



Optimal fermentation conditions for growth and recombinant protein production in *Pichia pastoris*: Strain selection, ploidy level and carbon source

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ABSTRACT

High-cell-density fermentation is a critical aspect of industrial protein production, requiring the selection of an optimal growth medium and carbon source. *Pichia pastoris*, a methylotrophic yeast, has been established as a widespread recombinant protein expression system in the food and pharmaceutical industries. The primary objective of this work was to create a superior platform for producing alternative proteins thus contributing to future innovation in these sectors. This study compared three wild-type strains, with two of them also analyzed in their diploid versions, using shake flasks and bioreactors. It investigated glucose and glycerol as carbon sources using mCherry as a protein model. Glycerol emerged as the preferred carbon source, resulting in over 40% increase in biomass concentrations compared to glucose across all strains. Notably, wild-type strain Y-7556 reached an exceptional biomass concentration of 244 g DCW/L in just 48 h, the highest reported to date, highlighting the potential of high-cell-density fermentation in *P. pastoris*. Regarding protein expression, the diploid version of Y-11430 produced >43% of purified mCherry protein after 123 h of fermentation, compared to the haploid counterpart. Our findings underscore the advantages of diploid strains, optimized fermentation media, and carbon source selection, effectively addressing crucial gaps in the literature.

1. Introduction

Pichia pastoris is a methylotrophic yeast established as a widespread recombinant protein expression platform in research and the industry (Schwarzshans et al., 2017). It can express high levels of functional proteins (Karbalaee et al., 2020), which makes it a popular choice for biotechnology and pharmaceutical applications. *P. pastoris* is a non-pathogenic, non-toxic industrial production organism with a long history of safe use dating back to the 1980s (Reyes et al., 2021) and has been used to produce a wide range of proteins, including enzymes (Li et al., 2015), hormones (Azadi et al., 2018), antibodies (Tir et al., 2022), and vaccines (Wang et al., 2016).

P. pastoris strains are available in different cell banks around the world. Various strains of *P. pastoris* have been isolated and characterized. However, little effort has been made to explore the growth ability of the different wild-type strains. There are differences between wild-type strains in growth rates, utilization of various carbon sources, the ability to produce ethanol, and several other parameters. Choosing a wild-type strain with the highest growth potential is essential to a

project's success. To date, there is one comprehensive assessment of the genomes and transcriptomes of nine strains of *P. pastoris* (Brady et al., 2020).

One of the characterized strains is NRRL Y-11430 (Kurtzman, 2009). Initially, strain Y-11430 underwent mutagenesis using nitrosoguanidine to produce the histidine auxotroph GS115. This strain gained popularity due to its ease of integrating heterologous genes into the genome through the complementation of HIS4. Subsequently, X-33 was developed by introducing HIS4 into GS115 to restore prototrophy, and it is now commercially available alongside GS115 (Brady et al., 2020). Strain X-33 is considered a wild type and is widely used in academics and industry but a license is required for commercial purposes from Research Corporation Technologies Inc (Tucson, Arizona) (<https://www.thermofisher.com/order/catalog/product/C18000>).

The Y-7556 strain is recognized for its utility in scientific research and biotechnological applications (Steimann et al., 2024). It is known for exhibiting a thick cell wall, which leads to poor transformation efficiency (Brady et al., 2020).

Strains Y-11430 and Y-7556 are available from the Agricultural Research Service Culture Collection (NRRL) and there are no restrictions

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Abbreviations:

WT –	Wild type
DCW -	dry cell weight
ARS -	Agriculture Research Service
PI -	Propidium iodide

on their use. However, any commercial or other unauthorized use of these strains without the consent of the depositor could involve patent infringements. The production strain MX0291, modified to over-express the gene encoding the soy leghemoglobin by Impossible Foods Inc., was derived from the parent strain NRRL Y-11430 (Fraser et al., 2018). Motif Food Works Inc., produced myoglobin from *Bos taurus* by fermentation of a modified *P. pastoris* derived from the parent strain NRRL Y-7556 (<https://www.fda.gov/media/153921/download>).

Like *Saccharomyces cerevisiae*, *P. pastoris* is an ascomycetous homothallic budding yeast capable of existing as haploid or diploid. *P. pastoris* typically maintains stability in the vegetative haploid form and remains haploid unless induced to mate under specific conditions like nitrogen starvation limitations (Chen et al., 2012). In contrast, most *S. cerevisiae* industrial strains are diploids or polyploids (Chen et al., 2012). The diploid form of *S. cerevisiae* exhibits distinct metabolic traits compared to the haploid form, such as elevated levels of glycolytic intermediates and amino acids, along with reduced changes in stress protectants when exposed to ethanol pressure. Additionally, diploid yeast shows greater tolerance to ethanol stress, whereas the haploid form is inherently more vulnerable to challenging environmental conditions (Ding et al., 2010). The number of sets of homologous chromosomes in a cell can alter gene regulation, cellular physiology, and the spectrum of acquired mutations (Todd et al., 2018). Previous work described mating between two haploid *P. pastoris* strains for synthesizing and secretion of recombinant hetero-multimeric proteins (Chen et al., 2012). Each haploid strain produced a subunit of the antibody and the target diploid strain produced the two subunits that form the fully assembled antibody. Moreover, the regulation of mating types in *P. pastoris* was investigated through phenotypic characterization of mating and transcript level analyses, shedding light on the roles played by *P. pastoris* MAT genes in mating and sporulation processes (Heisteringer et al., 2017). Nonetheless, the influence of ploidy level on recombinant protein production has not been investigated to date.

High-density fermentation of *P. pastoris* expression strains is particularly advantageous for producing secreted proteins because as cell density increases, the concentration of these proteins in the culture medium is expected to rise (Higgins and Cregg, 1998). *P. pastoris* can utilize glucose and glycerol as carbon sources for growth and metabolic activities. When grown on glucose, yeast produces ethanol under aerobic conditions (De Deken, 1966) which can be a drawback in some applications. However, glucose remains an appealing carbon source due to its lower cost than glycerol (Hang et al., 2009). The choice of carbon source can significantly impact the yeast's growth rate and productivity, as well as the expression of recombinant proteins.

P. pastoris is well-known for its ability to grow using methanol as a sole carbon source, thus enabling the production of single-cell protein (SCP) for use in food applications. Wu and colleagues have recently manipulated the cellular signaling pathways to obtain a superior phenotype with a high methanol conversion rate of 67.21% and crude protein yields of 0.46 gDCW/g. Furthermore, adaptive laboratory evolution has been employed by the group to overcome the low methanol utilization efficiency and intolerance to a temperature of 33 °C. An engineered strain produced high levels of SCP from methanol in a pilot-scale fed-batch culture resulting in a biomass of 63.37 g DCW/L, methanol conversion rate of 0.43 g DCW/g, and protein content of 0.506 g/g DCW (Gao et al., 2023; Meng et al., 2023).

As *P. pastoris* developed into a highly successful system for producing a variety of heterologous proteins (Cereghino and Cregg, 2000a), several promoters have been utilized with the most common being the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) and methanol-induced alcohol oxidase I (AOX1) (Cereghino and Cregg, 2000b; Várnai et al., 2014; Zhang et al., 2009).

The goal of the present work was to perform a systematic study of three common WT *P. pastoris* strains (X-33, NRRL Y-11430, NRRL Y-7556), to obtain conditions for reaching biomass levels of over 150 g DCW/L and to assess for the first time the influence of ploidy level on recombinant protein expression. The fluorescent protein mCherry was used as a model due to its ease of detection and quantification.

2. Materials and methods

2.1. Materials

Unless otherwise specified, chemicals and reagents were purchased from Merck (Darmstadt, Germany). Glucose was purchased from Sigma-Aldrich (St Louis, MO, USA). Potassium phosphate monobasic was from Supelco (Bellefonte, PA, USA). Peptone, Tryptone and yeast extract were purchased from Gibco, ThermoFisher Scientific (Waltham, MA, USA). Agar and YNB (yeast nitrogen base w/o amino acid and ammonium sulfate) were from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Glycerol, ethanol, methanol, acrylamide, ammonium hydroxide and sodium chloride were from BioLab (Jerusalem, Israel). Magnesium sulfate, riboflavin, pyridoxine hydrochloride, 4-aminobenzoic acid, and folic acid were purchased from Alfa-Aesar (Ward Hill, MA, USA). Calcium chloride was from Spectrum (Gardena, CA, USA). Ammonium sulfate was from Carlo Erba (Barcelona, Spain). Thiamine hydrochloride was purchased from Acros Organics (Geel, Belgium). Dextrose was purchased from Dagal Food Additives Ltd (Maanit, Israel). Potassium chloride was purchased from Frutarom (Haifa, Israel). Propidium iodide was purchased from Invitrogen by ThermoFisher (Carlsbad, CA, USA). Methanol, isopropanol and water for HPLC analysis were purchased from JT Baker, Avantor (Pennsylvania, USA). Coomassie protein stain, InstantBlue was purchased from Abcam (Cambridge, United Kingdom).

2.2. Strains and genes

All the strains used in this study are listed in Table 1. Y-11430 and Y-7556 were obtained from the Agriculture Research Service (ARS) culture collection, National Center for Agricultural Utilization Research (Peoria, IL, USA). X-33 was kindly provided by Prof. Niv Papo from Ben-Gurion University, Israel. All strains were stored at –80 °C in a medium containing 70% YPD medium (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 30% glycerol. The mCherry gene was kindly provided by Prof. Roei Amit from Technion, Israel.

2.3. Mating and sporulation of *P. pastoris*

The strains were inoculated onto Petri dishes containing YPD medium (2% peptone, 1% yeast extract, 2% glucose and, 2% agar) and incubated at 30 °C for 2–4 days. A full inoculation loop was then transferred to Petri dishes containing a mating-supporting medium (1% potassium chloride, 0.5% sodium acetate, 1% glucose, and 2% agar). The mating reaction proceeded at room temperature for 2–4 days (Chen et al., 2012). Cells were then seeded out to single colonies on YPD plates. Different colonies were screened under the microscope (Motic, Panthera L, China) for a larger cell size than their ancestor. Photos were taken using the imaging system of Motic Panthera L under 40× magnification. Selected colonies were then analyzed with flow cytometry analysis for ploidy level.

Table 1
Strains used in this study.

