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Enhanced pyruvic acid yield in an osmotic stress-resistant mutant of *Yarrowia lipolytica*

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ABSTRACT

Background: Pyruvic acid (PA), a vital α-oxocarboxylic acid, plays an important role in energy and carbon metabolism. The oleaginous yeast *Yarrowia lipolytica (Y. lipolytica)* has considerable potential for the production of PA. An increased NaCl concentration reportedly increases the biomass and PA yield of *Y. lipolytica*.
Results: To increase the yield of PA, the NaCl-tolerant *Y. lipolytica* A4 mutant was produced using the atmospheric and room temperature plasma method of mutation. The A4 mutant showed growth on medium containing 160 g/L NaCl. The PA yield of the A4 mutant reached 97.2 g/L at 120 h (0.795 g/g glycerol) in a 20-L fermenter with glycerol as the sole carbon source, which was 28.9% higher than that of the parental strain.
Conclusion: The PA yield from *Y. lipolytica* can be improved by increasing its NaCl tolerance.
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1. Introduction

Pyruvic acid (PA) plays a prominent role in the cell cycle and in carbon metabolism. PA is also used as a food additive and in cosmetics, nutraceuticals, and weight control supplements. Commercially, PA is used for the synthesis of various amino acids, such as L-tryptophan, L-tyrosine, and 3,4-dihydroxyphenylalanine. Furthermore, it acts as a substrate for estimating the activities of enzymes, such as pyruvate carboxylase, pyruvate dehydrogenase, and pyruvate decarboxylase [1]. PA is mainly produced by chemical, fermentation, and enzymatic processes using resting cells; of which, fermentation is the most popular method because of its sustainability and low cost [1,2,3,4,5]. PA can be produced using a number of microorganisms, including *Saccharomyces cerevisiae*, *Escherichia coli*, *Yarrowia lipolytica (Y. lipolytica),* and *Torulopsis glabrata* [6,7,8,9,10,11]. However, the fermentation methods for PA production suffer from low yield [6,9] or hypersensitivity to high glucose concentrations [11,12]. Although a higher yield of PA was achieved using a *S. cerevisiae* pyruvate decarboxylase-negative mutant, its low glucose resistance limited its potential for industrial application [12]. Therefore, to increase the yield of PA, substantially focus must be laid on individual factors in the synthesis process. The pH would not be a major reason for its low yield because the pH of the fermentation medium is typically stabilized using standard NaOH. As PA is produced as pyruvate sodium, a benign form, and thus the toxicity of PA is dismissed. Foregoing reports have shown that osmotic stress is vital for microbial PA synthesis [8,13].

Y. lipolytica can be used to produce PA from crude glycerol, an economical constituent for the fermentation process, thus suggesting that this microbe has potential for the industrial production of PA [6,7,14,15]. However, no report of improved osmotic resistance of *Y. lipolytica* to increase the yield of PA has been published to date. The atmospheric and room temperature plasma (ARTP) method induces mutations in microorganisms based on the effect of a plasma stream. ARTP facilitates rapid transformation, generates a variety of mutants, and is a straightforward and safe procedure [16,17,18].

We attempted to increase the PA yield of *Y. lipolytica* Po1g by producing NaCl-tolerant mutants using ARTP. The results revealed that enhancing the NaCl tolerance of *Y. lipolytica,* improves its production of PA in the fermentation process.

2. Materials and Methods

Pure chemicals were procured from Sigma-Aldrich (Shanghai, China) or from TaKaRa Bio (Hangzhou, China).

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2.1. Strains and media

The parental strain used was *Y. lipolytica* Po1g (Ura$^-$). The parental strain and the mutants generated by ARTP were cultivated in yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, and 1% yeast extract). To evaluate the growth rate and PA production in flasks, the strains were grown in yeast nitrogen glycerol (YNG) medium (0.67% yeast nitrogen base [YNB] and 50 g/L glycerol) for 108 h. For fermentation in flasks, the pH was adjusted to pH 5.5 and 40 g/L $CaCO_3$ was added. To scale up to a 20-L fermenter, the strains were initially grown in YPD medium, and subsequently shifted to a 20-L fermenter with a working volume of 12 L medium supplemented with 10 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 1.4 g/L $MgSO_4 \cdot 7H_2O$, 0.8 g/L $CaCl_2$, 0.5 g/L NaCl, 60 g/L glycerol, and 1 μg/L thiamine. Glycerol was the sole carbon and energy source, which was added periodically. The following bioreactor conditions were maintained during the fermentation process: pH 4.0 (using 20% NaOH), a dissolved O_2 concentration of 40%, and a temperature of 30°C.

