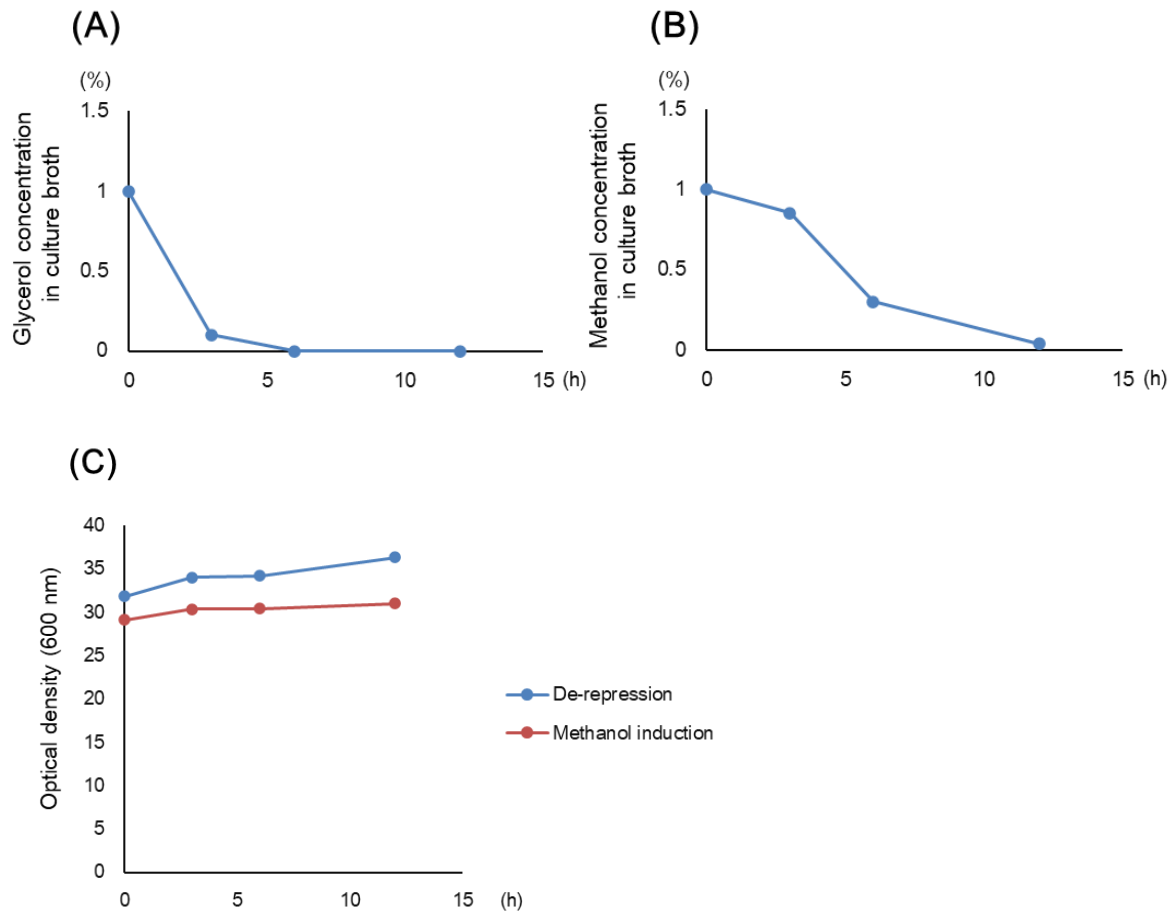


Fig. 2-2 (A) Glycerol concentration in the culture broth under de-repression conditions. (B) Methanol concentration in the culture broth under methanol induction conditions. (C) Optical density measurements at a wavelength of 600 nm (OD₆₀₀). The x-axis indicates the time points after carbon source bolus feeding.

The methanol consumption ability of *AOX1*-gene-deficient *O. minuta* mutant

Although recombinant HSA was effectively produced under methanol induction conditions, as shown in Fig. 2-1A, efforts were made to reduce the methanol consumption rate in order to enhance the industrial value of the heterologous protein production system in *O. minuta*. For this, an *AOX1*-gene-deficient mutant that does not consume methanol was generated. The Mut^S strain of *K. phaffii*, with disruption of the *AOX1* gene representing slow methanol utilization, and the Mut⁻ strain, with disruption of the *AOX1* and *AOX2* genes preventing growth on methanol as a sole carbon source, were reported as hosts for producing heterologous proteins (53, 54). In a similar attempt, a homology search for alcohol oxidase in the in-house genomic database of *O. minuta* was conducted and only one homologous DNA sequence was identified (GenBank accession number: AB242209.1). To disrupt this *AOX1* gene in *O. minuta*, pOMEA-Z1-10746 was introduced and processed as described in Fig. 2-3A. The resulting *AOX1*-disrupted (Δ aox) strain, tat10113, was confirmed by PCR using *AOX1* ORF amplification primers (Fig. 2-3B). Evaluating the ability of tat10113 to consume and grow on methanol was difficult due to the very slow growth rate caused by auxotrophic deficiency. Hence, I generated and used tat20313 for cultivation studies in test tubes. Although tat20313 hardly consumed methanol, the residual methanol was clearly reduced from its initial concentration (Fig. 2-3C). To assess the impact of methanol evaporation, a shaking study was conducted using a mixture of medium and methanol similar to the cultivation study, showing that the residual methanol remained unchanged (data not shown). Furthermore, the OD₆₀₀ of cultured samples was measured, showing that tat20313 hardly grew under medium conditions containing methanol as a sole carbon source (Fig. 2-4). These results might indicate that the generated Δ aox strain could utilize methanol at a reduced rate.

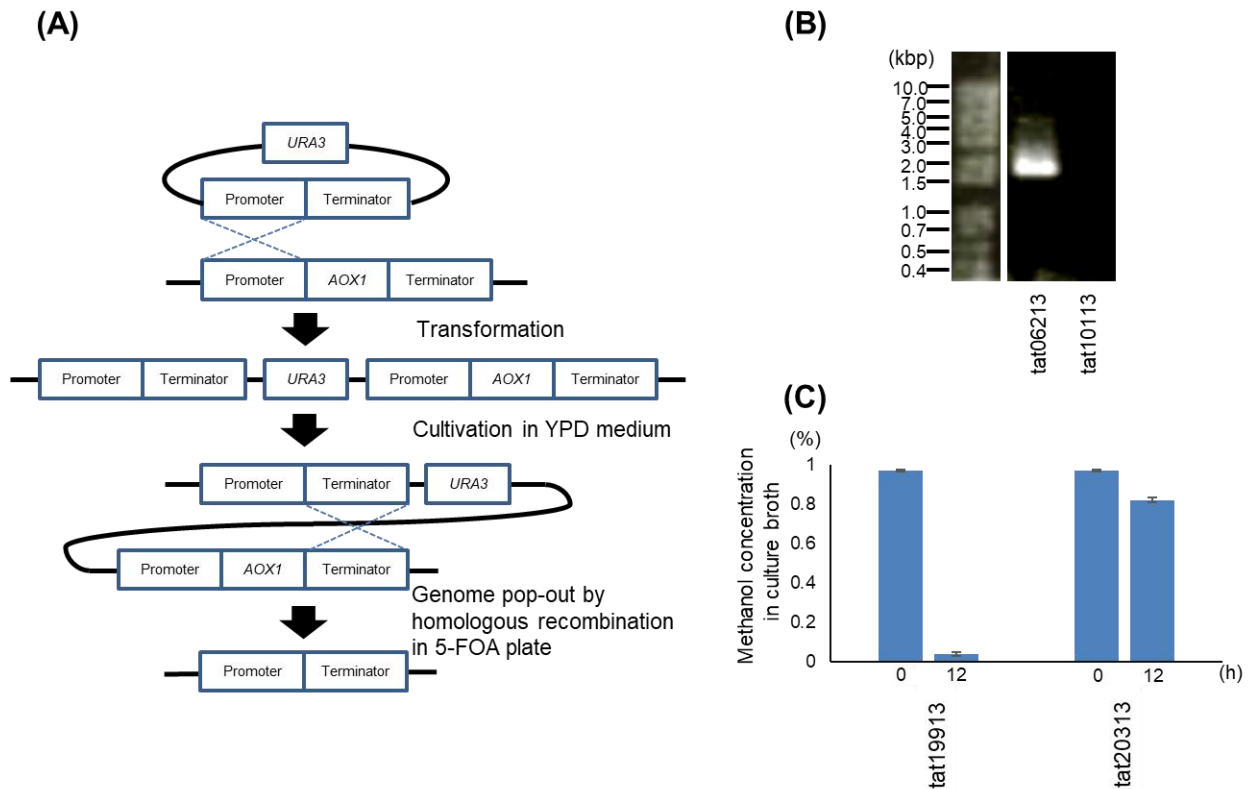


Fig. 2-3 Disruption of the *AOX1* gene in *O. minuta*. (A) Schematic overview of the *AOX1* gene disruption process. (B) PCR amplification of the *AOX1* ORF gene in tat06213 and tat10113. (C) Comparison of the methanol consumption rates of tat19913 and tat20313. Values are shown as mean $\pm$ S.D. of three independent experiments. The x-axis indicates the time points after carbon source bolus feeding.

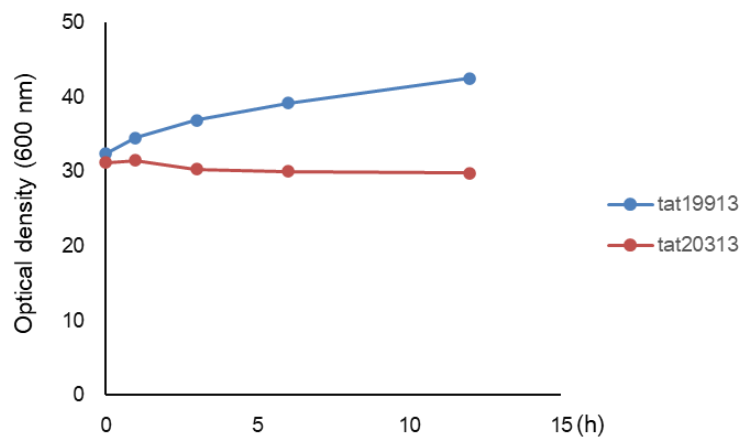


Fig. 2-4 Optical density measurements at a wavelength of 600 nm (OD_{600}). The x-axis indicates the time points after carbon source bolus feeding.

Adjustment of the feeding conditions for the Δ aox strain

As the Δ aox strain showed the elimination or reduction of methanol consumption, methanol could be used as an inducer only. As such, a feeding solution for *O. minuta* containing a mixture of glycerol and methanol was developed here. This approach was applied given that solutions containing a mixture of carbon sources have been widely studied in *K. phaffii* (47). The cultivation conditions with the mixed feed solution containing 0.5% methanol and 0.5% glycerol achieved greater secretion of HSA protein than the de-repression conditions. In detail, tat20313 was cultured in a test tube containing 2xYP-P6-GG medium. After overnight incubation, bolus feeding of glycerol solution was supplied at 1.0% (w/v) as de-repression conditions and bolus feeding of the mixture of 0.5% (v/v) methanol and 0.5% (w/v) glycerol was supplied as mixed feed conditions. tat19913 was cultured in a test tube containing 2xYP-P6-GG medium. After overnight incubation, bolus feeding of methanol was supplied at 1.0% (v/v) as methanol induction conditions (Fig. 2-5). Furthermore, the amount of HSA protein secreted by tat20313 under mixed feed conditions was comparable to that by tat19913 under methanol induction conditions (Fig. 2-5). For further investigation of the optimal culture conditions under mixed feed conditions, expression analysis was conducted by RT-qPCR with mRNA extracted at each sampling point as a template. As shown in Fig. 2-1B, the transcript level of *AOX1* exhibited a similar tendency to that of *HSA*. Therefore, the transcript level of *HSA* was used to evaluate the *AOX1* promoter activity. The transcript level of *HSA* in tat20313 under mixed feed conditions was higher than not only that in tat20313 under de-repression conditions but also that in tat19913 under methanol induction conditions at 3 h after bolus feeding (Fig. 2-6A). The OD₆₀₀ of cultured samples was measured over the course of fermentation (Fig. 2-6B). These results suggested that applying the tested mixed feed conditions to the Δ aox strain was effective for producing heterologous proteins.

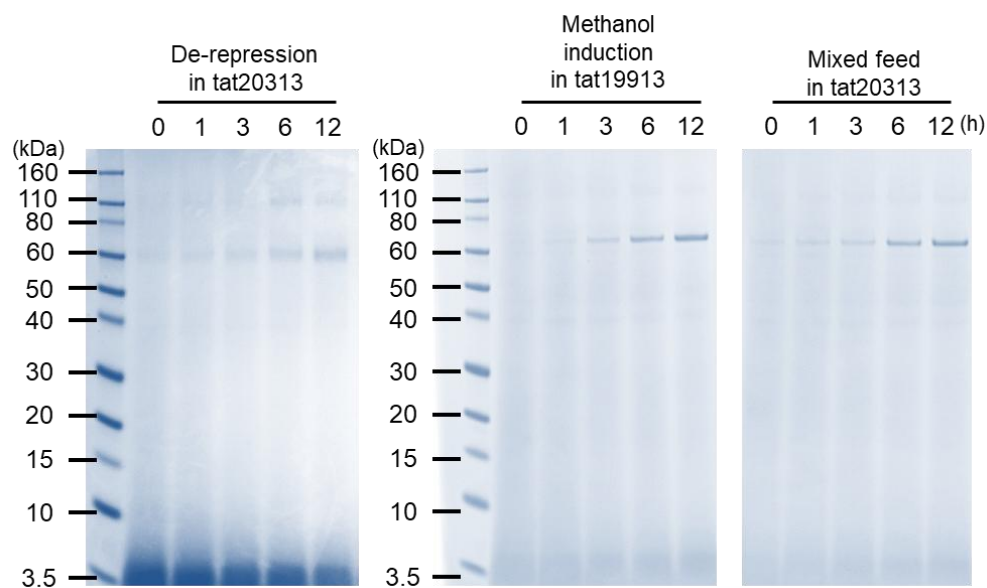


Fig. 2-5 CBB staining/SDS-PAGE analysis under reducing conditions of secreted recombinant HSA. Time points after carbon source bolus feeding (h) are indicated.

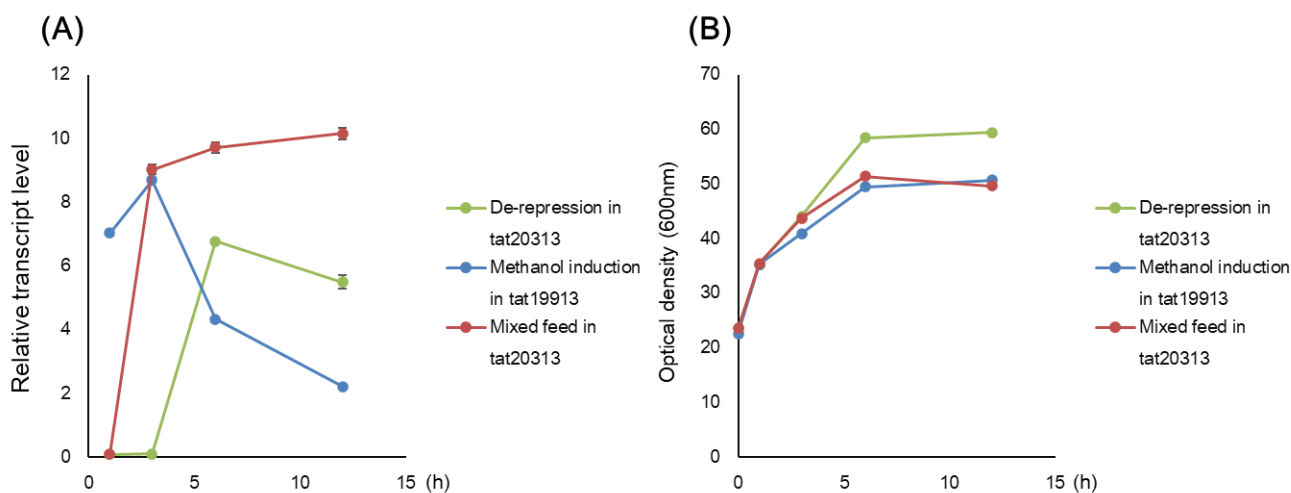


Fig. 2-6 (A) Comparison of the ratio of mRNA copy number by quantitative RT-PCR. The copy number of the gene encoding HSA was normalized by dividing by that of the gene encoding actin (*ACT1*) (gray: de-repression conditions in tat20313, blue: methanol induction conditions in tat19913, orange: mixed feed conditions in tat20313). Values are shown as mean $\pm$ S.D. of three independent experiments. (B) Optical density measurements at a wavelength of 600 nm (OD_{600}). The x-axis indicates the time points after carbon source bolus feeding.

Heterologous protein production by the wild-type strain and the Δ aox strain in a 3-L fermentor

The performance of the Δ aox strain was further investigated in a 3-L fermentor because this setting allows control of the concentration of carbon sources to achieve the appropriate regulation of promoter activity. CLuc, namely, luciferase secreted from the ostracod *Cypridina noctiluca* (20), was applied as a reporter protein to quantitatively evaluate heterologous protein production. In detail, methanol was continuously supplied at 4.5 g/h after bolus feeding of methanol at 1.0% for tat07313. Glycerol was continuously supplied at 3.3 g/h after bolus feeding of methanol at 1.0% for tat09513. tat07313 showed higher CLuc activity than tat09513 at 6 h after methanol induction. However, the activity of CLuc secreted from tat09513 reached almost the same level as that from tat07313 and continued to increase without reaching a plateau (Fig. 2-7). The OD₆₀₀ of cultured samples was measured over the course of fermentation (Fig. 2-8A). The methanol concentration in the culture of tat09513 decreased more slowly than that of tat07313 (Fig. 2-8B). The methanol concentration required by tat09513 under mixed feed conditions was markedly lower than that by tat07313 under methanol induction conditions (Fig. 2-8C). These results showed that the *O. minuta* Δ aox strain under mixed feed conditions could replace the simple methanol induction yeast system for producing heterologous protein.