Strains	Parent and Relevant Characteristics	Ploidy level	Sources
X-33	Wild type	Haploid	Lab stock
NRRL Y-11430	Wild type	Haploid	ARS
NRRL Y-7556	Wild type	Haploid	ARS
X-33_MAT1	Derived from X-33 through mating	Diploid	This study
Y-11430_MAT9	Derived from NRRL Y-11430 through mating	Diploid	This study
X-33_mC1	Derived from X-33. pPICZ α -mCherry	Haploid	This study
X-33_mC8	Derived from X-33. pPICZ α -mCherry	Haploid	This study
Y-11430_mC4	Derived from NRRL Y-11430. pPICZ α -mCherry	Haploid	This study
Y-11430_mC8	Derived from NRRL Y-11430. pPICZ α -mCherry	Haploid	This study
X-33_mC1_MAT73	Derived from X-33_mC1 through mating	Diploid	This study
Y-11430_mC4_MAT7	Derived from NRRL Y-11430_mC4 through mating	Diploid	This study

2.4. Flow cytometry analysis of fungal ploidy

The fungal ploidy level was determined according to published protocols with some modifications (Haase and Lew, 1997). All strains were inoculated onto Petri dishes containing YPD medium and incubated at 30 °C for one day. The cells were harvested and diluted with sterile dH₂O, counted using a hemocytometer, and about 1×10^7 cells were taken and centrifuged, and the supernatant was discarded. The cells were fixed with 5 mL 70% (v/v) ethanol. Then, the cells were centrifuged, and the ethanol was discarded. Next, the cells were washed with 1 mL dH₂O. The pellet was then resuspended in 450 μ L of Tris HCl 50 mM, pH 8 and 50 μ L of 20 mg/mL RNase A (in 40% glycerol) and incubated at 37 °C for 2 h. The cells were centrifuged, and the supernatant was discarded. Next, cells were resuspended in 500 μ L of PI (propidium iodide) solution (1.05% NaCl, 1.428% MgCl₂·6H₂O, 0.5% propidium iodide, 100 mM Tris, pH 7.5) overnight at 4 °C. An hour before the analysis, 100 μ L from the cell solution was diluted with 300 μ L of PI solution. Before the actual measurement, 70 μ L of the cell solution was placed in a polystyrene tube containing 500 μ L of dilution solution (0.5% PI, 50 mM Tris, pH 7.5). Control without PI was done with the same condition for each strain. The analysis was performed using a flow cytometer (BD, LSRFortessa, Becton Dickinson, USA) equipped with a 488-nm excitation laser. Measurements of the forward scatter and the side scatter were done to identify a single cell. Gates and regions were placed around populations of cells with common characteristics to exclude cell debris, apoptotic cells, and doublets from the analysis. Forward and side scatter of 30,000 cells per sample (~100–200 events per second) were measured. Data were analyzed with analysis software (BD, FlowJo, Becton Dickinson, USA).

2.5. Evaluation of growth in shake flasks

In order to evaluate growth using shake flasks, all strains were inoculated onto Petri dishes containing YPD medium and incubated at 30 °C for three days. A full inoculation loop was then transferred to 20 mL YPD (1% yeast extract, 2% peptone and 2% glucose) or YPG medium (1% yeast extract, 2% peptone and 2% glycerol) in 100 mL Erlenmeyer flasks and incubated in a shaker-incubator (MRC, orbital shaker incubator, Holon, Israel) at 30 °C, 250 rpm for 8 h. Samples were taken to determine the biomass (dry cell weight = DCW) – 1 mL samples were collected, centrifuged, washed twice with dH₂O, and dried in 90 °C until constant weight in disposable aluminum dishes (in duplicates). 1.5 mg of cells (based on the DCW) were transferred to 250 mL Erlenmeyer flasks containing 50 mL YPD or YPG media and incubated at 30 °C and 400 rpm for 49 h in a shaker-incubator, for the growth experiment. The experiment was done in duplicate, and samples were taken at 5, 15, 24, 31, and 49 h. Microscope visualization confirmed that growth was free from contamination. The pH was measured using a pH meter (Eutech Instruments, CyberScan, Singapore). For biomass analysis – 1 mL samples were collected and centrifuged, the pellet was washed twice with dH₂O and dried in a 100 °C oven in glass tubes (In duplicates). For HPLC

analysis, 1 mL samples were collected and centrifuged, and the supernatant was filtered using 0.22 μ m filter, and 2 μ L were injected to the instrument. Glucose, glycerol, and ethanol levels were determined using the Ultimate 3000 system (ThermoFisher Scientific, Waltham, MA, USA) equipped with a Phenomenex Rezex ROA- organic acid H+ (8%) LC column (300 \times 7.8 mm) and guard columns (50 \times 7.8 mm) of the same material (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Shodex RI-101, Showa Denko, NY, USA). The HPLC column was heated to 60 °C. The mobile phase comprised 0.005 N sulfuric acid in water at a flow rate of 0.6 mL/min. The concentrations were calculated from calibration curves for standard solutions.

2.6. High-cell density fermentation

High-cell density cultivations were conducted in a 3.7 L laboratory bioreactor (Bioengineering AG, KLF, Switzerland). First, all strains were inoculated onto Petri dishes containing YPD medium and incubated at 30 °C for three days. A full inoculation loop was then transferred to 50 mL YPG medium in 250 mL Erlenmeyer flasks to use as a starter flask. Glycerol was used to avoid the Crabtree effect in the flask. Starter flasks were incubated in a shaker-incubator at 30 °C, 400 rpm for 4 h 30 mL from the starter flask were transferred to 1 L Erlenmeyer flasks containing 200 mL YPG medium and incubated at 30 °C and 400 rpm for 7–8 h. Then, this culture was inoculated into 1 L fermentation medium held in a 3.7 L fermenter containing 20 g yeast extract, 9 g (NH₄)₂SO₄, 38 g KH₂PO₄, 18 g MgSO₄·7H₂O, 3 g CaCl₂·2H₂O, 42.5 mg ZnSO₄·7H₂O, 325 mg FeSO₄·7H₂O, 25 mg CuSO₄·5H₂O, 35 mg MnSO₄·4H₂O, 1 mL anti foam (Sigma 204), 0.8 mg biotin, 0.8 mg folic acid, 4 mg inositol, 4 mg pyridoxine hydrochloride, 0.8 mg p-aminobenzoic acid, 4 mg thiamine hydrochloride and 2 mg riboflavin. While glucose was used in the shake-flask experiments, dextrose was used in the fermentation trials to better mimic industrial settings. Dextrose or glycerol as a carbon source was fed from a 60% (w/v) sterile solution supplemented with 1.2% yeast extract. The fermentation operation was divided into two phases. A dextrose or glycerol batch phase (44 g of 60% dextrose or glycerol solution were fed to the fermenter at the beginning of the process) and a dextrose or glycerol fed-batch phase. The second phase started when the yeast culture consumed the dextrose or glycerol entirely, and the concentration was 0 g/L. The measurement of dextrose concentration in the broth was done using a urine stick (MACHEREY-NAGEL, Medi-Test, Düren, Germany) and the measurement of glycerol concentration in the broth was done using a refractometer (Soonda, digital glycerin refractometer, China). The temperature was maintained at 30 °C during the entire process. The pH was kept at 5 using a 10% ammonium hydroxide solution. Agitation was kept around 1200 rpm. The aeration was manually adjusted from 4.5 L air/min at the beginning of the fermentation to 5.0 L air/min when the biomass reached 80–100 g DCW/L and to 6.0 L air/min when the biomass reached 130–150 g DCW/L. In the first 16 h from the start of the second (fed-batch) phase, samples were taken regularly every hour to measure the concentration of dextrose or glycerol in the broth, and later, samples were taken only

once every few hours. The dextrose concentration inside the fermenter was kept between 0 and 1.5 g/L and the glycerol kept between 0 and 3 g/L. If the concentration was higher, the flow rate of the carbon source was decreased and vice versa. The dextrose, glycerol and ammonia solutions that were fed to the fermenter were measured using a weight (Pris, HT-NC, China). In addition, DCW was measured every few hours. The fermentation medium was checked under the microscope every day to confirm that it was free from contamination.

2.7. Cloning of mCherry in *P. pastoris*

In order to compare the expression and secretion of a recombinant protein by the WT strains (and diploid versions), mCherry was used as a model protein. The mCherry protein gene was cloned into pPICZα (Invitrogen, Carlsbad, CA, U.S.A.) between *Xho*I and *Not*I (New England Biolabs, Ipswich, MA, U.S.A.) restriction sites. The sequence from the *Xho*I site to the end of the α-signal was rebuilt using PCR. The mCherry protein gene sequence and the primers are listed in [Supplementary Table 1](#). The plasmids were then transformed into *E. coli* XL1 and selected on low-salt LB plates (1% tryptone, 0.5% NaCl, 0.5% yeast extract and 2% agar) with 50 µg/mL Zeocin. The open reading frame, with the yeast secretion signal peptide, was confirmed by Sanger sequencing. For gene transformation to *P. pastoris*, the selected *E. coli* XL1 were grown overnight at 37 °C in 200 mL of low-salt LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extract) with 50 µg/mL Zeocin and plasmids were extracted by NucleoBond Xtra Midi (MACHEREY-NAGEL, Düren, Germany). The plasmid was linearized at 37 °C with *Sac*I-HF (New England Biolabs, Ipswich, MA, U.S.A.). Agarose gel electrophoresis was used to check the linearization of the plasmid. Next, the linearized plasmid was purified by a PCR purification kit, NucleoSpin, gel and PCR clean-up, (MACHEREY-NAGEL, Düren, Germany) using dH₂O instead of the elution buffer. *P. pastoris* strains were prepared according to the pPICZα protocol (Invitrogen) with some modifications. Briefly, all strains were inoculated onto Petri dishes containing YPD medium and incubated at 30 °C for 3–4 days. A full inoculation loop was then transferred to 3 mL YPD medium in a 14 mL culture tube with a 2-stage cap and grown overnight at 30 °C in a suspension mixer (MRC, TMO-1701, Holon, Israel) at 150 rpm to use as a starter tube. 0.5 mL from the starter tube was transferred to 50 mL YPD medium in 250 mL Erlenmeyer flask and incubated at 30 °C and 250 rpm until the OD₆₀₀ reached 1–2. The cells were collected and centrifuged. The pellet was washed twice with dH₂O and once with 1 M sorbitol. 80 µL of 1 M sorbitol was added to the cells, which were placed to cool on ice. 80 µL of the cell's solution with 6 µg of the linearized plasmid were placed in electroporation cuvettes and was electroporated using the manufacturer's instructions for *S. cerevisiae* (Bio-Rad, MicroPulser Electroporation Systems, CA, USA) with a charging voltage of 1.5 kV. Next, 1 mL of ice cold 1 M sorbitol was added to the cuvettes and the cell solution was transferred to a sterile tube. After 1 h at 30 °C, the cells were seeded on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 18% sorbitol and 2% agar) containing 100 µg/mL Zeocin at 30 °C for 3 days.