2.2. Mutagenesis of the parental strain and selection of mutants

To select NaCl-resistant mutants, the parental strain was mutated using the ARTP breeding system (Si Qing Yuan Biotechnology Co., Ltd., Beijing, China). The screening techniques are depicted in Fig. 1. Pure helium was applied as an operating gas and was set at a flow rate of 10 standard liters per minute. The RF input power was 120 Watts. A gap (D) of 2 mm was maintained between the exit of the plasma torch nozzle and sample plate, and the temperature of the plasma jet was sustained at <30°C. The parental strain was grown in YPD medium until the optical density at 600 nm (OD_{600}) reached 1.0. Subsequently, 5 μL of 10% glycerol and 5 μL of cells were placed in a mini stainless-steel disk and subjected to the ARTP jet for 0 to 480 s. Next, the cells were transferred to the YPD medium containing 80 g/L NaCl overnight. To select NaCl-tolerant mutants, 1 mL of overnight-cultured cells was transferred to the YPD medium containing a higher concentration of NaCl and subsequently plated on YPD agar. Single isolated colonies on the agar were picked and inoculated into the YPD medium containing 160 g/L NaCl to verify their NaCl tolerance. To evaluate the PA yield of the mutants, colonies were inoculated into 250-mL flasks containing 50 mL of YNG medium and incubated for 108 h at 30°C. A specified quantity of sample was withdrawn through the sampling port and subjected to centrifugation. The PA concentration in the resulting supernatant was determined using high-performance liquid chromatography (HPLC). The mutants with increased PA production were used in subsequent experiments.

2.3. Evaluation of ARTP mutagenesis

The rate of lethality of the parental strain was evaluated according to Equation 1:

$$\text{Lethal rate}(\%) = \frac{A-B}{A} \times 100$$

where A represents the total number of colonies before ARTP and B represents the total number of colonies after ARTP. The number of *Y. lipolytica* cells is expressed as colony-forming units (CFU)/mL.

2.4. Analytical methods

The cell density of the fermented broth was evaluated by measuring the OD_{600} using a spectrophotometer (Lengguang Technology Co., Shanghai, China) after serial dilution. To assay PA concentration, fermentation broth was centrifuged at 10,000 × *g* for 10 min and the PA concentration was determined by HPLC (Agilent 1100 series, Santa Clara, CA, USA) with a Stable Bond C18 column (Shiseido Co. Ltd, Tokyo, Japan). The flow rate of the mobile phase (0.1% phosphoric acid aqueous solution) was 1 mL/min, the column temperature was 28°C, the injection volume was 10 μL, and detection was in the ultraviolet range of 210 nm. Values are means of at least three independent experiments.

3. Results

3.1. Effect of NaCl on the growth and PA yield of the parental strain

Significant osmotic stress is the predominant barrier to cell development and PA production [1,8]. The influence of NaCl on the growth and PA yield of the parental strain is depicted in Fig. 2. The highest OD_{600} (10.9, Fig. 2a) and the highest PA yield (5.1 g/L, Fig. 2b) were detected in the absence of NaCl. The cell concentration and PA yield decreased with increasing NaCl concentration in the culture broth. In the presence of 80 g/L NaCl, the cell density (OD_{600} 0.74) and PA yield (0.05 g/L) were reduced by 92.2% and 99.1%, respectively (Fig. 2). In the presence of >100 g/L NaCl, *Y. lipolytica* failed to grow and produce PA. Thus, the addition of NaCl during the initial stage of fermentation restricted the growth of *Y. lipolytica* and its production of PA in a concentration-dependent manner. This indicates that PA production is suppressed by osmotic stress. Therefore, the PA yield could be improved by increasing the tolerance of *Y. lipolytica* to NaCl stress.