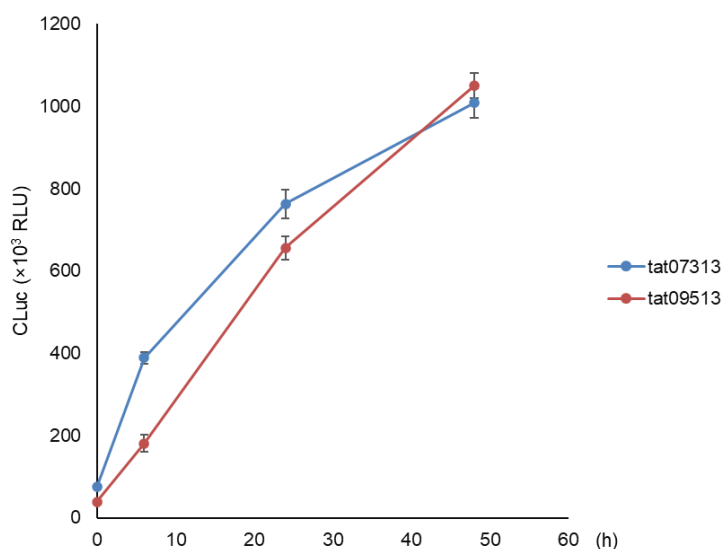


Fig. 2-7 Quantified CLuc expression levels of the cultivation study in a jar fermentor (blue: tat07313, orange: tat09513). Values are shown as mean $\pm$ S.D. of three independent experiments. The x-axis indicates the time points after methanol induction.

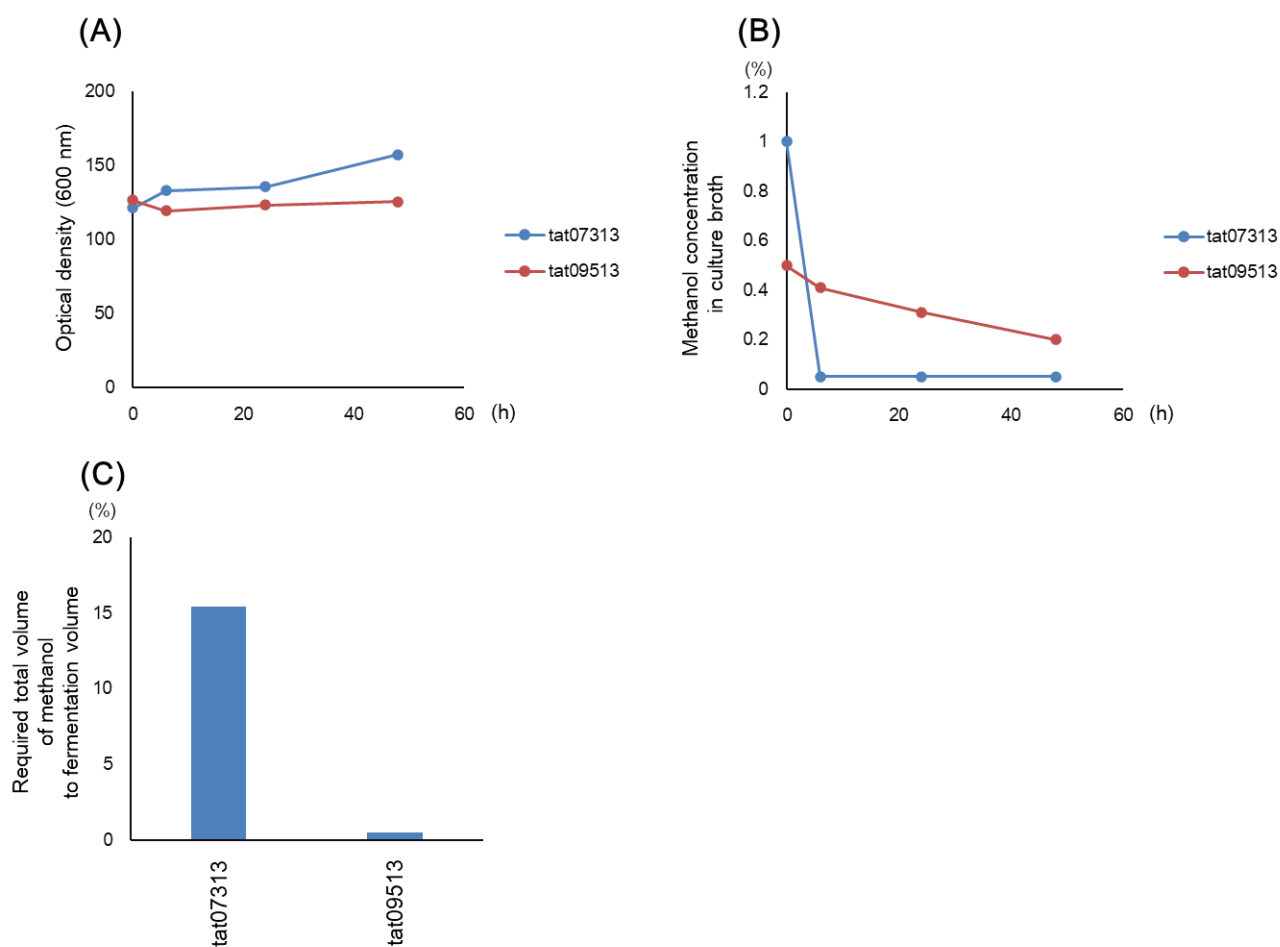


Fig. 2-8 (A) Optical density measurements at a wavelength of 600 nm (OD_{600}). (B) The concentration of methanol in culture broth (blue: *tat07313*, orange: *09513*). The x-axis indicates the time points after methanol induction. (C) The percentage of the required amount of methanol relative to the fermentation volume for 46 h of cultivation.

Discussion

Methylotrophic yeast has been identified as a promising host for producing heterologous proteins given its advantages including the availability of a methanol-inducible promoter (5). However, methanol is generally unfavorable for manufacturing on an industrial scale. For example, in 2000 L-scale fermentation, approximately 100 L of methanol would be required under 2% (v/v) methanol feeding for 3 days. There would thus be a need to construct a safe facility to be able to handle a large amount of methanol under explosion-proof and fire-proof conditions and to protect workers, which would be expensive. As such, reduction of the methanol consumption rate is one option that is highly anticipated to improve methanol-induction-based methylotrophic yeast systems including that of *O. minuta*.

To reduce the amount of methanol in methylotrophic yeast production systems using the *AOX1* promoter, methanol was used as an inducer only, not as a carbon source. In general, regulation of the *AOX1* promoter in response to carbon sources has been well investigated in *K. phaffii* (40). One of the unique features of the *AOX1* promoter of being strongly induced by methanol and repressed by some carbon sources such as glucose and glycerol is advantageous for producing heterologous proteins by enabling separation of the growth phase and the production phase. By adjusting the feeding rate of the carbon sources glycerol and sorbitol, the productivity with a de-repression promoter was improved in *K. phaffii* (47). In this study, I demonstrated the de-repression-based production by glycerol, resulting in productivity lower than that induced by methanol (Fig. 2-1A). The difference in productivity could be derived from the feeding mode, namely, bolus feeding or continuous feeding. However, because the comparison between the de-repression and methanol induction conditions was conducted using the same bolus feeding method, I concluded that the de-repression-based production by glycerol is not practical in *O. minuta*.

Although applying the methanol induction conditions would be an effective way of producing a large amount of protein, for manufacturing-scale production it is desirable to decrease the amount of methanol required. Therefore, a strain that uses less methanol was required and generated using the *AOX1* promoter, with the results confirming that this promoter was induced by a lower concentration of methanol. A homology search in the in-house genomic database revealed only one gene encoding alcohol oxidase, suggesting that *O. minuta* is closely related to *O. polymorpha*, which also has only one alcohol oxidase (*MOX1*) (52). One study reported the production of heterologous proteins in *K. phaffii* using the Mut⁻ strain, in which the two genes encoding alcohol oxidase (*AOX1*, *AOX2*) were disrupted (46). Meanwhile, in the methylotrophic yeast *Candida boidinii*, to use the peroxisome as the site for protein accumulation, alcohol oxidase, which is localized to the peroxisome, was disrupted;

the resulting alcohol oxidase-deficient strain showed 47-fold-higher productivity than the original strain (55). It is considered that the genomic region related to the MUT pathway is extremely highly expressed in response to methanol in yeast cells. Hence, disrupting this region might improve the heterologous protein productivity by saving the energy and space used up by the products of this genome expression.

To increase productivity, cultivation strategies in *K. phaffii* have been well investigated and reviewed (56, 57). Even with limited glycerol feeding, maximal protein production was achieved in this species. However, prolonged cultivation with the lowest glycerol feeding rate caused an imbalance of energy or amino acids in the cells, resulting in degradation of the produced protein. There was thus a need to refine the process by determining the appropriate ratio of methanol to glycerol in the fed substrate in a glycerol-methanol mixed feed strategy. As shown in Fig. 2-7, the mixed feed conditions using the *O. minuta* Δ ox strain achieved productivity comparable to that of the wild-type strain under methanol induction conditions. It seemed that the productivity using the *O. minuta* Δ ox strain continued to increase without reaching a plateau. Therefore, it was predicted that further refinement of the fermentation conditions, such as the methanol concentration, glycerol feeding rate, and supply of nitrogen bases, would be necessary to boost productivity upon prolonging the cultivation.

The concentration of methanol in the culture broth of the Δ ox strain gradually decreased during the fermentation period (Fig. 2-8B). This finding might have been due to the promiscuous activity of alcohol dehydrogenase (58). To maintain an appropriate methanol concentration for promoter induction, long-term fermentation could require supplementation with methanol at a much lower volume than for the wild-type strain under the methanol induction conditions.

It has been reported that several transcription factors are involved in regulating alcohol oxidase genes in methylotrophic yeasts. In *K. phaffii*, a strategy using engineered transcription factors was proposed as a methanol-independent process of producing proteins (48, 49). Yoko-o et al. (41) revealed that regulation of the methanol induction pathway is conserved among methylotrophic yeasts to some extent, but that *O. minuta* exhibits several specific features. Genetic engineering of the regulation of transcription factors might improve the methanol-free induction system in *O. minuta*.

Recently, single-use technologies (SUTs) have been widely adopted for manufacturing biologics by not only mammalian cells but also microorganisms as an alternative to conventional stainless jar fermentors (59). Methanol-induction-based production systems generally require a sufficient oxygen supply for the oxidation of methanol by alcohol oxidase as the first step in the MUT pathway (60). Furthermore, the methanol consumption was reported to result in overheating of the culture, especially

in large-scale fermentation (61). With regard to fitting the culture in a single-use bioreactor, it is preferable to use the Δ aox strain under mixed feeding conditions with restricted methanol and glycerol.

In this study, I modified the heterologous protein production system using an *O. minuta* alcohol oxidase-deficient strain (Δ aox) under mixed feed conditions. This system has the potential to replace the conventional methanol induction system.

Section I-3

Optimization of heterologous protein production process using genetically engineered *Ogataea minuta* toward industrial-scale manufacturing

Introduction

To improve the ability of yeasts to produce recombinant heterologous proteins, including biologic drugs and industrial enzymes, on an industrial scale, genetic engineering techniques have been widely adopted. In the emerging model yeast *Komagataella phaffii* (also known as *Pichia pastoris*), several strains that have been genetically engineered to exhibit phenotypes such as auxotrophy, protease deficiency, and methylotrophy, are widely available (62). With reference to insights gained from previous work on *K. phaffii*, I have developed the methylotrophic yeast *Ogataea minuta* as an attractive host for producing heterologous proteins including monoclonal antibodies via genetic engineering techniques (19, 51, 52, 63).

Although *Ogataea polymorpha*, which is genetically closed to *O. minuta*, is also widely utilized as an industrial protein production host, I selected *O. minuta* based on both literature precedents and its experimental compatibility (51, 64). In addition, previous studies have demonstrated that *O. minuta* could efficiently produce human-compatible Man₅GlcNAc₂-type *N*-glycans and suppress undesirable *O*-mannosylation (14, 19, 65). To produce the therapeutic biologics, it would be crucial to control glycosylation as an appropriate glycan structure. These features align with my purpose of useful host development for pharmaceutical products.

In the present study, I generated *O. minuta* double-mutant which lacks both the Prb1 protease gene (*PRB1*) and alcohol oxidase gene (*AOX1*), and evaluated its optimal fermentation condition. I also applied the established fermentation process to the production of a candidate biologic protein and finally achieved its industrial manufacturing in a 4,500-L bioreactor.

Materials and Methods

Yeast strains and media

All *O. minuta* strains used for protein production in this study are listed in Table 3-1. All the strains used for protein production are derived from *O. minuta* tat06213 (52) and tat10113 (63). The plasmids pOU1/PDER-PKA (19) and pOMEA-Z1-10746/HSA (52) were introduced into tat06213 and tat10113 to generate strains for human serum albumin (HSA) production with co-overexpressing chaperones (Pdi1, Ero1, and Kar2). The plasmid to produce ProteinX was also introduced into each strain with the plasmids pOU1/PDER-PKA (19). Transformation of *O. minuta* was performed as previously described (19). The electroporated cells were plated on synthetic dextrose (0.67% yeast nitrogen base without amino acids (Thermo Fisher Scientific, Waltham, MA, USA), 0.5% casamino acids (Thermo Fisher Scientific), 2% glucose (FUJIFILM Wako Pure Chemical, Osaka, Japan), 0.002% L-tryptophan (FUJIFILM Wako Pure Chemical)) with 2% agar. YPD (1% Difco™ yeast extract (Thermo Fisher Scientific), 2% Bacto™ peptone (Thermo Fisher Scientific), 2% glucose (FUJIFILM Wako Pure Chemical), 0.004% adenine (FUJIFILM Wako Pure Chemical)) was used for pre-culture cultivation, and YPD with 2% agar (Thermo Fisher Scientific) was used for cell growth on plate media. 2xYP-P6-GG medium (2% Difco™ yeast extract (Thermo Fisher Scientific), 4% Bacto™ peptone (Thermo Fisher Scientific), 0.5% glucose (FUJIFILM Wako Pure Chemical), 4% glycerol (FUJIFILM Wako Pure Chemical), 0.1 M potassium phosphate buffer, pH 6.0) and 3xYP-P6-GG medium (3% Difco™ yeast extract (Thermo Fisher Scientific), 6% Bacto™ peptone (Thermo Fisher Scientific), 0.5% glucose (FUJIFILM Wako Pure Chemical), 2% glycerol (FUJIFILM Wako Pure Chemical), 0.1 M potassium phosphate buffer, pH 6.0) were used to produce the HSA protein and the candidate biologic protein: ProteinX (lipocalin (66) derivative which is designed to attach to the calcitonin gene-related peptide). L-Histidine hydrochloride monohydrate was purchased from FUJIFILM Wako Pure Chemical. Representative clones were stored as suspensions in 17% (w/v) glycerol at –80 °C.

Table 3-1 Strains used in this study

Strain	Genotype	Harboring plasmids	Reference
tat06213	<i>ura3</i> Δ		Tsuda et al. (2023)
tat10113	<i>aox1</i> Δ <i>ura3</i> Δ		Tsuda et al. (2024)
tat19913	<i>ura3</i> Δ	pOU1/PDER-PKA, pOMEA-Z1-10746/HSA	Tsuda et al. (2024)
tat20313	<i>aox1</i> Δ <i>ura3</i> Δ	pOU1/PDER-PKA, pOMEA-Z1-10746/HSA	Tsuda et al. (2024)
tat20813	<i>ura3</i> Δ <i>prb1::aph(4)</i> ^a	pOU1/PDER-PKA, pOMEA-Z1-10746/HSA	This study
tat21213	<i>aox1</i> Δ <i>ura3</i> Δ <i>prb1::aph(4)</i> ^a	pOU1/PDER-PKA, pOMEA-Z1-10746/HSA	This study
tat21913	<i>ura3</i> Δ <i>prb1::aph(4)</i> ^a	pOU1/PDER-PKA, pOMEA-Z1-10746/ProteinX	This study
tat22313	<i>aox1</i> Δ <i>ura3</i> Δ <i>prb1::aph(4)</i> ^a	pOU1/PDER-PKA, pOMEA-Z1-10746/ProteinX	This study
tat24314	<i>aox1</i> Δ <i>ura3</i> Δ <i>prb1</i> Δ		This study
tat34315	<i>aox1</i> Δ <i>ura3</i> Δ <i>prb1</i> Δ	pOU1/PDER-PKA, pOMEA-Z1-10746/ProteinX, pOMEA-H-10746/ProteinX	This study

^a *aph(4)*: hygromycin B resistance gene, which encodes aminocyclitol phosphotransferase that inactivates hygromycin by covalently adding a phosphate group at C4-position. (GenBank Accession No. V01499.1)

Chemicals and recombinant DNA techniques

E. coli JM109, restriction enzymes, and DNA-modifying enzymes were purchased from Takara Bio (Kusatsu, Japan). The media, growth conditions, and general recombinant DNA techniques used in this study were in accordance with the work of Sambrook and Russell (22). Zeocin™ and hygromycin were purchased from Thermo Fisher Scientific and FUJIFILM Wako Pure Chemical, respectively. All oligonucleotides in this study were synthesized by FASMAC (Atsugi, Japan). Polymerase chain reaction (PCR) amplification was performed using the GeneAmp® PCR System 9700 (Thermo Fisher Scientific). Automated DNA sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (Thermo Fisher Scientific).

Plasmids used for protein production in this study

The plasmid *dprb1/pOMGPH1* was constructed as follows: part of the coding region of *OmPRB1* (accession number BAE91901.1), with HindIII and KpnI restriction enzyme sites attached at the 5'- and 3'-ends, respectively, was PCR-amplified from *O. minuta* genomic DNA using primers 1 and 2. All primers used in this study are listed in Table 3-2. The amplified *OmPRB1* partial coding region was introduced into *pOMGPH1* (65) at the XbaI and BamHI sites, resulting in *dprb1/pOMGPH1*.