2.8. *P. pastoris* clone selection

Two colonies of each transformation were picked into a 14 mL culture tube with a 2-stage cap containing 3 mL of YPG medium and grown for 24 h at 30 °C in a suspension mixer (MRC, TMO-1701, Holon, Israel) at 150 rpm. Next, the transformants were diluted and seeded on YPD agar plates containing 100, 500 and 1000 µg/mL Zeocin. The protein production was tested according to US 4,617,274 with some modifications ([Wegner, 1986](#)). Briefly, the transformants were inoculated into a 14 mL culture tube with a 2-stage cap containing 3 mL of growth media BMGY (0.9% yeast extract, 1.8% peptone, 1.2% YNB, 0.8% KH₂PO₄, 0.5% K₂HPO₄ supplemented with 0.75% (v/v) glycerol) and grown for 24 h at 30 °C in a suspension mixer at 150 rpm. Next, expression of the protein was induced with methanol by diluting the culture with 300 µL

of BMMY media (0.9% yeast extract, 1.8% peptone, 1.2% YNB, 0.8% KH₂PO₄, 0.5% K₂HPO₄ supplemented with 10% (v/v) methanol) every 12 h for 3 times. After another day, the broth was centrifuged, the yeast pellet was discarded and the growth medium's fluorescent absorbance was measured using a fluorescent microplate reader (Bio-Tek, Synergy H1, Winooski, Vermont, USA) with Ex/Em of 550/610 nm. Finally, one clone of each strain was used to create a diploid version as described and for large-scale protein expression in the 3.7 L laboratory bioreactor.

2.9. mCherry production on YPM plates

After the creation of the diploid strains, both the haploid and diploid versions of the Y-11430 and X-33 strains were evaluated for growth and mCherry production using YPM plates (2% peptone, 1% yeast extract, 2% methanol and, 2% agar, with methanol added after autoclaving to avoid evaporation). The yeast strains were seeded directly from –80 °C storage onto the same YPM plates and incubated for 4 days at 30 °C ([Kobayashi et al., 2000](#)). To assess mCherry production, images of the grown colonies were captured using a Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France) equipped with a Cy3 filter for fluorescence detection. The images were then analyzed using Evolution-Capt software, allowing for a detailed comparison of fluorescence intensity and mCherry production between the haploid and diploid strains. Fluorescence was further verified using an AXIOscope A1 fluorescence microscope (Zeiss, Munich, Germany) equipped with an mCherry filter, and the images were analyzed with ZEN 2.6 software to ensure that the observed intensity was not due to autofluorescence.

2.10. mCherry production in a 3.7 L fermenter

The fermentation operation was divided into three phases for protein production. A glycerol batch phase (44 g of 60% glycerol solution was fed to the fermenter at the beginning of the process), followed by the glycerol fed-batch phase. It continued in the same manner as described before until the biomass reached about 50–60% of the highest biomass of each WT strain. Finally, the induction phase started immediately after the glycerol in the media was consumed. In the induction phase, the carbon source was switched to 99% methanol solution supplemented with 17 mg/L ZnSO₄·7H₂O, 130 mg/L FeSO₄·7H₂O, 10 mg/L CuSO₄·5H₂O, 14 mg/L MnSO₄·4H₂O, 0.8 mg/L biotin, 0.8 mg/L folic acid, 4 mg/L inositol, 0.8 mg/L p-aminobenzoic acid, 4 mg/L pyridoxine hydrochloride, 2 mg/L riboflavin, and 4 mg/L thiamine hydrochloride and 10 g of yeast extract was fed into the fermenter.

For the X-33 clones, an additional 10 g of yeast extract was added 70 h after the start of the fermentation. The methanol solution was fed to the fermenter at a constant rate of 7.8–8.8 g/h. The fermentation operation of the diploid strain was similar to the haploid strains and the induction phase started with the same timing as the haploid. The aeration was manually adjusted from 4.5 L air/min at the beginning of the fermentation to 5.0 L air/min when the biomass reached 80–100 g DCW/L, to 5.5 L air/min at the beginning of the induction phase and to 6 L air/min after 35–40 h from the beginning of the induction phase. Before and during the induction phase, samples were taken, and protein secretion was evaluated by Bradford assay (Bio-Rad), medium's fluorescent absorbance, and native PAGE gels. In addition, ammonium concentration in the fermentation medium was checked every few hours using ammonium sticks (Quantofix Ammonium, MACHEREY-NAGEL, Düren, Germany). The fermentation medium was checked under the microscope every day to confirm that it was free from contamination.

Another set of fermentations for the Y-11430 and X-33 clones was carried out under the same conditions as described here, with the exception that for the X-33 clones, no additional yeast extract was added after 70 h.

2.11. Purification of mCherry from the medium

To purify mCherry from the fermentation medium, 250 μ L of the fermentation medium after 123 h was diluted with 250 μ L of dH₂O and transferred to a 10 kDa membrane tube (Millipore, Tullagreen, Carrigtwohill Co., Cork, Ireland). The mixture was centrifuged at 14,000 g for 20 min. After centrifugation, the retentate was diluted with water to a final volume of 5 mL and then processed using a 100 kDa membrane tube. The purification steps were followed using a native PAGE gel to ensure that mCherry was not lost during the process. The filtrate from the 100 kDa membrane, which contained the mCherry, was analyzed using the Bradford assay (Bio-Rad) to quantify the protein.

2.12. Poly acrylamide gels electrophoresis

Native PAGE gel was performed according to published protocols (Walker, 2012). Gels were imaged using the Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France) and to evaluate protein purity, gels were stained with Coomassie protein stain.

2.13. Statistical analysis

The students' *t*-test was used to compare the two sample groups statistically. Data and error bars are depicted as means and standard deviations derived from distinct experiments and/or culture replicates. Statistical significance levels were denoted as $p < 0.1$, $p < 0.05$, and $p < 0.01$ using a two-tailed distribution.

3. Results and discussion

3.1. Generation of diploid versions of WT strains

The correlation between higher ploidy level and heterologous recombinant protein expression is not straightforward and has not been definitively established and documented in the literature. It has been

reported that changing ploidy level alters the volume of *S. cerevisiae* cells, such that tetraploid cells have higher size than diploids, which have higher size than haploids (Mundkur, 1953). Mating was performed on a designated medium (Chen et al., 2012) and the cells were transferred to YPD plates for isolation seeding. Then, different colonies were picked and checked under the microscope to select bigger-looking cells. The cells that appeared to be larger than the WT cells were picked and analyzed by flow cytometry. The microscope images of the three WT strains (X-33, NRRL Y-11430, NRRL Y-7556) and the cells that were suspicious of undergoing mating (X-33_MAT1 and 11,430_MAT9) are shown in Fig. 1.

It is evident that the cells that successfully mated, X-33_MAT1 and 11,430_MAT9 (Fig. 1d and e), are bigger than the cells of their ancestors, X-33 and Y-11430 respectively (Fig. 1a and b). The diameters of the haploids were 1–6 μ m for X-33, 1–5 μ m for Y-7556 and 1–7 μ m for Y-11430. The diameters of the diploid were 2–8 μ m for X-33_MAT1 and 2–9 μ m for the Y-11430_MAT9 after 3 days on YPD Petri dishes. Attempts to obtain diploids of Y-7556 were unsuccessful although zygotes and spores were observed under the microscope in the mating-supporting medium as shown in Supplementary Fig. 1.

Next, flow cytometry was used to evaluate the DNA content within cells. The strains were grown on YPD Petri dishes to avoid heavy aggregates appearing after liquid media growth. About 1×10^7 cells were fixed with 70% ethanol, followed by RNase treatment and staining with propidium iodide (PI) (Haase and Lew, 1997). A total of 30,000 cells in each sample were counted in the cytometer (100–200 events per second) and every sample was measured twice. The population of the cells from the dot plot (forward scatter versus side scatter) was gated, and a histogram with a logarithmic fluorescence intensity scale of PI versus cell count was made. The overlay histogram analysis results of the total gated cells are presented in Fig. 2. In each histogram, the initial peak to the left signifies the G1 phase of the cell cycle, while the subsequent peak on the right denotes the G2 phase. The area between these peaks corresponds to cells in the S phase of the cell cycle (Todd et al., 2018). Bellon et al. reported on fluorescence flow cytometry analysis of

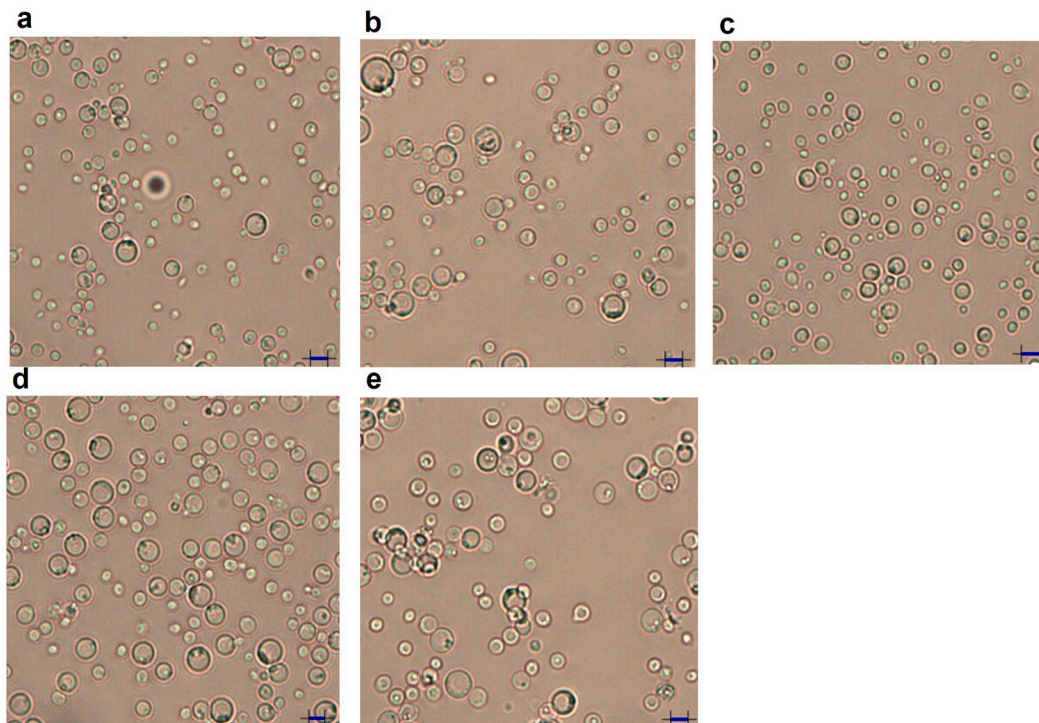


Fig. 1. Light microscopy images of WT (haploid) and diploid cultures. a, X-33; b, Y-11430; c, Y-7556; d, X-33_MAT1 (diploid version); e, Y-11430_MAT9 (diploid version). After growth on YPD plates. Scale bar = 5 μ m.