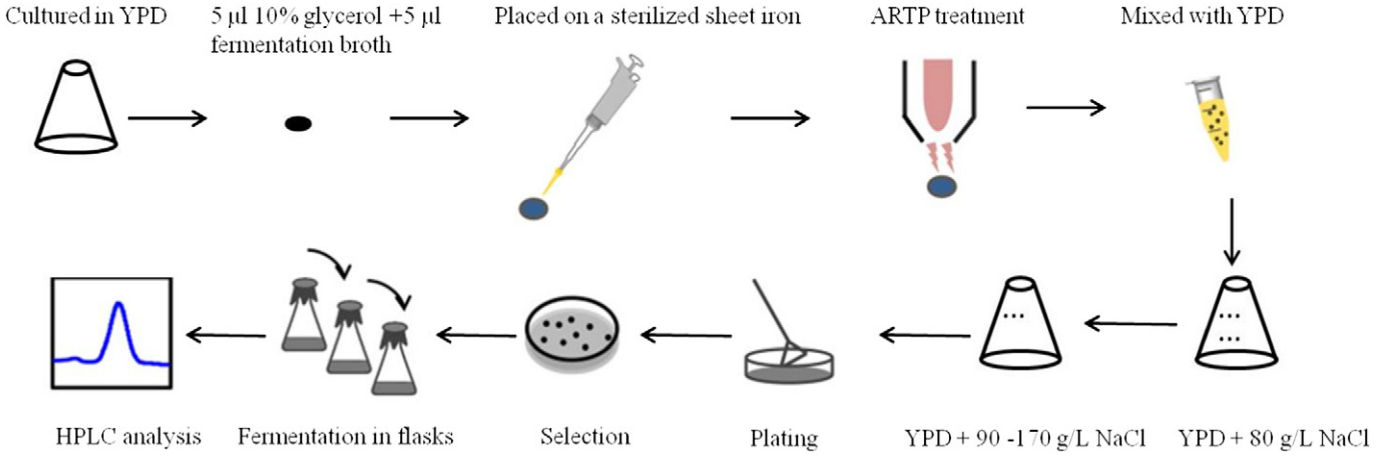

Fig. 1. Generation of mutants with a high yield of pyruvic acid (PA) by the atmospheric and room temperature plasma (ARTP) method.

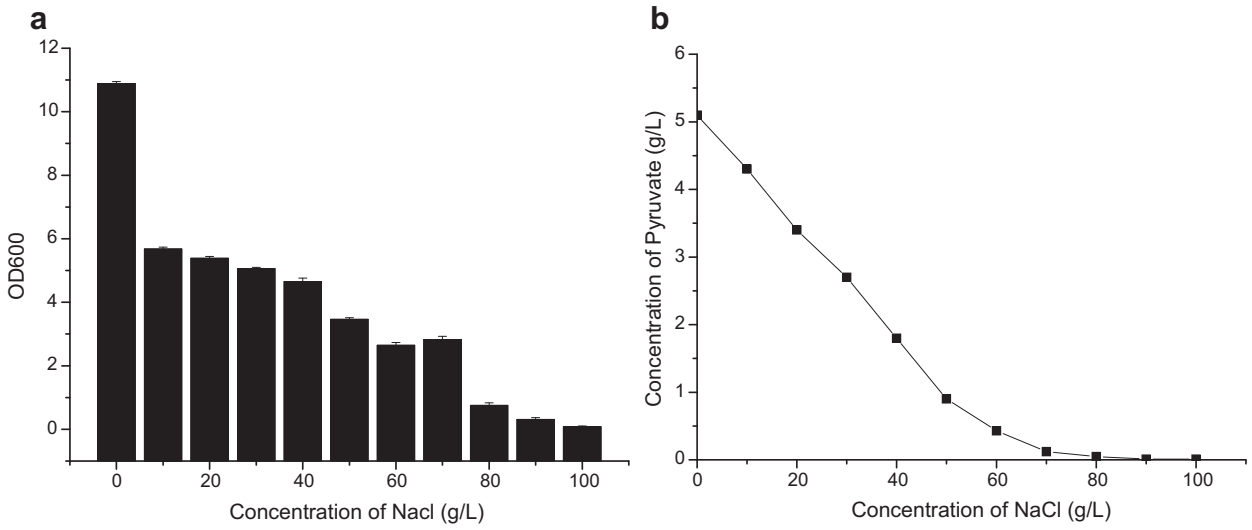

Fig. 2. Effects of NaCl on the growth and PA yield of the parental strain. (a) Cells grown in yeast extract peptone dextrose (YPD) containing the indicated concentrations of NaCl for 24 h in flasks. (b) Cells grown in yeast nitrogen glycerol (YNG) medium for 108 h in flasks.