The plasmid *prb1Δ/pOMU* was constructed as follows: flanking regions of *OmPRB1* were PCR-amplified using primers 3 and 4 for the deduced promoter region and primers 5 and 6 for the deduced terminator region. The fused *OmPRB1* deduced promoter-terminator fragment was amplified from a mixture of the above amplified fragments by overlapping PCR with primers 3 and 5. To remove the restriction sites HindIII and KpnI from the fused *OmPRB1* deduced promoter-terminator fragment, fragments were amplified using primers 3 and 7 for the partial promoter region and primers 5 and 8 for the partial promoter-terminator fragment. The partial fragment was amplified. The fused *OmPRB1* deduced promoter-terminator fragment without HindIII was amplified from a mixture of the above amplified fragments by overlapping PCR with primers 3 and 5. To remove the restriction site KpnI from the fused *OmPRB1* deduced promoter-terminator fragment without HindIII, a series of PCRs was performed using primers 3, 5, 9, and 10 in the same manner. The resulting fused *OmPRB1* deduced promoter-terminator fragment without HindIII and KpnI was introduced into *pOMEU* (52), resulting in the plasmid *prb1Δ/pOMU*.

The plasmid *pOMEA-Z1-10746/ProteinX* was constructed as follows: the ProteinX coding sequence (described as SEQ ID no. 89 in a patent by Kubota et al. (67)) fused with α -mating factor secretion signal sequence (α F signal) at the N-terminus and hexa-His tag at the C-terminus was designed and synthesized with XbaI and BamHI at the 5'- and 3'-ends, respectively, by GeneArt (Regensburg,

Table 3-2 Primers used in this study.

Primer #	Nucleotide Sequence (5' to 3')
1	CAAGCTTCGTTGGCAGCAGTGGAG
2	CGGTACCCGATGGAATCTCAGACA
3	AAGCTTGACATGCTGCTGTTCTCG
4	ACTGAATTGTTAGAACTTTGGTTCAAGTGGTTG
5	GGTACCAGCCGCAATCTCGGG
6	CAACCACTTGAACCAAAGTTTCTAACAATTCAG
7	GACATCGACGACATTCTCCTAGGGATAACGGGTCAGGAG
8	CTCCTGAACCCGTTATCCCTAGGAGAATGTCGTCGATGTC
9	ATTAGAGTCATTGCTTGAAGCTATTTTATGACTTTGGACA
10	TGTCCAAAGTCATAAAATAGCTTCAAGCAATGACTCTAAT
11	CTAATCGAACAATCAGCAACC
12	CAGGAAACAGCTATGAC

Germany). The synthesized fragment was introduced into pOMEA-Z1-10746 (52) at the XbaI and BamHI sites, resulting in the plasmid pOMEA-Z1-10746/ProteinX.

The plasmid pOMEA-H-10746/ProteinX was constructed as follows: the plasmid pOMEA-Z1-10746/ProteinX was double-digested with HindIII and KpnI restriction enzymes to generate the *AOX1* promoter-terminator cassette containing the ProteinX coding sequence. The resulting fragment harboring HindIII and KpnI restriction enzyme sites at the 5'- and 3'-ends was inserted into HindIII-KpnI-cleaved pOMGPH1 (65).

Disruption of *PRB1* gene in *O. minuta*

The plasmid dprb1/pOMGPH1 was introduced into protein production strains to generate *prb1* protease-deficient mutants (*prb1::aph(4)*). The transformed cells were regenerated on a synthetic dextrose plate containing 200 ng/mL hygromycin at 28 °C for 2 days. The transformants were transferred on the same plate to confirm that the transformation had been performed successfully. *prb1* protease-deficient mutants were confirmed by PCR amplification with primers 11 and 12.

prb1-null mutants (*prb1Δ*) were constructed by pop-in/pop-out method using *URA3* and 5-fluoroorotic acid (5-FOA) (FUJIFILM Wako Pure Chemical) as follows. The plasmid *prb1Δ*/pOMU was introduced into tat10113. The transformed cells were regenerated on synthetic dextrose plate medium at 28 °C for approximately 3 days. The transformants were transferred on the same selection medium to confirm that transformation had been performed successfully. The selected strain harboring

the *URA3* marker gene and duplicate promoter-terminator cassettes was cultivated in YPD medium in a test tube at 28 °C for approximately 2 days with reciprocal shaking at 300 rpm. Then, 0.1 mL of the culture broth was transferred onto 5-FOA plate medium and incubated at 28 °C for 4 days. The colonies growing on the 5-FOA plate medium were transferred onto another plate with the same medium to confirm 5-FOA resistance. The resulting clone as the *prb1*-knockout mutant was named tat24314.

Selection of strains for producing HSA and ProteinX

HSA and ProteinX production were carried out in accordance with the method of Tsuda et al. (52) with slight modifications. For the selection of protein of interest (POI)-producing cells, transformants were cultivated in individual wells of a 96-well deep-well plate at 28 °C for 4 days with reciprocal shaking at 300 rpm. After removal of the cells by centrifugation ($2,200 \times g$), the POI in the culture broth was quantified by dot blot analysis.

Dot blot analysis

Harvested supernatant was equally mixed with β -ME Sample Treatment for Tris SDS (Cosmo Bio, Tokyo, Japan) and heated at 99.5 °C. The heat-treated sample was then spotted onto nitrocellulose membranes, 0.45 μ m (Bio-Rad Laboratories, Hercules, CA, USA). Dried membranes were blocked with 2% ECL™ Advance Blocking Reagent (Cytiva, Uppsala, Sweden) in PBS with 0.1% (v/v) Tween 20 (PBS-T) for 1 h. Blocked membranes were washed with PBS-T and treated with Anti-Albumin, Human, Goat-Poly, HRP (Bethyl Laboratories, Montgomery, TX, USA) in PBS-T for HSA, and with Anti-His HRP Conjugates (Qiagen, Hilden, Germany) for ProteinX. After 1 h of treatment with the detection antibody, the resulting membranes were washed with PBS-T three times and analyzed using an ECL Advance Western Blotting Detection Kit (Cytiva).

Bioreactor cultivation

In a preculture, frozen stocks were thawed and streaked onto YPD plates. The plates were then incubated at 28 °C for 2 days. Colonies grown on the YPD plates were cultured in test tubes containing 5 mL of YPD at 28 °C with reciprocal shaking at 250 rpm for 24 h. Ten milliliters of collected cultured broth was transferred to baffled flasks containing 40 mL of 2xYP-P6-GG medium and cultured at 28 °C with rotary shaking at 250 rpm for 16 h. Fed-batch culture was then carried out in a 3-L bioreactor (Biott, Tokyo, Japan) containing 1.5 L of 3xYP-P6-GG medium. The inoculation volume was 5% (v/v) of the starting volume. The stirrer speed was varied to keep the dissolved oxygen concentration at 25% at a constant aeration rate. After 8 h of inoculation, glycerol solution was

continuously supplied for 12 h at 0.33 g/h. For the methanol-utilizing strains, methanol was continuously added at 0.3% (v/v) after bolus feeding of methanol at 1.0% (v/v). For the *aox1Δ* strain, glycerol was continuously added at 0.083% (w/v) after bolus feeding of methanol at 0.5% (v/v). To suppress generation of the revertant with active *PRB1* via pop-out of *prb1*-integrated plasmid, hygromycin was supplied at 50 ng/mL/day in the culture media. As a reinforced supply of dual nitrogen sources, Difco™ yeast extract and L-histidine hydrochloride monohydrate were supplied at 0.5%/day and 6.5 mM/day, respectively, as a mixed feeding solution. Under pH-controlled conditions, pH was adjusted to 7 using 25% ammonia solution (FUJIFILM Wako Pure Chemical) after methanol induction. The concentrations of carbon sources in the culture media were measured using a flow injection analyzer (BF-9; Oji Scientific Instruments, Amagasaki, Japan). In this system, the carbon sources were converted to hydrogen peroxide by the enzymes immobilized on columns, and the amount of generated hydrogen peroxide was measured by the electrochemical method.

Fermentation in large-scale bioreactors, including 30-L and 4,500-L scales, was carried out in a fundamentally similar manner, with the following modifications. In a preculture, Frozen cell bank was cultured in 2-L baffled flasks containing 250 mL of YPD at 28 °C with reciprocal shaking at 180 rpm for 24 h. Forty milliliters of collected cultured broth was transferred to 2.8-L baffled flasks containing 1.0 L of YPD medium and cultured at 28 °C with rotary shaking at 130 rpm for 24 h. The inoculation volume was 0.18% (v/v) of the starting volume.

SDS-PAGE

The cultured broth containing secreted HSA was heated at 70 °C for 10 min in NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA, USA). The heat-treated samples were analyzed with the NuPAGE® System (Invitrogen) using 10% Bis-Tris gels (12 wells, 7 cm wide, 1 mm thick) and NuPAGE® MES SDS Running Buffer (Invitrogen) for 30 min at a constant voltage of 200 V, followed by Coomassie Brilliant Blue (CBB) staining using EzStain Aqua (Atto, Tokyo, Japan). Stained HSA protein bands were analyzed by densitometry using Albumin, from Human Serum (FUJIFILM Wako Pure Chemical), as a standard.

Western blot analysis

Western blot analysis for antibodies was carried out in accordance with the method of Suzuki et al. (19). Anti-Albumin, Human, Goat-Poly, HRP (Bethyl Laboratories) was used as a detection antibody. Detection was performed using the ECL Advance Western Blotting Detection Kit (Cytiva).

RP-HPLC analysis to measure ProteinX concentration

Secreted ProteinX samples were quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis using YMC-Pack Ph Column (ID: 150 × 2.0 mm, particle size: 3 µm, pore size: 12 nm) (YMC, Kyoto, Japan). Analyses were performed using a Prominence (Shimadzu, Kyoto, Japan) at 50 °C. Mobile phase A was trifluoroacetic acid/water (1:1000 v/v), while mobile phase B was acetonitrile/water (50:50 v/v). The separation was obtained at a flow rate of 0.4 mL/min with a gradient program that allowed for 0 min at 35% B followed by a 12.5 min step that raised eluent B to 70%. Then, washing at 100% B and equilibration at 35% B were performed in a total analysis time of 20 min.

Evaluation of reactivity of ProteinX by cAMP detection assay

The cAMP detection assay using HitHunter[®] (Eurofins, Fremont, CA, USA) was conducted to evaluate the reactivity of ProteinX according to the manufacturer's instructions with minor modifications. In this assay, the mixture of calcitonin gene-related peptide (Peptide Institute, Inc., Osaka, Japan) and ProteinX was used as a test substrate, The human SK-N-MC -HTB-10 cell line (ATCC, Manassas, VA, USA) was used for testing.

Statistical analyses

Statistical significance of the difference in HSA productivity was tested using a two-tailed Tukey-Kramer test. Probability values (p) < 0.05 were considered statistically significant.

Results

Comparison of the generated mutants for heterologous protein production

In previous studies on the use of the methylotrophic yeast *O. minuta* for heterologous protein production, the alcohol oxidase-knockout mutant (*aox1*Δ) and the Prb1 protease-deficient mutant (*prb1::aph(4)*) were generated separately by genetic engineering and each mutation was functionally evaluated in an *O. minuta* (51, 52, 63). Therefore, in this study the *O. minuta* double mutant in which the *AOX1* and *PRB1* genes were disrupted was evaluated as a host for producing heterologous proteins. Recombinant HSA was used as a model protein and HSA-producing strains were generated and cultivated as described in the Materials and Methods. The secreted HSA was analyzed and quantified by dot blotting, showing that the double mutant secreted more HSA than the other strains (Fig. 3-1). Representative dot blot results are shown in Fig. 3-2. The cultured samples were analyzed by SDS-PAGE, which revealed that the 60 kDa band corresponds to the theoretical molecular weight of HSA (Fig. 3-3). The double mutant strain (tat21213) also produced 1.3-fold more HSA than tat19913 strain, which has intact *AOX1* and *PRB1* (designated as parent strain). These results were consistent with the findings of SDS-PAGE analysis under reducing conditions (Fig. 3-3). The quantified intensities of protein bands detected by SDS-PAGE were presented in Fig. 3-4. Meanwhile, samples obtained from the parent strain and *aox1*Δ strain (tat20313) harboring the *PRB1* gene showed faint protein bands corresponding to a molecular weight of around 40 kDa. This observation suggested the possibility that HSA might have been degraded by Prb1 protease.

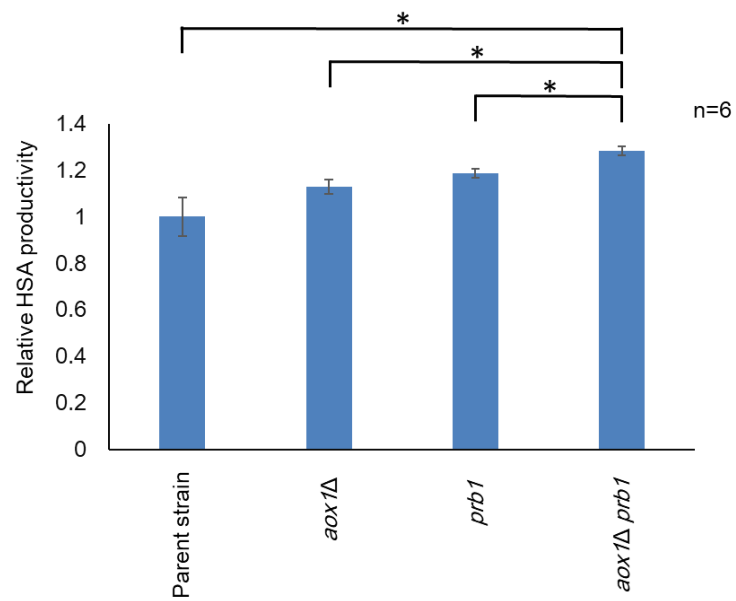


Fig. 3-1 Relative production levels of secreted recombinant HSA in culture broth determined by quantitative dot blot analysis. tat19913 (parent strain), tat20313 (*aox1Δ*), tat20813 (*prb1*), and tat21213 (*aox1Δ prb1*) cells were cultured using 2xYP-P6-GG medium at 28 °C for 4 days in 96-well deep-well plate, and culture supernatants were subjected to dot blot. Values are shown as mean ± S.D. of six independent experiments. * $p < 0.05$.

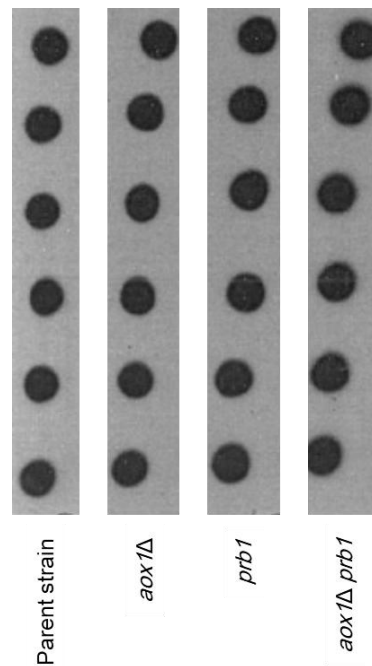


Fig. 3-2 Representative results of dot blot analysis of secreted recombinant HSA in culture broth. tat19913 (parent strain), tat20313 (*aox1Δ*), tat20813 (*prb1*), and tat21213 (*aox1Δ prb1*) cells were cultured using 2xYP-P6-GG medium at 28 °C for 4 days in 96-well deep-well plate, and culture supernatants were subjected to dot blot. n=6

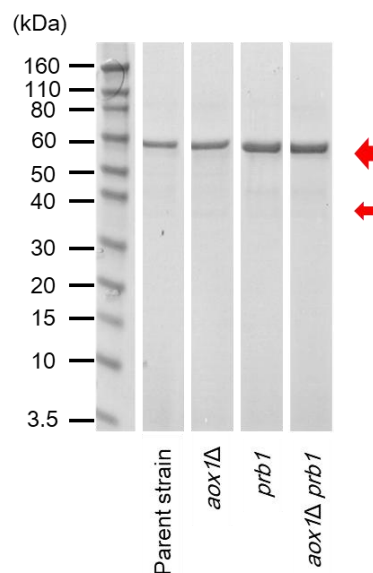


Fig. 3-3 CBB staining/SDS-PAGE analysis under reducing conditions of secreted recombinant HSA. tat19913 (parent strain), tat20313 (*aox1Δ*), tat20813 (*prb1*), and tat21213 (*aox1Δ prb1*) cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days, and culture supernatants were subjected to SDS-PAGE. The upper arrow and lower arrow indicate an intact and degraded HSA, respectively.

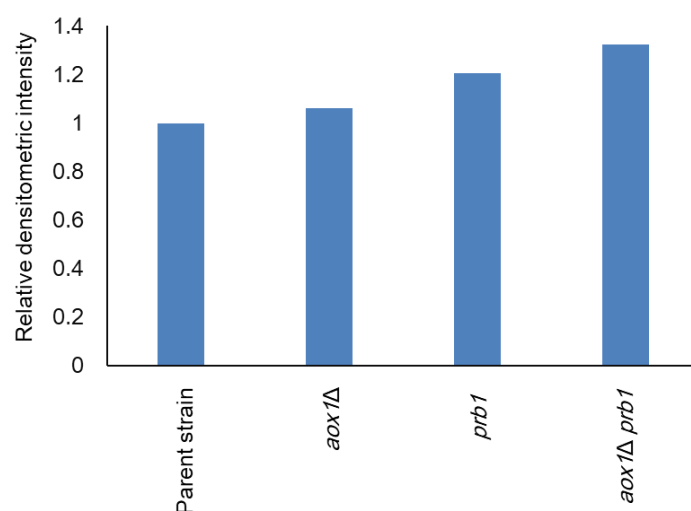


Fig. 3-4 Quantitative densitometric analysis of CBB Staining/SDS-PAGE under reducing conditions of secreted recombinant HSA. tat19913 (parent strain), tat20313 (*aox1Δ*), tat20813 (*prb1*), and tat21213 (*aox1Δ prb1*) cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days, and culture supernatants were subjected to SDS-PAGE.