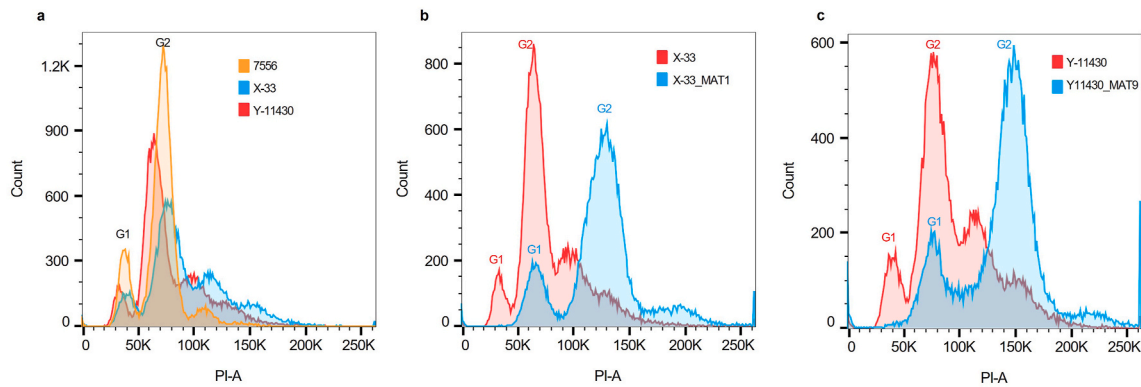


Fig. 2. Histogram of gated cell count by propidium iodide (area). **a**, WT strains; **b**, Haploid and diploid versions of X-33; **c**, Haploid and diploid versions of Y-11430. Two peaks are present (G1, G2), suggesting that the population of each strain contains only cells of a single ploidy.

haploid, diploid and tetraploid *S. cerevisiae* strains (Bellon et al., 2013). Each culture produced dual fluorescence peaks, with the second peak associated with cells in the process of DNA synthesis. The diploid and tetraploid control strains were easily identifiable, as non-dividing cells

exhibited peaks approximately twice and four times the fluorescence levels of the haploid strain, respectively (Bellon et al., 2013). In the haploid samples of X-33 and Y-11430, the G2 fluorescent peak contains two copies of the genome and aligns with the G1 fluorescent peaks of the

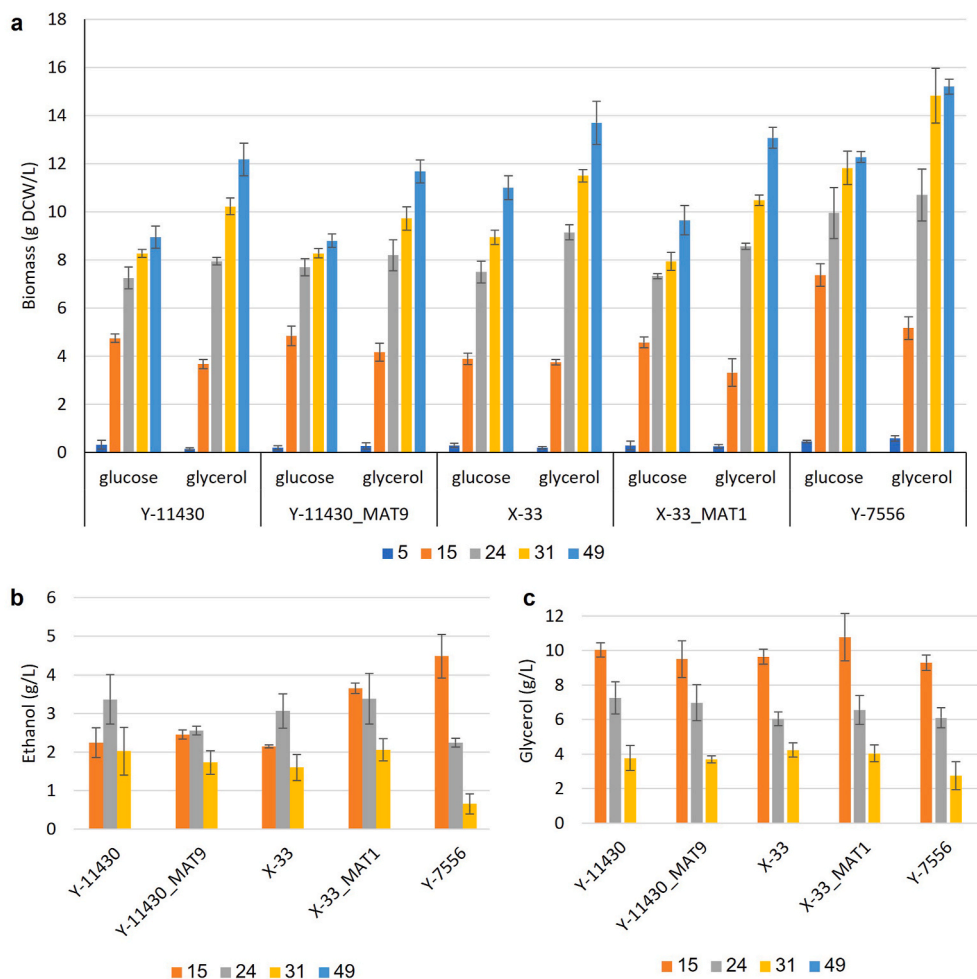


Fig. 3. Shake flask experiments used to determine growth rates of haploid and diploid WT strains using two carbon sources. WT strains Y-11430, Y-7556, X-33, and two diploid versions, Y-11430_MAT9 and X-33_MAT1, were grown in 250 mL flasks with glucose or glycerol (2% w/v) as carbon sources. **a**, Biomass concentration as a function of strain and carbon source with growth time (h). The biomass was measured periodically; **b**, Ethanol concentration measured with HPLC of WT and diploid strains. At 49 h the concentration was non-detectable; **c**, Glycerol concentration measured with HPLC of WT and diploid strains. At 49 h the concentration was non-detectable. Different colors represent different time points in hours. The results are an average of duplicates. The data are expressed as mean \pm SD. Statistical significance was determined using a *t*-test, and *p*-values are mentioned in the text depending on the comparisons described. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

X-33_MAT1 and Y-11430_MAT9 diploid samples (Fig. 2), similar to results received for the *S. cerevisiae* strains (Bellon et al., 2013). This is due to the fact, that haploid G2 and diploid G1 fluorescent peaks both contain two copies of the genome (Todd et al., 2018). From these results, it can be concluded that the formed X-33_MAT1 and 11,430_MAT9 are diploid versions of X-33 and Y-11430 and therefore confirm the successful creation of diploid versions of *P. pastoris* WT strains.

3.2. Determination of growth rates in shake flasks

The initial growth experiments were conducted in shake flasks, utilizing either glucose or glycerol as carbon sources, to determine the most suitable carbon source for achieving high biomass and rapid growth rates across all the WT haploid and diploid strains. These results are important for designing fermenter growth media, particularly when a high growth rate is critical, and time efficiency is essential to mitigate costs. Glycerol offers a distinct advantage by avoiding the Crabtree effect, a phenomenon in which yeast produce ethanol from glucose under aerobic conditions (De Deken, 1966). However, it is noteworthy that glycerol is more expensive than glucose (Hang et al., 2009). The use of glucose, while cost-effective, necessitates strict control of its concentration to prevent the onset of the Crabtree effect, a control that can be effectively managed in fermenters but not easily regulated in shake flasks.

The biomass (g DCW/L) obtained in shake flasks for all the strains using two different carbon sources is presented in Fig. 3a. The initial starting point in all flasks was 0.03 g DCW/L. Notably, Y-7556 exhibited faster growth and reached higher biomass concentrations than Y-11430, X-33, and their diploid versions within 15 h, a trend that persisted throughout the experiment ($p < 0.1$). In the first 15 h, Y-11430 and Y-7556 grew faster on glucose than on glycerol (for Y-11430 4.8 ± 0.2 vs. 3.7 ± 0.2 g DCW/L, and Y-7556 7.4 ± 0.2 vs. 5.2 ± 0.2 g DCW/L, respectively) ($p < 0.01$). However, after 31 h, glycerol enabled high biomass concentrations ($p < 0.05$). Furthermore, glucose measurements taken from the flasks after 15 h of growth indicated complete depletion in the case of Y-7556. The concentrations in the media were 3.6 ± 0.2 g/L for Y-11430, 2.6 ± 0.2 g/L for Y-11430_MAT9, 4.3 ± 0.4 g/L for X-33 and 3.0 ± 0.1 g/L for X-33_MAT1. For these strains, the glucose concentration reached zero between the time points of 15–24 h. The ethanol concentrations in the cultures grown on glucose, as measured using HPLC, are presented in Fig. 3b. The ethanol concentration after 15 h for Y-7556 was the highest compared to all strains, likely due to the high biomass. The ethanol decreased after 15 h for this strain due to a lack of glucose, unlike the other strains, which showed increased ethanol concentration from 15 h to 24 h. Representative HPLC chromatograms for glucose, ethanol and glycerol are presented in Supplementary Fig. 2.

WT X-33 reached the same biomass after 15 h of growth in glucose and glycerol (about 3.8 g DCW/L); after 24 h, the biomass in glycerol was higher ($p < 0.01$). After 15 h of growth, strain X-33 grew slower on glucose than Y-11430 and Y-7556 ($p < 0.05$). However, after 31 h the biomass of X-33 was slightly higher than Y-11430 on glucose and glycerol (for Y-11430 8.3 ± 0.2 on glucose and 10.2 ± 0.3 g DCW/L on glycerol, and for X-33 9.0 ± 0.3 on glucose and 11.5 ± 0.3 g DCW/L on glycerol) ($p < 0.1$). The glycerol concentrations in the respective experiments are presented in Fig. 3c and show a decline with time.