3.2. Screening of mutants

The optimum lethality rate is vital for successful mutation and the screening of positive mutants. The lethality rate of *Y. lipolytica* according to exposure duration is shown in Fig. 3a. The lethality rate increased by 86, 90, and 95%, respectively, after treatment for 300, 360, and 420 s. Exposure for ≥480 s resulted in the death of all *Y. lipolytica* cells. A lethality rate of >90% is optimum for inducing mutation [19,20]. Therefore, we performed ARTP treatment for 420 s in this study. As the parental strain barely grew in the YPD medium containing 80 g/L NaCl, ARTP-treated cells were initially grown in the YPD medium containing 80 g/L NaCl for enrichment, and were subsequently transferred to the YPD medium containing increasing concentrations of NaCl (90, 100, 110, 120, 130, 140, 150, 160, and 170 g/L). After eight rounds of mutation, the mutants exhibited good growth in the YPD medium containing 160 g/L NaCl but did not grow in the medium with 170 g/L NaCl. Subsequently, approximately 240 NaCl-tolerant mutants were grown and obtained from the solid YPD medium containing 160 g/L NaCl. Twelve of these mutants showed growth in tubes containing YPD medium with 160 g/L NaCl. The ability of the 12 mutants to produce PA was evaluated in YNG medium containing 50 g/L glycerol as a carbon source (Fig. 3b). The A4 mutant, which exhibited the highest PA yield of 7.7 g/L was selected. To confirm its NaCl tolerance, the A4 mutant and the parental strain were grown in the YPD medium containing 160 g/L NaCl. The results showed that the A4 mutant grew well in the YPD medium containing 160 g/L NaCl, but the parental strain showed no significant growth (data not shown). Therefore, the identified A4 mutant for its highest PA synthesis was used in subsequent experiments.

3.3. Effect of carbon source on PA yield

To determine whether glycerol is an appropriate carbon source for PA production, we evaluated YNB medias containing 50 g/L of glucose or glycerol or sucrose. The biomass of the A4 mutant increased to an