Establishment of fermentation conditions in a 3-L bioreactor

To enhance the production of biopharmaceuticals by yeast host cells, research has primarily focused on optimizing fermentation conditions, including media composition, temperature, pH, inoculation conditions, and production kinetics (68). As the double mutant in this study exhibited the highest production of HSA in small-scale cultivation (Fig. 3-1), optimization of fermentation conditions using the double mutant strain with the parent strain as the reference to compare was conducted on the scale of a bioreactor. I evaluated several fermentation conditions, including a reinforced supply of dual nitrogen sources and pH control. In detail, methanol as a carbon source was continuously supplied at 4.5 g/h after bolus feeding of methanol at 1.0% for the parent strain (tat19913) of *O. minuta* in Jar 1 and Jar 2. For *aox1Δ prb1* double mutant strain (tat21213) in Jar 3 and Jar 4, glycerol as a carbon source was continuously supplied at 3.3 g/h after bolus feeding of methanol at 1.0% and daily bolus feeding of methanol at 0.3% was provided as the fermentation time increased. For Jar 2 and Jar 4, a mixture of Difco™ yeast extract and L-histidine hydrochloride monohydrate was supplied at 0.5%/day and 6.5 mM/day, respectively, as a reinforced supply of dual nitrogen sources. For Jar 4, pH was adjusted to 7 using 25% ammonia solution after methanol induction. The results showed that the double mutant under the fermentation conditions including a reinforced supply of dual nitrogen sources and pH control in Jar 4 achieved the highest HSA production, as analyzed by SDS-PAGE and quantified

by densitometry. For the parent strain and the double mutant in Jar 1 and Jar 3, respectively, HSA secretion reached a plateau in the middle of the fermentation period, which lasted for 10 days. In contrast, in the parent strain cultured under a reinforced supply of dual nitrogen sources in Jar 2, the secreted HSA was rapidly degraded in the early phase of the fermentation period (Fig. 3-5). Meanwhile, in Jar 4, the amount of secreted HSA increased without reaching a plateau until 21 days after inoculation, with the concentration of secreted HSA peaking at around 7.5 g/L (Fig. 3-6A). The maximum concentrations of the secreted HSA in each bioreactor are summarized in Table 3-3. For further investigation of the quality of secreted HSA, Western blotting using anti-HSA antibody was conducted to detect multiple protein bands (Fig. 3-6B). Bands with a molecular weight of approximately 60 kDa corresponding to the theoretical molecular weight of HSA were identified, similar to the findings of SDS-PAGE analysis in small-scale cultivation (Fig. 3-2). Protein bands with molecular weights of 110 kDa and 40 kDa were also identified, which might reflect dimeric HSA and degraded HSA, respectively. To characterize the effect of the fermentation conditions on cultivation profiles, optical density (OD) at 600 nm (OD_{600}) of the cultured samples was measured over the course of fermentation (Fig. 3-6C). During fermentation, the OD_{600} values in Jar 2 and Jar 4 did not decrease, while those in Jar 1 and Jar 3 started to decrease 4 days after inoculation. These results indicated that the reinforced supply of dual nitrogen sources was effective at maintaining cell density, while it was not effective at suppressing protein degradation in the parent strain under methanol-induction conditions without pH control.

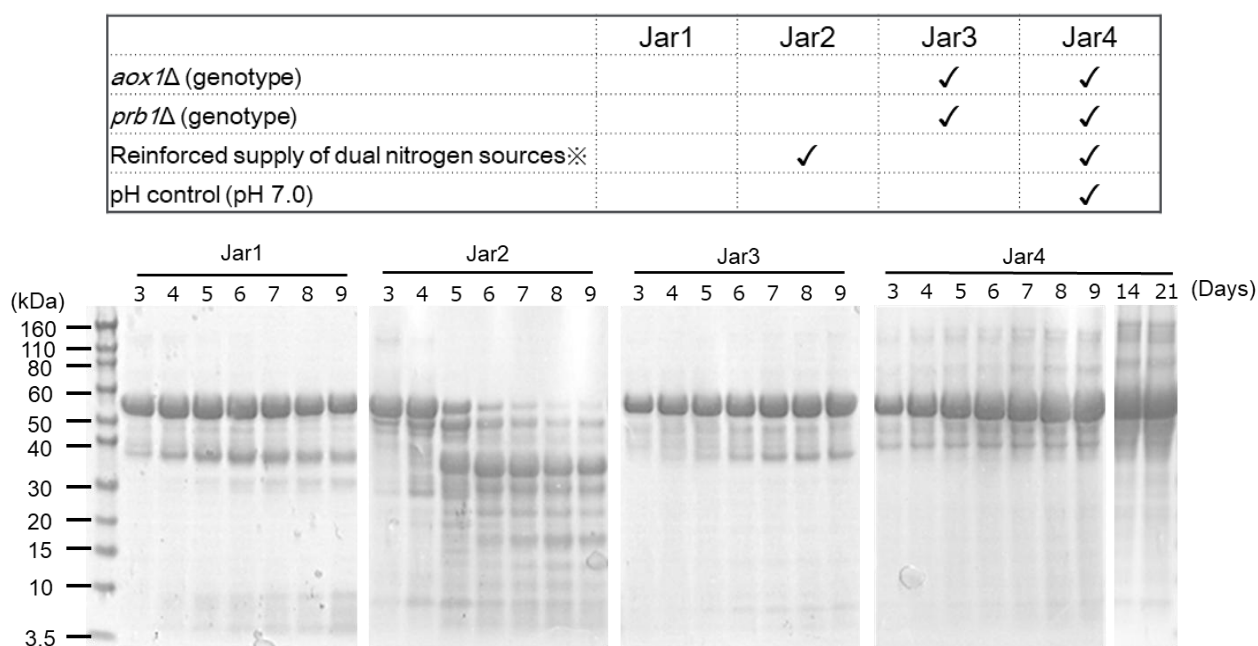


Fig. 3-5 CBB staining/SDS-PAGE analysis under reducing conditions of secreted recombinant HSA. Cells were cultured in 3xYP-P6-GG medium at 28 °C, and culture supernatants were subjected to SDS-PAGE. Days indicate sampling points after inoculation. Jar 1: Fermentation using tat19913 (parent strain) under standard fermentation conditions, Jar 2: Fermentation using tat19913 under optimized fermentation conditions such as supply of nitrogen bases, Jar 3: Fermentation using tat21213 (*aox1*Δ *prb1*) under standard fermentation conditions, Jar 4: Fermentation using tat21213 under optimized conditions such as supply of nitrogen bases and pH control.

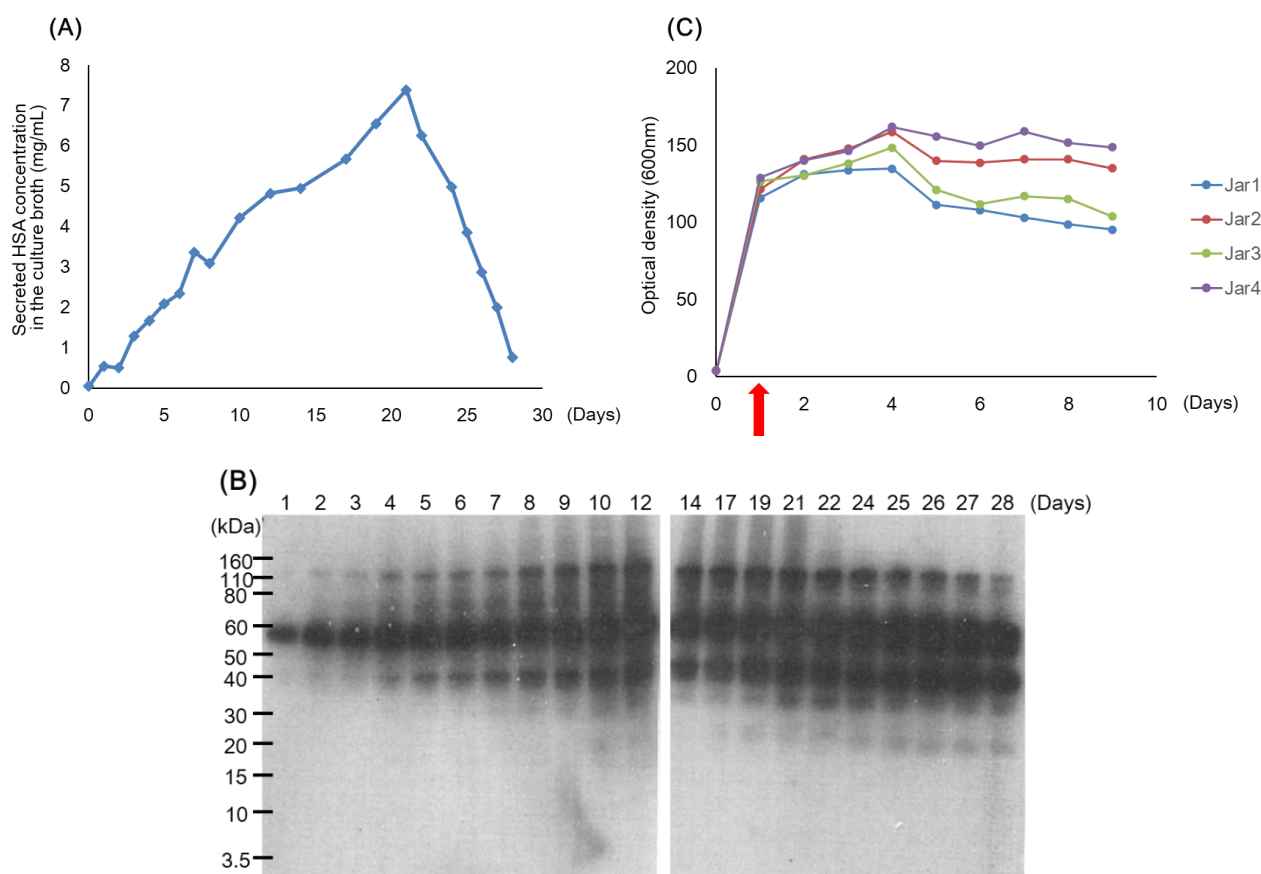


Fig. 3-6 (A) The concentration of secreted recombinant HSA in Jar4. The x-axis indicates the time points after inoculation. (B) Western blot analysis under reducing conditions of secreted recombinant HSA in Jar4. The x-axis indicates the time points after inoculation. (C) Optical density measurements at a wavelength of 600 nm (OD₆₀₀) in Jar 1, 2, 3, and 4. Days indicate sampling points after inoculation. The red arrow indicates the induction start point.

Table 3-3 The comparison of maximum concentrations of secreted HSA in each bioreactor

No.	Maximum concentration of secreted HSA (g/L)	Cultivation time (Days after inoculation)
Jar1	2.9	4
Jar2	2.4	3
Jar3	1.5	8
Jar4	7.5	21

Applying the established fermentation condition to the industrial-scale manufacturing of the candidate biologic

Since the fermentation condition using the *aox1Δ prb1* double mutant with optimized fermentation parameters achieved the highest production, I attempted to apply the condition to the manufacturing of a candidate biologic protein, ProteinX, on an industrial scale. To compare the productivity of ProteinX between the *prb1* protease-deficient mutant and *aox1Δ prb1* double mutant, a study of the small-scale production of ProteinX in 96-well deep-well plate was performed. The cultured samples were analyzed by SDS-PAGE, which revealed that the 20 kDa band corresponds to the theoretical molecular weight of ProteinX (Fig. 3-7).

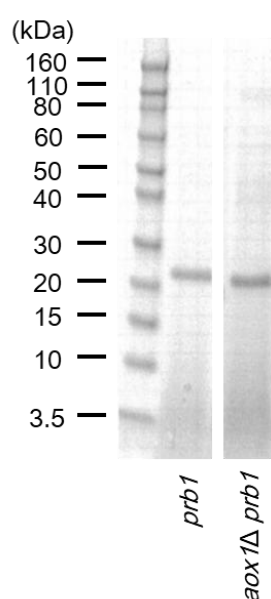


Fig. 3-7 CBB staining/SDS-PAGE analysis under reducing conditions of secreted ProteinX in 96-well deep-well plate. tat21913 (*prb1*) and tat22313 (*aox1Δ prb1*) cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days, and culture supernatants were subjected to SDS-PAGE.

As described in my previous study, a culture process using the *O. minuta aox1Δ* strain achieved higher heterologous protein production than the parent strain under low-methanol conditions (63). Hence, the double mutant was selected as a host for producing ProteinX. To avoid generation of the revertant with active *PRB1*, a *prb1*-null mutant (*prb1Δ*) was generated (tat24314) and transformed with plasmids for co-overexpressing chaperones and ProteinX production, resulting in the strain tat34315 for industrial-scale manufacturing, into which two different plasmids carrying the ProteinX gene were integrated.

Prior to the fermentation manufacturing on an industrial scale, a preliminary triplicate intermediate-scale (30-L) fermentation study was conducted to confirm the robustness of the established fermentation condition, including a reinforced supply of dual nitrogen sources and pH control (Fig. 3-8). From the results of the triplicate fermentation, similar growth curves and levels of ProteinX production were observed among the triplicate experiments. The cultured samples were analyzed by SDS-PAGE, which also revealed similar bands representing ProteinX among the triplicate fermentation samples (Fig. 3-9). These results confirmed the robustness of the fermentation condition.

Therefore, I adopted the established fermentation condition for the manufacture of ProteinX on an industrial scale. Specifically, the manufacturing of ProteinX in a 4,500-L bioreactor was carried out in accordance with the established fermentation condition with slight modifications with regard to the preculture to prepare a sufficient inoculation volume (Fig. 3-10). The optical density and the concentration of secreted ProteinX in the industrial-scale fermentation were lower than those observed in the intermediate-scale fermentation. All fermentation parameters were set as intended and monitored. Two parameters, pH and stirrer speed, are shown in the following graphs. (Fig. 3-11). There was little fluctuation in fermentation conditions such as temperature, dissolved oxygen, and air supply. The stirrer speed was increased in response to growing oxygen demand accompanying the growth of yeast cells. After the stirrer speed reached its maximum, methanol was supplied as an inducer of protein production. pH was adjusted to 7 after methanol induction and maintained throughout the fermentation period, which lasted for 6 days. The manufacturing process on an industrial scale was completed successfully, although the OD₆₀₀ and the amount of ProteinX produced were slightly lower than expected from the findings of the intermediate-scale experiment. As the result of fermentation, 1.87 kg of product was obtained successfully through centrifugation and filtration. Harvested crude product was purified and biological activity of purified ProteinX was confirmed by bioassay.

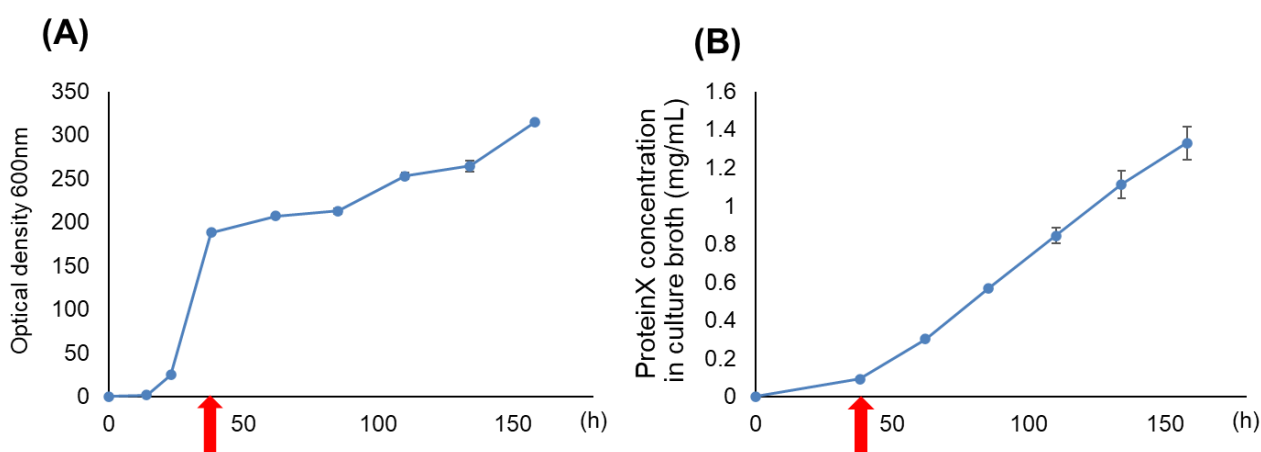


Fig. 3-8 Triplicate fermentation study performed in a 30-L bioreactor. tat34315 cells were cultured in 3xYP-P6-GG medium at 28 °C. (A) Optical density measurements at a wavelength of 600 nm (OD_{600}). Values are shown as mean $\pm$ S.D. of three independent experiments. (B) Secreted ProteinX concentration in culture broth measured by RP-HPLC. Values are shown as mean $\pm$ S.D. of three independent experiments. The x-axis indicates the time points after inoculation. The red arrow indicates the induction start point.

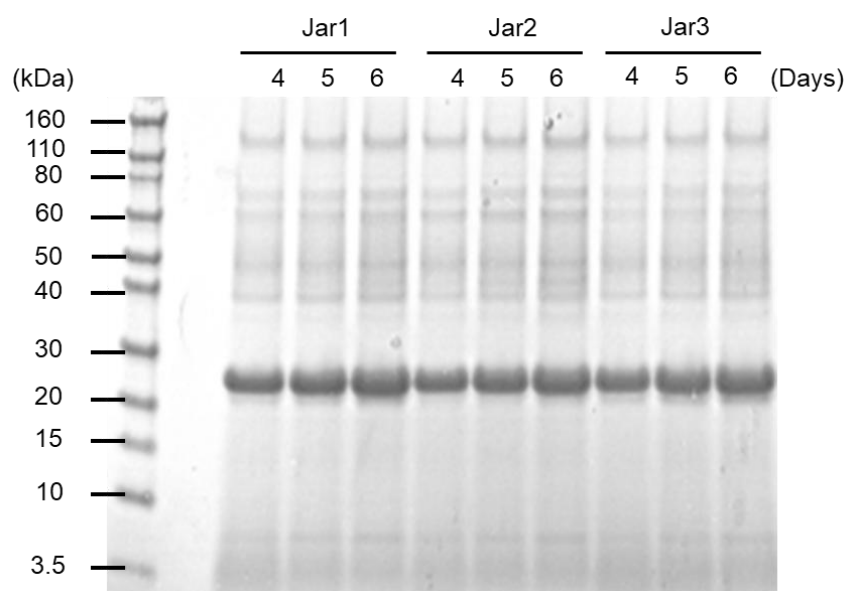


Fig. 3-9 CBB staining/SDS-PAGE analysis under reducing conditions of secreted ProteinX performed in triplicate. Days indicate sampling points after inoculation.