After 49 h from the start of the experiment, the ethanol and the glycerol were fully consumed in all the flasks (Fig. 3b and c). Y-7556 exhibited higher biomass yields, meaning that higher biomass was obtained for the same glucose or glycerol concentration, as shown in Fig. 3a. Furthermore, X-33_MAT1 (diploid) grew faster on glucose after 15 h than X-33 (haploid), 4.6 and 3.9 g DCW/L, respectively ($p < 0.05$). Y-11430 (haploid) and Y-11430_MAT9 (diploid) grew similarly with just a minor difference.

The initial pH of the medium was 6.8. In the tested condition, the growth in glucose correlated with a faster decline in pH than growth in glycerol in shake flasks, as shown in Supplementary Fig. 3. The pH after

49 h increased in all the flasks due to carbon source depletion as shown in the HPLC analyses.

3.3. High-cell density fermentation

High-cell-density fermentation is required for industrial production of proteins, peptides, and amino acids (Zhao et al., 2021). This typically involves selecting the appropriate growth medium, controlling the temperature and pH of the culture, and adding any necessary supplements (such as vitamins or minerals) (Choi et al., 2006). In order to develop an optimal growth medium suitable for the fermentation of *P. pastoris*, NRRL Y-11430 was used as a model. Dextrose was used to ensure easy monitoring of the carbon source during the fermentation process. Several experiments were conducted for this purpose focused on exploring different medium compositions to identify the one that could sustain the highest biomass yield (more than 150 g DCW/L in a short total fermentation time). The fermentation process was divided into two phases: a dextrose batch phase and a dextrose fed-batch phase that increases cell density (Kastilan et al., 2017; Liu et al., 2016). First, 44 g of the 60% solution of dextrose was fed into the fermenter. The dextrose fed-batch phase started immediately after the batch phase, and a 60% dextrose solution was fed into the fermenter during that phase. The feeding rate of the dextrose was increased depending on the remaining concentration in the broth. The end of the fed-batch phase occurred when the culture stopped growing. After two time points of constant biomass, the experiment was ended. The dextrose concentration inside the fermenter was kept between 0 and 1.5 g/L. If the concentration was higher, the flow rate of the dextrose was decreased and vice versa. The fermentation medium with the best performance ended after 35 h with a biomass of 161 g DCW/L. This medium was termed the “base” medium in subsequent experiments. The base medium contained 20 g yeast extract, 9 g $(\text{NH}_4)_2\text{SO}_4$, 38 g KH_2PO_4 , 18 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 42.5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 325 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 25 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 35 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1 mL anti foam, 0.8 mg biotin, 0.8 mg folic acid, 4 mg inositol, 4 mg pyridoxine hydrochloride, 0.8 mg *p*-aminobenzoic acid, 4 mg thiamine hydrochloride and 2 mg riboflavin.

3.4. Determination of growth rates in the fermenter

The “base” medium and methodology described above was used with all the haploid strains to compare their growth ability using dextrose and glycerol as carbon sources. The initial dextrose amount was consumed completely after ~12 h for Y-11430, 11 h for Y-7556, and 16 h for X-33. The glycerol was consumed completely after ~13.5 h for Y-11430, 12.5 h for Y-7556, and 18 h for X-33. The dextrose concentration inside the fermenter was kept between 0 and 1.5 g/L and the glycerol was kept between 0 and 3 g/L. If the concentration was higher, the flow rate of the carbon source was decreased and vice versa. The growth curves of all the WT strains are presented in Fig. 4a. Strain Y-7556 reached a biomass concentration of 244 g DCW/L after 48 h using glycerol and 169 g DCW/L after 41 h using dextrose. Strain Y-11430 reached a biomass concentration of 235 g DCW/L after 64 h using glycerol and 161 g DCW/L after 35 h using dextrose, and strain X-33 reached a biomass concentration of 179 g DCW/L after 62 h using glycerol and 93 g DCW/L after 61 h using dextrose. Higher biomass yield was obtained for all strains on glycerol as a carbon source by more than 40% as compared to dextrose. The cell pellet of 1 mL fermentation broth of Y-7556 after growth on glycerol is presented in Supplementary Fig. 4 providing visual evidence of the very high cell density obtained. It can be seen from Fig. 4a and Table 2 that during the first 23 h, all strains were in the growth phase and each strain had similar biomass on dextrose and glycerol (for Y-11430, 110 vs. 97 g DCW/L, Y-7556, 126 vs. 110 g DCW/L and X-33 40 vs. 34 g DCW/L, respectively). The biomass (X) yield as a function of the consumed carbon source (S) during growth (Y_x^s) was calculated in this time frame, and the results are summarized in Table 2. The yield is higher on glycerol than dextrose for all the strains. In addition, the yield on dextrose is

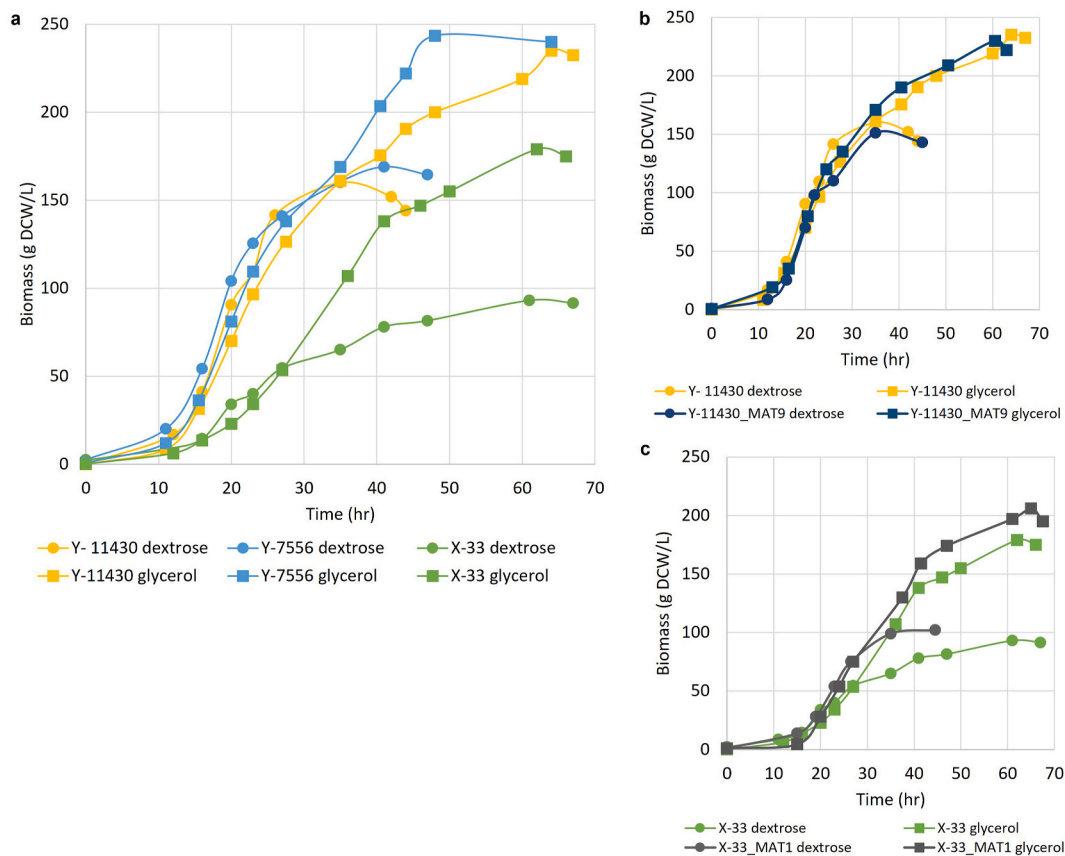


Fig. 4. Biomass concentration during the fermentation of *P. pastoris* WT strains and their diploid versions in a 3.7-L fermenter as a function of carbon source. Dextrose or glycerol as a carbon source was fed from a 60% w/v solution. The temperature was maintained at 30 °C during the entire process. The pH was kept at 5 using a 10% ammonium hydroxide solution. Agitation was kept around 1200 rpm and the aeration was manually adjusted from 4.5 L air/min to 6 L air/min. **a**, haploid *P. pastoris* WT strains; **b**, Y-11430 and Y-11430_MAT9; **c**, X-33 and X-33_MAT1.

Table 2

Comparison between WT strains after 23 h from the start of the fermentation and biomass concentration of WT strains at the end of the fed-batch phases in the 3.7 L fermentation.

		Biomass T23 (gDCW/L)	Dextrose/glycerol consumed (g/L)	^a Y_x^s (g/g)	Total fermentation duration (h)	Final biomass (g DCW/L)
Y-7556	Dextrose	126	254	0.50	41	169
	Glycerol	110	178	0.62	48	244
Y-11430	Dextrose	110	238	0.46	35	160
	Glycerol	97	149	0.65	64	235
X-33	Dextrose	40	109	0.37	61	93
	Glycerol	34	55	0.62	62	179

^a Biomass (X) yield as a function of the consumed carbon source (S) during growth (g/g).

higher for Y-7556 (0.5 g/g) and Y-11430 (0.46 g/g) than for X-33 (0.37 g/g).

Strains Y-7556 and Y-11430 reached a higher final biomass concentration, compared to X-33 (Table 2). These results are inconsistent with those obtained in flasks for strains X-33 and NRRL Y-11430. In flasks, after 31 h, the biomass of X-33 was slightly higher than Y-11430 on glucose and glycerol. In shake flask experiments, media with high nutrient concentrations are utilized to promote rapid cell growth. Nevertheless, the resulting biomass concentrations are generally much lower than that of fermenter experiments. This difference in biomass concentration can be attributed to variations in nutrient availability, oxygen transfer rates, and overall growth conditions.

A comparison between the haploid and the diploid was also made in the 3.7 L fermenter (Fig. 4b and c). The WT Y-11430 and the diploid version Y-11430_MAT9 grew similarly with just a minor difference. However, X-33_MAT1 reached the final biomass faster than WT X-33, as

shown in Fig. 4c. The biomass after 35 h using dextrose was 65 g DCW/L for X-33 and 99 g DCW/L for X-33_MAT1. The biomass after 62 h using glycerol was 179 g DCW/L for X-33 and 197 g DCW/L for X-33_MAT1.