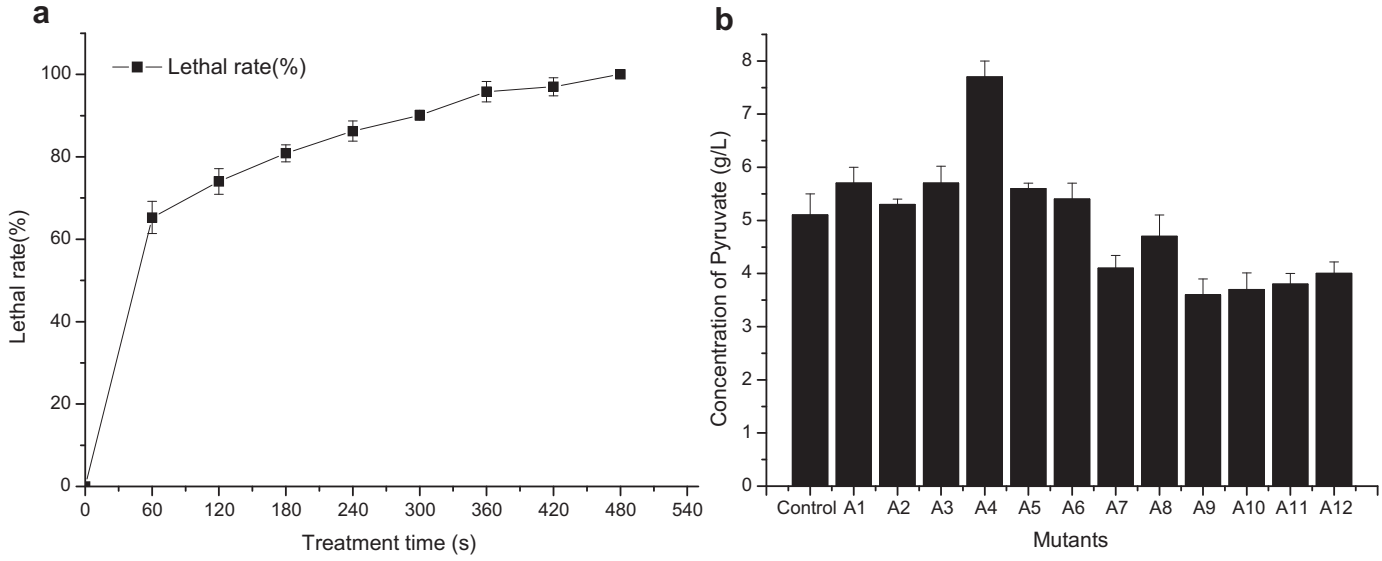

Fig. 3. Determination of the mutant-screening conditions. (a) Lethality rate of the parental strain due to ATRP. (b) Mutants were grown in YNG medium containing 50 g/L glycerol in flasks for 108 h.

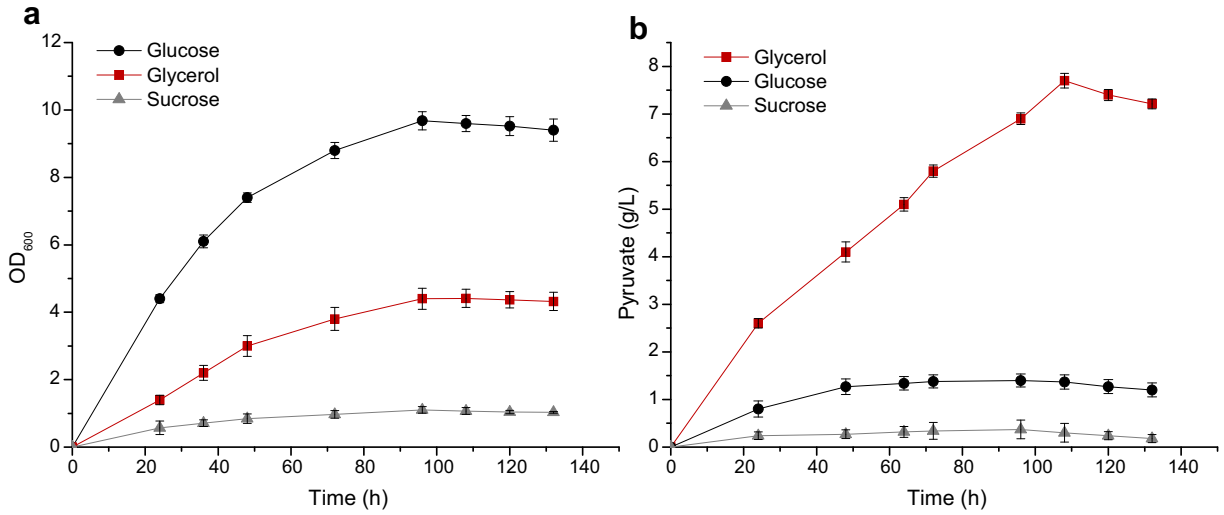

Fig. 4. Effects of carbon source on the biomass (a) and PA yield (b) of the A4 mutant.

OD_{600} of 9.68 when cultured in 50 g/L glucose, which was higher than that in 50 g/L glycerol (OD_{600} 4.41) (Fig. 4a**)**. However, the yield of PA was significantly higher when cultured with glycerol (7.7 g/L) compared to the culture containing glucose (1.4 g/L). (Fig. 4b**)**. The biomass and PA yield of the A4 mutant were 1.1 and 0.37 g/L, and were relatively low compared to other carbon source supplements suggesting that sucrose could be unsuitable for the growth of *Y. lipolytica* and also hindered the production of PA. Therefore, glycerol