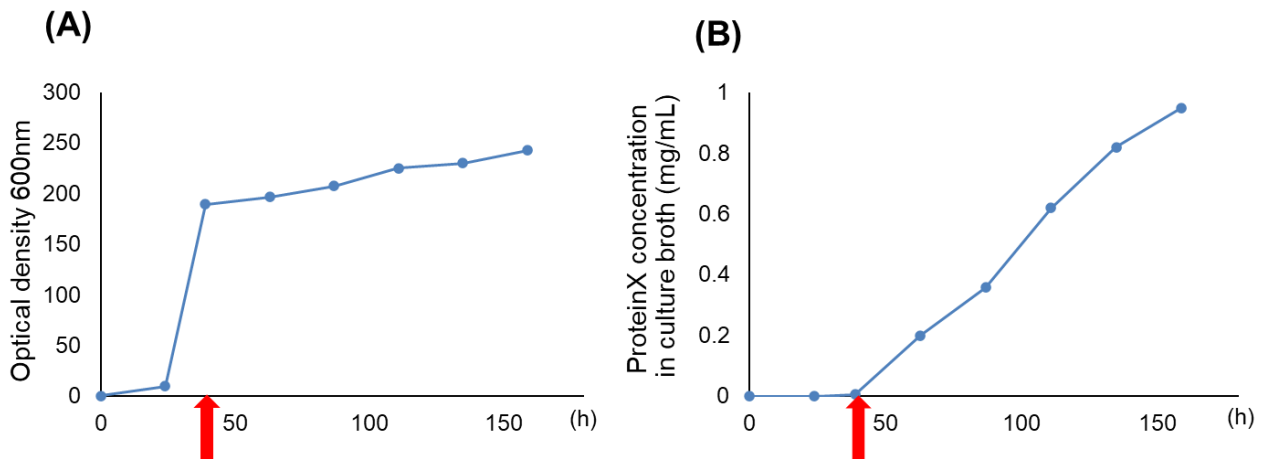


Fig. 3-10 Industrial-scale fermentation conducted in a 4,500-L bioreactor. tat34315 cells were cultured in 3xYP-P6-GG medium at 28 °C. (A) Optical density measurements at a wavelength of 600 nm (OD_{600}). (B) Secreted Protein-X concentration in culture broth measured by RP-HPLC. The x-axis indicates the time points after inoculation. The red arrow indicates the induction start point.

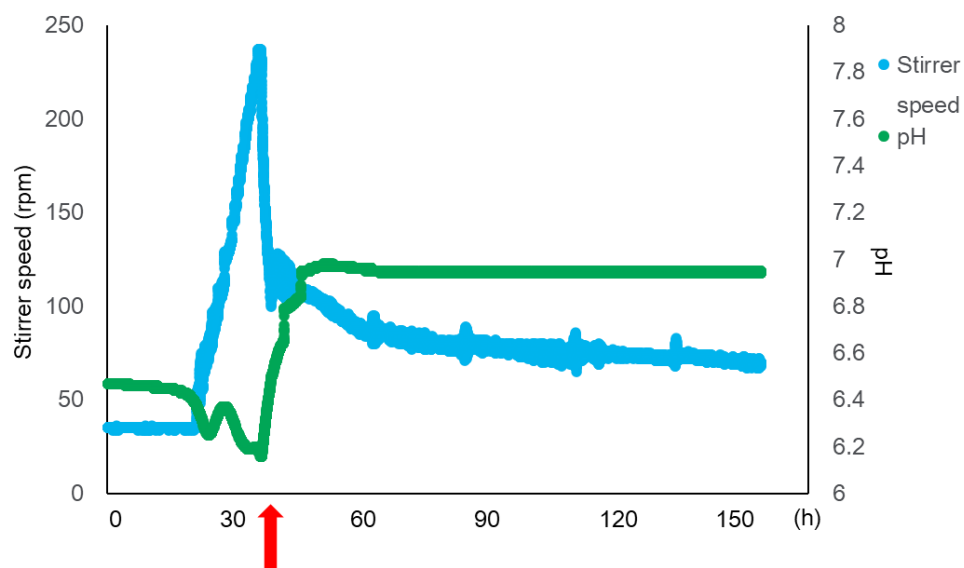


Fig. 3-11 Cultivation profile of industrial-scale fermentation conducted in a 4,500-L bioreactor. The x-axis indicates the time points after inoculation. The red arrow indicates the induction start point.

Discussion

The development of a process for producing heterologous recombinant proteins typically involves several key steps: selecting a suitable host, screening the constructed production strains, and optimizing the fermentation conditions. Yeast heterologous protein production systems are well known for high production yields, but yeast sometimes exhibits strong protease activity, which may cause degradation of the expressed POI. In *K. phaffii*, several multiple-protease-deficient strains have been generated to prevent this. Among them, the *pep4*- and *prb1*-knockout mutants are still the most effective and widely employed (11). In contrast, the types of protease-deficient hosts that consistently enhance heterologous protein production in *O. minuta* remain unclear. In one study, it was found that *O. minuta pep4*, *prb1*, and *yps1* protease-deficient strains were effective at maintaining antibody production intact (18), but generally protease-deficient strains show slower growth rates, lower transformation efficiency, greater susceptibility to mechanical stresses, and reduced viability (69). I have reported that Prb1 was identified as the major protease in the *O. minuta* culture broth and that use of the *prb1*-deficient mutant was effective for suppressing protein degradation (51). Although a *pep4*- and *prb1*-deficient mutant has been developed, it showed weaker growth and low productivity, contrary to my expectations (data not shown). It has been also reported that protease-deficient strains showed typically slower growth rates, lower transformation efficiencies and reduced viability (69). I thus adopted the *prb1* protease-deficient mutant as a standard protease-deficient mutant in *O. minuta*. In this mutant, methanol is available as the sole carbon source and the methanol-inducible promoter shows strong gene expression under methanol feeding conditions, as one of the most advantageous features of methylotrophic yeast. However, methanol and its metabolites, including formaldehyde and formic acid, are toxic and can cause acidosis and other clinical symptoms. To address the toxicity associated with methanol-based promoter induction, several approaches have been explored in *K. phaffii*, such as protein production under de-repression conditions, de-repressed promoter engineering, and transcription factor engineering (46, 47, 49).

Building on this previous work, I have reported that a culture process using the *O. minuta aox1Δ* mutant achieved higher heterologous protein production than the parent strain under low-methanol conditions (63). In this study, the *aox1Δ* mutant was thus employed to produce the POI under low-methanol conditions.

In previous work, I only developed the *prb1* protease-deficient mutant (51) and the *aox1Δ* mutant (63) separately; however, the *aox1Δ prb1Δ* double mutant had not been evaluated as a heterologous protein production host. This work was thus implemented to analyze this double mutant.

Efforts to enhance POI production in methylotrophic yeast systems have focused on optimizing culture conditions, including temperature, pH, inoculation, dissolved oxygen, and key substrates such as carbon and nitrogen sources (68). I have investigated the appropriate continuous feed rate of methanol and glycerol for parent strains and the *aox1* Δ mutant, to avoid the accumulation of these carbon sources in culture broth (63). In this study, I focused on optimization of the most promising strain and fermentation conditions, rather than performing a strict head-to-head comparison of strains under the same conditions. While this approach makes it difficult to evaluate effects of individual parameters, it reflects a practical strategy for process development. The combination of the different production host, reinforced supply of dual nitrogen sources, and pH control was evaluated, as shown in Fig. 3-5. As expected, a culture process using the double mutant under mixed-feed conditions with reinforced nitrogen supply and pH control demonstrated the highest production of HSA, resulting in a yield of 7.5 g/L. The HSA production level was markedly improved by medium optimization, while approximately 7.2 mL/L/24h of methanol was consumed because the strain that can utilize methanol as a carbon source (Mut⁺) was employed as the host. Meanwhile, only 0.3 mL/L/24 h of methanol was supplied in the culture process using the double mutant under mixed-feed conditions during fermentation. This reduced consumption of methanol in the culture process would be advantageous for the production of HSA. However, to apply HSA produced in *O. minuta* for medical use, it would be necessary to prove its comparability to the current HSA product manufactured using human plasma and its safety as a pharmaceutical product.

Regarding pH control during fermentation, the optimal pH varies among host strains and depending on the POI because of its impact on yeast cell growth, product production, protein folding, and protease activity (3). In *K. phaffii*, a reduction of the pH from 5 to 4 increased the production of cellulose binding module of *Candida antarctica* lipase B by 50% (70). In contrast, among intensive investigations of yeast proteases, it has been revealed that the major protease Pep4 is localized in vacuoles and has an acidic isoelectric point (71). To suppress acidic protease activity, pH was increased to 7 after methanol induction in fermentation conditions using the *aox1* Δ *prb1* Δ double mutant.

During the growth and production phase, it is essential to suitably supplement the nutrients consumed by the production host to prevent the depletion of the required nutrients. Supplementation of a chemically defined medium containing specific amino acids or metabolic intermediates has been shown to enhance heterologous protein production in *Saccharomyces cerevisiae* (72). In preliminary analysis of the concentration of free amino acids in cultured broth by LC/MS, histidine was markedly consumed and depleted as the fermentation continued (data not shown). To compensate for the loss of histidine and other nitrogen sources, a mixture of yeast extract and L-histidine hydrochloride

monohydrate was supplied as a reinforced supply of dual nitrogen sources, resulting in the improved production and secretion of HSA by the double mutant under pH-controlled conditions (Fig. 3-5).

Also, optimization of fermentation period is crucial parameter to develop the manufacturing process in industrial scale. It is well-known that prolonged fermentation duration sometimes caused proteolysis issue, especially during high cell density cultivation (11). The cultivation period should be set carefully to prevent undesirable events, including degradation of POI, filtration clogging caused by cell derived-debris, and other potentially undesirable outcomes.

As an alternative approach to maintaining the production system at the optimal state for improved productivity, continuous biomanufacturing has attracted attention. For example, in *K. phaffii*, a continuous fermentation process was developed using the Mut⁺ strain for improved production of hepatitis B surface antigen, in comparison with that in the fed-batch process (73). However, especially at the industrial scale, there are operational difficulties such as the need for tightly controlled conditions and robust monitoring methods, along with the risk of contamination. Additionally, a genetically stable host system would be required for long-term operation (74), so a fed-batch mode using a stirred tank, instead of continuous fermentation, has been adopted in the majority of industrial fermentation processes (75).

While industrial-scale production of valuable proteins, including enzymes and biologics, has been widely explored, challenges remain due to substantial differences in optimal conditions between small- and large-scale processes. Addressing these challenges requires a thorough understanding of key scale-up parameters and the impact of inherently fluctuating environmental conditions in large-scale fermentations. Differences in terms of nutrient concentrations, oxygen availability, pH, pressure, shear stress, and other parameters, compared with those in small-scale processes, would affect biomass accumulation, substrate yield, and productivity in industrial-scale fermentation (2). Among these parameters, oxygen availability is one of the most important factors for protein production using methylotrophic yeast hosts (76). Especially in the industrial large-scale cultivation, a sufficient oxygen supply is required for methanol oxidation by alcohol oxidase, which has critical role to utilize methanol as a carbon source in methylotrophic yeast. However, because I used *O. minuta* host strains, in which *AOX1* was inactivated, a culture under lower oxygen concentration compared to the parent strain could be efficiently applied in this study. Large-scale fermentation in particular also suffers from the issue of overheating of the culture associated with methanol consumption (61). On an industrial scale, it is thus preferable to use the *aox1*Δ strain with restricted methanol and glycerol. In a large-scale reactor, increased hydrostatic pressure leads to a higher dissolved CO₂ concentration. Notably, elevated dissolved CO₂ levels have been reported to raise the energy demand for maintaining homeostasis,

ultimately reducing biomass and product yield in *S. cerevisiae* (77). Thus, the increased energy for cellular maintenance might also result in ProteinX production in industrial-scale manufacturing being unexpectedly lower than that in intermediate-scale manufacturing.

In this study, I established the optimal fermentation conditions using the *O. minuta aox1Δ prb1Δ* double mutant under low-methanol conditions, a reinforced supply of dual nitrogen sources, and pH control. Moreover, I applied the fermentation condition to the manufacture of the candidate biologic protein ProteinX, which was successfully achieved in an industrial 4,500-L scale bioreactor. The HSA and ProteinX have been successively manufactured with the high potential by *O. minuta*-based production system for various heterologous protein. It was expected that this system would be applied to produce the other useful proteins based on the findings in this study.

Chapter II

Applications of developed *Ogataea minuta*-based heterologous protein expression system in biopharmaceutical protein production

Section II-1

Production of Kex2 proteinase in a genetically modified *Ogataea minuta* system

Introduction

Kex2 protease (Kex2) is a calcium-dependent serine protease homologous to the mammalian proprotein convertase family, including furin. This enzyme plays a key role in the maturation of biologically active proteins by cleaving precursor proteins at specific dibasic amino acid sequences (78). Its function is tightly regulated by its localization within the secretory pathway. Immunolocalization studies identified Kex2 in the trans-Golgi network (TGN) of *Saccharomyces cerevisiae* (79). The localization of Kex2 is mediated by a specific sorting signal in its C-terminal cytosolic tail. Studies indicate that clathrin inactivation affects Kex2 localization through mechanisms independent of this trans-Golgi network localization signal, suggesting a complex regulatory system (80). Furthermore, the proregion of Kex2 is essential for its biosynthesis and requires a C-terminal basic residue for proper function (81). The cleavage specificity of Kex2 has been extensively characterized; genetic assays have elucidated its recognition of the P2 position in substrates *in vivo* (82). This high specificity makes Kex2 a valuable tool in biotechnology, where it is used for both *in vivo* and *in vitro* processing of recombinant proteins, particularly for precise removal of fusion tags during purification and activation steps of prohormone or physiologically active peptides (83).

In this section, Kex2 expression was attempted using the *O. minuta*-based protein production system developed in Chapter I. Expressed Kex2 was subjected to SDS-PAGE and detected protein bands were compared by densitometry. Moreover, enzyme activity of expressed Kex2 was analyzed by Kex2 enzyme activity assay.

Materials and Methods

Yeast strains, plasmids, and media

O. minuta tat06213 (52) and tat10113 (63) was used as a host strain for protein production. The plasmid pOU1/PDER-PKA was introduced to generate strains that co-overexpress chaperones for protein production, namely, PDI1, ERO1, and KAR2, to improve the productivity of the protein of interest (19). Codon-optimized DNA sequences encoding C-terminal domain-deleted variant Kex2 protease, described by Magota et al. (84) (engineered from GenBank accession number: M22870.1), fused with the α -mating factor secretion signal (α F signal) sequence at N-terminus and 9His-tag at C-terminus were synthesized with XbaI and BamHI at the 5' and 3' ends, respectively, by GeneArt™ (Regensburg, Germany). The synthesized fragments were introduced into pOMEA-Z1-10746 (52) at the XbaI and BamHI sites, resulting in pOMEA-Z1-10746/Kex2. Plasmids pOU1/PDER-PKA and pOMEA-Z1-10746/Kex2 were introduced into tat06213 to generate the “parent” strain as a control. Plasmids pOU1/PDER-PKA, pOMEA-Z1-10746/Kex2, and dprb1/pOMGPH1 were introduced into *O. minuta* tat10113 to generate the “*aox1Δ prb1*” strain.

Chemicals and recombinant DNA techniques

E. coli JM109, restriction enzymes, and DNA-modifying enzymes were purchased from Takara Bio (Kusatsu, Japan). The media, growth conditions, and general recombinant DNA techniques used in this study were in accordance with the work of Sambrook and Russell (22). Zeocin™ and hygromycin were purchased from Thermo Fisher Scientific and FUJIFILM Wako Pure Chemical, respectively. All oligonucleotides in this study were synthesized by FASMAC (Atsugi, Japan). Polymerase chain reaction (PCR) amplification was performed using the GeneAmp® PCR System 9700 (Thermo Fisher Scientific). Automated DNA sequencing was performed on an ABI PRISM® 3700 DNA Analyzer (Thermo Fisher Scientific).

Cultivation in a 96-well deep-well plate

Kex2 production was carried out in accordance with the method of Tsuda et al. (85). Briefly, transformants were inoculated in 800 μ L 2xYP-P6-GG medium containing 0.5% glucose and 0.5% glycerol and cultivated in individual wells of a 96-well deep-well plate at 28 °C for 4 days with reciprocal shaking at 300 rpm. For the methanol-utilizing strains, methanol was added daily at 1.0% (v/v) via bolus feeding starting 2 days after inoculation. For the *aox1Δ* strain, methanol was first added at 0.5% (v/v) via bolus feeding starting 2 days after inoculation, followed by daily bolus feeding of

glycerol at 0.5% (w/v). After removal of the cells by centrifugation ($2,200 \times g$), the Kex2 in the culture broth was quantified by dot blot analysis and Kex2 activity assay.

Dot blot analysis

Harvested supernatant was equally mixed with β -ME Sample Treatment for Tris SDS (Cosmo Bio, Tokyo, Japan) and heated at 99.5 °C. The heat-treated sample was then spotted onto nitrocellulose membranes, 0.45 μ m (Bio-Rad Laboratories, Hercules, CA, USA). Dried membranes were blocked with 2% ECL™ Advance Blocking Reagent (Cytiva, Uppsala, Sweden) in PBS with 0.1% (v/v) Tween 20 (PBS-T) for 1 h. Blocked membranes were washed with PBS-T and treated with Anti-Albumin, Human, Goat-Poly, HRP (Bethyl Laboratories, Montgomery, TX, USA) in PBS-T for HSA, and with Anti-His HRP Conjugates (Qiagen, Hilden, Germany) for Kex2. After 1 h of treatment with the detection antibody, the resulting membranes were washed with PBS-T three times and analyzed using an ECL Advance Western Blotting Detection Kit (Cytiva).

SDS-PAGE

The cultured broth containing secreted HSA was heated at 70 °C for 10 min in NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA, USA). The heat-treated samples were analyzed with the NuPAGE® System (Invitrogen) using 10% Bis-Tris gels (12 wells, 7 cm wide, 1 mm thick) and NuPAGE® MES SDS Running Buffer (Invitrogen) for 30 min at a constant voltage of 200 V, followed by Coomassie Brilliant Blue (CBB) staining using EzStain Aqua (Atto, Tokyo, Japan). Band intensity was analyzed with Cool Saver 2 software (Atto).