It can be concluded from the above experiments, that glycerol is a preferred carbon source for the growth of all three *P. pastoris* WT strains and the diploid versions of X-33 and Y-11430, resulting in more than 40% higher biomass concentration. Arias et al. reported that using glycerol as a carbon source resulted in a biomass of 94.7 g DCW/L after 24 h of growth, while dextrose yielded a biomass of 95.2 g DCW/L after 32 h of growth (Arias et al., 2017). These findings suggest that glycerol led to faster growth and biomass accumulation compared to dextrose using the *P. pastoris* cloned strain SMD 1168 with a similar trend during the flask experiment.

Ultra-high cell densities are defined as >100 g/L DCW or >400 g/L wet cell weight for *P. pastoris* (Picanço-Castro and Swiech, 2018). Zhou et al. reported a final biomass concentration of 116.3 g DCW/L after 120

h using glycerol as the carbon source and methanol as an inducer with *P. pastoris* strain SMD 1168 (Zhou et al., 2014). Some papers reported on high biomass but used wet or unwashed cell weight. YaPing et al. reported 420 g wet cell weight/L after 84 h (YaPing et al., 2017). Others convert OD₆₀₀ to a DCW. For example, Dai et al. reached a maximum OD₆₀₀ of 420 and used the ratio of 1 OD₆₀₀ = 0.24 g DCW/L (Dai et al., 2023). This conversion ratio is also reported in previous work (Wang et al., 2021), which is not necessarily accurate in high-density and long fermentation processes as absorbance readings are sensitive to the size of cells (Lieberman, 2004), which is modulated by nutrients. Thus, cells growing in poor-nutrient medium can be nearly half the size of cells in rich media (Leitao and Kellogg, 2017) and temperature also induces variability in cell size (Zakhartsev and Reuss, 2018). Heyland et al. used the *P. pastoris* strain SMD 1168 and achieved an OD₆₀₀ of 990 after 66 h from the start of the fermentation. They correlated this OD₆₀₀ value with a 218 g DCW/L biomass concentration. However, 48 h from the start of the fermentation, the biomass was around 70 g DCW/L (Heyland et al., 2010). The marked increase in biomass from 70 g DCW/L to 218 g DCW/L within this time frame is not commonly observed in other studies involving *P. pastoris*.

The biomass of strain Y-7556 reached 244 g DCW/L within 48 h (Fig. 4a). The OD₆₀₀ at this time point was 1120. However, at the time point of 64 h (deep stationary phase), the OD₆₀₀ was 1260, although the biomass was 240 g DCW/L. To our knowledge, this is the highest biomass concentration reported to date for *Pichia pastoris*.

All in all, a comparison of three WT strains of *P. pastoris* was performed both in shake flasks and fermentation conditions. Strains Y-7556 and Y-11430 performed better than X-33. Glycerol was the favored carbon source over dextrose.

3.5. Clone selection of mCherry producers

The future goal of this work is to use the established platform to produce proteins of commercial interest, thus protein expression levels are a crucial parameter. *P. pastoris* is a methylotrophic yeast that utilizes methanol as the sole carbon source (Byrne, 2015). Protein production in *P. pastoris* is typically achieved by using the AOX1 promoter, which responds to methanol induction and has solid and regulatable characteristics (Chang et al., 2018). pPICZα vector contains the AOX1 promoter and is used to express and secrete recombinant proteins in *P. pastoris* which are expressed as fusions to a peptide encoding a secretion signal. It also contains the Zeocin resistance gene for *E. coli* and *P. pastoris* selection, which is driven by the constitutive translational elongation factor 1α promoter (TEF1) (<https://www.thermofisher.com/order/cat>

alog/product/V19520). mCherry served as a model to assess the protein production capabilities of the different strains. Cloning was successful for X-33 and NRRL Y-11430 but not for NRRL Y-7556, likely due to its thicker cell wall and poor transformation efficiencies, as previously reported (Brady et al., 2020). Some papers present high-efficiency transformation by electroporation of *P. pastoris* pretreated with lithium acetate and dithiothreitol (Wu and Letchworth, 2004). Attempts to obtain colonies with those methods from NRRL Y-7556 were unsuccessful.

After transformation, two colonies from each strain (mC1 & mC8 for X-33, mC4 & mC8 for NRRL Y-11430) were selected and seeded in YPG medium for 24 h at 30 °C. Subsequently, the transformants were diluted and plated onto YPD agar plates containing increasing concentrations of Zeocin. This method, which generates multicopy expression strains, is a known technique to enhance the production of recombinant proteins in *P. pastoris* (Lin-Cereghino et al., 2008). However, it is important to note that an increased vector copy number does not always correlate with higher protein yields (Hu et al., 2011), necessitating further evaluation of protein expression levels (Hu et al., 2011). Clones of X-33 were grown on plates containing 100 and 500 µg/mL Zeocin, while NRRL Y-11430 clones were grown on 1000 µg/mL Zeocin plates, as presented in Fig. 5a. Next, protein expression was induced using methanol. For the X-33 clones, the difference in protein expression was visually noticeable, with X-33_mC1 exhibiting a reddish color compared to X-33_mC4, as shown in Fig. 5b. Consequently, X-33_mC1 was selected for further work. In the case of NRRL Y-11430 clones, the yeast pellet was separated by centrifugation, and the growth medium was measured for fluorescence absorbance with excitation/emission wavelengths of 550/610 nm. The absorbance readings for the two clones, Y-11430_mC4 and Y-11430_mC8, were similar (~95,000) and Y-11430_mC4 was selected.

3.6. Generation of diploid versions mCherry-producing strains

The selected clones, X-33_mC1 and Y-11430_mC4, were then used to create diploid strains (X-33_mC1_MAT73 and Y-11430_mC4_MAT7) using the same methodology applied to non-expressing WT strains. The overlay histogram analysis results in Supplementary Fig. 5 provide evidence that diploid strains were successfully created.

Chen et al. created diploid versions of *P. pastoris* using two plasmids that contained different chains of anti-HER2 IgG1 monoclonal antibodies. One of the plasmids contained the mCherry gene with the anti-HER2 LC chain with the arsenic resistance selection marker and the other the GFP gene with the Anti-Her2 HC chain with nourseothricin resistance selection marker (Chen et al., 2012). In parallel, they also

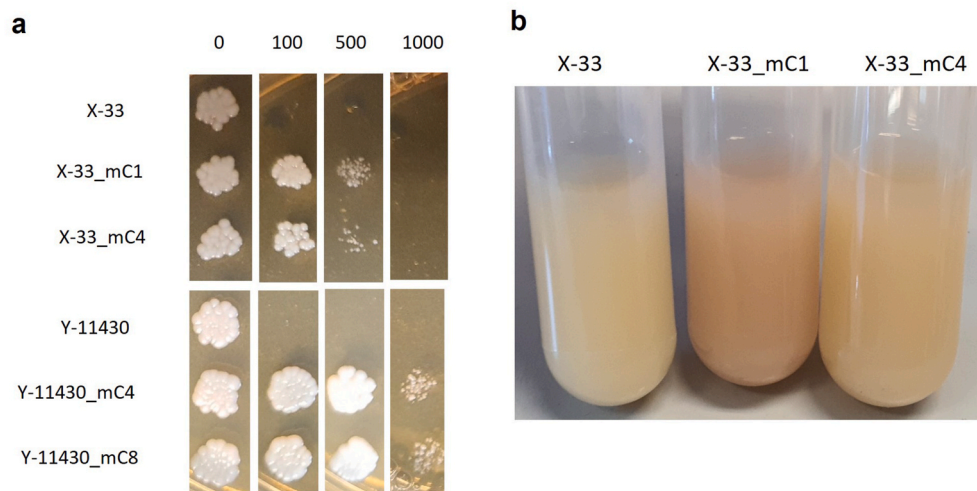


Fig. 5. Selection of mCherry-producing clones. **a**, Plating of 10⁻⁴ dilution of strains on YPD and YPD supplemented with different concentrations of Zeocin; **b**, X-33 clone selection by visual appearance.

created a haploid strain (YGLY13979) capable of producing both anti-HER2 IgG1 chains. This strain was rendered auxotrophic for uracil (YGLY19313) and arginine using URA5 as a selectable marker (YGLY19853). They successfully generated a diploid strain (YGLY19895) using mating between YGLY19313 and YGLY19853. This strain contained one copy of URA5 and ARG3 in its diploid genome (Chen et al., 2012). However, the assumption that introducing a suitable marker gene will result in complementation, creating a physiological identical to the reference strain, does not hold (Pronk, 2002). Çakar et al. observed discrepancies in final cell densities when they complemented auxotrophic strains with the plasmid based LEU2 selection marker compared to chromosomal expression of LEU2 in otherwise isogenic, prototrophic strains (Çakar et al., 1999). X-33 was reportedly created by complementation of HIS4 into GS115 to restore prototrophy (Brady et al., 2020). Brady et al. evaluated X-33 for histidine auxotrophy or bradytroph (slowed growth) by comparing it to the WT Y-11430 after growth on a synthetic medium (Brady et al., 2020). In addition, diploid strain resulting from auxotrophic complementation using mating can synthesize essential nutrients due to the complementation of genes from the parent haploid strains. However, these essential nutrients are still required for optimal growth of the diploid strain. As evidence, the biomass (wet cell weight/L) was 24% higher for the haploid (YGLY13979) than the diploid (YGLY19895) (Chen et al., 2012).

Here, we used mating that occurred among the progeny of a single haploid cell to ensure that the mCherry gene would be double in each diploid strain compared to the haploid with no other modifications.

3.7. mCherry production on YPM plates

The selected clones, X-33_mC1 and Y-11430_mC4, along with their diploid versions, were evaluated for growth and mCherry production efficiency after 4 days of incubation on YPM plates (Kobayashi et al., 2000). YPM plates contained methanol instead of glucose (as found in

YPD), which is crucial for inducing the AOX1 promoter in the plasmid used, enabling the assessment of mCherry production efficiency under methanol-induced conditions. Both haploid and diploid versions of the Y-11430 and X-33 strains were analyzed for colony size and fluorescence intensity, with all strains seeded on the same plate to ensure uniform environmental conditions (Fig. 6a). The Y-11430 strains, both haploid and diploid, formed larger single colonies compared to the X-33 strains.

To further assess mCherry production, fluorescence imaging was performed using the Fusion FX imaging system, followed by a detailed 3D analysis using Evolution-Capt software, as shown in Fig. 6b. In the 3D fluorescence images, the diploid strains exhibited significantly higher fluorescence intensity compared to their haploid counterparts in both Y-11430 and X-33 clones. This was visually represented by more intense colors and greater height peaks in the diploid strains, indicating higher levels of mCherry expression.