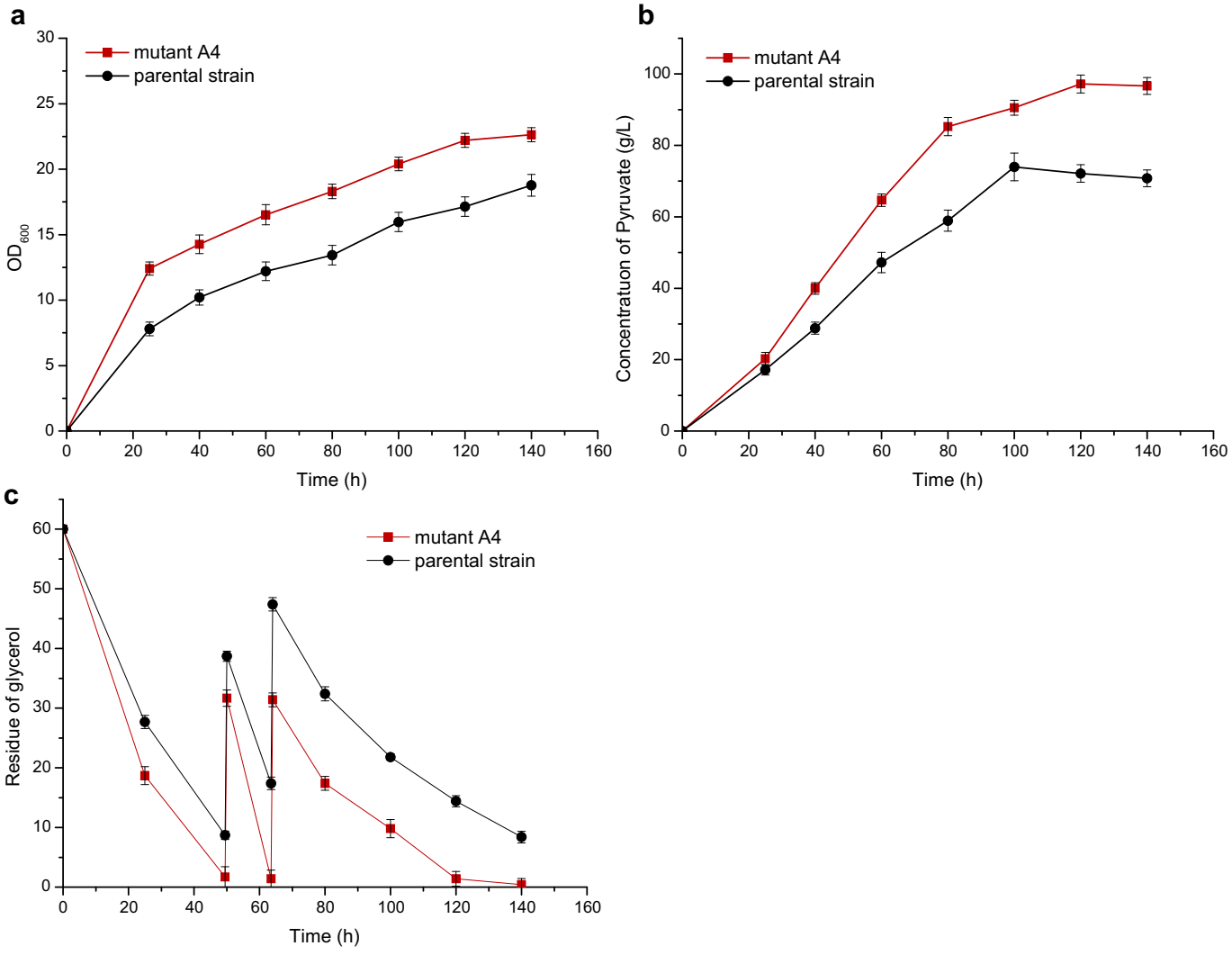

Fig. 5. Growth (a), PA yield (b), and residual glycerol level (c) of the A4 mutant and the parental strain in a 20-L fermenter.

was the optimum carbon source in terms of maximizing the PA yield of *Y. lipolytica*.

3.4. Scale-up fermentation in a 20-L bioreactor

The production of PA by the A4 mutant was scaled-up to a 20-L fermenter (Fig. 5). The initial glycerol concentration was 60 g/L and 30 g/L glycerol was added after 50 and 64 h of fermentation. The pH of the fermentation medium was maintained at 4.0 using a biosensor and 6 M NaOH. The cell density reached 22.2 g/L, which was 29.1% higher than the parental strain. Under optimal conditions, the A4 mutant produced the highest PA yield of 97.2 g/L (*i.e.*, 0.795 g/g glycerol) after 120 h of fermentation, and was 31.5% higher than that of the parental strain, which produced 73.9 g/L PA.