Kex2 Activity Assay

Kex2 activity was measured using the fluorescent substrate Boc-Leu-Arg-Arg-MCA (4-methylcoumaryl-7-amide) (#3140-v, Peptide Institute, Inc., Osaka, Japan). Reactions were performed in 100 mM Tris (pH 7.0) buffer with CaCl_2 and lubrol at 28°C for 30 minutes. The reaction was terminated with EGTA, and fluorescence was measured at 355 nm excitation and 460 nm emission. One unit of activity was defined as 1 pmol AMC (7-amino-4-methylcoumarin) released per minute.

Statistical analyses

Statistical significance of the difference in Kex2 productivity was tested using a T-test. Probability values (p) < 0.05 were considered statistically significant.

Results

Applying the established *O. minuta*-based production system to express the kex2 protease

To compare the productivity of Kex2 between the “parent” strain and the “*aox1Δ prb1*” mutant, a study of the small-scale production of Kex2 in 96-well deep-well plate was performed. The cultured samples were analyzed by Kex2 activity assay, which revealed that the double mutant strain (“*aox1Δ prb1*”) produced 1.6-fold more Kex2 than “parent” strain, which has intact *AOX1* and *PRB1* (Fig. 4-1).

The cultured samples were analyzed by SDS-PAGE, which revealed that the 70 kDa band corresponds to the theoretical molecular weight of engineered Kex2 (Fig. 4-2). The double mutant strain (“*aox1Δ prb1*”) produced 1.9-fold more Kex2 than “parent” strain, which has intact *AOX1* and *PRB1*. These results were consistent with the findings of SDS-PAGE analysis under reducing conditions (Fig. 4-2A). The quantified intensities of protein bands detected by SDS-PAGE were presented in Fig. 4-2B.

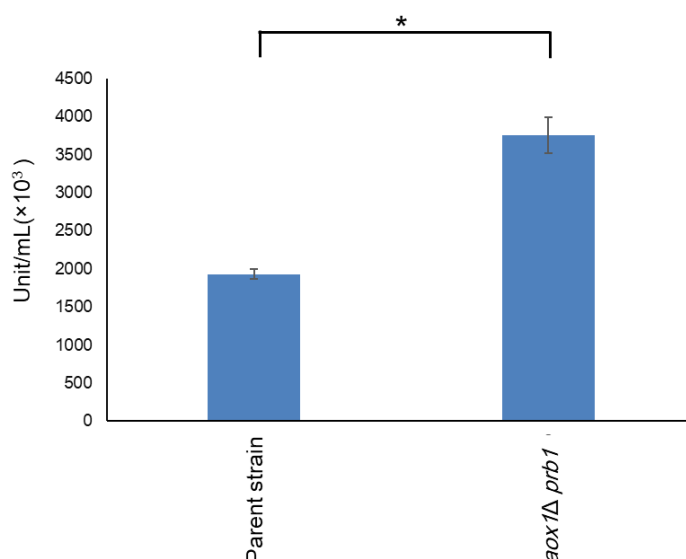


Fig. 4-1 Enzyme activity levels of secreted recombinant Kex2 in culture broth determined by Kex2 activity assay. Parent strain and *aox1Δ prb1* cells were cultured using 2xYP-P6-GG medium at 28 °C for 4 days in 96-well deep-well plate, and culture supernatants were subjected to dot blot. Values are shown as mean ± S.D. of six independent experiments. * $p < 0.05$.

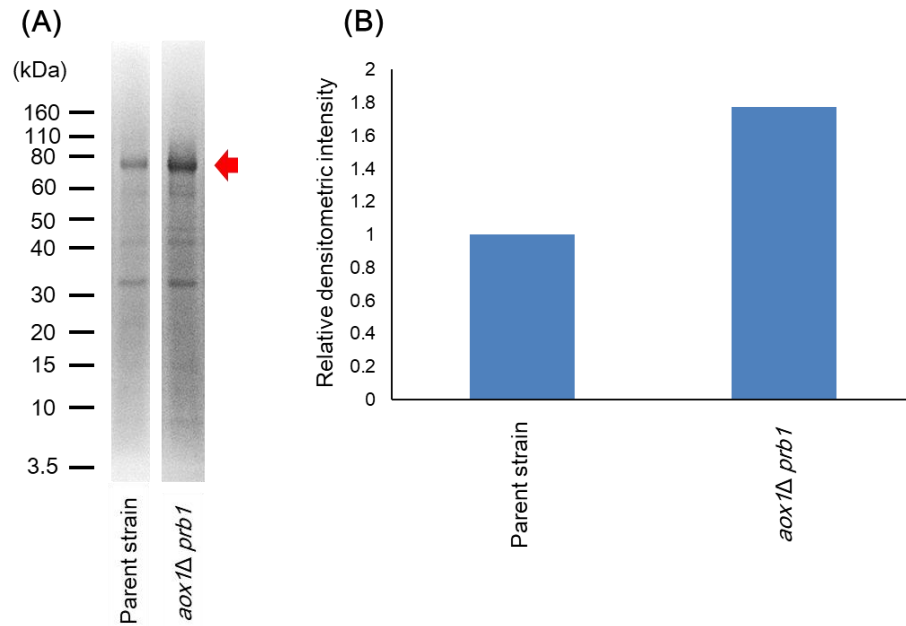


Fig. 4-2 (A) CBB staining/SDS-PAGE analysis under reducing conditions of secreted recombinant Kex2. Parent strain and *aox1Δ prb1* cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days, and culture supernatants were subjected to SDS-PAGE. The arrow indicates expressed Kex2. (B) Quantitative densitometric analysis of CBB Staining/SDS-PAGE under reducing conditions of secreted recombinant Kex2. Parent strain and *aox1Δ prb1* cells were cultured in 2xYP-P6-GG medium at 28 °C for 4 days, and culture supernatants were subjected to SDS-PAGE.

Discussion

Chimeric protein expression is a widely used strategy for producing bioactive peptides, with release of the target peptide typically achieved through chemical or enzymatic cleavage. Chemical methods, such as nitrous acid cleavage at asparagine residues or CNBr cleavage at methionine residues (86), often lead to unwanted modifications, high purification costs, and limiting their industrial applicability. On the other hands, enzymatic methods, including *Staphylococcus aureus* protease V8, offer improved specificity but generally recognize only a single amino acid (87). This cleavage capability of V8 protease may cause the limitation of their application, as the recognition site must be absent from the target peptide sequence to avoid off-target cleavage.

Prohormone convertases such as Kex2 represent a promising class of enzymes for in vitro peptide excision. Kex2, a calcium-dependent serine protease derived from *Saccharomyces cerevisiae*, recognizes dibasic residues, specifically Lys-Arg, Arg-Arg, and Pro-Arg sequences, and cleave at the C-terminal side (82, 88). This characteristic feature of Kex2 could allow more versatile and controlled cleavage of fusion proteins. Structurally, Kex2 comprises 814 amino acids, including an N-terminal signal peptide and a hydrophobic transmembrane domain at the C-terminus. Its intracellular localization in the trans-Golgi network reflects its physiological role in precursor processing (79). However, the intracellular localization domain must be deleted for efficient secretory production of functional Kex2. While expression of a C-terminal domain-deleted variant has been reported, the productivity was low, and it was uncertain whether stable secretory production could be achieved (89).

In the *O. minuta*-based production system, an expression vector harboring the coding sequence of a C-terminal domain-deleted variant of Kex2 was introduced, resulting in the successful secretion of Kex2 which shows an enzymatic activity (Fig. 4-1). In contrast, expression of full-length Kex2 did not yield any detectable enzymatic activity in the culture supernatant (data not shown). Based on the estimation from the result of SDS-PAGE (Fig. 4-2), the expression level of Kex2 is expected to reach several hundred mg/L in a larger bioreactor, indicating that this system could be considerable promise as an efficient platform for Kex2 production.

The addition of affinity peptide tags is a widely adopted strategy for the purification of expressed heterologous proteins. However, in cases where the tag may affect the activity of the target protein, it is often necessary to remove the tag after purification. In addition, for vaccine applications, tag-free proteins are reported to be preferable (90). To address such cases, it is a well-established strategy to incorporate a protease cleavage site into the expression construct in advance to remove the affinity tag following purification. Since Kex2 expressed in *O. minuta*-based system demonstrates both efficient proteolytic activity and high production yields, it is supposed to be applicable to such downstream

applications. This is also consistent with my objective of establishing a production platform for pharmaceutical-grade proteins.

Section II-2

Production and characterization of Norovirus VLPs vaccine candidates in a genetically modified *Ogataea minuta* system

Introduction

Developing effective vaccines against noroviruses has been challenging due to their diversity, the limited understanding of immune responses that they generate, and the inability to cultivate them using conventional cell culture systems (91). Noroviruses are nonenveloped viruses characterized by a positive-sense, single-stranded RNA genome of approximately 7.5-kb. The norovirus viral capsid is primarily composed of 90 dimers of the major structural VP1 protein (92). Noroviruses are regularly the causative agent of outbreaks of acute gastroenteritis, particularly affecting vulnerable populations such as children and the elderly. The development of an effective vaccine against them has been a major public health issue. Several vaccine candidates for Norovirus are known to be under development, including those based on virus-like particles (VLPs), mRNA, adenoviral vectors, or P-particles, which utilize only the P domain of the VP1 protein. Among them, VLPs are considered particularly promising vaccine candidates, due to their demonstrated safety, strong immunogenicity, and evidence of protective efficacy in clinical trials (93, 94). The heterologous expression of the VP1 protein results in the self-assembly of VLPs, which closely resemble the native form of the virus both structurally and in terms of antigenicity (95).

Various heterologous protein production systems have been established using representative host organisms including mammalian cells such as Chinese hamster ovary (CHO) cells, insect cells, yeast, and *Escherichia coli*. Among these systems, methylotrophic yeast has been proposed as a particularly promising host for the production of diverse heterologous proteins (3). Methylotrophic yeast systems are attractive in commercial protein production given their various advantages, including efficient protein secretion into the culture medium, eukaryotic protein quality control, post-translational modification capabilities, and rapid growth (9). Notably, these systems utilize methanol-inducible promoters, such as the alcohol oxidase 1 (*AOX1*) promoter. The strong induction of the *AOX1* promoter by methanol but its repression by glucose and glycerol make it particularly effective for controlling the expression of recombinant proteins (10, 11). Among methylotrophic yeast systems, *Komagataella phaffii* (also known as *Pichia pastoris*) is one of the most successful hosts for heterologous protein production in methylotrophic yeast systems. Genetically engineered strains of *K. phaffii* exhibiting various phenotypes such as auxotrophy, protease deficiency, and methylotrophy are widely available

(62). Intensive efforts to produce VLPs using *K. phaffii* protein expression systems have also been reported. These efforts have led to the successful expression of immunogenic VLPs and have shown promising results in preclinical studies (96, 97). Building on insights from previous studies of *K. phaffii*, I have established the methylotrophic yeast *Ogataea minuta* as a promising host for producing heterologous proteins, including monoclonal antibodies, through genetic engineering approaches (19, 51, 52). An *O. minuta*-based production system has been shown to efficiently express various heterologous proteins under low-methanol conditions, which offer advantages in terms of reducing the necessity of explosion-proof facilities and enabling more cost-effective manufacturing compared to *K. phaffii* systems that require higher methanol concentrations for induction (63, 85). In addition, the platform has been scaled up successfully, demonstrating its industrial feasibility and operational robustness (85).

In the present study toward industrial-scale social-implementation of *O. minuta*-based production system, Norovirus VP1 protein was expressed in a generated *O. minuta* strain and successfully purified through various steps. The VP1 protein was confirmed to be purified in the form of VLPs, as determined by both a transmission electron microscopy (TEM) assay and an antigen protein binding assay. Furthermore, to evaluate their biological activity *in vivo*, VLPs consisting of the purified VP1 protein were administered to mice and their antibody production responses were monitored. These findings demonstrated the potential of VLPs from the VP1 protein produced by *O. minuta* as a norovirus vaccine candidate.

Materials and Methods

Yeast strains and media

All strains used are derived from *O. minuta* tat10113 (63). The plasmid pOMEA-Z1/aF-VP1 or pOMEA-Z1/VP1 was introduced into tat24314 to produce VP1 protein as a subunit of norovirus VLPs. These plasmids contain the nucleotide sequence that encodes the VP1 protein, which was synthesized based on the amino acid sequence listed in GenBank under accession number BAQ55213.1. The plasmids pOU1/PDER-PKA (19) was also introduced into the recombinant strains to co-overexpress the chaperones Pdi1, Ero1, and Kar2 which were derived from *O. minuta*, to improve protein folding. Transformation of *O. minuta* was performed as previously described (19). 2xYP-P6-GG medium [2% Difco™ yeast extract (Thermo Fisher Scientific, Waltham, MA, USA), 4% Bacto™ peptone (Thermo Fisher Scientific), 0.5% glucose (FUJIFILM Wako Pure Chemical, Osaka, Japan), 4% glycerol (FUJIFILM Wako Pure Chemical), 0.1 M potassium phosphate buffer, pH 6.0] and 3xYP-P6-GG medium [3% Difco™ yeast extract (Thermo Fisher Scientific), 6% Bacto™ peptone (Thermo Fisher Scientific), 0.5% glucose (FUJIFILM Wako Pure Chemical), 2% glycerol (FUJIFILM Wako Pure Chemical), 0.1 M potassium phosphate buffer, pH 6.0] were used to produce the VP1 protein. Representative clones were stored as suspensions in 17% (w/v) glycerol at –80 °C.

Animals

Female BALB/c Cr Slc mice (5–6 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed under specific pathogen-free conditions. All animal experiments were conducted in accordance with the guidelines of Daiichi Sankyo Co., Ltd.

Adjuvant (Lipid A)

The chemical synthesis of the adjuvant (Lipid A) was performed in-house, with reference to the method described by Kobayashi, Bazin and Khalaf (98-100). The synthesized adjuvant was analyzed by mass spectrometry to confirm that its molecular size was as intended.

Chemicals and recombinant DNA techniques

E. coli JM109, restriction enzymes, and DNA-modifying enzymes were purchased from Takara Bio (Kusatsu, Japan). The media, growth conditions, and general recombinant DNA techniques used in this study were in accordance with the work of Sambrook and Russell (22). Zeocin™ was purchased from Thermo Fisher Scientific. All oligonucleotides used in this study were synthesized by FASMAC

(Atsugi, Japan). Polymerase chain reaction (PCR) amplification was performed using the GeneAmp[®] PCR System 9700 (Thermo Fisher Scientific). Automated DNA sequencing was performed on an ABI PRISM[®] 3700 DNA Analyzer (Thermo Fisher Scientific).

Construction of plasmids used for VP1 protein production

The plasmid pOMEA-Z1/aF-VP1 was constructed as follows: the VP1 protein coding sequence fused with α -mating factor secretion signal sequence (α F signal) at the N-terminus and hexa-His tag at the C-terminus was designed and the DNA fragment corresponding to the designed protein sequence was synthesized with XbaI and BamHI sites at the 5'- and 3'-ends, respectively, by GeneArt (Regensburg, Germany). The synthesized fragment was introduced into pOMEA-Z1-10746 (52) at the XbaI and BamHI sites, resulting in the plasmid pOMEA-Z1/aF-VP1. The plasmid pOMEA-Z1/VP1 for intracellular production of the VP1 protein was constructed in a similar manner using the VP1 protein coding sequence, which was fused with a hexa-His tag at the C-terminus. The DNA fragment corresponding to the designed protein sequence was again synthesized with XbaI and BamHI sites at the 5'- and 3'-ends, respectively, by GeneArt.

Selection of VP1 protein-producing strains

The VP1 protein production was carried out in accordance with a slightly modified version of a previously reported method (52). For the selection of protein of interest (POI)-producing cells, transformants were cultivated in individual wells of a 96-well deep-well plate using 2xYP-P6-GG medium at 28 °C for 4 days with reciprocal shaking at 300 rpm. After removal of the cells by centrifugation ($2,200 \times g$), the POI in the culture broth or the POI produced intracellularly. was quantified by dot blot analysis.

Dot blot analysis

The harvested supernatant or the mixture of cells and broth was mixed with an equal volume of β -ME Sample Treatment for Tris SDS (Cosmo Bio, Tokyo, Japan) and heated at 99.5 °C. The heat-treated sample was then spotted onto nitrocellulose membranes (0.45 μ m; Bio-Rad Laboratories, Hercules, CA, USA). Dried membranes were blocked with 2% ECL[™] Advance Blocking Reagent (Cytiva, Uppsala, Sweden) in PBS with 0.1% (v/v) Tween 20 (PBS-T) for 1 h. Blocked membranes were washed with PBS-T and treated with Anti-His HRP Conjugates (Qiagen, Hilden, Germany) in PBS-T. After 1 h of treatment with the detection antibody, the resulting membranes were washed with PBS-T three times and analyzed using an ECL Advance Western Blotting Detection Kit (Cytiva).

Bioreactor cultivation

To prepare the preculture, frozen stock was thawed and streaked onto YPD plates. The plates were then incubated at 28 °C for 2 days. Colonies grown on the YPD plates were cultured in test tubes containing 5 mL of YPD at 28 °C with reciprocal shaking at 250 rpm for 24 h. Ten milliliters of collected cultured broth was transferred to baffled flasks containing 40 mL of 2xYP-P6-GG medium and cultured at 28 °C with rotary shaking at 250 rpm for 16 h. Fed-batch culture was then carried out in a 3-L bioreactor (Biott, Tokyo, Japan) containing 1.5 L of 3xYP-P6-GG medium. The inoculation volume was 5% (v/v) of the starting volume. The stirrer speed was varied to keep the dissolved oxygen concentration at 25% at a constant aeration rate. After 8 h of cultivation, a continuous glycerol feed was initiated at 0.33 g/h for 12 h. Thereafter, methanol was added once to the culture to reach a final concentration of 0.5% (v/v) for methanol induction, as described previously (85). Following methanol induction, glycerol, which was prepared as a 60% (w/v) stock solution, was fed at a fixed feeding rate of 2.08 mL/h using a peristaltic pump, corresponding to 0.083% (w/v) relative to the culture volume.