The color intensity in the 3D images, particularly in the diploid strains, was much more pronounced, suggesting that these strains are more efficient at producing the fluorescent protein. This increased fluorescence, despite the colonies being of similar size for haploid and diploid versions of the same strain, highlights the enhanced protein production capabilities of the diploid strains. Additionally, it was observed that the Y-11430 clones have higher fluorescence intensity than the X-33 clones, further demonstrating the superior potential of Y-11430 over X-33 for such applications. This fluorescence was verified using fluorescence microscopy with an mCherry filter to ensure that the intensity was not due to autofluorescence, as shown in Fig. 6c and d for the clones Y-11430_mC4 and Y-11430_mC4_MAT7. The clone Y-11430_mC4_MAT7, after growth on a YPD plate, served as a control where no intensity was detected under the microscope.

3.8. mCherry fermentation

Fermentation is considered a preferred route for successfully

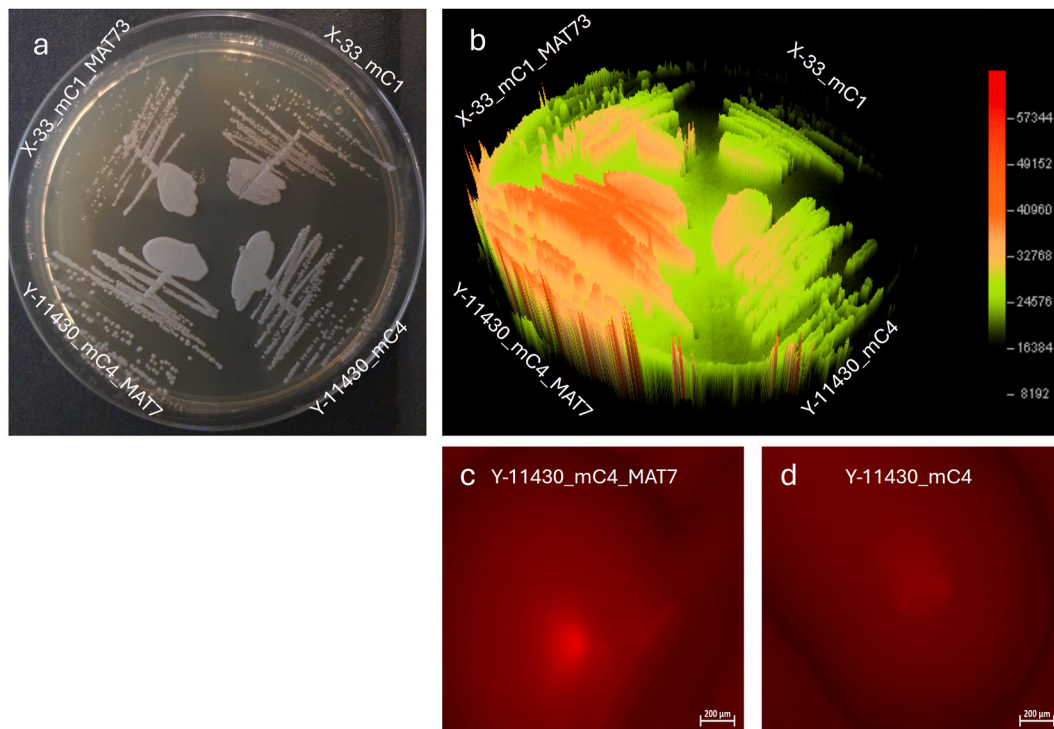


Fig. 6. Growth and fluorescence analysis of X-33_mC1 and Y-11430_mC4 clones, along with their diploid versions, on YPM plates. **a**, Representative image of yeast colonies after 4 days of incubation on YPM plates, showing the growth of both haploid and diploid versions of the X-33 and Y-11430 strains; **b**, 3D fluorescence analysis using Evolution-Capt software, depicting the fluorescence intensity of mCherry expression. The color scale on the right represents fluorescence intensity levels; **c**, Y-11430_mC4_MAT7 after growth on YPM plates under fluorescence microscopy; **d**, Y-11430_mC4 after growth on YPM plates under fluorescence microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

producing recombinant proteins using *P. pastoris* (Gao and Shi, 2013). Typically, AOX1-regulated cultivation of *P. pastoris* is divided into 3 phases: batch, fed-batch, and methanol induction phases (Liu et al., 2016). During the batch and fed-batch phases, glycerol was used as the carbon source, as it had proven to be the preferred choice for the growth of all three *P. pastoris* WT strains compared to dextrose. Since X-33 exhibited slow growth, an addition of 10 g of yeast extract was fed to the fermenter after 70 h from the start of the fermentation. Biomass was measured every few hours during the process, as shown in Fig. 7a and b. To sustain the vitality of the *P. pastoris* strains and ensure a continuous supply of nutrients in the growth media for the protein production phase, the fed-batch phase lasted until the biomass reached 50–60% of the maximum biomass observed in the WT strains (Y-11430_mC4 - 122 g DCW/L after 29 h and X-33_mC1- 89 g DCW/L after 36 h). At this point, glycerol feeding was stopped, and after complete consumption of the glycerol in the media, methanol feeding began at a constant rate. The biomass measured for the clones, X-33_mC1 and Y-11430_mC4, was similar to the WT strain (Fig. 7a and b) until the end of the fed-batch phase and the beginning of the induction phase. Afterwards, the

switch from glycerol to a slow-rate methanol feeding resulted in the biomass not reaching the same levels as the WT strain (Fig. 4). The fermentation progress for the diploid strains was similar to that of the haploid strains, with comparable glycerol and methanol feeding rates, resulting in similar biomass levels between the haploid and diploid versions. Samples were collected before and during the induction phase, and protein secretion was assessed (Fig. 7c and d) along with fluorescence measurement (Fig. 7e and f). To avoid the appearance of sporulation, the ammonium concentration in the fermentation medium was checked to ensure sufficient nitrogen throughout the process.

Fig. 8a and b presents native gels after staining with Coomassie blue for more precise visualization of protein bands. It is important to note that in native gel electrophoresis, there is no linear relationship between mass and migration. Fig. 8c provides native gel imaged using a Cy3 filter. The diploid Y-11430_mC4_MAT7 demonstrated superior protein expression compared to haploid Y-11430_mC4, as indicated by protein measurement (5548 ± 66 mg/L and 3381 ± 26 mg/L of protein after 112 h of fermentation) and fluorescence intensity ($1,512,750 \pm 6200$ and $1,255,950 \pm 4850$ after 112 h). Remarkably, diploid Y-

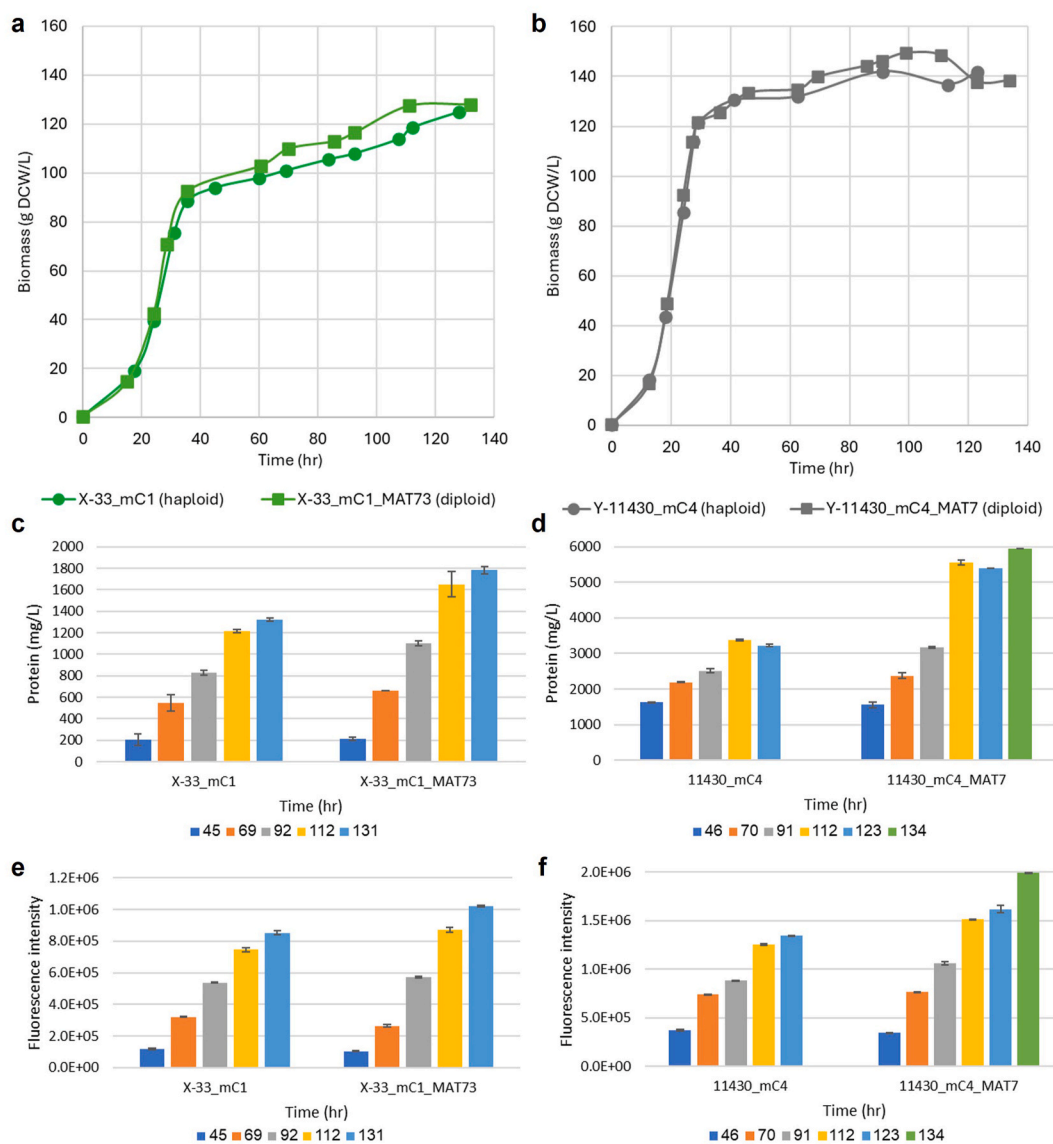


Fig. 7. MCherry production in a 3.7 L fermenter by the haploid and diploid versions of X-33 and Y-11430. **a**, Biomass concentration during the fermentation of *P. pastoris* clone X-33_mC1; **b**, Biomass concentration during the fermentation of *P. pastoris* clone Y-11430_mC4 and its diploid; **c**, Protein concentration of X-33 clone during the induction phase measured by Bradford assay kit; **d**, Protein concentration of Y-11430 clone during the induction phase measured by Bradford assay kit; **e** Fluorescence intensity during the induction phase of X-33 clones; **f**, Fluorescence intensity during the induction phase of Y-11430 clones.