4. Discussion

PA synthesis by fermentation has a long history but traditional strains are active solely when vital enzymes, vitamins, and additional cofactors are implemented to stabilize the process [21]. Literatures have since been emerging using various strains of yeasts, such as *Candida maltosa*, *Debaryomyces coudertii,* and *Torulopsis etchellsii,* but the PA yield of them were par low compared to *Y. lipolytica* [14]. Also, most of the studies have been performed with glucose as the sole carbon source. The use of *Y. lipolytica* for PA production dates back to 1974 with acetamide as the carbon source and 1.5 g/L PA yield [22]. Since then, many different strains of *Y. lipolytica* have been continuously screened under different growth conditions and carbon sources for maximizing the PA biosynthesis. A recent study identified *Y. lipolytica* VKM Y-2378 strain to be the best producer with 41g/L PA synthesis and a yield of 0.82 g/g of glycerol substrate [15].

The development of engineered strains has to be propelled to further maximize the product formation even at concealed fluctuations in the environmental factors. High osmotic pressure is known to strongly affect the product biosynthesis of *Y. lipolytica* cells especially when bases are applied in the pH standardization steps and when glycerol is used as the carbon source [23,24]. In this study, we not only developed the A4 mutant strain of *Y. lipolytica* to resist the osmotic pressure caused by NaCl, but also achieved the highest PA yield until date using this mutant cell in the fermentation process. Furthermore, the developed mutant strain was compared with the parental strain using various carbon sources and their cellular biomass and the PA yield were validated in a scale-up fermentation system.

Osmotic stress is the most prominent limiting factor suppressing cell growth and biomass yield during cultivation [25,26]. Therefore, both the cell growth and PA yield of the parental strain of *Y. lipolytica* were initially screened at different concentrations of NaCl and it was observed that both these factors drastically plunged at the lowest concentration of NaCl, and there was a complete cease in cell growth and PA synthesis with NaCl >100 g/L.

ARTP breeding system, an extensively used method to develop high-yield and fast-growing mutant strains for fermentation was applied at a sublethal dose of 420 s for eight rounds. After screening for their NaCl tolerance, 12 mutants were selected with the potential to survive NaCl up to 160 g/L compared to the parental strain, which barely survived 80 g/L. Among them, the best A4 mutant was identified to produce the highest PA yield of 7.7 g/L when cultured in tubes with 50 g/L glycerol-supplemented YNG medium. The best carbon source for the developed mutant has to be standardized as a microorganism, particularly the yeasts have varied abilities to utilize different carbon sources [27]. Glucose and sucrose are the traditionally used carbon sources and Glycerol is applied as an alternative lately [28]. In this study, although the cellular biomass was highest when cultured with glucose rather than glycerol, the PA synthesis was significantly higher in glycerol-supplemented culture with a yield of 7.7 g/L compared to glucose with a yield of 1.4 g/L and sucrose having negligible PA. Similar results were observed in various studies and glycerol has been proven to be a very good energy source for the biotechnological process involving *Y. lipolytica* [6,14]. Moreover, the use of glucose in the fermentation process is also known to produce other coproducts, which might complicate further downstream purification steps [15,29]. The lower PA yield despite the higher cellular biomass of the A4 mutant might be because of catabolite repression and is quite common when grown in a medium containing sugars particularly glucose [27].

The PA production by the A4 mutants were further analyzed in a scale-up reaction using a bioreactor system and was observed with an exorbitant cell density growth of 22.2 g/L and PA yield of 97.2 g/L. A very recent study using *Y. lipolytica* and different forms of glycerol as carbon sources has claimed highest PA biosynthesis with a yield of 124.4 g/L for 200 g/L glycerol supplied, which results in a potential yield of 0.62 g/g of synthesized PA to supplied glycerol [14]. The developed A4 mutant in this study surpasses by having a potential PA yield of 0.795 g/g glycerol and is almost 18% higher than the previous report. It is worth mentioning that the obtained PA yield to the supplied glycerol is the highest reported to date and the developed A4 mutant has the potential ability to withstand fluctuations of the osmotic pressure raised in the fermentation process. The developed stress-tolerant strain is capable of reducing the inhibition of PA fermentation caused by osmotic stress and has the competence to be implemented at the industrial level.

5. Conclusions

ARTP-mediated mutation of *Y. lipolytica* increased the yield of PA by 28.9% compared to that of the parental strain. The developed A4 mutant was able to withstand high osmotic pressure when grown with NaCl up to 160 g/L and the crude glycerol was identified as the best carbon source to obtain highest PA yield. Further work is needed to identify the mechanism underlying the enhanced NaCl tolerance of the *Y. lipolytica* A4 mutant.

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Conflict of interest

There is no conflict of interest.

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