Purification of the VP1 protein

The VP1 protein was purified after cultivation in the bioreactor as follows: Cells were pelleted from 50 mL of culture broth by centrifugation at $3,000 \times g$, and the resulting pellet was disrupted by glass beads. The disrupted lysate was filtered using a Stericup Durapore 0.22 mm PVDF filter (EMD Millipore Corp., Billerica, MA, USA). The filtered supernatant was equilibrated with binding buffer (20 mM sodium phosphate buffer, pH 7.4) and applied to a HisTrap HP (5 mL, Cytiva) for immobilized metal affinity chromatography (IMAC) purification. After washing with binding buffer containing 5 mM imidazole, the protein was eluted with elution buffer containing 250 mM imidazole. Eluted fractions were analyzed by CBB staining/SDS-PAGE and pooled for further purification. Following IMAC purification, the protein was further purified by anion exchange chromatography. The pooled IMAC elution solution was dialyzed with equilibrated buffer [Buffer A: 50 mM Tris-HCl (pH 8.0), 50 mM NaCl] overnight at 4 °C. The resulting solution was applied to a HiTrap Q HP (5 mL, Cytiva), which was equilibrated to Buffer A. Proteins were eluted from the HiTrap Q HP column using a linear gradient of NaCl. Elution was performed from 50 mM NaCl to 1 M NaCl in Tris-HCl pH 8.0. Eluted fractions were analyzed by CBB staining/SDS-PAGE and pooled for further analysis. Collected fractions were equilibrated to storage buffer (20 mM Tris-HCl containing 350 mM NaCl, pH 7.5).

SDS-PAGE

The sample was heated at 70 °C for 10 min in NuPAGE[®] LDS sample buffer (Invitrogen, Carlsbad, CA, USA). The heat-treated samples were analyzed with the NuPAGE[®] System (Invitrogen) using 10% Bis-Tris gels (12 wells, 7 cm wide, 1 mm thick) and NuPAGE[®] MES SDS Running Buffer (Invitrogen) for 30 min at a constant voltage of 200 V, followed by Coomassie Brilliant Blue (CBB) staining using EzStain Aqua (Atto, Tokyo, Japan).

Western blot analysis

Western blot analysis was carried out in accordance with the method of Suzuki et al. (19). Anti-His HRP Conjugates (Qiagen) diluted 1:2000 was used as detection antibodies. Detection was performed using the ECL Advance Western Blotting Detection Kit (Cytiva).

N-terminal analysis

The purified VP1 protein was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Cytiva). The protein band of interest was excised and subjected to N-terminal amino acid sequencing using the Procise cLC Protein Sequencing System (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's protocol.

Transmission electron microscope analysis

The purified VP1 protein samples were fixed with 2% glutaraldehyde (Nissin EM Co., Ltd., Tokyo, Japan) and underwent negative staining using the Exosome-TEM-easy Kit (Cosmo Bio Co., Ltd., Tokyo, Japan), in accordance with the manufacturer's protocol. The stained samples were then examined using a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

Evaluation of reactivity of the VP1 protein with the anti-Norovirus antibody and histo-blood group antigens (HBGAs) by ELISA assay

Enzyme-linked immunosorbent assay (ELISA) was performed as follows: Flat-bottom 96-well plates (Merck, Darmstadt, Germany) were coated with 25 µL of VP1 protein diluted in Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific) overnight at 4°C. After three washes with 180 µL of wash buffer [0.05% Tween20 in Tris-buffered saline (TBS) (Takara)], 150 µL of blocking buffer [25% Block Ace (FUJIFILM Wako Pure Chemical) in TBS] was added and incubated for 1 h at room temperature. After three washes with 180 µL of wash buffer, the appropriate primary detection reagent was added and incubated under the following conditions. For anti-Norovirus GII.4 monoclonal

antibody reactivity, 25 μ L of diluted anti-Norovirus antibody (2002-G5, 1:5000, Abcam, Cambridge, UK; or 17E11, 1:1000, Abcam) in wash buffer containing 10% Block Ace (FUJIFILM Wako Pure Chemical) was added and incubated for 2 h at room temperature. For HBGAs binding, 25 μ L of 10 μ g/mL Blood type B (tri)-PAA-biotin (Glycotect, Gaithersburg, MA, USA) diluted with wash buffer containing 10% Block Ace (FUJIFILM Wako Pure Chemical), or 10 μ g/mL H (type 3)-PAA-biotin (Glycotect) diluted with wash buffer containing 10% Block Ace (FUJIFILM Wako Pure Chemical) was added and incubated overnight at 4°C. After three washes with 180 μ L of wash buffer, 25 μ L of the appropriate HRP-conjugated secondary antibody and detection reagent was added. For antibody detection, HRP-conjugated anti-mouse antibody (1:2000; Southern Biotechnology Associates, Birmingham, AL, USA) in wash buffer containing 10% Block Ace (FUJIFILM Wako Pure Chemical) was added and incubated for 1 h at 37°C. For biotinylated antigen detection, Poly-HRP 40-Streptavidin (1:5000; Fitzgerald Industries, Acton, MA, USA) in wash buffer containing 10% Block Ace was added and incubated for 1 h at 37°C. After three washes with 180 μ L of wash buffer, 30 μ L of substrate solution (Sure Blue; KPL, Gaithersburg, MD, USA) was added and incubated for 20 min at room temperature. After the reaction, 30 μ L of the substrate solution (KPL) was added and measured at 450 nm using a microplate reader. Concentrations were calculated based on a standard curve.

Immunization for the intramuscular injection and sample collection

The VLPs were diluted in phosphate-buffered saline (PBS) to final concentrations of 320, 40, and 5 μ g/mL. Groups of mice (n=6 per concentration) received intramuscular injections of 50 μ L of the respective VLP solution. Immunizations were performed twice at a 3-week interval: The first dose was administered into the left quadriceps femoris muscle and the second into the right quadriceps. Two weeks after the second immunization, the mice were anesthetized with isoflurane and blood was collected via cardiac puncture. Blood samples were allowed to clot at room temperature for 35 min and then incubated at 4°C for 30 min. Serum was separated by centrifugation at 10,000 rpm for 10 min and stored at 4°C.

Mucosal immunization and sample collection

Mice (5–6 weeks old, n=4 per group) were immunized intranasally or sublingually with the VLPs (2 μ g/mouse) with or without adjuvant (Lipid A, 2.5 μ g/mouse). For each administration, the mice were anesthetized with isoflurane, and 2 μ L of antigen solution was applied either intranasally or sublingually. The immunization was performed twice on the same day at a 1 h interval, with food and water withheld for 1 h before the first administration and resumed 1 h after the second one. This

procedure was repeated three times at 1-week intervals. Two weeks after the third immunization, the mice were anesthetized with isoflurane and blood was collected by cardiac puncture. Blood samples were allowed to clot at room temperature for 35 min and centrifuged at $9,600 \times g$ for 10 min to obtain serum, which was stored at 4°C until analysis. Approximately 200 mg of cecal content was collected from each mouse as the fecal sample. Fecal samples were suspended in 1000 μ L of PBS containing protease inhibitor cocktail (Sigma, diluted 1:100), and incubated overnight at 4°C. After centrifugation at $16,200 \times g$ for 10 min at 4°C, the supernatant was collected as the fecal extract and stored at 4°C until use.

Evaluation of anti-Norovirus antibody using ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed as follows: Flat-bottom 96-well plates (Merck) were coated with 25 μ L of 1 μ g/mL purified VLPs in PBS (Thermo Fisher Scientific) overnight at 4°C. After three washes with 180 μ L of wash buffer, 150 μ L of blocking buffer was added and incubated for 85 min at room temperature. After three further washes with 180 μ L of wash buffer, 25 μ L of diluted sample was added and incubated for 1 h at 37°C. After another three washes with 180 μ L of wash buffer, 25 μ L of HRP-conjugated secondary anti-mouse antibody (1:2000; Southern Biotechnology Associates, Birmingham, AL, USA) in wash buffer containing 10% Block Ace (FUJIFILM Wako Pure Chemical) was added and incubated for 1 h at 37°C. Following a further set of three washes with 180 μ L of wash buffer, 30 μ L of the substrate solution (Sure Blue; KPL) was added and incubated for 20 min. After the reaction, 30 μ L of the substrate solution (KPL) was added and the absorbance was measured at 450 nm using a microplate reader. Concentrations were calculated based on a standard curve.

Statistical Analysis

The antibody titers were compared using one-way analysis of variance (ANOVA) for one independent variable or using two-way ANOVA for two independent variables. The antibody titers of the groups with and without adjuvant were compared using the Mann–Whitney U test. *p* values less than 0.05 were considered statistically significant.

Results

Expression of the VP1 protein using *O. minuta* heterologous protein production system

To generate a strain for the high production of VP1 protein, I employed an *O. minuta* double-mutant strain in which both the alcohol oxidase 1 gene (*AOX1*) and the Prb1 protease gene (*PRB1*) were disrupted. Previous studies showed that this double-mutant yeast system enhanced heterologous protein productivity under low-methanol-feeding conditions (63, 85). The VP1 protein-producing strains were generated and cultivated as described in the Materials and Methods. Initial attempts to produce VP1 as a secreted protein using the α F signal sequence resulted in very low secretion efficiency. However, a strong signal was confirmed in the cell extract fraction (Fig. 5-1, left panel). The same result was observed in the strain producing VP1 without the α F signal sequence (Fig. 5-1, right panel). Next, I selected transformants with the highest signal intensity from the two strains using dot blot analysis. I cultivated each strain in a 1.5-L bioreactor and measured the optical density at 600 nm (OD_{600}) of the cultured samples over the course of fermentation (Fig. 5-2). Western blotting revealed strong signals at approximately 70 kDa in the strain producing the α F-VP1 protein (Fig. 5-3, left panel) and at approximately 60 kDa in the strain producing the VP1 protein (Fig. 5-3, right panel).

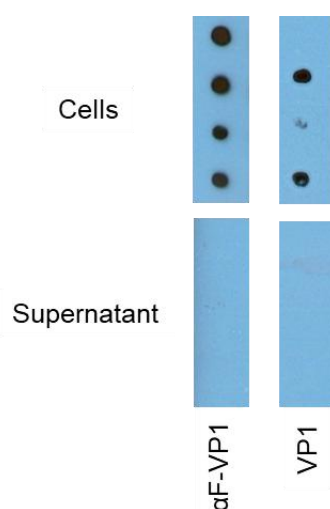


Fig. 5-1 Representative result of dot blot analysis under reducing conditions using anti-His antibody. α F-VP1 and VP1 refer to VP1 proteins with an α F signal sequence expected to induce secretion and with the native N-terminal amino acid sequence, respectively. Culture supernatants and protein solutions recovered from disrupted harvested cells were subjected to dot blot analysis (n=4).

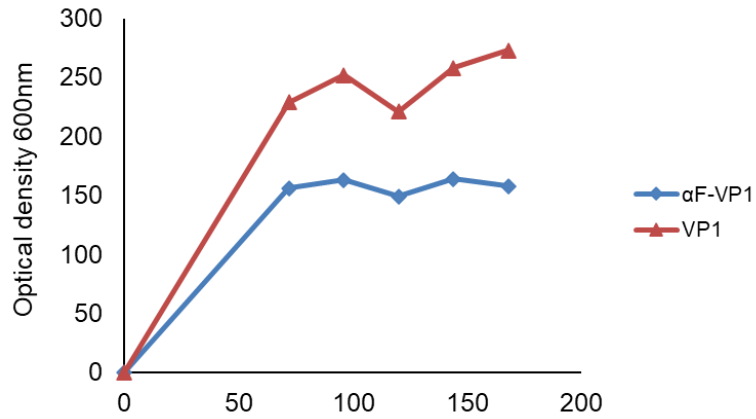


Fig. 5-2 The fermentation study performed in a 1.5-L bioreactor. α F-VP1 and VP1 refer to VP1 proteins with an α F signal sequence expected to induce secretion and with the native N-terminal amino acid sequence, respectively. Optical density measurements were performed at a wavelength of 600 nm (OD_{600}).

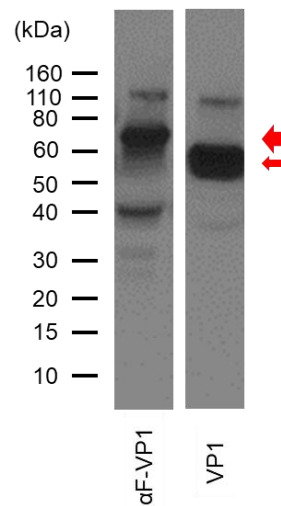


Fig. 5-3 Western blot analysis of the VP1 protein recovered from disrupted cells under reducing conditions using anti-His antibody. α F-VP1 and VP1 show the VP1 protein with α -factor signal sequence and with native N-terminal amino acid sequence, respectively. The upper and lower arrows indicate the expected α F-VP1 and VP1, respectively.

As the α F-VP1 protein was shown to potentially retain its uncleaved secretion signal, I instead selected the VP1 protein for vaccine production. The cell pellet was harvested after centrifugation of 50 mL of culture broth and disrupted to obtain the cell lysate. The recovered cell lysate was then applied to Ni-immobilized metal affinity chromatography (IMAC) (Fig. 5-4A). For further purification, IMAC elution fractions containing the VP1 protein were pooled and purified by anion exchange chromatography (Fig. 5-4B). Pooled fractions were buffer-exchanged into storage buffer. As a result of fermentation and purification, 13.4 mg of the VP1 protein was successfully obtained.

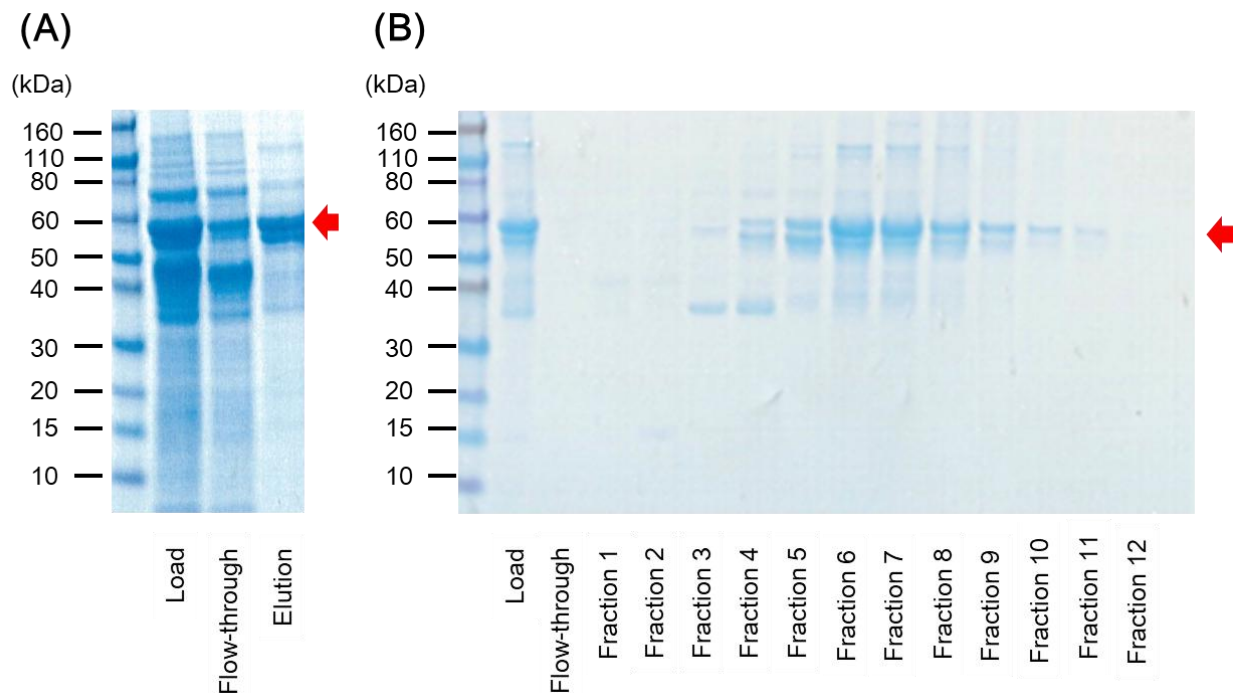


Fig. 5-4 Purification profiles of the produced VP1 protein. The samples purified by IMAC chromatography of the VP1 protein recovered from disrupted cell lysate were analyzed by SDS-PAGE with CBB staining (A). The IMAC elution fraction was further purified by anion exchange chromatography and analyzed by SDS-PAGE with CBB staining (B). Fractions 6 and 7 were pooled as the concentrated VP1 protein fraction. The arrow indicates the position of the expected VP1 protein.

Characterization of the purified VP1 protein

N-terminal amino acid sequencing of the purified VP1 protein was performed. The results showed that the expressed VP1 protein had the intended amino acid sequence (data not shown). Furthermore, TEM analysis revealed that the molecular structure of the purified VP1 protein was consistent with the typical structure of virus-like particles (VLPs), as previously reported (101). Generally, proteins are susceptible to structural changes upon freeze-thawing. Therefore, it is essential to assess the freeze-thaw stability of biologics (102). Freeze-thawed VP1 protein was subjected to TEM analysis, and I confirmed that it had maintained its molecular structure through a single cycle of freeze-thawing (Fig. 5-5).

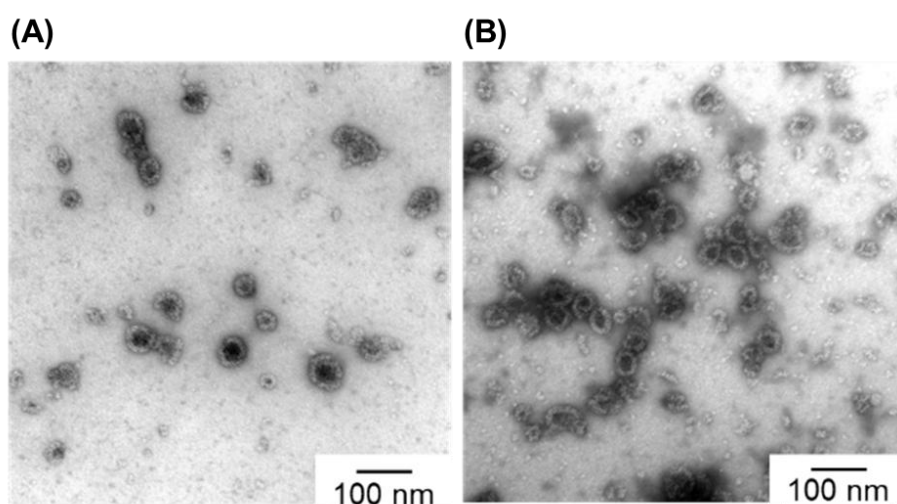


Fig. 5-5 Transmission electronic microscopy of VLPs. (A) Freshly purified VP1 protein sample after negative staining. (B) Freeze-thawed VP1 protein sample after negative staining.