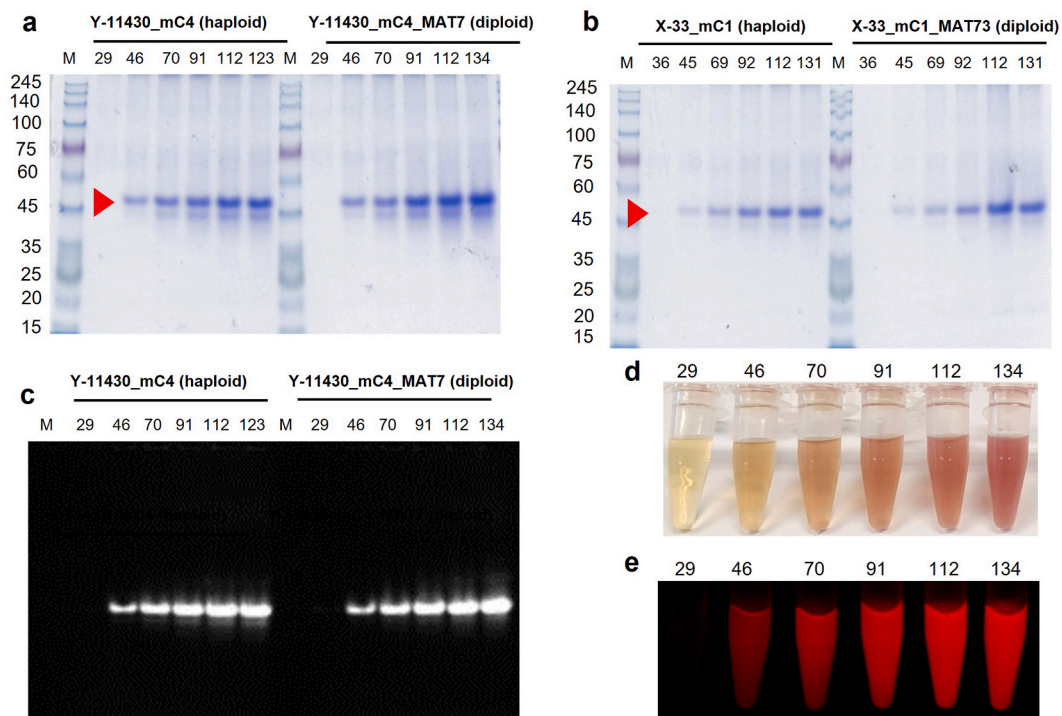


Fig. 8. MCherry production by haploid and diploid strains of Y-11430 and X-33 in a 3.7 L fermenter. **a**, Native PAGE gel Coomassie stain of media from *P. pastoris* Y-11430_mC4 and Y-11430_mC4_MAT7 expressing mCherry after 123 and 134 h from the start of the fermentation. Red arrowheads point at mCherry. Load: 2.5 μ L media; **b**, Native PAGE gel Coomassie stain of media from *P. pastoris* X-33_mC1 and X-33_mC1_MAT73 expressing mCherry after 131 h from the start of the fermentation. Red arrowheads point at mCherry. Load: 2.5 μ L fermentation media; **c**, Native PAGE gel imaged using a Fusion FX imaging system equipped with a Cy3 filter of media from *P. pastoris* Y-11430_mC4 and Y-11430_mC4_MAT7 expressing mCherry after 123 and 134 h from the start of the fermentation Load: 7.5 μ L med **d**, Visualization of the fermentation medium of Y-11430_mC4_MAT7 with increasing secreted mCherry concentrations; **e**, The fermentation medium of Y-11430_mC4_MAT7 under fluorescence binocular microscope. Numbers 29–134 indicate hours from the start of the fermentations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

11430_mC4_MAT7 continued to produce mCherry, reaching approximately 5945 ± 0 mg/L of secreted protein as measured by Bradford after 134 h, with a fluorescence intensity of $1,991,350 \pm 7700$. The same trend was recorded for the diploid X-33_mC1_MAT73 compared to the haploid X-33_mC1 (1325 ± 15 mg/L and 1781 ± 31 mg/L of protein and fluorescence intensity of $853,550 \pm 13,800$ and $1,020,050 \pm 5800$ after 131 h of fermentation).

These results highlight for the first time the advantage of diploid strains for enhanced protein production.

Regarding protein production, Y-11430_mC4 performed better than X-33_mC1, producing 3381 ± 26 mg/L of protein compared to 1219 ± 15 mg/L after 112 h as shown in Fig. 7c and d. This difference is further evidenced in fluorescence intensity ($745,500 \pm 10,650$ for X-33_mC1 and $1,255,950 \pm 4850$ for Y-11430_mC4 after 112 h), as shown in Fig. 7e and f. The same trend is evident in the native PAGE gels shown in Fig. 8a and b. The progressive increase in mCherry production by Y-11430_mC4_MAT7 is shown in Fig. 7e, where the fermentation medium gradually turns redder as the fermentation proceeds. After 134 h, a deep red color was obtained as visualized under a fluorescent binocular microscope (Fig. 8e).

The discrepancies in the increase in the expression levels observed between the fluorescence readings and the results from the Bradford assay can be explained by several factors. Firstly, fluorescent proteins can lose their fluorescence over time due to photobleaching or environmental conditions (Shaner et al., 2004). This loss of fluorescence is independent of ploidy levels and likely affected all samples. Additionally, the medium may contain a variety of proteins besides mCherry, which could further contribute to these differences. These other proteins can influence overall protein measurements, resulting in higher readings in assays that detect total protein content rather than mCherry

fluorescence specifically.

To confirm the results, two sets of fermentation experiments were conducted. The second trial for the Y-11430 clones is presented in Supplementary Fig. 6, and the repetition for the X-33 clones is presented in Supplementary Fig. 7. The data show consistent trends, with the haploid and diploid versions of the same strain exhibiting similar growth patterns, and the diploids demonstrating over 20% higher fluorescence in both Y-11430 and X-33. Additionally, the haploid and diploid versions of the same strain exhibited similar biomass.

3.9. Purification of mCherry from the medium

To confirm the superiority of the diploid as a protein producer, mCherry was purified from the fermentation broth at a small scale. As mCherry is a 26.5 kDa protein, a 10 kDa membrane was initially used to remove all small molecules, continuing with the retentate for further processing. Afterwards, a 100 kDa membrane was utilized, retaining the filtrate that contained the mCherry. The purification steps were carefully monitored using native PAGE gel to confirm that mCherry was retained throughout the process, with minimal loss during the different filtration stages, as shown in Supplementary Fig. 8. The results of the mCherry purification and quantification experiments on the clones of Y-11430 revealed a significant difference in mCherry production between the haploid and diploid strains after 123 h of fermentation. Specifically, the haploid strain produced 1245 mg/L of mCherry, while the diploid strain produced 1784 mg/L (values were extrapolated from the small-scale trials). The increase in mCherry yield by approximately 43% in the diploid strain confirms the results obtained by total protein content and fluorescence measurements. Overall, these results reinforce the conclusion that diploid *P. pastoris* strains, which grow at a similar rate as

haploid strains, are more effective in producing higher levels of recombinant proteins like mCherry. This conclusion was further validated with a recombinant canola protein (results not shown).

Chen et al. compared a diploid strain created using auxotrophies, multiple genetic modifications and selection markers (mating between two different auxotroph haploid strains) to a haploid strain that produced both chains of the IgG1 monoclonal antibodies, and the results were that the diploid strain had lower titer (920 mg/L for the diploid and 1048 for the haploid) (Chen et al., 2012). However, the comparison differs from our case since the haploids used to create diploids were not identical and included a knockout of genes and plasmid transformation using different markers (Chen et al., 2012). Expression of plasmids in yeast systems burdens cells, leading to reduced growth rates and lower production of desired metabolites compared to non-plasmid-bearing cells (Karim et al., 2013). Genetic modifications and selection markers introduced during the creation of the diploid strain may impact its growth characteristics, protein production capabilities, and overall fitness. Therefore, we suggest carefully considering these factors and evaluating the suitability of diploid strains created using those techniques.

4. Conclusions

This study aimed to compare three wild-type *Pichia pastoris* strains and their diploid versions concerning growth rates and protein expression capabilities. Successful diploid versions were identified for X-33 and Y-11430 through flow cytometry analysis. The investigation extended to flask and fermentation experiments, utilizing glucose and glycerol as carbon sources to assess biomass and yields across the different strains. Glycerol emerged as the preferred carbon source, most likely due to the avoidance of the Crabtree effect. Notably, wild-type strains Y-7556 and Y-11430 exhibited faster growth and higher biomass concentrations than X-33. Ultra-high cell densities are defined as >100 g/L DCW or >400 g/L wet cell weight. The superiority of glycerol over dextrose became evident, with Y-7556 reaching biomass concentrations of 244 g DCW/L after only 48 h from the start of the fermentation. To our knowledge, this is the highest biomass concentration reported to date for a WT strain. Cloning of mCherry succeeded for X-33 and NRRL Y-11430 but faced challenges with NRRL Y-7556. The fermentation process, divided into three phases, batch, fed-batch, and methanol induction, demonstrated enhanced protein production by diploid strains (X-33_MAT1 and Y-11430_MAT7). The enhanced protein production observed in the diploid strains can be attributed to their increased gene copy number compared to haploid strains, which contributed to the improved phenotypes in this study. In addition, the clones of Y-11430 showed better protein expression and growth potential than the clones of X-33.

To sum, this study successfully generated diploid variants of *P. pastoris* strains, verified their ploidy levels, and showcased the impact of ploidy on protein production. Additionally, the preference for glycerol over dextrose in promoting faster growth and higher biomass concentrations, achieving superior biomass exceeding 240 g DCW/L, contributes valuable insights for optimizing *P. pastoris* in industrial applications, particularly in recombinant protein production.

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CRediT authorship contribution statement

Paz Shemesh: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Ayelet Fishman:**

Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ayelet Fishman reports financial support was provided by Good Food Institute. Ayelet Fishman reports financial support was provided by Israel Ministry of Innovation, Science, and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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