To further analyze the antigenicity of the purified VP1 protein, I performed ELISA using anti-Norovirus GII.4 antibodies (2002-G5 and 17 E11). As shown in Fig. 5-6A, a concentration-dependent signal intensity curve was observed for both antibodies. Subsequently, ELISA was performed utilizing a recombinant form of the host receptor to assess the capacity of the purified VP1 protein to interact with its corresponding receptor proteins, HBGAs. As shown in Fig. 5-6B, the purified VP1 protein specifically bound to the target receptor protein in a dose-dependent manner. In summary, these results showed that the purified VP1 protein exhibited robust antigenicity and the capacity to bind specifically to its receptors.

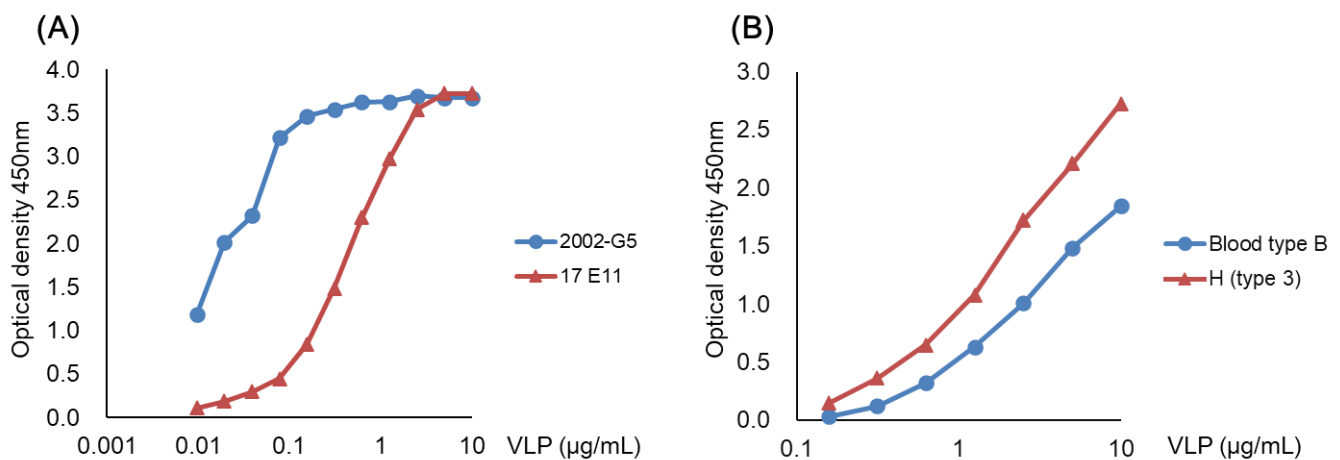


Fig. 5-6 (A) ELISA to evaluate the reactivity of VLPs with anti-Norovirus GII.4 monoclonal antibodies, 2002-G5 and 17 E11. Optical density measurements ($n=2$) were performed at a wavelength of 450 nm, with the mean values plotted on the graph. (B) ELISA to evaluate the reactivity of VLPs to human histo-blood group antigens (HBGAs), and blood types B and H (type 3). Optical density was measured ($n=1$) at a wavelength of 450 nm.

***In vivo* immunogenicity of purified VP1 protein administered via different routes**

To investigate the immunogenicity of purified VP1 proteins as VLPs *in vivo*, BALB/c mice were immunized via intramuscular, intranasal, or sublingual administration with or without Lipid A as an adjuvant. For the intramuscular immunization, 50 μ L of VLP antigen formulated at a protein-equivalent dose of 0.25, 2, or 16 μ g per dose was administered twice at a 3 weeks interval. There was no statistically significant difference in antibody titers among the low-, medium-, and high-dose groups ($p < 0.05$, one-way ANOVA) (Fig. 5-7), indicating that a lower antigen dose may be sufficient to achieve a robust immune response.

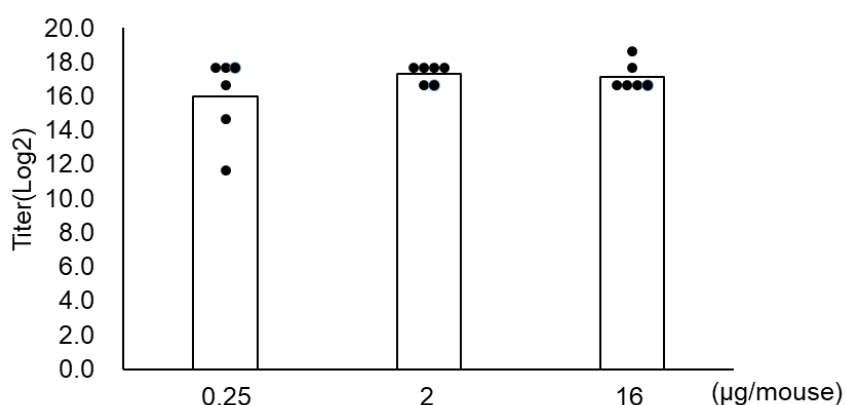


Fig. 5-7 Systemic antibody response induced by intramuscular immunization with VLPs.

Mice (n=6 per group) were immunized intramuscularly twice at a 2-week interval with 0.25, 2, or 16 μ g of VLPs. Two weeks after the second administration, serum anti-VLP antibody titers were measured by ELISA. The bar graph shows the mean values. Each dot represents an individual mouse (n=6).

To evaluate the immunogenicity of VLPs administered via mucosal routes, mice were also intranasally or sublingually immunized with VLPs with or without the addition of Lipid A as an adjuvant (Fig. 5-8). The mice administered VLPs alone showed elevated levels of antigen-specific IgG and IgA in the serum, as well as antigen-specific IgA in fecal extracts. Notably, the addition of Lipid A significantly enhanced the antibody responses. Specifically, both serum IgG and IgA titers in the Lipid A group were higher than those in the VLP-only group ($p < 0.05$, the Mann–Whitney U test) (Fig. 5-8A and 5-8B). Additionally, fecal IgA levels were also increased in the Lipid A group compared with those in the group with VLP alone ($p < 0.05$, the Mann–Whitney U test) (Fig. 5-8C). In contrast, antigen-specific IgG was undetectable in all fecal samples, remaining below the detection limit of the assay used (data not shown).

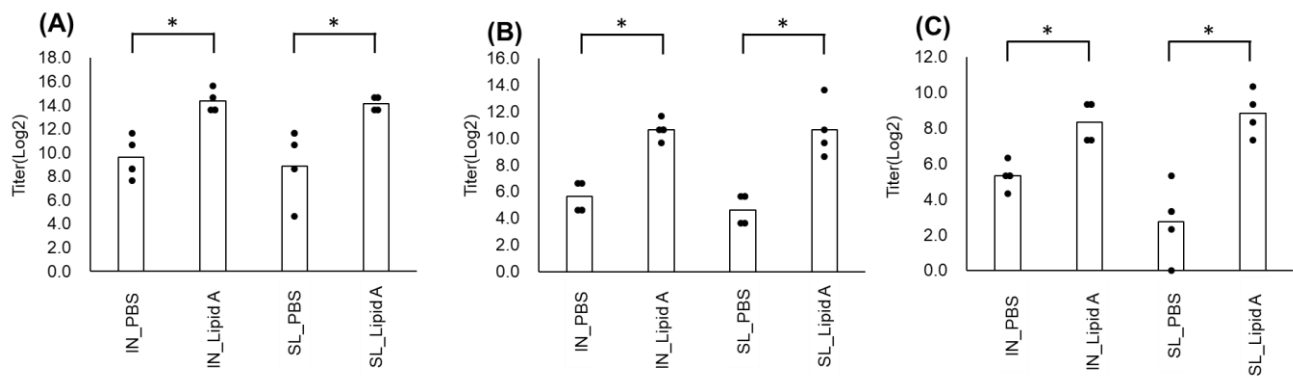


Fig. 5-8 Serum and fecal antibody responses after intranasal (IN) and sublingual (SL) administration of VLPs with or without Lipid A in mice ($n=4$). Two weeks after the third immunization, all samples were collected. Antigen-specific serum IgG (A), serum IgA (B), and fecal IgA (C) were measured by ELISA. The bar graph shows the mean values. Each dot represents an individual mouse; $*p < 0.05$.

Discussion

In this study, I successfully demonstrated the intracellular production of the VP1 protein of Norovirus using the *O. minuta aox1Δ prb1Δ* double mutant under low-methanol conditions. Although it was reported that the VP1 protein was produced as a secreted protein by *K. phaffii* (96), the initial attempt to produce the VP1 protein with α -factor signal sequence via yeast secretory system didn't work well (Fig. 5-1). As the produced VP1 protein retaining an α -factor signal sequence and lacking glycosylation was detected in the cell lysate, it was suggested that the VP1 protein might have been missorted to the cytoplasm (103). Therefore, I attempted to recover the VP1 protein produced intracellularly, resulting in approximately 15 mg of VP1 protein from 50 mL of fermented cells (Fig. 5-4).

While one advantage of yeast expression systems is their ability to utilize the secretory pathway for extracellular protein production (9), it is also possible to accumulate POI intracellularly in microbial heterologous protein production systems. In the methylotrophic yeast *Candida boidinii*, the peroxisome was used as the location for protein accumulation. A gene encoding alcohol oxidase localized to the peroxisome was disrupted in the original strain. The resulting alcohol oxidase-deficient strain showed 47-fold higher productivity (55). In my previous study, I found that the produced POI was degraded in the fermentation broth by proteases that were secreted or leaked out from potentially damaged cells (52, 63). Therefore, intracellular production in host cells would be a plausible approach to produce intact POI.

Although yeast heterologous protein production systems are known for high production yields, it has also been reported that these systems can exhibit significant protease activity, which may lead to degradation of the expressed recombinant proteins. Therefore, the protease-deficient host strain was applied to achieve high productivity of intact recombinant protein and employed for commercial production (62, 104). In this study, the *O. minuta aox1Δ prb1Δ* double mutant was employed to produce the VP1 protein to prevent protein degradation. As a result, N-terminal sequence analysis confirmed that the VP1 protein was not cleaved at the N-terminus. Furthermore, based on the successful result of the IMAC purification step, the hexa-His tag at the C-terminus would not degrade. These results indicate that the VP1 protein produced in this study was successfully produced as an intact form that could assemble into VLPs.

To develop VLPs as a potential candidate for Norovirus vaccines, evaluation across multiple critical features is necessary, including physicochemical characteristics, immunogenicity, and safety (106). Structural and stability analyses of the VP1 protein by TEM confirmed its robustness to freeze-thawing stress and uniform particle formation (Fig. 5-5). The appropriate capsid structure ensures the correct

presentation of neutralizing epitopes. Misfolded VLPs that lack or incorrectly present these essential epitopes would lead to the production of antibodies that cannot neutralize the virus or are associated with a generally weak immune response (105-107).

For further structural characterization of the VP1 protein, I evaluated its ability to bind specific anti-Norovirus antibodies and HBGAs, which play a pivotal role in host susceptibility and the initial stages of infection (Fig. 5-6). The results on the binding of several specific anti-Norovirus antibodies indicated that the peptide domains recognized by antibodies are located externally on the VLP, as in the native Norovirus capsid. This matched the findings of the binding of VLPs with HBGAs. This is a key finding given that the use of VLPs for a vaccine is dependent on them binding to HBGAs in the same manner as for the native Norovirus to induce effective antibodies that prevent infection (108).

To demonstrate the immunogenicity, I showed that an IgG response was induced by the intramuscular administration of VLPs in mice (Fig. 5-7). This coincides with a report describing that the intramuscular injection of GII4 VLPs elicited antibody responses and induced IgG (109). In clinical applications, establishing a minimal dose is crucial for vaccine development. These finding that a dose higher than 2 µg per mouse may not provide additional immunological benefit might allow the use of lower and more cost-effective doses in future clinical studies.

Since the induction of an antibody response by the VLPs was confirmed, I proceeded to study VLP administration intranasally and sublingually with or without a vaccine adjuvant, Lipid A. These results demonstrated that the intranasal and sublingual administration of VLPs induced robust systemic and mucosal immune responses, which were significantly enhanced by Lipid A (Fig. 5-8). Intranasal immunization is known to induce both systemic IgG and mucosal IgA responses. In a previous study on mice, intranasal delivery of Norovirus VLPs along with Toll-like receptor agonists resulted in significantly high fecal IgA and serum IgG levels (110). Furthermore, it has been reported that intranasal immunization with VLPs induces mucosal IgA and IgG levels that effectively block viral receptor binding, whereas intramuscular immunization fails to generate mucosal IgA (109). Physiologically, the presence of both serum IgG and IgA antibodies indicates the induction of a systemic immune response, while fecal IgA antibodies specifically highlight the generation of a mucosal immune response (111-112). This dual response is particularly desirable for vaccine candidates for pathogens such as noroviruses. These results, which successfully demonstrated antibody responses of serum IgG, serum IgA, and fecal IgA, strongly suggested that VLPs produced by *O. minuta* are comparable to other VLPs produced by other production hosts as vaccine candidates.

Recent studies have shown that intranasal administration induces significantly higher systemic IgG titers, whereas sublingual administration induces stronger fecal IgA responses (113). However, these

results revealed no significant differences in antibody titers of fecal IgA between the intranasal and sublingual administration routes ($p < 0.05$, two-way ANOVA) (Fig. 5-8A and 5-8C). One possible explanation for this is the limited statistical power of this study. Although trends in antibody titers were observed, the small sample size may have limited the ability to detect significant differences between the two administration routes.

To decrease the toxicity of the vaccine adjuvant, I have developed a derivative of CRX-547, a member of the aminoalkyl glucosaminide 4-phosphate class of synthetic lipid A mimetics. This lower toxicity is expected to be associated with a promising adjuvant profile that may enhance vaccine safety while maintaining robust immunogenicity (114). Indeed, the *in vivo* immunogenicity assay successfully demonstrated the adjuvant's ability to enhance the immunological effects of vaccine candidates (Fig. 5-8).

In this study, I produced the VP1 protein of Norovirus using an *O. minuta* production system, and then purified and characterized it. The VP1 protein purified in the form of VLPs showed various characteristics desirable for such particles, including in terms of physicochemical properties, immunogenicity, and safety. These findings demonstrate the potential of the VP1 protein produced by *O. minuta* as a Norovirus vaccine candidate.

Conclusion

I aimed to develop a recombinant protein production system, using *Ogataea minuta*, that enables high-level protein expression under low-methanol conditions.

In Chapter I Section I-1, I evaluated the protein expression capability of three *Ogataea minuta* var. *minuta* strains (NBRC 0975, 10402, 10746) to reveal that NBRC 10746 demonstrates the highest secretory protein production. The selected clone (#5-30, named tat06213) from NBRC 10746 was isolated as promising host for further development of the *O. minuta*-based system.

In Chapter I Section I-2, I generated an alcohol oxidase-deficient (Δaox) *O. minuta* strain and developed the *O. minuta*-based system to produce heterologous protein under low-methanol conditions. A mixed-feed culture process was developed in a jar bioreactor, enabling protein production levels comparable to the wild-type strain under low-methanol conditions, enhancing the system's industrial potential.

In Chapter I Section I-3, I improved the *O. minuta*-based system by optimizing the fermentation conditions, producing ~7.5 g/L of human serum albumin in 21 days. These conditions were successfully applied at industrial scale (4,500-L) to produce a lipocalin-based biologic. This study demonstrates the potential of *O. minuta* for large-scale protein production.

In Chapter II Section II-1, I produced the Kex2 protease successfully using the developed *O. minuta*-based system. Produced Kex2 showed efficient enzyme activity and improved productivity.

In Chapter II Section II-2, I produced the Norovirus The GII.4 capsid protein VP1 successfully as virus-like particles (VLPs) using the developed *O. minuta*-based system. These VLPs remained structurally stable after freeze-thawing and showed strong antigenicity with specific, dose-dependent binding to histo-blood group antigens. In mice, intramuscular and mucosal (intranasal/sublingual) administration induced robust systemic and mucosal immune responses, which were further enhanced by a Lipid A adjuvant. These results demonstrated the potential of the *O. minuta* system for producing effective Norovirus VLP vaccine candidates.

In the present study, an *O. minuta*-based heterologous protein production system was successfully established, enabling enhanced productivity under reduced methanol consumption conditions. While methanol is widely used as an inducer of gene expression in methylotrophic yeast systems, particularly through the *AOX1* promoter, its use presents safety and handling concerns at industrial scale. To address these issues, a production platform utilizing an alcohol oxidase-deficient strain and a mixed-feed fermentation strategy was developed. This approach achieved protein expression levels comparable to those observed under conventional high-methanol conditions.

This study presents the first successful case of industrial-scale production of a pharmaceutical biologic using the *O. minuta* expression system. This achievement demonstrates its potential as an efficient, scalable, and safer alternative for biomanufacturing. Furthermore, the versatility of the established platform was validated by the successful production of both a functional protease and a virus-like particle vaccine candidate. These results support the applicability of this system to future biopharmaceutical development and provide valuable insight into large-scale cultivation of methylotrophic yeasts for protein production.

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Publications

1. **Tsuda, M., Nakatani, Y., Baba, S., Tanaka, I., Ichikawa, K., Nonaka, K., Ito, R., Yoko-o, T. and Chiba, Y.**: Selection of the methylotrophic yeast *Ogataea minuta* as a high-producing host for heterologous protein expression, J. Biosci. Bioeng., **135**, 196–202 (2023).
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